Examination of Chlorophyllic Pigments Contents in Iranian Extra Virgin Olive Oil using High Performance Liquid Chromatography

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Abstract: In this study, extra virgin olive oils from provinces Gilan, Zanjan, Qazvin, Golestan , Fars, and Kermanshah were sampled and then chlorophyllic pigments and their derivatives contents in 7 oil samples were determined using high performance liquid chromatography with inverse phase. The results showed that olive oils from Zanjan and Gilan1 had the highest content of chlorophyll a, at 22.6 ppm, 26.5 ppm respectively, and the sample from Fars had the lowest amount at 12.4 ppm. Qazvin and Zanjan samples showed the highest amount of chlorophyll b at 15.8 and 11.3 respectively and Fars sample showed the lowest one at 5.5 ppm. Fars (64.79 ppm) and Gilan2 (61.3 ppm) samples showed the highest content of pheophytin a at 64.79ppm and 61.3ppm, respectively and Zanjan sample had the lowest amount at 49.18 ppm. The results also revealed that pheophytin a is the most abundant chlorophyllic pigment in Iranian extra virgin olive oil as it represents 58.02% of total chlorophyllic pigments and pyropheophytin a show the lowest contents (0.28% of total chlorophyllic pigments).
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1. Introduction

Natural olive oil predominantly is the only vegetable oil that is consumed as crude directly without any purification (Viola and Viola., 2009) and contains nutritional elements including silica, phosphorous, potassium, sulphur, copper, manganese, vitamins and antioxidants (Roca & Minguez-Mosquera., 2002).

Natural extra virgin olive oil, among different olive oil categories, has significant olive oil categories, has significant nutritional, therapeutic, and economic benefits. This type of olive oil has desirable taste and aroma with maximum free fatty acid at 1% on the basis of oleic acid and maximum peroxide value at 20 m Eq/Kg (IOC, 2008).

Iranian per capita consumption of olive oil is 150g annually representing one percent of European per capita consumption and three percents of global olive oil consumption. At the present, olive cultivation covers approximately 100,000 ha in Iran (Keshavarzi Jihad, 2010). Carotenoids and chlorophylls give the color of natural olive oil (Atenaa Poiana et al., 2009). Pigments contents in olive oil depend on the variety, olive ripeness, climatic conditions the kind of extraction process, and storage conditions (Oconnella et al., 2007). Green color of natural olive oil consists of chlorophyll a and b as well as the products of chlorophyll decomposition, i.e. pheophytin a, and b (Glorine and Fabietti, 2005).

The chlorophyllic pigments contents depend on olive variety, olive ripeness, and the kind of extraction, climatic conditions and oil storage conditions (Tsimidou and Psomiadou., 2001). Chlorophyll molecule consists of four pyrrole rings connected by methyl bridges with a magnesium ion in the center (Glorine and Fabietti., 2005). Chlorophyll content in natural olive oil ranges from 1.7 ppm to 27 ppm. Pheophytin content in natural olive oil ranges from 0.3 ppm to 21.7 ppm. Pheophytin a, is predominate ranging from 70 to 80 percent of total pheophytin (Giuffrida et al., 2007).

Chlorophyll and pheophytin serve as catalysts in the presence of light for production of single oxygen in the process of photo oxidation. (Cichelli and Pertesana., 2004, Cinquanta et al., 2001). Chlorophylls, thus, act as proxidant depending on their concentrations resulting in reduced oil stability (Fakourelis et al., 1987). The research on chlorophyll pigments and their derivatives in vegetables showed that pheophytin b and pheophorbide b had the most significant antioxidant activity against fat oxidation that was comparable with BHT (Lanfer et al., 2005). Chlorophyll pigment has an important role in removing harmful compounds from the body by liver. It also improves circulation, immune system function and may reduce cramps and infection. (Mario et al, 2007).

The level of chlorophyll is considered as one of the most important factors in olive oil. Chlorophyll plays a vital role in determining the olive oil color,

and the color plays a key role in acceptability among consumers. in fact ,many consumers preferred a deep green color in olive oil, as in virgin oils (Del Giovine and Fabietti, 2005). Color is an important attribute to consumers, who associate the green hues from the chlorophyll in the oil with freshness of product (Ryan et al., 1998). Climate also has an important role in the chlorophyll concentration in olive oil, as does the ripeness of the fruit. Pervious researchers have found that the concentration of chlorophyll is high, up to 80 mg/kg of oil at early stage of ripening period, and at very low (about 2 mg/kg oil) when fruit is very ripe (Salvador et al., 2001).

Mataeos and Garcia (2006) quantified chlorophyllic pigments and carotenoid content in olive oil by using three different methods of extraction, solid phase extraction by DLI cartridge, and high performance liquid chromatography with inverse phase and UV bipolar arrangement detector, liquid-liquid extraction with N, N'-dimethyl formamide, and solid phase extraction with C18 column (C18 SPE). The results showed higher percentage of pigments recovery by diol-SPE method (98.4%) as compared with LLE method (96.4%) and C18 SPE method (51.3%)

Tsimidio and Psomiadou examined pigments in olive oils from different parts of Greece, they showed that pheophytin a (>10 mgkg-1) was the main pigment. Lutein and beta-carotene were the main pigments of carotenoid (Tsimidio and Psomiadou, 2001).

Giuffrida et al, (2007) examined the pigments of 24 samples of natural olive oil from three Italian main varieties. The study showed that pheophytin a (25.04-36.19) was the main pigment in olive oil. Other pigments included beta carotene (27.16-8.06), pheophytin b (17.6-2.92), lutein (49.4-28.2), and neosantin (11.2 – 1.54). Beta-carotene and neosantin contents were higher than the other varieties likely because of different genetic factors (olive variety) or climatic conditions (Giuffrida et al., 2007)

Criado et al., (2005) examined the effect of temperature behavior of olive fruit on olive color by measuring main pigments of virgin olive oil. The results showed that lutein and beta-carotene contents increase as compared with control samples. Chlorophyllic compounds such as chlorophyll a and b as well as pheophytin a, and b also showed significant increase. High temperature resulted from the processes of oil extraction and cutting was the reason of high temperature behavior (Criado et.al., 2005).

Hashem pour and Fotouhi, (2010) were measured the quality indicators including pigments of olive oil from yellow, oily, and Mary varieties in Kazeroon district using spectrophotometer. The obtained results revealed that yellow and oily varieties had the highest amount of chlorophyll and carotenoid. In the present study, contents of chlorophyllic pigments and their derivatives in Iranian olive oils from different parts of Iran were examined (Hashem pour and Fotouhi, 2010).

2. Materials and Methods

2.1. Materials

Seven samples of extra virgin olive oil were collected from provinces Gilan, Qazvin, Zanjan, Golestan, Kermanshah, and Fars.

2.2. Physicochemical analysis

The amounts of chlorophyllic pigments and their derivatives in extra virgin olive oil samples were determined according to Mataeos and Garcia (2006) method and using high performance liquid chromatography with reverse phase as well as solid phase extraction by DL cartridge. The pigments were separated from olive oil samples (1g) by solid phase extraction using banded DL cartridge and then injected to high performance chromatography with reverse phase equipped with UV detector and then analyzed.

In order to confirm olive oil samples being extra virgin, in this study, acidity value test according to AOAC standard No. 940.28 was conducted in triplicates. Also, peroxide index of the samples was determined using iodometery method according to AOAC standard No. cd -8b- 90.

2.3. Solid phase extraction of pigments using DL cartridge

Banded DL cartridge is connected to vacuum washing system in order to prevent the column from drying and to facilitate solvent movement and then is conditioned by successive movements of 6ml of methanol and 6 ml of hexane. Olive oil sample (1.0±0.001) weighed and then dissolved in 4ml of hexane. The oil solution, then, transferred to the column and the solvent is collected in a volume flask. Then, 5 ml of hexane is added to the column and integrated with the previous hexane. Finally, the column is washed with 3 ml of acetone and the solvent is dried in a vacuum rotating evaporator at room temperature. The leftover is dissolved in 0.3 ml of acetone and then 20 µl of the final solution injected to high performance liquid gas -chromatography (Mataeos and Garcia, 2006).

High performance liquid gas-chromatography was characterized by Youglin model with UV detector, wavelength 660 nm for chlorophylls identification particle size 5µm with a column of ODS2 type, with

250 mm long, 4.2 mm internal diameter, and mobile phase speed at 1 mm/ min.

2.4 Experimental design and statistical analysis

One-way analysis of variance (ANOVA) and Tukey's test ($p \leq 0.05$) were used to analyze the results obtained from all the tests. The statistical analysis was performed using the Minitab version 14 (Minitab Inc., State College, PA, United States).

3. Results and discussion

3.1 Acidity means values of olive oil samples

In order to confirm being extra virgin, the acidity of the samples was measured. The results from Table 1 revealed that the acidity values of the experimental samples meet IOC standard being below 1 percent for extra virgin olive oil (IOC, 2008).

Table 1. Acidity value of extra virgin olive oil

Treatment	Acidity
Gilan2	0.72 ± 0.02^{b}
Qazvin	0.92 ± 0.01^{cd}
Fars	0.98 ± 0.01^{d}
Zanjan	0.84 ± 0.03^{c}
Gilan1	0.95 ± 0.02^{d}
Golestan	0.60 ± 0.01^{a}
Kermanshah	0.62 ± 0.01^{a}

3.2 Peroxide index mean values of olive oil samples

As shown in Table 2, peroxide index mean values of all extra virgin olive oils at 1st week of storage meet IOC standard for extra virgin olive oil, ≤20.

Table 2. Peroxide index (mEq/kg) of extra virgin olive oil

Treatment	Peroxide value
Gilan2	6.51 ± 0.25^{a}
Qazvin	11.43 ± 0.15^{c}
Fars	11.24 ± 0.12^{c}
Zanjan	11.07 ± 0.15^{c}
Gilan1	9.86 ± 0.06^{b}
Golestan	9.75 ± 0.12^{b}
Kermanshah	10.05 ± 0.10^{b}

3.3 chlorophyllic pigments contents and their derivatives in olive oil

Table 3, shows chlorophyllic pigments contents in olive oil from different parts of the country. As shown in Table 3, pheophytin a, is the most abundant chlorophyllic pigment in Iranian extra virgin olive oil, representing 58.02% of total chlorophyllic pigments.

Pyropheophytin shows the lowest amount of chlorophyllic pigments in Iranian extra virgin olive oil. As the results indicated that, pheophytin a shows the highest content, followed by chlorophyll a, and pyropheophytin and pheophytin a show the lowest contents.

As shown in Table 3, pheophytin a is the most abundant chlorophyllic pigment in Iranian extra virgin olive oil, representing 58.02% of total chlorophyllic pigments. Pyropheophytin shows the lowest amount of chlorophyllic pigments in Iranian extra virgin olive oil. As the results indicate, pheophytin a shows the highest content, followed by chlorophyll a, and pyropheophytin a and pheophytin a show the lowest contents.

As demonstrated in Table 4, variation coefficient (VC%) of different chlorophyllic pigments in extra virgin olive oil from different districts of Iran reveals that the pigments vary among different oil samples because of different factors including the climatic condition, variety, oil extraction method, stage of ripening, and irrigation management.

As show in Table 4, pheophytin a, and pheophytin b have the lowest VC% (10.3) and the highest VC% (45.5), respectively. The lower VC% of pheophytin a, implies that its content fluctuation is insignificant in the district of interest.

Pheophytin b is one of the chlorophyllic pigments in natural olive oil resulting from chlorophyll b decomposition. Its high CV% reveals its significant variation and content fluctuation. As indicated in Table 4, pheophytin a and pyropheophytin a show the highest (57.42 ppm) and the lowest (0.285) contents, respectively.

The obtained result corresponds with the findings of Tsimido, (2001), Giuffrida, (2007) and Criado, (2007) who suggested that pheophytin a among chlorophyllic pigments in olive oil showed the highest contents.

Table 3. Chlorophyllic pigments contents (ppm) of extra virgin olive oil from different parts of Iran.

Treatment	pheophytin a`	pheophytin b	pheophytin a	Pyropheophytin a	Chlorophyll b	Chlorophyll a
Kermanshah	3.46±0.04^{ab}	11.05±0.10^{b}	57.64±0.51^{b}	0.39±0.05^{a}	10.46±0.03^{b}	16.20±0.20^{cd}
Golestan	6.06±0.05^{cd}	12.63±1.89^{b}	59.70±0.25^{c}	0.00±0.00^{a}	6.70±0.07^{a}	15.05±0.25^{a}
Gilan1	8.39±0.8^{d}	0.00±0^{a}	49.46±0.26^{a}	0.00±0.00^{a}	6.81±0.53^{a}	22.60±0.23^{e}
Zanjan	3.18±0.68^{a}	9.72±0.5^{b}	49.18±0.23^{a}	0.00±0.00^{a}	11.30±0.30^{b}	26.50±0.18^{f}
Fars	2.90±0.27^{a}	13.00±0.32^{b}	64.17±0.12^{e}	0.78±0.27^{a}	5.50±0.53^{a}	12.40±0.11^{a}
Qazvin	4.70±0.5^{abc}	10.75±0.30^{b}	60.01±0.25^{cd}	0.83±0.32^{a}	15.80±0.30^{c}	15.11±0.20^{bc}
Gilan2	5.40±0.20^{bc}	11.19±0.30^{b}	61.30±0.12^{d}	0.00±0.00^{a}	5.72±0.28^{a}	16.28±0.23^{d}

Table 4. Mean, maximum and minimum values of chlorophyllic pigments of extra virgin olive oil from the districts of interest.

Index	pheophytin a`	pheophytin b	pheophytin a	Pyropheophytin a	Chlorophyll b	Chlorophyll a
Coefficient of variation (%)	40.00	45.50	10.30	13.40	42.70	27.90
Mean (ppm)	4.87	9.76	57.42	0.28	8.89	17.73
Maximum (ppm)	8.39	13.00	64.70	0.83	15.80	26.50
Minimum (ppm)	2.90	0.00	49.18	0.00	5.50	12.40

3.4 Examination of chlorophyllic pigments contents mean values in Iranian extra virgin olive oil

Diagram 1, shows chlorophyllic pigments contents mean values (ppm) of Iranian extra virgin olive oil obtained from different parts of Iran. As shown in Figure 1, pheophytin a, and pyropheophytin a were shown the highest and lowest content of mean value Iranian extra virgin olive oil respectively.

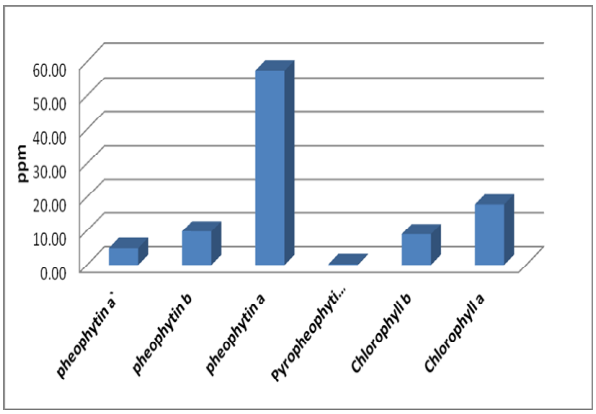

Figure 1. Mean values (ppm) of Iranian extra virgin olive oil obtained from different parts of Iran.

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