THE POLYCYCLIC AROMATIC HYDROCARBON CONTENT AND MUTAGENICITY OF THE RESIDUE FROM CANE BURNING AND VEHICLE EMISSIONS

by

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ABSTRACT

Polycyclic (or polynuclear) aromatic hydrocarbons (PAHs) are environmental pollutants produced during the incomplete combustion of organic matter. Since many of these compounds have been shown to be mutagenic and/or carcinogenic, an investigation was initiated into determining the PAH content and mutagenicity of the ash that remains after sugar cane crop burning, and the soot deposited on toll booths by vehicle exhaust emissions.

Due to the large amount of sugar cane farming in the Natal coastal region and that the favoured method of disposing unwanted leafy trash is crop burning, concern was expressed as to the nature of the residue that is formed. PAHs have been identified in the residues from combusted wood and straw and, due to their intrinsic similarity to sugar cane, it was considered that the burning of sugar cane could generate PAHs.

It is well documented that vehicle exhaust emissions exhibit mutagenic properties and PAHs have been identified as the major contributors of this observed mutagenicity. Since a toll plaza is an area of high traffic density, it was considered to be an ideal location for an investigation into the build-up of particles emitted by the passing vehicles, and to study to what extent the operators are

exposed to harmful compounds. In addition, this sample acted as a control, since the detection of PAHs and mutagenic activity in the soot would be an indication that the correct experimental techniques were being employed.

Samples were collected on site. The sugar cane ash was collected off a field immediately after burning had taken place, and the soot was collected either by scraping the toll booth walls and surrounding areas or by wiping the surfaces with cotton wool swabs. The organic portion of the samples was separated from the inorganic and carbonaceous substances by extraction into a suitable solvent; the use of both acetone and dichloromethane was investigated. The extracts were divided into two portions - one was used for the analysis of PAHs and the other for determining mutagenic activity. Analysis for PAHs involved subjecting the extracts to a sample clean-up routine and the use of a number of analytical techniques to characterise the components. The mutagenic properties of the samples were investigated by means of two bacterial mutagenicity tests: the *Salmonella typhimurium* assay (the Ames test) and a new commercially available test kit, the SOS Chromotest.

A number of PAHs were identified in the extracts by means of reverse phase high performance liquid chromatography (HPLC) with both ultraviolet and fluorescence detection, the latter being the more sensitive method. Mutagenic activity was detected for both samples in the Ames test and

for the toll booth soot in the SOS Chromotest, and this observed mutagenicity was attributed to the presence of the PAHs.

PREFACE

This thesis presents work carried out by the author and has not been submitted in part, or in whole, to any other university. Where use has been made of the work of others it has been duly acknowledged in the text.

The work described in this thesis was carried out in the Department of Chemistry and Applied Chemistry, University of Natal, King George V Avenue, Durban, 4001, from February 1991 to November 1992 under the supervision of Professor L.F. Salter.

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LIST OF ABBREVIATIONS

PAH	polycyclic aromatic hydrocarbon
DNA	Deoxyribonucleic Acid
B(a)P	benzo(a)pyrene
UV	ulraviolet
TLC	thin layer chromatography
GC-MS	gas chromatography-mass spectrometry
HPLC	high performance liquid chromatography
E. coli	*Escherichia coli*
DCM	dichloromethane
DMSO	dimethyl sulphoxide

CHAPTER 1

INTRODUCTION

The work reported in this thesis endeavours to demonstrate that the burning of sugar cane crops in the Natal coastal region, and the vehicle exhaust emissions to which toll booth operators are exposed, are sources of mutagenic particulate matter, and in particular polycyclic aromatic hydrocarbons (PAHs). The following introduction deals with a discussion of the discovery and nature of these compounds, the manner in which they exert their biological effects and the reasons for which they are of such environmental concern.

Cancer and mutations represent two of the most important hazards related to environmental pollution. In highly industrialised countries, 75 to 80% of all cancer incidences and almost all mutations are caused by environmental factors[1].

The first correlation between cancer and occupation was made in 1775[2] when a high incidence of scrotal cancer was noticed in chimney sweeps. This was later attributed to their pre-adolescent exposure to soot. In the 19th century[3], a high occurrence of skin cancer was reported amongst workers in the paraffin refining, shale oil and

coal tar industries. Early experiments attempting to produce tumours in animals by application of these materials onto the skin proved unsuccessful, and it was only in 1915 that tumours were successfully induced on rabbit ears by the repeated application (2-3 times a week for several months) of coal tar. In 1921, attempts were made by E. Kennaway[3] to characterise the carcinogen in coal tar. Based on the knowledge that the carcinogenic substances were present in the high boiling fractions, that they were essentially free of arsenic, sulphur and nitrogen, and that carcinogenic coal tars contained more aromatic compounds than non-carcinogenic ones, Kennaway concluded that the carcinogenic agent was some sort of polycyclic aromatic hydrocarbon (or PAH). Many PAHs were synthesised and their fluorescence spectra compared to that of the carcinogenic coal tar fraction, and in addition, were tested for carcinogenicity by application to mouse skin. In 1930, the first incidence of carcinogenic activity by a pure chemical compound was demonstrated when tumours were induced through the repeated application of dibenz(a,h)anthracene (shown in Figure 1.1(a)). However, the fluorescence spectrum of this compound did not match that of the tar fraction. Three years later, the carcinogenic material in the tar was isolated, purified and identified as benzo(a)pyrene, or B(a)P (shown in Figure 1.1(b)). Soon after this discovery[4], B(a)P was identified in domestic soot and ambient air particles, and by 1970, B(a)P and other related PAHs were recognised as being

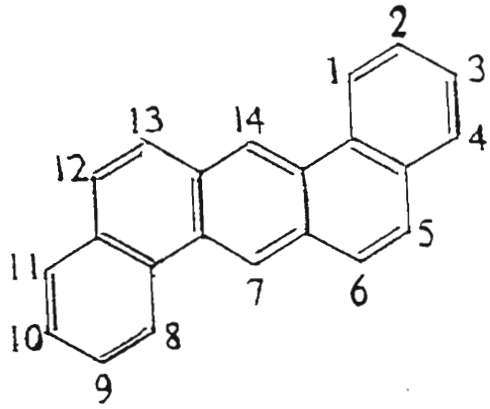


Figure 1.1 (a) Structure of dibenz(a,h)anthracene, the first pure chemical to show mutagenic properties.

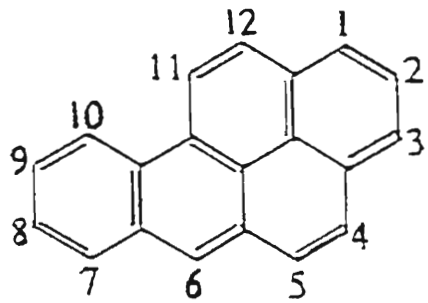
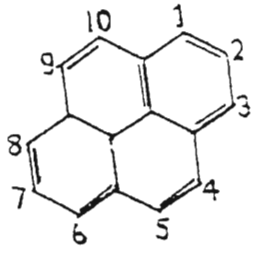


Figure 1.1 (b) Structure of benzo(a)pyrene, the first PAH to be isolated from a complex sample.

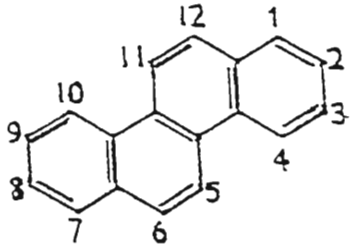
present in respirable ambient urban particles throughout the world. Since B(a)P is highly mutagenic, it is often used as a reference for PAH behaviour and atmospheric measurements of all PAHs[5].

Polycyclic (or polynuclear) aromatic hydrocarbons are formed during the incomplete combustion of organic matter[4,6] and are therefore present in the atmosphere through the burning of fossil fuels and vegetation, in cigarette smoke, and in smoked or braaied foods. Examples of commonly studied PAHs (in addition to those shown in Figure 1.1) are given in Figure 1.2.

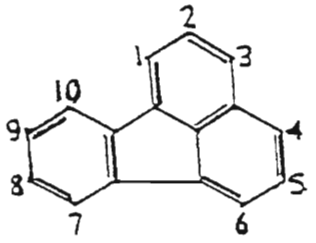
The mechanism for the formation of PAHs is thought to involve the production of free radicals by pyrolysis of organic matter at approximately 500 to 800°C in the chemically reducing zone of a flame burning with an insufficient supply of oxygen[4]. One proposed mechanism for PAH formation[7] is outlined in Figure 1.3. At high temperatures, the addition of vinyl to ethylene produces vinylethylene (C_4H_4), which, upon H-abstraction, produces the n-C_4H_3 radical. Addition of ethylene to this radical results in the formation of the first aromatic ring. At low temperatures, addition of ethylene to vinyl results in the formation of n-C_4H_5, which, upon addition of ethylene produces benzene. Benzene and phenyl are converted to one another by H-abstraction, or H-addition (see sheme 1). These rings then grow by a two-step process (see scheme 2)



(a) pyrene

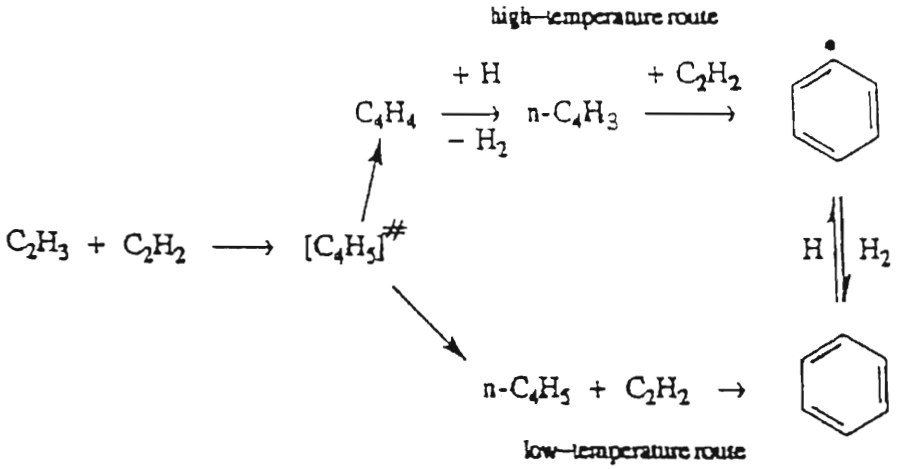


(b) chrysene

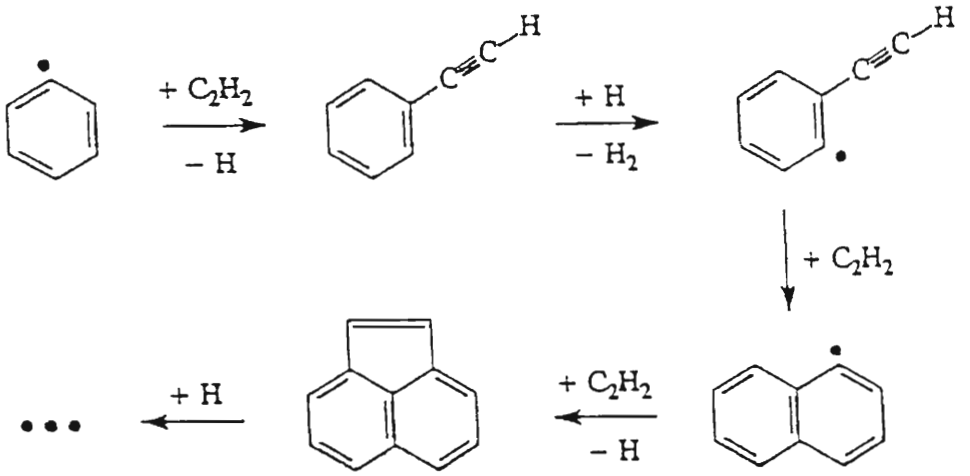


(c) fluoranthene

Figure 1.2 Structures of other commonly studied PAHs.



Scheme 1



Scheme 2

Figure 1.3 A proposed mechanism for the formation of PAHs from a non-aromatic source[7].

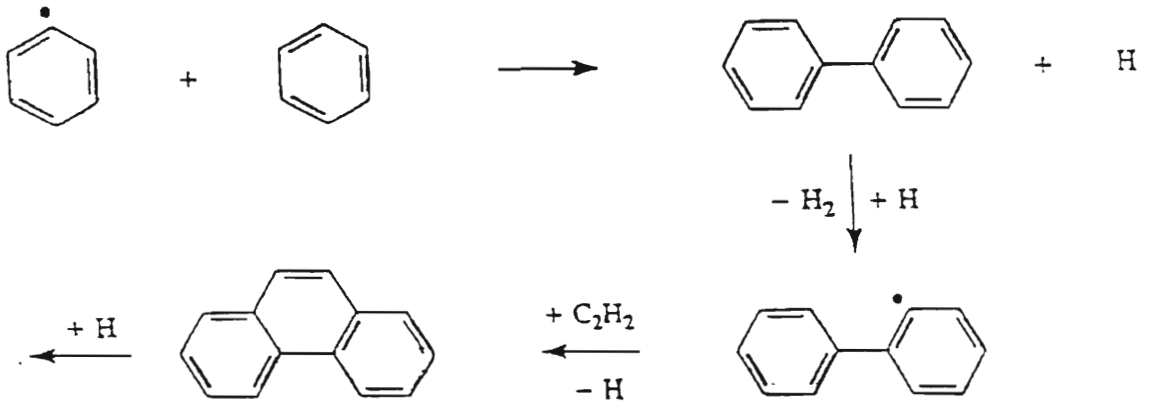


Figure 1.4 A proposed mechanism for PAH formation from a source containing aromatic substances[7].

involving H-abstraction to activate the aromatic molecule, followed by ethylene addition which leads to molecular growth and cyclization. If aromatic compounds are present in the substance being burnt, the initial production of PAHs is thought to occur through condensation of the intact aromatic rings (see Figure 1.4). However, as the reaction progresses, the initial benzene rings decompose to give primarily ethylene and the reaction mechanism reverts to that outlined in Figure 1.3.

PAHs remain in the gas phase at temperatures greater than 150°C, but condense onto particles at temperatures less than this. Therefore, at typical ambient temperatures they exist primarily in the particulate phase[8] and are normally associated with respirable particles (RSP), i.e. particles of diameter less than 2.5 μm[5]. These particles can penetrate deep into the gas exchange of the lung and have a deposition efficiency of 20-30%. The portion of RSP 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. This therefore provides an in-body source of PAHs in the system which can last for weeks.

Present evidence suggests that all chemical carcinogens are (or are converted by metabolism into) electrophilic reactants that exert their biological effects by covalent interaction with the DNA[6], usually through the modification of the purine or pyrimidine bases. On entering the body,

PAHs are metabolised to a number of metabolites[1,2,6] by the cytochrome P-450 enzyme system. The major metabolic compounds produced are phenols, dihydrodiols and glutathione conjugates and these are suspected to form via a reactive epoxide intermediate as outlined in Figure 1.5. The formation of the glutathione conjugate causes no further difficulties in the system as they are water soluble and can be excreted[2]. However, further activation of this epoxide intermediate to a diol epoxide can occur and this is considered to be the precursor to the product (the ultimate carcinogen) that can interact with macromolecules, and in particular the nucleic acid bases on the DNA. Figure 1.6 shows the structures of the four epoxides of B(a)P that can form, but it was discovered that only one of these isomers was further activated to the diol epoxide, and subsequently the ultimate carcinogen.

On further investigation it became evident that the carcinogenic PAHs were of similar size and structure, and two areas were identified as being important in elucidating the mechanism by which these PAHs exerted their mutagenic effects[2]. These areas were labelled the 'k-region' and the 'bay-region' and corresponded to the very active double bond region between atoms 9 and 10 in phenanthrene and the hindered region between the 4^{th} and 5^{th} positions of phenanthrene. Of course the position of these areas differs for each molecule and are indicated for phenanthrene and

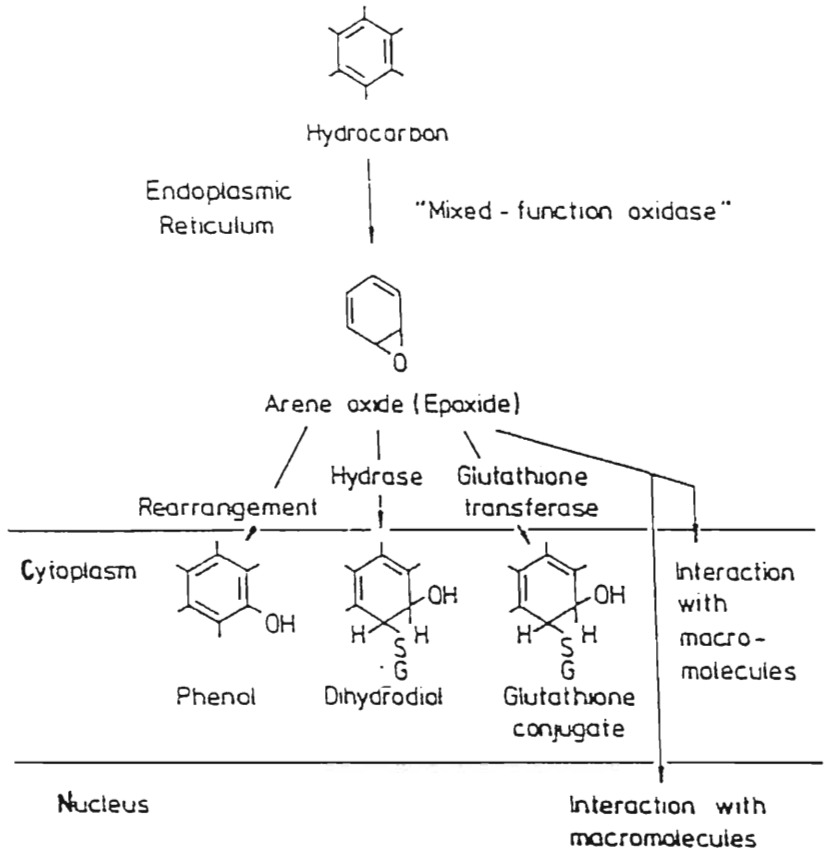


Figure 1.5 The metabolic pathway of PAHs showing the major products that are formed[1].

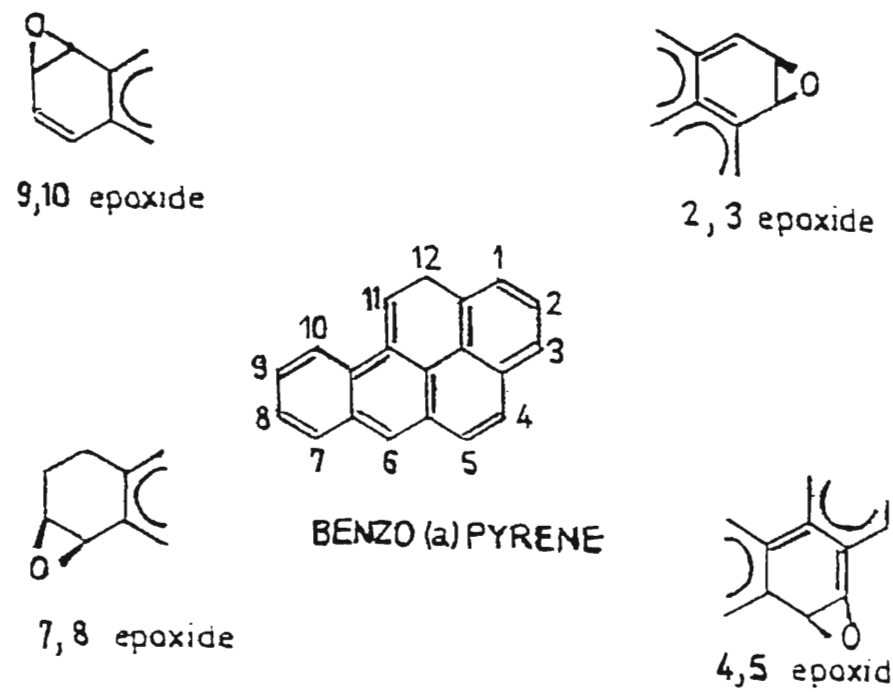


Figure 1.6 Structures of the four epoxides of benzo(a)pyrene[1] (B(a)P).

benzo(a)pyrene in Figure 1.7. It was originally thought that the k-region epoxides were responsible for the observed mutagenicity, but tests for carcinogenicity showed these compounds to be weaker carcinogens than the parent hydrocarbon[3]. Further research into the metabolic pathway of B(a)P (as given in Figure 1.8) revealed that it was the *trans*-7,8-epoxide isomer which was activated to the *trans*-7,8-dihydrodiol. This in turn was converted by epoxidation of the 9,10 double bond to the ultimate carcinogen, *anti*-7,8-diol-9,10-epoxide. The reactivity of this epoxide is due to the formation of the carbonium ion at the 10th position, adjacent to the bay-region of the molecule. Theoretical studies of a large number of PAHs possessing this bay-region have shown that carbonium ions formed on the benzylic carbon atom adjacent to this area are more reactive with DNA than those found in any other area[6].

Studies of the interaction of these diol epoxides with DNA have shown that the reaction takes place primarily with the exocyclic 2-amino group of guanine[3] resulting in the formation of the adduct shown in Figure 1.9. This bulky B(a)P substituent on the base will interfere with replication simply due to its size, but the exact nature of this adduct and the manner in which it causes a mutagenic event is still open to speculation[3].

The emission of PAHs into the atmosphere is also of concern due to the reactions which they undergo with other

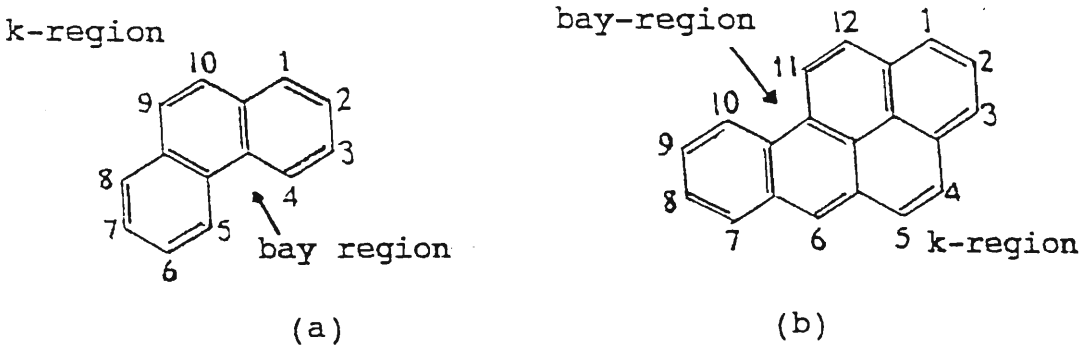


Figure 1.7 Positions of the 'k-region' and 'bay-region' in (a) phenanthrene and (b) B(a)P.

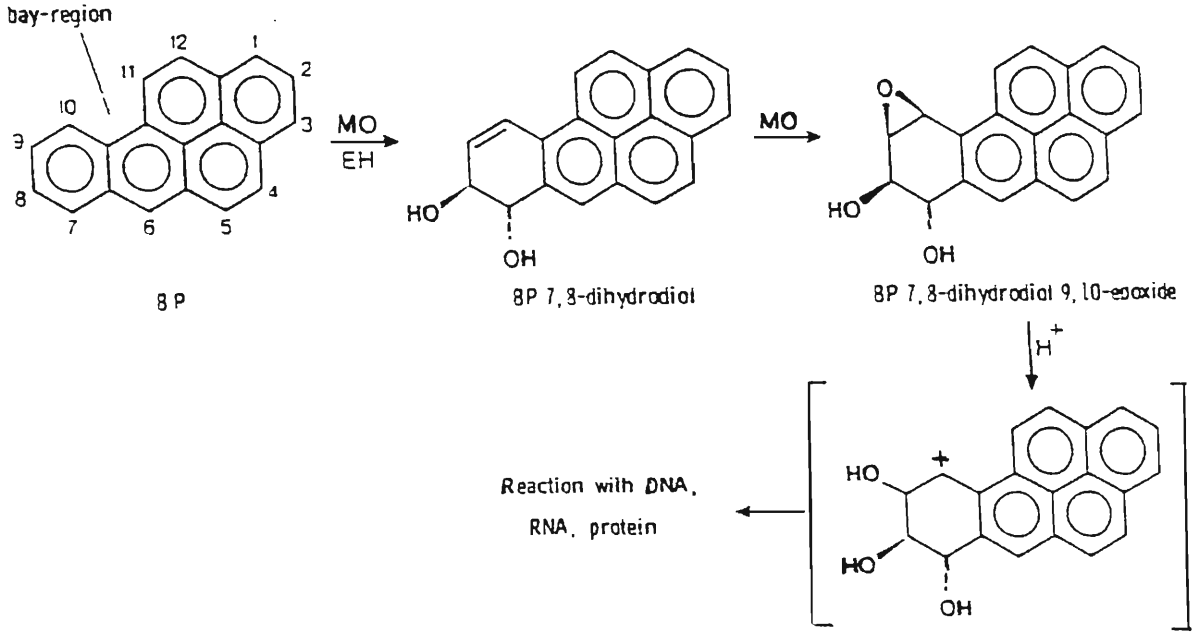


Figure 1.8 The major metabolic pathway of B(a)P[6], showing the formation of the carbonium ion adjacent to the bay-region.

BP = B(a)P, MO = mono-oxygenase, EH = epoxide hydrolase

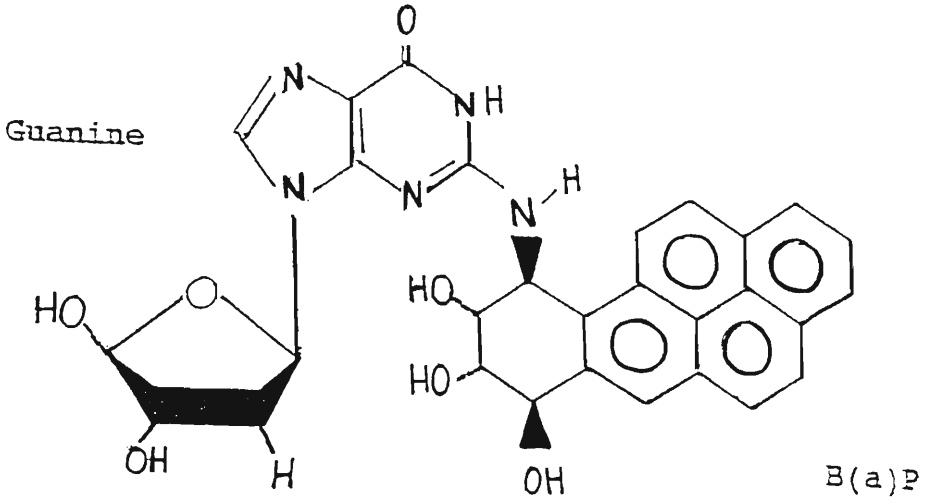


Figure 1.9 Structure of the adduct formed between B(a)P and guanine[3].

atmospheric components such as oxygen, ozone and nitrogen oxides since these reactions may lead to the production of compounds more mutagenic than the parent PAH[8]. For example, the reaction of B(a)P with oxygen (Figure 1.10) leads to the formation of the 1,6-, 3,6- and 6,12-B(a)P quinones, all of which exhibit mutagenic properties; and reaction with ozone leads to the production of a highly mutagenic compound, the 4,5-B(a)P oxide. Also of great concern are the reactions of PAHs in the atmosphere to produce nitro-PAH derivatives, as these compounds have been identified as the major cause of the observed mutagenicity in the *Salmonella typhimurium* mutagenicity assay[9-11] (or Ames test). For example[4], exposure of B(a)P to nitrogen dioxide (NO_2) in the presence of trace amounts of gaseous nitric acid leads to the formation of 6-NO_2-B(a)P and small amounts of the 1- and 3- isomers. The 6- derivative is a stronger precursor to the ultimate carcinogen than B(a)P itself, and the other isomers are powerful direct acting mutagens. Similar reactions take place with perylene and pyrene to give the direct acting mutagens 3-nitro-perylene and 1-nitro-pyrene (shown in Figure 1.11) In addition, reactions between PAHs and nitrogen pentoxide, N_2O_5, are possible and also lead to the production of mutagenic nitrogen compounds. Although these derivatives exhibit mutagenic properties in the Ames test, the effect of these compounds in a living system has not as yet been fully investigated.

The most common approach for the characterisation of

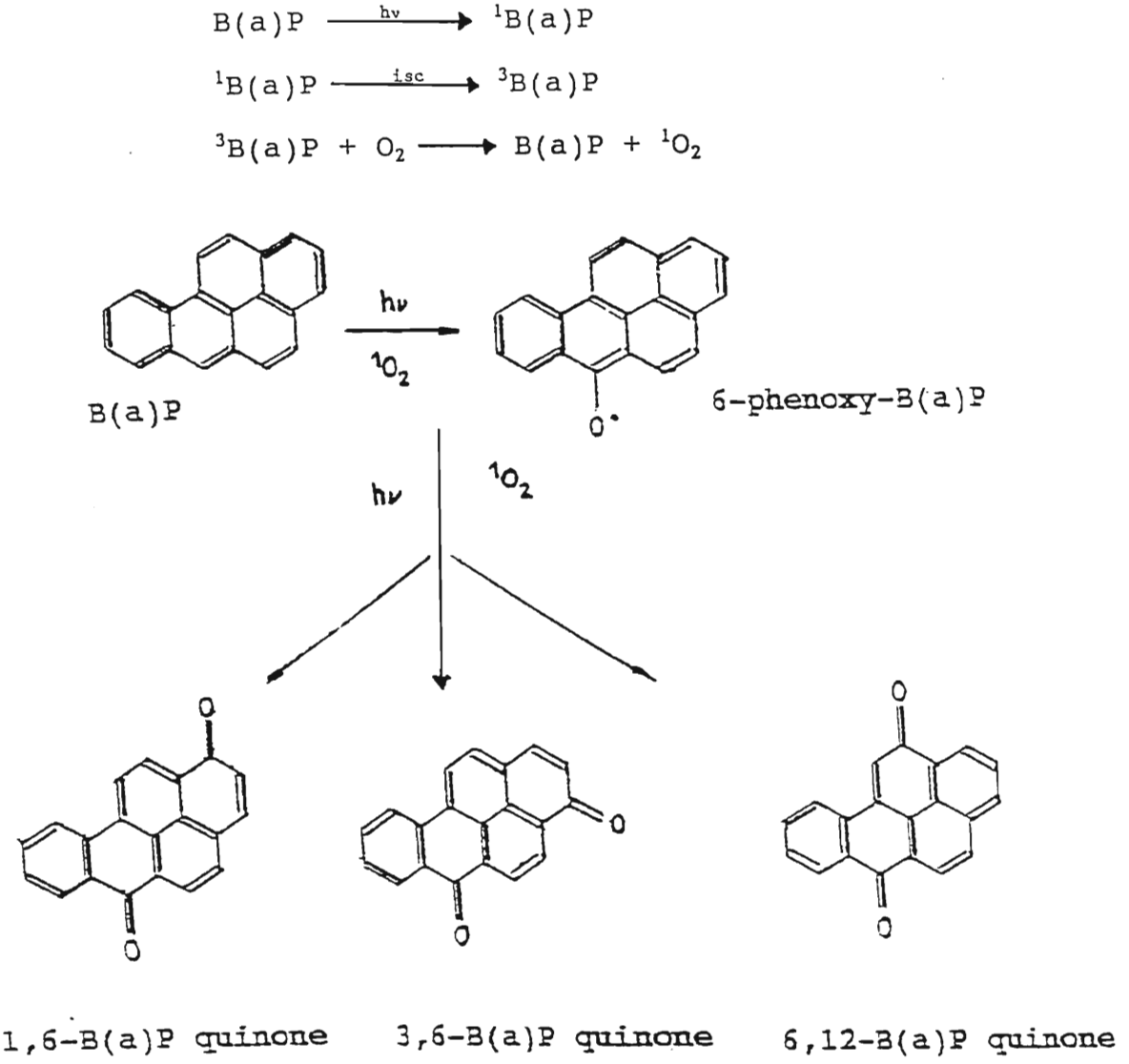
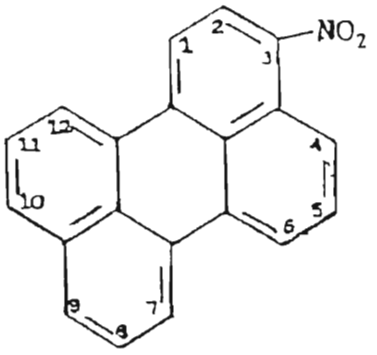


Figure 1.10 A proposed mechanism for the reaction of B(a)P with oxygen[8]. B(a)P acts as a photosensitiser to induce the formation of singlet oxygen, which in turn reacts with a ground state B(a)P molecule.

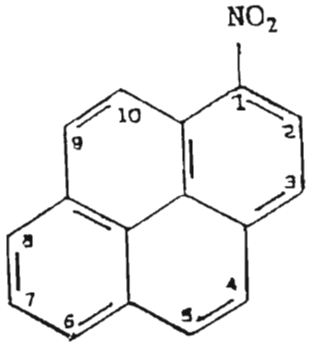
isc = intersystem crossing

^{1}B(a)P = singlet excited state

^{3}B(a)P = triplet excited state



(a) 3-nitro-perylene



(b) 1-nitro-pyrene

Figure 1.11 Structures of two mutagenic nitro-PAHs.

mutagens in a complex mixture[12] consists of chemical fractionation of the sample into groups with defined properties, followed by mutagenicity testing of the individual fractions. Various fractionation methods exist: the method of choice is dependent on the nature of the sample and the aim of the investigation. The fractions can then be analysed by a number of analytical techniques such as high performance liquid chromatography (HPLC), gas chromatography (GC) - either on its own or coupled with a mass spectrometer (GC-MS), and thin layer chromatography (TLC). Determination of the mutagenic properties of the sample involves subjecting the fractions to some short-term mutagenicity test. A number of short- term bioassays exist for the detection of carcinogens and mutagens[13], but by far the most commonly employed one is the *Salmonella typhimurium* microsomal mutagenicity assay (or Ames test). This is a bacterial revertant assay that uses specially constructed strains of *Salmonella* and is a rapid *in vitro* test for mutagens. It is routinely used for the screening of new chemical compounds and has provided valuable information with regard to the mutagenic properties of environmental samples[12] such as ambient air particles, vehicle exhaust emissions and the emissions from industrial processes.

As mentioned at the beginning of this chapter, the work reported in this thesis involves an investigation into determining the PAH content and mutagenic properties of the

ash remaining after sugar cane crop burning and the soot deposited on toll booths by vehicle exhaust emissions.

Due to the large amount of sugar cane farming in the Natal area and the favoured method of disposal of unwanted leafy trash being crop burning, concern was expressed as to the nature of the residue that was formed. PAHs have been identified in the residues from combusted wood and straw, and due to their intrinsic similarity to sugar cane, it was thought that the burning of crops could generate these mutagenic particulates.

It is well documented that vehicle exhaust emissions exhibit mutagenic properties and that PAHs have been identified as the major contributors to this mutagenicity. Since a toll plaza is an area of high traffic density, it was considered to be an ideal site for an investigation into the build-up of particles emitted by passing vehicles, and to determine to what extent the operators are exposed to harmful compounds.

A more detailed discussion of the reasons for undertaking this study, together with the techniques employed in preparing the samples for analysis is presented in Chapter 2. Chapter 3 covers the analytical techniques used to identify the PAHs present in the samples, and Chapter 4 presents a detailed discussion of the two bacterial tests used for the detection of mutagenic activity, namely the

Ames test and the SOS Chromotest. A discussion of the results obtained, and the conclusions, are given in Chapter 5. A list of all chemicals (together with grades and suppliers) and equipment used appears in Appendix A, and the procedures for the preparation of the stock solutions required in the Ames test are given in Appendix B.

CHAPTER 2

SAMPLE COLLECTION AND PREPARATION

The following chapter will deal with a discussion of the reasons for studying the residue remaining after sugar cane crop burning and the particulate matter deposited on toll booths by vehicle exhaust emissions. Information regarding the nature of the samples, the manner in which they were collected, and the techniques employed to prepare the samples for analysis is also presented.

2.1. SUGAR CANE SAMPLE

In order to easily harvest sugar cane crops, and to prepare fields for further plantations after cutting, it is necessary to dispose of the unwanted cane leaves, i.e. the trash. In sugar cane growing areas there are three existing methods[14] of handling this leafy trash: pre-harvest burning, post-harvest burning and trashing (i.e. no burning). Table 2.1 gives a list of the sugar cane growing countries and their trash disposal techniques. It is evident that pre-harvest burning is the most common practice.

Table 2.1 Summary of trash disposal methods[14] used in sugar cane growing countries.

Country	Pre-harvest	Post-harvest	Trashing
Argentina	x	x	x (25%)
Australia	x		x (30%)
Brazil	x		
Barbados			x(100%)
Columbia	x		
Dominican Republic		x*	x (85%)
Egypt			x(>90%)
Fiji		x	
India		x	
Indonesia		x	
Kalimantan	x		
South Africa	x		x (25%)
Taiwan	x		x (25%)
United States:			
Florida	x		
Hawaii	x		
Louisiana	x		
Texas	x		

* Only when ploughing a field for planting.

The advantages of burning as opposed to trashing are improvements in pest control, decreases in harvesting costs and cane losses during harvesting (due to less labour requirements and improved truck loads), higher processing efficiencies and higher sugar extraction. Trashing is advantageous in terms of weed control, better soil moisture

retention and less soil erosion, no fire hazards and no air pollution. Many of the areas using burning are investigating ways in which cane burning can be reduced and a greater amount of trashing implemented but it is estimated that this would involve an increase in harvesting costs of tens of millions of dollars and perhaps lead to segments of the sugar cane industry having to close down[14,15].

In South Africa the major sugar cane growing area is in the Natal province and it was estimated in 1990 that approximately 400 000 hectares of land are under cultivation. The areas can be sub-divided as follows[16]:

North Coast: Mount Edgecombe to the Tugela River - 111 000 ha.

Tugela River to Umfolozi River - 99 000 ha.

South Coast: Illovo - 5 000 ha.

Sezela - 40 000 ha.

Umzimkulu - 22 000 ha.

Midlands: Between Illovo, Mid-Illovo, Eston and Richmond - 25 500 ha.

Richmond, Alverston, Cato Ridge, Umlaas Road and Hillcrest - 15 500 ha.

Kraanskop - Pietermaritzburg - Greytown triangle - 50 000 ha.

Of these crops, approximately 44% (100 000 ha) on the North Coast and 61% (40 000 ha) on the South Coast are burnt annually.

Burning takes place mainly in winter from April to mid-December. This is an important consideration with regard to dissipation of particulate matter because the general meteorological conditions in Natal during winter do not favour dispersal of pollutants but rather re-circulation of contaminated air[16].

Most cane is burnt in the early morning before the dew has evaporated. This is known as a wet burn, and under these conditions a small amount of trash remains, i.e. complete combustion does not occur. Along the North Coast however, some fields are close to the roads and the reduced visibility associated with burning at this time poses a threat to early morning traffic. Burning can therefore also take place later in the day. Arson also accounts for a large amount of the crops being burnt as the people living in the area often set the fields alight during the night. The variety of possible burning times discussed above become an important consideration in terms of any assessment of pollution build-up and dispersion.

Analysis of the smoke produced during cane burning[17] has shown it to be composed mostly of water vapour and carbon dioxide with < 1.0% of carbon monoxide, < 0.1% of

hydrocarbons and < 0.1% of particulate matter being produced. The 0.1% hydrocarbons could contain PAHs. A study carried out in 1987 in Hawaii by the Environmental Protection Agency (EPA) found that the smoke contained approximately 20 organic compounds similar to those present in the smoke from combusted wood and other vegetation[14]. Thus sugar cane burning seems a possible contributor to ambient levels of PAHs in South Africa and may be significant in terms of its possible effects on humans in localized areas near to burning sites.

Apart from possibly forming PAHs, sugar cane crop burning has been identified as having other environmental consequences. In Brazil, large increases in the concentrations of carbon monoxide and tropospheric ozone have been observed during the sugar cane burning season[18,19] and these variations have been shown to be comparable to those in Africa and Hawaii[20]. Carbon monoxide and ozone production are detrimental in the troposphere because both gases make significant contributions to the greenhouse effect[20,21] and are toxic.

In South Africa a particular environmental consequence associated with the burning of crops along the Natal coast is the occurrence of line transmission failures in overhead power lines[16,22]. Sugar cane burning produces sucrose and carbon particles which can bond to the insulator surfaces, and the accumulation of these materials accounts for up to

30% of all transmission line failures. An estimated loss of R 150 000 to R 200 000 in energy sales per year has occurred due to these disruptions.

From this discussion it is evident that although sugar cane crop burning is the most advantageous method of disposing of trash it leads to a variety of pollution related problems. Of particular relevance to this work is the observation of organic compounds in smoke produced during burning. This suggests that PAHs could be formed. This thesis investigates the truth (or otherwise) of this hypothesis and the following sections deal with the analysis of the residue (or ash) that remains after crop burning with the particular aim of determining whether PAHs are present and whether the residue exhibits the mutagenic properties expected from their presence.

2.1.1. Site and Sample Information

Samples were collected from two sugar cane fields situated on the Northern Natal Coast, approximately 20 kilometers inland and away from any heavily used roads. The details of the cane are given in Table 2.2. Photographs of sugar cane fields before and after burning are shown in Figure 2.1.

Table 2.2 Details of the sugar cane samples used for analysis.

	Sample 1[a]	Sample 2[b]	Sample 3[c]
Date of collection	28/03/91	17/05/91	26/11/91
Variety[d]	N12	N12	N16
Age at collection (in months)	22	23.5	12
Rainfall received (mm)	1 895	2 005	837
Fertilizer: (kg/field) nitrogen phosphate potash	133 44 177	133 44 177	241 80 402
Soil type[e]	Inanda series	Inanda series	Inanda series

a: Fresh (unburnt) sugar cane leaves.

b: The ash that remains after crop burning. Collected from the same field as sample 1.

c: Ash collected from the same area as samples 1 and 2, but from a different field.

d: N12 and N16 refer to the manner in which the cane is bred at the experiment station. N12 is a slower maturing and harder cane than N16 and is more suitable for cultivation at higher altitudes.

e: This soil forms part of the Table Mountain sandstone mist belt; is a deep soil and has a low (30%) clay content.

(a)

(b)

Figure 2.1 Sugar cane fields (a) before and (b) after burning.

The fresh sugar cane leaves (sample 1) were taken to the laboratory and burnt in a covered steel drum with holes in the side to allow the smoke to escape. The resulting ash was collected and stored in zip-seal plastic bags in a sealed cardboard box (to prevent exposure to light) in the fridge below 4°C until analysis.

Samples 2 and 3 were collected on site immediately after burning of the sugar cane crops. The crops were burnt at 4 a.m. in the morning and the samples collected at 6 a.m. The ash was collected off the ground, placed in zip-seal plastic bags and stored in the dark in the fridge below 4°C until analysis.

2.2. TOLL BOOTH SAMPLE

The combustion of fuels in light gasoline and heavy duty diesel engines produces a large number of anthropogenic pollutants[23]. The main emissions are water and carbon dioxide, but other components include carbon monoxide (CO), sulphur dioxide (SO_2), nitrogen oxides (NO_x where x=1,2,3), lead, a large number of hydrocarbons (and oxygen and nitrogen derivatives thereof)[24] and soot composed of particles of respirable size[25]. These compounds may cause a spectrum of health effects varying from annoyance reactions, to bronchitis and to cancer in the respiratory

(and possibly other) organs. Deaths resulting from cardiovascular diseases in particularly sensitive individuals, have under certain circumstances been associated with exposure to ambient air pollution[26].

A toll plaza is an area that is susceptible to vehicle pollution build-up since vehicles spend extended periods of time either stationary (idling) or decelerating/ accelerating in low gears. This results in fuel being less efficiently burnt and hence higher concentrations of pollutants are emitted than at cruising speed[27].

The Mariannhill Toll Plaza (see Figure 2.2), situated on the national highway (N3) 22 km west of Durban (a large coastal town on the east coast of South Africa) en route to Pietermaritzburg, serves as a useful case study for an investigation into the effects of vehicle emissions on human health. Following complaints by the toll booth operators of headaches, nausea and general malaise, the Department of Geographical and Environmental Science at the University of Natal in Durban was approached by Tolplan Consortium (the owners of the toll plaza) to investigate the causative agents.

A joint study was undertaken by the Department of Chemistry and Applied Chemistry and the Department of Geographical and Environmental Science to monitor the levels of CO in the Toll Plaza vicinity. It was felt that CO was the

Figure 2.2 The Mariannhill toll plaza approaching from Pinetown.

pollutant most likely to be present in sufficiently high concentration to cause immediate effects. The effects of pollutants such as particulate mutagens were not considered to be responsible for the clinical signs reported since the interactions of such pollutants with human beings are of a different kind and more long term. It was found that the CO concentration vastly exceeded the limit that is regarded as being safe. Suggestions were made for improving the operating conditions. These included the replacement of the window scheme for accepting fees by a slide or slot operation and the installation of extractors that draw fresh air from an area away from the toll plaza so that the operators are exposed as little as possible to the immediate external ambient air[28].

The study now under discussion was initiated to determine the extent to which the build-up of vehicle emitted soot in the vicinity of the toll booths could be detrimental to the operators' health. It is well documented that particulate emissions from vehicles exhibit mutagenic properties as determined via the Ames *Salmonella typhimurium* histidine-reversion mutation assay (Ames test) and that PAHs are most likely responsible for this mutagenicity[26,29,30]. The following sections will deal with the collection of the soot from the Mariannhill Toll Plaza and the subsequent examination of its PAH content and mutagenicity.

2.2.1. Site and Sample Information

All samples were collected from the Mariannhill toll plaza situated on the N3 between Durban and Pietermaritzburg.

The soot deposited by the exhaust emissions of the passing vehicles was collected by scraping the toll booth walls, the railings surrounding the booths, the inside of the ceiling of the booths, the metal plates supporting the signals, and the concrete barriers that separate the lanes from one another as indicated in Figure 2.3. A sharpened metal spatula was used to scrape the particles into glass vials. In addition, cotton wool balls were used to wipe the surfaces because the layer of soot coating these areas was often very fine. The cotton wool was placed in zip-seal plastic bags. All samples, on being returned to the laboratory, were stored in the dark below 4°C until analysis.

Sample collections were made a number of times during this study due to the difficulty in obtaining large amounts of the particles.

Figure 2.3 A toll booth showing the areas from which soot was collected.

2.3. SAMPLE EXTRACTION

Once the samples had been obtained, the organic material was separated from the carbonaceous and inorganic matter by extraction into a suitable solvent. Two extraction methods were investigated: ultrasonic extraction and Soxhlet extraction.

All glassware used in the extraction and fractionation processes was washed with soap and water, rinsed with Milli-Q water and dried by rinsing with acetone and heating.

All samples and extracts were stored in the dark in the fridge to prevent possible photodegradation.

2.3.1. Choice of Solvent

The choice of extraction solvent depends on the nature of the particulates being extracted. Various studies[31,32] have reported acetone to be the most efficient extractor of mutagenic compounds from ambient air particulates whilst other researchers[33,34] have found dichloromethane (DCM) to be the most effective single solvent in extracting biologically active compounds from vehicle particulates. The use of other solvents such as cyclohexane[35], benzene[36], methanol[37] and toluene[38]

have been reported in literature but acetone or DCM are usually the solvents of choice and the use of both of these solvents was investigated in this study. Since organic solvents do contain some PAH impurities and can absorb them from the atmosphere, the DCM and acetone used to extract the samples were subjected to the same extraction and fractionation processes as the sugar cane ash and toll booth soot in order that solvent blanks were available for comparison.

2.3.2. Ultrasonic Extraction

The sugar cane ash or toll booth soot was weighed into a conical flask and either dichloromethane (DCM) or acetone was added. Typically, 300 ml of solvent per 15 g ash or 200 ml per 0,2 g soot was used. The mouth of the flask was covered with aluminium foil to prevent evaporation of the solvent. The sample was ultrasonically extracted for 2 hours, after which time the solvent was filtered through a Millipore 0,45 µm HV organic aqueous compatible filter to remove the particulates. Small portions of this "crude" extract were kept aside for mutagenicity testing in the Ames test and PAH analysis via HPLC, whilst the remainder was rotary evaporated to dryness in preparation for fractionation.

2.3.3. Soxhlet Extraction

The suitability of this method of extraction was investigated by extracting the sugar cane ash. The sugar cane ash was packed tightly into the Soxhlet thimble and covered with cotton wool. The thimble was extracted with DCM prior to packing, and was weighed before and after the addition of the ash so the mass of the sample could be determined. Approximately 7 g of ash was extracted at a time. The ash was extracted into 300 ml DCM for 16 to 18 hours after which time the extract was removed and portions retained for mutagenicity testing and PAH analysis. The remainder was evaporated to dryness with a rotary evaporator for fractionation. Due to the limited amount of sample that could be extracted at a time, this procedure was usually repeated a number of times and the extracts combined. Due to the limited amount of toll booth soot available, the Soxhlet extraction method was not performed on this sample.

Since ultrasonic extraction was far less time consuming and was found to give a greater recovery of soluble organic particulates as compared to Soxhlet extraction it was the method adopted for the extraction of both the sugar cane ash and toll booth soot.

2.4. SAMPLE FRACTIONATION

A number of approaches exist for the fractionation of complex samples prior to analysis[12,39]. These techniques can be used individually or in conjunction with each other and are discussed below:

(a) Sequential solvent extraction: This involves sequential extraction of the sample with solvents of increasing polarity to obtain non-polar, moderately polar and polar fractions. This technique is advantageous due to its simplicity, but does not always result in distinct chemical classes such as aliphatic, aromatic and oxidised hydrocarbons, as a great extent of overlap can occur between the fractions.

(b) Liquid-liquid partitioning: This involves separation into acidic, basic and neutral fractions by partitioning the sample between an immiscible organic solvent and an acidic or alkaline aqueous phase. This yields a good separation of chemical classes but contact with acids or bases can lead to undesirable reactions.

(c) Chromatographic techniques: These methods include thin layer chromatography (TLC), paper chromatography (PC), gel permeation chromatography (GPC), liquid chromatography in open columns (LC), and high performance liquid chromatography (HPLC). The disadvantages of these

methods are poor reproducibility of results and difficulty in separating the polar compounds from the rest of the system.

All of the above methods of sample fractionation were attempted and will be discussed in the following sections. Sequential solvent extraction was performed on the sugar cane ash and toll booth soot themselves, whilst the other fractionation processes discussed were performed on the dried crude extracts obtained from the ultrasonic extraction of the samples (see section 2.3).

2.4.1. Sequential Solvent Extraction

Initially, sequential solvent extraction was employed using cyclohexane, dichloromethane (DCM) and acetone in that order. The sample (i.e. the sugar cane ash or toll booth soot) was weighed into a conical flask and 50 ml of cyclohexane added. Typically, 0.5 g of ash and 0.1 g soot were used. Aluminium foil was placed over the neck of the flask to prevent loss of sample due to spillage and evaporation and the flask was shaken by means of a flask shaker. After 2 hours, the sample was filtered through a Millipore 0.45 μm filter and the filtrate stored in a glass vial in the fridge below 4°C. The residue was then taken up in 50 ml of dichloromethane, shaken for 2 hours, filtered and

the filtrate stored in the fridge. The same procedure was followed with 50 ml of acetone as the solvent. The flask shaker was later replaced by an ultrasonic bath which reduced the amount of sample loss.

2.4.2. Liquid-Liquid Partitioning in Conjunction with Chromatographic Techniques

The second method investigated was acid-base fractionation followed by the use of column chromatography to obtain sub-fractions of the neutral portion. This procedure is commonly used for the fractionation of ambient air and vehicle exhaust particles[36,12,40-44].

The fractionation procedure adopted in this study was a method modified from Teranishi, Hamada and Watanaba[36]. The crude sample extract (from the ultrasonic extraction process) was fractionated into 3 main fractions: acidic, basic and neutral. The neutral portion was then divided further by column chromatography on silica gel 60 into aliphatic, polyaromatic and oxygenated sub-fractions. A schematic outline of the fractionation process is shown in Figure 2.4.

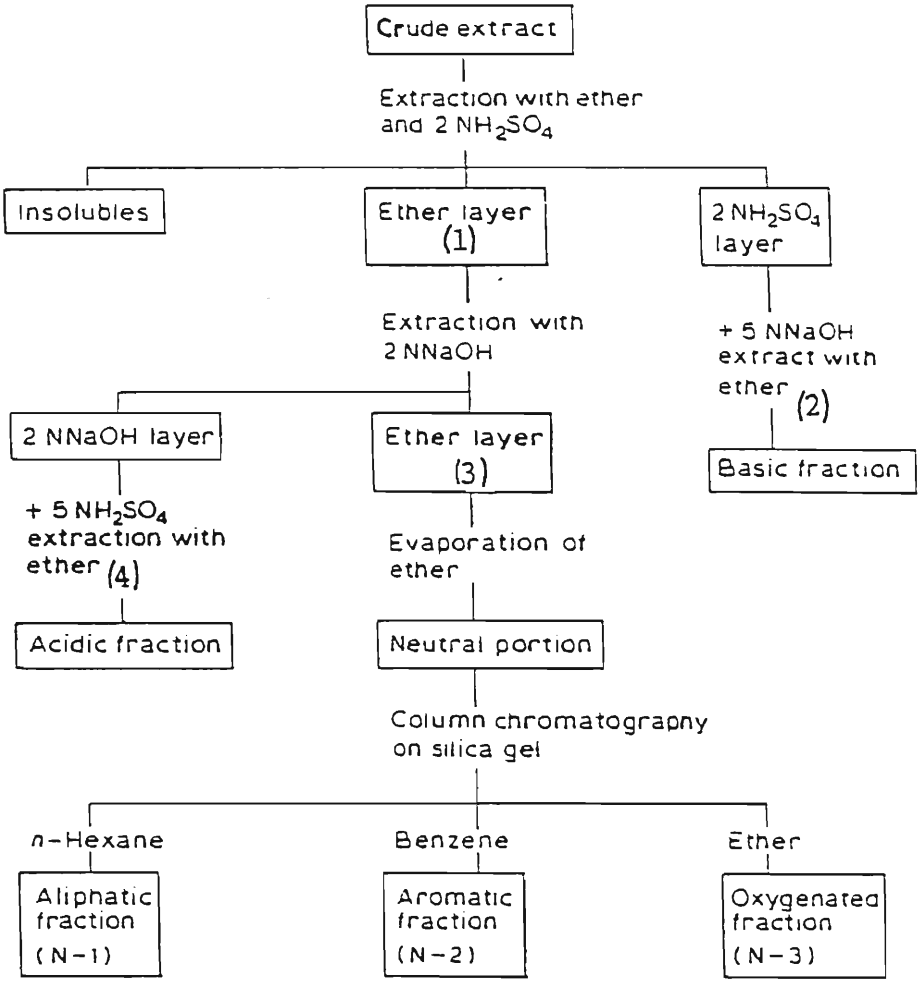


Figure 2.4 Procedure for the acid-base fractionation[36] of the crude extracts of the sugar cane ash and the toll booth soot.

2.4.2.1. Acid-base fractionation

Firstly, the crude sample extract (approximately 200-400 mg) was dissolved in 50 ml of diethyl ether to which 30 ml of 1 M H_2SO_4 was added. The mixture was shaken for 5 minutes. The aqueous layer was removed from the ether layer (1) and made alkaline with 30 ml of 5 M NaOH. This was followed by extraction into 50 ml ether. This ether layer (2) was transferred to a pre-weighed round bottom flask and evaporated to dryness using a rotary evaporator. This residue represented the basic fraction. The ether layer (1) from the H_2SO_4 extraction was extracted with 30 ml of 2 M NaOH and the aqueous layer removed. The remaining ether layer (3) was evaporated to dryness, the residue representing the neutral fraction. The separated aqueous layer was acidified with 30 ml of 2,5 M H_2SO_4 and extracted with 50 ml of ether. This ether layer (4) was evaporated to dryness, the residue representing the acidic fraction.

All extracts and fractions obtained from the sugar cane ash collected from the field (samples 2 and 3) were yellowish in colour, whereas the ash obtained from burning the leaves in the laboratory (sample 1) resulted in clear extracts and fractions.

The solvent extracts were initially dried by adding anhydrous Na_2SO_4 and left to stand. However, this caused a loss of sample during the removal of the sulphate by filtration. Careful removal of the aqueous layers from the separating funnel eliminated the need for this drying process.

2.4.2.2. Column chromatography

The neutral fraction obtained in the previous section was then separated into sub-fractions by use of column chromatography on silica gel.

A 1,0 cm diameter column was packed by plugging the end of the column with glass wool and a thin layer of acid-washed sand. Merck silica gel 60 (particle size 0.040-0.063 mm; 230-400 mesh) was added as a slurry in n-hexane and packed to a height of 10 cm. The top of the column was protected by another layer of acid-washed sand.

The residue was dissolved in a small volume of n-hexane and applied evenly to the top of the column with a Pasteur pipette. Each time the solvent was changed, the flask that contained the residue was rinsed with the new solvent before applying to the column to ensure complete transfer of the sample.

The column was first eluted with 100 ml of n-hexane to obtain the aliphatic fraction, followed by elution with 100 ml benzene to give the polyaromatic fraction, and finally by 150 ml of ether which gave the oxygenated fraction. Although the published method uses benzene, perhaps, since benzene is a known carcinogen, toluene should have been used instead. The fractions were evaporated to dryness with a rotary evaporator and stored in the dark at 4°C until analysis.

The aliphatic fractions from the sugar cane ash (samples 1, 2 and 3) were colourless, whereas the polyaromatic and oxygenated fractions were yellow in colour for samples 2 and 3, and clear for sample 1. It was also noted that the amount of residue produced from the aliphatic fraction was much smaller than those produced by the other fractions.

The dried crude extracts obtained from the ultrasonic extraction of the samples (i.e. the sugar cane ash and toll booth soot), the cyclohexane, DCM and acetone extracts obtained from the sequential extraction process, and the acidic, basic, neutral, aliphatic, aromatic and oxygenated fractions from the fractionation process, were all analysed for PAH content by a number of techniques. This analysis is discussed in the following chapter, Chapter 3. The mutagenic properties of the ash and soot were

investigated by testing the crude extracts obtained from the ultrasonic extraction of the samples in two bacterial mutagenicity assays. This will be discussed in Chapter 4.

CHAPTER 3

ANALYTICAL TECHNIQUES

The crude extracts and the various fractions of the sugar cane ash and toll booth soot, were analysed by a number of analytical techniques to investigate the presence of PAHs. In order that these compounds could be identified, a number of commercially available PAHs were purchased to serve as standards for comparison. The following sections will deal with a discussion on the preparation of the standard solutions and their analysis, and the analysis of the samples under investigation.

3.1. PREPARATION OF STANDARD SOLUTIONS

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. A list of the chemical grades and suppliers is given in Appendix A, section A.1.2. The above PAHs were chosen as standards because they were readily available and had been identified amongst those found in the residue from combusted wood and straw[45-47] and in vehicle emissions[29,30]. They were therefore thought to be useful for comparison

with the constituents of the samples being analysed here.

Solutions of 10^{-3} M concentration were prepared of each PAH by dissolving them in cyclohexane, acetone, dichloromethane (DCM) and a mixture of 50% (v/v) DCM-methanol since these were the solvents used for the extraction, fractionation and subsequent analysis of the samples. A standard PAH mixture was prepared by combining 1 ml aliquots of each solution. Since only qualitative analysis of the samples was being attempted it was not necessary to know the exact concentrations of each PAH in the mixture.

Due to the harmful nature of the compounds, all preparations of the standard solutions (weighing, diluting, etc.) were carried out in a fume cupboard and protective clothing such as a lab coat, gloves and a chemical mask was worn. All glassware was thoroughly cleaned by washing with detergent and rinsing with Milli-Q water, and dried by rinsing with acetone and heating. Fresh standards were prepared approximately every 4 months and stored in the dark between 0°C and 4°C at all times to prevent photodegradation.

The PAH standard mixture used in the analysis at Lancaster University in the United Kingdom contained 19 individual PAH compounds: namely, naphthalene, acenaphthene, fluorene, 1-methylphenanthrene, 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(ghi)perylene. These were used to prepare a stock standard solution, containing approximately 10 mg/ml of each compound, which was subsequently diluted down to a working standard level of 13 µg/ml of each PAH.

3.2. UV SPECTROSCOPY

This technique was employed to determine the wavelength region in which each PAH absorbs. This knowledge would allow tentative confirmation of the presence of PAHs in the samples by a comparison of the absorbance maxima of the standards to those of the sample extracts observed via multiple diode array detection after HPLC. It was also used as a means of checking the purity of the standard solutions and to ensure that no degradation had occurred. If any changes in the spectra were observed new solutions of the standards were prepared.

All spectra were obtained on a Varian DMS 300 double beam UV spectrophotometer using 1 cm pathlength quartz cuvettes.

The solutions of the standards were diluted in the cuvettes with the appropriate solvent and their absorbances measured against solvent blanks by scanning from 200 to 450 nm.

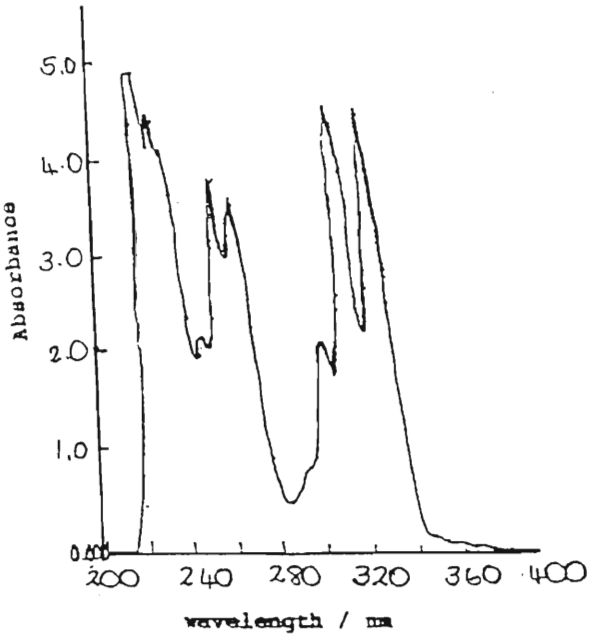
Examples of PAH absorbance spectra are presented in Figure 3.1. All standards showed strong absorbance in the 240 to 300 nm region.

To determine whether the sugar cane ash and toll booth soot extracts and fractions (obtained as described in sections 2.3 and 2.4) absorbed in the same region as the standards, the cyclohexane, DCM and acetone extracts from the sequential solvent extraction process (see section 2.4.1.) and the neutral, acidic and basic fractions from the acid-base fractionation process (see section 2.4.2.1.) were diluted with the appropriate solvents and the absorbances measured against solvent blanks by scanning from 200 to 450 nm.

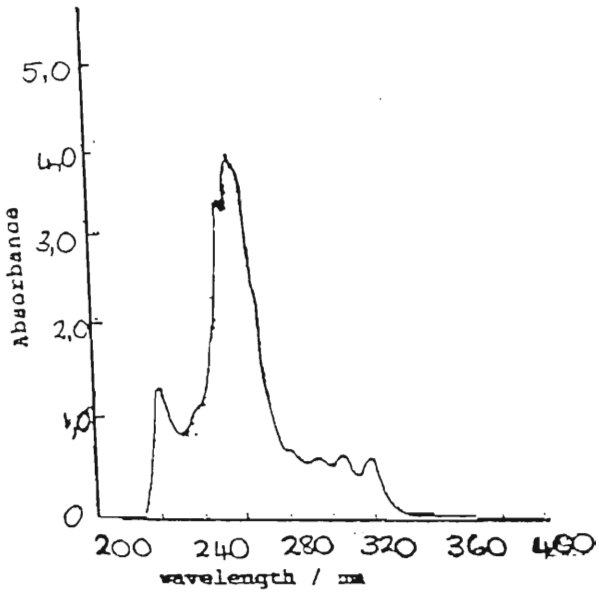
The DCM extract and the neutral, acidic and basic fractions all absorbed strongly in the 240 to 320 nm region thereby suggesting the possible presence of PAHs in the samples. The cyclohexane and acetone extracts showed only weak absorbances in this wavelength range which indicated that DCM was a more efficient extractor of polyaromatic compounds. Examples of sample spectra can be seen in Figure 3.2.

3.3. THIN LAYER CHROMATOGRAPHY (TLC)

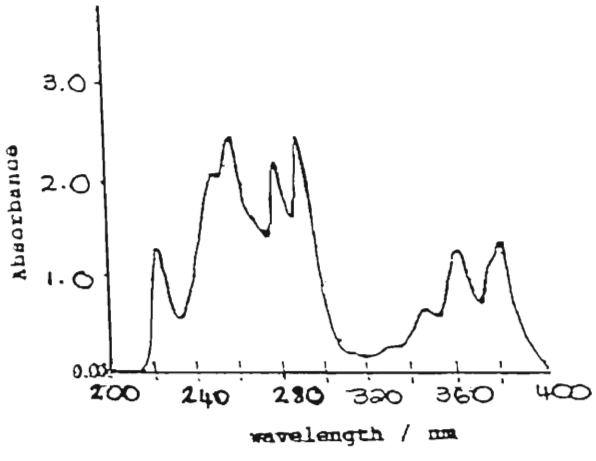
The use of TLC as a technique for detecting the presence of PAHs in the samples was also investigated. Merck silica gel



pyrene

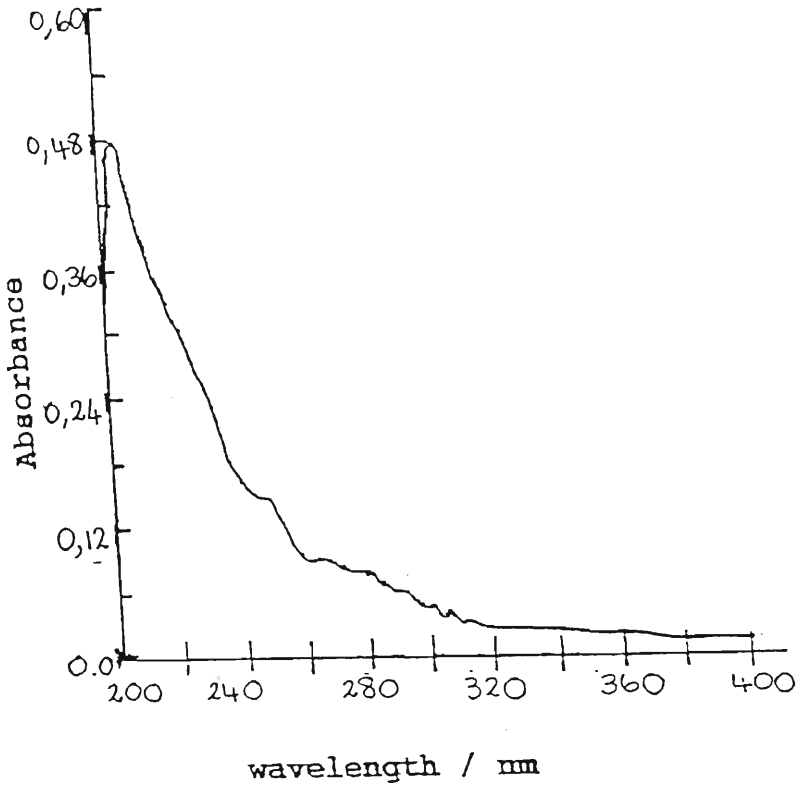


chrysene

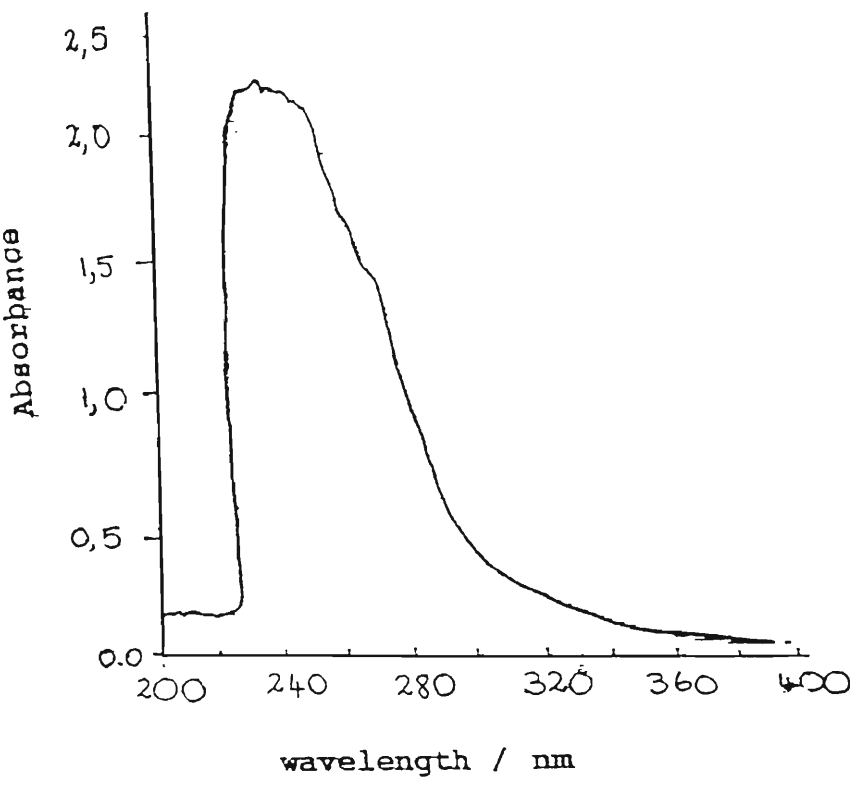


(B(a)P)

Figure 3.1 UV absorbance spectra of some standard PAHs.



(a)



(b)

Figure 3.2 UV absorbance spectra of the (a) cyclohexane, and (b) neutral fractions of the sugar cane ash extract.

60 aluminium backed F_{254} precoated TLC plates were used and 3 different solvent systems were investigated.

The standard solutions, the cyclohexane, DCM and acetone extracts and the neutral, acidic and basic fractions were spotted on the plates 1.5 cm from the bottom edge. The solvent system was poured into a chromatographic tank to a depth of approximately 1 cm, the tank was covered and the system left to equilibrate for an hour. The plate was placed in the tank at a slight angle and the solvent allowed to ascend to 1.5 cm from the top of the plate. The plate was removed from the tank, dried with a hair dryer and observed under an ultraviolet lamp for any development of spots.

The solvent systems investigated were a 50% (v/v) hexane-toluene mixture, a 50% (v/v) methanol-water mixture and a 80% (v/v) acetonitrile-water mixture. The first system was used because previous studies[10,48] of the extract of vehicle exhaust emissions had achieved separation with this system. The majority of the standard compounds eluted but all had the same retention times. No spots were observed for the samples. Similar results were obtained with the more polar systems investigated. These results therefore indicated that the solvent systems used had insufficient resolving powers, and that the extracts and fractions investigated were possibly too dilute for PAHs to be detected. TLC was therefore abandoned as an analytical technique.

3.4. GAS CHROMATOGRAPHY-MASS SPECTROMETRY (GC-MS)

Since GC-MS is considered to be one of the most sensitive techniques for the identification of PAHs in complex samples the standard PAH solutions and dried crude DCM sample extracts (i.e. before fractionation) were sent for analysis by GC-MS.

A Finnigan 1020 GC-MS equipped with a J & W Scientific DB5 30 m x 0.25 mm column was used with helium as the carrier gas. The operating conditions were established by first injecting the standard solutions to obtain a temperature profile. Once this had been achieved the samples were injected under the same conditions. The injector temperature was set at 280°C and the initial column (or oven) temperature at 100°C. This initial temperature was held for 5 minutes after which it was increased at a rate of 15°C per minute to a final temperature of 250°C. The interface region between the GC and the MS was set at 280°C with the MS manifold at 80°C. Injections were made with a 20 second split mode and scanned from 35 atomic mass units (amu) to 650 amu in 1.0 second.

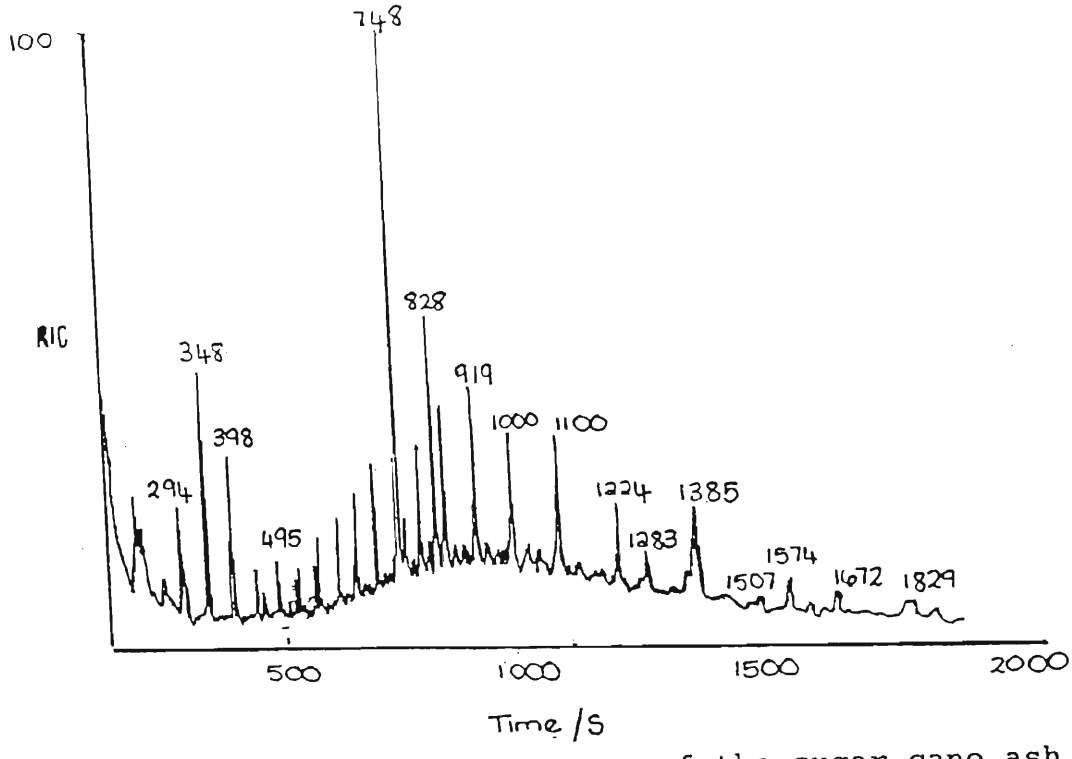
The crude extracts were taken up in a few drops of DCM and 1 µl injections of both the standards and the samples were made. The solutions were injected onto the GC column where separation of the components was achieved due to differences in volatility and interaction with the

stationary phase. Since the column was non-polar the PAHs were expected to elute fairly quickly. As the components, now in the gas phase, entered the mass detector region they were ionised by electron impact and the fragment ions analysed by the quadruple mass filter.

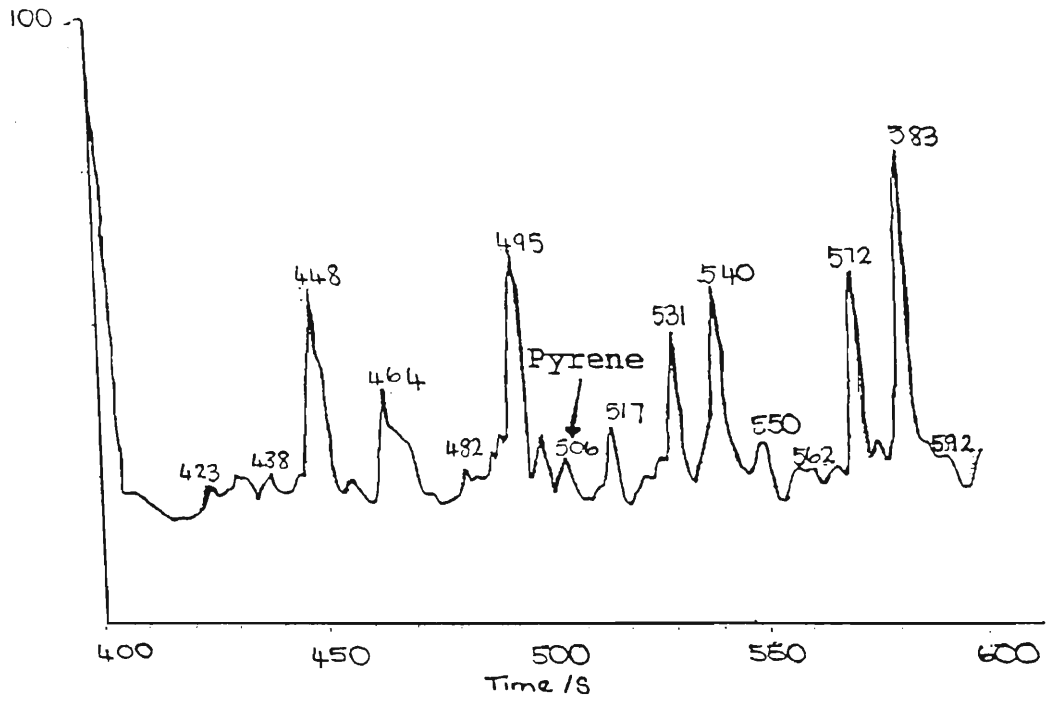
On comparing the retention times of the standards to those of the sample no similarities were noticed indicating the absence of PAHs. The technician therefore scanned the sample chromatogram, shown in Figure 3.3 (a), for the region in which a PAH such as pyrene of molecular mass 202.3 g/mol would most likely occur. The result, depicted in Figure 3.3 (b), was a peak at a retention time of 506 seconds of such low intensity that it was indistinguishable from the noise level. It was therefore concluded that the PAHs in the sample were present in such low concentrations that they were unable to be detected amongst the other components of the sample extracts by GC-MS.

3.5. HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

Sample extracts were analysed in this department by reverse phase HPLC coupled with UV detection. Since most PAHs are strong phosphors and each has its own characteristic pattern in both the excitation and emission spectra, fluorescence spectroscopy is a sensitive and selective detector for PAHs[49]. For this reason sample extracts were



(a) Total ion chromatogram of the sugar cane ash extract.



(b) Total ion chromatogram showing the pyrene peak - it is of the same intensity as the background noise.

Figure 3.3 Total ion chromatograms of the crude sugar cane ash extract showing the peak tentatively identified as pyrene.

also sent to Lancaster University in the United Kingdom where analysis was carried out at the Department of Environmental Science by means of reverse phase HPLC coupled with fluorescence detection.

3.5.1. HPLC Coupled with Fluorescence Detection

Crude, acidic, basic, neutral, aliphatic, aromatic and oxygenated sample extracts of the sugar cane leaves burnt in the laboratory, the sugar cane ash collected off the fields and the soot scraped from the toll booths were prepared as set out in section 2.4.2. The extracts were evaporated to dryness and sent to Lancaster University for analysis.

Analyses were carried out on a Perkin Elmer HPLC pump 250 with a Perkin Elmer LS 40 fluorescence detector. A Phase Separation Spherisorb PAH column, 4.6 mm x 150 mm and of 5 µ particle size was used. An optimum gradient for separation was established as 60%(v/v) acetonitrile-water for 15 minutes, increasing linearly to 100% acetonitrile over 10 minutes and held at 100% for 20 minutes at a flow rate of 1.5 ml/min.

The crude sample extracts were reconstituted in 1 to 5 ml DCM, filtered through a 0.5 µm syringe filter and 2 µl injections made. Quantification of the PAH

content was achieved by analysing a series of standards and calculating the area of the peaks. Prior to sample quantification a standard mixture containing approximately 1.25 ng of each PAH was injected and the calibration confirmed. Samples were then quantified against the standard mixture based on peak areas. This yielded a value in ng PAH/µl which was then converted to ng/sample.

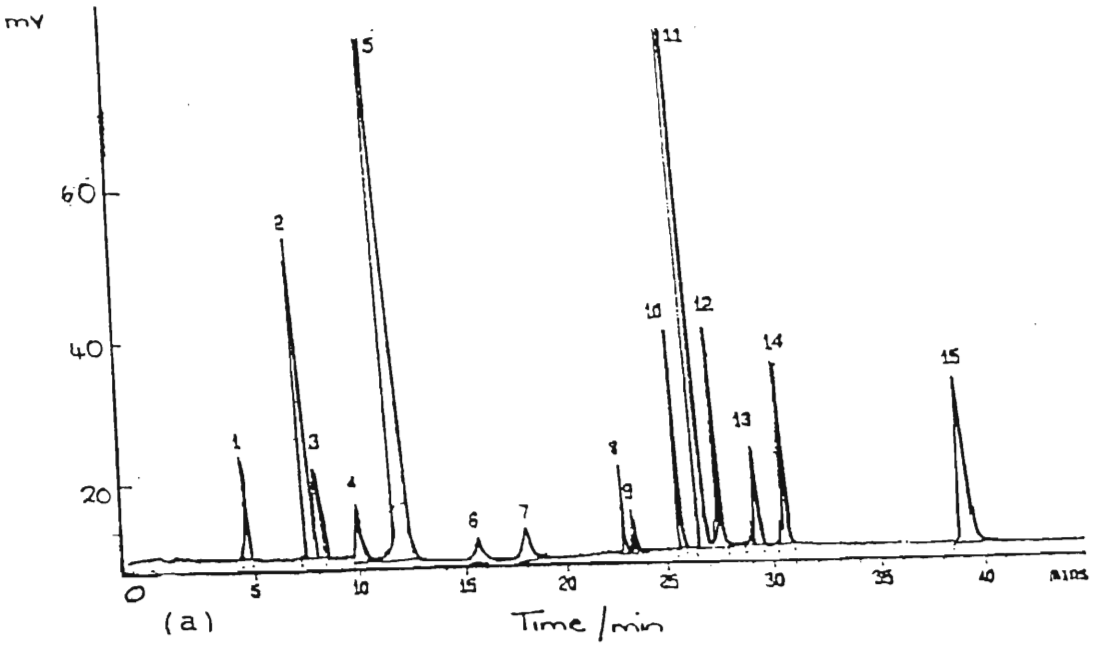
A list of the standard PAHs together with their respective excitation and emission wavelengths is given in Table 3.1. The HPLC chromatogram obtained for the PAH standard mixture, and a typical chromatogram of the crude extract of the sugar cane ash collected off the field (i.e. sample 2 from section 2.1.1), is shown in Figure 3.4. A number of PAHs were identified in the samples and a detailed discussion of the results will be presented in section 5.1.1.

3.5.2. HPLC Coupled with UV Detection

Since the analysis of complex environmental samples was new to this laboratory no established HPLC techniques existed and a large portion of this research was therefore devoted to the development of a HPLC routine that could satisfactorily separate the components in the sample extracts.

Table 3.1 A list of the excitation and emission wavelengths of the standard PAHs used in HPLC analysis with fluorescence detection at Lancaster University.

Standard PAH	Excitation λ / nm	Emission λ / nm
Naphthalene	280	340
Acenaphthene	280	340
Fluorene	280	340
Phenanthrene	240	400
Anthracene	240	400
Fluoranthene	305	430
1-Methylphenanthrene	305	430
Pyrene	305	430
Benzanthracene	305	430
Chrysene	305	430
Benzo(b)fluoranthene	305	430
Dibenz(ac)anthracene	305	430
Benzo(k)fluoranthene	305	430
Benzo(a)pyrene	305	430
Dibenz(ah)anthracene	305	430
Benzo(ghi)perylene	305	430
Coronene	305	500



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(ac)anthracene/Benzo(k)fluoranthene
12. Benzo(a)pyrene
13. Dibenz(ah)anthracene
14. Benzo(ghi)perylene
15. Coronene

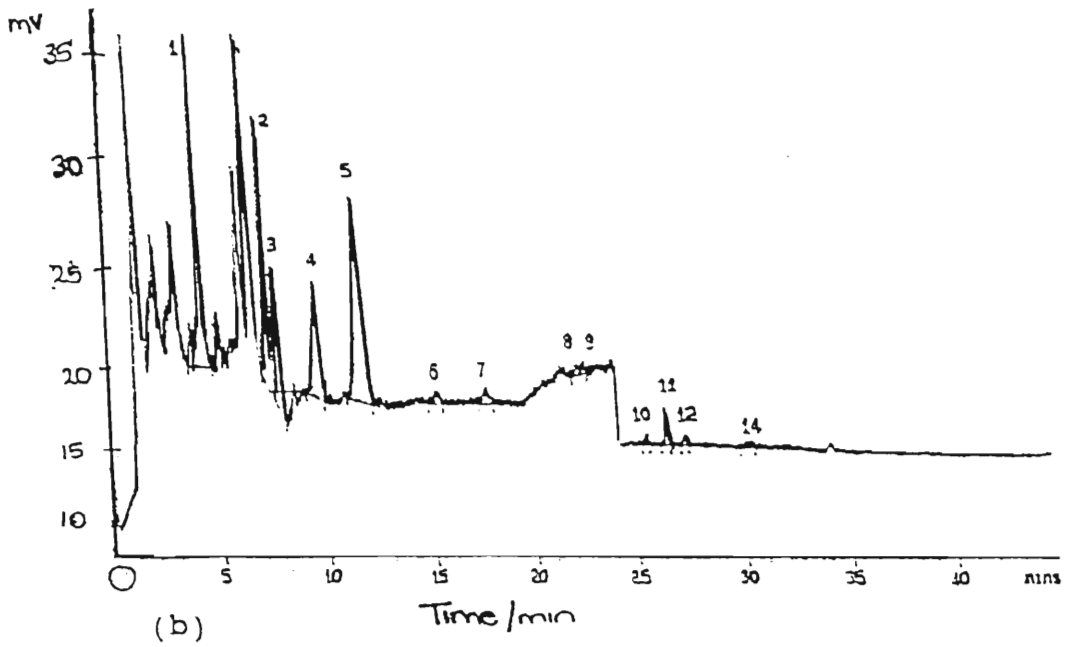


Figure 3.4 HPLC chromatograms obtained at Lancaster University in the United Kingdom for (a) the standard PAH mixture, and (b) the crude sugar cane extract of the ash collected off the field.

Analyses were performed on a Waters 600 multisolvent delivery system with a U6K variable injector. The multisolvent delivery system has programmable features which facilitate the use of complex gradient programs and clean-up procedures, as well as use in the isocratic mode. Detection was performed by a Waters 990 photodiode array which consists of 512 photodiodes with a range from the ultraviolet to the visible region. This permits a simultaneous scan of the UV/VIS absorption of the sample in the flow cell from 190 to 600 nm. In this way large amounts of chromatographic and spectral data may be acquired from a single analysis and stored on computer. The detector is linked to a NEC APCII personal computer and a Waters 990 plotter. All solvents used in the delivery system were filtered through Millipore 0.45 μm HV organic aqueous compatible filters in order to remove any dissolved gases and throughout the analyses the solvents were purged continuously with high purity helium gas to ensure that they remained degassed so as to prevent the formation of air bubbles in the pumps. In addition, a disposable μ-Bondapak C_{18} precolumn insert was placed between the solvent delivery system and the column to ensure no contamination of the column occurred due to particles that might not have been removed during the filtration. Prior to injection down the HPLC all

standards and samples were filtered through a Millex HV solvent resistant syringe filter to remove any particles that could precipitate on the column. Injections were made with a Hamilton microliter airtight syringe and were all monitored at 254 nm.

3.5.2.1. Choice of column

The first column used was a Partisil 10 ODS 2 since this was available in the laboratory. However, no satisfactory results were obtained using this column. Catalogues in which examples of standard environmental analyses were presented were consulted and it was decided that the most suitable column for this type of analysis was a Supelcosil LC-PAH column of dimensions 4.6 mm x 250 mm and 5 µm particle size. This is a C_{18} column designed specifically for the analysis of priority pollutant PAHs[50].

3.5.2.2. Development of HPLC conditions

The first mobile phase investigated was 60%(v/v) methanol-water at a flow of 0.7 ml/min since this was the solvent system used by the manufacturers to test the Supelcosil column. Each standard PAH solution and the standard mixture (all in DCM) were injected. Injection volumes were either 10 or 20 µl. This mobile phase was found to be too polar since

after 80 minutes only 5 of the 12 PAHs in the mixture had eluted. The use of a more non-polar solvent (acetonitrile) was therefore investigated.

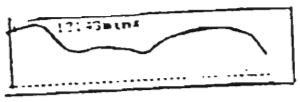
Table 3.2 summarises the various %(v/v) compositions of acetonitrile to water and flow rates that were tried. All resulted in poor separation of the PAH peaks.

Table 3.2 A summary of the mobile phase compositions (in %(v/v)) and flow rates investigated in the development of a HPLC routine for the separation of the standard PAHs.

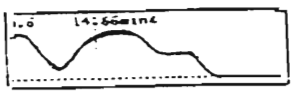
Mobile Phase Composition %(v/v)acetonitrile-water	Flow Rates /(ml/min)
100	0.7; 1.0; 0.5
70	0.7; 1.0; 0.5
35	0.7; 0.5

Besides giving poor separation of the standard PAH peaks it was noticed that the above injections of individual PAH solutions resulted in the elution of more than one peak. This was first thought to be due to impurities in the solids used to prepare the standard solutions but a study of the UV spectra

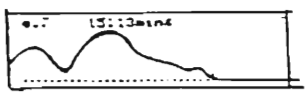
showed them to be pure. It was then realized that making 10 or 20 µl injections of the standards in DCM was causing precipitation of the compounds as they met the mobile phase; injection volumes of only 2 µl should have been made. This therefore accounted for the appearance of the other peaks. On referring to the Supelco catalogue[50] it was noticed that the standard PAH solutions used in their standard analysis were prepared in a mixture of 50%(v/v) DCM-methanol which allowed larger volumes, such as 10 or 20 µl, to be injected without sample precipitation occurring. Standard solutions were therefore prepared in this solvent mixture. Following the same procedure as given in this catalogue[50] the next solvent system investigated was 35%(v/v) acetonitrile-water held for 2 minutes and then increased linearly to 100% acetonitrile over 14 minutes at a flow rate of 2 ml/min. A mixture of 8 of the standard PAHs (prepared in the quantities as described in the catalogue[50]) in 50%(v/v) DCM-methanol was used to test this gradient. A list of these PAHs is given in Table 3.3. Good separation of the peaks was obtained and an example of a typical chromatogram can be seen Figure 3.5. Each standard was then injected individually to enable identification of the retention times and the UV spectra of the peaks.



1 Acenaphthylene

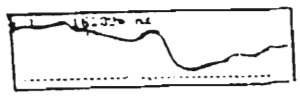


2. Fluorene

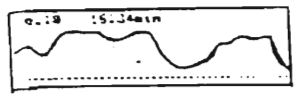


3. Phenanthrene

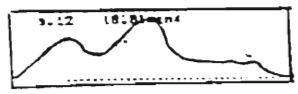
4. Anthracene



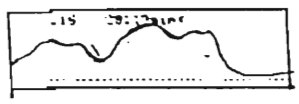
5. Fluoranthene



6. Pyrene



7. Chrysene



8. Benzo(a)pyrene

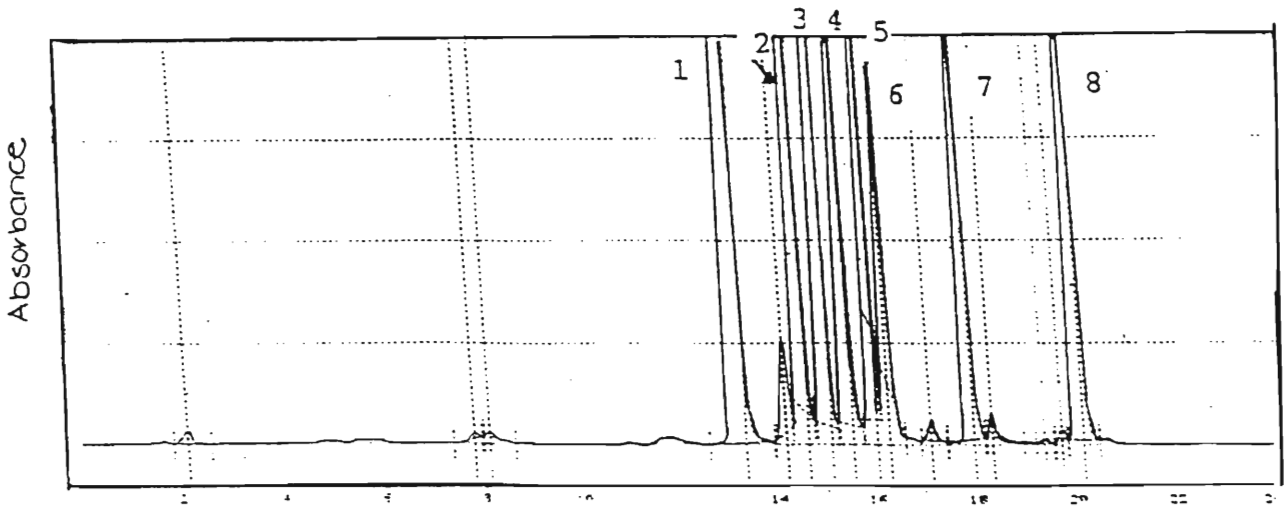


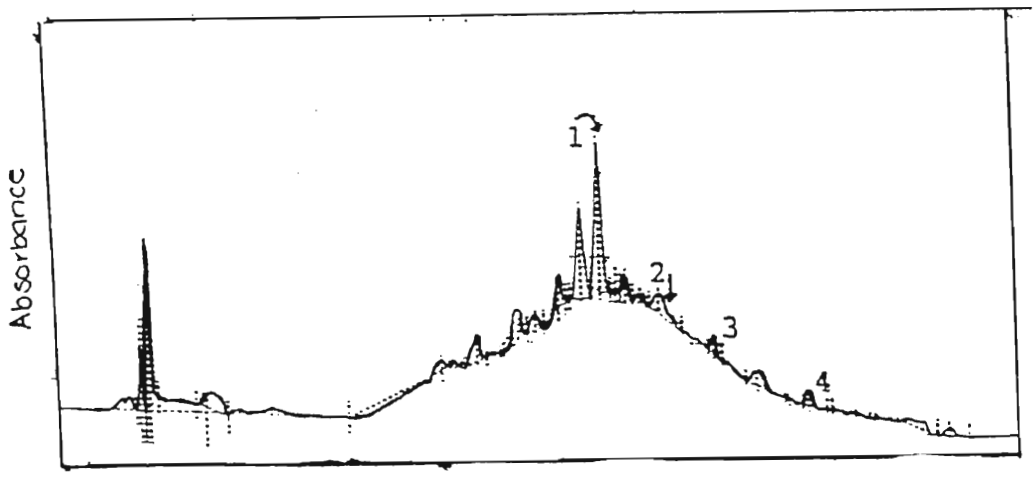
Figure 3.5 A HPLC chromatogram of the separation obtained of 8 standard PAHs on a gradient of 35%(v/v) acetonitrile-water held for 2 minutes, and increased linearly to 100% acetonitrile over 14 minutes, at a flow of 2 ml/min.

Table 3.3 The preparation of a standard mixture of 8 PAHs. The standards were weighed as indicated below and made up to 10 ml with 50%(v/v) DCM-methanol.

Standard PAH	Approximate Mass /g
Acenaphthylene	0.2
Anthracene	0.01
Fluorene	0.02
Phenanthrene	0.01
Fluoranthene	0.02
Pyrene	0.01
Chrysene	0.01
Benzo(a)pyrene	0.01

The neutral, acidic and basic fractions of the toll booth soot extract and the neutral, aliphatic and aromatic fractions of the sugar cane ash extract were reconstituted in 50%(v/v) DCM-methanol and injected onto the column under the conditions discussed above. Separation of the peaks was achieved and some PAHs were able to be identified by comparing the retention times and UV spectra to those of the standard PAH mixture. Examples of these chromatograms can be seen in Figure 3.6. After a number of injections, poor resolution of the peaks was obtained. A blank injection (i.e. an injection of the mobile phase only) showed the column to be

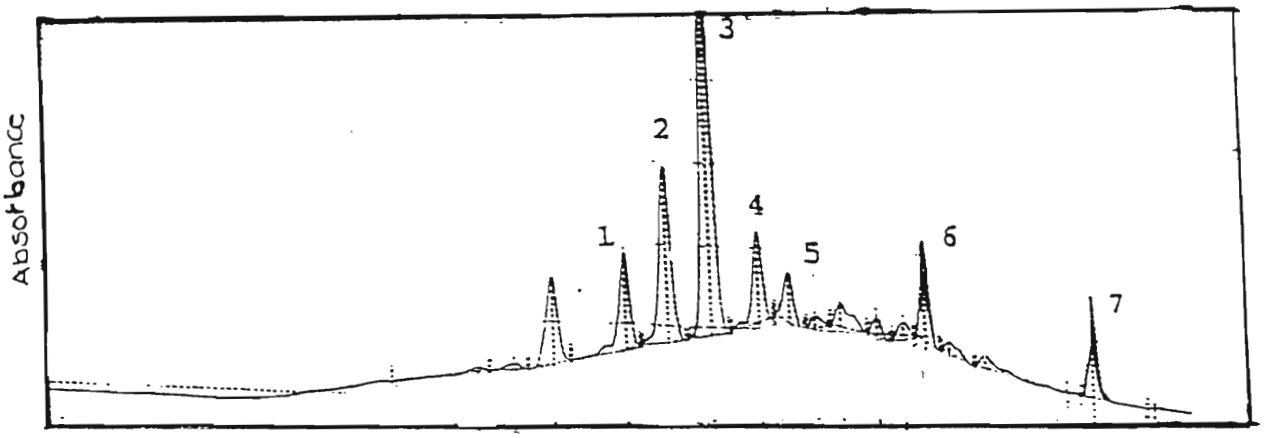
1. Anthracene
2. Pyrene
3. Chrysene
4. B(a)P



(a)

time / min

1. Fluorene
2. Phenanthrene
3. Anthracene
4. Fluoranthene
5. Pyrene
6. Chrysene
7. B(a)P



time / min

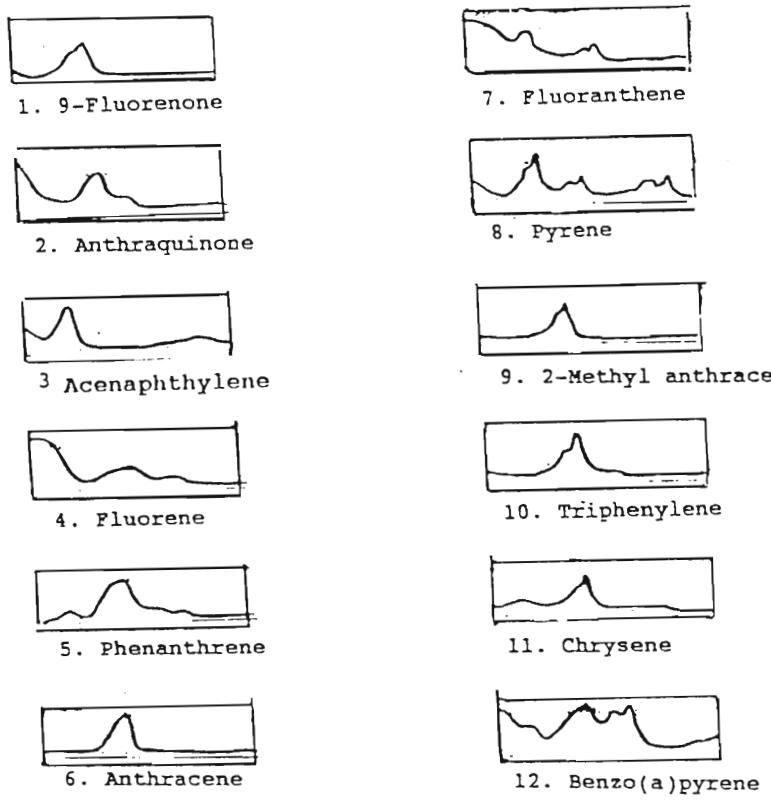
(b)

Figure 3.6 HPLC chromatograms obtained for the neutral fractions of (a) the sugar cane ash, and (b) the toll booth soot extracts on the same gradient as for Figure 3.5.

slightly contaminated as a number of peaks were obtained instead of a straight baseline. The column was therefore throroughly cleaned before further injections were performed (see section 3.5.2.5).

The above gradient was then used to separate a mixture of all 12 standard PAHs. Frequent use of the column at a flow rate of 2 ml/min had resulted in very high back pressures, and the flow rate was therefore reduced to 1.5 ml/min. An injection of the 12 standards resulted in the elution of only 11 peaks. In an attempt to improve the separation the gradient was changed to the following: 35%(v/v) acetonitrile-water held for 2 minutes and increased linearly to 100% acetonitrile over 20 minutes at a flow of 1.5 ml/min. All 12 PAHs were separated and identified by their UV spectra although two of the peaks were unable to be baseline resolved (see Figure 3.7). This gradient was therefore thought to be the best for the sample analysis.

The Department of Environmental Science at Lancaster University also gave some suggestions as to what solvent system to use for the analysis and the following gradient was investigated: 60%(v/v) acetonitrile-water for 15 minutes which was increased linearly to 100% acetonitrile over 10 minutes and held there for 15 minutes at a flow of



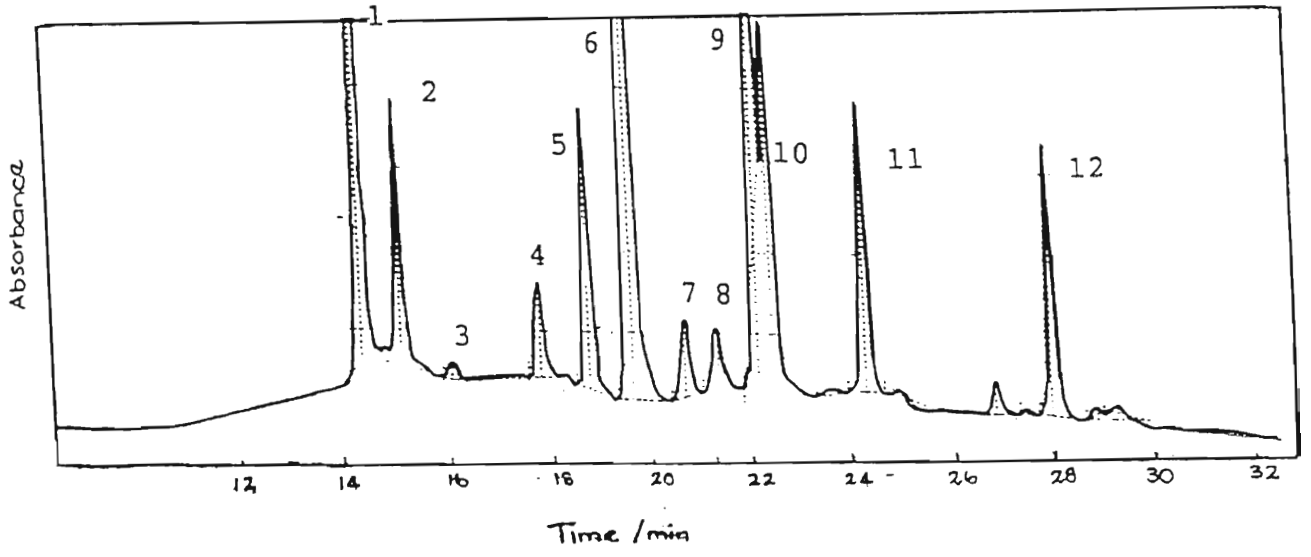


Figure 3.7 HPLC chromatogram of the separation obtained for 12 standard PAHs on a gradient of 35%(v/v) acetonitrile-water held for 2 minutes, and increased linearly to 100% acetonitrile over 20 minutes, at a flow of 1.5 ml/min.

1.5 ml/min. This resulted in the elution of only 11 peaks and even increasing the change over time to 25 minutes did not result in separation of all the peaks.

The Department of Environmental Science at Lancaster University analyses environmental samples frequently and the procedure followed by them involves the extraction of samples into DCM followed by a reduction of the extract volume under nitrogen. The extract is then cleaned-up by loading onto a Florisil column and eluting with DCM. It was therefore decided to concentrate on the crude DCM extracts using this same clean-up procedure. Toll booth soot and sugar cane ash were therefore extracted into DCM and cleaned-up on a Florisil column (see section 3.5.2.3). The eluants were evaporated to dryness under nitrogen and re-dissolved in 50%(v/v) DCM-methanol. Injections of these extracts were made under the conditions described previously as giving the best separation of PAHs. This did not result in as good a separation as achieved previously due to the presence of a number of peaks eluting very close to the area of interest which interfered with the identification of the PAHs (see Figure 3.8). These peaks were considered to be representative of more polar compounds than the PAHs since they eluted before the

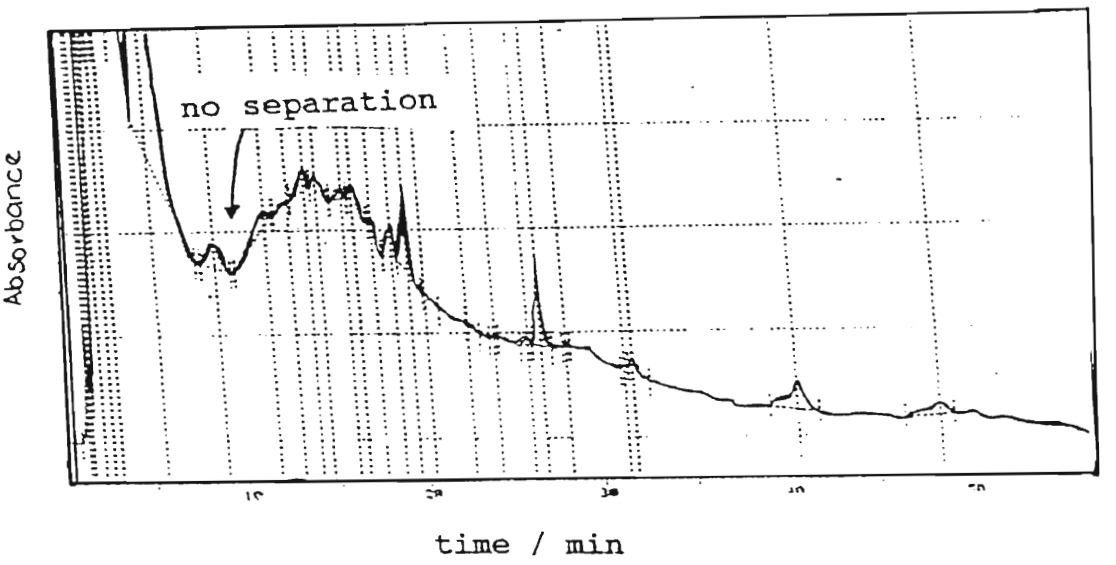


Figure 3.8 A typical HPLC chromatogram obtained of the crude sugar cane extract (obtained from ultrasonic extraction into DCM) after clean-up on Florisil, using the same gradient as for Figure 3.7.

PAH peaks. In an attempt to separate these peaks from those of interest water was included in the gradient. The follwing conditions were tested: 100% water held for 10 minutes, changed over 10 minutes to 35%(v/v) acetonitrile-water, held at this for 10 minutes and then changed over 20 minutes to 100% acetonitrile, at a flow of 1.5 ml/min. This resulted in separation of these peaks from the PAH peaks which aided in the identification of the various sample constituents (see Figure 3.9).

3.5.2.3. Sample clean-up

As mentioned in the previous section the crude sample extracts were subjected to a clean-up procedure on Florisil columns prior to analysis. The use of Florisil has been reported elsewhere[51] to give good and rapid recovery of neutral aromatic compounds. These columns were prepared in the laboratory by plugging a Pasteur pipette with glass wool and packing with 1 g Florisil (60 to 100 mesh). The sugar cane ash and toll booth soot were extracted into DCM as described in section 2.4.2 and the volumes of the extracts reduced by rotary evaporation to approximately 2 ml. The extracts were loaded onto the column by even application with a Pasteur pipette and eluted with DCM, 10 ml per gram Florisil. The eluents were then evaporated to

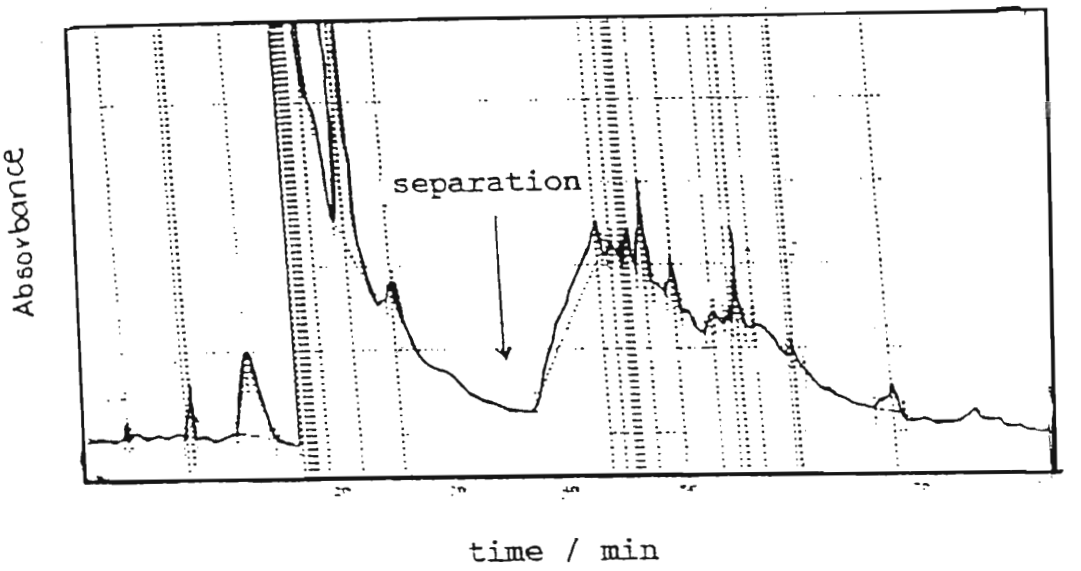


Figure 3.9 A HPLC chromatogram of the same crude sugar cane extract as in Figure 3.8, but with a gradient of water held for 10 minutes, changed over 10 minutes to 35%(v/v) acetonitrile-water, held for 10 minutes and increased linearly over 20 minutes to 100% acetonitrile, at a flow of 1.5 ml/min.

dryness under nitrogen and analysed via HPLC. If the sample extracts were very dirty larger quantities of Florisil were used.

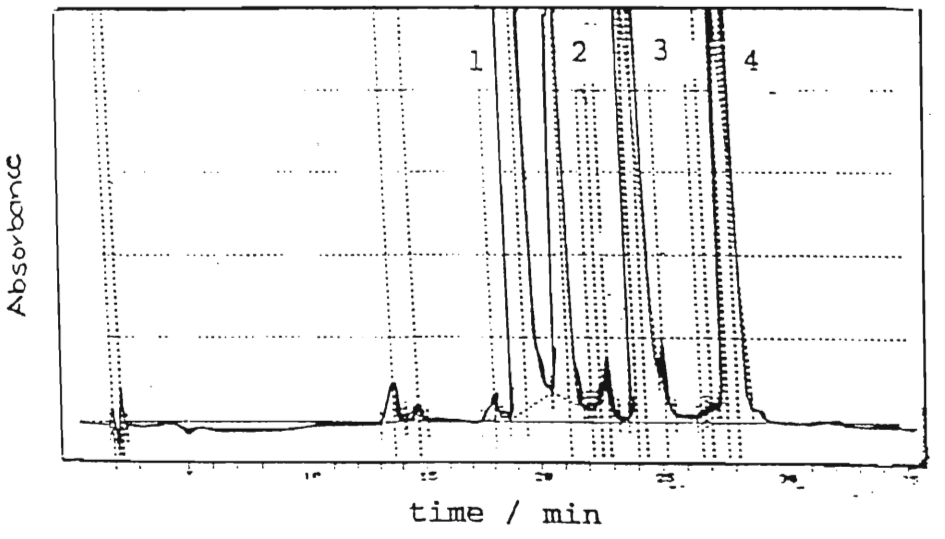
Since the efficiency of acetone as an extraction solvent was also being investigated, samples were also ultrasonically extracted into 200 ml acetone, filtered through a Millipore HV 0.45 μm filter and the filtrate volumes reduced by rotary evaporation to 2 ml. The extracts were loaded onto a Florisil column and eluted with 10 ml acetone. The eluents were evaporated to dryness under nitrogen and analysed via HPLC.

To ensure that no loss of PAHs was occurring during this clean-up procedure a solution of four standard PAHs was used to test the recovery efficiency of the column. The 4 standards: anthracene, pyrene, chrysene and benzo(a)pyrene were weighed into two flasks so that their concentrations would be 10^{-3} M if dissolved in 10 ml of solvent. They were ultrasonically extracted into 200 ml DCM for 2 hours and the solvent extracts were filtered through a Millipore 0.45 μm HV organic filter. One of the extracts was rotary evaporated to dryness and redissolved in 10 ml 50%(v/v) DCM-methanol and injected into the HPLC. The volume of the second extract was reduced to approximately 2 ml, loaded

onto a Florisil column and eluted with 10 ml DCM. The eluent was evaporated to dryness, redissolved in 50%(v/v) DCM-methanol and injected into the HPLC. A comparison of the two chromatograms (shown in Figure 3.10) revealed no apparent loss of any of the PAHs that were extracted.

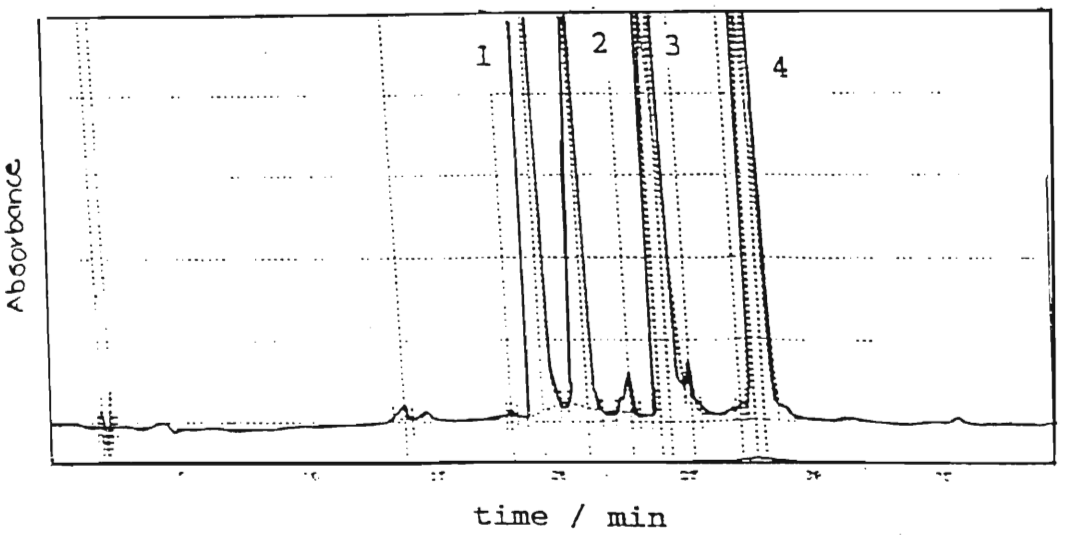
3.5.2.4. Sample analysis

Once the HPLC routine had been established all samples were analysed in the same manner. The sample extract residues were reconstituted in 1 to 2 ml 50%(v/v) DCM-methanol, filtered through a HV solvent resistant syringe filter to remove any undissolved particles and 10 µl injections were made onto the column. The extracts obtained from the fractionation process were eluted with the gradient of 35%(v/v) acetonitrile-water held for 2 minutes and then increased linearly to 100% acetonitrile over 20 minutes at a flow rate of 1.5 ml/min. The total analysis time was 60 minutes. The extracts obtained after clean-up of the crude extracts on Florisil were analysed with a gradient of 100% water held for 10 minutes, changed over 10 minutes to 35%(v/v) acetonitrile-water and held for 10 minutes, then increased over 20 minutes to 100% acetonitrile at a flow rate of 1.5 ml/min. Total run times were of the order of 100 minutes.



(a)

1. Anthracene
2. Pyrene
3. Chrysene
4. B(a)P



(b)

Figure 3.10 HPLC chromatograms of 4 standard PAHs (a) before, and (b) after clean-up on Florisil.

As mentioned in section 2.4 organic solvents can contain some PAH impurities and therefore it was important to subject the solvents used for the extraction, fractionation and clean-up processes to the same treatment and analyses as the samples in order to identify which peaks were due to solvent contamination.

The solvents (DCM and acetone) were either placed in flasks and sonicated for 2 hours or Soxhlet extracted with a clean thimble for 16 to 18 hours. The solvents were removed and filtered through a 0.45 µm HV organic filter. The filtrates were either rotary evaporated to dryness or the volumes were reduced to 2 ml, loaded onto a Florisil column, eluted with DCM and the eluent evaporated to dryness under nitrogen. All residues were reconstituted in 50%(v/v) DCM-methanol and 10 µl injections made down the HPLC using the same solvent gradient as used for the sample analyses. The chromatograms obtained from these injections were compared to the sample chromatograms and the peaks due to solvent contamination could be identified.

A detailed discussion of the results obtained from sample analysis via HPLC coupled with UV detection will be presented in section 5.1.2.

3.5.2.5. HPLC cleaning routine

As mentioned previously, due to the complex nature of the samples being analysed and the frequent use of the column, contamination and high back pressures were often experienced which interfered with sample analyses. To overcome these problems the column was thoroughly cleaned at various intervals during the study. This was achieved by running solvents of decreasing polarity through the column by means of gradient programs and then reversing the system to run the solvents in the opposite order, i.e. increasing polarity. For example, water would be run through the column for half an hour then changed by means of a gradient over 5 minutes to methanol and run for half an hour, then changed to acetonitrile and finally tetrahydrofuran (THF). This order would then be reversed and THF would be replaced by acetonitrile, methanol and finally water. A flow rate of 1.0 or 0.7 ml/min was usually employed.

If this did not result in a decrease in the back pressure of the column it usually meant that the column ends were blocked by dirt from a precipitated compound. To remove this dirt the column was connected backwards to the HPLC so that the solvent flow was reversed and the column was flushed for a few hours with THF at a low flow rate of 1 or 2

ml/min. If the pressure was still too high the column ends were removed (one at a time) and sonicated for 5 minutes in acetonitrile to remove any dirt that might be present. This procedure usually resulted in a drop in the back pressure and removal of the contaminants which was reflected by the straight baseline obtained from a blank injection.

A number of PAHs were identified in the sugar cane ash and toll booth soot extracts, and a discussion of these results will be presented in section 5.1. The crude sample extracts were also subjected to mutagenicity testing and this is described in detail in the following chapter.

CHAPTER 4

MUTAGENICITY TESTING

In this work, the residue from sugar cane crop burning and vehicle exhaust emissions were tested for their mutagenic potential. This chapter presents a detailed discussion of the mutagenicity assays employed.

Mutagenesis is defined as the process of producing an organism or gene that is different from the normal (or wild type) characteristics[52] (a mutant). This change is achieved by the addition of a physical agent or chemical reagent (a mutagen) to the organism which results in an alteration in the base sequence of the DNA, i.e. a mutation. The most common mutations are substitution, addition, rearrangement or deletion of one or more bases[53] (see Figure 4.1). When mutagenesis occurs in nature without the addition of a mutagen it is known as spontaneous mutagenesis, and conversely if a mutagen is added to cause the mutation it is called induced mutagenesis.

A number of short term carcinogenesis and mutagenesis bioassays exist for the detection of mutagens. These short term bioassays are conducted with micro-organisms such as bacteria and yeast, plants, insects, mammalian cells and, in some cases, whole animals. Different end points related

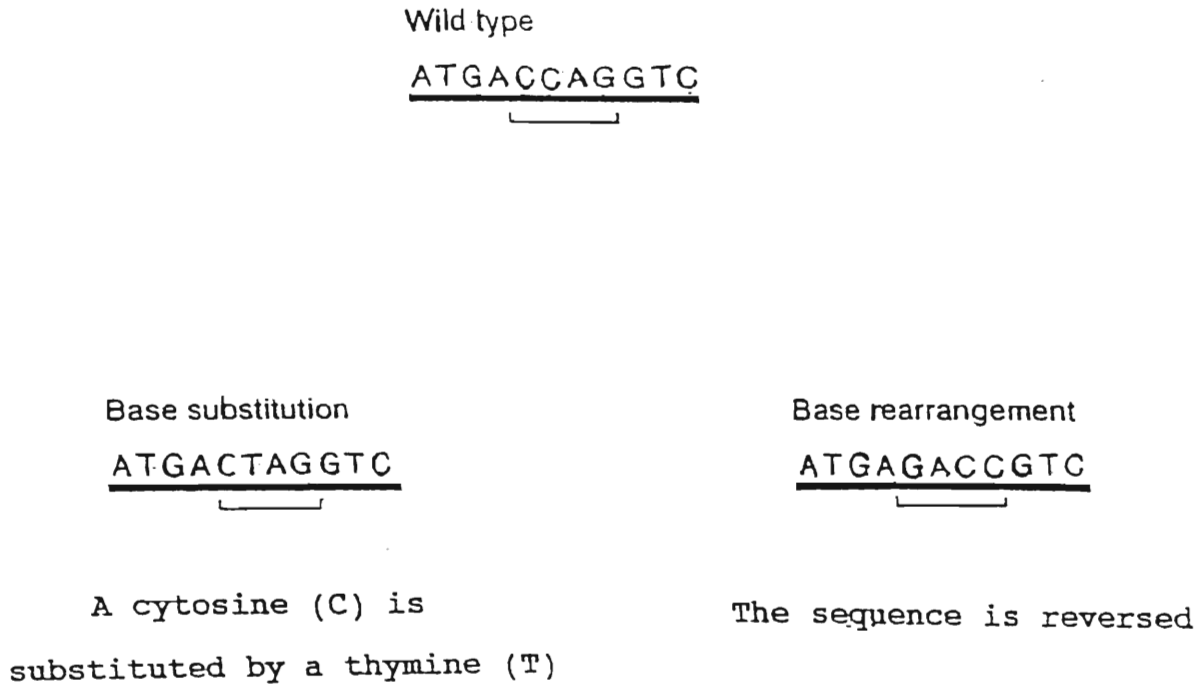


Figure 4.1 Mutations in the DNA[53] are caused by some alteration in the sequence of the nucleic acid bases. The area in which the changes are taking place is indicated by the square bracket.

to mutagenicity are detected such as gene mutation, DNA damage and repair, chromosomal effects and morphological cell transformations. The theoretical justification for substituting bacteria for mammalian cells is based on the fact that since cancer is caused by damage to the DNA (the cell's hereditary material), and both DNA lesions and the cellular processes that repair them are very similar in both bacteria and human cells, what is detrimental to bacterial DNA is most likely to harm human DNA. This theory is supported by both practical and experimental results [54].

The most widely employed short term bioassay is the *Salmonella typhimurium*/microsomal mutagenicity test, more commonly known as the Ames test, developed in the mid 1970's by Bruce Ames and his colleagues. This is a bacterial reversion assay that utilises 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[55-58] and to demonstrate the mutagenic potential of complex environmental samples such as coal tar[26,58], cigarette smoke[57,59], vehicle exhaust emissions[44,25,60,61] and water[62,63]. The use of a second bacterial mutagenicity assay, the SOS Chromotest, was also investigated. This is a commercially available test that uses strains of *E. coli* that have been specially modified to enable the detection of mutagens.

The following sections will present a discussion of the

basis of both the Ames test and the SOS Chromotest, the problems experienced in performing these assays and the application of these tests to detecting the mutagenic activity of the sample extracts under investigation.

In addition to testing the mutagenicity of the extracts, the toxicity was also studied by means of another commercially available test kit, the Toxi-Chromotest, and this will be discussed briefly in section 4.3.

4.1. THE AMES TEST

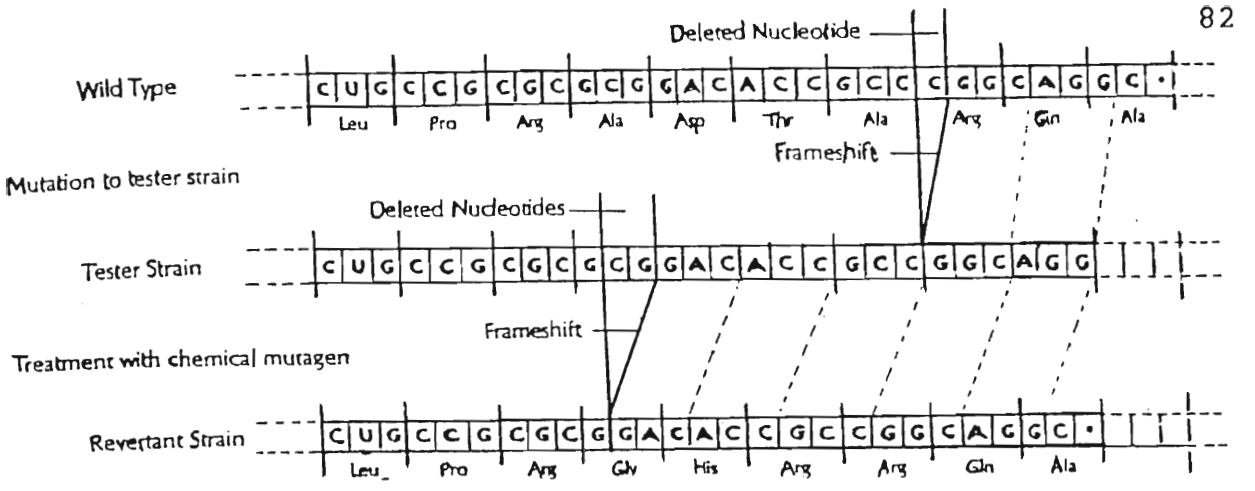
The Ames test was the first *in vitro* test that demonstrated the genetic relevance of chemical carcinogenesis. Previous *in vitro* tests had failed to show that carcinogens are mutagens; a hypothesis that is essential to the concept that the ætiology of cancer involves the formation of somatic tumours[64]. However, with the discovery by E. and J. Miller of the University of Wisconsin that most carcinogens are not active until they are metabolically converted in a living system, Ames was able to couple this finding with a modification of a reversion test for mutagenicity using *Salmonella typhimurium* to demonstrate that carcinogens are mutagens[55-57,59,65].

Normal *Salmonella typhimurium* bacteria (represented by his^+) do not require the presence of the amino acid histidine for

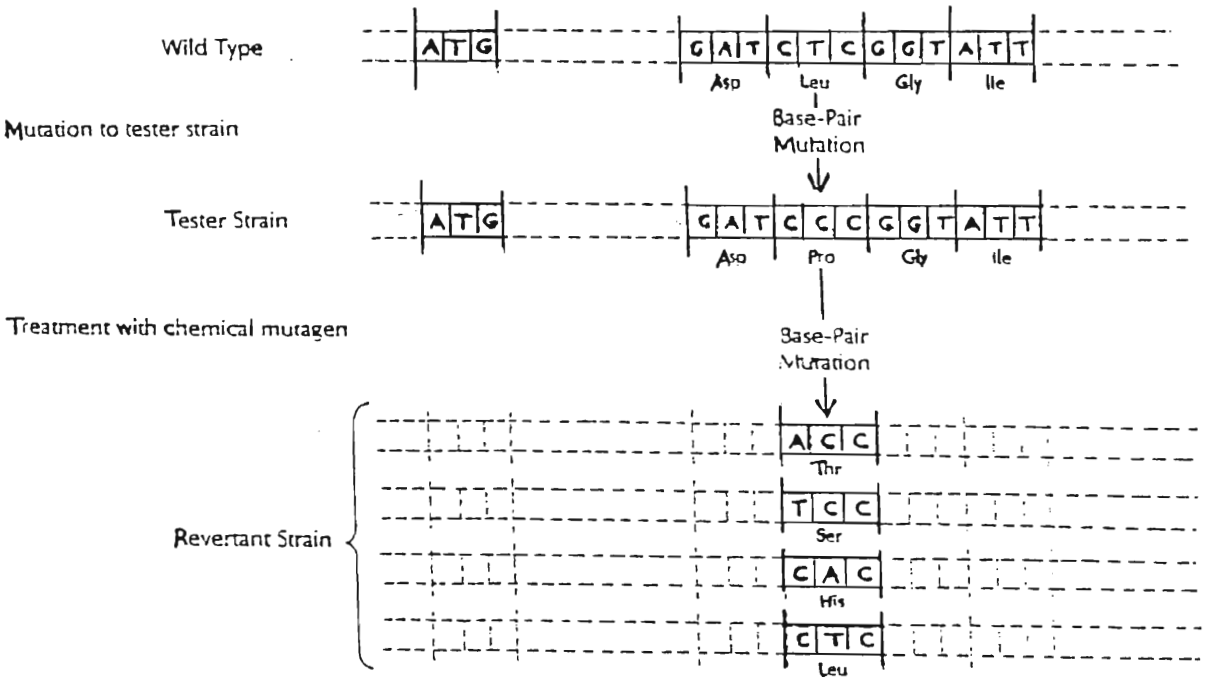
growth as they can produce their own. Ames and his colleagues constructed strains of the bacteria (his^-) that were mutated in such a way that one of the genes necessary for the biosynthesis of histidine was inactivated and these strains were unable to grow unless histidine was added to the growth medium[54,64,4].

The above mutations fall into two main categories: base pair substitution mutations and frameshift mutations. In base pair substitution mutations, one nucleotide base normally present in the series of matched nucleotides that make up each strand of DNA, is replaced for another. If strategically located this mismatching causes inactivation of the gene's protein product (see Figure 4.2 (a)). In frameshift mutations, a nucleotide is either added or deleted in a sequence of nucleotide base pairs. This results in every nucleotide in the sequence "down stream" from the mutation being mismatched with its counterpart on the other strand thus preventing the production of a functional protein (see figure 4.2(b)). This process of adding or deleting a base from the DNA is increased by the addition of an acridine-type compound such as a PAH that can intercalate in the DNA base pair stack and thereby stabilise the mismatching[66].

Treatment of these his^- strains with a mutagen will cause many different DNA sites, in different bacteria, to be mutated. Occasionally a mutation will occur at exactly the



(a) **Frameshift mutation:** Chemical deletion of a single cytosine (C) in a wild-type strain produces a tester strain with a shift in the normal reading frame, which is in codes of three letters each. Downstream from this frameshift mutation (i.e. to the right), all codes are incorrect, preventing gene product synthesis. Most mutagens that revert this strain put the reading frame "back in sync" not by replacing the missing C, but by deleting an upstream -CG- base pair.



(b) **Base-pair substitution mutation:** This involves the replacement of a single thymine (T) by a single C. This change produces proline instead of leucine, making the gene product nonfunctional. Some base-pair substitution mutagens restore the original -CTC- code, but others produce different codes (-ACC-, -TCC-, -CAC-) also allowing gene product synthesis.

Figure 4.2 Frameshift and base-pair substitution mutations[64] in *Salmonella*'s histidine gene.

same site as the original mutation (his^-) and the DNA is restored to its normal coding sequence which re-enables the bacteria to produce their own histidine (his^+). This process is called a reverse mutation and is detected by the ability of the his^- strain, on the addition of a suspected carcinogen or mutagen, to grow on a medium lacking in histidine.

Ames therefore developed the test for carcinogens by selecting a number of strains that were sensitive to reversion by a variety of base-pair substitution or frameshift mutagens and called these the "tester" strains, each of which contained different mutations. The most commonly used tester strains to date are TA 1535, TA 1537, TA 1538, TA 100 and TA 98. The strains TA 1535 and TA 100 are sensitive to reversion by base-pair substitution mutagens whereas TA 1537, TA 1538 and TA 98 detect frameshift mutagens. These latter bacterial strains (TA 1537, TA 1538 and TA 98) would therefore be expected to be particularly sensitive towards detecting any mutagenic activity of the samples under investigation because of the stabilizing effect that PAHs have on the frameshift mutation.

In order to enhance the sensitivity of the tester strains, Ames introduced a number of additional modifications. The first of these modifications (*uvr*B) was the deletion of the gene coding for the DNA excision repair system. This

system, normally present in every cell, enables the repair of certain lesions in the DNA before the damage can develop into an established mutation. The deletion of this gene therefore increases the sensitivity of the strains towards weak mutagens that might previously have been missed. Since this mutation occurs through the *bio* gene, biotin must be added to the growth medium. The second modification is the *rfa* mutation where the thick lipopolysaccharide outer coating of the bacteria is partially removed, thus enabling the diffusion of large molecules into the bacteria. The final modification was the introduction of an "R-factor" plasmid, pKM 101, into the tester strains. This plasmid, through error-prone DNA repair, enhances the mutagenic response of the host bacteria and these R-factor strains (TA 100 and TA 98) are reverted by a number of mutagens that are detected only weakly or not at all by the parent non-R-factor strains (TA 1535 and TA 1538 respectively)[64,65].

Many mutagens, for example benzo(a)pyrene, are known as promutagens. These compounds are not carcinogenic in their present forms but can be metabolised in a living system to reactive intermediates which then attack the DNA. In order to include this metabolising system in the test, an enzymatic system (the S9 mix) containing rat liver homogenate (the S9 fraction) is added to the growth medium. Some mutagens on the other hand do not require the addition of this microsomal activation as they are activated by

bacterial enzymes in *Salmonella* to their reactive intermediates[60] and these are called direct acting mutagens.

The most commonly followed test protocol, and the one followed in this study, is the plate incorporation test[57,64,65]. This involves the combination of the test compound, the bacterial tester strain and the S9 mix in soft agar containing a trace of histidine, not enough to allow growth of the bacteria but sufficient to permit expression of mutations. This mixture is then poured onto hardened minimal glucose plates and incubated for 48 hours at 37°C after which time they are removed and scored for revertant colonies. A schematic outline of the test can be seen in Figure 4.3. Included in the protocol are positive and negative control plates. Positive control plates contain a compound that is a known mutagen for that strain in place of the test chemical 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 and the S9 mix, and any colonies formed on these plates are known as spontaneous revertants. The number of spontaneous revertants is specific to each bacterial tester strain and deviations from the accepted range indicates a loss in the viability of the strain. Table 4.1 gives the ranges into which the number of spontaneous revertants for each strain should fall in the presence and absence of the S9 mix. These values may fluctuate from one laboratory to

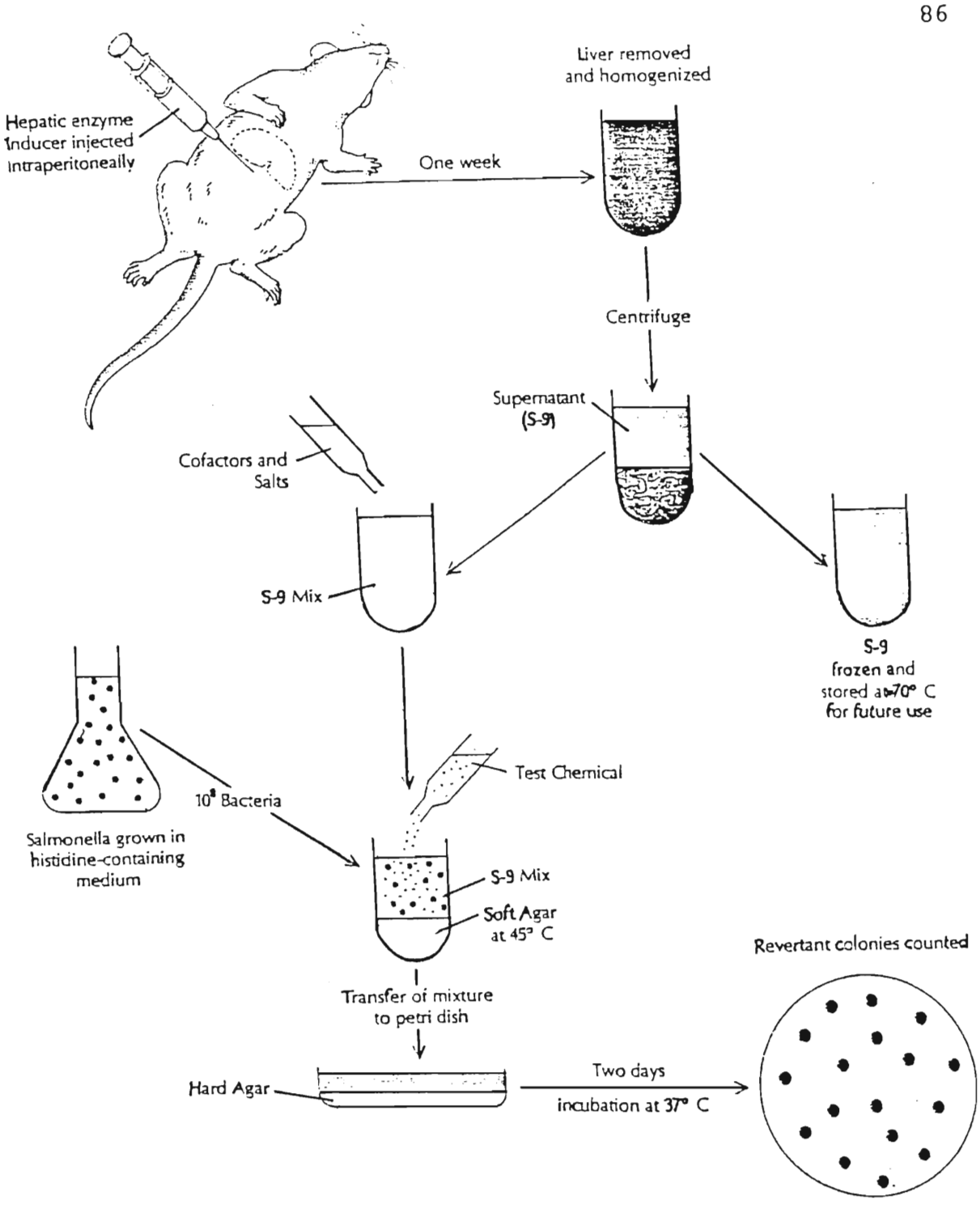


Figure 4.3 A schematic outline of the Ames test[64].

another but should remain consistant within one laboratory. If the number of revertant colonies on the plates containing the test chemical exceeds that on the negative control plates it is an indication of the mutagenicity of the test chemical. Subtraction of the spontaneous revertants from the revertants on the test plate gives the number of bacteria reverted by the chemical. Mutagenicity results are reported as revertants per μg test chemical in the form of a dose-response graph.

Table 4.1 Number of spontaneous revertants expected for the various tester strains in the presence (+S9) and absence (-S9) of the enzymatic system (S9 mix)[67].

Tester Strain	Spontaneous revertants per plate +S9	Spontaneous revertants per plate -S9
TA 1535	10-25	10-25
TA 1537	3-18	3-18
TA 1538	15-35	10-25
TA 100	140-180	150-200
TA 98	25-40	20-35

The following sections will describe in detail how the Ames test was carried out.

4.1.1. Acquisition of the Tester Strains and Arochlor 1254

The *Salmonella* tester strains used in the Ames test were obtained from a number of sources:

The first strains obtained were TA 1535, TA 1537, TA 1538, TA100 and TA 98. These were kindly donated by the Department of Microbiology at the University of Cape Town, and arrived in the form of slopes. However after performing the required tests for genotypes (see section 4.1.4) TA 100 and TA 98 were found to lack the necessary mutations and it was therefore impossible to include them in any testing procedures. Master plates and frozen permanents were prepared of TA 1535, TA 1537 and TA 1538 (see sections 4.1.5.2 and 4.1.5.3 for preparation) and these strains were used in the first few test procedures. However, due to the number of problems experienced in performing the mutagenicity assay, these strains were later omitted from the test procedure until the experimental techniques and conditions had been improved. To achieve the latter, more samples of the strains TA 98 and TA 100 were obtained from Martella Du Preez of the Regional Water Technology division at the Council for Scientific and Industrial Research (C.S.I.R.) in Pretoria (in the form of slopes). New strains were

obtained from this source from time to time during this study.

The tester strains ATCC 29629, 29630 and 29631 were also obtained from the American Type Culture Collection in the USA in the form of freeze-dried bacteria. These strains have the same modifications as the Ames cultures TA 1535, TA 1537 and TA 1538, but the suppliers could not guarantee that these strains would behave identically to the Ames strains. Rehydration of the strains was achieved by adding 0.5 ml of nutrient broth to the vials, mixing well and transferring the contents to a test tube containing 5 ml of the same broth. The cultures were aerated at 37°C by shaking overnight as before and the genotypes checked (see section 4.1.4). Frozen permanents were prepared from these bacteria but a test was never performed with these strains since the Ames strains arrived shortly after.

All the above tester strains were also obtained from Bruce Ames at the University of California in Berkeley in the form of sterile filter paper discs which had been dipped into fresh broth cultures of the appropriate bacterial strain. The discs were processed immediately after arrival by streaking on the appropriate agar plates (see Appendix B): ampicillin plates for the R-factor strains and

histidine-biotin plates for the non-R-factor strains, and the discs were dropped into sterile Oxoid nutrient broth. The plates were cross-streaked for single colony isolation and incubated at 37°C for 48 hours to serve as a back-up source of the strains. The broth cultures were grown up overnight by shaking at 37°C, the genotypes confirmed (see section 4.1.4) and frozen permanents prepared (as described in section 4.1.5.2) immediately. Once obtained and prepared, these frozen permanents served as the only source of the bacteria for further Ames testing.

The Arochlor 1254 solution was kindly donated by Dr W. C. A. Gelderblom of the Research Institute for Nutritional Diseases at the Medical Research Council in Cape Town.

4.1.2. Preparation of Stock Solutions and Media

All stock solutions and media required to carry out the Ames test were prepared according to Maron and Ames[65] and the recipes appear in Appendix B.

4.1.3. Procedure for Growing Cultures

The tester strain liquid cultures were prepared by inoculating a freshly prepared sterile solution of

Oxoid nutrient broth with the bacterial strains. The broth was prepared according to the manufacturers instructions (i.e. 25 g broth per litre), poured into a round 250 ml screw cap bottle and sterilized by autoclaving (121°C for 45 minutes). The amount of liquid culture required depended on the size of the test being performed and was based on 0.1 ml culture per plate. The sterile broth was inoculated from culture slopes, master plates or frozen permanents. These inoculation procedures are described below.

4.1.3.1. Inoculation from slopes

A wire loop was sterilized by heating in a Bunsen burner flame until red-hot. The lid of the bottle containing the culture was carefully removed and the mouth of the bottle passed over the flame to prevent contamination of the slopes from the outside air. The loop was cooled around the rim of the culture bottle, some bacteria removed by scraping the slope (see section for the 4.1.5.1 preparation of slopes) and placed in the sterile broth. Before re-closing the culture bottle, the mouth was once again flamed to ensure no contamination occurred.

4.1.3.2. Inoculation from master plates

A single piece of wire was heated in a flame until

red-hot. The master plate was opened and the wire cooled in the agar. A single colony was picked off the plate and placed in the sterile broth.

4.1.3.3. Inoculation from frozen permanents

A wire loop was sterilized as before, cooled and used to scrape some of the frozen culture and transfer it into the sterile broth. Alternatively, the frozen permanent was defrosted and the bacteria added to the broth by means of a micropipette. Typically, 20 µl bacteria per 5 ml broth was used.

All of the methods described above were used in this study. The inoculated broth, contained in round 250 ml screw cap bottles, was placed in a metabolic shaker set at 37°C with the bottle caps slightly loosened and aerated by shaking overnight (12 to 16 hours). The bacteria were grown up to a density of 1-2 x 10^9 cells/ml. Initially Oxoid nutrient broth code CM1 was used for growing the cultures but this consistently gave low bacterial densities when compared to a set of McFarland nephelometer standards[68]. These standards consist of a set of tubes containing a 1% sulphuric acid solution and a 1% barium chloride solution (both prepared in sterile broth) mixed in varying proportions to give

solutions of increasing optical densities. Each tube has a corresponding bacterial density value, and a visual comparison of the overnight liquid culture solution to these tubes gives an indication of the bacterial density. The procedure for the preparation of these tubes is given in Appendix B, section B.15. It was subsequently discovered that this broth (CM1) contained yeast extract which was causing bacterial death. The broth was replaced by Oxoid nutrient broth No. 2 which produced the required bacterial density of the overnight liquid cultures.

In performing the Ames test, only freshly grown up cultures were used and any remaining cultures were killed by sterilizing and discarded.

4.1.4. Confirming Genotypes of Tester Strains

It is possible that the his$^-$ *Salmonella typhimurium* tester strains could lose their mutations through sub-culturing or bad maintenance of stock solutions, therefore it is important to check these mutations or genotypes of the tester strains:

- immediately on receiving the cultures,
- when a new set of frozen permanents or master plates are prepared,
- when the number of spontaneous revertants fall out of the expected range, or

- when there is a loss of sensitivity to standard mutagens[65].

The checks involved testing for the his$^-$, *uvrB* and *rfa* mutations, and for the presence of the R-factor plasmid.

For these checks fresh broth cultures were used and all glassware, solutions, petri dishes and swabs were sterilized.

4.1.4.1. Histidine requirement

The his$^-$ character of the tester strains was confirmed by demonstrating the histidine requirement for growth on selective agar plates. Agar plates enriched with histidine and biotin, and control plates enriched only with biotin were prepared as given in Appendix B. Biotin is necessary for growth because of the *uvr*B mutation (see section 4.1). A flame sterilized wire loop was dipped into the freshly prepared liquid cultures (prepared as described in section 4.1.3) and a single sweep made across the biotin control plates and then across the histidine/biotin plates. The plates were incubated overnight in an oven set at 37°C and examined for growth. Colonies appeared only on the plates enriched with histidine and biotin, no growth was

observed on the control plates. This test therefore confirms the presence of the his$^-$ mutation as histidine was required for growth.

4.1.4.2. *uvr*B Mutation

The presence of this mutation is confirmed by demonstrating the sensitivity of the tester strains to ultraviolet light. In normal *Salmonella*, where the excision repair system is intact, irradiation by UV light does not affect bacterial growth. If however the *uvrB* mutation is present, the damage caused by exposure to UV light cannot be repaired, resulting in bacterial death. Sterile cotton wool swabs were dipped into fresh liquid cultures (prepared as described in section 4.1.3), the excess squeezed out on the sides of the bottles and the tester strains streaked across a nutrient agar plate in parallel lines. The R-factor strains were streaked on a different plate to the non-R-factor strains. The plate was covered with a piece of cardboard in such a way that half of each bacterial streak was covered. The plate containing the non-R-factor strains TA 1535, TA 1537 and TA 1538, was irradiated with a Hoefer Mighty Bright 230 V UV lamp for 6 seconds and the plate with the R-factor strains TA 98 and TA 100, was irradiated for 8 seconds. After incubation overnight at 37°C growth

was observed only on the side of the plate that had been covered which therefore indicated the presence of the *uvr*B mutation.

4.1.4.3. *rfa* Mutation

This mutation allows the permeation of large molecules through the bacterial cell wall and is confirmed by testing the sensitivity of the tester strains to the large crystal violet molecule. This compound is toxic to the bacteria and diffusion of this molecule into the cell wall will result in bacterial death. For each tester strain, 0.1 ml of fresh overnight broth culture (as prepared in section 4.1.3) was added to a sterile test tube containing 2 ml of molten top agar (see Appendix B, section B.3 for preparation) held at 45°C. Histidine and biotin are not necessary because of the presence of Oxoid nutrient broth in the agar plates used in this test. The test tube was swirled to cause mixing and the agar poured onto a hardened nutrient agar plate. The plate was tilted and rotated to distribute the top agar evenly, placed on a level surface and allowed to become firm. Crystal violet solution (10 µl) was then pipetted onto the centre of sterile filter paper discs (5 mm diameter) and one disc was transferred to each plate with a pair of sterile forceps. The disc was embedded slightly

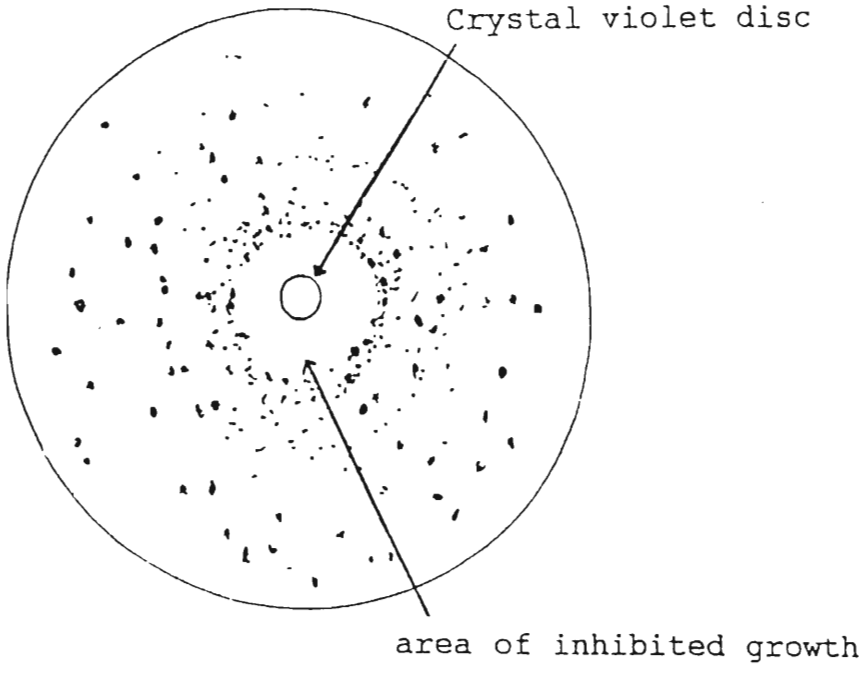


Figure 4.4 Outcome of the test for *rfa* mutation. The presence of the clear zone around the crystal violet filter disc indicates diffusion of the large molecules into the bacteria thereby causing cell death.

in the top agar, the plates inverted and incubated at 37°C. After 12 hours a clear zone (approximately 14 mm) of inhibited growth appeared around the disc (see Figure 4.4) thus indicating the presence of the *rfa* mutation which had permitted the large crystal violet molecule to enter and kill the bacteria.

4.1.4.4. R-factor

The R-factor strains, TA 98 and TA 100, should be tested regularly to check for the presence of the plasmid as it is unstable and can be lost from the bacteria[65]. This is achieved by checking the resistance of these strains to ampicillin. If the plasmid is present in the strains, the presence of ampicillin will have no effect on the bacteria. If however, the strain does not contain the plasmid, ampicillin will cause an inhibition of bacterial growth. A flame sterilized wire loop was dipped into fresh broth cultures (as prepared in section 4.1.3) and streaked across the surface of an ampicillin plate (see Appendix B for preparation). A non-R-factor strain was streaked on the same plate as a control for ampicillin activity. After incubation overnight at 37°C colonies had formed only along the streaks made with the R-factor strains and not along the non-R-factor control streak, indicating that the plasmid was still present in the R-factor strains.

4.1.5. Storage of the Tester Strains

As mentioned in the previous sections, the tester strains can be stored in the form of culture slopes, frozen permanents and master plates.

4.1.5.1. Culture slopes

Nutrient agar was prepared and culture bottles of approximately 10 ml capacity were quarter filled with the mixture. The bottles and agar were sterilized by autoclaving. They were removed from the autoclave and placed at an angle so that the agar hardened to form a slope. A sterile wire loop was dipped into fresh overnight cultures (see section 4.1.3) and wiped over the surface of the slopes. The bottles were incubated for 24 hours in an oven set at 37°C and then stored in the fridge until needed. New slopes were prepared every 3 weeks from the old slopes to prevent loss of bacterial activity.

4.1.5.2. Frozen permanents

Frozen permanent copies of the tester strains are stored at -80°C and can be kept for up to 3 years without loss of activity or genotypes. As mentioned previously, bacteria are removed by scraping the

surface of the permanent or by defrosting the permanent and adding a measured amount to the broth. Once thawed the bacteria must be discarded.

Frozen permanents were prepared from fresh overnight cultures (see section 4.1.3) to which dimethyl sulphoxide (DMSO) was added as a cryoprotective agent. Fresh broth cultures (50 ml) were grown to a density of 1-2 x 10^9 bacteria/ml as described in section 4.1.3. For each 1.0 ml of culture grown, 0.09 ml of spectroscopic grade DMSO was added. The culture and DMSO were combined in a sterile bottle and swirled gently to allow mixing. The mixture was transferred aseptically (using a micropipette with sterile tips) into labelled sterile 1.8 ml cryotubes (Nunc). The tubes were filled almost to capacity but allowing space for expansion during freezing. The tubes were placed upright in crushed ice until frozen and then stored at -80°C. Initially, liquid nitrogen was used for storing the cultures, but later a -80°C freezer became available.

For the bacteria obtained from the University of Cape Town, CSIR and the American Type Culture Collection, only 5 frozen permanents were prepared of each strain. These were removed from storage to inoculate broths and then returned to -80°C. This

however led to contamination of the cultures since they were opened a few times. Therefore, on obtaining the fresh samples of bacteria from Professor Bruce Ames, 20 frozen permanents of each strain were prepared. One permanent was defrosted each time broth was inoculated and any remainder was discarded.

4.1.5.3. Master plates

Master plates of the bacteria are prepared in addition to frozen permanents and are stored at 4°C. They are minimal glucose plates enriched with histidine and biotin, and ampicillin is added to the plates used for the R-factor strains. These plates are used routinely for the inoculation of overnight cultures thereby avoiding the frequent opening of frozen permanents and reducing the risk of contamination. It is important to reisolate the strains before preparing master plates as it ensures that the strains maintain their characteristic spontaneous reversion frequencies. Master plates are usually discarded after 2 months.

The strains were reisolated from the frozen permanents as follows. A permanent of the bacteria was thawed and a single drop applied to a histidine/biotin plate or ampicillin plate. Using a

sterile wire loop the plate was streaked out for single colony isolation as shown in Figure 4.5 and incubated in an oven set at 37°C for 48 hours. A well isolated colony was removed from the plate with a sterile wire loop and suspended in 0.3 ml or less of phosphate-buffered saline in a small culture tube. A sterile cotton swab was dipped into the bacterial suspension, the excess squeezed out and 4 to 5 parallel streaks made across the appropriate agar plate. The plates were incubated overnight at 37°C, removed and stored in the fridge at 4°C.

4.1.6. Preparation of Rat Liver Homogenate (S9 Fraction)

As mentioned previously, some compounds require metabolic activation in order to express their mutagenic activity. This activating system, the S9 mix, is derived from rat liver enzymes, the S9 fraction. This enzymatic system is prepared as follows.

All induction and liver excision procedures were performed by Fred Kruger of the Biomedical Resource Centre at the University of Durban-Westville.

Male Sprague-Dawley rats weighing approximately 200 g each were used. The liver enzymes were induced by injecting the rats with a polychlorinated biphenyl

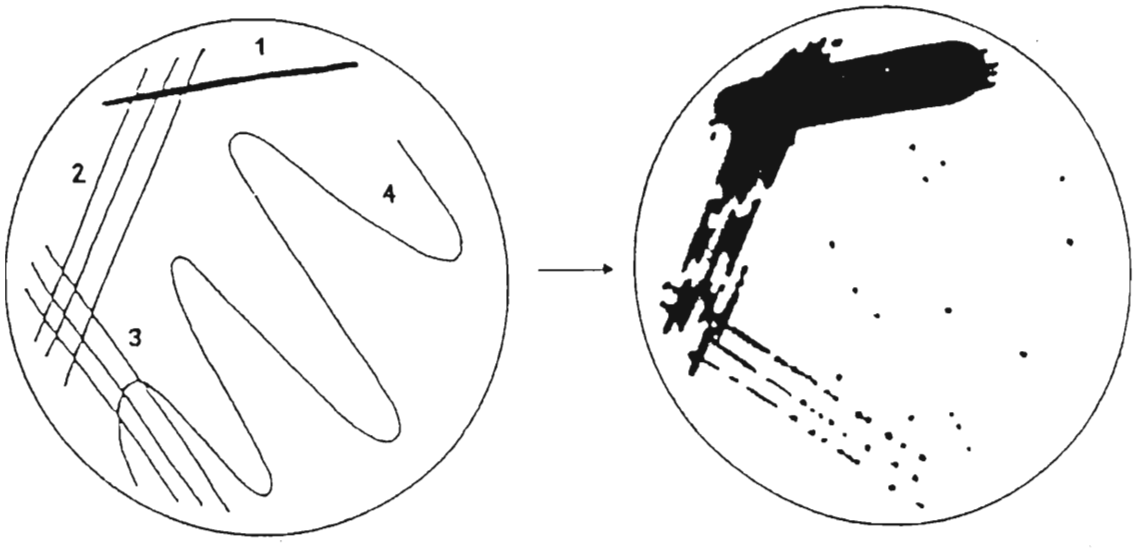
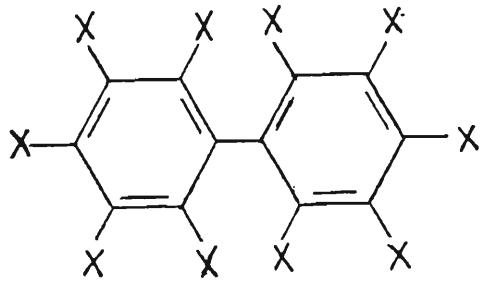


Figure 4.5 Procedure for streaking plates to form single bacterial colonies.

mixture, Arochlor 1254, five days prior to the removal of the livers. The structure of Arochlor 1254 can be seen in Figure 4.6. Arochlor 1254 was diluted in corn oil to a concentration of 200 mg/ml and each rat was injected intraperitoneally with a dose of 500 mg/kg. The rats were given water and Epol rat cubes until 12 hours prior to sacrifice when the food was removed. On the fifth day of induction the rats were killed by carbon dioxide suffocation and the livers removed aseptically using sterile surgical tools. Care was taken not to cut into the oesophagus or intestines as this would cause contamination of the liver homogenate.

The excised livers were placed in pre-weighed beakers containing approximately 1 ml of chilled (0-4°C) sterile 0.15 M KCl per gram of wet liver (one liver weighing approximately 10-15 g). After weighing, the livers were washed several times in fresh cold KCl solutions to ensure a sterile preparation and to remove haemoglobin which could inhibit the activity of the cytochrome P-450 enzymes. The washed livers were transferred to beakers containing 3 ml of cold 0.15 M KCl per gram wet liver, minced using sterile scissors and homogenized. Initially a sterile hand homogenizer with a glass pestle was used, but at a later date a Voss electric homogenizer with a teflon pestle



Average number of chlorine atoms per molecule = 4.96

Figure 4.6 Structure of Arochlor 1254[68].

became available. The homogenate was centrifuged for 10 minutes at 9000 rpm, the supernatant (the S9 fraction) decanted and transferred into 1.8 ml cryotubes (Nunc). The tubes were frozen quickly on crushed ice and stored in a -80°C freezer.

When performing an Ames test, only the required amount of S9 fraction was thawed and stored on crushed ice until incorporation into the S9 mix.

4.1.7. Preparation of S9 Mix

The components of the standard S9 mix are 8 mM $MgCl_2$, 33 mM KCl, 5 mM glucose-6-phosphate, 4 mM NADP, 100 mM sodium phosphate pH 7.4, and S9 fraction in a concentration of 0.04 ml per ml of mix. NADP and glucose-6-phosphate are required in the mix because the liver microsomal enzymes responsible for activating promutagens all require NADPH and in *in vitro* systems it is customary to add this NADPH-generating system[55]. The phosphate buffer and salts are necessary to ensure the stabilization of the S9 fraction in the top agar.

Calculation of the total volume of S9 mix required for an Ames test was based on the addition of 0.5 ml S9 mix per plate requiring S9 activation. For example, if 37 plates required the addition of S9

activation, then a total volume of 37 x 0.5 ml = 18 ml S9 mix was needed and 20 ml would be prepared as shown in Table 4.2. The S9 mix was prepared soon after the S9 fraction was thawed and kept on crushed ice throughout the test procedure. Any S9 mix remaining after the completion of a test was discarded. The sterility of this S9 mix preparation was determined by plating the mixture on a minimal glucose plate, incubating overnight at 37°C and examining the plate for any bacterial contamination. As contamination was observed, all S9 mix solutions were sterilized by filtering through a sterile 0.45 µm syringe filter before use.

Table 4.2 An outline of the preparation of the S9 mix.

Total vol S9 mix required in ml	10	20	30	35	45
vol 0.4 M $MgCl_2$/ml	0.2	0.4	0.6	0.7	0.9
vol 0.65 M KCl/ml	0.2	0.4	0.6	0.7	0.9
vol Buffer/ml	5.0	10.0	15.0	17.5	22.5
vol 1 M Glucose-6-phosphate/ml	0.05	0.1	0.15	0.17	0.225
vol 0.1 M NADP/ml	0.4	0.8	1.2	1.4	1.8
vol S9 fraction/ml	0.5	1.0	1.5	1.75	2.25
vol Milli-Q water/ml	3.65	7.3	10.9	12.7	16.42

The directions for the preparation of all the solutions used in the preparation of the S9 mix are given in Appendix B.

4.1.8. Preparation of Samples

The sugar cane ash and toll booth soot were extracted and evaporated to dryness in pre-weighed flasks as described in section 2.3 and the mass of the residues calculated. Stock solutions were prepared by calculating the maximum desired concentration (in µg/ml) of the samples and dissolving the dried extracts in the required volume of dimethyl sulphoxide (DMSO). The range of concentrations to be tested were then prepared by making serial dilutions of these stock solutions. The Ames test was performed a number of times with a variety of concentration ranges of both samples being tested before acceptable testing ranges were obtained. The development of these optimum conditions is discussed in section 4.1.9.2.

4.1.9. The Mutagenicity Test

As mentioned previously, the plate incorporation test procedure was followed. The preparations of all solutions are given in Appendix B. All solutions, glassware and micropipette tips used were sterile.

It was ensured that the petri-dishes purchased had previously been sterilized by radiation as plates sterilized by ethylene-oxide cause incorrect results because traces of this chemical are mutagenic to the bacterial strain TA 100[65].

The day prior to performing the Ames test, the required number of minimal glucose agar plates was prepared and placed in the oven overnight at 37°C to ensure that no contamination had occurred during the pouring process. Sterile Oxoid nutrient broth No. 2 was inoculated with bacterial strains TA 100 and TA 98 from defrosted frozen permanents as described in section 4.1.3.3 and grown up overnight by shaking at 37°C in a metabolic shaker.

The test procedure involved the setting up of covered sterile test tubes in a water bath held at 45°C. Top agar was melted by autoclaving and then 10 ml of 0.5 mM histidine/biotin solution per 100 ml was added. The agar was kept at 45°C by placing the bottle in the water bath. The volume of S9 mix required was calculated and prepared as shown in Table 4.2, filtered and stored on crushed ice. By means of a pipette, 2 ml of top agar was distributed into each test tube in the water bath. To this was added 0.1 ml of the overnight broth culture, 0.1 ml or less of each concentration of the sample being

tested in DMSO and 0.5 ml of S9 mix. Triplicate plates were poured for each sample concentration, both with and without the presence of the S9 mix. Negative and positive controls were also included in the assay.

Negative control plates consisted of the top agar, the bacterial strain and 0.1 ml of solvent (DMSO) but no sample extract. These plates were also triplicated and poured both in the absence and presence of the S9 mix.

Positive controls consisted of top agar, the bacterial strain and a chemical that is a known mutagen to that strain. For TA 100 sodium azide was used and 10 µl of a 1 mg/ml solution in water was added to the test tube. The presence of S9 mix was not required as this is a direct acting mutagen. 2-Aminofluorene was used for TA 98 and 10 µl of a 2 mg/ml solution in DMSO was added to the test tube. Since this chemical is a promutagen, S9 mix was also added. Only single plates were poured as positive controls for each strain.

The contents of the test tubes were mixed by swirling and poured onto the hardened minimal glucose plates. The plates were rotated to spread the mixture evenly and left to harden. The tubes

containing S9 mix were only allowed to remain at 45°C for a few seconds in order to prevent loss of viability. The mixing, pouring and distribution process took less than 20 seconds per plate which prevented the agar from hardening in mid-operation as this causes a rippling of the agar surface which makes counting of colonies difficult. Once the plates had hardened they were inverted and placed in an oven at 37°C for 48 hours.

A typical test procedure involved the pouring of approximately 80 plates per sample tested and an outline can be seen in Table 4.3.

Table 4.3 An outline of a typical Ames test.

	No. of plates TA 100		No. of plates TA 98	
	+S9	-S9	+S9	-S9
Spontaneous revertants	3	3	3	3
Conc. 1[a]	3	3	3	3
Conc. 2[a]	3	3	3	3
Conc. 3[a]	3	3	3	3
Conc. 4[a]	3	3	3	3
Conc. 5[a]	3	3	3	3
Positive control		1	1	

Total number of plates = 74

[a]Conc. 1, 2, 3, 4 and 5 refer to various concentrations of the sample tested in µg per plate.

4.1.9.1. Counting of colonies

This was done manually by counting each colony appearing on the plate. Because of the trace amount of histidine that was added to the top agar a fine haze of autotrophs (background bacterial lawn) appeared on the plates. If these autotrophs were not present the plate was discarded as this was an indication of bacterial toxicity.

The number of colonies appearing on the plates containing the samples were scored and an average taken of the 3 plates poured for each concentration. The average number of spontaneous revertants on the negative controls were also determined and the difference between the two gave the number of colonies formed due to the presence of the sample. These number of revertant colonies were then plotted against the concentrations of the samples added to the plates in µg/plate.

4.1.9.2. Optimization of sample concentration range

As mentioned previously a number of Ames tests were performed before the correct range of sample concentrations was achieved.

Sugar cane ash extract:

Mutagenicity tests were only performed on samples 2 and 3 (i.e. the ash collected off the fields) and these samples were combined prior to extraction with DCM. Sample 1 (i.e. the leaves burnt in the laboratory) was not subjected to mutagenicity testing due to the low concentration of PAHs present in the extract (see section 5.1.1).

In the first test with the sugar cane ash residue all the tester strains were used and only one concentration was tested. The mass of the dried crude residue was 11.1 µg and this was dissolved in 5 ml of DMSO to give a concentration of 2.22 µg/ml. Since the test involved the addition of 0.1 ml sample per plate, a concentration of 0.222 µg/plate was tested. Triplicate plates were poured for each strain with and without S9 activation as described in the previous section and the plates were incubated in an oven set at 37°C for 2 days (48 hours). On removal of the plates colonies were observed on some of the plates while others showed no bacterial growth. Even the positive control (sodium azide) for TA 100 and TA 1535 had not caused the bacteria to revert. It was therefore concluded that the problem lay in the techniques used during the test procedure and in the lack of

microbiological experience.

In the next test only tester strains TA 98 and TA 1535 were used. A sample extract of mass 0.1357 g was dissolved in 10 ml DMSO to give a concentration of 0.01357 g/ml (or 13 570 µg/ml). Various volumes of this stock solution were then added to the plates to obtain different concentrations. An outline of the test appears in Table 4.4.

Table 4.4 Outline of an Ames test performed on the sugar cane extract.

Vol.of sample added per plate/µl	Sample conc./(µg /plate)	No. plates TA 98		No. plates TA 1535	
		+S9	-S9	+S9	-S9
5	67.8	3	3	3	3
20	241.4	3	3	3	3
50	678.5	3	3	3	3
150	2035	3	3	3	3
500	6785	3	3	3	3
Spontaneous	-	3	3	3	3
Positive control	-	1			1

On removal of the plates from the oven it was impossible to count the colonies appearing on the

plates due to contamination by other bacterial growths. This was again attributed to a lack of experience in working under sterile conditions and until the laboratory techniques could be improved upon, further tests were performed using only strains TA 98 and TA 100.

For the next test the sugar cane residue was dissolved in 5 ml DMSO to give a concentration of 560 µg/ml. This stock solution was then diluted as shown in Table 4.5.

Table 4.5 Sample dilutions for the sugar cane ash extract.

Vol. stock /ml	Final vol. /ml	Vol. added/ (ml/plate)	Sample conc./ (µg/plate)
0.1	5	0.1	112
0.1	-	0.1	56
0.268	1.5	0.1	10
0.054	1.5	0.1	2
0.02	2	0.1	0.5

On removal of these plates from the oven, no contamination was observed and the colonies were counted. Unfortunately, very erratic counts were

obtained and the number of spontaneous revertants for both TA 98 and TA 100 were out of the accepted range. It was therefore thought that the frozen permanents used to inoculate the overnight broth cultures had become contaminated through frequent opening of the tubes.

After this test was performed, the bacterial strains arrived from Professor Bruce Ames and, as mentioned previously, 20 frozen permanents were prepared of each strain and one was sacrificed each time a test was performed.

The sugar cane ash residue was dissolved in DMSO to give a solution of concentration 1 005 µg/ml. This stock solution was then diluted in a similar manner to that shown in Table 4.5 to give a concentration range of 100, 20, 1, 0.5 and 0.1 µg/plate. The broth was inoculated from the new frozen permanents and both strains showed no visible contamination. After incubation of the plates for 2 days no other bacterial contamination was evident and the colonies were counted manually. However, the counts obtained were very similar to one another and close to the number of spontaneous revertants on the control plates.

This was thought to be due to one of three options:

(a) the sugar cane sample was not mutagenic,

(b) the concentration range was too high and the toxicity of the sample was causing cell death, or

(c) the concentration range was too low and mutagenic activity was not detectable at this level of concentration.

In order to test which of these possibilities was correct, two new tests were performed using lower and higher concentration ranges.

A stock solution of the sugar cane ash residue was prepared in DMSO and diluted to give a concentration range of 1, 0.8, 0.5, 0.2 and 0.1 μg/plate. For this test the counts on the test plates were less than the number of spontaneous revertants, thus indicating that either the concentration range was too low or the sample was not mutagenic. A higher concentration range was therefore investigated.

A sugar cane ash residue of mass 86.2 mg was partially dissolved in 8.6 ml DMSO to give an approximate concentration of 10 mg/ml. This stock solution was then either diluted to give lower concentrations, or various volumes of the stock were

added to give the desired concentrations (see Table 4.6 for an outline of the preparation).

Table 4.6 Sample dilutions for the sugar cane ash extract.

Vol. stock /ml	Final vol. /ml	Vol.added per plate /ml	Sample conc./ (µg/plate)
0.1	-	0.1	1000
0.08	-	0.08	800
0.06	-	0.06	600
1	2.5	0.1	400
1	5	0.1	200

The first attempt at testing the above concentration range failed due to contamination of the plates. However, on repeating the test, colonies were able to be counted and the results appear in Table 4.7. The plates were poured in duplicate rather that triplicate due to the lack of oven space available for incubation. Columns 2 and 3 give the number of revertants counted on each plate and column 5 is the average of these counts.

Table 4.7 Colony counts obtained for the sugar cane ash extract.

(a) TA 98 without S9 activation.

Conc./ (µg/plate)				Average no. of colonies
Spontaneous	12	15	11	13
1 000	21	15		18
800	36	-		36
600	32	35		34
400	26	11		19
200	10	-		10

(b) TA 98 with S9 activation.

Conc./ (µg/plate)				Average no. of Colonies
Spontaneous	24	20	21	22
1 000	34	12		23
800	54	56		55
600	48	63		56
400	36	56		46
200*	-	-		-

* No counts were available for this concentration as the plates were discarded due to the lack of a background autotroph.

(c) TA 100 without S9 activation.

Conc./ (µg/plate)				Average no. of Colonies
Spontaneous	91	102	104	99
1 000	53	61		57
800	85	65		75
600	82	78		80
400	83	89		86
200	22	23		23

No results were obtained for TA 100 with S9 activation as it was only noticed later that the filter used to sterilize the S9 mix had been sterilized by ethylene oxide which is mutagenic towards TA 100.

The number of revertant colonies formed due to the presence of the sugar cane ash extract was then calculated by subtracting the number of spontaneous revertants from the average number of colonies formed for each concentration. The results for TA 98 are given in Table 4.8. No results were calculated for TA 100 as the number of revertant colonies formed in the presence of the sugar cane ash extract were less than the number of spontaneous revertants. This indicated that the extract was not mutagenic

Table 4.8 The number of revertants formed due to the presence of the sugar cane extract with bacterial strain TA 98.

Concentration/ (µg/plate)	No. colonies +S9	No. colonies -S9
1 000	1	5
800	33	23
600	34	21
400	24	6
200	-	-3

towards TA 100. In other words, at the concentration levels being investigated, there were no detectable base-pair substitution mutagens present in the sample. This result was expected since PAHs have been identified in the extract (see section 3.5) and these types of compounds are frameshift mutagens which are detected by TA 98.

These revertant colonies were then plotted against concentration in µg/plate and the graph is depicted in Figure 4.7. At a concentration of approximately 800 µg/plate, a drop off in the graph is apparent. This is due to an increase in the toxicity of the sample at this and higher concentrations which results in bacterial cell death.

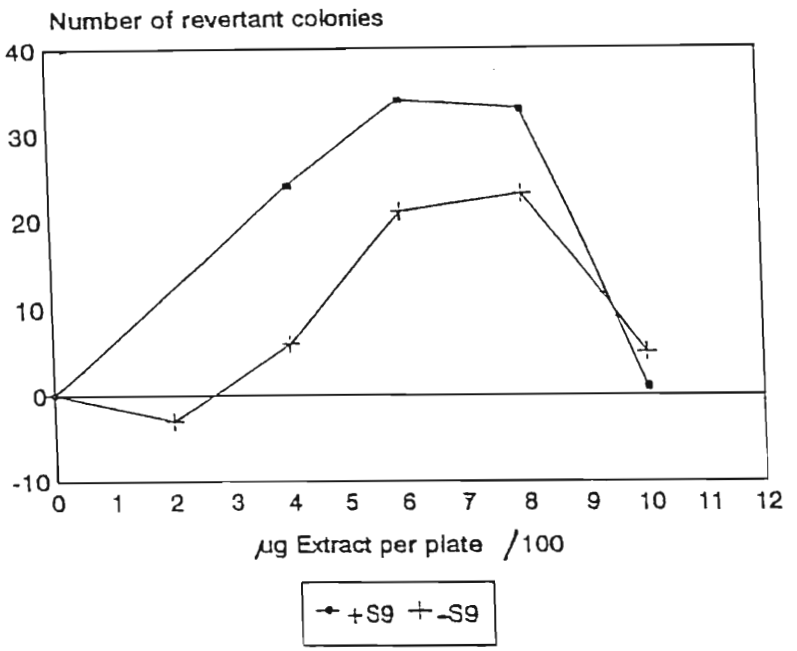


Figure 4.7 A dose-response curve obtained for the sugar cane ash extract for strain TA 98, with and without S9 activation.

As mentioned in section 2.3.1 the use of both DCM and acetone as extraction solvents was investigated. A further test was therefore performed with sugar cane ash extracted into acetone, evaporated to dryness and dissolved in DMSO, to determine whether acetone was more efficient at extracting mutagenic particles. However, this test was abandoned when the plates once again showed a large amount of contamination.

Toll booth soot extract:

Ames tests were only performed on the toll booth soot extract once the test procedure had become familiar as only a small amount of the sample was available. Tests were carried out with the tester strains TA 98 and TA 100 obtained from Professor Bruce Ames.

The first test that was performed on the toll booth extract was a comparison between soot extracted into DCM and soot extracted into acetone. Since it was not known what concentration range was required, the first range tested was 100, 10, 1 and 0,1 µg/plate. The soot was extracted into either acetone or DCM, the extract evaporated to dryness and redissolved in DMSO to give stock solutions which were then diluted

in a similar manner to the sugar cane ash extract to obtain the above concentration range. An outline of the test can be seen in Table 4.9.

Table 4.9 An outline of the Ames test performed on the DCM and acetone extracts of the toll booth sample.

Concentration/ (µg/plate)	No.of plates TA 98		No. of plates TA 100	
	+S9	-S9	+S9	-S9
Spontaneous	3	3	3	3
DCM: 100	3	3	3	3
DCM: 10	3	3	3	3
DCM: 1	3	3	3	3
DCM: 0.1	3	3	3	3
Acetone: 100	3	3	3	3
Acetone: 10	3	3	3	3
Acetone: 1	3	3	3	3
Acetone: 0.1	3	3	3	3
Positive control	1			1

The first test failed due to the presence of other bacterial contamination on the plates, but a second test was successful and colonies could be counted. An average of the counts for each concentration was calculated and the results appear in Table 4.10.

Table 4.10 Average number of revertant colonies obtained for the DCM and acetone extracts of the toll booth soot tested on strains TA 98 and TA 100, with and without S9 activation.

Concentration/ (µg/plate)		Av. no. of colonies TA 98		Av. no. of colonies TA 100	
		+S9	-S9	+S9	-S9
Spontaneous		25	24	96	93
DCM	100	87	57	155	141
DCM	10	35	24	111	120
DCM	1	17	19	108	78
DCM	0.1	20	23	-*	-*
Acetone	100	83	50	169	162
Acetone	10	35	27	112	90
Acetone	1	26	29	87	116
Acetone	0.1	33	25	95	88

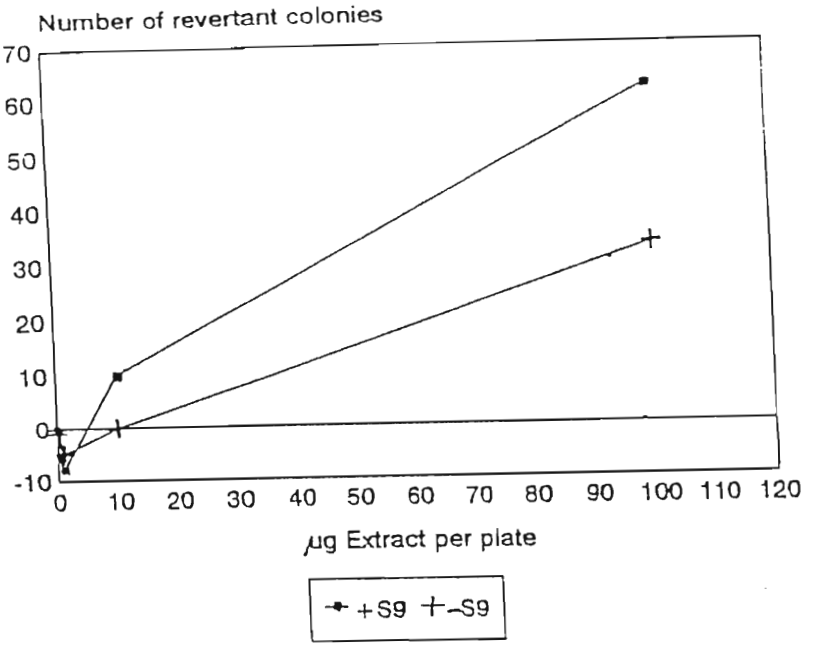
* These colonies could not be counted as there were far too many and it was not certain if they were all *Salmonella*.

The number of spontaneous revertants were then subtracted to obtain the number of revertant colonies formed due to the toll booth soot extract. These results are given in Table 4.11.

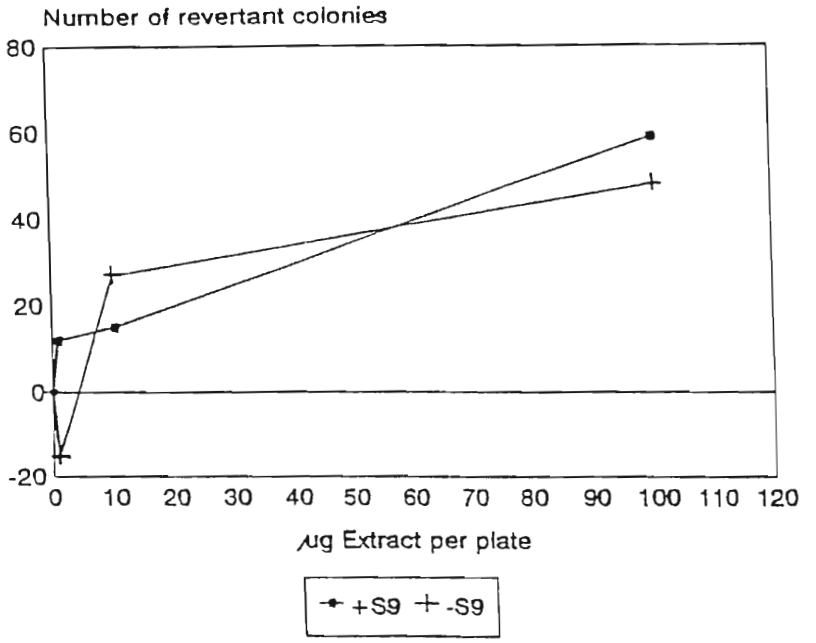
Table 4.11 Number of revertant colonies formed due to the toll booth extracts for TA 98 and TA 100, with and without S9 activation.

Concentration/ (µg/plate)		No. of Colonies TA 98		No.of Colonies TA 100	
		+S9	-S9	+S9	-S9
DCM	100	62	33	59	48
DCM	10	10	0	15	27
DCM	1	-8	-5	12	-15
DCM	0.1	-5	-1	-	-
Acetone	100	58	26	73	69
Acetone	10	10	3	16	-3
Acetone	1	1	5	-11	23
Acetone	0.1	8	1	-1	-5

Graphs were plotted of these results and are shown in Figures 4.8 and 4.9. From these graphs it is evident that insufficient data is available between 10 and 100 µg/plate. A test was therefore performed with a concentration range of 100, 80, 60, 40 and 20 µg/plate. Only the DCM extract was used in this test. Again, a stock solution was prepared in DMSO and the sample diluted and additions made as shown in Table 4.6 for the sugar cane ash extract. The first two attempts again failed due to bacterial contamination, but then two successful tests were

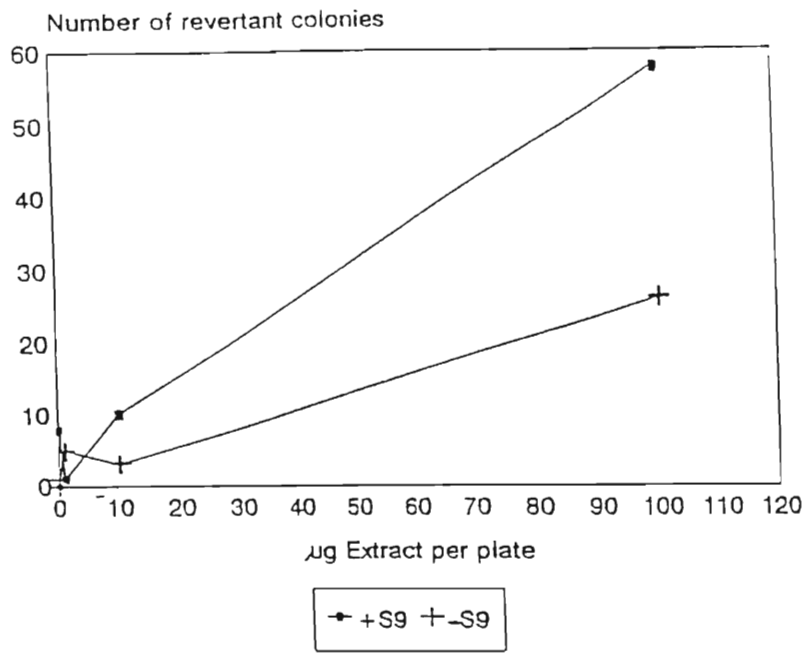


(a)

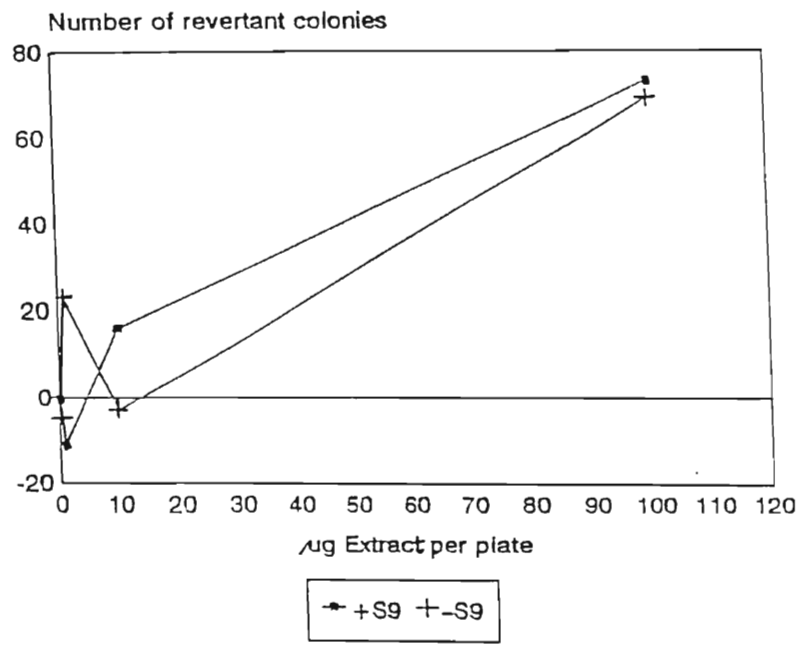


(b)

Figure 4.8 Dose-response curves obtained for the DCM extract of the toll booth soot for (a) TA 98, and (b) TA 100.



(a)



(b)

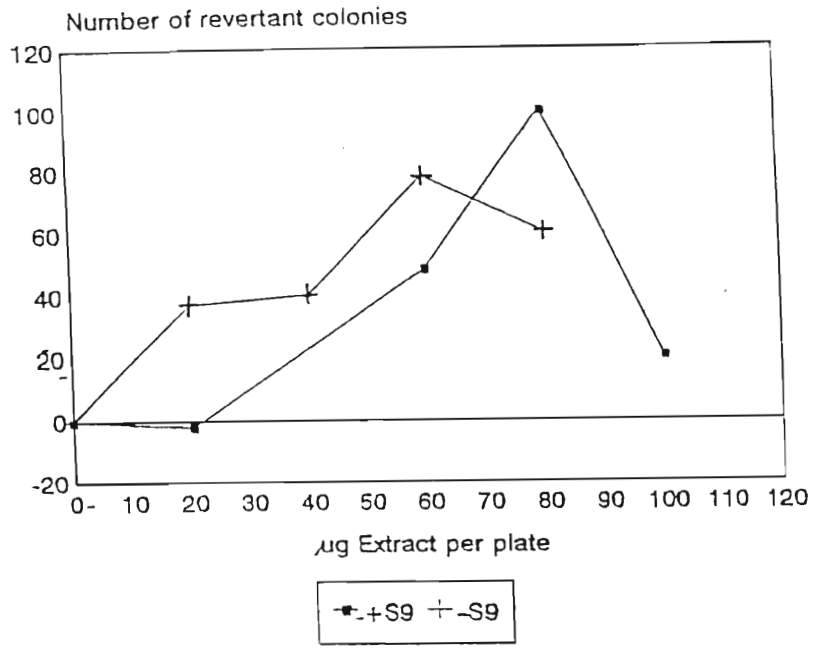
Figure 4.9 Dose-response curves obtained for the acetone extract of the toll booth soot with (a) TA 98, and (b) TA 100.

performed with TA 98. The number of revertant colonies formed due to the presence of the extract was calculated by subtracting the average number of spontaneous revertants from the average number of colonies formed for each concentration. The results are given in Table 4.12.

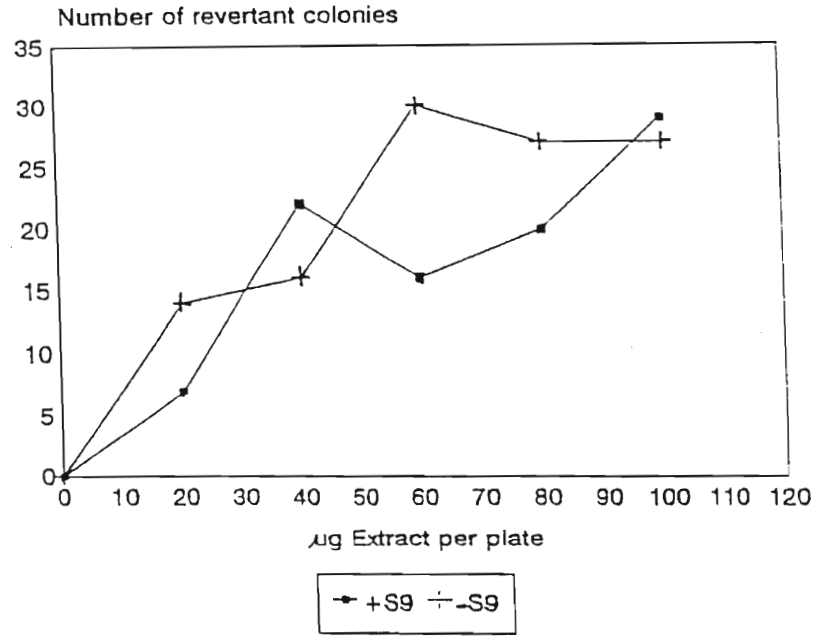
Table 4.12 Number of revertant colonies formed due to the DCM toll booth extract for TA 98, with and without S9 activation (for both tests).

Conc./ (µg/plate)	No. of Colonies +S9	No. of Colonies -S9
TEST 1		
100	20	-
80	100	60
60	48	78
40	-	40
20	-2	37
TEST 2		
100	51	40
80	42	40
60	38	43
40	44	26
20	29	27

Graphs were plotted of these results and are shown in Figure 4.10. No results were obtained for TA 100.



(i)



(ii)

Figure 4.10 Dose-response curves obtained for the DCM extract of the toll booth soot with TA 98 for (i) test 1, and (ii) test 2.

A further test was attempted with the same concentration range to compare the effect of using acetone as the extraction solvent but this failed due to bacterial contamination.

A detailed discussion of the results obtained for both the sugar cane and toll booth extracts will be given in section 5.2.1 together with a discussion on the problems experienced in performing the Ames test.

4.2. THE SOS CHROMOTEST

The second mutagenicity test investigated was the SOS Chromotest. This is a new commercially available test kit which is a quantitative bacterial colorimetric assay for mutagens and which uses a specially modified strain of *Escherichia coli*, *E. coli* PQ 37.

4.2.1. Basis of the SOS Chromotest Kit

In *E. coli* some of the responses induced by DNA damaging agents involve a set of functions known as the SOS responses. The bacterial strain contains a fusion of an operon which places *LacZ*, the structural gene for β-galactosidase, under the control of the *sfiA* gene, a SOS function that is

involved in cell division inhibition[70]. The β-galactosidase activity depends only on the *sfiA* activity and will therefore be produced if the SOS repair function is activated by a DNA damaging agent, i.e. a mutagen. The addition of a chromogenic substrate allows the production of this enzyme to be detected by a colour reaction.

As in the Ames test, the bacterial strain is further modified to increase the sensitivity of the bacteria to mutagenic activity[70,71]. The first modification is the *uvrA* mutation which removes the excision repair function thereby increasing the response to certain DNA damaging substances. The second mutation, the *rfa* mutation, involves the removal of the lipopolysaccharide barrier of the bacterial cells which allows better diffusion of certain chemicals into the cell.

4.2.1.1. The test procedure

The assay involves the addition of the bacterial strain to increasing concentrations of the sample being tested which is micropipetted into wells of a microplate. The test kit allows for the detection of promutagens as well as direct acting mutagens and this microsomal activation is achieved by the addition of a preparation modified from the S9 mix

in the Ames test. The samples are tested both in the presence and absence of this activating system. The plate is incubated for 2 hours at 37°C during which time the cells are exposed to mutagenic attack. This activates the SOS response thereby causing the production of the β-galactosidase enzyme. A blue chromogenic substrate is added and the plate incubated for a further 90 minutes after which time a blue colour develops in the wells, the intensity of which is related to the mutagenicity of the sample concentration. A schematic outline of the procedure is given in Figure 4.11. Also included in the assay are negative and positive controls. Negative controls are wells that contain the bacteria, the sample diluent and the S9 mix (or not), and the comparison of the colour in these wells to that in the test wells gives an indication of the mutagenicity of the sample. Two positive controls are provided with the kit, 4-nitroquinoline oxide (4NQO) as a control for direct acting mutagens, and 2-aminoanthracene (2AA) as a control for promutagens. These are included in the assay to check that the bacteria and the S9 mix are functioning correctly.

The amount of β-galactosidase produced is determined quantitatively by measuring the absorbance, or optical density (OD), of the solutions in each well

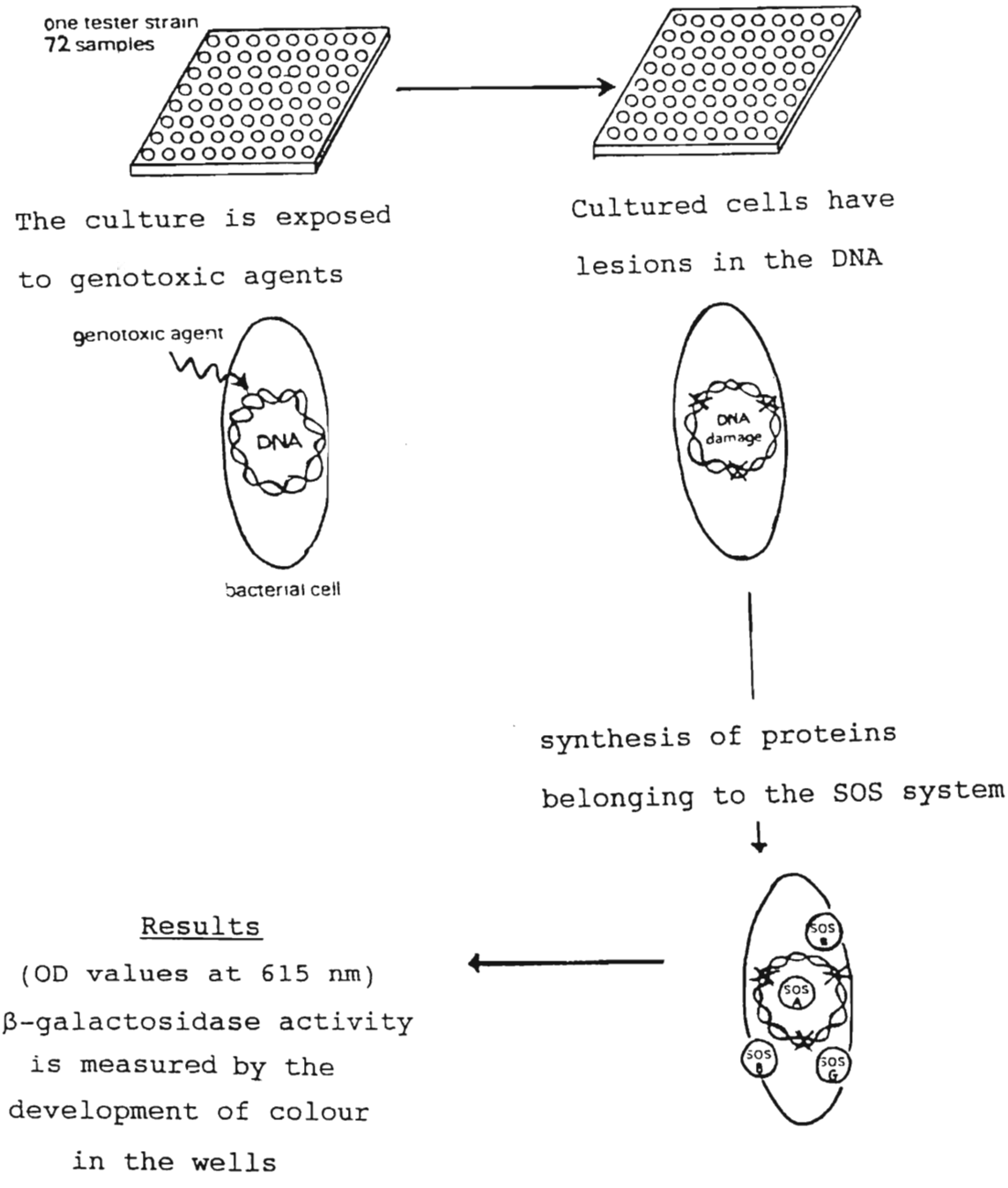


Figure 4.11 A schematic outline of the SOS Chromotest showing the test procedure and the corresponding cellular events[72].

at 615 nm, and blanking the spectrophotometer on the negative control solutions. Alternatively, a visual analysis can be performed. Included in the kit is a blue colour scale with approximate OD values at 615 nm. A comparison of the colour of the solutions in the test wells to that of the colour scale will give an indication of the mutagenicity.

Some samples are also toxic to the bacteria (they cause cell death) and this could result in false negatives. To correct for this, a method for determining the toxicity of the sample is included in the assay. This involves the measurement of the enzymatic activity of alkaline phosphatase, an enzyme not under the control of the SOS repair system. If a sample is toxic, this enzyme is produced and the addition of a chromogen substrate allows detection of the enzyme by a colour reaction.

A simultaneous determination of the mutagenicity and toxicity of the samples is possible by adding the blue chromogenic substrate and the alkaline phosphatase substrate at the same time. Incubation leads to the development of a green colour in the wells. The mutagenicity is determined by measuring the OD at 615 nm and the toxicity by measuring the OD at 405 nm. A simultaneous visual analysis is not possible.

4.2.1.2. Calculation of mutagenicity

The mutagenicity of the sample is calculated by determining what is known as the SOS Inducing Potency, or SOSIP, which is obtained from the slope of a plot of the OD of the sample at 615 nm versus the concentration of the sample in µg/ml. The toxicity of the sample may cause deviations from linearity and this is corrected for by calculating a 'correction factor' and multiplying all results by this value. This analysis will be discussed in section 5.2.2.1.

4.2.2. Acquisition of the SOS Chromotest Kit

The SOS Chromotest blue kit was purchased from Fransa in Johannesburg, the agents for Orgenics Ltd in Israel who developed the test kit together with the Institut Pasteur in France.

The kit was stored at 4°C immediately on acquisition and was used before the expiry date. Exposure to light was kept at a minimum due to the lability of some of the solutions. Sterile conditions were not necessary for the test procedure as the incubation periods were too short for any contamination to occur, but all precautions were taken to ensure that the work area was kept as clean as possible.

4.2.3. Contents of the SOS Chromotest Kit

The following solutions and media are provided in the kit in clearly labelled bottles.

Bottle A: Growth medium for the bacteria.

B: The dried bacteria *E. coli* PQ37.

C: 10% DMSO in saline: the SOS Chromotest diluent.

D: 10 µg/ml 4-nitroquinoline oxide in 10% DMSO in saline. The molar mass of 4NQO is 190.16 g/mol.

F: The blue chromogenic substrate.

G: The alkaline phosphatase substrate diluent.

H: The dried alkaline phosphatase substrate.

I: Stop solution. This is added to the wells after incubation of the bacteria, the sample and the chromogenic substrate, to stop the reaction from proceeding further.

P: 1 mg/ml 2-aminoanthracene in DMSO. The molar mass of 2AA is 193.22 g/mol.

DMSO: Pure DMSO is provided for dissolving samples that are insoluble in 10% DMSO in saline.

4.2.4. Preparation of the Bacteria

This procedure was performed as late as possible the evening before the test was to be performed. A bottle of growth medium (A) and the bacteria (B) were aseptically opened. The contents of A were poured into B and mixed. A piece of aluminium foil was sterilized in a flame and used to cover the bottle. The bacteria were incubated overnight at 37°C in an oven.

The bacterial suspension used in the test was required to have a final OD of 0.05 at 600 nm. In order to obtain this bacterial density, the bacteria were removed from the oven, diluted in a sterile test tube with one bottle of fresh medium (A), and the OD of the suspension measured at 600 nm against fresh medium in a 1 cm light path cuvette. The volume of the suspension required to obtain 10 ml of bacterial suspension with a final OD of 0.05 at 600 nm could then be calculated from equation 4.1:

$$\text{Required volume} = 0.5/\text{OD of suspension} \quad ..(4.1)$$

The calculated required volume of suspension was dispensed into a clean sterile test tube and made up to 10 ml with fresh medium. These bacteria were used for testing without S9 activation.

To prepare the bacteria for testing with S9 activation, the required volume was dispensed into a sterile test tube, 2.5 ml freshly prepared S9 mix (see section 4.2.5 for preparation) added, and the volume completed to 10 ml with fresh medium.

4.2.5. Preparation of S9 Mix

The S9 fraction required in the S9 mix was prepared from the livers of rats induced with Arochlor 1254 as described in section 4.1.6 of the Ames test. The volumes of each component required to prepare the S9 mix are given in Table 4.15. The S9 mix was prepared on the day of the assay and any remainder was discarded.

Table 4.15 The composition of the S9 mix used in the SOS Chromotest.

Component	Volume /ml
1.65 M KCl	0.1
0.4 M $MgCl_2$	0.1
0.5 M Glucose-6-phosphate	0.05
0.1 M Nicotine amide dinucleotide phosphate (NADP)	0.1
0.2 M tris HCl pH 7.4	2.5
Milli-Q water	2.05
S9 fraction	0.2

4.2.6. Preparation of Standards

Bottle D contained a 10 µg/ml solution of 4-nitroquinoline oxide (4NQO) dissolved in 10% DMSO in saline in a microfuge tube and this was used as the first dilution for the standard plot. Five additional 1 to 1 serial dilutions were prepared from this solution in 10% DMSO (bottle C) as shown in Table 4.16.

Bottle P contained a 1 mg/ml solution of 2-aminoanthracene (2AA) in a microfuge tube. This was diluted with 90 µl of diluent from bottle C in order to obtain the first dilution for the standard plot. Five additional 1 to 1 serial dilutions were prepared in 10% DMSO in saline (see Table 4.16). Since 2AA is very labile, the solution was only used if amber to brown in colour.

Table 4.16 Preparation of the standard solutions by serial dilutions in 10% DMSO in saline.

Dilutions	4NQO / (µg/ml)	2AA / (µg/ml)
1	10	100
2	5	50
3	2.5	25
4	1.25	12.5
5	0.625	6.25
6	0.313	3.13

Before preparing the standard solutions, the microfuge tubes were first centrifuged briefly to concentrate the liquid in the bottom of the tubes.

4.2.7. Preparation of the Samples

All sample solutions were prepared on the day of the assay. The sugar cane ash and toll booth soot were extracted into DCM as described in section 2.3 and evaporated to dryness in preweighed flasks. The crude residues were then redissolved in DMSO to obtain stock solutions and from these solutions, 15 serial 1 to 1 dilutions of the samples were prepared. Dilutions were prepared initially in 10% DMSO in saline and then also in 100% DMSO when it was observed that the samples were not completely soluble in 10% DMSO and tended to precipitate at the higher concentrations. The disadvantage of using 100% DMSO is that only 1 to 3 µl of the sample can be tested in a well as opposed to 10 µl if in 10% DMSO, because the higher concentration of DMSO is toxic to the bacteria. Tests were performed with the samples dissolved in both these diluents and the results compared.

As in the Ames test, the test was performed a few times before the ideal concentration range for testing was obtained. The optimization of the

testing conditions is presented in section 4.2.9.

Sample blanks contained only the bacteria, S9 mix and the diluent (i.e. either 10% DMSO in saline or 100% DMSO).

4.2.8. Preparation of the Colour Scale

In order that a visual analysis could be performed, the colour scale provided in the kit had to be prepared. The scale consisted of a strip of 8 wells with varying degrees of blue dye painted on the bottom of the wells. To each well, 100 µl of fresh medium (A) and 100 µl of milli-Q water were added and mixed to obtain blue colours of varying degrees of intensity. Approximate OD values at 615 nm were assigned to each well ranging from 2.0 (most intense) to 0 (colourless); i.e. 2.0, 1.75, 1.5, 0.75, 0.5, 0.1, 0.05 and 0.

4.2.9. Sample Testing

Initially a visual analysis of the SOS Chromotest was attempted.

The bacteria were grown up overnight and diluted as described in section 4.2.4 and the optical density at 600 nm measured. A reading of 0.109 absorbance

units was obtained and the "required volume" was calculated as given in equation 4.1:

$$\text{Required volume} = 0.5/0.109$$
$$= 4.59 \text{ ml}$$

Therefore, 4.59 ml of the bacterial suspension was diluted to 10 ml for testing without S9 activation; and 2.5 ml S9 mix was added to 4.59 ml suspension and diluted to 10 ml for testing in the presence of S9 activation.

Stock solutions of the sugar cane ash and toll booth soot extracts were prepared in DMSO to give concentrations of 1 002 µg/ml. Serial dilutions of the stock solutions were made by taking 0.1 ml and diluting to 0.2 ml in 10% DMSO in saline to give a concentration of 500 µg/ml; 0.1 ml of this solution was then taken and diluted to 0.2 ml (10% DMSO in saline) to give a concentration of 250 µg/ml. This was repeated a further 12 times until final concentrations of 0.05 µg/ml were obtained. The standards were prepared as described in section 4.2.7.

The SOS Chromotest was performed in a microplate which consisted of 12 columns (labelled 1 to 12) and 8 rows (labelled A to H), and therefore 96 wells. A

diagram is shown in Figure 4.12. The samples were dispensed into the microplate by pipetting 10 µl into each well as shown in Table 4.17. Columns 1 to 6 were for testing without S9 activation and columns 7 to 12 were for testing with the S9 mix. Using micropipettes, 100 µl of the bacteria was added to each well ensuring that columns 7 to 12 obtained the bacterial suspension containing the S9 mix. Column 1 contained the dilutions of 4-nitroquinoline oxide and column 7 the dilutions of 2-aminoanthracene. Columns 2, 3, 8 and 9 contained the sugar cane extract and columns 4, 5, 10 and 11 contained the toll booth soot extract dilutions. Row H was kept free for the negative control, i.e. each well in this row contained only the bacteria and the diluent. In the first test that was performed, the 2-aminoanthracene solution was clear in colour and could therefore not be used. The samples were therefore only tested without S9 activation.

Once the bacteria were added, the plate was incubated at 37°C for 2 hours in an oven. After this time, the plate was removed and 100 µl of blue chromogen substrate from bottle F was added to each well. Since a visual analysis was being performed, the alkaline phosphatase substrate was not added. The plate was incubated for a further 90 minutes after which time a blue colour had developed in the

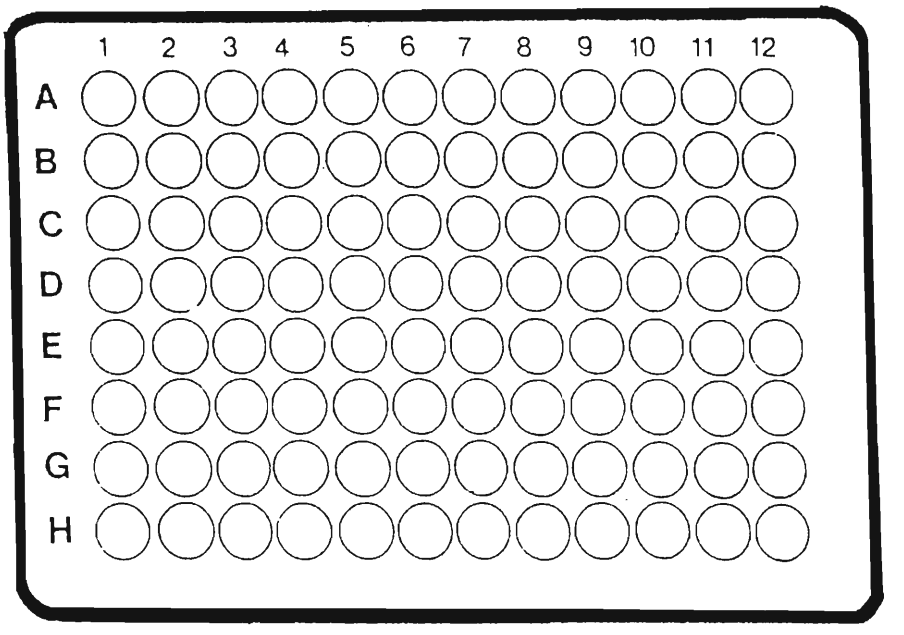


Figure 4.12 A microplate used in the SOS Chromotest.

Table 4.17 A typical outline of the SOS Chromotest, showing sample concentrations.

Without S9 Activation

	4NQO µg/ml	Sugar µg/ml	Sugar µg/ml	Toll µg/ml	Toll µg/ml	
	1	2	3	4	5	6
A	10	1002	4	1002	4	
B	5	500	2	500	2	
C	2.5	250	1	250	1	
D	1.25	125	0.5	125	0.5	
E	0.625	63	0.25	63	0.25	
F	0.313	32	0.13	32	0.13	
G	blank	16	0.06	16	0.06	
H	blank	8	blank	8	blank	blank

With S9 Activation

	2AA µg/ml	Sugar µg/ml	Sugar µg/ml	Toll µg/ml	Toll µg/ml	
	7	8	9	10	11	12
A	100	1002	4	1002	4	
B	50	500	2	500	2	
C	25	250	1	250	1	
D	12.5	125	0.5	125	0.5	
E	6.25	63	0.25	63	0.25	
F	3.13	32	0.13	32	0.13	
G	blank	16	0.05	16	0.06	
H	blank	8	blank	8	blank	blank

wells. However, on comparison of these to the blue scale in the kit, all wells had an approximate OD at 615 nm of 0.1 absorbance units. A higher concentration range of samples was therefore necessary.

Stock solutions of concentration 10 000 µg/ml were prepared of both the sample extracts. Serial dilutions were prepared in a similar manner as explained before so that concentration ranges of 10 000 to 0.13 µg/ml could be tested. Again a blue colour similar to the blank was obtained in all the wells except for the three highest concentrations. It was then thought that the fact that the samples were not completely soluble in 10% DMSO in saline could account for the negative results obtained. Therefore tests were performed with the samples dissolved in 10% DMSO in saline and 100% DMSO. A few positive results were obtained, but it was very difficult to accurately compare the colours in the wells to the standards, blanks and colour scale. An example of the results obtained for the toll booth extract is given in Table 4.18. The OD values are approximate values at 615 nm obtained by comparing the colours to the blue colour scale.

Table 4.18 Approximate OD values at 615 nm obtained from the visual analysis of the toll booth extract.

4NQO		Toll Booth Extract		
Conc./ (µg/ml)	OD	Conc./ (µg/ml)	OD 10% DMSO	OD 100% DMSO
10	2.0	10 000	0.1	0.75
5	2.0	5 000	0.25	0.75
2.5	1.5	2 500	1.0	0.75
1.3	1.0	1 250	1.0	0.75
0.6	0.75	625	0.75	0.5
0.3	0.5	312.5	0.5	0.5
blank	0.1	156	0.5	0.25
		78	0.5	0.25
		39	0.25	0.25
		19.5	0.5	0.25
		9.8	0.5	0.25
		4.9	0.5	0.25
		2.4	0.5	0.25
		1.2	0.5	0.25

As is evident from these results, a more accurate means of measuring the OD at 615 nm was required in order to obtain meaningful results.

The next test was performed on the same sample dilutions in both 10% DMSO in saline and 100% DMSO, but the optical densities of the solutions were

measured using a LKB Ultrospec IIE single beam spectrophotometer and a 50 µl cuvette of 1 cm light pathlength. A simultaneous determination of the mutagenicity and toxicity was performed. The bacteria were grown up overnight and diluted as described previously. The standard and sample dilutions were dispensed into the wells of the microplate (10 µl of the samples in 10% DMSO in saline and 3 µl of the samples in 100% DMSO), 100 µl of the appropriately prepared bacteria added and the plate incubated for 2 hours at 37°C. The blue chromogenic substrate (bottle F) was added to the alkyl phosphatase substrate (bottle H) and mixed well. The plate was removed from the oven and 100 µl of the mixture added to each well. The plate was returned to the oven for a further 90 minutes until a green colour developed in the wells. A volume of 50 µl of the stop solution (bottle I) was added to each well to halt the reaction process. The optical density of the standards and solutions were read at 615 nm against the blank wells to determine the β-galactosidase activity and hence the mutagenicity. The absorbance was also read at 405 nm to determine the toxicity of the samples, but no results could be obtained as the absorbance of the blank solution at this wavelength was so high that the spectrophotometer could not distinguish between it and the absorbance of the samples. No colour scale

was observed for 2-aminoanthracene which indicated that either the standard had undergone a chemical change or that the S9 mix was not functioning properly. For this reason, no results were obtained for the samples in the presence of the activating system. Results for 4NQO are given in Table 4.19 and the results for the toll booth and sugar cane extracts are given in Table 4.20.

Table 4.19 OD values at 615 nm obtained for 4-nitroquinoline oxide.

Conc./ (µg/ml)	Optical density
10	>3
5	>3
2.5	>3
1.25	2.154
0.625	0.685
0.313	0.266

Graphs of OD at 615 nm versus concentration (in µg/ml) could be plotted for 4NQO and the toll booth extract, both in 10% DMSO in saline and 100% DMSO, but the readings from the sugar cane ash extract were too close to one another and too erratic to produce a meaningful plot.

Table 4.20 OD values at 615 nm obtained for the sugar cane and toll booth extracts in both 10% DMSO in saline and 100% DMSO (without S9 activation).

Conc./ (µg/ml)	OD for Toll booth extract		OD for Sugar cane extract	
	10%DMSO	100%DMSO	10%DMSO	100%DMSO
10000	-0.15	0.583	-0.199	0.034
5000	0.2	0.372	-0.68	0.071
2500	0.547	0.211	-0.012	0.056
1250	0.356	0.158	0.026	0.089
625	-0.18	0.105	0.017	0.093
313	0.136	0.033	0.004	0.128
156	0.213	0.024	0.003	0.048
78	0.088	-	-0.094	-
39	0.064	-	-0.005	-
19.5	-0.02	-	0.017	-
9.8	-0.015	-	0.023	-
4.9	-0.061	-	0.009	-
2.4	-0.04	-	0.015	-
1.2	-0.025	-	-0.026	-

The analysis of these graphs (i.e. the calculation of SOSIP) and a discussion of the results will be presented in section 5.2.2.

4.3. THE TOXI-CHROMOTEST

The Toxi-Chromotest is a bacterial colorimetric assay kit for the determination of toxicity that utilises a highly permeable mutant of the *E. coli* strain[73].

4.3.1. Basis of the Toxi-Chromotest Kit

The bacteria are firstly exposed to stressing conditions, after which they are mixed with a cocktail that contains factors essential for the recovery of the bacteria from the stressed conditions and also a specific inducer for the enzyme β-galactosidase. If the cells have the ability to recover from the stress, this enzyme will be produced. The bacteria, together with the reaction cocktail are incubated for 2 hours at 37°C with varying concentrations of the samples being tested. All of these are pipetted into the wells of a microplate. After this period, the plate is removed from the oven, a blue chromogenic substrate added and the plate incubated for a further 90 minutes, during which time a blue colour should develop in the wells. If no colour is apparent, the plate is incubated for a longer period. The addition of the blue chromogenic substrate detects the activity of β-galactosidase by the formation of an easily detectable colour, which can be measured

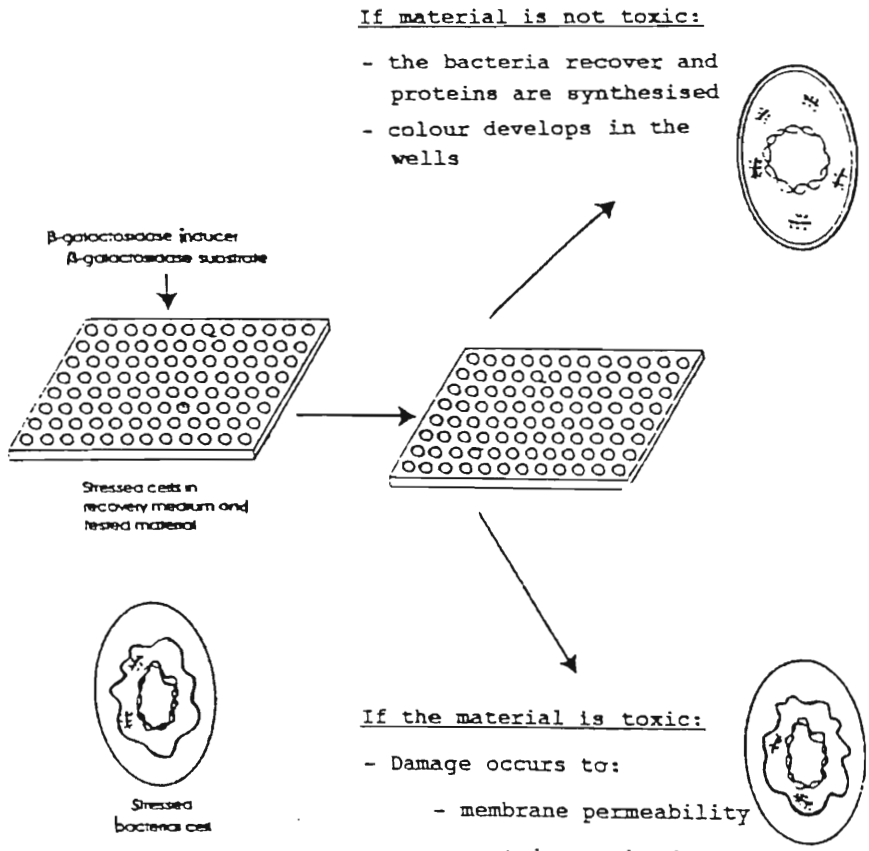
visually or by means of a spectrophotometer at 615 nm. If the substance being tested is toxic, it interferes with the ability of the cells to produce the enzyme thereby resulting in decreased colour formation. A schematic outline of the test is shown in Figure 4.13.

As in the SOS Chromotest, both negative and positive controls are included in the assay. Negative controls consist of wells to which only the sample diluent, the bacteria in the reaction cocktail mixture and the chromogenic substrate are added. The positive control is a 4 µg/ml solution of mercury chloride in water which is a known toxicant for the bacterial strain.

4.3.2. Analysis of the Results

Analysis of the toxic activity of the samples can be performed either visually or instrumentally. A positive toxic result is a colour density in the sample wells below that in the negative control wells.

Firsly, the colour in the wells of the standard toxicant, $HgCl_2$, is observed. The colour should appear in gradation, with the most colour in the negative control well (i.e. the least toxic). If



If material is mildly toxic:

- colour develops in proportion to toxicant dilution

If material is highly toxic:

- no colour develops in the wells

Figure 4.13 A schematic outline of the Toxi-Chromotest showing the test procedure and the corresponding cellular events[74].

this is not observed, it is an indication that the bacteria are not functioning properly and all results obtained are invalid.

For visual analysis, the colour densities in the sample wells are assigned + and - signs (i.e. =, -, +-, +, ++, +++, etc.) and compared to values obtained for the standard.

For instrumental analysis, the absorbance, or optical density, is measured at 615 nm with the spectrophotometer blanked on the wells containing the cocktail mixture, diluent and chromogenic substrate. A plot of OD at 615 nm versus concentration in µg/ml is then made. The toxicity of the sample causes the OD to be smaller than that of the wells without the sample (negative controls) and can be calculated from equation 4.2.

$$\% \text{ Toxicity} = [0.1 - (\text{OD sample}/\text{OD control})] \times 100 \quad \ldots.(4.2)$$

From this, the Minimal Inhibitory Concentration (MIC) is calculated. This is defined as the concentration of sample that causes 20% toxicity.

4.3.3. Acquisition of the Toxi-Chromotest Kit

The test kit was obtained from Fransa in Johannesburg, the agents for the manufacturer Orgenics Ltd in Israel.

4.3.4. Contents of the Toxi-Chromotest Kit

Each test kit contains sufficient solutions for 4 test procedures. On obtaining the kit, it must be stored at 4°C and used within 6 months. All solutions are supplied in clearly labelled bottles as follows:

Bottle A: This contains the reaction mixture - a cocktail that contains the co-factors necessary for the recovery of the bacteria from their stressed conditions, and the inducer for β-galactosidase.

B: This contains the lypholised bacteria - a highly permeable strain of *E. coli*.

C: This contains the solution required to re-hydrate the bacteria.

D: 4 µg/ml solution of mercury chloride in water - the standard toxicant.

E: Not supplied in the blue Toxi-Chromotest kit.

Bottle F: Blue chromogenic substrate.

G: Double distilled water - the diluent for the standard and samples.

Each time a test is performed, one bottle of A, B, C and F are used and any remaining solutions are discarded. Only one bottle of $HgCl_2$ is supplied and is used for all test procedures. Similarly, two bottles of the diluent (G) are supplied and used for all tests.

4.3.5. Preparation of Samples

The sugar cane ash and toll booth soot extracts were dissolved in DMSO to give stock solutions of concentration 10 000 µg/ml. The dilutions of the samples were made in the wells of the microplate and are described in the following section.

4.3.6. The Test Procedure

A typical test layout is shown in Table 4.22. As in the SOS Chromotest, the Toxi-Chromotest is carried out in a microplate consisting of 12 columns (labelled 1 to 12) and 8 rows (labelled A to H). Refer to Figure 4.12 for a diagram of the microplate. The first column in the microplate was used for the blank, i.e. only the reaction mixture

(bottle A), the diluent (bottle G) and the chromogenic substrate (bottle F). Column 2 was used for the standard toxicant, while columns 3 and 4 were used for the toll booth extract, and columns 5 and 6 for the sugar cane extract. Each well in the last row, row H, was used as a negative control, i.e. the reaction mixture and bacteria, the diluent and the chromogenic substrate.

A volume of 100 µl of the sample diluent (bottle G) was dispensed into each well of the microplate apart from wells A_2, A_3 and A_5. A volume of 200 µl of the standard toxicant, $HgCl_2$, was micropipetted into well A_2. From this well, 100 µl was transferred to well B_2 and mixed. Again, 100 µl was removed, transferred to well C_2 and mixed. This procedure was repeated in all the wells of column 2 until G_2, where 100 µl was removed and discarded. The toll booth and sugar cane extracts were prepared in a similar manner, and initially 200 µl of the extracts were pipetted into wells A_3 and A_5 respectively. Serial dilutions were made as for $HgCl_2$, and finally 100 µl of the extracts were discarded from wells G_4 and G_6 respectively. Row H was kept for the negative controls. The reaction mixture (A) was dispensed into each well of the first column to serve as the blank.

Table 4.22 Layout of the Toxi-Chromotest showing the concentration range of the samples that were tested.

	Blank 1	$HgCl_2$ µg/ml 2	Toll µg/ml 3	Toll µg/ml 4	Sugar µg/ml 5	Sugar µg/ml 6
A	blank	4	10 000	78	10 000	78
B	blank	2	5 000	39	5 000	39
C	blank	1	2 500	19.5	2 500	19.5
D	blank	0.5	1 250	9.8	1 250	9.8
E	blank	0.25	625	4.9	625	4.9
F	blank	0.13	313	2.5	313	2.5
G	blank	0.06	156	1.25	156	1.25
H	blank	N/C*	N/C	N/C	N/C	N/C

* N/C refers to negative control

The bacteria were rehydrated by adding the rehydrating solution (bottle C) to the bacterial preparation (bottle B) and mixed by shaking gently. Both bottles were kept cold prior to the addition. The mixture was kept at room temperature for 15 minutes, then 1 ml of the suspension was transferred to the reaction mixture in bottle A. This new reaction mixture (100 µl) was pipetted into each well of the plate except for the wells in the first column. The plate was incubated for 2 hours at 37°C. After this time, the plate was removed from the oven and 100 µl of the blue chromogenic substrate (bottle

F) was added to each well. The plate was incubated for a further 90 minutes after which time a blue colour should have appeared in the wells. After 90 minutes, this change was not observed and the plate was incubated for another hour. After this period, there was still no colour develpment in the wells including those for the standard toxicant. It was therefore assumed that the bacteria were not functioning properly and the test was abandoned.

Due to the high cost (approximately R 600.00) of the Toxi-Chromotest, further toxicity tests were not performed as it was felt that the information provided by this test did not warrant the extra expense. In addition, toxicity results are attainable from the SOS Chromotest kit.

CHAPTER 5

RESULTS AND DISCUSSION

The aim of the work carried out in this thesis was two-fold; firstly to determine, by various analytical techniques, whether PAHs are present in the organic extracts of the ash remaining after the burning of sugar cane crops and in the soot deposited on toll booths by vehicle emissions and secondly to investigate the mutagenicity of these extracts. The experimental techniques are described in detail in Chapters 2, 3 and 4. Briefly, the samples were collected on site (i.e. off a sugar cane field immediately after burning and by scraping toll booths), extracted into an organic solvent, fractionated or subjected to sample clean-up on Florisil, and analysed by UV, TLC, GC-MS and HPLC. The crude organic extracts were dissolved in DMSO and subjected to mutagenicity testing in the Ames test and the SOS Chromotest.

This chapter will present and discuss the results obtained for both the determination of PAH content and the mutagenicity tests of the sample extracts.

5.1. THE DETERMINATION OF PAH CONTENT

The analytical techniques employed to determine whether PAHs are present in the sample extracts included UV, TLC, GC-MS, and HPLC with both UV and fluorescence detection. The use of UV spectroscopy did not allow for the positive identification of PAHs in the samples, but did give an indication that perhaps these compounds were present since strong absorbance was detected in the wavelength range in which PAHs absorb (240-300 nm). TLC proved to be ineffective in separating the standard PAHs from one another and was therefore not pursued as a suitable analytical technique. Since the flame ionisation detector (FID) used in GC-MS is not a selective detector, any PAHs present in low concentrations in the extracts were masked by peaks due to the presence of other hydrocarbons. Pyrene was tentatively identified in the sugar cane extract, but in order that this technique be useful, a far more efficient sample clean-up routine needs to be developed. The technique that did prove to be effective in the identification of PAHs was reverse phase HPLC with fluorescence and UV detection. These results are discussed in the following sections.

5.1.1. HPLC with Fluorescence Detection

Samples of the extracts of the sugar cane ash collected off the fields and from the burning of

leaves at the laboratory, and extracts of the soot scraped from the inside of the toll booths and the surrounding areas, were sent to Lancaster University in the United Kingdom for analysis by HPLC using fluorescence detection. The ash and soot were extracted into DCM and fractionated as described in section 2.4 to give crude, acidic, basic, neutral, aliphatic, aromatic and oxygenated fractions. These extracts were evaporated to dryness and sent to Lancaster University, where they were reconstituted in DCM and injected into the HPLC. The sample chromatograms were compared to a chromatogram of the standard mixture and various PAHs identified (see Figure 3.4 in section 3.5.1). Tables 5.1 and 5.2 list the PAHs identified in the fractions of the cane ash (from the field and the laboratory) and the toll booth soot (inside and outside) respectively.

The numerical values shown in the tables are reported as ng PAHs per sample extract and were determined by quantifying the sample against the standard mixture based on peak areas (the standard mixture consisted of 1.25 ng of each PAH). Unfortunately, the exact masses of the samples are not known as it was not initially intended to perform a quantitative analysis, but they were approximately 0.1 g. Therefore, to obtain an idea of the mass of PAHs present per gram sample extract,

these values must be multiplied by a factor of ten. No solvent blanks were supplied for comparison, therefore these figures cannot be taken as absolute values as they may have contributions from other sources such as solvent impurities.

For both the cane ash and toll booth soot, no PAHs were detected in the acidic or basic fractions, possibly due to the low concentration of PAHs in these portions. In addition, the substances usually associated with these fractions[42], such as organic, sulfonic and sulphinic acids and phenols in the acidic fraction, and amines and N-heterocyclic compounds in the basic fraction, were not present in the standard mixture used for comparison.

On comparing the results obtained for the cane leaves burnt in the laboratory (Table 5.1(b)) and those for the ash collected off the field (Table 5.1(a)), it is evident that in the laboratory sample fewer PAHs are present and the concentrations are generally lower. This is thought to be due to the different degree of combustion that had taken place. Since a cane field is burnt very quickly, only partial combustion of the leaves takes place, whereas in the controlled situation (i.e. in the steel drum) the leaves were burnt completely, resulting in an ash consisting chiefly of carbon.

Table 5.1 PAHs identified in sugar cane ash by HPLC with fluorescence detection.

(a) Ash collected off the fields (i.e samples 2 and 3).

PAH	Crude	Neut	Aliph	Arom	Oxy
Acenaphthene	1970.4	1378.5	51.4	40.1	0.0
Fluorene	4343.2	2665.5	190.5	40.6	0.0
Phenanthrene	5166.2	3945.2	914.1	231.0	0.0
Anthracene	547.6	376.8	69.6	16.1	0.0
Fluoranthene/ Methyl phenanthrene	2474.4	1046.9	460.1	0.0	0.0
Pyrene	1092.4	950.9	231.3	0.0	0.0
Benzanthracene	542.5	396.8	71.4	0.0	0.0
Chrysene	942.0	1009.2	1773.3	0.0	0.0
Benzo(b) fluoranthene	146.4	105.9	50.1	37.4	0.0
Dibenz(ac) anthracene/ Benzo(k) fluoranthene	118.5	87.6	20.0	55.4	3.8
Benzo(a)pyrene	99.7	97.6	20.2	123.9	7.3
Dibenz(ah) anthracene	0.0	0.0	0.0	0.0	0.0
Benzo(ghi) perylene	71.4	86.2	23.4	70.4	16.0
Coronene	0.0	0.0	0.0	41.1	0.0

(b) Leaves burnt in the laboratory

(i.e. sample 1).

PAH	Crude	Neut	Aliph	Arom	Oxy
Acenaphthene	37.4	0.0	19.5	54.3	0.0
Fluorene	0.0	0.0	62.1	78.8	0.0
Phenanthrene	0.0	0.0	108.2	135.1	0.0
Anthracene	3.8	0.0	0.0	15.5	2.5
Fluoranthene/ Methyl- phenanthrene	397.8	0.0	0.0	0.0	0.0
Pyrene	0.0	0.0	0.0	0.0	0.0
Benzanthracene	0.0	0.0	116.6	0.0	0.0
Chrysene	0.0	0.0	103.9	0.0	0.0
Benzo(b) fluoranthene	7.4	0.0	0.0	19.4	0.0
Dibenz(ac) anthracene/ Benzo(k) fluoranthene	3.5	3.9	5.1	16.2	3.3
Benzo(a)pyrene	0.0	0.0	0.0	67.0	0.0
Dibenz(ah) anthracene	0.0	0.0	0.0	0.0	0.0
Benzo(ghi) perylene	0.0	10.9	0.0	25.7	31.4
Coronene	0.0	0.0	0.0	22.9	14.6

Table 5.2 PAHs identified in the toll booth soot by HPLC with fluorescence detection.

(a) Inside the toll booths.

PAH	Crude	Neut	Aliph	Arom	Oxy
Acenaphthene	0.0	0.0	0.0	145.2	0.0
Fluorene	0.0	0.0	0.0	168.9	0.0
Phenanthrene	489.7	367.9	436.8	158.6	0.0
Anthracene	54.3	35.5	28.9	10.4	0.0
Fluoranthene/ Methyl-phenanthrene	3672.1	1206.1	805.0	0.0	0.0
Pyrene	1217.5	721.0	799.6	0.0	141.1
Benzanthracene	819.4	149.9	270.6	0.0	34.6
Chrysene	988.2	162.7	404.9	0.0	135.5
Benzo(b) fluoranthene	293.3	61.8	213.9	71.7	58.3
Dibenz(ac) anthracene/ Benzo(k) fluoranthene	278.5	49.0	178.0	11.5	8.5
Benzo(a)pyrene	84.4	29.4	59.9	13.8	11.1
Dibenz(ah) anthracene	0.0	0.0	0.0	0.0	0.0
Benzo(ghi) perylene	171.5	33.6	139.9	15.1	75.6
Coronene	128.2	14.3	65.0	0.0	36.2

(b) Outside the toll booths.

PAH	Crude	Neut	Aliph	Arom	Oxy
Acenaphthene	0.0	0.0	0.0	129.0	0.0
Fluorene	0.0	0.0	0.0	99.6	0.0
Phenanthrene	1421.5	405.6	292.7	106.9	0.0
Anthracene	81.8	33.3	27.5	7.0	0.0
Fluoranthene/ Methyl- phenanthrene	2954.5	1288.2	965.4	0.0	0.0
Pyrene	1788.2	778.0	752.4	0.0	122.8
Benzanthracene	989.3	170.7	112.3	0.0	96.4
Chrysene	986.0	336.8	108.4	0.0	0.0
Benzo(b) fluoranthene	164.9	66.7	61.3	11.0	430.0
Dibenz(ac) anthracene/ Benzo(k) fluoranthene	108.2	46.7	42.4	11.6	7.3
Benzo(a)pyrene	60.1	21.9	14.4	31.6	43.9
Dibenz(ac) anthracene	0.0	0.0	0.0	0.0	0.0
Benzo(ghi) perylene	95.8	38.6	37.1	0.0	89.1
Coronene	0.0	15.1	22.9	0.0	35.1

The values obtained for the PAH content of the soot scraped from the toll booths both inside (Table 5.2(a)) and outside (Table 5.2(b)) are fairly comparable to one other. This indicates that the

booth operators are continuously exposed to high levels of various PAHs and demonstrates the importance of developing ways to reduce this harmful exposure.

5.1.2. HPLC with UV Detection

The development of this technique is described in detail in section 3.5.2.2. The sugar cane ash collected from the fields (i.e. samples 1 and 2) and the toll booth soot were ultrasonically extracted into DCM for 2 hours, the extracts filtered, and the volumes reduced by rotary evaporation (see also section 2.3.). These crude extracts were then subjected to a clean-up routine on Florisil columns as described in section 3.5.2.3. The resulting eluents were evaporated to dryness with a rotary evaporator and the residues dissolved in a 50%(v/v) DCM-methanol mixture. These solutions were then filtered through a 0.45 μm filter and injected onto the HPLC. The conditions that were found to give the best separation of the PAHs in the extracts involved the use of a Supelcosil LC-PAH column, 10 μl injection volumes of the standard solutions and sample extracts in 50%(v/v) DCM-methanol, and a gradient elution. The gradient used was Milli-Q water held for 10 minutes, changed over 10 minutes to 35%(v/v) acetonitrile-water, held at this mixture

for 10 minutes and then increased linearly over 20 minutes to 100% acetonitrile. The flow rate was 1.5 ml/minute. Good separation of the peaks was achieved with these conditions and examples of the chromatograms obtained are given in section 3.5.2.2.

The work carried out in this laboratory was intended only as a qualitative analysis and therefore no quantitation of the PAH content of the extracts was performed. Table 5.3 lists the PAHs identified in the sugar cane ash and toll booth soot extracts by HPLC with UV detection. No PAHs were identified in the extract of the cane leaves burnt in the laboratory due to their low concentrations and the decreased sensitivity of UV detection towards PAHs as compared to fluorescence detection.

A summary of all the PAHs identified in both sample extracts is given in Table 5.4. This list is by no means conclusive as other PAHs could be present but not identified in this study because of the limitation in the standards used for comparison.

Table 5.3 PAHs identified in the sugar cane ash (samples 1 and 2) and toll booth soot by HPLC with UV detection.

Sugar Cane Extract	Toll Booth Extract
Anthracene	Anthracene
9-Fluorenone	Fluorene
Chrysene	Fluoranthene
Pyrene	Chrysene
Phenanthrene	Pyrene
Triphenylene	Phenanthrene
2-Methyl anthracene	Triphenylene
Benzo(a)pyrene	2-Methyl anthracene
	Benzo(a)pyrene

Table 5.4 A summary of the PAHs identified in the sugar cane ash and toll booth soot extracts by HPLC with both fluorescence and UV detection.

Sugar Cane Extract	Toll Booth Extract
Acenaphthene	Acenaphthene
Fluorene	Fluorene
Phenanthrene	Phenenthrene
Anthracene	Anthracene
Fluoranthene	Fluoranthene
Methyl phenanthrene	Methyl phenanthrene
Pyrene	Pyrene
Benzanthracene	Benzanthracene
Chrysene	Chrysene
Benzo(b)fluoranthene	Benzo(b)fluoranthene
Dibenz(ac)anthracene	Dibenz(ac)anthracene
Benzo(k)fluoranthene	Benzo(k)fluoranthene
Benzo(a)pyrene	Benzo(a)pyrene
Benzo(ghi)perylene	Benzo(ghi)perylene
Coronene	Coronene
Triphenylene	Triphenylene
2-Methyl anthracene	2-Methyl anthracene
9-Fluorenone	

It is important to note the presence of a number of known carcinogens such as benzo(a)pyrene, fluoranthene, chrysene, benzanthracene, benzo(ghi)perylene and coronene. These extracts would therefore be expected to test positive in any mutagenicity study. The PAHs identified in this study

are comparable to those identified in the analysis of the residue from combusted wood and straw[45-47], e.g. fluoranthene, acenaphthene, anthracene, pyrene, benzo(a)pyrene, coronene and chrysene, and in extracts of vehicle exhaust emissions[23,29,30], e.g. fluoranthene, pyrene, phenanthrene, coronene, benzo(a)pyrene and chrysene.

A number of difficulties were encountered in using HPLC with UV detection for the analysis of PAHs. Due to the complex nature of the samples and the low concentrations of PAHs, there was a large amount of interference from peaks due to other compounds in the extracts which made identification difficult. A much more efficient sample clean-up routine is therefore necessary. In addition, PAHs have much stronger fluorescence spectra than UV absorbances and therefore the technique used in this laboratory was not the ideal method for detecting PAHs.

On comparing the results obtained for the sugar cane ash and toll booth soot (see Table 5.4), it is evident that the same PAHs were identified in both extracts. This does not however mean that similar quantities of PAHs are present in the two extracts. Initially, it was necessary to use a larger mass of sugar cane ash than toll booth soot for ultrasonic extraction in order that sufficiently high concentrations of PAHs be present in the resulting extract, i.e. 15 g ash compared to 0.2 g soot (see section 2.3.1). This would therefore indicate a higher concentration of

PAHs in the toll booth soot. However, quantitation of these results is necessary before any definite conclusions can be drawn as to which sample contains a greater amount of mutagenic compounds.

5.2. SAMPLE MUTAGENICITY

The mutagenicity of the sugar cane ash and toll booth soot extracts was investigated by means of two mutagenicity assays: the Ames test and the SOS Chromotest. Mutagenic activity was observed for both extracts in the Ames test, and for the toll booth extract in the SOS Chromotest. The following sections will deal with a discussion of these results.

5.2.1. The Ames Test

The results obtained from the Ames test (section 4.1.9.2) indicated that the crude extracts of both the sugar cane ash and toll booth soot exhibited mutagenic properties. This mutagenicity can be determined by calculating the slope of the linear region of the dose-response curve to give a value in terms of the number of revertant colonies per μg of sample. Figure 5.1(a) shows the graph obtained for the sugar cane extract and Figure 5.1(b) gives the graphs obtained for test 1 and test 2 for the toll

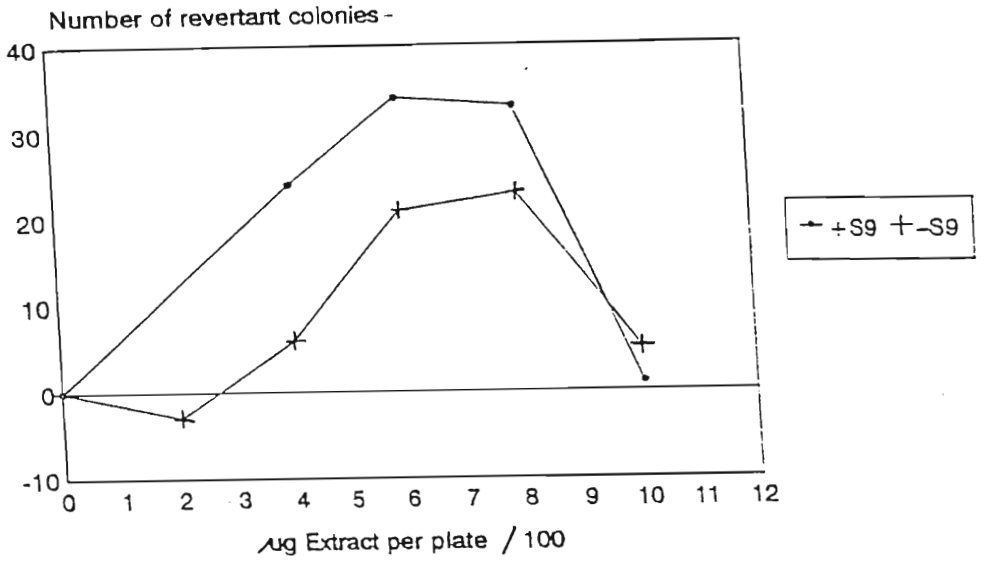
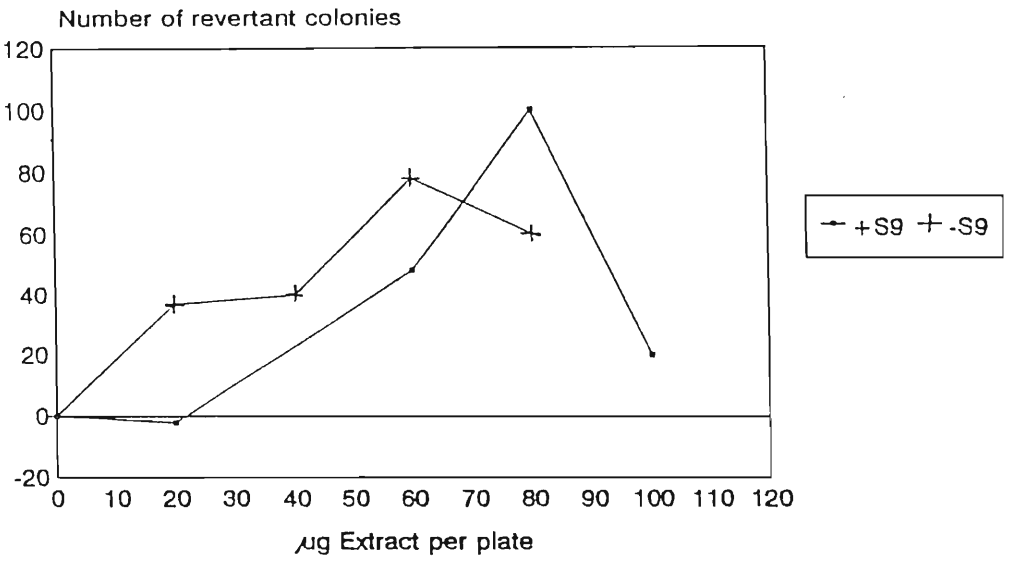


Figure 5.1 (a) The dose-response curve obtained for the sugar cane ash extract with TA 98.



(i)

(ii)

Figure 5.1 (b) The dose-response curves obtained for the toll booth extract with TA 98 for (i) test 1, and (ii) test 2.

booth extract for bacterial strain TA 98 (see also section 4.1.9.2). In order to obtain an indication of the mutagenicity, the slopes of the graphs were calculated by simple linear regression analysis of the linear region of the plots, although there were insufficient points to justify this. Therefore, the number of revertant colonies that were scored at the concentration where toxicity of the samples occurred were ignored (i.e. at 1 000 µg/plate for the sugar cane extract and 100 µg/plate for the toll booth extract in test 1). The values calculated for the slopes are given in Table 5.5.

Table 5.5 The mutagenicity of the sugar cane and toll booth extracts as calculated by linear regression analysis of the dose-response curves.

	Number of Revertants/µg extract	
	+S9	-S9
Sugar cane extract	0.044	0.035
Toll booth extract		
Test 1	1.250	1.185
Test 2	0.254	0.268

Therefore, the mutagenicity of the sugar cane ash

can be expressed as 0.044 (+S9) and 0.035 (-S9) revertants/μg extract, and that of the toll booth soot as 1.25 or 0.254 (+S9) and 1.18 or 0.268 (-S9) revertants/μg extract. The difference in the results obtained for the two tests on the toll booth extract is probably due to a different amount and proportion of mutagenic material being extracted for each test. If an average is taken of the two tests, the mutagenicity is calculated to be 0.75 (+S9) and 0.72 (-S9) revertants/μg extract. These values are comparable to those determined in another study of this type of environmental sample where a mutagenicity of 0.8 revertants/μg sample was reported[76].

However, due to the number of problems experienced in performing the Ames test, the samples were considered to be mutagenic simply if an increase in the number of revertant colonies was observed with an increase in the sample concentration.

The following sections will discuss the effect of sample toxicity and the addition of the S9 mix, the suitability of the tester strains used, and the problems experienced in performing the Ames test.

5.2.1.1. Sample toxicity

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. This decrease is a result of cell death. For the sugar cane extract this toxicity is apparent at approximately 800 µg/plate (see Figure 5.1(a)) and for the toll booth extract at approximately 60 µg/plate in the absence of the S9 mix and 80 µg/plate in the presence of the S9 mix (see Figure 5.1(b)). This therefore indicates that the toll booth soot extract is more toxic than that of the sugar cane ash.

5.2.1.2. Effect of the S9 mix

Addition of the S9 mix to the sugar cane extract resulted in an increase in the number of revertant colonies. This indicated the presence of compounds that require metabolic activation, i.e. promutagens. This result is however, inconsistent with those obtained for the mutagenicity of the residues from wood and straw furnaces where greater mutagenic activity was observed in the absence of S9[45-47]. This difference could be due to the following factors:

(a) a high concentration of promutagens in the extract of the cane ash, and

(b) since most direct acting mutagenicity is attributed to the presence of substituted PAHs such as nitro-PAHs[10,60], PAHs in the other studies could have undergone substitution during the burning process in the furnace or during sample collection on filters and therefore not contributed to the mutagenicity observed in the presence of the S9 mix.

Since the result reported here was obtained from only one successful test, it cannot be taken to be conclusive. Many more tests must be performed before any definite conclusions can be drawn as to the mutagenic activity of the sugar cane extract.

From the slopes of the graphs (see Table 5.5) it appears that for test 1, the mutagenicity of the toll booth extract is greater in the presence of the S9 mix. This result does not appear to be consistent with the other evidence obtained. If the number of revertants obtained at each concentration with and without S9 are compared, the numbers of revertants formed in the absence of S9 are always greater than those formed in its presence (apart from the value obtained at 80 µg/plate). This therefore indicates a greater degree of mutagenicity of the sample without S9. For test 2, the mutagenicity as

calculated from the slopes of the graphs is greater in the absence of S9. 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[10,60]. 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[44]. Again, more tests need to be performed until consistent results are obtained and a definite conclusion can be made as to the mutagenicity of this extract.

5.2.1.3. Suitability of the tester strains

The bacterial strain TA 98 proved to be the most effective strain for the detection of mutagenic activity for both samples investigated. This was to be expected as TA 98 is sensitive towards the detection of frameshift mutagens and the PAHs identified in the extracts (see section 5.1) are such mutagens. Although some mutagenicity was observed with the strain TA 100 for the toll booth extract (see section 4.1.9.2), this result could not be duplicated and no positive results were obtained for the sugar cane extract. This was possibly due to a low concentration of base pair substitution mutagens in the samples.

5.2.1.4. Spontaneous revertants

From what was mentioned in section 4.1.9, it is evident that the number of spontaneous revertants obtained for TA 98 and TA 100 in all the tests were less than those given in Table 4.1 as the accepted range. These latter values can however fluctuate from one laboratory to the next[65], and since the counts obtained in this laboratory were consistent from one test to another and the strains checked positive in the tests for genotypes, these counts were considered to be acceptable.

5.2.1.5. Problems experienced in performing the Ames test

There were numerous problems encountered in performing the Ames Test. The major disadvantage was the lack of experience in working with bacteria in this laboratory and therefore the experimental techniques were under developed. In addition, the conditions in the laboratory used were not ideal for sterile work even though precautions were taken to ensure that the area was frequently cleaned. The incubation period required for the test plates was 2 days at 37°C which presented ideal conditions for other bacteria to grow. These three factors contributed greatly to the problems encountered with

contamination of the bacterial strains both before and during the test procedure.

Problems were also experienced in counting the colonies on the plates. With no experience in microbiology, it was difficult to identify which colonies were *Salmonella* and whether the colonies were single or multiple growths. To try and overcome this problem and exclude any discrepancies between tests, all plates were counted in the same manner.

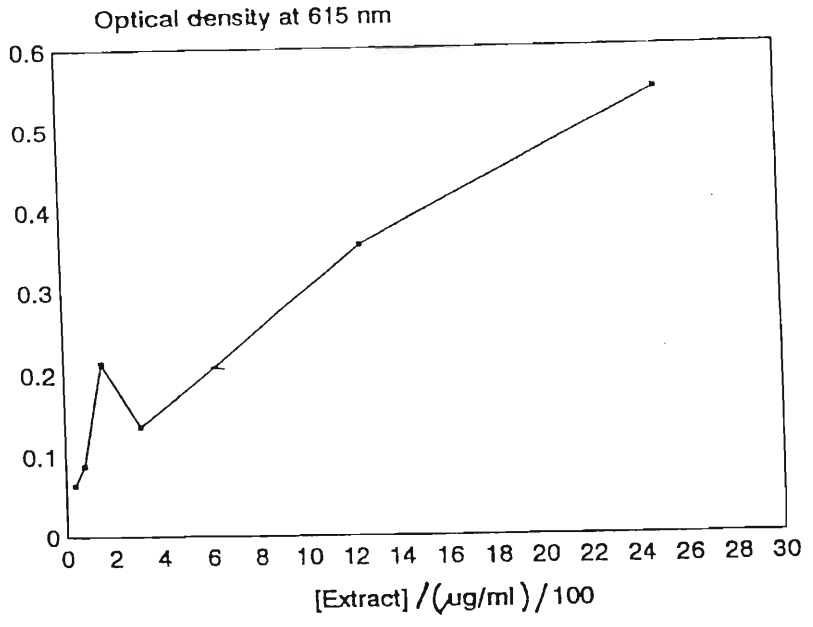
Therefore, although the Ames test is a far more rapid technique for determining mutagenicity than *in vivo* processes and is valuable in terms of assessing the effects of various chemicals, it is evident that it is necessary to have some microbiological experience and sterile laboratory conditions in order to obtain consistent results.

5.2.2. The SOS Chromotest

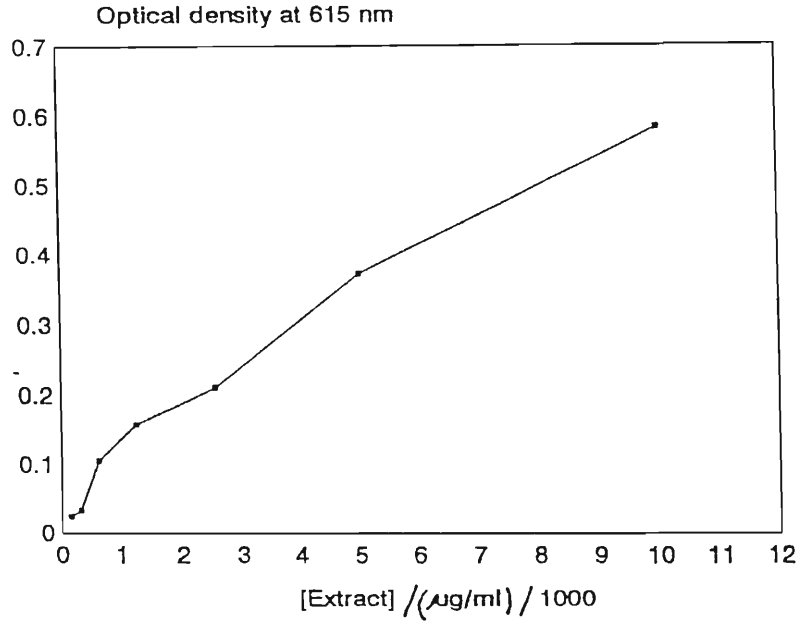
The second test used to measure mutagenicity of the extracts was a new commercially available test kit, the SOS Chromotest. Sample mutagenicity is detected by a colour reaction and measured by determining the optical density (OD) of the solutions at 615 nm. In order to obtain a quantitatitve measure of the

mutagenic activity, the OD results are plotted against the concentration of the sample in µg/ml. The results of the test will now be discussed.

The OD values at 615 nm that were obtained for the toll booth extract (see Table 4.20) were plotted against the concentration of the extract in µg/ml for both solvent media, i.e. 10% DMSO in saline and 100% DMSO, and are shown in Figure 5.2. The results for the standard 4-nitroquinoline oxide (4NQO) were also plotted to give the graph shown in Figure 5.3. No graph was plotted for the sugar cane extract as the OD values were too erratic and close to one another. This possibly arose because the concentration range used was too low for mutagenicity to be detected and that a higher concentration range needed to be tested. This seems consistent with results obtained from the Ames test where for the sugar cane ash extract a concentration range 10 times greater than that of the toll booth extract was necessary in order to obtain a positive mutagenic response. The test on the sugar cane ash extract was not repeated at these higher concentration values because it would have required the purchase of a new test kit which is very costly.



(a)



(b)

Figure 5.2 Plots of OD at 615 nm versus concentration in µg/ml for the toll booth extract in (a) 10% DMSO in saline, and (b) 100% DMSO.

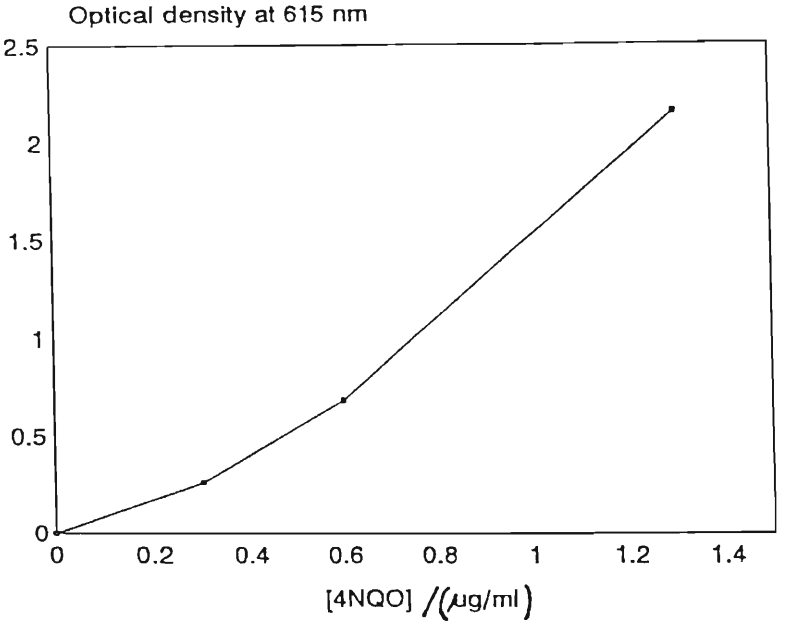


Figure 5.3 A plot of OD at 615 nm versus concentration in µg/ml for the standard mutagen 4-nitroquinoline oxide (4NQO).

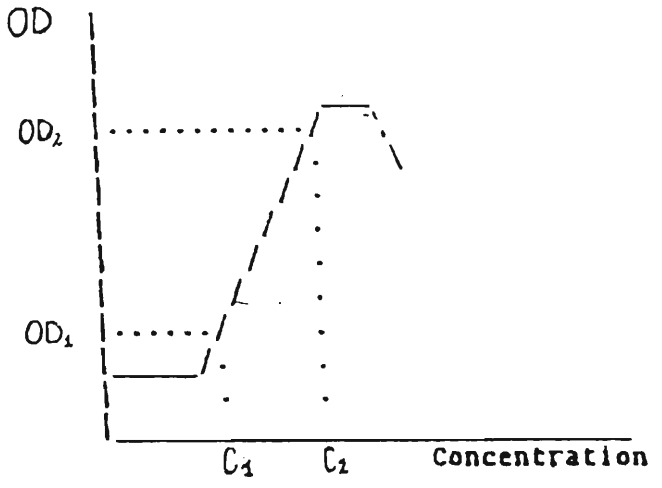


Figure 5.4 A graph of OD versus concentration to demonstrate the calculation of SOSIP[70].

5.2.2.1. Calculation of mutagenicity (SOSIP)

The mutagenicity of the sample is determined by calculating the SOS inducing potency or SOSIP. The OD at 615 nm versus sample concentration (in µg/ml) is plotted as shown in Figure 5.4 and the SOSIP is calculated from the slope of the graph according to equation 5.1:

$$SOSIP = 10 \times (OD_2-OD_1)/(C_2-C_1) \quad ...(5.1)$$

where C is concentration in nanomoles per reaction

well and is converted from µg/ml by equation 5.2:

$$C = (CONC \times vol)/ MM \quad \ldots\ldots\ldots\ldots (5.2)$$

where CONC is the concentration of the sample in µg/ml, vol the volume of the test compound solution in each well in µl, and MM the molar mass of the test compound.

The calculated SOSIP value may change over a period of time due to changing incubation conditions and age of the bacteria, and therefore the values are corrected according to the activity of a known standard such as 4-nitroquinoline oxide. The SOSIP is calculated for 4NQO as in equation 5.1 and divided by 71 to get a "SOSIP correction factor" (the value of 71 is the published result for 4NQO[71]). All SOSIP values calculated for the test samples are then divided by this correction factor to obtain SOSIP values that are comparable to previously published results.

The plots used to calculate SOSIP values may sometimes be non-linear and this needs to be corrected as explained in the following section.

5.2.2.2. Correction for non-linearity

This non-linearity may arise because the toxicity of the test sample interferes with the SOS response. Thus when a non-linear plot is obtained, the OD is corrected for the toxicity of the sample. The correction factor is calculated as in equation 5.3:

$$\text{Correction factor} = OD_0/OD_x \quad \ldots\ldots\ldots(5.3)$$

where OD_0 is the OD of alkaline phosphatase reaction in the absence of the tested sample, and OD_x the OD of alkaline phosphatase reaction at concentration "x" of the tested sample.

To obtain the corrected β-galactosidase OD the observed value is multiplied by this correction factor and the corrected values re-plotted. The SOSIP value is then calculated as described previously in section 5.2.2.1.

5.2.2.3. Calculation of SOSIP for 4NQO

Firstly, the SOSIP for 4NQO was calculated so as to be used as a comparison for the relative mutagenicity of the toll booth extract. The concentration of the standard was first converted to nanomoles per reaction well by using equation 5.2.

and the molar mass of 190.6 g/mol. The values obtained are listed in Table 5.6.

The graph shown in Figure 5.3 was replotted using these values and the SOSIP calculated from equation 5.1:

$$SOSIP = 10 \times (OD_2-OD_1/C_2-C_1)$$

where (OD_2-OD_1/C_2-C_1), the slope of the graph, was determined by simple linear regression analysis of the graph.

Table 5.6 The conversion of concentration in µg/ml to nanomols/reaction well for 4NQO in order to calculate SOSIP.

OD @ 615 nm	Conc./(µg/ml)	Conc./(nanomols/well)
0	0	0
0.266	0.3	1.57×10^{-2}
0.685	0.6	3.15×10^{-2}
2.154	1.3	6.82×10^{-2}

The slope of the graph was found to be 32.44.

Therefore:

$$SOSIP = 10 \times 32.44$$
$$= 324.4.$$

The correction factor was then calculated by dividing the SOSIP value by 71:

$$\text{Correction factor} = 324.4/71$$
$$= 4.57.$$

5.2.2.4. Calculation of SOSIP for toll booth extract

This calculation of SOSIP (as explained in the previous section) was however, inappropriate for the samples under investigation as they were very complex and of unknown molar mass. To compensate for this, the calculations were altered so that SOSIP values were determined in terms of µg/well rather than nanomoles/well. The conversion from µg/ml to µg/well was calculated by multiplying the concentration in µg/ml by the volume of sample added to each well in µl and using the appropriate conversion factors. In order that the SOSIP values calculated for the toll booth extract could be compared to the standard mutagen, the concentration values for 4NQO had to be recalculated. Table 5.7 gives the concentrations in µg/well for 4NQO.

The SOSIP value was then recalculated for 4NQO as shown in the previous section to give a value of 1.702×10^3.

Table 5.8 gives the concentrations in µg/well for the toll booth extract. A new plot was then made of OD of the toll booth extract measured at 615 nm versus extract concentration in µg/reaction well and is shown in Figure 5.5.

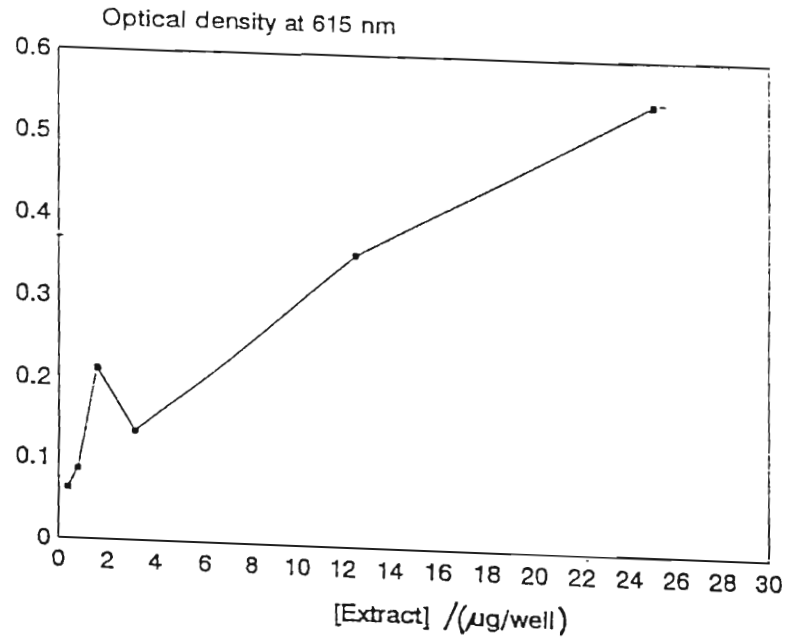
The SOSIP of the extract in both 10% DMSO in saline and 100% DMSO was then calculated as for 4NQO and the results are given in Table 5.9.

Table 5.7 The conversion of concentration of 4NQO in µg/ml to µg/well required to recalculate SOSIP.

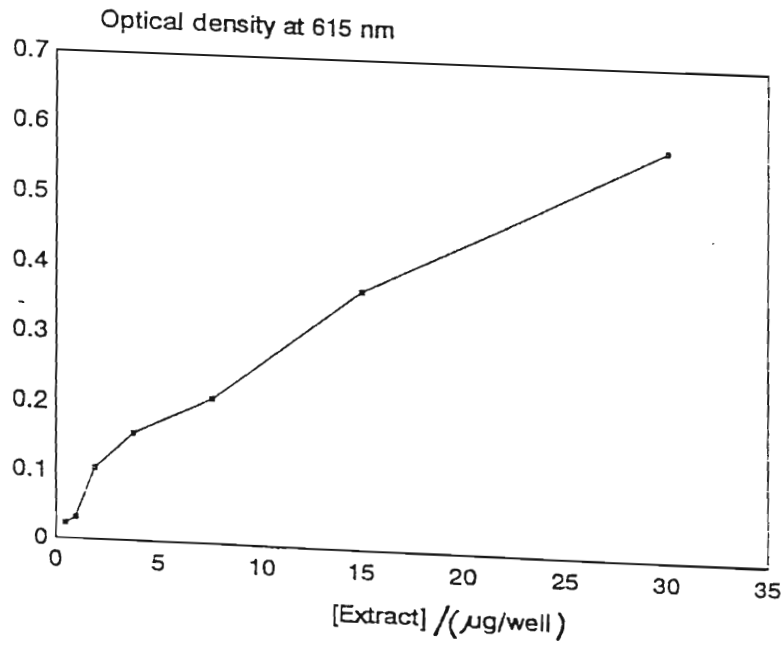
Conc./(µg/ml)	Vol./µl	Conc./(µg/well)
0	-	0
0.3	10	3×10^{-3}
0.6	10	6×10^{-3}
1.3	10	1.3×10^{-2}

Table 5.8 The conversion of concentration in µg/ml to µg/well for the toll booth extract used to calculate SOSIP.

Conc./ (µg/ml)	Conc. in 10% DMSO /(µg/well)	Conc in 100% DMSO /(µg/well)
10 000	100	30
5 000	50	15
2 500	25	7.5
1 250	12.5	3.75
625	6.25	1.88
312.5	3.13	0.94
156	1.56	0.47
78	0.78	0.23
39	0.39	0.18



(a)



(b)

Figure 5.5 Plots of OD at 615 nm versus concentration in µg/reaction well for the toll booth extract in (a) 10% DMSO in saline, and (b) 100% DMSO.

Table 5.9 The calculated SOSIP values for the toll booth extract in 10% DMSO in saline and 100% DMSO.

	10% DMSO	100% DMSO
Slope of graph	1.95×10^{-2}	1.91×10^{-2}
SOSIP	1.95×10^{-1}	1.91×10^{-1}

The SOSIP values calculated for the two solvents are in good agreement.

On comparing the SOSIP value of the standard mutagen, 4NQO, to those of the toll booth extract in 10% and 100% DMSO, it is evident that 4-nitroquinoline oxide is approximately 5×10^3 times more mutagenic. Although this difference seems large, it indicates that the toll booth extract is fairly mutagenic as only a small proportion of the compounds present in the extract exhibit mutagenic properties. Unfortunately, the results obtained for the toll booth soot extract could not be compared to those determined in other studies due to the different method employed in calculating SOSIP (i.e. using µg/well rather than nanomols/well).

5.2.2.5. Problems experienced in performing the SOS chromotest

The major drawback of the SOS Chromotest kit was that the test kits only supplied sufficient solutions for one test procedure. Therefore if any tests had to be repeated, further kits would need to be purchased which leads to high costs.

The 2-aminoanthracene standard provided in the kit as a control for mutagenicity in the presence of S9 was very labile and in this research no results were obtained for the mutagenicity of the samples in the presence of the activating system (S9 mix).

Another drawback was that, although the test was very sensitive and detected mutagenic activity in the toll booth extract at concentrations as low as 0.5 µg/well (or 150 µg/ml), the concentrations of the sugar cane sample were too low for any activity to be detected. In order to test a higher concentration range, a large amount of sugar cane ash would have to be extracted so that a stock solution of concentration greater than 10 000 µg/ml can be prepared. This is not always possible, as in the analysis of such complex environmental samples only limited amounts of sample are usually available.

In preparing the sample dilutions extreme care has to be taken to ensure accurate dilutions as any inaccuracies are detected by the spectrophotometer. This is therefore very time consuming, especially if a large number of samples are to be analysed.

Finally, this test was not suitable for the analysis of such complex samples where the components are largely unknown. The results obtained in this test could not be compared to any other previous test results as all SOSIP values depend on the molar mass of the sample being known.

Therefore, although the Ames test presented a number of problems in terms of lack of experience and contamination, it seems to be more valuable in assessing the mutagenicity of environmental samples than the SOS Chromotest kit. In addition, the Ames test is used extensively for the determination of the mutagenic properties of complex samples and therefore a large amount of documentation is available for comparison. The SOS Chromotest kit is an ideal assay for the determination of the mutagenic properties of pure chemicals, where the molar mass of the sample under investigation is known.

5.3. COMPARISON OF EXTRACTION SOLVENT

The use of both DCM and acetone as extraction solvents was investigated to determine which solvent was more efficient at extracting PAHs and mutagenic material from the environmental samples. Once the HPLC routine had been established, examination of the chromatograms showed that using DCM as the extraction solvent consistently resulted in the identification of PAHs in the samples, but analysis of the acetone extracts did not always indicate their presence. Therefore, in this study, DCM was considered to be the more efficient extraction solvent. Based on this observation, the DCM extracts were expected to give greater mutagenicity results in the Ames test than the acetone extracts. Unfortunately this comparison could not be made as a result of the problems experienced with contamination of the bacteria in the tests performed on the acetone extracts. Therefore, no definite conclusions can be made as to which solvent is more efficient at extracting mutagenic compounds.

5.4. CONCLUSIONS

In this study, the PAH content and mutagenic properties of two environmentally important samples were investigated: the ash remaining after the burning of sugar cane crops and the soot deposited on toll booths by vehicle exhaust emissions. The PAH content was determined by analysing the dichloromethane extracts of each of these samples by means of reverse phase HPLC with both uv and fluorescence detection. The mutagenic activity was investigated by subjecting these extracts to mutagenicity testing in two *in vitro* bacterial assays: the Ames *Salmonella* revertant assay and a new commercially available chromogenic test, the SOS Chromotest.

PAHs were identified in the extracts of both the sugar cane and toll booth samples, a number of which are known carcinogens. Quantification was not attempted at this stage although an indication of the PAH content of various fractions of the extracts was obtained from the HPLC analysis where fluorescence detection was employed. The values obtained show that a number of PAHs are present in fairly high concentrations which stresses the need for further research into this area.

The use of other analytical techniques such as UV spectroscopy, TLC and GC-MS were also investigated for the identification of PAHs in the samples. These techniques

were not sensitive enough to detect the low concentrations of PAHs in the sugar cane ash and toll booth soot and were therefore not pursued.

On subjecting these extracts to mutagenicity testing in the Ames test and the SOS Chromotest, it was evident that both samples exhibited mutagenic properties, the toll booth extract to a greater extent than that of the sugar cane. This observed mutagenicity was, at first, attributed only to the presence of the PAHs identified by comparison to the standard mixtures, the majority of which require metabolic activation in order to express their mutagenicity. However, the observation that the addition of the S9 mix to the toll booth extract resulted in a decrease in mutagenicity suggested the presence of other compounds that did not require the addition of this activating system, i.e. direct acting mutagens which can be activated to their reactive intermediates by the enzymes present in the *Salmonella* bacteria. The compounds that are thought to cause this direct acting mutagenesis are various derivatives of PAHs, especially the nitro-PAHs. The presence of these other compounds indicates the need for their inclusion into the standard mixtures that are used as a comparison for the identification of the components present in environmental samples. Therefore, Table 5.4 (section 5.1) cannot be taken to be a complete indication of the harmful substances present in the samples that were investigated in this study.

Due to the number of problems experienced during the mutagenicity testing of the extracts, the results obtained were not always reproducable. Further testing, with both the Ames test and the SOS Chromotest, is therefore necessary before any definite conclusions can be made as to the mutagenic properties of the samples, especially with regard to the effect of the S9 activating system. Once this has been achieved, the next step would be the quantification of the mutagenic activity of the samples and the determination of the extent to which the PAHs identified in this study are responsible for the observed mutagenicity.

Further research into the effects of sugar cane crop burning should focus on the analysis of smoke particulates produced during the burning process. In addition, the effects of the various fertilizers, the type of burn (i.e. wet or dry) and the breed of cane should be taken into consideration.

It is hoped that this research will provide a starting point for further investigation into the nature and mutagenic properties of the compounds produced during the combustion of sugar cane, and aid in encouraging the development of safer working conditions for toll booth operators.

REFERENCES

(1) Mercier M., Chromatography Symposia Series, 1 (1979) 37-58.

(2) Glusker J., Carcinogens and Carcinogen-DNA Interactions, in *Molecular Structure: Chemical Reactivity and Biological Activity*, Stezowski J.J., Huang J.L. and Shao M.C. (Eds), Oxford Science Publications, (1988) pp 201-220.

(3) Phillips D., Nature, 303:9 (1983) 468-472.

(4) Finlayson-Pitts B.J. and Pitts J.N. Jnr, *Atmospheric Chemistry: Fundamentals and Experimental Techniques*, John Wiley and Sons, New York, (1986) pp 871-958.

(5) Lioy P.J. and Greenberg A., Toxicology and Industrial Health, 6:2 (1990) 209-223.

(6) Phillips D., Chemical Carcinogenesis, in *The Molecular Basis of Cancer*, Farmer P.B. and Walker J.M. (Eds), Croom Helm, London, (1985) pp 135-180.

(7) Frenklach M. and Wang H., Preprints of Papers. American Chemical Society. Division of Fuel Chemistry, 36:4 (1991) 1509-1516.

(8) Lane D., The Fate of Polycyclic Aromatic Compounds in the Atmosphere and During Sampling, in *Chemical Analysis of Polycyclic Aromatic Compounds*, Vo-Dinh T. (Ed), John Wiley and Sons, New York, (1989) pp 31-58.

(9) Schuetzle D., Riley T., Prater T.J., Harvey T.M. and Hunt D.F., Analytical Chemistry, **54** (1982) 265-271.

(10) Pederson T.C. and Siak J.S., Journal of Applied Toxicology, **1:2** (1981) 54-60.

(11) Rosenkranz H., Mutation Research, **101** (1982) 1-10.

(12) Alfheim I., Bjørseth A. and Møller M., Critical Reviews in Environmental Control, **14:2** (1984) 91-150.

(13) Lewtas J., Environmental Health Perspectives, **47** (1983) 141-152.

(14) Hawaiian Sugar Planters Association Report, (1990) 21-25.

(15) Heinz D.J. and How K., Unpublished Hawaiian Sugar Planters Association Data, (1987) 1-5.

(16) Common S., Honours Project, Department of Geographical and Environmental Sciences, University of Natal, Durban, (1990) 35-37.

(17) Hawaiian Sugar Planters Association Report, (1985) 1-7.

(18) Kirchhoff V.W.J.H., Marinho E.V.A., Dias P.L.S., Calheiros R., André R. and Volpe C., Nature, **339** (1989) 264.

(19) Kirchhoff V.W.J.H., Marinho E.V.A., Dias P.L.S., Pereira E., Calheiros R., André R. and Volpe C., Journal of Atmospheric Chemistry, **12** (1991) 87-102.

(20) Kirchhoff V.W.J.H. and Rasmussen R.A., Journal of Geophysical Research, **95:D6** (1990) 7521-7532.

(21) Crutzen P., Heidt L., Krasnec J., Pollock W. and Seiler W., Nature, **282:5736** (1979) 253-256.

(22) Diab R., Common S. and Roberts L.M., Atmospheric Environment, **24A** (1990) 1-6.

(23) Benner B., Gordon G. and Wise S., Environmental Science and Technology, **23**:10 (1989) 1269-1278.

(24) Ulfvarson U., Alexandersson R., Aringer L., Svensson E., Hedenstierna G., Hogstedt C., Holmberg B., Rosén G. and Sorsa M., Scandinavian Journal of Work Environment and Health, **13** (1987) 505-512.

(25) Salmeen I., Pero A., Zator R., Schuetzle D. and Riley T., Environmental Science and Technology, **18** (1984) 375-382.

(26) Holmberg B. and Ahlborg U. (Ed), Environmental Health Perspectives, **47** (1983) 1-30.

(27) François K., Honours Project, Department of Geographical and Environmental Sciences, University of Natal, Durban, (1990) 1-3.

(28) Diab R., Foster S.J., François K. and Salter L.F., An Investigation of Ambient Carbon Monoxide Levels at Mariannhill Toll Plaza, unpublished results.

(29) Schuetzle D., Environmental Health Perspectives, **47** (1983) 65-80.

(30) Schuetzle D., Lee F., Prater T.J. and Tejada S.B., International Journal of Environmental Analytical Chemistry, **9** (1981) 93-144.

(31) Krishna G., Ong T., Whong W.Z. and Nath J., Mutation Research, **124** (1983) 113-120.

(32) Lee H., Law S.M. and Lin S.T., Toxicology Letters, **58** (1991) 56-67.

(33) Zweidinger R.B., Emission Factors from Diesel and Gasoline Powered Vehicles: Correlation with the Ames Test, in *Toxicological Effects of Emissions from Diesel Engines*, Lewtas J. (Ed), Elsevier Science Publishing Co., (1982) pp 83-96.

(34) Petersen B.A. and Chuang C.C., Methodology of Fractionation and Partition of Diesel Exhaust Particulate Samples, in *Toxicological Effects of Emissions from Diesel Engines*, Lewtas J. (Ed), Elsevier Science Publishing Co., (1982) pp 51-57.

(35) Møller M. and Alfheim I., Atmospheric Environment, **14** (1980) 83-88.

(36) Teranishi K., Hamada K. and Watanabe H., Mutation Research, **56** (1978) 273-280.

(37) Tokiwa H., Morita K., Takeyoshi H., Takahashi K. and Ohnishi Y., Mutation Research, **48** (1977) 237-248.

(38) McDow S.R., Giger W., Burtscher H., Schmidt-Ott A. and Siegmann H., Atmospheric Environment, **24A:**12 (1990) 2911-2916.

(39) Schuetzle D. and Lewtas J., Analytical Chemistry, **58:**11 (1986) 1060A-1071A.

(40) Guerin M.R., Ho C.H., Rao T.K., Clark B.R. and Epler J.L., International Journal of Environmental Analytical Chemistry, 8 (1980) 217-225.

(41) Schuetzle D., Jenson T.E. and Ball J.C., Environment International, **11** (1985) 169-181.

(42) Chan T.L., Lee S. and Siak J.S., American Chemical Society, **15:**1 (1981) 89-93.

(43) Hughes T.J., Pellizzari E., Little L., Sparacino C. and Kolber A., Mutation Research, 76 (1980) 51-83.

(44) Ohnishi Y., Kachi K., Sato K., Tahara I., Takeyoshi H. and Tokiwa H., Mutation Research, 77 (1980) 229-240.

(45) Ramdahl T. and Møller M., Chemosphere, 12:1 (1983) 23-34.

(46) Ramdahl T., Alfheim I., Rustad S. and Olsen T., Chemosphere, 11:6 (1982) 601-611.

(47) Nielsen P.A. and Jensen L., Chemosphere, 23:6 (1991) 723-735.

(48) Greenberg A., Wang Y., Darack F.B., Harkov R., Louis J. and Atherholt T., Polycyclic Aromatic Compounds, 1 (1990) 233-250.

(49) Obuchi A., Aoyama H., Ohi A. and Ohuchi H., Journal of Chromatography, 312 (1984) 247-259.

(50) Catalogue of Supelco Chromatography Products and Chemical Standards, (1992) p. 166.

(51) Garrigues Ph. and Bellocq J., Journal of High Resolution Chromatography, 12 (1989) 400-403.

(52) Freifelder D., *Essentials of Molecular Biology*, Jones and Bartlett Publishers, Boston, (1985) pp 172-188.

(53) Freifelder D., *Molecular Biology : A Comprehensive Introduction to Prokaryotes and Eukaryotes*, Jones and Bartlett Publishers, Boston, (1983) pp 339-357.

(54) Devoret R., Bacterial Tests for Potential Carcinogens, in *Readings from Scientific American, Genetics*, W.H. Freeman Company, San Francisco, (1981) pp 290-199.

(55) Ames B.N., Durston W.E., Yamasaki E. and Lee F.D., Proceedings: National Academy of Science, 70:8 (1973) 2281-2285.

(56) McCann J., Choi E., Yamasaki E. and Ames B.N., Proceedings: National Academy of Science, 72:12 (1975) 5135-5139.

(57) Ames B.N., McCann J. and Yamasaki E., Mutation Research, 31 (1975) 347-364.

(58) Alfheim I., Bergström J.G.T., Jenssen D. and Møller M., Environmental Health Perspectives, 47 (1983) 177-187.

(59) Ames B.N., Science, 204 (1979) 587-593.

(60) Wang Y.Y., Rappaport S., Sawyer R., Talcott R. and Wei E., Cancer Letters, 5 (1978) 39-47.

(61) Nachtman J., Xiao-bai Xu., Rappaport S., Talcott R. and Wei E., Bulletin of Environmental Contamination and Toxicology, 27 (1981) 463-466.

(62) Kfir R., Grabow W.O.K. and Hilner C.A., Bulletin of Environmental Contamination and Toxicology, 28 (1982) 641-646.

(63) Grabow W.O.K., Denkhaus R. and Rossum P.G., South African Journal of Science, 76 (1980) 118-123.

(64) McCann J., Hospital Practice (1983) 73-85.

(65) Maron D. and Ames B.N., Mutation Research, 113 (1983) 173-215.

(66) Ames B.N., A Bacterial System for Detecting Mutagens and Carcinogens, in *Mutagenic Effects of Environmental Contaminants*, Sutton H.E. and Harris M.I. (Eds), Academic Press, New York, (1972) pp 57- 66.

(67) De Flora S., Carcinogenesis, **2:4** (1981) 283-298.

(68) *Gradwohl's Clinical Laboratory Methods and Diagnosis*, Volume 2, 8th Edition, Sonnenwirth A.C., Jarett L. (Eds), The C.v. Mosby Company, St. Louis, (1980) p. 1363.

(69) The Merck Index, Budavari S. (Ed), 11th Edition, Merck and Co., (1989) p. 1203.

(70) Quillardet P. and Hofnung M., Mutation Research, **147** (1985) 65-78.

(71) Instructions for Use, SOS Chromotest Blue Kit, Version 5.3, pp 1-22.

(72) SOS Cromotest Advertising Brochure.

(73) Instructions for Use, Toxi-Chromotest Kit, Version 2.1, pp 1-6.

(74) Toxi-Chromotest Advertising Brochure.

(75) Wei E.T., Wang Y.Y. and Rappaport S., Journal of the Air Pollution Control Association, **30:3** (1980) 267-271.

(76) Brooks A.L., Li A.P., Dutcher J.S., Clark C.R., Rothenberg S.J., Kiyoura R., Bechtold W.E. and McClellen R.O., Environmental Mutagenesis, **6** (1984) 651-668.

APPENDIX A

This appendix lists all the chemicals and equipment used in the experimental sections (Chapters 2 to 4) of this thesis.

A.1. MATERIALS

Details of the chemicals used are summarized below in the order: name, chemical grade, supplier and percentage assay.

A.1.1. Chemicals for Extraction and Fractionation

Dichloromethane	(AR)	Saarchem	99,5%
Acetone	(AR)	Kleber	99,5%
Cyclohexane	(LAB)	Saarchem	98,5%
Diethyl ether	(AR)	BDH	95%
n-Hexane	(HPLC)	May and Baker	99%
Benzene	(AR)	BDH	99%
Sodium hydroxide	(CP)	Kleber	98%
Sulphuric acid	(AR)	BDH	95%
Silica gel 60 (230-400 mesh)		Merck	
Milli-Q water*			

A.1.2. Standard PAH Solutions

Acenaphthylene	(AR)	Aldrich	85%
Anthraquinone	(AR)	BDH	99%
Anthracene	(LAB)	BDH	95%
Benzo(a)pyrene	(SIGMA)	Sigma	98%
Chrysene	(SIGMA)	Sigma	95%
Fluoranthene	(AR)	Aldrich	98%
Fluorene	(SIGMA)	Sigma	98%
9-Fluorenone	(AR)	Aldrich	98%
2-Methyl anthracene	(SIGMA)	Sigma	98%
Phenanthrene	(LAB)	Fluka	90%
Pyrene	(SIGMA)	Sigma	98%
Triphenylene	(AR)	Aldrich	98%
Cyclohexane	(AR)	Hopkin and Williams	99.5%
Dichloromethane	(AR)	Saarchem	99,5%
Methanol	(HPLC)	Waters	99,9%

The following PAHs were used as standards for the HPLC analysis at Lancaster University in the United Kingdom. They were purchased individually from Aldrich, Prochem and Sigma as solids.

Naphthalene

Acenaphthene

Fluorene

Phenanthrene

Anthracene

Fluoranthene

1-Methyl phenanthrene

Pyrene

Benzanthracene

Chrysene

Benzo(b)fluoranthene

Dibenz(a,c)anthracene

Benzo(k)fluoranthene

Benzo(a)pyrene

Dibenz(a,h)anthracene

Benzo(g,h,i)perylene

Coronene

A.1.3. Chemicals for UV Spectroscopy

Cyclohexane	(LAB)	Saarchem	98,5%
Acetonitrile	(HPLC)	Waters	99,9%
Dichloromethane	(AR)	Saarchem	99,5%
Methanol	(HPLC)	Waters	99,9%

A.1.4. Chemicals for TLC

Hexane	(HPLC)	May and Baker	99%
Toluene	(AR)	Hopkin and Williams	92%
Acetonitrile	(HPLC)	Waters	99,9%
Methanol	(HPLC)	Waters	99,9%

Milli-Q water*

A.1.5. Chemicals for GC-MS

Dichloromethane	(AR)	Saarchem	99,5%
Methanol	(HPLC)	Waters	99,9%

A.1.6. Chemicals for HPLC

Dichloromethane	(AR)	Saarchem	99,55
Methanol	(HPLC)	Waters	99,9%
Florisil (60-100 mesh)	(LAB)	Saarchem	
Acetonitrile	(HPLC)	Waters	99,9%
Tetrahydrofuran	(HPLC)	Waters	99,9%

Milli-Q water*

A.1.7. Chemicals for Ames Test

Magnesium sulphate	(AR)	BDH	99,5%
Citric acid monohydrate	(AR)	PAL	99%
Sodium ammonium phosphate	(LAB)	BDH	99%
D-biotin	(SIGMA)	Sigma	98%
L-histidine hydrochloride monohydrate	(CCR)	Sigma	98%
Sodium chloride	(LAB)	Saarchem	99%
Potassium chloride	(LAB)	Saarchem	99%
Magnesium chloride	(LAB)	Saarchem	98%

Sodium dihydrogen phosphate	(LAB)	Riedel-de Haën	98,5%
Disodium hydrogen phosphate	(AR)	Saarchem	99%
Nicotine adenine dinucleotide phosphate	(SIGMA)	Sigma	98%
D-glucose-6-phosphate	(SIGMA)	Sigma	98%
Ampicillin trihydrate	(SIGMA)	Sigma	98%
Sodium hydroxide	(AR)	Kleber	98%
Crystal violet	(SIGMA)	Sigma	90%
D-glucose	(LAB)	BDH	95%
Sodium azide	(LAB)	BDH	99%
2-Aminofluorene	(AR)	Merck	98%
Dimethyl sulphoxide	(CCR)	Sigma	99%
Agar Agar		Merck	
Nutrient broth No.2		Oxoid	
Milli-Q water*			
Arochlor 1254	See section 4.1.5.		
Bacterial strains: TA 100, TA 98, TA 1535, TA 1537, TA 1538	See section 4.1.5.		

A.1.8. Chemicals for SOS Chromotest

Tris(hydroxymethyl)-aminomethane	(AR)	Saarchem	99,5%
Hydrochloric acid	(GP)	BDH	33%
Magnesium chloride	(LAB)	Saarchem	98%
Potassium chloride	(LAB)	Saarchem	99%
D-glucose-6-phosphate	(SIGMA)	Sigma	98%

Nicotine adenine dinucleotide phosphate	(SIGMA)	Sigma	98%
Dimethyl sulphoxide	(UVASOL)	Merck	99,5%
Milli-Q water*			

The following chemicals are supplied with the kit:

Growth medium

Lypholised bacteria (*E. coli* PQ37)

10% dimethyl sulphoxide in saline

4-Nitroquinoline oxide

Blue chromogenic substrate

Alkaline phosphatase substrate

Alkaline phosphatase diluent

2-Aminoanthracene

Stop solution for quenching the colour reaction

A.1.9. Chemicals for Toxi Chromotest

These chemicals are supplied with the kit:

Reaction mixture - a cocktail containing an inducer for the enzyme β-galactosidase, and cofactors required for the recovery of the bacteria from their stressed condition.

Lyophilized bacteria - a highly permeable mutant of *E. coli*.

Rehydration solution - to hydrate the bacteria.

4 µg/ml mercury chloride in water - a standard toxic substance.

Chromogenic substrate - blue or yellow.

Yellow chromogenic diluent.

Double distilled water.

Stop solution for quenching the colour reaction.

* NOTE: Milli-Q water refers to water that has been passed through the Millipore Milli-RO 4 water purification system which consists of a series of ion-exchange and organic removal resins.

A.2. EQUIPMENT

Details of the equipment used together with the names of the manufacturers are listed below.

A.2.1. Extraction and Fractionation

Gallenkamp flask shaker

Ultrasonic Manufacturing Company (UMC) ultrasonic bath model 20

Junker & Kunkel rotary evaporator

A.2.2. UV Spectroscopy

Varian DMS 300 Spectrophotometer

1 cm pathlength quartz cuvettes

A.2.3. TLC

Shandon Southern glass TLC Chromatank

Merck aluminium TLC sheets pre-coated with silica gel 60 F_{254} (0.2 mm thick); 20 x 20 cm

A.2.4. GC-MS

Finnigan 1020 GC-MS

Column: J & W Scientific DB5 30 m x 0.25 mm (id)
0.25 µm film thickness

Carrier Gas: Helium

A.2.5. HPLC

Waters 600 multisolvent delivery system

U6K variable injector

Waters 990 photodiode array detector

NEC APC II personal computer

Waters 990 plotter

Millipore solvent filtering system

Hamilton Microliter Gastight Syringe (10-100 µl)

Column: Supelcosil LC PAH; 25 cm x 4.6 mm; 5 µm

The following equipment was used for analysis at Lancaster University in the United Kingdom:

Perkin Elmer binary HPLC pump 250

Perkin Elmer LS 40 fluorescence detector

Column: Phase Separation Spherisorb PAH;
15 cm x 4.6 mm

A.2.6. Ames Test

All American electric steam sterilizer; model 25X

Tomy Autoclave; model S9 30N

Protea metabolic shaker; model 204

Vindon Solid State oven

Grant waterbath

Hoefer Mighty Bright UV TM-19 230V ultraviolet lamp

Beckman model J2-21 centrifuge

Voss S 30/CB electric homogenizer

Volac high precision micropipettes: 100-1000 µl
20-200 µl

Lida PRO-X 0.22 µm sterile filters

Promex petri dishes (90 mm diameter)

Intermed Nunc tubes 1.8 ml

Angelantoni Scientifica -80°C freezer

A.2.7. SOS Chromotest and Toxi-Chromotest

MLA metal micropipette: 10-50 µl

LKB Ultrospec IIE UV spectrophotometer

Helma Suprasil quartz 1 cm pathlength and 50 µl capacity cuvettes

Microtitre plates (or microplates)

Hettich Microliter micro-centrifuge

Vindon Solid State oven

APPENDIX B

This appendix lists the recipes for the stock solutions and media that are required in the Ames test (Chapter 4). All recipes are as given in the paper, "Revised Methods for the *Salmonella* Mutagenicity Test" by D.M. Maron and B.N. Ames[65]. Also included here is the procedure for the preparation of McFarland nephelometer tubes[68].

B.1. Vogel Bonner Medium E (50X)

Use: Minimal agar for plate incorporation test

Ingredient	Quantity per litre
Warm milli-Q water	670 ml
Magnesium sulphate ($MgSO_4.7H_2O$)	10 g
Citric acid monohydrate	100 g
Potassium phosphate (K_2HPO_4)	500 g
Sodium ammonium hydrogen phosphate ($NaHNH_4PO_4.4H_2O$)	175 g

The salts were added to the water contained in a 2 litre flask in the order indicated, allowing each salt to dissolve completely before adding the next. The solution was transferred to a 1 litre flask and the volume adjusted to 1 litre. The solution was then distributed into two 1 litre bottles and autoclaved loosely capped at 121°C for 45 minutes. Once cooled

the caps were tightened and the bottles stored in the fridge at 4°C.

B.2. 0.5 mM Histidine/Biotin Solution

Use: Added to top agar in mutagenicity test

Ingredient	Quantity per 250 ml
D-Biotin (m.m. 247.3 g/mol)	30.9 mg
L-Histidine.HCl (m.m. 191.7 g/mol)	24.0 mg
Milli-Q water	250 ml

The biotin was dissolved in the boiling water, the histidine added and the solution sterilized by autoclaving for 45 minutes at 121°C. The solution was stored in a glass bottle at 4°C.

B.3. Top Agar

Use: Mutagenicity assay

Ingredient	Quantity per litre
Agar	6 g
Sodium chloride	5 g
Milli-Q water	1 000 ml

The above substances were mixed and the agar dissolved by autoclaving briefly. The solutionn was then thoroughly mixed and 100 ml aliquots transferred into

glass bottles. The bottles were autoclaved loosely capped for 45 minutes at 121°C and once the agar had hardened, stored in the fridge,

B.4. Salt Solution (1.65 M KCl + 0.4 M $MgCl_2$)

Use: Preparation of S9 mix

Ingredient	Quantity per 500 ml
Potassium chloride (KCl)	61.5 g
Magnesium chloride ($MgCl_2.6H_2O$)	40.7 g
Milli-Q water	500 ml

The solutions of 1.65 M KCl and 0.4 M $MgCl_2$ were prepared separately and autoclaved as previously described. The solutions were stored in glass bottles at 4°C.

B.5. 0.2 M Sodium Phosphate Buffer (pH 7.4)

Use: Preparation of S9 mix

Ingredient	Quantity per 500 ml
0.2 M sodium dihydrogen phosphate ($NaH_2PO_4.H_2O$ 13.8 g/500 ml)	60 ml
0.2 M disodium hydrogen phosphate (Na_2HPO_4 14.2 g/500 ml)	440 ml

The pH was tested and if too low was adjusted by adding more 0.2 M disodium hydrogen phosphate. The

solution was sterilized by autoclaving and stored in glass bottles at 4°C.

B.6. 0.1 NADP Solution (nicotine adenine dinucleotide phosphate)

Use: Preparation of S9 mix

Ingredient	Quantity per 5 ml
NADP (m.m. 765.4 g/mol)	383 mg
Sterile milli-Q water	5 ml

The required amount of NADP was weighed into pre-weighed sterile nunc tubes and the water added aseptically. Only the amount of NADP required in a test was prepared at a time.

B.7. 1 M Glucose-6-phosphate

Use: Preparation of S9 mix

Ingredient	Quantity per 10 ml
Glucose-6-phosphate (G-6-P)	2.82 g
Sterile milli-Q water	10 ml

The amount of glucose-6-phosphate solution required was calculated, the powder weighed into a pre-weighed sterile nunc tube and the sterile water added.

B.8. S9 Mix (rat liver microsomal enzymes + cofactors)

Use: Mutagenicity assay

Ingredient	Quantity per 50 ml
Rat liver S9	2.0 ml
$MgCl_2$-KCl salts	1.0 ml
1 M G-6-P	0.25 ml
0.1 M NADP	2.0 ml
0.2 M phosphate buffer	25.0 ml
Sterile milli-Q water	19.75 ml

The ingredients were added to each other in the reverse order indicated so that the liver was added to the buffered solution. The $MgCl_2$ and KCl salts, prepared in section A.4 were mixed prior to their addition. The solution was prepared fresh for each mutagenicity assay and kept on ice throughout the test. Any remaining S9 mix was discarded.

B.9. Ampicillin Solution (8 mg/ml)

Use: For testing ampicillin resistance

Preparation of ampicillin plates for R-factor strains

Ingredient	Quantity per 100 ml
Ampicillin trihydrate	0.8 g
Sodium hydroxide (0.02 M)	100 ml

The ampicillin was dissolved in the sodium hydroxide solution and stored in a dark bottle at 4°C. The solution was sterilized by filtering through a sterile 0.22 µm syringe filter when required.

B.10. Crystal Violet Solution (0.1%)

Use: Testing for *rfa* mutation

Ingredient	Quantity per 100 ml
Crystal violet	0.1 g
milli-Q water	100 ml

The solution was stored at 4°C in a dark bottle which was covered with foil to protect from the light.

B.11. Minimal Glucose Plates

Use: Mutagenicity assay

Ingredient	Quantity per litre
Agar	15 g
Milli-Q water	930 ml
50X VB salts	20 ml
40%(m/v) glucose	50 ml

The agar and water were mixed in a 2 litre flask and a magnetic stirring bar added. The solution was autoclaved at 121°C for 30 minutes, removed and

sterile 50X VB salts and sterile 40% glucose added. The solution was mixed thoroughly and approximately 25 ml poured into each petri plate. Once the agar had hardened the plates were inverted and stored at 4°C until required.

B.12. Histidine/Biotin Plates

Use: Testing for histidine requirement

Master plates for non-R-factor strains

Ingredient	Quantity per litre
Agar	15 g
Milli-Q water	914 ml
50X VB salts	20 ml
40%(m/v) glucose	50 ml
Sterile histidine.HCl.H_2O (2 g/400 ml H_2O)	10 ml
Sterile 0.5 mM biotin	6 ml

The agar, water and a magnetic stirrer bar were placed in a 2 litre flask and autoclaved to dissolve the agar. The sterile glucose, 50X VB salts and histidine were added to the hot agar. The solution was allowed to cool slightly and the biotin was added. The solution was mixed thoroughly and approximately 25 ml poured into each plate. The glucose, 50X VB salts and histidine were all autoclaved separately before addition to the agar.

B.13. Ampicillin Plates

Use: Testing for ampicillin resistance

Preparation of master plates for R-factor strains

Ingredient	Quantity per litre
Agar	15 g
Milli-Q water	910 ml
50X VB salts	20 ml
40% glucose	50 ml
Sterile histidine.HCl.H_2O (2 g/400 ml)	10 ml
Sterile ampicillin solution (8 mg/ml 0.02 M NaOH)	3.15 ml

The agar and water were autoclaved. The sterile glucose, 50X VB salts and histidine solutions were added to the hot agar. The mixture was cooled to approximately 50°C and the sterile biotin and ampicillin solutions added aseptically (these solutions were sterilized by filtering through a 0.22 µm syringe filter). Approximately 25 ml of the mixture was poured into each plate, allowed to harden, inverted and stored in the fridge. The plates were discarded after 2 months.

B.14. Nutrient Agar Plates

Use: (1) Testing for genotypes:

(i) crystal violet sensitivity

(ii) UV sensitivity

(2) testing for viability of bacteria

Ingredient	Quantity per litre
Oxoid nutrient broth No. 2	25 g
Agar	15 g
Milli-Q water	1 000 ml

The ingredients were mixed in a 2 litre flask and autoclaved for 45 minutes at 121°C. The agar was cooled and the plates poured in the same manner as described previously.

B.15. McFarland Nephelometer Tubes

Use: Estimation of bacterial suspension densities.

Solutions of 1% H_2SO_4 and 1% $BaCl_2$ were prepared in sterile Oxoid nutrient broth no. 2 and mixed according to the above table. All tubes were of uniform size. The tubes were plugged with rubber stoppers and sealed with parafilm. The bacterial suspensions were compared with these standards to obtain an estimation of the density of the growth.

Tube No.	1% H_2SO_4 (in broth) volume/ml	1% $BaCl_2$ (in broth) volume/ml	Corresponding bacterial density - approx. 10^6/ml
1	9.9	0.1	300
2	9.8	0.2	600
3	9.7	0.3	900
4	9.6	0.4	1 200
5	9.5	0.5	1 500
6	9.4	0.6	1 800
7	9.3	0.7	2 100
8	9.2	0.8	2 400
9	9.1	0.9	2 700
10	9.0	1.0	3 000

APPENDIX C

The following papers were presented during the course of this work:

Godefroy, S.J., Salter, L.F., Martincigh, B.S.
The Identification of Polycyclic Aromatic Hydrocarbons in an Environmental Sample.
The Carman National Physical Chemistry Convention,
Durban, 29-31 January 1992.

Godefroy, S.J., Salter, L.F., Martincigh, B.S.
Mutagenicity Testing of Environmental Samples.
AECI, Modderfontein, 23 June 1992.

Godefroy, S.J., Salter, L.F., Martincigh, B.S.
The Identification of Polycyclic Aromatic Hydrocarbons in Environmental Samples.
The XI congress of the South African Biochemical Society,
Sun City, 28 June - 1 July 1992.

Godefroy, S.J., Salter, L.F., Martincigh, B.S., Raynor, M. *The Polycyclic Aromatic Hydrocarbon Content and Mutagenicity of Environmental Samples.*
South African Sugar Association, Mount Edgecombe Experiment Station, Mount Edgecombe, 2 December 1992.