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Analysis of very long chain polyunsaturated fatty acids using high-performance liquid chromatography – atmospheric pressure chemical ionization mass spectrometry

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Abstract

The presence and identity of very long chain polyunsaturated fatty acids from three freshwater crustacean species, *Bathynella natans, B. baicalensis* and *Baicalobathynella magna* from Lake Baikal and caves of central Europe were determined by means of liquid chromatography–mass spectrometry with atmospheric pressure chemical ionization (LC-MS with APCI). LC–MS with APCI enabled the identification of more than 50 very long chain polyunsaturated fatty acids. These acids were described in the crustaceans for the first time, predominantly 26:5n6, 28:7n6, 30:7n3 and 40:7n6. A hypothesis for the biosynthesis of these acids is proposed.

Keywords: Very long chain polyunsaturated fatty acids; High-performance liquid chromatography – atmospheric pressure chemical ionization mass spectrometry; *Bathynella*; *Baicalobathynella*; Crustacea

1. Introduction

The occurrence of very long chain polyunsaturated fatty acids (VLCPUFA) in biological materials has been reported (Řezanka, 1989). So far, they have been demonstrated primarily in animal retina and human tissues (e.g. brain suffering from Zellweger syndrome (Poulos, 1989)) and in marine vertebrate and invertebrate species such as the herring (Linko and Karinkanta, 1970) (Clupea harengus), seals or sponges

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(Dembitsky et al., 1993). The main problem in the identification of VLCPUFA in natural materials is their low concentration, which usually does not exceed a few tenths of percent, with a few exceptions (e.g. some sponges or the retina). Various procedures have been used for enriching fatty acids with VLCPUFA, such as thin layer chromatography on silver nitrate impregnated silica gel. Ion exchange HPLC has allowed the separation of fatty acids according to the number of unsaturated bonds (Řezanka, 1990a). Their identification is complicated by their sensitivity to heat and aerial oxygen.

RP-HPLC is usually used for separation of the fatty acids present in natural fats and oils (Gutnikov, 1995; Nikolova-Damyanova, 1997). The most efficient separations have been achieved with columns packed with chemically bonded octadecylsilyl phases, employing mobile phases consisting of acetonitrile with methanol, propionitrile or chlorinated solvents (Christie, 1987). Separation is based on both the chain length of the fatty acyls and the total number of double bonds in the molecule (Gutnikov, 1995; Nikolova-Damyanova, 1997). A range of detection methods, including many different detectors, has been employed in the analysis of fatty acid derivatives (Gutnikov, 1995; Christie, 1987, 1997). However, identification of individual components by these methods relies on relative retention times or collection of peaks followed by mass spectrometric analysis (usually by GC-MS). With very complex mixtures, the identification of fatty acids in this way is impractical. Therefore mass spectrometric detection, which provides detailed information on the fatty acid composition and allows the identification of partially resolved or co-eluting peaks, becomes increasingly widespread.

The MS and LC-MS procedures for the identification of fatty acids have relied mainly on electron impact (EI) or chemical ionization (CI) with gas or the mobile-phase solvent to give molecular fragments, molecular ions (M), and molecular ion adducts (Christie, 1997; Murphy, 1993). EI-MS produced complicated fragmentation with different abundances of M^+ (Murphy, 1993). CI-MS, with reagent gas, produced simple spectra, which conclusively identified fatty acids, but not double-bond positions (Murphy, 1993). However, the interface for CI-MS between HPLC and MS involved the introduction of eluents into the MS by either thermospray or the direct injection. The thermospray technique, which uses heat to remove the solvent, may alter the unsaturated fatty acids. Direct injection of the stream into the MS utilizes $\sim 1/100$ of it and may result in MS sensitivity problems for complex natural fatty acid mixtures.

Although a wide range of mass spectrometric ionization techniques is available, only a few of them are suitable for use in conjunction with HPLC systems. Atmospheric pressure chemical ionization (APCI) is a relatively recent mass spectrometric soft ionization technique, which has considerable potential for combined HPLC-quadruple MS analysis when coupled with HPLC. The eluent is constantly introduced into the APCI ion source where reactions promoted by a high-voltage corona produce protonated fatty acid molecular ions $[M + l]^+$ by means of the eluent. The APCI spectra of fatty acids are relatively simple and typically show a protonated molecule $[M + H]^+$, the adduct and some fragments, which make it possible to conclusively identify positional isomers, see e.g. Fig. 1. The mobile phase is ionized and transfers energy into eluted molecules of compounds.

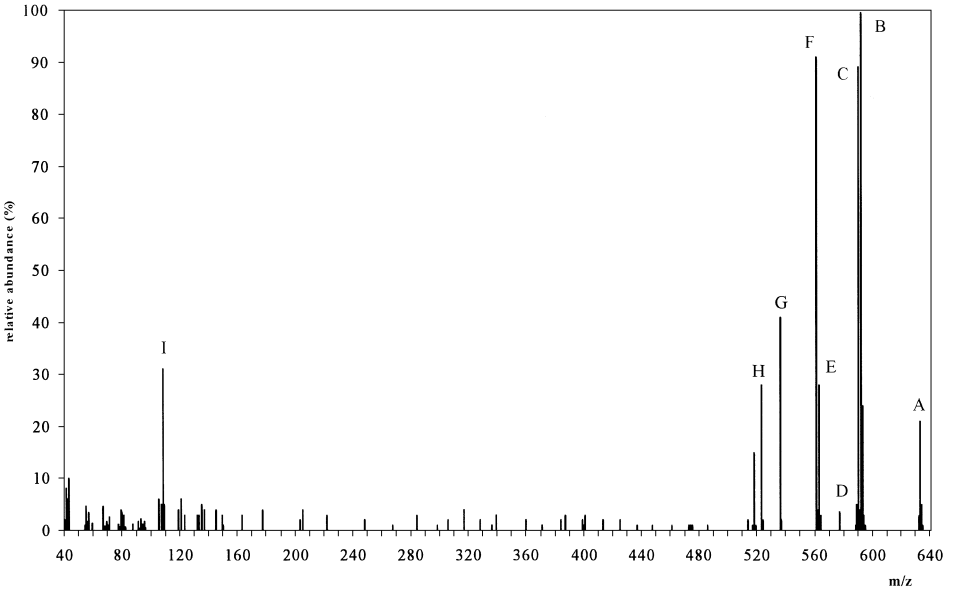

Fig. 1. LC-MS (APCI) of 40 : 8n3 FAME from *Bathynella natans*. Ions: A – $[M + H + CH_3CN]^+$, B – $[M + H]^+$ C – $[M\text{-}H]^+$, D – $[M + H\text{-}CH_3]^+$, E – $[M + H\text{-}C_2H_5]^+$, F – $[M + H\text{-}CH_3O]^+$, G – $[M + H\text{-}C_4H_9]^+$, H – $[M + H\text{-}C_5H_9]^+$, I – $m/z = 109$ (diagnostic ion, see text).

APCI uses a corona needle discharge to impart charge onto vaporized molecules, which are sprayed from a capillary inlet. These are swept into the vacuum region of the mass spectrometer through a capillary bleed. Also, APCI requires no buffers in solution in order to produce efficient fragmentation. This lack of need of a buffer allows neutral, nonpolar molecules to be analyzed as easily as more polar molecules that are ionizable in solution. An APCI interface allows direct introduction of HPLC column effluent at a rate of up to 2 ml/min.

We present the first report of an APCI interface for the direct mass spectrometric analysis of very long chain polyunsaturated fatty acids. We demonstrate that, because of minimal fragmentation, APCI is a suitable method for identification of VLCPUFA from lower organisms. In this study, we continued the determination of the fatty acid content of organisms living in unusual environments. As described in a previous paper (Řezanka and Dembitsky, 1999), animals of the class Bathynellacea live in unusual surroundings and these environments stimulate the biosynthesis and storage of unusual very long chain polyunsaturated fatty acids.

2. Experimental

2.1. Material

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 in the vicinity of Srbsko near Prague. The samples were stored and transported frozen. Fatty acid methyl esters (FAME) were prepared by the basic transesterification of the total lipids that had been extracted from the crustaceans using a mixture of $CHCl_3$–MeOH (Christie, 1989). Oxygen was removed from the HPLC 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.

2.2. Methods

Silver-ion-exchange HPLC (Řezanka, 1990b): Preparative HPLC was performed in a gradient LC system G-1 (Shimadzu, Tokyo, Japan) with two LC-6A pumps (4 ml/min), an SCL-6A system controller, an SPD UV detector (208 nm), an SIL-IA sample injector, and a CR3A data processor. A 30 cm × 7.9 mm semipreparative column packed with a strongly acidic cation-exchange resin ($-SO_3H$ groups, SCR-101 H, 10 μm spherical particles, Shimadzu, Tokyo, Japan) was employed. After 10 mg FAME was injected, a linear gradient was performed from 0% acetonitrile in methanol to 50% acetonitrile in methanol over 30 min (Řezanka, 1990a). The individual classes of FAME (from 0–4 [and more] unsaturated bonds) were collected, evaporated, and analysed by LC-MS (see below).

HPLC equipment consisted of a 1090 Win system, PV5 ternary pump and automatic injector (HP 1090 series, Hewlett Packard, USA) and two columns in series. Supelcosil narrow-bore columns, i.e. Supelcosil LC-18 250 × 2.1 mm ID, 3 μm phase particle, 120 Å pore size. A quadruple mass spectrometer system Navigator (Finnigan MAT, San Jose, CA, USA) was used which was fitted with an atmospheric pressure chemical ionization source (vaporiser temperature 400°C, capillary heater temperature 220°C, corona current 5 μA, sheath gas-high-purity nitrogen, pressure 55 psi, and auxiliary gas (also nitrogen) flow rate 15 ml/min. Positively charged ions with m/z 70 – 800 were scanned with a scan time of 0.5 s. The whole HPLC flow (0.45 ml/min) was introduced into the APCI source without any splitting. Fatty acid methyl esters were separated using a gradient solvent program with acetonitrile (ACN), dichloromethane (DCM) and propionitrile (PrCN) as follows: initial ACN/PrCN/DCM (60:20:20, vol/vol/vol); linear from 600 to 960 s ACN/PrCN/DCM (50:30:20, vol/vol/vol); held until 1560 s; linear from 1560 to 2100 s ACN/PrCN/DCM 30:40:30, vol/vol/vol); held until 2700 s; the composition was returned to the initial conditions over 480 s. A peak threshold of 0.3% intensity was applied to the mass spectra. Data acquisition and analyses were performed using PC with MassLab 2.0 for Windows NT 4.0 applications/operating software.

3. Results and discussion

Fatty acids isolated from all the three crustacean species have already been identified and quantified using a GC-MS method (Řezanka and Dembitsky, 1999). As mentioned in Mansour et al. (1999) and in our introduction, fatty acids with an

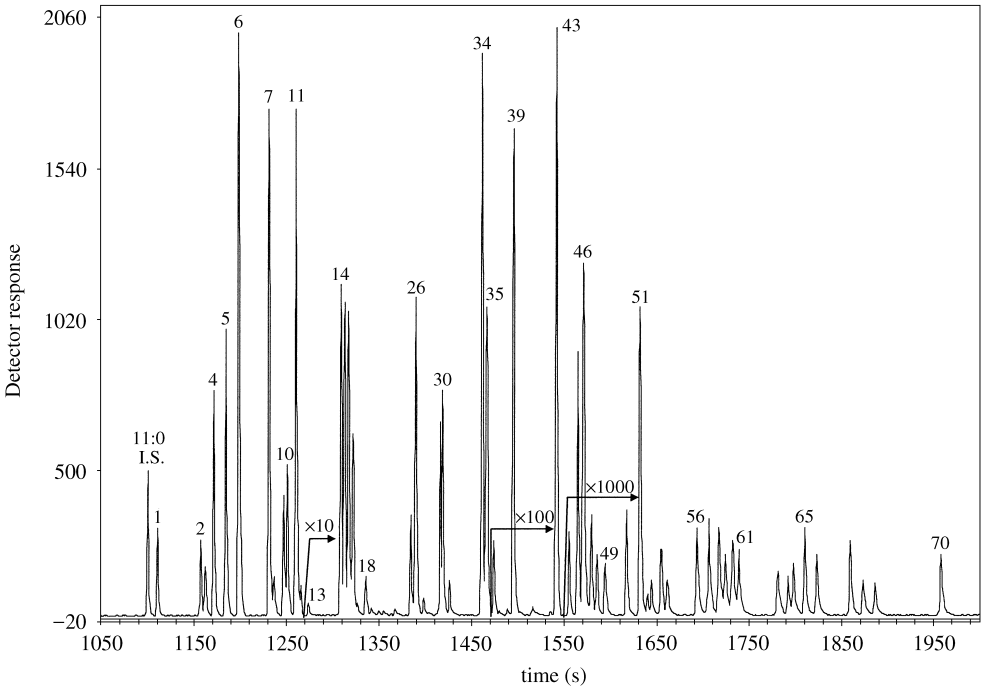

Fig. 2. Chromatogram (LC-MS with APCI) of VLCPUFA from *Bathynella baicalensis*.

unusual chain length and a higher number of double bonds in the chain are extremely sensitive to thermal degradation. The GC-MS determination of VLCPUFA requires the usage of a higher temperature. To minimize the possibility of thermal degradation we used two HPLC steps, where in the first Ag^+-HPLC the PUFA were concentrated and in the second (LC-MS) the separate molecular types of PUFA were separated, quantified and identified. Because of the Ag^+-HPLC preseparation, the amount of single PUFA was changed, but their ratio remained constant. In both chemical ionization (Řezanka, 1990a) and APCI (in fact CI), the intensity ratio of ions (*m*/*z* 109 and 151) was used for determining the position of double bonds in the FAME chain as described by Fellenberg et al. (1987). The determination of VLCPUFA identity was very difficult since no standards are available and the concentration of these acids in the samples was lower than 1% of total PUFA (see also Fig. 2). However, modern analytical methods including LC-MS enabled us to increase the sensitivity up to 1000× and thus allowed the identification of minor compounds.

In two recent papers, i.e. Mansour et al. (1999) and VanPelt et al. (1999b) gas chromatography was used for the separation of VLCPUFA. This method is suitable for the analysis of volatile compounds or compounds, which are volatile after derivatization. Unfortunately, after the separation of VLCPUFA there is also a problem to quantificate eluted peaks. From literature it is known (Řezanka and Mareš, 1991) that with increasing number of carbons and also of double bonds, the

discrimination of higher homologues also increases. The technique used in this paper, i.e. APCI, removes this discrepancy; see Řezanka (2000) in press). The authors of both the papers described apparently right results but in the paper of Mansour the classical method was used for the determination of higher homologues than usual. VanPelt, on the contrary, used a modern and progressive technique, in addition based on very precise theoretical results (VanPelt et al., 1999a,b). Unfortunately, not a single author analyzed higher homologues, i.e. VLCPUFA higher than 28 carbon atoms. It is very probable, on the basis of literature data (Řezanka, 1989) that these lower water microorganisms contained VLCPUFA having higher than 28 carbons. The method of Mansour however is significantly simple and makes it possible to analyze samples and interpret results even with less equipped laboratory than the method of VanPelt. This method requires equipment that enables both ionization (EI and/or CI) and also MS/MS.

In our preceding paper (Řezanka and Dembitsky, 1999) we have already used the GC-MS method for analysis of VLCPUFA up to 30:7. Unfortunately, further chromatography by means of the GC-MS has not been possible and therefore modern soft ionization techniques (APCI) connected with HPLC were used in this paper.

Methyl esters of saturated monoenoic, dienoic and polyenoic acids were separated using ion-exchange HPLC, and PUFA were found to constitute almost 50% of the total fatty acid content. This high content of PUFA is easy to explain. The temperature of subterraneous water in the caves as well as 1000 m deep in the Baikal lake is practically constant and does not deviate from 4°C. This is the reason why the cells predominantly contain PUFA.

70 different VLCPUFA were identified in the second chromatographic step (LC-MS with APCI). In accordance with already published data (Řezanka and Dembitsky, 1999) the 18:3n6, 18:4n3, 20:4n3, 20:5n3 and 22:6n3 acids were found to be the major polyenoic fatty acids, 22:6n3 being the most abundant one. Interestingly, the type of fatty acids content is affected not only by the taxonomy, but is also nutrient-dependent (Kakela et al., 1995). The differences are clearly visible in Table 1, where the 18:3n6 to 40:8n3 are similar in *Bathynella baicalensis* and *Bacialobathynella magna* as well as in the lake organism *Bathynella natans*, while the content of 26:3n3, 26:4n6, 30:5n6, 34:6n3 a 36:5n6 acids depends far more on the taxonomical relationship of *Bathynella* species (from both the lake and the cave). The scheme in Fig. 3 can be used to elucidate the biosynthetic relationships among particular PUFA. Although some of the biosynthetic products were not identified, it is possible to hypothesize from the scheme the likely biosynthetic pathways from 18:3n6 up to 40:8n3. With the increasing number of double bonds, their position is also changed from n6 to n3. With the key metabolite 30:7n3 the biosynthetic pathway divides and very unusual fatty acids with 8 methylene-interrupted double bonds are synthesized.

The presence of VLCPUFA was very unusual, as these acids occur very rarely in nature (Řezanka, 1989; Poulos, 1995). They are found predominantly in the animal kingdom (in the tissues of some specialized vertebrates) while their presence in plants is far less frequent. They were detected especially in marine vertebrates, e.g. fish and mammals. In fact they were found only in marine dinoflagellates (Mansour et al., 1999), and trace amounts were detected also in some lichens. VanPelt et al. (1999b)

Table 1
Fatty acid composition in different species of Bathynelaceae (% of total)[a]

Peak No.	FA	*B. natans*	*B. baicalensis*	*B. magna*
1	16:4n3	2.05	3.58	3.01
2	16:3n3	3.98	2.74	2.61
3	16:3n6	2.58	2.69	1.70
4	18:4n3	4.17	9.92	7.75
5	20:5n3	8.15	8.82	9.87
6	22:6n3	26.02	24.27	19.19
7	18:3n3	9.98	13.02	17.42
8	18:3n6	10.55	1.89	1.38
9	20:4n3	4.94	3.51	4.16
10	20:4n6	2.36	4.89	5.21
11	22:5n3	15.67	12.67	17.36
12	22:5n6	2.36	0.96	1.05
13	24:6n3	134.7[a]	51.9[a]	44.1[a]
14	20:3n3	853.0	2183.0	1141.0
15	20:3n6	357.0	1514.0	1086.0
16	20:3n9	956.0	753.0	1054.0
17	22:4n3	534.0	201.0	623.0
18	22:4n6	62.0	55.0	47.0
19	24:5n3	195.3	148.9	138.2
20	24:5n6	56.1	40.2	27.3
21	26:6n3	134.7	51.9	44.1
22	26:6n6	12.8	23.4	19.7
23	28:7n3	22.5	1.7	5.0
24	28:7n6	4.8	56.6	33.6
25	22:3n3	125.0	202.0	358.0
26	22:3n6	348.0	824.0	1092.0
27	24:4n3	62.6	63.6	62.3
28	24:4n6	46.5	11.8	10.6
29	26:5n3	12.8	10.0	7.6
30	26:5n6	19.2	190.8	187.1
31	28:6n3	3.2	115.1	12.2
32	28:6n6	6.4	10.7	9.1
33	30:7n3	12.8	3.3	1.5
34	24:3n3	1954.1	2996.4	1801.4
35	24:3n6	826.0	1024.0	1057.0
36	26:4n3	38.5	18.4	23.8
37	26:4n6	12.8	11.7	0.0
38	32:8n3	2.6	2.0	2.6
39	28:5n6	131.2	150.1	127.8
40	30:6n3	1.6	1.7	1.5
41	32:7n3	1.1	3.5	3.0
42	26:3n3	7.5	8.5	1.4
43	26:3n6	192.5	208.0	191.0
44	28:4n6	1.9	1.8	2.9
45	30:5n3	3.2	8.4	9.1
46	30:5n6	12.8	14.1	2.2
47	34:8n3	1.3	3.0	2.6
48	32:6n3	0.6	4.2	3.5

(continued on next page)

Table 1 *(continued)*

Peak No.	FA	*B. natans*	*B. baicalensis*	*B. magna*
49	32:6n6	1.4	2.5	2.1
50	34:7n3	1.9	4.0	3.8
51	30:4n6	1.8	15.1	10.6
52	28:3n6	2.1	2.0	3.6
53	32:5n3	0.3	1.2	0.8
54	32:5n6	0.8	1.5	1.2
55	34:6n3	2.4	2.3	0.4
56	38:8n3	1.3	4.0	3.3
57	34:6n6	3.8	5.4	3.0
58	36:7n3	2.6	2.2	2.2
59	32:4n6	2.7	3.2	3.0
60	30:3n6	3.2	1.5	1.2
61	34:5n3	1.4	2.8	2.1
62	34:5n6	2.7	2.7	2.3
63	40:8n3	3.4	1.7	1.4
64	36:6n6	0.8	3.5	3.0
65	36:5n3	0.6	2.3	1.8
66	38:6n3	1.8	0.6	0.5
67	36:5n6	2.1	2.7	4.6
68	40:7n3	1.1	2.4	2.2
69	36:4n6	1.2	1.3	1.1
70	40:6n6	2.4	3.5	2.1

[a]x 0.001.

determined using acetonitrile chemical ionization mass spectrometry the molecular weight and double-bond locations of an oclaenoic fatty acid 28:8n-3 from heterotrophic algae *Crypthecodinium cohnii*. The situation is completely different with invertebrates: marine as well as freshwater sponges contain non-methylene interrupted PUFA as well as "classical" VLCPUFA (30:4n6 and 30:5n3). The 24:4n6, 24:5n6, 24:6n3, 30:4n6 a 30:5n3 acids were identified also in a Baikal lake sponge (Dembitsky et al., 1993).

VCLCPUFA were identified in crustaceans for the first time but as already mentioned, their biosynthesis as well as their function remain unknown. Even though we tried to describe some possible biosynthetic pathways in Fig. 3, we probably documented only a small part of the potential combinations. Based on the biosynthetic pathways (Fig. 3), as well as on some already published data (Mansour et al., 1999), we suspect that the VLCPUFA in crustaceans come more likely from the feed than from being synthesized by the invertebrates. As mentioned above, the VLCPUFA (e.g. 28:7n6 and 28:8n3) were documented in marine dinoflagellates. There is no reason why they could not be found in freshwater plankton (the feed of the crustaceans under study). According to the currently accepted theory the VLCPUFA originate from C_{18} PUFA (in our case 18:3n6), via elongation and desaturation; however, not all precursors were identified; see also Fig. 3. Mansour et al. (1999) maintained that the precise determination of the biosynthetic pathway was not possible and that 28:7n6 and 28:8n3 were not synthesized via chain elongation, as the

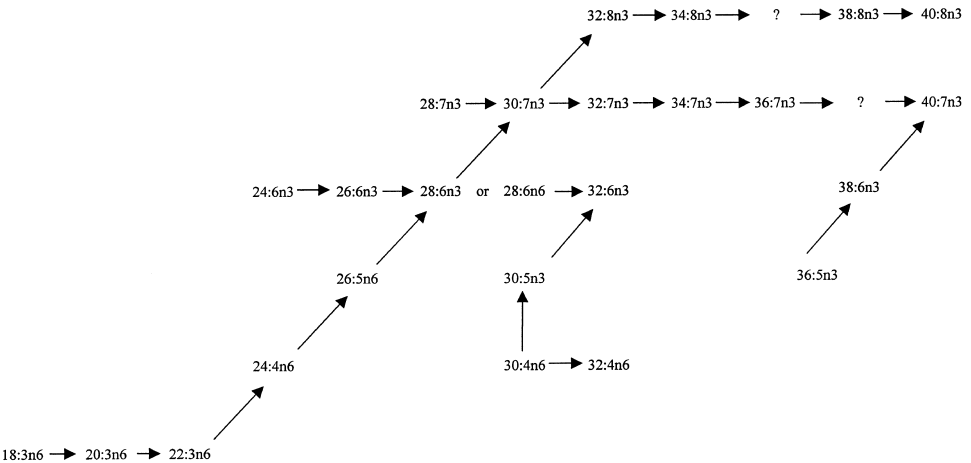

Fig. 3. Proposed pathway for the biosynthesis of VLCPUFA in *Bathynella* sp.

precursors were found in concentrations lower than 0.1% of total fatty acids. Mansour et al. (1999) published two hypotheses : One possibility is that 28:7n6 may be desaturated to 28:8n3, and because there is room for only one more methylene-interrupted double bond, by necessity it must be located in the n3 position. Alternatively, 28:8n3 could be formed and then hydrogenated to 28:7n6. This second possibility, although unconventional, could explain why two species have 28:8n3, but no detectable 28:7n-6. Our data nearly unambiguously support the first possibility. The last interesting fact is the double-bond shift, i.e. the desaturation connected with the creation of n3 double-bond.

We assume that the actual mechanism is desaturation of 28:7n6 to 28:8n3, although the authors support the other mechanism because no 28:7n6 was found in dinoflagellates. On the other hand, we suppose that 28:7n6 and other precursors are present in the feed, but their concentration is lower than 0.1% of the total FA, so that they are very difficult to detect.

VanPelt et al. (1999b) also suggest a possible biosynthetic mechanism of 18:8n3. This acid may be synthesized by a repeat of the pathway from 22:6n3 acid, and it is represented by classical elongation/desaturation steps.

Using the LC-MS method we increased the sensitivity of the method 1000 times and we were able to detect the majority of these biosynthetic precursors. Even with all its experimental and financial limitations, LC-MS is seen to be a useful and modern method for the determination of minor metabolic intermediates and can be expected to become more widespread in the future.

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