UNIVERSITY OF SÃO PAULO
FACULTY OF PHARMACEUTICAL SCIENCES
GRADUATE PROGRAM IN DRUG AND MEDICINES
AREA OF PHARMACEUTICAL PRODUCTION AND CONTROL

CAROLINE SAUR SANTOS

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

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Dissertation presented to the Faculty of Pharmacy of the University of São Paulo for the Master's degree.

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Advisor: Prof. Dr. Felipe Rebello Lourenço

Co-advisor: Prof. Dr. André Rolim Baby

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DEDICATION

I dedicate this work to my family who supported me throughout my academic journey, sparing no effort to help me complete this stage.

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SANTOS, Caroline Saur. **Quantificação simultânea de ácido 2-pirrolidona-5-carboxílico (PCA), ácido urocânico (UCA) e histidina (His) no estrato córneo por HPLC-PDA.** 2024. 64p. Dissertação (Mestrado em Fármacos e Medicamentos) – Faculdade de Farmácia, Universidade de São Paulo, São Paulo, 2024.

RESUMO

A pele é o maior órgão do corpo humano e possui a função de proteger o organismo do meio externo. Na parte mais superficial da pele se encontra o estrato córneo que tem a função de evitar a perda de água transepidermal (TEWL) e auxiliar da homeostase da pele. Tais funções se dão pela presença do Fator de Hidratação Natural (NMF), um conjunto de aminoácidos livres e seus derivados, como por exemplo o ácido pirrolidona-carboxílico (PCA) e o ácido urocânico (UCA), associados a outros componentes. Alguns estudos demonstram que baixos níveis de NMF estão, frequentemente, relacionados a peles secas, escamosas, psoríase e dermatites, por isso a importância em conseguir quantificar de forma mais precisa. A determinação e quantificação de componentes do NMF, com o uso de amostras *ex-vivo*, vem sendo utilizada nos últimos anos e é reportada no primeiro capítulo deste estudo. Destacam-se o uso de métodos cromatográficos, devido a frequência com que são utilizados. Entretanto, outros métodos também têm demostram resultados satisfatórios neste tipo de análise, como o Raman, eletroforese, espectroscopia confocal, entre outros. No segundo capítulo, é apresentado o desenvolvimento de um protocolo para quantificação simultânea de PCA, UCA e histidina por HPLC-PDA. Neste capítulo é dada ênfase ao público participante da pesquisa, isto é, estabelece a população que gera respostas mais claras e distintas à análise por HPLC-PDA. Aplicando-se um procedimento analítico validado de acordo com as diretrizes da AOAC, foi possível concluir que a população que melhor responde a este estudo foram pessoas com até 35 anos, de todos os fototipos, e preferencialmente mulheres.

Palavras-chave: Fator de hidratação natural; estrato córneo e HPLC-PDA.

SANTOS, Caroline Saur. **Simultaneous quantification of 2-pyrrolidone-5-carboxylic acid (PCA), urocanic acid (UCA), and histidine (His) in the stratum corneum by HPLC-PDA.** 2024. 64p. Dissertação (Mestrado em Fármacos e Medicamentos) – Faculdade de Farmácia, Universidade de São Paulo, São Paulo, 2024.

ABSTRACT

The skin is the largest organ of the human body and has the function of protecting the organism from the external environment. In the most superficial part of the skin lies the stratum corneum, which functions to prevent transepidermal water loss (TEWL) and assist in skin homeostasis. These functions are carried out by the presence of the Natural Moisturizing Factor (NMF), a combination of free amino acids and their derivatives, such as pyrrolidone carboxylic acid (PCA) and urocanic acid (UCA), associated with other components. Some studies demonstrate that low levels of NMF are often related to dry, scaly skin, psoriasis, and dermatitis, hence the importance of being able to quantify it more accurately. The determination and quantification of NMF components, using ex-vivo samples, have been used in recent years and are reported in the first chapter of this study. Chromatographic methods are highlighted due to their frequency of use. However, other methods have also shown satisfactory results in this type of analysis, such as Raman spectroscopy, electrophoresis, confocal spectroscopy, among others. In the second chapter, the development of a protocol for simultaneous quantification of PCA, UCA, and histidine by HPLC-PDA is presented. This chapter emphasizes the target population of the research, establishing the population that provides clearer and distinct responses to the HPLC-PDA analysis. Using an analytical procedure validated in accordance with AOAC guidelines, it was possible to conclude that the population that best responds to this study consists of individuals up to 35 years old, of all phototypes, preferably women.

Keywords: Natural Moisturizing Factor; stratum corneum; HPLC-PDA.

SUMMARY

1 INTRODUCTION

The skin is the largest organ of the human body and is responsible for protecting the body from external mechanical and chemical aggressions, as well as regulating body temperature (ABDALA *et al*., 2024; DARLENSKI *et al.*, 2009). The barrier formed by the skin, more specifically the epidermis, prevents water loss and protects the body from external allergens and pathogens (DARLENSKI *et al.*, 2009; KIM *et al*., 2021). The outermost layer of the skin, known as the stratum corneum, is located in the epidermis (RAWLINGS; HARDING, 2004; TSUKUI *et al.*, 2022). It is composed of multiple layers of corneocytes (SON *et al.*, 2015), which are anucleated/dead keratinized cells surrounded by extracellular lipids. Additionally, it is a heterogeneous and selectively permeable layer (DRISLANE; IRVINE, 2020; IRVING *et al.*, 2017; MAENO; SHIDA; SHIMADA, 2017). The stratum corneum is responsible for preventing transepidermal water loss (TEWL), thus assisting in skin homeostasis (DARLENSKI *et al*., 2009; VERDIER-SÉVRAIN; BONTÉ, 2007). This hydration balance generated by the stratum corneum is determined by intercellular lipids and corneocytes along with their hygroscopic content, known as Natural Moisturizing Factor (NMF) (SON *et al*., 2015; VERDIER-SÉVRAIN; BONTÉ, 2007). The NMF is formed by free amino acids and their derivatives, such as pyrrolidone carboxylic acid (PCA) and urocanic acid (UCA), as well as lactic acid, urea, citrate, and sugars (VERDIER-SÉVRAIN; BONTÉ, 2007; WATABE *et al.*, 2013).

The NMF is a result of the degradation of filaggrin, a key structure in barrier function (KEZIC *et al.*, 2009), which functions to aggregate keratin filaments in corneocytes, providing mechanical resistance to the stratum corneum (DRISLANE; IRVINE, 2020; KEZIC et al., 2009). When filaggrin is degraded, it is transformed into histidine and glutamine, which, upon reaching the stratum corneum, are metabolized into two hygroscopic substances, UCA and PCA, respectively (CHOI *et al*., 2017; FENG *et al*., 2014; SON *et al.*, 2015). Studies indicate that the degradation of filaggrin into NMF is dependent on the hydration of the stratum cornem and an inverse correlation between the water gradient of the stratum corneum and the gradient of NMF components (BJÖRKLUND *et al.*, 2014).

When it comes to skin hydration assessment, the Corneometer® has proven to be a valuable tool in efficacy testing for cosmetics and pharmaceutical industries, as well as universities (DE DECKER *et al*., 2023; LEITE E SILVA *et al.*, 2009; LI *et al., 2023)*. This equipment evaluates skin hydration through the capacitance of the

dielectric constant (COURAGE + KHAZAKA ELECTRONIC GMBH, 2017). As the hydration of the stratum corneum increases, the dielectric properties change. The measurement principle of the equipment, according to the manufacturer Courage + Khazaka Electronic GMBH, 2017, is based on the fact that water has a high dielectric constant compared to other substances. In other words, the equipment's resonance system detects the frequency of change in system oscillation, which is related to hydration (COURAGE + KHAZAKA ELECTRONIC GMBH, 2017).

This dissertation is organized into two chapters: Chapter I provides a literature review of different analytical methods used to identify and quantify dermal markers present in the NMF, with emphasis on pyrrolidone carboxylic acid, urocanic acid, and histidine. Among the methods discussed, chromatographic techniques, electrophoresis, Raman spectroscopy, confocal spectroscopy, and direct analysis in real time (DART) are highlighted. Chapter II is dedicated to the development of a protocol for the simultaneous quantification of pyrrolidone carboxylic acid, urocanic acid, and histidine ex vivo by HPLC-PDA. In order to complement the articles found in the review, the study aims to define the population that produces clearer and more distinct responses to the procedure, by analyzing data obtained from different age groups, phototypes, and genders of the participants.

Studying more precise ways to quantify NMF in the stratum corneum seems relevant when considering that there are studies reporting that low levels of NMF are often associated with dry and scaly skin, atopic dermatitis, and psoriasis (JOO *et al.*, 2012).

2 OBJECTIVES

The objective of this research was to establish a protocol for the quantification of PCA, UCA, and His by HPLC-PDA in samples of stratum corneum obtained by tape-stripping (*ex vivo*).

2.1 SPECIFIC OBJECTIVES

- Conduct a literature review on chromatographic methods and other analytical techniques used for the identification and quantification of NMF biomarkers.
- Quantify the dermal markers PCA, UCA, and His from the stratum corneum of the research participants.

- Validate the analytical procedure used in the determination by HPLC-PDA, according to established regulatory criteria.

- Considering gender, age range, and phototype characteristics, establish the population that yields clearer and more distinct responses results.

3 CHAPTER I

Analytical methods of identification and quantification of Natural Moisturizing Factor markers - a review

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Keywords: Natural Moisturizing Factor, stratum corneum, analytical methods, moisturizing.

ABSTRACT

The skin is our body's first defense barrier against external threats, as well as assists in the body's water balance. The main responsible for water retention is located in the skin, specifically in the stratum corneum, and is known as Natural Moisturizing Factor (NMF). Containing different components, the NMF is the target of a lot of research that identifies and quantifies it, although few aim to develop the techniques themselves. To gather the different methodologies published in recent years, this article considers different NMF markers to carry out this review, such as pyrrolidone carboxylic acid, urocanic acid, and histidine. In addition, we sought to focus on methodologies used in the last decade that were dedicated to analyses with dermal materials. The analytical methodologies used by researchers in recent years are discussed here and can be divided into chromatographic and non-chromatographic models. Despite the different existing methods, there was a predominance of those that use high-performance liquid chromatography and confocal Raman spectroscopy for such analyses. Improving identification and quantification methods of dermal markers can help in the diagnosis of pathologies in the development of dermatological research.

INTRODUCTION

The skin is considered the first defense barrier against external chemical, physical and microbial threats, in addition to being the tissue responsible for protecting the human body against environmental changes and preventing water loss (BOUSLIMANI *et al.*, 2019; HARDING *et al.*, 2000; RAWLINGS; HARDING, 2004). This activity is linked, more precisely, to the stratum corneum: the fourth and outermost layer of the epidermis in direct contact with the environment (FENG *et al.*, 2014; HARDING *et al*., 2000; MLITZ *et al.*, 2012).

The stratum corneum is the result of a process of cellular differentiation of the basal keratinocytes where, through mitosis, the new cells ascend and mature towards the stratum corneum (RICHTERS *et al.*, 2017). It is formed by corneocytes (keratinocytes of the stratum corneum) and an intercellular lipid matrix. Corneocytes are made up of keratin filaments that, throughout their differentiation process, form a cornified envelope (BÖHLING *et al.*, 2014; HARDING *et al*., 2000). In this last step, the keratinocytes from the stratum granulosum begin to contain keratohyalin granules, which contain profilaggrin, a precursor of the filaggrin protein (CANDI; SCHMIDT; MELINO, 2005; MOOSBRUGGER-MARTINZ *et al.*, 2022). Filaggrin, when degraded, forms the skin's Natural Moisturizing Factor (NMF) (CANDI; SCHMIDT; MELINO, 2005), a mixture of hygroscopic and low molecular weight components (BÖHLING *et al.,* 2014; DE SOUZA NETO *et al.*, 2023; HARDING *et al.*, 2000), such as free amino acids, pyrrolidone carboxylic acid (PCA), urocanic acid (UCA), urea, lactate, citrate, and sugars (MLITZ *et al.*, 2012; RAWLINGS; HARDING, 2004). In between corneocytes is the intercellular matrix, composed of lamellar lipids, which help to prevent excessive evaporation of water from the skin (BÖHLING *et al.*, 2014; HARDING *et al.*, 2000; RAWLINGS *et al.*, 1994).

The Natural Moisturizing Factor helps in the formation of the skin's water barrier and when hydrated and associated with keratin, it provides elastic properties to the skin (BÖHLING *et al.*, 2014; RAWLINGS *et al.*, 1994). This is due to the ionic interaction between keratin and the Natural Moisturizing Factor, associated with reduced water mobility and consequent reduction of intermolecular forces of keratin fibers (HARDING *et al.*, 2000).

Cutaneous dehydration can favor the entry of some allergens and pathogens into the stratum corneum, leading to the development of some pathologies, such as atopic dermatitis, dermal contact irritation, and contact allergies (LEE, 2020).

Some articles have demonstrated that hereditary mutations in the filaggrin gene may be associated with reduced filaggrin expression and, consequently, lead to the appearance of atopic dermatitis (LIMA *et al.*, 2021; MLITZ *et al.,* 2012; PALMER *et al.*, 2006). Atopic dermatitis is directly related to the reduction of amino acids present in the Natural Moisturizing Factor of the skin, such as pyrrolidone carboxylic acid (PCA), urocanic acid, and histidine (MLITZ *et al.*, 2012; WEI *et al.*, 2016). In this way, being able to identify and quantify these moisturizing markers may become an ally in the diagnosis of dermatological pathologies (MLITZ *et al.,* 2012; PALMER *et al.,* 2006).

In this research work, we revised the analytical methods used in the identification and quantification of three moisturizing markers of stratum corneum: UCA, PCA, and His (Figure 1) (HARDING *et al.*, 2000; RAWLINGS; HARDING, 2004).

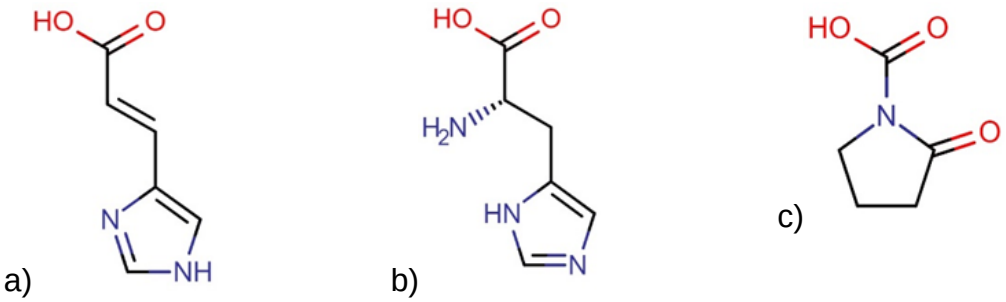

Figure 1. a) Urocanic acid (UCA) (CHEMICALIZE - INSTANT CHEMINFORMATICS SOLUTIONS, [s.d.]); b) Histidine (CHEMICALIZE - INSTANT CHEMINFORMATICS SOLUTIONS, [s.d.]); c) Pyrrolidone carboxylic acid (PCA) (CHEMICALIZE - INSTANT CHEMINFORMATICS SOLUTIONS, [s.d.]).

This review was carried out by addressing the most varied techniques used to determine pyrrolidone carboxylic acid, urocanic acid isomers, and histidine. The reference was given to specialized literature published in the last decade. Research that determined such substances in other organs was not included in this review, once the collection and preparation of samples did not apply to cutaneous samples. For this reason, the research of markers was always accompanied by the terms “skin” or “stratum corneum”.

PYRROLIDONE CARBOXYLIC ACID (PCA), UROCANIC ACID, AND HISTIDINE

Filaggrin is a protein present in the skin and the oral and nasal mucous membranes (PALMER *et al.*, 2006). In the skin, more specifically, in the stratum corneum, filaggrin is degraded and forms several amino acids that compound the

Natural Moisturizing Factor (NMF), including glutamine and histidine (KEZIC *et al.*, 2009; RAWLINGS *et al.*, 1994), responsible for the formation of pyrrolidone carboxylic acid (PCA) and urocanic acid, respectively (MLITZ *et al.*, 2012). Other amino acids abundant in NMF are L-histidine, serine, glycine, arginine, and alanine (HARDING *et al.*, 2000; RAWLINGS; HARDING, 2004).

Histidine, when degraded by histidase, forms urocanic acid and other metabolites such as histamine and 1-methylhistamine (HERMANN; ABECK, 2000). Urocanic acid, in turn, can be found not only in the skin but also in the liver (SIZOVS *et al.*, 2020). However, it is in the skin that this compound exerts a photoprotective function, being able to absorb ultraviolet radiation (UVB) (SIZOVS *et al*., 2020). Urocanic acid is naturally present in the skin on *trans* conformation (*trans*-urocanic acid) and, when exposed to UVB, its conformation changes to *cis* (*cis*-urocanic acid) (KEURENTJES *et al.*, 2020; SIZOVS *et al.*, 2020).

Pyrrolidone carboxylic acid (PCA), which is also found in the literature under the name of pyroglutamic acid (BERDYSHEV *et al.*, 2021; MONTENEGRO *et al.*, 2019), is the most hygroscopic constituent of the stratum corneum (FIUME *et al.*, 2019) and one of the most important components present in the Natural Moisturizing Factor (WEI *et al*., 2016), corresponding to approximately 12% of that (RAWLINGS; HARDING, 2004). Pyrrolidone carboxylic acid is present in the human epidermis, plasma, and cerebrospinal fluid in the L-PCA conformation. In urine, L- and D- isomers are present (FIUME *et al.*, 2019).

ANALYTICAL METHODOLOGIES

Chromatographic methods

Irving *et al.*, 2017 performed research that evaluated the action potential of urocanic acid (*cis*- and *trans*-) in mouse skin in the experimental model of autoimmune encephalomyelitis. The study used two dermal markers, *cis*-/*trans*-urocanic acid, which were analyzed by reversed-phase high-performance liquid chromatography (HPLC) associated with a photodiode array detector (PDA). The analysis was performed by gradient, starting with 100% water and followed by 70:30 water: acetonitrile (ACN), for 15 minutes (IRVING et al., 2017). More recently, Keurentjes *et al.*, 2022 studied the same markers to evaluate the skin of outworkers, using the methodology previously used by Keurentjes *et al*., 2020 and Keurentjes *et al.*, 2021. The approach of those

studies also used reversed-phase HPLC, but associated with an ultraviolet (UV) detector (KEURENTJES *et al.*, 2020, 2022, 2021). The collection of the stratum corneum by tape-stripping, according to Keurentjes *et al.*, 2020, is a promising skin *ex vivo* technique that is relevant in controlling exposure to UV radiation. In this study the mobile phase, an isocratic solution with low UV absorption was used (KEURENTJES *et al.*, 2020). According to the researchers, the technique may present limitations for monitoring high or repeated UV exposition due to the conversion of the *trans*- to *cis*- a fraction of urocanic acid. For this reason, these are more suitable markers for low and/or single UV exposure, as is the case of sunscreen evaluation (KEURENTJES *et al.*, 2020). A similar methodology was previously used by Dapic *et al.*, 2013; Simonsen *et al*., 2017; Soltanipoor *et al.*, 2017. The approaches by Dapic *et al.*, 2013 and Simonsen *et al.*, 2017 were determined, in addition to urocanic acid isomers, pyrrolidone carboxylic acid (PCA), and histidine.

In 2017, Choi *et al*. developed a methodology based on the approaches of Dapic *et al*., 2013; Kezic *et al.*, 2009; and Raj *et al.*, 2017. With this methodology, the researchers were able to quantify the dermal markers *cis*-/*trans*-urocanic acid and pyrrolidone carboxylic acid (PCA). Using the same technique, but with different sample and mobile phase treatment methods Raj *et al.*, 2016 and Raj *et al.*, 2017 also quantified pyrrolidone carboxylic acid (PCA) through HPLC with a UV detector. The details of the analytical conditions are presented in **Table 1**.

Wei *et al.*, 2016 made a parallel between the concentrations of moisturizing markers in volunteers in summer and winter. Therefore, the researchers were able to assess the dermal changes in different seasons (WEI *et al.*, 2016). The analysis of this research was performed using the reversed-phase HPLC coupled with tandem mass spectrometry. The removal of the stratum corneum was performed by tape-stripping. Once the collection was made, a protein content analysis was performed through optical absorption at 850 nm in a SquameScan™ 850A infrared densimeter. The objective of this process was to normalize the measurements for further statistical analysis (WEI *et al.*, 2016). A similar methodology was also used by Visscher et al., 2020 in the analysis of the markers histidine, *cis*-/*trans*-urocanic acid, and pyrrolidone carboxylic acid (PCA); and also in the analysis of pyrrolidone carboxylic acid in the study by Wei *et al.*, 2021.

Another methodology found to quantify pyrrolidone carboxylic acid and *cis*-/*trans*-urocanic acid is liquid chromatography by hydrophilic interaction coupled with

tandem mass spectrometry (HILIC-MS/MS). Pham *et al.*, 2017 and Jung *et al.,* 2020 presented studies that used the technique previously developed and validated by Joo *et al.*, 2012. The use of liquid chromatography by hydrophilic interaction coupled with tandem mass spectrometry is justified, according to Joo *et al.*, 2012, by highly polar analytes, with retention and separation difficulty of reversed-phase HPLC. Furthermore, the use of the mobile phase with high concentrations of organic solvent, such as acetonitrile, led to HILIC presenting a higher ionization efficiency (JOO *et al.*, 2012). For sample preparation it was used 30% water in acetonitrile (1:9) with alkaline phosphate buffer (PBS) as an extractor without derivatization (JOO *et al.*, 2012). Another advantage observed by Joo *et al.*, 2012 is that this methodology allowed the determination of analytes to occur simultaneously. The studies presented in this review that used this methodology of analysis had the human stratum corneum as a matrix, also removed by the tape-stripping (JOO *et al.*, 2012; JUNG *et al.*, 2020; PHAM *et al.*, 2017). Before carrying out the analysis of the analytes in question, the researchers separated the previously prepared aliquots and quantified the soluble proteins with a protein assay kit (JOO *et al.*, 2012; JUNG *et al.*, 2020; PHAM *et al.*, 2017). Then, the samples were incubated for 30 minutes at a temperature of 37ºC and then the absorbance of soluble proteins was measured in a Microplate Reader at 595 nm (JOO *et al.*, 2012; JUNG *et al.*, 2020; PHAM *et al*., 2017). To perform the analyses in the chromatograph, the internal standard of L-proline-13C5,15N and the acetonitrile solution were added to the previously prepared samples (JOO *et al.*, 2012). The mobile phase consisted of an elution gradient, as shown in **Table 1**. This methodology described here was validated by researchers (JOO *et al.*, 2012).

The study fulfilled by Leung *et al.*, 2019 used liquid chromatography coupled with a tandem mass spectrometer in the electrospray ionization mode (LC-ESI-MS/MS), within an approach on a mass spectrometer coupled with a UHPLC system. The researchers separated and quantified *cis*-/*trans*-urocanic acid, pyrrolidone carboxylic acid (PCA), and proline using a gradient elution in the mobile phase (LEUNG *et al.*, 2019). Amino acids were detected in positive ion mode, operating with charge mass ratio (m/z) 139.1 > m/z 121.1 (UCA), m/z 130.2 > m/z 83.9 (PCA) and m /z 122.1 > m/z 75.0 (U-[13C,15N] proline) (LEUNG *et al.*, 2019). A new study similar to this one was developed by Berdyshev *et al.*, 2021 years later and the entire methodology was reproduced again (BERDYSHEV *et al.*, 2021).

Kim *et al*. evaluated how much pollution can influence the formation of the NMF in the skin, and consequently the barrier function (KIM *et al*., 2021). The *cis*-/*trans*-urocanic acid and pyrrolidone carboxylic acid (PCA) dermal markers were analyzed again using the methodology used by Leung *et al*., 2019.

A summary of the chromatographic methods used for the identification and quantification of stratum corneum biomarkers, including the matrix, biomarkers, technique, sample preparation/biomarkers extraction, chromatographic column, mobile phase, and split separation condition/detection, is presented in **Table 1**.

TABLE 1

Table 1. Summary of the chromatographic methods used for the identification and quantification stratum corneum biomarkers, including the matrix, biomarkers, technique, sample preparation/biomarkers extraction, chromatographic column, mobile phase, and split separation condition/detection

Matrix	Markers	Technique	Samples Preparation/Biomarkers extration	Chromatogtaphic column	Mobile Phase	Split separation condition/detection	Reference
Human stratum corneum	*c*UCA, *t*UCA	Tape-stripping - HPLC/UV	Add to tha tapes 600µL of Milipore water	250x3mm, reversed-phase, Synergi 4µm Polar RP 80A column.	4,3mM hydrochloric acid; 0,1 mM sodium octane-1-sulfonate and 2% acetonitrile.	Isocration elution. Flow rate of 0,4mL/min; wavelenghs were monitored in 267nm.	KEURENTJES *et al*., 2019; KEURENTJES *et al.*, 2020; KEURENTJES *et al* 2022
Human stratum corneum	L-citrulline, glycine, L-ornithine, L-proline, 2-pyrrolidone-5-carboxylic acid, L-serine (free amino acids), tUCA and L-histidine.	Tape-stripping - HPLC-MS/MS	Add to the tapes 25µL of internal standard solution (L-citrullina-D7; glycine-D2, 15N; histidine-D3; L-ornithine-D6; L-proline-D3; 2-pyrrolidone-5-carboxylic-D5 acid; L-serine-D3; ácido cis-urocanic-13C3); 1,0mL of water with 0,1% formic acid and 0,1% heptafluorobutyric acid. Vortexed for 10s and sonificated 10min.	2,1mmx50 mm, 3-µm particles size, reverse-phase - Waters Atlantis T3 column	not presented	Gradient elution	WEI *et al*., 2016 and VISSCHER *et al*., 2020 apud WEI *et al*., 2016
Human stratum corneum	PCA, *t*UCA and *c*UCA, proline	Tape-stripping - LC-ESI-MS/MS in a	Tapes was let is Petri dish with 2ml water:methanol solution (9:1, v/v).	2,1mm x 100mm; 1,7 µm particles size - Acquity UPLC BEH amide column	Solvent A: acetonitrile. Solvent B: methanol:water:f	Gradient elution: 5%B until 0,5 min, then inscrease for 20%B at 1 min, followed by	LEUNG *et al*., 2019; and KIM *et al*., 2021 *apud* LEUNG *et al*., 2019

		UHPLC system	Floating particles were transferred and extracted by Bligh and Dyer. U-[13C,15N] proline was added to ensure the right quantification. To extract was added 0,5mL of methanol and 1mL of chloroform followed by adding of 1,5ml de chloroform and 1,35ml 2% of formic acid. Vortexed and centrigation (2.000g x 10min). Gather the superior phase, evapotated under nitrogen and redissolved in water/acetonitrile (1:1).		ormic acid (65:35:0,5, with ammonium formate).	60%B at 3 min. Hold 60%B until 4,1 min and descrease for 5%B for 4,5 min and hold 5%B until 5 min. Ion positive mode by followed transitions: mass to charge (m/z) 139,1 > m/z 121,1 (UCA), m/z 130,2>m/z 83,9 (PCA) e m/z 122,1> m/z 75,0 (U-[13C,15N] proline).	
Human stratum corneum	PCA, *t*UCA and *c*UCA	Tape-stripping/ HILC-MS/MS	The extraction: Glass vials contaning 5 mL 0,1% (w/v) sodium dodecyl sulphate/ 2% propylene glycol in phosphate buffer solution. 1 hour of sonification. Centrifuge at 12.000 rpm for 10	2,1mmx100mm, 1.7 µm particles size - Acquity UPLC BEH Amide column. Column temperature: 40ºC. Autosampler temperature: 10ºC.	Solvent A: deionized water. Solvent B: acetonitrile.	Gradient elution: 80%B for 0,5 min; followed by a 1,5 min gradient to 40%B and then mantain 40%B for 3,5 min. Turn back 80%B in 5 min. Flow rate: 0,3mL/min. Injection volume: 5 µL. Spetrometer ion ESI positive. Capillary voltage: 3,5kV; ions	PHAM *et al.*, 2017; and JUNG *et al.*, 2020 *apud*. JOO *et al.*, 2012

			min at 4ºC. For analyze: in HPLC vials - 100 µL of extration above, 50 µL internal standard (1µg/mg) and 850 µL water with 30% acetonitrile.			source temperature: 120ºC; desolvation temperature: 350ºC; desolvation gas flow rate: 750L/h and cone gas flow rate: 50L/h.	
Human stratum corneum	PCA, *t*UCA	Tape-stripping - HPLC/UV	Add to the tapes 750µL of 0,1M KOH solution. Shake during 2 hours and add 4,5 µL of perchloric acid.	250mmx4,6mm - Synergi 4µm POLAR-RP-80A LC column.	4,3mM hydrochloric acid, 0,1mM sodium octane-1-sulfonate and 2% acetonitrile at pH 2,4.	Isocratic elution. Injection volume: 15µL. Flow rate: 0,4mL/min. PCA and tUCA were monitored at 210nm.	CHOI *et al.*, 2017
Human stratum corneum	PCA	Tape-stripping - HPLC/UV	Add to the tapes 750µL 0,1mol/L KOH solution. Shake continuously for 2h in 1000rpm and 32ºC. Neutralize with 4,5µL 12M perchloric acid. Shake for 2h e filter through 0,2µm membrane.	4,6mmx150mm - Synergi 4µm POLAR-RP-80A column.	20mmol/L ammonium formate, 1,5 mmol/L tetrabutylammonium hydroxide and 3% acetonitrile at pH 7,3.	Isocratic elution. Injection volume: 10µL. Flow rate: 0,4 mL/min. Retention time: 7,5 min.	RAJ *et al.*, 2017
Mice's skin	*t*UCA and *c*UCA	HPLC/PDA	Bligh-Dyer method isolate UCA. Homogenized in cold water, add methanol/dichloromethane, dichloromethane and water again. Centrifuge for 15	3,9mmx150mm; 5 µm particles size - Waters Symmetry C18. Column temperature: 30ºC.	Water containing 0,1% trifluoroacetic acid (TFA); and acetonitrile.	Gradient elution: 100% water with TFA to 70:30 water:ACN run over 15 minutes. Flow rate: 0,5mL/min. Retention time: tUCA - 10,3min and cUCA 14,2min. Wavelength: 264nm.	IRVING *et al.*, 2017

			min at 3500rpm at 4ºC. Collect water phase and filtered first through 0,45 µm and again 0,22 µm. Dry under nitrogen and ressuspended in water with 0,1% trifluoroacetic acid.				
Human stratum corneum	PCA	Tape-stripping - HPLC/UV	Add to the tapes 750 µL 0,1mol/L KOH solution for 2h at 32º with 1000rpm continuous shaking. Neutralize with 4,5µL 12mol/L perchloric acid and shake again for 2h. Filter through 0,2 µm membrane.	150mmx4,60 mm; 4µm particles size - Synergi HPLC column 4µm POLAR-RP-80A	20 mmol/L ammonium formate, 1,5 mmol/L tetrabutylammonium hydroxide and 3% acetonitrile at pH 7,3.	Injection volume: 10µL. Flow rate: 0,4 mL/min. Retention time: 7,5min. Wavelength: 210nm.	RAJ *et al.*, 2016

Other techniques

In 2017, Maeno; Shida; Shimada developed an optimized methodology for the analysis of NMF amino acids. Through the technique of Direct Analysis in Real Time (DART) associated with a time-of-flight mass spectrometer (TOF-MS), it was possible to quantify the amino acids in a much shorter time, since they eliminated the pre-treatment of the samples (MAENO; SHIDA; SHIMADA, 2017). The DART was performed by equipment that has an ion source that ejects heated and excited molecules of helium gas, which reach the analyte that is on a surface in front and desorb the ions from it (MAENO; SHIDA; SHIMADA, 2017). The ions of the helium gas carry the ions from the analyte to the mass spectrometer, where the quantification of the analyte takes place (MAENO; SHIDA; SHIMADA, 2017). To perform this analysis, the researchers collected the stratum corneum of volunteers using the tape-stripping technique. Then, they exposed the tapes with the analytes in question (stratum corneum) to a DART ion source (MAENO; SHIDA; SHIMADA, 2017). To be able to quantify the analytes properly, the matrix effect and the fluctuation effects were eliminated through the isotope-stable-labeled (SIL) technique containing Glycine-^{15}N and lysine-^{15}N2, which was used as an internal standard (SIL-IS), and added directly to the sample (MAENO; SHIDA; SHIMADA, 2017). Thus, it was possible to normalize the analyte signal with the internal standard (MAENO; SHIDA; SHIMADA, 2017). To validate the results of this new methodology, the researchers performed an analysis in parallel, using an already established technique, gas chromatography coupled to the mass spectrometer (GC-MS). The results of this analysis, 12 amino acids were detected with good sensitivity and selectivity and 8 wasn't detected with this technique. However, changing the parameters of the mass detector, it is possible to quantify more 6 amino acids (MAENO; SHIDA; SHIMADA, 2017).

Later, the same researchers sought to improve the used technique to solve the problems encountered previously (MAENO, 2019; MAENO; SHIDA; SHIMADA, 2017). The new methodology used the technique of DART, but now with a triple quadrupole mass spectrometer (TQ-MS), associated with a LaboJet inkjet (MAENO, 2019). This association was carried out to improve the uniformity of the SIL-IS deposition and, consequently, its accuracy (MAENO, 2019). To increase the sensitivity of the method, the researchers used two monitoring methods, chosen according to the characteristics of each amino acid: multiple reaction monitoring (MRM) and selected ion monitoring (SIM) (MAENO, 2019). According to Maeno, 2019, the analytical method developed,

which associated DART-TQ/MS technique with the SIL-IS coating by LaboJet, reached the desired objective (MAENO, 2019). Also according to the researchers, this methodology allowed the analysis of approximately 100 samples to be performed in 2 hours (MAENO, 2019). This result means a significant reduction in analysis time compared to conventional techniques (MAENO, 2019). Both studies by Maeno, 2019 and Maeno; Shida; Shimada, 2017 made comparisons with a methodology already established and validated by Badawy; Morgan; Turner, 2008, who used gas chromatography to determine amino acids (BADAWY; MORGAN; TURNER, 2008). Although the study by Badawy; Morgan; Turner, 2008 was not designed to determine stratum corneum amino acids, Maeno, 2019 and Maeno; Shida; Shimada, 2017 obtained good results.

Confocal Raman spectroscopy is a non-invasive methodology capable of an in-depth assessment of skin composition (PERTICAROLI *et al.*, 2019). Furthermore, it is a technique that has been increasingly used in the evaluation of pharmaceutical and cosmetic products (PERTICAROLI *et al.*, 2019). Raman spectroscopy operates through the inelastic mirroring of radiation (FREDERICCI *et al.*, 2016) and, when associated with Confocal optics, allows the researcher to observe depth profiles of structured samples, such as the skin (PERTICAROLI *et al.*, 2019). To determine the wave size to be used in samples, researchers often use the International Laser Safety Standard (IEC 600285-1:2007) (PERTICAROLI *et al.*, 2019). In the studies by Perticaroli *et al*., 2019 and Yoon *et al*., 2019 two frequencies were used: 785 nm which is the fingerprint region of the spectrum (400-1800 cm^{-1}), and 671 nm for the high-frequency region (2400-4000 cm^{-1}). Spectra readings in volunteers are usually performed every 2 µm of depth, until reaching a depth of approximately 20 µm from the skin surface (PERTICAROLI *et al.*, 2019; YOON *et al.*, 2019). For Perticaroli *et al.*, 2019 using the Multivariate Curve Resolution with the alternate least squares method for data interpretation seems to be the best alternative. This association of methodologies has great potential for extracting information from the Confocal Raman analysis and reducing errors related to the estimation of system concentration (PERTICAROLI *et al*., 2019). The restriction presented in this model appears when the study variables have a strong tendency to covariate with each other (PERTICAROLI *et al.*, 2019).

In 2020, Ho *et al.* developed and patented the first portable Raman Confocal spectrometer system. The device was integrated with a library of skin spectra obtained

by Raman spectroscopy, as well as individual spectra and algorithms of machine learning (HO *et al.*, 2020). The quantitative analysis of this research used urocanic acid, water, and ceramides as stratum corneum markers. The performance of this device was confirmed using a traditional Raman Confocal spectrometer (HO *et al.*, 2020).

Another methodology that stands out, due to its sensitivity, is capillary electrophoresis (HERMANN; ABECK, 2000), however, during the execution of this article, we did not find recent articles. The research carried out by HERMANN; ABECK, 2000 and SUGAWARA *et al.*, 2012 were able to determine histidine, urocanic acid isomers, and pyrrolidone carboxylic acid (PCA), as shown in **Table 2**.

A summary of the non-chromatographic methods used for the identification and quantification of stratum corneum biomarkers, including the matrix, biomarkers, technique, sample preparation, and detection, is presented in **Table 2**.

TABLE 2

Table 2. Summary of the non-chromatographic methods used for the identification and quantification stratum corneum biomarkers, including the matrix, biomarkers, technique, sample preparation, and detection.

Matrix	Biomarkers	Technique	Sample Preparation	Detection	References
Human stratum corneum	amino acids (alanine, arginine, asparagine, aspartic acid, cysteine, glutamic acid, glutamine, glycine, proline, serine, tyrosine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, valine, ornithine, and citrulline), pyroglutamic acid, urocanic acid, urea, and lactic acid.	Tape-stripping/DART-TQMS with SIL-IS and inkjet-printing (LaboJet)	not required	Ion DART: positiv mode, 500 °C, ionizing helium gas, flow of 3L/min. Desolvation temperature: 250 °C.	MAENO, 2019

Human stratum corneum	keratin, NMF, water lipids	Confocal Raman spectroscopy	not required	Low-frequency laser: fingerprint region with signal to noise ratio > 20 (~1500). High-frequency laser: all perfiles > 3800 spectra.	PERTICAROLI *et al.*, 2019
Human stratum corneum	alanine, glycine, histidine, ornithine, serine, proline, *trans*-UCA, PCA, lactate, urea, ceramide e cholesterol.	Confocal Raman spectroscopy	not required	Laser 1: NMF and lipids measurement - wavelength of 785 nm. Laser 2: Moisturizing measurement - wavelenght of 761 nm; 30 µm depth.	YOON *et al*., 2019
Human stratum corneum	UCA	Handheld Confocal Raman spectroscopy	not required	Measurement was made each 10 µm until 100µm depthly. Laser was operated with wavelength of 785nm. Each 10s, another measure was made.	HO *et al.*, 2020

Human stratum corneum	UCA (*cis*- and *trans*-), His, Hid and MH	High-performace capillary electrophoresis (HPCE)	Skin samples extraction: glass funnel method - 3,0 mL of ethanol-water (1:1). The solution stayed in contact for 3 minutes on the skin. The extraction solution was dried using agitation and a vacuum centrifuge. Reconstitution was made using 0,5mL of HCl 0,001M and centrifuge for 3 minutes.	Column: fused-silica, 50cm x 75µL. Buffer: 0,05M NaH_2PO_4. Temperature: 25ºC. pH5 and 12KV. Wavelength: 214 e 267 nm. The samples was injected under pressure for 15s.	HERMANN; ABECK, 2000

Human stratum corneum	Alanine, arginine, asparagine, aspartic acid, cysteine, glutamic acid, glutamine, glycine, proline, serine, tyrosine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine.	Tape-stripping/DART-MS com SIL-IS	not required	DART: modo íon positivo; 450°C; gás ionizante: hélio; fluxo: 3 L/min. MS: voltagem capilar, -1500 V; deslocamento da placa final, -500 V; fluxo de gás de secagem, 8 L/min; e temperatura do gás de secagem, 180 °C.	MAENO; SHIDA; SHIMADA, 2017

CONCLUSION

For the last decade, many articles related to the identification and quantification of cutaneous markers were found, particularly urocanic acid, pyrrolidone carboxylic acid, and histidine. Through our review, we observed a predominance of the use of human stratum corneum obtained *ex vivo* by tape-stripping removal. The most used methodologies make use of HPLC with the use of a UV detector or a mass spectrometer. Another highlighted methodology was confocal Raman spectroscopy since comparatively, it seemed to be even less invasive when compared to tape-stripping, however, adding costs to the tests. This kind of analysis does not require sample preparation and has shown good results. The researchers also point out that this methodology, when applied to the stratum corneum, can vary and generate more errors in its results (YOON *et al.*, 2019).

Throughout this research, we observed that the methods of analysis of cutaneous markers did not vary much, in other words, few researchers explored new techniques or tried to optimize existing ones. This most likely happens because few articles aim to work on the technique itself. In addition, the identification and quantification of markers can help to develop dermatological research, for pharmaceutical and cosmetic industries, allowing the correlation of different methodologies with other equipment used in the clinical assessment of skin hydration, such as the capacitive method, for example. Due to discoveries relating the structures of the Natural Factor of the Skin with some pathologies, as: cancer (UCA), atopic dermatitis, o dry, scaly skin, atopic dermatitis, psoriasis (JOO *et al.*, 2012); developing new techniques or optimizing existing ones can help to clarify dermal pathologies that are not yet well established.

4 CHAPTER II

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

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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.

KEYWORDS: Histidine; Urocanic acid; Pyrrolidone carboxylic acid; HPLC-PDA; Stratum corneum

INTRODUCTION

The stratum corneum comes from keratinocytes and makes up the most superficial layer of the skin, located in the epidermis (RAWLINGS; HARDING, 2004; TSUKUI *et al.*, 2022; VERDIER-SÉVRAIN; BONTÉ, 2007). This barrier protects the body from dehydration, maintains water balance, and protects it from the external environment (RAWLINGS; HARDING, 2004; TREFFEL; GABARD, 1995; TSUKUI *et al.*, 2022). Composed of corneocytes and an intercellular lipid bilayer matrix, the stratum corneum depends on water to fulfill its functions and therefore, keeping it within the physiological range is essential (JOO *et al.*, 2012; TREFFEL; GABARD, 1995; VERDIER-SÉVRAIN; BONTÉ, 2007). Corneocytes are enucleated cells with keratin inside, in addition to amino acids and other small molecules that, together, are called Natural Moisturizing Factor (NMF) (VERDIER-SÉVRAIN; BONTÉ, 2007). NMF is a mixture of highly hygroscopic molecules derived from filaggrin, a protein that is rich in histidine (His) (**Figure 1**), which recovers keratin filaments (KOPPES *et al.*, 2017; VERDIER-SÉVRAIN; BONTÉ, 2007). 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) (**Figure 1**) (VERDIER-SÉVRAIN; BONTÉ, 2007). In addition to these components, NMF includes lactates, urea, and electrolytes (VERDIER-SÉVRAIN; BONTÉ, 2007). 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 (CELLENO, 2018; HAGEMANN; PROKSCH, 1996; LEITE E SILVA *et al.*, 2009). 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 (KOPPES *et al.*, 2017). In addition to being in skin (LEITE E SILVA *et al.*, 2009), filaggrin can also be found in oral and nasal mucosa (PALMER *et al.*, 2006). 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 (FIUME *et al.*, 2019). PCA is the most hygroscopic and one of the most important components of the NMF, corresponding to approximately 12% of NMF (RAWLINGS; HARDING, 2004). 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.*, 2013, Hermann; ABECK, 2001; Kezic *et al.*, 2009, Koppes *et al.*, 2017, 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 (FIUME *et al.*, 2019; HERMANN; ABECK, 2000; IRVING *et al.*, 2017; JOO *et al.*, 2012; PALMER *et al.*, 2006; RAJ *et al.*, 2017; VISSCHER *et al*., 2020). However, other techniques, such as Direct Analysis in Real Time (MAENO, 2019; MAENO; SHIDA; SHIMADA, 2017), Raman spectroscopy, and electrophoresis (HERMANN; ABECK, 2000), 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

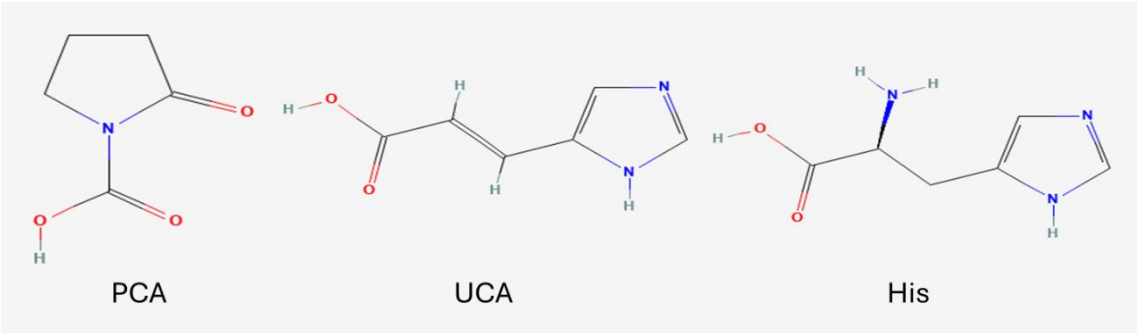

Figure 1. Chemical structure of 2-pyrrolidone-5-carboxylic acid (PCA), urocanic acid (UCA), and histidine (His) (CHEMICALIZE - INSTANT CHEMINFORMATICS SOLUTIONS, [s.d.], [s.d.], [s.d.]).

NMF represents 20–30% of the dry weight of the stratum corneum, which is composed of approximately 20% water (VERDIER-SÉVRAIN; BONTÉ, 2007). The percentage of water present in the stratum corneum is linked to the water

retention capacity of the NMF (VERDIER-SÉVRAIN; BONTÉ, 2007). Some studies report that low levels of NMF are often related to dry, scaly skin, atopic dermatitis, psoriasis (JOO *et al.*, 2012), 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).

MATERIAL AND METHODS

Urea was obtained from Petrobras (cosmetic grade); 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) 1M solution were obtained from Merck Brazil. Disodium EDTA was obtained from Shijiazhuang Jackchem Co. Ltd. from China; methylparaben was obtained from UENO Fine Chemical from Japan; and imidazolidinyl urea was from Wuhu Huahai Bio. Engineering Co., Ltd. from China, all supplied in Brazil by Embacaps Química e Farmacêutica LTDA, from Brazil. Ammonium acryloyldimethyltaurate/VP copolymer (Aristoflex® AVC) was obtained from Clariant and supplied in Brazil by Pharma Special. Analytical Grade acetonitrile was obtained from Honeywell Brazil. MilliQ® water was obtained from a Merck apparatus.

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) (MEDICAL; ASSOCIATION, 2013) 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 hours 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. Each participant provided

three samples of stratum corneum taken from different 2 x 5 cm sites randomly delimited on the forearms. One was from bare skin (control group), another with the application of a neutral gel (neutral group), and another with 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**.

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.

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 0.001N 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 gradient elution. The mobile phase of TEAP 0.01M 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..

Table 2. Gradient elution of the chromatographic runs.

Time	Triethylammonium phosphate (TEAP) 1M solution	Acetonitrile	Type
0–7 minutes	100	0	Isocratic
7–8 minutes*	100–0	0–100	Gradient
8–14 minutes*	0	100	Isocratic

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

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 were the same as those used to carry out the tape-stripping. Participants underwent a 30 min acclimatization period at a temperature of 20 ± 2 °C and relative humidity of 50 ± 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.

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 (INTERNATIONAL COUNCIL FOR HARMONISATION, 2022).

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 standard was weighed one-by-one and solubilized with HCl 0.001N. Then, dilutions were made until it became closer to the final required concentration. Then, exact volumes to achieve the desired concentration were obtained in a pool of the standard in 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 (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 (AOAC OFFICIAL METHODS OF ANALYSIS, 2002, 2016). 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 6

times (DOQ-CGCRE-008) (INSTITUTO NACIONAL DE METROLOGIA (INMETRO), 2020), the ratio of intercept standard deviation, and the slope of linear equation, respectively.

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).

$$u_c = \sqrt{u_b^{\,2} + u_p^{\,2}} = \sqrt{(R - 100)^2 + s_p^{\,2}} \qquad \textbf{Eq. 1}$$

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 50000 Monte Carlo simulations.

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 x 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.

RESULTS AND DISCUSSION

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 (**Figure 2A** and **Figure 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 (**Figure 2B** and **Figure 3B**). Finally, the tapes from participants were subject to chromatographic analysis. A representative chromatogram of stripped tapes from subjects is provided in **Figure 2C** and **Figure 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 (DE SOUZA NETO *et al*., 2023; JOO *et al.*, 2012), 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.

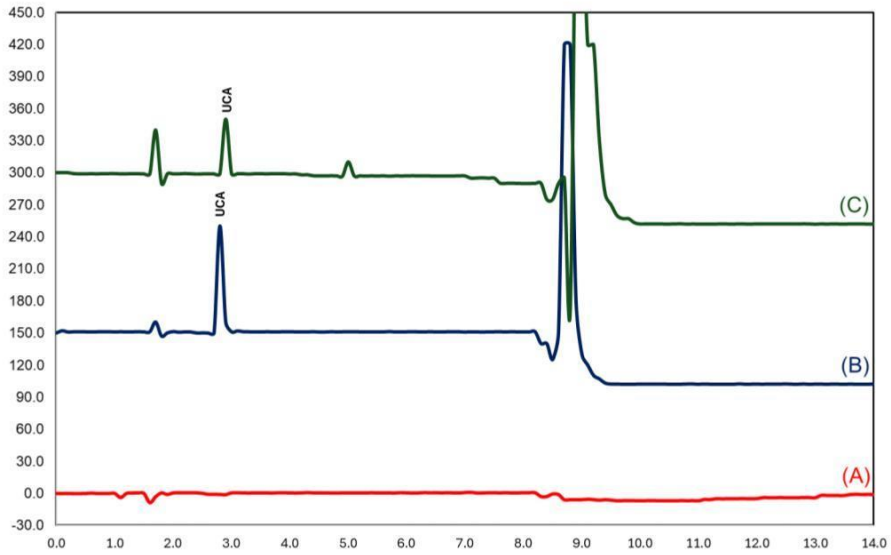

Figure 2. Chromatogram in a complete run (14 minutes) a 268nm: A) isolated tape-stripping; B) UCA standards (1µg/ml) in a presence of the tape; C) participant's tape-stripping skin (without the presence of gel).

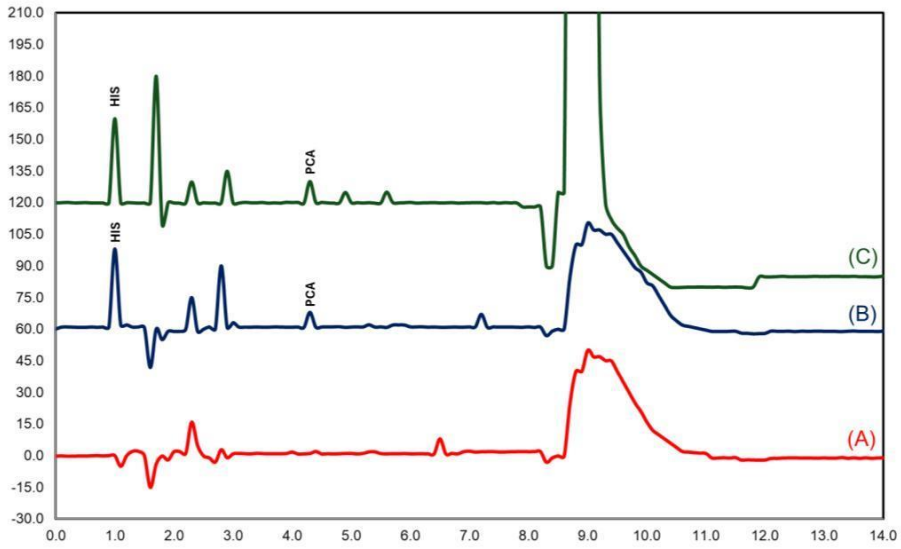

Figure 3. Chromatogram in a complete run (14 minutes) a 210nm: A) isolated tape-stripping; B) PCA and His standards (1µg/ml) in a presence of the tape; C) participant's tape-stripping skin (without the presence of gel).

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 (AOAC OFFICIAL METHODS OF

ANALYSIS, 2002, 2016; ICH, 2005). 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 (AOAC OFFICIAL METHODS OF ANALYSIS, 2002, 2016), must be between 75 and 120%. The mean recovery values 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 (AOAC OFFICIAL METHODS OF ANALYSIS, 2002), 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**.

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 R2 > 0.99 / 0.2 to 10 µg/mL	Y = 8800+135183X R2 = 0.9999 / 0.2 to 10 µg/mL	Y = -780+36230X R2 = 0.9999 / 0.2 to 10 µg/mL	Y = 26032+513058X R2 = 0.9999 / 0.2 to 10 µg/mL
Trueness (recovery)	0.2 µg/ml: 75–120%	100.2% (96.9–105.5%)	103.6% (92.7–113.6%)	105.6% (100.6–115.1%)
	1.0 µg/ml: 75–120%	98.3% (97.0–100.9%)	102.2% (96.8–107.4%)	98.6% (94.5–102.5%)
	5.0 µg/ml: 75–120%	102.1% (98.2–107.3%)	99.3% (94.4–101.3%)	98.3% (96.0–100.6%)
Precision (RSD)	0.2 µg/ml: 8% / 16%	1.7% / 2.1%	3.5% / 12.1%	4.8% / 5.6%
Repeatability / Intermediate precision	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*	-	0.06 µg/ml	0.18 µg/ml	0.12 µg/ml

Table 3. Summary of validation data results of specificity, linearity, trueness, precision, and LOD/LOQ. *The limits of detection and quantification were estimated as 3 and 6 times, respectively, for the ratio of intercept standard deviation and the slope of linear equation.

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).

Detailed evaluation of HPLC*-ex vivo *protocol according to 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 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 (SACHDEVA, 2009). 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.

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

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

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**).

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 (0.01)	4.27 (0.13)	3.39 (0.1)	1.14 (0.03)	0.33 (0.03)	0.23 (0.02)	0.04 (0)
Female	Low values	A	1.56 (0.03)	2.10 (0.05)	2.20 (0.05)	3.31 (0.1)	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 (0.00)	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 (0.10)	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 (0.05)	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 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 (*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

Table 6. Statistical comparisons (*p*-values*) for the concentration of His, PCA, and UCA for the 24 participants with different gender, phototype, and age.

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 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 **Figure 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 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 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 **Figure 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 **Figure 4C**.

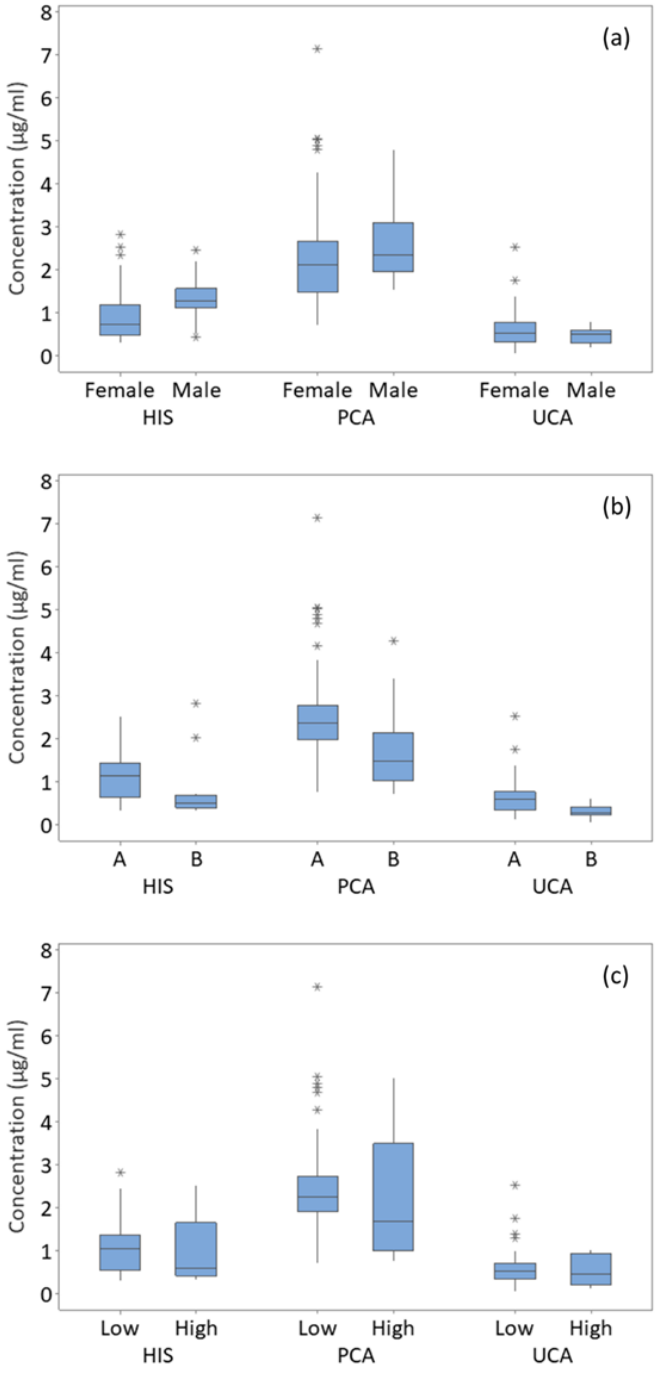

Figure 4. Box-plot graphs of HIS, PCA, and UCA concentration by (a) gender (female and male), (b) age (groups A and B), and (c) phototype (low and high).

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 postmenopausal females (LUEBBERDING; KRUEGER; KERSCHER, 2013). 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 (FENG *et al.*, 2014). Previous studies indicate that female skin can vary its hydration naturally up to 40 years (LUEBBERDING; KRUEGER; KERSCHER, 2013). 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 (LUEBBERDING; KRUEGER; KERSCHER, 2013). 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.

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.

5 CONCLUSION

In the literature review, various analytical procedures for the quantification of the dermatological markers PCA, UCA, and His were found. The most commonly used technique is high-performance liquid chromatography; however, other techniques (such as Raman spectroscopy, electrophoresis, among others) are also described in the literature. Although there are several analytical procedures described in the literature, we did not find studies on how population characteristics (such as gender, age range, and phototype) affect the determination of the dermatological markers in question.

Corneometer® was used as a reference in measuring skin hydration, and subsequently, it was compared with the results of quantifying the markers PCA, UCA, and His by HPLC-PDA in human stratum corneum extracted using the tape-stripping technique. The analytical procedure HPLC-PDA was validated for parameters of linearity, accuracy, repeatability/intermediate precision, LOD, and LOQ. All evaluated parameters showed results in accordance with the guidelines established by the AOAC.

Each participant provided 3 samples of stratum corneum, one from bare skin (control group), another with application of a neutral gel (neutral group), and another with application of gel containing 10% urea (urea group). The obtained values were analyzed using Monte Carlo Simulation and the non-parametric Mann-Whitney method. Although some results present limitations in terms of the number of participants, we obtained significant insights for refining a protocol for quantifying the biomarkers His, PCA, and UCA by HPLC-PDA from ex vivo human stratum corneum. Through statistical analyses, we can infer that the best audience for quantifying the dermal markers were individuals up to 35 years old, of all phototypes, preferably females.

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