International Food Research Journal 22(5): 1961-1967 (2015)
Journal homepage: *http://www.ifrj.upm.edu.my*

Effects of cultivar and ripening stage of Iranian olive fruit on bioactive compounds and antioxidant activity of its virgin oil

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Article history

Received: 19 September 2014
Received in revised form: 13 February 2015
Accepted: 7 March 2015

Keywords

Olive
Ripening stage
Fatty acid composition
Total phenolic content
Antioxidant activity

Abstract

The effects of different cultivars and ripening stages of Iranian olive fruits (*Olea europaea* L.) on fatty acid composition, ΔECN 42 value, total phenol, chlorophyll and carotenoid contents and antioxidant activity of olive oil were evaluated. In first stage of ripening stage, for all cultivars, oleic acid was major fatty acid. The ratio of monounsaturated to polyunsaturated, unsaturated to saturated fatty acids and ΔECN 42 were affected by cultivar and ripening stage. However, in all cases, ΔECN 42 was lower than 0.2. Total phenolic, chlorophyll and carotenoid contents were significantly different (p<0.05) for all cultivars whereas these variables were decreased during fruit ripening stages.

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Introduction

The olive (*Olea europaea* L.) tree has been cultivated all over the World, in particular, in the countries surrounding the Mediterranean Sea as well as the northern part of Iran. Climate conditions of these regions are very suitable for producing high quality olive fruit. Virgin olive oil which has been extracted from fresh and healthy fruits using cold press without any refining process is one of the oldest known valuable vegetable oils. The oil is rich in valuable, nutritional and health promoting compounds including oleic acid, polyphenols, tocopherols, chlorophyll and carotenoid pigments (Iconomou *et al*., 2010). Oleic acid which is located in two position of triacylglycerols reduces the penetration of fatty acids into the arterial walls resulted in decreasing coronary disease (Asık and Ozkan, 2011; Nabil *et al*., 2012). It was reported that phenolic compounds and natural pigments have great effect on stability, pungent taste and yellow-green color of olive oil (Boukachabine *et al*., 2011; Dag *et al*., 2011). Additionally, these compounds showed antioxidant activity in olive oil (Gallardo-Guerrero *et al.*, 2002). Several researches have revealed that quality and quantity of these valuable compounds influenced by several factors such as climatic conditions, cultivar, fruit ripeness and oil extraction method (Baiano *et al*., 2009; Dabbou *et al*., 2011). Studies on berries and avocado have also shown that cultivar and optimal harvesting time are the most important factors determine amount of fatty acids, triacylglycerol species (TAGs) and antioxidant activity of extracted oil (Ozdemir and Topuz, 2004; Yang and Kallio, 2004).

To date, there is no investigation on effect of cultivar and ripening stage on bioactive compounds and antioxidant activity of Iranian virgin olive oil. Therefore, the objectives of current research were to determine effects of different varieties and ripening stages of Iranian olive fruit (*Olea europaea* L.) on fatty acid composition (FAC), TAGs, total phenolic content, chlorophyll and carotenoid contents and antioxidant activity of its virgin olive oil extracted using screw press.

Materials and Methods

Materials

Different cultivars of Iranian olive fruits (*Olea europaea* L.) including Green, Mari and Shenge without infection or physical damages were handpicked from all sides of identified trees in traditional Iranian orchards of Tarom at three different ripening stages from September to November, 2012. Pure standard of fatty acids mixture and TAG

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were purchased from Sigma-Aldrich (Milwaukee, USA) and Larodan Fine Chemicals AB (Limhamn, Sweden), respectively. All reagents and solvents used were analytical grade and supplied by Merck (Darmstadt, Germany).

Oil extraction

Pilot-scale screw press (P500R, Germany) was used to extract olive oil at temperature of 35 and 40ºC.

Fatty acid analysis

Fatty acid methyl esters prepared using 0.1 g oil, 5 mL of methanolic solution of NaOH (0.5 N) and 1 mL n-hexane in a screw cap vial. The vial was heated in boiling water bath for 10 min. The vial was then removed from boiling water and allowed to cool to near room temperature. As catalyst 2.175 mL of Boron trifluoride was added and the vial was heated in boiling water bath. After 3 min, the vial was cooled and 1 mL of saturated sodium chloride solution and 1 mL n-hexane were added. Then, the vial was shaken vigorously (Metcalf *et al.*, 1966). The vial was allowed to stand for 5 min and then 0.2 mL of top

n-hexane layer containing the methyl esters was removed and injected to the gas chromatograph (GC; Unicam 4600, UK) equipped with a flame ionization detector (FID) and a fused-silica capillary column (BPX70 30 m × 0.25 mm i.d and 0.22 mm film thickness, SGE, Melbourne, Australia). The GC split ratio was 1:10 and volume of each injection was 0.2 mL. The FID detector and injector temperatures were set at 270 and 250°C, respectively. The initial column temperature was 160°C for 6 min and then raised to 180°C at a rate of 6°C/min and held for 9 min, and then the heating rate increased to 20°C/ min until it reached to 200°C. Helium was used as carrier gas with a flow rate of 1 mL/min. Fatty acid determination was done by comparison of sample retention times with pure standard mixtures. The peak areas were calculated and the percentages of the areas were obtained.

Determination of triacyclglycerol species (TAGS)

TAGS of olive oil samples were determined using reversed-phase high performance liquid chromatography (HPLC, Younglin, Acme 9000, Hogye, South Korea) equipped with a refractive index (RI) detector and a Lichrosphere RP C-18 column (25 cm × 4 mm × 4 µm) (Tecnokroma, S. Coop., Spain). The mobile phase was acetone: acetonitrile (60:40) at a flow rate of 0.6 mL/min. The column and detector temperatures were set at 35 and 40°C, respectively. 10 µL of the sample which was diluted with chloroform (5:95 v/v) was injected into the column. TAG peaks were identified using comparing the retention times with those of TAG standards (Zaringhalami *et al*., 2011).

Determination of total phenolic content (TPC)

Total phenols were isolated using water:methanol (60:40 v/v) from an oil-in-hexane solution (10 g/23 mL) according to the method described by Baccouri *et al.* (2007) with some modifications. Total phenolic content (TPC) was determined using Folin–Ciocalteu and colorimetric measurement at 725 nm. The results were expressed as mg of gallic acid (GAE) per Kg of oil.

Antioxidant activity assay

Antioxidant activity was evaluated using 1,1-diphenyl-2-picrylhydrazyl (DPPH) according to Koleva *et al*. (2002) with slight modifications. 2 mL of 0.1 mM methanolic solution of DPPH was added to 20, 40, 60, 80 and 100 µL of the oil samples and then 1 mL methanol was also added. The reaction mixture was thoroughly vortexed and then kept in dark for 30 min. Ascorbic acid at different concentrations (0.625, 1.25, 2.5, 5 and 10 (µg/ mL water) was considered as control. The absorbance of the mixture was recorded at 517 nm using spectrophotometer (Shimadzu UV-2550; Shimadzu, Kyoto, Japan) and percentage inhibition of the $DPPH^{\cdot}$ radical was calculated using the following equation:

$$DPPH^{\cdot}\ (\%) = [(\text{absorbance of the control} - \text{absorbance of the sample}) / \text{absorbance of the control}] \times 100$$

Determination of chlorophyll and carotenoid contents

Olive oil pigments (chlorophyll and carotenoid) were extracted according to Nabil *et al.* (2012). Briefly, 7.5 g of oil sample dissolved in cyclohexane completely and then adjust to 25 mL with same solvent. The maximum absorbance was recorded using a spectrophotometer (Shimadzu UV-2550; Shimadzu, Kyoto, Japan) at 670 and 470 nm for chlorophyll and carotenoid, respectively. The amounts of chlorophyll and carotenoid were calculated as follows:

$$\text{Chlorophyll (mg pheophytin 'a' kg}^{-1}) = (A_{670} \times 10^6) / (E_0 \times 100 \times d)$$

$$\text{Carotenoid (mg lutein kg}^{-1}) = (A_{470} \times 10^6) / (E_0 \times 100 \times d)$$

Where A is the absorbance, E_0 is coefficient of specific extinction which is 613 and 2000 for pheophytin 'a' and lutein, respectively and d is cell thickness (1 cm) of spectrophotometer.

Table 1. Fatty acid composition (percent) of oils extracted from three different cultivars of Iranian olive fruit during ripening stages

Ripening stage	Cultivar	Fatty acids				
		(C16:0)	(C18:0)	(C18:1)	(C18:2)	(C18:3)
September	*Green*	10.1±0.05c/C	2.5±0.02b/B	81.3±0.01a/A	4.2±0.04c/C	0.4±0.04ab/B
	Mari	13.2±0.06a/B	2.8±0.06b/A	73±0.02b/B	7.1±0.09a/B	0.5±0.04b/B
	Shenge	16.3±0.03a/A	2.5±0.04c/B	68.3±0.03c/C	9.10±0.03a/A	0.7±0.05b/A
October	*Green*	13.1±0.09a/B	3.0±0.04a/A	74.6±0.02c/A	7.4±0.02a/B	0.6±0.07a/AB
	Mari	12.6±0.04b/C	3.1±0.01a/A	74.5±0.04a/A	6.6±0.02b/C	0.5±0.06b/B
	Shenge	14.2±0.02b/A	2.9±0.03b/A	69.8±0.02b/B	9.00±0.07a/A	0.7±0.03b/A
November	*Green*	11.5±0.06b/B	1.8±0.03c/B	79.6±0.02b/A	6.7±0.04b/B	0.1±0.02b/B
	Mari	11.8±0.05c/B	3.1±0.01a/A	69.5±0.06c/C	6.8±0.06ab/B	0.8±0.04a/A
	Shenge	13.0±0.07c/A	3.3±0.02a/A	70.6±0.03a/B	8.8±0.06b/A	1.00±0.08a/A

Each value in the table represents the mean±standard deviation of triplicate analysis. Different uppercase and lowercase indicate significant statistical differences (p<0.05) between and within cultivars during ripening stages, respectively, by LSD test. C16:0 palmitic acid; C18:0 stearic acid; C18:1 oleic acid; C18:2 linoleic acid; C18:3 linolenic acid.

Statistical analysis

The experimental design applied was Completely Randomized Design (CRD). Analysis of variance (ANOVA) at significance level of 95% and LSD pair-wise comparison test was performed using Statistix 9.0 software (Tallahassee, FL, USA). All data were represented as mean value ±standard deviation (SD).

Results and Discussion

Fatty acid composition and triacylglycerol species

Major fatty acids of all samples at different ripening stages were presented in Table 1. Oleic, palmitic, linoleic and stearic acids were found to be major fatty acids in all cultivars during ripening stages. The content of oleic acid as the most important fatty acid in olive oil varies among different cultivars during ripening stages. The highest amount of oleic acid in the first stage of ripening was found for Green cultivar followed by Shenge and Mari cultivars. Among all of the oil samples oleic acid content increased only in Shenge cultivar during ripening stages. Linoleic acid content of all samples decreased at the end of ripening stage. An inverse relationship was found between the amounts of oleic and linoleic acids in all cultivars. Gecgel *et al*. (2007) and Zaringhalami *et al*. (2011) also reported that the lowest content of oleic acid was found in the highest content of linoleic acid in safflower and tea seed oils, respectively. The results revealed that the content of palmitic acid as another important fatty acid in olive oil was decreased at the end of the ripening stage. All of the findings on variation of major fatty acid contents for all of cultivars during ripening stages are in agreement with previous studies (Cossignani *et al*., 2001; Esmaeili *et al*., 2012).

∑MUFA/∑PUFA and ∑UFA/∑SFA show the ratios of monounsaturated to polyunsaturated fatty acids and unsaturated to saturated fatty acids, respectively. High value of these ratios which indicating high level of oleic acid and low levels of linoleic, palmitic or steraric acids resulted in high stability and nutritional value of oil obtained (Beltran *et al*., 2004; Desouky *et al.*, 2009; Diraman and Dibeklioglu, 2009). The results which are given in Table 2 revealed that these ratios are related to cultivar and ripening stages of the olive fruit. In addition, these ratios decreased during ripening stages of all cultivars. The equivalent chain number (ECN) determined by adding the actual number of carbon atoms in the triacylglycerol molecule and subtracting twice the number of double bonds in the molecule (Andrikopoulos *et al*., 2001; Boukachabine *et al*., 2011). The difference between theoretical ECN value which calculated from gas chromatographic determination of FAC and experimental ECN value which determined using measuring triacylglycerols by HPLC is called ΔECN value. Olive oil, unlike the most seed oils, has many triacylglycerols with ECN numbers of 40, 42, 44, 46, 48 and 50 (Aranda *et al*., 2004). ΔECN 42 in extra virgin olive oils must not exceed 0.2. Higher values confirm low quality and poor nutritional characteristics of olive oil (Aranda *et al.*, 2004; Boukachabine *et al*., 2011). According to Table 2, the ΔECN 42 values were affected by cultivar and ripening stage. This finding is in accordance with Aranda *et al.* (2004) and Matthaus and Ozcan (2011). They pointed out that variation of ΔECN 42 values

Table 2. Monounsaturated to polyunsaturated and unsaturated to saturated fatty acid ratios and ΔECN42 in the oils extracted from three different cultivars of Iranian olive fruit during ripening stage

Ripening stage	Cultivars	Σ MUFA / Σ PUF	Σ UFA/ Σ SFA	ΔECN42
September	*Green*	17.6±0.03a/A	6.8±0.03a/A	0.02
	Mari	9.3±0.01b/C	5.1±0.05a/C	0.19
	Shenge	11.7±0.04a/B	6.5±0.06a/B	0.19
October	*Green*	9.7±0.09b/B	5.0±0.05b/A	0.04
	Mari	10.5±0.09a/A	5.2±0.02a/A	0.14
	Shenge	9.10±0.04b/C	5.2±0.06b/A	0.09
November	*Green*	7.0±0.07c/A	4.2±0.01c/C	0.12
	Mari	7.2±0.04c/A	4.6±0.01b/B	0.13
	Shenge	7.2±0.03c/A	4.9±0.04c/A	0.14

Each value in the table represents the mean±standard deviation of triplicate analysis. Different uppercase and lowercase indicate significant statistical differences (p<0.05) between and within varieties during maturity period, respectively, by LSD test. MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids, UFA: unsaturated fatty acids, SFA: saturated fatty acids.

were lower than 0.2 and depended on cultivar, climate conditions and stage of fruit ripening. According to Table 2, ΔECN 42 values were lower than 0.2 and affected by cultivars and ripening stage. This finding is accordance with Aranda *et al.* (2004) and Matthaus and Ozcan (2011). They pointed out that variation of ΔECN 42 values depended on cultivar, climate conditions and stage of fruit ripening.

Total phenolic content

Phenolic compounds are strong antioxidants which protect biological system and edible oils from oxidation damages as well as produce the flavor characteristics of virgin olive oil (Gomez-Alonso *et al*., 2003; Carrasco-Pancorbo *et al*., 2005; Soni *et al*., 2006; Matos *et al*., 2007; Gomez-Rico *et al*., 2008; Dabbou *et al*., 2011; Nabil *et al.*, 2012). Several studies revealed that TPC of olive oils varies widely depending on variety, maturity of the fruit, climate conditions and oil extraction method (Aguilera *et al*., 2005; Shibasaki, 2005; Ceci and Carelli, 2007; Baccouri *et al*., 2008; Diraman and Dibeklioglu, 2009; Dabbou *et al.*, 2011). In the current research, utilization of extraction method resulted in high quality olive oil with respect to high amount of valuable compounds. The TPC of all experimented cultivars during ripening stage were depicted in Fig. 1. TPC of the oil samples varied between 0.35±0.08 to 2.07±0.08 mg GAE Kg^{-1} oil. The results confirmed TPC of the oil samples was closely related to cultivar and maturity of the fruit. The highest TPC was detected in Shenge followed by Mari and Green at the first stage of fruit ripening, respectively. These results were in accordance with several researchers

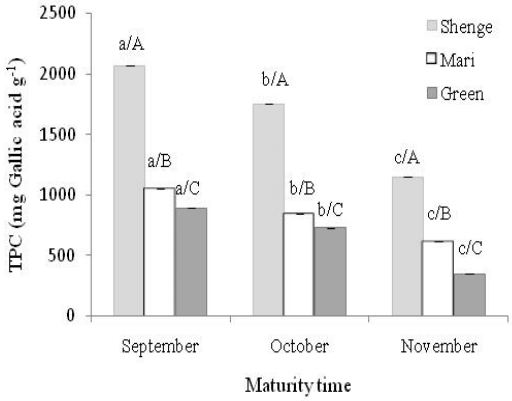

Figure 1. Total phenolic content (mg Gallic acid Kg^{-1} oil) of the oil extracted from three different cultivars of Iranian olive fruit during ripening stages.

reported that TPC of oil obtained from all cultivar of olive fruits decreased during ripening stage (Aranda *et al*., 2004; Shibasaki, 2005; Baccouri *et al.*, 2008; Dıraman and Dibeklioğlu, 2009; Dabbou *et al.*, 2011; Dag *et al.*, 2011; Matthaus and Ozcan, 2011).

Chlorophyll and carotenoids contents

Chlorophyll and carotenoids which are the most important pigments of olive oil also showed antioxidant activity (Cavalli *et al*., 2003; Matos *et al.*, 2007; Dabbou *et al*., 2011; Dag *et al*., 2011). Therefore, these pigments could have an important effect on quality and oxidation stability of olive oil. Numerous studies have shown that the amount of these valuable pigments varied widely depending on fruit cultivar, degree of ripeness and oil extraction method (Rotondi *et al.*, 2004; Aguilera *et al*., 2005; Desouky *et al.*, 2009; Dıraman and Dibeklioğlu,

Table 3. Chlorophyll (mg pheophytin 'a' kg^{-1}) and carotenoid (mg lutein kg^{-1}) contents of oil extracted from three different Iranian olive cultivars during ripening stage

Pigments	Cultivars	Ripening stage		
		September	October	November
Chlorophyll	*Green*	38.74±0.03a/A	31.81±0.02b/A	20.55±0.08c/A
	Mari	21.41±0.08a/C	16.72±0.03b/C	10.52±0.03c/C
	Shenge	27.16±0.01a/B	20.7±80.02b/B	13.38±0.07c/B
Carotenoid	*Green*	3.59±0.01ab/C	2.17±0.09b/C	1.65±0.08c/B
	Mari	3.45±0.06a/B	3.17±0.04b/B	2.5±0.03c/B
	Shenge	4.67±0.01a/A	4.59±0.02a/A	4.06±0.07b/A

Each value in the table represents the mean±standard deviation of triplicate analysis.
Different superscripts indicate significant statistical differences ($p < 0.05$) by LSD test

Table 4. Antioxidant activity (DPPH• %) in virgin olive oils extracted from three different cultivars of Iranian olive fruits at different ripening stages

Cultivars	Ripening stage		
	September	October	November
Green	92.4±0.02a/C	87.4±0.01b/B	74.00±0.02c/C
Mari	93.2±0.03a/B	87.5±0.01b/B	79.5±0.03c/B
Shenge	97.7±0.02a/A	95.2±0.02b/A	93.9±0.01c/A

Each value in the table represents the mean±standard deviation of triplicate analysis. Different uppercase and lowercase indicate significant statistical differences ($p<0.05$) between and within varieties during ripening stages by LSD test.

2009; Dabbou *et al.*, 2011; Dag *et al*., 2011). The effects of key parameters including cultivar and ripening stage on chlorophyll and carotenoids contents were evaluated and the results are presented in Table 3. According to Table 3, the oil of Green and Shenge cultivars showed the maximum content of chlorophyll and carotenoid pigments, respectively. The chlorophyll and carotenoid contents decreased throughout ripening stage and a significant difference ($p<0.05$) was observed. The results also showed that the disappearance of chlorophyll slightly greater than carotenoids. Our findings are in agreement with several previous researches (Baccouri *et al*., 2008; AL-Maaitah *et al.*, 2009; Asık and Ozkan, 2011).

Antioxidant activity

Antioxidant activity depends on amounts of bioactive compounds such as total phenols and pigments. Therefore, antioxidant activity varies due to amount of these compounds during ripening stage. As shown in Table 4, the highest antioxidant activity was measured for oil of Shenge followed by Mari and Green cultivars at the first of the ripening stage. As mentioned, it could be due to the positive correlation between amount of polyphenols, pigments content and antioxidant activity (Asık and Ozkan, 2011; Boukachabine *et al.*, 2011). Furthermore, antioxidant activity decreased significantly ($p<0.05$) during fruits ripening. Similar results were reported by Aguilera *et al.*, 2005, Baccouri *et al.*, 2008 Boukachabine *et al*., 2011; Dabbou *et al.*, 2011.

Conclusions

In the current research, several characteristics of Iranian virgin olive oil such as FAC, TAGs, TPC, chlorophyll and carotenoid contents and antioxidant activity were assessed during fruit ripening stages with respect to types of cultivar. It was revealed that in first stage of ripening stage (September), for all cultivars, oleic acid was major fatty acid. On the other hand, TPC, chlorophyll and carotenoids contents were significantly different ($p<0.05$) for all cultivars whereas these variables were decreased during fruit ripening stages. The data are important in providing information on some characteristics of olive oil to decide the proper harvesting time. From nutritional and industrial point of view, the oil obtained from any selected cultivar which harvested in September for the high amounts of unsaturated fatty acids and valuable compounds as antioxidant is useful to prevent many diseases and oil oxidation damages.

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