Application Note

Squalene identification and quantitation in adjuvanted vaccines enabled by a USP Analytical Reference Material

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Squalene ($C_{30}H_{50}$), a linear triterpene, is widely used in oil-in-water vaccine adjuvants and drug delivery emulsions due to its distinctive properties, including a high surface tension that enables the creation of small droplet-size emulsions. Emulsions containing squalene facilitate solubilization and cellular uptake of vaccines. MF59 (Novartis), AS03 (GSK) and AF03 (Sanofi) are examples of well-established, safe, and potent oil-in-water squalene-based emulsion adjuvants that have been licensed for use in seasonal influenza vaccines.[1]

Squalene, a naturally occurring compound in the isoprenoid family, is primarily sourced from shark liver oil and must be of high purity for use in vaccines, as impurities can affect safety and efficacy. Squalene's natural origin, favorable safety profile, and proven ability to enhance immune responses make it a valuable component in vaccine formulations. Rigorous quality control is essential to meet regulatory standards. Developing reliable analytical standards and methods for assessing squalene quality is vital for its continued use in vaccines and drug delivery systems.

Due to the natural variability of squalene sources and the complexities of its analytical assessment, accurate determination of squalene quantity and identity in formulations is needed. To this end, USP developed a Squalene Based Oil in Water Nano-Emulsion Analytical Reference Material (ARM) (Catalog # 1619550; CAS# 111-02-4; $C_{30}H_{50}$; M_w 410.7 Da) for the precise identification and quantitation of squalene in vaccine adjuvants.

This fit-for-purpose, well-characterized ARM supports the identification and quantitation of squalene in adjuvant and final vaccines from development to lot release including:

- Raw material quality control
- Internal control for system suitability testing of analytical methods
- Identification and quantitation of squalene in adjuvant sand final vaccine products
- Stability assessments

Feasibility study on commercial squalene demonstrates need for quality reference materials

The need for a reference material has been highlighted by a proof-of-concept (PoC) study conducted by USP. Squalene from several vendors was thoroughly assessed in its purified form, within an adjuvanted solution, and in the vaccine formulation. The quality attributes outlined in **Table 1** for evaluating squalene were assessed using the reverse phase HPLC/UV (RP-HPLC/UV) method outlined in **Table 2**.

A. Purified form of squalene

During the analytical testing of pure squalene samples from various suppliers, the observed chromatographic response (such as peak height and area under the curve) was consistent across all samples (**Figure 1**). However, the retention time of sample from Vendor C varied slightly, indicating possible differences in squalene quality. Using a reference material allows users to confirm both the quality and identity of the squalene more reliably.

Squalene is easily degraded with exposure to light. To demonstrate the possible impact of this variable, a forced degradation study was conducted and revealed squalene's volatility and susceptibility to UV-induced oxidation (**Figure 2**). The pre-peak in the chromatogram displays the degradation of the squalene. These findings underscore the importance of using a high quality reference material when analyzing squalene in vaccine adjuvants to help ensure accurate and reliable quantitation; regulators require manufacturers to provide the concentration determination on the final squalene in adjuvant.

Table 1. Quality attributes of squalene

Quality Attribute	Measurement
Identity	Retention time
Purity	% peak area
Quantity	Peak Area

Table 2. RP-HPLC Method Parameters for squalene

Sample Concentration	50 µg/mL
Calibration Curve Concentrations	5, 10, 20, 40, 60, 80, 100 µg/mL
Diluent	Isopropanol
Mobile Phase	Methanol
Column	C18, 4.6 mm x 250 mm, 5 µm
Detector	UV 214 nm
Method	Injection volume: 10 µL Flow rate: 0.5 mL/min Column temperature: 25 °C Sampler temperature: 5 °C Run time: 20 minutes

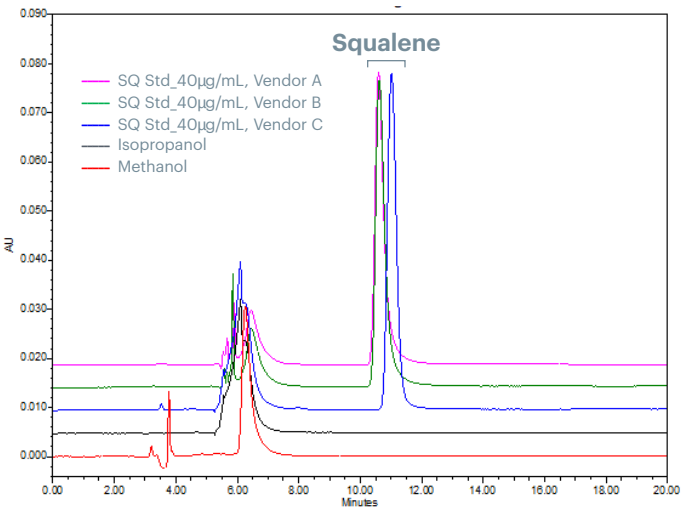
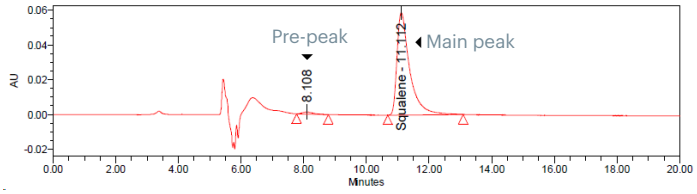


Figure 1. Chromatogram of three squalene samples from different vendors.

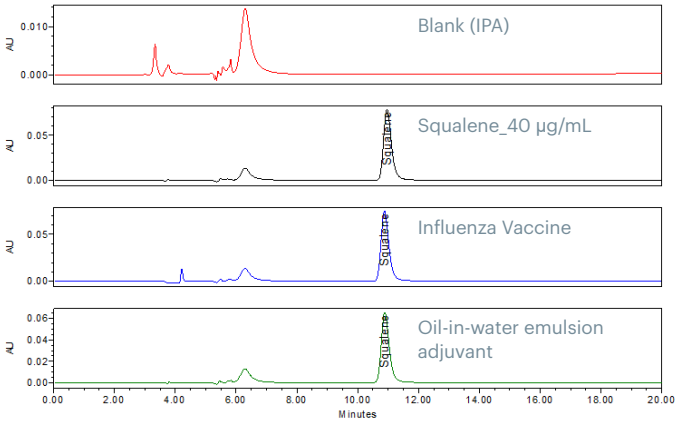
B. Comparison of peak area of three squalene sample types

The presence of squalene was assessed by HPLC (**Table 2**) in three sample types – squalene in isopropanol, in an adjuvanted influenza vaccine, and in an oil in water emulsion adjuvant. The retention time of squalene peaks was found to be consistent across the three sample types (**Figure 3**).



Description	% Pre-peak	% Main peak	Total Area
Control	NA	100.0	2301749
UV exposed (24 hrs)	1.71	98.3	1684700

Figure 2. Chromatographic profile of squalene sample exposed to UV light.



Sample no.	Sample ID	RT (min)
1	Blank (IPA)	NA
2	Squalene 40 µg/mL	10.96
3	Influenza Vaccine (Adjuvanted)	10.88
4	Oil-in-Water Emulsion Adjuvant	10.89

Figure 3. Presence of squalene in three sample types.

Analytical evaluation of *USP squalene based oil in water nano-emulsion for quantitation ARM*

Based on the PoC study conducted by USP using various commercially available sources and compositions, USP developed a squalene analytical reference material (ARM), named *USP Squalene Based Oil in Water Nano-Emulsion for Quantitation*[2]. This USP ARM, consisting of squalene in adjuvant form, was analyzed by HPLC (**Table 2**) for identity and concentration (**Figure 4 a & b**).

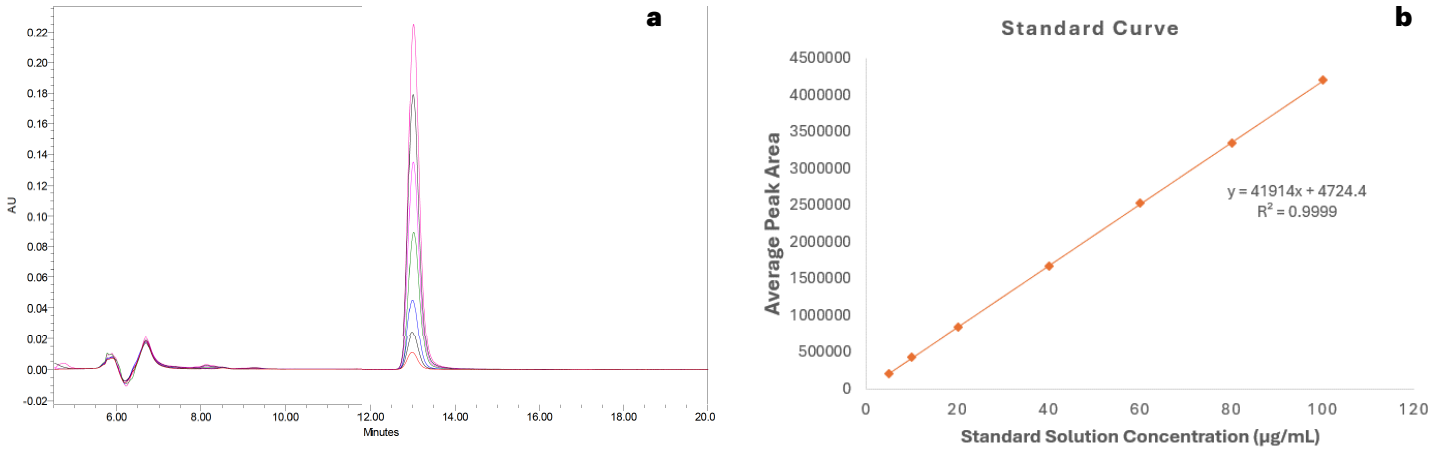


Figure 4. HPLC profile of USP Squalene ARM (a). Standard curve of USP Squalene ARM at 5, 10, 20, 40, 80, and 100 µg/mL (b).

The USP Squalene ARM can be used to identify squalene in test samples; it can also be used to create a standard curve to quantify squalene in test samples. The squalene concentration of the USP ARM (4% w/v squalene, 0.5% w/v sorbitan trioleate, 1.17% w/v polyoxyl 10 oleyl ether) is 41 mg/mL; the USP Squalene ARM was quantified using the EDQM Squalene CRS batch 1 (Catalog code: Y0002131)

Conclusion

Squalene, a key component in certain adjuvants, enhances the immune response when included in vaccine formulations. Analytical control of squalene is crucial to ensure the efficacy and safety of vaccines. Use of the USP Squalene Based Oil in Water Nano-Emulsion for Quantitation ARM supports the identity, quantity, and purity determinations and can be used in many aspects of vaccine development.

References

1. Nguyen-Contant P, Sangster MY, Topham DJ. Squalene-Based Influenza Vaccine Adjuvants and Their Impact on the Hemagglutinin-Specific B Cell Response. Pathogens. 2021 Mar 17;10(3):355. doi: 10.3390/pathogens10030355

2. Product Information Sheet for USP SQUALENE BASED OIL IN WATER NANO-EMULSION FOR QUANTITATION Analytical Reference Material (https://store.usp.org/product/1619550)

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