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HPLC quantification of the dihydrochalcones, aspalathin and nothofagin in rooibos tea (*Aspalathus linearis*) as affected by processing

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An HPLC method was developed for determination of the *C*-glucoside dihydrochalcones, aspalathin and nothofagin, in rooibos tea. Gradient separation of the phenolic fraction was achieved on a reversed-phase C_{18} column. The polyphenolic fraction was prepared by extraction of the phenolic compounds with hot water, followed by liquid–liquid extraction with ethyl acetate. The method was applied to unprocessed, partially oxidized (unfermented) and fermented rooibos tea as well as tea dried in the sun and under controlled conditions. Aspalathin and nothofagin oxidation occurred as soon as the tea leaves were comminuted which resulted in browning as indicated by CIELAB parameters. The drying method had no effect on the degree of oxidation of aspalathin and nothofagin.

INTRODUCTION

Rooibos tea is produced from the foilage and the thin stems of the indigenous South African plant, *Aspalathus linearis* (Brum. fil) R. Dahlgr. spp *linearis*. Traditional processing, which involves comminution, bruising, 'fermentation' and drying, takes place in the open air. It is believed by tea processors that sunlight is necessary for the development of the characteristic red–brown colour of rooibos tea. However, processing of rooibos tea under controlled conditions in the absence of sunlight does not affect the colour (CIELAB chromaticity parameters) of the tea leaves (Joubert, 1994).

According to Koeppen (1970) the dihydrochalcone, aspalathin (2′,3,4,4′,6′-pentahydroxy-3-*C*-*β*-D-glucopyranosyldihydrochalcone), which is unique to rooibos tea, contributes significantly to the characteristic red–brown colour of processed rooibos tea. Aspalathin is the principal monomeric flavonoid in unprocessed rooibos tea (Koeppen & Roux, 1966). Recently Rabe *et al.* (1994) isolated it for the first time from processed rooibos tea. *β*-Hydroxy-dihydrochalcones are rare in nature and, apart from aspalathin, only pterosupin and nothofagin have been reported to be present in plant material (Yoshida *et al.*, 1993). Nothofagin, structurally similar to aspalathin except for the hydroxylation pattern of the B-ring (Fig. 1), is also present in unprocessed rooibos tea (Ferreira, personal communication, 1994). It was first isolated from the heartwood of *Nothofagus fusca*, at present the only other natural source of nothofagin (Hillis & Inoue, 1967). Other phenolic compounds present in rooibos tea are flavones which include iso-orientin, orientin (Koeppen *et al.*, 1962; Koeppen & Roux, 1965), vitexin, iso-vitexin, chrysoeriol (Rabe *et al.*, 1994) and luteolin (Snyckers & Salemi, 1974). Its flavonols include rutin, isoquercitrin (Koeppen *et al.*, 1962) and quercetin (Snyckers & Salemi, 1974), while the phenolic acids comprise *p*-hydroxybenzoic, protocatechuic, vanillic, caffeic, *p*-coumaric and ferulic acids (Rabe *et al.*, 1994).

Koeppen & Roux (1966) found that, in the presence of oxygen and sunlight, the flavanones 2,3-dihydro-iso-orientin and 2,3-dihydro-orientin were formed in an ethanolic solution of aspalathin, but with prolonged exposure to sunlight the flavanones were converted to unknown brown products. No conversion of nothofagin to the corresponding flavanones occurred under these conditions, but the compound turned brown after it was exposed to sunlight for a few days (Hillis & Inoue, 1967).

Quantitative data on the aspalathin and nothofagin content of rooibos tea are needed to elucidate their role in the fermentation (oxidation) process of the tea. The purpose of this study was to develop an HPLC method for the quantification of aspalathin and nothofagin in green and processed rooibos tea. The change in the aspalathin and nothofagin content of rooibos tea with

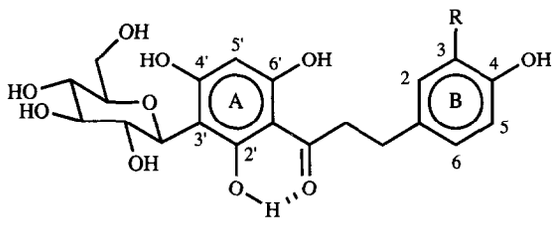

Fig. 1. Structure of the dihydrochalcones aspalathin (R = OH) and nothofagin (R = H) found in rooibos tea.

processing, as well as the effect of processing in the absence of sunlight, were also investigated.

MATERIALS AND METHODS

Sample preparation

Rooibos tea, differing in the degree of oxidation, was used for method development. Sample A consisted of green, unprocessed rooibos tea that was freeze-dried without first being comminuted. Partly oxidized tea was prepared by comminution of fresh, green tea allowing oxidation to take place during slow air drying at ambient temperature (sample B). No fermentation of sample B was carried out. Standard grade rooibos tea, obtained from the Rooibos Tea Board, was used as representative of fermented tea (sample C).

The following samples, prepared from a batch of freshly harvested rooibos tea, were used to determine the change in aspalathin and nothofagin content with processing: sample E (freeze-dried without comminution); sample F (comminuted *ca* 15 min before freezing at −30°C; then freeze-dried); sample G (comminuted *ca* 210 min before freezing at −30°C; then freeze-dried); and sample H (comminuted *ca* 210 min before fermentation; then fermented at 38°C for 14 h and air-dried at 40°C for 3 h).

The effect of drying in the absence of sunlight on the aspalathin and nothofagin content of rooibos tea leaves was studied using 16 batches of tea. Approximately 3.5 kg tea (65% moisture) were fermented at 40°C for 12–14 h, depending on the batch. A sample (250 g) of each batch was dried in a tray drier at 50°C for 3 h and sun-dried for 3 h, respectively.

Eleven batches of fresh, green rooibos tea, obtained from different farmers, were used to determine the change in leaf colour with comminution.

Reference standards

Authentic standards were obtained from Fluka (*p*-hydroxybenzoic acid, caffeic acid, ferulic acid), Sigma (protocatechuic acid, *p*-coumaric acid), Merck (rutin) and Dr Theodore Schuchart (vanillic acid). Aspalathin, vitexin, isoquercitrin, orientin and iso-orientin were isolated by the late Dr B. H. Koeppen from rooibos tea while 2,3-dihydro-iso-orientin was isolated by Koeppen after photochemical conversion of aspalathin (Koeppen & Roux, 1966). 2,3-Dihydro-iso-orientin isolated from processed rooibos tea (Rabe, 1992), and nothofagin (*ca* 80% purity) isolated from unprocessed rooibos tea, were obtained from the Research Unit for Polyphenol and Synthetic Chemistry of the University of the Orange Free State. All samples were dissolved in HPLC-grade methanol (BDH HiPerSolv) and stored at −18°C prior to injection.

Extraction of polyphenols from plant material

Polyphenols were extracted from samples A–C by pouring 250 ml hot, distilled water on 10 g tea and steeping it for 15 min on a steam bath. The water extracts were cooled to room temperature, made up to volume (250 ml) with distilled water, and filtered through Whatman No. 1 filter paper. An aliquot (200 ml) of each extract was pipetted into a liquid–liquid extraction apparatus and extraction carried out under reflux for 6 h with ethyl acetate (BDH Analar). The extracts were evaporated in vacuo to dryness at 38°C with a Büchi Rotavapor. The dry residues were dissolved in 20 ml HPLC-grade methanol (BDH HiPerSolv) and stored at −18°C until analyzed.

Extractions of ethyl acetate soluble solids of samples E–H were carried out in duplicate as described above, except that 10 g finely grounded tea was extracted with 400 ml water, followed by centrifugation (2100 **g**) before extraction. A similar procedure was used for the controlled-dried and sun-dried samples.

The soluble solid contents of the water extracts were determined as described by Joubert (1988). The scaled-down Folin–Ciocalteu assay (Singleton & Rossi, 1965) was used to determine total polyphenol content of the ethyl acetate extracts. Results were expressed as gallic acid equivalents (GAE).

Chromatographic conditions

Analyses were carried out with a Varian 5000 HPLC equipped with a UV-100 variable wavelength detector. Samples were injected with a manual Rheodyne valve (Model 7125) using a 10 μl loop. Peaks were monitored at 280 nm (0.05 AUFS) except when stated otherwise. A Hewlett-Packard 3390A integrator, operating at a chart speed of 0.2 cm min^{-1}, was used for peak integration. Separation was performed by solvent gradient elution (Table 1) at a flow rate of 0.4 ml min^{-1} on a Merck LiChrospher 100 RP-18 (5 μm) column (250×4 mm ID) with a guard column (Merck LiChrospher 100 RP-18; 5 μm). The column temperature was maintained at 38°C and reconditioning took place at a flow rate of 1.2 ml min^{-1}. Solvent A was pure HPLC-grade methanol (BDH HiPerSolv; UV transmission 98% at 260 nm). Solvent B consisted of 2% (v/v) formic acid (Merck; 88% m/v) in glass distilled water which was pretreated with a Norganic cartridge (Millipore) to remove traces of organic substances. The solvents were filtered (0.45 μm filter) and degassed under vacuum in an ultrasonic bath before use.

Table 1. Gradient elution[a] and reconditioning[b] programme employed for reversed-phase HPLC separation of polyphenols of rooibos tea

Time (min)	Solvent composition[c] (% methanol)
0	20
5	20
25	30
40	35
50	40
60	50
70	60
80	80
90	60
110	20
125	20

[a]0.4 ml min^{-1}.
[b]1.2 ml min^{-1}.
[c]Gradient of formic acid–water (2%, v/v) and methanol.

Peak identification

Tentative peak identifications were achieved by comparing retention times of the reference standards with sample peaks. Spiking of tea samples with the authentic standards and detection performed at 280 and 320 nm were also used for identification.

Quantification of aspalathin and nothofagin

Calibration curves for aspalathin and nothofagin, with a standard series containing 5–100 $\mu g\ ml^{-1}$ of both dihydrochalcones, were determined at 280 nm. Quantification of aspalathin and nothofagin in the tea samples was done according to the external standard method using a single standard solution (S_{std}) containing aspalathin (61 $\mu g\ ml^{-1}$) and nothofagin (59 $\mu g\ ml^{-1}$; not corrected for purity). Aliquots of the sample solutions were diluted to match the aspalathin peak areas of the standard solution and sample solutions. The quantities of dihydrochalcones were calculated from the peak

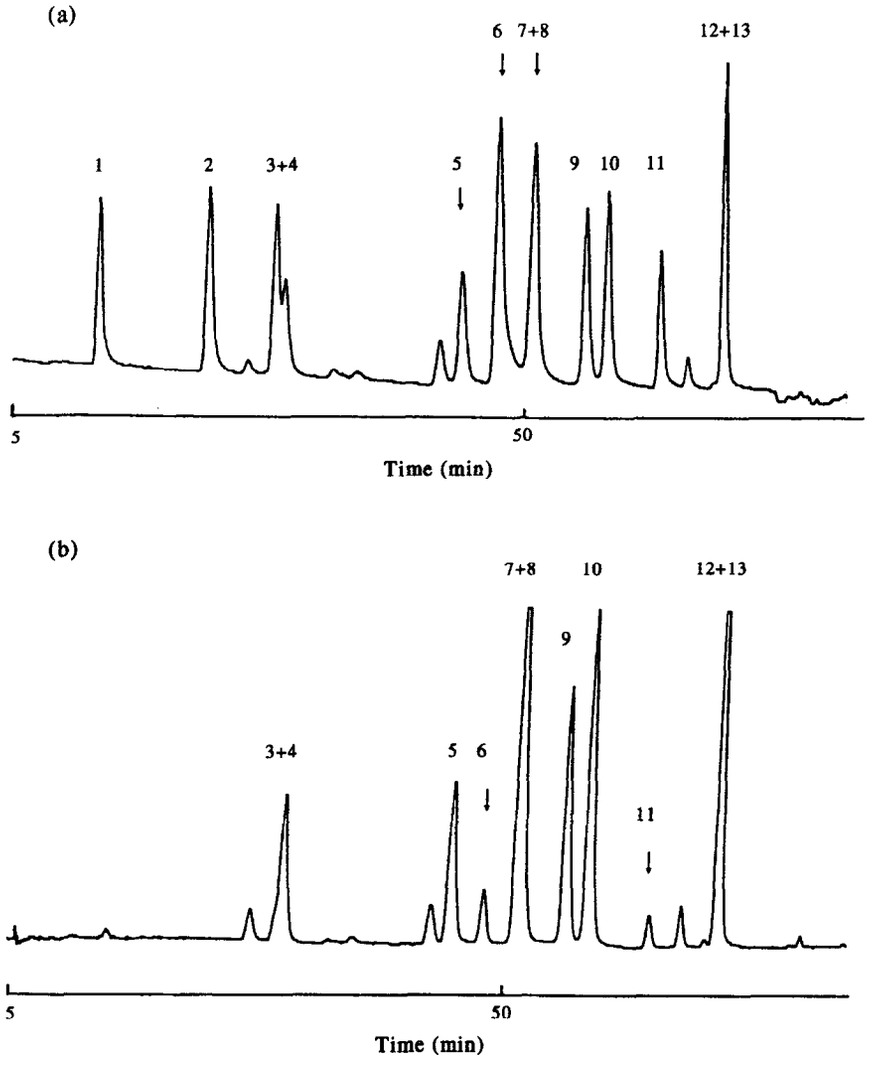

Fig. 2. Chromatograms of reversed-phase HPLC separation of standards of phenolic acids and flavonoids with UV detection at (a) 280 and (b) 320 nm. Peak identification: 1 = protocatechuic acid; 2 = *p*-hydroxybenzoic acid; 3 + 4 = vanillic + caffeic acid; 5 = *p*-coumaric acid; 6 = aspalathin; 7 + 8 = orientin + ferulic acid; 9 = iso-orientin; 10 = vitexin; 11 = nothofagin; 12 + 13 = rutin + isoquercitrin.

areas using the respective response factors of the standards. Quantification was carried out in triplicate.

Reproducibility of HPLC method

Ten aliquots of the standard solution (S_{std}) and five aliquots of samples A, B and C were injected over a period of 1 month to determine the reproducibility of the HPLC method.

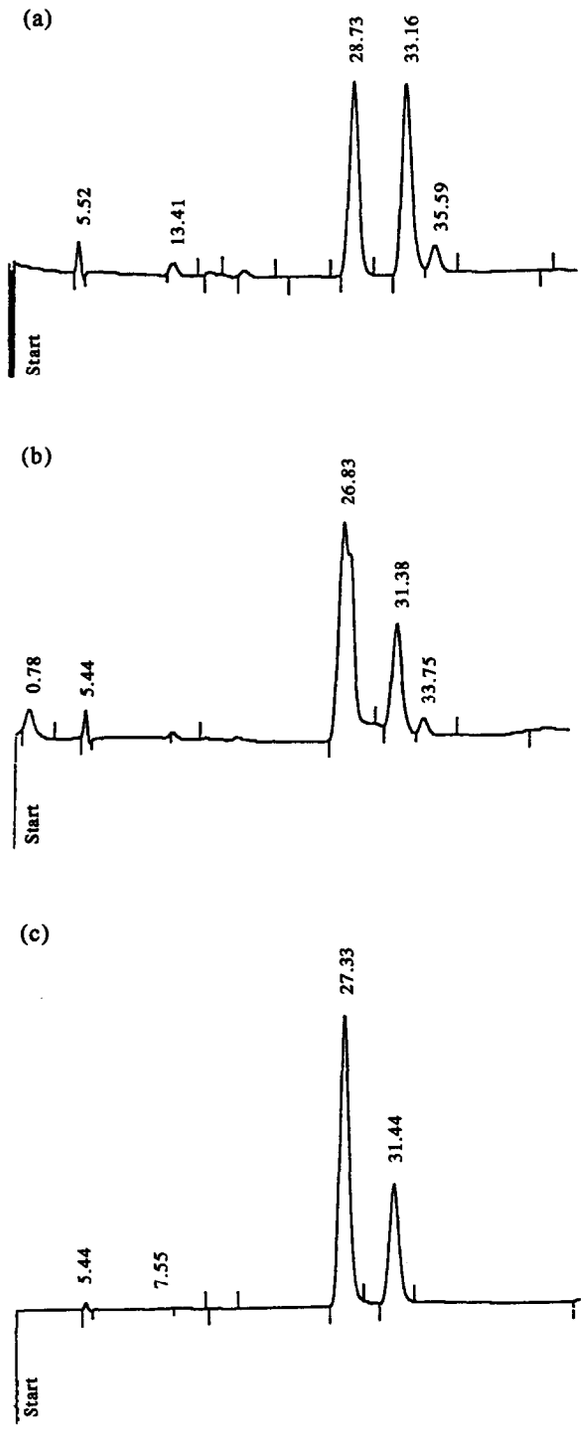

Fig. 3. Chromatograms of reversed-phase HPLC separation of (a) 2,3-dihydro-iso-orientin (isolated by Rabe, 1993), (b) 2,3-dihydro-iso-orientin (isolated by Rabe, 1993) spiked with vanillic and caffeic acids and (c) 2,3-dihydro-iso-orientin (isolated by Koeppen & Roux, 1966) with UV detection at 280 nm.

Extraction efficiency

Ethyl acetate soluble polyphenols (*ca* 250 mg), extracted from unprocessed rooibos tea (sample A), were dissolved in 25 ml methanol. An aliquot (20 ml), containing an estimated concentration of polyphenols similar to that given by 10 g unprocessed rooibos tea, was diluted to *ca* 200 ml with distilled water, extracted with ethyl acetate and further treated as described above. The aspalathin and nothofagin concentrations, determined by HPLC, were compared against those from direct injection of the initial solution of ethyl acetate soluble polyphenols in methanol.

Objective colour measurement

The change in leaf colour with comminution was determined with a Colorgard 2000/05 system (Pacific Scientific), using the CIELAB colour parameters, L^*, a^* and b^*. Colour measurement started within 10 min after comminution and the change in L^*, a^* and b^* was recorded over time for at least 240 min.

Statistical analysis

A two-sided *t*-test for paired samples was performed with SAS statistical software on the data of the different tea batches obtained by sun-drying and controlled-drying. Significance levels were calculated for the different variables.

RESULTS AND DISCUSSION

Separation of polyphenol standards

The separation of a mixture of different phenolic acids and flavonoids with UV detection at 280 and 320 nm is given in Fig. 2. Reasonably well resolved and symmetrical peaks were obtained in most cases with the acidified methanol–water solvent system. *p*-Coumaric acid separated into two peaks with the small peak eluting before the major peak (peak 5).

The retention time of vanillic acid was slightly less than that of caffeic acid but no complete separation of these acids was possible under the conditions employed. The sample of 2,3-dihydro-iso-orientin (Rabe, 1993) gave two peaks with the first peak co-eluting with vanillic and caffeic acid (Fig. 3). Since separation of diastereomers was unlikely under these separating conditions (Krause & Galensa, 1990), and no conclusive ^{1}H NMR spectra could be obtained for 2,3-dihydro-iso-orientin (Rabe, 1993), the peaks could possibly be 2,3-dihydro-iso-orientin and 2,3-dihydro-orientin. The sample prepared by photoxidation of aspalathin (Koeppen & Roux, 1966) also gave two peaks suggesting unequal quantities of the two products (Fig. 3). According to Koeppen & Roux (1966) 2,3-dihydro-iso-orientin forms more readily than 2,3-dihydro-orientin which resulted in a 2:1 ratio of these components. As

the ratio of the peaks (Fig. 3) also approximated 2:1 it was concluded that both 2,3-dihydro-orientin and 2,3-dihydro-iso-orientin were present in the samples and that 2,3-dihydro-iso-orientin eluted first. These flavanones elute before their corresponding flavones owing to their greater polarity and partial planarity compared with the more planar flavones (Wulf & Nagel, 1976).

Rutin, containing the disaccharide rutinose, did not separate from isoquercitrin which contains a glucoside. This is attributed to the shielding effect of some of the hydrophilic substituents by the sugars (Vande Casteele *et al.*, 1982). Nothofagin eluted after vitexin, its corresponding flavone. Aspalathin did not follow this pattern and eluted before orientin and iso-orientin, its corresponding flavones. This elution disparity could be explained in terms of the increase in polarity of aspalathin compared to that of nothofagin with addition of a hydroxyl group at the 3-position on the B-ring and its effect on the keto-enol equilibrium of dihydrochalcones. Aspalathin readily forms an enol which is favoured by polar conditions (Rabe *et al.*, 1994). The hydroxyl group of the enol form is more polar than the keto group which could explain the shorter retention time of aspalathin in relation to orientin compared with nothofagin and vitexin.

Peak identification of polyphenols in rooibos tea

Typical separations of ethyl acetate soluble polyphenols extracted from rooibos tea are shown in Fig. 4. Determination of relative retention times of the phenolic compounds known to be present in rooibos tea was necessary to ensure that one or more of these substances did not co-elute with aspalathin and nothofagin. Tentative identification of the peaks was based on the retention time of authentic standards, spiking of the sample with standards and the absorbance of the compounds at two different wavelengths. Spiking with standards resulted only in increased absorbance and no separation of the standard and peaks given by rooibos tea was observed for all the components.

The absorbance of sample peaks (Fig. 4) corresponding to peaks 1, 2, 3+4, 6, 11 (Fig. 2) and dihydro-2,3-iso-orientin (Fig. 3) decreased with absorbance at 320

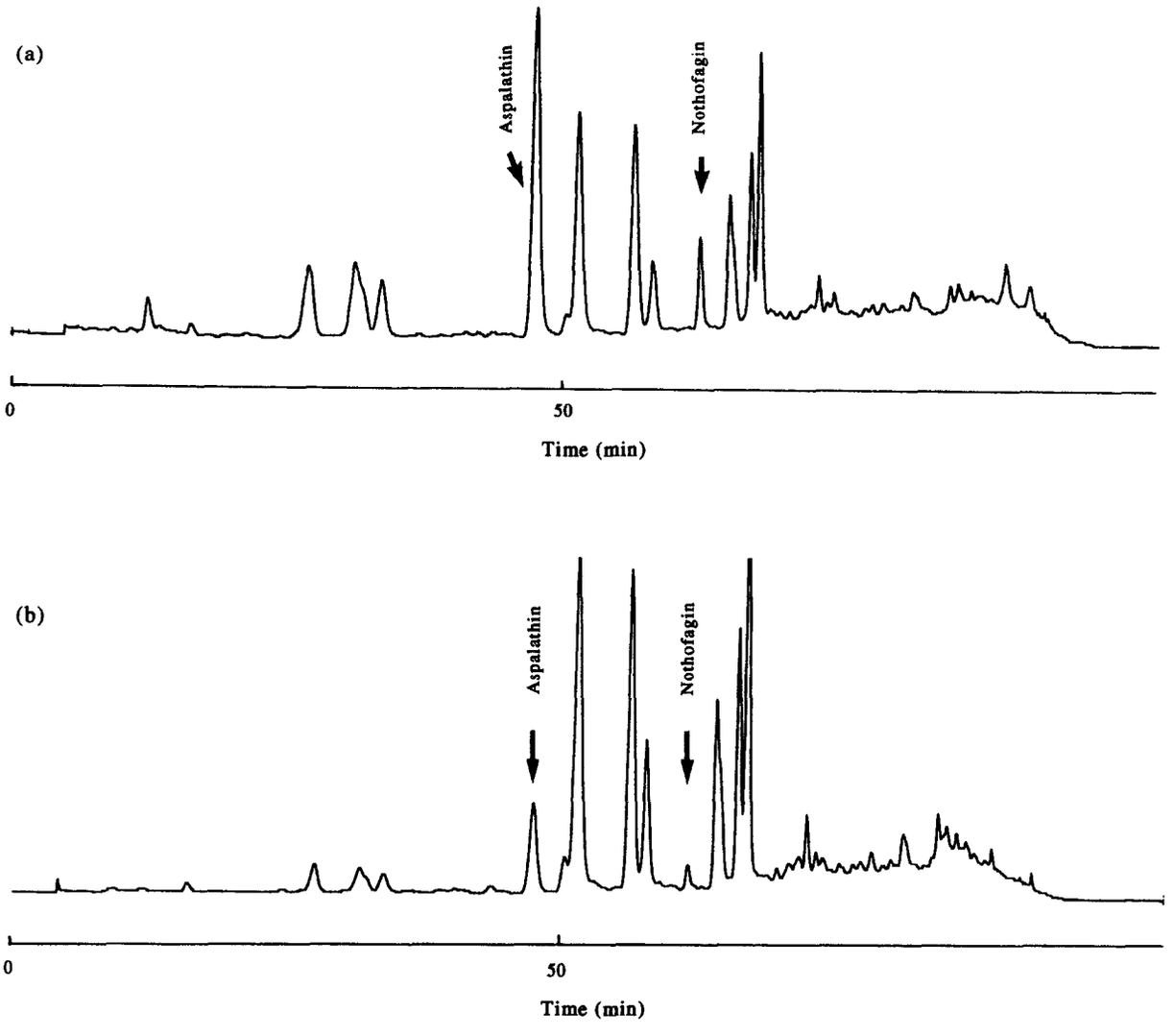

Fig. 4. Chromatograms of reversed-phase HPLC separation of phenolic acids and flavonoids of fermented rooibos tea with UV detection at (a) 280 and (b) 320 nm.

nm compared to that at 280 nm (Fig. 4). This corresponds with peaks given by protocatechuic acid, *p*-hydroxybenzoic acid, vanillic acid, aspalathin, nothofagin and 2,3-dihydro-iso-orientin. Both aspalathin and nothofagin exhibit maximum absorbance at 290 nm in ethanol owing to absorbance of the A-ring (Koeppen & Roux, 1966; Hillis & Inoue, 1967).

The peak given by rooibos tea (Fig. 4) which corresponds to peak 7+8 (Fig. 2) of the standard increased at 320 nm. This is attributed to increased absorbance of orientin since ferulic acid was present in low quantities, and ferulic acid and orientin in the tea sample were partly separated. Iso-orientin, vitexin, rutin and isoquercitrin (Fig. 4) which correspond to peaks 9, 10 and 12+13 (Fig. 2) also showed an increased absorbance at 320 (Fig. 4).

Calibration, reproducibility of determination and extraction efficiency of dihydrochalcones

Absorbance increased linearly over the concentration range 5–100 μg ml^{-1} for both aspalathin ($y = 66493 + 626170x$; $R = 0.99$) and nothofagin ($y = 25921 + 311530x$; $R = 0.99$). No correction for impurities in the nothofagin standard was made as the extinction coefficient for nothofagin was not available to compare the theoretical and calculated values. Since a non-zero intercept was obtained for both aspalathin and nothofagin and calibration, using more than one point was too time consuming, the standard and sample concentrations were matched for at least one of the compounds for accurate quantification as suggested by Delaney (1985). Good reproducible values were obtained for aspalathin and nothofagin determined in the various tea extracts and the standard polyphenol solution (Table 2). Recoveries of aspalathin and nothofagin were both 94%.

Changes in aspalathin and nothofagin content with processing

The change in the qualitative phenolic composition of rooibos tea with processing is shown in Fig. 5. It was found that aspalathin and nothofagin were the major ethyl acetate soluble polyphenols in samples E–G and that their content decreased relatively to the other compounds with fermentation (sample H). Results of quantitative data are given in Table 3. From this it is evident that approximately 19 g kg^{-1} of the dry matter of unprocessed rooibos tea consists of dihydrochalcones. This value decreased drastically for partially oxidized (sample G) and fermented tea (sample H). In practice even more time elapses between comminution and

Table 2. Reproducibility of reversed-phase HPLC determination of aspalathin and nothofagin

Sample[a]	No. of injections	Aspalathin			Nothofagin		
		Area[b] (integration units)	SD[c]	% CV[d]	Area (integration units)	SD	% CV
S_{std}	10	4 089 533	75 950	1.86	2 019 173	24 213	1.20
A	5	3 717 000	93 657	2.52	576 933	13 713	2.38
B	5	3 662 700	40 544	1.11	911 116	17 660	1.94
C	5	3 607 960	54 941	1.52	1 296 580	19 953	1.54

[a]S_{std}.
[b]Mean value.
[c]Standard deviation.
[d]Coefficient of variation.

Table 3. Effect of processing under controlled conditions on the aspalathin, nothofagin and total polyphenol content of rooibos tea

Sample[a]	Aspalathin		Nothofagin[b]			
	(g kg^{-1} dry tea)	(g kg^{-1} soluble solids)[e]	(g kg^{-1} dry tea)	(g kg^{-1} soluble solids)	TP[c] (g GAE[f] kg^{-1} dry tea)	DHC[d]/TP
E	14.65	77.3	4.31	23.2	16.24	1.17
F	11.38	54.8	3.01	14.5	16.00	0.90
G	4.75	23.0	1.53	7.4	11.42	0.55
H	1.02	5.5	0.35	1.9	7.28	0.19

[a]E, green, uncomminuted, unfermented, freeze-dried; F, comminuted *ca* 15 min before freezing, freeze-dried; G, comminuted *ca* 210 min before freezing, freeze-dried; H, comminuted *ca* 210 min before fermentation, fermented at 38°C for 14 h, air-dried at 40°C for 3 h.
[b]Values for nothofagin were not compensated for inpurities of the standard.
[c]Total polyphenol content of the ethyl acetate extract.
[d]Total amount of dihydrochalcones (aspalathin + nothofagin).
[e]Water-soluble solids.
[f]Gallic acid equivalents.

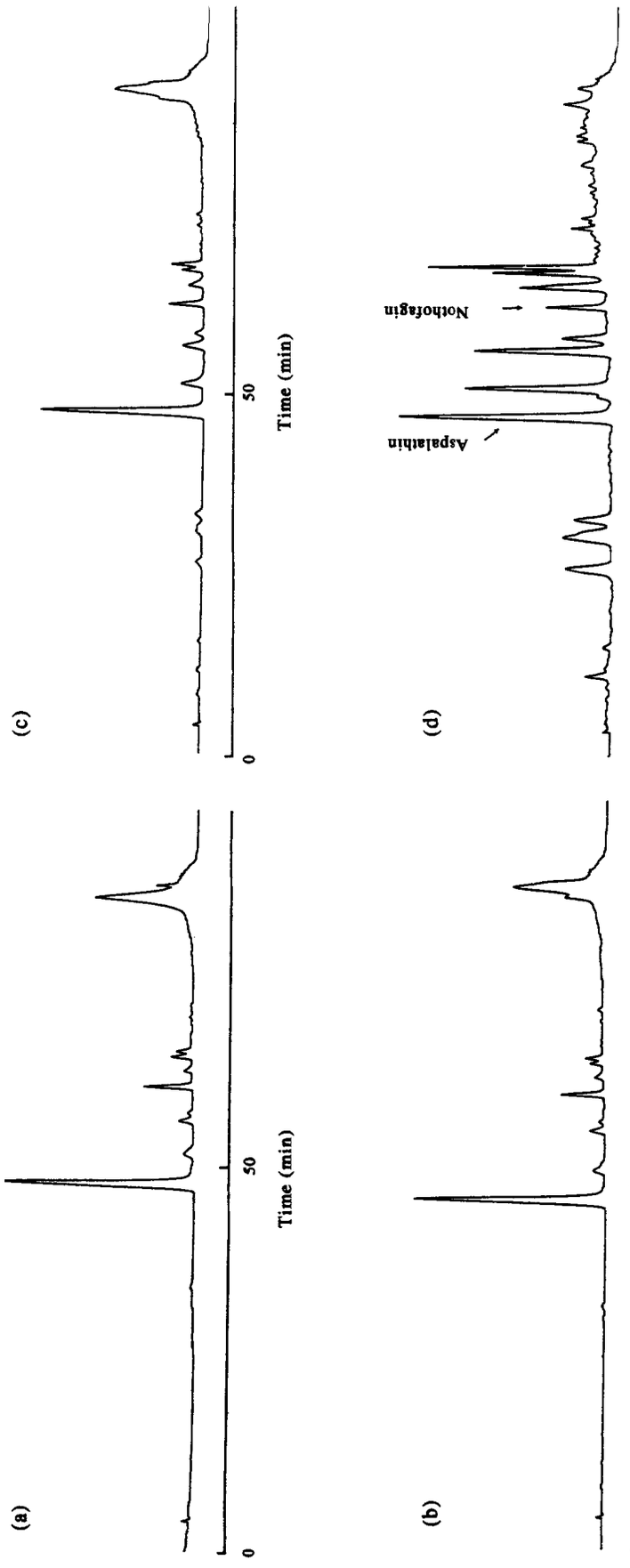

Fig. 5. Chromatograms of reversed-phase HPLC separation of rooibos tea polyphenols with UV detection at 280 nm: (a) sample E (green, uncomminuted, unfermented, freeze-dried; dilution—8:400); (b) sample F (comminuted *ca* 15 min before freezing, then freeze-dried; dilution—7:300); (c) sample G (comminuted *ca* 210 min before freezing, then freeze-dried; dilution—15:300); and (d) sample H (comminuted, fermented at 38°C for 14 h, then air-dried at 40°C for 3 h; dilution—160:400).

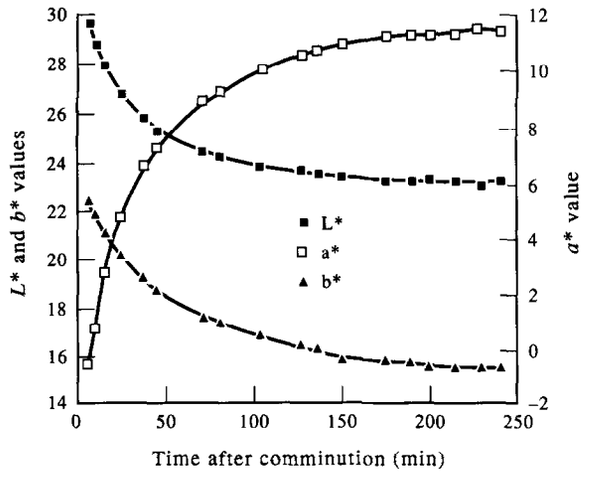

Fig. 6. Typical changes of CIELAB colour parameters after comminution of fresh, green rooibos tea.

fermentation. The fermented tea contained approximately 7% of the dihydrochalcones originally present in the green tea. The ratio of dihydrochalcones to total ethyl acetate soluble polyphenols also decreased with processing (Table 3) indicating its conversion to other ethyl acetate soluble polyphenols.

The decrease in dihydrochalcones can be attributed to enzymatic and chemical oxidation of polyphenols, which were initiated with comminution of the tea at which time exposure to oxygen and cell damage occurred. The nature of these reactions in rooibos tea is still unknown but enzymes such as polyphenol oxidase and peroxidase could play a role. Rathmell & Bendall (1972) and Wong & Wilson (1976*a*,*b*) demonstrated peroxidase-catalyzed oxidation of chalcones with further non-enzymatic oxidation of the initial reaction product.

The dihydrochalcone aspalathin is initially converted to 2,3-dihydro-iso-orientin and 2,3-dihydro-orientin in an ethanolic solution (Koeppen & Roux, 1966). Results of this study indicated that these conversions probably also occur in rooibos tea during fermentation. Chromatograms of extracts of fermented rooibos tea (Fig. 4) revealed that the two peaks which correspond with those of the 2,3-dihydro-iso-orientin samples (Fig. 3) were present in approximately equal amounts in the fermented tea (sample H). The relative quantities of these compounds, together with the flavones, flavonols and phenolic acids increased with processing (Fig. 5). Similar trends were obtained for all other rooibos tea extracts analyzed.

If aspalathin is oxidized via flavanones to polymeric brown substances as postulated by Koeppen & Roux (1966), it occurs rapidly in rooibos tea even in the absence of sunlight. Aspalathin could also react with other flavonoids to form polymeric substances since Gujer *et al.* (1986) demonstrated the formation of polymeric flavonoids from a chalcone and a flavonoid. Oxidation in rooibos tea resulted in almost immediate noticeable browning of the tea which is substantiated by the change in the CIELAB values illustrated in Fig. 6. A sharp decrease in L^* and b^* values was accompanied by a sharp increase in a^* during the initial stages after comminution, but it levelled off after *ca* 200 min. Similar trends were obtained with other batches of tea indicating that rapid browning of rooibos tea due to oxidation of polyphenols occurred after comminution.

Effect of controlled-dried vs sun-drying on the polyphenolic content

Similar elution patterns for different batches of tea as well as for sun-dried vs controlled-dried tea were obtained. The aspalathin, nothofagin and total polyphenol content of tea dried under controlled conditions did not differ significantly from that of sun-dried tea (Table 4). These results showed that sun-drying is not essential for degradation of dihydrochalcones. It is thus evident that other degradation mechanisms, such as enzymatic conversions, occur in the leaves to cause degradation of aspalathin and nothofagin in the absence of sunlight.

CONCLUSIONS

The study showed that reversed-phase HPLC with UV detection at 280 nm and gradient elution can be used for

Table 4. Effect of the drying method on the aspalathin, nothofagin and total polyphenol content of rooibos tea

Polyphenol content	Drying method		
	Sun-drying	Controlled-drying	Level of significance
Total polyphenols[a] (g GAE[b] kg^{-1} dry tea)	9.21	9.06	0.587
Aspalathin (g kg^{-1} dry tea)	0.80	0.87	0.147
(g kg^{-1} soluble solids[c])	4.5	4.8	0.083
Nothofagin[d] (g kg^{-1} dry tea)	0.36	0.37	0.434
(g kg^{-1} soluble solids)	2.0	2.1	0.366

[a]Total polyphenol content of the ethyl acetate extract.
[b]Gallic acid equivalents.
[c]Water-soluble solids.
[d]Values for nothofagin were not compensated for impurities of the standard.

the separation and quantification of the dihydrochalcones aspalathin and nothofagin in rooibos tea. Further method development is necessary for the determination of phenolic acids, flavanones, flavones and flavanols in the tea. The aspalathin and nothofagin contents decreased with processing and depended on the degree of oxidation of the tea. A major portion of the dihydrochalcones was oxidized during the period before fermentation. Controlled-drying compared to sun-drying did not affect the dihydrochalcone content of rooibos tea.

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