Available online at www.sciencedirect.com
SCIENCE DIRECT®
Pergamon
Biochemical Systematics and Ecology 31 (2003) 1125–1145
biochemical systematics and ecology
www.elsevier.com/locate/biochemsyseco

Variability of the fatty acids of the marine green algae belonging to the genus *Codium*

V.M. Dembitsky [a,*], H. Řezanková [b], T. Řezanka [c], L.O. Hanuš [a]

[a] *Department of Medicinal Chemistry and Natural Products, School of Pharmacy, P.O. Box 12065, Hebrew University of Jerusalem, Jerusalem 91120, Israel*
[b] *Department of Statistics and Probability, University of Economics, W. Churchill Sq. 4, 13067, Prague, Czech Republic*
[c] *Institute of Microbiology, Videnska 1083, 14220, Prague, Czech Republic*

Received 10 July 2002; accepted 3 January 2003

Abstract

Analysis of fatty acids by gas chromatography-mass spectrometry (GC-MS) using serially coupled capillary columns with different polarity of stationary phases is reported. More than 40 volatile compounds, including, low molecular, dioic and fatty acids were determined of two marine green algae *Codium dwarkense* and *C. taylorii*. There are large variations in individual fatty acid contents according to species, season and location of the genus *Codium*. Statistical analysis of variability of fatty acids belonging to the genus *Codium* is reported. Published by Elsevier Science Ltd.

Keywords: Fatty acids; *Codium*; GC-MS; Statistical analysis

1. Introduction

The scientific investigation of fatty acids of marine oils began in the first decade of the 20th century (Ackman, 1989). In the field of lipid chemistry, a great deal of investigation of fatty acids of marine plants has been carried out since the 1950s, when gas-liquid chromatography was developed. The taxonomic classification of many plants is assisted by chemical investigation of some secondary metabolites.

* Corresponding author. Tel.: +972-2-675-7549; fax: +972-2-675-8201.
E-mail address: dvalery@cc.huji.ac.il (V.M. Dembitsky).

0305-1978/03/$ - see front matter. Published by Elsevier Science Ltd.
doi:10.1016/S0305-1978(03)00043-7

Plant lipids, fatty acids, and also other volatile compounds provide interesting information for assignment of the taxonomic position, particularly of marine, but also of freshwater algal species (Dembitsky et al., 1990, 1991, 1992; Dembitsky and Rozentsvet, 1996; Rozentsvet et al., 1995). Fatty acids, sterols and hydrocarbons are also perfect biogeochemical markers for studies of fossil structures of ancient organisms. Marine algae are potential suppliers of fatty acids, sterols and hydrocarbons, which are found in marine sediments (Peters and Moldowan, 1993; Volkman et al., 1998).

Analysis by gas-chromatography-mass spectrometry is essential for identification of natural fatty acids, sterols and alkanes isolated from biological samples, including marine and freshwater algae, sediments and crude oil (Volkman et al., 1998; Christie, 1998a, Christie, 1998b; McClosky, 1971). Some papers have been published recently on the subject of optimising capillary GC separations through the use of serially coupled columns (Repka et al., 1989; Benicka et al., 1990; Engewald and Maurer, 1990; Maurer et al., 1990; Williams and Mitchell, 1991; Lou et al., 1993; Rastogi, 1993). A number of different variables have been manipulated to change the selectivity of the coupled system. However, the use of these approaches has not been fully investigated, for instance, in the separation of fatty, dioic, and carboxylic acids and other metabolites from marine algae.

In this paper we describe the application of serially coupled capillary columns with consecutive nonpolar and semipolar stationary phase coating for separation of fatty, dioic and low molecular acids isolated from two marine green algae *Codium dwarkense* and *C. taylorii*. The results obtained are discussed, together with literature data, to verify previously proposed fatty acid distributions for marine green algae belonging to the genus *Codium*.

2. Experimental

2.1. Algal material

Codium dwarkense Børgesen and *C. taylorii* P. Silva were collected from littoral rocks in the Mediterranean Sea near Ashdod and Ashkelon (Israel) during June–July 2000 at 2–5 m depth. Freshly collected algae were carefully cleaned of extraneous matter and only clean tissue was used for homogenisation in a high-speed unit (Dembitsky et al., 1990; Dembitsky and Rozentsvet, 1996).

2.2. Extracted procedure

Fresh material of *Codium dwarkense* and *C. taylorii* was treated with MeOH and heated for 6 hrs at 60 °C. After cooling the extract to room temperature, a cold mixture of *n*-C_5H_{12}–H_2O (3:6, v/v) was added. The mixture was shaken for 15 min, and then cooled under ice. The C_5H_{12} and methanol-water phases were separated. The methanol-water phase was extracted with CH_2CI_2 three times, and the phases were separated, and used for preparation of fatty acid methyl esters. The pentane and dichloromethane extracts were washed with 0.9% KCl solution three times. Then

the pentane and dichloromethane extracts were combined and evaporated under nitrogen at 10 °C. The remaining organic matter was kept at −20 °C for analysis by GC-MS.

2.3. Preparation of fatty acid derivatives

The fatty acids were converted to methyl esters by reaction with 5% methanolic HCl (overnight, 50 °C (Christie, 1989). After methylation, the mixture was cooled to room temperature, and the pentane-extract was washed three times with 0.9% KCl, and kept at -20 °C before analysis by GC-MS.

2.4. Apparatus and chromatographic conditions

Separation of hydrocarbons and fatty acid methyl esters was carried out with a Hewlett-Packard 5890 (series II) gas chromatograph (Palo Alto, CA), equipped with a 5971B MSD mass selective detector. Low molecular carboxylic, dioic and fatty acids were analyzed by gas chromatography using two coupled capillary columns with different stationary phases; a RTX-1 column 30 m, ID = 0.32 mm, film thickness 0.25 μm, (Restek, PA) coupled with a second capillary column RTX-1701 30 m, ID = 0.32 mm × 0.25 μm film thickness (Restek, PA), as described previously (Dembitsky et al., 1999). The GC oven program had an initial temperature of 40 °C for 2 min, 2 °C/min run to 300 °C and final hold at 300 °C (20 min). Injector temperature was kept at 180 °C (splitless), and carrier gas (helium) flow rate was 25 cm/sec. The MS detector was operated at 194 °C, scan range was from 30 to 650 m/z at 0.9 scan/sec scan rate. Solvent delay was 10 min. Fatty acids and other volatile compounds were identified by mass spectral library search (Wiley 7 Edition), followed by matching of MS data and comparison of some components with commercial standard fatty acids.

2.5. Statistical analysis

Statistical package STATISTICA 6.0 (Stat Soft, Inc, Tulsa, OK) was used for this purpose.

3. Results and discussion

The problem of the simultaneous analysis of lipid metabolites is a difficult one because of the large number of different simple lipids, low molecular acids (from C_2 to C_{10}), dioic acids, and fatty acids that may be present. As a rule, for the separation of such a complex mixture of lipid components, it is necessary to resort to preliminary separation of low molecular carboxylic and fatty acids and other products, using preparative TLC (thin-layer chromatography), or some other manipulations. This, however, results in the loss of a number of components, or their oxidation and other undesirables processes may happen. Low molecular acids ($C_{2-}C_{10}$),

dioic acids, and also other metabolites are practically detectable by GC analysis. Use of coupled columns with different degrees of polarity of stationary phases gives a satisfactory separation capability for all the above mentioned components without their preliminary separation.

The genus *Codium* attracts attention because these seaweeds contain unusual structures of some branched fatty acids (Aliya et al., 1992; Aliya and Shameel, 1993), rare and novel sterols (Sheu et al., 1995; Ahmad et al., 1993; Aknin et al., 1992a; Pollesello et al., 1992; Romeo and Toscano, 1983; Rubinstein and Goad, 1974), carotenoids (Pollesello et al., 1992; Egeland et al., 1997; Matsuno and Hirao, 1989), sulphated polysaccharides (Siddhanta et al., 1999), sulphated galactans (Matsubara et al., 2001), and halogenated and other bioactive metabolites (Dembitsky and Srebnik, 2002; Tringalli, 1997; Siddhanta and Shanmugam, 1999).

Representatives of the genus *Codium* collected in various regions of the world have been investigated regarding their content of various fatty acids (Aliya et al., 1991, 1992; Aliya and Shameel, 1993; Herbreteau et al., 1997; Vaskovsky et al., 1996; Aknin et al., 1992b; Shameel and Khan, 1991); the members of the genus investigated are presented in Table 1. Data of the quantitative analysis of fatty acids are shown in Tables 2–4, and in essence do not differ from published data. Special attention was given to dioic acids. The analysis of published data on *Codium* has shown that only Aliya and Shameel (1993) have found propane-1,3-dioic acid in *C. iyegarii*. With the usual columns, detection of dioic acids is rather problematic. However, in our experience use of coupled columns solves the problem perfectly. Doubtlessly, the greatest interest was taken in the composition of the saturated fatty acids (Table 2). The system used allows the separation and identification of the highly polar fatty acids.

The composition of monoenoic acids is shown in Table 3. Again, we came to the conclusion that our system enables the identification of considerably more monoenoic acids in the total lipid extract. It is possible to separate *trans*- and *cis*-isomers of various fatty acids. Polyenoic fatty acids (Table 4) present in various species of *Codium* are represented by a large spectrum of acids. It is difficult to interpret the absence of polyenoic acid in some species indicated by numbers 6, 10, 15. It is possible, that these are artefacts.

Special attention was paid to *C. fragile* (Table 5). This species is widespread in marine ecosystems from the shores of Australia up to northern Europe, Africa and the Black Sea. As shown in Table 5, *C. fragile* from different locations contains acid 18:4, as also do other species of this genus. The obtained results are of interest from the viewpoint of biochemistry and chemotaxonomy and also provide more complete data for the pharmaceutical industry (Ackman, 1981, 1989; Dembitsky, 1996a; Pohl and Zurheide, 1979).

Previously we described using package STATISTICA 6.0 for statistical analysis of fatty acids and triacylglycerols in vegetable and molluscs (Rezanka and Rezankova, 1999; Go et al., 2002), and showed that this procedure is effective for chemotaxonomic and related investigations. We applied this package to calculate the relationship between algal species inside the genus *Codium* grown in different marine locations from Australian, European and America coasts (Table 6).

Table 1
Codium species investigated for their content of fatty acids

DIVISION	**CHLOROPHYCOTA**		
Class	*Chlorophyceae*		
Order	Codiales		
Family	**Bryopsidaceae**		
Genus	*Codium*		
Species			
	Collected place	**Collected date**	**Reference**
1. *decorticatum*	Indian Ocean, Karachi, Pakistan	November 1988	Aliya and Shameel (1993)
2. *dichotomum*	Senegalese coast, Dakar, Senegal	July 1990	Aknin et al. (1992)
3. *duthiae*	Pacific Ocean, Victoria, Australia	July 1995	Xu et al. (1998)
4. *duthiae*	Pacific Ocean, South Australia	August 1995	Xu et al. (1998)
5. *dwarkense*	Mediterranean Sea, Israel	June 2000	Present study
6. *dwarkense*	Buleji Bay, Karachi, Pakistan	November 1989	Shameel and Khan (1991)
7. *elongatum*	Senegalese coast, Dakar, Senegal	July 1990	Aknin et al. (1992)
8. *elongatum*	North Sea, Germany	September 1966	Pohl and Zurheide (1979)
9. *flabellatum*	Buleji Bay, Karachi, Pakistan	November, 1988	Aliya and Shameel (1993)
10. *flabellatum*	Buleji Bay, Karachi, Pakistan	February 1990	Shameel and Khan (1991)
11. *galeatum*	Pacific Ocean, Victoria, Australia	February 1995	Xu et al. (1998)
12. *harveyi*	Pacific Ocean, Victoria, Australia	July 1995	Xu et al. (1998)
13. *harveyi*	Pacific Ocean, South Australia	August 1995	Xu et al. (1998)
14. *intractum*	Sea of Japan, Japan	July 1980	Kato and Ariga (1982)
15. *iyengarii*	Indian Ocean, Karachi, Pakistan	November 1988	Aliya and Shameel (1993)
16. *muelleri*	Pacific Ocean, South Australia	August 1995	Xu et al. (1998)
17. *muelleri*	Pacific Ocean, Tasmania, Australia	October 1995	Xu et al. (1998)
18. *pomoides*	Pacific Ocean, Victoria, Australia	July 1995	Xu et al. (1998)
19. *taylorii*	Mediterranean Sea, Israel	July 2000	Present study
20. *fragile*	North Sea, Germany	August 1962	Klenk et al. (1963)

(*continued on next page*)

Table 1 (*continued*)

DIVISION	**CHLOROPHYCOTA**		
Class	*Chlorophyceae*		
Order	Codiales		
Family	**Bryopsidaceae**		
Genus	*Codium*		
Species			
	Collected place	**Collected date**	**Reference**
21. *fragile*	Amur Bay, Sea of Japan, Russia	August 1991	Khotimchenko (1993)
22. *fragile*	Sea of Japan, Japan	June 1974	Sato (1975)
23. *fragile*	Sillon Bay, Cotes d'Amor, France	June 1993	Herbreteau et al. (1997)
24. *fragile*	Feodosiya Bay, Black Sea, Russia	July 1987	Dembitsky (1996)
25. *fragile*	Yellow Sea, China	October 1995	Vaskovsky et al. (1996)
26. *fragile*	Sea of Japan, Yamaguchi, Japan	May 1996	Kaneniwa et al. (1998)
27. *fragile*	Pacific Ocean, Victoria, Australia	July 1994	Xu et al. (1998)
28. *fragile*	Sea of Japan, Japan	July 1980	Kato and Ariga (1982)
29. *fragile*	Amur Bay, Sea of Japan, Russia	August 1991	Khotimchenko (1993)

Table 2
Comparison of the proportion of saturated and branched fatty acids obtained in the present study with data reported in literature for *Codium* species

Acid type	Systematic name	1[a]	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
	Ethane-1,2-dioic					0.17														0.11
	Propane-1,3-dioic															1.20				0.56
	Hexane-1,6-dioic					0.77														0.21
	Octane-1,8-dioic					0.18														0.38
	Nonane-1,9-dioic					0.32														0.31
	Total Dioic acids					**1.44**										**1.20**				**1.57**
C 5:0	2,3-Dimethyl-3:0	0.39								0.45										
C 4:0	Butanoic					0.16														0.11
C 6:0	Hexanoic					0.18														0.21
C 7:0	Heptanoic									2.73						2.07				0.33
C 8:0	Octanoic					0.23	0.49			1.12	0.48									0.27
C10:0	4,6-Dimethyl-8:0					0.27														0.24
C 9:0	Nonanoic									6.23										0.22
C10:0	Decanoic					0.25														0.31
C11:0	8-Methyl-10:0					0.24														0.18
C11:0	9-Methyl-10:0					0.21														0.26
C11:0	Undecanoic					0.20	0.98													0.19
C12:0	10-Methyl-11:0					0.19														0.17
C12:0	Dodecanoic					0.54			2.40		0.87									0.59
C14:0	Tetradecanoic		2.80	2.70	1.40	5.33	0.47	1.50	4.40		0.45	3.10	3.50	2.80	2.40	0.73	2.90	1.00	7.80	5.38
C15:0	9-Methyl-14:0					0.22														0.29
C15:0	13-Methyl-14:0					0.21														0.20
C15:0	Pentadecanoic	4.95	0.10	0.10	0.10	0.64		0.20					0.10	0.10			0.10	0.10		0.58

(*continued on next page*)

Table 2 (*continued*)

Acid type	Systematic name	1[a]	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
C16:0	Hexadecanoic	5.09	32.90	26.60	36.90	26.03	83.79	28.40	10.90	5.85	83.59	41.20	26.80	44.80	21.20	8.98	38.80	25.60	25.00	22.90
C17:0	2-Methyl-16:0					0.29														0.42
C17:0	14-Methyl-16:0					0.25														0.31
C17:0	Heptadecanoic	2.58		0.10	0.10	0.23		0.30		9.10		0.40				7.66			0.20	0.26
C18:0	Octadecanoic	4.60	0.70	1.10	1.20	1.67	2.53	1.10	2.00		2.67	2.50	0.90	1.70		3.53	1.10	0.40	1.00	1.19
C19:0	Nonadecanoic	9.59														4.40				0.24
C20:0	Eicosanoic		0.20	0.20	0.20	0.30				15.18		0.30	0.30	0.30			0.20	0.10	0.40	0.22
C21:0	Heneicosanoic					0.23				4.08						1.46				0.29
C22:0	Docosanoic	3.75		0.90	2.30				0.70	3.57		2.40	0.80	2.20		4.74	1.80	0.90		0.15
C23:0	Tricosanoic									4.75						2.07				0.18
C24:0	Tetracosanoic	3.64		0.40	0.20	0.31						0.80	0.30	0.80			0.20	0.30	0.30	0.36
C27:0	Heptacosanoic															5.30				
C29:0	Nonacosanoic	2.18																		
Total		**36.77**	**36.70**	**32.10**	**42.40**	**39.43**	**88.26**	**31.50**	**24.40**	**53.06**	**88.06**	**50.70**	**32.70**	**52.70**	**23.60**	**42.14**	**45.10**	**28.40**	**34.70**	**37.41**

[a] Species and references indicated in Table 1.

Table 3
Comparison of the proportion of monoenoic fatty acids obtained in the present study with data reported in literature for *Codium* species

Acid type	Systematic name	1[a]	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
C 9:1	(*E*)-2-Nonenoic					0.24														
C10:1	9-Decenoic	1.17																		0.16
C11:1	1D-Undecenoic	2.95														5.61				
C12:1	9-Dodecenoic	1.35					2.77				2.72					6.64				
C13:1	Tridecenoic	1.81														10.02				
C14:1	9-Tetradecenoic	8.40								1.98						3.88				
C14:1	5-Tetradecenoic		0.40					0.30												
C15:1	Pentadecenoic	2.07				0.09				3.13						0.64				0.16
C16:1	All isomers								10.20		1.75				3.20					
C16:1	5-Hexadecenoic			0.10								0.20	0.10					0.50	0.40	
C16:1	7-Hexadecenoic		2.00			0.66		3.00												0.63
C16:1	9-Hexadecenoic		0.90					1.10								2.15				
C16:1	(Z)-9-Hexadecenoic					3.62														4.83
C16:1	11-Hexadecenoic					1.32														1.07
C16:1	(*E*)-13-Hexadecenoic		0.50					1.30												
C16:1	*trans*-Hexadecenoic			1.90	2.50							2.30	2.60	2.30			4.00	0.50	0.50	
C17:1	Heptadecenoic	3.19								4.41						0.91				

(*continued on next page*)

Table 3 (*continued*)

Acid type	Systematic name	1[a]	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
C18:1	All isomers						8.92				7.43				22.90					
C18:1	7-Octadecenoic		1.00	1.70	1.20	2.83		0.90				2.80	2.70	2.80			2.40		1.40	2.91
C18:1	8-Octadecenoic					2.12														2.21
C18:1	9-Octadecenoic	4.85	14.50	13.60	9.50			11.30	18.30	2.58		12.70	13.10	15.80		6.83	19.60	18.30	15.40	
C18:1	(*Z*)-9-Octadecenoic					6.07														5.01
C18:1	(*E*)-9-Octadecenoic					0.71														0.49
C18:1	11-Octadecenoic					1.41														1.95
C19:1	Nonadecenoic									2.10										
C20:1	7-Eicosenoic			0.10	0.20							0.30	0.10	0.20			0.20	0.10	0.30	
C20:1	9-Eicosenoic	3.55	0.20	0.20					6.20	2.84		0.10						0.30	0.50	
C20:1	11-Eicosenoic		0.50																	
C21:1	Heneicosenoic									1.12										
C22:1	11-Docosenoic								0.70	4.74										
C23:1	Tricosenoic	4.20														4.57				
Total Monoenoic acids		**33.52**	**20.00**	**17.60**	**13.40**	**19.07**	**11.69**	**17.90**	**36.40**	**22.90**	**11.90**	**18.40**	**18.60**	**21.20**	**26.10**	**41.84**	**26.00**	**19.70**	**18.50**	**25.20**
C13:1	Tridecynoic															0.65				
C16:1	12-Methoxy-4-Hexadecynoic															9.22				
Total Monoynoic acids																**9.87**				

[a] Species and references indicated in Table 1.

Table 4
Comparison of the proportion of polyenoic fatty acids obtained in the present study with data reported in literature for *Codium* species

Acid type	Systematic name	1[a]	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
C11:2	3,8-Dimethyl-2,7-Nonadienoic									7.70										
C14:2	7-Ethyl-3-Methyl-Undecadienoic	2.02																		
C16:2	All isomers	2.70							3.00											
C16:2	6,9-Hexadecadienoic			1.80	0.50							0.30	1.70	0.20			1.40	3.50		
C16:2	7,10-Hexadecadienoic					0.46														0.70
C16:2	9,12-Hexadecadienoic		1.50			0.57		2.20												0.91
C17:2	Heptadecadienoic																			
C18:2	All isomers	3.80													8.90					
C18:2	8,11-Octadecadienoic					1.69														2.22
C18:2	9,12-Octadecadienoic		4.00	7.40	5.00	3.11		7.30	9.70			3.80	7.00	2.90			3.60	5.10	15.60	2.73
C18:2	10,13-Octadecadienoic					1.49														1.88
C18:2	11,14-Octadecadienoic					1.41														1.46
C20:2	11,13-Eicosadienoic					0.16														0.32
Total dienoic fatty acids		**8.52**	**5.50**	**9.20**	**5.50**	**8.89**	**0.00**	**9.50**	**12.70**	**7.70**	**0.00**	**4.10**	**8.70**	**3.10**	**8.90**	**0.00**	**5.00**	**8.60**	**15.60**	**10.22**

(*continued on next page*)

Table 4 (*continued*)

Acid type	Systematic name	1[a]	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
C11:3	Undecatrienoic	1.17																		
C12:3	3,7,10-Trimethyl-2,6,10-Dodecatrienoic	6.42								2.25										
C13:3	Tridecatrienoic	1.57																		
C16:3	7,10,13-Hexadecatrienoic		10.60		5.30	0.76		10.30				0.70	11.50	2.20			5.70	13.90	3.80	1.06
C17:3	Heptadecatrienoic	6.30								6.36										
C18:3	All isomers														21.00					
C18:3	9,12,15-Octadecatrienoic		12.00	12.70	12.20	2.65		14.60	16.40			1.50	12.30	4.30			8.00	20.80	3.60	3.41
C18:3	6,9,12-Octadecatrienoic		1.50	5.80	3.10			3.50				2.70	4.90	2.40			2.10	1.90	3.50	
C20:3	5,8,11-Eicosatrienoic					0.52														0.71
C20:3	11,14,17-Eicosatrienoic					0.75														1.07
C20:3	8,11,14-Eicosatrienoic		0.30	0.50	0.30			0.90				0.30	0.40	0.30			0.10	0.20	0.20	
C16:4	4,7,10,13-Hexadecatetraenoic		1.10					0.20		0.30										
C18:4	3,6,9,12-Octadecatetraenoic		1.40		4.40	5.48		1.80									1.80			8.09
C18:4	6,9,12,15-Octadecatetraenoic			1.40	3.40	0.26			4.90			1.90	1.30	3.30	1.50		1.20	1.30	0.30	

Table 4 (*continued*)

Acid type	Systematic name	1[a]	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
C20:4	5,8,11,14-Eicosatetraenoic		3.90	5.00	4.20	4.90		4.30	4.80			10.90	4.00	2.90	4.50		1.80	3.60	4.50	4.03
C20:4	8,11,14,17-Eicosatetraenoic		2.60	0.10	0.20			2.70					0.10					0.10	0.20	
C20:5	5,8,11,14,17-Eicosapentaenoic		2.10	1.20	1.90	5.48		1.70	3.60			2.00	1.10	0.60	4.00		0.60	1.50	4.50	4.25
C22:4	7,10,13,16-Docosatetraenoic			0.30	0.10								0.10	0.20			0.10		0.10	
C22:5	7,10,13,16,19-Docosapentaenoic		1.60	0.50				0.40				3.20								
C22:6	4,7,10,13,16,19-Docosahexaenoic			0.10				0.20				0.50								
C29:3	Nonadecatrienoic	5.65								7.70										
Total polyenoic fatty acids		**21.11**	**37.10**	**37.80**	**35.10**	**20.80**	**0.00**	**40.30**	**29.70**	**16.31**	**0.00**	**24.00**	**35.70**	**16.20**	**44.30**	**0.00**	**21.40**	**43.30**	**20.70**	**21.55**
Other and non-identified fatty acids		0.08	0.07	3.30	3.60		0.05	0.08	1.80	7.73	0.04	2.80	4.30	6.90	19.30	6.15	2.50		10.50	

[a] Species and references indicated in Table 1.

Table 5
Comparison of the proportion of fatty acids reported in literature for the *C. fragile* collected from different locations

Acid type	Systematic name	20[a]	21	22	23	24	25	26	27	28	29
C10:0	Decanoic				48.60						
C12:0	Dodecanoic				0.70	1.10					
C14:0	Tetradecanoic	0.80	1.50	3.60	2.50	3.25	1.30	1.60	5.30	2.30	1.30
C15:0	Pentadecanoic		0.10			0.68			0.20		
C16:0	14-Methyl-15:0		2.40			0.32					
C16:0	Hexadecanoic	28.10	26.20	38.00	19.70	19.96	22.40	34.60	40.50	17.90	17.50
C17:0	Heptadecanoic	0.10				0.33			0.30		
C18:0	Octadecanoic	0.90	0.90			1.25	0.90	1.20	0.70		
C20:0	Eicosanoic	0.50				1.14			0.20		
C22:0	Docosanoic	3.20	2.90				0.20	2.60	1.10		
C24:0	Tetracosanoic					0.29		1.40	0.50		
Total Saturated		**33.60**	**34.00**	**41.60**	**71.50**	**28.32**	**24.80**	**41.40**	**48.80**	**20.20**	**18.80**
C16:1	All isomers	1.60		7.20	2.30	2.86				3.20	2.90
C16:1	7-Hexadecenoic		1.50				3.30	2.50	2.30		
C16:1	9-Hexadecenoic		0.60								
C16:1	*trans*-16:1		0.50			0.70	1.90		0.80		
C18:1	All isomers			14.20	6.20	15.35				22.90	17.70
C18:1	5-Octadecenoic								0.20		
C18:1	7-Octadecenoic		1.00				1.10		2.10		
C18:1	9-Octadecenoic	10.80	9.90				5.20	12.50	23.30		
C20:1	7-Eicosenoic								0.70		
C20:1	9-Eicosenoic								0.90		
C22:1	Docosenoic	3.20									
Total Monoenoic		**15.60**	**13.60**	**21.40**	**8.50**	**18.91**	**11.50**	**15.00**	**30.30**	**26.10**	**20.60**
C16:2	All isomers	0.90				2.28		1.40	1.20		
C16:2	7,10-Hexadecadienoic		2.80				1.00				
C16:3	All isomers	12.20		10.20							
C16:3	7,10,13-Hexadecatrienoic		9.40				12.60		3.30		
C18:2	All isomers			4.00	3.60	5.78				8.90	11.00
C18:2	9,12-Octadecadienoic	5.50	8.90				5.40	5.10	4.00		
C18:3	All isomers	27.20		8.50	12.20	24.90				21.00	22.50
C18:3	9,12,15-Octadecatrienoic		15.10				19.10	15.40	5.60		
C18:3	6,9,12-Octadecatrienoic		2.30				2.60	1.90	0.90		

(*continued on next page*)

Fig. 1 shows the results of cluster analysis. Euclidean distance and Ward's method for joining clusters were used for standardized data. Euclidean distance is the geometric distance in a multidimensional space. For two objects i and i' it is computed as: $d_{ii'} = \sqrt{\Sigma_{j=1}^{p}(x_{ij} - x_{i'j})^2}$ Where p is a number of variables.

Table 5 (*continued*)

Acid type	Systematic name	20[a]	21	22	23	24	25	26	27	28	29
C20:3	8,11,14-Eicosatrienoic		0.40						0.20		
C18:4	All isomers			1.00		1.39				1.50	1.90
C18:4	6,9,12,15-Octadecatetraenoic	1.50	1.40				2.00	1.30	0.30		
C20:4	All isomers	3.00				12.47					
C20:4	8,11,14,17-Eicosatetraenoic		0.20		4.20		0.50	0.20	0.10		6.00
C20:4	5,8,11,14-Eicosatetraenoic		5.20	0.40			5.90	2.30	1.50	4.50	10.10
C20:5	All isomers					3.49					
C20:5	5,8,11,14,17-Eicosapentaenoic	1.90	3.40	0.40			8.40	1.80	0.50	4.00	
C22:5	7,10,13,16,19-Docosapentaenoic		0.90			0.94		0.30			
Total Polyenoic		**52.20**	**52.50**	**36.60**	**20.00**	**51.25**	**66.30**	**43.60**	**21.40**	**53.70**	**60.60**
Other and non-identified fatty acids			2.50	12.14		1.52	6.20	13.90	3.30	13.35	9.10

[a] Species of marine algae and references indicated in Table 1.

Ward's method uses the analysis of variance approach to evaluate the distances between clusters. This method attempts to minimize the sum of squares of any two (hypothetical) clusters that can be formed at each step.

Two important conclusions may be drawn from this figure. First, the expected taxonomical affinity of the same kinds of *Codium* sp. (predominantly *C. fragile*) was confirmed experimentally. The taxonomical affinity was independent of the time and place of sample collection. As an illustrative example, we can use similarities between *C. fragile* (24) found in the Black Sea and *C. fragile* (28) collected from the Pacific Ocean near Japan, or two samples of *C. fragile* (23 and 29) gathered from the coasts of the Atlantic (France) and Pacific Oceans (Far East of Russia). Secondly, the cluster analysis uncovered the bio-geographical affinity of the fatty acid content of *Codium* sp. An example is the similarity of a closed group of five different strains [*C. decorticatum* (1), *C. dwarkense* (5), *C. dwarkense* (6), *C. flabellatum* (9), and *C. flabellatum* (10)] gathered from the north coast of the Indian Ocean in Pakistan compared with distant relative strains of *C. fragile* (23, 29). Similarly, the strains of *C. dwarkense* (5) and *C. taylorii* (19) collected from the Mediterranean Sea show a distinct kinship in fatty acid content. The other isolated cluster constitutes the species to be found along the Atlantic Ocean coasts, such as *C. dichotomum* (2) and *C. elongatum* (7) from Senegal and *C. elongatum* (8) from the North Sea. In order to support our working hypothesis, we identified a close group of similar species that is located in the Pacific Ocean near Australia. The strains of *C. fragile* (21 and 25) serve as an exception to the rule. The fatty acid content of both strains

Table 6
Statistical analysis of selected fatty acids isolated from different species of *Codium*

Fatty acids	14:0	15:0	16:0	17:0	18:0	19:0	20:0	22:0	24:0	Saturated[b]	16:1[a]	18:1[a]	20:1[a]	Mono-enoic[b]	18:2	Dienolc[b]	16:3	18:3n3	18:3n6	18:4[a]	20:4[a]	20:5	Polyenoic[b]
Number of values	19	19	19	19	19	19	19	19	19	19	19	19	19	19	19	19	19	19	19	19	19	19	19
Minimum	0.0	0.0	5.10	0.0	0.0	0.0	0.0	0.0	0.0	23.60	0.0	2.58	0.0	11.69	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
25% Percentile	0.85	0.0	22.05	0.0	0.95	0.0	0.0	0.0	0.0	32.40	0.0	9.81	0.0	18.15	3.25	4.55	0.0	0.75	0.0	0.15	1.05	0.30	18.51
Median	2.70	0.10	26.60	0.10	1.19	0.0	0.20	0.80	0.20	37.41	0.20	15.30	0.20	19.70	5.00	8.52	0.76	4.30	1.50	1.40	4.10	1.50	21.51
75% Percentile	7.80	0.15	40.00	0.35	2.51	0.0	0.30	2.35	0.38	51.70	4.40	18.30	0.75	26.05	7.99	9.35	8.00	12.50	3.30	4.10	5.05	3.80	37.45
Maximum	7.80	4.98	83.79	9.10	4.60	9.59	15.18	4.74	3.64	88.26	10.20	22.90	6.20	41.84	15.60	15.60	13.90	20.80	5.80	8.09	10.90	5.48	44.30
Mean	2.582	0.374	31.330	1.102	1.573	0.748	1.037	1.274	0.401	43.16	1.912	13.59	0.836	22.04	5.268	6.933	3.464	7.129	1.65 3	2.299	3.681	1.817	24.49
Std. deviation	2.118	1.130	21.640	2.641	1.179	2.366	3.454	1.497	0.824	17.990	2.958	5.612	1.624	7.901	4.026	4.232	4.702	6.632	1.874	2.568	2.875	1.749	14.120
Std. error	0.486	0.259	4.965	0.606	0.270	0.543	0.79	0.343	0.189	4.126	0.678	1.287	0.373	1.813	0.924	0.971	1.079	1.522	0.429	0.589	0.659	0.401	3.240
Lower 95% Confidence Intervals	1.561	-0.171	20.900	-0.171	1.005	-0.391	-0.628	0.553	0.003	34.500	0.487	10.89	0.053	18.24	3.328	4.893	1.198	3.933	0.749	1.062	2.295	0.974	17.690
Upper 95% Confidence Intervals	3.602	0.919	41.770	2.374	2.141	1.889	2.701	1.996	0.797	51.830	3.338	16.300	1.619	25.850	7.208	8.973	5.730	10.330	2.556	3.537	5.066	2.660	31.300
KS Distance	0.1402	0.4032	0.1855	0.4469	0.2032	0.4799	0.4679	0.2303	0.3424	0.2012	0.3151	0.1460	0.3510	0.1810	0.1152	0.1725	0.2744	0.1915	0.2848	0.2460	0.1529	0.1727	0.1421
P value	>0.10	>0.004	>0.10	>0.001	>0.10	>0.0003	>0.0005	>0.10	>0.023	>0.10	>0.046	>0.10	>0.0185	>0.10	>0.10	>0.10	>0.10	>0.10	>0.10	>0.10	>0.10	>0.10	>0.10

[a] All isomers

[b] Total of each types of fatty acids

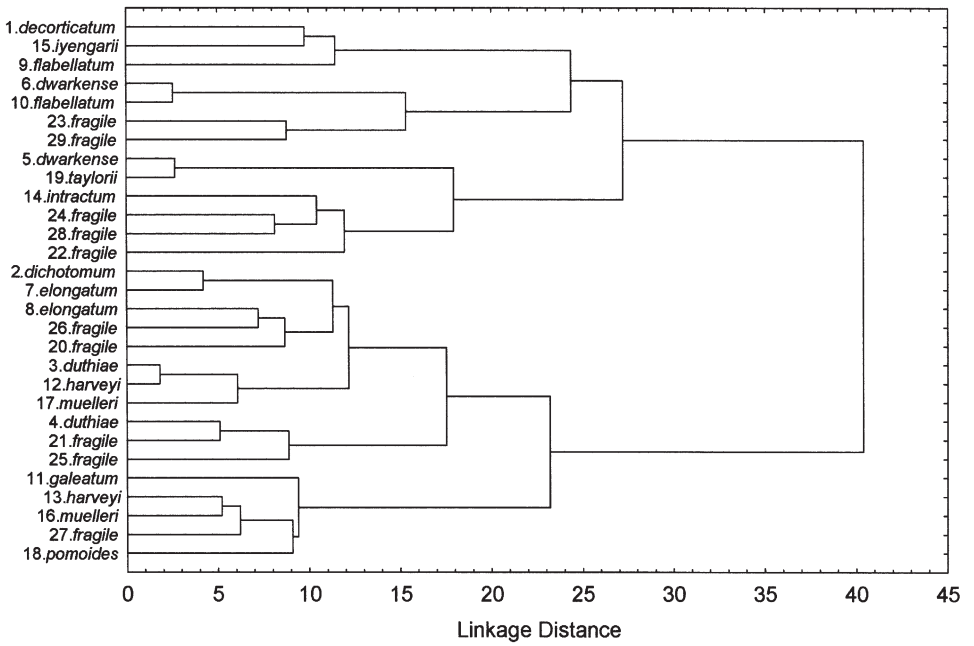

Fig. 1. Dendrogram from hierarchical cluster analysis of twenty nine species of *Codium*.

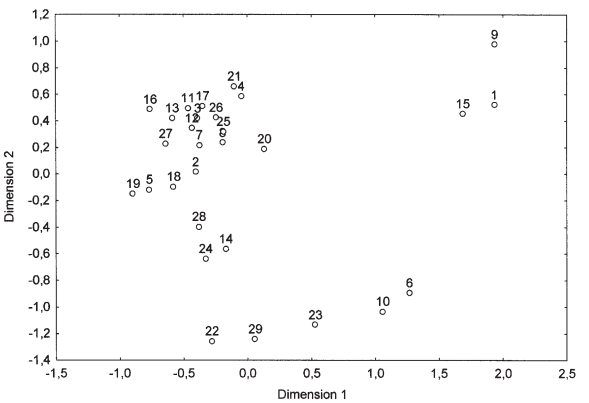

Fig. 2. Two-dimensional graph of the final configuration from a multidimensional scaling of twenty nine species of *Codium*.

matches the "Australian group". However, they were collected thousands of miles away. The strain *C. fragile* (21) was gathered from the coast of the Yellow Sea (China) and the other one in Russia (Japan Sea coast). To conclude, the results of cluster analysis revealed a strong relationship between bio-geographical conditions and fatty acid content. Environmental effects such as salinity, temperature, nutrition, solar radiation and taxonomical affinity are very important.

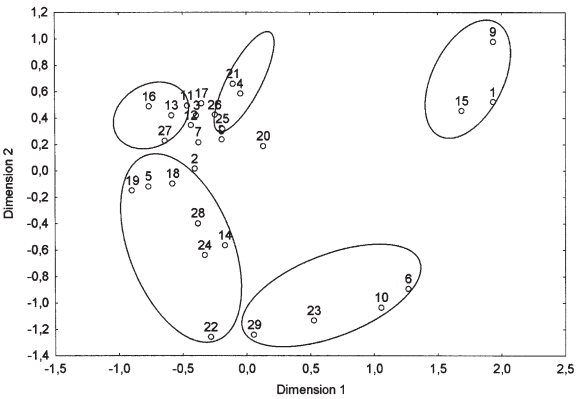

Fig. 3. Two-dimensional graph of a final configuration from the multidimensional scaling of twenty nine species of *Codium*. Circles correspond to some clusters in Fig. 1 (Some of them correspond to geographical places).

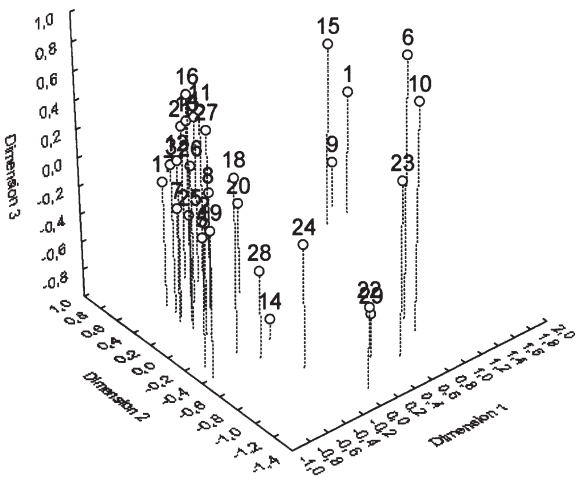

Fig. 4. Three-dimensional graph of a final configuration from the multidimensional scaling of twenty nine species of *Codium*.

Similar results were obtained by multidimensional scaling. The same proximity matrix as that for cluster analysis was used on the basis of the Euclidean distance for standardized data (Figs 2–4).

Acknowledgements

The authors thank Prof. I. Dor (Environmental Division, Hebrew University) for help in the preparation and identification of marine algae.

References

Ackman, R.G. (Ed.), 1989. Marine Biogenic Lipids, Fats and Oils. Vols. 1 & 2. CRC Press Inc, Boca Raton, FL.

Ackman, R.G., 1981. Algae as sources for edible lipids. In: Pryde, E.H., Princen, L.H., Mukheriee, K.D. (Eds.), New Sources of Fats and Oils. Am. Oil Chem. Soc, pp. 189–220.

Ahmad, V.U., Aliya, R., Perveen, S., Shameel, M., 1993. Sterols from the marine green alga *Codium decorticatum*. Phytochemistry 33, 1189–1192.

Aknin, M., Moellet-Nzaou, R., Cisse, E., Komprobst, J.M., Gaydou, E.M., Samb, A., Miralles, J., 1992a. Fatty acid composition of 12 species of Chlorophyceae from the Senegalese coast. Phytochemistry 31, 2739–2741.

Aknin, M., Moellet-Nzaou, R., Komprobst, J.M., Gaydou, E.M., Samb, A., Miralles, J., 1992b. Sterol composition of 12 Chlorophyceae from the Senegalese coast and their chemotaxonomic significance. Phytochemistry 31, 4167–4169.

Aliya, R., Shameel, M., 1993. Phycochemical examination of three species of Codium (Bryopsidophyceae). Botanica Marina 36, 371–376.

Aliya, R., Shameel, M., Usmanghani, K., Ahmad, V.U., 1992. Comparative composition of fatty acids of *Codium decorticatum* and *Codium flabellatum* (Bryopsidophyceae). Pakistan J Pharmaceutical Sci. 5, 87–99.

Aliya, R., Shameel, M., Usmanghani, K., Ahmad, V.U., 1991. Analysis of fatty acids from *Codium iyngaria* (Bryopsidophyceae). Pakistan J Pharmaceutical Sci. 4, 103–111.

Benicka, E., Krupcik, J., Kulovsky, P., Repka, D., Garaj, J., 1990. Computer assisted optimization of selectivity by tuning temperature and carrier gas-pressure drop in two GC capillary columns coupled in series. Mikrochim. Acta 3, 1–10.

Christie, W.W., 1998a. Gas chromatography mass spectrometry methods for structural analysis of fatty acids. Lipids 33, 343–351.

Christie, W.W., 1998b. Some recent advances in the chromatographic analysis of lipids. Analysis Magazine 26, M34–M40.

Christie, W.W., 1989. Gas Chromatography and Lipids—A Practical Guide. The Oily Press, Ayr, UK pp. 64-84.

Dembitsky, V.M., 1996. Alkoxylipids of the organic world: chemistry and biology. Moscow Academy of the Fine Chemical Technology, Moscow, pp. 334.

Dembitsky, V.M., 1996a. Betaine ether-linked glycerolipids: chemistry and biology. Prog. Lipid Res. 35, 1–51.

Dembitsky, V.M., Srebnik, M., 2002. Natural halogenated fatty acids: their analogues and derivatives. Prog. Lipid Res. 41, 315–367.

Dembitsky, V.M., Rozentsvet, O.A., Pechenkina, E.E., 1990. Glycolipids, phospholipids and fatty acids of brown algae species. Phytochemistry 29, 3417–3421.

Dembitsky, V.M., Pechenkina-Shubina, E.E., Rozentsvet, O.A., 1991. Glycolipids and fatty acids of some seaweeds and marine grasses from the Black Sea. Phytochemistry 30, 2279–2283.

Dembitsky, V.M., Rozentsvet, O.A., Zhuikova, V.S., Vasilenko, R.F., Kashin, A.G., 1992. Lipid composition of freshwater macrophytes from the Volga River estuary. Phytochemistry 31, 3259–3261.

Dembitsky, V.M., Shkrob, I., Dor, I., 1999. 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. J. Chromatogr. 862, 221–229.

Dembitsky, V.M., Rozentsvet, O.A., 1996. Distribution of polar lipids in some marine, brackish and freshwater green macrophytes. Phytochemistry 41, 483–488.

Egeland, E.S., Guillard, R.R., Liaaen-Jensen, S., 1997. Algal carotenoids. 63. Carotenoids from Prasinophyceae. 7. Additional carotenoid prototype representatives and a general chemosystematic evaluation of carotenoids in Prasinophyceae (Chlorophyta). Phytochemistry 44, 1087–1097.

Engewald, W., Maurer, T., 1990. Enhanced possibilities for identification by the use of series-coupled capillary gas-chromatographic columns. 1. General exposition and application of the retention index concept. J. Chromatogr. 520, 3–13.

Herbreteau, F., Coiffard, L.J.M., Derrien, A., De Roeck-Holtzhauer, Y., 1997. The fatty acid composition of five species of macroalgae. Bot. Marina 40, 25–27.

Go, J.V., Rezanka, T., Srebnik, M., Dembitsky, V.M., 2002. Variability of fatty acid components of marine and freshwater gastropod species from the littoral zone of the Red Sea, Mediterranean Sea, and Sea of Galilee. Biochem. Syst. Ecol. 30, 819–835.

Kaneniwa, M., Kaminishi, Y., Kunimoto, M., 1998. Fatty acid compositions of nineteen species of marine algae mainly obtained from the Yamaguchi prefecture coast. J. National Fish. Univ. 46, 191–195.

Kato, M., Ariga, N., 1982. Fatty acid compositions of some Japanese algal species. *Kyoyobu Kenkyu Hakoku* (*Gifu Daigaku*) 18, 53–62 (In Japanese).

Khotimchenko, S.V., 1993. Fatty acids of green macrophytic algae from the Sea of Japan. Phytochemistry 32, 1203–1207.

Klenk, K., Knipprath, W., Eberhagen, D., Koof, H.P., 1963. Inhaltsstoffe von algen. II: Über die unterschiedliche fettsäurezusammensetzung von salz- und süßwasseralgen. Hoppe-Seyler's Z. Physiol. Chem. 334, 44–59.

Lou, X., Liu, X., Zhou, L., 1993. Series-coupled capillary columns for the separation of N,(O)-trifluoroacetyl isopropyl derivatives of D,L-aspartic acid and L-hydroxyproline by gas chromatography. J. Chromatogr. 634, 281–288.

Matsubara, K., Matsuura, Y., Bacic, A., Liao, M.-L., Hori, H., Miyazawa, K., 2001. Anticoagulant properties of a sulfated galactan preparation from a marine green alga, *Codium cylindricum*. Internat. J. Biol. Macromol. 28, 395–399.

Matsuno, T., Hirao, S., 1989. Marine carotenoids. In: Ackman, R.G. (Ed.), Marine Biogenic Lipids, Fats and Oils. Vol. 1. CRC Press, FL, pp. 251–388.

Maurer, T., Engewald, W., Steinbom, A., 1990. Enhanced possibilities for identification using series-coupled capillary gas-chromatographic columns. 2. Retention indexes as an identification tool in selectivity tuning. J. Chromatogr. 517, 77–86.

McClosky, J.A., 1971. Mass spectrometry of fatty acid derivatives. In: Gunstone, F.D. (Ed.), Topics in Lipid Chemistry. Vol. 1. Logos Press, Ltd, pp. 369–440.

Peters, K.E., Moldowan, J.M. (Eds). 1993. The Biomarker Guide: Interpreting Molecular Fossils in Petroleum and Ancient Sediments, Prentice Hall, NJ, pp. 363.

Pohl, P., Zurheide, F., 1979. Fatty acids and lipids of marine algae and the control of their biosynthesis by environmental factors. In: Hoppe, H.A., Levring, T., Tanaka, Y. (Eds.), Marine Algae in Pharmaceutical Science. Walter de Gruyter, Berlin—N.Y, pp. 473–523.

Pollesello, P., Toffanin, R., Murano, E., Paoletti, S., Rizzo, R., Kvam, B.J., 1992. Lipid extracts from different algal species—^{1}H -NMR and ^{13}C-NMR spectroscopic studies as a new tool to screen differences in the composition of fatty acids, sterols and carotenoids. J. Appl. Phycol. 4, 315–322.

Rastogi, S.C., 1993. Gas chromatographic analysis of organic-solvent mixtures on capillary columns of different polarity. Chromatographia 37, 211–214.

Repka, D., Krupcik, J., Benicka, E., Leclercq, P.A., Rijks, J.A., 1989. Optimization of basic parameters in temperature-programmed gas-chromatographic separations of multi-component samples within a given time. J. Chromatogr. 463, 243–251.

Rezanka, T., Rezankova, H., 1999. Characterization of fatty acids and triacylglycerols in vegetable oils by gas chromatography and statistical analysis. Analytica Chimica Acta 398, 253–261.

Romeo, G., Toscano, M.A., 1983. Isolation and characterization of (24S)-24-methylcholesta-5,25-dien-3β-ol from the siphonous marine alga *Codium bursa*. J. Nat. Prod. 46, 187–189.

Rozentsvet, O.A., Dembitsky, V.M., Zhuikova, V.S., 1995. Lipids from macrophytes of the middle Volga. Phytochemistry 38, 1209–1213.

Rubinstein, I., Goad, L.J., 1974. Sterols of the siphonous marine alga *Codium fragile*. Phytochemistry 13, 481–484.

Sato, S., 1975. Fatty acid compositions of lipids in some species of marine algae. Bull. Jap. Soc. Sci. Fish. 41, 1177–1183.

Shameel, M., Khan, R., 1991. Fatty acid composition of nine green seaweeds. Botanica Marina 34, 501–504.

Sheu, J.H., Liaw, C.C., Duh, C.Y., 1995. Oxygenated clerosterols isolated from the marine alga *Codium arabicum*. J. Nat. Prod. 58, 1521–1526.

Siddhanta, A.K., Shanmugam, M., Mody, K.H., Goswami, A.M., Ramavat, B.K., 1999. Sulphated polysaccharides of *Codium dwarkense* Boergs from the west coast of India: chemical composition and blood anticoagulant activity. Internat. J. Biol. Macromol. 26, 151–154.

Siddhanta, A.K., Shanmugam, M., 1999. Metabolites of tropical marine algae of the family Codiaceae (Chlorophyta): Chemistry and Bioactivity. J. Indian Chem. Soc. 76, 323–334.

Tringalli, C., 1997. Bioactive metabolites from marine algae: recent results. Current Org. Chem 1, 375–394.

Vaskovsky, V.E., Khotimchenko, S.V., Xia, B., Hefang, L., 1996. Polar lipids and fatty acids of some marine macrophytes from the Yellow Sea. Phytochemistry 42, 1347–1356.

Volkman, J.K., Barrett, S.M., Blackburn, S.I., Mansour, M.P., Sikes, E.L., Gelin, F., 1998. Microalgal biomarkers: a review of recent research developments. Org. Geochem. 29, 1163–1179.

Xu, X-Q., Tran, V.H., Kraft, G., Beardall, J., 1998. Fatty acids of six Codium species from southeast Australia. Phytochemistry 48, 1335–1339.

Williams, R.G., Mitchell, H.D., 1991. Computer spreadsheet calculation of the optimum temperature and column lengths for serially coupled capillary gas-chromatographic columns. J. Chromatogr. 541, 59–74.