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Original Article

Headspace analysis and characterisation of South African propolis volatile compounds using GCxGC–ToF–MS

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ABSTRACT

Propolis also known as "bee glue or bee resin" is a resinous mixture of bee saliva or bee wax and exudate from tree trunks and flowers, produced by honeybees. The composition of propolis varies depending on the vegetation the bees can access. It is therefore expected that propolis obtained from various localities may have different chemical profiles. In this study, the headspace volatiles of propolis ($n = 39$) collected from various locations in South Africa (Gauteng, Northern Cape and Western Cape Provinces) were explored for the first time using GCxGC–ToF–MS. Several GCxGC parameters were optimised including; incubation time, temperature and modulation period. Multivariate data analysis techniques (principal component and hierarchical cluster analyses) were applied on the GCxGC–ToF–MS data to investigate trends and clustering patterns within propolis samples. The results demonstrated that headspace volatiles of propolis varied between locations. The volatile profiles were dominated by monoterpenes such as α-pinene (1.2–46.5%), β-pinene (2.0–21.8%), dihydrosabinene (trace-17.8%), limonene (trace-11.6%), *p*-cymene (0.1–5.3%), 1,8-cineole (0.1–11.0%), 2,7-dimethyl-3-octen-5-yne (trace-11.7%), *E*-β-ocimene (trace-17.8%), octanal (trace-12.9%), styrene (trace-13.5%) and α-thujene (trace-11.0%). Principal component analysis revealed chemical variation within propolis from the various locations. The heatmap of the averages revealed dehydrosabinene, isopropentyltoluene, *p*-cymene, acetophenone and α-thujene as chemical markers for the Northern Cape propolis, while λ-terpinene, propanoic acid, furfural, 2-methoxy benzyl alcohol and hexanoic acid methylester were filtered out as markers for Gauteng propolis. The propolis samples originating from the Western Cape Province were dominated by prenal, cinnamaldehyde styrene, 1,8-cineole, decanal, prenyl acetate and butanoic acid. Using GCxGC–ToF–MS in combination with chemometrics, it was possible to profile headspace volatile constituents of propolis and further identify marker compounds that differentiate propolis from various provinces in South Africa.

Introduction

Propolis is derived from the Greek word "pro" which means ïn defence ofänd "polis" meaning "city". This means that the ancient Greeks thought that propolis had the power to make them defend their cities. It is commonly known as "bee glue" or "bee resin" which is a honeybee product that has been used by humans for centuries (Bankosta et al., 2001). Propolis is a resinous mixture produced by bees through combining beeswax and exudate from tree buds, sap flows, leaves, flowers or fruits, with bee saliva. Propolis is composed of 50% resin (polyphenolic fraction), 30% wax, 10% essential oils, 5% pollen and other substances (Burdock, 1998; Huang et al., 2014). Traditionally, propolis has been used extensively for many years in many countries as a remedy for the prevention and treatment of colds, wounds, ulcers, rheumatism, heart disease, diabetes and dental caries. Recently, propolis has been included in various commercial products such as lotions, toothpastes and bath soaps, individually and in combination with other medicinal plants such as *Aloe* species.

Various biological properties have been ascribed to propolis and these include; antimicrobial, anti-oxidant, antitumour, anti-inflammatory, anti-ulcer, anesthetic and anti-HIV activities (Ito et al., 2001; Zhu et al., 2011a, 2011b; De Castro Ishida et al., 2011; Król et al., 2013; Suleman et al., 2015). In the past decade, many

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studies have highlighted the potential of propolis as a dietary supplement and food preservative due to the reported biological properties (Mu et al., 2006; Candir et al., 2009; Yang et al., 2010). The bioactivity of propolis is believed to be a result of its complex chemical composition, which harbours a wide range of bioactive compounds.

The chemistry of propolis varies greatly and these variations depend on various factors such as flora at the collection site, geographical location and the type of bee involved in the pollination process, among others. In the past few years, researchers have gained interest in investigating the chemical composition of propolis from various regions. In a recent review on the chemistry of propolis, over 300 compounds were reported to occur from diverse chemical groups such as flavonoids, phenylpropanoids, terpenenes, stilbenes, aldehydes, esters, lignans, coumarins, and their prenylated derivatives (Kaškonienė et al., 2014; Huang et al., 2014). A review published by Bankova et al. (2014) pointed out that the volatile constituents of propolis are diverse among countries. It was also noted in the review that samples from the same country are highly variable with regards to volatile constituents. However, the investigation into the volatile components of propolis has not been well documented and researchers have reported that the volatiles constitute a very low proportion of the propolis chemistry. The volatile chemistry of South African propolis has been understudied which prompted the investigatation on the headspace volatiles from propolis using two dimensional gas chromatography (GCxGC–ToF–MS).

Headspace analysis is a simple, non-destructive and solvent-free technique used to extract volatile compounds from both liquid and solid matrices. The technique has been widely used in the food and flavour industries to generate flavour profiles for a variety of foods and spices. The headspace solid phase micro-extraction has also been used to determine the volatile constituents of propolis (Pellati et al., 2013). Gas chromatography (1D GC) for headspace analysis is a method of choice for volatiles. However, this technique is limited by co-elution of compounds, while separation of some isomers may be difficult to achieve. In recent years, 2D GC (GCxGC–ToF–MS) has been introduced to overcome the limitations of the 1D GC method. In the GCxGC–ToF–MS, two columns (polar and non-polar) are assembled in series, which provides higher resolution allowing detection of more compounds. Furthermore, 2D GC has higher sensitivity compared to 1D GC analysis. Due to the multivariate nature of 2D GC data, there is a need to employ chemometric algorithms to unfold all the relevant chemical information in a comprehensive manner. This study therefore applied multivariate data analysis methods such as principal component analysis (PCA) and hierarchical cluster analysis (HCA) to investigate variation in propolis volatiles from various South African localities based on GCxGC–ToF–MS profiles.

Materials and methods

Sample preparation

Propolis samples ($n = 39$) were collected from various localities within South Africa and retention sample information is provided in Table 1. No sample processing was performed prior to headspace analysis.

Table 1
List of propolis samples, location and date of collection.

No.	Province	Locality	Collection date
P1	Gauteng	Walkerville	03/2013
P2		Springs	03/2013
P3		Bapsfontein	03/2013
P4		Johannesburg	03/2013
P5		Johannesburg	03/2013
P6		Johannesburg	03/2013
P7		Edenvale	04/2013
P8		Edenvale	04/2013
P9		Pretoria	05/2013
P10		Pretoria	05/2013
P11		Lydiana Gardens	05/2013
P12		Lydiana Gardens	05/2013
P13		Wilgerivier	06/2013
P14		Beaulieu	06/2013
P15		President Park	06/2013
P16		Devon – Sedibeng area	06/2013
P17	Western Cape	Beaufort West	06/2013
P18		Western Cape	04/2013
P19		Southern Suburbs	06/2013
P20		Southern Suburbs	06/2013
P21		Southern Suburbs	06/2013
P22		Western Cape	03/2013
P23		Somerset West	03/2013
P24		Botrivier	03/2013
P25		Graafwater	03/2013
P26		Lakeside/Westlake	03/2013
P27	Northern Cape	Douglas	03/2013
P28		Northern Cape	03/2013
P29		Northern Cape	03/2013
P30	Unknown	Unknown	05/2013
P31		Unknown	06/2013
P32		Unknown	Unknown
P33		Unknown	Unknown
P34		Unknown	Unknown
P35		Unknown	Unknown
P36		Unknown	Unknown
P37		Unknown	Unknown
P38		Unknown	Unknown
P39		Unknown	06/2013

GCxGC–ToF–MS and headspace analysis

Propolis samples were placed in headspace vials to sample the gaseous or vapour phase. The vials were heated at 45 °C with agitation for 5 min in the heating module to release the volatile constituents. After heating, 1 ml of the gaseous phase was collected using a 2.5 ml syringe and injected into the Leco Pegasus© 4 GCxGC system with a 5:1 split ratio. The GCxGC system consisted of a Multi-Purpose Sampler (Gerstel) which was operated in the headspace mode. The instrument consisted of an Agilent 7890 Gas Chromatograph with cryogenic thermal modulator and a secondary oven. A 30 m × 0.25 mm × 0.25 μm film thickness, Rxi-5Sil MS GC capillary column, was used as the first column, the second column was 0.790 m × 0.25 mm × 0.25 μm film thickness, Rxi-5Sil MS GC capillary column. Helium was used as the carrier gas at a constant flow rate of 1.50 ml/min, front inlet septum purge flow at 3 ml/min, purge valve time 60 s after the beginning of the run. The inlet temperature was 200 °C, from the beginning and throughout the run. The primary column was programmed with an initial oven temperature of 40 °C for 1 min, at the rate of 10 °C/min and was ramped to 220 °C for 2 min. The secondary column temperature program was set to an initial temperature of 60 °C for 30 s then ramped at 10 °C/min to 240 °C for 2 min. The thermal modulator initial temperature was set at 80 °C for 1 min, at the rate of 10 °C/min and was ramped to 260 °C for 2 min. Both the front inlet and transfer line temperature were constant at 200 °C and 225 °C, respectively. The total analysis time was 21 min. Various GCxGC parameters such as incubation time (1–5 min), incubation temperature (40–60 °C) and modulation period (1–4 s) were tested. The MS mass range was 35–400 *m/z* with an acquisition rate of 100 spectra/s. The ion source chamber was set at 200 °C.

Data pre-processing

Data were pre-processed using LECO ChromaToF® version 4.50 software. The minimum signal to noise ratio (S/N) was set at 500 based on "unique mass" which is the most specific mass extracted for the analyte after deconvolution of the mass spectra signal. Tentative identification of peaks was performed using the NIST Mass Spectral Library (NIST 11). Library similarity factors were reported on a scale ≥800 for both forward and reverse search. Authentic standards of some constituents (α-pinene, β-pinene, limonene, benzaldehyle, *p*-cymene, 1,8-cineole, myrcene, cinnamaldehyde, acetic acid) were obtained from Sigma® and used to confirm the identity of the compounds. Relative amount (% area) calculations were based on the ratio between the peak area of each compound and the sum of areas of all selected compounds. The data matrix for chemometrics was constructed using the relative % area of the prominent constituents (about 61) for each chromatographic profile. This was selected by using a high signal to noise ratio (S/N > 750), to determine compounds present in high amounts that could be used to differentiate samples from various locations. The data was exported to MetaboAnalyst (www.metaboanalyst.ca) for chemometric analyses.

Chemometric analysis

Data were analysed using the free web-based software MetaboAnalyst (www.metaboanalyst.ca), designed for high-throughput metabolomic data analysis. Data pre-treatment involved normalisation, scaling and spectral filtering to obtain the best model parameters. Unsupervised PCA and HCA were employed on the pre-processed data to investigate possible patterns within the dataset and detect important compounds contributing to the variation. Hierarchical cluster analysis using the Euclidean distance as dissimilarity measure was applied in order to estimate the distance between any pair of the clusters, and the Ward's linkage represented the clustering method. Both the 2D scores scatter plot and a dendrogram were constructed to display variation modelled using PCA and HCA, respectively. Furthermore, a heat map of variables was constructed for the different localities of South African propolis to determine marker compounds for the various groupings.

Results and discussion

GCxGC–ToF–MS and headspace analysis

The solvent free headspace technique, which is selective towards extraction of low boiling point compounds or volatile constituents successfully, extracted volatile constituents from propolis resin. The best GCxGC conditions for good chromatographic separation, peak detection and better resolution were obtained as follows; incubation time of 5 min, incubation temperature of 45 °C and modulation time of 2 s without wrap around effects. The use of GCxGC–ToF–MS enabled the detection of more than 150 volatile compounds in propolis demonstrating much greater sensitivity compared to GC–MS whose average detection of volatile propolis compounds from previous studies is less than 40 compounds (Bankova, 2005; Nunes and Guerreiro, 2012; Kaškonienė et al., 2014). Table 2 is a list of some of the detected compounds (61) for which identities could be verified using standards, literature searches and good match with NIST library. The identified compounds belong to various classes, such as alkenes, alcohols, esters, terpenes, aldehydes and ketones. The predominant compounds were; α-pinene (1.2–46.5%), β-pinene (2.0–21.8%), dehydrosabinene (trace-17.8%), limonene (trace-11.6%), *p*-cymene (0.1–5.3%), 1,8-cineole (0.1–11.0%), 2,7-dimethyl-3-octen-5-yne

Table 2
Variation in headspace volatile constituents of propolis collected from various localities in South Africa.

Peak number	Compounds	1st D RT (s)/2nd D RT (s)	Min	Max	Avg
1	3-Carene	265.5; 1.24	tr	3.9	0.6
2	2-Butenal	276.0; 0.60	tr	4.9	0.7
3	Hexanal	279.0; 0.69	tr	9.2	1.1
4	α-Pinene	280.5; 1.17	1.2	46.5	17.3
5	α-Thujene	295.5; 1.28	tr	11.0	2.2
6	2,7-Dimethyl-3-octen-5-yne (isomer)	300.0; 1.12	tr	11.7	3.8
7	Camphene	309.0; 0.45	0.2	4.2	2.0
8	Dehydrosabinene	355.5, 1.04	tr	17.8	2.7
9	β-Pinene	357.0; 1.14	2.0	21.8	5.0
10	Sabinene	358.5; 1.13	tr	7.7	1.5
11	Prenal	373.5; 0.58	tr	5.3	0.8
12	2-Heptanone	382.5; 0.75	tr	2.8	0.6
13	Heptanal	383.0; 0.76	tr	5.0	0.6
14	Hexanoic acid methyl ester	390.0; 0.82	tr	3.5	0.4
15	2,7-Dimethyl-3-octen-5-yne (isomer)	402.0; 1.16	tr	8.7	2.7
16	Myrcene	403.5; 1.03	tr	2.1	0.5
17	*E*-β-Ocimene	418.5; 1.08	tr	17.8	2.5
18	3-Methyl butenol	426.0; 0.50	tr	5.2	0.9
19	Pentanol	439.5; 0.53	tr	4.3	0.8
20	Prenyl acetate	448.5; 0.72	tr	5.2	1.3
21	1,8-Cineole	450.0; 1.10	0.1	11.0	2.4
22	Limonene	451.5; 1.03	tr	11.6	2.7
23	Styrene	453.0; 0.68	tr	13.5	2.1
24	2-Pentyl-furan	453.0; 0.90	tr	2.0	0.3
25	λ-Terpinene	499.5; 1.04	tr	7.8	1.1
26	α-Phellandrene	502.5; 1.09	nd	0.5	0.1
27	2-Methyl butanol	504.0; 0.50	tr	4	0.6
28	*p*-Cymene	508.0; 0.91	0.1	5.3	1.6
29	Octanal	513.0; 0.82	tr	12.9	1.8
30	Furfuraldehyde	604.5; 050	tr	1.9	0.3
31	4-Penten-2-ol	621.0; 0.43	tr	4.3	0.6
32	Acetic acid	627.0; 0.42	tr	61.7	4.8
33	Nonanal	637.5; 0.86	0.1	7.3	1.2
34	Furfural	640.5; 0.49	tr	11.3	2.1
35	Camphenol	655.5; 0.81	tr	0.3	0.1
36	α-Dimethyl styrene	675.0; 0.78	tr	3.4	0.6
37	3-Hepten-1-ol	679.5; 0.37	tr	5.8	0.5
38	Heptanol	681.0; 0.58	tr	0.5	tr
39	Benzaldehyde	717.0; 0.57	0.2	18.2	2.8
40	Campholenal	724.5; 0.79	tr	0.5	0.1
41	Propanoic acid	727.0; 0.43	tr	6.9	1.1
42	Decanal	762.0; 0.88	tr	12.3	0.8
43	Pinocarvone	795.0; 0.77	tr	0.3	tr
44	Butanoic acid	813.0; 0.44	tr	4.4	0.7
45	Acetophenone	850.5; 0.58	tr	1.0	0.3
46	Myrtenal	855.0; 0.75	tr	0.2	tr
47	Estragole	892.5; 0.69	tr	0.2	tr
48	*cis* Verbenol	918.0, 0.61	tr	0.5	tr
49	Furanone	921.0; 0.46	tr	0.4	tr
50	*trans* Bergamotene	922.5; 0.46	tr	0.5	tr
51	β-Caryophyllene	924.0; 1.17	tr	3.8	0.5
52	Bornyl acetate	934.0; 0.95	tr	3.2	0.7
53	Hexanoic acid	1030.5; 0.46	tr	2.1	0.2
54	Benzyl alcohol	1053.0; 0.47	tr	4.7	0.7
55	Phenylethyl alcohool	1093.5; 0.50	tr	1.2	0.2
56	2-Methoxy benzyl alcohol	1098.5, 0.78	tr	10.5	0.7
57	Phenol	1152.0; 0.43	tr	0.9	tr
58	Cinnamaldehyde	1200.0; 1.24	tr	3.6	0.6
59	Octanoic acid, methyl ester	1224.5, 0.48	tr	10.5	0.7
60	Caryophyllene oxide	1227.0; 0.89	tr	0.8	tr
61	Thymol	1338.0; 0.50	tr	0.7	0.1

tr, % area <0.05; 1st D RT(s), first dimension retention time in second; 2nd D RT (s), second dimension retention time in second; Max, maximum; Min, minimum; Avg, average.

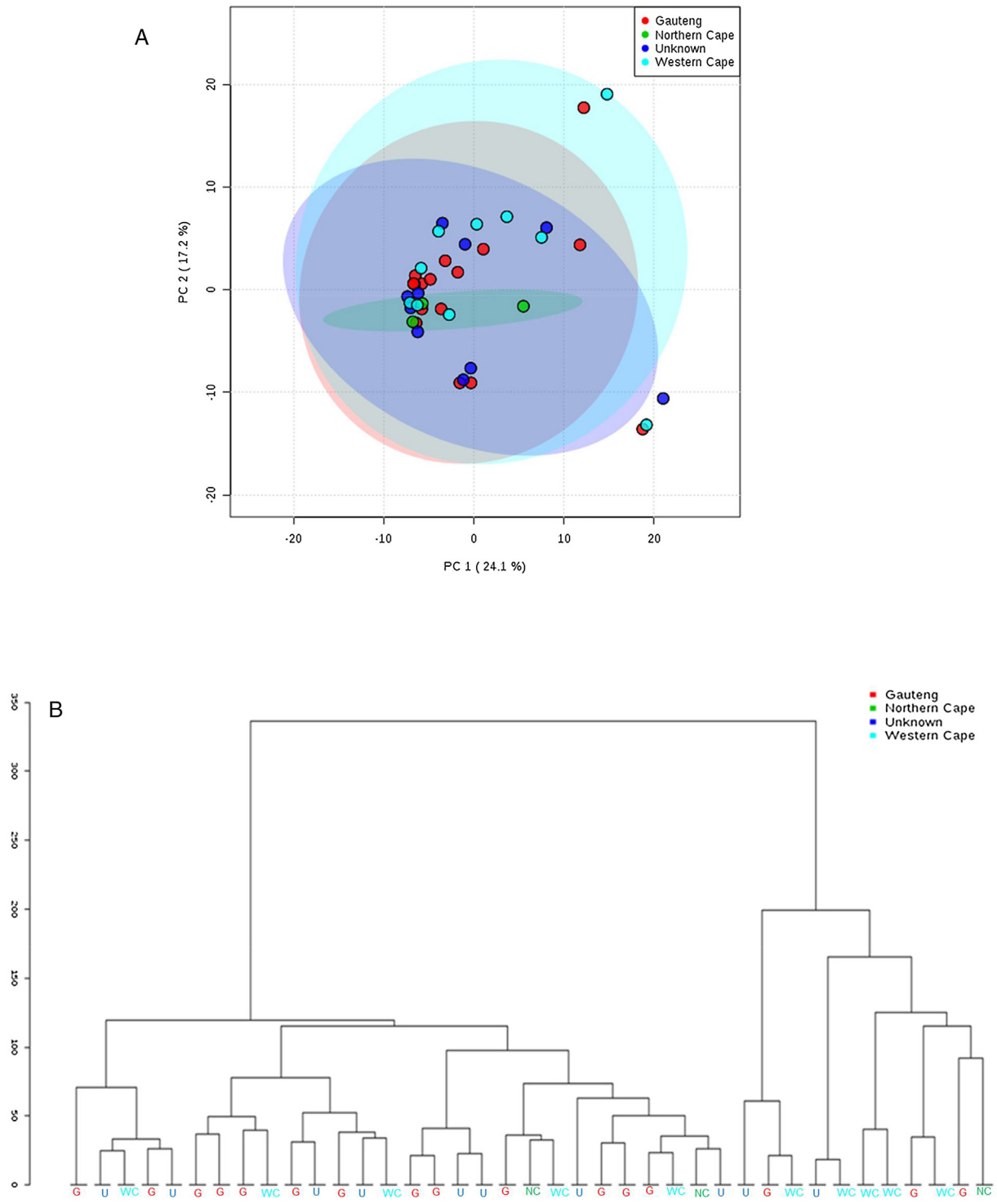

Fig. 1. (A) Score scatter plot and (B) dendrogram, indicating variation of South African volatile compounds from propolis from four different localities (G: Gauteng; U: unknown; WC: Western Cape; NC: Nothern Cape).

(trace-11.7%), *E*-β-ocimene (trace-17.8%), octanal (trace-12.9%), styrene (trace-13.5%) and α-thujene (trace-11.0%). Kaškonienė et al. (2014) investigated the static headspace volatiles of propolis originating from China, Uruguay, Estonia and Brazil and found that α-pinene and β-pinene were the major volatile constituents representing up to 77% of the total composition. Taking into account that propolis from Brazil is usually considered to be of superior quality (Suleman et al., 2015), our results demonstrate that some South African propolis have similar volatile chemical profiles when compared to Brazilian propolis which is high in α-and β-pinene content. Nunes and Guerreiro (2012) investigated headspace profiles of Green propolis (from Brazil) using GC–MS and ESI–MS methods. Only 24 compounds were identified and these included α-and β-pinenes, linalool, 1,8-cineole and sesquiterpenes such as germacrene D, farnesol, spathulenol, viridiflorene and β-caryophyllene. Although quantitative values were not provided, it was clear from the chromatograms that β-caryophyllene, linalool and farnesol were present in higher amounts as demonstrated by the large

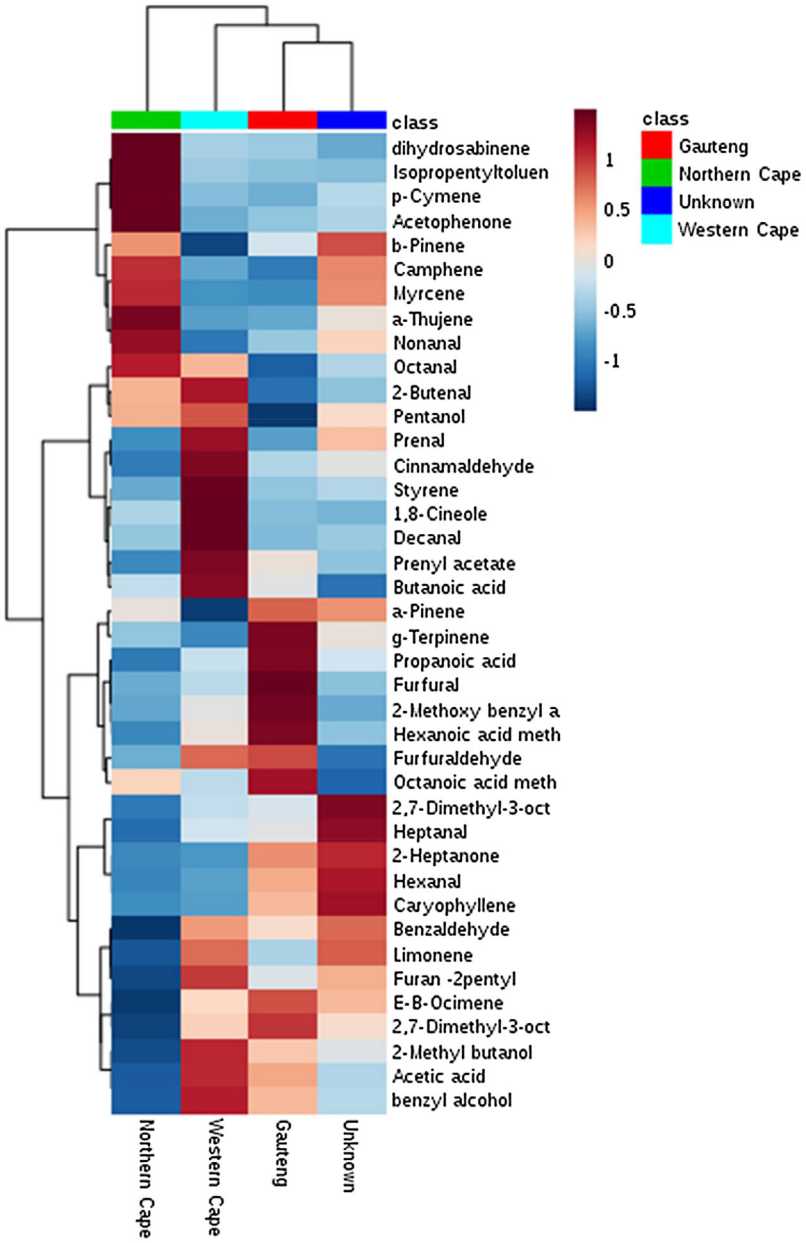

Fig. 2. Heatmap of the variables (averages) illustrating the different constituents and their occurrence in propolis from various localities.

peaks. In a study conducted by Pellati et al. (2013) who investigated nine Italian propolis using headspacesolid phase micro-extraction, 99 constituents were identified and the main compounds detected were benzoic acid (0.9–30.1%), benzyl benzoate (0.2–13.0%), benzyl salicylate (0.3–1.9%), benzyl cinnamate (0.3–3.2%), δ-cadinene (1.3–13.3%), λ-cadinene (1.4–8.9%), α-muurolene (0.8–6.6%), eudesmol (2.3–12.8%), T-cadinol (2.7–10.0%) and α-cadinol (4.8–9.7%). Vanillin (0.1–5.4%) was detected in the majority of the samples investigated. Various factors such as the type of vegetation, climatic conditions and geographical location play an important role in determining the chemical composition of propolis. For instance, the volatile constituents of propolis collected from different areas in North East Anatolia (Turkey) demonstrated high degree of chemical variation. The Yesilyurt propolis included phenylethyl alcohol (7.7%), benzyl alcohol (7.4%), decanal (6.7%), ethyl benzoate (6.5%), nonanal (5%) and cedrol (4.1%), while cedrol (15.6%) was the major constituent in Saricicek propolis. α-Bisabolol (14.3%), cedrol (7%), δ-cadinene (5.6%) and α-eudesmol (3.6%) were also identified as the major constituents in the propolis from Erzincan (Hames-Kocabas et al., 2013). There are various types of propolis based on the plant source and the type of pollinators and so far to the best of our knowledge, only standardisation of poplar type propolis and Brazilian green propolis are currently available.

Multivariate data analysis and identification of marker compounds

The peak areas of all 61 identified compounds in the 39 propolis samples were recorded and exported to SIMCA-P 14.0 for analysis. Principal component analysis was performed and a five principal component (PC) model was obtained displaying total chemical variation (R^2X_{cum}) of >90%. The first two PCs modelled 42.7% of total variance, which was enough to visualise the variation (qualitative or quantitative) between the 39 samples investigated. Several 2D score scatter plots were constructed, assessing different sets of

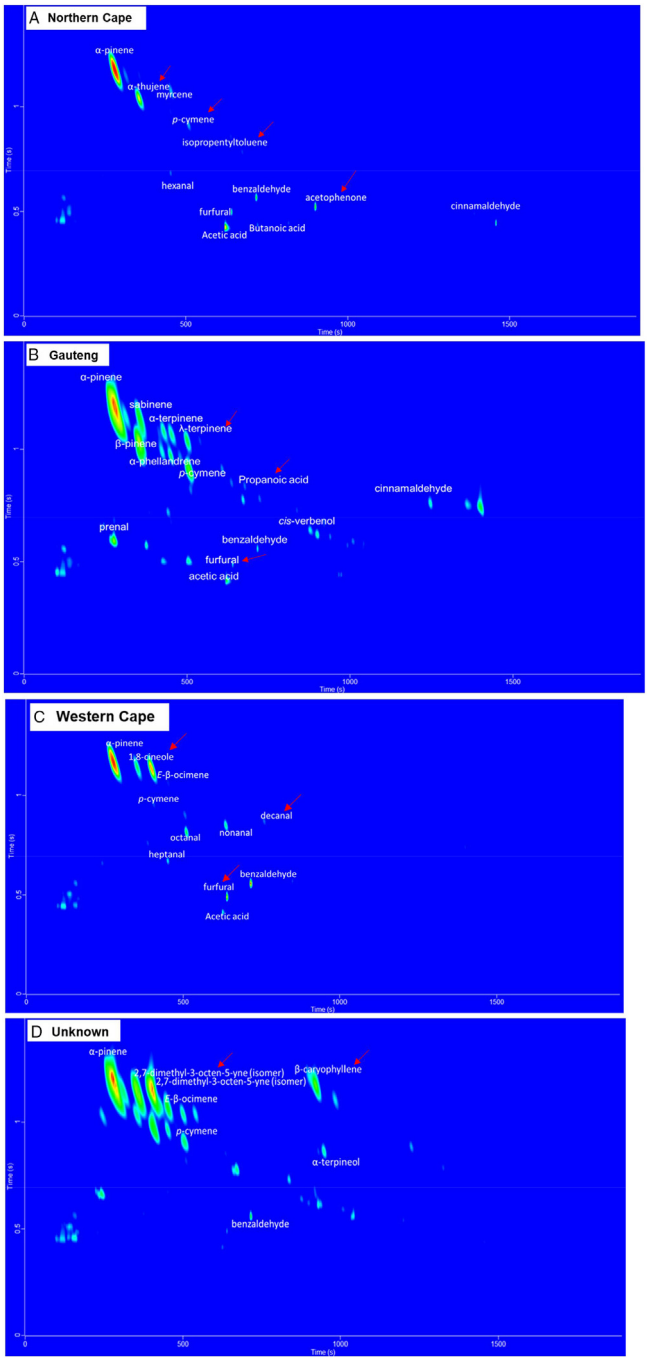

Fig. 3. Typical headspace GCxGC surface plot of propolis from (A) Northern Cape, (B) Gauteng, (C) Western Cape and (D) unknown, indicating some marker compounds.

components for possible patterns between South African propolis from various localities. Fig. 1A is a scores scatter plot (PC1 vs PC2) which shows variation among propolis samples from different localities and all the samples were distributed without specificity to a geographical location. Furthermore, the dendrogram (Fig. 1B) displays a pattern that is consistent with the scores plot indicating differences among propolis from various localities.

To investigate possible marker compounds in propolis from various locations, a heatmap of the averages was constructed (Fig. 2). The heat map shows correlations between variables (vertical dendrogram) and also enables biomarker identification by correlating variables to various classes/groupings (horizontal dendrogram) as displayed in Fig. 2. It is important to mention that quantitative and qualitative differences contributed to the identification of marker molecules and these markers were not necessarily the major compounds occurring in the samples. The marker compounds for the Northern Cape propolis were identified as dehydrosabinene (thujadiene), isopropenyltoluene (α-dimethyl styrene), *p*-cymene, acetophenone and α-thujene, as indicated by a colour amplitude value of 1. The findings were confirmed by analysing the 2D contour plots of Northern Cape propolis where some of the marker compounds were observed as indicated in the representative plot (Fig. 3A). The compounds λ-terpinene, propanoic acid, furfural, 2-methoxy benzyl alcohol and hexanoic acid methylester were filtered out for characterising Gauteng propolis according to the heatmap (Fig. 2). A representative 2D contour plot of Gauteng propolis revealed the presence of some of these marker compounds as displayed in Fig. 3B. Prenal, cinnamaldehyde styrene, 1,8-cineole, decanal, prenyl acetate and butanoic acid were the commonly occurring compounds in the Western Cape propolis as shown by the colour amplitude of 1. These compounds were identified in the representative 2D contour plot of Western Cape propolis shown in Fig. 3C. Finally, propolis from the unknown locality was represented by 2,7-dimethyl-3-octen-5-yne, heptanal and caryophyllene (Fig. 2). The corresponding 2D contour plot displays two of these marker molecules as illustrated in Fig. 3D. The results confirmed variation in the headspace volatile constituents of propolis and assisted in determining markers for use in the identification of different propolis chemotypes.

Conclusions

The direct headspace technique selectively targeted the volatile compounds from propolis samples for chromatographic analysis. Headspace GCxGC data in combination with chemometrics revealed variation in the chemical profiles of propolis and identified marker compounds representative of the different propolis chemotypes from various locations. The volatile compounds of South African propolis are highly variable. The monoterpene compounds constituted the major class of volatiles in propolis samples with pinene present in high amount in some samples. Two dimensional GCxGC has shown high sensitivity although not all compounds detected could be unambiguously identified.

Contribution of each of the authors

GK, performed the analysis, prepared the manuscript, addressed the reviewer comments, and submitted the manuscript; MS and ST, performed the metabolomic analysis and read the manuscript; SVV, provided the samples and read the manuscript; and AV, read the manuscript and provided the financial support through the South African National Research Foundation (SARChI program) and the South African Medical Research Council.

Conflicts of interest

The authors declare no conflict of interest

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