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Pharmacokinetics of Anthocyanins and Antioxidant Effects after the Consumption of Anthocyanin-Rich Açai Juice and Pulp (*Euterpe oleracea* Mart.) in Human Healthy Volunteers

Susanne U. Mertens-Talcott,*,†,§ Jolian Rios,# Petra Jilma-Stohlawetz,⊥ Lisbeth A. Pacheco-Palencia,§ Bernd Meibohm,|| Stephen T. Talcott,§ and Hartmut Derendorf⊥

Department of Veterinary Physiology and Pharmacology and Department of Nutrition and Food Science, Texas A&M University, College Station, Texas 77843; Food Science and Human Nutrition Department and Pharmaceutics Department, University of Florida, Gainesville, Florida 32610; and Pharmaceutical Sciences, University of Tennessee, Memphis, Tennessee 38163

The açai berry is the fruit of the acai palm and is traditionally consumed in Brazil but has gained popularity abroad as a food and functional ingredient, yet little information exists on its health effect in humans. This study was performed as an acute four-way crossover clinical trial with açai pulp and clarified açai juice compared to applesauce and a non-antioxidant beverage as controls. Healthy volunteers (12) were dosed at 7 mL/kg of body weight after a washout phase and overnight fast, and plasma was repeatedly sampled over 12 h and urine over 24 h after consumption. Noncompartmental pharmacokinetic analysis of total anthocyanins quantified as cyanidin-3-*O*-glucoside showed C_{max} values of 2321 and 1138 ng/L at t_{max} times of 2.2 and 2.0 h, and AUC_{last} values of 8568 and 3314 ng h L^{-1} for pulp and juice, respectively. Nonlinear mixed effect modeling identified dose volume as a significant predictor of relative oral bioavailability in a negative nonlinear relationship for açai pulp and juice. Plasma antioxidant capacity was significantly increased by the açai pulp and applesauce. Individual increases in plasma antioxidant capacity of up to 2.3- and 3-fold for açai juice and pulp, respectively were observed. The antioxidant capacity in urine, generation of reactive oxygen species, and uric acid concentrations in plasma were not significantly altered by the treatments. Results demonstrate the absorption and antioxidant effects of anthocyanins in açai in plasma in an acute human consumption trial.

KEYWORDS: Anthocyanins; açai berry; *Euterpe oleracea*; antioxidant properties; pharmacokinetics

INTRODUCTION

The intake of polyphenols has been inversely correlated to the incidence of several chronic diseases such as several types of cancer and cardiovascular disease as discussed in several recent studies (*1–3*). Polyphenolics are associated with increased antioxidant potential in plasma and generally show vascular protection (*1, 4*). Along with several antioxidant-rich fruits from around the world, of which absorption, antioxidant, and anti-inflammatory effects have been investigated (*5–10*), açai fruit is gaining popularity in worldwide markets as exports from Brazil increase annually. Common retail products made from açai fruit pulps include frozen treats, mixed fruit smoothies, sweetened pulps, and a variety of beverages featuring açai pulp or clarified açai juice. Açai fruit are obtained from the Amazon River basin and are manually harvested from wild açai palms (*Euterpe oleracea*) native to this area. Açai pulp is historically commonly consumed as a food in the form of a viscous pulp that has been associated with nutritional and medicinal properties including antidiarrheal activity (*11, 12*). However, its high concentrations of antioxidant polyphenolics, primarily from anthocyanin, provide it excellent potential as a functional food ingredient (*13, 14*). The phytochemical composition of açai fruit has been characterized (*15, 16*) revealing a diversity of hydroxybenzoic acids and flavan-3-ols along with cyanidin 3-*O*-

* Address correspondence to this author at the Department of Veterinary Physiology and Pharmacology, Department of Nutrition and Food Science, 415A VMRB, Mail Stop 4466, Texas A&M University, College Station, TX 77843-4466 (e-mail smtalcott@tamu.edu).

† Department of Veterinary Physiology and Pharmacology, Texas A&M University.

§ Department of Nutrition and Food Science, Texas A&M University.

Food Science and Human Nutrition Department, University of Florida.

⊥ Pharmaceutics Department, University of Florida.

|| Pharmaceutical Sciences, University of Tennessee.

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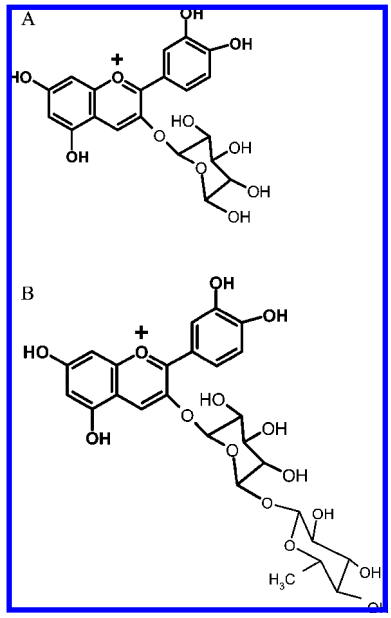

Figure 1. Chemical structures of cyanidin-3-*O*-glucoside (**A**) and cyanidin-3-*O*-rutinoside (**B**).

rutinoside and cyanidin 3-*O*-glucoside as the predominant anthocyanins.

Little evidence is available on the potential health benefits of açai, beyond its in vitro radical scavenging activities. Antioxidant capacity of açai has been demonstrated using different antioxidant assays including the oxygen radical absorbance capacity assay, the peroxynitrite averting capacity, and hydroxyl radical averting capacity. Açai was demonstrated to inhibit the generation of reactive oxygen species and the activity of cyclooxygenases 1 and 2 (*17*). In rats, an açai skin and seed extracts induced endothelium-dependent vasodilation (*18*), while a variety of polyphenolic extracts from açai pulp were shown to exhibit anticarcinogenic effects in an in vitro model with leukemia cells (*19*). In a variety of fruits, anthocyanins were shown to be absorbed intact in their glycosidic forms (*20*). The absorption of anthocyanins from red grape juice and red wine was assessed in nine healthy volunteers and indicated higher plasma absorption from the juice compared to the wine with a corresponding increase in plasma antioxidant capacity (*21*). The excretion of anthocyanins from elderberries and blueberries in elderly women revealed higher urinary excretion levels for major anthocyanins (cyanidin-3-glucoside and cyanidin-3-sambubioside) and four metabolites from elderberry as compared to similar compounds from blueberry. Plasma concentrations of anthocyanins were below the limit of detection in that study (*22*). Anthocyanins in freeze-dries black raspberries were determined in human plasma over a period of 0−12 h (*23*). Once absorbed from the diet, the effect of anthocyanins on plasma and urine antioxidant potential caused a reduction in oxidative cell damage (*24*) and a reduction in exercise-induced oxidative damage (*25*).

Limited information is available on the phytochemical absorption from açai in humans, which lead to this exploratory study on the absorption and kinetics of total anthocyanin absorption from açai pulp and clarified açai juice in relation to an applesauce and non-antioxidant beverage control. Differences in anthocyanin concentration between açai pulp and clarified açai juice were hypothesized to influence the rate and kinetics of absorption in human subjects and influence antioxidant-based biomarkers in plasma and urine.

MATERIALS AND METHODS

Study Subjects. This study was performed after approval by the University of Florida's Institutional Review Board. Subjects gave informed consent before participation in this study. Study subjects were healthy, nonsmoking volunteers recruited at the University of Florida, Gainesville, FL. Subjects were 21−31 years old (mean = 27.2 ± 1.1), with a body mass index range of 17.8−25.9 (mean = 24.6 ± 1.1). Initially 14 subjects were enrolled in this study, but 2 subjects withdrew from the study before starting due to scheduling and time-related aspects. One subject completed only two treatments due to scheduling difficulties. The remaining 11 subjects completed all three food regimens, which included 100% açai pulp, 100% clarified açai juice, and 100% applesauce. Of those 11 subjects, 7 completed the non-antioxidant control beverage study treatment. No adverse events, fatalities, or side-effects were observed for the entire study duration.

Study Treatments. Açai pulp utilized in this study was kindly donated by the Bossa Nova Beverage Group (Los Angeles, CA) from pasteurized, frozen açai pulp imported from Brazil. Chemical analysis of anthocyanins followed the procedures previously described (*26*). Acai pulp, clarified açai juice, and the non-antioxidant control beverage were each adjusted to 10 °Brix with sucrose prior to human consumption to match the soluble solids content of the applesauce. Clarified açai juice was manufactured directly from the açai pulp by centrifugal separation of insoluble solids and lipids from the juice. The recovered juice was filtered through a 1 cm bed of diatomaceous earth to clarify until visually free of lipids and sediment, leaving behind a purple residue of insoluble solids. The non-antioxidant control beverage was manufactured from deionized water and was free of phytochemical compounds. The beverage was likewise sweetened with sucrose, acidified to pH 4.0 with food-grade citric acid, and blended with FD&C artificial food colors to create a purple color that visually matched that of the clarified açai juice. Applesauce was included in the study as an anthocyanin-negative control food matrix which presents a commonly consumed fruit pulp in the United State. Applesauce was obtained commercially from a nationally distributed brand in the United States.

Study Design. Subjects were asked to undergo a 72 h dietary washout phase before each study treatment day. During this washout phase, subjects followed an equi-caloric diet low in antioxidants that excluded a majority of dietary polyphenolic sources. Subjects abstained from the intake of dietary supplements, excessive exercise, sleep deprivation, and the consumption of alcohol. Subjects were asked to fast overnight for at least 8 h prior to beginning of each study day. Subjects obtained 7 mL/kg of body weight of each study treatment. Blood samples were drawn at baseline prior to consumption and 0.5, 1, 2, 4, 6, and 12 h after consumption. Urine samples were collected for the periods 0−3, 3−6, 6−9, 9−12, and 12−24 h after consumption. Subjects were allowed to consume foods low in polyphenolics starting 4 h after the baseline time.

Plasma Concentrations of Anthocyanins. Plasma samples were acidified to pH 2.0 with trifluoroacetic acid and stored at −80 °C until analysis, whereby anthocyanins were extracted and isolated using a reversed phase C18 (OMIX) 96 well extraction plate (Varian, Palo Alto, CA, USA). Anthocyanins were eluted from the plate with 50% methanol and immediately analyzed by HPLC-PDA at 520 nm. Cyanidin-3-*O*-glucoside (Polyphenols Laboratories AS, Sandness, Norway) was used as an external standard for quantification and naringin (Sigma-Aldrich, St. Louis, MO) was used as internal standard. The recovery of anthocyanins was >90% for concentrations between 50 and 5000 ng/L with inter- and intraday variabilities of <3 and <2%, respectively. Sample preparation was conducted at 4 °C and samples were analyzed with an Agilent 1100 system with PDA detection with samples cooled to 4 °C. Samples were separated on a Pursuit XRS C18, 3 μm column, (Varian, Palo Alto, CA), with mobile phase A (water, pH 2.4 acidified with orthophosphoric acid), mobile phase B (70% methanol, 30% water, pH 2.4 acidified with orthophosphoric acid) using a gradient, from 100% −20% of mobile phase A at a flow-rate of 0.4 mL/min with a total analysis time of 20 min. Stability assessment for cyanidin-3-*O*-glycoside indicated stability within the 95th percentile for more than 48 h under the described conditions. Sample analysis was performed within 24 h of samples preparation. The lower limit of quantification (LLOQ) was 20 ng/L.

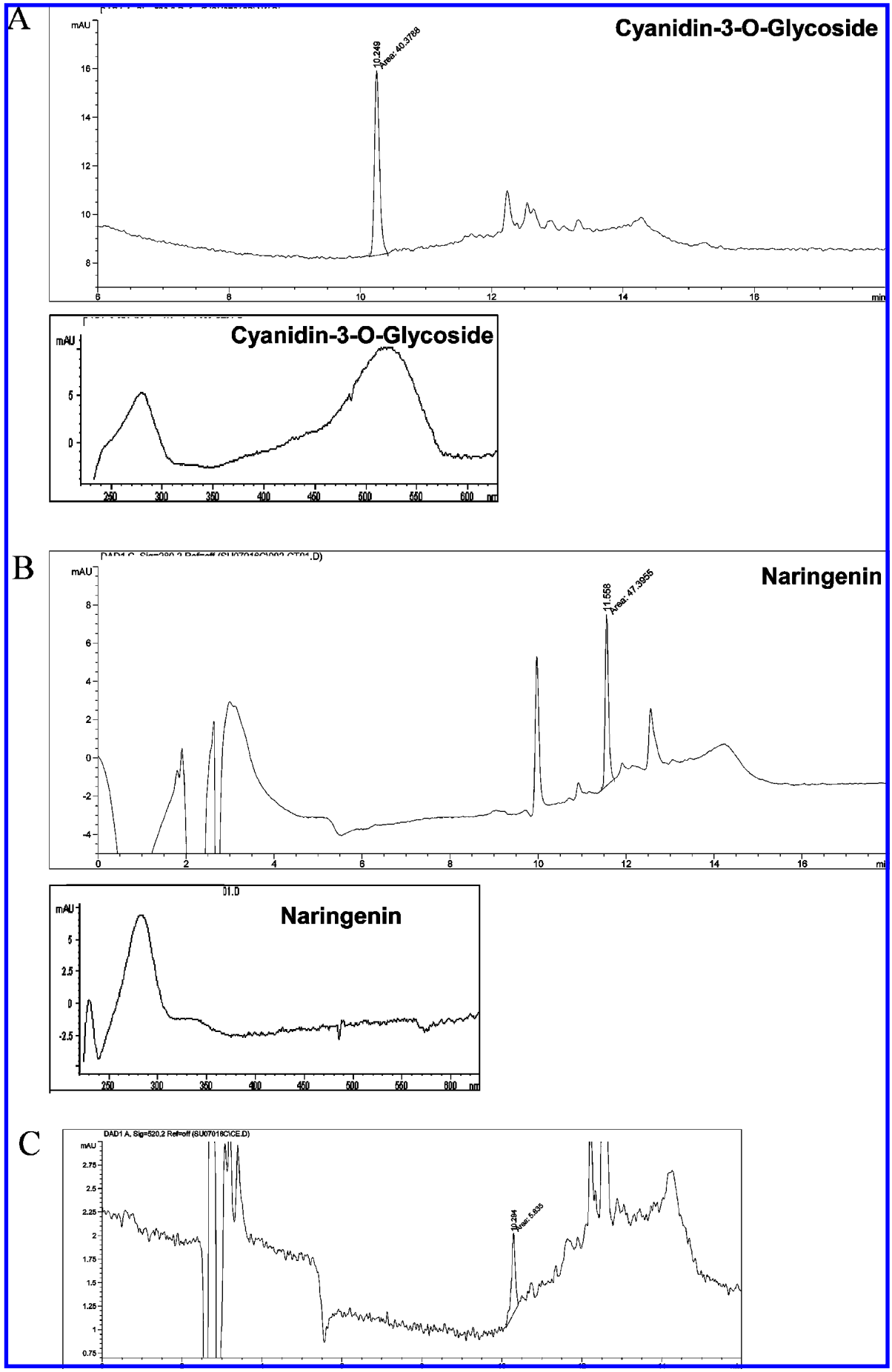

Figure 2. Blank plasma spiked with cyanidin-3-*O*-glucoside (**A**) and naringenin as internal standard (**B**) with compound spectra and plasma concentrations of anthocyanins after the consumption of açai pulp (**C**).

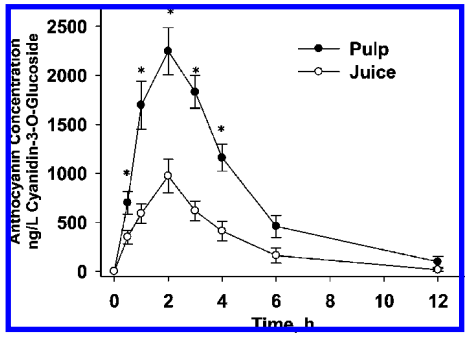

Figure 3. Representative plasma concentration of cyanidin-3-*O*-glucoside after the consumption of açai pulp and juice (subject 8). Asterisks indicate time points at which the anthocyanin concentrations of pulp are significantly different from those of juice, $p \leq 0.05$.

Antioxidant Capacity (ORAC Assay for Plasma and Urine Samples). Plasma and urine samples were acidified 3:1 with 0.44 M TFA, centrifuged, and the deproteinated supernatant diluted with phosphate buffered saline for the ORAC analysis as previously described by Cao et al. and subsequently modified by Ou et al. with fluorescein as fluorescent probe (*27, 28*). Peroxyl radicals were generated by 2,2′-azobis(2-amidinopropane) dihydrochloride and fluorescence monitored at 485 nm excitation and 538 nm emission on a Bio-Tek Synergy KC4 fluorescence plate reader (Bio-Tek Instruments, Winooski, VT).

Generation of Reactive Oxygen Species. In order to determine the extent of intracellular reactive oxygen species (ROS) induced in peripheral blood mononuclear cells, a fluorescence method using dichlorofluorescein-diacetate (DCFH-DA) as the probe was adapted from Wang and Joseph (*29*). Cell concentration was adjusted to 1×10^6 cells/mL. Cells were washed twice with DPBS and incubated with 10 μmol/L DCFH-DA for 30 min at 37 °C in order to preload cells with DCFH-DA substrate. After washing cells, cells were incubated with plasma and with 100 mmol/L hydrogen peroxide. Fluorescence was assessed at 60 min after the incubation on a Bio-Tek Synergy KC4 fluorescence plate reader (Bio-Tek Instruments).

Concentrations of Uric Acid in Plasma. Plasma was acidified with TFA and centrifuged. Supernatant was analyzed by HPLC-PDA at 252 nm as previously described (*30*).

Statistical and Pharmacokinetic Analysis. All values are means ± SEM unless otherwise indicated. Statistical analysis was performed with a one-way analysis of variance ANOVA with a Student's *t* test comparison of all means as posthoc analysis with the JMP software (SAS Institute, Cary, NC). Noncompartmental pharmacokinetic analysis of anthocyanin concentrations in plasma was performed using NCA model 200 of the WINNONLIN 3.1.168 software (Pharsight, Mountain View, CA). Plasma concentrations of anthocyanins were further analyzed by nonlinear mixed effects modeling with the NONMEM software package (v.5, level 1.1, Icon Development Solutions, Ellicott City, MD) using the first-order conditional estimation method with $\eta-\epsilon$ interaction. The concentration-time profile was best described by a one-compartment model with first-order input and lag-time. Between-subject variability was modeled by an exponential error model, residual variability by a combined additive and proportional error model.

The general linear model (GLM) and ANOVA were used to determine any significant predictors for antioxidant capacity (ORAC assay).

RESULTS AND DISCUSSION

Açai Chemical Composition. Açai pulp contained 972 ± 27 mg/kg of total anthocyanins as cyanidin-3-glucoside when exhaustively extracted with an equal volume of methanol, which was useful in solubilizing those anthocyanins bound to the cellular matrix. However, the water-soluble anthocyanins, as reflected during juice clarification, represented only 531 ± 0.2 mg/L indicating a large portion of the anthocyanins were bound to or trapped within the insoluble fiber of the pulp as evidenced from anthocyanins removed during centrifugation and diatomaceous earth filtration. Total anthocyanins by HPLC as a sum of cyanidin glucoside and rutinside (*26*) indicated total anthocyanins of 303.8 mg/kg for açai pulp and 165.9 mg/L for clarified açai juice. The difference between total anthocyanin concentrations quantified using a spectrophotometric assay versus HPLC are largely due to matrix effects that impact the visual color of açai juice, such as the presence of copigments and a nonhomogenous anthocyanin content. Additionally, the difference in anthocyanin concentrations between açai pulp and clarified juice demonstrate that anthocyanin concentrations can be increased in juices with food processing procedures that disrupt the insoluble solids matrix to liberate additional anthocyanins.

Table 1. Pharmacokinetic Parameters of Noncompartmental Analysis[a]

	pulp		juice	
	mean	SE	mean	SE
t_{max}	2.17	0.11	2.00	0.22
C_{max}	2321.35	230.30	1138.51	142.19
$t_{1/2}$	6.56	3.05	3.00	0.56
AUC_{last}	8568.30	972.08	3314.04	604.71
MRT_{last}	2.98	0.26	2.50	0.22

[a] t_{max} = time of maximum concentration; C_{max} = peak plasma concentration (ng/L); AUC_{last} (ng h L^{-1}) = area under the curve from 0 to 12 h; MRT = mean residence time.

Plasma Concentrations of Anthocyanins. Anthocyanins in plasma were quantified as cyanindin-3-*O*-glucoside using naringin as an internal standard (**Figure 2**). The oral dose of study treatments were adjusted to body weight at 7 mL/kg of body weight. Plasma concentrations were determined for all study treatments; however, no measurable concentrations were detected for the non-antioxidant control beverage or the applesauce (data not shown). Plasma anthocyanin concentrations were determined over a period of 0−12 h (**Figure 3**). The noncompartmental analysis (**Table 1**) determined a time of maximum concentration (t_{max}) of 2.2 and 2.0 h, maximum concentration (c_{max}) of 2321 and 1138 ng/L, half-life ($t_{1/2}$) of 6.56 and 3.00 h, and area under the curve$_{last}$ (AUC_{last}) of 8568 and 3314 ng h L^{-1}, respectively for açai pulp and clarified açai juice. C_{max} and AUC_{last} after the consumption of clarified açai juice was 49 and 39% of the respective values after the consumption of açai pulp. Overall, the anthocyanin concentrations for pulp were significantly higher than for clarified juice for time points from 0.5−4 h and as determined by HPLC and spectrophotometrically were 54% higher, indicating that anthocyanins bound or trapped within the water-insoluble cellular matrix of the pulp were liberated in the intestinal tract and were available for absorption as indicated by the slightly higher t_{max} for açai pulp compared to clarified açai juice.

Nonlinear mixed effect modeling (NONMEM) indicated a very low oral bioavailability of anthocyanins as demonstrated by large, supra-physiological values for both, oral clearance (CL/F) and volume of distribution corrected for bioavailability (V/F) (**Table 2**). The between-subject variability in oral clearance was 40.4% and at that relatively high, and was smaller for volume of distribution corrected for bioavailability (25.4%). The proportional term of the residual variability indicated a value of 32%, the additive term a standard deviation of 145 ng/mL.

Age, weight, height, BMI, gender, treatment (pulp vs juice) and dose volume were evaluated as potential predictors for pharmacokinetic parameters. Dose volume was identified as a significant predictor of relative oral bioavailability in a negative nonlinear relationship, with lower bioavailability at higher dose

Table 2. Nonlinear Mixed Effect Analysis, Estimates for Typical Values of Model Parameters, and Their Between-Subject Variability[a]

parameter	abbrev	units	estimate	rel SE (%)	BSV (% CV)
oral clearance	CL/F	L/h	19300	12.8	40.4
volume of distribution	V/F	L	45400	13.0	25.4
absorption rate constant	K_a	h^{-1}	1.05	17.8	
lag time	t_{lag}	h	0.288	10.9	

[a] BSV = between-subject variability; CV = coeeficient of variation; SE = standard error.

volumes. The relationship was modeled as $F = 1 \times (\text{DVol}/525)^{\theta}$, where F is the relative oral bioavailability, DVol is the dose volume, 525 is the median dose volume in mL, and θ is the estimated exponential factor of −1.14 (95% confidence interval −2.16 to −0.125). No other of the investigated covariates was found to be a predictor for any of the model parameters.

Previously, absorption pharmacometric parameters of anthocyanins were investigated after administering a dose of 45 g of freeze-dried raspberries containing 141 mg of cyanidin-3-*O*-glucoside (among other anthocyanins), including C_{max}, t_{max}, AUC, and $t_{1/2}$, after (*23*). After the consumption of the study treatment, an AUC_{last} of 6910 ng h/L, a C_{max} of 2150 ng/L, t_{max} of 1.09, and $t_{1/2}$ of 2.52 h were observed for cyanidin-3-*O*-glucoside. In this study the average dose of anthocyanins was 145 mg for the pulp and 110 mg for the juice, where the resulting pharmacokinetic data are within a similar range for AUC_{last} and C_{max} compared to the results for cyanidin-3-*O*-glucoside in the previously performed study using freeze-dried raspberries. t_{max} and $t_{1/2}$ in our study are higher in comparison, potentially caused by the different nature of the study treatment, juice and pulp vs a freeze-dried fruit product. Overall, the absorption of anthocyanins from fruits have been demonstrated in other human clinical trials, where pharmacokinetics were comparable with results found in this study (*31–33*).

Antioxidant Capacity in Plasma and Urine. Subjects were allowed to consume food after the 4 h blood draw therefore the analysis in plasma was only performed from 0 to 4 h, whereas the urine was collected and analyzed over 24 h. The antioxidant capacity of plasma indicated a high intersubject variability, which was decreased when the ratio plasma/antioxidant capacity (each time point to baseline at 0 h) was divided by the dose volume (L) administered to each patient. The rationale for this is based on our finding that dose volume was a negative correlative predictor for oral bioavailability (**Figure 4A**) and expressed as the ratio of Trolox-equivalents to baseline divided by dose-volume in L. The individual increase in antioxidant capacity was up to 3-fold for the açai pulp and up to 2.3-fold for the clarified açai juice. The t_{max} was reached after 3 h for applesauce, clarified açai juice, and açai pulp and 2 h for the non-antioxidant control beverage. The non-antioxidant control-beverage also caused an increase in the antioxidant capacity in plasma compared to the baseline, potentially based on contained fructose as previously reported (*34, 35*). One-way analysis of variance and repeated measures analysis for 0.5−4 h revealed that all treatments caused a significant ($p < 0.01$) increase in plasma antioxidant capacity compared to the non-antioxidant control beverage. Moreover, both applesauce and açai pulp induced significantly higher plasma antioxidant activities than açai juice ($p < 0.05$).

Antioxidant capacity of urine expressed in Trolox equivalents per liter were divided by urine volume (liters) and by the time period over which urine was collected and results expressed as micromoles of Trolox equivalents per hour. Results from urine

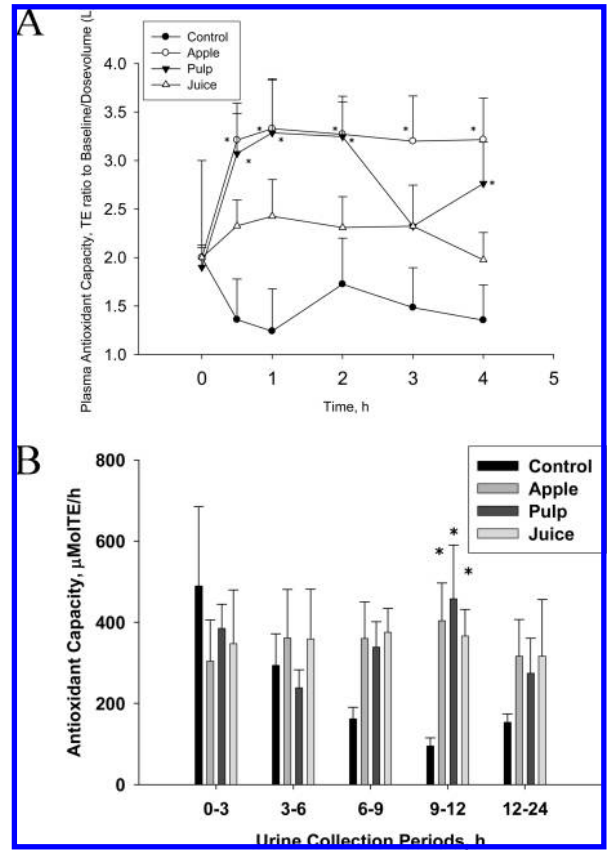

Figure 4. Antioxidant capacity in plasma (**A**) and urine (**B**) after the consumption of açai pulp and juice. Asterisks indicate time points at which the anthocyanin concentrations of treatments are significantly different from the control, $p \leq 0.05$.

analysis demonstrated that the antioxidant treatments were not significantly different across time overall. However, the antioxidant capacity for all treatments were significantly higher than the control at 9−12 h.

Previous studies have demonstrated an increase in plasma antioxidant capacity induced by antioxidant fruits or fruit or vegetable juices (*36–38*), yet other studies report a lack of antioxidant effects after the consumption of antioxidant fruit preparations (*39, 40*). These differences may in part be due to differences in methodology of antioxidant assays, administered amounts of antioxidants, as well as selection of a study population. In our study, the plasma antioxidant effects of açai were clearly demonstrated.

The antioxidant capacity in urine was not significantly increased after the consumption of antioxidant study treatments in relation to the non-antioxidant control. Previous studies have demonstrated an increase in urine antioxidant capacity after the consumption of antioxidant fruit preparations (*41*), where the amounts given were comparable to those administered in this study. Overall, antioxidant effects were observed in plasma induced by the açai pulp, a higher dose may be necessary in order to observe an increase in antioxidant capacity in urine.

Uric Acid Concentrations. Based on previous publications where the consumption of fructose containing foods has been demonstrated to increase plasma antioxidant potential (*34, 35*), we hypothesized that the consumption of sugar and fructose-containing study treatments such as apple sauce, non-antioxidant control beverage and the açai treatments would increase the plasma antioxidant potential based on increased uric acid concentrations in plasma. Applesauce was used as a fruit pulp in order to compare the antioxidant effects to açai pulp. Apples

naturally contain around 6−9% of fructose (*42*), where the concentrations in açai are reported to be 0.4 g in 100 g freeze-dried berries (*15*) and therefore present in very low concentrations (<0.1%) in fresh açai pulp and clarified açai juice. The total sugar content of all study treatments was normalized with sucrose, which ensured that all treatments were comparable in their fructose content. Uric acid concentrations were determined in plasma for the period of 0−12 h using HPLC-PDA. Results indicated, that concentrations were not significantly different between any of the study treatments(data not shown), which indicates that fructose overall may not have been a significant contributor to the differences in antioxidant effects observed between treatments in this study.

Generation of Reactive Oxygen Species (ROS). The generation of ROS was determined in peripheral blood mononuclear cells (PBMC) collected from each patient over 0−4 h blood sampling points. PBMC were incubated with 10% autologous plasma and treated with 100 mM hydrogen peroxide for 30 min. The generation of ROS was not significantly decreased by any of the study treatments (data not shown). Previously, polyphenols have been demonstrated to increase as well as decrease the generation of ROS (*43–45*). The concentrations of antioxidant polyphenolics in plasma did not seem to be high enough to reduce the hydrogen-induced generation of ROS.

In summary, anthocyanins from açai are bioavailable in healthy human volunteers upon the consumption of açai juice and pulp in moderate amounts and açai. Acai pulp caused a significant increase in the antioxidant capacity of plasma, which indicates the in vivo antioxidant potential of açai. Follow-up studies, investigating the metabolism of açai polyphenolics as well as further studying the antioxidant properties and health benefits are necessary to demonstrate the health benefits of açai.

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