TITLE:

Preparation of keratin hydrolysate from chicken feathers and its application in cosmetics.

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KEYWORDS:

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SHORT ABSTRACT:

The goal of the protocol is to prepare keratin hydrolysate from chicken feathers by alkaline-enzymatic hydrolysis and to test whether adding of keratin hydrolysate into a cosmetics ointment base improves skin barrier function (heighten hydration and diminishing transepidermal water loss). Tests are conducted on men and woman volunteers.

LONG ABSTRACT:

Keratin hydrolysates (KH) have established as standard components in hair cosmetics. Understanding the moisturising effects of KH is advantageous for skin-care cosmetics. The goals of the protocol are: (1) to process chicken feathers into KH by alkaline-enzymatic hydrolysis and purify it by dialysis, and (2) to test if adding KH into an ointment base (OB) increases hydration of the skin and improves skin barrier function - diminishes transepidermal water loss (TEWL). During alkaline-enzymatic hydrolysis feathers are firstly incubated at higher temperature in alkaline environment and then, under mild conditions, hydrolyzed with proteolytic enzyme. Solution of KH is dialyzed, vacuum dried and milled to a fine powder. Cosmetic formulations comprising from oil in water emulsion (O/W) containing 2, 4 and 6 weight % of KH (based on weight of the OB) are prepared. Testing the moisturizing properties of KH is carried out on 10 men and 10 women at time intervals of 1, 2, 3, 4, 24 and 48 h. Tested formulations are spread at degreased volar forearm sites. Skin hydration of stratum corneum (SC) is assessed by measuring capacitance of the skin, which is one of the most world-wide used and simple method. TEWL is based on measuring the quantity of water being transported per a defined area and period of time from the skin. Both methods are fully non-invasive. KH makes for an excellent occlusive; depending on the addition of KH into OB it brings about a 30 % reduction in TEWL after application. KH also functions as a humectant, as it binds water from the lower layers of the epidermis to the SC; at optimum KH addition into the OB up to 19 % rise in hydration in men and 22 % rise in women occurs.

INTRODUCTION:

Slaughterhouses, food industry and tanning industry annually produce immense amounts of solid keratin by-products – wool, feathers, bristles, hooves, claws, horns and the like. According to latest statistical data total live weight of chickens, turkeys, ducks and other slaughtered poultry in the USA is 62.5 billion pounds per year[1]; in the EU it is approx. 28.7 billion pounds per year. Considering that feathers make up to 8.5 % of total poultry weight, only the USA itself annually produces approx. 5.3 billion pounds of waste feathers[2].

Keratin is a protein exhibiting high chemical resistance because it is strongly cross-linked with disulfide bridges that render its processing difficult. Obtaining soluble products requires cleaving cross-links and possibly carrying out hydrolysis of peptide bonds[3]. Cleavage of disulfide bridges may proceed through a reaction of thiol anion according to following pattern[4, 5]:

$S_a^- + -S_bS_c- \leftrightarrow -S_b^- + -S_aS_c-$

With a very high pH level, hydrolysis of disulfide bridges also appears, in accordance with pattern[6]:

$-SS- + OH^- \rightarrow -S^- + -SOH$

Under mild conditions (pH approx. 8), even sulfitolysis takes place according to following pattern:

$-SS- + HSO_3^- \rightarrow -SH + -SSO_3^-$

The most economical way of degrading keratin is microbial breakdown which is characterized, by mild reaction conditions during processing and high breakdown efficiency (approx. 90 %)[7, 8]. Keratinases are produced by some bacteria isolated from soil and keratin waste[9]. Microbial keratinases hydrolyze rigid and strongly cross-linked keratin structures[10] and keratin hydrolysate prepared is rich in soluble proteins and no loss in essential amino acids was detected with it[11].

In order to incorporate a protein in cosmetic preparations (e.g. emulsions, lotions and gels), requirements ensure such proteins are soluble in water, the given systems are transparent and that re-aggregation of peptides is avoided due to hydrophobic interactions. Therefore, a common practice is to apply hydrolysates of proteins, such as hydrolyzed collagen, elastin and keratin. When adding hydrolysates into cosmetic emulsions, steps are taken to ensure that the hydrolysate is firstly dissolved in water. In some cases it is desirable that the protein - or the hydrolysate - is soluble in alcohol or other organic solvents[12].

KH normally features in shampoos, conditioners, lotions, and nutritive serums for hair, as well as mascaras, nail polish and eye make-up agents. The KH effects declared usually include forming a protective film, smoothing the hair or nail structure, heightened plasticity and appearance of the treated formation, regulating the consistency of products and encouraging the formation of foam[13, 14]. It has also been shown that KH reduces surface tension, hence supplementation in cosmetics can facilitate reduction in the amount of emulsifier added to stabilize creams. KH limit the effects of irritation triggered by cleaning agents (surfactants) to the skin, eyes and hair, thus reducing any potential side effects of cleaning agents on tissue (e.g. dehydration of the skin, hardness and decreased barrier function of the skin). The high

buffering capability of hydrolysates is also exploited to stabilize the pH of cosmetics; peptides of shorter length have a greater buffering effect[15, 16]. Although KH have become established as standard components in hair and nail cosmetics as well as being utilized in products for skin care, studies on the moisturising effects of KH do not appear among contemporary literature.

At our workplaces, alkaline-enzymatic technology has been developed for processing keratin by-products into KH, and active testing is in process on the effects of a number of cosmetic additives[17-22]. The advantage of 2-stage alkaline-enzymatic hydrolysis using microbial proteases for chicken feathers achieves high efficiency under mild reaction conditions and the quality of KH is very high in contrast to hydrolysis employed in strong acids or alkalis. In the first stage, feathers are incubated at higher temperature in alkaline environment which partially disrupt the keratin structure and swell the feathers; after adjusting pH the feathers are hydrolyzed with proteolytic enzyme under mild conditions in the second stage. Dialyzed KH possesses a high content of proteins.

The purposes of this method we describe here are processing poultry feathers into a KH through alkaline-enzymatic hydrolysis and testing the effect of moisturising properties of KH applied to O/W cosmetic emulsion. The moisturising properties are investigated by instrumental non-invasive methods *in vivo*. The most frequent methods for measuring skin hydration and barrier function of SC include measuring electrical properties of the skin (conductance or capacitance). Different methods for investigating SC hydration include near infrared multispectral imagining method (NIM), nuclear magnetic resonance spectroscopy, optical coherence tomography or transient thermal transfer[23]. Barrier function of SC correlates to TEWL of SC and it is measured by ventilated chamber method, unventilated chamber method and open chamber method [24].

Properties of model formulations are determined using Multi Probe adapter MPA 5 with three types of probes. The first one, corneometer CM 825, measures skin hydration by assessing changes in the electrical capacity of the skin's surface; the measuring capacitor shows changes in capacitance of the skin surface in corneometric units. The corneometer gives only a relative assessment of skin hydration[25]. For TEWL, the second probe, tewameter TM 300 is used for measuring the density gradient of water evaporation (open chamber instrument based on Fick's diffusion law) from the skin indirectly by the two pairs of sensors (temperature and relative humidity) indicating the quantity of water being transported per a defined area and period of time ($g/m^2/h$). This method can detect even the slightest disruption of skin barrier function[26]. Skin pH is one of the indicators of barrier and anti-microbial function of SC[27]. The acidity of the skin mantle was measured by a (third) skin PH 905 probe connected to MPA 5 station. This specially designed probe consists of a flat-topped glass electrode for full skin contact, connected to a voltmeter. The system measures potential changes due to the activity of hydrogen cations surrounding the very thin layer of semi-solid forms measured at the top of the probe. The changes in voltage are displayed as pH[28].

We present experiments divided into three sections. (1) Preparation of KH from chicken feathers by two-stage alkaline-enzymatic hydrolysis and its purification by dialysis (removing

salts and low-molecular fractions). (2) Preparation of cosmetic formulations containing 2, 4 and 6 % KH. (3) Testing the properties of KH by measuring skin hydration, TEWL and skin pH. Testing was carried out on 10 women with the mean age of 27.2 years and on 10 men with the mean age of 26.2 years. The method of selecting the volunteers and the testing itself were conducted in accordance with international ethical principles of bio-medical research utilising human subjects[29]; all persons gave their informed consent prior to inclusion in the study. Before testing commenced, the volunteers were asked to complete a questionnaire on their health status. The volunteers committed to avoid applying any cosmetic product to the test sites and surrounding regions during the 24 h prior to and during the test period; furthermore, they were only permitted brief evening washes with running water.

PROTOCOL:

1. Process chicken feathers into keratin hydrolysate

Collect chicken feathers from a poultry farm.

1.1. Wash out any insoluble impurities and blood remnants from the chicken feathers with a sufficient excess of fresh running (cold) water. Place the feathers on a flat plate and dry overnight at 50 °C.

Note: The protocol can be paused here.

1.2. Grind the dried feathers in a cutting mill (suitable for soft to medium-hard sample materials, and fibrous materials) into final fineness 1.0 mm. Alternatively, final fineness of grinded feathers could be higher, but not more than 3.0 mm.

Note: The protocol can be paused here.

1.3. Degrease feathers

The most effective and economic method of degreasing poultry feather is using a commercial lipolytic enzyme.

1.3.1. In a stainless steel 27-L boiler container with temperature control mix feathers with water preheated up to 40±2 °C in a weight ratio 1:75, add a lipolytic enzyme (activity 100 KLU/g) in a dose of 1.5-2.0 % (related to weighed-in dry feathers) and gently stir the content with overhead stirrer for 5 min.

1.3.2. Adjust the mixture pH to the level corresponding to the maximum activity of the lipolytic enzyme by adding 1-% NaOH or 1-% H_3PO_4 solution. In this case adjust pH to 9.0±0.2 by adding 1-% NaOH solution. Stir the mix for 5 min, check and re-adjust the pH level.

1.3.3. Gently stir the mixture with overhead stirrer for 24 h at 40±0.5 °C. Alternatively, incubate the mixture at 40±0.5 °C and during the first 6 h of incubation shortly stir the content in 1-hour intervals.

1.3.4. Filter the mixture through a fine sieve (100 µm size) and wash degreased feathers with a

stream of fresh running (cold) water and dry the feathers on a flat plate at 50 °C in chamber drier overnight.
Note: The protocol can be paused here.

1.4. Perform the first stage of chicken feathers hydrolysis. Mix feathers with 0.3-% KOH water solution in a weight ratio 1:50 and gently stir with overhead stirrer at 60±0.5 °C for 24 h. Level of pH in the mixture decreases from approx. 12.5 at the start of incubation to approx. 11.0 at the end of incubation. On finishing first stage of hydrolysis, adjust mixture pH to the level corresponding to the maximum activity of the proteolytic enzyme with 10-% H_3PO_4 (in this case to a level of 9.0±0.2).

1.5. Perform the second stage of chicken feathers hydrolysis. Add to the mixture proteolytic enzyme in a dose of 5.0 % (related to dry matter of quantity of feathers weighed-in at the beginning of the first stage of hydrolysis). Gently stir with overhead stirrer at 60±0.5 °C for 8 h and then heat the mixture (in the same stainless steel 27-L boiler container) to a boiling point (100 °C) and boil for 10 min to inactivate enzyme.

1.6. Separate solution of KH (prepared in step 1.5) from undissolved remnant by filtering it through low-density filter paper on a Buechner funnel with slight under-pressure; alternatively use a centrifuge.
Note: The protocol can be paused here for several days if a solution of KH is stored at 5±1 °C.

1.7. In the plastic bucket (26 cm in diameter and 26 cm high) dialyze keratin hydrolysate using 12K MWCO membrane to remove small peptides and salts. Pour 400 mL of solution of KH into dialysis tubing and dialyse it against 4 L of distilled water for 80 h at room temperature; after 18, 36 and 60 h change distilled water.

1.8. Cast a dialysed solution of KH on an anti-adhesive plate (e.g. silicone one) on ratio of 500 mL to 1,000 cm^2 plate area, vacuum dry it on a thin film at 40±0.5 °C for overnight, mill to form a fine powder and keep it in a closed vessel in a desiccator.
Note: The protocol can be paused here for several months if KH powder is stored in a dry place.

2. Prepare cosmetic formulations with keratin hydrolysate

Note: The ointment base used for testing was an commercial hydrophilic oil in water (O/W) cream base and comprised of aqua, paraffin, paraffin liquid, cetearyl alcohol, Laureth 4, sodium hydroxide, carbomer, methylparaben, propylparaben.

2.1. Prepare formulations containing 2, 4 and 6 % KH (in accordance with the base weight of the ointment). Weigh the amount of KH powder into a polyethylene vessel (7 cm in diameter and 10 cm high) and add the ointment base at an amount that ensured the total weight of the formulation equalled 50 g; see schedule in Table 1.

[Place Table 1 here]

2.2. Homogenize the mixture with a 3-bladed laboratory stirrer for 10 min at 134.16 g using a mechanical overhead stirrer. Maintain the prepared formulations at 5±1 °C and condition them at room temperature for 2 h prior to use.
Note: The protocol can be paused here for up to 5 months if formulations are stored at 5±1 °C.

3. Test the properties of KH by measuring skin hydration, TEWL and pH
Note: Perform all measurements in a conditioned room at 23±2 °C and the relative humidity of 56±3 %.

3.1. Place 5 strips of filter paper (size 2x4 cm) into the physiological solution (0.90 % NaCl) and left them for approximately 1 min in the solution.

3.2. Apply two strips to the inner side of the right forearm, and three to the inner side of the left forearm and fix them with adhesive plasters. This step is aimed to degrease the skin and eliminate individual characteristics of the skin at the site. After 4 h, remove the strips and demark the areas with a permanent pen, see Figure 1.

[Place Figure 1 here]

3.3. Apply 0.1 mL of the tested formulations at each spot of degreased forearm sites using syringes and spread it over the entire marked surface. On the left arm, left the first site clear (it is control), the OB apply to the second site and OB + 2 % KH put on the third area. OB + 4 % KH and OB + 6 % KH apply to the right arm.

3.4. Measure each sample at each site and each interval (1, 2, 3, 4, 24, and 48 h) and take 5 readings with corneometer CM 825 probe for skin hydration, 15 readings with tewameter TM 300 probe for skin TEWL and 1 reading with pH 905 probe for skin pH. Do not allow volunteers to apply any cosmetic product to the test sites and surrounding areas during the test period; they can only permit brief evening washes with running water.
Note: The protocol can be paused here.

3.5. Process the resultant readings by basic numerical characteristics of the descriptive statistics, using table processor. From the 5 hydration readings measured for each sample ignore the lowest and the highest readings and calculate only three readings for arithmetic mean and standard deviation. From the 15 TEWL readings measured for each sample ignore the first 5 and calculate only 10 readings for arithmetic mean and standard deviation.

REPRESENTATIVE RESULTS:
KH prepared according to procedure presented in Protocol 1 (see Figure 2) is yellow in color, easily soluble in water with high protein content (inorganic solids represent < 2.0 %); pH of 1.0% solution of KH is 5.3 and fulfils requirements for cosmetic-grade hydrolysates. Yield of KH from raw material is approx. 30 %. The molecular weight distribution of KH was determined by SDS-PAGE and is shown in Figure 3.

[Place Figures 2 and 3 here]

The hydration and TEWL values as measured were delineated in different volunteers, thus could not be compared to each other. Therefore, the values are expressed as change in per cent in comparison with the OB on the site treated with the formulations, the latter containing 2, 4 and 6 % additions of KH at intervals for measurement of 1, 2, 3, 4, 24 and 48 h. The pH values for skin are expressed as the arithmetic mean of the recorded values of skin pH for all volunteers. The results for alteration in hydration and TEWL in per cent, relative to the OB, and for pH levels of the skin are given in Table 2 for 10 men volunteers and in Table 3 for 10 woman volunteers.

[Place Tables 2 and 3 here]

Hydration of SC for men volunteers.

At short intervals of measurement (1-4 h) it is clear that the highest increases in hydration of the skin (12-19 %) were recorded for formulations supplementing the OB with 2 % of KH; the addition of 4 % KH showed a lesser rise (1-7 %) in hydration. Conversely, KH at 6 % reflected negatively on values for SC hydration (a decrease of 3-7 %). After 24 h of measurement, an increase of 11 % in SC hydration was discerned for all the additions of KH to the OB tested. The same trend continued even after 48 h, following with a slight increase in hydration still remained: a 15 % increase for KH at 2 %, a rise of 14 % for the 4 % addition of KH, and the drop of 17 % for KH at 6 %.

Hydration of SC for women volunteers.

It is observable that supplementing the OB with 2 % KH causes an approximate 22 % increase in SC hydration, relative to OB alone, as early as at 1 h of measurement; a 4 % addition of KH to the OB has, in fact, no effect on hydration; while, conversely, adding 6 % KH to the OB is reflected in an approximate 12 % decrease in hydration, as compared with pure OB. Similar tendencies are seen after 2, 3 and 4 h of measurement, at which hydration increases by 12-15 % for supplementation with 2 % KH; for larger additions of KH, hydration either stays the same or diminishes. After 24 h of measurement, hydration was recorded as higher than for the OB, this for all the tested KH additions; the greatest increase (14 %) in hydration occurred for the addition of 2 % KH, while the lowest increase (8 %) was seen for supplementation at 6 % of KH. Similar results are achieved after 48 h of measurement, wherein greater hydration than for pure OB was recorded for all samples with additions of KH; the biggest rise (18 %) in hydration occurred for the 2 % KH addition, while the lowest increase (10 %) was noted for KH supplementation at 6 %.

TEWL for men volunteers.

The results of TEWL make it clear that formulations supplemented with KH diminish TEWL, when applied to the skin, in comparison with pure OB. A heightened amount of KH exerted a positive effect on lower values of TEWL. An hour after applying the formulations, TEWL was recorded as 20 % lower than for pure OB, pertaining to a formulation with 2% KH; while KH at 4 % became manifest as a drop in TEWL of 28 %; whereas KH at 6 % resulted in a dramatic 36 %

decrease in TEWL. Indeed, diminished TEWL values were also observed at 2, 3 and 4 h of measurement for formulations supplemented with KH. After 24 and 48 h, TEWL was even significantly lowered on sites treated with the KH formulations. After 24 h, TEWL observed on the skin was 23 % lower for the OB with 2 % addition of KH than at the site for pure OB; KH at 4 % brought about a drop in TEWL by about 47 %, while KH at 6 % triggered TEWL to fall by 53 %. A similar trend is evident even after 48 h – the TEWL for skin treated with OB containing 2 % KH was 21 % lower than at the site for pure OB; KH at 4 % showed a TEWL at 36 % lower; and KH at 6 % brought about a drop of 54 %.

TEWL for women volunteers.

Similarly to men volunteers, it is evident that all the monitored additions of KH to OB shall be reflected in reduced TEWL. After 1 h of measurement, a significant decrease in TEWL was recorded for OB samples containing all the additions of KH; about a 32 % reduction in TEWL was seen for the 2 % addition of KH, around a 41 % decrease for 4 % KH, and even a 50 % drop in TEWL for 6 % supplementation of KH. Following 2, 3 and 4 h of measurement, the situation remains similar, i.e. there is a decrease in TEWL at such time intervals; the least drop in TEWL occurs for 2 % KH, while the greatest is seen for 6 % KH. After 2 h, TEWL decreases by 16 % for KH at 2 %, 37 % for KH at 4 % and 39 % for KH at 6 %. At 3 h, TEWL diminishes further by 12 % for KH at 2 %, by 24 % for KH at 4 % and 29 % for KH at 6 %. At 4 h, TEWL further decreases by 20 % for 2 % KH, 34 % for 4 % KH, and 39 % for 6 % KH. After 24 h, the least reduction in TEWL (16 %) occurred for the 6 % addition of KH, while the greatest (44 %) was seen for 4 % supplementation of KH; KH at 2 % was observed to cause a 35 % decrease in TEWL. At 48 h, the results stay similar and relatively balanced - the least decrease in TEWL (33 %) for the 6 % addition of KH, the greatest (38 %) for 4 % KH and for 2 % KH.

FIGURE AND TABLE LEGENDS:

Figure 1: Method for location of test formulations on the forearm of the left and right upper limbs.

Figure 2: Representative picture of keratin hydrolysate.

Figure 3: SDS-PAGE of keratin hydrolysate and protein standards; lane 1-ultra low range molecular weight Marker (3.5-26.6 kDa), lanes 2, 3 and 4-keratin hydrolyates prepared in 3 batches, lane 5-wide range molecular weight Marker (6.5-200 kDa)

Table 1: Weight-in quantities of ointment base and keratin hydrolysate to prepare cosmetic formulations.

Table 2: Results for change in hydration and TEWL and pH of skin of 10 men volunteers at the measurement intervals of 1, 2, 3, 4, 24 and 48 h.

Table 3: Results for change in hydration and TEWL and pH of skin of 10 women volunteers at the measurement intervals of 1, 2, 3, 4, 24 and 48 h.

DISCUSSION:

The advantage of alkaline-enzymatic hydrolysis is that it can be modified according to future applications of KH. For example, in hair-care cosmetics applications where a lightly brownish color of a product is not an obstacle a higher temperature in the hydrolysis can be applied leading to higher yield of KH. In addition, longer processing time during both stages of the

technological procedure significantly affects overall process efficiency – yield of KH rises up to 85 %.

Findings for hydration measurement make it evident that, during the monitored interval of measurement (1-48 h), the addition of 2 % KH to OB is optimum since it causes a 11-19 % increase in SC hydration for men volunteers, respectively a 12-22 % increase for woman volunteers. The KH that is added possesses a broad distribution of molecular weight. We suggest that a portion of low-molecular-weight fractions (Mr < 20 kDa) penetrates the epidermis following application to the skin. Increased hydration of the skin after putting on a formulation supplemented with KH is explained through KH binding water from lower epidermis layers to the structure of the SC, leading to the formation of H-bridges between KH molecules and water. This mechanism of action is also embraced by some authors[30]. The moisturising effect of KH is comparable with conventional moisturisers (e.g. glycerol, urea and hyaluronic acid) that were tested in emulsion and gel formulations[22].

TEWL measurements highlight that, during the observed time of measurement (1-48 h) at men volunteers, all the formulations with KH supplementation caused TEWL to decline after application. The 2 % addition of KH to the OB caused a reduction in TEWL of 11-23 %, in comparison with pure OB. When the OB was supplemented with 4 % KH, TEWL dropped by 28-47 %; while for KH at 6 % it diminished by as much as 31-54 %. For woman volunteers supplementing the OB with 4 % KH represents the best option, as there is 24-44 % decrease in SC TEWL. The significantly lower TEWL for formulations supplemented with KH can be explained by the process of higher-molecular-weight fractions of KH forming a protective film once applied to the epidermis, thereby preventing the loss of epidermal water. In fact, the highly positive effect of KH on TEWL is comparable or even exceeds, for the sake of comparison, values of TEWL recorded for cosmetic gels or emulsions supplemented with 5-10 % of glycerol and 1-5 % of sericin. Then same is in comparison with conventional mineral oils that diminish TEWL by approximately 25-30 %[31]. Additionally, the barrier properties of KH are better than, for example, those for urea and hyaluronic acid[22].

pH of the skin surface. For reference, pH 3.5 to 4.3 is an acidic skin surface, pH 4.4 to 5.5 is neutral in this respect, and pH 5.6 to 6.5 represents a basic skin surface[32]. We found that no significant changes were observed in the pH of the skin surface after putting on all of the tested formulations (OB + 2, 4 and 6 % KH); the pH of 4.6 to 5.0 (men volunteers), respectively 4.9 to 5.4 (woman volunteers) corresponds to a normal skin surface.

Longer-term analysis (more than 2 days) was not accomplished.

Modifications and troubleshooting: Processing of chicken feathers into KH is very easy, runs under atmospheric pressure and at mild temperature; the process can be favorably transformed from a laboratory scale to a pilot plant scale and an industrial scale. In the 2nd step of the protocol where KH is homogenized with the (O/W) emulsion base some modifications are possible. In industrial practice O/W and W/O emulsions are prepared by mixing water (W) phase (water + cosmetic ingredients soluble in water) and oil (O) phase (oil + cosmetic

ingredients soluble in oil). KH is soluble in water, so it is favorable to blend it directly into water phase of the system.

Limitations of the technique: localization of each spot for measuring hydration and TEWL and hammered pressure at measuring with corneometer skin probe.

Critical steps within the protocol lies above all in the 3rd step of the protocol (Testing the properties of KH by measuring skin hydration, TEWL and pH). Health state, individual differences, smokers/non-smokers, gender, age differences, menstruation and mental condition can influence measuring of skin hydration and TEWL. For acquiring representative results the same person should apply tested formulations on forearms and measure hydration and TEWL values. It is vital to perform all measurements in a conditioned room with a stable temperature and relative humidity. In case of measuring values at intervals of 24 and 48 h acclimatization of volunteers in a conditioned room for at least 30 min prior to measurement is necessary.

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DISCLOSURES:
The authors have nothing to disclose.

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Figure 1

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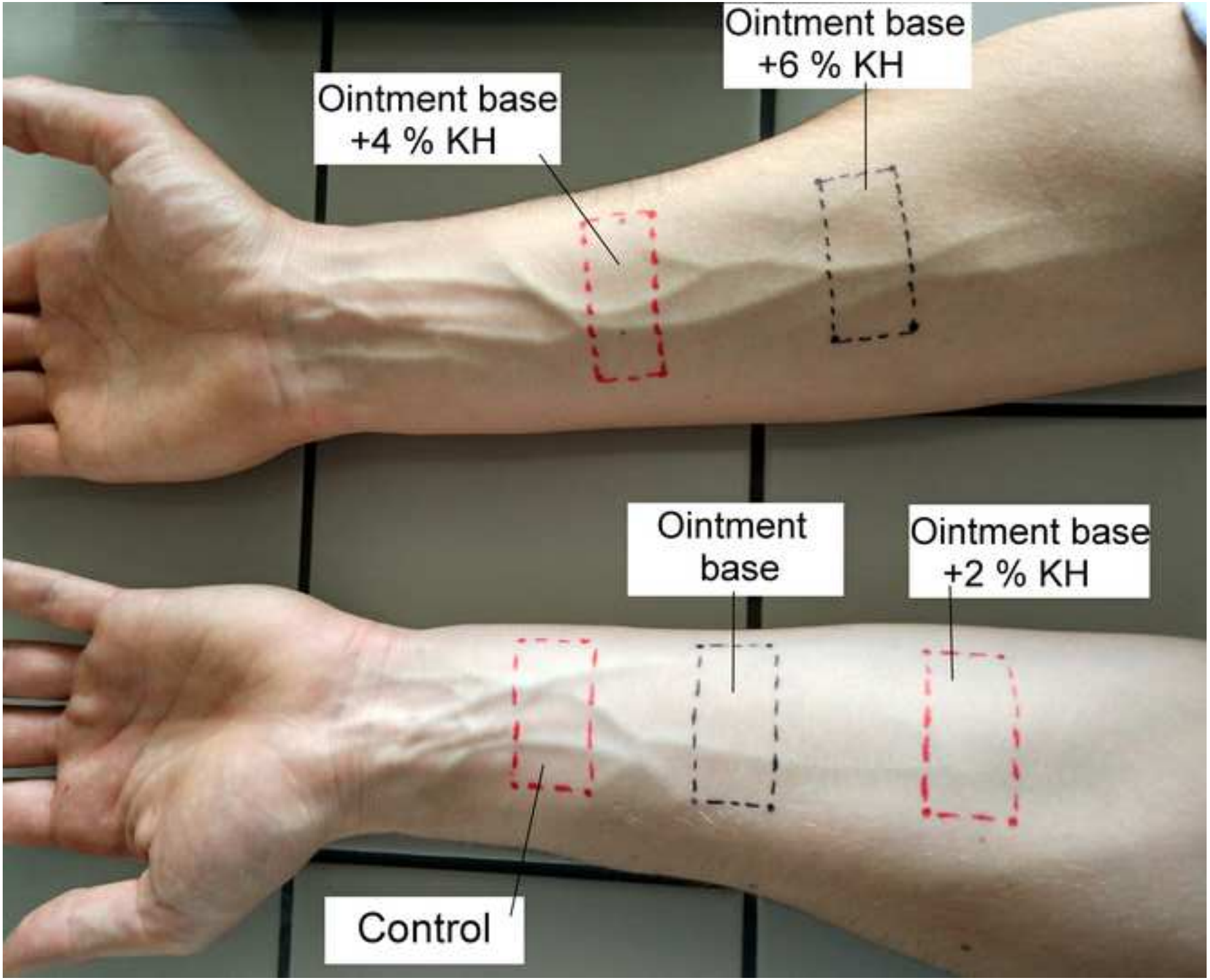

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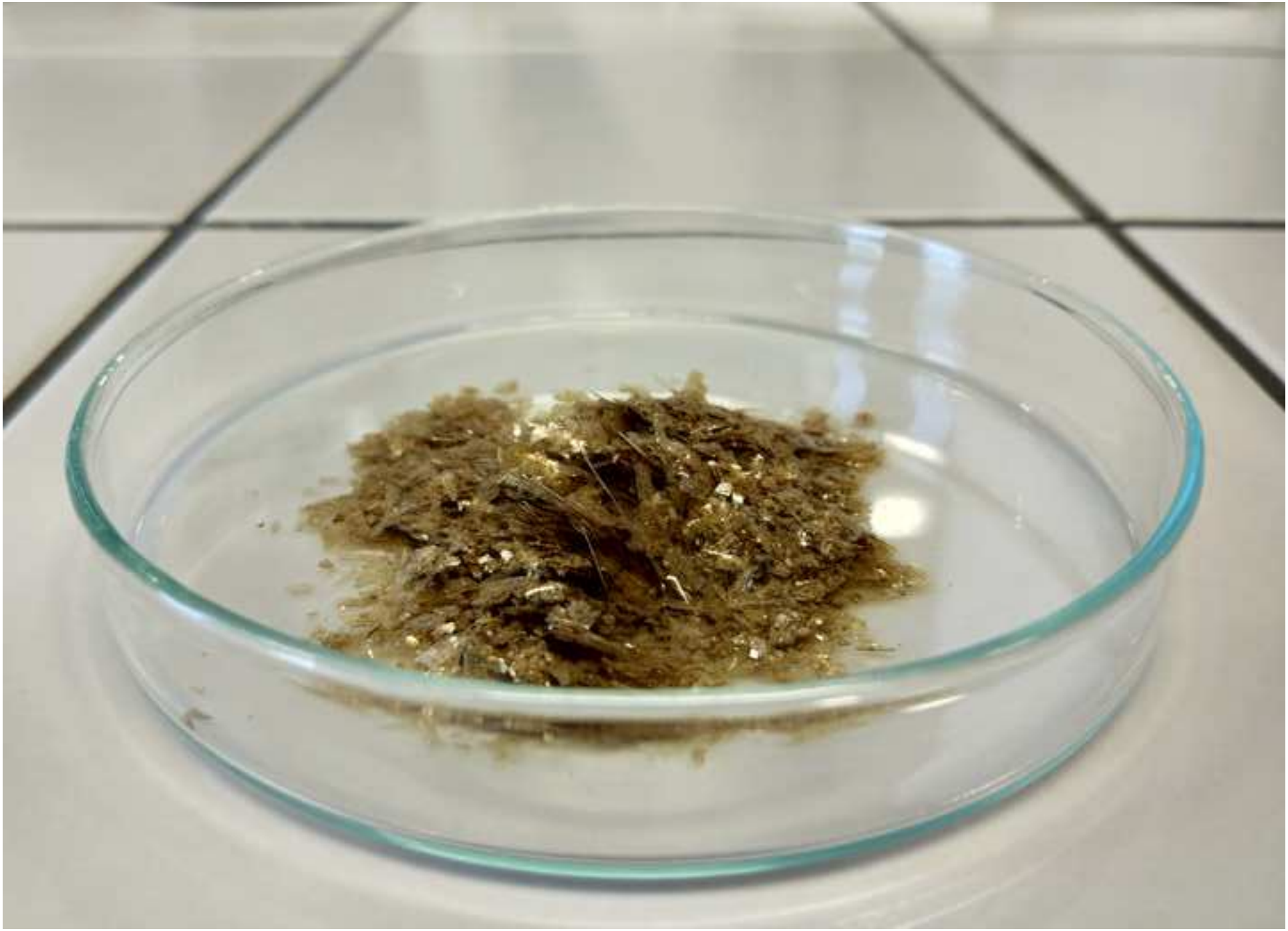

Figure 3

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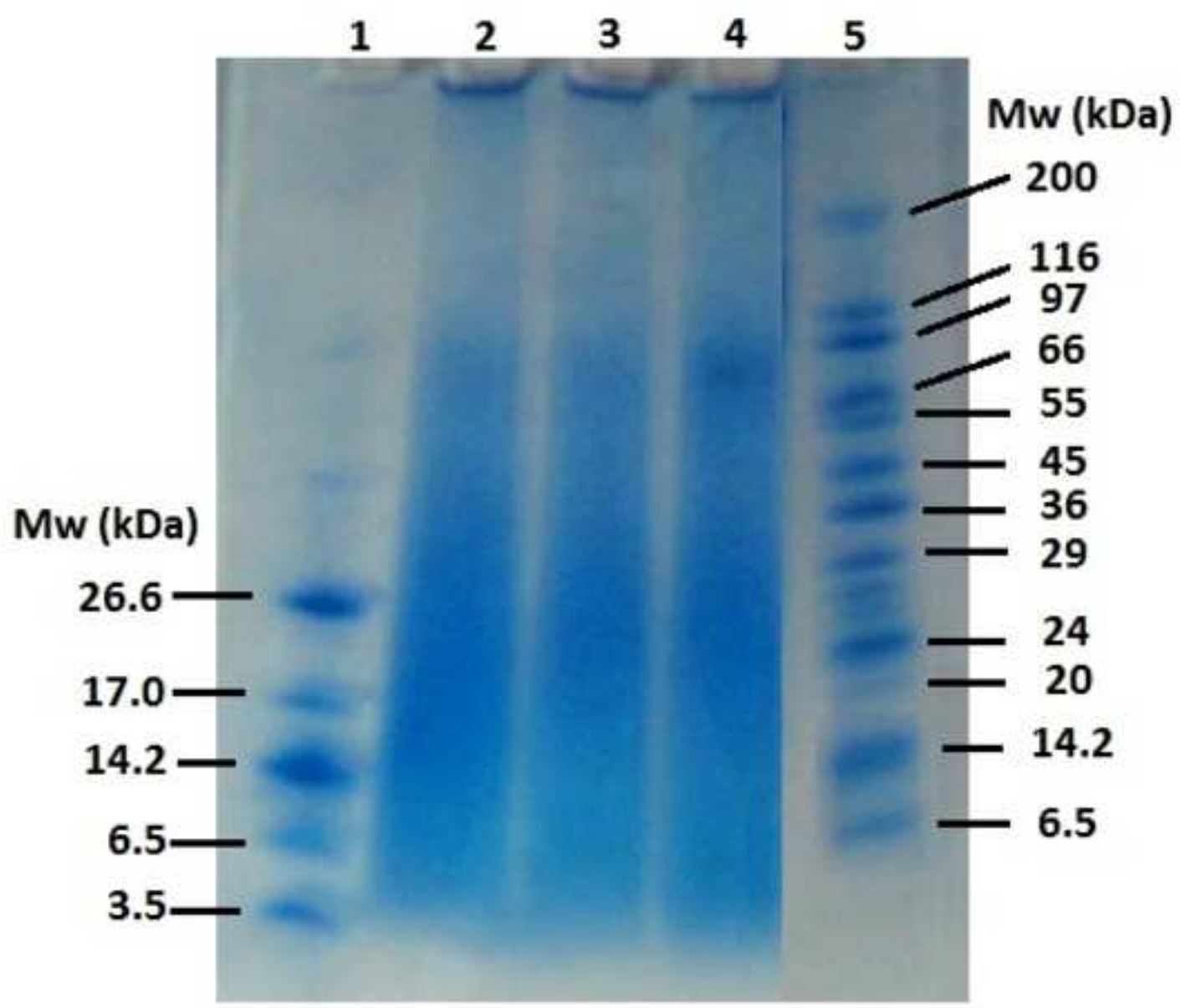

Table of Materials/Equipment

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Name of Material/ Equipment	Company	Catalog Number
Material or chemicals		
LIPEX 100T	Novozymes	LJP30020
Savinase Ultra 16L	Novozymes	PXN40001
Potassium hydroxide, KOH	Sigma-Aldrich	302510289
Phosphoric acid solution, H_3PO_4	Sigma-Aldrich	W290017
Sodium chloride physiological solution	Sigma-Aldrich	52455
Sodium hydroxide, NaOH	Penta s.r.o.	40216
AmiFarm (Cremor base-A)	Fagron	608425
Equipment		
IKA EUROSTAR POWER control-visc stirrers	IKA-labortechnik	Z404020
IKA Propeller stirrer, 3-bladed	IKA-labortechnik	R 1381
Dialysis tubing closures	Sigma-Aldrich	Z371017-10EA
Dialysis tubing cellulose membrane	Sigma-Aldrich	D9402-100FT
DOMO Pot with stailess, LCD	DOMO Elektronic	DO42325PC
Hettich zentrifugen Universal 32	Gemini bv	2770 GS1R
LT 3 shaking device	Fischer Scientific	6470.0002
KERN 440-47N	Kern	440-47N
KERN 770	Kern	770 -N
VENTICELL 222 - Komfort	BMT, MMM Group	C 131749
Vacucell 55 - EVO	BMT, MMM Group	B 050328
PULVERISETTE 19	Fritsch	19.1030.00
Multi Probe Adapter System MPA 5	Courage & Kazaka Electronic	10225237
Skin pH-meter PH 905 probe	Courage & Kazaka Electronic	
Corneometer CM 825 probe	Courage & Kazaka Electronic	
Tewameter TM 300	Courage & Kazaka Electronic	
Heidolph RZR 2020	Heidolph	13-225-007-03-1
Heidolph mechanical stirrer BR 10	Heidolph	Z336688-1EA
Fagor FS 12	Fagor	BTT-138
WTW bench pH/mV meter	WTW	Z313165

Container	RPC Superfos
Software	
Microsoft Office 2010	Microsoft
C+K software	Courage and Khazaka Electronic GmbH

Comments/Description

Lipex ® - enzyme produced by submerged fermentation of a genetically-modified microorganism, activity 100 KLU/g

Savinase® - enzyme produced by submerged fermentation of a genetically-modified microorganism, activity 16 KNPU-S/g

Potassium hydroxide, KOH, 97,0 %, Mr 56,11

Phosphoric acid solution, H_3PO_4, 85 wt. % concentration in water, Mr 98,00

Tablets of BioUltra NaCl physiological solution; 1 tablet in 1000 mL of water yields 0.9 % NaCl

Sodium hydroxide, NaOH, 97,0 %, Mr 40,00

Hydrophilic oil in water (O/W) cream base; the composition: aqua, paraffin, paraffin liquid, cetearyl alkohol, Laureth 4, sodium hydroxide, carbomer, methylparaben, propylparaben.

Digital laboratory stirrer, for tasks up to the high viscosity range, 230V, 1/cs

Propeller stirrer, 3-bladed, stirrer Ø: 45 mm, shaft Ø: 8 mm, shaft length: 350 mm

Dialysis tubing closures, red, size 110 mm

Dialysis tubing cellulose membrane, average flat width 76 mm (3.0 in.)

Preserving boiler stainless steel, 2000 W, 27-L container (diameter 37 cm, height 30 cm), temperature control 30-100 ° C, operation LCI

Mid bench centrifuge, speed 18000 rpm

Orbital shaking device

Laboratory balance

Laboratory analytical balance

Drying oven, temperature control 30-100 ° C, air circulation control

Vacuum drying oven, temperature control 30-100 ° C

Universal cutting mill, rotor with V-cutting edges and fixed knives

MPA 5 Station - equipment for measurement hydratation, TEWL and pH

Probe to specifically measure the pH on the skin surface or the scalp

Probe to determine the hydration level of the skin surface (Stratum corneum).

Probe for the assessment of the transepidermal water loss (TEWL)

Overhead stirrer, mechanical speed setting and stepless transmission; speed range 40-2000 rpm

Blade impeller crossed stirrer

Laboratory refrigerator with freezer space

High-performance bench pH and pH/conductivity meters for routine and high precision laboratory measurements in research or quality control laboratories

13-L plastic bucket, diameter 26 cm, height 26 cm

MPA 5 station operating software

D display

Table 1

Click here to download Table of Materials/Equipment Table 1.xlsx

Table 1

Cosmetic formulation	Weight of ointment base [g]	Weight of keratin hydrolysate [g]
Ointment base	50	0
Ointment base + 2 % KH	49	1
Ointment base + 4 % KH	48	2
Ointment base + 6 % KH	47	3

Total weight [g]
50

Table 2

Click here to download Table of Materials/Equipment Table 2.xlsx

Table 2

Men						
Time	1h	2h	3h	4h	24h	48h
	Hydration (% change vs. Oitment base) ± SD					
Ointment base + 2 % KH	+16±15	+14±16	+12±9	+19±14	+11±18	+15±9
Ointment base + 4 % KH	+6±19	+1±18	+5±10	+7±16	+11±9	+14±15
Ointment base + 6 % KH	-3±25	-4±14	-7±18	-4±17	+11±14	-17±14
	TEWL (% change vs. Oitment base) ± SD					
Ointment base + 2 % KH	-20±15	-20±22	-11±21	-20±21	-23±20	-21±17
Ointment base + 4 % KH	-28±12	-29±20	-28±20	-28±24	-47±20	-36±20
Ointment base + 6 % KH	-36±16	-41±21	-31±17	-36±17	-53±20	-54±17
	pH					
Control	4.7±0.5	5.1±0.4	4.9±0.4	5.1±0.3	4.6±0.5	4.8±0.7
Ointment base	4.8±0.5	5.1±0.3	4.9±0.3	5.0±0.4	4.6±0.4	5.0±0.6
Ointment base + 2 % KH	5.0±0.6	4.8±0.4	4.9±0.5	4.9±0.5	4.7±0.3	5.0±0.6
Ointment base + 4 % KH	4.8±0.5	4.9±0.3	4.8±0.4	4.8±0.3	4.7±0.5	4.8±0.5
Ointment base + 6 % KH	4.7±0.5	5.0±0.2	4.9±0.4	4.8±0.4	4.8±0.6	5.0±0.6

Table 3

Click here to download Table of Materials/Equipment Table 3.xlsx

Table 3

Women						
Time	1h	2h	3h	4h	24h	48h
	Hydration (% change vs. Oitment base) ± SD					
Ointment base + 2 % KH	+22±7	+15±6	+15±8	+12±9	+14±14	+18±9
Ointment base + 4 % KH	-0.4±4	-6±5	-2±5	+1±7	+10±13	+15±10
Ointment base + 6 % KH	-12±5	-14±2	-9±7	-5±9	+8±12	+10±9
	TEWL (% change vs. Oitment base) ± SD					
Ointment base + 2 % KH	-32±1.6	-16±3.0	-12±1.3	-20±0.9	-35±1.9	-38±1.6
Ointment base + 4 % KH	-41±1.1	-37±2.7	-24±0.8	-34±0.9	-44±1.5	-38±1.9
Ointment base + 6 % KH	-50±1.4	-39±2.2	-29±0.7	-39±0.9	-16±2.4	-33±2.1
	pH					
Control	5.0±0.7	5.3±0.3	4.9±0.7	5.0±0.5	5.0±0.8	4.7±0.7
Ointment base	5.2±0.6	5.3±0.3	5.2±0.7	5.0±0.4	5.1±0.8	4.8±0.7
Ointment base + 2 % KH	5.4±0.7	5.1±0.4	4.9±0.4	5.1±0.7	4.9±0.7	5.0±1.0
Ointment base + 4 % KH	5.2±0.7	5.1±0.3	5.0±0.4	4.9±0.4	5.1±0.6	5.1±0.2
Ointment base + 6 % KH	5.2±07	5.2±0.2	5.0±0.4	5.0±0.3	5.4±0.6	5.2±0.4

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Author(s): P. MOKREJŠ, M. HUTTA, J. PAVLAČKOVÁ, P. EGNER

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Rebuttal Comments - JoVE56254

Dear Editor and Reviewers,
Below you will find comments referring to the revisions made to manuscript **JoVE56254** ***Preparation of keratin hydrolysate from chicken feathers and its application in cosmetics***.

Revisions in the manuscript are highlighted in red color.

General Formatting:

1) JoVE is unable to publish manuscripts containing commercial sounding language…. Here are some examples from your manuscript - lipex, savinase, Excel 2010.

Revisions made in the manuscript:

„Lipex 100T“ was removed and general statement “lipolytic enzyme” is used

“Excel 2010” was replaced by “table processor”

“Savinase Ultra 16 L“ was removed and general statement “proteolytic enzyme” is used

2) If you are re-using figures from a previous publication…

Answer: no figurers from previous publications are included in the manuscript; all 3 figures are new ones.

3) Please copy-edit the entire manuscript for any grammatical errors you may find….

Answer: the language and grammar was reviewed.

4) Please provide email address of all authors on line 12

Answer: E-mail addresses of all co-authors were added.

5) Please add all the reagents and software to the Table of materials – cutting mill

Answer: software equipment (Microsoft Office 2010) and cutting mill (PULVERISETTE 19) are included in “JOVE_Materials” list.

Protocol:

1) Line 143 – was there any approval taken for use of animal products on humans? How were the individuals recruited? Please add the ethics statement here

Answer: There was no approval with respect to using animal products on humans. Keratin hydrolysate is a common cosmetic ingredient used in hair-care products (shampoos, conditioners etc.). Volunteers were recruited among employees and students of our university. The method of selecting was conducted according to “International Ethical Guidelines for Biomedical Research Involving Human Subjects. Council for International Organizations of Medical Sciences, Geneva (2002).“ This is stated in last paragraph of „Introduction“.

2) Line 147 – what is the approximate weight of feathers?

Answer: the weight of feathers depends on the amount of processed feathers. In a laboratory experiment the weight of feathers was approximately 50 g

3) Line 160 – what sized container is used for this? 2-liter beaker, 10 liter bucket, etc?

Answer: a stainless steel 27-L container is used; this is added in the manuscript.

4) Line 161 – what is used for stirring?

Answer: an overhead stirrer is used; this is added in the manuscript

5) Line 166 – how do you measure the pH?

Answer: pH is measured with a laboratory WTW bench pH/mV meter; this equipment is included in the material/equipment list.

6) Line 168 – is the stirring done using an automated magnetic stirrer?

Answer: no, the stirring is done with overhead stirrer; this is added in the manuscript.

7) Line 171 – what is the sieve size?

Answer: the sieve size is 100 µm; this is added in the manuscript.

8) Line 185 – what is the boiling point?

Answer: the boiling point was 100 ^{o}C; this is added in the manuscript.

9) Line 185 – how do you heat the mixture?

Answer: the mixture was heated in the same stainless steel 27-L boiler container; this is added in the manuscript

10) Do you refer to the solution prepared in step 1.5 as KH? Please specify

Answer: yes. This is specified in the text.

11) Line 192 – what is the size of the container?

Answer: plastic bucket 26 cm in diameter and 26 cm high is used; this is added in the manuscript.

12) Line 194 – what is the volume of water used for dialysis?

Answer: the volumes are as follows: 400 mL of solution of KH against 4 L of distilled water.

13) Line 203 – label as note

Answer: it was labeled as note in the manuscript.

14) Line 204 – alcohol

Answer: the misspelling was corrected.

15) Line 207 – please provide detailed instructions of how to prepare these formulations. You may include a separate table

Answer: Commercial hydrophilic oil in water (O/W) cream base was used for preparing cosmetic formulations with keratin hydrolysate; this is added in the manuscript. Separate table (Table 1) referring to preparation of formulation is included in the manuscript.

16) Line 209 – please provide an example of how to achieve this 50 g final formulation

Answer: This is clearly specified in Table 1.

17) Line 213 – what do you mean by "condition them"?¨

Answer: Conditioning formulations for 2 h after withdrawing from ice-box means to warm them up to a room temperature to simulate common usage of cosmetic formulations.

18) Line 218 – lable as note

Answer: it was labeled as note in the manuscript.

19) Line 231 and rest of the protocol – SI units mL

Answer: in all protocol "mL" instead of "ml" is used.

20) Notes should start on a new line. Do not highlight notes.

Answer: all notes start on a new line and are not highlighted.

Results:

1) line 249 – please provide a representative picture of KH

Answer: Figure 2 showing representative picture of keratin hydrolysate is added in the manuscript.

2) line 259 – how many men and women volunteers?

Answer: 10 men and on 10 women; this is added in the manuscript.

Figure legends:

1) Expand the figure legends, add information about number of volunteers, presentation of values [SD or SEM], was any statistical analysis performed?

Answer: required information is added. Readings we processed by basic numerical characteristics of the descriptive statistics. SD is calculated.

Discussion:

1) Please expand the discussion so that it covers the following in detail and in paragraph form: 1) modifications and troubleshooting, 2) limitations of the technique, and 3) critical steps within the protocol.

Answer: The discussion was expanded as suggested in paragraph forms.

2) Is it possible to provide long term analysis – 7 days or longer? If not, insert comments here
Answer: In "Discussion" there is a statement "Longer-term analysis (more than 2 days) was not accomplished."

Figures:

1) The difference between control, OB and other groups is not clear based on figure 1. will it be possible to include high resolution images for each group?
Answer: New Figure 1 with high resolution images for each group is included.

Reviewers' comments:

Reviewer #1:

Minor Concerns:

● The inclusion of electrophoresis or chromatography data showing the MW distribution would be very useful.
Answer: Gel electrophoresis data (SDS-PAGE) showing the molecular weight distribution of KH is shown in Figure 3.

● lines 160 - 162: The activity of the enzyme should be considered when determining how much to use. (related to weight of dry feathers)
Answer: the activity of enzyme is 100 KLU/g; this is added in the manuscript. The activity is added to "JOVE_Materials" list as well. Note: KLU = kilo lipase unit

● line 197: Give some suggestion on ratio of solution volume to plate area.
Answer: ratio of solution volume to plate area is 500 mL / 1,000 cm^2

●lines 212 - 213: Are there other options if a digital stirrer is not available? With nondigital stirrer, how can one estimate the correct speed, and how important is it?
Answer: Homogenizing the mixture of ointment base with keratin hydrolysate can be done with non-digital stirrer as well. On non-digital stirrer there are scales with approximate speed (in rpm) as well. Gentle stirring is the best way how to do this step.

● line 339: Where is the evidence for the broad MW distribution?
Answer: This is obvious from Figure 3 „SDS-PAGE of keratin hydrolysate and protein standards" which is included in manuscript.

● line 340: What is the basis for "We believe".
Answer: The basis is from the broad MW distribution of keratin hydrolysate. The higher values for

hydration of the skin can be assigned to low molecular weight fractions of keratin hydrolysate penetrating to the epidermis and helping to bind water in epidermis layer. On the other hand, higher molecular weight fractions of keratin hydrolysate create thin film on the outer layer of epidermis and thus enhance barrier properties of epidermis which leads to lower trans-epidermal water loss.
It might be better to replace “We believe” by “We suggest” – this is corrected in the manuscript.

Additional Comments to Authors:

The manuscript can be understood, but is not easily readable for several reasons. Please edit the language. Some suggestions, not all inclusive:

● lines 49-50: The numbers are too precise for what are really estimates, try 62.5 billion, etc.

Answer: The numbers were corrected.

● lines 59 and 61: mixed US and UK English usage in disulfide and sulphitolysis

Answer: The text was corrected for US English only.

● line 148: Place the feathers on a flat plate and dry overnight at 50 °C.

Answer: Our previous sentence was replaced by this suggested one.

● lines 192-193: Dialyze keratin hydrolysate using 12K MWCO membrane to remove small peptides and salts.

Answer: Our previous sentence was replaced by this suggested one.

● lines 224 - 225: Apply two strips to the inner side of the right forearm, and three to the inner side of the left forearm.

Answer: Our previous sentence was replaced by this suggested one.

● line 236: readings at 1, 2, 3, 4, 24 and 48 hours would be 6 points, make it clear that 5 readings are taken at each site and each interval.

Answer: The sentence was revised to make it clear as suggested.

● In several place, explanations are too wordy. Proof read the manuscript carefully, watch for American grammar, agreement of nouns and verbs with respect to singular and plural, consistency of verb tense, and spelling, especially of author's names in the reference list.

Answer: The manuscript was proofed again.

Reviewer #2:

Major Concerns:

● The pollution of hydrolysis with alkaline pretreatment would be a imperfection.

Answer: After hydrolysis keratin hydrolysate is dialyzed to remove slats (and small peptides) so there will be no problem with alkaline pollution. In addition, pH of 1.0% solution of KH is 5.3 and fulfils requirements for cosmetic-grade hydrolysates. This is added in the manuscript.

Minor Concerns:

● If there is fluorence of the keratin and it may have some effect for sun screen.

Answer: The effect of keratin hydrolysate for sun screen is the matter of further research.

Additional Comments to Authors:

● The mould proof may need for the keratin solution.

Answer: The mould proof for the keratin solution is not included. After hydrolysis, KH solution was heated to the boiling point to inactivate enzyme and to sterilize it. Then, KH solution is dried and in further steps dry powder is used.