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Hydrophilic gels containing chlorophyllin-loaded liposomes: development and stability evaluation

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Received April 21, 2004, accepted October 18, 2004

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Pharmazie 60: 588–592 (2005)

The aim of this study was to develop and characterize hydrophilic gels containing chlorophyllin(CHL)-loaded liposomes as well as to evaluate their stability. Two different CHL-loaded liposome dispersions using non-hydrogenated and hydrogenated soybean lecithin were prepared, characterized for their particle size, polydispersity index and trapping efficiency and incorporated in Carbopol® 940 NF hydrogel. The gels obtained were analyzed for flow properties, pH values and CHL content. The control liposome-free gel was obtained by incorporating the CHL solution in the hydrogel. The stability of the gels was evaluated in terms of rheological properties, pH values and CHL content during 6 months' storage at 20 ± 2 °C. Suitable gel formulations for topical use were obtained revealing shear-thinning plastic flow behaviour without significant thixotropy during the whole period of examination. High yield values of the samples during the whole period indicated a long-term stability of the gel formulations. The gel formulations expressed a mild acid value acceptable for topical preparations. After 6 months' storage the CHL content was highest in the gel containing non-hydrogenated lecithin liposomes, followed by the gel containing hydrogenated lecithin liposomes and liposome-free gel, indicating that the encapsulation of CHL in liposomes led to a greater stability of CHL.

1. Introduction

Chlorophyllin (CHL) is a water-soluble derivative of chlorophyll which has been used for the treatment of abnormal human conditions without toxicity. CHL has been shown to be protective against a wide variety of direct- and indirect-acting mutagens (Kimm 1982; Dashwood 1994). CHL also attenuated the genotoxicity of some antitumor drugs, such as cyclophosphamide, bleomycin, doxorubicin and cisplatin (Gentile 1998). In addition to the antimutagenic activity, anticarcinogenic properties of CHL have been demonstrated (Guo 1995; Park 1995, 1996). Recent studies revealed that CHL can exert anticarcinogenic activities at different stages of skin tumor development and during the promotional stage can regress skin tumors previously produced. The results indicated that CHL can inhibit both tumor promotion and the progression of papillomagenesis in the two-stage mouse skin carcinogenesis induced by a certain mutagen and carcinogen (Chung 1999).
The purpose of this study was to prepare and characterize hydrophilic gels containing two different liposome dispersions of CHL. For liposome preparation Phospholipon 80 and Phospholipon 80 H were used. The obtained liposome dispersions were characterized for their vesicle size, polydispersity index and encapsulation efficiency. Since a disadvantage of using liposomes topically could be the liquid nature of the preparation, the liposomes were incorporated in Carbopol 940 gel. The gels containing liposomes and the control gel with the free substance, i.e. with incorporated CHL solution were analyzed for their flow properties, pH values and CHL contents. In order to evaluate the stability of the test gels, rheological parameters, the pH values and CHL contents were determined after 1, 3 and 6 months' storage at room temperature (20 ± 2 °C). In addition, the CHL content was also determined in the diluted liposome dispersions during their 6 months' storage at room temperature.

2. Investigations, results and discussion

The method employed allowed liposome dispersions of CHL (5% w/w) to be obtained using different phospholipids: the unsaturated phospholipid Phospholipon 80 (10% w/w) and the saturated phospholipid Phospholipon 80H (10% w/w). The results of particle size analysis (Table 1) indicate that the mean sizes of the vesicles were about 200 nm as well as that the liposomes prepared with Phospholipon 80 and Phospholipon 80H were of similar mean diameter, indicating that the degree of saturation of the phospholipids used did not have an influence on the mean size of the vesicles. Regarding the homogeneity, since the polydispersity index may have a value between 0 and 1, the low values of the polydispersity index of the liposome dispersions (Table 1) indicate homogenous populations of liposomes.

Table 1: Characterization parameters of liposome dispersions prepared with different phospholipids (mean ± SD, n = 3)

Formulation labels	Particle size (nm)	Polydispersity index	Encapsulation efficiency
LD1[a]	193.7 ± 2.5	0.24 ± 0.01	97.1 ± 0.1
LD2[b]	207.0 ± 1.3	0.22 ± 0.01	95.8 ± 0.3

[a] LD1 liposome dispersion of CHL prepared using Phospholipon 80
[b] LD2 liposome dispersion of CHL prepared using Phospholipon 80H

To deliver an amount of drug sufficient to provide a therapeutic effect, a high trapping efficiency of drug in liposomes is required. Using this procedure liposome dispersions of high trapping efficiency, close to 100% were obtained, regardless of the phospholipid type which was used, indicating that the degree of saturation of the phospholipid did not have a marked impact on the values of the trapping efficiency.
The obtained CHL liposome dispersions were incorporated in Carbopol 940 hydrogel (1 : 10) at room temperature resulting in formulations with 1% (w/w) phospholipids and 0.5% (w/w) CHL. The hydrogel formulation without liposomes was prepared under the same conditions, i.e. by incorporating CHL solution in the gel (1 : 10); the obtained CHL concentration was 0.5% (w/w). This was the desired CHL concentration, since a previous study demonstrated that topical application of 0.5% CHL can inhibit tumor promotion and progression of papillomagenesis in a two-stage mouse skin carcinogenesis induced by certain mutagens (Chung 1999).
All the gels tested were homogenous and of semisolid consistency as required for skin application. In addition, these gels were smooth, glossy and of an intense green colour.
The pH values of the gels (Table 2) were in the range of 6.76 ± 0.03 – 6.94 ± 0.01 indicating that all samples expressed a mild acid value acceptable for topical preparations.
The rheological behaviour of the gels was studied since it plays an important role in the mixing and flow characteristics of the materials, their packaging into containers, their physical stability and even patient acceptability. The flow data for the different gels obtained 48 h after preparation are shown in the Fig. 1 and in Tables 3–4. From the flow curves it was deduced that all samples showed non-Newtonian behaviour, since their viscosities were not constant, but changed as a function of the shear rate. All gel formulations showed a shear-thinning behaviour according to the Casson model (excellent fitting $R > 0.999$) with continuously decreasing viscosity, thus indicating successive loss of polymer entanglement upon increasing shear stress. Shear-thinning is a desirable property of semisolid dosage forms, since they should be "thin" during application and "thick" otherwise (Pena 1994). Since the gel formulations possessed yield values, they also showed plastic behaviour, indicating that the gel networks exhibit a resistance to an external force before they start flowing.

Table 2: pH Values of gels (mean ± SD), as function of time (n = 3)

Formulation labels	pH values			
	After 48 h	After 1 month	After 3 months	After 6 months
LG1[a]	6.94 ± 0.01	6.97 ± 0.01	6.89 ± 0.00	6.90 ± 0.01
LG2[b]	6.76 ± 0.03	6.74 ± 0.03	6.75 ± 0.01	6.73 ± 0.03
LG3[c]	6.91 ± 0.03	6.90 ± 0.01	6.83 ± 0.01	6.86 ± 0.01

[a] LG1 gel containing the CHL liposome dispersion prepared using Phospholipon 80
[b] LG2 gel containing the CHL liposome dispersion prepared using Phospholipon 80H
[c] LG3 gel containing the CHL solution in buffer

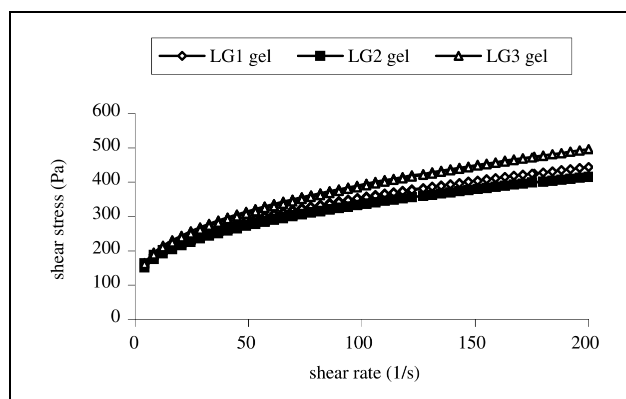

Fig.: Flow curves of the hydrogels LG1-LG3 obtained 48 h after preparation (initial flow curves)

To complete the evaluation of the gel formulations developed and keeping in mind their suitability for topical application, a long-term stability study was performed.
During the storage time the pH values remained almost unchanged (Table 2), indicating that neutralization of the polyacrylic acid by triethanolamine was complete as well as that no hydrolysis of the liposomal phospholipids in the liposomal gels occurred, which was very important for the long-term stability of the gel samples.
In order to investigate the stability of the samples stored for 6 months at room temperature (20 ± 2 °C), rheological measurements were performed after 1, 3 and 6 months' storage. The values of the rheological parameters, i.e. the values of the yield stress and the maximal apparent viscosity obtained after 1, 3 and 6 months' storage are presented in Tables 3–4. During 6 months' storage at room temperature the flow pattern of the gels LG1–LG3 did not change significantly, i.e. the flow curves of the gels obtained after 6 months' storage were quite similar to the initial flow curves presented in the Fig.

Table 3: Yield stress values of gels (mean ± SD), as function of time (n = 3)

Formulation labels	Yield stress values (Pa)			
	After 48 h	After 1 month	After 3 months	After 6 months
LG1[a]	50.8 ± 5.5	54.2 ± 4.0	55.0 ± 2.9	58.7 ± 3.6
LG2[b]	72.4 ± 1.4	71.6 ± 13.8	71.9 ± 37.2	82.9 ± 20.1
LG3[c]	36.6 ± 8.5	37.7 ± 3.0	45.5 ± 7.2	45.0 ± 10.6

[a] LG1 gel containing the CHL liposome dispersion prepared using Phospholipon 80
[b] LG2 gel containing the CHL liposome dispersion prepared using Phospholipon 80H
[c] LG3 gel containing the CHL solution in buffer

Table 4: Maximal apparent viscosities (at $4.08\ s^{-1}$) of gels (mean ± SD), as function of time (n = 3)

Formulation labels	Maximal apparent viscosities (Pas)			
	After 48 h	After 1 month	After 3 months	After 6 months
LG1[a]	39.5 ± 1.9	40.9 ± 0.3	41.2 ± 0.4	41.0 ± 0.6
LG2[b]	40.1 ± 1.8	43.2 ± 0.6	43.4 ± 1.0	44.5 ± 0.1
LG3[c]	40.5 ± 1.2	40.6 ± 1.4	46.8 ± 2.0	45.1 ± 0.7

[a] LG1 gel containing the CHL liposome dispersion prepared using Phospholipon 80
[b] LG2 gel containing the CHL liposome dispersion prepared using Phospholipon 80H
[c] LG3 gel containing the CHL solution in buffer

Thus, it was concluded that the gels maintained plastic behaviour which is appropriate for topical use. After 6 months' storage at room temperature the gels still displayed the properties of Casson type fluids.

Variations in rheological parameters were observed in all formulations during the examination period and can be explained by the structuration of the systems, which usually occurs after a couple of days or in some cases after a couple of months.

The yield stress can be used to evaluate the quality of a formulation and according to some authors it is the most reliable parameter for describing stability (Tamburić and Duncan 1995). For topical preparations it is desirable to posses the yield stress not only in terms of good stability but also because it describes the flow behaviour at small shear rates, i.e. before and after the application (when the formulation should not start flowing). From data presented in Table 3 it is evident that the yield stress of all gels increased during storage, but not significantly ($p < 0.05$). This can be explained by the structuration of the systems which yields a higher stability.

Regarding the values of maximal apparent viscosities, the formulation LG1 did not show a significant change ($p < 0.05$) in maximal apparent viscosity during storage for 6 months, indicating an optimal ratio between the hydrogel vehicle and the added liposome dispersion LD1, i.e. the addition of the liposome dispersion LD1 did not produce changes in the gel structure. Apparent maximal viscosity of the LG2 sample did not change significantly during the first 3 months. However, the value of apparent maximal viscosity of the sample LG2 obtained after 6 months' storage was significantly increased ($p < 0.05$) in comparison to the initial value, indicating a stronger gel structure of the aged sample LG2. A possible explanation for the difference in the rheological behaviour of the samples LG1 and LG2, i.e. for the increase in maximal apparent viscosity in the aged sample LG2, could be the significantly higher viscosity of the liposome dispersion LD2, which was added to the hydrogel vehicle in order to obtain the final gel sample LG2, in comparison to the viscosity of the liposome dispersion LD1.

The trend of increasing maximal apparent viscosity during storage was also observed for the liposome-free gel LG3, obtained after incorporation of the CHL solution in the hydrogel vehicle. However, the maximal apparent viscosity of the LG3 formulation was significantly increased already after 3 months. With elapsing the time of storage of up to 6 months this parameter remained increased, differing significantly from those obtained after 48 h and 1 months' storage ($p < 0.05$). This observation, which was unexpected and difficult to explain since the CHL solution was of smallest viscosity, indicated that ageing had an effect on the gel structure of the sample LG3 making the gel structure stronger.

During the storage time the CHL content decreased in all hydrogels. However, the CHL content was greater in the liposome hydrogels LG1 and LG2 than in the liposome-free hydrogel LG3 (Table 5) indicating that the encapsulation of CHL in liposomes led to a greater stability of CHL. The results obtained were in accordance with the results of a previous investigation, which demonstrated higher stability of retinol-palmitate in a liposomal carbomer gel than in a conventional carbomer gel (Arsić 1997). The CHL content was greater in the LG1 gel, obtained by incorporating the liposome dispersion LD1 – prepared using the non-hydrogenated soybean lecithin (Phospholipon 80) – in the Carbopol 940 gel, than in the LG2 gel,

Table 5: Contents of CHL in the hydrogels and the diluted liposome dispersions (mean ± SD), as function of time (n = 3)

Formulation labels	Contents of CHL (% of the initial content)			
	Initial content	After 1 month	After 3 months	After 6 months
LG1[a]	100	85.2 ± 0.3	73.2 ± 0.5	71.1 ± 0.5
LG2[b]	100	80.5 ± 0.6	69.7 ± 0.2	62.6 ± 0.4
LG3[c]	100	73.9 ± 0.2	61.0 ± 0.3	57.2 ± 0.4
DLD1[d]	100	84.7 ± 0.5	65.2 ± 0.1	60.5 ± 0.3
DLD2[e]	100	70.3 ± 0.1	62.2 ± 0.3	54.8 ± 0.6

[a] LG1 gel containing the CHL liposome dispersion prepared from Phospholipon 80
[b] LG2 gel containing the CHL liposome dispersion prepared from Phospholipon 80H
[c] LG3 gel containing the CHL solution in buffer
[d] DLD1 diluted LD1 liposome dispersion of CHL prepared from Phospholipon 80
[e] DLD2 diluted LD2 liposome dispersion of CHL prepared from Phospholipon 80H

obtained by incorporating the liposome dispersion LD2 – prepared using the hydrogenated soybean lecithin (Phospholipon 80H) – in the same gel vehicle. Also greater stability of CHL in the liposome dispersion DLD1 than in the liposome dispersion DLD2 (Table 5) was observed, both prepared by diluting the original liposome dispersions LD1 and LD2 with phosphate buffer saline pH 6.5 in order to achieve the concentration of CHL used in the gels. The lipid membranes of the liposomes DLD1, made of non-hydrogenated phospholipids, were in the fluid-state in contrast to the gel-state (rigid) lipid membranes of the liposomes DLD2, made of hydrogenated phospholipids. CHL since it has hydrophobic (vinyl, pyrrol and alkyl groups) groups, immerses in the phospholipid membranes of the liposomes. CHL immerses deeper in the fluid membranes than in the rigid ones and is hence better protected from degradation in the fluid-state liposomes of the dispersion DLD1 than in the liposomes of the dispersion DLD2.

Higher stability of CHL was demonstrated in the liposomal gels than in the liposome dispersions. This observed phenomenon was expected since immobilization of liposomes in a polyacrylic gel vehicle leads to higher physical stability of liposomes (Thoma and Jocham 1992), which may result in higher stability of the encapsulated substances (Arsić 1999).

In conclusion, this study showed the technological feasibility of the development of hydrogels containing CHL-loaded liposomes. The results obtained have shown that all samples exhibited non-Newtonian plastic flow without significant thixotropy in both fresh and aged states, i.e. all samples showed a similar flow pattern after preparation and after 6 months' storage. Regarding the rheological parameters it has been shown that the duration of storage had an influence on the maximal apparent viscosity of the samples LG2 and LG3, i.e. the values obtained after 6 months' storage were higher than the initial values. The increase in the maximal apparent viscosities after 6 months' storage of the gels was not marked, since the values were higher for approximatly 10% in comparison to the initial values. Since the yield stress can be used to evaluate the stability of a formulation, high yield stress values during the whole period of evaluation indicated a long-term stability of all gels. The CHL content decreased during storage in all hydrogel formulations. However, the CHL content after 6 months' storage was higher in the liposome hydrogels than in the liposome-free gel, being highest in the gel containing non-hydrogenated phospholipid liposomes, indicating the potential of liposomes to increase the stability of encapsulated substances. Keeping in

mind all the observed phenomena it was concluded that the gel containing non-hydrogenated phospholipid liposomes was the most physically and chemically stable gel formulation, since it showed the smallest changes in rheology and CHL content during 6 months' storage at room temperature.

3. Experimental

3.1. Materials

For the preparation of liposomes and topical gel formulations the following materials were used: non-hydrogenated soybean lecithin (Phospholipon® 80, Nattermann, Germany), hydrogenated soybean lecithin (Phospholipon® 80H, Nattermann, Germany), copper complex of 1,5,8-trimethyl-3-formyl(b)-4-ethyl-2-vinyl-9-keto-10-carbomethoxy-phorbinyl-7-propionate, disodium (Copper-chlorophyllin®, Dr. Marcus GmbH, Germany), Carbomer 940 (Carbopol® 940 NF, BFGoodrich, USA), phenoxyethanol (and) methylparaben (and) ethylparaben (and) propylparaben (and) butylparaben (Phenonip®, NIPA, USA), diazolidinyl urea (Germall®II, ISP Europe, UK), propylene glycol (BASF, Germany), kaliumdihydrogenphosphat (Sigma-Aldrich, Germany), sodium hydroxide (Sigma-Aldrich, Germany), triethanolamine (Sigma, USA), edetate disodium (Sigma, USA). All other chemicals were of analytical grade and the water used was double distilled.

3.2. Liposomes preparation and characterization

3.2.1. Preparation of chlorophyllin-loaded liposomes and the chlorophyllin solution

Liposomes were prepared by the following method. First, the CHL solution was prepared by dissolving CHL (5% wt) in phosphate buffer saline pH 6.5 and adding Phenonip (0.5% wt). This solution was vortexed for 1 min and then sonicated for 5 min. Phospholipon 80 (10% wt) was added at 30 °C to the CHL solution, while the Phospholipon 80H (10% wt) previously mixed with ethanol (in order to maintain the same composition of the dispersions since Phospholipon 80 is available on the market as a mixture with ethanol) was added to the CHL solution previously warmed at 65 °C, under agitate stirring (10000 rpm, 15 min) using the Ultra-Turrax T 25 mixer (Ika, Labortechnik, Germany). The liposome dispersion prepared using the Phospholipon 80 was labeled as liposome dispersion LD1, while the liposome dispersion prepared from Phospholipon 80H was labeled as liposome dispersion LD2.
These spontaneously formed multilamellar vesicles (MLV) were pressed through a 200 nm size pore polycarbonate membrane to get unilamellar liposomes with the help of Mini Extruder Lipofast extrusion device (Avestin Ottawa, Canada).
The CHL solution was prepared by dissolving CHL (5% wt) in phosphate buffer saline pH 6.5.

3.2.2. Liposomal size and polydispersity index

The diameter of vesicles and the polydispersity index were determined by photon correlation spectroscopy using the Zetamaster S (Malvern Instruments, UK). Samples were diluted with distilled water prior to the measurements. The diameter was calculated from the autocorrelation function of the intensity of light scattered from particles, assuming a spherical form of particles. The polydispersity index (PI) was used as a measure of a unimodal size distribution, which ranges from 0 (homogenous dispersion) to 1 (high heterogeneity).

3.2.3. Determination of trapping efficiency of CHL and CHL content in the samples

The trapping efficiency of the drug in liposomes was determined indirectly, on the basis of free CHL in the supernatant after dilution of the liposome dispersions with phosphate buffer saline pH 6.5 (1 : 10) and centrifugation (1 h, 35000 rpm/min, Beckman Ultracentrifuge L 7–55, Beckman, USA). The percentage of drug encapsulated (trapping efficiency) was calculated from the ratio of CHL in the liposomes, which was determined from the difference between the total and the free CHL concentration, to the total amount of CHL in the aqueous suspension. The concentration of CHL, both the total concentration and free, was determined spectrophotometrically (405 nm, Cintra 20 GBC Spectral, USA).
The concentration of CHL in the final gels and the diluted liposome dispersions was also determined spectrophotometrically.

3.3. Preparation and characterization of CHL gels

3.3.1. Preparation of the 0.5% CHL gels

As a vehicle for the incorporation of liposomes Carbopol 940 was used, since the compatibility of liposomes with acrylic acid polymers has been proven (Foldovari 1996). The gel (1% w/w) was prepared by the following procedure: Carbopol resin (1%) was dispersed in distilled water in which propylene glycol (5%), edetate disodium (0.05%) and the preservatives (Phenonip 0.2% and Germal II 0.3%) were previously added and left to stay over night. The mixture was stirred the next day (500 rpm/min, 5 min) until the Carbopol resin was homogeneously dispersed and then neutralized by dropwise addition of 10% (w/w) triethanolamine, until a transparent gel appeared.
Liposomes were incorporated into Carbopol 940 gel by mixing them into the gel by an electrical mixer at 200 rpm/min 5 min (Heidolph RZR 2020, Germany). The concentration of liposomes achieved in the gel was 10% (w/w, liposomal suspension/total), the phospholipio concentration was 1% (w/w), while the concentration of CHL was 0.5% (w/w). The control gel (Carbopol gel mixed with the CHL solution in buffer instead of liposomes) was prepared under the same conditions i.e. the concentration of CHL in all gels was 0.5% w/w.
The gel obtained by incorporation of the liposome dispersion LD1 in the hydrogel was labeled as LG1, the gel obtained by incorporation of the liposome dispersion LD2 was labeled as LG2 and finally the liposome-free gel obtained by incorporation of the CHL solution in the hydrogel was labeled as LG3. The composition of the gels LG1-LG3 is presented in Table 6.

Table 6: Composition of the model hydrogels LG1–LG3

Ingredients (% w/w)	Formulations		
	LG1	LG2	LG3
Carbomer (Carbopol® 940)	1.0 g	1.0 g	1.0 g
Diazolidinyl Urea (Germall® II)	0.3 g	0.3 g	0.3 g
Phenoxyethanol (and) Methylparaben (and) Ethylparaben (and) Propylparaben (and) Butylparaben (Phenonip®)	0.2 g	0.2 g	0.2 g
Propylene Glycol	5.0 g	5.0 g	5.0 g
Edetate disodium	0.05 g	0.05 g	0.05 g
Triethanolamine sol. 10%	1.35 g	1.35 g	1.35 g
Chlorophyllin sol. 5%	–	–	10.0 g
LD1 [a]	10.0 g	–	–
LD2 [b]	–	10.0 g	–
Purified water ad	100.0 g	100.0 g	100.0 g

[a] $LD1_{CHL}$ liposome dispersion of CHL prepared using Phospholipon 80
[b] $LD2_{CHL}$ liposome dispersion of CHL prepared using Phospholipon 80H

3.3.2. pH Determination of the gels

The pH values were determined directly in gels at room temperature (HI 8417, Hanna Instruments, USA) and served to evaluate the chemical stability.

3.3.3. Rheological evaluation of gels containing liposomes

A Rheometer (Rheolab MC 120, Paar Physica, Stuttgart, Germany) was used to determine flow properties of fresh (48 h old) Carbopol gels with incorporated liposomes. Measurements were performed at 20 ± 0.1 °C using the cone/plate MK 22 (radius of measuring cone 25 nm, angle of measuring cone 1°) measuring system. Continuous flow tests were carried out by increasing the shear rate from 0 to 200 s^{-1} and decreasing it back to 0 s^{-1}, each stage lasting 200 s. Under the same conditions the flow properties of fresh liposome-free control gels were examined. Three measurements were performed for each sample and the mean value was calculated.

3.4. Stability study of CHL hydrogels

After preparation, the hydrogels both with and without liposomes, were placed into glass opaque containers, tightly closed and stored at room temperature (20 ± 2 °C). For each formulation, the pH value, rheological parameters and CHL content were measured after 1, 3 and 6 months' storage at room temperature as described previously in 3.3.2, 3.3.3. and 3.2.3. Physical evaluation of the samples' stability was carried out by visual inspection and the aforementioned rheological tests, while the chemical stability was evaluated by pH determination.

3.5. Statistical analysis

Statistical analysis was carried out using One-Way Analysis of Variance. Significant differences were determined at $p < 0.05$.

Acknowledgements: The authors would like to thank Dr. Gabriele Blume and Michael Sacher from ROVI GmbH (Schlüchtern, Germany) for their help and support.

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