

## **In vitro tests with fresh cigarette smoke - Effects of charcoal filters on whole smoke and vapour phase mutagenicity and genotoxicity**

WIECZOREK, R.; RÖPER, W.

Imperial Tobacco Group, Reemtsma Cigarettenfabriken GmbH, Science & Stewardship, 22761 Hamburg, Germany.

*In vitro* analysis of fresh smoke is an important part of cigarette testing because the exposure methods employed may reflect human smoke exposure. Whole smoke is a dynamic system and has to be handled accordingly. In a few seconds smoke changes its properties. Therefore, the period of time until the smoke is in contact with cells has to be kept as short as possible. The same preconditions need to be considered for the vapour phase. For realistic assessment and comparison of biological effects of whole smoke and its vapour phase the conditions of the tests have to be the same.

Cigarette Smoke Condensates (CSC) tests reflect only part of cigarette smoke's biological effects. In particular, the effects of charcoal filters on vapour phase are difficult to detect with standard *in vitro* methods for cigarette testing (Health Canada methods T 501, T 503).

In this study fresh smoke mutagenicity and genotoxicity of two charcoal cigarettes was compared with a Cellulose Acetate (CA) filter cigarette. The three American Blend cigarettes had similar puff numbers and tar yields.

Fresh Whole Smoke (WS) and Vapour Phase (VP) were tested for mutagenicity and genotoxicity in the Ames test and the *In Vitro* Micronucleus assay (IVM, air-liquid system).

For the detection of mutagenicity with the Ames test our in-house fresh smoke treatment liquid system with *S. typhimurium* TA100 was used. The test showed high responsiveness to whole smoke and vapour phase. Charcoal filters decreased the whole smoke mutagenicity in comparison to a standard CA filter up to 46% and the vapour phase mutagenicity up to 61%.

The IVM tests with V-79 showed, dependent on quantity of charcoal, significant reductions of WS and VP activity compared with CA filter cigarettes.

## **Introduction**

Mutagenicity and genotoxicity testing is, besides toxicity testing, an essential part of cigarette smoke in vitro analysis. The exposure methods should take into account the dynamic nature of smoke.

As regards mutagenicity testing a modified Ames method was already presented at the CORESTA meeting 2007 in Jeju / Korea. The direct bubbling of *S. typhimurium* TA100 bacteria suspension in an impinger inserted between a 3 port adapter RM158 and a single port smoking machine guaranteed direct contact of bacteria with fresh smoke.

For the genotoxicity testing (IVM) the Bt020 smoke aerosol cell exposure apparatus (Burghart Tabaktechnik, Wedel, Germany) was used to mimic human smoke exposure. The period of time until the smoke comes into direct contact with cells is 4 seconds and thus very short. The high throughput of Bt020 allowed the testing of the three different cigarettes with the same cell preparation to get better reproducibility.

The influence of fresh whole smoke and vapour phase is an essential point for the correct evaluation of charcoal products with regard to their in vitro genotoxicity and mutagenicity.

## **Material and Methods**

### **Experimental cigarettes**

Mono-acetate filters (CA) and two charcoal filters (Dal = Dalmatian, Cav = Cavity) were manufactured at Filtrona UK. The carbon loading was 82.5 mg and 85 mg for the Dal and Cav filter, respectively. Pressure drop was adjusted by filter selection for achieving comparable tar and nicotine retention. For all charcoal filters we used one batch of PICA FA 60 10 H carbon; a commercial 30/70 mesh grade with 60 % CCl<sub>4</sub> activity and 10 % moisture content. An American Blend (AB) was processed in ITG's pilot plant without added tobacco ingredients. Cigarette samples with 93 mm length (25 mm filter and 68 mm rod length) and 24.5 mm circumference were made on a Protos 90 maker. All cigarettes were packed and stored at room temperature over a period of more than one year.

**Table 1:** Experimental cigarettes.

| <b>Code</b> | <b>Filter</b>                | <b>Total Particulate Matter<br/>TPM [mg/cig]</b> | <b>water free Part. Matter<br/>DPM [mg/cig]</b> | <b>H<sub>2</sub>O<br/>[mg/cig]</b> | <b>Nicotine<br/>[mg/cig]</b> |
|-------------|------------------------------|--------------------------------------------------|-------------------------------------------------|------------------------------------|------------------------------|
| <b>CA</b>   | acetate                      | 15.39                                            | 13.92                                           | 1.48                               | 1.07                         |
| <b>Cav</b>  | acetate, carbon<br>cavity    | 16.45                                            | 14.57                                           | 1.88                               | 1.11                         |
| <b>Dal</b>  | acetate, carbon<br>dalmatian | 15.98                                            | 14.31                                           | 1.67                               | 1.10                         |

Prior to use the cigarettes were stored in a conditioning chamber at 22°C and 60% relative humidity for 2-3 days.

Conditioning and smoking was performed under ISO conditions. Total Particulate Matter (TPM) was collected on a 92 mm Cambridge filter pad.

Immediately after smoking and weighing, the Cambridge filters were divided radially with a special cutting device, into 6 identical segments. Two opposite parts were analysed for nicotine and water in the TPM. The other 4 parts of the filter were extracted for 30 minutes at room temperature with DMSO, and the extract was immediately frozen.

### **Ames test**

*Salmonella typhimurium* strain TA100 used for the mutagenicity test was initially provided by Dr. Bruce Ames (University of California, Berkeley, CA). The Ames test was performed as recommended by Maron and Ames [1983]. The Ames test with fresh smoke was performed as presented by Wieczorek, R.; Röper, W.; Kahl, E. [2007]. For each petri plate 100 µl of bacteria suspension were used. All mutagenicity assays were conducted in the presence of metabolic activation (6.25% S9).

Revertant colonies were counted using an automatic colony counter (ARTEK Counter; Model 880)

### **Negative and positive controls**

Negative (DMSO) and positive controls (2-Aminoanthracene) were concurrently run with each test.

### **Preparation of bacteria suspension for Ames test**

Before starting the test 10 ml culture of *S. typhimurium* TA100 were incubated at 37°C overnight in Ampicillin (25 µg/ml) supplemented Nutrient Broth. Additionally 30 ml of fresh medium were added to the overnight culture and incubated for a further 2 hrs.

The ready-to-use bacteria suspension was prepared by centrifugation at 2300 x g for 10 minutes. The pellet was re-suspended in 40 ml of Ca, Mg free Dulbecco's PBS with 0.5% DMSO. The bacteria were prepared and treated at room temperature under protection from UV light.

### **Smoke treatment of bacteria**

Aqueous vapour phase or whole smoke extracts were prepared using a 3 port adapter RM158 (Burghart Tabaktechnik, Wedel, Germany) connected with a single port smoking machine (ISO smoking regime). A special Cambridge filter holder (92 mm) with reduced inner volume of only 14 ml was used.

Cigarette Smoke was bubbled in four consecutive runs, each with three cigarettes, through an impinger containing a tube with 12 ml bacteria suspension in PBS/DMSO. A flushing step with fresh air followed each puff of cigarette.

### **Determining dry particulate matter retained by the bacteria suspension**

The amount of particulate matter trapped in the medium was estimated in additional tests by measuring optical density (OD) at 370 nm. The concentration of dry particulate matter [ $\mu\text{g}/\text{ml}$ ] in whole smoke bubbled bacteria suspension was calculated based on the calibration curves generated with known DPM concentrations of CSC in DMSO and in PBS/DMSO - TA100 bacteria suspension.

### **Calculation of mutagenicity**

Initially, cigarette specific mutagenicity data were plotted as dose-response linear regressions with 95% confidence interval, mutagenicity [revertants per plate] vs. dose [cigarette equivalents per plate]. Data presented here refer to both, mutagenicity per cigarette, and per  $\mu\text{g}$  particulate matter (re-calculated).

CSC specific mutagenicity was directly determined with plates containing known amounts of TPM per plate (standard procedure when testing CSC).

For a reasonable comparison of (standard) CSC specific mutagenicity and whole smoke or vapour phase mutagenicity, it is necessary to know the percentage of particulate matter trapped by the smoke bubbled bacteria suspension (see above).

Based on the amount of particulates trapped by the bacteria suspension, new regression lines and new slopes [rev per  $\mu\text{g}$  trapped] were generated with GraphPad Prism 5.0. This data was used to calculate, based on standard CSC mutagenicity data [rev/ $\mu\text{g}$ ], the cigarette specific mutagenicity (theoretically originating from trapped particulate matter), and compared with whole smoke and vapour phase mutagenicity data.

### **IVM assay**

In this In Vitro Micronuclei assay test, the clastogenic and aneugenic potential of fresh smoke and their vapour phase can be assessed through its effects on the chromosomes. The IVM test was following the draft guideline provided by OECD (2007). V-79 cells, originally provided by ECACC (Cat No. 86041102), were cultured and treated in inserts placed in 24 multiwell plates.

### **IVM test with CSC**

The condensate tests were performed in Collagen I coated 24 multiwell plates (BD; #354408). The wells were filled with 500  $\mu\text{l}$  of  $5 \times 10^4$  V-79 cells per 1 ml. The condensates were incubated over 24 hours, and the cells harvested and prepared with cytospin on the microscope slides.

### **Preparation of inserts for fresh smoke testing**

For the tests 24 multiwell plates were filled with 250  $\mu\text{l}$ /well Dulbecco's modified eagle's medium supplemented with 10% of serum. Inserts with 3  $\mu\text{m}$  membrane (Nunc; #140627) were inserted into

the wells and filled with 400 µl of  $10 \times 10^4$  V-79 cells per 1 ml. The pre-incubation time was 20 hours at 37°C and 5% CO<sub>2</sub>. Directly before the smoke treatment, medium was removed and the inserts were transferred to another multiwell with 250 µl medium supplemented with HEPES buffer (20mM final concentration). The 24 multiwell Plates were fixed in the exposure chamber and treated with the smoke of 3 cigarettes.

Immediately after the treatment in the smoking mimic machine, (Bt020) 300 µl medium was carefully added to each insert and further incubated for a minimum of 20 hours.

### **Treatment with freshly generated smoke**

V-79 cells were exposed to freshly generated whole smoke aerosol and vapour phase.

The Bt020 smoke aerosol cell exposure apparatus (Burghart Tabaktechnik) is a single port smoking machine directly connected with the exposure device. The machine is equipped with smoke 'distributors' for 24 multiwell plates. Each well is provided with a separate smoke inlet and 2 suction ducts. When exposure starts (first puff) a stainless steel sliding lid covers the control wells.

Directly after puffing, whole smoke or vapour phase are diluted inside the syringe, by partly discarding smoke and diluting with charcoal filtered air. Within 4 seconds after the puff being taken the diluted smoke comes into contact with the cells. After 6 seconds of exposure, excess smoke from the wells is pushed upwards with 80 ml of air and exhausted directly through suction ducts in the smoke distributor. After 10 seconds, excess smoke is removed by suction through ducts on the periphery of the exposure chamber. During the puff interval, 10 flushing cycles of the chamber air are done (80 ml in 2 seconds) before the next puff is taken from the cigarette.

The 24 multiwell plates have 6 rows of wells with inserts. The first row containing inserts was covered during smoke exposure to measure standard growth (control). Thus, up to 5 rows (row 2 to 6) can be used for puff dependent exposure of cells. Each row was exposed to 2 puffs. Each of the rows subsequent to row 2, was treated with 2 puffs more than the previous one.

For each test 3 cigarettes were smoked onto one multiwell plate. The total exposure time was less than 30 minutes. Row 2 was exposed only to the first puff of the cigarettes and the last row of wells was exposed to all puffs of the cigarettes.

### **Calculation of genotoxicity**

Cigarette specific effects **of micronucleus formation (EC-MN)** were calculated from three replicate tests taking into account the total numbers of puff and dilution factors applied. The total number of puffs (per cigarette) was set to 100% smoke dose for a given cigarette. Hence, the calculated effect is independent of the number of puffs and allows cigarettes genotoxicity to be compared.

The counted micronuclei (as per cent of cigarette doses) of V-79 cells follow a sigmoidal curve in the dose-response diagram. The background level was calculated as 0.5% and the top level was set at 100%.

The effective concentration (EC-MN) is the concentration of smoke that results in 4-fold increase against background level of micronuclei (2%). The **EC-MN** were calculated with GraphPad Prism 5.

## **Results and discussion**

### **Calculation of trap dry particulate matter in the bacteria suspension**

In order to calculate the influence of condensate mutagenicity on whole smoke mutagenicity the amount of trapped dry particulate matter in the bacteria suspension has to be determined. For the tests all 3 cigarettes were tested for trapped particulates. Calibration curves of CSC in DMSO and TA100 in PBS/DMSO, respectively, were used to estimate the DPM yield and the amount of particulate matter trapped by the bacteria suspension. The concentration of DPM was determined by measuring optical density (OD) at 370nm.

No significant differences between the tested cigarettes as regards solubility of particulates in the bacteria suspension were found. After the treatment 2.4% of whole DPM were found in the bacteria suspension. The cigarettes had roughly the same yield of condensate (15.9 mg/cig +/- 0.5) with similar level of water in CSC (10.5% +/- 0.9).

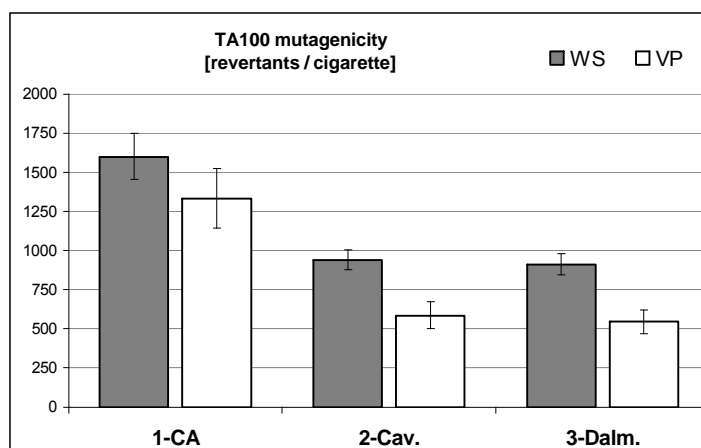
The data were compared with data from previous tests with cigarettes with different filters and blends. The percent of trapped particles from the cigarettes in suspension correlated with the content of water in CSC. The higher the water content in CSC the higher their solubility.

### **Cigarette specific WS, VP and condensate mutagenicity**

Both for preparation of vapour phase and whole smoke the filter holder was attached to the smoking machine. After each smoking run the holder was opened for a filter change or in case of whole smoke preparation to mimic filter change.

Three replicate tests were performed to determine mutagenicity of whole smoke, vapour phase and condensate. The cigarette specific mutagenicity [revertants per cigarette] and 95% confidence intervals were calculated with GraphPad Prism 5.0. The calculation was based on cigarette equivalents per ml PBS bacteria suspension with 0.5% DMSO.

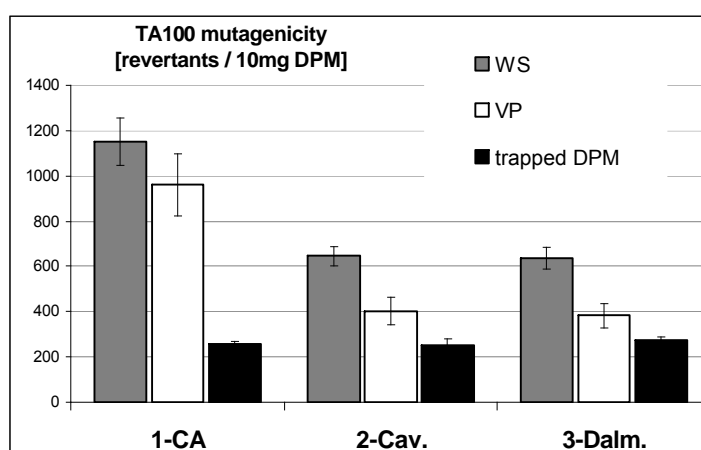
For carbon filter cigarettes whole smoke mutagenicity was higher than vapour phase mutagenicity. Normal acetate filter cigarettes did not show significant differences between the WS and VP mutagenicity.



**Fig. 1:** Whole Smoke (■ WS) and Vapour Phase (□ VP) cigarette specific mutagenicity with 95% confidence intervals.

### Comparison of condensate specific mutagenicity with whole smoke and vapour phase mutagenicity

For comparing the mutagenicity of the test cigarettes, whole smoke, vapour phase and standard CSC mutagenicity were determined with TA100. In order to determine mutagenicity of CSC, three replicate tests were done with CSC collected on a 92 mm diameter Cambridge filter (RM 20) and dissolved in DMSO. The mutagenicity of DPM was calculated as described above. No significant differences of the condensate mutagenicity were found. The numbers of revertants per 10 mg DPM were calculated. Cigarette specific mutagenicity as calculated from routine CSC testing was much higher in comparison to the directly measured response to fresh whole smoke (data not shown). In the smoke bubbled bacteria suspension 2.4% of the cigarette DPM was trapped. The corresponding contribution of trapped DPM on mutagenicity was calculated and compared to whole smoke and vapour phase mutagenicity.



**Fig. 2:** Whole Smoke (■ WS), Vapour Phase (□ VP) and water free Particulate Matter (■ DPM) condensate specific mutagenicity with 95% confidence intervals.

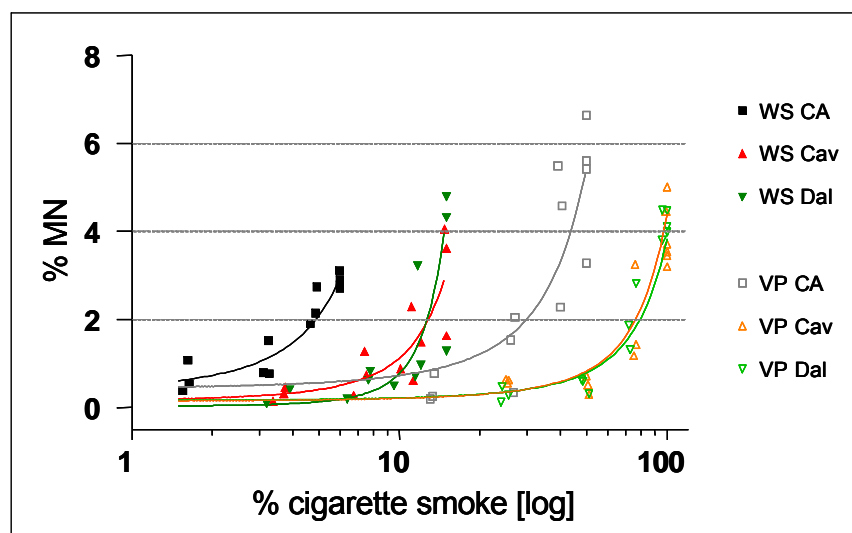
The TA100 mutagenicity of vapour phase from the control cigarette was only 17% lower than whole smoke mutagenicity in contrast to charcoal filtered cigarettes with 40% lower VP mutagenicity. The decreases of VP mutagenicity of charcoal cigarettes have a direct effect on the whole cigarette mutagenicity.

### Cigarette specific WS, VP and condensate (CSC) genotoxicity

Three replicate tests were performed to determine genotoxicity of whole smoke, vapour phase and condensate. In order to determine genotoxicity of condensate, IVM test with of CSC dissolved in DMSO were made. No significant differences of the condensate genotoxicity were found (data not shown).

For preparation of whole smoke the Cambridge filter holder was attached to the Bt020 cell exposure apparatus. After each smoking run the holder was opened to mimic filter change. The cells were treated with smoke of 3 cigarettes. Only 2% WS of from the control cigarette and 4% from the charcoal cigarettes were transferred into the inserts. For vapour phase testing, smoke was diluted 1:6 (control) and 1:3 (carbon filters), respectively. Thus, 16.6% and 33.3%, respectively, of total vapour phase were used to achieve equivalent genotoxic effects. For data comparison the concentration of smoke necessary to increase micronuclei up to 2% was calculated (EC-MN). The corresponding toxicity measured by a cell counter was ca. 60% for whole smoke and 30% for vapour phase and condensate in DMSO.

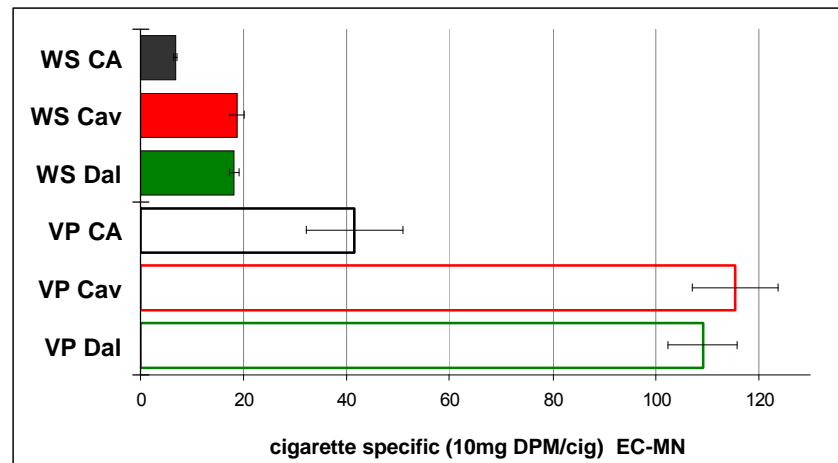
The cigarette whole smoke shows higher genotoxicity than the vapour phase. In comparison to the CA cigarette VP and WS genotoxicity of charcoal cigarettes are significantly lower.



**Fig. 3:** CA filter cigarette (■ CA), cavity filter cigarette (▲ Cav) and dalmatian filter cigarette (▼ Dal). Filled symbols refer to whole smoke (WS) and open symbols to vapour phase (VP).



The cigarette specific EC-MN for WS and VP genotoxicity were calculated. For correct comparison of cigarettes DPM specific EC-MN were used. The biological effects of cavity and dalmatian carbon filter cigarettes did not show significant differences. Both charcoal filters significantly decreased the smoke genotoxicity in comparison to a standard CA filter (up ca. 60%).



**Fig. 4:** CA filter cigarette (■ CA), cavity filter cigarette (■ Cav) and dalmatian filter cigarette (■ Dal) condensate specific genotoxicity with 95% confidence intervals. Filled columns refer to whole smoke and open to vapour phase.

### Conclusions

The testing of freshly generated smoke is an adequate method for a comprehensive biological evaluation of cigarette smoke. In the present study CA and charcoal filter cigarettes with similar condensate yields were compared. Ames and IVM tests show significant differences in fresh smoke mutagenicity and genotoxicity. In contrast no significant differences with CSC were found.

The mutagenicity was tested with the Ames test with *S. typhimurium* TA100. Freshly generated whole smoke and vapour phase were directly bubbled in the bacteria suspension. Within 1 second after the puff is being taken the smoke comes in contact with the bacteria.

In this test charcoal filter cigarettes in comparison to the control showed a significant decrease of whole smoke and vapour phase mutagenicity. Both types of filter, cavity or dalmatian, led to similar effects. Charcoal filters decreased whole smoke mutagenicity up 45% and 46% with cavity and dalmatian filter, respectively. The vapour phase mutagenicity decreased up 58% and 60% with cavity and dalmatian filter, respectively. In contrast, CSC from these cigarettes show neither with the TA100 nor the TA98 strain (data not show) significant differences. The decreases of mutagenicity of charcoal cigarettes come solely from the vapour phase.

Similar effects were found with the IVM test. The inserts with V79 cells were exposed in the Bt020 cell exposure apparatus. During the tests the cells are basally surfaced with medium and apically in direct contact with freshly generated whole smoke or vapour phase.

Cavity and dalmatian charcoal filters decreased the whole smoke and vapour phase genotoxicity (IVM) in comparison to a standard CA filter up 63%. In contrast to mutagenic effects (Ames test) the vapour phase influenced the whole smoke genotoxicity by only 16%.

The WS genotoxicity seems to mainly originate from the particulate matter in fresh whole smoke. The diluted condensates in DMSO did not show significant differences in the IVM test.

## **References**

**Maron, D.M.** and Ames, B. N. (1983): Revised methods for the *Salmonella* mutagenicity test. *Mutat.Res.* 113: 173-215.

**Wieczorek, R.;** Röper, W.; Kahl, E. [2007]: Mutagenicity of Cigarette Smoke Vapour Phase in the AMES Test. CORESTA Meet. Smoke Sci.-Prod. Techno Groups, Jeju Island, 2007. SSPT 42.

**OECD (2007)** OECD guideline for the testing of chemicals. Draft Proposal for a New Guideline 487:. In Vitro Mammalian Cell Micronucleus Test (MNvit), 3<sup>th</sup> version, December 13, 2007. 21pp. OECD, Paris.