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Preserving hesperetin nanosuspensions for dermal application

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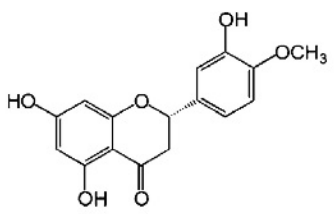
Nanosuspensions as aqueous formulations need to be preserved. However, preservatives could vitiate the physical stability of suspensions and to a greater extent nanosuspensions. The impact of six varied preservatives on the physical stability of previously prepared nanosuspensions was studied. The hesperetin nanosuspensions were stabilized using plantacare 2000. 30 cycles of high pressure homogenization (HPH) led to a mean photon correlation spectroscopy (PCS) diameter of 335 nm. The preservatives were, caprylyl glycol, Euxyl PE9010, Hydrolite-5, MultiEx naturotics, Phenonip and Rokonsal PB5. On one hand, aggregations were noticed after adding caprylyl glycol, MultiEx naturotics and Phenonip reaching PCS mean diameters of about 500, 1070, 800 nm, respectively. While on the other hand Euxyl PE9010, Hydrolite-5 and Rokonsal PB5 have not significantly affected the physical stability of the nanosuspensions with mean PCS diameters of about 365, 332, 350 nm, respectively. The obtained nanosuspensions were further characterized by measuring zeta potential. From the obtained data it was found that the lipophilicity of the used preservatives demonstrates major influence on the stability of the nanosuspensions, i.e. the higher lipophilicity of the preservative, the stronger the destabilizing effect. Briefly, highly hydrophilic preservatives are recommended to preserve hesperetin nanosuspensions in order to maintain their physical stability during storage.

1. Introduction

The most prominent aging theory is the free radical theory postulated by Harman (Harman 1956; Beckman and Ames 1998). Oxidative damage increases with age and contributes to the ageing phenotype as well as various diseases due to the fact that the activity of antioxidant enzymes and the level of non-enzymatic antioxidants decline with age, allowing oxidative damage to occur (Kohen and Gati 2000; Rabe et al. 2006).

Hesperetin is the aglycon of hesperidin, an antioxidant (Bonina et al. 1996; Choi 2008; Choi and Kim 2008). It possesses additionally anti-allergic, anti-inflammatory properties and even lowers the hepatic content of triaceylglycerol (Cha et al. 2001; Neung-Kee et al. 2004). Pharmaceutically it can be applied as protective agent against skin cancer because it acts as scavenger of free radicals (Bonina et al. 1996). Due to this hesperetin is under investigation as a dermally applied cosmetic ingredient in anti-aging but also for pharmaceutical products. As hesperetin is a very poorly water soluble compound it can be transferred to nanocrystals to make it biologically active (Longxiao and Jun 2008).



hesperetin (((S)-2,3-dihydro-5,7-dihydroxy-2-(3-hydroxy-4 methoxy-phenyl)-4H-1-benzopyran-4-one)

An increasing number of newly developed drugs are poorly soluble in water and simultaneously in organic media (Müller and Peters 1998). Estimates state that more than 50% of the drugs in the pipelines have solubility problems (Stegemann et al. 2007). There are many ways to increase the solubility of poorly soluble drugs. But these methods are limited to drugs with certain properties in regard to their chemistry (e.g. solubility in certain organic media) or for example to their molecular size or conformation (e.g. molecules to be incorporated into the cyclodextrin ring structure (Tommasini et al. 2004, 2005)). Apart from that, the usage of surfactants or cosolvents is also possible, but sometimes leads to increased side effects (e.g. Cremophor EL increases the toxicity of taxol, HP-β-cyclodextrin is the cause of nephrotoxicity of itraconazole in Sporanox® (Willems et al. 2001)).

Another approach to overcome the solubility obstacle is to render the drug particles into nanocrystals. Drug nanocrystals are pure solid drug particles with a mean particle size below 1 μm, mostly between 200 nm and 500 nm (Keck and Müller 2006). Nanocrystals not only can provide an increased dissolution velocity due to their large surface area but also increase the saturation solubility which in turn enhances the bioavailability (Müller et al. 1999; Keck et al. 2008). With regard to dermal application a prolonged contact time due to the adhesive properties of nanoparticulate materials on the skin can be achieved. Furthermore, the increased saturation solubility can promote penetration of the active ingredient due to an increased concentration gradient between the dermal formulation and the skin.

In order to obtain good microbial stability aqueous systems need to be preserved. Nanocrystals are provided as aqueous concentrates for incorporation into dermal formulations, for reasons

of safety preservation is essential. However, many preservatives can destabilize suspensions, especially highly dispersed systems as nanosuspensions. The influence of six preservatives with different chemical properties on the physical stability of hesperetin nanosuspensions was investigated to assess the physical stability. The particle size and particle size distribution were measured directly after the addition of the preservatives and over one month of storage at three different temperatures. Based on zeta potentials, observed data and by considering the chemical structure of the preservatives, a general guideline for the choice of preservatives for nanosuspensions should be outlined.

2. Investigations, results and discussion

2.1. Physical characterisation

All nanosuspensions were produced by high pressure homogenization (HPH) in purified water according to Müller et al. (1999) using an LAB 40 with a batch size of 40 g. The final formulations produced contained 5.0% (w/w) hesperetin and 1.0% (w/w) plantacare 2000, a stable hesperitin nanosuspension formulation previously developed by Mishra et al. (2009). At the end of the homogenization the preservatives were added to the nanosuspensions. This was in contrast to the procedure by Al Shaal et al. (2009), who added the preservatives to the macrosuspension prior to homogenization (Table 1).

To investigate the physical stability of the differently preserved nanosuspensions in order to identify the most suitable preservative, the nanosuspensions were stored at three different temperatures (4 °C, 25 °C, and 40 °C) for thirty days. Characterization was carried out on day 0, 14 and 30. Day 0 is the day of production after addition of the preservative. Each preservative is advised to be used in a concentration range specified by the manufacturer or in the literature.

The particle size achieved of hesperetin nanosuspension stabilized with plantacare 2000 after 30 cycles in a previous study was around 304 nm (Mishra et al. 2009). The final particle size after homogenization is normally determined by the applied pressure and the number of homogenization cycles (Müller et al. 1995). The stabilizer plays an important rule in stabilizing the fine produced nanocrystals. In this study a mean PCS diameter of 335 nm and a polydispersity index of 0.390 was achieved being close to the previously published data. PCS was implemented to investigate the alteration in particle size due to the fact that also minor changes in the mean particle size of the nanosuspensions can be detected by this method (standard deviation of optimized measuring conditions ±1%).

Figure 1 shows the particle sizes of the preserved nanosuspensions and the PDI right after admixing the preservatives to the nanosuspension.

With PCS measurements only particle sizes below approximately 3 μm can be detected. The advantage of laser diffractometry (LD) measurements has a board measuring range (e.g. 20 nm–2000 μm) which enables to detect large particles or aggregates beside small sized bulk populations.

Figure 2 demonstrates particle size distributions d(v)50%, d(v)90% and d(v)99% of the preserved nanosuspensions after admixing preservatives to the nanosuspension.

From the data obtained by PCS it can be seen that particle sizes of nanosuspensions preserved with Euxyl PE9010, Hydrolite-5 and Rokonsal PB5 remained nearly unchanged after preservatives addition. The particle sizes were 373 nm, 330 nm and 396 nm, respectively. On the other hand particle sizes of nanosuspensions preserved with caprylyl glycol, MultiEx naturotics and Phenonip

Table 1: Preservatives used: trade names, composition, effective concentration range and used concentration in the nanosuspensions

Trade name of preservative	Composition of preservative	Concentrations [%(w/w)]	
		Effective range	Used
Hydrolite-5	Pentylene glycol	2.0–5.0	2.0
Euxyl PE9010	90% Phenoxyethanol, 10% Ethylhexylglycerin	1.0	1.0
Rokonsal PB5	72% Phenoxyethanol, 14.5% Methylparaben, 5.8% Ethylparaben, 3.6% Butylparaben, 2.4% Propylparaben, 1.8% Isobutylparaben	0.3–1.2	0.75
Caprylyl glycol	1,2 - Octandiol	0.5–1.0	0.75
MultiEx naturotics	Mixture of natural plant extracts (e.g. Magnolol, Honokiol)	1.0–3.0	2.0
Phenonip	60–80% Phenoxyethanol, 13–17% Methylparaben, 4–6% Ethylparaben, 4–6% Propylparaben, 4–6% Butylparaben	0.25–1.0	0.75

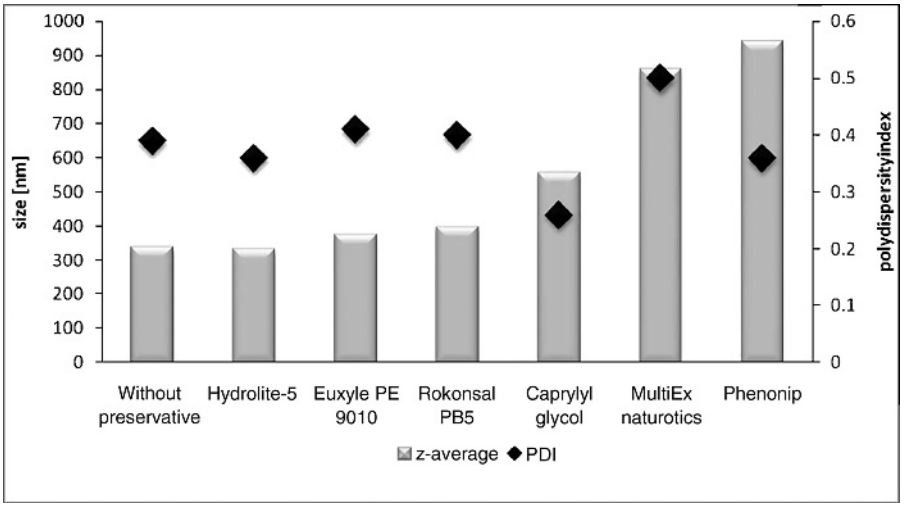


Fig. 1: PCS diameter and polydispersity index (PDI) of the preserved nanosuspension directly after the addition of the preservative

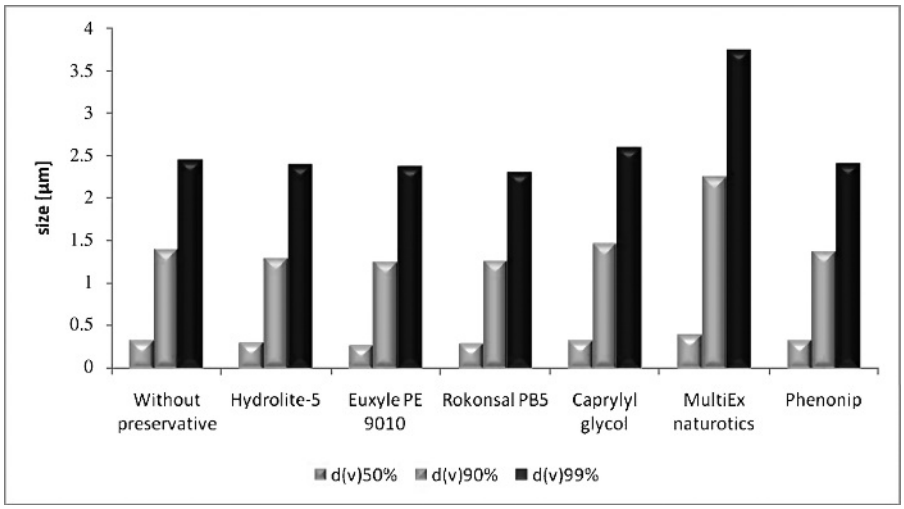


Fig. 2: LD diameters 50%, 90% and 95% of the preserved nanosuspensions directly after addition of the preservative

were distinctly increased due to significant aggregations with diameters of 553 nm, 858 nm and 939 nm, respectively (Fig. 3). The LD data surprisingly do not demonstrate large agglomerations like PCS data except the nanosuspension preserved with MultiEx naturotics (Fig. 2). This can be due to the fact that LD instrument has a build-in stirrer that de-aggregates loose agglomerations. That de-aggregation in the LD during the measurement took place could be proven when investigating

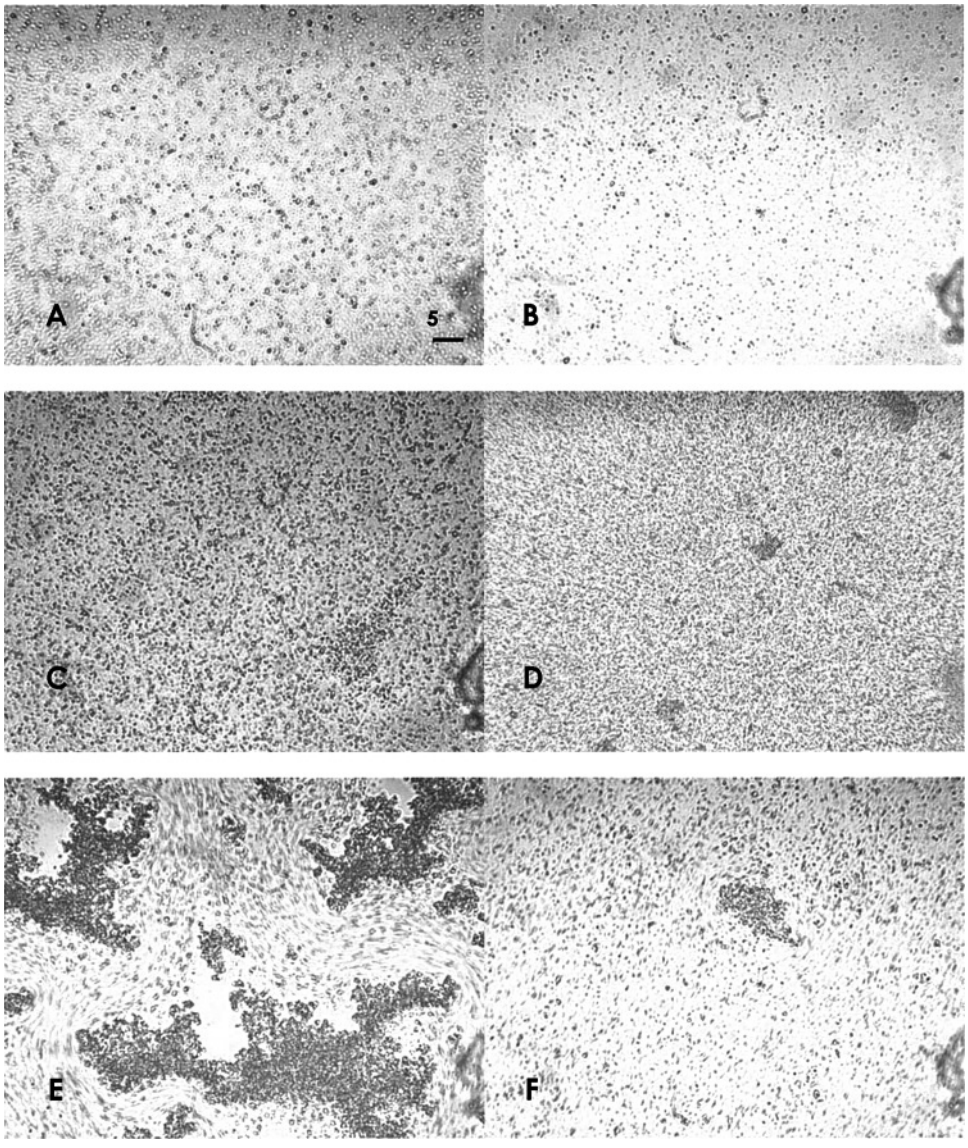


Fig. 3: Light microscopy images of nanosuspensions preserved with: (A) Hydrolie-5, (B) Euxyl PE9010, (C) Rokonsal PB5, (D) Caprylyl glycol, (E) MultiEx naturotics, (F) Phenonip, (Magnification 600 fold, bar = 5 µm)

the nanosuspensions by light microscopy. Light microscopy is not able to give precise size information of nanocrystals in the lower nanometer size range due to the detection limit of the microscope, but it allows nicely to check for the presence of crystals or aggregates larger than 1 μm. In Fig. 3 the pictures are put in order of increasing PCS diameters. Fig. 3A–C are the nanosuspensions which were found stable according to PCS and LD data, that means Hydrolite-5, Euxyl PE9010 and Rokonsal PB5. Fig. 3D–E show the microscopic pictures of the preserved nanaosuspensions being instable according to PCS, that means preserved with caprylyl glycol, MultiEx naturotics and Phenonip. The last three pictures show clear aggregates ranging from slight aggregation to very pronounced aggregation (MultiEx naturotics). Looking at the pictures one has the impression that the aggregates are not condensed, they seem to be rather loose which would be in agreement with the de-aggregation during the LD measurement. These data demonstrate at the same time how important it is to complement LD measurements by simple microscopic pictures, often forgotten.

The zeta potential was measured in distilled water in order to measure the Stern potential. The zeta potentials for all preserved nanosuspensions with Euxyl PE9010, Hydrolite-5, Rokonsal PB5 and Phenonip were around −58 mV (Table 2). MultiEx naturotics possessed a distinctly lower ZP value of −32.0 mV. Molecules adsorbed on a crystal surface can increase or decrease the zeta potential. In case of non-ionic compounds like the preservatives used, generally a decrease takes place due to the shift of the plane of sheer to a larger distance from the particle surface. The measured practical identical zeta potential of −58 mV for five of the preserved nanosuspensions could be interpreted this way, that dilution of the nanosuspension for the measurement in distilled water led to desorption of adsorbed preservatives. The charge of the original hesperitin nanocrystal is revealed being around −58 mV. For the nanocrystals preserved with MultiEx naturotics a lower zeta potential of −32.0 mV was observed. This could be explained by still adsorbed molecules of the preservative leading to the shift of plane of sheer and the reduced measured zeta potential. This appears likely when looking at the chemical structure of the preservatives. Hydrophobic compounds like magnolol and honokiol are likely to stay adsorbed on the hydrophobic crystal surface.

Measurements of ZP in the original dispersion medium are measurement of the diffuse layer. In order to obtain a stable nanosuspension an absolute ZP is to be greater than |30| mV. However, with steric stabilizers a value of 20 mV can be sufficient to stabilize the nanosuspension (Müller 1996). From Table 2 it can be seen that all preserved nanosuspensions have ZP values around −30.0 except the nanosuspension preserved with caprylyl glycol it was −10.5 mV. The zeta potentials above 30 mV for Hydrolyte-5, Euxyl PE9010 and Rokonsal PB5 are in agreement with the PCS data indicating stability. The lowest value of −10.5 mV for caprylyl glycol is also in agreement with the observed instability. However, the preservatives MultiEx naturotix and Phenonip show also a high potential around −30 mV, but were not stable. This indicates that the zeta potential is not the only stability determining parameter. Properties, e.g. rigidity and fluidity of the stabilising surfactant film seam also to play a role.

Table 2: ZP values of the preserved nanosuspensions in distilled water and in original dispersion medium

Preservative	Zetapotential [mV]	
	Distilled water (pH = 5.8, Conductivity 50 μS/cm)	In original dispersion medium
Hydrolite-5	**−59.0**	**−33.3**
Euxyl PE9010	**−57.5**	−29.0
Rokonsal PB5	**−58.9**	**−32.6**
Caprylyl glycol	**−55.3**	−10.5
MultiEx naturotics	−32.0	−28.2
Phenonip	**−58.6**	**−31.9**

2.2. Stability study

In order to estimate the stability of the preserved nanosuspensions on the long term during storage, a short-term stability was performed. Samples were stored at three different temperatures (4 °C, 25 °C and 40 °C). These temperatures were chosen due to the principle that storing samples at low temperatures may promote re-crystallization due to solubility reduction, with particle size augmentation. High temperatures may contrarily increase the solubility, therefore being accompanied with a reduction in particle size. However, small particles may dissolve and larger particles remain, leading to an increase in the larger particle population, Ostwald ripening (Keck 2006).

Figure 4 shows the z-average diameters from PCS and d(v)99% measured with LD of the six preserved nanosuspensions stored at different temperatures for one month. With PCS measurements small changes in the mean particle size of the bulk population can be detected, whereas changes occurred on the large particles can be detected with the d(v)99% measured with laser diffractometry. PCS mean diameters show no significant changes in nanosuspensions preserved with Euxyl PE9010, Hydrolite-5 and Rokonsal PB5 (Fig. 4A–C). In addition negligible changes were detected in the d(v)99% measurements. Therefore, they were all stable at the three different temperatures. In contrast, PCS mean diameters of nanosuspensions preserved with MultiEx naturotics, caprylyl glycol and Phenonip were significantly higher than the previously mentioned preserved nanosuspensions. PCS mean diameter fluctuations measured for the MultiEx naturotics nanosuspension and the Phenonip nanosuspension were due to the particles that tend to partially de-agglomerate during agitation in sample preparation. Particle sizes measured with PCS for the caprylyl glycol preserved nanosuspension were large and remained large during the stability study except for 40 °C which exhibited an increase in particle size during storage. LD measurements on the other hand did not show that fluctuations due to the stirrer-effect that loose the fragile aggregates. All preservatives used were water soluble preservatives. In order to act as a preservative it should possess a week lipophilic pole that enables it to interact with lipophilic membranes of bacteria. As hesperetin is a poorly water soluble substance the lipophilic pole of the preservatives tends to adsorb to the surface of hesperetin nanoparticles.

Hydrolite-5, which is pentylene glycol, is highly hydrophilic and this is why it is the least preservative that may interact with the particle surfaces and the film of the surfactant on the particles as well. Euxyl PE9010 contains 90% phenoxyethanol which in turn consist of a lipophilic benzene ring making it more affiliated to the surface of the particle.

Rokonsal PB5 and to a large extent Phenonip contain parabens. Parabens are hydrophobic materials which make the two mentioned preservatives less compatible with the nanosuspension than Euxyl PE9010 and Hydrolite-5. Phenonip contains more parabens than Rokonsal PB5 which make the incompatibility more pronounced.

Caprylyl glycol, which is octandiol, is a long carbon chain surfactant with high hydrophobicity which in turn promotes strong adsorption. MultiEx naturotics contains several substances, of which many are hydrophobic and may destabilize the nanosuspension.

2.3. Optimised addition of preservatives

In this study the preservatives were added after production of the nanosuspension. In the previous study by Al Shaal et al. (2009) the preservatives were added before homogenization that means they were present in the homogenization process. The adsorption layer around the nanocrystals was therefore not composed of stabiliser only, but of a mixture of stabiliser and adsorbed preservative. Of course, such mixed adsorbed layers can have different stabilising properties compared to a pure stabiliser adsorption layer.

To compare the effect of way of addition the PCS diameters of both methods were compared. For the comparison the PCS diameters after 4 weeks were taken, not immediately after homogenization/addition of the preservative. This makes sure that the systems are in equilibrium. Figure 5 shows that the PCS diameters are in most cases lower when the preservative is added after production. Obviously the preservatives present in the mixed adsorption layer had a negative effect on stability, that means they led to aggregation. Logically no difference was found for the preservative least interacting with the nanocrystal

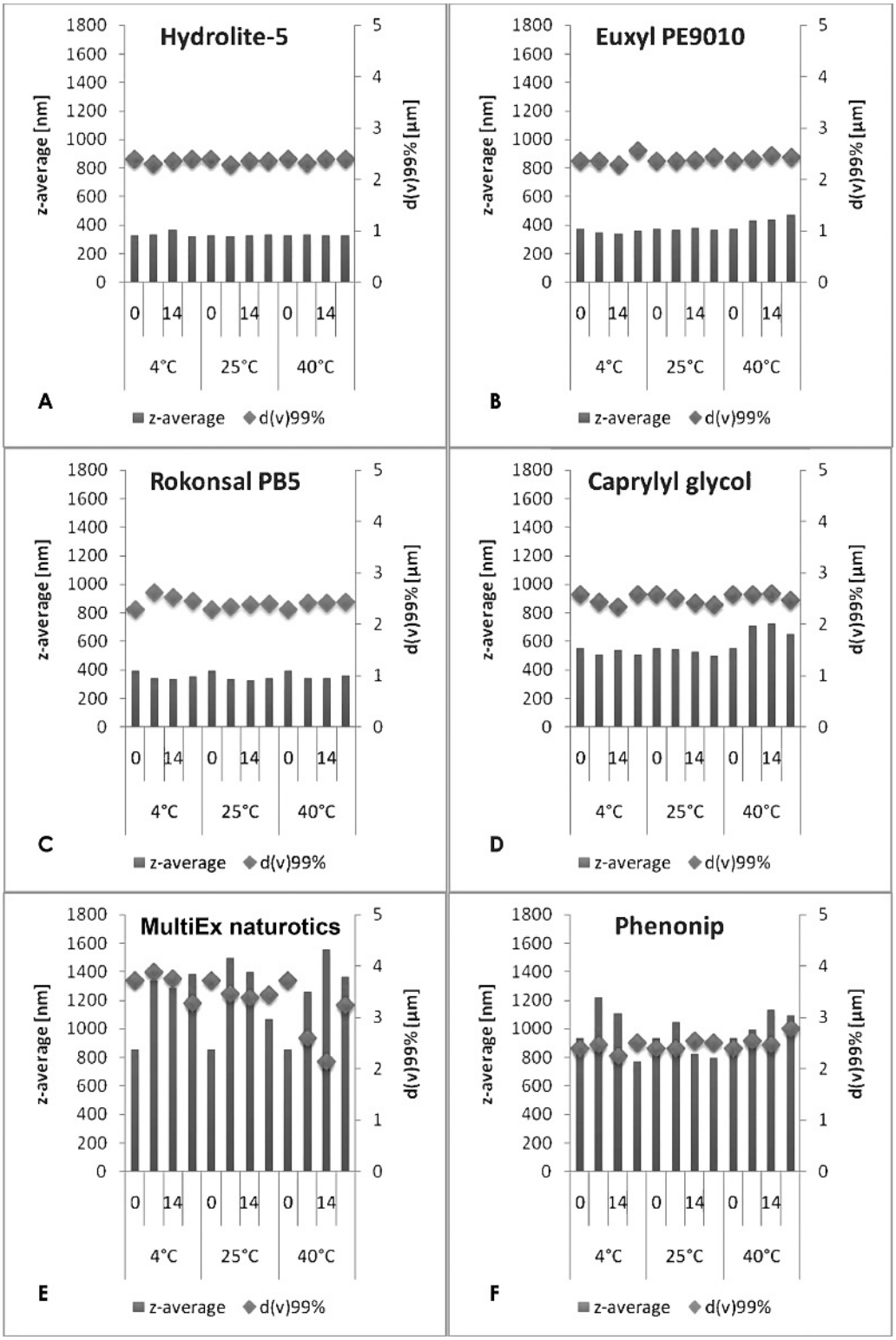


Fig. 4: PCS diameters (bars) and LD diameters d(v)99% (diamonds) of preserved hesperetin nanosuspensions as function of days (0–30) stored at different temperatures

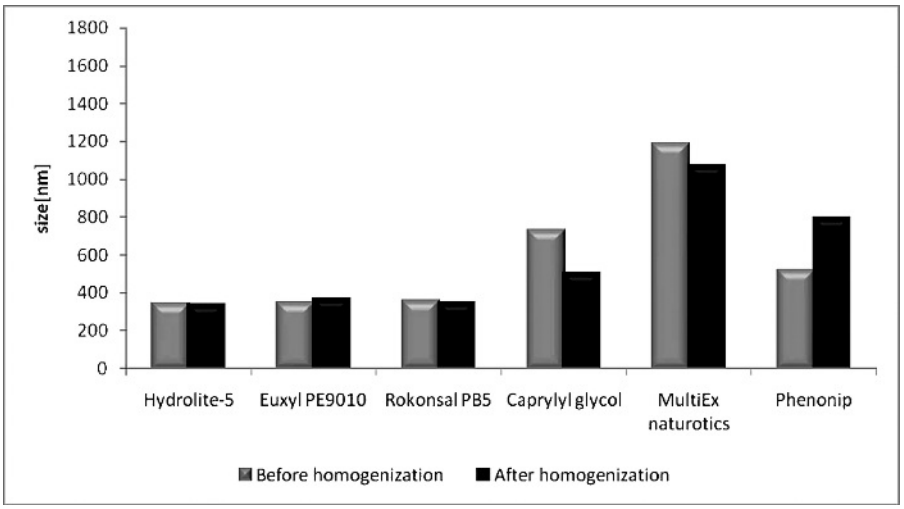


Fig. 5: Comparison of PCS diameters of preserved hesperetin nanosuspensions (added before or after homogenization) after one month stability stored at room temperature

surface, hydrolyte-5. From this it can be concluded, in case the nanosuspension is produced with a good stabiliser, the preservatives should be added after the production. In addition, the preservative should be added to the nanosuspension, not vice versa, to avoid that nanocrystals are added to a highly concentrated preservative solution.

As seeing from Fig. 4, after addition of the preservative the particle size increased immediately in case of the stabilising preservatives. There was no or only very limited further increase during the storage period of 30 days. That means for screening for optimal preservatives, just one day or only a few days of storage are sufficient to identify immediately the ones with a strong de-stabilising effect. The aggregation process is fast and then seems to rest on a certain aggregation level. These aggregates are obviously loose aggregates, as seen from the easy re-dispersion during the LD measurements (Fig. 2). Also the microscopic pictures rather suggest a loose and not a condensed aggregate structure (Fig. 3).

2.4. Conclusion

Based on these results it is suggested to produce the nanosuspensions and to preserve them using highly hydrophilic preservatives in order to obtain a long-term stabilized preserved nanosuspension.

Stability of the nanosuspension is not impaired when:

1. The preservative is relatively hydrophilic with low adsorption to nanocrystal surface (as indicated by high ZP values).
2. The ZP reduction caused by the preservative is small, i.e. ZP in the original dispersion stays above 30 mV.
3. Important: A high ZP of 30 mV does not necessarily guarantee a stable nanosuspension, obviously properties of the mixed adsorbed layer of stabilizer/preservative also play a role (rigidity, fluidity, thickness).
4. In general the preservatives should be added after production.

3. Experimental

3.1. Materials

The following materials were used in this study: Hesperetin (Exquim, S.A., Spain), plantacare 2000 (Cognis, Germany), freshly prepared double distilled and ultra purified water (milli-Q, Millipore GmbH, Germany), 0.9% sodium chloride solution (B. Braun Melsungen AG, Germany).

The six different preservatives were: Hydrolite-5 (Dragoco Gerberding & Co AG, Germany), Euxyl PE 9010 (Schülke & Mayr GmbH, Germany), Rokonsal PB5 (ISP Biochema Schwaben GmbH, Germany), Phenonip (Nipa Laborotories Inc., UK), caprylyl glycol (ACIMA AG für Chemische Industrie, Switzerland) and MultiEx naturotics (Biospectrum Inc., Korea).

3.2. Preparation of nanocrystals

The nanosuspensions were produced by high pressure homogenization, using an LAB 40 (APV Deutschland GmbH, Germany) equipped with a water jacket for the temperature control. Plantacare 2000 (0.8 g) was dissolved in purified water (35.2 g). Hesperetin powder (4.0 g) were dispersed in the (36.0 g) plantacare 2000 aqueous solution, pre-dispersed using an Ultra-Turrax T25 (speed: 10,000 rpm for 1 min, Janke and Kunkel GmbH, Germany) followed by 5 cycles of HPH at low pressures. The suspension was subjected to 30 cycles of high pressure homogenization at 1,500 bar. The whole homogenization process was performed at 1 °C. The concentrated nanosuspension was then diluted with preservatives aqueous solutions.

3.3. Physical characterization

The particle size was analyzed using dynamic and static light scattering. Light microscopy was especially employed to judge if possible larger particles found by static light scattering measurements are related to large drug crystals or to agglomerates. The surface charge of the nanocrystals was analysed by zeta potential measurements.

3.3.1. Photon Correlation Spectroscopy (PCS, dynamic light scattering)

PCS, was used to analyse the bulk mean diameter, as well as the width of the size distribution (Zetasizer Nano ZS, Malvern Instruments, UK).

3.3.2. Laser diffractometry (LD, static light scattering)

For LD measurements a Mastersizer 2000 (Malvern Instruments, UK) was used. The results were analyzed using Mie theory (real refractive index: 1.59; imaginary refractive index: 0.01).

3.3.3. Light microscopy

An Orthoplan (Leitz, Germany) equipped with a CMEX-1 digital camera (Euromex microscopes, Arnheim, Netherlands) connected to Image Focus software version 1.3.1.4. (Euromex microscopes, Arnheim, Netherlands) was used. Magnifications 16×10-fold, 40×10-fold, 63×10-fold and 100×10-fold were used.

3.3.4. Zeta potential (ZP)

The zeta potential was measured using a Zetasizer Nano ZS (Malvern Instruments, UK). Measurements were performed in water (distilled water with the conductivity adjusted to 50 µS/cm by NaCl solution) and in the original dispersion media. All measurements were performed at 25 °C with a field strength of 20 V/cm, the Helmholtz-Smoluchowski equation was used for data evaluation.

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