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Recovery of value-added anthocyanins from mulberry by a cation exchange chromatography

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ABSTRACT

Anthocyanins are often targets in polyphenol analysis. However, it's hard to effectively separate anthocyanin from copigments such as phenolic acid and flavanols due to their similar structure. Thus, a cation exchange chromatography with 001 × 7 has been developed, which is available for anthocyanins isolation both on a small and large scale. The optimal process condition of anthocyanins isolation was determined. Compared to the macroporous adsorbent resins and Strong Cation Exchange resin (SCX), 001X7 shows greater economic advantages in large-scale purification of anthocyanins. More than 95% purity of the anthocyanin fraction can be achieved through this approach. This method shows a path to provide large quantities of copigments-free anthocyanins from mulberry polyphenols for the further study of its biological effects and may be extended to other analytical methods of polyphenol isolation from other plant materials.

1. Introduction

Anthocyanins are a kind of natural flavonoids, which are also classified as polyphenols (Espada-Bellido et al., 2017). As a pigment, anthocyanins are widely found in fruits and other plants dark-colored, for instance, mulberry (Silva et al., 2017). Though abundant in content, the category of anthocyanins in mulberry is relatively single. The majority of anthocyanins in mulberry are cyanidin-3-*O*-glucoside (C3G), and cyanidin-3-*O*-rutinoside (C3R) (Du et al., 2008; Hassimotto et al., 2008). Anthocyanins are known to have varieties of functional bioactivities, such as antioxidants (Romano et al., 2022), anti-inflammatory (Zhao et al., 2021), anti-cancer (Li et al., 2021), anti-diabetes (Gowd et al., 2017) and regulations on other chronic diseases like nonalcoholic fatty liver disease. (Zhu et al., 2022).

In view of the ample anthocyanins in black mulberry, efficient and selective methods are required for anthocyanins isolation. Ion exchange chromatography for anthocyanin purification has attracted much research interest in recent years due to its controllability and high selectivity. KU-2-8 (Pismenskaya et al., 2020), SCX (Ahmadiani et al., 2019) and other cation exchange resins are used for laboratory-scale purification of anthocyanins from fruits and vegetables. Anthocyanins would convert to positively charged flavylium cations in an acidic environment because of the chemical nature of the hydroxyl group in the 3-position (Pismenskaya et al., 2020). Subsequently, the flavylium cations are retarded on the negatively charged sulfonic acid group of resin through the ionic interactions. However, since the pK_a of phenolic acid is approximately 4–5, they are predominately neutral (protonated) under acidic conditions when the pH is below the pK_a and predominately ionized (negatively charged) when the pH is above the pK_a (Blum, 2019). Therefore, these copigments are not likely to be adsorbed by the resin and are flushed with the eluent (pH < 3), resulting in the separation of anthocyanins from the other phenolic compounds.

Nevertheless, the yet available preparative separation procedures only allow a partial enrichment of anthocyanins but not a complete separation (Juadjur and Winterhalter, 2012). Furthermore, it is crucial to keep the cost down for the industrial purification of anthocyanins. Most adsorber and ion exchange processes which are established even on an industrial scale are based on empirical approaches and have only rarely been systematically optimized. There is still a lack of knowledge with regard to the systematic adaption of process parameters for

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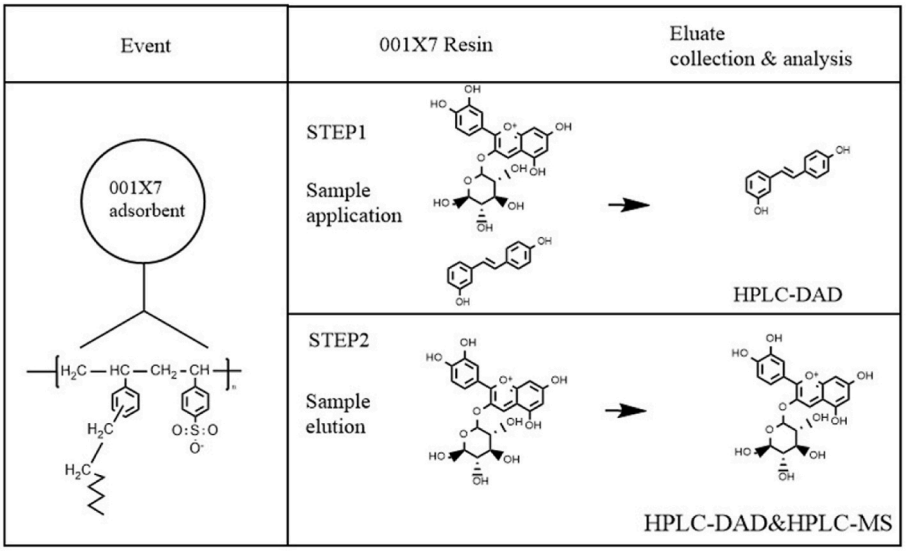

Fig. 1. Elution sequence of the purification process of anthocyanins in mulberry extract using 001X7 cation-exchange resin.

Table 1
Operation conditions for anthocyanins purification using 001X7.

pH of loading eluent	Flow rate of washing (mL/min)	Flow rate of elution (mL/min)	Concentration of cations (mol/L)
2.51	20	20	0.5
2.25	30	30	0.7
2.17	40	40	1
2.11	50	50	1.3
2.05	60	60	1.5

cost-efficiently and selectively enriching and purifying individual phenolic compounds from crude plant or by-product extracts (Kammerer et al., 2014). 001X7 resin is widely used for the preparation of high purity water, as well as catalysts and dehydrating agents in sugar industries because of its cost-effectiveness. To our best knowledge, the cation-exchange resin has not been used for the purification of natural pigments.

In the present study, we developed a new cation-exchange adsorptive chromatographic method to isolate anthocyanins from the mulberry extract by using a large-scale available column system (001X7 resin). A promising method for the purification of anthocyanins that can be developed into a large-scale purification process is proposed, and the application and optimization of the method are reported here.

2. Materials and methods

2.1. Chemicals

A standard (purity >98%) of cyanidin 3-*O*-glucoside was purchased from Chengdu Must Bio-Technology Co., Ltd (Chengdu, China). Chlorogenic acid, protocatechuic acid, quercetin, rutin, and isoquercitrin were purchased from Shanghaiyuanye Bio-Technology Co., Ltd (Shanghai, China). Methanol, acetonitrile, ethanol, formic acid, and acetic acid were high-performance liquid chromatographic (HPLC) grade. All other reagents were analytical reagents.

2.2. Sample preparation and adsorptive column chromatography

Fresh mulberries were purchased from Lejiguo (Guangdong, China). The fresh mulberry fruits were freeze-dried and fine powdered. The powder was extracted in the acidified 95% ethanol (0.1% trifluoroacetic acid v/v) two times and vacuum filtered. The combined supernatant was evaporated at 40 °C to remove the organic solvents. The anthocyanin content was determined by using the pH differential method (Lee et al., 2016).

To develop the column chromatography, a new strongly acidic cation exchange resin 001X7 was used in this study, which was usually used for wastewater treatment. The purification mechanism and process of anthocyanins in mulberry extract using 001X7 cation-exchange resin were illustrated in Fig. 1. After elution, the eluate was added with 1% acetic acid to stabilize the anthocyanins fraction. The copigments and anthocyanins eluate fractions were analyzed by HPLC-DAD and UPLC-MS.

To analyze the kinetics of the adsorption and desorption of resin 001X7 to anthocyanins in mulberry extract (both static and dynamic), the operation conditions for purification were optimized. As described in Table 1, the effect of pH of loading eluent, the flow rate during washing and elution, concentration of cations in desorbent were tested as single factors.

2.3. Laboratory-scale isolation of anthocyanins

In preliminary tests, optimal parameters of purification were determined for the laboratory-scale isolation of anthocyanins. Prior to the isolation, the resin had to be regenerated with the following steps: (1) rinsing with 2 bed volume (BV) of sodium hydroxide solution (1 M), (2) rinsing with 2 BV of hydrochloric acid (0.1 M) and (3) washing the resin by deionized water until the pH of eluate solution converts to neutral. After the equilibration, the resin could be loaded with the extract solution. The concentrated extract was dissolved in the acidified methanol (7% acetic acid v/v) to stabilize the anthocyanins, converting anthocyanins to flavylium cations. Furthermore, to remove the copigments, the resin was flushed with 2BV of acidified methanol (7% acetic acid v/v) at a flow rate of 40 mL/min. For the isolation of anthocyanins, the loaded resin was flushed with a mixture of methanol/NaCl solution (1 M) 1:1 (v/v) until the anthocyanins were totally eluted (through observing the color of eluate). The organic solvent was removed by rotary evaporation. The concentrated eluate was acidified by acetic acid (1% of the solution) to stabilize the anthocyanins. Subsequently, the resin could be regenerated again by repeating the above-mentioned steps.

2.4. Large-scale isolation of anthocyanins

For larege-scale isolation of anthocyanins, a larger column (12 cm × 120 cm, Yongcheng, Jiangsu, China) was used. The concentrated extract

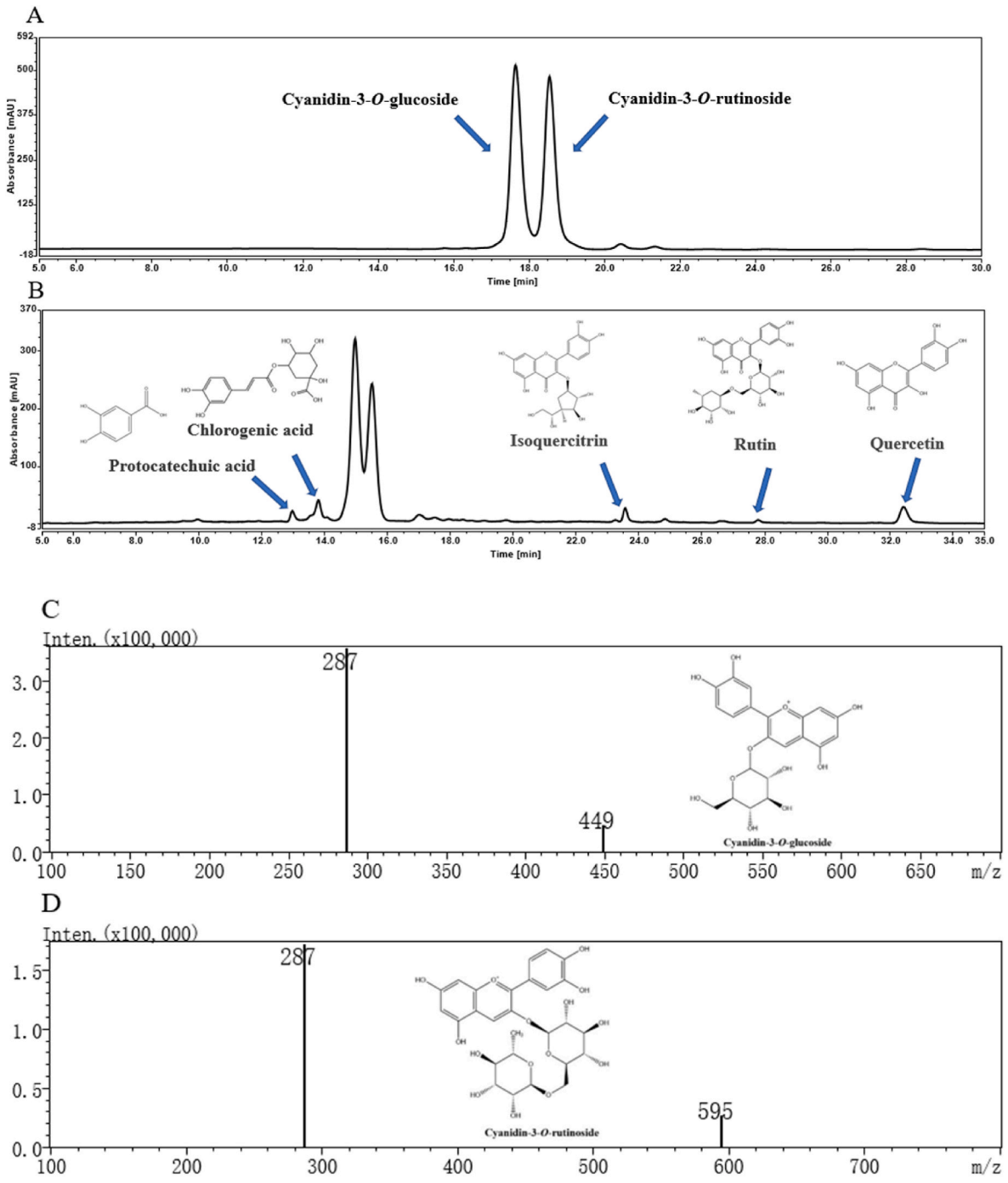

Fig. 2. Identification and quantification of anthocyanins and copigments from black mulberry. HPLC-DAD chromatograms of anthocyanins at 520 nm (A) and copigments at 280 nm (B); LC-MS spectras and structures of cyanidin-3-*O*-glucoside (C), cyanidin-3-*O*-rutinoside (D).

was dissolved in 1 L acidified methanol (7% acetic acid v/v). After the regeneration and equilibration using 2 L NaOH (1 M), 2 L HCl (0.01 M) and 1 L acidified methanol (7% acetic acid v/v), the resin was loaded with the concentrated extract and washed by acidified methanol (7% acetic acid v/v) at a flow rate of 100 mL/min to remove the copigments. In case of the remains of copigments, it was advisable to add approximately 1 L extra acidified methanol (7% acetic acid v/v) to wash the resin. Again, a mixture of methanol/NaCl solution (1 M) 1:1 (v/v) was used to elute the anthocyanins adsorbed in the column until the effluent was colorless. Sampling after every 200 mL eluent was analyzed by using an Ultimate 3000 HPLC system equipped with a diode array detector (HPLC-DAD) (methods see section 2.6 and 2.7). For further purification, the eluate was combined after HPLC analysis and the solvent was evaporated by rotary evaporation. Finally, the eluate was acidified by acetic acid (1% of the solution) to stabilize the anthocyanins.

2.5. Purification of anthocyanins with C18 column

For the removal of NaCl in the anthocyanins fraction, a column (60 mm × 134 mm, iLOK, Santai) filled with C18 was used. To equilibrate the column, it was washed and soaked in methanol overnight. 1 BV of deionized water was used for cleaning until the effluent was impurity free. The condensed fraction containing NaCl was loaded onto the column. To completely remove the NaCl, the column was rinsed with 5–6

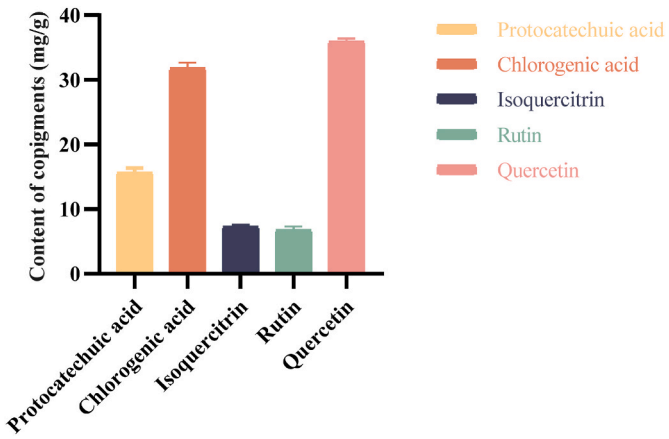

Fig. 3. Distribution of phenolic acid and flavonols in the mulberry extract. n=3, mean ± SD.

BV of deionized water with measuring salinity by a salinometer until the salinity of eluate was under 5%. Subsequently, the column was flushed with 2-3BV ethanol to elute the anthocyanins. After the elution, the organic solvent was removed by rotary evaporation. The anthocyanins fraction was freeze-dried. The method is also fit for the removal of cations in the polyphenol eluate.

2.6. Quantitation of anthocyanins and phenolic derivatives

All the fractionated phenol analyses were performed using HPLC-DAD system. The separation was carried out with an analytical scale Acclaim TM C18 column (5 μm 120A 4.6 mm × 250 mm, Thermo Scientific, Germany) and the column temperature was 30 °C. The aqueous eluent (A) consisted of formic acid and water (1:50 v/v), and acetonitrile (B). The flow rate of the mobile phase was 1.0 mL/min and the injection volume was 10 mL. All samples were filtered through a 0.22 μm syringe filter before analysis.

The linear gradient was: 0–28 min, 6–20% B; 28–30 min, 20–95% B, 30–35 min, 95% B; and then back to initial conditions during 5 min. Elution of anthocyanins was detected at 520 nm, 5 polyphenols were detected at 280 nm and they were all quantified using an external standard. Individual polyphenols were dissolved in HPLC-grade methanol and ultrapure water to generate the external standard calibration curves and the concentrations of standards were 0.025, 0.05, 0.1, 0.25, 0.5, and 1 mg/mL, which have a linearity of R^2>0.999 for all.

2.7. Identification of anthocyanins

For further identification of anthocyanins, analyses were performed by a Shimadzu LC-MS-8045 system (Japan) after filtration using a 0.22 μm syringe filter, which comprised a binary pump (LC-30AD), an autosampler (SIL-30AC), a diode array detector (SPD-M20A), a column oven (CTO-20AC), an ion trap mass spectrometer with an electron spray ionization source (ESI) (Li et al., 2020). The aqueous eluent (A) consisted of formic acid and water (1:100 v/v), and the organic eluent (C) consisted of acetonitrile. The flow rate was 0.2 mL/min and the injection volume was 5 μL. The linear gradient was: 0–3 min, 6% C; 3–6 min, 6–13% C; 6–26 min, 13–30% C; 26–28 min, 30–60% C; 28–30 min, 60% C; 30–32 min, then back to initial conditions during 2 min and maintain 6min.

ESI was performed as the following parameters: probe temperature, 300 °C; DL temperature, 250 °C; flow rate of atomization gas, 2.5 L/min; dry gas, 10 L/min, convert dynode, 10 kV, Detector voltage, 2.42 kV, scan range, m/z 100–1000. LabSolutions™ LCMS Software was for data collection and analyses.

3. Results and discussion

3.1. Analysis of anthocyanins and copigments

The content of anthocyanins in mulberry was 3.2 mg/g fresh weight. Only two main anthocyanins in the mulberry extract were characterized by HPLC-DAD and LC-MS, including cyanidin-3-*O*-glucoside (C3G), cyanidin-3-*O*-rutinoside (C3R) (Fig. 2). For more accurate quantification of anthocyanins, HPLC-DAD was used with the commercially available C3G standard (purity >98%), which turned out that the content of C3G in total anthocyanins was 59.7%. Fig. 2C&D shows the chemical structures of the two anthocyanins and their MS spectra.

The identification and quantification of copigments in the mulberry extract were performed by HPLC-DAD with chemical standard (purity

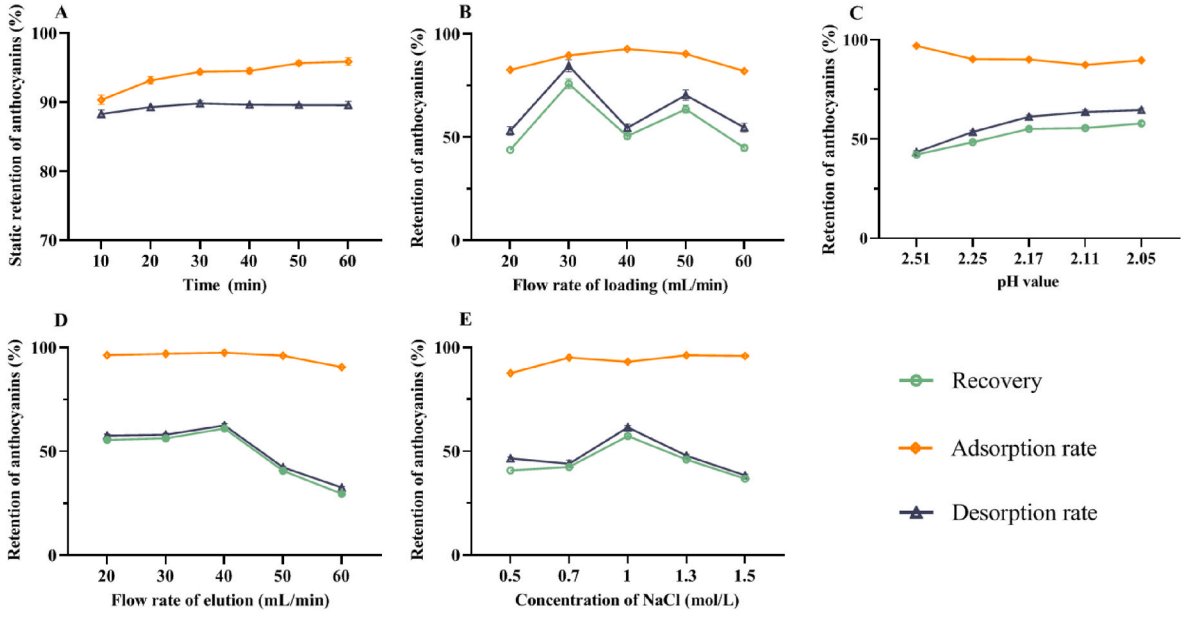

Fig. 4. Effect of different pH of loading eluate solution (A), flow rate during loading (B) and elution steps (C), concentration of NaCl in eluate solution (D) on recovery, adsorption and desorption rate, Static adsorption and desorption (E). n=3, mean ± SD.

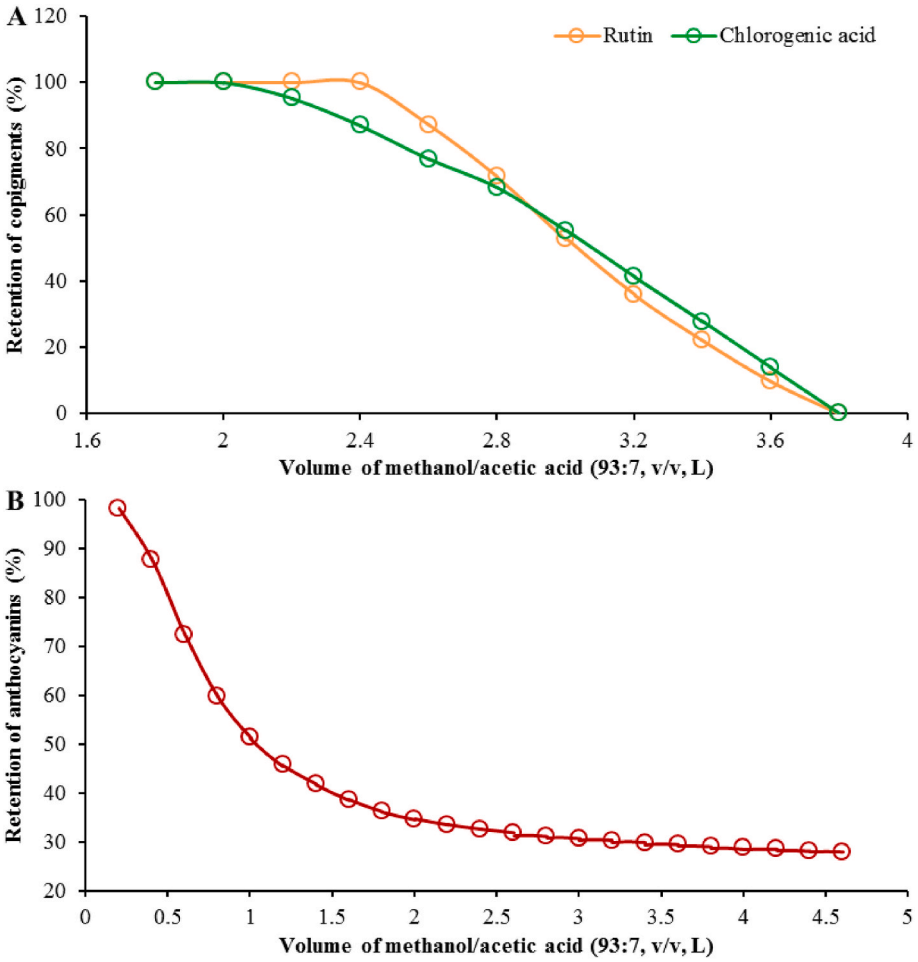

Fig. 5. Retention of copigments (A) and anthocyanins (B) during the column chromatographic separation (large scale). Extract dissolved in methanol/acetic acid 93:7 (v/v) and concentration were at 1.3 g/L.

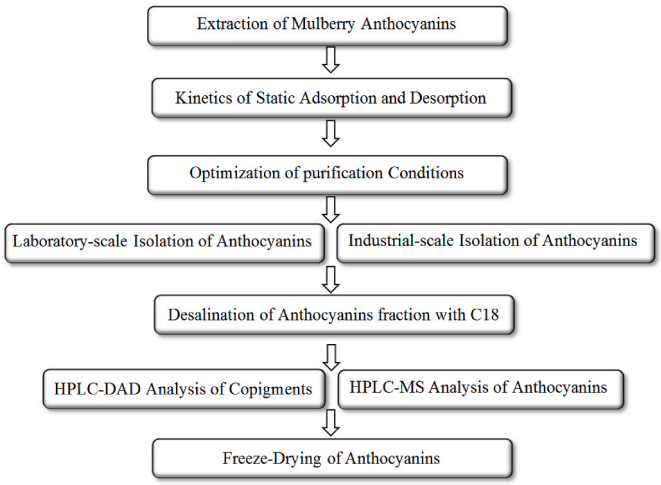

Fig. 6. Flowchart for the implementation of adsorptive column chromatographic method for anthocyanins fractionation.

>98%) at the wavelength of 280 nm (Fig. 2B). Five phenols were characterized and quantified as Fig. 3 shown. They were protocatechuic acid (16.0 mg/g), chlorogenic acid (32.7 mg/g), isoquercitrin (7.4 mg/g), rutin (6.9 mg/g), and quercetin (36.4 mg/g).

3.2. Kinetic test and adsorptive column chromatography

Kinetics of the adsorption and desorption both in static and dynamic of resin 001X7 were tested. Thus, we obtained results on the optimal separation conditions, including the pH of loading eluate solution, the flow rate during washing and elution, concentration and kinds of cations in eluate solutions (Fig. 4). In the first step, the adsorption and desorption of anthocyanins were studied using the static method. From Fig. 4A, it can be seen that anthocyanins were gradually adsorbed by the resin, and tended to equilibrium after 50 min, meaning that the adsorption of mulberry anthocyanin on the resin reached saturation. Therefore, the equilibrium time of static adsorption should be 50 min. In the meanwhile, the desorption rate of resin in saturated conditions did not change much after 50 min, which means that the desorption of mulberry anthocyanins was complete at 50 min. Therefore, the equilibrium time of static adsorption and desorption should be 50 min and 30 min, respectively.

As the washing step accelerated, the recovery was in a downward trend. From 20 to 30 mL/min, there was an uplift of recovery and reached the highest (approximately 78%) at the flow rate of 30 mL/min, then declined to 50% (Fig. 4B). When the flow rate is low, there is more time for the adsorbed molecules to interact with the active site of the resin. Though a high flow rate reduced time required for purification, it has a negative impact on the adsorption capacity due to the shorter residence time of the anthocyanin (Jampani et al., 2014). When the loading pH was between 2.51 and 2.17, the adsorption rate gradually increased as the pH continued to drop, then the adsorption of

Table 2
Comparison of large-scale anthocyanins purification using MAR, SCX and 001X7 in cost, revenue and profit[a]. (Based on ¥ 500 g^{-1} Mulberry).

Input (Cost)	MAR	SCX	001X7	Output (Revenue)	MAR	SCX	001X7
Raw materials	19	19	19	Anthocyanins	300 (60%)	13540 (98%)	220 (95%)
Extraction	130	130	130	Mulberry	130	130	130
Chemical	3	3	3	Polysaccharide	430	13670	350
Waste Water[b]	51	26	51	(30%)			
Press-filtration	6	6	6	Subtotal			
Filtrate-concentration	6	6	6				
Resin	162	16725	3				
Subtotal	377	16915	218				

[a] Calculation based on Fig. 6.
[b] Waste water management fee was calculated according to Chinese policy.

anthocyanins tended to level off (Fig. 4C). It was suggested that ionic interaction plays a significant role in adsorption and desorption of ionic resins (da Silva et al., 2017). As pH rises, with the weakening of the ionic interaction and the dissociation of hydrogen bonds and carboxyl groups, adsorption of anthocyanins decreases (Fu et al., 2005). The desorption rate varied with the flow rate during the elution, which slightly increased with the flow acceleration and peaked at 40 mL/min (Fig. 4D). It was revealed that a high desorption flow rate could lead to insufficient desorption. Though lower flow rate enhances the interactions between desorbent and the targets, long time consumption is not a good choice for an efficient desorption process (Sandhu & Gu, 2013). As for the effect of concentration of cations in desorbent, we could see a rise to 57% on desorption rate as the concentration of Na^+ reached 1.0 mol/L, then declined to 38% as the concentration continued to increase (Fig. 4E). Insufficient replacement capacity of the low-concentration sodium ions eluent could be related with the above results, leading to the incomplete desorption of adsorbed flavylium cations. While the high concentration of sodium ions and neutral pH conditions may have promoted the shift of the adsorption mechanism of anthocyanins to hydrophobic interactions with sulfonic acid groups (He & Giusti, 2011; Lima et al., 2017). On the understanding of that, the optimal conditions for the purification of anthocyanin by 001X7 resin are flow rate 30 mL/min when washing; 40 mL/min when elution; pH 2.17; 1.0 mol/L NaCl in desorbent (mixed with methanol in a ratio of 1:1 (v/v)). The eluted anthocyanins fraction was concentrated by rotary evaporation and desalinated by a C18 column. Then, the copigments-free anthocyanin aqueous solution was freeze-dried.

3.3. Scale-up of the separation and cost-benefit analysis

For large-scale isolation of anthocyanins, a larger column (12 cm × 120 cm) was used. After regeneration and equilibration using 2 L of 1 M NaOH, 2 L of 0.01 M HCl and 1 L of methanol/acetic acid 93:7 (v/v) (pH 2.17), 3.0 L extracts (concentration 1.3 g/L) were loaded into the column. During the loading, washing and elution, every 200 mL of the eluate was collected and analyzed by HPLC-DAD. To remove the copigments, methanol/acetic acid 93:7 (v/v) was used to wash at a flow rate of 100 mL/min. Through monitoring the washing-step eluate of absorption peak under 520 nm by HPLC-DAD, over 99.8% of the anthocyanins extract was adsorbed, for which only 0.02% of the original concentration of anthocyanins were detected. If the column was overloading, the unabsorbed anthocyanins in the copigments fraction could be isolated by second column chromatography. For copigments, most of them were not detected under 280 nm until 2.0 L of methanol/acetic acid 93:7 (v/v) was applied, specifically 2.4 L for rutin. (Fig. 5A). After rinsing the column with 4.0 L of extractant, the retained anthocyanin was desorbed with 4.6 L of a 1:1 (v/v) mixture of aqueous 1 M NaCl solution and methanol. From Fig. 5B, it could be observed that the majority of anthocyanins were eluted in the first 1.6 L. Again, 1% acetic acid was added for the stabilization of anthocyanins. Concentration and desalination were carried out as laboratory-scale purification described above.

Table 2 illustrated the cost and profit of large-scale anthocyanins purification using macroporous adsorbent resins (MAR), Strong Cation Exchange column (SCX) and 001X7. The profit increased from −3245 to 132.5 ¥ 500g^{-1} raw materials. MAR, a non-ionic adsorbent resin, was widely used for preliminary purification to remove the polysaccharide and protein in anthocyanins extract because of its good reticulation structure and large specific surface area, while the application process is cumbersome and time-consuming. It took 1 or 2 days to purify the extract, whereas only reached 60% purity (Chen et al., 2017). It is worth noticing that the whole purification process using 001X7 only takes 3–4 h and eventually reaches more than 95% purity. Though it is achievable to reach over 98% purity by using the SCX column to isolate the anthocyanins extract (He & Giusti, 2011), the much higher cost (16725¥ 500 g^{-1}) gets in the way of its industrialization. On the contrary, the lower cost of using 001X7 resin (2.5¥ 500 g^{-1}) shows a more profitable way to purify anthocyanins extract at a large scale.

4. Conclusions

This study aimed to propose a scale-up available column chromatographic method with a cation-exchange resin (001X7) to fractionate anthocyanins and copigments from the mulberry extract. Two anthocyanins (C3G and C3R) and five major copigments (protocatechuic acid, chlorogenic acid, isoquercitrin, rutin, and quercetin) were identified and quantified by HPLC. Recovery of value-added anthocyanins with high purity (>95%) from mulberry using the 001X7 cation exchange chromatography was achieved. Using 001X7 resin for the large-scale purification of anthocyanins is a promising approach for its effectiveness and profitability.

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CRediT authorship contribution statement

Zhiwei Liao: Conceptualization, Investigation, Writing – original draft. **Xuan Zhang:** Methodology, Validation, Visualization. **Xi Chen:** Visualization, Writing – review & editing. **Maurizio Battino:** Writing – review & editing. **Francesca Giampieri:** Writing – review & editing. **Weibin Bai:** Supervision, Writing – review & editing. **Lingmin Tian:** Conceptualization, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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