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Research Article

DEVELOPMENT AND VALIDATION OF A UV SPECTROPHOTOMETRIC METHOD FOR KOJIC ACID QUANTIFICATION BASED ON ITS ALUMINUM COMPLEXES

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ABSTRACT

Kojic acid (KA) is a fungal metabolite that is widely used as a depigmenting agent in cosmetics. Although there are sophisticated techniques to quantify this substance, such as HPLC and LC-MS, cheaper and more accessible methods employing electronic spectroscopy are still needed. We have developed and validated an UV spectrophotometric method for KA quantification that is based on the analyte's ability to form complexes with Al^{3+} ions. Superior selectivity is achieved with the presented method because KA-Al^{3+} complexes absorb at higher wavelengths than do non-complexed KA. This approach has proven to be applicable for detection of KA in both raw materials and cosmetic creams. Detection is possible even in the presence of hydroquinone, which is frequently combined with KA in cosmetic preparations. Method validation results at 305 nm indicated appropriate selectivity and linearity in the range of 5 to 50 µg/mL (r= 0.9998). Sensitivity evaluation showed a DL= 0.15 µg/mL and QL= 0.46 µg/mL. Accuracy (recovery) was near 100% (99,53 – 101,24%). Method precision was determined through repeatability (RSD 0.402%, 1.284% and 1.192% for 10, 15 and 20 µg/ mL, respectively) and intermediate precision (RSD 2.171%, 0.976% and 0.440% for 10, 15 and 20 µg/ mL, respectively). Robustness was evaluated by varying the pH, temperature and reading time.

Keywords: kojic acid, aluminium chloride, spectrophotometry, validation of analytical method, complexometry

INTRODUCTION

In the contemporary world, the preoccupation with physical appearance has been intensified because of a "need" to adapt to the standards of esthetics and beauty. Hyperpigmentations are one of several sources of concern, creating an increased demand for cosmetic products and, consequently, requiring an increase in the quality control of raw materials and cosmetic preparations.

Kojic acid (KA), 5-hydroxy-2-(hydroxymethyl)-4H-pyran-4-one, is an organic acid that was first isolated by Saito (1907) from cultures of *Aspergillus oryzae*[1]. Its name and structure were defined in 1924 by Jabuta [2]. KA has been utilized in the food industry as an antioxidant[3] and in the treatment of diseases such as β-thalassemia[4] and diabetes [5]. However, the main application of KA is related to the cosmetic industry, where it is utilized as a bleaching agent in creams[6]. The skin bleaching promoted by KA is based on its interference in the first and second steps of melanin synthesis, where tyrosinase is inhibited through chelation of a copper ion, an essential cofactor for the enzyme activation[7, 8]. The efficacy of kojic acid is mainly related to its active penetration capacity, which has been evaluated *in vitro* and *in vivo*[9]. KA has been shown to be an effective metal chelator, forming complexes with metal ions such as aluminum[4].

KA, as a raw material, can be quantified using UV spectrophotometry at 269 nm[10]; however, this wavelength is not considered selective for KA quantification in formulations because the commonly used preservatives (methylparaben or propylparaben) absorb in the same spectral range. Hydroquinone (HQ) (λ_{max}= 294 nm), a whitening agent commonly prescribed in conjunction with KA, also interferes with KA spectrophotometric quantification. This type of interference can be avoided by exploiting the ability of KA to form chelates to shift its main absorption bands to distinct wavelengths. In formulations, KA is currently quantified using methods such as UV with multivariate calibration[11], HPLC[12] and capillary electrophoresis[13].

This paper describes the development and validation of a complexation-based UV spectrometric method for KA quantification as a raw material and in cosmetic creams. This technique is simple and inexpensive. The presented results demonstrate that appropriate selectivity was achieved through the displacement of KA UV bands, which was promoted by Al^{3+} complexation.

MATERIALS AND METHODS

Chemicals

KA (≥98.0%) and D_2O were purchased from Sigma Aldrich Chem. Co. and methanol (analytical grade) from FMAIA®. Ultrapure water was obtained from a Milli-Q® Plus apparatus (Millipore®). The $AlCl_3.6H_2O$ solution, in HNO_3, was purchased from MERCK®. The cream (matrix), cream KA 2% and cream KA 2% + Hydroquinone (HQ) 4% were obtained from a local manipulation pharmacy. Commercial raw materials used were pharmaceutical grade. All other reagents used were analytical grade and were used as received.

Instrumentation

Spectrophotometric measurements were conducted using an UV/Vis AGILENT 8453E spectrophotometer and an UV/Vis Shimadzu 1601PC, both with a 1-cm quartz cuvette.

Development of the method

To evaluate the UV spectra of KA in the presence of different concentrations of aluminum chloride acidic solution, as well as the effect of the metal concentration on the absorbance intensity, 23 solutions were prepared in methanol. The concentration of KA was fixed at 15 µg/mL, while the concentration of $AlCl_3$ was varied in terms of ligand to metal ratio (KA:Al^{3+}) from 1:0 up to 1:136. The absorbance of each solution was measured at 269 and 305 nm.

Selectivity of the method

Determined by analyzing placebos and the sample matrix without the analyte. The system response was examined for the presence of interference or overlaps with the KA responses.

Validation of UV method / Spectrophotometric measurements

All validation work was performed using a KA standard solution (2000 µg/mL) and an acidic 0.2% solution of aluminum chloride (w/V) ([H^+]= 0.1 mol/L), both in methanolic solution.

Linearity

The linearity was estimated using a standard curve with mixtures of KA and acidic 0.2% aluminum chloride solution, yielding kojic acid solution standards that ranged between 1-100 µg/mL. The measurements were taken at 305 nm and performed in triplicate.

The correlation coefficient and the regression equation were assessed. In addition to the raw material, the linearity in cosmetic cream was also studied (KA 2%; KA 2% + HQ 4%) at 305 and 320 nm. The Kolmogorov-Smirnov test was applied to verify the normality and the comparison of variances by estimating the ratio of mean square regression and mean square residual with the F-value tabulated (95% confidence interval).

Sensitivity

Detection limits (LOD) and quantification limits (LOQ) were determined based on the standard deviation of responses and slope of the regression equation from the analytical curve.

Precision

The precision was determined by intra-day repeatability of samples at different concentrations of KA (10, 15 and 20 µg/mL; n= 6x3) and acidic 0.2% aluminum chloride solution analyzed using different equipment, different analysts on the same day. Intermediate precision was also verified using samples of KA (10, 15 and 20 µg/mL; n= 3x3) and acidic 0.2% aluminum chloride solutions analyzed on different days with different equipment and a different analyst. The same tests were performed for creams using solutions containing only KA and KA + HQ. The measurements were taken at 305 nm. For each concentration, intra-assay and inter-assay precision were determined using the variation coefficient and t-test for independent samples.

Accuracy

Accuracy was determined using a recovery test, and samples were analyzed according to the proposed method. Known amounts of either KA (2%) or KA (2%) with HQ (4%) were added to the matrix (chemynol 0.3%, imidazole 0.6%, propylene glycol 0.5%, lanette N 16.0%, liquid vaseline 5.0%, crystal butylated hydroxy toluene (BHT) 0.05%, nipazole 0.1%, white glycerin 5.0%, EDTA 0.1%, nipagin 0.2%, volatile silicone 2.0% and purified water) to yield samples that contained 10, 15 and 20 µg/mL of KA in methanolic acidic solution in the presence of 0.2% aluminum chloride (w/V) ($[H^+]$= 0.1 mol/L). All experiments were performed in triplicate at 305, 315 and 320 nm. Data were verified using a t-test for paired samples.

Robustness

KA (15 µg/mL) in acidic $AlCl_3$ solution was used to determine the effect of pH, reading time, temperature and metal concentration on the absorbance intensity. For the pH effect study, solutions were adjusted from pH 1 to 14. For reading time, measurements were conducted at 0, 24 and 72 hours after sample preparation. For the temperature effect evaluation, samples were maintained at 10, 20 and 30ºC and read after 4 hours. All experiments were executed in triplicate at 305 nm. Data were verified using a t-test for paired samples.

Data treatment

Statistical analysis was performed using SPSS 14.0 program (IBM, Chicago, USA).

RESULTS AND DISCUSSION

Development of the method

The KA UV spectrum in methanolic solution showed maximum absorption bands at 215 and 269 nm. With the addition of an acidic $AlCl_3$ solution, a new band appeared at 305 nm (Figure 1). KA-aluminum coordination likely occurs at the non-bonding electrons located at the KA enolic hydroxyl group and/or at the carbonyl group. KA and acidic solutions of $AlCl_3$ alone do not absorb in the 300 nm region. Thus, the appearance of a new absorption band can be explained by the formation of KA-aluminum complexes.

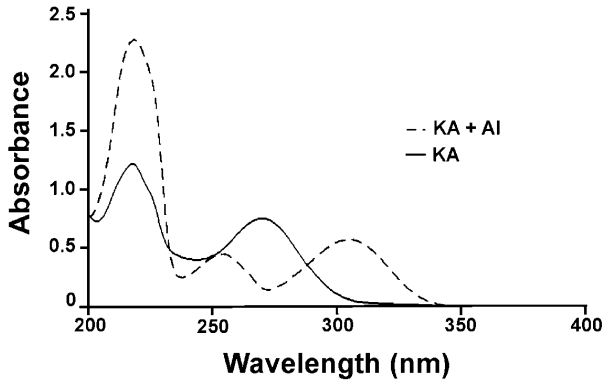

Figure 1: Kojic Acid Spectra (15 Mg/Ml) And Kojic Acid Complex Spectrum (15 Mg/Ml) with acidic solution Of 0.2% Aluminum Chloride (W/V) ($[H^+]$= 0.1 Mol.L^{-1}), both in Methanolic Solution

By alkalinizing the same solution with 0.1 mol/L KOH (from pH 3 to 12), the ionization changed the electron arrangement and, hence, the chromophoric group, which caused a bathochromic shift. However, the alkalinization process caused the solution to become turbid, due to the precipitation of aluminum, and the analyte could not be stabilized.

For a fixed concentration of KA (15 µg/mL), the maximum absorption intensity was observed when an equimolar KA:Al^{3+} proportion was reached (around 1:1). An excess of $AlCl_3$ did not interfere with the absorption intensity until the ligand-to-metal ratio reached 1:136. Based on the lack of absorption interference when using a high excess of aluminum and on the fact that it is necessary to have enough metal available to complex a wide concentration range of KA, the KA:Al^{3+} ratio was defined as 1:20

In assessing the selectivity of the method, the spectra of a KA standard solution, standard HQ solution, matrix (cream) and matrix with 2% KA (Figure 3) were compared. It was found that there was no matrix interference at 305 nm and no matrix or hydroquinone interferences at 320 nm.

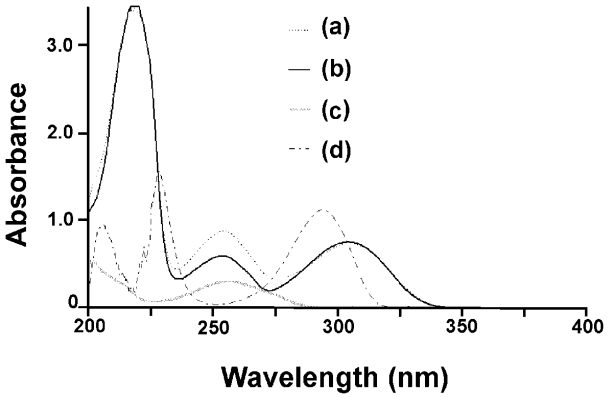

Figure 2: spectra of the cosmetic cream with ka 2% (a), standard kojic acid (15 µg/ml) (b), cosmetic cream (c), and cosmetic cream with ka (15 µg/ml) and hq (30 µg/ml) (d), to all solutions, alcl$_3$ 0.2% in acidic methanolic solution was added.

The p-values for each data set used for the comparison of KA (15 µg/mL) at 305, 315 and 320 nm, with or without the matrix, were higher than 0.05. This means that one should not reject the null hypothesis and that the values are statistically equal. After the addition of HQ to the matrix, the p-values at 269 and 305 nm were lower than 0.05, indicating that, at this wavelength, the data are statistically identical. However, the p-value at 320 nm was still higher than 0.05.

Validation of the method

Linearity

An appropriate linear relationship between concentration and absorbance was obtained for KA raw material (5-50 μg/mL) and for cosmetic cream containing KA (5-30 μg/mL), both with 0.2% $AlCl_3$ at 305 nm. For cosmetic cream containing both KA and HQ, linearity was evaluated for KA (5-30 μg/mL) at 320 nm. This method was considered linear because all of the correlation coefficients (r) were close to 1.00, which is in accordance with the recommendations found in the literature that should be greater than 0.99[14,15,16].

Normally, the linear correlation coefficient is used to evaluate the linearity but, according to BIDLINGMEYER (1993), a good linear correlation coefficient alone does not necessarily indicate a linear standard curve. Therefore, after plotting the measured signals against the different concentrations, the equation of the simple linear regression curve through the data was estimated using an unweighted linear least square regression. The residuals at every standard concentration were normally distributed. This was proven mathematically using the Kolmogorov–Smirnov test, where the calculated values for this test were smaller than the critical values at n = 10, for raw material, and n= 6, for cosmetic cream and α = 0.05. The minimum and maximum values of the standard residuals indicated that the residuals are normally distributed around zero. The ratio of the mean square regression and the mean square residual were higher than the F-value tabulated ($F_{0.05;\ 1.8}$= 5.32). Therefore, one can accept the linear model as appropriate (Table 1). The leverage vs. student residual graphic demonstrated that there were no anomalous samples.

Table 1 : Statistical Analysis For The Linearity Test

Sample	Kolmogorov-Smirnov Z	Standard residual		Mean square		F
		Min	Max	Regres	Resid	
KA*	0.321	-1.187	+1.343	2061.6	0.116	17839
KA **	0.311	-1.079	+1.102	369.9	0.023	16406
KA+HQ***	0.309	-0.937	+0.875	369.8	0.055	6683

raw material at 305 nm;** cosmetic cream with kojic acid at 305 nm; cosmetic cream with kojic acid and Hydroquinone at 320 nm**

LOD and LOQ

The LOD (0.15 μg/mL) and LOQ (0.45 μg/mL) indicate suitable sensitivity for the method.

Precision

With RSD values lower than 2.0 for KA at three different concentration levels (10, 15 and 20 μg/mL) the repeatability and intermediate precision assessments indicate that the method is precise to quantify KA in raw material (at 305, 315 and 320 nm), cream (at 305, 315 and 320 nm) and cream with HQ (at 320 nm).

In addition, *t*-tests for independent sample were used to compare the average obtained from the analyses. Regarding repeatability, the p-values for each concentration (10, 15 and 20 μg/mL) were 0.076, 0.357 and 0.777, respectively. For the intermediate precision (15 and 20 μg/mL), the p-values were 0.242 and 0.149. Both tests show (values higher than 0.05) that one should not reject the null hypothesis, which means that the values are statistically identical.

Accuracy

Recoveries were determined and gave values close to 100%. The RSD values were all lower than 1.0%, indicating good reliability and accuracy and consequently meets with the requirements of AOAC guidelines.

To solutions containing only KA the p-values for the 15 μg/mL concentration at 305, 315 and 320 nm were 0.560, 0.319 and 0.158, respectively. The values are higher than 0.05, therefore, the null hypothesis should not be rejected and the values are considered statistically identical to the true value.

To solutions containing KA and HQ the p-values for the 15 μg/mL concentration in 305, 315 and 320 nm were 0.000, 0.000 and 0.219, respectively. At 305 and 315 nm the p-value are lower than 0.05, meaning that the data are not equal to the true value. At 320 nm, the p-value is higher than 0.05, and therefore is statistically identical.

Robustness

The solution stability was investigated by verifying the influence of pH, reading time and temperature on the absorbance intensity. The assessment of pH was made only in the acidic range. In some of the solutions in the alkaline pH range, precipitation of aluminum as hydrolysis products was observed, which compromised the complexation measurements. A significant difference in absorbance values was not found at pH values of 1.0, 2.0 and 3.0. Additionally, when varying the reading time from 0 to 72 hours, a significant difference in the absorbance was not observed. The influence of temperature on solution stability was evaluated at 5, 20 and 30ºC, for over 4 hours, and no significant difference in the resulting absorbance values was observed. Thus, the present method was considered to be robust around those parameters.

The p-values for the concentration (15 μg/mL) used to study the effect of pH (1, 2 and 3), reading time (0, 24 and 72 h) and temperature (5, 20 and 30ºC) on the absorbance intensity were up to 0.05. Therefore, the null hypothesis should not be rejected and the values were statistically equal to the true value. The homogeneity of variances was determined using Levene's test. The results indicated that the variances are homogenous ($F_{calculated} < F_{(0.05;2;6)} = 5.14$) for the following parameters: pH (1, 2 and 3) F= 2.137, p-value= 0.174; reading time (0, 24 and 72 h) F= 1.980, p-value= 0.219 and temperature (5, 20 and 30ºC) F=1.980, p-value= 0.219. Additionally, an one-way analysis of variance (ANOVA) statistical method was used to detect any significant differences (p= 0.05). The results showed that there were no significant differences ($F_{calculated} < F_{(0.05;2;6)} = 5.14$) among the following parameters ($F_{calculated} < F_{(0.05;2;6)} = 5.14$): pH (1, 2 and 3) F= 1.108, p-value= 0.401, reading time (0, 24 and 72 h) F= 1.023, p-value= 0.415 and temperature (5, 20 and 30ºC) F= 1.030, p-value = 0.415. This test showed that systematic error was not produced by pH (1, 2 and 3) and reading time (0, 24 and 72 h) or temperature (5, 20 and 30ºC).

CONCLUSIONS

The UV spectrophotometric method developed in this paper is appropriate for the quantification of KA in raw material and in final pharmaceutical formulations. The method is selective to quantify KA in creams. According to the validation results, the method is linear (5-50 μg/mL), precise, accurate and robust. Additionally the method takes advantage of simple and accessible reagents and equipment, while having a low operating cost.

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