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**Chromatographic Isolation and Characterization of
beeswax hydrocarbons**

عزل الهيدروكربونات من شمع النحل وتشخيصها كروماتوغرافيا

A Thesis Submitted in Partial Fulfillment of the
Requirements of the Master Degree in chemistry

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DEDICATION

To the light of my life, to my dearest of all, to my parents.

To my brothers and sisters.

AKNOWLEDGMENT

First of all I would like to thank my god who gave me health, patience and strength to complete this work.

Great appreciation goes to my respectful supervisor Dr. Essa Esmail Mohammad Ahmad, for his support and help throughout this dissertation.

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Abstract

The objectives of this research were to isolate and characterize beeswax hydrocarbons. The physicochemical parameters of the bleached beeswax have shown that the sample contains ester constituents and unsaturated compounds. In addition, both peroxide and acid values were found to be zero. Silica gel column chromatography was used to fractionate the bleached beeswax components. Elution with n-hexane was carried out to separate the nonpolar constituents (fraction 1). Structural and thermal analyses were performed to characterize fraction 1. Fourier transform infrared (FT-IR) has demonstrated the successful isolation of the hydrocarbons because of the presence of pure absorption peaks of hydrocarbons. Furthermore, high temperature gas chromatography (HT-GC/FID) has displayed that fraction 1 contains eleven different hydrocarbons. They are linear and saturated even and odd numbered hydrocarbons. They range between C₂₃ to C₅₀. Differential scanning calorimetry (DSC) revealed that fraction 1 (hydrocarbons) is crystalline in nature.

ملخص البحث

هدفت هذه الرسالة الي عزل وتشخيص الهيدروكربونات الموجودة في عينة من شمع النحل. أحضرت العينة من السوق المحلي لمدينة الدمازين - ولاية النيل الازرق - السودان وتمت معالجتها اولا لازالة المتبقي من العسل والمواد الملونة . وقد أوضحت نتائج الخصائص الفيزيوكيميائية للعينة المعالجة علي وجود مركبات الاسترات ومركبات غير مشبعة . كما بينت النتائج ايضا ان قيمتي كل من رقم الحامض ورقم البيروكسيد مساوية للصفر . استخدمت كروماتوغرافيا العمود المعبأ بجل السليكا لفصل مكونات العينة المعالجة حيث تم إستخدام مذيب الهكسان كطور متحرك لفصل المكونات غير القطبية المفصولة . إجريت التحاليل لتحديد البنية وكذلك الخصائص الحرارية للمكونات غير القطبية المفصولة. برهنت نتائج الأشعة تحت الحمراء علي نجاح فصل الهيدروكربونات وذلك لظهور الإمتصاصات المميزة فقط لهذه المركبات في الطيف. كما بينت نتائج تحليل كروماتوغرافيا علي وجود احدي عشر مركب هيدروكربوني. تمتاز هذه المركبات بأنها خطية مشبعة ذات عدد زوجي وكذلك فردي لذرات الكربون وتقع في المدي C23 الي C50. بينت نتائج التحليل الحراري ان هذه المركبات ذات بنية بلورية.

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List of abbreviations and symbols

IR	Infrared spectroscopy
HT-GC	High temperature gas chromatography technique
GC-MS	Gas chromatography mass spectroscopy
LC	Liquid chromatography
ASTM D-5	American Society for testing and materials
MS	Mass spectroscopy
FTIR	Fourier transform infrared spectroscopy
XRD	X-ray diffraction
NMR	Nuclear magnetic resonance
HT-GC-MS	High temperature gas chromatography mass spectroscopy
C13 CP/Mas	Carbon13 Cross polarization/Magic angle spinning
Ti relaxation	The time constant of an exponential return of a system to equilibrium
	after disturbance
US-FDA	United States food and drug administration
DSC	Differential scanning calorimetry
TGA	Thermal gravimetric analysis
TA	Thermal analyzer
DP	Direct polarization
C13	Carbon13 Nuclear Magnetic Resonance
Ppm	Part per million
C13	Carbon13
M.wt	Molecular weight
n-Hexane	Normal Hexane

Introduction

In recent years, due to declining of petroleum reserves together with economical and environmental concerns attention has been shifted toward materials that are renewable, sustainable, recyclable and environmentally friendly. Materials derived from petroleum origin are non-renewable, non-sustainable, waste problem, and costly. In this context, renewable materials such as biomass, natural waxes, and vegetable oils have found considerable attention as promising substitutes for the petroleum based materials [1-3].

Natural waxes are those made from plants or animals material derived from vegetables, beans, jojoba, candelilla, carnauba, bees, spermaceti, and other sources. These materials have properties different from synthetic waxes derived from minerals or petroleum products. They have found many applications in many fields such as in food preservation, beverages, cosmetics, forming composite materials, preparation of nano-materials, manufacturing candles, industrial sector and in medicine [4].

Beeswax (white and yellow) is refined wax of honey combs. It is produced in wax glands located in the abdomen of bees. Generally, beeswax consists of a complex mixture of aliphatic hydrocarbons, mono-, di- and poly esters, hydroxy esters, free fatty acids, free fatty alcohols and minor of other compounds. It has been reported in the literature that the chemical composition of beeswax depends on its origin, age, and climatic conditions. In the earliest time it was exploited for various purposes such as preservation of mummies, square wax writing tablets, bending agent, and for sealing and waterproofing. Although beeswax is now partly replaced by synthetic or fossil products, it played an important role in a number of fields such as polymer technology, symbolic and artistic fields, preparation of cosmetics or medicinal commodities, food, pharmaceutical, and pesticides [5-16].

Our literature Survey has revealed that in almost all published research works beeswax has been used as a whole material for the specified application. Fractionation of beeswax into its different constituent components could further extend its application and make it value added material.

1.1 Research objectives

The objectives of this research work were to separate and characterize the hydrocarbons of a beeswax sample originated from Blue Nile State (Aldamazin-Sudan). Structural and thermal properties are chosen mainly because they are crucial for understanding the physical and chemical properties as well as the applications of the different beeswax fractions in different fields.

Chapter two

Literature review

2.1 Natural waxes: Definition and types

Natural waxes are naturally occurring compounds composed of a mixture of chemical compounds such as hydrocarbons, esters, fatty acids, alcohols, ketones, mono-, di-, tri-acylglycerols and sterol esters [17]. The two main classes of natural waxes are: synthetic and natural origins. They play a great role as raw materials in many industrial branches like pharmaceuticals and cosmetic industries as well as for applications in culture and art [18]. Natural waxes also are considered as lipids in nature mainly contain a wide variety of lipid components [17]. Natural waxes are further classified into four groups which are: plant, animal, mineral and petrochemical waxes [19].

2.2 Physical properties of natural waxes

Natural waxes consist of a group of chemical substances such as various long chain fatty acids beside other different components depending on their origin. Therefore each wax has significant physical and chemical properties which are exploited in a multitude of applications. Waxes are not soluble in water but soluble in organic non polar solvents [20-22]. In agreement with the above reported properties, Albert illustrated that waxes are insoluble in water, sparingly soluble in alcohol, and very soluble in chloroform, ether, and in fixed and volatile oils. They are partially soluble in cold carbon disulfide and completely soluble in it at temperatures of 30°C and above [12].

2.3 Chemical composition of natural waxes

Although, studying the chemical composition of natural waxes is not an easy task due to its complicated chemical structure but it has been studied through many analytical techniques which include (IR) infrared spectroscopy, gas chromatography (GC/FID), pyrolysis-gas chromatography, gas chromatography/mass spectrometry (GC/MS), and liquid chromatography (LC) [7-9,12,19, 24-28]. The findings of these studies could be

summarized in the fact that natural waxes whether they are from plant or animal source mainly represent a mixture of waxy esters containing other compounds with different quantities which are fatty acids, fatty alcohols and hydrocarbons too [19,29-31].

2.4 Beeswax

Beeswax is one of the natural waxy materials found in nature easily. The diversity of the natural waxes gives a range of usages [32, 33]. The most important commercial animal waxes are bees wax and wool grease.

Beeswax is an exudation of the abdominal glands of the honeybee. Because of its unique characteristics, is now being used in development of new products in various fields such as cosmetics, foods, pharmaceuticals, engineering and industry [5-6,34-38]. It is originally obtained from honeycombs of bees after honey removal, through the following procedure which includes hot water, steam or solar heat for combs melting then the impurities is removed and the liquid wax is cast into cakes for further purification to obtain food -grade yellow beeswax. This yellow beeswax can be converted to white beeswax by bleaching with hydrogen peroxide, sulfuric acid or sunlight [11]. Although the melting point of beeswax is about 60°C, wax is secreted in a liquid state at ambient temperatures. The liquid was crystallizes in this condition. In general, the form of the crystal changes depending upon physical parameters such as temperature, pressure, and cooling rate. Therefore, it is probable that a specific structure is present in crude beeswax [39]. The physical properties of some artificial and natural waxes are shown in the following table (Table 1) [28,40].

Lewis and Leclering [41-42] have studied the density and the melting point of beeswax. Their results showed that itis density equals 0.95 g/cm³ whereas its melting point ranges from 62 to 65°C [41,43].

Beeswax is completely insoluble in water due to its resistance to hydrolysis and natural oxidation. However, it is soluble in alcohol, chloroform, ether and oils. Its combustible and it is the properties remain unspoiled by time. Apart from the larvae of the wax moth, no animal has the digestive acids and juices to break it down [12]. At normal temperature beeswax is solid in appearance. It becomes brittle when temperature drops below 18°C however it quickly becomes soft and pliable at around 35 °C to 40 °C.

Table 1: The physical properties of some artificial and natural waxes

Type of wax	Melting point	Density	Acid number	Saponification number	Hardness ASTM D-5
Beeswax	61-65	0.950-0.965	17-24	87-100	15
Artificial waxes					
Ceresin	65-80	0.91-0.92	0	0	
Paraffin	45-70	0.88-0.91	0	0	
Stearin	52-55	0.89	205-209	-207-210	
Natural waxes					
Bayberry-myrtle	48-50	-0.875-0.980	4-30	205-217	7.5
Candelilla	65-69	0.97-0.99	-1-19	45-65	1.5
Caranday	82-85	0.99-1	3-10	62-80	1
Carnauba	82-86	0.99-1	2-11	-78-88	1
Castor bean wax	86	0.98-0.99	2	17	2
Japan wax	78	.99	24	70	1.5
Montan crude wax	50-56	0.97-1.06	6-20	217-237	8
Ouricury	85	0.99-1.00	45-50	88	
Shellac wax	72-86	0.97-0.98	2.25	45-85	2
Spermaceti	45-49	0.94-95	1.00	116-125	16
Sugar cane wax	75-79	0.98-.095	6-10	25-35	3

-2.5 Chemical composition of beeswax

The chemical composition of beeswax has been studied by many researchers [7-10,12,23-25,45]. In most of these studies chromatographic methods coupled with MS and the FTIR are the main characterization techniques. In other few studies, X-ray diffraction and NMR have been used for characterization purposes [30,39].

The chemical composition of beeswax is controlled by certain factors as it mentioned by Aichholz and lorbeer [9-10]. These factors can be concluded in subspecies of the bees, the age of the wax, and the weather circumstances of its production. However, this variation in the composition occurs mainly in the relative amounts of different components present, rather than in their chemical identity [11].

It has reported that beeswax is a complex mixture of hydrocarbon, carboxylic acids, esters, and alcohol [7]. Free fatty acids, free primary fatty alcohols, linear wax monoester and hydroxymonoesters, complex wax ester and odd-numbered, straight chain hydrocarbons represent the main five groups of chemical components that can be found in beeswax [11]. The major constituents of beeswax are esters (30%-80%) which consist of molecular species of C_{40} to C_{46} . Analysis of those materials was performed using liquid chromatography [8].

In another study beeswax of the honeybee (*Apis mellifera*) was investigated. It was found that the beeswax is a complex mixture of long-chain alkanes, alkenes, monoesters, diesters, hydroxymonoesters, and fatty acids as well as several minor components [23]. Much of the ester fraction contains palmitic, oleic, and tetracosanoic acid esters of alcohols ranging in length from 12 to 36 carbons [9].

Albert [12] also illustrates that beeswax consist primarily of five main groups of components. Free fatty acids (typically 12-14%) most of which are saturated (ca. 85%) and have a chain length of C_{28} - C_{32} and free primary fatty alcohols (ca. 1%) with chain length of C_{28} - C_{35} . Linear wax monoesters and hydroxymonoesters (35-45%) with chain lengths of C_{40} - C_{48} . The esters are derived almost exclusively from palmitic acid, 15-hydroxy palmitic acid, and oleic acid. The variation in total length chain of ester is mainly the result of the different chain length of the alcohol moiety (C_{24} - C_{34}). Complex wax ester (15-27%) containing 15-hydroxypalmitic acid or diols which through their hydroxy group are linked to another fatty-acid molecule. Tri and higher esters are also found. Odd numbered, straight chain hydrocarbons (12-16%) with a predominant chain length of C_{27} - C_{33} with increasing chain length, the

proportion of unsaturated species increases (above C₃₃ only unsaturated species are present) and alkadienes and-trienes have been reported at only very low levels. The reported concentrations of these main components vary somewhat upon the analytical procedures applied [12].

It has also been noticed that crude beeswax contains a large number of minor components such as terpenoids and flavonoids which were thought to be originated from plant [10]. In the chemical composition of beeswax volatile components have also been detected at low levels. Oxygenated compounds produced by the bees are also present amongst which decanal (ca.50% of the oxygenated volatiles), 1-decanol, nonanol, octanal, furfural, and benzaldehyde are responsible for the bouquet of the wax [24].

Maia and Nunes [25] used high-temperature gas chromatography technique coupled to mass spectroscopy (HT-GC/MS) for identification purposes and HT-GC-FID for quantification purposes to characterize beeswax. They found that more than 50 compounds were present. Some of these compounds were present only in trace amounts and were only detected by (HT-GC/MS) after extraction of characteristic ions while other were specifically detected in over-lapping peaks by extraction specific ions and quantified together by (HT-GC-FID). This study was clarified that beeswax compose of seven homologous series of lipid component, the odd number hydrocarbons, even number hydrocarbons, odd number mono-saturated hydrocarbon, and palmitate monoesters, oleate mono-esters and two series of hydroxyl palmitate monoesters with long chain alcohols. Odd number hydrocarbons with 17 to 35 carbon atoms were detected with the main hydrocarbon being heptacosane (CH 27:00), followed by nonacosane (CH 29:0) and hentriacontane (CH 31:0). The even number hydrocarbon 22 to 34 in a low abundance compare to odd number hydrocarbon.

In other studies [9-10,45] using GC/MS, it was found that beeswax contains 74 major and 210 minor components, about 40 of which were identified. It is also reported the identification of 24 previously unidentified compounds, including a new family of unsaturated ethyl esters from C₁₆ to C₃₄ chain length, although the absence of C₂₀ chain lengths. In addition, these studies showed that 47% of the wax was made up of esters which contain C₂₄-C₃₄ alcohols combined primarily with palmitic and oleic acids. It was also noticed the presence of odd-numbered alkenes, hydroxyl esters, and branched alkanes.

The composition and structure of beeswax were also studied using X-ray diffraction (XRD), Fourier transform infrared, and gas chromatography in combination with MS by Parva et al., [26]. XRD pattern of beeswax was illustrated that wax is a crystalline substance in nature and this was proved by the presence of sharp peaks in the XRD pattern. FTIR showed the presence of carbonyl groups, fatty acids, esters, CH₂ bending, C-C vibration, nucleic acid, =CH₂ bending and CH₂ rocking respectively, while GC/MS signifies that beeswax contains alkenes, acid and alkane groups.

Besides using the above spectroscopic techniques, NMR was also used for beeswax investigation. The Carbon-C¹³ chemical shift of solid material is known to be sensitive to the crystal form [27]. The C¹³CP/MAS have been used to determine the spectrum of beeswax by Basson and Reynhardt. But no detailed structural analysis was performed using C¹³ chemical shifts and this was attributed to poor separation of the peaks from the CH₂ region. Solid-state C¹³ NMR spectra were obtained for crude beeswax with direct polarization (DP) at room temperature. The assignment of the peaks was performed by comparison to references [27]. The strongest resonance is centered between 30 and 35 ppm, which are typical chemical shifts for internal-chain methylene (int-CH₂) carbons. The peak at 14.6 ppm is due to methyl carbons at the remains of alkyl chains.

Also the molecular structure of beeswax was investigated by using C¹³ measurements in this work chemical shift, cross –polarization rate and T₁ relaxation were observed. Liquid chromatography was also utilized as an analytical tool for analysis of waxes and it proved that the major constituents of beeswax are esters (30-80%), which consist of molecular species of C₄₀ to C₄₆ [17].

Zimnicka and Hacura have used FT-IR and Raman spectroscopy to investigate the structure of beeswax comb in the native state of the polish bees (*Apis mellifera*) from different country regions. It was observed that after 1 to 10 minutes from sample preparation there were changes in the spectral bands of the CH₂- groups. At first in this spectral range, there was only one central band whose intensity decreased in time. After 5 minutes, an increase of side shoulders at the finally splitted band arising from CH₂- group vibrations was observed. 10 minutes later, the splitting of the band near 1460 cm⁻¹ was 8.7 cm⁻¹. It is the same phenomena of forming a semi crystal local

structure of hydrocarbons, as reported for linear n- alkanes in binary mixtures in solid solution [46].

2.6 Applications of beeswax

Beeswax represents the most important natural waxes and that is due to its significant properties. Beeswax has useful applications in different fields such as medicine, food, cosmetics, industry as well as pharmaceutical products [47].

Beneficial of beeswax as a product starts from bees themselves as it considered an important structural material of honeybees and utilized for the formation of comb [24]. One study has shown that the beeswax is used by the worker to build out honey comb and also for the comb broadening [26].

Beeswax has widespread uses in medicine and this is attributed to its physical properties [48]. Beeswax is an essential component in food supplement capsules as filling material to fill about 40% of the capsule. Furthermore, beeswax is the most appropriate stabilizer for keeping oil-based capsule content in suspension. Also for certain food supplements tablets beeswax is used as stabilizer and realizing agent [12].

Beside the above applications of beeswax in food by mixing it with other ingredients such as oil, fats and other waxes, it serves as a glazing agent for confectionery (including chocolate), in small product of fine bakery were coated with chocolate, in snakes, nuts, coffee beans, dietary food supplements, and in certain fresh fruits for surface treatment at quantum sits levels . The US-FAD has affirmed that beeswax can be used in formulation of chewing gum base with concentration not exceed than 50mg/kg for the final product [12].

In medicine there is a significant use for long-chain aliphatic alcohols as dietary supplements or nutraceuticals in the treatment of various chronic diseases such as diabetes and hypercholesterolemia [12,13-14]. The composition of beeswax indicates that it is a natural and good source of the long-chain aliphatic alcohols [45].

Mankind have been using candles which are made of beeswax in religious festivals since very long time and this proved the usage of this natural product in industry field [15]. Candles are also produced from paraffin wax but the ones which are produced from beeswax has higher melting point and that is why it can remain straight at ambient temperature [48].

In cosmetics beeswax represent a main constituent in the recipes of creams and ointments since long time beside oil with different percentages according to the required consistency. Beeswax has many properties contribute to it is basic use in cosmetic field such as improving water binding of ointments and creams, increasing the protective action of sun creams, improve soap function (provide a protective film on skin and enhance it is elasticity), has antibiotic and thermo-storing properties and the desired effect of beeswax can be achieved often by little amounts of it 1-3% .It is also used in manufacturing of lipsticks gives (shine, consistency and color stabilization) [16].

Chapter Three

Experimental

3.1 Materials

Beeswax was purchased from Aldamazin local market, Blue Nile State, Sudan. It is yellow in colour and it appears as it was processed by melting.

n-Hexane (Assay 85%, density 0.655-0.665 g/cm³ at 20 °C, Mwt. = 86.16). Diethyl ether (Assay 98%, density 0.713-0.717 g/cm³ at 20 °C, Mwt. = 74). Silica gel (60-120 mesh for column chromatography), Activated charcoal, Potassium Iodide(Assay 99.5%, Mwt=166), Potassium Hydroxide(Assay 85%, Mwt= 56.1), Sodium Thiosulphate(Assay 99%, Mwt= 248.16), Hydrochloric acid(Assay 36.5%, Mwt =36.46, Density 1.18 g/cm³), Chloroform(Assay 99.5%, Mwt= 119.38, Density 1.48 g/cm³), Acetic acid(Assay 99.5%, Mwt= 60.05, Density 1.05 g/cm³), Iodine monochloride, Phenolphthalein indicator and the Starch).

3.2 Pretreatments of the raw beeswax

The beeswax was cleaned from the remaining honey by washing it with hot water, then filtered while it was at 40 °C and dried. The dried beeswax was dissolved in 50 ml n-hexane and charcoal was added to get rid of the coloured substances. The mixture was boiled at 70 °C, filtered while it was hot, washed by using hot n-hexane, then evaporated and dried. The bleached beeswax was used for the fractionation.

3.3 The physico-chemical parameters of beeswax sample

3.3.1 Saponification value

25 mL of 0.5 M alcoholic potassium hydroxide was added to 3 grams of the bleached beeswax sample and the mixture was refluxed for 90 minutes. The solution was cooled to room temperature and two drops of phenolphthalein indicator were added and then titrated against hydrochloric acid (HCl) 0.5 M till the pink color was disappeared and the volume of the titrant was recorded. A typical method was repeated for the blank solution. The saponification value was calculated using the following equation:

$$\text{Saponification value} = (B - S) \times N \times 56.1/W \dots\dots\dots (1)$$

Where B = Volume of titrant (mL) for the blank solution

S = Volume of titrant (mL) for the sample

N = Normality of HCl

56.1 = Molecular weight of the KOH

W = Weight of the sample

3.3.2 The iodine value

20 mL of chloroform was added to 0.3 g of the beeswax sample which followed by addition of 25mL of 25% iodine-monochloride solution. The mixture was left in a dark place for an hour and after that 25mL of 25% potassium hydroxide solution was added. The previous solution was titrated against sodium thiosulphate solution (0.1M) till the color changed to a pale yellow after which 2-3 mL of starch solution was added (as indicator) and the titration was continued till the blue color was completely disappeared. The volume of the titrant was recorded. A typical method was repeated for the blank solution.

The iodine value was calculated by using the following equation:

$$\text{The iodine value} = (B - S) \times N \times 126.9 \times 100/W \dots\dots\dots (2)$$

Where B = Volume of titrant (mL) for the blank solution

S = Volume of titrant for the sample solution

N = Normality of sodium thiosulphate

126.9 = Molecular weight of the iodine

W = Sample mass

3.3.3 The peroxide value

20 mL of chloroform and 15 mL of glacial acetic acid were added to 0.25g of beeswax sample. 5 mL of a 10% potassium iodide (KI) was added to the previous mixture and the resulting solution was left in darkness for one hour. Titration against sodium thiosulphate solution (0.1N) was carried out till the color changed to pale yellow and then 2 mL of the starch solution (as indicator) was added and the titration was continued till the disappearance of the blue color. The volume of the titrant was recorded. A typical method was repeated for the blank solution. The peroxide value was calculated using the following equation:

$$\text{Peroxide value} = (S - B) \times N \times 1000/W \dots\dots\dots (3)$$

S = Volume of titrant for the sample solution

B = Volume of titrant for the blank solution

N = Normality of Sodium thiosulphate solution

W = weight of the sample

3.3.4 Free fatty acid value

20 mL of chloroform was added to 0.25g of bleached beeswax sample then two drops of phenolphthalein indicator was added and the mixture was titrated against 0.5M sodium hydroxide (NaOH) till the presence of a pink color. The free fatty acid was calculated as follows:

$$\text{Free fatty acid (FFA)} = A \times N \times 56.1/W \dots\dots\dots (4)$$

Where A = Volume of 0.1N potassium hydroxide required for sample

N = Normality of potassium hydroxide solution

W = Weight in grams of the sample

3.4 Fractionation of beeswax hydrocarbons via column chromatography

Column chromatography was used to fractionate the components of the beeswax through their differences in polarity. The column was prepared first by filling it with silica gel, and then 1g of the bleached beeswax sample was dissolved in n-hexane and passed through the column using a solvents system which constitutes of n-Hexane/diethyl ether (95:5, v/v).

The percentage of the hydrocarbons in beeswax was calculated using the following equation:

$$\text{Hydrocarbons (\%)} = (\text{wt. of the fractionated hydrocarbons/wt. of the bleached wax}) \times 100$$

3.5 Methods of characterization

3.5.1 Fourier transform infrared spectroscopy analysis of the bleached beeswax and the fractionated hydrocarbons

Fourier transform infrared (FT-IR) spectroscopy is certainly one of the most important analytical techniques available today. One of its great advantages that sample in any state (gas, liquid, solutions, films, and surfaces) can be studied by a suitable choice of sampling technique [49]. The energy at which any peak in an absorption or transmission of FT-IR spectrum appears corresponds to the frequency of vibration of a part of the sample molecule. This provides information on the functional groups in the molecule. From the frequencies of the absorptions it is possible to determine whether various functional groups are present or absent. This forms the basis of analyses of the molecular structure of single and multi component materials [50].

The FT-IR spectra of the bleached beeswax and the fractionated hydrocarbons were obtained using a Shimadzu spectrophotometer in the wavenumber range of 4000–500 cm^{-1} . 2 mg of each sample and 200 mg of KBr were thoroughly mixed. The mixture was then squeezed to form transparent pellets which were examined by FT-IR.

3.5.2 High-temperature gas chromatography analysis of the fractionated hydrocarbons (GC/FID)

The flame ionization detector (FID) is the most useful GC detector available and by far the most commonly used in GC analysis. The FID has a very wide dynamic range, a high sensitivity, and (with the exception of a few low molecular weight compounds) detects all substances that contain carbon. In the GC-FID the ions which are produced in the flame are measured as opposed to the heat generated. Hydrogen is mixed with the column eluent and burned at a small jet. Surrounding the flame is a cylindrical electrode and a relatively high voltage is applied between the jet and the electrode to collect the ions that are formed in the flame. The resulting current is amplified by a high impedance amplifier and the output fed to a data acquisition system or a potentiometric recorder. The detector usually requires three separate gas supplies together with their precision flow regulator. The gases normally used are Hydrogen for combustion, Helium or Nitrogen as carrier gas and Oxygen or air as the combustion agent [51].

The GC-FID analysis was performed using a Chrompack CP9001-gas chromatograph. HT-AQ5 column which is 25m in length and has internal diameter equals 22mm was used for the analysis. The maximum temperature was between 420-480 °C.

3.5.3 Differential scanning calorimetry (DSC)

Differential scanning calorimetry is one of the most versatile thermal analysis techniques available. It can be used to study thermodynamic processes, transitions, specific heat capacity and kinetic events. DSC is a technique that measures the differences in the heat flow to a sample and a reference sample as a direct function of time or temperature under heating, cooling or isothermal conditions. In a DSC experiment the difference in energy input to a sample and a reference material is measured while the sample and the reference are subjected to a controlled temperature program. DSC requires two cells equipped with thermocouples in addition to a programmable furnace, recorder and gas controller. The DSC instrument is commercially available as a power compensating or as a heat-flux DSC. Also in DSC the measured energy differential corresponds to the heat content (enthalpy) or the specific heat of the sample. DSC is often used in conjugation with TA (Thermal Analyzer) to determine if a reaction is endothermic, such as melting, vaporization and sublimation or exothermic, such as oxidative degradation. It is also used to determine the glass transition temperature [52,53].

The DSC analysis was done by using differential scanning calorimeter-DSC1, Mettler-Toledo International Inc., (Germany). 5 to 10 mg of the sample were weighed and transferred into an aluminium pan, covered, and sealed securely. Empty aluminium pan was used as a reference in order to measure the power (heat energy per unit time) difference between them. The sample was analyzed under nitrogen gas flow (20 mL min⁻¹) from 0 °C to 80 °C at a heating rate of 5 °C min⁻¹.

Chapter four

Results and discussion

4.1 Physicochemical parameters of beeswax sample

Table 4.1 shows the physicochemical parameters of the beeswax sample. It could be observed from the table that the beeswax sample does not contain free fatty acids and also does not undergo any oxidation processes during storage and transportation because the acid and peroxide values were found to be zero. The absence of the free fatty acids was also confirmed by the FT-IR analysis (see 4.2) which did not show any strong, wide absorption band (-OH) in the range of 3200-2500 cm^{-1} and intense band (-C=O) in the range of 1700-1725 cm^{-1} . Although free fatty acids were reported in the literature [11-12] as one of the main components of beeswax. Furthermore, iodine value shows the presence of degree of unsaturation in the beeswax sample. In fats and oils, the saponification value measures the average molecular weight of the triacylglycerol in a sample. The smaller the saponification value the larger the average molecular weight of the triacylglycerols present. Previous studies [12,25] have shown that both the acids and the alcohols which constitute the esters in beeswax are long chain, high molecular weight compounds. Compared to previous studies [12,54], significant variations have been noticed in peroxide, acid, and saponification values. These variations could be attributed to the differences in point of origin of beeswax, environmental and geographical factors, and possible experimental errors.

Table 4.1: The physico-chemical parameters of the bleached beeswax sample

The physico-chemical parameter	The value
Acid value (mgKOH/g)	0.00
Peroxide value(meq/kg)	0.00
Iodine value (g/100g)	21.17
Saponification value (mgKOH/g)	46.75
Ester value (mgKOH/g)	46.75

4.2 FT-IR measurements of the beeswax sample and the fractionated compounds

Figure (1) represents the FTIR spectrum of the beeswax sample. As can be seen from the spectrum, the main absorption peaks are appeared at 2918 cm^{-1} , 2849 cm^{-1} , 1736 cm^{-1} , 1464 cm^{-1} , 1175 cm^{-1} , and 729 cm^{-1} . These wave numbers could be attributed to the stretching and bending vibrations of C-H symmetry, C-H asymmetric, aliphatic -C=O stretching, C-H bending, aliphatic C-O stretching, and CH_2 rocking respectively. It could be conclude that the beeswax sample contains different functional groups (various chemical compounds) which is indicative to its structural complexity. Similar results were reported by Prava *et.al*. Their results demonstrated that the beeswax is heterogeneous in nature [26].

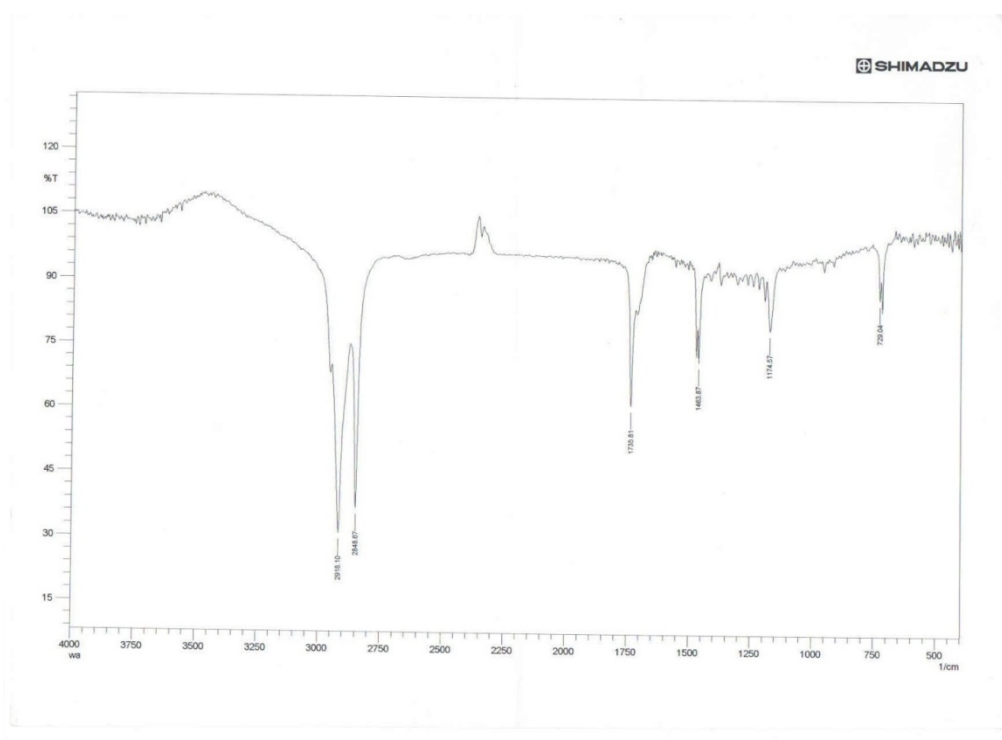


Figure 4.1: The FT-IR spectrum of the beeswax sample

Figure 4.2 represents the FT-IR spectrum of (n-hexane) fraction which is probably hydrocarbons according to the polarity of the solvent that was used (n-hexane) in the fractionation process, polarity of the silica gel and the polarity of the beeswax components. The comparison between figure 4.1 and 4.2 shows clearly the absence of the carbonyl and the -C-O absorption peaks and many other weak absorption peaks in the range of $1300\text{ to }1000\text{ cm}^{-1}$. The main absorption peaks in this spectrum are at

2916 cm^{-1} , 2840 cm^{-1} , 1463 cm^{-1} , and 719 cm^{-1} respectively. The absorption peaks at 2916 cm^{-1} and 2840 cm^{-1} are attributed to the stretching vibrations of SP^3 $-\text{C}-\text{H}$ whereas the one at 1463 cm^{-1} is due to the bending vibration of $-\text{C}-\text{H}$. Finally, the peaks at 719 cm^{-1} is due to the rocking vibration of $-\text{C}-\text{H}$. It has been reported in the literature [40] that the main absorption peaks of hydrocarbons are at 2870 cm^{-1} for asymmetric C-H stretching while at 2960 cm^{-1} for symmetric stretching. On the other hand, CH_2 shows asymmetric stretching at 2930 cm^{-1} and symmetric stretching at 2850 cm^{-1} . C-H bending gives rise to bands in the region below 1500 cm^{-1} , and CH_3 produces two bending bands, symmetric at 1380 cm^{-1} and asymmetric at 1470 cm^{-1} . Finally CH_2 give rise to four bending vibrations scissoring at 1465, rocking at 720 cm^{-1} , wagging at 1305 cm^{-1} and twisting at 1300 cm^{-1} . It is obvious from these facts that fraction 1 (figure 2) is pure hydrocarbons.

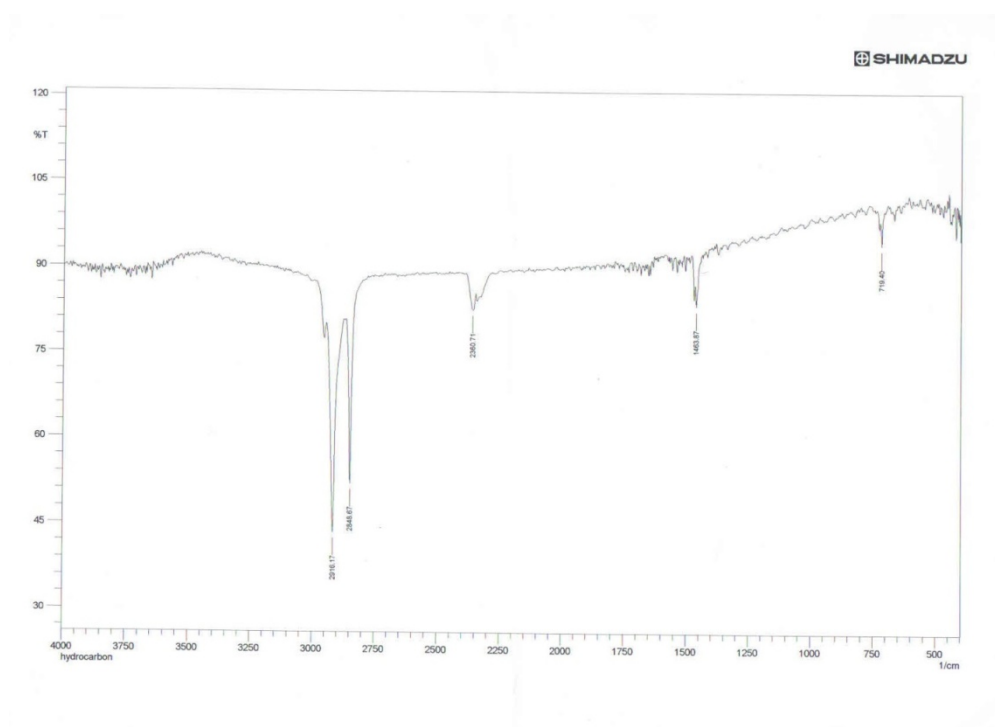


Figure 4.2: The the FT-IR spectrum of (n-hexane) fraction

4.3 HT-GC/FID analysis of the hydrocarbons fraction

In this study high temperature gas chromatography (HT-GC/FID) technique was used to determine the hydrocarbons in the beeswax sample. The results are tabulated in table 4.1. As can be seen from the table, both odd and even numbered hydrocarbons

(non-branched) are present in the sample. The most important observation from these results is that this the first time to report the presence of the even numbered hydrocarbons from C42 to C50. Obviously, the even numbered hydrocarbons are the dominant ones (approximately 52% of the total sample). Moreover, C27 hydrocarbon shows the highest percentage which is followed by C48, C42, C46, C44, C25, C31, C50 and C29. The percentages of C23 and C33 were found to be less than 2 %.

Table 4.2: The GC/FID results of the fractionated beeswax hydrocarbons

Component name	Area	Area%
n-C23	17058	1.922
n-C25	72442	8.164
n-C27	157957	17.80
n-C29	52284	5.892
n-C31	67280	7.582
n-C33	13768	1.552
n-C42	114549	12.91
n-C44	81759	9.214
n-C46	91649	10.329
n-C48	126780	14.288
n-C50	54595	6.153

These results showed some variations with the ones reported by Maia and Nunes [25]. They used high temperature gas chromatography technique coupled to mass spectroscopy (HT-GC-MS) for identification purposes and HT-GC-FID for quantification purposes to characterize beeswax. Their results showed the presence of odd and even numbered hydrocarbons. Odd number hydrocarbons with 17 to 35 carbon atoms were detected with the main hydrocarbon being heptacontane (C27), followed by nonacosane (C29) and hentriacontane (C31). Compared to odd numbered

hydrocarbons, the even numbered hydrocarbons (from 22 to 34) have shown lower percentages.

Other studies[23,45] have reported the presence of hydrocarbons as odd chain n-alkanes (C23-C31) are the predominant hydrocarbons in beeswax with heptacosane (C27), nonacosane (C29), hentriacontane (C31), pentacosane (C25) and triocosane (C23) being reported as the most abundant.

4.4 Differential scanning calorimetry (DSC) of the fractionated hydrocarbons

DSC analysis was carried out to examine whether the fractionated hydrocarbons are crystalline or amorphous in nature. The DSC cooling and melting curves for the fractionated hydrocarbons are shown in figure 4.3 and 4.4. As it appears from the DSC thermogram two melting peaks were observed at around 50 °C and 57 °C respectively.

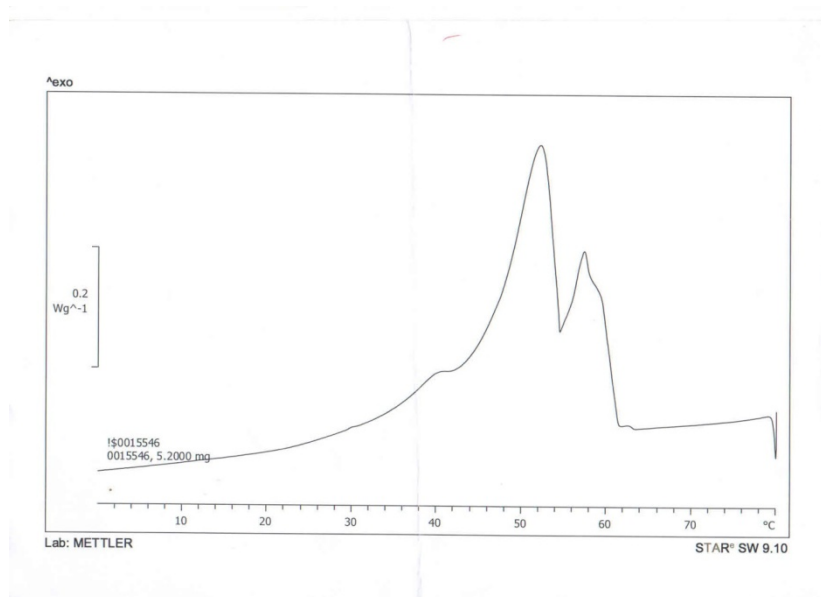


Figure 4.3: The DSC heating curves of the (n-hexane) fraction

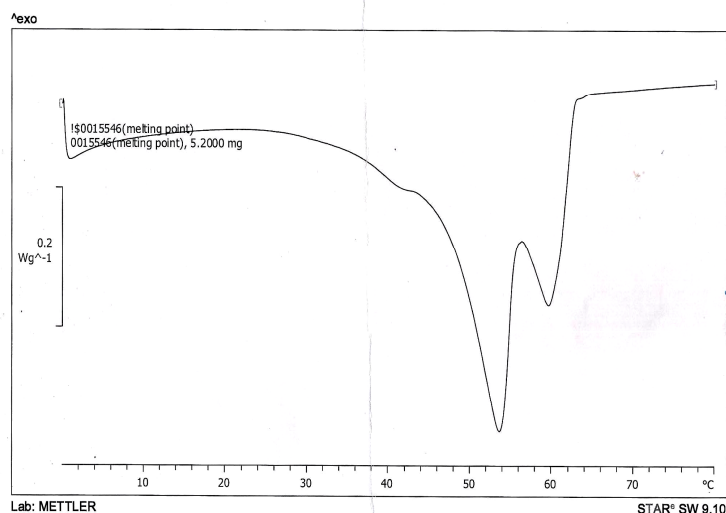


Figure 4.4: The DSC cooling curves of the fractionated beeswax.

The presence of a number of melting peaks for n-alkanes was extensively studied by Webber and Hutton[55]. They concluded that polymorphism is the main reason behind this phenomenon in n-alkanes (also in mixtures of n-alkanes and low-melting paraffin waxes). Depends on the number of carbon atoms and whether the hydrocarbons are odd or even numbered, different crystallite forms such as orthorhombic, monoclinic, and triclinic have been reported. Both even and odd numbered hydrocarbons were present in our sample (see table 4.1) and hence the two peaks most probably represent melting peaks of two different crystalline forms.

Conclusion

Beeswax is one of the important naturally occurring waxes. It has found diverse applications in many fields. It is used in pharmaceuticals, cosmetics, polymer composites, and food additives represent only some examples of its wide applications. Its applications could be further improved by utilizing each component of its mixture for specific purpose instead of using the whole wax. Fractionation and characterization of hydrocarbons from a beeswax sample was conducted in this study. Nonpolar solvent system (n-hexane) was utilized for elution of the hydrocarbons via a silica gel column chromatography. Almost pure hydrocarbons absorption peaks were noticed from the FT-IR spectrum of fraction 1. Eleven hydrocarbons (both even and odd-numbered hydrocarbons) were determined by GC/FID and thermal analysis has shown that these hydrocarbons are crystalline in nature.

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