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Antioxidant Ability and Stability Studies of 3-*O*-Ethyl Ascorbic Acid, a Cosmetic Tyrosinase Inhibitor

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Synopsis

3-*O*-ethyl ascorbic acid may be a good whitening ingredient in cosmetics. However, before it can be successfully used in cosmetics, its biofunctionality and stability need to be comprehensively investigated. The reduction and 2,2-Diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging ability of this compound were analyzed to assess its antioxidant potential. In addition, the tyrosinase inhibitory ability was analyzed to show the whitening capacity of 3-*O*-ethyl ascorbic acid. Response surface methodology (RSM) was used to determine the optimal conditions for the ascorbic acid derivative in cosmetics. Based on the DPPH radical scavenging ability results, the half-inhibitory concentration (IC_{50}) value of 3-*O*-ethyl ascorbic acid was 0.032 g/L. It also showed a good reducing ability at 1.5 g/L concentration. Based on the tyrosinase inhibition analysis, the IC_{50} value was 7.5 g/L. The optimal conditions to achieve the best stability were determined from the RSM as 36.3°C and pH 5.46.

INTRODUCTION

Ascorbic acid is known as a good whitening ingredient in cosmetics (1). It has also been used to protect the skin from damage caused by ultra violet (UV) rays (2,3). However, the degradation of ascorbic acid reduces the biofunctionality of cosmetics containing it. Therefore, a more stable derivative such as 3-*O*-ethyl ascorbic acid would be preferred. The chemical structures of these compounds are shown in Figure 1. Although a resonance structure is formed between two hydroxyl groups at the ortho position of ascorbic acid, ascorbic acid is a relatively unstable compound. It can be easily oxidized when oxygen is present. Because the ethyl group prevents 3-*O*-ethyl ascorbic acid from being oxidized, 3-*O*-ethyl ascorbic acid is more stable than ascorbic acid (4,5).

Because of their free radical scavenging ability, ascorbic acid and its derivatives are known as good antioxidants (6). In addition, Yen et al. reported that ascorbic acid has a powerful reducing ability (7). Because 3-*O*-ethyl ascorbic acid is proposed for use in cosmetics, its antioxidant ability has been evaluated in this study.

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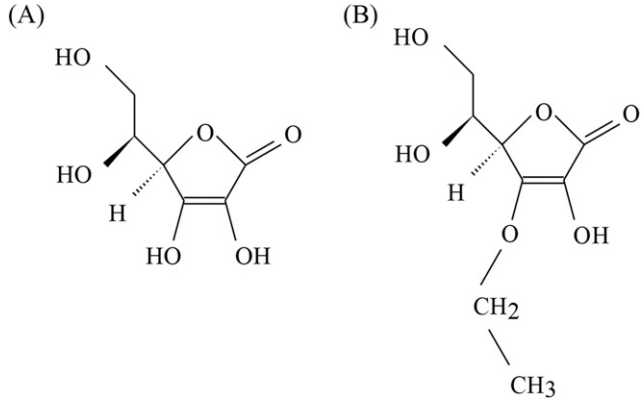

Figure 1. Chemical structures of (A) ascorbic acid and (B) 3-*O*-ethyl ascorbic acid.

Tyrosinase catalyzes the oxidation of L-tyrosine to 3,4-dihydroxyphenylalanine (DOPA), which can in turn transform into DOPAchrome (8). These catalyzed reactions result in the formation of melanin, which is responsible for skin pigmentation (9). The inhibitory activity of tyrosinase (EC 1.14.18.1) has been extensively studied (10-13). 3-*O*-ethyl ascorbic acid successfully inhibits the synthesis of melanin; however, its high water solubility hampers its permeation across the skin. In addition, the instability of this water-soluble compound leads to complications for formulation chemists (14).

Before 3-*O*-ethyl ascorbic acid can be successfully used in cosmetics, its stability needs to be comprehensively investigated. Storage temperature and pH are two factors that generally affect the stability of active ingredients in cosmetics. Changes in these two factors may cause the degradation of ingredients. Thus, optimal conditions need to be determined when using 3-*O*-ethyl ascorbic acid in cosmetics. In this study, response surface methodology (RSM) was used to study the stability of 3-*O*-ethyl ascorbic. RSM is a good technique to determine the optimal conditions for many applications. Huang et al. successfully used RSM models to obtain the optimal conditions for using ascorbic acid 2-glucoside, which is also used in cosmetics (15). Central composite design was used to establish a second-order RSM model to predict the stability of 3-*O*-ethyl ascorbic acid. The second-order regression model (equation 1) included linear, quadratic, and interactive components.

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i=1}^{k} \sum_{i<j}^{k} \beta_{ij} X_i X_j + \varepsilon, \quad (1)$$

where Y is the response value; X_i and X_j are the input variables; β_0 is the intercept; β_i is the linear coefficients; β_{ii} is the square coefficients; β_{ij} is the interaction coefficients; and ε is an error term.

This study aims to assess the antioxidant and reducing abilities of 3-*O*-ethyl ascorbic acid. The DPPH free radical scavenging ability, which is commonly used to represent the antioxidant ability, was evaluated. The tyrosinase inhibitory activity of 3-*O*-ethyl ascorbic acid was also investigated. The stability of this compound was studied using RSM. The optimal conditions to retain the best stability were determined.

MATERIALS AND METHODS

3-*O*-ethyl ascorbic acid was purchased from Cosmol (Gyeonggi-do, Korea). Citric acid was purchased from Nihon Shiyaku Reagent (Osaka, Japan). Methanol (High performance liquid chromatography (HPLC) grade) was purchased from Mallinckrodt (St. Louis, MO). Phosphoric acid (HPLC grade) was purchased from Wako Pure Chemical (Osaka, Japan). Methylparaben was purchased from Ueno (Hyogo, Japan). 2,2-Diphenyl-1-picrylhydrazyl, ascorbic acid, sodium phosphate monobasic, sodium phosphate dibasic anhydrous, butylated hydroxyanisole (BHA), and mushroom tyrosinase were purchased from Sigma-Aldrich (St. Louis, MO). Potassium ferricyanide ($K_3Fe(CN)_6$), iron (III) chloride ($FeCl_3$), and trichloroacetic acid (TCA) were purchased from Merck (Darmstadt, Germany). L-3,4-dihydroxyphenylalanine (L-DOPA) and kojic acid were purchased from Acros (Morris, NJ).

DPPH FREE RADICAL SCAVENGING ABILITY

The analytical method was based on the reports by Singh and Rajini and Chan et al. (16,17). The 3-*O*-ethyl ascorbic acid solution sample (50 μL) was mixed with 50 μL of 160 μM DPPH in ethanol. The mixture was kept in a dark room at 25°C for 30 min. The absorbance of the mixture was measured at 517 nm wavelength using an enzyme-linked immunosorbent assay (ELISA) reader (TECAN^R, Grödig, Austria). Each measurement was performed at least twice. The radical scavenging activity was calculated as follows:

$$\text{DPPH radical scavenging activity}\,(\%) = \left(1 - \frac{A_{\text{Sample}}}{A_{\text{Blank}}}\right) \times 100\%, \tag{2}$$

where A_{Sample} and A_{Blank} represented the absorbance of the sample and blank solutions, respectively. A low measured absorbance represented a strong DPPH radical scavenging activity.

The antioxidant ability of 3-*O*-ethyl ascorbic acid was further analyzed based on the kinetic model provided by Lai et al. (18). A first-order model was used to show the relation between the antioxidant ability and 3-*O*-ethyl ascorbic acid concentration.

$$\frac{dA}{dC} = -k\left(A^* - A\right), \tag{3}$$

$$-\ln(1 - X_A) = kC, \tag{4}$$

where A is the antioxidant ability of the sample, A^* is the maximum antioxidant ability of the sample, $X_A = A/A^*$, C is the 3-*O*-ethyl ascorbic acid concentration, and k is the rate constant. Equation 4 was used to generate a linear plot. The slope of the line corresponded to the rate constant.

REDUCING ABILITY ANALYSIS

The reducing ability of the samples was measured following the method described by Lee et al. (19). Samples of the 3-*O*-ethyl ascorbic acid solution (100 μL each) were placed into

vials. Each vial was added with 100 μL of 1% (w/v) $K_3Fe(CN)_6$ and 100 μL of 2 mM phosphate buffer (pH 6.6). These mixtures were placed in a water bath at 50°C for 20 min. After the temperature dropped to 25°C, 100 μL of the 10% (w/v) TCA solution was added to each vial. The mixtures were centrifuged at 3,000 rpm for 2 min. Then, 100 μL of the supernatant was transferred to a 96-well plate. Each well contained 100 μL of distilled water and 20 μL of the 0.1% (w/v) $FeCl_3$ solution. The absorbance was measured using the ELISA reader at 700 nm wavelength. A BHA solution was used as the internal standard. Each measurement was performed at least twice.

TYROSINASE INHIBITORY ABILITY ANALYSIS

Twenty microliter of 3-*O*-ethyl ascorbic acid (1.5, 6.0, 10.0, 16.0, and 20.0 g/L) was placed in a 96-well plate. Then, 40 μL of a tyrosinase solution (12 U/mL) and 0.1 mM of L-DOPA solution (dissolved in a sodium phosphate buffer at pH 6.8) were added. The mixed solutions were kept at 25°C for 25 min. The absorbance was measured at 475 nm wavelength using the ELISA reader (20). Kojic acid was used as the internal standard. The tyrosinase inhibitory rate (%) was calculated from equation 5:

$$\text{The inhibition rate}\,(\%) = \left(1 - \frac{\text{OD}_{\text{sample}}}{\text{OD}_{\text{control}}}\right) \times 100\% \tag{5}$$

The absorbance of the sample (OD_{sample}) and control ($OD_{control}$) was measured at 475 nm wavelength. The IC_{50} value was determined from a dose–response curve in which 50% of the target activity was lost.

STABILITY ANALYSIS

The stability study was based on the RSM experimental design. The concentration of the 3-*O*-ethyl ascorbic acid solutions was 3 g/100 mL. These prepared solutions were kept in an incubator. After 24-h incubation, the residual amount of 3-*O*-ethyl ascorbic acid was measured using HPLC. Methylparaben was the internal standard. The ingredient mobile phase including 0.25% (v/v) phosphoric acid and methanol was designed based on the method reported by Kang et al. and Nandhasri and Suksangpleng with slight modifications (21,22). The wavelength was set at 254 nm. The flow rate was 0.8 mL/min and the injection volume was 10 μL.

STATISTICAL ANALYSIS

The statistical evaluation was performed using analysis of variance (ANOVA) and regression calculations with STATISTICA^R (version 7.0, StatSoft, Inc., Tulsa, OK). All data are presented as mean ± standard deviation (SD). A difference was considered to be statistically significant when the p value was less than 0.05 ($p < 0.05$). The significance of the regression coefficients as associated probabilities was also evaluated. Regression coefficients were used to generate a mathematical model to predict the system responses. A 3-D mesh plot and a

contour map were drawn to point out the main effects of temperature and pH on 3-*O*-ethyl ascorbic acid.

RESULTS AND DISCUSSION

DPPH FREE RADICAL SCAVENGING ABILITY

Figure 2A shows the DPPH radical scavenging ability. When the concentration was 0.04 g/L, the DPPH radical scavenging ability of ascorbic acid and 3-*O*-ethyl ascorbic acid was 82.91% and 55.52%, respectively. Ascorbic acid had better radical scavenging ability

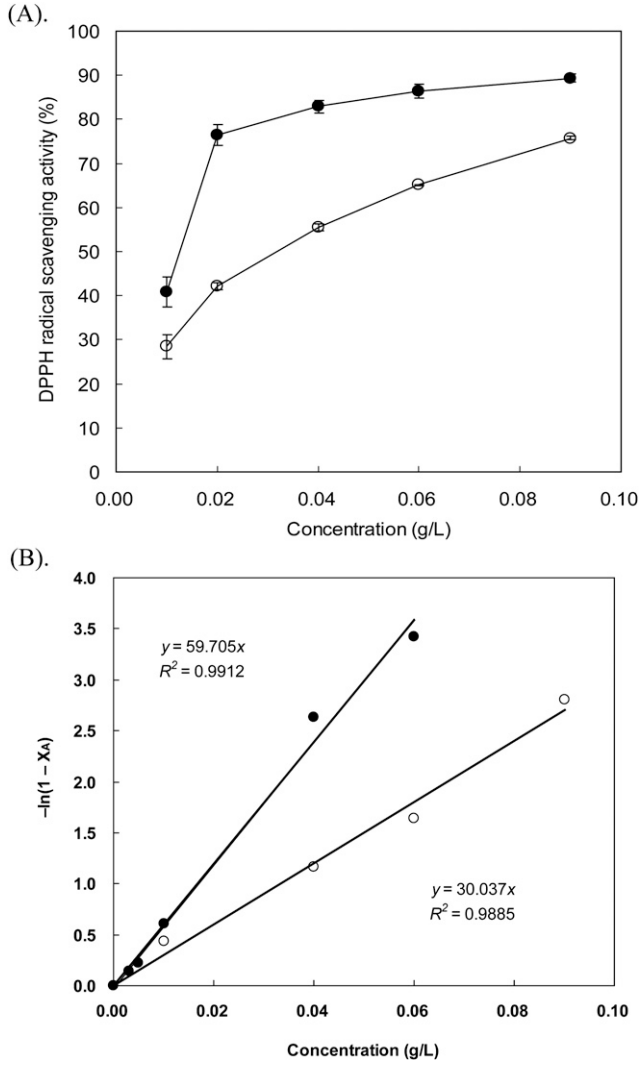

Figure 2. (A) DPPH radical scavenging ability. (B) First-order kinetic model (black filled circle: ascorbic acid; white filled circle: 3-*O*-ethyl ascorbic acid).

because it has more hydroxyl groups on the ring structure. However, when oxygen was presented, ascorbic acid easily degraded and resulted in a reduced biological ability. The ethyl group in the 3-*O*-ethyl ascorbic acid prevented it from being oxidized. Because of its better stability, this ascorbic acid derivative may be an ideal cosmetic ingredient.

Based on equation 4, linear equations were achieved from the regression of $-\ln(1 - X_A)$ plots (Figure 2B). The slope of the linear regressions represents the reaction rate constant (k). For ascorbic acid and 3-*O*-ethyl ascorbic acid, the k values were 59.70 and 30.03 L/g, respectively. The half-inhibitory concentration (IC_{50}) was calculated from equation 4. The IC_{50} values were 0.014 and 0.032 g/L for ascorbic acid and 3-*O*-ethyl ascorbic acid, respectively. A higher k value represented a better DPPH radical scavenging ability. Although the radical scavenging ability of the derivative was lower than that of ascorbic acid, 3-*O*-ethyl ascorbic acid was still a good antioxidant and could be used in cosmetics.

REDUCING ABILITY ANALYSIS

The ability of chemical compounds to provide electrons was indicated as the reducing ability. In this study, a higher absorbance measured at 700 nm wavelength represented a stronger reducing ability. Figure 3 shows the reducing ability of 3-*O*-ethyl ascorbic acid and BHA. Because BHA is a good electron donor and has the ability to reduce free radicals, it is often used as a standard for this analysis. As seen in Figure 3, when the concentration was higher than 1.5 g/L, the reducing ability reached a plateau. On the other hand, when the concentration was lower than 0.75 g/L, there was no significant difference between 3-*O*-ethyl ascorbic acid and BHA. Although the reducing ability of the ascorbic

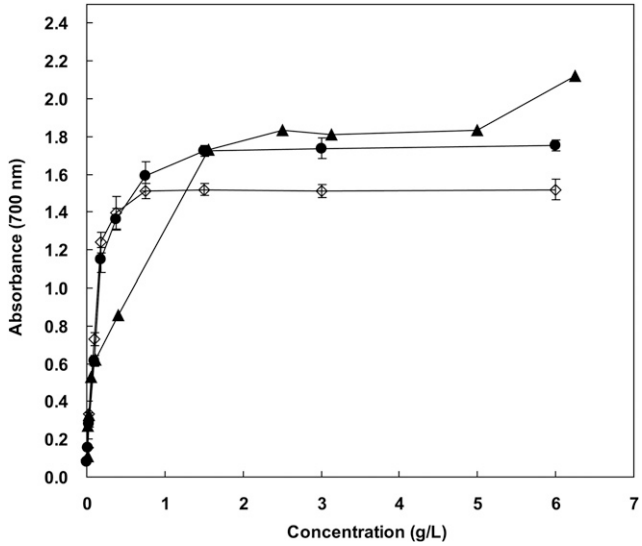

Figure 3. Reducing ability analysis of 3-*O*-ethyl ascorbic acid and BHA (black filled circle: BHA; white filled circle: 3-*O*-ethyl ascorbic acid; black filled triangle: ascorbic acid).

acid derivative was lower than that of the standard, 3-*O*-ethyl ascorbic acid still had good reducing ability.

TYROSINASE INHIBITORY ABILITY ANALYSIS

Tyrosinase catalyzes the formation of DOPAchrome from tyrosine. The product was detected at 475 nm wavelength. In this study, the tyrosinase inhibition rate when using a 20.0 g/L 3-*O*-ethyl ascorbic acid solution was 88.63%. The inhibition rate was also analyzed for 16.0, 10.0, 6.0, and 1.5 g/L solutions. The results are plotted in Figure 4. The linear regression was used to calculate the IC_{50} value (7.5 g/L) of 3-*O*-ethyl ascorbic acid. The IC_{50} value of kojic acid was 0.04 g/L (data are not shown). Kojic acid inhibits the ability of tyrosinase by chelating the copper ion, which is a cofactor of the enzyme (23). This compound has been widely used in Asia as a skin-lightening agent (24). Although kojic acid had a lower IC_{50} value, it has safety issues, which have been discussed in the past (25). Because 3-*O*-ethyl ascorbic acid was a stable and effective component, it could be safely used in most cosmetics.

STABILITY ANALYSIS

3-*O*-ethyl ascorbic acid solutions (3 g/100 mL) were kept in an incubator under specific temperature and pH for 24 h before running the HPLC analysis. The entire experimental design based on the RSM is shown in Table I. Two independent factors, including

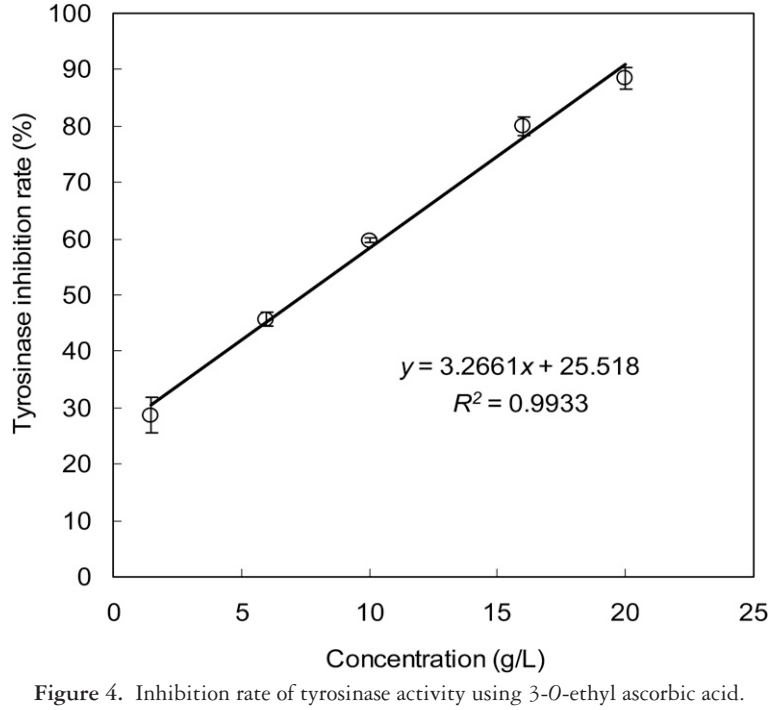

Figure 4. Inhibition rate of tyrosinase activity using 3-*O*-ethyl ascorbic acid.

Table I
Experimental Design and Observed Response Values (Area/IS) with Two Independent Factors, X_1 (Temperature, °C) and X_2 (pH)

	X_1	X_2	Response values
Run no.	Temperature	pH	Area/IS
1	45.0 (0)[a]	8.12 (+1.414)	2.176 ± 0.018
2	60.0 (+1)	7.50 (+1)	1.297 ± 0.009
3	66.2 (+1.414)	6.00 (0)	2.887 ± 0.036
4	60.0 (+1)	4.50 (−1)	2.891 ± 0.005
5	45.0 (0)	3.88 (−1.414)	2.962 ± 0.053
6	30.0 (−1)	4.50 (−1)	3.097 ± 0.005
7	23.8 (−1.414)	6.00 (0)	2.973 ± 0.024
8	30.0 (−1)	7.50 (+1)	2.754 ± 0.021
9	45.0 (0)	6.00 (0)	3.021 ± 0.013
10	45.0 (0)	6.00 (0)	2.977 ± 0.030

[a](−1.414), (−1), (0), (+1), and (+1.414) are coded symbols for levels of independent factors.
Response values are means of three replicates (means ± SD), and they represent the area ratio of 3-*O*-ethyl ascorbic acid and methylparaben (internal standard, IS).

temperature and pH, were involved in the RSM regression model. The response value was the peak area ratio of 3-*O*-ethyl ascorbic acid and methylparaben. Based on preliminary studies, the central point was set to 45°C and pH 6.00. After running the regression analysis, the coefficients of the second-order regression model were determined and substituted in equation 1.

$$Y = -3.8093 + 0.1063X_1 - 0.0004X_1^2 + 1.8378X_2 - 0.1222X_2^2 + 0.0139X_1X_2 \qquad (6)$$

This regression RSM model ($R^2 = 0.822$) was used to predict the system's response. Table II shows the ANOVA results of independent factors from the 3-*O*-ethyl ascorbic acid stability study. The *p* values and regression coefficients of the model are shown in Table III. Because the linear term of pH was a significant factor ($p < 0.05$), the peak area ratio was significantly affected by changes in the pH. Thus, 3-*O*-ethyl ascorbic acid was very sensitive to pH. However, temperature and pH were both included when running the regression analysis. Because the analysis included nonsignificant terms, the regression results might be affected by these nonsignificant terms and resulted in a reduction of R^2 value. Figure 5

Table II
Analysis of Variance for the Overall Effect of Factors on 3-*O*-Ethyl Ascorbic Acid Stability (X_1: Temperature, °C; and X_2: pH)

	SS	df	MS	F	*p* value
X_1 (linear)	0.3984	1	0.3984	3.1981	0.1482
X_1 (quadratic)	0.0408	1	0.0408	0.3273	0.5978
X_2 (linear)	1.1618	1	1.1618	9.3266	0.0379*
X_2 (quadratic)	0.3454	1	0.3454	2.7730	0.1712
$X_1 \times X_2$ (interactive)	0.3913	1	0.3913	3.1409	0.1510
Error	0.4983	4	0.1246		
Total SS	2.7981	9			

*Significant at $p \leqq 0.05$.

Table III
Regression Coefficients of the 3-*O*-Ethyl Ascorbic Acid Stability RSM Model

Regression coefficient	Estimate
Intercept, β_0	−3.8093
β_1	0.1063
β_{11}	−0.0004
β_2	1.8378
β_{22}	−0.1222
β_{12}	0.0139

shows the 3-D mesh plot of the RSM model. The surface curve of the figure represented the HPLC peak area ratio of 3-*O*-ethyl ascorbic acid and methylparaben. A larger peak area ratio indicated that more 3-*O*-ethyl ascorbic acid was detected. The highest calculated point of the surface curve in Figure 5 was 3.133, which occurred at 36.3°C and pH 5.46. When cosmetics were stored at the optimal conditions, 3-*O*-ethyl ascorbic acid had the best stability. Because this compound was very sensitive to changes in pH, buffers would be required for cosmetics to maintain the optimal pH (5.46). On the other hand, the stability of 3-*O*-ethyl ascorbic acid was not significantly affected by temperature. These results would be useful for cosmetic manufacturers who could use 3-*O*-ethyl ascorbic acid in their products.

CONCLUSIONS

Based on the reducing and DPPH radical scavenging ability analysis results, 3-*O*-ethyl ascorbic acid is a good antioxidant. Moreover, this compound inhibited the activity of tyrosinase to prevent the formation of melanin. Therefore, it could be used in cosmetics

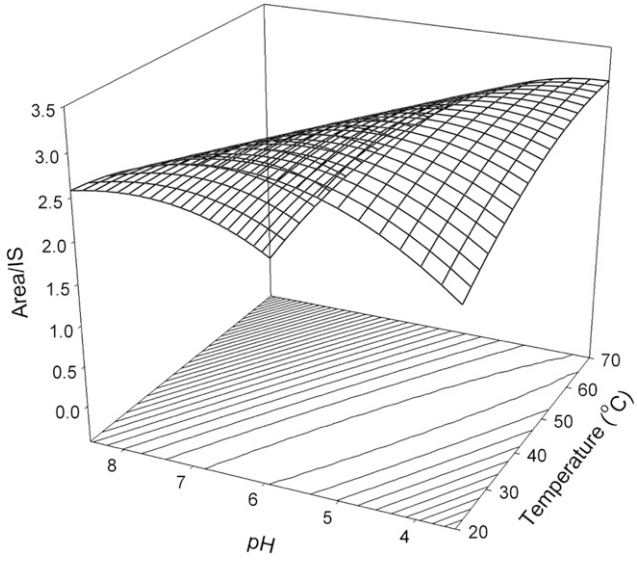

Figure 5. Response surface plot showing the effect of temperature (°C) and pH on the stability of 3-*O*-ethyl ascorbic acid.

as a whitening ingredient. The optimal conditions to retain the best stability were successfully determined using a RSM regression model. Because changes in pH significantly affected the stability of 3-*O*-ethyl ascorbic acid, buffers would be required to maintain the optimal pH (5.46). This ascorbic acid derivative is a stable and effective component that could be safely used in most cosmetics.

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