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Extraction of Squalene from Lebanese Olive Oil

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***Abstract*—**Squalene is a valuable component of the oil composed of 30 carbon atoms and is mainly used for cosmetic materials. The main concern of this article is to study the Squalene composition in the Lebanese olive oil and to compare it with foreign oil results. To our knowledge, extraction of Squalene from the Lebanese olive oil has not been conducted before.

Three different techniques were studied and experiments were performed on three brands of olive oil, Al Wadi Al Akhdar, Virgo Bio and Boulos. The techniques performed are the Fractional Crystallization, the Soxhlet and the Esterification.

By comparing the results, it is found that the Lebanese oil contains squalene and Soxhlet method is the most effective between the three methods extracting about 6.5E-04 grams of Squalene per grams of olive oil.

***Keywords*—**Squalene, extraction, crystallization, Soxhlet.

I. Introduction

As a country lying on the Mediterranean coast, Lebanon is one of the main producers of the high quality olive oil. Olive oil is extracted from the evergreen olive tree fruits (*Olea europaea*, family *Oleaceae*) by three common extraction procedures the pressure system, the centrifugation system and percolation. The milling and time of ripening affects the olive oil quality and composition. The composition of olive oil varies between different areas and altitudes. However, the main components are free fatty acids, pigments, tocopherols, carotenoids, glycerol, chlorophyls, sterols and mainly triacylglycerols [1]. Of these components, the main concern in this research is squalene.

Squalene is a hydrocarbon that is produced either by animals such as sharks or by plants such as olives, rice and soy beans [2]. It is composed of thirty carbon atoms and six conjugated double bonds that cause its degradation if subjected to high temperature conditions [3]. Squalene has several health benefits including cancer prevention, skin regeneration and cholesterol reduction [4]. The intake of dietary squalene will help reduce plasma cholesterol on the expanse of increasing triglycerides [5]. The amount of squalene is more abundant in the shark liver than in the olive oil; but nowadays, environmental regulations ban its extraction from this endangered marine species [6]. Therefore, eco-friendly sources are now under study including the olive oil. This is an advantage to the Lebanese industry due to the abundance of the olive trees on the shore and due to the enormous production of olive oil yearly. Squalene can be used as a major component in the production of many cosmetic products, and in case the Lebanese industry succeeded in its extraction, the cost of these products will be reduced and the profit will be increased.

Extraction of Squalene from the olive oil can be done using several methods. Supercritical fluid extraction is the most globally used due to its high rates of recovery [7] and because compounds sensitive to temperature will be extracted safely [8]. However, it is very costly in comparison with other methods like Soxhlet extraction [9] or fractional crystallization [10]. Three methods are used for the extraction of Squalene from the Lebanese commercial olive oil, and for this purpose, three brands of olive oil are used, Al Wadi Al Akhdar, Virgo Bio and Boulos.

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II. Materials and Methods

Three extra virgin olive oil brands were used through the experimental part; Al Wadi Al Akhdar, Boulos and Virgo Bio. All three were provided from the same local hypermarket. The oil glass bottles were preserved in a dark place at the ambient temperature. All samples were produced in the year 2013 and have an expiry date in 2015.

For the Soxhlet, the hexane used is analytical reagent grade produced by Fisher Scientific, UK, provided in a bottle of 2.5L. Its molecular weight is 86.18 g/mol. The silica gel is ultra-pure with a pore size of 60 - 200μm and a particle size of 60Å produced by Acros Organics. The thimble for the Soxhlet extraction is a Cellulose extraction thimble, single thickness with an internal diameter of 4.1mm and a length of 123mm produced by Whatman.

Methanol 99.8% obtained from Sigma-Aldrich was the reactant used for the esterification, tans-esterification method. Sulfuric acid 95–98% extra-pure packed in HDEP bottles provided from Scharlau, Spain was used as a homogenous catalyst. Silicon oil for heating and temperature control was also provided from Sigma-Aldrich.

The main chemical components used for the fractional crystallization are methanol and acetone. Methanol used was obtained from Sigma-Aldrich having purity of 99.8% which is ensured by a gas chromatography analysis stated on the label. Its molecular weight is 32.04 g/mol. The acetone is obtained from Alpha Chimie and is of 99.5% purity. The label on the bottle stated its composition such as 0.02% water, 0.003% acidity and 0.05% methanol. Its molecular weight is 58.08 g/mol. Both the acetone and the methanol are manufactured in France and their expiry date is in the year 2016.

A. Soxhlet Method

To dry off all water content in the silica gel, 60 g were put in a furnace at 150°C for 1 hour. 20 g of oil were mixed with 150 mL of hexane in a 500mL beaker then the 60g of dried silica gel were added to the mixture, all at room temperature.

The mixture was then magnetically stirred for 1 hour at a rate of 1500 rpm with aluminum foil covering the beaker to prevent any spilling or losses. The turbulent solution was put to rest for a few minutes until two distinct layers could be noticed and all the silica gel had settled at the bottom of the beaker. The clear liquid phase was filtered out and the oil-loaded silica gel was packed into an extraction thimble that was directly placed into the Soxhlet extractor. A condenser system was connected to a Soxhlet extractor and 350 mL of hexane were put in a round-bottom flask of 500 mL that is then also connected to the Soxhlet extractor and heated. The less polar lipids were extracted in this step. As the temperature increased, the hexane vapor went up the distillation arm then back into the chamber holding the thimble. The chamber where the thimble is place is slowly filled with hexane. When the chamber is completely filled, it was automatically emptied by a siphon side arm returning the hexane to the round-bottom flask. During each cycle, a part of the non-volatile compounds dissolves in the hexane solution. The process is kept for 18 hours which were enough to ensure that sufficient cycles took place so that the desired compounds are concentrated in the round-bottom flask. The hexane was then removed using the rotary vacuum evaporator set at a constant temperature of 60°C connected to a vacuum pump of 60 mBar. The remaining portion rich in squalene, which is one of the components that were absorbed by the hexane referred to as the nonpolar lipid fraction (NPLF), was taken for further HPLC analysis.

B. Esterification, Trans-Esterification Method of Extraction

The esterification, tans-esterification reaction conducted was done using 75g of oil. A volumetric methanol to oil ratio of 0.40 was used to guaranty an excess of methanol. Sulfuric acid used as a catalyst in a volume of 1% of the oil. The acid was first diluted in the methanol than added to the oil in a round bottom flask. A reflux vapor condenser was mounted on the top of the flask. The heat source used was a silicon oil bath set at a temperature of 60°C controlled using an electrical temperature probe. Magnetic stirring was set at 1200 rpm all along the experiment of 2 hour. The mixture obtained from the reaction was stored in glass vessels at 5°C temperature. The methanol in excess of the reaction was recovered using a rotary evaporator under vacuum set at a temperature of 60°C using a water bath. The product obtained after the recovery of the methanol was kept for 24 hour in a separator funnel. The bottom aqueous layer was taken for further analysis. The upper oil layer containing squalene, biodiesel and other component was kept stored at 5°C temperature. A vacuum distillation was attempted to recover the biodiesel failed due to low vacuum pressure provided by the available pumps. The oil was burned producing viscous black liquid, and therefore the samples discarded.

C. Fractional Crystallization

In order to perform the fractional crystallization, a mass of 0.5 grams of oil is weighed by means of a digital scale and placed in a 25 ml propylene tube. To the olive oil droplet a ratio of 7:3 by volume of methanol to acetone is added in such a way that the total added volume is 20 ml. therefore, 14 ml of methanol and 6 ml of acetone are added to the test tube and sealed tightly and are placed in a freezer for a minimum of 24 hours at a temperature of -25°C.

After the 24 hours, the sample becomes gel like in appearance and therefore it is shaken lightly and filtered in a vacuum filter using a cellulose filter paper. This step is essential to ensure that no impurities and small particles are present within the samples that will be further analyzed using HPLC. Any impurity can cause a clogging in the HPLC column. After the filtration, evaporation of the acetone and methanol is needed and for this purpose the tube is placed in a water bath at a temperature of 70°C for few minutes. The remaining residue is a small yellow droplet of oil like appearance. This small droplet is believed to contain the squalene and for the next steps it will be diluted in a 1ml of acetonitrile and hexane for 2 different samples. In total 6 samples were made 2 for each oil brand.

D. RP-HPLC Analysis of Squalene

Squalene detection was carried on a reverse phase Hypersil ODS C18 column (particle size 5µm, 250 × 4.6 mm i.d.) having a porosity of 120 Å and a volume of 4.1 ml. The column was provided from Agilent Technologies. HPLC apparatus is an Agilent Technologies (USA) series 1200 equipped with a quaternary pump, a DAD (Diode array detector) detecting UV-visible wave, a manual injection 4 way loop with a capacity of 20µl filled with a 50µl Hamilton syringe. The mobile phase used was a 100% acetonitrile HPLC grade (Scharlau, Spain) at a flow rate of 1.20 ml/min. The sample was filtered before injection using a 0.45µm Minisart (Sartorius stedim biotech) filter filled with a 3cc syringe. The column was kept at 26°C, and a safety maximum pressure of 400 bar. The UV-visible detector was set at 208nm wave length. Analytical standard having a purity of 99.3% was provided from Sigma-Aldrich in 1g dark glass vessel in order to have a calibration curve to bas our calculation on. The standard was diluted in hexane with a dilution factor of 10000.

III. Results

The concentration of the product obtained was evaluated using the data obtained from the HPLC analysis. The area formed by the peak of detection by the diluted standard was used as a reference for the calculation. The concentration of squalene could be evaluated knowing the standard concentration and the dilution factor. Linear regression was employed to evaluate the concentration of squalene in each sample. The area of the peaks was evaluated by the HPLC software. The retention time of squalene was also defined by the standard analysis. Depending on the dilution solvent, composition of the samples and the experiment conditions, the range of retention durations was defined from 19 to 23 min. For further details the HPLC detection signal versus time are provided in the Appendix A. The content of squalene of each sample in the form of gram squalene to gram oil ratio was then calculated using the concentration of the product, the volume obtained and the original quantity of oil used.

Going through the results obtained from the HPLC analysis it can be noticed that a consistency in the quantity extracted of squalene compared to the original oil weight when using the fractional crystallization method and the Soxhlet method. Al Wadi Al Akhdar oil revealed to contain the most quantity of squalene followed by Boulos and Virgo respectively. One sample prepared according to fractional crystallization method, diluted in acetonitrile presented an inconsistency that will be discussed later on.

TABLE I
CALIBRATION OF THE STANDARD SAMPLE WITH HPLC RESULTS

Dilution	Area	Concentration (mol/L)
1/10,000	334885.0	2.07E-04

TABLE II
HPLC RESULTS FOR THE ESTERIFICATION METHOD

Brand	Area	Concentration (mol/L)	g Squalene/g oil
Virgo Bio	13423.1	8.31E-06	**2.73E-07**
Boulos	10376.0	6.43E-06	1.20E-07
Al Wadi Al Akhdar	2008.0	1.24E-06	5.45E-08

TABLE III
HPLC RESULTS FOR THE FRACTIONAL CRYSTALLIZATION METHOD DILUTED IN ACETONITRILE

Brand	Area	Concentration (mol/L)	g Squalene/g oil
Virgo Bio	441145.3	2.73E-04	2.24E-04
Boulos	139830.0	8.66E-05	7.11E-05
Al Wadi Al Akhdar	711745.0	4.41E-04	**3.62E-04**

TABLE IV
HPLC RESULTS FOR THE FRACTIONAL CRYSTALLIZATION METHOD DILUTED IN HEXANE

Brand	Area	Concentration (mol/L)	g Squalene/g oil
Virgo Bio	131591.0	8.15E-05	6.70E-05
Boulos	139830.0	8.66E-05	7.11E-05
Al Wadi Al Akhdar	142815.0	8.85E-05	**7.27E-05**

TABLE V
HPLC RESULTS FOR THE SOXHLET METHOD

Brand	Area	Concentration (mol/l)	g squalene / g oil
Virgo Bio	24646.7	1.53E-03	2.82E-04
Boulos	31452.6	1.95E-03	5.37E-04
Al Wadi Al Akhdar	31953.5	1.98E-03	**6.10E-04**

Soxhlet extraction method is the best technique of extraction compared to the ones investigated. It represents the highest quantity of squalene extracted to oil mass ratio and the highest concentration of product obtained compared to fractional crystallization. The quantity of squalene extracted to oil mass ratio is in accord with the one showed in the literature. The values are in the same range, but the one obtained from the extraction are little less than the one in literature. This can be explained by the losses occurred during the extraction steps and the relative old age of the oil. Also in the literature, olive oil was obtained from different countries and its composition may be different. The extraction process involves the usage of moderate heat, knowing that squalene is heat sensitive; this can affect the quantity extracted. In addition, multi-step processes can sometimes be complex and affect the efficiency of extraction. The age of the oil also plays a role. In fact, as the oil gets older the quantity of Squalene present in it tends to decrease. In another perspective, the concentration of squalene in the product obtained is far away from the high purity squalene provided for calibration. This implies the necessity of a purification step in order to obtain pure squalene. The composition of the product needs to be further analyzed and their potential usage can be revealed useful depending on the application.

In the case of esterification method, the extraction failed for the reasons discussed in the experimental part. After the recovery of the excess methanol from the esterification, the oil layer obtained was left to decant in a separatory funnel for 24 hours. The formation of two layers was noticed after the decantation step. The bottom layer taken for HPLC analysis is the aqueous layer formed as a byproduct of the esterification reaction of the free fatty acids. Due to polarity differences, squalene will not be soluble in water. This was confirmed by the analysis conducted. Only traces of squalene were detected in the aqueous layer. This leads us to the conclusion that squalene is still present in the oil layer. The attempted method used to recover the squalene failed due to low vacuum pressure supplied by the pump available. In theory, the vacuum distillation step will recover the biodiesel obtained from the reaction and the residue of the distillation will be squalene rich. The conditions required for such separation are hard to achieve using normal laboratory glass distillation apparatus. That left us in a necessity to propose another separation method more practical and less challenging to perform. Another method proposed to purify the biodiesel is to filter the product obtained after the reaction and recovery of the water obtained and the alcohol used. The filtration step will be done using activated carbon as filtering material. The non-polar materials present in the biodiesel will adsorb on the activated carbon and the polar one will be collected in the filtrate. Knowing that squalene is nonpolar, it will be adsorbed on the filtering material. To recover the squalene from the filtering material, a desorption step will be required. In order to desorb the non-polar material from the filter a nonpolar solvent must be used, hexane presents the perfect candidate. Simple washing of the activated carbon using hexane will break the physical bond created between the nonpolar materials and the activated carbon. After a solvent recovery step we should obtain a product rich in squalene. This proposed procedure is theoretical and further testing for it in the laboratory is needed to confirm it efficiency and degree of recovery.

The fractional distillation is the less demanding and simplest method to execute. The main weakness of this method is the usage of small quantity of oil 0.5 grams to be exacted [11]. This, in fact, increases the margin of error during the experiment. Since the product obtained is very small in quantity and therefore needs to be diluted before it could be tested by its injection in the HPLC loop. One of the samples was diluted in acetonitrile and the other in hexane. The sample

diluted in acetonitrile was completely miscible. On the other hand, the one diluted in hexane was not completely miscible. Two layers could be clearly seen inside the dilution vessel. The main reason behind this difference in solubility is due to the composition of the product rich in polar compounds. This difference in solubility created a difference in the analysis results. We can notice that the quantity measured in the sample diluted in acetonitrile is higher than the one diluted in hexane. Regarding the sample of Boulos oil used diluted in acetonitrile creating a deviation in the results, it was revealed that after repetition of the sample that water was present in the oil. The water presence lets us use more time to evaporate the solvent that normally requires little time on a moderate temperature. The additional time caused the destruction of squalene molecules. The water addition to oil in a homogenous mixture is possible through the usage of food emulsifier additives.

One must keep in mind that in this article the studied samples are of Lebanese origin, which may differ in composition from the brands and samples studied on foreign olive oil. In addition, the olive oil used is a commercial oil and thus it is not extracted from the fruits by the traditional ways and therefore it may have lost some of its squalene during the final stages before it was bottled.

According to Table II, Virgo Bio oil shows the highest recovery of squalene of 2.73E-07 g squalene / g of oil followed by Boulos 1.2E-07 g squalene per gram oil. Al Wadi Al Akhdar ranked third in a recovery of 5.45E-08 g squalene / g oil.

According to Table III, Al Wadi Al Akhdar oil shows the highest recovery of squalene of 3.62E-04 g squalene / g of oil followed by Virgo Bio 2.24E-04 g squalene per gram oil. Boulos ranked third in a recovery of 7.11E-05 g squalene / g oil.

According to Table IV, Al Wadi Al Akhdar oil shows the highest recovery of squalene of 6.1E-04 g squalene / g of oil followed by Boulos 5.37E-04 g squalene per gram oil. Boulos ranked third in a recovery of 2.84E-05 g squalene / g oil.

To recapitulate, the Soxhlet method for Al Wadi Al Akhdar sample showed the highest recovery between all extractions in all three methods. However, unexpectedly, the esterification of Al Wadi Al Akhdar showed the lowest recovery. In general, the esterification method yielded the lowest recovery results while the Soxhlet method proved to be the best. Therefore, it can be concluded that regardless of the brand or origin of the olive oil, the Soxhlet method is the most effective for extraction of squalene because the lowest recovery obtained in the Soxhlet are higher than the highest recovery in the other methods.

Comparing the result obtained with the one found in literature for non-Lebanese Olive Oil, we can find that the quantities extracted are in the same range. The highest quantity extracted from Elias, S.A. virgin olive oil samples by is around 6.5E-04 grams of squalene from 1 gram of oil [11], [12]. On another hand another paper showed the extraction of 5.2E-04 grams of squalene from 1 gram of oil from one of the samples of Picual virgin olive oil and 2.8E-04 grams of squalene from 1 gram of oil from Arbequina olive oil [13]. The Arbequina and Picual olive oil are two variety of Spanish oil. Comparing the best quantity extracted found in the literature and the highest quantity extracted in this study (6.1E-04 grams of squalene from 1 gram of oil), we can conclude that the quality of Lebanese virgin olive oil regarding squalene content is very satisfying and should be considered of great importance. It can confidently compete with other Mediterranean olive oil.

REFERENCES

[1] B. Raina, "Olives", *Encyclopedia of food sciences and nutrition*, 2, 2003 4260 - 4267.

[2] G. Reglero, G. Torello, & C. Torres. "Extraction and enzymatic modification of functional lipids from soybean oil deodorizer distillate," Retreived from *http://www.intechopen.com/books/recent-trends-for-enhancing-the-diversity-and-quality-of-soybean-products/extraction-and-enzymatic-modification-of-functional-lipids-from-soybean-oil-deodorizer-distillate*, 2011.

[3] C. Gutiérrez, A. Siles, F. Chica, & A. Martin. "Kinetics of biofuel generation from deodorizer distillates derived from the physical refining of olive oil and squalene recovery," *Biomass & Bioenergy*, 62, 2014, 93-99. Doi: 10.1016/j.biombioe.2014.01.010.

[4] A. Raminez-Torrres, C. Gabas, & C. Barranquero. "Squalene: Current knowledge and potential," New York: *Nova Biomedical Books*, 2011.

[5] M. Tsimidoum. "Olives and olive oil in health and disease prevention (V. R. Preedy, & R. R. Watson, Eds)," *Academic Press*, New York, 2010.

[6] B. Wetherbee, & P. Nichols. "Lipid composition of the liver oil of deep-sea sharks from the chatham rise," *Comparative Biochemistry & Physiology Part B*, New Zealand., *125*, 1999, 511-521.

[7] P. Kraujalis & P. Venskutonis. "Supercritical carbon dioxide extraction of squalene and tocopherols fromamaranth and assessment of extracts antioxidant activity," *The Journal of Supercritical Fluids, 80*, 2013, 79-85.

[8] P. Dugic, M. Jotanovic, Z. Lepojevic. "Supercritical fluid extraction with carbon dioxide at differential pressures," *Original Scientific Papers*, 2011, 84-87. Doi:10.5767/anurs.cmat.110201.en.084M.

[9] S. Gunawan, N. Kasim, & Y. Ju. "Separation and purification of squalene from soybean oil deodorizer distillate," *Separation and Purification Technology*, *60*, 2007, 128–135.

[10] N. Nenadis, & M. Tsimidou. "Determination of squalene in olive oil using fractional crystallization for sample preparation," *JAOCS*, *79* (3), 2002, 257-259.

[11] M. Tsimidou, G. Blekas, & D. Boskou. "Olive Oil," In *Encyclopedia of food sciences and nutrition.* Retrieved from http://www.sciencedirect.com/science/article/pii/B012227055X01347X?np=y, 2003.

[12] D. Grigoriadoua, A. Androulaki, E. Psomiadoub, & M. Tsimidou. "Solid phase extraction in the analysis of squalene and tocopherols in olive oil," *Food Chemistry*, *105*, 2006, 675–680.

[13] A. Fernández-Cuesta, L. León, L. Velascoa, & R. De la Rosa. "Changes in squalene and sterols associated with olive maturation," *Food Research International*, *54*, 2013, 1885–1889.