Chemical characterization of the constituents of the aroma of honeybush, *Cyclopia genistoides*

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Abstract

A high-capacity headspace sample enrichment probe (SEP) was used in conjunction with gas chromatography–mass spectrometry (GC–MS) to analyse the volatile organic compounds present in the aroma of dry or infused, unfermented (green) and fermented *C. genistoides*, one of the South African *Cyclopia* species from which a herbal tea, known as honeybush tea, is made. Seventy-seven compounds were identified in the volatile fraction of the aroma of dry, green *C. genistoides*, comprising, *inter alia*, a large number of saturated and unsaturated alcohols, aldehydes and methyl ketones.

In the aroma of dry, as well as infused, fermented *C. genistoides,* 79 compounds were identified, 46 of which were terpenoids that were mostly present in much lower relative concentrations in the unfermented material. The methodology developed and the results obtained in the analysis of the aroma of *C. genistoides* provide a basis for ongoing comparative studies on the chemical composition of a series of prominent *Cyclopia* species with the view to developing a rapid screening device and protocol for honeybush tea evaluation.

Keywords: Aroma profile; *Cyclopia genistoides*; Headspace–GC–MS; Honeybush tea; Terpenoids; Volatile organic compounds.

Introduction

Honeybush tea, also known as “South Africa's sweetest tea”, is a herbal tea made from the leaves and twigs of *Cyclopia* spp., indigenous to the fynbos biome in the Western and Eastern Cape Provinces of South Africa. The pleasant sweet aroma and taste of fermented honeybush, its low tannin content and the absence of caffeine led to widespread interest during the mid-1990s in the commercial cultivation and processing of honeybush tea. However, poor and inconsistent quality, especially poor flavour or the presence of off-flavours, contributed to poor market share. The lack of good quality tea was identified as a major stumbling block in successful commercialization and advancement of the industry (Du Toit et al., 1998).

Major improvement in sensory quality was subsequently achieved through optimization and control of the fermentation and drying conditions (Du Toit and Joubert, 1998 and Du Toit and Joubert, 1999). As flavour of the herbal tea is only as good as the inherent flavour potential of the plant, improvement of plant material through breeding and selection and the application of certain horticultural practices (ARC Honeybush Research Programme, 2007), provide researchers with further opportunities to improve product quality. Large numbers of samples, generated in the course of the breeding and selection programme of the Agricultural Research Council (ARC), must be evaluated in terms of several criteria, *i.e.* growth and production parameters, composition (Joubert et al., 2006), bioactivity (Verhoog et al., 2007) and sensory characteristics, as determined by the intended use of the plant material.

The determination of optimum processing conditions (Du Toit and Joubert, 1998 and Du Toit and Joubert, 1999) by means of sensory analysis, is not a viable option due to its inherent drawbacks, *i.e.* lack of trained panelists, limited availability of potential panelists, panel continuity, panelist fatigue during testing sessions, limited number of

samples tested per session and the time-consuming nature of sensory testing. Clearly, an instrumental method suitable for rapid screening of the tea flavour is a key prerequisite for the success of the programme.

Although more than 20 species of honeybush grow in the wild, only a few species are commercially exploited for the manufacturing of tea, the more prominent species presently being *C. intermedia*, *C. subternata*, and *C. genistoides*. The latter species was chosen as representative species for this study with the view to applying the developed methodology to a comparative study of all the important *Cyclopia* species. The chemical characterization of the aroma of *Cyclopia* species has not yet been reported in the literature. We report here on the analysis and chemical characterization of the aroma of *C. genistoides*, which forms the basis of our efforts to develop a rapid screening device and protocol for honeybush tea and contributes to the comprehensive honeybush research programme conducted at ARC Infruitec-Nietvoorbij in South Africa (ARC Honeybush Research Programme, 2007).

Materials and methods

Plant material

Cultivated *Cyclopia genistoides* L. Vent, Fabaceae was harvested on Reins Farm near Albertinia in the South Western Cape, South Africa, by cutting the bushes to the ground and shredding the shoots to 2–3 mm lengths using a mechanised fodder cutter. The shredded plant material was divided into two batches of 3.6 kg each. One batch was used to prepare unfermented tea by drying it immediately in a thin layer to a moisture content of about 10% on 30 mesh stainless-steel drying racks at 40 °C for 6 h in a temperature-controlled dehydration tunnel with cross-flow air movement at 3 m/s.

Deionised water was added to the other batch to wet the leaves superficially, followed by fermentation at 90 °C for 16 h in a stainless-steel container, covered with aluminium foil in a laboratory oven. After fermentation, the tea was dried following the same method as described above for the drying of green tea. The dried tea was sieved, using a 1.4 mm Endecotts sieve. The fraction that was found to be smaller than 1.4 mm in size was collected and stored in a sealed glass jar at room temperature (22 °C) until it was subjected to headspace analysis, either directly as dry material or as an infusion.

Headspace sampling of dry plant material

Each sample (8 g) was placed in a capped 100-ml glass bottle and the volatile organic compounds present in the headspace sampled for 5 h at 40 °C by means of a sample enrichment probe (SEP) (Burger et al., 2006). The analytes were thermally desorbed in the injector of a gas chromatograph for subsequent gas chromatographic analysis.

Preparation and headspace sampling of honeybush infusions

Infusions of fermented honeybush tea were prepared by adding 200 ml boiling (100 °C) bottled spring water (Valprè, Fricona Valley, South Africa) to 8 g dry, fermented honeybush in an insulated flask, sealing the flask immediately and allowing the tea to brew for 10 min while swirling the contents of the flask. The leaves and twigs were removed by filtering, and for each analysis 50 ml of the tea infusion was transferred to a 100-ml glass bottle, sealed and incubated at 40 °C for 30 min after which the infusion headspace was sampled by means of a SEP at 40 °C for 5 h.

Gas chromatography (GC) and gas chromatography–mass spectrometry (GC–MS)

GC analyses were carried out on a Carlo Erba HRGC 5300 gas chromatograph fitted with a split/splitless injector and FID (Milan, Italy). The sorbed volatiles were thermally desorbed from the SEP at an injector temperature of 230 °C without cryotrapping and were analysed on a capillary column (40 m × 0.3 mm) with a 5-m integrated retention gap and coated with 0.25 µm apolar PS-089 phase (DB-5 equivalent), using a temperature programme of 2 °C/min from 40 to 180 °C. Hydrogen was used as carrier gas at a linear flow velocity of 50 cm/s, measured at an oven temperature of 40 °C. The injector was operated in the split mode with split flow at 10 ml/min.

Low-resolution electron-impact mass spectrometry was performed on a Carlo Erba QMD 1000 GC–MS system (Milan, Italy) using the GC column and conditions specified above, and helium as carrier gas at a linear velocity of 28.6 cm/s at 40 °C. The line-of-sight interface was kept at 250 °C, while the ion-source temperature was set at 180 °C. Mass spectra were recorded at 70 eV at a scan rate of 0.9 s/scan with an interscan time of 0.1 s. The identity of the compounds was assigned by comparison of their mass spectra and retention indices (relative to C_6–C_{18} *n*-alkanes) with those of authentic reference compounds that were obtained commercially or were synthesized.

The structures of these compounds were confirmed by means of data obtained from reference libraries of mass spectrometric data (NBS, 1990, NIST, 2005 and Adams, 2004) and retention indices (Adams, 2004, ESO, 2006 and Hochmuth, 2006). These databases were also used to identify components for which standard reference compounds were unavailable. The relative concentrations of the headspace components were computed as percent areas of the total ion current (area % TIC); the data were calculated as mean values of three analyses of each sample.

Results and discussion

Headspace sampling technique

A high-capacity sample enrichment probe (SEP) (Burger et al., 2006) developed for research on the aroma constituents of herbal teas derived from different South African plant species, was used in this study as an alternative to existing headspace sampling techniques, such as solid-phase micro-extraction (SPME) (Arthur and Pawliszyn, 1990), which is widely used, but lacks the capacity necessary for this specific application, and stir-bar sorptive extraction (SBSE) (Baltussen et al., 1999), which has the necessary capacity, but requires expensive instrumentation. SEP analysis does not involve organic solvents and does not require cryofocussing of the analytes desorbed from the enrichment device in the injector of the GC.

Aroma composition

Seventy-seven components were identified in green honeybush aroma, comprising, *inter alia*, a large number of saturated and unsaturated alcohols, aldehydes and methyl ketones that were mostly present in lower relative concentrations in the aroma of fermented honeybush (Table 1). Most of these compounds, including 6-methyl-5-hepten-2-one, identified as the major constituent (54% TIC) of green honeybush aroma, are known to have distinctly grassy odours (Arctander, 1969). In the volatile fraction of fermented *C. genistoides* 79 compounds were identified, 46 of which were terpenoids, namely hemiterpenoids, monoterpenoids, sesquiterpenoids, diterpenoids, and tetraterpenoids, that were mostly present in much higher relative concentrations than in the green honeybush aroma. Linalool (36%) was identified as the major constituent of fermented tea aroma, while 6-methyl-5-hepten-2-one (14%) and the terpenoids limonene (3%), *trans*-furanoid linalool oxide (2%), *cis*-furanoid linalool oxide (2%), α-terpineol (17%), nerol (3%), and geraniol (11%), most of which are known to have floral and sweet odours (Arctander, 1969), occur in significant relative quantities.

The terpenoids geranyl acetone, β-cyclocitral, and dihydroactinidiolide were present in significantly lower concentrations in fermented *C. genistoides* than in unfermented material from the same batch. The major components of the green and fermented tea, representing 81% and 91% of their respective total ion currents, are printed in bold-faced type in Table 1 and their aroma descriptors, obtained from the literature, are given in Table 2. A good honeybush tea is expected to have lower concentrations of the components contributing to the undesirable green notes and higher concentrations of those responsible for the characteristically sweet, honey-like notes.

Dry plant material vs. *infusion*

The fact that tea is enjoyed as an infusion has to be taken into consideration in the evaluation of the aroma of the plant material. The chemical composition of the aroma of dry, fermented *C. genistoides* (Table 1) was qualitatively, and to a very large extent also quantitatively, identical to that of infused, fermented *C. genistoides* (data not shown), and it could be concluded that the dry plant material can be used as substitute for infusions in further analyses. A rapid screening method will greatly benefit from the use of the dry plant material, which offers definite advantages such as the absence of water from the sample matrix, the elimination of a time-consuming step and the circumvention of precise control of parameters, such as infusion and holding temperatures and times and pH of the water.

Envisaged rapid instrumental screening method

Minor components are not necessarily unimportant in the context of the evaluation of tea flavour. A headspace sampling period of 5 h and a GC programming rate of 2 °C/min (run-time 60 min) were used in this study to identify the aroma volatiles as fully as possible. However, a much shorter sampling period and GC temperature programme, as well as a GC column with thinner phase coating, will be implemented for rapid analysis, and quantification can be done by integration of GC data instead of using GC–MS generated data. This complete chemical characterization of the honeybush aroma allows for the determination by GC-olfactometry of the contribution of minor constituents

to the aroma with a view to including them in the rapid screening method. This study provides the analytical and chemical information required for the development of a device and protocol that has to be sufficiently rapid for the large-scale screening of honeybush tea during the envisaged plant material improvement process.

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Tables

Table 1.
Volatile organic compounds identified by headspace–GC–MS analysis in the aroma of dry, green (unfermented), and dry, fermented honeybush, *Cyclopia genistoides*

Compound[a]	RI[b]	ID[c]	Unfermented		Fermented	
			Area %[d]	RSD (%, *n* = 3)	Area %	RSD (%, *n* = 3)
1-penten-3-ol	626	A	0.27	12	0.21	5
Pentanal	641	A	0.17	3	0.02	16
2-ethylfuran	653	B	0.05	16	0.03	8
1-pentanol	741	A	0.09	18	0.13	10
2-penten-1-ol[e]	744	B	0.20	4	0.14	12
Hexanal	773	A	**4.08**	**4**	**1.76**	**7**
(*E*)-2-hexenal	824	A	0.22	6	0.10	9
(*Z*)-3-hexen-1-ol	831	A	0.46	17	0.02	6
2-methylbutanoic acid[f]	837	A	0.06	22	0.04	17
1-hexanol	844	A	0.05	2	0.01	12
4-acetylcyclohexene[f]	858	C	0.01	9	–[g]	–
2-heptanone	858	A	0.06	7	0.05	3
4-heptenal[f]	863	C	0.03	7	–	–
ã-butyrolactone	866	A	0.08	13	0.10	5
Heptanal	868	A	0.08	8	0.04	5
2-acetylfuran	873	A	0.09	4	0.07	7
Tiglic acid	874	A	–	–	0.01	6
Benzaldehyde	923	A	0.26	9	0.09	6
6-methyl-5-hepten-2-one	970	A	**54.07**	**2**	**14.17**	**2**
2,4-heptadienal[e]	974	C	0.02	12	–	–
2-pentylfuran	979	A	0.36	3	0.41	10
trans-dehydroxy furanoid linalool oxide[f]	980	A	0.06	3	0.15	10
Hexanoic acid	927	A	–	–	0.04	6
Myrcene	982	A	0.10	3	0.35	8
(*E*, *E*)-2,4-heptadienal	993	A	0.58	6	0.02	10
cis-dehydroxy furanoid linalool oxide[f]	998	A	0.04	11	0.05	7
Decane	999	A	0.01	7	0.01	14
α-terpinene	1009	B	0.17	3	0.10	8
Unidentified	1010		0.48	13	0.23	8
3,4-dimethyl-2,5-furandione	1011	C	0.20	9	0.10	10
p-cymene	1014	B	0.58	5	0.34	5
Benzyl alcohol	1017	A	0.01	3	–	–
2,2,6-trimethylcyclohexanone	1019	B	0.41	5	0.12	6
Limonene[f]	1020	A	**4.60**	**2**	**3.15**	**3**
Hexan-4-olide	1026	A	0.18	7	0.05	13
(*Z*)-β-ocimene	1030	A	0.15	12	0.17	11
(*E*)-β-ocimene	1039	A	0.09	7	0.13	2

Compound[a]	RI[b]	ID[c]	Unfermented		Fermented	
			Area %[d]	RSD (%, *n* = 3)	Area %	RSD (%, *n* = 3)
Unidentified	1041		0.87	1	0.39	2
γ-terpinene	1047	A	0.20	4	0.12	6
3,5-octadien-2-one[f]	1052	C	**2.42**	**1**	0.50	3
***trans*-furanoid linalool oxide**[f]	1058	A	0.93	16	**2.29**	**2**
***cis*-furanoid linalool oxide**[f]	1073	A	0.81	1	**1.67**	**4**
Isoterpinolene	1075	B	0.86	4	0.56	2
6-methyl-3,5-heptadien-2-one[e]	1082	B	**1.43**	**3**	–	–
Linalool[f]	1088	A	**10.68**	**2**	**35.94**	**0**
2-phenylethanol	1090	A	0.07	6	0.08	8
4-ketoisophorone	1116	A	0.24	15	0.09	7
(*E*)-3-nonen-2-one	1122	A	0.11	20	–	–
2,6-nonadienal[e]	1134	B	0.09	8	0.01	13
2,6-dimethyl-5,7-octadien-2-ol (ocimenol)[e,f]	1139	C	–	–	0.01	16
2,2,6-trimethyl-1,4-cyclohexanedione	1139	C	–	–	0.01	22
Nerol oxide[f]	1141	B	0.04	23	0.12	7
cis-pyranoid linalool oxide[f]	1154	B	0.06	4	0.14	3
trans-pyranoid linalool oxide[f]	1160	B	0.03	1	0.07	11
Terpinen-4-ol[f]	1164	A	0.58	4	0.48	1
α-terpineol[f]	1180	A	**3.75**	**3**	**17.30**	**1**
Safranal	1183	A	0.12	10	0.05	7
Decanal	1196	B	0.04	16	0.02	11
p-menth-1-en-9-al (diastereomer) [f]	1201	C	0.02	16	0.02	11
Dodecane	1202	A	0.06	12	0.01	13
p-menth-1-en-9-al (diastereomer)[f]	1204	C	0.02	16	0.02	1
β-cyclocitral	1207	A	**1.47**	**2**	0.25	1
Unidentified	1219		0.35	10	0.13	5
Nerol	1223	A	0.34	8	**3.49**	**1**
Neral	1232	A	0.01	11	0.01	14
p-anisaldehyde	1238	A	–	–	0.01	14
Geraniol	1253	A	0.96	7	**10.80**	**2**
Geranial	1264	A	0.03	18	0.06	8
Unidentified	1273		0.26	16	–	–
Neryl formate	1281	B	0.01	10	0.05	11
2-undecanone	1296	A	0.02	7	0.01	8
Geranyl formate	1303	A	0.03	2	0.18	3
Tridecane	1311	A	0.02	6	0.01	5
Unidentified	1324		0.48	11	0.21	1
Hexyl tiglate	1330	B	–	–	0.02	1
Unidentified	1336		–	–	0.37	2
Nonan-4-olide	1346	A	0.02	7	0.02	0
Eugenol	1348	A	0.06	3	0.11	5

Compound[a]	RI[b]	ID[c]	Unfermented		Fermented	
			Area %[d]	RSD (%, *n* = 3)	Area %	RSD (%, *n* = 3)
Neryl acetate	1360	A	0.02	1	0.03	8
3-hydroxy-2,4,4-trimethyl-pentyl 2-methylpropanoate	1367	C	0.01	1	–	–
(*E*)-β-damascenone	1375	B	0.09	6	0.34	3
α-copaene	1375	B	0.10	6	0.03	3
Geranyl acetate	1377	A	0.07	10	0.08	6
Tetradecane	1402	A	0.02	20	0.02	3
(*E*)-β-damascone	1403	A	–	–	0.06	8
Geranyl acetone	1450	A	**2.33**	**11**	**0.59**	**2**
Oxoedulan	1478	C	–	–	0.02	10
Unidentified	1485		0.43	3	–	–
β-ionone[f]	1487	A	0.74	9	0.11	2
β-dihydroagarofuran[f]	1508	B	–	–	0.01	12
Dihydroactinidiolide[f]	1516	B	**1.02**	**12**	0.16	1
trans-calamenene[f]	1526	B	0.01	20	–	–
α-calacorene[f]	1542	B	–	–	0.02	5
(*Z*)-3-hexenyl benzoate	1563	A	0.02	20	0.02	10

a In order of elution from apolar PS-089 column (DB-5 equivalent).

b Retention index (RI), relative to C_6–C_{18} *n*-alkanes, on PS-089 column.

c Identification: A, mass spectrum and RI correspond to those of an authentic standard; B, comparison of mass spectrum and RI with published mass spectrometric and RI data; C, comparison with published mass spectrometric data.

d Average percent area calculated from TIC. The dominant compounds are indicated in bold.

e *E* / *Z*—Stereochemistry not determined.

f Absolute configuration of chiral compounds not determined.

g Not detected, or area % < 0.005.

Table 2.
Odour descriptions of the main volatile components identified by headspace–GC–MS analysis in the aroma of dry, green (unfermented), and dry, fermented honeybush, *Cyclopia genistoides*

Compound	RI	Unfermented	Fermented	Aroma descriptors[a]
		Area %	Area %	
Hexanal	773	4.08	1.76	Fatty, green grass
6-methyl-5-hepten-2-one	970	54.07	14.17	Oily, green grass, herbaceous
Limonene	1020	4.60	3.15	Citrus, sweet, orange, lemon
3,5-octadien-2-one	1052	2.42	0.50	–
trans-furanoid linalool oxide	1058	0.93	2.29	Sweet–woody, floral–woody–earthy
cis-furanoid linalool oxide	1073	0.81	1.67	Sweet–woody, floral–woody–earthy
6-methyl-3,5-heptadien-2-one	1082	1.43	–	Warm spicy, cinnamon-like
Linalool	1088	10.68	35.94	Refreshing, light, clean, floral
α-terpineol	1180	3.75	17.30	Fragrant, floral, sweet lilac
β-cyclocitral	1207	1.47	0.25	Minty, fruity, green
Nerol	1223	0.34	3.49	Sweet, floral
Geraniol	1253	0.96	10.80	Sweet, floral, rose, fruity
Geranyl acetone	1450	2.33	0.59	Floral, sweet-rosy, slightly green
Dihydroactinidiolide	1516	1.02	0.16	Sweet, floral, tobacco

a Arctander, 1969, Sigma-Aldrich, 2004 and Leffingwell, 2004.