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Use of Serially Coupled Capillary Columns with Different Polarity of Stationary Phases for the Separation of the Natural Complex Volatile Mixture of the Marine Red Alga *Corallina elongata*

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Abstract—Separation of a complex of natural volatile compounds using serially coupled capillary columns with different polarity of stationary phases by gas chromatography—mass spectrometry from the medicinal marine red alga *Corallina elongata* is reported. Nearly 200 hydrocarbons, halogen compounds, fatty acids, and other metabolites were found. Using this gas chromatography procedure we demonstrate the successful separation of different volatile organic compounds.

Key words: biochemical method, red alga, *Corallina elongata*, GC—MS, serially coupled capillary columns

The potential of GC—MS for the separation and identification of natural and/or synthetic drugs, its metabolites, and/or organic compounds has been evident for many years [1]. Analysis of essential oil from biological samples by GC—MS is one of the basic and most efficient methods [2, 3]. Many papers have been published on the subject of optimizing the parameters of serially coupled capillary columns, such as plate height equivalent, temperature, pressure, and time [4-8]. Length, internal diameter, and film thickness have also been investigated [9, 10]. However, the use of these approaches has not been fully investigated for the separation of natural complex mixtures [10]. Recently we demonstrated good separation of a mixture of natural complex volatile compounds from cultured cyanobacterium *Nostoc* sp. by GC—MS using serially coupled capillary columns with consecutive nonpolar and semipolar stationary phases [11]. The aim of this study was to establish the possibility of using serially coupled capillary columns with different polarity of stationary phases for separating a mixture of natural complex volatile compounds of the medicinal marine red algae *Corallina elongata*.

The red alga *Corallina elongata* is widely distributed along the Mediterranean cost of South Europe, Western Asia, and North Africa [12, 13]. This alga is frequently used for medicinal purposes, but information is limited about lipid and bioactive metabolites of this red alga [14-17].

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MATERIALS AND METHODS

Algae samples. The marine red alga *Corallina elongata* Ellis and Solander was collected at Ashdod (Israel) on the Mediterranean Sea coast in July 1998. The alga was carefully cleared of exegetic impurities and only clean alga was used for extraction.

Extraction of natural compounds. The fresh alga was homogenized in a high-speed unit, and successively percolated with pentane, CH_2Cl_2, and also benzene at 60°C, 2 h. Extracts were combined and solvents were removed under reduced pressure. The oil sample (200 mg) was dissolved in benzene, and 5% HCl in methanol was added. The mixture was left overnight in a stoppered tube at 50°C. After cooling to 5°C, water (10 ml) was added. Volatile compounds were extracted with pentane and then CH_2Cl_2. The pentane and CH_2Cl_2 fractions were combined. The solution was filtered and solvent removed under reduced pressure. The oil residue was dissolved in CH_2Cl_2 and stored at −20ºC prior to GC—MS analysis.

GC—MS analysis. A Hewlett-Packard 6890 (series II) gas chromatograph (USA) that was modified for a glass capillary column and an HP GC-mass selective detector (5971B MSD) were used. Volatile compounds were analyzed by gas chromatography on a serially capillary column [11]: RTX-1 (Restek, USA), 30 m, ID 0.32 mm, film thickness 0.25 μm, coupled with a second capillary column RTX-1701 (Restek), 30 m, 0.32 mm, 0.25 μm film. The GC oven was programmed: 40°C 2 min, 2°C/min to 300°C, 20 min at 300°C. Injector temperature was kept on

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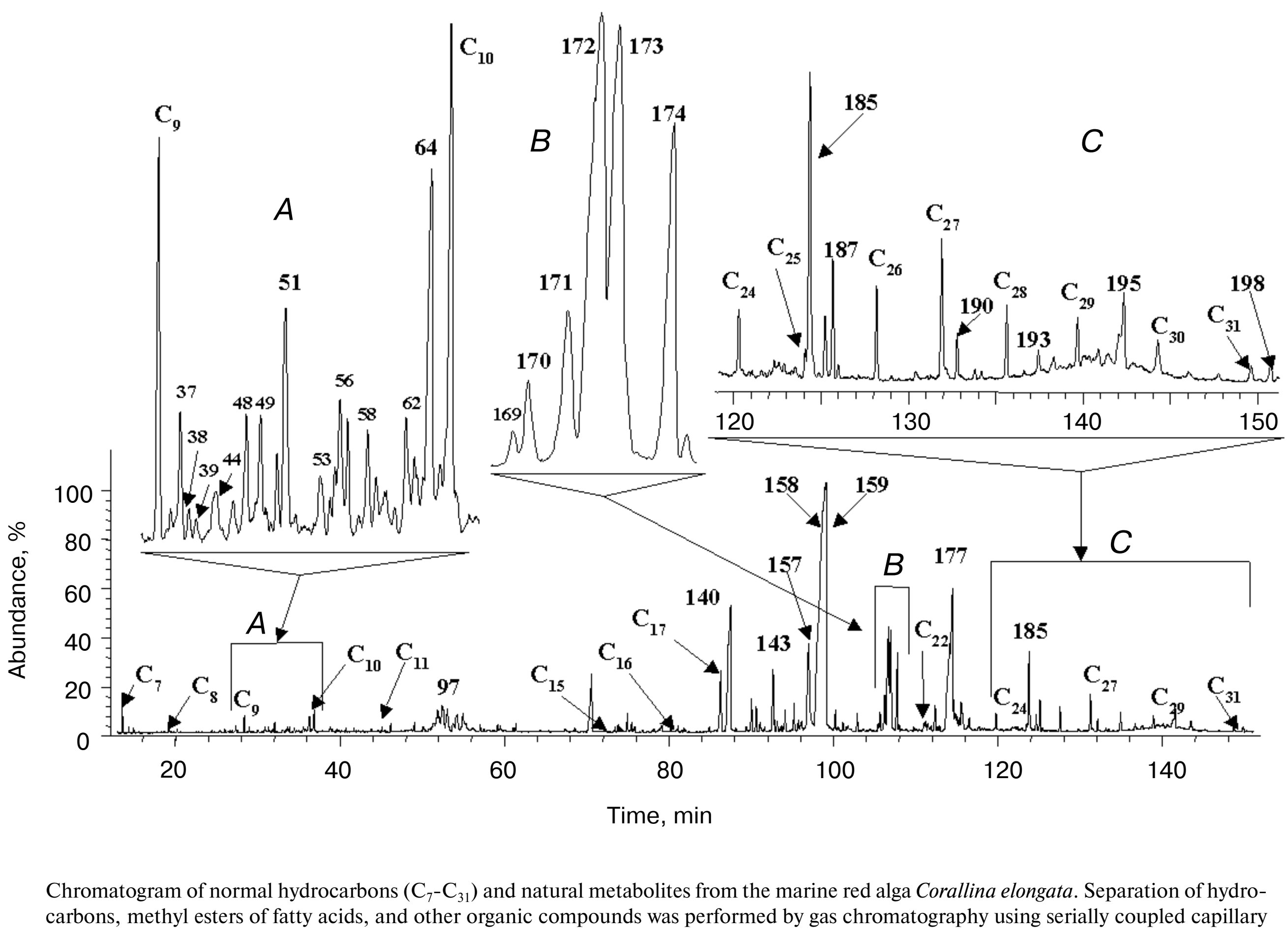
180°C (splitless). Flow rate of carrier gas (helium) was 2.5 ml/sec. The MS detector was operated at 194°C, ionization energy 70 eV. The scan range was from 30 to 650 m/z and scan rate 0.9 scan/sec. Solvent delay was 12 min. Volatile metabolites were identified by mass spectral search libraries (Wiley, 7th edition).

RESULTS AND DISCUSSION

The problem of the simultaneous separation of complex natural metabolites such as fatty acids, hydrocarbons, and other organic compounds by GC is a difficult one because of the large number of different compounds present. The use of serially coupled capillary columns with stationary phases of different polarity appears a practical approach to this problem. The subject has recently been reviewed [10, 18]. However, its application to the analysis of complex natural mixtures has not been widely applied. Our initial effort using serially coupled capillary columns with stationary phases of different polarity for the separation of a complex mixture of volatile compounds is represented in figure. Two hundred compounds were separated, both low molecular weight (part A) and higher molecular weight (part B) compounds being clearly discernible in the GC–MS trace (total run time was 165 min). All identified compounds are listed in Tables 1-4. Tables 1 and 2 contain normal, branched and cyclic, and unsaturated hydrocarbons, respectively. Positional isomers, i.e., trimethylcyclohexane (peaks 26, 27, 31, and 32) or, for instance, *cis*- and *trans*-isomers of 1-ethyl-2-methyl-cyclohexane (peaks 38 and 39) could also be separated. Table 3 includes alcohols, aldehydes, ketones, and halogen compounds. Table 4 includes dioic, carboxylic, and fatty acids.

The major natural compounds are the acids (79.8%, see Table 4), and secondary metabolites, which were identified as total hydrocarbons (14.9%, see Tables 1 and 2). Minor compounds (total content 5.2%) considered necessary secondary metabolites are indicated in Table 3, and include alcohols, aldehydes, ketones, and halogen compounds. The mass spectra of all volatile compounds



Chromatogram of normal hydrocarbons (C_7-C_{31}) and natural metabolites from the marine red alga *Corallina elongata*. Separation of hydrocarbons, methyl esters of fatty acids, and other organic compounds was performed by gas chromatography using serially coupled capillary columns with different polarity of stationary phases. A) Separation of low molecular weight compounds located between two *n*-alkanes C_9-C_{10}; B) an example of separation of methyl esters of saturated, mono-, di-, and trienoic fatty acids and their isomers; C) an example of separation of *n*-hydrocarbons C_{24}-C_{31} and other high molecular weight compounds. Overall run time was 165 min. Identification of peaks is given in Tables 1-4

Table 1. Normal and branched alkanes identified from the red alga *Corallina elongata*

Peak No.	Compound	Retention time, min	Molecular weight, daltons	Percentage of total volatile metabolites, %	Percentage of total *n*-alkanes, %
	***n*-Alkanes**			**6.046**	**100**
2	Heptane (C_7)	12.704	100	0.273	4.52
19	Octane (C_8)	19.452	114	0.058	0.96
36	Nonane (C_9)	26.695	128	0.249	4.13
65	Decane (C_{10})	36.326	142	0.497	8.22
84	Undecane (C_{11})	44.679	156	0.120	1.98
113	Tridecane (C_{13})	67.273	184	0.035	0.58
118	Pentadecane (C_{15})	73.935	212	0.083	1.38
128	Hexadecane (C_{16})	80.214	226	0.078	1.29
138	Heptadecane (C_{17})	86.354	240	1.997	33.03
175	Docosane (C_{22})	111.678	310	0.188	3.11
183	Tetracosane (C_{24})	120.344	338	0.383	6.33
184	Pentacosane (C_{25})	124.047	352	0.187	3.09
188	Hexacosane C_{26})	128.197	366	0.462	7.64
189	Heptacosane (C_{27})	131.927	380	0.786	13.00
192	Octacosane (C_{28})	135.635	394	0.124	2.05
194	Nonacosane (C_{29})	139.660	408	0.239	3.95
196	Tricontane (C_{30})	144.261	422	0.161	2.66
197	Hentriacontane (C_{31})	149.565	436	0.126	2.08
	Branched alkanes			**1.703**	
6	3-Methyl-hexane	14.602	100	0.008	
7	2,4-Dimethyl-hexane	14.752	114	0.014	
11	4-Methyl-heptane	16.928	114	0.022	
12	2-Methyl-heptane	17.048	114	0.009	
25	2,2,3,3-Tetramethyl-butane	22.357	114	0.009	
28	2,3-Dimethyl-heptane	24.051	128	0.021	
29	3-Methyl-octane	24.714	128	0.063	
30	2-Methyl-octane	25.316	128	0.052	
43	2,2,3,3-Tetramethyl-pentane	29.889	128	0.047	
44	3,6-Dimethyl-octane	30.287	142	0.124	
45	3,3-Dimethyl-octane	30.849	142	0.023	
48	3-Methyl-nonane	30.699	142	0.133	
49	2,5,5-Trimethyl-heptane	30.849	142	0.023	
51	3-Ethyl-2-methyl-heptane	31.440	142	0.236	
53	2,2,3,3-Tetramethyl-hexane	32.441	142	0.075	
56	4-Methyl-nonane	33.050	142	0.133	
57	4-Ethyl-octane	33.267	142	0.085	
58	3-Ethyl-octane	33.586	142	0.018	
59	3-Methyl-nonane	33.859	142	0.097	
67	3,5-Dimethyl-octane	38.071	142	0.021	
68	4-Methyl-decane	38.345	156	0.148	
70	3-Methyl-decane	38.777	156	0.040	
79	2,6-Dimethyl-nonane	41.461	156	0.039	
80	2-Methyl-decane	41.759	156	0.050	
81	3,5-Dimethyl-nonane	42.304	156	0.059	
123	2,6,7-Trimethyl-decane	78.124	184	0.069	
191	2,6,10,14,18-Pentamethyl-eicosane	134.230	352	0.085	

Table 2. Cyclic and unsaturated hydrocarbons identified from the red alga *Corallina elongata*

Peak No.	Compound	Retention time, min	Molecular weight, daltons	Percentage of total volatile metabolites, %
	Cyclic hydrocarbons			**3.414**
1	1,2-Dimethyl-cyclopentane	12.144	98	0.013
4	Methyl-cyclohexane	14.068	98	0.060
20	*trans*-1,3-Dimethyl-cyclohexane	19.854	112	0.013
22	*cis*-1,2-Dimethyl-cyclohexane	21.744	112	0.025
23	*trans*-1,2-Dimethyl-cyclohexane	21.985	112	0.012
24	Ethyl-cyclohexane	22.087	112	0.015
26	1,1,3-Trimethyl-cyclohexane	22.478	126	0.023
27	1,3,5-Trimethyl-cyclohexane	22.708	126	0.044
31	1,1,2-Trimethyl-cyclohexane	25.650	126	0.026
32	1,2,4-Trimethyl-cyclohexane	26.005	126	0.058
34	*trans*-1-Ethyl-4-methyl-cyclohexane	26.665	126	0.133
35	*cis*-1-Ethyl-4-methyl-cyclohexane	26.867	126	0.043
37	1α,2α,3α-Trimethyl-cyclohexane	28.057	126	0.024
38	*trans*-1-Ethyl-2-methyl-cyclohexane	28.337	126	0.107
39	*cis*-1-Ethyl-2-methyl-cyclohexane	28.586	126	0.026
41	Bicyclo[3,3,1]nonane	29.164	124	0.007
42	1-Methylethyl-cyclohexane	29.375	126	0.072
46	1-Ethyl-2,3-dimethyl-cyclohexane	30.191	140	0.062
50	1-Ethyl-2,4-dimethyl-cyclohexane	31.197	140	0.067
54	1,1,2,3-Tetramethyl-cyclohexane	32.756	140	0.028
55	2-Ethyl-1,3-methyl-cyclohexane	32.895	140	0.045
60	1-Methyl-4-[1-methylethyl]-cyclohexane	34.101	140	0.057
61	1-Methyl-2-propyl-cyclohexane	34.398	140	0.074
62	1-Ethyl-1,3-dimethyl-cyclohexane	34.658	140	0.028
63	1-Methyl-3-propyl-cyclohexane	34.983	140	0.136
73	2-Methylbutyl-cyclohexane	39.706	140	0.052
78	*cis*-Decahydro-naphthalene	41.165	138	0.032
83	Nonyl-cyclopropane	44.112	168	0.035
97	*cis*-1,4-Dimethyl-cyclooctane	52.727	140	1.075
99	Butyl-cyclooctane	53.710	168	0.278
104	1,2,5,6-Diepoxy-cyclooctane	57.172	140	0.086
127	Cyclotetradecane	79.663	196	0.035
187	3α,4α-Epoxy-cholestane	125.719	386	0.619
	Unsaturated hydrocarbons			**3.789**
8	2-Ethyl-acrolein	15.284	84	0.013
10	3,5-Dimethyl-1-hexene	16.564	112	0.010
15	1-Methoxy-2(E)-hexene	18.328	114	0.030
33	3(E)-nonene	26.245	126	0.031
74	6-Methyl-4(E)-decene	39.845	154	0.009
76	4(Z)-Undecene	40.444	154	0.031
88	9-Methyl-1-decene	47.959	154	0.049
91	2,9-Dimethyl-3,7-decadiene	48.678	166	0.199
93	2,4-Dimethyl-1-decene	50.395	168	0.091
94	6-Methyl-4-undecene	50.901	168	0.095
95	1-Tridecene	51.364	182	0.979
96	10-Methyl-1-undecene	52.057	168	0.978
101	5-Methyl-3-undecene	54.690	168	0.738
105	1-Dodecene-3-yne	58.889	164	0.286
124	1-Tetradecen-3-yne	79.015	192	0.098
135	1-Hexadecene	84.700	224	0.025
149	2,4-Diphenyl-4-methyl-2(E)-pentene	93.849	236	0.125

Table 3. Alcohols, aldehydes, ketones, and halogen metabolites identified from the red alga *Corallina elongata*

Peak No.	Compound	Retention time, min	Molecular weight, daltons	Percentage of total volatile metabolites, %
	Alcohols			**1.357**
9	3-Methyl-3-pentanol	15.640	102	0.006
17	3-Hexanol	18.725	102	0.039
18	2-Hexanol	19.061	102	0.030
47	4-Methyl-2-propyl-1-pentanol	30.287	144	0.123
52	3-Octyn-2-ol	31.736	126	0.023
72	1-Decanol	39.374	158	0.059
86	3,7-Dimethyl-1-octanol	46.208	158	0.057
92	1-Decanol	49.310	158	0.028
98	6-Dodecanol	53.410	186	0.397
102	1-Dodecanol	54.849	186	0.106
126	8-Amino-2-naphthalenol	79.395	159	0.022
134	2-Ethyl-1-dodecanol	84.484	214	0.021
137	Hexadecanol	85.364	242	0.025
151	3,7,11,15-Tetramethyl-2-hexadecene-1-ol	94.373	296	0.423
	Aldehydes			**0.616**
16	Hexanal	18.474	100	0.142
75	2(E)-Octenal	40.214	126	0.072
103	2(Z)-Decenal	56.750	154	0.145
106	2(E),4(E)-Dodecadienal	59.256	180	0.155
109	2,4-Decadienal	60.804	152	0.103
	Ketones			**0.381**
5	3(E)-Pentene-2-one	14.409	84	0.012
13	3-Hexanone	17.521	100	0.027
14	2-Hexanone	17.830	100	0.024
82	3(E),5(E)-Octadiene-2-one	43.149	124	0.028
112	2-Dodecanone	67.133	184	0.051
131	Benzophenone	81.837	182	0.075
148	9-Heptadecanone	93.665	256	0.072
150	6,10,14-Trimethyl-2-pentadecanone	94.091	268	0.092
	Halogen compounds			**2.886**
69	6-Bromo-1-hexene	38.568	162	0.067
71	1-Bromo-3-methyl-butane	39.114	150	0.079
77	1-Fluoro-nonane	40.750	146	0.025
87	3-Chloro-octane	46.738	148	0.048
89	3-Bromo-1,1,1-Trimethyl-propane	48.239	164	0.067
108	*cis*-1,3-Dichloro-cyclohexane	60.790	152	0.039
120	3-Bromo-cyclohexane	75.430	160	0.198
122	3-(Bromomethyl)-cyclohexane	77.941	174	0.034
145	1-Chloro-hexadecane	91.822	260	0.112
147	(1S)-Endo-(–)-3-bromo-camphor	93.324	230	0.263
152	1-Bromo-7-(tetrahydro-2-pyranyloxy)-heptane	95.109	278	0.052
153	(1R)-Endo-(+)-3-bromo-camphor	95.321	230	0.624
168	1-Bromo-4-phenyl-bicyclo[2,2,2]octane	104.677	264	0.061
193	1-Iodo-octadecane	137.483	380	0.318
195	3-Bromo-3β-cholest-5-ene	142.346	448	0.756
198	5β,6β-Epoxy-7α-bromo-cholestan-3β-ol	150.742	480	0.143

Table 4. Dioic, carboxylic, and fatty acids identified from the red alga *Corallina elongata*

Peak No.	Compound	Retention time, min	Molecular weight, daltons	Percentage of total volatile metabolites, %	Percentage of total acids, %
	Total of dioic, carboxylic, and fatty acids			**79.808**	**100.00**
3	Butanoic acid, ME	13.458	102	0.064	0.08
21	Pentanoic acid, ME	20.521	116	0.014	0.02
40	Hexanoic acid, ME	28.804	130	0.021	0.03
64	3-Methyl-pentanoic acid, ME	35.737	130	0.397	0.50
66	Heptanoic acid, ME	37.373	144	0.034	0.04
85	Octanoic acid, ME	45.737	158	0.163	0.20
90	Propionic acid, HE	48.437	158	0.016	0.02
100	1,2-Benzisothiazole-3-carboxylic acid	53.920	179	0.513	0.64
107	8-Nonynoic acid, ME	59.749	168	0.049	0.06
110	Decanoic acid, ME	61.171	186	0.143	0.18
111	Heptanedioic acid, DME	62.042	188	0.016	0.02
114	Undecanoic acid, ME	68.229	200	0.025	0.03
115	9-Oxo-undecanoic acid, ME	68.456	200	0.076	0.10
116	Octanedioic acid, DME	69.368	202	0.012	0.01
117	1,4-Benzenedicarboxylic acid, DME	70.185	194	2.198	2.75
119	Dodecanoic acid, ME	74.895	214	0.322	0.40
121	Nonanedioic acid, DME	75.813	216	0.077	0.10
125	12-Tridecynoic acid, ME	79.129	224	0.114	0.14
129	Tridecanoic acid, ME	81.132	228	0.105	0.13
130	2-Methyldodecanoic acid, ME	81.648	228	0.053	0.07
132	Decanedioic acid, ME	82.091	230	0.036	0.05
133	Cyclopropanenonanoic acid, ME	82.472	212	0.024	0.03
136	Tetradecanoic acid, ME	84.983	242	0.110	0.14
139	12-Methyl-tridecanoic acid, ME	87.346	242	2.576	3.23
140	Tetradecanoic acid, ME	87.624	242	4.479	5.61
141	Undecanedioic acid, DME	88.255	244	0.071	0.09
142	4,8,12-Trimethyltridecanoic acid, ME	90.151	270	0.635	0.79
143	Pentadecanoic acid, ME	90.756	256	0.469	0.59
144	12-Methyl-tetradecanoic acid, ME	91.199	256	0.280	0.35
146	9-Methyl-tetradecanoic acid, ME	92.756	256	1.348	1.69
154	4,7,10-Hexadecatrienoic acid, ME	95.938	264	0.374	0.47
155	10-Methyl-pentadecanoic acid, ME	96.201	270	0.432	0.54
156	9(Z)-Hexadecenoic acid, ME	97.054	268	4.733	5.93
157	7-Hexadecenoic acid, ME	97.526	268	0.251	0.31
158	Hexadecanoic acid, ME	99.243	270	28.957	36.28
159	13-Methyl-pentadecanoic acid, ME	99.398	270	3.846	4.82
160	14-Methyl-pentadecanoic acid, ME	100.531	270	0.428	0.54
161	Cyclopentaneundecanoic acid, ME	100.768	268	0.108	0.13
162	13-Methyl-hexadecanoic acid, ME	101.443	284	0.169	0.21
163	14-Methyl-hexadecanoic acid, ME	101.868	284	0.171	0.21
164	2-Hexyl-cyclopropaneoctanoic acid, ME	102.082	282	0.131	0.16
165	Heptadecanoic acid, ME	103.202	284	0.331	0.41
166	10,13-Dimethyltetradecanoic acid, ME	103.514	270	0.045	0.05
167	2-Hydroxy-hexadecanoic acid, ME	104.351	286	0.061	0.07
169	6,9,12-Octadecatrienoic acid, ME	105.755	292	0.196	0.24
170	9,12,15-Octadecatrienoic acid, ME	105.988	292	0.466	0.58
171	9(Z),12(Z)-Octadecadienoic acid, ME	106.568	294	1.085	1.36
172	9(Z)-Octadecenoic acid, ME	107.055	296	4.108	5.15
173	9(E)-Octadecenoic acid, ME	107.342	296	3.269	4.10
174	Octadecanoic acid, ME	108.156	298	1.877	2.35
176	Nonadecanoic acid, ME	112.704	312	0.706	0.88
177	5,8,11,14,17-Eicosapentaenoic acid, ME	114.587	316	5.000	6.26
178	5,8,11,14-Eicosatetraenic acid, ME	114.895	318	3.960	4.96
179	7,10,13-Eicosatrienoic acid, ME	115.089	320	0.558	0.70
180	10,13-Eicosadienoic acid, ME	115.756	322	0.280	0.35
181	11-Eicosenoic acid, ME	115.970	324	0.625	0.78
182	Eicosanoic acid, ME	116.955	326	0.432	0.54
185	13(Z)-Docosenoic acid, ME	124.477	352	2.105	2.64
186	Docosanoic acid, ME	124.204	354	0.391	0.49
190	Tetracosanoic acid, ME	132.796	382	0.256	0.32

Note: ME, methyl ester; DME, dimethyl ester; HE, hexyl ester.

were compared to the spectra in different mass spectral libraries (NIST 98 and Wiley, 7th edition). To reduce to a minimum any error in identification, each compound was compared to the spectra in both spectral libraries.

Many different metabolites have been isolated from red algae during the last 30 years and the data are partly reviewed [19-24]. The composition of fatty acids was determined using packed and/or capillary columns for study of red algae [22, 25]. Chloro-, bromo-, and iodo-derivatives widely occurring in marine algae were also isolated and identified [20-23, 26, 27].

Interesting data were obtained for *n*-hydrocarbons. According to the review on marine hydrocarbons [28], the major component among hydrocarbons is *n*-heptadecane (peak No. 138). Its concentration in different species of marine red algae varied from 37 up to 99%. The data obtained by us also confirms that heptadecane comprises 33% of the sum of *n*-hydrocarbons. However, there are also significant differences in the composition of total hydrocarbons. Our analysis indicated that the total content of *n*-hydrocarbons for *Corallina elongata* consists of 14.9% of total volatiles. This is several times higher than that found for other red algae reported previously [28]. The discrepancy, perhaps, is possibly due to the method of analysis. For example, thin-layer chromatography, or other similar methods, in particular, column chromatography [29], can result in the loss of a considerable proportion of hydrocarbon volatiles. Preparation of samples for GC–MS demands more careful sample-handling and constant refrigeration. In particular we have paid attention to the handling of methanolic extracts in the presence of HCl. Care was taken to assure that the vials were always tightly sealed to prevent loss of volatile components.

In this study we demonstrated the successful separation of natural complex mixtures of the red alga *Corallina elongata* using a serial capillary column system. This GC application could be use in biochemical investigations for the study of organic metabolites and/or lipid content of different biological samples.

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