Analysis and Characterisation of Anthocyanins in Mulberry Fruit

Chuanguang QIN, Yang LI, Weining NIU, Yan DING, Ruijie ZHANG and Xiaoya SHANG

Faculty of Life Science, Northwestern Polytechnical University, Shaanxi, P. R. China

Abstract

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The fruit of mulberry (*Morus alba* L., Moraceae) has been used as medicinal food in China for a long history. The pigment from the fruit extract is a kind of natural colourant for food processing and has potential medical and commercial values. This study focuses on the analysis and characterisation of anthocyanins from mulberry pigment. The fresh mulberry fruits were extracted with the solvent of 95% alcohol/0.1% HC l (1:1, ratio) at room temperature for 4 h in the dark. After the isolation using C-18 column, the pigment was identified with UV-Visible Spectroscopy, HPLC-PAD, LC-MS, and ^{1}HNMR. The results showed that the abundant anthocyanins in mulberry pigment are cyanidin 3-*O*-rutinoside (60%) and cyanidin 3-*O*-glucoside (38%). The minor anthocyanins (totally 2%) are pelargonidin 3-*O*-glucoside and pelargonidin 3-*O*-rutinoside.

Keywords: anthocyanin; mulberry fruit; spectroscopy characteristics; stability

Anthocyanins are glycosylated polyhydroxy and polymethoxy derivates of 2-phenylbenzopyrylium (flavylium) salt, which are natural pigments widely distributed in nature, accounting for the colours of innumerable edible fruits (Qin 1997; Tian *et al*. 2001; Lee 2002; Moyer *et al*. 2002; Kuskoski *et al*. 2003; Andersen *et al*. 2004; Longo *et al*. 2005; Longo & Vasapollo 2005, 2006; Dugo *et al*. 2006; McGhie *et al*. 2006), vegetables, flowers and other plants (Qin *et al*. 1997; Phippen & Simon 1998; Slimestad & Solheim 2002; Abdel-Aal *et al*. 2006; Mori *et al*. 2006). They differ from other natural flavonoids by the range of colours that can be derived from them and by their ability to form resonance structures through pH variation (Konga *et al*. 2003; Stintzing & Carle 2004; Torskangerpoll & Andersen 2005). Anthocyanins have been extracted from various plant sources and residual materials, notably from grape skins, to produce authorised food colourants, nutraceuticals, and drugs (Galvanoa *et al*. 2004; Jayaprakasam *et al*. 2005). The average intake of anthocyanins has been estimated at up to 180–215 mg/day, which is higher than that for other flavonoids such as flavonols. During the last two decades, there has been an increasing interest in phenolic phytochemicals due to their protection against cardiovascular diseases and certain

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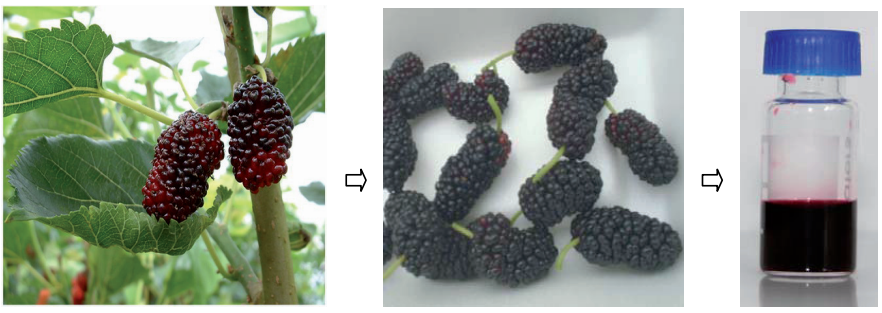
forms of cancer (Katsube *et al*. 2003; Cooke *et al*. 2005; Chen *et al*. 2006). Like other polyphenols, anthocyanins are potent antioxidants (Stintzing *et al*. 2002; Kahkonen & Heinonen 2003; Awika *et al*. 2004; Tsai *et al*. 2005). This function is related to their antiatherosclerotic, anticarcinogenic, and anti-inflammatory properties (McDougall *et al*. 2005; Mattivi *et al*. 2006).

Mulberry fruit changes the colour from green to black purple through red with maturity. Some varieties introduced from mid Asia have white fruits. Mulberry has been used as medicine since ancient times. Mulberry fruit contains anthocyanins (Yang & Tsai 1994), sugars, organic acids, free amino acids, vitamins, micronutrients, and other components. Mulberry fruit is consumed fresh and as mulberry juice. Canned fruit jam and mulberry wines have been developed. Mulberry fruit has also been used as important medicinal materials in China (Nomura 1999; Liu *et al*. 2001). Recently, comprehensive studies on nutrition components and cultivation technology of mulberry fruit have been conducted (Alizai *et al*. 1996; Maskan & Gogus 1998; Asano *et al*. 2001; Elmaci & Altug 2002). Some researches now focus on the bioactive components of mulberry. The absorption and purification of mulberry red pigment have been done with resin (Dugo *et al*. 2001). It has been found that mulberry fruit extract has an anti-oxidative property (Tsai *et al*. 2005) and antiradical capacity (Suh *et al*. 2004). On the other hand, the safety of synthetic colourants was questioned in the past years. The interest in natural pigments has significantly increased as a consequence of both the legislative action and consumer awareness of the use of safe additives in food industry. Mulberry fruit is a kind of natural pigment resource. However, the fruits are not utilised efficiently, because most of mulberry fruits are abandoned when mulberry tree was used to collect leaves for silkworm food. Therefore, it is necessary to strengthen the research into the mulberry fruit pigment and to promote the industrialisation of mulberry fruit products. This study focuses on the composition and analysis of anthocyanins in the fruit of mulberry (*Morus alba* L., Moraceae).

MATERIALS AND METHODS

Chemicals. HPLC grade solvents, such as methanol, acetonitrile, propan-2-ol, chloroform et al., were purchased from Fisher Scientific (Morris Plains, USA). Trifluoroacetic acid (TFA) and tetrahydrofuran (THF) of HPLC grade were purchased from Tedia Co. Inc. (Fairfield, USA). The other chemicals of analytical grade quality, such as ethanol, methanol, glycine, citric acid, Na_2HPO_4, sodium hydroxide, and concentrated hydrochloric acid (HCl), were purchased from Shanghai Chemical Reagent Co. (Shangai, CHina). Distilled water was used throughout the procedure. Buffer solutions: the solvents used for the buffer solutions were (A) 0.2M HCl, (B) 0.2M glycine, (C) 0.1M citric acid, (D) 0.2M Na_2HPO_4, (E) 0.2M NaOH. The following solvent proportions were used for pH 1.0: A-H_2O, 50.0:50.0; pH 3.0: A-B-H_2O, 25.0:5.7:69.3; pH 5.0: C-D-H_2O, 9.7:10.3:80.0; pH 7.0: C-D-H_2O, 3.5:16.5:80.0; pH 9.0: B-E-H_2O, 25.6:4.4:70; pH 11.0: B-E-H_2O, 25.0:23.0:52.0. The accurate pH values were measured with a Sartorius PH-10 pH-meter equipped with a Sartorius PY-P10 pH electrode (Sartorius AG, Goettingen, Germany).

Sample preparation. Ripe fruits of mulberry (*Morus alba* L., Moraceae) (Figure 1) were sampled



(a) (b) (c)

Figure 1. Images of the ripe mulberry fruits: (a) The fruits of mulberry (*Morus alba* L., Moraceae) in the tree; (b) Collected fruits of mulberry (*Morus alba* L., Moraceae), (c) The pigment extracted from fruits of mulberry (*Morus alba* L., Moraceae)

in Zhouzhi county, Shaanxi Province, China. After sampling, the fruits were immediately transported to laboratory, put in plastic bags, and stored below 4°C in a refrigerator. Their size was about 1–2 cm in the length and their colour was purplish black. After the stems were taken away, mulberry fruits were first washed carefully under running tap water, dried, and mashed with a DS21 tissue triturator (Shanghai, China). Then, the homogenised fruit sample was weighed and immediately transferred into a beaker for further processing.

Pigment extraction. Pretreated and well homogenised sample of 100 g was weighed in a 1-liter beaker and 500 ml of the extraction solvent was added immediately. The extraction solvents were added in the ratio of 1:5 (w/v). The samples were stirred with a glass rod every 5 min to ensure a well mixed extraction. After the extraction in the dark at room temperature for 4 h, the extract was centrifuged (5000 r/min) at 4°C for 20 min in a Optima L-100XP (Beckman Co., Windermere, USA). Then the supernatant was separated and filtered. This pigment solution was used for further purification and analysis.

Anthocyanin purification. A C18 Sep-Pak cartridge (Waters Associates, Milford, USA) was activated for 30 min with distilled water and HPLC grade methanol, respectively. The pigment solution was loaded onto the column. After successive washing with 5-fold volume of distilled water (acidified with 0.1% HCl) and ethyl acetate, anthocyanins were eluted with methanol containing 0.1% HCl. The anthocyanin solution was collected and condensed at 40°C using a N-1000V-W rotary evaporator (Eyela Co., Tokyo, Japan) under vacuum. For the spectroscopy and element analysis, a part of the condensed anthocyanin solution was dried for spectroscopy analysis with a CVE-2000 centrifugal evaporator under vacuum (Eyela Co., Tokyo, Japan).

Acid hydrolysis of anthocyanins. First, 5 ml of 2N HCl was added to 1 ml of the purified anthocyanin solution (3 mg/ml) in a screw-cap test tube, flushed with nitrogen, and capped. The pigments were hydrolysed at 100°C for 1.5 h; then, the solution was immediately cooled in an ice bath (Longo & Vasapollo 2005). The hydrolysate was purified using a 500 mg sorbent weight C-18 Sep-Pak cartridge (Waters) as previously described.

HPLC-PAD analysis and preparation. High-performance liquid chromatography (HPLC) was performed on a Waters RP C18 column (250 × 4.6 mm i.d., 5 µm C18, Waters Assoc., Milford, USA) using a Waters 2696 separation module equipped with a 996 photodiode array detector. A gradient mobile phase was used for elution: A – water containing 0.1% TFA (trifluoroacetic acid); B – acetonitrile containing 0.1% TFA. The elution profile was 0–60 min: 0 min, 10% B; 0–2 min, 10% B; 2–35 min, 10–90% B; 35–40 min, 90–100% B; 40–60 min, 100% B. The flow rate was 1.0 ml per minute.

Spectroscopy characteristics. UV-Visible absorption spectra of anthocyanins were recorded from 200–800 nm in steps of 2 nm using a U-3310 double-beam scanning UV-Visible spectrophotometer (Hitachi High-technologies Co., Tokyo, Japan) and an on-line photodiode array detector (Waters Assoc., Milford, USA). The mass spectra were obtained using a Micromass triple quadrupole ion-tunnel mass spectrometer equipped with a Zspray ESI source (Micromass UK Ltd., Manchester, UK). Approximately 100 µl of the eluate from HPLC (conditions were similar as with HPLC analysis above) was delivered to the ESI source by a micro splitter valve for ESI/MS and MS/MS analysis. Standard cyanidin 3-rutinoside was used to tune the instrument. For the precursor ion scan, the instrument was tuned to maximum abundance of the daughter ion (m/z 287) and molecular cation (m/z 595) signals (approximately in equal abundance). For the product ion scan, the precursor ion (m/z 595) was attenuated to 50%. The quadruple instrument was operated at the following settings: capillary voltage 3.0 kV; cone voltage 35 V; RF lense 1 50 V; desolvation gas temperature 500°C at a flow of 17 l/min; source temperature 105°C; collision gas (argon) pressure, 7 psi; collision energy was set at 18 eV. The relative amount of each anthocyanin was reported as the mean of three replicates. The detection limits (S/N > 3) of approximately 1 femtomol were obtained during LC/MS/MS analysis.

^{1}HNMR spectral data were recorded on a Bruker Advance 400 instrument operating at 400 MHz in CD_3OD/CF_3OD (9:1) with TMS (tetramethylsilane) as internal standard. The sample temperature was stabilised at 25°C.

Stability tests. The dry anthocyanin sample was weighed and dissolved in buffer (pH = 3) as stock solution (0.1 mg/ml). For the stability tests, the effects of the concentration, pH-value, temperature, and light were studied with a U-3310 double-beam scanning UV-Visible Spectrophotometer (Hitachi

High-technologies Co., Tokyo, Japan). 6 × 10 µl of anthocyanin stock solution was diluted 10 times with different buffers (pH = 1, 3, 5, 7, 9, 11) for pH stability. 1 ml of anthocyanin stock solution was diluted at different times with buffer (pH = 3) and UV-Visible spectra were measured. The diluted anthocyanin solution (50 µg/m, pH = 3) was divided and stored in small glass bottles for the test. Some samples were stored at 40°C to 100°C in water bath for testing the temperature effect. Other samples were exposed to the direct sunlight at room temperature for 28 days for testing the light effect.

Loss rate = Absorbance value of the pigment solution after the treatment/Absorbance value of the pigment solution before the treatment × 100% = (Absorbance value of the pigment solution before the treatment – Absorbance value of the pigment solution after the treatment)/Absorbance value of the pigment solution before the treatment × 100%

RESULTS AND DISCUSSION

HPLC-PAD analysis of anthocyanin from mulberry fruit pigment

The aqueous concentrate of the acidified ethanolic extract of mulberry was purified using C18 Sep-Pak cartridge column chromatography. The anthocyanins in the purified extract were separated and analysed on a Waters RP C18 column using HPLC with a Waters 2696 separation module equipped with a 996 photodiode array detector. The HPLC-PAD chromatogram at 520 nm (Figure 2a) shows that the mulberry fruit pigment consists of four different anthocyanins and abounds in two kinds of them, at the retention times of 9.6 min (peak 1, 38%) and 9.9 min (peak 2 60%), respectively. Peak 3 at a retention time of 10.4 min makes only 0.5% and peak 4 at a retention time of 10.7 min makes only 1.5%. After acid hydrolysis, the HPLC-PAD profile indicated that all the four anthocyanins produce only two different aglycones, peak 5 and peak 6 at retention times of 12.5 min and 13.9 min, respectively (Figure 2b). The UV-Visible absorptions of peaks 1, 2, 3, 4, 5, and 6, recorded with a 996 photodiode array detector, are listed in Figure 3. Compared with the standard anthocyanins (Figure 3e, h) from the reference (Longo & Vasapollo 2006), the

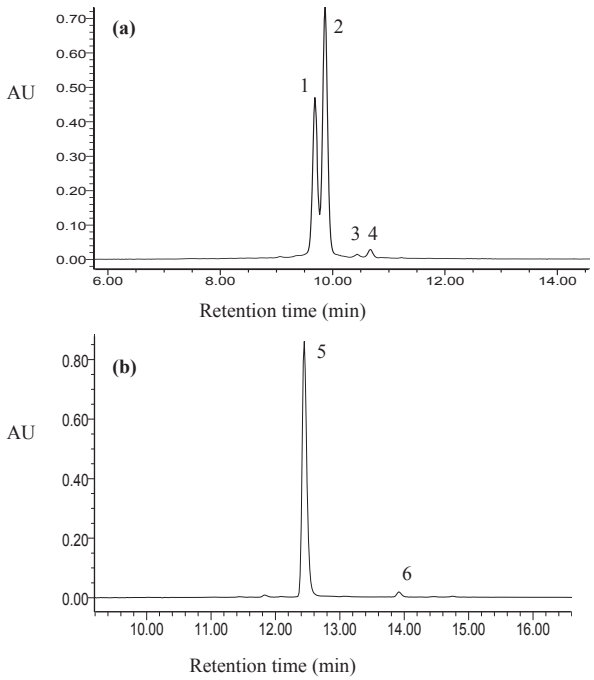


Figure 2. HPLC-PAD chromatogram files recorded at 520 nm corresponding to: (a) the purified extract of mulberry fruit; (b) the purified extract of mulberry fruit after acid hydrolysis

UV-Visible absorption characteristics of primary anthocyanins (1 and 2) from mulberry fruit pigment and their aglycone 5 from acid hydrolysis correspond to those of cyanidin 3-*O*-rutinoside, and those of the minor anthocyanins (3 and 4) and their aglycone 6 from acid hydrolysis correspond to those of pelargonidin 3-*O*-rutinoside. The result indicated that anthocyanins from mulberry fruit pigment probably contain two kinds of aglycone structure unit, cyanidin and pelargonidin.

Spectroscopy characteristics of anthocyanins from mulberry fruit pigment

The data of the mass spectra from HPLC-PAD-MS indicated that the molecular structure of anthocyanins from mulberry fruit pigment matched with cyanidin 3-*O*-rutinoside, cyanidin 3-*O*-glucoside, pelargonidin 3-*O*-glucoside, and pelargonidin 3-*O*-rutinoside (structures see Figure. 4). Peak 1 was identified as cyanidin 3-*O*-glucoside (1): MS *m*/*z* 449.17 for $M^+ = C_{21}H_{21}O^+_{11}$ (cacul. 449.1) 287.16 for M^+-glucosyl = $C_{15}H_{11}O^+_6$ (cacul. 287.1); Peak 2 was identified as cyanidin 3-*O*-rutinoside (2): MS *m*/*z* 595.23 for $M^+ = C_{27}H_{31}OO^+_{15}$ (cacul.

595.1), 449.20 for M^+-deoxyglucosyl = $C_{21}H_{21}O^+_{11}$ (cacul. 449.1), 287.18 for M^+-rutinosyl = $C_{15}H_{11}O^+_6$ (cacul. 287.1); Peak 3 was identified as pelargonidin 3-*O*-glucoside (3): 433.18 for $M^+ = C_{21}H_{21}O^+_{10}$ (cacul. 433.1), 271.33 for M^+-glucosyl = $C_{15}H_{11}O^+_5$ (cacul. 271.1). Peak 4 was identified as pelargonidin 3-*O*-rutinoside (4): MS *m*/*z* 579.25 for $M^+ = C_{27}H_{30}O^+_{14}$ (cacul. 579.1), 433.20 for M^+-deoxyglucosyl = $C_{21}H_{21}O^+_{10}$ 271.18 for M^+-rutinosyl = $C_{15}H_{11}O^+_5$ (cacul. 271.1). After acid hydrolysis, two kinds of aglycones (5 and 6) were found. Peak 5 was identified as cyanidin (5). MS *m*/*z* 287.20 for $C_{15}H_{16}O^+_6$ (cacul. 287.1); Peak 6 was identified as pelargonidin (6): MS *m*/*z* 271.15 for $C_{15}H_{11}O^+_5$ (cacul. 271.1).

Among the four anthocyanins detected in mulberry fruit pigment, the two major anthocyanins and their aglycone from acid hydrolysis (compounds 1, 2 and 5, Figure 2) were isolated by preparative HPLC and their identity and structures were confirmed on the basis of ^{1}HNMR spectroscopic data. The chemical shifts (δ) obtained from the ^{1}HNMR analysis of anthocyanins 1, 2 and 5 were reported as the following: ^{1}H NMR spectroscopic data of compound 1 [δ in CD_3OD/CF_3OD (9:1)]: H-4 (9.05, s), H-6 (6.68, d, J = 2.2), H-8 (6.89, brs), H-2' (7.95, d, J = 2.4), H-5' (7.03, d, J = 8.8), H-6' (8.15, dd, J = 2.3, 8.5), H-1" (5.32, d, J = 7.5), H-2", H-3", H-4", H-5" (3.20-3.55, m); ^{1}H NMR spectroscopic data of compound 2 [δ in CD_3OD/CF_3OD (9:1)]: H-4 (8.90, s), H-6 (6.70, d, J = 2.0), H-8 (6.87, brs), H-2' (7.92, d, J = 2.4), H-5' (7.05, d, J = 8.8), H-6' (8.15, dd, J = 2.3, 8.5), H-1" (5.30, d, J = 7.4), H-1"' (4.72, brs), H-2", H-3", H-4", H-5", H-2"', H-3"', H-4"', H-5"' (3.10-3.85, m), H-6' (2.85, dd, J = 2.4, 8.6); ^{1}H NMR spectroscopic data of compound 5 [δ in CD_3OD/CF_3OD (9:1)]: H-4 (8.98, s), H-6 (6.68, d, J = 2.0), H-8 (6.87, brs), H-2' (7.90, d, J = 2.3), H-5' (7.05, d, J = 8.6), H-6' (8.23, dd, J = 2.4, 8.7). The signals in the downfield of the spectra between δ 6.68 and 9.05 ppm were clearly attributable to the aromatic protons (A and B rings) of the aglycone molecule as previously reported (Longo & Vasapollo 2005) for these compounds. The signal doublets at around δ 5.32 corresponded to the protons on the anomeric carbon from the glucose residues, confirming that they were in position C-3 as also indicated by the $Abs_{440}/Abs_{\lambda_{max}}$ ratio values ranging from 29% to 32%. The β-configuration of this moiety was confirmed from the magnitude (J = 7.6 Hz) of the $J_{1'''2'''}$ coupling constant in the ^{1}HNMR spectra (Longo & Vasapollo 2005). The spectrum of compound 2 presented a doublet at δ 5.30 (d, J = 7.4 Hz, H-1 glucose) and a broad singlet at δ 4.65 (brs, H-1 rhamnose) confirming the presence of rutinose as the sugar moiety (Longo & Vasapollo 2005). It was not possible by means of NMR analysis to confirm the identity of anthocyanins 3, 4 and their aglycone 6 obtained by acid hydrolysis (Figure 2), which were characterised as glucoside and rutinoside derivatives of pelargonidin, respectively, by means of HPLC-PAD-MS analysis; their low concentrations in the mulberry fruit extract did not enable us to isolate preparatively these minor pigments for their NMR characterisation.

UV-Visible absorption spectra of anthocyanin from mulberry fruit pigment

The UV-Visible absorption spectra of anthocyanins from mulberry fruit pigment (pH = 3) at different concentrations ranging from 16 μg/ml to 128 μg/ml were recorded between 200 and 800 nm with a U-3310 double-beam scanning UV-Visible spectrophotometer (Figure 5a). The result indicated that the solution (pH = 3) of anthocyanins from mulberry fruit pigment has two absorption peaks (0.1% HCl-MeOH): one is λ_{max} = 280 nm in UV range and another is λ_{max} = 520 nm in visible range. Compared with the standard anthocyanins (Figure 3d) from the reference (Longo & Vasapollo 2006), the UV-Visible absorption characteristic of anthocyanins from mulberry fruit pigment correspond to that of cyanidin 3-*O*-rutinoside (structure see Figure 4). The result confirmed again that anthocyanins from mulberry fruit pigment probably contain the same structural unit as cyanidin.

Effect of pH-value on stability of anthocyanins from mulberry fruit pigment

For 10 μg/ml solutions of anthocyanins from mulberry fruit pigment, the UV-Visible absorption spectra at six different pH-values were measured on a U-3310 UV–Visible Spectrophotometer (Figure 5b). As blanks, the respective buffer solutions were used. The UV/Visible spectra indicated that the stability of anthocyanins from mulberry fruit pigment was strongly influenced by pH-value. The anthocyanins from mulberry fruit pigment were

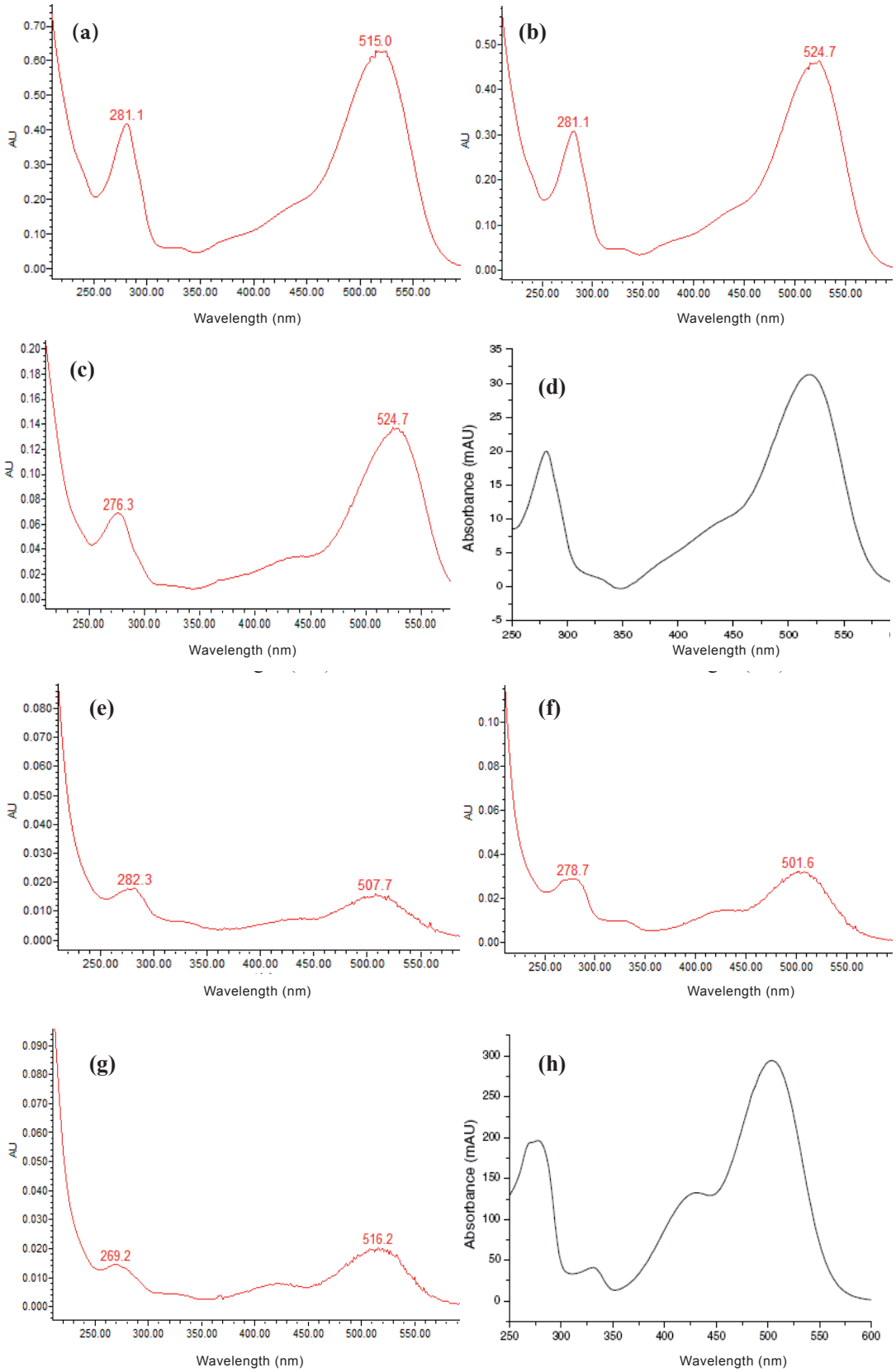


Figure 3. UV-Visible spectra recorded with a photodiode array detector: (a) peak 1; (b) peak 2; (c) peak 5; (d) standard cyanidin 3-*O*-rutinoside; (e) peak 3; (f) peak 4; (g) peak 6; (h) standard pelargonidin 3-*O*-rutinoside

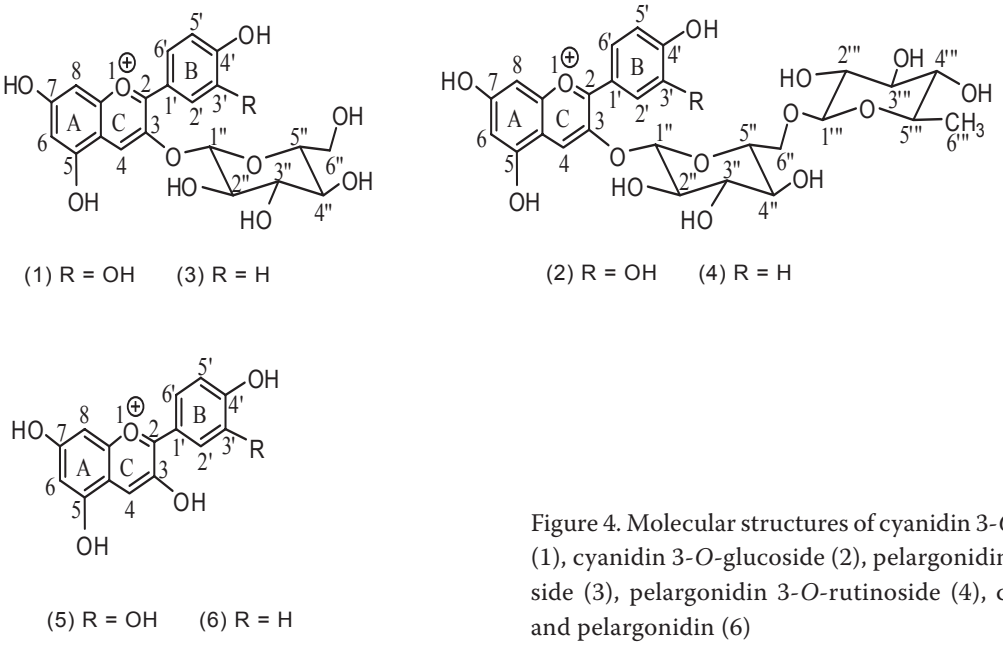


Figure 4. Molecular structures of cyanidin 3-*O*-rutinoside (1), cyanidin 3-*O*-glucoside (2), pelargonidin 3-*O*-glucoside (3), pelargonidin 3-*O*-rutinoside (4), cyanidin (5), and pelargonidin (6)

stabilised in acidic solution (pH < 5, from red to purple-black), but unstable in neutral or alkali solution (pH > 5, from colourless to blue). This is a typical property of anthocyanin compounds. The most common way to indicate anthocyanin colours is based on the presentation of visible λmax-values from UV/Visible absorption spectra. Since anthocyanins in rather strong acid solutions occur only as flavylium forms, the λ_{max}-values may be reasonably representative of colour at these pH values. However, when pH increases, each anthocyanin occurs as a mixture of various equilibrium forms (Figure 6) in proportions which are unknown for practically all anthocyanins. At these pH-values, the corresponding single λ_{max}-values may, to a lesser degree, be representative of the solution colour.

Effect of temperature and light on the stability of anthocyanins from mulberry fruit pigment

Four anthocyanin stock solutions (50 μg/ml) at pH = 3 were kept at different temperatures from 20°C to 100°C, respectively. The samples were taken periodically in every 15 min up to 100 min and their absorbance was determined at 520 nm.

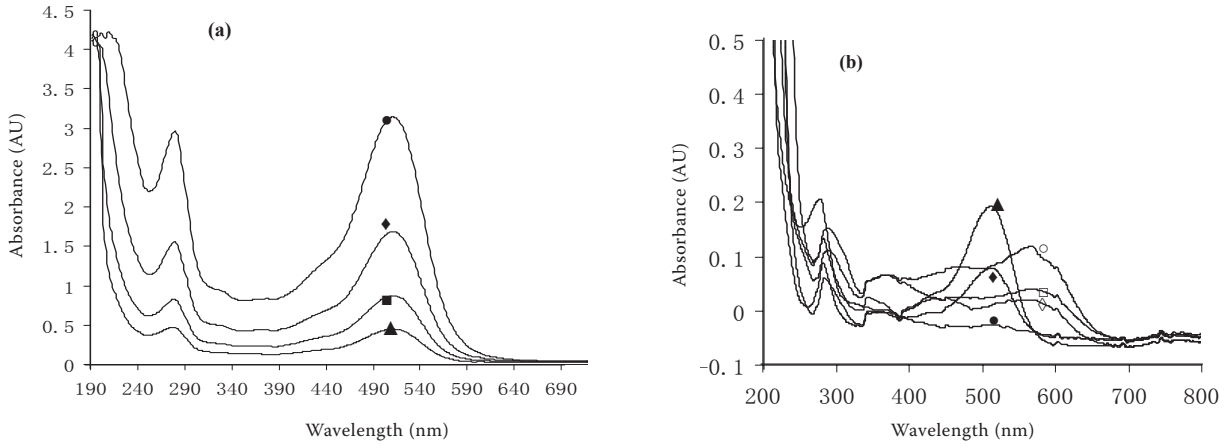
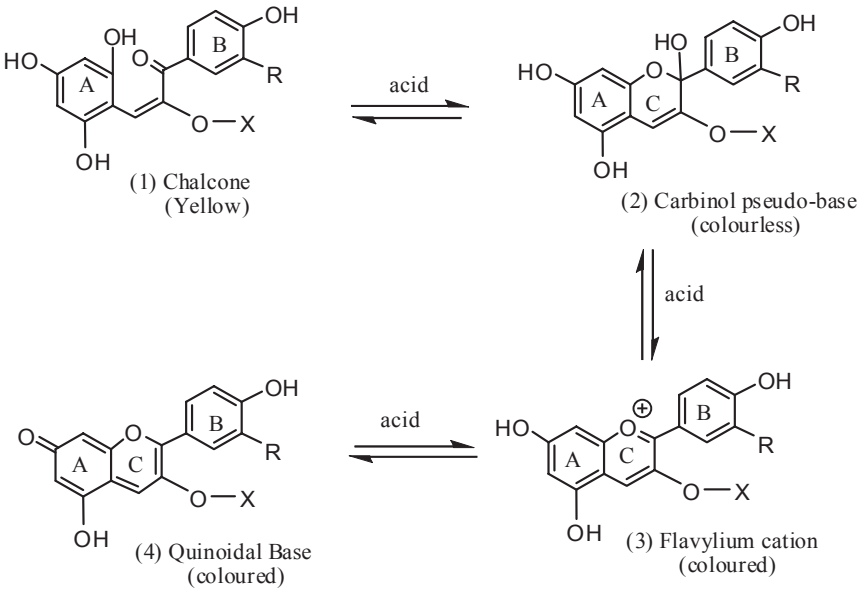


Figure 5. UV-Visible spectrum of total anthocyanins from mulberry fruit pigment. (a) With HCl-glycine-H_2O buffer, pH = 3, at different concentrations ranged from 16 μg/ml to 128 μg/ml : 16 μg/ml (▲), 32 μg/ml (■), 64 μg/ml (◆) 128 μg/ml (●); (b) 10 μg/ml of pigment at different pH-values: pH = 1 (▲), pH = 3 (◆), pH = 5 (●), pH = 7 (◊), pH = 9 (○), pH = 11 (□)



R = OH or H X = H, glucosyl or Rutinosyl

Figure 6. pH-dependent conformational rearrangement of the anthocyan molecule, shown here for anthocyanins bearing a sugar on C3. Which conformation predominates depends upon pH. At neutral pH, anthocyans occur as chalcones with an open C ring (1). Under mild acidic conditions, the ring is closed to form a carbinol pseudo base (2). In strong acid (pH 2), ring C acquires aromaticity involving a flavylium cation, which imparts intense colour on the molecule (3). In alkali, oxidation of ring A generates a quinoid structure with the elimination of the positive charge, this species is also coloured (4). The ring-opened chalcone can be reformed at neutral pH

It is obvious from Figure 7 that the absorbance decreased with time while the loss rate of the pigment colour, or percentage los, gradually increased. Furthermore, the rate of the colour loss of the pigment was most obvious at higher temperatures. When the pigment was kept at 100°C for 1.5 h, the loss rate reached 46.5%. The colour loss at 100°C was obviously higher than that at 80°C. The colour loss of the mulberry pigment solution was relatively stable at lower temperatures. On the other hand, the light had no significant effect on the stability of the mulberry pigment under the conditions tested.

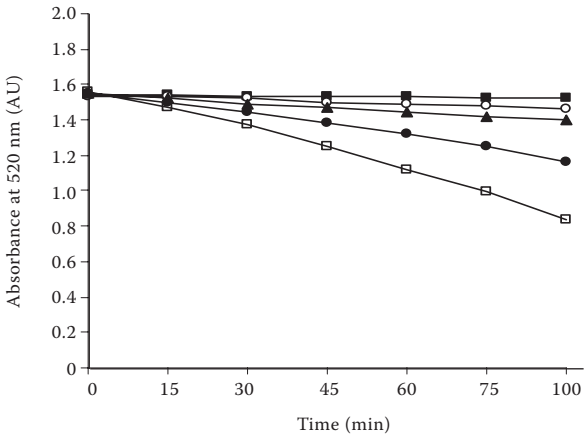


Figure 7. Changes in absorbance (HCl-glycine-H_2O buffer, pH = 3, 520 nm) at different temperatures for different times: 20°C (■), 40°C (○), 60°C (▲), 80°C (●), and 100°C (□)

CONCLUSION

The pigment in fruits of mulberry (*Morus alba* L., Moraceae) is a kind of natural pigment for food processing and has potential medical and commercial values. This study focused on the analysis and characterisation of anthocyanins from mulberry pigment. Fresh mulberry fruits were extracted with the solvent of 95% alcohol/0.1% HC l (1:1, ratio) at room temperature for 4 h in the dark. After the isolation with C-18 column, the pigment was identified by means of UV-Visible spectroscopy, HPLC-PAD, LC-MS, and ^{1}HNMR. The results showed that the abundant anthocyanins in mulberry pigment are cyanidin 3-*O*-rutinoside (60%) and cyanidin 3-*O*-glucoside (38%). The minor anthocyanins (totally 2%) are pelargonidin 3-*O*-glucoside and pelargonidin 3-*O*-rutinoside. At the

same time, the stability of the purified mulberry pigment at different concentrations, pH values and temperatures has been systematically tested using UV-Visible spectroscopy.

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Corresponding author:

Dr. Chuanguang Qin, Northwestern Polytechnical University, Faculty of Life Science, P.O. Box 707, 127 West Friendship Rd., Xi'an Shaanxi Province, 710072, P. R. China
tel.: + 86 29 884 918 40, e-mail: qinchg@nwpu.edu.cn