Characterization of Volatiles and Aroma-Active Compounds in Honeybush (*Cyclopia subternata*) by GC-MS and GC-O Analysis

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Abstract

Volatile organic compounds (VOCs) in fermented honeybush, *Cyclopia subternata*, were sampled by means of a high-capacity headspace sample enrichment probe (SEP) and analyzed by gas chromatography–mass spectrometry (GC-MS). Stereochemistry was determined by means of enantioselective GC-MS with derivatized β-cyclodextrin columns as chiral selectors. A total of 183 compounds, the majority of which are terpenoids (103; 56%), were identified by comparing their mass spectra and retention indices with those of reference compounds or tentatively identified by comparison with spectral library or literature data.

Of these compounds, 37 were determined by gas chromatography–olfactometry (GC-O), using detection frequency (DF) and aroma extract dilution analysis (AEDA), to be odor-active (FD ≥ 2). (*E*)-β-Damascenone, (*R*/*S*)-linalool, (*E*)-β-damascone, geraniol, (*E*)-β-ionone, and (7*E*)-megastigma-5,7,9-trien-4-one were identified with the highest FD factors (≥512). The odors of certain compounds, that is, (6*E*,8*Z*)-megastigma-4,6,8-trien-3-one, (6*E*,8*E*)-megastigma-4,6,8-trien-3-one, (7*E*)-megastigma-5,7,9-trien-4-one, 10-*epi*-γ-eudesmol, *epi*-α-muurolol, and *epi*-α-cadinol, were perceived by GC-O assessors as typically honeybush-like.

Keywords: *Cyclopia subternata*; honeybush tea; volatile organic compounds; terpenoids; odor-active compounds; headspace analysis; sample enrichment probe (SEP); gas chromatography−mass spectrometry (GC-MS); gas chromatography−olfactometry (GC-O).

Introduction

Honeybush tea is a sweet, honey-like herbal brew made from the leaves and twigs of *Cyclopia* spp. (family Fabaceae; tribe Podalyrieae), endemic to the fynbos biome in the Western and Eastern Cape Provinces of South Africa. It is one of the few indigenous South African plants that made the transition from the wild to a commercial product during the past 100 years.(1) The increasing popularity of honeybush can be ascribed not only to its pleasant, characteristic flavor but also to a low tannin content, the absence of caffeine, and health-promoting properties.(1, 2) Although more than 20 *Cyclopia* species of honeybush grow in the wild, only a few, that is, *Cyclopia intermedia*, *Cyclopia subternata*, and *Cyclopia genistoides*, are currently commercially exploited to manufacture tea.

Honeybush is mostly enjoyed in “fermented” (oxidized) form, but the “unfermented” (green) product also has a small market share.(1) The present research forms part of an ongoing comprehensive research program at the Agricultural Research Council (ARC) Infruitec-Nietvoorbij in South Africa, aimed at the development of a viable honeybush industry.(1) In the first phase of the research on the aroma compounds in *Cyclopia* spp., the analytical methodology was developed for the sampling and analysis of extremely low concentrations of volatile organic compounds (VOCs) in dry or infused unfermented (green) and fermented honeybush, using the commercial species, *C. genistoides*, as the representative species.(3)

Many of the terpenoids identified in *C. genistoides*,(3) for example, α-terpineol, hexahydrofarnesylacetone, 2,6-dimethyl-1,7-octadien-3,6-diol, *Z*- and *E*-geraniol, linalool, linalool oxide isomers, pseudoionone, β-damascone, and eugenol, are known to have floral, sweet, sweet-woody, floral-woody, or spicy odors.(4) Sensory descriptive analysis showed that *C. subternata* differs from *C. genistoides* with respect to their sensory profile with *C. subternata* predominantly having a fruity sweet and apricot jam-like flavor note as opposed to *C. genistoides* having a vegetative

sweet aroma.(5) Mainly for this reason, *C. subternata* was chosen as the representative species in the present phase of the research to determine the actual aroma-active constituents in honeybush by means of gas chromatography–mass spectrometry (GC-MS) in conjunction with gas chromatography–olfactometry (GC-O).

Solid-phase microextraction (SPME) is an elegant method for trapping VOCs from the headspace of solids and liquids, specifically aqueous samples, and has been applied successfully in analyses of the VOCs in a wide range of plant products, including teas.(6) However, it was found to lack the enrichment efficiency required for the analysis of VOCs in certain indigenous herbal teas.(3, 7) Stir bar sorptive extraction (SBSE), on the other hand, is a powerful, high-capacity technique for the enrichment of VOCs from similar media but requires expensive automated thermal desorption and cryofocusing instrumentation.

The sample enrichment probe (SEP)(7, 8) was developed specifically to fill a niche that exists for a moderately priced, high-capacity sampling method that can be used in applications that do not require automated, high-throughput sample handling.

Materials and Methods

Plant Material

Cultivated *C. subternata* was harvested on the farm Toekomst near Bredasdorp in the Western Cape Province of South Africa. About two-thirds of the shoot lengths were cut from the plants, and the shoots were shredded to 2–3 mm lengths using a mechanized fodder cutter. Deionized water was added to wet the plant material superficially, which was then placed in a stainless steel container, covered with aluminum foil, and allowed to ferment (oxidize) in a laboratory oven at 90 °C for 16 h.(9) After fermentation, the tea was dried, 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 of 3 m/s. The dried tea was sieved, using a 1.4 mm Endecotts sieve.

The fractions smaller than 1.4 mm were collected and stored in airtight glass jars fitted with screw caps lined with aluminum foil, in the absence of light at a controlled temperature (22 °C), until subjected to analysis of the headspace volatiles.

Preparation and Headspace Sampling of Brewed Honeybush

Brews of fermented honeybush plant material were prepared in batches by adding boiling water (220 mL per batch) to 30 g of the dry plant material in a 500 mL round-bottom flask. The leaves were infused by heating the flask at 100 °C for 5 min until boiling. The water was allowed to cool down to 90 °C, the flask was covered, and the plant material was allowed to brew for 9 h at this temperature. The leaves and twigs were then filtered off. For each low-resolution GC-MS (GC-LRMS) analysis, 50 mL of filtrate was transferred to a 100 mL glass bottle with adapted cap,(7) sealed, and incubated at 50 °C for 30 min, after which the headspace volatiles of the filtrate were enriched at 50 °C for 5 h using a SEP30 (MasChrom Analisetegniek, Stellenbosch, South Africa), which contains 30 mm polydimethylsiloxane (PDMS) tubing, equivalent to 28 mg of PDMS.(7, 8)

Longer enrichment periods of 17 h and a SEP60 (56 mg of PDMS) were used for GC-O and high-resolution GC-MS (GC-HRMS) analyses.

GC Columns

Most of the capillary columns used in this study were manufactured by the Laboratory for Ecological Chemistry (LECUS, Stellenbosch University) and were provided with integrated retention gaps of 1–2 m: column A [glass, 40 m × 0.25 mm i.d., coated with 0.25 µm of PS-089-OH (DB-5 equivalent)], column B [glass, 40 m × 0.25 mm i.d., coated with 0.25 µm of the polar stationary phase AT-1000 (FFAP equivalent)], enantioselective column C [glass, 30 m × 0.3 mm i.d., coated with 0.25 µm of OV-1701-OH containing 10% heptakis(2,3-di-*O*-methyl-6-*O*-*tert*-butyldimethylsilyl)-β-cyclodextrin], and enantionselective column D [glass, 30 m × 0.3 mm i.d., coated with 0.25 µm of OV-1701-OH containing 10% heptakis(2,3-di-*O*-acetyl-6-*O*-*tert*-butyldimethylsilyl)-β-cyclodextrin)].(10)
The glass columns were prepared according to methods adapted from those of Grob et al.(11) An Agilent HP5MS column (30 m × 0.25 mm i.d, coated with 0.25 µm 5% phenylmethylpolysiloxane) (Agilent JW Scientific, Folsom, United States) and a Supelcowax-10 column (60 m × 0.32 mm i.d., coated with 0.5 µm Carbowax 20 M phase)

(Sigma-Aldrich/Supelco, Bellefonte, PA) were used for GC-HRMS and gas chromatography–mass spectrometry–olfactometry (GC-MS-O) analysis, respectively.

GC-MS

GC-LRMS was performed on a Carlo Erba QMD 1000 GC-MS system (Milan, Italy) using helium as the carrier gas at a linear velocity of 28.6 cm/s (at a column temperature of 40 °C) and either apolar column A or polar column B. The VOCs sorbed in the PDMS of the SEP were desorbed at an injector temperature of 230 °C (split flow, 10 mL/min). The desorbed material was not cryofocused but was swept into the capillary column by the carrier gas and cold-trapped on the column at a temperature below 30 °C. The column temperature was then ballistically increased to 40 °C, after which temperature programs of 2 °C/min from 40 to 280 °C and 2 °C/min from 40 to 250 °C were used for columns A and B, respectively.

The final temperature was held for 20 min at either 280 or 250 °C. The line-of-sight interface was kept at 250 °C, while the ion-source temperature was set at 180 °C. Electron-impact (EI) mass spectra were recorded at 70 eV at a scan rate of 0.9 s/scan, with an interscan time of 0.1 s. GC-MS data processing was achieved using an NBS database (VG Masslab, VG Instruments, Manchester, United Kingdom) and NIST mass spectral library (version 2.0d, National Institute of Standards and Technology, United States).

GC-HRMS was performed on a Waters GCT Premier benchtop orthogonal acceleration time-of-flight instrument (Waters, MA). The volatiles were desorbed from the SEP at an injector temperature of 260 °C (splitless mode) and analyzed using helium as the carrier gas (1 mL/min) on an Agilent HP5MS column programmed at 2 °C/min from 40 to 280 °C. The ion-source temperature was set at 180 °C. Data were acquired in centroid mode, scanning from 35–650 amu, and using perfluorotri-*N*-butylamine as a reference for accurate mass determination. Mass spectra were recorded at 70 eV at a scan rate of 0.2 s/scan, with an interscan time of 0.05 s.

Mass differences of less than 5 mDa between the observed mass and the mass calculated for a specific ion were considered acceptable.

Enantioselective GC-MS Analysis

Enantioselective GC-LRMS with the enantioselective columns C and D was performed on a Fisons MD800 GC-MS system (Rodano, Milan, Italy). Helium was used as the 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, using a temperature program of 1 °C/min from 40 to 240 °C for column C and 1 °C/min from 40 to 200 °C for column D.

GC-O

GC-O analyses were performed on a conventional Carlo Erba HR gas chromatograph converted for GC-O use by installing a glass effluent splitter, a humidified air conduit, and a glass sniffing port. The GC capillary column was connected to the glass effluent splitter with two deactivated fused silica tubing outlets of equal lengths conducting the column effluent to the FID and to the sniffing device, according to the basic design described for gas chromatography–electroantennographic detection (GC-EAD) analysis by Burger et al.(12)

GC-O analyses were carried out using the analytical parameters described above for the GC-MS analyses. The chemical structures of the odor-active compounds were confirmed by GC retention time comparison with authentic reference samples.

Detection Frequency Method

The headspace volatiles of infused *C. subternata* were subjected to GC-O evaluation by a 15-membered panel of assessors who were required to individually sniff the GC effluent and report the results according to the detection frequency (DF) method.(13) To prevent sensory “fatigue”, each assessor was required to sniff the effluent during alternating first and second halves of consecutive analyses. The total number of panel members who could positively detect an odorant at a specific retention time was expressed as a percentage of the total number of assessors.

Aroma Extract Dilution Analysis

A brew of *C. subternata*, prepared as described above, was diluted stepwise (1:1 by volume) with boiled filtered water, and the individual dilutions were analyzed by GC-O by a single trained assessor who was required to sniff the effluent of each consecutive dilution and report which odorants could still be detected. Sniffing of the series of dilutions proceeded until no odorant could be detected by the assessor, and the previous dilution was recorded as the final dilution. Sniffing of all extract dilutions was repeated twice.

An averaged flavor dilution (FD) factor was calculated for each odorant by means of the formula FD = $R^{(n_1+n_2)/2}$, where n_1 (of first replicate) and n_2 (of second replicate) represent the last dilution in which the odorant was still detectable, and R is the factor by which the sample was sequentially diluted (in this case R = 2).(14)

GC-MS-O

GC-MS-O was performed on a Hewlett-Packard 5890 Series II gas chromatograph (Hewlett-Packard, Waldbronn, Germany), connected to a 5972 Series mass spectrometer (Hewlett-Packard), and equipped with an olfactometric port. The sorbed volatiles were thermally desorbed from the SEP at an injector temperature of 250 °C (splitless mode, 2 min) and analyzed on a Supelcowax-10 column (60 m × 0.32 mm i.d., coated with 0.5 µm Carbowax 20 M phase), using a temperature program of 2 °C/min from 40 to 220 °C. Helium was used as the carrier gas at a linear flow rate of 3 mL/min (at 40 °C).

Mass spectra were recorded at 70 eV at a scan rate of 2.36 scans/s, scanning from 30 to 350 amu, and compared to those in a Wiley 275 database (Wiley & Sons Inc., New York).

GC-MS Retention Index Determination

The tentative MS identification of honeybush VOCs, analyzed on both polar and nonpolar GC columns, was confirmed by GC-MS retention time comparison of these compounds with authentic reference compounds. GC-MS retention indices (RIs), determined relative to the C_5–C_{18} *n*-alkanes on nonpolar column A, were compared with those of the reference compounds and confirmed with published RI values.(15, 16)

These RI databases were also used to identify components for which standard reference compounds were not available.

Chemicals

The following reference compounds were purchased from the companies given in parentheses: 1-pentanol, 1-penten-3-ol, 2-ethylfuran, (*Z*)-2-penten-1-ol, pentanal, hexanal, (*Z*)-3-hexen-1-ol, (*E*)-2-hexenal, 2-methylbutanoic acid, heptanal, (*E*)-2-heptenal, benzaldehyde, 6-methyl-2-heptanone, 6-methyl-5-hepten-2-one, 2-pentylfuran, myrcene, octanal, (*E*,*E*)-2,4-heptadienal, α-terpinene, (*E*)-3-octen-2-one, *p*-cymenene, 3-thujanone, 4-acetyl-1-methylcyclohexene, 4-ketoisophorone, (*E*)-3-nonen-2-one, (*E*,*Z*)-2,6-nonadienal, (*E*)-2-nonenal, terpinen-4-ol, *p*-cymen-8-ol, α-terpineol, safranal, decanal, β-cyclocitral, nerol, (*Z*)-3-hexenyl 2-methylbutanoate, citral (neral and geranial), (*Z*)-3-hexenyl isovalerate, 2,6,6-trimethyl-1-cyclohexene-1-acetaldehyde, geraniol, 2-undecanone, theaspirane, undecanal, (*E*,*E*)-2,4-decadienal, (*Z*)-3-hexenyl (*E*)-2-methyl-2-butenoate, nonan-4-olide, 6,10-dimethyl-2-undecanone, dodecanal, α-ionone, jasmin absolute, decan-5-olide, geranylacetone, dodecanoic acid, caryophyllene oxide, *trans*-nerolidol, (*Z*)-β-ocimene, geranyl acetate, (*Z*)-3-hexenyl benzoate, and benzothiazole (Sigma Aldrich, Steinheim, Germany); 3-methylbutanoic acid, *p*-cymene, and dodecane (Merck, Darmstadt, Germany); 2-heptanone and methyl dodecanoate (Polyscience Corp., Evanston, IL); (*Z*)-4-heptenal, α-pinene, 1-octen-3-ol, α-phellandrene, 2,2,6-trimethylcyclohexanone, limonene, γ-terpinene, *trans*-furanoid linalool oxide, *cis*-furanoid linalool oxide, terpinolene, linalool, isophorone, borneol, *p*-anisaldehyde, eugenol, α-copaene, β-damascone, and (*E*)-β-ionone (Fluka, Buchs, Switzerland); (6*Z*)-2,6-dimethyl-2,6-octadiene, (6*E*)-2,6-dimethyl-2,6-octadiene, (3*E*)-6-methyl-3,5-heptadien-2-one, (*E*)-caryophyllene, and pseudoionone (ICN Pharmaceuticals Inc., Plainview, NY); decane, tetradecane, and pentadecane (Supelco, Bellefonte, PA); 2-phenylethanol, nonanoic acid, and camphene (BDH, Poole, United Kingdom); *allo*-ocimene (K&K laboratories, Plainview, NY); neryl acetate (Haarmann and Reimer, Springfield, United States); β-damascenone (Firmenich, Geneva, Switzerland); and geranyl formate (Dauphin, Bourgoin-Jallieu, France). (*E*)-β-Ocimene was a gift, originally purchased from Givaudan Corp. (Cincinnati, OH). *cis*-Pyranoid linalool oxide and *trans*-pyranoid linalool oxide were previously synthesized in our

laboratory.(17) Solutions of the reference componds were prepared in dichloromethane (Merck Residue Analysis grade, Darmstadt, Germany).

Syntheses

The following compounds were synthesized according to the literature cited (experimental details and NMR data are given in the Supporting Information): 2,6,6-trimethylcyclohex-2-enone,(18) (*E*,*E*)- and (*Z*,*E*)-3,5-octadien-2-one,(19) 5,6-epoxy-β-ionone,(20) hexyl tiglate, benzyl tiglate, 3,4-dehydro-β-ionone,(21) octan-5-olide,(22) hexahydrofarnesylacetone,(23) nerol oxide,(24) (+)-*p*-menth-1-en-9-al,(25) and *cis*- and *trans*-dehydroxylinalool oxide.(26)

Results and Discussion

The honeybush plant material was processed under controlled conditions simulating those used for commercially produced tea to ensure development of the same flavor profile. During processing and storage, contact with rubber and plastic materials, which could possibly be responsible for the absorption of headspace volatiles or could contribute to headspace impurities, was avoided. Commercial honeybush tea has a shelf life of a minimum of 2 years and lasts perfectly well even if exposed to air, light, and ambient temperatures. However, for the purpose of the study, we adhered to controlled storage conditions to ensure the preservation of the material over the period during which the study was conducted.

In addition, brewing, incubation and sampling times, and temperatures were standardized. A long brewing time was chosen to simulate traditional practice, entailing prolonged heating for sufficient release of flavor. Honeybush was known as “three day tea”, as the spent leaves could repeatedly be used by just adding water after decantation of the tea and keeping the brew warm, for example, on the side of a coal stove.(2) The VOCs present in the headspace of the brews of fermented *C. subternata*, chosen as representative honeybush species in this study on account of its characteristic heavy, sweet aroma, were sampled by means of a high-capacity SEP.

The analytes desorbed from the SEP were analyzed by GC-LRMS and GC-HRMS on both nonpolar and polar GC columns. Apart from supplying molecular formulas and elemental compositions of ion fragments, the high data acquisition rate of the GC-HRMS instrument also allowed improved deconvolution of overlapping peaks in the total ion chromatogram (TIC). The stereochemistry of chiral compounds was determined, as far as possible, by means of enantioselective GC-MS with derivatized β-cyclodextrin columns. A total of 183 compounds were detected, and most of them could be identified by combining a number of diagnostic techniques.

Comparison of mass spectra with those in commercial online and offline databases, combined with high-resolution molecular formula data, served as a tentative starting point. In most cases, the proposed structures were confirmed by GC-MS retention time comparison with authentic reference compounds. Furthermore, RIs, determined on the nonpolar column, were compared with those of the reference compounds and confirmed with published RI values. These RI databases were also used to identify components for which standard reference compounds were not available.

In some cases, it was necessary to revert to fundamental interpretation of mass spectra, aided by published diagnostic information (27) and previous mass spectrometric studies carried out in our laboratory. The majority of identified or tentatively identified compounds were terpenoids (103; 56%), comprising terpene ketones (27 constituents), terpenes (24), terpene ethers (20), terpene alcohols (18), terpene aldehydes (7), terpene esters (6), and a terpene lactone (1). Of the nonterpenoid compound classes found in the headspace of the brews of fermented *C. subternata*, aldehydes (20) are the most well represented, followed by ketones (12), hydrocarbons (11), esters (9), alcohols (6), lactones (5), furans (5), carboxylic acids (4), ethers (2), and a thiazole compound (1) (Table 1).

The qualitative results obtained in the present study correspond to those previously obtained for *C. genistoides*,(3) but the VOC profiles of the two species do differ quantitatively. This aspect will be highlighted in a future study comparing the aroma profiles of a number of *Cyclopia* species. Existing GC-O methodologies have been reviewed in detail by Delahunty et al.(13) In the present study, DF and aroma extract dilution analysis (AEDA) were chosen as

aroma evaluation techniques for the identification of the aroma-active compounds in fermented honeybush. A total of 37 components were found to be odor-active (FD ≥ 2) (Table 1, bold type).

A single trained assessor, who had also been a member of the DF panel, carried out two replicates of the AEDA experiment, and the respective FD factors were averaged. It was previously determined during the DF experiment that this particular assessor had no specific anosmia for any of the odor-active compounds identified by the panel as a whole, and she was able to detect each individual compound with an accuracy of 100%. GC-MS-O analyses using a polar column were carried out to confirm the results obtained by GC-O using a nonpolar column. The characteristic odor and flavor of honeybush is quite unlike that of any well-known fruit, flower, or tea.

Popular descriptions of the flavor of honeybush tea vary from that of hot apricot jam, floral, honey-like, and dried fruit mix with the overall impression of sweetness.(2) (*E*)-β-Damascenone, (*R*/*S*)-linalool, (*E*)-β-damascone, geraniol, (*E*)-β-ionone, and (7*E*)-megastigma-5,7,9-trien-4-one were identified in this study with FD factors higher than 512. The three odorants with highest FD factors, that is, (*E*)-β-damascenone (FD 32768), (*R*/*S*)-linalool (FD 16384), and (*E*)-β-damascone (FD 4096), were detected by all of the assessors in the DF experiment and therefore have reported DF values of 100, while geraniol (FD 512), (*E*)-β-ionone (FD 512), and (7*E*)-megastigma-5,7,9-trien-4-one (FD 512) all had DF factors ≥60.

Four of the mentioned compounds are generally associated with a sweet aroma, that is, (*E*)-β-damascenone (also honey-like, fruity, dried prune),(28-31) linalool (also floral, floral-woody),(4, 29) geraniol (also floral, floral-woody),(4, 29) and (*E*)-β-ionone (also floral, fruity).(4, 28, 32) (*E*)-β-Damascone and (7*E*)-megastigma-5,7,9-trien-4-one are not generally described as sweet but rather as tea-like and spicy with undertones of dried fruit.(28, 30) In a study on Grenache wine, β-damascenone, detected in the present study with the highest FD factor, has been qualified as an "aroma enhancer".

Although it had the second higest odor activity value by GC-O, results indicated that it was not a character impact compound but probably contributed a sweet background note.(14) (*E*)-β-Damascenone, (*R*/*S*)-linalool, and β-ionone have previously been identified as key aroma compounds in apricots.(33) Two other odorants identified with high FD or OAV values in apricot aroma(33) were also identified in the present study but with low FD values, namely, decan-5-olide (FD 2) and (*E*/*Z*)-2.6-nonadienal (FD 32). The GC-O assessors, all of whom are familiar with the aroma and taste of honeybush tea, singled out the compounds (6*E*,8*Z*)-megastigma-4,6,8-trien-3-one (FD 2), (6*E*,8*E*)-megastigma-4,6,8-trien-3-one (FD 8), (7*E*)-megastigma-5,7,9-trien-4-one (FD 512), 10-*epi*-γ-eudesmol (FD 64), *epi*-α-muurolol (FD 64), and *epi*-α-cadinol (FD 64) as typically honeybush-like.

Of these six compounds, only (6*E*,8*Z*)-megastigma-4,6,8-trien-3-one, (6*E*,8*E*)-megastigma-4,6,8-trien-3-one, and 10-*epi*-γ-eudesmol are generally described as sweet.(28, 31) The latter compound also has woody, floral descriptors,(30, 31) while the megastigmatrienones are also associated with a woody, tobacco-like aroma.(28, 30) Both *epi*-α-muurolol and *epi*-α-cadinol have herbaceous descriptors, while *epi*-α-muurolol is also considered to be slightly spicy.(31) A more comprehensive discussion of the role of the identified aroma-active compounds in honeybush flavor will be made possible in the future by an ongoing investigation into the association between the quantitative data obtained for the sensory attributes of several *Cyclopia* species and their volatile compounds using multivariate statistical analysis.

To our knowledge, the results reported here constitute the first comprehensive chemical and olfactometric characterization of the VOCs in a *Cyclopia* species.

Supporting Information
Comparison of SEP and SPME enrichment capacity, synthetic methods, and ^{1}H, ^{13}C NMR, and MS data of synthesized compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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Abbreviations Used	
VOCs	volatile organic compounds
SEP	sample enrichment probe
SPME	solid-phase microextraction
SBSE	stir bar sorptive extraction
PDMS	polydimethylsiloxane
GC-MS	gas chromatography–mass spectrometry
GC-LRMS	low resolution gas chromatography–mass spectrometry
GC-HRMS	high resolution gas chromatography–mass spectrometry
GC-FID	gas chromatography with flame ionization detection
GC-EAD	gas chromatography–electroantennographic detection
GC-O	gas chromatography–olfactometry
GC-MS-O	gas chromatography–mass spectrometry–olfactometry
DF	detection frequency
AEDA	aroma extract dilution analysis
FD	flavor dilution
RI	retention index
TIC	total ion chromatogram

Table

Table 1. VOCs in Honeybush (*Cylclopia subternata*) (Odor-Active Compounds in Bold Type)

	RI					
compound name[a]	**column A**[b]	**column B**[c]	**ID**[d]	**enantiomeric ratio (column)**[e]	**DF**[f]	**FD**[g]
1-penten-3-ol	639	1133	A	racemic [R_s = 0.60] (C)		
pentanal	649	1000	A			
2-ethylfuran	659	977	A			
1-pentanol	739	1204	A			
(*Z*)-2-penten-1-ol	743	1261	A			
hexanal	767	1054	A, D			
2-ethyl-5,5-dimethyl-1,3-cyclopentadiene	827	1545	B			
(*E*)-2-hexenal	828	1160	A			
(*Z*)-3-hexen-1-ol	838	1316	A			
3-methylbutanoic acid	857	1581	A, D		93	8
1,3,6-octatriene[h]	863		B			
(*R*)-2-methylbutanoic acid	866	1588	A, C	0*S*:100*R* [R_s = 0.76] (C)	73	2
2-heptanone	871	1105	A			
(*Z*)-4-heptenal	879	1167	A			
heptanal	882	1107	A			
α-pinene	923	1006	A	82(1*S*,5*S*):18(1*R*,5*R*) [R_s = 2.4](C)		
camphene	936	1037	A	15*R*:85*S* [R_s = 1.3](C)		
benzaldehyde	936	1426	A			
(*E*)-2-heptenal	938	1352	A			
6-methyl-2-heptanone	939	1221	A			
2,2,6-trimethyl-6-vinyltetrahydropyran[h]	960	1073	B			
1-octen-3-ol	969	1386	A	38*S*:62*R* [R_s = 1.5](D)		
6-methyl-5-hepten-2-one	971	1269	A, C			
(*E*,*Z*)-2,4-heptadienal	978	1384	B			
(6*Z*)-2,6-dimethyl-2,6-octadiene	981	1069	A			
2-pentylfuran	981	1164	A			
trans-dehydroxylinalool oxide (furanoid)[h]	981	1150	A			
myrcene	983	1116	A			
octanal	988	1221	A			
(2*Z*)-2-(2-pentenyl)furan	990	1229	B			
(*E*,*E*)-2,4-heptadienal	992	1409	A			
α-phellandrene	994	1135	A	20*R*:80*S* [R_s = 0.57] (C)		

	RI					
compound name[a]	**column A**[b]	**column B**[c]	**ID**[d]	**enantiomeric ratio (column)**[e]	**DF**[f]	**FD**[g]
cis-dehydroxylinalool oxide (furanoid)[h]	997	1185	A			
decane	997	1020	A			
α-terpinene	1007	1118	A			
p-cymene	1013	1199	A, D			
2,2,6-trimethylcyclohexanone	1019	1235	A	racemic [R_s = 3.6] (C)		
limonene	1019	1131	A	26*S*:74*R* [R_s = 3.1] (C)		
(*E*)-3-octen-2-one	1024	1333	A			
(*Z*)-β-ocimene	1030	1181	A, C		60	4
(*E*)-β-ocimene	1040	1193	A			
2,6,6-trimethylcyclohex-2-enone	1042	1316	A			
γ-terpinene	1049	1193	A, D			
(*Z*,*E*)-3,5-octadien-2-one	1054	1438	A			
trans-linalool oxide (furanoid)	1061	1366	A	23(2*R*5*R*):39(2*R*5*S*):20(2*S*5*S*):18(2*S*5*R*) [R_s = 1.14–11.4] (C)		
cis-linalool oxide (furanoid)	1076	1394	A			
p-cymenene	1076	1343	A			
(*E*,*E*)-3,5-octadien-2-one	1077	1491	A, C		93	4
terpinolene	1079	1208	A, C			
(3*E*)-6-methyl-3,5-heptadien-2-one	1088	1509	A			
linalool	1095	1489	A	53*R*:47*S* [R_s = 1.6] (D)	100	16384
hotrienol	1096	1540	B	38*R*:62*S* [R_s = 2.5] (C)		
2-phenylethanol	1098	1818	A, C		73	4
isophorone	1102	1490	A			
3-thujanone[h]	1104	1331	A			
cis-2-*p*-menthen-1-ol[h]	1110		B			
4-acetyl-1-methylcyclohexene[h]	1114	1457	A, C		67	4
4-ketoisophorone	1121	1592	A			
allo-ocimene	1122	1101	A			
dihydrolinalool[h]	1125	1474	B			
(*E*)-3-nonen-2-one	1126	1432	A			
lilac aldehyde isomer 1[h]	1134	1513	B, C			
(*E*,*Z*)-2,6-nonadienal	1137	1501	A		100	32

	RI					
compound name[a]	**column A**[b]	**column B**[c]	**ID**[d]	**enantiomeric ratio (column)**[e]	**DF**[f]	**FD**[g]
nerol oxide[h]	1144	1391	A			
(*E*)-2-nonenal	1145	1453	A, D		100	4
borneol	1152		A	0(1*S*2*R*4*S*):100(1*R*2*S*4*R*) [R_s = 1.5] (C)		
(*E*)-ocimenol	1153		B			
a dimethylbenzaldehyde	1155	1622	B			
cis-pyranoid linalool oxide	1158	1654	A	20(2*S*5*R*):22(2*S*5*S*):31(2*R*5*S*):27(2*R*5*R*) [R_s = 2.2–7.3] (C)		
trans-pyranoid linalool oxide	1164	1687	A			
terpinen-4-ol	1165	1516	A	40*R*:60*S* [R_s = 2.5] (D)		
dill ether isomer 1[h]	1171	1493	B			
p-cymen-8-ol	1172	1763	A			
α-terpineol	1181	1619	A, C	38*S*:62*R* [R_s = 1.4] (D)	93	2
safranal	1182	1542	A			
decanal	1194	1433	A			
(+)-*p*-menth-1-en-9-al	1198	1519	A			
dodecane	1199	1201	A			
benzothiazole	1200		A			
(+)-*p*-menth-1-en-9-al	1200	1519	A, C		93	2
β-cyclositral	1203	1522	A, C		40	2
nerol	1219	1727	A, C		67	8
(*Z*)-3-hexenyl 2-methylbutanoate[h]	1223	1408	A			
neral	1225	1626	A			
(*Z*)-3-hexenyl isovalerate	1228	1424	A			
***p*-anisaldehyde**	1232	1936	A, D		53	4
3,5,7-nonatrien-2-one	1241	1819	B			
2,6,6-trimethyl-1-cyclohexene-1-acetaldehyde	1241	1520	A			
2-(2-butenyl)-1,3,5-trimethylbenzene[h]	1241		B			
geraniol	1248	1783	A, C		93	512
(*E*,*E*,*Z*)-2,4,6-nonatrienal	1253		B			
geranial	1255	1647	A, C			
(*R*)-octan-5-olide	1259	1864	A, C	0*S*:100*R* [R_s = 1.23] (D)	60	4
4,8-dimethyl-3,7-nonadien-2-one[h]	1261		B			

	RI					
compound name[a]	**column A**[b]	**column B**[c]	**ID**[d]	**enantiomeric ratio (column)**[e]	**DF**[f]	**FD**[g]
(*E*,*E*,*E*)-2,4,6-nonatrienal	1262	1800	B			
neryl formate	1270	1596	B			
nonanoic acid	1272	2110	A			
limonen-10-ol[h]	1279		B			
2-undecanone	1283	1529	A			
component 162	1283	1790	C		40	2
theaspirane isomer 1[h]	1288		A			
geranyl formate	1291	1630	A		33	2
2,3,4-trimethylbenzaldehyde	1295		B			
undecanal	1295		A			
(*E*,*E*)-2,4-decadienal	1300	1721	A		33	64
theaspirane isomer 2[h]	1304		A			
(*Z*)-3-hexenyl (*E*)-2-methyl-2-butenoate	1312	1591	A			
component C178 ($C_9H_{14}O_2$)	1317	1988	C		60	512
2,5-epoxymegastigma-6,8-diene[h]	1326	1550	B			
nonan-4-olide	1337	1942	A	51*R*:49*S* [R_s = 2.7] (D)		
α-terpinyl acetate[h]	1337		B			
1,5,8-trimethyl-1,2-dihydronaphthalene[h]	1338		B			
1-(2-hydroxy-1-methylethyl)-2,2-dimethylpropyl 2-methylpropanoate[h]	1339	1780	B			
eugenol	1340	2090	A, D		80	4
2,3-dihydro-1,1,5,6-tetramethyl-1*H*-indene	1340		B			
α-ionene	1343		B			
(*Z*)-β-damascenone	1347		A			
neryl acetate	1353	1658	A			
3-hydroxy-2,4,4-trimethylpentyl 2-methylpropanoate[h]	1363	1790	B			
2,3-dehydro-α-ionone[h]	1366	1729	B, C		33	8
(*E*)-β-damascenone	1369	1722	A, C		100	32768
α-copaene[h]	1369	1423	A			
geranyl acetate	1372	1687	A			
6,10-dimethyl-2-undecanone[h]	1395	1628	A			
dodecanal	1398	1641	A			
tetradecane	1399	1403	A			

	RI					
compound name[a]	**column A**[b]	**column B**[c]	**ID**[d]	**enantiomeric ratio (column)**[e]	**DF**[f]	**FD**[g]
(*E*)-β-damascone	1399	1718	A, C		100	4096
1,3-dimethylnaphthalene	1401	1901	B			
4-(2,6,6-trimethyl-1,3-cyclohexadien-1-yl)-2-butanone	1403		B			
6-methyl-6-(5-methylfuran-2-yl)heptan-2-one	1410	1821	B			
(*E*)-caryophyllene[h]	1411	1509	A			
(*R*)-α-ionone	1413	1755	A	100*R*:0*S* [R_S = 2.14] (D)		
3,4-dehydro-γ-ionone[h]	1415	1847	B			
(*E*)-6-methyl-6-(5-methylfuran-2-yl)hept-3-en-2-one	1431	1888	B			
geranylacetone	1441	1784	A			
2,3-dehydro-γ-ionone[h]	1450	1805	B, C		87	32
cabreuva oxide B[h]	1452	1623	B			
9-*epi*-(*E*)-caryophyllene[h]	1452	1602	B			
(*S*)-(*Z*)-7-decen-5-olide	1465	2151	A, C	0*R*:100*S* [R_S = 1.2] (D)	93	2
3,4-dehydro-β-ionone	1467	1923	A, C		87	64
cabreuva oxide D[h]	1468	1663	B			
5,6-epoxy-β-ionone	1469	1911	A	racemic [R_S = 0.82] (D)		
(*R*)-decan-5-olide	1470	2099	A, C	0*S*:100*R* [R_S = 1.29] (D)	87	2
(*E*)-β-ionone	1471	1850	A, C		87	512
calamenene-1,11-epoxide[h]	1477	1784	B			
β-dihydroagarofuran[h]	1489	1616	B			
α-muurolene[h]	1492	1642	B			
pentadecane	1499	1502	A			
dihydroactinidiolide	1499	2201	B	52*R*:48*S* [R_S = 3.6] (D)		
γ-cadinene[h]	1504	1667	B			
bovolide	1504	2065	B, C		80	4
trans-calamenene[h]	1511	1738	B			
δ-cadinene[h]	1514	1672	B			
methyl dodecanoate	1516		A			
pseudoionone isomer (*E*,*Z*)	1516	1977	A			
α-calacorene[h]	1530	1814	B			
α-agarofuran[h]	1531	1773	B			

	RI					
compound name[a]	**column A**[b]	**column B**[c]	**ID**[d]	**enantiomeric ratio (column)**[e]	**DF**[f]	**FD**[g]
(6*Z*,8*Z*)-megastigma-4,6,8-trien-3-one	1542	2068	B			
dihydroagarofuran isomer[h]	1545	1723	B			
(*E*)-nerolidol	1554	2001	A	41*R*:59*S* [R_s = 1.2] (C)		
(*Z*)-3-hexenyl benzoate	1554	2044	A			
(6*Z*,8*E*)-megastigma-4,6,8-trien-3-one	1560	2105	B			
dodecanoic acid	1562		A			
caryophyllene oxide[h]	1568		A			
pseudoionone isomer (*E*,*E*)	1569	2069	A			
component C269(bergamotol-type comp.)	1586		C			
1-[2-(isobutyryloxy)-1-methylethyl]-2,2-dimethylpropyl 2-methylpropanoate[h]	1586	1821	B			
(6*E*,8*Z*)-megastigma-4,6,8-trien-3-one	1591	2168	B, C		67	2
geranyl 2-methylbutanoate[h]	1591		B		47	8
1-(2,3,6-trimethylphenyl)-3-buten-2-one	1592		B			
(6*E*,8*E*)-megastigma-4,6,8-trien-3-one	1604	2194	B, C		40	8
10-*epi*-γ-eudesmol[h]	1605	2009	B, C		40	64
***epi*-α-cadinol**[h]	1628		B, C		60	64
***epi*-α-muurolol**[h]	1629		B, C		60	64
α-cadinol[h]	1641		B			
cadalene	1659	2127	B		33	8
3,7,7-trimethyl-1-penta-1,3-dienyl-2-oxabicyclo[3.2.0]hept-3-ene isomer 1[h]	1661	2135	B			
3,7,7-trimethyl-1-penta-1,3-dienyl-2-oxabicyclo[3.2.0]hept-3-ene isomer 2[h]	1680	2168	B			
(7*E*)-megastigma-5,7,9-trien-4-one	1686		B		60	512
isopropyl myristate	1817	2029	A			
hexahydrofarnesylacetone[h]	1834	2103	A			

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

b RI, relative to C_5–C_{18}*n*-alkanes, on PS-089 column (DB-5 equivalent).

c RI, relative to C_5–C_{18}*n*-alkanes, on AT-1000 column (FFAP equivalent).

d Identification: A, comparison of mass spectrum and RI with those of an authentic reference compound; B (tentative identification), HRGC-MS data and comparison of mass spectrum and RI with NBS and NIST databases and published data;(15, 34-37) C, odor activity by GC-O and GC-MS-O; and D, odor activity by GC-O.
e Enantiomeric ratio determined on column C (OV-1701-OH containing 10% heptakis(2,3-di-*O*-methyl-6-*O*-*tert*-butyldimethylsilyl)-β-cyclodextrin) or column D (OV-1701-OH containing 10% heptakis(2,3-di-*O*-acetyl-6-*O*-*tert*-butyldimethylsilyl)-β-cyclodextrin).
f Detection frequency.
g FD factor determined by aroma extract dilution analysis.
h Stereochemistry not determined.

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