Fraunhofer Institut
Toxikologie und
Experimentelle Medizin

Final Report

Chamber Trials for the Combined Repeated-Dose Toxicity Study with the Reproduction/Developmental Toxicity Screening Test and Mammalian Erythrocyte Micronucleus Test via Inhalation with Roofing Asphalt Fume Condensate

Fraunhofer ITEM Study No. 02N07532

Number 2 of 2 Originals

Test Facility:
Fraunhofer Institute of Toxicology
and Experimental Medicine
Nikolai-Fuchs-Str. 1
30625 Hannover, Germany

Executive Director:
[redacted]

Sponsor:
PlasticsEurope
Av. E. Van Nieuwenhuyse 4/3
1160 Brussels
Belgium

This report consists of 27 pages

Contents

1 Statement of Study Director

Study no.: 02N07532

Test substance: asphalt fume condensate

Title: Chamber Trials for the Combined Repeated-Dose Toxicity Study with the Reproduction/Developmental Toxicity Screening Test and Mammalian Erythrocyte Micronucleus Test via Inhalation Exposure to Roofing Asphalt Fume Condensate

This non-clinical study was not conducted in compliance with the principles of GLP, but in the spirit of GLP.

I accept the responsibility for the validity of the study.

Date: 14.03.08

[redacted] [redacted]
Study Director

2 Introduction

2.1 Objectives of the study

The aim of the study was to demonstrate that:

1. The selected experimental atmosphere generation system that will be used for the inhalation exposures produces consistent target concentrations within the animals' breathing zones.
2. The monitoring system to be selected accurately measures test article concentrations and there is acceptable agreement between nominal and measured concentrations.
3. There is acceptable test material homogeneity throughout the chamber.

2.2 Guidelines for conduct of the study

This was a non GLP study. The principles of Good Laboratory Practice, however, were taken into consideration as far as possible (German Chemicals Law, Appendix 1, June 28, 2002), and the study was conducted in the spirit of Good Laboratory Practice.

2.3 Dates

Study initiation date:	Sept., 10, 2007
Experimental starting date:	Sept., 17, 2007
Experimental completion date:	Dec., 07, 2007
Study completion date:	March 14, 2008

2.4 Test item

The test item is roofing asphalt fume condensate.

Details on the roofing asphalt fume condensate used for the test are given in the report "Collection, Validation and Generation of Asphalt Roofing Fumes for Reproductive/ Developmental Toxicity Study", Heritage Research Group, 3 February 2006.

Density: 0.8745 mg/l
Kinematic viscosity: 8.3616 centistokes at 100 ℉
Refractive index: will be given by the sponsor
Expiry date: will be given by the sponsor

Certificates of the roofing asphalt fume condensate are given in Appendix A.

3 Methods

3.1 Determination of the UV fluorescence in mg/kg as diphenyl anthracene

Determination of the UV fluorescence intensity as diphenyl anthracene (DPA) was carried out according to the method provided by Heritage Research Group (Fraunhofer ITEM SOP No. 150401). 6 µl of roofing asphalt fume condensate (bottle 1 of 5, A 210/07) were dissolved in 20 ml dichloromethane. The measurement was carried out in a 1-cm cuvette using a Shimadzu RF 1501 spectrometer (high-sensitivity range, excitation wavelength 385 nm, emission wavelength 415 nm, bandwidth 10 nm). The value was corrected for solvent blank.

3.2 Determination of the factor used for quantification of asphalt fume by the BIA IR method

The asphalt fume concentration in the inhalation atmosphere was determined according to the method used by the German employers' liability insurance association (BIA Method 6305). This method is based on a comparison of the intensities of the C-H stretching vibrations of a standard oil and the sample (Fraunhofer ITEM SOP No. 150246). For determination of the factor between the absolute asphalt fume concentration and the concentration determined using the BIA method, five samples of the asphalt fume condensate were prepared in tetrachloroethylene and analyzed by the BIA method. Measurements were carried out with the Bruker FT-IR spectrometer Vector 22.

3.3 Determination of the boiling point distribution

The boiling point distribution was determined according to the ASTM standard method D 2887 - 04a (Fraunhofer ITEM SOP No. 150383). 20 mg of roofing asphalt fume condensate (bottle 1 of 5, A 210/07) were dissolved in 1ml dichloromethane. The following GC conditions were used:

GC:	HP 5890	
Detector:	FID, 330 °C	
Injector:	split/splittless, 330 °C	
Carrier gas:	helium	
Column:	DB 5 (J&W Scientific) 30 m, 0.53 mm i.d., d_f = 0.5 µm	
Temp. program:	initial temperature:	40 °C for 3 min
	rate:	10 °C/min
	final temperature:	315 °C for 11 min
Standard:	n-alkane standard mixture (C_8 -C_{40}, 500 µg per component in hexane, diluted to 10 µg/ml with dichloromethane)	

3.4 Determination of 16 EPA-PAH + benzo(e)pyrene and triphenylene

PAHs were determined according to SOP 150375 (Fraunhofer ITEM).

3.4.1 Column chromatography

For PAH analysis, a defined amount (0.1 ml) of the condensate sample was taken. The sample was then spiked with a solution of deuterated reference compounds in toluene, so that the concentration of the internal standard was in the same concentration range as that expected for the PAHs.

For silica gel chromatography, silica gel 60 was conditioned using 12.5 % w/w water (standing overnight). About 40 g of silica gel were suspended in cyclohexane and filled into a 25 x 400 mm glass column.

The sample was applied in a small volume of cyclohexane to the silica gel column and eluted with 240 mL of cyclohexane. Two fractions (50 and 190 mL) were collected. The first fraction was rejected. The second fraction was evaporated to about 10 ml. Thereafter, 2-propanol (2-3 ml) was added and the solution reduced in volume to about 1 mL using a TurboVap concentration workstation.

Silica gel chromatography was followed by chromatography on Sephadex LH 20. To this end, 5 g Sephadex LH 20 were suspended in 2-propanol. The suspension was allowed to stand for several hours (or overnight) for swelling before transferring it to the chromatographic column (25 x 150 mm). The sample dissolved in about 1 mL 2-propanol as described above was applied to the Sephadex LH 20 column and eluted with 165 mL 2-propanol in two fractions (15 and 150 mL). The second fraction of 150 mL was reduced to approx. 1 mL using a TurboVap concentration workstation and measured by GC/MS.

3.4.2 GC-MS analysis

The PAHs were quantified by the method of internal standardization using the following instrumental conditions:

GC: HP 6890 Series
Autosampler: Agilent 7683
Injector: split/splitless, 250 ℃
Injector purge-off time: 0.5 min
Injection volume: 1 µL
Carrier gas: helium, constant flow mode (1.4 ml)
Column: DB5, 60 m, 0.32 mm i.d., df = 0.32 µm
Temperature: Initial temperature 60 ℃, 5 ℃/min to 250 ℃, 4 ℃/min to 280 ℃ (20 min), 10 ℃/min to 300 ℃ (10 min)
Mass spectrometer: MSD 5973N
Ionization: Electron impact, EI (70 eV)
Source: 230 ℃
Transfer line: 280 ℃
Quadrupole: 150 ℃
Data acquisition: SIM with 1 mass per component

The analysis included the 16 priority PAHs defined in EPA method 610, in addition to triphenylene and benzo(e)pyrene. The PAHs and the m/z values of the ions used for acquisition and quantification are summarized in Table 1.

Table 1: Ions (m/z values) used for acquisition and quantification of PAHs.

Compound	m/z
Naphthalene	128
Naphthalene-d_8	136
Acenaphthalene	152
Acenaphthalene-d_8	160
Acenaphthene	154
Acenaphthene-d_{10}	164
Fluorene	166
Fluorene-d_{10}	176
Phenanthrene	178
Phenanthrene-d_{10}	188
Anthracene	178
Anthracene-d_{10}	188
Fluoranthene	202
Fluoranthene-d_{10}	212
Pyrene	202
Pyrene-d_{10}	212
Benz(a)anthracene	228
Benz(a)anthracene-d_{12}	240
Chrysene	228
Chrysene-d_{12}	240
Triphenylene	228
Benzo(b)fluoranthene	252
Benzo(b)fluoranthene-d_{12}	264
Benzo(k)fluoranthene	252
Benzo(k)fluoranthene-d_{12}	264
Benzo(e)pyrene	252
Benzo(a)pyrene	252
Benzo(a)pyrene-d_{12}	264
Indeno(1,2,3-cd)pyrene	276
Indeno(1,2,3-cd)pyrene-d_{12}	288
Dibenz(ah)anthracene	278
Dibenz(ah)anthracene-d_{14}	292
Benzo(ghi)perylene	276
Benzo(ghi)perylene-d_{12}	288

3.5 Sample collection and extraction

The fume concentration was determined by sampling from the nose-only unit using a combination of a glass fiber filter (type: MG, 37 mm) and an XAD absorption tube (3.2 g XAD-2) with a sample flow of 2 l/min. The material collected was extracted with tetrachloroethylene. The extracts of filter and XAD were analyzed separately by IR spectroscopy according to BIA guideline 6305 (see chapter 5.1.3).

For boiling point determination in the chamber the same sampling device was used. The material collected was extracted in an ultrasonic bath with dichloromethane (filter (2 x 50 ml) and XAD-2 tube (2 x 50 ml)). The solvents were combined and evaporated with a rotary evaporator (conditions: 35 ^{o}C/650 mbar) to 2 ml and analyzed by gas chromatography (see chapter 3.3).

4 Test system

4.1 Fume generator

The method developed by Koch (1993) for regeneration of asphalt fume basically uses a free jet to recondense hot vapor in a stream of cool air. Since in this system heat and mass transfer are mainly determined by the free jet, the particle generation process is very robust, not sensitive to external factors, and yields a stable fume in respect to its physical and chemical composition.

The asphalt fume test atmosphere is generated using the free jet principle by means of a laboratory setup developed at the Fraunhofer ITEM. In this apparatus (Figure 1), liquid asphalt fume condensate is evaporated and recondenses on a large number of condensation nuclei also generated from the condensate by the apparatus. This leads to a highly dispersed aerosol phase in equilibrium with the corresponding vapor phase.

In the free jet evaporation-condensation generator, a well-defined mass flux of vaporized material is issued together with a carrier (nitrogen) at high velocity through a nozzle into a stream of slowly flowing cool air. An expanding turbulent jet forms, as a result of the surrounding air mixing with the vapor stream. The asphalt vapor is cooled and diluted downstream of the nozzle, as the jet develops. The initial vapor phase contains very fine seed particles, originating from the vapor generation process. The vapor is generated by first nebulizing the condensate, using a pneumatic nebulization nozzle, and subsequently evaporating the droplets in a heated evaporation tube. The seed particles result from lower volatility constituents in the condensate. Due to the nonlinear temperature behavior of the saturation concentration, the saturation ratio, S, goes through a maximum as a function of distance, x, from the nozzle. Depending on the temperature of the surrounding air in the generator and the mass flux of the vaporized material, supersaturation ($S > 1$) of the vapor is eventually achieved, leading to formation and growth of liquid aerosol droplets by condensation of the vapor phase on the seed particles.

The setup of the fume generator is shown in Figure 1. A peristaltic pump, driven by a stepping motor, maintains the pump feed rate of the asphalt fume condensate. For the nose-only system used in the inhalation studies and the concentrations envisaged, the feed rate had to be of the order of 4.5 ml/h. The condensate is pumped via a stainless steel tube directly into the pneumatic dispersion nozzle. This nozzle is operated with heated nitrogen (160 °C) at a flow rate of 5 l/min and generates droplets with a mean diameter of about 6 µm. The droplets are fed directly into a tube heated at approx. 220 °C, where they evaporate. The vapor is then issued through the nozzle and is recondensed in the condensation section as described, by mixing with cool air.

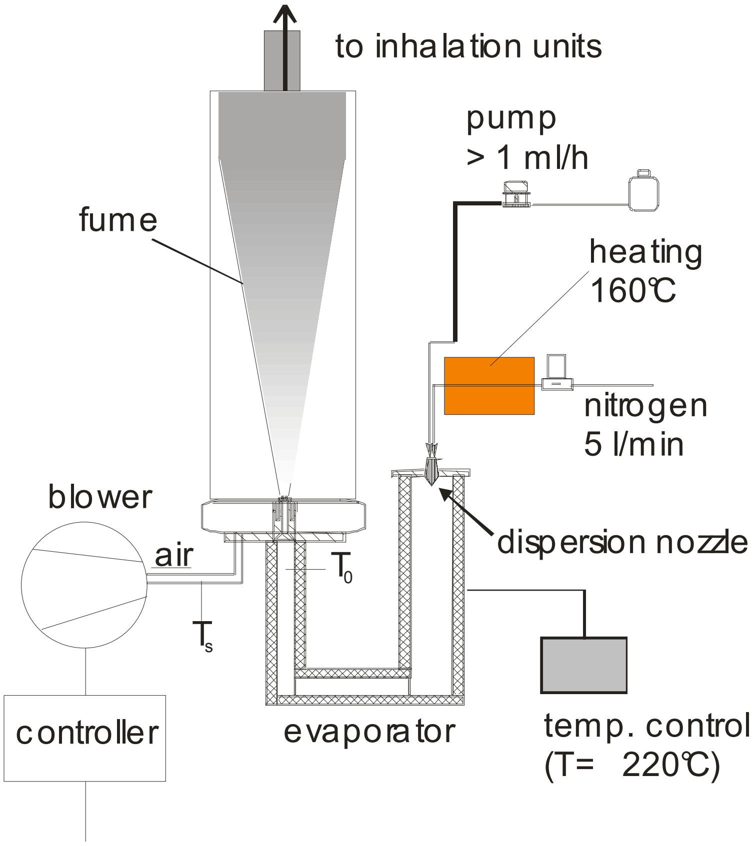

Figure 1: Aerosol generator. The red nitrogen heater has been added compared to the original setup described in Pohlmann et al., 2006.

4.2 Inhalation units and dilution

From the generator, the fume is directed through stainless steel tubes to the different inhalation units (Figure 2). Flow resistors control the flow of asphalt fume to each inhalation unit. The flow rate through these resistors is maintained by keeping a constant pressure difference between the inhalation units and the generator by controlling the flow rate of the cooling air in the generator. The final concentrations are achieved by mixing the asphalt fume with dilution air, regulated by mass flow controllers. The whole inhalation system is microprocessor-controlled and supervised by a central computer.

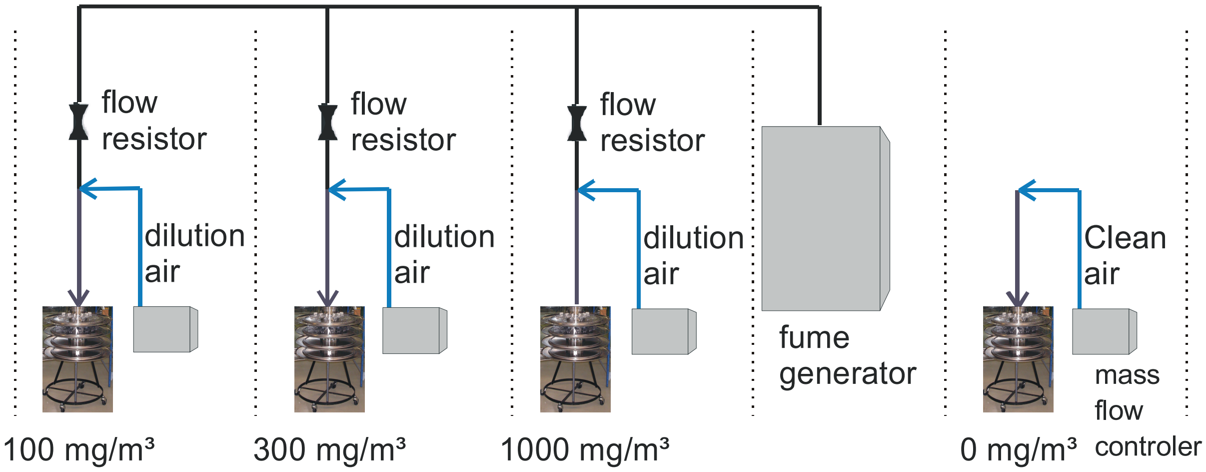

Figure 2: Scheme of the inhalation setup. Flow resistors control the flow rate of asphalt fume from the generator into the inhalation units.

The exposure to asphalt fume takes place in direct-flow nose-only inhalation exposure systems as shown in Figure 3. These inhalation exposure units are placed in closed laboratory hoods that are maintained at a constant pressure. Within each unit, fumes are supplied to each animal individually, and the exhaled air is exhausted immediately. Animals are placed around the exposure cylinder in tapered acrylic glass tubes with adjustable backstops. Nominal target concentrations at the different inhalation units are 0, 100, 300 and 1000 mg/m³.

Figure 3: Partial view of the direct-flow nose-only inhalation exposure system (inhalation unit). The picture on the left shows the upper part of the unit with the fume inlet installed, the inhalation ports and, as an example, one acrylic glass tube installed. The picture on the right shows a top view of the inhalation unit with the fume inlet removed, providing a view of the inner inlet cylinder with the stainless steel tubes directed to the inhalation ports as well as the outer cylinder that serves as exhaust drain.

5 Results

5.1 Test item

5.1.1 Identity of test item

Sample identity was confirmed by determination of density and refractive index of the roofing asphalt fume condensate (API identification "sample # 06-01") in all bottles provided by the sponsor.
The refractive index was determined according to ASTM D 1218-02 (Fraunhofer ITEM SOP No. 150399) with an Abbe refractometer Krüss AR 3-6. Density was determined gravimetrically. Results are summarized in Table 2.

Table 2: Refractive index and density of test item samples.

Sample # 06-01	Certificate of analysis		Fraunhofer ITEM	
	RI at 25 °C	Density at 22 °C [g/mL]	RI at 25 °C	Density at 21 °C [g/mL]
Bottle 1 of 5 (A 210/07)	1.4830	0.8766	1.4830	0.8763
Bottle 2 of 5 (A 211/07)	1.4831	0.8757	1.4829	0.8755
Bottle 3 of 5 (A 212/07)	1.4830	0.8754	1.4829	0.8762
Bottle 4 of 5 (A 213/07)	1.4831	0.8748	1.4830	0.8754
Bottle 5 of 5 (A 214/07)	1.4831	0.8765	1.4831	0.8758

5.1.2 Determination of UV fluorescence in mg/kg as DPA

UV fluorescence response as DPA equivalent: 320 mg/kg
Heritage certificate of analysis: 322 mg/kg

5.1.3 Determination of the factor used for quantification of asphalt fume by the BIA IR method

The mean factor for quantification of asphalt fume by the BIA IR method is: 1.26 ± 0.01. Detailed results are summarized in Table 3.

Table 3: Determination of the factor used for quantification of asphalt fume by the BIA method

Concentration of the prepared solution	IR response in standard oil equivalents	Factor
7.00 mg/10 ml	5.555	1.260
3.50 mg/10 ml	2.777	1.260
5.26 mg/10 ml	4.199	1.253
2.63 mg/10 ml	2.101	1.252
3.52 mg/10 ml	2.793	1.260
1.76 mg/10 ml	1.398	1.259

5.1.4 Determination of the boiling point distribution

The boiling point distribution curve is shown in Figure 4, the corresponding values are compared with the values given in the heritage certificate in Table 4.

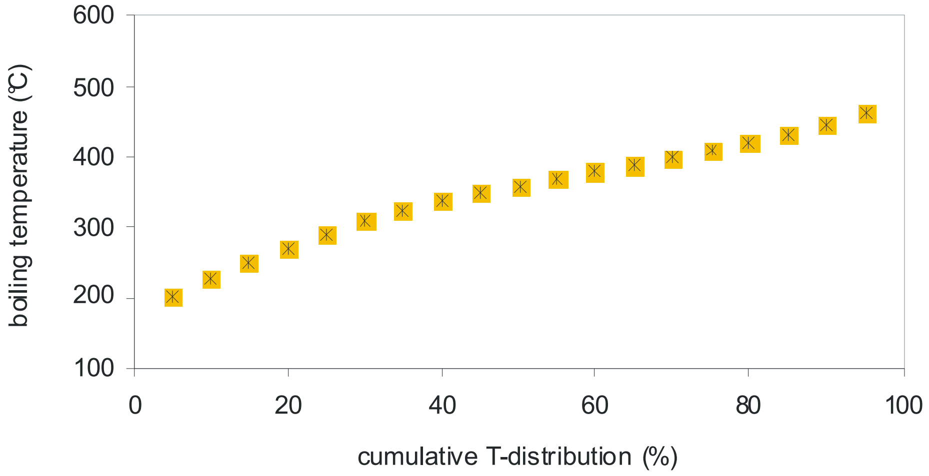

Figure 4: Boiling point distribution curve of roofing asphalt fume condensate, sample # 06-01 (bottle 1 of 5, A 210/07)

Table 4: Boiling points at different percentages of distillation for roofing asphalt fume condensate, sample # 06-01.

Percentage of distillation	Fraunhofer ITEM	Certificate of analysis
10 %	229	218 ℃
50 %	357	345 ℃
90 %	444	427 ℃

5.1.5 Determination of 16 EPA-PAH + benzo(e)pyrene and triphenylene

Results of the PAH analysis of the asphalt fume condensate samples are given in Table 5.

Table 5: Concentration of PAHs in the roofing asphalt fume condensate, API identification "Sample # 06-01", bottle 1 of 5 (A 210/07).

Compound	Analysis A [µg/g]	Analysis B [µg/g]	Certificate of analysis [µg/g]
Naphthalene	164.	165	180
Acenaphthylene	*)	*)	-
Acenaphthene	29.2	28.8	-
Fluorene	254	253	300
Phenanthrene	248	250	230
Anthracene	31.9	32.0	-
Fluoranthene	10.5	9.97	-
Pyrene	48.8	47.7	-
Benz(a)anthracene	7.81	7.99	-
Triphenylene	20.7	21.6	-
Chrysene	19.8	19.8	-
Benzo(b)fluoranthene	4.80	4.70	-
Benzo(k)fluoranthene	1.72	1.15	-
Benzo(e)pyrene	7.72	7.73	-
Benzo(a)pyrene	4.09	4.10	-
Indeno(1,2,3-cd)pyrene	0.70	0.60	-
Dibenz(ah)anthracene	1.41	1.37	-
Benzo(ghi)perylene	2.93	2.76	-

*) could not be determined because of overlapping signals

5.1.6 Stability of test item

Refractive index and fluorescence of the test item (bottle 1) were determined three times in the bottle used for test atmosphere generation. Determinations (methods see 1 and 2.1) were done once per month (09/2007 -10/2007-11/2007). Results are shown in Table 6.

Table 6: Results of stability test.

	Sample # 06-01	
Time point	RI at 25 ℃	DPA [mg/kg]
09/2007	1.4831	320
10/2007	1.4832	320
11/2007	1.4830	323

5.2 Optimization of fume generation, sampling, and analysis

Evaporation of the asphalt condensate for fume generation using the setup described in Pohlmann et al. (2006) did not yield sufficient efficiency, therefore, optimization of the evaporation process was necessary. First trials showed that only less than 50 % of the condensate could be transferred into the fume. Since under these circumstances the amount of condensate available would not be sufficient for the animal study, a better efficiency hat to be achieved.

For this purpose, in principle the temperatures of the evaporator would have to be raised to more than 300 ℃. Unfortunately, this temperature is too high for the generator to be used over a prolonged period. Therefore, a different technique was applied. In the actual setting, the nitrogen used for dispersion of the condensate is heated up to 160 ℃ (Figure 1, red part) prior to dispersion. This results in a much more effective evaporation, thus minimizing losses in the system to about 22 %.

In parallel to the inefficient evaporation, differences in the boiling point distributions of condensate and fume (Figure 5 and Figure 6) occurred. To figure out what accounted for these differences, diverse procedures for sampling and sample preparation were tried. The underestimation of light boiling material was found to be due to losses during sample preparation. Therefore, sample preparation was altered. Instead of using a flush blow method, evaporation is now conducted using a rotary evaporator with slightly negative pressure and temperature increase (650 mbar, 35 ℃). Additionally, the so-called BIA-sampler with pre-filter and a cartridge containing 2.5 g XAD2 is used for sample collection. These changes led to the results shown in Figure 7 and Figure 8, which show good agreement between the boiling point distributions of condensate and fume in the inhalation chamber.

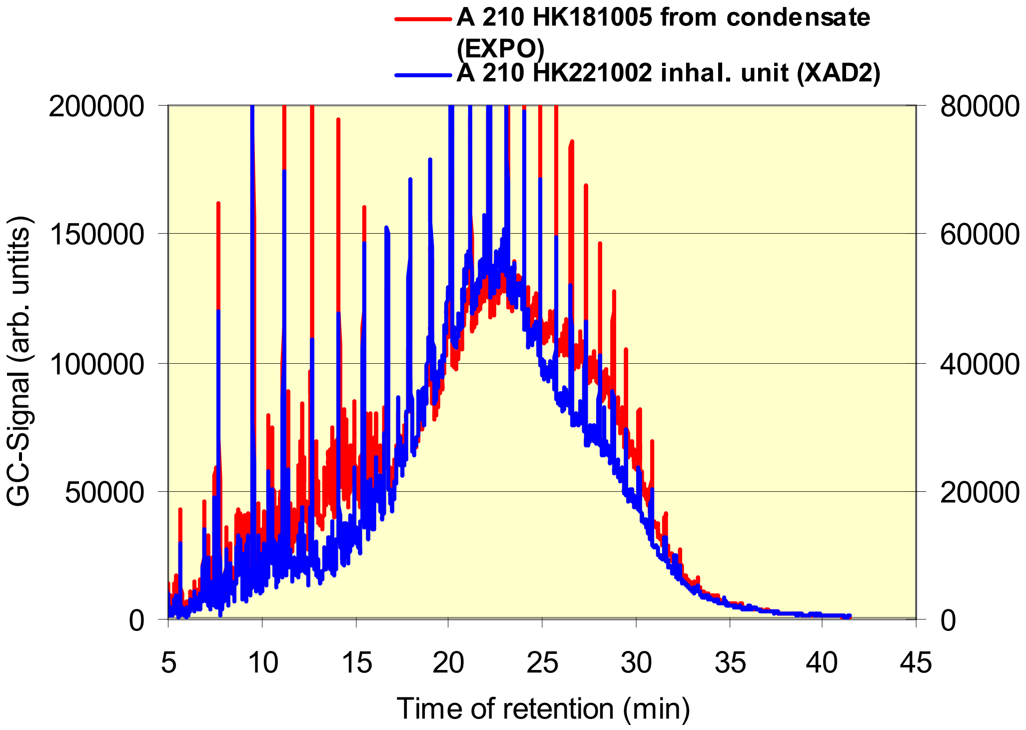

Figure 5: Comparison of chromatograms of the original condensate (red) and a sample from the inhalation unit on 1.5 g XAD with particle filter (high dose) before optimization.

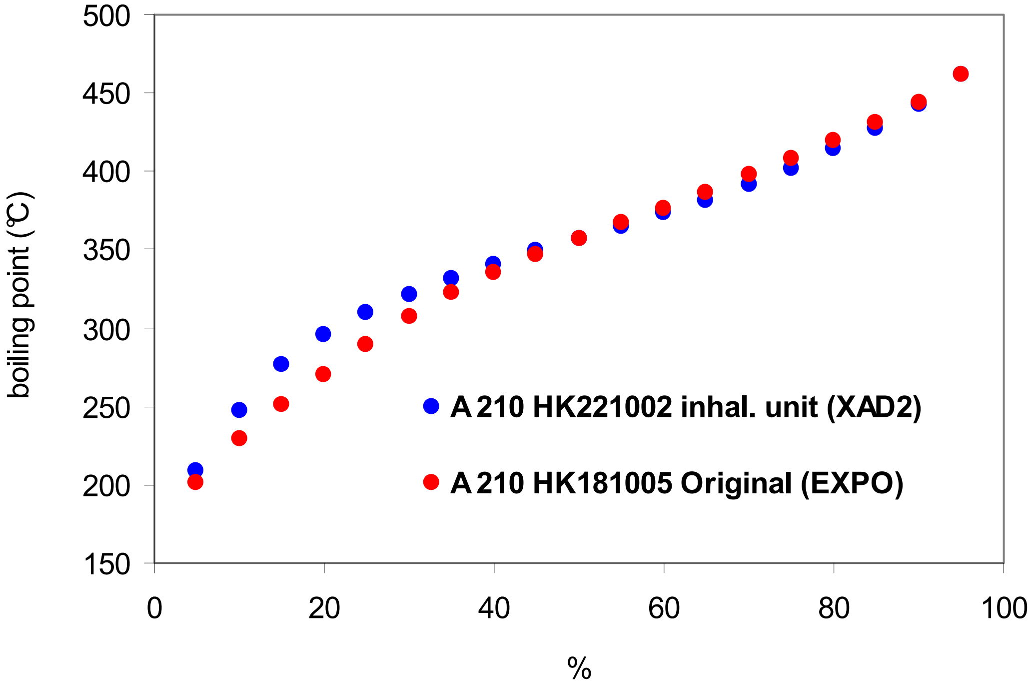

Figure 6: Comparison of boiling point distributions of the original condensate (red) and a sample from the inhalation unit on 1.5 g XAD with particle filter (high dose) before optimization.

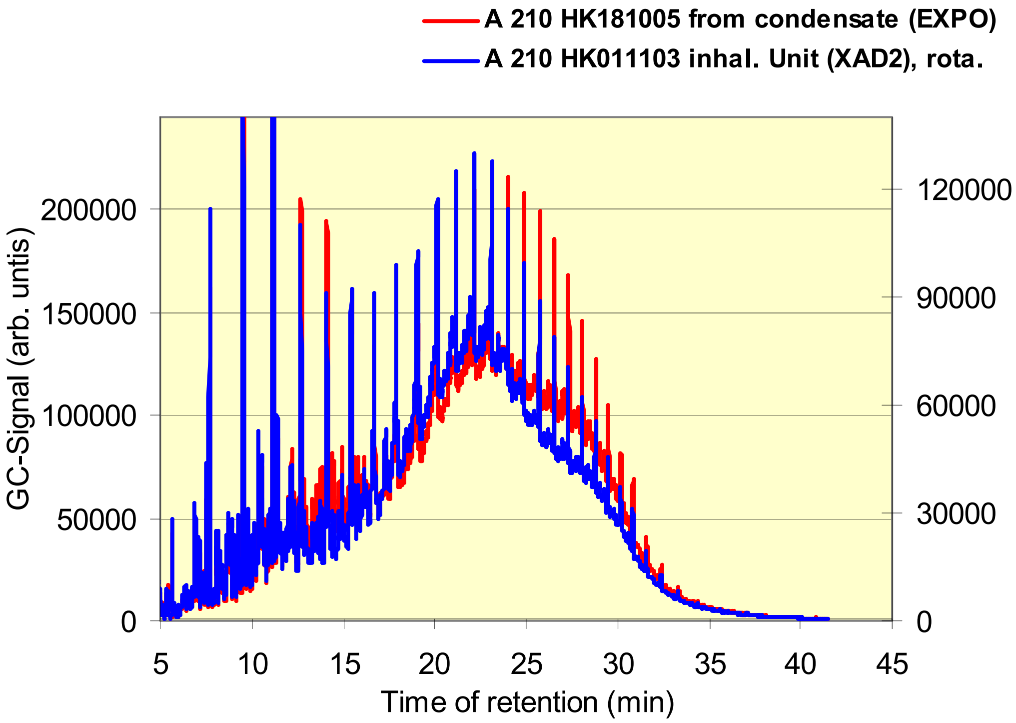

Figure 7: Comparison of chromatograms of the original condensate (red) and a sample from the inhalation unit (high dose) after optimization for evaporation, sampling (2.5 g XAD2 with filter), and sample preparation.

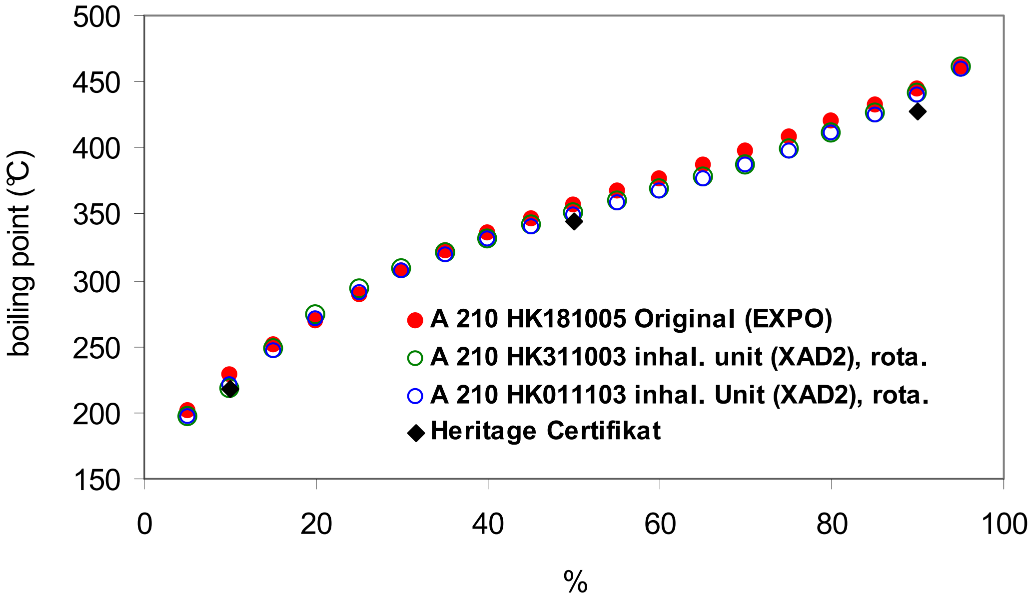

Figure 8: Comparison of boiling point distributions of the original condensate (red), two samples from the inhalation units (high dose, open green and red symbols), and the data from the certificate.

5.3 Selection of monitoring system

As already supposed in the study plan, it turned out that using a flame ionization detector (FID) necessitates a pre-filter in front of the FID. Since most of the fume is particulate (> 80 % by mass) because of the relatively low vapor pressure, FID signals are depleted. Furthermore, after a certain time blow-off from the filter mixes with the gas phase, resulting in a slow but steady increase in signal with time. After shutting down the fume generation, the signal decreases only slowly as compared to other on-line measurement methods.

For particles with (small) diameters d_p in the so-called Rayleigh-regime ($d_p << \lambda_{\text{light}}$), the scattering intensity (I) detected by an aerosol photometer is proportional to particle number concentration (C_N) times particle diameter to the power of six ($I \propto C_N d_p^6$). Since the particles generated by the current method are in this size range (compare Figure 9), photometer signals are sensitive to changes in particle size and therefore sensitive to shifts in the particle to gas equilibrium of the asphalt fume. On the other hand, the proportionality to particle number concentration allows for immediate detection of changes in particle number concentrations.

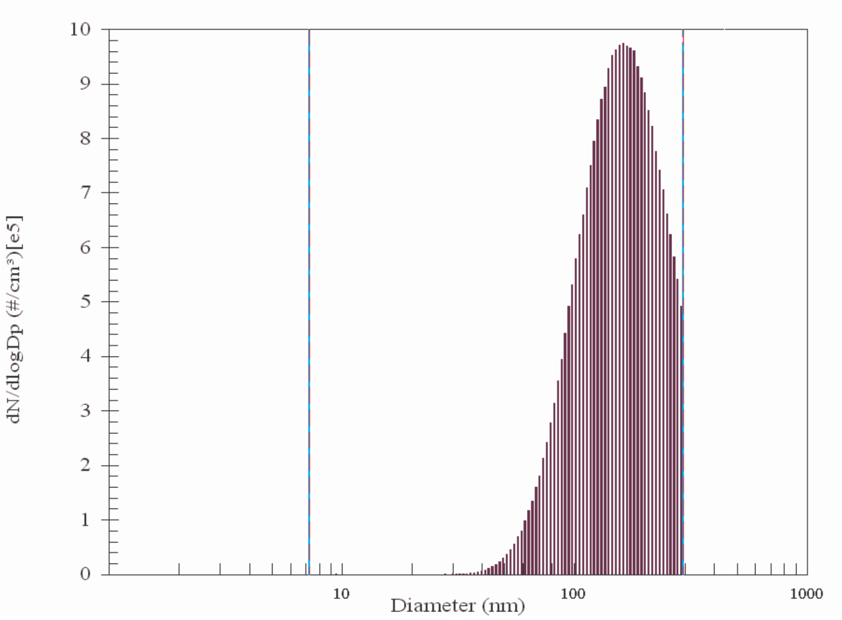

Figure 9: Particle size measured in the high-dose group using a Scanning Mobility Particle Sizer (SMPS with Model 3081 differential mobility analyzer and Model 3010 CPC, TSI Inc.).

Assuming spherical particles and if total number concentration and size distribution of an aerosol are known, total volume concentration of the particulate phase can be approximated simply by multiplying the volume of the particle with average mass with the number concentration. To assess total aerosol volume concentration as a function of time, we used data acquired with a Scanning Mobility Analyzer (SMPS with Model 3081 differential mobility analyzer and Model 3010 CPC, TSI Inc.) for comparison with FID and photometer measurements. Figure 10 shows an example of such a comparison. After starting the generator, total aerosol volume concentration shows a steep increase and reaches its final level after approx. 10 to 15 minutes. The relatively wide spread of the curve is due to measurement uncertainties of the SMPS and short-term oscillations in particle size. After shut-down of the aerosol generator, total aerosol concentration declines immediately down to about 25 % of the maximal value. Due to memory effects in the whole inhalation setup (tubing etc.) further decline is slower.

The FID signal (light grey, dashed line in Figure 10) follows the total volume curve with a time delay and a much less steep increase. After generator shut-down, the signal declines only slowly and not in accordance with total aerosol volume.

The increase in photometer signal is comparable to the increase for the FID, also not immediately following the volume concentration, but in this case for different reasons. The oscillations of the signal are due to comparable oscillations in particle size caused by the generator, which, despite the lower time resolution, can also be observed for the total aerosol volume. After generator shut-down, the photometer signal declines concurrently to the total aerosol volume. The slower increase of the photometer signal at start is due to the fact that the generator initially generates smaller particles with higher number concentrations (Figure 11) until number concentration and particle size reach a steady state. After several runs of the generator, the steady state is reached very soon and the comparison of the photometer signal with results from the chemical analysis (described in detail in chapter 5.5) shows good agreement (Figure 13).

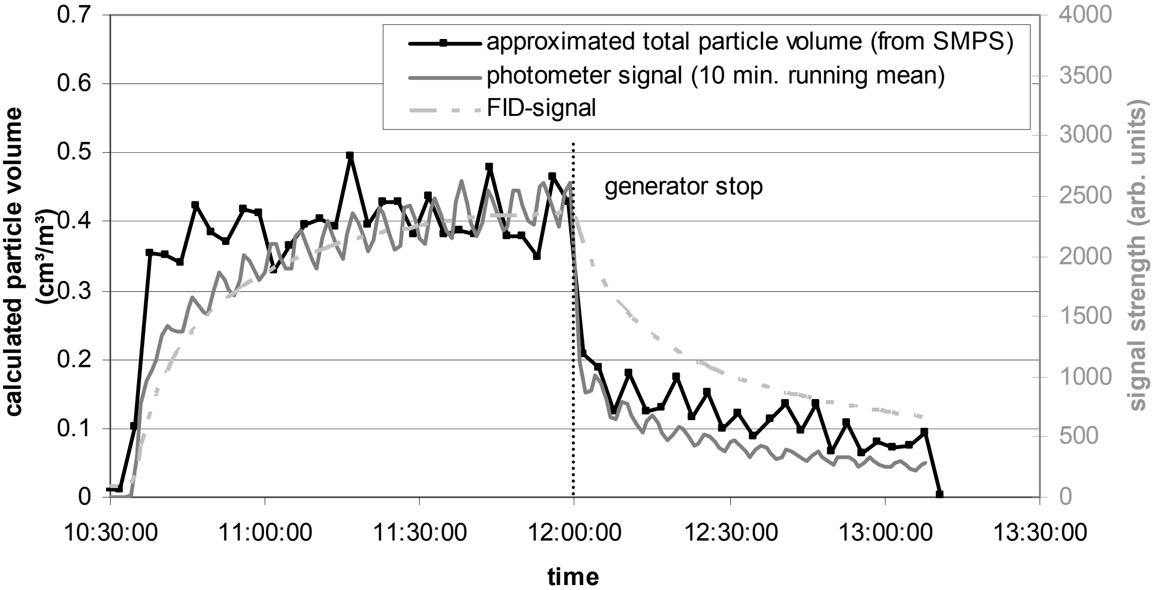

Figure 10: Comparison of output of two online monitors with total aerosol volume approximated from SMPS measurements.

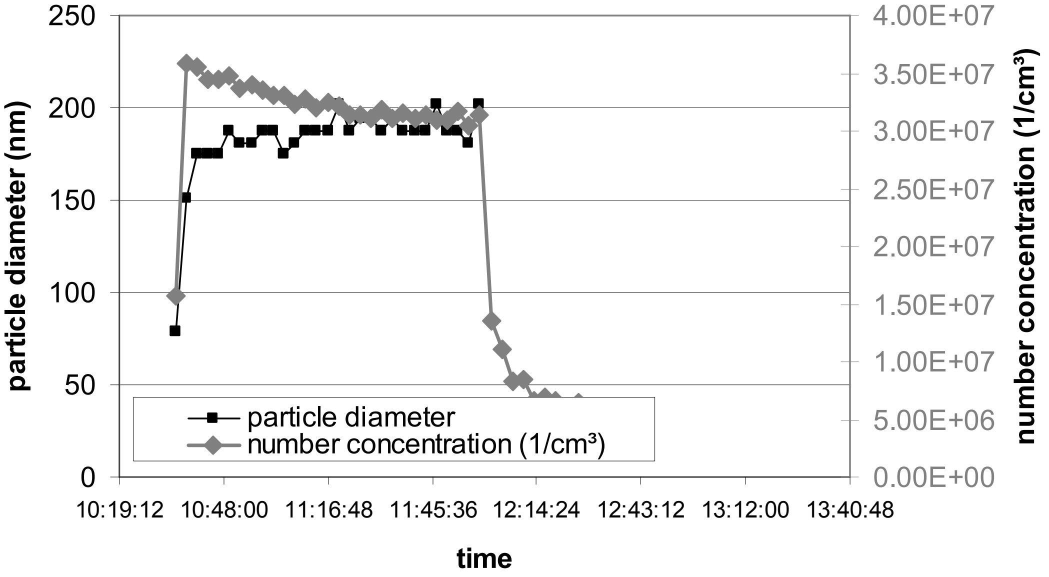

Figure 11: Total number concentration and particle size measured by SMPS.

The ideal instrument for online monitoring of asphalt fume should measure total fume or total aerosol volume like the FID or the SMPS system. Therefore, using the FID without pre-filter would be ideal but is not possible, whereas with filter the time response of the FID is poor. For technical reasons the SMPS cannot be used as an on-line monitoring system. Irrespective of the sensitivity to particle size the aerosol photometer has a short response time and high time resolution. During the inhalation study, parameters for the aerosol generator will be held constant and therefore major changes in particle size are not to be expected. Considering all arguments, the aerosol photometer is the best choice for on-line monitoring.

5.4 Target concentrations for the different dose groups

To optimize vaporization efficiency and adjust the concentrations several trials were carried out. Measurements were accomplished using sampling and measurement procedures according to BIA guideline #6305 and on-line monitoring. Because of the initially low evaporation efficiency (< 50 %) using an evaporator temperature of 220 ℃, further trials were conducted using an evaporator temperature of 280 ℃ (Table 7 and Table 8). These trials gave better evaporation efficiencies (ca. 78 %), but yielded unsatisfactory boiling point distributions, which were assumed to be due to chemical changes caused by the high evaporation temperature.

From Table 7 and Table 8 it can also be seen that it takes a certain time after generator start to reach final concentrations. So far, the nitrogen used for dispersion of the asphalt fume condensate had been kept at room temperature. Heating the pressurized nitrogen to 160 ℃ and simultaneously lowering the evaporator temperature results in the same evaporation efficiency, but also satisfactory boiling point distributions, as shown in Figure 8. Table 9 shows the results of the chemical analysis after introducing the nitrogen heating. All but the highest concentration show nearly exactly the target concentration. Before continuing with the measurements, the flow rate for the highest concentration was slightly adjusted to match

1000 mg/m³. With the current settings, photometer sensitivities are 0.44 (1000 mg/m³), 0.34 (300 mg/m³), and 0.055 (100 mg/m³) mV/(mg/m³). It should be noted though that according to experience slight shifts in sensitivity are possible and adjustments during the inhalation study based on the chemical analysis might be necessary.

Table 7: Concentrations for the different dose groups using an evaporator temperature of 280 ℃. Sampling 2 hours after start of the generator.

	Concentration [mg/m³]				
Sample #	Aerosol	Gas	Total	Actual/target	Aerosol fraction
VV2.03-9a	905	112	1017	102%	89%
VV3.03-5a	258	42.1	300	100%	86%
VV4.03-1a	78.4	14.5	93	93%	84%

Table 8: Concentrations for the different dose groups using an evaporator temperature of 280 ℃. Sampling 1 hour after start of the generator.

	Concentration [mg/m³]				
Sample #	Aerosol	Gas	Total	Actual/target	Aerosol fraction
VV2.04-9a	853	135	988	99%	86%
VV3.04-5a	217	43	260	87%	83%
VV4.04-1a	63.1	11.8	75	75%	84%

Table 9: Concentrations for the different dose groups using an evaporator temperature of 220 ℃ and preheated N_2 (160 ℃). Sampling 2 hours after start of the generator.

	Concentration [mg/m³]				
Sample #	Aerosol	Gas	Total	Actual/target	Aerosol fraction
VV2.27-9a	765	105	870	87%	88%
VV3.06-5a	259	42.2	301	100%	86%
VV4.06-1a	86.9	15.5	102	102%	85%

5.5 Monitoring system and intra-day concentration variation

On two consecutive days for 6 hours each, concentration measurements in the inhalation unit were conducted for the high-dose group. These runs were the first with a duration of 6 hours. After shut-down of the aerosol generator after the first 6-hour trial, all air flows were stopped immediately, so the saturation time for the tubing of the inhalation setup on the next day was reduced significantly to be clearly recognized by the much higher onset of the photometer signal on the second day (compare Figure 12 and Figure 13). Using the on-line monitoring system the whole time period was covered and 1-minute averages of the signals were stored on the computer. For comparison, three samples per day were taken from one port of the inhalation system, analyzed according to BIA guideline #6305, and compared to the on-line signal. The samples were taken at the beginning (1/2 h after start), in the middle (3 h after start), and at the end of the generation periods (5 ½ h after start).

The results of the chemical analysis are shown in Figure 12, Figure 13, Table 10, and Table 11. Average concentrations calculated from photometer measurements are 946 and 1043 mg/m³ for days 1 and 2, respectively. The corresponding average values from the chemical analysis are 990 and 1045 mg/m³, thus resulting in deviations of photometer from chemical values by 5 % (day 1) and < 1 % (day 2).

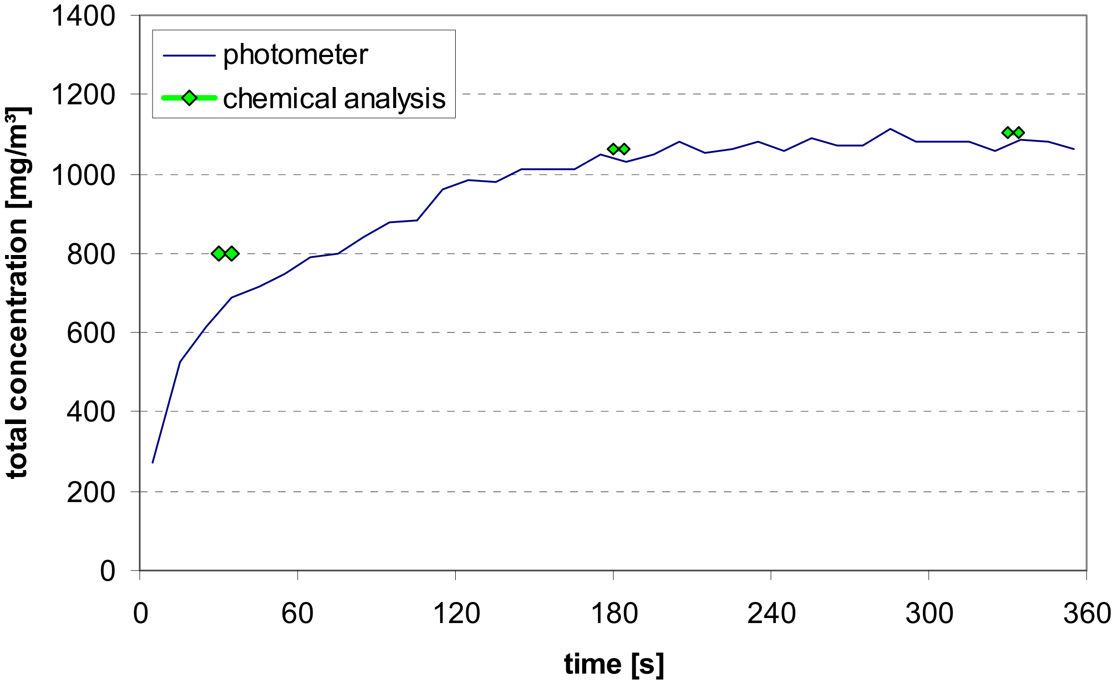

Figure 12: First trial of 6-hour concentration measurement with a photometer (calculated based on a sensitivity of 0.44 mV/(mg/m³)) and by chemical analysis. Note that in this case the whole system had been flushed for more than 1 h with clean air prior to conducting the trial. Therefore, initial photometer signals and concentrations were decreased.

Table 10: Concentrations determined by chemical analysis (BIA method) for the first 6-hour trial. Note that in this case the whole system had been flushed for more than 1 h with clean air prior to conducting the trial. Therefore, initial concentrations were decreased.

Start time (min)	Sample #	Concentration [mg/m³]			Actual/ target	Aerosol fraction
		Aerosol	Gas	Total		
30	VV2.28-9a	701	98	799	80%	88%
180	VV2.29-10a	942	122	1064	106%	89%
330	VV2.30-11a	989	117	1106	111%	89%

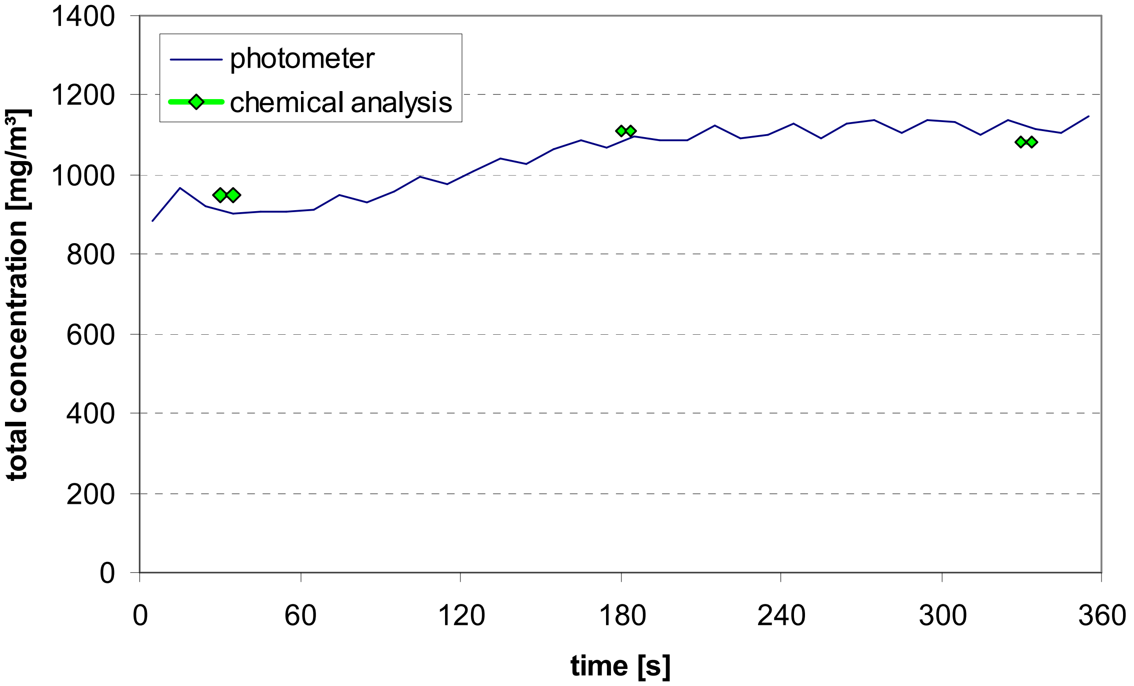

Figure 13: Second trial of 6-hour concentration measurement with a photometer (calculated based on a sensitivity of 0.44 mV/(mg/m³)) and by chemical analysis. Note that in this case the whole system had not been flushed prior to the trial. Therefore, initial photometer signals and concentrations were not decreased.

Table 11: Concentrations determined by chemical analysis (BIA method) for the second 6-hour trial. Note that in this case the whole system had been flushed for more than 1 h with clean air prior to conducting the trial. Therefore, initial concentrations were decreased.

Start time (min)	Sample #	Concentration [mg/m³]			Actual/ target	Aerosol fraction
		Aerosol	Gas	Total		
30	VV2.31-9a	849	98	947	95%	90%
180	VV2.32-10a	988	120	1108	111%	89%
330	VV2.33-11a	965	116	1081	108%	89%

5.6 Test material homogeneity

Each inhalation unit used in the repeated-dose toxicity study will consist of three levels with 16 inhalation ports each. To confirm test material homogeneity, the following parameters have been measured at four ports (at 0, 90, 180, and 270°) of each level in the three dose groups: concentration of aerosol and gas phases (by BIA method) and composition after simulated distillation. Tables 12 to 14 show the results of the chemical analysis as relative distributions. The relative distributions shown in the tables where calculated by dividing the values of each column by the column mean (=100%). The ratios of aerosol concentration to total concentration were measured to be 86 % ± 0.3 % (low-dose group, spread 86-87 %), 86 % ± 0.3 % (mid-dose group, spread 85-86 %), and 89 % ± 0.4 % (high-dose group, spread 88-89 %). In none of the groups a deviation from the average concentration greater than 7 % or significant differences between the ratios of aerosol to total concentration at the different positions and levels were detected, and hence no segregation of the vapor and aerosol phases takes place.

Table 12: Test material homogeneity in the low-dose group (100 mg/m³) expressed as relative concentrations measured by the BIA method.

		Relative distribution		
Level	Position (°)	Aerosol	Vapor	Total
3	0	100%	98%	99%
3	45	100%	99%	100%
3	90	100%	99%	100%
3	135	100%	100%	100%
2	0	97%	95%	97%
2	45	102%	108%	103%
2	90	101%	100%	100%
2	135	99%	104%	100%
1	0	100%	99%	100%
1	45	101%	101%	101%
1	90	101%	98%	100%
1	135	100%	99%	100%

Table 13: Test material homogeneity in the mid-dose group (300 mg/m³) expressed as relative concentrations measured by the BIA method.

		Relative distribution		
Level	Position (°)	Aerosol	Vapor	Total
3	0	102%	98%	101%
3	45	100%	101%	100%
3	90	99%	101%	100%
3	135	101%	100%	101%
2	0	97%	95%	97%
2	45	100%	105%	101%
2	90	100%	101%	100%
2	135	100%	100%	100%
1	0	100%	100%	100%
1	45	100%	101%	101%
1	90	100%	98%	100%
1	135	100%	101%	100%

Table 14: Test material homogeneity in the high-dose group (1000 mg/m³) expressed as relative concentrations measured by the BIA method.

		Relative distribution		
Level	Position (°)	Aerosol	Vapor	Total
3	0	104%	109%	104%
3	45	104%	100%	104%
3	90	101%	101%	101%
3	135	104%	101%	103%
2	0	101%	101%	101%
2	45	103%	96%	102%
2	90	93%	93%	93%
2	135	99%	101%	99%
1	0	98%	96%	98%
1	45	97%	103%	98%
1	90	98%	101%	98%
1	135	98%	96%	98%

Table 15 to Table 17 show the averages of the results of measurements by simulated distillation. The intra group deviations are very small, therefore only the averages and the absolute maximum and minimum values from all positions and levels are shown. In all groups no significant trends in regard to level or position have been observed. In Figure 14 the average boiling point distributions measured are compared. Intra group variations are so small that the error bars are smaller than the symbols for the values. The points for high and medium group overlap for the total distribution. For the low dose group small deviations for lower temperatures can be observed. These deviations are most probably due to the prolonged sampling period made necessary by the low concentration causing slight blow of from the XAD absorption tube.

Table 15: Test material homogeneity in the low-dose group (100 mg/m³) after simulated distillation.

SUM_T	Mean	ASD	RSD	min.	max.
(%)	T (℃)		(%)	T (℃)	
5	205	1.6	0.8	203	207
10	**237**	**2.5**	**1.1**	235	243
15	271	3.0	1.1	268	277
20	296	2.5	0.8	293	301
25	313	1.8	0.6	311	317
30	326	1.7	0.5	324	330
35	337	1.8	0.5	335	341
40	346	1.8	0.5	344	350
45	355	1.6	0.5	354	359
50	**364**	**1.8**	**0.5**	362	368
55	372	2.1	0.6	370	376
60	381	2.1	0.6	379	385
65	390	2.3	0.6	388	394
70	399	2.5	0.6	397	404
75	410	2.7	0.6	407	415
80	421	2.7	0.6	418	426
85	432	2.4	0.6	429	437
90	**445**	**2.5**	**0.6**	442	449
95	461	3.1	0.7	458	467

ASD = Absolut standard deviation,
RSD = Relative standard deviation

Table 16: Test material homogeneity in the mid-dose group (300 mg/m³) after simulated distillation.

SUM_T	Mean	ASD	RSD	min.	max.
(%)	T (℃)		(%)	T (℃)	
5	199	1.9	0.9	197	202
10	**228**	**1.6**	**0.7**	226	231
15	257	2.4	0.9	253	260
20	282	1.8	0.6	280	285
25	300	0.9	0.3	299	301
30	315	1.0	0.3	313	316
35	327	0.9	0.3	325	328
40	337	0.9	0.3	335	338
45	346	0.8	0.2	344	347
50	**355**	**0.7**	**0.2**	354	356
55	363	0.8	0.2	362	364
60	371	0.8	0.2	370	373
65	380	0.8	0.2	379	382
70	390	0.9	0.2	389	392
75	401	1.1	0.3	399	403
80	413	1.0	0.2	411	414
85	425	1.1	0.3	424	427
90	**439**	**1.0**	**0.2**	438	441
95	457	1.1	0.2	455	459

ASD = Absolut standard deviation,
RSD = Relative standard deviation

Table 17: Test material homogeneity in the high-dose group (1000 mg/m³) after simulated distillation.

SUM_T	Mean	ASD	RSD	min.	max.
(%)	T (°C)		(%)	T (°C)	
5	204	1.8	0.9	200	206
10	**233**	**1.7**	**0.7**	229	235
15	259	2.5	1.0	254	261
20	282	2.5	0.9	277	284
25	301	1.8	0.6	297	302
30	316	1.8	0.6	312	318
35	328	1.8	0.6	325	330
40	339	2.0	0.6	336	341
45	349	2.1	0.6	345	351
50	**358**	**2.1**	**0.6**	355	361
55	367	2.1	0.6	363	370
60	377	2.5	0.7	372	380
65	386	2.8	0.7	381	390
70	396	3.1	0.8	391	401
75	407	3.2	0.8	402	412
80	419	3.2	0.8	413	423
85	432	3.1	0.7	426	435
90	**445**	**3.1**	**0.7**	439	449
95	464	3.9	0.8	456	468

ASD = Absolut standard deviation,
RSD = Relative standard deviation

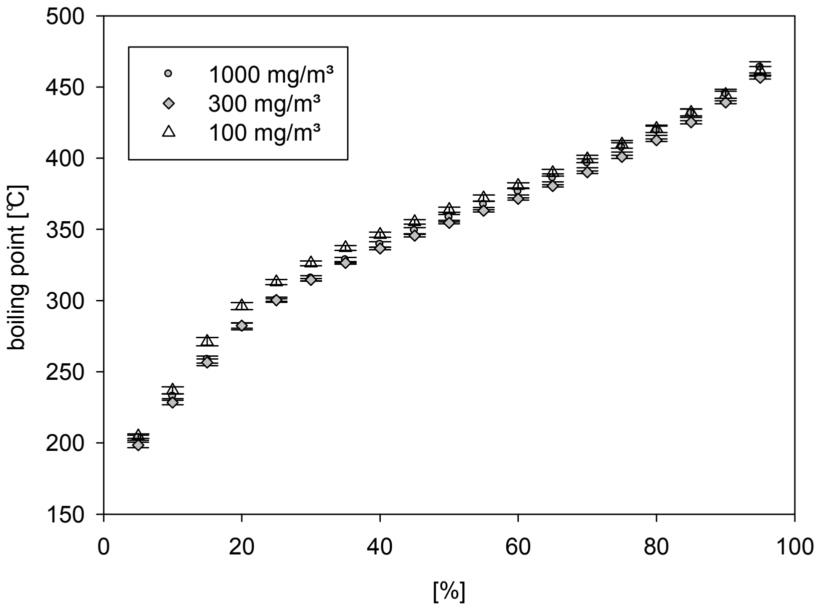

Figure 14: Inter group comparison of the average boiling point distributions.

6 Archiving

One original of the study plan will be stored at least for the period of time required by the GLP principles. Thereafter, the Fraunhofer ITEM and the Sponsor will have to agree on further archiving.

7 Summary and conclusions

The objective of this study was to demonstrate that:

1. The selected experimental atmosphere generation system that will be used for the inhalation exposures produces consistent target concentrations within the animals' breathing zones.
2. The monitoring system to be selected accurately measures test article concentrations and there is acceptable agreement between nominal and measured concentrations.
3. There is acceptable test material homogeneity throughout the chamber.

The test item used was verified and its stability assessed. No measurable changes of the test material during time have been detected.

Fume generation has been optimized, resulting in a generation efficiency of 78 % utilizing a pump feed rate of 4.3 g/h, pressurized nitrogen at 160 °C, and an evaporator temperature of 220 °C. After optimization of fume generation and sample procedures, comparison of the boiling point distributions of the original asphalt fume condensate with test samples shows good agreement.

Despite particle size dependency, the aerosol photometer turned out to be the best on-line monitor. In routine operation, the match between chemical sample analysis and daily averages of the photometer reading can be expected to be in the range of ± 5 % or better. Periodical check and optionally recalculation of the photometer sensitivity during the inhalation study using the results of chemical analyses is recommended.

A fine adjustment of the actual concentrations to match target concentrations is easily achieved by altering the dilution ratios. It has been shown that it is possible to control fume generation parameters in such a way that a match between nominal and actual daily averaged concentration within a tolerance of ± 1 0% is possible. Due to memory effects in the total inhalation system (pipes and tubes) concentration depletion during the first 1 to 2 hours after start of the system is possible, but can almost be prevented by not flushing the tubes with fresh air. In this case, concentration variation during 6 hours is in the range of ± 1 0%.

Test material homogeneity measured at 36 positions of the inhalation units in the three dose groups regarding measured concentrations are in the range of ± 4 %. The widest spread with 93-109 % was found in the high-dose group. The data give no clue to systematic deviations from the average depending on position or level within the inhalation units. No segregation of aerosol and vapor phases has been detected.

8 References

Pohlmann, G. , A. Preiss, W. Koch, H. Kock, M. Elend, M. Raabe (2006), "Collection, Validation and Generation of Asphalt Fumes for Inhalation Studies in Rats. Part 3: Regeneration of Asphalt Fumes, Inhalation Setup and Validation", Ann Occup Hyg., 50 (2006), Nr.8, S.813-819