Bull. Pharm. Sci., Assiut University, Vol. 45, Issue 1, 2022, pp.249-268.

Bulletin of Pharmaceutical Sciences
Assiut University
Website: http://bpsa.journals.ekb.eg/
e-mail: bullpharm@aun.edu.eg

ASSESSING THE QUANTITY AND QUALITY OF OZONATED OLIVE OIL AND STUDYING ITS SHELF-LIFE STABILITY

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Ozonated oils have received an increasing interest lately due to the importance of their products in medicine. Therefore, Quantitative and qualitative analysis of ozonated olive oil was applied, and its shelf-life stability study was also performed to ascertain its effectiveness. For qualitative determination, physical tests (density and viscosity), as well as 1H-NMR spectroscopy were applied. For quantitative determination, Ozone Efficiency method was used, after it was validated, throughout peroxide value. Ozone Efficiency method was basically used as a new method for monitoring the stability of the ozonated olive oil, and it was supported by Acidity Value method. Ozone Efficiency method was evaluated for linearity, accuracy, and precision according to ICH guidelines. Validation results showed that Ozone Efficiency method gave accepted linearity (R2= 0.9984), with excellent mean recovery values (98-102%), and precision with RSD% < 2%. Shelf-life stability study was conducted for six months, with storage temperature control. For stability study results, it was noticed that Ozone Efficiency and Peroxide values decreased while Acidity values increased. These changes were faster at higher temperature. Therefore, the temperature of storage should be lower, and appropriate excipients should be studied to be added to the ozonated olive oil. In addition, the results showed that Ozone Efficiency method is better and more effective than Acidity Value method in monitoring the stability of the ozonated oil.

Keywords: *1H-NMR, Ozone Efficiency, Peroxide Value, Ozonated olive oil, Shelf-life Stability*

INTRODUCTION

As it is known, ozone is a natural, unstable gas (in comparison to oxygen) that is found in the atmosphere[1]. It is composed of three oxygen atoms with a cyclic structure (O_3)**[1].** Ozone is a controversial gas because, although it is very useful in the stratosphere by absorbing dangerous B and C ultraviolet radiations, it is toxic for the pulmonary tract in the troposphere, especially in the presence of CO and N_2O_2[2]. Medical ozone that is usually used in treatment, however, is different from that found in the atmosphere; it is a mixture of ozone and oxygen with a percentage of no less than 95% O_2 and no more than 5% of O_3[3]. Since ozone is a super powerful oxidant, it has been used as a disinfectant of water at first[1&4]. After that, it has been used in medicine for treating diseases since 1894 due to ozone′s biocidal properties (antibacterial, antiviral, antifungal)[5].Ozone was first used for the treatment of gaseous gangrene in World War I in 1914 in dentistry and wound healing[3]. However, the domains of using ozone in medicine have been expanded in the 20thcentury to dermatology, gynecology, angiology, cardiology and cosmetology[4]**.** Consequently, it is very important to know what the ozone dose for treatment in order to avoid the toxicity. Ozone was widely used as a gaseous O_3 in the treatment of different skin ulcers, resulting from atherosclerosis, radiotherapy, and diabetes in particular along its incurable injuries (diabetic foot)[6] by using either a polythene-bag surrounding the leg or using an ozone-resistant plastic cup to be applied over other areas[1,7]. However, since this method of using gaseous ozone is difficult to apply by patients themselves in addition to the

Received in 14/11/2021 & Accepted in 17/1/2022

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fact that ozone itself is unstable, ozone has been widely used as ozonated oil. Whereas, Ozone as a gas has a short half-life of 40 min at 20 ºC[1] and Ozonated water, which was also used in treatment, has a half-life of about 10 hours at room temperature and five days in the fridge[3]. Ozonated oils are more stable because of their longer shelf-life and that comes from the reaction between ozone and the double bounds of the unsaturated fatty acids in oils according to the mechanism described by *Criegee* reaction forming ozonide[3,7&8] Figure1.

Because of this reaction, 1.2.3 trioxolane or molozonide forms at first, but this compound is unstable. Therefore, it tends, in the presence of aprotic solvent, to rearrange to *Criegee ozonide* (1.2.4-trioxolane), which is more stable throughout the formation of carbonyl oxide as an intermediate compound that reacts with aldehyde to form the stable ozonide. However, in the presence of water or any protic solvent, a carbonyl compound and hydroxyl-hydroperoxide are formed. In touch with living tissues that already contain water, hydroxyl-hydroperoxide decomposes into a peroxide H_2O_2 , which is classified as one of Reactive Oxygen Species (ROS_S), and a carbonyl fragment which is one of Lipid Oxidation Products (LOP_S)[2&9]. In general, ROS_S and LOP_S are both toxic. However, it was found that at low concentrations existing in "normal" cells, they play a role of a messenger for cell signaling to do acute oxidative stress and then create biological effects without toxicity[2,10&11]. Peroxide, which is considered as one of ROS_S, is an oxidative and non-radical molecule that is found in human body. it plays a role as a messenger of ozone and can activate multiple cell targets so it could be responsible for many immune responses[12&13]. There are many species of LOP_S and aldehyde is one of them in the presence of water[14].

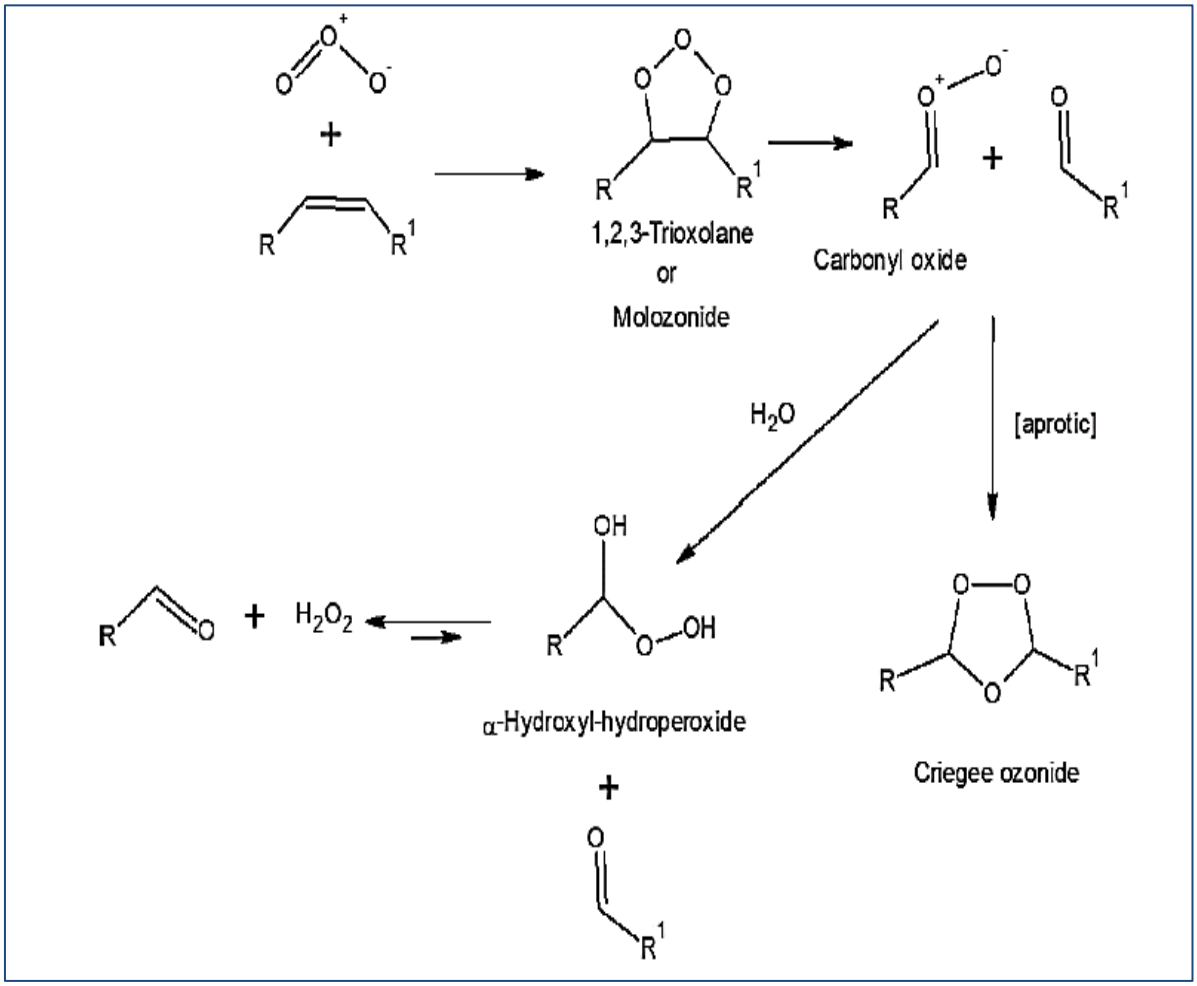


Fig. 1: Criegee mechanism explains the reaction between ozone and the double bonds in unsaturated fatty acids in vegetable oils[9].

In consequence, ozone remains more stable as ozonide in vegetable oils. As such, these oils act as a carrier of ozone. They also have an effect on wound healing due to their content, especially oleic, linoleic, and linolenic acids (Figure 2) that are responsible for the anti-inflammatory and antioxidant effect of these oils[15]. Moreover, ozone can be considered as a preservative that protect ozonated products from microbial contamination[16]. For these reasons, research has been heading towards studying ozonated oils, considering them natural products and ecofriendly cosmetics extracted from plants[15&17].

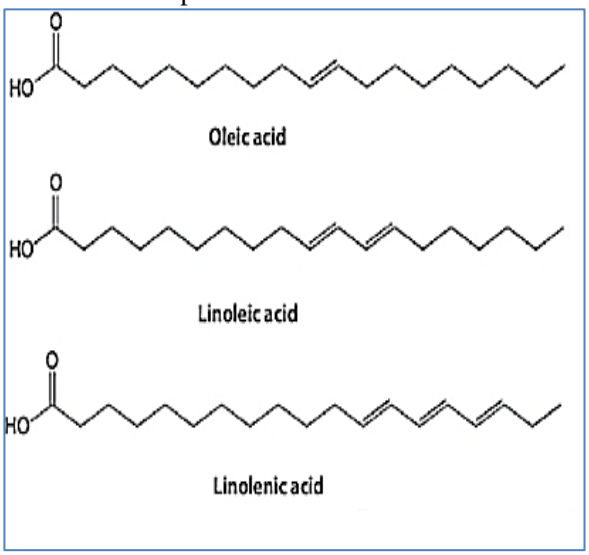


Fig. 2: Structures of most common fatty acids in vegetable oils.

This article aims to study ozonated olive oil quantitatively and qualitatively, as well as monitoring the stability of this ozonated oil using a new method. Qualitatively, physical tests: Density and viscosity were conducted, as well as ^{1}H-NMR considering it a precise analytical way without adding any changes to the sample or producing serious wastes[18]. Moreover, since 1993 NMR has become an official way according to American Oil Chemists Society (AOCS[19]). Quantitatively, Peroxide Value (PV) and Ozone Efficiency (OE) as well as Acidity Value (AV) were used. PV is considered the Standard Operation Procedure (SOP) by the International Scientific Committee of Ozone Therapy ($ISCO_3$[20]) as well as being one of the reliable analytical ways of (AOCS[21]). Taking peroxide value as a base, Ozone Efficiency (OE) was calculated and was used in this study as a quantitative method after it had been validated. OE represents an estimation of the amount of ozonated compound, as evaluated by (PV), to the amount of the applied ozone. Then, (OE) has been employed to monitor the stability of the ozonated olive oil on shelf because of ozonides that are known for their reactivity. According to our knowledge, this is the first work that studies the stability of ozonated oils using Ozone Efficiency (OE) as a new method. Acidity Value (AV) represents the number of mg of potassium hydroxide required to neutralize the free acids in 1g of fat or fatty oil[22].It was considered an additional analytical method that was used along with ozone efficiency method in quantitative study and in monitoring ozonated oils' stability as well.

MATERIALS AND METHODS

Materials

All used reagents were of analytical grade: Chloroform (SCP®, England), glacial acetic acid (SCP®, England), potassium iodide (MERCK®, Germany), sodium thiosulfate pentahydrate (MERCK®, Germany), starch (HiMedia®, India), Ether (SCP®), Potassium hydroxide (PROLABO®), Phenolphthalein (HiMedia®, India), Ethanol (Schalau SL®, Spain), distilled water. Olive oil samples (non-ozonated and ozonated) were locally supplied from Ozone Supportive Therapy Clinic in Aleppo-Syria, and were stored in well tight, opaque refills in the dark away from humidity. Samples were studied on shelf at ambient temperature.

Preparation of purified olive oil

An amount of water, equivalent to the amount of olive oil, was heated to about 70 $^{\circ}$C. Then, the water was poured over the virgin olive oil. The mix was well stirred then left to rest for the next day. After that, the upper layer was collected without mixing with water in the bottom.

Ozonation procedure

A mixture of ozone-oxygen was bubbled by a generator medical ozone (BOZON SPE ECONIKA®) within a container full of 5 L of purified olive oil throughout a column length of 13 cm with constant flow rate of ozone at 2 L/min and ozone concentration of 95 mg/L. The procedure was performed at 20 $^{\circ}$C for 28 days with an average of 12 hrs of ozonation a day.

Preparation of solutions

Sodium thiosulfate solution (0.01 M)

1.25 g of sodium thiosulphate pentahydrate was dissolved in a little amount of distilled water at first, and then it was completed to 500 mL in a volumetric flask.

Saturated potassium iodide solution

In 30 mL of distilled water, dissolve the most probable amount of potassium iodide until it is no more dissolvable in the water.

Potassium hydroxide solution (0.1 M)

3 g of potassium hydroxide was dissolved in a little amount of distilled water at first, and then it was completed to 500 mL in a volumetric flask.

Starch solution

10 g of starch was added to 30 mL lf distilled water, the mixture was heated until boiling and turning into a clear solution, then it was left to cool.

Physical Tests

Density and Viscosity Measurement

Density of olive oil was measured for two samples, before and after ozonation, using a 10 mL pycnometer at room temperature 25 °C**.** each sample was measured three time and the mean value was calculated.

Determination of viscosity was also conducted on olive oil samples before and after ozonation using Fungilab viscometer, type viscolead one at 30 °C. each sample was measured three time and the mean value was calculated.

NMR Spectroscopy

^{1}H- NMR experiments were performed on a Bruker Avance 500 spectrometer operating at 500.13 MHz equipped with a 5 mm cryoprobe at 298 K. The spectra were recorded on 600 µL samples in deuterated chloroform $CDCl_3$. Typical acquisition parameters were as follows: acquisition time 1.64 s, spectral width 10000 HZ, 32 K data points, relaxation delay 2 s and number of scans 128. Relaxation delay was lengthened to 3 s with a pulse width of 3.5 µs (flip angle ≈ 30°). The solvent used exhibits signal at 7.26 ppm.

Peroxide Value and Ozone Efficiency[7]

The basic principle of determination of peroxide value counts on the reduction of present hydroperoxides by potassium iodide as clarified in Figure 3[23].

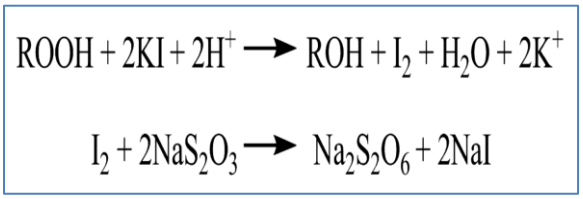


Fig. 3: Peroxide value determination reactions[23]

There are many different, adopted methods to evaluate PV. However, the official way described in Pharmacopoeia (European, British, United States Pharmacopoeia) was chosen[24-26] with a little change in the weight of the sample to be titrated (in this study the following weights from both non-ozonated and ozonated samples were used: 0.2 g, 0.25 g, 0,3 g, 0.35 g, 0.4 g). The sample was dissolved in 30 mL of a mixture of chloroform and glacial acetic acid (2:3). Then, 0.5 mL of saturated potassium iodide solution was added for only one minute with well agitation and the reaction was stopped by adding 30 mL of distilled water. Finally, the mixture was titrated with sodium thiosulphate solution in the presence of starch solution as an indicator.

The equation used to calculate the PV is the following:

$$PV=\frac{10.\ (V_1\text{-}V_0)}{W}$$

Where V_1 is the volume of sodium thiosulphate solution used for titration the sample (mL), V_0 is the volume of sodium thiosulphate solution used for the blank (mL), W is the weight of the sample (g). each titration was repeated three times and the mean volume was recorded.

After getting peroxide value results, Ozone efficiency (OE) was calculated by the following equitation:

$$OE = \frac{(PV_S - PV_0)}{1000} \times \frac{24}{OAD} \times 100$$

Where PV_S is the peroxide value of the ozonated sample, PV_0 is the peroxide value of the non-ozonated sample, OAD is Ozone Applied Dose (mg/g) and refers to the amount of ozone (mg) generated from 1mL of oxygen. OAD was calculated with ozone concentration

95 mg/L to be considered as it is explained as follows:

95mg/L = 0.095 mg/mL

Density of O_2 is 1.429 g/L =0.001429 g/mL →

$$OAD = \frac{0.095\ (\frac{mg}{mL})}{0.001429(\frac{g}{mL})} = 66.48\ mg/g$$

Acidity Value AV[22]

In order to determine this value, the sample was dissolved in 50 mL of a mixture of ethanol and ether (50%-50%). Then, the previous mixture was titrated with potassium hydroxide solution 0.1 M in the presence of phenolphthalein solution as an indicator until pink color can be observed and persists for 30 s. After that, AV was calculated according to the following equation:

$$AV = \frac{5.61\ V}{W}$$

Where: V is the volume of potassium hydroxide used for titration (mL), W is the weight of the sample (g). each titration was repeated three times and the mean volume was recorded.

Stability study of ozonated olive oil

Locally ozonated olive oil underwent a chemical stability study using basically OE method, and Acidity Value method as a supportive method as it was mentioned. It was started to prepare the studying batch in 24/9/2020 and was done in 22/10/2020 with the same ozonation conditions that was mentioned in ozonation procedure. Stability was controlled by taking the sample of non- ozonated olive oil at first and calculating its PV. Then, samples of ozonated olive oil were taken at particular points after different times of ozonation between 12 and 28 days (OO_{12}, OO_{15}, OO_{21}, and OO_{28}). OE value was calculated of each point during performing stability study which was continued for six months as the following: in the 1st month four points were measured with an integral of one week, in the 2nd month two points were measured with an integral of 15 days, in the 3rd-4th-5th-6th months one point was measured in each month.

OE method was supported by Acidity Value method for the same points. The ambient temperature was measured at each studying point.

RESULTS AND DISCUSSION

Density and Viscosity determination

The results of density and viscosity measurement are shown in Table 1.

Table 1: density and viscosity measurement of olive oil before and after ozonation

	Density (g/cm^3) ± SD	**Viscosity (cp) ± SD**
T (°C)	25	30
Non-ozonated olive oil	0.9183 [a] ± 0.0001	110 [a] ± 1.5
Ozonated olive oil	0.9561 [a] ± 0.0000	1038 [a] ± 2
[a] mean:n=3		

The results of density for non-ozonated and ozonated samples showed that there is a significant increasing. This increasing is due to the saturation of the double bonds after ozonation which leads to an increase of oil specific gravity[4]. Density of ozonated oils is a highly important parameter to know in manufacturing the ozonated product, while it helps to choose the compatible components for the best formulations[16].

Viscosity measurement results before and after ozonation also indicated that there is an increasing in the values which is caused by the saturation of the double bonds after ozonation. This modification of the unsaturated chains affects their mobility. Furthermore, polymerization happens during ozonation process and this increases the viscosity of the oil[27].

Results of density and viscosity indicated that they are acceptable and agree with previous studies[16,28]. Both density and viscosity can be observed from the change in the appearance of olive oil as in Figure 4.

Fig. 4: Olive oil before(on the left) and after ozonation (on the right) with the change in its density and viscosity.

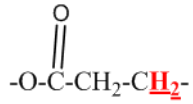
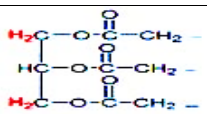
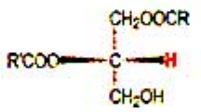
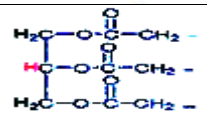
NMR analysis

As it was mentioned, ^{1}H-NMR was used in the analysis of non-ozonated and ozonated olive oil samples quantitatively.

Non-ozonated olive oil ^{1}H-NMR spectrum

Figure 5 represents non-ozonated olive oil spectra. The major peaks, which refer to the main components in the oil, are attached in Table 2 after making comparison with spectra in other studies[19&29-33].

Table 2: Peaks of non-ozonated olive oil NMR spectrum

Peak number	Functional group	H^{1} Chemical shift (ppm)	Attribution
1	**–C<u>H</u>$_3$**	**0.866**	**saturated, oleic and linoleic**
2	**-(C<u>H</u>$_2$)$_n$-**	**1.17-1.39**	
3		**1.51-1.65**	
4	**-C<u>H2</u>-CH=CH-C<u>H2</u>-** **-C<u>H2</u>-CH=CH-CH2-CH=CH-C<u>H2</u>-**	**1.91-2.10**	
5	**-OCO-C<u>H2</u>-**	**2.24-2.36**	
6	**=CH-C<u>H2</u>-CH=**	**2.72-2.82**	**Linoleic acid**
7-8		**4.04-4.35**	**Triglycerides**
9		**5.02-5.18**	***sn* 1,2-diglycerides**
10		**5.22-5.287**	**Triglycerides**
11	**-C<u>H</u>=C<u>H</u>-**	**5.287-5.40**	

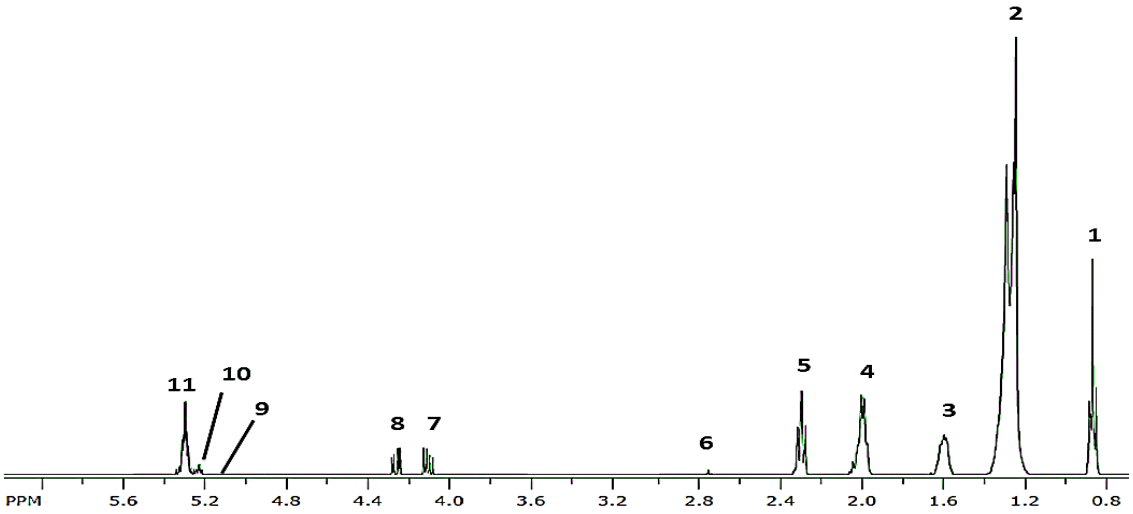


Fig. 5: Non-ozonated olive oil NMR spectrum and the main peaks.

The major components of olive oil are triglycerides 99%, while the minor components are free fatty acids (oleic, linoleic, and linolenic acid basically), mono- and di-glycerides as well as lipids such as hydrocarbons, sterols, aliphatic alcohols, tocopherols, and pigments, which can only be observed by ^{1}H-NMR[34].

The first peak (**1**) appears at 0.866 ppm and refers to methyl group of saturated oleic and linoleic fatty acids. Peak (**2**) which is basically two overlapped peaks, appears at range between 1.17-1.37 ppm referring to saturated acyl chains - $(CH_2)_n$- . Peak (**3**) turns on at signal range between 1.51-1.65 ppm indicating to protons of acyl group as well but in β position of carbonyl function -OCO-CH_2-CH_2-. However, the protons of acyl group in α position -OCO-CH2- are represented by peak (**5**) at the range between 2.24-2.36 ppm.

Peaks of acyl group, which are related to unsaturation or in other words double bonds, are very important to study ozonation procedure as it was mentioned. The first peak of those peaks is Peak (**4**) that turns up at the range 1.91-2.1 ppm and represents the presence of acyl group with protons in α position of the double bond of unsaturated fatty acids (oleic and linoleic acid) Figure 6.

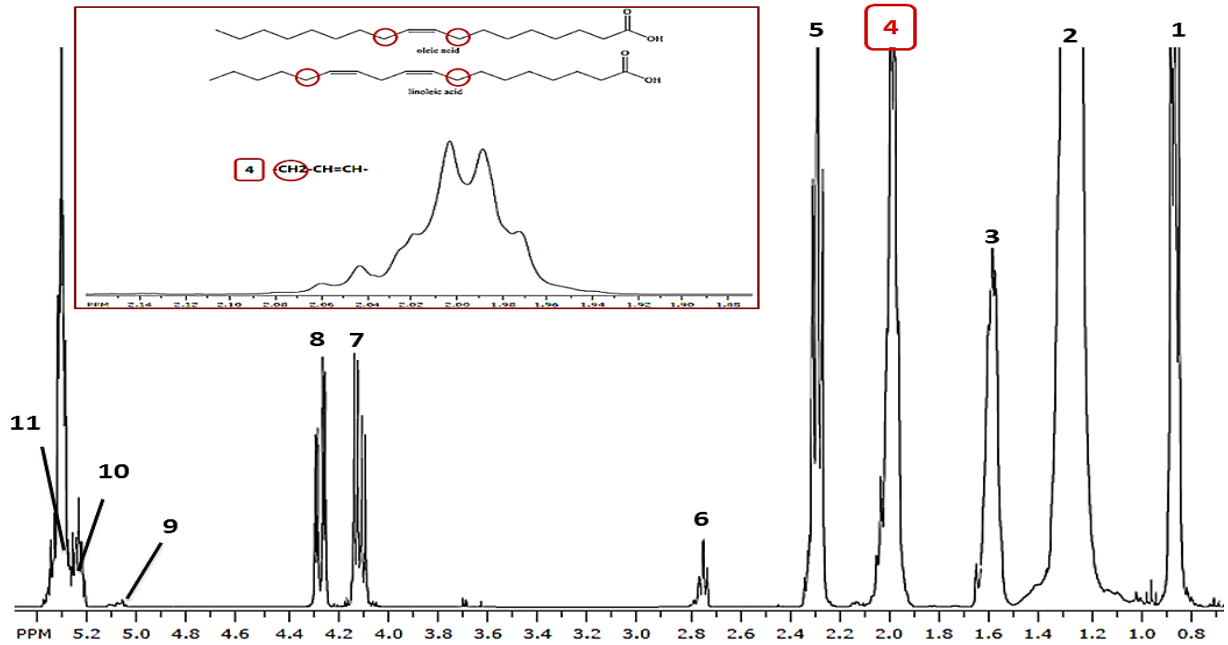


Fig. 6: Peak (4) acyl group in position α of the double bond.

The second peak is Peak (**6**) that turns up at the range 2.72-2.82 ppm referring to acyl group that is also located in α position of the double bond but between two double bonds at the same time. This situation is found in the structure of linoleic acid and linolenic acid Figure 7.

The last peak related to double bonds is Peak (**11**), which is the most important peak in this spectrum since the main reaction happens between ozone and the double bonds of the unsaturated fatty acids. Protons of the double bond in acyl group -CH=CH- show signals at the range 5.287-5.4 ppm Figure 8.

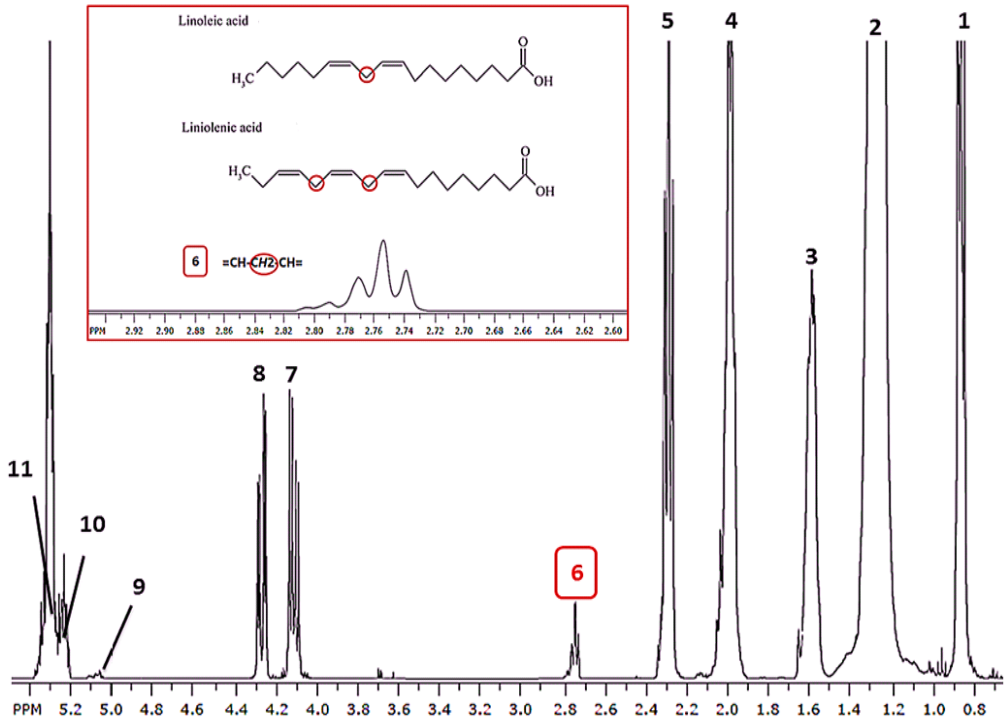


Fig. 7: Peak (6) represents acyl group between two double bonds in linoleic and linolenic acid.

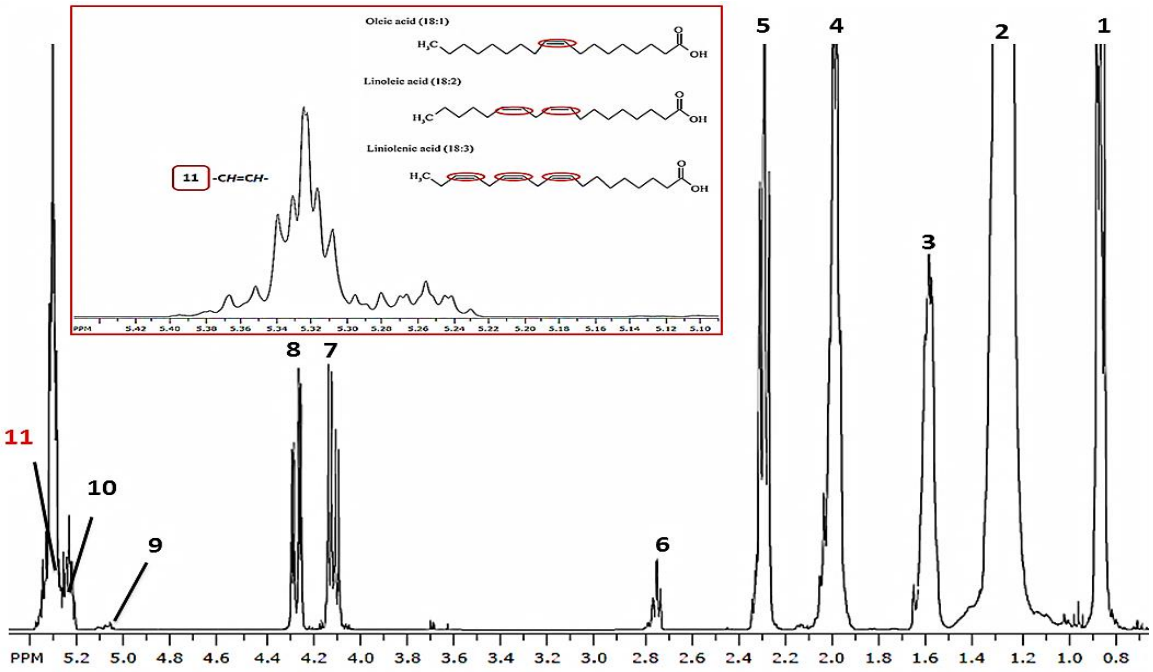


Fig. 8: Peak (11) acyl group of the double bond

Moving to protons of glyceryl group in triglycerides –CH_2OCOR that are represented as almost two symmetrical peaks (7)–(8) exhibited at the range 4.04-4.35 ppm Figure 9.

Moreover, another peak (10) also represents triglycerides at different range of signals 5.22-5.287 ppm that is related to different protons >CHOCOR. Final peak (9) shows up as a weak signal at 5.02-5.18 ppm and is related to protons of glyceryl group in sn-1,2-diacylglycerol –CH_2OH **Figure 10**.

Ozonated olive oil NMR spectrum

Figure 11 represents the spectrum of ozonated olive oil and shows the structural changes during the ozonation procedure with the main peaks in comparison with recent studies[4,17,30,32,35&36].

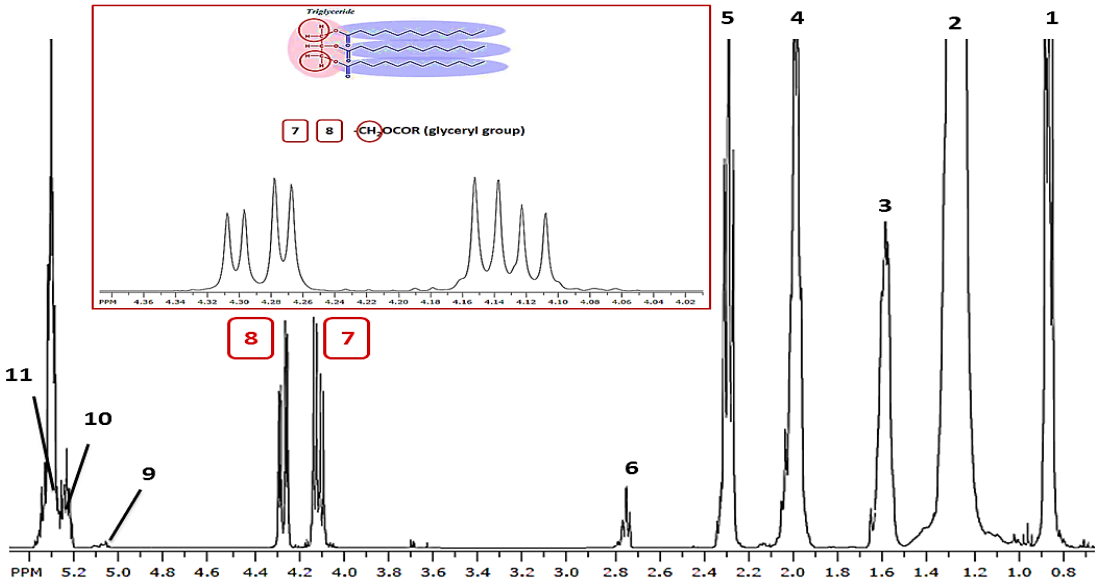


Fig. 9: Peaks (7)-(8) glyceryl group of triglycerides.

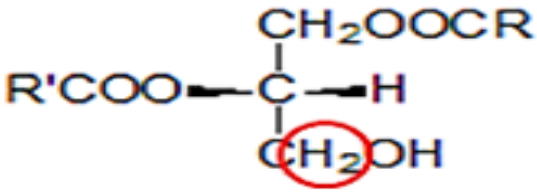


Fig. 10: Protons of glyceryl group in sn-1,2-diacylglycerol represented by Peak (9).

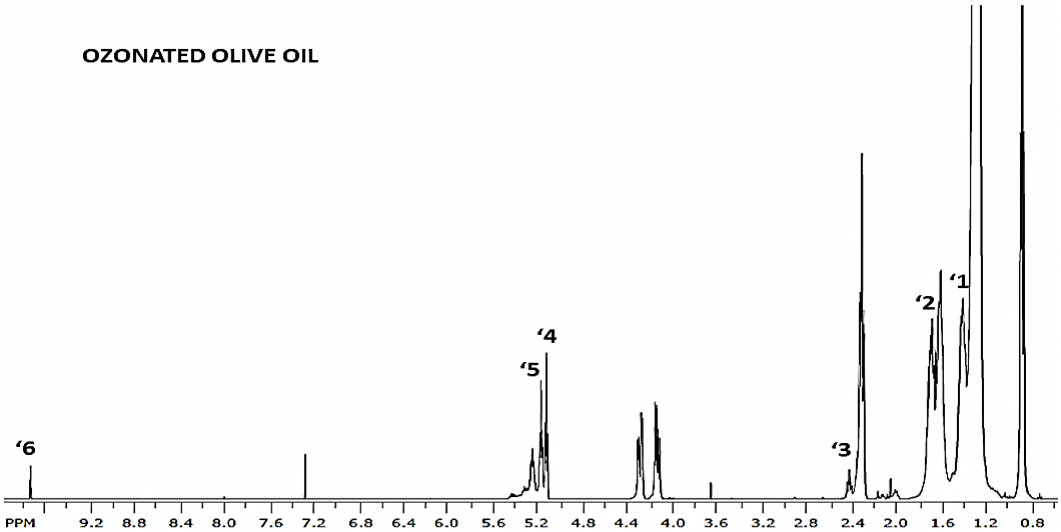
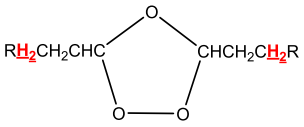
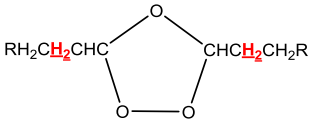
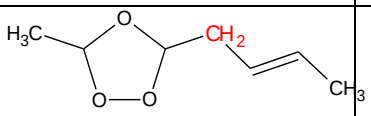
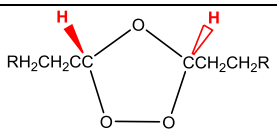
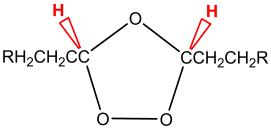
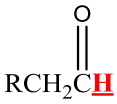


Fig. 11: Ozonated olive oil spectrum and the main peaks**.**

Table 3: Changes on olive oil after ozonation between disappearing and appearing peaks

Peak number	Functional group	Decreasing/Disappearing signals (ppm)	Newly appearing signals (ppm)	Attribution
4	**CH=CH-CH2**	**1.91-2.1 ppm**		
6	**CH=CH-CH2-CH=CH**	**2.72-2.82 ppm**		**Linoleic acid**
11	**CH2-CH=CH-CH2**	**5.287-5.4 ppm**		
'1			**1.36-1.44 ppm**	
'2			**1.65-1.90 ppm**	
'3			**2.36-2.42 ppm**	
'4			**5.10-5.15 ppm**	**trans 1,2,4 trioxolane**
'5			**5.15-5.21 ppm**	**cis 1,2,4 trioxolane**
'6			**9.74-9.80 ppm**	

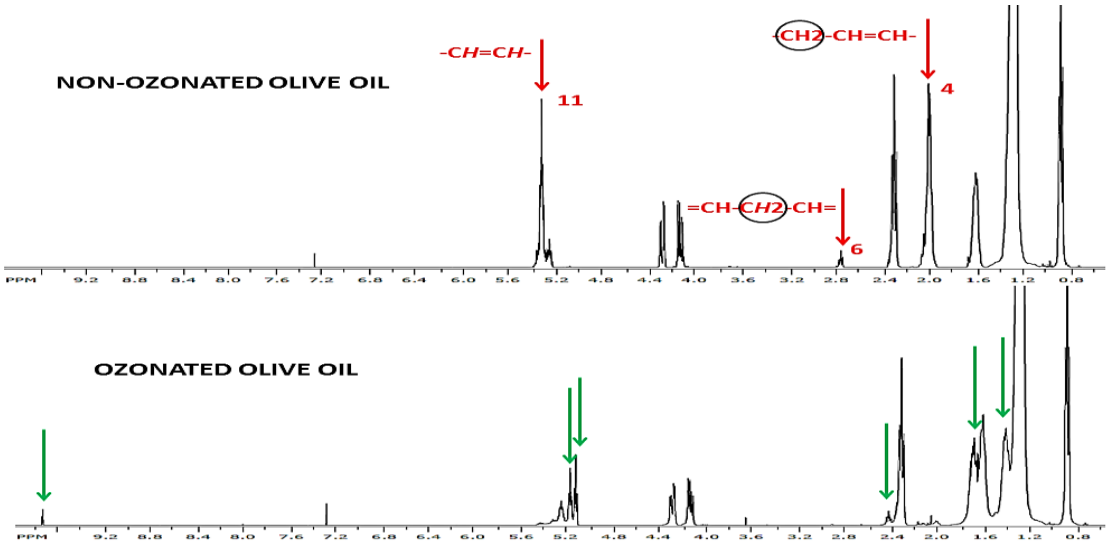


Fig. 12: Comparsion between the spectra before and after ozonation; ↓ disappeared peaks, ↓ newly appearing peaks.

As it was mentioned, the reaction contains breaking of the double bonds. Consequently, peaks of the double bonds will decrease or even disappear from the spectrum, and new peaks will appear, confirming the formation of ozonide (1, 2, 4-trioxolane). Figure 12 shows the differences between the spectra, before and after ozonation. As a result, it was noticed that three peaks almost disappeared, Peak (4) –Peak (6) – Peak (11) and these peaks refer to double bonds. At the same time, five peaks newly appeared, representing ozonides and aldehydes as primary results of ozonation. All the previous results are listed in Table 3.

Starting with peaks ('4) and ('5), they show up at a range of signals 5.10-5.15 ppm and 5.15-5.21 ppm respectively and represent the protons on oxolane ring carbons. This indicates to the formation of two isomeric forms of 1, 2, 4-trioxolanes: *cis* and *trans* represented by two symmetrical triplet peaks in Figure 13.

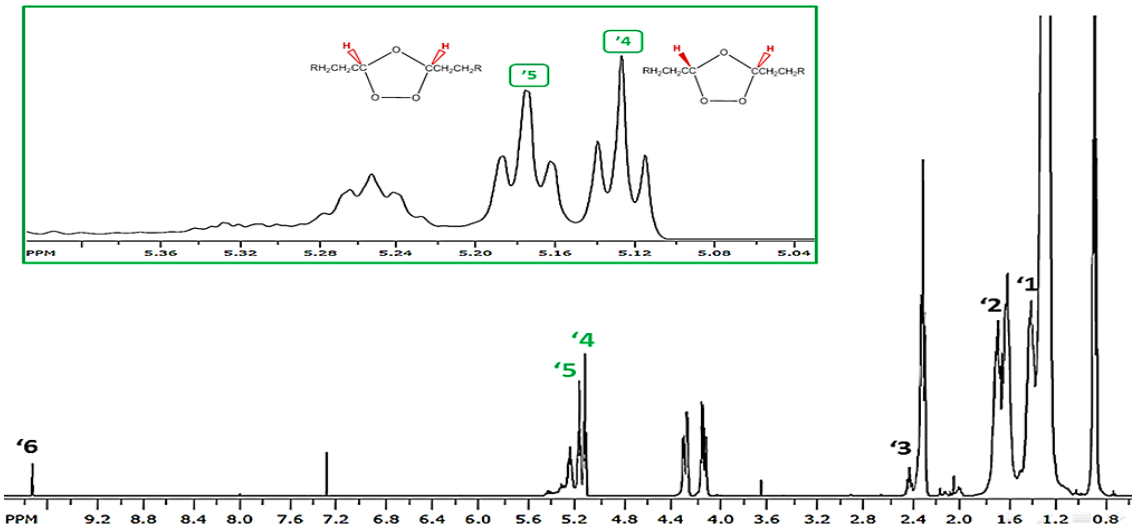


Fig.13: Peaks ('4,'5) of *cis* and *trans* 1, 2, 4-trioxolane in ozonated olive oil NMR spectrum.

Other peaks indicated the presence of ozonides as in peak ('2) with a chemical shift at 1.65-1.90 ppm that relates to α methylene protons of oxolane ring, and peak ('1) with a chemical shift at 1.36-1.44 ppm that relates to β methylene protons oxolane ring. Both peaks are showed in Figure 14 and Figure 15 in order.

Furthermore, the range of signals between 2.36-2.42 ppm represented by peak ('3) belongs to the methylene bridge protons which connects the 1, 2, 4-trioxolane ring to the double bond. Therefore, it will increase up in the beginning of ozonation then it will decrease after the attack of ozone on the double bond. Peak ('3) is illustrated in Figure 16.

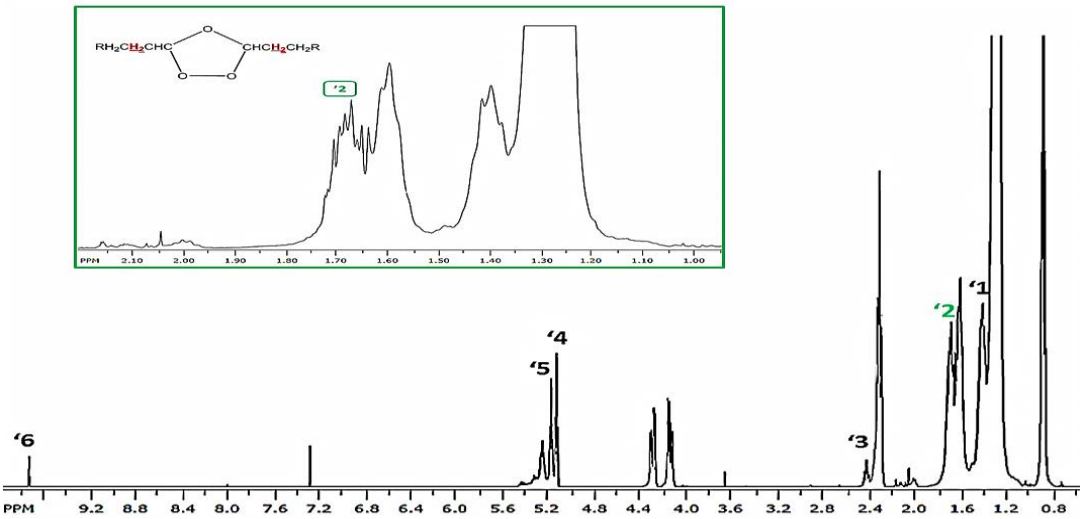


Fig. 14: Peak ('2) in ozonated olive oil NMR spectrum

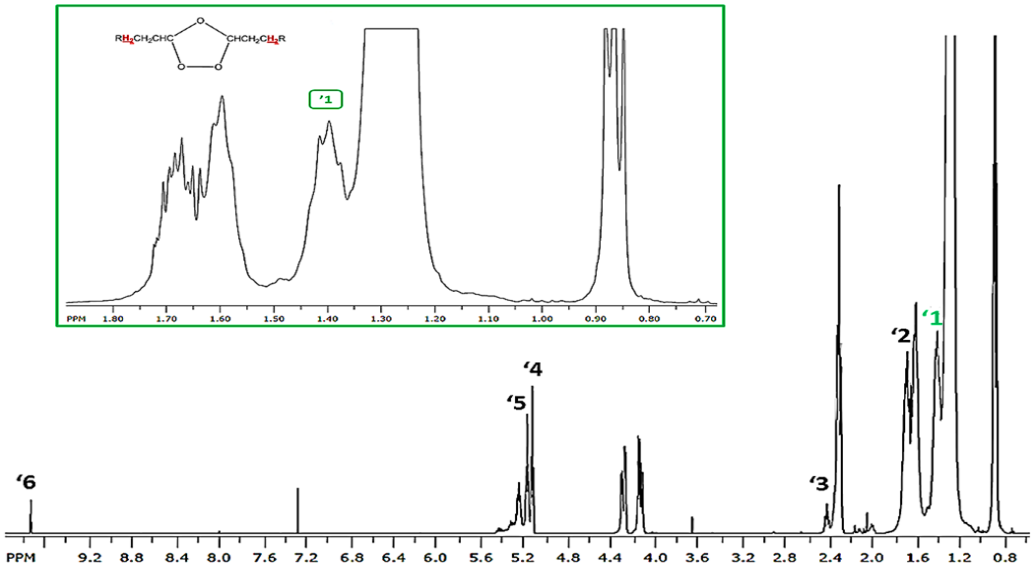


Fig. 15: Peak ('1) in ozonated olive oil NMR spectrum

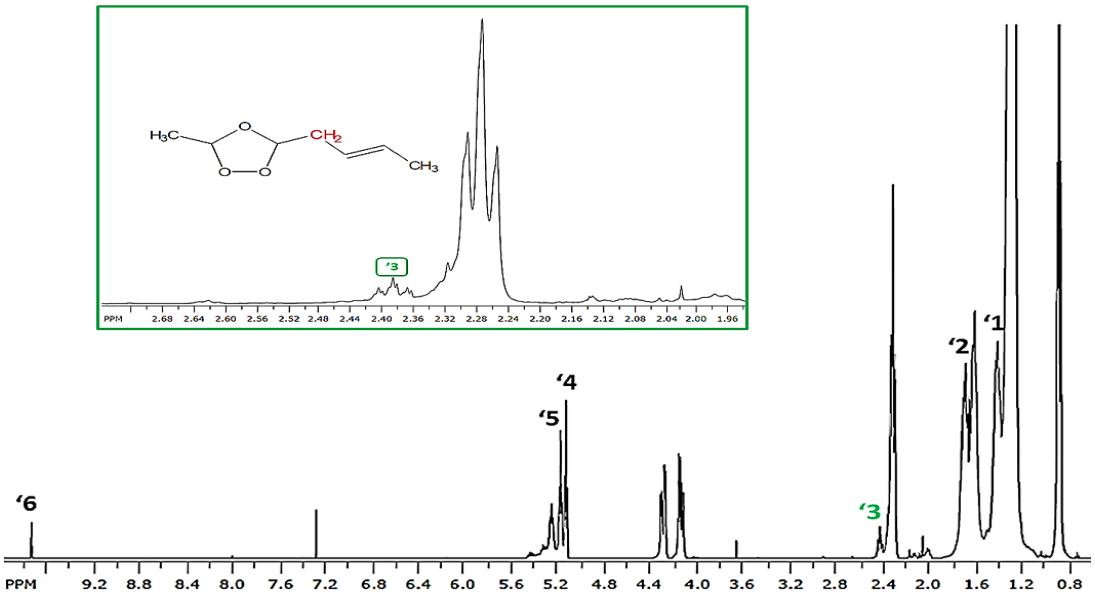


Fig. 16: Peak ('3) in ozonated olive oil NMR spectrum.

The last arising peak ('6) in ozonated olive oil spectrum is connected to protons of aldehydes-the second main compound of ozonolysis- with chemical shift at 9.74-9.80 ppm Figure 17.

Aldehydes may be found in ozonated oils due to either the presence of solvent (water) and the formation of hydropeoxides in Figure 1 which is an evidence of humidity, or the interaction between the two carbonyl oxide intermediates at the relatively "high" temperatures which is considered one of non-ozonide routes of conversion of carbonyl oxide intermediates[36] Figure 18.

Peroxide value PV and ozone efficiency OE

Peroxide values of the samples were calculated by the iodometric titration and then ozone efficiency had been reached throughout these values. Peroxide value, as it was mentioned, is an official analytical way that is found in Pharmacopoeias. However, ozone efficiency is not. Therefore, it needs to be validated and that what was done in this study in order to make it a novel and a reliable method in comparison with the recent studies which relied on PV value only.

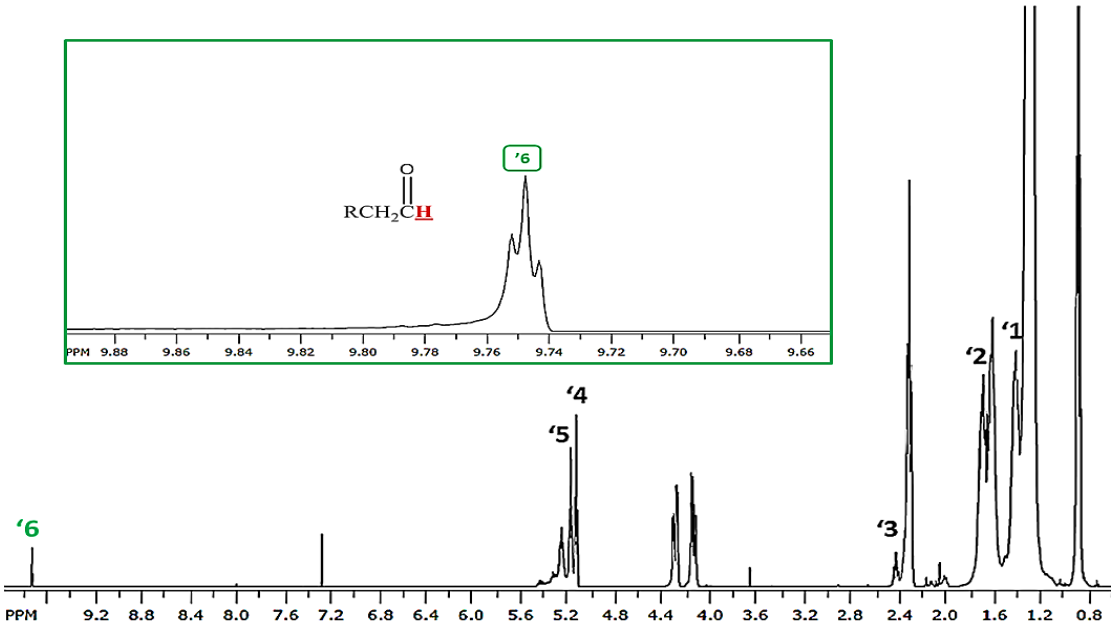


Fig. 17: Peak ('6) in ozonated olive oil NMR spectrum.

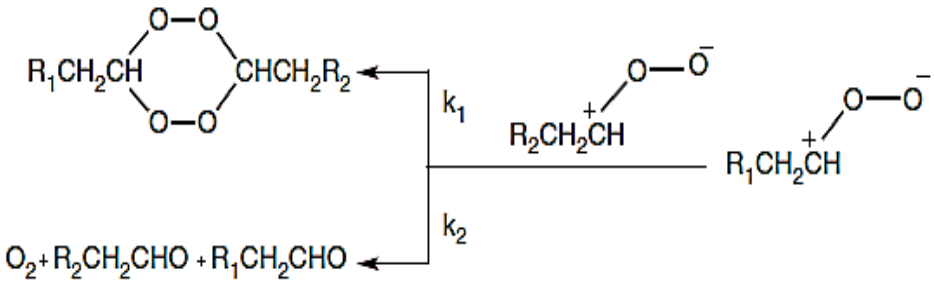


Fig. 18: Nonozonide routes of conversion of carbonyl oxide intermediates. Formation of diperoxides (k1), formation of two aldehydes and oxygen from two carbonyl oxide intermediates (k2)[36]

Ozone efficiency Method validation

Validation of this analytical method was performed with respect to the International Conference on Harmonization (ICH) recommendations by the following parameters: linearity, accuracy, and precision[37-40].

Linearity and range

Linearity was evaluated by linear regression analysis throughout the analysis of the non-ozonated and ozonated samples supplied from Ozone Supportive Therapy Clinic by their conditions of ozonation at five different weights within the working range (0.2-0.4 g). Each weight was titrated three times in one day.

The line that demonstrates the relation between the ozone efficiency (OE) and the corresponding weights (W) was set up in Figure 19. After that, the correlation coefficient (r) was calculated in order to evaluate the linearity of the method. The result equitation of the calibration curve was y= -9.56 x + 27.186 with (r) of 0.9992.

Therefore, these results exhibit very good correlations within the examined weights range and suggest the linearity of the proposed method.

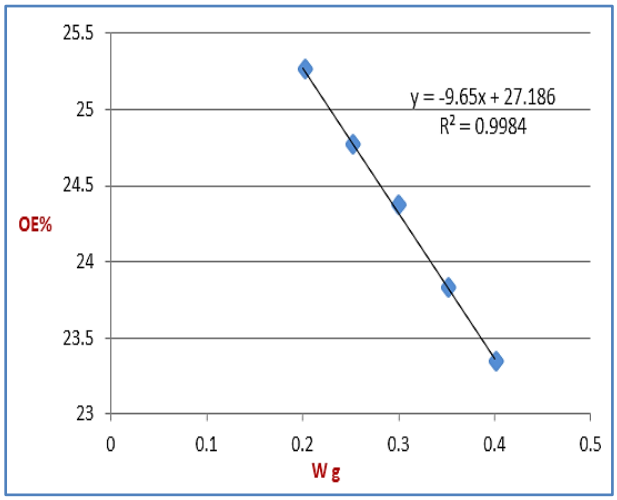


Fig. 19: Linearity line between OE and Weight.

Accuracy

Accuracy means the closeness of adjustment between the value that is accepted, either as a conventional true value or an accepted reference value, and the value found[37].

To study the accuracy of Ozone Efficiency OE method, three weights of each non-ozonated and ozonated olive oil samples were used within the range of linearity (0.3, 0.35, and 0.4 g). Each weight was titrated three times and the whole approach was performed in one day. Recovery results were determined by comparing the gained ozone efficiency with the theoretical one. As it is shown in Table 4, all recovery results as well as mean recovery were among the accepted range of accuracy (98-102%). Therefore, ozone efficiency method is accurate.

Table 4: Accuracy of the proposed ozone efficiency method

Weight (g)	0.3	0.35	0.4
Actual OE_S%	24.37	23.88	23.42
	24.37	23.89	23.4
	24.37	23.9	23.41
Mean[a]	24.37	23.89	23.41
Theoretical OE_S%	24.318	23.84	23.362
Recovery %	98.33	98.57	99
Mean recovery % ± SD		98.63± 0.0067	
RSD %	0	0.042	0.043
[a] n=3			

Precision

Precision means the closeness of adjustment between a series of measurement gained from many samples of the same identical sample under the prescribed conditions[37].

Starting with repeatability or the precision under the same conditions over a short interval time, the weight 0.3 g was used and was titrated six times, and the results were expressed as RSD% in Table 5. Ideally, RSD% value should be <1% and that was the actual result.

Then, intermediate precision was conducted in two days titrating two weights within the linearity 0.25 and 0.35 g. In the first day, each weight was titrated three times, and peroxide value and ozone efficiency were then calculated. In the second day, the same processes were conducted.

Table 5: Repeatability precision of OE method

Weight (g)	0.3
OE1%	24.37
OE2%	24.37
OE3%	24.37
OE4%	24.37
OE5%	24.37
OE6%	24.37
Mean[a] % ± SD	24.37 ± 0
RSD %	0
[a] n=6	

As it is noticed, the results also were expressed as RSD% which ideally should be <2% in the same day, and <5% in the two days together and that was proved by the results shown in **Table 6** which indicated that ozone efficiency method is precise.

Peroxide value and ozone efficiency application

After the method has been validated, three different samples related to different batches were tested according to the conditions supplied from Ozone Supportive Therapy Clinic and results were presented in Table 7.

The first thing to notice is that the peroxide value of the oil sample increases after ozonation due to the formation of ozonides and hydroperoxides. It is also important to notice that the results are different among the three preparations and this is a sign to consider that the conditions supplied from Ozone Supportive Therapy Clinic are not very stable. Therefore, OE method was very helpful for the clinic to monitor and adjust ozonation conditions.

Table 6: Intermediate precision of OE method

Intra-day				Inter-day	
Weight (g)	Theoretical OE %	Actual OE% [a] ± SD	RDS%	Actual OE% [*] ± SD	RDS%
0.25	24.80	24.82 ± 0.01	0.040	24.82 ± 0.008	0.033
0.35	23.84	23.89 ± 0.01	0.042	23.89 ± 0.0098	0.041
[a] mean: n=3, *mean: n=6					

Table 7: Peroxide value and ozone efficiency results

Lot Num.	Lot 1	Lot 2	Lot 3
W (g)	0.3	0.3	0.3
PV_0 [a] (mEq / Kg)	30	66.67	65.56
PV_S * (mEq / Kg)	733.33	1000	783.33
OE %	25.4	33.7	25.9
[a]mean: n=3, *mean: n=3			

Acidity Value determination

The same previous samples that had been tested by ozone efficiency method were examined by Acidity Value method as well. The following results that are listed in Table 8 show that acidity value of the oil sample increases after ozonation due to decomposition of hydroperoxides to carboxylic acids. These results also support OE method's results that indicated the instability of ozonation conditions.

Table 8: Acidity value method results

Lot Num.	Lot 1	Lot 2	Lot 3
W (g)	0.3	0.3	0.3
V_0* (mL)	1.2	1.5	2
AV_0 (mg KOH/g)	22.4	28.1	37.4
V* (mL)	7.5	8.8	10
AV (mg KOH/g)	140.25	164.56	187
*mean: n=3, V_0 and AV_0: non ozonated samples, V and AV: ozonated samples			

Stability study

In order to control the stability of locally ozonated olive oil, OE method was used throughout PV and this indicates the formation of peroxidic compounds, especially 1, 2, 4-trioxolane. Acidity value method was used as a supportive method at the same time for the same points and the results were summarized in Table 9.

There are several variables that can affect the results of PV and OE such as: temperature, the volume of the solvents used in titration, the amount of potassium iodide added, and the time of reaction. It was noticed that non-ozonated olive oil does not have OE value at the start of the ozonation process. Therefore, it was started with the first point **OO_{12}** with OE= 53.35% that increased during the ozonation process until the 15_{th} day of ozonation to reach OE= 55.35%. Then, OE started to decrease until the end of the ozonation process OE= 33.74 in the 21_{st} day and OE= 25.6% in the 28_{th} day. The decreasing in OE and PV during the ozonation process is possibly due to the formation of polymers or due to over-oxidation that leads to ozonides and hydroperoxides degradation to form secondary oxidation products[21&41] as illustrated in **Figure 20.**

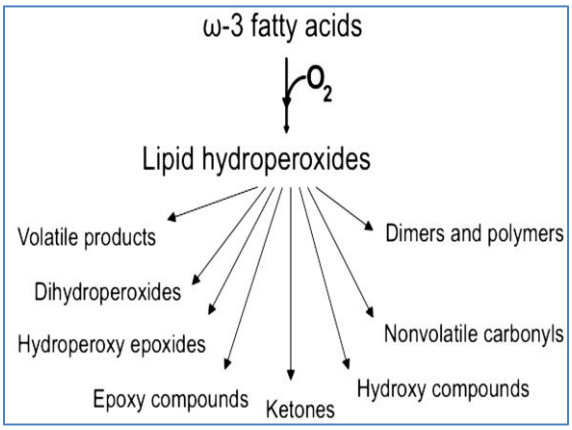


Fig. 20: Decomposition of hydrroperoxides and the formation of secondary oxidation products[21].

It was noticed that PV is directly proportional to OE. Therefore, it follows the same changes starting with PV_0= 33.33 mEq/Kg in the point OO_0, passing by PV_s= 1582.22 mEq/Kg in the point OO_{15} in the middle of ozonation procedure, down to PV_s= 774.44 mEq/Kg in the end of the process. After monitoring the stability of the studied samples, it was noticed according to the final results that OE of ozonated olive oil was decreasing during the storage on shelf and that was significantly affected by the storage temperature, meaning that the activity of the oil is reduced. PV and therefore OE values decreased faster at high temperature than at room temperature[42]**.** This decrease could be demonstrated by the disappearance of ozonides due to their thermal degradation[42]. Even though the storage temperature in this study did not exceed 29 ˚C, the results still show that the lower the storage temperature, the slower the decrease of OE values of ozonated oils. Observing all the points of the study, OE values were more stable in the range 16-18 ˚C and the decreasing was even less such as the point OO_{12}, OE was 9.21% in the 4^{th} month of the study and reached 6.62% in the 5^{th} month and 4.37% in the 6^{th} month. The same thing was for the other points OO_{15}, OO_{21}, OO_{28}. Figure 21 shows the behavior of OE of each point in the study during storage on shelf.

Table 9: Stability study of ozonated olive oil by OE, PV, and AV

Points	Measures	initial results	1st month				2nd month		3rd month	4th month	5th month	6th month
OO_0	T (°C)	29	29				20-22		18-19	17-18	16-17	17
	PV_0*	33.33	40	46.67	60	66.67	73.33	83.33	133.33	133.33	136.67	140
	AV^b	18.7	20.57	22.44	24.31	26.18	28.05	29.92	31.79	32.73	32.73	32.73
OO_{12}	T (°C)	22-29	22-29				19-22		17-18	16-17	17	17-18
	PV_0*	42.22	61.11	64.4	67.68	67.78	75.56	98.89	133.33	133.33	166.67	173.33
	PV_t*	1515.45	1441.11	1294.44	1258.89	1000	915.56	794.44	555.55	394.44	350	294.44
	OE %	53.19	49.83	44.65	43	34.34	30.32	25.11	15.24	9.21	6.62	4.37
	AV^b	74.8	76.67	80.41	84.15	86.02	89.76	95.37	99.11	100.98	101.92	103.79
OO_{15}	T (°C)	22-29	22-29				19-21		17-18	16-17	17	17-18
	PV_0*	48.89	63.33	64.44	66.67	67.78	83.33	100	133.33	133.33	166.67	183.33
	PV_t*	1582.22	1320	1234.44	1038.89	700	664.45	633.33	611.11	538.89	477.78	450
	OE %	55.35	45.37	42.24	35.1	22.82	21	19.25	17.25	14.64	11.23	9.63
	AV^b	82.28	86.02	89.76	93.5	95.37	99.11	102.85	104.72	106.59	107.53	108.46
OO_{21}	T (°C)	19-29	19-29				18-20		18	16-17	17	18-19
	PV_0*	65.56	65.56	66.67	71.11	80	100	100	133.33	166.67	176.67	183.33
	PV_t*	1000	670	581.11	533.33	494.44	350	332.22	310	300	277.78	213.34
	OE %	33.74	21.82	18.59	16.7	15	9	8.38	6.38	4.8	3.65	1.1
	AV^b*	138.38	142.12	145.86	149.6	151.47	155.21	158.95	160.82	162.69	163.63	165.5
OO_{28}	T (°C)	19-29	19-29				17-20		17	16	16-17	18-19
	PV_0*	65.56	66.67	70	81.11	83.33	100	133.33	133.33	136.67	140	150
	PV_t*	774.44	572.22	494.44	400	266.67	244.44	213.34	183.33	176.67	166.67	163.33
	OE %	25.6	18.28	15.32	11.51	6.62	5.23	2.9	1.8	1.44	1	0.5
	AV^b	192.61	196.35	200.09	203.83	205.7	209.44	213.18	215.05	216.92	217.86	221.6

*mean : n=3, ^{t}mean: n=3, ^{b}mean: n=3, PV_0 and PV_t (mEq / Kg), AV (mg KOH/g)

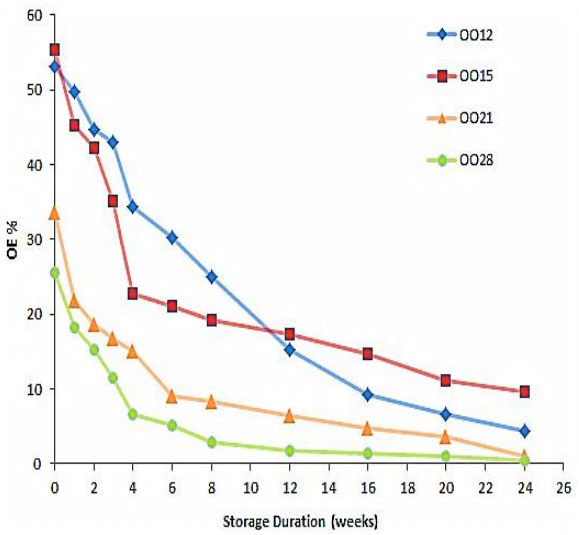


Fig. 21: Stability study of ozonated olive oil using OE method during storage on shelf.

PV_0 values of non-ozonated or purified olive oil were increasing during storage especially at a relatively high temperature 22-29 ˚C. The evolution of PV_0 values was slower in the lower temperature so that the oil was more stable (< 22 ˚C) and these results were almost similar to the results for C. Sanmartin et al. (2018)[43]. This behavior of PV_0 is due to the activation of the oxidation process by high storage temperature where hydroperoxides are formed[44].

For acidity value results of the same points of the study, it was noticed that there was also an increasing in AV values during the ozonation process starting with point OO_0: AV= 18.7 mgKOH/g, down to point OO_{28}: AV= 192.61 mgKOH/g. The increase of AV during ozonation is consistent as long as the process continues and this behavior can be demonstrated by the decomposition of hydroperoxides to form carboxylic acids as secondary oxidation results[9&45]. For the stability study results done by AV method, it was noticed that AV values of the ozonated olive oil increased during the storage on shelf and this was also affected by the storage temperature. The increasing was faster at temperature > 19 ˚C and was slower at lower temperature 16-17 ˚C. AV for all the points in the 1st month of the study and for OO_0, OO_{12}, OO_{15} in the 2nd month where T > 19 ˚C increased faster than the values in 3rd and 6th months where T=18-19˚C. While AV values are even slower to increase in the 4th and the 5th months where T= 16-17 ˚C. These results agree with those for *Sophie Moureu et al.*(2015)[42]. AV value of non-ozonated olive oil was high from the beginning (AV= 18.7 mg KOH/g) and follows the same behavior during the storage which is faster increasing at high temperature > 16-17˚C, and

slower at lower temperature ≤ 16-17˚C. Figure 22 illustrates the evolution of AV of each point in the study during storage on shelf.

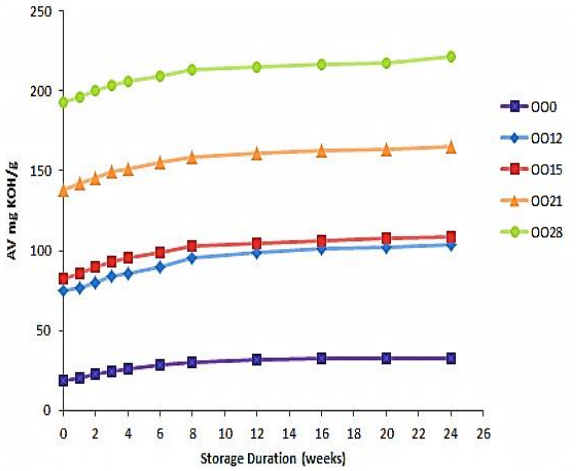


Fig. 22: Stability study of ozonated olive oil using AV method during storage on shelf.

Conclusion

Qualitative assessment of ozonated olive oil using physical tests (Density and viscosity), and ^{1}H-NMR indicated the changes in oil's composition and appearance. Whearas, the most of double bond signals disappeared or strongly decreased as well as ozonides' new signals appeared. It was found that Ozone Effeciency OE method, after the validation, is valuable and practical for the routine application in calibrating and calculating ozone percentage of ozonated oils according to ozonation conditions. Quantitatively, The results of OE and AV confirmed that the conditions of ozonation process are unstable so that more adjusment is needed to be taken into account. During ozonation process, it was noticed that OE and PV values of the ozonated olive oil increase until the 15_{th} day of ozonation. Then they started to deacrease no matter how much ozone was supplied. This behavior lead to reduce the days of ozonation which is conducted by Ozone Supportive Therapy Clinic in order to save time, effort, and money. Stability study results, that were accomplished using Ozone Efficincy OE as a non- previously used method, indicate that ozonated olive oil is unstable during storage on shelf and it can be more stable at lower storage temperature. Further stability studies are needed to determine the perfect storage temperature and the perfect excipients that can be added to the ozonated oils for the best stability. For AV results of monitoring the stability, it was noticed that the changes in all points are generally minor to be observed; hence, OE value is a more appropriate method to monitor the stability of the ozonated oils.

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Bull. Pharm. Sci., Assiut University, Vol. 45, Issue 1, 2022, pp. 249-268.

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التقييم الكمي والنوعي لزيت الزيتون المؤوزن ودراسة ثباته على الرف

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لاقت الزيوت المؤوزنة اهتماماً متزايداً في الآونة الأخيرة نظراً لأهمية منتجاتها في الطب, لهذا تم إجراء التحليل الكمي والنوعي لزيت الزيتون المؤوزن, إلى جانب دراسة ثباته أثناء التخزين على الرف بهدف تأكيد فعالية هذا الزيت. طُبقت الفحوصات الفيزيائية (الكثافة واللزوجة) إلى جانب طيف الرنين المغناطيسي النووي H-NMR١ من أجل التحديد النوعي, بينما استخدمت طريقة فعالية الأوزنة Ozone Efficiency OE , بعد أن تم التحقق من صلاحيتها, من أجل التحديد الكمي من خلال حساب قرينة البيروكسيد Peroxide Value. على مستوى دراسة ثبات زيت الزيتون المؤوزن, استخدمت بشكل أساسي طريقة فعالية الأوزنة OEكطريقة جديدة في دراسة ثبات هذا الزيت وتم دعمها بطريقة قرينة الحموضة Acidity Value AV. تم تقييم طريقة فعالية الأوزنة OE من حيث الخطية، الصحة, والدقة وذلك وفقاً للقواعد الإرشادية للـ ICH, فأظهرت نتائج التحقق من الصلاحية أن طريقة OE أعطت خطية مقبولة (R2 = 0.9984) , مع قيم استردادية وسطية ممتازة (٩٨-١٠٢%), ودقة ذات انحراف معياري نسبي RSD% أقل من ٢%. بعد ذلك أُجريت دراسة الثبات على الرف لمدة ستة أشهر مع مراقبة درجة حرارة التخزين. لوحظ من خلال نتائج دراسة الثبات أن قيم OE و PV انخفضت, بينما ازدادت قيم AV وكانت هذه التغييرات أسرع عند التخزين بدرجة حرارة مرتفعة. ولهذا يجب تأمين درجة حرارة أقل للتخزين ودراسة السواغات المناسبة من أجل إضافتها إلى زيت الزيتون المؤوزن. كما تبين من النتائج أن طريقة فعالية الأوزنة هي طريقة أفضل وأكثر فعالية من طريقة قرينة الحموضة في مراقبة ثبات الزيت المؤوزن.