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Use of Green Rooibos (*Aspalathus linearis*) Extract and Water-Soluble Nanomicelles of Green Rooibos Extract Encapsulated with Ascorbic Acid for Enhanced Aspalathin Content in Ready-to-Drink Iced Teas

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Heat-induced changes in aspalathin, iso-orientin, and orientin content of ready-to-drink (RTD) green rooibos iced tea formulations were investigated. An organic-solvent-based aspalathin-enriched extract prepared from green rooibos was used "as-is" or encapsulated with ascorbic acid in a water-soluble nanomicelle-based carrier system. The common iced tea ingredients, ascorbic acid, and/or citric acid were added to the iced tea containing green rooibos extract. Only citric acid was added to the iced tea containing the nanomicelles. Heat treatments consisted of pasteurization (93 °C/5 min and 93 °C/30 min), normal-temperature sterilization (NTS; 121 °C/15 min), and high-temperature sterilization (HTS; 135 °C/4 min). Pasteurization had little or no effect on the flavonoid content. NTS and HTS induced significant losses in the flavonoids. The addition of citric and ascorbic acids improved the stability of the flavonoids, but encapsulation of green rooibos extract with ascorbic acid in nanomicelles did not offer additional stability. The only benefit of using the water-soluble nanomicelles was the improved clarity of the RTD product. Iso-orientin and orientin contents were substantially less affected than aspalathin by the heat treatments, partially because of conversion of aspalathin to these flavones, which countered losses. 5-Hydroxymethylfurfural (HMF), a known dehydration product of hexoses under mild acidic conditions and also a degradation product of ascorbic acid, was observed in formulations containing citric and/or ascorbic acids.

KEYWORDS: Green rooibos; *Aspalathus linearis*; aspalathin; iso-orientin; orientin; iced tea; heat processing; nanomicelles

INTRODUCTION

Dietary flavonoids are considered major functional ingredients, because of their potential beneficial role in the "health and wellness" of consumers. Stability of functional ingredients in food products and beverages is of the utmost importance to ensure retention of their beneficial effects. Recent years has seen a growing market for ready-to-drink (RTD) iced teas, including products containing extracts of the herbal tea, rooibos. High levels of the dihydrochalcone *C*-glucoside, aspalathin (**Figure 1**), present in the rooibos plant (*Aspalathus linearis*) (*1*) has led to the production of aspalathin-enriched extracts, offering a means to deliver products with enhanced aspalathin content. Aspalathin is a potent antioxidant (*2*−*5*) with antimutagenic properties (*6*). In addition, recent studies demonstrated the *in vivo* glucose-lowering ability for aspalathin (*7*,*8*), furthering interest in this compound as a nutraceutical ingredient in food and beverage products.

"Fermented" (oxidized) rooibos is the most common form used by the food industry as herbal tea or in products such as RTD iced teas and yoghurt. The oxidation process, necessary for development of the characteristic rooibos flavor and color, however, greatly reduces the aspalathin content of the plant material (*9*) and leads to the formation of the two flavones, iso-orientin and orientin, as major products (**Figure 1**) (*10*). Aqueous spray-dried extracts of fermented rooibos usually contain less than 0.5% aspalathin (*11*,*12*). Knowledge of its stability during food and beverage processing is limited to our previous investigation on RTD rooibos iced tea, prepared with fermented rooibos extract (*12*). Sterilization required for RTD fermented rooibos iced tea with an extended shelf life was shown to be detrimental to the retention of aspalathin. Losses of between 10 and 78%, depending upon the severity of the heat treatment and the presence of other food ingredients, such as ascorbic and citric acids, were demonstrated (*12*). Commercial RTD rooibos iced tea products containing fermented rooibos extract were found to contain little or no aspalathin (*12*), indicating that the product should be improved in terms of its aspalathin content.

An alternative strategy is thus required to achieve higher levels of aspalathin in the final RTD product. The obvious strategy would be to use a green rooibos extract instead of a fermented rooibos extract because substantially higher aspalathin levels could be realized in the green rooibos extract (*13*). However,

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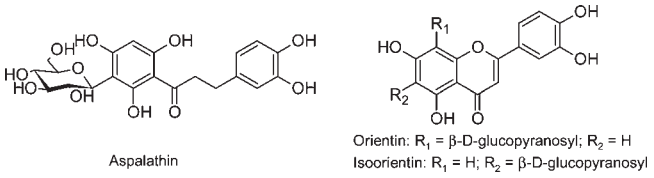

Figure 1. Structures of aspalathin, iso-orientin, and orientin.

the qualitative and quantitative phenolic composition of green rooibos extract, especially its high aspalathin content as opposed to fermented rooibos extract, could potentially lead to pro-oxidative conditions and, thus, destabilization of aspalathin. The use of ascorbic acid in the product formulation and the presence of trace metal ions, originating from the plant material, could lead to pro-oxidative conditions (*13*). Oxidation of ascorbic acid would lead to the formation of hydrogen peroxide (H_2O_2) (*14*) and, subsequently, hydroxyl radicals ($^{\bullet}OH$) through the Fenton reaction (*15*). Another potential source of H_2O_2 and, thus, $^{\bullet}OH$ in a RTD iced tea is the packaging. Trace quantities of H_2O_2 remain after sterilization of the packaging with H_2O_2. The Food and Drug Administration (FDA) regulation 21 Code of Federal Regulations (CFR) 178.1005 currently limits the residual H_2O_2 concentration to 0.5 mg/L.

The main focus of the present study was, thus, to determine the heat stability of aspalathin in RTD iced tea prepared with an organic-solvent-based aspalathin-enriched green rooibos extract. The availability of aspalathin-enriched green rooibos extract encapsulated with ascorbic acid in a water-soluble nanomicelle carrier system provided the opportunity to determine whether the stability of aspalathin could be improved when applied in this form in the RTD iced tea. The question of whether pro-oxidative conditions would play a role in aspalathin stability was addressed by adding a small quantity of H_2O_2 to some formulations. Heat treatments consisted of two pasteurization and two sterilization regimes. A low acidic product, such as RTD iced tea without added citric acid, requires sterilization to ensure a long shelf life. For the purpose of this study, in-container sterilization as opposed to continuous-flow sterilization was used to evaluate the stability of aspalathin at extreme heat conditions.

MATERIALS AND METHODS

Chemicals and Water Purification. Ascorbic acid and deuterated solvents (C_3D_6O and D_2O) were purchased from Sigma Chemical Co. (St Louis, MO). BDH acetonitrile ("far UV" for gradient analysis) was purchased from Merck (Darmstadt, Germany). H_2O_2 solution (30%, w/v) was purchased from Saarchem (Gauteng, South Africa). All other solvents were analytical-grade. Aspalathin (>95% purity), isolated from green rooibos, was obtained from the PROMEC Unit of the Medical Research Council (Parow, South Africa). Extrasynthese (Genay, France) and Carl Roth (Karlsruhe, Germany) supplied iso-orientin and orientin, respectively. Tap water was purified to laboratory grade for the preparation of the different beverage formulations by serial carbon, reverse osmosis, and deionizer treatment (Continental Water Systems Corporation, San Antonio, TX). High-performance liquid chromatography (HPLC)-grade water was obtained using a Milli-Q academic water purifier (Millipore, Bedford, MA). Food-grade ingredients for the preparation of rooibos iced tea comprised sucrose (Huletts, Rossburgh, South Africa) and citric acid (Warren Chem Specialties, Cape Town, South Africa).

Green Rooibos Extracts and Product Formulation. Powdered green rooibos extract, enriched in aspalathin through organic solvent extraction (*16*), was supplied by the Raps Foundation (Freising-Weihenstephan, Germany) for preparation of RTD beverages of three different formulations. These formulations were similar to those previously described for RTD beverages prepared from hot water extract of fermented rooibos (*12*). The formulations contained (1) green rooibos extract (E), (2) extract and citric acid (EC), and (3) extract, citric acid, and ascorbic acid (ECA).

Table 1. Aspalathin, Orientin, and Iso-orientin Contents of Green Rooibos Extract,[a] Solubilizate,[b] and Their Respective Formulations

compound	green rooibos extract[c]	formulation containing green rooibos extract[d]	solubilizate[c]	formulation containing solubilizate[d]
aspalathin	20.70	362.3	3.08	431.2
orientin	1.39	24.3	0.20	28.0
iso-orientin	1.55	27.1	0.21	29.4

[a] Aspalathin-enriched. [b] Aspalathin-enriched green rooibos extract and ascorbic acid encapsulated in a water-soluble nanomicelle carrier system. [c] g/100 g. [d] mg/L.

Formulations EC and ECA contained 1.2 g/L citric acid, while formulation ECA also contained 0.2 g/L ascorbic acid. All formulations contained 60 g/L sugar and 1.75 g/L green rooibos extract.

To test the stability of aspalathin when encapsulated with ascorbic acid, a water-soluble nanomicelle-based carrier system ("solubilizate") (NovaSol, Aquanova, Darmstadt, Germany), containing ca. 15% aspalathin-enriched green rooibos extract and 5% ascorbic acid (m/m), was used. The core of the micelle contained the extract and ascorbic acid, while its shell was formed by polyoxyethylene sorbitan monolaurate. The stability of two RTD beverage formulations was investigated. Both formulations contained 60 g/L sugar and 14 g/L solubilizate (equivalent to 2.1 g/L of green rooibos extract and 0.7 g/L ascorbic acid), which formed the base (NA). Formulation NAC also contained 1.2 g/L citric acid. The pH of all RTD formulations was determined, using a pH meter (Crison Instruments SA, South Africa). The iron content of the extract and solubilizate was determined by Bemlab (Somerset West, South Africa). The composition of the extracts and formulations in terms of aspalathin and its flavone analogues are given in **Table 1**.

Heat Treatment. The formulations were subjected to pasteurization, "normal"-temperature sterilization (NTS), and high-temperature sterilization (HTS), as previously described (*12*). For pasteurization, aliquots of the samples, sealed in 25 mL screw-cap glass vials, were heated in a water bath at 93 °C for either 5 or 30 min. Samples achieved the target temperature after ca. 2 min. For the NTS and HTS treatments, aliquots were sealed in 20 mL gas chromatography headspace vials. The NTS treatment included autoclaving the samples at 121 °C for 15 min, whereas the HTS treatment entailed autoclaving at 135 °C for 4 min. In both cases, the samples were in the autoclave for approximately 45 min, because of the duration of the heating and cooling cycles of the equipment. The samples were immediately placed on ice for 30 min, following their removal from the water bath and autoclave. Samples of all heat treatment × formulation combinations were taken before (control) and after heat treatment to determine the changes introduced by heating.

Hydrogen Peroxide. For this experiment, two green rooibos extract formulations were prepared, i.e., (1) extract and ascorbic acid (EA) and (2) extract, ascorbic acid, and citric acid (EAC). In the case of the solubilizate, the two formulations contained, respectively, (1) the solubilizate (NA) and (2) a mixture of the solubilizate and citric acid (NAC). No ascorbic acid was added because it was already present in the solubilizate. All other quantities were the same as for the heat treatment experiment, except that no sugar was added. H_2O_2 was added to all formulations to give a final concentration of 0.5 mg/L. The controls of each formulation contained no added H_2O_2. The reaction with H_2O_2 was allowed to proceed for 1 h at room temperature, wherafter aliquots were frozen for analysis at a later stage.

Absorbance Measurement. Browning of the iced tea samples was quantified in terms of the increase in absorbance at 420 nm (*17*) relative to a control (unheated). Samples were centrifuged at 18000 rpm for 4 min (Hettich Universal 16 centrifuge, Hettich, Tuttlingen, Germany) to remove any precipitate. Absorbance of 200 μL aliquots of the supernatants was then measured in 96-well round-bottom plates using a Biotek Synergy HT multiplate reader (Biotek Instruments, Winooski, VT).

HPLC Quantification of Aspalathin and Flavone Analogues. Quantification of the individual flavonoids in the extract, solubilizate, and RTD samples was performed by HPLC−diode array detection (DAD), as described previously (*12*). Separation took place on a 150 × 4.6 mm inner diameter, 5 μm, Agilent Zorbax Eclipse XDB-C18 column, protected with a 4 × 4 mm inner diameter RP/C18 guard column (Vici-AG International, Schenkon, Switzerland), using an Agilent 1200 series HPLC

system (quaternary pump, column thermostat, diode array detector, and Chemstation software for LC 3D systems). The mobile phases, i.e., (A) 2% acetic acid and (B) acetonitrile, were used in the following gradient: 0−10 min, 18−20% B; 10−13 min, 20−80% B; 13−16 min, 80−18% B; and 16−23 min, 18% B. The flow rate and column temperature were maintained at 0.8 mL/min and 38 °C, respectively. Injection volumes, adjusted according to the initial aspalathin content, were as follows: 5 μL of reconstituted extract, diluted solubilizate, and RTD formulations containing green rooibos extract, 3 μL of the RTD formulations containing the solubilizate, and 20 μL of the calibration standards. Chromatograms were recorded at 288 and 350 nm for quantification of the dihydrochalcone and flavones, respectively. Peaks were identified according to retention time and spectroscopic characteristics. A five-point calibration series was employed for each of the compounds.

Liquid Chromatography−Electrospray Ionization−Mass Spectrometry (LC−ESI−MS) and −Tandem Mass Spectrometry (MS/MS) Analyses. One sample each of the green rooibos formulation ECA subjected to HTS and a standard mixture of aspalathin, iso-orientin, and orientin were analyzed by LC−ESI−MS to confirm peak identity and purity. The same column and gradient as for HPLC−DAD were used, except that 2% acetic acid was replaced with 0.1% formic acid to improve ionization. The LC−ESI−MS system consisted of a Waters Acquity Ultra Performance LC system (Waters, Milford, MA) with a quaternary pump and an autosampler and a Waters API QTOF Ultima MS detector. Electrospray ionization in the positive mode was performed at the following settings: desolvation temperature, 350 °C; nitrogen flow rate, 350 L/h; source temperature, 100 °C; capillary voltage, 3.5 kV; cone voltage, 35 V; and collision energy, 5 arbitrary units. The settings for negative ionization were as follows: desolvation temperature, 370 °C; nitrogen flow rate, 370 L/h; source temperature, 100 °C; capillary voltage, 3.7 kV; cone voltage, 35 V; and collision energy, 5 arbitrary units. LC−ESI−MS/MS was performed at a collision energy setting of 20 arbitrary units.

Isolation of Compound 1. A total of 2 L of green rooibos iced tea (formulation ECA) was autoclaved for 40 min at 121 °C. An enriched fraction was prepared by liquid−liquid fractionation of the extract with six portions of 1 L ethyl acetate. The ethyl acetate fractions were pooled, evaporated under vacuum, and freeze-dried. Semi-preparative reversed-phase HPLC separation was performed on the Agilent 1200 series HPLC equipped with a fraction collector. Separation took place on a Gemini C18 (150 × 10 mm inner diameter; 5 μm particle size; 110 Å pore size) column, protected by a guard cartridge (10 × 10 mm inner diameter) with the same stationary phase (Phenomenex, Torrance, CA). The previous gradient used for analysis of the samples was adapted to shift the peak of compound **1** further from the void volume to allow for a higher loading with injection: 0−7 min, 3% B; 7−8 min, 3−50% B; 8−10 min, 50% B; 10−11 min, 50−3% B; and 11−21 min, 3% B, with (A) 0.1% formic acid and (B) acetonitrile as mobile phases. The flow rate and column temperature were maintained at 4.7 mL/min and 30 °C, respectively. The ethyl acetate fraction (1.02 g) was dissolved in dimethyl sulfoxide (DMSO) and diluted with water (1:4) to ca. 20 mg/mL. The solution was filtered through 0.45 μm Millex-HV hydrophilic polyvinylidene fluoride (PVDF) syringe filters (Millipore), and 100 μL was injected repeatedly. The peak corresponding to compound **1** was collected, and the fractions of repeated runs were pooled, evaporated under vacuum, and freeze-dried. Approximately 19 mg of compound **1** (purity by HPLC = 95%) was recovered and subjected to structure elucidation.

Gas Chromatography−Mass Spectrometry (GC−MS) Analysis. One sample each of the green rooibos extract formulations E, EC, and ECA and an aqueous solution of compound **1** were extracted with dichloromethane (1 mL sample extracted twice with 250 μL of dichloromethane). GC−MS analysis using electron impact ionization (positive mode) of the dichloromethane fraction was performed on a 30 m × 0.25 mm inner diameter, 0.25 μm film thickness, HP5 column (J&W Scientific, Folsom, CA), with a Waters GCT Premier GC−time-of-flight (TOF) apparatus. The oven temperature gradient was as follows: 40 °C for 5 min, 40−300 °C at a rate of 10 °C/min, and hold for 2 min. Other operational parameters were as follows: injector temperature, 260 °C; split ratio, 1:10; carrier gas, helium (1 mL/min); MS transfer line temperature, 250 °C; ionization energy, 70 eV; scanning mass range, m/z 35−500 (perfluorotri-*N*-butylamine as a mass reference).

^{1}H and ^{13}C Nuclear Magnetic Resonance (NMR) Analysis. Compound **1** was dissolved in C_3D_6O/D_2O and filtered through glass wool prior to analysis using a Varian ^{Unity}*Inova* 600 NMR spectrometer at a ^{1}H frequency of 600 MHz and a ^{13}C frequency of 150 MHz. A 5 mm inverse detection PFG probe was used to collect spectra. ^{1}H NMR spectra were referenced to the residual acetone peak at 2.05 ppm, and the ^{13}C spectra were referenced to the residual acetone peak at 29.9 ppm. ^{1}H and ^{13}C NMR data are consistent with those previously reported (*18*) for 5-hydroxymethylfurfural. ^{1}H NMR (C_3D_6O/D_2O, 25 °C) δ: 9.48 (s, 1H, CHO), 7.40 (d, J = 3.5 Hz, 1H, H-3), 6.57 (d, J = 3.5 Hz, 1H, H-4), 4.56 (s, 2H, H-6). ^{13}C NMR δ: 179 (s, C-1), 163 (s, C-2), 153 (s, C-5), 125 (s, C-3), 111 (s, C-4), 57 (s, C-6). GC−MS−EI = 126.03; MW calculated, 126.12 g/mol.

Statistical Analysis. All treatments were replicated 4 times. The actual values for the compounds and the percentage change in these values relative to their respective controls were subjected to analysis of variance (ANOVA), the Shapiro−Wilk test for normality ($p \geq 0.05$), and the Student t test, using SAS, version 9.3 (SAS Institute, Cary, NC). Values differing at the 5% level ($p < 0.05$) were considered significant.

RESULTS

Green Rooibos Extract. The pH values of the three iced tea formulations E, EC, and ECA (before heating) were 4.40, 2.80, and 2.80, respectively. A trace quantity of iron (94 mg/kg) was present in the extract, equaling 165 μg/L in the beverage. The extract did not fully dissolve, giving a slightly turbid solution that formed a fine precipitate upon standing.

Pasteurization for 5 min increased the absorbance at 420 nm of formulation E ($p < 0.05$) by 12.9%, while formulations EC and ECA were not affected. When subjected to a 30 min pasteurization period, the absorbance of all three formulations increased in excess of 50%. An even higher increase in absorbance was observed for the NTS and HTS treatments (**Figure 2A**).

Pasteurization for 5 min had no significant ($p \geq 0.05$) effect on the flavonoid content of the different formulations (panels **B−D** of **Figure 2**). The combination of citric and ascorbic acids (formulation ECA) subjected to 30 min of pasteurization produced an iced tea with a slightly (2.5%) but significantly higher ($p < 0.05$) aspalathin content than the control. However, the aspalathin content of formulation EC and the iso-orientin and orientin contents of formulations EC and ECA remained unaffected. While the impact of pasteurization on the flavonoids was minimal, even at the extended period of 30 min, both sterilization treatments led to substantial decreases in the aspalathin content of all formulations, ranging between 10 and 38% (**Figure 2B**). The retention of aspalathin was the lowest in formulation E, containing only green rooibos extract and sucrose as ingredients. The addition of ascorbic and/or citric acids improved aspalathin retention significantly ($p < 0.05$) during the NTS and HTS treatments. The addition of both citric and ascorbic acids was more effective than citric acid alone. The decrease in iso-orientin content of formulation E (NTS) was more pronounced than that for formulations EC and ECA and similar ($p \geq 0.05$) to that of the formulations subjected to HTS (**Figure 2C**). Overall, the percentage loss of this compound was less than that of aspalathin, irrespective of the heat treatment × formulation combination. Orientin was even less affected by heat treatment and, contrary to iso-orientin its content, remained stable in formulation E of both the NTS and HTS treatments (**Figure 2D**). The addition of ascorbic acid to formulation EC had no additional stabilizing effect on orientin as that achieved by citric acid alone (ECA versus EC).

The NTS and HTS treatments of the EC and ECA formulations resulted in the formation of a very prominent new peak (designated compound **1**; λ_{max}, 284), with a retention time of 2.6 min on the HPLC−DAD chromatogram (**Figure 3**). The same peak also appeared on the chromatograms of formulations EC and ECA subjected to pasteurization for 30 min. On the basis of

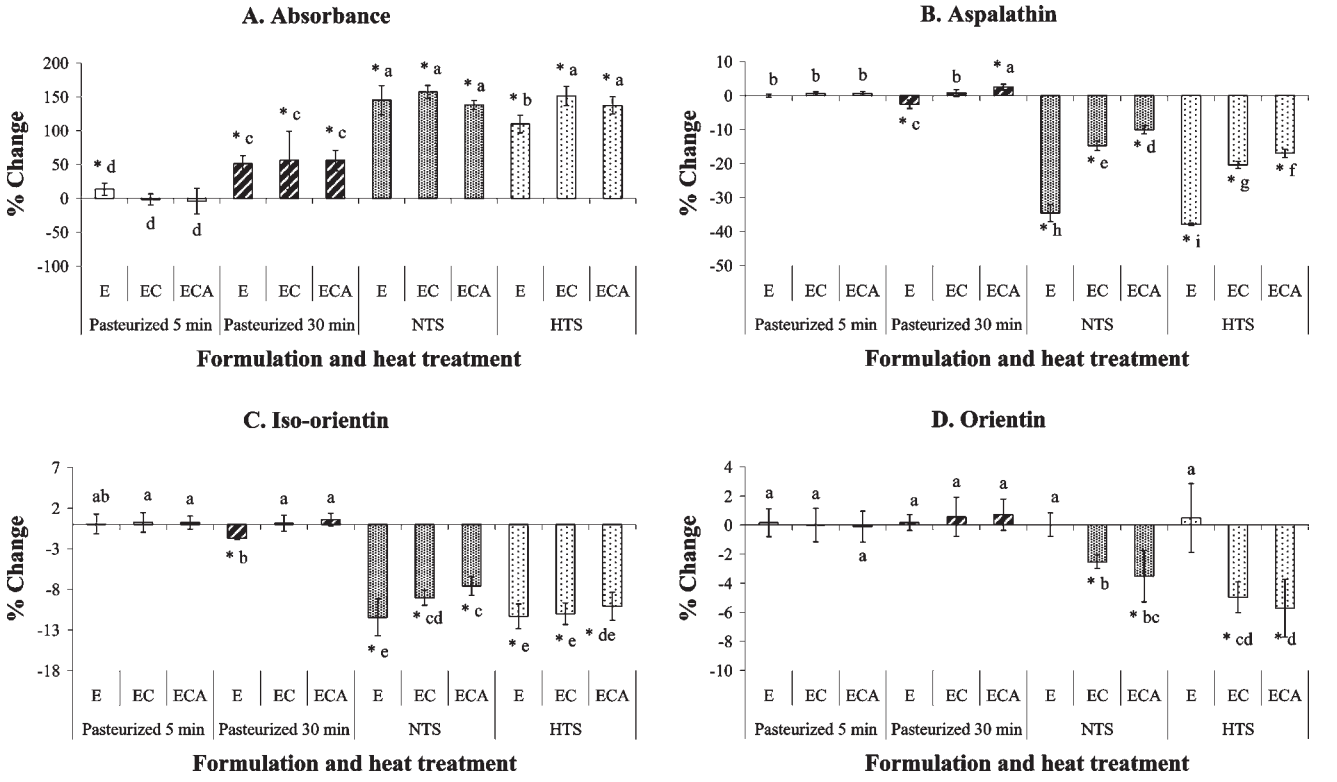

Figure 2. Effect of the formulation and heat treatment compared to the unheated control on the percentage change in (A) absorbance (420 nm) and (B) aspalathin, (C) iso-orientin, and (D) orientin contents [mean ± standard deviation (SD)] of RTD aspalathin-enriched green rooibos iced tea. Formulation E, green rooibos extract reconstituted in deionized water; EC, E + citric acid; ECA, E + citric acid + ascorbic acid; pasteurized, pasteurization for 5 or 30 min; NTS, normal-temperature sterilization; HTS, high-temperature sterilization. Bars labeled with ∗ differ significantly ($p < 0.05$) from controls (before heating). Different letters indicate significant differences ($p < 0.05$) between treatments.

peak area, very small quantities were formed during pasteurization compared to NTS and HTS treatments. Slightly higher quantities were present in the HTS- than NTS-treated formulations (data not shown).

Solubilizate. The pH of the rooibos iced tea containing only the solubilizate (formulation NA) was 3.45, while that of the iced tea with solubilizate and citric acid (formulation NAC) was 2.80. The solubilizate contained 14.4 mg/kg of iron, contributing 202 μg/L to the beverage. The product was clear, and no precipitate formed upon standing after preparation and heating.

An increase in the absorbance at 420 nm was observed for the formulations subjected to 30 min pasteurization, NTS, and HTS ($p < 0.05$) (**Figure 4A**). Pasteurization had very little effect on the flavonoid content of the two formulations, but the NTS and HTS treatments resulted in substantial losses (panels **B**−**D** of **Figure 4**). These losses were substantially higher for aspalathin than for the flavones, with orientin the least susceptible to degradation, as shown for the green rooibos extract. Sterilization of the RTD iced teas containing the solubilizate also led to the appearance of a new peak with retention time and UV−vis characteristics similar to those observed for compound **1** in the green rooibos iced teas. A higher concentration based on peak area was present in the NAC formulation compared to NA after NTS and HTS heat treatments.

Hydrogen Peroxide. The addition of 0.5 mg/L H_2O_2 had no significant ($p \geq 0.05$) effect on the flavonoid content of the formulations (**Table 2**).

Isolation and Identification of Compound 1. Compound **1** was identified as 5-hydroxymethylfurfural (HMF) by GC−MS and NMR spectroscopic methods. GC−MS analysis of the dichloromethane fractions of formulations E, EC, and ECA confirmed the presence of HMF in EC and ECA. The UV−vis spectrum of

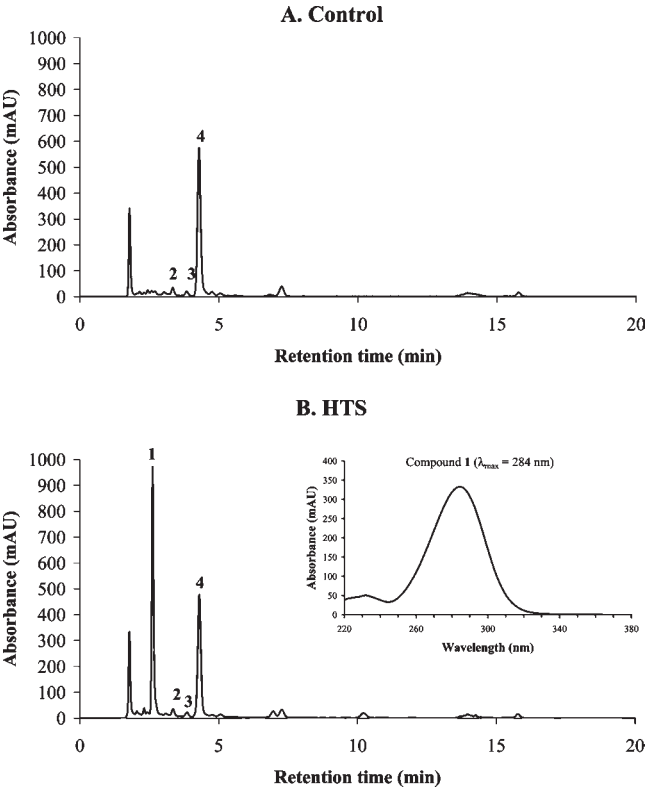

Figure 3. HPLC chromatograms of formulation ECA of RTD aspalathin-enriched green rooibos iced tea (A) before (control) and (B) after HTS at 288 nm. Indicated are compound **1** formed during heating (1), iso-orientin (2), orientin (3), and aspalathin (4). Abbreviations as given in **Figure 2**.

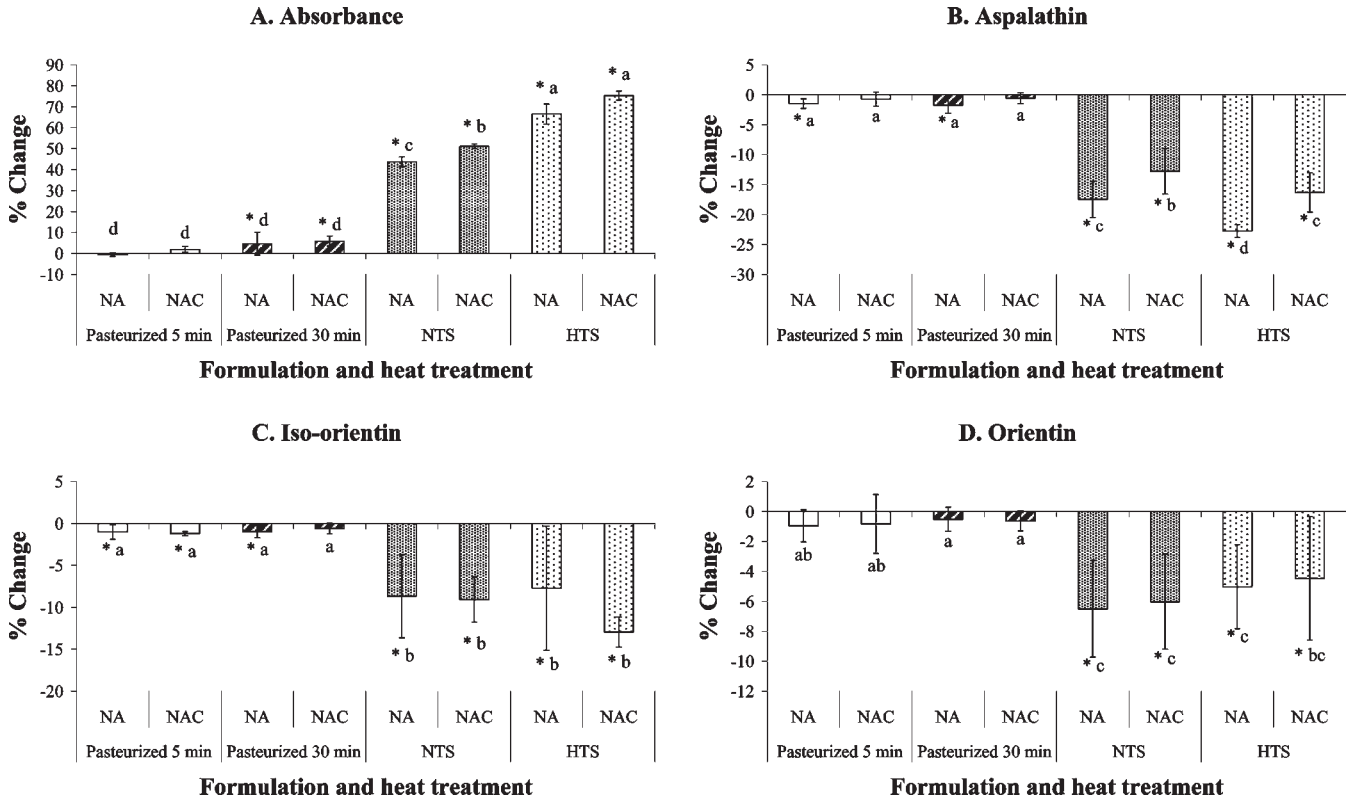

Figure 4. Effect of the formulation and heat treatment compared to the unheated control on the percentage change in (A) absorbance (420 nm) and (B) aspalathin, (C) iso-orientin, and (D) orientin contents (mean ± SD) of RTD iced tea containing solubilizate, i.e., aspalathin-enriched green rooibos extract and ascorbic acid encapsulated in a water-soluble nanomicelle carrier system. Formulation NA, solubilizate diluted in deionized water; NAC, NA + citric acid; other abbreviations as given in **Figure 2**. Bars labeled with ∗ differ significantly ($p < 0.05$) from controls (before heating). Different letters indicate significant differences ($p < 0.05$) between treatments.

Table 2. Effect of the Formulation and Addition of 0.5 mg/L H_2O_2 on the Aspalathin, Iso-orientin, and Orientin Content of RTD Iced Teas (without Sugar)

	aspalathin (g/100 g)		iso-orientin (g/100 g)		orientin (g/100 g)	
formulation	control[a]	treated[b]	control[a]	treated[b]	control[a]	treated[b]
EA[c]	19.340 ± 0.030 a[d]	19.349 ± 0.045 a	1.494 ± 0.003 a	1.492 ± 0.007 a	1.384 ± 0.002 a	1.382 ± 0.005 a
ECA[e]	19.305 ± 0.021 a	19.335 ± 0.014 a	1.498 ± 0.010 a	1.499 ± 0.002 a	1.384 ± 0.002 a	1.388 ± 0.001 a
NA[f]	2.723 ± 0.009 a	2.723 ± 0.005 a	0.250 ± 0.001 a	0.249 ± 0.001 a	0.227 ± 0.001 a	0.228 ± 0.001 a
NAC[g]	2.717 ± 0.002 a	2.721 ± 0.014 a	0.252 ± 0.001 a	0.253 ± 0.001 a	0.230 ± 0.000 a	0.232 ± 0.001 a

[a] Sample without H_2O_2. [b] Sample with H_2O_2. [c] Green rooibos extract in deionized water + ascorbic acid. [d] Means for control and treated samples with the same letter indicate no significant difference ($p \geq 0.05$) between them. [e] EA + citric acid. [f] Solubilizate, i.e., a water-soluble nanomicelle carrier system containing green rooibos extract and ascorbic acid, diluted in deionized water. [g] NA + citric acid.

compound **1** in these formulations (**Figure 3**) was consistent with that of HMF, giving a λ_{max} of 284 nm (*19*).

DISCUSSION

Aspalathin is of increasing interest to the food and nutraceutical industries, not only as an antioxidant (*2*−*5*) and antimutagen (*6*) but also for its glucose-lowering effect (*7*, *8*). It also imparts novelty value to rooibos and its products; to date, aspalathin has only been isolated from *A. linearis* (*20*). Despite this, information on the stability of aspalathin in food products is limited to our previous study on RTD fermented rooibos iced tea (*12*). The poor phenolic quality of commercial RTD rooibos iced teas prepared from fermented rooibos extracts and the extensive losses of aspalathin during sterilization of the product prompted the present study. An organic-solvent-based aspalathin-enriched green rooibos extract was chosen in favor of a water-based extract of green rooibos to deliver a RTD product with high aspalathin content. Typically, an industrial aqueous extract of green rooibos contains 7% aspalathin, which would only realize 125 mg/L aspalathin in the beverage before heat processing, compared to 362 mg/L achieved in the present study. Because of the lower solubility of the organic-solvent-based aspalathin-enriched green rooibos extract, a turbid product, which formed a precipitate upon standing, was obtained. The availability of this extract, encapsulated with ascorbic acid in water-soluble nanomicelles, provided an opportunity to address the problem of poor solubility of the extract. Improved water solubility and clarity of lipophilic compounds and extracts because of the nanoscale size of the particles of the carrier system are some of the advantages of nanotechnology (*21*). Another advantage is improved bioavailability of compounds (*22*−*24*), which could be an added benefit for aspalathin. The presence of a *C*-linked glucoside and its physicochemical properties indicate poor bioavailability of aspalathin (*25*). This was confirmed by human studies (*26*, *27*).

Pasteurization, even for 30 min, had little impact on the aspalathin content of the different formulations and would, therefore, be the preferred heat-processing method. A slight increase in aspalathin content of formulation ECA was also observed when fermented rooibos extract was used (*12*). This increase was attributed

to the release of aspalathin from association with polymers as a result of changes in or degradation of their structures because of heat treatment. In the case of the control, the polymer−flavonoid "complex" is removed during filtration required for HPLC analysis. The severity of the NTS and HTS treatments would nullify such an effect. In the case of the solubilizate, this "release" before filtration compensating for heat degradation during pasteurization is not relevant because the nanosized micelle will not be retained by the filter.

When beverages are pasteurized, pH becomes a critical factor in preventing the growth of food spoilage micro-organisms. The pH of formulation E is too near the upper limit for a microbially safe product with a shelf life of at least 3 months. If a RTD product with "natural taste" is required, lowering the pH with citric acid to create an environment unfavorable for growth of food spoilage micro-organisms is not an option. This would unfortunately also exclude the use of the solubilizate because it contains polyoxyethylene sorbitan monolaurate, even though the pH of the NA formulation makes it suitable for pasteurization. The use of preservatives could be perceived in a negative light, especially by consumers preferring preservative-free products. In such a case, sterilization would be required, but losses of aspalathin in excess of 30% could be expected. When fermented rooibos extract was used in the RTD product, very little aspalathin remained after heating (*12*). Using the aspalathin-enriched green rooibos extract instead, an aspalathin content of 231 mg/L could be attainable in the product directly after heating. The heat treatments, NTS and HTS, employed in this study represent the extreme. Normally heat exposure during continuous ultra-high temperature sterilization, combined with cold aseptic packaging, is in the order of seconds, compared to exposure times of 20 min and more for in-container sterilization. Under industrial conditions, higher levels of aspalathin retention could thus be expected.

Interestingly, the aspalathin was more stable when present in the RTD formulations containing the aspalathin-enriched green rooibos extract than when fermented rooibos extract was used. The previous study showed that aspalathin losses of 76−78% occurred when the latter product was subjected to the NTS and HTS treatments (*12*). This phenomenon needs further investigation, but compositional differences between the extract types could have played a role. Using pure compounds, Murakami et al. (*28*) demonstrated that the presence of chlorogenic acid protects rutin to some extent against heat degradation.

The addition of citric acid to formulation E was effective in reducing aspalathin losses during NTS and HTS to 15 and 20%, respectively. Lowering the pH conferred greater stability to aspalathin because of protonation of the OH groups. When both citric and ascorbic acids were added, aspalathin stability in NTS and HTS products could be further improved (10 and 17% loss, respectively). Encapsulation of the green rooibos extract with ascorbic acid in nanomicelles and the higher ratio of ascorbic acid/aspalathin in formulation NAC, however, did not offer any improvement in the stability of aspalathin compared to formulation ECA. The use of the solubilizate may be considered when a clear solution is required.

Improving the stability of aspalathin with ascorbic acid in the presence of citric acid indicates that conditions were not favorable for the Fenton reaction and the presence of excess aspalathin (*13*) and ascorbic acid (*29*) probably suppressed the Fenton reaction because of the scavenging of $^{\bullet}OH$ instead of its generation. Chelation of iron by citric acid would make generation of $^{\bullet}OH$ and its subsequent reaction site-specific. Even the addition of H_2O_2 did not result in a noticeable effect. Any destabilization of the RTD product because of the Fenton reaction could thus be ruled out.

Orientin was apparently more stable than iso-orientin during NTS and HTS. This was most likely due to the conversion of iso-orientin to orientin (*10*), which would have canceled out losses of orientin during heating. Results from the present study support this because the greatest loss in iso-orientin coincided with no change in orientin content (formulation E of NTS) (**Figure 2**). Conversion of aspalathin to iso-orientin would also have balanced out, to some extent, the decrease in iso-orientin content caused by its conversion to orientin. It was apparent from the results for formulation E (NTS) that the large decrease in aspalathin content did not coincide with concurrent increases in iso-orientin and orientin contents.

Apart from its conversion to the flavones, early stages of non-enzymatic oxidation of aspalathin are also accompanied by the formation of dimers (*30*), while high-molecular-weight brown products eventually form in solution (*10*, *31*). Futher insight into the browning of the beverage is provided by the increase in absorbance and the concomitant change or lack of significant change in the flavonoid content. Pasteurization for 30 min induced a substantial increase in absorbance (51%), irrespective of formulation, yet little or no change in flavonoid content was observed. On the other hand, for example, in the case of NTS, the increase in absorbance was also the same for formulations E, EC, and ECA yet their decrease in aspalathin content differed. This indicates that other factors contributed to browning. The identification of compound **1** as HMF and its presence in formulations EC and ECA, especially when subjected to sterilization, support this. HMF, one of the most common intermediate products of the Maillard reaction and a precursor of brown pigments, also forms upon heating of sugars in an acidic medium. As one of the decomposition products of ascorbic acid, it is used to evaluate the severity of heating during fruit juice processing (*32*). The presence of sucrose, citric acid, and ascorbic acid would explain the formation of HMF in the EC, ECA, NA, and NAC formulations.

The market growth in RTD rooibos and other iced teas during the past few years in South Africa showed that local consumers are willing to buy into the "health and wellness" marketing concept. It is, therefore, vital that reputable brands deliver the implied promise of a health-promoting product, such as rooibos. Pasteurized RTD green rooibos iced teas, containing ascorbic and/or citric acids, could very well serve as a vehicle to increase the dietary intake of flavonoids, such as aspalathin and its flavone analogues, by consumers.

ABBREVIATIONS USED

RTD, ready-to-drink; E, green rooibos extract in deionized water; EA, E + ascorbic acid; EC, E + citric acid; ECA, E + citric acid + ascorbic acid; NA; solubilizate, i.e., a water-soluble nanomicelle carrier system containing green rooibos extract and ascorbic acid, diluted in deionized water; NAC; NA + citric acid; HTS, high-temperature sterilization; NTS, normal-temperature sterilization; HMF, 5-hydroxymethylfurfural.

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LITERATURE CITED

(1) Manley, M.; Joubert, E.; Botha, M. Quantification of the major phenolic compounds, soluble solid content and total antioxidant activity of green rooibos (*Aspalathus linearis*) by means of near infrared spectroscopy. *J. Near Infrared Spectrosc.* **2006**, *14*, 213−222.

(2) Von Gadow, A.; Joubert, E.; Hansmann, C. F. Comparison of the antioxidant activity of aspalathin with that of other plant phenols of rooibos tea (*Aspalathus linearis*), α-tocopherol, BHT, and BHA. *J. Agric. Food Chem.* **1997**, *45*, 632−638.

(3) Joubert, E.; Winterton, P.; Britz, T. J.; Ferreira, D. Superoxide anion radical and α,α-diphenyl-β-picrylhydrazyl radical scavenging capacity of rooibos (*Aspalathus linearis*) aqueous extracts, crude phenolic fractions, tannin and flavonoids. *Food Res. Int.* **2004**, *37*, 133−138.

(4) Krafczyk, N.; Woyand, F.; Glomb, M. A. Structure−antioxidant relationship of flavonoids from fermented rooibos. *Mol. Nutr. Food Res.* **2009**, *53*, 635−642.

(5) Snijman, P. W.; Joubert, E.; Ferreira, D.; Li, X.-C.; Ding, Y.; Green, I. R.; Gelderblom, W. C. A. Antioxidant activity of the dihydrochalcones aspalathin and nothofagin and their corresponding flavones in relation to other rooibos (*Aspalathus linearis*) flavonoids, epigallocatechin gallate, and Trolox. *J. Agric. Food Chem.* **2009**, *57*, 6678−6684.

(6) Snijman, P. W.; Swanevelder, S.; Joubert, E.; Green, I. R.; Gelderblom, W. C. A. The antimutagenic activity of the major flavonoids of rooibos (*Aspalathus linearis*): Some dose−response effects on mutagen activation−flavonoid interactions. *Mut. Res.* **2007**, *631*, 111−123.

(7) Mose Larsen, P. M.; Fey, S.; Louw, J.; Joubert, L. An anti-diabetic extract from rooibos. International Patent Application WO2008/110551A1, 2008.

(8) Kawano, A.; Nakamura, H.; Hata, S.-I.; Minakawa, M.; Miura, Y.; Yagasaki, K. Hypoglycemic effect of aspalathin, a rooibos tea component from *Aspalathus linearis*, in type 2 diabetic model db/db mice. *Phytomedicine* **2009**, *16*, 437−443.

(9) Joubert, E. HPLC quantification of the dihydrochalcone, aspalathin and nothofagin in rooibos tea (*Aspalathus linearis*) as affected by processing. *Food Chem.* **1996**, *55*, 403−411.

(10) Krafczyk, N.; Glomb, M. Investigation of polyphenolic ingredients in rooibos by multilayer countercurrent chromatography. *J. Agric. Food Chem.* **2008**, *56*, 3368−3376.

(11) Joubert, E.; Schulz, H. Production and quality aspects of rooibos tea and related products. A review. *J. Appl. Bot. Food Qual.* **2006**, *80*, 138−144.

(12) Joubert, E.; Viljoen, M.; De Beer, D.; Manley, M. Effect of heat on aspalathin, iso-orientin, and orientin contents and color of fermented rooibos (*Aspalathus linearis*) iced tea. *J. Agric. Food Chem.* **2009**, *57*, 4204−4211.

(13) Joubert, E.; Winterton, P.; Britz, T. J.; Gelderblom, W. C. A. Antioxidant and pro-oxidant activities of aqueous extracts and crude polyphenolic fractions of rooibos (*Aspalathus linearis*). *J. Agric. Food Chem.* **2005**, *53*, 10260−10267.

(14) Bradshaw, M. P.; Prenzler, P. D.; Scollary, G. R. Ascorbic acid-induced browning of (+)-catechin in a model wine system. *J. Agric. Food Chem.* **2001**, *49*, 934−939.

(15) Lloyd, R. V.; Hanna, R. M.; Mason, R. P. The origin of the hydroxyl radical oxygen in the Fenton reaction. *Free Radical Biol. Med.* **1997**, *22*, 885−888.

(16) Grüner-Richter, S.; Otto, F.; Weinreich, B. Rooibos extract with increased aspalathin content, process for the preparation of such a rooibos extract, and cosmetic agent containing such a rooibos extract. U.S. Patent Application US2008/0247974 A1, 2008.

(17) Cilliers, J. J. L.; Singleton, V. L. Caffeic acid autoxidation and the effects of thiols. *J. Agric. Food Chem.* **1990**, *38*, 1789−1796.

(18) Caruso, T.; Vasca, E. Electrogenerated acid as an efficient catalyst for the preparation of 5-hydroxymethylfurfural. *Electrochem. Commun.* **2010**, *12*, 1149−1153.

(19) Spano, N.; Ciulu, M.; Floris, I.; Panzanelli, A.; Pilo, M. I.; Piu, P. C.; Salis, S.; Sanna, G. A direct RP-HPLC method for the determination of furanic aldehydes and acids in honey. *Talanta* **2009**, *78*, 310−314.

(20) Joubert, E.; Gelderblom, W. C. A.; Louw, A.; De Beer, D. South African herbal teas: *Aspalathus linearis*, *Cyclopia* spp. and *Athrixia phylicoides*—A review. *J. Ethnopharmacol.* **2008**, *119*, 376−412.

(21) Solans, C.; Izquierdo, P.; Nolla, J.; Azemar, N.; Garcia-Celma, M. J. Nano-emulsions. *Curr. Opin. Colloid Interface Sci.* **2005**, *10*, 102−110.

(22) Back, E. I.; Frindt, C.; Oćenášková, E.; Nohr, D.; Stern, M.; Biesalski, H. K. Can changes in hydrophobicity increase the bioavailability of α-tocopherol? *Eur. J. Nutr.* **2006**, *45*, 1−6.

(23) Wang, X.; Wang, Y.-W.; Huang, Q. Enhancing stability and oral bioavailability of polyphenols using nanoemulsions. In *Micro/Nanoencapsulation of Active Food Ingredients*; Huang, Q., Given, P., Qian, M., Eds.; Amercican Chemical Society: Washington, D.C., 2009; ACS Symposium Series, Vol. 1007, Chapter 13, pp 198−213.

(24) Huang, Q.; Yu, H.; Ru, Q. Bioavailability and delivery of nutraceuticals using nanotechnology. *J. Food Sci.* **2010**, *75*, R50−R57.

(25) Joubert, E.; Gelderblom, W. C. A.; De Beer, D. Phenolic contribution of South African herbal teas to a healthy diet. *Nat. Prod. Commun.* **2009**, *4*, 701−718.

(26) Stalmach, A.; Mullen, W.; Pecorari, M.; Serafini, M.; Crozier, A. Bioavailability of *C*-linked dihydrochalcone and flavanone glucosides in humans following ingestion of unfermented and fermented rooibos teas. *J. Agric. Food Chem.* **2009**, *57*, 7104−7111.

(27) Courts, F. L.; Williamson, G. The *C*-glycosyl flavonoid, aspalathin, is absorbed, methylated and glucuronidated intact in humans. *Mol. Nutr. Food Res.* **2009**, *53*, 1104−1111.

(28) Murakami, M.; Yamaguchi, T; Takamura, H.; Matoba, T. Effects of thermal treatment on radical-scavenging activity of single and mixed polyphenolic compounds. *J. Food Sci.* **2008**, *69*, FCT7−FCT10.

(29) Zhao, M. J.; Jung, L. Kinetics of the competitive degradation of deoxyribose and other molecules by hydroxyl radicals produced by the Fenton reaction in the presence of ascorbic acid. *Free Radical Res.* **1995**, *23*, 229−243.

(30) Krafczyk, N.; Heinrich, T.; Porzel, A.; Glomb, M. A. Oxidation of the dihydrochalcone aspalathin leads to dimerization. *J. Agric. Food Chem.* **2009**, *57*, 6833−6843.

(31) Koeppen, B. H.; Roux, D. G. *C*-Glycosylflavonoids. The chemistry of aspalathin. *Biochem. J.* **1966**, *99*, 604−609.

(32) Damasceno, L. F.; Fernandes, F. A. N.; Magalhães, M. M. A.; Brito, E. S. Non-enzymatic browning in clarified cashew apple juice during thermal treatment: Kinetics and process control. *Food Chem.* **2008**, *106*, 172−179.

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