

# Neutral Red Uptake (NRU) Cytotoxicity Analysis of Aerosol Generated from a Temperature-Regulated Nicotine Salt Pod System Utilizing Cotton Wicking Material

Bryant Hiraki<sup>1</sup>, Manoj Misra, Ph.D.<sup>1</sup>, I. Gene Gilman, Ph. D.<sup>2</sup>, David Cook<sup>1</sup>

<sup>1</sup>JUUL Labs, Inc., CA, USA. <sup>2</sup>Enthalpy Analytical LLC., VA, USA.



## Introduction

The JUUL Nicotine Salt Pod System (NSPS) is temperature-regulated to minimize the generation of combustion related degradation byproducts in the aerosol. The NSPS e-liquids contain 5 major ingredients: nicotine, benzoic acid, glycerol, propylene glycol, and flavorants. The assessed NSPS e-liquids, including 9 mg/mL nicotine concentration and flavors, are available in ex-US markets. Pods utilizing cotton wicking material to conduct e-liquid for aerosolization were evaluated for cytotoxic potential over a range of dosing concentrations relative to a 3R4F Kentucky Reference Cigarette. The Neutral Red Uptake (NRU) *in vitro* assay assessed the relative toxicity in CHO-K1 cell populations following 24-hour exposure to NSPS aerosol and 3R4F smoke using validated methodologies at an accredited ISO-17025 3<sup>rd</sup> party laboratory (Enthalpy Analytical).

## Materials and Methods

Cytotoxic potential was evaluated using the NRU *in vitro* assay, according to OECD guidelines<sup>1</sup>. CHO-K1 cells were treated with NSPS aerosol condensates, positive control, and vehicle control for 24 hours utilizing USP ethanol<sup>2</sup>. NSPS aerosol was tested for seven different formulations: Alpine Berry, Apple Orchard, Glacier Mint, Golden Tobacco, Mango Nectar, Royal Creme, Virginia Tobacco, all at a nicotine concentration of 9 mg/mL.

NSPS with cotton wicking material aerosol collection was prepared under a modified CORESTA recommended method<sup>3</sup>. 3R4F cigarette smoke collection was performed on a Rotary Smoking Machine, NSPS aerosol collection was performed on a linear machine using a square wave of 70 mL volume puffed over 3 seconds across 30 second intervals. Generated aerosol was collected on a Cambridge filter pad followed in series by an impinger constituted with USP ethanol. NSPS aerosol exposure thus consisted of both pad and impinger collections extracted in USP ethanol to a final concentration of approximately 60 mg/mL. Assay dosing was conducted for combined aerosol collected mass (ACM) and gas vapor phase (GVP) across a concentration range of (0-300 µg/mL).

Cell viability following NSPS aerosol exposure was subsequently compared against the combined mainstream Total Particulate Matter (TPM) and GVP from 3R4F Kentucky reference cigarettes, following similar sample preparation as NSPS, at a dose range of (0-150 µg/mL) extracted in USP Ethanol. 3R4F cigarette samples were prepared under the Health Canada defined "intense puffing regime" characterized as a 55 mL puff volume over a 2 second draw with a 30 second puff interval. Additionally, the cells were exposed to the positive control, SLS (Sodium Lauryl Sulfate) [CAS No. 151-21-3] at a dose range of 0-100 µg/mL, a physiological representative dose validated by the accredited 3<sup>rd</sup> party lab (Enthalpy Analytical).

## Results

Study results are presented as the relative percent cell viability and standard deviation (±SD) of the combined ACM and GVP of each test article relative to its respective vehicle control. At the doses tested, EC<sub>50</sub> for the NSPS aerosol and carrier control aerosol could not be calculated because cell viability was greater than 50% at all concentrations tested. Additionally, TPM from the 3R4F reference cigarette demonstrated expected toxicity with a calculated EC<sub>50</sub> of 59.46 µg/mL with an r<sup>2</sup> of 0.98.

Table 1 : Osmolarity for 2 Highest Doses Per Formulation Tested

Sample ID	Doses (µg/ml)	Osmolarity (mOsmol/kg)			pH		
		Average (n=3)	Std Dev	% RSD	Average (n=3)	Std Dev	% RSD
Media with 5% serum	N/A	351	0.69	0.20	7.33	0.01	0.14
Vehicle Control (0.5% USP grade EtOH)	N/A	434	6.66	1.53	7.39	0.02	0.27
Alpine Berry	300	431	2.19	0.51	7.38	0.04	0.59
	205	430	2.14	0.50	7.39	0.03	0.47
Apple Orchard	300	424	5.50	1.30	7.40	0.02	0.28
	205	429	3.36	0.78	7.44	0.02	0.21
Golden Tobacco	300	435	4.91	1.13	7.39	0.04	0.55
	205	431	0.19	0.04	7.40	0.07	0.96
Glacier Mint	300	431	2.12	0.49	7.35	0.02	0.28
	205	428	5.19	1.21	7.35	0.05	0.62
Mango Nectar	300	430	1.07	0.25	7.48	0.08	1.12
	205	431	2.78	0.64	7.48	0.07	0.91
Royal Creme	300	431	2.46	0.57	7.42	0.05	0.64
	205	436	5.00	1.15	7.44	0.02	0.28
Virginia Tobacco	300	427	0.96	0.23	7.43	0.02	0.23
	205	431	1.35	0.31	7.45	0.04	0.54

Fig. 1 : Comparison of Alpine Berry NSPS Aerosol with TPM from 3R4F Reference Cigarette

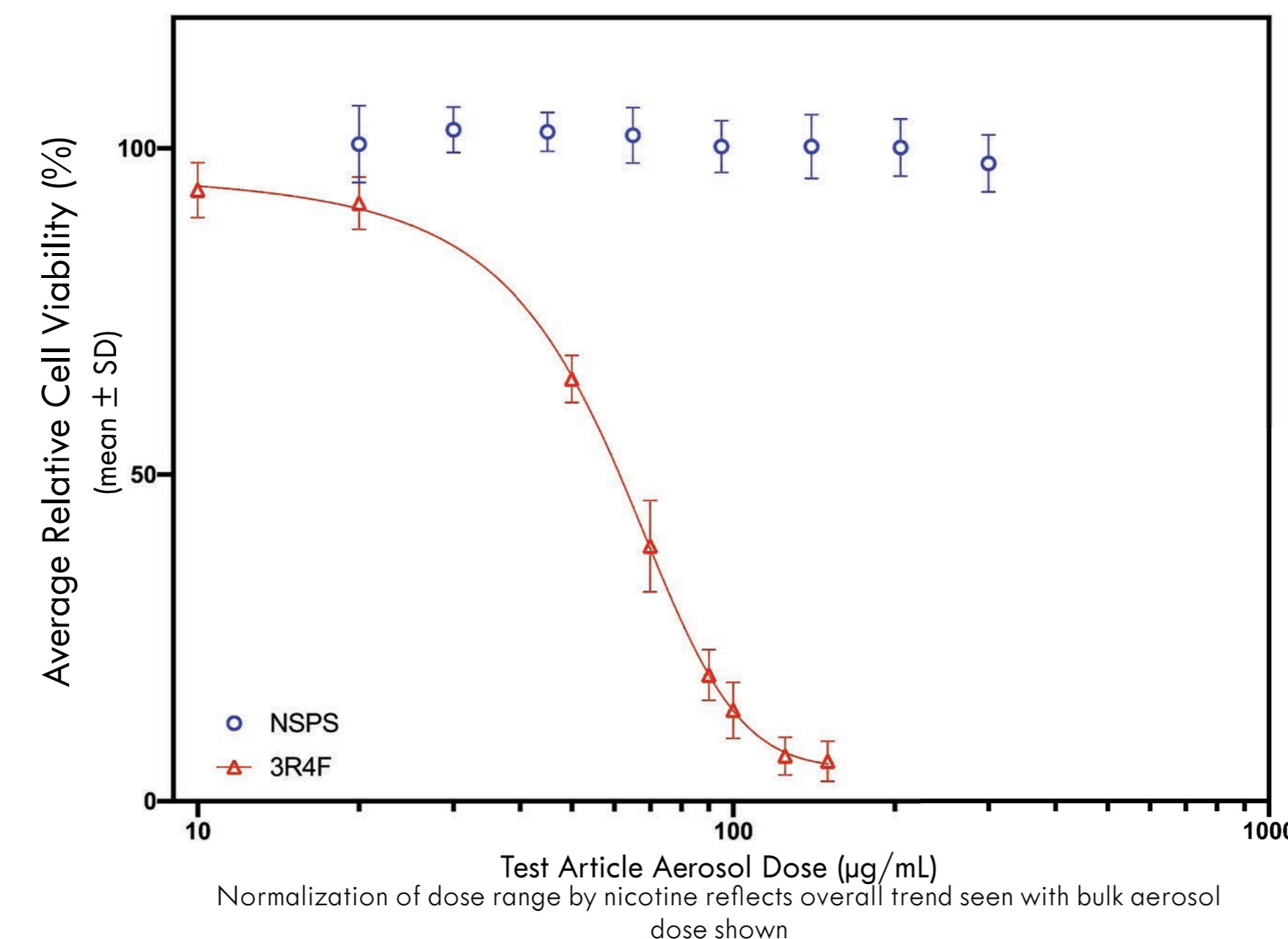


Fig. 2 : Comparison of Alpine Berry NSPS Aerosol with TPM from 3R4F Reference Cigarette

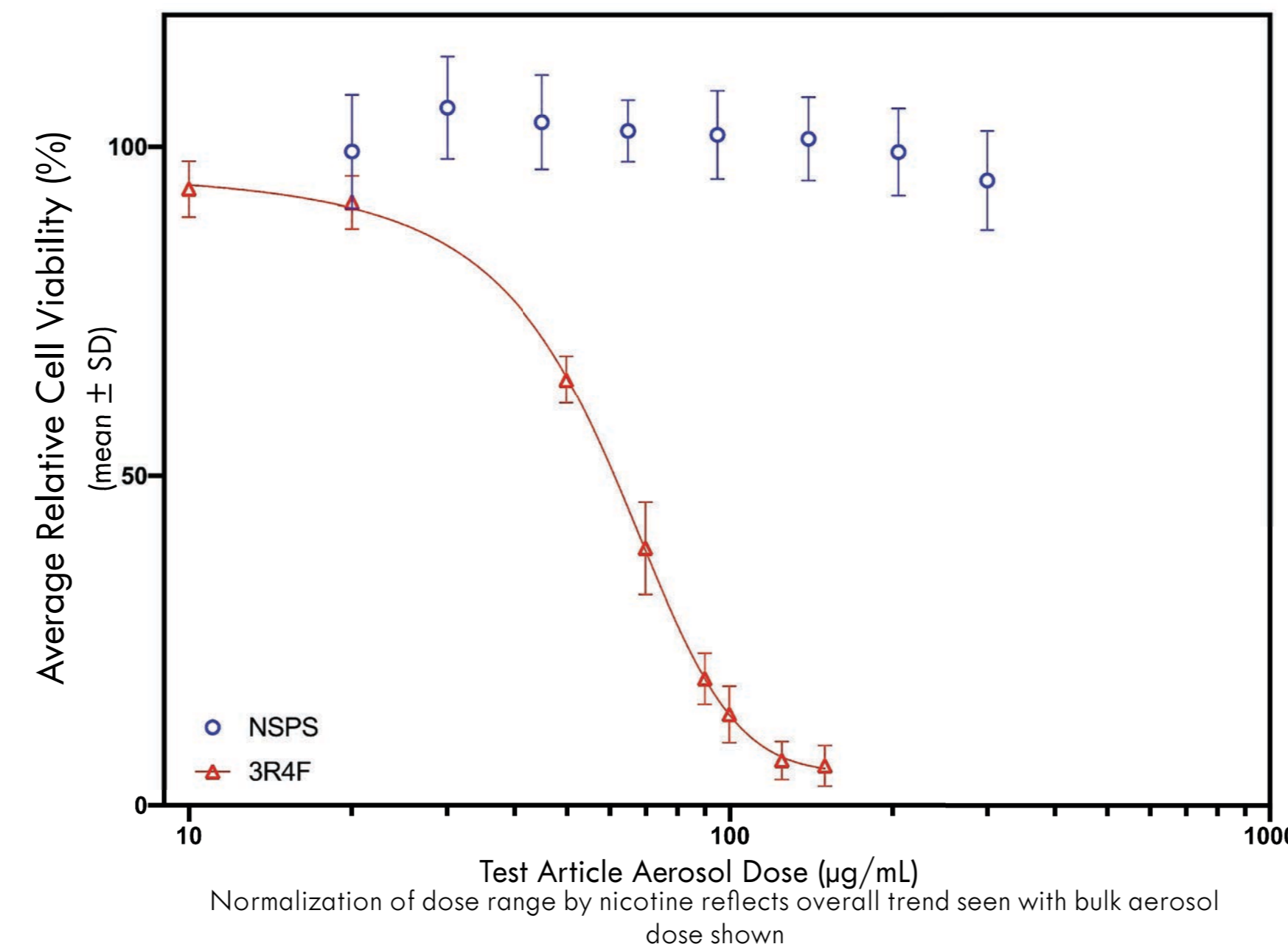


Fig. 5 : Comparison of Mango Nectar NSPS Aerosol with TPM from 3R4F Reference Cigarette

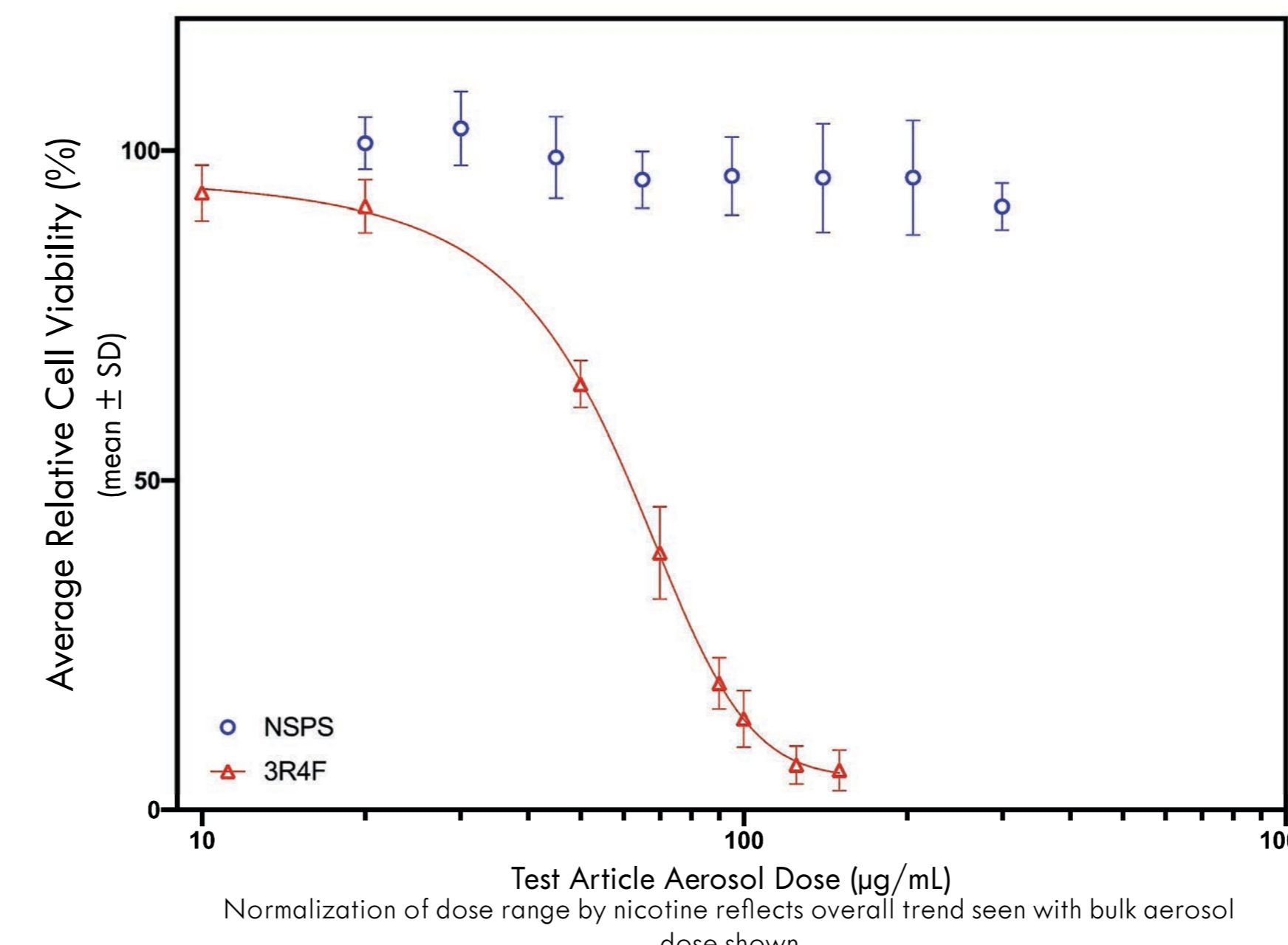


Fig. 4 : Comparison of Golden Tobacco NSPS Aerosol with TPM from 3R4F Reference Cigarette

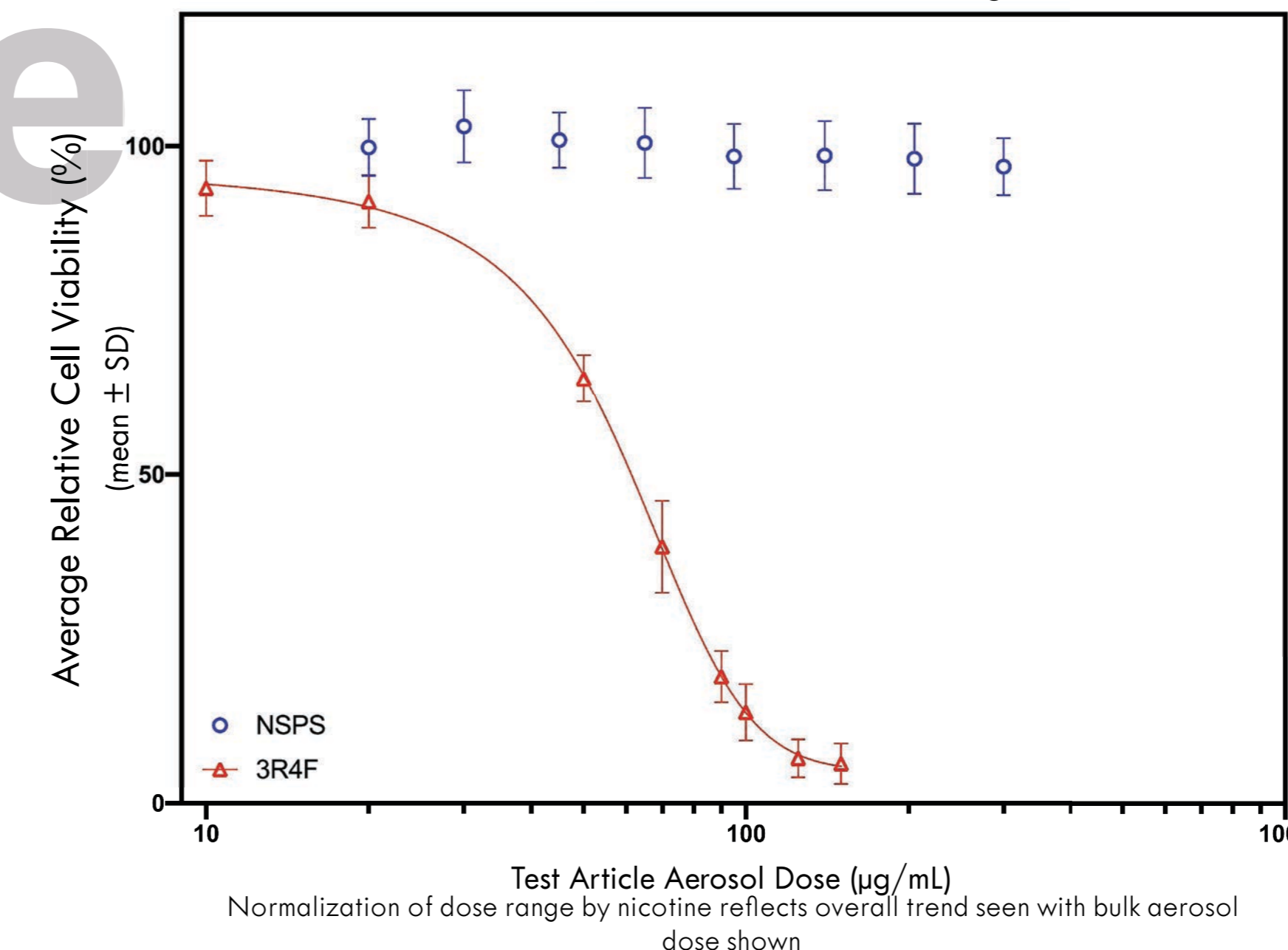


Fig. 7 : Comparison of Virginia Tobacco NSPS Aerosol with TPM from 3R4F Reference Cigarette

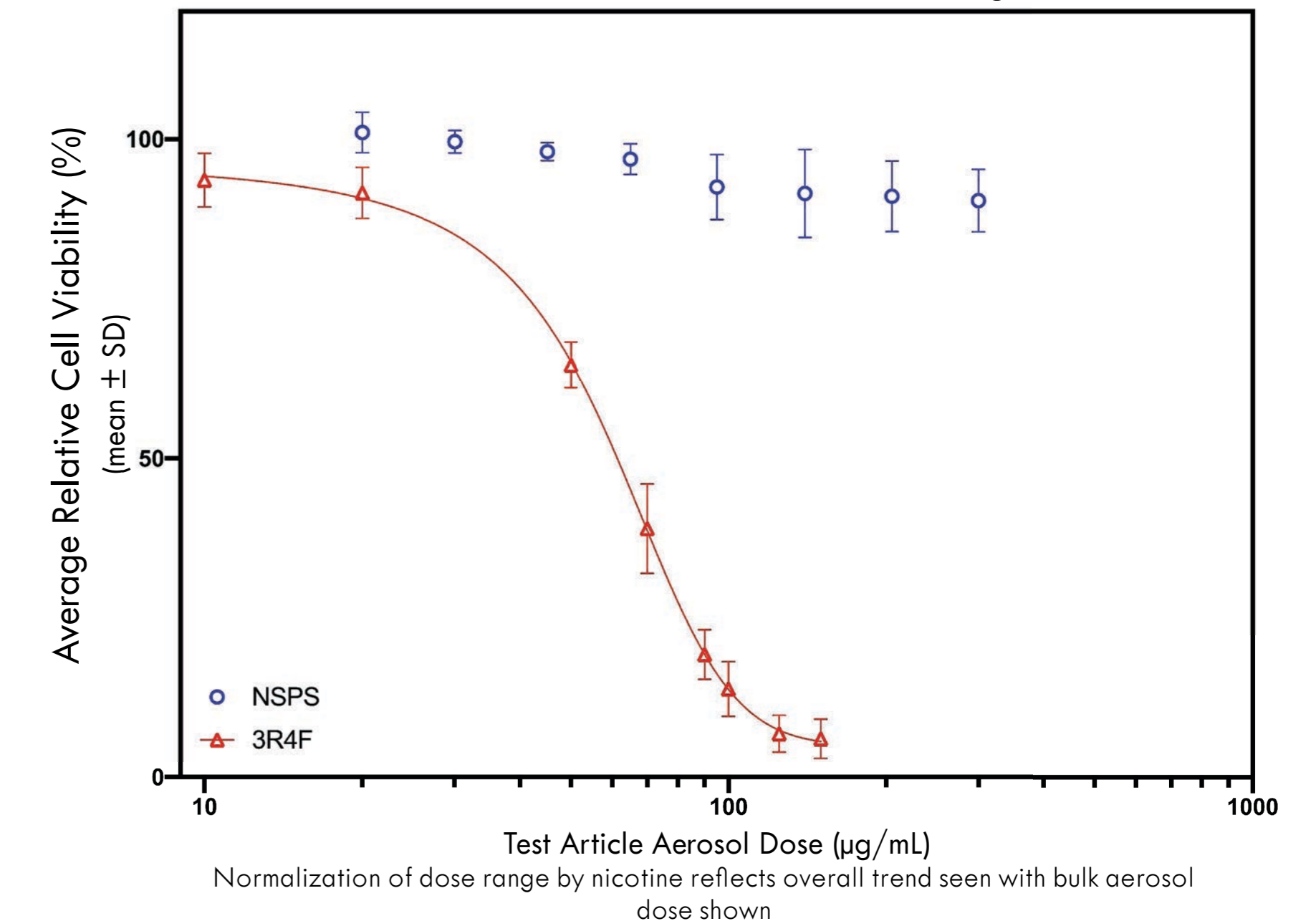


Fig. 3 : Comparison of Glacier Mint NSPS Aerosol with TPM from 3R4F Reference Cigarette

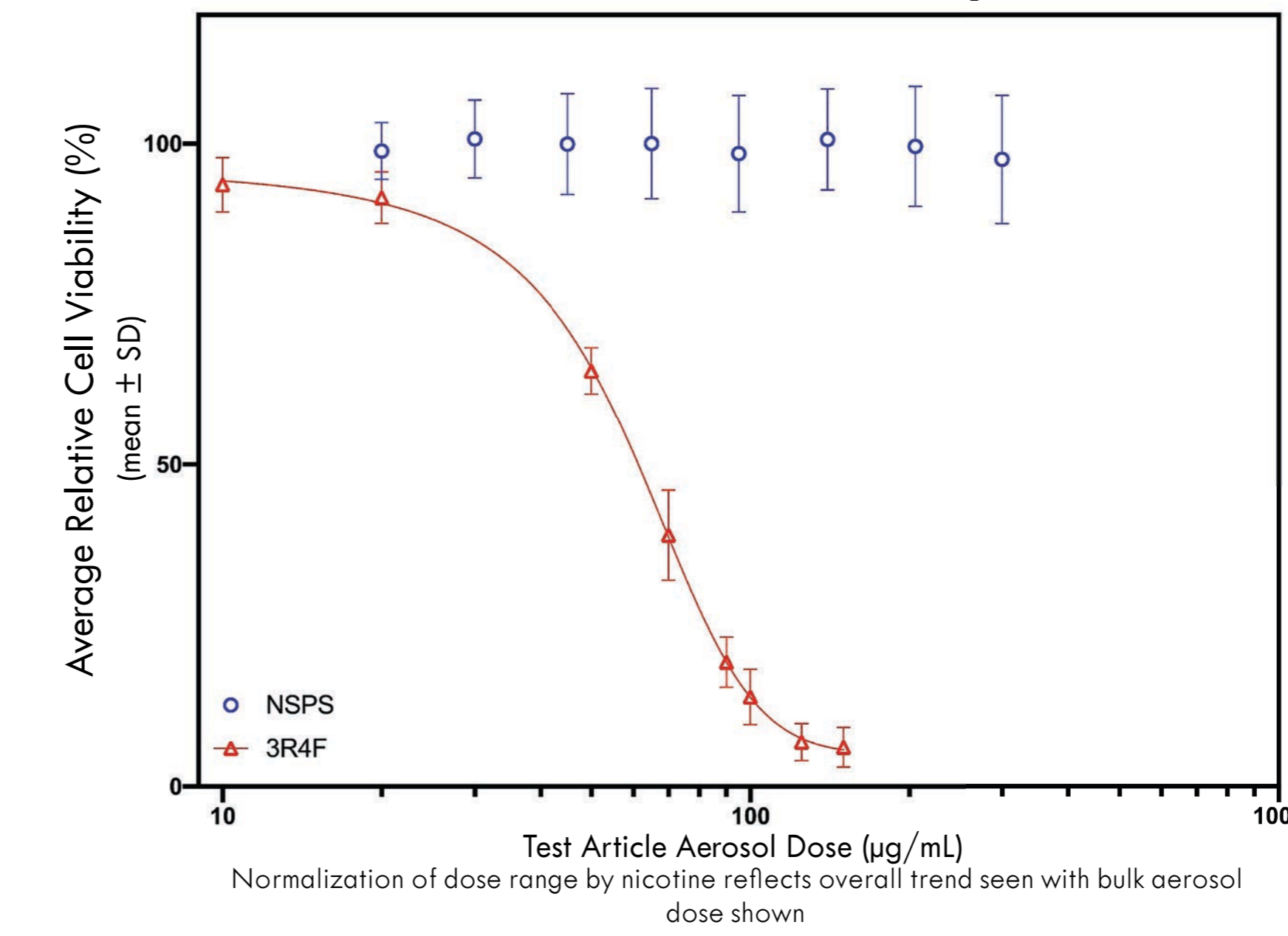
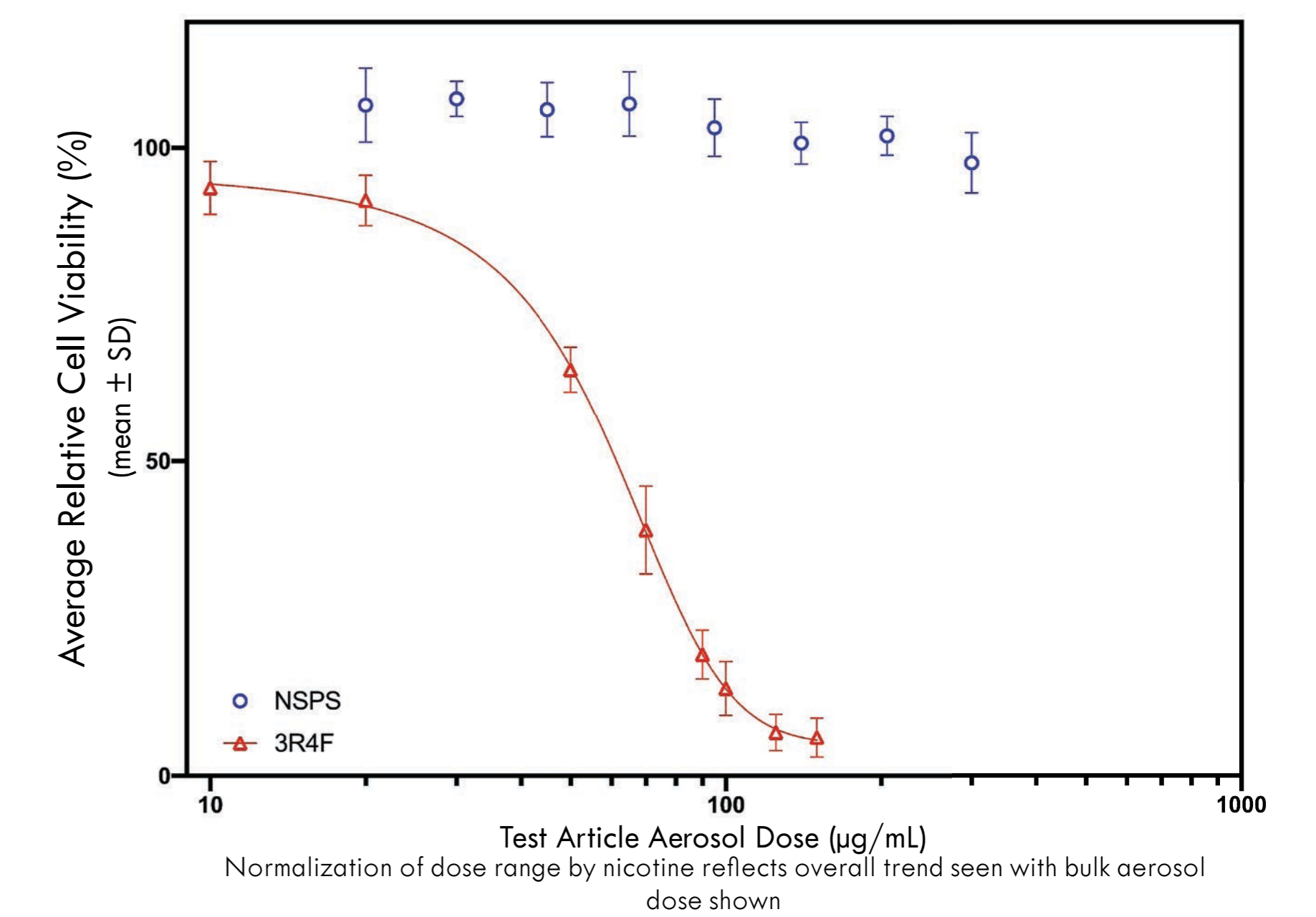


Fig. 5 : Comparison of Royal Creme NSPS Aerosol with TPM from 3R4F Reference Cigarette



## Conclusion

Under the experimental conditions and based on the criteria for Evaluation of Cytotoxic Response (ISO 10993-5), NSPS aerosol condensates generated from the 9 mg/mL NSPS test articles were found to be non-cytotoxic.

## References

1. OECD, "Guidance Document on Using Cytotoxicity Tests to Estimate Starting Doses For Acute Oral Systemic Toxicity Tests" (TG 129), July 2010.
2. M.Scian, "In Vitro Testing of an Ethanol Collection Method Combining Particulate and Gas-Vapor Phase Components: Neutral Red Assay", Sep. 2019.
3. CORESTA, "Routine Analytical Machine For E-Cigarette Aerosol Generation And Collection - Definitions And Standard Conditions" (Method No. 81), June 2015.
4. M. Misra, G. Gilman, P. Desai, "Neutral Red Uptake (NRU) Cytotoxicity Analysis of Aerosol Generated From a Temperature-Regulated Nicotine-Salt Based Ends Product", Tobacco Science Research Conference, Memphis, Tennessee, Dec. 2018.