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Application Note

From Fermentation to Vaccination

Non Animal Origin Squalene for High-Risk Applications

As consumers and industries increasingly prioritize responsibly sourced solutions, the demand for alternatives to traditional animal-derived ingredients is rising. One such ingredient, squalene, is well-known for its role as a component in vaccine adjuvants, where it enhances immune response. Traditionally, squalene has been sourced from shark liver oil, a practice that raises significant concerns, including overfishing and the depletion of shark populations.

Given these concerns, the need for animal free alternative (NAO) squalene has become critical. This application note explores NAO Squalene Emprove® Expert as a viable alternative to shark-derived squalene. We present comprehensive data on the purity and stability of NAO Squalene, both as a standalone raw material and within a nanoemulsion formulation. Notably, our findings demonstrate that the squalene-based nanoemulsion formulation exhibits identical stability and performance when utilizing either shark-derived or non-animal-origin squalene.

This underscores the efficacy of NAO Squalene as an alternative, aligning with the growing demand for responsible sourcing in the industry.

Applications of Squalene in Vaccine Formulation

Squalene has emerged as a critical component in the formulation of nanoemulsion vaccine adjuvants due to its stability enhancing effects and biocompatibility. Squalene-based emulsion adjuvants induce a strong innate immune response, enhancing antigen presentation both quantitively and qualitatively to generate strong B cell responses and antibody production. Squalene-based vaccine adjuvants, such as MF59 (Seqirus), AS03 (GlaxoSmithKline Biologicals), and AF03 (Sanofi), are included in seasonal vaccines against influenza viruses and are currently being considered for inclusion in several vaccines against SARS-CoV-2 and future pandemic threats.[1]

Table 1.

Squalene-based emulsion adjuvants and their applications.[2]

Adjuvant name	Squalene	Additional ingredients	Application	Marketed Product
MF59 (Seqirus)	✓	• polysorbate 80 • sorbitan trioleate	Seasonal influenza vaccine	FLUAD® FLUAD® Quadrivalent
			A/H1N1 pandemic influenza vaccine	Forcetria® Celtura®
AS03 (GlaxoSmithKline)	✓	• α-tocopherol • polysorbate 80	A/H1N1 pandemic influenza vaccine	Pandemrix® Prepandrix™
AF03 (Sanofi)	✓	• polyoxyethylene cetostearyl ether • mannitol • sorbitan oleate	A/H1N1 pandemic influenza vaccine	Humenza™

High Purity Squalene

Despite its beneficial applications, the traditional sourcing of squalene from shark liver oil raises ethical concerns. Overfishing and the depletion of shark populations have led to a pressing need for alternatives. About 3,000 sharks are required to extract 1 metric ton of squalene.[1] Consequently, research has shifted towards non-animal sources of squalene, like plant or vegetable oil and fermentation of microbes like yeast.

Plant-derived squalene still has an ecological impact due to reliance on agriculture and energy resources; it is estimated that approximately 20 hectares of olive trees are required to extract 1 metric ton of squalene.[1] Therefore, yeast-derived squalene, which is derived from renewable sources and relies only on bioreactor production, serves as a promising solution to meet the growing global demand while addressing sustainability and ethical considerations.

Table 2.

Sources of Squalene.

Source	Shark Liver Oil	Plant oil [e.g., amaranth, olive]	Yeast Fermentation
Sustainability	High ecological footprint, negative impact on shark populations and marine biodiversity	Rely on agricultural land, require significant energy resources, oil refining, and use pesticides	Ethical and renewable sourcing
Extraction	Mechanical methods or solvent extraction separate oil from biomass, then oil is refined to remove impurities		Engineered yeast grown in fermentation media metabolize sugars to produce squalene through biosynthetic pathway. Cells are disrupted to release intracellular squalene and efficiently extracted.
Efficiency	Less efficient and requires significant amounts of raw material to yield a smaller final quantity		
Scalability	Low; concentration and purity vary due to inconsistent raw material sources	Medium; less supply reliability due to seasonal sourcing or pesticide contamination	Medium; less supply reliability due to seasonal sourcing or pesticide contamination

Characteristics of Non-Animal Origin Squalene Emprove® Expert

Product Description

Non Animal Origin (NAO) Squalene Emprove® Expert is excipient GMP grade according to IPEC-PQG Pharmaceutical Excipients GMP Guide/EXCiPACT squalene derived from yeast fermentation. It is a high-purity Emprove® Expert product suitable for high-risk pharmaceutical applications, featuring low specified endotoxin (≤2.25 EU/mL) and bioburden levels. Furthermore, NAO Squalene Emprove® Expert complies with the analytical specifications outlined in the Ph. Eur. monograph 2805.

High Purity

There are two primary methods for testing squalene purity: Ph. Eur. gas chromatography assay (GC) section 2.2.28 and an additional validated GC test method that measures purity and impurity levels.

Vaccine-grade squalene derived from shark liver oil typically achieves a purity exceeding 99%. Similarly, NAO Squalene Emprove® Expert consistently maintains high purity of ≥99% (see **Figure 1** for example comparison data), making it a suitable option for vaccine formulations, and an acceptable alternative to Shark Squalene.

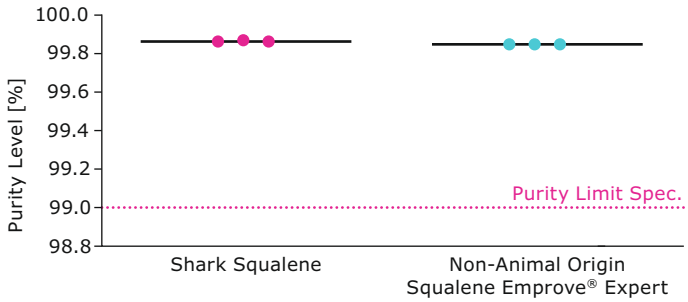

Figure 1.

GC-analysis of squalene purity from 3 batches of Shark Squalene and NAO Squalene using validated GC Test method.

Stability

Purity level and peroxide concentration of three batches of NAO Squalene Emprove® Expert stored at refrigerated conditions (2–8 °C) and 28 °C were measured in stability studies. NAO Squalene Emprove® Expert demonstrates a shelf life of at least two years at 2–8 °C, as forecasted by accelerated stability studies conducted in a nitrogen atmosphere at 28 °C for up to 6 months. The shelf life will be extended when additional time points are available. The studies also confirm consistently high purity and low impurity levels throughout the stability study period, measured using the validated GC method, along with low peroxide levels evaluated according to the Ph. Eur. peroxide assay (section 2.5.5), which has a limit of 5.0 $mEqO_2/kg$. Analysis of the three batches indicates batch-to-batch consistency in both purity levels and peroxide concentrations, with all batches consistently meeting the established quality criteria throughout the stability studies.

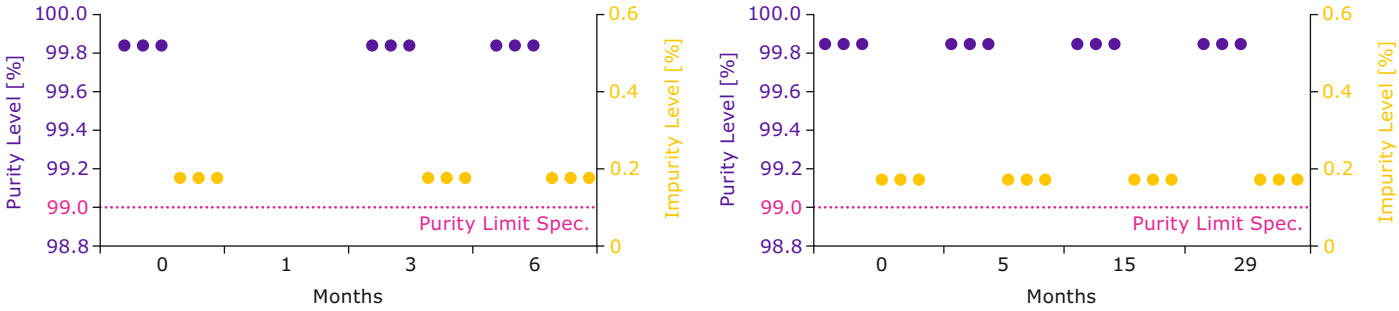

Figure 2.

Purity and impurity levels from 3 batches of NAO Squalene Emprove® Expert in a stability study at 5 °C (real-time, left) and 28 °C, providing accelerated estimates for real-time conditions at 5 °C (right).

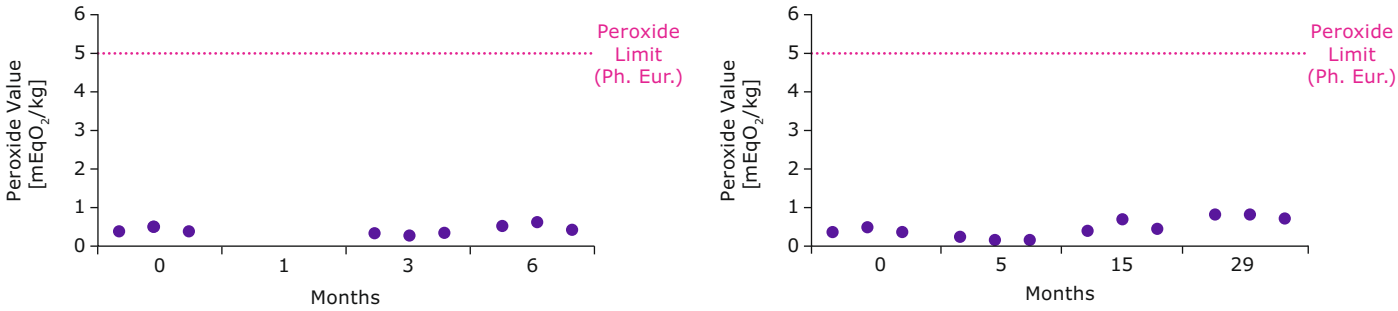

Figure 3.

Peroxide Value from 3 batches of NAO Squalene Emprove® Expert in a stability study at 5 °C (real-time, left) and 28 °C, providing accelerated estimates for real-time conditions at 5 °C (right).

Particle Size Distribution and Stability of Squalene-based Nanoemulsions

Stable nanoemulsions were obtained from high-pressure homogenization and polydispersity index (PDI) was evaluated along with particle size distribution using the dynamic light scattering method. The stable nanoemulsion derived from NAO Squalene Emprove® Expert meets the acceptance criteria for parenteral applications, featuring a particle size of less than 200 nm and a PDI value of up to 0.250, making it comparable to shark squalene.[3] A low PDI of less than 0.250 signifies a concentrated and narrow particle size distribution, contributing to enhanced stability. Both squalene-based nanoemulsions exhibited stability, maintaining consistent nanoemulsion size and PDI throughout an 8-week stability study at 5 °C.

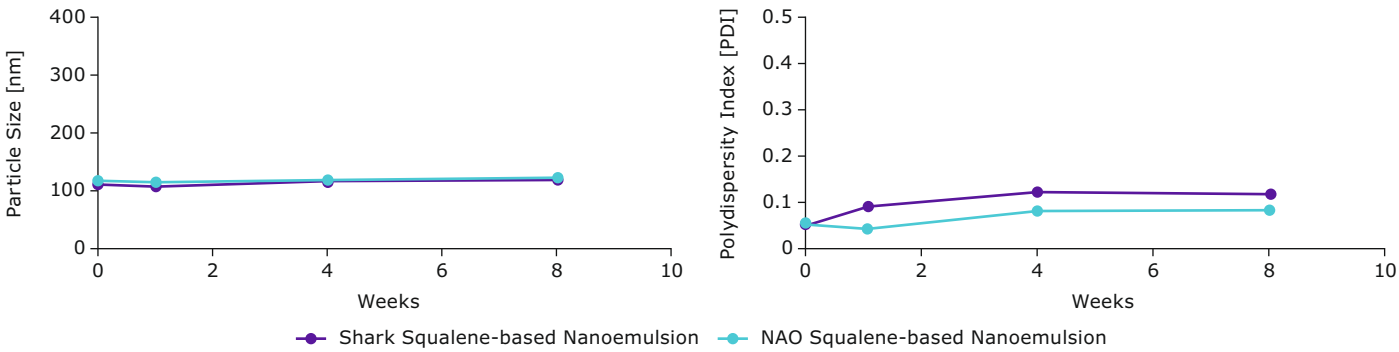

Figure 4.

Physical stability of nanoemulsions stored for up to 8 weeks at 5 °C, displaying nanoemulsion size (left) and PDI values (right).

In-Vitro Immunological Study of Stable Nanoemulsions

Immunological studies of stable squalene-based nanoemulsions were performed using *in vitro* cytokine stimulation assays. Interleukin (IL)-8, monocyte chemoattractantprotein-1 (MCP-1) were chosen as potential indicators of innate stimulation. All nanoemulsions exhibited activity on the secretion of IL8 and MCP-1. A secretion of IL8 and MCP-1 with medium control (represent as 0% v/v stable nanoemulsion) was not detectable; therefore, the window of activation was wide. Overall, NAO Squalene Emprove® Expert-derived nanoemulsions exhibited comparable cytokine stimulation to shark squalene-derived nanoemulsions.

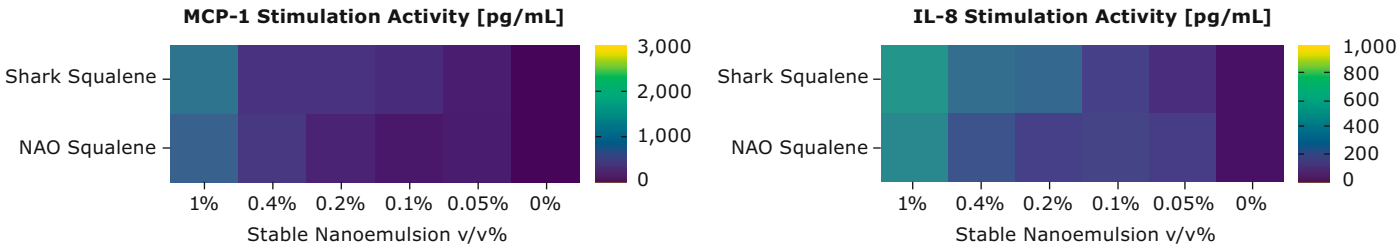

Figure 5.

Results of *in-vitro* immunological studies of squalene-based stable nanoemulsions were determined using *in vitro* cytokine stimulation assays, which demonstrated cytokine activity on MCP-1 (left) and IL-8 (right). Heat map illustrates the cytokine activity in human whole blood stimulated with various concentrations of nanoemulsions.

Conclusion

Non Animal Origin (NAO) Squalene Emprove® Expert consistently demonstrates high purity and stability supporting at least a 2-year shelf life at 2–8 °C. In addition, NAO Squalene forms stable nanoemulsions that effectively activate cytokine stimulation. Taken together, these results support the use of NAO Squalene as an animal free alternative for vaccine formulation especially as an adjuvant. Commercially available adjuvants contain squalene, highlighting the critical role NAO Squalene can play as a sustainable, non-animal derived alternative for modern vaccines.

References

1. Mendes, A.; Azevedo-Silva, J.; Fernandes, J.C. From Sharks to Yeasts: Squalene in the Development of Vaccine Adjuvants. Pharmaceuticals 2022, 15, 265.
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Merck KGaA
Frankfurter Strasse 250
64293 Darmstadt, Germany

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