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Article

Detection method of black goji berry anthocyanin content based on colorimetry

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SUMMARY

Black goji berries are rich in anthocyanin, which is a natural water-soluble pigment widely found in plants. Anthocyanin has functions such as an antioxidant and antimutagen, which are extremely beneficial for human health. Currently, analyzing black goji anthocyanin content requires professional instruments in laboratories, and the process is difficult and time-consuming. Anthocyanin appears red and is relatively stable in acidic solutions, while in basic solutions, it appears blue but is unstable. Utilizing this characteristic, we aimed to develop a method for detecting the anthocyanin content in black goji berries. We hypothesized that there is a one-to-one correspondence between the concentration of an anthocyanin solution and the RGB values of its color. We selected 60% ethanol with a pH value of 1 as the solvent to prepare a series of 30 anthocyanin solutions ranging from 50 mg/L to 1500 mg/L in 50 mg/L increments. By making and measuring color cards for these 30 solutions, we obtained a correspondence table between RGB values and anthocyanin concentrations. We designed and developed a detector composed of a color sensor and a microprocessor. We used the color sensor to measure RGB values and embedded the correspondence table into the microprocessor. The microprocessor searched for the color card with the smallest color difference to the solution being tested, read the corresponding anthocyanin concentration value, and converted it into the anthocyanin content of the sample. We further established a standard curve of RGB values versus anthocyanin concentrations according to Beer-Lambert's Law. We utilized this standard curve to improve the method for measuring anthocyanin concentration.

INTRODUCTION

Black goji berry is a perennial shrub, primarily distributed in East Asia and Europe (1). Its fruits are sweet and extremely nutritious, and they are believed to be beneficial to human health and capable of assisting in the treatment of certain illnesses, such as rheumatoid arthritis, fatty liver disease, and arteriosclerosis (2). The active ingredient in black goji berries is anthocyanin, which has anti-aging and immune-boosting effects (2). Many dark-colored fruits contain anthocyanin; however, black goji berry is currently known to have the highest content of anthocyanin (3). The higher the anthocyanin content in black goji berries, the more pronounced its antioxidant effect, the stronger its ability to regulate blood lipids, and the higher its price. Therefore, anthocyanin content is one of the important indicators for evaluating the quality of black goji berries (4). It is necessary to research a rapid method for detecting anthocyanin content in black goji berry.

Anthocyanin exhibits different colors in solutions with different pH values (5). Anthocyanin is relatively stable in acidic solutions, where the flavyilium cation I (red color) is its primary form (6). In neutral or alkaline solutions, the decrease in hydrogen ion concentration affects the charge distribution and chemical bond strength within anthocyanin molecules (6). This especially affects the fragile covalent bonds, such as hydrogen bonds between certain hydroxyl and carbonyl groups or glycosidic bonds connecting aromatic rings to sugar moieties, resulting in cleavage. When these crucial chemical bonds break, the molecular structure of anthocyanin changes, ultimately causing its color to fade away.

The methods for detecting anthocyanin include the pH differential method, UV-Vis spectrophotometry, and high-performance liquid chromatography (7). These methods require detection reagents and laboratory equipment (8). Colorimetry is a method that determines the content of a component in a solution by comparing or measuring the color intensity (9). The theoretical basis of colorimetry is Beer-Lambert's Law, which states that the absorbance is proportional to the concentration of the substance (10).

RGB is a color model that covers the full visible spectrum by varying ratios of red (R), green (G), and blue (B) (11). A unique color is defined by a specific RGB combination (11). If a color corresponds to a specific concentration, and we make a table to record the different concentrations corresponding to the RGB values of various colors, then by measuring the RGB values and consulting the table, we may be able to assess the content of anthocyanin.

We hypothesized that in an acidic solution with a constant pH value, as the concentration of anthocyanin increases, the absorbance of the solution also increases, and there would exist a clear one-to-one correlation between the concentration and the RGB values of the solution. We proposed a colorimetric method for calculating the concentration of anthocyanin solutions and developed a detection instrument equipped with a color sensor and a microprocessor. This instrument was capable of obtaining the concentration of anthocyanin solutions and converting it into the anthocyanin content in black goji berries.

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RESULTS

Spectral Characteristics of Anthocyanin in Black Goji Berries

We determined the optimal wavelength for pH differential method by measuring the absorbance of black goji berry extract in solutions with two different pH values, which assisted us in accurately measuring anthocyanin concentrations with the pH differential method and preparing anthocyanin solutions with equally spaced concentrations. We soaked the black goji berry powder in 60% ethanol (pH = 1) and obtained the anthocyanin solution through centrifugal extraction. We prepared a hydrochloric acid-potassium chloride buffer solution (pH = 1) and a sodium acetate-acetic acid buffer solution (pH = 4.5) to measure and compare the absorbance of anthocyanin in solutions with different pH values. We introduced aliquots of the anthocyanin solution into these two buffered solutions with distinct pH values (pH = 1 and pH = 4.5) and subsequently utilized a UV-visible spectrophotometer to determine the absorption spectra of the anthocyanin in these solutions at the specified pH values. The absorbance difference was greatest at 525 nm, which was the optimal measurement wavelength for the pH differential method (**Figure 1**, 1).

Study on Color Characteristics and Stability of Anthocyanin Solution

We then conducted a study on the color characteristics and stability of anthocyanin in solutions of different pH values to identify a suitable solvent for preparing solutions with equally spaced anthocyanin concentrations. We dissolved black goji berry powder in three solutions with different pH values, each at a concentration of 1 g/L, and let them stand for 5 minutes. The acidic solution (pH < 7) turned red, the neutral solution (7 < pH < 8) turned purple, and the alkaline solution (pH > 8) turned blue (**Figure 2A-B**). After being left undisturbed for 12 hours, the color of the acidic anthocyanin solution (pH < 7) remained red and the color of the neutral

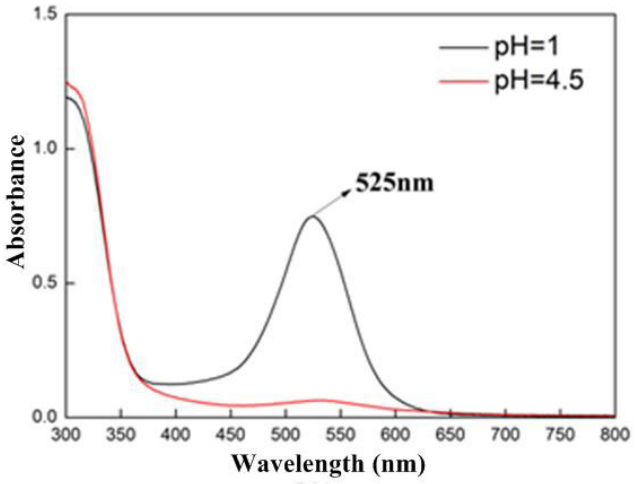

Figure 1: Absorption spectra of black goji berry extracts at pH levels of 1 and 4.5. We measured the absorption spectra of the black goji berry extracts in buffer solutions with pH values of 1 and 4.5 from 300 nm to 800 nm by a UV-Vis spectrophotometer and plotted the absorbance curves. The peak of the absorbance difference between the two solutions was observed at a wavelength of 525 nm.

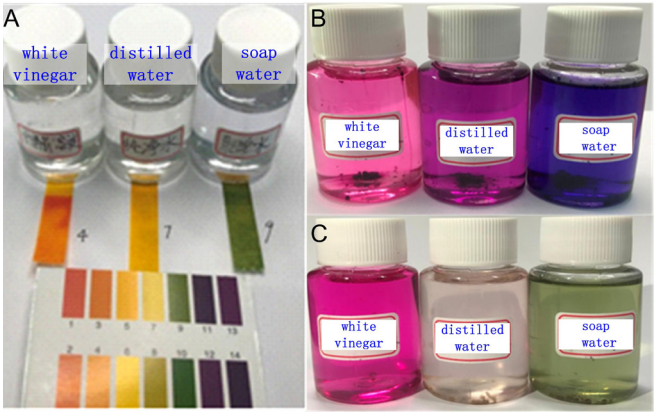

Figure 2: The color of black goji berry powder solutions at three different pHs changed over time. Black goji berry powder was added to solutions of acidic (white vinegar), neutral (DI water), and basic (soap water) pH (**A**). Solution colors were recorded after 5 minutes (**B**) and 12 hours (**C**).

anthocyanin solution (7 < pH < 8) turned from purple to pale pink, while the color of the alkaline anthocyanin solution (pH > 8) turned from blue to green (**Figure 2C**). The change in color over time indicated that anthocyanin was prone to decomposition in neutral and alkaline solutions, resulting in poor color stability, whereas in acidic solutions, anthocyanin was less susceptible to decomposition, leading to better color stability.

We further conducted experiments on anthocyanin solutions in 60% acidified ethanol (pH = 1) for different time durations, measuring the RGB values of the solution colors and comparing them to verify the stability of the coloration. For 5 minutes, 4 hours, 12 hours, 24 hours, 48 hours, and 72 hours, a 60% acidified ethanol solution containing 1 g/L goji berry extract was left standing. At each time point, the RGB values of the solution were measured by a commercial colorimeter. By calculating the color differences between the RGB values of the first time point and those of the subsequent five time points, we evaluated the degree of color change. Except for the slightly larger color difference between the last and the first time points, the color differences at the other time points were relatively small (**Figure 3**). The experimental results indicated that the color change of anthocyanin in a 60% acidic ethanol solution (pH = 1) within 72 hours was minimal.

The Relationship Between RGB Values and Anthocyanin Concentrations

Based on the stable color of anthocyanin in a 60% acidified ethanol solution (pH = 1) over a prolonged period, we selected this as the solvent to make color cards for 30 anthocyanin solutions with equal interval concentrations ranging from 50 mg/L to 1500 mg/L. In order to reduce the error caused by temperature in the measurement results, we maintained anthocyanin solutions at temperatures of 0, 25, 30, and 35 °C respectively, immersed filter paper in each solution, dried the paper to make color cards, and recorded the RGB values of each color card with a colorimeter (**Figure 4**). The RGB values were not found to vary much between the temperature conditions; therefore, we determined that it would be fine to average across the different temperatures. The final RGB

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Time	R	G	B	Distance	Color
5 minutes	91	18	66	0	
4 hours	89	17	41	25.1	
12 hours	80	13	48	21.7	
24 hours	85	15	65	6.78	
48 hours	87	28	52	17.7	
72 hours	90	51	65	33.0	

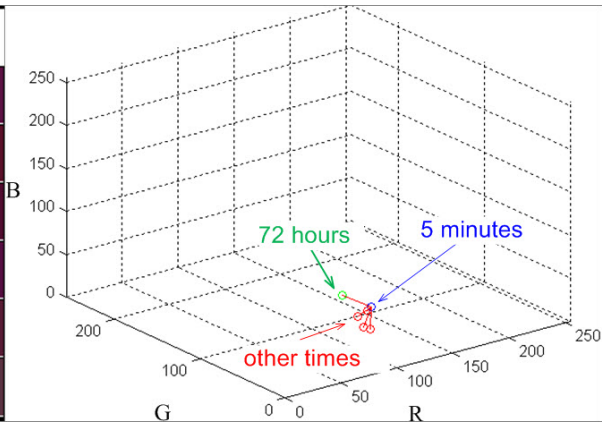

Figure 3: Changes in RGB values of anthocyanin color over time in an acidic ethanol solution (pH = 1). We prepared a goji berry extract solution in acidic ethanol and left the solution to stand for 5 minutes, 4 hours, 12 hours, 24 hours, 48 hours, and 72 hours. We measured the RGB values with a commercial colorimeter and calculated the RGB spatial distances between the last five time points and the first time point.

values of the color cards adopted were the average of the four measurements. There was a one-to-one correspondence between RGB values and the concentrations (**Table 1**). In the range where the concentration was less than 700 mg/L, the R, G, and B values were linear over concentrations, and the slope of G value was steepest (**Figure 5A**). This was likely because the anthocyanin exhibited the highest absorbance near a wavelength of 525 nm, which corresponded to the green color. Therefore, estimating the concentration based on the linear relationship between the G value and the anthocyanin concentration would be sensitive. 255 is the saturation value of the G value. The G value appeared to have a negative linear correlation with the anthocyanin concentration, while the value of 255-G demonstrated a linear positive correlation with concentration. Therefore, we fitted a standard curve for (255-G) versus concentrations. The (255-G) curve demonstrated good linearity within the range of anthocyanin concentrations less than 700 mg/L, with a relatively steep slope that provided excellent discrimination (**Figure 5B**).

Design and Implementation of Anthocyanin Content Detector

We designed a device to detect anthocyanin content. We input the correspondence table of RGB values and anthocyanin concentrations into a microprocessor. The device used a color sensor to measure the RGB values. The microprocessor calculated the RGB difference between the anthocyanin solution and the color cards to identify the most closely matched color card.

The detector we designed consisted of four functional modules: a color detection module, a color difference calculation module, a colorimetric module, and a result display module (**Figure 6**). The color detection module, composed of a color sensor, was responsible for measuring the RGB values of the filter paper made from the sample solution. The color difference calculation module and the colorimetric module were implemented by a microprocessor, which calculated the color difference between the RGB values of the sample and those of the standard color cards and identified the color card with RGB values closest to the sample. After obtaining the anthocyanin concentration value corresponding to that color card, the microprocessor converted it into the anthocyanin content in the black goji berries. The result display module showed both the concentration of the anthocyanin solution and the anthocyanin content in black goji berries.

Verification of the Method and Device

To verify the accuracy of our detector, we prepared 30 anthocyanin solutions with unknown concentrations. The pH differential method, which could precisely determine the

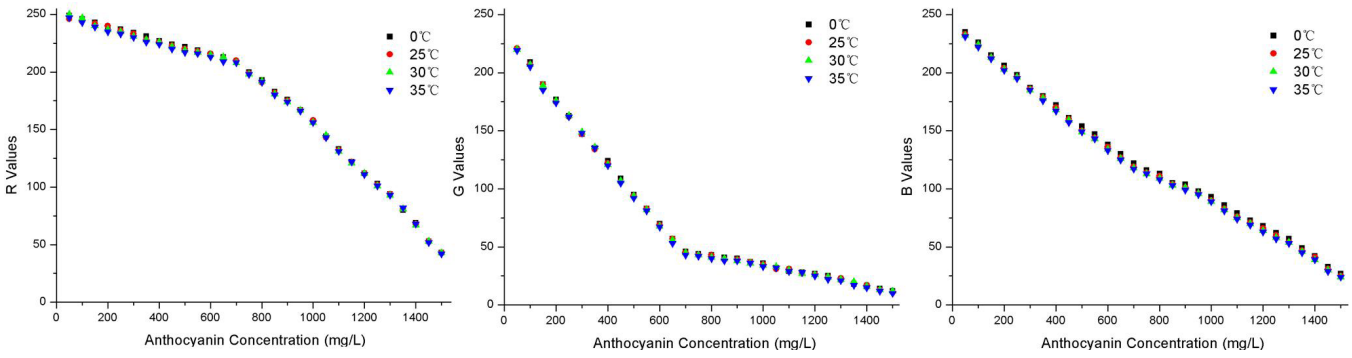

Figure 4: Variations of R, G, and B values across anthocyanin solution concentrations at four different temperatures. We measured the R, G, and B values of color cards made from anthocyanin solutions (pH = 1) with equal intervals of concentration at four different temperatures, and plotted the R, G, and B values across concentrations.

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No.	RGB	Color	mg/L	No.	RGB	Color	mg/L
1	248,220,233		50	16	192,42,110		800
2	245,207,224		100	17	182,40,104		850
3	241,189,213		150	18	175,39,101		900
4	238,176,204		200	19	167,37,96		950
5	235,163,196		250	20	157,35,90		1000
6	232,148,186		300	21	144,32,83		1050
7	229,135,178		350	22	132,30,76		1100
8	226,122,169		400	23	122,28,71		1150
9	223,107,159		450	24	112,26,65		1200
10	220,94,151		500	25	102,24,59		1250
11	218,82,144		550	26	94,22,55		1300
12	215,69,135		600	27	81,19,47		1350
13	212,56,127		650	28	68,16,40		1400
14	209,45,119		700	29	53,13,31		1450
15	199,43,114		750	30	43,11,25		1500

Table 1: Correspondence table of color RGB values and anthocyanin concentrations. The correspondence table provided RGB values for 30 color cards, each representing a 50 mg/L increment of anthocyanin concentration from 50 mg/L to 1500 mg/L. These color cards were made by dipping filter paper in the anthocyanin solution and then drying.

concentration of anthocyanin solutions, was used to measure the concentrations of these 30 solutions, and the results were taken as the "expected" values. We then created color cards for these 30 solutions and used our detector to measure their concentrations, which were considered as the "reported" values. When the concentration was less than 700 mg/L, the standard error between the “expected” and “reported” values was less than 10 mg/L. When the concentration exceeded 700 mg/L, the standard error was greater than 10 mg/L, and the “reported” values were lower than the “expected” values. Beer-Lambert's Law applied to solutions with lower concentrations; therefore, when the anthocyanin concentration was less than 700 mg/L, our detector demonstrated high accuracy. The correlation coefficient between the "expected" and "reported" values was 0.99983 (**Figure 7**).

DISCUSSION

In this study, we aimed to develop a method to determine anthocyanin concentration based on the RGB values of solutions. We further discussed methods to improve accuracy and techniques for measuring concentrations continuously. We hypothesized that under a certain pH condition, there existed a one-to-one correlation between the concentration of an anthocyanin solution and its color RGB values. This hypothesis was essentially based on Beer-Lambert's Law, which states that there is a linear relationship between the concentration of a solution and its absorbance. However, Beer-Lambert's Law is only applicable to solutions with low concentrations, especially when performing quantitative or colorimetric analysis (10). It is crucial to ensure that the solution concentration falls within an appropriate range to obtain accurate results. In this study, when the anthocyanin concentration was high, the predicted concentration based on RGB values had higher error. When the sample solution concentration was less than 700 mg/L, the standard error was relatively small. Therefore, to improve the measurement accuracy of the method and the detector, it is recommended to prepare the sample solution with a concentration of less than 700 mg/L.

To adapt to this change, the detector will be equipped with an input function that allows calculating the conversion coefficient from solution concentration to anthocyanin content by inputting the mass of the material used to prepare the sample solution and the volume of the solution. Currently, this coefficient was fixed at 0.05 and could not be changed in the detector.

If other substances contained in black goji berries also exhibit the color similar to anthocyanin under acidic conditions, it will impact the accuracy of measurement. To eliminate this possibility, we compared the spectral characteristic of standard anthocyanin with black goji berry extracts. The most common form of standard anthocyanin is cyaniding-3-glucoside, which exhibits maximum absorbance at 530 nm (7). The spectral characteristics of black goji berry

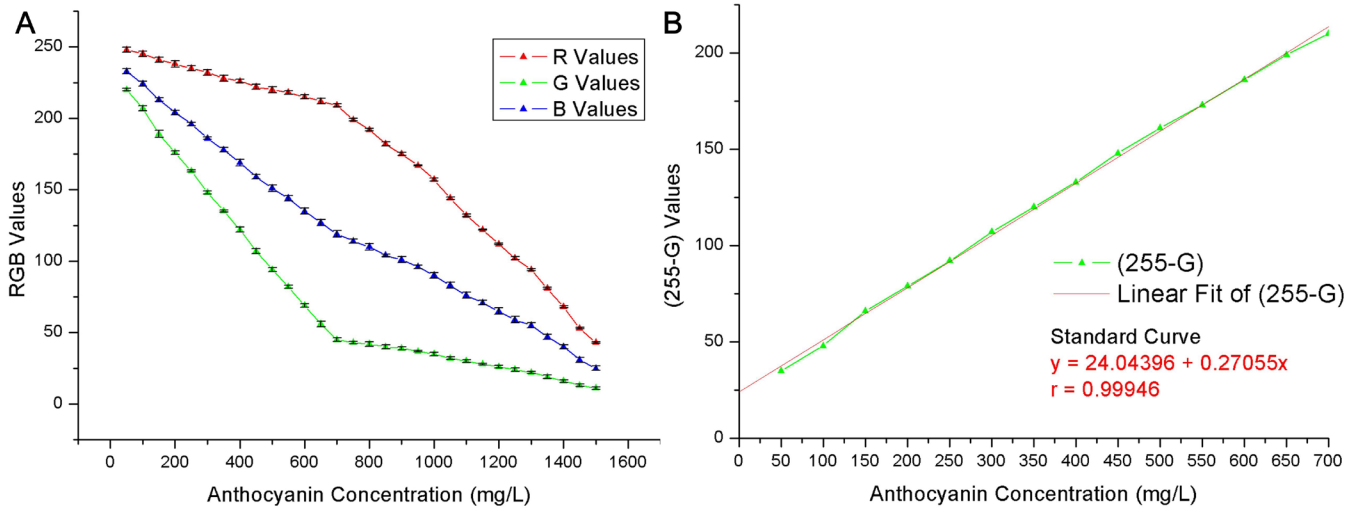

Figure 5: The R, G, and B values of anthocyanin solutions across concentrations and the linear fit of (255-G). (**A**) We plotted the curves of R, G, and B values of equal concentration interval anthocyanin solutions (pH = 1), along with error bars (n = 4). (**B**) We performed a linear fit for (255-G) within the range of 0 to 700 mg/L, obtaining a standard curve with a correlation coefficient of 0.99946.

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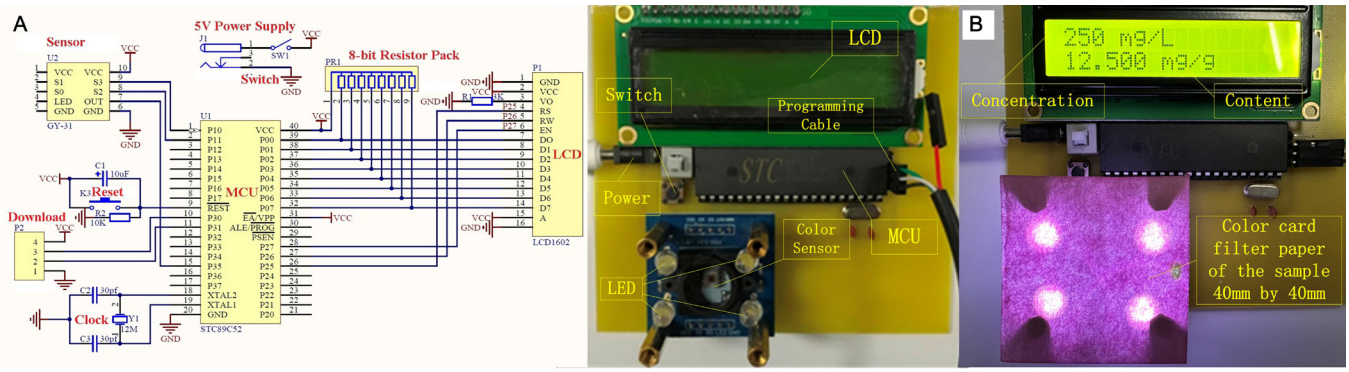

Figure 6: Circuit block diagram, and operational status diagram of the detector. (**A**) The detector comprised a color sensor, a microprocessor, an LCD, a power supply, and other components. (**B**) The color sensor measured the RGB values of the filter paper soaked in the sample solution, while the microprocessor searched for the most matching color card, getting the corresponding anthocyanin concentration (mg/L) from the card and converted it into the anthocyanin content (mg/g) in the sample. The LCD screen displayed the measurement results.

extracts were similar to those of pure anthocyanin reported in literature, indicating that the absorbance of black goji berry extracts was mainly caused by anthocyanin.

We programmed the color correspondence table into the detector. Since we obtained the RGB values in the table by measuring color cards, in actual measurements, the RGB values should also be acquired by measuring a color card produced from the sample solution to be tested. Otherwise, there will be a methodological inconsistency, which may lead to errors. Our approach was to make a piece of filter paper from the black goji berry extract solution, and measure the RGB values of the filter paper, which could maintain consistency in the methodology.

The RGB values measured by the color sensor may be affected by lighting or background conditions, and different RGB values correspond to different concentration values, which is a potential source of error. Therefore, it is necessary to ensure constant background conditions during the measurement process. We reduced the impact of lighting or background conditions on the measurement results by adding LED beads, setting the optimal measurement distance for the sensor, and fixing the specification of the filter paper (12).

Our method only produces measurement results that are integer multiples of the sampling step size. Because we created the correspondence table with intervals of 50mg/L, the measurement results were restricted to integer multiples of 50 mg/L. To improve the resolution, we can decrease the sampling interval to 25 mg/L, allowing the measurement results to be integer multiples of 25 mg/L instead.

In analytical chemistry, a standard curve is commonly used to derive the response value corresponding to unknown points. However, RGB values, which are a combination of three integers, cannot be directly subjected to linear regression. To overcome this limitation, if we imitate the method of a standard curve and identify a certain numerical value derived from RGB values to serve as the response value for estimating the unknown concentration, we can achieve continuous measurement of points that are not restricted to fixed intervals.

Within the range of less than 700 mg/L, there was a clear positive linear correlation between (255-G) and concentration. A standard curve was obtained by performing linear regression on (255-G), with a correlation coefficient r of 0.99946. We plan to program this standard curve into our detector in future research to measure anthocyanin solutions of any concentration within the range of 700 mg/L.

The UV-Vis spectroscopy and the pH differential method are costly, complex in operation, yet highly accurate (7). In comparison, our method offered convenience and cost-effectiveness but came with a slight reduction in accuracy. If an anthocyanin measurement is required within 10 minutes with lower costs, our method will be a suitable option. For instance, when purchasing black goji berries, rapidly measuring the anthocyanin content will help in selecting those with high anthocyanin levels, rather than relying on external characteristics such as size, shape, and color to judge their quality. This study proposed a new method for quickly, easily, and cost-effectively measuring the anthocyanin content in black goji berries, which will promote the application of black goji berries in more fields.

MATERIALS AND METHODS

Extraction of Anthocyanin from Black Goji Berries

After freeze-drying the black goji berries for 36 hours, we loaded them into a grinding jar and then placed the jar in a liquid nitrogen container to freeze it for 5 minutes. After removing the jar from the liquid nitrogen, the frozen samples were ground for 30 seconds. The ground samples were then sieved through a screen with 40 holes per inch and a mesh size of 0.425 mm, collected in wide-mouthed bottles, sealed, and stored in a dark place.

We weighed 1 g of black goji berry powder, placed it in a round-bottom flask, added 40 mL of 60% acidified ethanol (pH = 1), and macerated the mixture for 3 hours at 25 °C. We centrifuged the mixture, collected the supernatant, and added acidified ethanol to the remaining powder again for extraction. We repeated the extraction process three times, combined the supernatants, and diluted the solution to 250 mL with acidified ethanol.

Experimental Study on the Color Characteristics of Anthocyanin

We dispensed equal volumes of white vinegar solution, distilled water, and soap water into three transparent plastic bottles and measured the pH value of the liquid in each bottle with pH paper. An equal quantity of black goji berries was

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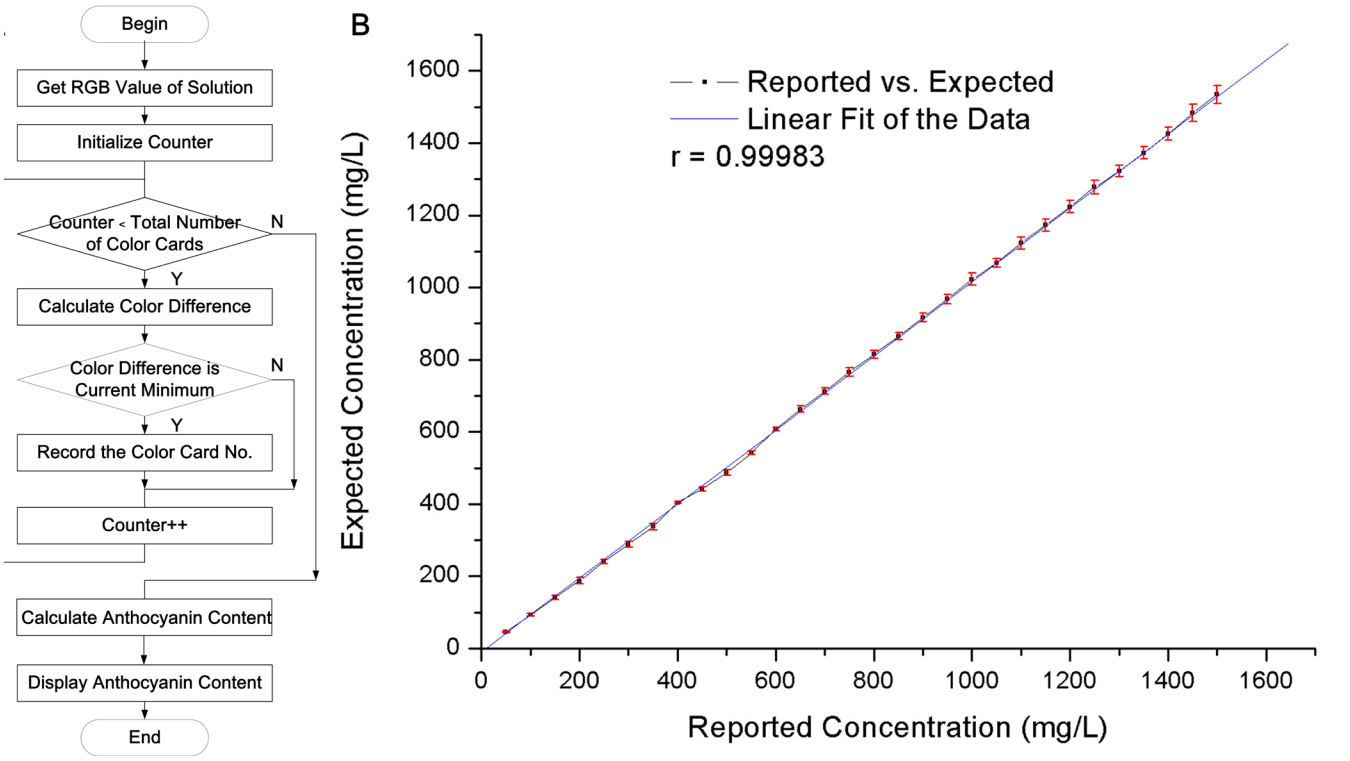

Figure 7: Software logic diagram and the deviation between reported values and expected values. (**A**) We drew a flowchart of the software in the detector. (**B**) We used the concentration of 30 anthocyanin solutions determined by pH differential method as the "expected value" and the concentration determined by our detector as the "reported value", plotted the curve of the "expected value" across "reported value" and the standard deviation, and performed linear fitting on the curve.

then added to each bottle. After 5 minutes and 12 hours, respectively, we observed and recorded the color change process of the solutions in each bottle.

We added hydrochloric acid to a 60% aqueous ethanol solution to adjust its pH to 1, then added black goji berry powder to the solution and let it stand for 5 minutes, 4 hours, 12 hours, 24 hours, 48 hours, and 72 hours. The RGB values of the solution were measured by a commercial colorimeter. We evaluated the degree of color change by calculating the color differences between the RGB values of the first time point and those of the subsequent five time points.

Determination of Anthocyanin Content in Black Goji Berry by pH Differential Method

We employed the pH differential method to determine the anthocyanin content of a black goji berry sample, with the aim of preparing anthocyanin solutions across various concentrations.

We prepared a 60% acidic ethanol solution and adjusted its pH to 1 as the extraction solution. Additionally, two buffer solutions with pH values of 1 and 4.5 were required by the pH differential method. For the pH 1 buffer solution, we measured 67 mL of 0.2 mol/L hydrochloric acid solution and added 25 mL of 0.2 mol/L potassium chloride solution to it, adjusting the pH to 1. For the pH 4.5 buffer solution, we weighed 18 g of sodium acetate and added 9.8 mL of acetic acid. We then diluted the mixture with water to 1 L and adjusted the pH to 4.5.

We weighed 1 g of black goji berry powder and placed it in a round-bottom flask, adding 40 mL of 60% acidic ethanol and soaking at 25 °C for 3 hours. We centrifuged the solution at 3000 rpm for 5 minutes and collected the supernatant. The extraction process was repeated three times. We took 1 mL of the supernatant and diluted it to 10 mL in a volumetric flask using the buffer solution with a pH value of 1. We then repeated the dilution process with the buffer solution with a pH value of 4.5.

We measured the absorbance of the two black goji berry extract solutions with pH values of 1 and 4.5 within the wavelength range of 300 to 800 nm by a UV spectrophotometer (Lambda 950 ultraviolet spectrophotometer). We identified that the maximum absorbance difference between the two solutions occurred at a wavelength of 525 nm. We then measured the absorbance difference at 700 nm for background correction and calculated the concentration of anthocyanin using Equation 1 (6).

$$C = \frac{\Delta A \times MW \times DF}{\varepsilon \times l} \quad \text{(Equation 1)}$$

C is the concentration of anthocyanin (g/L). ΔA is the absorbance difference of anthocyanin solution at pH values of 1 and 4.5 (Equation 2) (6).

$$\Delta A = (A_{525nm} - A_{700nm})_{pH1.0} - (A_{525nm} - A_{700nm})_{pH4.5} \quad \text{(Equation 2)}$$

MW is the molar mass of the effective component of anthocyanin, cyanidin-3-glucoside (MW = 449.2 g/mol). DF is the dilution factor. ε is the molar extinction coefficient of

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cyanidin-3-glucoside (ε = 26900 *L/(mol·cm)*). *l* is the optical path length of the cuvette (*l* = 1 *cm*).

Making Color Cards and Designing Anthocyanin Content Detector

We measured the concentration of anthocyanin in a black goji berry extract solution to be 1614.39 mg/L by the pH differential method. Diluting the solution, we prepared 30 anthocyanin solutions with concentrations ranging from 50 mg/L to 1500 mg/L, evenly spaced at intervals of 50 mg/L. We immersed filter paper into the solutions for 10 minutes and then placed them in environments at 0, 25, 30, and 35□ respectively for drying, and subsequently measured the RGB values of these color cards. The final RGB values we adopted for the color cards were the averages of the measurements obtained at the four temperatures.

The color cards included RGB values of 30 different colors and their corresponding anthocyanin concentration values. We used a color sensor (TCS3200, TAOS) on the detector to measure the RGB values of the color cards made from the anthocyanin solution. The microprocessor on the detector calculated weighted RGB color differences between the tested sample and the color cards by Equation 3. The weights (w_r, w_g, w_b) in the equation were set to (3, 4, 2), which were optimized values that had been validated by practice (13).

$$D(x_1, x_2) = \sqrt{w_r(r_1 - r_2)^2 + w_g(g_1 - g_2)^2 + w_b(b_1 - b_2)^2} \quad \text{(Equation 3)}$$

D stood for color difference, where x_1 and x_2 represented Color 1 and Color 2, respectively. r_1, g_1, and b_1 were the RGB values for Color 1, while r_2, g_2, and b_2 were the RGB values for Color 2. Furthermore, w_r, w_g, and w_b were the weight coefficients for red, green, and blue colors, respectively.

Our detector found the color card with the color closest to the tested sample and obtained its corresponding anthocyanin concentration (mg/L). Since we dissolved 1 g of black goji berry powder in 50 mL of solvent to prepare the solution for testing, we simply multiplied the measured anthocyanin concentration (mg/L) by 0.05 to calculate the anthocyanin content in the black goji berries (mg/g). We employed the STC89C52 microprocessor to carry out the search and perform the calculation.

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