TOWARDS TECHNICAL DEVELOPMENT OF NATURAL DYES FOR THE TEXTILE INDUSTRY IN KENYA: A CASE STUDY OF *Bixa orellana.L* SOLVENT EXTRACT.

BY

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DECLARATION

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DEDICATION

This thesis is dedicated to my sweet heart Rebecca and my children Nicole, Beryl and Debra.

ABSTRACT

The aim of the study is to report the dyeing of textile fibres using the natural dyes extracted from the seeds of *Bixa orellana Linn.* Natural dyes derived from plant materials have proved to be important alternatives to the use of synthetic dyes in the textile industry. Dyes extracted from annatto *(Bixa orellana.L*) seeds were used in this study. *Bixa orellana.L* is a tropical plant cultivated in coastal region of Kenya. Its seeds are covered by a red resin which contains a number of carotenoid compounds that constitute the main colouring agents; Bixin and Norbixin are the predominant colouring compounds. Annatto colouring compounds can be identified by (GC-MS) technique. Analyses of the essential oils were carried out using GC, GC-MS and GC co-injection (of some of the available essential oils with authentic samples).GC and GC co-injection were performed on capillary gas chromatograph Hewlett Packard (HP) 5890 A Series II equipped with a split-less capillary injector system, cross-linked Hewlett Packard Ultra Methyl Silicone (50m length, 0.22m internal diameter, 0.33µm Carbowax film thickness) capillary column and flame ionization detector coupled to Hewlett Packard 3396 series II integrator (fig 13 and 14). Hydrogen gas was used as a source of fuel, while nitrogen gas flowing at a speed of 0.8ml/min was used as carrier gas. Before the sample was injected a compensation run was made for a period of one hour. The dye was then used to dye cellulosic fibre by Exhaustion method. Dyeing was carried out using mordants through pre and post mordant in order to compare the fastness properties of the dye. Reflectance curves of the dyed materials were made using GretagMcBeth colour Eye 700 A^0spectrophotometer. From the results, the plant studied is a promising dye-yielding plant and produces a yellow orange colour.It could be exploited as a source of textile dyes and an economic plant. Additionally provide more in depth knowledge on behavior of annatto dyes and dyed cotton textile fibres.

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LIST OF ABBREVIATIONS AND ACRONYMS

CC- Column Chromatography

C-I- Chemical Ionization

E.I – Electron Ionization

Fig- Figure

g -Gramme

GC -Gas Chromatography

HP- Hewlett Packard

HPLC -High Performance Liquid Chromatography

IR- Infra-red

m/z- mass to charge ratio

mL-Millilitre

MS- Mass Spectrometry

NACOSTI- National Commission for Science, Technology and Innovation

nm- nanometer

NMR- Nuclear magnetic resonance

Rf- Ratio of Factor

Ri- Retention index

Rt- Retention time

TLC- Thin layer chromatography

UV-Vis- Ultraviolet-visible spectroscopy

DEFINITION OF KEY TERMS

Annatto : Is an orange red pigment derived from the seeds of Bixa plant

Carotenoids : Are widely distributed group of naturally occurring pigments usually orange or yellow. Divided to Carotene and Xanthophylls.

Chromatography: Is a set of laboratory techniques for separation of mixtures.

Cotton: Is soft fibre that grows in a boll or protective capsule around seeds of cotton plants

Dyeing: Is a process of adding colour to textile products like cotton fibre

Mordant: Is a substance used to set dyes on fabrics by forming a coordination complex with dye, which then attaches to fabric.

Natural dyes: Are colorants derived from plants or minerals.

Spectrophotometry: Is the quantitative measurement of the reflection or Transmission properties of a material as a function of wavelength.

Spectroscopy: Is the study of the interaction between matter and electromagnetic radiation.

Terpenes: Are large and diverse class of organic compounds produced by variety of plants. Are formally derived from Isoprene unit.

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CHAPTER ONE

INTRODUCTION

1.1Background information of the study

Traditionally, plants in Kenya have been utilized as a source of colorants in the making of mats, ropes and other home-based craft materials for a long time.

Natural dyes derived from plants have recently gained economic advantage over synthetic dyes because of their non-toxic non-carcinogenic and biodegradable (Bhuyan and Saikia.,2005).They are environmentally friendly making them a top priority for use in the textile industry, with a growing need to find suitable and less toxic alternative sources of natural dyes.

Various researches have shown that some dyes from plants generally possess desirable colour properties and good performance on natural fibres, which are comparable to some highly rated synthetic dyes (Siva,2007;Purohit *et al*.,2007).Studies in the last 10 years have characterized and promoted the use of natural dyes from different plant species, partly because of recent scientific developments in instrumental methods of colour measurements, analysis and structure determination (Bhuyan and Saikia.,2005).

The importance of natural dyes in the textile industry disappeared by the extensive use of synthetic dyes used in different fibres and still during the last ten years the use of natural dyes gained momentum in the world due to the demand for natural dyes by the food, pharmaceutical and cosmetic industries due to the new environmental laws established by different countries (Siva., 2007).

The use of natural dyes has been subject for applied research in textile industries. The increasing use of these dyes resulted in the increasing demand and in some cases there are problems to satisfy the world market. The natural dyes are widely looked for due to its biodegradability and low toxicity and can be employed in the dyeing of natural and synthetic fibres (Purohit *et al*., 2007).

Every year international events are organized in defense of the environment in order to reduce the environmental impact caused by man in the different areas of production and new laws are being put forward to use of less polluting materials and processes.

United Nations report called for attention on the danger that the lack of water threatens two-third of the world population. To process a ton of textile, one needs between 230 to 270 tonnes of water (Jondiko *et al*., 1989).

In attempt to identify plants with the potential dye-yielding, we chose to work on *Bixa orellana.L;* a plant that has been reported to have several essential oils of which geranylgenaniol and Ishwarane being the major components
(Lawrence and Hogg, 1973; Jondiko *et al*., 1989).

This plant is commercially grown for its red colouring matter referred to as annatto. Despite the abundance of essential oils in seeds, fruits and leaves, there is no scientific documentary evidence of the plant on its use as a textile dye in Kenya. Moreover, no laboratory experiments had been conducted using the plant`s
essential oil or other products to investigate the dyeing of fibres. The seeds were used as a source of natural dyes. The current work reports the dyeing of textile
fibres using *Bixa orellana.L* seeds for the first time. In addition, the composition of the carotenoids is also discussed.

1.2 Statement of the problem

Natural dyes derived from plant-based materials have proved to be important alternatives to the use of synthetic dyes in the textile industry in Kenya.

The use of synthetic dyes is a serious problem and a growing concern in the textile industry.

Research has shown that synthetic dyes are suspected to release harmful chemicals that are allergic, carcinogenic and detrimental to human health (Bhuyan *et al*., 2005).

In 1996, Germany became the first country to ban certain azo dyes.

Natural dyes are environment friendly though commercial practitioners feel natural dyes are non-viable on grounds of both quality and economics.

Little has been done on natural dyes in Kenya and hence there was need for this research to avail data for future reference and application.

1.3 Justification of the project

In Kenya, textile industries use synthetic dyes and yet it has approximately 600 plant species especially the dye-yielding plants which have not yet been characterized (Mayunga., 2007).They have neither been identified nor documented except for use in

local herbal medicine and represent an enormous reservoir of new molecules with potential dye activities which are waiting to be discovered. One such product from nature is annatto dye.

Bixa orellana.L is well known as a source of red pigment known as annatto (bixin) produced from the seeds. Bixa has long been used to colour food products like cheese, fish and salad oil (Lawrence and Hogg.,1973).

The dye stuff industry is considered as one of the most important branches of chemical industry in Kenya. Although the total value in terms of money is small; it represents the highest development of applied chemical research. Products of the dye stuff industry contribute to the strength of national economy towards the vision 2030.

This study will therefore be an effort towards this noble cause.

1.4 General Objective

The project has the main objective to dye textile fibres using natural dyes extracted from annatto seeds *(Bixa orellana.L).*

1.5 Specific Objectives

The specific objectives of this project were:

- **i.** To extract the essential oil from *Bixa orellana Linn* seeds by steam distillation.
- **ii.** To extract constituents of *Bixa orellana Linn* seeds by solvent extraction method.
- **iii.** To perform preliminary separation of *Bixa orellana* dye by column chromatography.
- **iv.** To tentatively identify the constituents in essential oils of *Bixa orellana* seeds using high resolution gas chromatography.
- **v.** To develop a process of dye extraction from annatto seeds and dyeing of cotton fabrics with the extracted dye.
- **vi.** To carry out analysis of the reflectance and fastness properties of the dyed fabrics.

CHAPTER TWO

LITERATURE REVIEW

2.1 Botany and Uses of *Bixa orellana.L*

Annatto, *Bixa orellana Linn*, is an ever green, profusely fruiting shrub or small tree that grows 5-10 m in height **(fig 1a)** .It grows throughout South and Central America, Mexico and Africa; particularly along the coastal regions. In Kenya it is cultivated in Kwale and Lamu districts.Annatto belongs to the order Violales and is the only species in bixaceae family (Ingram and Francis., 1969).

Approximately 50 seeds grow inside prickly reddish orange heart shaped pods or the end of the branches. The trees are literally covered by brightly colored pods.(**fig 1b** and **1c**).One small Bixa tree can produce up to 600 pounds of seeds.

The seeds are covered with reddish arid coat which is the source of the orange yellow dye. Traditionally, the crushed seed are soaked in water and then the water evaporates to make a brightly coloured paste. This paste is added to soups, cheeses and other foods to give it a bright yellow or orange colour.A great deal of annatto seeds are exported to North America and Europe where it is a food colorant,a source of natural dye for cloth and wool, cosmetics and soup industries (Schultes *et al*.,1990).Throughout the rain forest, the indigenous tribes used annatto seeds as body paint and as a fabric dye (Schuttes *et al*., 1990; Acero *et al*., 1979).Although mostly only the seed paste or seed oil is used today, the rain forest tribes used the entire plant as a medicine for centuries. Tea made with young shoots is used by Pierre tribes as an anti-dysentric, an aphrodisiac and to treat skin problems

The seeds are believed to be expectorant and the roots anti-fussive (Acero *et al*., 1979).Many Indians employed it as a principal coloring agent in foods, body paints and as a colouring for art crafts and murals (Heinerman., 1996).Today, in Brazilian traditional medicine, annatto is used to treat heart burn and stomach distress caused by spicy foods and as a mild diuretic and mild purgative (Heinermann., 1996)

Fig.1a annatto shrub

Fig .1b annatto pods

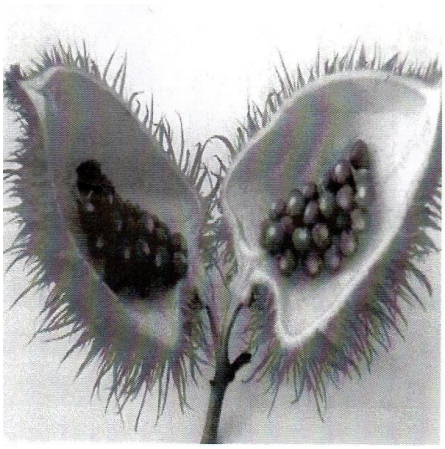
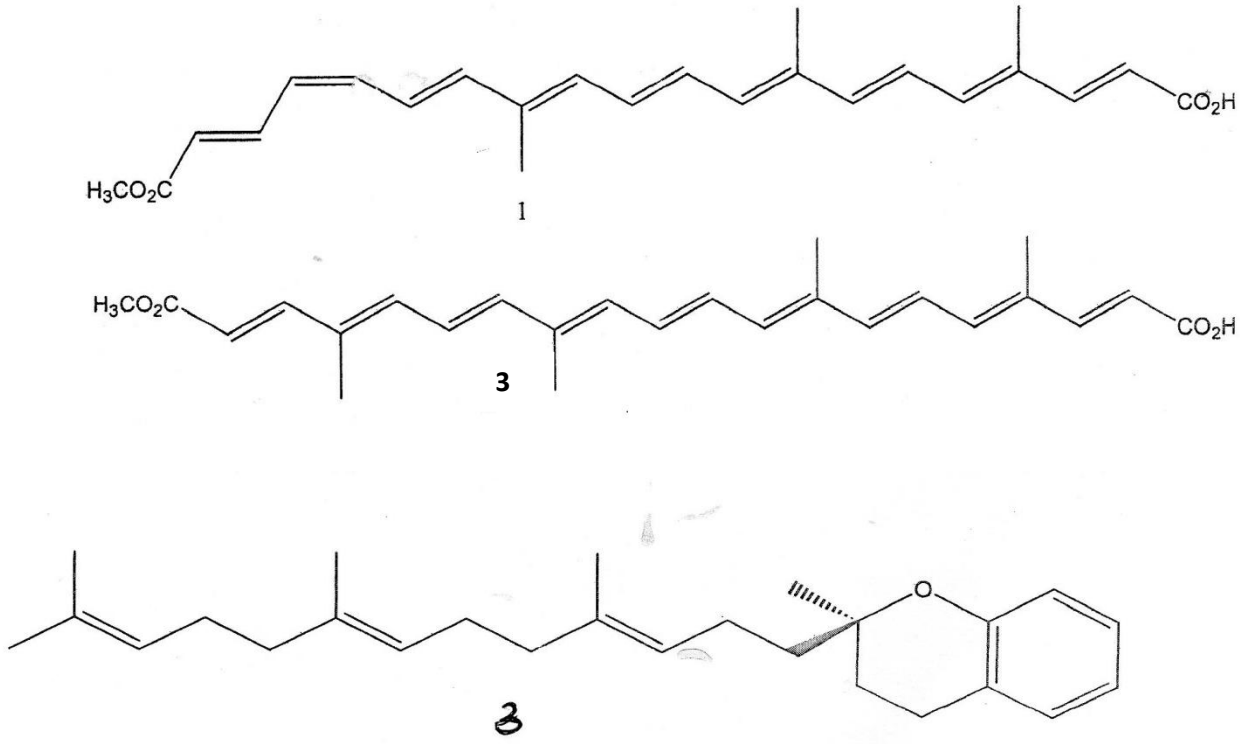


Fig. 1c an open annatto fruit with seeds.

Source: Author, 2014

Annatto also contains tannins, ethereal oils, saponins and mustard oil-like substances among others (Lawrence and Hagg., 1973).

Annatto oil extracted from the seeds is the main source of the pigments bixin**(1)** and norbixin**(2)** which are classified as carotenoids as well for production of tocotrienal**(3).**Bixin is used as a commercial colourant in foods.



2.2 Methods and Concepts in the Chemistry of Essentials Oils

2.2.1 The concept of essential oil

The term essential oil refers to highly volatile substances obtained by steam distillation from an odoriferous plant of a single botanical species. The oil bears the name of the plant from which it is derived(Gwinn,1985).This definition based on volatility and process of isolation is unsatisfactory since many other plant metabolites such as fats, coumarins, anthraquinones and certain alkaloids are also distillable.A more accurate definition has been proposed by Schleicher and Hegnaver (1986).It states that, essential oils are products or mixtures of products which are present in form of tiny droplets between cells; they are volatile and aromatic from a chemical point of view. Essential oils are mixtures of fragrant substances or mixtures of fragrant or odorless substance.

2.2.2 Source and Isolation of Essential oils

Almost all parts of odiferous plants contain essential oils like fruits, seeds, buds, flowers, leaves, stems, roots, barks or wood. The raw material from which essential oils are manufactured may either be fresh partly dehydrated or dried .Some of the methods used for isolation of essential oils are discussed below (Sudhir, 2003; Schleicher and Hegnaver, 1986)

2.2.3 Hydro distillation

This is one of the selective ways of extracting essential oils which involves boiling of the plant material in water and capturing the resultant steam condensing them into water and essential oils followed by separation of the two phases. This method requires well regulated heating system to avoid decomposition or rearrangement of terpenes and hence production of lesser quality of oils. The apparatus commonly used is called Clevenger hydrodistillator (Sudhir, 2003).

2.2.4 Steam distillation

This is the most common method used to capture essential oil. It involves placing the plant material onto a grill situated inside a steel tank followed by spraying pressurized steam through the plant material from below. The heat allows the essential oils to be released and carried out with the steam up into a condensation tube, where the steam is condensed to form an aqueous and essential oil phases in which the latter phase floats on top (Sudhir,2003).

Subsequently the essential oils are skimmed off the top or since most are less dense than water they are allowed to pour out into separate chamber. The water produced contains other important plant constituents mostly water soluble substances.

Most plant materials that are distilled are fresh. This method yields higher quality of essential oils than hydro distillation since the amount of plant material exposed to the steam is minimal thus reducing the deterioration of the essential oil components (Craveiro *et al.*, 1984).When a mixture of two immiscible liquids is distilled, the boiling point of the mixture falls well below the boiling point of each pure component because the vapour pressure exerted by each of the liquids in the mixture is independent of other. Since the liquids are immiscible, the liquids independently exert pressures against the common external pressure and when the sum of the two partial pressure and when the sum of the two partial pressures equals external pressure boiling occurs. This principle is made use of during steam distillation since the oils will

volatilize below 100^{o}c even if their boiling points are above 100^{o}c.This avoids decomposition of some high boiling oils which can occur even before the boiling point of such oils are attained because the oils volatilize at lower temperature (Craveiro *et al.,* 1984).The boiling point remains constant during steam distillation provided adequate amounts of both water and organic component are present to saturate the vapour phase. This method, previously used by (Pino and Correa 2003) to extract essential oils from annatto fruits and seeds and was used in this experiment to isolate essential oils(here in referred to as steam distillate) from commercial seeds of *Bixa orellana L*.

2.2.5 Solvent Extraction

This technique is used in order to increase yield of the oil or to extract products that cannot be obtained by other processes. The plant material is immersed in suitable solvents of various polarities such as hexane or diethyl ether to produce crude extract. In addition to the essential oils, this extraction method also yields substantial amount of other constituents such as resins, pigments, waxes and other non-volatile material and avoids the danger of artifact formation via isomerization, polymerization, and dehydration at raised temperatures involved in such methods as hydro-distillation and steam distillation. The excess solvent is removed from the crude extract under reduced pressure. Other workers have used this method for extraction of essential oils of *Bixa orellana.L* seeds (Jondiko and Pattaden., 1989). It was also used in this study.

2.2.6 Cold Expression

This involves mechanically pressing the plant material followed by spraying of water ensure that all oils and pulp are captured. The mixture is then centrifuged. This form of extraction is commonly used for citrus fruits (Curtis *et al*., 1994).

2.2.7 Super critical Carbon dioxide extraction

This method utilizes carbon dioxide to extract most of the constituents of the plant material. The plant material is placed in a stainless steel tank, the CO_2 is added and pressure is increased inside the tank. With high pressure and lower temperature, the CO_2 liquefies and acts as solvent extracting the oils and other plant constituents, and then the pressure is decreased to allow CO_2 to evaporate leaving a pure extract completely free of solvents. There are two types of CO_2 extracts; selective or total selective extracts;

Selective extracts are produced using lower pressures and contain the volatile oils of the plant, very much like steam distillation.

Total extracts contain other plant extracts besides the volatile essential oils such as waxes, pigments and fats are extracted using high pressure.

Although this method has been used to extract annatto products (Chao *et al*., 1991; Degna *et al*., 1991.Anderson *et al*., 1997).It was not used in this study due to high costs involved.

2.2.8 Maceration and effleurage

This method is used for extraction of essential oils from delicate flowers. The flowers are soaked in vegetables oil in a glass jar. After some time the flowers are removed and replaced by fresh bids where upon further absorption of essential oils into fat takes place. This process is continued and effleurage pomade of highly fragrant odour is obtained. The pomade is then repeatedly extracted with alcohol. The alcoholic solutions are then chilled allowing precipitation of flower wax which is removed by filtration. The filtrate is submitted to gentle vacuum distillation to recover the alcohol leaving a residue of effluent absolute. This method was not used during extraction of essential oil (Curtis *et al*., 1984).

2.2.9 Vacuum distillation

Many substances cannot be distilled satisfactorily in the ordinary way either because they boil at such high temperatures that decomposition occurs or they are sensitive to oxidation (Jondiko *et al*., 1989)

In such cases purification can be accomplished by distillation at very low pressure. Distillation under vacuum is done using a round bottomed pyrex ware and thick walled suction flasks that do not collapse. The round bottomed flask containing the material to be distilled is connected to a Clainsen distillator to head and a thermometer.

The condenser fits into a vacuum adapter that is connected to a receiver and via a heavy walled rubber tubing to a mercury manometer and hence to the trap and water aspirator. Anti-bumping stones are used to reduce liquid bumps during distillation process. The pot is heated using a suitable liquid bath such as paraffin oil, Silicon oil, cotton seed oil or molten liquid. The bath is heated at a temperature about 20^0 c higher than the temperature at which the substance is expected to distil. The bath temperature is kept constant throughout the distillation process. Heating of the flask is

done only after the system is evacuated to desired pressure; otherwise the liquid might boil suddenly at such reduced pressure. A rotary oil pump was used to create vacuum that was used to purify the solvent extract. The method has been used previously by (Jondiko *et al*., 1989) to extract compounds from *Bixa orellana.L*

2.3 Analysis of essential oils

Many methods have been used for studying chemical composition of essential oils (Massada, 1976; Karl-Heinz, 2002; Herbert *et al*., 1964; Robert *et al*., 1996)

Such methods include infra-red spectroscopy (IR), ultra-violet spectroscopy (UV-Vis), nuclear magnetic spectroscopy (NMR), chromatography like TLC, GC, HPLC and mass spectrometry (MS).

2.3.1 Chromatography

Chromatography is the separation of a mixture of two or more different compounds by distribution between stationary and mobile phases.

Various types of chromatography are possible and depending on the nature of two phases involved solid-liquid (column, thin layer and paper).

Liquid-liquid and gas-liquid (vapour phase) chromatographic methods are common. The choice of technique depends largely on differential solubilities or absorptivities and volatilities of the compounds involved with respect to the two phases between which they are to be partitioned. The underlying mechanism of adsorption chromatography is the partitioning of the moving compounds between phases and also their being reversibly adsorbed on the surface of the stationary phase. There are however various qualitative factors to be considered in the choice of adsorbents and solvents; size of the columns (both length and diameter) relative

to the amount of material to be chromatographed and the rate of elution or flow. These factors enable the chromatographic behavior of a substance to be predicted (Guiochon *et al*., 2001; Gupta *et al*., 1963).

2.3.2Thin layer chromatography (TLC)

This involves the use of a particulate sorbent spread on an inert sheet of a glass, plastic or metal as a stationary phase. The mobile phase (solvent) is allowed to travel up the plate carrying the sample that was initially spotted on the sorbent just above the solvent (Brenner.,1962).

Depending on the nature of the stationary phase, the separation can be either partition or adsorption chromatography. The results obtained are described by quoting the ratio

of front (RF) values which refer migration, relative to the solvent front which is related to the distribution co-efficient of the component thus:

$$RF = \frac{\text{Distance travelled by solute}}{\text{Distant travelled by solvent front}}$$

The ratio of fronts (RF) value is dependent upon and many variables which must be watched during the preparation and evaluation of the chromatogram if reproducible results are to be obtained.

Such variables include quality of the layer material, activation grade of the layer, layer thickness, chamber saturation, quality of solvent, development tank size, type of solvent used among others.(Brenner.,1962).

The advantage of TLC is that samples do not have to undergo the extensive clean-up steps and ability to detect a wide range of compounds using reactive spray reagents such as sulphuric acid and iodine crystals, non-destructive detection (fluorescent indicators in the plates, examination under ultra-violet lamp), also makes it possible for purified sample to be scraped of the plate and be analyzed by other techniques (Guiochon *et al*., 2001; Ohlenschlager., 1977 and Synder., 1979).Both analytical and preparative TLC was used during the analysis.

2.3.3 Column chromatography (CC)

This consists of a column of particulate material such as silica or alumina that has a solvent passed through it under atmospheric medium or low pressure.

The separation can be liquid-solid (adsorption) or liquid-liquid (partition) while the columns are usually glass or plastic with sinter frits to hold the packing. Most systems rely on gravity to push the solvent through, but medium pressure pumps are commonly used. In flash column chromatography, the sample is dissolved in solvent and applied to the front of the column (wet-packing)or adsorbed on a coarse silica gel (dry packing).Normally, the solvent is non-polar and the surface polar although there are a wide range of packing materials including chemically bound phase systems.(Synder., 1979).

Bonded phases usually partition mechanisms. The solvent is changed step wise and fractions are collected according to separation required as the eluting products are monitored by TLC. The technique is not efficient as relatively high volumes of solvent

are used and particle size is constrained by the need to have a flow of several milliliters per minute (mls/min).

The advantage is that no expensive equipment is required and the technique can be scaled up to handle sample sizes approaching gram amounts (Guiochon *et al*., 2001)

Column chromatography was utilized during this work to isolate compounds.

2.3.4 High pressure liquid chromatography (HPLC)

This is a development of a column chromatography, to improve resolution. HPLC columns are packed with small sized particles (3.5 or 10 µm) with a narrow size distribution. Flow rates and column dimensions can be adjusted. Pressures are supplied by pumps that can withstand the involved chemicals (Guiochon et al., 2001).

In addition to the normal phase columns (non-polar solvent and polar surface such as silica),there are reverse phase columns. Reverse phase columns involve use of a polar solvent such as water, methanol or acetonitrile and a non-polar surface. Reverse phase HPLC is the method of choice for longer nonvolatile molecules.

The commonly used detector is the ultra violet detector, which not only places constraint on solvents that can be used but also is limited to absorbing compounds. HPLC works best for compounds which can be detected in the ultra violet or visible regions of spectrum and provides a versatile method of quantitative plant analysis.

It is mainly used for analysis of non-volatile compounds like higher terpenoids, phenolics of all types, alkaloids, lipids and sugars (Guiochon *et al*., 2001)

2.3.5 Gas chromatography (GC)

In Gas chromatography a carrier gas to convey the sample in a vapour state through a narrow column made from fused silica tubes (0.1 to 0.3 mm internal diameter (ID) that have refined stationery phase films (0.1 to 0.5µm) bound to the surface and cross linked to increase thermal stability (Rodel *et al*., 1982).The column is installed in an oven that has temperature control and the column can slowly be heated up to 350^{o}c – 450^{o}c starting from ambient temperature to provide separation of a wide range of compounds. The carrier gas is normally hydrogen or helium under pressure and the eluting compounds can be detected in several ways including flames (flame ionization detector) changes in properties of the carrier (thermal conductivity detector) or mass spectrometry. In the present work a flame ionization detector was used because it is not sensitive to oxides of carbon, hydrogen and nitrogen which are common impurities in organic compounds. The sensitivity of flame ionization detector is also high.

Availability of modified cyclodextrins as stationery phases made it possible to separate enantiomers, the detection of enantiometric rations and absolute configurations. However, GC is restricted to molecules or derivatives that are sufficiently stable and volatile to pass through the GC system, intact at operating temperatures (Rodel *et al*., 1982). Gas chromatography has proved to be an efficient method of analysis of essential oil because it is very rapid, has high separation capacity and also has great sensitivity. Complexity of the sample especially essential oil is usually a challenge. Chromatographic separation allows quantification of a specific compound that may be indicative of positive or negative quality notes of the essential oils. The complexity of the essential oil is such that no single column is able to completely resolve all the components. Polymethylsiloxane column are usually preferred over polar stationery phases for both their longer lifetime and separation capacity (Bayer *et al*., 1958). Though gas chromatography is certainly a rapid method of separation since no preliminary separation are required and also a method of choice when only very small amount of sample is available it has several shortcoming; First there may be incomplete separation of compound which could be isolated by preliminary functional group separations.

Secondly when complete oil is injected in the gas chromatograph minor constituent may be too diluted for detection and if larger amount are introduced, the major constituents may overload the column and these peaks mask minor constituents. Thirdly, it may be time consuming if the aim of using it is to isolate minor constituents. Moreover the overloading of the column may also lead to loss of sensitivity of the column in the long run. Finally, some oils may contain high boiling material and if injected in a column it will take a very long time at higher temperature to get these compounds out of the columns. This result in poor separation since higher boiling points is usually contaminated with fats. Very often, part of the high boiling melts remain in the column and may damage it. Due to the above disadvantage GC is normally used alongside other separation and analytical techniques. This method was employed in the current study.

2.3.6 Mass spectrometry

This is an analytical technique that involves generating charged particles (ions) from molecules of the charged analyte (Robert *et al.,* 1996)

The most common mode of ionization involves electron impact (E.I) and the alternative is the Chemical Ionization (C.I). Chemical ionization is a milder method than electron ionization impact which leads to less fragmentation of the molecular ion (Robert *et al.*, 1996).

Other techniques developed to analyze complex and sensitive compounds includes fast atom bombardments (FAB), matrix assisted laser desorption (MALDI), time of flight (TOF) among others (Robert, 1996).

There are factors that determine the fragmentation process; First, weak bonds tend to break most readily.

Secondly, stable fragments not only ions but also the accompanying radicals and molecules are produced most readily.

Thirdly, some fragmentation processes depend on the ability of molecules to form cyclic transition states. Thus fragmentation processes naturally occur more often and ions thus formed give rise to strong peaks in the mass spectrum. Apart from giving the molecular weight information of a substance the molecule ion of a compound may provide additional isotope abundance like Bromine has two isotope, ^{79}Br 49% and ^{81}Br 51%, the relative intensities of the $[M]^+$, $[M+1]^+$ and $[M+2]^+$ ions show characteristic pattern depending on the elements which make up the ion (Robert *et al*., 1996).

The electron impact mass spectra of many sesquiterpene hydrocarbons and oxygynated derivatives of known structures are available as mass spectral libraries (Konig *et al*., 1999). The first step in examining a mass spectrum is the determination of the molecular ion. It is then easy to obtain the molecular formula and from it, the number of double bond equivalents of the molecule. It is helpful to note the major fragment ions and attempt to elucidate the main fragmentation pathways. This is best achieved by checking for σ metastable ions. Knowing the molecular weight and the main features of fragmentation of a compound it may be possible to make a tentative structural assignment. It should be noted that the appearance of a molecular ion depends on its stability. Double bonds, cyclic structures and aromatic ring stabilize the molecular ion and increase the probability of its appearance (Mac Lafferty *et al*., 1993).

On a mass spectrum the ordinate represent relative abundance of each ion. Assignment of relative abundance begins by assigning the most abundant ion a relative abundance of 100%. This peak is also known as the base peak. All the other ions are shown as a percentage of that of the abundant ion. If the molecular ion appears it will be the highest mass in an electron ionization spectrum, except for isotope peaks. In general bond cleavages take place at σ bonds or σ bonds next to hetero atom such as oxygen or σ bonds allylic to sites of saturation (MSDC., 1986).

In additional, cleavage may take place through rearrangement reactions such as retro Diels-Alder and Mac Lafferty rearrangements. Weaker bonds tend to break easily particularly next to structural features that support the stability of the formed ion through resonance in aromatic compounds and through inductive effects as well as donations of ion pairs of electrons. Functional groups and overall structures determine how portions of molecules will resist fragmentations while other portions fragment readily. Mass spectral patterns are reproducible. Mass spectra of compounds have been published and can be used to identify unknowns. Computers generally contain spectral libraries which can be searched for matches. During this work, a special library generated under identical experimental conditions was routinely used to identify known compounds (MSDC., 1986).

2.3.7 Gas chromatography-Mass spectrometry (GC-MS)

Coupling of a gas chromatograph to a mass spectrometer is a widely used technique that is routinely used for analysis of compositions of various essential oils and other volatile organic compounds in complex mixtures (Adams., 2001; Adams, 1995; Witte, 1986). In a GC-MS, normally the charged particles that are ions required for mass analysis are formed by electron impact technique. The gas molecules eluting from GC are bombarded by a high energy electron beam (70eV), a mixture of compounds to be analyzed is initially injected into a GC where the mixture is vaporized in a heated chamber. The gas mixture travels through a GC column carried by a carrier gas where compounds become separated as they interact with the stationary phase of the column. The separated compounds then immediately enter the mass spectrometer that generates the mass spectrum of the individual compounds. Nevertheless it has been reported that even where GC –MS is used for analysis assignment or identification of compounds cannot often be made on the basis of spectrometric data only since many terpenes have essentially identical mass spectra. This can be due to the initial similarity in structures

or due to various fragmentation and rearrangement after ionization hence some knowledge of retention characteristics is often required to compliment mass spectral data (Jenning *et al.*, 1980). This technique was employed during analysis of essential oils of *Bixa orellana.L.*

2.3.8 Retention Times

Gas chromatography as mentioned earlier is an analytical method used to separate and identify compound. Separation occurs due to different gas/liquid equilibrium constants which in turn depend on polarity and volatility of the analytes. The gas chromatographic retention time can be used as a property to characterize the compound because under constant chromatographic conditions the retention time of a compound is reproducible (Karlsen *et al*., 1975).

However in GC-MS automatic library searches rarely considers retention time thus precious information is lost. Since the chromatographic retention time and mass spectrum are independent experiments the combination of both drastically improves accuracy and hit quality of library searches (Witte., 1986). Identification based on retention time relies on knowing which compound elutes at a certain retention time of one's sample with table of previously recorded retention times. Thus reference compounds of all possible constituents have to be measured under exactly the same identical chromatographic conditions. Naturally different compounds might coincidentally co-elute at the same time which somewhat limits the scope of gas chromatography and gives rise to the need of GC-MS. The major problem of retention time base approach of identifying compound is the necessity of maintaining exactly identical conditions. A subtle temperature difference of $1^{o}C$, a slightly increased carrier pressure or a few seconds of delay when starting the acquisition may cause retention time range of several possible constituents. Further it is not possible with required degree of accuracy to compare the retention times of one GC with another system, neither in the same laboratory nor worldwide. In most cases system maintenance like shortening the column or installing a new column will change the retention times and require all reference retention times to be measured again. Routinely, references and samples are run in the same sequence shortly after each other (Breckler *et al*., 1970; Karlsen *et al*., 1975).

2.3.9 Relative retention times

One way of overcoming above limitations is to calculate the relative retention times that are dividing the retention times of a compound by the retention time of internal standard. Thus, slight variations of temperature can be compensated for because they have equal effect on both compound and relative retention times are to a certain degree comparable between different systems. However, delays of entirely different temperature programmes cannot be compensated for with this method.

Further the larger the retention time difference between the internal standard and the target compound less accurate is this kind of compensation and the more the disturbances may occur after the first compound eluted thus affecting only one of both substances, which in turn cannot be compensated for by this method (Massada.,1976).

2.3.10 Retention indices/Kovat indices

The limitations of using relative retention time can be overcome by calculating the relative retention times based on two internal standards, one shortly eluting before and the other shortly eluting after the target compound (Kovats.,1958 and Kovats., 1961). This quite a large number of standards are necessary to cover the complete time range and use of inert n-alkanes is established for this purpose. The difference between the retention times of two consecutive n-alkanes is divided in 100 parts and the so called retention index of an n-alkane itself is defined as 100n.

Definition of retention index of a compound X (RIx)

$RIx = 100no + 100 (RTx-Rtno)/(RTni-Rtno)$

Where X is the target compound

n_o is n-alkane $C_{no}H_{2no}+2$ directly

Eluting before x

n_i is n alkane $C_{ni}H_{2ni}+2$ directly

Eluting after x

RT is Retention time (in any unit such as minutes or seconds or scans).

RI is Retention index (without units).

Example of retention indices

RI (n-decane) = 1000

RI (n-undecane) = 1100

RIx = 1050 for any X that elutes exactly in the middle of n-decane and n-undecane.

A compound suspected to be Ishwarane by considering the MS spectra was found to have a retention time of 50.394 minutes eluting after tetradecane (retention time 44.551 minutes and pentadecane,retention time 53.305 minutes on an HP-ultra-methyl silicon column has retention index of 1467.

RIx = 1400+100(50.394-44.551)/(53.305-44.551)

= 1466.75 which is 1467 (round off)

Comparison of retention index with literature confirms that the compound is Ishwarane (RI = 1468+-1, a compound previously reported to occur in *Bixa orellana* leaves and seeds (Konig, 1988; Konig, 1999; Lawrence, 1973).

2.3.11 Application of retention Indices, RI

Retention indices are established worldwide and used by a large number of scientists and laboratories. Retention indices may be thought of as a kind natural property of a compound which is a complicated way related to the underlying gas/liquid equilibrium. Naturally, RI is dependent on the kind of stationery phase and different stationary phases give rise for different RI of the same compound. Thus the type of stationary phase should always be stated when stating retention indices. Besides this limitation, RI is extremely system independent, reliable and reproducible (Konig, 1999; Zenkerich, 1996, 1997; Kovats, 1988).

System independency is due to the fact that retention indices are independent from:

I. Delay to acquisition (absolute shift of time axis).
II. Unit of time measurement (min seconds scans).
III. Isothermal and linear temperature variations.
IV. Typical temperature programs with only linear section give only very slight RI variations.
V. Carrier gas pressure and flow rate (have no influence during one measurement if held constant).
VI. Column length.

VII. Column diameter stationary film thickness and pre-columns have no influence on RI.

Generally retention indices are reproducible on the same system with deviations of less than ±1RI and between typical systems with deviations of ±5RI. A system is typical if it has the same stationary phase, normal carrier gas velocity with respect to its column length, a linear or moderately fast temperature gradient and overall good chromatographic resolutions. This means that matching commercially available polysiloxane columns with proper installation and good resolution should afford retention indices of approximately ±5RI or better when compared with reference values (Konig., 1999). However retention indices may differ from reference values in cases where there is overloading effects, foreign column activity (contaminated column reactive parts, polar interactions) and interconverting reactive or decomposing compounds. Such factors must be controlled while making use of retention indices. Normally employment of retention indices uses n-alkanes as external standards. All the alkanes necessary to cover the desired range are mixed in equal amounts in a simple sample. This mixture is measured under the standard chromatographic condition to obtain the retention times of all relevant n-alkanes and the results are called alkane pattern (Konig., 1999)

2.3.12 Reference retention indices

Gas chromatographic retention indices (Kovats indices) are a valuable aid in identification of mono terpenes and sesquiterpenes in oils and related natural and synthetic products. Some 906 Kovats indices of 400 individual compounds on methyl silicone (dimethyl poxysiloxane) and on carbowax 20M liquid phase are summarized from general literature (Davies, 1990). Comparison of mass spectra and retention indices of a compound can also be made with those in the spectra library established under identical experimental conditions (Konig *et al*., 1998 mass finder software and Data bank, Hochmuth *et al*., 2006; online Willey and NIST mass spectral libraries). The GC-MS mass spectral data used in the structural elucidation of the essential oils in this study as shown in results section.

2.3.13 Other spectroscopic techniques

Infrared (IR) and ultraviolet and visible (UV-Vis) spectroscopic techniques are also useful in structural elucidation of compounds. IR is mainly used to detect functional groups in pure compounds. Main useful region of the IR spectrum ranges from 4000-

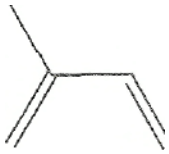
650cm^{-1} UV-Vis is primarily used to measure the multiple and or aromatic conjugation within molecules. The UV region extends from 1000-4000Á or 100-400 nanometers (nm) (Konig.,1999).

If from a GC-MS study, one is unable to easily identify a component by comparison with existing library of compounds or is doubtful he or she may need to isolate the product and compare their nuclear magnetic resonance spectrum (NMR) with that of an authentic sample (Konig., 1999).

Enantiometric composition is determined by enantio selective gas chromatography (Konig., 1999). An unknown compound needs a complete structure elucidation. Since sesquiterpenes do not crystallize, NMR spectroscopy is always the best method for determination of their structures. The interpretation of H^1-NMR, C^{12}-NMR, C^{13}-NMR, DEPT, HMQC, HMBC and NOESY spectra not only provides the constitution of the molecule but also the relative configuration. For complete phase one need phase sensitive COSY or gradient selected HMQC both of which enable the measurement of scalier coupling of over lapping signals (Konig., 1999).

2.4 Terpenes as constituents of essential oils

Terpene is the generic name of a group of natural products structurally based on isoprene (isopentenyl) units **(4)** linked together head to tail.



4

The term may also refer to oxygen derivatives of these compounds that are known as terpenoids. The theory that provided the first conceptual frame work for a common structural relationship among terpenes was first formulated by Wallach (1887) after carrying out structural investigations on several terpenes. His theory stated that terpenes are made up to one or more isoprene (2-Methyl-l, 3-diene) units joined together in a head to tail manner. Later on in 1950s, Ruzicka refined Wallach's of theory by his idea of formation of biogenic isoprene rule emphasizing mechanistic consideration of terpene synthesis in terms of electrophilic elongation (Wallach, 1887; Ruzicka, 1953).

This rule stated that "terpenes are compounds formed by the combination of isoprene units to aliphatic substances such as geraniol, farnesol geranylgeraniol,squalene and other ofsimilar kind and can be derived from these aliphatic precursors by accepted cyclization andin some cases by rearrangement mechanisms" (Ruzicka, 1959) . Taking the above information into account Barton and de Mayo (1957) proposed that the best definition of terpenes is that "it is a compound whose carbon skeleton is either theoretically constructed from isoprene units or has at some stage in its biogenesis had a carbon skeleton so constructed". Most natural terpenoids have cyclic structures with one or more functional groups (hydroxyl, carbonyl) hence the final steps in the synthesis involve cyclization and oxidation or other structural modification such as skel*etal* rearrangement. Terpenoids are of significance in plant growth, metabolism or ecology (Harborne, 1973 and 1991)

2.4.1 Classification of Terpenes

Terpenes are classified according to the number of isoprene units from which they are biogenetically derived. Hemiterpenes are made up to one isoprene unit and the best known hemiterpene is isoprene itself (Loonils, 1973). Monoterpenes contain two isoprene units andare widely distributed in nature particularly in essential oils (Croateau, 1981). Sesquiterpenes contain three isoprene units and are found particularly in higher plants. There are a vast number of sesquiterpenoid carbon skeletons which all arise from a common precursor farnesyl pyrophosphate by various modes of cyclizations followed by rearrangement. Diterpenes are derived from geranylgeranyl pyrophosphate and contain twenty carbons in their skeleton. They are made up of four isoprene units. Triterpenes are made up of six isoprene unit. They are believed to .derived from squalene which is in turn are formed up from head to head coupling of two sesquiterpenoid units. They may be tetracyclic or pentacylic and best classified biogenetically (Cane, 1981; Hanson, 1972 and Connolly, 1991). Tetraterpenes on the other hand are made up of eight isoprene units. They are formed by head to head coupling of geranylgeranyl pyrophosphate molecules; the important group of these terpenes is the carotenoids which contain forty carbons in their skeleton (Goodwin 1981; Spurgeon 1981; Goodwin, 1981). Some most common mono and sesquiterpene basic structures are shown (Devon *et al.* 1972). Only a few diterpenes were identified and not much of them are discussed here apart from the acyclic ones.

2.5.0 The chemistry of natural dyes

The dyes which form the group of Carotenoids originate from the seeds of annatto, (*Bixa orellana.Linn)* a plant which is grown in the coastal regions of Kenya. The Carotenoids

from one of the most important groups of natural dyes and are found in the plant kingdom. It was estimated that nearly 100 million tones are produced annually by nature. The carotenoids can be classified in carotenes (hydrocarbons) and xanthophylls (oxygen derivations). The annatto plant belongs to the family Bixaceae and is known botanically, *Bixa orellana Linn*. The fruits are in the form of capsules or pods covered with flexible thorns, the pods vary in size and appearance. The pods vary in size and appearance being rounded and elongated with pointed extremes (Ingram and Francis, 1969). Inside the pods normally divided into two halves with 10 to 50 small seeds, similar to the size of grape seeds and the dye is localized on the surface of the seeds. The more important red pigment present in these seeds is oxygenated carotenoid, alpha bixin, also known as cis-bixin or labile. The cis-bixin forms more than 80% of the total carotenoids (Wood *et al*., 1991)

Table 2.1 Carotenoid pigments in annatto seeds (Wood *et al*.,1991)

Name	Molecular formula	Molecular weight.
Alfa bixin (cisbixin or unstable bixin)	$C_{25}H_{30}O_4$	394
Betabixin (Trans bixin or stable bixin)	$C_{25}H_{30}O_4$	394
Alfa noprbixin (cisnorbixin or unstable norbixin)	$C_{24}H_{28}O_4$	380
Beta norbixin (Trans norbixin or stable norbixin)	$C_{24}H_{28}O_4$	380
Yellow product of thermal degradation	$C_{17}H_{20}O_4$	288

The molecular formula of this compound is shown in **Table 2.1** and the chemical structure **(1)** confirming that it a mono methyl of a dicarboxylic acid with nine-conjugated double bond of which one is with Cis-configuration.

Bixin is partially soluble in ethyl alcohol and insoluble in water, which could be solubilized in alkaline solutions where it transforms into norbixin (Heinermann., 1996)

Dyes are classified based on their chemical structure sources, method of application, color etc. The chemistry of important natural coloring used presently is;

a) Yellows

This group has colorants which are generally less fast than other groups.

Chemically subdivided into;

i) Flavonoids and related compounds
ii) Polyenes like Bixin (carotenoids)

b) Reds

These are Hydroxyanthroquinone derivative.

c) Blues and Purples

The main naturally recurring blue dye was indigo.

d) Black

The coloring matter is haematoxylin or its oxidation product haematin.

2.5.1 The chemical and Physical structure of textile fibres

Textile fibres are composed of molecules that are long and flexible. They are polymeric in nature. Their chemical structure has an effect on dyeing properties. Orientation crystallinity influences the kinetics and equilibrium uptake of dyes (Wood *et al.*, 1991)

Fibres are broadly classified into four classes namely:

a) Cellulosic like cotton. Composed principally of cellulose.

b) Protein like wool, silk. They are animal proteins.

c) Cellulose acetates

d) Synthetic fibres like nylon, acryllic etc.

2.5.2 Role of chemical bonds in textile dyeing

A chemical bond is an attraction between atoms that allows the formation of chemical substances that contain two or more atoms. The bond is caused by the electromagnetic force of attraction between opposite charges either between electrons and nuclei, or as the result of dipole attraction (Ibrahim *et al*., 1990).The reaction between the dyes and fibres must take into account the various types of forces exerted by one molecule upon another. All dying mechanisms can be divided into the following three groups:

a. A specific bond between the dye and fibre owing to the covalent bond, hydrogen bonds or other directed bonds.

b. Non-specific attraction between dye and fibre owing to ion-exchange or Van der waal's forces.

c. In absence of any interactions, dyes are only mechanically retained. This may be due to insolubilisation of the dye inside the fibre or may be due to self-association into the possibly quite large molecular aggregates following their entry into fibre. An aggregation is promoted by a high ratio of molecular weight to ionic group as well as by increase in length of the aromatic structure of the molecule, also by rise in concentration and the presence of inorganic salts like the common salt (Ibrahim *et al*., 1990).

Dye – fibre bonds that responsible for the substantivity of the dyes are broadly classified into:

1) Van der Waal's forces

These are very weak forces of attraction and are always present between the electrons of one atom and the nucleus of another in close enough proximity. Individually these atoms are very weak forces, but collectively they are considered to be of sufficient strength to the most important attractive forces between dye and fibres. These forces of attractions are known as Van der Waal`s forces. Disperse dyes are held in a polyester fibre by means of Van der waal's forces.

2) Hydrogen bonds

These forces of attraction are weak forces set up between certain atoms in the dye stuff molecule when they are close enough to other atoms in the fibre. Some of the direct and vat dyes are hydrogen bonded in the cellulose fibres.

3) Hydrophobic bonding

The hydrophobic groups especially alkyl chains, tend to associate together and escape from aqueous environment. The effect due to two simultaneous causes, the Van der waal's forces between the hydrogen groups and the hydrogen bonds between water molecules. Each set of forces causes respective assembly of molecules or groups to associate together and to exclude the other. Hydrophobic bonds occur when both the fibre and the dye contain a considerable portion of purely hydrocarbon aliphatic or aromatic, as with some dyes applied on the wool or most dyes applied on the polyester.

4) Ionic bonds

These bonds play an important part in dyeing fibre containing amino groups; wool, silk and nylon with ionic dyes. In the presence of water or diluted acids the amino group becomes protonated. Acid dyes being anionic in solution are attracted at the positive site of the fibre. As the fibre form zwitterions on ionization, a negative charge is also created on the fibre. This negative charge is responsible for the attraction towards basic dyes which forms cationic dye ions in solution. However basic dyes are now mostly applied on acrylic fibres which contain strong acidic sites. Due to ionization in acidic medium, the negative charged sites are created in fibre which attracts cationic dye ions.

5) Covalent bonds

Bonds that result in very strong chemical forces that are not easy to break are covalent bonds. The combination of cellulose fibres with reactive dye stuffs where the Hydroxyl group in the cellulose is covalently bonded to a suitable atom in the reactive dye.

Table 2.2 Comparison of the relative strength of dye – fibre bonds (Courtesy of Wood *et al.*, 1991)

Bond Type	**Relative strength**
Van der waal's force	1.0
Hydrogen bond	3.0
Ionic bond	7.0
Covalent bond	30.0

Table 2.3Fibres, Dyes and Dye-fibre bonds (Courtesy of Wood *et al.*,1991)

Fibre	**Dye class having affinity**	**Type of dye-fibre bond**
Cellulooose cotton	Direct vat sulphur dye	Van der waal's forces and hydrogen bonding
Protein wool,silk,nylons	Direct acid,m*etal*complex and basic Reactive	Ironic bond Covalent bond
Polyester	Disperse dye	Van der waal's forces and hydrogen bonding
Acryllic	Disperse dye	Van der waal's forces and hydrogen bonding

2.5.3 Advantages of Natural Dyes

Natural dyes are considered to be eco-friendly as these are obtained from renewable resources as compared to synthetic dyes which are derived from non-renewable petroleum resources. These are biodegradable and the residual veg*etal* matter left after extraction of dyes can be easily composted and used as fertilizers. They produce soft colours soothing to the eye which are in harmony in nature.

Many of natural dyes absorb in the ultra-violet region and therefore fabrics dyed with such dyes should offer good protection from ultra-violet light. Many natural dye

materials possess antimicrobial properties. Therefore textiles dyed with such materials are also likely to show antimicrobial properties and the same has been reported by many researchers (Ibrahim *et al*., 1990), have reported improvements in both ultraviolet protection and anti-microbial activity for Polymide σ fabrics after treatment with natural dyes.

Fabrics dyed with some natural dyes especially *Bixa orellana.L* have been reported by the wearers to be free of odor perhaps due to the anti-bacterial or bacteriostatic properties of natural dye materials. Users of natural dyed fabrics have also found such fabrics to be mosquito repellant (Jondiko and Patterden 1989).

Recently, cellulosic textiles treated with natural plant extract have been found to exhibit flame-retardant properties (Saxena *et al*., 1997). Many natural dyes possess curative properties and have been used in various traditional medicinal systems. Textiles dyed possess healing properties by absorption of medicinal compounds through the skin.

2.5.4 Drawbacks of Natural dyes

Natural dyes are considered to be an eco-friendly alternative for dyeing of textiles materials. However, there are many limitations in the usage of natural dyes as listed below:

2.5.5 Tedious Application Process

Natural dyes require a longer dyeing time in comparison with synthetic dyes as very often an additional mordanting step is required. Use of raw dye-bearing materials ensures authenticity but at the same time involves additional dye extraction steps that require time and separate set up. Natural dyes in this form are also not suitable for use in many commercial textile dyeing machines which makes the process labour intensive (Wood *et al*., 1991).

Exhaustion of most of the natural dyes on textile materials is poor in spite of using the mordants which leaves a large quantity in the dye bath after dyeing.

2.5.6 Limited shade range

Shade range of natural dyes is limited .Out of the three primary colours namely red, yellow and blue. Only few dyes can be applied in mixtures and differences in fastness properties further limit the choice.

2.5.7 Non reproducible shades

There difficulty in reproducing the shades. It is not possible to produce the same shade with a particular natural dye in every dyeing operation. Most of natural dyes are pH sensitive and tend to change due to change in pH.

2.5.8 Fastness properties

Color fastness to light and washing are most important parameters to evaluate the performance of a textile. Only few natural dyes possess fastness properties conforming to modern textile requirements.

Improvement and optimization of mordanting and dyeing procedures can help in solving this issue.

2.5.9 Safety Issues

Use of metallic mordants requires caution so as not to cause adverse health effects during handling. Precaution should also be taken to prevent pollution.

2.5.10 Characterization and certification issues

Dyeing of cotton fabrics with dyes from natural plants has been investigated though little information is available on identification and characterization of the natural dyes. Determination of dye content and characterization of dye material is important in the case of natural dyes. Absorption spectroscopy is very successfully used for measuring the dye content of synthetic dyes but has limited applicability for natural dyes as these dyes are usually not a single chemical entity but a mixture of closely related compounds and no clearly defined absorption maxima (Saxena *et al*., 1997)

Different techniques including high-performance thin-layer chromatography (HPLC) thin-layer chromatography (TLC) and mass spectroscopy have been employed in this project, for the purpose to characterize the *Bixa orellana.L* dyes.

2.6.0 Current scenario and sustainability issues in usage of

Bixa orellana.L natural dyes

Presently natural dyes are not in use for mainstream textile processing. Only about 1% of the total textiles produced are dyed by using natural dyes (Siva, 2007)

Traditional dyers, enthusiasts and hobby groups are the main users of natural dyes who work at the cottage level. Some small industries are using natural dyes and are selling as finely ground plant material as well as purified extracts, for example Bixa Kenya limited.

Sustainability is a complex multidimensional concept concerning the environment, economy, human health and social impact. It aims to meet the needs of the present generation without compromising the ability of future generations to meet their needs.

Greater emphasis on using natural dyes in the textile industry can make a valuable contribution to the environment sustainability in the 21stCentuary.Various sustainability issues involved in the present status of usage of natural dyes includes: (Saxena et al .,1997).

2.6.1 Renewable and Biodegradable

Natural dyes are obtained from natural resources mainly plants, and are renewed annually unlike petroleum resources.

Exploration of ways and means to utilize these resources for enhancing the availability of natural dye is much required as that would also provide additional income and empower farmers.

Natural dyes are biodegradable and can easily be degraded by microbial action (Gwinn., 1985).

2.6.2 Easy-to-Treat effluent

As natural dyes are biodegradable and complex auxiliaries and extreme PH conditions are not used in the dyeing process, effluent produced during their usage is considered to be easily treatable and expensive elaborate effluent treatment plants needed for synthetic dyes are not required (Gwinn, 1985)

The effluent produced by different natural dyes was found to have a BOD value of 40-85 mg/L only which is less than the limit of 100 mg/L as prescribed (Purohit *et al* .,2007)

2.6.3 Promote Vegetation

As natural dyes are mostly derived from plants, higher usage of natural dyes would lead to the planting of more dye- bearing plant materials which would lead to higher carbon fixation in the form of biomass synthesized by these plants.

2.6.4 Status in GOTS

All natural dyes are recommended for coloration of organic textiles according to the latest (3.0) version of Global Organic Textile standards (GOTS) (Siva., 2007)

All dyed textiles should fulfill the norms with respect to restricted heavy m*etal* content and colour fastness requirements and their production should not lead to environmental contamination (Purohit *et al* .,2007)

Natural-dyed textiles where chrome or tin is used as mordant would not be able to pass the test of restricted heavy metal content (Purohit *et al*., 2007)

2.6.5 Availability and Supply Issues

At the present level of Kenyan textile production natural dyes can only replace a fraction of total textile dye consumption. The tedious process of colour extraction and seasonal availability of natural dye sources are the reasons hindering their use in textile colouration. Better agronomic practices and high colour-yielding varieties of *Bixa orellana.L* needs to be developed.

2.6.6 Cost Considerations

Presently the cost of dyeing textiles with natural dyes is much higher in comparison to the cost of dyeing with synthetic dyes. Their application procedure is lengthy and complicated. They are not compatible with many industrial dyeing machines hence labour intensive.

CHAPTER THREE

METHODOLOGY

3.0 Plant materials, seeds.

Commercial seeds of *Bixa orellana* were obtained from Kenya Bixa Limited in Tiwi, Kwale County along Likoni-Ukunda road at Latitude 4.2384^{0}S, Longitude 39.59502 ^{o} E in April 2014. Air dried seeds of *Bixa orellana* were separately subjected to steam distillation and solvent extraction.

3.1 Steam Distillation of Bixa

Four kilograms of the seeds were put into a tank that has a wire mesh to suspend the seeds. Three liters of water was added to the tank. The tank was then covered. The lid of the tank has a hole to which Dean and Stock apparatus. The tank was then heated from below. The heat generates the steam below the wire mesh and breaks the oil globules thereby releasing the essential oils. The oil is then forced by the steam up through the dean stock apparatus to the condensation tube where the steam is condensed to form an aqueous phase and essential oil floating on top. The water and the essential oils were separated at the top of the dean-stock apparatus by draining the water first followed by the oil. During the process of distillation, the oil came out in three different colors, first light yellow oil, followed by light green oil and finally dark green oil.

Analysis of these oils by thin layer chromatography and GC-MS indicates that the three oils had the same components varying only in abundance of the extraction. Alternative method of extraction discussed below was then used.

3.1.1 Solvent Extraction of Annatto Dye

Dry commercial seeds of four kilograms were shaken in five liter conical flask using a rotary orbital shaker at a speed of 200 revolutions per minute under distilled hexane of three liters for four hours at room temperature. Aluminum foil was used to cover the flask to minimize light. The solution was then decanted and the seeds shaken further under two liters of hexane for two hours then decanted. The combined extract was then filtered and the solvent removed using a rotary evaporator at 60^{o}C leaving behind 114.5 grams of a deep red colored solution.

3.1.2 Column Chromatography of Bixa

Seven grams of yellowish green oil was chromatographed on a column packed with 150 grams of Silica gel. The column was the first eluted with 500 ml of 100% analar

grade 40-60^{o}C petroleum ether. Five fractions of 100 ml each were collected. The first three portions did not contain any compound as was revealed by analytical thin layers chromatography (TLC). The fourth and the fifth fractions resulted in a bluish liquid when it was later on concentrated. The column was then eluted with 500ml 90% petroleum ether and 10% ethyl acetate. Five other 100ml fractions were collected again. Each of them gave colorless solutions that became greenish when concentrated.

This was followed by a change of solvent system once more this time using 2.5 liters of 70% petroleum ether and 30% ethyl acetate. Twenty fractions each of 50ml were then collected and analyzed by TLC. The next fifteen fractions resulted in light yellow oil component which gave almost similar spots on TLC plates (two large peaks and some other smaller ones) while the remainder gave colorless solutions even after concentration. Concentration of the yellow solutions yielded 4.2 grams of oil. The column was then rinsed with another 500ml of 60% and 40% petroleum ether and ethyl acetate respectively.

Five fractions each of 100ml were once again collected. These fractions were colorless and the TLC did not reveal any major spots. The yellow fractions from the above column were re-chromatographed on a column packed with 130 grams silica gel because it was suspected that one of the major spots revealed by the TLC could have been bixin. The relative proportions and chemical compositions are discussed in the next chapter.

3.1.3 GC and GC-MS analysis of Bixa components

Analyses of the essential oils were carried out using GC, GC-MS and GC co-injection (of some of the available essential oils with authentic samples).

GC and GC co-injection were performed on capillary gas chromatograph Hewlett Packard (HP) 5890 A Series II equipped with a split-less capillary injector system,cross-linked Hewlett Packard Ultra Methyl Silicone (50m length, 0.22m internal diameter, 0.33µm Carbowax film thickness) capillary column and flame ionization detector coupled to Hewlett Packard 3396 series II integrator (fig **3** and **4**). Hydrogen gas was used as a source of fuel, while nitrogen gas flowing at a speed of 0.8ml/min was used as carrier gas. Before the sample was injected a compensation run was made for a period of one hour. Temperature programs consisted of an **(i)** initial temperature of 50^{o}C for 5 minutes then raised to 280 ^{0} c at a rate of 5^{0} per minute

finally maintained at 280^{o}C for 20 minutes for the steam distillate. Bixa fraction and Bixa solvent extract (Fig**2a**) and **(ii)** initial temperature of 60^{o}C for 5 minutes rose at 130^{o}C at a rate of 4^{o}C per minute, raised again to 175^{o}C at a rate of 1^{o}C per minute, then raised once more to 295^{o}C per minute at 30^{o}C per minute and finally held at a constant temperature of 295^{o}C for a period of 20 minutes Figure **2b.**

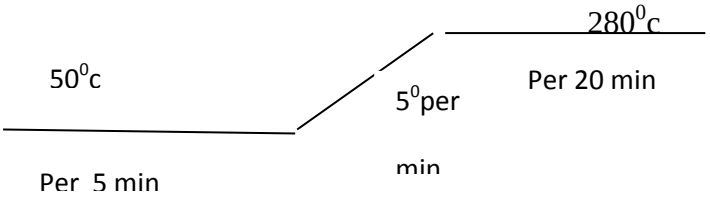


Fig. 2a: GC and GC-Ms temperature programme for steam distillate and solvent extracts

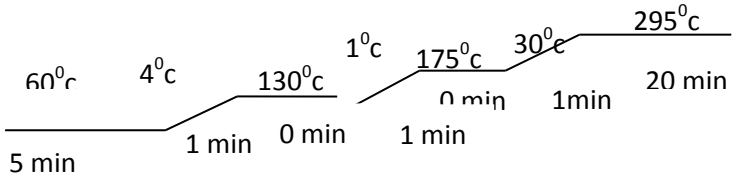


Fig 2 b: GC and GC-MS temperature programme for vacuum distillate

GC-MS analysis was carried out on a Hewlett Packard 5790A series II Gas chromatograph coupled to a VG analytical organic mass spectrophotometer manufactured by Micro mass, United Kingdom formally known as a VG Biotech. The mass spectrophotometer (Ms) was operated in the electron ionization (EI) mode at 70 electron volts (eV) and an emission current of 200 micro amperes (µA). The temperature of the source was held at 180^{o}C and a multiplier voltage of 300 volts. The pressure of the ion source and MS detector were held at 9.4x10^{-5} milliards respectively and1.4 x10^{-5} millibar respectively.

The MS had a scan cycle of 1.5 seconds (scan duration of IS and inter-scan delay of 0.5s). The mass and scan ranges were set at mass charge ratio (m/z) of 1-1400 and 38-650 respectively. The instrument was calibrated using heptacosafluortributyl amine. The column used for the GC-MS was the same as the one described for GC above except that the film thickness was 0.5µm. The temperature programme was similar to the ones described for GC above. Helium was used as a carrier gas. In both GC and GC-MS, high performance liquid chromatography (HPLC) grade dichloromethane (DCM) was used as a dilution solvent.

Sample volume for Bixa crude extract are steam distillate (referred to here as Bixa D.G) was 2µL while a dilution ratio of 1:200 µL was used. Sample volume for vacuum distillate referred to here as Bixa D.B) and all column chromatographic fractions was 6µL whereas the dilution ratio was 1:40. Sample volume for the non-polar hexane column chromatography eluent of oleoresin was 6µL while sample volume and sample dilution ratio was in a ratio of 1:40. The differences in sample size and sample dilution ratio were as a result of differences in complexity of essential oil components and therefore the sample volume and dilution ratios above were the ones that gave the best separation after several attempts.

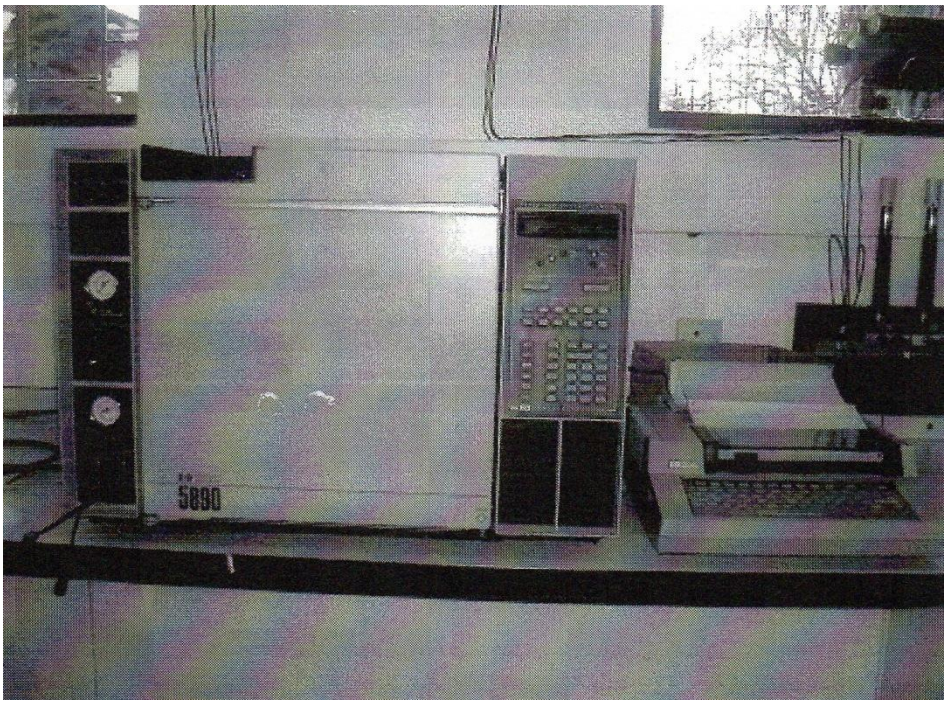


Fig 3.A HP 5890 series gas chromatograph with an integrator

(Source: Author, 2014)

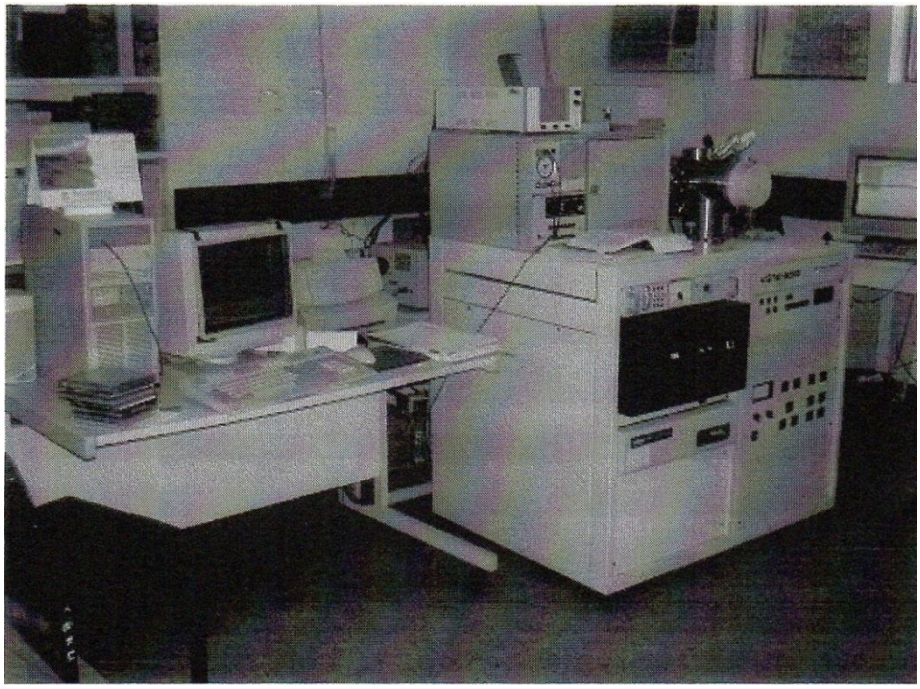


Fig.4 GC-MS integrated with computers

(Source: Author, 2014)

3.2.0 Dyeing Procedures

3.2.1 Collection of Plant Materials

Bixa orellana.L plant materials were collected from Kenya Bixa limited farms in Kwale County. All measurements were carried out at the Rivatex Chemical Laboratory, Moi University, Eldoret.

3.2.2 Dyeing fabric

Cotton (cellulose based) fabric articles 002A and 053A in both bleached and non bleached (100m state or grey cloth) conditions were obtained from the processing department within Rivatex for experimental dyeing.

Article 002A at Rivatex is constructed as follows; warp/weft count 43/43 nm; 13 picks/cm and 25 ends/cm while article 053A has its construction as warp/weft count 18/18 nm; 16picks/cm and 23 ends/cm.

3.2.3 Fabric scouring

The cotton fabric articles cut into small size pieces whose total weight was 100 grams (g) were first washed in hot water for about 10 minutes using a non-bleach washing

powder. A stainless steel pot was filled with 5 liters of water in which 35g soda ash was carefully added. The wet fabric articles were then put into the water and swished around using a stirring rod. The glass rod was left inside the pot to prop the lid slightly open to prevent the liquid from boiling over. The water was eventually brought to the boil (Tan, 2004).

The heat was adjusted to low boil or hard simmers and allowed the fiber to boil half covered for 2 hours. The fabric articles were stirred every 15 minutes to ensure adequate scouring. The sauce pan was removed from the heat source after 2 hours and the fabric articles allowed to cool down until they could safely be removed from the water. The fabric articles were finally rinsed in clean cold water (Tan, 2004)

3.2.4 Fabric mordanting

Mordanting was done in two sets as follows;

1. **Single mordanting in an Alum/Vinegar bath**

An alum [hydrated aluminium potassium sulphate],[$KAl(SO_4)_2.12H_2O$] mordant bath was prepared for this procedure. A solution containing 25g of alum, 30ml of vinegar (3% acetic acid) in three liters of water was prepared. The solution was brought to boil and then let to cool. The fabric articles were inversed into the cool mordant bath whose temperature was eventually brought to the range 80-90^oC by heating. This was simmered for 1 hour, removed and then let to cool (Siva., 2007).

2. **Tri-mordanting in three successive mordant baths of Alum, Tannin and alum**

Bath 1 (Alum mordant)

The dye pot was filled with 3 liters of water and nearly brought to boil. Alum 25g were first dissolved in a small container with boiling water and slowly added to the pot and stirred well.

Soda ash (Na_2CO_3) of 6g were then weighed out and added slowly and carefully to the water in the pot. The cleaned and scoured fabric articles were then added to the pot heated to simmering point and simmered for 1 hour. The fabric articles were stirred every fifteen minutes during simmering and eventually left in the pot for 24 hours. The

fabric article pieces were wringed well, dried in the open air and left to age for a week. The pieces were washed well before treating them in the second bath.

Bath 2 (Tannin mordant)

The dye pot was filled with 3 liters of hot water. Tannic acid (6g) were dissolved in a small container with boiling point and added to the pot to which the cotton fabric articles were added. Simmering was done for 1 hour and left for 24 hours. The cotton pieces were then wringed well, dried and left to age for a week. The pieces were washed well before treating them in the third bath.

Bath 3 (Second Alum Bath)

Alum mordanting was repeated as described in Bath 1.

3.2.5 Dye Extraction from *Bixa orellana*

50g of Annatto seeds in a flat bottomed flask together with 100ml of ethyl alcohol was agitated in a magnetic agitator for 15,30, 60 and 120 minutes. After agitation, the mixture was sieved using a 200 mesh screen. The filtrate was saved for future distillation purposes and the reuse of alcohol used. The filtered dye was dried at a temperature of 60^{o}C in an air circulating oven for 30 minutes. After drying, the dye was pulverized and conditioned in a suitable container (Siva, 2007).

3.2.6 Dyeing

The dye bath was prepared with 5% (W/W) annatto dye which was solubilised with a solution of 0.5% Na_2CO_3 filtered through a 200 mesh screen in order to eliminate any impurities. Cotton samples weighing 100g were dyed in a COLOUR PET 12 with a liquor ratio 100:1 and at temperature of 60^{o}C for 30, 60 and 90 minutes. Tartaric acid, tannin and Alum were used as mordants. The mordants were added at the beginning of dyeing at concentrations of 5 and 10% (W/W). After dyeing the samples were washed with distilled water at room temperature in the same equipment for 20 minutes. Later they were dried (Sudhir., 2003).

3.3.0 Testing dyed fabric

3.3.1 Fastness to washing

A soap solution made up of 5g soap and 2g of anhydrous sodium carbonate (Na_2CO_3) in one litre of distilled water in a beaker was prepared and heated to 60^{0}C.

Specimens of dyed cotton fabrics measuring 10 cm by 4 cm were cut out. They were separately placed between two pieces of undyed cloths free from finishing chemicals of cotton measuring 5cm by 4cm and each of the now three pieces held together by stitching round the edges leaving 5cm by 4cm exposed.

The specimens were then placed in a beaker for 30 minutes with constant stirring while maintain the temperature at 60^{o}C by steady heating. They were removed and rinsed in cold water for 10 minutes. The specimens were then squeezed and dried in air not exceeding 60^{o}C (Siva., 2007)).

3.3.2 Bleaching test on dyed fabric

Pieces of dyed fabrics were immersed into a 3.85% V/V solution of Sodium hydrochlorite (NaOCl) and left to stand for a period of one hour.

3.3.3 Testing for type of dye produced by *Bixa orellana*

One half of a liter of 5% W/V caustic soda (NaOH) solution was prepared and put in a saucepan. Pieces of dyed fabrics were added, then heated and brought to boil for a period of 2 minutes (Siva., 2007)

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 Steam Distillation of the seeds

It involved the placing of plant material on to a grill situated inside a steel tank followed by spraying pressurized steam through the seeds from below. The heat allowed the essential oils to be released and carried out with the steam up into a condensation tube, where the steam condensed and formed aqueous and essential oil phases.

Subsequently the essential oils were skimmed off one were allowed to pour out to separate chambers while the water produced contained water soluble plant constituents.

Analysis of the essential oils by thin layer chromatography indicated that three layers of oils had the same components varying only in abundance of the extraction.

4.2. Solvent Extraction of the dye

The extracted solution was decanted and seeds shaken further under two litres of hexane 95% purity for two hours then decanted. The combined extract was then filtered and solvent removed using rotary evaporator at 60^0C leaving behind 114.5 grams of a deep red coloured solution.

4.3 Column Chromatography

Seven grams of yellowish green oil was chromatographed on a column packed with 150 grams of Silica gel. Five fractions of 100 ml each were collected.

The first three portions did not contain any compounds as was revealed by thin layer chromatography (TLC).The Fourth and fifth fractions resulted in a bluish liquid.

Five other 100 ml fractions were collected again. Each gave colourless solutions that became greenish when concentrated. Five fractions each of 100 ml were once again collected. These fractions were colourless and the TLC did not reveal any major spots.

The yellow fractions from the above column were re-chromatographed on a column with 130 grams Silica gel were used and the suspected major spots revealed by the TLC could have been Bixin.

4.4 Gas Chromatographic Separation and Tentative Identification of constituents

Analysis of the essential oils was carried out using GC-MS and GC Co-injection and some of the available essential oils with authentic samples. The Spectra is shown on this Chapter.

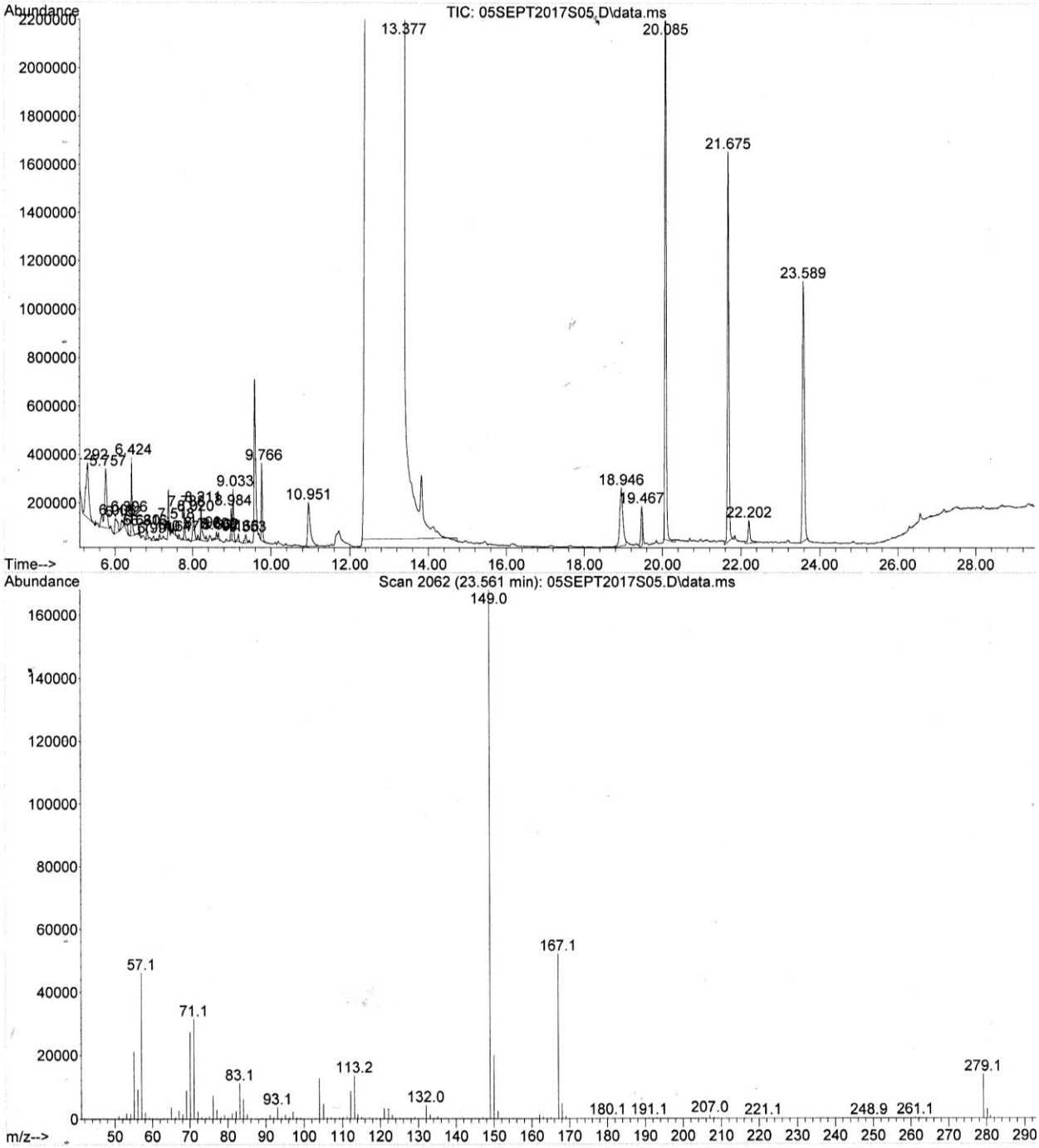


Fig. 5 The GC-MS Spectra for Bixa Solvent extract

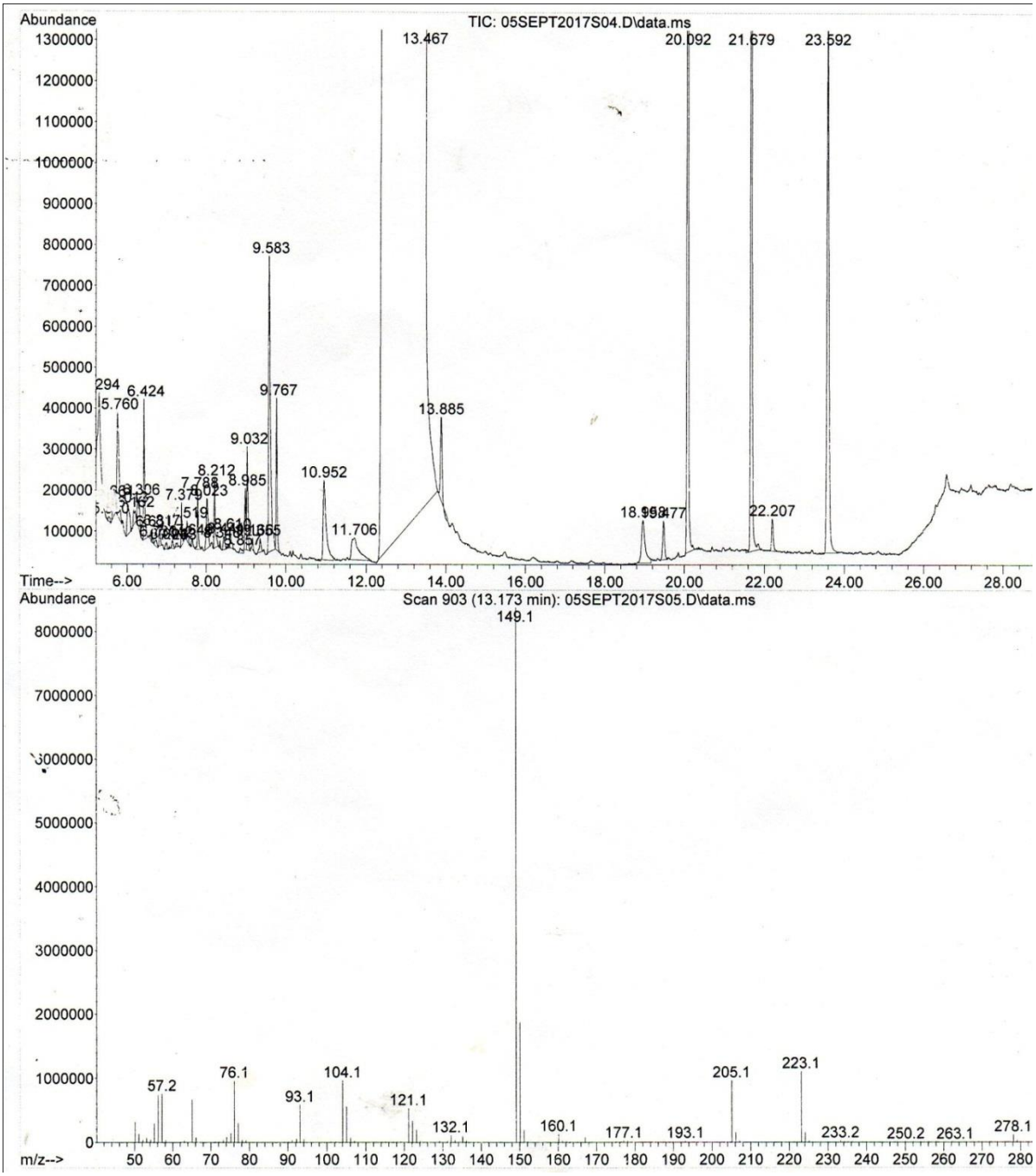


Fig. 6 The GC-MS Spectra of C_8 to C_{20} n-alkane standard and Ion Chromatogram of identified compunds at each retention time

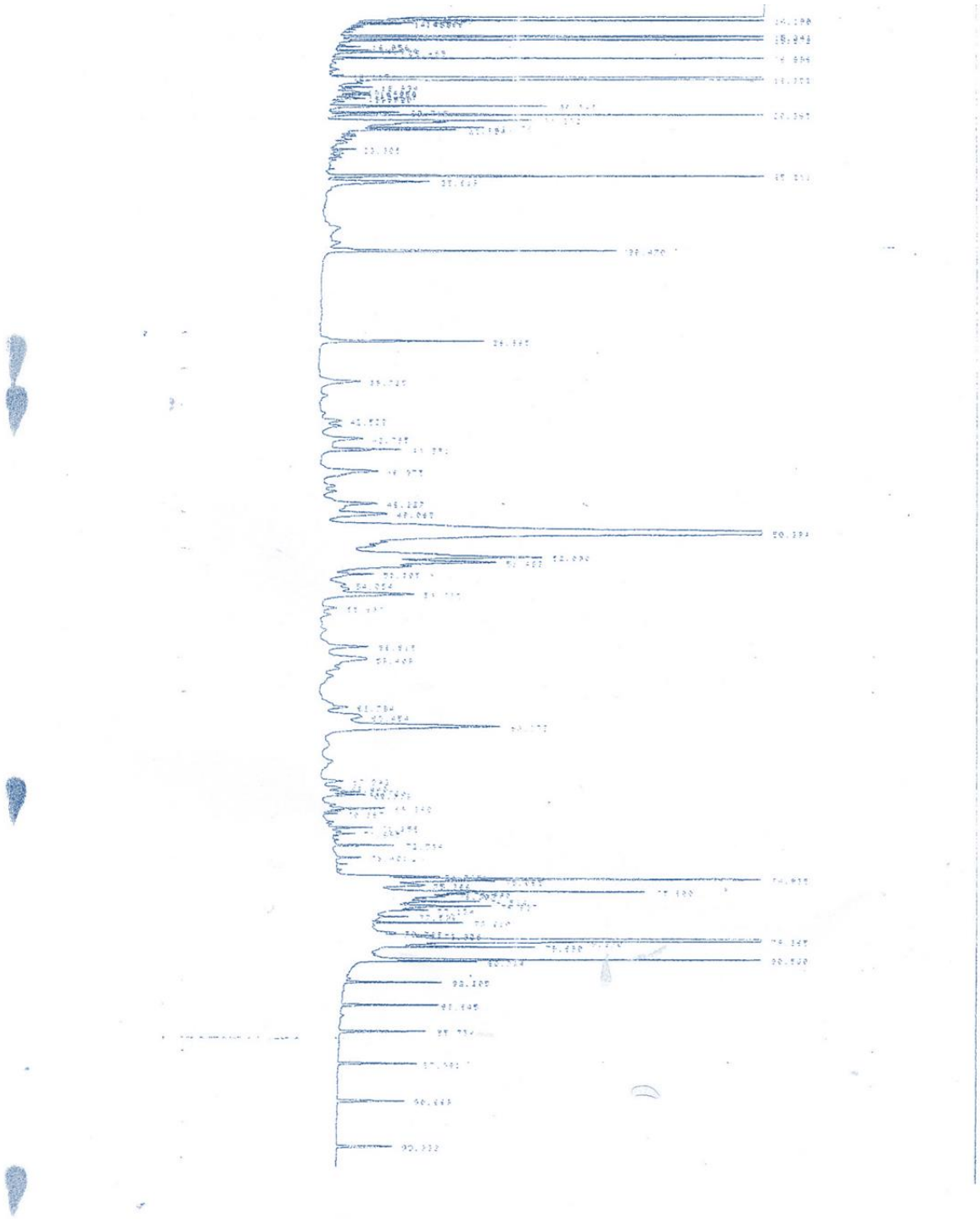


Fig.7 GC Co – injection spectrum of vacuum distilled essential oil with C_8 to C_{40} alkane standards

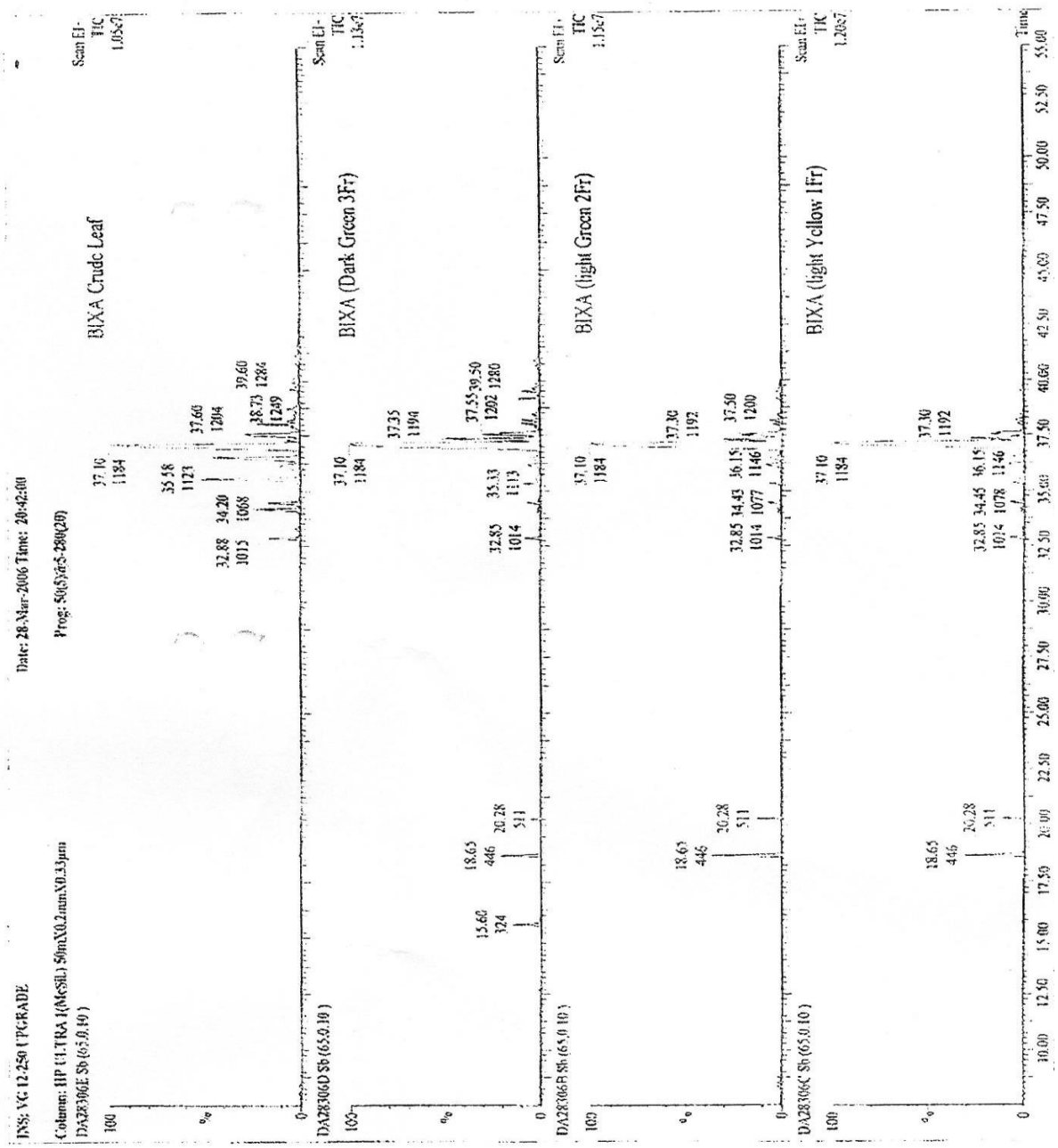


Fig.8 Comparative GC spectrum of steam distillate oils

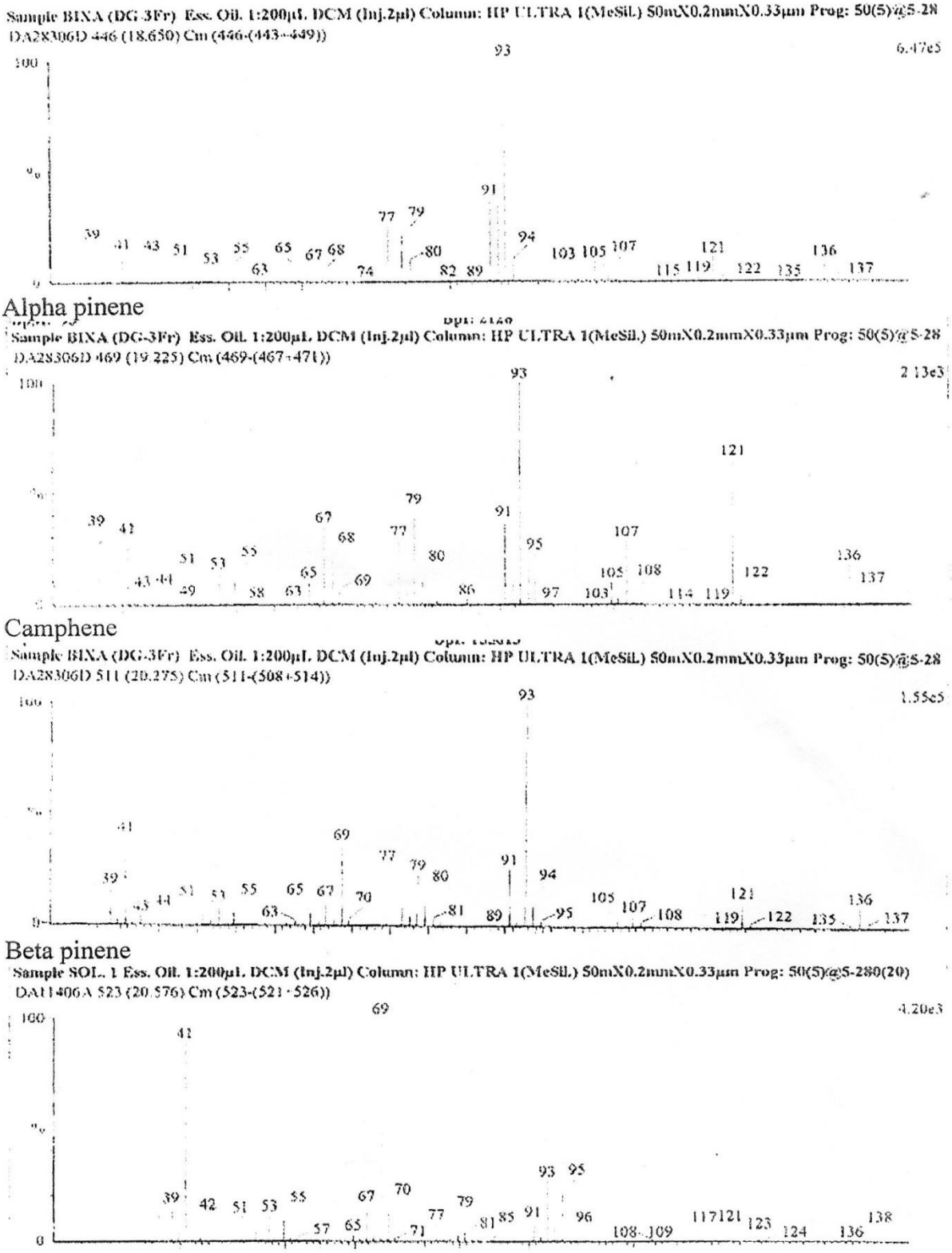


Fig. 9 Mass Spectra of some compounds of *Bixa orellana*

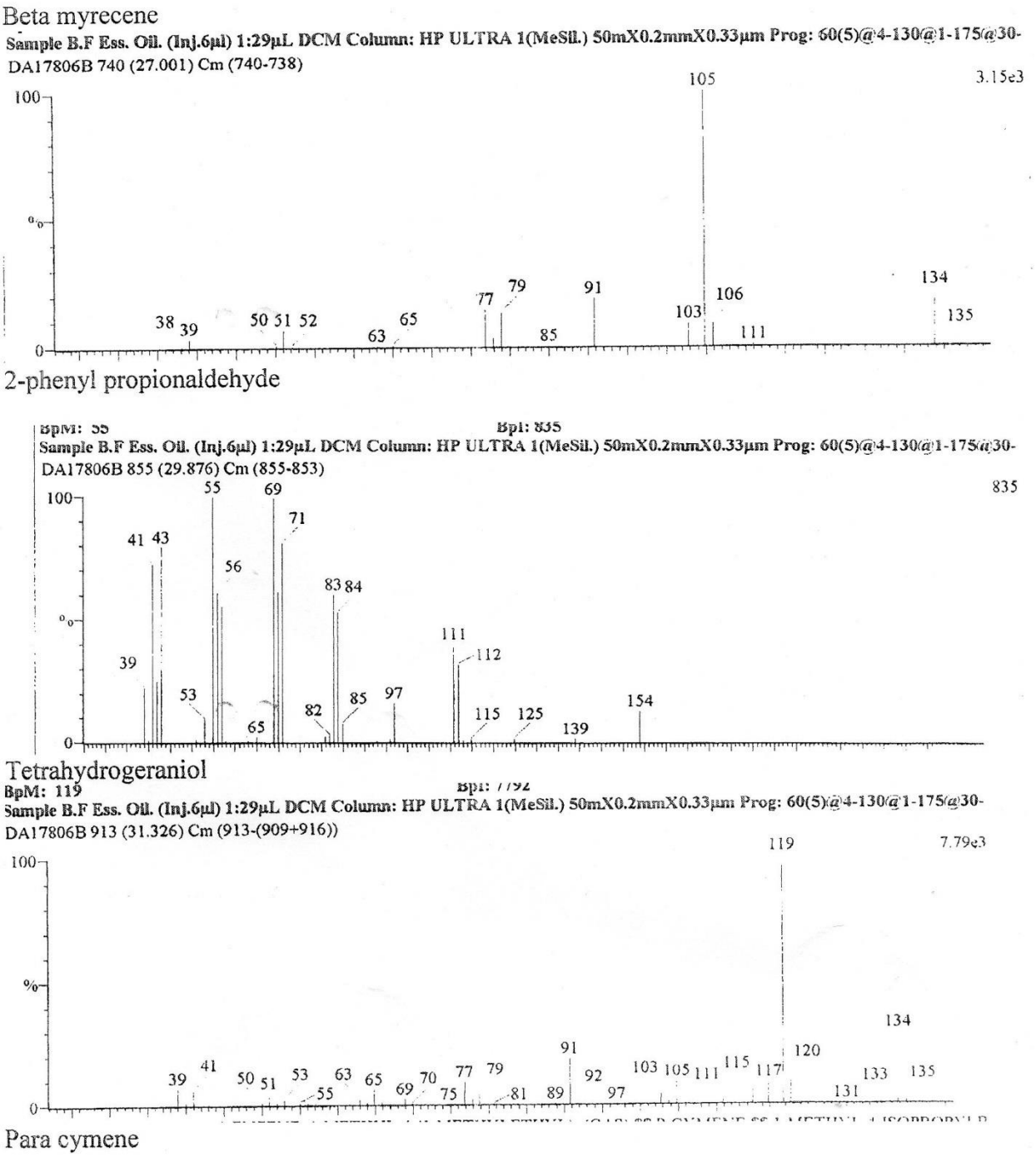


Fig.10 Mass spectra of some compounds of *Bixa orellana*

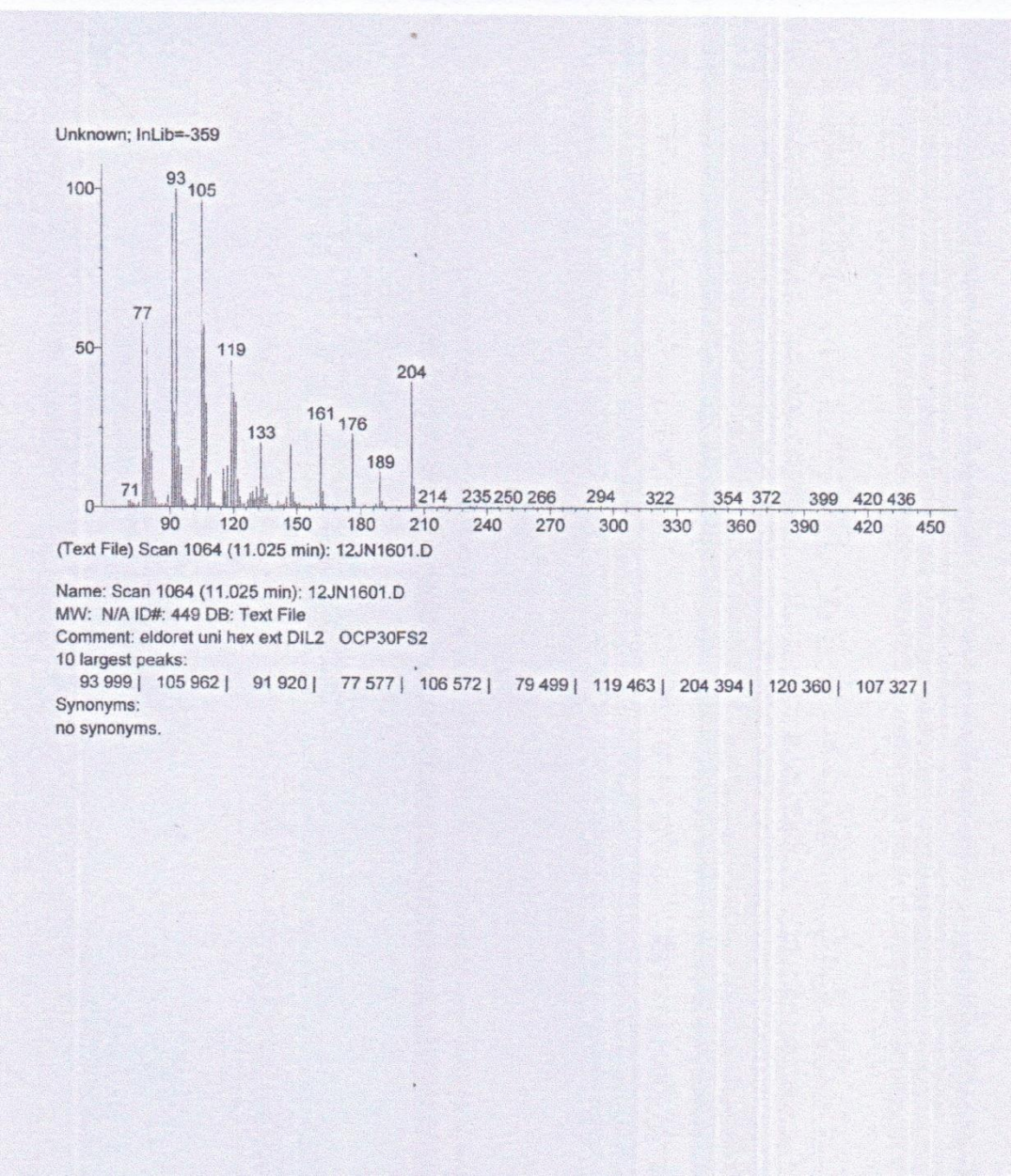


Fig.11 Ion Chromatogram of identified compounds at each retention time

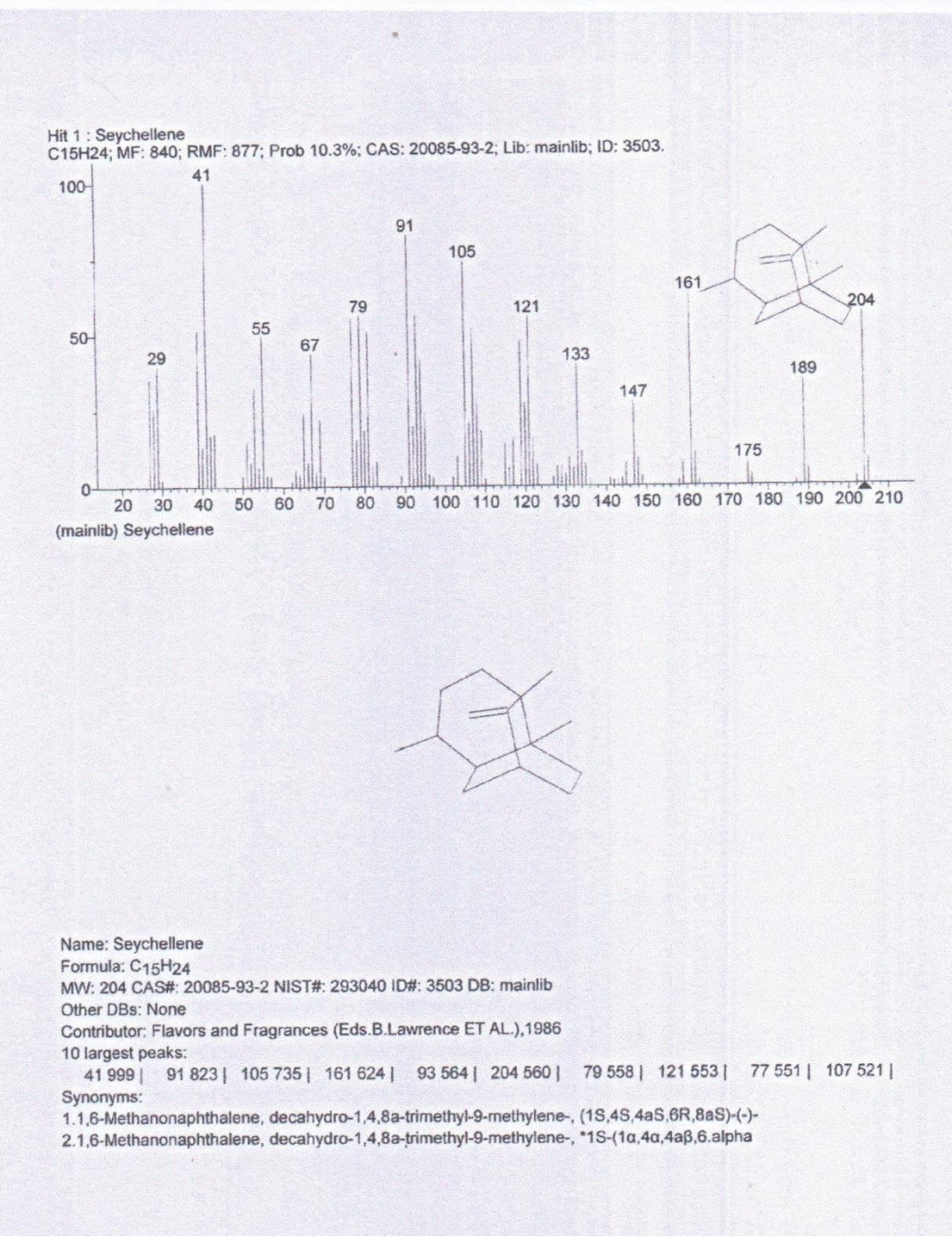


Fig.12 Ion Chromatogram of identified compounds at each retention time

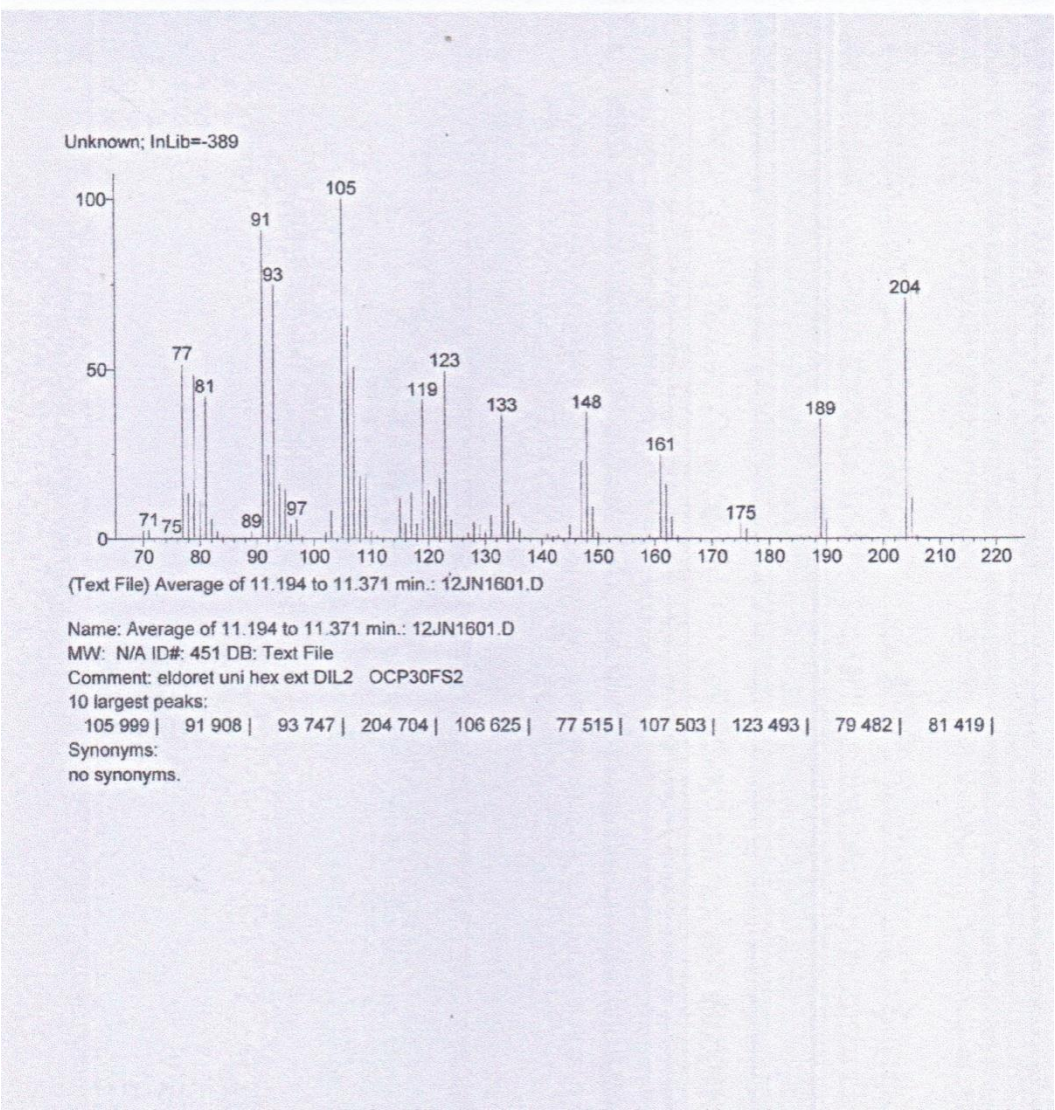


Fig. 13 Ion Chromatogram of identified compounds at each retention time

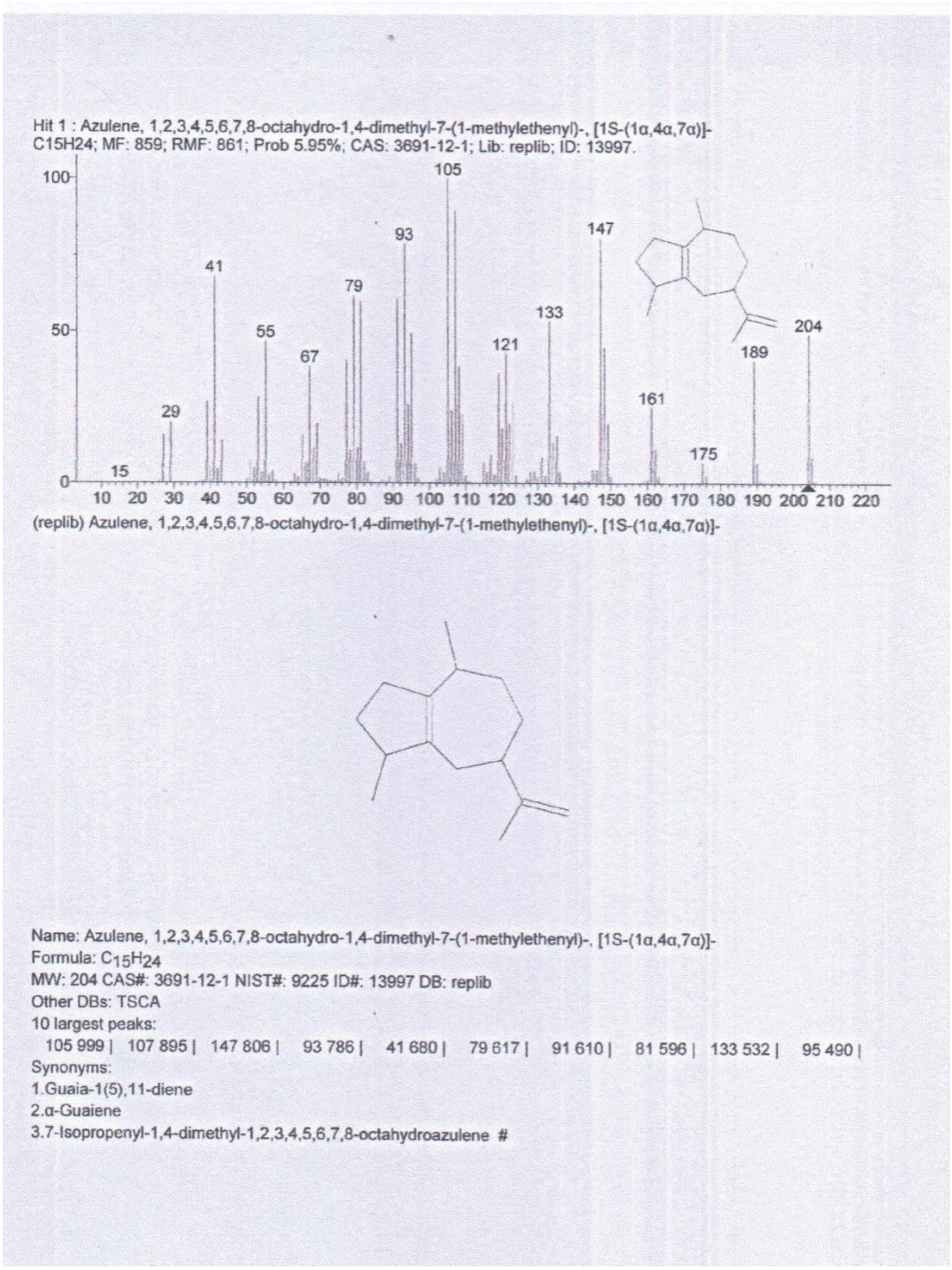


Fig. 14 Ion Chromatogram of identified compounds at each retention time

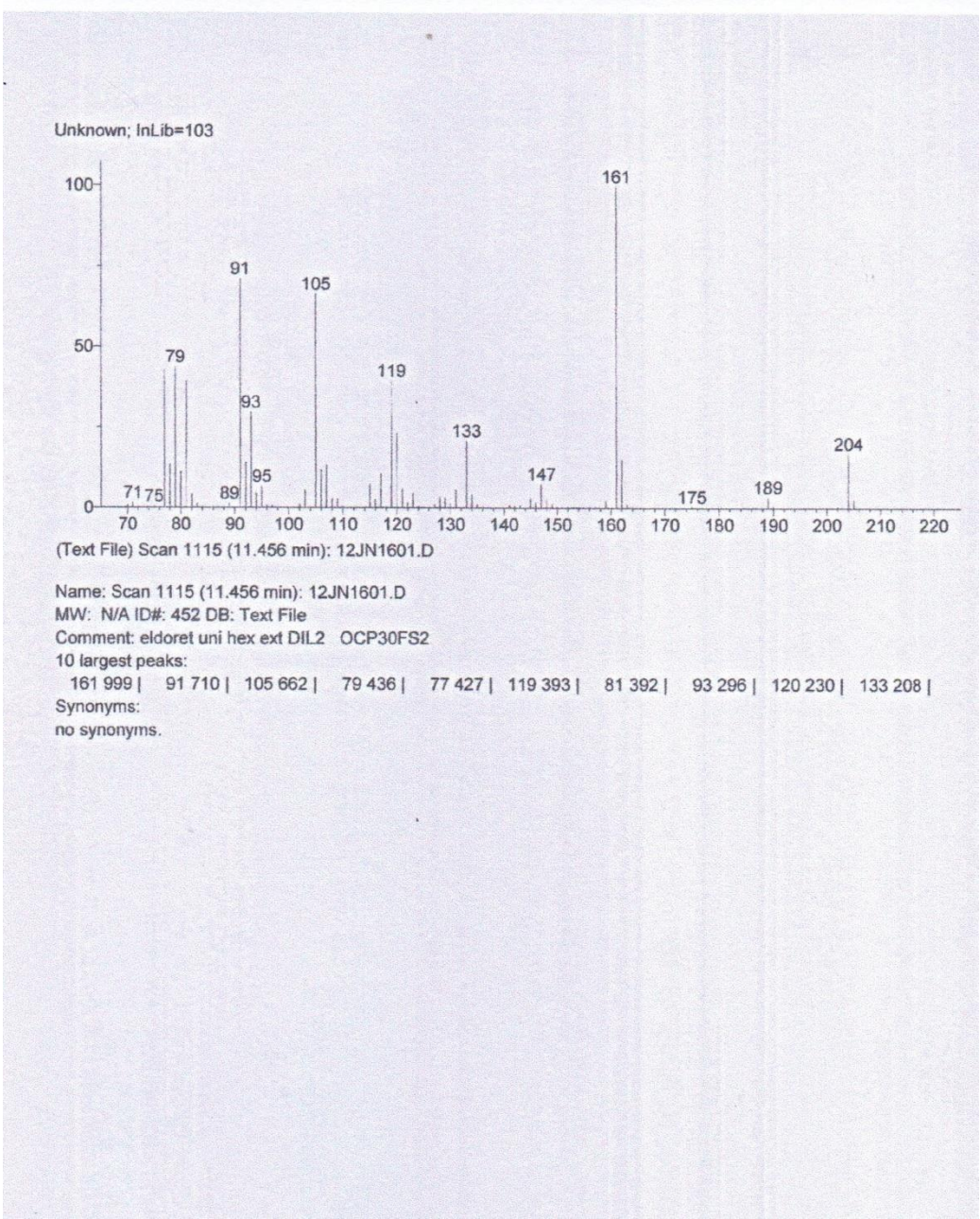


Fig. 15 Ion Chromatogram of identified compounds at each retention time

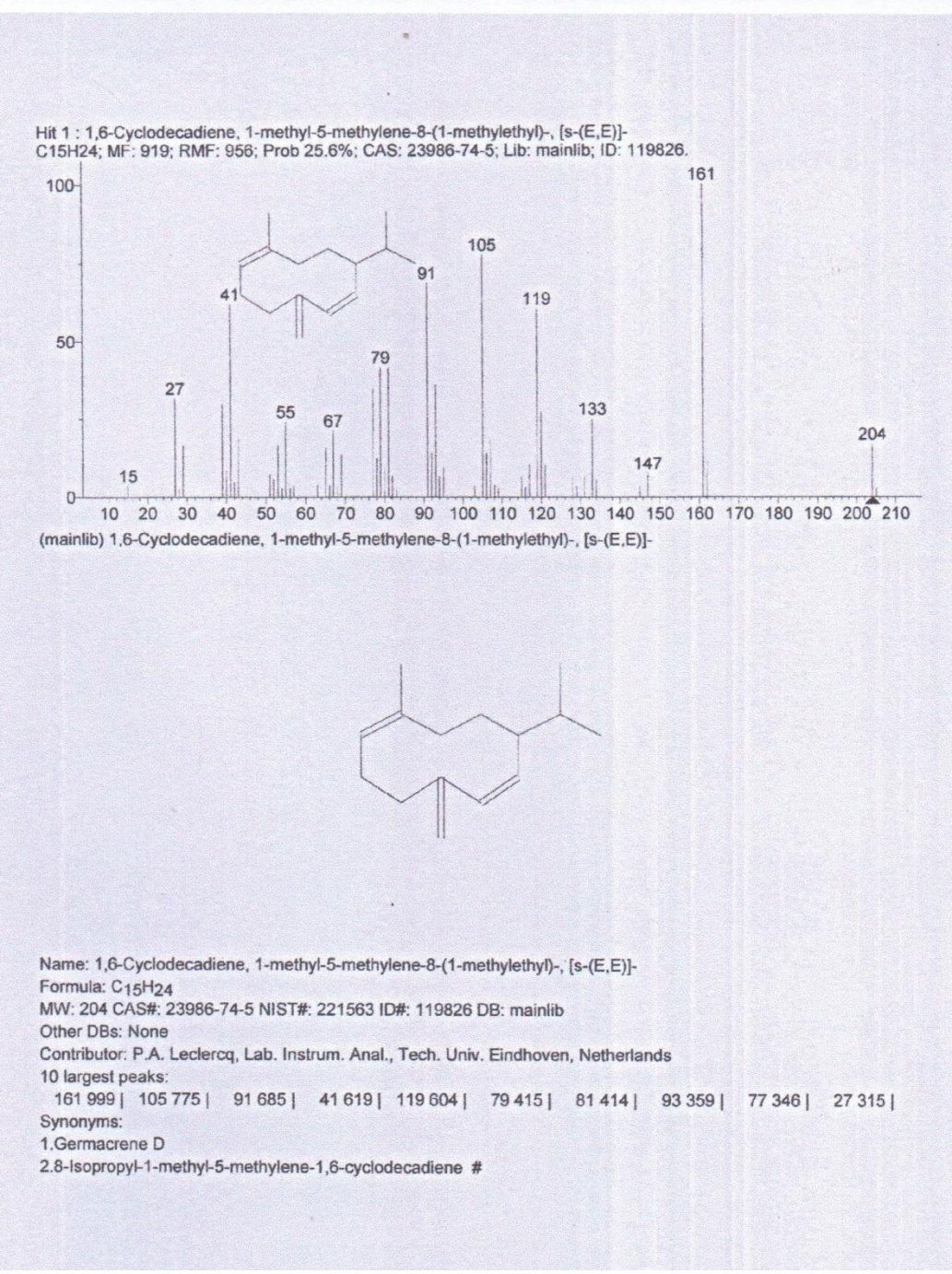


Fig.16 Ion Chromatogram of identified compounds at each retention time

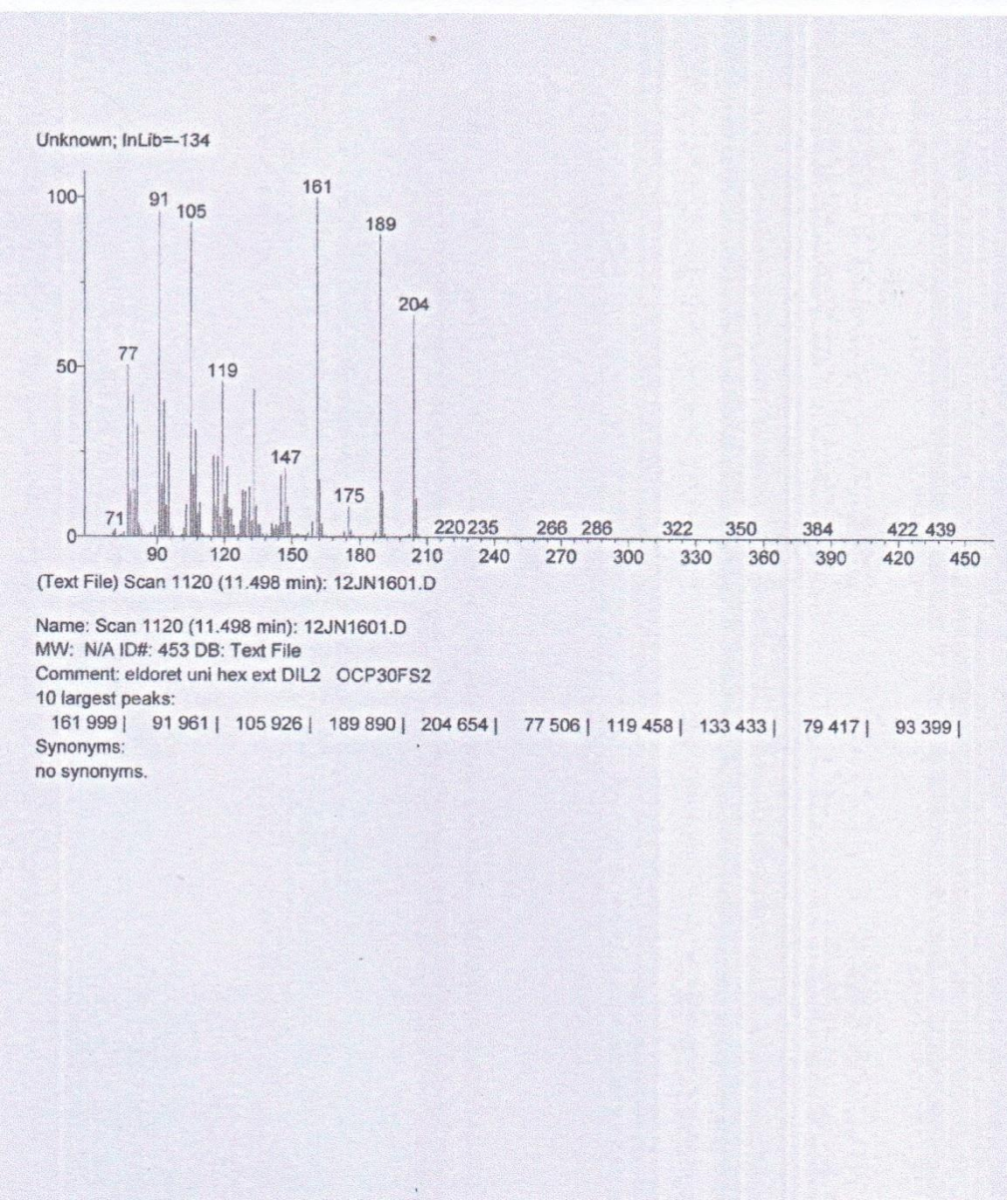


Fig.17 Ion Chromatogram of identified compounds at each retention time

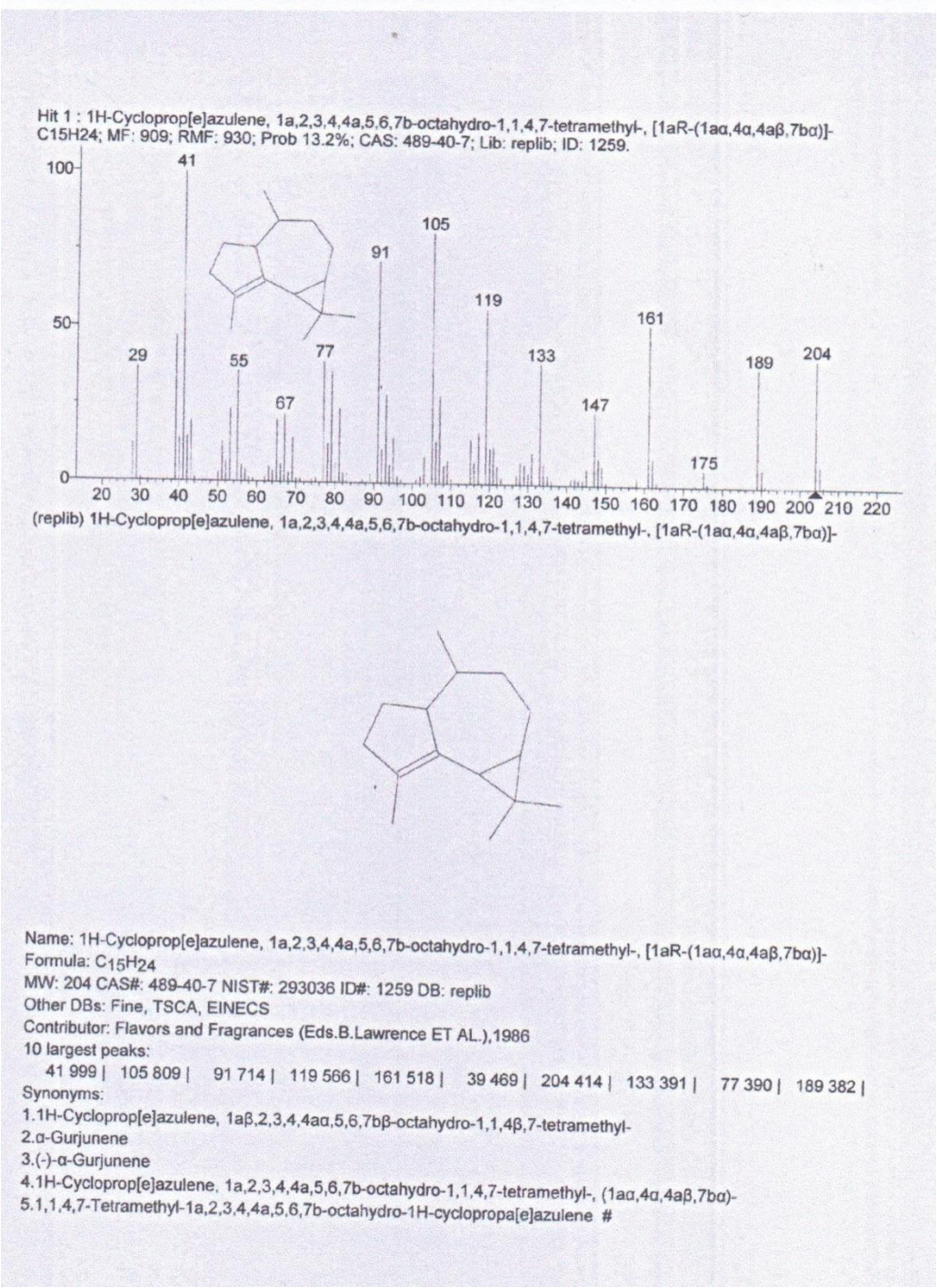


Fig. 18 Ion Chromatogram of identified compounds at each retention time

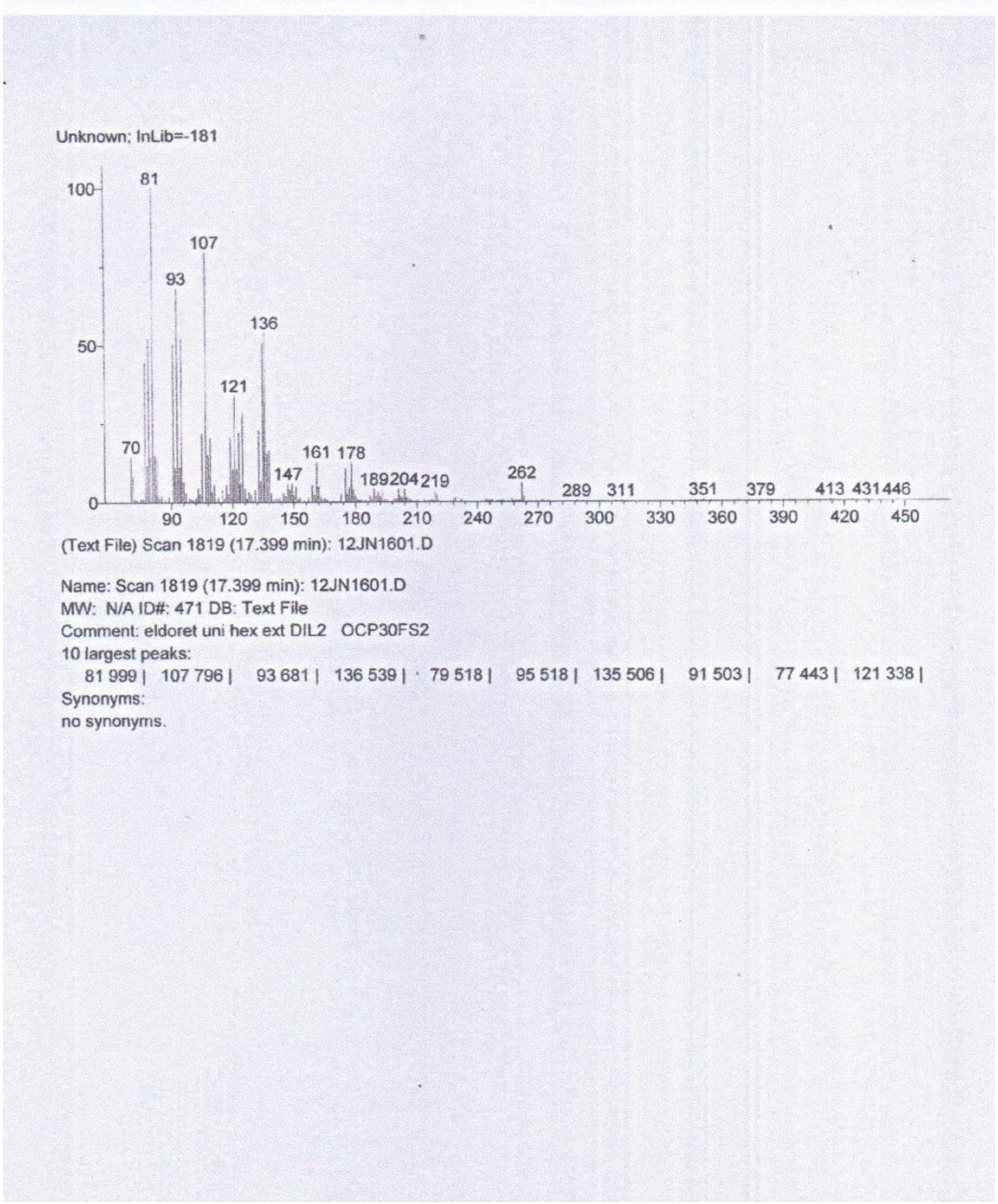


Fig. 19 Ion Chromatogram of identified compounds at each retention

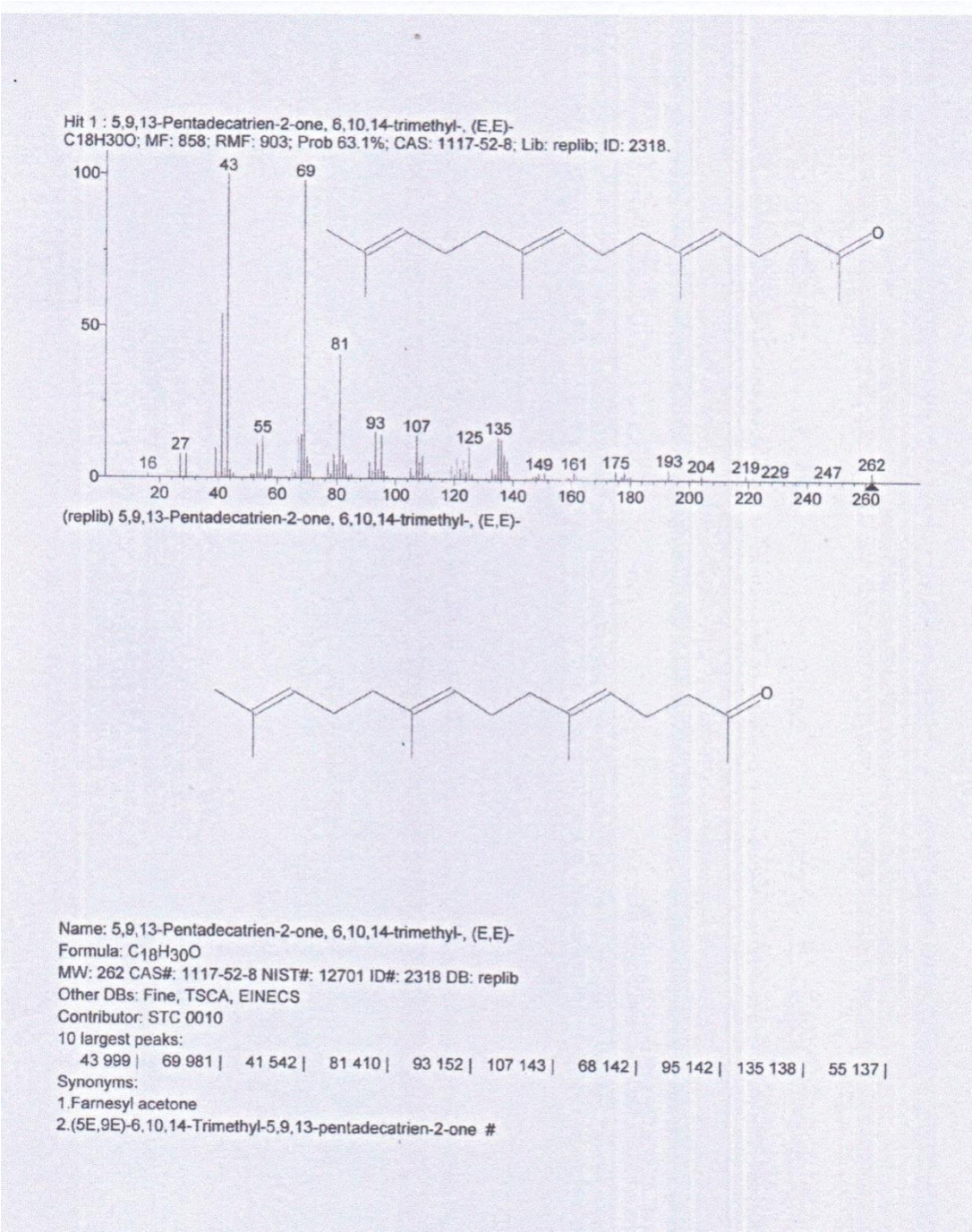


Fig. 20 Ion Chromatogram of identified compounds at each retention time

4.5 Dye extraction from *Bixa orellana.L*

The dye-bath obtained from the seeds had a yellow orange appearance as illustrated in Fig. 21.

Fig. 21 Bixa dye bath (Source; Author, 2014)

4.6 Fabric mordanting and dyeing

Conspicuous change of colour on fabrics was observed. The fabric articles took up a yellow range colour early in the dying process. Fabric articles tri-mordanted and dyed at room temperature and pressure and those at the range of 50 to 60^{o}C showed inconsistency in colour absorption especially at room temperature and pressure, Figures 22 and 23.

However all articles dyed in the temperature range of 80-90^{o}C exhibited uniform absorption of colour, Figures 24 and 25.Single mordanted articles were dyed in the temperature range of 80-90^{o}C, Figures 25.

There seemed to be no clear difference except for brighter colours for the tri-mordanted 002 articles compared with the single mordanted, Figures 24a and 25a.

However the single mordanted 053 bleached article exhibited a brighter shade compared with tri-mordanted one, Figures 24c and 25c.

This could be attributed to other prevailing factors and conditions during the dyeing process that brought about the differences.

Visual observations showed that unbleached fabric articles had a brighter intensity of colour as compared to the bleached ones especially for the articles 002 whereas brightness was greater in the bleached fabrics for both articles 002 and 053, Figures 22 to Figures 25.

The characteristic odour of *Bixa orellana.L* which could have repulsive effects to different people was totally absent after dyeing and drying the fabric articles. This is a very positive attribute indicating that factors contributing to the odour emanating from *Bixa orellana* plant prior to the dye extraction are absent in the dye produced.

Addition of caustic soda 8ml per liter to the dye bath and dyeing produced dark brown shade, Figure 21.

FIGURES:

Fig. 22 a Tri-mordanted cotton fabric bleached) article 002 dyed at rtp.Control on the left.

Fig 22 b.Tri-mordanted cotton fabric (un-bleached) article 002 dyed at rtp. Control on the left

Fig.22 c Tri-mordanted cotton fabric (bleached) article 053 dyed at rtp. Control on the right.

Fig. 22d Tri-mordanted cotton fabric (un-bleached) article 053 dyed at rtp. Control on the left.

Fig. 23a Tri-mordanted cotton fabric (bleached) article 002 dyed at 50- 60^0 C. Control on the left.

Fig. 23b Tri-mordanted cotton fabric (un-bleached) article 002 dyed at 50-60^0c. Control on the left.

Fig. 23c Tri-mordanted cotton fabric (bleached) article 053 dyed at 50-60^0 C. Control on the left.

Fig. 23d Tri-mordanted cotton fabric (bleached) article 053 dyed at 50-60^0 C. Control on the left.

Fig. 24 a Tri-mordanted cotton fabric (bleached) article 002 dyed at 80-90^0 C. Control on the right.

Fig. 24b Tri-mordanted cotton fabric (bleached) article 002 dyed at 80-90^0 C Control on the left.

Fig. 24c Tri-mordanted cotton fabric (bleached) article 053 dyed at 80-90^0 C. Control on the left.

Fig. 24d Tri-mordanted cotton fabric (bleached) article053 dyed at 80-90^0 C. Control on the left.

Fig. 25a Tri-mordanted cotton fabric (bleached) article002 dyed at 80-90^0 C. Control on the right.

Fig. 25b Single-mordanted cotton fabric (un-bleached) article 002 dyed at 80-90^0 C. Control on the left.

Fig.25c Single-mordanted cotton fabric (un-bleached) article 053 dyed at 80-90^0 C. Control on the right.

Fig. 25d Single-mordanted cotton fabric (un-bleached) article 053 dyed at 80-90^0 C. Control on the left.

Fig. 26a Bleached cotton fabric article 002 dyed at 80-90 ^{0}C.Control on the left.

Fig. 26 b Un-bleached cotton fabric article 002 dyed at 80-90 ^{0}C.Control on the left.

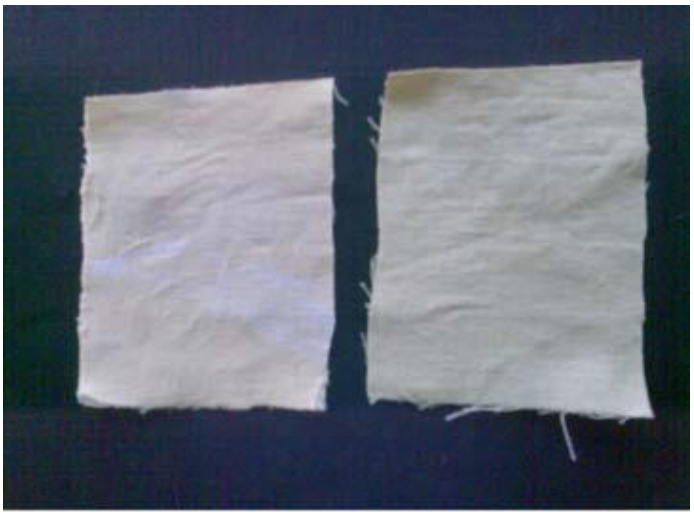


Fig .27 A fabric piece that has undergone bleaching.

Fig .28a Resulting faint yellow alkali solution after dyed fabric for 2 minutes.Unused alkali solution on the right.

Fig. 28b Resultant fabric after boiling with the dye removed. Control on the left.

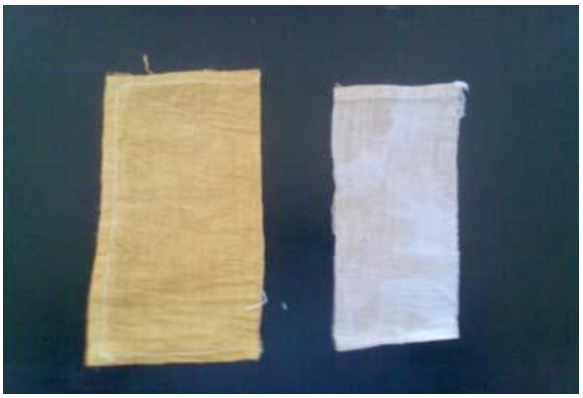


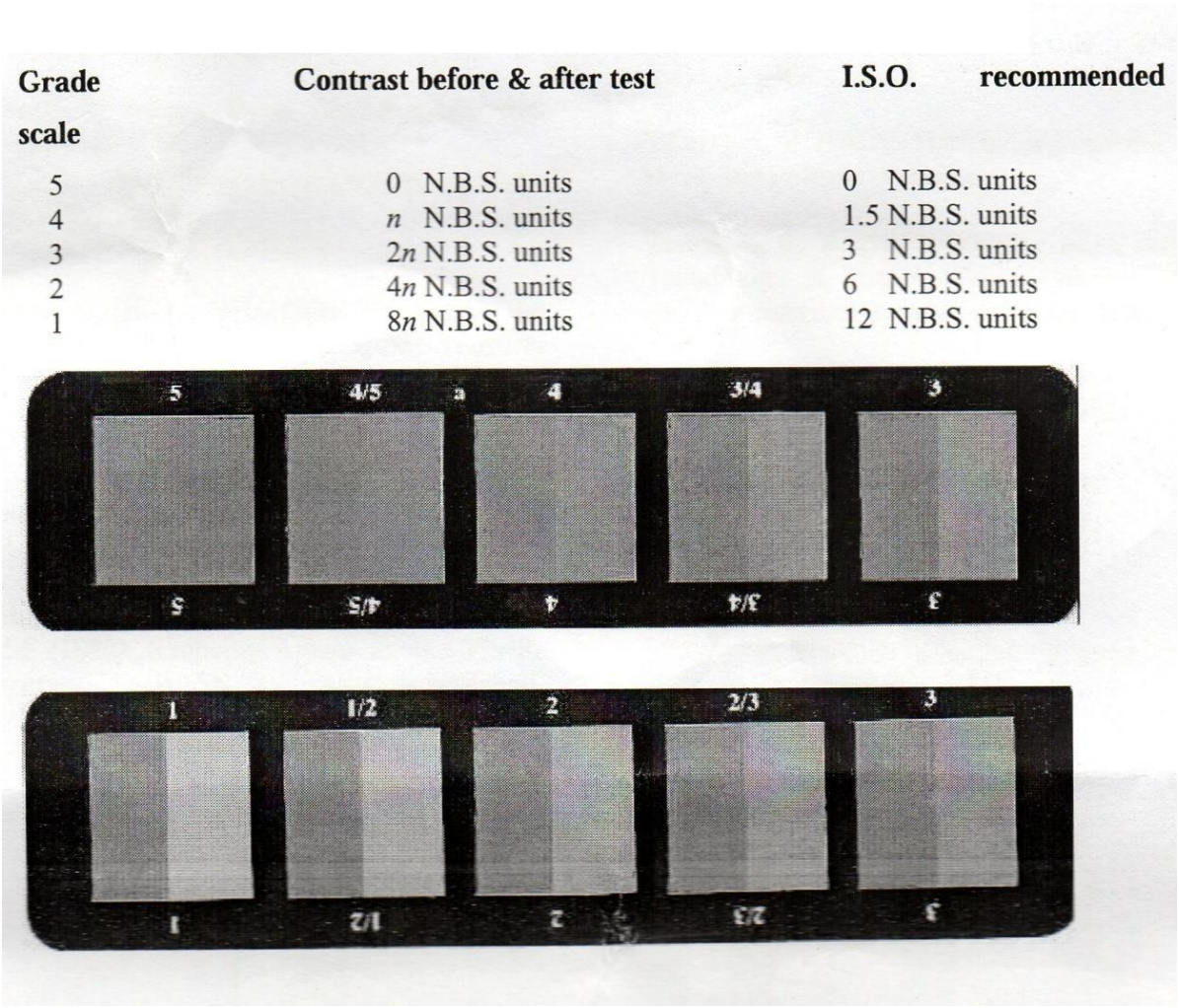
Fig. 29a Fabric stripped for 30 minutes. Note the un-stripped patches on the treated fabric. Control on the right.

Fig. 29b Fabric stripped for 60 minutes. Control on the left.

(Source; Author, 2014)

4.7 Physico-chemical testing of cotton fabrics

4.7.1 Bleaching test of dyed fabrics



Grade scale	Contrast before & after test	I.S.O. recommended
5	0 N.B.S. units	0 N.B.S. units
4	*n* N.B.S. units	1.5 N.B.S. units
3	2*n* N.B.S. units	3 N.B.S. units
2	4*n* N.B.S. units	6 N.B.S. units
1	8*n* N.B.S. units	12 N.B.S. units

Fig. 30 Geometric scales of dyed cotton material for colour fastness (Source; Lab manual)

There was little effect of NaOCl on dyed fabric when pieces were immersed in concentrated bleaching solution for one hour except for the brightening effect. Thus the dye is not easily bleached by ordinary bleaches because it sticks firmly to the fabrics

4.7.2 Testing for the dye obtained from *Bixa orellana*

There was considerable stripping on the dye from the dyed fabrics. Preliminary dye testing using 5% NaOH whereby not so much of the *Bixa orellana* dye was observed in the alkali solution Fig**. 28 a** though the fabric showed a considerable removal of the dye was a clear inference that the dye was a reactive dye.

4.7.3 Fastness to washing test

The fabric articles showed no fading in the shade acquired during the dying process after carrying out the fastness to washing test. Fastness test carried out on the fabric according to the McLaren (1952) grade scale gave grade 5 (Figure 30**)** (Textile laboratory manual

CHAPTER FIVE

CONCLUSION AND RECOMMENDATIONS

5.1Conclusion

The results showed that *Bixa orellana.L* plant is capable of producing viable dyes that can be used for dyeing cellulose-based fabrics.

Bixa orellana.L produces reactive yellow orange dyes as evidenced in this study. However, different colours could be produced on the fabrics depending on the type of mordant used in fixing the colours on to the fabrics. In this study it was found out that high temperatures of between 80-90^{o}C are optimal for dyeing. This is because higher temperatures with a component of pressure could give better results in terms of brightness and fastness. This calls for further research.

GC, GC-MS and co-injection remain the most appropriate methods of analysis of complex mixtures as evidenced from this study.

By modified GC parameter it was possible to identify several components in the seeds of *Bixa orellana.L.* The more important red pigment present in this seeds is oxygenated carotenoid alpha bixin or Cis-bixin which forms 80% of the total carotenoids. The annatto dye extracted has the potential of being used as natural dye because they show dyeing properties at relatively low concentrations and for relatively longer period of time.

5.2 Recommendations

It is therefore recommended to evaluate the potential of using the annatto dye as one of the means of dyeing in the textile industries in Kenya and especially in the coastal regions where *Bixa orellana.L* plant is grown as a commercial crop. Isolation of individual components of the Bixa dye was not successfully achieved. It is therefore recommended that other spectroscopic isolation procedures such as preparative HPLC be made use of to isolate the compounds.

Moreover, isolation of the individual compounds, bixin and norbixin, would make it possible to conduct further analysis to determine whether the activities demonstrated by the annatto dye is as a result of these major components or not. Alternatively, the

synthetic dyes similar to the components of the annatto dye from the seeds could also be used for comparison.

Since in this study, only short term toxicological effects of the components was conducted, this does not give clear picture of any serious dermatological effects the dye might have on the users of the dyed fabric. It is therefore recommended that a specialist in the area of toxicology to carry out more detailed study on this dye. This would ensure that the potential users of these dyes do not expose their skins to a health hazard resulting to diseases.

The findings of this study points to the fact that dye extracted from *Bixa orellana* plant is possible. The properties of the dye obtained compare favourably with commercial reactive dyes that are petro-chemicals in nature.

Bixa orellana dye is a primary colour, yellow which together with the other two red and blue could yield all other desired colours, for dyeing purposes.

This study is a milestone in the dyeing industry as the world seeks to protect environment from synthetic dyes.

The study however reflects the tip of the iceberg of a research that should be carried out in more detail and over a longer period of time.

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APPENDIX I

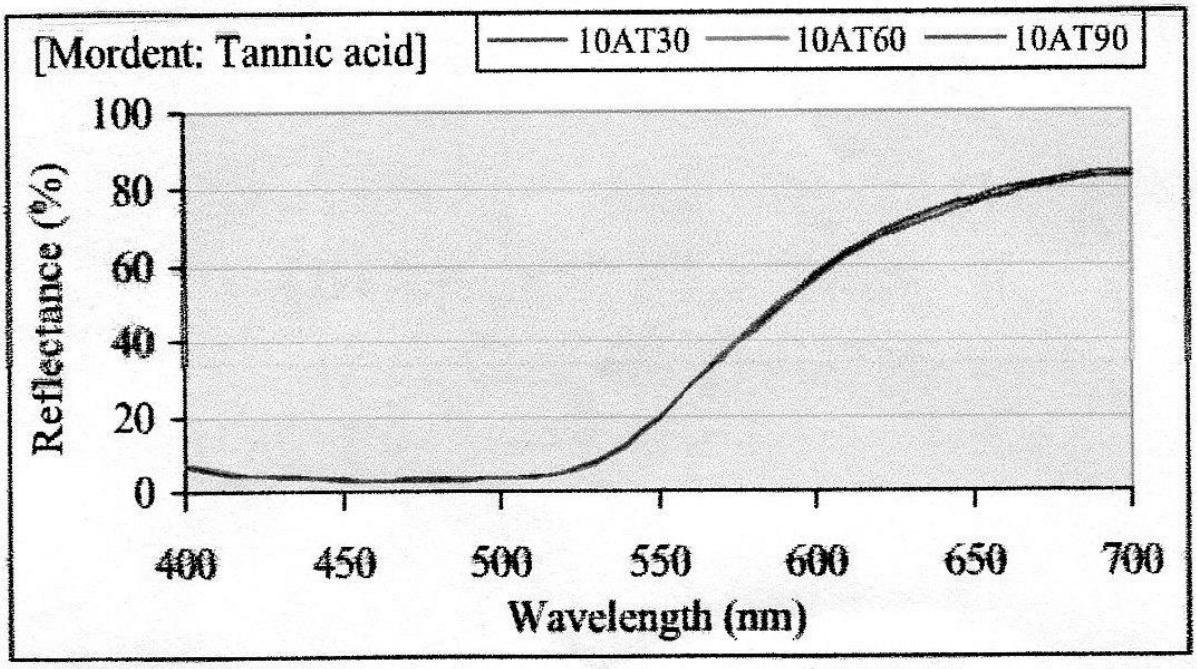

Fig. 31 Reflectance curve for the annatto dye using tannic acid mordent

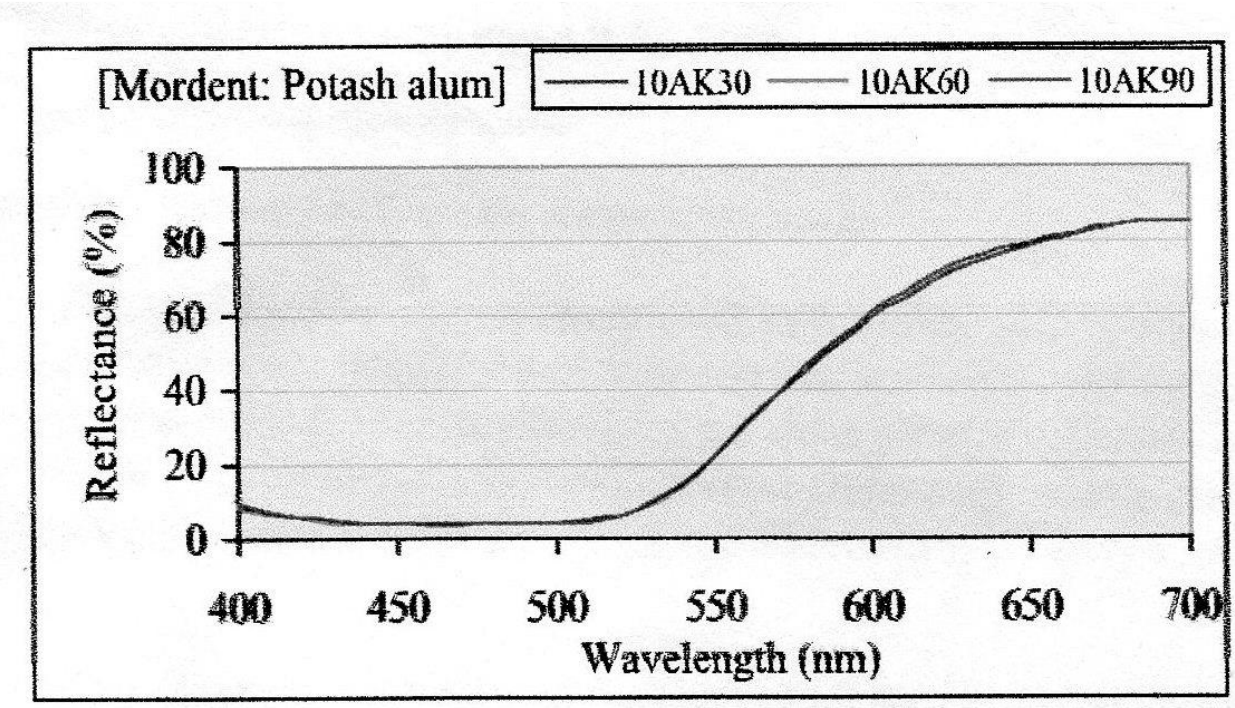

Fig. 32 Reflectance curves for the annatto dye using potash alum mordent

APPENDIX II

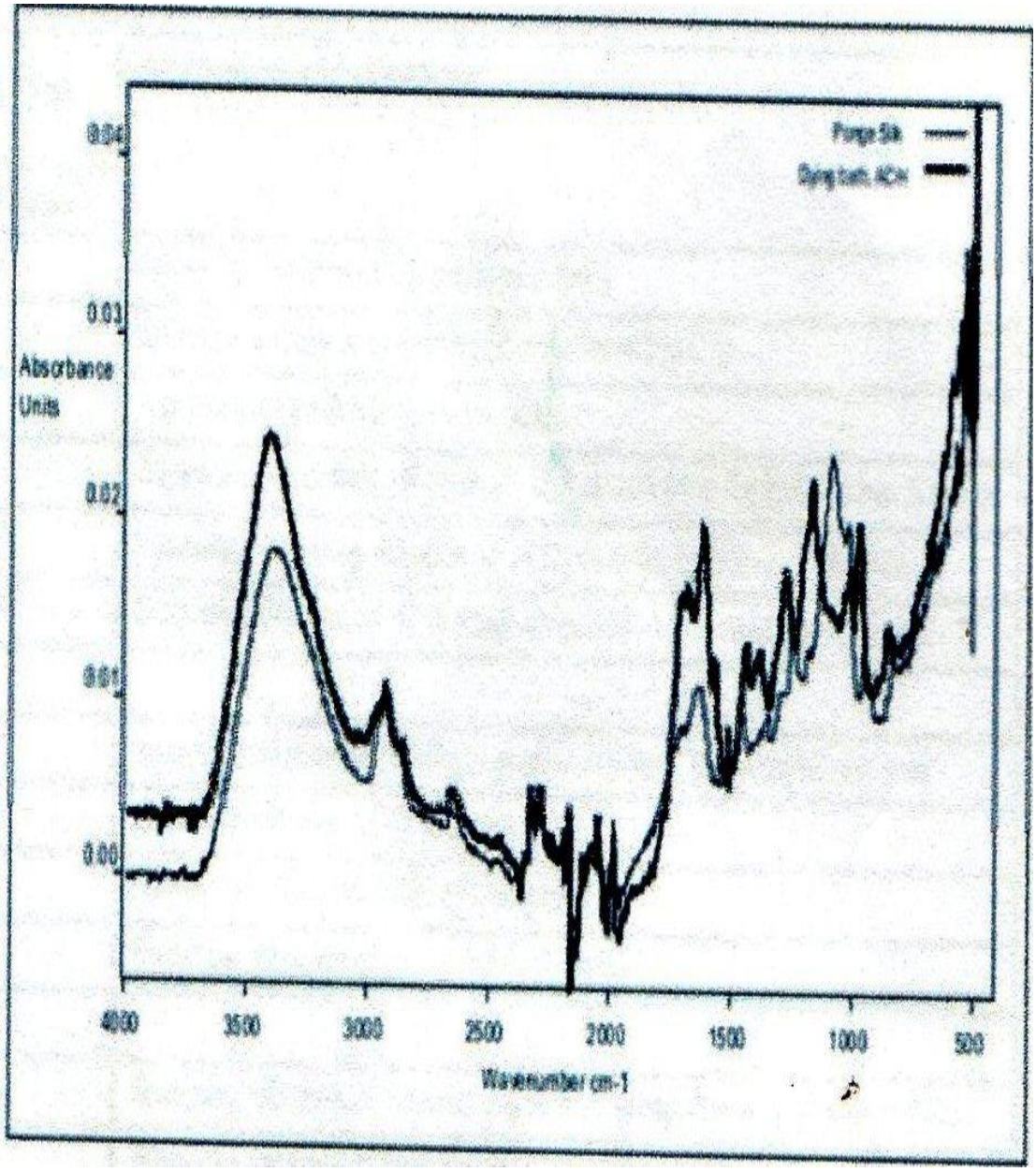

Fig. 33 FTIR spectra of cotton and annatto dyeing bath extract

(Source; Mercadante *et al.*, 1999)

APPENDIX III

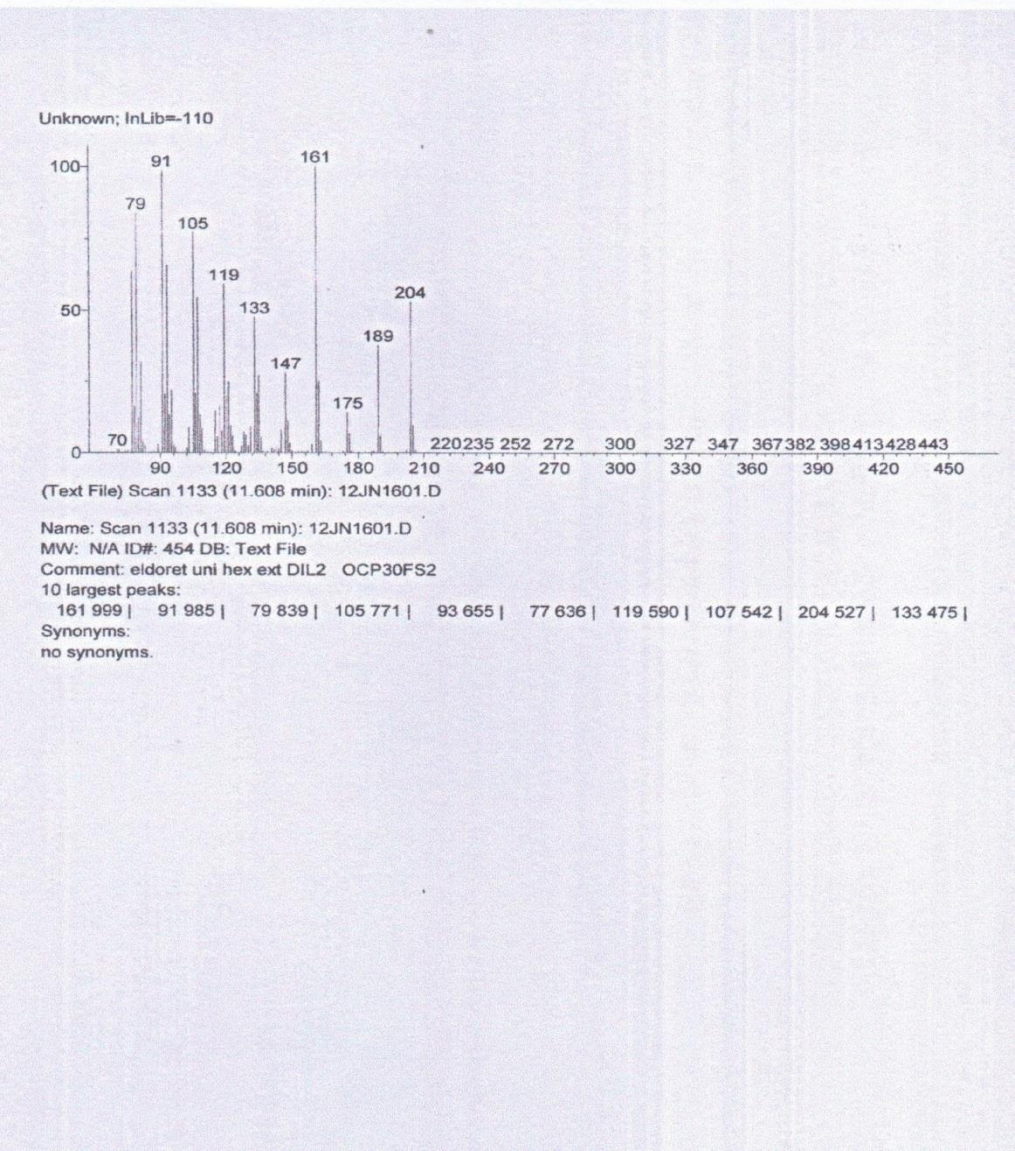

Fig.34 Ion Chromatogram of identified compounds at each retention time

APPENDIX IV

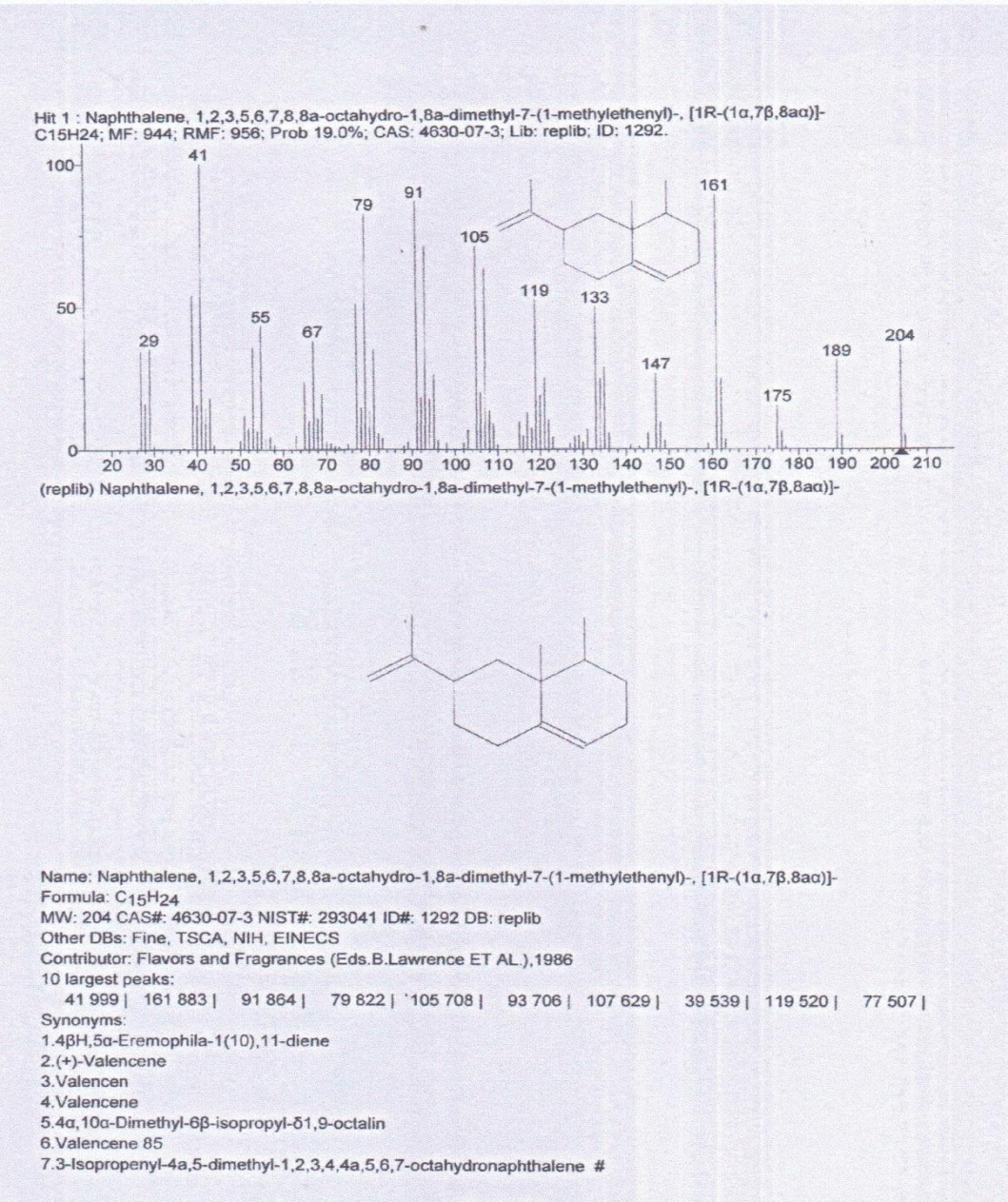

Fig.35 Ion Chromatogram of identified compounds at each retention time

APPENDIX V

Area Percent Report

Data File : C:\HPCHEM\1\DATA\12JN1601.D Vial: 1
Acq On : 12 Jun 2016 14:55 Operator: mirikau
Sample : eldoret uni hex ext DIL2 OCP30FS2 Inst : GC/MS Ins
Misc : madadi Multiplr: 1.00
Sample Amount: 0.00

MS Integration Params: autoint1.e

Method : C:\HPCHEM\1\METHODS\OCPPCBM1.M (Chemstation Integrator)
Title :

Signal : TIC

peak #	R.T. min	first scan	max scan	last scan	PK TY	peak height	corr. area	corr. % max.	% of total
1	8.991	790	823	866	BB	945492	36221024	1.41%	0.929%
2	9.902	911	931	968	VB	550680	22685573	0.88%	0.582%
3	10.400	979	990	1004	BV 3	225967	10886079	0.42%	0.279%
4	10.839	1023	1042	1055	VV	362379	19789092	0.77%	0.508%
5	11.017	1055	1063	1081	VV 4	308703	16859542	0.65%	0.433%
6	11.329	1081	1100	1107	VV	6730305	342953694	13.32%	8.800%
7	11.447	1107	1114	1127	VV 2	3036945	151629614	5.89%	3.891%
8	11.608	1127	1133	1169	VV 3	1084691	81171459	3.15%	2.083%
9	12.013	1169	1181	1190	VV 2	453245	21415888	0.83%	0.549%
10	12.638	1241	1255	1273	VV	360297	14523779	0.56%	0.373%
11	12.849	1273	1280	1327	VV	972130	44532814	1.73%	1.143%
12	13.676	1346	1378	1401	BV 2	1115373	58733215	2.28%	1.507%
13	17.399	1754	1819	1853	BB 2	242277	12416071	0.48%	0.319%
14	21.071	2161	2254	2277	BV	16193854	2574853559	100.00%	66.067%
15	21.485	2277	2303	2320	VV 9	435804	80769563	3.14%	2.072%
16	21.721	2320	2331	2336	VV 4	537136	37019077	1.44%	0.950%
17	22.186	2336	2386	2402	VV	1431320	299306123	11.62%	7.680%
18	22.363	2402	2407	2424	VV	1345877	53781630	2.09%	1.380%
19	25.427	2726	2770	2801	BV 5	162921	17816376	0.69%	0.457%

Sum of corrected areas: 3897364174

12JN1601.D OCPPCBM1.M Mon Jun 13 19:35:20 2016

Fig. 36 Ion Chromatogram of identified compounds at each retention time