A Thesis

entitled

ISOLATION AND CHARACTERIZATION OF ACTIVE INGREDIENTS OF "BETEL NUT" (ARECA CATECHU)

Presented by

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in part fulfilment of the requirements
for the degree of
MASTER OF PHILOSOPHY

DEPARTMENT OF CHEMISTRY
BANGLADESH UNIVERSITY OF ENGINEERING & TECHNOLOGY
DHAKA

TO MY PARENTS

THESIS APPROVAL SHEET

Thesis entitled " ISOLATION AND CHARACTERIZATION OF ACTIVE INGREDIENTS OF DETEL NUT (ARECA CATECHU) " by Mrs. Tahmina Sultana is approved for the degree of MASTER OF PHILOSOPHY.

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ACKNOWLEDGEMENTS

I would like to express heartiest gratitude to my Professor Enamul Huq, Head, Department of Chemistry, Bangladesh University of Engineering and Technology, Dhaka, for his indispensable guidance, keen interest and thoughtful suggestions during the progress of my research work. I would also like to thank Dr. Nazrul Islam, Assistant Professor, Department of Chemistry, BUET, Dhaka, for his guidance, suggestions and advice during my research work.

I would also like to thank Professor Mesbahuddin Ahmed, Department of Chemistry, Jahangirnagar University, Savar, Dhaka. for his kind co-operation in taking pmr, ir and Mass Spectra of the samples.

Thanks are also due to Dr. Monowarul Islam, Dr. Monimul Huq, Associate Professors, and Mr. Nurul Islam, Mr. M.A.Rashid, Assistant Professors, Department of Chemistry, BUET for their kind co-operation and advice during research work.

I also owe to my husband Dr. Harun-Ar-Rashid, for his constant co-operation during this long working period.

I express my sincere gratitude to the Bangladesh University of Engineering and Technology, Dhaka, for the grant.

Finally, I would like to thank the teachers and staff of this Department, for their encouragement and cordial co-operation.

Sultana 1.8.90.
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ABSTRACT

The work is divided into three chapters. The first chapter is entirely devoted to general reviews regarding the necessity of research of the indigeneous fruit Areca Catechu(Betel nut). This part also deals with the detailed description of the fruit and the plant bearing it. Besides, the medicinal use of the fruit was also discussed in detail. The objective of the project is also included in this chapter.

The second chapter contains the interpretation and discussion of the results of the isolated products from Betel nut. The petroleum ether extract and the elthanol extract were subjected to systematic study and from them the following compounds were isolated and characterized: a mixture of hydrocarbons, mixture of higher fatty acid esters. All the above compounds were characterized by ir, pmr, and mass spectra. The extensive use of modern methods in mass spectrometry was taken up for the confirmation of the isolated compounds. GC-ms analysis and as well as mass spectral fragmentation pattern established the presence of two alkanes in the hydrocarbon mixture. The mass

spectra also revealed the presence of alkanes having even and odd number of carbon atoms. The fatty acid ester fractions were proved to be a mixture of ethyl esters of fatty acids by its pmr spectra and also by its mass spectral fragmentation pattern.

The third chapter illustrates all experiments performed in this thesis work.

CONTENTS

CHAPTER I

INTRODUCTION

1.1. General Introduction

Arecanut also known as Betel nut is the fruit of Areca Catechu Linn. A small-genus, comprising about 20 pieces of slendor palms. The genus is essentially Indo - Malayan, distributed in tropical Asia, New Guinea and tropical Australia. Four species are known to occur in India and one is endamic in Srilanka(A. Concinna Thw.). A Catechu yields the areca or Betel nut. The seeds of A. Concinna, A. nagensis Griff. (Naga hills, Assam), and A triandra Roxb., (The Andaman Islands and Sumatra) are occasionally used as substitutes for Areca nuts.

Betel nut, an indigenous fruit, is very well known for its different types of medicinal effects. The changes which occur successively on the maturity of the Betel nut are of physiological interest and of biological importance in the nitrogen metabolism in the plant.

The Areca plam is considored to be a native of Malaya, where it is cultivated on an extensive scale. It is found throughout the East Indies and the Philippines. In India, it is cultivated in the coastal regions of Southern Bombay and Madras, Mysore, Bengal and Assam. Although it is a maritime species, it thrives in areas up to 250 miles from the coast and at altitudes up to 3,000'. It is also grown in Srilanka and Burma and it has been extended to Madgascar and East Africa.

The fruit is generally ovoid, about 1½ - 2" across, and 2 - 2½" long, and is brightly orange when fully ripe. The pericap (65%) is hard and fibrous, and the kernel (seed 35%), called the Areca nut, is about 1 - 1½" in diameter and greyish brown in colour. It is hard and has reddish brown lines because of its ruminate albumen. The fruits and nuts vary greatly in size and shape.

The hardness and astringency of the nuts also show considerable variatious. Some nuts are large, flat, and are almost bitter. Others are conical or spherical and so bland in taste as to be called 'sweet Areca nuts'. There are also nuts which produce tightness in the throat and profuse secretion of mucus. These are not restricted to any particular locality, and occasionally good and bad nuts are found on the same tree.

These and other differences are probably of a varietal character, or, due to the influence of soil and climate.[1]

This palm requires a moist tropical climate for luxuriant growth and is very sensitive to drought. It thrives in areas with heavy rainfall (200 inches) provided drainage is good, and also in drier areas (20 inches), if suitably irrigated.It is a shade Loving Plant, especially in earlier stages.Sometimes it is also grown in the midst of mango, jack, and guava trees.

Each tree yields 2 - 3 bunches a year, containing 200 - 250 fruits, weighing 3.2 - 4.9 lb. per 100 fruits. The yield per acre, with 400 trees in bearing, is 160,000 - 300,000 fruits or about 6 - 10 cwt. of dried Areca nuts.

Areca nut is used either raw or cured. The latter kind is used mostly in Southern India, and the former, in the rest of the country. For marketing raw nuts, only ripe fruits are collected. They are then husked, cut into two, dried and the half-nuts are removed from shells. Sometimes ripe fruits are dried in the sun for six to seven weeks and marketed as such and peeled before use.

The curing of nuts is a speciallity of some parts of India. Curing improves the colour of the nuts, their taste and keeping quality and it brings them to the correct degree of palatability by removing excess of tannin and mucilage. The percentage of tannin in raw nuts is 21.6 - 30.2 and in the cured nuts, 8.6 - 15.1. At the same time, during curing the nuts acquire a uniform colour by infusion of tannin into mottled parts and become sufficiently soft and tender for chewing.

Varieties of cured nuts are found in the market. Batlu (Plate) variety consists of kernels cut across into two halves. Pieces from embryo ends fetch a higher price. Chooru (pieces) consists of karnels sliced into smaller pieces and the best of them is lavanga chooru, which resembles cloves. Idi (whole) is from nuts boiled witbout slicing.

Areca nut is extensively used as a masticator throughout India, Burma, Srilanka and Malaysia. It is generally chewed along with pan (Leaves of piper betle) and a little slaked lime, to which katha (from acacia catechu), spices and tobacco are simetimes added.[2]

Ready-made chewing preparations like these, called beedas are sold in the bazar. Chewing develops salivation and the saliva is coloured red. It is supposed to prevent the decay of teeth,but its continued use blackens and loosens them. The fresh nuts have intoxicating properties and produce giddiness. The juice of tender nuts, in small doses, acts as a laxative. The burnt nut is used as a dentifrice.

A large quantity of husks is obtained during the preparation of kernels for the market. The husk contains 47.6% of cellulose on the dry basis and is composed of a mass of weak wolly fibers, mixed with coarser and stronger bristle-like fibers. Attempts at the utilization of husk for the preparation of paper have not proved successful. They may perhaps be employed with longer-fibred materials for the production of low grade-brown papers and cardboard.[3] The decomposition of husk in the soil is extremely slow, and even after a year the highly lighified fibres do not rot. They form a good mulching material.

A. catechu is one of the most handy forms of wood in rural parts. Its stems are used in various ways in house-construction;

pillars, joists, reapers, etc. The petioles, of the large expanded sheaths at the lower ends of leaves, are commonly used as packing material and for making kottes to protect the inflorescence, against excessive moisture. The spathe is tough and impervious to water and finds several domestic uses. The inflorescence is used in ceremonies on auspicious occasions. Cooking of A. catechu with a liquor of 19.0% active alkali as Na_2O for 2.0 h at 170^{o} gave pulp in 37.0% yield, with 2042H - factor and 27.6 kappa no. Fibre fractionation data revealed that fines fraction is high and fibre length is low in comparison to hardwood pulp. Physicomechanical properties of Areca Catechu pulp were more or less comparable to those of hardwood pulp.[4]

The preparation of pan acting chemically upon the saliva, colours it red. A decoction of the nut is used in dyeing and a kind of inferior cetechu is prepared from it. With tu'n (Cedrela Toona) it is said to give a red dye. Pan is also used in Dinajpur as a subsidiary in red dyeing with Morinda Tinctoria.

The spathe which covers the flowering axis may be used for paper making and so also might the fibrous pericap which is removed from the nut. The spathes are largely used in India for packing and in the preparation of small articles for personal use.

Young nut is said to possess astringent properties and is prescribed in bowel complaints and bad ulcers. Betel nut - a favourite stimulant in South Asia was investigated by Ernst.[5] It contains a large proportion of tannic and gallic acids, and hence its astringent property. Of the alkaoids of Areca nut, arecoline is the only one which exhibits toxic properties. It acts on the central and peripheral nervous system, producing paralysis which may be preceded by convulsions. Its hydrobromide is recognised by some continental pharmacopoeias, and is given hypodermically (dose, 1 grain) as a cathartic for horses. It is also employed as a taenicide in dogs in oral doses of $\frac{1}{10}$ - $\frac{1}{2}$ grain. The burnt nuts when powdered from an excellent dentifrice. It has also been found very useful in urinary disorders, and is reported to possess aphrodisiac properties. The dried nuts when chewed produce stimulant and exhilarant effects on the system.

The powdered seeds have also long been held in some reputation as an anthelmintic for dogs and Areca has now been introduced into the British pharmacopaeia on account of its supposed efficacy in promoting the expulsion of the tape-warm in the human subject. It is also reputed to be efficacious against round warm (Ascaris Lumbricoides).[6]

The nut is regarded as a nervine tonic and emmenagogue, and is used as an astringent Lotion for the eyes. The juice of the young leaves mixed with oil is said to be used externally in lumbago. The dry expanded petioles may be used as ready-made splints.

"Is useful in checking the pyrosis of pregnancey, control experiments; made with tincture of catechu showed the superiority of the nut would seem to demonstrate that this is not merely due to astringent action; possibly its property as a nervine stimulant enhances its utility. It is also used as an astringent for bleeding gums.

The nut is one of the indispensible ingredients which enter into the preparation of the pan or betel leaf, which is chewed so universally by natives of all classes. It is said to stimulate digestion, small pieces of the prepared Betel nut are rolled up with a little lime, cathecu, cardamons, cloves and even rose water with in the betel pepper leaf. This combination forms the pan which gives to the lips and teeth the red hue which the native admire. In the course of time, it has the effect however, of colouring the teeth black, at least along the edges, thus destroying the appearance of the teeth. The chewing of pan is supposed to prevent dysentry. It is said to dispel nausee, excite appetite and strengthen the stomach.

The green kermels yield an extract containing about 67% of tannin. Areca nuts contain: moisture, 31.3 ; protein, 4.9; fat (ether extract), 4.4; carbohydrates, 47.2; mineral matter, 1.0; Ca,0.05; P, 0.13%; Fe, 1.5 mg/100 g. (Hlth. Bull., No 23, 1941,43,). The following conatants are recorded for the petroleum ether extract; m.p., 36 - 38° ; sp. gr./15°, 0.973 ; sep. val., 234.6; iod. val 12.3 (Jamieson, 130). It consists mostly of the glycerides of lauric (ca. 50%), myristic acids (21% - 29%) (Wehmer, I, 123).

Catechin, $C_{15}H_{14}O_6$, $4H_2O$, m.p., 96° (anhydrous m.p., 175°) has been isolated from the nuts.

Of the several alkaloids present in the nuts, the most active is arecoline, $C_6H_{11}O_2N$ (about 0.1%), a strongly alkaline, colourless and odourless liquid, b.p., 220° [7]. The others are arecaidine, $C_7H_{11}O_2N$ guvacoline, $C_7H_{11}O_2N$, guvacine, $C_6H_9O_2N$, etc. According to continental pharmacopoeias, the seeds should contain not less than 0.4% of alkaloid calculated as arecoline.

The fatty acid composition of Arecanut[8] fat was determined by the usual esterification method, using an electrically heated and packed column for fractional distillation under high vacuum (0.2 mm). The glyceride structure was studied by crystallization of the neutral fat from acetone and ether; the composition of each of these glyceride fractions was studied by the fractionation method and the final possible glyceride composition computed therefrom. The Chief component acids are lauric (19.5%), myristic (46.2%) and palmitic (12.7%), and in the unsaturated portion oleic (6.2%), linolic (5.4%) and hexadecenoic acid (7.2%). Minor proportions of stearic, decanoic and of unsaturated monoethylenic C_{12} and C_{14} acids are also present.

The glyceride composition follows closely Hilditch's rule of widest distribution of acyl radicals in the glyceride molecules.

The fully saturated glyceride content of the fat, determined separately by the method of Hilditch and Lea[9], is 53.7%. Triglycerides of Arecanut were examined[10] at 5 stages of maturation, during which oil content increased from 1.8 to 14.5%. Similarly, coconuts were sampled at 3 stages of maturation (63.8 - 70.8% oil content). In Arecanut, ripening was associated with an increase in saturation from 35 to 85 mol% . In ripe nuts, the proportions of fully saturated and fully unsaturated triglycerides were greater than predicted by the random distribution rule. During coconut ripening, fatty acid saturation increased from 86 to 97 mol%, fully saturated and fully unsaturated triglycerides species in ripe coconuts were again present in greater amounts than predicted by the random distribution rule.

The tannin content of Arecanut [11,12] is about 8-15%. The Areca tannins are purely of the condensed type; the leathers tanned with them are found to be good in quality except for the defect of red colour developing with time. This mey be due to the presence of leucoanthocyanidins in the Arecanut tannins.

Similar observations have been made by Hillis [13,14,15] Vis-a-

-Vis Eucalyptus species. The nuts collected from vittal (South Kanara, Mysore State), one of the centres of Arecanut cultivation, have been investigated with reference to the polyphenolic constitutes. D-catechin and a leucocyanidin (5: 7: 3': 4' - tetrahydroxyflavan - 3: 4 diol) have been isolated.

The polyphenols[16] of Arecanut have been examined by use of paper chromatography and specific spray reagents. Besides (+) - catechin and leucocyanidin, a monomeric and some polymeric leucocyanidins have been shown to be present. The data on the high yield of the cyanidin from the polymeric compounds suggest that they contain readily cleavable linkages. Quantitative estimation of the different components shows a preponderance of the polymeric flavan - 3: 4 - diols, yielding cyanidin.

(+) - Catechin was reported to be present by Yamamoto and Muraoka[17] and has recently been isolated by Seshadri and Nagarajan[18]. The present of Leucocyanidin was indicated by Bate - Smith and by Sastry et.al[19-21] recently. Lecocyanidin has been isolated and identified by means of its derivatives and its physical properties. The latter workers indicated that other more complex polyphenolic substances are also present.

Vasoconstrictor effects of some extracts of A. Catechu have been described earlier[22]. It was also suggested that these actions may be due to the polyphenolic constituents of the nut. Since many polyphenols are known to potentiate the actions of adrenalin in vivo[23], the influence of Areca extract on adrendalin induced vaso-constriction has been studied.

The protein, fat, carbohydrate and tannins contents of Arecanut have been earlier recorded . The qualitative and quantitative analysis of the amino acid makeup of A. Catechu was reported. According to their communication the Arecanuts contain a large number of amino acids both in free and combined state. The changes which occur successively on maturity are of physiological interest and of biochemical importance in the nitrogen metabolism of the plant[24].

The oxytocic value of a given plant (Ecbolic properties of Indian Medicinal Plants) serves as an index of its proable ecbolic, including abortifacient and emmenagogue properties. Experimental results based on the very high oxytocic properties indicated that there is much scientific truth behind this practice to bring about abortion by local application of latex of green fruit or decoction of runipe seed. Oxytocic properties of crude extractions of plants[25] studied were ; Latex of calotropia gigantea 0.0027, twigs of Artemisia vulgaris 2.2, Arecanut (Catechu) 2.4, Allium cepa bulb 2.9, and Allium Sativum bulb 31-50 mg.= 0.003I.U. of oxytocin.

By using the principles of comparison and interpretation as described by Burn et.al[26], quantitative estimation of the oxytocic value of each crude drug (extract/emulsion) was determined. Experimental results shown that it has very low oxytocic properties (as much as 2.4 mg. of the drug is found to be equivalant to 0.003 I.U. of oxytocin.)

A specimen of Arecanut fat[28], 78-mol.% saturated acids, contained 54.9 fully saturated triglycerides (GS_3), 8.1 mol.% tri-unsatureted glycerides (GU_3) and for partly unsaturated triglycerides, 32.5 mol.% GS_2U and 4.7 GSU_2 compared with 46.7, 1.1, 40.5 and 11.7 mol.%, respectively, expected for random distribution. The maximum proportions of GS_3 and GU_3 possible by any mechanism of esterification are shown to be the same as the random distribution values. The unuaual structure of the fat is hence due to high order compositeness, i.e. it is produced by admixture of fats of widely differing saturated acid contents from different cells of the same tissue. Published data on the biogenesis of fat in ripening Arecanut support this concept. This heterogeneity was not evident from examination of the tissues.

Preliminary separation of the complex proanthocyanidins[29] has been made possible by solvent fractionation using ethyl acetate and ethyl acetate containing ethyl alcohol. Subsequent paper chromatographic separation has yielded good results in the estimation of individual components. Besides leucocyanidin, leucopelargonidin and catechin have been detected in polymeric components.

The polyphenols of Arecanut were studied by paper chromatography. The simple polyphenols separate well and have been identified as (+) Catechin and (+) Leucocyanidin[30-32]. However, during Chromatography, there was overcrowding of the areas that represented complex polyphenols, which constituted the major bulk of polyphenols. In recent years, proanthocyanidins from many plant sources were shown to be dimers of catechin and leucocyanidin[33,34,35]. Later studies also revealed occurence in plants of dimers of two flavan 3:4-diols, although the nature of linkage was not fully determined[36]. In the light of these developments in the field, the nature of the polymeric polyphenols of Arecanut were examined. The high incidence of oral cancer in the far East is generally atributed to the habitual use of betel nut "Quids" which may contain, as well as betel nut,cured tobacco and various other organic and inorganic materials[37-40]. Attempts to extract from betel nut a material carcinogenic for oral tissues have not been successful[41, 42],;possibly because extracts were prepared with solvents in which the carcinogenic materials were not soluble. The choice of DMSO was based on the evidence that it is an excellent solvent with a low toxicity[43] that it has great powers of penetration[44] and that it enhances the absorption of verious drugs through skin and mucous membranés without apparently affecting their pharmacological properties [45 - 48]. The Kailash, Suri, et.al have found that repeated, topical applications of DMSO extracts of betel nut to the mucosa of the buccal pouch of hamasters result in the development of leukoplakia and tumours[49, 50]. A review of research of the author and others[51] on the possible carcinogenic effects of nicotine and its metabolites, especially continine and of betel nut (Areca Catechu) alkoloids and their metabolities, particularly areooline, arecaidine, guvacine, and guvacoline. While the research is incomplete, there can be no doubt that both tobacco and the Arecanut contain substances carcinogenic to man[52].

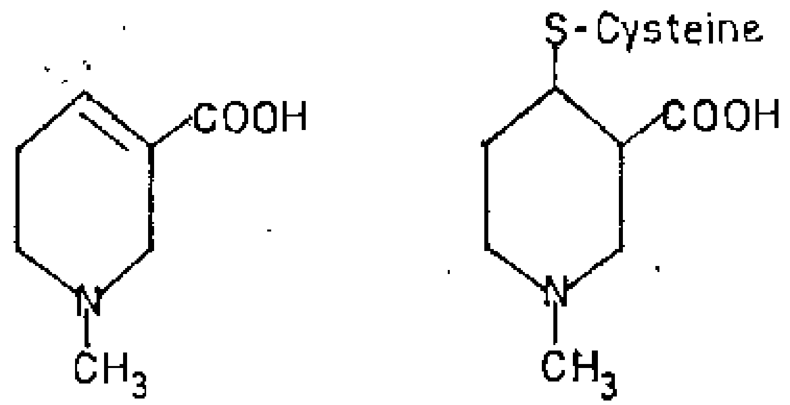
Willstattar and Stoll in 1928 determined chlorophyll contents of Ulvalactuca and number of different species of higher plante and found that the proportion of chlorophyll 'b' to 'a' was higher. Griffth et. al (1944), Arnon (1949), Ramakrishnan et.al (1969), Soni and Raudhawa (1969), Yadava (1969) and others have determined the pigments and organic acid contents from various species of different families and plants grown under a wide range of ecological conditions. No such data ere available for the chlorophyll and organic acid contents of Arecanut species grown in India.

Therefore, the present investigation were undertaken to determine the total chlorophyll 'a' and 'b' and organic acids in Arecanut species and the reaults are reported[53-62].

Among the species, A. trindra contained higher value of total chlorophyll while A. catechu has the minimum quantity. Restriction of GS_3 formation to a minimum in C_{16}-C_{18} acid vegetable seed fats has been attributed to the presence of a mechanism for prior selective esterification of position C-2 with C_{18} -unsaturated acids followed by random esterification of positions 1, 3 with remaining acids (2-unsaturated - 1,3 random rule)[63-64]. Two specimens of Arecanut fat later reported[65] were mixtures of fats randomly distributed.

Fats from ripening nuts of Arecanut (Areca Catechu L.) and coconut (Cocos nucifera L.) showed appreciable decrease in iodine value (from 100 to 36 and from 21 to 6 respectively), with progress in ripening. In Arecanut this decrease was accompanied by a change in the nature of the saturated acids from C_{16} - C_{18} acid type in the earliest stages to the C_{14} and lower in the later stages of ripening (Kartha and Narayanan, 1956, Kartha et.al, 1959).

The glyceride structures of Arecanut fats at different stages of ripening as determined by fully standardized oxidation procedures was reported[66]. The oils from ripening Arecanut were analysed for glyceride structure by the same procedure as used earlier for analysis of animal depot fats (Khan, 1972, Khan and kartha, 1974). The chewing of "Quids" composed of betel nuts (Areca catechu L.) lime, and occasionally, tobacco leaf, has been associated with an increased incidence of oral cancer in man. At least six reduced pyridine alkaloids are prsent in these nuts and of these, arecaidine and its methyl ester (arecoline) have received greatest attention as the possible carcinogenic agents[67]. Until recently, the only evidence to support this contention was that these alkaloids were present in the nuts and that each reacts with cysteine, both in vivo and in vitro, to produce a common cysteine B-alkylation adduct[68]. The cysteine adduct of arecolina has lost its methyl-ester grouping, an observation that is reflected in the ready hydrolysis by lime of arecoline to arecaidine. This indicates that arecaidine (fig.1) is probably a more likely carceinogenic principle than arecoline[69].



I II

Fig. 1 :- Structure of arecaidine (I) and its S-Cysteine adduct(II)

Two procyanidin tetramers,[70] two trimers, and a dimer which is a structural isomer of procyanidin B-1, along with (+)-catechin, (-) - epicatechin, and procyanidins A-1, B-1, and B-2, have been isolated pure from the seed of Areca Catechu L. and their ^{1}H and ^{13}C nmr spectral data, combined with degradative studies on their reactions with toluene - α - thiol, have established that they all, except for procyanidin B-2, have the C(4)-to-C (8) [or C (6)] - Linked (-)-epicatechin stereochemistry [C (2), C (3): Cis] in the upper units and the (+) - catechin stereochemistry [C (2), C (3) : trans] in the terminal (lower) units.

Fats from eight samples of Arecanuts, five mango Kernels and one each of phulwara and pisa Kernels were analysed for their characteristics and fatty acid composition by glc Glyceride composition of one each of the hard fats was determined by argentation tlc - glc Techniques.

Areca fat is a very light coloured hard fat having a slip point(S.P) of 39.5°C . The total saturated acid content(78.6-85.5% for the eight samples in the present study) is very high, and this is reflected in low I.V.(iodine value) of the fat (17.2 - 25.7%).

Inspite of the low I.V., significant amount of linolaic acid (3.3 - 8.4%) is present in the fat. The major saturated acid is myristic (46.2 - 52.5% which is followed by Laurio (15.9-20.2%) and palmitic acids (12.7 - 16.9%); predominanca of low molecular weight fatty acids results in high S.V. of the fat (224.8 - 229.2). The observed ranges[71] of characteristics and composition of eight samples of areca fats falls within the range of earlier reported values[72 - 90] but the present range is narrower. The odd chain fatty acids (17:0, 19:0, and 21:0) and some unsaturated acids (12:1, and 18:3) reported to be present in the fat by a few earlier workers could not be detected in the present study.

Among various tannin tested Areca II-5-C, a fraction isolated from seeds of Areca Catechu L.[91] , showed the most potent angiotensin-converting enzyme (ACE) (9015-82-1) inhibitory activity in vitro.Its antihypertensive activity was therefore investigated in normotensive and sponteneous hypertensive rats (SHR) after both oral and I.V. administration. The activity was compared with that of captopril, a potent ACE inhibitor. Oral administration of Areca II-5-C to SHR produced a lasting, dose related antihypertensive effect,and the responses obtained with doses of 100 and 200 mg/kg were comparable to these of captopril at dosee of 30 and 100 mg/kg. I.V. administrations of Areca II-5-C to SHR produced a rapid and marked reduction in blood pressure at doses of 10 and 15 mg/kg. The maximum hypertensive effect of Areca II-5-C in SHR, at an I.V. dose of 15 mg/kg, was about 5 times as large as that of captopril at the same dose.

Although the vasopressor reeponse to dl-norepinephrine and vaso depressor responses to brady kinin and acetyl choline were not appreciably changed by I.V. treatment with Areca-II-5-C at a dose of 5 mg/kg, it did produce dose -related inhibition of the pressor responses to angiotensin I and angiotensin II. It is suggeeted that Areca II-5-C has favourable properties as a hypotansive drug through its ability to inhibit the pressor responsea to both angiotensin I and II.

Total aquous extractione of ripe betel nuts[92] of unproceesed and processed varietias were administered to pregnant mice at dose levels of 1,3 and 5 mg/day/mouse through days 6-15 of gestation. The treatments resulted in increased resorptions as well as daad of fetuses. Fetal weight was advereely affected as indicated by the dose-related reduction in average body weight of live fetuses. No major Morphol., visceral and skeletal defects, apart from hematomas,curved tails and a few incidences of rib anomaliee, were observed. There was, however, a dose - related decrease in the number of fetuses possessing ossified coccygeal vatebrae and an increase in the no. of fetuses with unossified 5th metacarpels. This indicated a delay in skeletal maturity, particularly in those fetuses exposed prenatally to the betel nut extraction of the unprocessed variety.

The proton-induced X-ray emission (PIXE) method[93] was employed to study the concentration of mineral and trace elements in betel leaves (piper betel), Betel nuts (Areca Catechu) and mineral lime consumed in Bangladesh.

The concentration of 15 elements (K,Ca, Ti, V, Cr, Mn, Fe, Ni, Cu, Zn, Se, Br, Rb, Sr. and Pb) was measured by comparison with calibration curve constructed from the NBS orchard lead saturated SRM 1571.

Exfoliated mucosal cells were collected from the oral cavity of 3 groups at high risk for oral cancer. Indian Betel nut chewers, Phillipino inverted smokers (burning ends of cigarettes in mouth), and Indian Khainin Tobacco Chewers. DNA was extracted from these samples.

DNA was analyzed for the presence of aromatic DNA adducts using 32 p- post labelling analysis.

No adducts were found in high risk groups which did not also appear in control subjects was reported by Brucep et.al.[94].

The formation of reactive Oxygen species[95] (ROS) from Betel quid ingredients, namely Areca nut and tobacco, was studied using a chemi-luminescence (CL) Technique. Aqeous extractions of Areca nut were capable of generating super oxide anion and H_2O_2 at PH > 9.5.

In order to evaluated the effect of concurrent administration of Arecanut and sodium nitrite, a long term feeding study was conducted with 120 syrian hamsters.

The total tumor response nitrite together with Arecanut constituents appears to enhance the risk of developing malignancies[96].

New 5' -nucleotidase inhibitors[97] were isolated from the seeds of Areca Catechu.

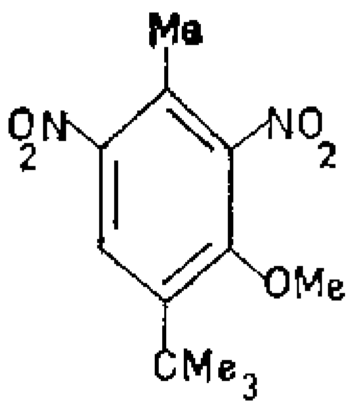
Four bioactive polyphenolic substances that inhibit the 5' -nucleotidase were isolated from the seeds of Areca Catechu. They are, polyphenolic compounds and inhibit 5'-nucleotidase from snake venom and rat liver membrane. They also showed antitumor activity as shown in same reference No.[97].

According to their physio chemical properties, the compounds are polyphenolic substances and showed significant - therapeutic activity.

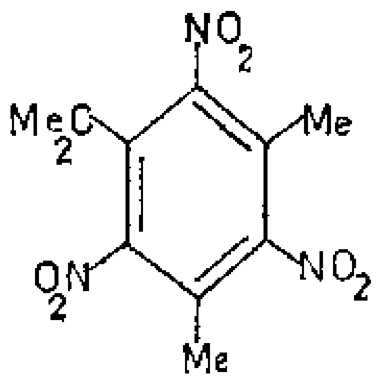
2- cyano ethyl diazohydroxide is a likely product of metabolic hydroxylation of 3 -(methyl nitros amino propionitrile (MNPN). The reaction of 2- (N-Carbethoxy-N-nitrosamino) propionitrile, a stable precursor of 2- cyano ethyl diazohydroxide, with deoxyguanoisine, catalyzed by porcine liver esterase, was investigated by Naylor Dana[98].

During N-nitrosamine analysis of extractions of betel quid[99] with tobacco and of the saliva chewers of betel quid with tobacco for N-nitrosamines using thermal energy analyzer, 2 unknown compounds were detected. They were indentified as synthetic nitro musks, musk ambrette (I) and musk Xylene (II), by gas chromatography, maes spectrometry and Fourier Transform NMR spectroecopy. These compounds were detected in several samples of betel quid with tobacco and in

Perfumed tobacco used for chewing in India in amounts ranging from 0.45 to 23.5 mg/g wet wight. Musk ambrette was mutagenic in salmonella Tryphimurium TA 100 requiring metabolic activation by ratliver postmitochondril supernatant but musk Xylene lacked mutagenicity.



I



II

A review with several references on methods of preventing oral cancer caused by chewing Betel nut, tobacco, or other materials was reported by Hans[100].

1.2. Objective of the Project

From the above review work which was carried out in this thesis work, it was revealed that, Areca Catechu (Betel nut) has been used as medicine to prevent dysentry and dispel nausea. The maturity of the Betel nut is of physiological interest and of biological importance in the nitrogen metabolism in the plant. Chemical investigatione on the fruit are quite appreciable as reported in the literature. It has been reported that Areca Catechu contain fatty acids, carbohydrates, catechin and several alkaloids.

Although investigation of the nut procured from the local market had been earlier reported[101] but complete and detailed spectroscopic analysis to elucidate the atructure of the isolated compounds was not much reported. With this end in view Areca Catechu linn was chosen for systematic study.

CHAPTER II

RESULTS AND DISCUSSIONS

2.1. Betel nut (Areca Catechu) an indigeneous fruit is very well known for its different types of medicinal effects. Recently, attention has been drawn to extensive investigation of its different solvent extractives to exploro its therapeutic importance. Although the presence of quite a number of triglycerides, alkoloids and other organic compounds present in Betel nut has been reported, but their presence in the fruits procured from our local market is less studied. It was, therefore, planned to carryout a systematic examination of the chemical constituents of Areca Catechu.

The Betel nut was procured locally and after drying in the sunlight was grinded to powder mechanically. This powder was used for all experimental purposes.

2.2. The percentage of moisture, ash and extractives were determined by standard methods as described in the experimental section. The results are given in Table 1 and 2. It is apparent from the table, that alcohol is more effective than petroleum ether for extraction. This is perhaps due to the presence of colouring and waxy materials which are easily extracted by alcohol.

Petroleum ether extract "C" on treatment with sodium bicarbonate solution (5%) resulted into two fractions "E" and "F".

2.3. Study of Fraction "E".

"E" was fractionated on silica gel 60(70-230 mesh ASTM) column (60 x 203 cm) as described in experiment No. 3.1.8 A pure fraction in substantial amount was isolated and identified with R_f value of 0.8 (pet-ether:ethyl acetate = 9:1). This fraction was labeled as compound T_1. The other fractions were found to be mixtures of two or three compounds having R_f values ranging from 0.98 to 0.37, 0.51, 0.74, 0.85(5:1), present in small quantity and hence further study could not be carried out.

2.4. Examination of Fraction "T_1".

Fraction T_1 (0.38%) was isolated as a light yellow waxy substance soluble in petroleum ether, chloroform, ethyl acetate. It gave only one spot on tlc plate (R_f 0.8 in pet-ether:ethyl acetate = 9:1). It showed strong ir absorption [Fig - 1] at ν_{max} 1750 cm^{-1} suggesting it to contain a ketonic group. Besides, other ir absorptions were observed at ν_{max} 2920, 2840 and 725 cm^{-1}

suggesting the presence of CH_3 and CH_2 groups. The pmr spectrum [Fig-2] of the compound showed a triplet in the methyl absorption region at δ 0.88 and a sharp singlet at δ 1.25, characteristic for methylene protons. There was a triplet at δ 2.3, characteristic for protons in the acyl portion of an ester and a multiplet at δ 4.28 showing the presence of protons of oxymethylene groups. The mass spectrum of the compound in general showed the presence of a mixture of two esters. The identification of the ester mixture was resolved by running a GC-Mass spectra of the compound on an OV-275 column at programmed temperature range. The presence of two long chain alkyl esters $CH_3(CH_2)_{31}COOCH_2CH_3$ and $CH_3(CH_2)_{20}$ - $COOCH_2CH_3$ were clearly established by this technique [Fig-3]. The molecular ions of the esters were established by running a CI mass spectrum of the mixture. The results were in complete accord with the mass spectra of the compounds which showed mass peaks at m/e 523 (M^{+} + 1) [Fig-4] and 367 (M^{+}-1) [Fig-5] their peaks are in agreement with molecular ions of the ethyl esters of tetratricontanoic acid $CH_3(CH_2)_{31}COOH$ and docosanoic acid $CH_3(CH_2)_{20}COOH$ respectively. Their fragentation showed pattern characteristic of an ethyl ester of long chain alkyl fatty acids.

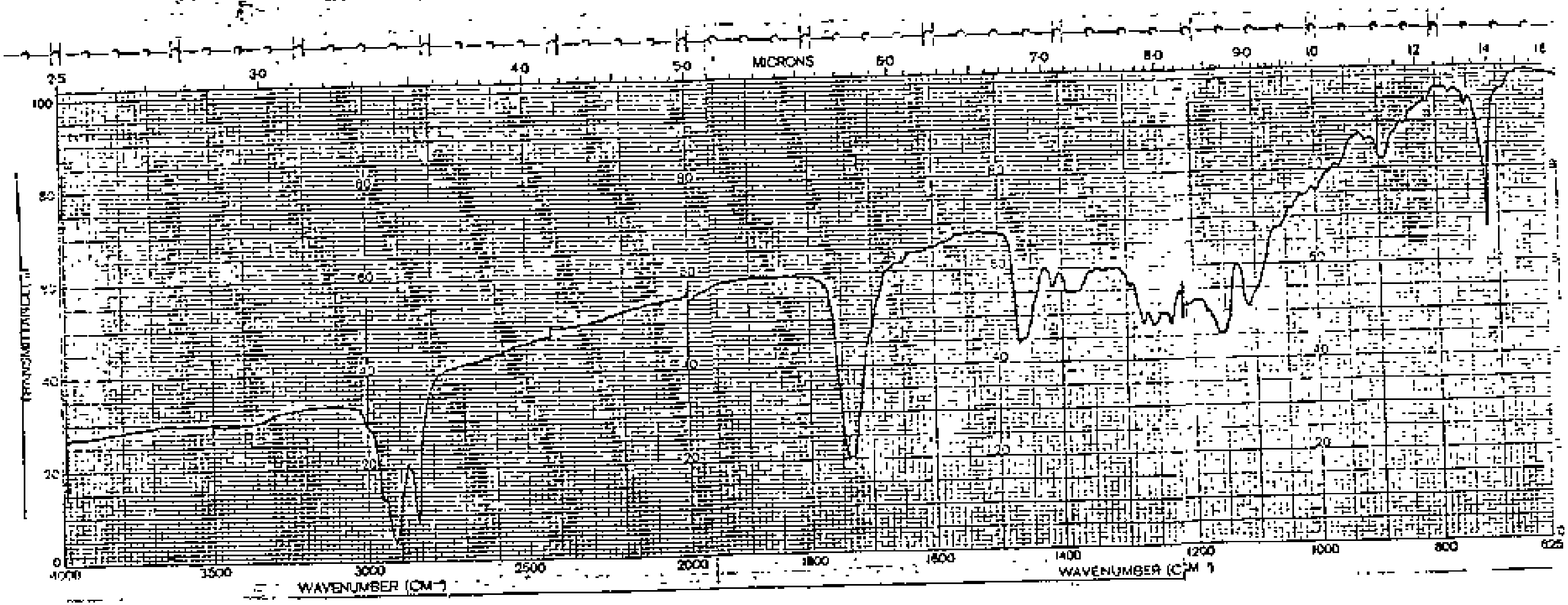
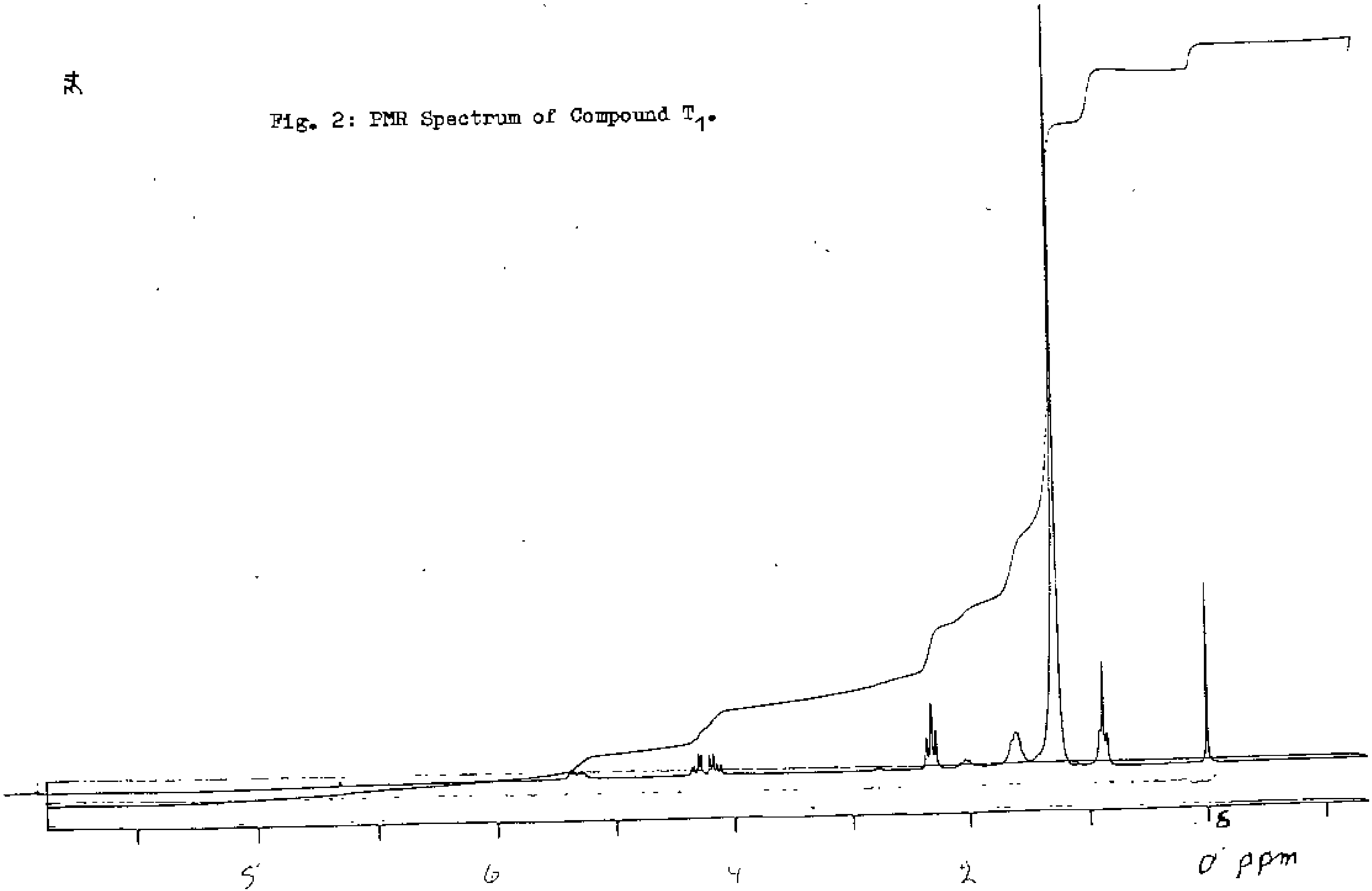


Fig. 1: Infra red spectrum of Compound T_1.

Fig. 2: PMR Spectrum of Compound T_1.



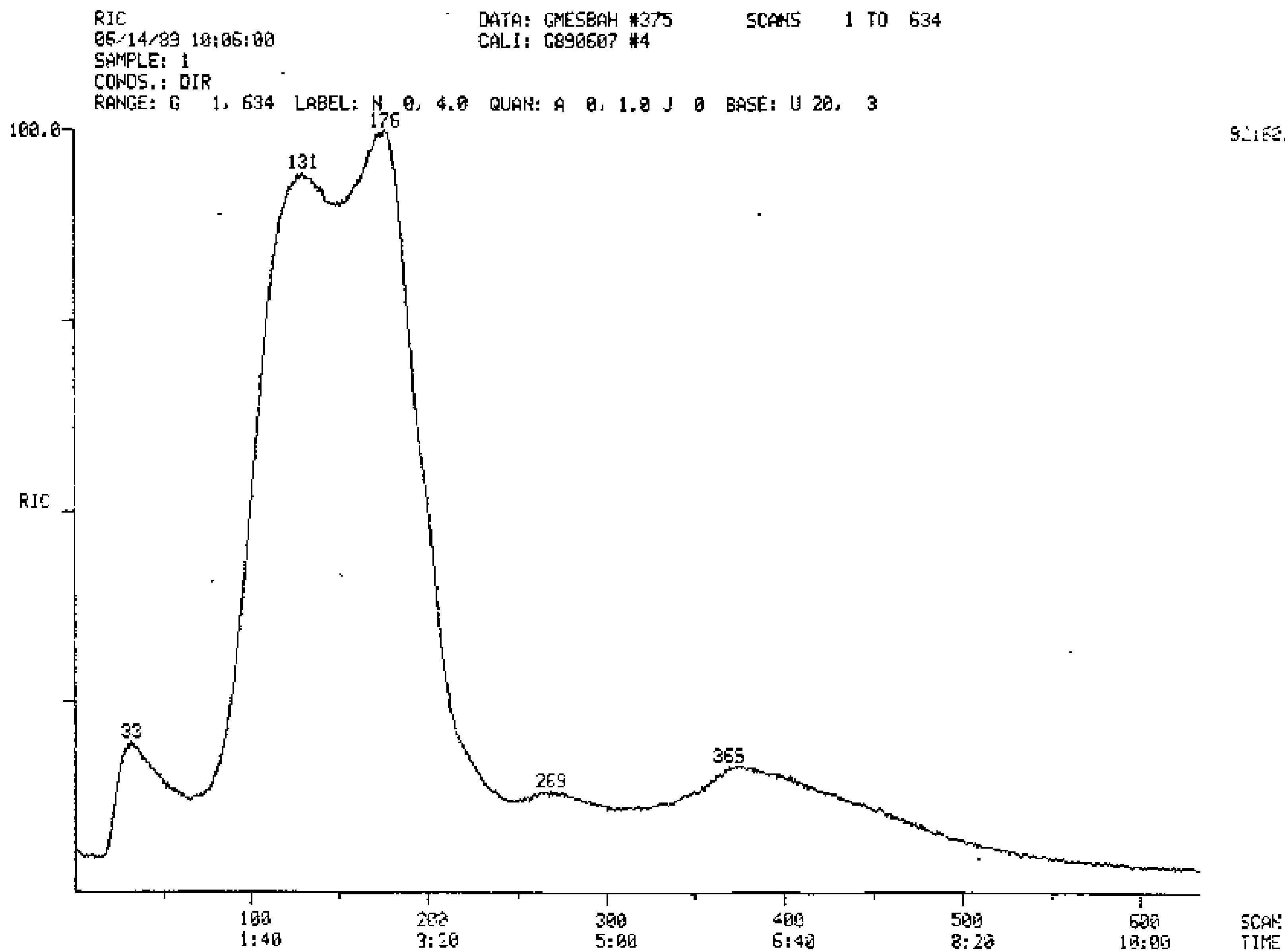


Fig. 3: GC-Mass spectra of Compound T_1.

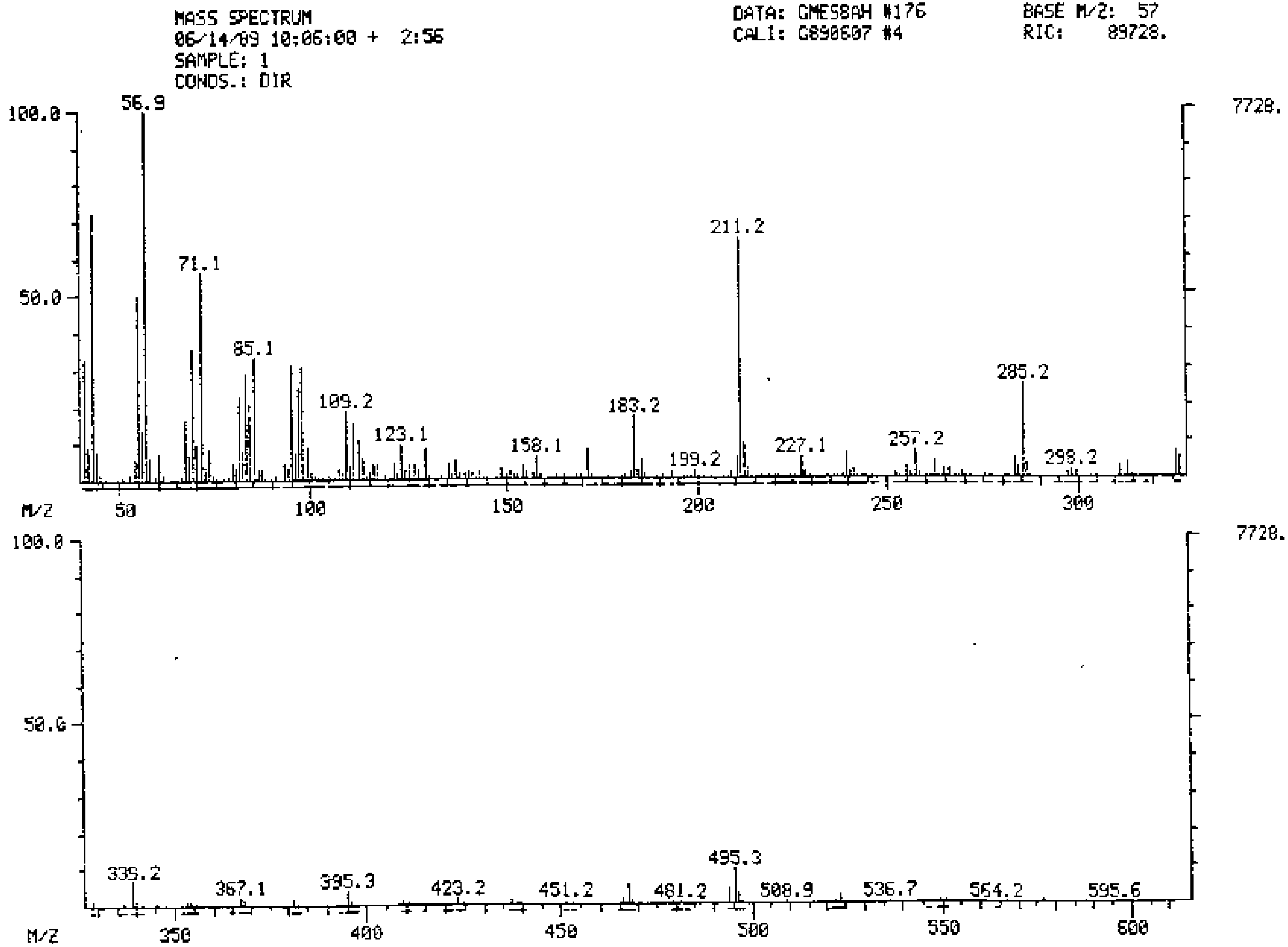


Fig. 4: Mass Spectrum of Compound T_1.

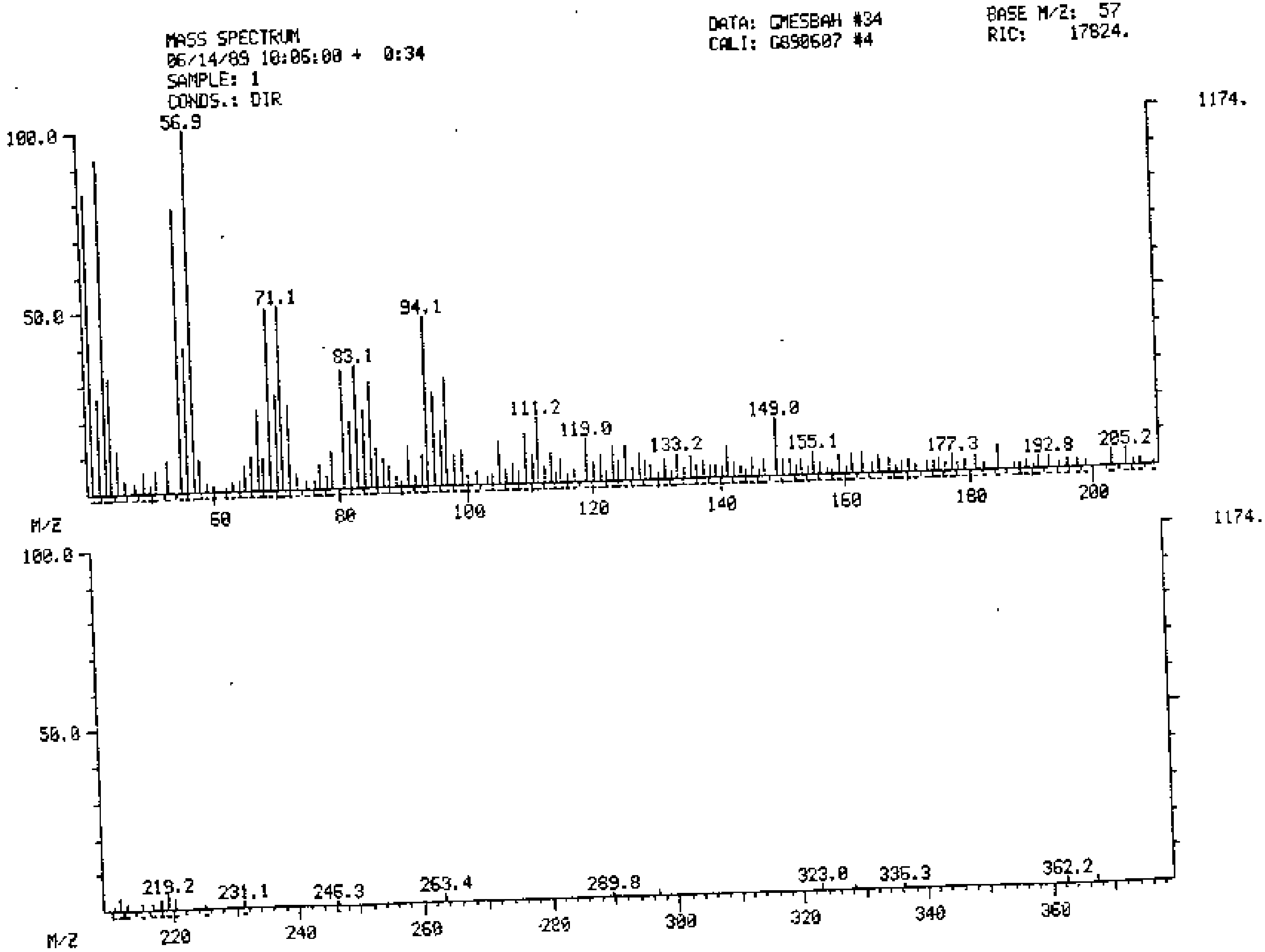


Fig. 5: Mass Spectrum of Compound T_1.

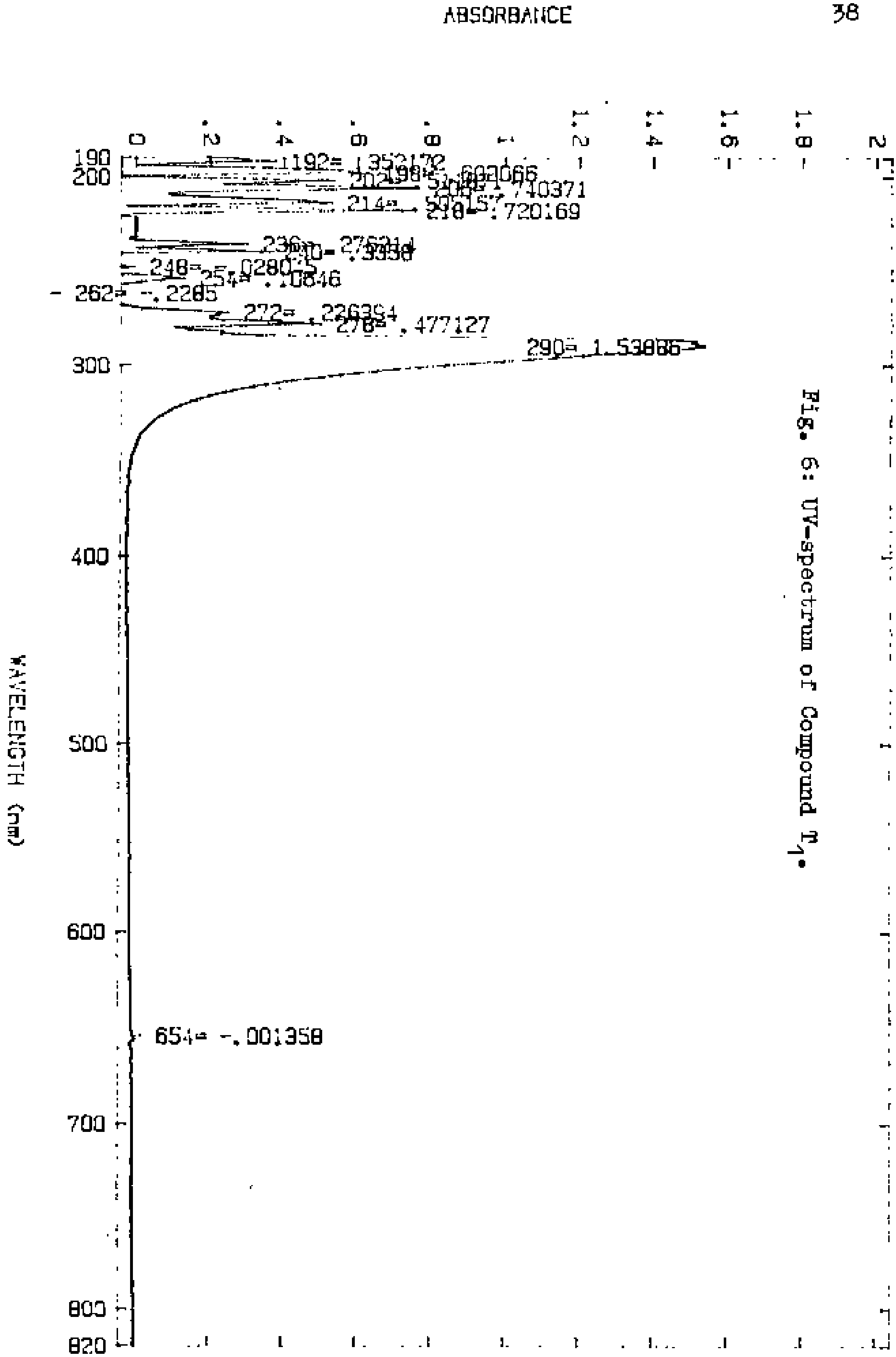


Fig. 6: UV-spectrum of Compound T_1.

The UV-spectrum [Fig - 6] of the compound showed an absorption in the near ultraviolet region with the maximum at λ_{max} 290 nm characteristic of a ketonic group of an ester. Thus, the compound T_1 appears to be a mixture of ethyl esters containing the $-\overset{O}{\overset{\|}{C}}-OC_2H_5$ group attached to an alkyl chain.

2.5. Study on Fraction "L".

Fraction "L" seperated from the alcohol extract on trituration, was dissolved in ethyl acetate and then run on a preparative thin layer chromatographic plate. On developement as described in section 3.1.14. four new fractions were seperated. One of the fractions was indicated as T_2 and the other as T_3.The fraction on the base was indicated as L_4.

2.6. Examination of Fraction "T_2"

Fraction T_2 (0.026%) was isolated as a yellowish white gummy substance soluble in ethyl acetate, chloroform, acetone, alcohol.It gave only one spot on tlc plates (R_f 0.55 in pet-ether: ethyl acetate = 24:1). The ir spectrum [Fig-7] of the fraction was taken in chloroform. It showed a strong ir absorption at ν_{max} 1740 cm^{-1} suggesting it to contain a C=O group of a long chain alkyl ester. Other important absorption bands were observed at ν_{max} 2920, 2850, 1460, 960 and 725 cm^{-1} suggesting the presence of methyl and methylene groups in the compound. The pmr spectrum [Fig - 8] of the fraction was apparently a mixture of two compounds. The pmr spectrum showed sharp triplet at δ 0.84 for protons of methyl groups. The sharp and strong singlet at δ 1.25 was characteristic for protons of methylene groups. Signals at δ 2.3 and δ 4.25 in the form of a triplet and multiplet respectively were characteristic of protons in the acyl part and oxymethylene part of an ester.

The weak multiplet at δ 5.26 showed absorption characteristic for vinyl protons of a compound.

GC-Mass spectra [Fig - 9] of the fraction T_2 showed two peaks. The molecular ion of the sub-fractions of fraction T_2 were established by running a mass spectrum. One of the sub-fractions showed a mass peak m/e 523 identical to the compound T_1 discussed as above in section 2.4 [Fig - 4]. The other sub-fraction showed highest m/e 285 ($M^{+\cdot}$ + 1). This peak is in agreement with molecular ion of the ethyl ester of palmitic acid $CH_3(CH_2)_{14}COOCH_2CH_3$ [Fig-10]. It's fragmentation showed characteristic pattern of an ester of a long chain alkyl fatty acid.

<u>Cleavage from the ester side:</u>

$$CH_3(CH_2)_{14}COOCH_2CH_3 \xrightarrow[-73]{-COOC_2H_5} 211 \xrightarrow[-14]{-CH_2} 197 \xrightarrow[-14]{-CH_2} 183\text{-etc}$$

($M^{+\cdot}$ + 1)284

<u>Cleavage from alkyl side:</u>

$$CH_3(CH_2)_{14}COOCH_2CH_3 \xrightarrow[-15]{-CH_3} 269 \xrightarrow{-28} 241 \longrightarrow \text{etc.}$$

($M^{+\cdot}$ + 1)284

The mass spectrum of the compound showed some peaks with different m/e values which could be due to the presence of some minor impurities.

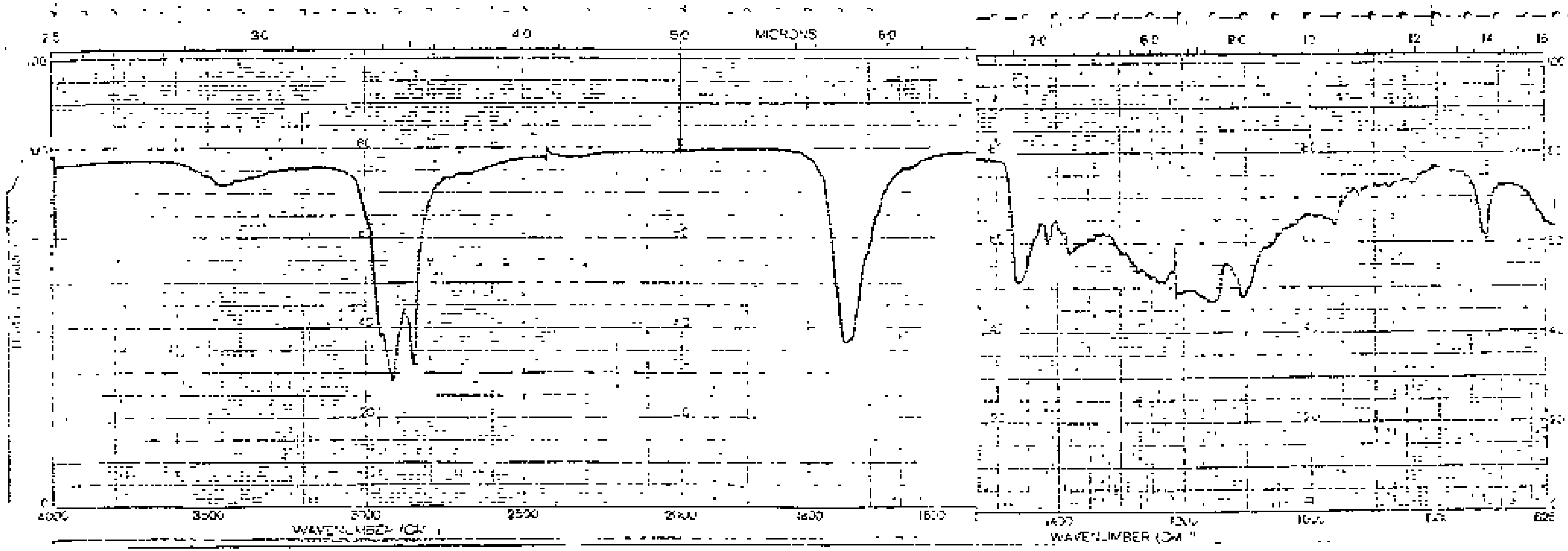


Fig. 7: Infra red spectrum of Compound T_2.

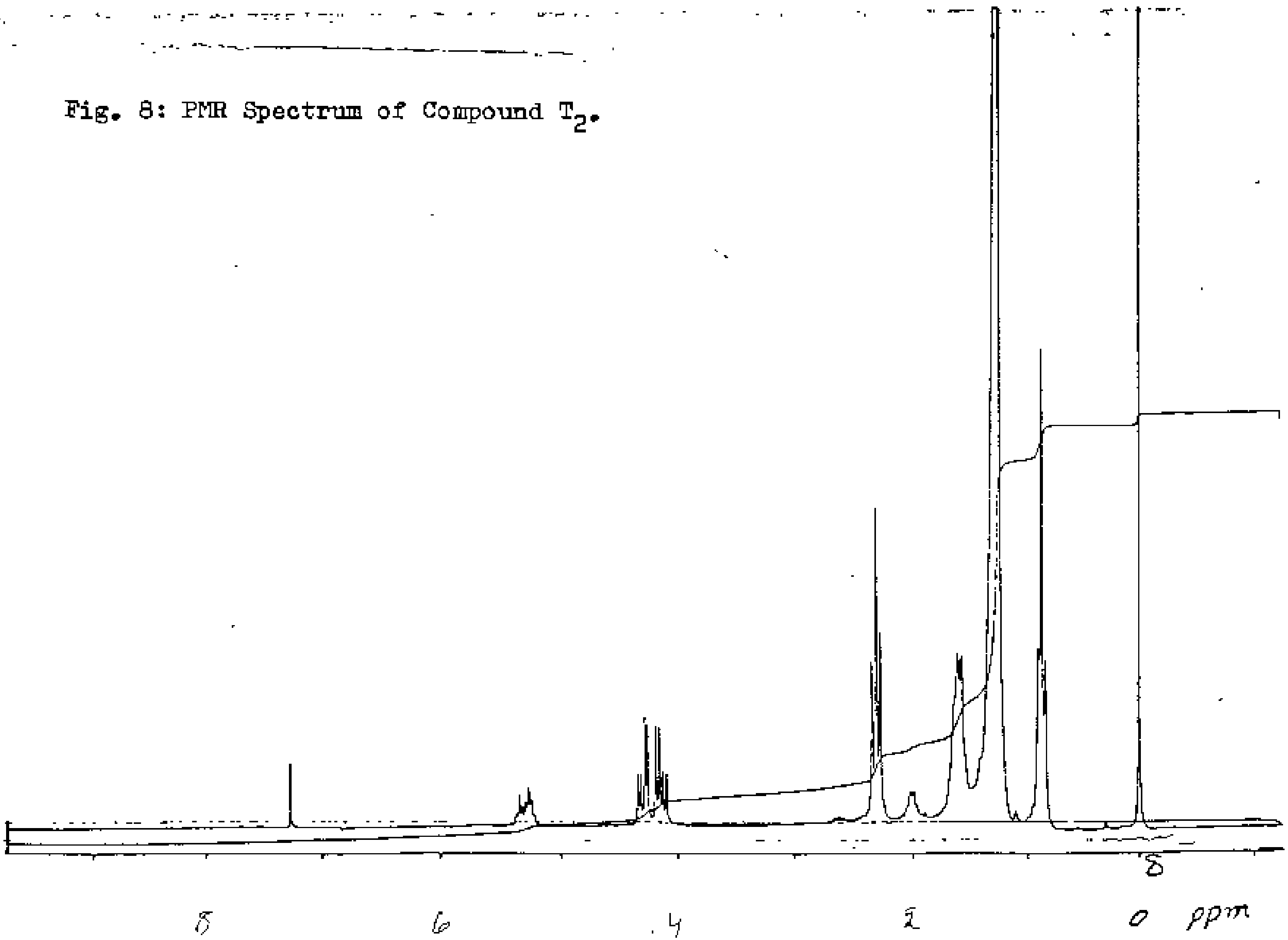


Fig. 8: PMR Spectrum of Compound T_2.

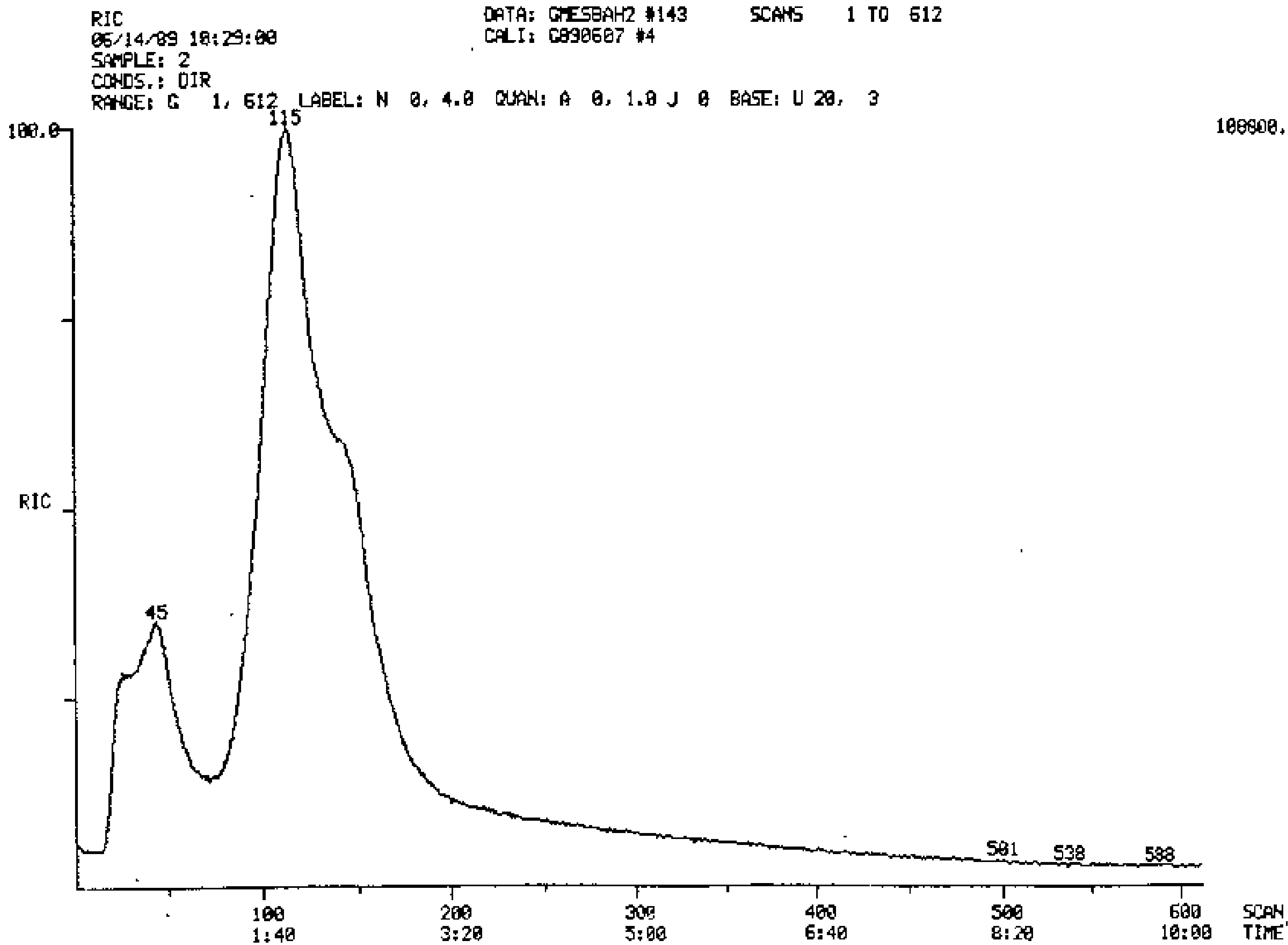


Fig. 9: GC-Mass spectra Compound T_2.

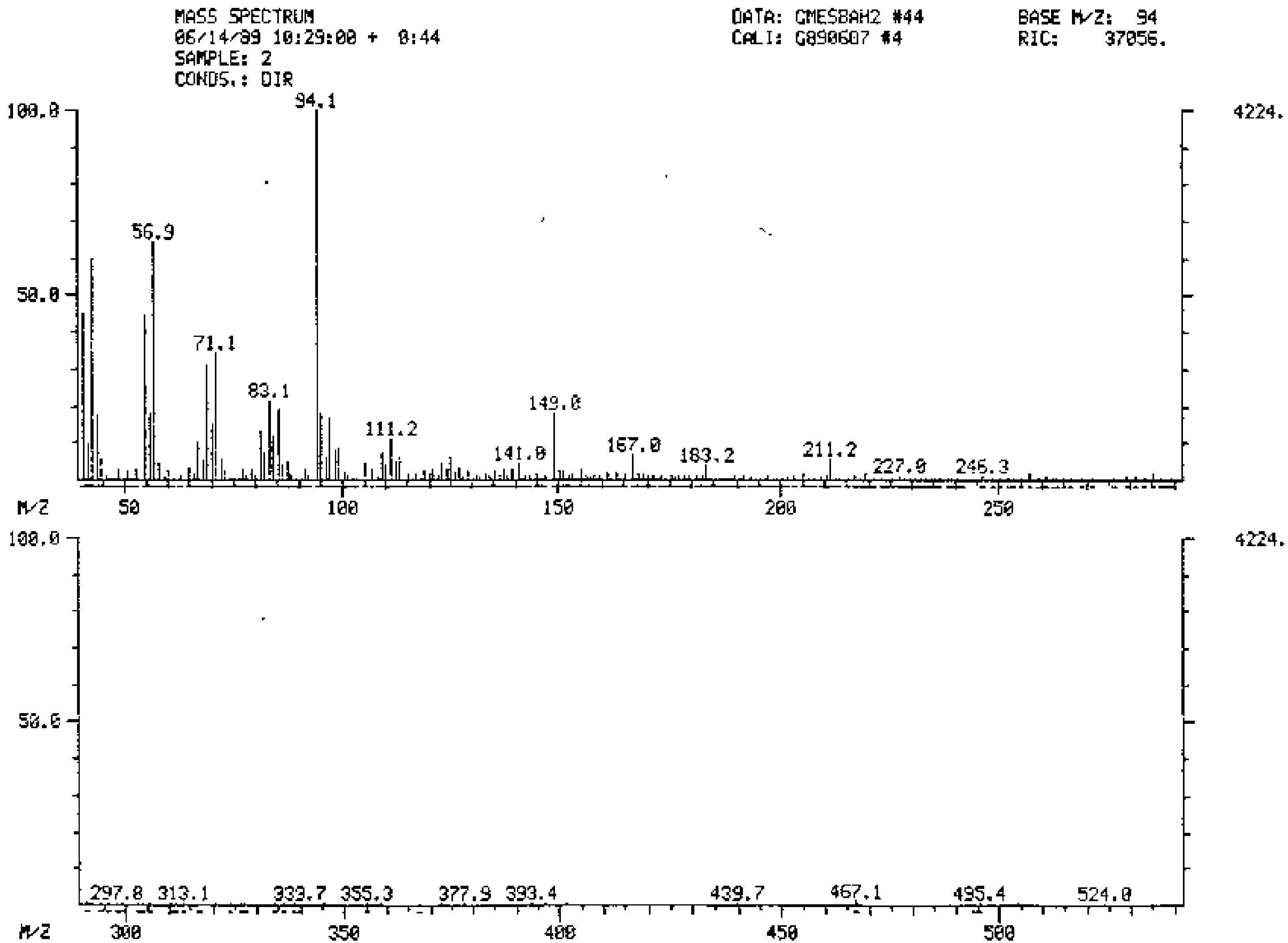


Fig. 10: Mass Spectrum of Compound T_2.

2.7. Examination of Fraction "T_3"

Fraction T_3 (0.00545%) was isolated as an oily substance being soluble in $CHCl_3$. Attempt to crystallize it from different solvents failed. When spotted on tlc plate and developed with solvent system pet-ether : ethyl acetate = 24:1, it gave a single spot with R_f 0.92. It showed an ir absorption [Fig-11] bands at ν_{max} 2920, 2850, 1460, 1380, 760 cm^{-1} characteristic for $-CH_2-$groups. The pmr spectrum [Fig -12] of the fraction showed a triplet centred at δ 0.88 characteristic for the protons of methyl group. A strong singlet at δ 1.25 showed the presence of protons of the methylene groups.

The nature of the ir and the pmr spectra indicated it to be a mixture of saturated hydrocarbons. The mass spectra of the compound also showed in general the presence of a mixture of saturated hydrocarbons. The identifications of the hydrocarbon mixture was resolved by running a GC-Mass spectra of the compound [Fig-13]. The molecular ions of the hydrocarbons were established by running a CI mass spectrum of the mixture. The results were in complete accord with the mass spectra of the compounds which showed mass peaks at m/e 366 [Fig-14] and m/e 322 [Fig-15]. The mass spectrum

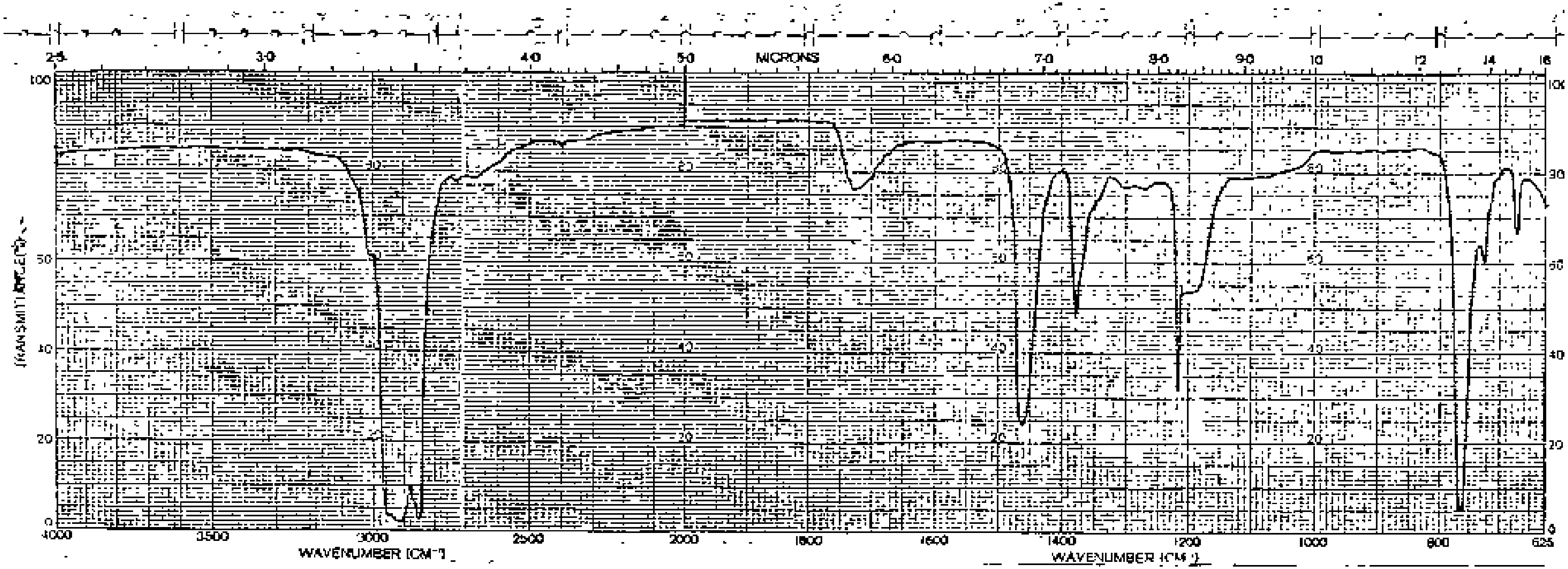
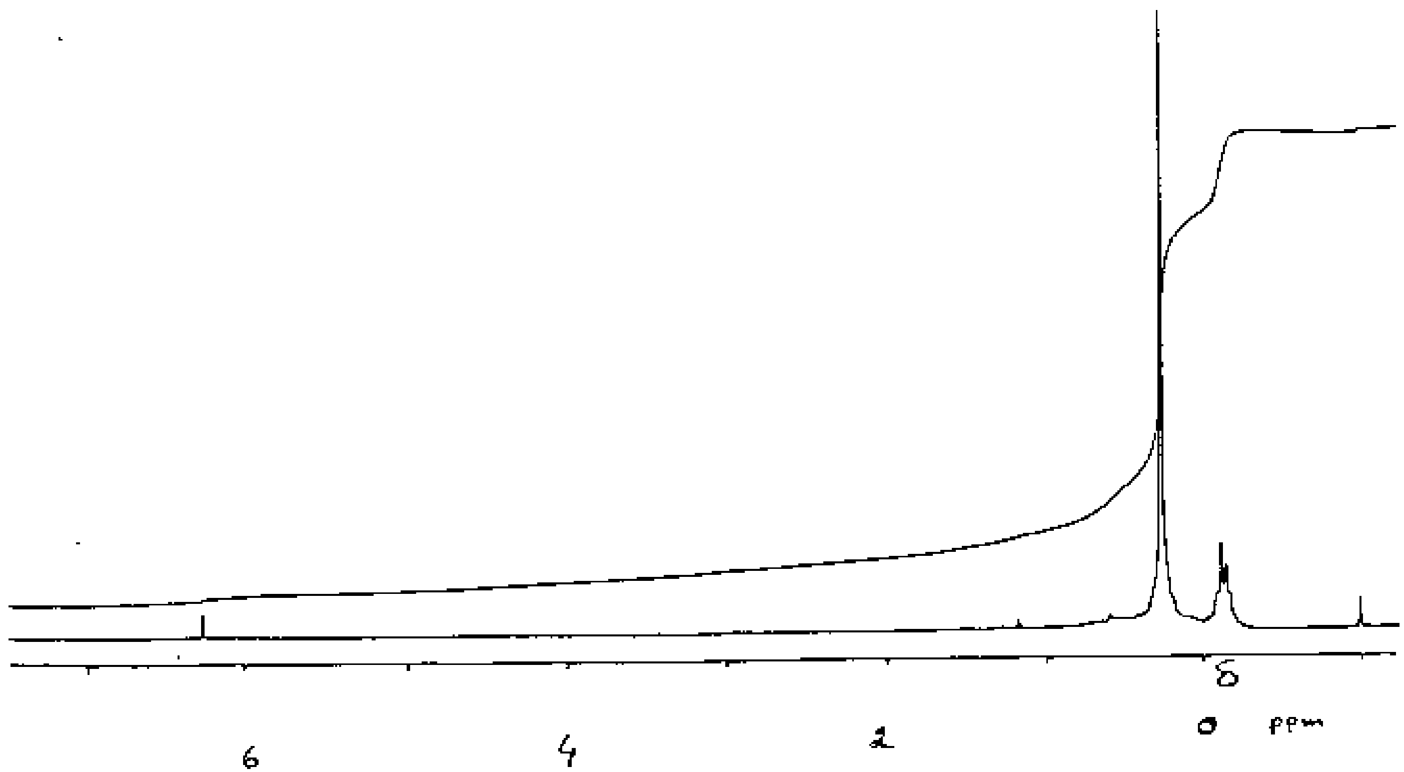


Fig. 11: Infra red spectrum of Compound T_3.

Fig. 12: PMR Spectrum of Compound T_3.



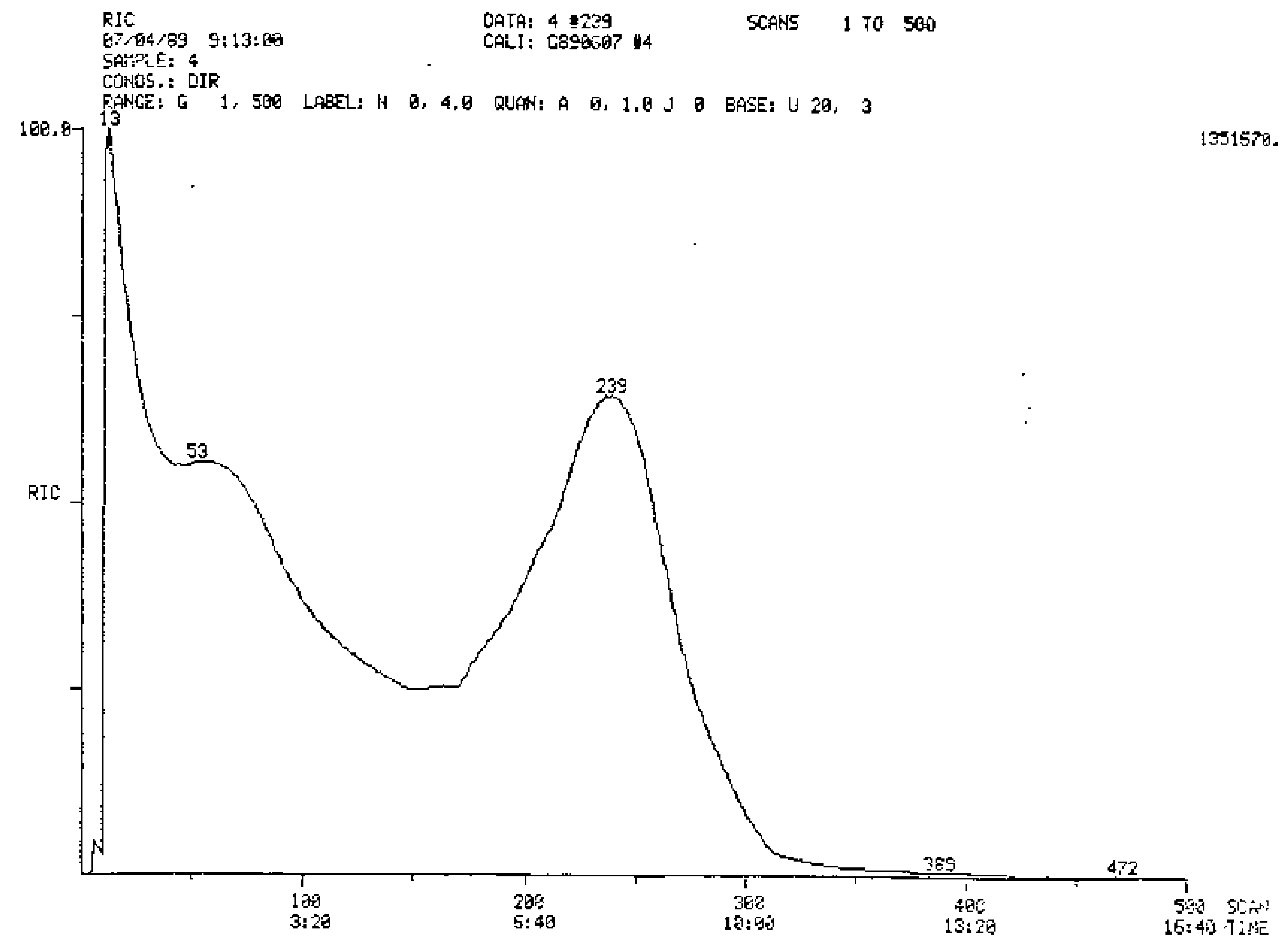


Fig. 13: GC-Mass Spectra of Compound T_3.

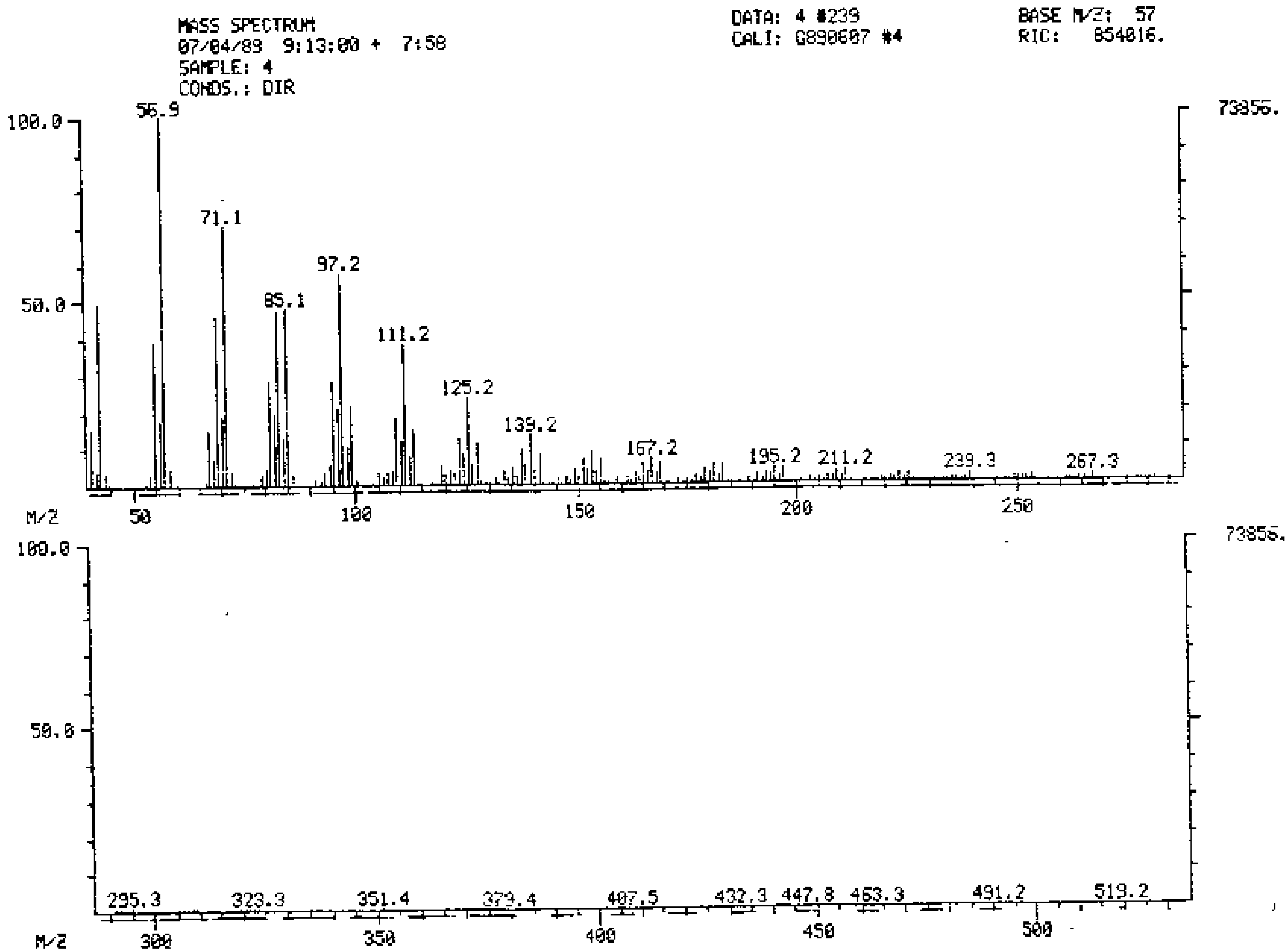


Fig. 14: Mass Spectrum of Compound T_3.

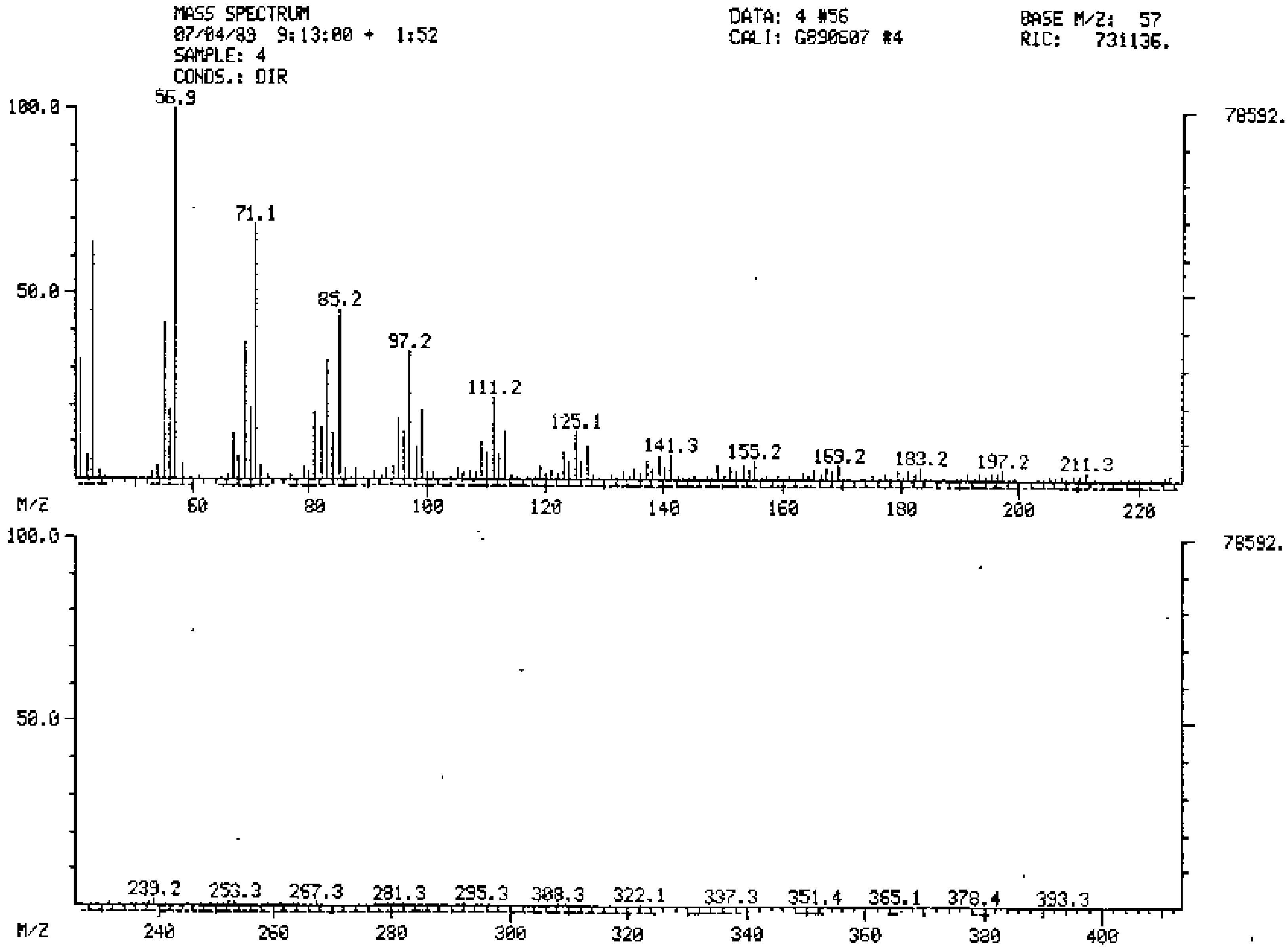


Fig. 15: Mass Spectrum of Compound T_3.

exhibited fragmentation pattern expected of long chain alkanes, i.e. m/e peaks at successive loss of 14 and 28 mass units. The m/e series 322, 309, 295, 281, 267, 253, 239, 211, 183, 169, etc. and 366, 351, 323, 295, 267, 239, 211, 183, 167, 139, 111, etc. indicated the presence of $C_{23}H_{48}$ (M^{+} - 2, 322) and $C_{26}H_{54}$ (M^{+} , 366) respectively. This assumption was not however, verified by the glc analysis due to the nonavailability of the authentic samples of higher alkanes.

2.8. Study on Mass "L_4"

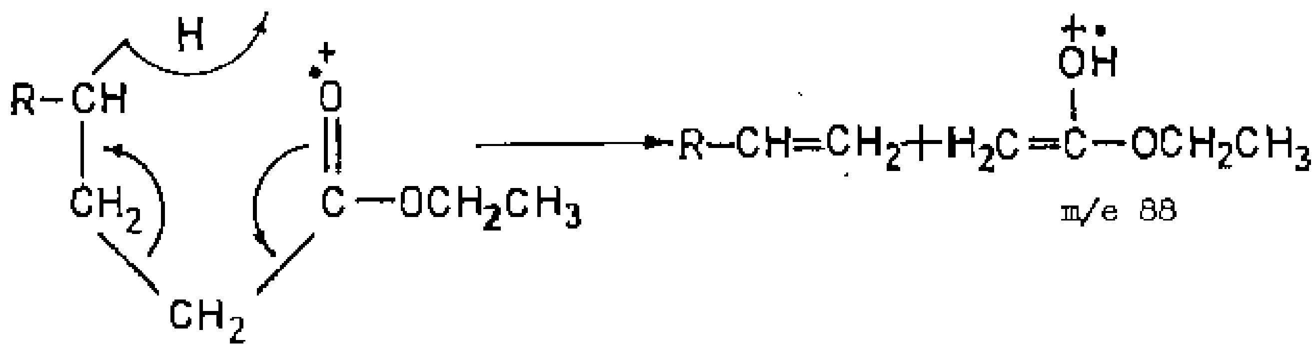
Fraction "L_4" was dissolved in ethyl acetate and then run on a preparative thin layer chromatographic plate with a solvent system ethyl acetate : pet-ether = 3.2 : 1.8, as described in section 3.1.17. On developement a pure fraction "N" was as later indicated as T_4 was seperated. Two other fraction "M" and "O" were found in very small quantity.

2.9. Examination of Fraction "T_4"

Fraction T_4(0.00971%) was isolated as an oily compound. Futile attempts were made to get it in the crystalline form. When spotted on tlc plate and developed with solvent system pet-ether: ethyl acetate = 2:1, it gave a single spot with R_f 0.44. The ir spectrum was recorded with the sample in plates [Fig-16]. It showed a strong ir absorption band at νmax 1740 cm^{-1} characteristic of a carbonyl group of an ester, other absorptions were observed at 2850, 1470, 1160, 1040, and 725 cm^{-1}. The pmr spectrum [Fig-17] of the fraction showed a triplet centred at δ 0.87 suggesting the presence of methyl groups. A strong singlet at δ 1.24 and δ 1.5 showed the presence of the methylene groups. The triplet at δ 2.2 was characteristic for protons in the acyl portion of an ester and the multiplet at δ 4.13 showed the presence of protons in oxymethylene group in the compound.

The nature of the ir and pmr spectra indicated it to be a long chain ester of a fatty acid. The mass spectrum of the compound showed in general the presence of an ester by running a GC-mass spectrum of the compound [Fig-18]. The moleular ion of the ester was established by running a CI mass spectrum. The result was in accordance with the mass spectrum of the compound with a molecular ion peak at m/e 312 [Fig-19] which is in agreement with the molecular ion of the ethyl ester of stearic acid $CH_3(CH_2)_{16}COOH$.

The presence of carbethoxy group ($- COOCH_2CH_3$) in this compound was clearly evidenced by the peak at m/e 88 which can arise from it's McLafferty Rearangement. The mass peak at m/e239 can arise from M^{+} 312 by the loss of $- COOCH_2CH_3$ group from the compound.



McLafferty Rearrangement of the ester.

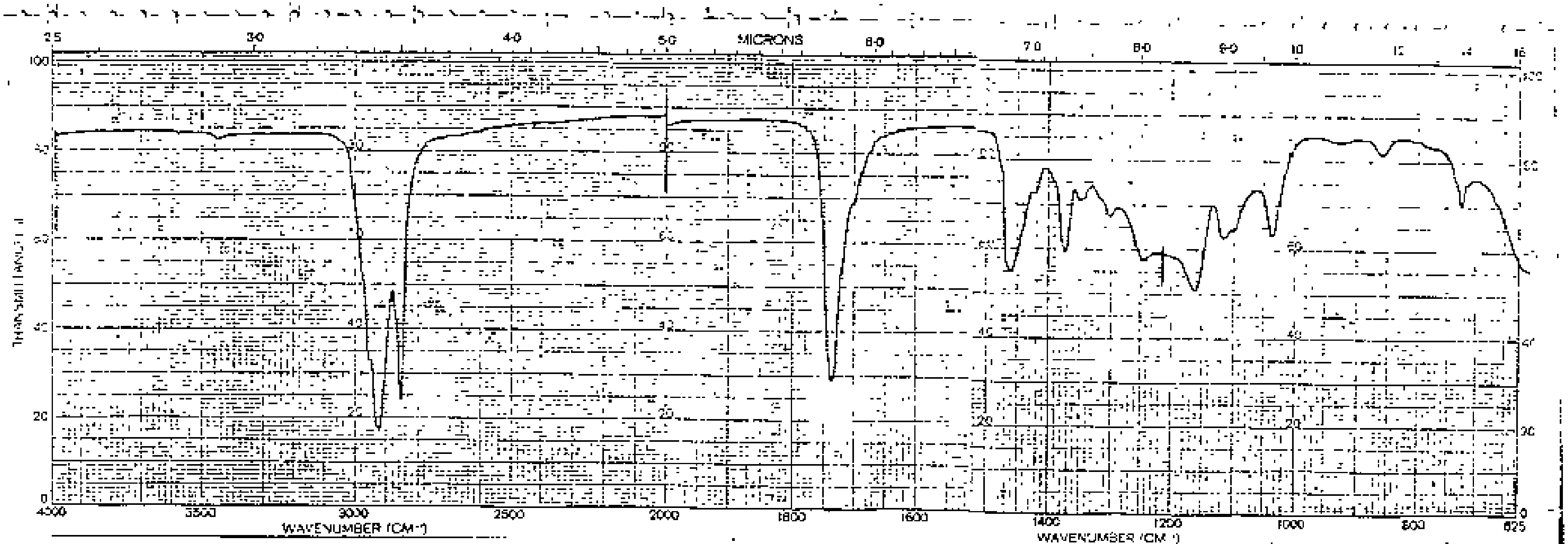
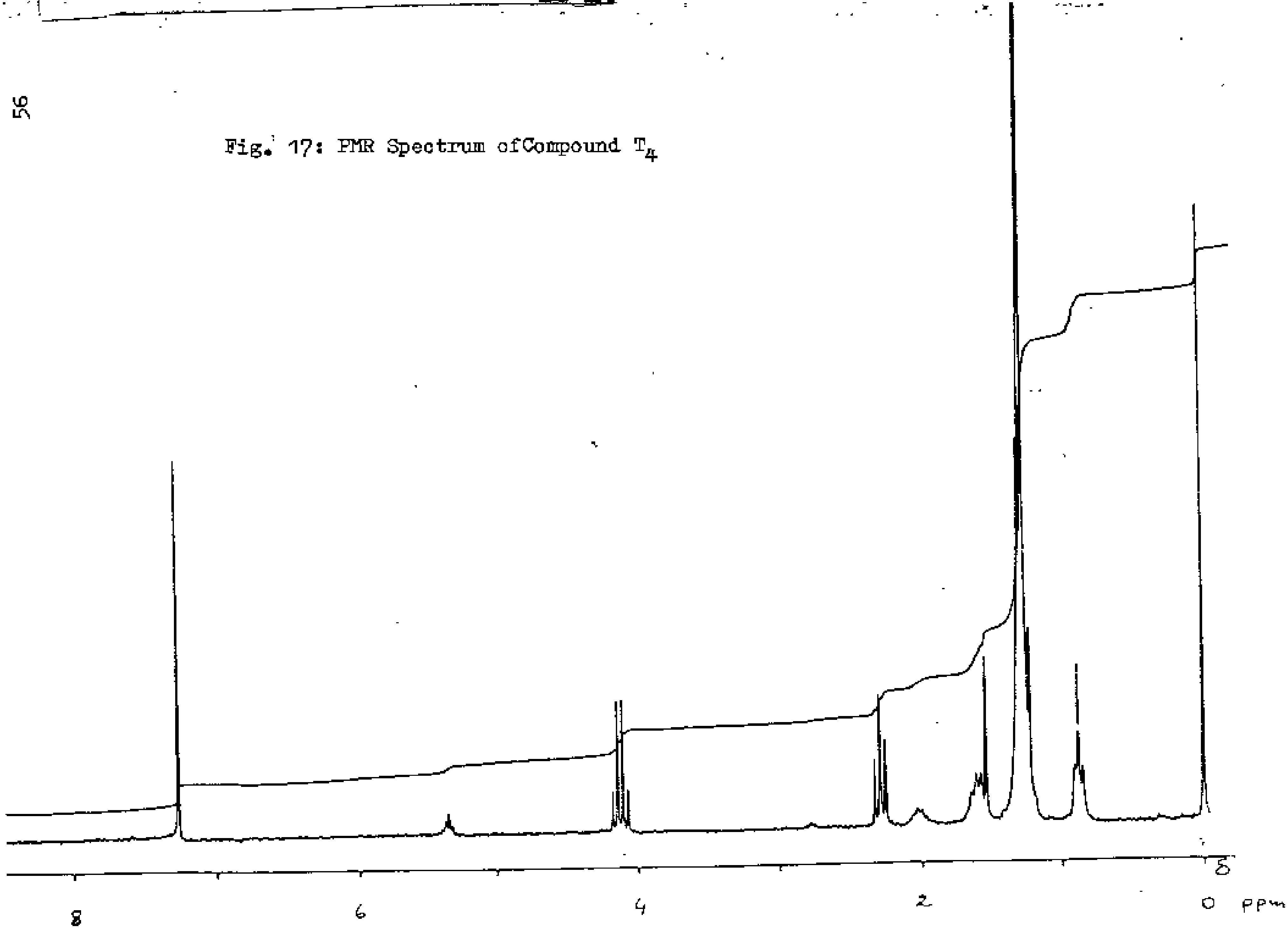


Fig. 16: Infra redspectrum of Compound T_4.

Fig. 17: PMR Spectrum of Compound T_4



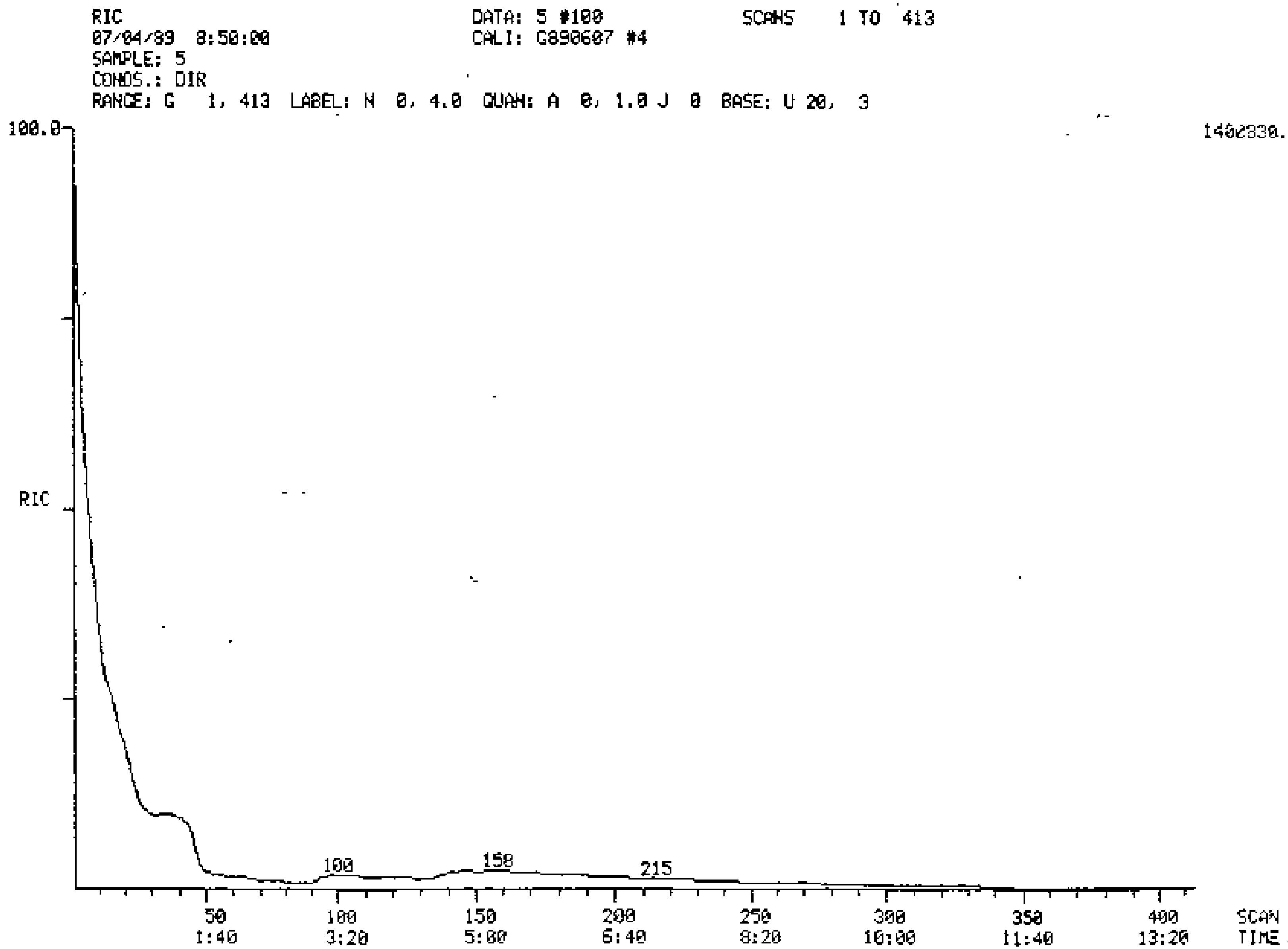


Fig. 18: GC-Mass Spectra of Compound T_4.

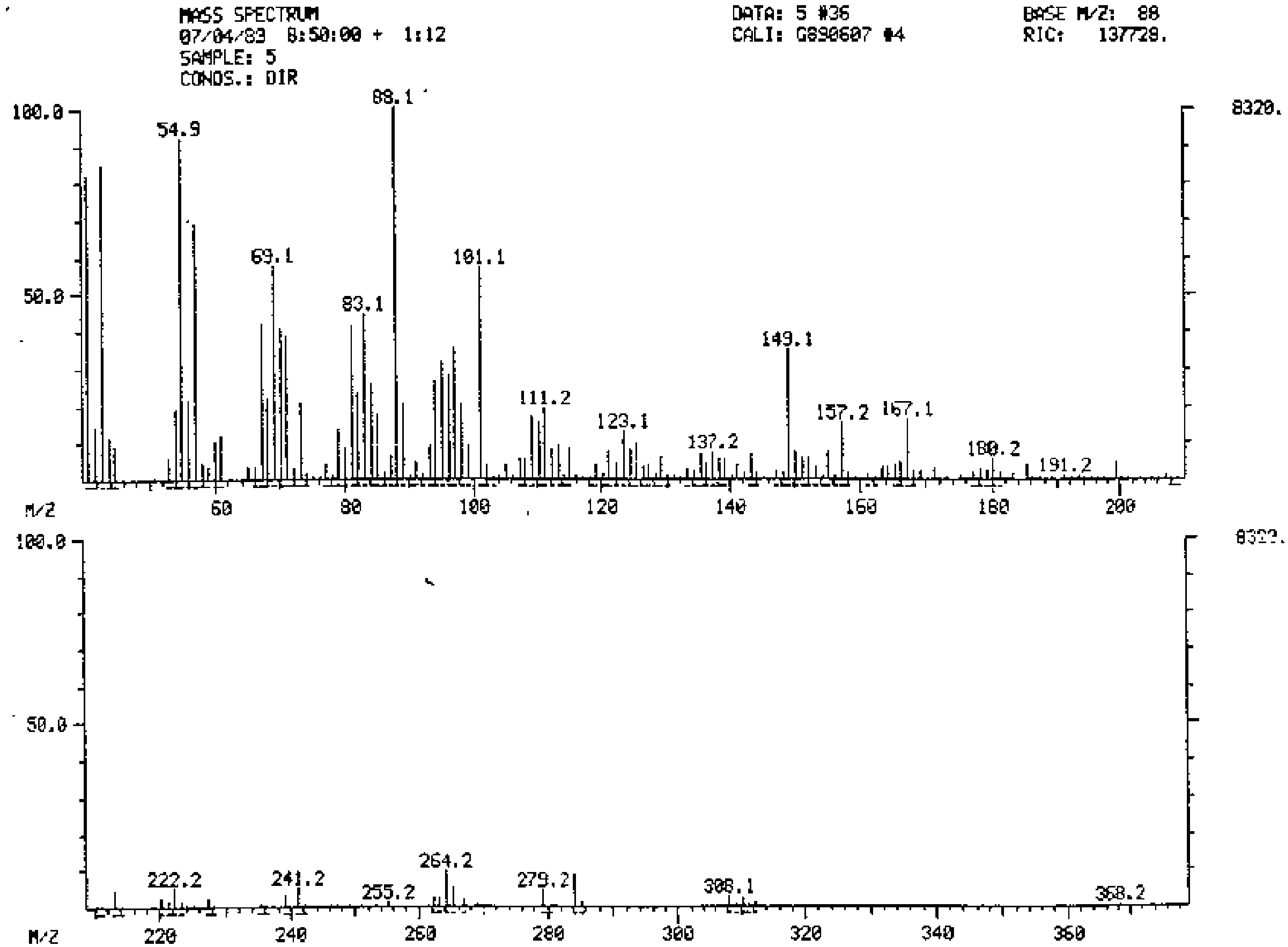


Fig. 19: Mass Spectrum of Compound T_4.

Other mass peaks expected from fragmentation from both the sides i.e. the alkyl and ester sides were also observed.

Cleavage from the ester side:

$$CH_3(CH_2)_{16}COOCH_2CH_3 \xrightarrow{-COOC_2H_5} 239 \xrightarrow{-28} 211 \xrightarrow{-28} 183 \longrightarrow \text{etc.}$$

(M^{+} 312)

Cleavage from alkyl side:

$$CH_3(CH_2)_{16}COOCH_2CH_3 \longrightarrow 241 \longrightarrow 227 \longrightarrow 213 \longrightarrow \text{etc.}$$

(M^{+} 312)

The mass spectrum of the compound showed some other mass peaks which appear due to the presence of some minor impurities. The pmr spectrum of the compound showed an absorption in the olefinic region as a triplet at δ 5.36. This may be due to the presence of some unsaturated fatty acid ester.

Further confirmation about the nature of the compound could not be established for want of suitable glc analytical facilities. The hydrolysis to convert the ester to acid and the analysis of the acid could not be done because of the meagre quantity of material obtained.

CHAPTER III

EXPERIMENTAL

3.1.1. General Experimental:

Thin Layer Chromatography(tlc):

The material used for thin layer chromatography was Kiesel gel 60 HF_{254} (MERCK). The plates (7.5 x 2.5 cm) were prepared by drawing a suspension of Kiesel gel 60 HF_{254} (8g.in 16 ml water) over the thoroughly cleaned plates. The plates were left in position at room temperature until the surface becomes completely dry. The plates were then allowed to stand for twenty four hours for activation and were ready for use.

Preparative Thin Layer Chromatography(ptlc):

Preparative thin layer chromatography was carried out on plates coated with Kiesel gel 60 HF_{254} (MERCK). The plates were prepared in the same manner as deacribed above but using larger glass plates (23 x 20 cm) and a larger spreader allowing thicker coating (0.75 cm). The plates were air dried at room temperature over night and then further activated by warming them at 110°C for half an hour.

Column Chromatography:

The column was prepared by slurry method, silica gel (70-230 mesh ASTM,MERCK) being the stationary phase.The column was made half filled with the appropriate solvent (the best running sovent was established by tlc), and the slurry was poured into it so that the packing was compact and uniform. Air bubble was avoided by making the column as quickly as possible and allowing the solvent to fall drop by drop through the stopcock of the column. The solvent was allowed to pass through the column for sufficient time and then the column was allowed to settle for about one hour. The mixture of compounds was then taken as a solution and was allowed to fall on the surface of the column. The column was then eluted with previously purified desired solvent system.

Preparation of Absolute Alcohol:

The alcohol was purified by the following way:Rectified spirit obtained by the distillation of wash contains about 95 percent ethyl alcohol. Since, a mixture of 95.6 percent alcohol with water boils at lower temperature(78.1°C) than the boiling point of pure alcohol (78.5°), it is impossible to get an alcohol of higher concentration by fractional distillation of rectified spirit. Anhydrous or absolute alcohol can be obtained by digesting the rectified spirit over quicklime for several days and then distilling. The first and the last runninge are rejected, and the main portion of the distillate is 100 percent or absolute alcohol.

A modern process is the azetropic distillation of rectified spirit with benzene. When distillation is carried after addition of a certain amounts of benzene, at first ternary mixture of water, alcohol and benzene comes over at 65^{o} till all the water is thus removed. Then the boiling point rises and the remaining benzene comes over as binary mixture with alcohol at 68^{o}. Finally absolute alcohol distils at 78.5^{o}.

Purification of Petroleum ether:

When the petroleum ether present in the mixture have their boiling points close to each other, the separation is best effected by fitting the distillation flask with a fractionating column which in turn is connected to the condenser. On heating, the vapours of the more volatile liquid A, along with a little of the vapours of less volatile liquid B, rise up and come in contact with the large cooling surface of the fractionating coulumn. The vapours of B condense first and that of A pass on. The condensed liquid flowing down the column meets the fresh hot ascending vapour. It snatches more of B from the vapour mixture and gives up any dissolved vapour of A. This process is repeated at every bulb of the fractionating column, so that the vapour escaping at its top consists almost exclusively of A and the condensed liquid flowing back into the distillating flask is rich in B. If necessary,the process can be repeated with the distillate and the liquid (i.e petroleum ether 40-60°C and 60-80°C)left in the distillation flask.In this way, the petroleum ether (40-60°C) and petroleum ether (60-80°C) were purified.

Soxhlet Extraction:

Soxhlet Extraction is used for the extraction of oils, fats, carbohydrates, and alkaloids from the powdered Betel nut. This apparatus ensures maximum extraction with a limited quantity of the solvent.

Mass Spectra(ms):

Mass spectra were recorded on a DS-55 Mass Spectrometer,

RF I EASF 3.20.

Infra red Spectra(ir):

Infra red spectra were recorded on a perkin-Elmer-237 spectrophotometer and PYE UNICAM SP3-200 spectrophotometer using either chloroform or Nujol mull or liquid film.

Proton Magnetic Resonance Spectra(pmr):

Proton Magnetic resonance spectra were recorded on a JEOL FX 90 spectrophotometer using deuterochloroform($CDCL_3$) as solvent with tetramethylsilane(TMS) as external standard.

Melting Point Determination(mp):

Micro melting point apparatue(Mettler FP5 + FP52) was used for the determination of melting point(mp).

The following abbreviations were used in describing Spectra:

ir : s, strong ; m, medium;
w, weak ; b, broad ;

pmr : s, singlet ; d, doublet;
t, triplet ; q, quarlet;
m, multiplet;dd, doublet of doublets; b, broad;
sh, sharp;

Fractional Recrystallisation:

Solid organic compounds when isolated from Betel nut, they were usually contaminated with small amounts of other compounds (impurities) which were produced along with the desired product. The purification of impure crystalline compounds is usually effected by crystallisation from a suitable solvent or mixture of solvents.

Solid substances were purified by recrystallisation. The solvents generally used for recrystallisation were benzene, petroleum ether (60 - 80°C).

Fractional Distillation Under Normal or Reduced Pressure:

Fractional distillation of small amounts of samples was carried out in a 2-vigreux column fitted with specially designed distilling head. An efficient rotary oil pump, Hitachi Ltd, and a manometer were connected with the system during the fractional distillations under reduced pressures.

Semi-micro and micro distillation of small amounts of compounds were carried out with equipments. In this way, the compounds were concentrated and dried.

3.1.2. Collection and Preparation of the samples:

Betel nut (Areca Catechu) was obtained from the local market and this was then dried and obtained as a sliced one. This nut was cut into small pieces and made mechanically powder herein after, these powders are called, the crude powder. The resulting samples was used for investigations.

3.1.3. Determination of Moisture Content:

The crude powder (18.35 gm) was taken in a weighing bottle which was previously cleaned, dried at constant temperature, (105°C) and weighed. It was then heated in an oven at temperature 105°C, for an hour, cooled in a desiccator and weighed, till a constant weight was obtained, heating, cooling and weighing were continued. The result is shown in Table - 1.

3.1.4. Determination of Ash Content:

The crude powder (10.75 gm) was taken in a cleaned, dried and accurately weighed porcelein crucible. It was then heated first slowly in a low flame to prevent any loss during charring and then strongly heated until only ash remained. The crucible was cooled in a desiccator and weighed to a constant weight. The result is shown in Table-1.

3.1.5. Extraction with Petroleum Ether:

The crude powder (693.03 gm) (b.p 40-60°C) which was packed in cloth bags was extracted with petroleum ether (b.p 40-60°C) in a glass soxhlet for six hours. The powder was then removed from the soxhlet, air dried and weighed. The petroleum ether extract was concentrated, dried and weighed. The amounts of extractive was calculated on the moisture free basis and the result is given in Table-I. This amount of extractive was indicated by "A".

3.1.6. Extraction with Absolute Alcohol:

The extractive free powder (623 gm) obtained after extraction with petroleum ether(b.p. 40-60°C) was then extracted with absolute alcohol in the sama way as described above, where the crude nut was extracted with petroleum ether. The absolute alcohol extract was concentrated. The amount of extractive was calculated by difference and the result is given in Table-I. This amount of extractive was indicated by "B".

Table No - 1

Batch - 1

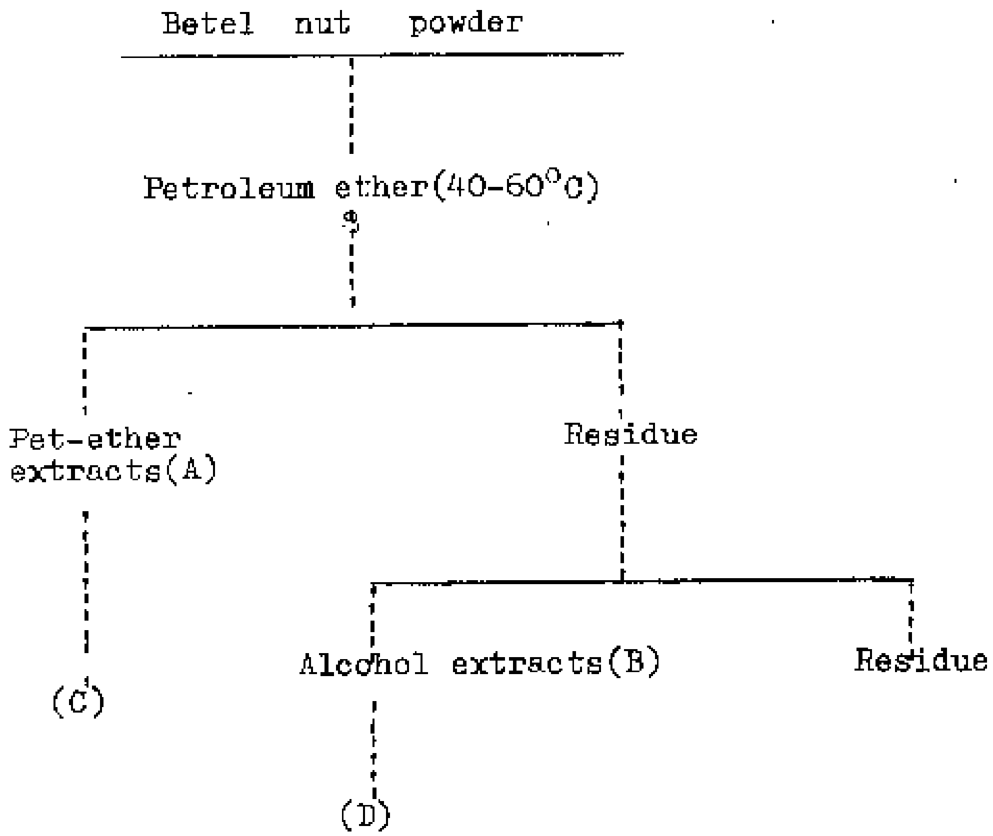
Proximate Composition of Betel Nut

% Constituents		
1.	Moisture	12.18
2.	Ash	1.56
3.	Petroleum ether extractives	8.08
4.	Absolute Alcohol extractives	10.10
5.	Compound T_1	0.38
6.	Compound T_2	0.026
7.	Compound T_3	0.00545
8.	Compound T_4	0.00971

3.1.7. Extractives obtained from "A" and "B":

The extractive obtained from "A" was evaporated on a rotary vacuum evaporator at about 40-45°C to drymass, cooled in a desiccator and weighed. It was marked as "C".

Similarly, the extractives obtained from "B" was evaporated to a concentrated mass and stored for analysis. It was marked as "D".



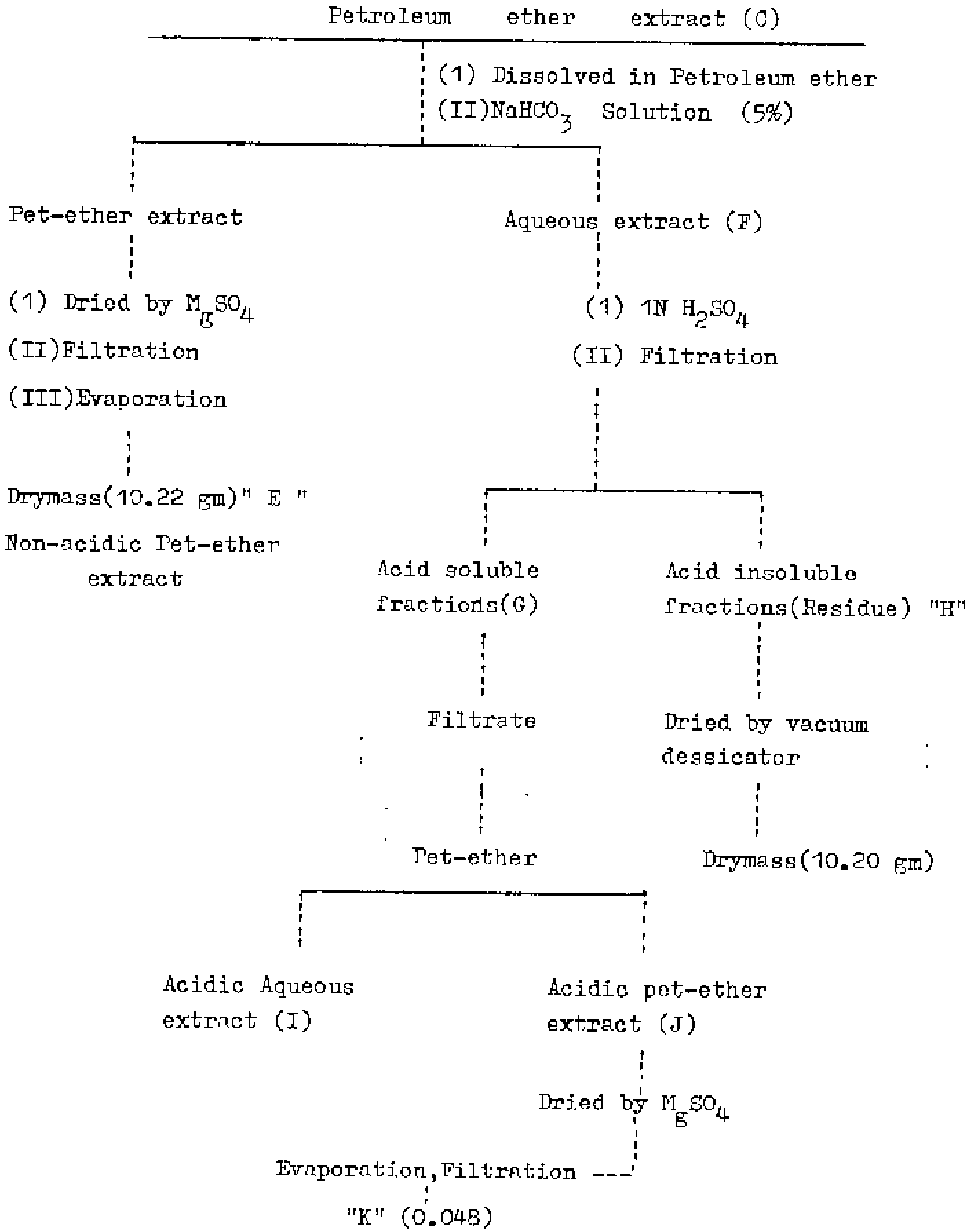
3.1.8. <u>Preliminary Examination of Petroleum Ether Extract"C":</u>

"C" (25.48 gm) was dissolved in petroleum ether. It was then treated with sodium bicarbonate solution(5%) and transferred into a separting funnel. After shaking, the resulting mixture was allowed to stand for separation. When mixture had settled, two distinct layers were visible.Upper layer was the petroleum ether layer and lower layer was the milky white aqueous layer. The two layers were then separated. Petroleum ether layer was washed with water twice. The petroleum ether layer was treated with anhydrous M_gSO_4 for about an hour. It was filtered and the filtrate was concentrated. It was finally dried over night in a vacuum desiccator and weighed (10.22 gm). The petroleum ether extract "E" was non acidic petroleum ether extracts.

The milky white aqueous extract "F", when treated with sulphuric acid (IN, 15 ml), yielded acid soluble fraction "G" and acid insoluble material "H". It was filtered and the residue was washed with water twice. It was dried overnight in a vacuum desiccator and weighed (10.20 gm).

"G" was transferred into a separating funnel and extracted with petroleum ether. After shaking, the resulting mixture was allowed to stand for half an hour. Upper layer was the acidic petroleum ether layer and lower layer was the aqueous layer. The lower fraction "I" was drained down. Acidic petroleum ether extract "J" was washed with water twice and treated with anhydrous M_gSO_4 for about an hour. It was filtered and the filtrate was concentrated. It was finally dried overnight in a vacuum desiccator and weighed (0.048 gm). It was indicated by "K".

The scheme of extraction is shown below:



3.1.9. Investigation on "E":

A small portion of dried "E" was dissoved in petroleum ether. The solution was spotted on a thin layer chromatographic plate and was placed in the solvent tank containing the solvent system pet-ether: chloromform (1:1,1:4) and alcohol : pet-ether (1:3,2:3, 1:1), no suitable separation was attained in this case. But the presence of a mixture of three compounds was visible. So the mixture was not separated in this way. From this investigation, it was found that the suitable solvent system was pet-ether: ethyl acetate(9:1).

3.1.10. Fractionation of "E":

A column (60 x 2.3 cm) wae packed with silica gel 60(70-230 Mesh ASTM,65 g) using petroleum ether as the solvent. A solution of "E" (3.012 gm) in 2 ml of petroleum ether and a few drops of ethyl acetate was carefully put on the top of the column with the help of pipette. The tip of the pipette was placed against the wall of the column just above the surface of the absorbent,while the sample solution was slowly drained from the pipette. Care was taken not to touch the packing material.

When the sample was adsorbed on the top of the silica gel, the vacant space above was filled with petroleum ether and ethyl acetate. About 5 ml fraction was collected in each teet tube. Each fraction was concentrated and spotted on tlc plate. Column was eluted with gradient elution with petroleum ether: ethyl acetate. Fraction-E_1 : 1 to 9 test tubes were blank.

Fraction-E_2 : 10 to 22 test tubes were combined and evaporated to dryness. But the amount was very small and further study was not possible. But the presence of a spot with R_f = 0.64 was noted.

Fraction-E_3 : 23 to 34 test tubes gave same material. They were combined and evaparated to dryness with R_f = 0.71. But the amount was so small, further investigation was not possible.

Fraction-E_4 : 35 to 55 test tubes were identical on tlc plates containing solvent system pet-ether: ethyl acetate (3.5:1.5) with R_f = 0.61, But the amount was so small, that further study was not possible.

Fraction-E_5 : 56 to 74 test tubes gave same material. They were combined and evaporated to dryness.

Fraction-E_6 : 75 to 185 test tubes gave same material. But they were mixture. So further study was not possible.

Fraction-E_7 : 186 to 194 test tubes gave same material. They were combined and evaporated to dryness (R_f = 0.88). Fractions E_1 - E_7 on evaporation, gave such a small amount of material, that further investigations could not be carried out.

Fraction-E_8 : 195 to 196 test tubes gave a single spot (R_f = 0.80) on tlc with petroleum ether: ethyl acetate(9:1). Solvent from the test tubes were combined, evaporated to dryness and weighed (2.6280 gm).

Fraction-E_9 : 197 to 199 test tubes gave tailed material. They were combined and evaporated to dryness.

Fraction-E_{10}: 200 to 275 test tubes gave same material. They were a mixture and these test tubes were combined.

Fraction-E_{11}: 288 to 325 test tubes gave same material. They were combined, evaporated to dryness and weighed.

Fraction-E_{12}: 326 to 365 test tubes gave same material.

Fraction-E_{13}: 366 to 375 test tubes gave same material.

Fraction-E_{14}: 376 to 445 test tubes gave same material. The material composed of three components were seen. But the amounts of material are so small, further study was not possible.

Fraction-E_{15}: 446 to 546 test tubes gave mixture of compounds and the column was washed with pet-ether.

Fraction-E_9-E_{15} contained trace amount of compounds. Hence, further study of them could not be carried out.

Fraction contained in test tube 490, on tlc containing solvent system(pet-ether:ethyl acetate) (5:1) showed four spots,each having R_f value = 0.37,0.51,0.74, 0.85 respectively. But the amount was so small, that the compounds could not be further separated.

3.1.11. Characterization of the Isolated Compound $E_8(T_1)$:

Compound - E_8

Physical Characteristics:Yellowish white waxy substance.

Thin layer Charomatography(tlc): E_8 was dissolved in petroleum ether spotted on tlc plate containing a solvent system, petroleum ether : ethyl acetate (9:1). The compound gave a single spot with R_f value 0.80. This compound was indicated as T_1.

Proton Magnetic Resonance Spectra(pmr)Examination of $E_8(T_1)$:

pmr spectra were recorded on a JEOL FX 90 spectrophotometer using deuterochloroform($CDCL_3$) as slovent with tetramethylsilane (TMS) as internal standard, showed the following signals:

(i) a triplet centred at δ 0.88

(ii) a singlet at δ 1.25

(iii) a triplet centred at δ 2.3

(iv) a multiplet centred at δ 4.28

UV-Spectrum: Absorption in the near ultraviolet with the maximum at λ_{max} = 290 nm characteristic of >C=O, of an ester.

Infra red Spectra(ir): The ir absorption spectrum of compound E_8 showed the following characteristic :

max ν cm^{-1}	Characteristic groups
2920	C-H Stretching
2840	
1750	C=O Stretching

1375	
1465	C-H Stretching of-CH_3
1450	
725	C-H Stretching of-CH_2

Mass Spectra : m/e 523, 495, 467, 423, 395, 367, 339, 285, 257, 211, 199, 183, 123, 109, 95, 71, 57,

m/e 367, 336, 323, 296, 289, 263, 246, 219, 205, 184, 177, 149, 119, 111, 94, 83, 71, 57.

Melting Point Determination(mp):

mp : 43 - 44°C. weight = 66mg(0.38%).

Soluble in pet - ether (40 - 60°C). It is a waxy type compound.

3.1.12. Trituration of Alcohol Extracts:

Alcohol extract (D), which was obtained after extraction of petroleum ether was dissolved in chloroform and kept for a few days. The chloroform solution was filtered and the filtrate was evaporated to dryness and gave a dark brown substance(0.7298 gm). When ethyl acetate was added to the above substance some dispersed material was seen to be present in the ethyl acetate solution. The dispersed material was seperated by centrifugation (0.6966 gm) and the ethyl acetate solution was evaporated to dryness (0.0332 gm). Ethyle acetate soluble portion on tlc plate showed a mixture of compounds which was difficult to seperate. The other part was indicated as "L" (0.6966 gm) and was used for further investigation.

3.1.13. Thin Layer Chromatography(tlc) of "L":

A small portion of "L" was dissolved in ethyl acetate and tlc was taken containing solvent system pet-ether:ethyl acetate (24:1). In this case, pet-ether : ethyl acetate was found to be the most suitable solvent system to separate four componente.

3.1.14. Preparative Thin Layer Chromatography of "L":

Fraction "L" (0.6966 gm) containing four components was separated by preparative thin layer chromatographic method."L" was dissolved in ethyl acetate and run on ptlc plate. This plate was put into pet-ether : ethyl acetate (24:1) solvent system and than dried for 2-3 hours and developed in the iodine chamber.The four components were then separated individually.tlc was taken from these individual fractions. All fractions showed a single individual spot.

Fraction - 1, was seperated from the upper part of the ptlc plate, this was indicated as T_3.

Fraction- 2, was seperated from the middle part of the plate, this was indicated as T_2.

Fraction - 3, was the third part of the sample and was present in small quantity, Hence, further study could not be carried out.

Fraction - 4, was in the base of the ptlc plate and was a mixture as observed from the tlc. This amount was used for further study. This fraction was obtained from the fraction "L" and hence was denoted as "L_4".

3.1.15. (a) Characterization of the Isolated Compound(T_2):

Physical characteristics : Yellowish white gummy substance.

Thin Layer chromatography : This substance was dissolved in ethyl acetate, spotted on tlc and developed with solvent system petroleum ether: ethyl acetate (24:1) and then developed in iodine chamber. A single spot was obtained. The compound gave a single spot with R_f value 0.55 . This compound was indicated by T_2.

Proton Magnetic Resonance Spectra(pmr):

pmr spectra, were recorded on a JEOL FX 90 spectrophotometer using deuterochloroform as solvent of teramethyl silane (TMS) as internal standard, showed, the following signals :δ0.84(t), 1.25(s),1.57(d), 2.3(t), 4.25(m), 5.26(m).

Infra red Spectra($CHCL_3$): νmax 2920, 2850, 1740, 1460, 1100, 960,and 725 cm^{-1}.

Mass Spectra: GC - Mass spectra of the fraction T_2 showed two peaks. The molecular ion of ths subfrections of T_2 were established by running a mass spectrum.

m/e 285, 257, 211, 183, 167, 149, 111, 94, 83, 71, 57.

m/e 523, 495, 467, 439, 423, 409, 395, 339, 285, 257, 211, 183, 129, 85, 71, 57.

Melting point : It was a gummy type compound, therefore, no melting point was taken.

3.1.15. (b) Characterization of the Isolated Compound(T_3):

Physical characteristics : Yellowish oily compound.

Thin Layer Chromatography: T_3 was dissolved in $CHCL_3$, spotted on tlc plate and developed with solvent system pet-ether: ethyl acetate (24:1). The compound gave a single spot with R_f value 0.92.

Infra red Spectra($CHCL_3$): νmax 2920, 2850(s), 1460, 1380, 1220, 760(s) cm^{-1}.

Proton Magnetic Resonance Spectra(pmr) ($CDCL_3$):

δ 0.88(d), 1.25(s), 7.25(s).

Mass spectra: m/e 366, 351, 295, 267, 239, 211, 167, 139, 125, 111, 85, 71, 57.

m/e 322, 295, 281, 267, 239, 211, 183, 169, 141, 111, 85, 71, 57.

3.1.16. Investigation of Fraction "L_4":

A small portions of fraction "L_4" was dissolved in ethyl acetate. The solution was spotted on a thin-layer chromatographic plate containing solvent system, pet-ether: ethyl acetate(3.5:1.5). Two individual spots were observed. By changing the solvent system to pet-ether:ethyl acetate(3.2:1.8), three spots were completely separated from one another.

3.1.17. Preparative thin layer chromatography of fraction "L_4":

Portion L_4 was dissolved in ethyl actate and was put on preparative thin layer chromatographic plate.The plate was dried and then placed in the solvent tank containing pet-ether : ethyl acetate (3.2.:1.8) and the plate was taken out before the solvent front reached the top of the silica bed. It was dried in the air and then developed in an iodine chamber when three spots were visible. One part(M) was in the solvent front, whose R_f = 0.89, other was in the middle (N) and the last part was in the base line(O), which was a mixture of two compounds were seen from tlc plate. Fraction "M" was in small quantity, hence this amount was not taken for further study. "O" was also in small quantity and hence was rejected. The fraction "N" was filtered and dried.

3.1.18. Characterization of "N":

Physical characteristics : Yellowish oily type compound.

Thin layer chromatography: "N" was dissolved in ethyl acetate, spotted on tlc plate and developed with solvent system pet-ether: ethyl acetate (3.0 : 1.5). The compound gave a single spot with R_f value 0.44. This was different from the compound T_3. In this case compound was indicated as T_4.

Proton Magnetic Resonance Spectra(pmr)($CDCl_3$):

δ 0.87(t), 1.24(t,sh), 1.5(t), 2.2(t), 4.13(q), 5.3(d), 7.25(s).

IR - spectra($CHCl_3$) : ν_{max} 2920, 2850, 1740, 1470, 1370, 1160, 1040cm^{-1}.

Mass spectra : m/e 312, 310, 284, 279, 269, 264, 241, 239, 227, 222, 213, 183, 167, 157, 149, 137, 123, 111, 101, 88, 83, 69, 54.

3.1.19. Thin layer chromatography of Alcohol extractives:

A small portion of dried alcohol extractives was dissolved in absolute alcohol. The solution when spotted on a thin layer chromatographic plate containing ethyl acetate: alcohol (1:1), gave tailing end. Different ratios of mixture of ethyl acetate and alcohol (4:1, 3:1) , of which athyl acetate: alcohol (2:1) was suitable to separate two components, but the tailing end persisted. Hence, further investigations were not carried out.

3.2.1. Collection and Preparation of the samples:

Betel nut (Areca catechu) was obtained from the region of Barisal as a finely sliced one and dried at room temperature. This was cut into small pieces and herein after called the crude nut. The resulting sample was used for investigations.

3.2.2. Determination of Moisture Content:

The crude nut (7.75 gm) was taken in a weighing bottle which was previously cleaned, dried at constant temperature (105°C) and weighed. The same procedure was applied as in Batch-1. The result is shown in Table-2.

3.2.3. Determination of Ash Content:

The crude nut(7.55 gm) was taken in a previously cleaned, dried and accurately weighed porcelein crucible. The same procedure was applied as in Batch-1, where the ash content was determined. The result is shown in Table - 2.

3.2.4. Extraction with Petroleum Ether (b.p 40-60°C):

The crude nut, which was packed in cloth bag,

(302.79 gm), was extractod with petroleum ether (b.p.40-60°C) in a glass soxhlet for six hours. The same procedure was applied as in the Batch - 1, where crude nut was extracted with petroleum ether (b.p. 40 - 60°C). The amount of extractives from pet- ether extraction is given in Table - 2.

3.2.5. Extraction with Absolute Alcohol:

The extractive free powder (301.15 gm) obtained after extraction with petroleum ether (40-60°C) was then extracted with absolute alcohol in the same way as described in experiment No.(3.2.4. The absolute alcohol extracts were combined and concentrated. The amount of extractives were calculated by difference and the result is given in Table - 2.

3.2.6. Extractives obtained from (3.2.4.):

The extractive obtained from (3.2.4) was evaporated on a rotary vacuum evaporator at about 40 -45°C to a dry mass, cooled in a dessicator, and weighed. It was marked as "P". Similarly, the extractives obtained from (3.2.5) was evaporated to a concentrated mass and stored for analysis. It was marked as "Q".

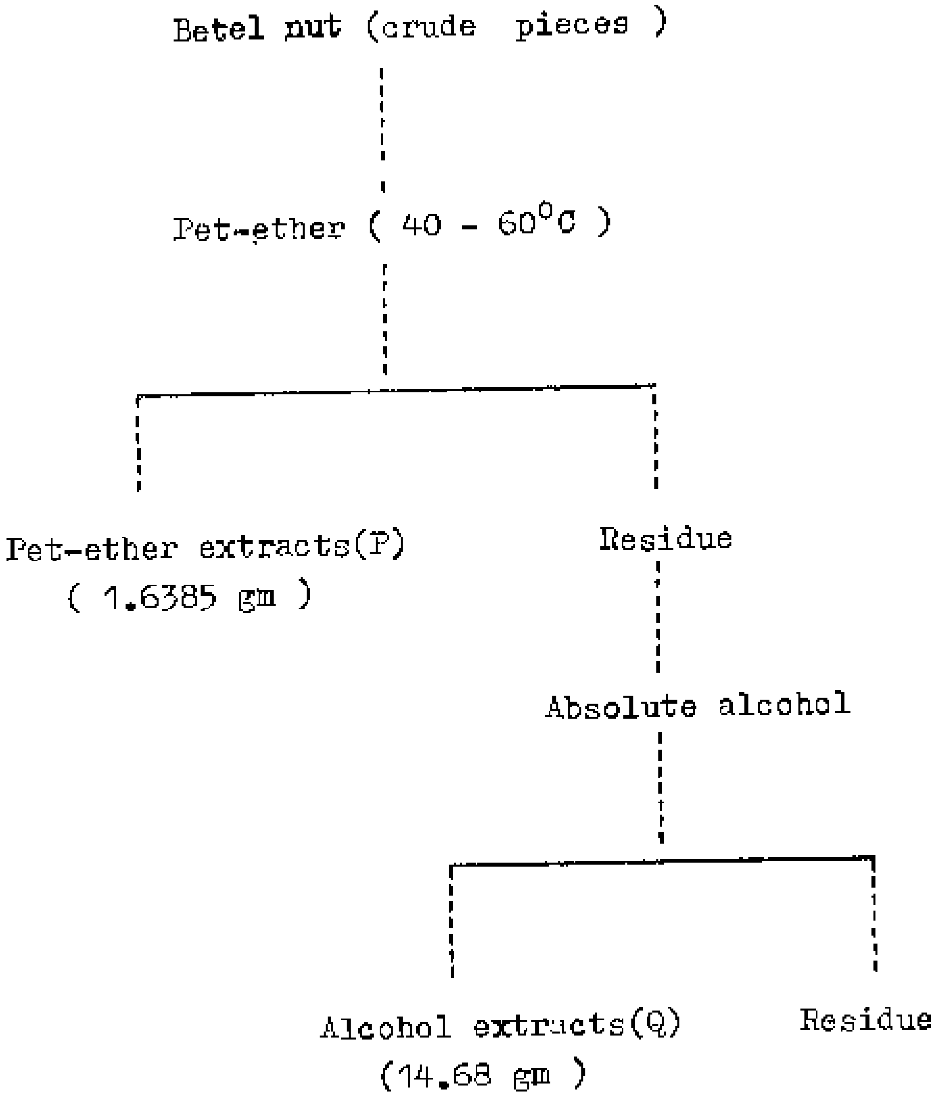


Table No - 2

Batch - 2

Proximate Composition of Betel Nut

% Constituents		
1.	Moisture	48.28
2.	Ash	1.24
3.	Petroleum Ether extractives	0.5411
4.	Absolute Alcohol extractives	4.85021

3.2.7. Preliminary Examination of Petroleum Ether Extracts "P":

P (1.6385 gm) was dissolved in petroleum ether. It was then treated with sodium bicarbonate solution (5%) and the extractives were transferred into a separating funnel. After shaking, a white precipitate settled at the bottom of the flask. The supernatant heavy liquid was carefully decanted off and the white solid mass R_1 was collected (1.02 gm). The heavy liquid was further concentrated at reduced pressure at 30°C when a white yellowish mass R_2 was obtained. This R_2 fraction was the mixture of several compounds on tlc plate. So, further study was not possible.

3.2.8. Study on Mass R_1

The white mass R_1 was a complicated mixture of more than three compounds as revealed by thin layer chromotography plate. Elution with pet-ether and ethyl acetate in different proportions yielded four fractions. One of the fractions was seperated as a pure compound and denoted as S. The other fractions were found to be mixtures of two or three compounds.

3.2.9. Examination of Fraction "S"

Fraction "S" (26 mg), melted at 36-38°C. It was completely soluble in petroleum ether, but partly soluble in carbon tetrachloride, ethyl acetate, chloroform, acetone on warming and insoluble in methanol and ethanol. It gave only one spot on tlc plate containing the solvent system pet-ether: chloroform (1:1). But further study was not carried out. The R_f value in pet-ether : chloroform (1:1) for this fraction is 0.43. Similarly, the R_f value in ethyl acetate : pat-ether (1:1) and pet-ether : chloroform (3:2) are 0.47 and 0.57 respectively.

3.2.10. Study on Mass "Q"

A small amount of 1.025 gm (Q_1), which was taken from (Q) was completely soluble in ethanol but insoluble in pet-ether, acetone, carbonterachloride, chlorofrom. The brown mass Q_1 was a complicated mixture of atleast 6 or 7 compounds as revealed by tlc plate. tlc was taken from Q_1 containing the solvent system pet-ether: ethyl acetate (1:1), alcohol : pet-ether (3:2), ethyl acetate: chloroform (5:2), ethyl acetate : alcohol (2:1). The tailing compounds are obtained, and hence no suitable separation was obtained in this case. So, further examination was not done.

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