

Natural moisturizing factors (NMF) in the stratum corneum (SC). I. Effects of lipid extraction and soaking

MARISA ROBINSON, MARTY VISSCHER, ANGELA LARUFFA,
and RANDY WICKETT, *The James L. Winkle College of Pharmacy,
University of Cincinnati, 3225 Eden Avenue, Cincinnati, OH 45267
(M.R., R.W.), and Skin Sciences Institute, Cincinnati Children's Hospital
Medical Center, 3333 Burnet Avenue, Cincinnati, OH 45229 (M.V., A.L.R.)*

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Synopsis

Natural moisturizing factor (NMF) is essential for appropriate stratum corneum hydration, barrier homeostasis, desquamation, and plasticity. It is formed from filaggrin proteolysis to small, hygroscopic molecules including amino acids. We hypothesized that common lipid extraction and soaking in water would alter the level of NMF in the upper SC and its biophysical properties. A novel method of measuring and quantifying the amino acid components of NMF is presented. Adhesive tapes were used to collect samples of the stratum corneum (SC) and were extracted with 6mM perchloric acid for analysis by reverse-phase HPLC. HPLC results were standardized to the amount of protein removed by the tapes. An increase in NMF was found with increased SC depth. Also, the combination of extraction and soaking was found to increase NMF loss relative to control or to extraction or soaking alone. Our results indicate that common skin care practices significantly influence the water binding materials in the upper SC. The findings have implications for the evaluation and formulation of skin care products.

INTRODUCTION

Natural moisturizing factor (NMF) is important for maintaining proper moisture levels in the stratum corneum. It is a degradation product of filaggrin, a histidine-rich protein found in the upper layers of the epidermis. Filaggrin aggregates keratin filaments in the cells of the stratum granulosum to form the keratin bundles responsible for the rigid structure of the cells of the stratum corneum (SC). In the stratum corneum, filaggrin is degraded into a number of low-molecular-weight, hygroscopic molecules such as urea, pyrrolidone carboxylic acid (1,2), glutamic acid, and other individual amino acids, which are collectively referred to as the natural moisturizing factor of the skin (NMF). Filaggrin degradation to NMF has been shown to relate to ambient humidity levels, with an optimum humidity range for NMF production of 80–95% (3). The role of NMF and its optimum levels *in vivo* are not well known, although it has been observed experimentally that filaggrin levels are lower in the skin of patients with atopic dermatitis, even in non-lesional areas (1,2,4-6).

Various investigators have linked mutations in filaggrin-encoding genes to such diseases as atopic dermatitis (1,2,5,7) and ichthyosis vulgaris (6), and even to self-perceived frequent dry skin (8). Surprisingly, the experimental data on the effects of common practices such as bathing or soaking on NMF levels *in vivo* is sparse. It has been shown that increased hydration of SC *ex vivo* increases membrane fluidity, thus increasing the permeability of the SC to endogenous compounds such as NMF as well as to exogenous ones (9). Our earlier reports have shown that bathing/soaking reduces skin hydration and the rate of stratum corneum moisturization in both infants and adults, while acetone/ether (A/E) extraction increases TEWL but does not affect MAT (10,11). Topical application of NMF reversed the dehydrating effects of soaking, but direct quantitative measures of NMF were not made. The A/E extraction procedure used in these studies was not expected to remove substantive quantities of water-soluble materials from the skin (12,13). A/E extraction, however, has been shown to disrupt the lipid lamellae of the SC in such a way as to reduce bound-water content and permit the liberation of large amounts of water-soluble material (13). Given our previous findings, we hypothesized that exposure to water (soaking) and solvent would reduce NMF levels in the outer stratum corneum relative to normal, non-exposed skin and that the combination of both water and solvent together would create a larger effect, as it impacts both the water-handling and lipid facets of skin hydration. We report a sensitive new method to quantify the amino acid components of NMF that uses reverse-phase high-performance liquid chromatography (HPLC) in conjunction with the collection of SC samples following *in vivo* exposure to water, solvent (acetone/ether), and combinations thereof.

MATERIALS AND METHODS

MATERIALS

Sampling tapes (D-Squame[®]) were from CuDerm (Dallas, TX). HPLC grade acetonitrile was from Acros Organics (Geel, Belgium), HPLC grade methanol from Fisher chemicals (Fairlawn, NJ), and the AccQ-Tag system (containing derivatizing reagent, buffers, and one HPLC liquid phase) from Waters Corp (Milford, MA). α -amino-n-butyric acid (AABA), amino acid standard solution (acidic and neutral), citrulline, and sodium lauryl sulfate came from Sigma Chemical Co., (St Louis, MO). Amino acid standard H, BCA protein assay kit, and 6 N hydrochloric acid came from Pierce Biotechnology Inc. (Rockford, IL), and urea was from Ameresco (Solon, OH). Samples were run on a Waters 2690 HPLC system using a C-18 reverse-phase column, 25 cm \times 4.6 mm, and a Waters 470 fluorescence detector, with Waters Millennium 32 acquisition software, Version 2.15.01.

SUBJECTS

Evaluations were performed on eleven healthy female subjects aged 23–55. Exclusion criteria included visually dry forearm skin and dermatological conditions such as psoriasis and eczema in the study areas. The Institutional Review Board of the University of Cincinnati Medical Center approved the protocols. All subjects provided informed consent.

EXPERIMENTAL PROCEDURE

Prior to entry into the studies, subjects refrained from using moisturizer on their forearms for 72 hours. Two 2 × 2-cm treatment sites were marked on each volar forearm. Panelists acclimated to environmental conditions (temperature 21° ± 1°C and relative humidity 31% ± 5%) for 30 minutes before initial measurements were collected. Baseline measurements of transepidermal water loss (TEWL, g/m²/hr) and the rate of moisture accumulation (MAT, cru/sec) were made for all sites. Biophysical measurements were collected again at 0.25, 0.5, and 4 hours following treatment.

TREATMENTS

The skin was exposed to three treatments: (a) acetone/ether, (b) water soak, and (c) acetone/ether followed by a water soak. Untreated skin served as the study control. The treatments were randomized both up and down and between arms.

Acetone/ether extraction. One site on each forearm was treated with a 1:1 mixture of acetone and ether (A/E) to remove surface and intercellular lipids from the outer layers of the SC. The sites were exposed to A/E for ten minutes using a glass extraction cup to hold the solvent. The areas were continuously rubbed with a cotton swab dipped in the A/E mixture during this time. Following extraction, the biophysical measurements were repeated on the extracted sites to quantify the immediate post-extraction state.

Soaking. Once the extraction was complete, one forearm was soaked in fresh water (temperature 40° ± 2°C) for ten minutes and blotted dry. The sites on the other, unsoaked, arm served as control. Biophysical measurements were repeated on all test sites 15 minutes, 30 minutes, and four hours after soaking.

NMF MEASUREMENTS

Skin surface sampling. At the end of the measurement period, four hours after soaking, and after collection of all biophysical measurements, SC samples were serially collected on all treatment and control sites. Two different sizes of tapes were used in these studies: for sample collection, 14-mm tapes were used; for desquamation between the collected samples (tapes 2, 4, 6–9, and 11–14), larger, 22-mm tapes were used to help ensure that the collected samples reflected a consistent SC depth. Each sampling tape was placed on the skin, rubbed once with the collector's thumb, and removed immediately by tweezers. The next tape was placed in the same location but removed from a different angle to ensure overall evenness of desquamation. The collected sampling tapes were placed in snap-cap centrifuge tubes and stored at -18 degrees Celsius until analysis.

Preparation of standards and calibration curves. The stock solution was diluted 1:100 with a further 10 mM HCl. A calibration curve for citrulline was prepared using the amino acid acidics and neutrals standards as follows: Four separate dilutions, 1/200, 1/200, 1/75, and 1/50, of the stock solution (2.5 µmol/ml) were prepared using 10 mM of HCl. A 2.5-µmol/ml solution of citrulline in 10 mM of HCl was prepared by weighing 4.38 mg of citrulline into 10 ml of 10 mM HCl and analyzed in triplicate against the acidics and

neutrals calibration curve following the same preparation for HPLC analysis as the samples, with the exclusion of the internal standard.

Once the citrulline concentration was validated, a calibration curve for the samples was generated using amino acid standard H, as seen in Table I. AABA internal standard stock solution was prepared by weighing 2.5 mg of AABA into 10 ml of 10 mM HCl. Two milliliters of this solution was diluted to 50 ml with a further 10 mM of HCl. All stock solutions were stored refrigerated until use.

Quantitation of NMF. The tapes were extracted with 300 μ l of 6mM perchloric acid spiked with 10 μ l of 2 μ mol/ml α -amino-n-butyric acid (AABA) at room temperature for three hours. After three hours, the extract was removed by micropipette to a fresh microcentrifuge tube, while the tape was reserved in the original tube for protein analysis. For HPLC analysis, 20 μ l of extract and 30 μ l of 0.2 M sodium borate buffer, pH 8.8, were added to HPLC microvials, derivatized with 10 μ l of AccQ-Fluor for fluorescence detection, and vortexed for five seconds. Samples were analyzed using the AccQ-Tag system (Waters Corp.) with gradient elution (Eluent A: Waters AccQ-Tag Eluent A [one part to ten parts deionized water]; Eluent B: 45% ACN, 15% MeOH, 40% H₂O [w/w]; see Table II) on a C-18 reverse-phase column (25-cm length, 4.6-mm internal diameter). The excitation wavelength was 250 nm with emission at 395 nm. The column temperature was 40° C with a run time of 40 minutes.

The HPLC results were standardized to the amount of protein removed by the individual tape using the Pierce BCA protein assay (14). After NMF extraction, the tapes were re-extracted using a solution of 300 mM urea + 2% w/w sodium lauryl sulfate. Three hundred microliters of this solution was added to each microcentrifuge tube and vortexed for one minute before being refrigerated overnight. The following morning, the samples were warmed back to room temperature, placed in a 60° C water bath for 30 minutes, sonicated for six minutes, then vortexed again for one minute. At this point, the spent tapes were discarded. A 96-well plate was filled, in triplicate, with 25 μ l of this protein extract and 200 μ l of Pierce working reagent in each well. A calibration curve with concentrations of 6.25, 12.5, 25, 50, 100, 200, and 400 μ g/ml was prepared by serial dilution of an 800- μ g/ml BSA stock solution (in water). The plates were incubated at 37°C for one hour, and then read on a spectrophotometer at 562 nm.

BIOPHYSICAL INSTRUMENTATION

Transepidermal water loss (TEWL, g/m²/hr) was determined using a DermaLab evaporimeter (Cortex Technology, Denmark). The rate of moisture accumulation (MAT, cru/sec)

Table I
HPLC Standard Curve Preparation Amounts

Target concentration	AA standard H	Citrulline stock	10 mM HCl
12.5 nM/ml	5 μ l	5 μ l	990 μ l
25 nM/ml	10 μ l	10 μ l	980 μ l
50 nM/ml	20 μ l	20 μ l	960 μ l
75 nM/ml	30 μ l	30 μ l	940 μ l
100 nM/ml	40 μ l	40 μ l	920 μ l
250 nM/ml	100 μ l	100 μ l	800 μ l
500 nM/ml	200 μ l	200 μ l	600 μ l

Table II
HPLC Gradient

Time (min)	Flow (ml/min)	% Eluent A	% Eluent B	Curve
0.00	1.0	98	2	
15.00	1.0	93	7	6
16.00	1.0	87	13	6
27.00	1.0	68	32	6
30.00	1.0	68	32	6
31.00	1.0	0	100	6
37.00	1.0	0	100	6
38.00	1.5	98	2	6
40.50	1.5	98	2	6
41.00	1.0	98	2	6
50.00	1.0	98	2	6
60.00	1.0	0	100	6
100.00	0.0	0	100	11

Curve type 6 represents a linear change of solvent concentration over the time period.

Curve type 11 is an immediate change of conditions.

was measured with a NOVA[®] Dermal Phase Meter 9003 (NOVA[®] Technology, Portsmouth, NH). The MAT uses changes in skin capacitive resistance (the ratio of charge to potential on an electrically charged isolated conductor) under occlusion by the probe to determine the extent of skin hydration (15). Transepidermal water accumulates under the sensor for twenty seconds and the value is calculated as the slope of the regression line (cru/s). The MAT method provides a dynamic measure of SC water handling, as previously reported (16).

STATISTICS

All data were examined using univariate GLM measures in SPSS (SPSS, Inc.). Prior to analysis, \log_{10} transformation of the NMF data was used to improve the normality of the data sets. The statistical assessment for the NMF data included tape number, treatment, treatment * tape number (interaction), and treatment * subject (interaction). MAT and TEWL are reported as estimates \pm confidence intervals. The model for analysis of the biophysical data included treatment, panelist, baseline TEWL, and baseline TEWL * treatment (interaction). A value of $p < 0.05$ was considered statistically significant. Treatments were compared using analysis of variance (ANOVA, $p < 0.05$, SigmaStat, SPSS, Inc.), and pairwise comparisons were made with the appropriate procedure (Tukey, Dunn, $p < 0.05$).

RESULTS AND DISCUSSION

UNTREATED CONTROL AND EFFECTS OF DEPTH

A significant increase in NMF levels corresponding to an increase in SC depth (as tape strip number) was found for tape strips 1, 3, and 5 for the untreated control. Tapes 10 and 15 were not significantly different from tape 5 and are not further discussed. This increase was found to be significant for the summed amino acids, as

well as for a large group of the individual amino acids: Glu, Gly, His, Arg, Cit, Thr, Lys, Leu, and Phe. This correlates to the depth gradient in NMF levels shown by Scott, Harding, Bowser, and Rawlings' group (17,18) and is supported by the isolated SC work done by Hashimotokumasaka *et al.* (19). The gradient increase shown here fits within the scale of resolution offered by confocal laser Raman spectroscopy, and the leveling off of NMF levels at deeper layers of the SC is consistent with results obtained by Caspers *et al.* (20–22) using *in vivo* confocal Raman microspectroscopy. Raman spectroscopy is limited in its depth by the models used to fit the spectra, which are unable to match changes in molecular composition in the lower SC. Due to this limitation, it can not be a truly quantitative method. Our method can detect smaller differences in NMF levels in the upper SC, e.g., as a function of treatment, due to its greater sample resolution. However, it is limited in depth and does not permit analysis down to Caspers' reported "band of stable filaggrin" due to subject trauma from repetitive tape stripping. Our method uses readily accessible HPLC technology and affordable and easily stored tapes for sample collection, and provides quantitative values for local NMF levels, rather than being limited to measuring the change in NMF levels at various strata.

Gradients in NMF levels were also found in the treated sites, with varying curvatures (Figure 1). Soaking the skin exacerbates the change in NMF levels with SC depth, making the decrease in NMF levels towards the surface of the skin more dramatic. No differences between treatments are observed at tape strip levels 10 and above.

These gradients were particularly well-defined for citrulline, which is an important marker for the NMF as a whole, since citrulline in the skin originates entirely from filaggrin degradation (3). A covariate analysis of the data over all tape strip depths shows a strong effect of the subject, not surprising given the high person-to-person variability observed in the raw data. Significant differences in citrulline were shown between the soaked and control sites over all samples, however, and a strong trend towards significance ($p=0.053$) was observed in the summed amino acid samples, with the soaked site having lower levels than the control.

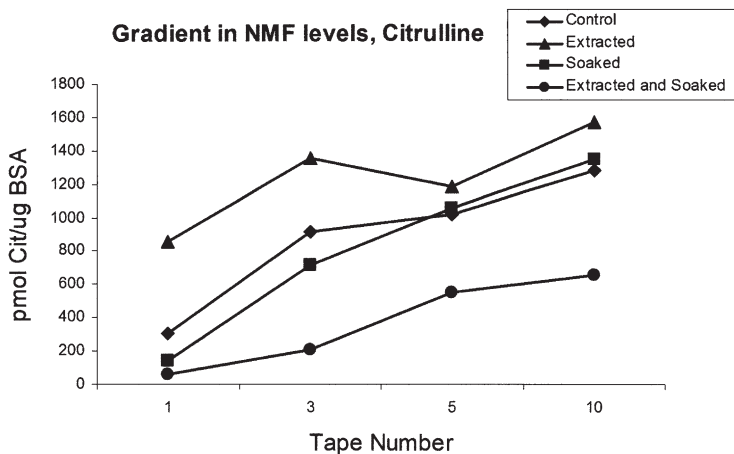


Figure 1. Citrulline levels increased significantly with depth in the SC, and varied with treatment, with the extracted and soaked site showing the greatest loss of NMF.

amino acids other than those from filaggrin proteolysis are likely, we believe that the present method measures NMF, since citrulline is not generated in SC other than from filaggrin proteolysis, which is the source of the bulk of the free amino acids found in SC (23–25). In addition, the other amino acids, as well as the sum, follow the same patterns of significance as citrulline in these studies.

TREATMENT EFFECTS

Significant differences between the treatments were also observed (Figure 2). For the amino acids of greater prevalence in NMF and the SC, the findings were as follows: (a) for citrulline at tape 1, all treatments were significantly different; (b) for the summed AAs at tape 1, the control was significantly different from the extracted and soaked (ES) site; and (c) the extracted site was different from all others. For tape 3, (a) significant differences between ES and the other three treatments as well as between the extracted (E) and soaked (S) sites were seen for the summed AAs, Cit, His, and Phe; (b) for leucine, ES was significantly different from C, S, and E, and for serine, ES was significantly different from E. At strip 5, the summed AAs, His, Leu, and Phe all showed a significant difference between ES and the other three sites; for citrulline, all comparisons were significant.

Because all treatments converged by tape 10, tapes 1, 3, and 5 were summed to examine treatment effects over the upper portion of the SC. For the summed AAs, the sum of tapes 1, 3, and 5 confirms the trends shown in the individual tape strips, with site ES differing from all others and E differing from S, but not from C. E and ES are different for serine. ES is different from all for histidine. For citrulline, E is different from all. For both leucine and phenylalanine, E is different from all and S and ES are different. Table III provides a summary of the significant differences in NMF.

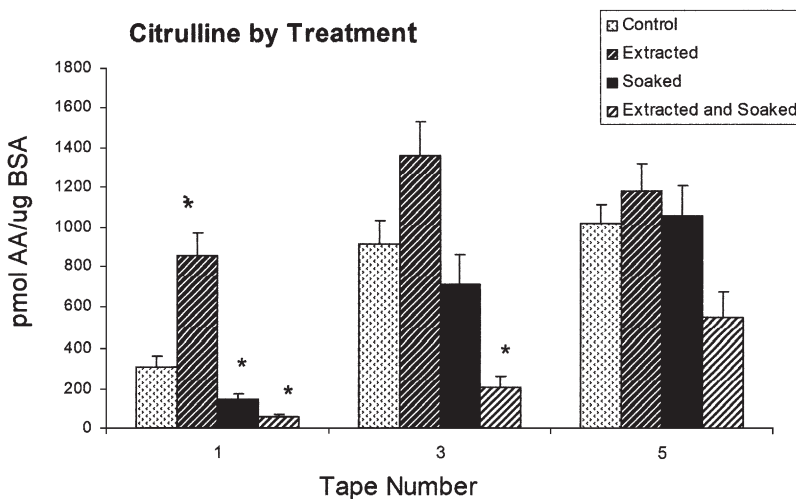


Figure 2. Significant reduction in NMF levels was caused by extraction and soaking, with the combination producing especially low levels of NMF. *Indicates significant difference from control, $p < 0.001$.

Table III
Significant NMF Results

	All AAs	Ser	Gly	His	Arg	Cit	Phe
Strip 1	E vs S, ES, C ES vs C	E vs ES	E vs ES, S	E vs S, ES, C ES vs C	NS	E vs ES, C, S C vs S	E vs S, ES, C ES vs C
Strip 3	ES vs C, E, S E vs S	E vs ES	E vs ES	ES vs S, E, C E vs S	ES vs E, C	E vs C, S, ES E vs S	ES vs C, E, S E vs S
Strip 5	ES vs S, E, C	None	ES vs ES	ES vs E, C, S	ES vs E, S	E vs ES	ES vs E, C, S
Sum 1, 3, 5	ES vs E, C, S E vs S	E vs ES	ES vs E, C	ES vs E, C, S	E vs ES	E vs ES, C, S	E vs ES, C, S ES vs S
Sum 1, 3, 5, 10	ES vs E, C, S 1 vs 3, 5, 10 3 vs 10	NS 1 vs 3, 5, 10	E vs ES 1 vs 3, 5, 10	ES vs E, C, S 1 vs 3, 5, 10 3 vs 10	ES vs E, C, S 1 vs 3, 5, 10	ES vs E, C 1 vs 3, 5, 10 3 vs 10	ES vs E, C, S 1 vs 3, 5, 10 3 vs 10
Extracted	1 vs 3, 5, 10	1 vs 3, 5, 10	1 vs 3, 5, 10	1 vs 3, 5, 10	1 vs 3, 5, 10	1 vs 3, 5, 10	1 vs 3, 5, 10
Soaked	1 vs 3, 5, 10 3 vs 5, 10	1 vs 3, 5, 10 3 vs 5, 10	1 vs 3, 5, 10 3 vs 5, 10	1 vs 3, 5, 10 3 vs 5, 10	1 vs 3, 5, 10 3 vs 10	1 vs 3, 5, 10 3 vs 10	1 vs 3, 5, 10 3 vs 10
Extracted and soaked	1 vs 5, 10 3 vs 5, 10	1 vs 5, 10 3 vs 10, 5 vs 10	1 vs 3, 5, 10 3 vs 5, 10 5 vs 10	1 vs 3, 5, 10 3 vs 5, 10 5 vs 10	1 vs 3, 5, 10 3 vs 5, 10	1 vs 3, 5, 10 3 vs 5, 10 5 vs 10	1 vs 3, 5, 10 3 vs 5, 10

C = control; E = extracted only; S = soaked only; ES = extracted and soaked site. Tape strip numbers are listed as numbers only, e.g., 1 for tape strip 1. All comparisons listed are significant at $p < 0.05$.

INSTRUMENTAL RESULTS

Extraction of normal forearm skin for ten minutes with 1:1 acetone/ether did not significantly change TEWL or MAT (Tables IV and V) relative to the initial skin condition on the measurement sites. Exposure of the sites to a fresh-water soak alone did not cause significant changes in TEWL over the four-hour duration of the study, but did cause a significant decrease in MAT on the soaked site for the first 30 minutes post-soaking and on the soaked and extracted site for the duration of the study. In fact, TEWL showed no significant changes with treatment or time that could not be simply explained by continued water evaporation from the soaked arm, indicating that neither extracting the skin nor soaking it caused significant barrier damage.

It is clear, then, that although no clinical barrier damage is done by either brief water soaking or by acetone/ether extraction, significant changes in the NMF result from these treatments. The combination of these treatments causes particularly great NMF loss, which is important, as we speculate that soaking and extraction mimic conditions caused by bathing with soap. Soap and surfactant use cause a loss of lipid in the SC not caused by water use, although water alone is sufficient to cause dryness (26). Soap use has been shown to disrupt the lipid lamellae of the SC (27), as does A/E extraction.

The present study quantifies NMF levels in the upper stratum corneum and examines the relationship between NMF levels and biophysical measures of barrier integrity (TEWL) and moisture accumulation. It confirms the conclusions previously drawn (10), that a simple ten-minute fresh-water soak is capable of removing significant amounts of the free amino acid components of the stratum corneum. It also is consistent with our conclusion that SC recovery to its baseline condition after soaking is relatively slow. We also present a relatively simple, quantitative method for the analysis of the amino acid components of the NMF that discriminates the effects of common SC treatments. We believe this method will have important use for the evaluation and formulation of skin care products.

SUPPLEMENTARY INFORMATION

The complete, numeric NMF data sets are available by correspondence.

Table IV
Transepidermal Water Loss (TEWL)

Study 1	Baseline	.25 Hours post-soak	.5 Hours post-soak	4 Hours post-soak ^a
Control	6.3 ± 1.1	6.2 ± 0.3	6.3 ± 0.2	6.7 ± 0.2
Extracted	6.5 ± 1.9	6.5 ± 0.3	6.5 ± 0.2	7.2 ± 0.2
Soaked	6.6 ± 1.5	8.4 ± 0.3*	7.1 ± 0.2*	6.7 ± 0.2
Extracted and soaked	6.6 ± 1.6	9.7 ± 0.3*	7.9 ± 0.2*	7.2 ± 0.2

TEWL (g/m²/hr) was measured for each site prior to any treatment (baseline), following the A/E extraction; and 15 minutes, 30 minutes and four hours after the fresh-water soak. The post-treatment values reported are estimates ± standard error, n = 11. Baseline values are the actual values ± standard error, n = 11.

*Indicates significant difference from baseline at $p < 0.05$.

^aCovariates appearing in the model are evaluated at the following value: BTEWL = 6.5.

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REFERENCES

- (1) C. N. Palmer *et al.*, *Nature Genetics*, **38**, 441–446 (2006).
- (2) S. Weidinger *et al.*, *J. Allergy Clin. Immunol.* **118**, 214–219 (2006).
- (3) I. R. Scott and C. R. Harding, *Dev. Biol.*, **115**, 84–92 (1986).
- (4) T. Seguchi, C. Chang-Yi, S. Kusuda, M. Takahashi, K. Aisu, and T. Tezuka, Decreased expression of filaggrin in atopic skin, *Arch. Dermatol. Res.*, **288**, 442–446 (1996).
- (5) J. N. Barker *et al.*, *J. Invest. Dermatol.*, **126**, 127(3), 564–567 (2006).
- (6) A. Sandilands *et al.*, *J. Invest. Dermatol.*, **126**, 1770–1775 (2006).
- (7) G. M. O'Regan, A. Sandilands, W. H. McLean, and A. D. Irvine, *J. Allergy Clin. Immunol.*, **122**, 689–693 (2008).
- (8) R. S. Ginger, S. Blachford, J. Rowland, M. Rowson, and C. R. Harding, *Arch. Dermatol. Res.*, **297**, 235–241 (2005).
- (9) A. Alonso, N. C. Meirelles, V. E. Yushmanov, and M. Tabak, *J. Invest. Dermatol.*, **106**, 1058–1063 (1996).
- (10) M. O. Visscher, G. T. Tolia, R. R. Wickert, and S. B. Hoath, *J. Cosmet. Sci.*, **54**, 289–300 (2003).
- (11) R. R. Wickert, G. Tolia, B. Fugitt, M. O. Visscher, and S. B. Hoath, Bioengineering evaluation of the water handling capacities of stratum corneum in vivo, *Proc. 2001 IFSCC. Intl. Conf., Taipei, Taiwan*, 37–46 (2001).
- (12) T. Yamamura and T. Tezuka, *J. Invest. Dermatol.*, **93**, 160–164 (1989).
- (13) G. Imokawa, H. Kuno, and M. Kawai, *J. Invest. Dermatol.*, **96**, 845–851 (1991).
- (14) P. K. Smith *et al.*, *Analyt. Biochem.*, **150**, 76–85 (1985).
- (15) G. B. Jemec and J. Serup, *Acta Derm. Venereol. (Stockh.)*, **70**, 245–247 (1990).
- (16) P. Treffel and B. Gabard, *Arch. Dermatol. Res.*, **287**, 474–479 (1995).
- (17) I. Scott and C. Harding, *Dermatology 2000*, 773 (1993).
- (18) A. V. Rawlings, I. R. Scott, C. R. Harding, and P. A. Bowser, *J. Invest. Dermatol.*, **103**, 731–741 (1994).
- (19) K. Hashimoto-kumasaka, I. Horii, and H. Tagami, *Arch. Dermatol. Res.*, **283**, 342–346 (1991).
- (20) P. J. Caspers, G. W. Lucassen, E. A. Carter, H. A. Bruining, and G. J. Puppels, *J. Invest. Dermatol.*, **116**, 434–442 (2001).
- (21) P. J. Caspers, G. W. Lucassen, R. Wolthuis, H. A. Bruining, and G. J. Puppels, *Biospectroscopy*, **4**, S31–S39 (1998).
- (22) P. J. Caspers, G. W. Lucassen, and G. J. Puppels, *Biophys. J.* **85**, 572–580 (2003).
- (23) J. Kubilus, R. W. Waitkus, and H. P. Baden, *Biochim. Biophys. Acta*, **581**, 114–121 (1979).
- (24) J. Kubilus *et al.*, *J. Invest. Dermatol.*, **85**, 513–517 (1985).
- (25) I. R. Scott, C. R. Harding, and J. G. Barrett, *Biochim. Biophys. Acta*, **719**, 110–117 (1982).
- (26) A. Bornkessel, M. Flach, M. Arens-Corell, P. Elsner, and J. W. Fluhr, *Skin Res. Technol.*, **11**, 53–60 (2005).
- (27) J. L. Leveque, J. de Rigal, D. Saint-Leger, and D. Billy, *Skin Pharmacol.*, **6**, 111–115 (1993).