

Separate Versus Combined Aerosol Fractions for Electronic Nicotine Delivery Systems (ENDS) *in vitro* Toxicological Evaluations

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INTRODUCTION

Regulatory *in vitro* assay batteries are widely used for hazard characterization of tobacco and nicotine products. For electronic nicotine delivery systems (ENDS), these evaluations have largely relied on aerosol collection approaches originally developed for combustible cigarettes, in which the particulate phase — aerosol collected matter (ACM) for ENDS, or total particulate matter (TPM) for cigarettes — and the gas–vapor phase (GVP) are trapped, prepared, and tested as separate fractions.

Although well established, separate-fraction testing evaluates each phase in isolation and does not fully reflect the whole-aerosol exposure that occurs during real-world product use. Because ENDS aerosols are inhaled as a mixture of particulate and vapor constituents, a test sample that replicates similar exposure may offer a more representative basis for *in vitro* hazard characterization.

To address this limitation, an alternative approach was investigated whereby ACM and GVP fractions were generated independently and subsequently combined at a 1:1 (v/v) ratio for *in vitro* assessment. This methodology has previously been described for combustible products in the Health Canada Neutral Red Uptake method (T-502).¹ Its performance in three *in vitro* assays was evaluated in parallel with the well-established separate fraction testing strategy.

AIMS

- Analytically characterize the exposures achieved with each strategy in terms of aerosol mass and nicotine delivery across products and assays, together with assessing the comparability of primary constituents and selected carbonyls.
- Determine whether the combined-fraction strategy provides hazard characterization comparable to the standard separate-fraction approach while more closely reflecting whole-aerosol exposure by comparing the toxicity profiles of a reference combustible cigarette (1R6F) and three commercially available ENDS using three established regulatory *in vitro* assays: the Ames assay (mutagenicity), the Neutral Red Uptake assay (cytotoxicity), and the *in vitro* Micronucleus assay (genotoxicity).

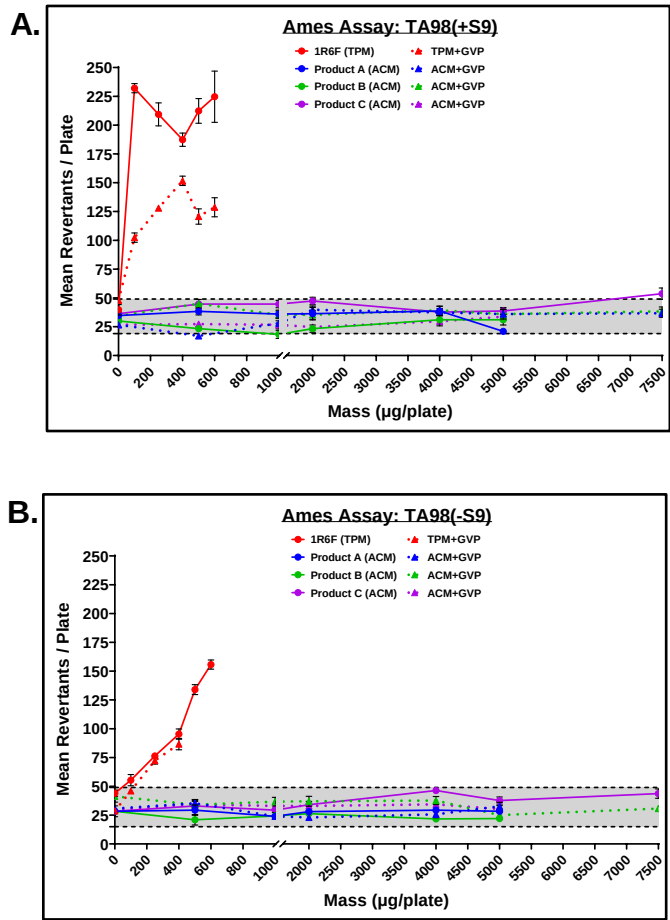
METHODS

- For separate fraction testing:**
 - TPM test samples were collected at concentrations up to 40 mg TPM/mL in DMSO and GVP test samples at concentrations up to 40 mg TPM equivalent/mL in PBS.
 - ACM test samples were collected at concentrations of 200 mg ACM/mL in DMSO and GVP test samples at concentrations of 200 mg ACM equivalent/mL in PBS.
- For combined fraction testing:**
 - TPM/ACM and GVP test samples were collected in the same manner as for separate fraction testing and then combined at a 1:1 ratio (v/v) to give a final stock concentration of up to 20 mg TPM equivalent/mL for 1R6F or 100 mg ACM equivalent/mL for market ENDS products in DMSO:PBS. To account for the dilution resulting from combining the fractions at a 1:1 ratio (v/v), the total volume applied in the *in vitro* assays was doubled to match the exposures achieved using separate fractions.
 - The maximum test sample concentration evaluated in the *in vitro* assays was either limited by toxicity or by solvent restrictions.
 - All test samples were characterized for nicotine and glycerol (TPM, ACM and TPM/ACM+GVP) and select carbonyls (GVP and TPM/ACM+GVP).

RESULTS

Figure 1. Ames Assay.

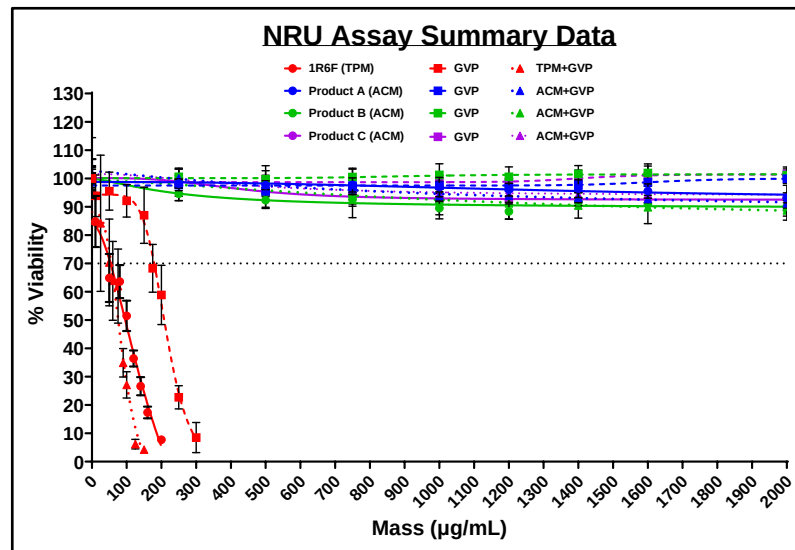
TPM/ACM, GVP (data not shown) and TPM/ACM+GVP test samples were tested at concentrations up to 1000 µg/plate (1R6F), and up to 10000 µg/plate (ENDS). Data are shown for TPM/ACM and TPM/ACM+GVP test samples in strain TA98 **A.** with (+S9) and **B.** without (-S9) metabolic activation. Data are normalized to mass.



Mutagenicity

- 1R6F TPM test samples** induced mutagenic responses in TA98 (+/-S9) and TA1537 (+S9).
- 1R6F TPM+GVP test samples** induced mutagenic responses in TA98 (+/-S9) and TA1537 (+/-S9).
- Exposure** in terms of mass was comparable across both test sample formats for 1R6F and ENDS test items.
- ENDS test samples** were negative in the Ames assay even at 10-15-fold higher concentrations than 1R6F.
- All GVP fractions were negative (data not shown).

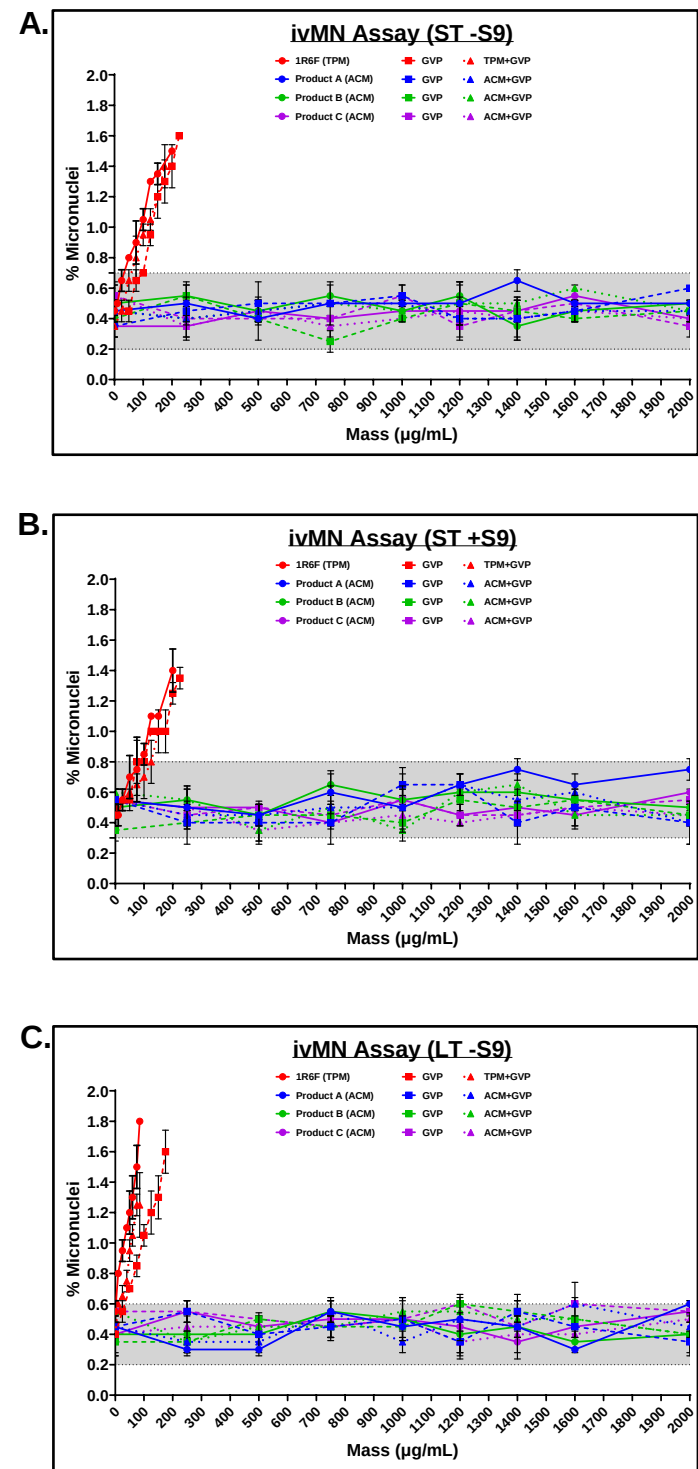
Figure 2. Neutral Red Uptake (NRU) Assay. TPM and TPM+GVP test samples were tested at concentrations up to 200 µg/mL and 300 µg/mL for GVP test samples (1R6F). All ENDS test samples were tested up to 2000 µg/mL. Cytotoxicity results are shown for all test samples. Data are normalized to mass.



Cytotoxicity

- 1R6F TPM, GVP, and TPM+GVP test samples** induced dose-dependent reductions in viability with IC₅₀ values of 96.0 µg/mL, 207.8 µg/mL, and 78.6 µg/mL.
- Exposure** in terms of mass was comparable across both test sample formats for 1R6F and ENDS test items.
- ACM, GVP, & ACM+GVP test samples from all ENDS test items** were non-cytotoxic at up to 10-fold higher concentrations than 1R6F.

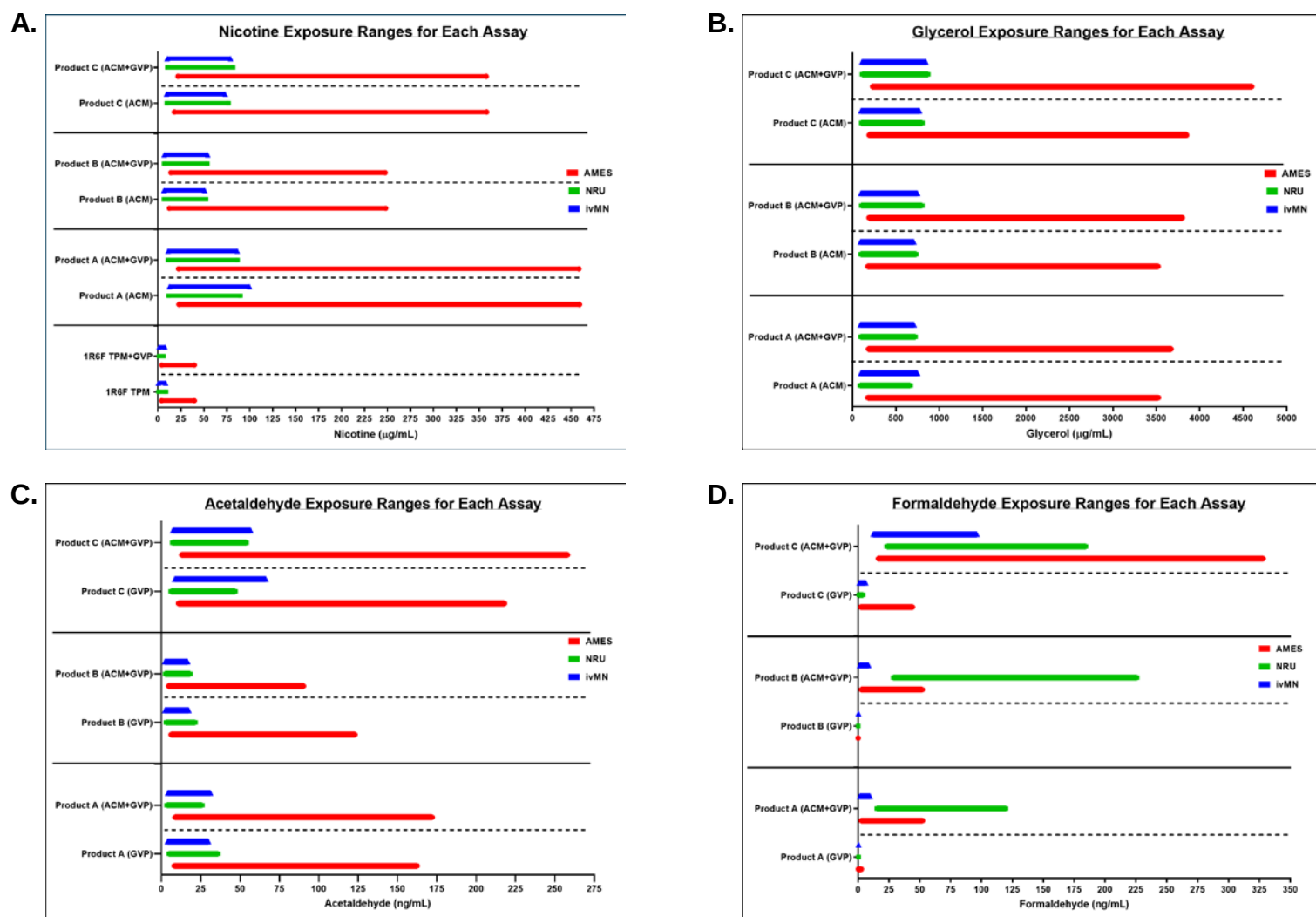
Figure 3. *In vitro* Micronucleus (ivMN) Assay. TPM/ACM, GVP, and TPM/ACM+GVP test samples were tested at concentrations up to 225 µg/mL (1R6F) and up to 2000 µg/mL (ENDS). Test samples were assessed across three schedules and are shown below for **A.** Schedule I (short term without (-S9) metabolic activation); **B.** Schedule II (short term +S9); **C.** Schedule III (long term -S9). Data are normalized to mass.



Genotoxicity

- 1R6F TPM, GVP, & TPM+GVP test samples** induced genotoxicity in all three treatment schedules.
- Exposure** in terms of mass was comparable across both test sample formats for 1R6F and ENDS test items.
- ACM, GVP, & ACM+GVP test samples** from tested ENDS products induced no genotoxicity even when tested at up to 10-fold higher concentrations than 1R6F.

Figure 4. Primary Constituent and Carbonyl Exposure Analysis. TPM/ACM and TPM/ACM+GVP test samples were analyzed for the primary constituents **A.** nicotine and **B.** glycerol while GVP and TPM/ACM+GVP test samples were for select carbonyls including **C.** acetaldehyde and **D.** formaldehyde to compare their corresponding assay exposure concentrations for each *in vitro* assay.



Chemistry Analysis

- The minimum and maximum exposure concentrations for **nicotine and glycerol** were comparable across test sample formats for all test products in each assay. (Glycerol values for 1R6F were much lower compared with ENDS products and are therefore not shown.)
 - Measurement of select carbonyls revealed significantly lower levels of acetaldehyde and formaldehyde for all ENDS products compared with 1R6F (data not shown).
 - Acetaldehyde exposure ranges were well-aligned between test sample formats with respect to *in vitro* exposures for all products tested. (Acetaldehyde values for 1R6F are not shown.)
 - Formaldehyde** values appeared significantly higher in the ACM+GVP test sample format for 1R6F (data not shown) and tested ENDS products.*
- (* Results due to high background levels arising from DMSO reagent blank and DMSO+PBS air blank, see Study Limitations)

CONCLUSIONS

- Comparable exposure to TPM/ACM, primary constituents, and select carbonyls was achieved between the test sample formats across all *in vitro* assays.
- 1R6F was considered mutagenic, cytotoxic, and genotoxic, while all tested ENDS products were considered non-mutagenic, non-cytotoxic, and non-genotoxic; these results were independent of test sample format.
- Collectively, both sample formats revealed the same conclusion: ENDS products have substantially lower biological activity in the *in vitro* assays compared to 1R6F.
- Without any loss of potential biological activity as assessed in these three assays, combined fraction testing provides a more representative real-world exposure sample format while reducing the number of test samples, testing time, and associated study costs.

STUDY LIMITATIONS

- Determination of formaldehyde exposure in the *in vitro* assays was negatively impacted in the combined fractions.
- Root cause analysis for the high formaldehyde background revealed the source was the DMSO reagent used for sample preparations and chemical analyses.
- To mitigate the elevated background formaldehyde levels observed, subsequent studies will employ a DMSO grade with improved biological and chemical compatibility.

REFERENCES

- Health Canada, Official Method T-502, Neutral Red Uptake Assay for Mainstream Tobacco Smoke, 2017.
- ISO 20778:2018 Cigarettes — Routine analytical cigarette smoking machine — Definitions and standard conditions with an intense smoking regime.
- ISO 20768:2018 - Vapour Products — Routine Analytical Vaping Machine — Definitions and Standard Conditions.
- OECD Guideline for Testing Chemicals, Bacterial Reverse Mutation Assay No. 471, adopted on 26 June 2020.
- OECD Guideline for Cytotoxicity Tests to Estimate Starting Doses for Acute Oral Systemic Toxicity Test, No. 129, adopted on 20 July 2010.
- OECD Guideline for In Vitro Mammalian Cell Micronucleus Test, No. 487, adopted on 3 July 2023.



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