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High-Performance Liquid Chromatography with Photodiode Array Detection (HPLC−DAD)/HPLC−Mass Spectrometry (MS) Profiling of Anthocyanins from Andean Mashua Tubers (*Tropaeolum tuberosum* Ruíz and Pavón) and Their Contribution to the Overall Antioxidant Activity

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Mashua (*Tropaeolum tuberosum* Ruíz and Pavón), an Andean tuber with high antioxidant activity, has sparked interest because of its traditional medicinal use. In this study, we evaluated the anthocyanin composition for three purple mashua genotypes and their contribution to the overall antioxidant activity of the tuber. Mashua anthocyanins, total phenolics, and 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) antioxidant activity ranged from 45.5 to 131.9 mg of cyanidin 3-glucoside equivalents/100 g fresh weight (FW), 174.9 to 275.5 mg of gallic acid equivalents/100 g of FW, and 16.2 to 45.7 μmol of Trolox equivalents/g of FW, respectively. The high-performance liquid chromatography with photodiode array detection (HPLC−DAD) and HPLC−electrospray ionization tandem mass spectrometry (ESI/MS−MS) profiles revealed the presence of 11 different anthocyanins. The two major pigments (56.4−73.0% total area range at 520 nm) were identified as delphinidin 3-glucoside-5-acetylrhamnoside and delphinidin 3-sophoroside-5-acetylrhamnoside. Other pigments were delphinidin 3-glucoside-5-rhamnoside, delphinidin 3-sophoroside-5-rhamnoside, delphinidin 3-glucoside, cyanidin 3-sophoroside, and cyanidin 3-sophoroside-5-rhamnoside. Cyanidin 3-glucoside and cyanidin 3-rutinoside were only found in two genotypes, while pelargonidin 3-sophoroside and pelargonidin 3-sophoroside-5-rhamnoside were only found in the third one. Anthocyanins from mashua were the major contributors to the total ABTS values for only one of the three genotypes, suggesting that other phenolics present are playing a major role in the antioxidant power of mashua tubers. Results from this study provide important information for the Nutraceutical and Functional Food Market for the use of mashua anthocyanins not only as a source of natural colorants but also as a source of phytonutrients.

KEYWORDS: Mashua; anthocyanins; *Tropaeolum tuberosum*; antioxidant activity; HPLC−DAD; HPLC−ESI/MS−MS; total phenolics

INTRODUCTION

Mashua (*Tropaeolum tuberosum* Ruíz and Pavón), an Andean tuber, has been cultivated for centuries in Peru, Bolivia, Ecuador, Venezuela, and Colombia (*1*). Mashua tubers are commonly yellow, but varieties with an intense purple coloration surrounding the skin and dispersed throughout the tuber also exist. Health-promoting properties in mashua tubers thus far investigated have been associated mainly with glucosinolates (*2*) and phenolic compounds (*3*). A recent study aiming at comparing the antioxidant potential of certain Andean crops including colored potatoes, olluco, and oca showed that mashua tubers had the highest antioxidant activity and purple-colored mashua tubers had an 8−10 times higher antioxidant activity than the

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yellow-colored mashua tubers (*3*). This higher antioxidant activity was suspected to be correlated with the high anthocyanin content.

Anthocyanins are natural pigments widely distributed in the plant kingdom. They are water-soluble pigments responsible for the orange, red, blue, and purple colors found in most fruits, flowers, leaves, and cereal grains (*4*−*6*). The anthocyanin molecule consists of the aglycone based on the flavylium nucleus, one or more sugars, and often an organic acid (*6*). The aglycones or anthocyanidins most commonly found in nature are cyanidin, delphinidin, peonidin, petunidin, malvidin, and pelargonidin. In plants, anthocyanidins occur as glycosylated forms or anthocyanins. D-glucose, D-galactose, L-rhamnose, D-xylose, and D-arabinose are the most prevalent sugars found as 3-glycosides or 3,5-diglycosides (*7, 8*). In addition, the sugar residues may occur acylated with cinnamic acids (e.g., caffeic, *p*-coumaric, ferulic, or sinapic acid) and/or aliphatic acids (e.g., acetic, malic, malonic, oxalic, or succinic acid). These acyl substituents are commonly bound to the C-3 sugar through an ester bond with the 6-OH or less frequently to the 4-OH group of the sugar (*7*).

Many biological activities are attributed to anthocyanins. Antioxidant, antimutagenic, and anticarcinogenic activities of anthocyanins have been extensively reported (*9*−*16*). In addition, anthocyanins have also been utilized in the treatment of various blood circulation disorders resulting from capillary fragility (*17*), inflammatory processes (*18*), or excessive platelet aggregation (*19*).

Preliminary determinations performed by our group have highlighted a high anthocyanin content (85−205 mg of cyanidin 3-glucoside/100 g) in mashua tubers (*3*). This is comparable to those reported for other important anthocyanin sources such as blueberries (82−420 mg of cyanidin 3-glucoside equivalents/100 g), cranberries (60−200 mg of cyanidin 3-glucoside equivalents/100 g), and black currant (130−400 mg of cyanidin 3-glucoside equivalents/100 g) (*7*). However, the anthocyanin composition of mashua has not been reported before. Because the type of anthocyanins present in mashua may influence their color intensity and stability (*20*), antioxidant activity (*17, 21*), and other health-related properties, chemical identification of these compounds is needed. Reverse-phase high-performance liquid chromatography with photodiode array detection (HPLC−DAD) has been extensively used for anthocyanin identification (*4*). However, one drawback of this technique is that some anthocyanins show similar retention time and spectroscopic characteristics, which make their identification difficult (*22*). Electrospray ionization mass spectrometry (ESI−MS) and tandem mass spectrometry (MS−MS) analysis may thus be used as a complementary tool for anthocyanin identification (*23*).

Considering the major questions highlighted above, the objectives of this study were to identify the anthocyanins present in three purple-colored mashua genotypes by means of HPLC−DAD and HPLC−ESI/MS−MS and to determine the contribution of total anthocyanins to the overall antioxidant activity of these genotypes.

MATERIALS AND METHODS

Plant Material. Three purple-colored mashua (*T. tuberosum* Ruíz and Pavón) genotypes, DP 0224, ARB 5241, and AGM 5109, were kindly provided by the International Potato Center (CIP, Lima, Peru) in June 2004. All mashua tubers were harvested at 7.5 months of maturity stage (full maturity). Harvested tubers were immediately lyophilized and stored at −20 °C.

Reagents and Standards. Trolox (6-hydroxy-2,5,7,8-tetramethyl chroman-2-carboxylic acid), 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 2 N Folin−Ciocalteu, and sugar standards, D-glucose, rhamnose, and galactose, were purchased from Sigma Chemicals Co. (St. Louis, MO). Anthocyanin standards, delphinidin 3-glucoside, delphinidin 3-rutinoside, cyanidin 3-glucoside, cyanidin 3-rutinoside, and peonidin 3-glucoside, were purchased from Extrasynthèse (Genay, France). Anthocyanin aglycone standards, delphinidin, cyanidin, peonidin, pelargonidin, and malvidin, were purchased from ChromaDex (Santa Clara, CA). HPLC-grade acetonitrile and methanol were purchased from J.T. Baker, Inc. (Philipsburg, NJ). Other solvents and reagents were purchased from Merck (Darmstadt, Germany).

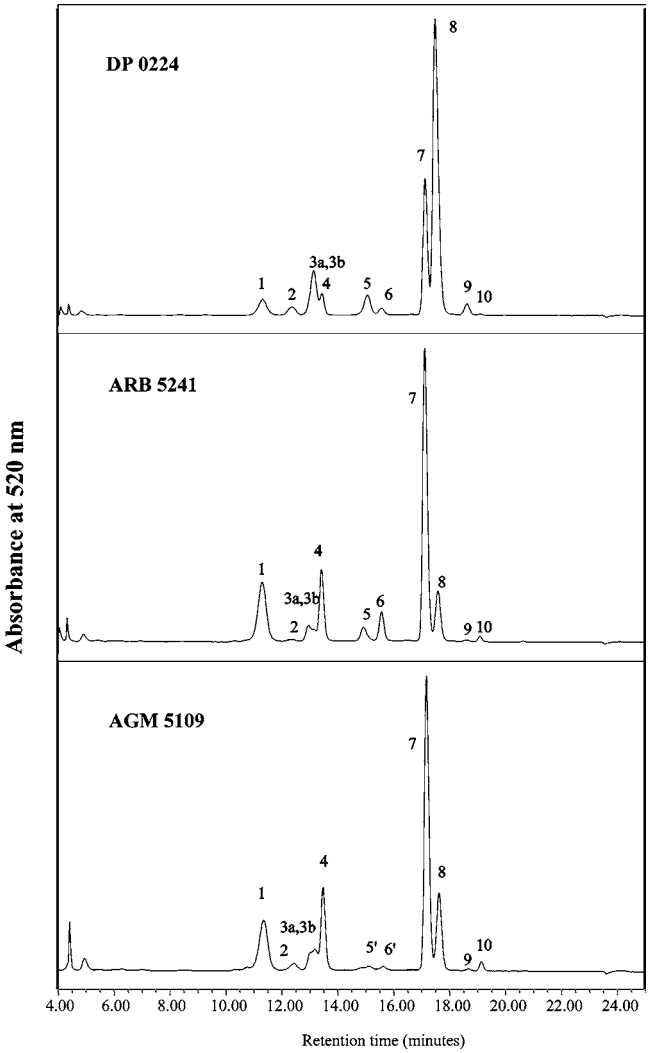

Figure 1. Anthocyanin HPLC–DAD profiles for the three mashua genotypes recorded at 520 nm. Peaks identified for the standards that were available were delphinidin 3-glucoside (peak 2) for the three genotypes evaluated and cyanidin 3-glucoside (peak 5) and cyanidin 3-rutinoside (peak 6) for DP 0224 and ARB 5241 genotypes.

Preparation of Extracts. Approximately 0.7 g of a lyophilized mashua sample was placed in a screw-cap vial wrapped with aluminum foil and extracted with 42 mL of 0.1% HCl in 90% MeOH. The mixture was vortexed and flushed with nitrogen for 3 min. The vial was kept in a water bath at room temperature for 60 min under agitation (200 rpm). Then, the extract was centrifugated at 27000*g* for 10 min at 4 °C, and the supernatant was collected. The above extraction was repeated on the pellet with 20 mL of solvent for 15 min. The supernatants were combined and evaporated in a rotary evaporator at 40 °C. The residue was diluted with acidified water (0.01% HCl) for later anthocyanin purification and identification. Extractions for each mashua genotype were carried out in triplicate.

The extracts were further processed by two different methods, depending upon the objective pursued. Method 1 was performed for the identification of anthocyanins present, whereas method 2 was used

for the evaluation of their contribution to the antioxidant activity of the mashua phenolic extracts.

Purification of Anthocyanin Extracts. The purification protocols were different for the two processes mentioned above. In method 1, the purification (clean up procedure) was carried out using solid-phase extraction (SPE) cartridges (*3*). A total of 3 mL of the extract was passed through a 500 mg, 3 mL, C_{18} Sep-Pak cartridge (Waters, Milford, MA) previously activated with methanol and acidified water (0.01% HCl). The cartridge was then rinsed with acidified water (0.01% HCl) to remove sugars, acids, and other interfering substances. The elution of anthocyanins was performed with acidified methanol (0.01% HCl). Then, the methanolic eluate was concentrated under vacuum (40 °C) to remove the solvent, and the residue was redissolved in 0.01% aqueous HCl to a final volume of 3 mL. The extract was kept at −20 °C until analyzed by HPLC−DAD and HPLC−MS. In method 2, the purification combined the use of a C_{18} Sep-Pak cartridge (*24*) with Sephadex LH-20 (Sigma, St. Louis, MO) gel-permeation chromatography (*25*). A total of 3 mL of the extract was passed through a C_{18} Sep-pak cartridge previously activated as described in purification method 1. Anthocyanins and phenolics were absorbed onto the column. Sugars, acids, and other polar compounds (F-I) were eluted and collected with 6 mL of acidified water (0.01% HCl). The phenolic fraction (F-II), excluding the anthocyanin, was then eluted with 6 mL of ethyl acetate. Ethyl acetate from the eluate was then removed under vacuum (40 °C), and the residue was redissolved in 3 mL of methanol. The anthocyanin-rich fraction (F-III) was then eluted and collected with 9 mL of acidified methanol (0.01% HCl). The methanol was evaporated under vacuum (40 °C), and the residue was redissolved in 3 mL of acidified water (0.01% HCl). A total of 1 mL of the F-III was further applied on a 140 × 13 mm i.d. Sephadex LH-20 column. The compounds present on the column were eluted with 18 mL of 20% methanol, 36 mL of 60% methanol, and 306 mL of 100% methanol, at a flow rate of 1 mL/min. A total of 180 fractions of 2 mL were collected. Compound elution was monitored at 280, 320, and 520 nm using a Genesys-5 UV/vis spectrophotometer (Milton Roy, New York, NY). For the evaluation of the antioxidant capacity, the fractions were then assembled in three groups according to the elution profiles. These pools were then concentrated under vacuum (40 °C) and redissolved in acidified methanol (0.01% HCl) to a final volume of 6 mL. The fractions were stored at −20 °C until analysis. Anthocyanin, phenolic, and ABTS recovery values were accounted, taking into consideration their initial values and following their variation during the purification process based on a mass balance.

Alkaline and Acid Hydrolysis of Anthocyanins. Anthocyanin acid and alkaline hydrolysis were carried out in triplicate according to the procedure proposed by Hong and Wrolstad (*22*) and Durst and Wrolstad (*4*), respectively. For the alkaline hydrolysis (saponification), approximately 5 mg of purified pigment (method 1) was added to 10 mL of 10% aqueous KOH in a screw-cap test tube, flushed with nitrogen, capped, and placed in darkness for 8 min at room temperature. The mixture was then neutralized with 2 N HCl, and the resulting solution was purified using a C_{18} Sep-Pak cartridge and analyzed by HPLC−DAD for the identification of the anthocyanin glucosides.

For the acid hydrolysis, approximately 0.5 mg of purified pigment (method 1) was mixed with 10 mL of 2 N HCl in a screw-cap test tube, flushed with nitrogen, and capped. The tube was placed at 100 °C for 60 min in the dark and then cooled in an ice bath. The resulting solution was purified through a C_{18} Sep-Pak cartridge and analyzed by HPLC−DAD for identification of anthocyanidins.

The sugars released after acid hydrolysis were evaluated by qualitative thin-layer chromatography (TLC) analysis (*26*). A small amount (5 μL) of concentrated hydrolyzed sample was applied to the silica gel 60 F254 plates (Merck, Darmstadt, Germany). Sugars were separated using propanol/water (85:15, v/v) as a solvent. The presence of separated sugars was revealed by submerging the prepared TLC plates in diphenylamine−aniline−phosphate solution. Sugars were identified by comparing their R_f values to known sugar standards run in parallel.

HPLC−DAD Conditions. Identification of anthocyanins, saponified anthocyanins, and anthocyanidins were carried out according to a modified procedure proposed by Chandra et al. (*27*). Anthocyanins were separated by using a reversed-phase HPLC on a 2695 Separation Module (Waters, Milford, MA) equipped with an autoinjector, a 996 photodiode array detector (DAD), and the Empower software. Spectroscopic data from 200 to 700 nm were recorded during the whole run. A 150 × 4.6 mm i.d., 5 μm Atlantis C_{18} column (Waters, Milford, MA) and a 4.6 mm × 2.0 mm guard column were used for anthocyanin separation at 40 °C. The mobile phase was composed of (A) water/phosphoric acid (100:0.85, v/v) at pH 2.17 and (B) water/acetonitrile/acetic acid/phosphoric acid (50.3:49.1:0.4:0.2, v/v/v/v) at pH 2.27. The solvent gradient was from 20−38% B in 13 min, followed by an increase to 40% B in 1 min and an increased to 50% B in 11 min. A flow rate of 0.7 mL/min was used, and 20 μL of sample was injected. Peak elution was monitored at 520 nm. Samples and mobile phases were filtered through a 0.22 μm filter, type GV (Millipore, Bedford, MA) prior to HPLC injection. The anthocyanins and anthocyanidins were identified by comparing their retention times and UV/vis spectroscopic data to known, previously injected standards. Each extract was analyzed in triplicate.

Table 1. Anthocyanins, Total Phenolics, and Antioxidant Activity for Three Purple Mashua Genotypes[a]

genotype	anthocyanin[b]	total phenolics[c]	ABTS[d]
DP 0224	131.9 ± 2.5 a	275.5 ± 3.9 b	29.6 ± 0.9 b
ARB 5241	123.4 ± 2.7 b	374.4 ± 7.6 a	45.7 ± 0.3 a
AGM 5109	45.5 ± 3.1 c	174.9 ± 1.0 c	16.2 ± 1.2 c

[a] $x \pm$ SD ($n = 3$). [b] Total anthocyanin content expressed as milligrams of cyanidin 3-glucoside equivalents/100 g of mashua FW (CGE/100 g of FW). [c] Total phenolic content expressed as milligrams of gallic acid equivalents/100 g of mashua FW (GAE/100 g of FW). [d] Antioxidant capacity expressed as micromoles of Trolox equivalents/g of mashua FW (TE/g of FW). Values within a column with different letters are significantly different at $p \leq 0.05$.

HPLC−ESI/MS−MS Conditions. Separation of anthocyanins was conducted on a 75 mm × 4.6 mm i.d., 3.5 μm Symmetry C_{18} column (Waters) using a 2695 HPLC separation module. Detection was performed using both the DAD for some of the runs and a triple quadrupole ion tunnel mass spectrometer equipped with Masslynx V3.5 software and a Z-spray ESI source (Quattro Ultima, Micromass, Manchester, U.K.). The mass spectrometer is very sensitive to the solvent used in the runs. Thus, a new mobile phase was developed for the analysis. Solvent A was water containing 10% formic acid, and solvent B was acetonitrile. A linear gradient from 5 to 15% B in 20 min and a flow rate of 0.8 mL/min were used. Absorption spectra of anthocyanins were recorded from 200 to 600 nm with the in-line DAD. Approximately 100 μL/min of the HPLC eluate separated by a microsplitter valve (Upchurch Scientific, Oak Harbor, WA) was delivered to the ESI source. The quadrupole instrument was operated at the following settings: capillary voltage, 3.0 kV; cone voltage, 35 V; RF lens 1, 50 V; desolvation gas temperature, 420 °C at a flow of 17 L/min; source temperature, 105 °C; collision gas (argon) pressure, 7 psi; collision energy, 25 eV.

Antioxidant Activity Evaluation. Antioxidant activities were determined by the ABTS procedure proposed by Arnao et al. (*28*). Samples (150 μL) were mixed with 2850 μL of $ABTS^{+\cdot}$ solution prepared as described by Awika et al. (*29*). This mixture was reacted at 20 °C until a steady absorbance was reached. Methanol was used as a control. The Genesys-5 UV/vis spectrophotometer was blanked with methanol, and the decrease in absorbance because of antioxidants was recorded at 734 nm. The antioxidant capacity was calculated as micromoles of Trolox equivalents (TE)/1 g of mashua fresh weight (FW) from a standard curve developed with Trolox.

Total Phenolics and Total Anthocyanins. Total phenolics were determined according to the Folin−Ciocalteu procedure (*30*). Absorbance was measured at 755 nm, and the results were expressed as milligrams of gallic acid equivalents (GAE)/100 g of mashua FW. Total anthocyanins were measured using the pH differential method (*31*). Absorbance was measured at 520 and 700 nm in pH 1.0 and 4.5 buffers. A molar extinction coefficient of 26 900 L cm^{-1} mol^{-1} and a molecular weight of 449.2 were used for anthocyanin calculation. Results were

Table 2. Chromatographic and Spectroscopic Characteristics of Anthocyanins Detected in Mashua Tuber Extracts

peak number	peak area (%) HPLC–DAD[a]	retention time (min)[a] HPLC–ESI/MS–MS	molecular ion: M^+ *m/z*	fragment ions in MS *m/z*	anthocyanin assignment
DP 0224					
1	4.9	10.10	773	627, 303	delphinidin 3-sophoroside-5-rhamnoside
2	2.7	10.65	465	303	delphinidin 3-glucoside
3a	8.6	11.35	611	287	cyanidin 3-sophoroside
3b		11.90	611	465, 303	delphinidin 3-glucoside-5-rhamnoside
4	3.1	12.63	757	611, 287	cyanidin 3-sophoroside-5-rhamnoside
5	3.6	13.76	449	287	cyanidin 3-glucoside
6	1.3	14.57	595	287	cyanidin 3-rutinoside
7	19.8	16.95	653	465, 303	delphinidin 3-glucoside-5-acetylrhamnoside
8	53.2	17.42	815	627, 303	delphinidin 3-sophoroside-5-acetylrhamnoside
9	2.1	17.71	815	627,303	similar to peak 8
10	0.4	18.78	653	465, 303	similar to peak 7
ARB 5241					
1	18.5	11.02	773	627, 303	delphinidin 3-sophoroside-5-rhamnoside
2	0.6	10.51	465	303	delphinidin 3-glucoside
3a	4.4	11.38	611	287	cyanidin 3-sophoroside
3b		11.81	611	465, 303	delphinidin 3-glucoside-5-rhamnoside
4	10.7	12.55	757	611, 287	cyanidin 3-sophoroside-5-rhamnoside
5	3.2	13.81	449	287	cyanidin 3-glucoside
6	4.9	14.62	595	287	cyanidin 3-rutinoside
7	47.2	16.89	653	465, 303	delphinidin 3-glucoside-5-acetylrhamnoside
8	9.2	17.33	815	627, 303	delphinidin 3-sophoroside-5-acetylrhamnoside
9	0.3	17.68	815	627, 303	similar to peak 8
10	1.0	18.70	653	465, 303	similar to peak 7
AGM 5109					
1	16.1	10.08	773	627, 303	delphinidin 3-sophoroside-5-rhamnoside
2	2.1	10.45	465	303	delphinidin 3-glucoside
3a	6.1	11.40	611	287	cyanidin 3-sophoroside
3b		11.68	611	465, 303	delphinidin 3-sophoroside-5-rhamnoside
4	11.9	12.23	757	611, 287	cyanidin 3-sophoroside-5-rhamnoside
5′	1.6	12.56	595	271	pelargonidin 3-sophoroside
6′	0.8	13.71	741	595, 271	pelargonidin 3-sophoroside-5-rhamnoside
7	45.4	16.92	653	465, 303	delphinidin 3-glucoside-5-acetylrhamnoside
8	13.8	17.34	815	627, 303	delphinidin 3-sophoroside-5-acetylrhamnoside
9	0.4	17.69	815	627, 303	similar to peak 8
10	1.8	18.71	613	465, 303	similar to peak 7

[a] Mean of three repetitions.

expressed as milligrams of cyanidin 3-glucoside equivalents (CGE)/100 g of mashua FW.

Statistical Analysis. Quantitative data were presented as mean values with the respective standard deviation values corresponding to three replicates. Antioxidant capacity, total phenolic, and total anthocyanin results were processed by the one-way variance analysis (ANOVA). Differences at $p < 0.05$ were considered to be significant. SPSS for Windows 14.0 (SPSS, Chicago, IL) was used for all statistical tests.

RESULTS AND DISCUSSION

Total Anthocyanins and Total Phenolics. The total anthocyanin and total phenolic contents for the three purple-colored mashua tuber genotypes are shown in **Table 1**. Total anthocyanins ranged from 45.5 to 131.9 mg of CGE/100 g of FW, which is comparable to the values reported for commodities such as red raspberry (10−60 mg of CGE/100 g of FW), strawberry (20−90 mg of CGE/100 g of FW), red cabbage (30−90 mg of CGE/100 g of FW) (*32*), and banana bracts (32 mg of CGE/100 g of FW) (*33*). The total phenolic content ranged from 174.9 to 374.4 mg of GAE/100 g of FW, which is in the same range as the contents reported for cranberry, blackberry, black raspberry, and red raspberry (315, 226, 267, and 234 mg of GAE/100 g of FW, respectively) (*34*) but higher than the values found in strawberries (103 mg of GAE/100 g of FW).

Interestingly, genotype ARB 5241 had the highest total phenolic content (374.4 mg of GAE/100 g of FW) but not the highest anthocyanin content. Genotype DP 0224 had the highest anthocyanin content (131.9 mg of CGE/100 g of FW). Genotype AGM 5109 was the lowest in both anthocyanin and total phenolic contents.

HPLC−DAD Analysis of Anthocyanins. The mashua extracts corresponding to the three different genotypes were cleaned-up by SPE with a C_{18} Sep-Pak cartridge (purification method 1) before HPLC analyses. Minimal clean up is recommended prior to anthocyanin characterization by HPLC to avoid changing the relative proportion of the anthocyanins present in the samples (*24*). In addition, the presence of impurities in the samples may modify the UV/DAD spectra of the anthocyanins and hinder their identification by a comparison with the UV/DAD spectra of the reference substances. **Figure 1** and **Table 2** show that the three mashua genotypes contain roughly the same anthocyanins but in different proportions as revealed by the differences in the relative percentage area of the different pigments. Peak 1, 4, 7, and 8 appear to be the main anthocyanins present at least for two of the three genotypes.

Important structural information can be obtained from anthocyanin spectroscopic data, such as the nature of the aglycone, the position of the attachment of the sugar molecule, and a possible acylation with aromatic organic acids (*22*, *32*). The UV/vis characteristics of the different pigments were determined using the DAD. The $Abs_{440}/Abs_{\lambda max}$ ratios calculated for each anthocyanin ranged from 26.3 to 31.9% for all peaks in the three mashua genotypes. These values are typical of a glycosidic bond at position C-3 of the anthocyanidin (*22*, *32*). The low absorbance at $\lambda_{310-320\ nm}$ for peaks 1−10 indicated that anthocyanins were not acylated with hydroxylated aromatic organic

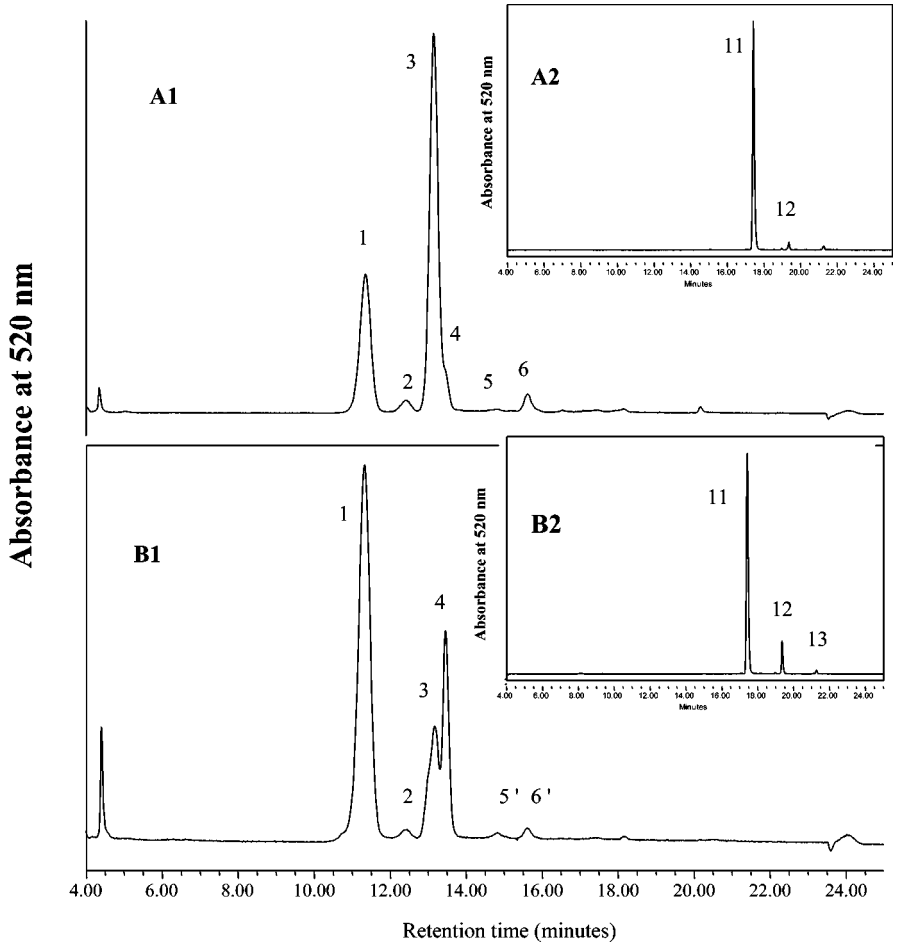

Figure 2. HPLC–DAD chromatograms for DP 0224 and AGM 5109 mashua genotypes obtained after basic (A1 and B1) and acid (A2 and B2) hydrolysis, respectively. Peaks 1–6 correspond to the peaks of **Figure 1**. Peaks were identified through a comparison with anthocyanidin standards: delphinidin (peak 11), cyanidin (peak 12), pelargonidin (peak 13).

acids (*22*, *25*). A comparison of retention times and UV/vis data with known standards revealed the presence of delphinidin 3-glucoside (peak 2) for the three genotypes evaluated. Cyanidin 3-glucoside (peak 5) and cyanidin 3-rutinoside (peak 6) were only found in DP 0224 and ARB 5241 genotypes. After alkaline hydrolysis, peaks 7−10 disappeared, while the relative proportions of peaks 1 and 3 increased (**Figure 2**) for the three mashua genotypes. The disappearance of peaks 7−10 after saponification indicates that these anthocyanins were acylated. The resulting increase in relative proportion of peaks 1 and 3 suggest that the difference between peaks 7−10 and peaks 1 and 3 is due to acylation. The low absorbance at 310−320 nm suggested that the acylation of the anthocyanins was with an aliphatic organic acid (*7*, *22*).

Acid hydrolysis revealed the presence of delphinidin (peak 11) and cyanidin (peak 12) for all genotypes, with delphinidin being the most abundant. In addition, a third aglycone, namely, pelargonidin (peak 13), was found in the AGM 5109 genotype (**Figure 2**). The identity of these anthocyanidins was confirmed by comparing retention times and UV/vis data with previously injected authentic anthocyanin aglycones. The presence of released glucose and rhamnose from mashua anthocyanins was confirmed with the qualitative TLC analysis of sugars (data not shown). To confirm these results and to further characterize the remaining nonidentified mashua anthocyanins, we proceeded with the HPLC−ESI/MS−MS identification.

HPLC−ESI/MS−MS Analysis of Anthocyanins. The HPLC−ESI/MS−MS analyses revealed that peak 3 corresponded to the coelution of two different anthocyanins (3a and 3b) that were not distinguished by the DAD. The molecular masses obtained from precursor ion analyses indicated the presence of different aglycones corresponding to delphinidin (*m*/*z* 303), cyanidin (*m*/*z* 287), and pelargonidin (*m*/*z* 271) (*23*). Genotypes DP 0224 and ARB 5241 showed similar pigment profiles. Pelargonidin was only found in the AGM 5109 genotype, confirming the results obtained with HPLC−DAD.

MS−MS has been reported as a powerful technique that provides clear and characteristic fragmentation patterns that facilitate anthocyanin characterization (*23*). These fragmentation patterns may allow for a rough determination of the location of the glycosidation and acylating groups. In combination with HPLC, ESI/MS and MS−MS represent a very powerful tool for anthocyanin characterization and were used to aid on mashua anthocyanin characterization.

Peaks 1, 2, 3b, and 7−10 all produced a major fragment at *m*/*z* 303, indicating that they were delphinidin derivatives. Peaks 1 and 3b exhibited molecular ions at *m*/*z* 773 and 611, respectively. These molecular ions were fragmented into two major fragments at *m*/*z* 627 and 303 and *m*/*z* 465 and 303, respectively. The molecular ions at *m*/*z* 627 and 303 were possibly produced by the loss of one rhamnose (146u) and one sophorose (321u), and the molecular ions at *m*/*z* 465 and 303 were produced by the loss of one rhamnose (146u) and one glucose (162u), respectively (*35*). Therefore, peaks 1 and 3b were identified as delphinidin 3-sophoroside-5-rhamnoside and delphinidin 3-glucoside-5-rhamnoside, respectively. Peak 2

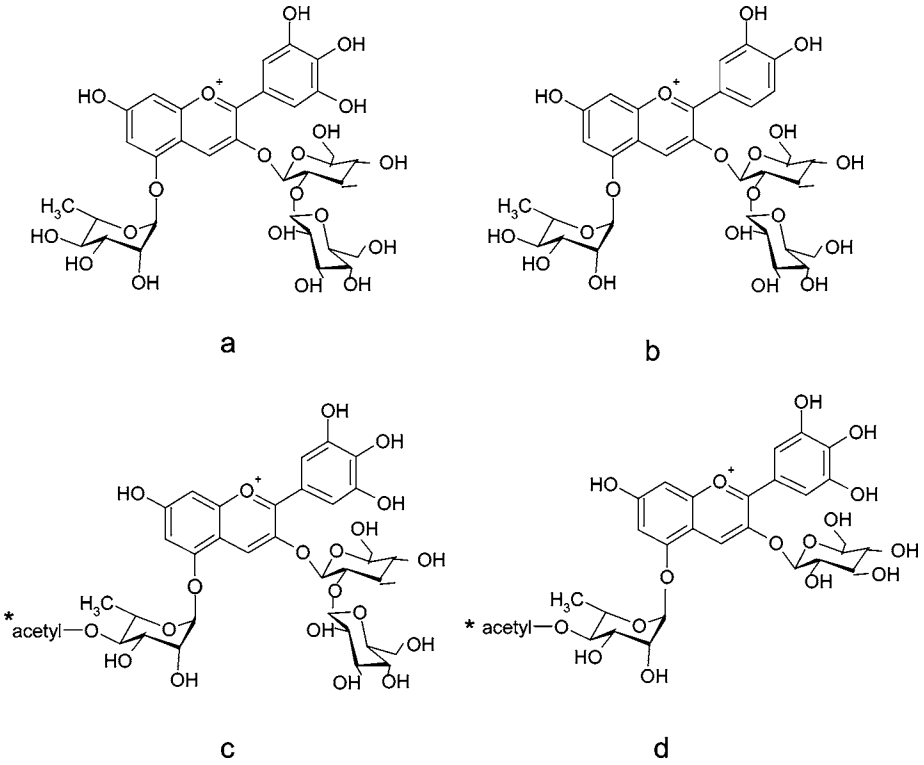

Figure 3. Chemical structures for the main identified anthocyanins in mashua (a) delphinidin 3-sophoroside-5-rhamnoside, (b) cyanidin 3-sophoroside-5-rhamnoside, (c) delphinidin 3-sophoroside-5-acetylrhamnoside, and (d) delphinidin 3-glucoside-5-acetylrhamnoside. Asterisks identify the tentative position of the acetyl group on 4-OH.

presented a molecular ion at *m*/*z* 465. The loss of 162u corresponded to one molecule of glucose. As a result, peak 2 was identified as delphinidin 3-glucoside. Peak 7 revealed a molecular ion at *m*/*z* 653 and fragment ions at *m*/*z* 465 and 303. The fragment *m*/*z* 465 was produced by the loss of one rhamnose linked to an acetyl group (146 + 42u), and *m*/*z* 303 was fragmented by the loss of one glucose (162u) (*35*). Peak 8 exhibited a molecular ion at *m*/*z* 815 and fragments at *m*/*z* 627 and 303. For this particular case, two losses were observed. The first loss corresponded to 188u (rhamnose linked to an acetyl group) and 324u (sophorose). The signals produced were very similar to the ones observed for peaks 1 and 3b, with the only difference being the loss of 42u (one acetyl group) for peaks 7 and 8. As a result, peaks 7 and 8 were identified as delphinidin 3-glucoside-5-acetylrhamnoside and delphinidin 3-sophoroside-5-acetylrhamnoside. Additionally, peaks 9 and 10 showed similar spectroscopic characteristics to peaks 8 and 7, respectively. These peaks might be the respective isomers of the former. These results are in agreement with our findings in HPLC−DAD alkaline hydrolysis for the three mashua genotypes that showed the disappearance of peaks 7−10 after saponification. Peaks 3a and 4−6 all produced a fragment of *m*/*z* 287, indicating that they were all cyanidin derivatives. Peak 3a exhibited a molecular ion at *m*/*z* 611, which was fragmented to *m*/*z* 287 (cyanidin). The loss of 324u corresponded to one molecule of sophorose. Peak 3a was thus identified as cyanidin 3-sophoroside. Peak 4 showed spectrometric data at *m*/*z* 757 and fragment ions at *m*/*z* 611 and 287. It was thus identified as cyanidin 3-sophoroside-5-rhamnoside. Peaks 5 and 6 displayed major signals at *m*/*z* 449 and 595, respectively. Fragmentation led to the loss of 162u (glucose) and 308u (rutinose), respectively. Therefore, peaks 5 and 6 were identified as cyanidin 3-glucoside and cyanidin 3-rutinoside. Main identified anthocyanin structures are shown in **Figure 3**.

Peaks 5′ and 6′ found only in the AGM 5109 genotype showed molecular ions of *m*/*z* 595 and 741, respectively. Fragmentation of the molecular ion *m*/*z* 595 produced only one fragment at *m*/*z* 271, typical of pelargonidin, because of the loss of 324u (sophorose). Fragmentation of the molecular ion *m*/*z* 741 produced two ions at *m*/*z* 595 and 271, because of the loss of 146u (rhamnose) and 324u (sophorose). Thus, the identities proposed for peaks 5′ and 6′ were pelargonidin 3-sophoroside and pelargonidin 3-sophoroside 5-rhamnoside.

Antioxidant Activity of Mashua Extracts and Purified Mashua Anthocyanins. ABTS antioxidant activity for the three purple mashua genotypes are shown in **Table 1**. Mean antioxidant activity ranged from 16.8 to 45.7 μmol of TE/1 g of mashua FW. The highest ABTS values were obtained for genotype ARB 5241, the same one that showed the highest total phenolic content but second highest in anthocyanins. Mashua extracts were semi-purified and fractionated with a C_{18} Sep-Pak cartridge and through chromatography on a Sephadex LH-20 column to obtain a purified anthocyanin fraction with the aim of evaluating its contribution to the overall antioxidant activity of the tuber. The total phenolic and anthocyanin contents as well as the ABTS activity for each fraction obtained after the purification method 2 are shown in **Figure 4**.

In the first step (SPE), most of the anthocyanins were present in the anthocyanin-rich fraction (F-III, **Figure 4**), as expected. The percentage of anthocyanin recovery for the three mashua genotypes was 96−97%. Minimal quantities of anthocyanins (0.29−0.5 mg of CGE/100 g of mashua FW) were found in the phenolic fraction (F-II, **Figure 4**). Anthocyanins were not present in the sugar/acid fraction (F-I, **Figure 4**). The anthocyanin-rich fraction also had the highest total phenolic values (143.2−258.2 mg of CGE/100 g of mashua FW) with respect to the phenolic fraction (18.5−49.5 mg of CGE/100 g of mashua FW) and sugar/acid fraction (19.3−45.7 mg of CGE/100 g of mashua FW). The anthocyanin-rich fraction appeared as the main contributor to the antioxidant capacity (65−85%) followed

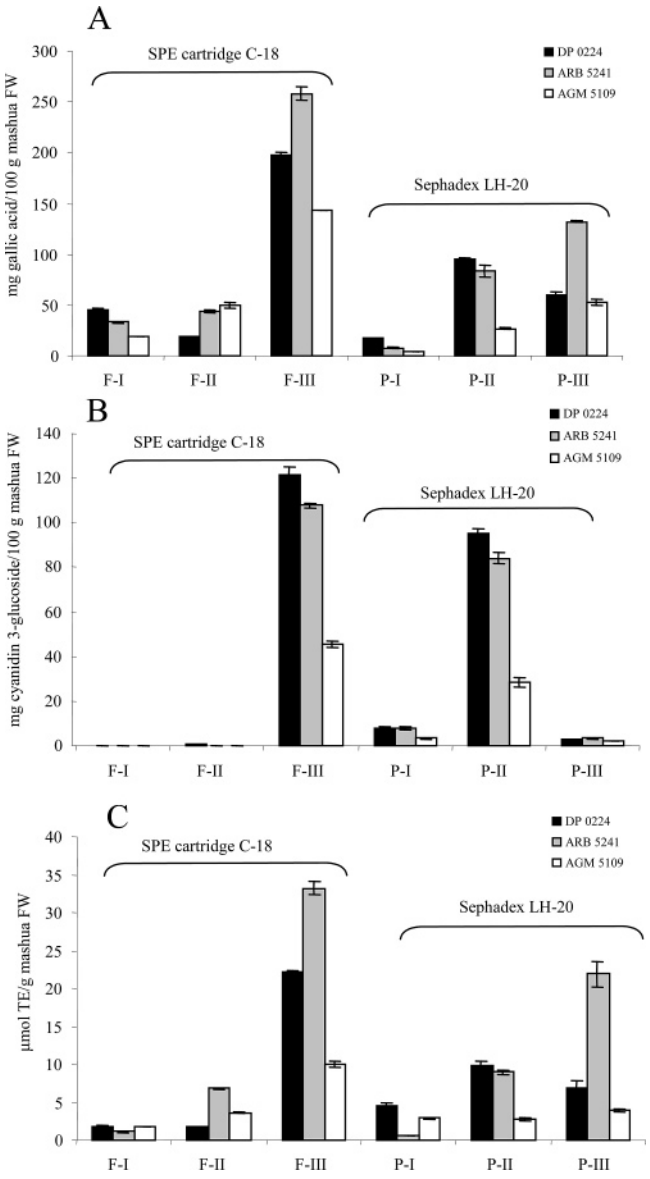

Figure 4. Comparison among the (A) total phenolic content (milligrams of gallic acid equivalents/100 g of mashua FW, GAE/100 g of FW), (B) anthocyanin content (milligrams of cyanidin 3-glucoside equivalents/100 g of mashua FW, CGE/100 g of FW), and (C) ABTS antioxidant activity (micromoles of Trolox equivalents/g of mashua FW, TE/g of FW) for the fractions obtained from purification method 2 for the three mashua genotypes. F-I, sugar/acid fraction; F-II; phenolic fraction; F-III, anthocyanin-rich fraction; and P-I, P-II, and P-III correspond to the subfractions obtained after Sephadex LH-20 gel-permeation chromatography. Bars indicate mean values ± standard deviation (SD) for two replicates.

by the phenolic fraction (7−23%) and finally by the sugar acid fraction (2−11%). The antioxidant capacity of the sugar/acid fraction could be attributed to the presence of water-soluble constituents in the mashua extract, such as sugars, acids, ascorbic acid, glutathione, and other water-soluble compounds, which also possess an antioxidant capacity (*21*). Interestingly, an appreciable amount of ascorbic acid (77.5 mg/100 g) in mashua tuber has been reported in comparison to other Andean tubers (*36*). Ascorbic acid contributes both in the measurement of total phenolic compounds when using the Folin−Ciocalteu method and in the measurements of antioxidant activity.

The relationship between anthocyanins or phenolic compounds and antioxidant activity (ABTS values) was determined. The anthocyanin-rich fraction presented a correlation coefficient

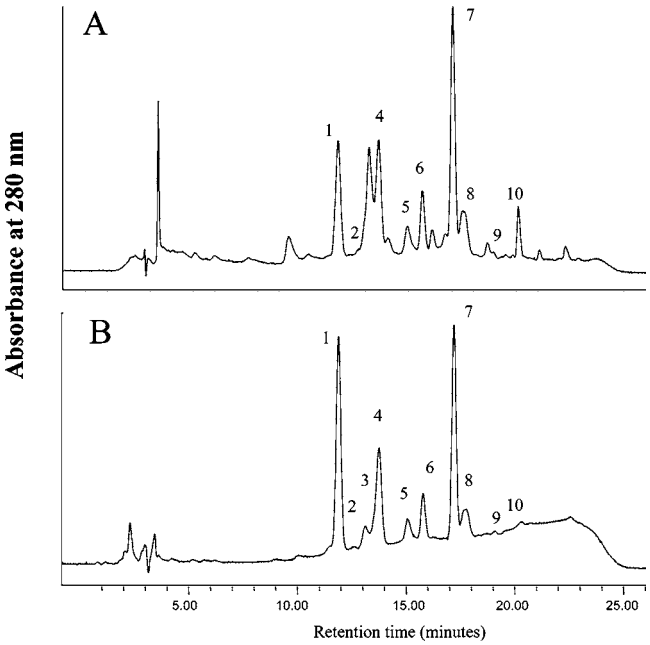

Figure 5. HPLC chromatogram of two fractions, anthocyanin-rich fraction (FIII) (A) and P-II (B), derived from the ARB 5241 genotype extract through purification method 2.

of 0.6379 ($y = 3.053x_1 + 23.006$) for ABTS (y) versus anthocyanin (x_1), whereas for ABTS (y) versus phenolics (x_2), the correlation coefficient was 0.9873 ($y = 6.872x_2 + 35.468$). These results suggest that phenolic compounds were directly responsible of the ABTS antioxidant activity in the anthocyanin-rich fraction. In contrast, anthocyanins present in the anthocyanin-rich fraction were not totally responsible for the overall antioxidant activity. Other remaining phenolic compounds most likely contributed to the ABTS values. This was confirmed by evaluating the anthocyanin-rich fraction in the HPLC−DAD at 280 nm. The presence of other phenolic peaks appeared near the anthocyanin peaks (**Figure 5A**).

A second step was used to further purify the anthocyanin-rich fraction by means of a Sephadex LH-20. The chromatographic profiles are shown in **Figure 6** for the two mashua genotypes. Fractions of 2 mL were collected, and their absorbance was measured at three different wavelengths. The fractions were then assembled in three pools (P-I, P-II, and P-III) on the basis of the absorbance data. As shown in **Figure 4**, the majority of the anthocyanins were recovered in pool P-II as expected from previous studies on anthocyanin purification (*25*, *37*, *38*), with values of 28−95 mg of CGE/100 g of mashua FW (70−78% of recovery). Similar results were found in the fractionation of blueberry and cranberry anthocyanins (67−100% of recovery) using the same method (*25*). In P-I and P-III, recoveries of 6−8 and 3−5% of anthocyanins were obtained, respectively. In a similar study using the same anthocyanin purification process (*25*), phenolic acids were found in P-I and procyanidins were present in P-III. The antioxidant activity was highest in P-III for the AGM 5109 and ARB 5241 genotypes (3.9 and 21.9 μmol of TE/1 g of mashua FW, 39−65%, respectively), whereas it was highest in P-II for the DP 0224 genotype (9.8 μmol of TE/1 g of mashua FW, 44.3%). The recoveries for total phenolics, anthocyanins, and antioxidant activity were 84−86, 86−88, and 94−95%, respectively.

The Sephadex LH-20 profiles at different wavelengths are shown in **Figure 6**. The wavelength of 280 nm was used to detect flavanols and benzoic acids, while wavelengths of 320 nm were used to detect cinamic acids and flavonols and the

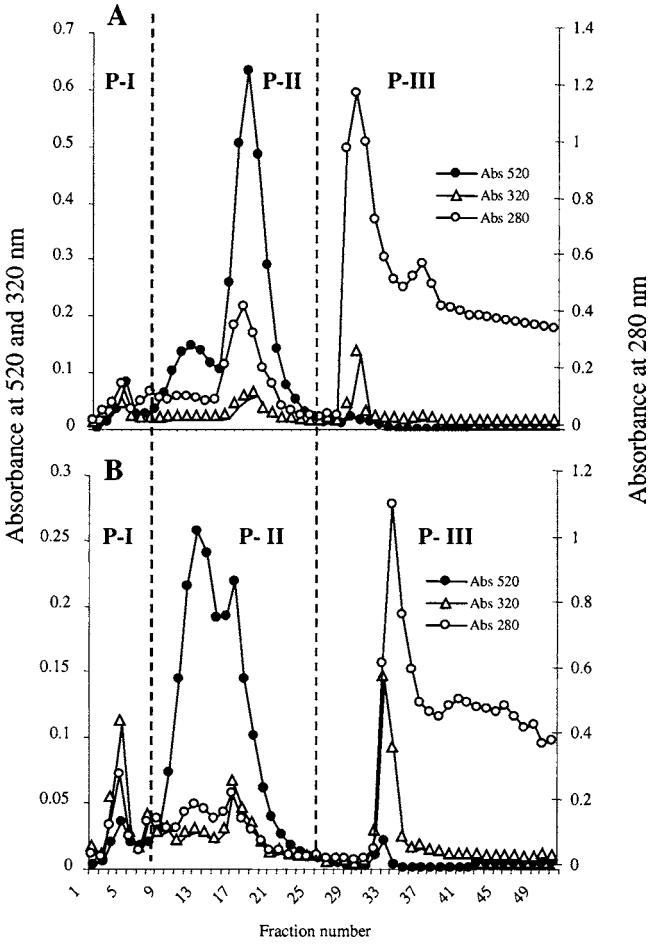

Figure 6. Gel-permation chromatography profiles of phenolic compounds present in the anthocyanin-rich fraction for the mashua genotypes (A) DP 0224 and (B) ARB 5241. (●), Abs 520 nm, corresponds to the anthocyanin recovery in each fraction; (△) and (○) correspond to other phenolic compounds.

wavelength of 520 nm was used to detect anthocyanins. The profile of phenolic compounds in P-II (**Figure 6**) monitored at 280, 320, and 520 nm presented the same trend, which suggested that P-II was mainly composed of anthocyanins. To confirm this result, P-II was tested in the HPLC−DAD at 280 nm. The chromatogram obtained at 280 nm for the ARB 5241 genotype showed the presence of only anthocyanins (**Figure 5B**). When the relationship between anthocyanin or phenolic compounds versus ABTS antioxidant activity was plotted for the P-II, a very good correlation was obtained. The correlation coefficient was 0.9973 ($y = 9.318x_1 + 1.5159$) for ABTS (y) versus anthocyanin (x_1) and 0.9875 ($y = 9.189x_2 + 3.177$) for ABTS (y) versus phenolics (x_2). These results suggest that the phenolics in P-II were mainly anthocyanins and that the antioxidant capacity of this fraction corresponded largely to anthocyanins.

The ABTS values of the P-II fraction (mainly made up of anthocyanins) were 2.7, 9.1, and 9.8 μmol of TE/1 g of mashua FW for the AGM 5109, ARB 5241, and DP 0224 mashua genotypes, respectively, and accounted for up to 26.8, 28.8, and 43.1% of the total ABTS values, after accounting for the losses during anthocyanin purification. These results suggest that anthocyanins contribute quite significantly to the antioxidant activity of the mashua tubers investigated. The anthocyanin contribution to the total antioxidant activity in blueberries, cranberries, chokeberries, and lingonberries has been reported to be 56.3, 54.5, 53.1, and 54.6% [oxygen radical absorbance capacity (ORAC) values], respectively (*21*).

Assuming that the ABTS antioxidant activity in P-II was mostly taken into account, the anthocyanins lead to ABTS values of 10.3, 10.7, and 9.6 μmol of TE/mg of anthocyanin for genotypes DP 0224, ARB 5241, and AGM 5109, respectively. Similar values were thus found among genotypes. Values of 14.8 and 12.6 μmol of TE/mg of anthocyanin (ORAC) have been reported for cyanidin 3-glucoside and delphinidin 3-glucoside, respectively (*21*). Interestingly, the fraction that eluted with 100% methanol (P-III) presented a high content of phenolic compounds (**Figure 4**), and its contribution to the overall antioxidant activity in relation to the type of phenolic compounds should be further studied. Using a Sephadex LH-20 column and 100% methanol elution, Prior et al. (*25*) obtained a procyanidin-rich fraction from berries. Mashua tubers may present the same type of phenolic compounds in the 100% methanol fraction. These unknown compounds appear in any case to be major contributors to the antioxidant activity of the tuber. To our knowledge, there is unfortunately no information available regarding the identification of nonanthocyanin phenolic compounds in mashua tubers.

In conclusion, this is the first study focused on the identification of mashua anthocyanins. The major anthocyanins found in the different genotypes investigated were delphinidin di- and triglycosides acylated with acetic acid. Differences among genotypes were in the proportion of individual pigments, as well in the identity of minor peaks. The sequential use of C_{18} SPE followed by chromatography using Sephadex LH-20 was an efficient technique to fractionate mashua phenolics and purify mashua anthocyanins. Anthocyanins contributed to a significant content to the total antioxidant activity of the mashua tubers, but other phenolics, possibly procyanidins, are also contributors to the antioxidant activity of purple mashua tubers. The phenolic composition of purple mashua tubers should thus be further studied.

ABBREVIATIONS USED

ABTS, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid); TE, Trolox equivalents; FW, fresh weight; GAE, gallic acid equivalents; CGE, cyanidin 3-glucoside equivalents.

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NOTE ADDED AFTER ASAP PUBLICATION

The original posting of August 18, 2006, contained a typographic error in the author name, Betalleluz. The correct version is shown in the posting as of August 21, 2006.

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