

Skin efficacy of liposomes composed of internal wool lipids rich in ceramides

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Synopsis

Ceramides from intercellular lipids of skin stratum corneum are known to play an essential role in maintaining and structuring the lipid barrier of the skin. Internal wool lipids (IWL), which are also rich in ceramides, have a composition similar to that of the stratum corneum lipids. IWL extracted with chloroform/methanol azeotrope at the laboratory scale have been shown to be capable of forming liposomes with a stable bilayer structure. Furthermore, topical application of these IWL liposomes on intact and compromised skin has been demonstrated to improve barrier skin properties.

In this study we evaluated the effect on human skin repair of different IWL extract compositions obtained by two extraction methodologies. The formation and characteristics of the liposomes prepared were greatly influenced by the IWL composition, primarily the sterol sulfate content. The IWL liposomes improved skin barrier integrity and increased skin hydration when applied onto intact skin. These improvements were slightly enhanced in the case of IWL liposomes that were richer in polar lipids.

INTRODUCTION

Wool is a natural fiber that is mainly comprised of protein. It contains external lipids (lanolin) and a small amount of internal lipids (1.5%). Internal wool lipids (IWL) arouse considerable interest given their high proportion of ceramides. IWL are rich in cholesterol, free fatty acids, cholesteryl sulfate, and ceramides; and they resemble those from membranes of other keratin tissues such as human hair or stratum corneum (1–4).

Intercellular lipids of stratum corneum, mainly ceramides, play an important role in the barrier function of the skin by preventing penetration of external agents and controlling transepidermal water loss to maintain the physiological skin water content (5). Recent studies have shown that formulations, especially ceramide supplementation, containing lipids that resemble the natural components of the skin can improve compromised skin conditions (6,7).

IWL have been extracted from wool on account of their high proportion of ceramides. IWL extracted by Soxhlet extraction at the laboratory scale has been shown to be capable

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of forming liposomes with a stable bilayer structure (8,9). This vesicular structure, which mimics the organized lipid structures of the stratum corneum, offers a suitable strategy for achieving an accurate vehiculization of a particular compound and for incorporating additional lipid content that may reinforce the barrier function of the skin (10–12). Furthermore, earlier studies (13,14) have demonstrated the ability of these IWL liposomes to improve skin barrier properties when applied onto intact and compromised skin. Slightly better results have been obtained with these liposomes, containing a mixture of natural ceramides, when compared with stratum corneum liposomes (modeling stratum corneum lipids using synthetic lipid mixtures), which had only one type of ceramide present in the formulation. Accordingly, IWL could be regarded as a new natural extract that is beneficial to topical application and suitable for incorporation into pharmaceutical or cosmetic formulations in the treatment and care of skin (15).

Therefore, IWL have been extracted at the pilot plant scale both by organic solvent extraction (OSE) using methanol or acetone (16) and by supercritical fluid extraction (SFE) with CO₂, using 10% methanol or ethanol as polarity modifiers (17). In the present work, liposomes containing IWL extracts with different lipid compositions were formed. Vesicular diameter, polydispersity index, and stability were determined. The efficacy of these IWL liposomes when applied topically onto intact skin on a long-term basis was studied. To this end, *in vivo* changes in transepidermal water loss (TEWL) were measured as an index of barrier repair, whereas the water-holding capacity was measured as changes in skin capacitance. Finally, the protection of intact skin against detergent action was evaluated after topical application of the IWL liposome samples by measuring the aforementioned parameters.

MATERIALS AND METHODS

EXTRACTION PROCEDURES

Raw Spanish Merino wool samples supplied by SAIPEL (Terrassa, Spain) were used for lipid extraction. Prior to the extraction, raw wool was industrially cleaned following a procedure previously described (16). IWL were extracted at the pilot plant scale by OSE using methanol. The extraction procedure consisted of a pump-forced reflow system. Five kilograms of wool were extracted for four hours at 56°C (16). IWL were also obtained at the pilot plant scale by SFE using a CSFF (Iberfluid Instruments/ICP-CSIC, Spain). Fifty grams of wool were extracted with CO₂ at 60°C and 160 atm using 10% methanol or ethanol as polarity modifiers of CO₂ (17). IWL extracts obtained at the pilot plant scale were concentrated and stored in chloroform/methanol (2:1, v/v) at –20°C until their analysis.

LIPID ANALYSIS

The quantitative analysis of the samples was performed by thin-layer chromatography coupled to an automated flame ionization detector (TLC-FID), an Iatroscan MK-5 analyzer (Iatron, Tokyo, Japan). Samples were applied on silica gel S-III Chromarods using an SES (Nieder-Olm, Germany) 3202/15-01 sample spotter. The determination of the composition was made using an optimized TLC-FID protocol to analyze lipid content (16). The rods were developed initially to a distance of 10 cm with n-hexane/diethyl ether/formic acid (53:17:0.3, by vol) to separate apolar and polar lipids. After a partial

scan of 85% to quantify and eliminate the apolar lipids, a second development, again to a distance of 10 cm, was performed with chloroform/n-hexane/methanol/acetone (55:5:3:7, by vol) to separate the ceramides. Following a partial scan of 85% to quantify and eliminate the ceramides, a third development, again to a distance of 10 cm, was performed with chloroform/methanol/formic acid (57:12:0.3, by vol) to separate and quantify, after a total scan of 100%, the glycosilceramides and sterol sulfate. After each elution, the rods were heated for 5 min at 60°C to dry the remaining solvent.

LIPOSOME FORMATION AND EVALUATION

IWL liposomes were prepared with the three extracts by evaporating the organic solvent to dryness under a stream of dry nitrogen to form a thin film on the flask. The film was hydrated with 0.9% NaCl solution to give a final lipid concentration of 2% for each extract. Liposomes were formed by sonication of the suspension in a sonicator, a Labsonic 1510 (B. Braun, Melsungen, Germany), at 100W for about 15 min, with the temperature maintained at 65°C.

Evaluation of vesicle size distribution and the polydispersity index of liposomes was carried out at 25°C by dynamic light scattering, employing an Autosizer IIc photon correlation spectrometer (Malvern Instruments Limited, Malvern, UK). Samples were diluted from 20 to 0.1 mg/ml. Quartz cuvettes were filled with the samples, and all the experiments were thermostatically controlled (25°C). The samples were measured at a scattering angle of 90°. Data thus obtained were analyzed using a version of the program CONTIN provided by Malvern Instruments.

SUBJECTS

Nine healthy Caucasian volunteers (all females), phototype (Fitzpatrick skin type) III–IV with a mean age of 29.9 ± 5.0 years (range 24–39 years), participated in all studies. All the subjects were advised to avoid the use of topical drugs or moisturizers on the volar forearm for one week prior to the experiments. To obtain reliable measurements, the volunteers were acclimatized for 15 min in a conditioned room (20°C, 60% RH) before the experiments.

NON-INVASIVE BIOPHYSICAL MEASUREMENTS

The effect of topically applied liposomes on skin properties was evaluated by non-invasive biophysical techniques. TEWL is a sensitive index of skin barrier integrity. This parameter evaluates the water loss in $\text{g}/\text{m}^2\text{h}$, measured using the Tewameter™ 210 (Courage & Khazaka, Cologne, Germany). Moreover, skin hydration was determined using a Corneometer CM 85 (Courage & Khazaka), which measures skin capacitance in arbitrary units (AU). Both parameters were recorded in accordance with established guidelines (18–21).

EFFICACY OF LIPOSOMES ON INTACT HUMAN SKIN

A long-term study was performed to test the effect of the liposome solutions when applied repeatedly to intact skin. Baseline measurements of TEWL and skin capacitance

were taken before 10 μ l of the solutions was topically applied onto five marked zones of the volar forearm: three zones for topical treatment (the three IWL liposome samples), one zone for the placebo solution (0.9% NaCl solution), and one untreated zone (control). The placebo and liposome solutions were randomly applied (10 μ l) onto marked areas of 4 cm² using an Exmire microsyringe (ITO Corp., Fuji, Japan). After 24 h (day 1), both biophysical parameters, TEWL and skin capacitance, were evaluated and then 10 μ l of the solutions was applied again. The procedure was repeated once daily for two more days and the parameters were measured (days 2, 3, and 4).

PROTECTION OF INTACT HUMAN SKIN AGAINST DETERGENT ACTION

A test was performed to evaluate the protective effect of IWL liposomes applied to intact skin followed by sodium lauryl sulfate (SLS) exposure. The baseline measurements of TEWL and skin capacitance were taken, in this study, in the five treated areas of the volar forearm of the volunteers submitted to the efficacy study described above. The five zones were then exposed to a 2% SLS aqueous solution for two hours (see SLS exposure), and the resultant irritation reaction was assessed 2.5 hours after SLS exposure by measuring TEWL and skin capacitance (22).

SLS EXPOSURE

SLS was chosen as a surfactant to provoke a chemical disruption of healthy skin since it is frequently present in detergent-based products of daily use such as household or body cleanser products. Ten microliters of an aqueous solution of 2% SLS was pipetted onto a layer of filter paper placed in each of several aluminium chambers (d=12 mm, large Finn chambers, Epitest Oy, Finland). The chambers were fixed to the skin for two hours with adhesive tape. Upon removal of the patch, the skin was gently rinsed with water and allowed to dry.

DATA TREATMENT

The mean values were calculated and Dixon's test was used to detect outliers, which were excluded from the data. The parameters were normalized, with each value divided by the baseline value. Parameter changes in the figures were simultaneously evaluated versus control and placebo values. ANOVA variance analysis was used to determine significant differences between values obtained from different treatments (significance level accepted: $p < 0.05$) using the Statgraphics program.

RESULTS AND DISCUSSION

LIPID EXTRACTION AND ANALYSIS

Raw Spanish Merino wool was extracted at the pilot plant scale in order to obtain a lipid extract rich in ceramides. Merino wool from Spain was used because its internal lipid composition resembles that found in the skin stratum corneum (23). The extraction was

performed by OSE with pure methanol and by SFE with CO₂, using 10% methanol or ethanol as polarity modifiers, at the conditions described in the experimental procedure section (16,17). These solvents were chosen as extractors to avoid the use of chlorinated solvents, which were previously used at laboratory scale (23).

Quantitative analysis of the three different lipid extracts was carried out by TLC-FID following the methodology previously reported (16). The different lipid families were quantified as sterol esters (ST-ES), free fatty acids (FFA), sterols (ST), ceramides and glycosilceramides (CER), and sterol sulfate (ST-SUL). The amount of these components analyzed, expressed in percentages of total lipid analyzed (% o.l.a.), is shown in Figure 1.

Comparison of the three extracts obtained at the pilot plant scale showed little variation in the percentage of the three main lipid families (free fatty acids, sterols, and ceramides). However, it should be noted that the percentage of sterol sulfate for the extract obtained by OSE with pure methanol (about 10%) is much higher than the sterol sulfate content in the two other extracts obtained by SFE (about 1%).

LIPOSOME CHARACTERISTICS AND STABILITY

IWL extracted with chloroform/methanol azeotrope by Soxhlet extraction at laboratory scale, whose composition resembles that in the stratum corneum, have been demonstrated to be capable of forming liposomes with a stable bilayer structure (8,9). In the present work, liposomes were formed using the different IWL extracts obtained at the pilot plant scale by evaporating the organic solvent to dryness, hydrating with 0.9% NaCl solution, and by sonication as detailed in the Experimental section. Liposomes were easily formed with the IWL extract obtained by OSE with pure methanol. However, some lipids remained as aggregates in the flask when liposomes were formed with the IWL extracts obtained by SFE using 10% methanol or ethanol as modifiers. These aggregates, analyzed by TLC-FID, were richer in free fatty acids and ceramides, whereas sterol and sterol sulfate were practically absent. Figure 2 shows the lipid composition of liposomes really

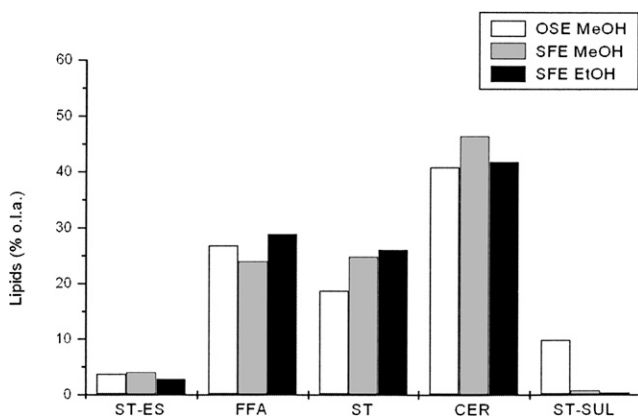


Figure 1. Amount of sterol esters (ST-ES), free fatty acids (FFA), sterols (ST), ceramides and glycosilceramides (CER), and sterol sulphate (ST-SUL) quantified in the three different IWL extracts, expressed as percentages of the total lipid weight analyzed (% o.l.a.).

formed with the three wool lipid extracts, expressed in percentages of total lipid analyzed (% o.l.a.)

The increase in percentage of sterol in the two extracts obtained by SFE should be pointed out. As a consequence, percentages of free fatty acids and ceramides in these SFE extracts decreased. Therefore, the composition of liposomes prepared with IWL extracted by OSE became richer in polar lipids such as ceramides, glycosilceramides, and sterol sulfate.

The ceramides alone are known to be insufficient for bilayer formation, perhaps because they are not ionized at physiological pH. However, free fatty acids and cholesteryl sulfate, which are also present in stratum corneum lipids, are ionized at physiological pH and their presence together with ceramides is necessary for bilayer formation (24). Therefore, the fact that the formation of liposomes with lipids extracted by SFE is more complicated than that of liposomes with lipids extracted by OSE may be due to the lower amount of sterol sulfate contained in lipids extracted by SFE (Figure 1). Besides, the composition of lipids extracted by OSE using pure methanol is very similar to that obtained with laboratory-scale Soxhlet extraction using chloroform/methanol azeotrope, which has been demonstrated to form stable liposomes (8,9).

The vesicle size distribution and polydispersity index of liposomes were determined by dynamic light scattering in order to characterize these liposomes and to study their stability for 14 days. The results are indicated in Table I.

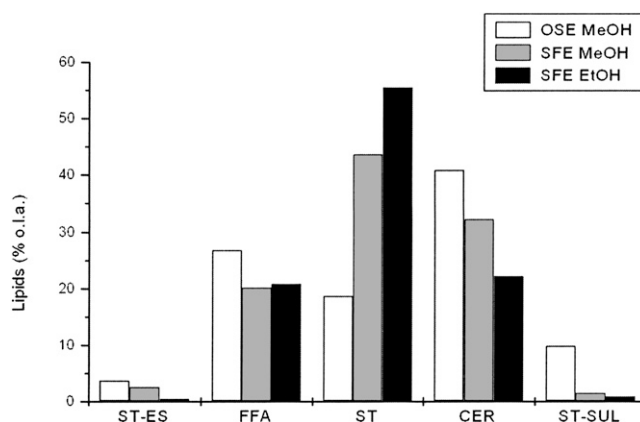


Figure 2. Amount of sterol esters (ST-ES), free fatty acids (FFA), sterols (ST), ceramides and glycosilceramides (CER), and sterol sulphate (ST-SUL) quantified in the three different IWL liposome samples, expressed as percentages of the total lipid weight analyzed (% o.l.a.).

Table I
Characteristics and Stability of Liposomes: Vesicular Diameter (d) and Polydispersity Index (P.I)

Extract	Day 0		Day 7		Day 14	
	d (nm)	P.I.	d (nm)	P.I.	d (nm)	P.I.
OSE MeOH	173.2	0.284	186.4	0.231	189.7	0.256
SFE MeOH	233.8	0.573	266.1	0.556	297.8	0.711
SFE EtOH	298.7	0.620	610.6	0.728	636.8	0.756

The characteristics of IWL liposomes were determined one hour after vesicle preparation (day 0). A smaller vesicle diameter and a lower polydispersity index were measured for liposomes formed with IWL extracted by OSE rather than by SFE. Moreover, higher values of both parameters were detected for liposomes formed with extracts obtained by SFE using ethanol as a modifier instead of methanol.

The stability results show that liposomes prepared from lipids extracted with OSE using pure methanol are stable for 14 days because the vesicular diameter and polydispersity index are maintained throughout the study. However, an increase in the vesicular diameter was detected in liposomes formed with lipids extracted by SFE. This increase is more significant when the lipids used were extracted with 10% ethanol (from 298.7 nm to 636.8 nm 14 days after their formation) than with 10% methanol (from 233.8 nm to 297.8 nm 14 days after their formation).

This difference in behavior of liposomes prepared with IWL obtained by OSE using methanol and SFE using 10% methanol or ethanol as modifiers may be due to the higher percentage of free fatty acids and sterol sulfate present in the IWL liposomes formed with lipids extracted by OSE using pure methanol (Figure 2). Moreover, the concentration of free fatty acids, sterols, and ceramides is more equimolar in OSE than in SFE liposome composition. It is well known that the intercellular lipid domain of the stratum corneum is composed of roughly equimolar concentrations of these components and that this equilibrium is fundamental for membrane-forming lipids (25).

EFFICACY OF LIPOSOMES ON INTACT HUMAN SKIN

A long-term study was performed on intact skin to determine the efficacy of the three IWL liposome samples. Evaluation of TEWL and skin capacitance was performed 24 hours after daily application during the treatment period (days 1, 2, 3, and 4). Figures 3 and 4 show the variation in skin capacitance and TEWL, respectively.

The results of skin capacitance measurement indicate that there is a trend of increased hydration for the three IWL liposome samples (Figure 3). This is more marked in the case of liposomes prepared with lipids extracted by OSE using methanol. The skin capacitance change is about 5% at the end of the treatment period despite not being statistically significant. As for the TEWL parameter, the results show a consistent trend towards improving skin barrier integrity in the zones treated with the three IWL liposome samples (Figure 4). This parameter undergoes a significant decrease of about 10% for almost all measurements during the treatment period. These findings agree with the results obtained in earlier work (13,14), in which IWL liposomes, with a composition similar to that of stratum corneum lipids, when applied onto skin reinforce skin barrier integrity and increase its water-holding capacity.

In Figures 3 and 4 it can be observed that the zones treated with the IWL liposome sample prepared with lipids extracted by OSE using pure methanol yield lower TEWL values and higher skin capacitance values than the IWL liposome samples formed with lipids obtained by SFE with methanol and even more with ethanol. This could be due to the different lipid composition and to the smaller size of the liposomes formed with lipids extracted by OSE using methanol. These smaller liposomes could, therefore, penetrate the stratum corneum more easily and could modify the lamellar bilayer on which the barrier function depends.

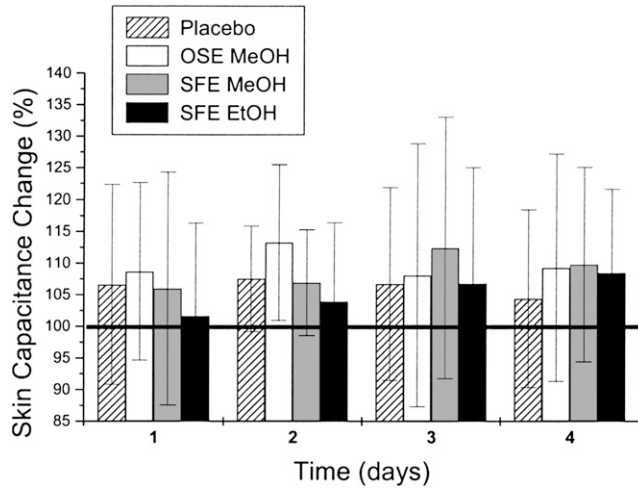


Figure 3. Variation in skin capacitance after sample application during the treatment period ($*p < 0.05$, calculated between samples and placebos). Changes were evaluated versus baseline and control values.

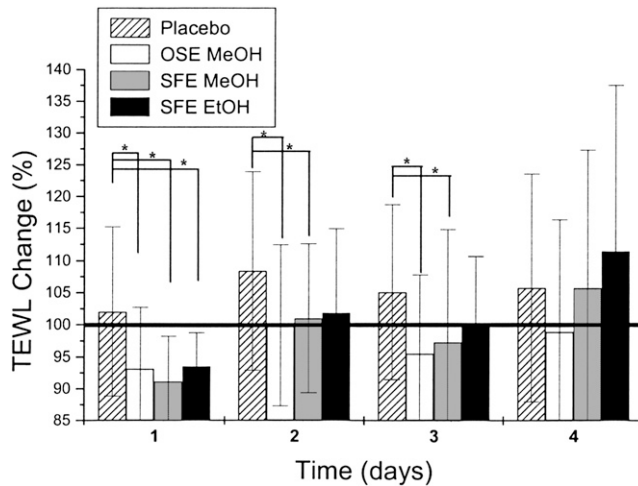


Figure 4. Variation of TEWL after sample application during the treatment period ($*p < 0.05$, calculated between samples and placebos). Changes were evaluated versus baseline and control values.

PROTECTION OF INTACT HUMAN SKIN AGAINST DETERGENT ACTION

In order to evaluate the protection of healthy human skin against detergent action, an irritant reaction was caused by SLS exposure after the application of IWL liposome samples in the previous long-term study. Biophysical parameters were measured before and after SLS exposure to provide data on the possible reinforcement and protection of the lipid barrier due to exogenous lipid application.

As for skin capacitance, the results do not show significant differences in the zones treated with IWL liposome samples with respect to untreated and placebo-treated zones. However,

SLS exposure has a much greater effect on TEWL (Figure 5), which reflects the skin barrier function. TEWL values decrease in the zones treated with the three IWL liposome samples with respect to control and placebo zones, which is indicative of the recovery of the skin barrier function. The variation in TEWL after SLS exposure in the zones treated with liposomes formed with IWL extracted by SFE is significant, with a decrease of about 10% in TEWL. This effect could be attributed to the larger size of the liposomes prepared with lipids extracted by SFE. These liposomes could remain in the external layers of the stratum corneum, thereby preventing some of the damaging effects of the surfactant insult. Moreover, the larger amount of sterol present in liposomes formed with SFE extracts could induce a decrease in the fluidity of the lipid bilayer (26), which could lead to decreased penetration of these liposomes in deeper skin layers.

In summary, different lipid mixtures extracted from wool fibers have been demonstrated to form stable liposomes. Their application onto skin increases skin hydration and improves skin barrier integrity. Therefore, wool lipid mixtures could provide a new approach to treatments of skin pathologies characterized by structural alterations in the stratum corneum, resulting in a loss of barrier function (25).

CONCLUSIONS

IWL extracted by OSE using pure methanol were richer in sterol sulfate than IWL extracted by SFE with 10% methanol or ethanol as modifiers, whereas a similar percentage of the three main lipid families (free fatty acids, sterols, and ceramides) was analyzed in the three IWL extracts. Both the formation and the characteristics of the liposomes prepared were considerably influenced by the IWL composition. The lower amount of sterol sulfate contained in lipids extracted with SFE hinders the formation of liposomes. Liposomes prepared from IWL extracted by OSE were richer in polar lipids such as ceramides, glycosilceramides, and sterol sulfate. These liposomes presented a smaller vesicular size,

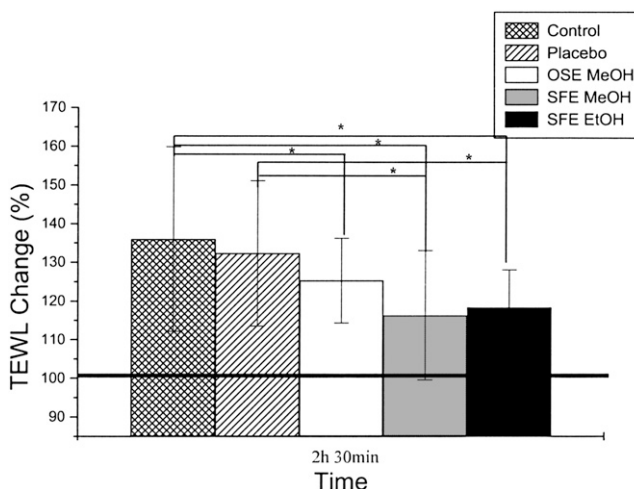


Figure 5. Variation of TEWL after SLS exposure. The intact skin was previously treated with IWL liposomes ($*p < 0.05$, calculated between samples and control and placebos). Changes were evaluated versus baseline values (SLS-treated skin).

a lower polydispersity index, and greater stability than liposomes formed with IWL obtained by SFE, which were richer in sterol.

The modification of the properties in intact skin after daily application of IWL structured as liposomes was investigated. The IWL liposomes improved skin barrier integrity and increased skin hydration when applied onto intact skin. These results were slightly enhanced when IWL liposomes richer in polar lipids were applied. Moreover, protection of intact skin against detergent action was confirmed for the three samples, the results being slightly better in the case of IWL liposomes that were richer in sterol. These results support the beneficial effects of skin lipid supplementation given that IWL resemble those in the stratum corneum both in composition and in organization.

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