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## Nanostructured lipid carriers (NLC) on the basis of Siberian pine (*Pinus sibirica*) seed oil

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Nanostructured lipid carriers (NLC) are new drug systems composed of physiological lipid materials. The possibility of including different types of lipids into the NLC structure revealed the wide prospects for using biologically active natural oils for the development of the cutaneous preparations. In this study the formulation parameters of NLC on the basis of Siberian pine seed oil were evaluated including concentration of lipids, types of surfactants and storage conditions (4 °C, 20 °C, 40 °C). Size distribution and storage stability of formulations produced by hot high pressure homogenisation were investigated by laser diffractometry and photon correlation spectroscopy. The NLC were characterised by their melting behaviour using differential scanning calorimetry. The obtained data indicated the high physical stability of the developed NLC formulations.

### 1. Introduction

In recent years, nanotechnology has been intensively studied in many science and engineering fields as well as in pharmaceutical technology and cosmetology. In the pharmaceutical and cosmetology field, several advantages of drug and biologically active compound delivery systems with nanosize range have been shown including increasing solubility, enhancing dissolution rate and improving bioavailability (Müller et al. 2000a). Nanoparticles can be prepared using different kinds of materials, for example biodegradable and biocompatible polymers, phospholipids, surfactants and lipids (Jenning et al. 2000; Müller et al. 2002).

At the beginning of the 1990s solid lipid nanoparticles (SLN) were introduced as a novel carrier system for cosmetic active ingredients and pharmaceutical drugs (Müller et al. 1995; Siekmann and Westesen 1996; Mehnert and Mäder 2001; Trotta et al. 2003).

Based on the experiences with these nanoparticles composed of solid lipids, at the turn of the millennium a new type of lipid nanoparticles has been developed by incorporating spatially different lipids and oils in the solid matrix of said particle, the so-called nanostructured lipid carriers (NLC) (Müller et al. 2000; Radtke and Müller 2001; Wissing et al. 2003).

In contrast to SLN being produced from solid lipids only, the NLC are produced by controlled mixing of solid lipids with spatially different liquid lipids leading to special nanostructures with improved drug incorporation and release properties.

The tendency to use medical preparations and cosmetic ingredients based on natural raw materials attracts an attention to oils containing biologically active polyunsaturated fatty acids. Siberian pine (*Pinus sibirica*) seed oil is a concentrate of powerful antioxidants, mono- and polyunsaturated fatty acids, complex of vitamins and minerals necessary for people in their

everyday life. Siberian pine seed oil with high medicinal value, traditionally used to cure ingested or applied externally—a range of dermatological disorders (Artamonov 1990; Cedar: a magnificent healer 2002).

Siberian pine seed oil is widely used to stimulate cell proliferation, prevent hypertension, decrease blood lipid and blood sugar, and inhibit allergic reactions (Rubchevskaya 1999). Apart from medicine, pine seed oil is used in cosmetics, beauty products, and as a high end massage oil. Massage with Siberian pine seed oil relieves fatigue, increases vitality, improves the blood circulation. Usage of pine seed oil ensures a high-grade care for skin. It can be used for cleaning, nourishment and protection of the skin on any sites of a body. This oil is useful for people suffering from skin diseases, baldness, increased fragility of nails and hair (Siberian pine seeds 1979; Chemistry of natural and biologically active compounds 1987; Zhukova et al. 2005). Siberian pine is considered a symbol of youth and longevity, the use of the pine nut oil confirms it vividly.

Using this oil for creation of NLC formulations reveals wide prospects for cosmetology and pharmacology. In this study NLC on the basis of Siberian pine seed oil have been developed. The formulation parameters affecting the stability of NLC have been evaluated including different lipid concentration, type of surfactants and storage conditions.

### 2. Investigations, results and discussion

#### 2.1. Investigation of the fatty acids composition of Siberian pine seed oil

The Siberian pine seed oil used in this work for NLC production comes from wild-harvested Siberian cedar seeds (Siberian pine seeds) from Baikal region (Siberia, Russia) growing in taiga zones remote from influence of powder-gas emissions of the

**Table 1: Fatty acid composition of Siberian pine seed oil**

Fatty acids (Cfa:DB)*	Fatty acid name	Concentration (%)
14:0	Myristic	0.03
16:0	Palmitic	4.61
16:1n9	<i>cis</i> -7-Hexadecenoic	0.02
16:1n7	Palmitoleic	0.04
18:0	Stearic	2.98
18:1n9	Oleic	27.33
18:1n7	<i>cis</i> -Vaccenic	0.37
18:2n9	6- <i>cis</i> ,9- <i>cis</i> -Octadecadienoic	1.83
18:2n6	Linoleic acid	41.33
18:3n6	$\gamma$ -Linolenic acid	18.73
18:3n3	$\alpha$ -Linolenic acid	0.16
20:0	Arachidic	0.33
20:1n11	9- <i>cis</i> -Eicosenoic acid	1.12
20:1n9	11- <i>cis</i> -Eicosenoic acid	0.09
20:2n6	11- <i>cis</i> ,14- <i>cis</i> -Eicosadienoic	0.49
20:4n6	Arachidonic	0.51

\* – number of carbon atoms of fatty acid chains (Cfa); number of double bonds (DB)  
 nX – number of carbon atom for the first double bond counted from the end of the fatty acid chain

industrial enterprises, on soil not exposed to chemical fertilizers, pesticides and herbicides.

Investigation of the pine seed oil fatty acid composition by gas chromatography has revealed a wide spectrum of biologically active mono and polyunsaturated fatty acids, including linoleic (41.3%), linolenic (18.9%), arachidonic (0.5%) acids (Table 1). A distinctive feature of the Siberian pine seed oil is the high concentration of oleic (27.3%), linoleic and linolenic (18:3n6 and 18:3n3) acids. These polyunsaturated fatty acids are the energy substrate for the intracellular respiration process and enters into phospholipid membrane structure (Lehninger 1979; Efremov 1998). Rather large percent from the sum of all acids make monounsaturated oleic acid and polyunsaturated acids - linoleic and linolenic, which concern to group of vitamin F, raising body resistance to radioactive radiation, stimulates healing and renewal of the tissues, and provides a local anti-inflammatory effect (Artamonov 1990; Gil 2002; Cakir 2004; Pintaeva et al. 2006). Unsaturated fatty acids have excellent moisturizing, nourishing and revitalizing properties, are helpful for inflammation, dehydrated and sensitive skin, and show remarkable skin penetrating and softening properties (Tanojo et al. 1994; Naik et al. 1995; Touitou et al. 2002). The

high concentration of the polyunsaturated fatty acids, including essential acids, contributes to the high biological activity of Siberian pine seed oil for cutaneous application.

## 2.2. Characterization of the investigated formulations

In this study 10% NLC were produced, the 10% are the sum of solid lipid and oil. The concentration ratio of solid lipid Dynasan 118 and liquid lipid Siberian pine seed oil, changed from 7.5%:2.5% to 5.0%:5.0%. In addition, different types of surfactant were also selected to stabilize lipid nanoparticles including Tego® Care 450 1.2% and 1.8% (w/w), PlantaCare® 2000 1.2% (w/w), Poloxamer 188 1.2% (w/w) and Tween® 80 1.2% (w/w).

### 2.2.1. Physical stability

The measurement of the zeta potential (ZP) value allows predictions about the stability of colloidal aqueous dispersions (Komatsu et al. 1995). Usually, particle aggregation is less likely to occur for charged particles with high ZP (>30 mV) due to electric repulsion (Levy et al. 1994). In general, lipid nanoparticles are negatively charged on the surface (Schwarz and Mehnert 1999). The determination of ZP was performed from aqueous NLC dispersions stored at room temperature. The ZP and standard deviation (SD) values of NLC are shown in Table 2.

The zeta potential value for developed NLC formulation was revealed to be basically dependent on the type of surfactants. NLC stabilized by Tego® Care 450 and PlantaCare® 2000 possessed the highest value. Tween® 80 showed the lowest value of ZP, however, this value was still around –30 mV for NLC containing 5.0% Dynasan 118 and 5.0% pine seed oil (between –29.8 and –32.9 mV) indicating good physical stability. Moreover, in case of non-ionic surfactants, steric hindrance is another additional effect which increases the stability of colloidal dispersions (Lim and Kim 2002). The obtained data showed that during storage time, the ZP value of the lipid nanoparticles changed a little. The comparison of the NLC formulations with the same surfactants revealed that the increasing of the pine seed concentration in the lipid phase generally enhanced the ZP value of the developed NLC slightly, except for NLC stabilized by Tego® Care 450 after 28 days of storage. All the formulations were stored in the cold (4 °C), at room temperature (20 °C) and at 40 °C to demonstrate their physical stability. Particle size analysis was performed by PCS and LD on days 1, 14 and 28.

**Table 2: Mean zeta potential (ZP) values of the developed NLC, measured on day 1, 14 and 28 days after production, room temperature**

Lipid concentration	Surfactant	Surfactant concentration (%)	ZP (mV)		
			Day 1	Day 14	Day 28
Dynasan 118 – 7.5% Pine seed oil – 2.5%	Tego® Care 450	1.2	−52.5 ± 2.8	−50.2 ± 1.9	−55.7 ± 1.0
		1.8	−46.5 ± 2.3	−50.8 ± 3.2	−53.5 ± 3.7
	PlantaCare® 2000	1.2	−48.4 ± 2.1	−49.3 ± 3.3	−45.5 ± 4.1
	Poloxamer 188	1.2	−28.9 ± 0.5	−28.5 ± 1.6	−31.9 ± 2.5
	Tween® 80	1.2	−23.4 ± 2.5	−22.9 ± 1.2	−26.8 ± 0.9
Dynasan 118 – 5.0% Pine seed oil – 5.0%	Tego® Care 450	1.2	−52.4 ± 1.7	−49.9 ± 1.2	−47.1 ± 3.7
		1.8	−48.5 ± 1.3	−49.7 ± 3.4	−51.7 ± 1.6
	PlantaCare® 2000	1.2	−56.4 ± 2.9	−56.5 ± 3.6	−58.3 ± 2.9
	Poloxamer 188	1.2	−35.7 ± 1.2	−33.9 ± 1.3	−34.4 ± 1.6
	Tween® 80	1.2	−29.8 ± 0.6	−30.3 ± 0.7	−32.9 ± 1.1

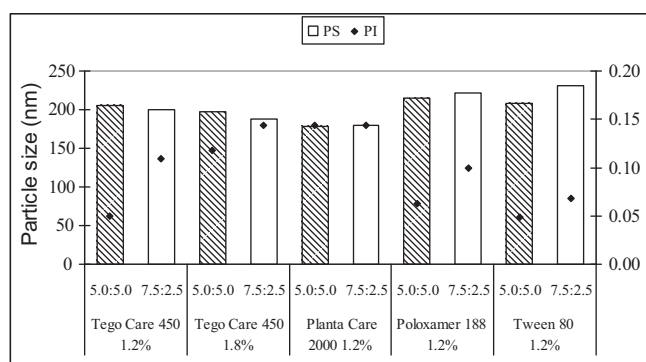


Fig. 1: Particle size (PS) and polydispersity index (PI) of developed NLC after one day storage at room temperature (20 °C), lipid ratio of Dynasan 118: Pine seed oil was 5.0%: 5.0% and 7.5%: 2.5%, respectively

Data obtained from PCS 1 day after production, indicated mean particle size values between 175 nm for NLC containing 5.0% of pine seed oil stabilized by PlantaCare® 2000 and 231 nm for NLC containing 2.5% of pine seed oil stabilized by Tween® 80 (Table 3). The polydispersity index (PI) for all formulations was lower than 0.15 indicating narrow particle size distribution for the obtained NLC formulations (Fig. 1).

Additionally, after 14 days storage at room temperature (20 °C), 4 °C and 40 °C, the mean particle size did not significantly change and was less than 232 nm for all formulations (Table 3). The lower range size value 175 nm remained for NLC containing 5.0% of pine seed oil with PlantaCare® 2000. The largest particle size value of the NLC containing 2.5% of pine seed oil with Tween® 80 also remained unchanged. The mean particle size was in range 181–232 nm on 28 day after production. Concerning the effect of storage temperature and liquid oil concentration on the mean particle size, no clear correlation was observed during this storage period. NLC dispersions stabilized by Tego® Care 450 and PlantaCare® 2000 showed the lowest particle size formulation irrespective of pine seed oil concentration in the lipid phase.

Analysis of PCS and LD results revealed the dependence of NLC particle size from the type of surfactants. Liquid oil concentration and storage condition had only a minor impact on the mean particle size. LD analysis demonstrated the size distribution with d50% (50% of the particles are smaller than the said value) around 180 nm and with d95% – less than 600 nm for obtained NLC formulations after one day storage at room temperature, except the NLC with Poloxamer 188 (Figs. 2, 3). The NLC dispersions stabilized with Poloxamer 188 one day after production had particle sizes of d50% about 260 nm, d90%

**Table 3: Particle size analysis of NLC determined by PCS, measured on day 1, 14 and 28 days after production at different storage temperatures (4 °C, 20 °C, 40 °C)**

Lipid concentration	Surfactant	Temperature (°C)	Particle size (nm)*		
			Day 1	Day 14	Day 28
Dynasan 118 – 7.5% Pine seed oil – 2.5%	Tego® Care 450, 1.2%	4	194	201	194
		20	199	199	195
		40	191	192	190
	Tego® Care 450, 1.8%	4	188	190	188
		20	187	190	188
		40	185	184	187
	PlantaCare® 2000, 1.2%	4	180	180	181
		20	179	181	181
		40	180	179	226
	Poloxamer 188, 1.2%	4	223	221	222
		20	221	223	221
		40	226	224	216
	Tween® 80, 1.2%	4	230	228	225
		20	231	232	232
		40	219	217	215
Dynasan 118 – 5.0% Pine seed oil – 5.0%	Tego® Care 450, 1.2%	4	200	205	200
		20	205	201	201
		40	192	189	192
	Tego® Care 450, 1.8%	4	198	200	199
		20	197	198	198
		40	195	193	197
	PlantaCare® 2000, 1.2%	4	176	176	177
		20	178	178	210
		40	175	175	175
	Poloxamer 188, 1.2%	4	214	209	212
		20	215	217	211
		40	212	208	199
	Tween® 80, 1.2%	4	210	209	200
		20	208	208	203
		40	204	201	197

\* – Standard deviations were typically in the range  $\pm 2$ –4 nm

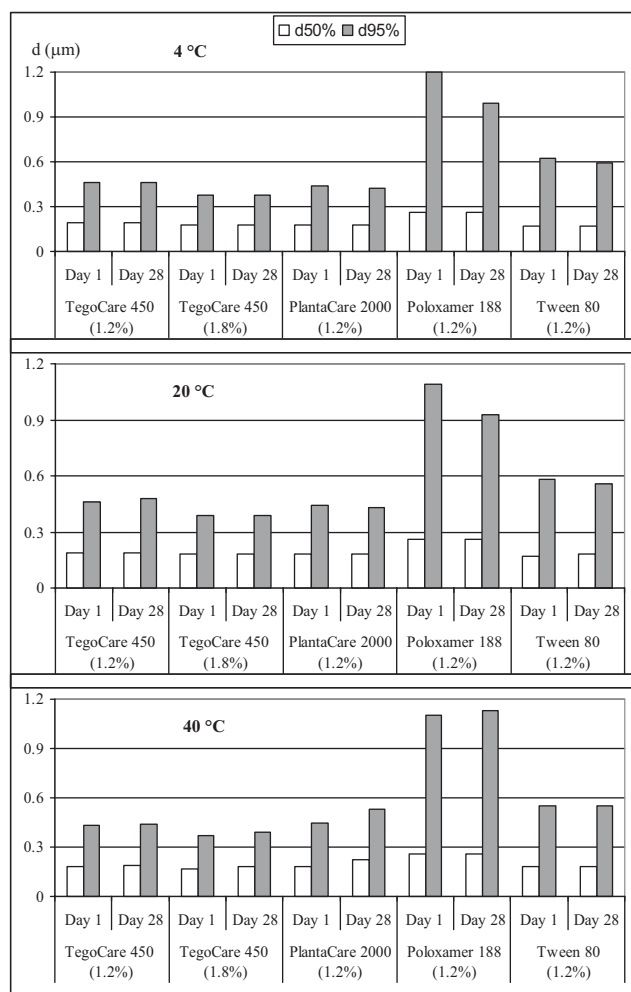


Fig. 2: LD diameters (d50%, d95%) of NLC at different temperature storage measured on day 1 and 28 days after production, lipid phase composition: Dynasan 118 was 7.5%, Pine seed oil was 2.5%

of 650 nm. The d95% for the NLC with pine seed content of 2.5% was up to 1.2  $\mu\text{m}$ , NLC dispersions with pine seed content of 5.0% had a d95% around 0.9  $\mu\text{m}$  (Fig. 3).

After 28 day storage at room temperature 50% of the particles were below 300 nm for all tested formulations. The NLC formulation stabilized by Tego<sup>®</sup> Care 450, PlantaCare<sup>®</sup> 2000 and Tween<sup>®</sup> 80 demonstrated a particle size of d95%

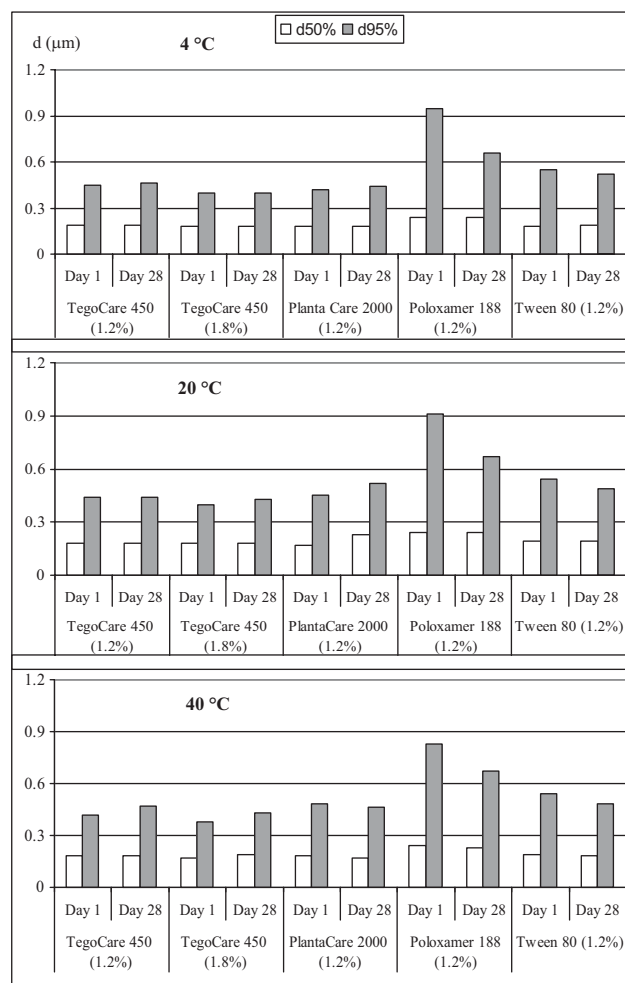
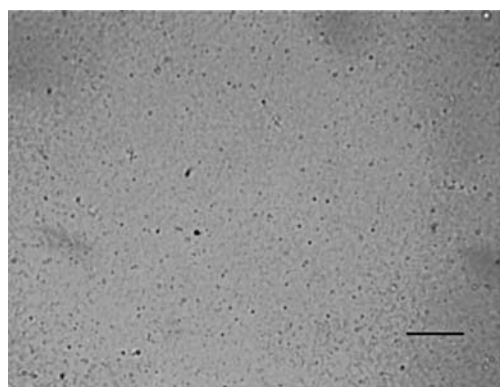


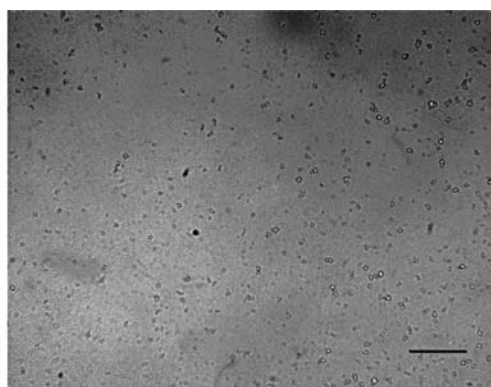
Fig. 3: LD diameters (d50%, d95%) of NLC at different temperature storage measured on day 1 and 28 days after production, lipid phase composition: Dynasan 118 was 5.0%, Pine seed oil was 5.0%

less than 600 nm irrespective of surfactant and liquid oil concentrations at 28 days after production, while d95% values for the NLC dispersions stabilized by Poloxamer 188 remained up to 1.1  $\mu\text{m}$  (Figs. 2, 3). This indicates high stability.

Light microscope pictures confirmed the data above and demonstrate the absence of microparticles for NLC formulation with



Dynasan 118 – 5.0 %  
Pine seed oil – 5.0 %  
Tego<sup>®</sup> Care 450 – 1.8 %



Dynasan 118 – 5.0 %  
Pine seed oil – 5.0 %  
Poloxamer 188 – 1.2 %

Fig. 4: Light microscope picture magnified 1000-fold for NLC stored at room temperature (bar refers to 10  $\mu\text{m}$ ) at 28 days after production

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

Lipid composition	Surfactant	Days	DSC parameters			
			Onset (°C)	Peak (°C)	Enthalpy of lipid phase (J/g)	Enthalpy of solid lipid (J/g)
Dynasan 118	–	–	71.77	72.71	201.4	201.4
Dynasan 118 – 7.5% Pine seed oil – 2.5%	Tego® Care 450, 1.2%	3	63.11	67.56	192.7	250.5
		30	63.59	67.53	140.3	182.4
	Tego® Care 450, 1.8%	3	63.46	67.15	146.0	189.8
		30	62.99	67.08	103.1	134.0
	PlantaCare® 2000, 1.2%	3	62.72	67.48	139.1	180.8
		30	66.03	69.10	128.5	167.1
	Poloxamer 188, 1.2%	3	62.88	67.97	174.0	226.2
		30	63.84	68.31	154.2	200.5
	Tween® 80, 1.2%	3	63.79	67.45	158.2	205.7
		30	64.29	67.63	146.2	190.1
Dynasan 118 – 5.0% Pine seed oil – 5.0%	Tego® Care 450, 1.2%	3	59.70	65.23	96.0	192.0
		30	59.55	64.82	76.9	153.8
	Tego® Care 450, 1.8%	3	59.19	64.56	59.2	118.4
		30	59.28	64.48	59.7	119.4
	PlantaCare® 2000, 1.2%	3	59.22	64.56	96.0	192.0
		30	63.05	66.82	90.0	180.0
	Poloxamer 188, 1.2%	3	59.78	64.99	90.5	181.0
		30	60.26	65.58	78.3	156.6
	Tween® 80, 1.2%	3	60.45	65.06	101.9	203.8
		30	60.80	65.30	84.6	169.2

The enthalpy was calculated for the lipid phase of the particles (solid lipid and oil–10% in suspension) and in addition related to the solid lipid only (i.g. being 7.5% and 5%, respectively). For Dynasan bulk, both values are identical (201.4 J/g)

5.0 % liquid oil loading stabilized by 1.8% Tego® Care 450. Some particles around 1 µm were found for NLC stabilized by Poloxamer 188 (Fig. 4).

Practically unchanged particle size values were found for the NLC formulations according to the PCS and LD measurements. Such consistency in particle size and PI up to 0.15 as well as d50% and d95% values obtained from LD indicated the high physical stability of the developed formulations.

### 2.2.2. DSC analysis

The solid state of the particles and the existence of NLC were investigated using DSC method. The method gives a qualitative and quantitative insight into the melting behavior and on the polymorphic state of crystalline materials like lipid nanoparticles. The physical state of the particles is very important from the technological as well as from the biopharmaceutical point of view.

Table 4 shows the DSC parameters including melting point, onset and after 3 and 30 days of storage at room temperature (20 °C) enthalpy. For developed NLC, the melting point values decreased compared to the bulk Dynasan 118 (72.7 °C), but still all formulations showed onset and melting point of higher than 40 °C which is the prerequisite when lipid nanoparticles are applied for skin delivery (Saupe et al. 2005).

The DSC parameters for developed NLC formulation were dependent on the composition of the lipid phase and surfactant type. There was a clear reduction in the melting point and melting enthalpy as the amount of pine seed oil increased. All formulation with 2.5% Pine seed oil in the lipid phase have the melting point value higher than 67 °C and melting enthalpy values from 139.1 to 192.7 J/g. The NLC with 5% pine seed oil have the peak around 65 °C and maximum enthalpy value

101.9 J/g. The type and concentration of surfactants mainly influence enthalpy. The value of melting enthalpy for developed NLC stabilized by 1.2% and 1.8% of Tego® Care 450 decreased from 192.7 J/g to 146.0 J/g, respectively, for formulations containing Dynasan 118 7.5% and Pine seed oil 2.5% (values at 3 days after production). It decreased from 96.0 J/g to 59.2 J/g for NLC dispersions with 5% pine seed oil loading.

Dynasan 118 as a triacylglycerol can crystallize in three different modifications:  $\alpha$ ,  $\beta'$  and  $\beta$  (most thermodynamically stable). Running a DSC with two cycles reveals the three modifications (Fig. 5). The heating curve of the first cycle shows the  $\beta$  modification with a peak maximum at 72.7 °C and the second cycle reveals the  $\beta'$  modification with its melting point of 62.9 °C. Crystallisation of bulk material from the melt after cooling usually occurs in the metastable  $\alpha$ -form which transforms via the  $\beta'$ - into the stable  $\beta$ -form upon heating or storage

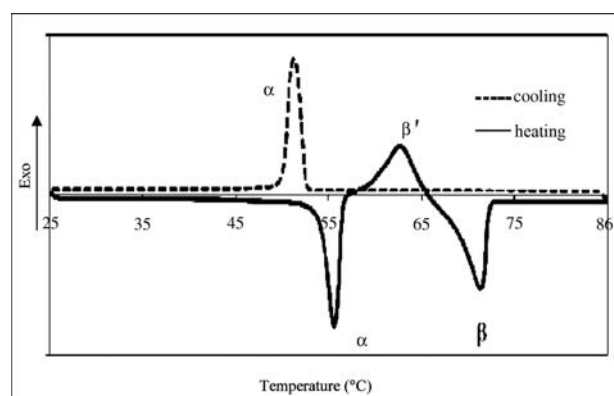


Fig. 5: DSC thermograph of Dynasan 118 bulk material (second heating) revealing the three different crystalline modification of the lipid

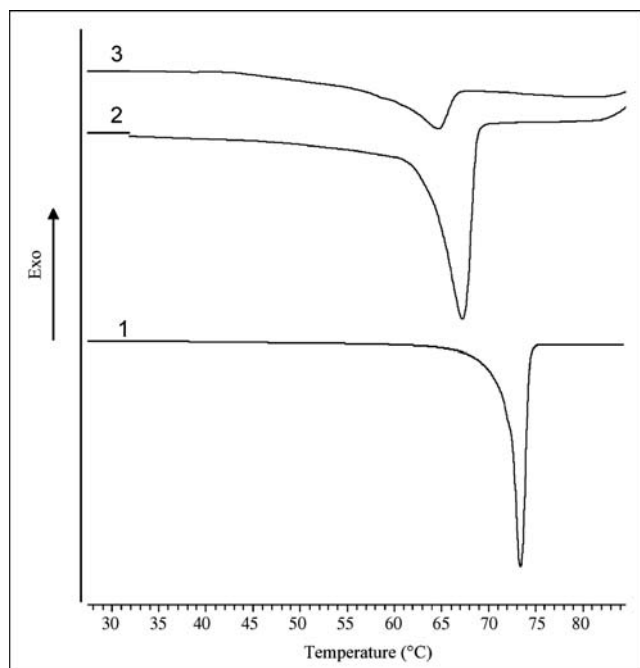


Fig. 6: DSC thermograph of Dynasan 118 bulk material (1 – first heating) and obtained NLC with composition: 2 – Dynasan 118 7.5%, Pine seed oil 2.5%, Tego® Care 450 1.2%; 3 - Dynasan 118 5.0%, Pine seed oil 5.0%, Tego® Care 450 1.8%

(Bunjes et al. 1996). Increasing of pine seed oil concentration led to decreasing crystallisation and melting temperatures of the particle matrix and accelerates the transition of the Dynasan 118 into the stable  $\beta$ -polymorph after crystallisation. The peak for the  $\beta'$ -modification is not pronounced for the NLC formulation, only the stable  $\beta$ -modification, melting peak is recognizable (Fig. 6).

The melting enthalpy values of the NLC lipid phase with pine seed oil concentration of 2.5% was up to 192.7 J/g after 3 days of storage, compared to 201.4 J/g for the bulk lipid Dynasan 118. Taking into account that Dynasan 118 concentration was only 7.5% of the NLC dispersion, the melting enthalpy increased to 250.5 J/g, which means that particles in NLC (Dynasan 118 7.5%, pine seed oil 2.5%, Tego Care 450 1.2%), have the higher degree of crystallinity. The lowest enthalpy value 59.2 J/g (Dynasan 118 – 5.0%, pine seed oil – 5%, Tego Care 450 – 1.8%) correspond to 59% of Dynasan crystallinity.

After storage for 28 days, the melting point value remains higher than 64 °C which is important for NLC formulations for dermal application to maintain the solid state of the particles when applied to the skin. From this result it can be concluded that the particles remained in the solid state throughout the study period.

### 2.3. Conclusions

It has been shown that NLC formulation on the basis of Siberian pine seed oil can be considered a promising system for topical administration from a physical point of view. An excellent stability of NLC could be obtained by selecting suitable types of surfactant and lipid phase composition. Increasing of pine seed oil loading in NLC lipid matrix up to 5% does not lead to instability of the developed formulation. Investigation of short-term stability of the obtained dispersion demonstrated the high physical stability of the developed formulations irrespective of storage temperature conditions. Moreover, the high concentrations of the biologically active polyunsaturated fatty acids in the developed NLC open wide prospects for dermal application of the obtained formulation.

## 3. Experimental

### 3.1. Materials

The following materials were used from the indicated sources without further purification procedures. Dynasan 118 was acquired from Sasol (Hamburg, Germany). Siberian pine seed oil was purchased from Taiga-Product (Angarsk, Russia). Tego® Care 450 (polyglyceryl-3 methylglucose distearate) was donated from Goldschmidt (Essen, Germany). PlantaCare® 2000 (C8-C16 fatty alcohol polyglycoside) was obtained from Cognis (Düsseldorf, Germany), Tween® 80 (polyoxyethylene sorbitan monooleate) – from Uniqema (Everberg, Belgium), Poloxamer 188 (Pluronic® F68, Polyethylene-Polypropylene Glycol) – from (BASF, Germany). Ultra purified water was obtained from a MilliQ Plus system, Millipore (Schwalbach, Germany).

### 3.2. Fatty acid analysis

Siberian pine seed oil was methanolized/extracted in a 1-step procedure (Grahl-Nielsen and Barnung 1985) by treatment with 0.5 ml anhydrous methanol containing HCl at a concentration of 2 mol/l for 2 h in an oven at 90 °C. The fatty acids methyl esters were analysed by 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 polyethyleneglycol as stationary phase (thickness of 0.2  $\mu$ m; CP-WAX 52CB Chrompack) and helium at 20 psi as mobile phase. The injector 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 rate of 30 °C/min, followed by an increase of 3 °C/min to 225 °C. The detected peaks 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 was melted approximately 10 °C above the melting point of the solid lipid (Dynasan 118). Then, the lipid phase was dispersed and admixed to a hot aqueous surfactant solution (85 °C) using an Ultra-Turrax T25 (Janke & Kunkel GmbH and Co. KG, Staufen, Germany). The obtained pre-emulsion 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 o/w nanoemulsion was cooled to room temperature leading to the lipid phase recrystallization 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 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 50  $\mu$ S/cm with a solution of 0.9% sodium chloride. PCS yields the mean particle size and 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)

The solid state of the particles and the existence of NLC were investigated using a Mettler DSC 821 apparatus (Mettler Toledo, Giessen, Switzerland). The samples were weighed for approximately 1–2 mg in 40  $\mu$ l aluminum pans. Heating curves were performed from 25 °C to 90 °C at the 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 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.

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