Composition Analysis and Structural Identification of Anthocyanins in Fruit of Waxberry

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Abstract

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Anthocyanin pigments in the fruit of waxberry (*Myrica rubra* Sieb. et Zucc.), were extracted with 0.1% HCl in ethanol, and the crude anthocyanin extract was purified by C18 Sep-Pak cartridge open-column chromatography. High-performance liquid chromatography (HPLC) with photodiode array detection (PAD) and matrix-assisted laser desorption/ionisation-time of flight-mass spectrometry (MALDI-TOF-MS) was applied for the separation and identification of anthocyanins in the fruit of waxberry and their aglycones resulting from acid hydrolysis. Three anthocyanins were found in the fruit of waxberry and identified as Cyanidin 3-*O*-β-galacopyranoside (14.8%), Cyanidin 3-*O*-β-glucopyranoside (60.5%), and petunidin 3-*O*-β-glucopyranoside (24.7%), respectively, using spectroscopic methods (UV-Vis and MS). The three anthocyanins were isolated and purified by preparative HPLC, and their chemical structures were further characterised by H^1 NMR. On the basis of chromatographic data, the total anthocyanin content was 286 mg/g in fresh fruit of waxberry.

Keywords: waxberry (*Myrica rubra* Sieb. et Zucc.); anthocyanin; natural product; pigment identification; composition analysis

Anthocyanins are plant pigments, widely found in many berries, in dark grapes, cabbages and other pigmented foods, plants, and vegetables. Chemically, they belong to the widespread class of phenolic compounds collectively named flavonoids and have molecular structures based on the 2-phenylbenzopyrylium (or flavylium) cation. The differences between the individual anthocyanins are related to the number of hydroxyl groups, the nature and number of sugars, and the position of these attachments (Kong *et al.* 2003; Andersen & Jordheim 2006; Longo & Vasapollo 2006; McGhie *et al.* 2006; Tian *et al.* 2006; Qin *et al.* 2009, 2010). Anthocyanins accumulate to high concentrations in some foods, for example berry fruit, and their consumption ranging up to several 100 mg/serving can be achieved. However, despite the identified *in vitro* therapeutic properties of anthocyanins and their widespread presence in the diet, the studies to date indicate that the apparent bioavailability is very low compared with other polyphenolics and flavonoids (McGhie *et al.* 2003; Manach *et al.* 2005; Škerget *et al.* 2005), which suggests that anthocyanins might not exhibit a therapeutic effect when consumed as part of the diet. Although anthocyanins have been widely studied because of their central role in determining the colour of the plant organs, they also have biochemical properties that are believed to be beneficial to human health. Depending on

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the nutritional habits, the daily intake of anthocyanins in humans has been estimated to be up to 200 mg/day (Kuhnau 1976). Anthocyanins have been found to be absorbed unmodified from the diet (Cao *et al.* 2001; Nielsen *et al.* 2003), and to be incorporated into cultured cells, both into the plasma membrane and into the cytosol (Youdim *et al.* 2000). Since a moderate consumption of such compounds through the intake of the products such as red wine or bilberry extract is associated with a lower risk of coronary heart disease, it has been proposed that anthocyanins may exert therapeutic activities on human diseases associated with oxidative stress, e.g. cancer and coronary heart disease (Tsuda *et al.* 2000, 2003; Lazze *et al.* 2004; Cooke *et al.* 2005; Chen *et al.* 2006). The antioxidant properties of anthocyanins have been demonstrated by both *in vitro* and *in vivo* experiments (Kahkonen & Heinonen 2003; Xu *et al.* 2005). It has also been suggested that anthocyanins play an important role in the prevention of mutagenesis and carcinogenesis mediating some physiological functions related to cancer suppression. Anthocyanins show inhibitory effects on the growth of some cancer cells and also inhibit cell transformation. As a class of compounds, anthocyanins have a diverse range of molecular structures that are likely to play important roles in determining their biological activities and associated factors such as bioavailability and metabolism.

Waxberry is an evergreen tree grown in the subtropical area and becomes a unique cultivated fruit tree in China and international market. The previously reported research works indicated that the ripe fruits of waxberry are typically rich in natural pigment, anthocyanin components such as cyanidin 3-*O*-glucoside (Du & Wang 2008; Du *et al.* 2008). Like other flavonoids, total anthocyanins in waxberry are polyphenolic compounds and exhibit substantial antioxidant capacity *in vitro* (Li *et al.* 2009). Additionally, anthocyanins have been shown to have a number of potential therapeutic properties such as anti-inflammatory, cancer chemopreventive, and antiobesity effects, and vasoprotection (Jayaprakasam *et al.* 2005). We are particularly interested in the composition of anthocyanins in the ripe fruit of waxberry (*Myrica rubra* Sieb. et Zucc.), which is a kind of traditional Chinese herb medicine and has been used in folk remedies for the treatment of pains, carbuncles, inflammations, and cancers for a long time. In this paper, the profile of anthocyanins in waxberry fruit is reported for their potential application as natural pigments and antioxidant agents by the food, pharmaceutical, and cosmetic industries.

MATERIALS AND METHODS

Chemicals and standards. HPLC grade solvents, such as methanol, acetonitrile, isopropanol, choloroform, et al., were supplied from Fisher Scientific (USA). Triflouroacetic acid (TFA) and tetrahydrofuran (THF) of HPLC grade were purchased from Tedia (Fairfield, USA). α-Cyano-4-hydroxycinnamic acid, *d*-triflouroacetic acid (CF_3COOD, 99.8%), d^4-methanol (CD_3OD, 99.8%), and trimethylsilicane (TMS) were purchased from Sigma Chemical (St. Louis, USA), and cyanidin 3-*O*-glucoside was obtained from Extrasynthese (Genay, France). Deionised water was used to prepare all solutions.

Sample collection. Fresh waxberry fruits from Yuyao, China (Figure 1a) were purchased from a market in Xi'an, China, during June 2007, placed in polyethylene bags, and stored at –20°C until use. The plant was classified at the Department of Biology, Northwest University, China, as *Myrica rubra* Sieb. et Zucc.

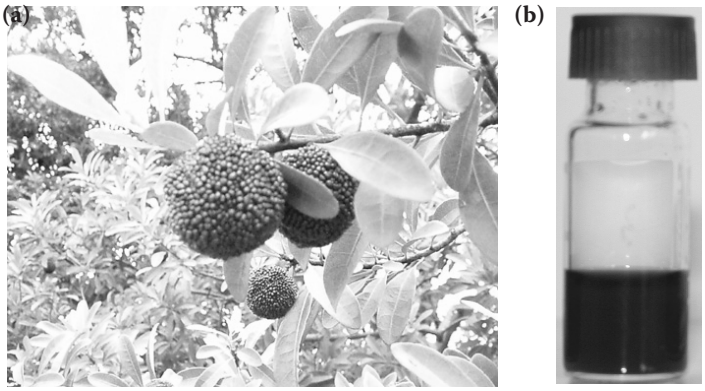


Figure 1. Pigment from the fruit of waxberry (*Myrica rubra* Sieb et Zuce.) (a) natural waxberry fruits in the tree; (b) pigment extracted from fresh waxberry fruits

Extraction of anthocyanins. Milled fresh waxberry fruits (500 g) were extracted in the dark by stirring with 1000 ml of 0.1% HCl (v/v) in ethanol at room temperature for 20 hours. The samples were filtered on a Buchner funnel, and the solid residue was washed with an additional 50 ml of 0.1% HCl (v/v) in ethanol. The filtrates were combined and dried using a rotary evaporator at 30°C. The remaining solid was dissolved in 0.01% HCl (v/v) in deionised water and successively purified.

Purification of anthocyanins. The anthocyanin aqueous solution obtained from the extraction procedure described before was passed through a 5 g sorbent weight C-18 Sep-Pak cartridge (Waters, Milford, USA), previously activated with methanol followed by 0.01% aqueous HCl (v/v). Anthocyanins and other polyphenolics were adsorbed onto the Sep-Pak column, while sugars, acids, and other watersoluble compounds were removed by washing the cartridge with 2 volumes of 0.01% aqueous HCl (v/v). Less polar polyphenolics were subsequently eluted with 2 volumes of ethyl acetate. Anthocyanins were then eluted with methanol containing 0.01% HCl (v/v). The acidified methanol solution was evaporated using a rotary evaporator at 30°C. The remaining solid was dissolved in 0.01% HCl (v/v) aqueous solution to obtain a defined concentration of the solution (2 mg/ml) and immediately analysed. This solution (Figure 1b) was stored at –20°C until used for subsequent acid hydrolysis.

Acid hydrolysis of anthocyanins. 5 ml of 2N HCl was added to 1 ml of the purified anthocyanin solution (2 mg/ml) in a screw-cap test tube, flushed with nitrogen, and capped. The pigments were hydrolysed at 100°C for 2 h; then, the solution was immediately cooled in an ice bath (Chaovanalikit *et al.* 2004). The hydrolysate was loaded onto a C-18 Sep-Pak cartridge (0.5 g sorbent weight, Waters, Milford, USA) and the filtrate passing through the column was collected as the sample for glycosyl identification. The anthocyanidins adsorbed on the column were washed and eluted using the same method as described above for the purification of anthocyanins.

HPLC-PAD analysis. The high-performance liquid chromatography (HPLC)-photodiode array detection (PAD) analytical analyses were performed using a Waters 2696 separation module equipped with a 996 photodiode array detector (PAD). Chromatographic separation was carried out using a 250 × 4.6 mm i.d., 5 μm Kromasil C18 column (No. NC-2546-06251151) with a 4 × 3 mm *i.d.* Phenomenex C18 guard cartridge, both thermostated at 32°C. The mobile phase was composed of 0.1% trifluoroacetic acid (TFA) in water (solvent A) and 0.1% TFA in acetonitrile (solvent B) and applied at a flow rate of 1 ml/min. The following gradient was used: 0 min – 10% B; 0–2 min – 10% B; 2–35 min – 10–90% B; 35–40 min – 90–100% B; 40–60 min – 100% B. The absorbance spectra were recorded every 2 s between 200 nm and 600 nm, with a bandwidth of 4 nm, and chromatograms were acquired at 520 nm, 440 nm, 310 nm, and 280 nm. The wavelength used for quantification was 520 nm (Hong & Wrolstad 1990; Ordaz-Galindo *et al.* 1999). The calibration curve was produced by the integration of the absorption peaks generated from the analysis of dilution series of cyanidin 3-glucoside.

HPLC preparation. The major anthocyanins were isolated by a preparative HPLC system consisting of a Shimadzu model LC-8A pump, a Shimadzu SCL-10A VP system controller, a manual injector fitted with a 1 ml sample loop, and a Shimadzu model SPD-10A UV/vis detector equipped with a preparative flow cell. A 250 × 21.2 mm *i.d.*, 7 μm Zorbax Stable Bond-C18 preparative column, coupled to a 50 × 21.2 mm *i.d.*, 5 μm Zorbax Stable Bond-C18 guard column (Shimadzu, Kyoto, Japan), was used for the separation and isolation of anthocyanins. The mobile phase was formic acid/water/methanol (10:75:15 v/v). The isocratic flow rate was 20 ml/min, and the detector was set at 520 nm. The anthocyanin fractions were isolated, and their purity was checked by analytical HPLC-PAD analysis as previously described. Each fraction was evaporated using a rotary evaporator at 30°C to remove methanol completely and then loaded on a 5 g sorbent weight C-18 Sep-Pak cartridge (Waters, Milford, USA) previously activated with methanol followed by 0.01% aqueous HCl (v/v). The cartridge was rinsed with 5 volumes of 0.01% aqueous HCl (v/v), and the adsorbed anthocyanin was eluted with 0.1% HCl (v/v) in methanol. The eluent solution was evaporated to dryness using a rotary evaporator at 30°C, and the resulting solid was resolubilised in a solvent suitable for MS and NMR analysis.

MS analysis. The anthocyanin fractions obtained by means of HPLC preparation above were dissolved in 0.1% trifluoroacetic acid (TFA) (v/v) in methanol. 2 μl of the anthocyanin solution was mixed well with 3 μl of the saturated α-cyano-4-hydro-

xycinnamic acid solution in a solvent mixture (CH_3CN/H_2O/TFA, 50:50:0.1), and 1 µl was loaded onto a sample plate for the matrix-assisted laser desorption/ionisation-time of flight-mass spectrometry (MALDI-TOF-MS). Mass spectral data of the anthocyanin fractions were read with an AXIMA-CFR^{TM} plus MALDI-TOF Mass Spectrometer (Shimadzu, Japan – Kratos Group, Wharfside, UK).

NMR analysis. H^1 NMR spectral data were recorded on an INOVA-400 instrument (Varian Inc., Palo Alto, USA) operating at 400 MHz in the solvent CD_3OD-CF_3OOD (9:1) with TMS as internal standard. Sample temperature was stabilised at 25°C.

Glycosyl identification. HPTLC was carried out using silica gel 60 layers (5 × 5 cm; Merck) according to the procedure previously described (De Pascual-Teresa *et al.* 2002; Di Paola-Naranjo *et al.* 2004). The chromatoplate was impregnated with 0.02M boric acid and activated at 100°C for 30 min before use. An aliquot (5 µl) of each sample was spotted on the plate and was horizontally developed using *n*-propanol-water (7:1, v/v) as solvent. After drying, the sugar spots were located by spraying with diphenylamine-aniline reagent (4 g diphenylamine, 4 ml aniline, 20 ml phosphoric acid 85% and 200 ml acetone) and maintaining the sprayed plate at 85°C for 10 minutes. Standards of D(+)-glucose, D(+)-galactose, L(+)-arabinose, α-L-rhamnose (Sigma-Aldrich Chemie, Steinhein, Germany) and D(+)-xylose (Merck, Darmstadt, Germany) at 1 mg/ml each were run simultaneously for the identification of individual sugars by comparison with the standard monosaccharides.

RESULTS AND DISCUSSION

The anthocyanin composition of the fruit of waxberry was determined by means of HPLC-PAD analysis. After purification through the column of C-18 Sep-Pak cartridge, the chromatogram obtained of the purified anthocyanin extract from the fruit of waxberry, recorded at 520 nm, is shown in Figure 2a. As can be seen, there are three peaks in the chromatogram at the retention time range of 8–10 min, indicating the presence of three

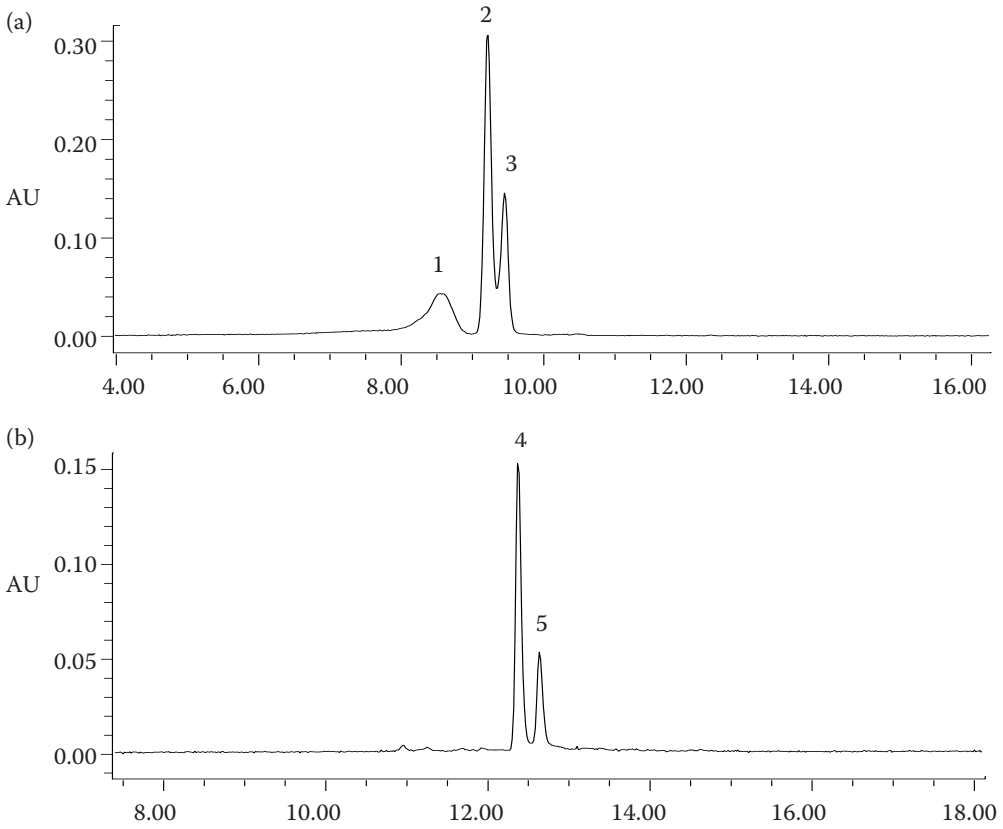
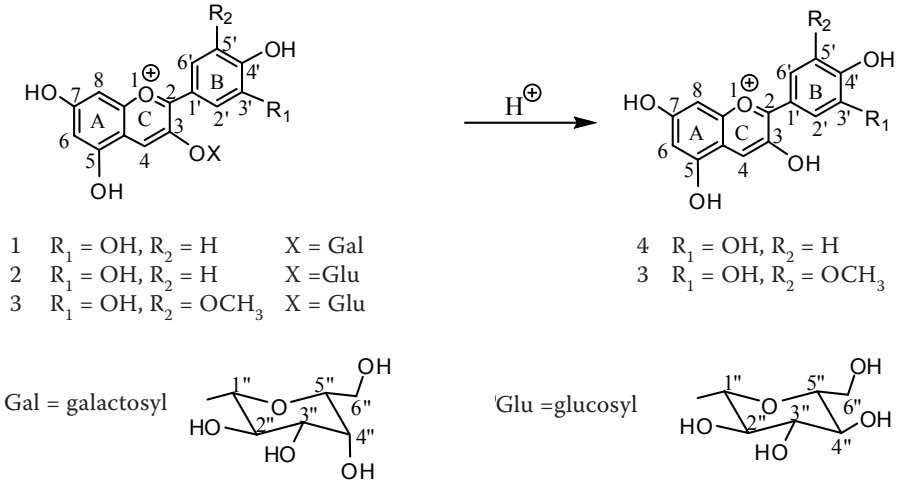


Figure 2. HPLC-PAD chromatogram recorded at 520 nm corresponding to (a) the purified extract from the fruit of waxberry; (b) the purified extract from the pigment of waxberry after acid hydrolysis



1 – cyanidin 3-*O*-galactoside (Cy-3-Gal); 2 – cyanidin 3-*O*-glucoside (Cy-3-Glu); 3 – petunidin 3-*O*-glucoside (Pt-3-Glu); 4 – cyanidin (Cy); 5 – petunidin (Pt)

Figure 3. Chemical structures of the anthocyanins identified in the fruit of waxberry and their aglycones after acid hydrolysis

different anthocyanins in the fruit of waxberry. The chromatogram of the purified product after acid hydrolysis of the anthocyanin extract, recorded at 520 nm (Figure 2b), showed that only two different aglycones could be obtained from the three anthocyanins in waxberry fruit. These three anthocyanins and their two corresponding aglycones, the structures of which are shown in Figure 3, were identified by comparison of the HPLC retention times, elution order, photodiode array UV/vis spectroscopic and MALDI-TOF-MS spectrometric data (Table 1).

The three major anthocyanins corresponding to peaks 1, 2 and 3 (Figure 2a) represented about 14.8%, 60.5%, and 24.7%, respectively, of the total peak area revealed at 520 nm. Peak 1 at a retention time of 8.6 min was identified as cyanidin 3-*O*-galactoside on the basis of its λmax of 518.6 nm and the mass spectrum comprising a M^+ at *m*/*z* 449 and a fragment ion at *m*/*z* 287 resulting from the loss of the glucose molecule (M^+ 162) and corresponding to the molecular ion of the aglycone cyanidin 4. Peak 2 at a retention time of 9.2 min was identified as cyanidin 3-*O*-galactoside on the basis of its λmax of 518.6 nm and the mass spectrum comprising a M^+ at *m*/*z* 449 and a fragment ion at *m*/*z* 287 resulting from the loss of the glucose molecule (M^+ 162), and corresponding to the molecular ion of the aglycone cyanidin 4. The MALDI-TOF-MS profiles of the peak 3 presented the molecular ions M^+ at *m*/*z* 479 and the fragment ions at *m*/*z* 317 resulting from the loss of the glucose molecules, and corresponding to the molecular ion of the aglycone petunidin 5. Peak 3 at a retention time of 9.5 min was therefore identified as petunidin 3-*O*-glucoside (Figure 4). The UV-Vis absorbance spectra of these compounds (Figure 5) confirmed their identity.

Table 1. Chromatographic, spectroscopic, and spectrometric characteristics of the anthocyanins found in the fruit of waxberry and their aglycones after acid hydrolysis

Peak No. (Figure 1)	t_R (min)	λ_{max} (nm)	$A_{440\,nm}/A_{\lambda_{max}}$	M calcd.)	M^+(found)	M^+-X (*m*/*z*)	Peak assignment
1	8.6	519 (281)	32.6	449.1	499.1	287.2	Cy-3-Gal
2	9.2	519 (281)	31.1	449.1	499.4	287.3	Cy-3-Glu
3	9.5	524 (281)	30.6	479.1	479.4	317.2	Pt-3-Glu
4	12.4	524 (275)	25.2	287.1	287.2	–	cyanidin (Cy)
5	12.6	526 (278)	26.7	317.1	317.3	–	petunidin (Pt)

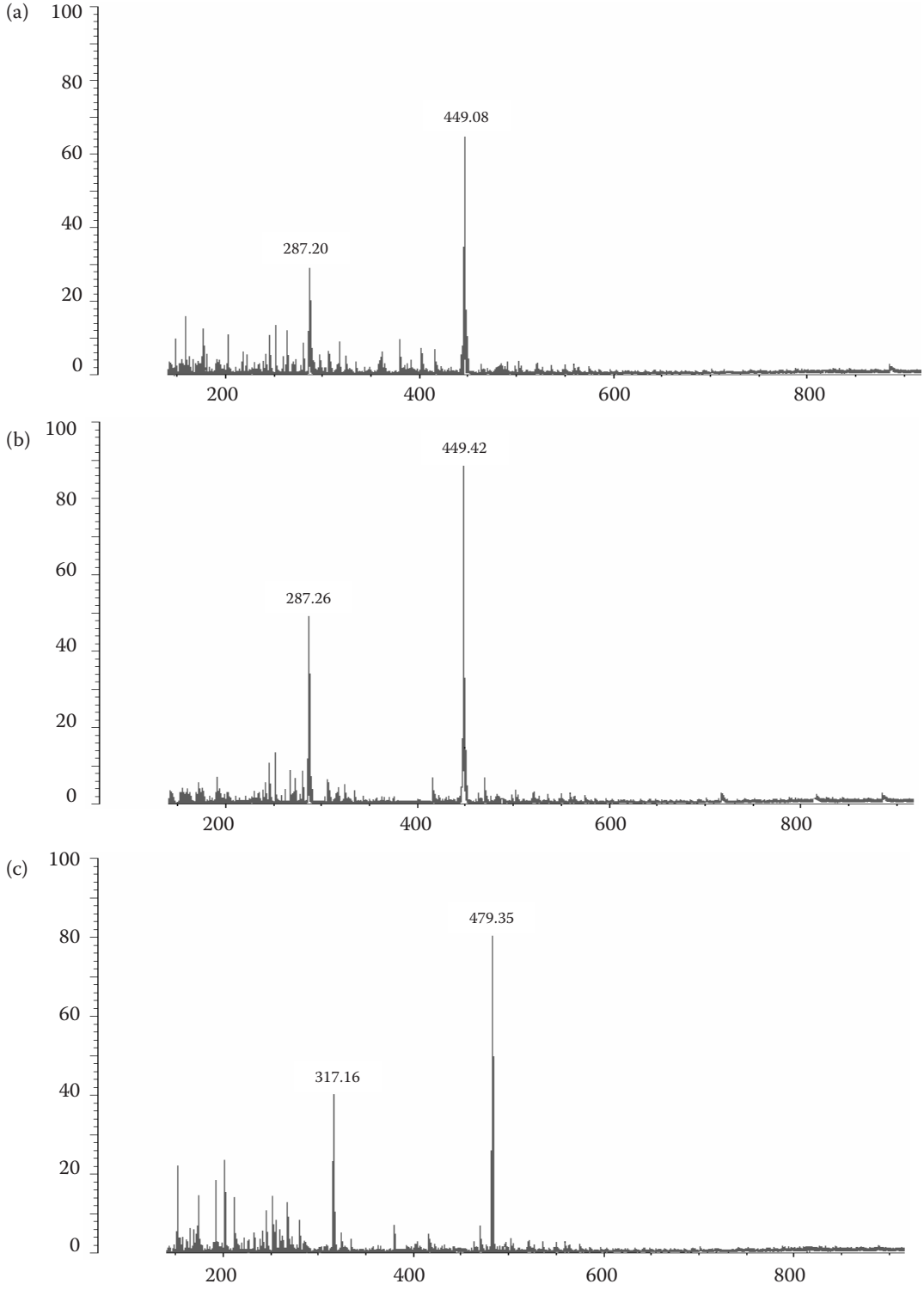


Figure 4. MALDI-TOF-MS of peaks 1–3: (a) peak 1; (b) peak 2; (c) peak 3

The $Abs_{440}/Abs_{\lambda_{max}}$ ratio values calculated for each anthocyanin, ranging from 31% to 33%, indicated a substitution in the C-3 position of the flavylium ring (Giusti *et al.* 1999). It is well known that anthocyanins with glycosidic substitutions at position 3 exhibit a ratio of the absorbance at 400–440 nm to the absorbance at the visible maximum wavelength (520 nm) that is almost twice as large as that for anthocyanins with glycosidic substitution at positions 5 or both 3 and 5 (Harbone 1976). In addition, the obtained $Abs_{280}/Abs_{\lambda_{max}}$ (67–100%) and $Abs_{310}/Abs_{\lambda_{max}}$ (13–22%) ratios

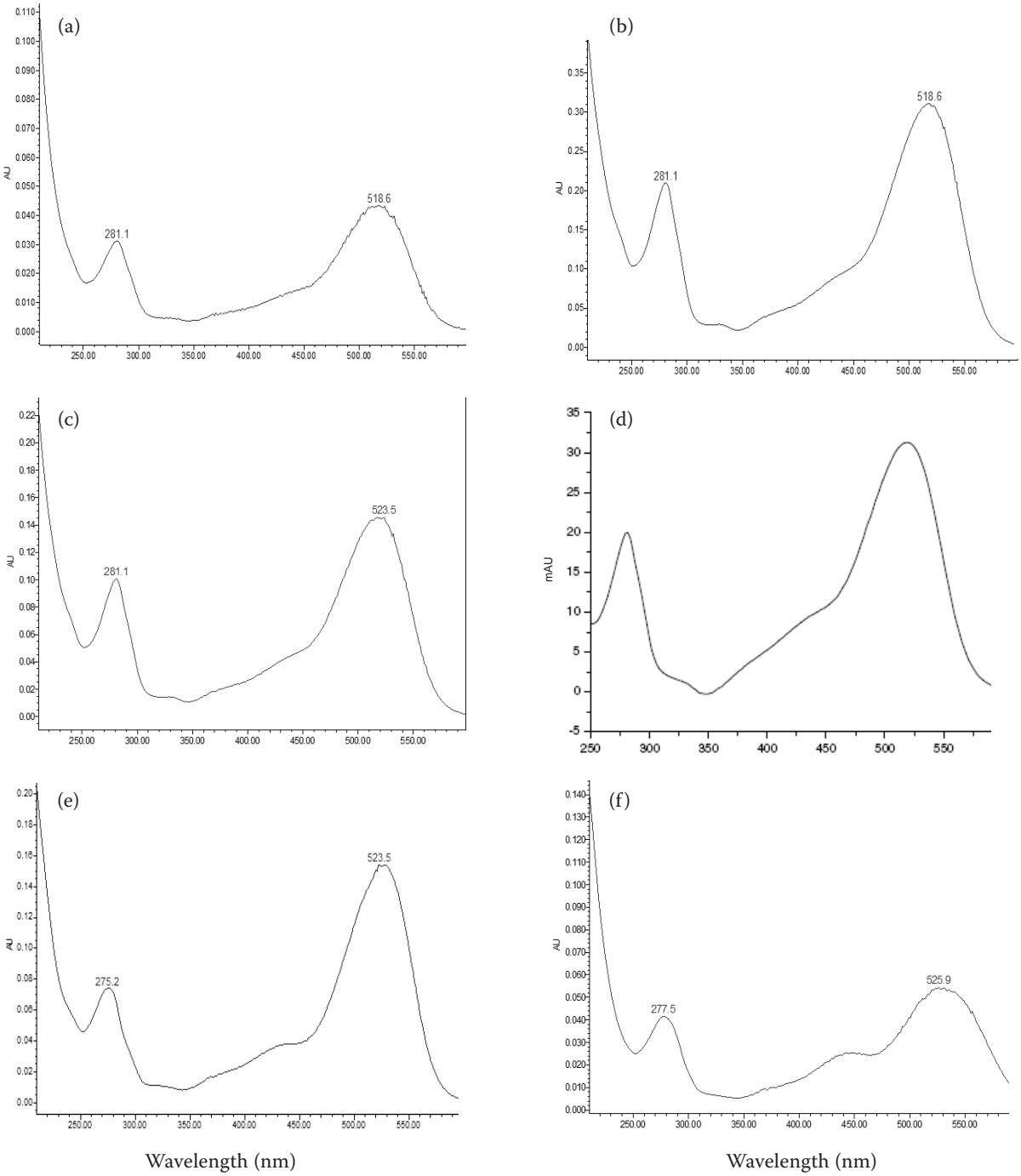


Figure 5. UV-Visible spectrum of peaks 1–5 recoded with a photodiode array detector (PAD) from 200 nm to 600 nm: (a) peak 1; (b) peak 2; (c) peak 3; (d) standard cyanidin 3-*O*-glucoside (Cy-3-Glu); (e) peak 4; (f) peak 5

confirmed that waxberry anthocyanins were simple anthocyanin molecules without acylation of the glycoside with aromatic acids (Woodall & Steward *et al.* 1998; Mozetic *et al.* 2002).

Acid hydrolysis of the purified anthocyanins produced three peaks (4 and 5), as shown in the chromatogram in Figure 2b. The MALDI-TOF-MS profiles of these compounds presented the molecular ions M^+ at *m*/*z* 287 (4) and 317 (5) corresponding to the molecular ions of cyanidin and petunidin, respectively. The absorbance spectra of these compounds confirmed their identity.

The three major anthocyanins (compounds 1–3 ; Figure 2a) were isolated by preparative HPLC and their identity and structure were confirmed on the basis of H^1 NMR spectroscopic data. The chemical

Table 2. H1 NMR spectroscopic data of two anthocyanins (1 and 3) isolated from the extract of waxberry fruit [δ in CD_3OD-CF_3COOD (9:1) at 25°C]

Anthocyanin	1 δ (J in Hz)	2 δ (J in Hz)	3 δ (J in Hz)
Aglycone			
H-4	8.98 s	9.02 s	9.05 s
H-6	6.68 d (2.1)	6.65 d (2.1)	6.69 d (1.8)
H-8	6.85 brs	6.89 brs	6.90 brs
H-2'	8.06 d (2.4)	8.06 d (2.4)	8.05 d (2.5)
H-5'	7.03 d (8.7)	7.05 d (8.5)	
H-6'	8.29 dd (2.5, 8.8)	8.31 dd (2.5, 8.8)	8.35 dd (2.4, 8.5)
O-CH_3			3.92 s
3-Glycosyl	3-galactosyl	3-glucosyl	3-glucosyl
H-1"	5.25 d (7.6)	5.31 d (7.8)	5.30 d (7.8)
H-2", H-3", H-4", H-5", H-6"	3.15-3.50 *m*	3.20-3.55 *m*	3.18-3.52 *m*

shifts (δ) obtained from the H^1 NMR analysis of anthocyanins 1, 2, and 3 are reported in Table 2. The signals in the downfield of the spectra between δ 6.7 and 9.0 ppm were clearly attributable to the aromatic protons (A and B rings) of the aglycone molecule as previously reported for these compounds (Pridham 1964; Kuskoski *et al.* 2003). The signal doublets at δ 5.3 corresponded to the protons on the anomeric carbon from the glucose residues, confirming that they were in position C-3 as also indicated by the $Abs_{440}/Abs_{\lambda max}$ ratio values. The β-configuration of this moiety was confirmed from the magnitude (J = 7.6 Hz) of the $J_{1"2"}$ coupling constant in the H^1 NMR spectra (Pale *et al.* 1997; Froytlog *et al.* 1998). The spectrum of compounds 2 and 3 presented a doublet at δ 5.29 (d, J = 7.6 Hz, H-1 glucose) confirming the presence of glucose as sugar moiety (Gonnett & Fenet 2000; Fossen *et al.* 2003).

The total amount of anthocyanins in the fruit of waxberry, determined on the cyanidin 3-glucoside basis, was 286 mg/g of stoned fresh fruits of waxberry. Cyanidin 3-*O*-glucoside was the most predominant anthocyanin (173 mg/g) followed by petunidin 3-*O*-glucoside (70.6 mg/g). The amount of Cyanidin 3-*O*-galactoside was 42.4 mg/g. This indicated that the composition of anthocyanins in waxberry fruit here did not match with the results reported previously (Du *et al.* 2008). The difference is probably due to different methods of extraction, processes of separation, and conditions of purification and analysis.

To our knowledge, waxberry has traditionally been used as Chinese herb medicine in folk remedies for the treatment of pains, carbuncles, inflammations, and cancers, and it occurs in great abundance in most regions of China. It could be used for the extraction of anthocyanins intended to be employed as food colorants and antioxidant agents by the food, pharmaceutical, and cosmetic industries.

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