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Very long chain polyunsaturated fatty acids in crustacea of the order Bathynellacea

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Abstract

Total lipids of three crustacean species, *Bathynella natans*, *B. baicalensis* and *Baicalobathynella magna* from Lake Baikal and the caves of Central Europe were analyzed for very long chain polyunsaturated fatty acids (VLCPUFA) using TLC (silver nitrate impregnated silica gel) and gas chromatography–mass spectrometry (GC–MS). The VLCPUFA in the total lipids were mainly n-6 and n-3 PUFA, i.e. 24 : 3n6, 24 : 3n3, 24 : 5n3, 26 : 3n6, 26:5n6 and 28 : 7n6. The major VLCPUFA components in all three samples were 24 : 3n6 (0.4–0.5% of total fatty acids) and 24 : 3n3 (~1%), but 26 : 5n6 (0.08%) was also prominent in the species from Lake Baikal. The total lipids of the Lake Baikal species contained considerably more 26 : 5n6 and 28 : 7n6 than those from caves. The differences among the VLCPUFA in these species appear to be due mainly to different environmental conditions, dietary habits and geographical spreading.

Keywords: Very long chain polyunsaturated fatty acids; Gas chromatography–mass spectrometry; *Bathynella*; *Baicalobathynella*; Crustacea

1. Introduction

Studies of very long chain fatty acids (chain length $> C_{22}$) have been reported (Řezanka, 1989; Jie et al., 1997). The occurrence in biological materials of VLCPUFA that have more than two unsaturated bonds has also been recorded (Řezanka, 1989; Poulos, 1995; Djerassi and Lam, 1991). In 1970, the presence of VLCPUFA in herring (*Clupea harengus*) was first mentioned (Linko and Karinkanta, 1970) and, within 25 years, these compounds were detected in other vertebrate tissues (Poulos, 1995; Moser

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and Moser, 1996). VLCPUFA having chains as long as C_{30} have been found in marine sponges (Bergquist et al., 1984; Lawson et al., 1984).

As part of a study of organisms that live under unusual conditions we investigated crustacean species belonging to the Syncacarides that are characterised by a primitive organisation and ancient origin. All the living syncarids are fresh-water animals. They live in alpine lakes, rivers and groundwater, i.e. in the depths of limestone caves. Representatives of the class Bathynellacea are among the most characteristic syncarids. Their small size (usually up to 2 mm, *Bacialobathynella magna* up to 5.4 mm), thin vermicular body and short extremities enable them to pass through narrow pores and among subsoil particles.

Together with groundwater they penetrate into wells, cave water reservoirs and sometimes also into the natural water layers in great lakes. When the water temperature is low enough, they can live even under conditions like these. In 1886 the Czech zoologist F. Vejdovský discovered the first Bathynellacea in Prague – *Bathynella natans* (Vejdovsky, 1888). For another 28 years nobody succeeded in finding these crustacea again. Later, in connection with the improvement of methods for investigation of ground water, Bathynellacea were discovered in many countries and found to be widespread. Nowadays they are known from many places in Europe, Africa, Madagascar, Asia (except its northern part), Japan, New Zealand, Australia, northern of USA and South America. In Russia they were found in caves in the Caucasus, in central Asia and the Far East, in wells close to the river Don and in the Baikal Lake. So far 72 species and subspecies of Bathynellacea have been described. The majority of them were discovered at places where the groundwater penetrates under the surface. In the Baikal Lake *Bathynella baicalensis* and *Bacialobathynella magna* live at great depths.

Crustacea are known to contain large amounts of PUFA, but VLCPUFA are recorded for the first time in the above mentioned very primitive species of the order Bathynellacea.

2. Experimental

Bathynella baicalensis and *Bacialobathynella. magna* from Lake Baikal (Russia) were collected in 1997 and sent to Prague in sealed ampoules in methanol. *Bathynella natans* was collected in caves near Srbsko near Prague. The samples were stored and transferred frozen.

Fatty acid methyl esters (FAME) were prepared by the basic transesterification of the total lipids that had been extracted from the crustacea using a mixture of $CHCl_3$–MeOH (Christie, 1989). The oxazolines were prepared by modification of the literature methods (Fay and Richli 1991). Oxygen was removed from the TLC mobile phase by bubbling with helium. Butylated hydroxytoluene was added to the other solvents (70 mg/L). All the vessels and the rotary evaporator were flushed with nitrogen before use. For qualitative study, preparative TLC was employed (10 mg of esters was separated on a 20×20 cm plate coated with 0.5 mm silica gel containing 10% (w/w) silver nitrate) using the solvent system n-hexane-diethyl ether (2 : 3, by vol). After

visualising under UV (254 nm), the components with 4–7 double bonds (fraction IV) were eluted from the adsorbent with $CHCl_3$–MeOH (9 : 1, v/v). The fraction contaminated with silver ions was eliminated from the extract by washing with dilute ammonia (pH ~ 9). However, only the original fatty acid samples were used for quantitation.

Gas chromatography–mass spectrometry–chemical ionisation. The total FAMEs, total oxazolines and fraction IV after separation on silver nitrate impregnated silica layers were identified using a Finnigan MAT 1020 B spectrometer (Finnigan MAT, San Jose, CA). A 60 m × 0.25 mm Supelcowax 10 column with a film thickness of 0.25 μm (Supelco, Gland, Switzerland) was used. Injection temperature was 100°C. The temperature program was as follows: 100°C for 1 min, then 100°C–230°C over 7 min, 230°–280°C over 25 min and 280°C for 10 min. The carrier gas was helium at a linear flow rate of 70 cm/s. The spectra were scanned within m/z 60–700.

3. Results and discussion

Fatty acids isolated from all three genera of crustacea were identified and quantified in the form of both methyl esters and oxazolines by using GC–MS. Unfortunately, it is well known that the majority of fatty acid derivatives are eluted at higher temperatures, that may cause their thermal degradation (Christie, 1989). This affects the response of the detector and thereby the data on their quantitative abundance, especially in the case of PUFA. This is the reason why the quantification was performed in all cases using methyl esters. On the other hand, oxazolines were used for qualitative assessment in the part of the sample containing fatty acids up to 22 carbon atoms in length. This method has been successfully employed many times (Yang et al., 1989; Yu et al., 1989; Dobson and Christie, 1996) for similar types of fatty acids. The structure of fatty acids, with the exception of VLCPUFA, was determined on identification of mass spectra of oxazolines.

The rest of the fatty acids were determined qualitatively as methyl esters in the enriched PUFA fraction (see Experimental). These methyl esters were identified using the method described by (Fellenberg et al., 1987). Methyl esters of a number of different fatty acids of the n3 family are reported to give a characteristic fragment at $m/z = 108$, while those of the n6 series give a prominent ion at $m/z = 150$. Fatty acid methyl esters of the n9 family would therefore be expected to have an abundant ion at $m/z = 192$. Because of their relatively low abundance this method was used for qualitative identification of the enriched fraction, while their quantitative abundance was determined, as was mentioned above, just in the case of total methyl esters.

More than 100 fatty acids were determined (see Table 1 and also Fig. 1). The most frequent are fatty acids traditionally present in all organisms, i.e. 16 : 0, 16 : 1, 18 : 0, 18 : 1n9, 18 : 2n6 and 18 : 3n3 acids. Together with the common acids all three species examined also contain less common acids. These are especially branched fatty acids of both the iso and anteiso type. The presence of pristanic and phytanic acids can be

Table 1
Fatty acid composition of three Syncarides (crustacea)

Peak no.	Fatty acid	*B. natans*	*B. baicalensis*	*B. magna*
1	i-12:0	0.184	0.135	0.131
2	12:0	0.722	0.305	0.306
3	12:1n5	0.848	0.121	0.160
4	i-13:0	0.339	0.121	0.124
5	ai-13:0	0.088	0.163	0.211
6	13:0	0.332	0.475	0.517
7	13:1n5	0.162	0.085	0.051
8	i-14:0	0.140	0.362	0.357
9	14:0	0.523	2.269	2.083
10	14:1n5	0.965	1.163	0.920
11	i-15:0	0.405	0.255	0.255
12	14:2n5	0.007	0.206	0.248
13	ai-15:0	0.192	0.468	0.466
14	15:0	0.862	1.255	1.238
15	15:1n9	0.184	0.461	0.350
16	15:1n7	0.737	0.581	0.677
17	i-16:0	0.287	0.269	0.342
18	pristanic	0.111	0.007	0.007
19	16:0	4.466	5.886	5.280
20	16:1n9	4.333	2.106	2.527
21	16:1n7	0.649	2.510	2.345
22	t-16:1n13	0.007	0.007	0.000
23	i-17:0	0.339	0.085	0.080
24	ai-17:0	0.177	0.199	0.197
25	16:3n6	1.187	1.142	0.816
26	i-17:1n9	0.442	0.099	0.138
27	16:2n4	1.245	2.716	1.668
28	ai-17:1n9	0.074	0.064	0.087
29	phytanic	0.560	0.539	0.554
30	17:0	0.354	0.220	0.218
31	17:1n9	0.059	0.121	0.102
32	17:1n7	0.007	0.206	0.248
33	16:3n3	1.828	1.163	1.253
34	16:4n3	0.943	1.518	1.442
35	18:0	2.837	1.681	1.326
36	18:1n11	1.245	2.716	1.668
37	18:1n9	10.123	3.014	3.627
38	18:1n7	0.442	2.291	1.020
39	18:2n6	5.070	8.346	8.456
40	ai-19:0	0.066	0.057	0.036
41	18:3n6	4.349	0.801	0.663
42	19:0	0.700	0.496	0.488
43	19:1n8	0.162	0.064	0.058
44	18:3n3	4.584	5.517	8.339
45	18:4n3	1.916	4.205	3.700
46	18:4n1	0.914	0.427	0.393
47	20:0	1.069	1.801	1.581
48	18:5n3	0.627	0.491	0.585
49	20:1n11	0.273	0.511	0.400
50	20:1n9	0.501	0.631	0.575
51	20:2n6	0.442	2.291	1.020
52	20:3n9	0.435	0.319	0.503

—*continued*

Table 1—*continued*

Peak no.	Fatty acid	*B. natans*	*B. baicalensis*	*B. magna*
53	20:3n6	0.162	0.638	0.517
54	21:0	0.162	0.085	0.087
55	20:4n6	1.083	2.071	2.498
56	21:1n8	0.044	0.021	0.073
57	20:3n3	0.391	0.922	0.546
58	20:4n3	2.270	1.489	1.996
59	20:5n3	3.744	3.737	4.712
60	22:0	0.413	0.191	0.597
61	22:1n11	4.333	2.106	2.527
62	22:1n9	0.649	2.510	2.321
63	22:2n6	0.162	0.064	0.058
64	22:3n6	0.162	0.340	0.524
65	22:4n6	0.027	0.020	0.021
66	22:3n3	0.044	0.298	0.168
67	23:1n9	0.848	0.121	0.160
68	22:5n6	1.083	0.408	0.505
69	22:4n3	0.243	0.085	0.299
70	22:5n3	7.264	5.406	6.535
71	24:0	1.688	2.447	2.200
72	22:6n3	11.370	9.618	9.614
73	24:1n11	0.162	0.085	0.071
74	24:1n9	0.884	0.993	1.092
75	24:3n6	0.383	0.443	0.510
76	24:4n6	0.021	0.003	0.005
77	24:3n3	0.960	1.163	0.925
78	24:5n6	0.026	0.017	0.013
79	24:4n3	0.029	0.027	0.030
80	24:5n3	0.094	0.063	0.066
81	24:6n3	0.062	0.022	0.021
82	26:1n11	0.037	0.033	0.007
83	26:1n9	0.516	0.425	0.369
84	26:2n6	0.007	0.006	0.005
85	26:3n6	0.088	0.078	0.017
86	26:4n6	0.006	0.006	0.000
87	26:5n6	0.009	0.086	0.080
88	26:4n3	0.018	0.008	0.014
89	26:6n6	0.006	0.010	0.009
90	26:5n3	0.006	0.004	0.004
91	26:6n3	0.005	0.008	0.008
92	28:3n6	0.001	0.006	0.003
93	28:4n6	0.007	0.015	0.017
94	28:5n6	0.007	0.004	0.001
95	28:6n6	0.003	0.005	0.004
96	28:7n6	0.002	0.020	0.022
97	28:6n3	0.001	0.006	0.006
98	28:7n3	0.010	0.001	0.001
99	30:4n6	0.001	0.006	0.005
100	30:5n6	0.006	0.006	0.001
101	30:5n3	0.001	0.004	0.004
102	30:6n3	0.001	0.001	0.001
103	30:7n3	0.006	0.001	0.001
	Unknown	0.980	0.927	0.894

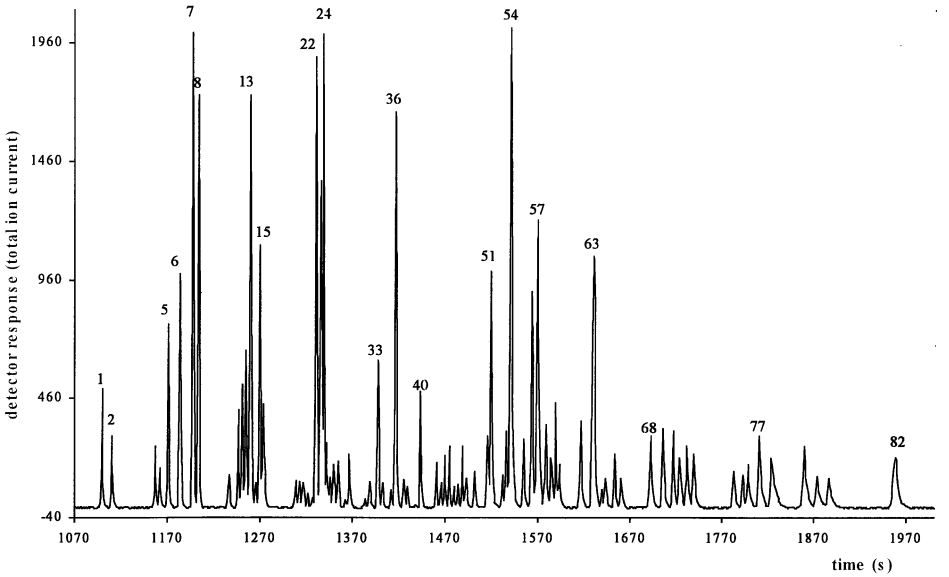

Fig. 1. Gas chromatography–mass spectrometry of fatty acid methyl esters from *Bathynella natans*, peak numbers, see Table 1.

explained on the basis of the animals diet (bacteria, cyanobacteria, unicellular algae, etc.). Syncarids are almost at the end of the food chain and thus they accumulate some food components in their bodies.

In terms of amount, the most abundant group is PUFA. The acid 14:2n5 is one of the shortest dienoic fatty acids. Trienoic and even tetraenoic fatty acids containing 16 carbon acids were also found. Their presence is not so surprising when one takes into account the place where the organisms live and their life style. All three species live in water whose temperature is almost constant, oscillating slightly around 4°C. They also live in absolute darkness; *Bathynella natans* lives in caves and *B. baicalensis* and *Baicalobathynella magna* live in lake depths greater than 1 km. Almost all the positional isomers of PUFA with 18, 20 and 22 carbon atoms were also identified. It is interesting that food and the geographical occurrence affect the content of fatty acids in these crustacea, but not the taxonomic relationship. This can be easily documented, e.g. on the abundance percentage of 18:4n3 and 20:4n6, and even of the minor 22:2n6, 20:3n6 and 22:5n6.

The discovery of VLCPUFA was very unusual as they are quite rare in nature. They occur only in a few animals, usually in specialised tissues (Kakela et al., 1995; Řezanka, 1989; Poulos, 1995; Linko and Karinkanta, 1970) while they have been observed only very rarely in the plant kingdom and in fungi. Only very few examples can be quoted. Lichens of the genus *Cladonia* and other lichens contained 24:2n3 and 26:3n3 acids (Dembitsky et al., 1991). Specialised tissues from vertebrates contain VLCPUFA in high concentrations; for instance the VLCPUFA content of the eye retina as well as the brain of patients suffering with genetic disorders reaches tenths of

1% of total lipids (Aveldano and Sprecher, 1987; Řezanka, 1989; Poulos, 1995; Moser and Moser, 1996). By contrast, among crustacea they are known to be present in only a few cases. Marine sponges were shown to contain especially demospongic acids, i.e. nonmethylene interrupted PUFA, mainly 5,9,16–26 : 3, 5,9,21–28 : 3, 5,9,23–29 : 3 and 5,9,23–30 : 3; classical VLCPUFA (30 : 4n6, 30 : 5n3) were detected as well (Litchfield et al., 1978). The 24 : 4n6, 24 : 5n6, 24 : 6n3, 30 : 4n6 and 30 : 5n5 acids were identified in a sponge from Lake Baikal (Dembitsky et al., 1993).

Fatty acids from crayfish and crab tissues show a very similar profile of major fatty acids, but the minor fatty acids have never been identified by other authors (Okajima et al., 1984; Pakrashi et al., 1989; Ishii et al., 1988). VLCPUFA were of special interest to us (see Table 1). In particular monoenoic fatty acids were found, i.e. positional isomers 24 : 1 and 26 : 1. The predominance of n9 over n11 is clear. The major VLCPUFA were identified to be 24 : 3n6, 24 : 3n3, 24 : 5n3, 26 : 3n6, 26 : 5n6 and 28 : 7n6. 24 : 3n6 (0.4–0.5% of total fatty acids) and 24 : 3n3 (1%) these were the most abundant acids in all three species.

We observed a very interesting phenomenon, the content of some fatty acids was similar irrespective of either geographic or taxonomic parameters. For instance the content of 26 : 3n6 was almost the same in all three species. On the other hand, the content of 24 : 4n6, 26 : 5n6, 28 : 7n6 and 28 : 7n3 was found to be geographically related and thus presumably is dependant mainly on food composition. The 26 : 3n6, 26 : 4n6 and 30 : 5n6 content is in good accordance with taxonomic relationships.

The VLCPUFA content of all three species reaches almost 2% of the total lipids and the qualitative abundance of VLCPUFA among all three crustacea is nearly the same. Almost all positional isomers of acids from 24 up to 30 carbon atoms long with 3–7 double bonds were found.

The predominance of n6 acids in freshwater organisms as opposed to n3 acids in marine animals remains to be clarified. Kakela et al. (1995) studied this problem in VLCPUFA from seals. They confirmed that the well-known rule that the freshwater animals contain more n6 than n3 PUFA. However, it is necessary to point out that seals, a mammal predator, occupy a niche at least two steps higher in the food chain. That is why these conclusions cannot be fully adopted in our case.

Even though the presence of VLCPUFA in crustacea has not been described previously, they will probably be increasingly detected due to the advances in physico-chemical separation and identification methods as well as improvement of methods of sample collection and also due to the currently increased interest in invertebrates.

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