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Variation in Phenolic Content and Antioxidant Activity of Fermented Rooibos Herbal Tea Infusions: Role of Production Season and Quality Grade

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ABSTRACT: Data are required to calculate the dietary exposure to rooibos herbal tea flavonoids and phenolic acids. Representative content values for the principal phenolic compounds and total antioxidant capacity of fermented rooibos infusion, taking into account variation caused by production seasons (2009, 2010, and 2011) and quality grades (A, B, C, and D), were determined for samples (n = 114) from different geographical areas and producers. The major phenolic constituents were isoorientin and orientin (>10 mg/L), with quercetin-3-*O*-robinobioside, phenylpyruvic acid glucoside, and aspalathin present at >5 mg/L. Isovitexin, vitexin, and hyperoside were present at <3 mg/L. Rutin, ferulic acid, and isoquercitrin were present at <2 mg/L. Nothofagin was present at <1 mg/L. Only traces of luteolin-7-*O*-glucoside and the aglycones quercetin, luteolin, and chrysoeriol were present. Substantial variation was observed in the individual content values of the phenolic compounds and total antioxidant capacity within production seasons and quality grades.

KEYWORDS: *Aspalathus linearis, rooibos, phenolic compounds, total antioxidant capacity, DPPH, ORAC*

INTRODUCTION

The herbal tea, rooibos, made from the indigenous South African fynbos plant *Aspalathus linearis* (Burm. F) Dahlg. (Fabaceae), has gained tremendous popularity on South African and global markets during the past 20 years. It is available on the market in green and "fermented" (oxidized) forms, with the latter comprising the bulk of production and exports (>95% of exports). Cultivation through seedlings takes place mainly in the Greater Cederberg Biodiversity Corridor, an area covering sandy plains to mountains but with dry, hot summers, essential for on-farm open-air processing, including sun-drying.[1]

A major contributing factor to increasing market share of rooibos is greater consumer awareness of its potential health-promoting properties, among others its antioxidant activity.[1] Recently, rooibos was included in an antioxidant food table containing data on the total antioxidant content of more than 3100 foods, beverages, spices, herbs, and supplements procured worldwide, developed by a Finnish research group.[2] Their antioxidant activity data are based on the ferric reducing antioxidant potential (FRAP) of the products. Wanjiku[3] compiled a database of antioxidant capacities of selected South African beverages, including rooibos, based on three assays, i.e., oxygen radical absorbance capacity (ORAC), FRAP, and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical cation scavenging activity. Quantitative data for the beverages were limited to content values for phenolic groups determined with spectrophotometric assays.

Databases are essential to calculate polyphenol intake by target populations and to determine the association with health and disease in epidemiological studies. Databases that have been developed specifically for the content of individual polyphenols and flavonoids in the diet include the United States Department of Agriculture (USDA) database on flavonoids, Phenol-Explorer, EuroFIR-Basis, and the Brazilian flavonoid database.[4−7] With regard to tea and tea-like infusions, data are currently restricted to green, black, and oolong tea prepared from *Camellia sinensis* plant material.[4,6,7]

To date, no epidemiological studies have been undertaken to confirm the health benefits of rooibos consumption. Insufficient data are available to calculate intake values for rooibos and its relative contribution to the dietary intake of specific polyphenols compared to other food sources. Rooibos is the only known source of aspalathin, a dihydrochalcone *C*-glucoside, one of its major flavonoids and antioxidants. Aspalathin also plays an essential role as a precursor in the color formation of fermented rooibos.[8] Occurrence of the phenylpropenoic acid glycoside, phenylpyruvic acid glucoside (PPAG) in beverages, is limited to rooibos.[9] This rooibos constituent has gained prominence for its antidiabetic properties.[10]

Estimating the intake of individual flavonoids and phenolic acids when consuming a hot water infusion of fermented rooibos is the first step toward documenting their protective effects against the risk inherent in lifestyle diseases. Quantitative

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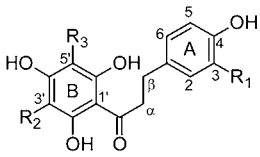
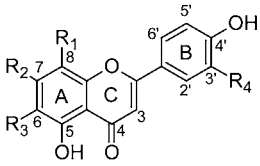
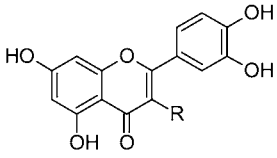
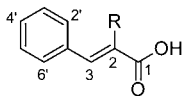
Table 1. Structures of the Rooibos Phenolic Compounds

General structure	Phenolic compound	Substituents
	**Dihydrochalcones**	
	nothofagin	R_1 = H, R_2 = *C*-β-D-glucosyl
	aspalathin	R_1 = OH, R_2 = *C*-β-D-glucosyl
	Flavones[a]	
	orientin (luteolin-8-*C*-glucoside)	R_1 = *C*-β-D-glucosyl, R_2, R_4 =OH, R_3 = H
	isoorientin (luteolin-6-*C*-glucoside)	R_1 = H, R_2, R_4 =OH, R_3 = *C*-β-D-glucosyl
	vitexin (apigenin-8-*C*-glucoside)	R_1 = *C*-β-D-glucosyl, R_2 = OH, R_3, R_4 = H
	isovitexin (apigenin-6-*C*-glucoside)	R_1, R_4 = H, R_2 = OH, R_3 = *C*-β-D-glucosyl
	luteolin	R_1, R_3 = H, R_2 = R_4 = OH
	luteolin-7-*O*-glucoside	R_1, R_3 = H, R_2 = *O*-β-D-glucosyl, R_4 = OH
	chrysoeriol	R_1, R_3 = H, R_2 = OH, R_4 = OCH_3
	Flavonols	
	quercetin	R = OH
	isoquercitrin (quercetin-3-*O*-glucoside)	R = *O*-β-D-glucosyl
	hyperoside (quercetin-3-*O*-galactoside)	R = *O*-β-D-galactosyl
	rutin (quercetin-3-*O*-rutinoside)	R = *O*-β-D-rutinosyl
	quercetin-3-*O*-robinobioside	R = *O*-β-D-robinobiosyl
	Phenylpropanoid	
	phenylpyruvic acid-2-*O*-glucoside (PPAG)	R = 2-*O*-β-D-glucosyl

[a]General numbering of the C skeleton of the flavones is also applicable for those of the flavonols.

data for infusions available in the literature are limited to a few flavonoids[11] or a small number of samples.[12] Factors such as production season and quality grades are usually not taken into account. Quality grading, performed by expert graders from industry, entails evaluating the appearance of the dry and infused rooibos leaves and the overall color and flavor of the rooibos infusions.[13] The different parameters carry different weights, with flavor being the most important. Grades A and D represent the highest and lowest grades, respectively. Most of the rooibos produced annually are of grades B and C. Grade D is mostly sold for extract production. Joubert and De Beer[11] demonstrated that quality grade affects phenolic composition and total antioxidant capacity (TAC) of hot water fermented rooibos extracts, prepared according to simulated industrial conditions.

Therefore, the aim of this study was to generate representative content values for the principal monomeric phenolic compounds present in a "cup-of-tea" rooibos infusion as normally consumed. Infusions of a large number of fermented rooibos production batches of different production seasons and quality grades, prepared according to recommended practice of the South African Rooibos Council at a "one-cup-serving" strength, were analyzed by a new high-performance liquid chromatography (HPLC)−diode array detector (DAD) method that we recently developed specifically for routine quantification of the 15 principal rooibos phenolics (Table 1).[14] Validation of this method is also described in the present paper. The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging and ORAC assays were employed to determine the TAC of the infusions. Samples were obtained from different geographical areas and different producers to capture as much potential variation in the phenolic composition and TAC as possible, to create a representative data set suitable for inclusion in food composition databases.

MATERIALS AND METHODS

Chemicals and Water. Deionized water, prepared using an Elix (Millipore, Milford, MA) water purification system, was further purified to HPLC-grade using a Milli-Q academic (Millipore) water purification system. HPLC-gradient-grade acetonitrile was purchased from Merck (Darmstadt, Germany), while analytical-grade reagents and chemicals, i.e., Folin−Ciocalteu reagent, DPPH, 2,2′-azobis-(2-methylpropionamide)dihydrochloride (AAPH), sodium fluorescein, (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), ascorbic acid, and glacial acetic acid, were supplied by Merck or Sigma-Aldrich (St. Louis, MO). Isovitexin, hyperoside, orientin, luteolin, and chrysoeriol standards were obtained from Extrasynthese (Genay, France). Vitexin, isoorientin, and luteolin-7-*O*-glucoside were obtained from Roth (Karlsruhe, Germany). Gallic acid, Trolox, ferulic acid, rutin, quercetin, and isoquercitrin were obtained from Sigma-Aldrich. Enolic phenylpyruvic acid-2-*O*-glucoside (PPAG) was isolated [purity > 95% by HPLC and liquid chromatography−mass spectrometry (LC−MS)] and supplied by the Post-Harvest and

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Table 2. Characteristics of Calibration Curves Obtained for HPLC Analysis of Rooibos Phenolic Standards

standard	wavelength (nm)[a]	linearity range (μg injected)	regression equation[b]	correlation coefficient, r^2
aspalathin	288	0.026−0.650	$y = 2205.64x - 1.34$	0.9999
nothofagin	288	0.025−0.619	$y = 1849.25x + 0.58$	0.9999
orientin	350	0.051−1.275	$y = 2983.38x + 0.16$	0.9999
isoorientin	350	0.050−1.256	$y = 2205.12x - 0.08$	0.9999
vitexin	350	0.025−0.630	$y = 2467.00x - 1.80$	0.9999
isovitexin	350	0.025−0.631	$y = 2425.36x - 1.46$	0.9999
isoquercitrin	350	0.025−0.623	$y = 1907.43x - 1.89$	0.9999
hyperoside	350	0.025−0.619	$y = 2148.55x - 1.00$	0.9999
rutin	350	0.025−0.628	$y = 1533.91x + 0.32$	0.9999
PPAG[c]	288	0.025−0.625	$y = 2796.95x - 1.80$	0.9999
ferulic acid	350	0.029−0.719	$y = 1828.60x - 0.59$	0.9999

[a]Wavelength used for quantification. [b]y = peak area (mAU), and x = amount of standard compound injected (μg). [c]Phenylpyruvic acid-2-*O*-glucoside.

Wine Technology Division of the Agricultural Research Council of South Africa (ARC Infruitec-Nietvoorbij, Stellenbosch, South Africa). Aspalathin and nothofagin (purity > 95% by HPLC and LC−MS) were supplied by the PROMEC Unit of the Medical Research Council of South Africa (Cape Town, South Africa).

Rooibos Plant Material. Unrefined, fermented rooibos [*Aspalathus linearis* (Brum. F) Dahlg.; family, Fabaceae; tribe, Crotalarieae] samples of different production batches (n = 114) of the cultivated Nortier type[1] were randomly selected during three production seasons (2009, n = 39; 2010, n = 40; and 2011, n = 35) from each of four quality grades (A, B, C, or D) to obtain 30 grade A and grade B samples each, 29 grade C samples, and 25 grade D samples. The different batches of rooibos were produced at different geographical locations. The samples were kindly supplied by a rooibos processing and marketing company. The plant material was sampled after processing by individual farmers but prior to refinement (sieving) and steam pasteurization by the processing and marketing company. Quality grading was performed according to their in-house grading system.[13]

Preparation of Steam-Pasteurized, Refined Rooibos Plant Material. In accordance with industry practice, unrefined fermented rooibos plant material (400 g) was passed through 10- and 40-mesh sieves for 1.5 min at 190 rpm using a SMC Mini-sifter (JM Quality Services, Cape Town, South Africa). The fraction >40 mesh and <10 mesh represents refined rooibos. A portion of each sample comprising the refined fraction was subjected to steam treatment at ca. 96 °C/2 min, followed by drying at 40 °C/20 min, as previously described,[11] simulating the pasteurization treatment used by industry.

Preparation of "Cup-of-Tea" Rooibos Infusions. Duplicate infusions were prepared according to a "one-cup-serving" strength by infusing 2.5 g of refined, pasteurized plant material for 5 min with 200 mL of freshly boiled, deionized water, without agitation. The infusions were decanted through a tea strainer, filtered through Whatman No. 4 filter paper (Whatman International, Ltd., Maidstone, U.K.), and cooled to room temperature. Aliquots (ca. 1.5 mL) of the infusions were stored at −20 °C until analysis.

Determination of Soluble Solids Content, Total Polyphenol Content, and TAC. The soluble solids content of the infusions was determined gravimetrically in triplicate using 20 mL aliquots.[11] The total polyphenol content of the infusions was determined in triplicate using the Folin−Ciocalteu method adapted for 96-well microplates.[15] The TAC of the infusions was determined in triplicate using the DPPH radical scavenging[15] and the ORAC[16] assays in 96-well microplate format. For the ORAC assay, 300 μL of deionized water was added to the outside wells of each plate to create a thermal barrier. A BioTek Synergy HT microplate reader with Gen5 software for data acquisition (Winooski, VT) was employed for absorbance and fluorescence readings.

HPLC Method Validation. HPLC−DAD analyses were performed on an Agilent 1200 system (Agilent, Santa Clara, CA) equipped with an in-line degasser, quaternary pump (maximum pressure of 400 bar), autosampler, column thermostat, and DAD (standard 13 μL flow cell and 10 mm path length), controlled by Chemstation software (Agilent Technologies, Waldbronn, Germany). Briefly, chromatographic conditions described in detail by Beelders et al.[14] were as follows: Separation was performed at 37 °C on a 100 × 4.6 mm, 1.8 μm Agilent Zorbax SB-C18 column protected with an Acquity ultra-performance liquid chromatography (UPLC) in-line filter (Waters, Milford, MA) and a 5.0 μm SB-C18 guard column (Agilent). The flow rate was 1.0 mL/min, and a multilinear gradient was performed as follows: 10% B (0−2 min), 10−14.8% B (2−19 min), 14.8−36.8% B (19−34 min), 36.8−100% B (34−37 min), 100% B isocratic (37−42 min), 100−10% B (42−45 min), and 10% B (45−50 min), with solvents A and B being 2% (m/v) acetic acid in water and acetonitrile, respectively.

Stock solutions of the phenolic standards were prepared in dimethylsulfoxide (DMSO) at concentrations of approximately 1 mg/mL and diluted with water according to experimental requirements. All diluted solutions were filtered through 0.22 μm polyvinylidene difluoride (PVDF) filters (Millipore) prior to use. Three standard calibration mixtures, each containing a different combination of phenolic standards at concentrations ranging between 0.012 and 0.026 μg/μL, and a randomly selected fermented rooibos infusion were used for method validation. Stability of the compounds in the standard calibration mixtures and a rooibos infusion was determined in the presence and absence of ascorbic acid. Ascorbic acid, added to prevent oxidative degradation, was added at final concentrations of 0.5 and 0.9 μg/μL in the standard mixtures and infusion, respectively.

Nine-point calibration curves were set up for all standards to test the linearity of the ultraviolet (UV)−DAD response. UV spectra were recorded between 200 and 700 nm with selective wavelength monitoring at 288 and 350 nm. The dihydrochalcones and PPAG were quantified at 288 nm, while the flavones, flavonols, and ferulic acid were quantified at 350 nm. The calibration mixtures were injected at different injection volumes (2, 5, 10, 15, 20, 25, 30, 40, and 50 μL), leading to levels of 0.025−1.20 μg on-column. Concentration ranges were selected to cover the different quantities of the compounds present in aqueous rooibos infusions. Linear regression, using the least-squares method (Microsoft Excel 2003, Microsoft Corporation, Redmond, WA), was performed on the calibration curve data for each compound to determine the slope, y intercept, and correlation coefficients (r^2).

Stability of the phenolic compounds, both as part of standard calibration mixtures and as a fermented rooibos infusion, was tested by repeated injection (n = 8) over 27 h (20 μL of standard calibration mixtures and 50 μL of rooibos infusion) with and without the addition of ascorbic acid. The percentage change (% change) in the peak areas over the 27 h period and the percentage relative standard deviation (% RSD) over eight time points during the period were used to evaluate the stability of the compounds.

Intra- and interday precision was determined by injecting the standard calibration mixtures (20 μL) and the rooibos infusion (50 μL), containing ascorbic acid, 6 times each on 3 consecutive days. The % RSD was determined for replicate injections on each day (intraday precision) and for mean values per day (interday precision) by considering the respective peak areas.

Quantification of Monomeric Phenolic Compounds. Aliquots of the rooibos infusions were thawed at room temperature and filtered through 0.22 μm PVDF filters (Millipore). Subsequently, 1 mL of the filtrate was pipetted into 1.5 mL amber autosampler vials, and 0.100 mL of 10% ascorbic acid (Sigma) in water (v/v) was added. All samples were analyzed within 24 h of sample preparation. The infusions were injected in duplicate using an injection volume of 50 μL. Phenolic compounds were identified by comparing their retention times and UV−visible (vis) spectra to those of authentic standards. Calibration curves were constructed weekly, as described in the preceding text. Quercetin-3-*O*-robinobioside was quantified using the calibration curve for rutin (quercetin-3-*O*-rutinoside), because no standard was available for this compound.

Statistical Analysis. Quantitative data were subjected to univariate analysis of variance (ANOVA) using SAS, version 9.2 (SAS Institute, Cary, NC). The Shapiro−Wilk test was performed to test for normality. Student's *t* test was used to calculate the least significant difference (LSD) at the 5% level to facilitate a comparison of mean values.

RESULTS AND DISCUSSION

Method Validation. Method validation was performed to ensure that the HPLC−DAD method was capable of producing reliable and reproducible results. Method validation parameters included linearity and range of the compounds in the standard calibration mixture, as well as stability and intra- and interday analytical precision of the compounds in the standard calibration mixture and rooibos infusion. Linearity of response, assessed by performing single measurements at nine analyte concentrations, was excellent (Table 2), with correlation coefficients >0.9999. The *y*-intercept values were also relatively low.

Data presented in Table 3 indicate that the stability of the phenolic compounds in the standard calibration mixtures without ascorbic acid was very poor. The percentage change in the respective peak areas over the 27 h period ranged between −7.01 and −24.45%, with the greatest loss observed for aspalathin. The % RSD ranged between 2.70% (isoorientin) and 9.49% (aspalathin), which signifies instability of the phenolic compounds when ascorbic acid is omitted from the standard calibration mixtures.

Conversely, the stability of the phenolic compounds in the aqueous rooibos infusion without ascorbic acid was remarkably better. A compound present in the rooibos infusion, tentatively identified as quercetin-3-*O*-robinobioside by LC−MS[14,17] and unequivocally by nuclear magnetic resonance (NMR),[18] was also stable over the considered time period. The change in peak area and RSD values calculated for most compounds were slightly higher than or equal to 2%. The exceptions were isoquercitrin and aspalathin, for which the changes were calculated as 7.1 and −18.9%, respectively. The apparent "increase" in isoquercitrin may be due to its small peak area and, hence, integration inaccuracy. The large decrease in the peak area of aspalathin indicated that this compound was particularly prone to oxidation, as previously demonstrated by Joubert.[19] The reason for the improved stability of the phenolic compounds as constituents of the rooibos infusion, as opposed to the standard calibration mixtures, may be attributed to matrix affects; i.e., unidentified constituents of the rooibos infusion may protect identified compounds from oxidation.

Table 3. Stability[a] of Phenolic Compounds as Constituents of Standard Calibration Mixtures and an Aqueous Infusion of Fermented Rooibos

		without ascorbic acid		with ascorbic acid	
compound	amount injected (μg)	% change[a]	% RSD[b]	% change[a]	% RSD[b]
Calibration Mixtures					
aspalathin	0.260	−24.45	9.49	0.52	0.19
nothofagin	0.248	−9.24	3.34	0.40	0.14
orientin	0.510	−8.06	2.90	1.23	0.38
isoorientin	0.504	−7.01	2.70	1.36	0.71
vitexin	0.252	−19.42	7.20	1.20	0.42
isovitexin	0.252	−19.56	7.24	0.42	0.21
isoquercitrin	0.249	−7.54	2.93	3.86	1.44
hyperoside	0.248	−7.47	2.90	3.55	1.29
rutin	0.251	−8.24	2.96	1.47	0.49
PPAG[c]	0.250	−15.07	5.66	3.47	1.15
ferulic acid	0.288	−7.68	3.15	0.06	0.18
Rooibos Infusion					
aspalathin	0.202	−18.90	7.52	0.85	0.49
nothofagin	0.074	−1.88	2.45	2.73	1.34
orientin	0.553	−0.02	0.52	−1.76	1.15
isoorientin	0.827	−0.74	0.49	−1.71	0.84
vitexin	0.120	−0.14	1.17	−3.43	1.80
isovitexin	0.148	−0.29	0.97	−2.52	1.25
isoquercitrin	0.018	7.10	4.24	1.23	1.44
hyperoside	0.087	0.67	0.74	−1.40	0.91
rutin	0.056	2.34	1.34	−0.69	1.87
ferulic acid	0.130	0.17	0.85	−2.40	1.26
PPAG	0.314	−0.34	0.47	−2.16	1.02
quercetin-3-rob[d]	0.404	−0.52	0.62	−1.45	0.93

[a]Percentage change in peak area. [b]Percentage relative standard deviation over eight time points during a 27 h period. [c]Phenylpyruvic acid-2-*O*-glucoside. [d]Quercetin-3-*O*-robinobioside quantified as rutin equivalents.

The stability of the phenolic compounds in the standard calibration mixtures and rooibos infusion containing ascorbic acid was very good (Table 3). The change in the respective peak areas of each compound over the 27 h period was less than 4%. The RSD values over the eight time points were all less than 2%, which indicates that no major decrease in any of the compounds occurred during this period. Note specifically the improved stability of aspalathin with the addition of ascorbic acid. Practically, the improved stability gained with the addition of ascorbic acid enables the preparation of a large number of samples simultaneously for injection with an autosampler, which greatly simplified sample preparation during subsequent routine analysis.

Precision criteria are usually RSD < 2%,[20] but values lower than 5% were deemed acceptable within the context of this study. Intra- and interday precision values were thus acceptable for all phenolic compounds (RSD < 4.4%; Table 4), from which it was concluded that the analytical precision of the HPLC−DAD method is good.

Quantification of Monomeric Phenolic Compounds. The optimized and validated HPLC−DAD method[14] was used for analysis of the infusions of fermented rooibos (n = 114), prepared at "one-cup-serving" strength to obtain data suitable

Table 4. Intra- and Interday Analytical Precision (% RSD) for HPLC Analysis of Phenolic Compounds as Constituents of Standard Calibration Mixtures and an Aqueous Infusion of Fermented Rooibos[a]

	calibration mixtures				rooibos infusion			
	intraday			interday	intraday			interday
compound	day 1 (n = 6)	day 2 (n = 6)	day 3 (n = 6)	pooled (n = 3)	day 1 (n = 6)	day 2 (n = 6)	day 3 (n = 6)	pooled (n = 3)
aspalathin	3.61	0.99	0.04	4.29	0.56	0.44	0.32	0.64
nothofagin	0.15	2.13	0.16	3.10	0.98	0.96	0.39	2.43
orientin	0.08	2.15	0.05	3.23	0.23	0.19	0.28	1.31
isoorientin	0.76	0.14	0.07	1.89	0.05	0.14	0.14	1.35
vitexin	3.30	0.84	0.05	4.28	0.87	0.97	0.83	0.91
isovitexin	3.33	0.89	0.03	4.33	0.57	0.65	0.59	1.09
isoquercitrin	0.75	0.48	0.22	1.15	2.49	3.51	1.80	2.10
hyperoside	0.79	0.43	0.18	1.03	0.62	0.68	1.01	1.43
rutin	0.18	2.19	0.23	3.44	0.82	1.67	1.61	1.26
PPAG[b]	2.86	0.63	0.22	3.59	0.54	0.51	0.65	0.64
ferulic acid	0.73	0.62	0.31	1.07	0.35	0.38	0.20	0.33
quercetin-3-rob[c]	*d*	*d*	*d*	*d*	0.17	0.23	0.40	0.60

[a]For concentrations of the compounds, refer to Table 2. [b]Phenylpyruvic acid-2-*O*-glucoside. [c]Quercetin-3-*O*-robinobioside quantified as rutin equivalents. [d]No quercetin-3-*O*-robinobioside in calibration mixtures.

for food composition databases (see Figure 1 for a typical chromatogram). Because all rooibos sold to consumers is

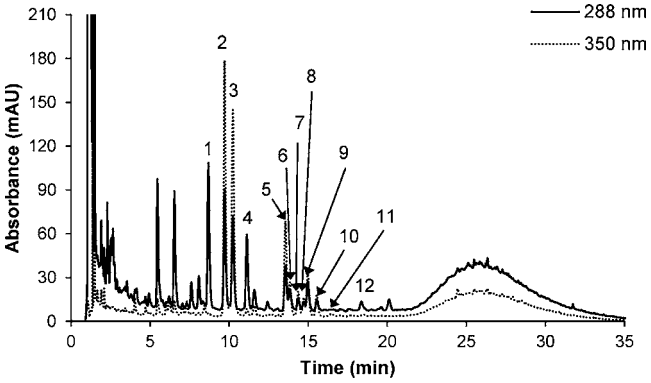

Figure 1. Typical HPLC chromatogram at 288 and 350 nm of a fermented rooibos infusion (1, phenylpyruvic acid-2-*O*-glucoside; 2, isoorientin; 3, orientin; 4, aspalathin; 5, quercetin-*O*-robinobioside; 6, vitexin; 7, hyperoside; 8, rutin; 9, isovitexin; 10, isoquercitrin; 11, luteolin-7-*O*-glucoside; and 12, nothofagin).

steam-pasteurized prior to packaging,[21] pasteurized rooibos was used for preparation of the infusions. Traditionally, loose-leaf rooibos was brewed for an extended period at low heat, but modern day consumers, largely with convenience in mind, prefer infusing one tea bag (ca. 2 g) per cup with freshly boiled water for 2−5 min to release flavor and color. For this reason, the rooibos industry has standardized on a 5 min infusion of 2.5 g of rooibos per 200 mL when compositional data of a "one-cup-serving" are required.

On the basis of mean values presented in Table 5, the major phenolic constituents of steam-pasteurized, fermented rooibos infusions are the flavone *C*-glycosides, isoorientin (average concentration of 15.03 mg/L) and orientin (average concentration of 10.84 mg/L). The higher value for isoorientin is in agreement with previous results from a small sample set (n = 20) of rooibos samples from the 2009 production year.[11] Bramati et al.,[12] on the other hand, found the orientin content of a fermented rooibos infusion higher than that of isoorientin. Quercetin-3-*O*-robinobioside, PPAG, and aspalathin were also present in relatively large average quantities of 8.34, 6.91, and 5.84 mg/L, respectively. Nothofagin was present at much lower quantities than aspalathin, as previously demonstrated.[11,12] Both of these dihydrochalcones are very susceptible to oxidation during fermentation of the plant material.[19] Recent papers provided greater insight into the mechanism and oxidation products of aspalathin, among others orientin and isoorientin.[22,23] However, the flavones also occur naturally in the unfermented plant material.[24] Aspalathin is expected to remain relatively stable during preparation of the infusion as the aspalathin content of a ready-to-drink rooibos iced tea containing no additives, such as ascorbic acid or citric acid, except sugar, decreased by less than 5% during pasteurization at 93 °C for 5 min.[25] Ferulic acid was present at the low average concentration of 1.51 mg/L in the infusions. This value is slightly overestimated, because partial coelution of ferulic acid and an unknown compound occurred for some of the samples. Luteolin-7-*O*-glucoside and the aglycones, quercetin, luteolin, and chrysoeriol, were only present in trace amounts and could therefore not be quantified accurately. This is in accordance with Toyoda et al.,[26] who reported small quantities of these aglycones in rooibos tea.

In the present study, substantial variation was observed in the individual content values of the phenolic compounds (Table 5). For instance, the minimum and maximum values for aspalathin and quercetin-3-*O*-robinobioside differed by factors of more than 15 and 20, respectively. This range was smaller for the other phenolic compounds (Table 5). Various factors known to influence the phenolic composition of plant material, i.e., environmental stress conditions and seasonal effects, could contribute to variation in the phenolic content, in addition to processing,[19] and the exclusive use of seedlings to propagate rooibos.[1]

Because the aim of this study was to characterize the phenolic composition of rooibos infusion at a "one-cup-serving" strength, the large degree of variation observed merely emphasizes the importance of using a large sample set. The samples of the present study were obtained from different geographical locations and were harvested over three production seasons to capture as much variation as possible. Different production seasons would allow for age of the bush

Table 5. Mean, Minimum, and Maximum Values[a] for the Phenolic Compounds, Total Polyphenols, Soluble Solids, and TAC of Aqueous Infusions of Fermented Rooibos, Including Values for Different Production Years

parameter	total (n = 114)[b]	2009 (n = 39)	2010 (n = 40)	2011 (n = 35)
aspalathin	5.84 ± 3.26 (nd[c]−15.66)	5.85 a[d] ± 2.25	5.48 a ± 3.59	6.17 a ± 3.89
nothofagin	0.95 ± 0.51 (nd−2.76)	1.10 a ± 0.37	1.02 a ± 0.63	0.69 b ± 0.40
orientin	10.84 ± 1.58 (10.33−14.31)	10.96 a ± 1.24	11.02 a ± 1.93	10.49 a ± 1.49
isoorientin	15.03 ± 2.50 (7.40−20.47)	14.85 b ± 1.73	14.33 b ± 2.75	16.05 a ± 2.67
vitexin	2.33 ± 0.37 (1.30−3.32)	2.27 a ± 0.31	2.42 a ± 0.46	2.31 a ± 0.32
isovitexin	2.40 ± 0.39 (1.35−3.29)	2.29 b ± 0.28	2.43 ab ± 0.45	2.50 a ± 0.39
isoquercitrin	1.08 ± 1.00 (nd−5.79)	1.23 a ± 0.98	0.96 a ± 0.85	0.90 a ± 0.86
hyperoside	2.22 ± 1.16 (nd−6.79)	2.01 a ± 1.28	1.53 ab ±1.20	1.39 b ± 1.11
rutin	1.65 ± 1.22 (nd−5.71)	2.25 a ± 1.00	2.24 a ± 1.18	2.15 a ± 1.34
PPAG[e]	6.91 ± 2.03 (2.72−14.81)	6.48 b ± 1.18	5.65 c ± 1.31	8.84 a ± 2.07
ferulic acid	1.51 ± 0.84 (nd−3.07)	1.35 a ± 0.62	1.55 a ± 0.58	1.48 a ± 0.72
quercetin-3-rob[f]	8.34 ± 3.66 (0.89−18.41)	8.97 a ± 3.16	8.42 a ± 3.82	7.54 a ± 3.94
total polyphenols[g]	296 ± 49 (159−471)	307 a ± 39	289 a ± 49	291 a ± 58
soluble solids	1172 ± 143 (843−1666)	1183 a ± 126	1218 a ± 150	1115 b ± 159
TAC_{DPPH}[h]	2205 ± 396 (1081−3464)	2199 a ± 292	2271 a ± 425	2227 a ± 453
TAC_{ORAC}[h]	10030 ± 1702 (5682−14636)	10199 ab ± 1224	10321 a ± 1783	9510 b ± 1976

[a]mg/L ± standard deviation. [b]Number of samples. [c]Not detected. [d]Means in the same row with the same letter are not significantly different (p > 0.05). [e]Phenylpyruvic acid-2-*O*-glucoside. [f]Quercetin-3-*O*-robinobioside quantified as rutin equivalents. [g]Gallic acid equivalents. [h]Total antioxidant capacity determined according to DPPH radical scavenging and ORAC assays expressed as micromolar Trolox equivalents.

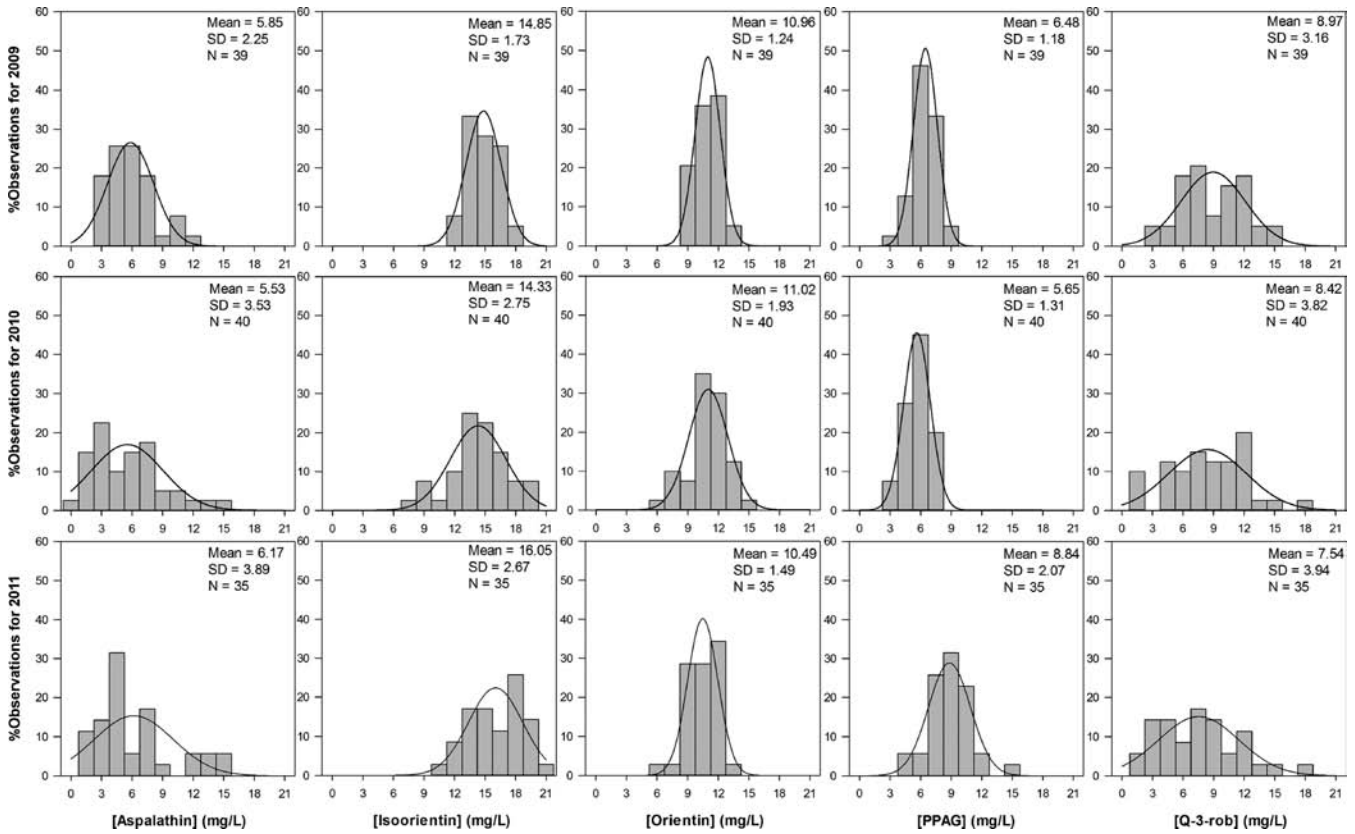

Figure 2. Distribution of the major phenolic constituents of steam-pasteurized, fermented rooibos infusions as a function of the production season (PPAG = phenylpyruvic acid-2-*O*-glucoside).

and influence of climate on the plant to be taken into consideration. The reported content values (Table 5) could therefore be considered representative of the amounts of phenolic compounds present in a typical "cup-of-tea" rooibos infusion.

Effect of Production Season. To investigate the effect of the production season on the phenolic composition of aqueous rooibos infusions, the samples were divided according to year, and mean values for the contents of the individual phenolic compounds are summarized in Table 5. No distinct trends were observed. The aspalathin, orientin, vitexin, isoquercitrin, ferulic acid, rutin, and quercetin-3-*O*-robinobioside contents were not significantly ($p \geq 0.05$) affected by the different production seasons. Mean values for nothofagin and hyperoside were

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Table 6. Mean Values[a] for Phenolic Compound, Total Polyphenol and Soluble Solids Contents, and TAC of Aqueous Infusions of Fermented Rooibos of Different Quality Grades

parameter	grade A (n = 30)[b]	grade B (n = 30)	grade C (n = 29)	grade D (n = 25)
aspalathin	7.70 a[c] ± 3.33	5.84 b ± 2.83	5.83 b ± 3.30	3.53 c ± 2.24
nothofagin	1.04 a ± 0.38	1.02 a ± 0.52	1.04 a ± 0.57	0.64 b ± 0.48
orientin	11.37 a ± 1.38	11.15 a ± 1.29	10.72 ab ±1.60	9.95 b ± 1.80
isoorientin	15.92 a ± 2.35	15.55 a ± 1.93	14.95 a ± 2.73	13.44 b ± 2.37
vitexin	2.45 a ± 0.36	2.42 a ± 0.26	2.22 b ± 0.39	2.21 b ± 0.42
isovitexin	2.53 a ± 0.35	2.49 a ± 0.32	2.35 ab ±0.43	2.22 b ± 0.40
isoquercitrin	1.82 a ± 1.11	0.98 b ± 0.65	0.69 b ± 0.55	0.59 b ± 0.62
hyperoside	3.22 a ± 1.41	2.06 b ± 0.67	1.66 b ± 0.78	1.83 b ± 0.97
rutin	2.47 a ± 1.50	1.57 b ± 0.88	1.38 b ± 0.95	1.08 b ± 1.01
PPAG[d]	7.29 a ± 2.09	7.39 a ± 1.98	6.86 a ± 2.27	5.94 b ± 1.40
ferulic acid	1.22 a ± 0.56	1.56 a ± 0.55	1.56 a ± 0.70	1.48 a ± 0.72
quercetin-3-rob[e]	11.07 a ± 3.58	8.17 b ± 2.83	6.89 b ± 3.00	6.95 b ± 3.70
total polyphenols[f]	332 a ± 47	298 b ± 35	282 bc ±36	266 c ± 52
soluble solids	1258 a ± 157	1170 b ± 137	1116 b ± 133	1146 b ± 138
TAC_{DPPH}[g]	2465 a ± 377	2234 b ± 314	2137 b ± 337	1939 c ± 394
TAC_{ORAC}[g]	10908 a ± 1808	10113 ab ±1378	9913 b ± 1558	9014 c ± 1583

[a]mg/L ± standard deviation. [b]Number of samples. [c]Means in the same row with different letters are significantly different ($p < 0.05$). [d]Phenylpyruvic acid-2-*O*-glucoside. [e]Quercetin-3-*O*-robinobioside quantified as rutin equivalents. [f]Gallic acid equivalents. [g]Total antioxidant capacity determined according to DPPH radical scavenging and ORAC assays expressed as micromolar Trolox equivalents.

highest for the 2009 production season, differing significantly ($p < 0.05$) from the values obtained for samples of the 2011 production season. Isoorientin and isovitexin were present at highest levels in 2011. PPAG was the only phenolic compound that exhibited significant differences ($p < 0.05$) over all three production seasons, with the highest content in 2011. Collectively, these results signify that data obtained from a single production season are, to a certain extent, representative. This was, in part, due to the random selection of samples employed in this study. Climatic conditions were similar during the respective production seasons. Decreasing rainfall and increasing temperatures, attributed to global warming, are experienced by the rooibos production area. This may, in the long term, result in greater dependence upon other ecotypes with greater resistance to drought.[27] Large variation has been demonstrated for the chemical composition of wild types of rooibos with some not containing aspalathin.[28]

The distributions of the contents of the major phenolic constituents (aspalathin, isoorientin, orientin, PPAG, and quercetin-3-*O*-robinobioside) as a function of the production season are illustrated in Figure 2. The Gaussian distributions for orientin and PPAG and, to a lesser extent, for isoorientin were narrow, with standard deviations (SDs) mostly less than 2. Conversely, the distributions for aspalathin and quercetin-3-*O*-robinobioside were broader (SD mostly >3), which were also exemplified by their minimum and maximum values (Table 5). This is indicative of larger natural variation in the concentration of these two major phenolic compounds in fermented rooibos plant material.

The production season had no significant effect on the total polyphenol content and TAC_{DPPH}, whereas the highest values for soluble solids content and TAC_{ORAC} were observed for the 2010 production season (Table 5). Similar values of TAC were obtained for a smaller sample set, comprising only samples of the 2009 production season.[11] The soluble solids content is useful to calculate "cup-of-tea" equivalents when rooibos extract is used in food products, such as yogurts and ready-to-drink beverages.

Effect of Quality Grade. The higher quality grade samples tend be to associated with higher levels of the phenolic compounds (Table 6). Grade A samples had the highest mean values for most phenolic compounds, excluding PPAG and ferulic acid. For the latter phenolic constituent, grade A samples had the lowest mean content value, although it did not statistically differ from the other quality grade samples. Grade A samples contained significantly ($p < 0.05$) higher levels of aspalathin, isoquercitrin, rutin, hyperoside, and quercetin-3-*O*-robinobioside than the other grades. The nothofagin, orientin, isoorientin, isovitexin, and PPAG contents of grades A, B, and C were not significantly ($p \geq 0.05$) different, while the vitexin content of grades A and B was significantly ($p < 0.05$) higher than that of grades C and D. It is therefore possible that, on the basis of the current quality grading system, these phenolic constituents are contributors to the positive quality attributes of the rooibos infusions. Conversely, the grade D samples had the lowest mean content values for most phenolic compounds, excluding hyperoside, quercetin-3-*O*-robinobioside, and ferulic acid. Mean values for hyperoside and quercetin-3-*O*-robinobioside were lowest in grade C samples, while the mean value for ferulic acid was lowest in grade A samples.

Considering mean values for total polyphenol content and TAC over the different quality grade samples (Table 6), it is evident that grades A and D are associated with the highest and lowest values, respectively. Grade B and C samples were not significantly different ($p \geq 0.05$). These grades constitute the largest percentage of the total production. As observed for the production season, large variation within a quality grade exists (Figure 3). Considering all parameters, including the individual phenolic compounds, the consumer could potentially benefit from drinking grade A rooibos tea. Whether these differences have a significant health impact needs to be determined. In the case of grades B and C, these parameters make no distinction between grades, and sensory quality would thus be the deciding factor. Koch et al.[13] reported that grade C rooibos has a higher "green" note than grade B rooibos, indicating possible under-fermentation.

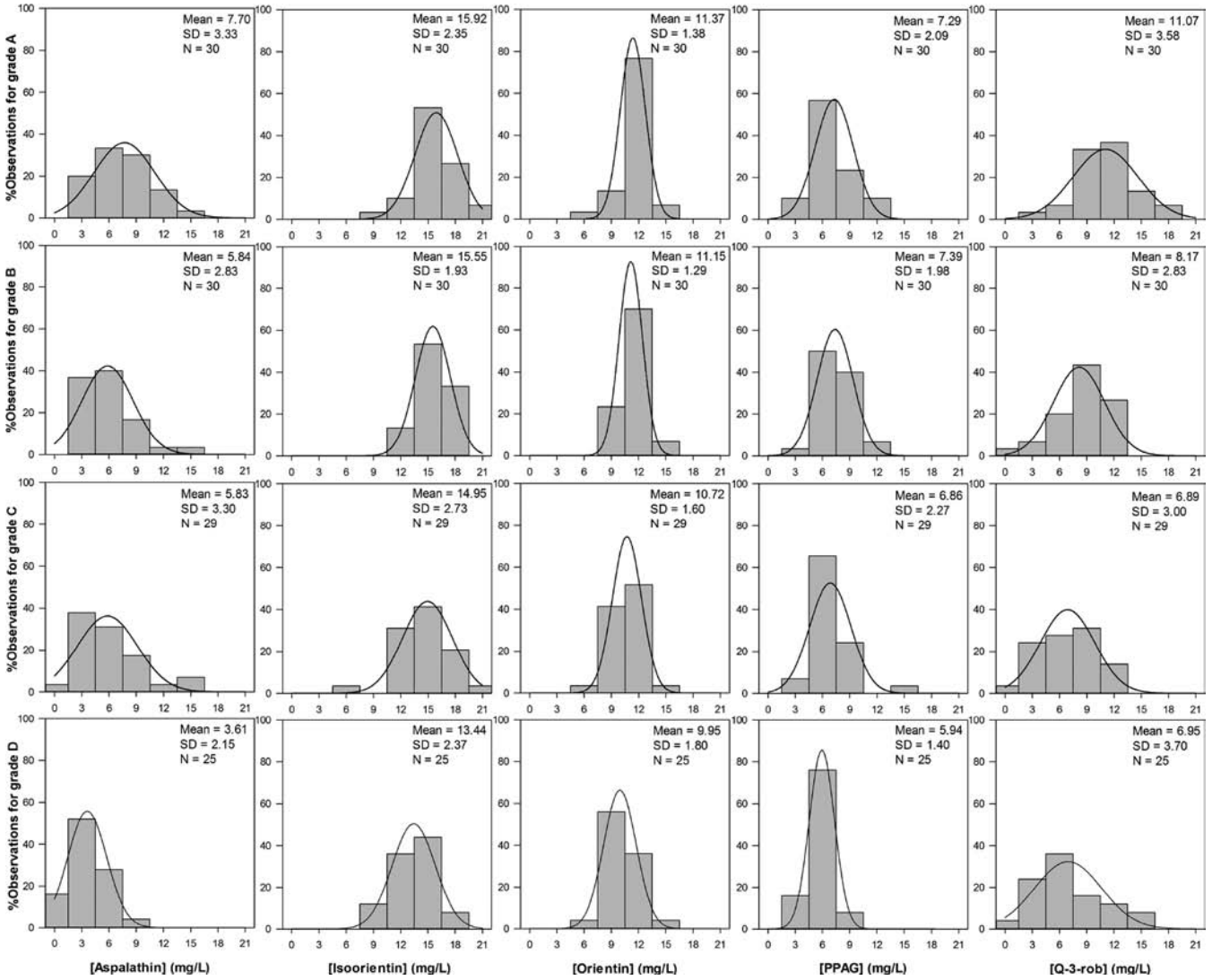

Figure 3. Distribution of the major phenolic constituents of steam-pasteurized, fermented rooibos infusions as a function of quality grade (PPAG = phenylpyruvic acid-2-*O*-glucoside).

The values presented for individual phenolic compounds captured the variation in phenolic composition of rooibos. Owing to the large number of representative samples analyzed, the data accurately reflect natural variation and are thus suitable for inclusion in food composition tables and calculation of dietary intake.

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Notes

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