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# Nanosuspensions of hesperetin: preparation and characterization

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Nanosuspensions are a smart formulation principle for dermal applications, as they increase the penetration of poorly soluble substances into the skin. Because microbial stability is a pre-requisite for dermal formulations, water containing formulations need to be preserved. Preservatives are known to possibly impair the physical stability of disperse systems, i.e. by causing agglomeration. These aggregation phenomena might occur during storage of the final product, but can already occur during the production process itself. Therefore, in this study the influence of six different preservatives on the diminution efficiency during the production of hesperetin nanocrystals was investigated. Particles with and without the addition of preservatives were produced by high pressure homogenization (HPH) and the final particle size was analysed and compared to the non-preserved suspension. All preservatives influenced the diminution progress during production and the final particle sizes obtained. The non-preserved suspension yielded a particle size of about 300 nm. Preservation with Hydrolite, Euxyl PE9010, Rokonsal and Phenonip led to sizes of about 400 nm. Preservation with Caprylyl glycol and MultiEx did not lead to nanoparticles (size > 1 μm) and caused a slight agglomeration of the nanosuspensions. Based on zeta potential measurements it was found that the impairment is related to the lipophilicity of the presverative, i.e. the lower the lipophilicity, the less is the impairment. In conclusion, preservatives impair the diminution efficacy during the production of nanosuspensions. Therefore, if possible, preservatives should be added to nanosuspensions after the production process. If preservatives are required during production, highly hydrophilic preservatives, e.g. Hydrolite E, should be used.

## 1. Introduction

Nanocrystals are used for the formulation of poorly soluble drugs, to overcome their oral bioavailability problems (Merisko-Liversidge et al. 2003). Since 2000 more than five products entered the pharmaceutical market (e.g. Emend, Tricor etc.), about 20 are presently in clinical phases (Rabinow 2004). Alternatively the nanocrystals can be dispersed in isotonic aqueous solution (so called nanosuspensions) and injected intravenously. The principle of nanocrystals is the increase in saturation solubility, surface area, and consequently dissolution velocity. In addition the concentration gradient at biological membranes/barriers is increased. These effects lead to an increased penetration into or permeation through membranes (Müller et al. 2011; Rabinow 2004).

At the beginning this was only exploited to transport drugs more efficiently across the gut wall for oral delivery. The barrier skin and dermal delivery was completely forgotten until recently. It could be shown that dermal application of rutin and hesperidin increased the bioactivity in the skin by up to a factor 500, compared to water soluble derivatives of the original molecules (Petersen 2006). The first cosmetic product with rutin nanocrystals entered the market in 2007 (line JUVEDICAL, Juvena Switzerland). The same principle can also be employed for the improved delivery of dermal drugs.

For incorporation into dermal products, the nanocrystals are admixed as a concentrated nanosuspension to the water phase of the dermal formulation. Thus, in general nanosuspensions are produced as intermediate products to be sold to cosmetic or dermal pharmaceutical companies. As nanosuspensions contain water, of course these nanosuspensions need to be preserved. Preservation is also important in drug delivery and pharmaceutical industry, i.e. nanosuspensions are also used for oral delivery (e.g. for flexible dosing and/or convenient administration for paediatric or elderly patients). In multi-dose vials also these oral nanosuspensions need to be preserved. For example, the oral nanosuspension Megace® ES contains sodium benzoate as preservative (Allmaier et al. 2008). Many preservatives are known to impair the physical stability of disperses systems. Hence, upon the addition of preservatives even physically stable systems can agglomerate. The effect is even more pronounced for small sized systems, i.e. nanoparticles. Therefore, the formulation of preserved nanosized systems remains challenging.

Preservation of nanosuspensions can be obtained by either adding the preservatives to the nanosuspension after the production process or by adding the preservatives directly to the water phase prior to the homogenization process. The advantage of the first procedure is that there will be no impairment during the production process itself. Therefore this kind of preservation procedure was performed until now. Nevertheless, the addition

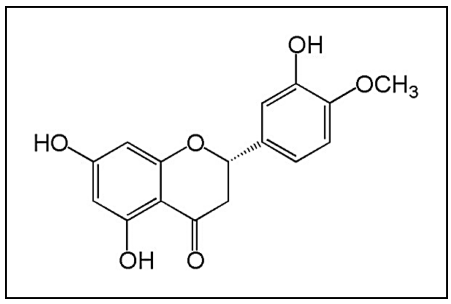


Fig. 1: Chemical structure of the flavaone hesperetin - (((S)-2,3-dihydro-5,7-dihydroxy-2-(3-hydroxy-4 methoxy-phenyl)-4H-1-benzopyran-4-one).

of any compound to a finally processed nanosuspension, might disturb the equilibrium of the system, which can contribute to Ostwald ripening and a decreased physical stability of the nanosuspensions (Lindfors et al. 2007). In case of preservatives, which are highly adhesive to surfaces (European Pharmacopoeia 6.0 2008; Nürnberg and Surmann 1991; Schuber 2010), the stability is also influenced by adhesion of the preservatives to the surface of the particles. The adhesion can lead to a reduction in the zeta potential and thus to a decreased electrostatic repulsion of the nanocrystals. To minimize the destabilizing effect of added preservatives, non-ionic stabilizers should be used for the preservation of nanosuspensions (Al Shaal et al. 2010). The type of preservative used needs to be identified case by case (Kobierski et al. 2009). In contrast to this nothing is known about the impact upon the addition of preservatives prior the production process. The addition of preservatives prior the production process would have the clear advantage that bacterial growth can be avoided also during the production itself. In addition disturbance of the established equilibrium during the homogenization process can be avoided. At the other hand it can be expected that the preservatives will impair the production process due to their high affinity to surfaces. Therefore the aim of this study was to investigate to which extent preservatives impair the production process of nanosuspensions and finally to judge if the addition of preservatives is more appropriate prior or after the production process. For a better understanding of the mechanisms different preservatives were used. To identify potential disturbance/impairment of the production process itself, the impairment was monitored by size measurements and light microscopy during the production process and after the homogenization process and compared to the same formulation without added preservatives. For a final selection of the optimal preservative in addition also the short term-stability was monitored at three different storage temperatures (room temperature, 4 °C and 40 °C).

Hesperetin (Fig. 1) is the aglycon of Hesperidin. Both compounds show strong anti-oxidative properties (Cho 2006; Miyake et al. 1998). However, in contrast to hesperidin, hesperetin also possesses anti-inflammatory properties. Therefore, it is of high interest for dermal application (e.g. increased photoprotection of skin) (Balestrieri et al. 2003). In a previous study a physically stable hesperetin nanosuspension with a particle size of about 300 nm was developed. In this study high pressure homogenization with 30 homogenization cycles at 1550 bar and Plantacare 2000 with a concentration of 1 % (w/w) were found to be the most efficient production parameters and stabilizer, respectively (Mishra et al. 2009). In this study the previously developed formulation was used and the influence of six different preservatives on the production efficacy was investigated. Only non-ionic preservatives were selected, initially avoiding ionic preservatives due to their stability reducing effect (e.g. zeta potential reduction by electrolytes).

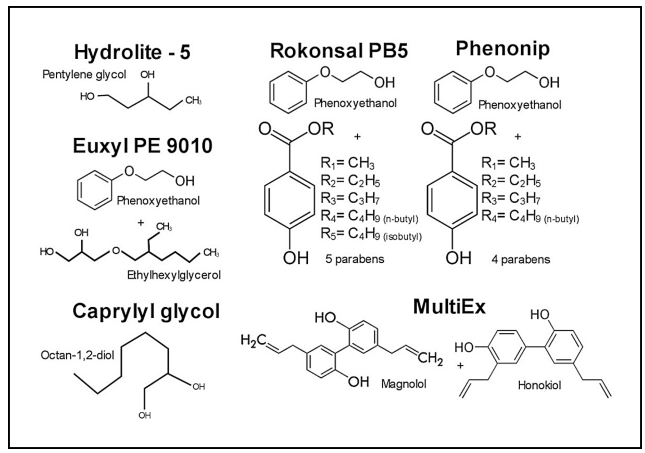


Fig. 2: Chemical composition and structures of the preservatives: Hydrolite, Euxyl PE9010, Rokonsal, Phenonip, Caprylyl glycol and MultiEx.

## 2. Investigations, results and discussion

### *2.1. Selection criteria for preservatives*

Suspensions are stabilized by various superimposing effects, e.g. electrostatic repulsion (zeta potential), reduced interfacial energy by the surfactants, rigidity of the surfactant layer (e.g. density of surfactants adsorbed on the surface of the particles), and steric stabilization effects. Added preservatives have the potential to interact with many of these stabilizing effects. Electrolytes reduce the zeta potential and thus the repulsion of the particles, whereas non-ionic compounds do not. Therefore, to avoid a strong interaction of stabilizer and preservative, only non-ionic preservatives were selected. Among them, molecules were selected which are currently preferred for use by manufacturers of cosmetic or dermal products (e.g. having less reports of undesired side effects, such as allergy). This resulted in the six preservatives and preservative mixtures listed in Table 1, the corresponding chemical structures are depicted in Fig. 2.

For each of the preservatives there is a suggested concentration or concentration range specified by the manufacturer or in the literature (cf. Table 1, 3rd column). Clearly, the anti-microbial efficiency is higher at high concentrations, but at the same time the potential stability impairment increases as well. For example the pentylene glycol (Hydrolite 5) competes for the hydration water with steric stabilizers (e.g. Poloxamer). With less hydrated stabilizer layers the stabilization of a suspension will be reduced. Therefore it was decided to use a concentration somewhere in the middle of the recommended concentration range (cf. Table 1, 4th column).

### *2.2. Production – effect on bulk population*

The preservatives were added to the macrosuspension prior to the homogenization process for the production of the nanosuspension. The rationale behind was to have microbial safety also during the production process. Even though this is not critical for lab scale production, because the actual homogenization step is very fast, in large scale production the suspension might be processed for hours or up to 2 days (depending on batch size), which gives bacteria time to contaminate the product and to multiply. For highest microbiological quality, all nanosuspensions were prepared with sterile water.

PCS was applied to investigate changes of the size of the bulk population as a function of homogenization cycle numbers. PCS is a sensitive method allowing to monitor even small changes in the mean particle size (e.g. changes at higher homogenization cycles, where normally only small size decreases occur). The

**Table 1: List of preservatives used**

| Preservative | Composition of preservative | Effective concentration for preservation [%(w/w)] | Concentration used in this study [%(w/w)] |
|---|---|---|---|
| **Hydrolite-5** | Pentylene glycol (1,3-Pentanediol) | 2.0−5.0 | 2.0 |
| **Euxyl PE9010** | Phenoxyethanol 90%, ethylhexylglycerin 10% | 1.0 | 1.0 |
| **Rokonsal PB5** | Phenoxyethanol 72%, Methylparaben 14.5%, Ethylparaben 5.8%, Butylparaben 3.6%, Propylparaben 2.4%, Isobutylparaben 1.8% | 0.3−1.2 | 0.5 |
| **Phenonip** | Phenoxyethanol 60-80%, Methylparaben 13–17%, Ethylparaben 4–6%, Butylparaben 4–6%, Propylparaben 2.4% | 0.25−1.0 | 0.75 |
| **Caprylyl glycol** | 1,2-Octanediol | 0.5−1.0 | 0.75 |
| **MultiEx Naturotics** | **Mixture of natural plant extracts from**: Magnolia Biondii bark, Propolis, Camellia Sinensis leaf, Thujopsis Dolabrata, Citrus Grandis (Grapefruit), Chamomilla Recutita, Salix Alba (willow) bark<br>Examples for antimicrobial actives: Magnolol, Honokiol (see Fig. 2), Hinokitiol<br>**Examples for other actives**: Tropolone, Epigallocatechin gallate, Azulene, Salicin | 1.0 – 3.0 | 2.0 |

Percentage refers to total nanosuspension, consequently the concentration in the water phase is slightly higher due to the solid content of the suspension).

final nanocrystal size obtained in the production process only depends on the production conditions and the properties of the coarse material, hence the final particle size of a material upon homogenization will be the same, independent on the stabilizer used. Only if a stabilizer is not efficient and not able to sufficiently stabilize the produced fine crystals, larger sizes will be measured by PCS.

In the previous hesperetin study without added preservative, by applying the same production conditions, the nanocrystal sizes were about 300 nm for the stabilizers Inutec, Plantacare and Poloxamer 188 stabilized nanosuspensions, respectively (after 30 cycles). A size of 347 nm was obtained when Tween 80 was used as stabilizer (Mishra et al. 2009). Based on these theoretical considerations, a reduced efficiency of the stabilizer due to a preservative present will lead to a more pronounced aggregation during the production process itself. Hence, the decrease in size from one cycle to the next will be less, when compared to the same suspension, being produced without preservative. The reason for this phenomenon is that the freshly diminuted fine crystals leaving the homogenization gap will be less efficiently stabilized, leading to some re-aggregation of the particles. In addition, also the ultrafine nanocrystals after cycle 30 might not be efficiently stabilized. Due to aggregation the finally measured size will be above the 300 nm obtained previously without preservative.

After pre-milling the nanocrystal size of the preservative-free hesperetin nanosuspensions were in the range of about 1000 nm (Mishra et al. 2009). Figure 3 shows that there is a strong interference of the preservatives with the pre-milling process, all PCS sizes after pre-milling (PM) are higher than 1,800 nm. Slightly higher sizes to about 2,000 nm were found for Euxyl PE9010, Rokonsal and Phenonip, and just above 3,000 nm for Hydrolite. These preservatives were classified as "group 1" having the least interference. A very pronounced increase was found for both caprylyl glycol and MultiEx: to about 8,000 nm (actually outside the reliable measuring range of PCS). This classification was confirmed by looking at the PCS sizes after 5 homogenization cycles at 1,500 bar. The preservative-free hesperetin nanosuspension stabilized with Plantacare exhibited a PCS size of 600 nm at cycle 5, the group 1 in this study sizes of around 1,000 nm (detailed data are shown in Table 2). The nanosuspensions preserved with caprylyl glycol had a PCS size of almost 2,000 nm, the one with MultiEx a size of about 5,000 nm, respectively (Table 2).

Even though the polydispersity indices are above 0.5, data have to be treated with care. A clear trend was observed. In general the group 1 showed a steady decay in size and polydispersity index with increasing cycle number, having lowest sizes and lowest PDI after 30 cycles (Fig. 3 A-D). In contrast to this, there was practically no size decrease in the nanosuspensions preserved with caprylyl glycol and MultiEx, the PCS sizes stayed around 1,000 and 3,000 nm, respectively. In addition also the PDI showed no decrease (Fig. 3 E and F).

Table 3 shows the detailed PCS data after 20 and 30 homogenisation cycles. These results could be used to place the best four preservatives in an order of least affecting the stability and final size. After 20 cycles of high pressure homogenization the preservatives Hydrolite and Euxyl PE9010 exhibited the lowest sizes,

**Table 2: PCS diameters and polydispersity indices (PDI) of the preservative-free hesperetin nanosuspension and the preserved nanosuspensions after pre-milling (PM) and homogenization cycles 1 and 5**

| | Pre-milling z-average | PDI | Cycle 1 z-average | PDI | Cycle 5 z-average | PDI |
|---|---|---|---|---|---|---|
| **no preservative** | 1016 | 0.607 | 618 | 0.515 | 531 | 0.493 |
| **Hydrolite** | 3304 | 0.896 | 2618 | 0.951 | 931 | 0.701 |
| **Euxyl PE9010** | 1887 | 0.888 | 1698 | 0.862 | 1231 | 0.760 |
| **Rokonsal** | 2156 | 0.840 | 2077 | 0.976 | 975 | 0.735 |
| **Phenonip** | 2187 | 0.733 | 1280 | 0.735 | 1003 | 0.676 |
| **Caprylyl glycol** | 8075 | 0.898 | 3390 | 0.983 | 2042 | 0.914 |
| **MultiEx** | 7657 | 0.718 | 6373 | 0.942 | 5429 | 0.736 |

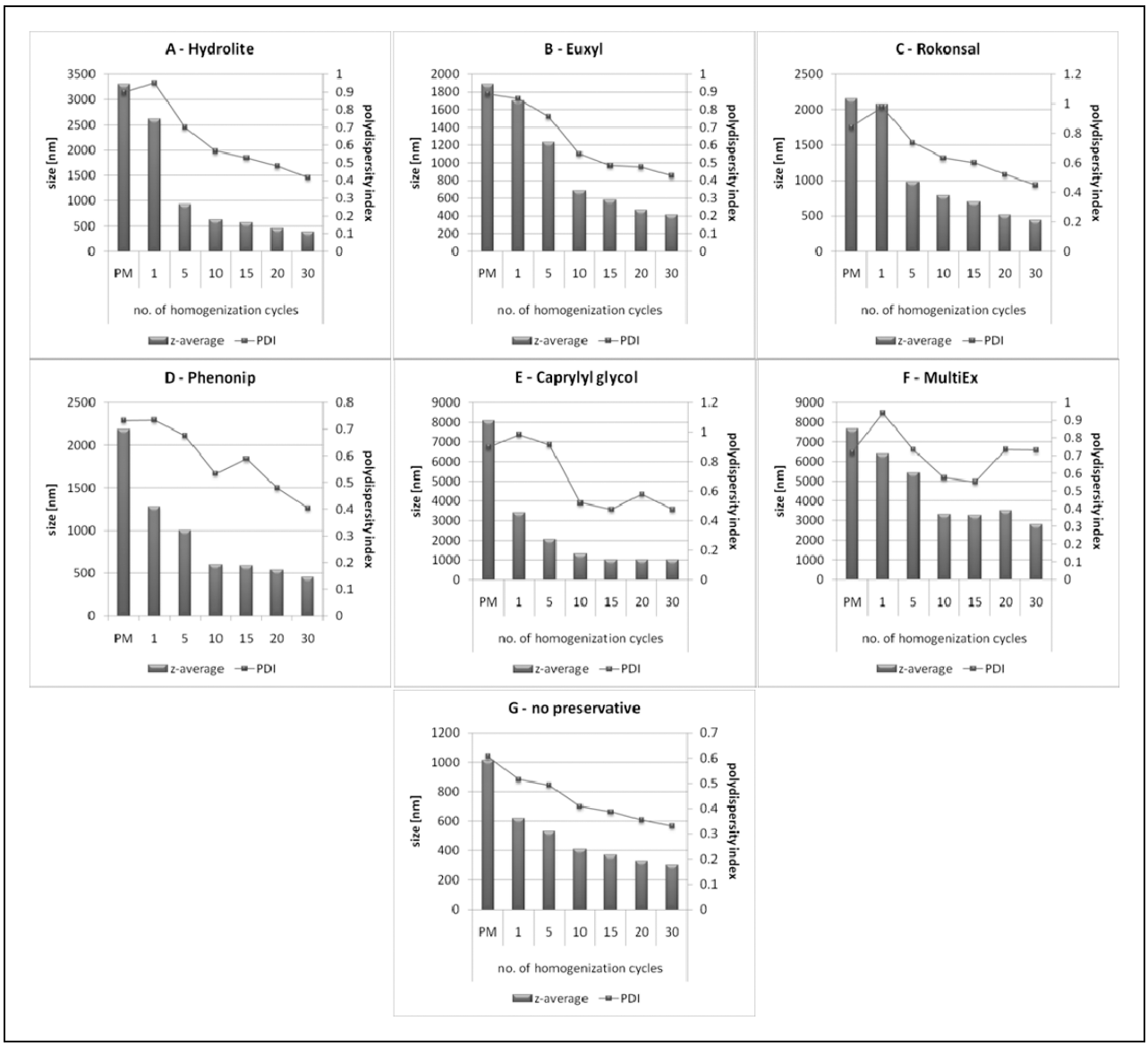


Fig. 3: PCS diameters and polydispersity indices (PDI) of the pre-served nanosuspensions after pre-milling (PM) and after homogenization cycles 1, 5, 10, 15, 20 and 30.

being 452 nm and 464 nm, respectively. The Rokonsal preserved nanosuspension had a PCS diameter of 510 nm, and Phenonip 540 nm. However, the polydispersity index of the suspension stabilized with Rokonsal after 20 cycles was still above 0.5. Therefore, PCS data of this suspension are not trustworthy at that point.

However, after 30 cycles of homogenization for all group 1 preserved suspensions PI values below 0.5 were obtained. The order in size after 20 cycles was confirmed by the sizes after 30 cycles, being 386 nm, 408 nm, 438 nm and 452 nm, respectively. Increasing the cycle number to 30 further reduced the size (in line with the theory) but the small size of 301 nm of the preservative-free nanosuspensions could not be reached. It might be reachable after another 10 or 20 cycles, but such a high cycle number is not applicable in an industrial production process, and was therefore not further investigated. Increasing the cycle number from 20 to 30 had no effect for the caprylyl glycol preserved system (1,023 nm and 1,007 nm) and only a limited effect in the MultiEx system (3,518 nm and 2,791 nm).

**Table 3: PCS data after 20 and 30 homogenization cycles**

| | Cycle 20 z-average | PDI | Cycle 30 z-average | PDI |
|---|---|---|---|---|
| no preservative | 331 | 0.354 | 305 | 0.330 |
| Hydrolite | 452 | 0.481 | 386 | 0.416 |
| Euxyl PE9010 | 464 | 0.475 | 408 | 0.426 |
| Rokonsal | 510 | 0.519 | 438 | 0.448 |
| Phenonip | 540 | 0.480 | 452 | 0.404 |
| Caprylyl glycol | 1023 | 0.578 | 1007 | 0.475 |
| MultiEx | 3518 | 0.735 | 2791 | 0.731 |

### *2.3. Production – effect on particles > 1 μm*

Figure 4 shows the decrease in the LD diameters with increasing cycle number. The d(v) 50% changes relatively little from cycle 10 to cycle 30. This is confirmed when looking at the detailed d(v) 50% data listed in Table 4. The diameters 95% and 99% are a sensitive measure of remaining larger particles, large crystals and/or aggregates. They show a nice exponential decay with increasing cycle number, with in general lowest values after 30 cycles (Fig. 4). Exception is the MultiEx suspension with identical values at 20 and 30 cycles (around 4.4 μm). Surprisingly the decay in the LD diameters is very similar for all investigated nanosuspensions. In contrast to the PCS data, the LD data do not reflect the clear difference between the group

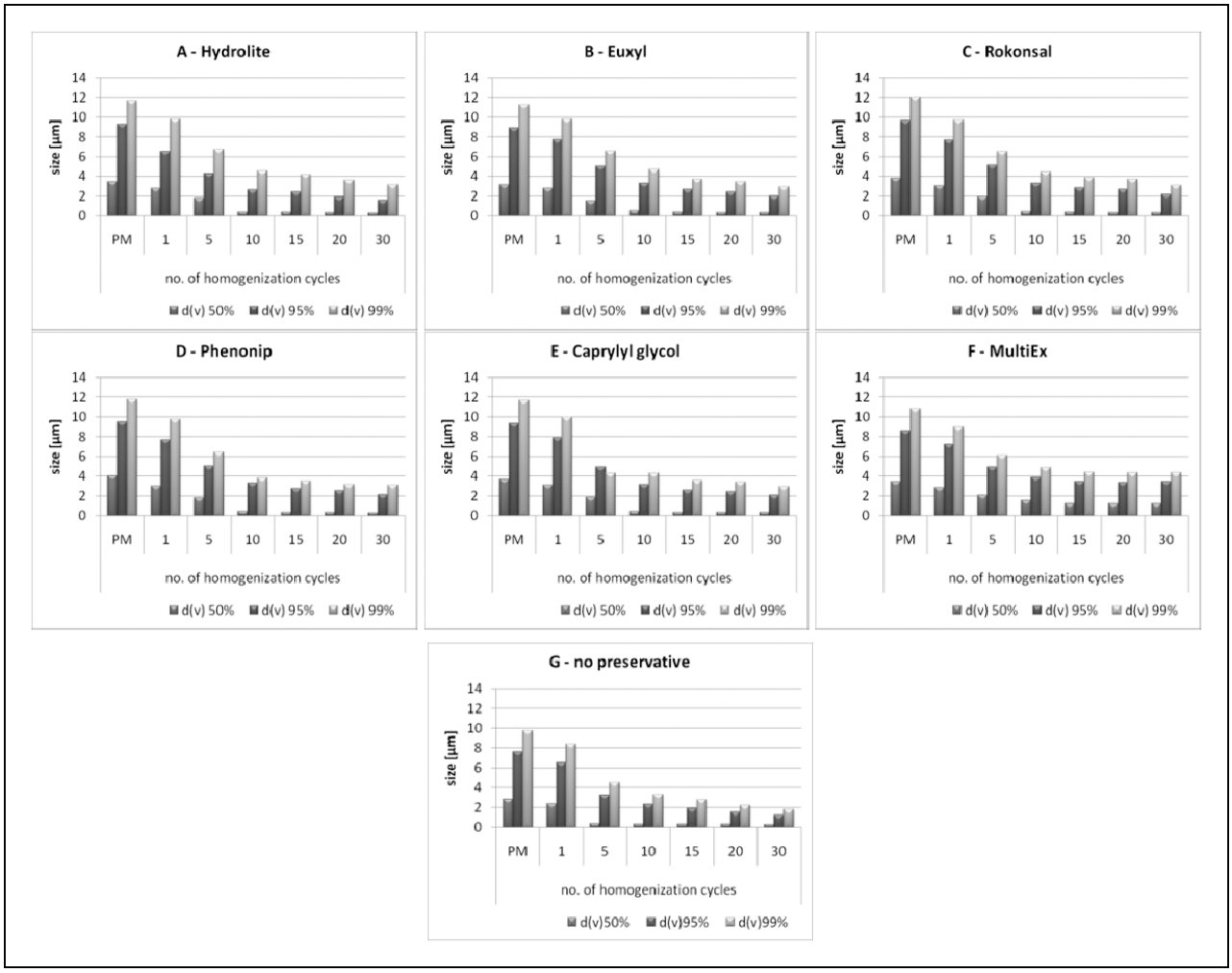


Fig. 4: LD diameters 50%, 95% and 99% of the pre-served nanosuspensions after pre-milling (PM) and increasing number of homogenization cycles from 1 to 30.

of the four least interfering preservatives (group 1) and caprylyl glycol and MultiEx, which dramatically affect the stability of the Hesperetin nanocrystals. A potential explanation is that the aggregates are relatively loose. The LD instrument has a build-in stirrer, which needs to be switched on during the measurement. This stirring can de-aggregate loose aggregates, thus making them not accessible to the analysis. This potential interference is a well-known problem (Acar Kübart and Keck 2013). In contrast, there is no stirring in the PCS measuring cell. This leads to a clear differentiation by the measured sizes. Light microscopy is another method to clearly discriminate differently agglomerated systems. The images obtained from light microscopy could therefore prove this assumption (Fig. 5). Fig. 5 represents images of the nanosuspension preserved with Hydrolite (Fig. 5 left) as a representative for group 1 preservatives and the images obtained from the suspensions preserved with capryly glycol (Fig. 5, middle) and MultiEx (Fig. 5, right). No agglomerates were found for Hydrolite, some agglomeration is detected for the suspension preserved with caprylyl glycol and heavy agglomeration is seen for the MultiEx preserved suspension.

### *2.4. Physical stability*

The influence of the different preservatives on the short- physical stability of the nanosuspension was assessed. The data obtained are shown in Fig. 6. After storage of 30 days almost all suspen-

| | | | | | | |
|---|---|---|---|---|---|---|
| **Caprylyl glycol** | 3.102 | 1.841 | 0.435 | 0.348 | 0.346 | 0.339 |
| **MultiEx** | 2.859 | 2.08 | 1.528 | 1.25 | 1.21 | 1.209 |

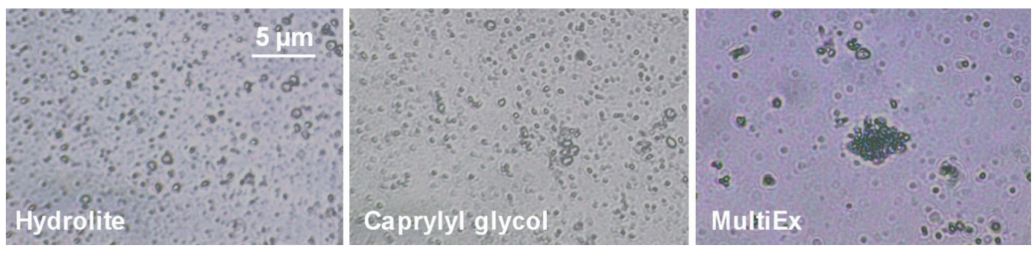


Fig. 5: Light microscopy images of nanosuspensions preserved with Hydrolite, caprylyl glycol and MultiEx (from left to right, magnification 1,000 fold, bars = 1 µm).

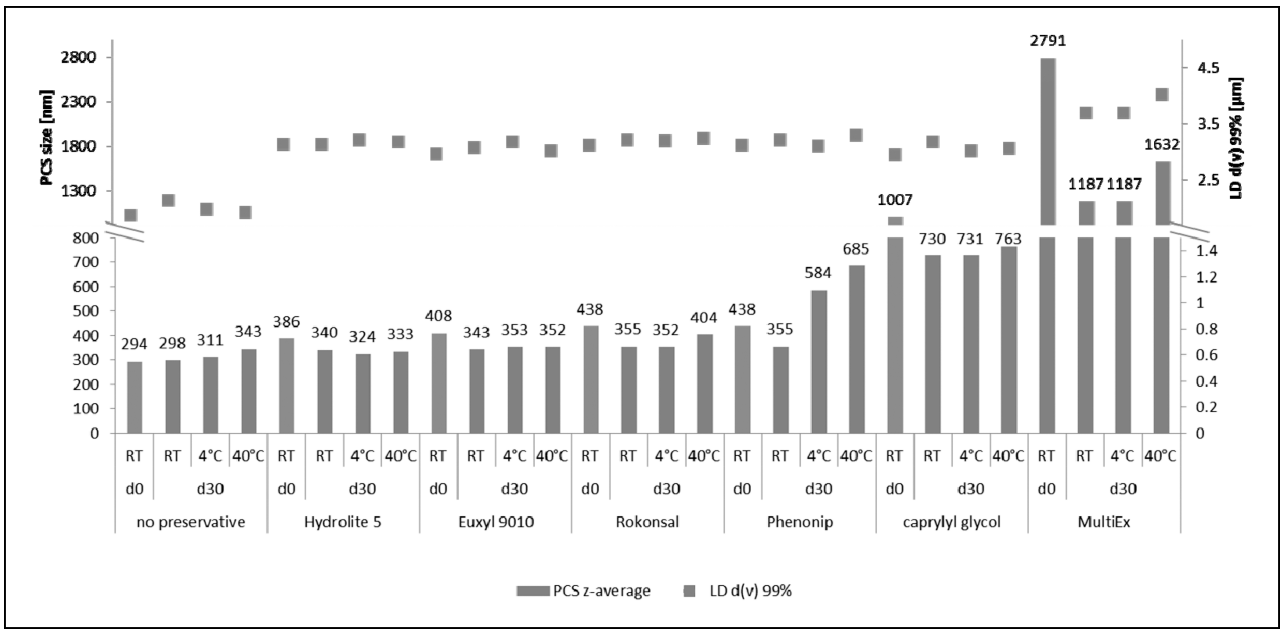


Fig. 6: Physical stability (1 month) of the suspensions stored at room temperature, 4 °C and 40 °C. The most stable suspensions are the suspensions stabilized with Hydrolite and Euxyl.

sions remained stable. After the observed changes after 96 h no significant changes for the z-average (PCS data) and the LD d(v) 99% were observed for all the suspension stored at room temperature. Except from the suspension preserved with Phenonip also storage at 4 °C led to stable suspensions. Storage at 40 °C led to increases in the PCS mean diameter for the suspensions preserved with Rokonsal, Phenonip and MultiEx, however the d(v) 99% was not or only little affected. From these results it can be concluded, that Hydrolite-5 and Euxyl PE9010 do not influence the physical stability in a longer term, whereas the other four do, either by an increase in size over time (Rokonsal and Phenonip) or by an ineffective diminution, leading to large particles (caprylyl glycol and MultiEx).

### *2.5. Zeta potential*

The zeta potential measured in distilled water is a measurement of the Stern potential (at these measuring conditions the zeta potential is set equal to the Stern potential). The Stern potential is related to the surface potential (Nernst potential), it is a measure for the surface charge, i.e. the amount of repulsive forces on the crystal surface. Interestingly, the Stern potentials of group 1, the four least interfering preservatives, are practically identical to the zeta potential measured for the non-preserved nanosuspension, i.e. around -50 mV (Table 5).

This supports that the preservatives, potentially adsorbed on the crystal surface (inner Helmholtz layer), are desorbed in praxi from the surface of the particles. In this case the pure Nernst potential is measured, and thus it should be identical for all formulations (because the crystals consist of the same milled material). Nevertheless, the Stern potential of the de-stabilizing preservatives caprylyl glycol and MultiEx are reduced distinctly (-43.7 mV and -26.9 mV), most pronounced for MultiEx, Table 5). This indicates that upon dilution of the nanosuspension with water for the measurement, non-ionic preservative remained adsorbed, reducing the measured potentials. These data are an indication, that the two preservatives have a high affinity to the crystal surface. The presence of these preservatives, caprylyl glycol and MultiEx, leads to the formation of a mixed film with the stabilizer Plantacare, reducing the particle charge, distorting the film and finally reducing the stability (Fig. 7).

The measurement of the zeta potential in the original dispersion medium is a measure for the thickness of the diffuse layer. Large thickness and thus high zeta potentials support the stability of suspensions. In general, an absolute zeta potential of > 30 mV (positive or negative) is considered to be required for a stable suspension, which is solely electrostatically stabilized. In case of additional steric stabilization, around 20 mV proved to be suf-

**Table 4: Decrease in particle size (d(v) 50 % values, LD data) after 1, 5, 10, 15, 20 and 30 homogenization cycles for the differently preserved nanosuspensions.**

| | no. of homogenization cycles | | | | | |
|---|---|---|---|---|---|---|
| | 1 | 5 | 10 | 15 | 20 | 30 |
| no preservative | 2.368 | 0.393 | 0.324 | 0.321 | 0.321 | 0.303 |
| Hydrolite 5 | 2.814 | 1.801 | 0.414 | 0.401 | 0.331 | 0.302 |
| Euxyl PE9010 | 2.769 | 1.561 | 0.575 | 0.382 | 0.368 | 0.323 |
| Rokonsal | 3.038 | 1.96 | 0.472 | 0.379 | 0.337 | 0.324 |
| Phenonip | 3.021 | 1.82 | 0.429 | 0.352 | 0.346 | 0.312 |
| Caprylyl glycol | 3.102 | 1.841 | 0.435 | 0.348 | 0.346 | 0.339 |
| MultiEx | 2.859 | 2.08 | 1.528 | 1.25 | 1.21 | 1.209 |

A pronounced decrease in size occurs during the first 10 homogenization cycles, during the $10^{th}$ and the $30^{th}$ cycle only small changes are observed.

**Table 5: Zeta potentials measured in water (conductivity adjusted to 50 µS/cm, left) and in the original dispersion media (right)**

| preservative | Zetapotential [mV] | |
|---|---|---|
| | in water (pH 5.8, conductivity 50 µS/cm) | in original dispersion medium |
| no preservative | *−48.3* | *−21.1* |
| Hydrolite | −50.5 | −24.8 |
| Euxyl PE9010 | −51.5 | −6.35 |
| Rokonsal | −51.5 | −18.2 |
| Phenonip | −48.4 | −18.6 |
| Caprylyl glycol | −43.7 | −10.3 |
| MultiEx | −26.9 | −3.14 |

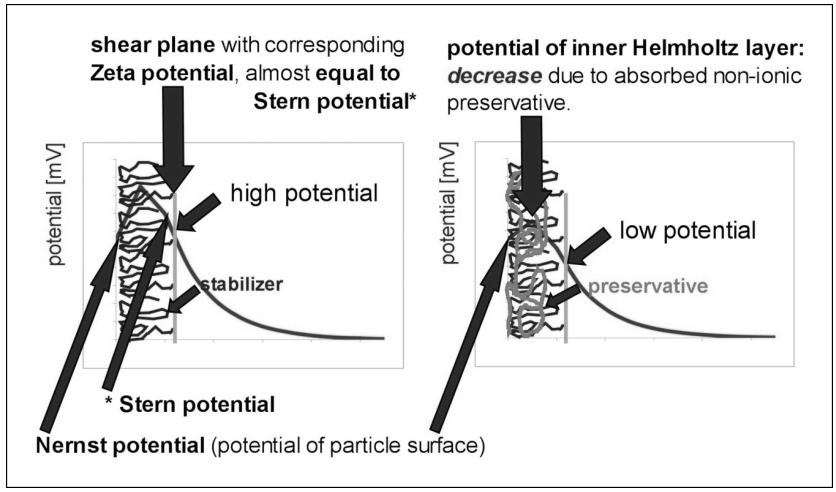


Fig. 7: Measurement of zeta potential in distilled water with conductivity adjusted to 50 μS/cm by addition of NaCl. Left: due too low electrolyte concentration the diffuse layer is thick, potential decay slow and the zeta potential practically identical with the Stern potential. The Stern potential is related to the Nernst potential (increase in Nernst potential also increases the Stern potential. Right: Non-ionic preservative remains adsorbed in the inner Helmholtz layer, thus reducing the potential of the inner Helmholtz layer and consequently the measured Stern potential. The reduction in Stern potential proves the remaining adsorption of non-ionic species on the crystal surface.

ficient in many cases (Müller 1996). The group 1 preservatives with little effect on the stability all exhibited a zeta potential in the original dispersion medium of around 20 mV. In accordance with the theory, this nicely explains the stability of the nanosuspensions (Fig. 8).

Zeta potential values of 10 mV and below, especially in the range 0–5 mV indicate highly instable suspensions. Caprylyl glycol exhibited a potential of -10.3 mV, the most de-stabilizing MultiEx a potential as low as 3.1 mV. These data are an explanation for the observed aggregation in the preserved nanosuspensions. Figure 8 explains the differences in the potential courses in the model of the electrical double layer and related stability.

### *2.6. Theory of the de-stabilizing effects*

Non-ionic surfactants were chosen to avoid a zeta potential reduction due to electrolytes being present (e.g. avoiding the "compression" of diffuse layer). Preservatives need to act in the water phase, where the bacteria are present. Thus preservatives need to be water soluble. In addition they also need to possess a certain lipophilicity to interact with lipophilic membranes of bacteria and to inhibit their growth. Of course this lipophilicity leads also to adsorption onto surfaces, especially onto hydrophobic surfaces. Hesperetin is a lipophilic, poorly soluble compound, therefore representing an ideal substrate for adsorption of lipophilic molecules.

Hydrolite is pentylene glycol, relatively hydrophilic and should possess the lowest affinity to the hesperetin surface. Therefore it showed least interaction with the surfactant and least impairment of the stability. Euxyl PE9010 consists to 90% of phenoxyethanol, due to the lipophilic benzene ring the interaction with the hydrophobic crystal surface will be slightly stronger, placing the preservative as number 2. The second component, ethyl glycerin is hydrophilic and should therefore adsorb only little onto the hydrophobic surface of the nanocrystals.

Rokonsal and Phenonip consist also of phenoxyethanol (72% and 60–80%, respectively), but additionally contain parabens (28% and 40–20%), being even more hydrophobic in character. This might be a reason why these preservatives were placed in position 3 and 4.

Caprylyl glycol is octanediol, the long hydrophobic, surfactant like carbon chain (as in SDS) has surfactant character promot-

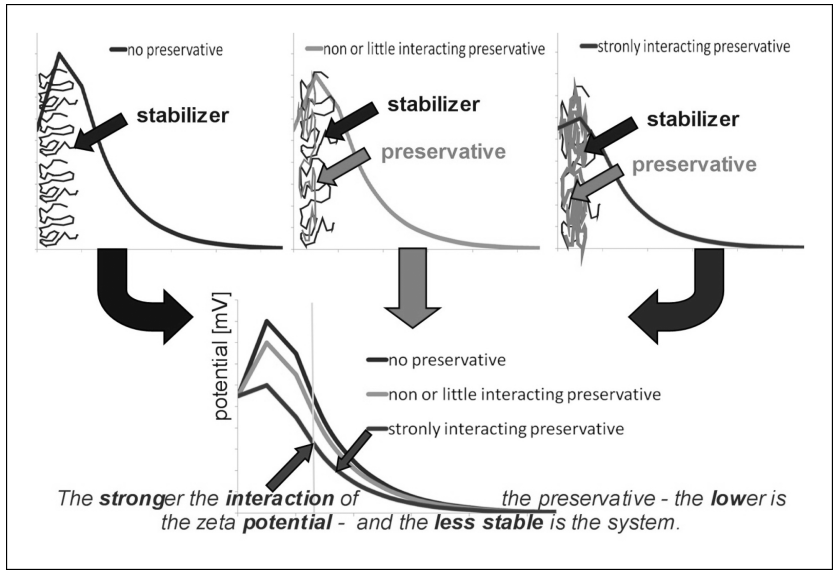


Fig. 8: Differences in the potential courses in the model of the electrical double layer and related stability. *Upper:* By interaction of a preservative with the nanocrystal surface the Nernst potential is decreased and thus the potential course of the potential will change. *Lower:* The stronger the interaction the lower is the measured zeta potential and the lower is the stability of the nanosuspension.

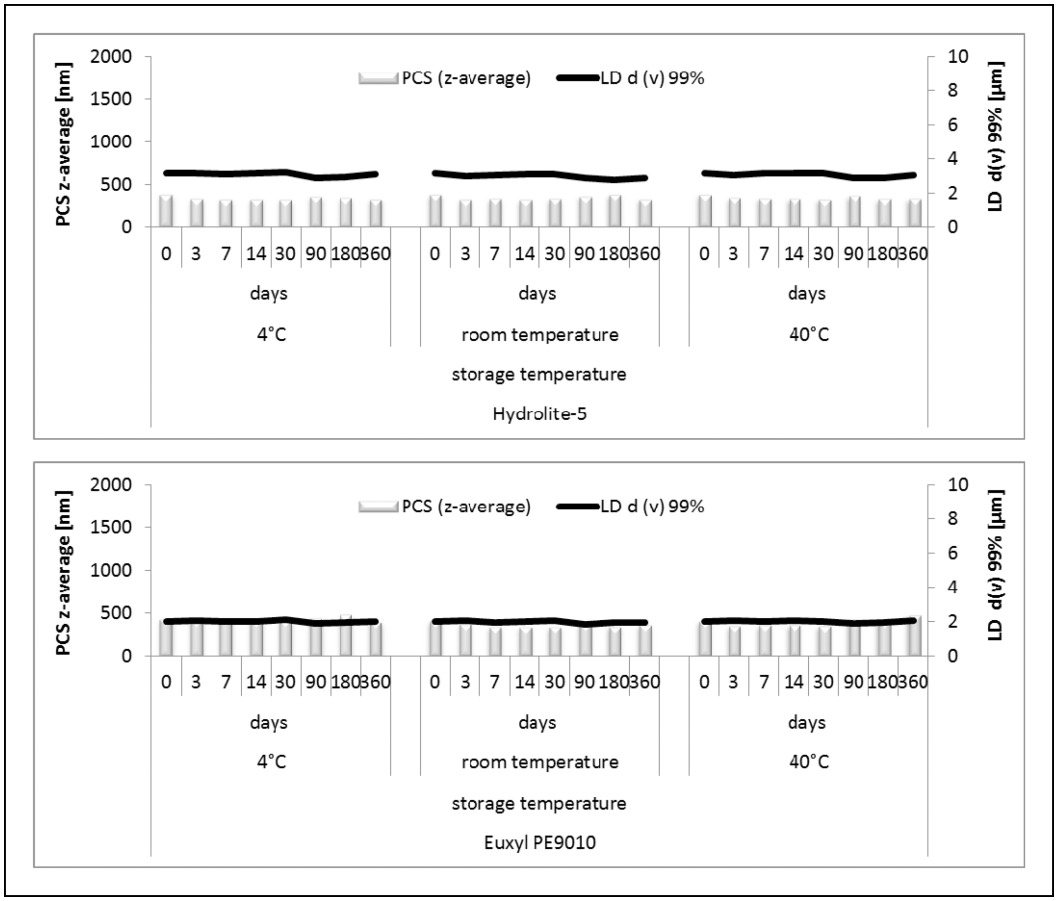


Fig. 9: PCS diameters (nm) and LD diameters 99% after 1 year of storage of the nanosuspensions preserved with Hydrolite, Euxyl PE9010, Rokonsal and Phenonip (from top to bottom), stored at three different temperatures.

ing strong adsorption. MultiEx contains various compounds, many of them being highly hydrophobic (Table 1), the interaction of these hydrophobic molecules can be discussed referring to the basic hydrophobic molecular structure of Magnolol and Honokiol (Fig. 2). These aspects might explain the strong destabilising effect of these two preservatives.

The physical stability of the suspensions is the first pre-requisite for selection of a preservative, the second selection criterion is that the preservative has a sufficient anti-microbial activity in the concentration applied. The activity of a preservative depends on the system to be preserved, being reflected by concentration ranges specified for each preservative. Depending on the affinity of the preservative to adsorb onto surfaces (more lipophilic preservatives have a higher tendency of adsorption), size of surface (nanosuspension versus macrosuspension), and ability to remain in the water phase (more hydrophilic preservatives such as Hydrolite) concentrations in the upper or lower recommended range are required. In this study in most cases medium concentrations were investigated. For Euxyl PE9010, besides Hydrolite one of the suitable preservatives, a microbial challenge test was performed using hesperidin (= glycoside of hesperetin). Hesperidin nanosuspensions preserved with 1% Euxyl PE9010 where shown to sufficiently prevent bacterial growth. They are meanwhile marketed in different commercial products (SmartCrystal - Lemon Extract, e.g. in Juvedical - Eye Optimizer by Juvena/Switzerland, in Edelweiss - wrinkle fighter by Audorasan/Germany, ageLine wo/man one, Eye Lifting Serum by ipam®/Germany).

### *2.7. Long-term stability 1 year*

From the size decrease in the production process, the least 4 destabilizing / not destabilizing preservatives were placed in group 1. From the size decrease they were placed in the order of least destabilizing effect being Hydrolite and Euxyl PE9010, then Rokonsal and with a slight tendency to aggregate Phenonip. This classification was performed on the basis of the PCS data, because the LD data could not differentiate, possibly due to de-aggregation during the dispersion in the measuring cell.

The short term storage result confirmed the clear superiority of the 4 preservatives in group 1, the destabilizing preservatives caprylyl glycol and MultiEx showed large sizes. Two preservatives of group 2 did not destabilize (Hydrolite, Euxyl PE9010), Rokonsal showed a negligible increase at 40 °C, Phenonip however clear indications of slight aggregation. The interesting question now was, if a long-term stability is predictable on these short term data, and based on the theoretical considerations behind the choice and action of the preservatives.

These four most stable preserved nanosuspensions were stored for 1 year at the three different temperatures. Figure 9 shows the PCS data and LD diameters 99%. The differentiation within group 1 from the short term storage was confirmed. Phenonip classified as slightly destabilizing in group 1 showed a further increase in the PCS diameter to about 1,500 nm. The LD diameter remained unchanged, again attributed to the loose character of any formed aggregates.

Rokonsal classified as number 3 out of the 4 with slight destabilizing effect showed an increase in PCS size to about 500 nm, confirming the classification from size decrease in production and short term stability. For the 2 best, not de-stabilizing preservatives Hydrolite and Euxyl PE9010 the prediction based on short term storage was confirmed.

From this it can be concluded that one month short term stability data allow selection of suitable preservatives for nanosuspensions, when using sensitive PCS measurements, in combination of course with light microscopy.

Looking at the data in more detail reveals that Euxyl PE9010 shows still a very small increase in PCS size, whereas Hydrolite shows no change at all over 1 year. Therefore long-term storage seems to allow fine differentiation in between suitable preservatives, Hydrolite being clearly the one least affecting nanosuspension stability. However, suitable for the preservation of commercial dermal nanosuspensions are also Euxyl PE9010 and Rokonsal.

### *2.8. Conclusions*

Four preservatives (group 1) impaired the stability very little. They could be placed in order of least impairment being 1. Hydrolite 5, 2. Euxyl PE9010, 3. Rokonsal PB 5 and 4. Phenonip. Pronounced stability impairment showed caprylyl glycol and especially MultiEx. The stability impairment could be explained by high affinity of the non-ionic constituents to the particles surface, thus reducing the electrostatic repulsion (zeta potential, thickness of diffuse layer). At the same time these non-ionic constituents had no compensatory sterically stabilizing effect.
However, also the group 1 interacted with the homogenisation process, as indicated by the less efficient size reduction with increasing homogenization cycles, compared to the preservative-free Hesperetin nanosuspension. There is obviously a distortion in the stabilising film properties of Plantacare during the stress of the homogenization process. From microbiological considerations, addition of the preservatives before beginning of the production process is desired. However, based on this study it is recommended to produce the nanosuspensions preservative-free, then adding the preservatives after production of the ultrafine nanosuspension. The benefit of a more narrowly sized nanosuspension with better long-term stability outweighs the advantage of early preservation.
Results from the size reduction performance in nanosuspension production in combination with 1 month short term stability data are a basis for the identification of suitable preservatives for nanosuspension stabilization.

## 3. Experimental

### *3.1. Materials*

Hesperetin was purchased from Exquim, S.A**.** (Spain). As stabilizer Plantacare 2000 (alkyl polyglycoside, Cognis, Germany) was used with a concentration of 1% (w/w). The influence on size and stability of the Hesperetin nanosuspension was investigated by using the following six different preservatives:

1. Hydrolite-5 (Dragoco Gerberding & Co AG, Germany)
2. Euxyl PE 9010 (Schülke & Mayr GmbH, Germany)
3. Rokonsal (ISP Biochema Schwaben GmbH, Germany)
4. Phenonip (Nipa Laborotories Inc., UK)
5. Caprylyl glycol (ACIMA AG für Chemische Industrie, Switzerland)
6. MultiEx Naturotics (Biospectrum Inc., Korea)

The chemical composition of the preservatives and the concentrations used are shown in Table 1.

### *3.2. Methods*

#### *3.2.1. Production of nanosuspensions*

The nanosuspensions, containing 5% (w/w) hesperetin were produced by high pressure homogenization (HPH), using an LAB 40 (APV Deutschland GmbH, Germany). After pre-milling of the macrosuspension by using an Ultra-Turrax T25 (speed: 10,000 rpm for 1 min, Janke and Kunkel GmbH, Germany) and 5 cycles at low pressures, the suspension was subjected to 30 cycles of high pressure homogenization at 1,500 bar. As dispersion media, purified water was used, in which the stabilizer and the respective preservative had been dissolved before (cf. 3.1.).

#### *3.2.2. Characterization*

The particle size was analyzed using dynamic and low angle static light scattering. Light microscopy was especially employed to judge if possible larger particles found from 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.

##### *3.2.2.1. Dynamic light scattering*

Dynamic light scattering, also known as photon correlation spectroscopy (PCS, Zetasizer Nano ZS, Malvern Instruments, UK), was used to analyse the bulk mean diameter, as well as the width of the size distribution. PCS yields the z-average, which is an intensity weighted mean diameter, and the polydispersity index (PDI), as a measure of the width of the size distribution (the smaller the PDI, the narrower is the distribution). The particle size was analysed by using the general purpose mode. The size given is the average of 10 subsequent measurements.

##### *3.2.2.2. Low angle static light scattering*

The Zetasizer Nano (for PCS–measurements) has an upper detection limit of 6 μm. In fact, particles > 6 μm are not reliably detected via this method. The advantage of low angle static light scattering, also known as laser diffraction (LD), is its broad measuring range (e.g. 20 nm – 2000 μm), enabling also the detection of larger particles besides a small sized bulk population. For the analysis a Mastersizer 2000, Malvern Instruments, UK) was used. The results were analyzed using the Mie theory (real refractive index: 1.59; imaginary refractive index: 0.01). The results are presented as median volume diameters (d(v)).

##### *3.2.2.3. Light microscopy*

An Orthoplan (Leitz, Germany) was used. Images were taken at a small magnification (160x), to detect possible larger particles in the systems and at a large magnification (1000x), to investigate the morphology of the fine crystals. To detect possible large crystals, the samples were also observed by using polarized light and a 160 fold magnification.

##### *3.2.2.4. Zeta potential*

The zeta potential was analysed using the Zetasizer Nano ZS (Malvern Instruments). Measurements were performed either in water (conductivity adjusted to 50 μS/cm) or in the original dispersion media (i.e. surfactant solution containing 1% (w/w) Plantacare 2000 with or without preservative). All measurements were performed at 25 °C and a field strength of 20 V/cm. The Zetasizer analyses the electrophoretic mobility of the particles, the conversion into the zeta potential was performed using the Helmholtz-Smoluchowski equation.

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