

SUSTAINABLE SQUALENE SYNTHESIS: ETHANOLYSIS OPTIMIZATION OF GMELINA ARBOREA LEAF BIOMASS USING RESPONSE SURFACE METHODOLOGY

HARUNA IBRAHIM; FATIMA HUSAINI; & ABUBAKAR M. ALI

Department of Chemical Engineering, Kaduna Polytechnic, Kaduna- Nigeria

Corresponding Author: ibrahimhauna@gmail.com

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Abstract

Squalene is a high-value triterpene widely utilized in pharmaceutical formulations, vaccine adjuvants, nutraceuticals, cosmetics, and specialty industrial applications. Growing environmental concerns associated with shark-derived squalene have intensified the search for sustainable, plant-based, and biomass-derived alternatives. This study investigates the potential of *Gmelina arborea* leaf biomass as a renewable feedstock for squalene production using a green ethanolysis approach under mild thermal conditions. A Box-Behnken Response Surface Methodology (RSM) was employed to evaluate the effects and interactions of temperature (40-60 °C), reaction time (40-60 min), and Ca(OH)₂ catalyst loading (1-2%) on squalene yield. Seventeen experimental runs were conducted, and products were analyzed using GC-MS after derivatization with TMSH and TEA. The results revealed that catalyst loading was the most significant linear factor ($p = 0.0083$), while reaction time exhibited a notable quadratic effect ($p = 0.0347$). The quadratic model was statistically significant ($F = 4.12$; $p = 0.0377$) with an R^2 of 0.841, indicating strong predictive capacity. The highest squalene yield of 123.7 mg/g was obtained at 50 °C, 40 min, and 2% catalyst loading, confirming the effectiveness of ethanolysis as a greener alternative to methanolysis, which had previously yielded 171.98 mg/g under harsher conditions (90 °C). Significant variability among center-point runs highlighted the sensitivity of the process to catalyst concentration and thermal conditions. Overall, this study provides the first optimized ethanolysis pathway for producing squalene from *Gmelina arborea* leaves, demonstrating a sustainable, efficient, and environmentally compatible route for renewable squalene synthesis. The findings offer strong potential for scale-up and integration into biomass valorization and green chemical production systems.

Keywords: Triterpenes, Catalytic optimization, Sustainable squalene production, Non-traditional biomass, Lignocellulosic resources

Introduction

Squalene is a naturally occurring triterpene hydrocarbon (C₃₀H₅₀) widely recognized as a key precursor in sterol biosynthesis across plants, animals, and microorganisms, including the formation of cholesterol, phytosterols, and other essential sterols (Permadi & Wilson, 2024; Micera *et al.*, 2020). First identified in shark liver oil, where it occurs in exceptionally high concentrations, squalene is also present in various plant oils such as olive, amaranth, and rice bran, as well as in diverse terrestrial and marine organisms (Permadi & Wilson, 2024; Kumar *et al.*, 2023; Mendes *et al.*, 2022; Gopakumar & Gopakumar, 2020; Shahidi *et al.*, 2019). Its highly unsaturated structure, characterized by six conjugated double bonds, imparts strong antioxidant capacity, chemical stability, and exceptional biocompatibility, enabling its extensive use as an emollient in pharmaceutical, cosmetic, nutraceutical, and vaccine adjuvant formulations (Dasari *et al.*, 2025; Maxim *et al.*, 2024; Morgan *et al.*, 2024; Kumar *et al.*, 2023). However, the historical reliance on shark-derived sources has raised major environmental and ethical concerns, prompting a global shift toward sustainable, plant-based, microbial, and biomass-derived alternatives (Leurs *et al.*, 2024; Bustamante, 2023). Despite progress, the relatively low yields from non-shark sources have continued to drive research into biotechnological synthesis, renewable feedstocks, and green extraction and catalytic processes to improve yield, purity, and environmental sustainability. As global demand for natural antioxidants and vaccine adjuvants increases, developing efficient and eco-friendly production pathways for squalene remains a significant scientific and industrial priority.

Squalene, the principal component of skin surface polyunsaturated lipids, provides multiple dermatological benefits owing to its emollient, antioxidant, hydrating, and antitumor properties (Zhang *et al.*, 2025; Ahmed & Mhammad, 2024; Morgan *et al.*, 2024; Pavlou *et al.*, 2021). It is widely incorporated into topical formulations-including lipid emulsions and nanostructured lipid carriers (NLCs)-to enhance stability and delivery efficiency (Mishra *et al.*, 2025; Tang *et al.*, 2023; Dobрева *et al.*, 2020). As a key constituent of vaccine adjuvants such as MicroFluidized 50 (MF59), squalene also serves important immunostimulatory and antioxidant roles in vaccine technology (Chen *et al.*, 2025; Mendes *et al.*, 2022; Nguyen-Contant *et al.*, 2021; Dasari *et al.*, 2025; Suseno *et al.*, 2024; Uysal, 2024). Its therapeutic potential extends to cancer research and drug delivery applications (Mavridi *et al.*, 2025; Bidooki *et al.*, 2024; Adriouach *et al.*, 2019). Owing to its excellent skin penetration, it is used broadly as a moisturizer, lubricant, and emollient in creams, lotions, sunscreens, anti-aging formulations, and lip balms (Morgan *et al.*, 2024; Maxim *et al.*, 2024; Budzianowska *et al.*, 2025; Veludurthi & Vadaga, 2024; Khanal *et al.*, 2024; Oderinde *et al.*, 2023). Its oxidative stability further supports its use as a formulation stabilizer (Mirmiranpour *et al.*, 2022; Musakhanian *et al.*, 2022; Kumar *et al.*, 2017). Orally, squalene is consumed as an antioxidant supplement and for its roles

in supporting immune function and regulating lipid homeostasis (Ahmed & Mhammad, 2024; Gao et al., 2022; Micera et al., 2020; Du et al., 2024; Mirmiranpour et al., 2022; Ibrahim et al., 2021; Sánchez-Quesada et al., 2018). Beyond biomedical applications, squalene serves as a natural antioxidant in food preservation and is incorporated into edible oils to enhance their nutritional profile and oxidative stability (Permadi & Wilson, 2024; Micera et al., 2020; Lozano-Grande et al., 2018; Hwang et al., 2024; Teh & Lau, 2023; Gutiérrez-Luna et al., 2022; Kumar et al., 2021). It also finds use in specialty lubricants, biodegradable formulations, and as an intermediate in the industrial synthesis of sterols such as cholesterol and phytosterols (Pichler et al., 2025; Dasari et al., 2025; Shalu et al., 2024; Permadi & Wilson, 2024; Gohil et al., 2019; Senbagalakshmi et al., 2019).

Shark liver oil, particularly from deep-sea sharks, is the most concentrated natural source of squalene, containing 40-80% by weight (Kumar et al., 2023; Revill et al., 2022). Historically, this has been the primary commercial source. However, reliance on sharks is environmentally unsustainable due to overfishing and their characteristically slow reproduction rates (Finucci et al., 2024; Permadi & Wilson, 2024; Porcher & Darvell, 2022). Consequently, significant research and industry focus have shifted toward identifying and developing alternative, sustainable sources.

Plant-Based and Renewable Alternatives

Alternative sources are primarily derived from plant oils and agricultural byproducts; though their squalene content is generally lower than that of shark liver oil. (Kumar et al., 2023; Muthukrishnan et al., 2019; Rosales-García et al., 2017).

- I. **Primary Plant Oil Sources:** Olive oil, a well-known dietary source, containing 0.2-0.7% squalene (Lozano-Grande et al., 2018; Martínez-Beamonte et al., 2020). Amaranth seed oil is considered one of the richest botanical sources, with a concentration of 5-8% squalene, making it a leading plant-based alternative (Hussain, 2023; Sattar et al., 2024). Rice bran oil contains a lower but notable amount, typically 0.1-0.3% squalene (Shimizu et al., 2019; Xu et al., 2021). Wheat germ oil is also a natural source, though specific concentration ranges are less frequently quantified in the recent literature (Paško et al., 2025).
- II. **Production from Agricultural and Industrial Byproducts:** A promising strategy involves extracting squalene from waste streams of existing industries, enhancing sustainability and value. Squalene is industrially recovered from palm fatty acid distillate (PFAD), a byproduct of crude palm oil refining (Jalani et al., 2025; Nurfatimah et al., 2021). It can be isolated and purified from soybean oil deodorizer distillate (SODD), another processing residue (Chakrabarty, 2003; Gunawan et al., 2006). Advanced extraction techniques, such as supercritical fluid extraction, can be employed to obtain squalene from Grape seed oil source (Gitea et al., 2023).

- III. **Emerging and Novel Sources:** Research continues to explore unconventional feedstocks, such as extracting squalene from the lignocellulosic biomass of plants like *Gmelina arborea* (Ibrahim & Ali, 2023), which could further diversify sustainable production pathways. Microbial lipids do contain squalene, a vital triterpenoid that serves as a precursor to steroids and hopanoids (Shalu *et al.*, 2024; Maswal *et al.*, 2023; Paramasivan & Mutturi, 2022).
- IV. **Microbial and Biotechnological Sources:** Yeasts such as *Saccharomyces cerevisiae* and *Yarrowia lipolytica* engineered via metabolic pathways to overproduce squalene (Zuo *et al.*, 2025; Liu *et al.*, 2024; Chai *et al.*, 2024). Microalgae (e.g., *Botryococcus braunii*) naturally accumulate high quantities of squalene but grow slowly (Tan *et al.*, 2025; Miyazaki *et al.*, 2025; Cao *et al.*, 2019). Another squalene production source is through bacteria engineered to channel carbon flux through the mevalonate pathway (Zhu *et al.*, 2025; Chai *et al.*, 2024; Shalu *et al.*, 2024).

Although squalene is widely recognized for its biomedical, nutraceutical, cosmetic, and industrial relevance, current sustainable production routes remain limited by several constraints. Plant-derived oils such as olive, amaranth, and rice bran contain squalene, but their concentrations are comparatively low and highly variable across cultivars, environmental conditions, and extraction methods. Industrial byproducts such as PFAD and SODD improve availability, yet their recovery requires energy-intensive refining and multistage purification processes that reduce economic viability for small-scale or decentralized production systems. Advances in microbial and metabolic engineering have demonstrated promising yields, but these systems require sophisticated fermentation infrastructure, high energy inputs, and are not yet cost-competitive for widespread industrial adoption.

A further gap exists in the exploration of non-traditional biomass sources, particularly lignocellulosic residues, which remain severely under-investigated despite their abundance and low cost. Previous work by Ibrahim and Ali (2023) demonstrated the potential of *Gmelina arborea* leaves as a novel substrate for squalene production via barium chloride-catalyzed methanolysis, achieving comparatively high yields. However, little is known about how alternative alcohol media, green catalysts, or mild processing conditions influence squalene recovery from this biomass. Specifically, ethanolysis-despite its lower toxicity, better environmental profile, and compatibility with green-chemistry-principles has not been systematically investigated for squalene generation from *Gmelina arborea* leaf material.

Moreover, no previous studies have applied a statistically rigorous optimization framework, such as the Box-Behnken Response Surface Methodology (RSM), to evaluate how temperature, reaction time, and catalyst concentration interact to influence squalene yield from this biomass. Existing reports lack detailed kinetic interpretation,

factorial interactions, reproducibility assessment, and predictive modelling necessary for process scale-up.

This study aims to develop and optimize a green catalytic ethanolysis process for the production of squalene from *Gmelina arborea* leaf biomass under mild thermal conditions. To achieve this aim, the work investigates the individual and interactive effects of temperature, reaction time, and $\text{Ca}(\text{OH})_2$ catalyst loading on squalene yield, employing a Box-Behnken Response Surface Methodology (RSM) to model process behaviour and identify statistically significant factors. The study further seeks to determine optimal reaction conditions using ANOVA-based model evaluation, compare the efficiency of ethanolysis with previously reported methanolysis pathways, and establish a sustainable, scalable approach for valorizing *Gmelina arborea* leaves as a renewable non-traditional feedstock for high-value squalene synthesis.

Materials and Methods

A Box-Behnken experimental design, implemented as a Response Surface Methodology (RSM), was employed to conduct 17 experimental runs as shown in Table 1. Dry leaves of *Gmelina arborea* were collected from the Kaduna Polytechnic premises, cleaned, pulverized, and sieved to obtain a uniform particle size according to the method of Ibrahim *et al.* (2025a) & Ali & Ibrahim (2023a). The catalyst solution was prepared by dissolving 1.0 g of $\text{Ca}(\text{OH})_2$ (equivalent to 2.0% w/w of the feed) in 500 mL of ethanol, after which 50 g of the processed *Gmelina arborea* leaf powder was added. The mixture was heated at 60 °C for 50 minutes on a Gallenkamp hot plate equipped with a magnetic stirrer. The resulting product was first filtered through a filter cloth and subsequently through Whatman filter paper following the procedure of Ali & Ibrahim (2023b). The filtrate was weighed and a 5 g aliquot of the filtrate was then subjected to GC-MS analysis for both qualitative and quantitative evaluation following the method of Ibrahim *et al.* (2024). This procedure was repeated for all runs specified in the design matrix presented in Table 1.

Table 1: Box Behken Design of Experiment

Run	Factor 1 A: Temperature (°C)	Factor 2 B; Time (min)	Factor 3 C; Catalyst load (%)	Response Yield (%)
1	50	60	2	
2	50	50	1.5	
3	40	50	1	
4	40	50	2	
5	50	50	1.5	
6	50	60	1	
7	60	50	2	

Run	Factor 1 A: Temperature (°C)	Factor 2 B; Time (min)	Factor 3 C; Catalyst load (%)	Response Yield (%)
8	50	50	1.5	
9	60	50	1	
10	50	50	1.5	
11	40	60	1.5	
12	60	60	1.5	
13	50	50	1.5	
14	40	40	1.5	
15	60	40	1.5	
16	50	40	2	
17	50	40	1	

For derivatization, 200 μL of the standard solution was combined with 100 μL of Trimethyl sulfonium Hydroxide (TMSH) and 20 μL of Triethylamine (TEA), and then heated at 70 °C for 1 h in sealed vials before GC-MS analysis following the method of Ibrahim *et al.* (2025b). The analyses were carried out using a Varian 3800/4000 gas chromatograph-mass spectrometer fitted with a DB-5 capillary column (30 m $\times$ 0.25 mm $\times$ 0.25 μm). Nitrogen served as the carrier gas, with the column head pressure maintained at 10 psi. The oven was programmed to start at 100 °C with a 3-minute hold, then increase at 8 °C min^{-1} to a final temperature of 300 °C. The transfer line temperature was set to 290 °C, and the VG 7070E magnetic sector mass spectrometer operated under electron impact ionization conditions. Mass spectra were acquired over an m/z range of 40-800 at 20 scans s^{-1} , with a 330-s solvent delay to prevent detector overload. Continuous monitoring of the signal ensured consistent and accurate detection.

Results and Discussion

Table 2 presents the weights of the filtered product, squalene yield in % as obtained from GC-MS, and the converted yield in mg/g. The highest yield was obtained from Run 14 (123.7 mg/g) at 50°C, 40 min, 2% Catalyst. This is the optimal combination in this experimental set. The lowest yield was obtained in Run 15 (46.4 mg/g) at 50°C, 50 min, 1.5% Catalyst. This is notably one of the center point runs, showing significant variability at these mid-level conditions.

Table 2: The yields of squalene in % and mg/g from ethanolysis of Ga leaf biomass

Run	Temp (°C)	Time (min)	Catalyst (%)	Filtered Product (g)	Squalene (%)	Yield (mg/g)
1	60	50	2	221.7	80.85	100.0
2	50	40	1	176.7	64.43	79.7
3	40	50	2	258.9	94.42	116.7

Run	Temp (°C)	Time (min)	Catalyst (%)	Filtered Product (g)	Squalene Yield (%)	Squalene Yield (mg/g)
4	40	40	1.5	249.0	90.81	112.3
5	50	60	2	224.9	82.03	101.4
6	50	60	1	165.2	60.22	74.5
7	50	50	1.5	138.4	50.47	62.4
8	50	50	1.5	195.9	71.42	88.3
9	40	60	1.5	138.1	50.36	62.3
10	50	50	1.5	162.7	59.34	73.4
11	60	40	1.5	255.2	93.08	115.1
12	40	50	1	143.6	52.37	64.8
13	50	50	1.5	139.3	50.81	62.8
14	50	40	2	274.2	100.00	123.7
15	50	50	1.5	102.9	37.54	46.4
16	60	60	1.5	222.9	81.32	100.5
17	60	50	1	175.1	63.86	78.9

Effect of Individual Factors:

Catalyst Concentration: This appears to be the most influential factor. Consistently, higher catalyst loads (2%) lead to higher yields (Runs 1, 3, 5, 14). Lower catalyst loads (1%) consistently give lower yields (Runs 2, 6, 12, 17). The jump from 1% to 2% has a dramatic positive effect.

Temperature: The effect is less straightforward and seems to interact with other factors. At high catalyst levels (2%), a moderate temperature (50 °C, Run 14) is best. However, at a mid-catalyst level (1.5%), a higher temperature (60 °C, Run 11) yields better results than a lower one (40 °C, Run 9). This indicates a significant interaction between Temperature and Catalyst.

Time: Time shows the least clear direct effect. For example, at 50 °C and 2% catalyst, a shorter time (40 min, Run 14) is better than a longer one (60 min, Run 5). However, at 40 °C and 1.5% catalyst, a longer time (60 min, Run 9) performs similarly poorly to a mid-time (Run 4). This suggests that time's effect is secondary and dependent on other conditions.

Analysis of the Centre Points (Runs 7, 8, 10, 13, 15):

All centre points are at 50 °C, 50 min, 1.5% Catalyst. The yields vary widely, ranging from 46.4 to 88.3 mg/g. This large variation (range of ~42 mg/g) is crucial. It indicates one or more of high sensitivity to uncontrolled variables (e.g., feedstock heterogeneity, precise catalyst mixing, analytical error) and potential for a strong nonlinear (curved) relationship near the centre of the experimental region. The process might be a "sweet spot" or a "ridge" where small changes cause large effects. The presence of significant

interaction effects (e.g., Temp vs Catalyst) means the impact of one factor depends on the level of another, which can lead to variability at middle settings.

Evidence of Interactions:

Temp x Catalyst: Compare Runs 11 (60 °C, 1.5% Cat = 115.1 mg/g) and 4 (40 °C, 1.5% Cat = 112.3 mg/g). Yield is similar. Now, compare Run 14 (50 °C, 2% Cat = 123.7 mg/g) and Run 3 (40 °C, 2% Cat = 116.7 mg/g). The effect of changing temperature depends entirely on the catalyst level. In the case of Time vs Catalyst, Run 14 (40 min, 2% Cat) is best. Run 5 (60 min, 2% Cat) is lower. This suggests that with high catalyst, a shorter time might be sufficient or even preferable, possibly because the reaction goes to completion quickly or because longer times lead to degradation.

Catalyst concentration is the key variable for maximizing squalene yield. Increasing it from 1% to 2% provides a consistent and substantial boost. Optimal Condition based on this data set, the best combination tested is 50 °C, 40 min, and 2% catalyst. The process is not very robust around the centre point (50 °C, 50 min, 1.5%), as shown by the high variability in replicates. Tight control of factors, especially catalyst amount, is critical for reproducible results.

The ANOVA table presented in Table 3 shows the results of a quadratic model used to analyze the yield of squalene from *Gmelina arborea* leaves under different ethanolysis conditions.

Overall Model Significance

Model F-value = 4.12, p-value = 0.0377, implies that this is statistically significant ($p < 0.05$), meaning the quadratic model adequately explains the variation in yield compared to noise.

$$R^2(model) = \frac{Model\ SS}{Total\ SS} = \frac{7239.04}{8606.50} = 0.841$$

where Model SS is the Model sum of squares, and Total SS is the Total sum of squares.

Thus, about 84.1% of the yield variability is explained by the model

Significant Factors

Only terms with $p < 0.05$ are typically considered significant here: C (Catalyst loading): $p = 0.0083$ implies that the catalyst has a highly significant linear effect, B^2 (Time squared): $p = 0.0347$ implies that time squared has significant quadratic curvature effect of time, and B (Time): $p = 0.0526$ implies that time has marginally significant linear effect (often noted at $0.05 < p < 0.10$).

All other linear, interaction, and quadratic terms are not significant at the 0.05 level, meaning their effects on yield are not clearly distinguishable from random variation within this experimental setup.

Table 3: ANOVA analysis of the yield of squalene

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	7239.04	9	804.34	4.12	0.0377	Significant
A - Temperature	184.32	1	184.32	0.9435	0.3637	
B - Time	1060.30	1	1060.30	5.43	0.0526	
C - Catalyst loading	2588.40	1	2588.40	13.25	0.0083	
AB	313.29	1	313.29	1.60	0.2459	
AC	237.16	1	237.16	1.21	0.3070	
BC	73.10	1	73.10	0.3742	0.5601	
A ²	720.64	1	720.64	3.69	0.0962	
B ²	1335.19	1	1335.19	6.83	0.0347	
C ²	451.70	1	451.70	2.31	0.1722	
Residual	1367.45	7	195.35			
Lack of fit	410.22	3	136.74	0.5714	0.6632	Not significant
Pure error	957.23	4	239.31			
Cor total	8606.50	16				

Lack of Fit Test: Lack of Fit F-value = 0.5714, p-value = 0.6632 implies not significant. This means the quadratic model fits the data well; there's no evidence that a more complex model is needed.

Residuals & Error: Pure error (from replicated runs) variance = 239.31 and Residual mean square = 195.35, close to pure error; this suggests model adequacy.

Practical Interpretation: Catalyst loading (C) is the most influential linear factor, Time (B) shows quadratic behavior (optimal time exists for yield), Temperature (A) alone is not significant, nor are most interaction effects (AB, AC, BC), and Since lack of fit is not significant, the quadratic model is suitable for prediction/optimization within the studied range.

Lozano-Grande *et al.* (2018) reported squalene levels of 564 mg/100 g in olive oil and 9.9 mg/100 g in soybean oil. Popa *et al.* (2015) documented squalene concentrations of 0.03-0.2 g/kg in sunflower oil, while Skwaryło-Bednarz *et al.* (2020) noted that amaranth oil contains comparatively high amounts, ranging from 3.2% to 8.0%. Xu *et al.* (2021) quantified squalene in rice bran at 2962 mg/kg, and Widiputri *et al.* (2020) reported a 23.94% squalene yield following saponification of Palm Fatty Acid Distillate (PFAD) derived from crude palm oil refining. Ibrahim and Ali (2023) achieved a maximum yield of 171.98 mg/g of squalene from *Gmelina arborea* leaves at 90 °C for 30 minutes using barium-chloride-catalyzed methanolysis. In the present ethanolysis model, *Gmelina arborea* leaves yielded 123.7 mg/g at 50 °C, 40 minutes, and 2% catalyst loading. These comparisons are clearly presented in Table 4.

Table 4: Squalene Content and Yields from Various Sources

Source	Material	Squalene Content / Yield	Conditions / Notes
Lozano-Grande <i>et al.</i> (2018)	Olive oil	564 mg/100 g	
Lozano-Grande <i>et al.</i> (2018)	Soybean oil	9.9 mg/100 g	
Popa <i>et al.</i> (2015)	Sunflower oil	0.03–0.2 g/kg	
Skwaryło-Bednarz <i>et al.</i> (2020)	Amaranth oil	3.2–8.0%	
Xu <i>et al.</i> (2021)	Rice bran	2962 mg/kg	
Widiputri <i>et al.</i> (2020)	PFAD	23.94% yield	After saponification
Ibrahim & Ali (2023)	Gmelina arborea leaves	171.98 mg/g	90°C, 30 min, BaCl ₂ methanolysis
This study	Gmelina arborea leaves	123.7 mg/g	50°C, 40 min, 2% catalyst, ethanolysis

Conclusion

This study confirms that *Gmelina arborea* leaf biomass is a promising renewable feedstock for squalene production via Ca(OH)₂-catalyzed ethanolysis under mild thermal conditions. Using Box–Behnken RSM, temperature, reaction time, and catalyst loading were systematically optimized, with catalyst concentration identified as the most significant factor and reaction time showing notable quadratic effects. The quadratic model demonstrated good predictive accuracy ($R^2 = 0.841$), and the highest squalene yield of 123.7 mg/g was achieved at 50 °C, 40 minutes, and 2% catalyst loading. These results show that ethanolysis offers a greener and effective alternative to previous methanolysis routes. Although some variability occurred at center-point conditions, the optimized process provides a solid basis for future scale-up and supports the development of sustainable, biomass-based pathways for high-value squalene production.

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