DETERMINATION OF POLYCYCLIC AROMATIC HYDROCARBONS IN ROAST MEAT AND SMOKED FISH BY COMPUTER ASSISTED HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

Paul Mumo Kirai

A thesis submitted in partial fulfillment of the requirements for a Masters Degree in Environmental Chemistry in the University of Nairobi.

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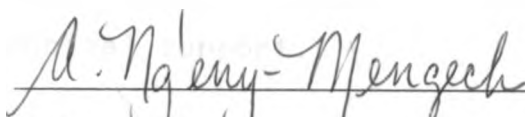This thesis is my original work and has not been presented for a degree in any other University.

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Paul Mumo Kirai

This thesis has been submitted for examination with our approval as University Supervisors.

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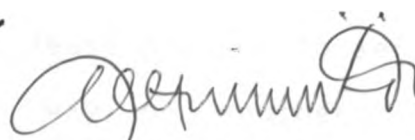
Dr. (Mrs) Annalee N. Mengech

Senior Lecturer

Chemistry Department

University of Nairobi

_____

Dr. Alex R. Tindimubona

Senior Lecturer

Chemistry Department

University of Nairobi.

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Some times one feels like giving up an idea especially when the goal appears seemingly invisible. This was almost the case with me at some difficult moments during the study period. However, I did not give up due to the support I received both from within and without the University. Key players were Dr. (Mrs) A. Mengech and Dr. A. R.Tindimubona to who I will always be indebted for their useful guidance and encouragement.

Much gratitude goes to the chairman of the Chemistry Department and his entire staff for their academic and technical support.

To my entire family, especially my wife and children: thank you for your patience and encouragement during this long period.

Last but not least are all those not mentioned above who in one way or another contributed to the achievement of the targeted objectives of this research.

DEDICATION

To Milka, Eunice and Joseph.

ABSTRACT

Many methods of analysis have been employed in the determination of levels of Polycyclic Aromatic Hydrocarbons (PAHs), present in various environmental samples, each with its own distinct advantages and limitations. The study reported here involved the development of a chromatographic method that was suitable for the analysis of these compounds in roast meat and smoked fish, and further, one that would be easily applied for the analysis of similarly complex environmental samples.

A gradient elution High Performance Liquid Chromatograph (HPLC), was interfaced with a micro-computer and the necessary hardware and software for data collection, storage and subsequent chromatographic analysis was developed.

The PAHs were extracted in methanol and methylene chloride, followed by clean-up on XAD-2. Removal of soluble protein was achieved by cooling the methanol extracts to -20^0C and then filtering under suction. The samples were then analysed by reverse phase HPLC employing a Vydac TP5 analytical column, coupled with a precolumn (Vydac 201TP 300A), and a gradient elution programme; 50:50(water:acetonitrile) for 3min then 100% acetonitrile over 7min linear

gradient. The chromatograph was interfaced with a microcomputer for data collection and analysis.

It was observed that the system and method of analysis was versatile and well suited to this type of operations. As many as eleven PAHs were identified in the roast meat and smoked fish samples. In all the samples analysed two compounds, phenanthrene and benzo(b)flouranthene were always present, the latter being an established carcinogen, though there was no correlation in their concentrations. Benzo(a)pyrene (BaP), which is one of the most carcinogenic PAH, was identified in only two roast meat samples at levels of 15 and 24ug/kg. Smoked fish was observed to contain most of the PAHs found in roast meat and in case of dibenz(a,h)anthracene, the levels obtained were much higher than in roast meat. However there were significant levels of other PAHs of various carcinogenic potency in all the samples of roast meat and smoked fish analysed.

CHAPTER ONE: INTRODUCTION

1. Polycyclic Aromatic Hydrocarbons (PAHs)

1.1a General Properties

Polycyclic aromatic compounds including both polycyclic aromatic hydrocarbons (PAHs) and heterocyclic compounds form one of the most heavily studied classes of environmental pollutants [1,2,3,4,]. This widespread interest stems from the demonstrated carcinogenic activity of many of these compounds (2,5,6,7). Animal experiments have revealed that a good number of these substances are carcinogenic and/or mutagenic and furthermore, they can act as tumor initiators or promoters [8]. In fact, the polycyclic aromatic hydrocarbons are the largest class of chemical environmental carcinogens known today [1,3].

The PAHs are largely non-polar compounds with more than one fused benzene ring and although they can be found as substituted derivatives, the study reported in this thesis pertains to the unsubstituted analogues.

The majority of PAHs in the atmosphere will be found adsorbed on solid particles but the lighter ones with only three to four fused benzene rings have

high vapour pressures and occasionally may be found free in the air. Examples of low molecular weight PAHs include anthracene, phenanthrene and fluorene all of which have boiling points below 400° C. However they form stable solids at room temperature and pressure. The structures of some common PAHs are shown in fig.1.1.

1.1b Occurrence of PAHs

PAHs are by-products of virtually all pyrolysis processes including for instance, wood burning, petrol combustion, industrial processes such as foundries, coking plants, ethylene cracking plants and many others [1,3,9,10]. They are thus widely distributed and are found in air, water, soil, foods and in almost every type of environmental sample matrix conceivable [1,3,4,8]. In the atmosphere they are usually found adsorbed on particulate matter, but some of the volatile ones can be found free – the latter including anthracene, naphthalene fluorene, phenanthrene and some others of lower molecular mass. Not only are PAHs the most extensively studied components of airborne particulate matter but their occurrence in other media is of great interest [2].

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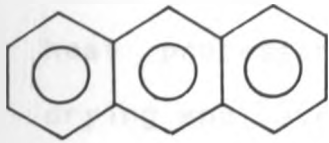
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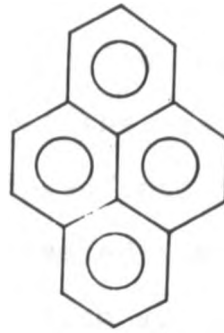
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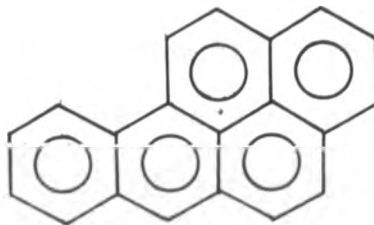
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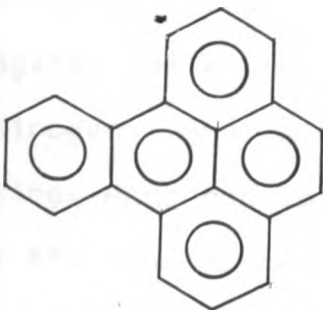
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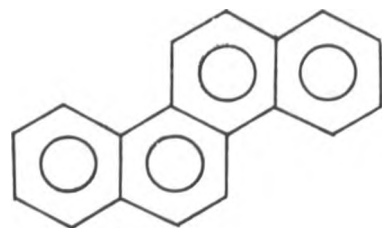
Chrysene

Fig 1.1. Structures of some common polycyclic Aromatic Hydrocarbons (PAHs).

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1.2 PAHs In Cooked Foods

Lijinsky and Shubik [11] showed in 1964 that some benzo(a)pyrene (BaP) and other PAHs are present on the surface of charcoal-grilled meat which they attributed to adsorption onto the meat of smoke from pyrolysed fat that had dripped onto the fire. Later studies have, however, revealed that all forms of heat processing of foods such as smoking, direct drying and even cooking, introduces substances with mutagenic activity. The formation of PAHs on cooking as measured by mutagenic activity varies with the nature and type of food. For example, high protein food such as meat has been observed to form more mutagenic products than starchy foods [10,12,13,14]. The nature of the food including water and fat content also affect the formation of PAHs on heating. On heating proteins, many nitrated PAHs are formed [12,15], some of which are highly carcinogenic and are believed to act as direct mutagens, while other PAHs may play the role of co-carcinogens or activators [7]. Even without heating, PAHs contamination of foods can also occur from the environment − water, air, or soil − and many unprocessed foodstuffs including vegetables and fruits have been found to contain these compounds [2]. PAHs prevalence in foods is such that some studies by Santodonato, Howard and colleagues [16] have estimated that food intake might actually

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surpass tobacco smoking as a major contributor to PAHs exposure. The levels of PAH content of some foods are shown in Tables 1.1 and 1.2.

Table 1.1   The concentration of PAHs found in charcoal broiled steaks [source [17]].

Compound	Concentration ug/kg
Anthracene	2.0
Benzo(ghi)perylene	4.5
Dibenz(a)anthracene	4.5
Dibenz(a,h)anthracene	0.2
Benzo(e)pyrene	6.0
Fluoranthene	20.0
Anthanthrene	2.0
Pyrene	11.0
Phenanthrene	18.0
Benzo(a)pyrene	8.0
Chrysene	1.4
Cororene	2.3
Perylene	2.0

Table 1.2 The concentrations of three selected
PAHs in various food products.
Source – [17]   (Conc.in ug/kg)

Food	Benzo(a) pyrene	Benz(a) anthracene	Chrysene
Cereals	0.19-4.13	0.40-6.80	0.8-14.2
Potato Peelings	0.36	–	–
Potato Tubers	0.09	–	–
Grain	0.73-2.30	–	–
Flour (dried)	4.40	–	–
Flour (untreated)	0.73	–	–
Bread ,	0.23	–	–
Bread (Toasted)	0.39-0.56	–	–
Lettuce	2.8-12.6	6.1-15.4	5.7-26.5
Tomatoes	0.21	0.3	0.5
Spinach	7.4	16.1	28.0
Coffee (Roasted)	0.3-15.8	0.5-42.7	0.6-19.1
Tea	3.9-21.3	2.9-36.0	4.6-63.0
Whisky	0.04	0.04-0.08	0.04-0.08

1.2a   Cooking Methods and PAHs Formation

Very little is known about the reaction pathways
which lead to PAH formation but some authors support
a free radical mechanism as a possible option
Larson [18].   In smoked foods, formation of PAHs is
thought to proceed through complex pyrosynthetic
chemistry in which fuel components are cracked to
small fragments and then reformed through

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predominantly free radical reactions [18]. The method of cooking can have profound effects on PAH production owing to the fact that the intensity and directness of heat transfer from the source to the food governs the rate and extent of PAH formation especially in meat products [10,13,15,18]. Many other parameters however govern the formation of PAHs in foods and all are interactive. These include the nature of cooking surface, duration of cooking, temperature, water and fat content of the food [10].

Investigations by Hatch [13] and Bjeldanes [19] revealed that stewed, simmered, boiled or deep fried meat produces low PAH levels while higher levels were obtained through roasting and baking. Variations in levels of PAHs were also observed with change of frying surfaces with teflon, enamel or ceramic surfaces giving the lowest levels and stainless steel and aluminium surfaces producing the highest levels [10] These observations indicate that the temperature of contact, i.e. the surface temperature, affects PAH formation. The surface of a steel pan or grill can rise to much higher temperatures than enamel or teflon surfaces thus prompting formation of more PAHs.
During boiling or deep frying, the food is not in direct contact with the cooking surface and hence

the food temperatures may not be very high which explains the low PAH levels observed. Felton, Healy Berry et al [20] noted a general increase in mutagen formation with increase in cooking temperature though not in a simple linear relationship. Most of the PAHs on the surfaces of smoked foods are believed to originate from the fire and smoke rather than from heat-induced reaction in the foods [21].

As noted earlier, water and fat content, including the rate of dehydration, affect the formation of PAHs in meat though the relationship is somewhat unclear [10]. Hatch [13] observed that lean red meat produced mutagens readily, followed by lighter meats and fish, but Weisburger [22] found a more complex relationship between fat content and mutagen formation. He noted that very low fat content led to decreased mutagen formation which was at variance with the observation by Hatch. He proposed that increased fat content may enhance the rate of heat transfer from the source to the meat and thus speed up PAH formation. However the results obtained indicated that the hypothesis only held in the lower ranges and when fat content rose to a certain level, total mutagen formation decreased with increased fat which supported Hatch's observation.

It would appear that at higher temperatures production of many different types of compounds

occurs which catalyze mutagen formation in foods. Glycerol, which may be produced by the thermal decomposition of fat, has been found to stimulate mutagen formation as does iron (III) [22]. Other findings indicate that it is possible to suppress mutagen formation on meat by adding substances such as ethylene diamine tetraacetic acid (EDTA) or soy protein before cooking [22]. However most workers concede that PAHs will appear on all major protein foods if cooked to a "well done" state [15].

The effect of time, i.e. the duration of cooking on the formation of mutagens has been studied and the relation found to be a complex one. At certain times during the cooking at certain temperatures, PAH formation was found to reach a maximum and to gradually decrease after prolonged cooking [10,15]. The loss of mutagens after prolonged cooking was attributed to degradation of the PAHs and to possible evaporation losses [15].

1.3 PAHs And Environmental Carcinogenesis

By the turn of the century, scientists had noted that a particular type of cancer — cancer of the scrotum — was predominant among 'chimney sweeps'. In 1915, Yamagiwa and Ichiwaka [23] demonstrated

that repeated contact with coal tar could induce cancer in mice. In 1962 Rocky, Speer, Ahn et al [24] managed to induce carcinomas in dogs throat by direct application of tar to the bronchi. Earlier in 1932 benzo(a)pyrene (BaP) had been isolated from coal tar and another PAH – Dibenz(a,h)anthracene – had been synthesized pure and both compounds had proved carcinogenic. The study of environmental carcinogens has greatly advanced since then and mutagens and/or carcinogens have been identified from many sources such as foods [2,10,15,20,25 – 29], air particulate [3,4,6,7,8], fossil fuels [21,30,31] and soils [9,32,33].

Procedures for fast assessment of the mutagenic activity of the many thousands of chemicals have been developed. Short term tests depend for their effectiveness on the universal nature of deoxyribonucleic acid (DNA) which in higher organisms is incorporated in the chromosomes. The (Bruce) Ames test [34] is the most common procedure employed for assessing mutagens. A mutation may be defined as a stable heritable change in a DNA nucleotide sequence and is usually assayed by methods which detect either forward or reverse mutations. The Ames test is designed to detect reverse mutations which are by their nature more specific. Reverse mutation assays use bacteria that

are already mutated at an easily detectable loci and the frequency at which the test chemical abolishes the effect of the pre-existing mutation is then determined. The bacteria used are strains of <u>Salmonella typhinurium</u> which require the addition of histidine to grow. During the assay of a particular chemical, the number of bacteria reverted back to an ability to grow without addition of histidine is measured by counting the revertant colonies after 48h of incubation. A chemical is positive in a test if it displays a reproducible dose-dependent response in at least one tester strain.

In many cases, metabolic activation of the substance is necessary before it can interact with DNA and in mammals these metabolic enzymes are supplied by the liver. In the Ames test <u>in vivo</u> conditions can be mimicked by adding an activating system (S9 mix) derived from rat liver which has been treated with the appropriate co-factors [12]. Other metabolizing systems can be derived from mouse or hamster liver.

Although the Ames test is a very useful one, confirmation of a carcinogen may not be possible until the substance has been tested in a mammalian system, sometimes over a long period of time [12,13,29,35]. However most mutagens identified so far have been shown to be carcinogenic and the evidence indicates that there is a strong

correlation between mutagenic and carcinogenic
compounds [12,35]. One study reported by Sugimura
[12] tested four hundred (400) chemicals on
Salmonella typhinurium TA 98. The results are
illustrated in Fig. 1.2
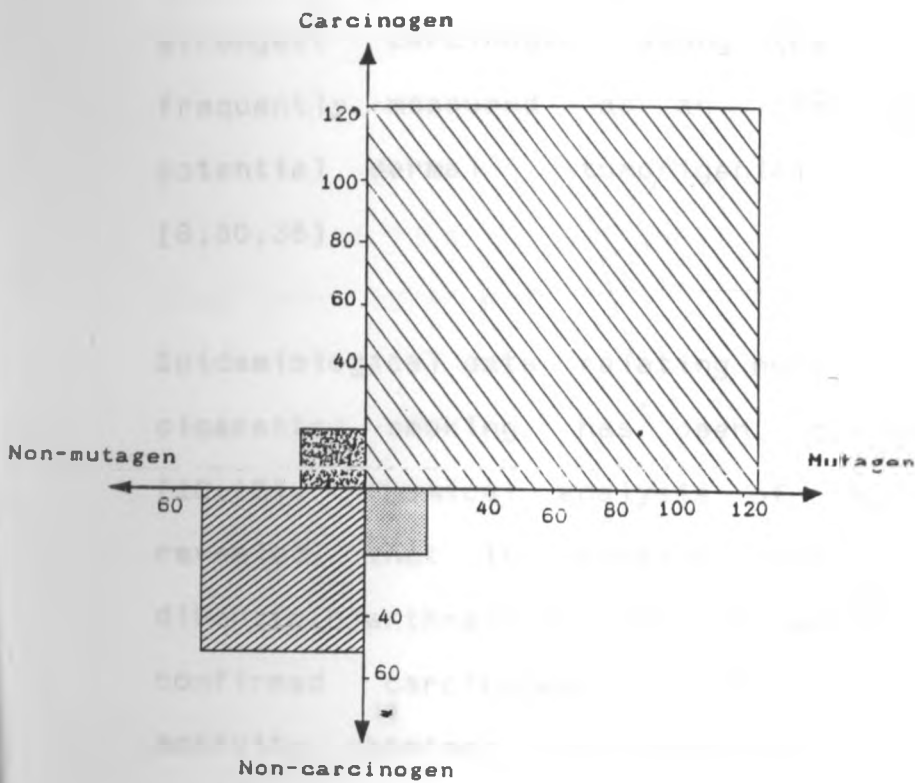
Figure 1.2 The results of a study to evaluate the
correlation between mutagenic and carcinogenic
compounds.

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One hundred and forty of these were found to be carcinogenic and sixty nine to be non-carcinogenic. 87% of the carcinogens were mutagenic while 73% of the non-carcinogens were also non-mutagenic.

Most workers agree that the correlation between mutagenicity and carcinogenicity is greater for aromatic amines and PAHs than for any other class of compounds [15]. Benzo(a)pyrene (BaP) is the strongest carcinogen among the PAHs and is frequently measured as an "indicator" of the potential dermal tumorigenicity of a sample [8,30,36].

Epidemiological data relating human lung cancer and cigarette smoking has been generally accepted [10,12]. Chemical analysis of cigarette tar has revealed that it contains PAHs such as BaP, dibenz(a,h)anthracene and chrysene, which are confirmed carcinogens. However the mutagenic activity obtained from cigarette smoke condensate vastly exceeds that which can be explained in terms of the content of BaP and other known carcinogens present, thus prompting the suggestion that other unknown substances present may also be active.

Screening of smoke condensate obtained by charcoal broiling of various types of fish gave the mutagenic activity levels which indicated that the smoke had a

high activity even without metabolic activation. Again it was observed that the activities obtained were higher than could be explained by BaP content.

In another experiment a sample of charcoal roasted steak was subjected to the mutagenic screening procedure. It was observed that 5g off the surface of 190g of beef steak had an activity equivalent to that of 855ug BaP which works out at 4,600ug BaP/kg of roast steak [12]. Some PAHs such as pyrene and fluoranthene possess properties which enable them to function as co-carcinogens and hence enhance the role of BaP as a carcinogen. It has been suggested that this synergistic action may be the reason for the observed high mutagenic activity of samples of roast meat and which can not be explained in terms of BaP content alone or other known carcinogens.

1.3a Relation of Diet to Cancer Occurrences

Though epidemiological data has long associated high incidence of lung cancer with cigarette smoking, the causes of most human cancers are still unknown [12,37]. There is, however, a lot of evidence indicating some relationship between diet and some types of cancer. Studies on Japanese immigrants in the United States of America demonstrated that human stomach cancer is probably related to diet and the

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same may be true for colon and pancreatic cancers [12]. Krepinsky [38] showed that dietary fat intake and beer consumption are correlated with large intestine cancer. This finding has been emphasized by Weisburger [22] who showed that several mutagens formed during cooking were carcinogenic to rodents. The affected organs in the rodents are the colon, breast and pancreas the same as in humans on western diets. He also found that enhancement of this group of cancers is associated with diets high in fats and cholesterol. T. Sugimura [28] identified mutagens in pyrolysates of proteins as well as in ordinary fried foods. All these substances were later found to be carcinogenic to rodents. Studies by the same worker [35] have associated increased cancer risk with excessive intake of calories, alcohol, salty foods, very hot beverages and char on food. In other parts of the world, similar studies have revealed similar trends. Lawrence and Weber identified 18 PAHs in a variety of selected foods available in Canada including smoked fish, fried and charcoal roasted meat. Eleven of the PAHs identified are known carcinogens. Smoking of fish has been shown to be the cause of the presence of BaP in the product [39].

The aforegoing gives indication as to the extent of the correlation of some cancers with dietary habits

and the following examples illustrate the situation
further.

Out of fourteen thousand cancer deaths in Japan over
a period of 50 years, Hiriyama [40] found that
stomach cancer had the highest incidence followed by
lung, liver and cervical cancer. He further
observed that breast cancer was associated with meat
eating and smoking while cancers of the mouth,
pharynx, esophagus and prostrate were correlated
with alcohol consumption. Though smoking was the
most important overall risk factor, meat eating had
a higher risk than smoking for esophageal and
pancreatic cancer. In attempting to relate the
lifestyle and the risk involved he found that the
lowest cancer risk (0.40) was associated with the
lifestyle of non-smoking, non-alcohol or meat
consumption but with plenty of vegetables and
fruits. The highest risk (1.00) was just the
opposite. It was correlated to a lifestyle of
smoking, alcohol and meat consumption with few or no
vegetables at all. He estimated that 33% of the
cancers were associated with smoking, 35% with diet,
5-6% with alcohol consumption and less than 3% with
occupation.

Carcinogenesis involves a balance between the
initiation and promotion processes. Both are dose

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dependent and the latter process is reversible when the stimuli is removed [22]. Experiments have shown that promoters increase the incidence of cancer and reduce the latent period after initiation. Typical promoters are sodium chloride(NaCl) and croton oil [12]. It is to be noted however that there are substances which lower the cancer risk. 'Dismutagens' as they are called, inhibit the action of, or even directly inactivate mutagens and promutagens. Extracts from many fruits and vegetables are dismutagenic. These include peroxidases from cabbage and broccoli, vegetable fibre (which absorbs many mutagens), and reducing agents such as vitamin C [42]. Negative correlation to cancer risk has been observed with high intakes of fruits and vegetable fibre [28,43,44,45], thus stressing the need for a diet rich in these foods.

1.4 Analysis of PAHs in Food

Methods of analyzing PAHs in food have evolved over many years but even the simplest of them invariably involves more than one step. In general there are at least two steps in preparing a sample for the final stage, which is analysis by either chromatography or a biological technique. These steps are:

a) Extraction of PAHs from the sample

b) Clean-up from interfering substances

c) Analysis by gas chromatography, HPLC,
   mutagenic assay, etc.

These steps vary in complexity and duration and none can be assumed to be of less importance than the other.

1.4a Extraction of PAH's From Food Samples

PAH extraction from foods has been achieved by using aqueous acid or base [10], mixtures of water and miscible organic solvents [2,25,26], organic solvents alone [20], or water [29,33]. In any of the methods above, the food was first broken into small fragments, then homogenized with the solvent and the mixture filtered to capture the filtrate. Though the filtrate contains the PAHs, it may also carry along soluble proteins and other interferences which need to be removed.

One of the earliest extraction methods to be commonly adopted was developed by Commoner and co-workers [45]. In this method the cooked food was homogenized with 0.1N HCl then filtered and soluble proteins were precipitated by ammonium sulphate,

$(NH_4)_2 SO_4$, before a final filtration. When the filtrate was adjusted to pH2 it was extracted with methylene chloride $(CH_2 Cl_2)$ then adjusted to pH10 followed by a final extraction with methylene chloride. Use of $(NH_4)_2 SO_4$ in this method was later observed to cause artificially high mutagenic activities [47] and was later replaced by sodium hydroxide (NaOH) [48]. Many variations and modifications of this method were utilized in a bid to improve the extraction efficiency but even then the recoveries reported were only about 3% at the highest [48].

A later development was the use of a mixture of water and a miscible organic solvent such as methanol, acetone or acetonitrile. This method has been used by Weber and Lawrence [26] to determine PAH levels in Canadian smoked fish and meat products [2]. They added potassium hydroxide (KOH) to mixture of water and methanol (1:9) and saponified the sample under reflux for 3-4 hours. High recoveries were reported and a total of 18 PAHs detected.

Extraction with undiluted organic solvents has proved to be a success, more so because of the ability of such solvents - methanol, acetonitrile etc.- to precipitate proteins, which is desirable

[20, 25, 29]. Food samples are homogenized with the solvent and the mixture then filtered and subjected to further clean-up procedures. High recoveries have been reported, especially when a suitable clean-up procedure was employed. Kato, Kikugawa and Hayatsu [27] report the use of water alone as an extraction media in their determination of mutagens from Japanese smoked fish and other smoke dried products. The sample was homogenized with distilled water, then heated and the mixture filtered.

In this type of extraction however, the main interest was in the determination of the general mutagenic activity and not in the PAH content of the sample. Quite a large number of the mutagens contained in foods are polar compounds – substituted PAHs which have appreciable solubility in water [7, 15, 27].

The above are some of the methods employed to extract PAHs from their enclosing matrix which form only the first step in their analysis. A second step is required to further free these compounds from other interfering substances and to lend them accessible for sensitive analysis. It is this second stage, i.e. the clean-up stage, which in conjunction with an appropriate extraction procedure that seems to govern the extraction efficiency to a large extent.

1.4b   Clean-up or Pre-fractionation

The selective isolation of PAHs from interfering compounds is of great importance and much effort has been devoted to this challenge [33]. Numerous methods for this purpose have been applied to purify PAHs from various samples such as air particulate [4,6], soil extracts [9,32,33] food [2, 26, 27] and water [25]. Some form of chromatography is utilized in all the separation techniques and TLC has found wide acceptance. It was used in the early studies [10] and is till common in recent ones [1,4,6,31] In TLC the sample is spotted along with pure standards and after development of the plate, the PAH fraction is scrapped off and redissolved in methanol for further analysis on gas chromatography, HPLC or another analytical technique.

New developments have occurred in this area giving rise to many other clean-up methods among the chromatography of the crude extract on a basic alumina column which has been found to yield good results [7,10,49]. Hagraves and Pariza [10] utilized a silica gel column, Adsorbosil-5, and reported satisfactory results. In this column, the non-polar compounds are eluted with n-hexane while the polar ones are eluted with methylene chloride. The Sephadex-LH20 which is a gel permeation column was often used in conjunction with a silica gel

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column to achieve sufficient purification with methanol or a combination of methanol: hexane: chloroform being the mobile phase [10]. This method has been criticized for being extremely slow [50] and preparative HPLC has been found to serve the same purpose and more efficiently [10]. Lawrence and Weber [2,26] utilized another commercial column - the Fluorosil column - to prefractionate PAH extracts from smoked foods using toluene as the mobile phase. Crosby and colleagues [25] employed the Sep-pak column packed with silica gel and elution with a combination of solvents to clean up a crude PAH extract from food before a final prefractionation on TLC. As mentioned above, preparative HPLC can be used for clean-up and is highly suitable for very sensitive analytical systems such as GC-MS (gas chromatography-mass spectroscopy). Tomkins and Griest [30] employed a preparative HPLC column - Partisil PAC 10 in their determination of BaP at parts per billion concentrations in highly refined fuels.

A more recent development in the field of PAH analysis and which has roused great interest is the Amberlite XAD-2 resin. This is a polyvinyl butadiene co-polymer that is highly specific to mutagens and especially PAHs and it has found wide acceptance and application. It has been employed

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with much success to collect air particulates for PAH analysis [8,51] and to concentrate PAHs in liquids such as urine [52] to mention a few. Many workers have used XAD-2 for clean-up of PAHs from interfering compounds [9,10,27,32,33] and found it efficient, reproducible and what's more, its separation efficiency increases with increased use especially with a careful choice of mobile phase. Clean-up on XAD-2 is done in various stages usually requiring more than one eluting solvent [9,32,33]. Some of the solvents that have been used to elute different fractions are as shown below.

- the non-polar fraction: hexane or pentane.
- the polar fraction: ethanol, methanol or acetonitrile.
- the PAH fraction: acetone or toluene.

The portion containing PAHs is then concentrated for analysis.

An alternative procedure is to use a single solvent such as acetone on an XAD-2 column. The various components will have different retention times and by carefully taking off aliqots of the eluent, the PAH fraction can be isolated [10]. This method was used by Kato, Kikugawa and Hayatsu [27] in the analysis of total mutagens by the Ames <u>Salmonella</u>

assay.   Using XAD-2 for clean-up is reported to
yield high recoveries with losses as low as 1%
[8,51].

1.5   Chromatographic analysis of PAHs
As is the case in most classes of compounds, the
biological activity of PAHS is isomer-specific [21].
This   is clearly illustrated   by the fact that
benzo(a)pyrene is one of the most potent carcinogens
among the PAHs,   while its isomer benzo(e)pyrene is
completely non-carcinogenic (see fig 1.1). The need
arises,   therefore,   for   a   clear   unambiguous
identification of the PAH_s present in   a sample
before any useful conclusions can be drawn.

Chromatography,   coupled   with   some   form   of
spectroscopy, is the most extensively employed mode
of PAH analysis and identification. A sample after
purification is introduced into a chromatographic
column and eluted with a mobile phase of choice.   A
suitable detector, (UV or fluorescence) which has
been adjusted for optimum sensitivity, is placed at
the end of the column and as the elution progresses,
components of the mixture are detected and the
results plotted on a chart.   Identification is
easily achieved by comparison of retention times

with those of known compounds or by subjecting the
components through a mass spectrometer.

1.5a Types of chromatography

The most common forms of chromatography employed for
PAHs analysis are gas chromatography (GC), or liquid
chromatography in the form of HPLC. In most
environmental PAH studies, GC was widely used with
either a glass or capillary column coupled to a
flame ionization detector (FID). Ciccioli and co-
workers [7] report of their work on atmospheric
samples where two preparative HPLC columns were used
to pre-fractionate the crude sample. When fractions
from these columns were applied to capillary GC, up
to twenty PAHS were identified from one fraction.
The GC was coupled to a mass spectrometer and a
complete analysis of each PAH was performed. This
combined GC-MS technique appears to be quite popular
for PAH analysis [2,7,26].

Recent development of high pressure pumps and
microparticulate columns for HPLC has resulted in a
steady increase in the use of HPLC for PAH
analysis. This form of chromatography usually
employs a UV or fluorescence detector and has been
employed on a wide range of samples including air

particulate (1,4,36,53) smoke condensate (10) and food (2,26). Most of the work has been carried out on reverse phase columns which have C-8 or C-18 supports [37] but there exists various other columns for HPLC work. Reverse phase is a special case of partition (liquid-liquid) chromatography though it is sometimes regarded as a separate category. In normal adsorption chromatography the stationary phase, usually silica gel or alumina, has active sites for which the molecules of the mobile phase and the sample components compete. Typical mobile phases used would comprise hexane or dichloromethne as a principal solvent, to which is added a second modifying solvent usually a polar one in minor proportions. Polar components in the sample are retained more in the stationary phase and hence they are last to elute. In a reverse phase chromatographic system, the mobile phase is more polar than the stationary phase. The stationary phase is commonly a C_{18} hydrocarbon chemically bonded to a support by the action of e.g. an octadecylchlorosilane. Mobile phases used in this case are based on water to which a water- miscible organic solvent is added to modify the elution characteristics. Unlike in normal phase systems, it is the more polar components of a sample that are first eluted followed by the less polar ones. Common mobile phases are water mixed with either

acetonitrile or methanol.

Columns with chemically modified support, have also been successful in resolving PAHs as well [1].

Of equal importance to the choice of column is the mobile phase and flow rate to enable separation of closely related compounds such as PAHs. A non-polar solvent such as n-hexane is suitable for chemically modified column supports such as Lichrosorb NH2 or Nucleosil No2 [1] while on reverse-phase columns, C-8 and C-18, polar solvents such as acetonitrile or methanol gives best results [2,26,30,49]. Introduction of the gradient elution technique has greatly improved on the resolution previously obtained under isocratic conditions. Gradient elution also lessens the time required for analysis as the change in solvent helps the slow components through the column much faster. Inclusion of a pre-column before the analytical column also has the effect of improving resolution and further provides protection to the column against harmful particulate in the sample.

Use of super-critical fluid chromatography (SCF) for separation of PAHs has been reported by Takeuchi and colleagues [54] and by Hirata and colleagues [55]. Both groups of workers employed fused silica packed columns but the former used carbon dioxide as

the mobile phase while the later used n-hexane. It was observed that SCF has the advantage of short analysis time and efficiency over other chromatographic systems. However, the instrumentation involved is much more elaborate and hence expensive.

1.5b Detection

On HPLC the detection of PAHs is effected by utilizing some of the optical characteristics of these compounds. Aromatic compounds are strong chromophores and hence absorb ultraviolet (UV) light over a wide band of wavelengths. After clean-up to remove other substances that might absorb UV light, the sample is introduced into a HPLC column at the end of which is attached a UV absorption spectrophotometer that has been set to a suitable wavelength, commonly 254nm. This technique is widely used [1,7,31].

PAHs are strong phosphors as well, and this characteristic is exploited in fluorescence detectors. When light of high intensity impinges on these compounds, they absorb it and subsequently

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emit light of lower intensity, i.e. they fluoresce. By selecting suitable excitation and fluorescence wavelengths, PAHs can be detected with high selectivity. Fluorescence detectors, though more expensive than UV absorbance detectors, are more suitable for PAH detection due to their high selectivity and sensitivity. They are thus frequently employed [31,53] Rossi, Desilers and Pardue [56] demonstrated the use of a multiwavelength detector for PAH detection. This detector consists of a diode array spectrophotometer in which the diodes emit UV light at different wavelengths and as a sample progresses along this array, a UV absorbance scan of the constituents is obtained. Since a sample may contain many UV - absorbing PAHs, some of them not well resolved, the spectra obtained is usually quite complicated and may not be easy to interpret. However this detector has the advantage that after interpreting the spectrum with the aid of a microcomputer, a clear and unambiguous identification of each constituent can be obtained since each compound has a unique UV absorbance pattern.

Amperometric or cyclic voltammetry methods have been used for identification of PAHs, by Khaled and Dorsy [3], using liquid chromatography. Amperometric detectors are very selective and when used in

conjunction with a UV absorbance detector, a clear identification of components can be achieved.

A more recent development in PAH detection using liquid chromatography is the High Resolution Sphol'skii Spectroscopy (HRSS) [21]. The authors reported total and unambiguous identification using this method which utilizes the luminescence characteristics of the compounds and combines the use of a micro-computer to analyse the complex spectra generated.

1.6 Use of microcomputers in chemical analysis

The demands of modern research are such that precision and accuracy are of prime importance, while at the same time great emphasis is laid on speed and efficiency. Consequently, analytical instruments have had to grow in sophistication and invariably the volume and complexity of data generated has increased proportionally. In order to meet the high standards and to achieve the set objectives in research the modes of handling data have had to change accordingly.

A development that has aided this process greatly is the advent of the microprocessor. The

microprocessor has made it possible for low-cost computers to be available for acquiring and processing complex data sets thus enabling more effective analysis than was previously possible [59]. Most modern analytical instruments now have on-board microprocessors or computers for performing functions such as instrument control, data logging and analysis. Use of computer systems on analytical instruments has greatly simplified the task of chemical analysis and has enabled researchers to make more effective use of several steps in chemical determination than was previously practical.

Chromatographic analysis is one such area where computers have found great applicability and especially in the analysis of complex mixtures. Though many modern materials for column chromatography that are capable of separating a wide range of compounds, including isomers of on-compound, have been developed, rarely doe separation occur with 100% resolution. Othe variables such as temperature, electrica fluctuations, solvent composition add to the complexity of the spectra obtained thus making it difficult to interpret. A computer system can very effectively be employed to resolve peaks of comple spectra by mathematical methods [57,58] and at the same time keep track of the changes or drift in the baseline. Further, the system can obtain heights,

peak areas and retention times of the various components and calculate their concentrations. The system may obtain other parameters if instructed and it can be used to control the numerous functions pertaining to the instrument and hence reduce human intervention. The net effect is to minimize possible errors, increase reproducibility and save on time. In the study reported in this thesis, a micro computer was interfaced with a HPLC mainly for the purposes of data collection and analysis. It was thus possible to acquire data, remove random noise, store data on disk, analyse it for peak areas and retention times, resolve merged peaks, display and print results. The chromatograms included in this text are computer print-outs generated by the system.

1.7 Proposed objective

The importance of PAHs in the process of carcinogenesis has been demonstrated and hence the great interest which these compounds arouse. As they have different potency levels, it is necessary to identify and quantify their occurrence and especially in foods. Though many such studies have been carried out in other parts of the world [2,10,20,25 - 29], none had yet been done in Kenya.

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Meat roasting is a much enjoyed social activity in
Kenya, whereas smoked fish is a popular diet among
some peoples of Kenya and as such it was important
to identify and quantify the PAHs that might be
present in these two foods . This was thus the
project's main objective.

CHAPTER TWO: EXPERIMENTAL

2.1 Reagents and chemicals

All solvents used were either of HPLC grade or glass
-distilled analytical grade. Each was scanned under
a UV spectrophotometer to ascertain its purity.

HPLC-grade acetonitrile and water were purchased
from Aldrich Chemical Co. as was n-pentane, methanol
and acetone, all of (99.9%+) purity. Analytical
grade methylene chloride (99.9%) was obtained from
Alpha Chemicals Co. Except for anthracene, all
other PAH standards and the XAD-2 resin were
obtained from Aldrich Chemical Co. Anthracene was
obtained from the Department of Chemistry
laboratories and recrystallized twice from methanol.

2.2 Instrumentation

The HPLC instrument available was a Gradient Liquid
Chromatograph Model 332 which employed two Model
110A high pressure pumps and a Model 210 sample
injector with a 20$\underline{u}$ml sample loop, all from
Beckmann. The detector was a Hitachi model 100-40
UV spectrophotometer of variable wavelength which
was fitted with a flow cell of pathlength 1cm.
The analytical column was a reverse phase Vydac TP5,

packed with C18 hydrocarbon bonded on an inert support, 25cm long and 4.6mm I.D which was protected by a guard column Vydac 201TP C18 300A with a 2.0 micron filter. For data collection and analysis a BBC Model B micro-computer was used. It was connected to the detector output through an interface which had been constructed earlier for this purpose comprising of a zener diode and a resistor. Access to the computer was through the analog port which contained an on-board analog to digital converter (ADC). This feature (the ADC) made the BBC micro-computer an ideal system for interfacing unlike other computers that do not possess this facility.

To protect the ADC input circuit, the interface was designed to "cut off" voltages outside the range -ve 0.3 V - +ve 2.3 V. The operation of the interface depended on the programs (software) that were developed to perform the following:-

i) **Sampling of the detector output.** This was done at the rate of 10 data points per second but only the average of each five successive points was sent to the computer memory, hence in one second, two points were recorded. These were found to be sufficient data points from which to reconstruct a continuous chromatogram.

ii) Removal of random noise from the data,

iii) Saving of chromatogram on magnetic diskette,

iv) Analysis of spectra,

2.3 Preparation of standards

Not all the standards were readily soluble in methanol and some of them needed to be dissolved in methylene chloride before dilution to the required volume with methanol. The peak due to methylene chloride on HPLC did not interfere with the detection of the PAHs as its absorbance was low and its retention time was less than that of any PAH analysed. One of the standards available, benz(b)anthracene, was not utilized in the analysis due to its poor solubility in any of the solvents in use such as methanol, methylene chloride, pentane or acetone and hence could not be analysed on HPLC.

Once the standards were prepared individually in a stock solution, a mixture containing all the standards was then made in methanol in the various concentrations shown in Table 2.1.

The various PAHs had to be prepared in different concentrations as they all had different extinction co-efficients at the wavelength chosen for detection (254nm). Suitable concentrations were prepared to obtain comparable peak heights on the chromatogram.
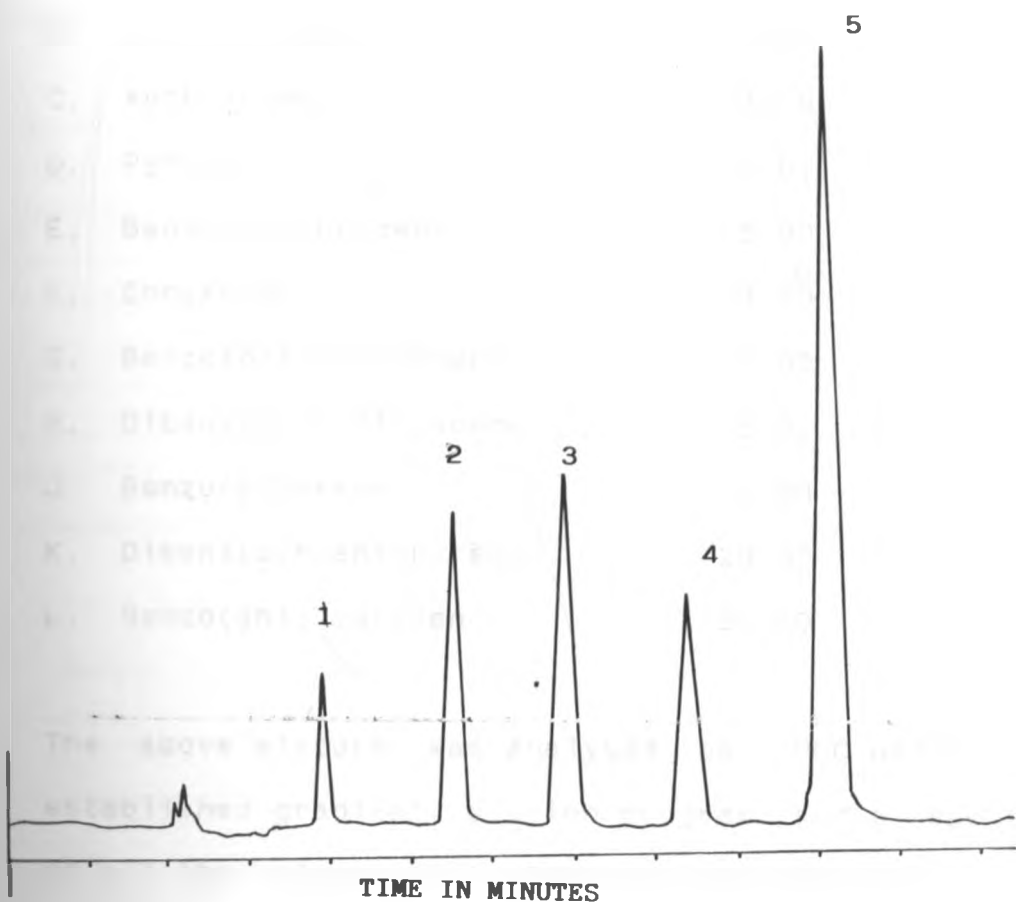
Figure 2.1   The HPLC   trace of a standard   mixture
                for   verification   of   the   column
                efficiency

1= Benzene, 2= Naphthalene, 3= Biphenyl, 4= Fluorene, 5=Phenanthrene,

Column: Vydac TP5, 25cm long, I.D. 4.6mm. Pre-column: Vydac 201 TP C18 300A
Flow   rate: 1.0ml/min.   chart   speed: 1cm/min.   Gradient   elution: 3min
(acetonitrile:water)(1:1)   then   100%   acetonitrile   over   7min   linear   gradient.

Table 2.1   Concentration of various PAHs in the
standard mixture prepared.

Compound	Conc. in ug/ml.
A. Fluorene	5.00
B. Phenanthrene	2.00
C. Anthracene	4.00
D. Pyrene	5.00
E. Benz(a)anthracene	5.00
F. Chrysene	4.00
G. Benzo(b)fluoranthene	5.00
H. Dibenz(a,c)anthracene	5.00
J. Benzo(a)Pyrene	5.00
K. Dibenz(a,h)anthracene	20.00
L. Benzo(ghi) perylene	20.00

The above mixture was analysed on HPLC using the
established gradient elution program and a spectra
of all the eleven (11) standards was obtained.   The
mixture was then enriched (doped) with a single
standard and again analysed on HPLC to enable the
determination of the retention time of that
particular compound in the standard mixture, figures
3.1a and 3.1. .   This was repeated for every
standard.

Table 2.2   The retention times (R.T.) and relative retention times (R.R.T) of some selected standards in a mixture.

Compound	(Ave) R.T.(min)	+(−)	(Ave) R.R.T.(min)	+(−
Fluorene	9.23	0.15	5.73	0.06
Phenanthrene	10.05	0.08	4.90	0.05
Dibenz(ah)anthracene	17.18	0.10	2.21	0.03
Benzo(ghi)perylene	17.95	0.11	3.00	0.05

The R.R.T was calculated w.r.t. Benzo(b)fluoranthene whose R.T. was about. 15min.

The relationship between concentration and the area under a peak was obtained by analyzing different concentrations of the PAH and plotting a calibration curve. Figure 2.2 illustrates this relationship for phenanthrene.
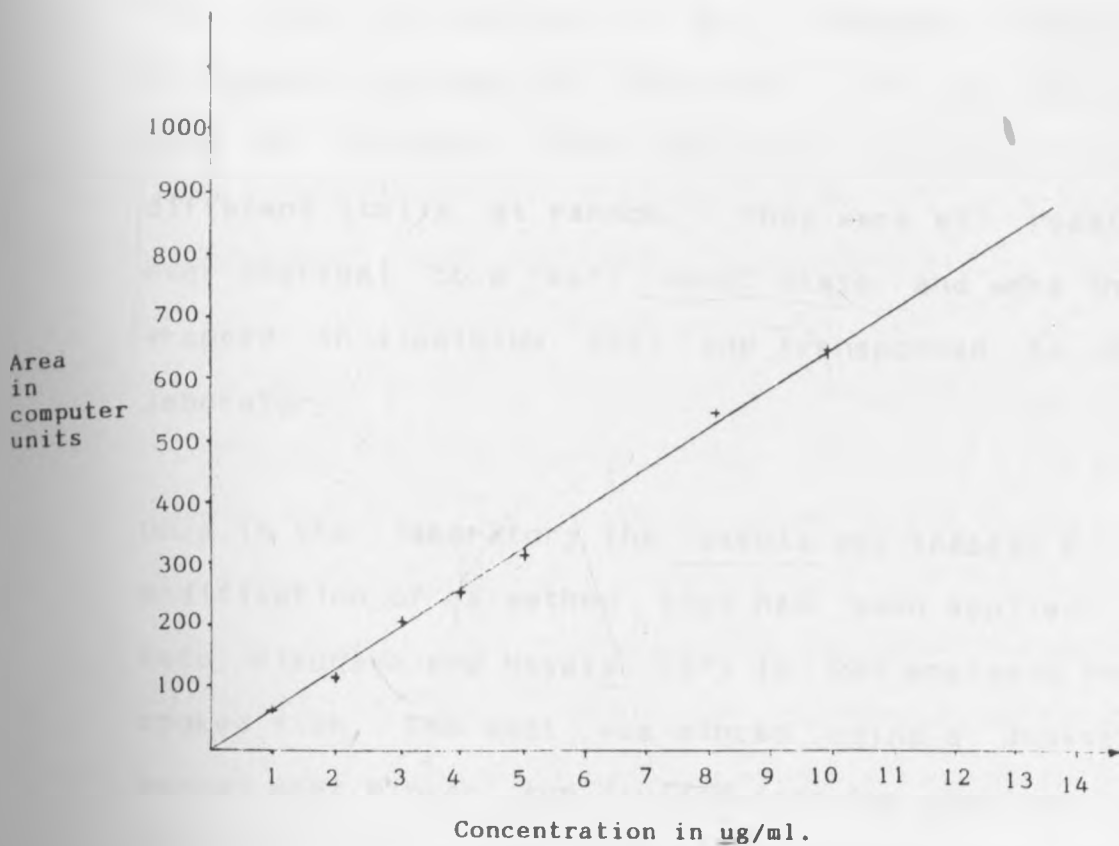
Figure 2.2 • Calibration curve for Phenanthrene

2.4 Sample preparation

Samples of roast beef were purchased from three well-known meat-roasting centers in Nairobi at different times of the day, i.e., before lunch (11.00a.m. - 12.30p.m.), during lunch (12.30p.m. -

2.00p.m.) and after lunch (2.00p.m. - 3.30p.m.). The three centers were: Kariokor, Kenyatta and Dagoretti Markets. Care was taken not to prejudice the study by posing as an ordinary customer throughout the sampling exercise. All the samples were of boneless meat and were purchased from different stalls at random. They were all roasted over charcoal to a "well done" state and were then wrapped in aluminium foil and transported to the laboratory.

Once in the laboratory the sample was treated by a modification of a method that had been applied by Kato, Kikugawa and Hayatsu (27) in PAH analysis from smoked fish. The meat was minced using a domestic manual meat mincer and divided into two portions of 100g each, which were placed in beakers and 100m methanol added to both. The beakers were the covered with aluminium foil and kept in the dark for several hours to allow the methanol to permeate the fibre. The sample was transferred into a blender along with 200ml methanol and the mixture blended over a period of 5min. It was then filtered under suction over clean glass wool and the residue again blended with 200ml methanol for another 5min.
After filtration the filtrates were combined and the volume reduced to about 20ml using a rotary evaporator. The concentrated mixture was then

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quantitatively transferred to a small sample bottle which was then tightly sealed and protected from light. It was left overnight in a deep freezer at minus 20^0 C.

A liquid and solid phase appeared after freezing and these were quickly separated over No.1 Whatman filter paper under suction. Cold methanol was used to wash the residue that comprised of gelatinous matter which quickly melted on warming. The excess solvent was removed over a rotary evaporator and the residue taken up in deionized water and transferred to a separating funnel. The mixture was extracted three times in aqueous methylene chloride $(CH_2 Cl_2 : H_2 O)$ (1:5). All the methylene chloride extracts were combined and the solvent removed on a rotary evaporator. The sample was taken up in 3ml methanol and was now ready for the next stage, i.e. clean-up.

2.5 Sample Clean-up on XAD-2

A column of 2cm I.D was filled up to 10cm with the Amberlite XAD-2 resin. The packing was first washed with 50ml acetone followed by 50ml methanol and finally 25ml n-pentane at a flowrate of 4 ml/min. 1ml of the methanol extract from the previous stage

was applied at the top of the column and a reservoir of solvent was placed above it. The flow rate was adjusted to 2 ml/min and the column was not allowed to dry out at any time during the clean-up process. The following fractionation procedure which is based on the method by T. Spitzer [32] for elution of PAHs was applied.

Solvent Used	Compounds Eluted
25ml methanol _____	Polar compounds
15ml n-pentane followed by	
10ml methanol _____	N o n - p o l a r compounds
10ml acetone followed by	
20ml methanol _____	PAHs

Flow rate: 2ml/min
Column: 10cm long, 2cm I.D

The column was then washed with 15ml acetone followed by 15ml methanol at flowrate 3ml/min and was then ready for the next sample. The total time spent on each clean-up operation was 50min.

The PAH fraction was then reduced in volume and adjusted to 2ml with methanol. It was immediately

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analysed on HPLC or stored overnight in a cold dark place. All samples were done in duplicate and the average value in ug/kg of PAH present recorded.

Smoked fish samples were purchased from Gikomba Fish Market in Nairobi. It was not possible to determine how long the fish had stayed in that state or how they had been smoked but it was certain that they were Tilapia from the Lake Victoria Region. Three batches were purchased and taken to the laboratory where the flesh was carefully separated from the bones.
All the fish had flesh of about 100g and thus each sample was divided into two equal portions of 50g each and the extracting solvent reduced proportionally. The rest of the extraction procedure was as for roast beef. The concentrations obtained were converted to ug/kg.

2.6 Determination of extraction efficiency
Eight (8) of the available standards were utilized in the determination of extraction efficiency. They were selected to represent the most polar, least polar and the medium polarity PAHs. The use of BaP was avoided due to difficulties in handling. The eight standards were as follows;

A. Fluorene

B. Phenanthrene

E. Benz(a)anthracene

F. Chrysene

G. Benzo(b)fluoranthene

H. Dibenz(a,c)anthracene

K. Dibenz(a,h)anthracene

L. Benzo (ghi) perylene

20ml of a standard mixture of these compounds in methanol was added to the residue of extracted meat sample in a 250ml beaker. After thorough mixing, the beaker was loosely covered and placed in a dark cabinet to allow the methanol to evaporate. A similar "sample" was placed into a beaker with pure methanol and was treated alongside as a control. Once the methanol had evaporated 24h later, the "samples" were re-extracted using the procedure previously described. The resulting extracts were analysed on HPLC to determine the concentration of PAHs. The process was repeated using a different concentration of standards and the results obtained are shown in Table 2.3.

Table 2.3 shows the percentage recoveries of selected PAH standards extracted from meat sample residues:- Conc.in ug/ml.

Standard	Initial Conc.	Observed Conc.	Recovery %
A	5.0	4.0	80
B	2.0	1.8	90
E	5.0	3.4	68
F	4.0	3.5	87
G	5.0	4.1	82
H	5.0	4.4	88
K	20.0	17.3	86
L	20.0	16.9	84

AVERAGE RECOVERY   83.2%

From the results, the average recovery was hence taken to be 83%.

2.7 HPLC Operating Conditions

The HPLC column was always kept in methanol. Before analysis would begin acetonitrile was pumped through the column to flush out the methanol and afterwards water was gradually introduced until a ratio of 50:50 (water:acetonitrile) was established at a flow

rate of 1.5 ml/min. With the detector on, the system was left to stabilize over a period of 1hr.

When stability had been attained, the sample was introduced to the column through the sample injector which had a 20uml sample loop. This was the beginning of data collection by the computer and also the start of the gradient elution program which was as follows: the 50:50 (acetonitrile:water) composition was allowed to continue for the first 3min, then the ratio of acetonitrile was increased to 100% linearly over the next 7min. There were no more changes in the mobile phase composition until the end of the elution when the system was programmed to revert to 50:50 (acetonitrile:water) over a linear gradient of 3min and then allowed to stabilize for 10min before the next sample was introduced. Meanwhile the data collected by the computer was saved as a digital spectrum on diskette after removal of random noise.

CHAPTER THREE:
RESULTS AND DISCUSSION

3.1 Extraction and clean-up

The extraction and clean-up procedure employed here yielded satisfactory results as can be inferred from the observed 83% extraction efficiency. Hatc, Felton and Bjeldanes [70] managed 85% efficiency using direct methanol extraction and employing a final extraction into methylene chloride.

Throughout the extraction, the pH was maintained neutral and it was not found necessary to vary the pH as other workers had done for determination of mutagens [20]. In meat, the polar ends of the amino acids bind to the polar ends of PAHs but this happens to a larger extent when the PAH is substituted by a polar group say; NO_2 or NH_2. The bond is much weaker for a non-substituted PAH such as BaP or anthracene. Varying the pH of the extracting solution from neutral to pH2 then to pH10 has been found to contribute significantly to the level of mutagenic activity observed in a sample [29]. This variation in pH helps release the polar groups bound to the amino-acid chains. While this may be a necessary step in total mutagen determination, it may not be so in the analysis of unsubstituted PAHs which were of main interest in

this study due to their low polarity. Figures 3.2 and 3.3 show the results of extraction by both methods. They are derived from one sample which was divided into two equal parts and each portion was extracted using a different method. The same set of PAHs were identified in both chromatograms but there is significantly better resolution in Fig 3.3. This may be a result of less interference from the polar compounds which may have been extracted along with the PAHs using the pH variation method in Fig 3.2. However the extraction of compound G — (benzo(b)fluoranthene) — appears to have been more complete in Figure 3.2 where the pH variation method was employed but the observed peak is nevertheless too high to be explained in terms of extraction efficiency of compound G. A close comparison of the two chromatograms helps to explain the difference. In Fig 3.3 the peak attributed to compound G is fairly small and well resolved from the preceding unidentified one and the successive peak due to compound H. In Fig 3.2 all these three peaks are poorly resolved and in such cases, the tendency is to complement each others' peak heights resulting in one high peak. As stated above many other substances are extracted along with the PAHs, in the pH variation method, and it is possible that some of these compounds contributed to increase the peak height at RT=15 min.
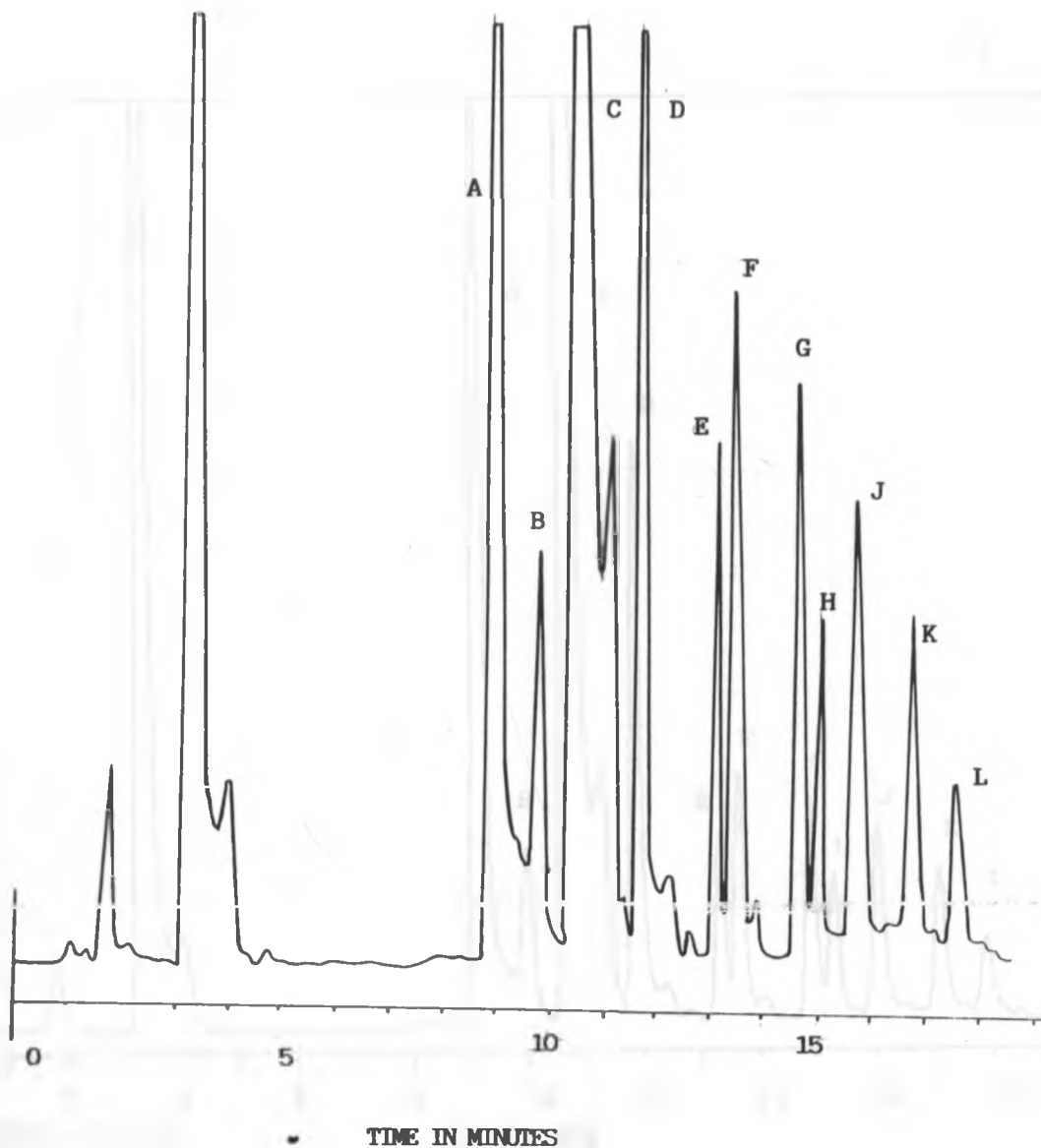
Figure 3.1a   The HPLC trace of a standard mixture
enriched with anthracene   (Original
trace from chart plotter).

A =Fluorene, B= Phenanthrene, C= Anthracene, D=Pyrene, E=benz(a)anthracene, F=Chrysene,
G=Benzo(b)fluoranthene, H=Dibenz(a,c)anthracene, J=Benzo(a)Pyrene,
K=Dibenz(a,h)anthracene, L=Benzo(ghi)perylene

Column: Vydac TP5, 25cm long, I.D. 4.6mm. Pre-column: Vydac 201 TP C18 300A. Flow rate:
1.5ml/min. Gradient elution: 3min (acetonitrile:water)(1:1) then 100% acetonitrile over
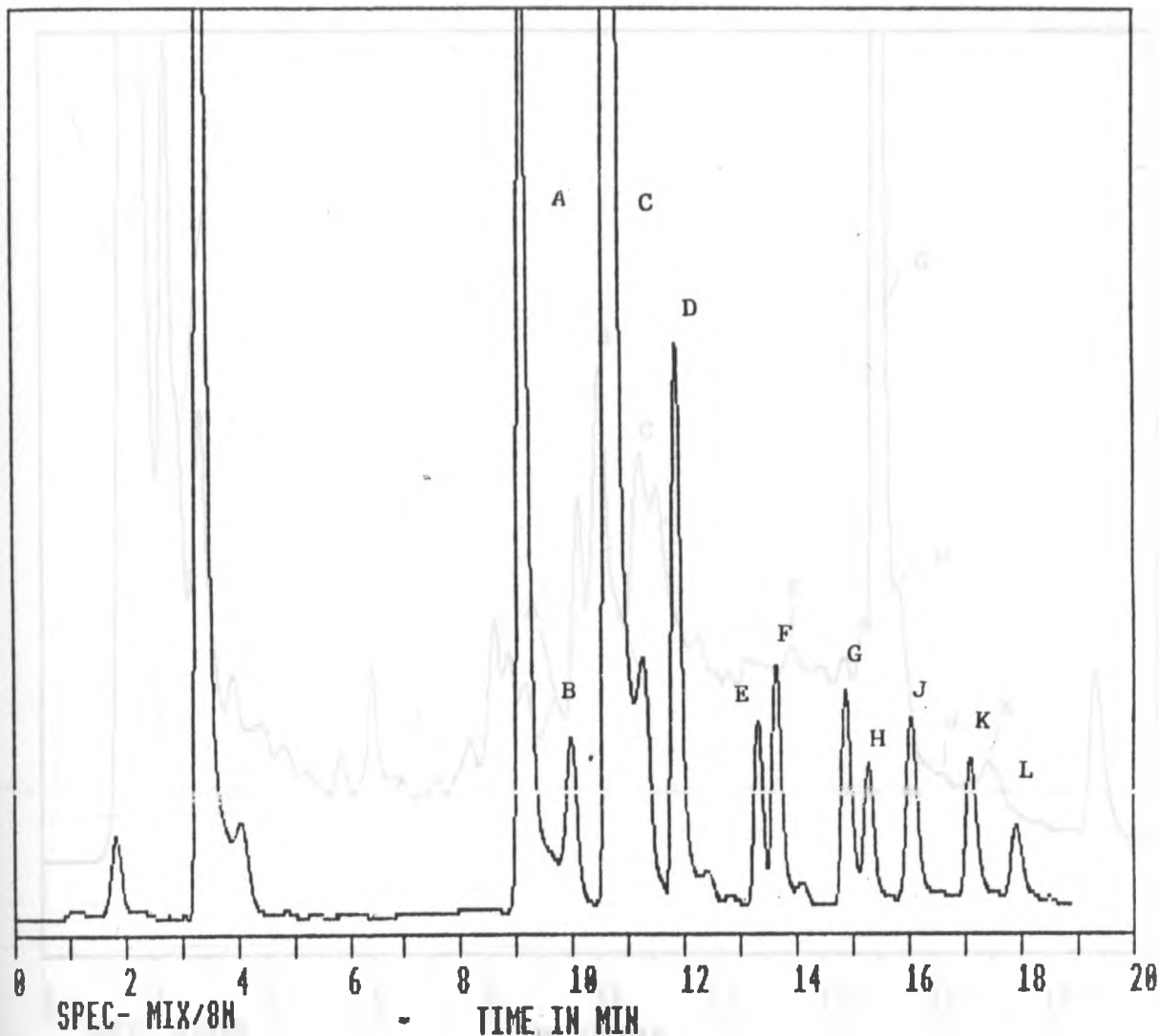7min linear gradient.

Figure 3.1   The HPLC chromatogram of a standard

mixture enriched with anthracene

(Computer print out).

A =Fluorene, B= Phenanthrene, C= Anthracene, D=Pyrene, E=benz(a)anthracene, F=Chrysene,
G=Benzo(b)fluoranthene, H=Dibenz(a,c)anthracene, J=Benzo(a)Pyrene,
K=Dibenz(a,h)anthracene, L=Benzo(ghi)perylene

Column: Vydac TP5, 25cm long, I.D. 4.6mm. Pre-column: Vydac 201 TP C18 300A
Flow rate: 1.5ml/min. Gradient elution: 3min (acetonitrile:water)(1:1) then 100%
acetonitrile over 7min linear gradient.

Figure 3.2 The HPLC chromatogram of PAHs from roast meat sample extracted by the pH variation method.

A =Fluorene, B= Phenanthrene, C= Anthracene, E=benz(a)anthracene,
G=Benzo(b)fluoranthene, H=Dibenz(a,c)anthracene, J=Benzo(a)Pyrene,
K=Dibenz(a,h)anthracene,

Column: Vydac TP5, 25cm long, I.D. 4.6mm. Pre-column: Vydac 201 TP C18 300A
Flow rate: 1.5ml/min. Gradient elution: 3min (acetonitrile:water)(1:1) then 100%
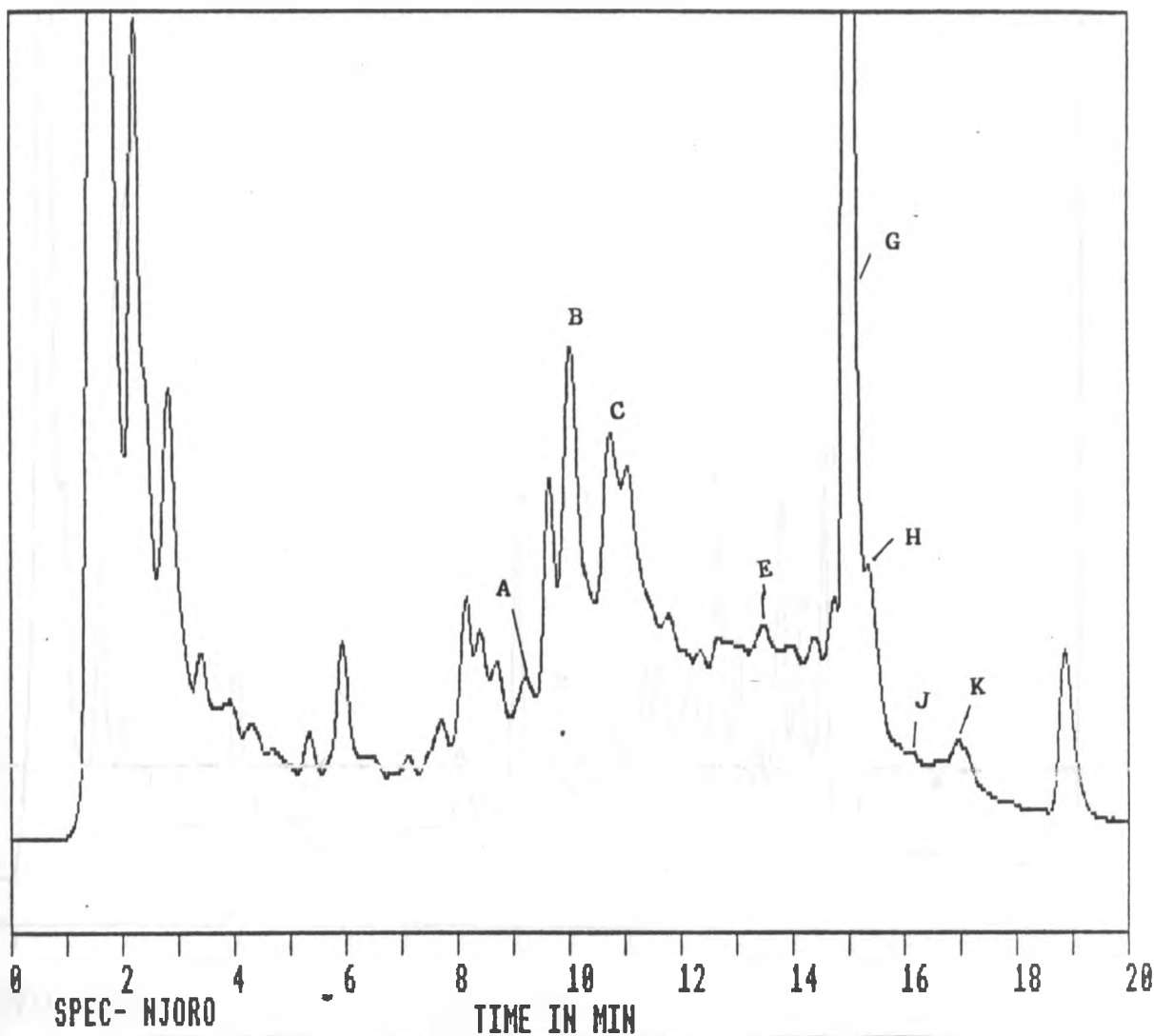acetonitrile over 7min linear gradient.

Figure 3.3   The HPLC chromatogram of PAHs from roast meat sample extracted by the neutral pH method.

A =Fluorene, B= Phenanthrene, C= Anthracene, E=benz(a)anthracene, F=Chrysene,
G=Benzo(b)fluoranthene, H=Dibenz(a,c)anthracene, J=Benzo(a)Pyrene,
K=Dibenz(a,h)anthracene,

Column: Vydac TP5, 25cm long, I.D. 4.6mm. Pre-column: Vydac 201 TP C18 300A
Flow rate: 1.5ml/min. Gradient elution: 3min (acetonitrile:water)(1:1) then 100%
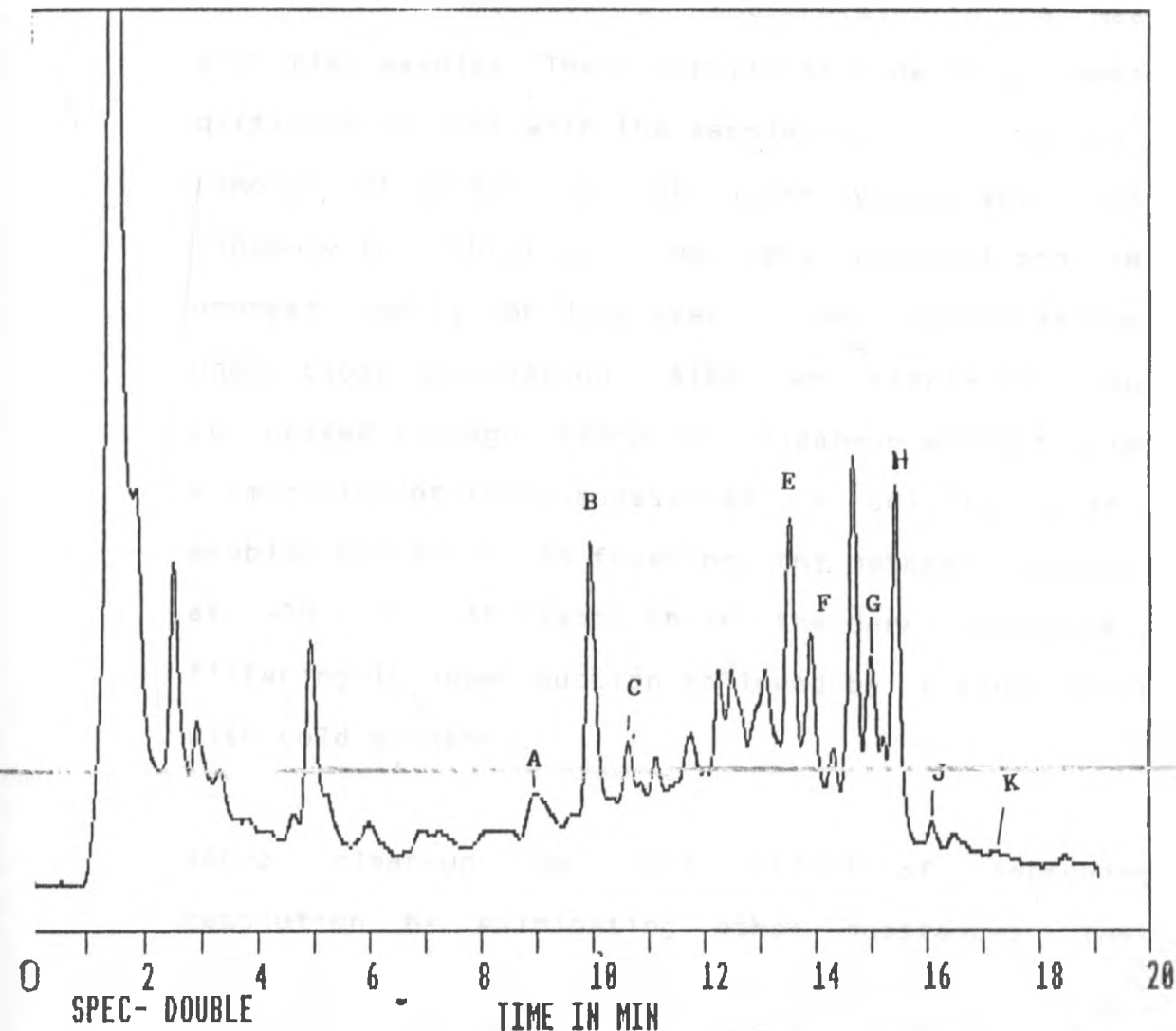acetonitrile over 7min linear gradient.

A major problem in the extraction and clean-up processes which was not documented in most studies was that of dissolved fat and proteins in the meat and fish samples. These substances made it extremely difficult to work with the sample especially during removal of excess solvent under vacuum where the tendency to "shoot out" was very frequent and the process had to be done over a long period of time under close observation. Also, the sample could not be passed through XAD-2 for clean-up without prior elimination of these substances. A solution to this problem was found in freezing the methanol extract at -20^0 C for at least 6h in the dark and quickly filtering it under suction followed by a final rinse with cold methanol.

XAD-2 clean-up had the effect of improving resolution by eliminating other substances that might absorb UV at the same wavelength, and further protecting the HPLC analytical column from non-polar compounds that would otherwise ruin it by binding on the active sites of the stationary phase. The process was, however, lengthy and tedious requiring almost 1h under close supervision.

As mentioned earlier, this fractionation procedure was adopted from the one employed by Spitzer [32] in the GC analysis of PAHs in soil. Where he had

used ethanol, methanol was used instead and acetone
was utilized in place of toluene. These solvents
were found to give satisfactory separation and the
use of acetone was advantageous in that solvent
removal was much easier on rotary evaporation than
would have been with toluene which has a boiling
point of 110^0C. Toluene also has a high extinction
co-efficient at 254nm and its presence interfered
greatly with the detection of PAHs that had R.T less
than 12 minutes.

The procedure employed can be summarized as shown
below:

                    Crude extract
                        | .
                        |
                        |
    25ml Methanol ____ Polars
                        |
                        |
    15ml n-pentane
    10ml methanol ____ Non-polars
                        |
                        |
  ▪   10ml acetone
      20ml methanol ____ PAHs
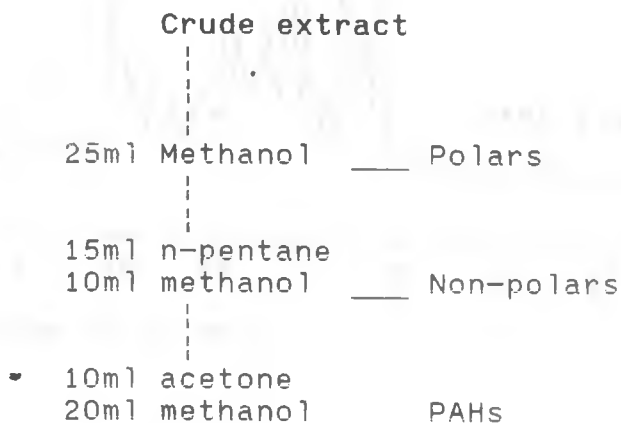
Figure 3.4 shows the results of the clean-up of a
sample on XAD-2 using the above procedure.

Non-Polar fraction

Polar fraction

PAHs fraction

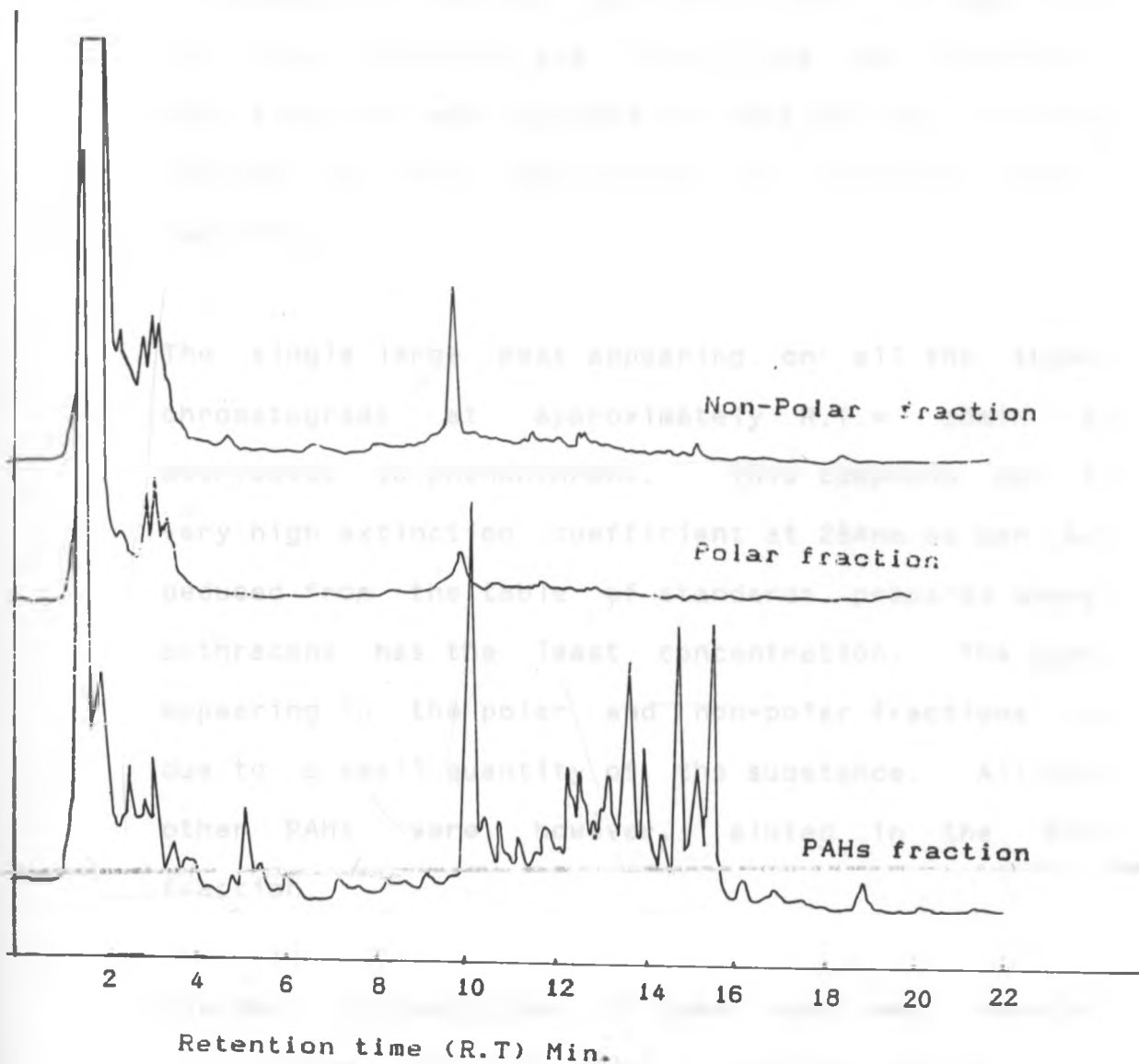Retention time (R.T) Min.

Fig.3.4   The HPLC chromatogram of all the three
fractions obtained after the clean—up of a sample on
XAD—2 and the above procedure.

Clean—up Column: 10cm long,   2cm I.D

Flow rate: 2ml/min

HPLC Column; Vydac TP5, 25cm long, I.D. 4.6mm. Pre-column; Vydac 201 TP C18 300A

Flow rate: 1.5ml/min. Gradient elution: 3min (acetonitrile:water)(1:1) then 100%

acetonitrile over 7min linear gradient.

The non-polar fraction was eluted first followed by the polar fraction and finally the PAH fraction. Each fraction was analysed on HPLC and the results plotted on the same chart to illustrate their relation.

The single large peak appearing on all the three chromatograms at approximately R.T.= 10min is attributed to phenanthrene. This compound has a very high extinction coefficient at 254nm as can be deduced from the table of standards prepared where anthracene has the least concentration. The peak appearing in the polar and non-polar fractions is due to a small quantity of the substance. All the other PAHs were, however, eluted in the PAH fraction.

The HPLC chromatograms of some roast meat samples from three different meat roasting centres in Nairobi are shown in Figures 3.5 - 3.7.
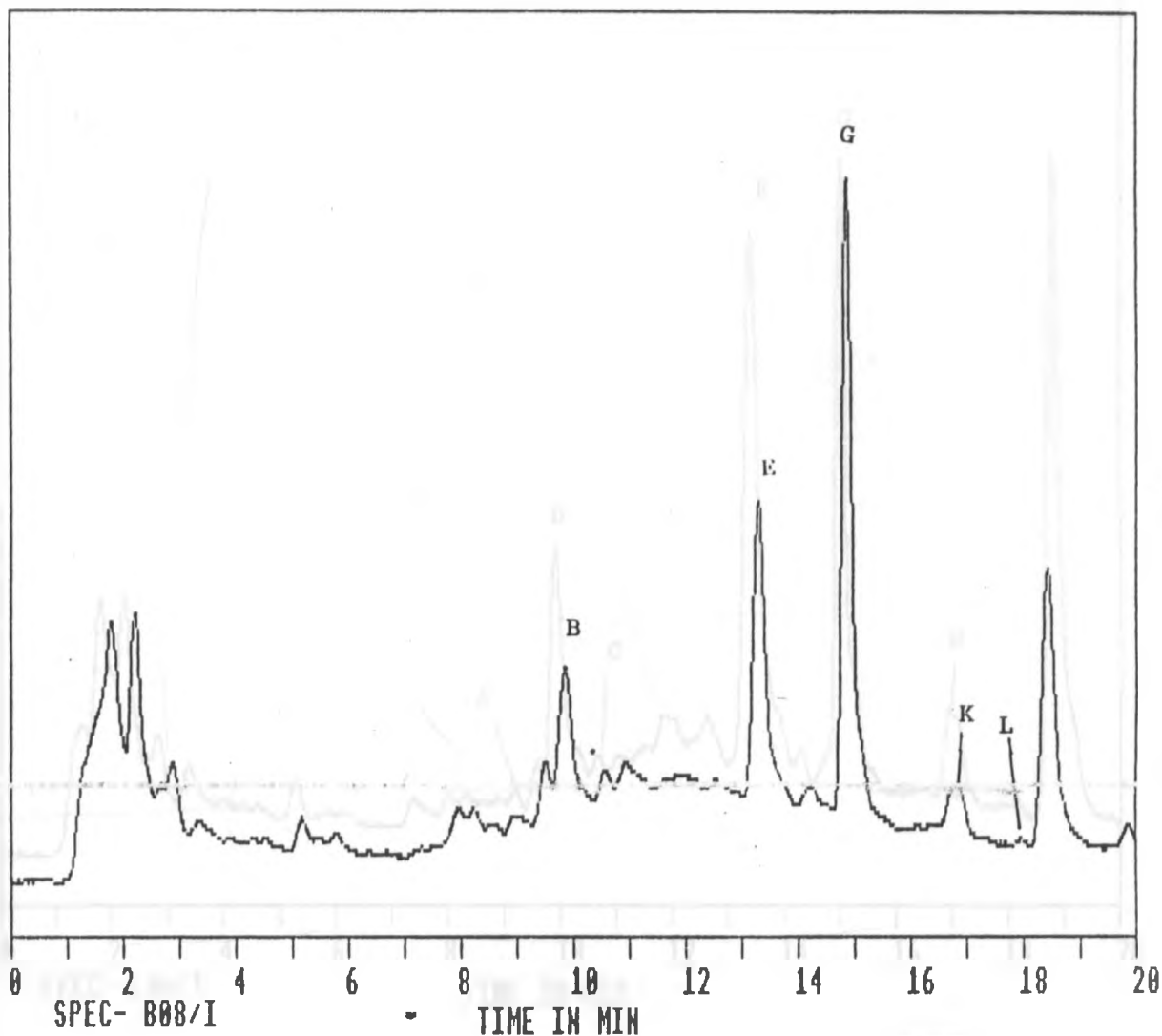
Figure 3.5   The HPLC chromatogram of PAHs in roast meat sample from Kenyatta Market.

B= Phenanthrene, E=benz(a)anthracene, G=Benzo(b)fluoranthene, K=Dibenz(a,h)anthracene, L=Benzo(ghi)perylene

Column: Vydac TP5, 25cm long, I.D. 4.6mm. Pre-column: Vydac 201 TP C18 300A
Flow rate: 1.5ml/min. Gradient elution: 3min (acetonitrile:water)(1:1) then 100%
acetonitrile over 7min linear gradient.
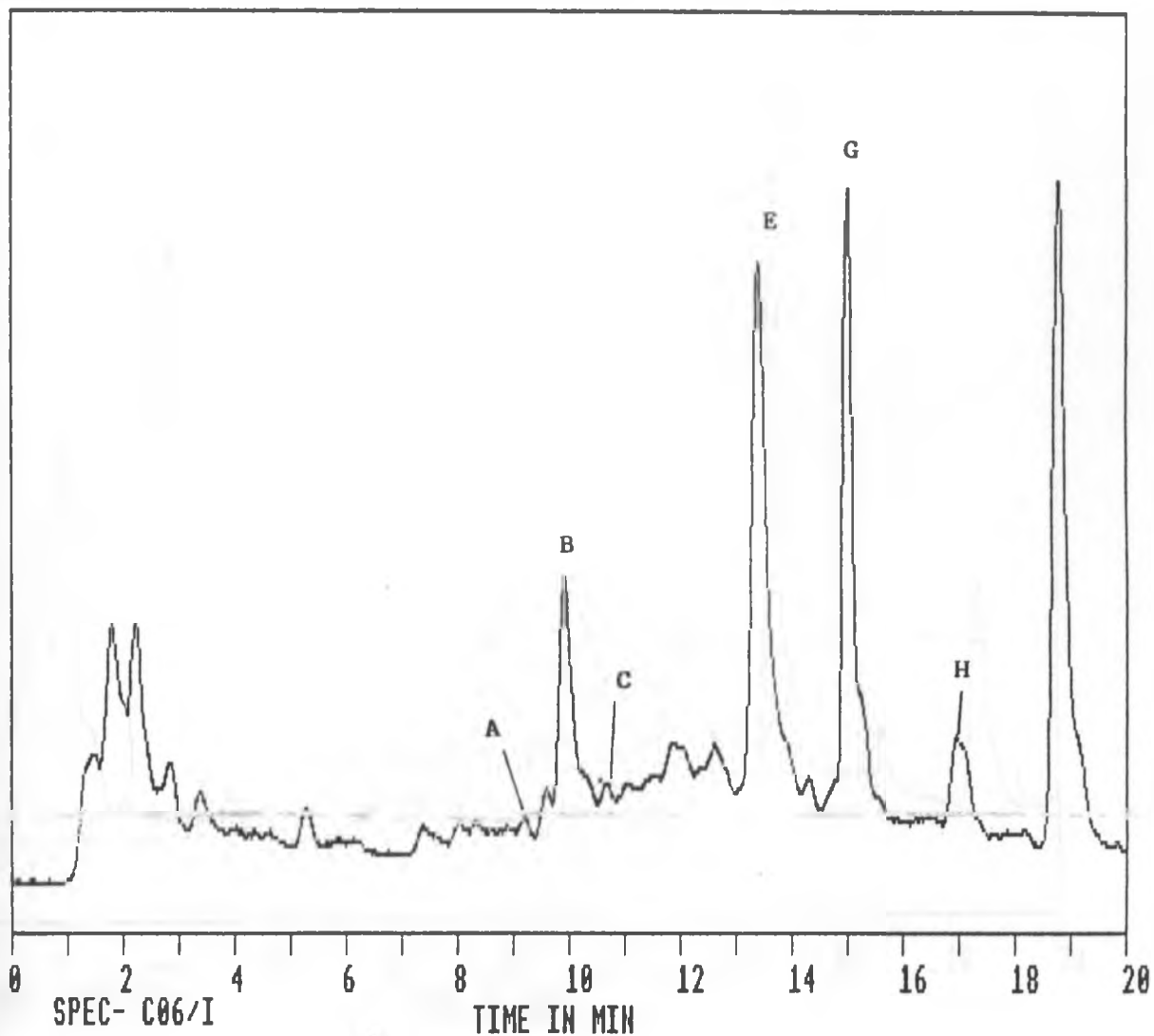
Figure 3.6   The HPLC chromatogram of PAHs in

roast meat sample from Dagoretti

Market.

A =Fluorene, B= Phenanthrene, C= Anthracene, E=benz(a)anthracene, G=Benzo(b)fluoranthene.
H=Dibenz(a,c)anthracene,

Column: Vydac TP5, 25cm long, I.D. 4.6mm. Pre-column: Vydac 201 TP C18 300A
Flow rate: 1.5ml/min. Gradient elution: 3min (acetonitrile:water)(1:1) then 100%
acetonitrile over 7min linear gradient.

Figure 3.7   The HPLC chromatogram of PAHs in
              roast meat sample from Kariokor
              Market.

A =Fluorene, B= Phenanthrene, C= Anthracene, G=Benzo(b)fluoranthene,
K=Dibenz(a,h)anthracene,

Column: Vydac TP5, 25cm long, I.D. 4.6mm. Pre-column: Vydac 201 TP C18 300A
Flow rate: 1.5ml/min. Gradient elution: 3min (acetonitrile:water)(1:1) then 100%
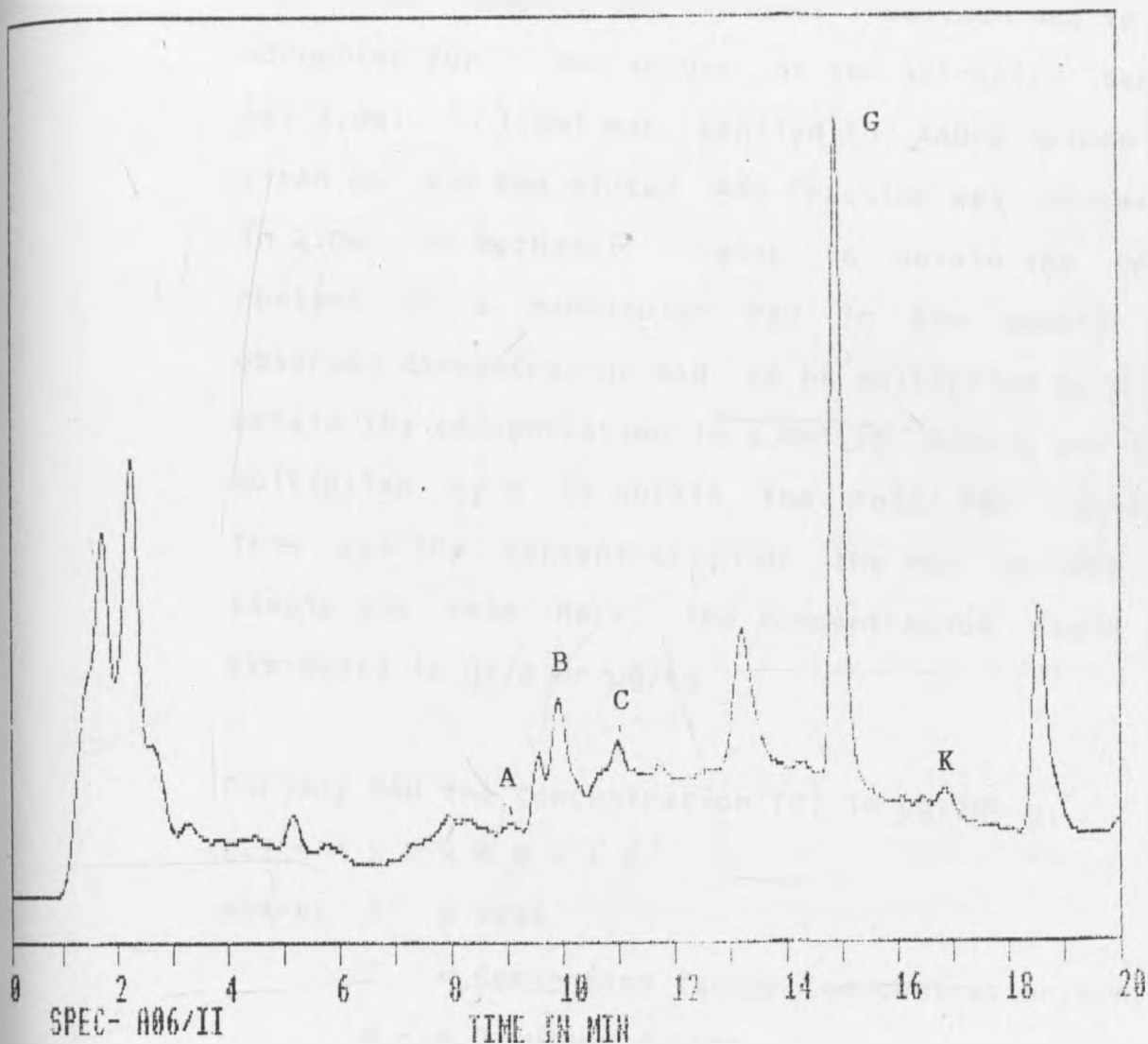acetonitrile over 7min linear gradient.

In calculating the concentration of PAHs in a particular sample, the volumes involved had to be accounted for. The volume of the extracted sample was 3.0ml. 1.0ml was applied to XAD-2 column for clean up and the eluted PAH fraction was contained in 2.0ml of methanol. Hence to obtain the total content of a particular PAH in the sample the observed concentration had to be multiplied by 2 to obtain the concentration in 1.0ml of sample and then multiplied by 3 to obtain the total PAH content. This was the concentration of the PAH in 100g of sample and from here, the concentration could be expressed in ug/g or ug/kg.

For any PAH the concentration [C] in ug/100 g,

[C] = A x F x 6.0 x 1.2

where: A = Area

F = Conversion factor (concentration/Area)

6 = Dilution factor

1.2 = Recovery co-efficient (100/83)

Therefore [C] = 7.2 x A x F

3.2 PAH Analysis of Roast Meat Samples

In all the meat samples analysed, the salient feature was the certain presence of

benzo(b)fluoranthene and phenanthrene. These two compounds were identified in all the samples tested, but were present in varying concentrations. The product moment correlation obtained for corresponding concentrations was 0.064 which is to be compared with the figure of 1.000 for perfectly correlated products. Hence the concentration of these two compounds were not correlated.

There were in total eleven PAHs identified in the samples but this number is by no means exhaustive as it was possible to identify only those compounds whose standards were available for HPLC analysis.

Table 3.1 shows the levels of the PAHs detected in all the roast meat samples analysed. They are classified according to 'market or center from which they were got and the time of day. The values given are the average of two determinations.

Table 3.1 Concentration of various PAHs found in roast meat from three meat roasting centres in Nairobi.

Sample No.		COMPOUND										
		A	B	C	D	E	F	G	H	J	K	L
	A03	1	115	-	-	-	22	1065	-	-	21	-
	A04	-	190	2.4	-	541	-	1353	-	15	-	-
@	A05	4	112	-	-	-	13	269	-	-	145	-
@	B01	-	169	16	-	610	-	1200	-	24	-	-
@	B02	-	34	-	-	67	-	362	-	-	-	-
	C01	3.7	187	-	-	23	-	857	-	-	-	-
	C02	2	230	1	-	-	145	1018	73	-	-	-
	C03	-	152		-	-	-	557	68	-	79	-
	A06	2	101	3	-	-	-	1965	-	-	47	-
	A07	2.5	157	-	-	21	-	774	-	-	-	-
	A08	1	140	1	-	-	-	970	27	-	105	-
#	B04	-	168	-	6	-	-	711	-	-	-	-
#	B05	1.2	162	-	-	262	-	795	-	-	-	-
#	C04	4	119	-	-	-	-	698	-	-	29	-
	C05	6	224	2.4	6	-	-	641	-	-	-	-
	C06	1.1	212	3.4	9	786	-	668	150	-	-	-
	C07	-	235	1.8	56	90	-	2213	14	-	-	-

TABLE 3.1 Continued.....

Sample		A	B	C	D	E	F	G	H	J	K	L
	A09	-	172	-	6	-	-	1636	-	-	82	-
	A10	3	191	1.6	10	-	-	2380	-	-	98	-
&	B07	-	81	1	3	237	-	241	22	-	-	-
&	B08	-	160	-	-	214	-	759	20	-	162	32
&	B09	1.4	45	3.7	-	-	-	2980	-	-	100	-
	B10	1.3	176	-	-	372	-	1291	-	-	23	-
	C08	6.3	309	-	-	-	132	1329	-	-	106	-
	C09	4	210	2.7	25	-	-	791	-	-	15	-
	C10	2.4	120	3.3	-	27	-	766	-	-	-	-

Sampling period.

@ = 11.00a.m — 12.30p.m.

= 12.30p.m — 2.00p.m.

& = 2.00p.m — 3.30p.m.

Samples: **AO** =Kariokor Market, **BO** =Kenyatta Market,

CO =Dagoretti Market.

Key to compounds:

A = Fluorene, B= Phenanthrene, C = Anthracene, D = Pyrene, E = benz(a)anthracene, F = Chrysene, G = Benzo(b)fluoranthene,   H = Dibenz(a,c)anthracene,   J = Benzo(a)Pyrene, K = Dibenz(a,h)anthracene L = Benzo(ghi)perylene

Tables 3.2 — 3.4   depict   other characteristics of the results obtained.

Table 3.2   The mean levels of PAHs obtained in meat from the three centres.   (Concentration in ug/kg.)

Compound	Kariokor Market	Kenyatta Market	Dagoretti Market
Fluorene	1.7	0.5	3.2
Phenanthrene	147.0	124.0	199.8
Anthracene	1.0	2.5	1.5
Pyrene	2.0	1.1	9.6
benz(a)anthracene	·70.2	220.2	92.6
Chrysene	4.4	0.0	28.7
Benzo(b)fluoranthene	1302.0	1042.0	954.0
Dibenz(a,c)anthracene	3.4	5.3	30.5
Benzo(a)pyrene	1.9	3.0	0.0
Dibenz(a,h)anthracene	62.3	36.0	23.0
Benzo(ghi)perylene	0.0	4.0	0.0

Table 3.3   Variations in the concentration of PAHs in roast meat from the three Nairobi Markets (conc.in ug/kg).

Compound	minimum	maximum	mean
Fluorene	0.0	6.3	1.8
Phenanthrene	34.0	309.0	160.0
Anthracene	0.0	16.0	1.6
Pyrene	0.0	56.0	4.7
benz(a)anthracene	0.0	786.0	125.0
Chrysene	0.0	145.0	12.0
Benzo(b)fluoranthene	241.0	2980.0	1088.0
Dibenz(a,c)anthracene	0.0	150.0	14.0
Benzo(a)pyrene	·0.0	24.0	1.5
dibenz(a,h)anthracene	0.0	162.0	39.0
Benzo(ghi)perylene	0.0	32.0	1.2

One of the main factors affecting PAH formation in roast meat is the rate of heat transfer from the source to the meat. PAH formation is also dependent on the smoke emanating from the fire as observed in cases of fish smoking where most of the PAHs were attributed to the smoke [12]. During the day, variations were expected in the intensity and

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character of the roasting fires, and indeed such variation were observed. At some times of the day (11.00a.m. – 12.30p.m.) the fires were fresh and levels of smoke from the charcoal were higher during this period but from 12.30 – 2.00p.m. the fires settled to bright glowing embers with little smoke from the charcoal and it was at this period that the fires were hottest. Later in the day (2.00 – 3.30p.m.), some fires were refuelled depending on requirements, and the fresh charcoal did introduce some smoke, while others were left to continue without addition of more fuel. The condition of the grill at the later hours of the day was mainly greasy. An attempt was made to observe the variation in levels of PAHs detected in the roast meat samples against the time of sampling and the results are given in Table 3.4 below. These figures are averages of the values from the three markets.

Table 3.4 : Average concentrations of PAHs in relation to time of sampling (Conc.in ug/kg).

Compound	Time of Day		
	11.00–12.30	12.30–2.00	2.00–3.00
Fluorene	1.3	2.0	2.0
Phenanthrene	149.0	169.0	163.0
Anthracene	2.4	1.3	1.4
Pyrene	0.0	8.5	4.9
benz(a)anthracene	155.0	129.0	94.4
Chrysene	22.5	0.0	0.0
Benzo(b)fluoranthene	835.0	1048.0	1353.0
Dibenz(a,c)anthracene	17.6	21.0	4.6
Benzo(a)pyrene	4.9	0.0	0.0
dibenz(a,h)anthracene	30.6	20.1	65.1
Benzo(ghi)perylene	0.0	0.0	3.5

The levels of the different compounds were generally different throughout the sampling period.

Fluorene levels were however almost constant during the whole period and the same could be said for phenanthrene and anthracene. Chrysene and Benzo(a)pyrene were detected only in the early hours (11.00a.m. – 12.30p.m.) and are probably due to

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smoke from the fires. Levels of phenanthrene, pyrene and dibenz(a,c)anthracene were highest at the peak hours (12.30 - 2.00p.m.). Pyrene was non-existent during the 11.00a.m. - 12.30p.m. period while dibenz(a,c)anthracene was lowest in the late afternoon session. It is also noted that levels of 1,2-benzanthracene diminished progressively with the time of sampling while those of benzo(b)fluoranthene increased. Benzo(ghi)perylene was only detected in samples taken between 2.00 - 3.30p.m. The sharp differences observed illustrate that there are many factors that govern PAH formation in roast meat and many of these are interrelated. Though it was difficult to retain constant conditions over the fires for long periods ·(I could not ask for this without divulging my real intentions) there were some general characteristics that were somewhat constant. These have been described above such as the smoky state of the fires during the hours before 12.30p.m. and the general stability of the fires from 12.30 - 2.00p.m. The compound BaP and chrysene were only detected in samples taken before 12.30p.m. suggesting that the source may have been the smoke from fresh charcoal. On the other hand benzo(ghi)perylene was only found in samples taken between 2.00 - 3.30p.m. Other PAHs such as phenanthrene, fluorene, anthracene, benz(a)anthracene, and benzo(b)fluoranthene were

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present in all samples and did not show marked variations with the time of sampling.

3.3 PAH Analysis in Samples of Smoked Fish

Samples of smoked fish bought from a popular Nairobi fish market (Gikomba) were analysed for PAHs using the procedure previously described. The levels obtained are shown in Table 3.5

Table 3.5 Levels of PAHs in samples of smoked fish obtained from a Nairobi fish market. (conc.in ug/kg)

Compound	Fish 1	Fish 2	Fish 3
Fluorene	. 0.0	0.0	180.0
Phenanthrene	274.0	322.0	194.0
Anthracene	15.0	0.0	0.0
Pyrene	57.0	70.0	0.0
benz(b)anthracene	0.0	0.0	0.0
Chrysene	0.0	31.0	0.0
Benzo(b)fluoranthene	1497.0	1206.0	965.0
Dibenz(a,c)anthracene	0.0	0.0	98.0
Benzo(a)pyrene	0.0	0.0	0.0
Dibenz(a,h)anthracene	0.0	743.0	446.0
Benzo(ghi)perylene	0.0	0.0	0.0

Not all the PAHs that were detected in meat samples could be identified in the smoked fish. Notable among these was the absence of BaP, benzo(ghi)perylene and benz(b)anthracene. Some compounds were detected only once in the three samples tested. These were fluorene, anthracene, chrysene and dibenz(a,c)anthracene. Of significance was the presence of both phenanthrene and benzo(b)fluoranthene in all the fish samples just like in the roast meat samples, but the levels of phenanthrene were even higher in the smoked fish samples. It is noteworthy that there is sufficient evidence for the fairly high carcinogenicity of benzo(b)fluoranthene, (see Tables 3.7 and 3.8). The amount of dibenz(a;h)anthracene, which is also an established carcinogen, detected in two of the samples was much higher than had been observed in any roast meat sample. The observed low values or total absence of some of the PAHs could probably be due to the long storage periods of the fish.

Two HPLC chromatograms of fish samples are shown in Figures 3.8 and 3.9.
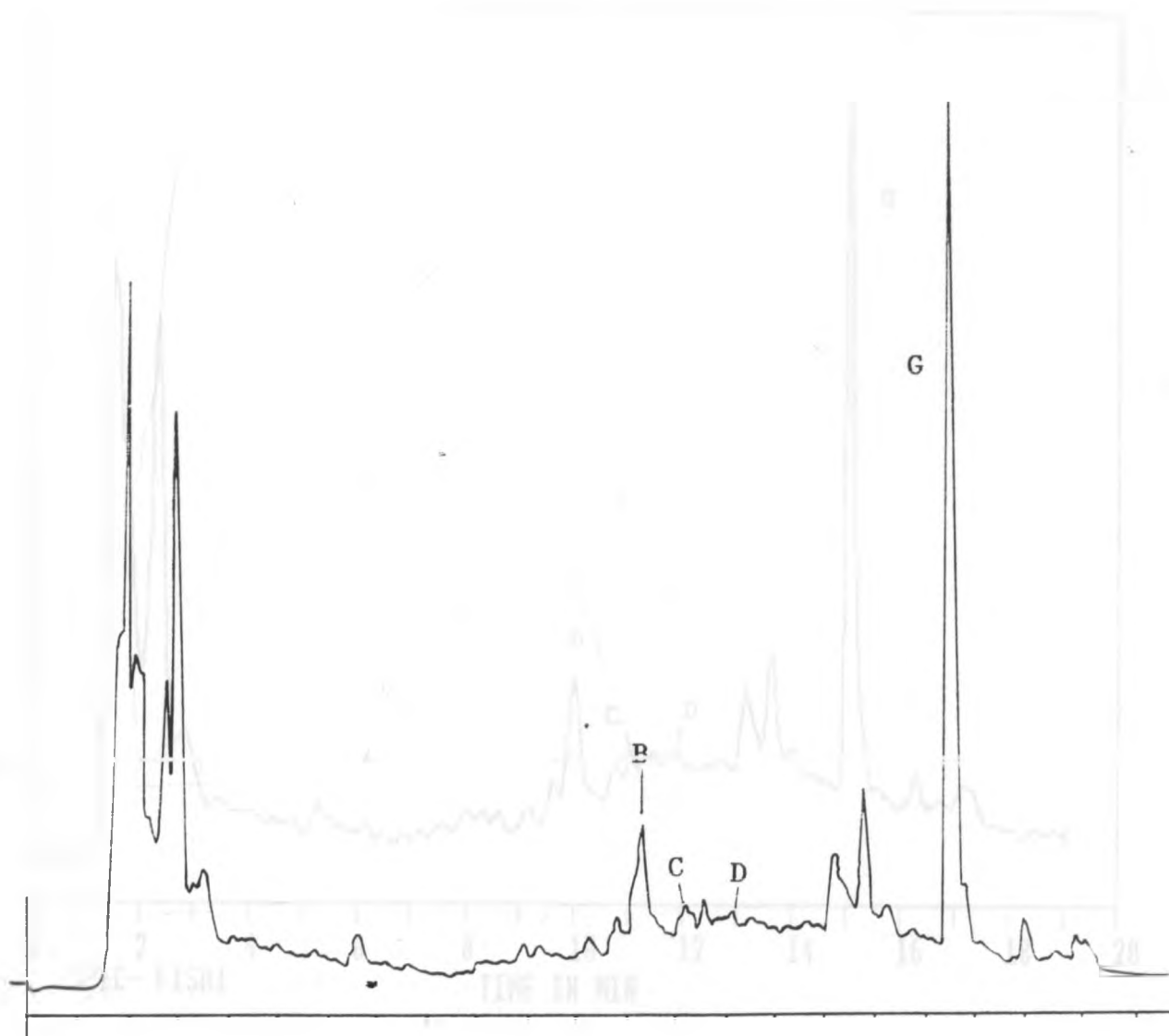
Figure 3.8a   The HPLC trace of PAHs in smoked fish
sample from Gikomba Market Nairobi
(trace from original plot).

B= Phenanthrene, C= Anthracene, D=Pyrene, G=Benzo(b)fluoranthene,

Column: Vydac TP5, 25cm long, I.D. 4.6mm. Pre-column: Vydac 201 TP C18 300A
Flow rate: 1.5ml/min. Gradient elution: 3min (acetonitrile:water)(1:1) then 100%
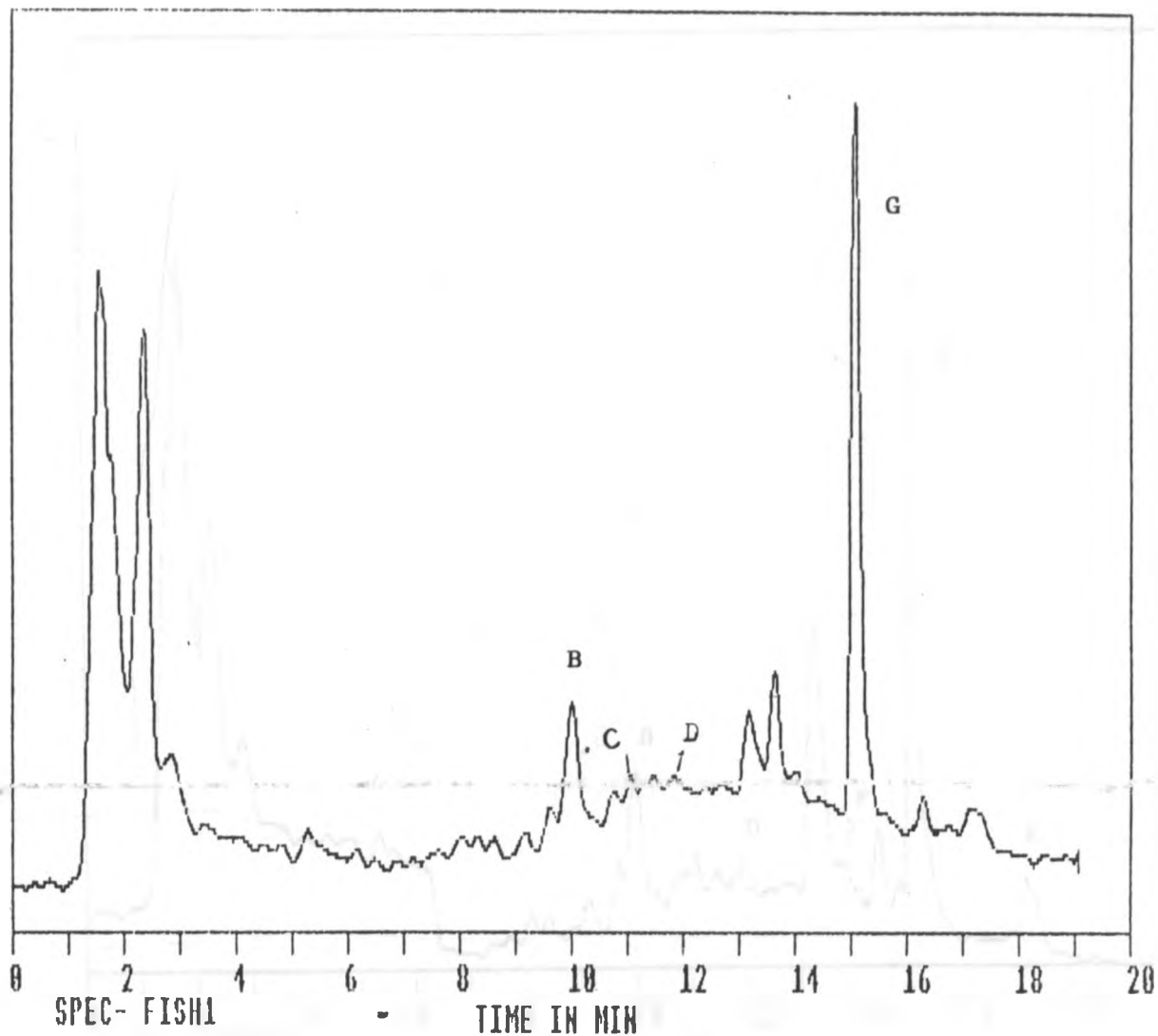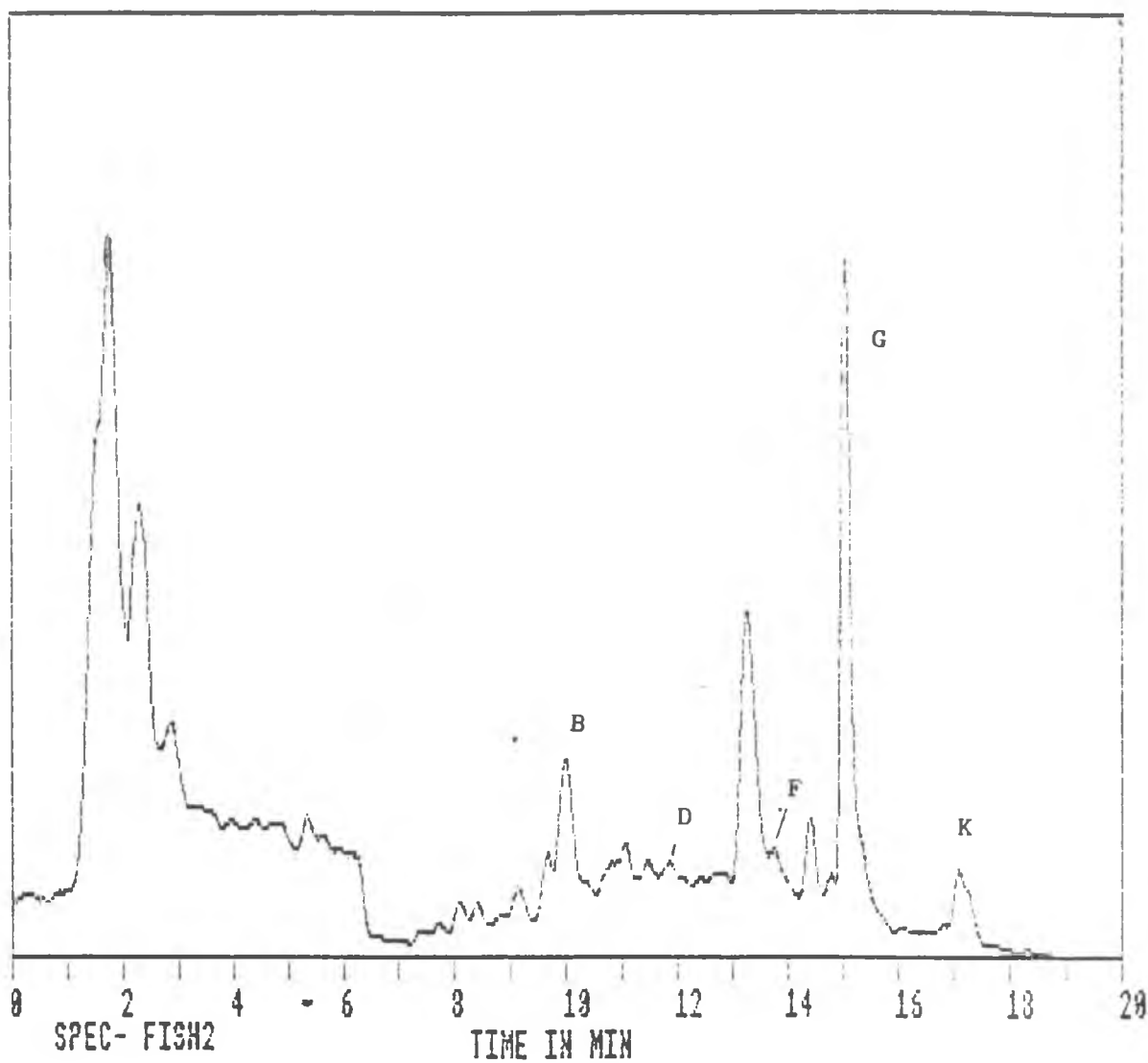acetonitrile over 7min linear gradient.

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Figure 3.8   The HPLC chromatogram of PAHs in smoked fish sample from Gikomba Market Nairobi.

B= Phenanthrene, C= Anthracene, D=Pyrene, G=Benzo(b)fluoranthene,

Column: Vydac TP5, 25cm long, I.D. 4.6mm. Pre-column: Vydac 201 TP C18 300A
Flow rate: 1.5ml/min. Gradient elution: 3min (acetonitrile:water)(1:1) then 100%
acetonitrile over 7min linear gradient

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Figure 3.9   The HPLC chromatogram of PAHs   in
              smoked   fish   sample   from   Gikomba
              Market Nairobi.

B= Phenanthrene, D=Pyrene, F=Chrysene, G=Benzo(b)fluoranthene, K=Dibenz(a,h)anthracene,

Column: Vydac TP5, 25cm long, I.D. 4.6mm. Pre-column: Vydac 201 TP C18 300A
Flow rate: 1.5ml/min. Gradient elution: 3min (acetonitrile:water)(1:1) then 100%
acetonitrile over 7min linear gradient.

3.4   Computer Assistance

Right from the start, the project was intended to be one that would feature the application of computers in the solution of common chemical analysis problems. Computers have been widely used to solve many chemical problems and are indeed available for the solution of many others as long as the mode of communication to tap the computer's "intelligence" can be established. This communication link is called an interface which normally comprises of a piece of hardware and a set of instructions (software) to operate it. At the beginning of the project, the Department of Chemistry had a microcomputer but without the necessary interface to link it with the various analytical instruments in the laboratory. As a result of my interest in computers I undertook to construct an interface and develop the operating software that would facilitate communication between the computer and a HPLC instrument to accomplish the following:-

- enter data into the computer from the detector,
- store data on magnetic disk for future manipulation,
- perform chromatographic analysis i.e. peak integration and determination of retention times
- produce various displays of data,, results or spectra for ease of reference and analysis.

The hardware requirements were few but a lot was required by way of software. The data was to be obtained by tapping the output voltages of the detector. Since this output voltage varied between a minimum of -ve 0.5V and +ve 1.0V, there was no need for signal amplification and the interface hardware consisted of a resistor and zener diode configured to protect the input port of the computer from excessive voltages.

In developing the software the following factors had to be taken into account:

a)   electronic noise from the instrument,

b)   the size of the microcomputer memory (storage capacity).

The signal to noise ratio (SNR) at the detector output was at a poor level and required some modification in order to free the signal from the noise. Some of this noise could be eliminated by increasing the attenuation on the spectrophotometer flowcell but this was unfortunately accompanied by a corresponding loss in sensitivity. A criterion for handling the noise problem was established after studying the detector output over a long period of time and under different conditions. As previously explained, the computer lumped five consecutive

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points and recorded the average which was taken as the true point on the curve and in this way, 2 points were recorded every second. Even after this initial noise reduction, another "smoothing" operation had to be done before saving the spectra. This was achieved by employing a 20 points box car average. It was noticed that after this "smoothing" operation the retention times of the peaks in the spectra were slightly altered and an adjustment in the software had to be incorporated to counter this shift.

The size of the computer memory was also one of the limiting factors in the development of the software. As the BBC microcomputer model B had a memory size of only 32 Kilobytes (32K RAM) and not all of which is available for data storage, measures had to be taken to conserve memory as much as possible.

Two data points per second were found to be the minimum possible points that if stored in the computer would allow a faithful reproduction of a chromatogram. Any given peak spanned at least 10 seconds. Another measure taken to conserve memory was to write (poke) the data directly into specific memory locations instead of leaving the computer to assign memory automatically. A lot of space in the memory was conserved in this way as the computer

normally reserves five memory spaces (bytes) for
each datum which sometimes is in excess of
requirement. Only one byte is needed to store a
point whose digital value does not exceed 255, and
two bytes for higher values but which do not exceed
255 x 255. The values entering the computer were
'controlled' not to exceed the latter value and
hence only a maximum of two bytes were required per
datum. Up to 1h. of chromatography could be stored
in the computer memory by this method. The saving
in memory was accompanied by a slight loss in
operation speed of the computer but not of a
magnitude to hamper the fluent collection of data.
Making use of the BBC microcomputer second processor
enhanced the memory to 64K from 32K and it was
possible to collect up to 36 minutes of
chromatography without having to write directly into
memory and therefore without loss of operation
speed. This arrangement was very useful during the
analysis of the chromatogram where speed was
essential.

3.6a   Obtaining areas and retention times
Quantification in chromatography requires the
determination of the size of the peak either in
units of area or weight, both of which are related

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to concentration and in some instances, peak heights
are used. These determinations are sometimes
complicated by the inability of the chromatographic
system to fully resolve all the components in a
sample, thus resulting in poorly defined merged
peaks and further, by the instability of the
baseline.

Since the introduction of computers into
chromatography various methods have been developed
for peak area determination. These range from the
simple ones such as the height times width (at half
height) and the trapezium methods, to elaborate
gaussian curve-fitting methods [57,58]

A fairly simple method was employed in our study to
obtain peak areas and to resolve merged peaks. This
involved the summation of consecutive heights along
a curve and after substraction of the baseline, the
values obtained were directly proportional to the
area of the peak. As this method follows the actual
curve, all points along it are taken into
consideration and is hence more accurate than the
trapezium or the height X width (at half height)
methods. For resolving merged peaks, the
perpendicular drop method [58] was adopted.

It was also possible to obtain areas of peaks that

on an ordinary chromatogram chart would be considered out of range and hence not quantifiable. With the computer system, peaks exceeding the maximum peak height of the chart could be stored in memory and later reconstructed for the purposes of analysis or display.

Identification in chromatography relies on the location of the peak in the chromatogram i.e. the retention time which is a factor of the polarity of the substance. Retention times were easily determined even for broad peaks as the program could pick out slight changes in the gradient. Where ambiguity or doubt arose in the identification of a peak, the sample was spiked with the suspected compound and an increase in height confirmed the inference.

It was noted that the retention time of a compound in a mixture was slightly different from the one obtained by putting the single compound through the HPLC. The R.T. for all standards were found to be very consistent and this made identification of the sample components a less tedious task.

The computer assisted HPLC method developed here proved to be a versatile system for performing HPLC analysis. It has since then been employed for the analysis of PAHs in wood smoke, pyrethrins from

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pyrethrum extract and even drug metabolites. It has also been used for instruction in course work and in a workshop on applications of HPLC for natural products analysis to mention a few.

As the system is not confined entirely to HPLC work and can be easily adopted for GC, IR or NMR its applications are expected to expand. Though it took a considerable length of time to develop the system the gains in efficiency and accuracy were worth the while and as other workers can make use of it, its usefulness is enhanced and this further justifies the amount of time spent on its development. Such a system is ideal for low budget laboratories found in many institutions which are mainly stocked with non-computerized instruments. The system extends to these instruments all the benefits of a computer but at the same time the computer retains its independence and is available for other tasks. The end result is a great deal of efficiency and flexibility at a low cost.

3.5 Health Effects of Eating Roasted Meat and Smoked Fish

Roast meat and smoked fish have been shown to be potential sources of PAH exposure to man [10,12,39]. Since the time when Percival and co-workers [59] observed the high incidence of epithelioma of the scrotum among 'chimney sweeps', the study of chemical carcinogenesis has widely progressed [60]. The successful induction of tumors in animal models [61] opened the way to experimental carcinogenesis and soon BaP was identified as the main active compound in the carcinogenic activity of coal tar [63]. Evidence exists for carcinogenicity of BaP to animals and in short term tests with bacteria but, ironically, the evidence is insufficient when applied to human beings [63]. Assessment of the risk due to BaP is difficult since human populations are also exposed simultaneously to mixtures of other compounds of known or possible carcinogenicity including (but not limited to) other PAHs.

There has been some attempts to quantify the carcinogenic risk to humans due to BaP based on its observed activity in animals and bacteria. One such estimate has postulated that exposure to 10ng BaP can potentially produce 10-40 total cancers per one million persons [64].

Many of the PAHs that were found in roasted meat and smoked fish have been identified in wood smoke as well. The results of the analysis of kitchen smoke from a Kenyan household are shown in table 3.6.

Table 3.6. Concentration of various PAHs in kitchen smoke from a Kenyan household. (Conc. ng/m^3)

Compound	Mean	Max	Min
1. Fluoranthene	254	779	34
2. Pyrene	77	2387	103
3. Benz(a)anthracene	273	838	36
4. Chrysene	262	801	34
5. Benzo(b)fluoranthene	106	324	14
6. Benzo(k)fluoranthene	36	109	5
7. Benzo(a)pyrene	86	263	11
8. Benzo(ghi)perylene	252	771	33
9. Dibenz(a,h)anthracene	143	438	19
10. Indeno(1,2,3-cd)pyrene	51	158	7

Source [49]

An earlier study done in the Kenyan Highlands had found the concentration of BaP in the total suspended particulate in a smoky house to be 145 ng/m^3 [65]

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As in the case of diet, the evidence on the relationship between exposure to biomass smoke and cancer risk is sparse [66]. There have been a few studies that have indicated as possible the relationship between exposure to biomass smoke and nasopharyngeal cancer but in general, however, the link between cancers and biomass smoke has not been established in spite of the large quantities of known carcinogens in biomass smoke [66]. Some established characteristics of some PAHs are shown in Tables 3.7 and 3.8.

Table 3.7   The established degree of evidence of carcinogenicity for some PAHs. (source [63]).

Compound	Degree of Evidence of Carcinogenicity for experimental Animals
Anthracene	No evidence
Benz(a)fluoranthene	Sufficient
Benzo(b)fluoranthene	" "
Benzo(k)fluoranthene	" "
Benzo(ghi)fluoranthene	Inadequate
Benzo(ghi)perylene	" "
Benzo(c)Phenanthrene	" "
Benzo(a)pyrene	Sufficient
Benzo(e)pyrene	Inadequate
Chrysene	Limited

Table 3.7 cont.........

Dibenz(a,c)anthracene	Limited
Dibenz(a,h)anthracene	Sufficient
Fluoranthene	No evidence
Fluorene	Inadequate
Indeno(1,2,3-cd)pyrene	Sufficient
Perylene	Inadequate
Phenanthrene	" "
Pyrene	No evidence

Table 3.8   The relation between PAHs carcinogenic activity and their effect on the immune system. Source [68].

Compound.	Carcino-genicity	Immuno suppression(%)
Naphthalene	−	−
Anthracene	−	−
Chrysene	−+	26
Benz(a)anthracene	+	56
Dibenz(a,c)anthracene	+	55
Dibenz(a,h)anthracene	+++	91
Benzo(a)pyrene	++++	64
Benzo(e)pyrene	−	−
Perylene	−	−
Pyrene	−	−

Key: No effect −, Very weakly active +−, Weakly active +, Active ++, Very active +++, Extremely active ++++.

Shown in Table 3.8 is the effect of the PAHs on the immune system of the body. It has been observed that all known carcinogenic PAHs, e.g. BaP, also suppress the immune system whereas the non-carcinogenic PAHs such as anthracene are devoid of immunosuppressive activity [68]. It is also known that some PAHs act as co-carcinogens which activate other PAHs that would otherwise be dormant and cause them to become carcinogenic.

3.6 Conclusion.

Roasted meat and smoked fish have been shown in this study and in others [10,12,39] to contain PAHs many of which are carcinogenic. Many of these PAHs have also been identified in biomass smoke and it is likely that these compounds end up in the foods treated over open fires. Eating roasted meat (nyama choma) is a celebrated social activity in many parts of the county and smoked fish is very popular with the people of the Lake Victoria Region. In view of the rising number of cancer deaths in Kenyan Hospitals [41] and also the growing evidence of the relationship of some types of cancer to diet, a more intensive investigation is required to clearly elucidate the correlation, if any, of roast meat and smoked fish consumption and cancer. As has been shown in this study, the compound, dibenz(a,h)anthracene is present in smoked fish in

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not quite insignificant levels and so is BaP in roast meat among others. Evidence exists relating these compounds to cancer occurrence and high immunosuppression (see Tables 3.7 and 3.8). There is also a need to evaluate the relationship between emission of PAHs from wood fuel and the levels of these compounds that are to be found in roasted meat or smoked fish.

There are however other factors that make the evaluation of the carcinogenic potential of a particular food even more complicated. This is the presence of dismutagens' in some of the foods consumed along with the suspected carcinogenic foods. These compounds exhibit antimutagenic properties and act by either directly inactivating mutagens or by inhibiting metabolic activation of promutagens as well as blocking the formation of mutagens from precursors [42]. Extracts from many fruits and vegetables are dismutagenic. These include peroxidases from cabbage and broccoli vegetable fibre (which absorb many mutagens) and reducing agents such as vitamin C. In assessing carcinogenic potential of a diet, it is necessary to take all the food components into consideration, and to mention the need, in the light of the above, of eating a balanced diet and to avoid foods with high levels of carcinogens.

LIST OF FIGURES

LIST OF TABLES

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COMPUTER PROGRAMMMES

```
>LOAD"MENU"
>L.
  100 SOUND 1,-12,150,2
  110 MODE1
  120 PRINT:PRINT
  130 PRINT:PRINT
  140  PRINT:PRINT
  150 ON ERROR GOTO 100
  160 REM VDU 19,4,2,0,0,0
  170 COLOUR 129
  180 PRINT "    YOU CAN CHOOSE ANY     "
  190 PRINT "  OF THE FOLLOWING OPTIONS"
  200 VDU 19,2,4,0,0,0
  210 COLOUR 130
  220 PRINT:PRINT
  230 PRINT
  240 PRINT "  (1) ENTER NEW SPECTRUM   "
  250 PRINT
  260 PRINT "  (2) DISPLAY SPECTRUM     "
  270 PRINT
  280 PRINT "  (3) OBTAIN PLOT ON PAPER"
  290 PRINT
  300 PRINT "  (4) ANALYSE SPECTRUM     "
  310 PRINT
  320 PRINT "  (5) QUIT"
  330 PRINT:PRINT
  340 COLOUR 129
  350 PRINT "  For ANALYSIS You Will need the"
  360 PRINT "  SECOND PROCESSOR              "
  370 PRINT:PRINT " If it is not ENGAGED Put it on "
  380 PRINT" and START again"
  390 COLOUR 130
  400 PRINT
  410 PRINT "    ENTER OPTION    ";
  420 C=GET
  430 IF C=(49) THEN 490
  440 IF C=(50) THEN 500
  450 IF C=(51) THEN 510
  460 IF C=(52) THEN 520
  470 IF C=(53) THEN 560
  480 GOTO 420
  490 CHAIN"COLLECT"
  500 CHAIN"PLOTC"
  510 CHAIN"PLOT3"
  520 CLS
  530 PRINT TAB(12,10)"PRESS   (f0)"
  540 VDU21
  550 *KEY 0 *FX 142,10!M :CH."SUMM"!M
  560 PRINT:END
```

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```
>LOAD"COLLECT"N
>L.
  100 MODE7
  110 DIM M 10000
  120 REM PROGRAMME TO ENTER DATA TO MEMORY AND ONTO DISC
  130 REM THIS PROGRAM DOES NOT USE THE SECOND PROCESSOR
  140 ON ERROR GOTO980
  150 REM THE PROGRAM COLLECTS 2 POINTS EVERY SECOND
  160 PD=2
  170 SOUND 1,-15,100,2
  180 PRINT TAB(5,24);"PRESS";CHR$(130);"SPACE BAR          ";CHR$(135);"TO CONTINUE
  "
  190 PRINT TAB(1,2);CHR$(129);"** THE SPECTRA MUST NOT EXCEED 30. Min"
  200 B$=GET$:IF B$<>CHR$(32) THEN 200
  210 SOUND 1,-15,150,4
  220 PRINT TAB(1,4);CHR$(130);"DO NOT USE THE SECOND PROCESSOR "
  230  B$=GET$:IF B$<>CHR$(32) THEN 230
  240 SOUND 1,-15,100,2
  250 PRINT TAB(1,6) CHR$(131);"CHECK THE FOLLOWING ON THE DETECTOR"
  260 B$=GET$:IF B$<>CHR$(32) THEN 260
  270 SOUND 1,-15,100,2
  280 PRINT:PRINT " 1. TIME CONSTANT SETTING IS 0.3          "
  290 B$=GET$:IF B$<>CHR$(32) THEN 290
  300 SOUND 1,-15,100,2
  310 PRINT:PRINT " 2. THE DETECTOR OUTPUT IS ABOUT +0.014          "
  320 B$=GET$:IF B$<>CHR$(32) THEN 320
  330 SOUND 1,-15,100,2
  340 PRINT:INPUT"    ENTER RANGE SETTING (0.01 _ 0.1)          ";RS:IF RS<.01 OR
  RS>.1 THEN 330
  350 IF RS=.01 THEN PD=20
  360 IF RS=.02 THEN PD=10
  370 IF RS=.05 THEN PD=4
  380 IF RS=.1 THEN PD=2
  390 SOUND 1,-15,100,2
  400 REM B$=GET$:IF B$<>CHR$(32) THEN 360
  410 SOUND 2,-10,20,15
  420 CLS:PRINT:PRINT ;CHR$(129);        "ENTER TITLE OF CHROMATOGRAM
  'NOT MORE THAN 7 CHRS'"
  430 PRINT:PRINT ;CHR$(130);:INPUT F$
  440 K=LEN(F$):IF K<1 OR K>7 THEN 410
  450 PRINT:PRINT:PRINT
  460 ?M=0:X%=0:N%=0:Q%=0
  470 CLS:PRINT:PRINT;CHR$(130);" TYPE IN 'SET' WHEN ABOUT TO START "
  480 PRINT TAB(2,10);CHR$(141);CHR$(129);"YOU WILL HAVE EXACTLY FIVE SECONDS"
  490 PRINT TAB(2,11);CHR$(141);CHR$(129);"YOU WILL HAVE EXACTLY FIVE SECONDS"
  500 PRINT TAB(2,16);CHR$(141);CHR$(129);"BEFORE SAMPLE INJECTION"
  510 PRINT TAB(2,17);CHR$(141);CHR$(129);"BEFORE SAMPLE INJECTION"
  520 INPUT TAB(15,13) P$:IF P$<>"SET" THEN 470
  530 Z%=5
  540 CLS:REPEAT:TIME=0
  550 SOUND 1,-15,150,1
  560 PRINT TAB(10,10)CHR$(141);CHR$(130);Z%
```

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```
570 PRINT TAB(10,11)CHR$(141);CHR$(130);Z%
580 REPEAT UNTIL TIME>=100
590 Z%=Z%-1:UNTIL Z%=0
600 *FX16,1
610 SOUND 1,-15,150,10
620 MODE4:PRINT:PRINT" RUNNING SAMPLE    ";F$
630 VDU24,0;0;1230;720;
640 ON ERROR GOTO 710
650 PRINT:PRINT "  TO STOP PRESS ESCAPE"
660 TIME=0.0:@%=131850:S%=0:PRINT TAB(2,7);"TIME ELAPSED="
670 FOR E%= 0 TO 1230
680 S%=0:FOR A%=0TO 4:REPEAT:UNTIL TIME>=10*N%:N%=N%+1:S%=S%+ADVAL(1)DIV PD:NE
XT
690 Y%=S% DIV5:?(M+X%)=Y% DIV128:?(M+X%+1)=Y% MOD128:DRAW E%,Y%:X%=X%+2:PRINT
TAB(17,7);TIME/6000:NEXT
700 VDU 16:MOVE 30,Y%:GOTO 670
710 @%=10:PRINT
720 VDU12:PRINT TAB(2,10);"DO YOU WISH TO SAVE SPECTRA? Y/N":INPUT D$:IF D$="Y
" THEN 750 ELSE 740
730 PRINT:PRINT
740 VDU12:INPUT TAB(2,10);"SURE YOU DONT WANT TO SAVE Y/N";Y$:IF Y$="Y" THEN 9
70 ELSE 720
750 G%=0:ON ERROR OFF
760 SOUND 1,-15,100,2
770 CLS:PRINT:PRINT "USE A DISKETTE WITH AMPLE SPACE"
780 PRINT:PRINT "PRESS SPACE TO CONTINUE"
790 C$=GET$:IF C$<>CHR$(32) THEN 790
800 SOUND 1,-15,100,2
810 ON ERROR GOTO 760
820 IF G%=1 THEN 920
830 CLS:PRINT:PRINT:PRINT:PRINT:PRINT:PRINT "THE DATA IS BEING SMOOTHED BEFORE
          STORAGE"
840 PRINT:PRINT:PRINT"THIS IS A SLOW PROCCESS BE PATIENT"
850 REM A 20 POINT MOVING AVERAGE
860 G%=1
870 C%=0
880 AV%=0:W%=0
890 IF C%+40>X% THEN 920
900 FOR I%=0 TO 39 STEP 2:W%=W%+(?(M+C%+I%)*128)+?(M+C%+I%+1):NEXT
910 AV%=W% DIV 20:?(M+C%)=AV% DIV128:?(M+C%+1)=AV% MOD128:C%=C%+2:GOTO 880
920 CLS:PRINT:PRINT "SAVING FILE ON DISC"
930 N=OPENOUT (F$)
940 FOR I%=0 TO C% STEP 2:PRINT£N,(?(M+I%)*128)+?(M+I%+1):NEXT
950 CLOSE£N:PRINT
960 PRINT"RECORDED AND FILE CLOSED"
970 ON ERROR OFF
980 VDU7
986 CLS:PRINT:PRINT:PRINT:PRINT"WANT TO ENTER ONOTHER SPECTRA ? Y/N"
990 ON ERROR GOTO980
1000 P$=GET$:IF P$=CHR$(89)THEN 1010 ELSE 1020
1010 MODE7:GOTO 120
1020 CHAIN"MENU":END
```

```
>  LOAD"SUMM"
>L.
  100 MODE4
  110 VDU 19,0,4,0,0,0
  120 REM PROGRAM TO FIND AREA UNDER CHROMATOGRAM PEAKS,R.T AND RRT
  130 VDU6
  140 ON ERROR GOTO 850
  150 DIM Y%(4000),V%(4000)
  160 VDU3:CLS:MOVE 0,0:PRINT:REM ROUTINE TO READ DATA FROM DISC
  170 VDU24,0;0;1279;800;
  180 VDU7
  190 PRINT:PRINT"ENTER DATA FILE"
  200 PRINT:INPUT R$
  210 @%=&20209
  220 B=OPENIN (R$):V%=0
  230 ON ERROR GOTO 170
  240 REPEAT
  250 DRAW 0,800:DRAW 1279,800:DRAW 1279,0:DRAW 0,0
  260 FOR D%=0TO 1278
  270 INPUT£B,Y%(V%):Y%=Y%(V%):DRAW D%,Y%(V%)
  280 ON ERROR GOTO 310
  290 IF V%>=3800 THEN 310
  300 V%=V%+1:NEXT:VDU16:MOVE0,Y%(V%):UNTIL EOF£(B)
  310 CLOSE£B:VDU12
  320 ON ERROR GOTO 810
  330 SOUND 1,-13,110,2
  340 S=0
  350 P%=0:X%=0:BL%=Y%(X%+2):PE%=2:X%=X%+2:Q%=0
  360 PRINT:PRINT"    PEAK INTEGRATION"
  370 PRINT:PRINT:PRINT:PRINT "DO YOU WANT TO PRINT RESULTS? Y/N"
  380 C$=GET$:IF C$=CHR$(89)THEN 410
  390 IF C$=CHR$(78)THEN 450
  400 GOTO 380
  410 PRINT :PRINT"MAKE SURE THE PRINTER IS READY AND PRESS   SPACE"
  420 K$=GET$:IF K$<>CHR$(32) THEN 420
  430 CLS
  440 VDU2::PRINT"        _____"
  450 PRINT:PRINT:PRINT "      SAMPLE ----";R$
  460 PRINT:PRINT
  470 IF S>0 THEN 510
  480 PRINT TAB(4);"PEAK";TAB(14);"RETENTION"
  490 PRINT TAB(5);"No";TAB(14);"TIME(Min)";TAB(28);"AREA"
  500 GOTO 520
  510 PRINT TAB(3);"PEAK";TAB(13);"R.T";TAB(20);"R.R.T.";TAB(30);"AREA"
  520 PRINT
  530 REM PEAK SEARCHING ROUTINE
  540 IF Q%=1 THEN 620
  550 REPEAT
  560 IF X%>V% THEN 810       •
  570 B%=Y%(X%):G%=Y%(X%+1):D%=Y%(X%+2)
  580 X%=X%+1:UNTIL D%>G% AND G%>B%
  590 PS%=X%-1:PROCbaseln
  600 REM NOTE THAT B% IS THE START OF A PEAK AND N% IS AN INCREAMENT COUNTER
  610 REM ROUTINE TO TRACK PEAK MAX AND RT.
  620 N%=0
  630 REPEAT
```

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```
640 J%=Y%(X%):K%=Y%(X%+1):L%=Y%(X%+2)
650 IF X%>V% THEN 810
660 X%=X%+1:N%=N%+1:UNTIL J%>K%
670 MIN=(X%+4)/120:RRT=MIN-S
680 REM ROUTINE TO TRACK FALLILNG EDGE AND PEAK END.
690 REPEAT:J%=Y%(X%):K%=Y%(X%+1):L%=Y%(X%+2)
700 IF X%>V% THEN 810
710 X%=X%+1:N%=N%+1:UNTIL (J%-K%) < 1 AND (K%-L%)<1
720 REM THIS IS THE PEAK END AND START OF PEAK INTEGRATION
730 PE%=X%
740 PROCfall
750 PROCint
760 IF A%<=300 THEN 530
770 P%=P%+1
780 IF S>0 THEN 800
790 PRINT TAB(5);P%;TAB(15);MIN;TAB(28);A%/10;TAB(36):GOTO 530
800 PRINT TAB(3);P%;TAB(13);MIN;TAB(20);RRT;TAB(30);A%/10:GOTO530
810 VDU3:PRINT:PRINT"   Press 'R' to Obtain R.R.T."
820 K$=GET$:IF K$<>CHR$(82) THEN 850
830 VDU7
840 PRINT:INPUT"   Enter Reference R.T.   ";S:GOTO350
850 @%=10
860 PRINT:PRINT"DO YOU WANT ANOTHER RUN Y/N"
870 A$=GET$:IF A$=CHR$(89) THEN 160
880 IF A$=CHR$(78) THEN 900
890 GOTO 870
900 CHAIN"MENU2"
910 END
920 DEFPROCbaseln
930 IF ABS(B%-BL%)>150 THEN 1020
940 IF PS%-PE% <10 THEN 1020
950 U%=Y%(PS%):W%=Y%(PE%)
960 IF W%-U% >=40 THEN 1020
970 BL%=U%
980  FOR M%=0 TO (PS%-PE%)
990  R%=Y%(X%-M%)
1000  IF R%<BL% THEN BL%=R%
1010  NEXT
1020 ENDPROC
1030 DEFPROCfall
1040 Q%=0:IF K%-L%<0 THEN Q%=1
1050 ENDPROC
1060 DEFPROCint
1070 A%=0
1080 FOR I%=0 TO N%
1090 A%=A%+(Y%(X%-I%))-BL%
1100 NEXT
1110 ENDPROC
```

```
>LOAD"PLOT3"
>L.
  100 *LOAD "SDUMP" A00
  110 MODE0
  120 REM ON ERROR GOTO 420
  130 PRINT: PRINT "   THIS PROGRAM DISPLAYS A SPECTRA "
  140 PRINT:PRINT"   AND ALSO PLOTS IT ON PAPER"
  150 PRINT:PRINT"   MAKE SURE PRINTER IS READY!"
  160 VDU5
  170 VDU24,0;0;1250;1000;
  180 N=0.0:S%=1
  190 MOVE 20,800:DRAW 900,800:DRAW 900,680:DRAW 20,680:DRAW 20,800
  200 PRINT:INPUT "    ENTER THE DATA FILE";F$
  210 PROCRUB
  220 INPUT "  ENTER LENGTH OF PLOT IN MIN. ";L:P%=L*120
  230 PROCRUB
  240 PRINT"  Press (A) to Alter Scale else RETURN"
  250 K$=GET$
  260 IF K$=CHR$(65)THEN 290
  270 IF K$=CHR$(13)THEN 310
  280 GOTO 250
  290 PROCRUB
  300 INPUT "  ENTER NEW SCALE   2 _ 10 ";S%
  310 CLS
  320 MOVE 5,100:DRAW 1205,100:DRAW 1205,1000:DRAW 5,1000:DRAW 5,100
  330 FOR I%=0 TO 1200 STEP 60:MOVE 5+I%,80:DRAW 5+I%,100:NEXT
  340 FOR I%=0 TO 1200 STEP 120:MOVE I%,70:PRINT;N:N=N+L/10:NEXT
  350 PRINT "   SPEC- ";F$
  360 MOVE 500,30:PRINT"TIME IN MIN"
  370 ON ERROR GOTO 520
  380 B= OPENIN(F$):X%=0
  390 MOVE 5,100
  400 Q%=1024*S%
  410 REPEAT
  420 INPUT£B,Y%
  430 A%=(Y%/Q%)*900:X%=X%+1
  440 DRAW 5+1200*X%/P%,100+A%
  450 IF X%=P% THEN 470
  460 UNTIL EOF£(B)
  470 CLOSE£B
  480 REM SCREEN DUMP
  490 ON ERROR OFF
  500 CALL &A00
  510 VDU4
  520 PRINT"  ONOTHER RUN ? Y/N"
  530 J$=GET$
  540 IF J$=CHR$(89) THEN 110
  550 IF J$=CHR$(78) THEN 570
  560 GOTO 530
  570 CHAIN"MENU"
  580 END
  590 DEFPROCRUB
  600 VDU7
  610 MOVE 830,775
  620 T%=3:REPEAT:VDU127:T%=T%+1:UNTILT%=52
  630 ENDPROC
```

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