Investigating the Effect of High Hydrostatic Pressure Processing on Anthocyanins Composition of Mulberry (*Morus moraceae*) Juice

Narku Felix ENGMANN, Yong-Kun Ma, Xu YING and Ye QING

School of Food and Biological Engineering, Jiangsu University, Zhenjiang, P.R. China

Abstract

Engmann N.F., Ma Y.-K., Ying X., Qing Y. (2013): **Investigating the effect of high hydrostatic pressure processing on anthocyanins composition of mulberry (*Morus moraceae*) juice**. Czech J. Food Sci., **31**: 72–80.

Anthocyanins are potent natural antioxidants with acclaimed health benefits and are also used as industrial colourants. These functions are based on the types and amounts of anthocyanins present in the food material. We identified and characterised mulberry fruit anthocyanins before and after high hydrostatic pressure (HHP) treatment. Three separate samples were differently treated at 200, 400, and 600 MPa for 20 min, respectively. Anthocyanins were identified and characterised using high-performance liquid chromatography (HPLC), electrospray ionisation mass spectrometry (ESI/MS), and the literature data. Cyanidin-3-*O*-glucopyranoside (55.56%) and cyanidin-3-*O*-coumaroylglucoside (44.44%) were detected in the untreated sample, while two new anthocyanins [pelargonidin-3-*O*-coumaroylglucoside (0.46%) and delphinidin-3-*O*-coumaroylglucoside (5.8%)] were identified in the sample treated at 200 MPa for 20 minutes. One new anthocyanin, delphinidin-3-*O*-coumaroylglucoside (5.38%), was detected in the juice treated at 400 MPa for 20 minutes. At 600 MPa for 20 min, no new anthocyanins were detected.

Keywords: mulberry fruit; antioxidants; health benefits; HPLC-ESI-MS

Processing foods using high hydrostatic pressure (HHP) has lethal effect on microorganisms, inactivates enzymes, and in some cases modification takes place in biomolecules like protein leading to the development of new products (Ahmed *et al*. 2003; Rodriguez *et al*. 2004). Because covalent bonds are not destroyed during HHP treatment, organoleptic properties of food are largely preserved (Cheftel 1992). It has been found that mulberry fruit extract has anti-oxidative, antimicrobial, and anti-inflammatory properties (Tsai *et al*. 2005; Sadiq Butt *et al*. 2008), and exhibits also antioxidant/antiradical properties (Suh *et al*. 2004). These health benefits have been attributed to anthocyanins reported to be potent antioxidants and also improve visual acuity (Mazza & Miniati 1993). Anthocyanins have also been observed to possess antineoplastic, radiation-protective, vasotonic, vasoprotective, anti-inflammatory, and chemo- and hepato-protective activities (Kamei *et al*. 1998; Smith *et al*. 2000; Wang *et al*. 2000). All these properties depend on the type and amount of anthocyanins present (Stintzing *et al*. 2002; Liu 2003; Matsumoto *et al*. 2003). Specifically, mulberry anthocyanin extract has been reported to have antimetastasis activity in inhibiting the migration of B16-F1 cells (Huang *et al*. 2008). Mulberry fruits contain higher amounts of anthocyanins than other well-known fruits such as elderberry and blackberry (Song *et al*. 2009). Anthocyanins are known to be affected by factors such as pH, temperature, salts and other chemicals, and possibly pressure (Türker & Erdoğdu 2006). In spite of the increasing commercial use of HHP in processing a variety of food materials, research into its effect on the anthocyanin content of fruit

juice, particularly mulberry fruits, is not extensive. The objective of this study was to identify and characterise mulberry fruit anthocyanins before and after various HHP treatments.

MATERIAL AND METHODS

Reagents. The reagents used were of analytical or HPLC grade and were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Deionised water was used throughout.

Sample preparation. Ripe mulberry fruits were harvested in farms along the Yangtze River basin in the Jiangsu Province of P.R. China. They were washed with potable water and frozen at –8°C before being used. Sampled frozen fruits were thawed and homogenised using a kitchen blender.

Pigment extraction. Anthocyanin pigment extraction was done using the method described by Giusti and Wrolstad (1996) with a few modifications. To 10 g of the homogenised mulberry fruit in a beaker, 40 ml acetone (1:4 w/v) was added. The mixture was sonicated whilst being stirred continuously for 10 min before vacuum filtration through a Büchner funnel with No. 1 Whatman filter paper. The residue was washed with 70% acetone. The extract (45 ml) was transferred to a separating funnel and 90 ml of trichloromethane was added, and 10 min was allowed for the phases to separate. The pigmented extract containing anthocyanins was collected for further purification.

Extract purification. The extract was purified as described by Rodriguez-Saona and Wrolstad (2001) using the solid phase extraction method with Sep-Pak C_{18} cartridge (Waters Co., Milford, USA) as the stationary phase, and 0.01% HCl in methanol (50 ml), ethyl acetate (50 ml), and 0.01% HCl in deionised water (50 ml) being the mobile phase. Methanol in the extract was evaporated using a rotary vacuum evaporator (Model RE 2000; Biochemical and Instrument Factory, Shanghai, China). Deionised water (5 ml) was used to dissolve the purified sample and the solution was packaged in a polythene bag.

HHP treatment. The HHP equipment (Intelligent Super High Pressure Food Processing Device, Jiangsu University, China) had an internal diameter of 150 mm and height of 440 mm, with an operational volume of 6.5 liters. Di-octyl sebacate was used as the pressurizing fluid. The maximum operational pressure of 600 MPa was reached in about 95 s while the depressurisation time was approximately 10 seconds. The packaged mulberry juice samples were divided into three separate batches and these were respectively subjected to pressures of 200, 400, and 600 MPa for 20 minutes. The samples were introduced into the high-pressure equipment at a temperature of 20°C and during pressurisation reached a maximum temperature of 25°C due to adiabatic heating. Just after depressurisation, the samples were transferred into an ice water bath, and then stored under refrigeration (4 ± 1°C) overnight before analysis.

HPLC-ESI-MS analysis. The treated and untreated samples were filtered through a 0.45-μm Millipore filter, type HV (Millipore Corp., Bedford, USA) before anthocyanin determination using HPLC-DAD (HP Agilent 1100 Series) and ESI-MS Bruker Esquire LC-MS ion trap multiple mass spectrometer (Bremen, Germany) in positive ionisation mode analysing ions from *m/z* 100 to *m/z* 1200. The HPLC features and working conditions were: DAD detector (G1315A): 200~700 nm full scan, quarternary pump system (G1311A), autosampler (G1313A). The column used was Agilent 20RBAX-SB C18 4.6 mm × 250 mm, 5 μm (Agilent, Santa Clara, USA). Solvent A (methanol) and solvent B (3% formic acid in deionised water) were membrane-filtered (0.45 μm) and de-aerated by sonication at 25°C for 40 minutes. The linear gradient elution was carried out as follows; 0~15 min A – increased from 5% to 25%; 15~25 min A – increased from 25% to 32 %; 25~35 min A-increased from 32% to 60%; 35~45 min A – increased from 60% to 80%; 45~55 min A – increased from 80% to

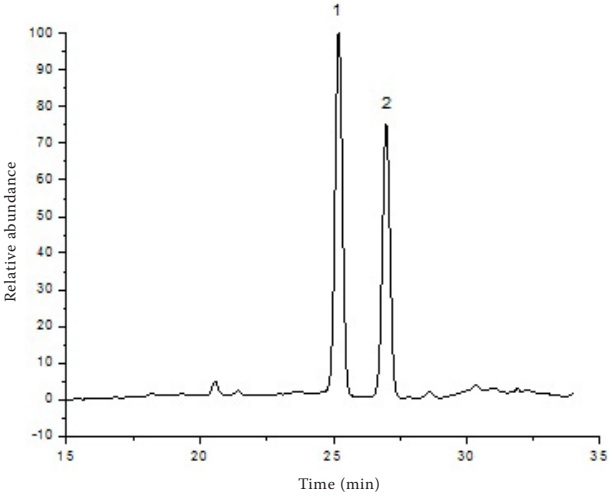

Figure 1. HPLC-DAD recorded at 520 nm for untreated mulberry juice

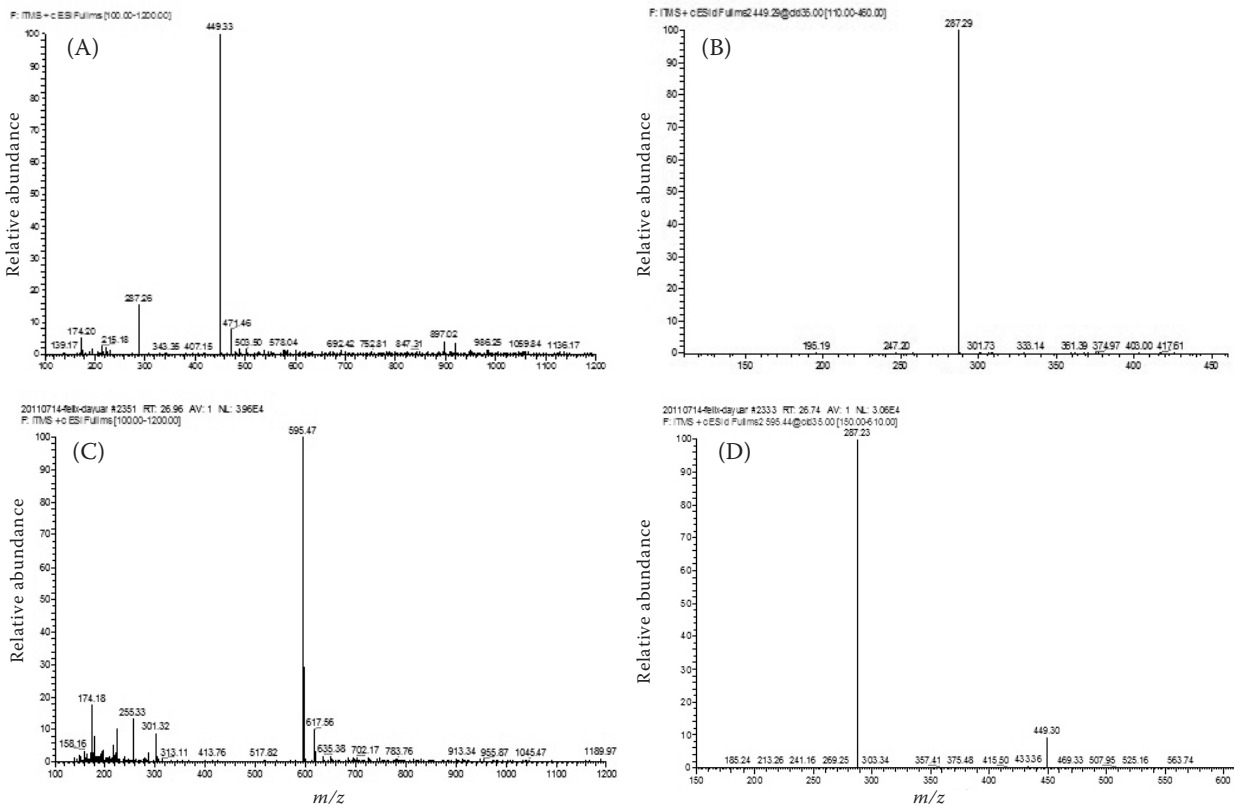

Figure 2. Positive ion ESI mass spectra of anthocyanin detected in control (Peak 1)

100%. The flow rate was 0.9 ml/min and injection volume was 5 µl. The column temperature was 25°C. The conditions of ESI-MS were as follows: ESI source voltage 4.5 kV, capillary voltage 30 V, sheath gas flow rate 30 arbitrary units, auxiliary gas flow rate 7 arbitrary units, tube lens voltage, 120 V, and capillary temperature 300°C.

The system was equipped with Xcalibur 2.0.7 SP1 software (Thermo Fisher Scientific Inc., Waltham, USA), which was used to create and edit the mass spectrometry data for the precursor and fragment ions, as well as the area under the individual peaks, used to determine the relative amounts of anthocyanins.

RESULTS AND DISCUSSION

Control sample

Anthocyanins were tentatively identified by matching their molecular ions ($M+H^+$), obtained on fragmentation by LC–ESI– MS and LC-MS/MS methods, with the theoretical molecular weights from the literature data (Maatta-Riihinen *et al.* 2004). The analyses utilised the positive ion mode (m/z $M+H^+$) for the detection of anthocyanins. The peaks in the HPLC–DAD chromatogram were monitored at 280 and 520 nm, which are the absorbance wavelengths typical of anthocyanins. For the control sample, two chromatographic peaks (Figure 1) were obtained at 520 nm, indicating the presence of two main anthocyanins.

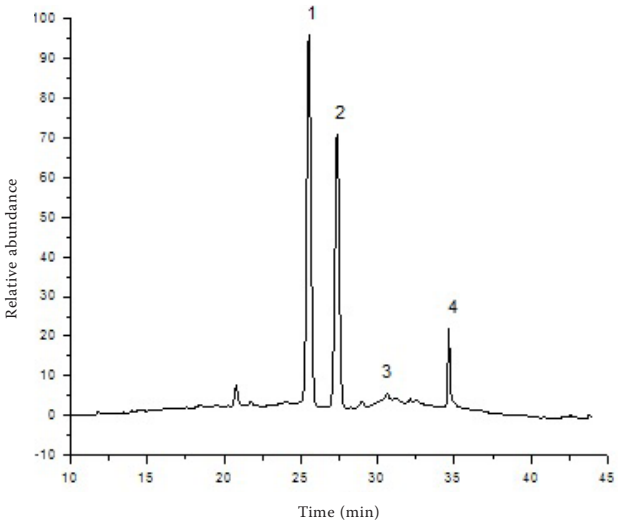

Figure 3. HPLC-DAD HPLC-DAD recorded at 520 nm for mulberry juice treated at 200 MPa for 20 minutes

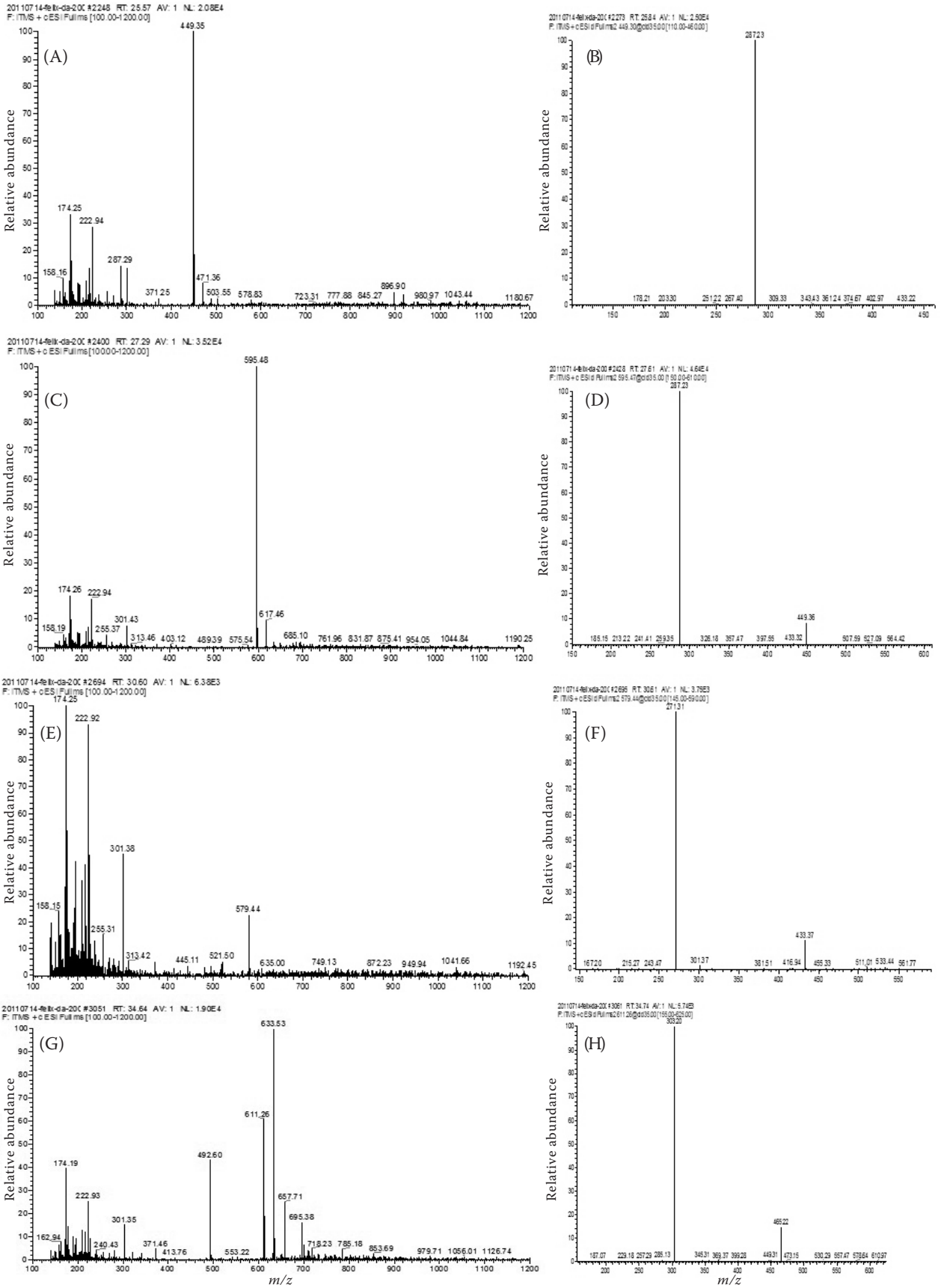

Figure 4. Positive ion ESI mass spectra of anthocyanin A and B – Peak 1, C and D – Peak 2, E and E – Peak 3, G and H – Peak 4 detected in sample treated at 200 MPa for 20 minutes

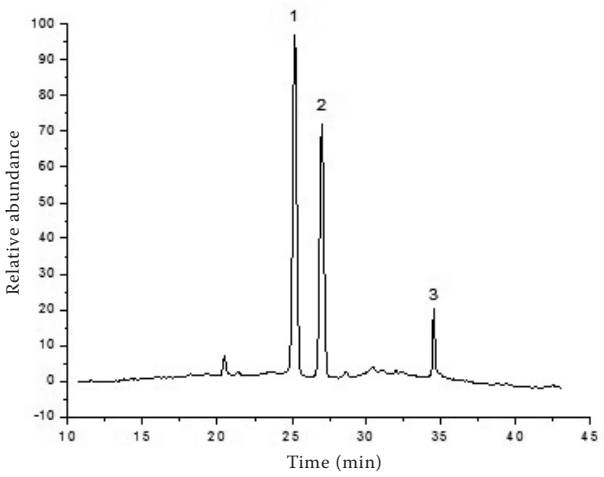

Figure 5. HPLC-DAD recorded at 520 nm for mulberry juice treated at 400 MPa for 20 minutes

Positive ion ESI mass spectra of peak 1 in the chromatograms (Figure 1) showed a molecular ion (M^+) at *m/z* 449 and a fragment ion at *m/z* 287 (Figures 2 A and B, respectively), corresponding to a cyanidin aglycon obtained by the loss of 162 amu, likely to be a hexose moiety. Peak 1, of the control sample, was identified as cyanidin-3-*O*-glucoside. This was confirmed by comparison with the mass spectra taken from the literature (Aaby *et al.*2005; Wu & Prior 2005; Steimer & Sjöberg 2011). Peak 2 of the control showed a molecular ion at *m/z* 595 (Figure 2C) and a fragment ion at *m/z* 449 (Figure 2D), which indicated the loss of a glycosyl unit (146 amu). Also, a major fragment ion occurred at *m/z* 287 (Figure 2D) which compared with cyanidin and the loss of a rutinosil unit (308 amu). Peak 2 was thus identified as cyanidin-3-*O*-rutinoside. Suh *et al.* (2003) and Song *et al.* (2009) also identified cyanidin 3-glucoside and cyanidin 3-rutinoside as the major anthocyanins in mulberry fruit.

Sample treated at 200 MPa for 20 minutes

Four chromatographic peaks (Figure 3) were obtained at 520 nm in the sample treated at 200 MPa for 20 min, indicating the presence of four anthocyanins.

Peak 1 in this chromatogram (Figure 3) showed a molecular ion (M^+) at *m/z* 449 and a fragment ion at *m/z* 287 (Figures 4 A and B, respectively), analogous to a cyanidin aglycon attained from the loss of 162 amu, likely to be a glucose unit. Peak 1, of Figure 3, was accordingly identified as cyanidin-3-*O*-glucoside. For peak 2 of Figure 3, a molecular ion at *m/z* 595 and a fragment ion at *m/z* 449 (Figures 4 C and D, respectively), implied the loss of 146 amu and a major fragment ion at *m/z* 287 (Figure 4D) indicative of cyanidin and the loss of a rutinosil unit (308 amu). Peak 2 was thus identified as cyanidin-3-*O*-rutinoside. The third peak in Figure 3 showed two major signals: the molecular ion at *m/*z 579 and the fragment ion at *m/z* 271 (Figures 4 E and F) corresponded to the related aglycon pelargonidin, being derived from the loss

Table 1. HPLC-DAD and ESI-MS chromatographs data for control and HHP treated mulberry juice samples

Treatment	Peak	t_R (min)	$[M+H]^+$ (*m/z*)	Fragment ions (*m/z*)	UV/visible data λ_{max} $A_{440}/A_{lvis-max]}$	Compound	Relative amount (%)
Control	1	25.20	449	287	517 28.57	Cy3-G	55.56
	2	26.98	595	287, 449	519 31.25	Cy3-R	44.44
200 MPa	1	25.52	449	287	516 30.95	Cy3-G	52.77
	2	27.36	595	284, 449	518 31.03	Cy3-R	40.97
	3	30.62	579	271, 433	510 35.29	Pl3-Co	0.46
	4	34.64	633	303, 465	–	Dp3-Co	5.80
400 MPa	1	25.18	449	287	517 28.57	Cy3-G	53.25
	2	27.00	595	287, 449	519 29.03	Cy3-R	41.37
	3	34.50	633	303, 465	–	Dp3-Co	5.38
600 MPa	1	25.48	449	287	517 31.58	Cy3-G	56.57
	2	27.33	595	287, 449	518 32.14	Cy3-R	43.43

Peak list including retention times (t_R) for $DAD_{520\ nm}$, precursor ions $[M+H]^+$ and assigned compounds: Cy3G – cyanidin-3-*O*-glucoside; Dp3Co – delphinidin 3-*O*-coumaroylglucoside; Cy3-R – cyanidin-3-*O*-rutinoside; Pl3-Co – pelargonidin-3-*O*-coumaroyl glucoside

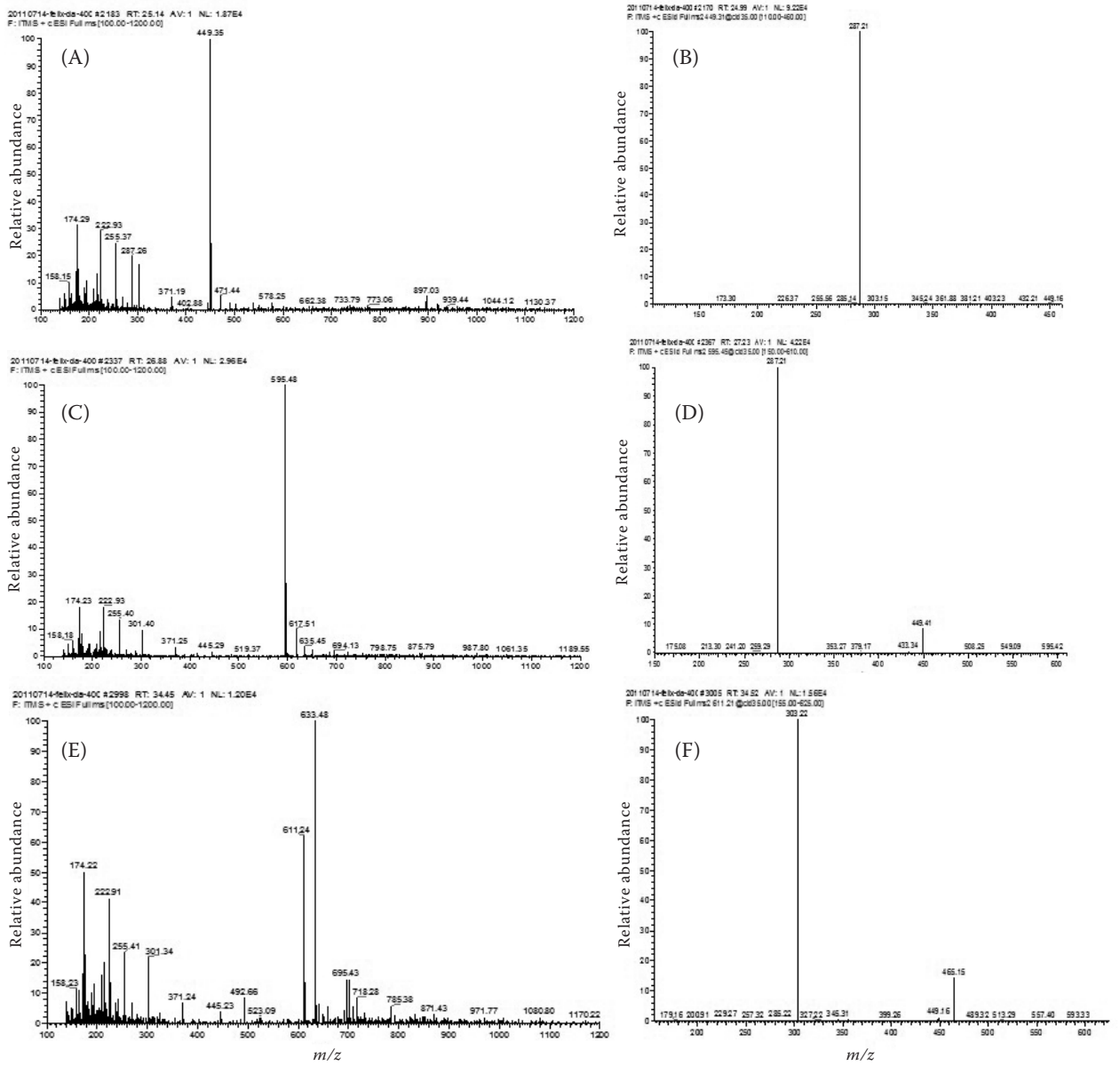

Figure 6. Positive ion ESI mass spectra of anthocyanin A and B – Peak 1, C and D – Peak 2, and E and F – Peak 3 detected in sample treated at 400 MPa for 20 minutes

of a glucose unit esterified with *O*-coumaric acid (308 amu). Peak 3 was identified as pelargonidin-3-*O*-coumaroyl glucoside. Gallori *et al*. (2004) identified the same anthocyanin with similar data.

The mass spectra of peak 4 (Figure 3) showed three major signals: the peak corresponding to the molecular ion at *m/z* 611 (Figure 4G) and the fragment ion at *m/z* 303 (Figure 4H) equivalent to the related aglycon delphinidin, and derived from the loss of a glucose unit esterified with *O*-coumaric acid (308 amu). The other fragment ion at *m/z* 465 (Figure 4H) further confirmed the esterification of *O*-coumaric acid with a glucose unit. Peak 4 was identified as delphinidin-3-*O*-coumaroylglucoside. The two new anthocyanins formed might be due to the effect of pressure at 200 MPa. The pressure could lead to the rearrangement of hydroxyl and hydroxide ions at the R_1 and R_2 positions on the aglycone cyanidin, leading to the formation of these new anthocyanins. HHP is known to affect non-covalent bonds such as hydrogen bonds, hydrophobic bonds, and ionic bonds (Cheftel 1992).

Sample treated at 400 MPa for 20 minutes

Three chromatographic peaks (Figure 5) were obtained when the pressure was increased to 400 MPa

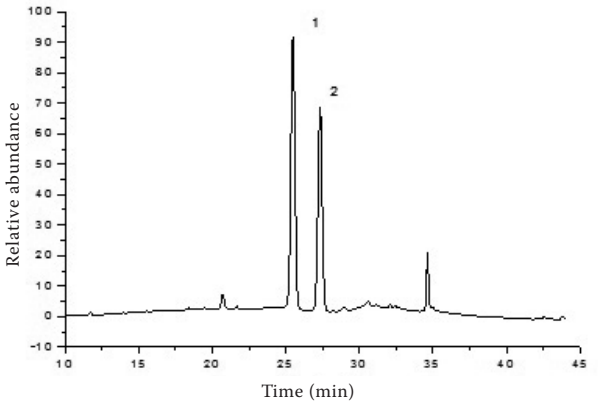

Figure 7. HPLC-DAD recorded at 520 nm for mulberry juice treated at 600 MPa for 20 minutes

for 20 minutes. Peak 1, with the molecular ion at *m/z* 449 (Figure 6A) and the fragment ion at *m/z* 287 (Figure 6B) indicated the presence of cyanidin aglycon. This ensued from the loss of 162 amu, likely to be glucose, and peak 1 was identified as cyanidin-3-*O*-glucoside.

The mass spectra acquired for peak 2 showed three major signals: the peak corresponding to the molecular ion at *m/z* 595 (Figure 6C) and the fragment ion at *m/z* 449 (Figure 6D), indicating the loss of a glycosyl sugar unit (146 amu). Again, a major fragment ion occurred at *m/z* 287 (Figure 6D) which corresponded to cyanidin and the loss of a rutinosil unit (308 amu). Peak 2 was identified as cyanidin-3-*O*-rutinoside.

Peak 3 showed the molecular ion at *m/z* 611 (Figure 6E) and the fragment ion at *m/z* 465, indicating the loss of a glycosyl unit (M-146) and the major fragment ion at *m/z* 303 (Figure 6B) which corresponded to delphinidin esterified with a glucose unit (308 amu). Peak 3 was identified as delphinidin-3-*O*-coumaroylglucoside.

Sample treated at 600 MPa for 20 minutes

Two peaks were identified in the sample treated at 600 MPa for 20 min (Figure 7). Positive ion ESI mass spectra of peak 1 with the molecular ion (M^+) at *m/z* 449 (Figure 8A) and the fragment ion at *m/z* 287 (Figure 8B) corresponded to a cyanidin aglycon obtained by the loss of a single glucose unit (162 amu). Peak 1 was therefore identified

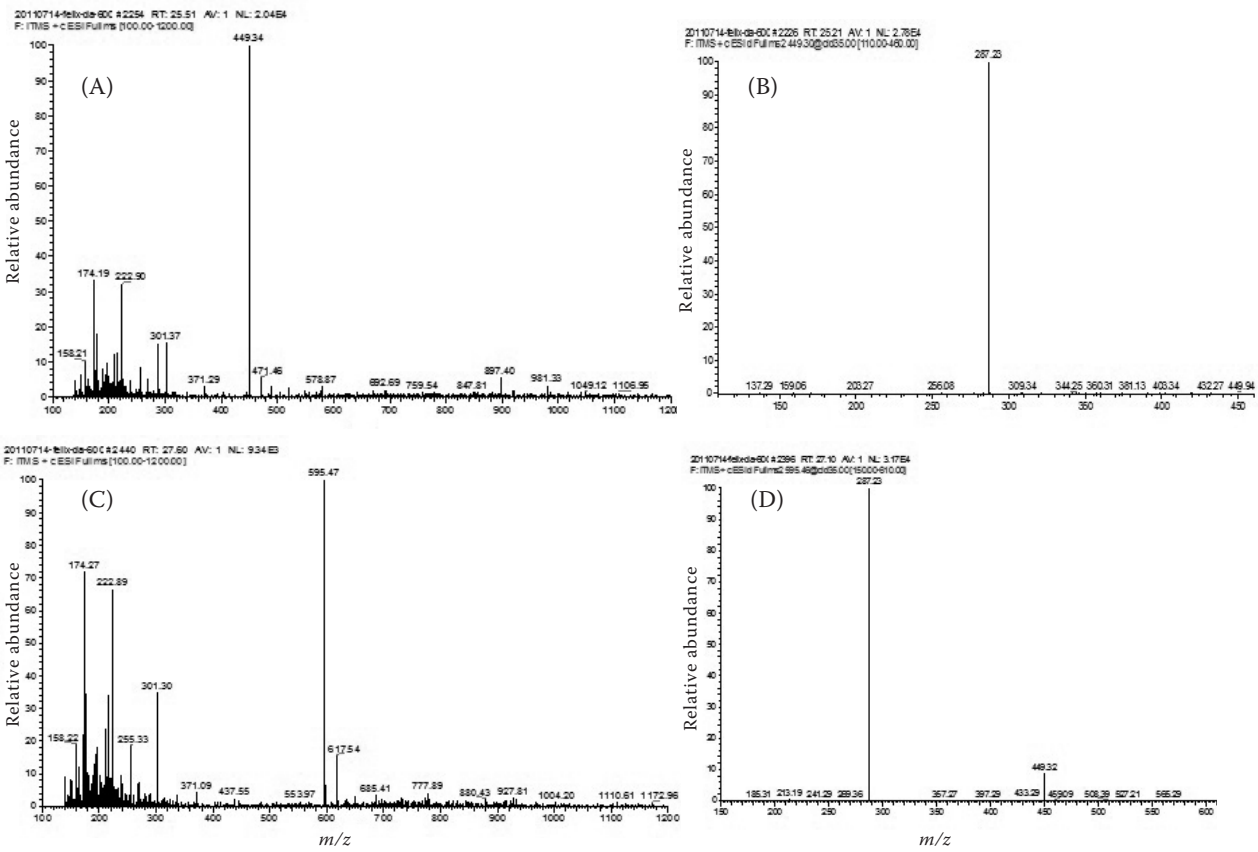

Figure 8.A and B: Positive ion ESI mass spectra of anthocyanin A and B – Peak 1 and C and D – Peak 2 detected in sample treated at 600 MPa for 20 minutes

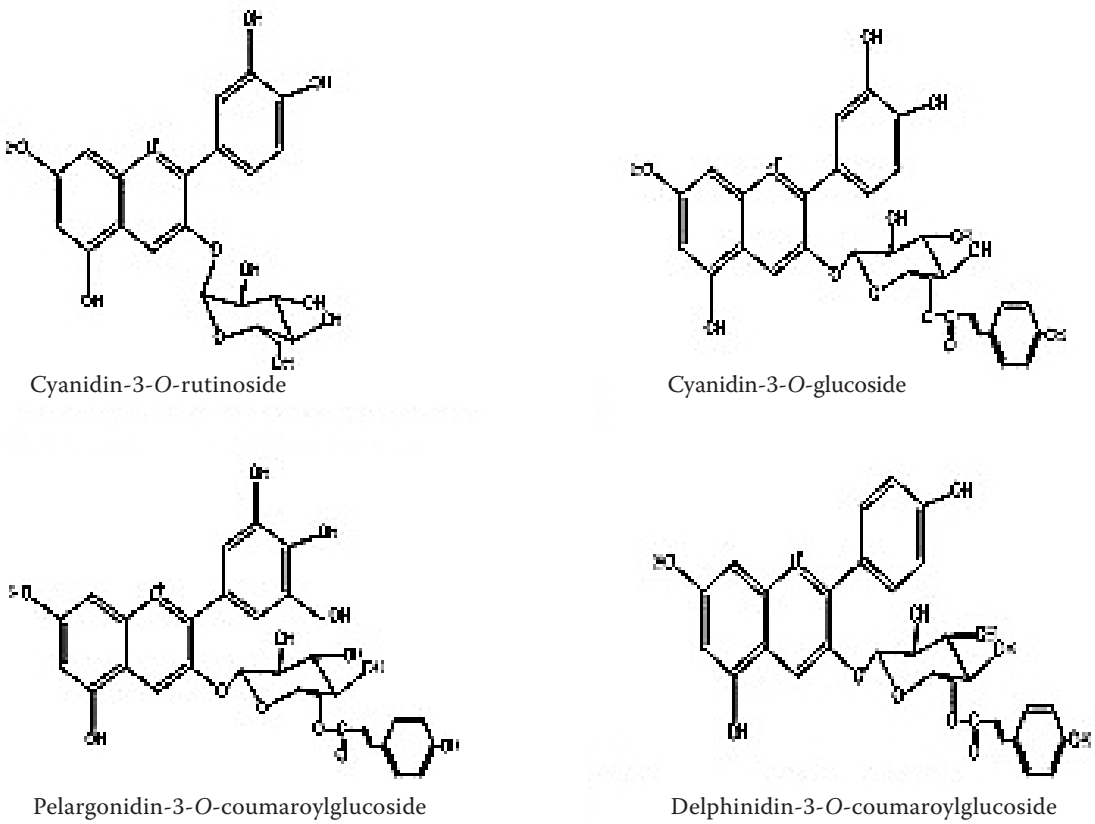

Figure 9. Structures of identified anthocyanins in mulberry juice under different HHP treatments

as cyanidin-3-*O*-glucoside. Peak 2 (Figure 7) with the molecular ion at *m/z* 595 (Figure 8C) and the fragment ion at *m/z* 449 (Figure 8D), indicated the loss of a glycosyl unit (146 amu). Also, a major fragment ion occurred at *m/z* 287 (Figure 8D) which compared with cyanidin and the loss of a rutinosil unit (308 amu). Peak 2 was identified as cyanidin-3-*O*-rutinoside. No new anthocyanins were formed, but the relative amount of cyanidin-3-*O*-glucoside increased as compared to all the other samples (Table 1).

CONCLUSION

The relative amount of cyanidin-3-*O*-glucoside increased with the increase in pressure, and vice versa as concerns cyanidin-3-*O*-rutinoside. Two new anthocyanins, delphinidin-3-*O*-coumaroylglucoside and pelargonidin-3-*O*-coumaroylglucoside were formed at 200 MPa for 20 min, but at 400 MPa for 20 min only delphinidin-3-*O*-coumaroylglucoside was formed. Therefore, depending on the pressure applied, the types and relative amounts were modified of anthocyanins in mulberry juice.

References

Aaby K., Skrede G., Wrolstad R.E. (2005): Phenolic composition and antioxidant activities in flesh and achenes of strawberries (*Fragaria ananassa*). Journal of Agricultural and Food Chemistry, **53:** 4032–4040.

Ahmed J., Ramaswamy H.S., Alli I., Ngadi M. (2003): Effect of high pressure on rheological characteristics of liquid egg. LWT-Food Science and Technology, **36**: 517–524.

Cheftel J.C. (1992): Effect of high hydrostatic pressure on food constituents: An overview. In: Balny C., Hayashi R., Hereman K., Masson P.J. (eds): High-Pressure and Biotechnology. Libbey Eurotext, London: 195–209.

Gallori S., Giaccherini C., Bilia A.R., Mulinacci N., Vincieri F.F. (2004): Identification of anthocyanins in *Amorphophallus titanum* (Becc.). Chromatographia, **59**; 647–651.

Giusti M.M., Wrolstad R.E. (1996): Characterization of red radish anthocyanins. Journal of Food Science, **61**: 322–326.

Huang H.P., Shih Y.W., Chang Y.C., Hung C.N., Wang C.J. (2008): Chemo inhibitory effect of mulberry anthocyanins on melanoma metastasis involved in the Ras/PI3K pathway. Journal of Agricultural and Food Chemistry, **56**: 9286–9293.

Kamei H., Hashimoto Y., Koide T., Kojima T. Hasegawa M. (1998): Anti-tumor effect of methanol extracts from red and white wines. Cancer Biotherapy and Radiopharmacology, **13**: 447–452.

Liu R. (2003): Health benefits of fruit and vegetables are from additive and synergistic combinations of phytochemicals. The American Journal of Clinical Nutrition, **78**: (Suppl 3): 517S–520S.

Maatta-Riihinen K.R., Kamal-Elin A., Torronen A.R. (2004): Identification and quantification of phenolic compounds in berries of *Fragaria* and *Rubus* species (family Rosaceae). Journal of Agricultural and Food Chemistry, **52**: 6178–6187.

Matsumoto H., Nakamura Y., Tachibanaki S., Kawamura S., Hirayama M. (2003): Stimulatory effect of cyanidin glucosides on the regeneration of rhodopsin. Journal of Agricultural and Food Chemistry, **51**: 3560–3563.

Mazza G., Miniati E. (1993): Anthocyanins in Fruits, Vegetables, and Grains. CRC Press, Boca Raton: 85–130.

Rodriguez A.C., Larkin J.W., Dunn J., Patazca E., Reddy N.R., Alvarez-Medina M., Tetzloff R., Fleischman G.J. (2004): Model of the inactivation of bacterial spores by moist heat and high pressure. Journal of Food Science, **69**: 367–74.

Rodriguez-Saona L.E., Wrolstad R.E. (2001): Unit F1.1: Anthocyanins. extraction, isolation and purification of anthocyanins. In: Wrolstad R.E. (ed.): Current Protocols in Food Analytical Chemistry. John Wiley & Sons, New York 1–11.

Sadiq Butt M., Nazir A., Tauseef Sultan M., Schroen K. (2008): *Morus alba* L. nature's functional tonic. Trends in Food Science and Technology, **19**: 505–512.

Steimer S. SjÖberg P.J.R. (2011): Anthocyanin characterization utilizing liquid chromatography combined with advanced mass spectrometric detection. Journal of Agricultural and Food Chemistry, **59**: 2988–2996.

Smith M., Marley K., Seigler D., Singletary K., Meline B. (2000): Bioactive properties of wild blueberry fruits. Journal of Food Science, **65**: 352–356.

Song W., Wang H.J., Bucheli P., Zhang P.F., Wei D. Z., Lu Y.H. (2009): Phytochemical profiles of different mulberry species from China. Journal of Agricultural and Food Chemistry, **57**: 9133–9140.

Stintzing F., Stintzing A., Carle R., Frei B., Wrolstad R. (2002): Color and antioxidant properties of cyanidin-based anthocyanin pigments. Journal of Agricultural and Food Chemistry, **50**: 6172–6181.

Suh H., Noh D.O., Kang C.S., Kim J. M., Lee S.W. (2003): Thermal kinetics of color degradation of mulberry fruit extract. Nahrung/Food, **47**: 132–135.

Suh H., Kim J.M., Lee H., Lee S.W., Choi M. (2004): Thermal kinetics on antiradical capacity of mulberry fruit extract. European Food Research and Technology, **219**: 80–83.

Tsai P.J., Delva L., Yu, T.Y., Huang Y.T., Dufosse L. (2005): Effect of sucrose on the anthocyanin and antioxidant capacity of mulberry extract during high temperature heating. Food Research International, **38**: 1059–1065.

Türker N., Erdoğdu F. (2006): Effects of pH and temperature of extraction medium on effective diffusion coefficient of anthocynanin pigments of black carrot (*Daucus carota* var. L.): Journal of Food Engineering, **76**: 579–583.

Wang C., Wang J., Lin W., Chu C., Chou F., Tseng T. (2000): Protective effect of *Hibiscus* anthocyanins against *tert*-butyl hydroperoxide-induced hepatic toxicity in rats. Food and Chemical Toxicology, **38**: 411–416.

Wu X., Prior R.L. (2005) Systematic identification and characterization of anthocyanins by HPLC-ESI-MS/MS in common foods in the United States: Fruits and berries. Journal of Agricultural and Food Chemistry, **53:** 2589–2599.

Recieved for publication December 30, 2011
Accepted after corrections May 16, 2012

Corresponding author:

Felix Narku Engmann, Jiangsu University, School of Food and Biological Engineering, 301 Xuefu Road, Zhenjiang, P.R. China; E-mail; felitexgh@yahoo.com