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Flavonols and Flavones Changes in Pomegranate (*Punica granatum* L.) Fruit Peel during Fruit Development

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ABSTRACT

Four Chinese pomegranate (*Punica granatum* L.) cultivars were analyzed for their individual flavonols and flavones (in fruit peel extracts) using High Performance Liquid Chromatography (HPLC), with changes in flavonols and flavones as occurring during fruit development. The results revealed the presence of kaempferol, quercetin, myricetin, luteolin, and apigenin in all the four cultivars. In addition, the concentrations of flavonols were found out as always higher than those of the flavones in different cultivars and throughout the different fruit developmental phases. Kaempferol was the most prominent flavonol in Hongbaoshi, Lvbaoshi and Shuijingtian, while myricetin the major and dominant flavonol in Moshiliu. The pattern of kaempferol was similar to quercetin in each individual cultivar, but quite differing among the four cultivars. The level of luteolin was higher than that of apigenin in all the cultivars. During test course, similar change patterns for luteolin and apigenin were revealed in each individual cultivar. This is the first report regarding the flavonols and flavones concentration changes in pomegranate peel extracts. The results indicated that the changes in flavonols and flavones were significantly affected by cultivar and its developmental stages. Moshiliu proved to be an excellent pomegranate cultivar with rich flavonol contents. It can be further studied for its potential uses being more fully exploited.

Keywords: Change patterns, Flavones, Flavonols, Pomegranate.

INTRODUCTION

Pomegranate (*Punica granatum* L.) is famed as one of the oldest known edible fruits. It is believed that the fruit is native to central Asia and consequently partly to Iran, and from where introduced to the Mediterranean regions (Levin, 1994). Today, pomegranate is cultivated throughout the world in many subtropical and tropical countries (Holland *et al*., 2009). Pomegranate has presently gained great economic importance due to substantial increase in its consumption. The fruit has been termed 'superfruit' due to its such biological actions as antioxidativity, and as well antidiabetic, antitumoral, antimicrobial, and anti-inflammatory properties (Seeram *et al*., 2006; Viuda-Martos *et al*., 2010; Mena *et al*., 2011). All these health-promoting properties have been ascribed to the polyphenol compounds present in various parts of the pomegranate fruit (Kaplan *et al*., 2001; Singh *et al*., 2002; Huang *et al*., 2005; Lansky and Newman, 2007; Surveswaran *et al*., 2007; Sestili, *et al*., 2007).

Pomegranate fruit peel is characterized by containing substantial levels of phenolic compounds, including hydrolyzable tannins

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(punicalin, pedunculagin, punicalagin, gallic and ellagic acid) (Madrigal-Carballo *et al*., 2009; Çam and Hışıl, 2010; Fischer *et al*., 2011), as well as flavonoids (anthocyanins, flavonols, and flavones) (Nawwar *et al*., 1994; Van Elswijk *et al*., 2004; Fischer *et al*., 2011; Zhao *et al*., 2013). Previous studies have demonstrated that pomegranate peel extracts exhibit markedly higher antioxidant capacity than the pulp, juice, and seed extracts (Guo *et al*., 2003; Li *et al*., 2006; Yasoubi *et al*., 2007; Ardekani *et al*., 2011; Orak *et al*., 2012). Therefore, there is a growing interest in the potential use of pomegranate peels as natural food preservatives and nutraceuticals (Çam and Hışıl, 2010; Qu *et al*., 2010; Viuda-Martos *et al*., 2010). Also, *in vitro* and *in vivo* studies have evidenced antibacterial (Negi and Jayaprakasha, 2006), antimicrobial (Al-Zoreky, 2009), anticancer (Dikmen *et al*., 2011), anti-inflammatory and antiallergic (Panichayupakaranant *et al*., 2010) properties of pomegranate peel extracts.

There is widespread concern over hydrolyzable tannins in pomegranate peels (Ismail *et al*., 2012), while little attention has been paid to flavonoids, another large group of polyphenols. The flavonoids are of particular interest due to their high prevalence in such foods as fruits, vegetables, and tea. Many flavonoids have been discerned as beneficial to human health (Yao *et al*., 2004). Flavonoids may be divided into five subclasses namely: 1- flavonols, 2- anthocyanidins , 3- flavanones, 4- flavones and 5- isoflavones (Maron, 2004). In the present study, it has been planned to focus on the subgroups of flavonols and flavones, as these two flavonoids occur ubiquitously in plants and are the ones which have been most frequently attended to.

The presence of flavonols and flavones in pomegranate has been reported in pomegranate peels (Van Elswijk *et al*., 2004; Taskeen *et al*., 2011; Mansour *et al*., 2013), leaves (Nawwar *et al*., 1994), and juices (Poyrazoğlu *et al*., 2002; Guo *et al*., 2008). Unfortunately, in many cases, investigators have simply separated some flavonoids' components, failing to perform quantification of flavonols and flavones, even with regard to the change patterns during fruit development. Due to the plentiful genetic diversity in Chinese pomegranate (Yuan *et al*., 2007), four cultivars of distinct fruit color selected for their flavonoids' assay. The aim of the current study is to quantify the flavonols and flavones in the four cultivars, by means of HPLC, and to evaluate the profiles of flavonols and flavones of each cultivar across their developmental stages.

MATERIALS AND METHODS

Sample Preparation

Four pomegranate cultivars, Hongbaoshi, Lvbaoshi, Shuijingtian, and Moshiliu, were selected as the study materials (Figure 1). Three trees from each cultivar were randomly selected from a commercial orchard in Yicheng, Shandong, China, where the trees were grown under homogeneous conditions. The fruits were

Figure1. Four cultivars of pomegranate: (A) Hongbaoshi; (B) Lvbaoshi; (C) Shuijingtian, and (D) Moshiliu.

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harvested within ten-day intervals from July 15 to September 13. A sample of 12 representative fruits was collected in each of the four tree orientations (east, west, south and north) from each cultivar. The samples were placed in a cooler, transported to the laboratory, peeled freshly. The peels from each individual cultivar of each sampling date were mixed together to assure uniformity. Tissues were frozen immediately in liquid nitrogen and stored at -80°C for subsequent testings.

Standards and Reagents

Reference standard of myricetin was purchased from Sigma-Aldrich (St. Louis, USA); apigenin was purchased from ChromaDex™ Corporation (Irvine, CA); quercetin, kaempferol and luteolin were purchased from National Institutes for Food and Drug Control (Beijing, China). Methanol and formic acid were in HPLC grade. The water used was treated through a Milli-Q water purification system (Millipore, Bedford, MA).

Extraction and Hydrolysis of Flavonoids

Extraction and acid hydrolysis of pomegranate peel was performed as previously described, with only a limited number of modifications (Justesen *et al*., 1998). In short, the frozen tissue was grinded in liquid nitrogen using a mortar and pestle. A quantity of a 0.3 g peel powder was then added to 15 mL of 62.5% aqueous methanol containing BHA (2 g L^{-1}) as antioxidant. To this, 3 mL of 6M HCl was added carefully. The extraction mixture was then heated to 90°C on a steam bath and refluxed for 2 hours, allowed to cool at room temperature, diluted to 25 mL with methanol, and finally sonicated for 5 min to form the final extract. All samples were then passed through 0.45 µm filters prior to HPLC analysis. For identification and quantification, extractions were performed in two replicates.

HPLC-DAD Analysis

HPLC analysis was performed using an Agilent 1200 series HPLC system equipped with an autosampler and Diode Array Detector (DAD). Separations were performed on a Zorbax SB-C18 column (150 mm×4.6 mm ID, 5 µm). The column temperature was set at 30°C. The mobile phase consisted of 2% aqueous formic acid solution (phase A) and methanol (phase B).The gradient was 80% A in 0 min and 20% A in 45 minutes at a flow rate of 1.0 mL min^{-1}. The injection volume for all the samples was 10 µL. Spectra were recorded from 200 to 600 nm, and chromatograms recorded at 365 nm.

Qualitative and Quantitative Analysis of Flavonoids

The qualitative identification of flavonoids was performed by comparing the retention times, elution order and UV-Vis spectra with those of the pure standards. Quantitative analysis was performed by means of external standard curve calibration.

Method Validation

Precision and accuracy (recovery) were employed to investigate the performance of the method. The precision was analyzed with five repeated assays of the mixed standard solutions. They were evaluated calculating the Relative Standard Deviation (RSD) of individual peak areas and elution retention times. Analytical recovery of individual flavonols and flavones were performed by adding known quantities of standards to four peel samples for hydrolysis and extraction, assaying as above. Recovery was determined by comparing the amounts of flavonoids added to the sample

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(subtracted from the endogenous one) to the corresponding amounts of standards.

Statistical Analysis

Data were analyzed through Analysis of Variance (ANOVA) to determine significant differences ($P < 0.05$) in total flavone and flavonol in different cultivars and at their different developmental stages using LSD test.

RESULTS AND DISCUSSION

Method Validation

Flavonoids extraction is an important factor in successful HPLC qualitation and quantification. In plants, flavonoid compounds are present in general in glycosylated forms. Aglycones (the forms lacking sugar moieties) occur less frequently. Various sugar molecules binding to various positions in the parent flavonoid lead to large numbers of flavonoids throughout the flora of the world, but most glycosylated flavonoid standards are not easily obtained commercially. It would be an impossible task to determine all glycosides flavonoids separately, thus, hydrolysis of flavonoid glycosides to corresponding aglycones offers the most practical approach (Hertog *et al*., 1992b). An additional advantage of this method is that after hydrolysis, low concentrations of individual glycosides with the same parent compound will add up and ease detection. Presently, hydrolysis of the glycosylated flavonoids has been applied to most flavonoids studies (Hollman *et al*., 1996; Justesen *et al*., 1998; Chu *et al*., 2000; Häkkinen and Törrönen, 2000; Škerget *et al*., 2005; Repollés *et al*., 2006). In the present study, the same acid hydrolysis assay was employed to isolate the flavonols and flavones in the pomegranate peel extracts.

The reliability of HPLC method was validated through the precision and recovery. The RSD value in elution times for myricetin, quercetin, luteolin, kaempferol, and apigenin were 0.281, 0.260, 0.274, 0.264, 0.263%, respectively. The lowest value of RSD in peak areas was obtained in luteolin (1.11%), whereas the results obtained led to the maximal value of RSD (2.21%) in quercetin. All RSDs obtained were satisfactory. The recovery of standards were 94.27-99.82% for myricetin, 94.0-100.1% for quercetin, 86.4-98.0% for luteolin, 92.4-104.0% for kaempferol, and 88.4-102.0% for apigenin, indicating excellent recovery.

Qualitation Analysis of Flavonols and Flavones

Based on the established method, three flavonols quercetin, myricetin, kaempferol, and two flavones apigenin and luteolin, were detected in all of the pomegranate samples (Figure 2). An identification of flavonols and flavones in pomegranate has previously been described. Van Elswijk *et al.* (2004) reported phytoestrogenic flavonoids (luteolin, kaempferol and quercetin) in pomegranate peels; apigenin and luteolin were identified in leaves (Nawwar *et al*., 1994); only quercetin was detected in Turkish and Tunisian pomegranates (Poyrazoğlu *et al*., 2002; Mansour *et al*., 2013); and when Mousavinejad *et al.* (2009) studied eight Iranian pomegranate juices, no flavonols were detected. These results implied that flavonols and flavones composition varied with different cultivars and plant tissues.

Major Flavonol and Flavone in Pomegranate

From Figure 3 it may be seen that in Hongbaoshi, Lvbaoshi and Shuijingtian cultivars, the level of kaempferol was the highest, followed by quercetin. In Moshiliu, myricetin was the most prominent, with the concentration significantly higher than those of kaempferol and quercetin throughout the entire test period. For flavones, the level of luteolin was always higher than that of apigenin in cultivars during their developmental stages (Figure 4).

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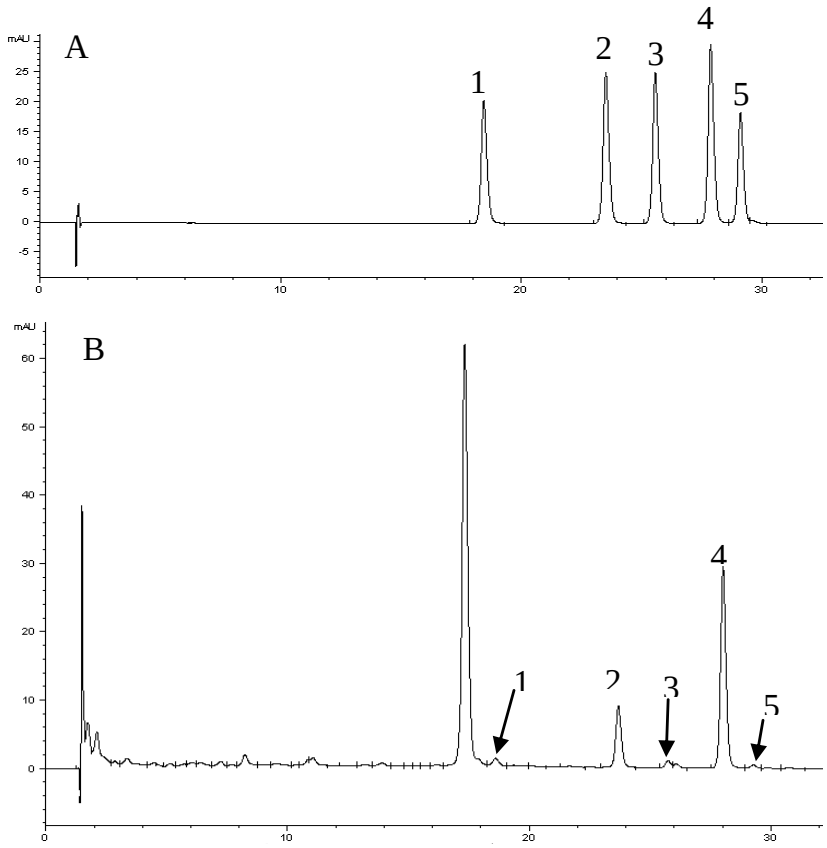

Figure 2. Chromatograms of flavonols and flavones: 1: Myricetin; 2: Quercetin; 3: Luteolin; 4: Kaempferol, 5: Apigenin and (A) Chromatogram of reference standards, (B) Chromatogram of Hongbaoshi sample of Sept. 3.

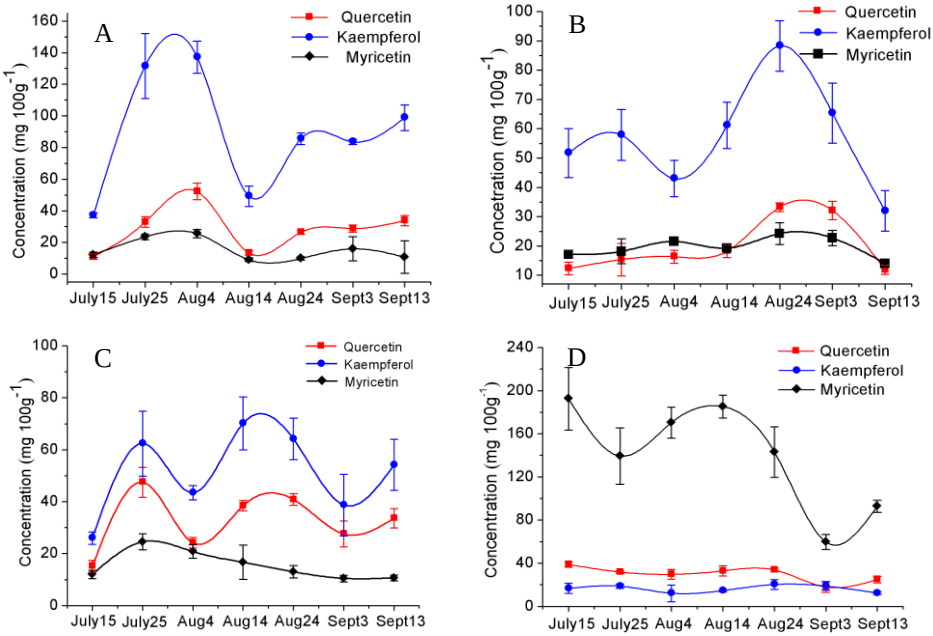

Figure 3. Change patterns of flavonols fruit development time among the four cultivars. Error bars represent ±SD (*n*= 2). (A) Hongbaoshi; (B) Lvbaoshi; (C) Shuijingtian, and (D) Moshiliu.

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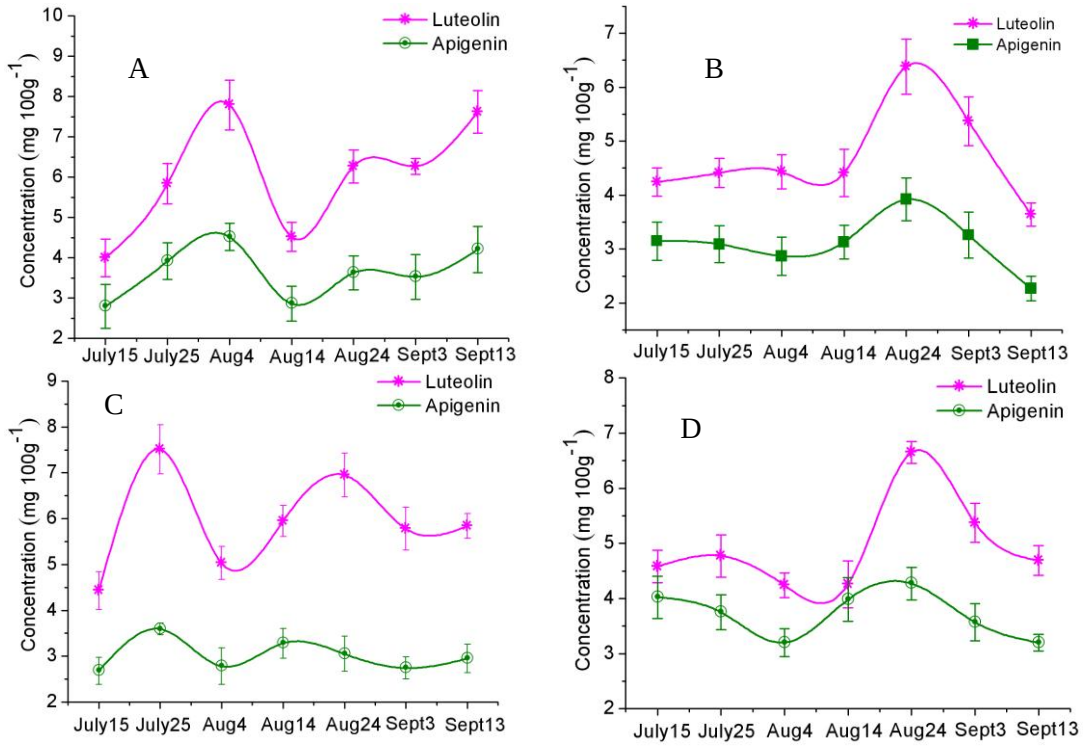

Figure 4. Change patterns of flavones with fruit development time among the four cultivars. Error bars represent ±SD (*n*= 2). (A) Hongbaoshi; (B) Lvbaoshi; (C) Shuijingtian, and (D) Moshiliu.

Previous studies on flavonols and flavones in such vegetables, fruits, and beverages as cauliflower (*Brassica oleracea*), red chili (*Capsicum annum*), black tea (*Camellia chinensis*), onion (*Allium odorum*), lettuce (*Lactuca sative* 'Capitata'), strawberry (*Fragaria ananassa*), apple (*Malus pumila*), and grape (*Vitis vinifera*) indicated that quercetin constituted their most ubiquitous and abundant flavonoid (Hertog *et al*., 1992b; Miean and Mohamed, 2001; Huber *et al*., 2009). However, the present study's results were different from the mentioned reports. It was determined that the concentration of quercetin was not the highest, myricetin was the major flavonol in Moshiliu, while kaempferol was the most abundant in the other three cultivars. Other studies have shown that kaempferol was also prominent in New Zealand spinach (*Amaranthus gangeticus*) (Huber *et al*., 2009), rucula (*Eruca sativa*) (Justesen *et al*., 2008; Huber *et al*., 2009), broccoli (*Brassica oleracea*) (Hollman and Arts, 2000), brinjal (*Solanum melongena*), pumpkin (*Cucurbita maxima*), carrot (*Daucus carota*), and bunga kantan (*Phaeomeria speciosa*) (Miean and Mohamed, 2001). In addition, luteolin was shown to be abundant in olive trees (Škerget *et al*., 2005). These studies demonstrated that the major flavonols and flavones were varied according to either species or cultivars.

Quantification Analysis of Flavonols and Flavones in Pomegranate

Figure 3 shows that the concentration of kaempferol in Hongbaoshi, Lvbaoshi and Shuijingtian was within the ranges of 37.15 ~137.15 mg $100g^{-1}$ fw (fresh weight), 31.98~88.29 mg $100g^{-1}$ fw , and 25.98~70.12 mg $100g^{-1}$ fw. The highest concentrations of quercetin in these three

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cultivars reached 52.24, 33.25, and 47.46 mg 100 g^{-1} fw, while myricetin had a lower concentration and varied slightly across the developmental stages. Myricetin concentration stood in the range of 59.78 to 185.27 mg 100g^{-1} fw in Moshiliu. Compared with flavonols, the levels of the flavones luteolin and apigenin in the four cultivars were much lower (Figure 4).

The results obtained implicated that cultivar and fruit developmental stages affected flavonoids content of pomegranate on tree. As shown in table 1, in Hongbaoshi, Shuijingtian, and Moshiliu, there were no significant changes in late stages for total flavonols and flavones, but in Lvbaoshi these two flavonoids' contents were both significant in late stages. In early stages, flavonols and flavones in Lvbaoshi and in Moshiliu were insignificant. As expected, total flavonol and flavone of pomegranate were significantly affected by cultivars and developmental stages according to ANOVA test results.

Quantification studies on flavonoids in pomegranate had previously led to different results. Taskeen *et al*. (2011) showed the quercetin content in pomegranate peel was 88.6 mg 100 g^{-1}, and myricetin 10.66 mg 100 g^{-1}. Guo *et al*. (2008) determined that pomegranate had the highest levels of flavonoids in common Chinese fruits, with the concentrations of quercetin, myricetin, and luteolin all higher than 15 mg 100 g^{-1} fw. The results' discrepancies may have initiated from extraction conditions, fruit ripeness, as well as cultivars.

Change Patterns of Flavonols and Flavones during Fruit's Developmental Stages

As shown in Figure 3, there was a similar pattern of kaempferol and quercetin for each cultivar, but there were various patterns for different cultivars. For flavones in Figure 4, the concentration change of luteolin and apigenin followed a similar trend over time in each individual cultivar. This diversity of

Table 1. LSD test for total flavonol and flavone content (mg 100^{-1} fw) among fruit developmental stages.[a]

	Hongbaoshi		Lvbaoshi		Shuijingtian		Moshiliu	
	Flavonol	Flavone	Flavonol	Flavone	Flavonol	Flavone	Flavonol	Flavone
July 15	60.49±1.39d	6.80±1.01b	81.10±9.28bc	7.39±0.09b	53.15±2.98d	7.11±0.71c	248.24±36.35a	8.60±0.68bc
July 25	187.90±22.02ab	9.76±0.95ab	91.37±18.47bc	7.50±0.07b	134.37±15.25a	11.10±0.41a	190.01±25.48ab	8.52±0.70bc
Aug. 4	215.02±2.27a	12.31±0.95a	80.85±9.73bc	7.30±0.04b	88.39±7.31bcd	7.81±0.76bc	212.29±17.34a	7.44±0.03c
Aug. 14	71.71±8.98d	7.38±0.80b	98.65±7.17bc	7.54±0.13b	125.22±5.66ab	9.23±0.02abc	232.88±13.94a	8.24±0.03bc
Aug. 24	121.94±3.52c	9.90±0.83ab	145.77±10.88a	10.30±0.11a	117.99±8.16abc	10.00±0.86ab	197.23±17.08ab	10.92±0.10a
Sept. 3	128.45±7.10c	9.80±0.36ab	120.22±4.48ab	8.63±0.88b	76.70±5.66de	8.52±0.23bc	96.72±2.31c	8.94±0.02b
Sept. 13	143.52±21.71bc	11.83±0.05a	57.84±7.55c	5.91±0.02c	98.39±7.25e	8.79±0.58bc	130.03±7.23bc	7.89±0.11bc

[a] The values with the same letter in a column are not significantly different ($p< 0.05$).

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change patterns revealed that flavonols and flavones profiles was decided by cultivars. So far as we know, this interesting results have never been reported. In fact, the variation of flavonols and flavones may reflect the underlying coordinate regulation of some related genes in their biosynthetic pathway. Further research may carried out to uncover the potential biosynthetic and regulation mechanism of flavonols and flavones in pomegranate on molecular levels.

Pomegranate is Rich in Flavonols

The total flavonols in the pomegranate peel were always recorded at a much higher level than flavones throughout the tests independent of cultivar. Previous studies had shown that the flavonols' level in onion was deemed to be the highest (120 mg 100 g^{-1} fw) (Manach *et al*., 2004). In such fruits as blueberry, gallon, and apple, flavonols' levels were shown to be 16, 7, and 4 mg 100 g^{-1} fw, respectively (Manach *et al*., 2004). Flavones were only found in a few such products, as parsley (185 mg 100 g^{-1} fw) and celery (14 mg 100 g^{-1} fw) (Manach *et al*., 2004). Compared with these data, pomegranate is shown to be rich in flavonols, especially the Moshiliu cultivar, which bears a higher concentration even than that in onion.

Though there were several reports on quantitative analysis of flavonols and flavones in pomegranate, no change patterns with fruit development have yet been reported. Our reports on the developmental variations in flavonols and flavones may provide useful preliminary information for further future studies on flavonoid metabolism pathway. Furthermore, in most instances, pomegranate peel is deemed as a low value by-product of the fruit, often directly disposed of in the field, which may even cause environmental inconveniences. The results of the present study show that pomegranate peel is a good source of flavonols, especially that of Moshiliu cultivar. In view of the potential antioxidative properties of flavonols (Hopia and Heinonen, 1999), pomegranate could be further exploited and utilized for human health-care purposes.

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تغییرات فلاونولها (Flavonols) و فلاون‌ها (Flavones) در پوست میوهٔ انار (*Punica granatum* L.) در خلال رشد و تکامل میوه

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چکیده

چهار رقم چینی انار (*Punica granatum* L.) برای برآورد فلاونول‌ها و فلاون‌های موجود در عصارهٔ پوست میوه (در مراحل متعدد رشد) با استفاده از کروماتوگرافی مایع پیشرفته (با کارائی بالا) High Performance Liquid Chromatography (HPLC) مورد تجزیه و تحلیل قرار گرفتند. نتایج وجود کامپفرول (Kaempferol)، کوئرستین (Quercetin)، میریستین (Myricetin) لوتئولین (Luteolin)، آپیجنین (Apigenin) را در هر چهار رقم میوه نشان داد. به علاوه، محتوای فلاونولها در مورد تمامی ارقام میوه و در خلال تمامی مراحل رسیدن آنها از فلاون‌ها بیشتر بود. کامپفرول مهمترین فلاونول در ارقام هنگ بائوشی (Hongbaoshi)، ال وی بائوشی (Lvbaoshi) و شوئی جینگ تیان (Shuigingtian) و حال آنکه میریستین فلاونول غالب در رقم موشیلیو (Moshiliu) تشخیص داده شد. زمینهٔ مربوط کامپفرول در تک تک کولیتوارها (Cultivars) مشابه کوئرستین بود، اما این وضعیت در هریک از کولیتوارها با کولیتوار دیگر متفاوت بود. سطح لوتئولین در تمامی کولیتوارها از اپی گنین بالاتر بود. در خلال مدت آزمایش دامنهٔ تغییرات مشابهی در مورد لوتی الین و اپی‌گنین‌ها وجود داشت. روند تغییرات کامپفرول ، کوئرستین ، لوتئولین و آپیجنین در ارقام هنگ بائوشی، ال وی بائوشی و شوئی جینگ‌تیان مشابه بود. این اولین گزارش از غلظت فلاونول‌ها و فلاون‌ها در عصارهٔ پوست انار است. نتایج نشان داد که تغییر در محتوای این ترکیبات به رقم و مرحلهٔ رسیدگی و تکامل میوه بستگی دارد. رقم موشی لیو از نظر محتوای فلاونول‌ها بهترین رقم تشخیص داده شد که می‌توان مطالعات بیشتری را در رابطه با این پتانسیل و بهره‌گیری از آن معمول داشت.

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