Journal of Chromatography B, 897 (2012) 55–63

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

# 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

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ARTICLE INFO

*Article history:*
Received 17 November 2011
Accepted 4 April 2012
Available online 12 April 2012

*Keywords:*
Pyroglutamic acid
*cis*-/*trans*-Urocanic acid
Human stratum corneum
Natural moisturizing factor (NMF)
HILIC–MS/MS

ABSTRACT

A rapid, sensitive and specific hydrophilic interaction liquid chromatography coupled to tandem mass spectrometric (HILIC–MS/MS) method for the simultaneous determination of pyroglutamic acid, *cis*- and *trans*-urocanic acid in human skin stratum corneum (SC) were developed and validated. This method was carried out without derivatization or addition of ion-pair additives in mobile phase. The analytes were extracted by PBS buffer solution and analyzed using an electrospray positive ionization mass spectrometry in the multiple reaction monitoring (MRM) mode. Chromatographic separation was performed on an AQUITY UPLC amide column using gradient elution with the mobile phase of water and acetonitrile. The standard curves were linear over the concentration range of 1.0–250 ng/mL with a correlation coefficient higher than 0.999 with an LLOQ of 0.5 ng/mL. The lower limits of detection (LLOD) of these analytes were lower than 0.2 ng/mL. The intra- and inter-day precisions were measured to be below 7.7% and accuracies were within the range of 94.3–102.6%. The validated method was successfully applied to determine the level of pyroglutamic acid and *cis*-/*trans*-urocanic acid in the SC samples from forearm and forehead region of 19 human volunteers.



## 1. Introduction

Water in skin stratum corneum (SC) is essential to the maintenance of mechanical integrity of the skin [1]. Natural moisturizing factor (NMF) which has high hygroscopicity and water-retaining capability is critical in preserving water in SC. NMF is mainly composed of free amino acids and their metabolites such as pyroglutamic acid (PGA) and urocanic acid (UCA) [2]. Abnormally low level of NMF is frequently observed in the dry and scaly skins [3], atopic dermatitis and psoriasis [4]. Accordingly, the measurement of amino acids and their metabolites in human SC can give important information on the health of skin.

PGA and UCA are the major components of NMF and commonly used as indicators of NMF level in SC since they are the terminal metabolites of amino acids. PGA is a metabolite of glutamine [5] and UCA is an imidazole-acrylic acid derivative that is produced from histidine [6]. Recently, additional biological roles of PGA and UCA have been enlightened that include the suppression of microorganism, UV-blockade and immune-suppression. Especially, on exposure to UV, *trans*- to *cis*-photoisomerization of UCA occurs [7]. Each UCA isomer has distinct biological activities [8], raising the need for a sensitive analytical method for the respective UCA isomers.

Bioanalytical methods for amino acids and amino acid derivatives have been extensively investigated such as HPLC-UV, fluorescence detection or coulometric detection, gas chromatography (GC) [10], thin-layer chromatography [10], capillary electrophoresis [9], GC–MS [10,11] and LC–MS [12]. However, in order to apply to biological matrices, these methods require time-consuming and laborious pre- or post-column derivatization. In addition, some derivatives are unstable and they are inapplicable to PGA since derivatizing reagents do not react with tertiary amines. Recently, ion-pair reagents such as trifluoroacetic acid, tetrabutylammonium hydroxide, heptafluorobutyric acid and pentadecafluorooctanoic acid (PDFOA) have been tried for LC–MS/MS-based amino acid analysis [13]. Addition of volatile ion-pair reagents to mobile phase improves separation of underivatized amino acids on a reverse-phase silica-based C8 or C18 columns. They also increase the MS

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http://dx.doi.org/10.1016/j.jchromb.2012.04.008

signal of amino acids and improve peak shape. However, ion-pair reagents frequently affect assay durability [13] and the sensitivity decreases due to ion suppression.

Many attempts have been made to develop analytical method to determine PGA [4,14] and UCA [9,15,16] in human SC. However, analytical method for PGA has not been fully validated to our best knowledge. For the analysis of UCA, ion-pair reagents like sodium heptasulfonate or derivatization with dabsyl chloride have been tried but they entail difficulties like column stabilization and sample preparation as described above. In addition, derivatization reagent can react with other amino acids and amines in the biological samples, demanding long chromatographic run times for the proper separation of analytes. Most importantly, these methods have low specificity and poor sensitivity. With ion-pair reagent method, LOD for *cis*- and *trans*-UCA was 277 ng/mL and 485 ng/mL [15], respectively. With derivatization method, UCA was detectable as low as 13.86 ng/mL [16]. With high performance capillary electrophoresis, LOD was far higher ranging 69.3 ng/mL [9].

Here, we developed a versatile, highly sensitive and selective LC–MS/MS method for the analysis of PGA and *cis*-/*trans*-UCA in the tape-stripped SC samples from human, using hydrophilic interaction liquid chromatography (HILIC). HILIC is employed for the analysis of highly polar analytes that are hard to retain and separate with reversed-phase HPLC [17]. HILIC increases ionization efficiency with the use of mobile phases containing a high proportion of organic solvents such as acetonitrile. The extraction using PBS buffer without derivatization or ion-pair reagents was sufficient for the sample preparation. With this method, PGA and *cis*-/*trans*-UCA were quantified in the SC samples from forearm and forehead region of 19 volunteers with LOD lower than 0.2 ng/mL. We compared their levels with skin barrier function to assess the applicability of this analytical method for biological studies.

## 2. Experimental

### 2.1. Chemicals and reagents

Pyroglutamic acid (PGA), *trans*- and *cis*-urocanic acid (UCA) were purchased from Aldrich (St. Louis, MO). L-proline-$^{13}C_5$, $^{15}N$ (IS) was purchased from Cambridge Isotope Laboratories (Andover, MA). HPLC grade acetonitrile was purchased from Fisher Scientific (Fair Lawn, NJ). Sodium dodecyl sulfate was obtained from Sigma (St. Louis, MO, USA) and trifluoroacetic acid (TFA), heptafluorobutyric acid (HFBA) and propylene glycol were purchased from Fluka (Burchs, Switzerland). Protein assay kit was purchased from Pierce (Rockford, IL). The water used were ultra-pure deionized water (18.2 MΩ cm) produced from Millipore Milli-Q Gradient system (Millipore, Bedford, MA). All other reagents used were of highest grade available.

### 2.2. Preparation of standards solution

Standard stock solutions of PGA, *cis*- and *trans*-UCA were prepared in water at concentration level of 1 mg/mL, respectively, and stored at −20 °C. The mixed working standard solution containing PGA, *cis*- and *trans*-UCA was serially diluted with phosphate buffered saline (pH 7.4) containing 0.1% (w/v) sodium dodecyl sulfate/2% (w/v) propylene glycol and 30% water in acetonitrile (1:9) to obtain concentrations for calibration curve standards (0.5, 1.0, 2.5, 5.0, 10.0, 25.0, 50.0, 100, 250 ng/mL).

Quality control samples were prepared to 1.0, 50 and 200 ng/mL with PBS buffer solution and 30% water in acetonitrile (1:9). 1 mg/mL internal standard stock solution of L-proline-$^{13}C_5$, $^{15}N$ was prepared in water and the working solution of IS was diluted to get a final concentration of 1 μg/mL in 30% water in acetonitrile. This solution was added to standard working solutions to a final concentration of 50 ng/mL.

**Table 1**
Gradient condition of mobile phase.

| Time (min) | Flow rate (mL/min) | A (%) | B (%) | Curve |
|---|---|---|---|---|
| 0.0 | 0.3 | 20 | 80 | 6 |
| 0.5 | 0.3 | 20 | 80 | 6 |
| 1.5 | 0.3 | 60 | 40 | 6 |
| 3.5 | 0.3 | 60 | 40 | 6 |
| 5.0 | 0.3 | 20 | 80 | 1 |

A: deionized water; B: acetonitrile.

### 2.3. Sample preparation

#### 2.3.1. Tape stripping

To minimize the effect from temperature and humidity on Trans-epidermal water loss (TEWL), the subjects (19 healthy and young Koreans) were undergone a standardized washing procedure (soap washing) and retained in humidity- and temperature-controlled room (24 ± 2 °C, 40 ± 2%) for 30 min before measuring TEWL and collecting SC samples. After measuring TEWL with VapoMeter (Delfin, Finland) on inner forearm and forehead, SC specimens were obtained with a D-squame standard tape (Cuderm, Dallas, TX, diameter, 2.2 cm). Ten consecutive tapes were collected from each volunteer (27–40 years old, 7 males and 12 females, Asian). The tapes were stored at 4 °C until use.

#### 2.3.2. Protein analysis of the tape strips

For protein quantification, 5 tapes were put in glass vials containing 5 ml of 0.1% (w/v) sodium dodecyl sulfate/2% (w/v) propylene glycol in PBS buffer solution and then sonicated for 1 h to obtain soluble proteins. The solutions were centrifuged at 12,000 rpm for 10 min at 4 °C. The supernatant was separated and aliquots of solutions were used to quantify the amount of protein. The concentration of soluble protein was assayed with protein assay kit (Pierce, Rockford, IL). The sample plates were incubated for 30 min at 37 °C after which the absorbance was measured with the Microplate Reader (Spectramax190, MDA) at 595 nm.

#### 2.3.3. Preparation of PGA, UCA sample solutions

100 μL of the soluble protein extract obtained in the above procedure was added to 50 μL of internal standard (1 μg/mL) and 850 μL of 30% water in acetonitrile into HPLC vial. The sample solutions for PGA, UCA analysis were mixed and stored −20 °C until analysis.

### 2.4. Liquid chromatography

The chromatographic separation was carried out using ACQUITY UPLC system (Waters Co., Milford, MA). The column was ACQUITY UPLC BEH Amide column (1.7 μm, 2.1 mm × 100 mm). The column temperature and autosampler tray temperature were maintained at 40 °C and 10 °C, respectively. The mobile phase consisted of deionized water (solvent A) and acetonitrile (solvent B). Gradient elution was as follows: isocratic elution with 80% B for 0.5 min, followed by a 1.5 min gradient to 40% B, isocratic elution with 40% B in 3.5 min, then returned to 80% B in 5 min (Table 1). The flow rate was 0.3 mL/min and injection volume was 5 μL.

### 2.5. Mass spectrometry

LC–MS analysis was performed using Waters Micromass Quattro Premier XE triple quadrupole mass spectrometer. The mass spectrometer was operated in the positive ESI mode with following

**Table 2**
MRM transition parameters for PGA, *cis*-, *trans*-UCA and internal standard (L-proline-$^{13}C_5$, $^{15}N$).

| Analytes | Precursor ion (*m*/*z*) | Product ion (*m*/*z*) | Capillary voltage (kV) | Cone voltage (V) | Collision energy (eV) |
|---|---|---|---|---|---|
| PGA | 129.7 | 83.7 | 3.3 | 25 | 14 |
| *cis*-UCA | 138.6 | 120.7 | 3.3 | 20 | 12 |
| *trans*-UCA | 138.7 | 120.7 | 3.3 | 30 | 16 |
| L-proline-$^{13}C_5$, $^{15}N$ | 121.8 | 74.8 | 3.3 | 25 | 15 |

operation conditions. Capillary voltage, 3.5 kV; ion source temperature, 120 °C; desolvation temperature, 350 °C; desolvation gas flow rate, 750 L/h; cone gas flow rate, 50 L/h. The optimum cone voltages for PGA, *cis*-, *trans*-UCA and L-proline-$^{13}C_5$, $^{15}N$ (IS) were set to different voltages according to *m*/*z* (Table 2). The multiple reaction monitoring mode (MRM) was used for analysis with dwell time 0.05 s per channel. We controlled the data acquiring and process with MassLynx Version 4.1 (Waters Co.).

### 2.6. Method validation

#### 2.6.1. Matrix effect

The matrix effect was investigated by comparing the peak areas of analytes spiked into five different blank tape extraction (post-extraction) solutions versus the peak areas of standard solution at the same concentrations. Each samples were prepared at low (1 ng/mL) and high (200 ng/mL) concentrations. The matrix effect was reported as the percentage values.

#### 2.6.2. Linearity, lower limit of quantification, accuracy and precision

The method was validated by linearity, the lower limit of detection (LLOD), the lower limit of quantification (LLOQ), intra- and inter-day accuracy and precision. The lower limit of detection (LLOD) was determined as the concentration when signal to noise was 3. The lower limit of quantification was determined as the lowest concentration point of standard curve when signal to noise ratio was 10. To evaluate of linearity, five sets of calibration standards ranging from 1.0 to 250 ng/mL were prepared and assayed. The calibration curves were constructed with linear or quadratic least-squares regression using weighting factor (1/*x*). The intra- and inter-day accuracy and precision were assayed by determining 5 replicates of QC samples at three concentration levels (1.0, 50, 100 ng/mL) in a single run for 3 days. Accuracy was expressed by (measured concentration)/(spiked concentration) × 100 and precision was calculated by % RSD (relative standard deviation). Acceptance criteria were within 15% both for precision and accuracy except at LLOQ (not exceed 20%).

The extraction recoveries of analytes were determined by comparing the peak area of obtained from QC samples that had been spiked with the analytes prior to extraction with those for recovery standards which were prepared by adding analytes to blank tape extracts.

## 3. Results and discussion

### 3.1. Method development

#### 3.1.1. Liquid chromatography

Using LC–MS/MS, we developed a method to determine three amino acid metabolites, pyroglutamic acid (PGA) and *cis*/*trans*-urocanic acid (UCA) in the skin stratum corneum, simultaneously (Fig. 1). Three LC modes were tested for the measurement of these analytes including reversed phase, ion-pairing and partitioning – weak cation exchange modes.

PGA and UCA are small hydrophilic molecules with molecular weights below 200 (Fig. 2). These hydrophilic compounds were poorly retained on the C18 column. Volatile ion-pairing reagents were added to improve the separation of hydrophilic compounds. Perfluorinated carboxylic acids such as TFA and HFBA were tested for the analyte separation on the ACQUITY BEH C18 column (2.1 mm × 100 mm, 1.7 μm). When 0.05% TFA was tested as modifier, PGA was not well retained on C18 column and UCA showed a peak tailing although it was well retained. To improve retention time and peak shape, we used a combination of 0.1% formic acid and 0.05% HFBA as modifier in the mobile phase. But PGA was eluted too quickly and the peak of UCA was broad (Fig. 3A) In addition, higher concentrations of HFBA not only caused ion suppression on ESI(+) MS, but also induced peak tailing of UCA. Different C18 column such as ACQUITY HSS T3 column (2.1 mm × 100 mm, 1.8 μm) that promotes polar compound retention and aqueous mobile-phase compatibility was also tested with the same condition. In case of using HSS T3 column, PGA and UCA were well retained, but UCA was split into two broad peaks (Fig. 3B) and interference was observed from blank injections when analyzing UCA at the low pH (below 3). Using 5 mM ammonium acetate/acetic acid buffer, the pH of mobile phase was increased to 5.8. By increasing pH, the contamination in the blank injections was eliminated. However, although the peak shapes of UCA and PGA were acceptable (Fig. 3C), *cis*- and *trans*-isomer form was not completely resolved (data not shown).

The HILIC (amide) approach was tried for UCA isomer separation and PGA quantification since HILIC is known to provide sufficient retention for very polar compounds [18]. Separation on the HILIC column is by partitioning and weak cation exchange mechanism. Previously, weak anion-exchange amino column has been successfully applied to the analysis of PGA in urine by Bishop et al. [19] even though it was not tested for UCA

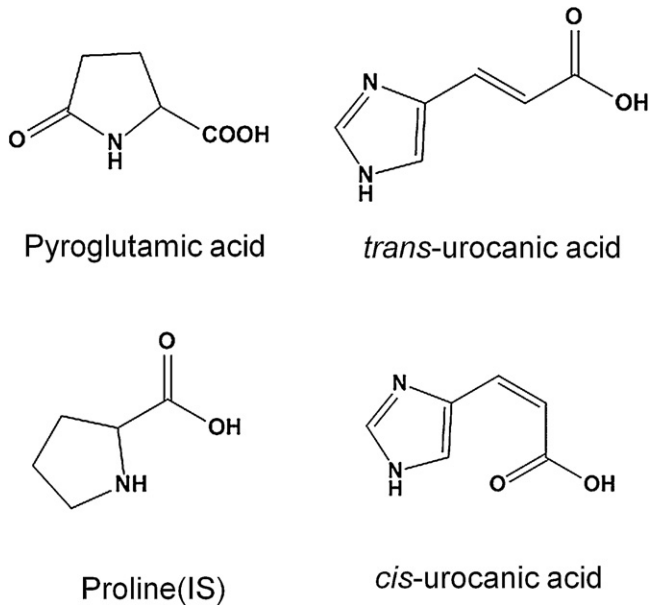


**Fig. 1.** Chemical structures of pyroglutamic acid (PGA), *trans*-urocanic acid (*trans*-UCA), *cis*-urocanic acid (*cis*-UCA) and proline (IS).

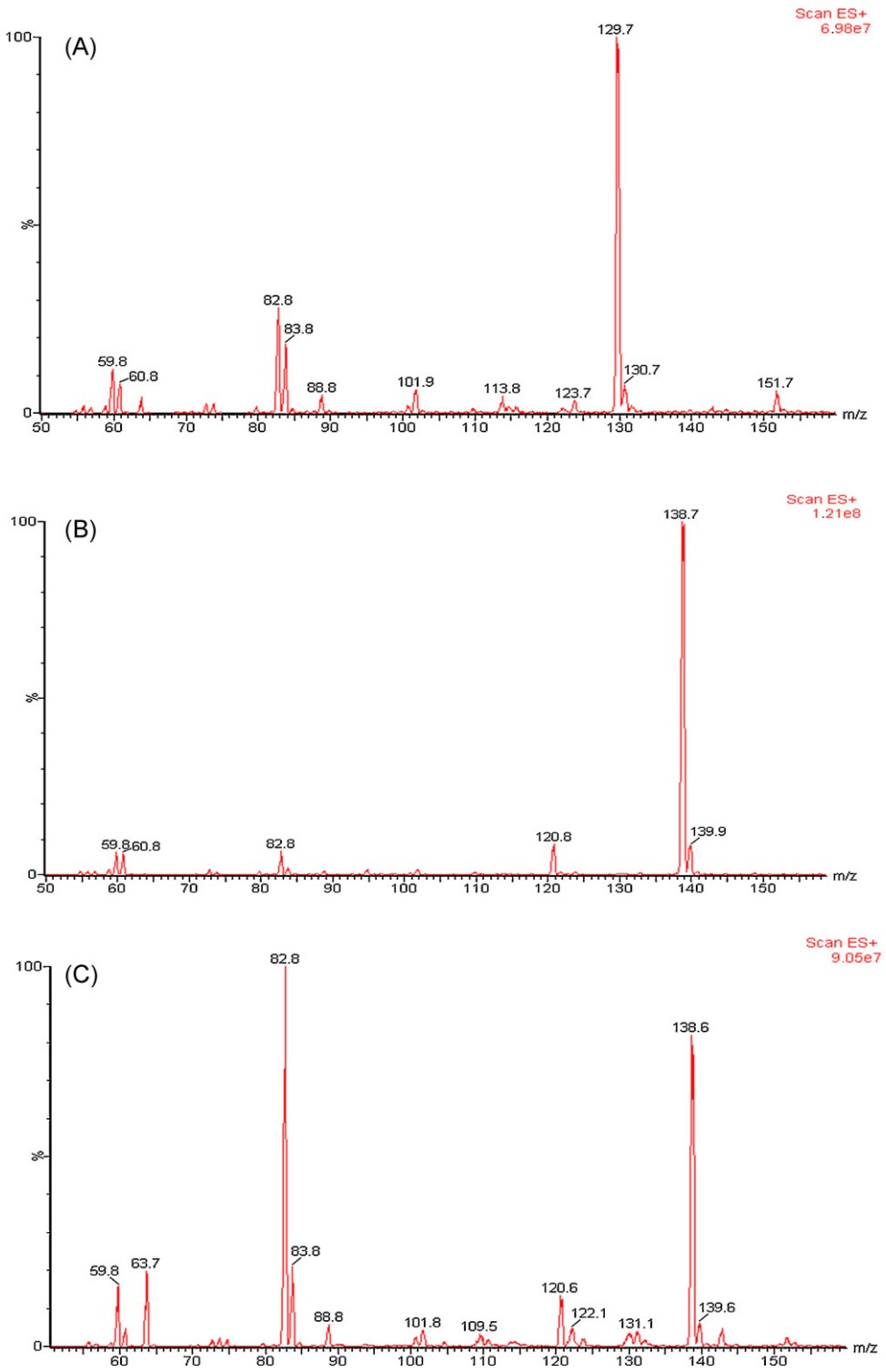


**Fig. 2.** Representative MS spectrum: (A) pyroglutamic acid, (B) *trans*-urocanic acid, (C) *cis*-urocanic acid.

isomers. The use of the mobile phase containing a high organic portion leads to higher MS sensitivity because of increased ionization efficiency. In the gradient elution of acetonitrile with acetic acid/5 mM ammonium acetate, peak-splitting and tailing were observed. When the composition of mobile phase was changed to acetonitrile–water, it showed a sufficient separation and good peak shapes of analytes on the BEH amide column (2.1 mm × 100 mm, 1.7 μm). In our method, a gradient elution program was employed with 80% organic phase as the initial concentration of gradient program (Table 1). With the acetonitrile–water gradient program, proper separation and retention of the analytes and internal standard were achieved in a total run time 5 min. The retention times were 1.1, 1.4, 1.8 and 1.9 min for PGA, *cis*-, *trans*-UCA and proline (IS), respectively. Representative chromatograms for blank and standard at LOQ are shown in Fig. 4.

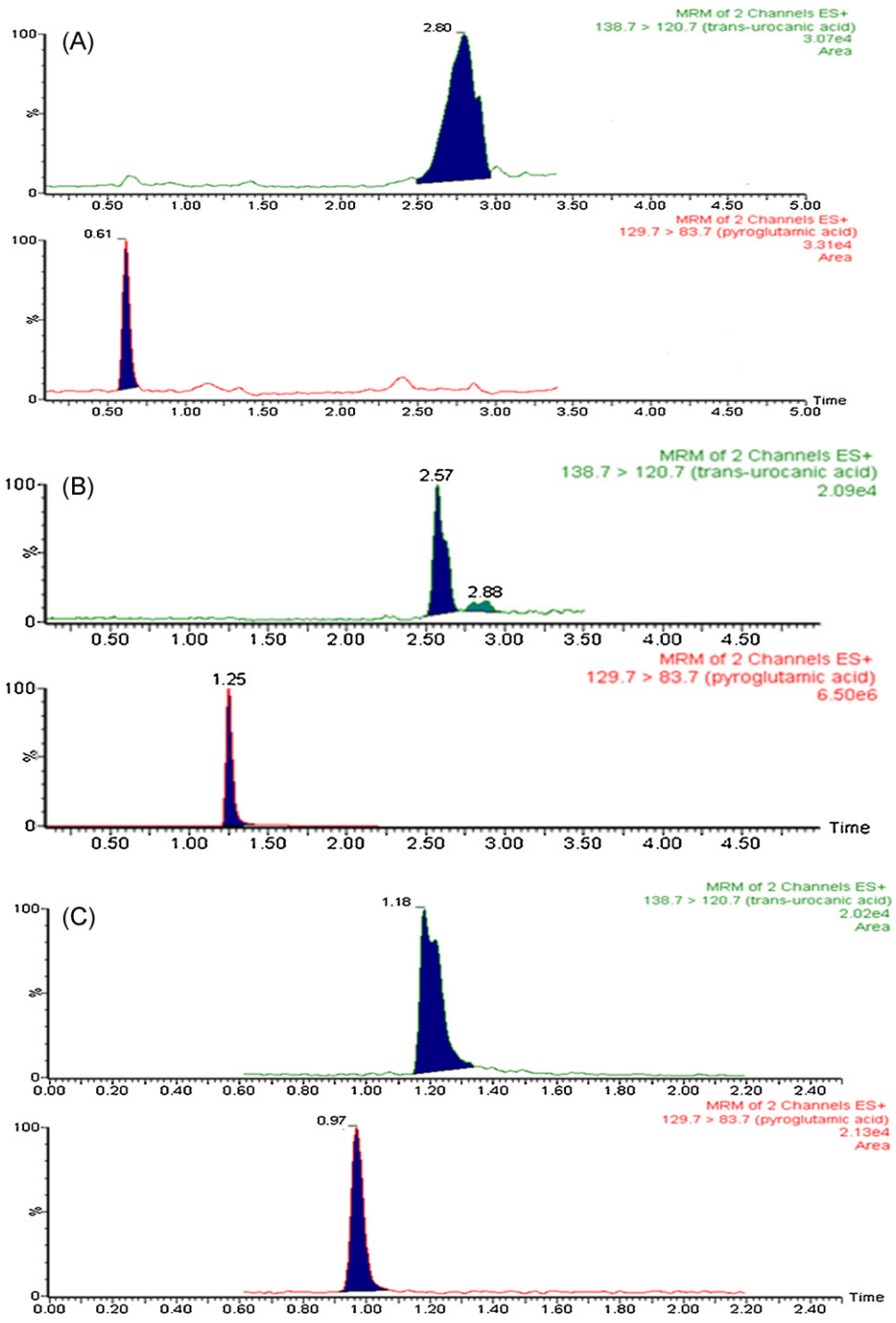


**Fig. 3.** MRM chromatograms of PGA, *trans*-UCA. Chromatographic conditions: (A) column: BEH C18 (2.1 mm × 50 mm, 1.7 μm), mobile phase A: 0.1% formic acid/0.05% heptafluorobutyric acid in DW, B: 0.1% formic acid/0.05% heptafluorobutyric acid in acetonitrile; (B) BEH HSS T3 (2.1 mm × 100 mm, 1.8 μm), mobile phase A: 0.1% formic acid/0.1% heptafluorobutyric acid in DW, B: 0.1% formic acid/0.1% heptafluorobutyric acid in acetonitrile; (C) BEH HSS T3 (2.1 mm × 100 mm, 1.8 μm), mobile phase A: 5 mM Ammonium acetate (pH 5.8), B: acetonitrile.

### 3.1.2. Mass spectrometry

The mass spectrometric conditions were optimized to obtain the maximum sensitivity of PGA, *cis*-, *trans*-UCA and proline (IS) using direct infusion of 1 μg/mL in a 50:50 mobile phase mixture. The MS responses to ESI were evaluated by measuring the full scan mass spectra in positive ion mode and negative ionization modes. The maximum sensitivity of PGA, *cis*-, *trans*-UCA and proline (IS) was showed in positive mode, where the analytes gave a protonated molecular ion $[M+H]^+$ at *m*/*z* 129.7, 138.6/138.7 and 121.8 for PGA, *cis*-, *trans*-UCA (Fig. 2) and L-proline-$^{13}C_5$, $^{15}N$, respectively. Proline was selected as internal standard because of its similarity in chemical structure, retention time, ionization and extraction efficiency. The most abundant fragment ions obtained by LC–MS/MS are summarized in Table 2. They were used for MRM transitions.

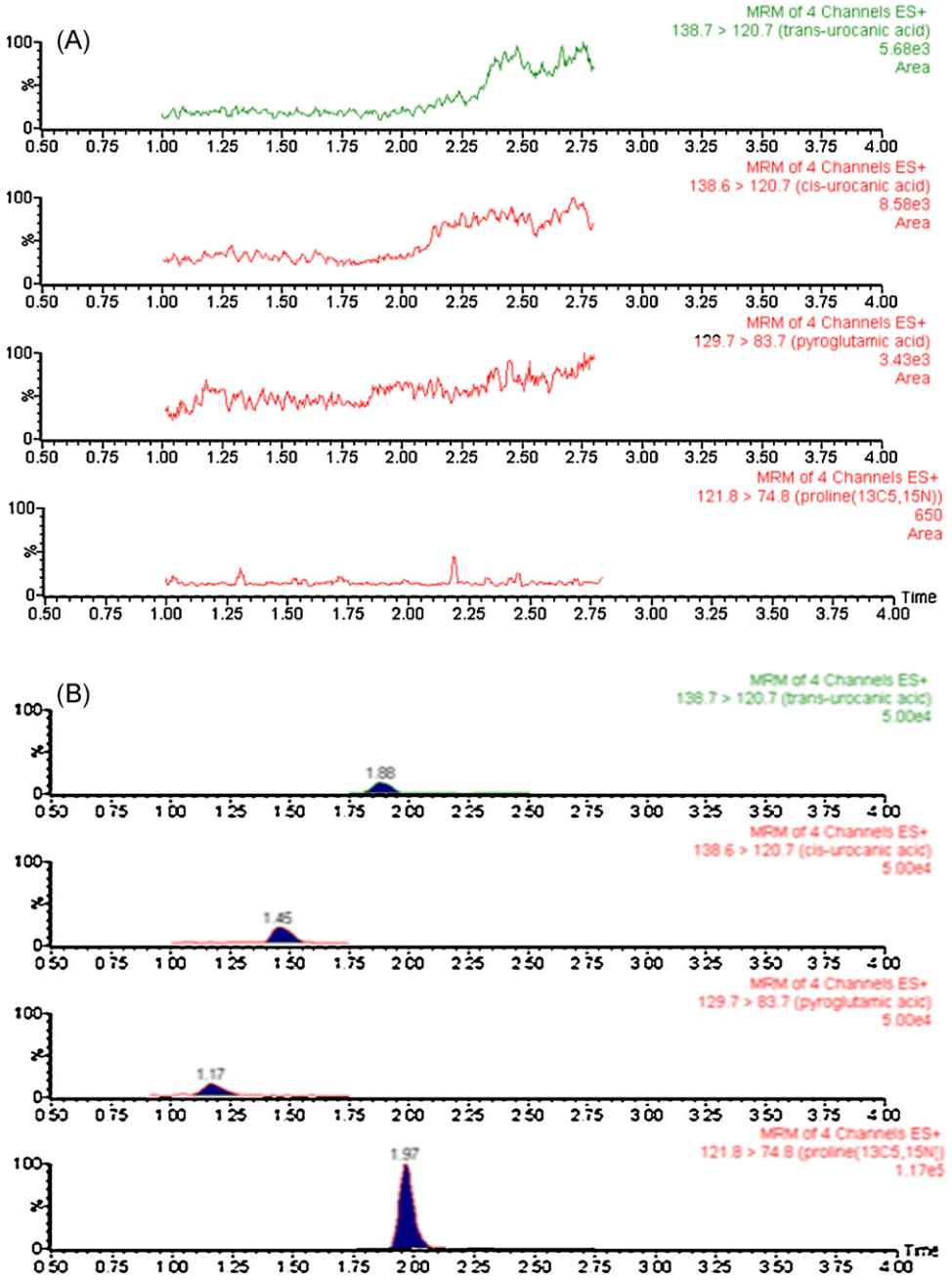


**Fig. 4.** Representative MRM chromatograms: (A) blank tape extract samples; (B) standard sample spiked with of pyroglutamic acid, *trans*-urocanic acid, *cis*-urocanic acid and IS at the lower limit of quantification (0.5 ng/mL).

### 3.2. Method validation

#### 3.2.1. Matrix effect

Five sets of blank tape extractions were spiked with PGA, *cis*- and *trans*-UCA and the variations due to matrix effects were calculated. As shown in Table 3, there were no significant matrix effects for all analytes at 1 ng/mL and 200 ng/mL.

#### 3.2.2. Specificity, linearity, lower limit of quantification, accuracy and precision

The specificity of the method was demonstrated by comparing MRM chromatograms of PGA, *cis*-, *trans*-UCA and the IS (L-proline-$^{13}C_5$, $^{15}N$) for a blank sample (Fig. 4A) with a spiked standard (Fig. 4B). Both the analytes and internal standard could be detected on their own ion chromatograms without any significant interference.

Linearity could be obtained from 1 to 250 ng/mL for PGA, *cis*- and *trans*-UCA with a correlation coefficient ($R^2$) value greater

**Table 3**
Matrix effect.

| Analyte | Analyte concentration (ng/mL) | Matrix effect (%) | Mean ± *SD* (%) (*N* = 5) |
|---|---|---|---|
| PGA | 1.0 | 97.5 | 102.0 ± 6.37 |
| | 200.0 | 105.0 | |
| *cis*-UCA | 1.0 | 93.5 | 95.8 ± 5.51 |
| | 200.0 | 97.3 | |
| *trans*-UCA | 1.0 | 106.5 | 101.7 ± 6.49 |
| | 200.0 | 98.5 | |

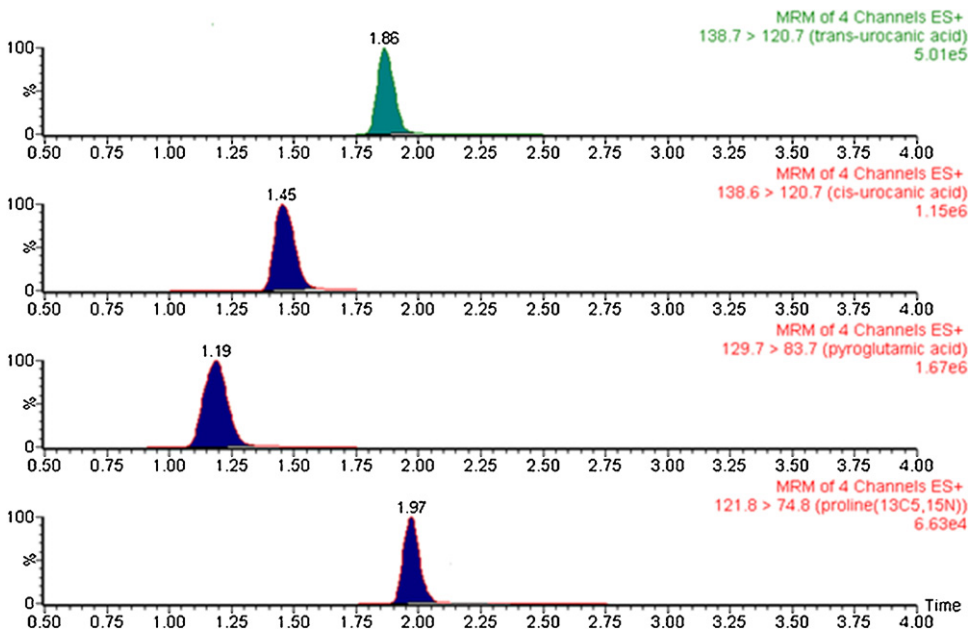


**Fig. 5.** Representative MRM chromatograms of sample obtained by tape stripping.

than 0.999 as listed in Table 4. The linear regression of the ratio of the areas of the analytes and IS vs. the concentration was weighted by 1/*x*. The LLOQs (lower limit of quantitation) were established at 0.5 ng/mL of PGA, *cis*- and *trans*-UCA with the accuracy and precision of 104.2, 104.4, 97.6 and 9.13, 5.15, 9.41%, respectively. The lower limits of detection (LLOD) of these analytes were 0.1–0.2 ng/mL. Table 5 summarized the intra- and inter-day precisions and accuracies of PGA, *cis*- and *trans*-UCA at three concentration levels. As shown in Table 5, the intra- and inter-day accuracies of these analytes were within the range of 94.3–102.5% and 95.1–102.6%, respectively. The intra- and inter-day precision was less than 7.7% for each QC samples. These data demonstrated that the method we developed has accurate and acceptable analytical performance.

The extraction recovery for three analytes was evaluated by comparing the absolute peak areas of analytes-added pre-extraction with those of post-extraction. The results showed that the extraction recoveries for all analytes were more than 85% and the mean recovery were 86.3%, 90.5% and 87% for PGA, *cis*- and *trans*-UCA, respectively.

### 3.3. Application to the measurement of PGA and UCA in human stratum corneum samples

With the developed method, levels of PGA, *cis*- and *trans*-UCA were determined in SC samples of healthy volunteers to explore the utility of this analytical method. A typical chromatogram in the extracted sample is shown in Fig. 5. Disquame-tape stripped

**Table 4**
Linear range, LLOQ, LLOD for PGA, *cis*- and *trans*-UCA.

| Analytes | Linear range (ng/mL) | Linearity | LLOQ (ng/mL) | LLOD (ng/mL) | Curve fitting | Weighting factor |
|---|---|---|---|---|---|---|
| PGA | 1.0–250 | 0.9994 | 0.5 | 0.2 | Linear | 1/*x* |
| *cis*-UCA | 1.0–250 | 0.9997 | 0.5 | 0.1 | Quadratic | 1/*x* |
| *trans*-UCA | 1.0–250 | 0.9991 | 0.5 | 0.1 | Quadratic | 1/*x* |

**Table 5**
Accuracy and precision of PGA, *cis*-UCA and *trans*-UCA spiked in surrogate matrix ($N = 5$).

| Spiked concentration (ng/mL) | Intra-day | | | Inter-day | | |
|---|---|---|---|---|---|---|
| | Measured concentration (ng/mL) | Accuracy (%) | Precision RSD (%) | Measured concentration (ng/mL) | Accuracy (%) | Precision RSD (%) |
| PGA | | | | | | |
| 1.0 | 0.94 ± 0.06 | 94.3 | 6.0 | 0.95 ± 0.07 | 95.1 | 7.7 |
| 50.0 | 50.7 ± 2.5 | 99.2 | 5.0 | 51.5 ± 3.1 | 102.6 | 6.0 |
| 100.0 | 95.6 ± 6.21 | 95.6 | 6.5 | 95.3 ± 5.4 | 95.4 | 5.6 |
| *cis*-UCA | | | | | | |
| 1.0 | 0.94 ± 0.04 | 94.3 | 4.3 | 0.96 ± 0.07 | 96.1 | 7.4 |
| 50.0 | 50.8 ± 3.3 | 101.5 | 6.5 | 50.8 ± 2.1 | 101.6 | 4.1 |
| 100.0 | 95.2 ± 6.2 | 95.2 | 6.5 | 96.8 ± 5.1 | 96.8 | 5.3 |
| *trans*-UCA | | | | | | |
| 1.0 | 0.95 ± 0.05 | 99.3 | 7.0 | 0.95 ± 0.05 | 95.3 | 5.0 |
| 50.0 | 51.2 ± 3.4 | 102.5 | 6.6 | 48.9 ± 2.0 | 97.9 | 4.2 |
| 100.0 | 96.1 ± 5.5 | 96.1 | 5.7 | 95.6 ± 4.2 | 95.6 | 4.4 |

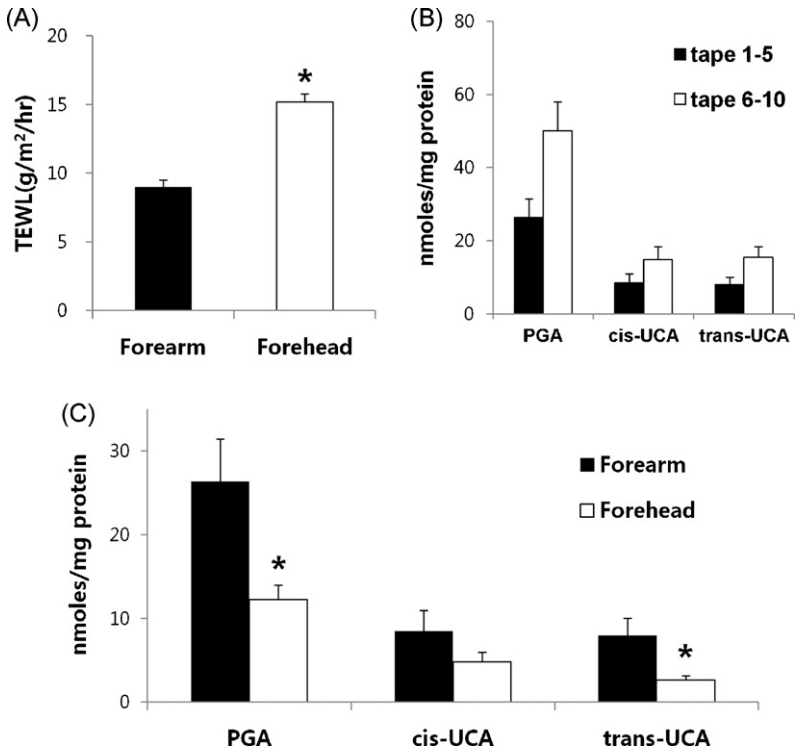


**Fig. 6.** Determination of PGA, *cis*- and *trans*-UCA in human stratum corneum of forehead and forearm region. (A) Trans-epidermal water loss (TEWL) of forehead and forearm region. (B) Amounts of PGA, *cis*- and *trans*-UCA in forearm area by skin depth ($N = 19$). (C) Amounts of PGA, *cis*- and *trans*-UCA in forearm and forehead regions (tape strips 1–5). * statistically different between forearm and forehead, Student's *t*-test, $P < 0.05$.

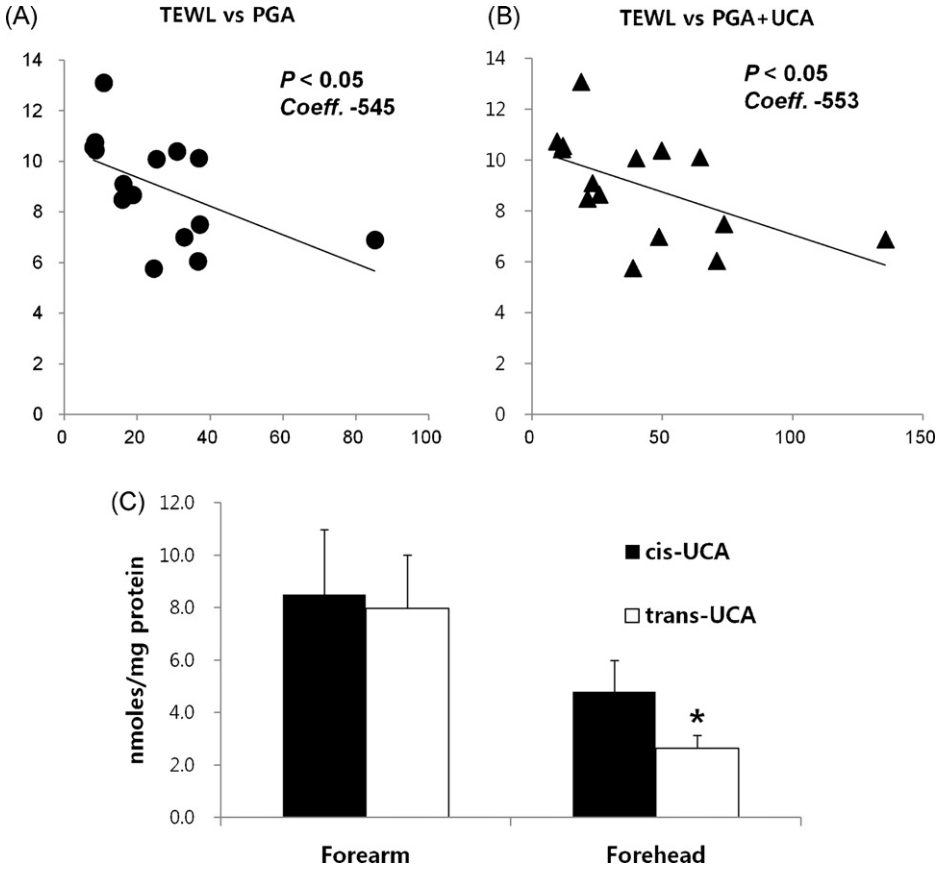


**Fig. 7.** Comparison of skin physiology with the concentration of PGA, *cis*- and *trans*-UCA. (A) Correlation of individual TEWL value with amount of PGA in forearm area. (B) Correlation of individual TEWL value with sum of PGA and UCA in forearm area (C). Comparison of *cis*- and *trans*-UCA in forearm and forehead regions (tape strips 1–5). Correlation was analyzed by Pearson's correlation analysis. * statistically different, Student's *t*-test, $P < 0.05$.

**Table 6**
The levels of total urocanic acid (UCA) and pyroglutamic acid (PGA) obtained by tape stripping normalized for protein amount by skin depth for different skin region ($N = 19$).

| Skin depth | Skin region | |
|---|---|---|
| | Forearm PGA/UCA sum (nmol/mg protein) | Forehead PGA/UCA sum (nmol/mg protein) |
| Tape 1–5 | 42.8 ± 33.4 | 19.7 ± 11.8 |
| Tape 6–10 | 80.3 ± 46.7 | 51.4 ± 34.7 |

SC samples can be easily obtained with minimally invasive procedure. SC samples were obtained from forearm and forehead regions of healthy human volunteers, the representative sites of body surface with high and low water holding properties [20] respectively to compare the levels of PGA and UCA. As shown in Fig. 6A, forearm showed significantly lower trans-epidermal water loss (TEWL), an inverse index of skin barrier function, suggesting that a higher water holding capacity of forearm when compared to forehead region.

Repeated tape-stripping could result in larger amounts of PGA and UCA (Fig. 6B), indicating that PGA and UCA are more enriched in the deeper layer of skin (Table 6). Comparison of PGA and UCA between forearm and forehead regions demonstrated that forearm had significantly larger amounts of PGA and UCA (Fig. 6C). Plotting of TEWL against PGA, UCA or their sum demonstrated that TEWL was significantly correlated with PGA and UCA (Pearson's correlation analysis, $p < 0.05$, Fig. 7A and B). UCA occurs as *trans*-form from the deamination of histidine but *trans*-UCA transforms into *cis*-isomer upon the exposure to UV light, i.e., undergoes photoisomerization. Comparison of *cis*- and *trans*-UCA between forearm and forehead regions showed that photoisomerization occurred more prevalently in forehead region. This agreed well with the every-day exposure of facial region to UV light.

## 4. Conclusion

In the present study, rapid and sensitive UPLC–ESI-MS/MS method using HILIC column for the determination of PGA, *cis*- and *trans*-UCA was developed and validated. This method was proved to be selective, precise, accurate and reliable for the simultaneous determination of PGA, *cis*- and *trans*-UCA within 5 min of single chromatographic run. The use of HILIC (amide) column for LC separation of three analytes and ESI-MS/MS with a simple single step solvent extraction was sufficient without any cleaning procedure or the use of ion-pair additives.

More importantly, this method was successfully applied to the simultaneous measurement of PGA and *cis*-/*trans*-UCA in the tape-stripped SC samples from human subjects and their levels were significantly correlated with the skin barrier function, indicating that this method can be practically applicable to the study of dermatology and skin physiology.

## Conflict of interest

None.

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