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EFFECTS OF VARIOUS HARVESTING TIMES ON BIOACTIVE COMPOUND IN *CITRUS PARADISI* (SHAMBER)

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Abstract

The antioxidant potential of isolated bioactive compounds of Citrus *paradisi* cv related to seasonal variation, explored in this article. The results revealed significant differences in physical and description of maturity of fruits, regarding to the accumulation of bioactive compounds. It establishes that contents of total phenolic compounds, total antioxidants, total carotenoids, total flavonoids, total pectin proteins as well as taste compounds directly related to maturity stage. The contents of carotenoids, Luteoxanthin, Lycopene, alpha-Carotenoids and beta Carotenoids found to be increased in the December harvested fruits, while non-significant differences were recorded to other harvested periods. The antioxidants potential of these compounds were determined through 2,2-Diphenyl-1-picrylhydrazyl (DPHH), Total Antioxidant Capacity (TAC), Antioxidants index (AI) and Ferric reducing antioxidant power (FRAP) assay method, found to be increased from early to late harvested periods. Pearson correlation coefficient matrix were determined at five different harvesting times followed by determination of the correlation of four essential phytochemicals like Total tannins (TT), Acy and gallic acid with harvesting times. It concluded the proper harvesting time is vital for the effective concentration of bioactive compounds in fruits.

Key words: Bioactive compounds; Harvesting times; Antioxidants; *Citrus paradisi* shamber.

Introduction

Grapefruit (*Citrus paradisi* Macf.) has a unique value of essential nutrients which are effective against several chronic diseases of human body (Patil *et al.,* 2004). Grapefruit (*Citrus paradisi* Macf.) is a cross product of seeds of pummelo and some other pollen donors like sweet orange (Tanaka *et al.,* 2000). It is a famous fruit crop for its distinctive taste and nutritional values, mostly consumed as fresh juice due to its unique health-related compounds (Gorinstein *et al.,* 2017). Depending on the growing season and harvesting time, these environmental conditions could be more or less limiting factors for synthesis of bioactive compounds, their accumulation, and formation (Patil *et al*., 2004; Duthie &. Crozier, 2000). The bioactive compounds in plants referred as secondary plant metabolites, having more than 9000 various structures (Contreras *et al.,* 2015; Özgen *et al*., 2018). Carotenoids and polyphenolic compounds derived from 2-phenylchromane, commonly found in many plants and flower. Grapefruit contain flavonoids, polyphenols, and antioxidants in a massive amount in different parts such as peel, rags, juice, and seeds (Chaovanalikit & Wrolstad, 2004; Deena *et al.,* 2010; Yousef *et al.,* 2012). Fruits contain sugars, organic acids (citric, malic, etc.), mucilage, tannin, dye (cyanine), pectin and vitamin C contents. The antioxidant and chemical contents of fruits are affected by many agents; in particular, ecological conditions and genotype characteristics, showed a significant impact on the diversification of these chemicals contents in black mulberry (Karafakioglu & Aksoy, 2019). Polyphenolic compounds and carotenoids are capable of scavenging free radicals through hydrogen atom donation (Seeram, 2008). The maturity is a critical factor in the accumulation of these bioactive compounds which induce changes in fruit quality. Seasonal variation put an impact on the quality of grapefruit also (Seeram, 2008). It reported by Jia *et al*., (1999) that environmental conditions may affect the various quality parameters of citrus fruits. The quality is affected through different abiotic stress or by changing in environmental conditions (Andreotti *et al.,* 2012). The matured fruits contained highest amount of the active compounds.

Grapefruit (*Citrus paradisi* Macf.) contains a lower amount of cholesterol that helps diabetes patients (Helyes et *al.,* 2018).Currently monitoring of the physical and chemical variations in pomegranate fruit revealed that the composition of minerals and bioactive compounds vary markedly different among the three ripening stages. Yousef *et al.,* (2012) reported that the phenolic compounds, removed free radicals which caused diseases in the human body. Recently; the studies on physicochemical changes in fruit crops, revealed the impact of short-term weather conditions on variations of bioactive compounds during their growing seasons (Hsu *et al.,* 2006; Gorinstein *et al*., 2004). Helyes *et al.,* (2018) reported the marked difference in the accumulation of bioactive compounds in different citrus crops which was similar to the changes in sweet lime and valentine orange Rizzolo *et al*., (2006).

In Pakistan, the Grapefruit (*Citrus paradisi Macf.)* harvested often early in the season, which may affect the various quality attributes in grapefruit (Zerbini *et al.,* 1999). At the early stage of ripening, the exponential and cell expansion phase of cell division may lead to lesser amounts of phytochemicals in the different parts of grapefruit (Bajwa Anjum, 2007). The aims and objective of present research was to evaluate the impact of harvesting time on

concentration of bioactive compounds in relation to their antioxidants activities and taste compounds through physical analysis. The standardization of proper harvesting times of grapefruit also discussed in the relevant section of this paper.

Material and Methods

Plants sampling and experimental site: The fresh fruits collected at various harvesting time for analysis of bioactive compounds in the year 2015 to 2016. They were transported and preserved in Pomology Lab., Institute of Horticultural Sciences, University of Agriculture, and Faisalabad, Pakistan by the method described by Ahmed *et al.,* (2018).

Maturity and physical characterization of grapefruit (*Citrus paradisi* Macf.): The fruit length and diameter measured with the help of digital Vernier caliper, the mathematical relationship used for shape index and fruit volume as described by the method of Al-Sadi *et al*., (2012). The shape index measured by using a formula of Shape index (SI) = (L/D).

Analysis of carotenoids using HPLC-DAD technique: The extract prepared from samples (5 mL) evaporated till complete dryness then obtained residue dissolved in 2 mL of DMSO solution. This DMSO extracts used for the HPLC analysis. Chromatographic separations were carried out using an X Bridge C18 (3.5 µm, 4.6 x 250 mm) column from waters company. The mobile phase used; consist of 0.25% orthophosphoric acid, and 1.50% tetrahydrofuran in water (solvent A) and methanol (solvent B). The gradient conditions were as follows; 0-15 min 30-70% A, 15-30 min 70-100% A, 30-35 min 100% A, 35-36 min 100-30% A, and 36-50 min 30% A with a total run time of 50 min. The column equilibrated for 10 min before each analysis (Justesen *et al.,* 1998).

Potential antioxidant activity: The reducing power of extracts measured through calorimetric methods as reported by Benzie & Strain, (1996). The reduction of the Fe^{3+} 2, 4, 6-Tripyridyl-S-triazin TPTZ complex into a colored Fe^{2+} 2, 4, 6-Tripyridyl-S-triazin TPTZ complex in the extract measured at 593 nm spectrophotometrically. The TAC (mM TE/mL PJ) antioxidant capacity based on Quanti Chrom assay methods, the result measured through a formula of total anthocyanin (µg/mL) = [(*A* x MW x DF)/ε x L)]. Total antioxidants activities of the grapefruit juice were assessed by measuring their scavenging abilities to 2, 2-diphenyl-1-picrylhydrazyl stable radicals as described by Amira *et al.,* (2012). DPPH antioxidant capacity based on DPPH assay and Antioxidants index (%) calculated in grapefruit juice.

Determination of total flavonoids and phytochemicals in grapefruit: Flavonoids contents in fruit sample were mesuared by the method of Kim *et al.,* (2003). Total Phenolic contents (TPC) calculated using the Folin-Ciocalteu reagent as reported by Ainsworth & Gillespie (2007). The extraction of high-methoxyl pectin from the fruit peel of grapefruit studied according to Uthai (2011). Total carotenoids contents estimated according to the method of Lichtenthaler & Buschmann (2001).

Determination of proximate analysis of grapefruit shamber: The ash contents were determined by ignited 5 ml of citrus juice in the muffle furnace at 550-600 °C for 8 hrs (Anon., 2000). The Crude fiber (CF) in juice was measured by sequential extraction with dilut acid and alkali as indicated by the method of Anon., (2000). The crude nitrogen (CN) content determined by the equation of crude protein (%), Nitrogen content 6.25 (%). The titratable acidity determined by the method, described by Hortwitz, (1960). Juice samples were titrated against 0.1N NaOH using two to three drops of phenolphthalein as an indicator, and the results expressed in percentage.

$$\text{T.A. (\%)} = \frac{\text{0.1N NaOH used x 0.0064}}{\text{The volume of the sample used}} \text{ x 100}$$

Pearson correlation study in grapefruit (shamber): Pearson correlation was used to investigate the properties of antioxidants and phenolic compounds of grapefruit juice. Furthermore; the positive correlation found in this correlation related to the changes in phenolic compounds during the growing seasons. The polyphenolic compounds were increased in the juice of *Citrus paradisi* cv. Shamber related to its maturity stages therefore reasonably hypothesize that there is a positive correlation noted in these bioactive compounds.

Data analysis: The data subjected to statistical analysis through software Statistics 8.1 by applying the CRD design. The significance of the data was also checked through calculations of Least Significant Difference (LSD) which give us mean lettering of the measured data. The graph was prepared by a software Graph Pad Prism 7.02. While the software of Chrom gate v 3.31 Knauer applied for the HPLC-DAD analysis of the compounds (Williams, 2010).

Results

HPLC-DAD analysis of cartonoids contents in relation to harvesting times: The HPLC-DAD chromatogram of individual carotenoids presented in Fig 1. The individual components of cartonoids, i.e., Luteoxanthin, Lycopene, Alpha carotenoids, Beta carotenoids showed significant differences related to the harvesting time (Table 1) which were found to be upsurges in December harvested fruits followed by lower contents in September reaped crops.

Proximate analysis of juice: The maximum ash contents (10%), crude protein, crude fibers (12%), and total sugar contents (11%) recorded in the fruits harvested in December in comparison of other harvesting dates (Table 2) like September, October, November, and January harvested fruits (Table 2) while the total acid reduced in late harvesting period in comparison of early harvest season.

Table 1. Means of Carotenoids in grapefruit (*Citrus* × *paradisi*) cv. Shamber at various harvested dates (mg/100g FW) through HPLC analysis.

Carotenoids	Harvesting dates				
	Sep. 1	Oct. 1	Nov. 1	Dec. 1	Jan. 1
Luteoxanthin	13e	14d	16b	17a	15c
Lycopene	14de	15c	16b	18a	16b
Alpha carotenoids	11e	12d	13c	15a	14b
Beta carotenoids	14e	15d	16c	18a	17b
LSD	0.02				

The results are presented as mean based on n=3, LSD = Least significant difference ($p<0.05$). Different letters within column indicate significant difference between harvesting times ($p<0.05$)

Table 2. Proximate analysis in grapefruit (*Citrus* × *paradisi*) cv. Shamber at various harvested dates (mg/100g FW).

Proximate analysis (mg/100g FW)	Harvesting dates				
	Sep. 1	Oct. 1	Nov. 1	Dec. 1	Jan. 1
Ash	7 e	8d	9c	10a	9b
Crude protein	9 d	10c	11b	12a	11b
Crude fiber	9 d	10c	11b	12a	11b
Total sugars	8 d	9c	10b	11a	10b
Total acids	8a	7b	6.5b	5c	6ab
LSD	0.012				

The results are presented as mean based on n=3, LSD = Least significant differences ($p<0.05$). Different letters within column indicate significant difference between harvesting times ($p<0.05$)

Table 3. Means of Phytochemicals in grapefruit (*Citrus* × *paradisi*) cv. Shamber at various harvested dates (mg/100g FW).

Phytochemicals	Harvesting dates				
	Sep. 1	Oct. 1	Nov. 1	Dec. 1	Jan. 1
Total phenolic compound	10d	11c	12b	13a	12b
Total antioxidants	6d	7d	8c	10b	9b
Total carotenoids	5d	6c	7c	9a	8b
Total flavonoids	12e	13d	14c	16a	15b
Total pectin	7d	8c	9b	10a	10a
LSD	0.011				

The results are presented as mean based on n=3, LSD = Least significant differences at ($p<0.05$). Different letters within column indicate significant difference between harvesting times ($p<0.05$)

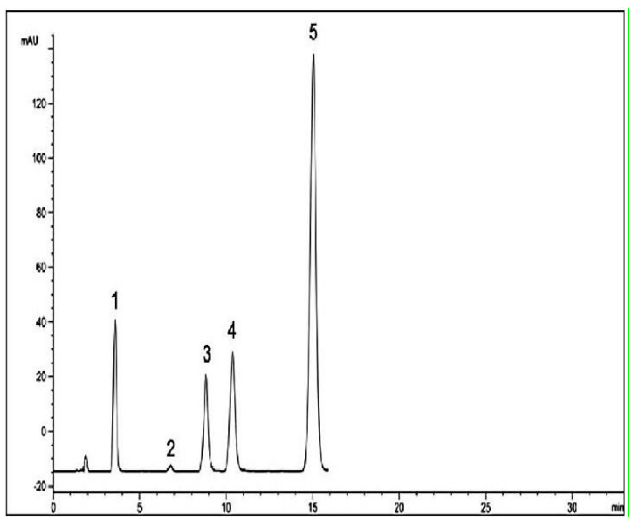

Fig. 1. Chromatogram of Carotenoids ethanolic extract of grapefruit obtained by HPLC DAD (1) Luteoxanthin; (2) Lycopene; (3) Alpha carotenoids; (4) Beta carotenoids at 100 nm.

Phytochemicals in relation to the harvesting times: The significant variations recorded in phenolic compounds with the harvesting time which were maximum in December harvested fruits while lower (TPC 10 mg100g^{-1} FW) in September harvested periods. The total antioxidants properties were least but statistically significant results were observd, like 10% DPPH in December followed by 9% DPPH during January (Table 3). The similar trends found for total carotenoids and total flavonoids from same harvested fruits in December (Table 3). The higher contents of methylesterase pectin were noted in December and January, while lower contents of methylesterase Pectin found in September harvested fruits.

Maturity and physical analysis of (C*itrus paradisi*) cv. shamber: The linear model for maturity and physical characterization of grapefruit juice reflect that the September harvested fruits showed an immature size and quality followed by the same comparisons of fruits on several other harvested time. The higher diameter of grapefruit (R^2 in 0.913), length (0.92 volume), and shaped index (0.903, 0.842) observed in December harvest fruits. While the Y- variable of all physical parameters was shown in (Tables 4 & 5) and the maturity stage denoted in (S1-S5). The analysis showed that the best and suitable harvesting time for full mature and ripens was December.

Table 4. Liner model for physical analysis of (*Citrus* × *paradisi)* cv Shamber.

Variable (X)	Y liner model	R^2
Diameter	Y = 49x + 66	0.913
Length	Y = 18.8x + 80.6	0.902
Volume	Y = 4x + 144.6	0.903
Shape index	Y = 2.3x + 14.3	0.842

Liner model of variables in physical analysis

Table 5. Fruit maturity samples during (*Citrus* × *paradisi*) cv. Shamber at various harvested dates.

Stage of maturity	(DAFB) days after full bloom	Description of maturity stages
S1	Sep.1	Immature
S2	Oct. 1	Mature slightly ripen
S3	Nov. 1	Mature semi ripen
S4	Dec. 1	Mature/full-ripen
S5	Jan. 1	Over ripen

The potential antioxidant activity in relation to the harvesting times: The potential antioxidants activities of bioactive compounds were measured through DPPH assay, antioxidant capacity based on Quanti, Chrom assay, antioxidants index, FRAP and antioxidant capacity based on FRAP assay (Table 6) where higher range of antioxidant potentials were observed in late harvested fruits. The antioxidant potential based on DPPH (2.7mM AAE /mL PJ) was higher in December harvested fruits but lower in September harvested fruits. Quanti Chrom assay methods also showed the higher antioxidant capacity in December harvested fruits, which were lower in the month of September harvested time. Similarly, the antioxidants index (4.48%) was higher in December harvested fruits while lower values measured in September harvested fruits followed by the same trend in the antioxidant capacity based on (FRAP) 4.51 (mM TE/mL PJ).

Pearson correlation coefficient matrix of phytochemicals: Total tannins (TT), Proanthocyanidins (PCy), Total anthocyanins (Acy), Gallic acid (GA) as phytochemical in grapefruit juice measured through the Pearson correlation of various matrixes which showed the correlation in between TT and Acy. All phytochemicals showed a relation of alteration in these compounds which presented in Table 7. The harvesting time showed a significant role. The correlation coefficient of gallic acid and total anthocyanins measured with a range of 0.721.

Table 6. Various methods of Antioxidant potential of (*Citrus* × *paradisi*) cv. Shamber at various harvested dates.

HD	DPHH [a] (mM AAE /mL PJ)	TAC [b] (mM TE/mL PJ)	Antioxidants [c] index (%)	FRAP [d] (mM TE/mL PJ)
Sep.1	2.1 ± 0.012	3.1 ± 0.012	4.1 ± 0.011	4.1 ± 0.012
Oct. 1	2.3 ± 0.013	3.13 ± 0.011	4.23 ± 0.021	4.21 ± 0.011
Nov. 1	2.4 ± 0.021	3.81 ± 0.021	4.31 ± 0.011	4.4 ± 0.011
Dec. 1	2.7 ± 0.011	3.91 ± 0.212	4.48 ± 0.012	4.51 ± 0.013
Jan. 1	2.5 ± 0.012	3.88 ± 0.021	4.35 ± 0.013	4.32 ± 0.012

a DPPH = antioxidant capacity based on DPPH assay; b TAC = Antioxidant capacity based on Quanti Chrom assay, AI= Antioxidants index FRAP, antioxidant capacity based on FRAP assay values (mean ± S.E) in the same column followed by different letter(s) are significantly different (p<0.05) according to Duncan's multiple range test

Table 7. Pearson correlation coefficient matrix of different phytochemicals studies of grapefruit cv. Shamber.

Variables	TT	Pcy	Acy	GA
Pcy	0.771*	0.712**		
TT	0.762**	0.723**	0.743*	0.712**
GA	0.761**	0.701**		
Acy	0.762**	0.702**		

95% Confidence interval ns = Non-significant, * = p<0.05 and ** = p<0.01 (2-tailed), TT, Total tannins, PCy = Proanthocyanidins, Acy = Total anthocyanins GA = Gallic acid

Discussion

To extend the season and supply over a more comprehensive period of the year gives significant advantages to the growers as well as consumers to the entire year. The synthesis of primary or secondary plant metabolites linked with harvesting time during the growing season. The times of harvest which was examined in *Citrus paradisi* (grapefruit) cv. Shamber reflected that the commercial harvest time started with early and mid-winter (Sep and Oct) in agro-climatic regions like "Sargodha," Pakistan. The contents of crude proteins and crude fiber were varied significantly during different maturity stages in the different harvested season (Table 1) which were similar to reports of Bermejo *et al*., (2012) who analyze 10 to 11 mg^{-1} 100g FW in cv. Ray ruby. Naderiboldaji *et al*., (2008) in their investigation reported the crude fiber and protein were in the range of 10-12, % in ray ruby grapefruit in full matured fruits. While the nutritional attributes mainly sugar; total soluble solid content increased and moisture content decreased (Kriedemann, 1969; Bermejo *et al*., 2012; Bronner & Beecher, 1995). The current investigation showed that the phenolic compounds associated with maturity stages and immensely synthesized in matured fruits (Table 2). The increased level of total phenolic compounds and total antioxidants in December showed higher antioxidant activity over early harvested fruit in the season or later. The changes of phenolic acids in plants are primarily derived from the phenylpropanoid biosynthetic pathway (Jinhe *et al.*, 2009; Ghasemi *et al.*, 2009; Karimi *et al*., 2012; Barbara *et al*., 2005). PAL functions in the entry point of the phenolic acid pathway observed through catalyzing phenylalanine to cinnamic acid during fully mature stages of fruits. The lower levels of phenolic

contents during early growth and development stages can be related to the immature state of the fruit which begins to accumulate in higher range at the end of ripening period. The results of current research are similar to previous reports of (Nair *et al*., 2004; Vielma *et al.,* 2008; Ghosh *et al.,* 2015) who reported that the initial decline could be in the fragment, due to the decrease in tannin content, which is in accordance of earlier workers like Andreotti *et al.,* (2008); Remorini *et al*., (2008). These particular antioxidants can pause and grab free radical chain reaction during oxidation and form stable free radicals, which inhibit the further oxidation process (Layina-Pathirana *et al*., 2006). The variations in DPPH, total phenolics, total antioxidant activity, total flavonoids and pectin in current investigation at the end of maturity are similar to previous studies of Layina-Pathirana *et al.,* (2006). The DPPH is stable free radicals which are extensively used to evaluate the antioxidant potential of a large number of plants metabolites as it is more stable than hydroxyl and superoxide radicals and more effective in assessing antioxidant (Remorini *et al*., 2008; Layina-Pathirana *et al.,* 2006).

Phenolic compounds and total antioxidants capacity of the fruit is directly related to the proper harvesting time which increases with maturation. The current study reported maximum contents of total flavonoids and total carotenoids in the December harvested fruit which were similar to other studies of (Grierson & Tucker, 1983; Goodner *et al*., 2001; Ye *et al*., 2009). The high enzymatic activities in matured fruits enhance the pectin esterase also, which is responsible for several cell wall variations (Goodner *et al.,* 2001). It is cellulose constitutes of the plant cell wall and plays a vital role in changing the texture of fruit tissue (Hakkinen *et al*., 1999). Early harvested fruit showed a reduction in the pectin-related enzyme whereas immature fruit displayed a reduced activity of the pectin-related enzyme (Goodner *et al.,* 2001).

The Lycopene, γ-carotene, and β-carotene were the major carotenoids found in the juice of a grapefruit found to be increase with the maturity stage or from unripe to fully ripened stage. The results in the present study are in line with the previously investigated studies; demonstrated that the degree of growth, maturity, and environmental factors affect the level of carotenoids in harvested fruit (Hakkinen *et al*., 1999: Landrault *et al*., 2001; Trichopoulou *et al*., 2000). During the fruit growth and development stage, the chlorophyll contents decreases at climacteric respiration and the level of carotenoids increased at fully ripened stage (Trichopoulou *et al*., 2000; Ahmad, *et al*., 1992). Deena *et al*., (2010) found that bioactive compounds present in different citrus members such as oranges, lemons, tangor, and mandarin significantly increased in the mature harvested fruits.

Conclusion

It concluded that the quality attributes in grapefruit (Shamber) juice significantly influenced by the harvested time. These were maximum in full matured fruits. Furtermore; the proximate analysis, individual carotenoids, phytochemicals and antioxidant potential through five different methods found to be increased in December harvested fruits while some in early harvested fruits. It also concluded that the harvest time and maturity level influence the level of the bioactive compound including their maximum accumulation in fruits. This study also proved that the purple-fleshed fruits (December harvest in Sargodha, Pakistan) have a tremendous antioxidant potential of naturally synthesized bioactive compounds.

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