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# Variability of Hydrocarbon and Fatty Acid Components in Cultures of the Filamentous Cyanobacterium *Scytonema* sp. Isolated from Microbial Community "Black Cover" of Limestone Walls in Jerusalem

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**Abstract**—The hydrocarbon and lipid components of four strains of the filamentous cyanobacterium *Scytonema* sp. isolated from microbial community "Black Cover" of limestone walls in Jerusalem were identified by gas chromatography–mass spectrometry using serially coupled capillary columns. The dominant compounds were: 1-heptadecyne (1.5-8%), hexadecanoic acid (14-36%), (*Z*,*Z*)-9,12-octadecadienoic acid (12-30%), (*Z*,*Z*,Z)-9,12,15-octadecatrienoic acid (6-12%), *n*-heptadecane (4-16%), and 1-heptadecene (1.5-8%). In addition to unsaturated alkanes and fatty acids, the very long-chain ($C_{30}$-$C_{32}$) hydrocarbons, squalene (2.4-3.0%), and branched 4,8,12-trimethyl-$C_{13:0}$ acid were also isolated. Two major hydrocarbons were detected in the cyanobacteria species using GC-MS and $^{13}$C-NMR.

*Key words*: hydrocarbons, fatty acids, *Scytonema* sp., cyanobacterial biofilm, GC-MS, $^{13}$C-NMR

Microbial biofilms are extreme microbial ecosystems and consist of a complex community of bacteria, cyanobacteria, grazing protozoa, fungi, microalgae, and lichens [1, 2]. Jerusalem is about 5,000 years, and for more that 3,000 years hard Turonian massive limestone rocks have been the main source of building stones [3]. The black-colored biofilms "Black Cover" consisting of euendolithic coccoid cyanobacteria and cyanophilous lichens occupy most of the surface walls [4]. They were classified and studied for the principal biodeterioration processes in the facing of old buildings and hard limestone walls in Jerusalem [4, 5]. According to Dor [5] only two species among the genus *Scytonema* were identified as living on rocks and in the soil in Jerusalem and surrounding areas: *Scytonema drilosiphon* (Kütz.) and *S. hofmanii* Ag.

Cyanobacteria are common inhabitants of pristine terrestrial and aquatic environments on a global scale and natural populations of the organisms can occur away from human influence [6]. Filamentous cyanobacteria belonging to the genus *Scytonema* are widespread in nature and found in cyanobacterial biofilms "Black Cover" on rocks, stones, concrete walls, and in drains in the subtropical part of Taiwan [7]. Ethanolic extracts of *Scytonema* biofilm as reported [7] show the strongest inhibitory activity against α-glucosidase.

*Scytonema* species produce many different neuro-, hepato-, and cytotoxins [8-10], and these toxins are potential risks to human health [6, 11, 12]. Cyanobacterial toxins include the secondary amine alkaloid neurotoxins, anatoxin-A and homoanatoxin-A, which are postsynaptic cholinergic nicotine agonists and neuromuscular-blocking agents [6, 13]. Hepatotoxins include cyclic peptides, a cyclic guanidine alkaloid, and cylindrospermopsin [6, 11, 12] which act on humans. Symptoms of poisoning include diarrhea, vomiting, pallor, weakness, recumbence, and labored breathing. Despite the importance of cyanobacteria as possible sources of different alkanes in terrestrial and aquatic environments, analysis of the lipid contents of individual cultured species are relatively few [13-15], although there are have been biochemical studies of various species of living and fossil cyanobacterial mats [16-22].

Information about hydrocarbon compounds isolated from terrestrial cyanobacterial biofilms is limited [23]. This report continues our previous studies of cyanobacterial compounds [24-28] and describes the hydrocarbon and fatty acid compositions of the filamentous cyanobacterium *Scytonema* sp. identified by GC-MS and $^{13}$C-NMR.

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## MATERIALS AND METHODS

**Cyanobacterial samples.** Four strains of the filamentous cyanobacteria *Scytonema* sp. (Nostocales, Scytonemataceae) were isolated from a microbial community of Jerusalem building limestones: strain EK1 (isolated from the microbial community of building limestones around Ein Ha-Kerem), strain GRC (Givat Ram Campus, Hebrew University), strain OCJ (Jerusalem Old City), and strain GIA (Gilo Aleph). Chasmoendolithic cyanobacteria were collected together with a small amount of rock during the summer of 1998. All isolated strains of *Scytonema* sp. were cultivated in the Laboratory of Hydrobiology (Division of Environmental Sciences). It was cultured on dry agar and then on medium BG-11 at 27°C as described previously [29], under illumination of cool white fluorescent lights of 30 $\mu E \cdot m^{-2} \cdot sec^{-1}$ at the surface of the flasks for nine months. Strains GIA and GRC of *Scytonema* sp. were additionally cultivated at 35°C using a monochromatic UV-B lamp (312 nm, Vilber Laurmat, T 15M 7C) at 16 $W/m^2$ in a special UV protected box for two and four months, respectively.

The cells were harvested by centrifugation, lyophilized, and stored in a deep freezer. The lyophilized cells of four strains of cyanobacterium *Scytonema* sp. were isolated, classified, and cultivated by Prof. I. Dor. Voucher specimens of all strains of the cyanobacterium *Scytonema* sp. are on file at the Laboratory of Hydrobiology (Division of Environmental Sciences).

**Extraction of hydrocarbons and fatty acids.** Lyophilized cells of each strain were added to 100 ml mixture of methanol–$H_2O$–HCl (90 : 9 : 3 v/v) warmed to 55°C for 6 h. After cooling to 10°C temperature, 150 ml of a mixture of $H_2O$–pentane (100 : 50 v/v) was added. The layers were separated. The pentane layer was concentrated to dryness *in vacuo* at 5°C. The residue was extracted with 150 ml of dichloromethane. Pentane and dichloromethane extracts were combined and dried *in vacuo* at 10°C under reduced pressure, and then dissolved in 2.5 ml of a cold mixture of pentane–dichloromethane (1 : 1 v/v), which was used for GC-MS analysis.

**Gas chromatographic-mass spectrometry analysis.** A Hewlett-Packard 5890 (series II) gas chromatograph, modified for a glass capillary column was used coupled to a HP GC-mass selective detector (5971B MSD). Hydrocarbons and methyl esters of fatty acids were analyzed by gas chromatography on serially coupled capillary columns as described previously [28], using: HP-5, 10 m; ID, 0.32 mm; film thickness, 0.25 μm; coupled with a second capillary column RTX-1701 (Restek, USA), 30 m; ID, 0.32 mm; film thickness, 0.25 μm; and coupled with a third capillary column HP-FFAP, 30 m; ID, 0.32 mm; film thickness, 0.25 μm. The GC oven was programmed as follows: 40°C 2 min, 2°C/min to 300°C, 20 min at 300°C. The injector temperature was kept at 180°C (splitless). The flow rate of the carrier gas (helium) was 25 ml/sec. The MS detector was operated at 194°C, with an ionization energy of 70 eV. The scan range was from 30 to 650 m/z and the scan rate 0.9 scan/sec. Solvent delay was 10 min. Hydrocarbons and methyl esters of fatty acids were identified by comparison to the masses in a mass spectral library search (Wiley, 7th Edition). Heptadecane, 1-heptadecene standards were obtained from Sigma Israel, Ltd.

**HPLC analysis.** High performance liquid chromatography separations were performed on an ISCO HPLC system (model 2350 pump and model 2360 gradient programmer). A C18 reversed phase column (7.8 × 250 mm, Supelco, USA) was employed. A linear gradient of 20% $H_2O$ and 80% acetonitrile to 1% water and 99% acetonitrile over 26 min, flow rate 2 ml/min was used to separate all the compounds in the crude extract. Hydrocarbons were detected by UV absorption at 208 nm.

**$^{13}$C-NMR analysis.** NMR spectra were recorded in $CDCl_3$, on a Bruker DRX-250 spectrometer at 37.5 MHz. All the chemical shifts were recorded relative to $CDCl_3$. All solvents used were spectral grade.

## RESULTS AND DISCUSSION

The photosynthetic organisms of the epilithic microbial community which include microalgae and cyanobacteria are in many ways the most significant mediators of limestone deterioration in that they are responsible for supporting the activities of potential deteriogens like heterotrophic fungi, lichens, and bacteria [1, 2, 30, 31].

Twenty five hydrocarbons and fatty acids were identified in the total lipid extracts of four strains of *Scytonema* sp. The distribution of hydrocarbons and fatty acids was determined in *Scytonema* strains (Table 1). Figure 1 demonstrates the separation of hydrocarbons and methyl ester fatty acids in two strains. GC-MS analysis of four strains showed that their fatty acids are dominated by saturated ($C_{16:0}$), branched (4,8,12-trimethyl-$C_{13:0}$), and unsaturated fatty acids such as (*Z*,*Z*)-9,12-$C_{18:2}$ and (*Z*,*Z*,*Z*)-9,12,15-$C_{18:3}$. Fatty acid composition of *Scytonema* sp. is in good agreement with the fatty acid content of other cyanobacteria species published previously ([32] and cited herein).

Separation by serially coupled capillary columns of heptadecane family is presented in Fig. 2. Two major hydrocarbons were isolated by HPLC. The $^{13}$C-NMR analysis one of two major hydrocarbons (Figs. 1b and 2) from strain GRC had the following peaks: (δ) C-1 (68.09), C-2 (84.78), C-3 (18.49), C-4 (28.55), C-5 and C-13 (31.93), C-6 (29.19), C-8-C-13 (29.74), C-14 (29.45), C-16 (22.75), and C-17 (14.09). From the data we concluded that the major hydrocarbon was 1-heptadecyne. The $^{13}$C-NMR data for 1-heptadecene is: (δ) C-1 (114.14), C-2 (139.20), C-3 and C-5 (33.93), C-4

**Table 1.** Hydrocarbon and fatty acid compositions of four strains of the cyanobacterium *Scytonema* sp. (wt. % of total volatile compounds)

| Peak number | Compound | RT | MW | EK1 | GRC | OCJ | GIA |
|---|---|---|---|---|---|---|---|
| 1 | heptadecane | 63.952 | 240 | 1.240 | 16.042 | 5.743 | 7.123 |
| 2 | 8-heptadecene | 64.201 | 238 | 0.313 | 2.790 | 0.170 | 0.835 |
| 3 | 1-heptadecene | 64.495 | 238 | 2.439 | 9.208 | 3.302 | 5.914 |
| 4 | 1-heptadecyne | 64.977 | 236 | 1.594 | 8.056 | 2.013 | 6.097 |
| 5 | 5-octadecene | 69.504 | 252 | 0.994 | 0.825 | 1.020 | 0.632 |
| 6 | tetradecanoic acid, methyl ester | 69.897 | 242 | 3.139 | 0.261 | 3.158 | 1.496 |
| 7 | 3-octadecene | 70.221 | 252 | 0.228 | 0.104 | 0.607 | 0.524 |
| 8 | 4,8,12-trimethyltridecanoic acid, methyl ester | 73.168 | 270 | 2.348 | 8.344 | 3.203 | 6.011 |
| 9 | 9-methyltetradecanoic acid, methyl ester | 74.517 | 256 | 0.476 | 3.662 | 0.495 | 0.327 |
| 10 | 12-methyltetradecanoic acid, methyl ester | 75.019 | 256 | 0.115 | 0.326 | 0.131 | 0.342 |
| 11 | pentadecanoic acid, methyl ester | 75.655 | 256 | 0.828 | 3.073 | 2.273 | 1.417 |
| 12 | 9-nonadecene | 77.369 | 266 | 0.159 | 2.617 | 0.578 | 2.019 |
| 13 | (*Z*)-7-hexadecenoic acid, methyl ester | 80.517 | 268 | 2.040 | 1.296 | 2.600 | 2.238 |
| 14 | hexadecanoic acid, methyl ester | 80.868 | 270 | 36.027 | 14.714 | 34.455 | 14.387 |
| 15 | heptadecanoic acid, methyl ester | 84.648 | 284 | 1.242 | 0.136 | 1.505 | 0.594 |
| 16 | octadecanoic acid, methyl ester | 88.371 | 298 | 1.139 | 0.543 | 1.083 | 0.748 |
| 17 | (*Z*)-9-octadecenoic acid, methyl ester | 90.022 | 296 | 8.774 | 2.278 | 8.910 | 4.238 |
| 18 | (*Z*,*Z*)-9,12-octadecadienoic acid, methyl ester | 90.550 | 294 | 18.594 | 12.173 | 17.034 | 30.050 |
| 19 | (*Z*,*Z*,*Z*)-9,12,15-octadecatrienoic acid, methyl ester | 91.758 | 292 | 10.799 | 9.528 | 5.978 | 12.014 |
| 20 | squalene | 114.298 | 410 | 3.012 | 2.498 | 2.747 | 3.023 |
| 21 | triacontane (C30) | 111.753 | 422 | 0.404 | 0.355 | 0.905 | 0.743 |
| 22 | hentriacontane (C31) | 115.184 | 436 | 0.486 | 0.376 | 1.008 | 0.621 |
| 23 | dotriacontane (C32) | 118.487 | 450 | 0.310 | 0.293 | 0.537 | 0.315 |
| 24 | tritriacontane (C33) | 121.761 | 464 | 0.300 | 0.502 | 0.428 | 0.292 |

Note: RT, retention time (min); MW, molecular weight. Strains: EK1 (isolated from microbial community of building limestone of Ein Ha-Kerem); GRC (from Givat Ram Campus, Hebrew University); OCJ (from Jerusalem Old City); GIA (from Gilo Aleph).

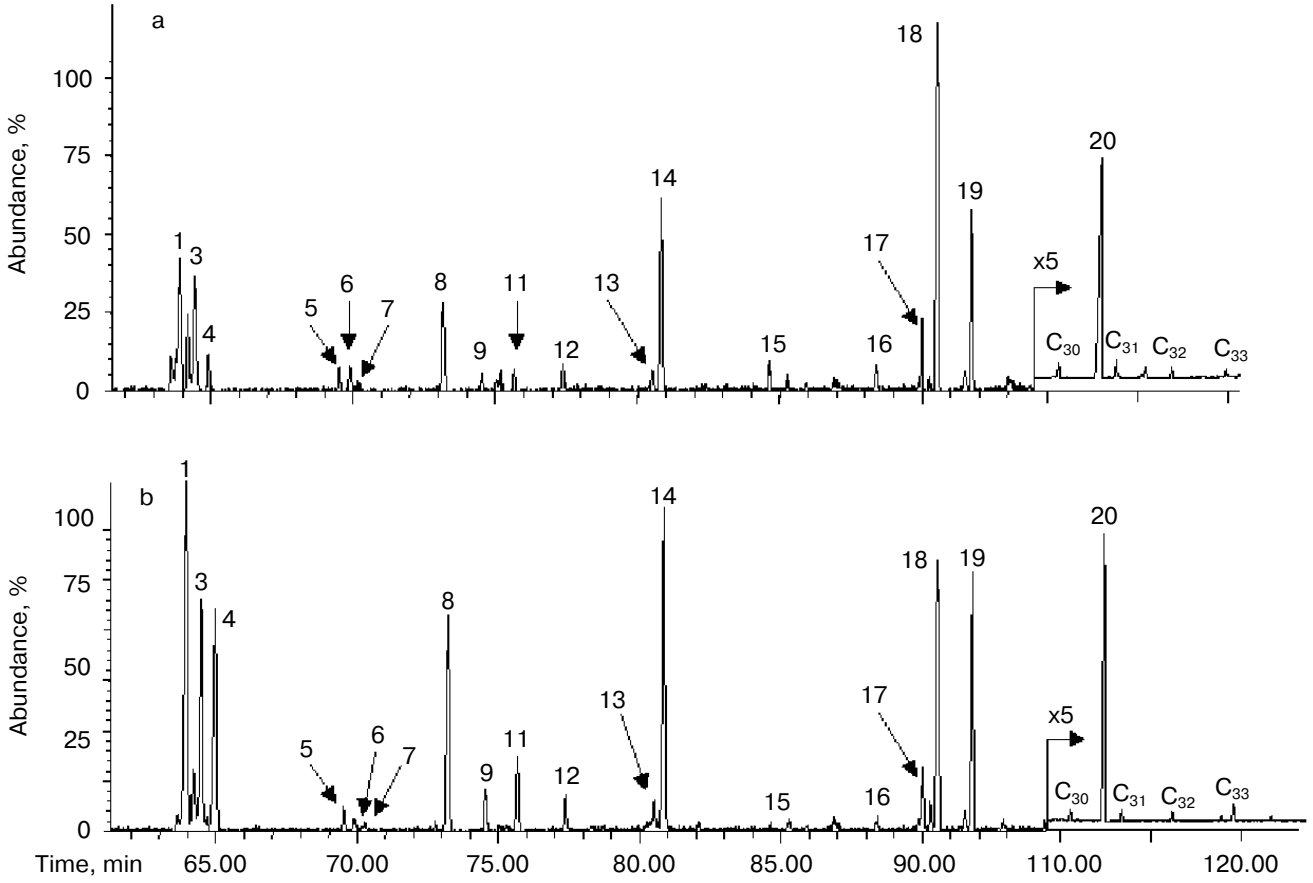


**Fig. 1.** GC-MS chromatogram of hydrocarbons and fatty acids of the cyanobacterium *Scytonema* sp. Separation of hydrocarbons and fatty acid methyl esters by gas chromatography using serially coupled capillary columns with stationary phases of different polarity. Full chromatogram (run up to 130 min). Parts of chromatogram running from 60 to 120 min. Strain GIA (a) and strain GRC (b) were additionally cultivated under a monochromatic UV-B lamp. Peak numbers on the chromatogram are given in Table 1.

(29.08), C-6 (29.63), C-7-C-13 (29.76), C-14 (29.44), C-15 (32.04), C-16 (22.75), and C-17 (14.11). Mass spectra of natural heptadecane family compounds are given in Fig. 3. This is the first time that high amounts of 1-heptadecyne from cyanobacterial species were identified. However, 5-hexadecyne occurred in *Microcystis aeroginosa* [33] when the cyanobacterium was exposed to direct sunlight for 9 h in media containing either low or high concentration of iron, and not found when exposure was less than 9 h. In addition, it was observed that the amount of *n*-heptadecane and *n*-octadecane increased threefold after 9 h exposure [33]. Our results confirm that two cultivated strains, GRC and GIA, of *Scytonema* sp. under UV-B irradiation also have increased contents of *n*-heptadecane, 1-heptadecene, and 1-heptadecyne, compared to the strains EK1 and OCJ used as standard cultures grown without additional exposure to UV-B irradiation (Table 1).

Most cyanobacterial species contain small amounts of short-chain hydrocarbons in the range $C_{13}$-$C_{21}$, often with either *n*-$C_{17}$ or $C_{17:1}$ predominating [32, 34, 35]. Nevertheless, some cyanobacterial species produce very long-chain and highly branched hydrocarbons $C_{19}$-$C_{29}$ [16-20]. Cyanobacteria produced hydrocarbons *via* decarboxylation of fatty acids. Thus, Han et al. [36] demonstrated that *Nostoc muscorum* culture enzymatically decarboxylated stearate to *n*-heptadecane. Also, McInnes et al. [37] described the pathway of *n*-heptadecane formation, and synthesized it using *Anacystis nidulans* culture. The complex hydrocarbons and volatile compounds produced by cultured cyanobacterial photobiont *Nostoc* sp. isolated from lichen *Collema* sp. was reported [28]. More than 130 volatile compounds including cyclopentane (11 isomers), cyclohexane (41 isomers), short-chain hydrocarbons ($C_7$-$C_{14}$), and also long-chain hydrocarbons ($C_{15}$-$C_{30}$) were identified by GC-MS. In

**Table 2.** Comparative distribution of heptadecane family and other hydrocarbons in cyanobacterial species (wt. %)

| Species | 15 : 0 | 16 : 0 | 17 : 0 | 17 : 1 | x-Me-17 : 0 | 18 : 0 | 19 : 0 | 19 : 1 | 19 : 2 | Squalene | References |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *Agmenellum quadruplicum* | | | | | | | | 95.0 | 4.0 | | [38] |
| *Anabaena cylindria* | | | 20.0 | 70.0 | 10.0 | | | | | | [39] |
| *Anabaena variabilis* | | | 15.0 | | 84.0 | | | | | | [40] |
| *Anacystis cyanea* | | | 87.0 | | | 13.0 | | | | | [34] |
| *Anacystis montana* | | | | 11.5 | | | | | | | [34, 41] |
| *Anacystis montana* | 93.0 | 0.9 | 0.2 | 0.3 | | | | | | | [42] |
| *Anacystis nidulans* | 23.3 | 7.9 | 43.8 | 20.0 | | 2.5 | | | | | [34] |
| *Anacystis nidulans* | 45.0 | 5.0 | 45.0 | 5.0 | | | | | | | [39] |
| *Anacystis nidulans* | 20.6 | 2.5 | 73.7 | 3.0 | 0.3 | | | | | | [43] |
| *Anacystis nidulas* | 21.0 | 5.0 | 68.0 | 5.0 | | | | | | | [38] |
| *Calothrix scopulorum* | | | 0.7 | | 99.0 | | | | | | [16] |
| *Calothrix* sp. | | | 47.4 | | | 30.9 | 10.4 | | | | [43] |
| *Chlorogloea fritschii* | | | 87.3 | | 12.3 | | | | | | [34] |
| *Chroococcus turgidus* | | | 32.0 | | 22.0 | | | | | | [42] |
| *Coccochloris elabens* | | | | 1.0 | | | | 85.0 | 13.0 | | [38] |
| *Limnothrix redekei* | | | + | + | + | | | | | | [44] |
| *Lyngbya aestuarii* | 2.0 | 6.0 | 35.0 | | 38.0 | 1.0 | | | | | [34] |
| *Lyngbya lagerhaimii* | 1.0 | 4.0 | 86.0 | 1.0 | 8.0 | | | | | | [38] |
| *Microcoleus chthonoplastes* | 2.0 | 4.0 | 92.0 | | | | | | | | [38] |
| *Microcoleus lyngbyaceus* | 3.7 | 3.8 | 84.0 | 3.1 | | | | | | | [43] |
| *Microcoleus lyngbyaceus* | 9.0 | 20.0 | 70.0 | 1.0 | | | | | | | [34] |
| *Microcoleus vaginatus* | | | 12.0 | 0.2 | 10.1 | 0.4 | | | | 0.4 | [25] |
| *Microcoleus vaginatus* | | | 24.6 | 0.6 | 19.5 | | | | | 1.0 | [26] |
| *Microcoleus vaginatus* | | | 14.4 | 23.5 | 0.3 | | | | | 2.2 | [26] |
| *Microcoleus vaginatus* | | | 7.5 | 15.5 | 1.5 | | | | | 1.6 | [26] |
| *Microcoleus vaginatus* | | | 23.0 | 3.4 | 10.6 | | | | | 1.2 | [26] |
| *Microcystis aeruginosa* | 18.0 | 5.0 | 69.0 | | | 6.0 | 2.0 | | | | [33] |
| *Nostoc commune* | 1.6 | 1.5 | 50.4 | 3.2 | 24.2 | | | | | | [43] |
| *Nostoc endophytum* | | | 80.0 | | 20.0 | | | | | | [39] |
| *Nostoc muscorum* | | | 83.0 | | 16.0 | | | | | | [36] |
| *Nostoc* sp.[a] | | | 2.4 | | 3.5 | | | | | | [28] |
| *Oscillatoria* f. *granulata* | | | + | | | | | | | | [45] |
| *Osillatoria williamsii* | 9.0 | | 91.0 | | | | | | | | [38] |
| *Osillatoria woronichinii* | 93.0 | 2.0 | 2.0 | | | | | | | | [46, 47] |
| *Phormidium luridum* | | | 96.0 | | 4.0 | | | | | | [40, 47] |
| *Planktothrix agardhii* | | | + | + | + | | | | | | [44] |
| *Planktothrix mougeotii* | | | + | + | + | | | | | | [44] |
| *Planktothrix suspense* | | | + | + | + | | | | | | [44] |
| *Planktothrix rubescens* | | | + | + | + | | | | | | [44] |
| *Plectonema terebrans* | 6.0 | 3.0 | 90.0 | 1.0 | | | | | | | [38] |
| *Prochloron* sp. | 6.3 | 1.2 | 81.7 | | | | | | | 10.8 | [48] |
| *Scytonema* sp.[b] | | | 31.3 | 37.0 | | | | | | 11.4 | * |
| *Scytonema* sp.[c] | | | 37.0 | 26.3 | | | | | | 5.5 | * |
| *Scytonema* sp.[d] | | | 22.9 | 30.1 | | | | | | 10.8 | * |
| *Scytonema* sp.[e] | | | 22.3 | 33.5 | | | | | | 9.4 | * |
| *Spirulina platensis* | 2.7 | 3.7 | 66.9 | 3.4 | | 0.6 | 0.9 | | | | [43] |
| *Spirulina platensis* | 3.7 | 3.8 | 84.0 | 3.1 | | 0.5 | 1.3 | | | | [43] |
| *Spirulina platensis* | 2.9 | 2.8 | 71.7 | 5.2 | | 0.7 | 1.9 | | | | [43] |
| *Spirulina platensis* | 10.0 | 20.0 | 70.0 | 1.0 | | | | | | | [34] |
| *Spirulina platensis* | | | 3.4 | 84.9 | | | | | | | [49] |
| *Synechococcus bacillaris* | 4.0 | 2.0 | 38.0 | 53.0 | | | | | | | [47] |
| *Synechococcus* sp. | | | | | | | | 17.8 | 82.1 | | [50] |
| *Synechocystis* UTEX 2470 | | | 69.0 | 13.0 | | 19.0 | | | | | [15] |
| *Trichodesmium erythraeum* | | 2.0 | 95.0 | | | | | | | | [38] |

[a] Symbiotic cyanobacterium isolated from lichen: 24:0 (11.9%); 26:0 (11.5%); 25:0 (11.1%); 27:0 (10.6%).
[b] 1-Heptadecyne, 17:1A (6.1%).
[c] 1-Heptadecyne, 17:1A (23.2%).
[d] 1-Heptadecyne, 17:1A (12.0%).
[e] 1-Heptadecyne, 17:1A (19.1%).
+, detected by GC-MS, the amounts have not been calculated.

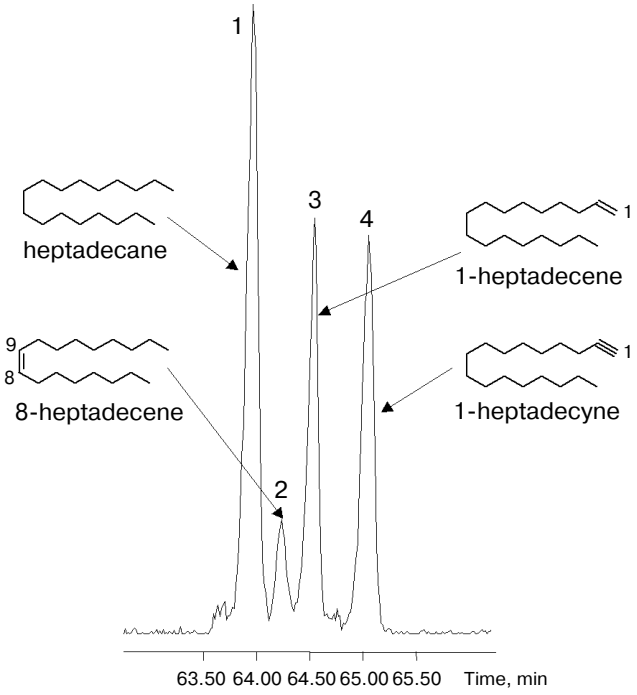


**Fig. 2.** GC-MS chromatogram separation of major *n*-hydrocarbons of the filamentous cyanobacterium *Scytonema* sp. (strain GRC) isolated from microbial community of limestone walls in Jerusalem. Parts of chromatogram running from 63 to 65 min.

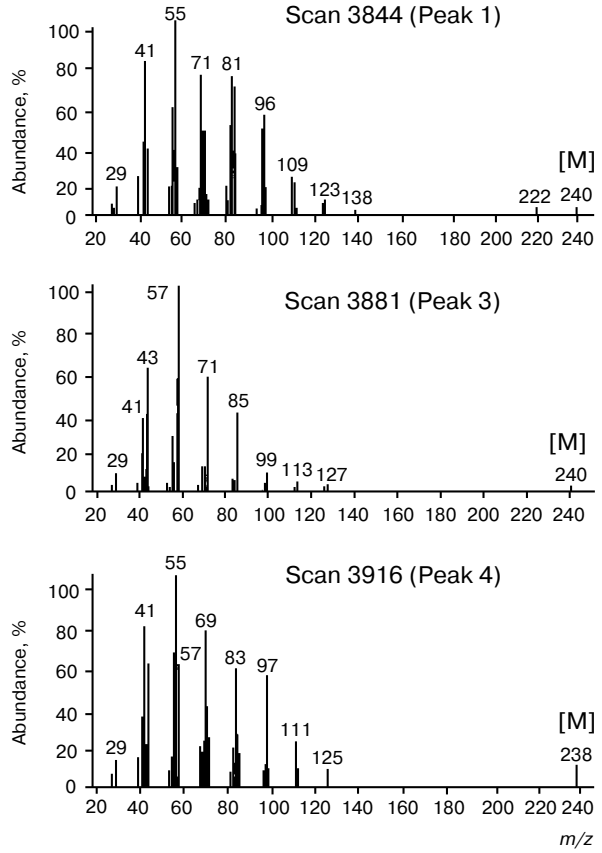


**Fig. 3.** Mass spectra of the major natural hydrocarbons isolated by HPLC from the strain GRC of *Scytonema* sp. Peaks: *1*) heptadecane; *3*) 1-heptadecene; *4*) 1-heptadecyne.

this study the very long-chain hydrocarbons $C_{30}$-$C_{32}$ with $C_{17}$ family predominating were identified. Table 2 illustrates the comparative distribution of hydrocarbon types identified from cyanobacterial species. The isoprenoids phytane, pristane, and also squalene have been reported frequently, suggesting that they are normal constituents of cyanobacteria species [35].

In conclusion, alterations in the production of *n*-heptadecane, 1-heptadecene, and also 1-heptadecyne were observed in cultured cyanobacterium *Scytonema* sp. exposed to UV-B irradiation. Furthermore, the concentration of some components increased or decreased, for instance, 4,8,12-trimethyl-$C_{13:0}$, and hexadecanoic, (*Z*)-9-octadecenoic acids, respectively.

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