Saudi Journal of Biological Sciences 24 (2017) 1094–1103

Contents lists available at ScienceDirect

Saudi Journal of Biological Sciences

journal homepage: www.sciencedirect.com

Original article

Chemical compositions and characteristics of organic compounds in propolis from Yemen

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ARTICLE INFO

Article history:
Received 3 March 2016
Revised 1 December 2016
Accepted 6 December 2016
Available online 23 December 2016

Keywords:
Propolis
Yemen
Triterpenoids
GC–MS

ABSTRACT

Propolis is a gummy material made by honeybees for protecting their hives from bacteria and fungi. The main objective of this study is to determine the chemical compositions and concentrations of organic compounds in the extractable organic matter (EOM) of propolis samples collected from four different regions in Yemen. The propolis samples were extracted with a mixture of dichloromethane and methanol and analyzed by gas chromatography–mass spectrometry (GC–MS). The results showed that the total extract yields ranged from 34% to 67% (mean = 55.5 ± 12.4%). The major compounds were triterpenoids (254 ± 188 mg g^{-1}, mainly α-, β-amyryl and dammaradienyl acetates), n-alkenes (145 ± 89 mg g^{-1}), n-alkanes (65 ± 29 mg g^{-1}), n-alkanoic acids (40 ± 26 mg g^{-1}), long chain wax esters (38 ± 25 mg g^{-1}), n-alkanols (8 ± 3 mg g^{-1}) and methyl n-alkanoates (6 ± 4 mg g^{-1}). The variation in the propolis chemical compositions is apparently related to the different plant sources. The compounds of these propolis samples indicate that they are potential sources of natural bio-active compounds for biological and pharmacological applications.

1. Introduction

Honeybees collect resin/gummy materials from different parts of plants to make a sticky substance known as propolis (Ghisalberti, 1979; Parolia et al., 2010; Simone-Finstrom and Spivak, 2010) and utilize it for sealing cracks in hives and protecting their hives from infection by bacteria and fungi (Banskota et al., 2001; Simone-Finstrom and Spivak, 2010). Egyptians, Greeks and Romans used propolis to treat some diseases (Marcucci, 1995; Sforcin and Bankova, 2011). Currently, researchers are interested in the chemical components and biological activities of propolis because of its remedial properties (Sforcin and Bankova, 2011; Bankova, 2005; Castaldo and Capasso, 2002; Sforcin, 2007; Bankova et al., 2014). The geographical locales, plant species, collecting season and bees selective behavior are the dominant attributes to the assortment of the chemical compositions of propolis (Sforcin and Bankova, 2011; Isidorov et al., 2014). The major chemical constituents of propolis include flavonoids, aromatic acids, diterpenoid acids and triterpenoids, and phenolic compounds are often the major components (Bankova et al., 2000; Chen et al., 2008; Cursta-Rubio et al., 2007; Daugsch et al., 2008; Kumazawa et al., 2008; Markham et al., 1996; Márquez Hernández et al., 2010; Popova et al., 2010). Some of these compounds are responsible for its biological activities (Bankova et al., 2000; Barros et al., 2007; Bassani-Silva et al., 2007; Bufalo et al., 2009; Cvek et al., 2007; Orsatti et al., 2010a,b; Orsi et al., 2005; Silva et al., 2009; Zamami et al., 2007). The three possible sources of the organic compounds in propolis include plants, secreted substances from honeybee metabolism, and materials that are introduced during propolis formation (Marcucci, 1995). The typical bulk composition of propolis is vegetation resin and balsam (gum) (50%), wax (30%),

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Peer review under responsibility of King Saud University.

Production and hosting by Elsevier

http://dx.doi.org/10.1016/j.sjbs.2016.12.012
1319-562X/

essential and aromatic oils (10%), pollen (5%) and other substances (5%) (Cirasino et al., 1987; Monti et al., 1983). The majority of the chemical compositions and bio-activity effects of propolis were described for samples from Europe and Latin America (e.g. Bankova et al., 1992, 2000; Daugsch et al., 2008; Barros et al., 2007; Monti et al., 1983; Márquez Hernández et al., 2010), whereas few studies have been reported on propolis from the Arabian Peninsula (Abd El-Mawla and Osman, 2011; Almutairi et al., 2014; Fahmi et al., 2001; Jerz et al., 2014; Popova et al., 2013; Alqarni et al., 2015), with none from Yemen. Yemen is located in south of the Arabian Peninsula with different climatic and physiographic conditions that endow the country with about 3700 species belonging to 140 families of plants (Alhammadi, 2010). Most beekeepers in the country focus only on honey production and there is a potential for producing huge amounts of propolis that contains various biologically active substances. Apparently, no work has reported the chemical compositions of propolis from Yemen.

Thus, the objective of this study is to determine the chemical compositions, characteristics and concentrations of organic compounds in the solvent extractable organic matter of propolis samples collected from different regions in Yemen.

2. Materials and methods

2.1. Sampling

Nine propolis samples were collected from the southeastern and central parts of Yemen representing different geographical regions. The specific areas were: Tarim (samples T1 and T2; 16°03′00.39″N and 19°00′00.53″E; Altitude = 614 m), Wadi Adem (sample WA; 15°18′17.67″N and 48°38′45.50″E; Altitude = 630 m), Seiyun (samples S1 and S2; 15°56′27.88″N and 48°46′48.49″E; Altitude = 640 m), Seiyun Shahoh (sample SH), Amran (sample AM; 16°16′13.32″N and 43°56′17.53″E; Altitude = 1918 m), and Thebi-Tarim (samples TT1 and TT2; 16°01′19.01″N and 48°59′33.55″E; Altitude = 615 m) (Fig. 1). All the samples were collected in November 2011, except for the sample TT1, which was collected in August 2011. The major vegetation of the southeast region of Yemen is comprised of different Acacia species such as *Acacia tortilis*, *Acacia hamulosa* around Adem Village, *Prosopis juliflora*, *Azadirachta indica* around Tarim and Seiyun. The Amran region is characterized by the Acacia species *Ziziphus spina-christi*. The propolis samples were collected using a stainless steal spatula (~30 g of each) in a Teflon-caped glass container, labeled and kept in a freezer until analysis.

2.2. Extraction and derivatization

Each sample (210 ± 40 mg) was broken up and extracted three times using ultrasonic agitation for successive 15 min periods with a of dichloromethane (DCM) and methanol (MeOH, 40 mL, 3:1 v:v) mixture. The solvent mixture was used to extract both polar and nonpolar compounds from propolis. The extraction was carried out in a precleaned beaker. The undissolved particles in the extract were then removed using a filtration unit containing an annealed glass fiber filter, dried and weighed. The filtrate was first concentrated on a rotary evaporator and then reduced using a stream of dry nitrogen gas to a volume of approximately 2 mL. The total extracts were analyzed by gas chromatography-mass spectrometry (GC–MS). Because underivatized polar compounds have low detection by GC–MS, an aliquot (100 μL) of each total extract was dried under a flow of nitrogen and reacted with silylating reagent [N,O-bis(trimethylsilyl)trifluoroacetamide, BSTFA, Pierce Chemical Co.] for 3 h at 70 °C. Then, the BSTFA was evaporated under a flow of nitrogen and the sample was dissolved in n–hexane before analysis by GC–MS. This derivatizing agent replaces the H on hydroxyl groups with a trimethylsilyl [$(CH_3)_3Si$, i.e. TMS] group for better GC resolution of polar compounds.

2.3. Chemical analysis

Instrumental analysis by GC–MS was carried out with an Agilent 6890 gas chromatograph coupled to a 5973 Mass Selective Detector, using a DB-5MS (Agilent) fused silica capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness) and helium as carrier gas. The GC was temperature programmed from 65 °C (2 min initial time) to 310 °C at 6 °C min^{-1} (isothermal for 55 min final time) and the MS was operated in the electron impact mode at 70 eV ion source energy. Mass spectrometric data were acquired and processed using the GC–MS ChemStation data system.

2.4. Identification and quantification

The compounds were identified by comparison with the chromatographic retention characteristics and mass spectra of authentic standards, literature mass spectra and the mass spectral library of the GC–MS data system. The mass spectra of unknown compounds were interpreted based on their fragmentation patterns. The identification of triterpenoids, n-alkenes, n-alkanes, n-alkanoic acids, long chain wax esters, n-alkanols, and methyl n-alkanoates are based primarily on their mass spectra (i.e. key ions at *m/z* 191/189/218, 97, 85, 117 (TMS), 257, 103 (TMS), and 87, respectively). Compounds were quantified using total ion current (TIC) peak area, and converted to compound mass using calibration curves of external standards (tetracosane for n-alkanes; hexadecanoic acid for n-alkanoic acids, alkyl alkanoates and n-alkanols; sitosterol for triterpenoids; glucose for monosaccharides; and sucrose for disaccharides), assuming similar responses for both standards and sample compounds. It was also assumed that the individual compounds used to prepare the calibration curves and the compounds in the propolis mixture have similar responses. Four different standards were prepared from stock solutions (500 ppm tetracosane, 100 ppm hexadecanoic acid, 500 ppm sitosterol, and 1000 ppm sucrose). The least square method was used to test the linearity of the calibration curves and to fit the concentrations of the different standards versus their relative responses. The correlations between the relative responses and the concentrations were high with coefficients (R^2) ranging from 0.91 to 0.98. The calibration curve standards and a procedural blank were run in sequence with propolis samples. No significant background interferences were found.

3. Results

The major extractable organic compound concentrations are listed in Table 1 and the features of the GC–MS results for the propolis samples are shown in Fig. 1. The main compounds were sugars (not listed in Table 1), triterpenoids, n-alkenes, n-alkanes, n-alkanoic acids, long chain wax esters, n-alkanols and methyl n-alkanoates (Table 1). Triterpenoid concentrations ranged from 5.3 mg g^{-1} to 443.8 mg g^{-1}. The major triterpenoids were α- and β-amyryl acetate (1.1–386.9 mg g^{-1}), and α- and β-amyrin (0.02–23.0 mg g^{-1}) (Fig. 1 and Table 1). The total concentrations of n-alkenes ranged from 13.5 mg g^{-1} to 236.5 mg g^{-1} and tritriacontene (8.9–263.2 mg g^{-1}) was the major compound (Table 1 and Fig. 2). For n-alkanes, the total concentrations ranged from 16.9 to 111.1 mg g^{-1}, where the major compounds were pentacosane (0.8–8.9 mg g^{-1}), heptacosane (6.5–70.6 mg g^{-1}), nonacosane

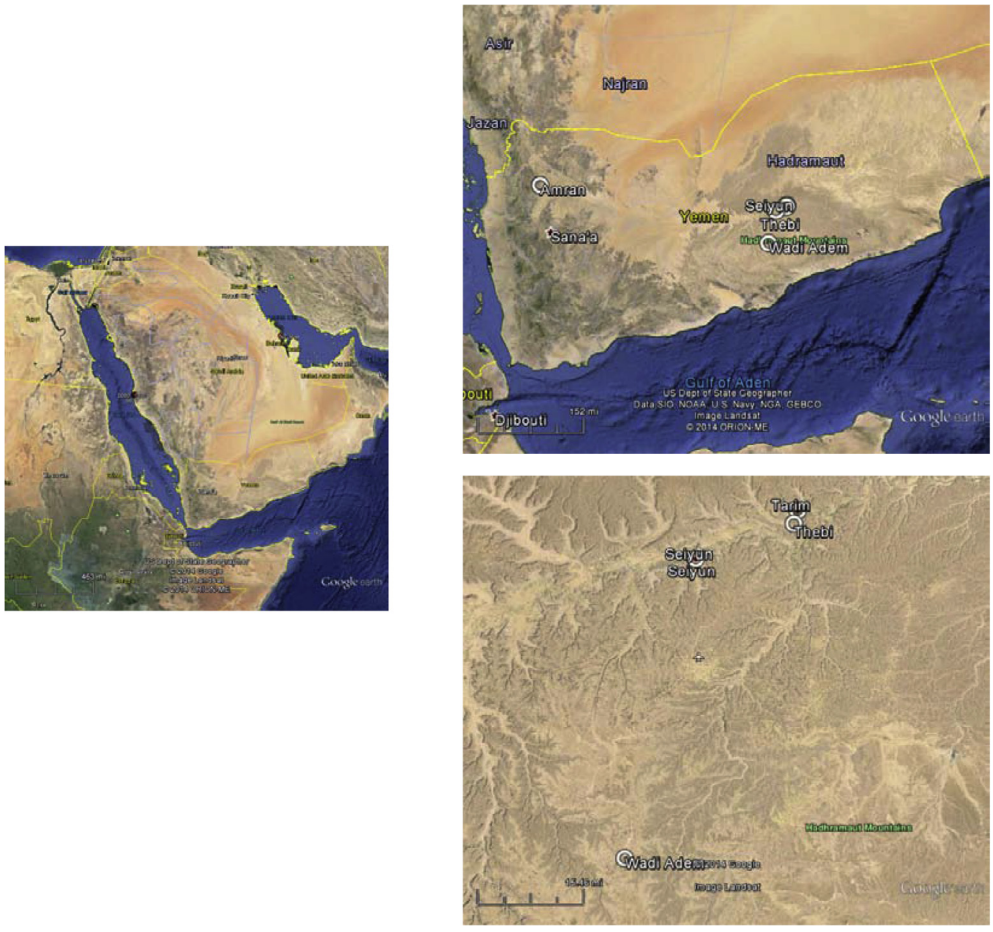

Figure 1. Map showing the locations of the propolis sample collection.

(3.7–18.2 mg g^{-1}), and hentriacontane (4.7–17.3 mg g^{-1}) (Table 1, Fig. 2). The concentrations of n-alkanoic acids ranged from 1.1 to 85.8 mg g^{-1} and the major compound was hexadecanoic acid (1.0–46.8 mg g^{-1}, Fig. 3). The total concentration of wax esters ranged from 8.6 to 59.9 mg g^{-1} and the major compound was tetracosyl hexadecanoate (7.1–65.12 mg g^{-1}; Table 1). For n-alkanols, the total concentrations ranged from 4.9 to 12.4 mg g^{-1} with tetracosanol (2.0–4.7 mg g^{-1}) as the major compound (Fig. 3). The methyl n-alkanoate concentrations ranged from 1.4 to 12.8 mg g^{-1} and methyl hexadecanoate (0.1–5.6 mg g^{-1}) was the major compound (Table 1).

4. Discussion

Obviously, the chemical compositions of propolis samples vary between different samples (Popova et al., 2010, 2011; Sforcin and Bankova, 2011; Bankova et al., 2014; Trusheva et al., 2003; Rushdi et al., 2014). The major compounds determined in the samples from Yemen include triterpenoids, n-alkenes, n-alkanes, n-alkanoic acids, wax esters, n-alkanols and methyl n-alkanoates. Flavonoids, which were detected in European, Brazilian, Chinese and Russian propolis (Bankova et al., 1983, 2014; Chang et al., 2002; Volpi and Bergonzini, 2006 and references therein), were not detected in these samples. The compound groups vary between different samples and locations. To investigate the similarity between the different samples, hierarchical cluster analysis (HCA) by the Ward's method was used. The output of the cluster analysis is shown in Fig. 4 where two separate sample clusters are recognized. The first cluster includes two (WA and AM) propolis samples, and the second includes the rest of the propolis samples (S1, S2, SH, T1, T2, TT1 and TT2). The second cluster shows that the samples TT1 and S2 are deviate from S1, SH, TT2, T1 and T2. This indicates that sources of poroplis components from Wadi Adem and Amran are different from the samples collected from Seiyun, Seiyun-Shahoh, Tarim and Thebi-Tarim.

To better understand the relationships between the variables of the different propolis samples, the Spearman correlation multivariate statistical approach and principal component analysis (PCA) were used. The output of the Spearman correlations is shown in Table 2. The analysis shows that there are significant correlations ($R > 80$) between T1 and TT1; T2, S1, S2, SH, and TT2 (Tarim, Thebi/Tarim, Seiyun and Seiyun-Shahoh); and WA (Wadi Adem) and AM (Amran). The output of the PCA, which was followed by varimax rotation, is shown in Table 3 and Fig. 5. The PCA identifies two significant components (C1 and C2), explaining 83.45% of the variance with an Eigen value of >1. Factor loadings of >0.80 are used for variables to interpret the data. C1 explains 58.64% of the variance, where T2, TT2, SH, S1 and S2 are dominant, indicating potential similar propolis components. It also shows relatively high loadings for T1 (0.78) and TT1 (0.64); thus, they can be considered similar to T2, TT2, SH, S1 and S2. C2 includes 24.81% of the variance and the significant factor loadings are for samples WA and AM, signifying similar propolis constituents. The similarity among the different samples is shown in Fig. 5, which demonstrates two clusters along axes 1 and 2 and the separation confirmed dissimilarity among the different propolis samples indicating different sources of local plants. Generally, the ordination plots of the propolis sam-

Table 1
The yields (%) and concentrations (mg g^{-1}) of the various lipid compound groups of propolis samples from different regions of Yemen[1].

			Sample									
			T1	**T2**	**WA**	**S1**	**S2**	**SH**	**AM**	**TT1**	**TT2**	**Mean**	**SD**
Total EOM (%)			64.88	66.58	59.88	33.64	43.97	41.31	63.65	65.23	60.64	55.53	12.40
Compound	**Composition**	**M.W.**											
Triterpenoids													
β-Amyrone	$C_{30}H_{48}O$	424	0.00	0.00	0.00	0.00	0.00	0.00	0.59	0.00	0.00		
β -Amyrin	$C_{30}H_{50}O$	426	3.33	1.25	0.96	1.65	0.18	1.69	10.80	2.52	25.58		
α-Amyrone	$C_{30}H_{48}O$	424	0.00	0.00	0.00	0.00	0.00	0.00	1.39	0.00	0.00		
β -Amyryl acetate	$C_{32}H_{52}O_2$	468	49.70	28.03	107.99	4.50	1.13	10.16	55.76	165.32	18.50		
α-Amyrin	$C_{30}H_{50}O$	426	2.29	0.36	0.00	1.07	0.27	1.02	22.07	0.00	34.92		
α-Amyryl acetate	$C_{32}H_{52}O_2$	468	34.12	18.69	386.94	2.78	3.12	6.05	335.35	104.82	29.11		
β -Amyryl pentanoate	$C_{35}H_{58}O_2$	510	12.34	6.47	0.00	0.61	0.14	3.46	0.00	3.58	11.80		
α-Amyryl pentanoate	$C_{35}H_{58}O_2$	510	9.92	4.65	0.00	0.44	0.00	2.56	0.00	2.96	9.19		
Amyryl hexanoate	$C_{36}H_{60}O_2$	524	0.98	0.10	0.00	0.00	0.00	0.23	0.00	0.12	1.24		
β -Amyryl hex-5-enoate	$C_{36}H_{58}O_2$	522	10.43	2.51	0.00	0.00	0.00	1.92	0.00	2.20	9.82		
α-Amyryl hex-5-enoate	$C_{36}H_{58}O_2$	522	4.22	0.82	0.00	0.00	0.00	0.81	0.00	0.00	4.35		
Moretenol	$C_{30}H_{50}O$	426	1.40	0.86	0.00	0.84	0.00	0.53	0.00	0.26	10.96		
Dammaradienol	$C_{30}H_{50}O$	426	6.30	2.81	0.00	0.81	0.00	2.64	0.00	1.38	19.46		
3β -Lupenyl acetate	$C_{32}H_{52}O_2$	468	13.81	10.52	8.69	3.18	0.44	4.80	0.04	1.18	6.77		
3α-Lupenyl acetate	$C_{32}H_{52}O_2$	468	145.38	95.47	0.98	2.15	0.25	29.78	0.00	144.75	21.20		
Dammaradienyl pentanoate	$C_{35}H_{58}O_2$	510	35.80	20.51	0.00	1.24	0.13	10.51	0.00	3.06	16.43		
Dammaradienyl hex-5-enoate	$C_{36}H_{58}O_2$	522	13.80	3.57	0.00	0.06	0.00	2.64	0.00	0.89	1.14		
Total			**343.81**	**196.61**	**505.56**	**19.33**	**5.67**	**78.80**	**426.01**	**433.07**	**220.47**	**247.71**	**188.58**
(%)			52.99	29.53	84.42	5.75	1.29	19.08	66.93	66.39	36.36	40.30	29.15
n-Alkenes													
Pentacosene	$C_{25}H_{50}$	350	1.30	2.41	0.15	1.91	1.85	1.86	0.78	0.86	1.30		
Hexacosene	$C_{26}H_{52}$	364	0.24	0.14	0.03	0.19	0.08	0.67	0.32	0.12	0.39		
Heptacosene	$C_{27}H_{54}$	378	10.64	20.68	1.20	16.94	10.42	8.88	5.33	7.33	10.15		
Octacosene	$C_{28}H_{56}$	392	1.58	0.87	0.19	0.45	0.14	0.46	0.70	0.10	0.21		
Nonacosene	$C_{29}H_{58}$	406	4.82	4.79	0.77	6.33	2.92	3.05	4.31	4.57	4.75		
Triacontene	$C_{30}H_{60}$	420	7.22	0.69	0.56	1.62	0.46	0.28	7.32	1.01	0.40		
Hentriacontene	$C_{31}H_{62}$	434	3.64	6.01	0.31	3.60	26.03	3.73	2.51	3.18	4.54		
Dotriacontene	$C_{32}H_{64}$	448	3.38	1.66	0.45	1.76	1.66	2.44	5.39	1.15	1.30		
Tritriacontene	$C_{33}H_{66}$	462	80.86	187.93	8.89	92.41	281.67	98.82	31.22	67.92	124.40		
Tetratriacontene	$C_{34}H_{68}$	476	3.12	0.86	0.51	1.75	0.33	2.33	4.96	0.63	0.67		
Pentatriacontene	$C_{35}H_{70}$	490	1.85	3.33	0.47	1.85	7.89	6.97	2.73	2.22	6.09		
Total			**118.67**	**229.37**	**13.53**	**128.79**	**333.46**	**129.49**	**65.57**	**89.08**	**154.23**	**140.24**	**93.83**
(%)			18.29	34.45	2.26	38.29	75.85	31.35	10.30	13.66	25.43	27.76	21.59
n-Alkanes													
Eicosane	$C_{20}H_{42}$	282	0.00	0.00	0.07	0.00	0.00	0.00	0.00	0.00	0.00		
Heneicosane	$C_{21}H_{44}$	296	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00		
Docosane	$C_{22}H_{46}$	310	0.00	0.00	0.09	0.00	0.00	0.00	0.00	0.00	0.00		
Tricosane	$C_{23}H_{48}$	324	1.20	2.05	0.15	0.91	1.05	1.00	0.35	0.52	0.86		
Tetracosane	$C_{24}H_{50}$	338	0.62	0.26	0.06	0.16	0.10	0.09	0.35	0.11	0.42		
Pentacosane	$C_{25}H_{52}$	352	4.41	8.59	0.83	6.18	5.04	4.79	2.63	2.76	4.57		
Hexacosane	$C_{26}H_{54}$	366	0.76	0.77	0.17	0.65	0.42	0.40	1.42	0.40	1.41		
Heptacosane	$C_{27}H_{56}$	380	36.03	68.46	6.54	52.74	25.84	24.58	16.25	23.18	34.88		
Octacosane	$C_{28}H_{58}$	394	0.72	0.47	0.29	0.68	0.03	0.02	0.25	0.22	1.91		
Nonacosane	$C_{29}H_{60}$	408	13.60	12.99	3.68	16.54	5.08	4.83	10.86	11.94	13.19		
Triacontane	$C_{30}H_{62}$	422	0.00	0.49	0.32	0.63	0.09	0.08	5.21	0.20	1.69		
Hentriacontane	$C_{31}H_{64}$	436	12.59	13.70	4.72	15.71	12.23	11.63	8.40	16.92	11.69		
Total			**69.92**	**107.78**	**16.94**	**94.21**	**49.86**	**47.42**	**45.72**	**56.24**	**70.64**	**62.08**	**27.29**
(%)			10.78	16.19	2.83	28.01	11.34	11.48	7.18	8.62	11.65	12.01	7.03
n-Alkanoic Acids													
Hexadecanoic acid	$C_{16}H_{32}O_2$	256	23.93	12.14	5.89	18.68	0.97	24.37	46.75	10.72	10.84		
Octadecenoic acid	$C_{18}H_{34}O_2$	282	7.16	7.48	2.10	0.96	0.02	9.43	3.37	11.94	12.07		
Octadecanoic acid	$C_{18}H_{36}O_2$	284	0.00	0.00	0.13	0.00	0.00	0.00	0.00	0.00	0.00		
Eicosanoic acid	$C_{20}H_{40}O_2$	312	0.00	0.00	0.18	0.12	0.11	0.10	0.00	0.00	0.00		
Tetracosanoic acid	$C_{24}H_{48}O_2$	368	1.96	37.98	1.12	0.88	0.00	51.93	0.00	26.17	26.46		
Total			**33.05**	**57.60**	**9.42**	**20.64**	**1.10**	**85.84**	**50.12**	**48.83**	**49.38**	**39.55**	**26.34**
(%)			5.09	8.65	1.57	6.13	0.25	20.78	7.87	7.49	8.14	7.33	5.84
Wax Esters													
Octadecyl hexadecanoate	$C_{34}H_{68}O_2$	508	0.09	0.11	0.02	0.07	0.07	0.06	0.01	0.02	0.11		
Eicosyl hexadecanoate	$C_{36}H_{72}O_2$	536	0.29	0.51	0.00	0.26	0.27	0.29	0.01	0.05	0.76		
Dodecasanyl hexadecanoate	$C_{38}H_{76}O_2$	564	0.57	0.80	0.36	0.64	0.38	0.66	0.79	0.06	1.24		
Tetracosanyl hexadecanoate	$C_{40}H_{80}O_2$	592	24.49	37.59	7.07	43.92	18.01	35.66	14.54	6.57	61.21		
Hexacosanyl hexadecanoate	$C_{42}H_{84}O_2$	620	5.19	5.47	2.31	9.50	3.57	8.06	6.16	1.80	14.83		
Octacosanyl hexadecanoate	$C_{44}H_{88}O_2$	648	1.01	0.42	0.03	0.13	0.28	0.32	0.36	0.05	0.45		
Total			**31.63**	**44.89**	**9.79**	**54.51**	**22.59**	**45.05**	**21.86**	**8.55**	**78.60**	**35.28**	**22.78**
(%)			4.88	6.74	1.64	16.21	5.14	10.91	3.43	1.31	12.96	7.02	5.21

(*continued on next page*)

Table 1 (*continued*)

			Sample										
			T1	**T2**	**WA**	**S1**	**S2**	**SH**	**AM**	**TT1**	**TT2**	**Mean**	**SD**
n-Alkanols													
Octadecanol	$C_{18}H_{38}O$	270	0.07	0.07	0.12	0.07	0.06	0.05	0.05	0.15	0.13		
Eicosanol	$C_{20}H_{42}O$	298	0.29	0.29	1.53	0.15	0.08	0.15	0.89	0.90	0.79		
Docosanol	$C_{22}H_{46}O$	326	0.46	0.46	1.79	0.24	0.13	0.27	0.19	1.36	1.19		
Tetracosanol	$C_{24}H_{50}O$	354	2.20	2.19	4.66	3.49	3.81	2.07	1.97	3.54	3.09		
Hexacosanol	$C_{26}H_{54}O$	382	0.73	0.73	1.08	1.19	0.82	0.80	1.19	2.21	1.93		
Octacosanol	$C_{28}H_{58}O$	410	0.39	0.39	0.39	0.66	0.30	0.51	0.97	1.32	1.15		
Triacontanol	$C_{30}H_{62}O$	438	0.56	0.56	0.71	0.95	0.51	0.84	0.33	1.72	1.50		
Dotriacontanol	$C_{32}H_{66}O$	466	0.21	0.21	0.49	0.26	0.21	0.28	0.65	1.23	1.08		
Total			**4.92**	**4.90**	**10.77**	**7.01**	**5.91**	**4.97**	**6.25**	**12.43**	**10.86**	**7.56**	**2.97**
(%)			0.76	0.74	1.80	2.08	1.34	1.20	0.98	1.91	1.79	1.40	0.51
Methyl n-Alkanoates													
Methyl dodecanoate	$C_{13}H_{26}O_2$	214	0.03	0.03	0.01	0.03	0.01	0.08	0.00	0.00	0.00		
Methyl tetradecanoate	$C_{15}H_{30}O_2$	242	0.07	0.07	0.02	0.03	0.01	0.05	0.03	0.01	0.03		
Methyl hexadecanoate	$C_{17}H_{34}O_2$	270	5.57	4.33	0.88	2.02	0.54	1.78	0.06	0.50	1.37		
Methyl octadecenoate	$C_{19}H_{36}O_2$	296	0.30	0.39	0.09	0.18	0.07	0.33	2.68	0.14	0.20		
Methyl octadecanoate	$C_{19}H_{38}O_2$	298	0.37	0.34	0.10	0.15	0.05	0.21	0.00	0.07	0.19		
Methyl eicosanoate	$C_{21}H_{42}O_2$	326	0.13	0.14	0.05	0.05	0.01	0.05	0.44	0.01	0.00		
Methyl docosanoate	$C_{23}H_{46}O_2$	354	0.23	0.21	0.06	0.12	0.04	0.09	0.19	0.03	0.06		
Methyl tetracosanoate	$C_{25}H_{50}O_2$	382	3.78	3.01	0.69	1.57	0.52	1.45	0.16	0.34	0.71		
Methyl hexacosanoate	$C_{27}H_{54}O_2$	410	1.08	0.78	0.20	0.46	0.17	0.53	1.70	0.12	0.23		
Methyl Octacosanoate	$C_{29}H_{58}O_2$	438	0.64	0.43	0.12	0.26	0.11	0.42	0.63	0.09	0.08		
Methyl Triacontanoate	$C_{31}H_{62}O_2$	466	0.39	0.26	0.06	0.15	0.07	0.34	0.44	0.08	0.08		
Methyl Dotriacontanoate	$C_{33}H_{66}O_2$	494	0.19	0.12	0.04	0.10	0.04	0.17	0.00	0.00	0.00		
Total			**12.79**	**10.10**	**2.31**	**5.14**	**1.64**	**5.50**	**6.75**	**1.38**	**2.95**	**5.40**	**3.95**
(%)			1.97	1.52	0.39	1.53	0.37	1.33	1.06	0.21	0.49	0.99	0.64

[1] The quantification of the propolis components assumes a similar response for the external standards, compounds of the calibration curves, and the sample compounds.

ples showed a slight separation between the samples from Tarim and Seiyun (T and S samples) and a clear separation for the samples from Wadi Adem (WA) and Amran (AM).

4.1. Triterpenoids

Triterpenoids have been reported to occur in diverse plant species as resin or gum constituents (Cursta-Rubio et al., 2007; de Castro Ishida et al., 2011). They are rarely found in fungi and animals (Lutta et al., 2008). Therefore, the major source of triterpenoids is terrestrial vegetation (Hernández-Vázquez et al., 2010; Manguro et al., 2009; Moreau et al., 2009; Ramadan et al., 2009). They are found in plant leaves, bark and resins (Ramadan et al., 2009; van Maarsenveen and Jetter, 2009; Feng et al., 2010; Manguro et al., 2009; Hernández-Vázquez et al., 2010; Vouffo et al., 2010; Rosas-Acevedo et al., 2011; Wang et al., 2011). Their concentrations vary and depend on the plant species. For example, α- and β-amyrin are found in Protium sp. *Byrosonima fagifolia* and *Byrosonima crassifolia* (Hernández-Vázquez et al., 2010), only α-amyrin is present in *Cassia obtusifolia* (Sob et al., 2010) and moretenol is found in *Ficus macrophytta* and *Ficus delloidea* leaves (Galbraith et al., 1965; Lip et al., 2009). Periera et al. (1996) detected lupenone, lupeol, lupenyl palmitate and lupenyl cinnamate in *Acacia dealbata*.

Triterpenoids were major compounds of propolis samples from Yemen. The relative concentrations of these substances ranged from 1.2 to 84.4% with a mean value of 41.6 ± 28.9%. They were mainly α- and β-amyryl acetates and α- and β-lupenyl acetates. It is worth mentioning that moretenol (Fig. 6) was detected as a major compound in sample TT2 (Thebi/Tarim) collected in August and minor amounts in other samples collected in November (Table 1). The highest triterpenoid concentrations were observed in the propolis from the Wadi Adem (84.4%), Amran (69.7%) and Thebi/Tarim (66.4%) areas, where the major vegetation is dominated by Acacia species (*Acacia tortitils* and *Acacia hamulosa* in Wadi Adem, and *Acacia origena* and *Acacia gerradii* in Amran). α- and β-Amyryl acetates were the major triterpenoids in all samples except those from the Tarim (T1 and T2) area (Table 1), where α- and β-lupenyl acetates were dominant. Dammaradienol and dammaradienyl pentanoate were also present in significant amounts in the samples from the Thebi-Tarim area (Table 1). These triterpenoids were also reported for propolis samples from Brazil, Cuba, Egypt and Ethiopia (El-Hady and Hegazi, 2002; Hegazi and El-Hady, 2002; de Castro Ishida et al., 2011; Márquez Hernández et al., 2010; Rushdi et al., 2014). Ugur et al. (2011) reported that the major components of *Onopordum caricium* were triterpenoids including lup-20(29)-ene-3β,28-diol and β-amyryl acetate. Diterpenoids were found as the major compounds in propolis samples from Europe (Popova et al., 2009, 2011; Trusheva et al., 2003). Thus, the results show that these propolis extracts include primarily lipid compounds from terrestrial plant sources as reported before (Bankova et al., 2000; Cursta-Rubio et al., 2007; Campo Fernandez et al., 2008; Melliou and Chinou, 2004; Salatino et al., 2005).

The presence of triterpenoids (mainly amyryl and lupenyl acetates) can act as antibacterial and antitumor agents (Simone-Finstrom and Spivak, 2010; de Castro Ishida et al., 2011). The main source of triterpenoids in propolis is obviously the surrounding vegetation. Therefore, the determination of the chemical compositions of the regional vegetation should be considered, because it will be useful for investigating the pharmacologically active components of local plants as well as of propolis.

4.2. n-Alkenes and n-alkanes

The concentrations of the n-alkenes ranged from 13.5 to 311.7 mg g^{-1} with a mean of 145.0 ± 89.3 mg g^{-1}. The highest concentration was found in the propolis sample from Seiyun (S2) area and the minimum in the samples from Wadi Adem (WA). The n-alkenes ranged from C_{25} to C_{35} with a C_{max} at 33. The distribution of n-alkenes with major concentrations of the odd numbered homologues and C_{max} at 33 supports an origin from insect wax (Jackson, 1972; Jackson and Baker, 1970), possibly from alteration

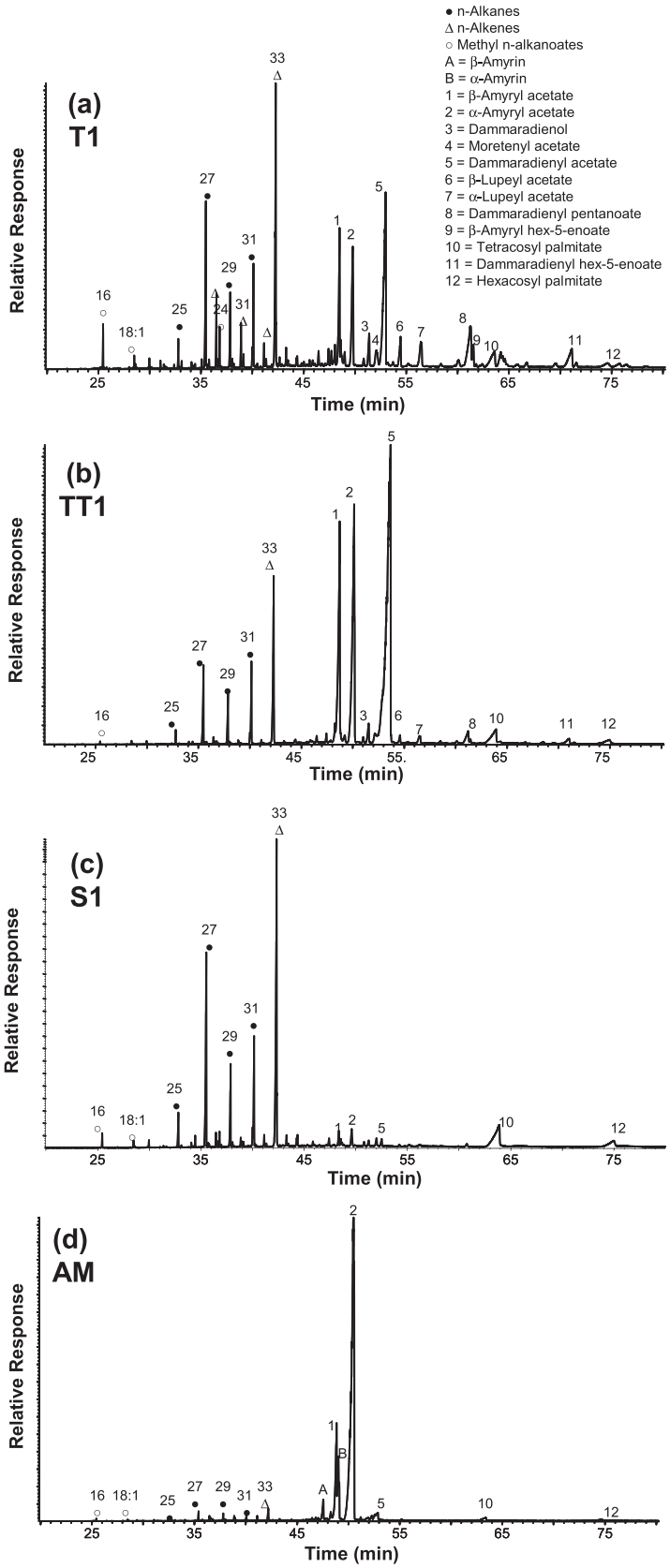

Figure 2. Total ion current (TIC) traces of total extracts showing the major organic tracers in propolis samples collected from the: (a) Tarim, (b) Thebi-Tarim, (c) Seiyun, and (d) Amran localities in Yemen (numbers above the symbols indicate the carbon number).

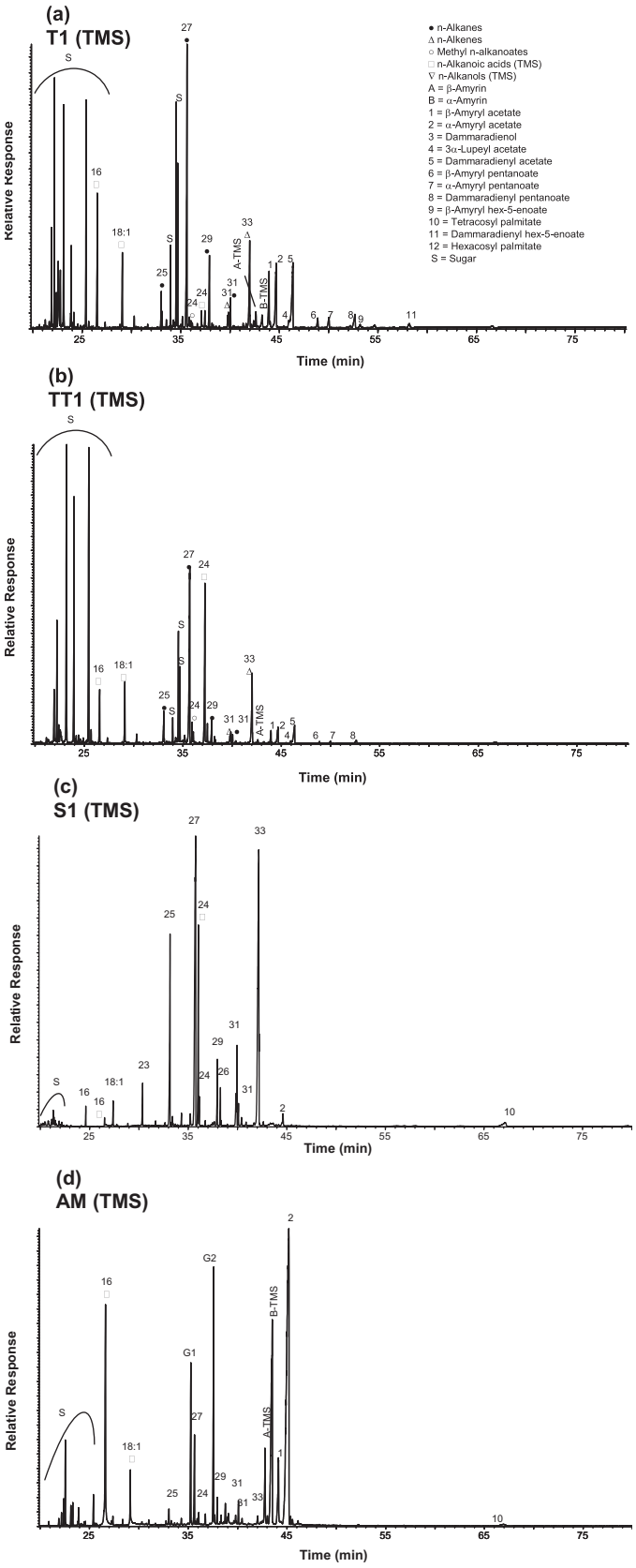

Figure 3. TIC traces showing the major compounds in the derivatized total extracts of propolis samples of the: (a) Tarim, (b) Thebi-Tarim, (c) Seiyun, and (d) Amran localities in Yemen (numbers above the symbols indicate the carbon number).

of long chain n-alkanols. n-Alkenes were major compounds with relative concentrations ranging from 2.3% to 70.9% and an average of 28.9 ± 20.8% of the total extract. Recent work showed that n-alkenes were present in both bee wax and propolis (Negri et al., 1998, 2000; Custadio et al., 2003). They were mainly $C_{31:1}$ and $C_{33:1}$ (Negri et al., 1998), indicating that the sources of the n-alkenes in propolis were bee wax.

The concentrations of n-alkanes in these propolis samples ranged from 46.4 to 111.1 mg g^{-1} of the total extracts with a mean of 65.2 ± 7.29 mg g^{-1} (Table 1). The lowest concentration was measured in the propolis from the S2 (Seiyun) sample, while the highest concentration was in the sample from the T2 (Tarim) area. The dominant n-alkanes were in the range of C_{20} to C_{31}, with a carbon number maximum concentration (C_{max}) at 27 (e.g. Fig. 2). Plant wax n-alkanes generally have a C_{max} in the range of C_{25}–C_{31}, which varies depending on the plant species as well as the season and locality (e.g. Eglinton and Hamilton, 1967). n-Alkanes are also a major fraction in propolis (Negri et al., 1998; Custadio et al., 2003; Rushdi et al., 2014; Alqarni et al., 2015). Bee wax n-alkanes generally have a C_{max} in the range from 21 to 33 depending on the age and color (Tulloch, 1971; Nadmar et al., 2007). The relative concentration of n-alkanes ranged from 2.8 to 16.7% with an average of 12.7 ± 7.8% of the total extract. Thus, the odd carbon number preference of the C_{21}-C_{31} n-alkanes and the C_{max} at 27 indicate the major source of these n-alkanes is likely from bee wax (Tulloch, 1971) with some contribution from plant wax.

4.3. n-Alkanoic acids

The total concentrations of n-alkanoic acids (fatty acids) in the propolis samples ranged from 1.1 to 85.8 mg g^{-1} with a mean value of 39.6 ± 26.3 mg g^{-1} (Table 1). The lowest concentration was found in the propolis from the S1 (Seiyun) area and the highest concentration was in sample SH (Seiyun-Shahoh). The n-alkanoic acids of flora and fauna have mainly even carbon chain length homologues and usually range from C_{12} to C_{32}. They are generally unsaturated in flora and saturated in fauna. The C_{18} mono-, di- and tri-unsaturated compounds are the major fatty acids in plants, whereas polyunsaturated fatty acids are more common in algae (Eglinton and Hamilton, 1967; Kolattukudy, 1976).

The n-alkanoic acids (silylated) in all propolis samples ranged from C_{16} to C_{24}, with a C_{max} at 16 for T1, WA, S1, S2 and AM or at 24 for T2, SH and TT1 (Table 1). The concentrations of hexadecanoic and tetracosanoic acids ranged from 1.0 to 46.8 mg g^{-1} and from 0.0 to 51.9 mg g^{-1}, respectively. The presence of n-alkanoic acids, with only an even carbon number predominance and unsaturated (octadecenoic) acids, plus C_{max} at 16 or 24, indicate sources from mainly vascular plants.

4.4. Long chain wax esters

Significant amounts of long chain wax esters were also detected in these samples with concentrations of 8.6 to 83.6 mg g^{-1} (mean = 37.8 + 25.5 mg g^{-1}), consisting mainly of docosanyl-, tetracosanyl-, hexacosanyl- and octacosanyl hexadecanoates. The major compound of the wax esters was tetracosanyl hexadecanoate in all samples (Table 1, Fig. 2). The relative concentration ranged from 1.3 to 17.8% with a mean value of 6.9 ± 6.0% of the total extract. Waxes secreted by bees contain more than 15% of wax esters (Katzav-Gozansky et al., 1997). Bee wax esters generally

Table 2
The Spearman correlation coefficients (R) for the different propolis samples.

	T1	T2	WA	S1	S2	SH	AM	TT1	TT2
T1	1.000	0.798	0.225	0.480	0.431	0.626	0.241	**0.807**	0.585
T2		1.000	0.096	**0.842**	**0.842**	**0.921**	0.151	0.577	**0.868**
WA			1.000	0.023	0.012	0.049	**0.980**	0.562	0.189
S1				1.000	**0.836**	**0.832**	0.114	0.260	**0.868**
S2					1.000	**0.809**	0.081	0.252	**0.821**
SH						1.000	0.123	0.440	**0.887**
AM							1.000	0.512	0.268
TT1								1.000	0.428
TT2									1.000

Table 3
The principal component factors for the different propolis samples.

Component Matrix		
	Component	
	C1	C2
T2	**0.97**	−0.17
TT2	**0.92**	−0.13
SH	**0.92**	−0.24
S1	**0.86**	−0.31
S2	**0.83**	−0.33
T1	0.78	0.17
TT1	0.64	0.55
WA	0.28	**0.92**
AM	0.34	**0.87**
Total variance (%)	58.64	24.81
Cumulative (%)	58.64	83.45

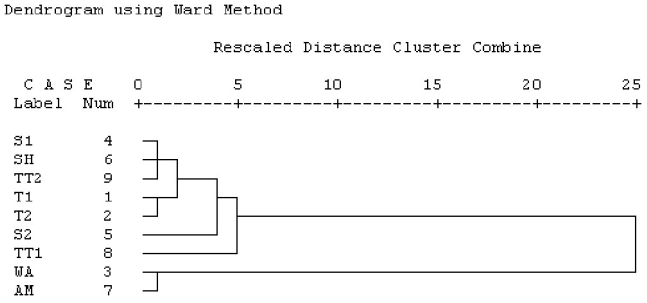

Figure 4. Plot showing the statistical outputs of cluster analysis.

include tetradecyl-dodecanoate, tetradecanoate and hexadecanoate, as well as hexadecyl-tetradecanoate and hexadecanoate (Katzav-Gozansky et al., 1997). Likely, they are derived from waxes secreted by the bees (Tulloch, 1971), from lipid components of terrestrial plants (Baker, 1982; Kolattukudy, 1976; Hamilton, 1995) of the region or both. The components of waxes in some younger plants are generally alcohols (40%) and they are mainly wax esters (42%) in older plants (Avato et al., 1990; Bianchi et al., 1989). The composition of vegetation wax esters depends also on the geographical location (Sforcin and Bankova, 2011).

4.5. n-Alkanols

The total concentrations of n-alkanols (fatty alcohols) ranged from 4.9 to 12.4 mg g^{-1} with an average of 7.6 ± 3.0 mg g^{-1}. The lowest concentration was measured in the samples T1 and T2 and the highest for TT1 (Table 1). They ranged from C_{18} to C_{32}, with a C_{max} at 24 which varied from 2.0 to 4.7 mg g^{-1}. The n-alkanols, found mainly in plants, have predominantly even number chains because they are biosynthesized from fatty acids by enzymatic reduction (Lehninger, 1970; More, 1993). The n-alkanol distributions in these propolis samples indicate an input from vascular plant wax of semitropical to tropical environments.

4.6. Methyl n-alkanoates

The concentrations of methyl n-alkanoates were relatively low in the range from 1.4 to 12.8 mg g^{-1} with a mean of

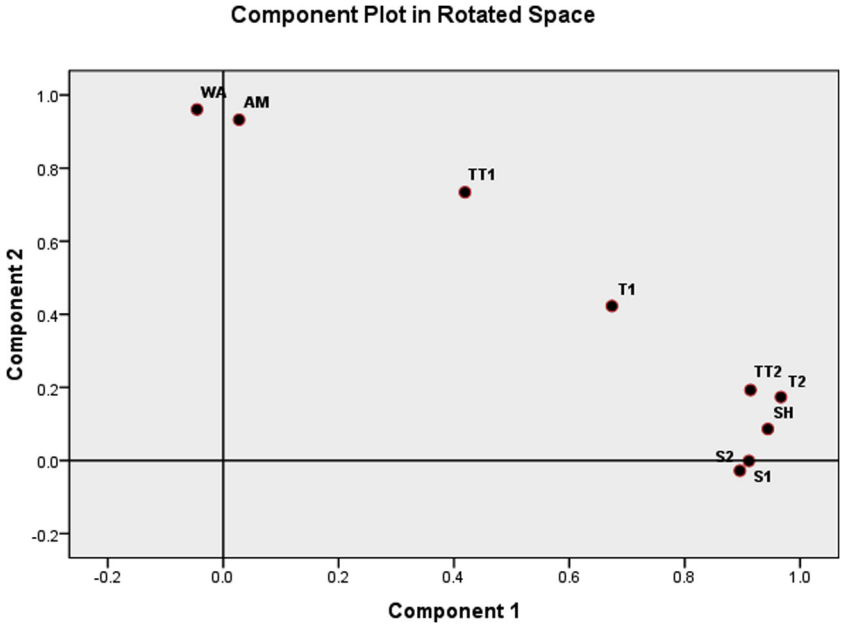

Figure 5. Plot showing the statistical outputs of the principal component analysis (PCA).

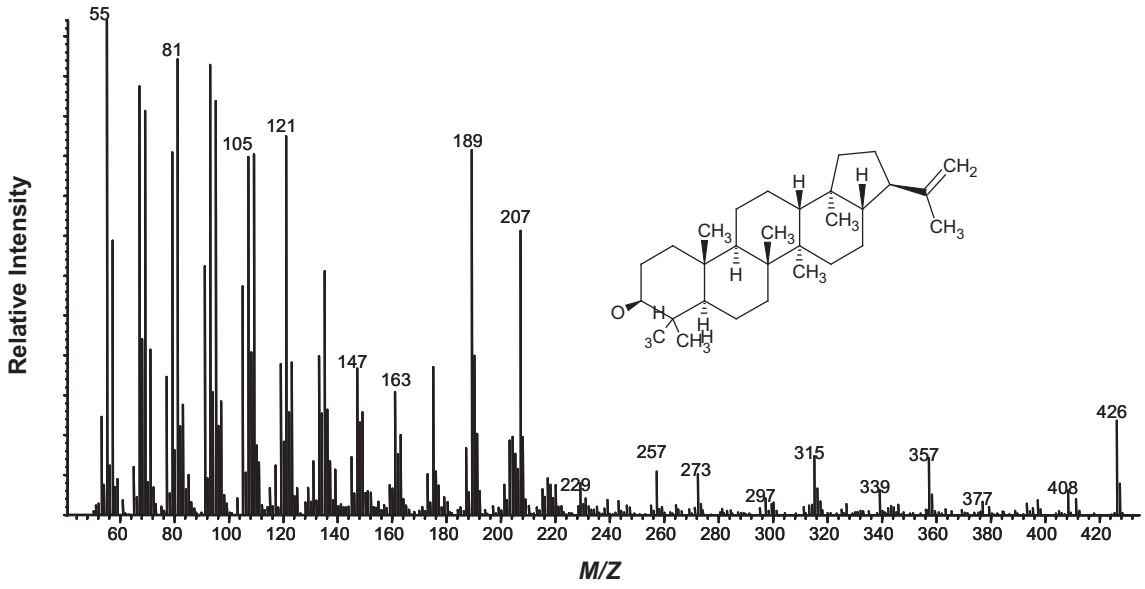

Figure 6. Mass spectrum of moretenol.

5.7 ± 4.0 mg g^{-1} (Table 1). They extend from C_{13} to C_{23} with a C_{max} at 17 and 25 (as acids C_{max} = 16 and 24). Methyl n-alkanoates may be natural or form by transesterification of n-alkanoic acids during extraction with methanol as indicated by their low relative concentrations. The highest concentration was found for the propolis sample from Tarim (T1) and the lowest from Thobi-Tarim (TT). The methyl n-alkanoates of these samples have a strong even carbon number predominance (as the alkanoic acids, Table 1), indicating that they are originally from natural biota (Harwood and Russell, 1984). The concentrations of methyl n-alkanoates were low relative to other compounds and ranged from 0.2 to 2.0% with an average of 1.0 ± 0.6% of the total extract.

5. Conclusion

The dichloromethane: methanol solvent-extractable organic matter of propolis samples from four regions in Yemen have been characterized by GC–MS methods. The major compounds were in order: triterpenoids > n-alkenes > n-alkanes > n-alkanoic acids > long chain wax esters > n-alkanols > methyl n-alkanoates. The predominant triterpenoids were α- and β-amyrins, α- and β-amyryl acetates, followed by α- and β-lupenyl acetates. n-Alkenes and n-alkanes ranged from C_{25} to C_{35} and C_{21} to C_{31}, with C_{max} at 33 and 27, respectively. Long chain wax esters were also present in significant amounts. Methyl n-alkanoates were relatively minor components in these samples. The sources of the major triterpenoids are from the regional Acacia waxes and gums, whereas the sources of n-alkenes and n-alkanes are likely from bee wax.

Acknowledgement

The project was financially supported by King Saud University, Vice Deanship of Research Chairs.

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