Coloration, anthocyanin profile and metal element content of Yunnan Red Pear (*Pyrus pyrifolia*)

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Abstract

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The pigmentation response, coloration components and the metal elemental content of Yunnan Red Pear were studied. Light is indispensable for peel pigmentation. With increasing duration of illumination of fruit, the area of skin colour and colour intensity of peel increases due to accumulation of anthocyanin. The red anthocyanin component of Yunnan Red Pear skin is cyanidin-3-*O*-galactoside. Other phenolic compounds in pear skin are chlorogenic acid, isorhamnetin-3-*O*-galactoside and isorhamnetin-3-*O*-6"-malonylgalactoside; or isorhamnetin-3-*O*-6"-malonylglucoside; or isorhamnetin-3-*O*-malonylgalactoside. The elements Ca, Mg and Fe are abundant in Yunnan Red Pear flesh, and Zn, Mn, Cu were also identified. These results will aid red pear breeding and pear nutrition research, as well as increase understanding of the regulatory mechanisms underlying pear fruit colouration.

Keywords: red peel pear; exocarp coloration; LC-MS/MS; anthocyanins; elemental analysis

The pigmentation of pear fruit skin varies from green, yellow, brown and red, of which red pears are most valued for health and consumer popularity (Allan et al. 2008). Therefore the selection of red skinned pears is a breeding goal of many programs (Zhang et al. 1997). The world's red pear germplasm resources are scarce, with the Yunnan Province of China having good germplasm resources because of its unique geographical and climatic conditions. Seven varieties of red pears were bred since 1986 beginning with the paternal red 'torch pear' of Yunnan (Wang et al. 1997). Subsequently, a number of red pears were generated from sports and by hybridization (Jiao et al. 1999; Guo et al. 2001).

Yunnan Red Pear belongs to *Pyrus pyrifolia*, with four varieties selected for cultivation based on the maturity; early-maturing cv. Zaobaimi, mid-matur-

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ing cv. Mantianhong and cv. Meirensu and late-maturing cv. Yunhong-1. Yunnan Red Pear is known for its large shape, thin exocarp, white flesh, sweet texture and appealing appearance. The late-maturing variety Yunhong-1 colors most easily, followed by mid-maturing varieties, while the early-maturing variety colors with difficulty. Skin color is not only an important genetic trait, but it is a key factor determining their commercial value. Anthocyanin accumulation is responsible for pigmentation in many fruits (Allan et al. 2008). At present, less is known about what contributes to the red component of Yunnan Red Pear's peel, such as what are the compounds that cause skin pigmentation and anthocyanin composition, and the metabolic pathways and regulatory mechanisms involved.

The anthocyanin composition of red pears was shown to be mainly cyanidin-3-*O*-galactoside and cyanidin-3-*O*-arabinoside (Francis 1970). Using TLC (Thin Layer Chromatography) and HPLC methods the anthocyanin composition of red pear cv. Sensation Red Bartlett was identified as containing major cyanidin-3-*O*-galactoside and minor peonidin-3-*O*-galactoside (Dussi et al. 1995). In 2007, the anthocyanin composition of *Pyrus communis* was determined as cyanidin-3-*O*-galactoside and cyanidin-3-*O*-arabinoside (Fischer et al. 2007). Using the LC-MS/MS method the red pear cultivars Red D'Anjou, D'Anjou and Seckel all contained major cyanidin-3-*O*-galactoside and minor peonidin-3-*O*-galactoside, while cv. Comice contained only cyanidin-3-*O*-galactoside (Lin, Harnly 2008). Using the HPLC method it was shown that Red D'Anjou peel contains major cyanidin-3-*O*-galactoside, minor peonidin-3-*O*-galactoside and trace amounts of cyanidin-3-*O*-arabinoside, cyanidin-3-*O*-glucoside and peonidin-3-*O*-glucoside (Ngo et al. 2009). Therefore, the anthocyanin component varies among different varieties of red pears.

Bagging and debagging techniques are commonly used in fruit production to protect the fruit from pests and pesticide residues and to enhance fruit pigmentation. In this study, bagging and debagging was used for treatment of pear and the relative anthocyanin content of Yunnan Red Pear peel was measured during fruit ripening. Anthocyanin and other phenolics were identified by HPLC with diode array and electrospray ionization/mass spectrometric detection (LC-DAD-ESI/MS_2) method. Trace metal elements are important for human health and plant nutrition, therefore the content of Yunnan Red Pear flesh metal elements Mn, Cu, Zn, Ca, Mg and Fe were determined by inductively coupled plasma atomic emission spectroscopy (ICP-AES). These results lay a foundation for red pear breeding and development of Yunnan Red Pear.

MATERIAL AND METHODS

The fruit peels of the red pear varieties used in this experiment were all collected from the orchards of the Yunnan Red Pear Science and Technology Development Company, China. Fruits were bagged with white single-layer bags after fruit set and replaced with double black sacks after fruit became too large for white bags. The peels were collected after removal of the bags at days 0, 1, 2, 3, 5 and 7, respectively, which was approximately 7 days before usual harvest date. The skin was peeled off and immediately placed into liquid nitrogen and stored at –80°C before use.

Reagents used were: acetonitrile (HPLC grade, Merk, Darmstadt, Germany); ultra-pure water; methanol (HPLC grade, Merk , Darmstadt, Germany); formic acid (HPLC grade, Merk, Darmstadt, Germany); perchloric acid (GR grade, Sinoreagent, Beijing, China); aqua fortis (GR grade, Sinoreagent, Beijing, China).

Pigment extraction and measurement. Using an adapted method (Zhang et al. 2011), 0.25 g of pear peel sample was fully ground in a mortar filled with liquid nitrogen, then 1 ml of 0.01N hydrochloric acid methanol (v/v = 1:999) added and continued grinding until thoroughly mixed. The mixture was transferred and centrifuged at 14,000 rpm under 4°C and filtered using a sterile 0.22μM filter. Finally the extract was stored at –20°C until use.

The method of peel phenolic compounds extraction for LC-DAD-MS_2 analysis was as above, except 1 g of pear peel sample was ground, then 3 ml of 0.1N hydrochloric acid methanol (v/v = 1:99) was added. The mixture was transferred into another clean centrifuge tube, and held at 4°C overnight. The next day it was centrifuged and filtered using a sterile 0.22μM filter. Finally the extract was stored at –20°C until use.

LC-DAD and ESI-MS conditions for analysis of pear peel. The LC-DAD consisted of a Waters 600 HPLC (Waters, Milford, USA). An Xterra Phenyl 5 μm (4.6 × 250 mm) column (Waters, Wexford, Ireland) was used at a flow rate of 1.0 ml/min. The column oven temperature was set at 25°C. The mobile phase consisted of a combination of A (10%

formic acid in water) and B (10% formic acid + 40% acetonitrile + water). The protocol was 20 to 85% B (v/v) in 40 min, 85 to 20% B for 20 minutes. The DAD was set at 530 nm. The software was Millenium 32 (Waters, Milford, USA).

The LC-DAD-ESI/MS_2 consisted of an Agilent 1200 HPLC (Agilent, USA) coupled to a diode array detector and HCT mass spectrometer (Bruker, Bremen, Germany). An Xterra Phenyl 5 μm (4.6 × 250 mm) column (Waters, Wexford, Ireland) was used at a flow rate of 1.0 ml/min. The column oven temperature was set at 25°C. The mobile phase consisted of a combination of A (0.1% formic acid in water) and B (100% methanol). The protocol is 5% B for 1 min, to 100% B (v/v) in 14 min, 100% B for 3 min, to 5% B for 10 s, 5% B for 2 min. The DAD was set at 280, 310, 330, 350, 440, and 530 nm. To record the peak intensity, and UV/visible spectra were recorded from 190 to 800 nm for phenolic component identification. Mass spectra were simultaneously acquired using electrospray ionization in the negative ionization (NI) modes over the range of 100 to 2,000 *m/z*. A drying gas flow of 10 l/min, a drying gas temperature of 330°C, a nebulizer pressure of 25 psi, and capillary voltages of 4,000 V for NI were used. The Software is Bruker Compass Data Analysis 4.0 (Bruker, Bremen, Germany).

Metal elements determination by ICP-AES. To determine the flesh metal elements of pear, a modified wet oxidation protocol (Zhang et al. 2007) was applied. The flesh of three fruits with similar size and from three different trees of cvs Zaobaimi, Meirensu, Mantianhong and Yunhong-1 were peeled off after washing with ddH_2O. Five g of each flesh was measured in 150 ml breaker, and was sealed by film after 2 ml of perchloric acid ($HClO_4$) were added. After reaction for 2 h at room temperature, 8 ml of concentrated nitric acid (HNO_4) were added into these samples, then heated by electromagnetic oven with shaking at intervals until the solutions were clear. Heating continued until white salts appeared. After cooling, these salts were dissolved into 50 ml of ddH_2O. The control was treated as above without pear flesh. Samples were analysed by Direct Reading Echelle (DRE) inductively coupled plasmatomic emission spectrometry (ICP-AES) (Leeman Lab, Hudson, USA). The working conditions were frequency: 40.68 MHz, generator: RF, power: 1.1 kW, cooling air: 18 l/min, auxiliary gas: 0.5 l/min, atomizer pressure: 372.6 KPa, sample uptake rate: 1.3 l/min. The argon used was analytical reagent grade (> 99.99%). The wavelength for Mn, Cu, Zn, Ca, Mg and Fe were 257.610, 324.754, 206.200, 317.933, 285.213 and 259.940 nm, separately. The detection limit for Mn, Cu, Zn, Ca, Mg and Fe were 0.93, 3.6, 3.9, 6.7, 1.1 and 4.1 mg/l, separately.

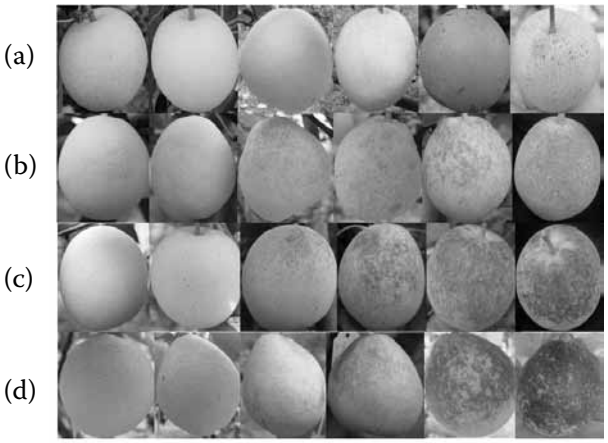


Fig.1. Yunnan Red Pear used for anthocyanin content measurement: (a) Zaobaimi, (b) Meirensu, (c) Mantianhong, (d) Yunhong-1

RESULTS AND DISCUSSION

Relationship between skin color change of Yunnan Red Pear and its anthocyanin content during ripening

As shown in Fig. 1, the peel of Yunnan Red Pear without solar illumination during the ripening period is pale, indicating that light is indispensable for coloration of peel. As the illumination time increases, the skin area and color intensity of pear peel increases, which is consistent with previous results (Huang et al. 2009). Cv. Yunhong-1 colours easily, while cv. Zaobaimi achieves pigmentation with difficulty. Peel skin pigmentation is due to accumulation of anthocyanin, therefore the relative content of peel anthocyanin was measured. In the ripening period, the relative content of the peel anthocyanins increases slowly within 3 days after solar illumination, and a rapid increase appears in 5^{th} or 7^{th} day (Fig. 2). These results suggest that the appearance of the peel is consistent with its relative anthocyanin content during fruit ripening.

HPLC analysis of anthocyanin composition of the red pear peel

Preliminary analysis for peel anthocyanin composition was done by HPLC analysis using cvs Yun-

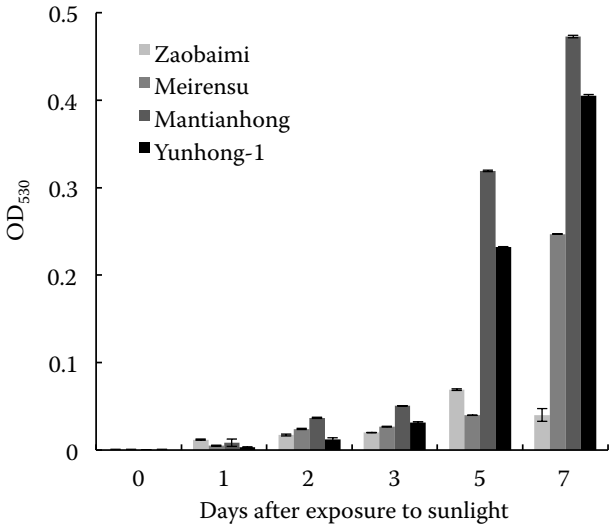


Fig. 2. Relative anthocyanin content of Yunnan Red Pear cvs Zaobaimi, Mantianhong, Meirensu and Yunhong-1's pericarp after solar illumination for 0, 1, 2, 3, 5 and 7 days

hong-1, Mantianhong, Meirensu and Zaobaimi after solar illumination of 0 and 7 days. The results showed that anthocyanin extracts of 0 and 7 days were colourless and red, respectively. And compared to the negative control (colourless), a major and a minor peak was detected in the peel anthocyanin extract of four red pear varieties after 7 day illumination, sharing the same retention time (data not shown). Therefore, combining the reported documents these four varieties of Yunnan pear may share the same pigment ingredients, major cyanidin-3-*O*-galactoside and minor peonidin-3-*O*-galactoside.

To further identify the anthocyanin composition of the red pear's peel, the UV-visible spectrum analysis was conducted. As shown in Table 1, anthocyanin extracts in hydrochloric acid methanol of four kinds of pears' peel has a 7.7 min retention peak with typical absorption at 280 and 530 nm, indicative of anthocyanin. The value of the A_{440}/A_{max} was 34–36%, suggesting that the substance belongs to anthocyanin-3-*O*-glycosides. The $A_{310}/A_{max\text{-}vis}$ of cvs Yunhong-1 and Mantianhong were about 40%, indicating that the substance does not have an acyl group, while the A_{310} and A_{440} of cvs Zaobaimi and Meirensu were not detected, possibly due to the low level of anthocyanin. After a small amount of $AlCl_3$ was added into anthocyanin extract a red shift was detected, indicating that the anthocyanin had o-phenolic hydroxyl groups, and it may be unacylated cyanin-3-*O*-glycoside, petunin-3-*O*-glycoside or delphinin-3-*O*-glycoside.

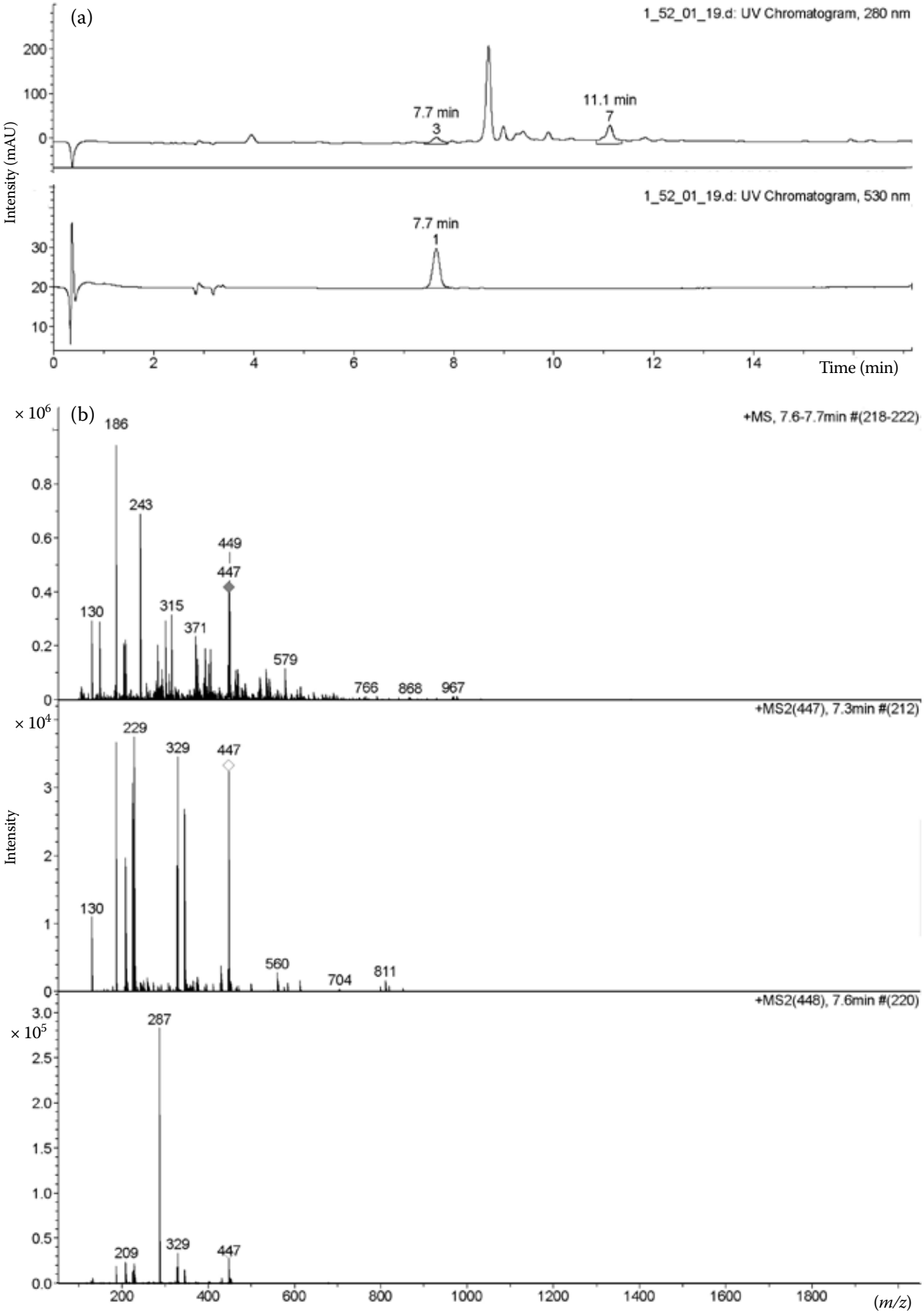
LC-MS/MS analysis of the anthocyanin composition of red pear's peel

In order to identify the peel's pigment composition, samples of cvs Yunhong-1, Mantianhong, Meirensu and Zaobaimi, after illumination for 7 days, were examined using LC-MS/MS analysis. The LC results showed that the four kinds of pear anthocyanin extracts shared the same retention time at 7.7 min, with typical absorption peaks at both 530 and 280 nm (Fig. 3a) and absorption peaks in 230, 259 and 343 nm, and a molecular weight of 447 and (or) 449 Da were detected in their corresponding $[M + H]^+$ (Fig. 3b), and a molecular weight of 287 was detected in the cleavage fragment in their $[A + H]^+$ (Fig. 3b) except cv. Zaobaimi. The expected molecular weight of cyanidin-3-*O*-galactoside was 448 Da, and the absorption peak of cyanidin-3-*O*-glucoside are 282 and 518 nm (Marina et al. 2010), so the anthocyanin of the peak at 7.7 min is cyanidin-3-*O*-galactoside. The peak with the retention time at 11.1 min and absorption peak at 350 nm (Fig. 3a), was also identified in cv. Yunhong-1. Both molecular weight of 465 and 463 Da were detected in their corresponding $[M + H]^+$ and $[M + H]^-$, and the expected molecular weight of peonidin-3-galactoside was 463 Da, which is different from the expected, and its $[A + H]^+$ and $[A + H]^-$ were detected in the molecular weight of

Table 1. UV and mass spectrum parameters of the main anthocyanin of Yunnan Red Pear peel

Anthocyanin	Retention (min)	Maximum absorption in UV (nm)	Maximum absorption in visible (nm)	A_{440}/A_{max}	$A_{310}/A_{max\text{-}vis}$	Red Shift	(*m/z*)
Yunhong-1	7.6	280	530	36	40.79	yes	449.287
Mantianhong	7.6	280	530	35.33	40.57	yes	449.287
Meirensu	7.6	280	530	33.90	–	yes	449.287
Zaobaimi	7.6	280	530	–	–	yes	449.n.d.

n.d. – not detected


(a)
1_52_01_19.d: UV Chromatogram, 280 nm
7.7 min
3
11.1 min
7
1_52_01_19.d: UV Chromatogram, 530 nm
7.7 min
1
Intensity (mAU)
Time (min)
(b)
+MS, 7.6-7.7min #(218-222)
+MS2(447), 7.3min #(212)
+MS2(448), 7.6min #(220)
Intensity
(m/z)

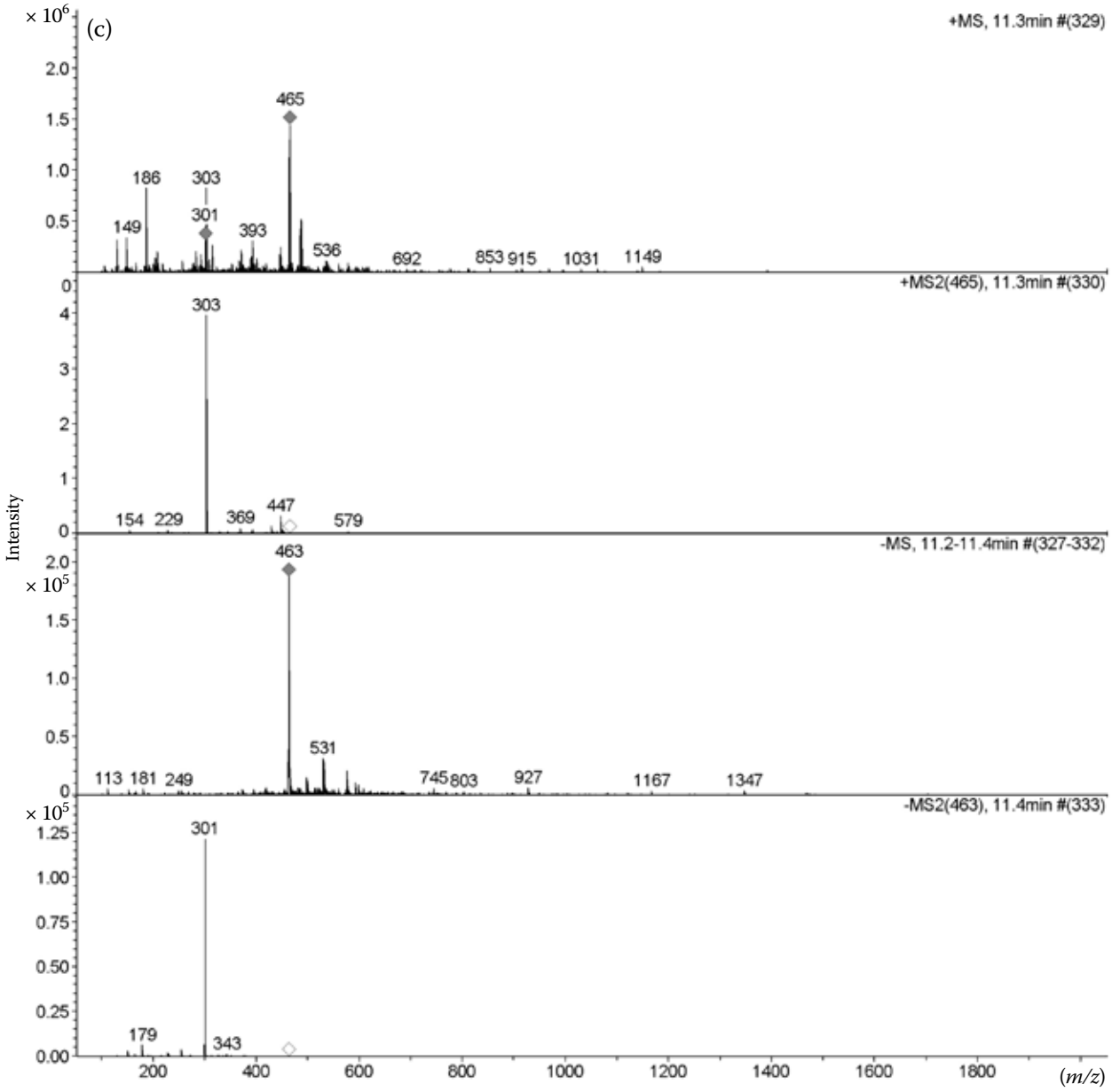


Fig. 3. LC-MS-MS results of the anthocyanin extract of Yunnan Red Pear. (a) LC results of cv. Yunhong-1; (b) Cv. Yunhong-1's MS/MS results of the peak at 7.7 min; (c) cv. Yunhong-1 MS/MS results of the peak at 11.1 min

303 and 301 Da (Fig. 3c). It is noteworthy that although the molecular weight of 449 Da in the $[M + H]^+$ was detected in cv. Zaobaimi, its $[M - H]^+$ cannot be carried out due to its low peak at 530 nm, this may be due to its low levels of anthocyanin in fruit peel. These results showed that the peak at 7.7 min was cyanidin-3-*O*-galactoside while the peak at 11.1 min was quercetin-3-*O*-galactoside or quercetin-3-*O*-glucoside (Lin et al. 2008), which shows that anthocyanin composition of Yunnan Red Pear peel was cyanidin-3-*O*-galactoside. Therefore, Yunnan Red Pear share the identical anthocyanin with previously reported red pear cv. Comice, whose red anthocyanin component is far less complicated than cv. Red D'Anjou (Ngo et al. 2009).

LC-MS/MS analysis of other phenolic compounds composition of red pear's peel

In order to analyze other phenolic compounds of Yunnan Red Pear, extracts of the four pear types were subjected to LC-MS/MS analysis. The LC result shows that all four pear varieties share similar peaks.

Table 2. LC-MS/MS characterization of main phenolic compounds of Yunnan Red Pear peel

Peak	RT (min)	$[M+H]^+/[M-H]^+$ (*m/z*)	$[A+H]^+/[A-H]^+$	UV λ_{max}(nm)	Compounds
1	2.9	478	134, 183, 314	230, 259, 293, 347	unknown
2	3.2	411	217	210, 254, 293, 345	unknown
3	3.9	567	295	222, 260, 284, 346,	unknown
4	7.4	449	287	230, 259, 286, 343	cyanidin-3-*O*-galactoside
5	8.7	355/353	163/191	217, 238sh, 297sh, 326	chlorogenic acid
6	9.4	377	359	230, 259sh, 287, 340	unknown
7	10.3	369/367	163/179	232, 294sh, 330sh	unknown
8	11.1	465	303	232, 254, 293, 343	quercetin-3-*O*-glucoside or quercetin-3-*O*-galactoside
9	11.8	479	317	233, 254, 292, 348	isorhamnetin-3-*O*-galactoside
10	12.8	565	317	235, 254, 292, 350	isorhamnetin-3-*O*-6"-malonylgalactoside isorhamnetin-3-*O*-6"-malonylglucoside isorhamnetin-3-*O*-malonylgalactoside

sh – shoulder peak

Phenolic extracts of typical cultivar cv. Yunhong-1 was analyzed by MS(n). Peak 5 is the most abundant in red pear skin, follow by peak 7, 8 and 9 (data not shown). The peak 4 is cyanidin-3-*O*-galactoside with the molecular weight of 449$[M + H]^+$/287$[A + H]^+$ and its absorption peak in 230, 259, 286, 343, 530 nm, while peak 8 may be quercetin-3-O-glucoside or quercetin-3-O-galactoside with the molecular weight of 465$[M + H]^+$/303$[A + H]^+$ and the absorption characterization (Table 2) (Lin et al. 2008). Peak 5 is chlorogenic acid with the molecular weight of 355$[M + H]^+$/163$[A + H]^+$and 353$[M + H]^-$/191 $[A + H]^-$, and it is the most abundant in red pear, which is consistent with previously reported results (Table 2) (Lin et al. 2008); it also indicates that Yunnan Red Pear possess strong antioxidant capacity as the antioxidant capacity was correlated with the content of chlorogenic acid (Galvis et al. 2003). Peak 9 is isorhamnetin-3-*O*-galactoside with the molecular weight of 479$[M + H]^+$/317[A + H] and absorption peaks in 233, 254, 292, 348 nm (Table 2) (Lin et al. 2008). And with the molecular weight of 565$[M + H]^+$/317[A + H] and absorption peaks in 233, 254, 292, 350 nm, peak 10 may be isorhamnetin-3-*O*-6"-malonylgalactoside; or isorhamnetin-3-*O*-6"-malonylglucoside; or isorhamnetin-3-*O*-malonylgalactoside (Table 2) (Lin et al. 2008). As few HPLC-MS-MS data of pears were reported, the other peaks are unknown, and they need further identification. The phenolic extracts of cvs Zaobaimi, Meirensu and Mantianhong share the similar LC characterization as cv. Yunhong-1, therefore they share the similar or identical phenolic composition with cv. Yunhong-1.

Elemental determination by ICP-AES

Many trace metal elements are beneficial for human health, so the content of metal elements (Mn, Cu, Zn, Ca, Mg and Fe) was measured. The results are shown in Fig. 4. The metal element content varied much among different trees. In general, the content of Ca, Mg and Fe are abundant in Yunnan Red Pear. Mg is the most abundant metal element, followed by Ca and Fe. Trace amounts of Mn, Cu and Zn were also identified in the four pear varieties. Vanadium and cobalt were not identified in four kinds of pears. Among the four pear varieties, cv. Mantianhong had the most metal content.

The functions of these elements are versatile in human body. Mg, which is the cofactor for many en-

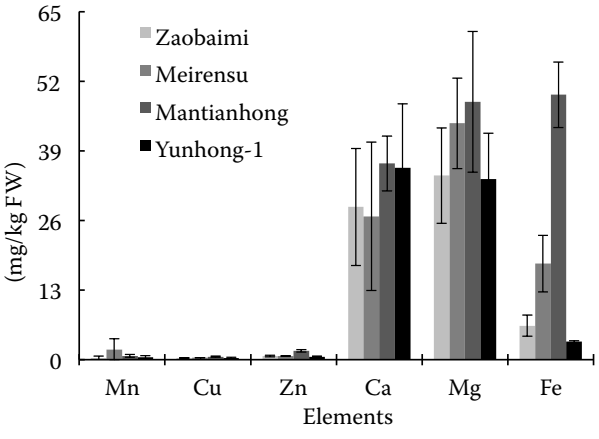


Fig. 4. The content of metal elements Mn, Cu, Zn, Ca, Mg and Fe of Yunnan Red Pear cvs Zaobaimi, Mantianhong, Meirensu and Yunhong-1 flesh were measured by ICP-AES Each sample was repeated for three times.

zymes, is vital for the cardiovascular functions. Ca is the essential component of human bone. Fe, which is present in hemoglobin, myoglobin and some enzymes, can fight against pneumonia of children. Zn can improve human immunity and promote growth and development. Cu is a component of many enzymes and it can affect electron transport and oxidation-reduction etc. Mn is involved in the metabolism of carbohydrate and fatty acids, which can affect growth and development. These results can provide useful information for nutritional application and rational application of fertilizer.

CONCLUSIONS

The coloration results of the study show that Yunnan Red Pear's pigmentation increased with solar exposure, as did the anthocyanin content. Light is indispensable for peel pigmentation. This study shows that anthocyanin in Yunnan Red Pear is cyanidin-3-*O*-galactoside. Other phenolic compounds are chlorogenic acid, isorhamnetin-3-*O*-galactoside and isorhamnetin-3-*O*-6"-malonylgalactoside; or isorhamnetin-3-*O*-6"-malonylglucoside; or isorhamnetin-3-*O*-malonylgalactoside. The elements Ca, Mg and Fe are abundant in Yunnan Red Pear, and Zn, Mn, Cu were also identified. These results will lay a foundation for red pears breeding, regulation mechanism of red exocarp of Yunnan Red Pear and pear nutrition research. The further study will be focused on how the complex PyMYB-PybHLH-PyWD40 responses to light, temperature, adverse stress, to regulate the biosynthesis of cyanidin-3-*O*-galactoside.

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