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TOXICOLOGICAL ANALYSIS OF SOUTH AFRICAN PARAFFIN

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TOXICOLOGICAL ANALYSIS OF SOUTH AFRICAN PARAFFIN

by

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DECLARATION

This study provides an overview and results of chemical analysis and toxicity of domestic South African paraffin. The analysis of the chemical composition of paraffin was conducted at Intertek Testing Services (ITS) while the toxicology studies were conducted at the University of KwaZulu Natal, Nelson R. Mandela School of Medicine. The work was carried out under the supervision of Prof N. Gqaleni.

These studies have not been submitted in any form to another University. This is the original work of the author and it has been acknowledged in the text where work of the others was used.

Nonhlanhla Dlamini

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ABSTRACT

There is little information available on the relationship between the chemical composition and toxicity of South African paraffin. The aim of this study was therefore to determine an association, if any, between the chemical composition and toxicity of South African paraffin. The objectives were to analyze the chemical composition and potential toxicity of paraffin using an A549 lung cell line. There were two phases to the study. In the first phase of the study the chemical composition of seven (7) paraffin samples was tested at accredited InterTek Testing Services (ITS) according to SABS protocol. In the second phase of the study toxicology studies were conducted using the MTT and Annexin assays to establish the toxicity of the samples. The experiments included dissolving paraffin in a constant volume of ethanol. Results of the chemical analysis of paraffin from local refineries indicated that the major components were aliphatic hydrocarbons (>75%, v/v), olefins (1-8%, v/v), aromatics (1-20%, v/v) and sulphur (<0.1%, v/v). Cytotoxicity tests indicated that there were significant ($p<0.001$) differences in the level of toxicity of the paraffin samples. The chemical composition or formulation was the single most important factor, which determined the degree of toxicity. The toxicity of paraffin dissolved in ethanol was significantly ($p<0.001$) more toxic when compared to that of undissolved paraffin. Paraffin samples also induced apoptosis and necrosis. It is therefore recommended that the chemical composition of paraffin must be standardized to a consistent less toxic product to ensure the safety of the South African public.

To My Mother

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LIST OF ABREVIATIONS

AHC	-	Aliphatic Hydrocarbons
AIP	-	Australian Institute of Petroleum
API	-	American Petroleum Institute
ATCC	-	American Type Culture Collection
b/d	-	barrels per day
bw	-	Body weight
CO	-	Carbon Monoxide
COEH	-	Centre for Occupational and Environmental Health
COHb	-	Carboxyhaemoglobin
COPD	-	Chronic Obstructive Pulmonary Disease
CuD	-	Carnitine Uptake Defect
DMSO	-	Dimethylsulphoxide
DNA	-	Deoxyribonucleic acid
DPK	-	Dual Purpose Kerosene
ELISA	-	Enzyme-linked Immunoabsorbent Assay
EMEM	-	Eagle's Minimum Essential Medium

ET	-	Embryo transfer
FCS	-	Foetal Calf Serum
GC/MS	-	Gas chromatography/Mass spectrometry
HBSS	-	Hank's Balanced Salt Solution
HPLC	-	High Performance Liquid Chromatography
ITS	-	Intertek Testing Services
kg	-	Kilogram
L	-	Litre
LD_{50}	-	Lethal Dose
MD	-	Middle Distillate
mg	-	Milligram
mm^2/s	-	Millimitre square per second
MSDS	-	Material Safety Data Sheets
MTT	-	Methyl Thiazol Tetrazolium
NEAA	-	Non-Essential Amino Acids
NO_2	-	Nitrogen Dioxide
PAH	-	Polycyclic Aromatic Hydrocarbons
PASASA	-	Paraffin Safety Association of South Africa
PS	-	Phosphatidylserine

SA	-	South Africa
SABS	-	South African Bureau of Standards
SO_2	-	Sulphur Dioxide
SSA	-	Statistics South Africa
TMB	-	Trimethylbenzenes
U/ml	-	Units per millilitre
USA	-	United States of America
µl	-	Microlitre
WHO	-	World Health Organization

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CHAPTER ONE

1.0 GENERAL INTRODUCTION

Domestic paraffin is also known as illuminating paraffin or kerosene (Truran *et al.*, 2004). Commonwealth countries tend to use "paraffin" while Canada and the United States of America (USA) use "kerosene". In South Africa, paraffin is not treated with the respect that it deserves despite the fact that it is a hazardous substance used in the households of millions as an energy source (Truran *et al.*, 2004). This is probably because there are no better financial incentives associated with selling paraffin low income communities.

According to Statistics South Africa (SSA, 2000), there are about 5 million households in South Africa that depend on paraffin for domestic applications. South Africa is still a developing, economically challenged country hence the high number of people who continue to use potentially harmful cheap sources of energy (like paraffin) in their homes, even with the introduction of electricity, known to be safer from a health point of view. It is therefore the responsibility of both the South African government and petrochemical industry to make sure that the health of paraffin users is not compromised by making necessary interventions leading to the improvement of the quality and safety of paraffin.

The current high number of South African people exposed to paraffin, on a daily basis and the fact that the majority of paraffin related accidents involve small children, as a result of ingesting paraffin accidentally, make it a necessity to study the health implications of domestic paraffin in South Africa.

There are three routes through which people can be exposed to paraffin components in their homes. The routes include accidental ingestion, skin contact and inhalation of paraffin components when paraffin is burned. For a number of years the American Petroleum Institute (API) has sponsored research on the carcinogenic activity of the various streams produced in the refining of crude oil (Broddle *et al.*, 1996) and one of these streams is paraffin. It was discovered that some of the components could be responsible for the carcinogenic activity of paraffin (WHO, 1996). Early toxicological studies of paraffin used animal models, however, studies investigating the toxicity of paraffin *in vitro* later followed with the introduction of cell culture. In the present study the toxicity of paraffin will be determined *in vitro* using a cell line.

In South Africa, our knowledge is limited when it comes to understanding the relationship between the chemical composition and toxicity of paraffin due to a lack of studies. The current study was therefore employed to determine the chemical composition and toxicity of South African paraffin *in vitro* using a cell line in an attempt to find an association if any between the chemical formulation and toxicity of paraffin. One significant problem with tissue culture systems is the necessity for solubilizing the compound of choice into aqueous media. Paraffin is a complex mixture that may contain both hydrophobic and hydrophilic components, which can be adequately solubilized with ethanol (Grant *et al.*, 2000). As a result the study included experiments with both unsolubilized and solubilized paraffin. Since the most common route of paraffin exposure is inhalation, the toxicity of paraffin was determined *in vitro* using a lung cell line.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Nature of paraffin

Paraffin is a clear water-like or colourless liquid that can be sourced from crude oil, coal and sea. It can be produced from crude oil by a refining process and from coal and sea by a process known as organic synthesis (Truran *et al.*, 2004). Crude oil is a viscous, dark brown liquid. It is formed in the Earth's crust over the course of millions of years by the decomposing action of anaerobic bacterial organisms on animal and vegetable matter. Crude oil deposits are widely distributed throughout the world, mainly in Africa, China, North and South America and the Middle East (Chang *et al.*, 1991).

Annexure 2.1 shows crude oil deposits found from different parts of the world. It further demonstrates that the characteristics of crude oil are influenced by geographic origins. The geologic history of a particular crude oil is found to be most important in the determination of its characteristics. The characteristics include its colour and chemical composition. For an example, some crude oils from Louisiana (USA) and Nigeria are similar because both are formed in similar marine deposits. In parts of the Far East, crude oil generally is waxy, black or brown, and low in sulphur. It is similar to crude oils found in central Africa because both are formed from non-marine sources. In Middle East, crude oil is black but less waxy and higher in sulphur. Crude oil from Western Australia can be a light, honey coloured liquid while that from the North Sea typically is a waxy, greenish-black liquid. Many kinds of crude oils are found in the United States because there is a great variety in the geologic history of its different regions (Chevron, 2002). Crude oil deposits from the United Kingdom, Sudan, Utah, Illinois and Texas are included in Annexure 2.1.

The actual colour and composition of crude oil varies with location (Chang *et al.*, 1991). It can range from a pale yellow liquid to a heavy black liquid. It is also a rich source of higher molecular weight hydrocarbons, such as; alkanes, cycloalkanes, aromatic hydrocarbons and may contain sulphur, nitrogen and oxygen compounds (Routh *et al.*, 1971). In addition to energy, crude oil also supplies numerous organic chemicals used to manufacture drugs, clothing and many other products (Chang *et al.*, 1991). Crude oil produces paraffin including other products from a refining process.

2.2 The refining process

The existence of crude oil has been known for centuries and has been used for many purposes but mostly as a fuel. However it was not until much later that mankind was able to separate it into its hydrocarbon components. The process known as distillation is used to separate crude oil into its hydrocarbon fractions (Routh *et al.*, 1971). In the petroleum industry, this process is called the refining process. In this process heat is used to break forces of attraction found between individual hydrocarbons. Energy is required to break up these forces of attraction. Energy required depends on the strength of the bonds, e.g., if the forces of attraction found between individual hydrocarbons are small therefore low energy (low boiling point) is required to break them up. However if the forces of attraction found between bonds are high, high energy (high boiling point) will be required to break them up (Routh *et al.*, 1971). This principle is used to separate crude oil components during distillation. The petroleum industry normally uses fractional distillation. In this process, crude oil is separated by distillation into several fractions possessing different distilling temperatures by the use of a fractionating column (Routh *et al.*, 1971). The different fractions can be removed from the fractionating column using proper methods.

The refining process using a fractionating column is illustrated in Annexure 2.2. The first products given off during the refining process are the gaseous hydrocarbons containing from 1 to 5 carbon atoms. These hydrocarbons are both saturated and unsaturated and are usually separated from each other by chemical methods. The unsaturated are used in the production of aviation gasoline, synthetic rubber and other organic compounds while the saturated hydrocarbons are liquefied and sold as bottled gas (Routh *et al.,* 1971). The second products to be produced during the refining process are petroleum esters. They are mainly consists of pentanes, hexanes and heptanes, and are used as fat solvents and dry cleaning agents (Routh *et al.,* 1971). Gasoline, which is a third product to be given off during a refining process, is probably the best known component of petroleum. It is also made up of volatile hydrocarbons. It mostly contains alkanes, cycloalkanes and a few aromatic hydrocarbons. Some of these compounds are far more suitable for fuelling than others, and herein lies the problem of the further treatment and refinement of gasoline (Chang *et al.,* 1991). The paraffin fraction which is used as diesel and jet fuel follows the gasoline (Routh *et al.,* 1971). Gas oil or fuel oil fraction contains a mixture of hydrocarbons whose smallest members have 15 carbon atoms. Fuel oil can be used in furnaces that burn oil, whereas diesel oil is used in diesel engines. This fraction may also be cracked to produce gasoline (Routh *et al.,* 1971).

The characteristics of uses of the crude oil distillation fractions from a fractionating column are summarized in Annexure 2.3. Of all the fractions referred to in Annexure 2.3, paraffin has been the most important fraction used in the early days of the petroleum industry. It was used for lighting, cooking and heating purposes but with the advent of the electric light and cars, its demand decreased and that of petrol increased (Routh *et al.*, 1971). However, the demand continued in most developing countries.

2.3 Physical and chemical properties of paraffin

2.3.1 Physical properties of paraffin

Paraffin is a clear liquid with a characteristic smell. In South Africa it can be produced by refining crude oil or synthetically from natural gas and/or coal. It has a minimum flash point of 43^0C at atmospheric pressure in South Africa. The flash point is the temperature at which vapour from a liquid can be ignited. Paraffin has a very low viscosity, which means it is very runny or fluid and spreads like water (Truran *et al.*, 2004).

2.3.2 Chemical properties of paraffin

Paraffin is made up of thousands of different hydrocarbons but chiefly consists of 80% (v/v) aliphatic hydrocarbons (AHC) and 20% (v/v) aromatic hydrocarbons (Ebbing *et al.*, 1999; Tsujino *et al.*, 2002). Hydrocarbons have different boiling points. This allows them to be separated during refinery distillation. Paraffin distils between approximately 150 – 300^0C (Truran *et al.*, 2004). The classification of hydrocarbons is demonstrated in Annexure 2.5. Other components which may be present in small concentrations include olefins and sulphur. Sulphur is not a very abundant element. It constitutes about 0.06% of the Earth's crust by mass (Chang *et al.*, 1991). It enters the refinery as crude oil feed. Sulphur content differs from oilfield to oilfield. Different countries have national standards, which restrict or control the sulphur content in paraffin or jet fuel (WHO, 1996).

The major physical properties and chemical components of paraffin are summarized in Annexure 2.4 while the classification of hydrocarbons (section 2.3.2.1 and 2.3.2.2) is illustrated in the form of a diagram in Annexure 2.5 (Ebbing *et al.*, 1999).

2.3.2.1 Aliphatic Hydrocarbons (AHC)

Hydrocarbons are made up of two elements, hydrogen and carbon. They are divided into aliphatic and aromatic classes on the basis of their structure. Aliphatic hydrocarbons do not contain the benzene ring whereas aromatic hydrocarbons do. A benzene ring is the formation where six benzene atoms are arranged in a ring, which has alternating single and double bonds, and a hydrogen atom, attached to each carbon atom (MCAT, 2006). The aliphatic hydrocarbons are further divided into alkanes, cycloalkanes, alkenes and alkynes. They differ in the number of bonds present (alkanes have one bond, alkenes have two and alkynes have three bonds). The cycloalkanes are alkanes whose carbons are joined in rings. The aliphatic hydrocarbons are the major components of paraffin (Chang *et al.*, 1991).

2.3.2.2 Aromatic hydrocarbons

Benzene happens to be the parent compound of this family. Aromatic hydrocarbons are very stable because of their structure (Chang *et al.*, 1991). They are not related to aliphatic hydrocarbons. Their boiling and melting points are higher than those of aliphatic hydrocarbons, which mean they are stable. They are quite flammable (Routh *et al.*, 1971).

A group of aliphatic hydrocarbons demonstrated in Annexure 2.6 contain alkanes as they have only one bond while the group of aromatic hydrocarbons found in the same annexure contain a benzene ring (AIP, 2002).

2.4 Paraffin standards

In South Africa, petrochemical companies, since the 1970's, have been relying on government purchasing specification, **CKS 78 illuminating paraffin,** for domestic paraffin quality as a result of having no national standard for domestic paraffin. A project to develop a national standard for paraffin can be considered a positive development because the main difference between the government-purchasing specifications and national standards is that government-purchasing specifications are guidelines that are not enforced while standards are enforceable (Annexure 2.12). This simply means adherence to these specifications is not compulsory however standards on the other hand are compulsory and will force any South African company to produce a product that meets the South African Bureau of Standards (SABS) requirements prior to being sold to the public. Meeting the requirements will therefore mean being familiar with SABS testing methods. The specifications for South African paraffin are presented in Annexures 2.7 and 2.8 while the national standards for paraffin from Canada, Malaysia and the United States of America, including the proposed South African standard are demonstrated in Annexures 2.9 to 2.12.

Canadian, Malaysian and USA standards cover two types of paraffin; low sulphur and regular grade sulphur paraffin. On the other hand South Africa has a standard for one type of paraffin. The sulphur content is set at 0.04 in other countries while in South Africa it is at 0.05. A flash point limit for the South African standard is set at 43 ^{0}C, which differs from the flash point limit for the other standards (Canada, Malaysia and USA) observed at 38 ^{0}C. Interestingly all standards lack the specification for the viscosity except for the American standard which states that the viscosity of paraffin should not be below 1.0 and not above 1.9. Low viscosity leads to the aspiration of paraffin into the lungs when it is ingested. It would therefore be useful for paraffin standards to include the viscosity of paraffin and to reconsider increasing the viscosity of paraffin, as it appears to prevent aspiration. The noticeable

differences are a measure of how we compare to international countries as far as paraffin standard is concerned.

2.5 Paraffin usage and public health implications

2.5.1 Paraffin usage in South Africa

The main energy sources used by South African households for cooking, heating and lighting are indicated in Annexures 2.13 to 2.15 while Annexures 2.16 to 2.17 indicate paraffin using households by province and race group. According to Statistics South Africa (SSA, 2001) paraffin is still among the most preferred energy sources in South Africa after electricity and it is mostly used for cooking followed by heating and lighting. Paraffin usage differs by province and is significantly higher among Black and Coloured households compared to Indian and White households.

It is estimated by the Paraffin Safety Association of South Africa (PASASA) that, between 40 and 50% of South African households use paraffin for some part of their domestic energy requirements (Truran *et al.*, 2004). These are low-income households (both rural and urban) living either in informal dwellings or small houses. Some of these households do have electricity but use it only for lighting and prefer to cook with paraffin. This also raises concern with the design of Reconstructive and Development Program (RDP) housing, which is geared towards the use of electricity and not designed for the use of paraffin stoves in spite of this reality (Truran *et al.*, 2004).

Paraffin is sold to the consumer by retailers, such as petrol filling stations, general dealers, public wholesalers and spaza shops. The price of paraffin is regulated by the government but is not strictly enforced (Truran *et al.*, 2004). Nearly 800 million litres are

manufactured and sold each year. It is sold in bulk liquid format (Truran *et al.*, 2004). The primary providers of paraffin, who happen to be the general dealers and spaza shops, store the fuel in large 210 litre drums or 2000 litre tanks above the ground. Consumers purchase paraffin in small quantities of one to five litres (Lloyd *et al.*, 1999; Monga *et al.*, 2000). It is often stored in cold drink bottles, milk bottles, juice bottles or other containers (Abrahams *et al.*, 1994; Ellis *et al.*, 1994).

There is a very complicated, unregulated and diverse supply chain between oil company depots and the consumer (Truran *et al.*, 2004). As a result paraffin may become prone to contamination with other products. The sales of major petroleum products, including paraffin, in South Africa from 1999 to 2004 are shown in Annexures 2.18 to 2.20. Petrol was the most sold petroleum product in South Africa in 1999 to 2004 followed by diesel and jet fuel while paraffin was the 4th petroleum product sold in South Africa in the same period.

2.5.2 Public health implications of paraffin use in South Africa

Paraffin ingestion is the most common cause of accidental childhood poisoning in South Africa. Children from the lower socio-economic group are affected the most. They drink paraffin in the summer months from bottles, mistaking it for water or cold drink (Ellis *et al.*, 1994). Hospital based studies have been conducted in order to investigate paraffin poisoning in South Africa and some of these studies are unpublished data. They provide evidence which accounts paraffin poisoning for a large percentage of acute accidental poisoning in children. Ellis *et al.* (1994) have reported that paraffin poisoning accounted for 78% of acute accidental poisoning in 1992 at Garankuwa hospital, located north of Pretoria. A similar study conducted at Red Cross Children's Hospital in the Western Cape found paraffin poisoning to be responsible for 22 to 30 % of all poisonings (CAPFSA, 2002). Clearly the

observations differed from hospital to hospital possibly because different hospitals serve different communities.

Reports by Krug *et al.* (1994) have highlighted summer as the season of greatest risks for children, possibly due to increased thirst. Interestingly, there had been discrepancies in the findings when gender with regards to paraffin ingestion is concerned. Some studies have found the incidence of paraffin ingestion to be more prevalent in males than females (Ellis *et al.*, 1994) while others have observed no difference (Singh *et al.*, 1992). The mean age of children affected by paraffin poisoning is 24 months (Ellis *et al.*, 1994). The most common clinical presentations of paraffin ingestion are coughing, tachypnea and fever (Abu-Ekteish *et al.*, 2002) however others may include respiratory symptoms, gastrointestinal symptoms, fever and neurological manifestations (Abu-Ekteish *et al.*, 2002; Majeed *et al.*, 1981). Paraffin ingestion may cause complications which may prove to be lethal. Small amounts of paraffin as little as 25 ml might be detrimental when ingested (Stones *et al.*, 1987). The development of respiratory complications is related to vomiting which results in paraffin being aspirated into the lungs (Majeed *et al.*, 1981).

Hospital case fatality rates due to paraffin ingestion ranging from 0.72% to 2.1% (Crisp *et al.*, 1986; Krug *et al.*, 1994; Joubert *et al.*, 1990; Simmank *et al.*, 1998) have been reported in South Africa. Often the cause of death following paraffin ingestion is chemical pneumonia. Chemical pneumonia is the most serious complication following paraffin ingestion (Chibwana, *et al.*, 2001; Majeed *et al.*, 1981).

There are cost implications associated with paraffin ingestion. The average cost of hospitalization for a patient in an urban hospital, north of Pretoria was R348 per day (Ellis *et al.*, 1994). In another study conducted in the Cape Peninsula the paraffin poisoning treatment

cost per patient varied from R144.00 – R410.00. However, this excludes the cost of treating children who are not hospitalized. It also excludes direct and indirect costs of paraffin ingestion.

Paraffin also threatens public health from an indoor air pollution perspective. Paraffin is burned in order to produce energy used for cooking, heating and lighting purposes. The burning of paraffin is also known as the combustion process. In this process oxygen reacts with the hydrocarbons of paraffin to produce carbon dioxide, water and heat energy, which are non-toxic. The combustion reaction described above is complete combustion. However, when there is insufficient oxygen or combustion conditions are not optimized, incomplete combustion occurs with the formation of varying amounts of carbon monoxide (CO) instead of carbon dioxide (CO_2). This is detrimental in two ways – carbon monoxide is highly toxic and the amount of heat produced is less (Odian *et al.*, 1994). If the room in which the appliance is burning is well ventilated, the effects of this pollution can be minimized. However, in low income dwelling situations, the stove often doubles as a heater and room ventilation is minimized to keep out the cold, damp and wind. In these sorts of situations carbon monoxide alone can at best cause dizziness, drowsiness and headaches and at worst, death (Truran *et al.*, 2004). Soot also produced as a result of incomplete combustion contains various polycyclic aromatic hydrocarbons and aliphatic hydrocarbons. Soot is associated with genotoxicity (Lohani *et al.*, 2000).

2.6 Toxicity of paraffin

Currently South Africa lacks the understanding of paraffin toxicity due to the unavailability of toxicological data. Our knowledge is limited to toxicological studies conducted by international organizations or institutions. Toxicological studies have used both animal models and cells *in vitro*.

2.6.1 Mammalian toxigenicity

2.6.1.1 Short-term acute exposure

In general paraffin was found to demonstrate a low order of acute toxicity after oral, dermal and inhalation administration. Acute LD_{50} values are summarized in Annexure 2.21. The data in Annexure 2.21 indicates that inhalation is the route through which paraffin exert its toxicity to animals (>0.1 mg/L) followed by dermal exposure (>2000 mg/kg body weight) and, lastly, ingestion (>5000 mg/kg body weight). The degree of toxicity appears to be dependent on the type of species exposed and according to the data in Annexure 2.21 oral ingestion is the least toxic route. Straight- run and hydrodesulphurised refers to the form of processing.

2.6.1.2 Long-term exposure

Paraffin is irritating (features include blanching, subcutaneous haemorrhage and fissuring (cracking) at the site of application) to the skin of animals and humans and may confound evaluation of toxicity for other endpoints (Schreiner et al., 1997; CONCAWE, 1995; Deininger *et al.*, 1991; API, 1982). It appears that dilution with mineral oil or hydrodesulphurisation (removal of the sulphur component) reduces the extent of irritation. Straight-run paraffin produced effects around hair follicles followed by epidermal degeneration in mice (Ingram *et al.*, 1993). Bioavailability studies indicated that the reduced irritation observed in samples diluted with mineral oil was not due to decreased skin penetration (CONCAWE, 1995).

Several studies have reported the tumorigenic potential of paraffin, as evaluated on the mouse skin resulted in significant numbers of skin tumours produced by non-genotoxic means

(Ingram and Grasso, 1991). The causal factors of tumour formation are important for hazard classification and human health risk assessment. Deininger *et al.* (1991) have studied the relationship between skin tumorigenesis and straight-run or cracked paraffin. The studies associated some components of paraffin with the tumorigenesis. Mutagenic activity correlated with the presence of three-to-seven ring polycyclic aromatic hydrocarbons when compared with the presence of one-to-two ring polycyclic aromatic hydrocarbons. The straight-run paraffin, which does not contain detectable levels of polycyclic aromatic hydrocarbons, was found to be non-mutagenic (McKee *et al.,* 1994). Cracked paraffin on the other hand, which contains detectable levels of polycyclic aromatic hydrocarbons, was found to be mutagenic. The data suggest that straight run paraffin produce cancer through a process that does not involve mutation (i.e., a non-genotoxic process) and cracked paraffin produce cancer through a process that involves mutation (i.e., genotoxic process) (McKee *et al.,* 1994). Later studies (Nessel *et al.* 1998) confirmed these findings. Bingham and Horton (1966) demonstrated that processing history is a critical determinant of carcinogenic activity and that those steps which might be expected to reduce or eliminate polycyclic aromatic hydrocarbons also reduced or eliminated carcinogenic activity.

2.6.1.3 Toxicity of paraffin components

Toxicological studies of paraffin demonstrated a potential association between the toxicity of paraffin and its chemical composition, and as a result much of the toxicological concern has focused on the toxicity of the individual components of paraffin. The systemic distribution of trimethylbenzenes and aliphatic hydrocarbons in blood and tissues following dermal exposure has been investigated by McDougal *et al.* (1990, 2000) and Tsujino *et al.* (2002) with higher trimethylbenzenes detected in tissues than those of aliphatic hydrocarbons. This suggested that after dermal exposure the components of paraffin are absorbed and that benzenes are absorbed at a faster rate compared to aliphatic hydrocarbons. Once absorbed, the

components are distributed to various organs via the blood. The absorption of trimethylbenzenes is influenced by the total amount of paraffin applied rather than the area exposed, whereas, aliphatic hydrocarbons are influenced by both the amount of paraffin applied and the unit area of skin exposed (Tsujino *et al.*, 2002).

2.6.2 Cytotoxicity

Given the proper environment some kinds of cells will survive and multiply in a tissue culture dish. Cell culture techniques were developed for use in research, but they have found many practical applications. Cell culture permits researchers to grow populations of cells to study (Arms and Camp *et al.*, 1995). Cell culture techniques have found application in a number of fields which include:

1. Agriculture and Forestry

The cell and tissue culture techniques are already used practically for the propagation of crop seedlings and for plant breeding. The embryo culture and another culture techniques as well as mutants appearing in a cell culture are used for plant breeding. These methods are used in particular for the breeding of new varieties of rice, vegetables and fruit trees. Seventy percents of strawberry seedlings are now produced virus-free and some 150 facilities have been established for this purpose (MAFF, 2000).

2. Livestock

The application of the embryo transfer (ET) technique to the cow has progressed in Japan. The number of calves born through ET is increasing and some 15,000 calves were born in 1997. Research on in vitro fertilization and nuclear transplantation is also carried out. Some

2,100 calves were born through in vitro fertilization in 1997, and successful experiments for producing clone calves through nuclear transplantation were reported in 1997 (MAFF, 2000).

3. Food Industry

The cell fusion technique has been applied for the development of new koji (rice mold) and other yeasts for the production of shochu (Japanese spirit), sake and bread. High fructose syrup, cyclo-dextrin (CD) and erythritol have been produced in a bioreactor containing fixed enzymes and microorganisms. rDNA microorganisms have been used for the production of chymosin, an enzyme used for cheese production in Europe and North America. A cheese made from rDNA chymosin is imported in Japan (MAFF, 2000).

Cytotoxicity is used to determine the toxicity if any possessed by a substance. Cytotoxicity is determined by cell death. Cell death may be defined in physiological terms as the irreversible breakdown of the energy dependent functions of the cell. Cell death could also mean a series of morphological changes, which occur following lethal injury. The morphological features seen in dead cells vary depending on which of the two processes (enzymatic digestion or protein denaturation) is dominant (Woolf *et al.*, 1986). Two types of cell death are evaluated in the current study, apoptosis and necrosis.

2.6.2.1 Apoptosis

Apoptosis refers to controlled cell death that keeps the intracellular content of the dying cell sequestered (Hickman *et al.*, 1999). It has been given the name apoptosis, to distinguish it from cell death that results from injury or similar destruction (Arms and Camp *et al.*, 1995). The cell is fragmented into membrane bound particles during apoptosis, and the particles are eliminated by phagocytosis. Alternatively apoptosis can be referred to as a

physiological suicide mechanism that preserves homeostasis and occurs during normal tissue turn over (Klein *et al.*, 1996).

The process appears to take place in two stages. The cell separates first from its neighbours and both the nucleus and the cytoplasm become condensed. Secondly it breaks up into a number of fragments. These fragments can either shed from epithelium-lined surfaces or are phagocytosed by other cells (Woolf *et al.*, 1986). The cell can go through a number of changes during apoptosis, which include:

1. Nuclear Changes

Characterization of morphologic events that take place during the apoptotic process have improved since the original description (Geske and Gerschenson *et al.*, 2001). Investigations have revealed that DNA breaks observed during apoptosis can involve the formation of high molecular weight DNA fragments (Oberhammer *et al.*, 1993). Later investigations revealed the second type of DNA breaks which involves cleavage of DNA into 180 - base pairs (bp) fragments that form a "ladder" upon separation on an agarose gel (Desjardins *et al.*, 1995).

2. Cell Membrane Changes

The early event in apoptosis (Kravtsov *et al.*, 1999) involves the presence of phosphatidylserine (PS) on the outer cell membrane as a mechanism for recognition and removal by apoptotic cells (Fadok *et al.*, 1992).

3. Mitochondrial Changes

The disrupted mitochondria during apoptosis release a proapoptotic protein called apoptosis-inducing factor and it rapidly induces apoptosis (Susin *et al.*, 1997).

4. Proteolytic Changes

The proteolysis of structural and regulatory proteins of the dying cell probably drives many of the morphologic features of an apoptotic phenotype (Geske and Gerschenson *et al.*, 2001). This proteolysis has been attributed to the activation of a family of cysteine proteases called caspases (Alnemri *et al.*, 1996). It was determined that the *ced-3* gene was required for the 131 specific programmed cell deaths that occur during development (Ellis *et al.*, 1991). *Ced-3* is homologous to the mammalian interleukin 1β- converting enzyme. This fact suggested that the death inducing properties of *ced-3* depended on proteolytic activity (Yuan *et al.*, 1998).

2.6.2.2 Necrosis

Necrosis refers to uncontrolled cell death. This is when cell death occurs in part of an organ or tissue and where continuity with neighbouring viable tissue is preserved and various morphological forms exist. The morphological type may provide a clue to the cause of tissue injury (Woolf *et al.*, 1986). Characteristics of the cell affected by necrosis include swelling, cell membrane lysis, inflammatory response, oedema and damage to mitochondria.

2.6.3 Toxicity of combustion products of paraffin

Incomplete combustion of paraffin can lead to the release of harmful components to the air, which in turn can be inhaled by exposed human beings. The combustion products of paraffin and their effects are summarized in Annexure 2.22. Carbon monoxide is hazardous because it is colourless and odourless. Toxicologically important as it competes with oxygen for haemoglobin binding, resulting in the formation of carboxyhemoglobin. Hemoglobin carbon monoxide saturation levels of 50 to 80 % are associated with unconsciousness and death. Chronic exposure to low levels of carbon monoxide produces medical symptoms

associated with many other disease states, including chronic fatigue, headache, dizziness, nausea and mental confusion and is often mistaken for other illnesses (Ritchie *et al.*, 2003).

2.6.4 Summary

In summary, paraffin is the primary fuel used for personal heating, cooking and illumination applications in a number of developing countries allowing extensive opportunity for repeated exposure to raw fuel, vapour/aerosols, or combustion products (Majeed *et al.*, 1981; Dutta *et al.*, 1998). It sometimes causes death in conjunction with other factors (Tsujino *et al.*, 2002).

In order to gain knowledge on its toxicity, studies have exploited many different animal models. Toxicological studies found paraffin to exert its toxicity through inhalation, dermal and oral ingestion routes. Presently our country relies on toxicological data investigated by international organizations since there is no published data in our country as far as understanding the mechanisms involved in the toxicity of paraffin is concerned. This is very ironical for a number of reasons:

1) Most of these international organizations are from well developed countries, which in recent years have focused on the toxicity of jet fuel which is chemically related to paraffin, as with the advent of the electric light and cars, the demand for paraffin in developed countries decreased and that of petrol and jet fuel increased (Routh *et al.*, 1971).

2) Our country is one of the developing countries around the world with the highest number of paraffin users and incidences associated with using paraffin.

To reach a better understanding of South African paraffin toxicity, the toxicity of paraffin produced from South African refineries needs to be investigated and further investigations should focus on associating or linking the toxicity observed with the chemical formulation. This will not only help us to understand the mechanisms involved in the toxicity of paraffin but it will also assist in the identification of the components of paraffin responsible for toxicity.

CHAPTER THREE

3.0 AIM AND OBJECTIVES

3.1 Aim

The aim of the current study is to determine an association, if any, between the chemical composition of paraffin and its toxicity to the cells.

3.2 Objectives

The objectives of the study are:

1) To determine the chemical composition of paraffin samples.
2) To determine the toxicity of the paraffin samples.

CHAPTER FOUR

4.0 METHODOLOGY

4.1 Ethical approval

Ethical approval of the study was obtained from the Research Ethics and Higher Degrees Committees of the University of KwaZulu Natal.

4.2 Chemical composition of South African paraffin

4.2.1 Paraffin samples

A sample of paraffin and its corresponding material and safety data sheet (MSDS) was requested from each refinery. Caltex (Cape Town), Engen (Durban), Natref (Sasolburg), PetroSA (Cape Town) and Sasol (Sasolburg), each provided a sample (2-5 litres) of paraffin. Since Shell has introduced odorless paraffin, they provided an additional sample therefore two samples of paraffin were received from Shell. In total seven samples were received for this study and labeled 1 - 7. The samples were sent to Intertek Testing Services (ITS), Durban, for laboratory analysis. This is an independent laboratory familiar with the South African Bureau of Standards (SABS) requirements for paraffin.

Seven paraffin samples obtained from South African refineries (as shown in Annexure 4.1) were used while South Africa's Petroleum Refineries were shown in Annexure 4.2.

4.2.1.1 Data Analysis: Chemical Composition of Paraffin Samples

Descriptive statistics for the chemical composition of South African paraffin samples were carried out and the results presented according the variables relating to the following: Flash Point, Final Boiling Point, Residues and Smoke point. Frequency statistics was calculated and presented for the Sulphur, Olefin, Aromatic and Aliphatic content of the paraffin samples.

4.2 Toxicity of South African paraffin

4.2.1 Cell culture

4.2.1.1 Cell lines

The A549 cell line was used for the current study. Annexure 4.3 shows the description of the A549 cell line. The A549 (human lung carcinoma cells) (ATCC CCL185) was obtained from the American Type Culture Collection and was kindly provided by the Department of Physiology, University of KwaZulu Natal.

4.2.1.2 Growth and maintenance

4.2.1.2.1 Culture medium

The culture medium used for cell growth was Eagle's Minimum Essential Medium (EMEM) containing 25 mM Hepes (BioWhittaker, Maryland, United States of America). The medium was supplemented with Foetal Calf Serum (FCS) (Delta Bioproducts, Johannesburg, South Africa) since EMEM on its own is not sufficient enough to sustain the growth of cells. Cells require nutrients and other growth factors in order to grow and these are provided by the

FCS. The cells were grown in 10% (v/v) serum concentration and maintained in 2-5% (v/v) serum concentration. The antibiotic used in order to prevent contamination was the penicillin/streptomycin mix (BioWhittaker) penicillin (100 U/ml) and streptomycin (100 U/ml) while the Non Essential Amino Acid (NEAA) used was L-Glutamine (BioWhittaker). Aseptic procedures were used to add the serum, antibiotics and NEAA to the medium.

The complete culture medium (EMEM supplemented with FCS, antibiotics and NEAA) was checked on a daily basis for microbial growth.

4.2.1.2.2 Growth requirements

Cells were grown in 25 or 75 cm^2 tissue culture flasks (Corning, New York, United States of America) equilibrated with complete culture medium. The cells were incubated at 37^0C. A light microscope was used to observe cells daily and the cell culture medium was changed every two days in order to:

1) Replace the nutrients of the culture medium as they are metabolized by the cells;
2) Remove unattached or dead cells; and
3) Maintain the pH of the culture medium.

4.2.1.2.3 Subcultivation

The cell monolayers were split once 80% confluence was reached. They were split either into two flasks for further growth or into a Petri dish for use in experiments. The process of splitting the cells (also known as trypsinization) was as follows:

1) Cells were washed twice with Hank's Balanced Salt Solution (HBSS) with phenol red (BioWhittaker) when they were confluent.

2) The second HBSS wash was decanted and trypsin (Gibco Laboratories, New York, United States of America) enough to cover the surface of the flask was added. The cells were incubated at 37^0C until they were seen to round up and detach from the flask surface. The flask was gently shaken to aid detachment.

3) Once detached, cells were immediately resuspended in complete culture medium (40 ml) after which 20 ml of this medium containing cells was transferred to a second flask.

4) The cells were counted (section 4.2.2.2.4) and the appropriate concentration of cells was determined.

5) The cells were incubated at the optimal growth requirements described above.

4.2.1.2.4 Cell count

The haemocytometer counting chamber was used for cell count. Since the haemocytometer counting chamber fails to differentiate between living and non-living cells, the Trypan Blue exclusion method was used for differentiation purposes. Non- viable cells take up the Trypan Blue stain because their cell membranes are damaged whereas viable cells exclude the dye since their cell membranes are intact.

The trypsinized cell suspension was stained with 1% Trypan Blue Stain (Merck, Darmstadt, Germany) by adding 100 µl of cell suspension to 100 µl of Trypan Blue stain, followed by mounting 10 µl of this mixture onto the haemocytometer. The mixture was mixed gently by pippeting up and down before a sample was drawn. The cell count was done under a light microscope at a magnification of 40.

The number of cells per ml was calculated as follows:

$$\frac{10^4 . n . y}{5}$$

Where:

10^4 = correction factor for volume (ml) over each large square

n = total number of cells counted in 5 chamber squares

y = dilution factor

5 = number of chamber squares counted

4.2.1.2.5 Cryopreservation

Healthy cells were frozen and stored at -70^{0}C until required. The method of cryopreservation was as follows:

1) A healthy confluent monolayer was trypsinized (section 4.2.2.2.3).

2) The cell suspension was re suspended in the cryoprotective medium (EMEM containing 10% FCS (v/v) and 10% dimethyl sulphoxide (DMSO) (v/v) (Merk, Gauteng, South Africa).

3) The cells in the cryoprotective medium were aliqouted in 1.0 ml amounts into sterile cryogenic vials (Corning).

4) Freezing was performed by allowing the cells to freeze slowly at -70^{0}C.

4.2.2 Cytotoxicity assays

In the current study Methyl Thiazol Tetrazolium (MTT) (Sigma, St Louis, United States of America) and Annexin - V - Fluos (Roche, Gauteng, South Africa) assays were used to determine the toxicity of paraffin samples.

4.2.2.1 Methyl Thiazol Tetrazolium (MTT)

4.2.2.1.1 Determination of a sample size

In order to determine the acceptable sample size of replicates required to show a statistical significance relationship between paraffin samples and cell toxicity a trial experiment was conducted as follows:

1) Cells were grown in 75 cm^2 tissue culture flasks and once confluence was reached they were trypsinized and re suspended in 15 ml complete culture medium.

2) The constant volumes of cells (100 µl) were seeded into each well of a 96 well microtitre plate (Corning).

3) The paraffin samples were added to each well (n = 3) at concentrations ranging from 1 – 100 µl (Table 4.1) and incubated for 24 hours.

Table 4.1. Details of dilutions of paraffin samples used in the MTT assay.

Sample	Media w/o cells	Cells	Final Dilution
100	0	100	1:2
50	50	100	1:4
20	80	100	1:10
5	95	100	1:40
1	99	100	1:200
0	100	100	0

4) After the incubation period, the medium was removed from the wells, replaced with 10 µl solution of tetrazolium salt and 100 µl of EMEM supplemented with 10 % FCS and incubated for 4 hrs at 37^0C.

5) The tetrazolium salt was decanted and cells were incubated a further 1 hour with dimethyl sulphoxide (100 µl) at 37^0C in order to dissolve formazan crystals.

6) Untreated cells were used as controls (n = 3).

7) Results were read using an ELISA reader at an absorbance of A_{570} nm and a reference filter of A_{650} nm. The percentage cell viability at each dilution was calculated as follows:

$$\% \text{ Cell Viability} = \frac{\text{Absorbance of test sample}}{\text{Absorbance of untreated cells}} \times 100$$

4.2.2.1.2 Toxicity of paraffin

Determination of the appropriate number of replicates to give statistically significant results was determined. The toxicity of paraffin samples was determined using the MTT assay (section 4.2.3.1.1). The experiment was repeated thrice on three different days to check consistency.

4.2.2.1.3 Determination of a suitable solvent

The second phase of the assay involved dissolving paraffin samples in a suitable solvent. Paraffin is a complex mixture that may contain both hydrophobic and hydrophilic components (Grant *et al.*, 2000). Three solvents (dimethyl sulphoxide, ethanol and methanol) were used in order to investigate a suitable solvent that can adequately solubilise paraffin. This was achieved by mixing paraffin with the solvents using different ratios. The mixtures were mixed thoroughly and allowed to settle. Miscibility was observed and assessed visually by checking layers or phases separation formed after mixing paraffin with the solvents.

4.2.2.1.4 Determination of the least toxic ratio

Paraffin samples were solubilized with ethanol. However, prior to determining the toxicity of solubilized paraffin, the ratio of a mixture of paraffin and ethanol that produces the least toxicity to the cells was determined since ethanol alone exerts toxicity to the cells and in this study we are interested in toxicity exerted by paraffin samples not ethanol. This was achieved using the following method:

1) Cells were grown, trypsinized and seeded into a 96 well microtitre plates.

2) Paraffin was dissolved in ethanol using various ratios.

3) The prepared mixtures were used to treat the cells and incubated overnight at 37^0C.

4) The MTT assay (section 4.2.3.1.1) was performed and the results read.

5) The ratio, which produced the least toxicity to the cells was identified and used in section 4.2.3.1.5.

4.2.2.1.5 Toxicity of solubilized paraffin

Paraffin samples were solubilized with ethanol using a pre-determined ratio (section 4.2.3.1.4). The toxicity of solubilized paraffin samples was determined using the MTT assay (section 4.2.3.1.1). The same method used in section 4.2.3.1.4 was followed. The experiment was repeated thrice on three different days to check consistency.

4.2.2.1.6 Data Analysis: Toxicity of South African paraffin

Multivariate analysis using a general linear regression model for cell toxicity was developed to establish the toxicity of the paraffin samples. The model included sample number and dilution variables. The ANOVA test was done to compare the means of samples to determine if there was any significant difference in the levels of toxicity. The level of significance was taken to be 0.05.

4.2.2.2 Annexin -V- Fluos assay

4.2.2.2.1 The principle

This assay is useful for the identification of apoptotic and necrotic cells. Initial stages of apoptosis involve plasma membrane alterations at the cell surface. Translocation of phosphatidylserine (PS) to the outer layer of the cell membrane is one of these alterations. The PS is normally located at the inner layer of the cell membrane. Annexin V is a phospholopid binding protein with a high affinity for PS. Therefore it can be used as a sensitive probe for PS exposed by apoptotic cells. However, the Annex V can also enter the necrotic cells and bind to the PS exposed on the inner cell membrane due to the loss of cell membrane integrity. Staining with propidium iodide (PI), a DNA stain that is not cell membrane permeable will help distinguish apoptotic cells from necrotic cells (Roche, 2002).

The stain can be used to quantify and analyse apoptotic and necrotic cells by flow cytometry and fluorescence microscopy.

4.2.2.2.2 Analysis by flow cytometry

Cells were grown in 25 cm^2 tissue culture flasks. Once confluent, they were treated with paraffin samples using 1:10 dilution. They were incubated for 6, 12 and 24 hours at 37^0C. Untreated cells were included and used as controls. Cells were trypsinized and stained with 100 µl of Annexin stain (Roche) before being analysed and quantified by flow cytometry. A549 are adherent cells and these tend to form clusters, which interferes with the flow cytometry analysis. In order to rectify this problem, an enzyme (DNAse, Grade 1) (Roche) was used before the analysis. This enzyme caused monodispertion of the clustered cells.

4.2.2.2.3 Analysis by fluorescence microscopy

Cells were grown in 75 cm^2 tissue culture flasks. Once confluent, they were trypsinized. Constant volumes of cells (1000 µl) were seeded in the 24 well tissue culture plates and incubated overnight at 37^0C. Cells were treated with paraffin samples using 1:10 dilution. Untreated cells were also included and used as controls. The number of untreated cells was the same as the number of treated test cells. Cells were incubated for 24 hours at 37^0C. After the incubation period, the media was removed and cells were stained with Annexin stain (100 µl) for 15 minutes at room temperature. Samples were analysed under a fluorescence microscope at a magnification of 40 and areas of interest and significance photographed.

CHAPTER FIVE

5.0 RESULTS

5.1 Chemical composition of South African paraffin

Tables 5.1 and 5.2 show the chemical composition and physical properties of South African paraffin. The results of the chemical composition and physical properties of South African paraffin are demonstrated in Table 5.1, while Table 5.2 shows data from the MSDS sheets of paraffin samples received from each refinery.

Table 5.1. Chemical composition and physical properties of South African paraffin samples.

	REFINERY						
FEATURE	1	2	3	4	5	6	7
*Flash Point (^{0}C)	61	61	54	57	56	70	52
Final Boiling Point (^{0}C)	255	256	210	251	246	231	233
Residue (%w/w)	1.0	1.2	1.2	1.0	1.5	1.0	0.5
Sulphur (%w/w)	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
**Smoke Point	27	28	40	23	25	>50	41
Olefins (%)	1.9	3.6	8.5	2.4	2.3	1.2	2.4
Aromatics (%)	11.7	19.7	1.9	17.3	18.4	1.5	9.1
Paraffin (%)	86.4	76.4	89.6	80.3	79.3	97.3	88.5

*Flash point: The lowest temperature at which a test flame ignites the sample vapor under test conditions

**Smoke point: The maximum flame height in millimeters (mm) at which paraffin burns without smoking

Table 5.2. Data from the MSDS sheets of paraffin samples received from each refinery.

FEATURE	1	3	4	5	6	7
Appearance	Clear, waterlike liquid	Clear Liquid	Clear & bright liquid mild	Clear white	Clear white	Colourless
Odour	-	Petroleum hydrocarbon	-	Characteristic	Characteristic	Characteristic
Boiling Point (^{0}C)	-	150 – 280	-	150 – 300	150 – 300	-
Flash Point (^{0}C)	-	>43	>46	>38	>38	-
Density (kg/l)	0.79 – 0.80	0.78 – 0.84	0.79	0.77 – 0.84	0.77 – 0.84	0.80
Viscosity @ 40^{0}C	1.2	1.5 – 3.5	1.5	-	1 – 2	-
Sulphur mg/kg	-	-	-	<10	<10	-
Total Aromatics (%v/v)	-	-	-	0.5	<0.5	-
Benzene (%v/v)	-	<0.01	-	-	-	-
N - Hexane	-	<0.01	-	-	-	-
Polycyclic compounds (%v/v)	-	<0.01	-	-	-	-

Table 5.1 revealed that paraffin derived from coal (sample 6) had the lowest level of aromatic hydrocarbons. Other samples with less than 10% aromatic content included sample 3 and paraffin derived from the sea (sample 7). Apart from sample 1 which had about 12%

aromatic content, the rest of the samples were found to have similar compositional limits for the concentration of aromatics (20%). The order of aromatic content was as follows: sample 6 < sample 3 < sample 7 < sample 1 < sample 4 < sample 5 < sample 2. Interestingly, sample 3, one of the samples with less than 10 % aromatic content had the highest levels of olefins unlike the rest of the samples. Paraffin derived from coal was found to have the least olefins. The sulfur content on the other hand was the same in all samples at less than 0.1 %. Physical properties including appearance, odor, density and viscosity features are shown in Table 5.2.

5.2 Toxicity of South African paraffin

5.2.1 Sample size determination

The number of replicates of each sample at each dilution required to show statistical significance with a precision of 0.05 were 52. Alternatively the common standard deviation was calculated as 0.013. Since it was very large, it was halved to 0.0065, which translated to a sample size of 26 replicates. Seven (7) paraffin samples, 5 different dilutions in triplicates and 1 cell line were used.

5.2.2 Cytotoxicity Studies

5.2.2.1 Toxicity of paraffin

The following graphs (Figures 5.1 – 5.3) show the results of the toxicity of South African paraffin using the MTT assay. The experiments were repeated thrice on three different days in order to check consistency.

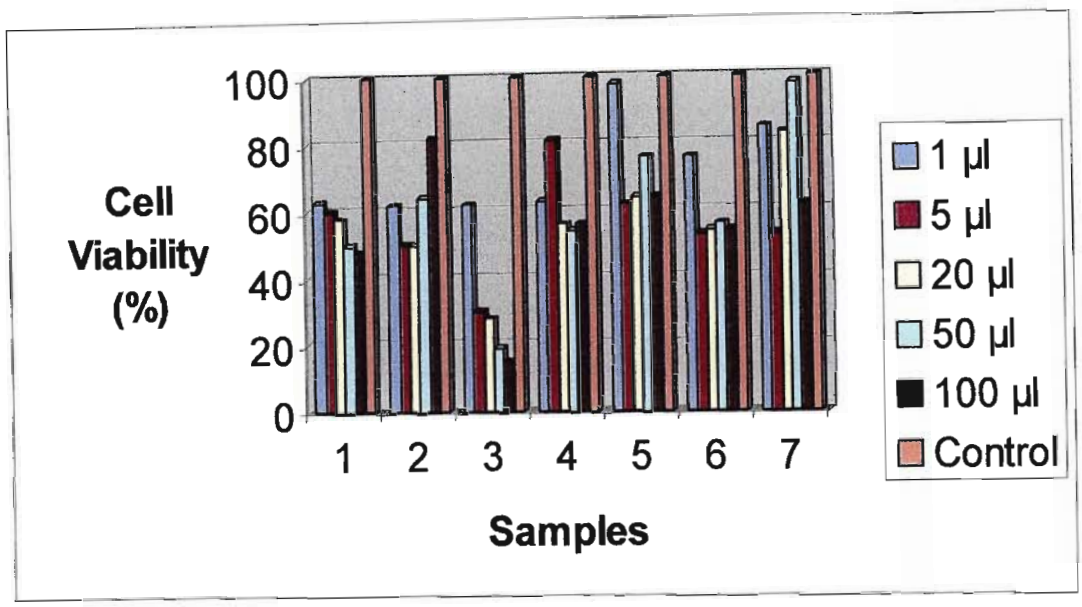

Figure 5.1. Toxicity of South African paraffin using the MTT assay.

At the most concentrated dilution (1:2, 100 µl), the paraffin sample number 3 proved highly toxic with only 11 % of the cells viable, followed by sample numbers 1 and 4. On the other hand paraffin sample number 2, followed by sample numbers 5 and 7 were the least toxic. However at the least concentrated dilution (1:200, 1µl), samples numbers 5 and 7 were the least toxic and the most toxic were samples 1, 2, 3 and 4 (Fig. 5.1).

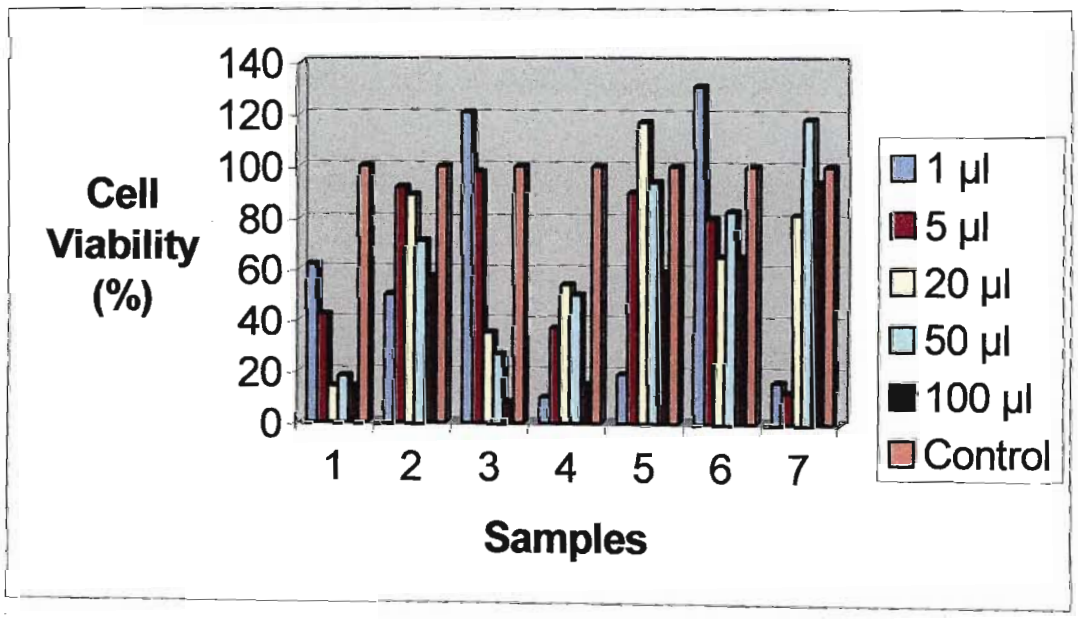

Figure 5.2. Toxicity of South African paraffin using the MTT the assay.

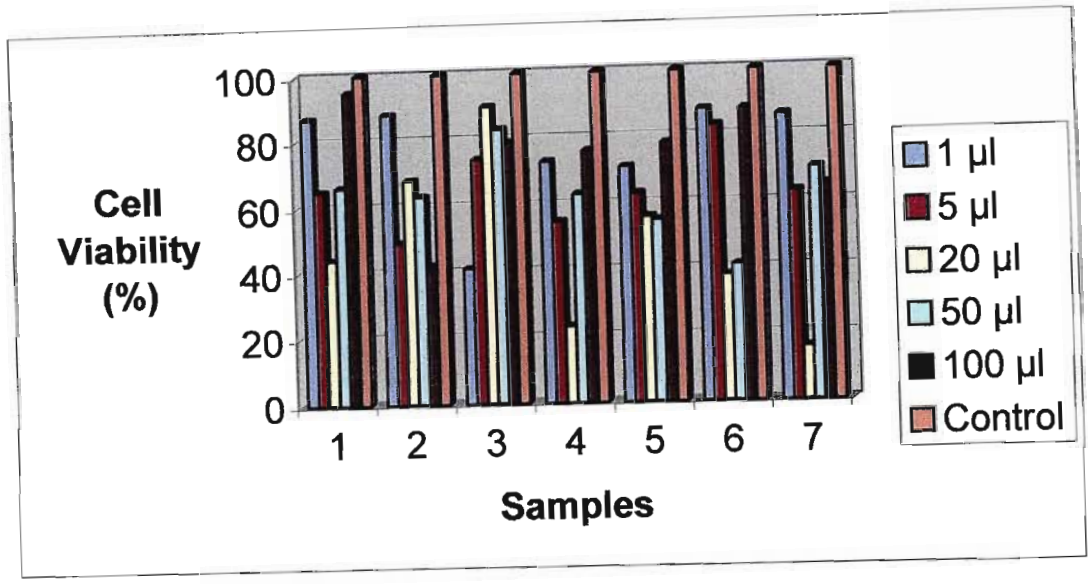

Figure 5.3. Toxicity of South African paraffin using the MTT assay.

At the most concentrated dilution, again samples 1, 3 and 4 were found to be most toxic and interestingly samples 2, 5, 6 and 7 were again the least toxic. Samples 4, 5 and 7 were found to be most toxic at the least concentrated dilution and samples 3 and 6 were the least toxic (Fig. 5.2).

Fig. 5.3 on the other hand shows that at the most concentrated dilution, the most toxic was sample number 2 and the least toxic were samples number 1 and 6. At the least concentrated dilution sample 3 was the most toxic and samples 1, 2, 6 and 7 were the least toxic (Fig. 5.3).

All paraffin samples exerted toxicity to the lung cell line however the degree appeared to be determined by the dilution and type of paraffin. All samples exerted toxicity to the cells even at the least concentrated dilution. General trend in all three experiments was observed. In two out of three experiments conducted, sample 1, 3 and 4 were the most toxic samples at the most concentrated dilution. Interestingly samples 3 and 4 were still found to be amongst the

most toxic samples at the least concentrated dilution. On the other hand, the least toxic samples at the most concentrated dilution were samples 2, 5 and 6 while at the least concentrated dilution sample 6 and 7 were the least toxic. Overall based on the above results samples 3 and 4 were associated with high toxicity and sample 6 with the least toxicity.

5.2.2.2 Results of statistical analysis

A statistical analysis of the results indicated that there were significant differences ($p<0.001$) in the levels of toxicity between the paraffin samples as well as between the dilutions used. The data presented indicate that the toxicity exerted by samples at different dilutions differed significantly however there were no significant differences in the toxicity exerted within the individual samples. This data indicates that the type or formulation of paraffin is the single most important factor, followed by dilution, determining the degree of toxicity. The toxicity of paraffin samples was increased when samples were dissolved in ethanol.

Table 5.3. Comparison of the toxicity of samples

Multiple Comparisons

Sample (dependent variable)	**Sample (independent variable)**	**Mean diff.**	**P**
1	3	0.02250	0.000
	8	-0.3389	0.000
2	3	0.02581	0.000
	6	0.00840	0.019
	8	-0.03059	0.000
3	1	-0.02250	0.000

	2	-0.02581	0.000
	4	-0.01922	0.000
	5	-0.02304	0.000
	6	-0.01741	0.000
	7	-0.02470	0.000
	8	-0.05639	0.000
4	3	0.01922	0.000
	8	-0.03717	0.000
5	3	0.02304	0.000
	8	-0.03335	0.000
6	2	-0.00840	0.019
	3	0.01741	0.000
	7	-0.00729	0.041
	8	-0.03899	0.000
7	3	0.02470	0.000
	6	0.00729	0.041
	8	-0.03169	0.000
8	1	0.03389	0.000
	2	0.03059	0.000
	3	0.05639	0.000
	4	0.03717	0.000
	5	0.03335	0.000
	6	0.03899	0.000
	7	0.03169	0.000

*. The mean difference is significant at the 0.05 level.

Table 5.4. Comparison of the toxicity of samples at each dilution

Sample (Dependent variable)	Sample (independent variable)	Mean diff.	Significance
S1 100 µL	2	-0.02288	0.000
	3	0.02215	0.000
	8	0.01506	0.006
S2 100 µL	1	0.02288	0.000
	3	0.04504	0.000
	4	0.02335	0.000
	5	0.01573	0.000
	6	0.02187	0.000
	7	0.01929	0.000
	8	0.03794	0.000
S3 100 µL	1	-0.02215	0.000
	2	-0.04504	0.000
	4	-0.02169	0.000
	5	-0.02931	0.000
	6	-0.02317	0.000
	7	-0.02575	0.000
S4 100 µL	2	-0.02335	0.000
	3	0.02169	0.000
	8	0.01460	0.007
S5 100 µL	2	-0.01573	0.000
	3	0.02931	0.000

	8	0.02221	0.000
S6 100 µL	2	-0.02187	0.000
	3	0.02317	0.000
	8	0.01608	0.003
S7 100 µL	2	-0.01929	0.000
	3	0.02575	0.000
	8	0.01865	0.001
S8 100 µL	1	-0.01506	0.006
	2	-0.03794	0.000
	4	-0.01460	0.007
	5	-0.02221	0.000
	6	-0.01608	0.003
	7	-0.01865	0.001

*. The mean difference is significant at the 0.05 level.

5.2.2.3 Toxicity of solubilized paraffin

5.2.2.3.1 Suitable solvent

Ethanol adequately solubilized paraffin compared to the other two solvents (dimethyl sulphoxide and methanol) used in the study (Table 5.5).

Table 5.5. Solubility of paraffin.

Solvent	**Solubility**
Dimethyl sulphoxide	A ring observed separating dimethyl sulphoxide and paraffin with paraffin remaining on top therefore miscibility was not complete.
Ethanol	Paraffin and ethanol mixed completely, no ring separating the two was observed.
Methanol	A ring observed separating methanol and paraffin with paraffin remaining on top therefore miscibility was not complete.

The following graphs (Figures 5.4 – 5.6) show the toxicity of solubilized paraffin samples. The experiments were conducted thrice on three different days.

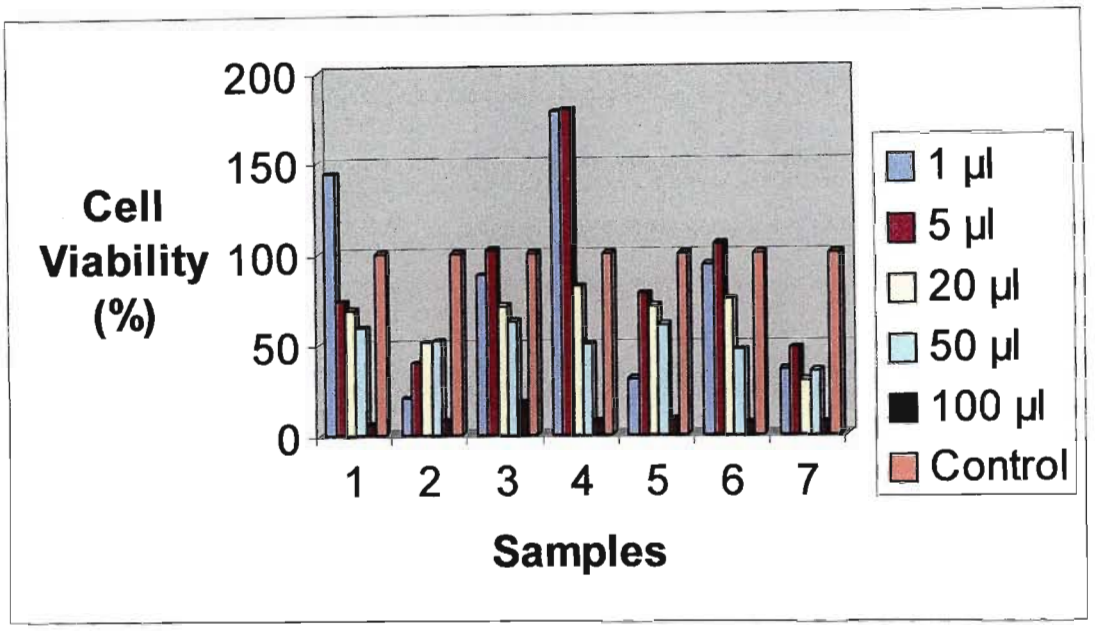

Figure 5.4. Toxicity of South African paraffin dissolved in ethanol using the MTT assay.

At the most concentrated dilution all the samples were highly toxic to the cells and only sample 2 followed by 5 and 7 were the most toxic at the least concentrated dilution, the least toxic being samples 1 and 4 at the same dilution (Fig. 5.4).

Figure 5.5 shows that the most toxic samples at the most concentrated dilution were samples 1, 3, 6, 5 and 7 and the least were samples 2 and 4 at the same dilution. At the least concentrated dilution the least toxic were samples 1, 3, 5 and 6.

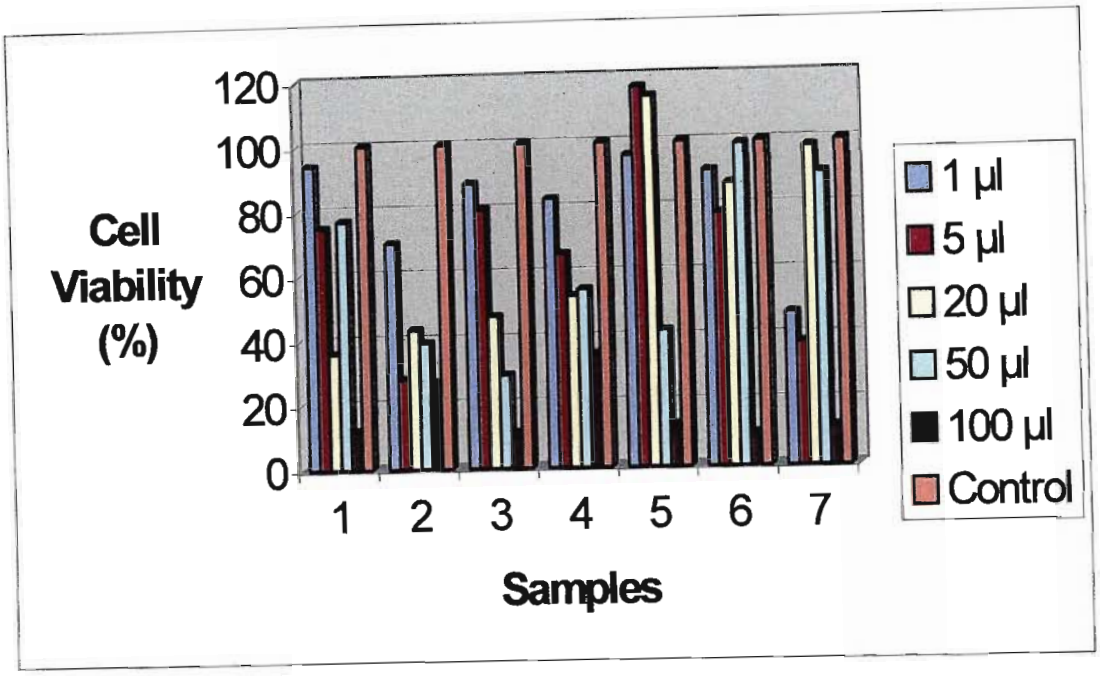

Figure 5.5. Toxicity of South African paraffin dissolved in ethanol using the MTT assay.

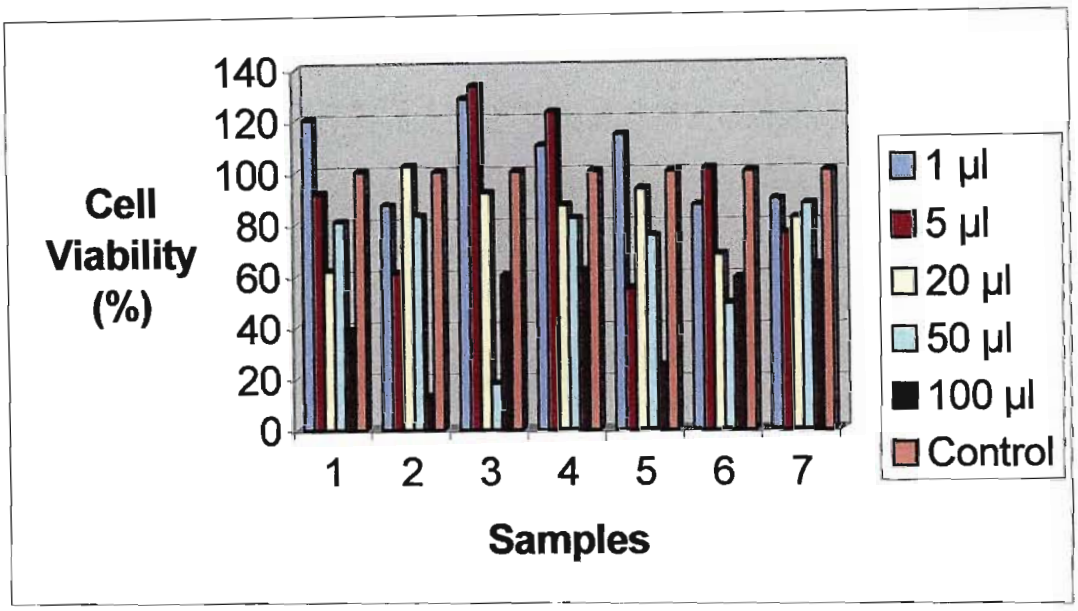

Figure 5.6. Toxicity of South African paraffin dissolved in ethanol using the MTT assay.

Most of the samples were highly toxic to the cells at the most concentrated dilution and at the least concentrated dilution the most toxic were samples 2, 6 and 7 while the least were samples 1, 3 and 5 (Fig 5.6).

Toxicity to the lung cell line was increased at most dilutions when paraffin samples were dissolved in ethanol compared to undissolved paraffin samples. There were very few dilutions where solubilized paraffin samples appeared to be decreasing the toxicity instead of increasing as observed in most dilutions. And most of these were low dilutions.

5.2.2.4 Analysis by flow cytometry

Quantification of Annexin stained apoptotic and necrotic cells, was determined using a flow cytometry. Graphs 5.7 and 5.8 demonstrate the percentage of apoptotic and necrotic cells induced by paraffin samples after 6, 12 and 24 hours.

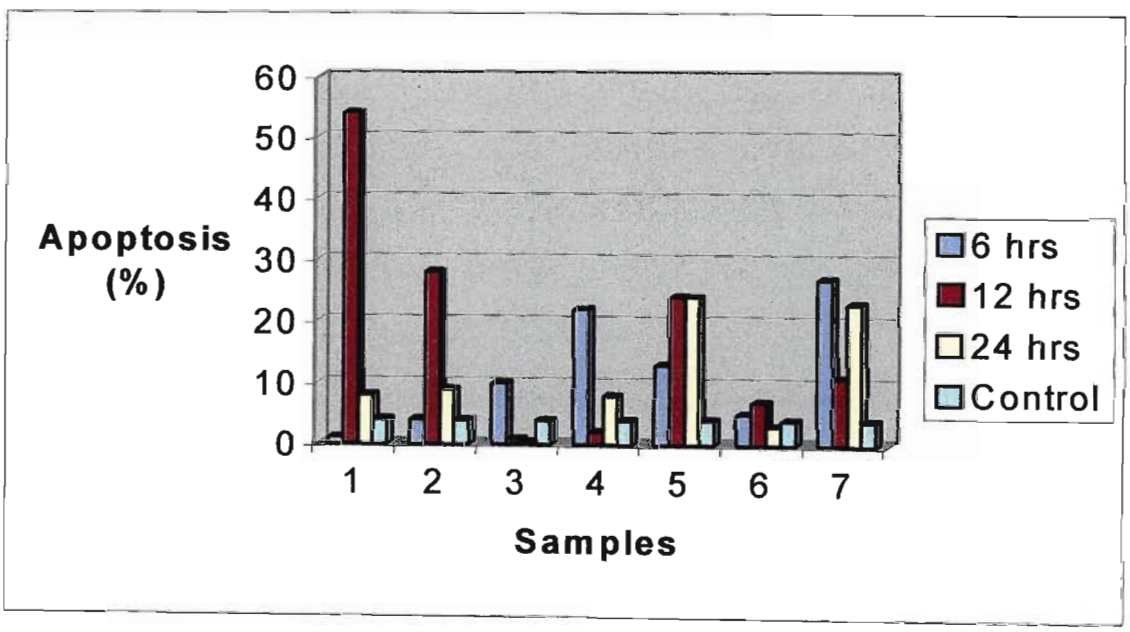

Figure 5.7. Percentage apoptotic cells after treatment with paraffin samples for 6, 12 and 24 hrs.

Apoptosis detected in the control was very low (3.6%) compared to paraffin treated cells. Even though the highest percentage (54%) apoptosis was detected after 12 hours in sample 1, samples 5 and 7 were responsible for producing the most apoptosis when all time incubations were considered while sample 6 produced the least apoptosis. Sample 7

produced the most apoptosis while samples 1, 2 and 6 produced the least apoptosis after 6 hrs. Sample 1 produced the most apoptosis after 12 hrs while samples 3, 4 and 6 produced the least. Samples 5 and 7 induced most apoptosis after 24 hrs while samples 3 and 6 induced the least (Fig. 5.7).

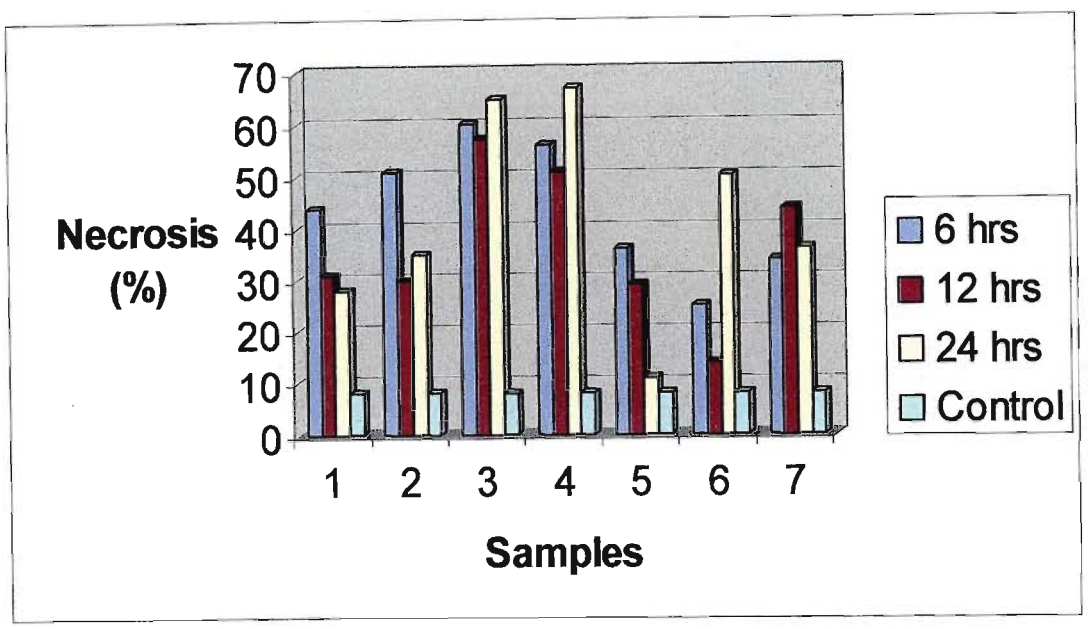

Figure 5.8. Percentage necrotic cells after treatment with paraffin samples for 6, 12 and 24 hrs.

Low necrosis (8.6%) was observed in the control. Samples 3 and 4 were responsible for inducing most necrosis while samples 5 and 6 were associated with the least necrosis. The most necrotic sample after 6 hrs was sample 3, followed very closely by sample 4 and the least being sample 6. Both samples were again found to be responsible for inducing most of the necrosis to the lung cell line after 12 hrs and sample 6 produced the least necrosis. At 24 hrs samples 3 and 4 produced the most necrosis (Fig. 5.8).

Necrosis was more marked than apoptosis even with the controls. Necrosis and apoptosis detected in untreated cells (controls) were very low compared to paraffin treated

cells. The level of apoptosis and necrosis did not appear to increase with time exposure as might be expected. In some of the samples, highest cell death was detected after 6 hours and as time of exposure was increased cell death detected seemed to be decreasing. At this stage it is not clear what could be the reason. However for samples 3 and 4, necrosis detected decreased slightly after 12 hours and then increased again after 24 hours. Apoptosis detected in cells treated with sample 5 appeared to be increasing with time of exposure.

5.2.2.5 Microscopy

5.2.2.5.1 Light microscopy

When viewed microscopically using light microscopy, a difference was observed between treated and untreated cells in terms of their morphology. Paraffin treated cells showed signs of dissociating from neighbouring cells. Their structure was altered as cells shrunk becoming rounder and smaller in size. Paraffin treated cells were floating in the medium (meaning they are dead) and not attached to the surface of the flask. A morphological change was observed even in cells treated with the least concentration of paraffin. The damage was more intense with an increase in paraffin concentration. In some cases the structure of paraffin treated cells was severely damaged to a point that cells looked like dots. The untreated cells on the other hand, were firmly attached to the surface of the flask and their structure or morphology was not affected.

5.2.2.5.2 Fluorescence microscopy

Cells treated with paraffin samples (1 – 7) including a control, are demonstrated in Figure 5.9. Examination of Annexin stained A549 lung cell line by fluorescence microscopy revealed that after 24 hours of incubation all samples induced apoptosis and necrosis. Apoptosis and necrosis were also observed in untreated cells however they were very mild in

untreated cells. Damaged cellular membranes and leaky cellular contents were visible. The PS was detected on the cell membranes indicating apoptosis. Stained DNA of the necrotic cells was evident. Untreated cells (C); Paraffin samples (1-7).

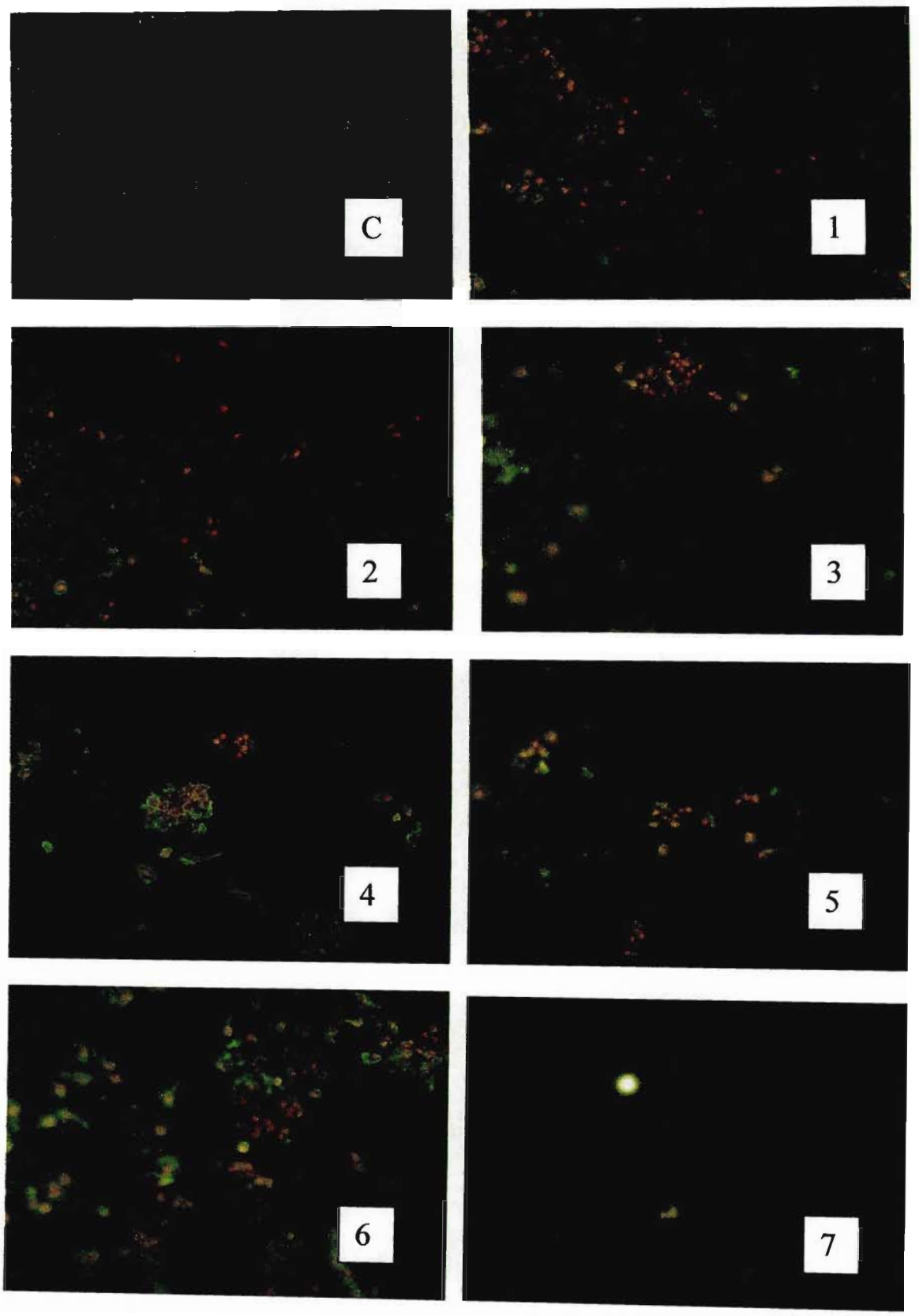

Figure 5.9: Apoptosis and necrosis detected by fluorescence microscopy.

CHAPTER SIX

6.0 DISCUSSION

6.1 Chemical composition of South African paraffin

The results of this study have shown that aliphatic and aromatic hydrocarbons are the predominant components of South African paraffin. Olefins and sulphur components are also present at lower concentrations with sulfur being the lowest, at less than 0.1 %. The aromatic content was found to decrease with the increase of aliphatic content and vice versa, thus showing an inverse relationship between aliphatic and aromatic hydrocarbons. The chemical composition differed from sample to sample indicating that paraffin produced in South Africa is chemically not the same however paraffin with less aromatic hydrocarbons would be ideal in unventilated indoor environment from environment's health point of view. The results in this study have further shown that having a high aliphatic content may not be necessarily safe as one might have originally thought. Samples with highest smoke point were found to have the highest content of aliphatic hydrocarbons and the least aromatic content, with the highest smoke point found in paraffin produced from coal which also had the highest level of aliphatic hydrocarbons. High aliphatic content was associated with an increase in smoke point.

The results of this study are in accordance with (Tsujino *et al.*, 2002; McDougal *et al.*, 2000) findings which found paraffin to be chiefly consists of 80% aliphatic hydrocarbons in the range of $C_9 - C_{16}$ and 20% aromatic hydrocarbons. The results of the smoke point were also in accordance with literature review which states that the smoke point is important because it determines the degree of illumination possible from a given paraffin. It is an indicator of the combustion qualities of paraffin and therefore contributes to indoor air

pollution. The smoke point is related to the hydrocarbon composition of paraffin. It is highest with aliphatic hydrocarbons, lower with naphthenes and even lower with aromatics (Petroleum Bazaar, 2001).

Paraffin had a minimum flash point of 52 ^{0}C at atmospheric pressure. This means that a paraffin lamp or stove will only ignite once the paraffin is soaked at a temperature of 52 ^{0}C or higher. The fact that paraffin ignites at a temperature above 52 ^{0}C should make it relatively safe unless contaminated with liquids such as petrol which ignite easily (Truran *et al.*, 2004). Paraffin has a low viscosity. This means it is very runny or fluid and it spreads easily like water (Truran *et al.*, 2004). The viscosity results were provided by MSDS received from the refineries.

6.2 Toxicity of South African paraffin

The results of the current study suggest, firstly, that paraffin samples are toxic to the lung cell line (Fig. 5.1 – 5.3) and secondly solubilising the paraffin samples further increases their toxicity (Fig. 5.4 – 5.6) to the cells. This is probably because by solubilising the samples one is making them more available to the cells. Of all the solvents used in the study, ethanol was found to solubilise paraffin adequately. This finding correlated with Grant *et al.* (2000) who also found that paraffin is a complex mixture containing hydrophobic and hydrophilic components that can be adequately solubilised by mixing with absolute ethanol. The individual aliphatic hydrocarbons by themselves are completely insoluble in water (Allen *et al.*, 2001) whereas the aromatics are soluble which might contribute to their high toxicity observed in previous studies. Solubilising samples grants access of the previously insoluble components to the cells therefore they are able to increase the toxicity to the cells.

The degree of toxicity produced by paraffin samples differed from sample to sample. The results of the MTT assay which is a simple, reliable and easy to used method developed by Mosmann (1983) but later modified by Hansen (1989) indicate that samples 3 and 4 produced high toxicity to the cells while sample 6 produced the least. The assay measures cell viability by quantifying the conversion of the tetrazolium salt (MTT) to purple crystals. The results are read using a spectrophotometer.

Statistical analysis of the results indicates that there were significant differences ($p<0.001$) in the levels of toxicity between the paraffin samples as well as between the dilutions used. The interaction between the type of paraffin used and the specific dilution of paraffin resulted in statistically significance difference in toxic effects ($p<0.001$). This data indicates that the type or formulation of paraffin is the single most important factor, followed by dilution, determining the degree of toxicity. The increase in toxicity of paraffin dissolved in ethanol was confirmed by statistical analysis of the data, which showed significant differences ($p<0.001$) between the samples dissolved in ethanol and those not dissolved.

There are no studies reporting on the role of paraffin on apoptosis and necrosis. Staining procedures can be useful in the determination of apoptosis or necrosis. There are many approaches to analysis of apoptosis in varying pathologic conditions, but none can exceed morphologic examination. Nuclear shrinkage and budding, loss of cell shape and blebbing are all hallmarks of the apoptotic cell (Hengartner *et al.*, 2000). These are features of late stage apoptotic cells (Geske *et al.*, 2001). Helpful additional methods in analysis include the terminal deoxynucleotidyl transferase-mediate dUTP nick end labeling (TUNEL) assay for identification of DNA fragmentation, annexin-V assays for detection of externalised PS and recently antibodies. They also identify later stages of apoptosis (Kravtsov *et al.*, 1999).

In the current study the apoptotic and necrotic cells induced by paraffin samples after 6, 12 and 24 hrs were therefore investigated. Apoptosis and necrosis were both induced in paraffin treated cells samples however necrosis was more marked than apoptosis.

Flourescence microscopy showed that paraffin samples were able to induce apoptosis and necrosis to the cells. Morphological characteristics observed when examinations were made by microscopy lead to the identification of the type of cell death (apoptosis or necrosis). The structure of the cell membrane appeared to be damaged or altered, causing the cell to become leaky. This observation was confirmed by the detection of PS in the outer layer of the cell membranes. It was further confirmed by the detection of the leaky contents of the cell.

The aim of this study was to determine an association if any between the predetermined chemical composition and toxicity of South African paraffin since in South Africa there are no studies demonstrating a relationship between the chemical composition and toxicity of paraffin.

The chemical composition which has been established in this study as the most important factor determining the degree of toxicity can in turn be influenced by the source and processing of paraffin. This makes evaluation and comparison of the sources and processes of paraffin currently in use critical and important for the determination of the association between the components and toxicity.

As a first step towards determining an association between the chemical composition and toxicity of paraffin, the chemical composition of samples found to be producing high toxicity and those producing the least toxicity to the cells was compared.

Increased aromatic and olefin content is associated with increased toxicity based on the findings. Reducing only one of these components is not enough to guarantee minimum toxicity. Both components (aromatics and olefins) are potentially toxic and should be minimized.

There was no association determined between aliphatic hydrocarbons and toxicity exerted on cells. However, an association between aliphatic hydrocarbons and a potential indoor air pollution threat was observed. The association between sulphur and toxicity was not considered at this stage, as it was the same in all samples and at very low levels.

The results of the study provide compelling evidence that the chemical composition and degree of toxicity produced by South African paraffin manufactured at different refineries differs. Their differences have the potential to lead us to the identification of toxic components of paraffin and at the same time advance our understanding on their role on the mechanisms involved in the toxicity of paraffin.

Understanding the toxicity of South African paraffin is therefore very important for its eventual improvement of quality and safety. Currently our understanding is very limited because of lack of research. While we know that paraffin poisoning as a result of ingestion may cause either mild symptoms or if there are complications, may prove to be lethal (Stones *et al.*, 1987), however we still need to know the mechanisms involved in the toxicity of paraffin that might possibly be responsible for symptoms and complications caused by paraffin poisoning. This is only possible through research.

Major routes of paraffin exposure include inhalation and dermal absorption (Koschier *et al.*, 1999). However most toxicology studies have focused on its capacity for pulmonary

toxicity (Allen *et al.*, 2001) and have used animal models. In the present study, the lung cell line was used to study the toxicity of paraffin *in vitro*.

Based on the type of processing paraffin may be categorized into two general classes: straight-run and cracked. Straight run paraffin is produced from the distillation of crude oil at atmospheric pressure. Alternatively, it is possible to produce paraffin by processes, which break down larger and more complex molecules by catalytic or thermal methods (generically referred to as "cracking") (Nessel *et al.*, 1998).

Studies have indicated that straight-runs generate skin tumors through a non-genotoxic mechanism while cracked paraffin may also produce tumors but through a genotoxic mechanism. Straight-runs produce skin tumors through a non-genotoxic mechanism since they have low or no activity in the mutagenicity assay (McKee *et al.*, 1989, 1994; Deininger *et al.,* 1991), which means they lack tumor-initiating activity (McKee *et al.*, 1989). This is due to low concentrations of carcinogenic polycyclic aromatic hydrocarbons (PAH) present in straight-runs (McKee *et al.*, 1994). However cracked paraffin or products have been associated with producing tumors through a genotoxic mechanism because they contain substantial levels of carcinogenic PAH, making cracked paraffin potentially more toxic than straight-run paraffin. Straight-run paraffin contains low concentrations of aromatics compared to cracked paraffin because it is produced using lower boiling points while cracked paraffin is produced using higher boiling points (McKee *et al.*, 1994).

This provides evidence that associates aromatic compounds with high toxicity. It further provides evidence making the type of processing a determinant of the degree of paraffin toxicity as it determines the type and level of chemical components that will constitute paraffin.

Shell petroleum, has introduced odourless paraffin, which is produced from coal. Claims that producing or synthesizing paraffin from coal reduces the aromatic content were proven by the results of the current study in which paraffin produced from coal was found to have the least aromatic content when compared to the rest of the paraffin samples. This qualifies the source of paraffin as one of the determinants of paraffin toxicity.

Studies focusing on the toxicity of the chemical components of paraffin have the ability to provide clarity as far as the mechanism involved in the toxicity of paraffin is concerned. Such studies can lead to sound recommendations to the South African petroleum industry supported by reliable scientific evidence.

Studies including Tsujino *et al.* (2002) and McKee *et al.* (1994) have researched on the toxicity of the major components of paraffin and a majority of these studies have associated aromatics with high toxicity. Therefore we have hypothesized that removing the aromatics from paraffin will make it less toxic and therefore safer.

Since dermal exposure is another major route of exposure to paraffin, distribution of the major components (aliphatic and aromatic hydrocarbons) of paraffin following dermal exposure has been evaluated. The aliphatic and aromatic hydrocarbons (benzenes) were absorbed through the skin and detected in blood and tissues. However the fractions of aromatics absorbed through the skin were greater in quantity than those of aliphatic hydrocarbons (Tsujino *et al.*, 2002), possibly due to their solubility characteristics. Their results accorded with those reported by Kimura *et al.* in which the fractions of aliphatic hydrocarbons detected in blood were much less than those of aromatics in rats that had experimentally inhaled paraffin (Kimura *et al.*, 1991). These were further confirmed by

McDougal *et al.* whose results also demonstrated that aromatic components penetrated rapidly and all aliphatic hydrocarbons were identified in the skin when paraffin based fuel was used (McDougal *et al.*, 2000).

CHAPTER SEVEN

7.0 CONCLUSION

7.1 Concluding summary

The present investigation has advanced the understanding of toxicity of South African paraffin. Research has involved the use of fluorescent microscopy and some biochemical techniques to reveal morphological and perhaps physiological changes that cells undergo upon exposure to South African domestic paraffin.

Decreasing both levels of aromatics and olefins is necessary in order to minimize the toxicity of paraffin. The role of aliphatics in cytotoxicity is not clear from the current study. However, their association with air pollution was established. An increase in aliphatic hydrocarbons minimizes the smoke given off by paraffin therefore lowering the potential for pollution.

7.2 Recommendations

The recommendations for producing less toxic paraffin are to:

a) Decrease aromatic hydrocarbons from paraffin to levels less than 2%.

b) Decrease olefins from paraffin to levels less than 2%.

c) Keep sulphur content at levels of less than 0.1%.

d) Keep the present levels of aliphatic hydrocarbons until further research.

e) Sources and processes of paraffin leading to the production of paraffin with the least aromatic and olefin components are encouraged.

f) Increase the viscosity of paraffin.

7.3 Further Studies

Fully understanding the toxicity of paraffin means being able to identify the constituents of paraffin responsible for the toxicity and also determining at what levels these constituents become toxic. It also means understanding the mechanisms of toxicity involved and which components are responsible for each. Before one can fully understand the above so that applicable and sound recommendations can be made, major research priorities must include the following:

a) Obtain data of the exact aromatics, olefins and aliphatic hydrocarbons and their levels that have been determined in the paraffin samples as this would account for the differences in toxicity between these samples.

b) Determining the toxicity of the individual components identified, using cell lines.

c) Finally, determine the toxicity of South African paraffin using skin cell lines, as skin contact is another major route of exposure.

CHAPTER EIGHT

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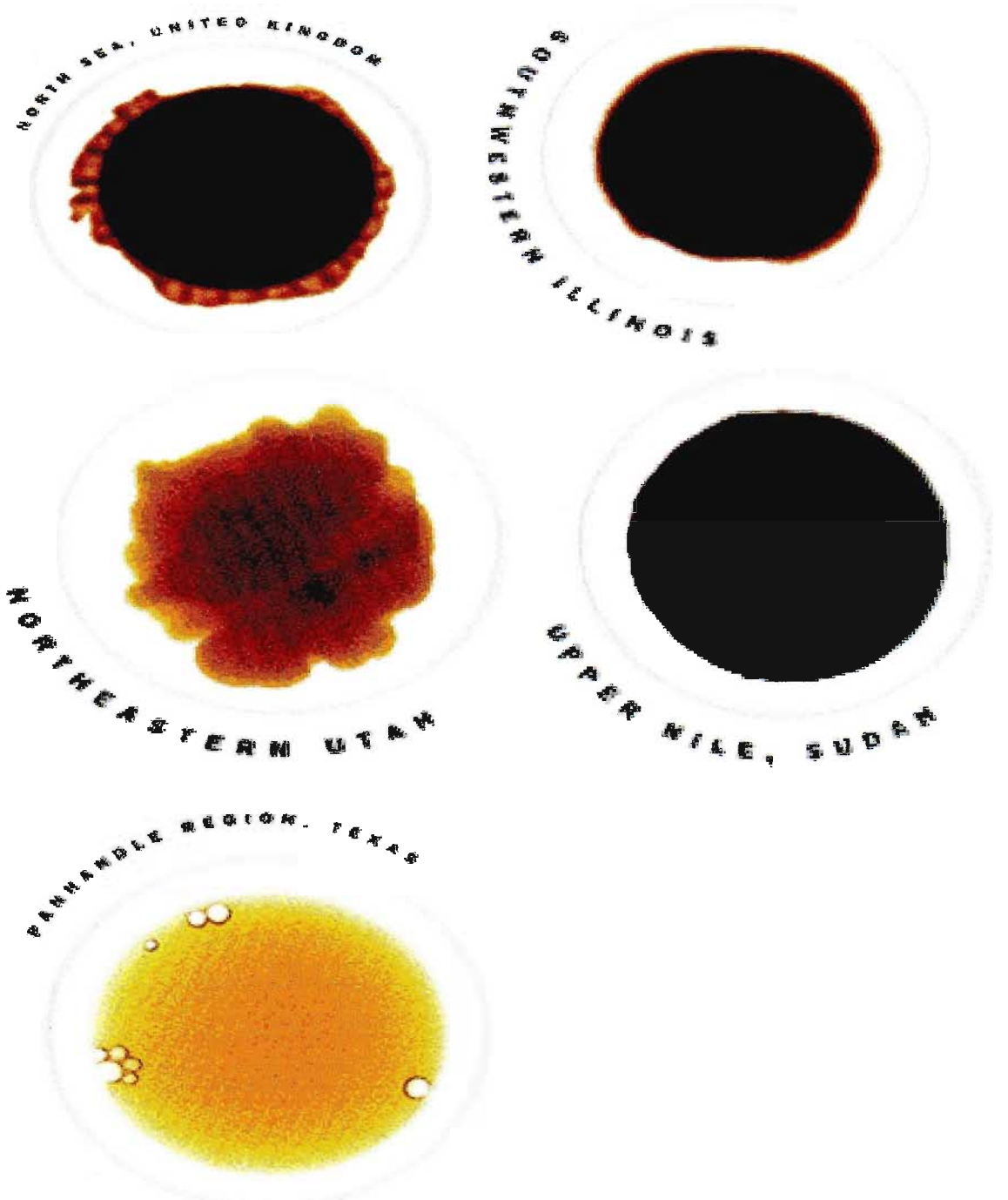
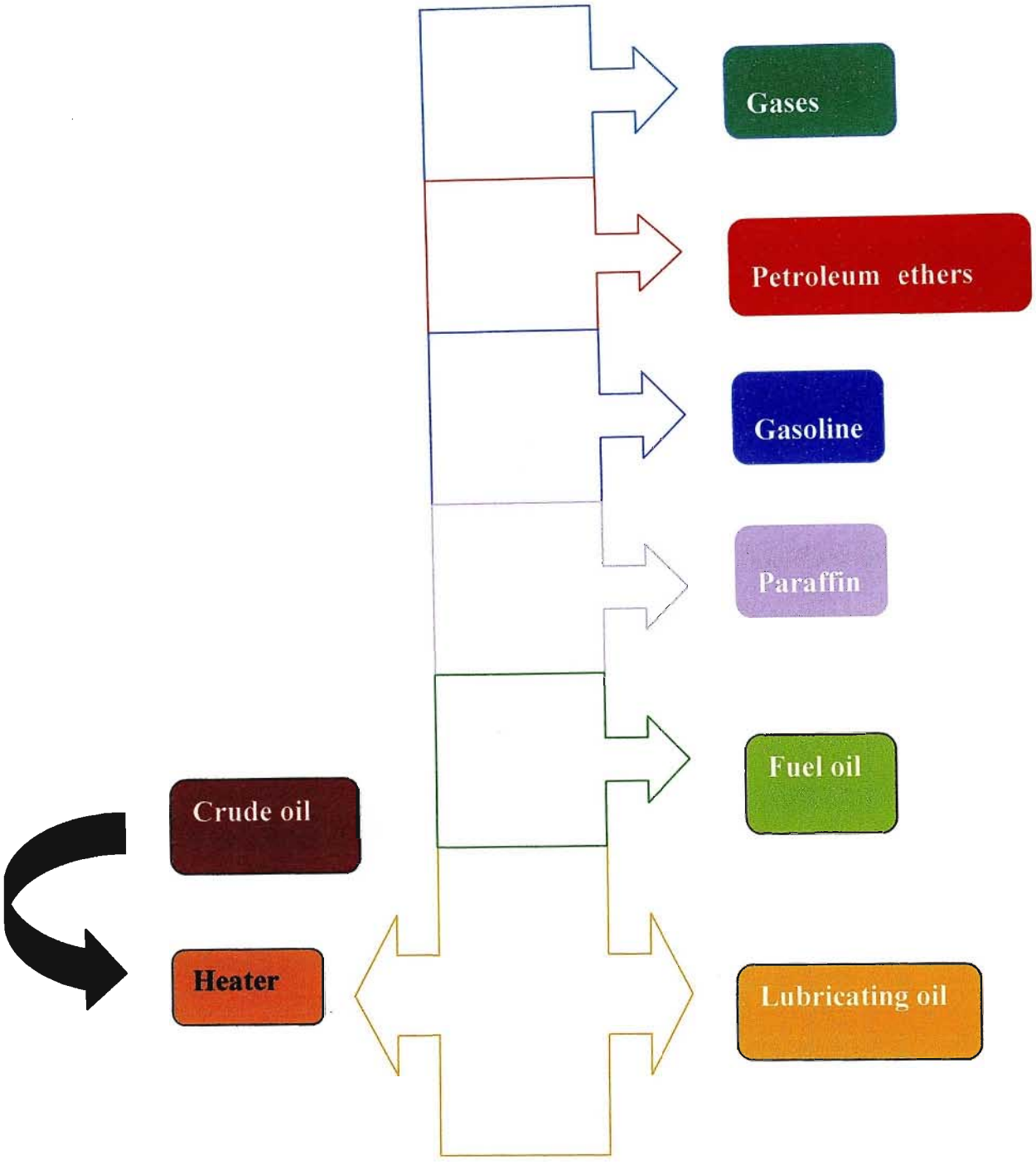
CHAPTER NINE

9.1 ANNEXURES



Annexure 2.1. Crude oil deposits found from different parts of the world (Chevron, 2002)



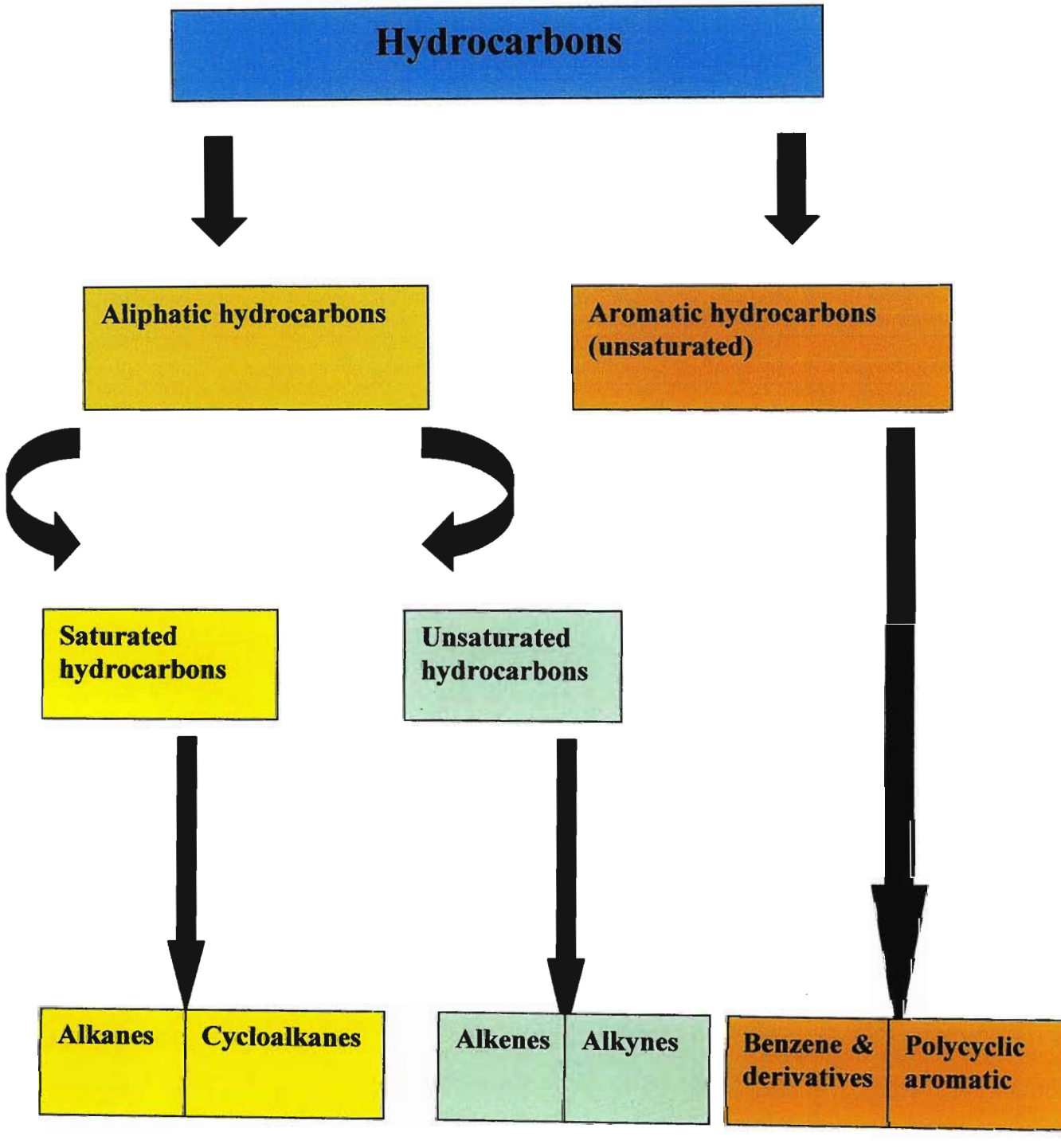
Annexure 2.2. Distillation fractions produced during the refining process (Routh *et al.*, 1971)

Annexure 2.3. Summary of characteristics of uses of crude oil distillation fractions from a fractionating column (Routh *et al.*, 1971)

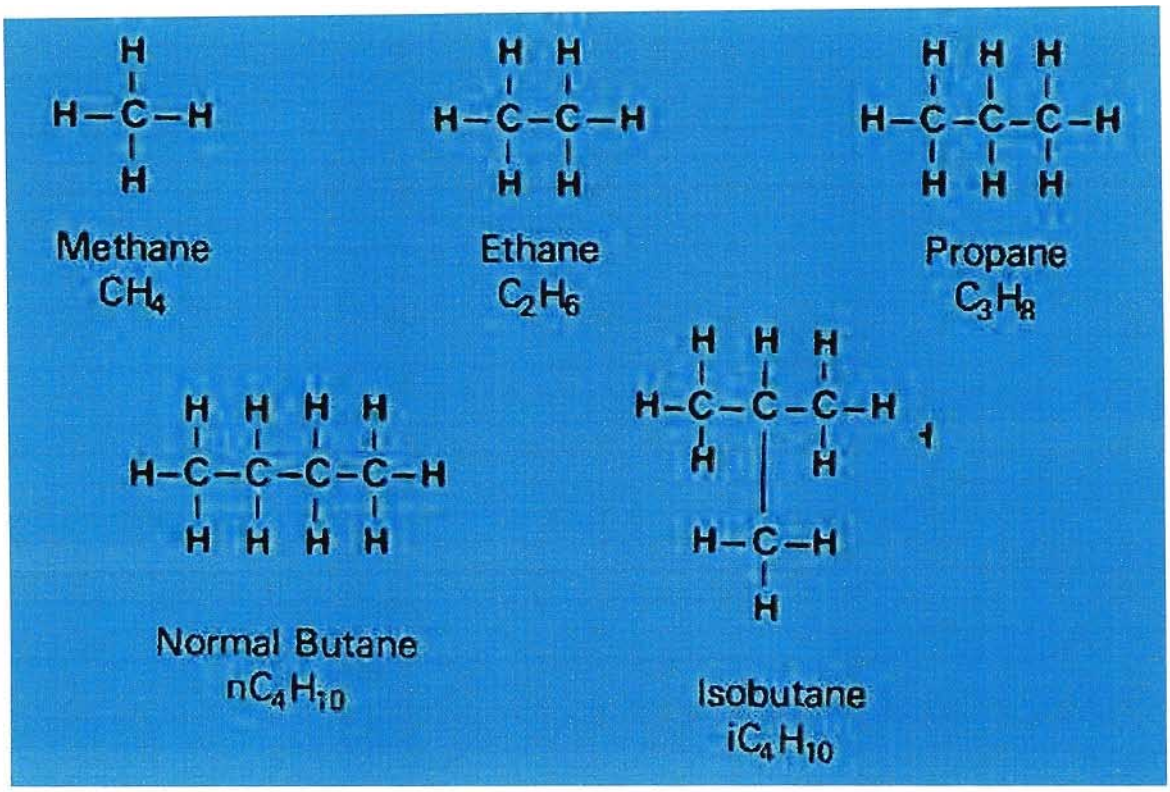
NAME	COMPOSITION (%) & BOILING RANGE (^{0}C)	MOLECULAR SIZE	USES
Gases	2 (0)	$C_1 - C_5$	Fuel
Petroleum ethers	2 (30 –110)	$C_5 - C_7$	Solvents
Gasoline	32 (30 –200)	$C_6 - C_{12}$	Motor fuel
Paraffin	18 (175 –275)	$C_{12} - C_{15}$	Diesel & Jet fuel
Fuel oil	20 (250 –400)	C_{15} -	Fuel
Lubricating Oils	- (300 -)	C_{19} -	Lubricants, Petrolatum

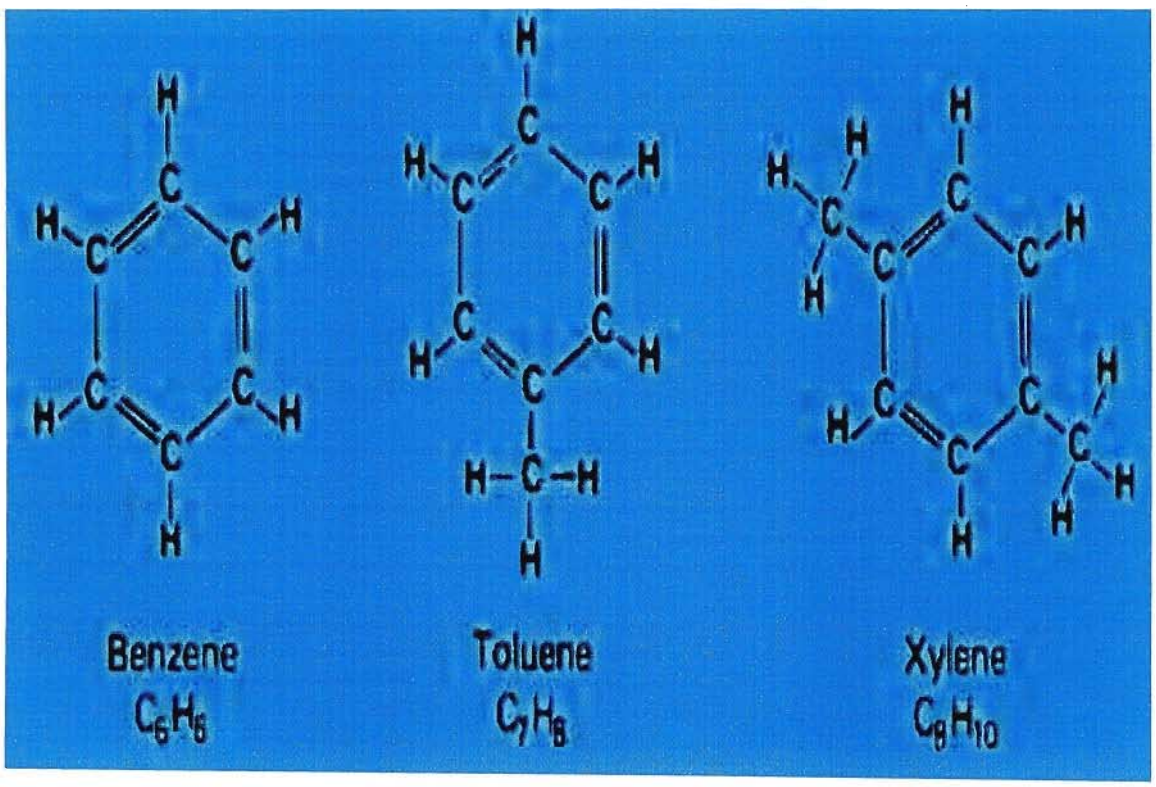
Annexure 2.4 Major physical properties and chemical components of paraffin (Ritchie *et al.*, 2003)

Distillation temperature	340 – 572 ^{0}F
Physical state	White to light yellow liquid
Odor description	Light hydrocarbon odor
Range	$C_6 - C_{16}$
Paraffins	55.2 % (w/w)
Naphthenes	40.9 % (w/w)
Aromatics: Aromatics: Benzene Toluene *p*-, *m*-, *o*-Xylenes Trimethylbenzenes	3.9 % (w/w) 17 – 25 % (v/v) 0.1 – 1 % 0.8 – 0.9 % 3.5 % < 2 %
Sulphur	< 0.05 %
Naphthalene	1 – 3 %
n – Hexane	< 0.1 %
Applications	Heating, cooking and illuminating fuel



Annexure 2.5. Classification of hydrocarbons (Ebbing *et al.*, 1999)





Annexure 2.6. Group of aliphatic and aromatic hydrocarbons (AIP, 2002)

Annexure 2.7. Requirements for South African paraffin (SABS CKS 78-1972)

Feature	Requirements
Distillation Final boiling point, ^{0}C, max Residue, % v/v, max	 280 2.0
Flash point Abel, ^{0}C, min TAG, ^{0}C, min	 43 43
Sulphur content, % m/m, max	0.1
Smoke point	See Table 2.4

Annexure 2.8. Requirements for smoke point of paraffin (SABS CKS 78-1972)

Sulphur content, % m/m			**Smoke point, mm, min**
0	–	0.015	25
0.016	–	0.032	27
0.033	–	0.049	29
0.050	–	0.066	31
0.067	–	0.083	33
0.084	–	0.01	35

Annexure 2.9. Canadian requirements for paraffin (CAN/CGSB - 3.3 - 99)

Feature	**Limit**
Flash point, ^{0}C, min	38
Distillation end point, ^{0}C, max	300
Sulphur, % (w/w), max Type No. 1-K Type No. 2-K	 0.04 0.3
Smoke point, mm, min	19.0

Annexure 2.10. Requirements for Malaysian paraffin (MS 117: 1998)

Feature	**Limits**
Appearance	Bright and clear
Flash point, ^{0}C, min Grade 1 Grade 2	 38 38
Distillation Recovery at 200 ^{0}C, % by mass, min Grade 1 Grade 2	 15 15
Sulphur, % by mass, max Grade 1 Grade 2	 0.15 0.2
Smoke point, mm, min Grade 1 Grade 2	 25 20

Annexure 2.11. Specifications for USA paraffin (ASTM D3699 - 02)

Feature	**Limit**
Flash point, ^{0}C	38
Distillation temperature (^{0}C) 10 % volume recovered, max Final boiling point, max	 205 300
Kinematic viscosity at 40 ^{0}C, mm^2/s min max	 1.0 1.9
Sulphur, % mass No. 1-K No. 2-K	 0.04 0.30

Annexure 2.12. Requirement for South African paraffin as proposed by SABS (SABS 1913: 200X)

Feature	**Limit**
Flash point, ^{0}C, min	43
Distillation Final point, ^{0}C, max Residue, % (by volume), max	 280 2.0
Sulphur content, % (by mass), max	0.05
Smoke point, min	37

Annexure 2.13. Main energy sources used by households for cooking in 1999 (Statistics South Africa, 2001)

ENERGY SOURCE	**% of HOUSEHOLDS**
Electricity	53
Paraffin	**21.1**
Wood	19.5
Other fuels	6.4

Annexure 2.14. Main energy sources used by households for heating in 1999 (Statistics South Africa, 2001)

ENERGY SOURCE	**% of HOUSEHOLDS**
Electricity	48
Wood	21.9
Paraffin	**13.5**
Other fuels	16.6

Annexure 2.15. Main energy sources used by households for lighting in 1999 (Statistics South Africa, 2001)

ENERGY SOURCE	% of HOUSEHOLDS
Electricity	69.8
Wood	20.1
Paraffin	**9.8**
Other fuels	0.3

Annexure 2.16. Paraffin using households by province lighting in South Africa (Statistics South Africa, 2001)

Province	No. of households	% of households
Eastern Cape	514,529	38.6
Free State	223,265	35.7
Gauteng	379,994	19.3
KwaZulu Natal	296,017	17.8
Mpumalanga	104,321	17.3
Northern Cape	33,091	17.7
Northern Province	241,400	24.6
North West	264,253	36.7
Western Cape	135,272	13.8
Total	2192142	24.2

Annexure 2.17. Paraffin using households by race group in South African (Statistics South Africa, 1996)

Race Group	No of households	% of households
Black	1886002	28.9
Coloured	49117	6.6
Indian	1228	0.5
White	1714	0.31

Annexure 18. The sales of major petroleum products in S.A. in 1999 (South African Petroleum Industry Association, 2004)

Product	**Quarters (Volumes in m/L)**				**Total/Year**
	1st	**2nd**	**3rd**	**4th**	
Petrol	2685	2674	2725	2777	10861
Diesel	1395	1455	1546	1597	5993
Jet Fuel	498	499	494	504	1995
Paraffin	**242**	**282**	**290**	**240**	**1054**
Fuel oil	137	144	145	135	561
LPG	114	131	160	135	540
Bitumen	55	59	50	49	213
TOTAL	5126	5244	5410	5437	21217

Annexure 19. The sales of major petroleum products in S.A. in 2003 (South African Petroleum Industry Association, 2004)

Product	**Quarters (Volumes in m/L)**				**Total/Year**
	1st	**2nd**	**3rd**	**4th**	
Petrol	2598	2586	2640	2843	10667
Diesel	1724	1759	1830	1950	7263
Jet Fuel	527	493	545	534	2099
Paraffin	**168**	**197**	**218**	**186**	**769**
LPG	127	143	159	129	558
Fuel oil	127	132	143	126	528
Bitumen	63	70	70	69	272
TOTAL	5334	5380	5605	5887	22156

Annexure 20. The sales of major petroleum products in S.A. in 2004 (South African Petroleum Industry Association, 2004)

Product	Quarters (Volumes in m/L)				Total/Year
	1st	2nd	3rd	4th	
Petrol	2698	2662	2769		
Diesel	1816	1882	2006		
Jet Fuel	529	497	505		
Paraffin	**183**	**210**	**231**		
LPG	118	144	166		
Fuel oil	108	162	166		
Bitumen	65	75	65		
TOTAL	5517	5632	5908		

Annexure 2.21. Acute toxicity of paraffin (WHO, 1996)

Paraffin	**Species**	**Route**	**Dose**
Paraffin	Rat	Oral	50 000 mg/kg bw
Paraffin	Guinea-pig	Oral	16 320 mg/kg bw
Paraffin	Rabbit	Oral	22 720 mg/kg bw
Straight-run Paraffin	Rat	Oral	> 5000 mg/kg bw
Hydrodesulphurised Paraffin	Rat	Oral	> 5000 mg/kg bw
Straight-run Paraffin	Rabbit	Dermal	> 2000 mg/kg bw
Deodorised Paraffin	Rat	Inhalation	0.1 ml/L (saturation)

Annexure 2.22. Summary of effects of combustion products of paraffin (Ritchie *et al.*, 2003)

Pollutant	Mechanism	Potential Health Effects
Carbon monoxide	Binding with Hb to produce COHb, which reduce O_2 delivery to key organs and developing fetus	Low birth weight (fetal COHb 2-10 %) Increase in perinatal deaths Asphyxiation high
Nitrogen dioxide	Acute exposure increases bronchial reactivity of asthma Longer term exposure increases susceptibility to bacterial and viral lung infections	Wheezing and exacerbation Respiratory infections
Soot	Causes biochemical and pathological changes in the heart and lungs	Lung and heart disorders Genetic disorders
Sulphur dioxide	Acute exposure increases bronchial reactivity Longer term difficult to dissociate from particulate effects	Wheezing and exacerbation of asthma Exacerbation of COPD, CuD
Polycyclic aromatic	Metabolised by cytochrome	Presents a human cancer risk
Hydrocarbons	P_{450} to toxic eletrophilic intermediates that mediate cell damage	Oral surfaces, lung, skin

Annexure 4.1. Paraffin samples

Sample	Refinery	Location	Source of paraffin
1	Natref	Sasolburg	Crude oil
2	Caltex	Cape Town	Crude oil
3	Sasol	Sasolburg	Crude oil
4	Engen	Durban	Crude oil
5	Shell; DPK	Durban	Crude oil
6	Shell; odourless	Durban	Coal
7	PetroSA	Cape Town	Sea

DPK: Dual Purpose Kerosene

Annexure 4.2. South Africa's Petroleum Refineries (Pennwell International Petroleum Encyclopaedia; Mbendi Information Services, 2004)

Refinery	Location	Owner	Capacity (b/d)	Catalytic Cracking (b/d)	Catalytic Reforming (b/d)
Sapref	Durban	Shell/BP	172,000	35,000	29,000
Calref	Cape Town	Caltex	110,000	22,300	15,000
Enref	Durban	Engen	104,000	22,600	20,750
Natref	Sasolburg	Sasol/Total	87,547	22,460	14,642
Total			**473,547**	**102,360**	**79,392**

Key: Sapref: South African Petroleum Refiners

Calref: Caltex Refinery

Enref: Engen Refinery

Natref: National Petroleum Refiners of South Africa

Annexure 4.3. Description of the A549 cell line

Cell Line	Origin	Species	Lineage	Cell Type
A549	ATCC CCL185	Human	Lung Carcinoma	Epithelial

ATCC: American Type Culture Collection