Journal of Zhejiang University-SCIENCE B (Biomedicine & Biotechnology)
ISSN 1673-1581 (Print); ISSN 1862-1783 (Online)
www.jzus.zju.edu.cn; www.springerlink.com
E-mail: jzus@zju.edu.cn

Phenolic compounds participating in mulberry juice sediment formation during storage*

Bo ZOU, Yu-juan XU, Ji-jun WU, Yuan-shan YU, Geng-sheng XIAO†‡

(*Sericultural & Agri-Food Research Institute, Guangdong Academy of Agricultural Sciences / Key Laboratory of Functional Foods, Ministry of Agriculture / Guangdong Key Laboratory of Agricultural Products Processing, Guangzhou 510610, China*)

†E-mail: 244520460@qq.com

Received Oct. 11, 2016; Revision accepted Mar. 28, 2017; Crosschecked Sept. 15, 2017

Abstract: The stability of clarified juice is of great importance in the beverage industry and to consumers. Phenolic compounds are considered to be one of the main factors responsible for sediment formation. The aim of this study is to investigate the changes in the phenolic content in clarified mulberry juice during storage. Hence, separation, identification, quantification, and analysis of the changes in the contents of phenolic compounds, both free and bound forms, in the supernatant and sediments of mulberry juice, were carried out using high performance liquid chromatographic system, equipped with a photo-diode array detector (HPLC-PDA) and HPLC coupled with quadrupole-time of flight mass spectrometric (HPLC-QTOF-MS/MS) techniques. There was an increase in the amount of sediment formed over the period of study. Total phenolic content of supernatant, as well as free phenolic content in the extracts of the precipitate decreased, whereas the bound phenolic content in the sediment increased. Quantitative estimation of individual phenolic compounds indicated high degradation of free anthocyanins in the supernatant and sediment from 938.60 to 2.30 mg/L and 235.60 to 1.74 mg/g, respectively. A decrease in flavonoids in the supernatant was also observed, whereas the contents of bound forms of gallic acid, protocatechuic acid, caffeic acid, and rutin in the sediment increased. Anthocyanins were the most abundant form of phenolics in the sediment, and accounted for 67.2% of total phenolics after 8 weeks of storage. These results revealed that phenolic compounds, particularly anthocyanins, were involved in the formation of sediments in mulberry juice during storage.

Key words: Mulberry juice; Phenolic compounds; Sediment; Anthocyanin; Antioxidant
http://dx.doi.org/10.1631/jzus.B1600461 **CLC number:** TS255.1

1 Introduction

The appearance of a beverage is very important as it forms the first impression of the consumer and has an impact on its acceptability. The stability and clarity of clarified juices are crucial factors that influence the appearance of beverages. Sediments in a bottle of juice decrease its commercial value and thus affect its sales. Therefore, the key issue is to retain the stability of the clear juice. Amongst the wide range of chemical compounds and mechanisms that affect stability, phenolic compounds are probably the most important factors that lead to the development of haze and eventually precipitation (Prakash *et al.*, 2016).

Mulberry (genus *Morus*, family Moraceae) plants are widely spread over tropical and subtropical regions (Pérezgregorio *et al.*, 2011). In the past, leaves from the plants of the genus *Morus* were fed to silkworms and were also used in sericulture, whereas their fruits gained little attention (Jia *et al.*, 1999). However, black mulberry is now cultivated in China mainly for its fruit rather than its foliage (Yu *et al.*, 2014). The fruit of the new *Morus* species, such as

‡ Corresponding author
* Project supported by the China Agriculture Research System (No. CARS-22-02A), the National Natural Science Foundation of China (No. 31501541), the Natural Science Foundation of Guangdong Province (No. 2015A030312001), and the Science and Technology Program of Guangzhou (No. 201510010063), China
ORCID: Geng-sheng XIAO, http://orcid.org/0000-0001-7137-3179

Da10, is now being cultivated, and its fruits have a very high demand in the market. Apart from being delicious, they abound with phenolic compounds (Imran *et al.*, 2010), which have good health benefits (Du *et al.*, 2008; Özgen *et al.*, 2009). The black mulberry fruit is rich in phenolic compounds, especially anthocyanins (Özgen *et al.*, 2009), which have antioxidant properties and also exhibit anti-glucosidase (Yu *et al.*, 2014), hypolipidemic (Yang *et al.*, 2010), anti-inflammatory (Liu and Lin, 2014), and neuroprotective activity (Kang *et al.*, 2006). Mulberry fruit has a short harvesting period, and it is susceptible to spoilage (Liu *et al.*, 2014). To prolong shelf-life, mulberries need to be processed into products such as jams, juices, wines, and vinegars.

Recently, demand for mulberry juice has increased several-fold. The instability of the juice is the biggest problem associated with its production. During storage, the dark red sediment that is formed is undesirable in terms of appearance. Reports suggest that polyphenols are the principal compounds present as sediments in juice (Fang *et al.*, 2006). Also, the stages in the formation of precipitates in wine are strongly associated with the presence of tannins (Prakash *et al.*, 2016). Black mulberry has a high content of cyanidin 3-*O*-glucoside (C3G) and cyanidin 3-*O*-rutinoside (C3R) (Yu *et al.*, 2014), along with a large number of phenolic acids and flavonoids, such as rutin (Zhang *et al.*, 2008; Natic *et al.*, 2015; Sánchezsalcedo *et al.*, 2015; Bao *et al.*, 2016; Mena *et al.*, 2016). This leads to the speculation that phenolic compounds are involved in the precipitation of mulberry juice.

To the best of our knowledge, no previous study has focused on analyzing the different phenolic compounds in mulberry juice sediment. The antioxidant activity of the precipitates is also unclear. Thus the present study aimed to determine the relationship between the sediment formation and phenolics. Therefore, the formation of the dark red sediment in mulberry juice during storage was investigated. The free and bound phenolic compounds in the supernatant and sediments of mulberry juice were identified and quantified. The changes in the antioxidant activity of the juice during storage were also investigated. The study may lead to industrial processes for improvement or prevention of sediment formation in mulberry juice.

2 Materials and methods

2.1 Chemicals

Standard preparations of gallic acid, protocatechuic acid, chlorogenic acid, neochlorogenic acid, caffeic acid, *p*-coumaric acid, quercetin, rutin, quercetin 3-glucoside, cyanidin 3-*O*-glucoside, cyanidin 3-*O*-rutinoside, pelargonidin 3-*O*-glucoside, and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were purchased from Sigma-Aldrich (St. Louis, USA). Folin-Ciocalteu reagent was obtained from Yuanye Bio-Technology Co., Ltd. (Shanghai, China). Fluorescein disodium salt, 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH), and 2,2-diphenyl-1-picrylhydrazyl radical (DPPH•) were obtained from Sigma-Aldrich. Acetonitrile and formic acid were purchased from Merck (Darmstadt, Germany). Water for the high performance liquid chromatographic system with photo-diode array detector (HPLC-PDA) and the HPLC with quadrupole-time of flight mass spectrometric (HPLC-QTOF-MS/MS) analyses was purified using a Milli-Q® system (Millipore, Bedford, USA).

2.2 Mulberry juice preparation and storage

Ripe black mulberry fruit of Da10 (*Morus atropurpurea* Roxb.) was harvested at the farm of our Research Institute (Guangzhou, China). Soon after collection, the fruit was stored at −80 °C until further analysis. The frozen mulberries were thawed and crushed in a pulper (Midea brand, Guangzhou, China). The puree was passed through filter cloth (100 mesh), centrifuged at 5000*g* for 5 min and the clear supernatant was collected. The mulberry juice was thermally treated at 95 °C for 1 min, cooled to room temperature, and then stored at 30 °C for 8 weeks. The mean summer temperature of Guangzhou, the region in which this process is carried out, is about 30 °C. Hence, temperature for storage was chosen as 30 °C.

2.3 Isolation of mulberry juice supernatant and sediment

The juice was centrifuged (5000*g* for 10 min at 25 °C) and the supernatant was passed through a 0.22-μm filter and used for analysis. To remove the soluble component, the sediment was washed twice with pure water and centrifuged (10000*g* for 10 min

at 4 °C). Subsequently, the sediment was lyophilized to yield a dry dark-red powder.

2.4 Extraction of phenolic compounds from sediment

Soluble free phenolics (F-P) and bound phenolics present in the ethyl acetate fraction (B-E) and in the water soluble fraction (B-W) of the sediment were extracted based on the method proposed by Sun *et al*. (2002), with some modification. F-P of the sediment were extracted as follows: 0.05 g of lyophilized sediment was soaked in 5 ml of 80% acetone (1:100, w/v) and shaken for 10 min. The mixture was centrifuged (10000*g* for 10 min at 4 °C), and the supernatant was evaporated under nitrogen, and then dissolved in 4 ml of 80% methanol. The residue that remained after the extraction of F-P was hydrolyzed with 5 ml of 4 mol/L NaOH at 25 °C in a nitrogen atmosphere. After 1 h, the pH of the mixture was adjusted to 2 with concentrated hydrochloric acid and extracted 6 times with ethyl acetate. The organic phase was evaporated to dryness on a rotary evaporator under reduced pressure at 35 °C and the residue was dissolved in 4 ml of 80% methanol. This fraction of the solution containing phenolic compounds was designated as B-E. The aqueous fraction containing the phenolic compounds was designated as B-W. Traces of ethyl acetate from the aqueous fraction were evaporated under vacuum at 35 °C and then recovered with 5 ml of water.

2.5 Determination of total phenolic content

Total phenolic content was determined by the Folin-Ciocalteu method as described previously (Kim *et al.*, 2013). In brief, appropriate dilutions of extracts (1 ml) were mixed with Folin-Ciocalteu reagent (2 ml). After 5 min, 2 ml of sodium carbonate (10 g/100 ml) was added to each mixture. The mixtures were allowed to stand at room temperature in the dark for 1 h. The absorbances were recorded at 760 nm on a UV-Visible (UV-Vis) spectrophotometer (Shimadzu, Tokyo, Japan). Gallic acid was used as the standard, and content was expressed as mg gallic acid equivalent (GAE) per milliliter or gram. Analysis of each sample was carried out in triplicate.

2.6 HPLC-PDA analysis of phenolics

The phenolic acid, anthocyanin, and flavonol contents were calculated from HPLC-PDA (LC-20AT; Shimadzu, Japan) assays, according to the method of Sokolletowska *et al*. (2014), with some modification. Separation of individual phenolics was carried out on a C18 Kinetex column (150 mm×4.6 mm, 2.6 μm; Phenomenex, Torrence, USA) at 35 °C. Solution A was 4.5% formic acid in water and solution B was 100% acetonitrile. The gradient elution program was as follows: 0–5 min, 5% B; 5–25 min, 5%–25% B; 25–30 min, 25%–35% B; 30–31 min, 35%–100% B; 31–36 min, 100% B; 36–37 min, 100%–5% B; 37–57 min, 5% B. The flow rate was 0.45 ml/min and the injection volume was 4 μl. Chromatographic data were acquired from 200 to 800 nm. Detection wavelengths were as follows: 320 nm for hydroxycinnamic acids, 280 nm for hydroxybenzoic acids, 520 nm for anthocyanins, and 360 nm for flavonol. The individual phenolic content was quantified using external calibration curves of standards.

2.7 HPLC-QTOF-MS/MS analysis of phenolics

Identification of phenolic compounds was performed using an HPLC-QTOF-MS/MS instrument (SCIEX, Massachusetts, USA). The separation of phenolics was carried out by the same method as above. The main parameters for the QTOF-MS were: cone voltage, 40 V; capillary voltage, 4.5 kV; source temperature, 130 °C; desolvation temperature, 350 °C; cone voltage, 30 V; desolvation gas (nitrogen) flow rate, 600 L/h; MS scan from *m*/*z* 100 to 1000 and MS/MS from *m*/*z* 50 to 1000.

2.8 Determination of DPPH radical-scavenging activity

The DPPH radical-scavenging activity of the extracts was evaluated according to the method reported by Yen and Chen (1995) with some modification. Briefly, a solution of DPPH was freshly prepared in ethanol to a concentration of 200 μmol/L. A 50-μl sample of extract was mixed with 150 μl DPPH solution and allowed to stand at room temperature in the dark for 20 min. The absorbance was recorded at 517 nm using a microplate reader (Tecan Austria GmbH, Grödig, Austria). The results were expressed as Trolox equivalent (TE) using the calibration curve plotted against different concentrations of Trolox.

2.9 Determination of oxygen radical absorbance capacity

The oxygen radical absorbance capacity (ORAC) assay was performed using an Infinite®

M200 PRO microplate reader according to the method of Wolfe *et al*. (2008). The sample (20 μl) was placed in each microplate well. This was followed by addition of 80 μl of a sodium fluorescein solution (1.25 μmol/L in 75 mmol/L phosphate buffer, pH 7.4) into each well and then incubation for 5 min at 37 °C. Then, 100 μl of freshly prepared AAPH (140 mmol/L in 75 mmol/L phosphate buffer, pH 7.4) was added to each well. Excitation and emission spectra were recorded at 485 and 520 nm, respectively, every 2.5 min for 35 cycles at 37 °C. The ORAC results were expressed as TE using the calibration curve plotted against different concentrations of Trolox.

2.10 Statistical analysis

All experiments were run in triplicate, and the results were presented as mean±standard deviation (SD). Data were evaluated by one-way analysis of variance (ANOVA) of SPSS 18.0 (Chicago, USA) with Tukey's multiple-range test. Differences were considered to be statistically significant at $P<0.05$.

3 Results and discussion

3.1 Formation of sediment during mulberry juice storage

The juice appeared virtually clear during the initial stages of storage. After 4 weeks, the juice turned hazy along with deposition of sediment. As the period increased from 2 to 8 weeks, the amount of dark red precipitate increased significantly ($P<0.05$), from 0.15 to 0.93 g/L (Fig. 1). These observations clearly indicated that sediments were formed in the fruit juice during storage.

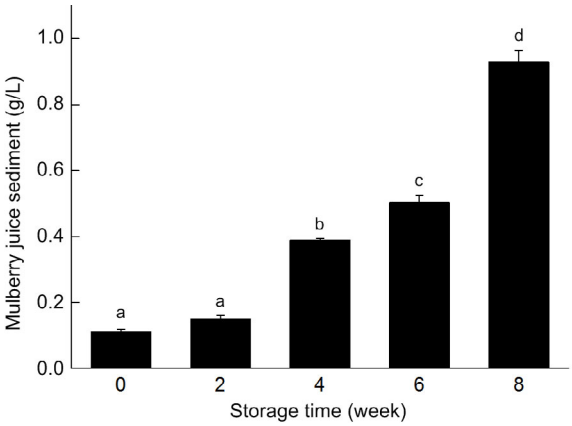

Fig. 1 Amount of mulberry juice sediment formed during storage

Data are expressed as mean±SD (n=3). Different alphabets indicate significant differences ($P<0.05$)

3.2 Total phenolic contents in the supernatant and the sediment of stored mulberry juice

The chemical composition of juice triggers the development of haze and is followed by sediment formation. It was reported that for clarified apple juice, there was an increase in turbidity and a decrease in tannin content of the supernatant (Tajchakavit *et al.*, 2001). However, analysis of the sediment was not carried out. According to some recent studies, polyphenols were present in the precipitates of bayberry juice (Fang *et al.*, 2006), blackberry juice (Siriwoharn *et al.*, 2005), and red wine (Prakash *et al.*, 2016). The analysis of phenolics in insoluble sediment is a challenging task. Therefore, bound phenolics were extracted by either alkaline or acidic hydrolysis (Acostaestrada *et al.*, 2014). In the present study, the bound phenolic compounds were extracted by alkaline hydrolysis.

The total phenolic content in the supernatant of mulberry juice showed a slight decrease with time (Fig. 2a), but no significant changes ($P>0.05$) were observed from 2 to 8 weeks. Similar results were obtained in the mulberry juice during ultra-high-pressure homogenization (UHPH) and pasteurization processes (Yu *et al.*, 2014). In the juice sediment, phenolic compounds that were present included F-P, B-E, and B-W. As shown in Fig. 2b, the amount of F-P decreased with storage time, especially after 8 weeks. In contrast to this, the total phenolic content in B-E increased during storage, from 8.17 to 12.35 mg GAE/g (Fig. 2c). In the water fraction, the total phenolic content increased from 2.31 to 19.50 mg GAE/g (Fig. 2d). This was obvious, since some phenolics could have been linked to other compounds such as proteins, which resulted in particles large enough to be perceived as haze and eventually precipitated in the juice. As reported by Mouls and Fulcrand (2012), during storage phenolics can become oxidized to form polymeric compounds, which have a sufficiently large molecular weight and separate from the juice. The oxidized phenolics can also remain incorporated within the protein and result in sediment

formation (Prakash *et al.*, 2016). The increase in total phenolic content was higher in B-W than in B-E. This can be explained by the fact that anthocyanins, the water-soluble pigments, were the main phenolic compounds in mulberry (Özgen *et al.*, 2009). They could be oxidized to polymers or linked to other compounds during their storage period. After alkaline hydrolysis, the anthocyanins were released and dissolved in water. Hence, the above results suggest that the phenolics may have participated in the formation of sediments.

3.3 Identification of phenolic compounds

To determine the changes in the amount of individual phenolics during the formation of sediments in mulberry juice, the compositions of phenolic compounds in the supernatant and sediment were analyzed. Twelve phenolic compounds including, 2 hydroxybenzoic acids, 4 hydroxycinnamic acids, 3 anthocyanins, and 3 flavonoids were identified in the samples. Identification was based on matching the retention time (t_R), the UV-Vis spectral profiles at 200–800 nm, mass spectra, and MS/MS analyses with commercial standards. Table 1 summarizes the identified phenolic compounds.

3.3.1 Hydroxybenzoic acids

The HPLC-QTOF-MS/MS analysis of the supernatant and sediment suggested the presence of two hydroxybenzoic acids in the negative ion mode (Table 1). Peak 1 had a pseudo-molecular ion at *m*/*z* 169, which showed a fragmentation peak at *m*/*z* 125, due to the loss of carboxyl (44 Da) residue (Natic *et al.*, 2015). Based on its retention time and UV-Vis spectra, this compound was tentatively identified as gallic acid. Peak 2 was tentatively identified as protocatechuic acid, based on its molecular formula $C_7H_5O_4^-$ and a fragment at *m*/*z* 109 (Table 1).

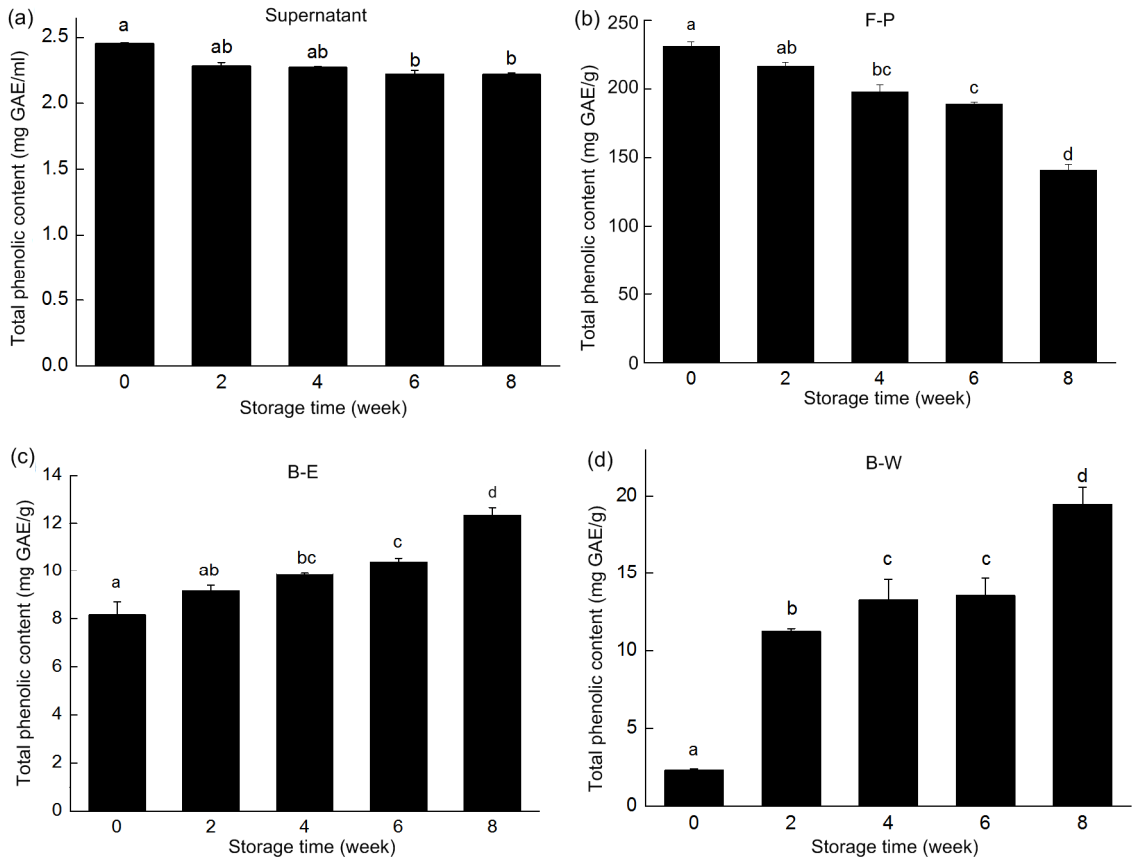

Fig. 2 Total phenolic content in mulberry juice during storage

(a) Supernatant in juice; (b) Soluble free phenolics in sediment, F-P; (c) Bound phenolics in ethyl acetate fraction, B-E; (d) Bound phenolics in water fraction, B-W. Data are expressed as mean±SD (n=3). Different alphabets indicate significant differences (P<0.05)

Table 1 Identification of phenolic compounds in mulberry juice

Peak	t_R (min)	λ_{max} (nm)	$[M-H]^-/[M+H]^+$ (*m*/*z*)	MS/MS (*m*/*z*)	Molecular formula	Identification
1	4.54	240, 270	169.01406	125	$C_7H_5O_5^-$	Gallic acid
2	7.23	259, 293	153.02166	109	$C_7H_5O_4^-$	Protocatechuic acid
3	8.05	247, 324	353.08794	191, 179, 173	$C_{16}H_{17}O_9^-$	Neochlorogenic acid
4	16.74	247, 324	353.08798	191, 179, 173	$C_{16}H_{17}O_9^-$	Chlorogenic acid
5	17.70	247, 322	179.03460	135	$C_9H_7O_4^-$	Caffeic acid
6	22.98	279, 515	449.10706	287, 137	$C_{21}H_{21}O_{11}^+$	Cyanidin 3-*O*-glucoside
7	23.88	280, 517	595.16503	449, 287	$C_{27}H_{31}O_{15}^+$	Cyanidin 3-*O*-rutinoside
8	24.89	275, 501	433.11260	271	$C_{21}H_{21}O_{10}^+$	Pelargonidin 3-*O*-glucoside
9	25.38	309	163.03984	119	$C_9H_7O_3^-$	*p*-Coumaric acid
10	29.27	255, 354	609.14807	343, 301, 255	$C_{27}H_{29}O_{16}^-$	Rutin
11	30.10	242, 254, 354	463.08809	301, 179, 151	$C_{21}H_{19}O_{12}^-$	Quercetin 3-*O*-glucoside
12	39.01	233, 254, 369	301.03455	273, 179, 151	$C_{15}H_9O_7^-$	Quercetin

3.3.2 Hydroxycinnamic acids

The chromatograms indicated the presence of four hydroxycinnamic acids. Peaks 3 and 4 yielded $[M-H]^-$ at *m*/*z* 353, and showed the characteristic fragmentation peak of a quinic acid derivative at *m*/*z* 191. By comparing the retention time and UV-Vis spectra of standards, these compounds were identified as neochlorogenic acid (peak 3) and chlorogenic acid (peak 4), respectively (Table 1). Peak 5 was identified as caffeic acid (Álvarezfernández *et al.*, 2016) due to $[M-H]^-$ at 179 and a principal MS/MS fragment at *m*/*z* 135 (Table 1). Peak 9 had an $[M-H]^-$ at *m*/*z* 163 with molecular formula $C_9H_7O_3^-$, which fragmented to *m*/*z* 119, corresponding to the loss of carboxyl (44 Da) residue. This compound was identified as *p*-coumaric acid.

3.3.3 Anthocyanins

Three anthocyanins were identified in the positive ionization mode (Table 1). The MS/MS fragmentation suggested that $[M+H]^+$ at *m*/*z* 287 was due to cyanidin (peaks 6 and 7), which indicated the presence of cyanidin-glycosides. Peak 6, with *m*/*z* at 449, was identified as cyanidin 3-*O*-glucoside, and peak 7, with pseudo-molecular cation at *m*/*z* 595, which showed characteristic fragmentation peaks at *m*/*z* 449 and 287, due to the loss of rhamnose (146 Da) and glucose (162 Da) residues, was identified as cyanidin 3-*O*-rutinoside. Peak 8, with *m*/*z* at 433, which showed a fragmentation peak at *m*/*z* 271, was identified as pelargonidin 3-*O*-glucoside.

3.3.4 Flavonoids

Three flavonoids were identified (Table 1). Peak 10, exhibiting $[M-H]^-$ at *m*/*z* 609 with molecular formula $C_{27}H_{29}O_{16}^-$, which showed a fragmentation peak at *m*/*z* 301, due to loss of rhamnose (146 Da) and glucose (162 Da) residues, was identified as rutin. Peak 12, exhibiting $[M-H]^-$ at *m*/*z* 301 with fragments as a typical ion cluster at *m*/*z* 179 and 151, was identified as quercetin. Peak 11, with *m*/*z* at 463, which had fragmentation peaks at *m*/*z* 301, due to loss of glucose (162 Da) residue, was identified as quercetin 3-*O*-glucoside.

Several studies have shown that the amounts of anthocyanins, hydroxybenzoic acids, hydroxycinnamic acids, and flavonoids are dependent on the variety and cultivation conditions (Bao *et al.*, 2016). According to Sánchezsalcedo *et al*. (2015), 3 hydroxybenzoic acids, 6 hydroxycinnamic acids, 3 flavonoids, and 4 anthocyanins were found in the black mulberry, grown in the southeast region of Spain. The ultra-HPLC-MS analysis had allowed the tentative identification of 4 anthocyanins, 4 hydroxybenzoic acids, 13 hydroxycinnamic derivatives, 20 flavonols, 6 flavanones, 2 flavan-3-ols, 1 flavone, 1 flavanonol, and 1 dihydrochalcone (Mena *et al.*, 2016). On the other hand, 14 hydroxycinnamic acid esters, 13 flavonol glycosides, and 14 anthocyanins were identified in the mulberry fruits grown in Vojvodina, North Serbia (Natic *et al.*, 2015). However, only a total of 15 phenolics were identified in the mulberry fruits grown in Zhejiang Province, China (Bao *et al.*, 2016). The

varieties of phenolic compounds identified in the present study are similar to those reported previously (Isabelle *et al.*, 2008; Bao *et al.*, 2016), but are fewer than in other studies (Natic *et al.*, 2015; Sánchezsalcedo *et al.*, 2015; Mena *et al.*, 2016). The discrepancies could probably be attributed to fruit cultivar, environmental conditions, and the degree of maturity. Furthermore, the phenolic compounds of mulberry juice may be affected by the technological processing parameters. The analysis of mass balance of raspberry juice and press cake had indicated that many phenolics, mostly tannins (98.0%) and flavanols (87.7%), were retained in the press cake (Sojka *et al.*, 2016). The different types of flavonoids in mulberry juice were fewer than those in mulberry fruit in previous studies, probably because of great losses during filtration and centrifugation.

3.4 Changes in the amount of individual phenolic compounds during storage

Little information appears to be available on the changes in both free and bound forms of individual phenolic compounds in mulberry juice during storage. Most studies were concerned with the amount of free phenolics in mulberry (Fazaeli *et al.*, 2013; Tomas *et al.*, 2015; Wang *et al.*, 2015). This is the first study to show the changes in the concentrations of individual phenolics in the sediment of mulberry juice during storage.

The predominant phenolic compounds in Da10 mulberry fruit were anthocyanins, particularly cyanidin 3-*O*-glucoside and cyanidin 3-*O*-rutinoside. As shown in Table 2, anthocyanins (cyanidin 3-*O*-glucoside, cyanidin 3-*O*-rutinoside, and pelargonidin 3-*O*-glucoside) in the supernatant of the juice decreased rapidly during storage, from 938.60 to 2.30 mg/L. The amounts of cyanidin 3-*O*-glucoside and pelargonidin 3-*O*-glucoside reached levels below the detection limit after storage for 8 weeks. In case of F-P in the juice sediment, anthocyanins dropped by 99% after storage for 8 weeks. Interestingly, anthocyanins were not observed in B-E or B-W initially, but they were detected on storage from weeks 2 to 8. This suggested that anthocyanins in juice supernatant were highly susceptible to degradation during storage at 30 °C. The degradation was possibly caused by oxidation and condensation reactions with other phenolics (Reque *et al.*, 2014). Simultaneously, some degraded anthocyanins could have been changed to bound phenolics in the juice sediment. This shows that anthocyanins were associated with the formation of the sediment.

Protocatechuic acid content of the supernatant increased with time, reaching a value 35.9 times its initial value after 8 weeks in B-E. However, the change in protocatechuic acid content of F-P was not significant. The increase in protocatechuic acid content in supernatant could be attributed to the degradation of anthocyanins, as protocatechuic acid is a constituent of the B-ring of anthocyanins (Sadilova *et al.*, 2006). It was reported that anthocyanins were susceptible to thermal degradation and were readily transformed into the intermediate chalcone, which in turn instantly cleaved to form protocatechuic acid (Sadilova *et al.*, 2007). Our results were consistent with previous studies (Sadilova *et al.*, 2007;

Table 2 Changes in amount of individual phenolics in mulberry juice during storage

Phenolics	Week	Supernatant (mg/L)	F-P (mg/g)	B-E (mg/g)	B-W (mg/g)
Cyanidin 3-*O*-glucoside	0	450.40±11.30^{d}	150.7±10.6^{e}		
	2	82.30±5.10^{c}	38.6±4.3^{d}	0.48±0.03^{a}	2.13±0.02^{a}
	4	13.70±0.62^{b}	9.3±0.8^{c}	1.08±0.05^{b}	11.75±0.46^{d}
	6	3.26±0.16^{a}	2.7±0.6^{b}	1.07±0.04^{b}	9.40±0.72^{c}
	8		0.9±0.1^{a}	1.08±0.02^{b}	6.08±0.42^{b}
Cyanidin 3-*O*-rutinoside	0	458.80±15.10^{e}	76.83±6.99^{e}		
	2	106.40±6.30^{d}	16.05±0.65^{d}	0.44±0.02^{a}	2.17±0.06^{a}
	4	22.82±1.07^{c}	5.40±0.43^{c}	0.68±0.08^{c}	11.36±0.17^{d}
	6	6.36±0.17^{b}	1.92±0.15^{b}	0.66±0.08^{c}	9.46±0.24^{c}
	8	2.30±0.16^{a}	0.74±0.024^{a}	0.57±0.02^{b}	6.49±0.37^{b}
Pelargonidin 3-*O*-glucoside	0	29.30±0.80^{c}	8.07±0.74^{e}		
	2	6.92±0.17^{b}	2.27±0.06^{d}	0.35±0.06^{b}	1.07±0.09^{b}

To be continued

Table 2

Phenolics	Week	Supernatant (mg/L)	F-P (mg/g)	B-E (mg/g)	B-W (mg/g)
Pelargonidin 3-*O*-glucoside	4	1.52±0.11^{a}	0.77±0.02^{c}	0.23±0.01^{a}	1.34±0.09^{c}
	6		0.21±0.01^{b}	0.23±0.01^{a}	0.99±0.04^{b}
	8		0.085±0.004^{a}	0.22±0.02^{a}	0.75±0.02^{a}
Gallic acid	0			0.041±0.004^{a}	
	2			0.402±0.023^{b}	
	4			0.761±0.074^{c}	
	6			0.842±0.054^{c}	
	8			1.448±0.095^{d}	
Protocatechuic acid	0	22.96±1.25^{a}	0.195±0.014	0.138±0.073^{a}	
	2	51.54±3.69^{b}	0.171±0.013	2.022±0.116^{b}	
	4	53.77±0.89^{b}	0.181±0.090	2.450±0.096^{bc}	
	6	62.82±1.09^{c}	0.180±0.094	2.792±0.053^{c}	
	8	78.59±4.09^{d}	0.168±0.018	4.954±0.240^{d}	
Neochlorogenic acid	0	18.34±1.10	0.0598±0.0029^{d}	0.0351±0.0012^{c}	
	2	20.68±2.75	0.0253±0.0023^{ab}	0.0302±0.0005^{c}	
	4	18.64±0.91	0.0412±0.0015^{c}	0.0154±0.0024^{b}	
	6	17.80±1.13	0.0305±0.0017^{bc}	0.0134±0.0007^{b}	
	8	17.47±0.83	0.0155±0.0011^{a}	0.0077±0.0002^{a}	
Chlorogenic acid	0	319.9±31.3^{c}			
	2	274.7±34.5^{bc}			
	4	261.9±33.1^{b}			
	6	223.7±12.7^{a}			
	8	220.0±8.6^{a}			
p-Coumaric acid	0	13.37±4.41^{b}	0.0804±0.0073^{c}		
	2	10.01±1.81^{b}	0.0340±0.0018^{ab}		
	4	5.47±0.93^{a}	0.0202±0.0021^{a}	0.017±0.001^{a}	
	6	4.69±1.14^{a}	0.0060±0.0005^{b}	0.020±0.001^{b}	
	8	4.42±0.41^{a}	0.0050±0.0006^{b}	0.024±0.002^{c}	
Rutin	0	352.8±48.2^{d}	2.34±0.06^{b}		
	2	314.1±16.8^{c}	1.63±0.08^{b}	0.041±0.006^{a}	
	4	293.1±14.0^{bc}	0.14±0.03^{a}	0.158±0.010^{b}	
	6	265.4±22.3^{ab}	0.19±0.01^{a}	0.161±0.008^{b}	
	8	241.6±10.8^{a}	0.22±0.01^{a}	0.164±0.005^{b}	
Quercetin 3-*O*-glucoside	0	6.73±0.76^{d}	0.080±0.002^{a}		
	2	5.95±0.90^{bc}	0.120±0.002^{ab}		
	4	5.57±0.27^{b}	0.142±0.007^{b}		
	6	5.00±0.26^{ab}	0.142±0.008^{b}		
	8	4.38±0.22^{a}	0.118±0.008^{ab}		
Quercetin	0		0.096±0.005^{a}		
	2		0.895±0.064^{b}		
	4		0.870±0.026^{b}		
	6		0.937±0.036^{b}		
	8		0.909±0.029^{b}		
Caffeic acid	0			0.021±0.002^{a}	
	2			0.097±0.007^{b}	
	4			0.169±0.041^{c}	
	6			0.203±0.023^{c}	
	8			0.239±0.018^{c}	

F-P, soluble free phenolics in sediment; B-E, bound phenolics in ethyl acetate fraction; B-W, bound phenolics in water fraction. Data are expressed as mean±SD (n=3). Different alphabets in the same phenolics along the same column indicate significant differences (P<0.05)

Álvarezfernández *et al.*, 2016). The other hydroxybenzoic acid (gallic acid) was detected only in B-E, which increased significantly to 35.3 times its initial value after storage for 8 weeks. This result indicated that gallic acid existed as insoluble bound complexes. In our previous study, gallic acid was determined in mulberry juice only after hydrolysis with NaOH (Yu *et al.*, 2014). Previous studies reported that gallic acid in Chinese bayberries increased gradually during storage over a period of 6 d (Wang *et al.*, 2009). However, the reason for the increase in gallic acid was unclear.

Four hydroxycinnamic acids in mulberry juice were also quantified. Neochlorogenic acid content in the supernatant showed no significant changes, while chlorogenic acid and *p*-coumaric acid content decreased gradually during storage. Only 6.2% of *p*-coumaric acid was retained in F-P of the supernatant within the first 2 weeks. It was also observed that caffeic acid, a primary hydrolysis product of chlorogenic acid (Farah and Duarte, 2015), was detected in B-E, and its amount increased during storage. *p*-Coumaric acid concentration reached a level below the detection limit in B-E in the initial and second weeks, and it then increased with time. These results suggested that the free hydroxycinnamic acids could have combined with other compounds to form the sediment. As Rentzsch *et al*. (2007) reported, there was a direct reaction between free hydroxycinnamic acids and anthocyanins leading to the formation of hydroxyphenyl-pyranoanthocyanins. In the present study, an increase in bound forms of anthocyanins and hydroxycinnamic acids was found to be associated with a decrease in the free forms.

In the supernatant of the juice, rutin and quercetin 3-*O*-glucoside content decreased (31.5% and 33.1%, respectively) after storage for 8 weeks, while in the free phenolic portion of the sediment, an increase in quercetin content was observed. In addition to this, quercetin 3-*O*-glucoside in F-P increased until 6 weeks of storage and then decreased. These results indicated that the flavonoids could have been hydrolyzed to release quercetin (Álvarezfernández *et al.*, 2016). Furthermore, in B-E, an increase in rutin was observed during storage time. Based on these results, it can be said that the flavonoids participated in the formation of precipitate.

During storage of the juice, all the phenolic compounds in the supernatant degraded, except protocatechuic acid and neochlorogenic acid, with anthocyanins showing the greatest losses. Our results were consistent with the findings of Reque *et al*. (2014). It was reported that the pasteurization treatment facilitated the rupture of cellular structures, thereby enhancing the exposure of substrates to nonenzymatic oxidations during storage (Oliveira *et al.*, 2014). This was one of the main reasons for the loss of free phenolics in the supernatant and sediment. Moreover, it must be highlighted that the concentration of certain bound phenolics in sediment, such as protocatechuic acid, caffeic acid, and rutin, increased during storage. Anthocyanin content also increased in the first 4 weeks and then decreased. Anthocyanins were not detected at the beginning of storage, but the content was higher than other phenolic compounds in the later stages. It is proposed that these phenolics contributed to the sediment formation by interacting with other chemical compounds (e.g. protein), through mechanisms involving polymerization or oxidation. This resulted in the formation of high molecular weight complexes that precipitated out as sediments. Therefore, it can be concluded that phenolic compounds, particularly anthocyanins, participated in sediment formation during storage. Indeed, the sum of the identified individual phenolic compounds in F-P (B-E, B-W) after storage for 8 weeks was 3.13 mg/g (8.17 mg/g, 13.32 mg/g), accounting for 2.2% (70.5%, 68.3%) of the total phenolic content of the Folin-Ciocalteu assay. The HPLC profiles of F-P and B-E showed a hump and many resolved peaks between retention time of 40 and 46 min in the sediment (data not shown). The results suggested that some of the phenolics in the sediment could be complex polymers (Prakash *et al.*, 2016) or unidentified compounds. Our results were consistent with the sediments of clarified bayberry juice (25.5% of identified individual phenolics) (Fang *et al.*, 2006) and black juice (10.8% of identified individual phenolics) (Siriwoharn *et al.*, 2005). Further purification steps and optimization of HPLC-MS/MS analysis are still necessary to gain further insight into the structures of these compounds.

3.5 Effect of storage time on antioxidant capacity

Fig. 3 shows changes in the DPPH free radical-scavenging activity and ORAC during storage. The DPPH and ORAC results indicated that the antioxidant activity of the supernatant and F-P decreased

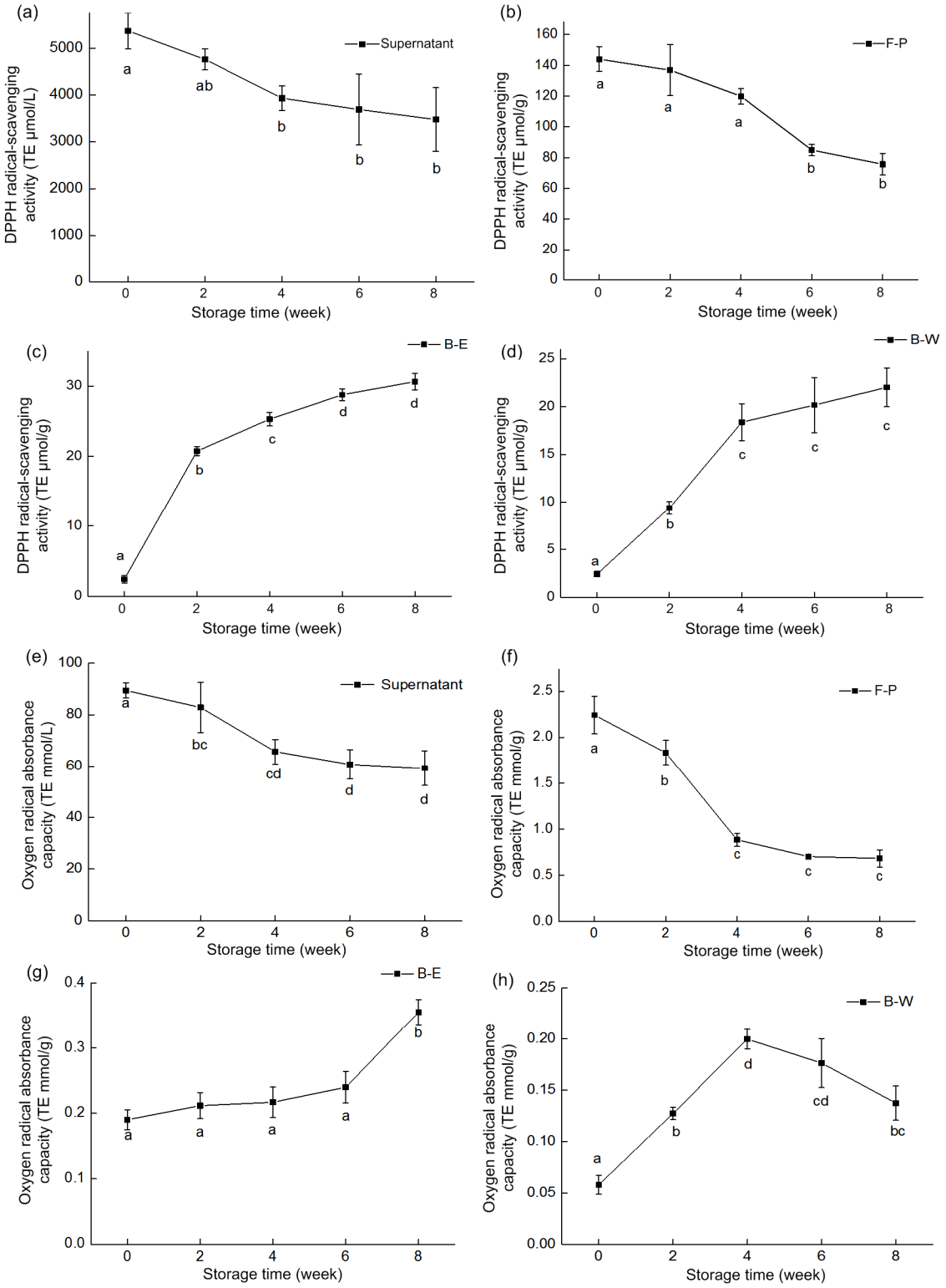

Fig. 3 Changes in DPPH radical-scavenging activity (a–d) and oxygen radical absorbance capacity (ORAC) (e–h) of mulberry juice during storage

Data are expressed as mean±SD (n=3). Different alphabets indicate significant differences (P<0.05). F-P, soluble free phenolics in sediment; B-E, bound phenolics in ethyl acetate fraction; B-W, bound phenolics in water fraction

gradually. Similar results were previously observed in grapefruit juice (Lacava and Sgroppo, 2015). The decrease in antioxidant activity could be attributed to the decrease in phenolic content. Interestingly, progressive increases in B-E and B-W were observed in the DPPH assay. In the case of the ORAC assay, the same trends were observed in the supernatant, F-P, and B-E. A similar behavior was reported in the analysis of antioxidant activity in sour cherry puree stored at 30 °C (Nowicka and Wojdyło, 2016). The increase in the overall antioxidant activity could probably be attributed to the formation of bound phenolics of the sediment from supernatant free phenolic compounds. However, the antioxidant activity of B-W increased until 4 weeks of storage and then decreased, which was not consistent with the DPPH results. These results were similar to those for a beverage obtained from strawberry fermentation (Álvarezfernández *et al.*, 2016). The discrepancy between DPPH and ORAC results could be due to differences in the reaction mechanisms in both cases.

4 Conclusions

This study investigated the changes in phenolic contents in clarified mulberry juice during storage. The results suggested that anthocyanins were degraded to a great extent in the supernatant, from 938.60 to 2.30 mg/L. On the other hand, non-anthocyanin phenolic compounds underwent fewer changes. The changes in free phenolics in sediment were similar to that in the supernatant, and the total phenolic content decreased by 39.0%. However, nine phenolic compounds were identified as bound phenolics in the sediment. Certain phenolics, such as gallic, protocatechuic, and caffeic acid showed increase in their content during storage. In addition to this, the content of bound anthocyanins was higher than hydroxybenzoic acids and hydroxycinnamic acids. After 8 weeks of storage, the amount of anthocyanins in the sediment accounted for 67.2% of the sum of individual phenolics. The results clearly indicated that phenolic compounds were responsible for sediment formation in mulberry juice. Although the present study focused on the phenolics of the sediment, in fact other components such as proteins, pectin, and cell wall fragments could participate in that formation sediment. The mechanism of interactions between phenolics and other chemical compounds needs further research. Taken together, our results provide significant information for improving the quality of mulberry juice.

Compliance with ethics guidelines

Bo ZOU, Yu-juan XU, Ji-jun WU, Yuan-shan YU, and Geng-sheng XIAO declare that they have no conflict of interest.

This article does not contain any studies with human or animal subjects performed by any of the authors.

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中文概要

题　目：酚类物质参与桑果汁贮藏期间沉淀形成的研究

目　的：探讨澄清型桑果汁贮藏期间酚类物质的变化规律，明确沉淀形成的主要物质基础。

创新点：首次研究了桑果汁贮藏期间上清和沉淀中酚类物质的变化规律，阐明了酚类物质参与桑果汁沉淀形成的机制，明确了花色苷是沉淀形成的主要物质基础。

方　法：对贮藏期间桑果汁的上清和沉淀进行了分离，提取了游离态和结合态多酚，对总酚含量的变化进行了研究，并采用高效液相色谱法和高效液相色谱质谱联用法对酚类化合物的组成进行了定性和定量分析。

结　论：随着贮藏时间的延长，桑果汁的沉淀量逐渐增加（图 1），果汁上清液和沉淀游离酚的总酚含量逐渐下降，而沉淀结合酚则呈现上升趋势（图 2）。上清液和沉淀中游离态花色苷分别从 938.60 和 235.60 mg/L 下降到 2.30 和 1.74 mg/L（表 2），上清中黄酮类物质也有所下降，而沉淀中结合态的没食子酸、原儿茶酸、咖啡酸和芦丁的含量则呈上升趋势（表 2）。桑果汁贮藏 8 周后，沉淀中的花色苷占酚类物质总量的 67.2%（表 2）。综上所述，酚类物质参与了桑果汁沉淀的形成，其中花色苷起主要作用。

关键词：桑果汁；酚类物质；沉淀；花色苷；抗氧化