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Study on Extraction Technology and Stability of Red Pigment in *Red Plumeria*

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Abstract: Background: Pigments, especially for those red color are widely used in various products and are closely related to human health, however, the study on screening optimal conditions for extracting red pigment from red frangipani is less reported. Objective: This study is aimed to decipher the effects of these factors on the stability of the pigment. Method: We developed an optimized extraction method of red pigment from *Plumeria rubra L* leaves through different combinations of extractants, incubation time and temperature. Results: Results show that the largest productivity of red pigment is found under the condition of 10% citric acid as extractant at 80°C for 90 min compared to other conditions. The pigment is sensitive to high light and appears to have strong reducibility rather than oxidability. The pigments production is also sensitive to pH value, as well as metal ion strength, such as Al^{3+}, Cu^{2+}, Fe^{3+}, Mn^{2+}, Zn^{2+}, K^{+}, Mg^{2+}, Na^{+}, and Ca^{2+}, whereas the production is inhibited by adding sucrose, salt and soluble starch. Conclusion: We concluded that red pigment is sensitive to external environmental stimulus and internal ion and carbohydrates concentrations, among these factors, a combination of 10% citric acid extranctant together with 80°C for 90 min treatments ensures to obtain the largest productivity of red pigment. This study provides a fundamental basis for the production of natural red plumeria red pigment and the screening for edible pigment varieties.

Keywords: Productivity, *Plumeria rubra L*, Pigment, Stability, Extract

1. Introduction

Pigments are extensively applied in various products and are closely related to our health. Nowadays, increasing attention has been paid on the security issues of pollution-free, healthy, environmental protection, and the safety of the use of pigments. In this regard, it has been made great efforts to develop natural pigments, instead of artificial pigments in terms of food, cosmetics and other industries [1]. Although natural pigments have the advantages of high safety, natural coloring, and easy to use, they are sensitive to various external factors (such as temperature, humidity, etc.), and their features to maintain stability is undesirable [2–4]. Even though there are abundant natural pigment resources in China, it is still urgent to need overseas seed companies to provide most of the excellent natural pigment sources. However, imported pigments are not only costly, but also it cannot guarantee the normal supply of provenance. Therefore, natural pigment raw materials insufficient supply increases the production cost of related products [3]. Therefore, expanding the source of natural pigments is of great significance to the industry of natural pigments in China.

Among various natural pigments, plant-derived pigments are very abundant and have unique physiological functions, therefore, which are regard as the primary option for modern natural pigments industries. Notably, among these natural pigments, red pigment is a very important type, of which the bright red color is helpful to stimulate the brain, a key component of analeptic, can also promote appetite [2, 4]. At present, extensive attempts on the research of red pigment

extraction technology and stability in rose [5], gerbera [6] and camellia [7] were reported using the red plumeria as the raw material. However, specific reports on extracting red pigment and its stability were less documented.

Red plumeria (*Plumeria rubra L*), also known as staghorn tree, thumping hammer flower, egg yolk flower, Burmese gardenia, big season flower, etc. [8-10]. Red frangipani, mostly small trees or shrubs, are native to tropical regions of the Americas such as Venezuela, Mexico and the West Indies [11, 12]. In some countries, red plumeria is commonly applied to make perfume and cosmetic raw materials as well as some food additives. Therefore, it is increasingly popular to make tea or eaten after dehydration in some cities of China, such as Fujian, Guangdong, Guangxi, and Yunnan. The trend has the promising prospect towards extensive development and utilization [13]. Thereby, it is a natural pigment resource, thanks of its bright red and beautiful flowers, that worth exploring and developing in red frangipani.

This study is aimed to provide the solution for the further expand the market of natural red plumeria red pigment, as well as the development of China's natural edible pigment varieties and pigment commodity industry. We systematically explored the extraction and stability of red frangipani red pigment, figure out the optimal extraction method of red frangipani pigment, and uncover the effects of external factors such as light conditions, pH, reducing agents, oxidants, various metal ions and three common food additives on stability of red plumeria red pigment.

2. Materials and Methods

2.1. Materials and Growth Conditions

The red frangipani was collected from the main campus of Guangdong Ocean University, China. The petal of uniform shape with red color was used in this study.

The pretreatment procedure for extraction of red frangipani was as follows: take fresh and clean red frangipani, cut off the yellow parts of the petals, keep the red parts, and remove the defective petals. Wash the petals and dry them. Place it in the baking tray of the oven and dry at a temperature of 80°C. After drying, use a coffee grinder to grind it into a fine powder and bottle it for later use.

2.2. Spectral Characteristics of Red Plumeria Red Pigment

~0.01 g dried petal powder of red frangipani in a test tube were taken into a total of 24 copies, followed by 10 mL of distilled water and shake was added, then cooled down at room temperature for 30 min, and then filtered it through a 0.45 m PTFE filter. The filtrate was transferred to a 50 mL volumetric flask maintaining a constant volume with distilled water. The final red plumeria red pigment extract was obtained. Using distilled water as a blank solution, the absorbance was measured at 280-350 nm with an ultraviolet spectrophotometer at room temperature. The recorded OD value was analyze to examine the optimal absorption wavelength, so that the point where the absorption value reaches the peak.

2.3. System Construction for Red Pigment Extraction from Red Plumeria

2.3.1. Screening Extractants

To screen the extractants, we took ~0.01 g of dried petal powder of red frangipani in a test tube, a total of 21 copies (7 levels, 3 repetitions), and then added 10 mL ethyl acetate, 10 mL acetone, 10 mL absolute ethanol, 10 mL distilled water, 10 mL 30% ethanol, 10 mL 50% ethanol, 10 mL 10% citric acid, followed by brief shaking. It was then cooled down at room temperature for 30 min, and filtered as mentioned above. The color of the extract and the extraction conditions were compared with each other to find the optimal extractant.

2.3.2. Screening Extraction Concentration

To uncover the optimal extraction concentration, we took ~0.01 g dried petal powder of red frangipani in a test tube into a total of 21 copies (7 levels, 3 repetitions), and then added 10 mL of extractant with concentration of 1%, 5%, 10%, 15%, 20%, 25% and 30% respectively, with brief shaking. The mixture was then cooled down at room temperature for 30 min, filtered, and the filtrate was transferred to a 50 mL volumetric flask maintaining constant volume with distilled water. We also used the extractant as a blank solution, simultaneously measured the OD value with the suitable absorption wavelength in the ultraviolet spectrophotometer to determine the best extractant concentration.

2.3.3. Screen the pH of the Extractant

The optimal concentration of the pigment extraction solvent, obtained by the above method is prepared with a low concentration hydrochloric acid solution and a sodium hydroxide solution to keep the pH of the solution as 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10, respectively. We took 0.01 g of dried petal powder of red frangipani in a test tube, a total of 33 copies (11 levels, 3 repetitions), and then added 10mL of the best extractant with different pH accordingly followed by gently shaking. The solution was then cooled down at room temperature for 30 min, and filtered as mentioned above. After that, the filtrate was transferred to a 50 mL volumetric flask and kept constant volume with distilled water. We used the extractant as a blank solution, and measured the OD value with the optimal absorption wavelength in the ultraviolet spectrophotometer to determine the best extraction pH.

2.3.4. Screening Extraction Temperature

To screen extraction temperature, we took ~0.01 g of dried petal powder of red frangipani in a test tube, i.e., a total of 24 parts (8 levels, 3 repetitions), and added 10 mL of the best extractant and shake. It was then placed in room temperature, 30°C, 40°C, 50°C, 60°C, 70°C, 80°C and 90°C water baths for 30 min, and filtered sequentially. Followed by that, we transferred the cooled filtrate to a 50 mL volumetric flask and make up to volume, we used the extractant as a blank solution, and measured the OD value with the best absorption wavelength in an ultraviolet spectrophotometer to determine the optimal extraction temperature.

2.3.5. Screening and Extraction Time

Take 0.01 g of dried petal powder of red frangipani in a test tube, a total of 18 parts (6 levels, 3 repetitions), and then added 10 mL of the pigment extraction solvent with brief shaking. Under the optimal extraction temperature obtained by the above method, the extraction duration is set to 15 min, 30 min, 45 min, 60 min, 75 min and 90 min and then filtered with 0.45 m PTFE filter. Transfer the cooled filtrate to a 50 mL volumetric flask and make up to volume. Using the extractant as a blank solution, measure the OD value with the best absorption wavelength in an ultraviolet spectrophotometer to find the optimal extraction duration.

2.4. Orthogonal Test

The tests mentioned above for extracting pigment conditions are all single-factor tests. It is of great interest to determine whether there is an interaction between the extraction factors, with an effect on the extraction of red pigment from red plumeria. Therefore, a 3-factor 3-level orthogonal test of L9 (3^4) was set to select the optimal conditions for extracting red pigment from red plumeria. In this regard, we used the extractant as a blank solution, and the OD value was simultaneously measured with the optimal absorption wavelength in an ultraviolet spectrophotometer. The optimal conditions for extracting the pigment were selected as shown in Table 1.

Table 1. *Orthogonal test on three factors for red frangipani extraction.*

Factor levels	A (Con./%)	B (Temp./ °C)	C (Time/min)
1	1	70	45
2	5	80	60
3	10	90	90

2.5. Red Plumeria Red Pigment Stability Study

The red plumeria red pigment stock solution is prepared by combining the optimal extraction conditions obtained by the above method.

2.5.1. Effect of Light on Red Pigment of Red Frangipani

Measure 100 mL of red plumeria red pigment extract in a 200 mL glass tube with a tap, 6 portions in total, seal and label it. Distribute it evenly in black plastic sealed bags under either natural outdoor light or darkness. Within 7 days, we determined the OD value at the optimal absorption wavelength of the pigment every day and observed the color change of solution.

2.5.2. Effect of pH on Red Pigment of Red Plumeria

A solution with a pH of 1~10 was prepared with 1 mol/L HCl, 1 mol/L NaOH, 0.1 mol/L HCl and 0.1 mol/L NaOH. We took 5 mL of pigment stock solution, and added sequentially 3 mL solutions with different pH values as mentioned above, and then shake. After incubation for 30 min, the OD value was measured with the optimal absorption wavelength.

2.5.3. Effect of Reducing Agent on Red Pigment of Red Plumeria

To unravel the effect of reducing agent on red pigment of red plumeria, we transferred 3 mL of $NaHSO_3$ solution of different concentrations (0.05 mol/L, 0.10 mol/L, 0.15 mol/L, 0.20 mol/L, and 0.25 mol/L) into 5 mL of pigment stock solution and briefly shake. After incubation for 30 min, we recorded the color change of the solution and measured the OD value at the optimal absorption wavelength.

2.5.4. Effect of Oxidants on Red Pigment of Red Plumeria

In total, 3 mL of H_2O_2 solutions of different concentrations (4%, 8%, 12%, 16%, 20%) was transferred into 5 mL of pigment stock solution and shake. After incubation for 30 min, we observed the color change of the solution and measured the OD values at the optimal absorption wavelength.

2.5.5. Effect of Various Metal Ions on Red Pigment of Red Plumeria

Effect of various metal ions on red pigment of red plumeria was examined through transferring 3 mL of 0.1 mol / L of various metal ion solutions (Fe^{3+}, Ca^{2+}, Mn^{2+}, Mg^{2+}, Na^{+}, Cu^{2+}, Zn^{2+}, Al^{3+}, K^{+}) into 5 mL of pigment stock solution and shake. After incubation for 30 min, we recorded the color change of the solution and measured the OD values at the optimal absorption wavelength.

2.5.6. Effect of Common Food Additives on Red Pigment of Red Plumeria

To examine the effect of common food additives on red pigment of red plumeria, we transferred 3 mL of sucrose solutions of different concentrations (0.5%, 1%, 1.5%, 2.0%, 2.5%, and 3.0%) into 5 mL of pigment stock solution and briefly shake. After incubation for 30 min, we observed the color change of the solution and measure the OD value at the optimal absorption wavelength.

For different concentrations (0.5%, 1%, 1.5%, 2.0%, 2.5%, and 3.0%) of common salt solution, 3 mL solution was transferred to 5 mL of pigment stock solution and shake. After incubation for 30 min, we recorded the color change of the solution and measure the OD value at the optimal absorption wavelength.

In order to examine the effects of soluble starch solutions, 3 mL different concentrations (0.5%, 1%, 1.5%, 2.0%, 2.5%, and 3.0%) were transferred into 5 mL of pigment stock solution and shake. After incubation for 30 min, we recorded the color change of the solution and measure the OD value at the optimal absorption wavelength.

2.6. Statistical Analysis

LSD method was employed in the DPS data processing system software to perform statistical analysis on the data. Figures were generated via SigmaPlot software.

3. Results

3.1. Spectral Characteristics of Red Pigment in Red Plumeria

As shown in Figure 1, from the wavelength of 280 nm to 350 nm, the peak is reached at 300 nm, and the absorbance

values on both sides of 300 nm gradually decreased. Results also show that among the 8 wavelengths, there are extremely significant differences between each wavelength except 330 nm and 290 nm (Table 2). At a wavelength of 300 nm, the mean value is greater than other wavelength and is significantly different from other wavelengths (P<0.05). These evidences suggest that the optimal absorption wavelength for red pigment in *red plumeria* is around 300 nm.

Table 2. *Significant difference analysis of the absorbance value of red egg pigment at different wavelengths.*

wavelength (nm)	Absorbance	5% sig. levels	1% sig. levels
300	0.9263±0.0116	a	A
310	0.8977±0.0032	b	B
320	0.8100±0.0090	c	C
330	0.6840±0.0040	d	D
290	0.6787±0.0059	d	D
340	0.5330±0.0056	e	E
350	0.3910±0.0046	f	F
280	0.2297±0.0035	g	G

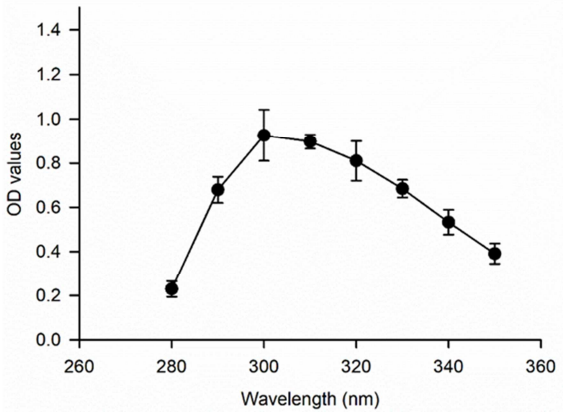

Figure 1. *Absorbance curve of red pigment in red egg flower at different wavelengths. Means are values ± SE (n=3).*

3.2. Extraction Process and Stability of Red Pigment from Red Plumeria

The red pigment of red plumeria is insoluble in ethyl acetate, acetone, and absolute ethanol, but it is soluble in distilled water, 30% ethanol, 50% ethanol, and 10% citric acid (Table 3). Therefore, these suggest that red plumeria red pigment could be a water-soluble pigment. The red pigment of red frangipani is extracted with distilled water, the dissolution speed is rapid, and the extraction is also sufficient. The initial pigment solution is pink, while the pigment is unstable, and the color of the solution will gradually fade down after a period of time. When extracting red plumeria red pigment with 30% ethanol and 50% ethanol, the speed is also fast and sufficient. The color of the solution is similar to the color of petals, which is light pink. However, when 10% citric acid was used to extract the pigment, the dissolved speed was fast, the color of the pigment solution was obviously the darkest. In addition, the solution color was not altered after incubation for a period of time. Therefore, the optimal extracting agent for red pigment of red plumeria, determined in this study is citric acid.

The absorption curve reached the absorption peak when the citric acid concentration was 10% (Figure 2A). The red pigment absorption value of red plumeria increases with the increase of citric acid concentration. After the citric acid concentration is 10%, the citric acid concentration increased while the absorbance value decreased. The absorbance value of 10% citric acid solution is significantly greater than that of other concentration solutions (Table 4). At the same time, the average absorbance value of 10% citric acid solution appear to be the largest, suggesting the best treatment at this condition. Therefore, the optimal concentration of citric acid to extract red pigment from red frangipani is around 10%.

Table 3. *Color alteration of red pigment of red egg flower treated with different extractants.*

Extractants	Filter residue	Filtrate	Extraction effect
Distilled water	White	Light pink	Easy
30% ethanol	White	Light pink	Easy
50% ethanol	White	Light pink	Easy
10% citric acid	White	Light pink	Easy
ethyl acetate	Pink	Colorless	Difficult
100% ethanol	Pink	Colorless	Difficult
acetone	Pink	Colorless	Difficult

Table 4. *Significant difference analysis between different concentrations of extractant treatment.*

extraction concentration (%)	Absorbance	5% sig. levels	1% sig. levels
10	0.8720±0.0056	a	A
5	0.8143±0.0059	b	B
1	0.7960±0.0020	c	C
15	0.7827±0.0125	d	C
20	0.7540±0.0040	e	D
25	0.7357±0.0108	f	E
30	0.7210±0.0036	g	E

In the range of pH 1~6, the absorption value gradually increases to the peak and then gradually decreases (Figure 2B). The first absorption peak is at the original pH solution. In the range of pH 6~10, there is another absorption peak, but the peak of the highest peak is significantly lower than the peak of the highest peak of the first absorption peak. In Table 5, when the pH value is low, the pigment solution preferably retains the original color. When the pH value gradually increases, the color of the pigment solution changes, the original color becomes lighter to none, and then changes to other colors. This evidence shows that as the pH value increases, the pigment is degraded. Therefore, weak acid and alkaline extractants are not suitable for extracting red pigment from red plumeria.

The absorbance of the original pH solution is significantly different from that of other pH solutions (Table 5). Results imply that different pH values could alter the extraction effect of the pigment solution, and notably, the extraction effect is less than the original pH solution. Therefore, we proposed that given that does not adjust the pH value for extraction, the best option is a citric acid solution.

As the extraction temperature increases, the red plumeria red pigment's absorbance value gradually increases (Figure 2C). At 80°C, the absorbance value reaches the maximum. Subsequently, as the temperature increases, the absorbance

value decreases. In Table 6, the difference between the treatment with a temperature of 80°C and other temperature treatments is significant. At the same time, the maximum mean value of the measured absorbance is at a temperature of 80°C, so this process is optimal. In summary, the optimal extraction temperature for the extraction of red pigment from red plumeria is 80°C. The red plumeria red pigment's absorbance value gradually increased with the prolonged time, then suddenly dropped at 75 min, and rised again at 90 min (Figure 2D). The OD values under 90 min treatment is very significantly different from that at 15 min, 30 min, 45 min, and 75 min, but not significantly different from that at 60 min (Table 7). Moreover, the maximum average value of the absorbance measured by the pigment solution is at 90 min, suggesting that optimal treatment time of the pigment could be 90 min.

Table 5. *Results of red egg anthocyanin extracted by different pH value extractants.*

citric acid pH	Solution color	Absorbance	5% sig. levels	1% sig. levels
Orig. pH (ck)	Pink	0.8707±0.0040	a	A
2	Pink	0.8410±0.0026	b	B
1	Pink	0.8283±0.0031	c	C
8	Yellow	0.8080±0.0026	d	D
3	Pink	0.8023±0.0038	de	DE
7	Light yellow	0.7963±0.0035	e	E
4	colorless	0.7813±0.0051	f	F
9	Light yellow	0.7370±0.0040	g	G
5	colorless	0.7157±0.0055	h	H
10	Dark yellow	0.7053±0.0067	i	I
6	colorless	0.6827±0.0031	j	J

Table 6. *Results of red egg anthocyanin extracted by different temperature conditions.*

Temperature (°C)	Absorbance	5% sig. levels	1% sig. levels
80	0.9087±0.0060	a	A
90	0.8940±0.0030	b	B
70	0.8883±0.0015	bc	BC
60	0.8863±0.0070	bcd	BC
50	0.8833±0.0042	cd	BC
40	0.8807±0.0021	cd	C
30	0.8793±0.0025	d	C
25 (room temperature)	0.8613±0.0060	e	D

Table 7. *Significance analysis among different extraction time treatments.*

Time (min)	Absorbance	5% sig. levels	1% sig. levels
90	0.9247±0.0032	a	A
60	0.9150±0.0075	ab	AB
45	0.9130±0.0066	b	AB
30	0.9087±0.0060	b	B
75	0.8900±0.0062	c	C
15	08243±0.0055	d	D

Table 8. *Statistical analysis on significance and range according to orthogonal test results.*

# Number	Treatment methods			Absorbance	5% sig. levels	1% sig. levels
	A (Con./%)	B (Temp/℃)	C (Time/min)			
1	1	1	1	0.8610	d	C
2	1	2	2	0.9060	abc	AB
3	1	3	3	0.9097	ab	AB
4	2	2	3	0.9160	a	A
5	2	3	1	0.8953	c	B
6	2	1	2	0.8517	d	C
7	3	3	2	0.9153	a	A
8	3	1	3	0.9117	ab	AB
9	3	2	1	0.9010	bc	AB
k1	0.8922	0.8748	0.8858			
k2	0.8877	0.9077	0.8910			
k3	0.9093	0.9068	0.9124			
R	0.0217	0.0329	0.0267			

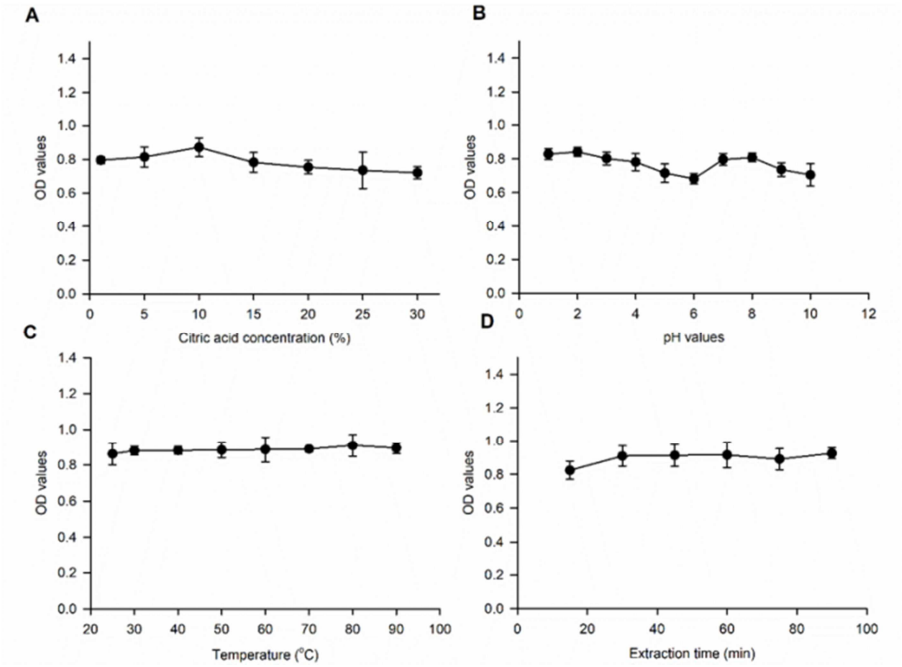

Figure 2. *Extraction process and stability of red pigment from red plumeria using different citric acid concentration (A), pH values (B), and temperature (C) and extraction time (D). Means are values ± SE (n=3).*

Among the measurement results of the orthogonal test, i.e., the three influencing factors of extractant concentration (A), extraction temperature (B), and extraction time (C), the effect level of the extraction effect of red pigment on the red plumeria was ranked as: extraction temperature (B) > extraction time (C)> extractant concentration (A) (Table 8). The optimal extraction combination of red plumeria on red pigment is the 10% citric acid, 80°C, and 90 min extraction time.

3.3. Stability of Pigments with Different Environmental Stimulus

The absorbance values for red pigment of red frangipani under different conditions, gradually decreased on days 1~4, but suddenly increased on the 5th day, and then declined continuously (Figure 3A). The overall increase or decrease in light conditions is more pronounced than that under dark conditions. This suggests that with the extension of the storage time, the red pigment of red plumeria is very likely continued to degrade, and other substances will appear first and then gradually degrade. The absorption value of the red pigment of red frangipani that has not been placed under light conditions is extremely significant, compared to the absorption values of other red plumeria flowers that have been placed for a period of time (Table 9). The absorption value of the red pigment of the red frangipani placed on the 3rd day under dark conditions was only significant on the days of 0, 1, and 2. Under light conditions, the dark pink solution faded to light pink during 7 days storage. The treatment in dark conditions for 7 days faded from the dark pink solution to pink. Our findings as mentioned above show that the red pigment of red frangipani is relatively stable under dark conditions, and other substances may be produced during the storage process. Therefore, it is essential to pay great attention to protect the pigment from light during exaction process.

As shown in Figure 3B, when the pH is maintained between 1 and 3, the light absorption value of the pigment decreases continuously, while given that pH is higher than 4, there are two absorption peaks. This reveals that the changes of pH lead to the alteration of substance in the solution. There is a significant difference in pH within each absorption peak (Table 10). The findings documented above show that the anthocyanin of red egg should be kept at proper pH value, when it is developed and used.

Table 9. *Statistical analysis on red pigment solution of red egg flower in light and dark environment.*

Days (d)	Light			
	Absorbance	**Color**	**5% sig. levels**	**1% sig. levels**
0	0.9227±0.0035	Dark pink	a	A
1	0.8370±0.0010	Dark pink	b	B
5	0.8337±0.0042	Pink	b	B
2	0.8317±0.0078	Dark pink	b	BC
6	0.8147±0.0045	Light pink	c	CD
3	0.8077±0.0090	Dark pink	c	D
4	0.7893±0.0115	Pink	d	E
7	0.7683±0.0119	Light pink	e	F

Table 9. *Continued.*

Days (d)	Darkness			
	Absorbance	Color	5% sig. levels	1% sig. levels
0	0.9223±0.0021	Dark pink	a	A
1	0.9193±0.0015	Dark pink	ab	A
5	0.9173±0.0040	Pink	ab	A
2	0.9167±0.0031	Dark pink	ab	A
6	0.9143±0.0032	Pink	b	A
3	0.8997±0.0093	Dark pink	c	B
7	0.8973±0.0040	Pink	c	BC
4	0.8893±0.0021	Dark pink	d	C

Table 10. *Statistical analysis on difference significance of absorbance value of pigment solution after adding different pH solutions.*

pH	Absorbance	5% sig. levels	1% sig. levels
5	0.6337±0.0025	a	A
1	0.6310±0.0030	ab	A
6	0.6260±0.0026	bc	AB
9	0.6217±0.0031	cd	BC
4	0.6197±0.0042	d	BC
7	0.6167±0.0064	d	C
2	0.6163±0.0015	d	CD
10	0.6087±0.0040	e	DE
3	0.6073±0.0021	e	E
8	0.6067±0.0021	e	E

Table 11. *Statistical analysis on difference of absorbance values of pigment solution after adding different concentrations $NaHSO_3$ solution.*

Con. reducing agent (mol/L)	Absorbance	Solution color	5% sig. levels	1% sig. levels
0.25	1.5330±0.0026	Colorless Obvious changes	a	A
0.20	1.5050±0.0062		b	B
0.15	1.4943±0.0053		c	B
0.10	1.4480±0.0025		d	C
0.05	1.2830±0.0053		e	D
0.00	0.9130±0.0020		f	E

The absorbance values of red pigment of red egg flower rises with the enhanced $NaHSO_3$ concentration, and the increase of absorbance value slows down with the increased concentration of $NaHSO_3$ (Figure 3C). As seen in Table 11, each treatment with $NaHSO_3$ of different concentrations has significant difference, compared with the control group, while the color change of the solution is not obvious. The results showed that the absorbance values of red pigment increased; however, the color did not change obviously after $NaHSO_3$ was added, hence the reducing agent had little effect on the stability of red pigment.

The red plumeria red pigment's absorbance value increases with promoted H_2O_2 concentration, and appear to be a plateau at 15~20% H_2O_2 concentration (Figure 3D). The absorbance of solutions when added with various concentrations of H_2O_2 is extremely significant, compared to the control group (Table 12). In addition, with the increase of the added H_2O_2 concentration, the extraction solution gradually faded from dark pink to colorless. Therefore, following increase in the concentration of H_2O_2, the absorbance of the pigment also increases simultaneously, but the color of the solution gradually becomes lighter. This is very likely due to the fact that H_2O_2 reacts with the red pigment of red plumeria, indicating that the pigment has weak oxidation resistance and should be prevented from incubating with the oxidizing agent.

Table 12. *Statistical analysis on the difference of absorbance value of pigment solution after adding different concentration of H_2O_2.*

Con. oxidants (%)	Absorbance	Solution color	5% sig. levels	1% sig. levels
20	1.5897±0.0051	Faded from dark pink to colorless	a	A
16	1.5400±0.0046		b	B
12	1.4630±0.0026		c	C
8	1.2817±0.0038		d	D
4	1.0557±0.0025		e	E
0	0.9150±0.0092		f	F

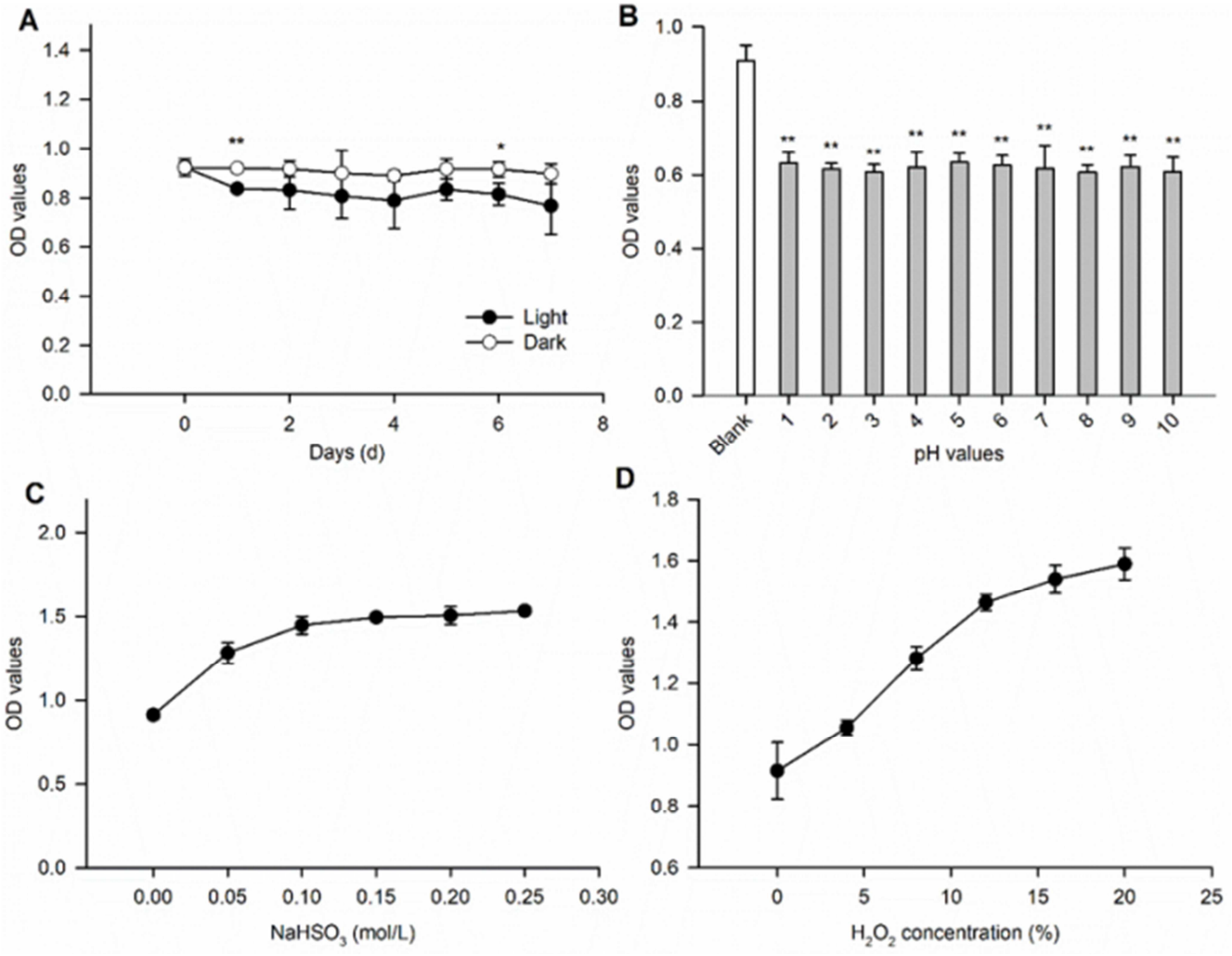

Figure 3. *Effects of factors on red pigment of red egg flower. A, light and dark; B, different pH values; C, reducing agent; D, oxidant. For panels A, B, means are values ± SE (n=3). Symbols "*", "**", and "***" represent P value < 0.05, 0.01 and 0.001, respectively, according to Student's t-test.*

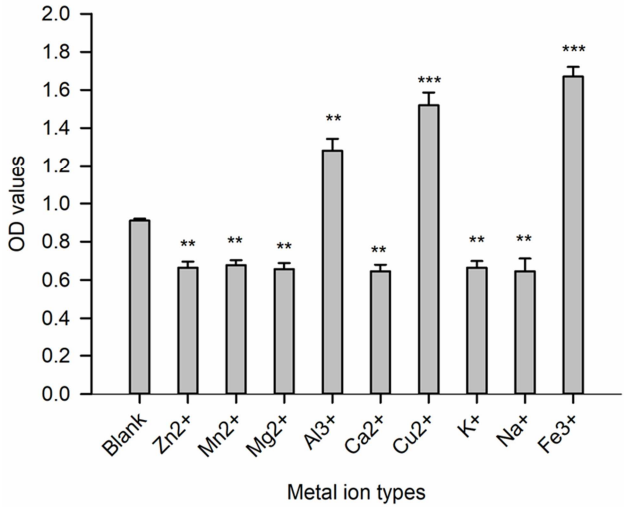

Figure 4. *Effect of metal ions on red pigment of red egg flower. Means are values ± SE (n=3). Symbols "*", "**", and "***" represent P value < 0.05, 0.01 and 0.001, respectively, according to Student's t-test.*

Compared with the control (CK), the absorption values of the pigment extraction solution increased after adding Al^{3+}, Cu^{2+}, Fe^{3+} (Figure 4). The absorption values of the pigment extraction solution increased significantly; while adding Mn^{2+}, Zn^{2+}, K^{+}, Mg^{2+}, Na^{+}, Ca^{2+} Compared with the control, the absorbance value of the pigment extraction solution is reduced. As shown in Table 13, after adding metal ions, the primary color of the metal ion solution replaces the color of the extraction solution, indicating that the addition of metal ions has a direct effect on the color of the extraction solution. There were significant differences among Mn^{2+}, Al^{3+}, Cu^{2+}, and Fe^{3+}, while there was no significant difference between Zn^{2+}, K^{+}, and Mg^{2+}, and it is the case (no significant difference) between Na^{+} and Ca^{2+}, although it was significant difference between each metal ion and the control. We hence proposed that each metal ion has a great influence on the pigment extraction, and it is highly recommended that extracting the pigment solution should avoid directly contacting with these metal ions.

Table 13. *Statistical analysis on the difference of absorbance value of red egg pigment solution after adding various metal ions.*

Metal ion	Metal color	Mean Absorbance	Solution color after adding metal ion	5% sig. levels	1% sig. levels
Fe^{3+}	Yellow	1.6707±0.0050	Light yellow	a	A
Cu^{2+}	Blue	1.5197±0.0067	Light blue	b	B
Al^{3+}	Colorless	1.2810±0.0062	Colorless	c	C
CK	Colorless	0.9110±0.0010	Pink	d	D
Mn^{2+}	Colorless	0.6770±0.0026	Colorless	e	E
Zn^{2+}	Colorless	0.6647±0.0031	Colorless	f	F
K^{+}	Colorless	0.6643±0.0035	Colorless	f	F
Mg^{2+}	Colorless	0.6573±0.0031	Colorless	f	F
Na^{+}	Colorless	0.6460±0.0066	Colorless	g	G
Ca^{2+}	Colorless	0.6447±0.0035	Colorless	g	G

The absorbance of the pigment solution increases with enhanced soluble starch concentration (Figure 5). The absorbance values of the pigment solution first gradually increases with the sucrose concentration, when the concentration reaches 2%, the absorbance value decreases, and then the absorbance value rises again (Figure 5). The absorbance value of the pigment solution first gradually increases with the salt concentration, and it changes when the concentration reaches up to 2%, and the absorbance value begins to decrease.

There is a significant difference between the absorbance values of sucrose pigment solutions, when adding with different concentrations and the control group (Table 14). Similarly, there is a significant difference between the absorbance value of the salt solution with different concentrations and the control group (Table 15). We also found that except for the soluble starch concentration of 1%, there is a significant difference in absorbance between the control group and other pigment solutions treated with different concentrations (Table 16). The absorbance of soluble starch differs significantly between different concentrations. Collectively, the addition of different concentrations of sucrose, table salt, and soluble starch all changed the color of the pigment solution and became colorless. In summary, none of sucrose, salt, and soluble starch can be mixed with red plumeria red pigment to make commodities.

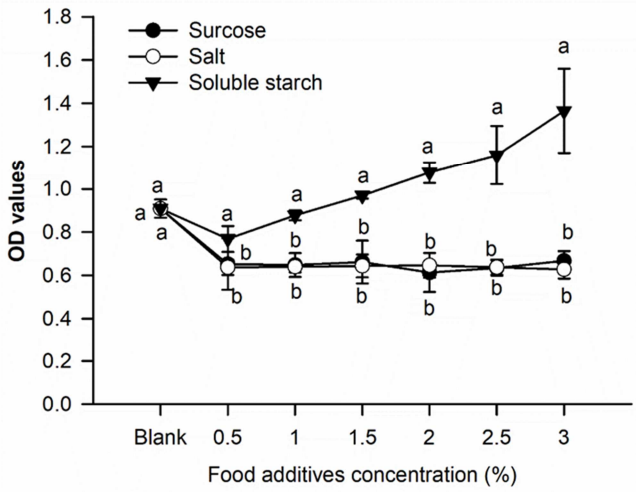

Figure 5. *Effect of common food additives on pigment extraction. Means are values ± SE (n=3). Different alphabet letters represent significant differences among different food additive for each concentration at P value < 0.05 according to one-way ANOVA.*

Table 14. *Statistical analysis on the difference of absorbance value of pigment solution with different concentration of sucrose.*

Con. (%)	Solution color	Mean Absorbance	5% sig. levels	1% sig. levels
CK	Pink	0.9093±0.0042	a	A
3.0	Colorless	0.6677±0.0045	b	B
1.5	Colorless	0.6617±0.0099	bc	BC
0.5	Colorless	0.6527±0.0119	cd	BCD
1.0	Colorless	0.6483±0.0055	d	CD
2.5	Colorless	0.6343±0.0035	e	D
2.0	Colorless	0.6137±0.0099	f	E

Table 15. *Statistical analysis on the difference of absorbance value of pigment solution after adding different concentration of salt solution.*

Con. (%)	Solution color	Mean Absorbance	5% sig. levels	1% sig. levels
CK	Pink	0.9093±0.0042	a	A
2.0	Colorless	0.6467±0.0057	b	B
1.5	Colorless	0.6440±0.0053	bc	B
1.0	Colorless	0.6397±0.0025	bc	B
2.5	Colorless	0.6377±0.0035	c	BC
0.5	Colorless	0.6373±0.0035	c	BC
3.0	Colorless	0.6277±0.0042	d	C

Table 16. Statistical analysis on the difference of absorbance value of pigment solution after adding different concentration of soluble starch solution.

Con. (%)	Solution color	Mean Absorbance	5% sig. levels	1% sig. levels
3.0	Colorless	1.3650±0.0195	a	A
2.5	Colorless	1.1593±0.0136	b	B
2.0	Colorless	1.0773±0.0549	c	C
1.5	Colorless	0.9703±0.0015	d	D
CK	Pink	0.9093±0.0042	e	E
1.0	Colorless	0.8783±0.0023	e	E
0.5	Colorless	0.7687±0.0060	f	F

4. Discussion

It is extensive reported that effective extraction for the pigment can be achieved through solvent extraction method [14–16]. In this study, we applied this approach with constraining the principle of single variable. The effects of different extractants, concentrations, pH conditions, temperature, and time on the extraction of red plumeria red pigment were systematically studied and analyzed. In order to determine whether there is an interaction between the extraction factors, we further employed an orthogonal test to screen out the optimal pigment extraction conditions. The optimal conditions for extracting red pigment from red frangipani are screened by timely measuring the absorbance value, and eventually the stability of the extracted red pigment from red frangipani is discussed.

Extensive studies have shown that the red anthocyanins in the strong acid solution, the pigment solution appear as the color as original shows [17, 18]. When the pH value is greater than 4, the pigment is red-shifted and the color becomes lighter. In contrast, when the pH is greater than 7, the pigment gradually turns to be orange yellow, yellow-green to yellow. Therefore, it is reasonable to propose that red pigment in red plumeria contains anthocyanins.

4.1. The Best Extraction Process of Red Pigment in Red Plumeria

In current study, in order to facilitate the approachable operation steps and further improve the extraction effects, the petals were dried and ground into a fine powder with a grinder. As used in many studies regarding extractant, their concentration, pH, extraction temperature and time are generally used as screening conditions for mining optimal extraction process [19, 20]. Among various options of solvents, red plumeria red pigment is insoluble in ethyl acetate, acetone, and absolute ethanol, but soluble in distilled water, 30% ethanol, 50% ethanol, and 10% citric acid. We here proved that the extraction speed of the pigments extracted with distilled water, ethanol, and citric acid is fast and sufficient.

In the research of other pigment extraction, water and ethanol are commonly used as extractants. For examples, water can be used as an extractant for natural gardenia yellow pigment [21], while ~ 60% ethanol was used to extract the pigment of red orange peel [22]. They found that the extraction effect was the best, but citric acid is rarely used for pigment extraction, and is generally used for color preservation of pigments. When the absorbance value was measured, it was found that the color of the solution was more obvious than that of the distilled water extract. Strikingly, the extract of the pigment extracted with distilled water had the highest absorbance value, and the citric acid pigment extract was obviously stable [23]. These results suggest that citric acid may play an important role in anti-oxidation and other color-preserving functions. Meanwhile, compared with alcohol-soluble aqueous solution, the cost of acidic solution is lower and closer to the ingredients of the food itself, therefore, we proposed that citric acid is finally selected as the extractant.

In terms of the optimal pH conditions, it was found that the pH changes its color by affecting the structure of anthocyanins [24]. The pigment can be extracted under strongly acidic conditions, and the resulting pigment solution maintains the original red plumeria color. As increases in the pH of the extractant, the higher the pH, the rate of anthocyanins degraded faster, leading to more obvious the color change of the pigment [25]. In our study, we found that adding other reagents to adjust the pH of the solvent would change the concentration and composition of the extractant, which reduces the effect of citric acid on extracting pigments. Therefore, unadjusted pH extractant could be the best option in this case. During the pigment extraction in response to temperature, we found that the optimal extraction temperature is 80 °C, indicating that the pigment is relatively heat-resistant and can be developed and applied to general food and beverage additives.

4.2. Stability Analysis of Red Pigment in Red Plumeria

Followed by prolong incubation time, as observed in our study, natural pigments turns to be degrade, and this process is further accelerated under light conditions. The absorbance of the pigment solution gradually decreased within 4 days, and the color gradually faded. This may be related to an increase in light exposure time, the increase in the number of photons, the increase in the number of excited pigment molecules, and the continuous photodegradation reaction [26, 27]. Therefore, when using pigments, it is necessary to prevent direct sunlight as much as possible. Interestingly, the absorbance value suddenly increased, and then continued to decrease at the 5th day. Meanwhile, it was found that there were translucent impurities in the pigment solution in the later stage. It is speculated that there is some kind of bacteria, therefore it is highly recommended that it should be sterilized before it is

used in practice.

In this study, the red plumeria red pigment solution's absorbance value changes continuously with the change of pH value. This is due to the alteration of pH of the solution, as well as the chemical structure of anthocyanin. Anthocyanins exist in the form of yellow salt cations, quinoid bases, pseudoalkali, and chalcone in the aqueous solution. The above four forms could be reversibly changed due to the alteration of pH regimes of the aqueous solution. Meanwhile, the color of the solution also changes due to alteration of composition [28]. Therefore, the pigment solution should be stored under suitable acid-base conditions.

In addition, the red pigment of red frangipani had better resistance to reductant agents, as observed in our study; however, it is feasible to oxidants. During the study of the stability of H_2O_2 to the red plumeria red pigment solution, the color of the solution gradually faded. This is because the pretense salt structure of anthocyanins is susceptible to nucleophilic attack by oxidants, and becomes unstable, non-ionic, namely epicatechin, and fades to colorless [29, 30]. Therefore, it is essential to avoid the simultaneous use of oxidant during pigment storage.

Interestingly, we found that all kinds of metal ions and common food additives broke the stability of the red pigment of red plumeria. For example, $A1^{3+}$, Zn^{2+}, Cu^{2+}, Fe^{3+} characterized with large molecular weight, high atomic valence states, therefore, metal ions with metal activity will react with natural pigments, which leads to the fade or precipitation of pigments. Therefore, K^+, Ca^{2+}, Na^+ and other metal ions do not react with it [22]. To explain the replacement of the color of the pigment solution by the primary color of the metal ion solution, we proposed that the pigment easily reacts with the metal ion, or it may be due to the interference of the color of the metal ion aqueous solution that can exhibit color. However, it requires further studied to prove whether there are other metal ions or food additives that can be stored more stably with the pigment.

Taken together, the red pigment of red frangipani has certain stability with a great development prospect. If the pigment is to be used in actual production, it should also be comprehensively studied to improve the properties of other aspects to ensure its population-free, effectiveness and high-extraction efficiency.

5. Conclusion

The red pigment of red plumeria can be dissolved in water and alcohol, rather than organic solvents, therefore, it is a water-soluble pigment with anthocyanin pigments, with the absorption peak at 300 nm. The optimal combination of pigment extraction is to use citric acid with a concentration of 10% as the extractant, the temperature is 80 ℃, and the time is 90 min. The red pigment of red frangipani is light-sensitive, but resistance to reductant agent, rather than to oxidation. The red pigment of red frangipani changes with various pH value as well as metal ion, including Al^{3+}, Cu^{2+}, Fe^{3+}, Mn^{2+}, Zn^{2+}, K^+, Mg^{2+}, Na^+, and Ca^{2+}. We also proposed that sucrose, salt and soluble starch should not be mixed when extracting red plumeria red pigment.

Author Contributions

Conceptualization, T. W., Y. Y.; Methodology, T. W., Y. Y.; Investigation, Q. L., B. C.; Writing, T. W.; Funding Acquisition, T. W.; Resources, T. W.; Supervision, T. W.

Declaration of Interest

The authors declare that they have no competing interests.

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