



Extraction and Purification of Squalene from Virgin Olive Oil via Catalytic Transesterification and Molecular Distillation

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Squalene, a highly valuable compound abundant in various natural sources, shows great potential in pharmaceutical, cosmetic, and nutraceutical applications. This research article outlines a comprehensive methodology for extracting and purifying squalene from virgin olive oil, a rich source of the compound. The extraction process begins with degumming, which involves heating the olive oil to 60-70°C to reduce viscosity, followed by the addition of 2-3% warm water to hydrate

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phospholipids. Food-grade phosphoric acid is then added to react with the phospholipids, forming precipitates. The mixture is stirred for 20-30 minutes and allowed to rest for an additional 20-30 minutes, enabling impurities to settle. The upper layer of degummed oil is separated via decantation or centrifugation and washed with warm water for pH adjustment. Next, transesterification is performed by mixing 100 ml of virgin olive oil with 25% methanol (w/w) and a catalyst (0.5% sodium methoxide or PTSA), and heating the mixture to 80-90°C under reflux for 1-2 hours. Following transesterification, the solvent and acetone are distilled out, and acetone precipitation is repeated 2-3 times to remove unsaponified matter, which is then filtered and evaporated. The concentrated oil undergoes molecular distillation at 180°C and 0.0033 bar pressure for 1 hour, yielding the distillate and residue for further analysis. Qualitative analysis using Thin Layer Chromatography (TLC) involves Merck TLC plates with silica gel 60 F254 and a hexane: chloroform (9:1) mobile phase. Spots are developed with a 10% HCl solution, confirming the presence of squalene with an RF value of 0.93. Quantitative analysis via High-Performance Thin-Layer Chromatography (HPTLC) employs Merck TLC plates with cyclohexane as the mobile phase and CAMAG at 254 nm and 366 nm wavelengths, revealing a squalene purity of 67% and a recovery rate of 69.8%. The initial purification through transesterification facilitated the conversion of ester groups, yielding squalene-rich fractions, while acetone precipitation effectively removed saponified matter. Molecular distillation further enhanced squalene purity. TLC analysis confirmed the qualitative presence of squalene, and HPTLC provided precise quantitative measurements. The obtained squalene purity of 67% significantly enriches the initial content in virgin olive oil, though further optimization could enhance purity and yield. Complementary techniques like GC-MS or HPLC could validate the purification process. This study presents an efficient, replicable procedure for extracting and purifying squalene from virgin olive oil, with significant implications for pharmaceutical, cosmetic, and nutraceutical industries. The findings support a sustainable and ethical shift towards vegetable-derived squalene, meeting market demands while ensuring high-quality production.

Keywords: *Squalene; virgin olive oil; transesterification; molecular distillation; TLC; HPTLC; purification.*

1. INTRODUCTION

Virgin olive oil, renowned for its health benefits and culinary significance, harbours a notable content of squalene, ranging from 1.2 mg to 2.5 mg per gram of oil. Squalene, a triterpene compound, holds considerable promise across various industries due to its diverse applications and beneficial properties [1].

In pharmaceuticals, squalene serves as a crucial ingredient in vaccine adjuvants, enhancing the body's immune response to antigens. Additionally, squalene finds extensive utilization in the cosmetic industry, where its emollient and moisturizing properties make it a sought-after component in skincare formulations. Moreover, the nutraceutical sector recognizes squalene for its potential in dietary supplements, attributed to its antioxidant properties and purported health benefits [2].

In recent years, the importance of squalene in vaccine production has garnered significant attention. Vaccine adjuvants, such as squalene, play a pivotal role in vaccine efficacy by stimulating a robust immune response [3].

Traditionally, squalene derived from shark liver oil has been utilized in vaccine formulations [4]. However, concerns regarding environmental sustainability and ethical considerations have led to a shift towards vegetable-derived squalene.

The vaccine industry's adoption of vegetable squalene over shark liver squalene is driven by several factors. Firstly, sourcing squalene from vegetable oils, such as virgin olive oil, aligns with sustainable practices and reduces reliance on marine resources vulnerable to overexploitation [5]. Furthermore, vegetable squalene offers a purer and more consistent composition compared to its shark-derived counterpart, mitigating potential variability in vaccine formulations. Additionally, vegetable squalene avoids the ethical dilemmas associated with harvesting squalene from shark populations, contributing to the industry's commitment to ethical sourcing and environmental stewardship [6].

Squalene, characterized by the molecular formula C₃₀H₅₀, plays a pivotal role as an intermediate in the synthesis of phytosterols or cholesterol in both plant and animal organisms,

exhibiting widespread distribution across the biological spectrum [7]. Recent research suggests that squalene may have been crucial for early life forms on Earth, as microorganisms and, later, higher organisms during the Precambrian era contained significant amounts of squalene in their cellular membranes, possibly aiding their survival in oxygen-deficient environments [8].

Interestingly, it wasn't until the latter half of the 20th century that scientists uncovered the presence of squalene in trace amounts within the human body. New-borns demonstrate the highest concentrations of squalene in their bloodstream, with levels gradually diminishing after reaching adulthood, notably between the ages of 30 and 40. In humans, the liver primarily synthesizes squalene, which is then secreted in notable quantities by the sebaceous glands. Notably, squalene accounts for about 12% of the lipids produced by these glands and does not undergo conversion into cholesterol [9]. Skin lipids exhibit the highest concentrations of squalene in humans, averaging approximately 500 µg/g, followed by adipose tissue with concentrations around 300 µg/g. Organs where active biosynthesis occurs, such as the liver and small intestine, generally exhibit lower squalene concentrations, typically ranging from 42 µg/g to 75 µg/g [10].

The credit for discovering squalene goes to Dr. Mitsumaru Tsujimoto, a Japanese researcher specializing in oils and fats at the Tokyo Industrial Testing Station. In 1906, Dr. Tsujimoto isolated the unsaponifiable fraction from shark liver oil, known as "kuroko-zame," leading to the identification of a highly unsaturated hydrocarbon [11]. A decade later, Dr. Tsujimoto successfully isolated an unsaturated hydrocarbon with the chemical formula C₃₀H₅₀ through fractional vacuum distillation of liver oil from deep-sea shark species, naming it "squalene" after the family name of sharks, Squalidae [11].

Subsequent studies have confirmed Dr. Tsujimoto's proposed chemical formula for squalene, providing further insights into its chemical structure and elucidating its chemical and physical properties [11]. These findings have contributed significantly to our understanding of squalene's roles and potential applications across various fields, including pharmaceuticals and cosmetics.

Squalene has been detected in various plant oils, displaying different concentrations. Olive oil was

the first vegetable oil where squalene was identified. Recent research by Frega et al. indicated squalene concentrations in olive oil at 564 mg/100 g, soybean oil at 9.9 mg/100 g, grape seed oil at 14.1 mg/100 g, hazelnut oil at 27.9 mg/100 g, peanut oil at 27.4 mg/100 g, and corn oil at 27.4 mg/100 g [12]. In Europe, commonly produced vegetable oils exhibit squalene concentration ranges of 0–0.19 g/kg in sunflower oil, 0.03–0.2 g/kg in soybean oil, 0.1–0.17 g/kg in corn oil, and 1.7–4.6 g/kg in olive oil. Among pseudograins, *Amaranthus* sp., introduced more recently in Europe, is recognized for having the highest squalene concentration in the plant kingdom, at 4.16 g squalene/kg seed. An extensive study encompassing 104 genotypes of 30 *Amaranthus* species revealed squalene concentrations ranging from 10.4 to 73.0 g/kg in amaranth oil [13].

1.1 Squalene Biosynthesis

Squalene, present in both mammalian and plant tissues, serves as a crucial intermediary in the synthesis pathways of cholesterol and sterols, essential for the functioning of organisms. The biosynthesis of squalene commences with the enzyme thiolase catalyzing the fusion of two Acetyl-CoA molecules to yield Acetoacetyl-CoA. Subsequently, HMG-synthase facilitates the addition of another Acetyl-CoA molecule, leading to the formation of β-Hydroxy-β-Methylglutaryl-CoA (HMG-CoA) [14]. Through the action of HMG-CoA reductase, Mevalonate is generated. Mevalonate-5-phosphotransferase and phosphomevalonate kinase then facilitate the addition of two phosphates from Adenosine Triphosphate (ATP), resulting in the conversion to Dimethylallyl pyrophosphate. Following this, phenyl-transferase mediates the formation of two head-to-tail unions and three isoprene units, yielding Farnesyl pyrophosphate. Finally, squalene synthase polymerizes Farnesyl pyrophosphate to produce squalene, liberating inorganic Pyrophosphate (PPi) in the process [14].

1.2 Different Methods of Extraction Squalene

Various methods are employed to extract lipids from biological materials and isolate specific compounds. Soxhlet extraction, also known as organic solvent extraction, is widely utilized as the standard method, ensuring complete extraction of target substances. Hexane is commonly employed as the solvent for large-

scale extractions due to its cost-effectiveness and high efficiency [15]. Organic solvents are typically involved in lipid extraction, with cold press techniques often preferred on an industrial scale to preserve thermolabile compounds. However, these methods may yield lower quantities, prompting the exploration of new high-pressure techniques to enhance yield and efficiency. Ultrasonic extraction in combination with organic solvents has shown promise in achieving higher yields [15].

Cold press methods, incorporating innovative mechanisms to replace traditional hammer crushers, have achieved significant extraction rates, with reported squalene yields of up to 65g/kg. Comparisons between cold press, organic solvent, and supercritical fluid extractions have demonstrated the superiority of supercritical fluid extraction in terms of both yield and purity. Another separation method involves silver ion complexation, which has been applied to Camellia oil extraction, resulting in squalene extracts with a purity of 37.8% [16]. While this method offers advantages such as cost-effectiveness and reagent recycling, it necessitates pre-treatment steps and the removal of chemical residues post-extraction [16].

Supercritical fluid extraction (SCFE) presents an alternative approach for extracting polar compounds. SCFE utilizes fluids with gas-like diffusivity and liquid-like solvation power, making them capable of penetrating solid materials. CO₂ is commonly employed as a solvent in SCFE due to its non-toxic, non-flammable nature and ease of removal [17]. SCFE has been particularly effective in extracting squalene, yielding high-purity extracts despite being considered an expensive technology. Amaranth seeds have been a focus of squalene SCFE studies, with varying conditions such as pressure, temperature, and extraction time influencing yield and efficiency. Optimal conditions for squalene extraction from *Amaranthus* species include pressures ranging from 30 to 55 MPa and temperatures between 40°C and 100°C, highlighting the versatility and efficacy of SCFE methods [18].

In light of these considerations, the vaccine industry stands to benefit significantly from transitioning towards vegetable-derived squalene, leveraging the abundant and sustainable sources available. By harnessing the potential of squalene from virgin olive oil and other vegetable sources, the vaccine industry

can uphold both efficacy and ethical standards in vaccine development, ensuring a sustainable and equitable approach to global healthcare.

2. MATERIALS AND METHODS

Olive oil: Virgin grade olive oil is purchased from the market

Degumming:

Heating: Heat the olive oil to a temperature between 60°C to 70°C (140°F to 158°F). This temperature range helps in lowering the viscosity of the oil and facilitating the degumming process.[19]

Mixing with Water: Add a predetermined amount of warm water (typically around 2-3% of the oil weight) to the heated olive oil. The water helps in hydrating the phospholipids present in the oil, allowing them to be separated more easily.[19]

Addition of Phosphoric Acid: Slowly add food-grade phosphoric acid to the oil-water mixture while stirring continuously. The phosphoric acid reacts with the phospholipids, forming precipitates that can be separated from the oil.

Mixing and Resting: Continue stirring the mixture for around 20 to 30 minutes to ensure thorough mixing and reaction. After this, allow the mixture to rest for an additional 20 to 30 minutes. During this resting period, the phospholipids and other impurities will settle at the bottom of the container.

Separation: After the resting period, the mixture will separate into distinct layers. The upper layer consists of degummed olive oil, while the lower layer contains the precipitated impurities.

Decantation or Centrifugation: Carefully decant or separate the upper layer of degummed olive oil from the lower layer of impurities. Alternatively, the mixture can be centrifuged to accelerate the separation process.

Washing and PH adjustment: The degummed olive oil can be washed with warm water to remove any residual impurities and PH is adjusted

Transesterification: 100 ml of virgin olive oil was taken. Methanol (25% w/w) was added as a solvent. Sodium methoxide or PTSA (0.5%) was added as a catalyst. The solution was stirred continuously. The mixture was heated to 80-90 degrees Celsius under reflux for 1-2 hours. [20, 21, 22]

Acetone Precipitation: After transesterification, the solvent and acetone were distilled out. Acetone precipitation was repeated two to three times until unsaponified matter was left. The unsaponified matter was filtered out, and acetone was evaporated completely [23].

Molecular Distillation: The concentrated oil obtained after acetone evaporation was subjected to molecular distillation. Molecular distillation was conducted at 180 degrees Celsius and 0.0033 bar pressure for 1 hour or until the process completed. The distillate and residue were collected for further analysis [24].

Qualitative analysis by Thin layer chromatography: After molecular distillation, the obtained squalene underwent qualitative analysis via Thin Layer Chromatography (TLC) using 6x10 cm Merck TLC plates coated with Al silica gel 60 F254 as the stationary phase. The mobile phase consisted of hexane: chloroform (9:1). Standard squalene and extracted squalene samples were spotted on the TLC plates and allowed to run for a distance of 9 cm. Subsequently, the TLC plates were dried and sprayed with a 10% hydrochloric acid (HCl) solution. [25]

Quantitative Analysis of Squalene: HPTLC analysis was performed using Merck TLC plates silica gel 60 F254 (100x100 mm) with a mobile phase of cyclohexane. The process is performed using CAMAG. The saturation time was set to 20 minutes, and wavelengths of 254 nm and 366 nm were utilized [26]. The squalene purity was determined using a standard purchased from DKSH (100%).

3. RESULTS

The extraction and purification process yielded promising results in the isolation of squalene from virgin olive oil. Initial transesterification of the oil with methanol and catalysts facilitated the conversion of ester groups, resulting in the formation of squalene-rich fractions. Acetone precipitation effectively removed saponified matter, leaving behind unsaponified components, including squalene. Subsequent molecular distillation further refined the squalene-rich fraction, enhancing its purity.

Thin layer chromatography was conducted utilizing hexane: chloroform (9:1) as the mobile phase. Following the migration of the solvent phase for a distance of 9 cm, the TLC plate was dried and subjected to spraying with a 10% HCl solution. Gentle heating was applied, resulting in

the appearance of pink-coloured spots, indicative of the presence of terpene squalene. Comparative analysis with the DKSH standard confirmed the RF (retention factor) value of 0.93, thereby affirming the qualitative assessment of the extracted squalene

Quantitative analysis of squalene purity using High-Performance Thin-Layer Chromatography (HPTLC) revealed a purity level of 67% in the obtained distillate. The recovery rate of squalene was determined to be 69.8%, indicating efficient extraction and purification processes. These results demonstrate the feasibility of isolating squalene from virgin olive oil using the proposed methodology.

Amount of sample applied on plate = 9.08 µg

Amount of squalene detected on sample plate = 6.084 µg

% of Squalene sample detected on HPTLC method = $(6.084/9.08) \times 100 = 67\%$

4. DISCUSSION

The obtained squalene purity of 67% represents a significant enrichment compared to the initial squalene content in virgin olive oil, underscoring the efficacy of the extraction and purification procedures employed. This substantial increase in purity highlights the effectiveness of the combined processes of degumming, transesterification, acetone precipitation, and molecular distillation. However, for pharmaceutical and cosmetic formulations where higher purity levels are often necessary, further optimization of the purification process may be warranted. Enhancing the purity to meet specific application requirements could involve refining various parameters within the existing methodology, such as adjusting reaction times, temperatures, and catalyst concentrations during transesterification and distillation stages.

The recovery rate of 69.8% indicates a satisfactory yield of squalene from the virgin olive oil feedstock. This efficiency suggests that the method is effective in isolating a substantial portion of the squalene available in the initial material. Nevertheless, there is always room for improvement, particularly in terms of yield efficiency and sustainability. Reducing energy consumption and minimizing environmental impact are critical considerations in refining the process [15,16,17]. This could involve optimizing reaction conditions to reduce the need for high temperatures and prolonged reaction times, thus making the process more energy-efficient.

Additionally, exploring alternative, less harmful catalysts or solvents could further enhance the environmental sustainability of the process.

The utilization of High-Performance Thin-Layer Chromatography (HPTLC) for quantitative analysis provided valuable insights into the squalene content and purity of the obtained distillate [26]. HPTLC's effectiveness in measuring the purity levels underscores its utility in monitoring and validating the extraction and purification process. However, to ensure the robustness and reliability of the results, complementary analytical techniques should be considered. Gas chromatography-mass spectrometry (GC-MS) [27] and high-performance liquid chromatography (HPLC) [28] are two such techniques that can offer additional characterization and confirmation of squalene purity. These methods provide higher sensitivity and specificity, potentially uncovering impurities that HPTLC might miss and thus offering a more comprehensive assessment of the purification process's efficacy.

The transition towards vegetable-derived squalene, as exemplified by the extraction from virgin olive oil, reflects a broader trend towards sustainable and ethical practices in the pharmaceutical and cosmetic industries [1,2,29]. Amaranthus-derived squalene, obtained through solvent extraction and subsequent purification processes, is another promising source, offering high yields and purity levels [30]. Additionally, methods such as supercritical CO₂ extraction [31,32] and ultrasound-assisted extraction have been explored for squalene extraction from Amaranthus, providing efficient and environmentally friendly alternative. By

prioritizing environmentally friendly sourcing and production methods, stakeholders can uphold ethical standards while meeting market demand for high-quality ingredients. This shift not only reduces the reliance on animal-derived squalene, primarily sourced from shark liver, but also aligns with consumer preferences for sustainable and cruelty-free products. Vegetable-derived squalene offers a viable alternative that does not compromise product quality or efficacy, thus supporting the industry's move towards more sustainable and ethical practices. Plant-derived squalene supplementation enhances growth performance and helps mitigate the adverse effects of acute oxidative stress on growth. This natural compound supports overall health and resilience in organisms facing oxidative challenges [33,34,35]

The extraction and purification of squalene from virgin olive oil, achieving a purity of 67% and a recovery rate of 69.8%, demonstrates a promising approach for obtaining a highly valuable compound with diverse applications. While the current method is effective, further optimization is recommended to enhance purity and yield, ensuring the process meets the stringent requirements of pharmaceutical and cosmetic industries. Complementary analytical techniques such as GC-MS and HPLC should be utilized to validate the findings and ensure the robustness of the purification process. The broader adoption of vegetable-derived squalene aligns with the industry's shift towards sustainable and ethical practices, offering a viable alternative to animal-derived sources without compromising on quality.

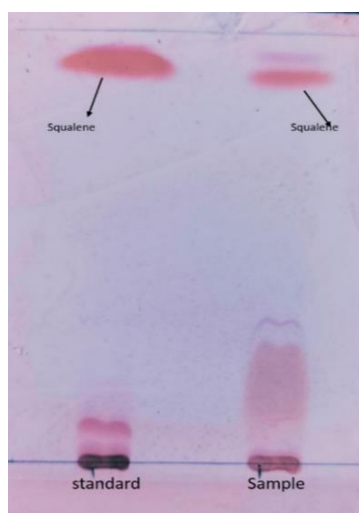


Fig. 1. TLC squalene Rf 0.93

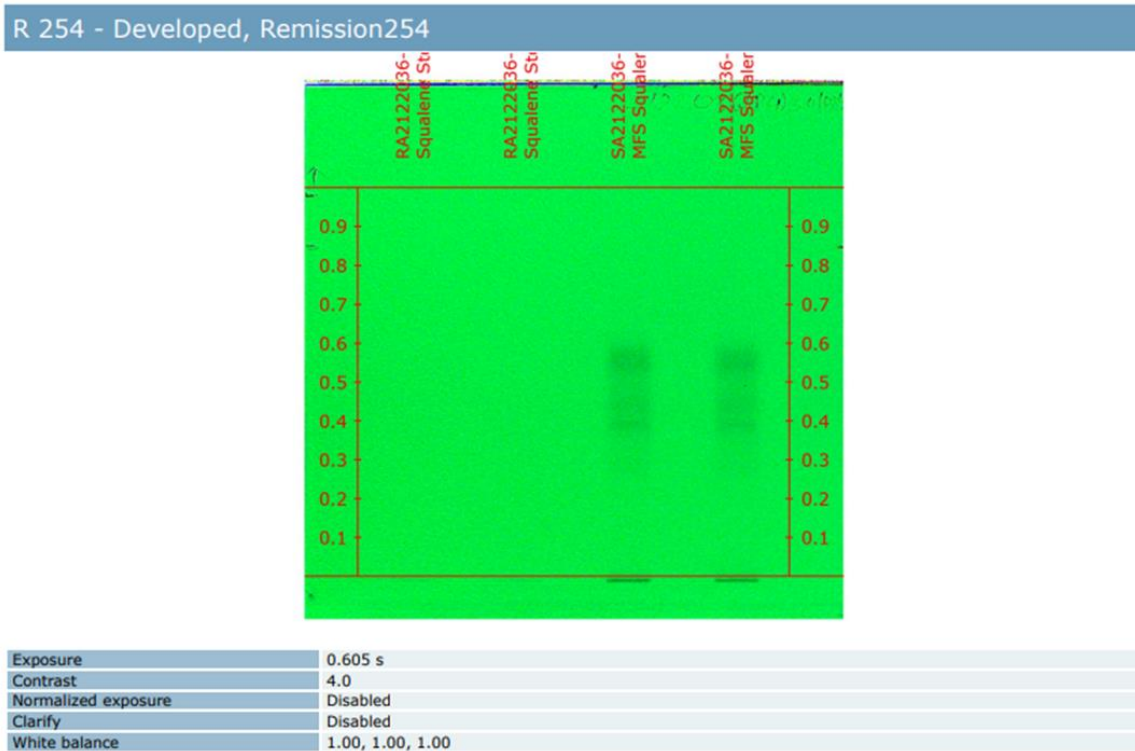


Fig. 2. HPTLC chromatogram of standard squalene & sample extracted at 254 nm

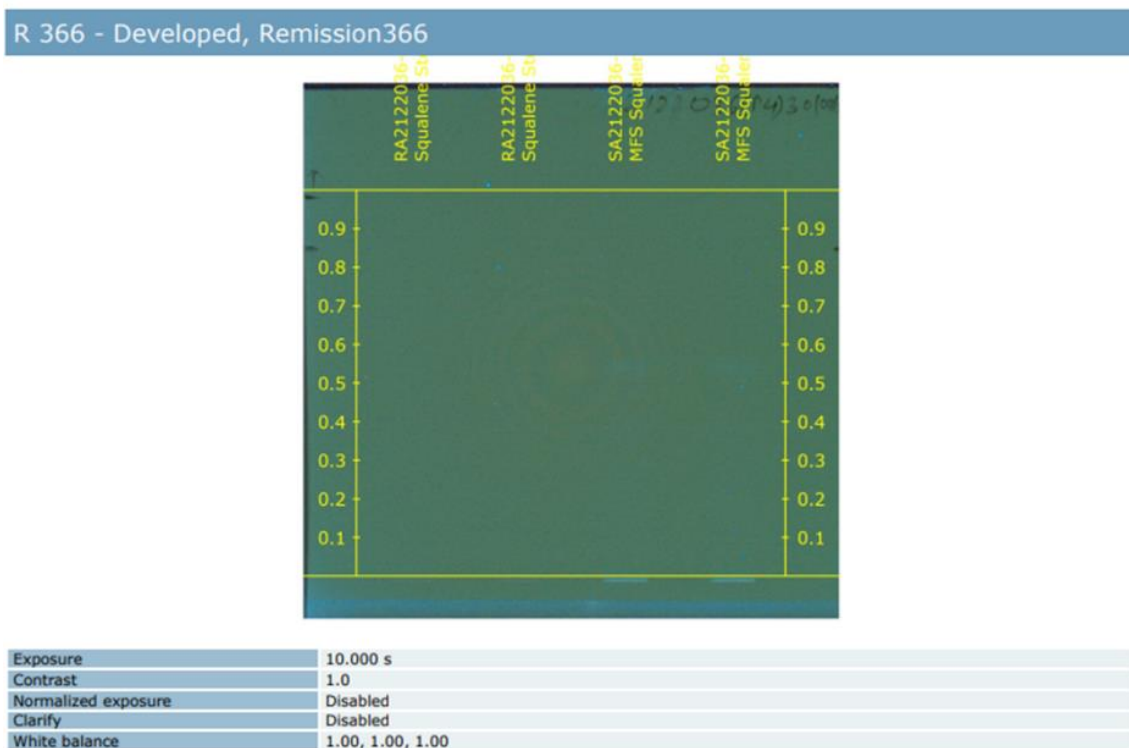
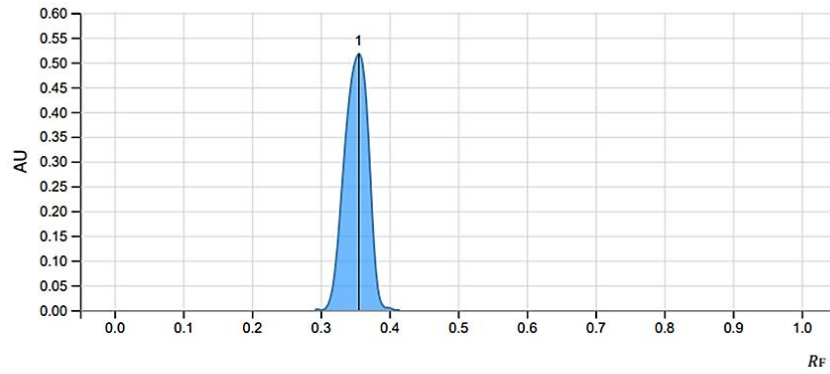
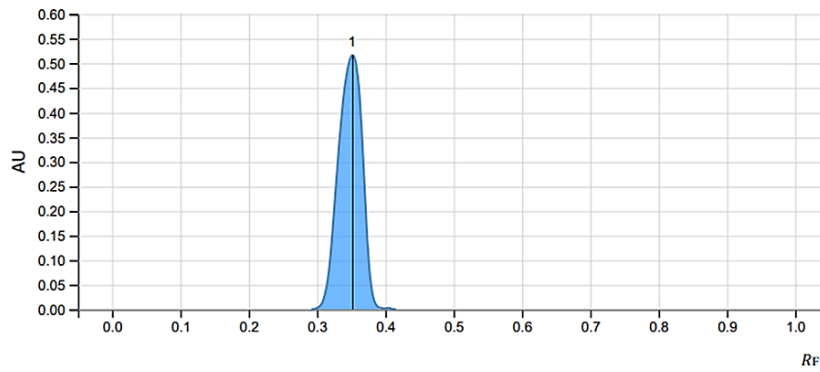


Fig. 3. HPTLC chromatogram of standard squalene & sample extracted at 366 nm



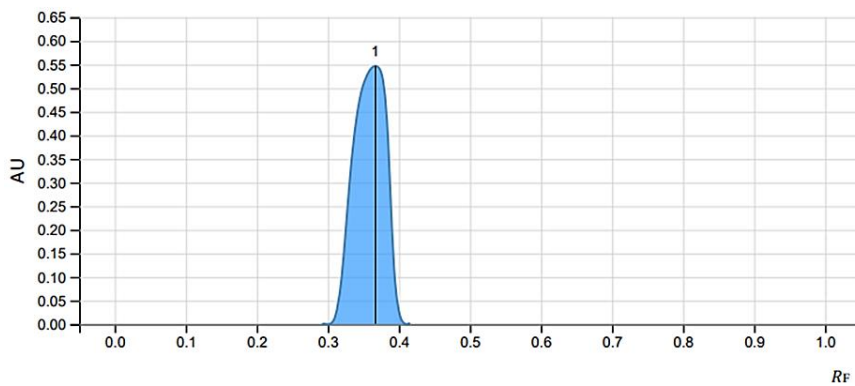
Peak #	Start		Max			End		Area		Manual peak	Substance Name
	R _F	H	R _F	H	%	R _F	H	A	%		
1	0.301	0.0000	0.356	0.5170	100.00	0.408	0.0000	0.02156	100.00	No	SQUALENE

Fig. 4. quantitative chromatograph of standard squalene spot 1



Peak #	Start		Max			End		Area		Manual peak	Substance Name
	R _F	H	R _F	H	%	R _F	H	A	%		
1	0.293	0.0000	0.351	0.5161	100.00	0.397	0.0018	0.02149	100.00	No	SQUALENE

Fig. 5. quantitative chromatograph of standard squalene spot 2



Peak #	Start		Max			End		Area		Manual peak	Substance Name
	R _F	H	R _F	H	%	R _F	H	A	%		
1	0.301	0.0000	0.367	0.5466	100.00	0.411	0.0000	0.03173	100.00	No	SQUALENE

Fig. 6. quantitative chromatograph of sample squalene spot 1

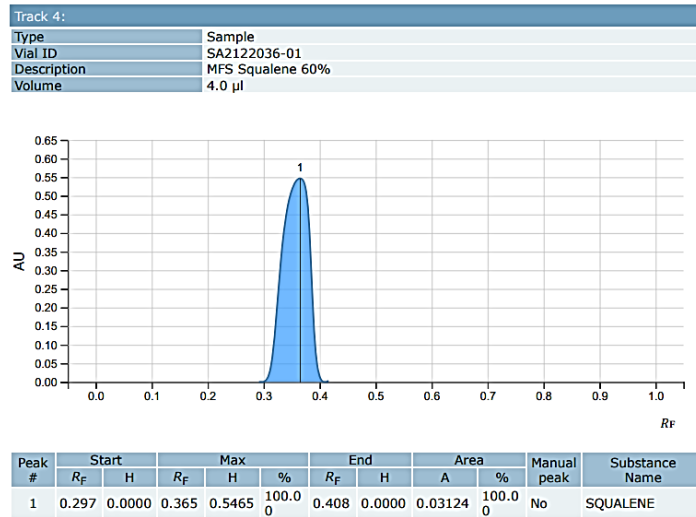
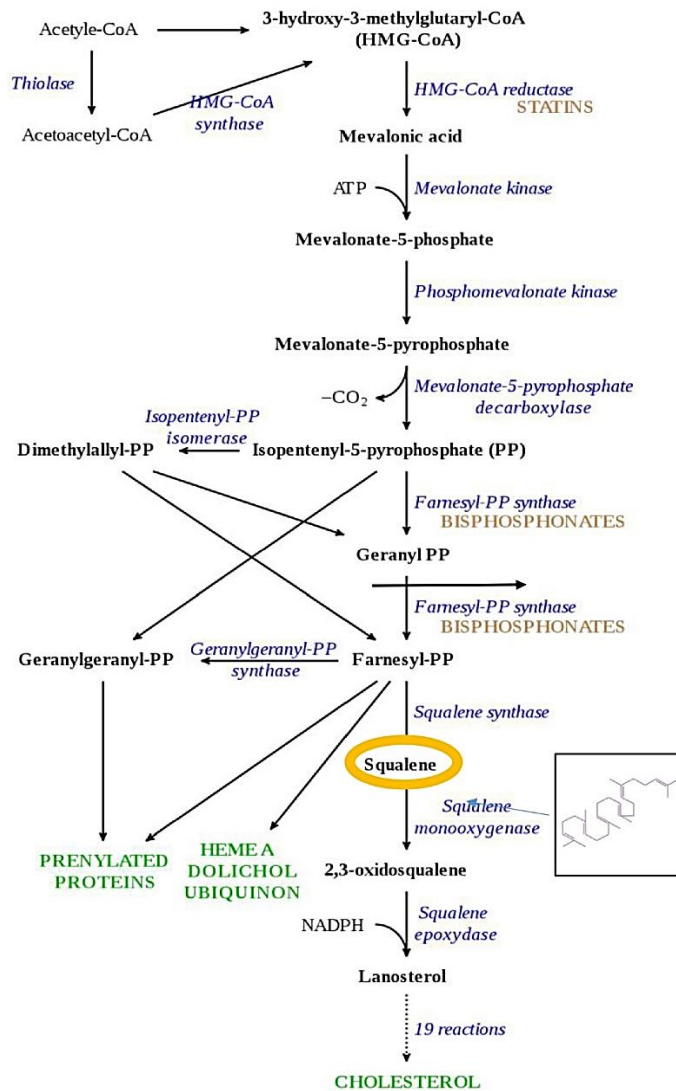


Fig. 7. quantitative chromatograph of sample squalene spot 2



5. CONCLUSION

The extraction and purification of squalene from virgin olive oil present a promising avenue for obtaining a highly valuable compound with diverse applications in pharmaceuticals, cosmetics, and nutraceuticals. The comprehensive procedure outlined in this research article, involving Degumming, transesterification, acetone precipitation, and molecular distillation, demonstrates an effective method for obtaining squalene with a purity of 67% and a recovery rate of 69.8%. While the initial purification steps have yielded satisfactory results, further refinement through molecular distillation is recommended to achieve higher purity levels required for specific applications.

The utilization of High-Performance Thin-Layer Chromatography (HPTLC) for quantitative analysis of squalene purity provides valuable insights into the efficiency of the purification process. Moreover, the proposed methodology offers a systematic approach that can be replicated and optimized for large-scale production, ensuring consistent and reliable outcomes.

Overall, the successful extraction and purification of squalene from virgin olive oil underscore its potential as a valuable resource for various industries. By harnessing the benefits of squalene, researchers and practitioners can explore new opportunities in pharmaceutical formulations, cosmetic products, and nutritional supplements. As advancements in extraction and purification techniques continue to evolve, the future holds promising prospects for maximizing the utility of squalene in improving human health and well-being.

CONSENT AND ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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