UTILIZATION OF A WHOLE SMOKE EXPOSURE SYSTEM FOR THE COMPARISON OF MAINSTREAM CIGARETTE SMOKE, GAS VAPOR PHASE AND TAR MUTAGENICITY

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ABSTRACT #2091

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This study was conducted to further optimize smoke exposure procedures, measure the mutagenicity of mainstream cigarette whole smoke (WS) and the contribution the gas vapor phase (GVP) and wet total particulate matter (WTPM) impart to the WS activity, as determined by the *Salmonella* Reverse Mutation (Ames) Assay. WS, GVP and WTPM were prepared from Kentucky Reference 3R4F cigarettes smoked under ISO puff profile (35 mL volume, 2 second puff duration and 1 minute puff interval) on a VITROCELL® VC10 smoking robot. TA98 and TA100, in the presence (S9+) and absence (S9-) of metabolic activation, were exposed to WS or GVP from three (3) 3R4F cigarettes via the VITROCELL® Dilution / Distribution System with dilution air flow rates set at 1, 2, 4 and 8 L / minute, allowing the delivery of four doses of WS or GVP to the Ames exposure modules during each exposure. For GVP experiments, a Cambridge filter pad was placed in-line prior to the puffing syringe in order to remove the smoke particulate fraction, which was subsequently extracted in dimethylsulfoxide (DMSO) for use in WTPM exposures. Quantification of several carbonyls verified the delivery of GVP to the bacteria. WTPM exposures (S9+/S9-) utilized a 30 minute preincubation with DMSO limited to 2.5% v/v final concentration. WS mutagenicity was detected in both strains (S9+/S9-); however, TA100 S9- WS activity was approximately 30% of TA100 S9+ WS activity while TA98 S9- WS activity was considerably lower at 3% of measured TA98 S9+ WS activity. No GVP mutagenicity was detected in both strains (S9+/S9-). Lack of GVP activity was not due to cytotoxicity since no significant decrease in cell viability was observed over the delivered GVP dose range. WTPM activity was detected in both strains (S9+ only) at approximately 67% and 94% of the WS activities measured in TA100 and TA98, respectively. Under the exposure conditions used in this study, the majority of the WS mutagenic activity was found to reside in the particulate fraction, with no apparent contribution to WS activity coming from GVP.

MATERIALS & METHODS

CIGARETTE SMOKE PREPARATIONS & EXPOSURES:

Kentucky Reference 3R4F cigarettes conditioned at least 18 hours in packs; 60% relative humidity (RH), ~23°C prior to smoking.

All cigarettes smoked (8 puffs / cigarette) on a VITROCELL® VC10 smoking robot (Figure 1) following ISO puff profile: 35 mL puff volume, 2 second draw, 1 minute puff interval.

Wet Total Particulate Matter (WTPM) collected on Cambridge filter pads, extracted in dimethylsulfoxide (DMSO) to a final concentration of 24 mg / mL, stored at -80°C prior to analysis.

Whole Smoke (WS) and Gas Vapor Phase (GVP) exposures performed on a VITROCELL® VC10 with smoke dilution system and Ames Exposure Modules. Smoke dilution air flows ranged from 0.1 L / min – 8.0 L / min as specified in figure legends.

AMES ASSAY[1, 2]:

Post-mitochondrial supernatant, Aroclor 1254-induced male Sprague-Dawley rat liver in 0.15M KCl (Moltox; Boone, NC).

S9-Mix: 33mM KCl, 8mM $MgCl_2$, 5mM Glucose-6-phosphate, 4mM NADP, sodium phosphate buffer (0.1M, pH 7.4), 5% v/v S9-fraction.

Preincubation Ames assays (WTPM only): TA98 or TA100 @ ~2 - 4X10^9 bacteria / mL in 200 µL S9-mix (or PBS) dosed with 5 µL WTPM or DMSO (vehicle control), mixed, preincubated for 30 minutes @ 37°C shaking @ 250 rpm in capped tubes and plated onto 0.4% minimal glucose agar plates (35 mm) supplemented with 0.05 mM Histidine / Biotin.

WS & GVP Exposures: TA98 or TA100 @ ~2 - 4X10^9 bacteria / mL in 200 µL S9-mix (or PBS) were spread on 0.4% minimal glucose agar plates (35 mm) supplemented with 0.05 mM Histidine / Biotin, exposed to WS or GVP from three 3R4Fcigarettes.

Revertant colonies counted after 48 hours of incubation @ 37°C.

Activity reported as revertants per cigarette was calculated from the linear portion of the dose response curve and compared using GraphPad Prism v. 5.02 (slope analysis, two tailed; for comparisons, statistical significance @ $p < 0.05$).

VIABILITY ASSAY:

Viability assays were conducted as in the Ames Assays with the following differences:
1) TA98 and TA100 were at a concentration of ~1000 – 2000 bacteria / mL (plating 200 µL = ~ 200 – 400 bacteria / plate).
2) Utilized nutrient agar plates instead of the minimal glucose agar plates.

Bacteria exposed as above and viable colonies were counted after 48 hours of incubation @ 37°C.

SMOKE DELIVERIES

WS: Exposures conducted as in the Ames Assays. VITROCELL® Laser Photometers positioned between dilution system and exposure module. 35 mm plates with 4 mL DMSO. Dilution air flow rates 0.1 – 8.0 L / min. Ex / Em: 355 / 485 measurements of smoke-exposed DMSO extrapolated to standard curve to determine µg captured in DMSO[1]. Plot laser photometer measurements versus DMSO-captured particulate (V vs µg) to determine delivered dose during WS exposures (Figure 2).

GVP: Exposures conducted as in the Ames Assays (GVP). Smoke delivered determined by WTPM delivery, dilution air flow rate, number of cigarettes and number of puffs per cigarette (calculation spreadsheet provided by VITROCELL®).

Carbonyls: Exposures conducted as in the Ames Assays for GVP (5 cigarettes, dilution air flow rates 1.0 – 4.0 L / min). 35 mm plates with 4 mL DNPH (2,4-Dinitrophenylhydrazine) in exposure modules. Captured carbonyls quantified by HPLC.

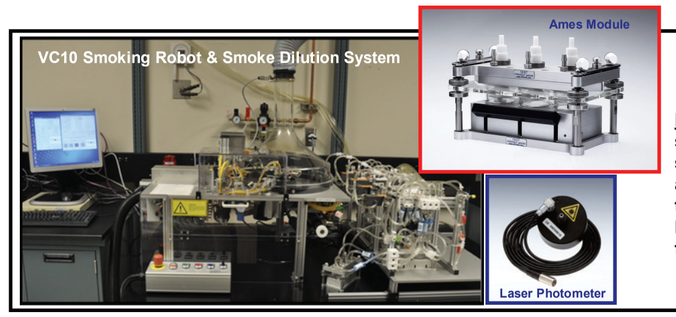

Figure 1: VITROCELL® VC10 smoking robot, smoke dilution system, Ames exposure module and laser photometer utilized in this study. Photos of Ames Module and Laser Photometer from www.vitrocell.com.

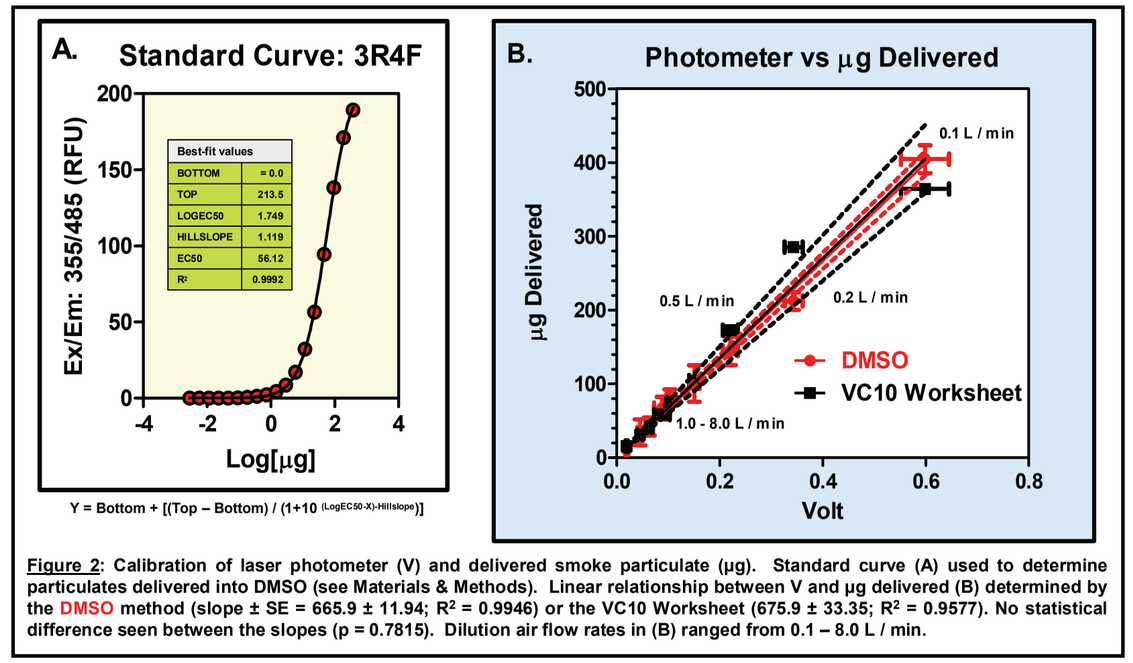

Figure 2: Calibration of laser photometer (V) and delivered smoke particulate (µg). Standard curve (A) used to determine particulates delivered into DMSO (see Materials & Methods). Linear relationship between V and µg delivered (B) determined by the DMSO method (slope ± SE = 665.9 ± 11.94; R^2 = 0.9946) or the VC10 Worksheet (675.9 ± 33.35; R^2 = 0.9577). No statistical difference seen between the slopes (p = 0.7815). Dilution air flow rates in (B) ranged from 0.1 – 8.0 L / min.

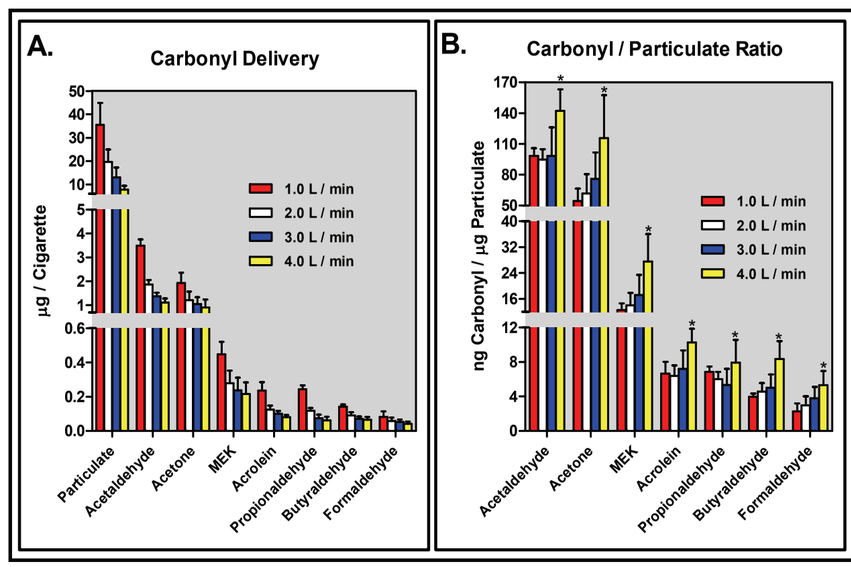

Figure 3: Quantification (± SD) of delivered carbonyls per cigarette (A) and the ratio of delivered carbonyls (ng) per µg delivered smoke particulates (B). As expected, with increasing dilution (dilution air flow rates ranged from 1.0 – 4.0 L / min) there is a decrease in delivered carbonyls. The ratio of carbonyl to particulate increased with increasing dilution, significantly (*$p < 0.05$) at highest dilution. This suggests differences exist in deposition and/or flow kinetics of particulate versus gas phase components in relation to dilution air flow rate.

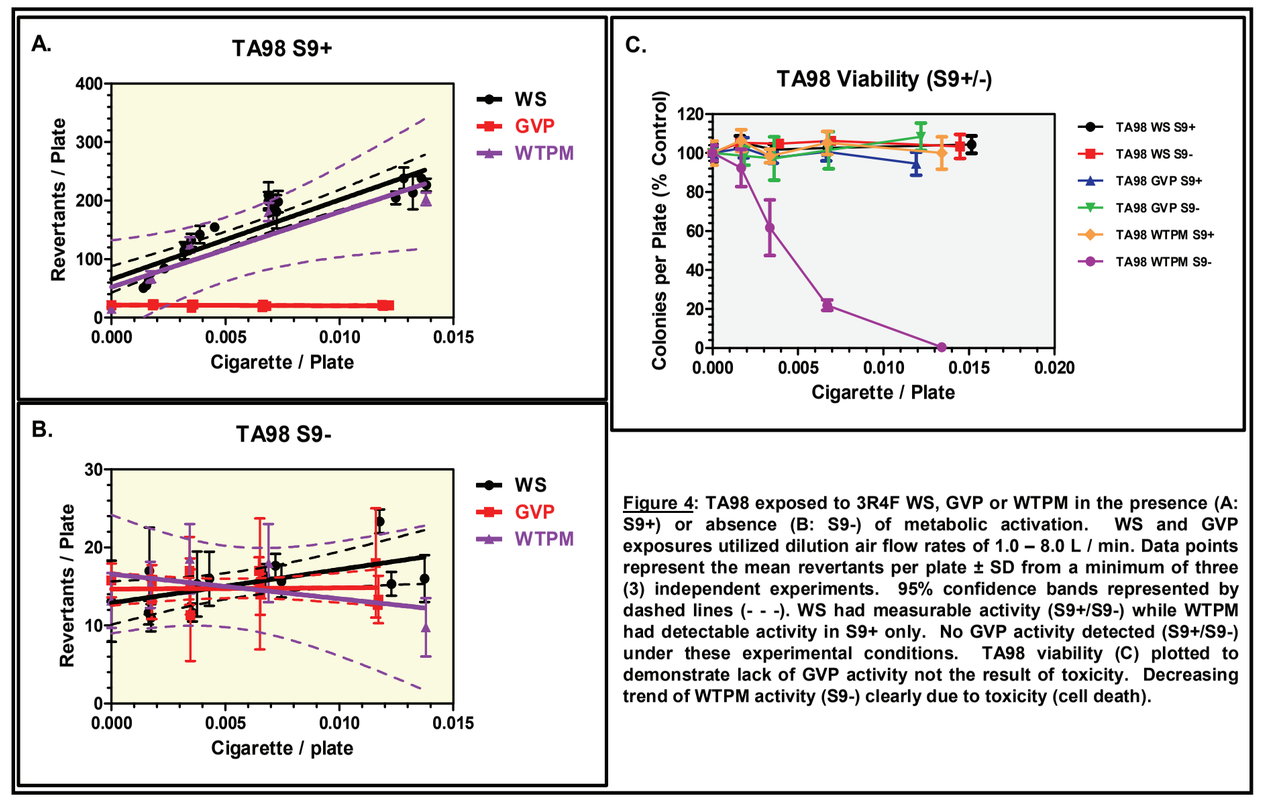

Figure 4: TA98 exposed to 3R4F WS, GVP or WTPM in the presence (A: S9+) or absence (B: S9-) of metabolic activation. WS and GVP exposures utilized dilution air flow rates of 1.0 – 8.0 L / min. Data points represent the mean revertants per plate ± SD from a minimum of three (3) independent experiments. 95% confidence bands represented by dashed lines (- - -). WS had measurable activity (S9+/S9-) while WTPM had detectable activity in S9+ only. No GVP activity detected (S9+/S9-) under these experimental conditions. TA98 viability (C) plotted to demonstrate lack of GVP activity not the result of toxicity. Decreasing trend of WTPM activity (S9-) clearly due to toxicity (cell death).

RESULTS

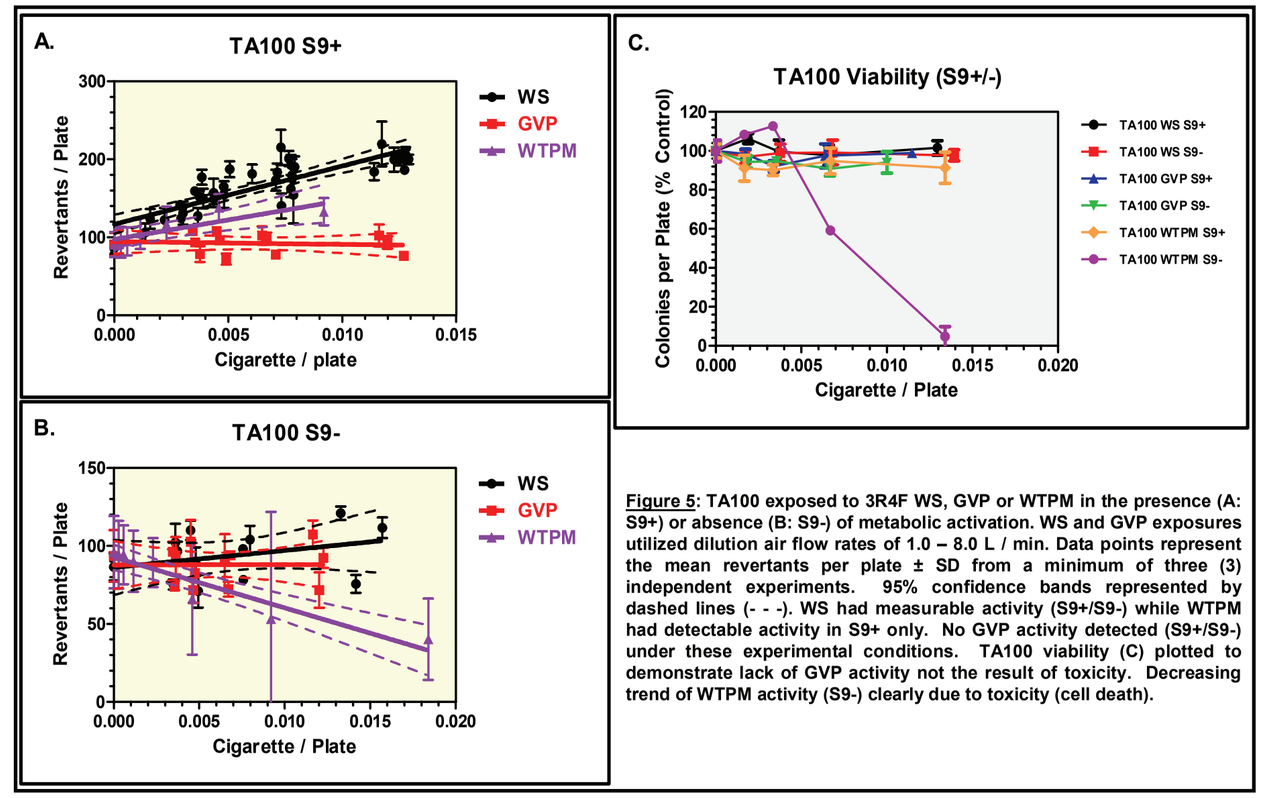

Figure 5: TA100 exposed to 3R4F WS, GVP or WTPM in the presence (A: S9+) or absence (B: S9-) of metabolic activation. WS and GVP exposures utilized dilution air flow rates of 1.0 – 8.0 L / min. Data points represent the mean revertants per plate ± SD from a minimum of three (3) independent experiments. 95% confidence bands represented by dashed lines (- - -). WS had measurable activity (S9+/S9-) while WTPM had detectable activity in S9+ only. No GVP activity detected (S9+/S9-) under these experimental conditions. TA100 viability (C) plotted to demonstrate lack of GVP activity not the result of toxicity. Decreasing trend of WTPM activity (S9-) clearly due to toxicity (cell death).

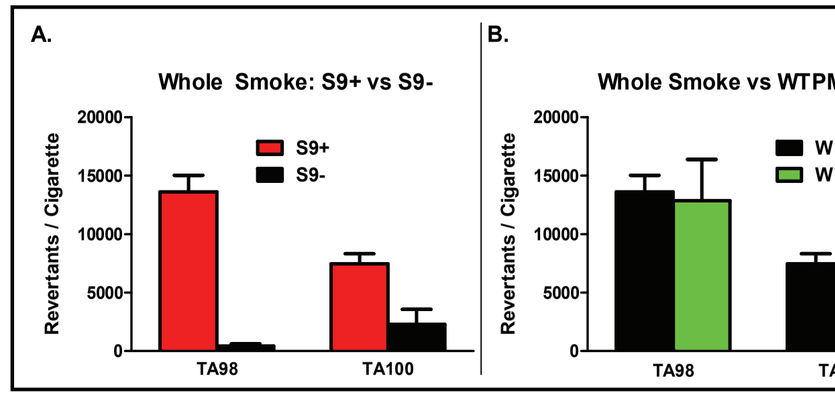

Figure 6: Comparing WS S9+/S9-activity (A) or WS vs WTPM (B: S9+ only). WS activity is predominately S9-dependent in both strains. WTPM activity appears to account for the majority of WS activity (TA98, ~94%; TA100, ~67%).

Revertants / Cigarette (Mean ± SEM)			
Strain	Whole Smoke		WTPM
	S9+	S9-	S9+
TA98	13,590 ± 1425	429 ± 174	12,842 ± 3335
TA100	7,466 ± 839	2,271 ± 1289	5,000 ± 1318

SUMMARY

- "Real-time" measurement of smoke delivery has been established with in-line laser photometers. VC10 worksheet calculation of 3R4F smoke delivery is highly representative of the measurements achieved with the laser photometers at the smoke dilutions used in this study (Figure 2).
- Quantification of several gas vapor phase constituents (carbonyls) demonstrated a decrease in delivery with increased dilution air flow rates; however, the ratio of carbonyl / particulate deliveries changed, suggesting differences in deposition and / or flow dynamics of particulate versus gas phase constituents with increasing dilution air flow rates (Figure 3).
- WS (S9+ / S9-) and WTPM (S9+) exposures in both strains resulted in an increase of revertant colonies in a dose dependent manner (Figures 4 & 5). The inability to detect any WTPM (S9-) mutagenicity was due to toxicity (cell death), most likely an effect of the preincubation exposure method. Detecting WS (S9-) activity, albeit at low levels, strengthens the argument for utilizing WS exposure methods.
- Under the experimental conditions utilized in this study, no GVP activity (S9+ / S9-) was detected in both strains (Figures 4 & 5).
- Under the exposure conditions used in this study, the majority of the Whole Smoke mutagenic activity was dependent on metabolic activation (S9+) and found to reside in the smoke particulate fraction (Figure 6), with no apparent contribution to Whole Smoke activity coming from the Gas Vapor Phase.

REFERENCES

1. Aufderheide, M and Gressmann, H. (2007) A modified Ames assay reveals the mutagenicity of native cigarette mainstream smoke and its gas vapour phase. *Experimental and Toxicologic Pathology*, 58, 383 – 392.
2. Maron, D. M. and Ames, B. N. (1983) Revised methods for the *Salmonella* mutagenicity test. *Mutation Research*, 113, 173 - 215.

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