Increasing the *Salmonella typhimurium* Reverse Mutation Assay's Sensitivity Following Exposure to Fresh Aerosols

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1. Introduction

The aerosol generated during tobacco combustion contains both total particulate matter (TPM) and a gaseous vapour phase (GVP). The particulate matter can be trapped on Cambridge filter pads and extracted from there using dimethylsulfoxide which can be tested for its mutagenic properties in a standard Ames assay setup (DeMarini et al., 2008). However, using this methodology might underestimate the products tested because the GVP cannot contribute to the biological activity. Hence it is crucial to develop systems that are able to quantify the mutagenicity of whole aerosols additionally to the conventional testing of the extracted TPM only (Kilford et al., 2014). Here we show that our system is able to quantify mutagenicity of mainstream whole smoke (WS) in OECD recommended tester strains TA98, TA100 and TA102 with the latter two also showing dose responses following the exposure to the filtered GVP from combusted tobacco products. The above mentioned principle of two phases holds also true for the vapour generated by electronic vapour products (EVP, e-cigarettes). We present the general applicability of the modified exposure system in combination with whole and filtered vapour from an EVP loaded with a formaldehyde (FA) spiked base liquid solution. FA was chosen as a representative carbonyl of EVPs (Grana et al, 2014). Its mutagenic activity in TA100 has already been shown (Dillon et al. 1998).

2. Materials and Methods

- Setup: The fresh whole smoke / aerosol is directed through the bacteria suspension placed in an impinger (see Fig. 1). A single-port RM 1 smoking machine adapted to smoke up to 3 test articles at a time is used for aerosol / vapour generation.

- Tester strains and cell suspension concentrations used: The OECD recommended *Salmonella typhimurium* strains TA100 and TA98 were used to assess the mutagenicity of WS / GVP from research cigarette 3R4F and CM7 as well as of whole vapour and the gas phase of vapour from EVP. In our procedure the overnight cultures are centrifuged and re-suspended in phosphate buffered saline to obtain increased cell densities (2.5 up to 12.5-fold, see Fig.2). In the main experiments 10ml of 10-fold concentrated bacteria suspensions were treated with aerosol and incubated in the presence and absence of external metabolic activation system S9. For screening purposes TA 102 (Fig.5) , 1535 and 1537 (not shown) were also exposed to WS and GVP from CM7. No clear dose response was found for TA1535 and TA1537.

- Aerosols and smoking regime: The experiments with tobacco products were performed using the CORESTA Monitor Test Piece 7 (CM7) and the Kentucky Reference Cigarette 3R4F. They were smoked according to ISO conditions (35/2/60). For the assessment of WS and GVP from CM7, 6 smoking runs with 1 cigarette each were performed consecutively. In the 6 smoking runs for WS and GVP from 3R4F 1 and 2 cigarettes were used respectively.
The EVP was smoked according to intense conditions (55/2/30). Per smoking run 2 EVPs were used at a time and puffed with 150 puffs each (total of 300 puffs was directed through the bacterial suspension).

- E-vapour positive control: The EVP cartomisers were loaded with 1ml base liquid (PG : Glycerol) spiked with 0.37% Formaldehyde (FA). 150puffs (corresponding to about 170mg liquid) from each of two EVPs where directed through the bacteria suspension. Using whole and filtered vapour enabled us to show that both the particles / droplets and GVP of the E-vapour do reach the bacteria effectively.

3. Setup and Results

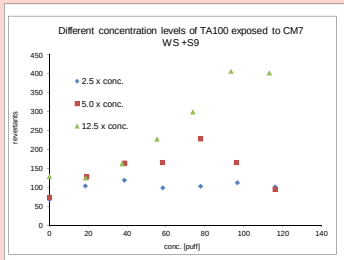

Fig.2: Initial experiments displaying dose response curves after "bubbling" of TA100 cell suspensions with whole smoke from CM7. The factors indicate the fold increases of bacteria density in relation to an overnight culture. Subsequent experiments unveiled that the concentration factor of 10 delivered highest reproducibility.

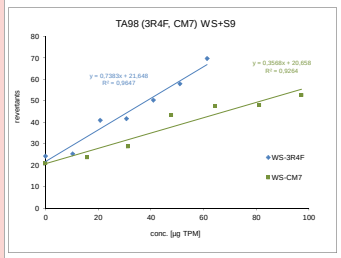

Fig.6: Dose response following exposure of TA98 to WS from CM7 and 3R4F in the presence of S9. Slopes are significantly different ($p<0.05$) on a TPM basis. TPM equivalents were calculated using historical TPM yields of the test items.

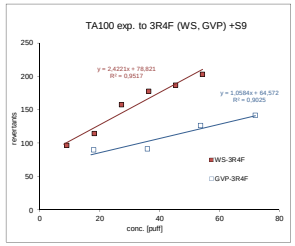

Fig.3: Linear dose response following exposure of TA100 to WS and GVP from 3R4F in the presence of S9.

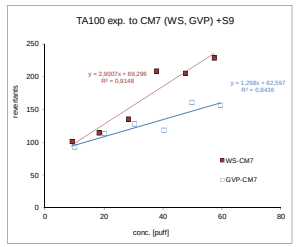

Fig.4: Linear dose response following exposure of TA100 to WS and GVP from CM7 in the presence of S9.

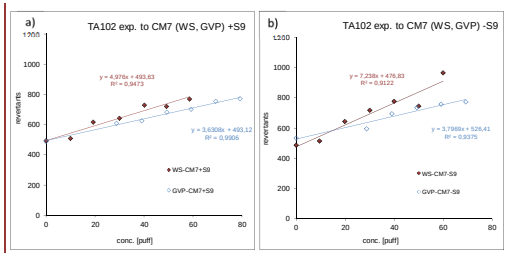

Fig.5: Linear dose response following exposure of TA102 to WS and GVP from CM7 in (a) the presence and (b) in the absence of S9.

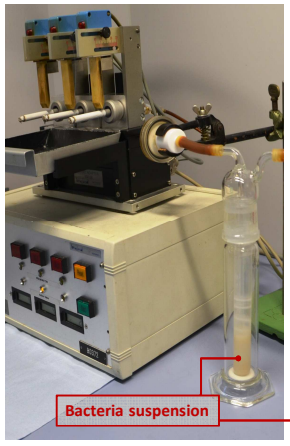

Fig.1: Experimental setup showing the adapted smoking machine and the exposure of bacteria suspension located in the impinger.

Results obtained with EVP positive control

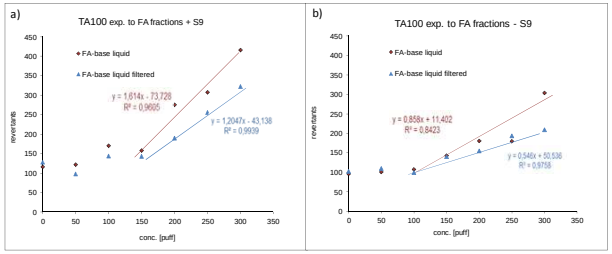

Fig.7: Dose responses following exposure of 10x concentrated TA100 to unfiltered and filtered vapour generated from Formaldehyde spiked base liquid. Under both conditions (a) with and (b) without S9 positive slopes are obtained with the unfiltered vapour delivering stronger responses as determined from the linear part of the dose response curves. The vapour dose level which marked the start of the linear part of the dose response curve corresponds to ~85mg FA-spiked base liquid.

4. Conclusions

The modification in terms of higher cell concentrations increases the sensitivity to mutagenic compounds and reduces the toxic effects of the test aerosol. Thereby, enlarged dose ranges of aerosols can be tested to increase the probability to detect mutagenic effects. Using this principle we show that the presented test system is suitable for the mutagenicity assessment of whole smoke with OECD recommended *S. typhimurium* strains. We found positive dose responses following exposure of TA98 and TA100 to whole smoke from CM7 and 3R4F with most pronounced effects in the presence of S9 (Fig.3, 4 and 6). TA100 also showed positive results when exposed to the filtered GVP of both 3R4F and CM7 (Fig.3 and 4). First results with tester strain TA102 showed positive dose responses in the presence and absence of S9 after exposure to WS and GVP of CM7 (Fig.5). Furthermore, a FA containing E-vapour positive control demonstrated the general applicability of the system in combination with EVPs (Fig.7). This adapted fresh smoke exposure system for Ames test using OECD recommended strains has high potential for practical applicability regarding the assessment of smoke and vapour mutagenicity.

5. References

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