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Resveratrol nanosuspensions for dermal application – production, characterization, and physical stability

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Nanosuspensions of the anti-oxidant resveratrol (5%) were produced for dermal application. Production was performed by high pressure homogenization, applying 1.500 bar up to 30 cycles. Four nanosuspensions were investigated using the stabilizers Tween 80, Poloxamer 188, Plantacare 2000 and Inutec SP1, 1% and 2% respectively. The nanosuspensions were characterized regarding size (photon correlation spectroscopy, laser diffraction), zeta potential and crystallinity. Nanocrystal sizes were about 150 nm (Poloxamer, Plantacare) and about 200 nm (Tween, Inutec); no amorphous fraction could be detected in the nanocrystals. In a short-term stability study (30 days, room temperature), the nanosuspensions with 2% stabilizer proved to be either less stable or at least had no stability advantage over the 1% formulations. All formulations with 1% stabilizer were stable in the short-term study, Plantacare and Inutec showing best stabilization. The stabilization is attributed solely or mainly to steric stabilization, because the measured zeta potentials in the original dispersion media were close to zero (−1 to −5 mV, Tween, Poloxamer, Plantacare) or around −20 mV (Inutec).

1. Introduction

Nanocrystals are applied in pharmacy as formulation principle to improve the oral bioavailability of poorly soluble drugs, preferential class II drugs of the biopharmaceutical classification system (BCS) (Müller and Keck 2004). The transfer of μm-sized powders to the nanodimension changes their physico-chemical properties. The saturation solubility c_s increases, besides the surface area A, thus increasing the dissolution velocity dc/dt of the drug (Buckton and Beezer 1992; Hecq et al. 2005; Müller et al. 2003; Sasson et al. 2007). Therefore nanocrystals are an ideal formulation for poorly soluble drugs for which dissolution velocity is the rate limiting step of oral bioavailability (Jia et al. 2002; Liversidge and Cundy 1995; Müller and Keck 2004). In addition – due to the small size of the nanocrystals (typical mean diameter 200–600 nm) – they can also be injected intravenously as an aqueous suspension (so called nanosuspension). Per definition, this makes any drug 100% bioavailable. Injection as intravenous nanosuspension can also reduce side effects, e.g. nephrotoxicity of itraconazole, compared to the commercial cyclodextrine-based product Sporanox (Rabinow et al. 2007). The makeability of the nanocrystal concept is meanwhile proven by five oral products on the market (Müller and Junghanns 2006; Rabinow 2004).

The principle of nanocrystals is that they improve the transport of drugs across a barrier/membrane. Surprisingly pharmaceutical attention focussed only on oral and i.v. administration. Other interesting areas such as dermal and ocular administration were completely neglected. This changed in 2005 with the filing of the first patent application for dermal delivery of cosmetic and pharmaceutical actives (Petersen 2006). It could be shown that the bioactivity in the skin of the original, poorly soluble plant molecule rutin is 500 times higher when compared to its water-soluble derivative rutin-glycoside. The first dermal products were placed on the market as cosmetic products by Juvena Switzerland in March 2007 (product line JUVEDICAL, product "DNA skin optimizer fluid" and "eye optimizer cream"). Incorporation of nanocrystals into dermal products is very simple. A concentrated nanosuspension is added to the water phase of creams or lotions. By now relatively little is published about dermal nanocrystals (Keck 2008; Keck and Müller 2008; Kobierski et al. 2008; Müller et al. 2007; Piao et al. 2008).

This study describes the production of resveratrol nanocrystals for dermal delivery. Resveratrol is well known from red wine and the French paradoxon (Jang and Surh 2001; Renaud and de Lorgeril 1992, 1993; Renaud and Gueguen 2007; Renaud and Ruf 1994). It has anti-oxidative properties, but is too poorly soluble to be efficiently applied in dermal formulations. As a cosmetic active, resveratrol is offered encapsulated in polymeric microparticles. They can be admixed to cosmetic creams, which overcomes the problem of incorporation in creams, because the active cannot be dissolved in the water or in the oil phase. However, it is not logic to encapsulate a poorly soluble active, which has already problems to dissolve and a too low solubility, in a prolonged release system such as a microparticle. This further reduces the amount of dissolved active in the cream, being available for penetration

into the skin. The aim therefore was to produce a resveratrol formulation with nanocrystals, which can be easily incorporated in the water phase of creams, and penetrate better into the skin because of their dissolution properties.
Production of the nanosuspensions was performed by high pressure homogenization (Müller et al. 1999a). The size of the nanosuspension was studied as a function of concentration and type of stabilizer. Apart from size, the nanocrystals were characterized by zeta potential and differential scanning calorimetry (DSC) measurements and their short-term stability was studied for first assessment of the stabilising properties of the various stabilizers.

2. Investigations, results and discussion

2.1. Preparation and particle size analysis of nanosuspensions

Identical to macrosuspensions, nanosuspensions need to be stabilized by surfactants (e.g. Tween) or steric stabilizers (e.g. polymers or macromolecules, in this case Poloxamer, Inutec and Plantacare). The four stabilizers selected for the study are non-ionic surfactants, which are generally considered as safe for human use. In general, non-ionic surfactants are better tolerated by the skin as ionic surfactants. These surfactants have been used in the formulation of several dermatological preparations and are known to possess low mucous and dermal irritation potential (Effendy and Maibach 1995; Mehling et al. 2007).
The industrial production technologies currently used for nanocrystals are top down methods, mainly pearl milling (Liversidge and Cundy 1995; Liversidge et al. 1992) and high pressure homogenization, either in water (Müller et al. 1999a, b) or in water mixtures or non-aqueous dispersion media (Müller et al. 2000). In this study, high pressure homogenization in aqueous stabilizer solution was applied. As first production step, the macrosuspensions were pre-milled at increasing pressures (2 cycles at 300 bar, 2 cycles at 500 bar, 1 cycle at 1,000 bar), before being homogenised at 1,500 bar (30 cycles). Pre-milling considerably reduced the particle size of the raw drug material, thus preventing blockage by particles of the very small homogenizer gap. The gap is approximately 3 μm at 1,500 bar. For instance particle diameters d(v)50%, d(v)90%, d(v)95% and d(v)99% (LD data) for the raw material (resveratrol drug powder) in 1% w/w poloxamer 188 solution were about 20 μm, 47 μm, 57 μm and 78 μm, respectively. After pre-milling, the corresponding diameters were reduced to about 5 μm, 9 μm, 10 μm and 12 μm.
Particle size diminution is improved if high homogenization pressures and an increased number of homogenization cycles are applied. At a given pressure, the size decreases with an increasing number of homogenization cycles. Figure 1 shows the PCS data (mean particle size, polydispersity index (PI)) of the resveratrol nanosuspensions stabilised with the four different stabilizers as a function of cycle numbers. Even after just one homogenization cycle, all suspensions apart from the one stabilized with 1% Tween 80 are in the nanometer range (appr. 700–800 nm).
However, besides the decrease in size, the saturation solubility c_s increases with an increasing number of homogenization cycles. Therefore, the size should be as small as possible to achieve the best penetration into the skin. With

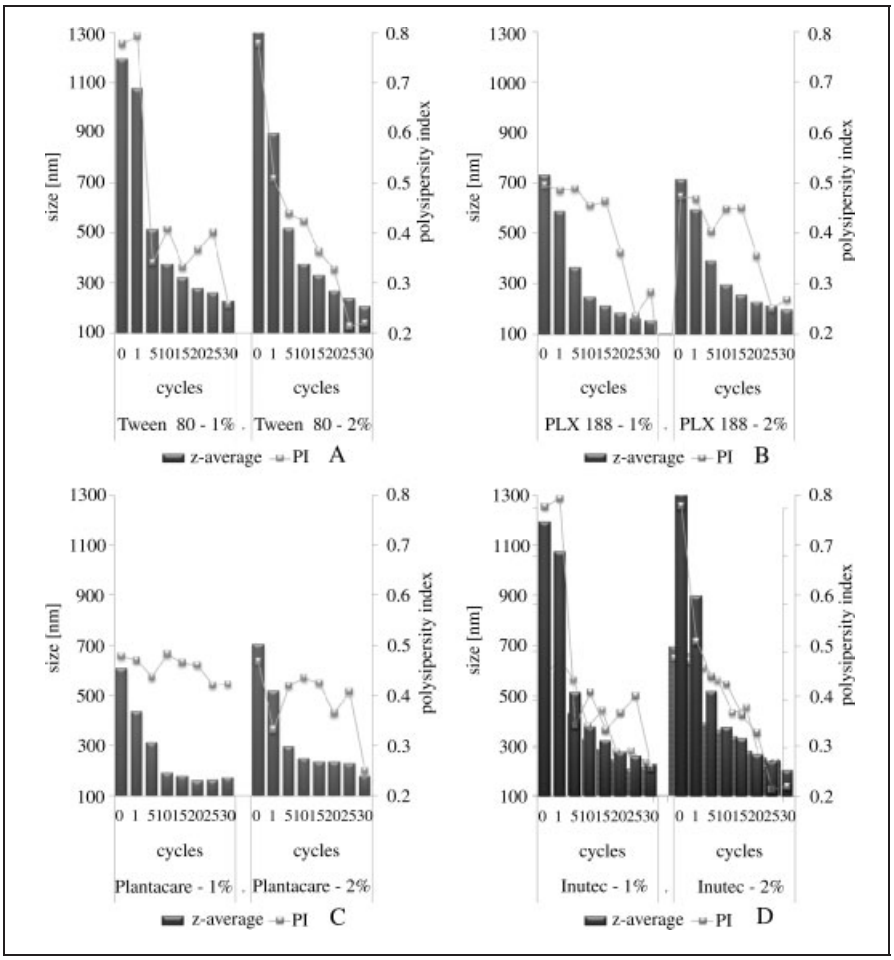

Fig. 1:
Particle diameter (PCS – z-average) and polydispersity index (PI) of resveratrol nanosuspensions stabilised with Tween 80 (A), Poloxamer 188 (PLX 188, B), Plantacare 2000 (C) and Inutec SP1 (D), either with 1% or 2% of the respective stabilizer; as a function of cycle numbers; cycle 0 denotes to the size after pre-milling

Table 1: Particle diameters (PCS – z-average (left) and LD d(v)99% (right)) of resveratrol nanosuspensions at cycle 20, 25 and 30 of high pressure homogenisation at 1,500 bar

Sample		Particle size					
Stabilizer	Concentration of stabilizer	z-average [nm] after			d(v)99% [μm] after		
		20 cycles	25 cycles	30 cycles	20 cycles	25 cycles	30 cycles
Tween 80	1%	276	260	226	4.083	3.287	3.099
	2%	267	237	203	4.779	3.721	3.529
Poloxamer 188	1%	182	162	153	4.474	4.019	3.284
	2%	225	210	195	5.142	4.483	3.869
Plantacare 2000	1%	157	159	169	3.391	3.204	2.745
	2%	231	225	177	3.501	3.224	2.750
Inutec SP1	1%	247	210	216	3.457	3.194	3.260
	2%	284	255	244	3.084	2.811	2.578

an increasing cycle number, the size of the 4 nanosuspensions decreased in the form of an exponential function, showing a similar shape for all 4 nanosuspensions (Fig. 1). It must be noted, however, that for a given drug particle at constant homogenization parameters, only a certain level of size diminution can be achieved (Fichera et al. 2004; Keck and Müller 2006). After a certain number of cycles the maximum dispersity (= smallest achievable size) is reached. Crystals preferentially break at imperfections. Thus, with a decrease in size the number of imperfections is getting less, as the crystals are getting more perfect. Consequently, with each homogenization cycle more force is necessary to break the crystals further. Finally the crystals are so perfect (and hard) that the pressure applied cannot break them anymore. Even when running more cycles, the bulk diameter will not change. This can be nicely seen in the size decrease of the four nanosuspensions.

After 20 cycles, there is only a limited further decrease up to cycle 30 in the size of the bulk population, i.e. the PCS data (Tween, Poloxamer, Plantacare 2% and Inutec). Plantacare 1% reaches the minimum size already after 20 cycles (Table 1). The PCS diameters are 157 nm after 20 cycles, 159 nm after 25 cycles and 169 after 30 cycles respectively, indicating that a further energy input rather leads to particle aggregation than to a further size reduction.

Increasing the cycle numbers from 20 to 25 and 30 cycles has only limited effect on the size of the bulk population (Fig. 1), but it further reduces the amount of the remaining larger crystals. This can be seen by the continuous decrease in the diameter d(v)99%, being a measure for the larger crystals present (Fig. 2). There is still a distinct decrease by about 1 μm in d(v)99% from 20 to 30 cycles, e.g. from 4.08 μm to 3.10 μm in case of Tween 1% (Table 1). The increase in homogeneity of the suspension is also reflected by the decrease of the polydispersity index (PI) with increasing cycle number (Fig. 1). The exception is Plantacare, where the decrease in d(v)99% little affects the PI.

Theoretically, the particle size of nanosuspensions produced via high pressure homogenization is not dependent on the type and the concentration of the stabilizer used. Rather, the final size of the drug nanocrystals is dependent on the hardness of the drug material (crystalline structure, number of imperfections), the power density of the homogenizer (homogenization pressure), and the number of homogenization cycles applied. The shape of nanocrystals is also independent of surfactant type or concentration, rather it is said to be a function of the crystalline structure of the starting raw materials used (Keck and Müller 2006). Therefore one should obtain similar sizes directly after production, assuming that the stabilizer is able to stabilize the produced nanosuspension sufficiently for some time (time required between production and performing the size measurement). However, surfactants will differ in their ability to preserve a size of a nanosuspension. In fact, after production the size will remain unchanged during storage in case of effective stabilizers, or will increase slowly or fast in case of less suitable stabili-

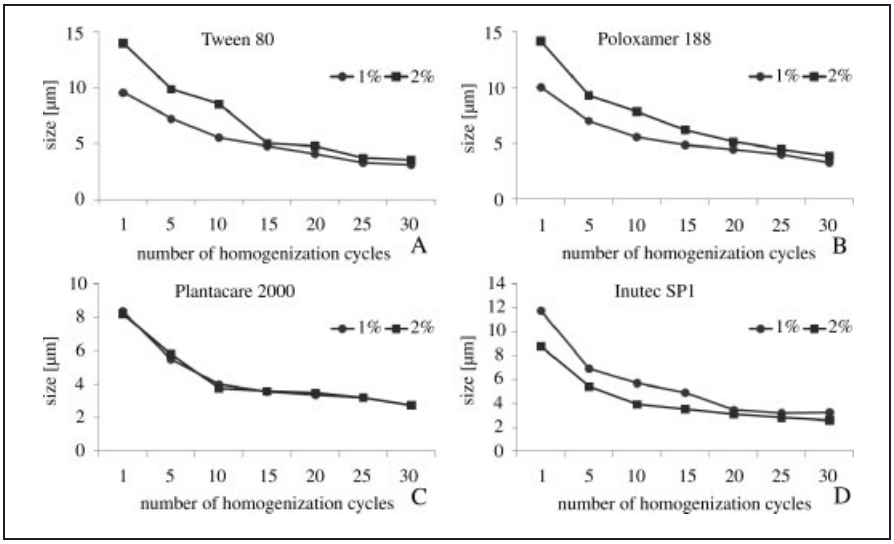

Fig. 2:
Particle diameter (LD d(v)99%) of resveratrol nanosuspensions stabilised with Tween 80 (A), Poloxamer 188 (B), Plantacare 2000 (C) and Inutec SP1 (D), either with 1% or 2% of the respective stabilizer; as a function of cycle numbers

zers. Therefore, in this study size measurements were performed immediately after production, and as a function of storage time.

Looking at the PCS sizes of the nanosuspensions (1% stabilizer) after 30 cycles reveals diameters of the bulk population of 216 nm (Inutec) and 226 nm (Tween). Sizes as small as 153 nm were obtained with Poloxamer and 157 nm for Plantacare (please note: for Plantacare size of cycle 20 was taken, because energy input up to cycle 30 aggregated again the nanosuspension to 169 nm). In summary, the nanocrystal size for all formulations is roughly about 200 nm. However, there is a small difference of about 50 nm. During the production in the homogenizer a very slight aggregation may have been occurred, indicating that Inutec and Tween might be slightly less effective in stabilization (cf. below, short-term stability).

Comparing the sizes of the 1% and 2% stabilized nanosuspensions the increase in stabilizer concentration in general reveals little or no reduction in the size of the bulk population (PCS), measured at the different cycle numbers (Fig. 1 and Table 1, Tween). In most cases, slightly bigger sizes were obtained after 30 cycles when using 2% stabilizer (e.g. Poloxamer, Plantacare and Inutec,). This might be attributed to some bridging effects of these higher molecular weight stabilizers. For polymers such as Poloxamer a strong dependency of the stabilizing ability as function of concentration is described (Karmarkar 2008). At low concentration, the coverage of the particle surface is insufficient, the sterically stabilizing layer too thin and the suspension not stable. At medium concentration, the layer is optimal in thickness and the suspension is stable. At higher concentrations, bridging can occur but also flocculation by depletion. Hence, the principle "the more, the better" is not valid. The optimal concentrations of higher molecular weight stabilizers need to be fine-tuned. Parameters affecting the optimal stabilizer concentration are e.g. the solubility of the polymer in the dispersion medium (quality of solvent), affinity to adsorb onto the particle surface, and of course, the particle concentration itself, as this affects the size of the surface area to be covered by the polymer/stabilizer.

The fact that the increase in stabilizer concentration does not lead to a significant size reduction can also be confirmed by the laser diffraction (LD) data (Fig. 2). At 2% stabilizer concentration after 30 cycles, the diameters d(v)99% are higher, when compared to the 1% nanosuspensions (Tween, Poloxamer), similar (Plantacare), and only smaller in case of the stabilizer Inutec (but PCS diameter is larger, 244 nm with 2% vs. 216 nm with 1%).

To summarize, increasing the stabilizer concentration had little positive effect, sizes were rather larger, and therefore the 1% stabilizer concentration is considered optimal.

2.2. Zeta potential

The zeta potential is a stability determining parameter. Zeta potentials above the absolute value of 30 mV are required for storage stability of a charge-stabilized dispersion (Müller 1996; Müller and Heinemann 1991). Zeta potential must be measured in the original dispersion medium. Measuring the zeta potential in water yields the Stern potential, which is correlated to the surface charge, the Nernst potential. For an optimal stability of a suspension, it is ideal to have a Nernst/Stern potential as high as possible. The higher the Stern potential, the higher will be the zeta potential in the original dispersion media, e.g. in media containing zeta potential reducing electrolytes.

The nanosuspensions were measured in distilled water having the conductivity adjusted with NaCl to 50 μS/cm. The adjustment of the conductivity was done to minimize effects of fluctuating conductivities in the distilled water (Müller 1996). The Stern potentials of most of the nanosuspensions, independent of the stabilizer concentration, were around −30 mV, (Table 2). This is at the lower limit for an electrostatically stabilized suspension. Exceptions are 2% Inutec and Plantacare 1% and 2%. The Stern potential of the Inutec stabilized nanosuspension decreases from −31 mV to −23 mV when increasing the Inutec concentration from 1% to 2%. This can be explained by a thicker adsorption layer of Inutec, leading to a shift of the plane of shear to a larger distance from the particle surface, and thus to a reduction in the measured potential (Fig. 3) (Müller 1996).

Plantacare both at 1% and at 2% shows a higher Stern potential of about −43 mV. This effect was observed previously with other nanosuspensions stabilized with Plantacare (e.g. Hesperetin, −48.3 mV). A potential explanation is that Plantacare (or some electrolytes in the surfactant product acting as anti-flocculant) adsorb in the inner Helmholtz layer, stay adsorbed during dilution of the sam-

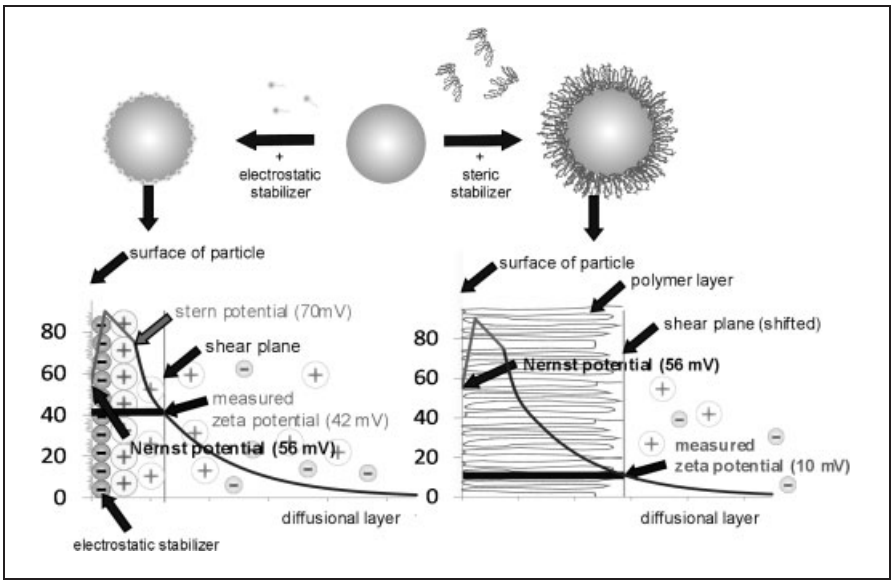

Fig. 3:
Composition of Stern layer and diffuse layer, course of potential in these layers, and plane of shear in an electrostatically stabilized particle (left) and a particle with an adsorbed polymer layer, shifting the plane of shear to a larger distance from the particle (right). The measured zeta potential is lower despite identical Stern and Nernst potential

Table 2: Zeta potentials of the four different resveratrol nanosuspensions measured in distilled water (conductivity adjusted to 50 µS/cm, middle column) and in the original dispersion medium, stabilizer solutions (right column)

Sample		Zeta potential [mV]	
Stabilizer	Concentration of stabilizer	in distilled water	in original dispersion medium
Tween 80	1%	−28.0 ± 2.1	−0.9 ± 0.1
	2%	−27.2 ± 0.1	−0.5 ± 0.1
Poloxamer 188	1%	−32.3 ± 1.3	−5.0 ± 3.6
	2%	−29.1 ± 0.1	−4.5 ± 1.7
Plantacare 2000	1%	−43.3 ± 3.5	−4.3 ± 0.8
	2%	−42.9 ± 1.3	−4.3 ± 0.4
Inutec SP1	1%	−31.0 ± 1.4	−22.3 ± 0.3
	2%	−22.7 ± 0.5	−13.8 ± 0.2

ple in water for the measurement, thus increasing the measured Stern potential.

Analysis of the zeta potential in the original dispersion media (stabilizer solutions) reveals for all nanosuspensions – with the exception of Inutec – values close to zero (about −5 mV to 0 mV). A purely electrostatically stabilized dispersion would aggregate within hours or a few days. However, the employed stabilizers act as steric stabilizers, and they can even be stable at no electrostatic contribution at all. In low electrolyte media, such as distilled water, the nanocrystals stabilized with these stabilizers possess a charge. This is reduced close to zero in the original dispersion media. An exception is Inutec, having still zeta potentials of about −22 mV and −14 mV (1% and 2%, resp.). From theoretical considerations, Inutec nanosuspensions could possess a better long-term stability (combined steric and electrostatic stabilization). Nevertheless, for a final judgement of the comparative stability of the four stabilizers, the short-term stability data need to be considered.

2.3. Crystalline state of the nanosuspensions

In general amorphous materials possess a higher saturation solubility than crystalline materials (e.g. c_s of amorphous griseofulvin is 44 µg/l compared to crystalline griseofulvin having only a solubility of 8 µg/ml) (Mosharraf and Nystrom 2003). Based on this, the ideal drug particle with best solubility properties should be in the nanosized range, being amorphous and not crystalline. This was realized in Nanomorph, a technology by the company Soliqs (drug delivery company of Abbott). However, amorphous drugs in formulations can lead to a reduced bioavailability during the shelf life of a product, in case they re-crystallize. To avoid this, the produced nanosuspensions should be crystalline, and of course very small sized for an optimal solubility. To verify the crystalline nature, DSC analysis of the nanosuspensions was performed. The aqueous nanosuspensions were dried and analyzed by DSC, and compared to the bulk material. Resveratrol bulk material exhibited a melting peak at 270 °C, and a corresponding melting enthalpy of 254.9 J/g. When interpretating the data it needs to be considered that the purity of the bulk material was only 90%, due to be extracted from natural plant material. The nanosuspensions exhibited melting peaks at 260.6 °C (Tween), 259.2 °C (Poloxamer), 260.2 °C (Plantacare) and 260.8 °C (Inutec). The melting point depression from 270 °C (bulk) to about 260 °C was attributed to the size reduction of the crystals to the nanometer range, as described by the Gibbs-Thomson equation (Sun and Simon 2007). The melting enthalpies of the Tween and Poloxamer stabilized nanosuspensions were 223.2 and 205.2 J/g, respectively. No amorphous transition was observed. Considering the content of up to 10% impurity, these nanosuspensions can be considered as crystalline. The melting peak of the nanosuspensions stabilized with Plantacare exhibited – besides the maximum at 260 °C – a second maximum at 255.3 °C, the one stabilized with Inutec two additional maxima at 248.0 °C and 232.0 °C. The corresponding melting enthalpies of the complete peaks were 360.0 J/g (Plantacare) and 284.2 J/g (Inutec), versus the bulk material with 254.9 J/g. A detailed interpretation of the peaks is not accessible due to the 10% impurity; at least the suspensions are crystalline.

2.4. Physical short-term stability

A short-term stability study was performed for final assessment of the comparative stabilization efficiency of the type and concentration of stabilizers. PCS is a very sensitive method to detect even minor changes in the size of the bulk population. Therefore, even from a short-term study, stabilizers can be placed in order of their stabilizing capacity. In addition, LD was employed for detection of aggregates, which might leave the measuring range of PCS (appr. 1 nm–6 µm).

Comparing 1% and 2% stabilizer concentration, the interpretation from the sizes obtained in the production was confirmed. Nanosuspensions with 2% stabilizer show either an increase in PCS diameter during storage time (e.g. 2% Tween 80, less stable than 1%), or show at least no better stability than 1% (Plantacare, Fig. 4). Therefore not only from the identical sizes obtained with 1% and 2% stabilizer in production, but also from the stability data, the use of 2% stabilizer is not sensible.

The nanosuspensions stabilized with Tween and Inutec, 1% respectively, remained unchanged around 220 nm. They were stable despite the zeta potential in the original dispersion medium was close to zero for Tween. The 1% poloxamer stabilized nanosuspension increased within one week from 153 to about 180 nm and then stayed unchanged. It seems that a kind of ripening might have ta-

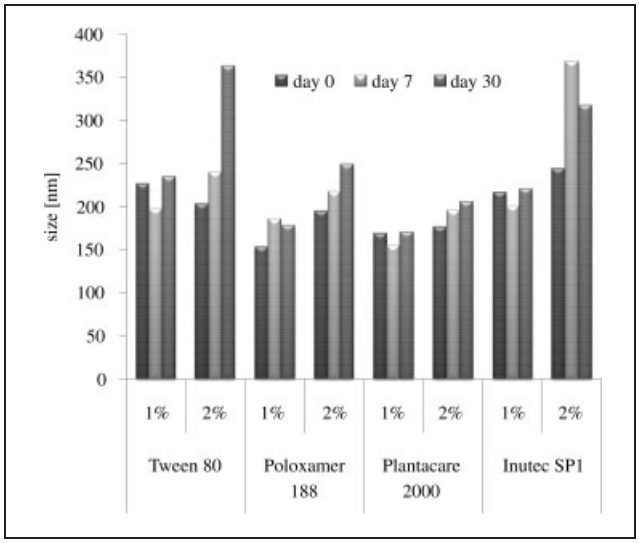

Fig. 4: Particle diameters (PCS) of resveratrol nanosuspensions stabilised with Tween 80, Poloxamer 188, Plantacare 2000 and Inutec SP1; showing different stability during storage at room temperature

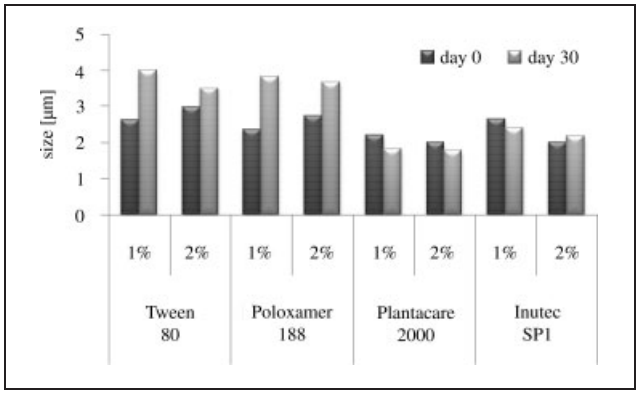

Fig. 5: Laser diffraction (LD) diameters d(v)95% of resveratrol nanosuspensions stabilized with Tween 80, Poloxamer 188, Plantacare 2000 and Inutec SP1, as a function of storage time (room temperature)

ken place leading to a stable nanosuspension after reaching its equilibrium. Plantacare seems to be most efficient, the very small size of about 170 nm after production remained unchanged over one month of storage. This interpretation is confirmed when looking at the diameters d(v)95% from LD analysis (Fig. 5). For all suspensions no aggregation and no formation of crystals was detected, as all diameters are below 5 µm, indicating a good stability of all suspensions. Also from this set of data it can be concluded, that there is no advantage of the 2% stabilizer suspensions over the 1% stabilizer suspensions. By looking at the efficiency of the different stabilizers, it seems that Plantacare and Inutec are superior than Tween and Poloxamer, because no changes in the d(v)95% are detected during the short-term study. However, only a slight increase in the d(v)95% was observed for the Tween and Poloxamer stabilized suspensions, which should not affect the bioactivity of the resveratrol nanosuspensions.

In Conclusions, Nanosuspensions in the low nanometer range (150–220 nm), thus possessing highest solubility and highest dissolution velocity, could be produced by high pressure homogenization. All the four stabilizers investigated yielded stable nanosuspensions at a concentration of 1%. A concentration of 2% did not stabilize better or rather de-stabilized the nanosuspensions, and should not be used. The nanosuspensions are solely (Tween, Poloxamer, and Plantacare) or mainly (Inutec) stabilized by steric stabilization, because the measured zeta potentials in the original dispersion media are close to zero (no electrostatic contribution) or around −20 mV (Inutec), indicating limited electrostatic stabilization/repulsion. The nanocrystals are crystalline (within the detection limit of DSC), and thus no stability problems originating from a too large amorphous fraction are expected. Based on these results a long-term stability of up to one or two years is predicted for the most efficient stabilizers Plantacare and Inutec. This is currently being investigated in a long-term stability study.

3. Experimental

3.1. Materials

Resveratrol was purchased from E. Denk Feinchemie GmbH (Munich, Germany), Tween 80 (polysorbate 80) from Uniqema (Everberg, Belgium); Lutrol F68 (Poloxamer 188) was provided by BASF (Ludwigshafen, Germany) and Plantacare 2000 (alkyl polyglycoside) by Cognis GmbH (Düsseldorf, Germany). Inutec SP1 (inulin lauryl carbamate) was purchased from Orafti Bio Based Chemicals (Tienen, Belgium). 0.9% sodium chloride solution was purchased from B. Braun (Melsungen, Germany). Freshly prepared double distilled and ultra purified water (milliQ, Millipore GmbH, Schwalbach, Germany) was used as dispersion medium.

3.2. Methods

3.2.1. Preparation of nanosuspensions

Aqueous formulations of resveratrol (5% w/w) and surfactant/stabilizer (1% w/w or 2% w/w) were prepared. The surfactants used were Tween 80, Poloxamer 188, Plantacare 2000 and Inutec SP1. The surfactants were either dissolved in water or dispersed in warm water (approx. 40 °C) with the use of an Ultra Turrax T25 (Janke and Kunkel GmbH, Staufen, Germany) until complete dissolution was obtained. The resveratrol powder was added to the aqueous surfactant solution and mixed with the Ultra Turrax for 1 min at 9,500 rpm. The well-mixed microparticulate suspensions were subjected to high pressure homogenization using an APV Gaulin Micron Lab 40 homogenizer (APV Deutschland GmbH, Unna, Germany). The discontinuous production technique was employed, as the homogenizer had to be refilled with the previously homogenised suspension after each cycle. The microsuspensions were pre-milled by applying two homogenization cycles at 300 bar, followed by two cycles at 500 bar, and finally, one cycle at 1,000 bar. After pre-milling, 30 homogenization cycles at 1,500 bar were applied to obtain the final product. Particle size analysis was performed after pre-milling and after 1, 5, 10, 15, 20, 25 and 30 homogenization cycles at 1,500 bar.

3.2.2. Particle size analysis

Particle sizes were determined by photon correlation spectroscopy (PCS) using a Malvern Zetasizer Nano ZS (Malvern Instruments, UK), and by laser diffractometry (LD) using a Mastersizer 2000 (Malvern Instruments, UK). PCS yields the z-average (z-ave), which is the intensity weighted mean diameter of the particle bulk population, and the polydispersity index (PI) as measure for the width of the size distribution. The PI can range from 0–1, the smaller the PI value, the more monodisperse and uniform the particles, and the higher the PI value, the wider the size distribution. The nanosuspensions were diluted with double distilled water to achieve the appropriate dilution before PCS analysis. Laser diffractometry sizes were analysed as volume size distribution using the diameters d(v)50%, d(v)95% and d(v)99% as characterization parameters for the nanosuspensions. The diameter d(v)50% represents the size where 50% of the particles are below the given size, it represents the mean particle size of the particle population. The diameters d(v)95% and d(v)99% are very important characterization parameters, as they represent larger particles within the sample and are meaningful to quantify e.g. larger crystals remaining in the suspension during the homogenization process or larger crystals and/or aggregates which might occur due to instability during storage. LD results have been analysed using Mie theory with the optical parameters 1.69 for the real refractive index and 0.02 for the imaginary refractive index.

3.2.3. Physical stability studies

The produced nanosuspensions (30 cycles at 1,500 bar) were divided into three parts to be stored at three different temperatures (room temperature, 4 °C and 40 °C, respectively). Particle size analyses of the samples using PCS and LD were carried out on day 0 (day of production), day 7, and day 30. PCS analysis gave the mean particle bulk diameter and the polydispersity index, while LD provided the volume diameters d(v)95% and d(v)99%.

3.2.4. Zeta potential measurements

Zeta potential measurements were performed using the Malvern Zetasizer Nano ZS (Malvern Instruments, UK). Measurements were performed in either water (adjusted to a conductivity of 50 µS/cm using 0.9% NaCl solution) or in the original dispersion medium/stabilizer solution of the suspensions. The measurements were performed by applying a field strength of 20 V/cm. The measurements yield the electrophoretic mobility, which was converted into the zeta potential using the Helmholtz-Smoluchowski equation.

3.2.5. Differential scanning calorimetry (DSC)

DSC measurements in the range from 25 °C–280 °C were performed using a Mettler DSC 822e1200 (Mettler Toledo, Germany) with a scanning rate of 10 °C/min in standard aluminium sample pans of 40 µl. The instrument was calibrated using indium as calibration standard. The analysis of the nanosuspensions was performed in dry state. For that the pans were filled with the aqueous nanosuspensions and then placed in a heated oven at 40 °C and dried to weight constancy. Measurements were performed with a nitrogen purge of 20 ml/min and an empty pan was used as a reference.

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