Baikal Institute of Nature Management[1], Siberian Branch of Russian Academy of Science, Ulan-Ude, Russia;
Department of Pharmaceutics, Biopharmaceutics and Biotechnology[2], Free University of Berlin, Berlin, Germany

Physical and chemical stability of nanostructured lipid drug carriers (NLC) based on natural lipids from Baikal region (Siberia, Russia)

E. S. Averina[1], R. H. Müller[2], D. V. Popov[1], L. D. Radnaeva[1]

Received October 29, 2010, accepted November 16, 2010

Elena S. Averina, Ph.D., Baikal Institute of Nature Management, Siberian Branch of the Russian Academy of Science, Sakhyanovoy st. 6, 670047 Ulan-Ude, Russia
averlen@yandex.ru

Pharmazie 66: 348–356 (2011) *doi: 10.1691/ph.2011.0326*

At the turn of the millennium, a new generation of lipid nanoparticles for pharmacology was developed, nanostructured lipid carriers (NLC). The features of NLC structure which allow the inclusion of natural biologically active lipids in the NLC matrix open a wide prospect for the creation of high performance drug carriers. In this study NLC formulations were developed based on natural lipids from the Siberia region (Russia): fish oil from Lake Baikal fish; polyunsaturated fatty acid fractions and monounsaturated and saturated fatty acid fractions from fish oil and Siberian pine seed oil. Formulation parameters of NLC such as as type of surfactant and storage conditions were evaluated. The data obtained indicated high physical stability of NLC formulated on the basis of pure fish oil stabilized by Tween® 80 and NLC formulated on the basis of free fatty acids stabilized by Poloxamer 188. The good chemical stability of the lipid matrix and the high concentrations of the biologically active polyunsaturated fatty acids in the NLC developed open wide prospects for their use in pharmaceutics and cosmetics.

1. Introduction

During recent years considerable attention has been focused on the development of novel and controlled release drug delivery systems. At the beginning of the 1990 s, the first generation solid lipid nanoparticles (SLN) were developed (Lucks et al. 1996). They were produced from a solid lipid only. In the second generation technology of nanostructured lipid carriers (NLC), the particles are produced using a blend of a solid lipid with a liquid lipid, this blend also being solid at body temperature (Müller et al. 2000a). This nanostructure improves drug loading and firmly incorporates the drug during storage. Like polymeric nanoparticles they possess a solid matrix, protecting chemically labile active compounds and giving the ability to modulate drug release. Like nanoemulsions and liposomes they are composed of well-tolerated lipids, accepted by regulators, and can be produced easily on a large industrial scale (e.g., by high pressure homogenisation). Moreover, features of the NLC structure open the possibility of creating a lipid matrix which not only allows drug targeting but also increases the efficiency of drugs, e.g., by the inclusion of biologically active lipids to the NLC matrix. These biologically active lipids can be natural lipids, for example triglycerides or polyunsaturated acids of plant or animal origin.
Lake Baikal is an ancient rift lake located in the centre of the great Siberian internal drainage basin framed by rugged mountain ranges. Although it does not cover the greatest area it holds the most water of any lake in the world, and it is the world's oldest (25 million yr), deepest (1637 m) and largest (23000 km^3) freshwater reservoir, with over 20% of the world's resources of surface fresh water. The Baikalian fauna diverged in the Tertiary period, and numbers more then 2000 species.
The most interesting Baikal fish is the Golomyanka (fat fish) of the *Comephoridae* family. It contains mainly lipids with triacylglycerol dominating (about 90% of total lipids), and is rich in vitamin A (Kozlova et al. 1993; Ju et al. 1997). There was a time when Tibetian monks came to Baikal and caught golomyanka on its shores. Its fat was used as a remedy for many diseases. Native Siberians used it to treat rheumatism and atherosclerosis and for healing wounds. The biomass of the golomyanka population is estimated to be somewhere between one hundred thousand and one hundred and fifty thousand tons, making it one of the most numerous forms of vertebrate life in Lake Baikal.
The particular interest in studying the tissue composition of marine and freshwater organisms is the biologically active lipids, especially polyunsaturated fatty acids. In the past three decades, views about polyunsaturated fatty acids, especially, ω3 fatty acids, have moved from speculation about their functions to solid evidence that they are not only essential nutrients but also may favorably modulate many diseases. ω3 Fatty acids are important fatty acids, necessary from conception through pregnancy and infancy (retinal and brain development) and, undoubtedly, throughout life (Neuringer et al. 1986; Makrides et al. 1994; Nordoy et al. 1993). The highly polyunsaturated ω3 fatty acids eicosapentaenoic acid (EPA; 20:5ω3) and docosahexaenoic acid (DHA; 22:6ω3) are vital components of the phospholipids of cellular membranes, especially in the brain and retina, and are necessary for their proper functioning. They have a beneficial effect on atherosclerosis, coronary heart disease, inflammatory disease, and behavioral disorders (Kang et al. 1996; Siscovick et al. 1995; Goodnight et al. 1982; Connor 2000). These very-long-chain and highly polyunsaturated ω3 fatty acids are abundant in fish, shellfish, and sea mammals and are scarce or absent in land animals and plants. EPA and DHA are synthesized by phytoplankton, which are plants in water and are the base of the food chain for marine life.
However, land plants also provide a rich source of other polyunsaturated fatty acids which may confer health benefits. For

Table 1: Relative mean contents, as % of sum ± SD, of different fatty acids in fish oil; fractions of polyunsaturated fatty acids (fish PUFA), monounsaturated and saturated fatty acids (fish MSFA) from fish oil; fractionated fatty acids from Siberian pine seed oils (pine PUFA and pine MSFA)

Fatty acids (Cfa:DB)†	Baikal fish oil	Fish MSFA	Fish PUFA	Pine MSFA	Pine PUFA
14:0	4.55 ± 0.18	7.21 ± 0.43	0.25 ± 0.04		
14:1ω5§	1.02 ± 0.06	1.54 ± 0.22	0.57 ± 0.11		
15:0	0.86 ± 0.07	1.25 ± 0.05	0.13 ± 0.02		
16:0	8.63 ± 0.62	14.41 ± 1.03	0.16 ± 0.03	11.44 ± 1.24	
16:1ω9	0.23 ± 0.07	0.27 ± 0.01			
16:1ω7	16.87 ± 1.04	19.29 ± 1.27	5.39 ± 1.01		
16:1ω5	0.54 ± 0.04	0.68 ± 0.03			
16:2ω6	0.89 ± 0.03		4.87 ± 0.98		
16:3ω4	0.65 ± 0.06		4.60 ± 1.00		
17:0	0.25 ± 0.01	0.96 ± 0.07			
18:0	1.01 ± 0.07	2.88 ± 0.26		10.37 ± 1.15	
18:1 ω9	23.19 ± 2.13	36.92 ± 2.78	5.04 ± 0.65	62.79 ± 4.01	1.70 ± 0.33
18:1 ω7	5.98 ± 1.02	8.60 ± 1.31	0.18 ± 0.01		
18:2 ω9					3.62 ± 0.06
18:2 ω6	4.52 ± 0.83	0.93 ± 0.07	10.52 ± 1.09	3.89 ± 0.45	55.47 ± 2.61
18:3ω3	2.99 ± 0.12	0.45 ± 0.06	7.02 ± 1.01		1.36 ± 0.07
18:3ω6	0.30 ± 0.01		1.20 ± 0.32	1.98 ± 0.17	33.42 ± 1.33
18:4 ω3	2.72 ± 0.09	0.33 ± 0.07	6.73 ± 1.04		
20:0				4.38 ± 1.16	
20:1 ω11				4.86 ± 1.09	
20:1ω7	0.51 ± 0.02	1.84 ± 0.19			
20:2 ω6	0.56 ± 0.06	0.82 ± 0.06		0.12 ± 0.02	1.02 ± 0.03
20:4 ω6	2.15 ± 0.09	5.08 ± 1.16	2.55 ± 0.04		
20:4 ω3	0.55 ± 0.02	1.41 ± 0.23			
20:5 ω3	5.49 ± 0.94	0.88 ± 0.16	12.24 ± 1.04		
22:4ω6	0.32 ± 0.02	1.00 ± 0.01			
22:5ω6	1.08 ± 0.15	0.09 ± 0.01	2.95 ± 0.28		
22:5 ω3	3.35 ± 0.82	0.28 ± 0.02	7.45 ± 1.23		
22:6 ω3	10.05 ± 1.03	0.8 ± 0.16	22.16 ± 2.14		
∑SAFA	15.30 ± 0.65	26.71 ± 1.15	0.54 ± 0.05	26.19 ± 2.05	
∑MUFA	48.34 ± 2.58	69.14 ± 3.87	11.08 ± 1.21	67.65 ± 4.15	1.70 ± 0.33
∑PUFA	35.62 ± 1.84	3.76 ± 0.60	88.15 ± 3.59	5.99 ± 0.48	97.44 ± 2.93
ω3	25.15 ± 1.62	2.74 ± 0.24	57.11 ± 3.05		1.36 ± 0.07

† number of carbon atoms of fatty acid chains (Cfa): number of double bonds (DB)

§ ωX – number of carbon atom for the first double bond counted from the end of the fatty acid chain

example, linoleic acid (LA) and alpha-linolenic acid (ALA) are widely distributed in plant oils (Connor 2000). Since they cannot be made in the body from other substances and must be supplied in food, they are called essential fatty acids. Mammals lack the ability to introduce double bonds in fatty acids beyond carbon atoms 9 and 10. Hence linoleic acid and linolenic acid are essential fatty acids for humans. In the body, essential fatty acids are primarily used to produce hormone-like substances that regulate a wide range of functions, including blood pressure, blood clotting, blood lipid levels, the immune response, and the inflammation response to injury infection (Gil 2002).

Siberian pine (Siberian cedar) seeds yield oil with a high medicinal value, traditionally used to cure a wide array of ailments – ingested (decreasing blood pressure, boosting immune system resistance, etc.) or applied externally (a range of dermatological disorders) (Siberian pine seeds 1979). Pine seed oil contains the polyunsaturated fatty acids, pinolenic, linolenic and linoleic acids, and is marketed in various countries as a means to stimulate cell proliferation, prevent hypertension, decrease blood lipid and blood sugar, and inhibit allergic reactions (Asset et al. 2001; Zhukova et al. 2005). A valuable property of cedar oil is its high content of polyunsaturated fatty acids, minerals and vital amino acids and vitamins (Efremov 1998; Pintaeva et al. 2006; Deineka et al. 2003). The Siberian pine seed oil used in this work comes from Siberian cedar seeds (Siberian pine seeds) harvested from the wild in the Baikal region (Siberia, Russia) and grown in taiga areas remote from the effects of industrial particulate and gas emissions, on soil not exposed to chemical fertilizers, pesticides and herbicides.

Thus, using Siberian pine seed oil and Baikal fish oil to create NLC formulations can be seen to have a wide range of prospects in pharmacology and cosmetology to develop new cosmetic and medicinal preparations. Furthermore, by controlling the fatty acid composition during the development of NLC, it is possible to create medicinal products with a specific orientation of action, with higher efficiency. For such purposes it is very desirable to utilise various fractions of definite fatty acid composition. Thus, NLC formulations based on fish oil and also on polyunsaturated acid concentrates, and fractions containing monounsaturated and saturated acids from fish and pine seed oils have been developed and investigated in this work. The formulation parameters affecting the stability of NLC were studied and perspectives for pharmacology and cosmetology evaluated.

2. Investigations, results and discussion

2.1. Fatty acid composition

Study of the fatty acid composition of fish oil by gas chromatography revealed 26 fatty acids, including 35.6% polyunsaturated fatty acids (PuFA) and 25.2% ω3 acids (Table 1). Concentrations of very-long-chain and highly polyunsaturated ω3 fatty acids EPA (20:5ω3) and DHA (22:6ω3) were 5.5% and 10.1%,

respectively. These acids have great importance for metabolic function and are abundant only in marine and freshwater organisms and absent in land animals and plants. The EPA and DNA content in the PUFA fraction was as high as 12.2% and 22.3%, respectively. Concentrating PUFA using the method of forming complexes with urea allows the fish oil polyunsaturated acid content to be enriched to 88% and ω3 acids to 57.1%.

There is no doubt that ω3 fatty acids are important in human nutrition. They are significant structural components of the phospholipid membranes of tissues throughout the body and are especially rich in the retina, brain, and spermatozoa, in which DHA constitutes ≤36.4% the of total fatty acids (Neuringer et al. 1986; Lin et al. 1993). In addition, ω3 PUFA exhibit anti-inflammatory properties in many inflammatory diseases and are beneficial in the treatment of IBD, eczema, psoriasis and rheumatoid arthritis (Gil 2002). Thus, ω3 fatty acids are essential fatty acids, necessary from conception through pregnancy and infancy and, indeed, throughout life.

Moreover, concentrations in the PUFA fraction of two essential fatty acids that cannot be made in the body and must be supplied in food, LA (18:2ω6) and ALA (18:3ω3), are more than twice as high.

Thus, the PUFA fish oil fraction obtained is a concentrate of biologically active fatty acids that opens wide prospects its for use in pharmacology and cosmetology.

Investigation of the fatty acid composition of pine seed oil has been discussed previously (Averina et al. 2010) and the wide spectrum of biologically active fatty acids, including essential and polyunsaturated acids, was described. Concentrating PUFA allows the PUFA content to be increased to 97.4%, with 55.5% of LA and 33.4% of pinolenic acid. Pinolenic acid is an isomer of gamma-linolenic acid and like the essential FA, it forms biologically active metabolites in the presence of cyclooxygenase or lipoxygenase. These metabolites can partially relieve some of the symptoms of essential FA deficiency (Elliott et al. 1985). Moreover, recent research has shown its potential use in weight loss by curbing the appetite. Pinolenic acid causes the triggering of two hunger suppressants – cholecystokinin and glucagon-like peptide-1 (GLP-1) (Causey 2006; Manrique et al. 2005). Pinolenic acid can be used in the form of a food supplement, as a pharmaceutical composition or as part of a food composition as an anti-inflammatory agent (Cain et al. 2002). In addition, pinolenic acid lowers cholesterol levels and has LDL-lowering and blood pressure normalising properties (Lee et al. 2004; Sugano et al. 1994).

It is interesting to consider the composition of the monounsaturated fatty acids (MSFA) fractions. The main component of the MSFA fractions is oleic (18:1ω9) acid: 36.9% for fish MSFA fraction and 62.8% for MSFA extracted from Siberian pine seed oil. Oleic acid has been found to be effective in reversing the inhibitory effect on insulin production of the inflammatory cytokine TNF-α, in lowering the risk of a heart attack or arteriosclerosis, and as an aid to cancer prevention (McDonald 1991; Vassiliou et al. 2009). Moreover, oleic acid is frequently used in the field of dermal research and for the development of transdermal delivery products as a skin penetration enhancer (Touitou et al. 2002; Tanojo et al. 1999; Naik et al. 1995).

Thus, by regulating the lipid composition for NLC creation it is possible to produce highly effective preparations for drug targeting.

2.2. Characterization of the formulations investigated

NLC formulations based on pure Siberian pine seed oil had been developed previously (Averina et al. 2010) and the optimum NLC composition to give the best physical stability of the NLS dispersion obtained was found: lipid concentration and ratio [solid lipid: liquid oil], and surfactant type and concentration. So, using the data obtained, in this study 10% NLC were produced, 10% being the sum of 5% solid lipid (Dynasan 118) and 5% oils (fish oil and fractions). We chose Poloxamer 188 (1.2% w/w) and Tween® 80 (1.2% w/w) as the surfactants not only because of our earlier investigations, but also due to their wide spectrum of use including dermal, oral and intravenous administration.

2.2.1. Physical stability

Measurement of the zeta potential (ZP) allows the stability of colloidal aqueous dispersions to be predicted (Komatsu et al. 1995). Usually, particle aggregation is less likely to occur for charged particles with a high ZP (>30 mV) due to electrical repulsion (Levy 1994), but in the case of non-ionic surfactants, steric hindrance is another additional effect which increases the stability of colloidal dispersions (Lim et al. 2002). In general, lipid nanoparticles are negatively charged on the surface (Schwarz et al. 1999). The determination of ZP was carried out on aqueous NLC dispersions stored at room temperature. The data obtained showed that during storage, there was no great change in the ZP of the lipid nanoparticles, which remained mainly under –30 mV or a little lower (Table 2). This confirmed the high stability of the formulations developed. Moreover, the ZP values of the NLC formulations developed were shown to depend on the type of both the surfactant and the liquid oil used. Thus, with fractionated oils (free fatty acids), a higher ZP value is observed for NLC stabilized by Tween® 80, while for pure fish oil (mainly triglycerides) NLC with Poloxamer 188 possessed the highest ZP.

All the formulations were stored in cold conditions (4 °C), at room temperature (20 °C) and at 40 °C to demonstrate their physical stability. Particle size analysis was performed by photon correlation spectroscopy (PCS) and laser diffractometry (LD) on days 1, 14 and 28.

Data obtained from PCS at room temperature 1 day after production gave mean particle size values between 192 nm, for NLC containing pine PUFA fraction stabilized by Poloxamer 188, and 370 nm, for NLC with fish PUFA fraction and Tween® 80 (Fig. 1). Storage temperature had no great significance for NLC particle size on day 1.

After 14 days' storage at different temperatures (20 °C, 4 °C and 40 °C), the mean particle size did not change significantly and was in the range from 181 nm, for NLC based on fish MSFA fraction and Poloxamer 188, to 370 nm, for NLC with fish PUFA and Tween® 80 (Fig. 1). The mean particle size of the NLC formulations generally changed only slightly after 28 days of storage, except for the NLC containing fish MSFA fraction stabilized by Tween® 80 (403.9 nm). The polydispersity index (PI) for all formulations was lower than 0.25 at day 1 indicating a narrow particle size distribution for the NLC formulations obtained, but 28 days after production the PI for NLC with fish MSFA and Tween® 80 had risen to 0.40, indicating a broader size distribution (Fig. 1).

Analysis of PCS and LD results revealed the dependence of NLC particle size on the type of surfactant and liquid oil. It is of interest that the particle sizes of the NLC formulations based on fractionated oils stabilized by Poloxamer 188 are much smaller compared with the same dispersions stabilized by Tween® 80 (Fig. 1, Table 3). Thus, all NLC with free fatty acids (PUFA, MSFA) stabilized with Poloxamer 188 have a size distribution with d50% (50% of particles smaller than the given value) around 200 nm and with d95% up to 300 nm during the whole storage period at different temperatures. As regards dispersions

Table 2: Mean zeta potentials (ZP ± SD) of NLC, measured on days 1, 14 and 28 after production, room temperature

Liquid lipid	Surfactant	ZP [mV]		
		Day 1	Day 14	Day 28
Fish oil	Poloxamer 188	–30.8 ± 2.4	–34.9 ± 1.0	–38.3 ± 2.8
	Tween® 80	–25.9 ± 1.5	–29.0 ± 1.2	–33.7 ± 1.1
Fish PUFA	Poloxamer 188	–20.2 ± 3.6	–23.0 ± 1.5	–25.1 ± 1.2
	Tween® 80	–38.7 ± 2.6	–34.3 ± 1.9	–32.5 ± 1.1
Fish MSFA	Poloxamer 188	–31.9 ± 3.7	–29.4 ± 1.3	–26.1 ± 1.5
	Tween® 80	–43.8 ± 0.9	–39.4 ± 2.0	–36.2 ± 2.3
Pine PUFA	Poloxamer 188	–32.8 ± 3.6	–32.9 ± 1.7	–28.6 ± 2.0
	Tween® 80	–38.7 ± 2.6	–39.4 ± 0.8	–32.6 ± 0.4
Pine MSFA	Poloxamer 188	–31.7 ± 1.2	–29.2 ± 0.6	–28.6 ± 0.1
	Tween® 80	–44.4 ± 1.3	–42.8 ± 1.8	–37.0 ± 0.6

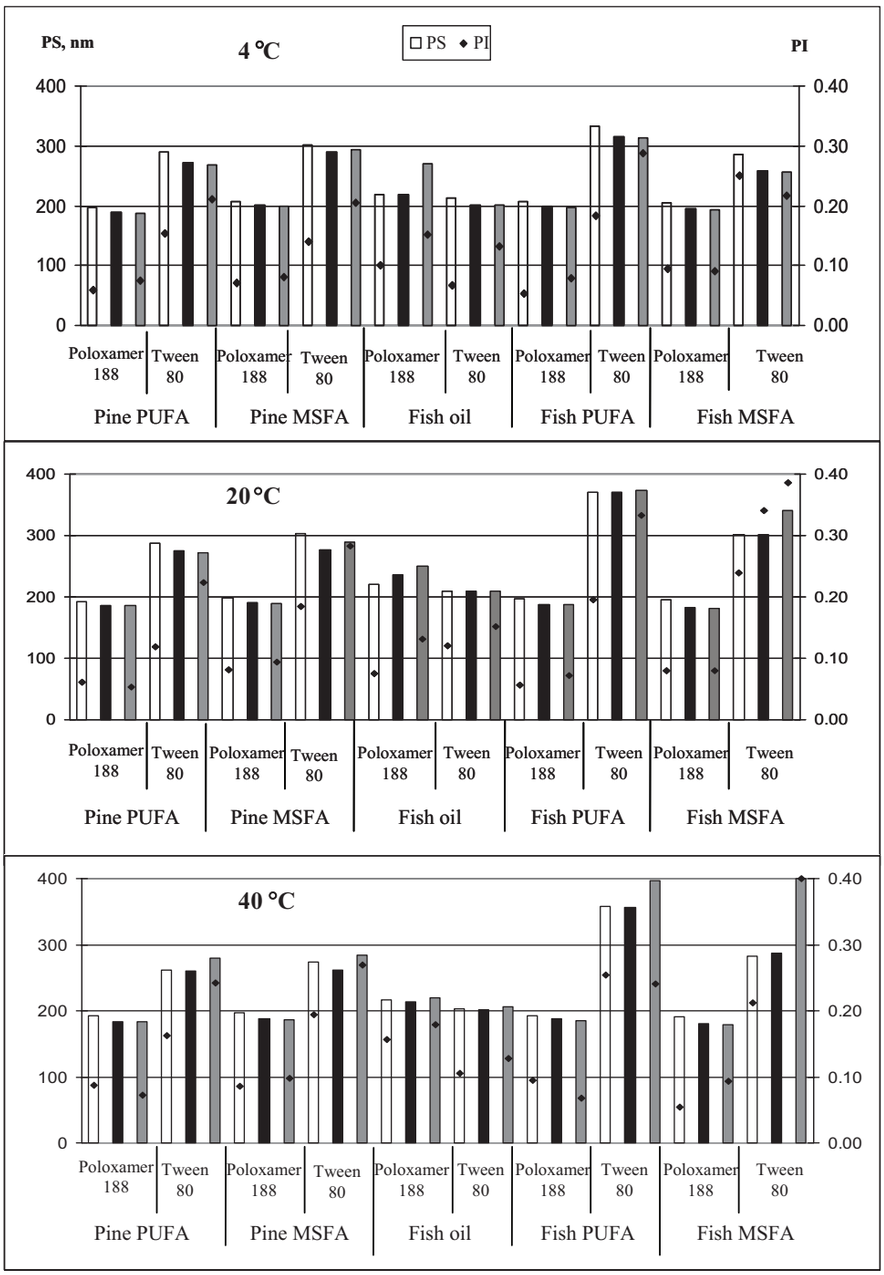

Fig. 1: Particle size (PS) and polydispersity index (PI) of developed NLC at different temperature storage measured on day 1 (white columns), 14 (shaded columns) and 28 (grey columns) days after production

Table 3: LD diameters (d50%, d90%, d95%) of NLC stored at different temperatures on days 1, 14 and 28 after production

Liquid oil	Surfactant	Storage tempera-ture [°C]	LD diameter [μm]†								
			Day 1			Day 14			Day 28		
			d50%	d90%	d95%	d50%	d90%	d95%	d50%	d90%	d95%
Pine PUFA	Poloxamer 188	4	0.2	0.3	0.3	0.2	0.3	0.3	0.2	0.3	0.3
		20	0.2	0.3	0.3	0.2	0.3	0.3	0.2	0.3	0.3
		40	0.2	0.3	0.3	0.2	0.3	0.3	0.2	0.3	0.3
	Tween 80	4	0.2	0.6	3.3	0.2	0.6	4.4	0.2	1.2	7.6
		20	0.2	0.6	3.9	0.2	0.6	4.7	0.2	0.7	7.6
		40	0.3	0.5	0.6	0.3	0.5	0.6	0.3	0.6	1.7
Pine MSFA	Poloxamer 188	4	0.2	0.3	0.3	0.2	0.3	0.3	0.2	0.3	0.3
		20	0.2	0.2	0.3	0.2	0.2	0.3	0.2	0.2	0.3
		40	0.2	0.3	0.3	0.2	0.3	0.3	0.2	0.2	0.3
	Tween 80	4	0.2	0.6	5.3	0.2	0.5	3.5	0.2	0.5	4.9
		20	0.2	3.7	8.2	0.2	5.4	10.9	0.2	8.3	13.6
		40	0.2	0.4	0.54	0.2	0.4	0.5	0.2	0.5	0.6
Fish oil	Poloxamer 188	4	0.3	0.6	0.9	0.3	0.6	0.8	0.4	0.8	1.1
		20	0.3	0.6	0.9	0.3	0.6	0.8	2.8	31.8	38.7
		40	0.3	0.6	0.9	0.3	1.8	5.4	0.9	24.1	57.7
	Tween 80	4	0.2	0.4	0.6	0.2	0.5	0.6	0.2	0.5	0.7
		20	0.2	0.4	0.6	0.2	0.5	0.6	0.2	0.5	0.7
		40	0.2	0.5	0.6	0.2	0.5	0.6	0.2	0.5	0.7
Fish PUFA	Poloxamer 188	4	0.2	0.3	0.3	0.2	0.3	0.3	0.2	0.3	0.3
		20	0.2	0.2	0.3	0.2	0.2	0.3	0.2	0.2	0.3
		40	0.2	0.2	0.3	0.2	0.2	0.3	0.2	0.2	0.3
	Tween 80	4	0.3	4.3	7.5	0.3	3.3	6.8	0.3	3.8	6.9
		20	0.3	0.7	0.9	0.3	0.8	3.6	0.3	0.8	3.2
		40	0.3	0.6	0.7	0.3	0.6	0.7	0.3	0.6	0.7
Fish MSFA	Poloxamer 188	4	0.2	0.2	0.3	0.2	0.3	0.3	0.2	0.3	0.3
		20	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
		40	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
		4	0.2	5.1	10.1	0.2	9.1	15.1	5.4	10.4	19.7
	Tween 80	20	0.2	0.5	3.9	0.2	0.5	5.7	0.3	5.9	39.5
		40	0.2	0.4	0.5	0.2	0.4	0.5	0.2	0.5	2.6

† Standard deviations were typically in the range ± 0.001–0.005 μm

stabilized with Tween® 80, the first aggregates could already be detected one day after production when stored at room temperature and at 4 °C (Table 3). At room temperature the LD diameter d50% was about 200 – 300 nm, d90% was in the range 500 – 700 nm, and d95% was up to 3.9 μm, except for NLC that contained MSFA from Siberian pine seed oil. The particle size distribution for NLC based on pine MSFA and Tween® 80 had a d95% of about 8.2 μm after 1 day's storage at 20 °C. After storage for 28 days at room temperature, 50% of the particles were below 300 nm for all the formulations tested which were stabilized with Tween® 80. The size distribution of these particles showed d95% in the range from 3.2 μm for NLC with fish PUFA to 39.5 μm with fish MSFA. The absence of aggregates at 40 °C in most cases (Table 3) can be explained by the high adsorption of non-ionic surfactants at elevated temperatures (Holmberg et al. 2003). Thus, NLC formulations based on fish and pine fatty acids stabilized by Poloxamer 188 demonstrated better stability.

In contrast, the reverse was found for NLC formulations based on pure fish oil. LD analysis of dispersions stabilized by Tween® 80 showed a size distribution with d50% about 200 nm during the whole test period, with a d95% of 600 nm on days 1 and 14, and about 700 nm on day 28 after production (Table 3). However, the dispersions stabilized with Poloxamer 188 had a particle size distribution with d50% about 900 nm after 1 day and 38.7 μm after 28 days' storage. The same was observed for NLC formulations based on pure Siberian pine seed oil (Averina et al. 2010).

Agglomerates can form due to insufficient coverage of newly generated surfaces by surfactant, during the homogenization procedure (Müller et al. 2002). The coverage of the surfaces of the NLC particles by surfactants in the dispersions is determined by interactions of surfactants with NLC surfaces and their structures. Such interactions are regulated by the critical packing parameter (Holmberg et al. 2003) and depend on the properties of the surfactants such as diffusion velocity and affinity to the particle surface (Müller et al. 2002). Light microscopy confirmed the data above and demonstrated the presence of particle agglomerates for NLC formulations with fish oil loading stabilized by Poloxamer 188, and for NLC with fish PUFA stabilized by Tween® 80 (Fig. 2).

2.2.2. DSC analysis

Differential scanning calorimetry is frequently used to provide information on the physical properties of a compound or formulation, by measuring the heat loss or gain resulting from physical or chemical changes within a sample as a function of the temperature. The method gives a qualitative and quantitative insight into melting behavior and into the polymorphic state of crystalline materials like lipid nanoparticles. The physical state of the particles is very important from a technological as well as a biopharmaceutical point of view.

Table 4 shows the DSC parameters including melting point, onset and enthalpy following storage at different temperatures (4, 20 and 40 °C) for 5 days after production. For all

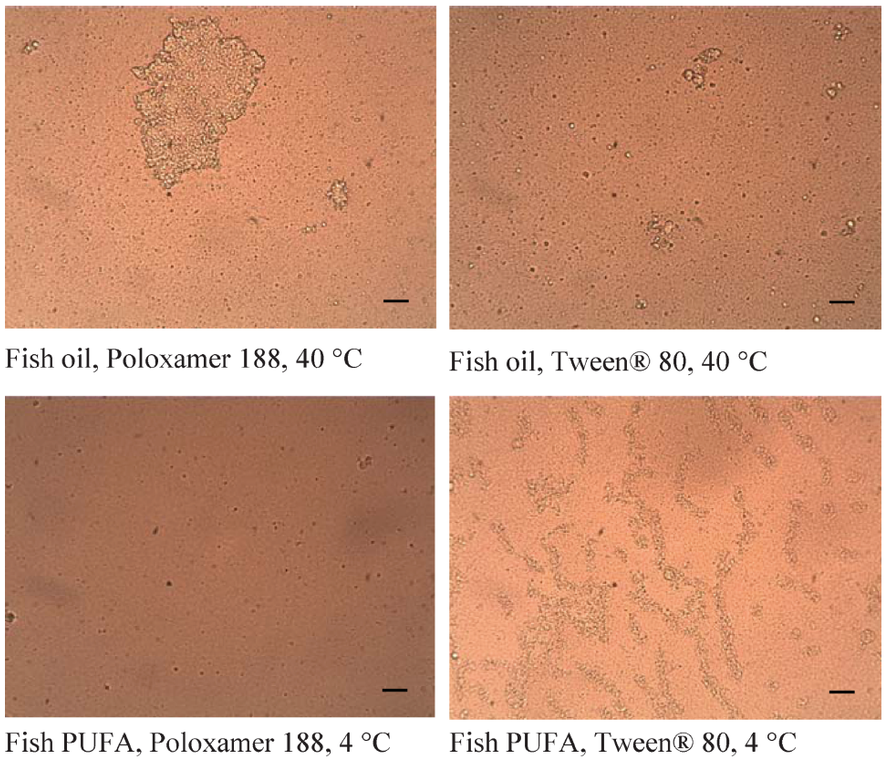

Fig. 2: Light microscope picture magnified 630-fold for NLC stored at different temperature (bar refers to 10 μm) at 28 days after production

the NLC formulations developed, the melting point values decreased compared to bulk Dynasan 118 (72.7 °C), which clearly indicates the association of the two lipids within the single nanoparticles after crystallization (Povey 2001; Saupe et al. 2005; Bunjes et al. 1996). Nevertheless, all the formulations an showed onset and melting point higher than 40 °C which is the prerequisite when lipid nanoparticles are applied, or example, for skin delivery (Saupe et al. 2005). Types of liquid oil and surfactant had a minor impact on the DSC parameters. However, it is of interest to note the higher values of melting enthalpy for NLC with fish oil at 20 °C in comparison with the free fatty acid loaded NLC (Table 4). The same features were observed for NLC with pure pine seed oil: 181.0 J/g for dispersions stabilized with Poloxamer 188 and 203.8 J/g for those with Tween® 80 (Averina et al. 2010). Thus, NLC based on pure oils were characterized by a higher degree of crystallinity compared with NLC formulations with free fatty acids.

Dynasan 118 and fish and pine seed oils, as triacylglycerols, can crystallize in three major polymorphic forms, α, β′ and β (in order of increasing thermodynamic stability) which are characterized by different subcell packing of the lipid chains, different angles of tilt of the lipid chains with respect to the molecular glyceride layers and different densities (Bunjes et al. 1996). Running a DSC scan for Dynasan 118 with two cycles reveals the three modifications: the heating curve of the first cycle shows the β modification with a maximum peak at 72.7 °C and the second cycle the β′ modification with its melting point of 62.9 °C (Averina et al. 2010). Free fatty acids can exist in four different polymorphic forms termed A, B, C and E (Kaneko 2001; Sato 2001). All the forms can be obtained upon crystallization from solution but only the C form grows upon crystallization from the melt (Bunjes et al. 2007).

The addition of liquid oils (pure and fractionated) leads to acceleration of the transition of Dynasan 118 into the stable β-polymorph after crystallisation. The peak for the β'-modification is not pronounced for the NLC formulation, only the stable β-modification melting peak being recognizable (Fig. 3).

In addition, it should be noted that NLC dispersions with fatty acids from fish oil (PUFA and MSFA) have higher melting enthalpy values at 4 °C in comparison with the same NLC formulations at 20 and 40 °C (Table 4). That is probably due to the crystallization behavior of the long-chain fatty acids from the PUFA and MSFA fractions that are absent in pine seed oil.

2.2.3. *Chemical stability*

Storage of NLC dispersions at various temperatures and the effect of oxygen in the air may cause heat and time-dependent losses of ω3 FA (Kolakowska et al. 2006). Long-chain polyunsaturated fatty acids are very sensitive to air (oxygen) and temperature due to the occurrence of numerous methylene-

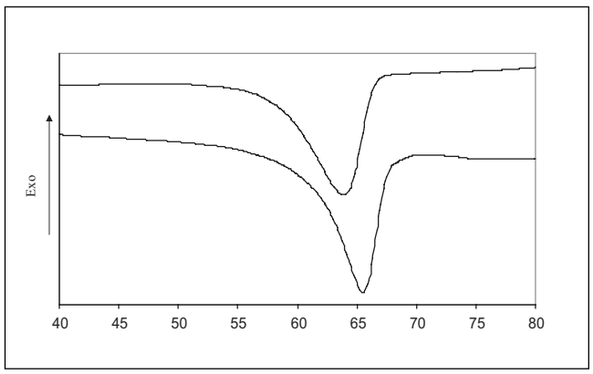

Fig. 3: DSC thermograph of obtained NLC with composition: 1 – Dynasan 118 5%, fish oil 5%, Tween® 80 1.2%; 2 - Dynasan 118 5%, fish MSFA 5%, Poloxamer 188 1.2%

Table 4: DSC results for Dynasan 118 bulk material and NLC formulation, at room temperature

Liquid oil	Surfactant	Tempera-ture [°C]	DSC parameters			
			Onset [°C]	Melting point [°C]	Enthalpy of lipid phase [J/g]	Enthalpy of solid lipid [J/g]
Dynasan 118	–	–	71.77	72.71	201.4	201.4
Fish oil	Poloxamer 188	4	60.18	65.57	72.8	145.6
		20	59.96	65.32	99.8	199.6
		40	60.53	65.71	77.0	154.0
	Tween 80	4	60.85	65.14	93.6	187.2
		20	61.10	65.23	94.3	188.6
		40	61.35	65.47	83.8	167.6
Fish PUFA	Poloxamer 188	4	58.44	63.88	71.6	143.2
		20	61.02	64.56	35.4	70.8
		40	61.18	64.64	35.4	70.8
	Tween 80	4	61.75	65.51	69.3	138.6
		20	62.34	66.08	62.0	124.0
		40	63.13	66.39	57.8	115.6
Pine PUFA	Poloxamer 188	4	56.58	63.48	76.1	152.2
		20	57.66	63.57	77.0	154.0
		40	58.60	64.16	84.2	168.4
	Tween 80	4	59.72	64.56	72.7	145.4
		20	60.80	64.82	68.5	137.0
		40	61.29	65.39	78.9	157.8
Fish MSFA	Poloxamer 188	4	58.34	63.89	83.8	167.6
		20	60.73	64.61	46.1	92.2
		40	60.87	64.99	49.9	99.8
	Tween 80	4	59.80	64.81	82.3	164.6
		20	62.58	66.10	51.6	103.2
		40	62.81	66.33	59.8	119.6
Pine MSFA	Poloxamer 188	4	58.72	63.79	69.4	138.8
		20	59.17	64.15	82.1	164.2
		40	59.99	64.83	72.9	145.8
	Tween 80	4	60.93	65.23	70.3	140.6
		20	61.52	65.50	72.1	144.2
		40	62.05	65.89	77.4	154.8

Enthalpy calculated for lipid phase of particles (solid lipid and oil – 10% in suspension) and also in relation to solid lipid only (i.e. 5%). For bulk Dynasan, both values are identical (201.4 J/g)

interrupted ethylenic double bonds. As a result of these degradation processes the level of polyunsaturated fatty acids may decrease. This can be detected most obviously in NLC containing PUFA fractions. The concentrations of fatty acids have been evaluated in the NLC developed after 3 months storage at different temperatures (Table 5).

Levels of polyunsaturated fatty acids (ΣPUFA) and ω3 fatty acids are slightly decreased after 3 months storage. The lowest changes occurred in NLC lipid matrix (with fish PUFA) at 4 °C: about 81 % of ΣPUFA and about 53 % of ω3 FA, compared to 88 % and 57 %, respectively, for initial fish oil PUFA fractions. Monounsaturated FA concentrations increased, possibly due to PUFA conversion during storage. The concentrations of the long-chain polyunsaturated fatty acids EPA and DHA decreased by about 2 %. Levels of the essential fatty acids LA (18:2ω6) and ALA (18:3ω3) did not significantly change either in fish or pine seed PUFA loaded NLC. The level of polyunsaturated FA (ΣPUFA) in NLC loaded pine seed PUFA remained almost unchanged during 3 months storage. In general, the changes in fatty acid composition were more marked in NLC based on fish PUFA stored at 40 °C.

2.3. Conclusions

It has been shown that NLC formulations developed on the basis of natural oils from the Siberia region as appear to be promising systems for creating new preparations use in for pharmacology and cosmetics. Highly stable NLC could be obtained by selecting suitable types of surfactant and storage conditions. Investigation of the short-term physical stability of the dispersions obtained demonstrated the high stability of the formulations developed irrespective of storage temperature conditions. Moreover, the good chemical stability of the lipid matrix and the high concentrations of the biologically active polyunsaturated fatty acids in the NLC developed open wide prospects for innovative applications.

3. Experimental

3.1. Materials

The following materials were used from the sources indicated without further purification procedures. Dynasan 118 was obtained from Sasol (Hamburg, Germany). Tween® 80 (polyoxyethylene sorbitan monooleate) was donated by Uniqema (Everberg, Belgium) and Poloxamer 188 (Pluronic® F68, polyethylene-polypropylene glycol) from BASF (Germany). Ultra purified water was obtained from a MilliQ Plus system from Millipore (Schwalbach, Germany). Siberian pine seed oil for fractionating was purchased from Taiga-Product (Angarsk, Russia).

Fish oil from Lake Baikal fish of the *Comephoridae* family, Golomyanka, was extracted by simple homogenization of muscle tissues and filtration due to their unique muscle composition consisting primarily of lipids. That permits avoiding the use of organic solvents during the production process. The concentrate of polyunsaturated fatty acids (PUFA) and the fractions containing monounsaturated and saturated fatty acids (MSFA) from pine seed and fish oils was obtained by the method of complex formation with urea (Wanasundara et al. 1999; Swern 1955).

3.2. Fatty acid analysis

The oils obtained were methanolyzed/extracted in a 1-step procedure (Grahl-Nielsen et al. 1985) by treatment with 0.5 ml anhydrous methanol containing

Table 5: Fatty acid composition (% of sum±SD) of the obtained NLC based on polyunsaturated fatty acids fractions from fish oil (fish PUFA), and Siberian pine seed oils (pine PUFA), 3 months storage (without Dynasan 118)

Fatty acid (Cfa:DB)†	Fish PUFA			Pine PUFA		
	4 °C	20 °C	40 °C	4 °C	20 °C	40 °C
14:0	0.32 ± 0.09	0.34 ± 0.06	0.35 ± 0.06			
14:1ω5§	1.04 ± 0.39	2.21 ± 0.43	2.23 ± 0.65			
15:0	0.10 ± 0.03	0.13 ± 0.03	0.08 ± 0.01			
16:0	0.20 ± 0.08	0.49 ± 0.12	0.62 ± 0.24			
16:1ω7	8.99 ± 1.05	9.87 ± 1.15	8.96 ± 1.09			
16:2ω6	4.61 ± 0.74	4.72 ± 0.69	4.68 ± 0.41			
16:3ω4	3.84 ± 0.68	3.75 ± 0.77	3.70 ± 0.96			
18:1ω9	6.64 ± 0.93	7.31 ± 0.62	7.39 ± 0.93	2.63 ± 0.99	2.23 ± 0.63	2.37 ± 0.35
18:1ω7	1.01 ± 1.12	1.18 ± 1.00	1.88 ± 1.13			
18:2ω9				3.04 ± 0.81	2.05 ± 0.56	2.14 ± 0.29
18:2ω6	9.73 ± 1.23	9.62 ± 1.07	8.63 ± 1.38	55.95 ± 4.56	55.79 ± 4.00	55.71 ± 3.72
18:3ω3	6.83 ± 1.09	6.12 ± 0.99	6.87 ± 1.02	0.70 ± 0.12	0.66 ± 0.20	0.60 ± 0.17
18:3ω6	1.11 ± 0.23	1.13 ± 0.31	1.09 ± 0.21	35.60 ± 3.01	36.02 ± 3.26	36.28 ± 3.11
18:4ω3	6.36 ± 1.07	6.21 ± 1.00	6.23 ± 1.02			
20:2ω6	0.56 ± 0.21	0.66 ± 0.25	0.47 ± 0.09	0.42 ± 0.19	0.43 ± 0.23	0.45 ± 0.12
20:4ω6	4.88 ± 1.14	4.85 ± 1.10	4.86 ± 1.02	1.60 ± 0.81	1.87 ± 0.90	1.81 ± 0.41
20:4ω3	0.96 ± 0.23	0.93 ± 0.39	0.89 ± 0.26			
20:5ω3	11.72 ± 1.56	10.80 ± 2.01	10.94 ± 2.11			
22:4ω6	0.84 ± 0.09	0.82 ± 0.16	0.94 ± 0.14			
22:5ω6	2.93 ± 0.62	2.80 ± 0.96	2.80 ± 0.75			
22:5ω3	6.08 ± 1.01	5.94 ± 1.63	6.15 ± 1.39			
22:6ω3	20.66±2.89	19.60 ± 2.59	19.55 ± 2.50			
$\sum$SAFA	0.61 ± 0.12	0.96 ± 0.14	1.05 ± 0.24			
$\sum$MUFA	17.69 ± 1.84	20.58 ± 1.70	20.45 ± 1.94	2.63 ± 0.99	2.23 ± 0.63	2.37 ± 0.35
$\sum$PUFA	81.09 ± 4.30	77.94 ± 4.48	77.79 ± 4.41	94.26 ± 5.59	94.77 ± 5.28	94.85 ± 4.88
ω3	52.60 ± 3.77	49.60 ± 3.94	50.63 ± 3.84	0.70 ± 0.12	0.66 ± 0.20	0.60 ± 0.17

† number of carbon atoms of fatty acid chains (Cfa): number of double bonds (DB),

§ $_{\omega}$X–number of carbon atom for first double bond counted from end of fatty acid chain

HCl at a concentration of 2 mol/l for 2 h in an oven at 90 °C. The fatty acid methyl esters were analysed using a gas-liquid chromatograph equipped with flame ionisation and mass detectors (GC-FID and GC-MS, respectively) (6890N network GC system with autosampler, FID detector and 5973 mass selective detector, Agilent, USA), on a 25 m × 0.25 mm fused-silica column, with polyethylene glycol as the stationary phase (thickness of 0.2 μm; CP-WAX 52CB Chrompack) and helium at 20 psi as the mobile phase. The injection temperature was 260 °C. The temperature of the column was kept at 90 °C for 4 min after injection and thereafter increased to 165 °C at a rate of 30 °C/min, followed by an increase of 3 °C/min to 225 °C. The peaks detected were integrated and the mass spectra extracted using Agilent Chemstation software. Samples were analyzed in random order with a standard solution (GLC-68D from Nu-Chek-Prep; Elysian, Minnesota, USA) containing 20 FAME and MS libraries NIST D.04.00.

3.3. Preparation of nanostructured lipid carries (NLC)

The preparation of aqueous NLC dispersions was carried out according to Müller et al. (2000a, b). Briefly, aqueous dispersions of NLC, composed of 10% (w/w) of lipid phase were produced using the hot high pressure homogenization technique. The mixtures of solid and liquid lipids were melted at approximately 10 °C above the melting point of the solid lipid (Dynasan 118). Then the lipid phase was dispersed and admixed in to a hot aqueous surfactant solution (85 °C) using an Ultra-Turrax T25 (Janke & Kunkel GmbH and Co. KG, Staufen, Germany). The pre-emulsion obtained was subsequently homogenized at 85 °C by a high pressure homogenizer for two cycles at 800 bar using an APV Micron Lab 40 (GEA Niro Soavi Deutschland, Lübeck, Germany). The hot oil/water nanoemulsion was cooled to room temperature leading to the recrystallization of the lipid phase and finally the NLC were formed.

3.4. Particle size analysis

All samples were kept in siliconized glass vials at different temperatures (4 °C, 20 °C and 40 °C). Particle size analysis was performed by photon correlation spectroscopy (PCS) using a Zetasizer Nano-ZS (Malvern Instruments, Malvern, UK) and laser diffractometry (LD) using a Mastersizer 2000 (Malvern Instruments). The surface charge of the NLC was determined by measurement of zeta potential (ZP) of the lipid nanoparticles according to Helmholtz–Smoluchowski from their electrophoretic mobility. ZP was also determined using the Zetasizer Nano-ZS in MilliQ water adjusted to a conductivity of 50 μS/cm with 0.9% sodium chloride solution. PCS gave the mean particle size and the polydispersity index (PI) as a measure of the width of the particle size distribution. Prior to the particle size measurement, the NLC formulation was diluted with double distilled water.

3.5. Differential scanning calorimetry (DSC)

Differential scanning calorimetry analysis was used to characterize the state and the degree of crystallinity of the lipid dispersions. The method allows a closer look at the melting and crystallization behaviour of crystalline materials such as lipid nanoparticles. In the present work, DSC measurements were carried out using a Mettler DSC 821 apparatus (Mettler Toledo, Giessen, Switzerland). The samples were weighed for approximately 1–2 mg in 40 μl aluminum pans. Heating curves were obtained from 25 °C to 90 °C at a heating rate of 5 K/min. An empty aluminum pan was used as a reference. The DSC parameters including onset, melting point and melting enthalpy were evaluated using STARe Software (Mettler Toledo, Switzerland). The melting enthalpy values of the NLC dispersions were recalculated for the 10% lipid phase of NLC and for the solid lipid only taking into account the solid lipid concentration in the NLC formulations.

3.6. Light microscopy

A light microscope (Leitz, Wetzlar, Germany) equipped with a CMEX-1 digital camera (Euromex microscopes, Arnheim, Netherlands) connected to Image Focus software (Euromex microscopes, Arnheim, Netherlands) was used.

Acknowledgements: The authors would like to acknowledge the financial support obtained from DAAD (Deutscher Akademischer Austauschdienst) for the present research.

References

Asset G, Baugé E, Wolff RL, Fruchart JC, Dallongeville J (2001) Effects of dietary maritime pine seed oil on lipoprotein metabolism and atherosclerosis development in mice expressing human apolipoprotein B. Eur J Nutr 40: 268–274.

Averina ES, Seewald G, Müller RH, Radnaeva LD, Popov DV (2010) Nanostructured lipid carriers (NLC) on the basis of Siberian pine (*Pinus sibirica*) seed oil. Pharmazie 65: 25–31.

Bunjes H, Westesen K, Koch MHJ (1996) Crystallization tendency and polymorphic transitions in triglyceride nanoparticles. Int J Pharm 129: 159–173.

Bunjes H, Unruh T (2007) Characterization of lipid nanoparticles by differential scanning calorimetry, X-ray and neutron scattering. Adv Drug Del Rev 59: 379–402.

Cain FW, Parmar P, Powell JR, Rogers JS (2002) Compositions containing pinolenic acid and its use as a health component. United States Patent 6479070.

Causey JL (2006) Korean pine nut fatty acids [pinolenic acid] induce satiety-producing hormone release in overweight human volunteers. American Chemical Society National Meeting & Exposition, March 26–30, Atlanta, GA.

Connor WE (2000) Importance of ω3 fatty acids in health and disease. Am J Clin Nutr 71: 171S–175S.

Deineka VI, Deineka LA (2003) Triglyceride composition of *Pinus sibirica* oil. Chem Natur Comp. 39: 2–10.

Efremov AA (1998) Prospects of cedar seeds processing for products of food and technical purposes. Khim. Rastit. Syrya 3: 83–86.

Elliott WJ, Sprecher H, Needleman P (1985) Physiologic effects of columbinic acid and its metabolites on rat skin. Biochim Biophys Acta 835(1): 158–160.

Gil A (2002) Polyunsaturated fatty acids and inflammatory diseases. Biomed Pharmacother 56: 388–396.

Grahl-Nielsen O, Barnung T (1985) Variation in the fatty profiles of marine animals caused by environmental and developmental changes. Mar Environ Res 17: 218–222.

Goodnight SH Jr, Harris WS, Connor WE, Illingworth DR (1982) Polyunsaturated fatty acids, hyperlipidemia and thrombosis. Arteriosclerosis 2: 87–113.

Holmberg K, Joensson B, Kronberg B, Lindman B (2003) Surfactants and polymers in aqueous solution, John Wiley and Sons, Ltd., England.

Ju S-J, Kucklick JR, Kozlova T, Harvey R (1997) Lipid accumulation and fatty acid composition during maturation of three pelagic fish species in Lake Baikal. J Great Lakes Res 23: 241–253.

Kaneko F (2001) Polymorphism and phase transitions of fatty acids and acylglycerols. In: Garti N, Sato K (ed.) Crystallization Processes in Fats and Lipid Systems. New York, pp. 53–97.

Kang JX, Leaf A (1996) Antiarrhythmic effects of polyunsaturated fatty acids. Circulation 94: 1774–1780.

Kolakowska A, Domiszewski Z, Bienkiewicz G (2006) Effect of biological and technological factors on the utility of fish as a source of n-3 PUFA. In: Teale MC (ed.) Omega 3 Fatty Acid Research. New York, pp. 83–108.

Komatsu H, Kitajima A, Okada S (1995) Pharmaceutical characterization of commercially available intravenous fat emulsions: estimation of average particle size, size distribution and surface potential using photon correlation spectroscopy. Chem Pharm Bull 43: 1412–1415.

Kozlova T, Khotimchenko S (1993) Fatty acid composition of endemic Baikal fish and crustacea. Comp Biochem Physiol 105B (1): 97–103.

Lee JW, Lee KW, Lee SW, Kim IH, Rhee C (2004) Selective increase in pinolenic acid (all-cis5,9,12–18:3) in Korean pine nut oil by crystallization and its effect on LDL-receptor activity. Lipids 39(4): 383–387.

Levy MY, Schutze W, Fuhrer C, Benita S (1994) Characterization of diazepam submicron emulsion interface: role of oleic acid. J Microencapsul 11: 79–92.

Lim SJ, Kim CK (2002) Formulation parameters determining the physicochemical characteristics of solid lipid nanoparticles loaded with all-trans retinoic acid. Int J Pharm 243: 135–146.

Lin DS, Connor WE, Wolf DP, Neuringer M, Hachey DL (1993) Unique lipids of primate spermatozoa: desmosterol and docosahexaenoic acid. J Lipid Res. 34: 491–499.

Lucks JS, Müller RH (1996) Arzneistoffträger aus festen Lipidteilchen — feste Lipid Nanosphären (SLN). European Patent 0 605 497.

Makrides M, Neumann MA, Byard RW, Simmer K, Gibson RA (1994) Fatty acid composition of brain, retina, and erythrocytes in breast- and formula-fed infants. Am J Clin Nutr 60: 189–194.

Manrique C, Lastra G, Whaley-Connell A, Sowers JR (2005) Hypertension and the cardiometabolic syndrome. J Clin Hypertens 7(8): 471–476.

McDonald BE (1991) Monounsaturated fatty acids and heart health. Can Med Assoc J 145(5): 473.

Müller RH, Mäder K, Lippacher A, Jenning V (2000a) Fest-flüssig (halbfeste) Lipidpartikel und Verfahren zur Herstellung hochkonzentrierter Lipidpartikeldispersionen, PCT-application PCT/EP00/04565.

Müller RH, Mäder K, Gohla S (2000b) Solid lipid nanoparticles (SLN) for controlled drug delivery – a review of the state of the art. Eur J Pharm Biopharm 50: 161–177.

Müller RH, Jacobs C (2002) Buparvaquone mucoadhesive nanosuspension: preparation, optimisation and long-term stability. Int J Pharm 237: 151–161.

Naik A, Pechtold LARM, Potts RO, Guy RH (1995) Mechanism of oleic acid-induced skin penetration enhancement *in vivo* in humans. J Control Release 37: 299–306.

Neuringer M, Connor WE, Lin DS, Barstad L, Luck S (1986) Biochemical and functional effects of prenatal and postnatal omega-3 fatty acid deficiency on retina and brain in rhesus monkeys. Proc Natl Acad Sci USA 83: 4021–4025.

Nordoy A, Hatcher LF, Ullmann DL, Connor WE (1993) Individual effects of dietary saturated fatty acids and fish oil on plasma lipids and lipoproteins in normal men. Am J Clin Nutr 57: 634–639.

Pintaeva ETs, Shiretorova VG, Hanturgaev AG, Radnaeva LD, Zvontsov IV (2006) Chemical composition of Cedar oil. East-Sib Technol Univ Proc, Ulan-Ude (Russia) 10: 130–136.

Povey MJW (2001) Crystallization of oil-in-water emulsions. In: Garti N, Sato K (ed.) Crystallization Processes in Fats and Lipid Systems. New York, pp. 177–209.

Sato K (2001) Crystallization of fats and fatty acids. In: Garti N, Sato K (ed.) Crystallization Processes in Fats and Lipid Systems. New York, p. 227–263.

Saupe A, Wissing S, Lenk A, Schmidt C, Müller RH (2005) Solid lipid nanoparticles (SLN) and nanostructured lipid carriers (NLC) — structural investigations on two different carrier systems. Biomed Mater Eng 15: 393–402.

Schwarz C, Mehnert W (1999) Solid lipid nanoparticles (SLN) for the controlled drug delivery. II. Drug incorporation and physicochemical characterization. J Microencapsul 16: 205–213.

Siberian pine seeds (1979) Ed. N. E. Sudachkova, Nauka, Novosibirsk (Russia).

Siscovick DS, Raghunathan TE, King I (1995) Dietary intake and cell membrane levels of long-chain n3 polyunsaturated fatty acids and the risk of primary cardiac arrest. JAMA 274: 1363–1367.

Sugano M, Ikeda I, Wakamatsu K, Oka T (1994) Influence of Korean pine (Pinus koraiensis) seed oil containing cis-5,cis-9,cis-l2-octadecatrienoic acid on polyunsaturated fatty acid metabolism, eicosanoid production and blood pressure of rats. Br J Nutr 72(5): 775–783.

Swern D (1955) Urea and thiourea complexes in separating organic compounds. Ind Eng Chem 47 (2): 216–21.

Tanojo H, Boelsma E, Junginger HE, Ponec M, Boddeĭ HE (1999) *In vivo* human skin permeability enhancement by oleic acid: a laser Doppler velocimetry study. J Control Release 58: 97–104.

Touitou E, Godin B, Karl Y, Bujanover S, Becker Y (2002) Oleic acid, a skin penetration enhancer, affects Langerhans cells and corneocytes. J Control Release 80: 1–7.

Vassiliou EK, Gonzalez A, Garcia C, Tadros JH, Chakraborty G, Toney JH (2009) Oleic acid and peanut oil high in oleic acid reverse the inhibitory effect of insulin production of the inflammatory cytokine TNF-α both *in vitro* and *in vivo* systems. Lipids in Health and Disease doi:10.1186/1476-511X-8-25, http://www.lipidworld.com/content/8/1/25.

Wanasundara UN, Shahidi F (1999) Concentration of omega 3-polyunsaturated fatty acids of seal blubber oil by urea complexation: optimization of reaction conditions. Food Chem 65: 41–49.

Zhukova EE, Budaeva VV, Egorova EU, Erohina GV, Vizukova AV (2005) Pine seed oil and opportunities of its usage. Oils Fats 2: 9–15.