

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

Maritha Le Roux,<sup>\*,†</sup> J. Christel Cronje,<sup>†</sup> Barend V. Burger,<sup>†</sup> and Elizabeth Joubert<sup>‡,§</sup>

<sup>†</sup>Laboratory for Ecological Chemistry, Department of Chemistry and Polymer Science, and <sup>‡</sup>Department of Food Science, Stellenbosch University, Private Bag X1, Matieland (Stellenbosch), 7602, South Africa

<sup>§</sup>Post-Harvest & Wine Technology Division, ARC Infruitec-Nietvoorbij, Private Bag X5026, Stellenbosch, 7599, South Africa

**S** Supporting Information

**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  $\beta$ -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  $\geq$  2). (*E*)- $\beta$ -Damasconone, (*R/S*)-linalool, (*E*)- $\beta$ -damascone, geraniol, (*E*)- $\beta$ -ionone, and (*7E*)-megastigma-5,7,9-trien-4-one were identified with the highest FD factors ( $\geq$ 512). The odors of certain compounds, that is, (*6E,8Z*)-megastigma-4,6,8-trien-3-one, (*6E,8E*)-megastigma-4,6,8-trien-3-one, (*7E*)-megastigma-5,7,9-trien-4-one, 10-*epi*- $\gamma$ -eudesmol, *epi*- $\alpha$ -muurolol, and *epi*- $\alpha$ -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 Podalyriaceae), 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.<sup>1</sup> 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.<sup>1,2</sup> 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.<sup>1</sup> 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.<sup>1</sup>

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.<sup>3</sup> Many of the terpenoids identified in *C. genistoides*,<sup>3</sup> for example,  $\alpha$ -terpineol, hexahydrofarnesylacetone, 2,6-dimethyl-1,7-octadien-3,6-diol, *Z*- and *E*-geraniol, linalool, linalool oxide isomers, pseudoionone,  $\beta$ -damascone, and eugenol, are known to have

floral, sweet, sweet-woody, floral-woody, or spicy odors.<sup>4</sup> 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.<sup>5</sup> 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.<sup>6</sup> However, it was found to lack the enrichment efficiency required for the analysis of VOCs in certain indigenous herbal teas.<sup>3,7</sup> 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)<sup>7,8</sup> was developed specifically to fill a niche that exists for a moderately priced, high-capacity sampling method that can be used in applications

Received: November 24, 2011

Revised: February 24, 2012

Accepted: February 26, 2012

Published: March 6, 2012

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.<sup>9</sup> 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,<sup>7</sup> 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 Analisetegnik, Stellenbosch, South Africa), which contains 30 mm polydimethylsiloxane (PDMS) tubing, equivalent to 28 mg of PDMS.<sup>7,8</sup> 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 enantioselective 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].<sup>10</sup> The glass columns were prepared according to methods adapted from those of Grob et al.<sup>11</sup> 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.<sup>12</sup> 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.<sup>13</sup> 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  $R = 2$ ).<sup>14</sup>

**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).

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

| compound name <sup>a</sup>                            | RI                    |                       | ID <sup>d</sup> | enantiomeric ratio (column) <sup>e</sup>                      | DF <sup>f</sup> | FD <sup>g</sup> |
|---|-----------------------|-----------------------|-----------------|---|-----------------|-----------------|
|   | column A <sup>b</sup> | column B <sup>c</sup> |                 |   |                 |                 |
| 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               |   |                 |                 |
| <b>3-methylbutanoic acid</b>                          | 857                   | 1581                  | A, D            |   | 93              | 8               |
| 1,3,6-octatriene <sup>h</sup>                         | 863                   |                       | B               |   |                 |                 |
| <b>(R)-2-methylbutanoic acid</b>                      | 866                   | 1588                  | A, C            | 0S:100R [ $R_s = 0.76$ ] (C)                                  | 73              | 2               |
| 2-heptanone   | 871                   | 1105                  | A               |   |                 |                 |
| (Z)-4-heptenal  | 879                   | 1167                  | A               |   |                 |                 |
| heptanal  | 882                   | 1107                  | A               |   |                 |                 |
| $\alpha$ -pinene                                      | 923                   | 1006                  | A               | 82(1S,5S):18(1R,5R) [ $R_s = 2.4$ ](C)                        |                 |                 |
| camphene  | 936                   | 1037                  | A               | 15R:85S [ $R_s = 1.3$ ](C)                                    |                 |                 |
| benzaldehyde  | 936                   | 1426                  | A               |   |                 |                 |
| (E)-2-heptenal  | 938                   | 1352                  | A               |   |                 |                 |
| 6-methyl-2-heptanone                                  | 939                   | 1221                  | A               |   |                 |                 |
| 2,2,6-trimethyl-6-vinyltetrahydropyran <sup>h</sup>   | 960                   | 1073                  | B               |   |                 |                 |
| 1-octen-3-ol  | 969                   | 1386                  | A               | 38S:62R [ $R_s = 1.5$ ](D)                                    |                 |                 |
| 6-methyl-5-hepten-2-one                               | 971                   | 1269                  | A, C            |   |                 |                 |
| (E,Z)-2,4-heptadienal                                 | 978                   | 1384                  | B               |   |                 |                 |
| (6Z)-2,6-dimethyl-2,6-octadiene                       | 981                   | 1069                  | A               |   |                 |                 |
| 2-pentylfuran   | 981                   | 1164                  | A               |   |                 |                 |
| trans-dehydroxylinalool oxide (furanoid) <sup>h</sup> | 981                   | 1150                  | A               |   |                 |                 |
| myrcene   | 983                   | 1116                  | A               |   |                 |                 |
| octanal   | 988                   | 1221                  | A               |   |                 |                 |
| (2Z)-2-(2-pentenyl)furan                              | 990                   | 1229                  | B               |   |                 |                 |
| (E,E)-2,4-heptadienal                                 | 992                   | 1409                  | A               |   |                 |                 |
| $\alpha$ -phellandrene                                | 994                   | 1135                  | A               | 20R:80S [ $R_s = 0.57$ ] (C)                                  |                 |                 |
| cis-dehydroxylinalool oxide (furanoid) <sup>h</sup>   | 997                   | 1185                  | A               |   |                 |                 |
| decane  | 997                   | 1020                  | A               |   |                 |                 |
| $\alpha$ -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               | 26S:74R [ $R_s = 3.1$ ] (C)                                   |                 |                 |
| (E)-3-octen-2-one                                     | 1024                  | 1333                  | A               |   |                 |                 |
| <b>(Z)-<math>\beta</math>-ocimene</b>                 | 1030                  | 1181                  | A, C            |   | 60              | 4               |
| (E)- $\beta$ -ocimene                                 | 1040                  | 1193                  | A               |   |                 |                 |
| 2,6,6-trimethylcyclohex-2-enone                       | 1042                  | 1316                  | A               |   |                 |                 |
| $\gamma$ -terpinene                                   | 1049                  | 1193                  | A, D            |   |                 |                 |
| (Z,E)-3,5-octadien-2-one                              | 1054                  | 1438                  | A               |   |                 |                 |
| trans-linalool oxide (furanoid)                       | 1061                  | 1366                  | A               | 23(2R5R):39(2R5S):20(2S5S):18(2S5R) [ $R_s = 1.14-11.4$ ] (C) |                 |                 |
| cis-linalool oxide (furanoid)                         | 1076                  | 1394                  | A               |   |                 |                 |
| p-cymenene  | 1076                  | 1343                  | A               |   |                 |                 |
| <b>(E,E)-3,5-octadien-2-one</b>                       | 1077                  | 1491                  | A, C            |   | 93              | 4               |
| terpinolene   | 1079                  | 1208                  | A, C            |   |                 |                 |
| (3E)-6-methyl-3,5-heptadien-2-one                     | 1088                  | 1509                  | A               |   |                 |                 |
| linalool  | 1095                  | 1489                  | A               | 53R:47S [ $R_s = 1.6$ ] (D)                                   | 100             | 16384           |
| hotrienol   | 1096                  | 1540                  | B               | 38R:62S [ $R_s = 2.5$ ] (C)                                   |                 |                 |
| <b>2-phenylethanol</b>                                | 1098                  | 1818                  | A, C            |   | 73              | 4               |
| isophorone  | 1102                  | 1490                  | A               |   |                 |                 |
| 3-thujanone <sup>h</sup>                              | 1104                  | 1331                  | A               |   |                 |                 |
| cis-2-p-menthen-1-ol <sup>h</sup>                     | 1110                  |                       | B               |   |                 |                 |
| <b>4-acetyl-1-methylcyclohexene<sup>h</sup></b>       | 1114                  | 1457                  | A, C            |   | 67              | 4               |
| 4-ketoisophorone                                      | 1121                  | 1592                  | A               |   |                 |                 |
| allo-ocimene  | 1122                  | 1101                  | A               |   |                 |                 |

Table 1. continued

| compound name <sup>a</sup>   | RI                    |                       | ID <sup>d</sup> | enantiomeric ratio (column) <sup>e</sup>                                   | DF <sup>f</sup> | FD <sup>g</sup> |
|--|-----------------------|-----------------------|-----------------|--|-----------------|-----------------|
|  | column A <sup>b</sup> | column B <sup>c</sup> |                 |  |                 |                 |
| dihydrolinalool <sup>h</sup>   | 1125                  | 1474                  | B               |  |                 |                 |
| ( <i>E</i> )-3-nonen-2-one   | 1126                  | 1432                  | A               |  |                 |                 |
| lilac aldehyde isomer 1 <sup>h</sup>   | 1134                  | 1513                  | B, C            |  |                 |                 |
| ( <i>E,Z</i> )-2,6-nonadienal  | 1137                  | 1501                  | A               |  | 100             | 32              |
| nerol oxide <sup>h</sup>   | 1144                  | 1391                  | A               |  |                 |                 |
| ( <i>E</i> )-2-nonenal   | 1145                  | 1453                  | A, D            |  | 100             | 4               |
| borneol  | 1152                  |                       | A               | 0(1S2R4S):100(1R2S4R) [ <i>R</i> <sub>s</sub> = 1.5] (C)                   |                 |                 |
| ( <i>E</i> )-ocimanol  | 1153                  |                       | B               |  |                 |                 |
| a dimethylbenzaldehyde   | 1155                  | 1622                  | B               |  |                 |                 |
| cis-pyranoid linalool oxide  | 1158                  | 1654                  | A               | 20(2S5R):22(2S5S):31(2R5S):27(2R5R) [ <i>R</i> <sub>s</sub> = 2.2–7.3] (C) |                 |                 |
| trans-pyranoid linalool oxide  | 1164                  | 1687                  | A               |  |                 |                 |
| terpinen-4-ol  | 1165                  | 1516                  | A               | 40R:60S [ <i>R</i> <sub>s</sub> = 2.5] (D)                                 |                 |                 |
| dill ether isomer 1 <sup>h</sup>   | 1171                  | 1493                  | B               |  |                 |                 |
| <i>p</i> -cymen-8-ol   | 1172                  | 1763                  | A               |  |                 |                 |
| <b>α-terpineol</b>   | 1181                  | 1619                  | A, C            | 38S:62R [ <i>R</i> <sub>s</sub> = 1.4] (D)                                 | 93              | 2               |
| safranal   | 1182                  | 1542                  | A               |  |                 |                 |
| decanal  | 1194                  | 1433                  | A               |  |                 |                 |
| (+)- <i>p</i> -menth-1-en-9-al   | 1198                  | 1519                  | A               |  |                 |                 |
| dodecane   | 1199                  | 1201                  | A               |  |                 |                 |
| benzothiazole  | 1200                  |                       | A               |  |                 |                 |
| (+)- <i>p</i> -menth-1-en-9-al   | 1200                  | 1519                  | A, C            |  | 93              | 2               |
| <b>β-cyclostril</b>  | 1203                  | 1522                  | A, C            |  | 40              | 2               |
| <b>nerol</b>   | 1219                  | 1727                  | A, C            |  | 67              | 8               |
| ( <i>Z</i> )-3-hexenyl 2-methylbutanoate <sup>h</sup>                          | 1223                  | 1408                  | A               |  |                 |                 |
| neral  | 1225                  | 1626                  | A               |  |                 |                 |
| ( <i>Z</i> )-3-hexenyl isovalerate   | 1228                  | 1424                  | A               |  |                 |                 |
| <b><i>p</i>-anisaldehyde</b>   | 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 <sup>h</sup>                              | 1241                  |                       | B               |  |                 |                 |
| geraniol   | 1248                  | 1783                  | A, C            |  | 93              | 512             |
| ( <i>E,E,Z</i> )-2,4,6-nonatrienal   | 1253                  |                       | B               |  |                 |                 |
| geraniol   | 1255                  | 1647                  | A, C            |  |                 |                 |
| ( <i>R</i> )- <b>octan-5-olide</b>   | 1259                  | 1864                  | A, C            | 0S:100R [ <i>R</i> <sub>s</sub> = 1.23] (D)                                | 60              | 4               |
| 4,8-dimethyl-3,7-nonadien-2-one <sup>h</sup>                                   | 1261                  |                       | B               |  |                 |                 |
| ( <i>E,E,E</i> )-2,4,6-nonatrienal   | 1262                  | 1800                  | B               |  |                 |                 |
| neryl formate  | 1270                  | 1596                  | B               |  |                 |                 |
| nonanoic acid  | 1272                  | 2110                  | A               |  |                 |                 |
| limonen-10-ol <sup>h</sup>   | 1279                  |                       | B               |  |                 |                 |
| 2-undecanone   | 1283                  | 1529                  | A               |  |                 |                 |
| <b>component 162</b>   | 1283                  | 1790                  | C               |  | 40              | 2               |
| theaspirane isomer 1 <sup>h</sup>  | 1288                  |                       | A               |  |                 |                 |
| <b>geranyl formate</b>   | 1291                  | 1630                  | A               |  | 33              | 2               |
| 2,3,4-trimethylbenzaldehyde  | 1295                  |                       | B               |  |                 |                 |
| undecanal  | 1295                  |                       | A               |  |                 |                 |
| ( <i>E,E</i> )-2,4-decadienal  | 1300                  | 1721                  | A               |  | 33              | 64              |
| theaspirane isomer 2 <sup>h</sup>  | 1304                  |                       | A               |  |                 |                 |
| ( <i>Z</i> )-3-hexenyl ( <i>E</i> )-2-methyl-2-butenate                        | 1312                  | 1591                  | A               |  |                 |                 |
| <b>component C178 (C<sub>9</sub>H<sub>14</sub>O<sub>2</sub>)</b>               | 1317                  | 1988                  | C               |  | 60              | 512             |
| 2,5-epoxymegastigma-6,8-diene <sup>h</sup>                                     | 1326                  | 1550                  | B               |  |                 |                 |
| nonan-4-olide  | 1337                  | 1942                  | A               | 51R:49S [ <i>R</i> <sub>s</sub> = 2.7] (D)                                 |                 |                 |
| <b>α-terpinyl acetate<sup>h</sup></b>  | 1337                  |                       | B               |  |                 |                 |
| 1,5,8-trimethyl-1,2-dihydronaphthalene <sup>h</sup>                            | 1338                  |                       | B               |  |                 |                 |
| 1-(2-hydroxy-1-methylethyl)-2,2-dimethylpropyl 2-methylpropanoate <sup>h</sup> | 1339                  | 1780                  | B               |  |                 |                 |
| <b>eugenol</b>   | 1340                  | 2090                  | A, D            |  | 80              | 4               |
| 2,3-dihydro-1,1,5,6-tetramethyl-1 <i>H</i> -indene                             | 1340                  |                       | B               |  |                 |                 |
| <b>α-ionene</b>  | 1343                  |                       | B               |  |                 |                 |
| ( <i>Z</i> )- <b>β-damascenone</b>   | 1347                  |                       | A               |  |                 |                 |

Table 1. continued

| compound name <sup>a</sup>   | RI                    |                       | ID <sup>d</sup> | enantiomeric ratio (column) <sup>e</sup> | DF <sup>f</sup> | FD <sup>g</sup> |
|--|-----------------------|-----------------------|-----------------|--|-----------------|-----------------|
|  | column A <sup>b</sup> | column B <sup>c</sup> |                 |  |                 |                 |
| neryl acetate  | 1353                  | 1658                  | A               |  |                 |                 |
| 3-hydroxy-2,4,4-trimethylpentyl 2-methylpropanoate <sup>h</sup>                        | 1363                  | 1790                  | B               |  |                 |                 |
| <b>2,3-dehydro-<math>\alpha</math>-ionone<sup>h</sup></b>                              | 1366                  | 1729                  | B, C            |  | 33              | 8               |
| <b>(E)-<math>\beta</math>-damascenone</b>  | 1369                  | 1722                  | A, C            |  | 100             | 32768           |
| $\alpha$ -copaene <sup>h</sup>   | 1369                  | 1423                  | A               |  |                 |                 |
| geranyl acetate  | 1372                  | 1687                  | A               |  |                 |                 |
| 6,10-dimethyl-2-undecanone <sup>h</sup>  | 1395                  | 1628                  | A               |  |                 |                 |
| dodecanal  | 1398                  | 1641                  | A               |  |                 |                 |
| tetradecane  | 1399                  | 1403                  | A               |  |                 |                 |
| <b>(E)-<math>\beta</math>-damascone</b>  | 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               |  |                 |                 |
| <b>(E)-caryophyllene<sup>h</sup></b>   | 1411                  | 1509                  | A               |  |                 |                 |
| <b>(R)-<math>\alpha</math>-ionone</b>  | 1413                  | 1755                  | A               | 100R:0S [ $R_s = 2.14$ ] (D)             |                 |                 |
| <b>3,4-dehydro-<math>\gamma</math>-ionone<sup>h</sup></b>                              | 1415                  | 1847                  | B               |  |                 |                 |
| <b>(E)-6-methyl-6-(5-methylfuran-2-yl)hept-3-en-2-one</b>                              | 1431                  | 1888                  | B               |  |                 |                 |
| geranylacetone   | 1441                  | 1784                  | A               |  |                 |                 |
| <b>2,3-dehydro-<math>\gamma</math>-ionone<sup>h</sup></b>                              | 1450                  | 1805                  | B, C            |  | 87              | 32              |
| cabreuva oxide B <sup>h</sup>  | 1452                  | 1623                  | B               |  |                 |                 |
| <b>9-epi-(E)-caryophyllene<sup>h</sup></b>   | 1452                  | 1602                  | B               |  |                 |                 |
| <b>(S)-(Z)-7-decen-5-olide</b>   | 1465                  | 2151                  | A, C            | 0R:100S [ $R_s = 1.2$ ] (D)              | 93              | 2               |
| <b>3,4-dehydro-<math>\beta</math>-ionone</b>   | 1467                  | 1923                  | A, C            |  | 87              | 64              |
| cabreuva oxide D <sup>h</sup>  | 1468                  | 1663                  | B               |  |                 |                 |
| <b>5,6-epoxy-<math>\beta</math>-ionone</b>   | 1469                  | 1911                  | A               | racemic [ $R_s = 0.82$ ] (D)             |                 |                 |
| <b>(R)-decen-5-olide</b>   | 1470                  | 2099                  | A, C            | 0S:100R [ $R_s = 1.29$ ] (D)             | 87              | 2               |
| <b>(E)-<math>\beta</math>-ionone</b>   | 1471                  | 1850                  | A, C            |  | 87              | 512             |
| calamenene-1,11-epoxide <sup>h</sup>   | 1477                  | 1784                  | B               |  |                 |                 |
| $\beta$ -dihydroagarofuran <sup>h</sup>  | 1489                  | 1616                  | B               |  |                 |                 |
| $\alpha$ -muurolene <sup>h</sup>   | 1492                  | 1642                  | B               |  |                 |                 |
| pentadecane  | 1499                  | 1502                  | A               |  |                 |                 |
| dihydroactinidiolide   | 1499                  | 2201                  | B               | 52R:48S [ $R_s = 3.6$ ] (D)              |                 |                 |
| $\gamma$ -cadinene <sup>h</sup>  | 1504                  | 1667                  | B               |  |                 |                 |
| <b>bovolide</b>  | 1504                  | 2065                  | B, C            |  | 80              | 4               |
| <b>trans-calamenene<sup>h</sup></b>  | 1511                  | 1738                  | B               |  |                 |                 |
| $\delta$ -cadinene <sup>h</sup>  | 1514                  | 1672                  | B               |  |                 |                 |
| methyl dodecanoate   | 1516                  |                       | A               |  |                 |                 |
| pseudoionone isomer (E,Z)  | 1516                  | 1977                  | A               |  |                 |                 |
| $\alpha$ -calacorene <sup>h</sup>  | 1530                  | 1814                  | B               |  |                 |                 |
| $\alpha$ -agarofuran <sup>h</sup>  | 1531                  | 1773                  | B               |  |                 |                 |
| <b>(6Z,8Z)-megastigma-4,6,8-trien-3-one</b>  | 1542                  | 2068                  | B               |  |                 |                 |
| dihydroagarofuran isomer <sup>h</sup>  | 1545                  | 1723                  | B               |  |                 |                 |
| <b>(E)-nerolidol</b>   | 1554                  | 2001                  | A               | 41R:59S [ $R_s = 1.2$ ] (C)              |                 |                 |
| <b>(Z)-3-hexenyl benzoate</b>  | 1554                  | 2044                  | A               |  |                 |                 |
| <b>(6Z,8E)-megastigma-4,6,8-trien-3-one</b>  | 1560                  | 2105                  | B               |  |                 |                 |
| dodecanoic acid  | 1562                  |                       | A               |  |                 |                 |
| caryophyllene oxide <sup>h</sup>   | 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 <sup>h</sup> | 1586                  | 1821                  | B               |  |                 |                 |
| <b>(6E,8Z)-megastigma-4,6,8-trien-3-one</b>  | 1591                  | 2168                  | B, C            |  | 67              | 2               |
| <b>geranyl 2-methylbutanoate<sup>h</sup></b>   | 1591                  |                       | B               |  | 47              | 8               |
| 1-(2,3,6-trimethylphenyl)-3-buten-2-one  | 1592                  |                       | B               |  |                 |                 |
| <b>(6E,8E)-megastigma-4,6,8-trien-3-one</b>  | 1604                  | 2194                  | B, C            |  | 40              | 8               |
| <b>10-epi-<math>\gamma</math>-eudesmol<sup>h</sup></b>                                 | 1605                  | 2009                  | B, C            |  | 40              | 64              |
| <b>epi-<math>\alpha</math>-cadinol<sup>h</sup></b>                                     | 1628                  |                       | B, C            |  | 60              | 64              |



Table 1. continued

| compound name <sup>a</sup>  | RI                    |                       | ID <sup>d</sup> | enantiomeric ratio (column) <sup>e</sup> | DF <sup>f</sup> | FD <sup>g</sup> |
|---|-----------------------|-----------------------|-----------------|--|-----------------|-----------------|
|   | column A <sup>b</sup> | column B <sup>c</sup> |                 |  |                 |                 |
| <i>epi-α-muurolool</i> <sup>h</sup>   | 1629                  |                       | B, C            |  | 60              | 64              |
| <i>α-cadinol</i> <sup>h</sup>   | 1641                  |                       | B               |  |                 |                 |
| <b>cadalene</b>   | 1659                  | 2127                  | B               |  | 33              | 8               |
| 3,7,7-trimethyl-1-penta-1,3-dienyl-2-oxabicyclo [3.2.0]hept-3-ene isomer 1 <sup>h</sup> | 1661                  | 2135                  | B               |  |                 |                 |
| 3,7,7-trimethyl-1-penta-1,3-dienyl-2-oxabicyclo [3.2.0]hept-3-ene isomer 2 <sup>h</sup> | 1680                  | 2168                  | B               |  |                 |                 |
| (7 <i>E</i> )- <b>megastigma-5,7,9-trien-4-one</b>                                      | 1686                  |                       | B               |  | 60              | 512             |
| isopropyl myristate   | 1817                  | 2029                  | A               |  |                 |                 |
| hexahydrofarnesylacetone <sup>h</sup>   | 1834                  | 2103                  | A               |  |                 |                 |

<sup>a</sup>In order of elution from apolar PS-089 column (DB-5 equivalent). <sup>b</sup>RI, relative to C<sub>5</sub>–C<sub>18</sub> *n*-alkanes, on PS-089 column (DB-5 equivalent). <sup>c</sup>RI, relative to C<sub>5</sub>–C<sub>18</sub> *n*-alkanes, on AT-1000 column (FFAP equivalent). <sup>d</sup>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;<sup>15,34–37</sup> C, odor activity by GC-O and GC-MS-O; and D, odor activity by GC-O. <sup>e</sup>Enantiomeric ratio determined on column C (OV-1701-OH containing 10% heptakis(2,3-di-*O*-methyl-6-*O*-*tert*-butyldimethylsilyl)- $\beta$ -cyclodextrin) or column D (OV-1701-OH containing 10% heptakis(2,3-di-*O*-acetyl-6-*O*-*tert*-butyldimethylsilyl)- $\beta$ -cyclodextrin). <sup>f</sup>Detection frequency. <sup>g</sup>FD factor determined by aroma extract dilution analysis. <sup>h</sup>Stereochemistry not determined.

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<sub>5</sub>–C<sub>18</sub> *n*-alkanes on nonpolar column A, were compared with those of the reference compounds and confirmed with published RI values.<sup>15,16</sup> 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-pentene-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,  $\alpha$ -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,  $\alpha$ -terpineol, safranal, decanal,  $\beta$ -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-butenolate, nonan-4-olide, 6,10-dimethyl-2-undecanone, dodecanal,  $\alpha$ -ionone, jasmin absolute, decan-5-olide, geranylacetone, dodecanoic acid, caryophyllene oxide, *trans*-nerolidol, (*Z*)- $\beta$ -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,  $\alpha$ -pinene, 1-octen-3-ol,  $\alpha$ -phellandrene, 2,2,6-trimethylcyclohexanone, limonene,  $\gamma$ -terpinene, *trans*-furanoid linalool oxide, *cis*-furanoid linalool oxide, terpinolene, linalool, isophorone, borneol, *p*-anisaldehyde, eugenol,  $\alpha$ -copaene,  $\beta$ -damascone, and (*E*)- $\beta$ -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);  $\beta$ -damascenone (Firmenich, Geneva, Switzerland); and geranyl formate (Dauphin, Bourgoin-Jallieu, France). (*E*)- $\beta$ -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.<sup>17</sup> Solutions of the reference compounds 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,<sup>18</sup> (*E,E*)- and (*Z,E*)-3,5-octadien-2-one,<sup>19</sup> 5,6-epoxy- $\beta$ -ionone,<sup>20</sup> hexyl tiglate, benzyl tiglate, 3,4-dehydro- $\beta$ -ionone,<sup>21</sup> octan-5-olide,<sup>22</sup> hexahydrofarnesylacetone,<sup>23</sup> nerol oxide,<sup>24</sup> (+)-*p*-menth-1-en-9-ol,<sup>25</sup> and *cis*- and *trans*-dehydroxylinalool oxide.<sup>26</sup>

## 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.<sup>2</sup>

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  $\beta$ -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<sup>27</sup> 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*,<sup>3</sup> 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.<sup>13</sup> 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  $\geq 2$ ) (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.<sup>2</sup> (*E*)- $\beta$ -Damascenone, (*R/S*)-linalool, (*E*)- $\beta$ -damascone, geraniol, (*E*)- $\beta$ -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*)- $\beta$ -damascenone (FD 32768), (*R/S*)-linalool (FD 16384), and (*E*)- $\beta$ -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*)- $\beta$ -ionone (FD 512), and (7*E*)-megastigma-5,7,

9-trien-4-one (FD 512) all had DF factors  $\geq 60$ . Four of the mentioned compounds are generally associated with a sweet aroma, that is, (*E*)- $\beta$ -damascenone (also honey-like, fruity, dried prune),<sup>28–31</sup> linalool (also floral, floral-woody),<sup>4,29</sup> geraniol (also floral, floral-woody),<sup>4,29</sup> and (*E*)- $\beta$ -ionone (also floral, fruity).<sup>4,28,32</sup> (*E*)- $\beta$ -Damascenone 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.<sup>28,30</sup> In a study on Grenache wine,  $\beta$ -damascenone, detected in the present study with the highest FD factor, has been qualified as an “aroma enhancer”. Although it had the second highest odor activity value by GC-O, results indicated that it was not a character impact compound but probably contributed a sweet background note.<sup>14</sup> (*E*)- $\beta$ -Damascenone, (*R/S*)-linalool, and  $\beta$ -ionone have previously been identified as key aroma compounds in apricots.<sup>33</sup> Two other odorants identified with high FD or OAV values in apricot aroma<sup>33</sup> 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,8Z*)-megastigma-4,6,8-trien-3-one (FD 2), (6*E,8E*)-megastigma-4,6,8-trien-3-one (FD 8), (7*E*)-megastigma-5,7,9-trien-4-one (FD 512), 10-*epi*- $\gamma$ -eudesmol (FD 64), *epi*- $\alpha$ -muurolol (FD 64), and *epi*- $\alpha$ -cadinol (FD 64) as typically honeybush-like. Of these six compounds, only (6*E,8Z*)-megastigma-4,6,8-trien-3-one, (6*E,8E*)-megastigma-4,6,8-trien-3-one, and 10-*epi*- $\gamma$ -eudesmol are generally described as sweet.<sup>28,31</sup> The latter compound also has woody, floral descriptors,<sup>30,31</sup> while the megastigmatrienones are also associated with a woody, tobacco-like aroma.<sup>28,30</sup> Both *epi*- $\alpha$ -muurolol and *epi*- $\alpha$ -cadinol have herbaceous descriptors, while *epi*- $\alpha$ -muurolol is also considered to be slightly spicy.<sup>31</sup>

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.

## ■ ASSOCIATED CONTENT

### ● Supporting Information

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

## ■ AUTHOR INFORMATION

### Corresponding Author

\*Tel: +27 21 808 3327. Fax: +27 21 808 3360. E-mail: [mlr3@sun.ac.za](mailto:mlr3@sun.ac.za).

### Funding

Funding for the research by Stellenbosch University and the National Research Foundation, Pretoria, South Africa, is acknowledged.

### Notes

The authors declare no competing financial interest.

## ACKNOWLEDGMENTS

Van Zyl Joubert of Toekomst farm in the Overberg area provided the plant material.

## 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

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