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# Squalene Extraction: Biological Sources and Extraction Methods.

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***Abstract*—***Squalene is a terpenoid with great importance in cosmetic, food and pharmaceutical industry; it was originally isolated from shark liver oil but is easily found in animals, vegetables and microorganisms. Nowadaysis shark fishing is prohibited in some countries, that is the main reason to use renewable sources forsqualene extraction to protect marine life, since last decade, squalene is extracted from different sources and methods to achieve best yields at lower possible cost. Traditional extraction methods usually involve organic solvents as hexane which left residues on the extracted matrix, that can limit material use for human consumptionafter extraction. Separation and purification stages after extraction can elevate operations cost, one of the most interesting technology to obtain squalene from biological matrix is supercritical fluid extraction with $CO_2$as solvent because of economic, safe and easy removal characteristics.*

***Keywords*—*Extraction, Renewable sources, Squalene, scale-up.***

## I. INTRODUCTION

Squalene is a very valuable compound common to found in vegetables and animal cells, because of its an intermediate on phytoesterolsand cholesterol biochemist-pathways and highly appreciated by its biological importance [1]. Squalene market is mainly divided un three industry sectors, cosmetics (69.2%), food (22.8%) and pharmaceutical (8%) (Fig. 1A) during 2014, squalene demand was about 267 000 ton that represents 102.4 billion dollars. Europe is the main squalene consumer followed by Asia Pacific and North America (Fig. 1B)[2]. Several investigations have been done to search new sources of squalene by different extraction methods to achieve greatest yield at lower possible cost. The aim of this work is to gather information about common and uncommon available animal, vegetal and microbial sources to extract squalene and methods or techniques to extract it as well and scaling-up experiments.

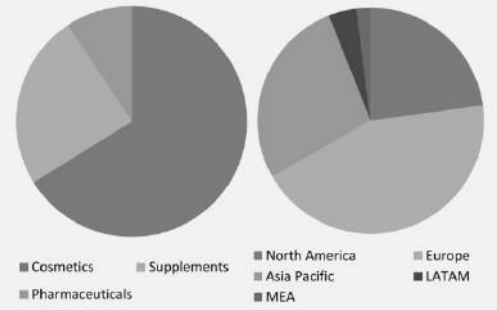


*Fig.1:* Market by industrial sector (A) and geographical area (B)[2].

## II. SQUALENE

In 1916 by MatsumaruTsujimoto[3], identified a highly unsaturated hydrocarbon was identified from liver oils of the squaloid sharks, he proposes the name 'Squalene'. Squalene, is anhydrocarbonated chain ($C_{30}H_{50}$), a triterpene containing six unsaturated bonds with antioxidant nature[4]. Squalene has applications in various end-user industries such as cosmetic, food supplements, pharmaceuticals, and in other applications like high grade lubrication and fiber coating additives, however,the major data of commercial is referred to Shark Liver Oil (SLO). In USA SLO was used for vitamin A production but now is highly recommended in alternative medicine and ointment[5].In Europe, the cosmetic industry demanded SLO, as mentioned before, since product as lotions, eyeliner, eyeshadows, eye makeup remover and perfumes contains 0.1-10%squalene and foundation, lipsticks and other faces preparations contains up to 50% squalene[6],pharmaceutical, textile and leather industry also demands squalene. In Africa SLO is mainly use on fishing boats maintenance[7].

### *2.1 Squalene importance*

Squalene is a molecule with a long carbon chain it tends to have hydrophobic properties that is of particular interest in industry because it can be used to transport liposoluble compounds in an effective and economic ways.[8] Squalene participates in the formation of steroid hormones, bile acids, steroids, and sterols synthesized though mevalonic acid pathway [9].

Human epidermal sebum is composed by triacylglycerides, free fatty acids (57%), wax esters (26%)

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and squalene (12%), the use of compounds present in human sebum as squalene in cosmetics reduces the possibility of allergies [10] and is highly appreciated in cosmetic industry due its emollient and antioxidant properties [1]. Squalene prevents $H_2O_2$ induced oxidative injury and protect against oxidative DNA damage [11]. Alcohol produces lipid peroxidation although, squalene reduces fetus retina during pregnancy [12]Squalene reduces serum cholesterol due this triterpenoid may act as a substrate for HMG-CoA reductase (3-hydroxy-3-methylglutaryl Co-A) [13]. Squalene has been studied along the years and has been reported with biological activity as antioxidant [11,14,15]chemopreventive[16]. The use of squalene in combination with antitumor drugs has been shown to decrease cancer cells growth [17,18].

2.2 *Squalene biosynthesis*

Squalene is found in both mammals and vegetable tissues because is an intermediary in cholesterol and sterols pathway, very important to any organism. Squalene biosynthesis initiates with enzyme thiolase that joins 2 units of Acetyl-CoA to form Acetoacetyl-CoA and by addition of another Acetyl-CoA by HMG-synthase, β-Hydroxy-β-Methylglutaryl-CoA (HMG-CoA) is formed, and by HMG-CoA reductase take place Mevalonate; then Mevalonate-5-phosphotranferse and phosphomevalonate kinase, 2 phosphates from Adenosine Triphosphate (ATP) are added and changes into Dimethylallyl pyrophosphate, Next phenyl-transferase made 2 head-to-tail unions and 3 isoprene units named as Farnesyl pyrophosphate that polymerizes by squalene synthase form squalene realizing inorganic Pyrophosphate (PPi) [19–21]. Those reactions can be observed in Fig. 2

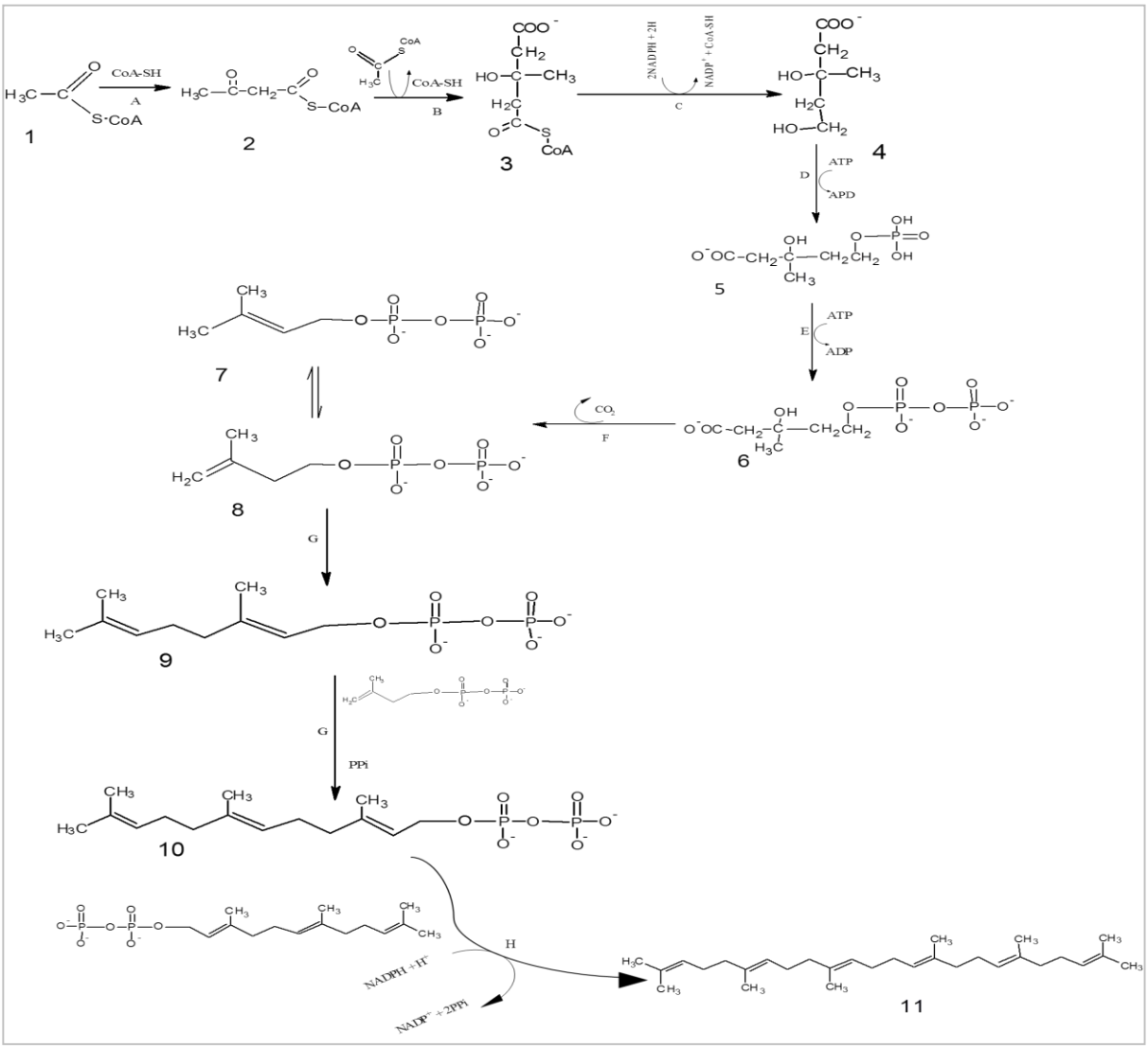


*Fig. 2: Squalene synthesis pathway, adapted from reference [22]*

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Intermediates and enzymes names, involved in squalene biosynthesis are listed in Table 1.

*Table 1: intermediates and enzymes name involved in squalene biosynthesis*

| Intermediate | | Enzymes | |
|---|---|---|---|
| 1 | Acetyl-CoA | A | Thiolase |
| 2 | Acetoacetyl-CoA | B | HMG-CoA synthase |
| 3 | β-hydroxi-β-Methylglutaryl-CoA | C | HMG-CoA reductase |
| 4 | Mevalonate | D | Mevalonate 5-phoosphotransferase |
| 5 | 5-Phosphomevalonate | E | phosphomevalonate kinase |
| 6 | 5-Pyrophosphomevalonate | F | Pyrophosphate mevalonate descarboxylase |
| 7 | Isopentenyl pyrophosphate | G | Prenyl transferase |
| 8 | Dimethyally pyrophosphate | H | Squalene sintase |
| 9 | Geranyl pyrophosphate | | |
| 10 | Farnesyl pyrophosphate | | |
| 11 | Squalene | | |

## III. SQUALENE SOURCES

### *3.1 Shark Liver Oil*

The richest source of squalene is abyssal shark livers even though shallow sharks' livers had lower squalene content than cod livers. New Zealander sharks livers contains about 50% by weight squalene[23]. Past decades studies were focused about shark livers and its squalene content. Some of these species are listed in Table 2.

*Table 2. Squalene content in different shark liver oil*

| Shark specie | Squalene liver content (%) | Reference |
|---|---|---|
| *Centroscymnuscrepidater* | 35.7-59.4 | 24 |
| *Centroscymnusowstoni* | 37.1-53.1 | |
| *Centroscymnuscoelolepis* | 31.1-47.1 | |
| *Deaniacalcea* | 43.4-66.1 | |
| *Etmopterusbaxteri* | 14.3-51.5 | |
| *Etmopterus sp. nov.* | 20.8 | |
| *Dalatiaslicha* | 43.4 | |
| *Centrophorussquamosus* | <0.01 | |
| *Centroscymnusplunketi* | 0.9* | 25 |
| *Etmopterusgranulosus* | 50.3-60.5* | |
| *Deaniacalcea* | 69.6* | |
| *Centroscymnuscrepidater* | 73* | |
| New Zelander shark | 50-55* | 26 |
| *Centrophorussquamosus* | 65.5 | 27 |
| Cuban sharks | 0.03 | 28 |

*Expressed as Hydrocarbon (predominantly squalene)

Cuban sharks, squalene determination was performed from a liver mixture of three species *Ginglymostomacirratum*, *Carcharhinuslongimanus*andCarcharhinus*falciformis*

Nowadays trade volumes of fishing sharks are close to exceed sustainable levels[29]. Onwards it become necessary to extract squalene fromrenewable sources[30].

### *3.2 Vegetable Sources*

Squalene is present in all vegetable oils but in small amounts[31]. Olive is a well know squalene source and its content depends of it is associated with fruit maturation as autumn begins reach the higher concentration of squalene and the end of season there are no significant changes[32]. Nowadays olive oil become one of most vegetable squalene source commercially exploited, but its content is not enough to satisfy the demand[33]. Deodorized olive oil contains about 28% squalene[34]. Olive pomace which has been considered like a by-product in the olive oil production has residual (0.0023%) amount of squalene [35]. In other hand olive leaves that were found containing 0.0038-0.0152% squalene in hexane extracts[36]. Other products as palm oil contains only 1.8-2.3 % of squalene however, it is produced in huge mounts and so it can be use as squalene source [37]

Recently some other crops have been tested as possible new source of squalene. Cucurbit seeds squalene content reported is 10.97-40.27%, differences are due to variety of cucurbit, although is suggested that cucurbit seeds can have hypocholesterolemic effect on human diet [38].

Tobacco plant that, contains approximately 2% but; like it continues growing it accumulates up to 20 % in 8 years [39].

Residues from wineryindustry (lees)may be also valorized trough squalene recovery, yield achieved was 0.06± 0.008%, although seasonal production of raw material, labor requirement may limits its potential as a squalene source[40].

In Asia, ginseng is important because not need to grow in warm weather, and seeds oil content between 514-569 mg/100g squalene and represents about 60% of unsaponificable matter. [41] Nuts are an excellent source of vegetable oil; but some of them such as brazil, pecan, pine, pistachio and cashew nuts have a great squalene content due this nuts should be added to dairy diet. [42]

Essential oil obtained by hydrodistillation of the *Strychnosspinosa* leaves contains about 0.5% of squalene in oil fraction. [43] Using deodorized soy oil can be extracted 100% squalene content and up to 93% purity by solvent modified extraction[44]Rice bran is a co-product of milled rice and its contains approximately 20% and about 8.5% of squalene[45]. Bee pollen of lotus (*Nelumbonucifera*Gaertn) content 0.0084% of squalene extracted by supercritical fluid extraction[46]. Some

authors[30] have explored unconventional squalene sources as pumpkin, amaranth seeds, borageand walnut reported0.52, 5.22, 0.022, 2.83,% squalene in oil respectively.

In contrast there are crops that have been underestimated with industrial purposebut in some communities is common to cultivate as cultural heritage and it consumption is local, as amaranth, a pre-Columbian crop that contains relatively high amounts of squalene[47]. *Amaranthuscruentus*, oil extracts reports squalene content 6.95, 5.0 and 8%[30,48,49], respectively.

Five varieties of *A. cruentus* were cultivated at different altitudes and reported different squalene content ranged from 2.26 to 5.94% of the oil and statistical analysis showed significant difference for localities but not for varieties of plant so it is suggested that environmental conditions, such as temperature and water availability, may lead to a greater accumulation of squalene in the grain. [50]

*Table 3: Squalene vegetable sources*

| Oil source | Squalenecontented in oil (%) | Reference |
|---|---|---|
| Olive oil deodorized | 28 | 34 |
| Cucurbit seeds | 10.97-40.27 | 38 |
| Olive pomace | 0.0023 | 35 |
| Olive leaves | 0.0038-0.0152 | 36 |
| Tobacco plant | 2.00-20.00 | 39 |
| Wine lees | 0.06 ± 0.008 | 40 |
| Gingseng seed | 0.51-0.56 | 41 |
| Brazilian nut | 137.78 | |
| Pecan nut | 15.7 | |
| Pistachio | 9.14 | 42 |
| Cashews | 8.94 | |
| Pine seed | 3.95 | |
| *Strychnos spinose* | 0.5 | 43 |
| Deodorized Soy oil | 1.83 | 44 |
| Rice bran | 11.75 | 45 |
| *Nelumbonucifera*Gaertn | 0.0084 | 46 |
| Palm oil | 1.8-2.3 | 37 |
| Olive oil | 0.5-0.65 | 33 |
| *Camellia olifeira* | 7.62 | 52 |
| Pumpkin seeds | 0.52 | |
| Amaranth seeds | 5.22 | 30 |
| Borage | 0.022 | |
| Walnut | 2.83 | |
| *Amranthuscruentus* | 5-8 | 30,48,49 |
| *Amaranthushybridus* | 5.27-7.21* | 51 |

*Expressed as unsaponifiable matter

*Amaranthushybridus* is reported to have between 5.27± 0.47–7.21±0.57% of unsaponifiable matter and can be assumed that it should content squalene[51]. *Table 3* summarizes squalene content in vegetables sources previously mentioned.Some of these renewable sources are not widely harvested or used industrially even when its squalene content is important, and others are wildly produced and made them better alternatives than shark livers.

### *3.3 Microorganism sources*

Microorganism are an interesting squalene source since not need to be harvested in huge portions of land. Microalgae (*Schizochytriummangrovei*) represents a viable alternative source of squalene reach 33 mg/g of cell dry weight, even when biomass is a residue from biodiesel production[53].

A novel yeast strain classified in *Pseudozyma* genus,isolated from seawater is also an interesting squalene source produces 340.52 mg squalene/L with 40 g/Lof glucose and sodium nitrogen as nitrogen source[54].

The strain *Schizochytrium* sp. CCTCC M209059 reports similar squalene content as in fish oil. Due its fast growing and productivity is an alternative source to obtain squalene. High aeration is recommended to increase squalene synthesis, same authors determined squalene keeps oil stable[55].

Wild-type *Saccharomyces cerevisiae* can accumulate between 0.62 mg/L of squalene during the stationary growth phase and 3.4 mg/L of squalene until the exponential growth but an engineered strain (named FOH-2) can accumulate more than wild-type strain, since squalene biosynthesis mechanism is overexpressed[56].

### 3.4 Squalene Localization

Squalene(and other polyisoprenes)has the function of inhibit proton leakage through cell membrane, but its localization in cell membrane, was no clear until neutron diffraction experiments were performed[57]. Cell membrane is composed by hydrophobic/hydrophilic lipid bilayer, where squalane, a structural analogue of squalene with same number of carbon atoms ($C_{30}H_{62}$) as squalene,was found to be located between membrane monolayers (*Fig. 3*). Due squalane is a saturated compound may haveless stability between the bilayer than unsaturated molecule as squalene[57].According to this,during squalene extraction, saturated and unsaturated fatty acids could also be extracted.

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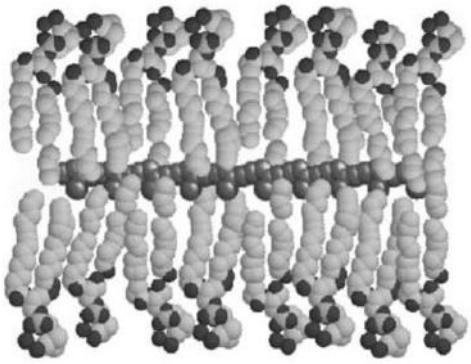

*Fig. 3: Schematic representation of squalane in the middle the bilayer of the cell membrane*[57]

Evidence of squalene enzymes obtained by immunofluorescence microscopy, suggested that squalene is synthesized in the smooth endoplasmic reticulum subsequently accumulated in small vesicles, some this material is incorporated to plasma membrane[58].Some squalene vegetables sources as amaranth seeds (*Amaranthushypochondriacus*), lipids fraction have been identified in embryonic cells (*Fig 4*), surrounding protein bodies (Pb) and cell nucleus (N). A considerable lipid fraction identified with selective colorants as Sudan Black B[59]. is possible to content squalene lipid bodies

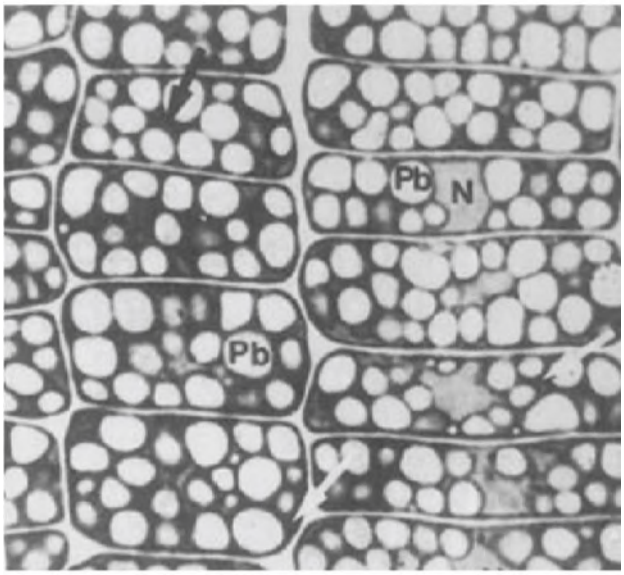


*Fig. 4 Light micrograph of amaranth cells peripheric embryo of cytoplasm surrounding the protein bodies is full of lipids (arrows) which stains dark with Sudan Black B*[59]

Identifying lipids reserves in vegetables matrix, which probably contains squalene are important to select suitable methods to extract squalene.

## IV. EXTRACTION METHODS

Many techniques can be used to recover lipids from biological matrix, and obtain specific compounds[60].Soxhlet extraction (organic solvent extraction) is the most common method used as standard and extract is considered to be 100 % of extractable matter[61].Hexane is typically the solvent used for large scale extractions due to its relatively low cost and high extraction efficiency[62].

Lipid extraction usually involves organic solvents, at industrial scale is commonly used cold press to avoid thermolabile compounds degradation, since this methods are at low pressures, yield might be low, so development of new techniques at higher pressures may aid to increase yield and process time[63]. Ultrasonic extraction combined with organic extraction can achieve higher yield[64].

Cold press with new mechanisms that replaces hammer crusher achieved 90.1% extraction and oil reported till 65g/kg of squalene[33]. Cold press, organic solvent and Supercritical fluids extraction, were tested in order to compare its yields and the conclusion was supercritical fluids extraction reached the highest yield and purity[31].

Other separation method, is silver ion complexation based on the complexation reaction between $Ag^+$ and unsaturated carbon double bonds, it was tested on Camellia oil obtained from seeds of *C. oleifera*, optimal condition was 70% methanol (v/v),0.6 mol/L AgNO3, for12 h, at 0 °C. Purity of squalene extract reach 37.8%. advantages of this method are low cost, recycle of reagents and continuous operation[65],an disadvantage of this method, is saponification and esterified before extraction and chemical reagents removed from extract after extraction.

Supercritical fluid extraction (SCFE) can be used to extract polar compounds. Supercritical fluids have diffusivity as gas so can penetrate solid materials, high density and solvation power as liquids, these fluids are compressible and little pressure changes its properties. SCFE have been studied due its advantages against conventional extraction and extract have better quality, biostability and easy to remove from extracted matrix[66]. $CO_2$ is used to extract oil due its convenience characteristics as non-toxic, non-flammable, easy to remove and economic solvent and also reduces thermolabiledegradation in extracted compounds[48]. Squalene SCFE have been performed by several researchers even when is considered as an expensive technology and achieved extract with high purity. Amaranth seeds have been mainly tested by squalene SCE, some conditions are the next: 35MPa and yield was 0.305% and by adding a co-solvent is possible obtain more squalene[67]. In other work, $CO_2$ were used at 50°C and 300 bar reach 7.95% in oil[61] other optimal conditions reported to extract squalene were 30MPa and 40°C by 90-120 min in order to allow highest and faster oil and squalene extraction from *Amaranthuscruentus*. [48] At

higher temperature (100°C) best yield is reported to be at 55 MPa and 1.5 h extraction time from *Amaranthuspaniculatus*.

## V. SCALED-UP EXPERIMENTS

At bench scale,embryonic tissue (as bran from amaranth) was separated from hole seed but amaranth bran was fine thin, therefore pellets were obtained by extrusion to be extracted. Large amounts of pellets (15 Kg) was immersed in hexane for 10 min, solvent was removed at vacuum at 65°C and then filtered[68] oil recovery reach up to 97.7%.

Scale up studies allows to establish methodology translate SCFE process from laboratory-scale to industrial scale, this behavior is not always approached or predicted, this is the mainly reason to observe differences at studies to avoid serious under or over estimates[69].Solubility of volatile solutes increases with temperature due an effect in their vapour pressure, this effect is pronounced multicomponent systems than binary systems. Pressure, temperature and solvent density had an effect on the extraction yield, due to the "enhanced solubility effect".SCFE Laboratory-scale units have a bed length/diameter of vessel ratio relatively high with those greater capacity units, these may affect overall lipid yield, reduced superficial velocity by increasing retention time consequently solvent is saturated [61]. Maintaining optimum condition extraction, solvent flow and biomass ratio not affect significantly the process efficiency even at 8 fold scaled-up[70].

Scaling-up SCFE process depends on extraction efficiency, a model capable to predict the extraction process and time operation which also depends of extracted matrix, batch size, retention time, time for load and unload extraction matrix and cycle of pressuring and depressing extractor to calculate extraction time cycle.[71]

## VI. CONCLUSION

Squalene is a natural antioxidant very valuable in cosmetic, food and pharmaceutical industries, squaleneis also an important intermediate in animal and vegetables cells pathways, accordant to this there are several alternative sources for squalene extraction, many investigations have focused on obtaining best yield as possible, some of the most profitable squalene sources, would be by-products from industrial processes, since squalene is mostly used in human products for human consumption, is important to consider safe extraction methods as supercritical fluid extraction. Scaling-up experiments are important to estimate extraction yield and extract cost.The best source of squalene extraction will depend on the bioproduct and the available technology.

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