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Measurements of Polycyclic Aromatic Hydrocarbons and Genotoxicity in Soot Deposited at a Toll Plaza near Durban, South Africa

Susan J. Godefroy, Bice S. Martincigh* and Leo F. Salter‡

School of Chemistry, University of KwaZulu-Natal, Howard College Campus, Durban, 4041 South Africa.

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ABSTRACT

This research was designed to examine the presence of polycyclic aromatic hydrocarbons (PAHs) in soot deposited at the Mariannhill toll plaza situated on the N3 highway in KwaZulu-Natal, South Africa. Samples were collected from the toll plaza either by scraping the toll booth walls and surrounding areas, or by wiping the surfaces with cotton wool swabs. The organic component was separated by ultrasonic extraction into dichloromethane and analysed for PAHs by reverse phase high performance liquid chromatography with both fluorescence and ultraviolet detection. The genotoxicity was investigated by means of two bacterial assays: the Ames test and the SOS Chromotest. A number of PAHs were identified and genotoxic activity was observed in both of the assays.

KEYWORDS

Polycyclic aromatic hydrocarbons, toll plaza, genotoxicity.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental pollutants that are of health concern because of their mutagenic and carcinogenic properties. They are formed during the incomplete combustion of organic matter[1,2] and are present in the atmosphere through the burning of fossil fuels and vegetation, in cigarette smoke, and in smoked or barbecued food.

PAHs remain in the gas phase at temperatures greater than 150°C, but condense onto particles at lower temperatures. Consequently, at typical ambient temperatures they exist primarily in the particulate phase[3] and are normally associated with respirable particles, i.e. particles of diameter less than 2.5 μm.[4] The portion of respirable particles that has the greatest abundance of PAHs is able to penetrate to the thoracic region of the lung, where it has a higher deposition efficiency and provides an in-body source of PAHs which can last for weeks.

On entering the body, PAHs are metabolized to a variety of metabolites[2,5,6] via a reactive epoxide intermediate. Further activation to a diol epoxide can occur, and this is considered to be a precursor to the product that can interact with the nucleic acid bases on the DNA. The reaction takes place primarily with the exocyclic 2-amino group of guanine, and this bulky PAH substituent on the DNA base interferes with replication merely because of its size.[7]

The emission of PAHs into the environment is also of concern because they can undergo reactions with constituents of the atmosphere – such as oxygen, ozone and nitrogen oxides – to produce compounds that are more mutagenic than the parent PAH.[3]

It is well documented that particulate emissions from vehicles exhibit mutagenic properties as determined via the *Salmonella typhimurium* histidine-reversion mutation assay (the Ames test), and that PAHs are the major contributors of this observed mutagenicity.[8–10] Since a toll plaza is an area of high traffic density, it is an ideal location for an investigation into the build-up of particles emitted by vehicles, and for a study of the extent to which toll booth workers are exposed to harmful compounds. The Mariannhill toll plaza situated on a national highway (N3) approximately 22 km west of Durban, South Africa, was chosen for this study. The toll booths at this plaza contained visible deposits of particulate soot-like material. It is known that soot from combustion sources, such as vehicular emissions, consists primarily of fine particles which tend to have a higher PAH content than coarse particles.[11,12] This paper will describe the extraction of the toll booth soot, PAH analysis by reverse phase high performance chromatography (HPLC) with both fluorescence and ultraviolet (UV) detection, and genotoxicity testing by means of the Ames test and the SOS Chromotest. The approach followed here differs from that of others in that deposited particulate matter was analysed rather than airborne particles collected on sampling filters. In addition, the sparsity of actual pollutant level measurements at toll plazas, and in South Africa in particular, and the lack of PAH monitoring in South Africa, add importance to this work.

2. Experimental

2.1. Sample Preparation

The deposited soot was collected by scraping the toll booth walls, ceilings and surrounding areas with a spatula into glass vials, or by wiping the surfaces with cotton wool and storing this cotton wool in zip-seal plastic bags. All samples were stored in the dark at 4°C.

In order to separate the organic matter from the inorganic and carbonaceous, the soot was ultrasonically extracted into dichloromethane (DCM). Typically, 200 mL of DCM was used per 0.2 g of soot. The samples were extracted for 2 h, filtered through a Millipore 0.45 μm HV organic aqueous compatible filter to remove the particles, and the filtrate (the crude extract)

* To whom correspondence should be addressed. E-mail: martinci@ukzn.ac.za
‡ Present address: Cornwall College, Cornwall TR15 3RD, United Kingdom.

was divided into three portions. Two portions were rotary evaporated to dryness, weighed and stored in the dark at 4°C for analysis in the genotoxicity assays. The remaining extract was reduced to 2 mL by rotary evaporation, loaded onto Florisil columns and eluted with DCM. The combined eluents were evaporated to dryness under nitrogen and stored in the dark at 4°C prior to analysis by reverse phase HPLC with UV detection. This procedure was also performed on DCM as organic solvents do contain some PAH impurities and can adsorb them from the atmosphere: it is important therefore to have a solvent blank. In order to assess sample loss by the ultrasonic extraction and Florisil clean-up procedure, a solution containing four of the PAH standards was used to test the recovery efficiency. From the HPLC analyses it appeared that this sample preparation procedure did not result in any loss of the PAHs extracted. Samples of the crude extract prior to clean-up on Florisil were also sent to the Department of Environmental Science at Lancaster University in the United Kingdom, where they were analysed by reverse phase HPLC with fluorescence detection.

2.2. Preparation of Florisil Columns

These were prepared in the laboratory by plugging Pasteur pipettes with glass wool and packing with 1 g of Florisil (60–100 mesh). Each column was loaded with 1 to 2 mL of the crude extract and eluted with 10 mL of DCM. This procedure has been reported to give good and rapid recovery of neutral aromatic compounds.[13]

2.3. Standards and Solvents

The 12 PAHs obtained commercially for use as standards were anthracene, acenaphthylene, anthraquinone, pyrene, phenanthrene, fluorene, fluoranthene, 9-fluorenone, chrysene, triphenylene, 2-methyl anthracene and benzo(a)pyrene. These were chosen as standards because they are readily available and have been identified amongst those found in vehicle exhaust emissions.[9,10] Solutions of 10^{-3} M concentration were prepared of each such PAH in a mixture of 50% (v/v) DCM-methanol, and a standard PAH mixture was prepared by combining 1 mL of each solution. The PAH mixture used as a standard at Lancaster University contained 17 individual PAHs: namely, napthalene, acenaphthene, fluorene, 1-methyl phenanthrene, benzanthracene, chrysene, coronene, benzo(b)fluoranthene, dibenz(a,c)anthracene, anthracene, fluoranthene, pyrene, benzo(k)fluoranthene, benzo(a)pyrene, dibenz(a,h)anthracene, phenanthrene and benzo(g,h,i)perylene. These were used to prepare a stock standard solution containing approximately 10 mg mL^{-1} of each compound, which was subsequently diluted to give a working standard level of 13 μg mL^{-1} of each PAH.

All PAHs purchased were stated to be more than 98% pure and all solvents were of Analar or HPLC grade.

2.4. HPLC Analysis

Analysis in this laboratory was performed on a Waters 600 multisolvent delivery system with a U6K injector linked to a Waters 990 photodiode array detector. All injections were monitored at 254 nm. A Supelco LC-PAH 4.6 × 250 mm (5 μm particle size) C_{18} column specific for PAHs was used. The condition that was found to give the best separation of the 12 PAHs was a gradient elution involving 100% water for 10 min, changed over 10 min to 35% (v/v) acetonitrile-water held for 10 min and then changed over 20 min to 100% acetonitrile at a flow rate of 1.5 mL min^{-1}. The extract from the Florisil columns was reconstituted in 2 mL of 50% (v/v) DCM-methanol, and both the standards and the sample were filtered through a 0.45 μm syringe filter prior to injecting onto the HPLC column. Injections of 10 μL of the sample and standard mixture were made and those PAHs for which standards were available were identified from their retention times and characteristic UV spectra.

Analysis at Lancaster University was performed on a Perkin Elmer binary HPLC pump 250 with a Perkin Elmer LS 40 fluorescence detector. A Spherisorb 150 × 4.6 mm (5 μm particle size) PAH cartridge was used. A gradient of 60% (v/v) acetonitrile-water, held for 15 min and increased linearly to 100% acetonitrile over 10 minand held for a further 20 min at a flow rate of 1.5 mL min^{-1}, was employed as the mobile phase. The crude extract was dissolved in DCM, filtered through a 0.5 μm syringe filter and volumes of 2 μL injected.

2.5. The Ames Test

This is a bacterial revertant assay developed in the mid-1970s by Bruce Ames and his colleagues. It utilizes specially mutated strains of *Salmonella typhimurium* (a colon bacterium) and has been used extensively as a rapid *in vitro* test for the detection of chemical carcinogens[14–17] and to demonstrate the mutagenic potential of complex environmental samples. The procedure of Maron and Ames[18] was followed here. The bacterial tester strains TA98 (which detect frameshift mutagens) and TA100 (which detect base-pair substitution mutagens) were grown up overnight at 37°C in Oxoid nutrient broth No. 2. The dried crude extract was dissolved in dimethylsulfoxide (DMSO) to yield the maximum desired concentration in μg $plate^{-1}$, and serial dilutions were prepared from this stock to give concentrations of 0.1, 1, 10, 20, 40, 60, 80 and 100 μg $plate^{-1}$. Since unsubstituted PAHs are not mutagenic in their original forms, and require metabolic activation in order to express their mutagenic activity (these are called promutagens), an activating system (S9-mix) derived from rat liver enzymes (S9 fraction) was added to the test plates. The preparation is described in detail by Maron and Ames.[18] The plate incorporation test procedure was followed. This involved the combination of 0.1 mL or less of the test chemical (toll booth extract), 0.5 mL of the S9-mix and 0.1 mL of the bacterial tester strain in 2 mL of soft agar containing a small amount of histidine and biotin (10 mL per 100 mL of agar), held at 45°C. This mixture was poured onto hardened minimal glucose agar plates and incubated for 48 h at 37°C, after which time the plates were removed and scored for revertant colonies. Triplicate plates were prepared for each concentration of extract and tests performed in the presence and absence of the S9-mix. Included in the assay were positive and negative control plates. Positive controls contain a compound that is a known mutagen for that strain (10 μL of 2 mg mL^{-1} 2-aminofluorene with S9-mix for TA98, and 10 μL of 1 mg mL^{-1} sodium azide without S9-mix for TA100), and are included in the test to check that the bacteria are responding in the correct manner. Negative controls consist only of the bacterial strain, the solvent DMSO and the presence or absence of S9-mix; any colonies formed on these plates are known as spontaneous revertants. The sample was considered to be mutagenic when the number of revertants was greater than twice the number of spontaneous revertants.

2.6. The SOS Chromotest

This is a commercially available test kit that detects DNA damage caused by genotoxins and is particularly suitable for testing environmental samples. It uses a specially modified strain of the intestinal bacterium *Escherichia coli, E. coli* PQ 37, that contains a fusion of an operon which places *lacZ,* the structural gene for β-galactosidase, under the control of the *sfiA* gene, a

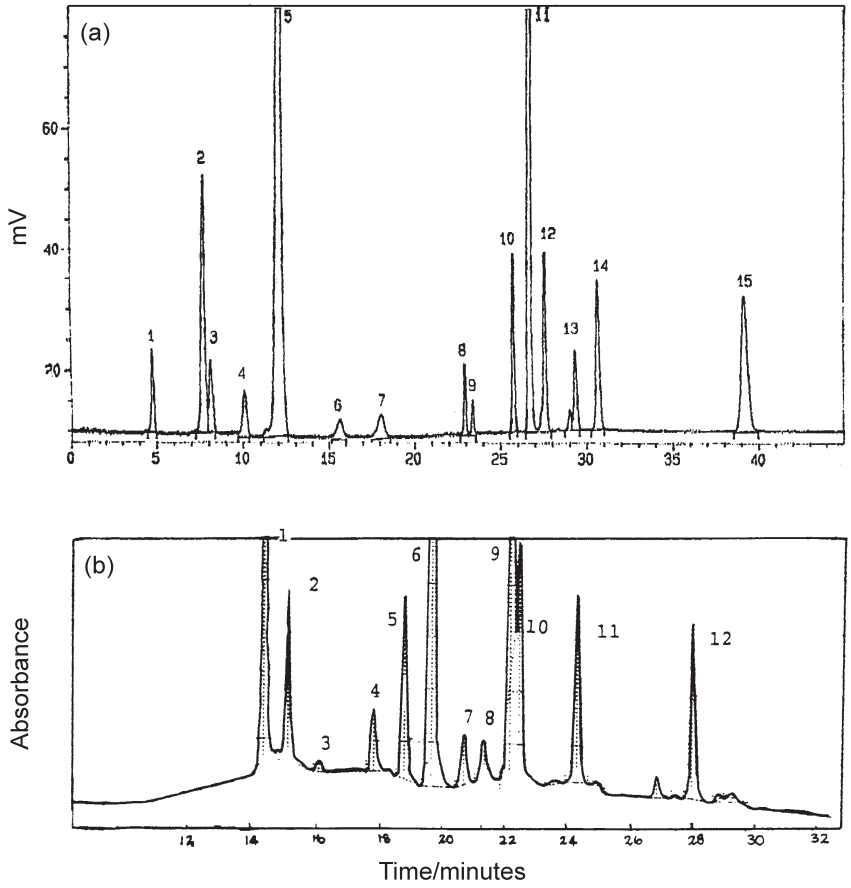

Figure 1 (**a**) Typical chromatogram obtained with fluorescence detection for the 17 standard PAHs. Peaks: 1: naphthalene, 2: acenaphthene, 3: fluorene, 4: phenanthrene, 5: anthracene, 6: fluoranthene/1-methyl phenanthrene, 7: pyrene, 8: benzanthracene, 9: chrysene, 10: benzo(b)fluoranthene, 11: dibenz(a,c)anthracene/benzo(k)fluoranthene, 12: benzo(a)pyrene, 13: dibenz(a,h)anthracene, 14: benzo(ghi)perylene, 15: coronene. HPLC conditions are as described in Section 2.4. Separation of fluoranthene from 1-methyl phenanthrene, and dibenz(a,c)anthracene from benzo(k)fluoranthene could not be achieved.

(**b**) Typical chromatogram obtained with UV detection for the 12 standard PAHs. Peaks: 1: 9-fluorenone, 2: anthraquinone, 3: acenapthylene, 4: fluorene, 5: phenanthrene, 6: anthracene, 7: fluoranthene, 8: pyrene, 9: 2-methyl anthracene, 10: triphenylene, 11: chrysene, 12: benzo(a)pyrene. HPLC conditions are as described in Section 2.4.

SOS function that is involved in cell division inhibition.[19] The β-galactosidase activity depends only on the *sifA* activity and will therefore be produced if the SOS repair function is activated by a DNA-damaging agent. The addition of a chromogenic substrate allows the production of this enzyme to be detected. An underestimate of the β-galactosidase activity is obtained if the substances being tested inhibit protein synthesis.[20] To correct for this possible error alkaline phosphatase activity is measured concurrently in order to assess general protein synthesis. This enzyme is not under the control of the SOS repair system and is therefore not inducible by DNA-damaging substances.

All solutions required for the test were supplied in the commercially available test kit (Orgenics Ltd, Israel), except for the S9-mix which was prepared as for the Ames test. The bacteria and the standard solutions were prepared as described in the instruction manual provided with the kit. The toll booth extract was dissolved in DMSO to give a solution of concentration 10 000 μg mL^{-1}. Serial 1 to 1 dilutions were made of this stock solution to obtain 14 solutions ranging in concentration from 10000 μg mL^{-1} to 1.2 μg mL^{-1}. The extract in DMSO (3 μL) was pipetted into the wells of the microplate, 100 μL of the bacteria added or 100 μL of the bacteria containing S9-mix added to the half of the plate that was being tested for promutagens. The plate was incubated for 2 h at 37°C. The blue chromogenic substrate was added to the alkyl phosphatase substrate and mixed well. A volume of 100 μL of this mixture was added to each well and the plate returned to the oven for a further 90 min, when a green colour developed in the wells. The plate was removed and 50 μL of the stop solution added to each well to halt the reaction. An aliquot of 50 μL was taken from each well and the absorbance read at 615 nm against a negative control in order to determine the β-galactosidase activity. The negative control contained the bacteria, the sample diluent and the S9-mix (if used). The absorbance was also read at 405 nm to determine the alkaline phosphatase activity. However, no meaningful results were obtained for this latter measurement as the blank solution had a larger absorbance value than any of the samples. Two positive controls were also included: 4-nitroquinoline oxide (4NQO) as a control for direct-acting mutagens (–S9), and 2-aminoanthracene as a control for promutagens (+S9). The genotoxic effect is expressed as the SOS-inducing potency (SOSIP), which is the slope of the linear portion of the dose-response relationship. The results of the SOS Chromotest were considered positive if the SOSIP was positive.

3. Results and Discussion

3.1. HPLC Analysis

Since PAHs absorb light in the 200–400 nm region and also strongly fluoresce, HPLC with either UV or fluorescence detection is widely used for their analysis.[21] Both detection systems were used in this work. Figure 1 shows typical

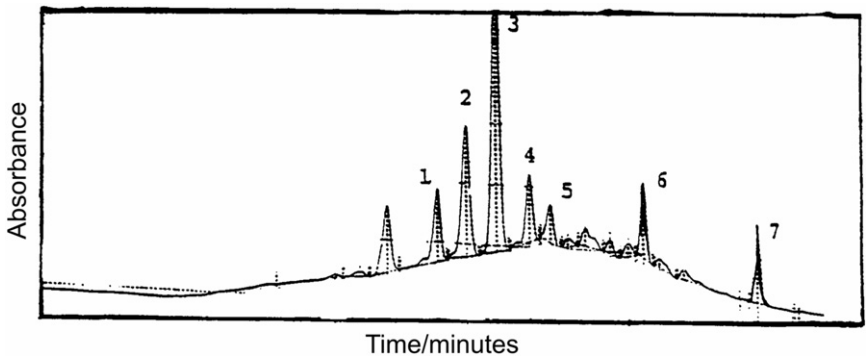

Figure 2 Typical chromatogram of the toll booth extract obtained using absorption at 254 nm. Peaks: 1: fluorene, 2: phenanthrene, 3: anthracene, 4: fluoranthene, 5: pyrene, 6: chrysene, 7: benzo(a)pyrene. HPLC conditions are as described in Section 2.4.

chromatograms obtained with these detection systems for the standard PAHs. In general, fluorescence detection gives improved limits of detection. In this work, UV detection suffered from interference from other UV-absorbing compounds that were also extracted, and the samples were sent to Lancaster University for confirmatory analysis by fluorescence detection. A typical chromatogram obtained with UV detection for the toll booth extract is displayed in Fig. 2.

Table 1 lists the PAHs found in the toll booth extract by HPLC with both UV and fluorescence detection. As can be seen from Table 1, a variety of PAHs were identified, a number of which are known carcinogens, e.g. benzo(a)pyrene, fluoranthene, chrysene, benzanthracene, benzo(g,h,i)perylene and coronene. These results are comparable to those obtained by other workers[9,10,22] in their investigations and analysis of vehicle exhaust emissions.

Although no exact quantitation was performed, the relative amounts of the various PAHs identified by HPLC with fluorescence detection are shown in Fig. 3 for samples of soot taken from inside and outside the toll booths. The PAH contents of the soot scraped from inside and outside the toll booths are fairly comparable. Takada *et al.*[12] found similarly that the PAH content and composition of Tokyo street dusts showed no consistent difference between inside and outside of tunnels, nor between asphalt-paved and concrete-paved streets. They therefore concluded that atmospheric fallout and asphalt particles are only minor contributors to PAHs in street dusts in heavily trafficked roads, where the PAHs are derived mainly from vehicle exhausts. Two pairs of PAHs, namely fluoranthene and methylphenanthrene, and dibenz(ac)anthracene and benzo(k)fluoranthene, could not be separated from each other with the HPLC separation procedure used at Lancaster University. Since the calibration mixture contained the same masses of all the PAH standards, the results for these two pairs are given as a mixture of the two PAHs in a mass proportion of 1:1. The results in Fig. 3 indicate that fluoranthene/methylphenanthrene and pyrene were the most abundant PAHs found in the toll booth soot. This is in keeping with the results of Sin *et al.*,[23] who monitored the urban atmosphere of Hong Kong for PAHs and concluded that vehicular exhausts were the predominant local emission sources of PAHs. Similarly Takada *et al.*[12] found that street dust collected from the Tokyo Metropolitan area contained predominantly three- and four-ring PAHs, namely phenanthrene, fluoranthene and pyrene. In order to characterize the sources of PAH emissions, concentration ratios of PAHs that are characteristic of the sources are often used. A ratio of benzo(a)pyrene to benzo(ghi)perylene of about 0.6 is an indicator of the presence of traffic emissions,[23] as is a ratio of 1.5 for coronene to benzo(a)pyrene.[24] In this study, the ratio of benzo(a)pyrene to benzo(ghi)perylene was found to be 0.49–0.63 and that of coronene to benzo(a)pyrene was 1.52. This indicates that the PAHs are most likely derived from vehicular emissions in the highly trafficked area of the toll plaza. A recent report[25] has shown that exposure levels of PAHs for toll booth workers can be correlated to vehicle flow rates and hence their emissions.

Table 1 PAHs identified in toll booth soot by using HPLC with both UV and fluorescence detection.

PAH
acenaphthene
fluorene
phenanthrene
anthracene
fluoranthene
methyl phenanthrene
pyrene
benzanthracene
chrysene
benzo(b)fluoranthene
dibenz(ac)anthracene
benzo(k)fluoranthene
benzo(a)pyrene
benzo(ghi)perylene
coronene
triphenylene
2-methyl anthracene

3.2. The Ames Test

Initially, concentrations of 0.1, 1.0, 10 and 100 μg of extract per plate were tested for both the TA98 and TA100 bacterial strains, and the results obtained are summarized in Fig. 4. As can be seen, more data were required between 10 and 100 μg plate^{-1} and therefore concentrations of 20, 40, 60, 80 and 100 μg plate^{-1} were tested. As the bacterial strain TA100 was not very responsive, TA100 was not tested further. The results for the further doses tested with TA98 are shown in Fig. 5.

The toll booth extract exhibited mutagenic properties, both in the presence and absence of S9-mix, as the higher concentrations showed a significant increase in the number of histidine revertants above the solvent controls.

Most mutagens are toxic to the bacteria at some concentration, and this is indicated by a decrease in the number of revertant colonies with increasing concentration of the mutagen as a result of cell death. For the toll booth extract this toxicity is apparent at approximately 80 μg plate^{-1} in the presence of the S9-mix and at 60 μg plate^{-1} in the absence of S9-mix (see Fig. 5).

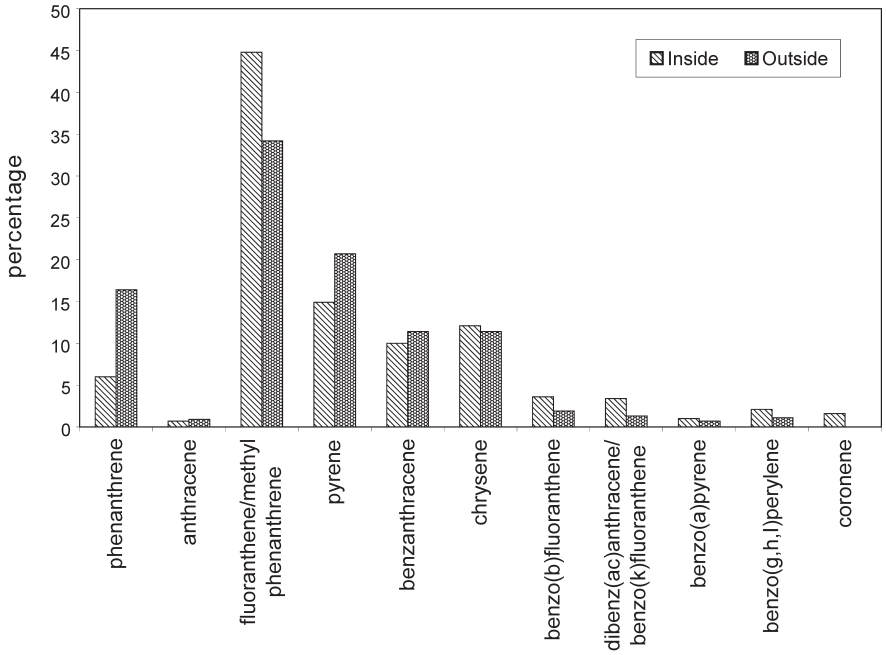

Figure 3 PAH distribution inside and outside the toll booths as a percentage of the total PAHs identified by HPLC with fluorescence detection.

As can be seen from Fig. 5, the number of revertant colonies is greater in the absence of the S9-mix than in its presence. This result is consistent with other studies of vehicle exhaust mutagenicity where the mutagenic activity is thought to be due to direct-acting mutagens such as nitro-PAHs.[26–28] These compounds are activated by nitro-reductase in *Salmonella* to their reactive intermediates, a process which may be masked in the presence of the S9-mix.[29]

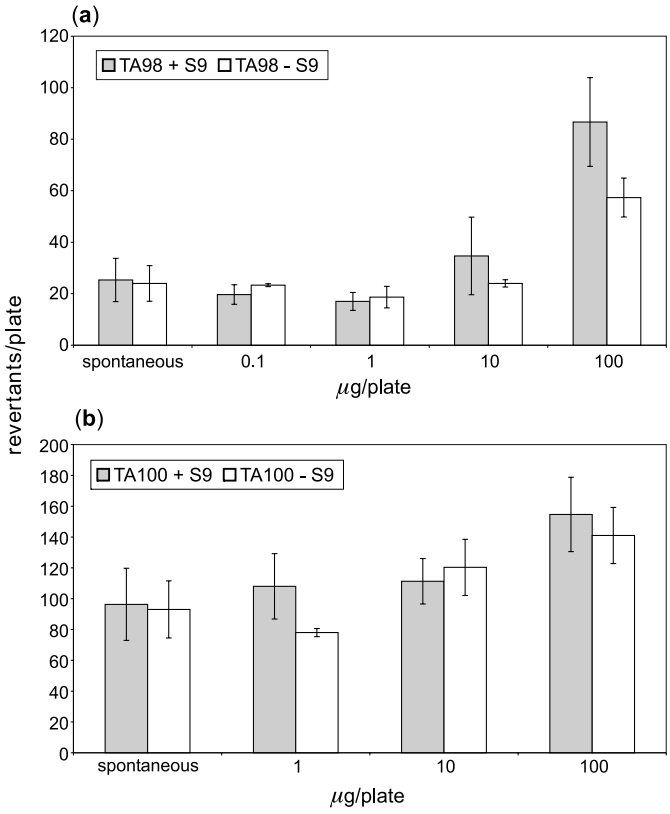

Figure 4 Mutagenicity expressed as the mean number of histidine revertants per plate (together with the standard deviation) for each of the dilutions of the toll booth extract tested with the bacterial strain: (**a**) TA98 and (**b**) TA100, in the Ames test.

3.3. The SOS Chromotest

The absorbance values at 615 nm for the toll booth extract were plotted against the concentration of the extract in μg well^{-1} as shown in Fig. 6. The results for the standard 4NQO were also plotted to give the graph shown in Fig. 7. No results were obtained in the presence of the S9-mix as the standard did not give the expected colour scale, indicating either that it had undergone a chemical change or that the S9-mix was not functioning properly.

The genotoxicity of the sample was determined by calculating the SOSIP value from the slopes of the graphs in Figs 6 and 7. The SOSIP for 4NQO was calculated to be 1.1×10^2 units of β-galactosidase activity per μg per well, whereas that for the toll booth extract was found to be 1.7×10^{-2}, which indicates genotoxicity because it is positive. On comparing these values it is evident that 4NQO is approximately 6.5×10^3 times more genotoxic. Although this difference seems large, it does not imply that the toll booth extract is not genotoxic as only some of the compounds present in the extract exhibit genotoxic properties. It must also be borne in mind that genotoxins in a mixture do not necessarily exert an additive effect,[30] and that non-carcinogenic compounds can enhance the carcinogenic potential of carcinogens.[31]

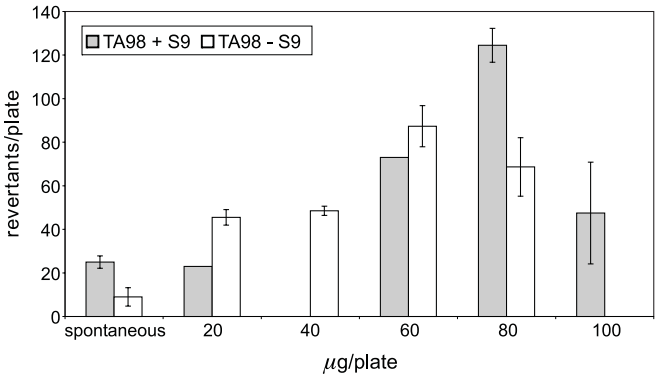

Figure 5 Mutagenicity expressed as the mean number of histidine revertants per plate (together with the standard deviation) for each of the further dilutions of the toll booth extract tested with the bacterial strain TA98 in the Ames test.

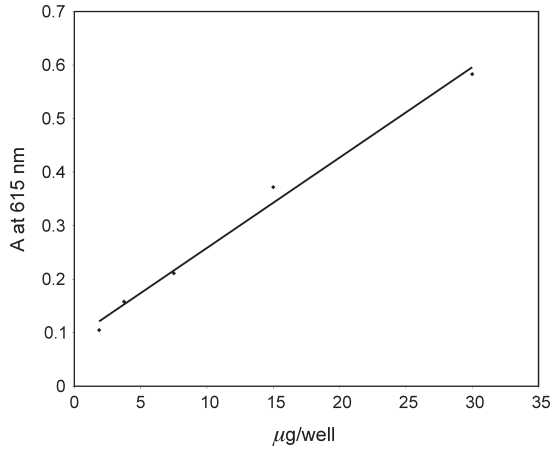

Figure 6 The dose-response curve used to obtain the SOS-inducing potency for the toll booth extract in the SOS Chromotest.

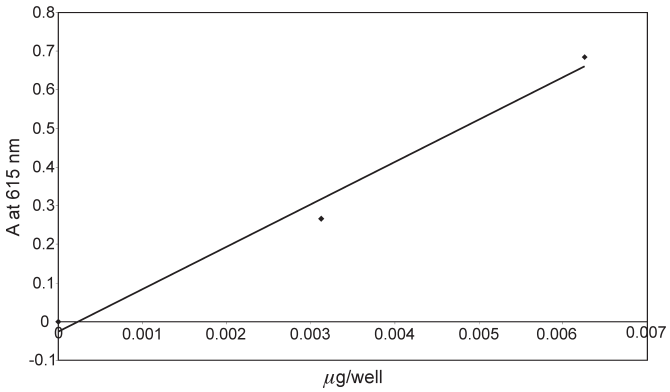

Figure 7 The dose-response curve used to obtain the SOS-inducing potency for the standard, 4-nitroquinoline oxide, in the SOS Chromotest.

4. Conclusions

This study was conducted to determine the PAH content and genotoxicity of the soot deposited in or near toll booths on a busy highway. A number of PAHs were identified in the dichloromethane extracts of the soot by HPLC with both UV and fluorescence detection. Some of these PAHs are well-known carcinogens. The profile of the PAHs indicates that they are derived from vehicle exhaust emissions. The observed mutagenicity in the Ames test and the genotoxicity in the SOS Chromotest was, at first, attributed only to those PAHs identified by comparison to the standard mixtures, the majority of which require metabolic activation in order to express their mutagenic activity. However, the observation that the addition of the S9-mix resulted in a decrease in mutagenicity suggested the presence of other compounds that did not require this activating system (i.e. direct-acting mutagens), such as nitro-substituted PAHs. The presence of such compounds indicates the need for their inclusion in the standard mixtures that are used for comparison. Therefore, Table 1 cannot be taken to be a complete indication of the harmful substances present in the toll booth extract.

This work indicates the need to encourage the development of safer working conditions for toll booth operators, so that they are not excessively exposed to this soot in their working environment. The importance of this matter is underlined by the findings of Tsai *et al.*[25] who from personal monitoring samples, found that toll booth attendants are exposed to total PAH levels higher than asphalt road paving workers, which places toll booth operators at a relatively high risk.

Acknowledgements

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