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Development of a Method to Produce A Concentrate of Polyunsaturated Fatty Acids

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Fat of Baikal seal contains a large amount of polyunsaturated fatty acids (PUFAs) and is a promising source for the production of biologically active supplements. The article describes a method for producing PUFAs concentrate from fat of Baikal seal and the study of fatty acid composition of the produced concentrate. The overall level of unsaturated fatty acids in the produced concentrate amounts for 92.67%, including É-3 PUFAs in amount of 12.2% and ω-6 PUFAs in amount of 15.91% that testifies high biological value of the concentrate.

Key words: Polyunsaturated fatty acids (PUFAs), arachidonic acid (AA), Eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), concentrate of PUFAs.

According to the World Health Organization, cardiovascular diseases (CVD) are the most common among the general diseases of population and are the main cause of death in most countries of the world[1,2]. The results of numerous population-based studies indicate the existence of an inverse relationship between the content of ω-3 PUFAs in the diet of a human and the risk of cardiovascular disease. It is shown that increased consumption of dietary supplements, containing PUFAs, significantly reduces mortality in patients with CVD[3, 4].

Foodstuff, enriched with ω-3 PUFAs of marine origin, containing eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), can be defined as a functional substance that affect human health[5]. These acids are essential; they are vital, though are not synthesized in the human body and must be ingested with food. Having antiatherosclerotic and hypocoagulation action, ω-3 fatty acids have a pronounced cardioprotective effect and are indispensable substances, providing the normal functioning of the cardiovascular system[6, 7].

The two major groups of PUFAs, performing important biological functions, include the acids of ω-6 and ω-3 families. The ω-6 fatty acids are contained in almost all vegetable oils and nuts, ω-3 fatty acids are also found in some vegetable oils (linseed oil, cruciferous seed oil and soybean oil)[8]. The main dietary sources of ω-3 fatty acids are fatty kinds of fish and some seafood[9-11]. Among ω-6 family PUFAs, a special place holds linoleic acid, which is a precursor substance to the most physiologically active acid of this family,

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namely arachidonic acid. Arachidonic acid is predominant representative of PUFAs in the human body[12].

According to the recommendations of experts of the International Society for the Study of Fatty Acids and Lipids, physiological need for ω-3 and ω-6 families fatty acids in adults should be 8-10 g/day (ω-6 fatty acids) and 0.8-1.6 g/day (ω-3 fatty acids) or 5-8% of the daily caloric value for ω-6 PUFAs and 1-2% of daily caloric value for ω-3 PUFAs, including EPA+DHA in amount not less than 500 mg/day[13, 14]. In 2004, the US Food and Drug Administration decided to declare a food, rich in ω-3 fatty acids, as pertaining to a group of functional foods. This organization has also suggested that consumption of EPA+ DHA should not exceed 3 grams per day, because exceeding this limit may lead tounwanted effects in terms of higher glycemic index, increased bleeding time and increase of LDL cholesterol (low-density lipoprotein cholesterol). In Brazil, the Scientific Health Surveillance Agency requires that foods enriched with ω-3 fatty acids should be provided at least in amount of 0.1 g of EPA and/or DHA per meal or per 100 grams (ml), to be able to talk about their functional properties[15].

Use of such amount of ω-3 fatty acids in diet is difficult. Alternatively, we can successfully use dietary supplements, which are concentrates with a high content of EPA and DHA. Produced PUFAs concentrate should primarily contain sufficient amount of ω-3 fatty acids to cover the daily requirement of EPA and DHA in human[16]. Also, in the formulations there must be relatively low concentration of ω-6 PUFAs, which are natural biological competitors of ω-3 PUFAs; otherwise pharmacodynamic effect will be neutralized by competitive action of arachidonic acid metabolites[17].

A unique feature of the world's largest freshwater lake Baikal is the availability in its water the only mammal, namely the Baikal seal (*Pusa (Phoca) sibirica Gmel.*)[18]. For preserving biodiversity and biological balance of Baikal ecosystem, Baikal seal population is maintained within certain limits by sanitary catching a strictly limited number of fish, ranging from 1-3 to 6 thousandzooids per year. At that, the fat and internal organs, constituting 50% of the fish body weight, is almost not used,whereas the study of the chemical composition of Baikal seal fat have shown that their fat is rich in unsaturated fatty acids, including polyunsaturated ones (the contents of EPAand DHA are 2.65% and 6.28%, consequently)[19]. This circumstance provides a high biological value of fat and perspectives of its use as a source for the production of biologically active supplements.

The aim of current study was to develop a method for producing a PUFAs concentrate out of Baikal seal fat with optimal content of ω-6 and ω-3 fatty acids.

METHODS AND MATERIALS

Among the various methods for producing PUFAs concentrate, the simplest one is formation of a complexwith urea, which is fast, inexpensive, reliable, most efficient and environmentally friendly method for producing ω-3 PUFAs concentrate in the form of free fatty acids[20-22].

The first stage to produce free fatty acids is based on use of alcoholysis. The process of fats inters etherification proceeds by exchange of radicals between triglyceride and an alcohol[23]. At that, strong alkali (KOH and NaOH) are conventionally employed as catalysts in the alcoholysis reaction. After alkaline hydrolysis, resulting soap is decomposed by an acid solution, and the separated fatty acids are extracted with hexane.

To assess the fat and PUFAs concentrate we determined acid-degree value using titration method (GOST R 52110-2003), peroxide value - by endpoint-based potentiometric method (GOST R ISO 27107-2010), and iodine value- by titration method (GOST R ISO 3961-2010).

Fatty acid composition of seal fat samples and PUFAs concentrate was determined by gas-liquid chromatography using a mass spectrometer 5973/6890 NMSD/DS Agilent Technology (USA). Capillary column HP-INNOWAX (30<× 250mkm ×0.50mkm) was used for the separation. Polyethylene glycol was a stationary phase. Helium at gas flow rate of 1 ml/min was eluent phase. Evaporator temperature was 250°C, ion source temperature was 230°C and the temperature of the line connecting the chromatograph with a mass spectrometer was 280°C. Scan range was 41-450 amu. Injected sample volume amounted 1 µl,

flow separation was 5:1. Chromatography was performed in isocratic mode at 200^{0}C. Records were done by total ion current (SCAN mode).

RESULTS AND DISCUSSION

Iodine, peroxide and acid values of the feedstock, namely Baikal seal fat, were investigated in the first stage. Indices of the acid value (3.7 mg KOH/g) and the peroxide value (0.5 mmol of active oxygen/kg) indicate a high quality fat. These indices are within the allowable range of values, specified by Sanitary Regulations and Standards (SanPiN) 2.3.2.2401-08 (acid value not exceeding 4 mg KOH/g, and peroxide value not exceeding 4 mmol of active oxygen/kg).

The next stage of the experiment was production of PUFAs concentrate. Production process included two stages:

Production of free fatty acids

The fat was saponified by heating with backflow condensing for 1 hour at the mixture boiling temperature of (50±2°C), using a mixture

Table 1. Characteristics of the Baikal seal fat

Indices	Fat of Baikal seal
Iodine value, % I_2	187
Peroxide value, mmol of active oxygen/kg	0.5
Acid value, mgKOH/g	3.7

Table 2. Fatty acid profile of fats, PUFAs concentrate, and saturated and monounsaturated fatty acids complex with urea, % of total fatty acids

No	Fatty acid name	Fatty acid index	Seal fat	Concentrate	Complex with urea
1	Lauric acid	12:00	0.224	0.06	0.217
2	Iso-tetradecanoic acid	14:00	4.223	0.185	8.073
3	Iso-pentadecanoic acid	15:0i	2.206	0.313	0.768
4	Pentadecanoic acid	15:00	0.619	0.264	1.069
5	Palmitic acid	16:00	7.543	4.573	20.556
6	Palmitooleic acid	16:1 (n-7)	19.729	22.853	14.371
7	Hexadecadienoic acid	16:2 (n-6)	0.837	1.02	0.113
8	Heptadecanoic acid	17:00	0.881	0.223	0.689
9	Iso-heptadecanoic acid	17:0i	2.118	1.205	0.418
10	Stearic acid	18:00	1.348	0.507	2.552
11	Oleic acid	18:1(n-9)	39.887	41.195	48.68
12	Cis-vaccenic acid	18:1(n-7)	-	4.954	-
13	Linoleic acid	18:2(n–6)	6.631	5.543	0.768
14	γ-linolenic acid	18:3(n-6)	1.042	0.516	0.123
15	α-linolenic acid	18:3(n-3)	2.714	1.776	0.143
16	Steoridic acid	18:4(n-3)	2.815	1.186	0.172
17	Arachidic acid	20:00	-	-	0.207
18	Gondoinic acid	20:1(n-9)	0.291	0.517	0.991
19	Eicozadienoic acid	20:2(n-6)	0.038	0.46	0.09
20	Dihomo-γ-linolenic acid	20:3 (n-6)	0.244	2.126	-
21	Arachidonic acid	20:4 (n-6)	1.638	1.186	-
22	Eicozatrienoic acid	20:3(n-3)	0.442	4.398	-
23	Eicosapentaenoic acid	20:5(n-3)	0.637	1.36	-
24	Clupanodonic acid	22:5(n-3)	0.541	1.14	-
25	Docosapentaenoic acid	22:5(n-6)	0.169	0.1	-
26	Docosahexaenoic acid	22:6(n-3)	3.183	2.34	-
	Sum of saturated fatty acids	19.162	7.33	34.549	
	Sum of monounsaturated fatty acids	59.616	64.565	64.042	
	Sum of polyunsaturated fatty acids	21.222	28.105	1.409	
	Sum of ω-3 PUFAs	10.332	12.2	0.315	
	Sum of ω-6 PUFAs	9.762	15.905	1.094	
	The ratio of ω-6/ω-3	0.94:1	1.3:1	3.47:1	

($NaOH+C_2H_5OH$). Water was added to the saponifaction product. Then hexane was added to the mixture, which wasfurther extracted and after settling out, the lower layer was poured into the flask. Extract of unsaponified matters was repeated twice. The aqueous layer containing the saponified matters was acidified with 10% H_2SO_4 (pH=1.0). The released fatty acids were converted into hexane. The mixture was transferred to a separating funnel. The liberated fatty acids were extracted with hexane. The hexane layer, containing free fatty acids, was washed several times with water in a separating funnel and poured into a conical flask, then dried over anhydrous sodium sulfate. The solvent was then distilled off at 50^0C on a 10 digital IKA HV rotary evaporator with a diaphragm vacuum pump. The yield of fatty acids was 81%.

Production of PUFAs concentrate

Fatty acids and urea were placed in a conical flask and dissolved in ethanol in an amount enabling obtaining a solution, saturated at 50^0C. After vigorous stirring, the solution was left for crystallization. The crystals precipitated upon cooling were sucked off on a glass filter and washed with a small amount of cold alcohol. The alcohol was distilled from the filtrate and the hexane was added to the residue. All substances were transferred to a separating funnel and washed with water. The hexane solution was separated and dried with anhydrous sodium sulfate. Unsaturated acids were weighed after distilling of the hexane.

The crystalline precipitate remaining on the filter (urea complex), was treated with hot water to dissolve the resulting adduct. The cooled solution was shaken with hexane to extract the saturated fatty acids. They were separated from solution in the same way as unsaturated fatty acids. Next, we investigated the iodine values of the produced products. Iodine value gives an indication of the degree of fractions separation into the polyunsaturated and saturated fatty acids. A large value of iodine concentrate (289% I_2), in comparison with the original fat, indicates a high degree of unsaturation of the concentrate that further was confirmed by analysis of fatty acid composition. Iodine value in the urea complex amounted for 66% I_2.

As a result of the conducted experiment, we have produced PUFAs concentrate out of the Baikal seal fat. Examining the fatty acid profile of the fat, PUFAs concentrate, complex of saturated and monounsaturated fatty acids with urea, it was found that the produced concentrate had a high content of PUFAs (28.105%), monounsaturated fatty acids (64.565%), as well as a low content of saturated fatty acids (7.33%). Complex with urea contained a high proportion of saturated fatty acids (34.549%), monounsaturated fatty acids (64.042%) and a minor amount of polyunsaturated fatty acids (1.409%).

The most important polyunsaturated ω-3 fatty acids are the following: α-linolenic acid ($C_{18:3}$, n-3), from which the tissue cells can synthesize long-chain n-3 PUFAs, eicosapentaenoic acid ($C_{20:5}$, n-3) and docosahexaenoic acid ($C_{22:6}$, n-3) with an efficiency of about 5% in men and slightly higher efficacy in women. The possibilities of DHA and EPA synthesis in the body are very limited; therefore they must be ingestedfrom exogenous sources. Inaging process, as well as at certain diseases, the ability of a human to synthesize DHA and EPA is completely lost. Furthermore, it is necessary to take into account that the chain extension reaction and ω-3 and ω-6 fatty acids desaturation reactions are catalyzed by the same enzymes, while fatty acids in these reactions compete for the enzymes. Therefore, an excess of fatty acidsbelonging to the same family, for example, arachidonic acid ($C_{20:4}$, n-6), will suppress the synthesis of the corresponding acid of another family, such as, for example, eicosapentaenoic acid ($C_{20:5}$, n-3). This effect emphasizes the importance of a balanced ratio between ω-3 and ω-6 PUFAs in the diet[17].

Thus, the accumulation of long-chain EPA and DHA in the tissues is the most effective, when they are ingested directly from food, or when competing amounts of ω-6 analogs are low. It was revealed that the easiest way to reduce the synthesis of ω-6 eicosanoids is consumption ofa large amount of ω-3 PUFAs. Dietary administration of EPA and DHA blocks synthesis of eicosanoids from both arachidonic acid, and endogenous eicozatrienoic acid ($C_{20:3}$, n-9). However, if one completely excludes arachidonic acid (AA) from the diet of a healthy person, it will bring only negative result, as metabolites of EPA will not be able to perform fully the functions that perform the metabolites of AA. This is confirmed by the results of epidemiological studies: the inhabitants of the

coastal regions, who feed exclusively sea foods, do not suffer from atherosclerosis, but they have increased bleeding sickness and low blood pressure[10].

Balanced ratio of ω-6/ω-3 PUFAs in the human body plays a key role in the full metabolism of prostaglandins. In the modern dietary ω-6/ω-3 is in a ratio of 30:1, instead of the required 5:1[13]. Comparison of fatty acid composition of the Baikal seal fat and PUFAs concentrate shows that the optimal ratios of ω-6/ω-3 are equal to 0.94:1 and 1.3:1, respectively. The overall level of unsaturated fatty acids in the resulting concentrate is 92.67%, including ω-3 PUFAs (12.2%) and ω-6 PUFAs (15.905%) that indicates the high biological value of the concentrate. The results show the promising use of the produced concentrate for dietary supplements. Found physical and chemical indicators of PUFAs concentrate comply with the hygienic safety requirements and nutritional value of food products.

CONCLUSION

We have developed a method enabling producing a PUFAs concentrate out of seal fat with optimal ratio of ω-6/ω-3 PUFAs. The resulting concentrate can be recommended for the production of dietary supplements that compensate the essential fatty acid deficiency.

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