FATTY ACID COMPOSITION OF THE MAIN PHOSPHOLIPIDS OF THE ANTARCTIC KRILL *Euphausia superba*

K. Yu. Gordeev, V. N. Filarin, S. V. Bondarenko, M. A. Kirpichenok, N. A. Gordeeva, I. I. Grandberg, and S. G. Batrakov

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The Antarctic krill Euphausia superba Dana contains about 5% of its natural weight of extractable lipids, more than half of which are in the form of phospholipids – phosphatidylcholine (33-36% of the sum of the lipids), phosphatidylethanolamine (15-17%), lysophosphatidylcholine (3-4%), and others (2-3%) – while among the phosphorus-free components triacylglycerols predominate (32-35%). In the first two phospholipids the dominating fatty acid residue is the arachidonic acid residue (more than 40% of the sum of the acyl residues) and the amounts of residues of eicosapentaenoic acid ($C_{20:5\omega3}$) are about 13 and 28%, respectively. A simple procedure for isolating the phosphatidylcholine and phosphatidylethanolamine is proposed. For the analysis of the fatty acid a new method was used, consisting of the HPLC of their (7-diethylaminocoumarin-4-yl)hydrazides, with fluorometric detection.

It is known that the lipids of marine organisms (fish, invertebrates, algae) are capable of favorably affecting the lipid metabolism of mammals, including man [1]. "Marine fats" lower the level of cholesterol and triglycerides in blood serum and normalize the lipoprotein spectrum of the plasma, and this is responsible for their prophylactic value and therapeutic effect in hypercholesterolemia, hyperglyceridemia, and atherosclerosis. An antithrombic and hypotensive activity of these substances has been found. The pharmacological properties of the acids under consideration are due to their high content of free and bound polyenic fatty acids (FAs) and, in particular, eicosapentaenoic ($C_{20:5\omega3}$) and docosahexaenoic ($C_{22:6\omega3}$). According to information in the literature [2-4], a rich source of the latter is the Antarctic krill Euphausia superba Dana (Crustaceae) which is caught on the industrial scale. A therapeutic action of the phospholipids of krill has been detected in experimental hypercholesteremia in laboratory animals [4].

In order to study the biological activity of the individual lipid fractions, we have performed an analysis of the phospholipids of E. superba and have developed a simple method for isolating the main phospholipids – phosphatidylcholine (PC) and phosphatidylethanolamine (PE). To determine the fatty acid compositions of these substances we used, in particular, high-performance liquid chromatography (HPLC) of new fluorescing derivatives of the FAs.

The total lipids were extracted from previously comminuted freshly frozen krill with a mixture of chloroform and methanol (2:1; here and below the volume ratios of solvents are given). The extract was freed from water-soluble impurities by the method of Folch et al. [5]. The amount of lipids extracted was 4.9-5.2%* on the weight of the natural krill. Their analysis with the aid of TLC and HPLC on silica gel showed that about 75% of the total lipids consisted of phospholipids – PC (33-36%), PE (15-17%), lyso-PC (3-4%) and unidentified phosphatides (2-3%); the phosphorus-free lipids consisted mainly of triacylglycerols (32-35%), while neutral glycolipids, sterols and their esters, free FAs, and diacylglycerols were present as minor components. The results obtained are in harmony with those of other authors [2].

*The text and Table 1 show the limiting values of the amounts and yields of lipids relative to the amount of FAs from the results of an investigation of five samples of krill; in the Experimental part, one of the experiments on the isolation of the total lipids, the PC, and the PE is described.

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The extremely limited set of polar lipids permitted the suggestion of a simple method of isolating individual fractions of PC and PE which consisted in the chromatography of the total lipids on silica gel with the use of its ion-exchange properties. Chromatography was conducted on silica gel L (Lachema, Czechoslovakia) previously treated with acetic acid. The acid and weakly polar neutral lipids were removed from the sorbent with chloroform–methanol (2:1) and then a mixture of the same solvents in a ratio of 1:1 eluted the PE, after which the PC was eluted by the chloroform–methanol–conc. aqueous ammonia (50:50:7) system [6]. By this method, 1 kg of natural krill yielded 15.3-16.0 g of PC and 7.3-7.5 g of PE (completeness of extraction about 90%), each with a purity of not less than 96%. On chromatography, no fine fractionation of the eluate with analysis of the fractions was required, but it was necessary to maintain the optimum volume of each mixture of solvents, which was equal to 6-8 times the volume of the sorbent. When the amount of eluents was decreased, the yield and degree of purity of the phospholipid fractions fell; an increase in volumes, although it had no appreciable effect on the yield and purity of the PE, led to the appearance of a substantial contamination of the PC fraction with lyso-PC.

The amounts of the O-alkyl and O-alk-1-enyl forms in the phospholipids isolated were determined by a method described previously [7] – each fraction was subjected successively to alkaline and acid methanolysis, and the methanolysates were analyzed with the aid of HPLC. In the PC fraction, the above-mentioned forms were present in amounts of 0.5 and 0.3%, and in the PE fraction 0.2 and 0.1%, respectively, which agrees well with figures in the literature [8].

The methyl esters (MEs) of the FAs formed on alkaline methanolysis were analyzed with the aid of GLC using the cyanopropylsilicone SP-2330 as the stationary phase. The results of the analysis are shown in Table 1. This also gives a comparison of the fatty acid compositions of the PC and PE from E. superba according to Fricke et al. [2]; basically similar sets of fatty acid residues were found in the total phospholipids of the same species [3, 4], and also some species of North Atlantic krill [9]. Attention is attracted by the extremely high level of arachidonic acid ($C_{20:4\omega6}$) in the PC and PE investigated, its relative amount among FAs in krill lipids usually not exceeding 3%. To check the results of the gas-chromatographic determination we therefore used chromatographic methods based on different principles of separation. In particular, we carried out an estimate of the composition of mixtures of FAMEs by reversed-phase TLC and by TLC on silica gel impregnated with silver nitrate, with parallel chromatography of standard compounds on the same plates. In both cases it was found that the proportion of the components of the mixtures migrating together with an authentic sample of methyl arachidonate was not less than one third of the total amount of MEs.

For quantitative analysis we used a new method, developed by the authors of the present communication, consisting in the HPLC of the (7-diethylaminocoumarin-4-yl)hydrazides of the FAs (I) with fluorometric detection. The phospholipids isolated – PC and PE – were subjected to alkaline hydrolysis and the FAs obtained were condensed with 7-diethylamino-4-hydrazinocoumarin (II) in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDCD) and pyridine in ethanol. Derivatives (I) were formed in the course of 20 min with a yield of not less than 95%. In experiments with the individual FAs shown in Table 2 and with standard mixtures it was established that the lengths of the hydrocarbon chain and the number of double bonds in the molecules of the C_{14}-C_{22} FAs had no appreciable influence on the yield of hydrazides (I); no losses of polyenic FAs due to oxidation during derivatization was observed, either.

The analysis was carried out under conditions close to those described in [10] for the HPLC of FA 2,4-dinitrophenylhydrazides. The sorbent was the modified silica gel Ultrasphere octyl (Altex, USA), and the eluent was acetonitrile–water (82:18, pH 4.5). The derivatives (I) obtained from the FAs listed in Table 2 were used for calibrating the column. The fluorescence spectra of all these compounds were characterized by an excitation maximum at 368 nm and an emission maximum at 454 nm; elution was monitored at the same wavelengths. In the interval of concentrations of the hydrazides (I) from 1 to 1000 nmole/ml a linear relationship between their molar concentration and the area under the corresponding peak on the chromatogram was maintained. From the ratios of these areas we calculated the relative amounts of the compounds in the mixture. Table 2 gives the retention times of the derivatives (I) under the conditions of chromatography, and Table 1 gives the results of a quantitative determination of the FAs in hydrolysates of the PC and PE. It can be seen from Table 1 that the results of the analysis of the fatty acid compositions of the PC and PE by the GLC

TABLE 1. Fatty Acid Compositions of the PC and PE of the Antarctic Krill E. Superba

Fatty acid	Relative amount, mol. %					
	PC			PE		
	Results of the present work*		according to [2]**	Results of the present work*		according to [2]**
	according to GLC	according to HPLC		according to GLC	according to HPLC	
14:0	7,1±0,4	6,7±0,3	4,5 2,8	1,7±0,3	1,8±0,3	—/2,9
16:0	10.6±0,4	9,4±0,4	43.7 25,7	3 6±0,3	4,2±0,3	42,7 24,5
16:1ω7	4,1±0,3	4,5±0,2	3,7/2,2	1,8±0,2	1,6±0,2	2,0 1,9
18:0	—	—	1,8 1,5	—	—	3,2/2,9
18:1ω7 } 18:1ω9 }	1,1±0,3	0,9±0,1	7,7,6,1 9,2/5,4	1,2±0,3	1,2±0,2	15,0/16,3 5,4 6,8
18:2ω6	1,0±0,3	0,7±0,1	1,6/1,1	0,9±0,2	0,5±0,1	1,0 1,0
18:3ω6 } 18:3ω3 }	1,2±0,3	0,9±0,1	—/2,7 —/0,8	0,9±0,3	0,6±0,1	—/0,7 —/—
20:1ω9	—	—	0,6/0,9	—	—	0,4/0,8
20:4ω6	40,2±0,7	40,1±0.5	—/—	43,0±0,9	42,0±0,5	—/—
20:5ω3	13,0±0,6	13,0±0,4	10.7 29,9	29,2±0,8	27,3±0,3	10,5/21,1
21:5ω3	—	—	1,0 1,1	—	—	— 0,7
22:1ω7	—	—	—/0,9	—	—	—/—
22:1ω9	—	—	—/1,7	—	—	—/—
22:5ω3	3,4±0,6	3,4±0,3	0,9/0.6	5,6±0,8	6,3±0,3	—/0,9
22:6ω3	12,0±0,8	12,5±0,4	6,2/11,5	11,7±0,9	12,5±0,4	7,6 19,2
Unidentified	6,0±0,6	7,7±0,5		1,0±0,3	1,7±0.4	

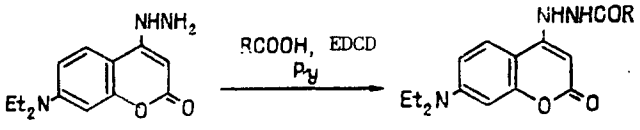
*—Less than 0.5 rel. mol.% of the acid.
**The numerator gives figures for krill caught in December, 1977, and the denominator the figures caught in March, 1981, in the same region.

TABLE 2. Retention Times of the (7-Diethylaminocoumarin-4-yl)hydrazides of FAs (I) in HPLC (for conditions see the Experimental part)

Fatty acid	Retention times, min	Fatty acid	Retention times, min
20:5ω3	3,76	20:4ω6	5,24
18:3ω3	3,97	18:2ω6	5,94
14:0	4,27	16:0	7,34
22:6ω3	4,64	18:1ω9	7,91
16:1ω7	4,84	18:0	10,02
22:5ω3	5,06	20:0	12,15

and HPLC methods agree satisfactorily. This relates, in particular, to the arachidonic acid. Thus, the more than 40% level of arachidonic acid residues among the acyl residues of the PC and PE of the twelve samples investigated must be regarded as an authentic fact. The discrepancy in this ratio from the results of other authors is most probably due to different seasonal and geographic conditions for the collection of the biomaterial; interyear differences in the fatty acid composition of the krill lipids cannot be excluded, either.

In a comparison of the results of our work with information published previously on the phospholipids of E. superba (see Table 1), attention is attracted by the fact that the increase in the proportion of arachidonic acid in the PC and PE fractions studied have taken place at the expense of a sharp fall in the amount of C_{16} and C_{18} saturated and monoenic acids.



for RCOOH - see Table 2

EXPERIMENTAL

Materials and General Methods. Five samples (of 200-600 g each) of the krill E. superba caught in August-September, 1987, in the Antarctic part of the Atlantic Ocean close to South Georgia were investigated. The biomaterial was stored at −20°.

For TLC we used high-resolution plates with dimensions of 5 × 10 cm coated with silica gel G (Merck, FRG). The chromatograms were run in the following solvent systems: in the analysis of the phospholipids – $CHCl_3$–MeOH–water (65:25:4 and 65:35:8) and $CHCl_3$–MeOH–water–conc. NH_4OH (55:25:4:2); in the analysis of weakly polar lipids – hexane–Et_2O–AcOH (85:15:1), hexane–Et_2O (1:1), and $CHCl_3$–MeOH (10:1). The substances were detected on the chromatograms: by nonspecific methods – spraying with 50% H_2SO_4 followed by heating them at ∿280°C, and spraying with a 5% solution of molybdophosphoric acid in ethanol and heating at 105°C; and with specific reagents – a reagent for phospholipids [11]; a 0.3% solution of ninhydrin in ethanol; the Dragendorff reagent [12]; the Libermann–Burchard reagent [12], and the anthrone reagent for glycosides [12]. For the analysis of the FAMEs we used, in addition, the above-described plates that had been treated with a 20% solution of $AgNO_3$ [13], and also plates with dimensions of 20 × 10 cm coated with a reversed phase (RP-8, Merck). In the first case, the chromatograms were run in the hexane–Et_2O (4:1 and 1:1) systems, and in the second case in the MeCN–Me_2CO (1:1) system. On the argentized plates the substances were detected in UV light after spraying with a solution of Rhodamine B in ethanol and also by spraying with 2% aqueous $KMnO_4$; on the reversed-phase plates detection was carried out with a solution of molybdophosphoric acid.

HPLC was conducted at 25°C in a modular system (Altex, USA): a model 70-11 device for the introduction of the sample having a 5-μl loop (for the analysis of the FA derivatives) and a 50-μl loop (for the analysis of the phospholipids); a model 164 UV detector with a floating wavelength regulator; two model 110 piston pumps; and a model 400 programmer; phospholipids were analyzed in a steel column with dimensions of 250 × 4.6 mm (internal diameter) filled with the silica gel μ-Porasil, 5 μm (Du Pont, USA), and the hydrazides (I) on the above-described column filled with the modified silica gel Ultrasphere octyl having a particle size of 5 μm (Altex). Phospholipids were chromatographed in the isopropanol–hexane (8:6)–water system with a linear increase in the proportion of the latter of 0 to 10% over 20 min, and then in the isocratic regime for 20 min; the rate of elution was 1.0 ml/min and detection was carried out from the optical density of the eluate at 207 nm. The FA derivatives (I) were chromatographed in the isocratic regime with elution by MeCN–water (82:18, pH 4.5); the pH of the eluent was established by the addition of 0.1 M hydrochloric acid. For detection a fluorimeter with a 9-μl flow-through cell (Gilson, France), was used. All the solvents were degassed with a stream of helium.

The GLC of the FA methyl esters was performed on a Perkin-Elmer chromatograph (model F-30, USA) fitted with a flame-ionization detector and a glass column with dimensions of 2 × 6000 mm filled with Chromosorb NAW 100-120 mesh impregnated with 10% of the cyanopropylsilicone SP-2330 (Supelco, USA). Chromatography was performed in the isothermal regime (210°C) at a rate of flow of helium of 20 ml/min.

Neutral lipids and phospholipids isolated from hens' egg yolk and cattle brain by the usual methods [14, 15], individual FAs, and standard mixtures of FAs and FAMEs (Supelco, USA) were used as standards in chromatographic analysis.

The acid and alkaline methanolysis and also the alkaline hydrolysis of the PC and PE were carried out in an atmosphere of argon by procedures described previously [7, 15].

Preparation of Silica Gel for Column Chromatography. A mixture of 200 g of silica gel L 100/250 (Lachema, Czechoslovakia) and 1 liter of 4% aqueous AcOH was stirred at 20°C for 30 min, after which the solid was filtered off, washed on the filter with 500 ml of distilled water, and dried at 20°C for 12 h and then at 105°C for 8 h.

Extraction of the Total Lipids. The frozen krill (247 g) was ground in a mincing machine and was stirred with 1.5 liter of $CHCl_3$–MeOH (2:1) at 5°C in an atmosphere of argon for 40 min. The mixture was filtered, the residue was washed on the filter with 200 ml of the same solvent mixture, and the filtrate was shaken with 350 ml of distilled water. The lower phase of the two-phase system that formed was separated off and was evaporated to dryness (here and below the solvents were distilled off at a water bath temperature not exceeding 28°C). The residue was dried at 30°C/0.02 mm for 6 h, giving 12.5 g of total krill lipids.

Isolation of the PC and PE. The total krill lipids (12.4 g) were dissolved in 100 ml of $CHCl_3$–MeOH (2:1), and the solution was transferred to a column filled with 110 g of silica gel that had been treated by the method described above. Elution was carried out successively with: 1) 900 ml of the same solvent; 2) 800 ml of $CHCl_3$–MeOH (1:1); and 3) 700 ml of $CHCl_3$–MeOH–conc. NH_4OH (50:50:7). After the evaporation of the eluates obtained with mixtures 2 and 3 and the drying of the residues at 30°C/0.02 mm for 2 h, 4.4 g of PC and 2.1 g of PE with purities of 96.8 and 96.2%, respectively (according to HPLC) were obtained.

FA (7-Diethylaminocoumarin-4-yl)hydrazides (I). An ethanolic solution (50 ml) of 50-300 μg of FAs obtained as the result of the alkaline hydrolysis of the phospholipids was treated with 100 μl of a 20 mM solution of 4-hydrazino-7-diethylaminocoumarin (II), 100 μl of a 3% solution of pyridine in ethanol, and 100 μl of a 0.25 M solution of EDCD hydrochloride (Sigma, USA) in ethanol. The mixture was heated at 60°C for 30 min, after which it was treated with 50 μl of a 2.5 M solution of KOH in 80% ethanol and heating at the same temperature was continued for 15 min. After cooling, the mixture was shaken with 1.5 ml of hexane and 2 ml of 0.3 M sodium phosphate buffer (pH 6.8), the resulting mixture was centrifuged at 1500 g for 5 min, and the upper phase was separated off and evaporated to dryness. The residue was dissolved in 1.0 ml of MeCN–water (82:18, pH 4.5). Aliquots of 1-5 μl were taken for analysis by the HPLC method. The derivatives (I) were obtained from standard FAs and their mixtures similarly.

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LITERATURE CITED

1. V. K. S. Shukla, J. Clausen, H. Egagaard, and E. Larsson, Fette, Seifen, Anstrichm., 82, 193 (1980); J. Dyerberg and K. A. Jorgensen, Prog. Lipid Res., 21, 255 (1982); G. G. Bruckner, B. Lokesh, B. German and J. E. Kinsella, Thromb. Res., 34, 479 (1984); G. J. Nelson and R. G. Ackman, Lipids, 23, No. 11, 1005 (1988).
2. H. Fricke, G. Gercken, W. Schreiber, and J. Oehlenschläger, Lipids, 19, No. 11, 821 (1984).
3. N. R. Bottino, Comp. Biochem. Physiol., 50B, No. 3, 479 (1975); T. E. Ellingson, and T. E. Mohr, in: 11th Scandanavian Symposium on Lipids, 1981, Marcus, Goeteborg (1982), p. 110; F. M. Rzhavskaya, E. A. Sakaeva, and T. A. Dubrovskaya, Krill Processing Technology. A Collection of Scientific Papers [in Russian], VNIRO, Moscow (1981), p. 24.
4. O. A. Artyukova, The Lipids of the Antarctic Krill (Euphausia superba Dana) and Their Therapeutic Effect in Experimental Hypercholesteremia [in Russian], Inst. Pitanya AMN SSSR, Moscow (1988).
5. J. Folch, M. Lees, and G. H. Sloane-Stanley, J. Biol. Chem., 226, No. 2, 497 (1957).
6. S. G. Batrakov, G. A. Ermolin, V. A. Vnukov, O. G. Sakandelizdze, and T. K. Mitrofanova, USSR Inventor's Certificate No. 1102603; Byull. Izobret., No. 26 (1984).
7. V. M. Dembitskii, Ukr. Biokhim. Zh., 59, No. 1, 50 (1987).
8. S. Nobukazu, Nippon Suisan Gakkashi, 49, No. 2, 259 (1983); H. Fricke, G. Gercken, and J. Oehlenschläger, Comp. Biochem. Physiol., 85B, No. 1, 131 (1986).
9. O. Saether, T. E. ELlingson, and V. Mohr, J. Lipid Res., 27, 274 (1986).
10. H. Miwa, M. Yamamoto, and T. Nishida, Clin. Clim. Acta, 155, No. 1, 95 (1986).
11. V. E. Vaskovsky [Vaskovskii] and V. I. Svetashev, J. Chromatogr., 65, 451 (1972).
12. J. G. Kirchner, Thin Layer Chromatography, Wiley-Interscience, New York, 2nd edn., (1978) [Russian translation, Mir , Moscow (1981), Ch. 7].
13. L. D. Bergelson, E. V. Djatlovitskaja [Dyatlovitskaya], and V. V. Voronkova, J. Chromatogr., 15, 191 (1964).
14. L. D. Bergel'son, E. V. Dyatlovitskaya, Yu. G. Molotkovskii, S. G. Batrakov, L. I. Barsukov, and N. V. Prokazova, The Preparative Biochemistry of Lipids [in Russian], Nauka, Moscow (1981).
15. M. Kates, Techniques of Lipidology, American Elsevier, New York (1972).