Simultaneous quantification of 2-pyrrolidone-5-carboxylic acid (PCA), urocanic acid (UCA), and histidine (His) in the stratum corneum by HPLC-PDA

Caroline Saur Santos, André Rolim Baby, Felipe Rebello Lourenço *

Pharmacy Department, Faculty of Pharmaceutical Sciences, University of São Paulo Av. Prof. Lineu Prestes, 580 – Bloco 15 – CEP 05508-000, São Paulo, SP, Brazil

ARTICLE INFO

ABSTRACT

Located in the epidermis, the stratum corneum is the most superficial layer of the skin, acting as a barrier against aggression from the external environment, preventing dehydration, and maintaining the water balance of the skin. The stratum corneum contains the Natural Hydration Factor (NMF), a mixture of hygroscopic molecules derived from filaggrin. The NMF includes 2-pyrrolidone-5-carboxylic acid (PCA), urocanic acid (UCA), and histidine (His), target biomarkers that were extracted by tape-stripping from the stratum corneum of participants for quantification in HPLC-PDA. By extracting the stratum corneum, we developed a protocol to optimize, through audience definition, the quantification of NMF biomarkers. Chromatographic analysis was performed using a YMC-Triart C18 chromatographic column, with a gradient elution of mobile phase composed of triethylammonium phosphate and acetonitrile mixture, and a photodiode array detector. HPLC-PDA procedure was selective, linear (in the range from 0.2 to 5.0 μg/mL), accurate (recovery from 92.7 to 115.1 %), precise (RSD from 0.3 to 12.1 %), and with proper detection and quantification limits. The measurement uncertainty was evaluated from validation data, with combined standard uncertainty values of 0.025–0.12 μg/mL (2.1–5.6 %), 0.004–0.28 μg/mL (2.4–12.6 %), and 0.016–0.16 μg/mL (3.2–7.9 %) for His, PCA, and UCA, respectively. Statistical analyses were performed using Monte Carlo simulation and the Mann-Whitney test, as our results were not homoscedastic and deviated from normality. The results indicate that the best audience for quantifying biomarkers were participants up to 35 years old, with all phototypes, and, preferably, female.

1. Introduction

The stratum corneum comes from keratinocytes and makes up the most superficial layer of the skin, located in the epidermis [1–3]. This barrier protects the body from dehydration, maintains water balance, and protects it from the external environment [1,2,4]. Composed of corneocytes and an intercellular lipid bilayer matrix, the stratum corneum depends on water to fulfill its functions; therefore, keeping it within the physiological range is essential [3–5]. Corneocytes are enucleated cells with keratin inside, in addition to amino acids and other small molecules that, together, are called Natural Moisturizing Factor (NMF) [3]. NMF is a mixture of highly hygroscopic molecules derived from filaggrin, a protein that is rich in histidine (His) (Fig. 1), which recovers keratin filaments [3,6]. Filaggrin, upon reaching the uppermost layer of the skin, undergoes proteolysis, forming free amino acids that will later compose NMF amino acids and their derivatives, such as 2-pyrrolidone-5-carboxylic acid (PCA) and urocanic acid (UCA) (Fig. 1) [3]. In addition to these components, NMF includes lactates, urea, and electrolytes [3]. Urea, another compound of NMF, is also a well-established active ingredient. It has been used for many years to hydrate skin and treat dermatological disorders [7–9]. In this study, we use a urea gel formulation as a potential stimulator for the biomarkers PCA, UCA, and His.

Some components of NMF may be present in other tissues, such as filaggrin. In addition to being in skin [6], filaggrin can also be found in oral and nasal mucosa [10]. Urocanic acid is naturally present in the skin in the *trans*-conformation (*t*UCA), and upon contact with UVB radiation, it transforms into *cis*- (*c*UCA). In this way, urocanic acid has the function of protecting from UVB radiation. In this study, only *t*UCA was considered. PCA is found in the skin as well as plasma and cerebrospinal fluid [11]. PCA is the most hygroscopic and one of the most important components of the NMF, corresponding to approximately 12 % of NMF [1].

---

* Corresponding author.
E-mail address: feliperl@usp.br (F. Rebello Lourenço).

Although the components are present in different human body tissues, only methodologies restricted to dermal analysis were considered to enable optimization of NMF analyses. To develop this study, research by Dapic et al. [12], Hermann and Abeck, [13]; Kezic et al. [14], and Koppes et al. [6] were used as methodological references and adaptations were made as necessary.

The literature contains various analytical procedures quantifying the dermatological markers PCA and UCA. The most commonly used technique is high-performance liquid chromatography [5,10,11,15–18]. However, other techniques, such as Direct Analysis in Real Time [19,20], Raman spectroscopy, and electrophoresis [15], are also described in the literature. Although several analytical procedures are described in the literature, studies were not found that focus on how population characteristics (such as gender, age range, and phototype) affect the determination of the dermatological markers in question to optimize the analysis.

NMF represents 20–30 % of the dry weight of the stratum corneum, which is composed of approximately 20 % water [3]. The percentage of water present in the stratum corneum is linked to the water retention capacity of the NMF [3]. Some studies report that low levels of NMF are often related to dry, scaly skin, atopic dermatitis, psoriasis [5], and even skin cancer (in the case of UCA), demonstrating the importance of developing new approaches to detecting NMF components. In this investigation, we aimed to develop a protocol to optimize, through audience definition, the determination of pyrrolidone carboxylic acid, urocanic acid, and histidine in the stratum corneum using high-performance liquid chromatography with photodiode array detector (HPLC-PDA).

2. Material and methods

Urea (cosmetic grade) was obtained from Petrobras (Rio de Janeiro, Brazil), and 2-pyrrolidone-5-carboxylic acid, 4-imidazoleacrylic acid (HPLC grade), L-histidine (analytical grade), hydrochloric acid (analytical grade), and triethylammonium phosphate (TEAP) (analytical grade) 1 M solution were obtained from Merck Brazil (São Paulo, Brazil). Disodium EDTA was obtained from Shijiazhuang Jackchem Co. Ltd. (Shijiazhuang, China); methylparaben was obtained from UENO Fine Chemical (Osaka, Japan); and imidazolidinyl urea was from Wuhu Huahai Bio. Engineering Co., Ltd. (Wuhu, China), all supplied in Brazil by Embacaps Química e Farmacêutica LTDA (São Paulo, Brazil). Ammonium acryloyldimethyltaurate/VP copolymer (Aristoflex® AVC) was obtained from Clariant (Muttenz, Switzerland) and supplied in Brazil by Pharma Special (Campinas, Brazil). Analytical Grade acetonitrile was obtained from Honeywell Brazil (São Paulo, Brazil). MilliQ® water was obtained from a Merck apparatus.

2.1. Study sample

Twenty-four participants (7 men and 17 women) between 19 and 58 years old were enrolled in the study. None of the participants had dermatological disorders or allergies. The study protocol was performed following the Declaration of Helsinki (2013) [21] and was previously approved by the Ethics Committee of the FCF-USP (CAAE: 45264021.1.0000.0067). Furthermore, all participants were asked not to apply any moisturizing product to the skin of their forearms for at least 24 h before the study. The subjects remained in an environment at room temperature (25 °C) throughout the analysis. A gel with urea was used to stimulate skin hydration and facilitate the visualization of HPLC of stratum corneum biomarkers during analysis. The forearm of each participant was divided into 3 delimited areas of 2x5 cm. From these 3 randomly delimited areas, each participant provided one sample of stratum corneum from untreated skin (control group), another sample of stratum corneum after the application of a neutral gel (neutral group), and another sample of stratum corneum after the application of a gel containing 10 % urea (urea group). Urea is one of the components of NMF, corresponding to approximately 7 % of it. When used as an active ingredient in moisturizing formulas, urea regulates transepidermal water loss, in addition to attracting water and maintaining skin. The gel placebo and urea gel formulas used were prepared as described in Table 1.

After 2 h, tape-stripping was performed using 10 tapes at each site. To avoid sample loss, all tapes were used in quantification analysis. The tapes were placed in Falcon tubes with 5 ml of 0.001 N HCl solution and shaken (Merse) for 1 min, followed by an ultrasound bath (Solidteel) for 20 min. Then, the samples were filtered using a 0.22 μm filter and analyzed (in triplicates) using a high-performance liquid chromatography (HPLC) (Thermo Scientific Accela) equipped with a quaternary pump, an autosampler, and a photodiode array detector (PDA detector). A YMC-Triart C18 chromatographic column (100 x 3.0 mm, 1.9 μm), with pre-column, was used. The chromatographic runs were performed using stepwise gradient elution. The mobile phase of TEAP 0.01 M was used from minutes 1 to 5. However, minutes 0 to 5 were analyzed to identify the UCA, PCA, and His markers. Between minutes 5 to 7, we identified some interfering factors, which led us to use acetonitrile from minutes 8 to 14 (Table 2). This prevented the interfering factors from influencing the next reading. A 5 mL sample was injected with a flow rate of 400 μL/mL. The samples were monitored at 210 nm for His and PCA and at 268 nm for UCA.

2.2. Measurement with Corneometer®

Tests with a Corneometer® were conducted by an external company with 12 people (men and women) aged 22 to 53 with phototypes II to IV, according to the Fitzpatrick scale. The inclusion and exclusion criteria

Table 1
The gel placebo and 10% urea gel formulas used in the stratum corneum study.

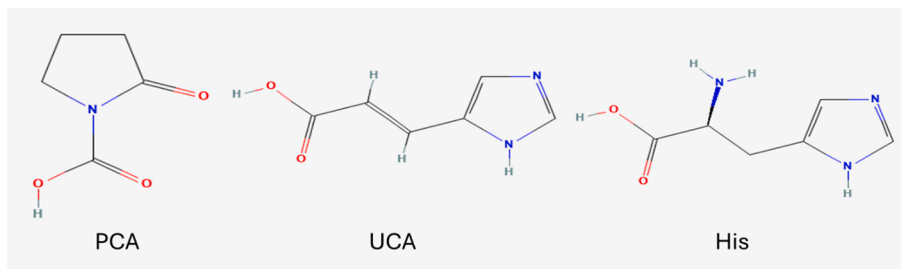|	Placebo gel (neutral group)	10 % urea gel (Urea group)
Aristoflex® AVC	1.0 %	1.0 %
Urea	–	10.0 %
Disodium EDTA	0.1 %	0.1 %
Methylparaben	0.1 %	0.1 %
Imidazolidinyl Urea	0.5 %	0.5 %
Water	q.s.p	q.s.p.

Fig. 1. Chemical structure of 2-pyrrolidone-5-carboxylic acid (PCA), urocanic acid (UCA), and histidine (His) [29–31].

Table 2
Gradient elution of the chromatographic runs.

Time	Triethylammonium phosphate (TEAP) 1 M solution	Acetonitrile
0–7 min	100	0
7–8 min*	100–0	0–100
8–14 min*	0	100

*Used for cleaning the chromatographic column after the elution of biomarkers.

were the same as those used to carry out the tape-stripping. Participants underwent a 30 min acclimatization period at a temperature of $20 \pm 2\,°C$ and relative humidity of $50 \pm 5\,\%$ before the start of measurements. For this test, two sites were defined on the forearm (right or left) of each volunteer, one for the application of the neutral gel and the other for the gel with urea, each evaluation site measuring 3 x 3 cm. Next, baseline corneometry measurements were performed in quintuplicate to assess skin hydration, using a Corneometer® probe coupled to the Multi Probe Adapter, MPA 580, equipment (CK electronics, Germany). The unit of hydration assessment is given in arbitrary corneometric units. After the baseline measurement, the test products were applied, and new hydration measurements were taken after 2 h in quintuplicates. A coefficient of variation between measurements (quintuplicate per site per time) of a maximum of 10 % was accepted. Otherwise, the measurement was repeated.

Corneometer® was used as a reference in measuring skin hydration, which was subsequently compared with the results of quantifying the markers PCA, *t*UCA, and His by HPLC-PDA in human stratum corneum extracted using the tape-stripping technique.

2.3. HPLC-ex vivo protocol analytical procedure validation

The HPLC-PDA procedure was validated by checking specificity, matrix effect, linearity/linear range, trueness (recovery), precision (repeatability and intermediate precision), carry-over, stability, and limits of detection and quantification (LOD/LOQ), according to the ICH guideline M10 on bioanalytical method validation and study sample analysis [22].

To verify the method specificity, a mixture of PCA, UCA, and His, in the presence and absence of tape-stripping (Magic Scotch), were subject to HPLC analysis. Linearity was checked by analyzing PCA, UCA, and His standard solution at concentrations of 0.2, 0.5, 1.0, 2.0, 4.0, 8.0, and 10 μg/mL. The HIS, PCA, and UCA standards were weighed individually and dissolved in 0.001 N HCl. Serial dilutions were then performed to obtained calibration solution with a pool of standards (HIS, PCA, and UCA) at different concentrations (0.2, 0.5, 1.0, 2.0, 4.0, 8.0, and 10 μg/mL). These standards were added to Falcon tubes containing 50 cm tapes and sample matrix to assess the matrix effect. This effect was evaluated by analyzing three replicates prepared using a matrix from different sources at low (0.2 μg/mL), medium (1.0 μg/mL), and high (5.0 μg/mL) concentrations. Recovery was determined by the ratio of the average area of the spiked samples (at three levels – 0.2, 1.0, and 5.0 μg/mL) and the average area of standard solutions. The standard deviations of spiked samples (at three levels – 0.2, 1.0, and 5.0 μg/mL) were calculated to assess the precision, with repeatability (nine replicas analyzed by same analyst, same instrument, same day) and intermediate precision (same instrument, same analyst, on two different days) conditions. Trueness and precision reference values were defined from AOAC [23,24]. A carry-over study was performed by testing a blank sample following the highest calibration standard. The stability of sample solutions was tested for 24 h at room temperature. The limits of detection and quantification were estimated as 3 and 10 times, the ratio of intercept standard deviation, and the slope of linear equation, respectively.

2.4. Measurement uncertainty evaluation

Measurement uncertainty evaluation was performed using a top-

down approach based on method validation data. Combined standard uncertainty (u_c) was calculated as described in Eq. 1, based on uncertainty from trueness (u_b, from recovery studies of spiked simulated samples) and uncertainty from precision (u_p, from repeatability/intermediate precision studies).

$$u_c = \sqrt{u_b{}^2 + u_p{}^2} = \sqrt{(R - 100)^2 + s_p{}^2} \quad (1)$$

where R is the mean recovery value and s_p is the standard deviation from repeatability/intermediate precision.

Biomarker concentrations (PCA, UCA, and His) obtained for the urea, control, and neutral groups for each volunteer were compared using Monte Carlo simulation. Monte Carlo method was implemented in MS-Excel, using the equation "=INV.NORM(RAND();X;uX)", where X and uX are the measured value and respective measurement uncertainty of each biomarker (PCA, UCA, or His) from each group (urea, control, or neutral) of each volunteer. P-value of pairwise comparisons (between urea and control groups, urea and neutral groups, and control and neutral groups) were obtained from 50,000 Monte Carlo simulations.

2.5. Moisturizer application and measurement protocol

The participants were acclimatized to room temperature $(+/-25\,°C)$ for approximately 30 min. On the volar part of the forearm, local cleaning was carried out only with a dry gaze to avoid sample loss, and then three locations were delimited, measuring 2×5 cm each. To allow the measurement of hydration itself over time, 20 mg of placebo gel (neutral group) or 10 % urea gel (urea group) was applied on the predetermined sites, and the third predetermined site was kept without any product. After 2 h, stratum corneum samples were removed from each of the three sites with 10 strips, totaling 30 strips per volunteer. To carry out the test, all options were considered to avoid sample loss.

3. Results and discussion

3.1. Chromatographic method for simultaneous quantification of PCA, UCA, and His in ex vivo samples

First, samples from the tape-stripping tapes were subject to chromatographic analysis (Figs. 2A and 3A) to identify possible interference. Then, stripped tapes spiked with PCA, UCA, and His reference standards in the range from 0.2 to 10 μg/mL were subject to chromatographic analysis (Figs. 2B and 3B). Finally, the tapes from participants were subject to chromatographic analysis. A representative chromatogram of stripped tapes from subjects is provided in Figs. 2C and 3C. In the results from the chromatogram, the peaks associated with dermal markers could be distinguished from the interfering peaks through different retention times. Therefore, the method presented satisfactory specificity. Using previous studies as a reference [5,25], the peaks after a retention time of 6 min were considered interfering; thus, we chose to use a gradient with acetonitrile to avoid possible errors in the following analyses. To keep the column free from interference, acetonitrile was used after 8 min of running, therefore, the peaks presented after this time are related to it. For this reason, chromatograms with standards and samples from subjects were not analyzed after 8 min.

The HPLC-PDA *ex vivo* procedure was validated considering the specificity, matrix effect, linearity, trueness (recovery), precision (repeatability and intermediate precision), carry-over, stability, and LOD/LOQ according to criteria defined by AOAC and ICH guidelines (Table 3) [22–24]. No analyte response alteration was detected due to interfering components in the sample matrix. Table 3 presents the regression equations (Y) and the coefficient of determination (R2) obtained for His, PCA, and UCA in the 0.25 to 10 μg/ml range. The values demonstrated that the linear model used was adequate since the coefficient of determination was greater than 0.99. The accuracy of the method for concentrations from 0.2 to 5.0 μg/ml, according to the AOAC [24], must be between 75 and 120 %. The mean recovery values
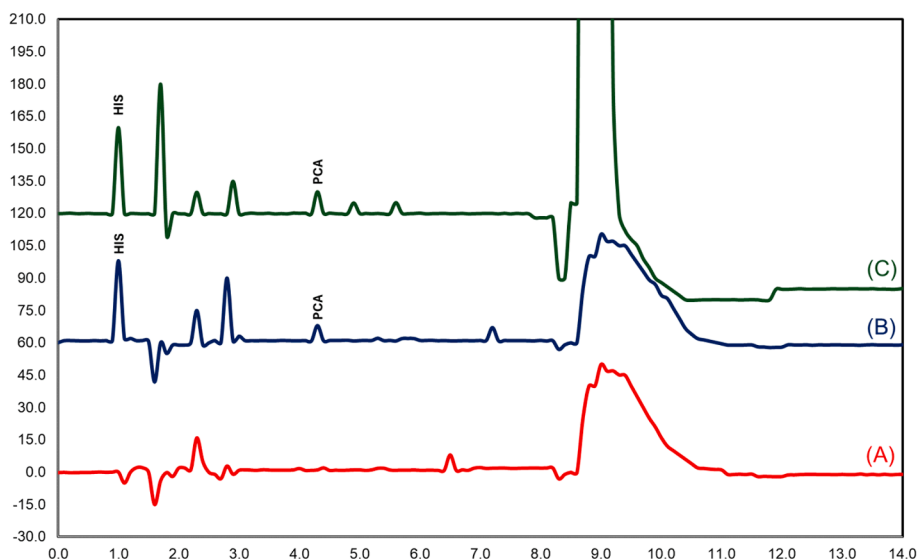
Fig. 2. Chromatogram in a short run (8 min) of 268 nm: A) isolated tape-stripping; B) UCA standards (1 µg/ml) in the presence of the tape; C) participant's tape-stripping skin (without the presence of gel).
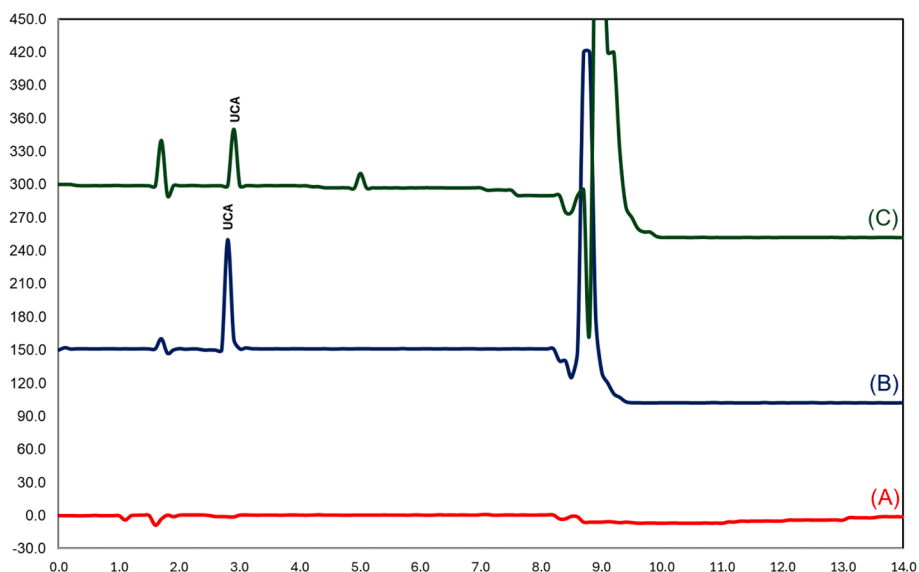

Fig. 3. Chromatogram in a short run (8 min) of 210 nm: A) isolated tape-stripping; B) PCA and His standards (1 µg/ml) in the presence of the tape; C) participant's tape-stripping skin (without the presence of gel).

obtained at different concentrations for His, PCA, and UCA were within the value required by AOAC, ranging from 100.2 to 102.1 % for His, 99.3 to 103.6 % for PCA, and 98.3 to 105.6 % for UCA (Table 3). According to the AOAC Guidelines [24], relative standard deviation (RSD) under repeatability and intermediate precision conditions should be less than 8 and 16 %, respectively. Thus, the values obtained for the three analytes at different levels revealed that the HPLC-PDA procedure was precise (1.3–5.2 %, 0.3–12.1 %, and 0.3–5.6 % for His, PCA, and UCA, respectively). Sample solutions were stable for at least 24 h at room temperature. Carry-over in the blank sample followed by the highest calibration standard was below 5 %. The LOD and LOQ were estimated to be 3 to 6 times, respectively, of the ratio of the standard deviation of the intercept at the linear search orientation. A summary of validation data is provided in Table 3.

3.2. Measurement uncertainty evaluation

Measurement uncertainty (i.e., total error) values were found to be 0.025–0.12 µg/mL (2.1–5.6 %), 0.004–0.28 µg/mL (2.4–12.6 %), and 0.016–0.16 µg/mL (3.2–7.9 %) for His, PCA, and UCA, respectively. Bias uncertainty contributed to 1–58 % of overall uncertainty, while the precision uncertainty component was responsible for 42–99 % of overall uncertainty. Concentrations of His, PCA, and UCA obtained from the tape-stripping of participants were expressed with expanded measurement uncertainty value, considering a 95 % coverage level (k = 2).

3.3. Detailed evaluation of HPLC-ex vivo protocol according to the volunteers' profile

The results obtained by both the Corneometer and the HPLC-*ex-vivo* protocol did not demonstrate, in general, that urea has a moisturizing effect when vehiculated in this type of gel. A summary of Corneometer

Table 3
Summary of validation data results of specificity, linearity, trueness, precision, and LOD/LOQ.

Parameter	Criteria	His	PCA	UCA
Specificity	No interference of stripping tape on the determination of His, PCA, and UCA	No interference	No interference	No interference
Matrix effect	No analyte response alteration due to interfering components in the sample matrix	No matrix effect	No matrix effect	No matrix effect
Linearity /Linear range	Linear equation $R^2 > 0.99$ /0.2 to 10 µg/mL	Y=8800 + 135183X $R^2 = 0.9999$ / 0.2 to 10 µg/mL	Y=−780 + 36230X $R^2 = 0.9999$ / 0.2 to 10 µg/mL	Y=26032 + 513058X $R^2 = 0.9999$ / 0.2 to 10 µg/mL
Trueness (recovery)	0.2 µg/ml: 75–120 %	100.2 % (RSD=2.7 %)	103.6 % (RSD=2.7 %)	105.6 % (RSD=3.3 %)
	1.0 µg/ml: 75–120 %	98.3 % (RSD=1.1 %)	102.2 % (RSD=3.9 %)	98.6 % (RSD=0.5 %)
	5.0 µg/ml: 75–120 %	102.1 % (RSD=0.9 %)	99.3 % (RSD=0.4 %)	98.3 % (RSD=0.4 %)
Precision (RSD)				
Repeatability / Intermediate precision	0.2 µg/ml: 8 % / 16 %	1.7 % / 2.1 %	3.5 % / 12.1 %	4.8 % / 5.6 %
	1.0 µg/ml: 8 % / 16 %	1.4 % / 1.4 %	4.6 % / 2.0 %	0.3 % / 4.9 %
	5.0 µg/ml: 8 % / 16 %	1.3 % / 5.2 %	0.3 % / 2.3 %	0.3 % / 2.7 %
Carry-over	Carry-over below 5 %	<5%	<5%	<5%
Stability	Sample solution stable for 24 h at room temperature	Sample solution stable for 24 h at room temperature	Sample solution stable for 24 h at room temperature	Sample solution stable for 24 h at room temperature
LOD*	–	0.03 µg/ml	0.09 µg/ml	0.06 µg/ml
LOQ*	–	0.1 µg/ml	0.3 µg/ml	0.2 µg/ml

*The limits of detection and quantification were estimated as 3 and 10 times, respectively, for the ratio of intercept standard deviation and the slope of linear equation.

and HPLC-*ex-vivo* protocol results is presented in Table 4. These results led to the design of a more precise protocol for this test, in which we segmented the subjects' results into gender, phototype, and age group (up to 35 years old and over 35 years old). The phototype was separated according to the Fitzpatrick scale, considering low-value phototype I to IV and high-value V to IV [26]. In this study, we only worked on *t*UCA; however, the presence of *c*UCA may influence the results, as the participants may have had different sun exposure times.

As the data showed deviations from normality and lack of homoscedasticity, we compared the results using two different approaches. The first approach was the Monte Carlo simulation, where the values presented involve the concentration of each biomarker (His, PCA, or UCA, in µg/mL) for each volunteer, followed by the standard deviation value (Table 5). The second approach employed the non-parametric Mann-Whitney test, where p-values are presented and considered a significance of 5 % (95 % confidence level) (Table 6).

Considering the gender comparisons, the concentration of His was significantly different (p-value < 0.05) between the control and neutral groups for 100 % (17 in 17) of females and 43 % (3 in 7) and males. Comparing the control and urea groups, the difference in the concentration of His was significant (p-value < 0.05) for 76 % (13 in 17) of females and 100 % (7 in 7) of males. When comparing the neutral and urea groups, the difference in the His concentration was significant (p-value < 0.05) for 100 % (17 in 17) of females and 71 % (5 in 7) of males. Furthermore, the concentration of PCA was significantly different (p-value < 0.05) between the control and neutral groups for 82 % (14 in 17) of females and 86 % (6 in 7) of males. When comparing the control and urea groups, the difference in PCA concentrations was significant (p-value < 0.05) for 76 % (13 in 17) of females and 71 % (5 in 7) of males. The comparison between neutral and urea groups indicated that the difference in the PCA concentration was significant (p-value < 0.05) for 82 % (14 in 17) of females and 71 % (5 in 7) of males. Finally, in the comparison between genders, the concentration of UCA was significantly different (p-value < 0.05) between the control and neutral groups

Table 4
Summary of Corneometer and HPLC-ex vivo protocol results.

Parameter	Urea *vs.* Neutral group response (IC 95 %) (n)	*p*-valor
Corneometer	0.285 (−1.85; 2.83) (12)	0.931
HPLC-*ex vivo* protocol		
− histidine	0.1076 (−0.0732; 0.2687) (24)	0.244
− PCA	0.2394 (−0.2632; 0.6506) (24)	0.348
− UCA	0.0468 (−0.0536; 0.1707) (24)	0.327

for 76 % (13 in 17) of females and 57 % (4 in 7) of males. In the comparison of the control and urea groups, the difference in the concentration of UCA was significant (p-value < 0.05) for 82 % (14 in 17) of females and 43 % (3 in 7) of males. When comparing the neutral and urea groups, the difference in the UCA concentration was significant (p-value < 0.05) for 82 % (14 in 17) of females and 43 % (3 in 7) of males.

We also compared the medians of biomarker concentration for the control, neutral, and urea groups considering gender. The comparisons of the concentrations of His between females and males were not significant when divided by the control (p-value $= 0.057$), neutral (p-value $= 0.228$), or urea (p-value $= 0.066$) groups. For PCA, the comparisons between females and males were not significant when divided by the control (0.446), neutral (p-value $= 0.899$), and urea groups (p-value $= 0.253$). Finally, the comparisons of the concentrations of UCA between females and males were not significant when divided by the control (p-value $= 0.568$), neutral (p-value $= 0.374$), or urea (p-value $= 1.000$) groups. Analyzing the concentrations of biomarkers (His, PCA, and UCA) according to gender elucidated that females tend to respond better to this type of study. The box-plot graphs for gender (female and male) comparisons were presented in Fig. 4A.

In the second section, the results obtained from the participants were compared according to their respective phototypes. The concentration of His was significantly different (p-value < 0.05) between the control and neutral groups for 80 % (16 in 20) of lower values of phototypes and 100 % (4 in 4) of higher values of phototypes. When comparing the control and urea groups, His concentration was significantly different (p-value < 0.05) for 80 % (16 in 20) of lower phototypes and 100 % (4 in 4) of higher phototypes. The comparison between the neutral and urea groups indicated that His concentration was significantly different (p-value < 0.05) for 90 % (18 in 20) of lower phototypes and 100 % (4 in 4) of higher phototypes. Furthermore, the concentration of PCA was significantly different (p-value < 0.05) between the control and neutral groups for 85 % (17 in 20) of lower phototypes and 75 % (3 in 4) of higher phototypes. The comparison between the control and urea groups indicated that the PCA concentration was significantly different (p-value < 0.05) for 75 % (15 in 20) of lower phototypes and 75 % (3 in 4) of higher phototypes. When comparing the neutral and urea groups, the PCA concentration was significantly different (p-value < 0.05) for 80 % (14 in 20) of lower phototypes and 75 % (3 in 4) of higher phototypes. Finally, the UCA concentration was significantly different (p-value < 0.05) between the control and neutral groups for 70 % (14 in 20) of lower phototypes and 75 % (3 in 4) of higher phototypes. The comparison between the control and urea groups indicated that UCA

Table 5

Concentration of His, PCA, and UCA and their respective measurement uncertainty (expressed as expanded uncertainty, with a 95 % confidence level, k = 2) for 24 participants with different gender, phototype, and age (phototype values → low: I-IV and high: V-VI; and age: A≤35 years old and B>35 years old).

Gender	Phototype	Age	His (µg/ml)			PCA (µg/ml)			UCA (µg/ml)		
			Control	Neutral	Urea	Control	Neutral	Urea	Control	Neutral	Urea
Female	Low values	B	1.31 (0.03)	1.38 (0.03)	1.12 (0.02)	2.40 (0.07)	2.70 (0.08)	2.37 (0.07)	0.41 (0.03)	0.52 (0.04)	0.46 (0.04)
Male	Low values	B	1.12 (0.02)	0.98 (0.02)	1.10 (0.02)	2.05 (0.06)	2.11 (0.06)	2.50 (0.07)	0.30 (0.02)	0.34 (0.03)	0.48 (0.04)
Male	Low values	B	0.75 (0.02)	0.98 (0.02)	0.75 (0.02)	1.60 (0.05)	2.07 (0.06)	1.68 (0.05)	0.59 (0.05)	0.76 (0.06)	0.63 (0.05)
Male	Low values	B	0.51 (0.01)	0.61 (0.01)	0.46 (0.01)	2.11 (0.06)	2.65 (0.08)	2.00 (0.06)	0.70 (0.06)	0.90 (0.07)	0.71 (0.06)
Male	Low values	A	0.31 (0.01)	0.52 (0.01)	0.31 (0.01)	1.02 (0.03)	2.12 (0.06)	1.47 (0.04)	0.23 (0.02)	0.51 (0.04)	0.34 (0.03)
Male	Low values	A	2.80 (0.06)	2.00 (0.04)	0.60 (0.10)	4.27 (0.13)	3.39 (0.10)	1.14 (0.03)	0.33 (0.03)	0.23 (0.02)	0.04 (0.01)
Female	Low values	A	1.56 (0.03)	2.10 (0.05)	2.20 (0.05)	3.31 (0.10)	4.67 (0.14)	4.78 (0.14)	0.49 (0.04)	0.64 (0.05)	0.64 (0.05)
Male	High values	B	0.52 (0.01)	0.69 (0.01)	0.63 (0.01)	0.74 (0.09)	0.98 (0.12)	0.81 (0.10)	0.10 (0.01)	0.17 (0.01)	0.15 (0.01)
Male	Low values	B	0.62 (0.01)	0.48 (0.01)	0.51 (0.01)	1.99 (0.06)	1.90 (0.06)	2.49 (0.07)	0.41 (0.03)	0.52 (0.04)	0.86 (0.07)
Male	Low values	B	1.88 (0.04)	2.33 (0.05)	1.32 (0.03)	2.12 (0.06)	2.44 (0.07)	1.36 (0.04)	0.33 (0.03)	0.33 (0.03)	0.17 (0.01)
Female	Low values	B	0.52 (0.01)	0.50 (0.01)	0.42 (0.01)	1.79 (0.05)	1.92 (0.06)	1.53 (0.05)	0.17 (0.01)	0.18 (0.01)	0.20 (0.02)
Male	Low values	A	0.37 (0.01)	0.46 (0.01)	0.60 (0.01)	0.71 (0.09)	1.01 (0.03)	1.46 (0.04)	0.06 (0.01)	0.10 (0.01)	0.21 (0.02)
Male	High values	B	1.96 (0.04)	2.09 (0.05)	2.51 (0.05)	3.75 (0.11)	4.16 (0.12)	5.01 (0.12)	0.88 (0.07)	1.00 (0.08)	1.01 (0.05)
Male	High values	B	0.39 (0.01)	0.45 (0.01)	0.31 (0.01)	1.96 (0.06)	2.69 (0.08)	1.58 (0.05)	0.61 (0.05)	0.95 (0.08)	0.46 (0.04)
Male	Low values	A	0.49 (0.01)	0.71 (0.02)	0.40 (0.01)	1.79 (0.05)	2.54 (0.08)	1.30 (0.04)	0.40 (0.03)	0.61 (0.05)	0.27 (0.02)
Female	Low values	B	2.43 (0.05)	1.35 (0.03)	2.14 (0.05)	3.82 (0.11)	2.17 (0.06)	3.43 (0.1)	0.65 (0.05)	0.32 (0.03)	0.55 (0.04)
Female	Low values	B	1.22 (0.03)	1.27 (0.03)	1.33 (0.03)	2.15 (0.06)	2.33 (0.07)	2.72 (0.08)	0.24 (0.02)	0.27 (0.02)	0.33 (0.03)
Male	Low values	B	0.45 (0.01)	1.48 (0.03)	0.93 (0.02)	1.96 (0.06)	7.13 (0.17)	4.88 (0.15)	0.68 (0.06)	2.51 (0.13)	1.74 (0.09)
Female	Low values	B	0.88 (0.02)	1.55 (0.03)	1.18 (0.03)	1.58 (0.05)	2.87 (0.09)	2.30 (0.07)	0.41 (0.03)	0.77 (0.06)	0.58 (0.05)
Male	Low values	B	0.92 (0.02)	1.94 (0.04)	2.06 (0.04)	2.56 (0.08)	4.78 (0.14)	5.04 (0.12)	0.71 (0.06)	1.38 (0.07)	1.28 (0.07)
Female	Low values	B	1.23 (0.03)	1.22 (0.03)	1.08 (0.02)	1.96 (0.06)	1.99 (0.06)	1.90 (0.06)	0.53 (0.04)	0.58 (0.05)	0.57 (0.05)
Male	High values	A	0.68 (0.01)	0.38 (0.01)	0.46 (0.01)	1.75 (0.05)	1.01 (0.03)	1.36 (0.04)	0.42 (0.03)	0.26 (0.02)	0.34 (0.03)
Male	Low values	B	0.85 (0.02)	0.75 (0.02)	0.91 (0.02)	2.78 (0.08)	2.53 (0.08)	2.52 (0.08)	0.76 (0.06)	0.70 (0.06)	0.99 (0.08)
Male	Low values	B	1.17 (0.03)	1.34 (0.03)	1.16 (0.03)	2.37 (0.07)	2.76 (0.08)	2.46 (0.07)	0.69 (0.06)	0.87 (0.07)	0.33 (0.03)

Table 6

Statistical comparisons (*p*-values*) for the concentration of His, PCA, and UCA for the 24 participants with different gender, phototype, and age.

Gender	Phototype	Age	His (p-values)			PCA (p-values)			UCA (p-values)		
			Control *vs.* Neutral	Control *vs.* Urea	Neutral *vs.* Urea	Control *vs.* Neutral	Control *vs.* Urea	Neutral *vs.* Urea	Control *vs.* Neutral	Control *vs.* Urea	Neutral *vs.* Urea
Female	Low values	B	0.0661	0.0000	0.0000	0.0028	0.3622	0.0011	0.0164	0.1453	0.1340
Male	Low values	B	0.0000	0.2165	0.0001	0.2201	0.0000	0.0000	0.1769	0.0002	0.0012
Male	Low values	B	0.0000	0.4864	0.0000	0.0000	0.1291	0.0000	0.0128	0.2778	0.0467
Male	Low values	B	0.0000	0.0002	0.0000	0.0000	0.1036	0.0000	0.0149	0.4554	0.0200
Male	Low values	A	0.0000	0.3144	0.0000	0.0000	0.0000	0.0000	0.0000	0.0002	0.0001
Male	Low values	A	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0013	0.0000	0.0000
Female	Low values	A	0.0000	0.0000	0.0531	0.0000	0.0000	0.2839	0.0063	0.0067	0.4967
Male	High values	B	0.0000	0.0000	0.0025	0.0599	0.3019	0.1391	0.0000	0.0003	0.2210
Male	Low values	B	0.0000	0.0000	0.0068	0.1455	0.0000	0.0000	0.0142	0.0000	0.0000
Male	Low values	B	0.0000	0.0000	0.0000	0.0004	0.0000	0.0000	0.4554	0.0000	0.0000
Female	Low values	B	0.1880	0.0000	0.0000	0.0445	0.0002	0.0000	0.2828	0.0705	0.1870
Male	Low values	A	0.0000	0.0000	0.0000	0.0006	0.0000	0.0000	0.0000	0.0000	0.0000
Male	High values	B	0.0234	0.0000	0.0000	0.0084	0.0000	0.0000	0.1236	0.0620	0.4520
Male	High values	B	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0002	0.0042	0.0000
Male	Low values	A	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0003	0.0003	0.0000
Female	Low values	B	0.0000	0.0000	0.0000	0.0000	0.0047	0.0000	0.0000	0.0595	0.0000
Female	Low values	B	0.1077	0.0036	0.0754	0.0240	0.0000	0.0003	0.1410	0.0020	0.0328
Male	Low values	B	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
Female	Low values	B	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0019	0.0077
Male	Low values	B	0.0000	0.0000	0.0276	0.0000	0.0000	0.0867	0.0000	0.0000	0.1520
Female	Low values	B	0.3708	0.0000	0.0000	0.4004	0.2187	0.1516	0.2251	0.2884	0.4210
Male	High values	A	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0270	0.0075
Male	Low values	B	0.0000	0.0104	0.0000	0.0128	0.0103	0.4698	0.2319	0.0087	0.0011
Male	Low values	B	0.0000	0.4243	0.0000	0.0002	0.1813	0.0032	0.0221	0.0000	0.0000

*p-values were determined using Monte Carlo simulations based on the comparisons of the concentration values and their respective measurement uncertainty values. Group A indicates age ≤ 35, and Group B>35.

concentration was significantly different (p-value < 0.05) for 70 % (14 in 20) of lower phototypes and 75 % (3 in 4) of higher phototypes. Comparing the neutral and urea groups indicated that UCA concentration was significantly different (p-value < 0.05) for 75 % (15 in 20) with lower phototypes and 50 % (2 in 4) of participants with higher phototypes.

When comparing the medians of biomarker concentrations in the control, neutral, and urea groups, considering the phototypes, none of the comparisons was statistically different. The p-values for the comparisons of the His concentration between lower and higher phototypes were 0.561, 0.201, and 0.462 when divided by the control, neutral, and urea groups, respectively. When comparing the PCA concentrations
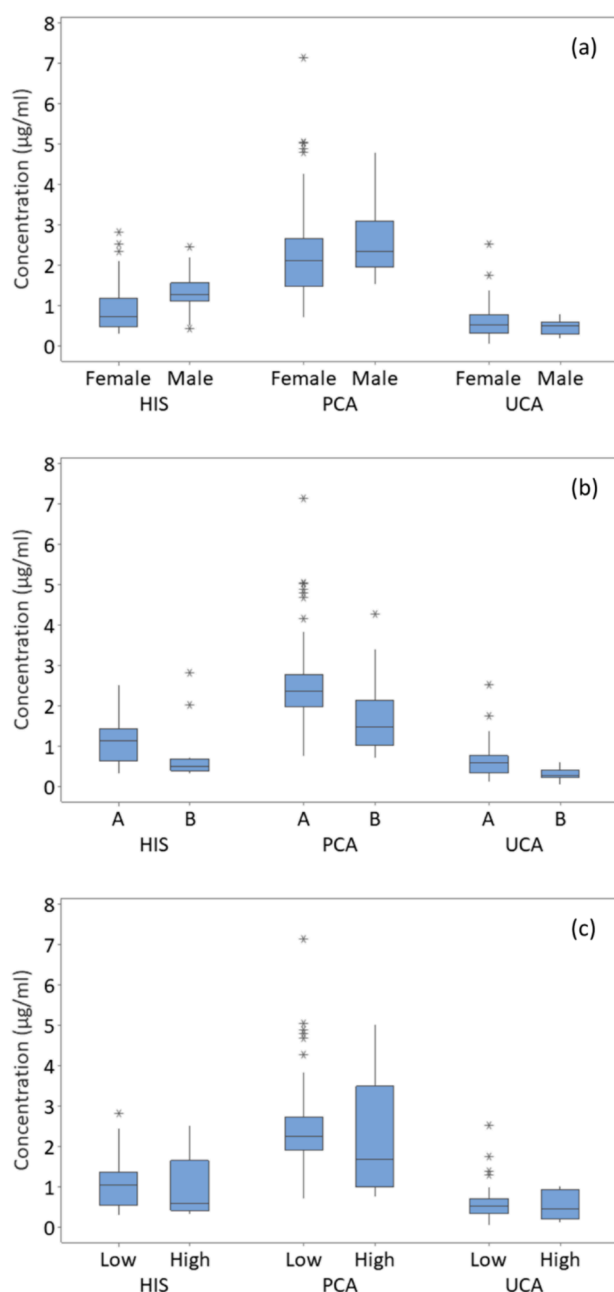
Fig. 4. Box-plot graphs of HIS, PCA, and UCA concentration by (a) gender (female and male), (b) age (groups A and B), and (c) phototype (low and high).

between lower and higher phototypes, the *p*-values were 0.416, 0.510, and 0.333, when divided by the control, neutral, and urea groups, respectively. Finally, the *p*-values for the comparisons of the UCA concentration between lower and higher phototypes were found to be 0.670, 1.000, and 0.727, when divided by the control, neutral, and urea groups, respectively. In this second section, no statistical significance was observed in the comparisons between the medians. However, there was a slight tendency toward more significant results in subjects with higher phototype values. As the difference between the lower and higher phototype groups was small, and as the group of participants with a higher phototype was small, it was not possible to verify any difference between the groups. The comparisons between low and high phototype were presented in Fig. 4B.

In the last section, the results were compared according to two age groups: up to 35 years old (group A) and over 35 years old (group B). The concentration of His was significantly different (*p*-value < 0.05)

between the control and neutral groups for 100 % (5 in 5) of group A and 79 % (15 in 19) of group B. When comparing the control and urea groups, His concentration was significantly different (*p*-value < 0.05) for 80 % (4 in 5) of group A and 84 % (16 in 19) of group B. The comparison between the neutral and urea groups indicated that the His concentration was significantly different (*p*-value < 0.05) for 100 % (5 in 5) of group A and 89 % (17 in 19) of group A. In addition, the PCA concentration was significantly different (*p*-value < 0.05) between the control and neutral groups for 100 % (5 in 5) of group A and 79 % (15 in 19) of group B. The comparison between the control and urea groups indicated that the PCA concentration was significantly different (*p*-value < 0.05) for 100 % (5 in 5) of group A and 68 % (13 in 19) of group B. When comparing the neutral and urea groups, the PCA concentration was significantly different (*p*-value < 0.05) for 100 % (5 in 5) of group A and 74 % (14 in 19) of group B. Finally, the UCA concentration was significantly different (*p*-value < 0.05) between the control and neutral groups for 100 % (5 in 5) of group A and 63 % (12 in 19) of group B. The comparison between control and urea groups indicated that UCA concentration was significantly different (*p*-value < 0.05) for 100 % (5 in 5) of group A and 63 % (12 in 19) of group B. When comparing the neutral and urea groups, UCA concentration was significantly different (*p*-value < 0.05) for 100 % (5 in 5) of group B and for 63 % (12 in 19) of group B.

Some statistical differences were found in medians of biomarker concentrations in the control, neutral, and urea groups, considering the age groups. The *p*-values for the comparisons of His concentration between groups A and B were found to be 0.115 and 0.118, when divided by the control and neutral groups, respectively. However, the *p*-value for the comparisons of His concentration between group A and group B was significant (*p*-value = 0.013), when divided by the urea group. When comparing the PCA concentrations between groups A and B, the *p*-values were found to be 0.155 and 0.320, when divided by the control and neutral groups, respectively. However, the *p*-value for the comparisons of PCA concentration between groups A and B was significant (*p*-value = 0.004), when divided by the urea group. Finally, the *p*-values for the comparisons of UCA concentration between groups A and B were found to be statistically significant (0.047, 0.047, and 0.028, when divided by the control, neutral, and urea groups, respectively). Considering these results, we can conclude that, even though it is a small sample, people up to 35 years old seemed to respond better to this study. The box-plot graphs for age (groups A and B) comparisons were presented in Fig. 4C.

The results demonstrate that the public that responded best to the study were people age 35 or under, with all skin types and, preferably, females. The fact that participants under 35 years of age responded better to the study may be related to a greater natural concentration of biomarkers in the stratum corneum of this population. Although not yet fully elucidated, studies indicate that hormonal factors tend to reduce the concentration of NMF after the age of 40–50, especially in post-menopausal females [27]. As we noticed that the urea gel did not increase skin hydration, as verified in the Corneometer and HPLC tests, these markers remained low throughout the study period. Previous studies demonstrate that hydrated skin has a greater concentration of PCA, the largest component in NMF [28]. Previous studies indicate that female skin can vary its hydration naturally up to 40 years [27]. In the case of males, hydration tends to remain stable until the age of 50, and after that, it begins to lose its water retention capacity [27]. This allows us to conclude that the slight trend shown by females may be related to age and, consequently, a greater concentration of biomarkers generating greater dermal hydration.

4. Conclusions

Optimizing biomarker quantification techniques through the development of a protocol allows studies to be more assertive and provide an understanding of certain pathologies. Although some results indicated limitations due to the number of participants, we obtained significant responses for the improvement of a protocol for quantifying the

biomarkers His, PCA, and UCA by HPLC-PDA from the human stratum corneum *ex vivo*. The statistical analyses indicate that the best audience for quantifying dermal markers were people up to 35 years old, of all phototypes, and preferably females. The fact that urea gel samples did not present statistically relevant results is a limiting factor in studies involving dermatological changes. Future investigations should seek a better understanding of the hydration of the gel containing urea in the stratum corneum and expand the audiences with different phototypes.

CRediT authorship contribution statement

Caroline Saur Santos: Writing – original draft, Validation, Methodology, Investigation, Formal analysis. **André Rolim Baby:** Writing – review & editing, Resources, Project administration, Methodology, Data curation, Conceptualization. **Felipe Rebello Lourenço:** Writing – review & editing, Supervision, Software, Resources, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Felipe Rebello Lourenco reports financial support was provided by State of Sao Paulo Research Foundation.

Data availability

The data that has been used is confidential.

References

[1] A.V. Rawlings, C.R. Harding, Moisturization and skin barrier function, Dermatol. Ther. 17 (2004) 43–48, https://doi.org/10.1111/j.1396-0296.2004.04S1005.x.

[2] K. Tsukui, T. Kakiuchi, M. Suzuki, H. Sakurai, Y. Tokudome, The ion balance of Shotokuseki extract promotes filaggrin fragmentation and increases amino acid production and pyrrolidone carboxylic acid content in three-dimensional cultured human epidermis, Nat. Products Bioprospect. 12 (2022), https://doi.org/10.1007/s13659-022-00353-0.

[3] S. Verdier-Sévrain, F. Bonté, Skin hydration: a review on its molecular mechanisms, J. Cosmet. Dermatol. 6 (2007) 75–82, https://doi.org/10.1111/j.1473-2165.2007.00300.x.

[4] P. Treffel, B. Gabard, Stratum corneum dynamic function measurements after moisturizer or irritant application, Arch. Dermatol. Res. 287 (1995) 474–479, https://doi.org/10.1007/BF00373431.

[5] K.M. Joo, J.Y. Han, E.D. Son, G.W. Nam, H.Y. Chung, H.J. Jeong, J.C. Cho, K. M. Lim, Rapid, simultaneous and nanomolar determination of pyroglutamic acid and cis-/trans-urocanic acid in human stratum corneum by hydrophilic interaction liquid chromatography (HILIC)-electrospray ionization tandem mass spectrometry, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 897 (2012) 55–63, https://doi.org/10.1016/j.jchromb.2012.04.008.

[6] S.A. Koppes, P. Kemperman, I. Van Tilburg, F. Calkoen-Kwa, K.A. Engebretsen, G. J. Puppels, P.J. Caspers, S. Kezic, Determination of natural moisturizing factors in the skin: Raman microspectroscopy versus HPLC, Biomarkers 22 (2017) 502–507, https://doi.org/10.1080/1354750X.2016.1256428.

[7] L. Celleno, Topical urea in skincare: A review, Dermatol. Ther. 31 (2018) 1–5, https://doi.org/10.1111/dth.12690.

[8] I. Hagemann, E. Proksch, Topical treatment by urea reduces epidermal hyperproliferation and induces differentiation in psoriasis, Acta Derm. Venereol. 76 (1996) 353–356, https://doi.org/10.2340/0001555576353356.

[9] V.R. Leite e Silva, M.A. Schulman, C. Ferelli, J.M. Gimenis, G.W. Ruas, A.R. Baby, M.V.R. Velasco, M.E. Taqueda, T.M. Kaneko, Hydrating effects of moisturizer active compounds incorporated into hydrogels: in vivo assessment and comparison between devices, J. Cosmet. Dermatol. 8 (2009) 32–39, https://doi.org/10.1111/j.1473-2165.2009.00421.x.

[10] C.N.A. Palmer, A.D. Irvine, A. Terron-Kwiatkowski, Y. Zhao, H. Liao, S.P. Lee, D. R. Goudie, A. Sandilands, L.E. Campbell, F.J.D. Smith, G.M. O'Regan, R.M. Watson, J.E. Cecil, S.J. Bale, J.G. Compton, J.J. DiGiovanna, P. Fleckman, S. Lewis-Jones, G. Arseculeratne, A. Sergeant, C.S. Munro, B. El Houate, K. McElreavey, L. B. Halkjaer, H. Bisgaard, S. Mukhopadhyay, W.H.I. McLean, Common loss-of-

[11] function variants of the epidermal barrier protein filaggrin are a major predisposing factor for atopic dermatitis, Nat. Genet. 38 (2006) 441–446, https://doi.org/10.1038/ng1767.

[11] M.M. Fiume, W.F. Bergfeld, D.V. Belsito, R.A. Hill, C.D. Klaassen, D.C. Liebler, J. G. Marks, R.C. Shank, T.J. Slaga, P.W. Snyder, L.J. Gill, B. Heldreth, Safety assessment of PCA (2-pyrrolidone-5-carboxylic acid) and its salts as used in cosmetics, Int. J. Toxicol. 38 (2019) 5S–11S, https://doi.org/10.1177/1091581819856568.

[12] I. Dapic, I. Jakasa, N.L.H. Yau, S. Kezic, A. Kammeyer, Evaluation of an HPLC method for the determination of natural moisturizing factors in the human stratum corneum, Anal. Lett. 46 (2013) 2133–2144, https://doi.org/10.1080/00032719.2013.789881.

[13] K. Hermann, D. Abeck, Analysis of histidine and urocanic acid isomers by reversed-phase high-performance liquid chromatography, J. Chromatogr. B Biomed. Sci. Appl. 750 (2001) 71–80, https://doi.org/10.1016/S0378-4347(00)00416-3.

[14] S. Kezic, A. Kammeyer, F. Calkoen, J.W. Fluhr, J.D. Bos, Natural moisturizing factor components in the stratum corneum as biomarkers of filaggrin genotype: Evaluation of minimally invasive methods, Br. J. Dermatol. 161 (2009) 1098–1104, https://doi.org/10.1111/j.1365-2133.2009.09342.x.

[15] K. Hermann, D. Abeck, Determination of histidine and urocanic acid isomers in the human skin by high-performance capillary electrophoresis, J. Chromatogr. B Biomed. Sci. Appl. 749 (2000) 41–47, https://doi.org/10.1016/S0378-4347(00)00376-5.

[16] A.A. Irving, S.J. Marling, L.A. Plum, H.F. DeLuca, Suppression of experimental autoimmune encephalomyelitis by ultraviolet light is not mediated by isomerization of urocanic acid, BMC Neurosci. 18 (2017) 2–9, https://doi.org/10.1186/s12868-016-0323-2.

[17] N. Raj, R. Voegeli, A.V. Rawlings, S. Doppler, D. Imfeld, M.R. Munday, M.E. Lane, A fundamental investigation into aspects of the physiology and biochemistry of the stratum corneum in subjects with sensitive skin, Int. J. Cosmet. Sci. 39 (2017) 2–10, https://doi.org/10.1111/ics.12334.

[18] M.O. Visscher, A.N. Carr, J. Winget, T. Huggins, C.C. Bascom, R. Isfort, K. Lammers, V. Narendran, Biomarkers of neonatal skin barrier adaptation reveal substantial differences compared to adult skin, Pediatr. Res. 89 (2020) 1208–1215, https://doi.org/10.1038/s41390-020-1035-y.

[19] K. Maeno, Direct quantification of natural moisturizing factors in stratum corneum using direct analysis in real time mass spectrometry with inkjet-printing technique, Sci. Rep. 9 (2019) 17789, https://doi.org/10.1038/s41598-019-54454-x.

[20] K. Maeno, Y. Shida, H. Shimada, Direct quantitative analysis of the natural moisturizing factor (NMF) in the stratum corneum by direct analysis in real time mass spectrometry (DART-MS), Anal. Methods 9 (2017) 4851–4857, https://doi.org/10.1039/C7AY01177F.

[21] W. Medical, Association, World Medical Association Declaration of Helsinki, in: JAMA, 2013: p. 2191. doi: 10.1001/jama.2013.281053.

[22] International Council for Harmonisation, M10 Guideline for Bioanalytical method validation and study sample analysis, (2022) 0–47. https://www.ich.org/page/multidisciplinary-guidelines.

[23] AOAC Official Methods of Analysis, Appendix F: Guidelines for Standard Method Performance Requirements, 2016. https://www.aoac.org/wp-content/uploads/2019/08/app_f.pdf.

[24] AOAC Official Methods of Analysis, AOAC Guidelines for Single Laboratory Validation of Chemical Methods for Dietary Supplements and Botanicals, 2002. https://s27415.pcdn.co/wp-content/uploads/2020/01/64ER20-7/Validation_Methods/d-AOAC_Guidelines_For_Single_Laboratory_Validation_Dietary_Supplements_and_Botanicals.pdf.

[25] A.V. de Souza Neto, D.Q. Balla, T.M. Candido, C. Rosado, A.R. Baby, F.V.L. S. Pessoa, Effect of an Emollient Emulsion Containing 15.0% of Caprylic/Capric Triglyceride on the Urocanic Acid of the Stratum Corneum, Life 13 (2023), https://doi.org/10.3390/life13040876.

[26] S. Sachdeva, Fitzpatrick skin typing: Applications in dermatology, Indian J. Dermatol. Venereol. Leprol. 75 (2009) 93–96, https://doi.org/10.4103/0378-6323.45238.

[27] S. Luebberding, N. Krueger, M. Kerscher, Skin physiology in men and women: In vivo evaluation of 300 people including TEWL, SC hydration, sebum content and skin surface pH, Int. J. Cosmet. Sci. 35 (2013) 477–483, https://doi.org/10.1111/ics.12068.

[28] L. Feng, P. Chandar, N. Lu, C. Vincent, J. Bajor, H. McGuiness, Characteristic differences in barrier and hygroscopic properties between normal and cosmetic dry skin. II. Depth profile of natural moisturizing factor and cohesivity, Int. J. Cosmet. Sci. 36 (2014) 231–238, https://doi.org/10.1111/ics.12118.

[29] Chemicalize - Instant Cheminformatics Solutions, Pyrrolidonecarboxylic acid, (n. d.). https://chemicalize.com/app/calculation/pyrrolidonecarboxylic acid (accessed May 3, 2021).

[30] Chemicalize - Instant Cheminformatics Solutions, Urocanic acid, (n.d.). https://chemicalize.com/app/calculation/urocanic acid (accessed May 3, 2021).

[31] Chemicalize - Instant Cheminformatics Solutions, Histidine, (n.d.). https://chemicalize.com/app/calculation/histidine (accessed March 5, 2021).