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EFFECT OF MATURITY ON PROXIMATE COMPOSITION, PHENOLICS AND ANTIOXIDANT ATTRIBUTES OF CHERRY FRUIT

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Abstract

In this study, the proximate composition, antioxidant activity as well as the phenolics profile of cherry fruit at three maturity stages were investigated. The moisture, protein, total sugars and total organic acids contents were increased as maturity progressed while those of ash and crude fiber decreased. The extraction yield of antioxidant components (6.32-23.65%), total phenolics content (176.38-687.68 mg GAE/100g DW), total flavonoids content (36.61-51.80 mg CE/100g DW) and DPPH radical scavenging activity (44.32-72.99%) increased as maturity progressed from un-ripened to fully-ripened stage. HPLC analysis of the phenolics revealed a significant ($p<0.05$) increase in the amounts of flavonols namely myricetin (15.52-40.26 mg/100g DW), quercetin (11.29-24.74 mg/100g DW) and kaempferol (2.55-4.86 mg/100g DW), and phenolic acids i.e. *p*-hydroxybenzoic (27.31-42.83 mg/100g DW), vanillic (8.56-21.54 mg/100g DW), *p*-coumaric (13.68-21.33 mg/100g DW), ferulic (2.46-14.32 mg/100g DW), chlorogenic (4.22-12.14 mg/100g DW) as the fruit maturity progressed. Overall, we concluded that the fully matured cherry fruits can be explored as a promising source of natural sugars and organic acids, and high-value antioxidants (flavonol and phenolic acids) suggesting their uses as ingredients of functional foods.

Introduction

It is widely accepted that several degenerative diseases can be prevented through the use of optimal nutrition which in addition to providing basic nutrients also imparts physiological benefits. The decline of dietary antioxidants due to the westernization of diet has led to enhance in vulnerability to oxidant damage and inflammation, resulting in more prevalence of diseases (Seaton *et al*., 1994). Research has proven that consuming the proper level of natural antioxidants into our bloodstream reduces the risk of cancer and several other oxidative-stress related disorders (Majhenic *et al*., 2007; Hussain *et al.,* 2011).

Soft fruits are promising crops with high economic value, not only due to their desired taste and food value but also for their known health-promoting properties. The quality of soft fruits, in terms of taste and consumers acceptance, is fundamentally based on the biochemical composition of the fruit and hence the balance between sugar and acid content within the fruit, referred as sugar/acid ratio, can be used as an important index of consumer acceptability (Perez *et al*., 1997; Terry *et al*., 2005), optimum maturity (Perez *et al*., 1997) overall fruit quality and taste e.g, riper fruit have higher sugar content. To satisfy current consumer demand together with the requirements of a more competitive market, a better understanding is required on how different cultivation or processing conditions affect the taste and the composition of medicinally important bioactives of fruits (Zainudin *et al.,* 2012).

Sweet cherry (*Prunus avium* L.) is one of the most popular temperate fruits. It is widely consumed due to its good taste, sweetness and plenty of nutrients. It belongs to the family Rosaceae, genus *Prunus*, and is regarded as one of the important fruit crops world over (Esti *et al*., 2002; Demirsoy & Demirsoy, 2004). The subgenus is native to the temperate regions of the Northern Hemisphere, with two species in America, three in Europe and the remaining in Asia.

The world over total production of cherry was around 2,015 million metric tons in 2010. The major producing countries are Turkey, United States and Iran. In Pakistan: Quetta, Pishin, Ziarat, Kalat, Zhob, Mastung, Loralai and Swat are ideal zones for commercial cherry production. In Balochistan, cherry was grown on about 897 ha on commercial basis with an annual production of about 1, 507 tons (Anon., 2002-2003).

Antioxidant activity of the fruits may be affected by several factors including maturity at harvest, season of maturity, genetic differences, pre-harvest environmental conditions, post-harvest storage conditions and processing (Prior *et al.,* 1998; Mirdehghan & Rahemi, 2007; Dragovic-Uzelac *et al*., 2007).

To the best of knowledge, detailed investigation on the valuable nutrients and antioxidant properties of cherry fruit during ripening stages have not yet been conducted. The present work therefore was planned to appraise the proximate parameters, phenolics profile and antioxidant attributes of local cherry fruit harvested at three different maturity stages (unripe, semi-ripe and full ripe).

Materials and Methods

Collection of samples: Fruit samples of cherry (*Prunus avium* L.) were collected at three different stages of maturity, un-ripened (green), semi-ripened (red) and fully-ripened (violet) in polythene bags from Kalat region in 2009 and transported to the laboratory of the Department of Chemistry and Biochemistry, University of Agriculture Faisalabad, Pakistan.

Dry matter determination: In view of the varying contents of moisture of the fruits from different stages, dry matters were determined. For dry matter determination, procedure (method 925.10) of Official Methods of the Association of Official Analytical Chemists (AOAC) was used (Anon., 2000). Briefly, a

known amount (*ca.* 5.0g) of the fruit sample was heated in an electric-oven (Memmert, Germany) at 105°C, until the complete loss of moisture and constant weight achieved.

Proximate analysis of the fruit: Proximate composition (moisture, ash, crude fiber, total sugar, total acidity) of the fruits was determined according to the Official Methods of the Association of Official Analytical Chemists (Anon., 2000), Nitrogen content (N) of the sample was estimated following Kjeldahl method (1983) and crude protein was calculated as by formula, N ×6.25 (Imran *et al*., 2008). All the calculations were carried out on dry matter basis.

Sample extraction for antioxidant activity: The fresh cherry fruit (20g) harvested at each maturity phase was extracted separately with 200mL of pure methanol (100% methanol) for 6 hours at room temperature using an orbital shaker (Gallenkamp, UK). The extracts were filtered to separate the residues using Whatman No. 1 filter paper. The residues, left over, were re-extracted with appropriate volume of fresh extracting solvent and the 2 extractions obtained pooled. The pooled extracts were made solvent-free by distilling off under reduced pressure in a rotary evaporator (EYELA, SB-651, Rikakikai Company Limited, Tokyo, Japan). Finally, the crude extracts recovered were quantitatively weighed to calculate the yield.

Determination of total phenolics content (TPC): Amount of TP was assessed using a reported method of Chaovanalikit & Wrolstad, (2004).One mg of crude extract was taken and mixed well with 1.0mL of 2N Folin-Ciocalteu reagent (10 fold diluted) and 0.5mL de-ionized water. After placing the mixture at ambient temperature(10 min), 0.8mL of sodium carbonate (7.5% w/v) was added and the mixture incubated in a water bath at 40°C for 20 min and then cooled in an ice bath; absorbance was measured at 760nm using a spectrophotometer (Spectrophotometer U-2001, Hitachi Instruments Inc., Tokyo, Japan). Amounts of TP were calculated using gallic acid calibration curve (R^2 = 0.9980). The results were calculated based upon gallic acid equivalents (GAE g/100g dry weight basis).

Determination of total flavonoid contents (TFC): TFC were determined colorimetrically (Dewanto *et al*., 2002). Extract of each selected fruit (5.0 mg/mL) was diluted with 5 mL distilled water in a 10 mL test tube. Initially, 0.3 mL of 5 % $NaNO_2$ was added to each test tube. After 5 min, 0.6mL of 10% $AlCl_3$, after further 5 minutes, 2mL of NaOH (1.0M) were added. Absorbance of the final reaction mixture was recorded at 510nm using the above specified spectrophotometer. TFC were calculated based upon catechin equivalents g/100g dry weight basis.

DPPH$^·$ radical scavenging assay: 1, 1-diphenyl 2-picrylhydrazyl (DPPH) free radical scavenging activity of fruit extracts was assessed using procedure reported earlier (Chiou *et al*., 2007). Briefly, to 1.0mL of extract, containing 1mg/mL of dry matter in methanol, 4.0mL of deionized water and 1.0mL of freshly prepared solution of DPPH (0.025g/L) were added and mixed well. Absorbance, after incubation for 10 min at room temperature, of the final reaction mixture was taken at 515 nm using a spectrophotometer and thus percent DPPH radical (DPPH$^·$) scavenging activity calculated.

Determination of reducing power: The redox potential of the extracts towards reducing ferric ions into ferrous ions via acting as electron donors was monitored according to the procedure described by Bae & Suh (2007), with minor modification. Briefly, variable concentration of the extract with in the range of 1.0 - 5.0 mg in distilled water was mixed with sodium phosphate buffer (2.5mL, 0.2M, pH 6.6) and potassium ferricyanide (2.5mL, 1.0%); the mixture was incubated at 50°C for 20 min. Then 2.5mL of 10% trichloroacetic acid was added and the mixture centrifuged at 3000 × *g* for 10 min. The upper layer of the solution (2.5mL) was decanted and diluted with 2.5mL of distilled water and ferric chloride (0.5mL, 0.1%), and absorbance of the final mixture recorded at 700nm using a spectrophotometer.

Sample preparation for HPLC analysis: For HPLC analysis, flavonols and phenolic acids were extracted/hydrolysed according to a reported method of Tokusoglu *et al*., (2003). Briefly, 25mL of acidified methanol containing 1% (v/v) HCl and 0.5mg/mL BHT (as preservative) was mixed with the dried fruit material (5g) in a refluxing flask followed by addition of 5mL HCl (1.2M). The mixture was refluxed/hydrolysed at 90°C for 120 min so as to produce aglycons of flavonol glycosides and to transform bound phenolics into respective free forms. Subsequently, the extracts were cooled to room temperature followed by centrifugation at 5000 x *g* for 10 min. The upper layer was recovered and sonicated for 5 min to free from the air-bubbles, if applicable. The final extract was filtered using a 0.45µm (Millipore) membrane before being injected into HPLC column. Phenolic acids and flavonols were analyzed as per following protocol.

Experimental Conditions used for flavonols analysis: A specified HPLC column from Merck Company (Merck KGaA, 64271 Darmstadt, Germany), namely Hibar® RP-C18 column (250mmx 4.6mm; 5µ particle size) operated at 25ºC temperature was used for separation of targeted flavonol compounds such as myricetin, quercetin and kaempferol. Mobile phase constituting a mixture of 50% Trifluoroacetic acid (0.3%), 30% Acetonitrile and 20% Methanol, was delivered at the flow rate of 1.0mL/min. The mobile phase was filtered through membrane filter (0.45mm) prior to elution and degassed via ultra sonication. Isocratic elution mode was employed while the targeted flavonols were detected at 360nm.

Experimental conditions used for phenolic acids analysis: A specified HPLC column from Merck Company (Merck KGaA, 64271 Darmstadt, Germany), namely Hibar® RP-C18 column (250mmx 4.6mm; 5µ particle size) maintained at 25ºC, was employed for the separation of targeted phenolic acids. Mobile phase mixture comprised of 40% Trifluoroacetic acid (0.3%),

40% Acetonitrile and 20% Methanol, was flushed at the flow rate of 1.0mL/min. The mobile phase was filtered through Nylon membrane filter (0.45 mm) and then degassed via ultra-sonication before being used. Gradient elution mode was selected and the target phenolic acids were monitored at 280nm.

Instrumentation: HPLC chromatographic analysis was carried out on an Agilent 1100-series HPLC system fitted with a Quaternary pump (G1311A Quat pump), vacuum degasser (G1379A), auto-sampler/auto-injector (G1313A ALS), column compartment (G1316A Colcom) and diode array detector (DAD) (G1315B DAD). An Agilent Chem Station was used to process chromatographic data.

Statistical analysis: Univariate analysis of variance (ANOVA) was employed for the fruit harvested at each maturity stage using Minitab 2000 Version 13.2 statistical software (Minitab Inc., State College, PA, USA). A probability value of $p<0.05$ was considered to denote the statistically significant difference among maturity stages.

Result and Discussion

Table 1 reveals the proximate composition of cherry fruits collected at three maturity stages. The moisture and crude protein contents (%) in cherry fruits gradually increased as maturity progressed while ash and crude fiber contents (%) decreased significantly. The amount of ash, crude fiber and crude protein in cherry fruit were determined to be 5.36-4.21%, 2.20-1.80% and 4.23-5.91%, at un-ripened, semi-ripened and fully-ripened stages, respectively. Brindza *et al*., (2009) investigated protein content of cornelian cherry in Slovakia between 0.34 and 0.50%. In another study the moisture contents of six sweet cherry cultivars ranged from 74.41- 83.33% (Naderiboldaji *et al.,* 2008). The contents of total sugars and total organic acids were established to be 1.10, 2.01 and 2.83 mg/100g FW and 14.46, 25.95 and 38.06 mg/100g FW at un-ripened, semi-ripened and fully-ripened stage, respectively. Overall trends represented an increase in the sugars and organic acids content as fruit maturity progressed.

Table 1. Proximate composition of Cherry fruits at different maturity stages.

	Maturity stages		
	Un-ripened	**Semi-ripened**	**Fully-ripened**
Moisture (%)	74.84 ± 4.72^{c}	78.26 ± 3.65^{b}	81.57 ± 4.29^{a}
Ash (%)	5.36 ± 0.12^{a}	5.23 ± 0.31^{a}	4.21 ± 0.08^{b}
Crude protein (%)	4.23 ± 0.57^{b}	5.11 ± 0.97^{a}	5.91 ± 0.58^{a}
Crude fiber (%)	2.20 ± 0.12^{a}	1.98 ± 0.11^{b}	1.80 ± 0.22^{b}
Total sugars (mg/100g FW)	1.10 ± 0.02^{c}	2.01 ± 0.10^{b}	2.87 ± 0.11^{a}
Total acids (mg/100g FW)	14.46 ± 0.26^{c}	25.95 ± 0.68^{b}	38.06 ± 0.61^{a}

Values (mean ± SD) are average of three samples of the fruit, analyzed individually in triplicate ($p<0.05$)

Different letters in superscript within the same row indicate significant difference among ripening stages

Phenolics, as a well known class of plant secondary metabolites, are effective free radical scavengers and also show multiple medicinal and biological functions in animals as well as in plant (Manach *et al*., 2005). Extraction yield of antioxidant components from cherry fruit obtained by using 100% methanol as extraction solvent ranged from 6.32-23.65% for un-ripened to fully-ripened stage samples while total phenolics (176.38-687.68 mg GAE/100g DW) and flavonoids content (36.61-51.80 mg CE/100g DW) (Table 2) also showed an increasing trends as maturity progressed. Goncalves *et al*., (2004) investigated total phenolics in four cherry cultivars at two ripening stages and found lowest TPC in *cv*. Van at partially-ripe stage (69 mg/100 g of FW), while highest in *cv.* Saco at fully-ripened stage (264 mg/100 g of FW). In agreement to our present findings, the results showed that as maturity progressed TPC also increased. In red-colored fruits, total phenols generally increased during ripening stage, due to the maximal accumulation of anthocyanins and flavonols (Gerasopoulos & Stavroulakis, 1997; Zadernowski *et al*., 2005). Liu *et al*., (2011) reported that TFC in red-colored cherries Burlat" were significantly higher (253.32 mg RE/100 g FW) than those in bicolored "Hongyan" (7.97 mg RE/100 g FW).

It is well known that antioxidants can break and seize free radical chain reaction during oxidation and form stable free radicals, which would not initiate or propagate further oxidation. DPPH is stable free radicals and is extensively being employed to assess the antioxidant potential of huge number of plants. The use of DPPH free radical is important in assessing antioxidant effectiveness because it is more stable than some other radicals such as hydroxyl and super oxide radicals (Layina-Pathirana *et al*., 2006). The percent DPPH radical scavenging activity of cherry fruit recorded at un-ripened (44.32%), semi-ripened (53.83%) and at fully-ripened stage (72.99%) is shown in Table 2. The results showed significant increase in DPPH scavenging activity as maturity progressed. The Fig. 1 represents the reducing power at different maturity stages of cherry fruit. Significant increase in reducing potential was observed in relation to the fruit maturity/ ripening.

Table 2. Extraction yield, total phenolics and total flavonoids and DPPH radical scavenging activity of Cherry fruits at different maturity stages.

	Maturity stages		
	Un-ripened	**Semi-ripened**	**Fully-ripened**
Extraction Yield (%)	6.32 ± 0.84[c]	19.72±2.19[b]	23.65±2.19[a]
Total phenolic (mg GAE/100g DW)	176.38 ± 7.22[c]	371.71 ± 16.20[b]	687.68 ± 37.90[a]
Total flavonoids (mg CE/100g DW)	36.61 ± 2.32[b]	47.36 ± 3.22[a]	51.80 ± 2.45[a]
DPPH Scavenging activity (%)	44.32± 4.71[b]	53.83± 5.01[b]	72.99 ± 6.982[a]

Values (mean ± SD) are average of three samples of the fruit, analyzed individually in triplicate ($p<0.05$)
Different letters in superscript within the same row indicate significant difference among ripening stages

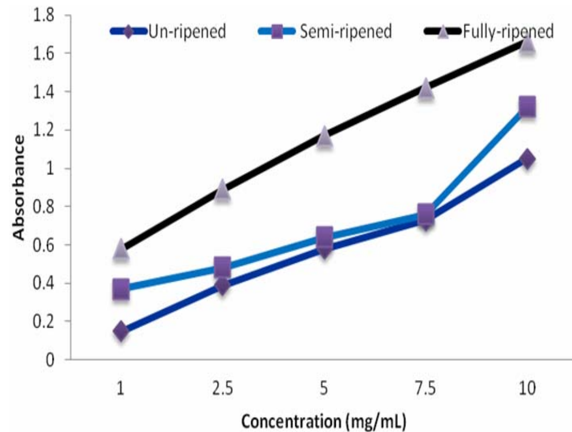

Fig. 1. Reducing power of cherry fruit at different maturity stages.

The data for the quantitative analysis of flavonols (kaempferol, quercetin and myricetin) in cherry fruit at different maturity stages are presented in Table 3. Flavonol compounds were analyzed by HPLC-DAD method and detected at 360 nm. In cherry, the concentrations of myricetin, quercetin and kaempferol were found to be 15.52-40.26, 11.29-24.74 and 2.55-4.86 mg/100g DW, respectively as the fruits maturity progressed. The contents of these flavonols were mainly increased as the fruit maturity progressed from un-ripened to fully-ripened stage. The concentration of kaempferol and quercetin has been reported to be 0.7-0.9mg/100g and 0.4-0.7mg/100g, respectively in the fresh ripe berries fruit (Hakkinen *et al*., 1999). Based upon flavonoids comparison, the cultivars analyzed in the present study can be more promising with regard to their beneficial effects on health, due in part to their higher content of flavonols. According to Jakobek *et al*., (2009) the amount of main flavonol (quercetin-3-rutinsoide) in sweet cherries was in the range of 8-37 mg/kg. Sakakibara *et al*., (2003) reported the level of quercetin- glycoside in sweet cherry to be 13 µmol/100g. Variation in flavonol content in fruits is strongly influenced by extrinsic factors such as fruit type and growth, season, climate, degree of ripeness, food preparation, and processing (McDonald *et al*., 1998; Lakenbrink *et al*., 2000; Trichopoulou *et al*., 2000; Vuorinen *et al*., 2000). Kevers *et al.,* (2007) quantitatively measured flavonol in cherry fruit by HPLC, cherry had kaempferol (242 µg/100g of FW) and quercetin (102 µg/100g of FW), however it was devoid of myricetin.

Table 3. Flavonols and phenolic acids (mg/100g DW) composition of Cherry fruits at different maturity stages.

Flavonols	Maturity stages		
	Un-ripened	**Semi-ripened**	**Fully-ripened**
Myricetin	15.52 ± 0.09[c]	23.09 ± 0.73[b]	40.26 ± 1.20[a]
Quercetin	11.29 ± 0.59[b]	22.71 ± 0.25[a]	24.74 ± 1.09[a]
Kaempferol	2.55 ± 0.09[b]	4.38 ± 0.06[a]	4.86 ± 0.17[a]
Total flavonols	29.36	50.99	69.86
Phenolic acid			
p-coumaric	13.68 ±1.01[b]	15.26 ± 1.23[b]	21.33 ± 1.65[a]
p-hydroxy-benzoic	27.31± 1.53[c]	31.05 ± 2.11[b]	42.83 ± 2.05[a]
Chlorogenic	4.22 ± 0.23[c]	9.58 ± 0.65[b]	12.14 ± 1.03[a]
Ferulic	2.46 ± 0.31[b]	13.90 ± 0.74[a]	14.32 ± 0.85[a]
Gallic	ND	5.47 ± 0.41[b]	9.65 ± 0.85[a]
Vanillic	8.56 ± 0.85[c]	11.08 ± 0.97[b]	21.54 ± 1.12[a]
Σ HBA	42.12	71.23	104.29
Σ HCA	42.07	56.65	82.93
Σ PHA	84.19	127.88	187.22

Values (mean ± SD) are average of three samples of the fruit, analyzed individually in triplicate ($p<0.05$)
ND = not detected
Different letters in superscript within the same row indicate significant difference with in ripening stages
Σ HBA = sum of benzoic acid derivatives
Σ HCA = sum of cinnamic acid derivatives
Σ PHA = sum of phenolic acids

As the fruit maturity progressed, the amount (mg/100g DW) of phenolic acids in cherry fruit increased i.e., *p*-hydroxybenzoic acid (27.31-42.83), vanillic (8.56-21.54), *p*-coumaric (13.68-31.33), ferulic (2.46-14.32), chlorogenic (4.22-12.14) and gallic (5.47-9.65) acids. The distribution of individual phenolic acids has been studied by Jakobek *et al*., (2009) in sweet cherry cultivar of Croatia. The chlorogenic (26-48 %) had the highest amount in the total phenolic acids. Only in sweet cherries grown on the 'PiKu 1' rootstocks, the portion of *p*-coumaric acid derivatives was higher (52%) than in sweet cherries grown on other rootstocks (20-27%). Usenik *et al*., (2008) found similar phenolic compounds distribution in 13 sweet cherry cultivars. *Para*-coumaric acid comprised 5-31%, and chlorogenic acid 3-15% of the total phenolics. The neochlorogenic, chlorogenic and *p*-coumaric acid derivatives, found as the major phenolic acids in all sweet cherry cultivars (cv. Lapins) (Kim *et al*., 2005; Mozetic *et al*., 2004; Usenik *et al*., 2008), were in agreement with the present results except neochlorogenic acid which was not detected in our analysis. In some earlier studies, the chlorogenic acid in different cherry cultivars (Hartland, Hedelfinger, Regina, Black Gold) was found to be in the range of 1.3-6.5 mg /kg of FW (Kim *et al*., 2005), while in other cultivars (Lambert, Bing, Stella Compact, Napoleon and Petrovka) 75-507mg/kg of FW (Mozetic *et al*., 2002). *Para*-coumaric acid derivatives, determined in the present study, were quite comparable with those (10-68 mg/kg) investigated in different cultivars of cherry by Kim *et al*., (2005).

Moreover, all of the phenolic compounds which comprise a significant part in the total phenols of sweet cherries of Lapins variety (neochlorogenic acid, chlorogenic acid, *p*-coumaric acid derivatives and quercetin-3-rutinsoide) showed strong antioxidant activity (Lien *et al*., 1999; Soobrattee *et al*., 2005). Phenolics are one of the most important bioactives and their composition and content defines the quality and flavor characteristics of fruits (Voca *et al*., 2008, 2009). Some earlier studies (Hakkinen *et al*., 1999; Kim *et al*., 2005; Kosar *et al*., 2005) reveal that cherry fruit consumption is linked with beneficial effects on the human health, which might be attributed to the occurrence of polyphenolics in this valued fruit.

Conclusions

This study presents the data on the proximate composition, phenolics and antioxidant attributes of Pakistani sweet cherry fruit at different maturity stages. We conclude that the composition of valuable nutrients including total sugars and organic acids as well as phenolics and antioxidant activity of the cherry fruit is strongly influenced during ripening stages. Based on the results of these findings, it can be concluded that the fully repined cherry fruit can serve as a potential source for functional food development to supporting the supply of high-value components such as phenolics and antioxidants in human diet.

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