IN VITRO TESTING OF AN ETHANOL COLLECTION METHOD COMBINING PARTICULATE AND GAS-VAPOR PHASE COMPONENTS: BACTERIAL REVERSION MUTATION (AMES) ASSAY

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BACKGROUND

Health Canada (HC) guidelines (T-502) for the collection and testing of cigarette smoke are used frequently for *in vitro* testing (1). The PP is collected in DMSO and can be tested for cytotoxicity (NRU assay), mutagenicity (Ames assay), and clastogenicity (MN assay), while the GVP phase is collected in PBS and only tested using the NRU assay. PBS has limited trapping efficiency of volatile or non-water-soluble compounds and must be used within 60 minutes of collection. These limitations could be overcome by collection of the PP and GVP together in Ethanol that might enhance trapping and stability of GVP components. We have tested the use of ethanol to collect PP and GVP components using the Health Canada (HC T-501 guideline) for Ames assay (2). Reference 3R4F cigarettes were used. Five strains of Salmonella bacteria (TA98, TA100, TA102, TA1535 and TA1537) were tested in the absence and presence of S9.

OBJECTIVES

Compare the mutagenicity and cytotoxicity of 3R4F condensates collected using Health Canada method and Enthalpy Analytical (EA) method in Bacterial Reversion Mutation (Ames) assay utilizing five strains of Salmonella tester strains.

METHODS

1. Collection of TPM, GVP&TPM (PBS) and GVP&TPM (EtOH)

Health Canada (HC) guidelines (T-502) was used for 3R4F cigarettes to collect Particulate Phase (PP) in DMSO, Gas phase in Phosphate Buffer Saline (PBS) and 1:1 mix of PP and Gas Phase (PP+GVP) as described in Figure 1. The same number of 3R4F cigarettes were smoked to collect PP and Gas phase together in ethanol. For Ames assay, PP, GVP+PP (1:1) and GVP+PP (Ethanol) were tested in increasing dose of TPM upto 250 ug per plate.

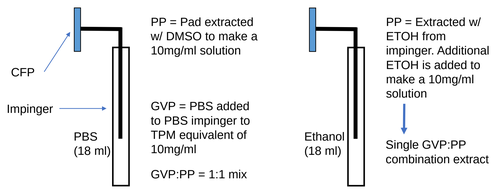


Figure1. Graphical representation of the extraction methods tested. CFP = Cambridge Filter Pad; PP = particulate phase; GVP = gas vapor phase; PBS = phosphate buffer solution; TPM = total particulate matter

2. Bacterial Reversion Mutation Assay to test the 3R4F condensates

Bacterial Reversion assay was used to determine the mutagenicity and cytotoxicity of the condensates. The five Salmonella strains used in this study is described in Table 1. The assays were performed using pre-incubation method as described earlier (3,4). Approximately 2 x 10^8 bacterial cells mixed with seven non-zero dosages of TPM collected from the two methods in the absence or presence of 5% S9 mix. As an incubator control, an appropriate amount of positive, vehicle, laboratory reference control (KY3R4F), smoked in ISO regime were also performed in the absence or presence of 5 % S9 mix. The positive control used in the assay is listed in Table 1. The mean number of revertants and bacterial background lawn thinning were determined after 48 hours of incubation at 37 °C.

Mutagenic Response:- more than 2-fold significant increase in mean number of revertants in treatment group as compared to vehicle control for TA100 and TA102 or at least 3-fold significant increase for TA98, TA1535 and TA1537.

Cytotoxic Response:- is determined by significant decrease in mean number of revertants and/or observing thinning or absence of bacterial lawn using Olympus inverted light microscope.

Table 1. Genetic Modification to the TA Salmonella strains used in the Ames Assay

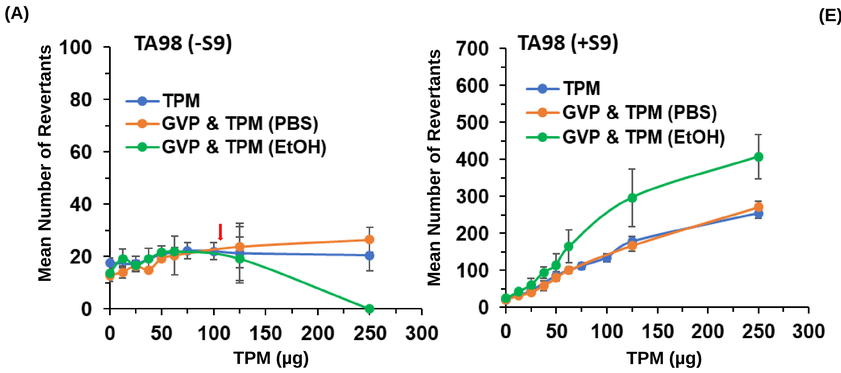
S. typhimurium Strain	Target allele	DNA repair	LPS	Biotin requirement	R-factor plasmid	Type of mutation detected
TA 98	hisD3052	uvrB	Rfa	bio-	pKM101	frameshift
TA 100	hisG46	uvrB	Rfa	bio-	pKM101	base pair substitution
TA 1535	hisG46	uvrB	Rfa	bio-	-	base-pair substitution
TA 1537	hisC3076	uvrB	Rfa	bio-	-	frameshift
TA 102	hisG428	-	Rfa	bio	pKM101	base-pair substitution

Table 2: Strain specific positive controls used

Tester Strain	S9 Mix	Positive Control	Concentration (µg/plate)
TA 98	+	Benzo[a]pyrene	2.50
TA 98	-	2-Nitrofluorene	1.00
TA 100	+	Benzo[a]pyrene	2.50
TA 100	-	Sodium azide	2.00
TA 1535	+	2-Aminoanthracene	2.50
TA 1535	-	Sodium azide	2.00
TA 1537	+	Benzo[a]pyrene	2.50
TA 1537	-	ICR-191	2.00
TA 102	+	2-aminoanthracene	15.00
TA 102	-	Mitomycin C	1.00

RESULTS

Comparison of TPM, GVP&TPM (PBS) and GVP&TPM (EtOH) using Bacterial Reversion Mutation (Ames) Assay



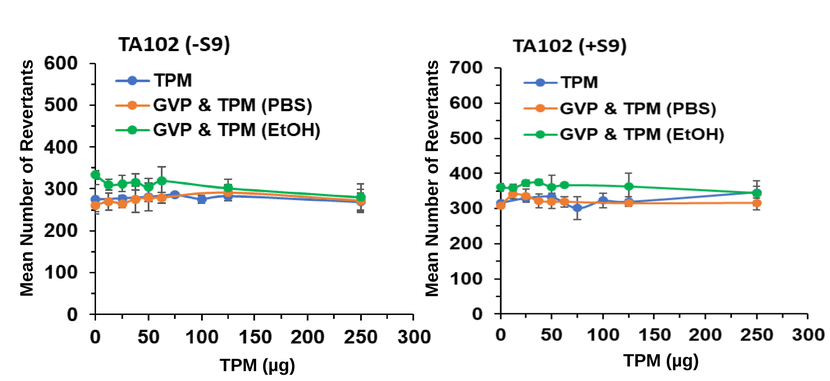


Figure 2: Mutagenicity and cytotoxicity of TPM, GVP&TPM (PBS) and GVP&TPM (EtOH). Mean number of revertants (average of three independent assays) in the absence of S9 or presence of S9 are shown as line graph. (A) TA98; (B) TA100; (C) TA1535; (D) TA1537; (E) TA102. TPM- PP fraction extracted in DMSO; GVP&TPM (PBS)- GVP+PP = 1:1 mix and GVP&TPM (EtOH)- condensates extracted in ethanol. Red arrow indicates the dose showing cytotoxicity.

Table 3. Incubator controls for Salmonella Ames strains*

(A) In the absence of S9 (-S9)

Treatment	TA98	TA100	TA1535	TA1537	TA102
Veh Control	14.44 ± 0.69	112.89 ± 19.25	9.22 ± 0.38	8.22 ± 1.84	300.0 ± 29.16
Ref TPM**	21.11 ± 1.64	109.33 ± 12.41	13.44 ± 1.26	8.56 ± 0.77	260.00 ± 6.64
Pos Control#	256.67 ± 31.64	605.11 ± 60.77	662.11 ± 22.71	2007.33 ± 675	1642.00 ± 275.40
Spon Rever	20.33 ± 3.51	109.00 ± 12.86	11.33 ± 6.26	11.33 ± 4.62	299.44 ± 15.11

(B) In the presence of 5%S9 (+S9)

Treatment	TA98	TA100	TA1535	TA1537	TA102
Veh Control	19.56 ± 2.69	107.44 ± 7.41	7.44 ± 1.39	5.33 ± 1.33	323.33 ± 10.97
Ref TPM**	200.78 ± 19.67	165.11 ± 1.64	15.00 ± 3.18	28.00 ± 5.49	349.33 ± 12.71
Pos Control#	336.33 ± 31.86	848.11 ± 46.86	176.78 ± 44.94	102.22 ± 2.80	999.00 ± 46.28

*- Mean number of revertants with SD from three independent assays
*- Ref TPM- 3R4F combustible prepared under ISO regime
#- Positive control chemicals for each Ames strains is indicated in Table 2

CONCLUSION

- In the absence of S9, ethanol condensate collecting PP+GVP together resulted in increased bacterial lawn cytotoxicity in TA98, TA100, TA1535 and TA1537. DMSO-extracted PP showed toxicity in TA98, TA100, TA1537 while PP+GVP in PBS only showed toxicity in TA1537.
- With metabolic activation, TPM+GVP in ethanol induced an 18-fold and 11-fold increase in the number of revertants in TA98 and TA1537 respectively. In comparison, DMSO extracted PP induced a 16-fold and 8-fold increase, while TPM+GVP in PBS induced a 14-fold and 6-fold increase in the same strains In the presence of S9, GVP & TPM-EtOH caused slightly more increase in revertants in TA98, TA100 and TA1537 as compared to TPM or GVP & TPM-PBS.
- Based on the Ames assay, we conclude that ethanol method allows for combined collection of PP+GVP components yielding a single whole-smoke extract significantly increasing the mutagenic and cytotoxic effects of the 3R4F condensates.

REFERENCES

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