

Determination of Fatty Acid Content in Irradiated and Non-Irradiated Syrian Olive Oil During Storage

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Received: April 01, 2019

Accepted: July 02, 2019

Published: July 05, 2019

Citation: Al-Bachir M, Koudsi Y. 2019. Determination of Fatty Acid Content in Irradiated and Non-Irradiated Syrian Olive Oil During Storage. *J Food Chem Nanotechnol* 5(3): 43-48.

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Abstract

This study was carried out on Syrian olive oil (SOO) produced from *Olea europaea* L. cv Kaissy, to determine the influence of different doses of gamma irradiation treatments (0, 1, 2 and 3 kGy) and various storage periods (0, 6, 12, 24 and 36 months) on fatty acid (FA) content. As a result, the composition of FAs was determined as palmitic acid (C16:0) (13.11%); palmitoleic acid (C16:1) (0.90%); stearic acid (C18:0) (3.08%); oleic acid (C18:1) (71.37%); linoleic acid (C18:2) (10.31%) and linolenic acid (C18:3) (1.22%), in order of relative abundance. Gamma irradiation significantly increased ($p < 0.01$) the saturated fatty acid (SFA) and decreased ($p < 0.01$) the unsaturated fatty acids (USFA). These results indicated that the USFA/SFA ratio of the virgin olive oil was markedly decreased by irradiation at 1, 2 and 3 kGy. Long storage has a significant ($p < 0.05$) effect in SFAs and USFAs in both irradiated and non-irradiated olive oil samples.

Keywords

Gamma irradiation, Fatty acids, Gas chromatography, Olive oil, Storage time

Introduction

Olive fruit (OF) and olive oil (OO) are very important foodstuffs worldwide since they are rich in nutrients and have an anti-oxidative activity which reduces the incidence of some diseases [1]. The Food and Drug Administration (FDA) of the USA reported the benefits of OF and OO on the risk of several diseases including coronary disease by consuming about 23 g of OO daily, due to the presence of monounsaturated fatty acids (MUFAs) in OO [2].

Olive fruits contains about 20% (wt./dry wt) of lipid consisting largely of USFAs [3]. The composition of OO is primarily triacylglycerols (99%) and secondary free fatty acids (FFAs), mono- & diacylglycerols, and an array of lipids such as sterols, hydrocarbons, tocopherols, pigments and aliphatic alcohols [4]. Triglycerides are the most dominant compounds in OO, which render the main physical and chemical properties of oil. On the other hand, the dominant FA in olive oil is oleic acid and a number of other FAs are present in tiny amounts [5]. The most important part in OO is the FAs that include stearic (18:0), oleic (18:1), linoleic (18:2), linolenic acids (18:3), palmitic (16:0), palmitoleic (16:1), and Myristic (14:0), However, eicosanoic and heptadecanoic acids are found in trace amounts [1, 4]. The role of minor components present in OO has been taken into consideration, since these compounds are able to improve their biological action when VOO is consumed freshly [6]. Initially, the richness of MUFAs, mainly oleic acid, was considered as the main healthful property of VOO. After the observation that other nutrients rich in MUFA, as sunflower, rapeseeds and soybean, were not comparable as healthful food with VOO [7, 8].

Processing of foods and foodstuffs by specific ionizing radiations including gamma irradiation improves microbiological safety and storability is one the most extensively studied technology of the 20th century [9, 10]. Ionizing radiation has been widely used to decontaminate both foods and spices [11]. Many studies have found that irradiation produce moderates in the FA profile in food rich in fat [12-14]. Britoet et al. [15] and Yilmaz and Gecel [16] showed that trans FAs were generated in ground beef treated with gamma irradiation. However, little information is available in the literature about the influence of gamma irradiation treatment on OFs or OOs. Also, in our knowledge there is no information about the effects of gamma irradiation treatment on FA components in OFs or OOs. Therefore, the objective of this study was to examine the effects of gamma irradiation treatments on the FA composition of OOs produced in Syria from local cultivar named Kaissy after irradiation treatment and storage period. Since the content of FAs as well as the ratio between USFAs and SFAs are important parameter for determination of nutritional value of certain oil.

Materials and Methods

Production of olive oil

Olive fruits of Kaissy cultivar, with good quality were harvested during 2010 growing season, from grove located at Deer Al Hajar research station, south Syrian region near Damascus. The oils from OFs were extracted using mechanical and physical processes [17]. The OO was extracted using the method described in the previous work [18]. Olive processing consisted of the following stages: milling and slowly mixed for about 30 min at 27 °C. Then, the paste mix was centrifuged at 3000 rpm for 3 min to extract the oil. Afterwards, the OOs were decanted and immediately transferred into dark glass bottles (500 ml) and stored at room temperature (20 - 25 °C) for analyzing. FA composition of OO samples were carried out immediately after irradiation, and after 6, 12, 24 and 36 months of storage.

Irradiation treatment

The OOs were packed in dark glass bottles and then irradiated with doses of 0, 1, 2 and 3 kGy, at room temperature (20 - 25 °C) and under normal atmospheric pressure, at a dose rate of 9.913 kGy h⁻¹ using a ⁶⁰CO irradiator (ROBO, Tech snab export, Moscow, Russia). The absorbed dose was monitored using alcoholic chlorobenzene dosimeters [10].

Fatty acids (FA) determination

The analysis was carried out using the method described in the previous work [18]. FA analysis of the samples which convert to methyl ester were made in the model of 17 Shimadzu gas chromatography apparatus (Shimadzu Corp., Kyoto, Japan) equipped with a flame ionization detector and a capillary column (CBP20-S25- 050, Shimadzu, Australia). The FA percentages were calculated by means of the CLASS - VP 4.3 program (Shimadzu Scientific Instruments, Inc., Columbia, MD, USA).

Statistical analysis

Three replicates of each treatment were used, and the entire assay was carried out in triplicate. The results were expressed as mean value and standard division (SD). Data regarding each parameter were analyzed using the SUPERANOVA computer package (Abacus Concepts Inc., Berkeley, CA, USA; 1998). The differences among means at p<0.05 were compared by using Fisher test [19].

Results and Discussion

Fatty acid profile of Syrian olive oil (SOO)

The fatty acid (FA) contents of VOO were measured by gas chromatography, and the obtained data of the SOO samples under the study regarding FAs content (%) were shown in tables 1, 2 and 3. Six main FAs were determined and divided into three group; saturated SFA, MUFA and polyunsaturated fatty acids (PUFA). Two main FAs composed in SFA group: palmitic acid (C16:0) (13.11%), and stearic acid (C18:0) (3.08%). MUFA was the most dominant group of FAs in the analyzed samples. Within this group, the oleic acid (C18:1) remained the most dominant, showing a mean amount of 71.37%.

Table 1: Changes of saturated fatty acids (palmitic (C16:0) and stearic (C18:0) content (%) on Syrian olive oil (SOO) during gamma irradiation and storage period.

Treatment	Control	1KGY	2KGY	3KGY	P-level
C16:0					
0	13.11 ± 0.14 ^{abc}	14.36 ± 0.05 ^{ab}	14.40 ± 0.13 ^{ab}	14.23 ± 0.15 ^A	**
6	13.21 ± 0.01 ^{bc}	14.21 ± 0.16 ^{ab}	14.14 ± 0.30 ^{ab}	14.15 ± 0.14 ^A	**
12	13.96 ± 0.17 ^A	14.92 ± 0.34 ^{ab}	14.52 ± 0.14 ^A	14.46 ± 0.03 ^A	**
24	13.51 ± 0.05 ^B	14.64 ± 0.17 ^B	14.21 ± 0.08 ^B	14.49 ± 0.36 ^A	**
36	13.87 ± 0.04 ^{BA}	15.11 ± 0.04 ^{BA}	14.55 ± 0.02 ^{BA}	14.32 ± 0.13 ^{CA}	**
P-level	**	**	*	NS	
C18:0					
0	3.08 ± 0.29 ^A	3.07 ± 0.38 ^A	2.21 ± 0.40 ^{BC}	2.28 ± 0.30 ^B	
6	1.96 ± 0.06 ^{BC}	2.31 ± 0.21 ^C	2.13 ± 0.20 ^{BC}	2.05 ± 0.13 ^{BC}	*
12	2.66 ± 0.01 ^{AB}	2.98 ± 0.01 ^A	2.89 ± 0.02 ^{BA}	2.77 ± 0.07 ^A	**
24	2.36 ± 0.22 ^B	2.51 ± 0.11 ^B	2.35 ± 0.08 ^{BC}	2.27 ± 0.24 ^B	NS
36	2.39 ± 0.02 ^{AB}	2.68 ± 0.05 ^B	2.62 ± 0.01 ^{BA}	2.47 ± 0.01 ^{AB}	**
P-level	**	**	*	**	

^{abc} Significant difference between irradiation treatments are presented with different superscript (* p<0.05, **p<0.01).

^{ABC} Significant difference between storage periods are presented with different superscript (* p<0.05, ** p<0.01).

NS: not significant.

The total SFAs, total USFA), and the ratio USFA/SFA of the SOO are presented in table 4. The results showed that the total SFA of SOO was found in a low amount (16.19%). While the amount of USFA and TUSFA/TSFA of the same sample was found in high level 83.81% and 5.18, respectively.

The value of oleic acid obtained in the present study from Kaissy variety of olive was comparable to the standard values

of oleic acid reported for olive oil in the literature [20]. The presence of high content of the essential oleic acid suggests that olive oil is highly nutritious. Palmitoleic acid (C16:1) (0.90%) was the second MUFA. PUFA was the third group of FAs present in SOO samples. Within this group, linoleic acid (C18:2) was the most dominant compound with average of 10.31%. Linolenic acid (C18:3) was, in order of relative abundance, the second most dominant analyst from the PUFA, with a mean concentration of 1.22%. It is well known fact that the oils of plant origin contain very small stearic acid fraction. The FA profiles of SOO revealed in our study were in synchronization with a number of already published reports on oil extracted from different commodities [21-25].

stearic and 0.4-0.8% linolenic acids.

The MUSFA have great importance because of their nutritional impact and their effects on oxidative stability of oils [30]. High PUFA percentage contributes to a healthier product, however it is very important to be aware of the possible reduced storage stability and problems related to fat oxidation [31].

Effect of gamma irradiation on FA profile of Syrian olive oil (SOO)

The effect of gamma irradiation doses (0, 1, 2 and 3 kGy) in FA content of Syrian olive oil (SOO) was determined. Data presented in tables 1 - 4, showed that the single FA content, total SFAs, total USFAs, and the ratio USFA/SFA of the olive oil of Kaissy cultivar were significantly ($p < 0.05$) changed by gamma irradiation. All used doses of gamma irradiation (1, 2 and 3 kGy) increased significantly ($p < 0.01$) the percentage of palmitic acid (C16:0). While, only the higher doses of gamma irradiation (2 and 3 kGy) significantly ($p < 0.01$) decreased the percentage of stearic acid (C18:0) (Table 1). Regarding the MUSFAs, 1, 2 and 3 kGy doses of gamma irradiation significantly ($p < 0.01$) increased the percentage of palmitoleic acid (C16:1) and decreased ($p < 0.01$) the percentage of oleic acid (C18:1) (Table 2). Finally, used doses of gamma irradiation significantly ($p < 0.01$) increased the percentage of linoleic acid (C18:2) and decrease ($p < 0.05$) the percentage of Linolenic acid (C18:3) (Table 3). In addition, gamma irradiation significantly ($p < 0.01$) increase the total SFAs and decreased

Table 2: Changes of monounsaturated fatty acids (palmitoleic (C16:1) and oleic (C18:1)) content (%) on Syrian olive oil (SOO) during gamma irradiation and storage period.

Treatment	Control	1kGy	2kGy	3kGy	P-level
Storage time (Months)					
C16:1					
0	0.90 ± 0.02 ^{AB}	1.23 ± 0.08 ^A	1.15 ± 0.06 ^{BB}	1.15 ± 0.02 ^{2A}	**
6	0.95 ± 0.10 ^A	1.30 ± 0.04 ^A	1.26 ± 0.12 ^A	1.21 ± 0.13 ^A	**
12	0.87 ± 0.04 ^{AB}	1.32 ± 0.16 ^A	1.09 ± 0.01 ^{1B}	0.97 ± 0.09 ^{2B}	**
24	0.83 ± 0.08 ^B	1.23 ± 0.04 ^A	1.04 ± 0.05 ^{1B}	1.01 ± 0.05 ^{1B}	**
36	0.91 ± 0.01 ^{1AB}	1.25 ± 0.03 ^A	1.09 ± 0.02 ^{1B}	1.00 ± 0.03 ^{1B}	**
P-level	*	NS	*	*	
C18:1					
0	71.37 ± 0.17 ^{AB}	68.79 ± 0.33 ^C	70.57 ± 0.23 ^{1B}	70.94 ± 0.12 ^{2A}	**
6	73.62 ± 0.27 ^A	69.96 ± 0.12 ^B	71.10 ± 0.10 ^{1A}	71.55 ± 0.51 ^{1A}	**
12	71.00 ± 0.18 ^{1B}	67.67 ± 0.07 ^{1D}	68.82 ± 0.14 ^C	69.33 ± 0.24 ^{1B}	**
24	73.26 ± 0.38 ^A	70.41 ± 0.18 ^A	71.21 ± 0.37 ^{1A}	71.44 ± 0.52 ^{1A}	**
36	73.08 ± 0.05 ^A	70.16 ± 0.07 ^{1AB}	70.91 ± 0.05 ^{1AB}	71.48 ± 0.11 ^{1A}	**
P-level	**	**	**	**	

^{abc} Significant difference between irradiation treatments are presented with different superscript (* $p < 0.05$, ** $p < 0.01$).

^{ABC} Significant difference between storage periods are presented with different superscript (* $p < 0.05$, ** $p < 0.01$).

NS: not significant.

Olive oil is nutritionally considered one of the best salad vegetable oil due to the highest MUFA content (75-77%), which is mainly due to the predominant presence of oleic acid [26]. The fatty acids concentration in olives vary depending on the olive variety, the ecological and environmental conditions of the location where the olives are produced, and the cultivation methods that are employed [27]. Samia Dabbou et al. [28] reported that fatty acids composition of Tunisian olive oils, palmitic acid was within the range of 9.45-11.25%, stearic acid from 2.6-2.95%, oleic acid from 66.21-72.81%, and linoleic acid from 10.92-14.92%. Aparico and Luna [29] reported that the contents of the major FAs in olive oil from Coratina, Koroneiki and Picual varieties varied between 78.1-80.3% oleic, 9.7-11.6% palmitic, 4.8-5.7% linoleic, 2.2-2.4%

Table 3: Changes of polyunsaturated fatty acids (linoleic (C18:2) and Linolenic (C18:3) acids) content (%) on Syrian olive oil (SOO) during gamma irradiation and storage period.

Treatment	Control	1kGy	2kGy	3kGy	P-level
Storage time (Months)					
C18:2					
0	10.31 ± 0.07 ^{1A}	11.52 ± 0.01 ^{1A}	10.89 ± 0.10 ^{1A}	10.64 ± 0.11 ^{1A}	**
6	9.38 ± 0.10 ^{1C}	11.19 ± 0.30 ^{1B}	10.54 ± 0.07 ^{1B}	10.17 ± 0.13 ^{1C}	**
12	9.60 ± 0.05 ^{1B}	10.95 ± 0.23 ^{1B}	10.63 ± 0.11 ^{1B}	10.42 ± 0.16 ^{1B}	**
24	9.35 ± 0.12 ^{1C}	10.60 ± 0.25 ^{1C}	10.52 ± 0.19 ^{1B}	10.14 ± 0.38 ^{1D}	**
36	9.19 ± 0.02 ^{1D}	10.23 ± 0.06 ^{1D}	10.16 ± 0.02 ^{1C}	10.02 ± 0.01 ^{1D}	**
P-level	**	**	**	*	
C18:3					
0	1.22 ± 0.32 ^{1B}	1.04 ± 0.11 ^{1AB}	0.79 ± 0.02 ^{1B}	0.77 ± 0.01 ^{1BC}	*
6	0.89 ± 0.01 ^{1C}	1.02 ± 0.24 ^{1B}	0.83 ± 0.20 ^{1B}	0.88 ± 0.15 ^{1B}	NS
12	1.91 ± 0.01 ^{1A}	2.15 ± 0.06 ^{1A}	2.06 ± 0.05 ^{1A}	2.00 ± 0.01 ^{1A}	**
24	0.69 ± 0.03 ^{1CD}	0.60 ± 0.06 ^{1C}	0.67 ± 0.04 ^{1B}	0.66 ± 0.03 ^{1BC}	*
36	0.56 ± 0.02 ^{1D}	0.57 ± 0.08 ^{1C}	0.67 ± 0.01 ^{1B}	0.71 ± 0.04 ^{1C}	*
P-level	**	**	**	**	

^{abc} Significant difference between irradiation treatments are presented with different superscript (* $p < 0.05$, ** $p < 0.01$).

^{ABC} Significant difference between storage periods are presented with different superscript (* $p < 0.05$, ** $p < 0.01$).

NS: not significant.

($p < 0.05$) the total USFAs and the ratio of TUSFA to TSFA. In the present study, this ratio was 5.18 prior to irradiation of SOO decreasing to 4.74, 5.02 and 5.06 after irradiation of oil with 1, 2 and 3 kGy, respectively (Table 4).

Table 4: Changes of total saturated fatty acids (SFA), total unsaturated fatty acids (USFA), (TUSFA/SFA), and (PUSFA/SFA) on Syrian olive oil (SOO) during gamma irradiation and storage period.

Treatment	Control	1kGy	2kGy	3kGy	P-level
Storage time (Months)					
SFA					
0	16.19 ± 0.26 ^{jb}	17.43 ± 0.36 ^{aAB}	16.61 ± 0.28 ^{8b}	16.50 ± 0.19 ^{8bC}	**
6	15.16 ± 0.06 ^{bd}	16.52 ± 0.31 ^c	16.27 ± 0.17 ^c	16.20 ± 0.25 ^c	**
12	16.61 ± 0.16 ^a	17.91 ± 0.35 ^a	17.41 ± 0.16 ^a	17.23 ± 0.10 ^a	**
24	15.88 ± 0.17 ^c	17.15 ± 0.07 ^{ab}	16.56 ± 0.16 ^{8bC}	16.76 ± 0.14 ^{8b}	**
36	16.27 ± 0.06 ^{db}	17.79 ± 0.05 ^a	17.18 ± 0.01 ^{ba}	16.79 ± 0.13 ^b	**
P-level	**	**	**	**	
USFA					
0	83.81 ± 0.26 ^c	82.57 ± 0.36 ^c	83.39 ± 0.28 ^{ab}	83.50 ± 0.19 ^{aB}	**
6	84.84 ± 0.06 ^a	83.48 ± 0.30 ^a	83.73 ± 0.17 ^c	83.80 ± 0.24 ^a	**
12	83.39 ± 0.16 ^{bd}	82.09 ± 0.35 ^{cd}	82.59 ± 0.16 ^a	82.71 ± 0.14 ^c	**
24	84.12 ± 0.17 ^{ab}	82.85 ± 0.07 ^b	83.45 ± 0.16 ^{8bC}	83.25 ± 0.14 ^{8b}	**
36	83.73 ± 0.06 ^c	82.21 ± 0.05 ^{cd}	82.82 ± 0.01 ^a	83.21 ± 0.12 ^b	**
P-level	**	**	**	**	
USFA/SFA					
0	5.18 ± 0.10 ^c	4.74 ± 0.12 ^{8bC}	5.02 ± 0.10 ^{ab}	5.06 ± 0.07 ^{aB}	**
6	5.60 ± 0.03 ^a	5.05 ± 0.11 ^{ba}	5.15 ± 0.07 ^a	5.17 ± 0.09 ^a	**
12	5.02 ± 0.06 ^{bd}	4.59 ± 0.11 ^c	4.74 ± 0.05 ^c	4.80 ± 0.03 ^c	**
24	5.30 ± 0.17 ^b	4.83 ± 0.02 ^b	5.04 ± 0.06 ^{8aB}	4.97 ± 0.05 ^{8b}	**
36	5.15 ± 0.02 ^c	4.62 ± 0.02 ^c	4.82 ± 0.002 ^c	4.96 ± 0.04 ^{8b}	**
P-level	**	**	**	**	
PUSFA/SFA					
0	0.71 ± 0.03 ^a	0.72 ± 0.02 ^a	0.70 ± 0.02 ^b	0.69 ± 0.02 ^b	NS
6	0.68 ± 0.01 ^{8b}	0.74 ± 0.03 ^a	0.70 ± 0.02 ^{8b}	0.68 ± 0.01 ^{8b}	**
12	0.69 ± 0.01 ^{8aB}	0.73 ± 0.03 ^a	0.73 ± 0.01 ^a	0.72 ± 0.01 ^{8a}	*
24	0.63 ± 0.01 ^c	0.65 ± 0.01 ^{8b}	0.68 ± 0.004 ^c	0.64 ± 0.02 ^c	**
36	0.60 ± 0.004 ^{bd}	0.61 ± 0.004 ^{8c}	0.63 ± 0.002 ^{8d}	0.64 ± 0.01 ^c	**
P-level	**	**	**	**	

^{abc} Significant difference between irradiation treatments are presented with different superscript (* $p < 0.05$, ** $p < 0.01$).

^{ABC} Significant difference between storage periods are presented with different superscript (* $p < 0.05$, ** $p < 0.01$).

NS: not significant.

The value of polyunsaturated fatty acids to saturated fatty acids (P/S) indexes of tested SOO are shown in table 4. The P/S index of the investigated SOO varied depending on the treatments. The P/S indexes of the analyzed SOO samples (irradiated at 0, 1, 2 and 3 kGy and stored for 0, 6, 12, 24 and 36 months) ranged from 0.60 and 0.74. The P/S index value of the control sample of SOO is 0.71, and this value decreased with increasing the storage time. There are no significant

differences in P/S index value between the irradiated and un-irradiated olive oil samples.

The concentration of total USFAs decreased significantly ($p < 0.01$), and the total SFAs increased ($p < 0.05$) throughout the storage period in the SOO samples analyzed. This leads to a corresponding decrease in total USFA/total SFA (oxidation index) throughout storage period for SOO (Table 4).

Tables 1, 2 and 3 show the trend of FA contents of irradiated and un-irradiated olive oil during storage. There were significant ($p < 0.05$) differences in the single fatty acid composition, total SFAs, total USFAs, and the ratio USFA/SFA of irradiated and un-irradiated SOO samples stored for 0, 6, 12, 24 and 36 months.

The results of this study indicate that irradiation induced decomposition of the USFAs. Free radicals generated by irradiation react with the double bonds of FAs [32, 33]. Moreover, radical such as thiyl radicals, which are generated by ionizing radiation treatment during the repair of any radical, may interact with USFAs [34]. Furthermore, the cis configuration is less stable the trans configuration [35, 36]. Similar results were previously observed [37, 38]. Irradiating at 5 kGy decreased total amount of USFAs and increased the total amount of SFAs in beef lipids [39]. Another study reported that the decrease in USFAs during the irradiation process of oil is mainly due to a molecular structure moderate in fatty acids [40]. The ratio of USFA/SFA was used to predict the shelf life of hazel nuts; indication that the lower the ratio, the longer was product shelf-life [41]. On other hand, irradiation of sesame peanut and sunflower seeds at doses of 3, 6 and 9 kGy did not significantly affect the FAs percentages. However, the USFAs, SFAs and the ratio of SFAs to USFAs (TU/TS) were changed upon irradiation [42].

The relationship between PUSFAs and SFAs content is expressed as P/S index. This value is very important parameter for evaluation the nutritional value of certain oil. Oils and fats with higher value of P/S index than one is considered to have high nutritional value [26].

The behavior of the FAs during storage in SOO reported in our study was found to be in harmony with Sanchez-Bel et al. [43] and Jubeen et al. [25]. However, the individual FAs percentage of all analyzed SOO samples falls within the recommended International Oil Council [20].

Conclusion

The irradiation doses 0, 1, 2 and 3 kGy and storage period 0, 12, 24 and 36 months applied to Syrian olive oil (SOO) induce significant statistical differences ($p < 0.05$) in FA content. It was found that, for all analyzed samples, the palmitic acid (C16:0) ranged from 13.11 to 15.11%; stearic acid (C18:0) ranged from 1.96 to 3.08%; palmitoleic acid (C16:1) ranged from 0.83% to 1.32; oleic acid (C18:1) ranged from 67.67 to 73.62%; linoleic acid (C18:2) 9.19 to 11.52%; and Linolenic acid (C18:3) varied from 0.56 to 2.15%. These fall within the recommended International Oil Council [20] for olive oils which indicate that: palmitic acid (7.50-20.00); stearic acid

(0.50–5.00%); palmitoleic acid (0.30 - 3.50); oleic acid (55.00 - 83.00%); and linoleic acid (2.50 - 21.00%). Therefore, this study supports the use of gamma irradiation (up to 3 kGy) as safety treatment for SOO (stored up to 36 months) and calls for further investigations to elucidate its influence on the other chemical and physical characteristics and constituents of the oil.

Acknowledgements

The authors are grateful wish to the Director General of the Atomic Energy Commission of Syria (AECS) and the staff of food irradiation group.

Conflict of Interest

The authors report no conflicts of interest. The author alone is responsible for the content and writing of the manuscript.

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