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Original article

Development of a green two-dimensional HPLC-DAD/ESI-MS method for the determination of anthocyanins from *Prunus cerasifera* var. *atropurpurea* leaf and improvement of their stability in energy drinks

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Summary This study aimed to develop a green two-dimensional HPLC-DAD/ESI-MS method for analysing anthocyanins from *Prunus cerasifera* var. *atropurpurea* leaf and improve their stability in energy drinks by the addition of phenolic acids. Ethanol and tartaric acid solutions were used as mobile phases for one-dimensional HPLC-DAD for quantitative analysis of anthocyanins, and the primary anthocyanins were identified as cyanidin-3-*O*-galactoside, cyanidin-3-*O*-glucoside and cyanidin-3-*O*-rutinoside using two-dimensional HPLC-MS. Method validation showed that the developed method was accurate, stable and reliable for the analysis of *P. cerasifera* anthocyanins. The effects of gallic, ferulic and caffeic acid on the stability of cyanidin-3-*O*-galactoside, cyanidin-3-*O*-glucoside, cyanidin-3-*O*-rutinoside and total anthocyanins from *P. cerasifera* leaf in energy drinks were evaluated, and the degradation of *P. cerasifera* anthocyanins ideally followed a first-order model ($R^2 > 0.98$). Gallic acid showed stronger protective effects on *P. cerasifera* anthocyanins in energy drinks, and adding/increasing ferulic and caffeic acids accelerated the degradation reactions.

Keywords Anthocyanins, degradation kinetics, gallic acid, green analytical chemistry, natural pigments, two-dimensional liquid chromatography.

Introduction

Prunus cerasifera var. *atropurpurea* (Rosaceae) is an ornamental tree species and is known as 'purple-leaf plum' because of its year-round purple or red leaves (Figure S1) (Zhang *et al*., 2013). *P. cerasifera* leaves are rich in bioactive constituents, such as phenolic acids, flavonoids and saponins (Wei *et al*., 2015), and its extract possesses potential antioxidant activity (Hu *et al*., 2002). *P. cerasifera* leaf is also an excellent source of natural edible pigments (Hou *et al*., 2011), and anthocyanins are the main constituents of red pigments (Gao *et al*., 2014). The extraction of anthocyanins from the *P. cerasifera* leaf has been investigated (Zhang *et al*., 2013); however, there are few reports on the further application of these anthocyanins. Therefore, the development of *P. cerasifera* anthocyanins is desired.

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Currently, anthocyanins are being developed as food additives in beverages due to their attractive colour and potential health benefits (Aguilera *et al*., 2016; Chung *et al*., 2017; He *et al*., 2017). The application of anthocyanins as natural food colourants is limited by relatively low stability, and some constituents have been used to increase anthocyanin stability (Xu *et al*., 2015; Chung *et al*., 2017; Qian *et al*., 2017). Phenolic acids are considered powerful copigments of anthocyanins, such as gallic acids, ferulic acids and caffeic acids (Eiro & Heinonen, 2002; Rein & Heinonen, 2004; Zhang *et al*., 2015; Navruz *et al*., 2016). Anthocyanins from the *P. cerasifera* leaf can be used in energy drinks to improve their acceptability and nutritional value. Studies are necessary to increase the stability of *P. cerasifera* anthocyanins.

With the development of green analytical chemistry, green chromatography has gained more attention from scientists and stresses the reduction in solvent consumption and replacement of toxic and environmentally

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hazardous solvents with more benign alternatives (Płotka *et al*., 2013). Liquid chromatography has been frequently used in food and medicine fields, and many HPLC methods have been developed for analysing target constituents such as isoflavonoids, sweeteners, caffeine, alkaloids, statins and clonazepam using some benign mobile phase additives (e.g. ethanol, cyclodextrins and deep eutectic solvents); however, acetonitrile and methanol were used in most of these analytical processes (Sik, 2012; Zeng *et al*., 2012a, 2012b; Eldin *et al*., 2014; Assassi *et al*., 2015; Tan *et al*., 2016). Acetonitrile, methanol, trifluoroacetic acid and high concentration of formic acid solutions were frequently used as HPLC mobile phases to analyse anthocyanins from various sources (Welch *et al*., 2008; Barnes *et al*., 2009; Zheng *et al*., 2011; Su *et al*., 2016). Sang *et al*. (2017a) have used ethanol-based mobile phase and investigated the effects of acids on HPLC analysis of anthocyanins; however, 5% (v/v) formic acid aqueous solution was still used in the developed analytical method. Formic acid is highly volatile, has a pungent odour and can damage health. For developing more work-safe mobile phase additives, Sang *et al*. (2017b) have investigated the effects of various fruit acid aqueous solutions on the chromatographic behaviours of anthocyanins and demonstrated that ethanol and a tartaric acid aqueous solution can be used as HPLC mobile phases for the determination of anthocyanins from haskap berry, mulberry and blackberry. To date, no detailed studies have been published for accurate and reliable HPLC analysis of *P. cerasifera* anthocyanins. Therefore, a green and efficient analytical method should be developed for qualitative and quantitative analyses of anthocyanins from the *P. cerasifera* leaf.

Two-dimensional liquid chromatography provides enlarged peak capacity and enhanced selectivity and is a potentially suitable approach for the separation and identification of complex samples (Zeng *et al*., 2012a, b). In this study, a green two-dimensional HPLC-DAD/ESI-MS method was developed for qualitative and quantitative analyses of anthocyanins from *P. cerasifera* leaf using work-safe and environmentally friendly mobile phases. *P. cerasifera* anthocyanins were used in energy drinks, and the effects of gallic, ferulic and caffeic acids on their stability were determined using an accelerated thermal stability assay and a first-order reaction kinetic model.

Materials and methods

Plant material and reagents

Prunus cerasifera leaf was collected from Xi'an, China. The material was ground using a grinder and stored at −20 °C. Cyanidin-3-*O*-glucoside (purity ≥ 98%) was purchased from Chengdu Must Bio-Technology Co., Ltd. (Chengdu, China). DL-tartaric acid (≥98.5%) was purchased from the Aladdin Industrial Corporation (Shanghai, China). HPLC-grade formic acid was purchased from Tianjin Kermel Chemical Reagent Co., Ltd. (Tianjin, China). HPLC-grade ethanol was purchased from Fisher Chemicals Co., Ltd. (Loughborough, Leicestershire, UK). The commercial energy drink was purchased from Danone Food & Beverages Ltd. (Xi'an, China). All other chemicals and reagents were of analytical grade, and deionised water was used in all experiments.

Preparation of anthocyanins and model solutions

Extraction of anthocyanins was performed according to a previous report (Zhang *et al*., 2013). Briefly, 100 g of material was ultrasonically extracted (300 W, 30 kHz) using 5100 mL methanol (0.1% HCl, v/v) for 5 min at 78 °C, and the extract was centrifuged at 3000 r min^{-1} for 5 min. The supernatant was concentrated using a rotary evaporator at 40 °C to remove methanol. The concentrate was dissolved using 255 mL water (0.1% HCl, v/v) and loaded onto a LS-32 macroporous resin column (16 × 50 mm; bed volume of 60 mL) at a flow rate of 1 mL min^{-1}. The adsorbate-laden column was washed with 240 mL of deionised water (0.1% HCl, v/v), and 180 mL of 50% aqueous ethanol (0.1% HCl, v/v) was used to desorb anthocyanins from LS-32 macroporous resin. The desorption solution was concentrated using rotary evaporation at 40 °C for later experiments and dissolved using a commercial energy drink to a final concentration of total anthocyanins of 0.22 mg mL^{-1}. Subsequently, various phenolic acids (gallic acid, ferulic acid and caffeic acid) were added and dissolved in the model solutions (pH = 3.0).

Thermal treatment

The thermal stability of *P. cerasifera* anthocyanins in the model solutions was determined using an accelerated thermal stability assay in a water bath at 90 °C. An aliquot of 0.5 mL of model solutions was collected with an interval of 30 min and analysed using HPLC-DAD after cooling in an ice bath for 5 min. A first-order model was used to evaluate the degradation of *P. cerasifera* anthocyanins, and the reaction rate constant (k) and half-life ($t_{1/2}$) were calculated using Eq. (1) and (2) (Hernández-Herrero & Frutos, 2011; Sang *et al*., 2017c).

$$\ln(C_t/C_0) = -k \times t \quad (1)$$

$$t_{1/2} = -\ln 0.5/k \quad (2)$$

where C_0 is the initial concentration of anthocyanins, and C_t is the concentration of anthocyanins after t min of heating.

HPLC-DAD analysis of anthocyanins

Anthocyanins were analysed using an Ultimate 3000 HPLC (Thermo Fisher Scientific Co., Ltd., Waltham, MA, USA) equipped with a quaternary gradient pump, autosampler, diode array detector and thermostatically controlled column oven with a six-way valve. An Agilent ZORBAX Eclipse Plus C18 column (100 × 4.5 mm, 3.5 μm particle, Agilent Technologies Co., Ltd., Santa Clara, CA, USA) was used as an analytical column; a mixture of ethanol and tartaric acid solution (0.25 mol L^{-1}) at a 13:87 (v/v) ratio was used as the mobile phase; the analytical process was 7 min; injection volume was 5 μL; detection wavelength was 520 nm; flow rate was 1 mL min^{-1}; and column temperature was 35 °C. A cyanidin-3-*O*-glucoside standard was used to quantify anthocyanins from the *P. cerasifera* leaf, and the content of total anthocyanins was approximated using the sum of the peak areas of the HPLC-DAD chromatogram.

HPLC-MS/MS analysis of anthocyanins

Identification of anthocyanins was performed using an Agilent 1290 series HPLC coupled to an Agilent 6460C triple quadrupole mass spectrometer with an AJS electrospray ionisation source (Agilent Technologies Co., Ltd.). An Agilent ZORBAX Eclipse Plus C18 column (100 × 4.5 mm, 3.5 μm particle, Agilent Technologies Co., Ltd.) was used as an analytical column. The mobile phase was a mixture of ethanol and aqueous formic acid (0.1%, v/v), and a gradient elution programme was performed as follows: (i) 10–15% of ethanol, from 0 to 10 min; and (ii) 15–20% ethanol, from 10 to 15 min. Other analytical conditions were a flow rate of 0.5 mL min^{-1}, injection volume of 20 μL, detection wavelength of 520 nm, column temperature of 35 °C, capillary voltage of 3500 V, sheath gas temperature of 350 °C, sheath gas flow rate of 12.0 L min^{-1}, nebuliser pressure of 45 psi, drying gas temperature of 300 °C, drying gas flow rate of 6.0 L min^{-1} and scan range of 100–1000 m/z in the positive mode.

Statistical analysis

Statistical analyses were performed using Microsoft Office Excel 2007 and GraphPad Prism v.5.0 (GraphPad Software, Inc., San Diego, CA, USA). One-way analysis of variance and Tukey's multiple comparison test were used to determine the statistical significance; $P < 0.05$ was considered significant.

Results and discussion

Anthocyanin profiling

Four anthocyanins were profiled using an HPLC-MS/MS method, and all were cyanidin derivatives (MS/MS = m/z 287) (Fig. 1 and Figure S2). The first (retention time = 7.993 min) and second (retention time = 8.853 min) anthocyanins exhibited a similar fragmentation pattern ($MS^+ = m/z$ 449; MS/MS = m/z 287) (Figure S2a and b), and the neutral loss of 162 Da may be a hexose residue. MS could not distinguish the glycosidic isomers, such as glucoside and galactoside (162 Da) (Sang *et al.*, 2017a), and other methods were desired for further identification. By comparing the retention times of the *P. cerasifera* anthocyanins and cyanidin-3-*O*-glucoside standard, the second anthocyanin (retention time = 8.853 min) was determined as cyanidin-3-*O*-glucoside. As a result, the first anthocyanin (retention time = 7.993 min) was identified as cyanidin-3-*O*-galactoside. The third anthocyanin (retention time = 9.923 min; $MS^+ = m/z$ 595) gave a fragment ion of m/z 287 via the neutral loss of 308 Da (Figure S2c) and was identified as cyanidin-3-*O*-rutinoside (Gao *et al.*, 2014; Garcia-Herrera *et al.*, 2016). According to the MS data ($MS^+ = m/z$ 419; MS/MS = m/z 287) (Figure S2d), the fourth anthocyanin (retention time = 9.923 min) was determined as cyanidin-3-*O*-arabinoside (Gao *et al.*, 2014; Garcia-Herrera *et al.*, 2016).

Two-dimensional HPLC-DAD/ESI-MS analysis of anthocyanins

A green HPLC-DAD method was developed for quantitative analysis of *P. cerasifera* anthocyanins using a mixture of ethanol and tartaric acid solution as the mobile phase (Fig. 2). Peaks 1, 2 and 3 accounted for 54.5%, 32.2% and 11.2% of the total anthocyanins, respectively. A six-way valve was used to isolate anthocyanin peaks 1, 2 and 3, and the interval range of the three target peaks was 3.5–4.0 min, 4.1–4.6 min and 4.7–5.2 min, respectively (Fig. 2a–c). According to the retention time (Fig. 1a–c) and MS data (Fig. 3), peaks 1 ($MS^+ = m/z$ 449; MS/MS = m/z 287), 2 ($MS^+ = m/z$ 449; MS/MS = m/z 287) and 3 ($MS^+ = m/z$ 595; MS/MS = m/z 287) were determined as cyanidin-3-*O*-galactoside, cyanidin-3-*O*-glucoside and cyanidin-3-*O*-rutinoside, respectively.

Method validation of HPLC-DAD method

Linearity and limits of detection and quantification

Cyanidin-3-*O*-glucoside standard solutions (0.02, 0.04, 0.08, 0.12, 0.16 and 0.20 mg mL^{-1}) were used to prepare a calibration curve. The calibration curve was given by Y (peak area) = 285.94 × X (cyanidin-3-*O*-glucoside concentration) – 1.2629 ($R^2 = 0.9995$). The limits of detection (signal-to-noise ratio of 3) and quantitation (signal-to-noise ratio of 10) were 0.000358 mg mL^{-1} and 0.001196 mg mL^{-1}, respectively.

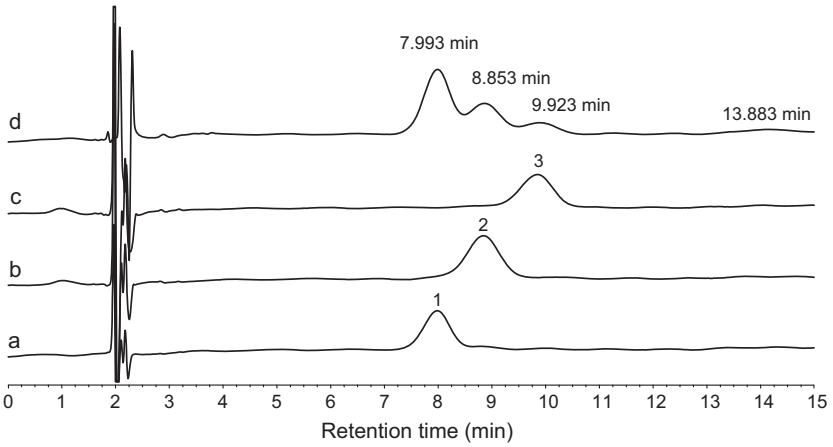

Figure 1 HPLC-MS chromatograms of cyanidin-3-*O*-galactoside (a), cyanidin-3-*O*-glucoside (b), cyanidin-3-*O*-rutinoside (c) and total anthocyanins (d) from *P. cerasifera* leaf. The mobile phase was a mixture of ethanol and aqueous formic acid (0.1%, v/v), and a gradient elution programme was performed as follows: (i) 10–15% of ethanol, from 0 to 10 min; and (ii) 15–20% ethanol, from 10 to 15 min. The flow rate was 0.5 mL min^{-1}; injection volume was 20 μL; detection wavelength was 520 nm; and column temperature was 35 °C.

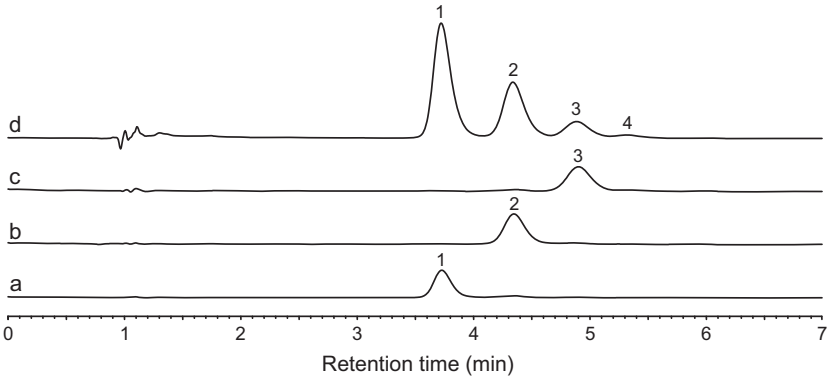

Figure 2 HPLC-DAD chromatograms of cyanidin-3-*O*-galactoside (a), cyanidin-3-*O*-glucoside (b), cyanidin-3-*O*-rutinoside (c) and total anthocyanins (d) from *P. cerasifera* leaf. The mobile phase was a mixture of ethanol and tartaric acid solution (0.25 mol L^{-1}) at a 13:87 (v/v) ratio; the analytical process was 7 min; injection volume was 5 μL; detection wavelength was 520 nm; flow rate was 1 mL/min; and column temperature was 35 °C.

Precision, repeatability, stability and recovery

The intraday precision and interday precision of the HPLC-DAD method were investigated by analysing a sample in triplicate on two consecutive days. The repeatability was evaluated by analysing six replicate samples. Stability was determined by analysing a sample at various times (0, 30, 60, 90, 120, 150 and 180 min) after preparation, respectively. For recovery (%), the known amounts of cyanidin-3-*O*-glucoside (0.1, 0.2 and 0.4 mg) were spiked into samples, and the mixtures were analysed using the HPLC-DAD method (Figure S3) (Sang *et al.*, 2017b). The results of intraday precision, interday precision, repeatability, stability and recovery are shown in Table 1, and the RSD (relative standard deviation) was all below the recommended value of 5%. In addition, the mean recovery was above 97.50%. Therefore, the developed HPLC-DAD method was accurate, stable and reliable for analysing anthocyanins from *P. cerasifera* leaf.

Effects of phenolic acids on anthocyanin stability

The effects of gallic, ferulic and caffeic acid on the stability of *P. cerasifera* anthocyanins in energy drink model solutions were evaluated using an accelerated stability test and a first-order reaction kinetic model. Table 2 shows the degradation kinetic parameters of cyanidin-3-*O*-galactoside, cyanidin-3-*O*-glucoside, cyanidin-3-*O*-rutinoside and total anthocyanins from the *P. cerasifera* leaf in model solutions with or

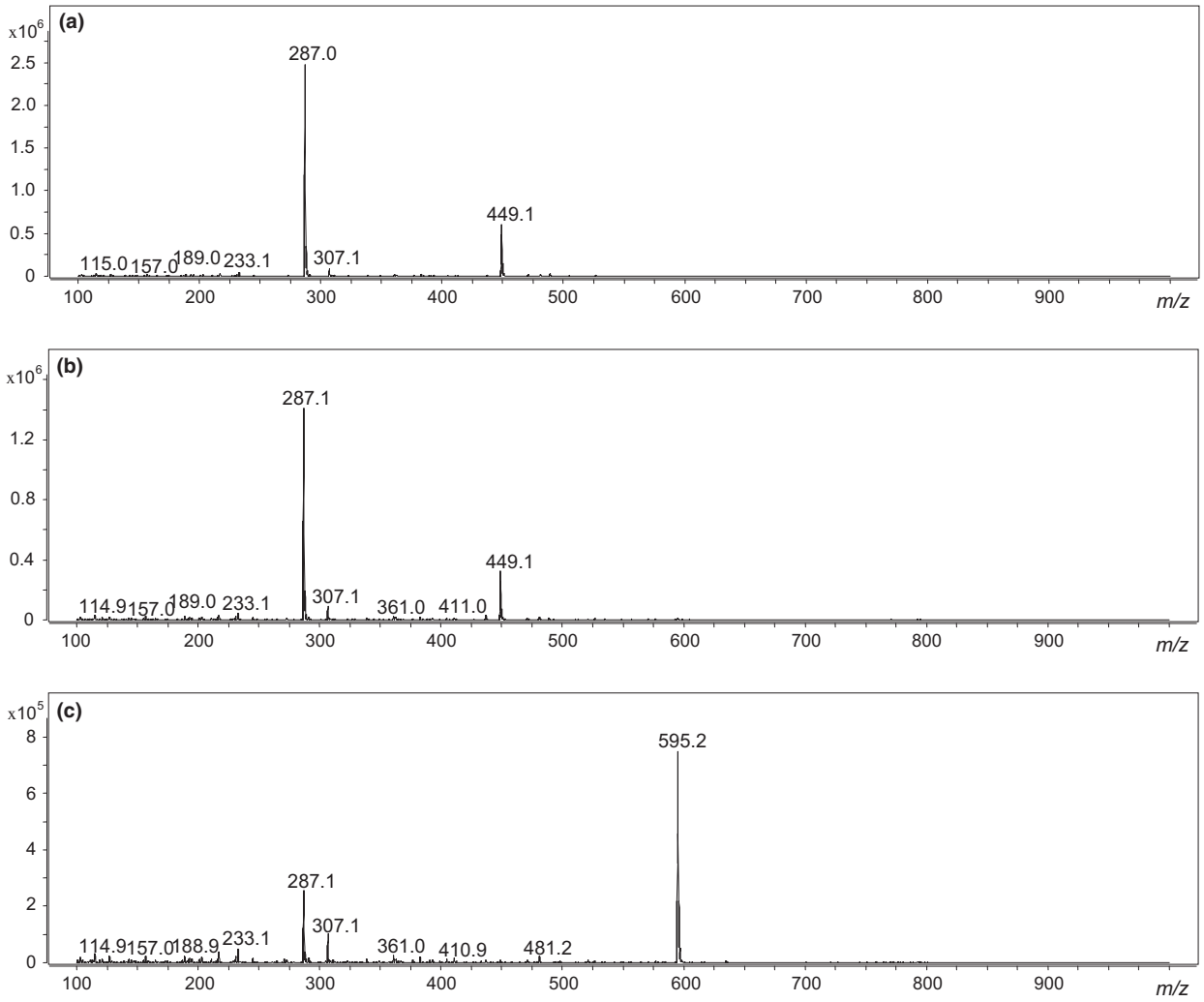

Figure 3 Mass spectra of cyanidin-3-*O*-galactoside (a), cyanidin-3-*O*-glucoside (b) and cyanidin-3-*O*-rutinoside (c) from *Prunus cerasifera* leaf identified using two-dimensional HPLC-DAD/ESI-MS.

Table 1 Intraday precision and interday precision, repeatability, stability and recovery of anthocyanins from *Prunus cerasifera* leaf

	Precision (RSD%)				Recovery (%) (n = 3)	
Anthocyanins	**Intraday precision (n = 3)**	**Interday precision (n = 6)**	**Repeatability RSD% (n = 6)**	**Stability RSD% (n = 6)**	**Mean**	**RSD**
Cyanidin-3-*O*-galactoside	1.28	1.09	4.45	1.21	–	–
Cyanidin-3-*O*-glucoside	1.06	1.11	4.24	1.19	97.51	2.17
Cyanidin-3-*O*-rutinoside	1.07	1.80	3.70	0.72	–	–
Total anthocyanins	2.34	2.19	4.27	1.21	99.94	2.45

without phenolic acids. The thermal degradation of *P. cerasifera* anthocyanins ideally follows a first-order model ($R^2 > 0.98$). In model solutions with or without phenolic acids, cyanidin-3-*O*-rutinoside showed the highest thermal stability, followed by cyanidin-3-*O*-glucoside, total anthocyanins and cyanidin-3-*O*-galactoside. Rubinskiene *et al.* (2005) have reported that the thermal stability of cyanidin-3-rutinoside was

Table 2 Thermal degradation kinetics of *Prunus cerasifera* anthocyanins in energy drink model solutions

		Cyanidin-3-*O*-galactoside			Cyanidin-3-*O*-glucoside			Cyanidin-3-*O*-rutinoside			Total anthocyanins		
Phenolic acids	**Concentration (mg/100 mL)**	k (min^{-1})	$t_{1/2}$ (min)	R^2	k (min^{-1})	$t_{1/2}$ (min)	R^2	k (min^{-1})	$t_{1/2}$ (min)	R^2	k (min^{-1})	$t_{1/2}$ (min)	R^2
Control	0	0.0090	77.00	0.9876	0.0056	123.75	0.9883	0.0049	141.43	0.9863	0.0069	100.43	0.9883
Gallic acid	20	0.0086	80.58	0.9878	0.0054	128.33	0.9870	0.0046	150.65	0.9928	0.0067	103.43	0.9844
	40	0.0085	81.53	0.9923	0.0052	133.27	0.9932	0.0045	154.00	0.9965	0.0065	106.62	0.9919
	80	0.0081	85.56	0.9908	0.0050	138.60	0.9910	0.0044	157.50	0.9859	0.0062	111.77	0.9897
Ferulic acid	20	0.0086	80.58	0.9904	0.0055	126.00	0.9916	0.0047	147.45	0.9871	0.0067	103.43	0.9908
	40	0.0087	79.66	0.9943	0.0056	123.75	0.9956	0.0049	141.43	0.9919	0.0069	100.43	0.9948
	80	0.0090	77.00	0.9916	0.0059	117.46	0.9921	0.0051	135.88	0.9812	0.0071	97.61	0.9918
Caffeic acid	20	0.0092	75.33	0.9974	0.0060	115.50	0.9971	0.0054	128.33	0.9823	0.0073	94.93	0.9965
	40	0.0092	75.33	0.9935	0.0061	113.61	0.9934	0.0055	126.00	0.9807	0.0074	93.65	0.9926
	80	0.0098	70.71	0.9939	0.0066	105.00	0.9965	0.0057	121.58	0.9978	0.0077	90.00	0.9920

higher than cyanidin-3-*O*-glucoside. Likewise, Sui *et al*. (2014) made a similar observation that cyanidin-3-*O*-rutinoside showed higher stability than cyanidin-3-*O*-glucoside in aqueous solution. A current study investigated the degradation kinetics of mulberry anthocyanins during hot air-drying and vacuum-drying and reported that cyanidin-3-*O*-rutinoside had a higher $t_{1/2}$ than cyanidin-3-*O*-glucoside under all studied conditions (Zhou *et al*., 2017). This phenomenon could be explained by the additional glycosylation present in cyanidin-3-*O*-rutinoside which conferred a stabilising effect (Sui *et al*., 2014).

Adding gallic acid increased the stability of cyanidin-3-*O*-galactoside, cyanidin-3-*O*-glucoside, cyanidin-3-*O*-rutinoside and total anthocyanins from the *P. cerasifera* leaf (Table 2). The results were in agreement with previous studies showing that gallic acid could stabilise anthocyanins (Hou *et al*., 2011; Pedro *et al*., 2016). Increasing gallic acid concentration increased the $t_{1/2}$ of *P. cerasifera* anthocyanins, and compared to lower gallic acid concentrations, 80 mg per 100 mL of gallic acid more efficiently stabilised anthocyanins with an 11.11%, 12.00%, 11.36% and 11.30% increase in the $t_{1/2}$ of cyanidin-3-*O*-galactoside, cyanidin-3-*O*-glucoside, cyanidin-3-*O*-rutinoside and total anthocyanins, respectively (Fig. 4). Previous studies reported that ferulic acid was a powerful copigment of anthocyanins in an aqueous solution, juice, red wine model solution and spray-dried sample (Eiro & Heinonen, 2002; Rein & Heinonen, 2004; Zhang *et al*., 2015; Weber *et al*., 2017). However, we observed that various ferulic acid concentrations had different effects on the stability of *P. cerasifera* anthocyanins in the model solutions (Fig. 4). A lower ferulic acid concentration (20 mg per 100 mL) stabilised cyanidin-3-*O*-galactoside, but increasing ferulic acid concentration decreased the protective effects on cyanidin-3-*O*-galactoside (Fig. 4a). For cyanidin-3-*O*-glucoside, cyanidin-3-*O*-rutinoside and total anthocyanins from *P. cerasifera* leaf, 20 mg mL^{-1} of ferulic acid increased their stabilities; 40 mg mL^{-1} of ferulic acid had few effects on their stabilities; and 80 mg per 100 mL of ferulic acid decreased their $t_{1/2}$ by 5.08%, 3.92% and 2.81%, respectively (Fig. 4b–d). Caffeic acid has also been reported as a promising copigment of anthocyanins in many publications (Eiro & Heinonen, 2002; Gordillo *et al*., 2012; Yan & Wang, 2015; Zhang *et al*., 2015); however, a recent study suggested that caffeic acid accelerated the degradation of anthocyanins from purple sweet potato (Qian *et al*., 2017). Our results show that caffeic acid decreased the stability of cyanidin-3-*O*-galactoside, cyanidin-3-*O*-glucoside, cyanidin-3-*O*-rutinoside and total anthocyanins from the *P. cerasifera* leaf, and increasing caffeic acid concentration accelerated the degradation of *P. cerasifera* anthocyanins. For example, 20 mg per 100 mL of caffeic acid decreased the $t_{1/2}$ of cyanidin-3-*O*-galactoside, cyanidin-3-*O*-glucoside, cyanidin-3-*O*-rutinoside and total anthocyanins by 2.17%, 6.67%, 9.26% and 5.47%, respectively; 80 mg per 100 mL of caffeic acid decreased the $t_{1/2}$ of these anthocyanins by more than 8%, 15%, 14% and 10%, respectively (Fig. 4). Qian *et al*. (2017) investigated the effects of gallic/ferulic/caffeic acids on the stability of purple sweet potato anthocyanins and suggested that gallic acid might protect the anthocyanidin nucleus from hydration better than ferulic acid and caffeic acid due to the shortest distance of the aromatic ring to the anthocyanin panel. Self-association reactions of anthocyanins can protect their molecular stability; but additional phenolic acids might interrupt anthocyanin self-stacking or self-association and accelerate anthocyanin degradation (Qian *et al*., 2017). Gómez-Míguez *et al*. (2006) reported that some copigments have few improved effects on the stability of anthocyanins, as similar degradation rates have been observed in the presence and absence of the copigments (Zhang *et al*., 2015). The protective effect of copigments on anthocyanins was not only

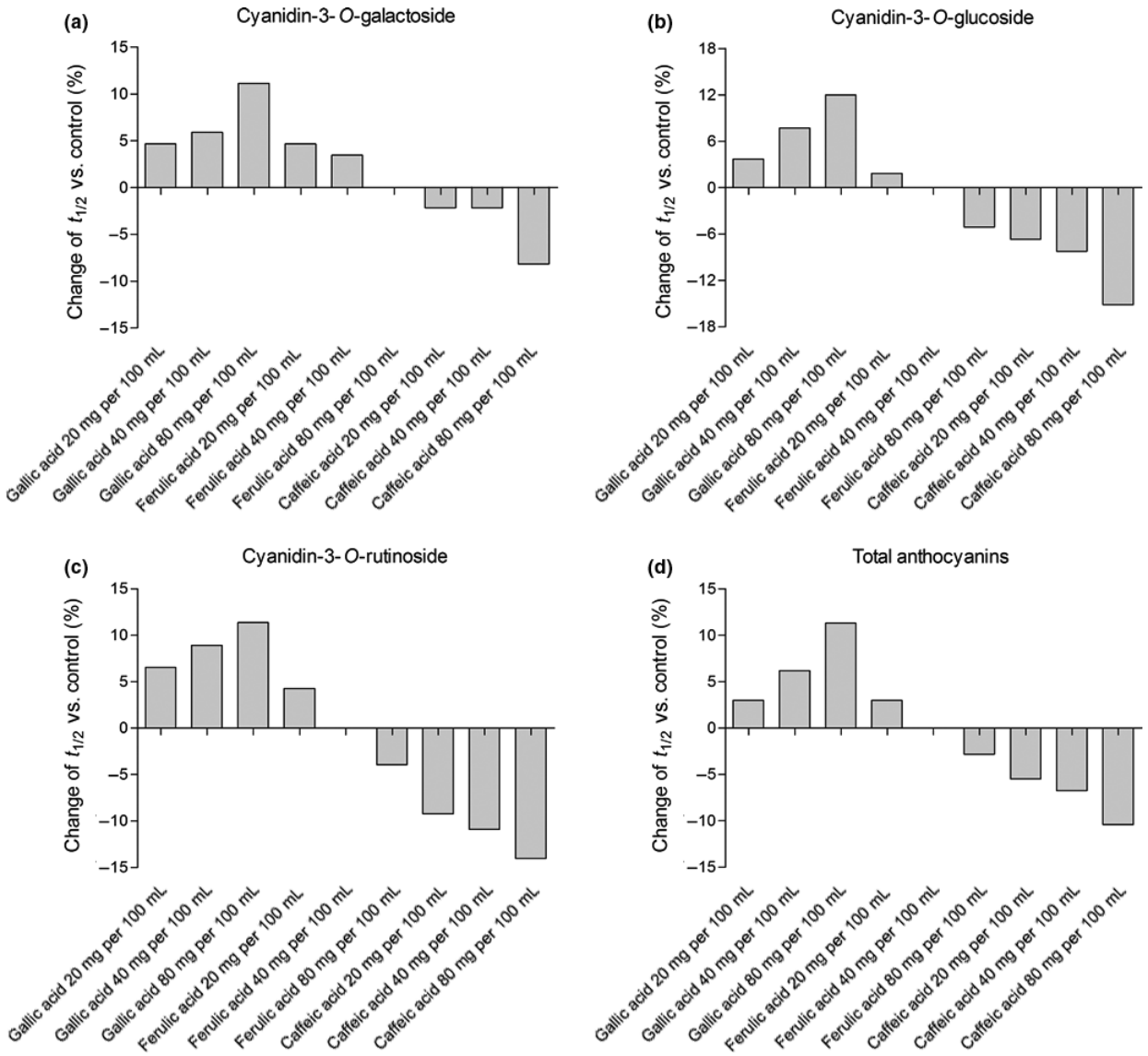

Figure 4 Effects of gallic, ferulic and caffeic acid on the $t_{1/2}$ of cyanidin-3-*O*-galactoside (a), cyanidin-3-*O*-glucoside (b), cyanidin-3-*O*-rutinoside (c) and total anthocyanins (d) from *P. cerasifera* leaf in energy drink model solutions.

correlated to the concentration of copigments but also strongly depended on their molecular structure. In the present study, gallic acid showed potential protective effects on *P. cerasifera* anthocyanins, and 80 mg per 100 mL of gallic acid was efficient to stabilise anthocyanins in the energy drink model solutions.

Conclusions

In the present study, a green analytical method was developed for the determination of cyanidin-3-*O*-galactoside, cyanidin-3-*O*-glucoside, cyanidin-3-*O*-rutinoside and total anthocyanins from *P. cerasifera* leaf. One-dimensional HPLC-DAD was used for quantitative analysis of anthocyanins from *P. cerasifera* leaf using ethanol and tartaric acid solution as mobile phases, and the two-dimensional HPLC-MS was used for anthocyanin identification using ethanol-based mobile phases. To develop the new natural pigments, *P. cerasifera* anthocyanins were used in energy drinks, and the effects of gallic, ferulic and caffeic acids on stability were determined using the first-order model ($R^2 > 0.98$). Gallic acid showed potential protective effects on *P. cerasifera* anthocyanins in the energy drink model solutions. The

developed green two-dimensional HPLC-DAD/ESI-MS method can be used to analyse *P. cerasifera* anthocyanins, and *P. cerasifera* leaf is a new source of natural functional pigments that is promising for use in the food industry.

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Conflict of interest

All authors declare that they have no conflict of interest to disclose.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Leaves of *Prunus cerasifera* var. *atropurpurea*.

Figure S2. Mass spectra of anthocyanins from *P. cerasifera* leaf profiled using HPLC-MS/MS (For details see Fig. 1d).

Figure S3. HPLC-DAD chromatograms of recovery determination of cyanidin-3-*O*-glucoside (peak 2) from *Prunus cerasifera* leaf, namely, extract without added standard (a), extract with added 0.1 mg (b), 0.2 mg (c) and 0.4 mg (d) of standard and cyanidin-3-*O*-glucoside standard (e).