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ORIGINAL PAPER

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Reversed-phase HPLC determination of mangiferin, isomangiferin and hesperidin in *Cyclopia* and the effect of harvesting date on the phenolic composition of *C. genistoides*

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Abstract A reversed-phase HPLC method for separation of polyphenols in honeybush tea (*Cyclopia* spp.) is presented. Separation of eriodictyol, luteolin, medicagol, formononetin, mangiferin, isomangiferin, hesperetin and hesperidin was investigated. A C12 stationary phase was required to separate mangiferin and isomangiferin. The method was used to quantify the three major polyphenols (mangiferin, isomangiferin and hesperidin) in *C. genistoides*, *C. intermedia*, *C. maculata* and *C. sessiliflora* and to study the effect of harvesting date on these compounds in two types of *C. genistoides*. The highest levels of the xanthones, mangiferin (3.61 g/100 g) and isomangiferin (0.54 g/100 g), and the flavanone, hesperidin (1.74 g/100 g), were found for *C. genistoides* (both xanthones) and *C. intermedia*, respectively. *Cyclopia sessiliflora* contained the lowest levels of mangiferin (1.04 g/100 g) and hesperidin (0.29 g/100 g). The mangiferin content of both the Overberg and West Coast types decreased with harvesting date ($P<0.05$). The Overberg type contained more mangiferin, but hesperidin was more prominent in the West Coast type.

Keywords Xanthones · Flavanones · Mangiferin · Isomangiferin · Hesperidin

Introduction

Utilization of the genus *Cyclopia* (Fabaceae family) for its medicinal value spans a few centuries [1, 2]. It is unknown at what stage *Cyclopia*, endemic to South Africa, was first processed and became popular as a beverage (honeybush tea) for everyday use and a substitute for tea, rather than being used for medicinal purposes. The aerial parts of *Cyclopia* contain flavones, flavanones, xanthones, isoflavones and coumestans [3, 4, 5]. Antioxidant activity has been demonstrated for aqueous extracts of several *Cyclopia* species [6, 7], which has stimulated interest in utilizing this plant for preparation of antioxidant-rich extracts. A prerequisite for preparation of these extracts on a commercial scale is the presence of high levels of the compounds of interest. De Nysschen et al. [3] estimated the major flavonoids of unprocessed *Cyclopia* species to be the xanthone, mangiferin, and the flavanones, hesperetin and isosakuranetin. However, no quantitative data were supplied. Ferreira et al. [4] found a relatively large quantity of hesperidin in the methanol extract of processed *C. intermedia*. Mangiferin is much more effective as a hydrogen donor and superoxide radical scavenger than hesperetin and its rutinoside, hesperidin [6, 8]. Based on structure-activity relationships in a free radical scavenging test system [9], it is to be expected that isosakuranetin without a 3'-hydroxyl group will also be less effective than mangiferin.

Fermentation, i.e. the chemical oxidation step during processing needed for formation of the sought-after brown colour and sweet flavour of the honeybush beverage [10], has been found to reduce antioxidant potency of extracts [6, 7]. Hubbe [7] showed that species differed in terms of antioxidant potency, and that the combined mangiferin and isomangiferin contents of a methanol extract of *C. intermedia* decreased substantially with fermentation. For the purpose of producing antioxidant-rich extracts from *Cyclopia*, unfermented plant material, as well as species with a high mangiferin content should therefore be selected.

In this paper the development of an HPLC method for the separation of the flavonoid fraction of *Cyclopia* and its application to quantifying mangiferin, isomangiferin and hesperidin is described. Four species, *C. intermedia*, *C. sessiliflora*, *C. genistoides* and *C. maculata*, presently under investigation for commercial cultivation, were

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analysed. Variation in the contents of these compounds as effected by the harvesting dates of two varieties of *C. genistoides* was also investigated.

Materials and methods

Plant material. Shoots (6–10 kg) of *C. intermedia* (Simondium), *C. maculata* (Genadendal) and *C. sessiliflora* (Simondium) plants were harvested in January 2000 at different locations from experimental plantations and one commercial plantation (*C. genistoides*; West Coast type) in the Western Cape Province. The plant material was dried at 40 °C under forced-air circulation in a drying tunnel and pulverized with a Retch mill before analysis. The thick stems of *C. maculata* were removed before drying. To study the effect of harvesting date *C. genistoides* was also harvested from two commercial plantations established in 1996 (West Coast type) and 1997 (Overberg type) in the Pearly Beach area. Nine months elapsed between the previous harvest and commencement of harvesting for experimental purposes. The plants were harvested every 5 weeks over a period of 15 weeks, beginning at the end of March 2001. On each occasion, approximately one quarter of the shoots (ca. 300 g) of each of five plants per type was harvested. Harvesting was terminated before full flowering of the plants occurred, but small flower buds were already present on some of the bushes during the June harvest. The plant material was dried and pulverized as described above.

Chemicals and standards. All solvents for sample preparation were of analytical grade, while solvents used for chromatographic separations were of HPLC grade (Merck, Darmstadt, Germany). For use as HPLC eluent, deionized water was further purified by means of a Milli-Q 185 Plus water purification system (Millipore, Eschborn, Germany). Mangiferin (2-β-D-glucopyranosyl-1,3,6,7-tetrahydroxy-9H-xanthen-9-one), hesperetin (5,7,3'-dihydroxy-4'-methoxy-flavanone), hesperidin (5,3'-dihydroxy-4'-methoxy-7-O-rutinosylflavanone), eriodictyol (5,7,3',4'-tetrahydroxyflavanone), formononetin (7-hydroxy-4'-methoxyisoflavone) and luteolin (5,7,3',4'-tetrahydroxyflavone) for use as analytical standards were purchased from Extrasynthese, Genay Cedex, France. Isomangiferin (4-β-D-glucopyranosyl-1,3,6,7-tetrahydroxy-9H-xanthen-9-one), and medicagol (3-hydroxy-8,9-methylenedioxycoumestan), isolated from *C. intermedia*, were kindly provided by Prof. EV Brandt of the University of the Orange Free State, Bloemfontein, South Africa.

HPLC analysis and method development. The HPLC system consisted of a ternary solvent pump (Gynkotek model 480), autosampler (Gynkotek Gina 50), decade electrochemical detector with a glassy carbon electrode (Antec) and a diode array detector (Gynkotek 340S). Gynkosoft software V5.60 was used to control the HPLC system and for data acquisition and analysis. The equipment was supplied by Dionex Softron (Idstein, Germany). Three columns, i.e. Multosphere C18 (3 µm; 125×4 mm ID), Phenomenex Synergy MAX-RP C12 80A with TMS end-capping (4 µm; 150×4.6 mm ID) and Phenomenex Synergi Polar RP (ether linked phenyl phase with polar end-capping) were tested for the chromatographic separation of the above-mentioned substances. The Multosphere column was purchased from CS, Langerwehe, Germany and Phenomenex, Aschaffenburg, Germany supplied the Phenomenex columns. Peak identity was determined by means of retention time and UV spectra that were recorded for all samples in the range 200–400 nm. During method development, three solvent gradients were tested: program I: 0–6 min (12% B), 7 min (18% B), 14 min (25% B), 19 min (40% B), 24 min (50% B), 29 min (12% B) (solvent A=2% acetic acid in aqueous solution (v/v) and solvent B=acetonitrile); program II: 0 min (5% B, 5% C); 4,5 min (6,5% B, 5% C), 7 min (18% B), 14 min (25% B), 19 min (40% B), 24 min (50% B), 30 min (5% B, 5% C) [solvent A=2% acetic acid in aqueous solution (v/v), solvent B=acetonitrile and solvent C=tetrahydrofurane (THF)];, program III: identical to Program I except that solvent A was purified water buffered to pH 4 with citrate buffer. This program, in combination with the Synergy MAX-RP C12 column, was used exclusively for electrochemical detection. In all cases a flow rate of 1 ml/min was used. The injection volume was 20 µl for each analysis and separations were carried out at room temperature.

Sample preparation and quantification. After preliminary testing of the extraction efficiency of ethanol, methanol and deionised water for mangiferin, isomangiferin and hesperidin, methanol combined with sonication was selected for extraction of the plant material. The dried, pulverized plant material (0.25 g) was extracted for 30 min with 30 ml methanol (100%) in an Erlenmeyer flask with a ground joint in an ultrasonic bath. Subsequently, a small volume of the extract was filtered through a 13 mm membrane filter (0.45 µm; polypropylene) directly into an HPLC sample vial for injection without further dilution. Analysis of plant material was carried out in duplicate.

Program I and the Synergy MAX-RP C12 column, in combination with the diode-array detector, were used for all quantitative determinations of mangiferin, isomangiferin and hesperidin. UV absorptions of the different compounds (mangiferin, isomangiferin and hesperidin) were determined at 258, 280, 320 and 368 nm. Quantification, based on peak area and using external standards, was carried out at 280 nm. A sufficiently pure isomangiferin standard was not available, and based on the assumption that mangiferin and isomangiferin have the same extinction coefficients, mangiferin was used to quantify isomangiferin. Linear calibration lines for mangiferin and hesperidin were compiled for each compound separately, using standard series of six dilutions between 4.7 and 98.3 µg/ml. The repeatability of the HPLC method was determined by ten injections of the same sample of extracted *C. intermedia*. The reproducibility of the complete assay was tested by means of ten sample preparations from a finely pulverized *C. intermedia* sample.

Statistical analyses of data (effect of harvesting date). The experimental design was a completely randomized design with two main plot treatments (plant type), four sub-plots (harvesting date) and five replications (bushes). An experimental unit was a quarter of a bush. The data were subjected to split-plot analysis of variance to determine whether type and harvest date significantly affected composition and regressions were fitted over time. Student's *t*-least significant difference was used to compare means for types and harvest dates (P=0.05). SAS Release version 8.2 was used for all statistical analyses.

Results and discussion

Of the different reversed-phase columns tested, the non-polar, end-capped C18 stationary phase (Multosphere) showed strong tailing of the polyphenolic substances and failed to separate the isomeric compounds, mangiferin and isomangiferin. Separation of the isomers was obtained when modifying the mobile phase with 5% THF. However, the tailing of the standard compounds remained unacceptable. By using the comparatively more polar C12 reversed phase material (Synergy MAX-RP), tailing of the compounds was strongly reduced and good separation of mangiferin and isomangiferin as well as of all other standard compounds was achieved (Fig. 1). Similar results, except for luteolin and eriodictyol, were achieved when using the Synergy Polar-RP column. These two substances, differing only in the C3-C4 position, could not be separated on the Polar-RP material.

Substances containing hydroxyl groups often show antioxidative properties. For this reason, the use of an

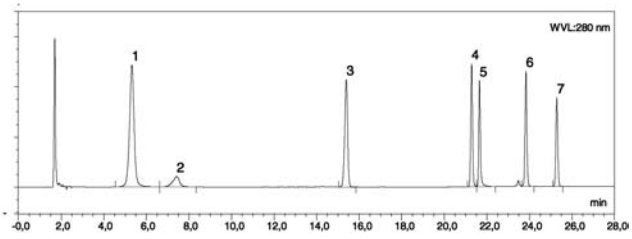

Fig. 1 Chromatogram of a standard mixture of commercially available polyphenols present in honeybush tea samples according to literature [4]. *1* Mangiferin, *2* unidentified impurity of mangiferin, *3* hesperidin, *4* eriodictyol, *5* luteolin, *6* hesperetin, *7* formononetin. Isomangiferin and medicagol would appear in the same chromatogram at 6.5 and 27.5 min, respectively. Gradient separation was carried out on a Synergy MAX-RP C12 column with acetonitrile and 2% acetic acid in water as eluents. Detection was at 280 nm

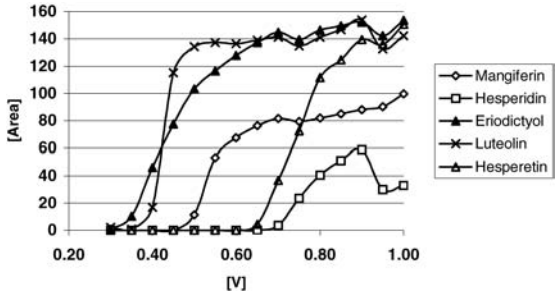

Fig. 2 Hydrodynamic voltammograms of *Cyclopia* -related polyphenols

electrochemical detector as a more sensitive alternative to the diode array detector was considered. Hydrodynamic voltammograms, showing the detector response for a given compound as a function of the working electrode potential, were recorded for all standards. The lower the electrochemical potential that has to be applied for the measurement of a sample, the more reproducible and smaller the effect of parameters such as changing eluent composition or complex sample composition on the measurement [11]. Under the chosen conditions, luteolin and eriodyctiol showed a good response at a relatively low potential of +0.5 V (Fig. 2). Reliable determination of mangiferin was possible at a potential of +0.6 V, even though the sensitivity of the measurement was not superior to the diode array. A reproducible determination of hesperidin was only possible above the working electrode potential of +0.9 V, showing a comparatively low sensitivity. Even at this relatively high potential, no peaks for formononetin and medicagol appeared in the chromatogram. As a consequence samples of unfermented honeybush have to be measured at +0.9 V, because hesperidin is a major component [4]. Under these conditions the baseline no longer remained a flat line during a run, which may be due to the constantly changing eluent composition of the gradient program. Furthermore, quantitative results of a real honeybush sample would be subject to a higher standard deviation.

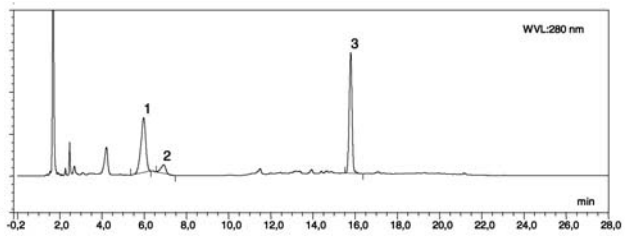

Fig. 3 Chromatogram of a non-fermented honeybush sample: *1* Mangiferin, *2* isomangiferin, *3* hesperidin. Gradient separation was carried out on a Synergy MAX-RP C12 column with acetonitrile and 2% acetic acid in water as eluents. Detection was at 280 nm

Table 1 Quantitative data for mangiferin, isomangiferin and hesperidin in unfermented *Cyclopia* species (UV detection at 280 nm). Results expressed as grams per 100 g dry weight. *M* Mangiferin, *I* isomangiferin, *H* hesperidin

Cyclopia species	M	I	H	Σ (M+I+H)
C. intermedia	1.691	0.215	1.764	3.669
C. maculata	1.626	0.316	0.724	2.665
C. sessiliflora	1.040	0.303	0.294	1.636
C. genistoides	3.607	0.537	1.504	5.648

Due to its sufficient sensitivity, the easier handling and the possibility of substance identification by means of UV spectra, the diode array detector was preferred for this type of sample.

Mangiferin and isomangiferin showed similar UV spectra with maxima at 258, 320 and 368 nm. These compounds gave the highest absorption at 258 nm, while 280 nm gave better sensitivity for hesperidin and other polyphenols. A typical HPLC chromatogram of a methanol extract of unfermented *C. intermedia* with detection at 280 nm is depicted in Fig. 3. Both xanthones can be found in very high quantities in unfermented honeybush species, so that the extra sensitivity obtained at 258 nm was not necessary and all quantification was subsequently carried out at 280 nm. Linear absorbance was obtained for both mangiferin (y=0.2155x –0.5108; R^2=0.9985) and hesperidin (y=0.3957x+0.2809; R^2=0.9998) over the concentration range tested. The detection limit, determined as 3 times the height of the baseline noise, was approximately 1.6 µg/ml for both substances. Repeated injection of the same sample gave low coefficients of variation for mangiferin (1.73%), isomangiferin (3.63%) and hesperidin (0.93%). The coefficient of variation for the reproducibility of the complete assay was 8.05% for mangiferin, 8.80% for isomangiferin and 8.75% for hesperidin.

Table 1 gives the mangiferin, isomangerin and hesperidin contents of the different *Cyclopia* species. The mangiferin content of *C. genistoides* was more than twice than that of the other species. *Cyclopia sessiliflora* had the lowest mangiferin content with *C. maculata* and *C. intermedia* more or less the same amounts. The isomangiferin contents of *C. genistoides* and *C. intermedia*

Table 2 Changes in mangiferin, isomangiferin and hesperidin contents with harvesting date of *Cyclopia genistoides*. Values are the averages for the types, Overberg and West Coast. Harvesting started at the end of March and was repeated every 5 weeks for 15 weeks. Results are expressed as grams per 100 g dry weight. *LSD* least significant difference. *NS* Not significant ($P>0.05$)

Harvesting date	Mangiferin	Isomangiferin	Hesperidin
1	5.942	1.100	3.908
2	5.792	1.123	3.509
3	5.657	1.133	3.749
4	5.208	1.070	3.705
LSD (P =0.05)	0.335	0.062	0.259
Regression	P <0.001	NS	NS

Table 3 Mangiferin, isomangiferin and hesperidin content of the *Cyclopia genistoides* types, Overberg and West Coast. Results are expressed as g per 100 g of dry weight

Type	Mangiferin	Isomangiferin	Hesperidin
Overberg	6.369±0.603	1.163±0.105	2.227±1.019
West Coast	4.931±0.728	1.051±0.210	5.209±0.423
P	0.004	0.310	<0.001

were the highest and lowest, respectively, of the different species. However, *C. intermedia* contained the most and *C. sessiliflora* the least hesperidin. Overall, *C. sessiliflora* had the lowest total concentration of these polyphenols. *Cyclopia sessiliflora*, a re-sprouter, is a very slow-growing species in comparison to the other species, especially *C. genistoides* and *C. maculata,* and flowering also occurs earlier than for the other species. Hubbe and Joubert [6] showed that aqueous extracts of unfermented and fermented *C. sessiliflora* contain the highest level of total polyphenols and flavonoids as determined by their Folin-Ciocalteu reactivity. This discrepancy with the present results could be partially explained by the difference in solubilities of phenolic compounds such as polymeric substances due to the solvent used, but the age of the plantation and growth and leaf to stem ratio could affect phenolic content. However, the effects of these aspects need to be investigated.

The same trend for the effect of harvesting date on the polyphenol content of the two types of *C. genistoides* was obtained, and averages for the types are presented in Table 2. Isomangiferin and hesperidin contents did not change significantly ($P>0.05$), but the mangiferin content decreased significantly ($P<0.001$) from the end of March to mid-July. The Overberg type contained significantly more mangiferin ($P=0.004$), but less hesperidin ($P<0.001$) than the West Coast type (Table 3). No significant difference ($P>0.05$) between the two types was obtained for isomangiferin content. The Overberg type would therefore be the plant material of choice if it were to be used for isolation of mangiferin and isomangiferin. Several pharmacological properties are attributed to xanthones [12], which could open up the possibility of nutraceutical/pharmaceutical products. However, the specific bioactivity of the two types needs to be studied to include the effects of minor, but potent compounds, before selection based on activity could be made.

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