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Short communication

Separation and identification of hydrocarbons and other volatile compounds from cultured blue-green alga *Nostoc* sp. by gas chromatography–mass spectrometry using serially coupled capillary columns with consecutive nonpolar and semipolar stationary phases[☆]

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Abstract

The complex hydrocarbons and volatile compounds produced by cultured blue-green alga *Nostoc* sp. were separated by serially coupled capillary columns with consecutive nonpolar and semipolar stationary phases. More than 130 metabolites including, cyclohexane, cyclopentane, normal saturated hydrocarbons (C₇–C₃₀), fatty acids and benzene derivatives were identified by GC–MS. The most abundant family of hydrocarbons identified were derivatives of cyclohexane (41) and cyclopentane (11). Most of these compounds have not been reported previously in blue-green algae studies. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: *Nostoc*; Blue-green alga; Column coupling; Hydrocarbons; Volatile compounds; Fatty acids; Cyclohexane; Cyclopentane

1. Introduction

Analysis by gas chromatography–mass spectrometry (GC–MS) is essential for identification of natural organic compounds isolated from blue-green

algae and other marine, freshwater and symbiotic microalgae, sediments and crude oil. Over the past decade, hundreds of compounds have been identified in crude oils using GC–MS and other techniques. Some of these compounds have distinctive chemical structures which are closely related to the organic compounds produced by cyanobacteria and microalgae. These “biomarker” compounds can also be valuable for identifying sources of petroleum contamination in the environment, for recognising natural constituents and as tracers for physical and biological processes that affect hydrocarbon distribu-

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tions such as biodegradation, biotransformation, dissolution, evaporation, and uptake by biota [1–5].

Many papers have been published recently on the subject of optimizing capillary GC separation through the use of serially coupled columns [6–11]. However, the use of these approaches have not been fully investigated, for instance, in the separation of natural compounds from blue-green algae and/or microalgae.

Species of the genus *Nostoc* are the most widespread of all nitrogen-fixing cyanobacteria. *Nostoc* is a genus of filamentous cyanobacteria that can form microscopic or macroscopic colonies and is common in both aquatic and terrestrial habitats [12,13].

The taxon is ancient and was found also in late Precambrian fossils [14]. Fossil cyanobacteria showing considerable morphological diversification have been found dating back at least 2.5 billion years and filamentous stromatolitic microfossils are known as far back as 3.5 billion years [15–17].

Nostoc-containing lichens are the dominant primary producers in some landscapes [18]. The *Nostoc* cyanobiont exists in three genera of lichens: *Nephroma*, *Peltigera* and *Collema* [19–21]. Little information is available about metabolites produced by *Nostoc* lichen — cyanobiont [22].

In this paper we describe the application of serially coupled capillary columns with consecutive nonpolar and semipolar stationary phase coating for the separation of complex hydrocarbons and other metabolites produced by blue-green alga *Nostoc* sp.

2. Experimental

2.1. Plant material

Collema sp. lichen was collected from rock at the Negev Desert in summer 1996. Photobiont stock was isolated and cultivated according to Refs. [23,24]. All cultures were inoculated by a single step transfer of the cells from the stock culture. Blue-green alga *Nostoc* sp. was isolated from lichen symbionts on a BG-11 agar medium (for details see Ref. [23]). Agar plates were incubated at 24°C for 4 months under continuous illumination with 1.0 lux fluorescent light (Leuci, T12, 2600, Mexico).

2.2. Extraction procedure

Fresh culture of blue-green alga *Nostoc* sp. (960 mg) was mixed with 10 ml MeOH–water (90:10, v/v) and heated for 50 min at 55°C. After cooling the solution to room temperature, 20 ml of a cold mixture of *n*-C₅H₁₂–water (3:5, v/v) was added. The mixture was shaken for 15 min, and then cooled under ice. The C₅H₁₂ and methanol–water phases were separated. The methanol–water phase was extracted three times by CH₂Cl₂ (10 ml×3), and the phases were separated. The pentane and dichloromethane extracts were washed three times with 0.9% KCl (10 ml×3). Then the pentane and dichloromethane extracts were combined, dried over anhydrous sodium sulphate, and evaporated under nitrogen at 10°C. The remaining organic matter was resolved in 0.5 ml CH₂Cl₂ and kept at –10°C for GC–MS analysis.

2.3. Preparation of fatty acid derivatives

The free carboxylic and fatty acids in the MeOH–water phase (20 ml) were converted to methyl esters by reaction with 5% methanolic HCl (20 ml) (overnight, 80°C) [25]. After methylation, the mixture was cooled to room temperature and extracted with pentane (10 ml×3). The pentane-extract was washed three times with 0.9% KCl (10 ml×3). The organic phase was dried over anhydrous sodium sulphate, evaporated under nitrogen, resolved in 0.5 ml CH₂Cl₂, combined with pentane–CH₂Cl₂ extracts and kept at –10°C before analysis by GC–MS.

2.4. Apparatus and chromatographic conditions

Separation of hydrocarbons, volatile compounds and fatty acid methyl esters, was carried out with a gas chromatograph Hewlett-Packard 5890 (series II) (Palo Alto, CA, USA), equipped with a 5971B MSD mass selective detector. GC–MS analyses were done with an ionization energy of 70 eV.

Hydrocarbons and volatile metabolites were analyzed by GC using coupled two capillary columns with different stationary phases: RTX-1 column 30 m×0.32 mm ID, film thickness 1 μm, (Restek) coupled with a second capillary column RTX-1701 30 m×0.32 mm ID, 0.25 μm film thickness (Restek).

Table 1
Hydrocarbons and metabolites identified from cultivated blue-green alga *Nostoc* sp. lichen^a

Peak	Compound	RT (min)	M.W.	Abundance (%)
1	Cyclopentane, 1,2-dimethyl-	12.067	98	0.048
2	Heptane (C ₇)	12.642	100	2.241
3	Cyclohexane, methyl	13.991	98	0.469
4	Heptane, 2-methyl-	14.519	114	0.040
5	Cyclopentane, ethyl-	14.667	98	0.113
6	3-Pentanol, 3-methyl-	15.531	102	0.088
7	1-Heptene, 3-methyl-	15.635	112	0.050
8	Hexane, 2,3-dimethyl-	16.463	114	0.051
9	Pentane, 3-ethyl-2-methyl-	16.589	114	0.062
10	Heptane, 4-methyl-	16.839	114	0.116
11	Heptane, 3-methyl-	16.941	114	0.037
12	Hexane, 2,4-dimethyl-	16.961	114	0.047
13	3-Hexanone	17.424	100	0.234
14	2-Hexanone	17.713	100	0.334
15	2-Hexanone, 4-methyl-	17.730	114	0.289
16	Cyclopentanol, 1-methyl-	18.201	100	0.274
17	3-Hexanol	18.605	102	0.311
18	2-Hexanol	18.954	102	0.368
19	Cyclohexane, 1,4-dimethyl-	19.217	112	0.052
20	Octane (C ₈)	19.345	114	0.119
21	Cyclohexane, 1,3-dimethyl-, <i>trans</i>	19.757	112	0.140
22	Cyclohexane, 1,2-dimethyl-, <i>cis</i>	21.638	112	0.210
23	Cyclohexane, 1 α ,3 α ,5 α -trimethyl-	21.889	126	0.104
24	Cyclohexane, ethyl-	21.996	112	0.144
25	Heptane, 2,5-dimethyl-	22.255	128	0.106
26	Cyclohexane, 1,1,3-trimethyl-	22.382	126	0.131
27	Cyclohexane, 1 α ,3 α ,5 α -trimethyl-	23.621	126	0.464
28	Cyclohexane, 1,2,4-trimethyl-	23.955	126	0.218
29	Octane, 4-methyl-	24.629	128	0.603
30	Octane, 3-methyl-	25.237	128	0.558
31	Cyclohexane, 1 α ,2 β ,4 β -trimethyl-	25.565	126	0.266
32	Cyclohexane, 1 α ,3 α ,5 β -trimethyl-	25.665	126	0.244
33	Cyclohexane, 1 α ,2 α ,3 β -trimethyl-	25.927	126	0.371
34	Cyclohexane, 1 α ,2 β ,3 α -trimethyl-	26.163	126	0.314
35	Cyclopentane, 1-methyl-2-propyl-	26.418	126	0.202
36	Cyclohexane, 1-ethyl-4-methyl-, <i>trans</i>	26.598	126	0.992
37	Cyclohexane, 1-ethyl-4-methyl-, <i>cis</i>	26.793	126	0.459
38	Cyclopentanol, 3,?-dimethyl-	27.002	114	0.168
39	Nonane (C ₉)	27.658	128	2.513
40	Cyclohexane, 1,1,2-trimethyl-	27.845	126	0.068
41	2-Pentene, 3-ethyl, 4,4-dimethyl-	27.973	126	0.315
42	Cyclopentane, 1,1,3,3-tetramethyl-	28.115	126	0.142
43	Cyclohexane, 1-ethyl-4-methyl-, <i>cis</i>	28.282	126	0.989
44	Heptane, 4-ethyl-	28.743	128	0.079
45	Pentalene, octahydro-2-methyl-	29.275	124	0.778
46	Cyclohexane, (1-methylethyl)-	29.486	126	0.085
47	Cyclohexane, 1-ethyl-2-methyl-, <i>trans</i>	29.510	126	0.059
48	Pentane, 2,2,3,3-tetramethyl-	29.810	128	0.418
49	Cyclohexane, 1-ethyl-2-methyl-, <i>cis</i>	30.125	126	0.217
50	Cyclohexane, propyl-	30.220	126	1.383
51	Cyclopentane, butyl-	30.477	126	0.261
52	Octane, 3,6-dimethyl-	30.620	142	0.953

(continued on next page)

Table 1 (continued)

Peak	Compound	RT (min)	M.W.	Abundance (%)
53	Heptane, 5-ethyl-2-methyl-	30.788	142	0.172
54	Cyclohexane, 1-ethyl-2,3-dimethyl-	31.113	140	0.632
55	Heptane, 3-ethyl-2-methyl-	31.307	142	0.750
56	5(<i>E</i>)-Decene-	31.617	140	0.258
57	Cyclopentane, (2-methylbutyl)-	31.643	140	0.238
58	2-Octene, 2,6-dimethyl-	31.912	140	0.265
59	Cyclohexane, 2-ethyl-1,3-dimethyl-	32.200	140	0.151
60	Octane, 2,3-dimethyl-	32.384	142	0.630
61	Cyclohexane, 1,1,2,3-tetramethyl-	32.630	140	0.241
62	Nonane, 5-methyl-	32.823	142	0.454
63	Nonane, 4-methyl-	32.974	142	1.322
64	Nonane, 3-methyl-	33.203	142	0.785
65	Heptane, 2,5,5-trimethyl-	33.517	142	0.203
66	Nonane, 2-methyl-	33.787	142	0.839
67	Cyclohexane, 1-methyl-4-(1-methylethyl)-	34.028	140	0.579
68	Cyclohexane, 1-methyl-3-(1-methylethyl)-	34.310	140	0.705
69	Cyclohexane, diethyl-	34.560	140	0.252
70	Cyclohexane, 1-ethyl-1,3-dimethyl-, <i>trans</i>	34.578	140	0.239
71	Cyclohexane, 1-methyl-3-propyl-	34.899	140	1.155
72	Cyclohexane, 1-methyl-2-propyl-	35.160	140	0.818
73	Cyclohexane, 1-ethyl-2,4-dimethyl-	35.384	140	0.378
74	Cyclopentane, pentyl-	35.743	140	0.118
75	Cyclohexane, 1-ethyl-1,4-dimethyl-, <i>trans</i>	35.914	140	0.379
76	Decane (C ₁₀)	36.297	142	4.369
77	Cyclooctane, ethyl-	37.010	140	0.107
78	Cyclohexane, butylidene-	37.151	138	0.113
79	Cyclohexane, 1-ethyl-2,3-dimethyl-	37.376	140	0.090
80	Nonane, 2,5-dimethyl-	37.523	156	0.488
81	Cyclopentane, 1-methyl-1-(2-methyl-2-propenyl)-	37.782	138	0.207
82	Hexane, 2,2,3,3-tetramethyl-	38.002	142	0.145
83	Nonane, 2,6-dimethyl-	38.266	156	1.028
84	Cyclohexane, (1-methylpropyl)-	38.475	140	0.318
85	Octane, 6-ethyl-2-methyl-	38.684	156	0.282
86	Cyclohexane, butyl-	39.038	140	0.591
87	Cyclopentane, 1-butyl-2-ethyl-	39.274	154	0.448
88	Nonane, 3,7-dimethyl-	39.621	156	0.597
89	4-Octene, 2,3,6-trimethyl-	40.375	154	0.251
90	Octane, 5-methyl-2-methyl-	40.680	156	0.181
91	Cyclohexane, 1,1-dimethyl-2-propyl-	40.870	154	0.166
92	Decane, 5-methyl-	41.106	156	0.416
93	Decane, 4-methyl-	41.377	156	0.295
91	Octane, 2,3,7-trimethyl-	41.669	156	0.281
92	Cyclohexane, (1,2-dimethylpropyl)-	41.880	154	0.130
93	Decane, 3-methyl-	42.222	156	0.283
94	4(<i>E</i>)-Decene, 6-methyl-	43.836	154	0.109
95	Undecane (C ₁₁)	44.598	156	0.715
96	Benzothiazole	53.802	135	0.854
97	Hexanedioic acid, dimethyl ester	54.357	174	0.242
98	2,5-Cyclohexadiene-1,4-dione-2,6-bis (1,1-methylethyl)-	71.797	220	0.340
99	Unknown	74.115	234	0.253

Table 1 (continued)

Peak	Compound	RT (min)	M.W.	Abundance (%)
100	Nitrofurantoin	79.016	238	0.111
101	Heptadecane (C ₁₇)	86.241	240	0.843
102	Benzene, 1,1'-(3,3-dimethyl-1-butenylidene)bis-	86.422	236	0.379
103	Benzoic acid, 4-phenoxy-, methyl ester	86.698	228	0.605
104	Tetradecanoic acid, methyl ester	87.062	242	0.964
105	1H-Indene, 2,3-dihydro-1,1,3-trimethyl-3-phenyl-	87.532	236	1.241
106	Heptadecane, 8-methyl-	88.698	254	0.556
107	Dodecane 2-cyclohexyl, 2-cyclohexyl-	89.490	252	0.681
108	Tetradecanoic acid, 11-methyl-, methyl ester	90.705	256	0.432
109	Tetradecanoic acid, 12-methyl-, methyl ester	91.161	256	0.608
110	Octadecane (C ₁₈)	91.703	254	0.201
111	Pentadecanoic acid, methyl ester	92.685	256	0.532
112	Unknown	93.883	258	0.286
113	1,2-Benzenedicarboxylic acid, dipropyl ester	94.868	250	0.490
114	Nonadecane (C ₁₉)	97.351	268	0.185
115	Hexadecanoic acid, methyl ester	98.166	270	1.402
116	1,2-Benzenedicarboxylic acid, butyl 2-methylpropyl ester	99.924	278	0.962
117	Hexadecanoic acid, 14-methyl-, methyl ester	101.753	284	0.461
118	Heptadecanoic acid, methyl ester	103.125	284	0.391
119	Eicosane (C ₂₀)	105.121	282	0.850
120	Unknown	105.925	304	0.881
121	9-Octadecenoic acid, methyl ester	106.726	296	0.527
122	Heptadecane, 2,6,10,15-tetramethyl-	107.041	296	0.680
123	Octadecanoic acid, methyl ester	107.956	298	0.649
124	Unknown	108.285	302	0.560
125	Heneicosane (C ₂₁)	111.691	296	1.634
126	Docosane (C ₂₂)	116.104	310	2.064
127	Unknown	118.423	314	0.578
128	Tricosane (C ₂₃)	120.346	324	2.762
129	Tetracosane (C ₂₄)	124.346	338	4.179
130	Unknown	125.260	330	3.862
131	Unknown	125.731	386	4.373
132	Pentacosane (C ₂₅)	128.265	352	3.888
133	Hexacosane (C ₂₆)	131.986	366	4.046
134	Heptacosane (C ₂₇)	135.700	380	3.703
135	Unknown	136.678	384	2.261
136	Octacosane (C ₂₈)	139.745	394	2.880
137	Nonacosane (C ₂₉)	144.354	408	1.877
138	Unknown	146.000	418	0.233
139	Triacontane (C ₃₀)	149.688	422	1.744
140	Unknown	151.179	428	0.115

^a Abbreviations: RT, retention time (min); M.W., molecular weight; Abundance, percentage of total hydrocarbons and volatiles.

GC oven program was initial temperature 40°C for 2 min, 2 C°/min run to 300°C, final hold at 300°C (20 min). Injector temperature was kept at 180°C (split-less). The carrier gas was helium at a linear flow-rate 25 cm/s. The MS detector was operated at 194°C. Scan range was from 30 to 650 *m/z* at 0.9 scan/s

scan rate. Solvent delay was 12 min. To check on purity of each GC peak, MS were taken at various parts of each peak. Hydrocarbons, volatile compounds, and fatty acids were identified by mass spectral library search (NBS75 and Wiley138K), followed by matching of MS data and comparison of

some components with commercial standards (fatty acids, cyclohexane derivatives and hydrocarbons from C₇ to C₃₀).

2.5. Reagents

Pentane and dichloromethane (both, high purity) were obtained from Baker (USA) and were checked by GC–MS before the analysis for volatile compounds and hydrocarbons.

3. Results and discussion

The composition of the hydrocarbons and volatile metabolites is presented in Table 1. More than 130

hydrocarbons and metabolites and were identified from blue-green alga *Nostoc* sp. including 41 cyclohexane and 11 cyclopentane derivatives which were found among the volatile compounds.

Separation of compounds isolated from *Nostoc* sp. were conducted with two coupled columns (60 m). The first column allows reasonable retention of the low volatile compounds, but other components are not separated and pass into the second column while the volatile compounds are detected. Fig. 1 illustrates the chromatograms obtained for the C₇–C₁₁ hydrocarbons and the volatile metabolites. The columns separation of many compounds, including some *cis*- and *trans*-isomers of 1-ethyl-4-methyl-cyclohexane (peaks 36 and 37) or, for instance, the positional isomers, 1 α ,2 α ,3 β -trimethyl-cyclohexane and

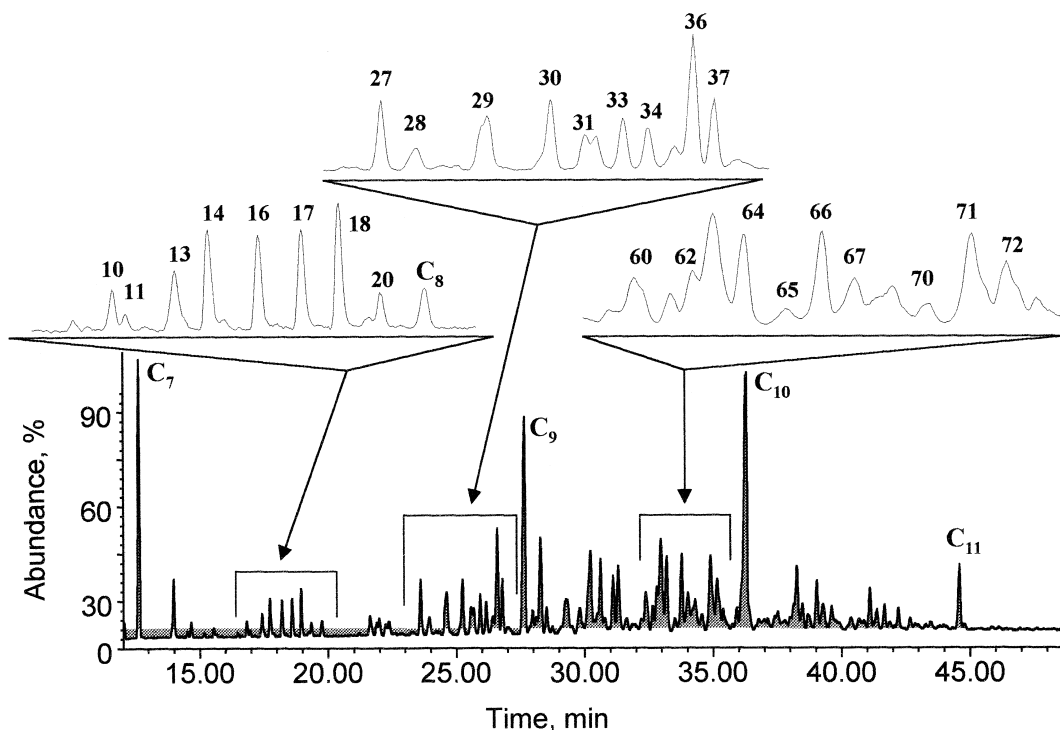


Fig. 1. The parts of gas chromatogram (full run is 162 min) of volatile metabolites and light hydrocarbons (C₇–C₁₀) identified from cultured culture of blue-green alga *Nostoc* sp. isolated from *Collema* sp. lichen. Some identified peaks show on three parts. Left part, peaks: 10, 4-methyl-heptane; 11, 3-methyl-heptane; 13, 3-hexanone; 14, 2-hexanone; 17, 3-hexanol and 18, 7-hexanol. Central part, separation of cyclohexane derivatives, peaks: 27, 1 α ,3 α ,5 α -trimethyl-cyclohexane; 28, 1,2,4-trimethyl-cyclohexane; 31, 1 α ,2 β ,4 β -trimethyl-cyclohexane; 32, 1 α ,3 α ,5 β -trimethyl-cyclohexane; 33, 1 α ,2 α ,3 β -trimethyl-cyclohexane; 34, 1 α ,2 β ,3 α -trimethyl-cyclohexane; 35, 1-methyl-2-propyl-cyclopentane; 36, *trans*-1-ethyl-4-methyl-cyclohexane, 37, *cis*-1-ethyl-4-methyl-cyclohexane. Right part, gas chromatographic separation of nonane isomers, peaks: 62, 5-methyl-nonane; 63, 4-methyl-nonane; 64, 3-methyl-nonane and 66, 2-methyl-nonane. For other peak identification refer to Table 1.

1 α ,2 β ,3 α -trimethyl-cyclohexane (peaks 33 and 34). Only a few components were eluted between 45 and 80 min.

The most important and unexpected data were obtained with *Nostoc* sp. These results essentially differ from published data, concerning first of all the alkanes. For example, it was stated [26,27] that cyanobacteria, including also *Nostoc* sp., contain only three alkanes C₁₅, C₁₆ and C₁₇. The content of C₁₇ and 7- and 8-methyl-C₁₇ is dominating and varies from 60 up to 97% of the total hydrocarbons. In a previous study of the biosynthesis of alkanes in *Nostoc muscorum*, it was shown, that *n*-hydrocarbons in the C₁₅–C₁₈ range with a predominance of C₁₇ are presented [28].

According to our results we identified alkane hydrocarbons from C₇ to C₃₀ with predominance of C₂₄–C₂₇ (Fig. 2A). The quantitative content of

alkanes is comparable to the content of fatty acids. Fig. 2B demonstrates the distribution of alkanes in lichen *Collema*. The main question which required an answer is which of the two bionts synthesizes alkane hydrocarbons — blue-green alga *Nostoc* or are they synthesized by mycobiont? We analyzed the metabolites of the lichen *Collema* and the mycobiont isolated from lichen. According to our results we conclude, that the mycobiont synthesizes alkane hydrocarbons from C₂₂ up to C₂₈, with dominating C₂₃–C₂₅. Their hydrocarbon content is rather low in comparison with photobiont hydrocarbons. As can be seen from the chromatograms (Fig. 2A,B), the greatest contribution from two bionts is introduced by photobiont.

It is known that some *Nostoc* sp. such as *N. muscorum* [29,30] and *Nostoc linckia* [31] produce cyclohexane derivatives; some of them were iden-

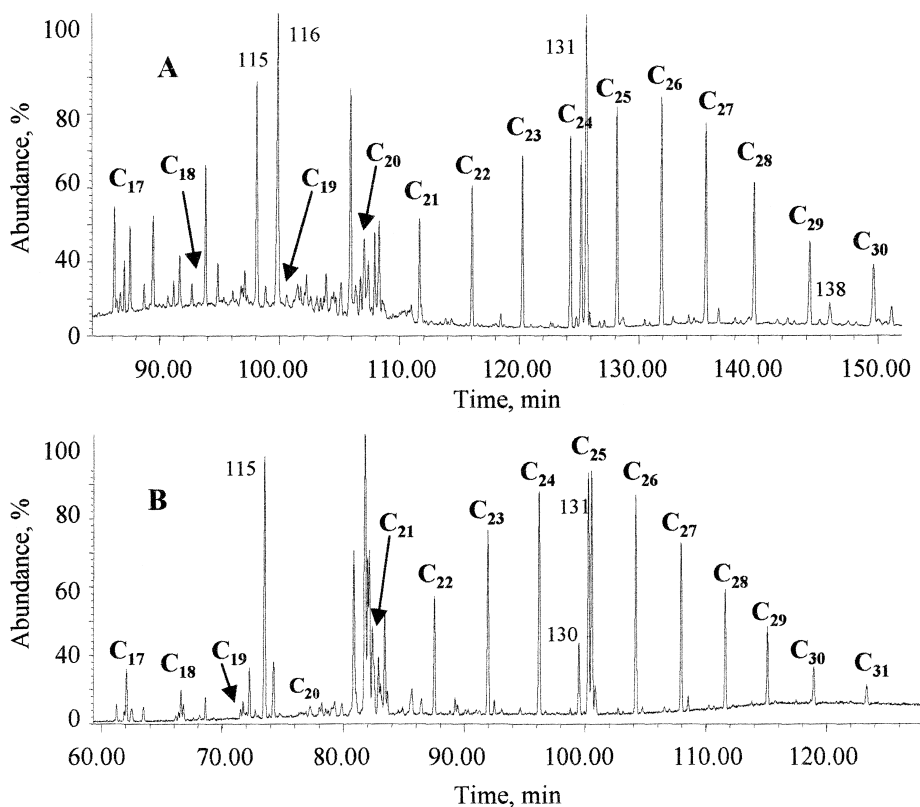


Fig. 2. Gas chromatographic separation of alkanes from C₁₇ to C₃₀ of the blue-green alga *Nostoc* sp. (A) and alkanes of lichen *Collema* sp. (B). The separation of *Collema* compounds (full run is 130 min) was done using RTX-I column, 30 m×0.32 mm ID. GC oven program was initial temperature 40°C for 2 min, 5°C min to 100°C, 2°C to 300°C. Final hold at 300°C 20 min.

tified. More than 50 volatile metabolites, including cyclohexane derivatives and saturated hydrocarbons were isolated from the lichen *Evernia prunastri* [32]. Solberg and Hattori [33,34] studied and identified six alkanes having carbon chain lengths in the range from C₁₂ to C₂₈. According to Gaskell et al. [35] and Catalano et al. [36] who studied the hydrocarbons of five lichen species, aliphatic hydrocarbons in the range C₁₇–C₃₃ are abundant. Surface alkanes were isolated from lichen *Xanthoria parietina* grown in two different valleys in Italy [37]; main *n*-alkanes such as C₂₇–C₃₁ were found. Some unsaturated hydrocarbons such as 17:1, 17:2, 22:1, 20:3 and a highly unsaturated hydrocarbon 20:5 were identified in some lichen species [32,33,36]. Interestingly a C₂₇ polyunsaturated, branched hydrocarbon, dominated in *Lobaria pulmonaria* (76%) and *Usnea hirta* (4%) [32]. *Nostoc* sp. isolated from *Collema* sp. which were cultivated in our laboratory produced two unidentified polyunsaturated branched hydrocarbons: peak 130 (*m/z*=330) and 131 (*m/z*=386); these compounds were found also in lichen *Collema* sp. (see Fig. 2). Identification studies of these hydrocarbons is in progress.

4. Conclusions

In this study, we described the successful separation of the hydrocarbons and other volatile compounds using serially coupled capillary columns with different consecutive nonpolar and semipolar stationary phases. This report presents information on cyclohexane, cyclopentane, alkane hydrocarbons and volatile metabolites produced by laboratory culture *Nostoc* sp.

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