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ARTICLE

Effect of Heat on Aspalathin, Iso-orientin, and Orientin Contents and Color of Fermented Rooibos (*Aspalathus linearis*) Iced Tea

Elizabeth Joubert,*[,†,§] Melvi Viljoen,[§] Dalene De Beer,[†] and Marena Manley[§]

[†]ARC Infruitec-Nietvoorbij, Private Bag X5026, Stellenbosch 7599, South Africa, and [§]Department of Food Science, Stellenbosch University, Private Bag X1, Matieland (Stellenbosch) 7602, South Africa

The phenolic quality of commercial South African fermented rooibos iced teas in terms of aspalathin, iso-orientin, and orientin contents in comparison to a "cup of tea" was shown to be inferior. The role of the different manufacturing stages of powdered extract used in iced tea formulation and, more specifically, the impact of pasteurization and sterilization on the color and phenolic content of the beverage, were assessed as potential causes of its inferior phenolic quality. Aspalathin and its corresponding flavones, iso-orientin and orientin, were found to be present at all stages of the powdered extract production process. Spray-drying did not significantly ($P \geq 0.05$) alter the aspalathin, iso-orientin, or orientin content of concentrates. Simulated normal-temperature sterilization (NTS at 121 °C/15 min) and high-temperature sterilization (HTS at 135 °C/4 min), but not necessarily pasteurization (93 °C/30 min), significantly ($P < 0.05$) reduced the aspalathin, iso-orientin, and orientin contents of different iced tea formulations. Heat-induced losses of iso-orientin and orientin were lower than those for aspalathin. Conversion of aspalathin to the flavones is implicated. The addition of ascorbic acid and/or citric acid to the base iced tea formulation containing only rooibos extract and sugar proved to be beneficial, especially for the retention of aspalathin. Browning, that is, absorbance at 420 nm, was significantly ($P < 0.05$) increased in the base formulation. In the case of the formulations also containing ascorbic acid and/or citric acid, absorbance remained unchanged or decreased when subjected to NTS and HTS treatments. This was attributed to removal of brown polymers from solution as the pH values of these formulations were lower than that of the base formulation.

KEYWORDS: Rooibos; *Aspalathus linearis*; iced tea; heat processing; aspalathin; iso-orientin; orientin

INTRODUCTION

The popularity of ready-to-drink iced tea prepared from the herbal tea, "fermented" rooibos (*Aspalathus linearis*), has led to a number of branded products on the South African and international markets. In most cases powdered aqueous extracts form the basis of these products. Part of the popularity of rooibos is its health-promoting properties, of which antioxidant activity has been the main focus (*1*). The major antioxidant in rooibos is the dihydrochalcone, aspalathin (**Figure 1**). This compound is very susceptible to oxidation, especially under the conditions prevalent during processing of the plant material, required for development of the characteristic sweet flavor and red-brown color of the traditional herbal tea (*2*). Koeppen and Roux (*3*, *4*) proposed a mechanism by which aspalathin oxidation to the flavones, orientin and iso-orientin, occurs via the respective intermediary flavanones, dihydroiso-orientin and dihydro-orientin. Brown polymers were also noted to form in an ethanolic solution. This pathway of aspalathin conversion was recently confirmed when oxidative conversion took place in a phosphate-buffered medium of pH 7.4 at 37 °C (*5*).

Given the instability of aspalathin under oxidative conditions, the question arose whether substantial losses of aspalathin also occur during the manufacture of powdered extract and to what extent heat processing during the manufacture of iced tea affects aspalathin degradation or conversion. Trace amounts or undetectable quantities of aspalathin could be indicative of either the use of overoxidized raw material for extract preparation or poor stability during extract manufacture and/or during iced tea manufacture and storage. As oxidation products, orientin and iso-orientin should be more stable and have potential as indicators of product authenticity as they are not commonly present in other iced tea ingredients, such as fruit juice. Should these compounds survive processing, their presence could serve to verify the use of rooibos extract in rooibos iced tea products. The objectives of the present study were to confirm the retention of aspalathin, iso-orientin, and orientin during the manufacture of rooibos extract powder and to determine the stability of these compounds when using powdered extract as the source of rooibos during ready-to-drink iced tea manufacture. Because heat treatment is essential to achieve a product with an extended shelf life,

*Author to whom correspondence should be addressed (telephone +27 21 809 3444; fax +27 21 809 3430; e-mail joubertL@arc.agric.za).

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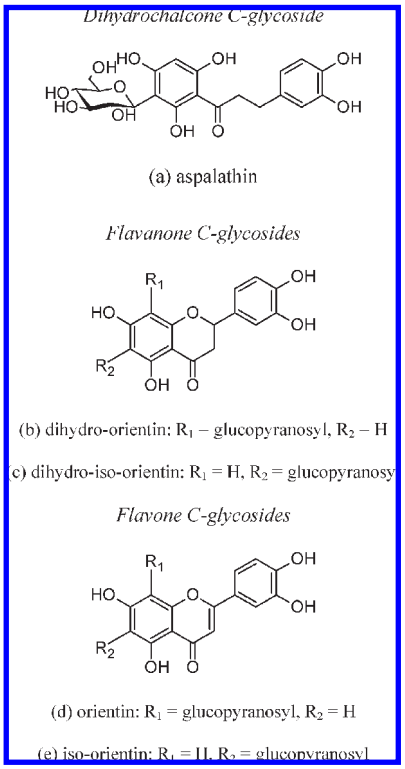

Figure 1. Structures of (**a**) aspalathin and its oxidation products (**b**) dihydro-orientin, (**c**) dihydroiso-orientin, (**d**) orientin, and (**e**) iso-orientin.

the effect of heat was investigated by subjecting the rooibos iced tea to different heat treatments, that is, pasteurization, normal-temperature sterilization (NTS), and high-temperature sterilization (HTS). As citric acid is usually an ingredient of rooibos iced tea, and sometimes ascorbic acid as well, different iced tea formulations were subjected to the heat treatments so that their effect on product stability could be established.

MATERIALS AND METHODS

Chemicals and Water Purification. The chemicals required for HPLC analysis were glacial acetic acid (Fluka, Buchs, Switzerland), HPLC grade acetonitrile (Merck, Darmstadt, Germany), and dimethyl sulfoxide and ascorbic acid (Sigma Chemical Co., St. Louis, MO). Aspalathin was obtained from the PROMEC Unit of the Medical Research Council (Parow, South Africa), whereas iso-orientin and orientin were supplied by Extrasynthese (Genay, France) and Roth (Karlsruhe, Germany), respectively. A mixture of flavanones, dihydroiso-orientin and dihydro-orientin, isolated from rooibos (isolation by B. H. Koeppen; available from the compound library of the Agricultural Research Council) was used for tentative confirmation of their presence or absence. Deionized water was prepared using a Modulab water purifier (Continental Water Systems Corp., San Antonio, TX). HPLC grade water was obtained by further purification using a Milli-Q academic water purifier (Millipore, Bedford, MA). The reagents required for the quantification of the total polyphenol content of the powdered extract used for preparation of the different iced tea formulations were Folin−Ciocalteu's phenol reagent (Merck), anhydrous sodium carbonate (Saarchem, Gauteng, South Africa), and gallic acid (Sigma Chemical Co.). Food grade ingredients for the preparation of rooibos iced tea comprised sucrose (Huletts, Rossburgh, Durban, South Africa) and citric acid (Warren Chem Specialities Cape Town, South Africa). Analytical grade ascorbic acid (Sigma Chemical Co.) was used in the iced tea formulation.

Sampling during Commercial Powdered Extract Manufacture. Samples were collected during the manufacture of a commercial rooibos powdered extract. The first samples (n = 10) were collected directly after high-temperature extraction, coarse filtration, and cooling. Following cooling, the extract was subjected to microfiltration. The microfiltered extracts (n = 9) were continuously fed into a holding tank, where a minimum of four batches of extract were collected before reverse osmosis commenced. Three batches of the subsequent semiconcentrated extract were sampled online before pooling in another holding tank with other production batches. The pooled, semiconcentrated extract was further concentrated under vacuum in a flow-through vacuum evaporator. Sampling (n = 7) took place online, directly after concentration. Several batches of concentrate were pooled in the holding tank before spray-drying commenced. Due to the large quantities required for operation of different units, necessitating pooling of batches, consecutive samples of a more advanced processing stage do not exactly represent the previous sample. However, in the case of spray-drying, sample sets (n = 5) were composed of the concentrate fed to the spray-dryer and its corresponding spray-dried product. During spray-drying, rooibos concentrate was exposed to an inlet temperature of 210 °C and an outlet temperature of 95 °C.

The collected extracts and concentrates were freeze-dried in an Edwards Modulyo freeze-drier (Edwards, Sussex, U.K.). The freeze-dried soluble solids were pulverized for 15 s in a ball mill (Retsch MM301, Retsch GmbH, Haan, Germany) to ensure homogeneity. Samples were stored at room temperature in sealed transparent plastic tubes in a desiccator in the dark until analysis.

Commercial Rooibos Iced teas/Fruit Teas. Eight brands of commercial rooibos iced teas (A−H), packed in either Tetrapak, aluminum cans, or PET bottles, were purchased from local supermarkets. All products listed rooibos extract as an ingredient. Brands A−D and H contained ascorbic acid. Several brands contained one or more fruit juices such as apple, pear, sour cherry, and cranberry. The pH of the iced tea was lowered by using either citric acid or malic acid. Two formulations of brand D were purchased: one containing sucrose (D_1) and the other an artificial sweetener (D_2). Except for brands G (4 samples) and H (16 samples), 3 bottles of each brand of iced tea (different production batches) were purchased. Brand H represented a major brand of which the basic formulation was used as a basis for the preparation of the experimental rooibos iced teas, investigated in the present study. Product H was pasteurized for 15 s at 80 °C. No information is available on the pasteurization or sterilization conditions of the other products.

Effect of Heat and Product Formulation on the Flavonoid Composition and Color of Experimental Rooibos Iced Tea. Three iced tea formulations were prepared using a spray-dried extract obtained from Afriplex (Paarl, South Africa): base (B, rooibos extract reconstituted in deionized water); base + citric acid (BC); and base + citric acid + ascorbic acid (BCA). All formulations contained 60 g/L sugar and 1.75 g/L rooibos extract. Formulations BC and BCA both contained 1.2 g/L citric acid, whereas formulation BCA also contained 0.2 g/L ascorbic acid.

The heat treatments were a pasteurization-like heat treatment (to be referred to as pasteurization), NTS, and HTS. Pasteurization was achieved by pumping the iced tea with a peristaltic pump at a flow rate of ca. 13.8 mL/s through a 3 m stainless steel coil (5 mm inner diameter) in a temperature-controlled water bath at 93 °C. The iced tea was continuously collected in a vacuum flask and withdrawn during the duration of the experiment, subjecting the iced tea to contact with the atmosphere. The sample was recirculated for 30 min before being collected in sealable 25 mL glass vials and cooled on ice for 30 min.

A benchtop autoclave sterilizer (Sturdy Industrial Co. Ltd., Taiwan) capable of a maximum temperature of 135 °C was used for NTS and HTS treatments of the iced tea formulations. Aliquots were placed in 20 mL gas chromatography headspace vials and sealed with aluminum caps containing PTFE/butyl rubber septa. NTS involved autoclaving at 121 °C for 15 min, whereas the HTS treatment entailed autoclaving at 135 °C for 4 min. Due to the duration of heating and cooling, samples were in the autoclave for approximately 45 min. Following removal from the autoclave, the samples were immediately placed on ice for 30 min.

A second pasteurization treatment was carried out by heating the iced tea formulations in sealed 19 mL Corning Pyrex glass tubes (i.d. = 15 mm) in a water bath (93 °C) for 5 or 30 min. Samples achieved a temperature of 93 °C after ca. 2 min. The vials were filled to the neck to limit the headspace.

To assess the effect of heat treatment on flavonoid content and color, samples were taken before (control) and after completion of a treatment. All treatments were independently replicated four times.

Total Polyphenol Content and pH Determination. The total polyphenol content of the commercial rooibos powdered extract used for preparation of the experimental iced tea was determined according to the Folin−Ciocalteu method of Singleton and Rossi (*6*), scaled down for a microplate reader. Results were expressed as grams of gallic acid equivalents (GAE) per 100 g of extract. The pH of the respective iced tea formulations was determined with a Crison GLP 21 pH-meter (Crison Instruments SA, South Africa).

Color Measurement. The brown color of the iced tea was determined by measuring the absorbance at 420 nm (*7*) on a Biotek Synergy HT multiplate reader (Biotek Instruments, Winooski, VT). Due to the formation of a precipitate, samples were centrifuged at 18000 rpm for 4 min (Hettich Universal 16 centrifuge, Hettich, Tuttlingen, Germany) prior to color measurement.

HPLC Quantification of Flavonoids. Quantification of aspalathin, orientin, and iso-orientin was performed by HPLC-DAD on an Agilent 1200 system (Agilent, Santa Clara, CA) comprising a quaternary pump, an autosampler, an in-line degasser, a column thermostat, and a diode array detector. Chemstation software for LC 3D systems (Agilent) was used to control the system and record data. Separation was performed at 38 °C on an Agilent Zorbax Eclipse XDB-C18 (5 μm, 150 × 4.6) mm column protected with an RP/C18 guard column (Vici-AG International, Schenkon, Switzerland). Conditions were selected to obtain rapid separation of aspalathin, iso-orientin, and orientin at a flow rate of 0.8 mL/min. A binary gradient system comprising 2% acetic acid and acetonitrile was used. The gradient in terms of acetonitrile was as follows: 0 min, 18%; 10 min, 20%; 13 min, 80%; 16−18 min (reconditioning), 18%. Chromatograms were recorded at 288 nm for the quantification of aspalathin and at 350 nm for the quantification of orientin and iso-orientin. Injection volumes were 50 μL for the experimental iced tea samples and 100 μL for the commercial products.

Spray- and freeze-dried extract samples were prepared for HPLC analysis by disolving ca. 88 mg of extract in 50 mL of deionized water containing 0.5% ascorbic acid. Iced tea samples (commercial and experimental) were prepared by mixing 1.5 mL of iced tea with 100 μL of 10% ascorbic acid. All samples were filtered before HPLC analysis using 33 mm Millex-HV 0.45 μm syringe tip filters (Millipore). Stock solutions of the standards were prepared with dimethyl sulfoxide and aliquots frozen until analysis. The calibration curve consisted of five concentrations of aspalathin (1.9−95.5 μg/mL), orientin (1.5−74.4 μg/mL), and iso-orientin (1.6−80.8 μg/mL). Sample peaks were identified as aspalathin, orientin, or iso-orientin according to retention time and spectral characteristics.

Statistical Analyses. The data were subjected to analysis of variance (ANOVA) using SAS version 9.3 (SAS Institute, Cary, NC) and analyzed for normality ($P \geq 0.05$) using the Shapiro−Wilk test for non-normality. The Student t test was used to ascertain whether there were significant differences between (1) actual values of control and treated samples and (2) percent change values of formulation × treatment combinations. Differences with a significance level of 5% ($P < 0.05$) were considered to be significant.

RESULTS

Flavonoid Content of Rooibos Extracts at Different Stages of Manufacture. Direct comparison of the extract at the various processing stages (e.g., microfiltration vs reverse osmosis) was not possible due to pooling of production batches between processing steps. However, the data clearly show that aspalathin, iso-orientin, and orientin were present in all samples and that major losses of these compounds did not occur during processing (**Table 1**). In the case of spray-drying, the five sample sets comprising the concentrate fed to the spray-dryer and its corresponding spray-dried product could be directly compared. Spray-drying did not significantly ($P < 0.05$) affect the flavonoid composition (**Table 1**).

Flavonoid Content of Commercial Rooibos Iced Teas/Fruit Teas. All six production batches of brand D tested negative for the presence of aspalathin, iso-orientin, and orientin (**Table 2**). Brands A, E, G, and H contained aspalathin, iso-orientin, and orientin in all of the analyzed production batches. One of the production batches of brand B lacked both aspalathin and iso-orientin. None of the production batches of brands C and F contained aspalathin, but all contained orientin, and two production batches of brand C also contained no iso-orientin.

Brand H was investigated more extensively than the other brands; that is, 16 bottles of various production dates were analyzed for their aspalathin, iso-orientin, and orientin contents. No distinct pattern with respect to the production date could be observed for any of these compounds (**Figure 2**).

Table 1. Aspalathin, Iso-orientin, and Orientin Contents[a] of Aqueous Extracts of Fermented Rooibos[b] Taken at Different Stages of the Commercial Extract Production Process

	aspalathin	iso-orientin	orientin
after extraction (n = 10)	0.07−0.37 (0.22 ± 0.09)	0.43−0.71 (0.58 ± 0.07)	0.76−1.09 (0.89 ± 0.10)
after microfiltration (n = 9)	0.14−0.35 (0.24 ± 0.08)	0.54−0.74 (0.66 ± 0.08)	0.81−1.21 (1.10 ± 0.13)
after reverse osmosis (n = 3)	0.23−0.44 (0.35 ± 0.11)	0.70−0.74 (0.71 ± 0.02)	1.15−1.21 (1.19 ± 0.03)
after concentration (n = 7)	0.18−0.38 (0.32 ± 0.09)	0.68−0.74 (0.71 ± 0.03)	1.16−1.20 (1.18 ± 0.01)
before spray-drying (n = 5)	0.18−0.28 (0.23 ± 0.04 a[c])	0.51−0.74 (0.59 ± 0.09 a)	1.01−1.21 (1.10 ± 0.08 a)
after spray-drying (n = 5)	0.21−0.34 (0.26 ± 0.05 a)	0.56−0.79 (0.64 ± 0.09 a)	1.04−1.26 (1.16 ± 0.08 a)

[a] Results expressed as g/100 g of extract. [b] All samples, except after spray-drying, were freeze-dried and reconstituted directly before analysis. [c] Averages in the same column with different letters differ significantly at the 5% level of significance ($P < 0.05$).

Table 2. Aspalathin, Iso-orientin, and Orientin Contents[a] of Nine Commercial, Fermented Rooibos Iced Teas

brand	aspalathin	iso-orientin	orientin
A (n = 3)[b]	0.11−0.15 (0.13 ± 0.02)[c]	0.62−0.64 (0.63 ± 0.01)	1.14−1.16 (1.15 ± 0.01)
B (n = 3)	nd[d],[e]−0.16 (0.13 ± 0.04)	nd[e]−0.29 (0.29 ± 0.00)	0.38−0.39 (0.39 ± 0.00)
C (n = 3)	nd	nd[f]−0.29	0.41−0.42 (0.41 ± 0.01)
D_1[g] (n = 3)	nd	nd	nd
D_2[g] (n = 3)	nd	nd	nd
E (n = 3)	0.29−0.34 (0.32 ± 0.03)	0.03−0.86 (0.54 ± 0.44)	1.26−1.41 (1.36 ± 0.09)
F (n = 3)	nd	0.21−0.22 (0.22 ± 0.00)	0.33−0.35 (0.34 ± 0.01)
G (n = 4)	0.10−0.69 (0.43 ± 0.24)	0.77−0.93 (0.85 ± 0.07)	1.07−1.29 (1.20 ± 0.10)
H (n = 16)	0.24−0.52 (0.41 ± 0.09)	0.59−1.20 (0.93 ± 0.17)	0.95−1.72 (1.38 ± 0.19)

[a] Results expressed in mg/100 mL of iced tea average. [b] Number of samples. [c] Average. [d] Not detected. [e] Compound not detected in one of the three samples. [f] Compound not detected in two of the three samples. [g] Two formulations of brand D were purchased: D_1, containing sucrose; and D_2, containing an articial sweetener.

In some cases, bottles sampled after their expiration date (i.e., 92 days) had higher aspalathin, iso-orientin, and orientin concentrations than bottles sampled after a shorter storage period (i.e., 37 and 44 days, not expired).

Effect of Heat Treatment and Product Formulation on Flavonoid Content and Color of Experimental Rooibos Iced Teas. The average composition of the rooibos extract powder used for preparation of the experimental iced teas was 0.90% aspalathin, 0.65% iso-orientin, and 1.06% orientin. Its total polyphenol content was 26.30 g of GAE/100 g of extract. The average aspalathin, iso-orientin, and orientin contents, as well as absorbance and pH of the iced tea formulations B, BC, and BCA (before heating = control), are listed in **Table 3**.

All three heat treatments led to a significant ($P < 0.05$) increase in the absorbance (i.e., browning) of formulation B (**Figure 3a**). NTS caused a significantly ($P < 0.05$) larger increase in the absorbance of formulation B than pasteurization. Pasteurization also significantly ($P < 0.05$) increased (8.5%) the absorbance of formulation BC. The absorbance of BC and BCA was unaffected by NTS, but significantly ($P < 0.05$) decreased by HTS (12.3 and 12.4%, respectively).

The aspalathin content of all three formulations was significantly ($P < 0.05$) reduced after NTS and HTS treatments (**Figure 3b**). In both cases, the largest reductions were observed for formulation B, that is, 78.5 and 78.1% for NTS and HTS, respectively. Pasteurization, on the other hand, led to a small, but significant ($P < 0.05$) reduction (5.7%) in the aspalathin content of formulation B, whereas its content unexpectedly increased significantly ($P < 0.05$) by 7.2 and 11.0% in formulations BC and BCA, respectively. No significant difference ($P \geq 0.05$) was observed between BC and BCA subjected to pasteurization and NTS.

The iso-orientin content of formulations B, BC, and BCA also decreased significantly ($P < 0.05$) with NTS and HTS (**Figure 3c**). Overall, loss of this compound was less than that of aspalathin, irrespective of the heat treatment × formulation combination. Losses varied between 3.7 and 9.8%. Small but significant ($P < 0.05$) increases in the iso-orientin content of all formulations, varying between 2.4 and 5.2%, were observed in pasteurized iced tea. However, formulation had no effect on the percentage change in the case of pasteurized iced tea. The iso-orientin content of some formulations within a heat treatment, for example, formulations BC and BCA of NTS and formulations B and BC of HTS, did not differ significantly ($P \geq 0.05$).

NTS and HTS treatments of formulations BC and BCA (**Figure 3d**) led to small but significant ($P < 0.05$) decreases in their orientin content (1.3−3.7%). Pasteurization, on the other hand, either had no effect (formulation BC) or led to significantly ($P < 0.05$) increased values (1.2% for B and 2.7% for BCA). The percentage change observed for formulation B was not affected by heat treatment.

Figure 4 shows the chromatographic profile of formulation BCA at 288 and 350 nm before (**Figure 4a**) and after (**Figure 4b**) HTS. This treatment resulted in a substantial increase in the peak area of an unknown compound (compound **1**) eluting at 2.5 min (λ_{max} = 285 nm) (**Figure 4c**). Compound **1** did not absorb at 350 nm, and its UV−visible spectrum did not resemble that of the flavanones, dihydro-orientin (**Figure 4c**) and dihydroiso-orientin (not shown). The increase in peak area of compound **1** was less prominent in formulation BC and not very obvious on the chromatogram of formulation B. NTS treatment, but not pasteurization, produced similar changes in the chromatogram of rooibos iced tea. Peak 5 is presumed to be ill-defined polymeric phenolic substances on the basis of the retention time of an isolated rooibos tannin fraction (unpublished results).

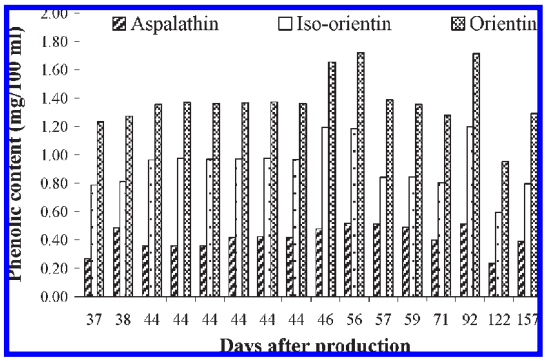

Figure 2. Aspalathin, iso-orientin, and orientin contents of commercial fermented rooibos iced tea (brand H) arranged by production date. Each bar represents a single iced tea sample.

Table 3. Absorbance, pH and Aspalathin, Iso-orientin, and Orientin Contents of the Different Fermented Rooibos Iced Tea Formulations before Heat Treatment (Controls)

parameter	B[a]	BC[b]	BCA[c]
aspalathin[d]	0.89 ± 0.01	0.89 ± 0.01	0.90 ± 0.01
iso-orientin[d]	0.67 ± 0.01	0.64 ± 0.00	0.64 ± 0.01
orientin[d]	1.07 ± 0.01	1.07 ± 0.01	1.05 ± 0.00
absorbance[e]	0.67 ± 0.01	0.45 ± 0.02	0.43 ± 0.03
pH	5.05 ± 0.02	2.95 ± 0.03	2.95 ± 0.02

[a] Base = rooibos extract in deionized water. [b] Base + citric acid. [c] Base + citric acid + ascorbic acid. [d] Results expressed in g/100 g of extract. [e] Measured at 420 nm.

The unexpected increase in the aspalathin content of certain iced tea formulations after pasteurization (first heating experiment) prompted reinvestigation. A closed system was simulated with the use of sealed containers, eliminating the possibility of evaporation as a cause for an increase in flavonoid content. After the 5 min pasteurization treatment, the absorbance of formulation B increased significantly ($P < 0.05$), whereas the values of BC and BCA remained unchanged. However, after the 30 min heat treatment, the absorbance values of all formulations were significantly ($P < 0.05$) increased (**Figure 5a**). The increase in absorbance for formulations containing citric acid was significantly ($P < 0.05$) more than for formulation B, as well as the 5 min pasteurization for formulation B. The aspalathin content of formulation B decreased only slightly but significantly ($P < 0.05$) after 5 min (4.0%), whereas BC and BCA remained unaffected. Pasteurization for 30 min reduced the aspalathin content of formulation B by 39.7%, whereas that of BC remained unchanged and BCA increased significantly ($P < 0.05$) (3.0%) (**Figure 5b**).

Pasteurization for 5 min led to a small (2% or less) but significant ($P < 0.05$) reduction in both the iso-orientin and orientin contents of formulations B, BC, and BCA (**Figure 5c**,**d**). Only the iso-orientin content was significantly ($P < 0.05$) affected by heat treatment for 30 min. The respective increases in iso-orientin contents of BC and BCA were 1.6 and 1.9%.

DISCUSSION

Commercial Rooibos Extracts and Iced Teas. Several brands of fermented rooibos iced teas are on the market in South Africa. The actual rooibos extract content of these beverages is not yet regulated by law. Subsequently, no tests are carried out by the regulatory authority to ensure that products reaching the market contain rooibos as indicated on the label or that a certain

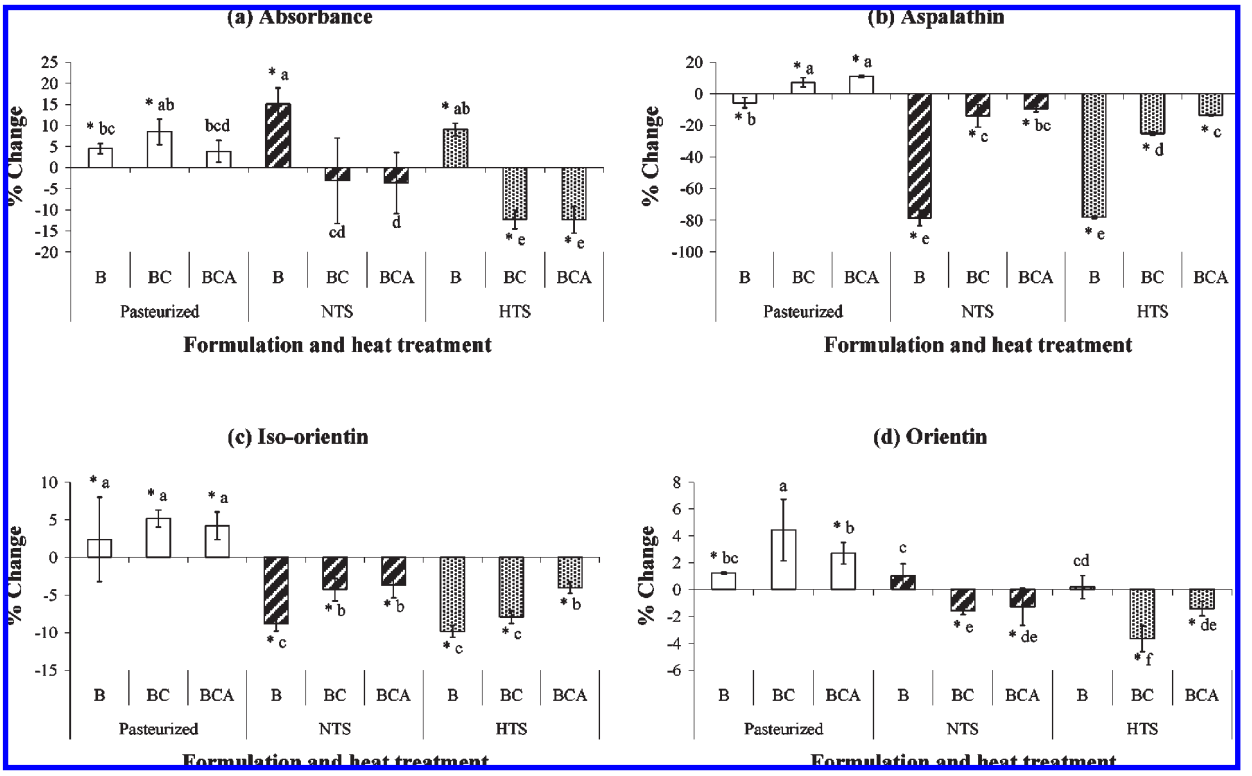

Figure 3. Effect of formulation and heat treatment compared to the unheated control on the percentage change in (**a**) absorbance (420 nm) and (**b**) aspalathin, (**c**) iso-orientin, and (**d**) orientin contents of fermented rooibos iced tea. B, base (rooibos extract in deionized water); BC, base + citric acid; BCA, base + citric + ascorbic acid; pasteurized, pasteurization (93 °C/30 min); NTS, normal-temperature sterilization (121 °C/15 min); HTS, high-temperature sterilization (135 °C/4 min). Bars labeled with an asterisk (∗) indicate values differing significantly ($P < 0.05$) from controls as a result of heat treatment, whereas treatment × formulation combinations differing significantly ($P < 0.05$) are indicated by different letters.

minimum quantity is present. The consumer therefore has no guarantee that the beverage actually contains rooibos extract. Analysis of a number of the major branded products showed that one brand (D) did not contain detectable levels of aspalathin, iso-orientin, or orientin, whereas some of the other brands either contained no detectable levels of aspalathin and/or one of the flavones.

The use of a small quantity of extract or of extract containing low concentrations of the flavonoids in question may be responsible for the inferior phenolic quality of these beverages. The total absence of detectable levels of the flavonoids even suggested that, in some instances, no rooibos extract had been added. Samples collected at the various stages of the commercial manufacturing process of the powdered rooibos extract all contained aspalathin, confirming that this compound should be present in extracts used for the production of commercial rooibos iced tea and, thus, in the iced tea itself. Another factor determining the extract quality is the phenolic content of the raw material. Analysis of a large number of samples of fermented rooibos plant material indicated that aspalathin is present in various quantities (*8*), which would subsequently affect the flavonoid content of the powdered extract. Large variation in iso-orientin and orientin contents is also to be expected. Screening of raw material for extract production on the basis of flavonoid content could thus ensure production of extract with sufficient levels of flavonoids.

Although brands A, G, E, and H all contained rooibos flavonoids, their concentrations were extremely low, mostly less than would be found in a cup of rooibos tea. Typically, 100 mL of rooibos beverage at "tea cup strength" may contain 0.70−2.06 mg of aspalathin, 0.32−1.67 mg of orientin, and 0.11−1.39 mg of iso-orientin (*8*). Closer inspection of a number of production samples of brand H produced on different dates revealed that no pattern exists with respect to flavonoid content. Both higher and lower values were observed shortly after production (well before expiration), as well as shortly prior to expiration. This indicates that aspalathin will survive a shelf-life period of at least 3 months. Factors other than storage time contribute to this absence of a pattern with regard to flavonoid content. This aspect requires further investigation.

Because aspalathin is unique to rooibos, its presence in a product is an indication that the product contains measurable quantities of rooibos extract. However, its absence does not necessarily imply that the product contains no rooibos extract, because the stability of aspalathin is a deciding factor. A better indication of the presence of rooibos is the presence of iso-orientin and orientin. Not only do they occur naturally in rooibos plant material (*9*), but as oxidation products of aspalathin (*5*) they will be more stable during processing and storage. Although these compounds occur in other plants as well, they are not present in other ingredients normally used in rooibos iced tea production. They therefore have potential as marker compounds for testing of product authenticity.

Effect of Heat and Product Formulation on Phenolic Content and Color of Experimental Rooibos Iced Teas. The pH values of the experimental iced teas containing ascorbic and/or citric acid were low enough so that pasteurization could be used to produce shelf-stable products. However, the samples were also subjected to extreme heating conditions, not normally employed for this type of product. Not only is a change in phenolic composition and thus color more likely, but retention of aspalathin, iso-orientin, and orientin under such extreme conditions would be a further confirmation that it would not be unreasonable to expect their presence in commercial rooibos iced teas.

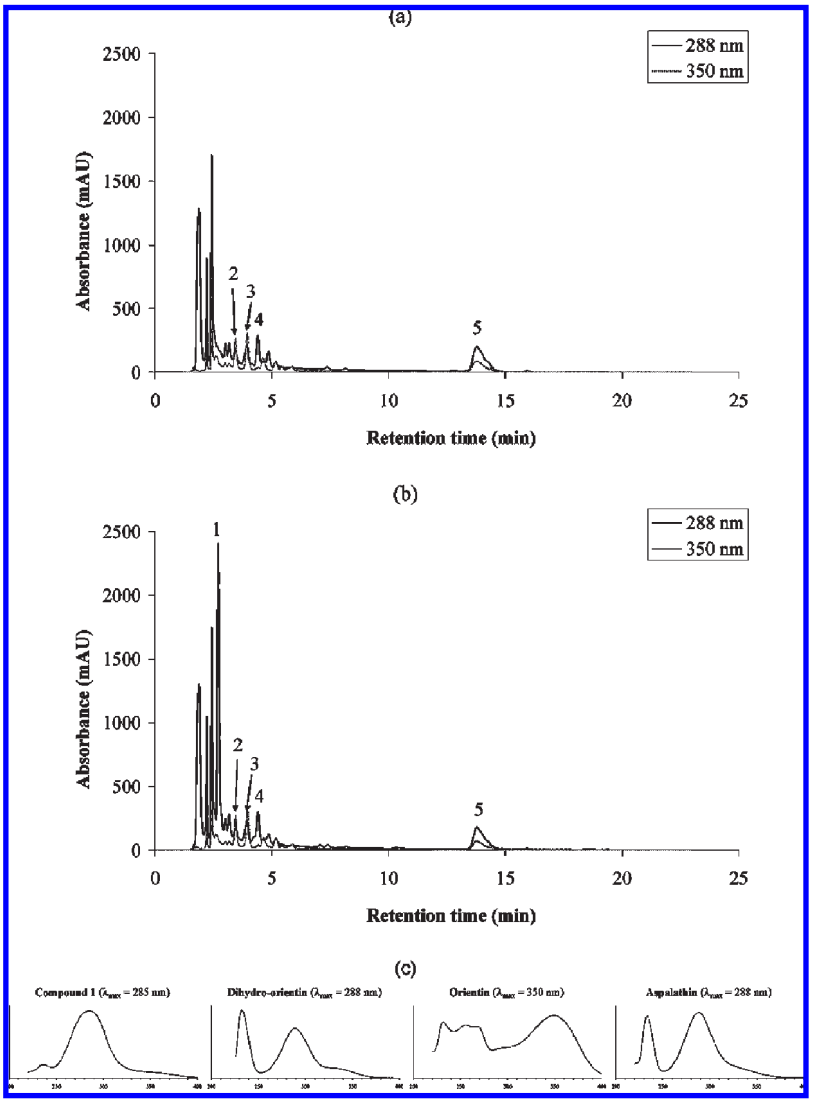

Figure 4. Chromatograms of formulation BCA of fermented rooibos iced tea (**a**) before and (**b**) after HTS, as well as (**c**) spectra of compound **1**, dihydro-orientin, orientin, and aspalathin. Peaks indicated on the chromatograms are (1) compound **1**, (2) iso-orientin, (3) orientin, (4) aspalathin, and (5) polymeric compounds (tentative identification).

The severity of the heat treatments, as well as formulation, clearly affected the extent and even the direction of absorbance change. Formulation B, irrespective of heat treatment, exhibited increased absorbance (browning) with heating. This increase in absorbance indicated a change in phenolic composition, that is, oxidative changes and possibly also formation of brown polymers. Conversion of aspalathin in solution at room temperature takes place slowly, eventually resulting in the formation of ill-defined brown products (*3*, *4*). Incubation of aspalathin in a phosphate buffer solution (pH 7.4) for 48 h at 37 °C also leads to the formation of uncharacterized, brown material (*5*). Furthermore, in a complex mixture such as rooibos extract, which contains a large number of phenolic compounds, it is possible that aspalathin could react with other flavonoids, including orientin and iso-orientin, to form polymeric substances. The formation of polymeric compounds from a chalcone and an unidentified flavonoid has been demonstrated (*10*). The main oxidation products of phloridzin, a dihydrochalcone glucoside, present in apples, were yellow monomers (*11*).

With the addition of ascorbic acid and/or citric acid absorbance either remained unchanged (no significant browning) or decreased when the iced tea was subjected to NTS and HTS, respectively. This could only be explained if it is assumed that, at the lower pH values of these solutions compared to formulation B, the polymers are to some extent less soluble, leading to their precipitation and subsequent removal by centrifugation prior to absorbance reading. Condensed tannin solubility is lower at lower pH due to suppression of ionization (*12*). Rooibos tannin is of the procyanidin type (*13*). No change would be observed when the increase in absorbance due to the formation of polymers is balanced by a decrease in absorbance as a result of their precipitation as for NTS. The severity of HTS, however, would not necessarily result in increased color formation, but could increase the formation of insoluble polymers, leading to the observed decrease in absorbance. During the manufacture of apple concentrate, procyanidins can polymerize to form insoluble compounds, which precipitate (*14*).

As for absorbance, pasteurization did not affect the aspalathin, iso-orientin, or orientin content of rooibos iced tea as greatly as NTS or HTS. Of the different formulations, the aspalathin content of B was the most negatively affected. Generally, the heat stability of dihydrochalcones (and phenolic compounds in general) is greater at lower pH values (*15*, *16*). At less acidic pH values deprotonation and subsequent oxidation of flavonoids proceed more easily (*17*). Dihydrochalcones appear to be relatively resistant to mild heat treatments.

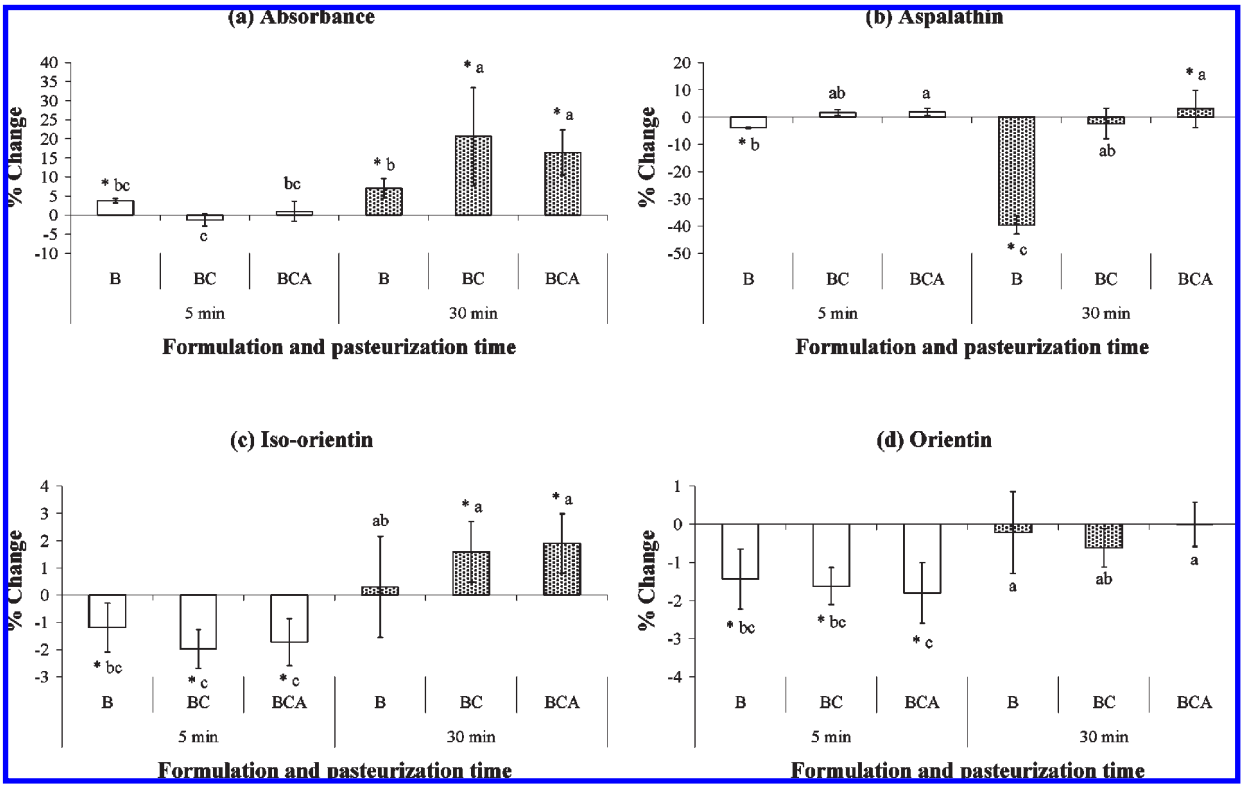

Figure 5. Effect of formulation and pasteurization time (in a closed system) compared to the unheated control on the percentage change on (**a**) absorbance (420 nm) and (**b**) aspalathin, (**c**) iso-orientin, and (**d**) orientin contents of fermented rooibos iced tea. B, base (rooibos extract in deionized water); BC, base + citric acid; BCA, base + citric + ascorbic acid. Bars labeled with an asterisk (∗) indicate values differing significantly ($P < 0.05$) from controls as a result of heat treatment, whereas treatment × formulation combinations differing significantly ($P < 0.05$) are indicated by different letters.

Neohesperidin dihydrochalcone did not undergo hydrolysis in juice-based drinks after 1 h of heating at 90 °C or after pasteurization at temperatures ranging from 60 °C (4 h) to 100 °C (45 min) (*18*). Furthermore, the presence of ascorbic acid (a reducing agent) in formulation BCA may have limited oxidation. The decrease in aspalathin content of formulation B with pasteurization (in the open system) may explain some of the observed increase in iso-orientin and orientin contents, considering conversion of aspalathin to iso-orientin and subsequently to orientin (*5*). However, with ascorbic acid and citric acid protecting aspalathin against extensive degradation, the increase in aspalathin content observed in formulations BC and BCA with pasteurization suggests its release from an association with other compounds (perhaps proteins) in the rooibos matrix upon heating, which would otherwise have been removed during filtration before HPLC analysis. Release is also a possibility for the flavones, which together with partial conversion of aspalathin, would explain the increase in their content in the BC and BCA formulations during pasteurization. Currently, however, there is no evidence for this, and this aspect needs further investigation.

Another factor that could contribute to an apparent increase in flavonoid content is evaporation during pasteurization, as the circulating system was not sealed. The use of sealed vials in the second pasteurization experiment, however, precluded loss of water through evaporation. Under these conditions the percentage change in the aspalathin content was either less compared to pasteurization in the open system or not significant, indicating that although evaporation could have contributed to the apparent increase in the first experiment, release from association with other compounds in the extract also played a role to balance out some of the loss with heating. Iso-orientin showed a similar trend for both pasteurization experiments (heating for 30 min), with the percentage increase in its content more pronounced with the open system. However, orientin content was not significantly affected in the closed pasteurization system, suggesting that its formation due to conversion of iso-orientin and any loss due to degradation or oxidation were canceled out. To establish unequivocally the stability of iso-orientin and orientin during heat treatment, and to exclude the role of aspalathin, and the extent of conversion of iso-orientin to orientin, model solutions containing the individual compounds, and not mixtures, will be investigated in the future.

The large decrease in the aspalathin, iso-orientin, and orientin contents of the iced tea samples subjected to NTS and HTS is most likely a consequence of the severity and duration of these heat treatments. The much smaller losses of iso-orientin and orientin compared to aspalathin may partly be attributed to the conversion of aspalathin to these flavones. Formation of orientin from iso-orientin (*5*) could explain why the percentage change in orientin content was less than for iso-orientin. The formation of new compounds, among others, compound **1** with a UV−visible spectrum similar to that of a phenolic acid, suggests most likely the degradation of aspalathin.

On the basis of the results it may thus be speculated that industrial pasteurization would not have a great effect on the phenolic composition of rooibos iced tea. This again emphasizes the importance of using rooibos extracts of good phenolic quality to produce iced teas with health-promoting properties comparable to those of a cup of freshly brewed rooibos.

ABBREVIATIONS USED

B, base (rooibos extract in deionized water); BC, base + citric acid; BCA, base + citric acid + ascorbic acid; GAE, gallic acid equivalents; HTS, high-temperature sterilization; NTS, normal temperature sterilization; TP, total polyphenol.

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