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Stability and Antioxidant Activity of Anthocyanins from Flowers of *Rhododendron pulchrum* Sweet.

Mei ZHANG*, Benyong LOU, Yanjie ZHANG, Haoxi FU

Department of Chemical Engineering and Materials, Ocean College, Minjiang University, Fuzhou 350108, China

Abstract The effects of light, temperature, pH, oxidant and reducing agents, metal ions and food additives on the stability of anthocyanins from flowers of *Rhododendron pulchrum* Sweet., as well as their scavenging capacity for ·OH, O^{2-}· and DPPH· free radicals and reducing power were investigated. The results showed that the anthocyanins from flowers of *R. pulchrum* were relatively stable in the room with natural light, at temperature lower than 45℃, and in the solution with pH ≤ 4. The addition of Na^+, K^+ Ca^{2+} metal ions, glucose, sodium benzoate, acetic acid and salt had little effect, but Cu^{2+}, Zn^{2+}, Fe^{3+}, Al^{3+}, starch and sodium glutamate showed adverse effects on the stability of the anthocyanins. The anthocyanins from flowers of *R. pulchrum* had a good antioxidant effect, and their scavenging capacity for ·OH, O^{2-}·, DPPH· was better than that of the same concentration of Vc.

Key words *Rhododendron pulchrum* Sweet., Anthocyanins, Stability, Antioxidant activity

1 Introduction

With the rapid development of society and the continuous improvement of people's living standards, people's awareness of environmental protection, safety and health is increasing, and the requirements for food additives are getting higher and higher. At the same time, a large number of research reports indicate that almost all synthetic pigments do not provide nutrients to the human body, even with varying degrees of toxicity, and some even cause cancer, endangering human health[1-2]. In the near future, new products with natural pigments will inevitably exceed those with synthetic pigments. Therefore, natural pigments with high safety and high nutritional value, especially plant anthocyanins are favored by people[3]. *Rhododendron pulchrum* Sweet. is a Rhododendron species with abundant resources. It is gorgeous in color and contains a lot of natural pigments, and is a good new resource for the development of anthocyanins, with broad development and application prospects[4-5]. In this paper, the stability and antioxidant power of anthocyanins from the flowers of *R. pulchrum* were studied systematically to provide a reliable reference for them as coloring additives in food, pharmaceutical and cosmetic fields.

2 Materials and methods

2.1 Materials and instruments The perennial *R. pulchrum* cultivated on campus was selected as the experimental material. During the full blooming period, the flowers were collected. They were dried at 55℃, smashed, passed through a 60-mesh sieve, and stored in ziplock bags for use. The reagents such as O-phenanthroline, ferrous sulfate, pyrogallol, potassium ferricyanide, 1, 1-diphenyl-2-trinitrophenylhydrazine, and potassium chloride were all analytically pure.

The used instruments and equipment included KQ-250B ultrasonic cleaning instrument, UV-2550 UV-visible spectrophotometer, TDL80-2B desktop centrifuge, BS124S electronic analytical balance, HH-Z2 thermal water bath, *etc.*

2.2 Experimental methods

2.2.1 Extraction of anthocyanins from flowers of *R. pulchrum*[5]. Several parts of the powder of purple flowers of *R. pulchrum*, about 0.1 g for each were weighed. Under the solid to liquid ratio of 120 mL/g, extractant of 80% acidified ethanol, extraction temperature of 40℃, and ultrasonic time of 50 min, the stock solution of anthocyanins was obtained after centrifuging for 15 min. It was diluted by 10 folds with 80% ethanol of pH 1 for determination.

2.2.2 Stability determination[6-8]. (i) Determination of the effect of different light source on the stability of anthocyanins from flowers of *R. pulchrum*. Certain volumes (12 mL for each) of the anthocyanin extract were placed in dark room, indoor natural light, outdoor sunlight, and 5-W UV light, respectively. The absorbance of the anthocyanin solution at 530 nm after 0, 1, 2, 3, 4, 5, 6, 7 and 8 h was determined in turn. Each determination was repeated three times.

(ii) Determination of the effect of temperature on the stability of anthocyanins from flowers of *R. pulchrum*. Certain volumes (12 mL for each) of the anthocyanin extract were placed in water bath at 25, 35, 45, 55, 65 and 75℃, respectively. The absorbance of the anthocyanin solution at 530 nm after 0, 1, 2, 3, 4 and 5 h was determined in turn.

(iii) Determination of the effect of pH on the stability of anthocyanins from flowers of *R. pulchrum*. Certain volumes (12 mL

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* Corresponding author. E-mail: zhangmei11@163.com

for each) of the anthocyanin extract were taken, and their pH values were adjusted to 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10, respectively. After 30 min, the absorbance of the anthocyanin solution was determined, and the color change of the solution was observed.

(iv) Determination of the effects of oxidant and reducing agents on the stability of anthocyanins from flowers of *R. pulchrum*. Certain volumes (12 mL for each) of the anthocyanin extract were placed in tubes, which were added with 4-mL 1% reducing agents Na_2SO_3 and Vc and oxidant H_2O_2, respectively. After 30 min, the absorbance of the anthocyanin solution was determined.

(v) Determination of the effects of metal ions on the stability of anthocyanins from flowers of *R. pulchrum*. Certain volumes (12 mL for each) of the anthocyanin extract were placed in tubes, which were added with 4-mL 0.5 mol/L metal ions, respectively. After 30 min, the absorbance of the anthocyanin solution was determined.

(vi) Determination of the effect of food additive on the stability of anthocyanins from flowers of *R. pulchrum*. Certain volumes (12 mL for each) of the anthocyanin extract were placed in tubes, which were added with 4-mL 1% food additives, respectively. After 30 min, the absorbance of the anthocyanin solution was determined.

2.2.3 Determination of antioxidant activity[9-11]. (i) Determination of the ability to scavenge hydroxyl radicals (· OH). A certain volume (2.0 mL) of phosphate buffer (pH 7.4) was mixed with 3 mL of phenanthroline solution (2.5 mmol/L), followed by 0.5 mL of $FeSO_4$ solution (15 mmol/L). Then, the mixture was diluted to 10 mL with distilled water. After incubating at 37℃ for 1 h, the solution was added with certain volumes (1 mL for each) of different concentrations of the anthocyanin extract, respectively. After adding 1 mL of H_2O_2 (1.0 mol/L), the absorbance of the new solution was determined at 510 nm. The calculation formula for the ability to scavenge hydroxyl radicals (· OH) was as follows:

Scavenging rate of · OH (%) $= (A_2 - A_1) \times 100\% / (A_0 - A_1)$

Where, A_0 represents the absorbance without H_2O_2 and the extract; A_1 represents the absorbance with H_2O_2 but without the extract; and A_2 represents the absorbance with H_2O_2 and the extract.

(ii) Determination of the ability to scavenge superoxide anion radicals (O^{2-} ·). A certain volume (4.5 mL) of Tris-HCI buffer solution was preheated in a water bath (20℃) for 20 min, and added with 0.4 mL of pyrogallol solution (25 mmol/L). Then, the solution was added with certain volumes (1 mL for each) of different concentrations of the anthocyanin extract, respectively. After heated in water bath (25℃) for 5 min, the solution was added with 1 mL of HCl solution (8 mol/L) to terminate the reaction. The absorbance A_1 was measured at 299 nm. Distilled water was used as blank control, and its absorbance A_0 was determined. The calculation formula for the ability to remove superoxide anion radicals (O^{2-} ·) was as follows:

Scavenging rate of O^{2-} · (%) $= (A_0 - A_1) \times 100\% / A_0$

(iii) Determination of ability to scavenge DPPH · . Certain volumes (2 mL for each) of different concentrations of the anthocyanin extract were placed in tubes, which were added with 2 mL of DPPH · solution (2×10^{-4} mol/L), respectively. The solutions were placed in dark for 30 min, and then their absorbance at 517 nm was determined. The calculation formula for the ability to scavenge DPPH · was as follows:

Scavenging rate of DPPH · (%) $= [A_0 - (A_1 - A_2)] \times 100\% / A_0$.

Where, A_1 represents the absorbance of the mixture of DPPH · solution (2 mL) and the test solution (2 mL); A_2 represents the absorbance of the mixture of absolute ethanol (2 mL) and the test solution (2 mL); and A_0 represents the absorbance of the mixture of absolute ethanol (2 mL) and the test solution (2 mL).

(iv) Determination of reducing ability. A certain volume (2.5 mL) of phosphate buffer (pH 6.6) was added with 2.5 mL of the test solution of different concentrations, respectively. The mixture was then added with 2.5 mL of 1% potassium ferricyanide solution, incubated at 50℃ for 20 min, and added with 2.5 mL of 10% trichloroacetic acid solution in success. A certain volume (5 mL) of the new solution was added with 5 mL of distilled water and 1 mL of 0.1% $FeCl_3$ solution. Finally, the absorbance of the solution at 700 nm was determined. The greater the absorbance was, the stronger the reducing power was.

3 Results and analysis

3.1 Stability analysis

3.1.1 Effect of light on the stability of anthocyanins from flowers of *R. pulchrum*. As shown in Fig. 1, after the anthocyanin extract was preserved in the dark, indoor natural light and 5-W UV light for 8 h, the change of absorbance value was not obvious, indicating that the stability of the anthocyanins was good. However, after outdoor solar radiation, the absorbance decreased rapidly, indicating poor stability. It was indicated that the stability of the anthocyanins from flowers of *R. pulchrum* was easily affected by solar radiation. When preserving anthocyanin solution, long-time solar radiation should be avoided to prevent degradation of anthocyanins.

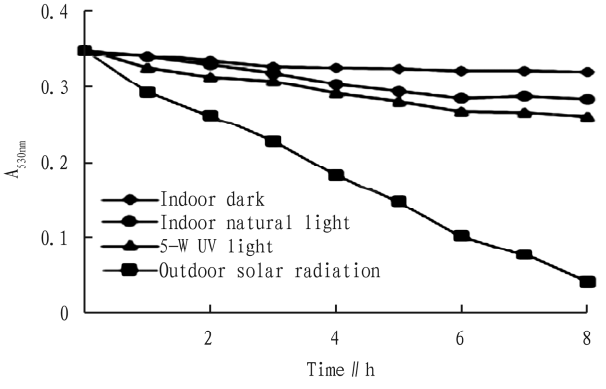

Fig. 1 Effect of light on the stability of the anthocyanins from flowers of *Rhododendron pulchrum* Sweet.

3.1.2 Effect of temperature on the stability of anthocyanins from flowers of *R. pulchrum*. As shown in Fig. 2, the absorbance of the

anthocyanins gradually decreased with increasing temperature. At 35℃ or less, the absorbance of the anthocyanins did not change significantly. After treatment at 35℃ for 5 h, it only decreased by 8.8%. When the temperature exceeded 55℃, the absorbance decreased rapidly. After treatment at 75℃ for 5 h, the absorbance decreased by 79.2%. It indicated that the anthocyanins were relatively stable at temperature below 35℃, and an increase in temperature accelerated the degradation of the anthocyanins.

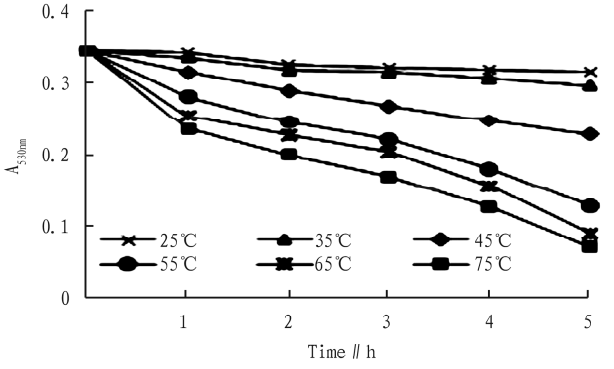

Fig. 2 Effect of temperature on the stability of the anthocyanins from flowers of *Rhododendron pulchrum* Sweet.

3.1.3 Effect of pH on the stability of anthocyanins from flowers of *R. pulchrum*. As shown in Fig. 3, the absorbance of the anthocyanin solution of different pH was different. With the increase of the pH, the absorbance of the anthocyanins decreased first and then increased. Under different pH, the anthocyanins exhibited different colors and states. At room temperature, under acidic conditions with pH changing greatly, the anthocyanin solution gradually changed from bright red to light red; and under alkaline conditions, the anthocyanin solution gradually changed from blue to green and yellow. This is an important property of anthocyanins. Under different pH, anthocyanins have different structural forms, and the difference in H^+ concentration in the medium causes different reversible changes in the structure, thus presenting different colors[12]. When pH ≤ 4, the anthocyanins were relatively stable; and when pH ≥ 5, the anthocyanin solution was unstable, with obvious color change. The experimental results showed that the anthocyanins from flowers of *R. pulchrum* were relatively stable under acidic conditions.

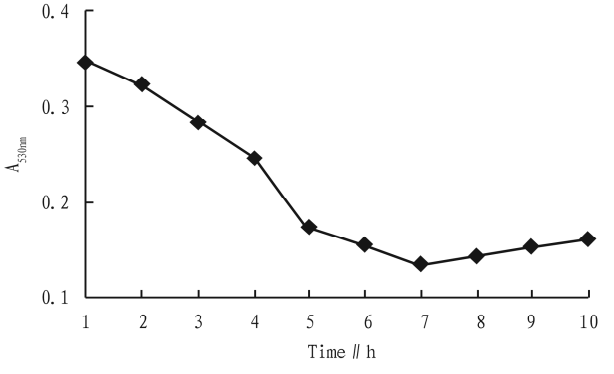

Fig. 3 Effect of pH on the stability of the anthocyanins from flowers of *Rhododendron pulchrum* Sweet.

3.1.4 Effects of oxidant and reducing agents on the stability of anthocyanins from flowers of *R. pulchrum*. As shown in Table 1, different redox agents in the test showed different degrees of damage to the stability of the anthocyanins. Among them, the most influential one was H_2O_2. The preservation rate was only 34.9%, and the color turned light yellow. Vc had the lest effect on the absorbance of the anthocyanins, and the color did not change much. When Na_2SO_3 was added, the color of the anthocyanins gradually faded to orange. It indicated that the anthocyanins from flowers of *R. pulchrum* had poor anti-oxidant and anti-reducing capacity. When using anthocyanins, they should be avoided from contacting with oxidizing or reducing substance. However, adding a small amount of Vc had little effect.

Table 1 Effects of oxidizer and reducers on the stability of the anthocyanins from flowers of *Rhododendron pulchrum* Sweet.

Redox agents	Absorbance	Solution color
Control	0.318	Red
H_2O_2	0.111	Pale yellow
Na_2SO_3	0.183	Orange
Vc	0.283	Red

3.1.5 Effects of metal ions on the stability of anthocyanins from flowers of *R. pulchrum*. As shown in Table 2, compared with the control group, the absorbance of the anthocyanins changed slightly after added with Na^+, K^+ or Ca^{2+} ions, suggesting that these ions had little effect on the stability of the anthocyanins and they even had a certain color protection effect. The metal ions such as Cu^{2+}, Zn^{2+}, Fe^{3+}, and Al^{3+} had adverse effects on the stability of the anthocyanins, leading to discoloration. They may even cause turbidity in the solution, which was probably because that the anthocyanins formed complexes with metal ions. Therefore, in practical applications, the anthocyanins should be avoided from contacting with Cu^{2+}, Zn^{2+} and Fe^{3+}.

Table 2 Effects of metal ions of the stability of the anthocyanins from flowers of *Rhododendron pulchrum* Sweet.

Metal ions	Absorbance	Solution color
Control	0.334	Red
Na^+	0.342	Red
K^+	0.332	Red
Ca^{2+}	0.361	Red
Cu^{2+}	0.482	Purplish blue turbidity
Zn^{2+}	0.448	Pale red turbidity
Fe^{3+}	0.875	Yellow brown turbidity
Al^{3+}	0.403	Orange

3.1.6 Effects of food additives on the stability of anthocyanins from flowers of *R. pulchrum*. As shown in Table 3, the food additives such as glucose, sodium benzoate, acetic acid and salt had little effect on the absorbance of the anthocyanin solution, and the color of the solution was close to the color of the anthocyanin stock solution. Starch had a certain effect on the absorbance of the anthocyanin solution, and it caused turbidity. Sodium glutamate had a great influence on the stability of the anthocyanins, and the color changed from red to pale yellow. Therefore, when the anthocya-

nins from flowers of *R. pulchrum* are applied in food, attention should be paid to the addition of food additives.

Table 3 Effects of food additives on the stability of the anthocyanins from flowers of *Rhododendron pulchrum* Sweet.

Food additives	Absorbance	Solution color
Control	0.351	Red
Glucose	0.331	Red
Sodium benzoate	0.366	Red
Starch	0.476	Pale red turbidity
Acetic acid	0.342	Red
Sodium glutamate	0.148	Pale yellow
Salt	0.338	Red

3.2 Antioxidant activity analysis

3.2.1 Ability of scavenge hydroxyl radicals (· OH). As shown in Fig. 4, the anthocyanins from flowers of *R. pulchrum* and Vc had better ability to scavenge · OH, and the scavenging capacity increased as the sample concentration increased. However, with the increase of concentration, the scavenging capacity of the anthocyanins for · OH became gradually better than that of Vc, and the difference reached a significant level. At the concentration of 0.5 mg/L, the scavenging capacity of the anthocyanins for · OH was 2.4 times that of Vc.

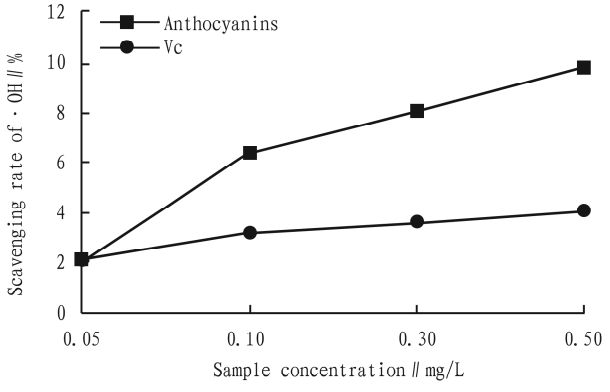

Fig. 4 Scavenging capacity for · OH

3.2.2 Ability to scavenge superoxide anion radicals (O^{2-} ·). As shown in Fig. 5, the anthocyanins and Vc both had significant ability to scavenge O^{2-} · . In the concentration range of 0.05 – 0.1 mg/L, the scavenging capacity of the anthocyanins increased most rapidly. When the concentration reached 0.3 mg/L, the scavenging rate of the anthocyanins for O^{2-} · reached 100%, but that of Vc was only 72.3%. It indicated that the scavenging capacity of the anthocyanins from flowers of *R. pulchrum* for O^{2-} · was slightly better than that of Vc.

3.2.3 Ability to scavenge DPPH · . As shown in Fig. 6, the anthocyanins and Vc both had strong scavenging capacity for DPPH · . However, with the increase in the concentration of the anthocyanin solution, the scavenging capacity for DPPH · increased correspondingly. At the concentration of 0.5 mg/L, the scavenging rate of DPPH · reached 98.8%. However, the scavenging capacity of Vc for DPPH · did not increase with the increased concentration.

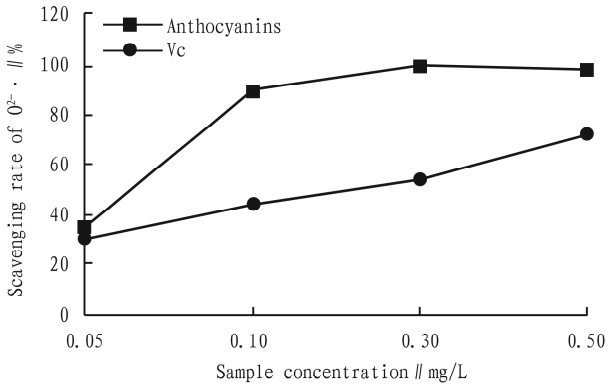

Fig. 5 Scavenging capacity for O^{2-} ·

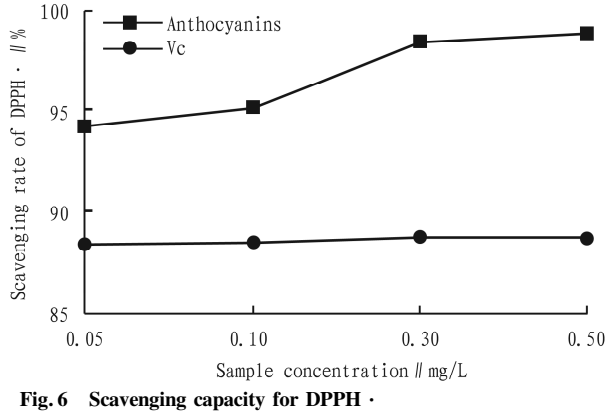

Fig. 6 Scavenging capacity for DPPH ·

3.2.4 Reducing ability. As shown in Fig. 7, the reducing power of the anthocyanins and Vc increased with the increase of concentration. When the concentration exceeded 0.1 mg/L, the reducing power rapidly increased with the increase of concentration. At the concentration of 0.18 mg/L, the reducing power of Vc began to exceed that of the anthocyanins. Various studies have shown that reducing power has a great correlation with antioxidant capacity. In practical applications, the anthocyanin concentration can be increased to increase its antioxidant capacity to the receptor.

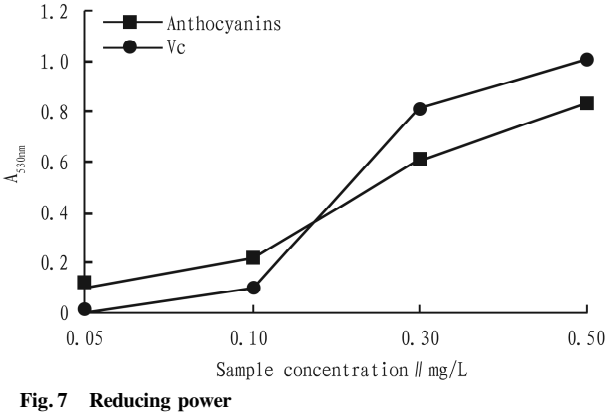

Fig. 7 Reducing power

4 Conclusions

In indoor natural light, the color and absorbance of the anthocyanins from flowers of *R. pulchrum* did not change significantly, with certain light stability. However, they should be avoided from solar radiation during storage and transportation. At temperature

below 45℃, the anthocyanins were relatively stable. However, when the temperature exceeded 55℃, the degradation of the anthocyanins was accelerated. The color of the anthocyanins was bright and stable under acidic conditions, and they can be used for acidic foods when coloring. The anthocyanins have poor antioxidant and reducing power, and should be avoided from contacting with oxidizing or reducing substances when using. The metal ions such as Na^+, K^+ and Ca^{2+} had little effect on the stability of the anthocyanins, but the metal ions such as Cu^{2+}, Zn^{2+}, Fe^{3+}, and Al^{3+} had adverse effects on the stability of the anthocyanins, causing discoloration and turbidity. Glucose, sodium benzoate, acetic acid and salt had little effect on the absorbance of the anthocyanins, but starch and sodium glutamate had a great influence on the stability of the anthocyanins. The scavenging capacity of the anthocyanins from flowers of *R. pulchrum* for ·OH, O^{2-}· and DPPH· was better than that of Vc. The reducing power of the anthocyanins was equivalent to that of Vc, and they both increased with the increase of concentration. It indicates that the anthocyanins from flowers of *R. pulchrum* have a good antioxidant effect.

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the economic benefits of the olive grove. This study found that Koroneiki and Arbequina have early fruiting and high yield, so they can be vigorously promoted in the olive-producing areas.

In this study, the fruit traits such as single fruit weight and flesh ratio, moisture content of fresh fruit, and oil content of fresh fruit were compared preliminarily among different olive cultivars. In order to more comprehensively reflect the differences in the traits of different cultivars, experimental research on the quality of oil of olive pulp and the active ingredients of single-varietal olive oil needs to be carried out. Hu Qiong *et al.*[10] concluded that fruit shape index, total soluble sugar, vitamin C and mineral element content are important indicators for evaluating the quality of fresh jujube fruit, which is very referenceable for future research. This is also the direction of next research.

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