1969

Minor Constituents of the Essential Oil of Anemopsis Californica

Dilip Ramrau Sanvordeker
University of the Pacific

MINOR CONSTITUENTS OF THE ESSENTIAL OIL OF ANEMOPSIS CALIFORNICA

A Thesis

Presented to

The Graduate School

University of the Pacific

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

---

by

Dilip Ramrau Sanvordeker

This thesis, written and submitted by

______________________,

is approved for recommendation to the Graduate Council, University of the Pacific.

Department Chairman or Dean:

Ivan W. Rowland

Thesis Committee:

______________________, Chairman

Donald K. Wedegaertner

____

____

Dated May 12, 1969

ACKNOWLEDGEMENT

The author wishes to express his sincere gratitude to Dr. Chaubal for his encouragement and guidance throughout this work. Withour Dr. Chaubal's assistance this work could not have been done. Sincere thanks are also expressed to Dr. Donald K.Wedegaetner for his valuable help in obtaining and interpretation of NMR spectra. Thanks are also due to Dr. James C.King for his help in the preparation of the manuscript.

A gift of some authentic samples from Fritzsche Brothers Inc., is gratefully acknowledged.

Last but not the least, financial assistance from the University of the Pacific is deeply appreciated.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

Anemopsis californica (Nutt.) Hook and Arn. (family Saururaceae), commonly known as Yerba del Manza, is widely distributed in the United States and northern provinces of Mexico. The extract of the roots and rhizomes is used by the natives of Mexico in the treatment of rheumatic disorders, for asthma and also as a blood purifier (1).

Early work on the plant by Horton and Paul (2) and Childs and Cole (3) showed methyleugenol to be the major constituent present in the essential oil isolated from the plant roots and rhizomes. Acharya (4) further studied the essential oil of *Anemopsis* in our laboratory in order to determine its composition and whether any therapeutic compounds were present in the essential oil. The author observed the presence of at least 20 compounds in the essential oil and identified two constituents, namely thymol and piperitone, while confirming the presence of methyleugenol as the major constituent. Also reported was the presence of an unsaturated aromatic ether as one of the unidentified constituents (5). The aim of the present

investigation was to isolate and characterize the remaining unidentified constituents in the essential oil of Anemopsis californica. The essential oil was extracted from the air dried roots and rhizomes, prefractionated, and characterized by gas-liquid chromatography(GC). Several constituents were isolated by gas-liquid chromatography in a pure state and were identified by physical methods of structure determination. The combination of GC and infrared spectroscopy was especially useful for this purpose.

The experimental methods, results of the experiments and conclusions based on these results are given in the following chapters.

CHAPTER II

A REVIEW OF LITERATURE AND PROCEDURES

Extraction of Essential Oils

Essential oils are heterogenous plant constituents which are complex mixtures of compounds, which none-the-less are biosynthetically related to one another (6). The major types of compounds present in the essential oils are:

I. Hydrocarbons - a. monocyclic, bicyclic and open chain terpenes; and b. aromatic

II. Oxygenated Derivatives - alcohols, esters, aldehydes, ketones, phenols.

Guenther (7) has given a review of the various procedures used for the essential oil extraction, used both in industry and research.[1] The major process of extraction is that of distillation with steam or water. The advantages of steam or hydrodistillation are that the procedure is simple, economical, exposure to heat is relatively short and thus usually less likely to cause decomposition in the constituents of the essential oils. Other processes such as solvent extraction, cold expression and enfluerage have limited specialized applications. The

yields in these procedures are relatively low and they are used in the extraction of oils which command a high price in the market.

The problem of unwanted changes in the constituents of the essential oil during extraction of the oil and isolation of the constituents is ever present and must be faced by a researcher. The complexity of the number and types of constituents present in the essential oils and their wide range of physical properties makes the isola - tion of these compounds a difficult task. In a few cases if a compound is present in large amount, it can be isolated by simple distillation; as for example methyl- salicylate from the oil of wintergreen. However, the compounds in small amounts or in traces present considerable difficulties in their detection and isola - tion. The advent and progress in gas-liquid chromato - graphy has enabled investigations to analyze essential oils in great detail and to isolate and identify even trace compounds previously unknown to be present in a particular essential oil.

A fractionation of the essential oil prior to GC analysis is often quite advantageous. Such procedures yield various fractions in which the constituents of the essential oil are separated and may lead to the

concentration or enrichment of trace compounds in some of the collected fractions. The major constituents are also concentrated in some of the fractions and hopefully removed from others so that the problem of peak overlapping in GC is reduced. Such concentration and enrichment of trace compounds also facilitates their detection by chemical means. Acharya has pointed out that piperitone could not be detected in the total oil of Anemopsis californica but that its presence could be shown only after adsorption chromatographic prefractionation.

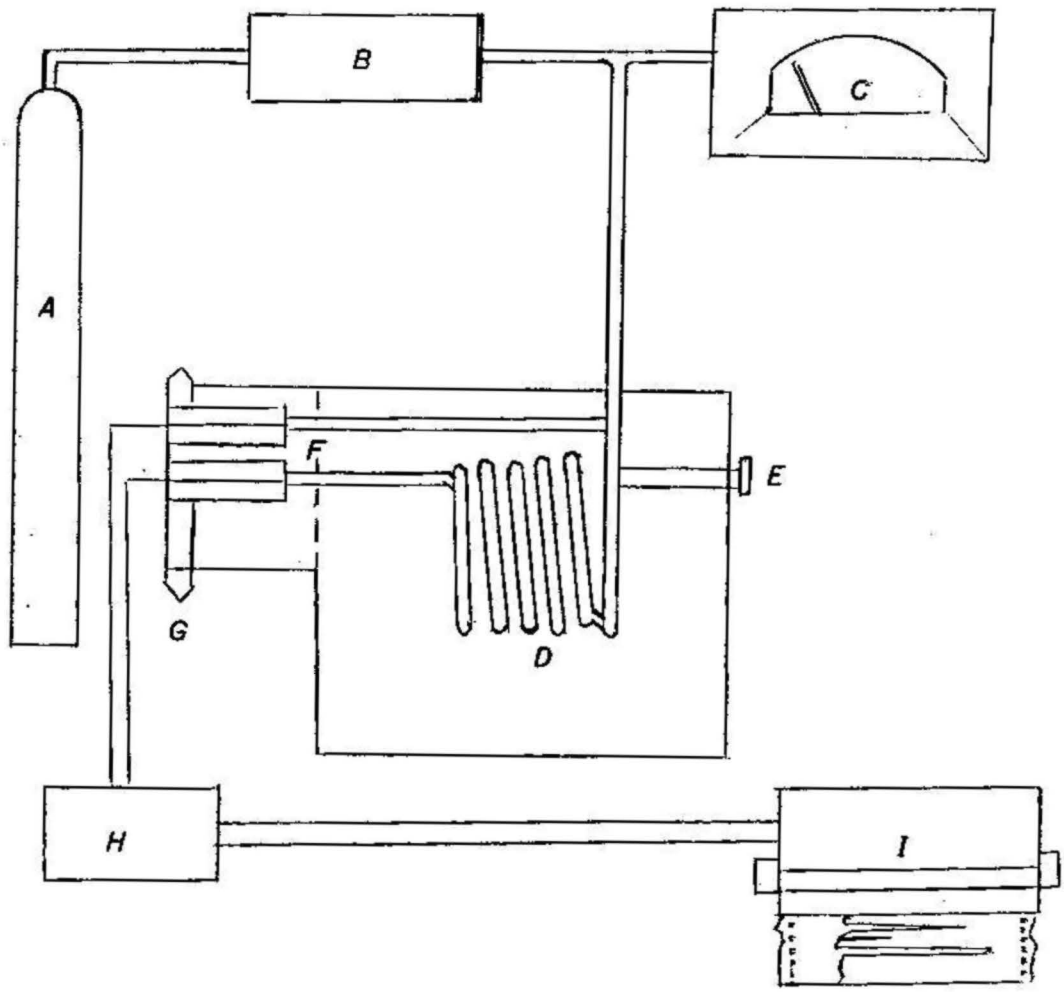
The well established methods of simple distillation and fractional distillation under vacuum, both using high efficiency distillation columns, are used commercially to concentrate the desired components of the essential oils. The procedure of adsorption chromatography can also be used for prefractionation, e.g. to remove terpene hydrocarbons from lemon oil. Preparative GC is a more recent and a quite useful procedure. Prefractionation using heat poses the question of heat degradation of compounds and polymerization of terpenes, as well as loss of sample. Adsorption chromatography on active alumina has also been shown by Nigam et al. (8) to cause isomerization of epoxides and thus again, raise the problem of alteration of the original composition of the essential oil.

Gas-Liquid Chromatography(GC)

Speed, sensitivity and versatility are the main advantages of GC. GC was applied early to the analysis of essential oils, and has been a great impetus to the study of essential oils and perfumes as well as flavors and aromatic substances present in beverages (9).

The technique of GC may be described as a chromatographic method of separation wherein the mobile phase is a vapor percolating through the liquid stationary phase by the use of an inert carrier gas such as helium or nitrogen under pressure. The stationary phase in GC is an active partitioning liquid which has a low vapor pressure at the temperature of operation, adsorbed on a suitable inert solid support in certain known proportion. A schematic diagram of a gas chromatograph is shown in Fig. 1. The mixture to be analyzed is instantly vaporized in the heated "injector" end of the column and passed through the "column" by the carrier gas. The column consists of a suitable glass or metal tubing packed with the stationary phase, the effluent end being connected to the "detector chamber".

The detector chamber consists of the detector which indicates the presence presence and measures the amount of



A- Gas Tank ; B- Pressure Regulator ; C- Manometer

D- Column ; E- Injector Port ; F- Detector Chamber,

G- Outlet Port ; H- Bridge ; I- Recorder

Fig. 1. Schematic Diagram of a Gas Chromatograph

components in the column effluent. A good detector has a high sensitivity and a fast linear response to the effluent gas. The diferential types of detectors are most commonly used (10). In this type of detector, some property of the effluent is continually compared with that of the carrier gas. Thermal conductivity, electrical conductivity, dielectric and ionization constants are some of the physical properties used in differential types of detectors. In this research, the "katharometer" or thermal conductivity detector was used. The detector chamber of such a detector system consists of a set of two katharometers. The diferential response of the two katharometers is recorded on a strip chart recorder, which gives various peaks corresponding to each of the component as it successively elutes out of the column into the detector chamber. These peaks are recorded on a time axis so as to give the retention time(RT) of each of the peaks of a mixture. A complete chart of the peaks of a mixture constitutes a "chromatogram".

The most important consideration for the separation of a mixture by GC is the choice of the liquid stationary phase. Selection of the proper column is based on the prior broad knowledge of the chemical nature of the components of the mixture to be analyzed. The stationary

phase must have a certain solvent power for the components of the mixture. These components will then be diferen - tially partitioned between the gaseous(mobile) phase and the liquid(stationary) phase and be retarded or retained on the column in a diferential manner,thus bringing about their separation. Keulemans (11) has discussed the various types of stationary phases used in GC. Langer and Sheehan (12) have more recently discussed the theory of the choice of stationary phases. A certain compatibility between the components of a mixture and the stationary phase is required. Thus, if the components were non-polar a non-polar stationary phase will effectively give a normal separation; a polar phase will efficiently separate polar compounds. Some semi-polar phases such as phthallic esters can be used for a wide variety of compounds. Keulemans recommends such semi-polar phases for the effective separation of mixtures of compounds of non-polar, polar and semi-polar nature.

Essential oils are mixtures of compounds ranging in polarity from non-polar hydrocarbons to polar aromatic alcohols. Some 50 stationary phases have been used for the analysis of essential oils (13, 14). Von Rudloff (15, 16, 17, 18) has reported on the use of polyglycols

(carbowaxes[a]) for the separation of essential oil constituents. Nigam and his co-workers (19, 20, 21, 22) have effectively used polypropylene glycol adipate (Reoplex-400[a]) for the analysis of various plant essential oils.. The semi-polar nature of these stationary phases enables one to separate the varied types of constituents of the essential oils effectively.

The second important consideration in gas chromatography is the selection of the column temperature. Purnell (23) has thoroughly discussed this parameter. The effective and efficient resolution of the varied compounds of essential oils as regards to the time for each chromatographic run poses considerable difficulties. If low column temperatures were selected, the peaks eluting out early are sharp while the later peaks tend to be broad and their RT are inordinately high. High column temperatures yield poor resolution of the early peaks although the later peaks are sharp and the chromatographic run takes shorter time. Griffith and co-workers (24) first used the technique of Programmed Temperature Gas

---

[a] Wilkens Instrument & Research Inc., Walnut Creek, California.

chromatography(PTGC) for the separation of mixtures of low and high boiling alkyl halides. Starting with low column temperatures, these authors raised the column temperature at a constant rate during the chromatographic run, keeping the carrier gas flow-rate constant. This technique improves the resolution of components and economizes the time for a chromatographic run. The technique of PTGC has since been developed into the following five variations:

1. Linear - temperature is increased at a prechosen linear rate.
2. Stepwise - increase in temperature is in steps, being held isothermal for a short period at each increment.
3. Multilinear - several linear rates of temperature increase are used during a chromatographic run.
4. Linear-isothermal - a chosen linear increase in temperature is used for a set period and then allowed to remain isothermal at that higher value until the end of the run.
5. Isothermal-linear - the initial temperature is maintained for a set period after which it is raised at a chosen rate until the end of

the run. These variations are shown diagramatically in Fig. 2.

Gas Chromatographic Identification by Retention

Recently an extensive review on the techniques and problems involved in the identification of the peaks in a gas chromatogram was given by Purnell (25). Dal Nogare and Juvet (26) and Crippen and Smith (27) have also described various identification procedures in detail. Under a definite set of operational conditions, the retention volume(V_R) or RT is characteristic of a certain compound. Thus in analogy with Rf values of paper chromatography, the retention values can be used in the identification of the compounds resolved by gas chromatography. However, the certainty with which a peak can be identified depends upon the accuracy with which the retention volume/time can be measured, and the number of closely eluting compounds with which a peak can be confused. In the case of a complex mixture, giving rise to many peaks (such as an essential oil), interferance is particularly liable to occur. The requirements of absolutely constant operating conditions in a series of chromatographic runs is almost impossible in practice to attain. The rather common practice of relying entirely on the retention coincidence

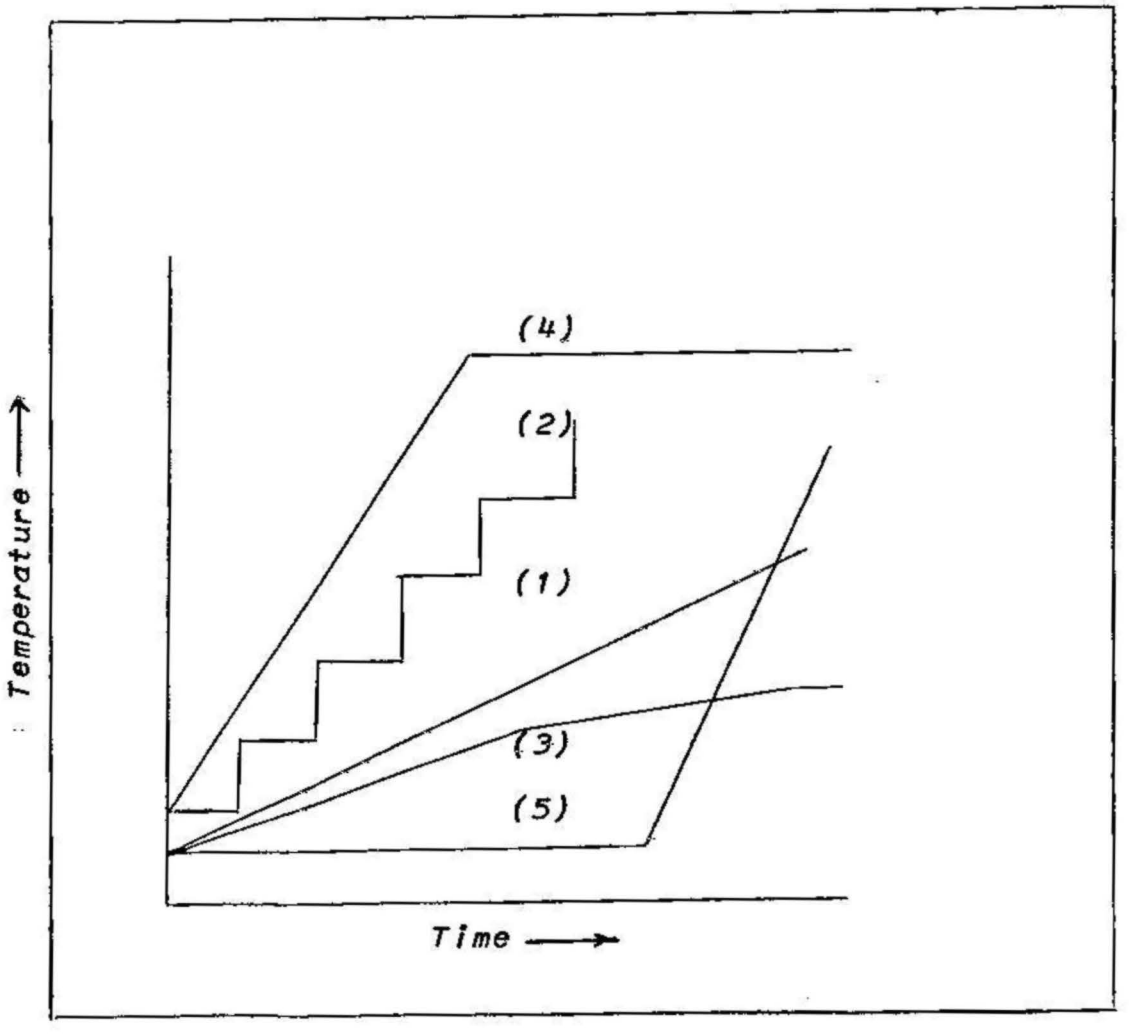


Fig. 2 Types of Programmed Temperature GC Techniques. (1) Linear PTGC (2) Stepwise PTGC (3) Multilinear PTGC (4) Linear-isothermal PTGC and (5) Isothermal--linear PTGC

methods have undoubtedly led to much qualitative error (25). The retention coincidence method of identification consists of the addition of a supposed component, i.e. an authentic sample, to the unknown mixture and the GC analysis of this artificial mixture under identical operating conditions. An increase in the height or area of any particular peak of the mixture is noted and it is assumed that these two, the unknown peak and the added authentic compound, are one and the same. Under very favorable conditions, it is possible to achieve the qualitative and quantitative analysis of the mixture on the basis of the retention data alone. However, the ambiguities in this method are too numerous and one must resort to other methods of characterization.

Retention data, can however, be a valuable aid in the characterization of compounds in a mixture if considered in conjunction with other data. The relative retention times(RRT) help one in the correction of the small but important variations in the operational parameters in a series of runs (19, 20). A known authentic compound in a known volume is chromatographed along with the unknown mixture and the chromatogram is established. The RT of all the peaks in a mixture are normalized with respect to the retention times(RT) of this known standard compound. The

assumption is that any change in parameters will affect the standard RT as well as the unknown RTs to the same degree and their effects cancel out in the normalized RRT values.

Alternately, a peak in the center of the chromatogram of unknown can be identified and chosen as the standard for normalizing the RT values of all the other peaks. Linalool (21, 22), d-limonene (21, 22) and d-camphor (17, 18) have been used to measure the RRT values of the constituents of essential oils.

The retention coincidence method of GC identification of components of a complex mixture, becomes more useful when one carries out multi-column GC. Keulemans states that "for the gas chromatographic analysis of a complex mixture, it is a rule rather than an exception that such mixtures be run on two or more different stationary phases", i.e. columns. The use of several columns reduces the chances of mistaken identification, particularly if the columns are widely different in their polarities. Multi-column GC involves relating the peaks in one chromatogram with those of all other chromatograms in order to determine the retention values of an unknown compound on all the columns employed. For this purpose, the unknown compound peak on column A may be isolated and rerun on column B to establish its retention values on different columns.

Trapping of Gas Chromatographic Peaks

As previously indicated, multi-column gas chromatography of an unknown mixture involves the isolation of individual peaks on one column and rechromatography of each of these isolated fractions on other different columns. The isolation involves the condensation or trapping of each of the GC peaks coming out of the column at the effluent port. Such trapped fractions can be used not only for rechromatography on the same column for further purification, or on different columns for establishing retention data, but also for other physical and chemical characterization.

The designs of the traps for collection of GC fractions involves either a scrubbing of the effluent gas by a suitable solvent, or the condensation of the compounds in the effluent gas by cooling so as to reduce the vapor pressure of the elute. The method involving solvent scrubbing is quite convenient, simple and frequently used, especially where physical means such as IR or UV spectroscopy are to be used for identification. Kroman and Bender (28) point out, however, that the amount of solvent compared to that of the GC fraction is more likely to interfere in subsequent treatment of the collected fractions. For most purposes, the low temperature trap is most

suitable. The effluent from the detector is passed through a suitable trap cooled by dry ice, liquid nitrogen or cooling mixtures such as salt and ice, to bring about rapid cooling of the eluant vapor. The compound condenses in fine drops on the walls of the trap while the carrier gas escapes. Such rapid cooling often produces aerosols and, unless the trap is designed properly, the condensed droplets may escape along with the carrier gas. Various designs of traps are available from preparative gas chromatograph manufacturers and accessories suppliers, which aim at reducing or eliminating aerosol formation and promoting efficient condensation of the effluent fraction.

Condensation of milligram quantities of fractions in preparative scale GC is not too difficult using the available traps. The submicro quantities of fractions encountered in analytical GC, however, pose considerable difficulties. Even if such submicro quantities were trapped, the recovery and handling of these for physical methods of identification, such as IR, UV and NMR spectroscopy leads to the severe losses of the sample unless special precautions are taken. Whenever possible, direct monitoring of the effluent fraction is desirable. Thus, Nigam and co-workers (29) have used a method of direct condensation of the effluent fraction of α-pinene on a salt

plate cooled in a dry ice jacket. This salt plate can then be placed directly in the IR spectrometer for analysis. Giuffrida (30) has also described such a technique. Thomas and Dwyer (31) have described the technique of condensing the eluate on 25 u cellulose membranes which can be placed directly in an IR spectrometer for obtaining the spectrum. A technique of collecting the effluent fraction in a simple hypodermic syringe, cooled in a jacket of dry ice has been described by Edwards and Fagerson (32). Oxygenated and sesqui-terpenes were collected with good efficiencies by Teranishi (33) in simple five inch lengths of glass tubes drawn out at one end and packed with clean stainless steel wool.

Physical Methods of Characterization of GC peaks

It has been pointed out previously that although the retention data, especially those obtained with multi-column GC, are a valuable aid in the characterization of GC peaks, reliance cannot be placed on such data alone. Other data, such as those obtained by the usual procedures of characterization of organic compounds have to be obtained also. The small, often microgram quantities of the GC fractions, preclude the use of such destructive methods as elemental analysis, functional

group determination, preparation of derivatives, etc. Physical methods of characterization such as the spectroscopic methods are invaluable aids for this purpose. The UV, IR, MS and NMR data are often the only data available for the determination of the structure of unknown compounds obtained in pure state by GC. Structural information which can be obtained by means of the various spectroscopic methods, is complimentary to each other. For example, the problem of determination of structure on the basis of IR spectrum becomes much more tractable if a MS is also available. UV data may also permit the clarification of important structural details and an NMR spectrum may be able to furnish information which could not be obtained by means of the three previous techniques. While a few hundred microgram quantities are sufficient for an IR, UV and a MS, larger samples are usually needed for running an NMR spectrum.

A molecule placed in the path of a monochromatic beam of electromagnetic radiations absorbs those frequencies which correspond to one of the natural frequencies of the molecule and momentarily attains high energy content. This absorption of energy is a highly specific property of the molecular structure and the frequency range within which energy can be absorbed is specifically dependent on

the molecular structure of the absorbing compound. The frequencies absorbed and the intensity of the emergent beam can be recorded by a suitable device placed in the emergent beam. Instrumentation is now available which uses each of the various regions of the electromagnetic spectrum. However for the determination of the structure of organic compounds the ultraviolet, 200 - 400 mμ; infrared, 2.0 - 16 μ; microwaves(ESR), 0.04 - 25 cm. and radiowaves(NMR), 25 cm, are most commonly used radiations. The infrared spectroscopy has been the most widely used of the four, as the absorption of IR radiations by molecules gives the most relevant information about the molecular structure. The UV, ESR and NMR spectral data are more or less complimentary to the IR spectrum.

Infrared Spectroscopy

Freeman (34) has pointed out that the identification of a compound by IR spectroscopy alone is a simple problem when the unknown compound is actually a known substance whose IR spectrum has been reported in the literature or is otherwise available to the investigator. However, after having matched the spectrum of an unknown with a published one, it is generally wise to obtain an authentic sample of the compound and record its spectrum in conditions identical with those previously employed for

the unknown sample. If the IR spectrum of the unknown compound and that of a known are found to be almost but not completely identical, before considering that the two compounds may only be closely related rather than identical, a purity check may be performed. Occasionally the published spectrum may be that of a less pure sample than the unknown compound under investigation, especially when GC has been used to isolate the unknown.

When the spectrum of the unknown cannot be matched with any available recorded spectrum, it must be treated as a true unknown and its structure may be postulated by carefully examining the IR spectrum. The identification of such a compound will require accumulation of many more physical and chemical data.

Almost since the begining of GC, IR spectroscopy has been used as a means of identification of GC fractions (25). Various techniques of preparation of samples for IR spectroscopy have been devised. The microamounts available after GC separation pose a number of problems in sample handling and no one method can be used for all types of samples. The physical nature of the sample, such as its volatility or non-volatility and whether it is a solid or a liquid determines the method of sample collection and preparation for IR.

A GC fraction condensing as a solid presents little problem. Repeated injections and condensation of the fractions may yield enough sample to press a KBr pellet for IR spectroscopy. Or small amounts of the fraction may be condensed in a suitable solvent and used to fill a micro-cavity sodium chloride cell. The micro-samples may require the use of a beam condenser and a reference beam attenuator which enables one to obtain IR spectra with good base-line characteristics. The handling of liquid GC fractions is quite difficult, especially if these fractions are volatile, as transfer of these samples from the trap to a suitable IR cell may lead to intolerable losses of an already meager amount. Volatility of a sample may preclude pressing out even a micro-KBr disc. The sample can be transfered to a NaCl micro-cavity cell with some difficulty of manipulation. The use of a suitable solvent to transfer the micro-sample to the IR cell is not without problems. Solvent absorption interferes and the solution may be too dilute to get a good spectrum. Various devices have been designed and are commercially available which aim at the direct collection and transfer of a GC fraction to an IR cell.

Barnes Engineering Company[b] manufactures a device based on the technique of Edwards and Fagerson (32). The condensed droplets are tranfered into the cell by placing the assembly into a centrifuge for a few minutes at low speed.

Adequate spectra may be obtained with as little as 0.2 u; of the sample. Wilks Scientific Corporation,[c] Model 15, GC-IR analyzer is a capillary internal reflectance cell in which the sample is collected and analyzed in the same cell. The same maunufactureralso supplies a vapor phase GC GC collector, Model 41. In this the sample is collected and kept heated in the cell in vapor phase. The complete heated assembly attaches to a sample holder of an IR spectrometer. One problem with this technique is that the vapor phase IR spectra may not be available for comparison. The cost of such devices is also a factor which must be considered especially when these devices may not be useful for a particular sample which a researcher is handling at the moment. He may have to devise some other techniques of his own.

As previously stated, the UV, NMR and Mass

---

[b] Barnes Engineering Company, Stanford, Connecticut.

[c] Wilks Scientific Corporation, South Norwalk, Connecticut.

spectra are also valuable in characterizing the GC fractions. The limited structural information of an UV spectrum, the cost, and often the unavailability of an NMR and MS, limit their application. NMR requires somewhat larger samples(> 1 mg.) although good spectra may be obtained using micro-samples with the recent introduction of computer averaging of multiple scans. Larger sample limits the application to post-trapping techniques, i.e. sufficient fraction is first trapped and then transfered in a suitable solvent to an NMR cell. NMR spectra can provide very valuable information in cases where the interpretation of the IR spectra of the unknown leads to more than one possible structure. It is also valuable when the sample is a true unknown compound and the IR alone cannot lead to a possible structure for the compound.

Purnell (25) has pointed out that the MS is one of the few detectors which simultaneously provides both qualitative and quantitative information about the GC effluent fraction. A GC-MS instrument, in which the GC effluent is directly led into a fast-scan MS is now available. MS is perhaps the more useful than IR or UV in characterizing the GC peaks and its spectra lend themselves to the interpretation and cataloging. The GC-MS combination is the most versatile all-purpose analytical

system available. This versatility is, however, obtained at great cost and even if the cost may be reduced, the particular requirements of the project can often be met in some other less expensive ways. Thus, the high cost of the GC-MS system limits the application of this most versatile technique to GC analysis.

GC Quantitative Analysis

The response of almost all the detectors used in GC is a function of sample size as well as some molecular property (25). Thus a chromatogram gives not only qualitative data but also quantitative information of the composition of the sample mixture.

Gill and his co-workers (35) have divided the quantitation process into the following three steps:

(a) Generation of the analog signal (Chromatography): involves the chromatograph and its components such as injector, column, detector and associated hardware.

(b) Analog to digital conversion (Integration): involves the recording and integration apparatus which can range from a simple recorder plus a ruler to a complex electronic integrator.

(c) Relation of the digital data to composition (Calculation):
involves the calculating apparatus which may range from simple pencil and paper or a slide rule to even a computer.

Gill and McNair (36) have summarized the sources of error in quantitative GC. With conventional GC systems, accuracy is affected most by recorder characteristics and the operator's skill. Some of the factors listed by these authors are as follows:

(a) Sampling Technique: Sample should be introduced without bias.

(b) Chromatograph Performance Factors: Column separation, detector characteristics, electrometer or TC bridge circuit accuracy.

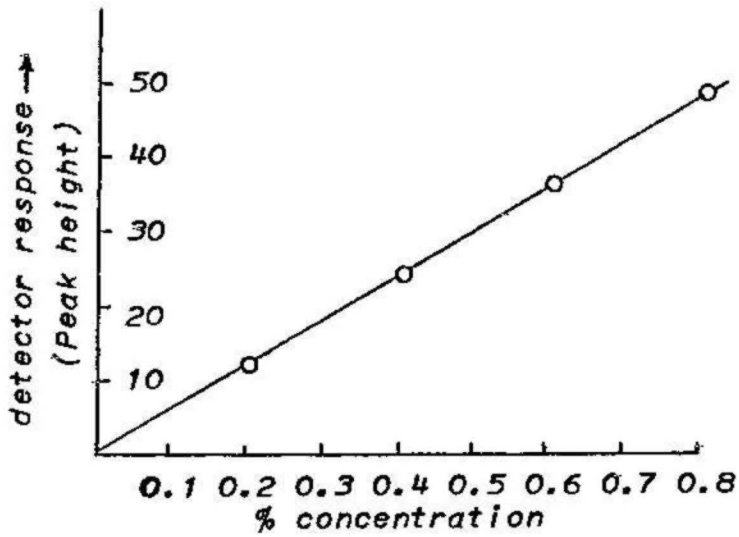
(c) Recorder Characteristics: Linearity, pen speed, dead band, electrical zero stability, etc., can affect the accuracy of chromatograph tracing. All quantitative techniques except electronic integration are based on the recorder trace.

(d) Human Errors: Operator's skill in introducing samples, operating the chromatograph, counting integrals and performing calculations all affect accuracy.

Thus the variables are numerous and hence the only acceptable practice is to determine the characteristics and performance of each instrument with known standard mixtures under study (35).

The detector response is both a qualitative and quantitative measure of a component of a mixture. This factor can be used in quantitative analysis by GC. The peak height obtained under stable operating conditions is a measure of the concentration of the compound. Thus, a detector response curve can be established. Known, varied concentrations of the compound under study are injected and separate chromatograms are established for each concentration under constant, stable operating conditions. A plot of concentration vs. peak height is then drawn and, from this standard curve, one can obtain the concentration of the compound under consideration in an unknown mixture as shown in the following diagram. This procedure requires that the compound be identified and that a pure authentic sample be available to establish the peak height - concentration standard curve.

The percent composition of an unknown mixture can be obtained by measuring the area (A) of each peak and relating these areas to the total area under the chromatogram, thus:

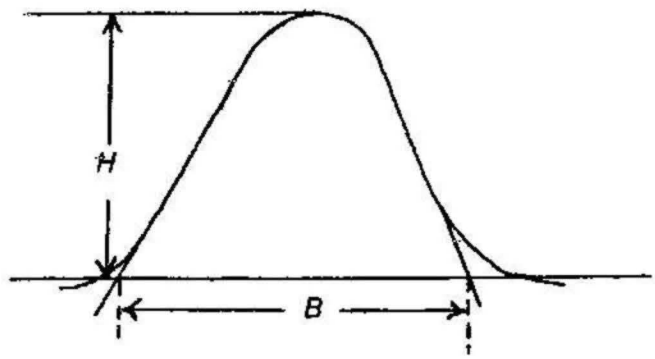


$$\frac{100 \times \text{corrected area A}}{\text{Total corrected area}} = \%\ v/v\ .$$

Correction factors may be determined for each given system by the GC of standard known mixtures. One of the following several methods may be utilized to determine the areas under the peaks.

1. Triangulation:

A triangle is constructed by drawing tangents to the slopes of the peaks and the intersection of these tangents with the baseline gives the base, B. Then A = BH/2. The slopes

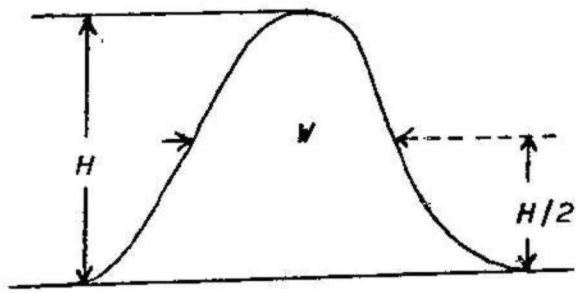


of the peaks and operator's skill affect this measurement.

However, the triangle formula gives only about 97% of the true peak area, at best.

2. Height x Width at Half-height:

The triangulation method suffers from inaccuracy caused by the baseline shift due to tailing or adsorption. The area is calculated by the formula: $A = HW$. However, for very narrow peaks W may not be accurately measurable. For



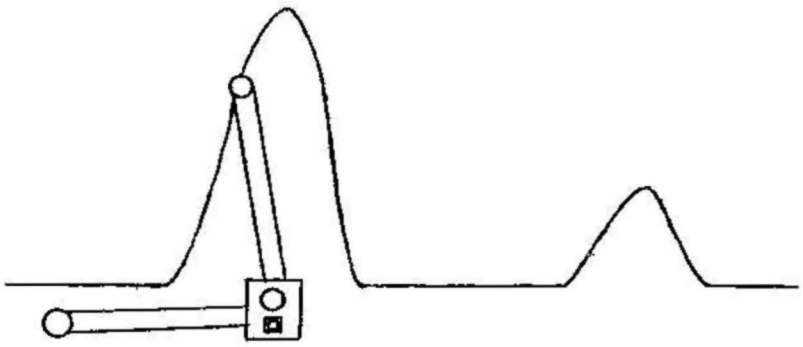
symetrical peaks, one can achieve reasonable precision but non-symetrical peaks give poor results.

3. Cut and Weigh:

The chromatographic peak can be cut from the tracing, the cut piece weighed, and then the weight ratio of the peak weight to the total chromatogram weight obtained. This procedure destroys the chromatogram and factors such as constancy of thickness and moisture content of the chart paper, as well as accuracy in cutting and weighing of the peak affect precision and accuracy.

4. Planimetry:

The peak can be traced manually with a planimeter, a manual analog digital device, and the peak area read on the dial. The procedure is time-consuming and accuracy



will depend on operator skill in tracing the peaks.

5. Integrators:

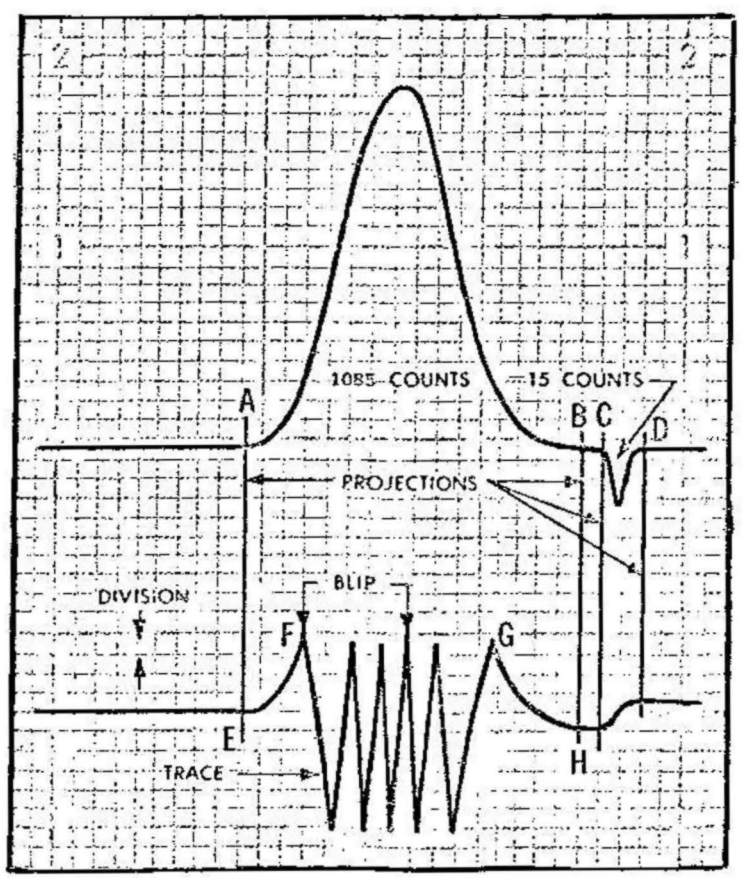
Various electronic instruments, such as ball and disc, analog and voltage integrating devises, have been designed for direct attachment to the recorder so that integration is performed with the recording of the chromatogram. The Disc Integrator[d] is, by far, the most widely used. The degree of precision and accuracy obtained with the Disc Integrator is limited by the mechanical performance of the recorder and, hence, a careful adjustment of the recorder

---

[d] Disc Instruments Inc., Santa Anna, Callfornia.

performance is necessary.

The Disc Integrator attached to the recorder is so arranged as to yield a pen trace which is continuously and automatically recorded below the peak trace as shown in the diagram below. To read the integrator trace, first,



the start and the end of the peaks are determined and the lines are projected downwards to the integrator pen trace.

The value of the interval is calculated by counting the chart graduations crossed by the integration trace. Each

interval has an arbitrary value of ten and a complete traverse produces a blip. The 600 counts between successive blips make it easier to read the trace. The percent composition of a peak is obtained in the following manner.

$$\frac{100 \times \text{counts under a peak}}{\text{total counts under all peaks}} = \%\ v/v$$

Electronic integrators eliminate the dependence on the recorder and human factors. However, the cost of such integrators is a limiting factor in their use. Gill, et al. (33) have given the following figures as an estimate on the precision obtained with various methods of area measurement. Typical standard deviations have been found to be:

Planimetry	4.0%
Triangulation	4.0%
H x W	2.5%
Cut & Weigh	1.7%
Disc Integrator	1.3%
Electronic Integrator ..	0.4%

CHAPTER III

EXPERIMENTAL AND RESULTS

Extraction and Fractionation of Essential Oil of Anemopsis

The process of hydrodistillation as employed by Acharya with modifications as described below, was employed to obtain the basic materials investigated in this study. The major constituents piperitone, thymol and methyleugenol had already been identified and were of no interest in this work. In fact these interfered in obtaining good GC separation of the minor constituents of the essential oil of Anemopsis. Acharya's attempt at fractionation of the oil by the use of Spinning Band Fractionator[e], had yielded four fractions. The first fraction of these four was enriched in the compounds of interest. However, the application of heat during distillation resulted in intolerable loss of sample and, as previously indicated, introduced the possibility of heat artifacts and alteration in the original constituents of the essential oil.

---

[e] Nester/Faust Manufacturing Corporation, Newark, Delaware.

During preliminary work on the hydrodistillation of the essential oil of Anemopsis, it was observed that the initial amounts of the oil which were condensed in the Clevinger tube were colorless and the oil gradually changed color to a deep blue towards the end of the hydrodistillation period. This lead us to believe that the various constituents of the oil distilled over at different periods of distillation and that, if small amounts of fractions of oil were collected separately at various time periods during the hydrodistillation, one may obtain fractions enriched in some of the constituents of interest. This assumption seemed logical, as Acharya had already indicated that the constituents ranged in boiling points of a few degrees above room temperature to that of thymol(253°), and these could be expected to distill over at different time periods during the total distillation period.

In order to test this hypothesis, two separate 500 g. batches of the roots and rhizomes of Anemopsis[f] were ground to a 40 mesh powder in a Straub mill.[g] One batch served as a control and was hydrodistilled with 3.5 l of

---

[f] Lot No.- RM-67-91, Hathaway Allied Products, Los Angeles, California

[g] Model-4E, The Straub Company Inc., Philadelphia, Penn.

water in a five liter round bottom flask. The hydrodistillation was performed continuously for 30 hours at the end of which the oil collected in the Clevinger tube was withdrawn and kept overnight over anhydrous sodium sulfate to remove water.

The second batch was also hydrodistilled with 3.5 l of water for 30 hours, but in an interrupted manner. After the initial short period of distillation, the flask was cooled and the amount of condensate in the Clevinger tube withdrawn to yield fraction #1. The distillation was resumed for another short period followed by cooling and withdrawal of the condensate to yield fraction #2, and so on. In this manner seven separate fractions were collected during the distillation period. Each fraction was dried overnight over anhydrous sodium sulfate. The data on these two batches and the seven fractions from batch II are given in Table I and II.

The control batch of <u>Anemopsis</u> was used to determine relative yield of the two batches and to establish a general chromatogram of the total oil on the two stationary phases which had been selected (see section on Gas Chromatography). The fractions 1, 3, 4, 5 and 7 were monitored by GC on Reoplex-400 column at 160° column temperature. Chromatograms of these fractions, obtained under the same

operating conditions are given in Fig. 3 - 7. Peaks 4 to 25 were of interest as these constitute the minor constituents of the essential oil of Anemopsis. An examination of the chromatogram showed that complete isolation of any one constituent in any one fraction had not been accomplished. The major constituents which are indicated by peaks 1, 2 and 3 were present in all the fractions. This also seemed to be the case when Acharya had employed a Spinning Band Fractionator. However, it was obvious that the major constituents were present in considerably lower concentration in fraction 1, as compared to other fractions, and that the other constituents of interest in this work were comparatively enriched in this fraction. Therefore fractions 1 and 2, obtained from batch II of the hydrodistillation, were used for the GC isolation and identification of the constituents as described in the following sections. The other fractions were not examined further.

Gas Chromatography of Essential Oil of Anemopsis

As previously stated, Reoplex-400 and carbowaxes have been successfully used for the analysis of various essential oils. These two, with their semi-polar nature, were thought to be best suitable for this study.

Table I. Data on the hydrodistillation of two batches of Anemopsis.

	Batch I Control	Batch II
Grams of Drug	500	500
Hydrodistillation Period	30 hr	29.25 hr
Total Oil (ml.)	33.5	31.0
No. of Fractions	1	7
Percent Yield	6.7	6.2

Table II. Data on Hydrodistillation of Batch II of Anemopsis.

Fraction #	Distillation Period(hr)	Volume(ml.) Collected	Color
1	2.5	2.5	colorless
2	2.0	2.5	pale yellow
3	3.75	4.0	pale yellow
4	4.5	4.0	light green
5	3.5	4.0	greenish blue
6	7.5	8.0	greenish blue
7	6.0	6.0	bright blue

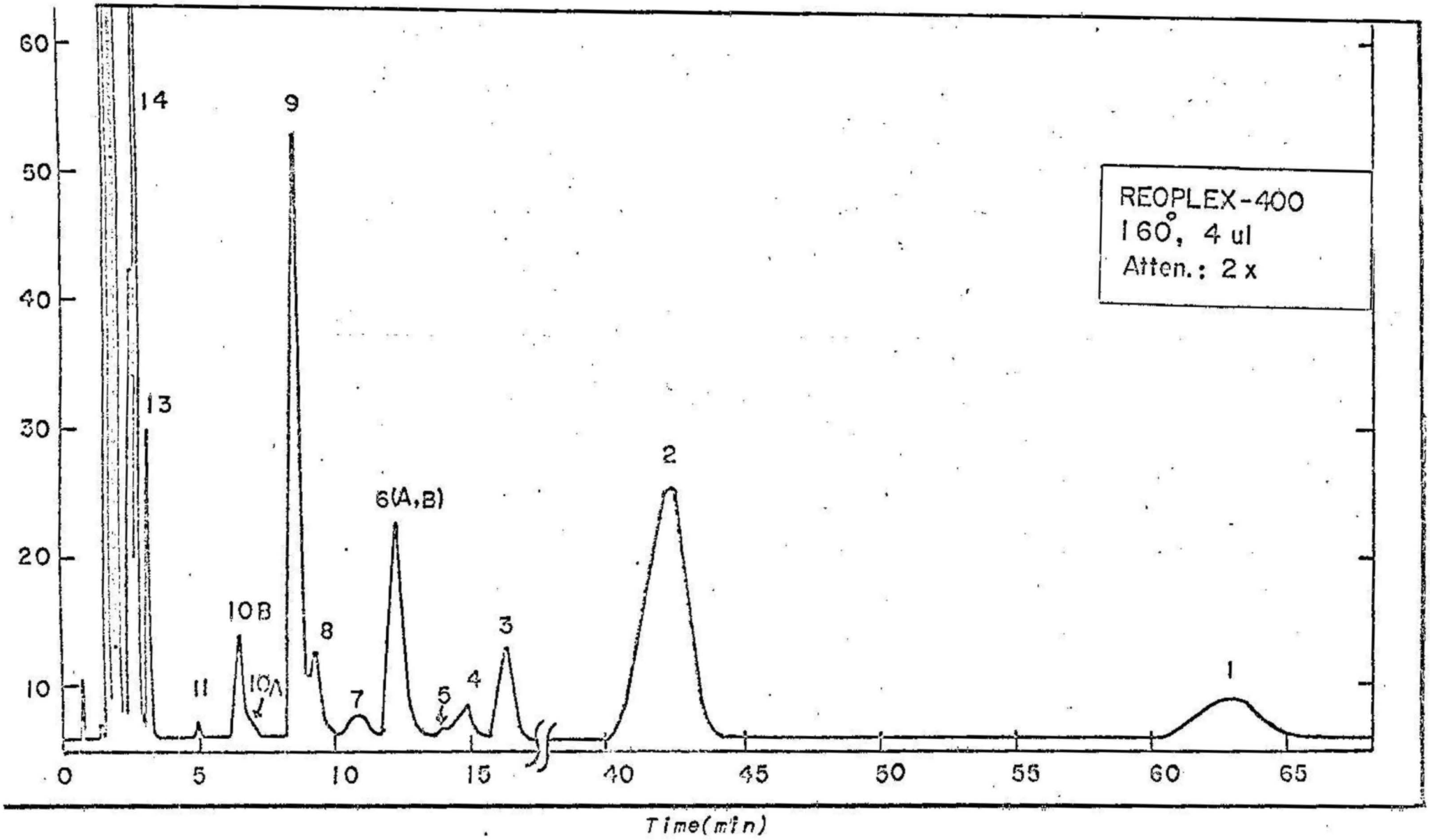


Fig. 3. Gas Chromatogram of Fraction 1 of the Essential Oil of Anemopsis.

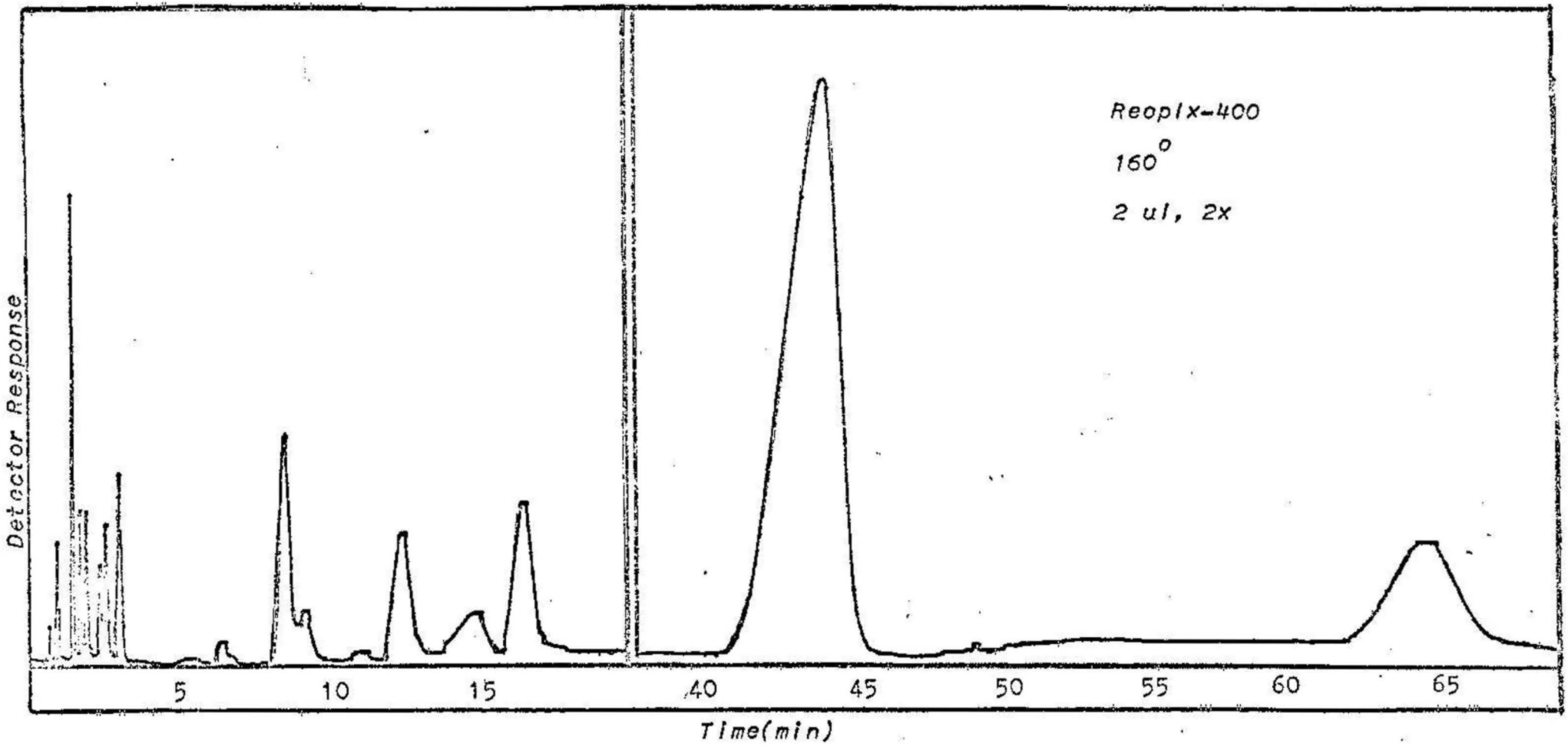


Fig. 4. Gas Chromatogram of Fraction 3 of the Essential Oil of Anemopsis.

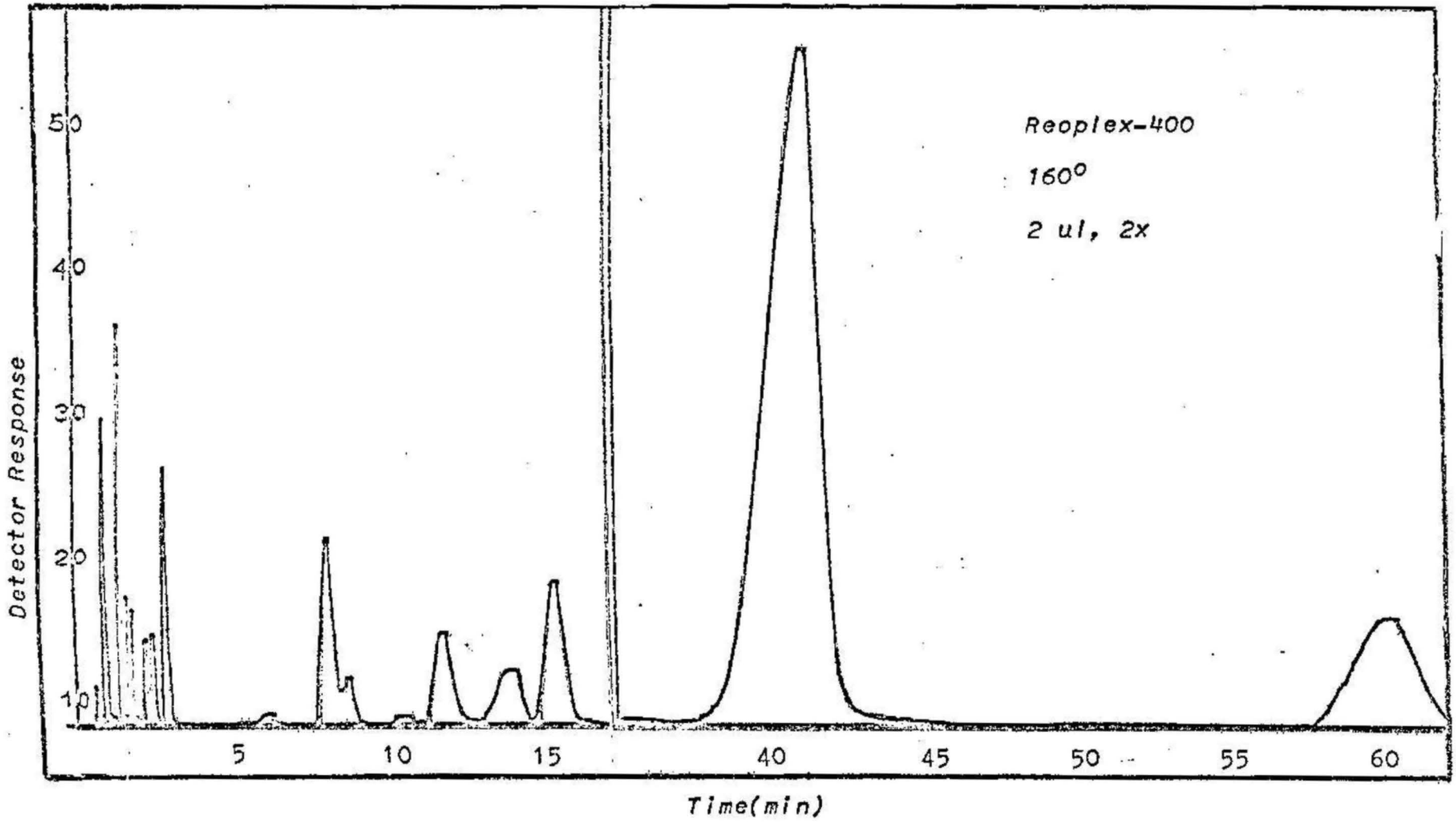


Fig. 5. Gas Chromatogram of Fraction 4 of the Essential Oil of Anemopsis.

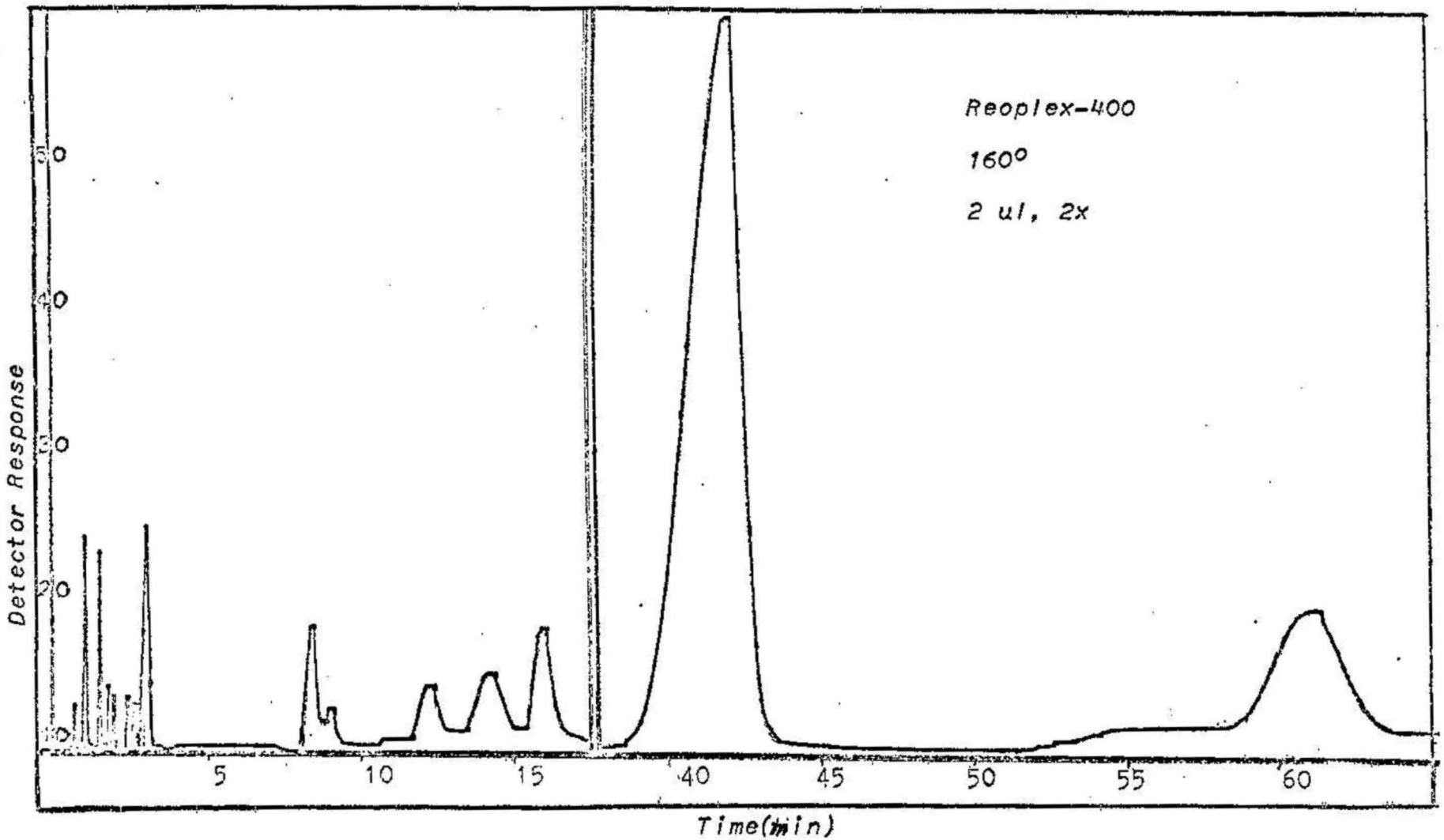


Fig. 6. Gas Chromatogram of Fraction 5 of the Essential Oil of Anemopsis.

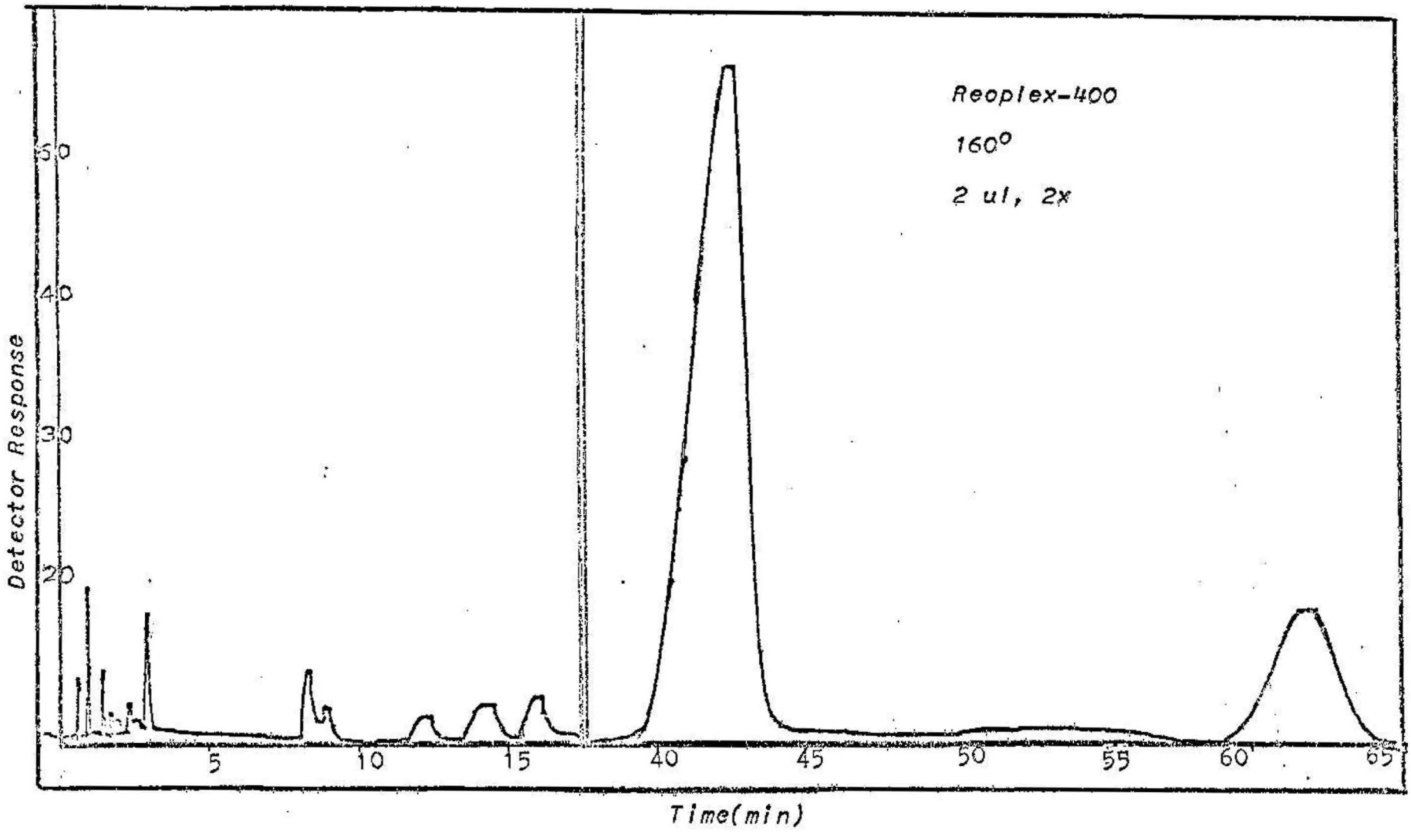


Fig. 7. Gas Chromatogram of Fraction 7 of the Essential Oil of Anemopsis.

Reoplex-400 was the stationary phase used most for finger-printing, resolution and collection of the constituents. Carbowax-20M gave resolution simmilar to Reoplex-400 and was used for specific separation of close lying peaks, as described later. Two columns, one each of Reoplex-400 and Carbowax-20M were prepared by the procedure which follows.

Ten g. of stationary phase were weighed in a tared beaker and dissolved in 50 ml. of chloroform. This solution and the washings(100 ml.) were then transferred to a 500 ml. round bottom flask. An additional 200 ml. of chloroform were added to the solution, followed by 50 g. of acid washed Chromosorb W(Chromosorb W, A/W,60-80 mesh[a]).

The slurry was shaken continuously for eight hours to coat the inert support uniformly after which the solvent was completely removed at 45° under water-aspirator vacuum with the aid of a rotary evaporator. The dry material was gently sieved by tapping through a 100 mesh sieve before it was stored in a tightly closed wide-mouth jar.

A 10 foot length of aluminium tubing, 1/4" i.d., was cut and straightened. With the lower end of the column plugged with selanised glass wool[h], a small funnel was attached to the upper end of the column which was then

---

[h] Analabs Inc., Hamden, Connecticut.

packed with the pre-coated inert support prepared as described above. After each addition of small portions, the column was vibrated by a portable electric vibrator[i] to insure uniform packing. Approximately 16 g. of the pre-coated support was required to pack the column. Finally, the upper end was plugged with another pledget of selanised wool and the packed column coiled so as to fit in the chromatograph oven. Swaglock[j] fittings were attached to the two ends for connection to the inlet and outlet ports.

Both the Reoplex-400 and Carbowax-20M columns were pre-conditioned before chromatography of the oil by heating at 170° overnight while a slow stream of helium was passed through them.

The general operating conditons of chromatography are shown in Table III, designed according to the recommendation of the U.S. Essential Oil Association. Variations in any parameters are given in figures of the chromato-grams.

A study of the chromatogram obtained at 160° (isothermal) on Reoplex-400 showed 25 peaks and these

---

[i] Burgess Vibrografters Inc., Grayslake, Illinois.

[j] Crawford Fitting Company, Solon, Ohio.

Table III. Operating Parameters for the Gas Chromatography of the Essential Oil of Anemopsis californica.

Sample: Oil of Anemopsis californica *Chromatograph: Autoprep, Model A 700*
Columns *Material: Aluminium Length: 20' I.D.: 1/4"* *Support: Chromosorb W 60/80* *Stationary Phase : Column A- Reoplex-400 (20% w/w)* *Column B- Carbowax-20M(20% w/w)*
Carrier Gas: Helium Gas Flow Rate: 75 ml/min
Conditions *Column Temperatures:* *Column A-(1) 160°, isothermal and* *(2) 110°-170°, isothermal-linear PTGC* *Column B-(1) 180°, isothermal and* *(2) 110°-190°, isothermal-linear PTGC*
Detector *Type: Thermal Conductivity, hot wire type* *Temperature : 240° Filament Current: 150 ma*
Recorder *1. Sargent[k], Model SRL; Chart Speed: 1"= 5 min* *2. Varian[l], Model 20, with Disc Integrator* *Chart Speed: 1"= 3 min*
[k] E.H.Sargent Company, Anaheim, California. *[l] Varian Associates Inc., Palo Alto, California.*

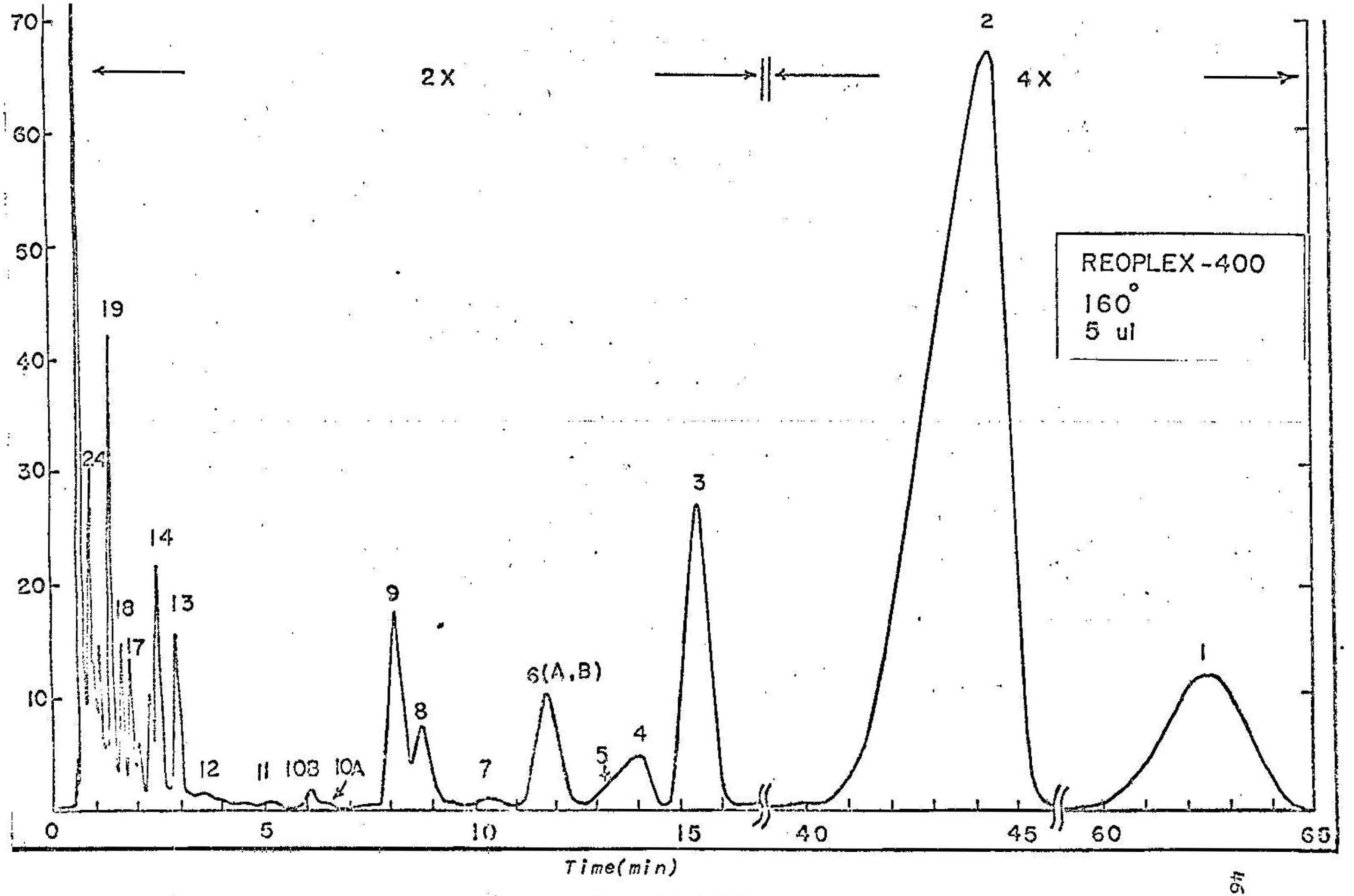
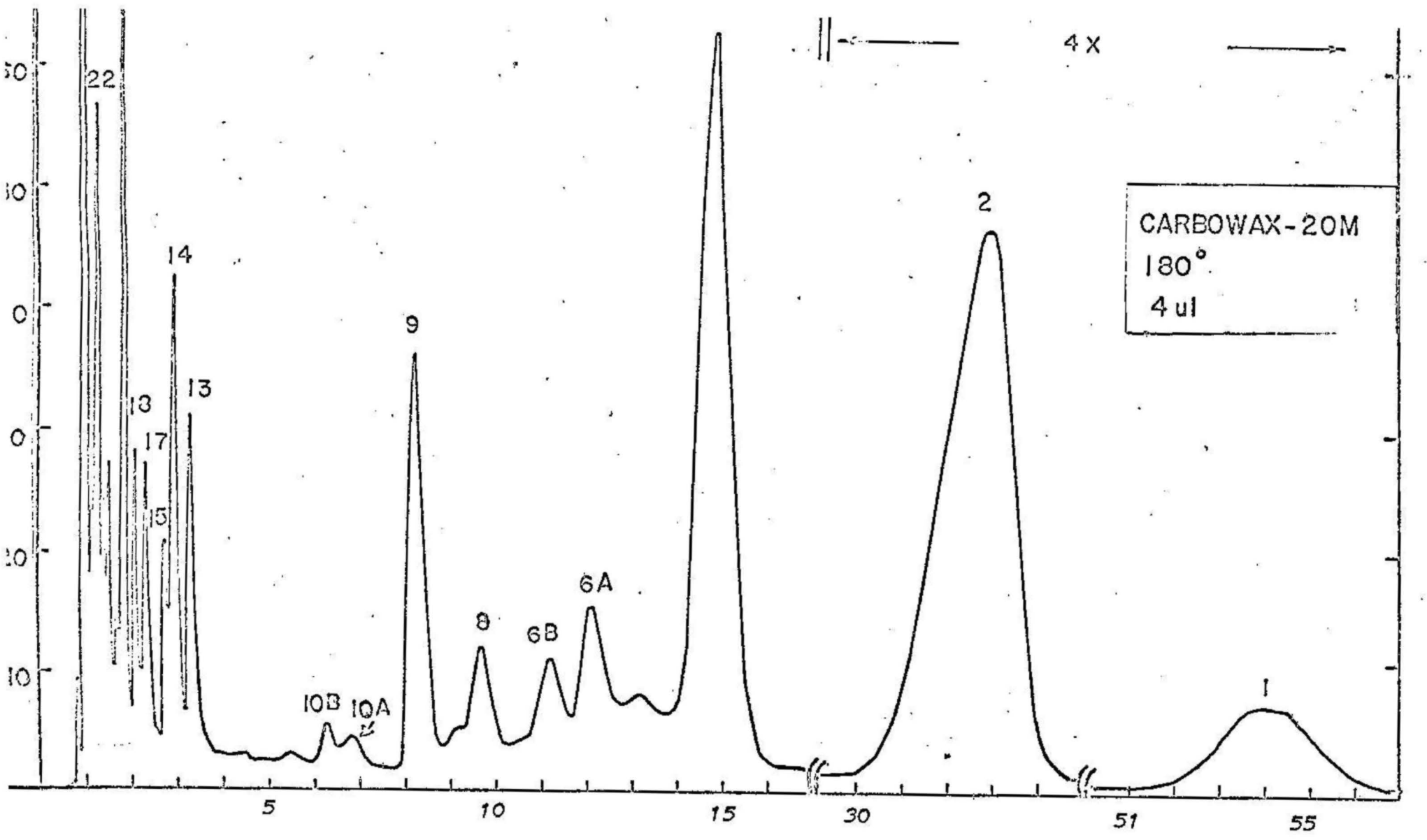


Fig. 8. Isothermal Chromatogram of Essential Oil of Anemopsis.


4 X
CARBOWAX-20M
180°
4 ul
22
14
13
13
17
15
9
8
6B
6A
10B
10A
2
1
5
10
15
30
51
55

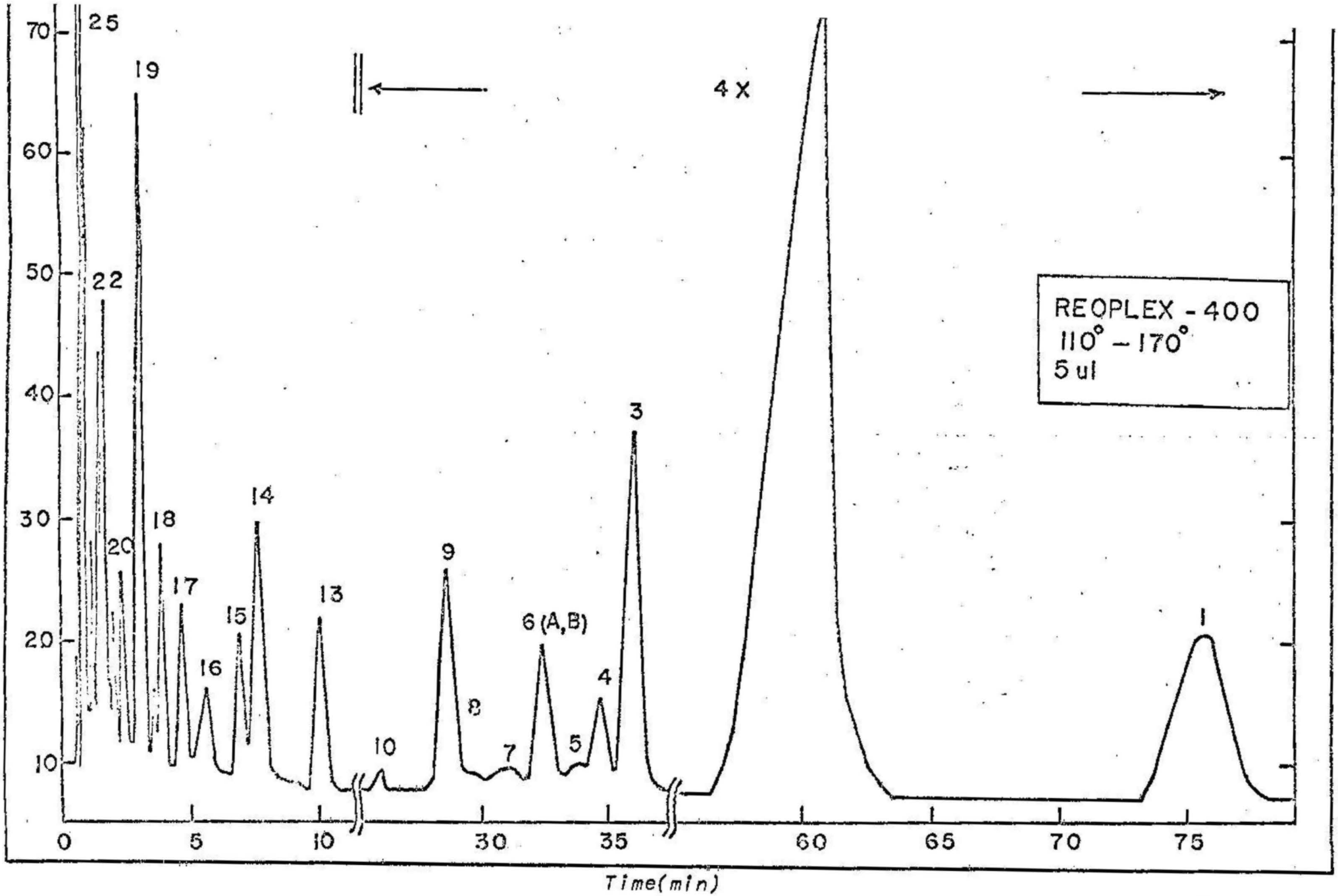


Fig. 10. Isothermal-linear PTGC of Essential Oil of Anemopsis.

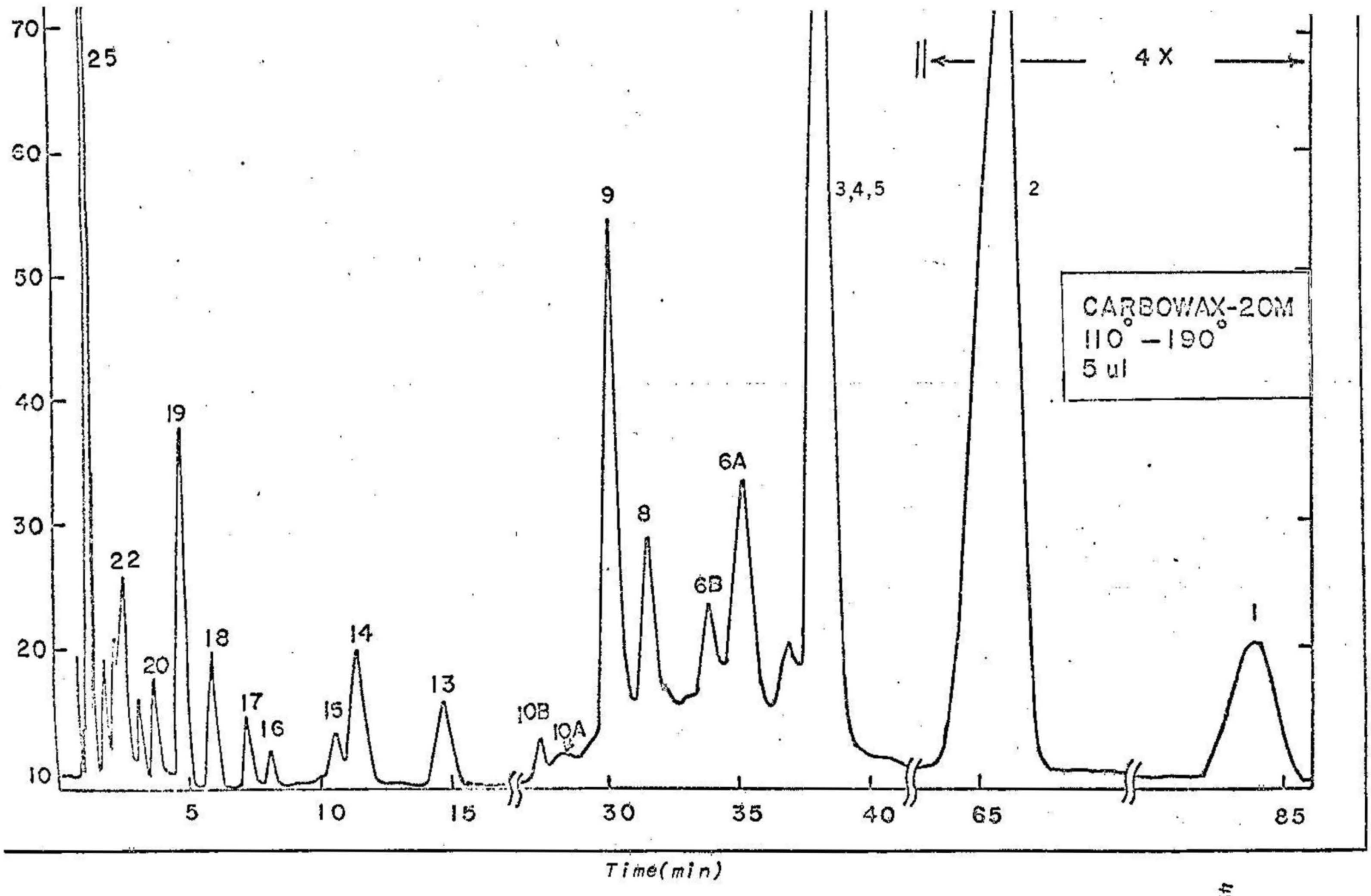


Fig. 11. Isothermal-linear PTGC of Essential Oil of Anemopsis.

could be divided into three regions. Peaks 10 to 25 eluted out within eight minutes of injection, constituting the early region. The middle region consisted of peaks 3 to 9 which eluted out within 10 to 20 minutes. Only two peaks, 1 and 2, constituted the late region, eluting out from 40 to 65 minutes following injection.

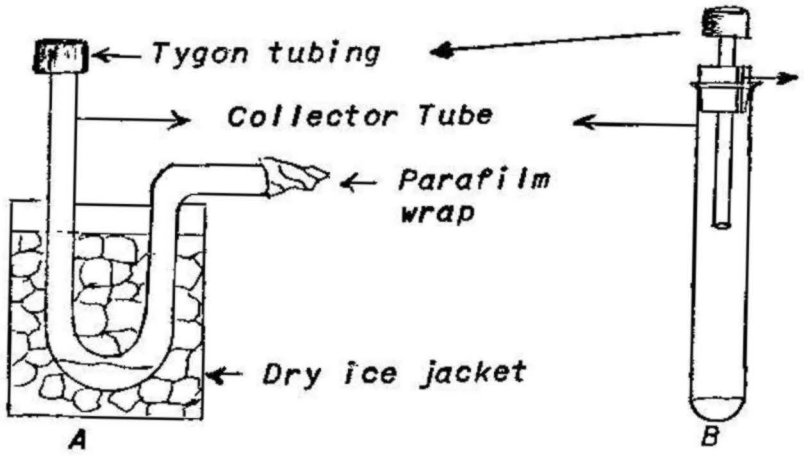
It was determined early in this work that peaks 1, 2 and 3 were the already identified thymol, methyleugenol and piperitone respectively and hence were of no further interest. The peaks eluting out within 20 minutes of injection were yet the unknown constituents. It seemed that low column temperature will resolve these peaks better and allow for their convenient trapping. However, a preliminary run at 110°, isothermal, showed that the total time for the run would approach several hours and, infact, one of the thymol was eluted out of the column at this low temperature. For good resolution of the peaks of interest and tolerable chromatographic time, PTGC was necessary.

Linear-isothermal PTGC was used to trap the constituents for identification. Initially the column was maintained at 110° when the sample was injected. This temperature was held for 15 minutes after injection and then raised linearly by the manual programmer on the chromato-

graph to the final temperature. The rate of increase of temperature was not determined. Isothermal mode of operation was used only to establish the retention data and for quantitative analysis. The isothermal-linear gas chromatograms are given in Fig. 10 and 11.

Trapping of GC peaks from the Essential Oil of Anemopsis

After some experimentation, it was found that simple traps prepared in the laboratory would work just as well as those commercially available. The two types of traps used in this work are shown below. Trapping was carried out manually although the chromatograph was a preparative unit with automatic fraction collection arrangement.



Trap A consisted of a 9" length of 1/8" diameter glass tubing bent into a U shape. The straight collector end

was attached to the chromatograph heated outlet by means of a short length of 1/8" diameter tygon tubing while the exit bent end was wrapped with a small strip of Parafilm.[m] The U portion was kept cooled in a dry ice bath. The Parafilm strip covering the exit end effectively prevented the contamination of the condensed fraction by atmospheric moisture. A number of such traps were made in the laboratory and one trap was used to condense each peak as it eluted out of the chromatograph. Peaks 10 to 19 were collected in this type of trap.

Trap B was a small, 8.5 cm. x 1 cm. test tube fitted with a small cork carrying an inlet tube and a side slit for escape of the carrier gas. This trap could be cooled if required. The non-volatile peaks 6 and 9, Fig. 8, were condensed in such traps at room temperature. A similar trap was used to collect the total fraction under peaks 20 to 25. These six peaks eluted out very close together and could not be collected individually. These six constituents were very volatile and therefore could not be condensed by the trap A in dry ice alone. Therefore a small volume(0.5 - 1 ml.) of carbon tetrachloride was placed in the bottom of a test tube to scrub the effluent

---

[m] American Can Company, Neenah, Wisconsin.

gas.

In order to obtain sufficient sample of each peak for identification, 40 - 45 ul. of fraction 1 and 2 (Table-II) were repeatedly injected and the individual peak fractions collected after each injection in the same marked trap. Each trap was kept cooled between successive collections. The trap was held to the outlet port of the chromatograph when the recorder pen had reached the straight ascending portion of a peak and removed when the pen had reached the 2/3 descending portion. This procedure reduced the chances of contamination of the condensed peak by another close lying constituent. Ten to twelve successive injections were required to collect sufficient quantities of each fraction for IR analysis. NMR analysis required larger samples and for this purpose, between 25 to 30 successive injections and collections were required.

Identification of the Constituents of Essential Oil of Anemopsis

The identification of the peaks was achieved by obtaining and correlating the retention data, internal standard addition and spectral data. Retention correlation was used as an aid in preliminary, broad identification.

Definite identity was assumed only when IR data were interpreted and compared either with those of authentic samples or with published spectra. In all cases, the peak condensates and authentic samples were purified by GC on Reoplex-400. The fractions were rechromatographed after first collection before spectral analysis.

The retention data were obtained both on Reoplex-400 and Carbowax-20M, under isothermal conditons. The two chromatograms are given in Fig. 8 and 9. Relative retention times(RRT) were computed based on the retention time of d-Limonene as unity; using the following formula:

$$RRT = \frac{RT\ compound}{RT\ d\text{-}Limonene}$$

d-Limonene was chosen as a standard because it has been identified as a component of the oil and because it had been used previously by von Rudloff and Nigam *et al.* to report the RRT values for the essential oil constituents. Thus we could compare our RRT values directly with those published and come to a preliminary decision as to the identity of the various peaks. After some of the peaks had been identified, their retention times, as determined from the chromatogrmas of the oil, were also checked by the injection of authentic, GC-pure samples under identical operating conditions. The following authentic samples

were available: α-Pinene and 1:8-Cineole[n], Camphene and Linalool[o] and p-Cymene and d-Limonene[p].

Internal standard addition technique was also used to determine the possible identity of some of the peaks. For this purpose, artificial mixtures of the essential oil with individual, GC-pure, authentic samples were chromatographed under identical conditions. An increase in the peak height of any one of the peaks of the oil indicated that the authentic sample and this particular peak were most likely the same compound. The various retention data are given in Table IV.

IR Identification of the GC Peaks of Essential Oil of Anemopsis

Although the above retention correlation data were valuable in giving a clue to the possible identity of the GC peaks, the major emphasis was placed on the spectral identification, especially IR analysis. In order to achieve good resolution of the peaks and efficient collection of the effluent compounds, isothermal-linear mode of

---

[n] Matheson, Coleman & Bell Co., Los Angeles, California

[o] Distillation Products Industries Inc., Rochester, N.Y.

[p] Fritzsche Brothers Inc., New York, N.Y.

Table IV. Composition of the Hydrodistilled Oil of Anemopsis californica.

Peak No.	Compound	Percent v/v	Retention Data							
			Reoplex-400				Carbowax-20M			
			110°		160°		110°		160°	
			RT	RRT	RT	RRT	RT	RRT	RT	RRT
1	Thymol	13.8	--	--	62.3	26.3	--	--	54.0	18.9
2	Methyleugenol	57.0	--	--	43.5	26.3	--	--	34.0	11.9
3	Piperitone	8.0	--	--	16.4	6.8	--	--		
4	Ketone ?	1.80	--	--	14.7	6.1	--	--	15.5	5.3
5	Unknown	0.70	--	--	13.6	5.7	--	--		
6A	sec.Alcohol[a] ?	1.8	--	--			--	--	12.1	4.2
6B	Esdragol[a]	1.32	--	--	12.2	5.2	--	--	11.2	3.9
7	Ketone(cyclic?)	0.34	--	--	10.8	4.6	--	--	--	--
8	Ester(cyclic,formate?)	1.50	--	--	9.0	3.8	--	--	9.8	3.4
9	Thymolmethylether[a]	2.96	--	--	8.5	3.6	--	--	8.3	2.85
10A	Unknown	0.20	--	--	6.3	2.6	--	--	6.9	2.4
10B	Linalool	0.32	--	--	6.1	2.58	--	--	6.3	2.2
11	Unknown	Trace	--	--	5.1	2.16	--	--	5.6	1.95
12	Unknown	Trace	--	--	3.6	1.5	--	--	4.5	1.6
13	p-Cymene	1.1	9.9	1.48	3.2	1.24	14.7	1.4	3.35	1.12
14	1,8-Cineol	1.35	7.55	1.09	2.5	1.26	11.5	1.1	3.0	1.05
15	d-Limonene	0.93	6.9	1.0	2.36	1.0	10.6	1.0	2.8	1.0
16	Unknown	0.1	5.6	0.81	1.9	0.81	8.3	0.78	2.3	0.81
17	β-Pinene	0.4	4.7	0.68			7.3	0.69		
18	Camphene	0.44	3.95	0.57	1.6	0.67	6.08	0.57	2.1	0.74
19	α-Pinene	1.4	3.08	0.45	1.5	0.62	4.9	0.46	1.95	0.68
20	Hydrocarbons	4.5	2.4	0.35	1.25	0.53	3.85	0.36	1.65	0.58
21			2.05	0.30	1.1	0.45	3.20	0.30	1.50	0.53
22			1.8	0.26	1.0	0.44	2.65	0.25	1.3	0.46
23			1.6	0.20	0.98	0.43	2.21	0.21	1.5	0.4
24			1.2	0.18	0.80	0.36	1.9	0.18		
25			0.98	0.14	0.70	0.30	1.25	0.11	1.0	0.32

RT = Retention Time(min). RRT = Relative Retention Time (d-Limonene reference). All compounds identified by IR Spectra.

a NMR in addition.

PTGC was employed (see Fig. 10 & 11). The condensed compounds were rechromatographed to obtain GC-pure samples before IR analysis. After the IR data were interpreted and the possible structures for the various constituents had been determined, authentic samples of as many of these compounds as possible were acquired. These authentic samples were also purified by GC. After this, the IR spectra of the GC-pure constituetnts and that of the authentic samples were obtained under identical conditions on the same chart paper. If the IR spectra of these two were identical in every respect, it was concluded that the two were the same compound. In only two cases, namely Esdragol (peak 6B) and β-Pinene(peak 17), the IR spectra of the peak condensates were compared with the published spectra (37, 38). Authentic samples of these were not available.

Using the Perkin-Elmer IR spectrometer,[q] Models 137B and 337B, the IR spectra of the compounds were obtained in a simple KBr micro-cell. Two thin, clear, 13 mm KBr pellets were pressed, each from approximately 150 mg. KBr. One of the pellets was placed in the IR pellet holder, 2 to 5 ul. of the sample were placed on the top and the holder cap carefully fastened. The IR spectra have been

---

[q] Perkin-Elmer Corporation, Norwalk, Connecticut.

reproduced in the following pages.

The IR spectra were taken with KBr micro-cell described as above except that of the total fraction under peaks 20 - 25(Fig. 31). This fraction was obtained by scrubbing with carbon tetrachloride and this solution in a sodium chloride cell was used to take the IR spectrum with pure carbon tetrachloride in the reference beam. Most of the fractions were resolved on Reoplex-400 column. Two peaks 6A and 6B were resolved only on Carbowax-20M column.

In the following discussion of the results of spectral analysis, the peak numbers cited are shown in the two isothermal-linear chromatograms (Fig. 10 & 11) and the correlation of the peak numbers and compounds identified has been shown in Table-IV. References may please be made to these.

Peaks 1, 2 and 3 were thymol, methyleugenol and piperitone, respectively. A critical examination of the isothermal-linear chromatograms on Reoplex-400 and Carbowax-20M showed that our initial choice of Reoplex-400 as the major column for isolation has been fortuitous. Carbowax-20M chowed only peak 3, whereas Reoplex-400 showed that there were two more peaks (4 and 5) eluting out

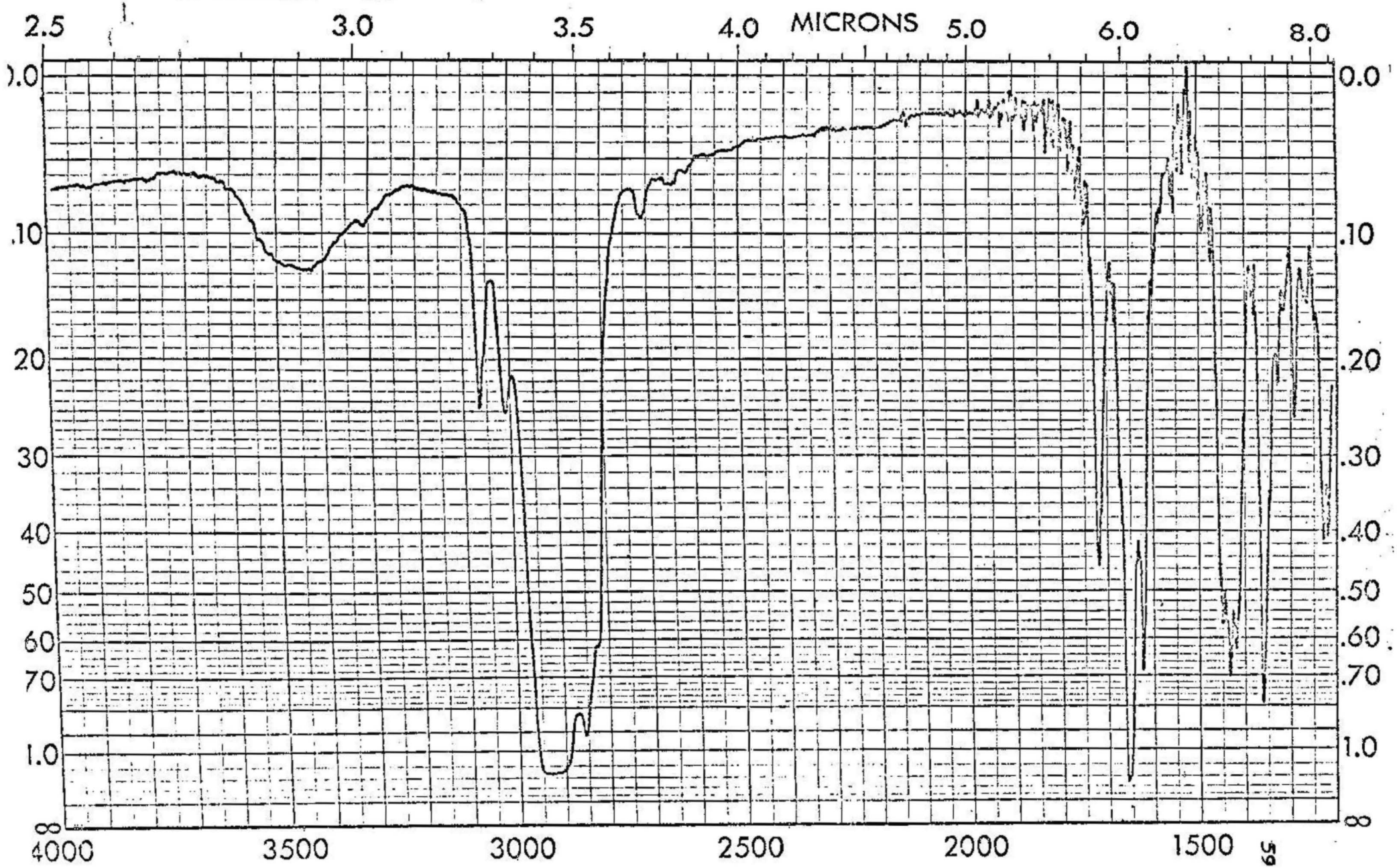


Fig. 12A. IR Spectrum of Peak 4 of Essential Oil of Anemopsis.

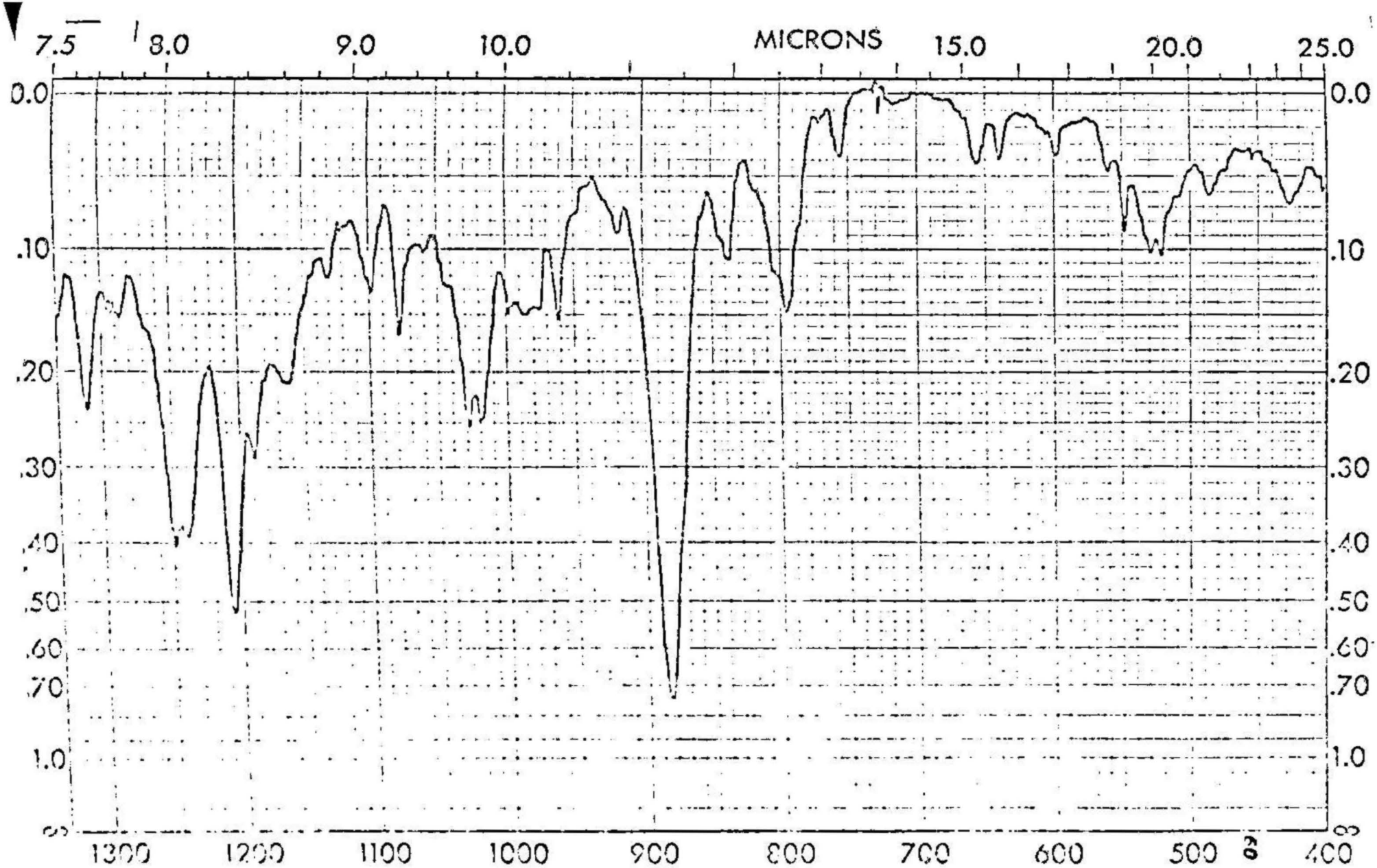


Fig. 128. IR Spectrum of Peak 4 of Essential Oil of Anemopsis.

quite close to peak 3. A further study confirmed this observation. When about 10 ul. of the fraction under peak 3 from Carbowax-20M column were condensed and then this fraction was rechromatographed on Reoplex-400, a clear resolution into three peaks (3, 4 & 5) was observed (Fig. 15). Peak 4 IR spectrum(Fig. 12A & B) showed the following features:

Absorption (cm^{-1})	Assignment
1665 (s)	>C=O, cyclic unsaturated ketone
3010 (m), sharp	vinyl (- $CH=CH_2$)
887 (s)	isopropenyl (- $CH^{=CH_2}_{-CH_3}$)

Thus peak 4 appears to be an α,β unsaturated ketone with an isopropenyl group. The compound could not be further identified as comparison spectra were not available and larger sample for NMR analysis could not be collected. Peak 5 appeared in traces and sufficient sample for spectral analysis could not be obtained.

Peak 6, isolated from Reoplex-400 gave an IR spectrum with the following features(Fig. 14):

Absorption (cm^{-1})	Assignment
3450 (s), 1055 (m)	2^0- alcohol (diameric)
3010 (w), 2860 (s)	aromatic ether(Ar-OCH_3?)
1600 (m), 1450 (s)	

1500 (s), 1248 (s)	aromatic ether
1380 - 1370 (m) doublet	gem-dimethyl($-CH(CH_3)_2$)

In order to obtain further information about the possible identity of peak 6, sufficient sample (40 mg.)* was collected and subjected to NMR analysis. NMR spectra were run on Varian, Model A 60, 60MHz NMR spectrometer with carbon tetrachloride as the solvent and TMS as internal standard. The NMR spectrum(Fig. 15) indicated that the sample was probably a mixture of at least two compounds. Rechromatography of the fraction on Reoplex-400 was not successful in resolving this mixture. Therefore the same fraction was rechromatographed on Carbowax-20M column yielding clearly resolved peaks 6A and 6B(Fig. 16). Peak 6B condensed as a pale yellow liquid with a sweet odor. Its IR spectrum showed the following features(Fig. 17):

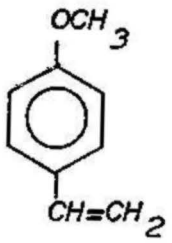
Absorption (cm^{-1})	Assignment
3010, 1640 (m), 1430 (s)	$-CH=CH_2$
1600 (m), 1450, 1500 (s), 1248 (s)	aromatic ether ($Ar-OCH_3$?) &

---

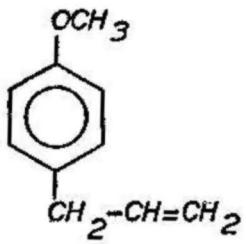
* Thanks are due to Dr. G.E.Pollard of Shell Research Lab., Modesto, California.

815 (m), broad	p-substituted aromatic nucleus

These further suggested a compound such as p-methoxy-styrene (I) or methylchavicol (II) (esdragol, estragol, isoanethole).



(I)



(II)

A literature search for published spectra of these compounds produced two spectra for esdragol (37, 39) which were identical with that of peak 6B. The NMR spectrum of the peak also gave values identical to those reported by Scheuler and Wester (40), thus establishing the identity of this peak with that of esdragol.

Peak 6A condensed as a white solid, with a melting point 76°- 77° (uncorrected). Its IR spectrum showed the following features(Fig. 18):

Absorption	Assignment
3615 (sharp), 3450, 1052 (broad)	2°- diameric alcohol
1380 & 1365 (m),doublet	gem-dimethyl($-CH-(CH_3)_2$

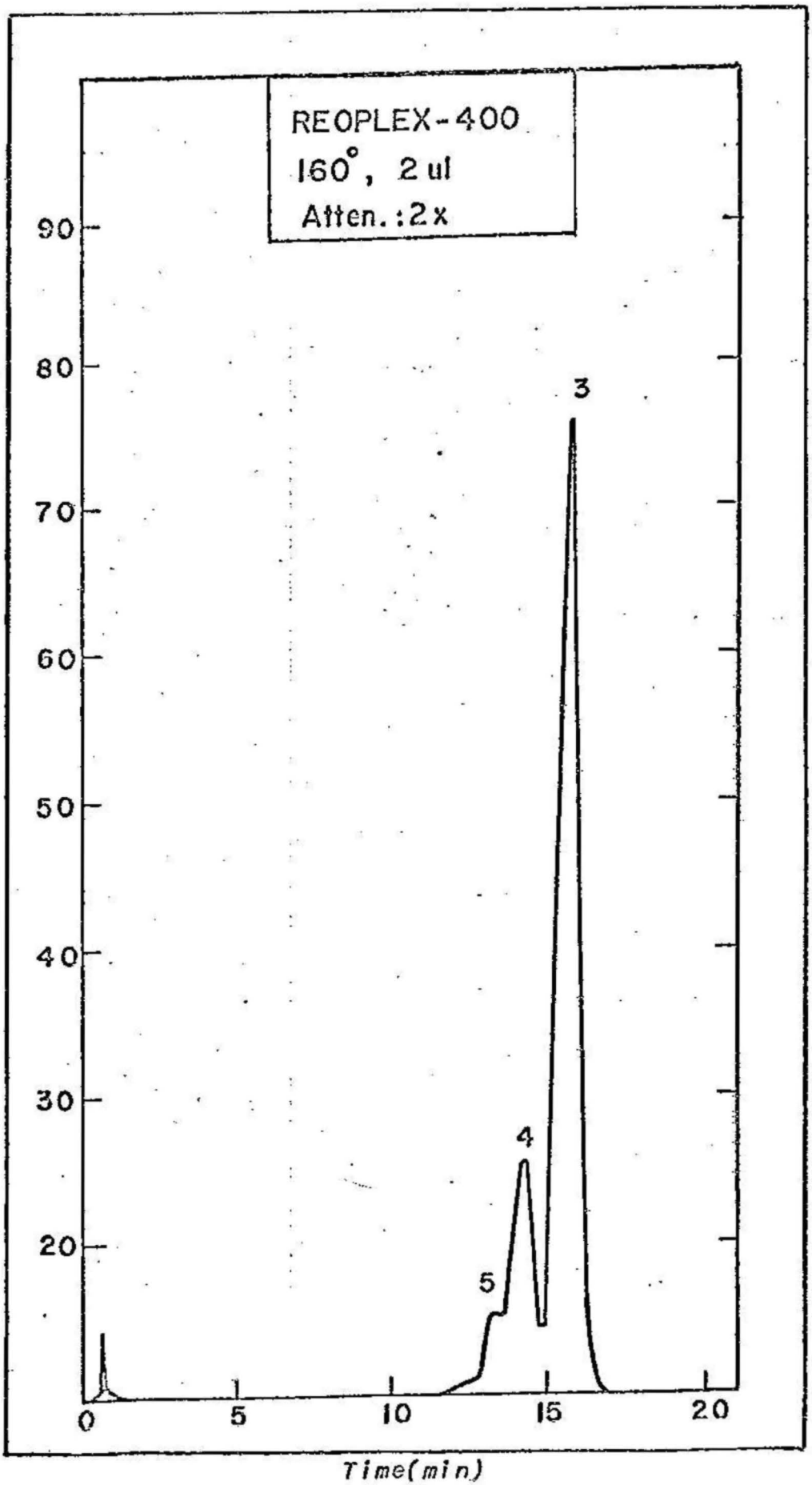


Fig. 13. Rechromatography of Peak 3 from Carbowax-20M on Reoplex-400.

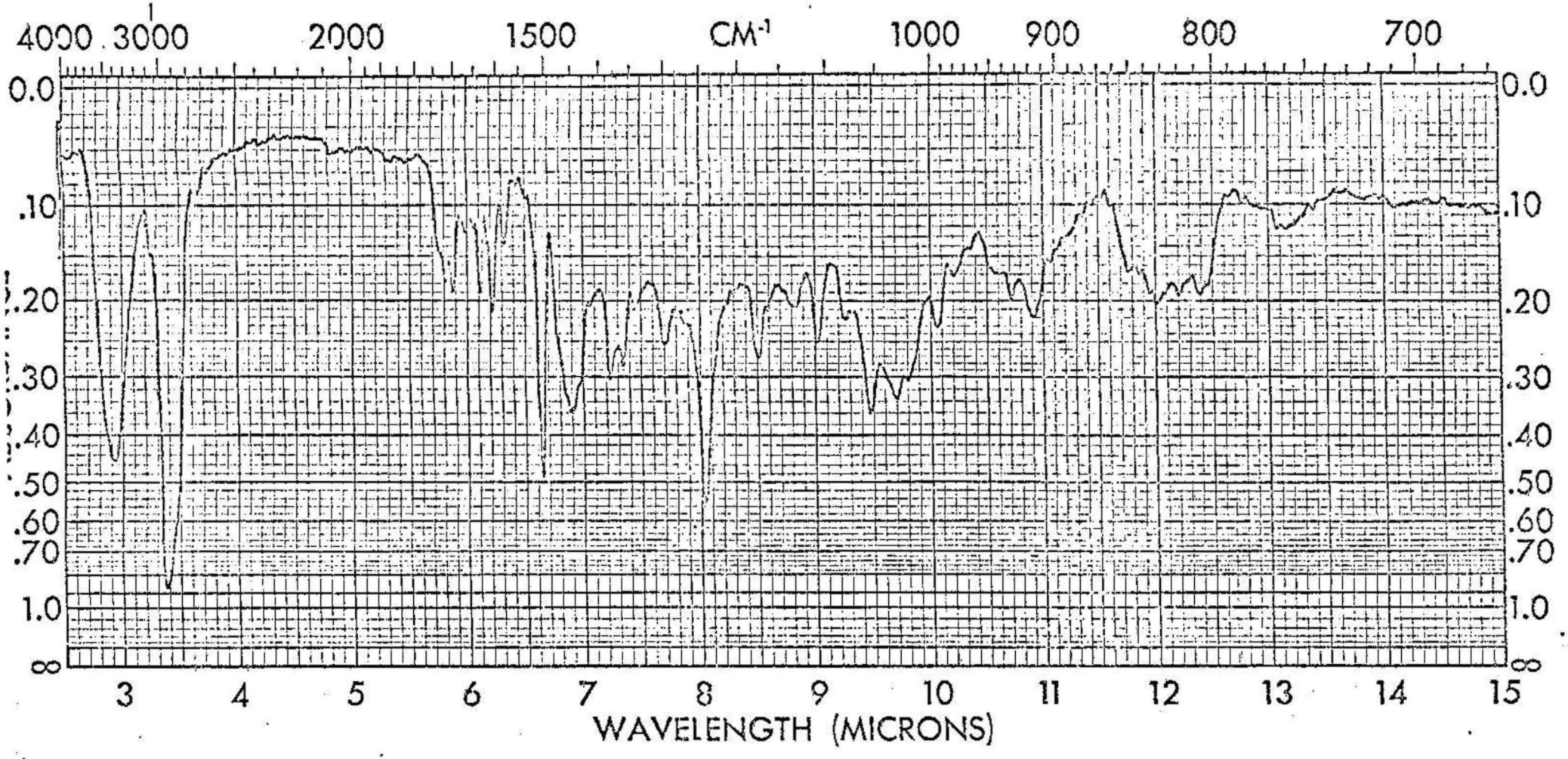


Fig. 14. IR Spectrum of Peak 6(A+B) of Essential Oil of Anemopsis

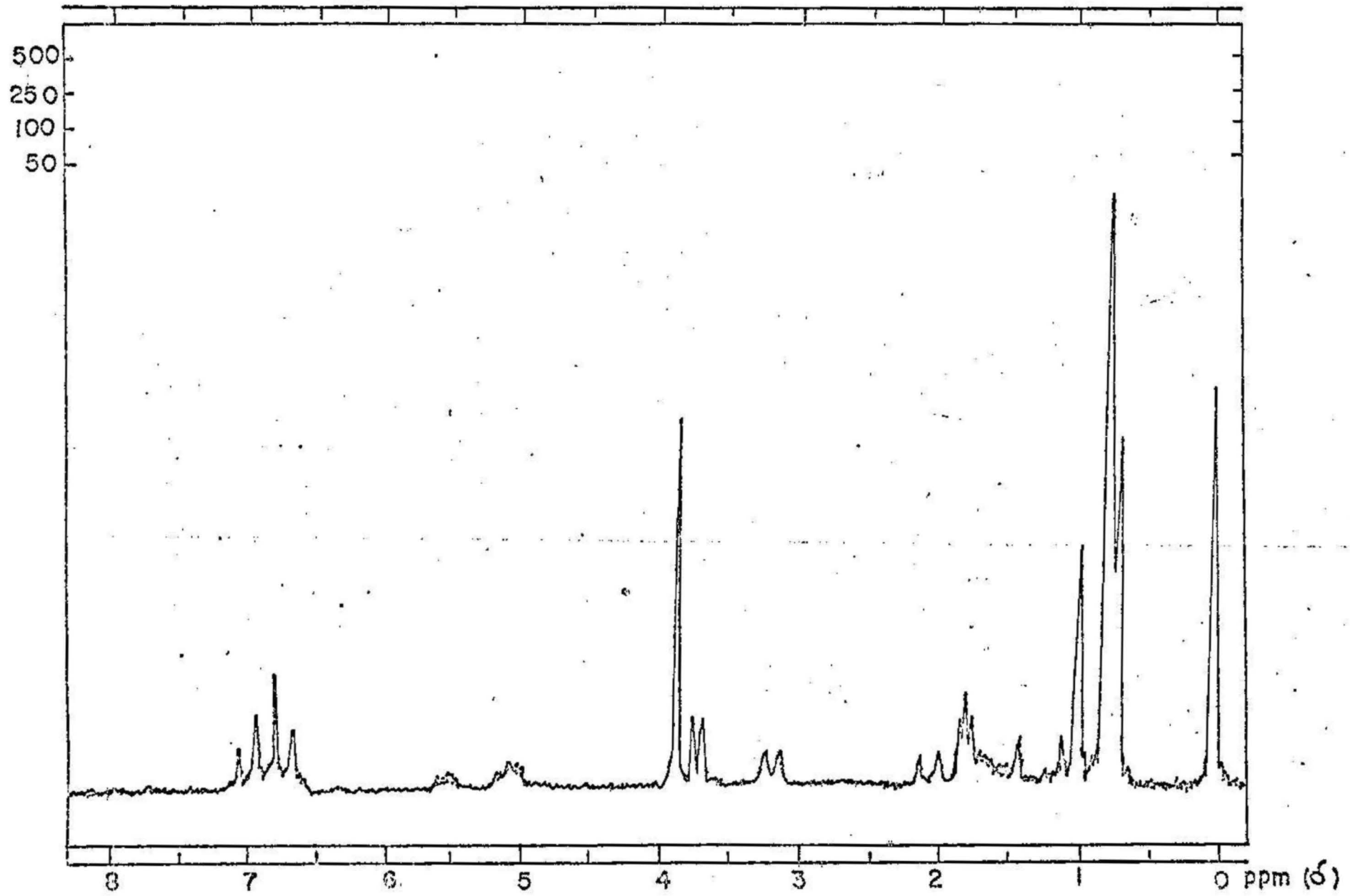


Fig. 15. NMR Spectrum of Peak 6 (A+B) of Essential Oil of Anemopsis.

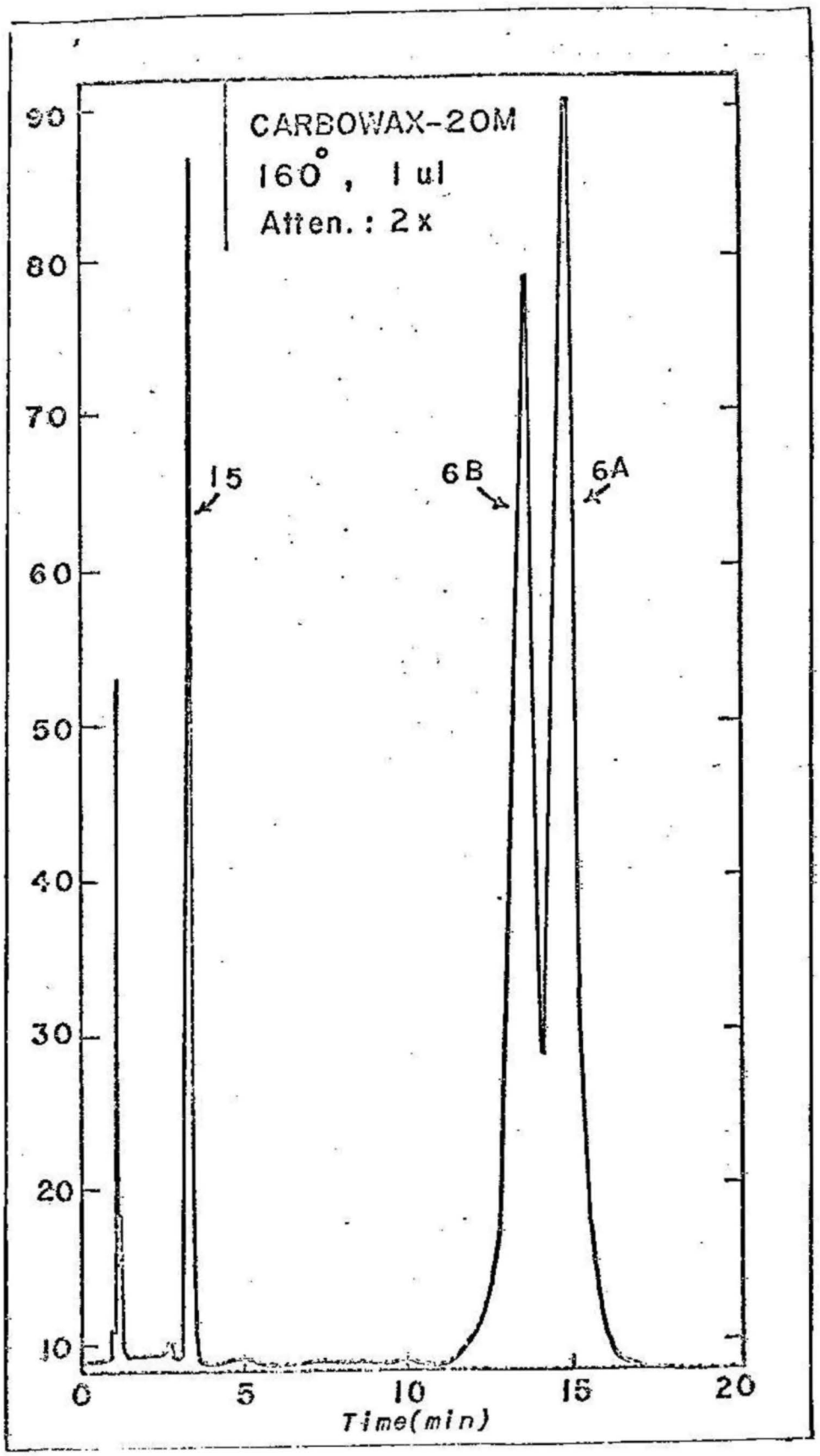


Fig. 16. Rechromatography of Peak 6 from Reoplex-400 on Carbowax-20M.

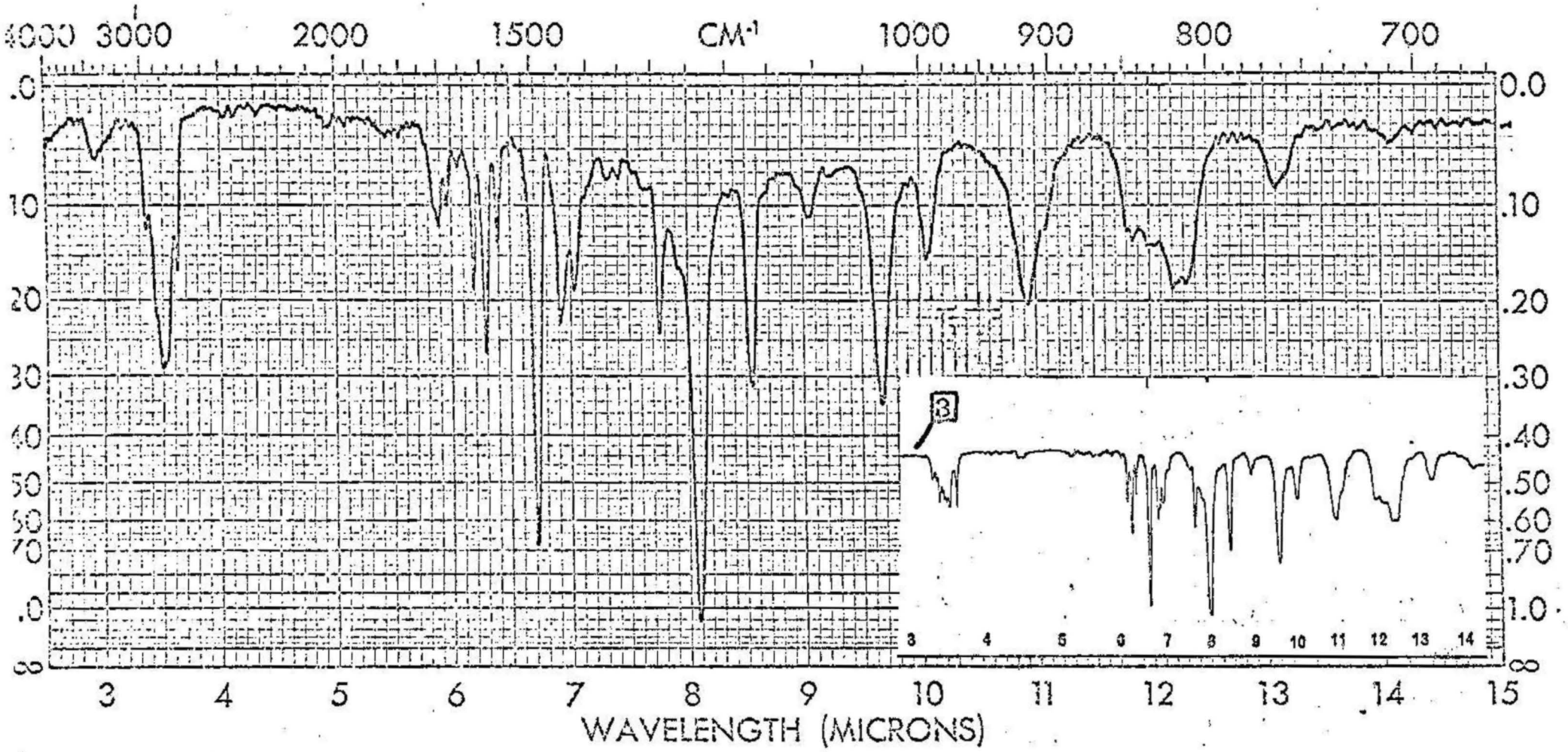


Fig. 17. IR Spectra of Peak 6B (A)(Carbowax-20M) & of Esdragol (B),(Ref.- 38).

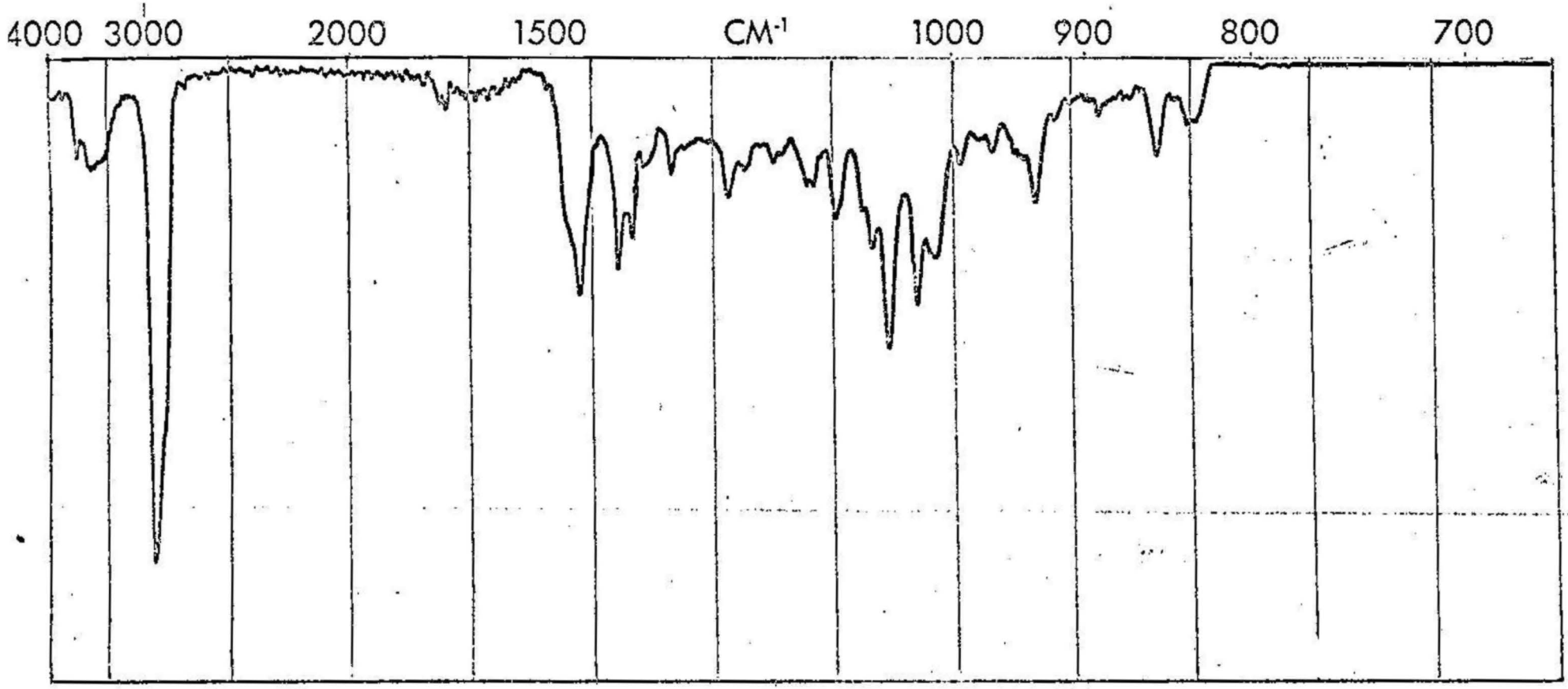
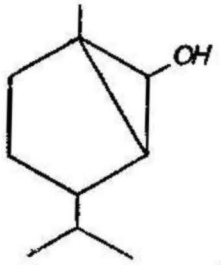


Fig. 18. IR Spectrum of Peak 6A(Carbowax-20M) of Essential oil of Anemopsis.

The IR spectrum of this compound resembles that of borneol. However, many absorption bands showed a shift and the melting point was lower than that of borneol. The NMR spectrum of the pure compound was therefore obtained yielding the features outlined in Table. V, as suggested by the spectrum given in Fig. 19. These NMR and IR data led to the following possible structure for peak 6A.



1-methyl-4-isopropyl-6-hydroxy-bicyclo(3:1:0)hexane

A literature search has failed to reveal any compound of this type having been isolated. It appears to be a new compound.

IR analysis of the very scant yield(5 - ul.) of the peak 7 fraction showed the following features, as indicated in Fig. 20 :

Absorption (cm^{-1})	Assignment
1375 (s), 1325 (m)	cyclic saturated ketone
1380 and 1365 (m)	gem-dimethyl($-CH(CH_3)_2$)

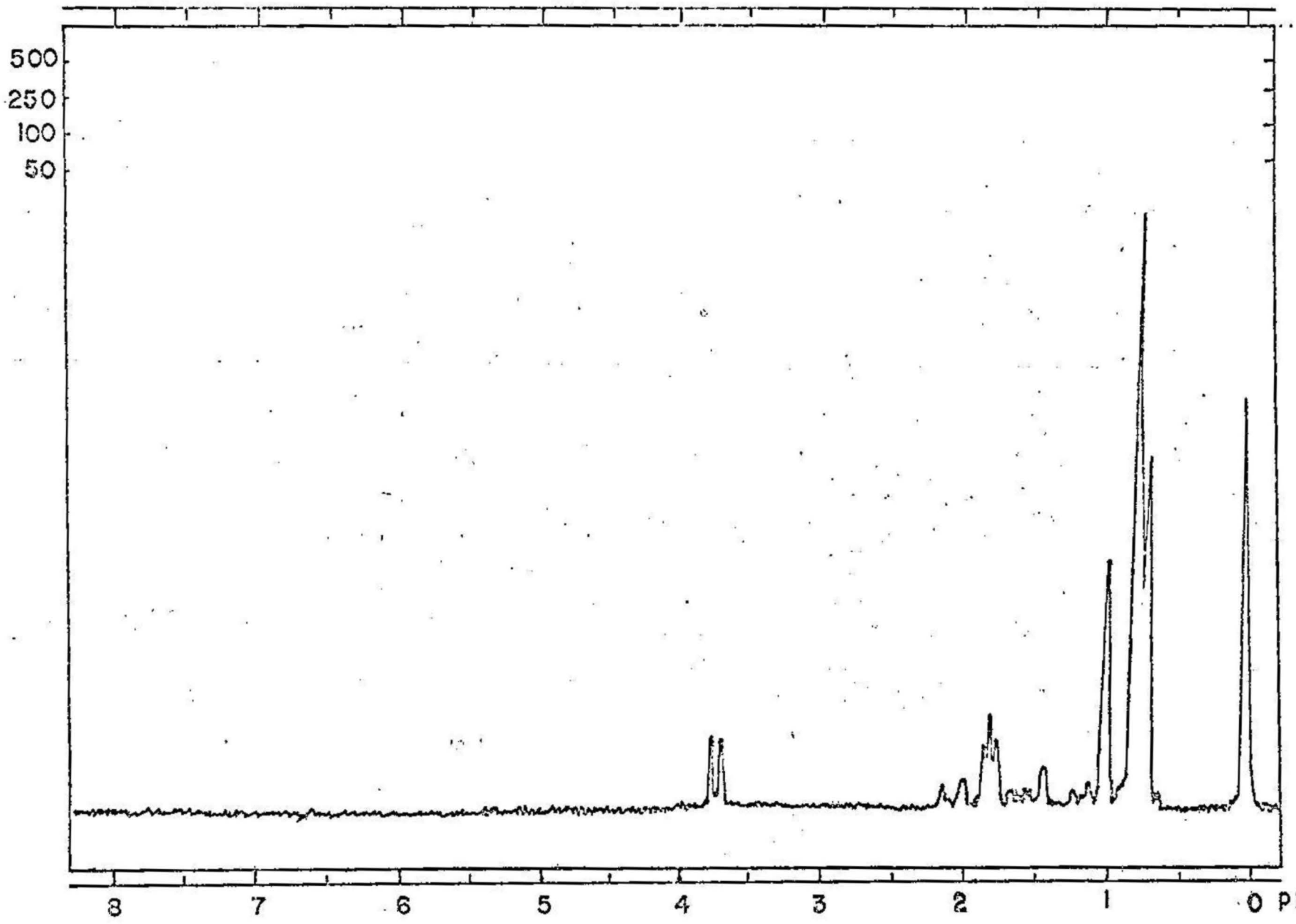


Fig. 19. NMR Spectrum of Peak 6A(Carbowax-20M) of Essential Oil of Anemopsis.

Table V. NMR Data for Peak 6A (Carbowax-20M)

TMS (ppm)	Splitting	Coupling constant (J)	Number of Protons	Assignment
0.85	doublet	3	6	1^{o}-alkyl($-CH(\underline{CH}_3)_2$)
1.05	singlet	-	3	1^{o}-alkyl($-\underline{CH}_3$)
1.2	multiplet	-	2	2^{o}-alkyl($-\underline{CH}_2-$)
1.45	singlet	-	1	2^{o}-OH (CH-$\underline{OH}$)
1.55	multiplet	-	2	3^{o}-alkyl($-\underline{CH}-$)
1.75	triplet	-	2	2^{o}-alkyl($-\underline{CH}_2-$)
2.0	multiplet	-	1	3^{o}-alkyl($-\underline{CH}$)
3.65	doublet	4	1	$-\underline{CH}$ - OH
			Total Protons: 18	

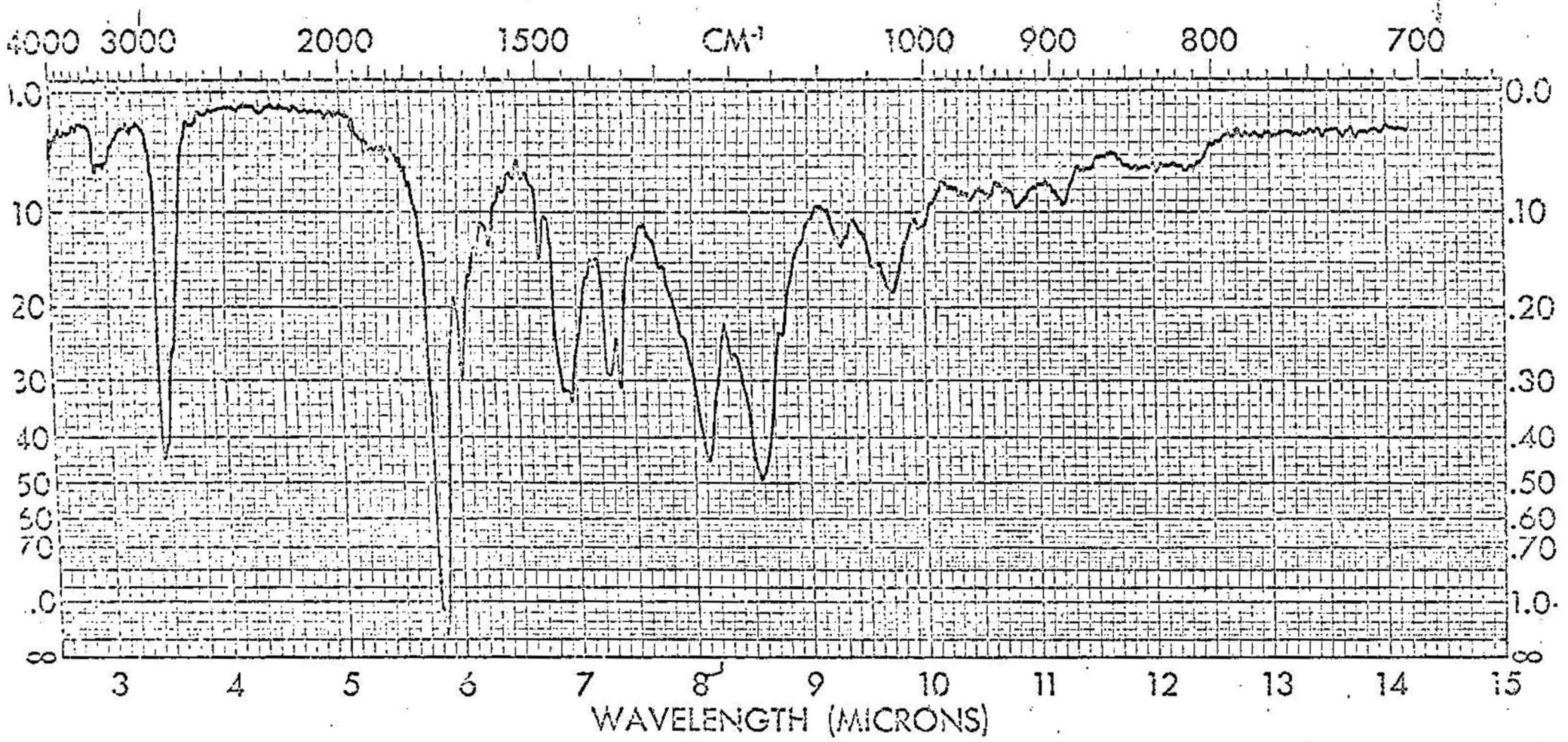


Fig. 20. IR Spectrum of Peak 7 of Essential Oil of Anemopsis.

Based upon the following features of the IR spectrum, Fig. 21, peak 8 appears to be a saturated cyclic ketone, most likely to be a formate with an isopropyl substituent.

Absorption (cm^{-1})	Assignment
1727 and 1127 (s)	cyclic ester(formate ?)
1375 and 1365 (m)	gem-dimethyl(-CH(CH_3)(CH_3))

Peak 9 showed the following features(Fig. 22):

Absorption (cm^{-1})	Assignment
3058, 1600, 1500 & 1450 (s)	aromatic
2860 (m), 1258 (s)	aromatic ether
993 (m), 813 (m)	1:2:4 tri-substituted aromatic ?
1385 and 1365 (m)	gem-dimethyl(-CH(CH_3)(CH_3))

The NMR spectrum gave the data as shown in Table VI. These spectral data led to the following six possible structures for peak 9. Comparison spectra could not be located. However on a biogenetic basis, thymolmethyl-ether would appear to be the most probable of these, as thymol has already been shown to be present in the oil. A synthetic sample of thymolmethylether was therefore purified by GC to obtain IR and NMR spectra. The peak 9

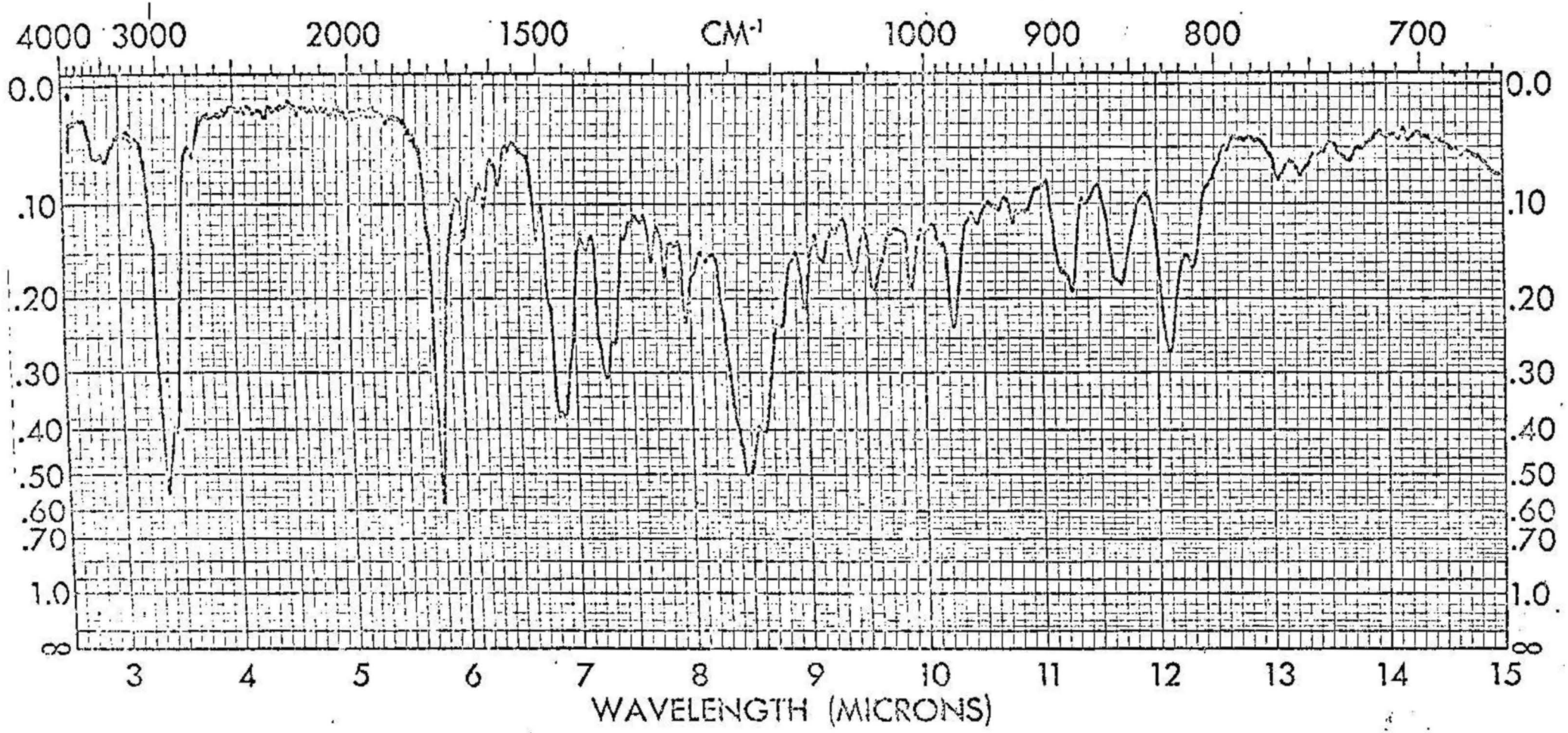


Fig. 21. IR Spectrum of Peak 8 of Essential Oil of Anemopsis.

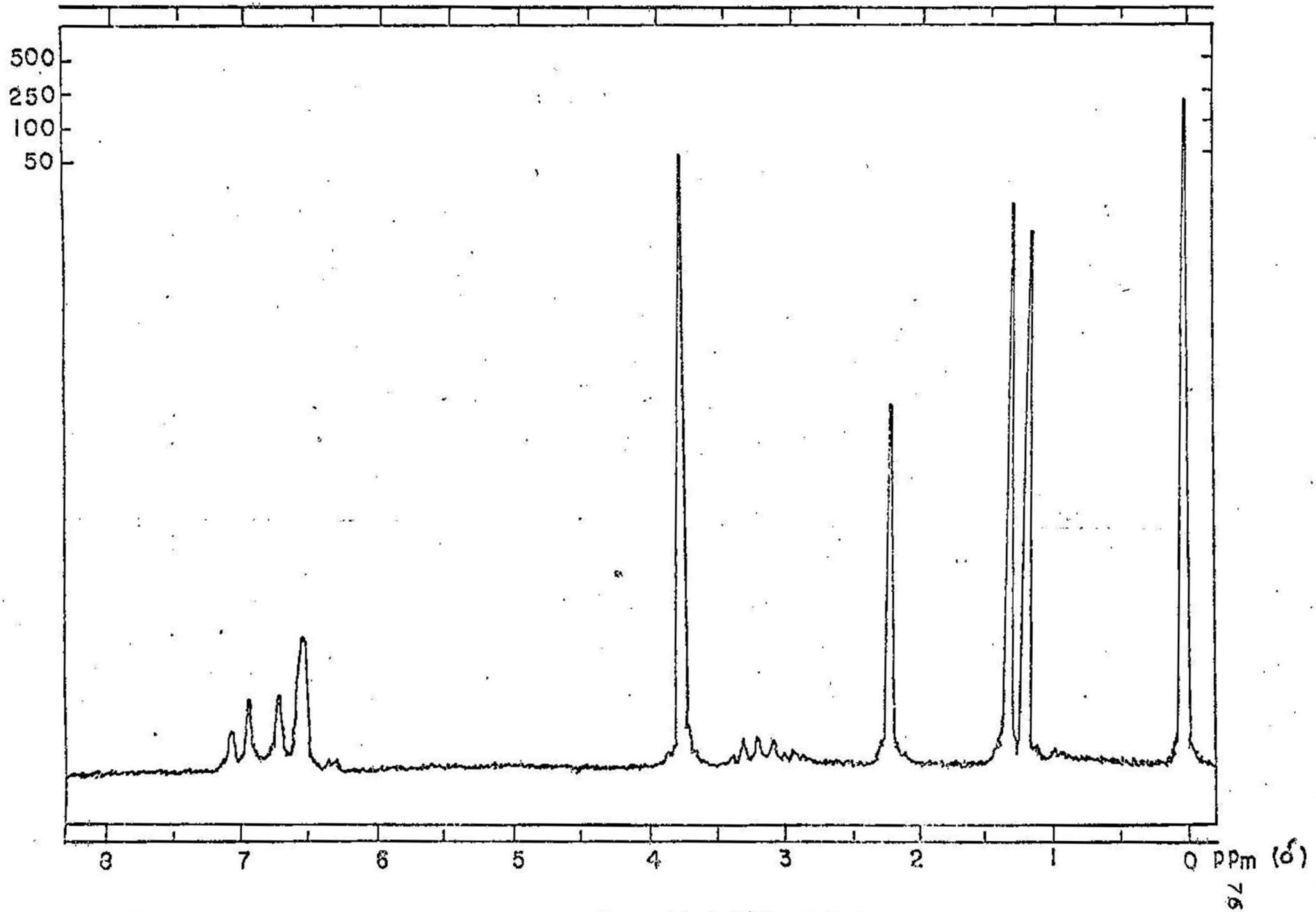
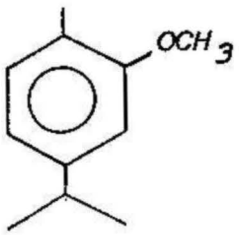


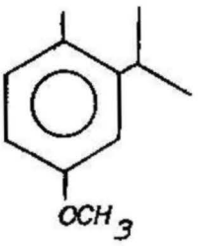
Fig. 22. NMR Spectrum of Peak 9 of Essential Oil of <u>Anemopsis</u>.

Table. VI. NMR Data of Peak 9

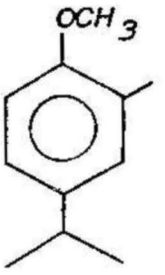
TMS δ (ppm)	Splitting	Coupling constant (J)	Number of Protons	assignment
6.8	quartet	9.1	3	aromatic-C<u>H</u>
3.8	singlet	-	3	1^{o}-alkyl
3.3	septet	6.0	1	3^{o}-alkyl
2.3	singlet	-	3	benzylic-C<u>H</u>
1.2	doublet	6.0	6	1^{o}-alkyl



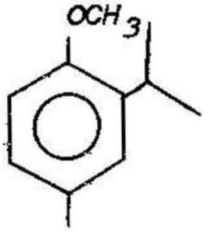
1-methyl-2-methoxy-4-isopropyl-benzene



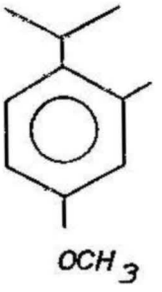
1-methyl-2-isopropyl-4-methoxybenzene



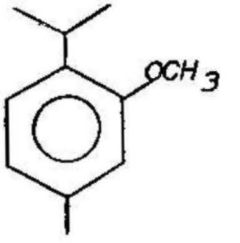
1-methoxy-2-methyl-4-isopropyl bezene



1-methoxy-2-isopropyl--4-methylbenzene



1-isopropyl-2-methyl-4-methoxy benzene



1-isopropyl-2-methoxy--4-methylbenzene (Thymolmethylether)

spectra were identical with those of GC-pure thymolmethyl-ether.

The isolation and identification of the peaks

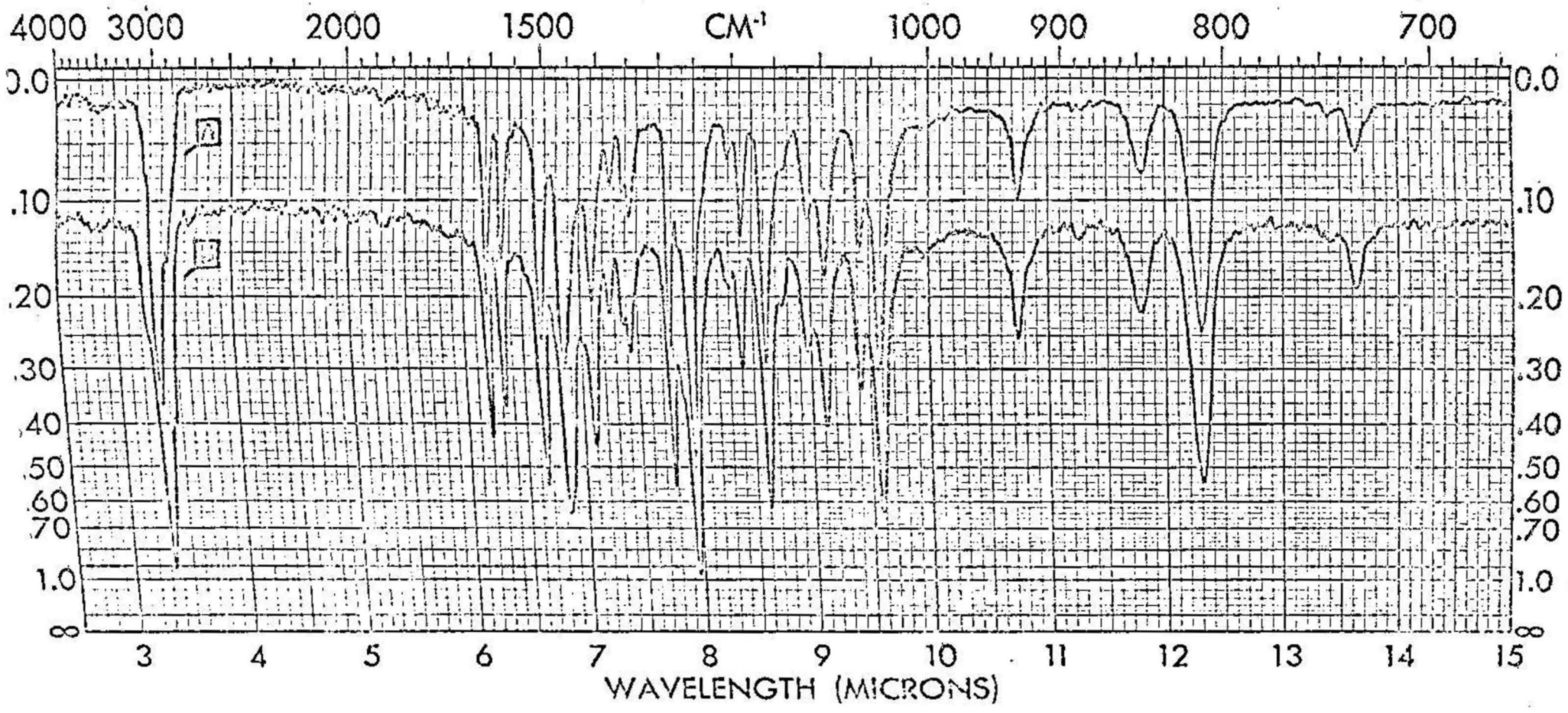


Fig. 23. IR Spectra of Peak 9 (A) of Essential Oil of Anemopsis and of Thymolmethylether (B).

10B (linalool), 13 (*p*-cymene), 14 (1:8-cineole), 15 (*d*-limonene), 17 (β-pinene), 18 (camphene) and 19 (α-pinene) were relatively a simple matters. Their RRT values had given a clue to their identification and authentic samples were available for preparing comparison spectra. The IR spectra of the authentic samples were identical in all respects. Peak 17 IR spectrum was identical to a published spectrum of β-pinene(38).

Peaks 10A, 11, 12 and 16 could not be collected as they were either present in traces or were very volatile. These peaks therefore remain unidentified. The total fraction under the peaks 20 to 25 gave an IR spectrum (Fig. 31.) with absorption bands indicative only of hydrocarbons. No functional groups were observed. The structures of compounds shown to be present in the essential oil of *Anemopsis* are shown in Fig. 32.

Quantitation of GC peaks in Essential Oil of Anemopsis

The isothermal mode of operation was employed with Reoplex-400 to obtain the percent v/v composition of the essential oil. The Varian recorder equipped with a Disc Integrator, Model 224, was used for quantitation and the data obtained from the chromatogram are given in Table VII.

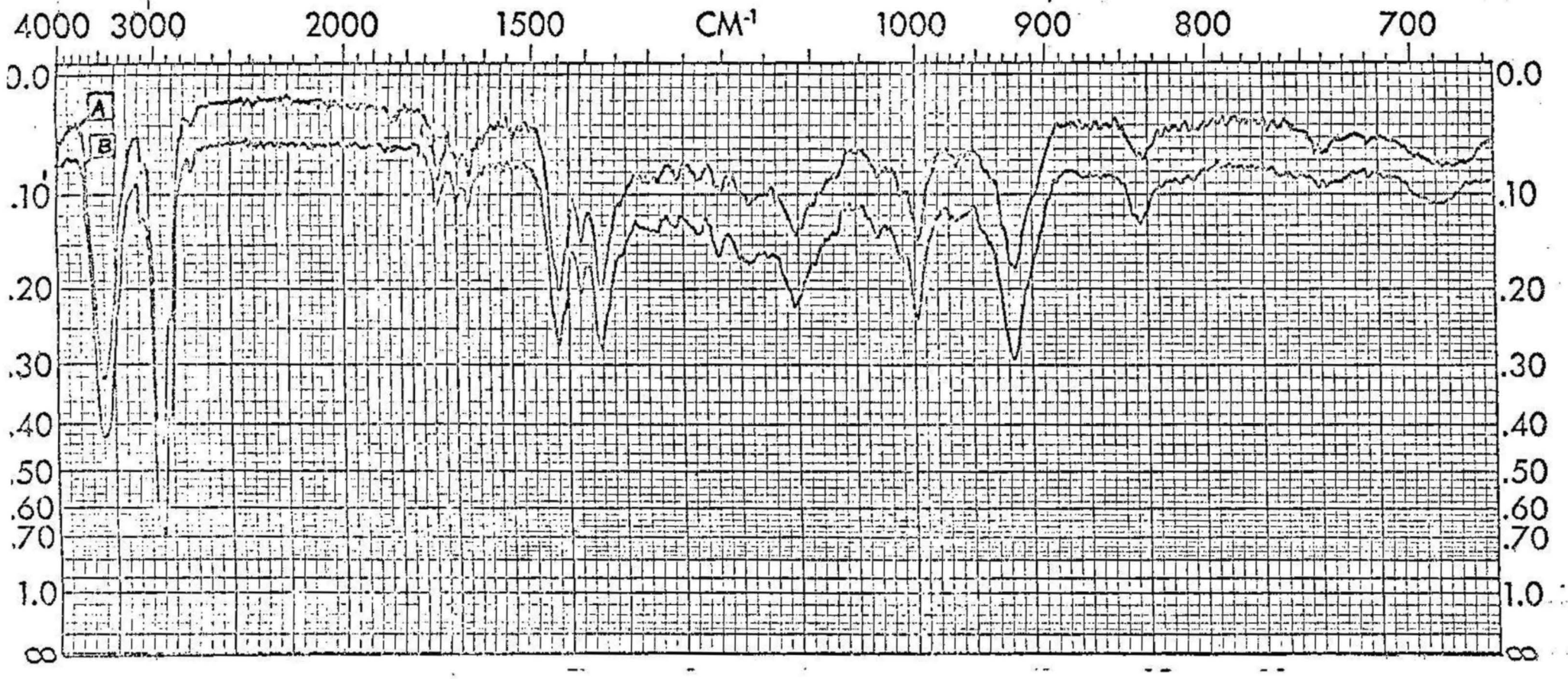


Fig. 24. IR Spectra of Peak 10B (A) of Essential Oil of <u>Anemopsis</u> and of Linalool(B).

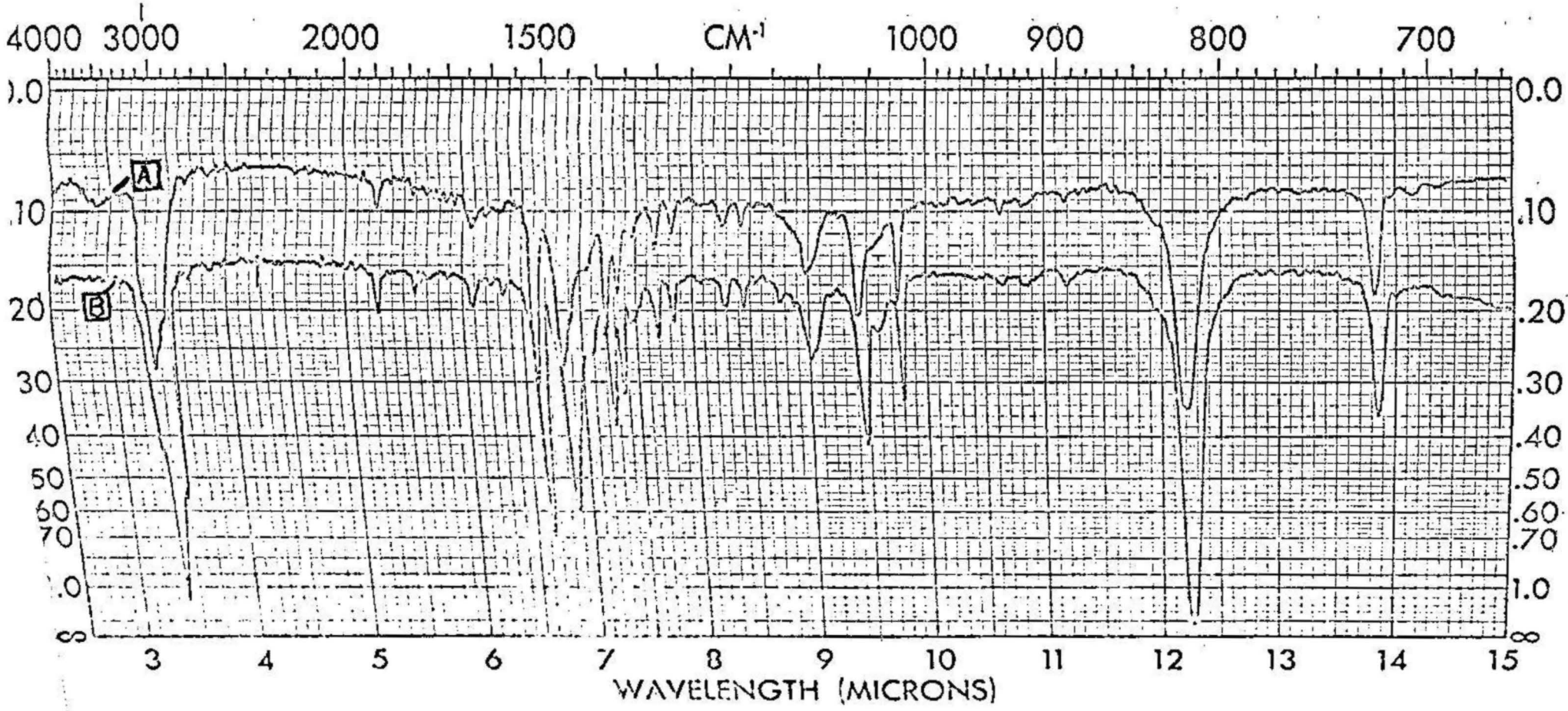


Fig. 23. IR Spectra of Peak 13 (A) of Essential Oil of *Anemopsis* and of *p*-Cymene (B).

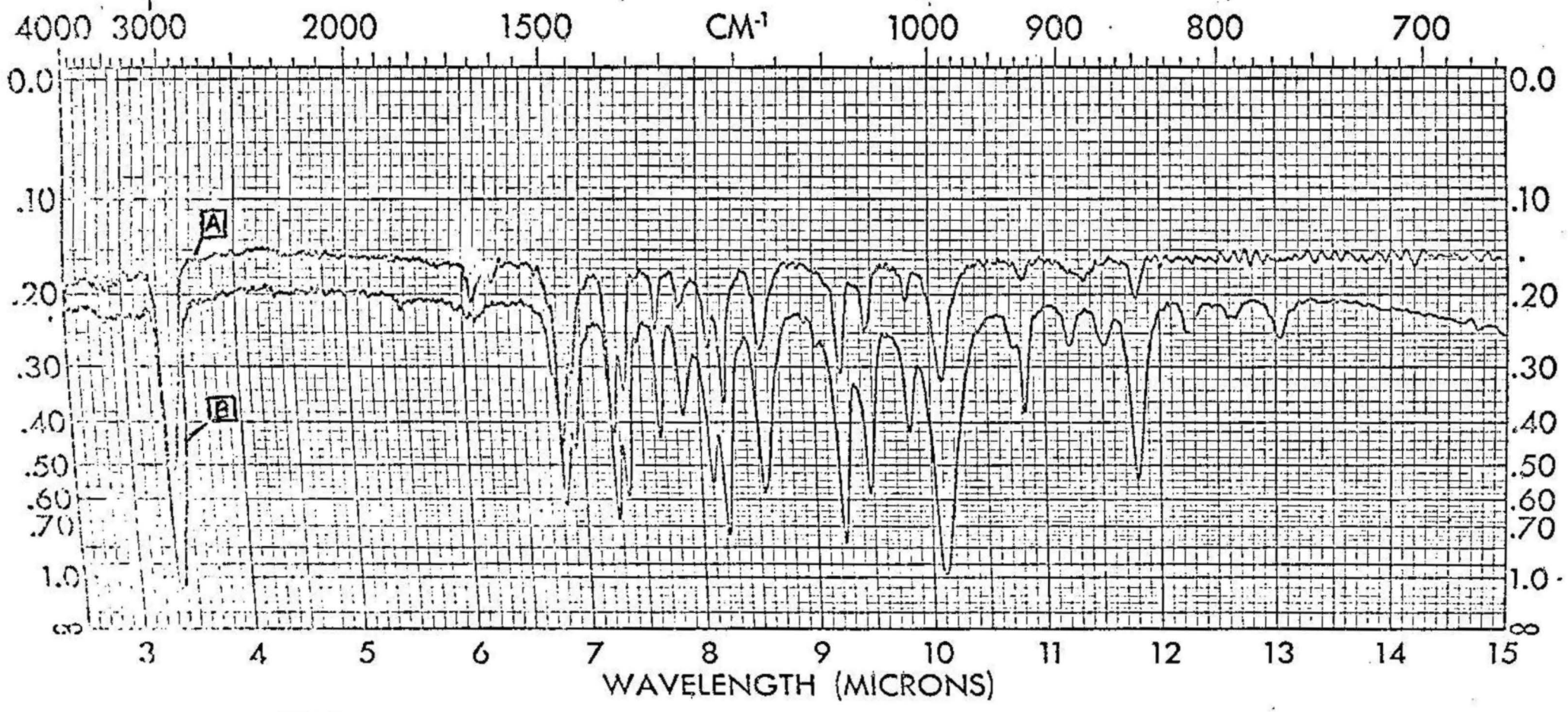


Fig. 26. IR Spectra of Peak 14 (A) of Essential Oil of *Anemopsis* and of 1:8-Cineole (B).

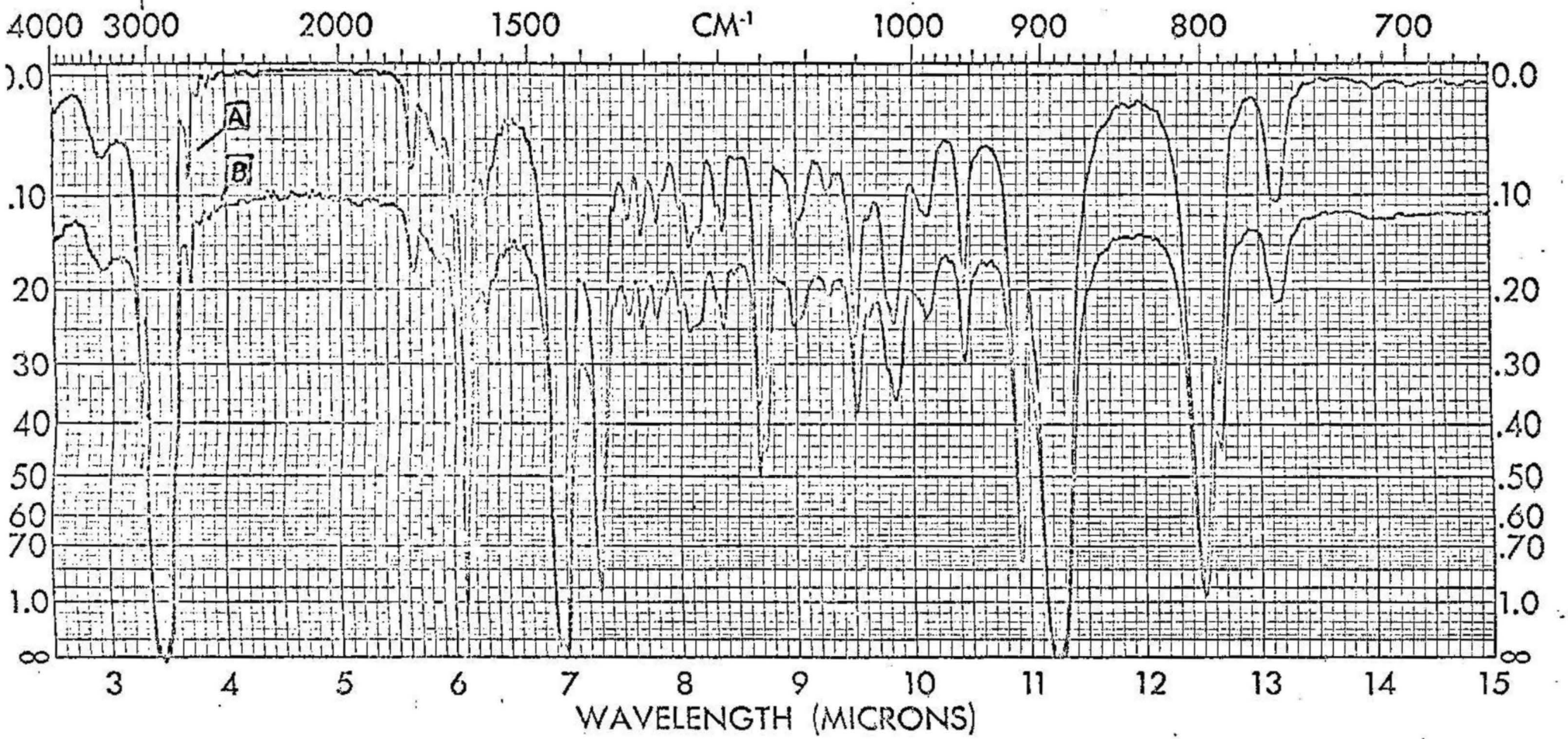


Fig. 27. IR Spectra of Peak 15 (A) of Essential Oil of Anemopsis and of d-Limonene (B).

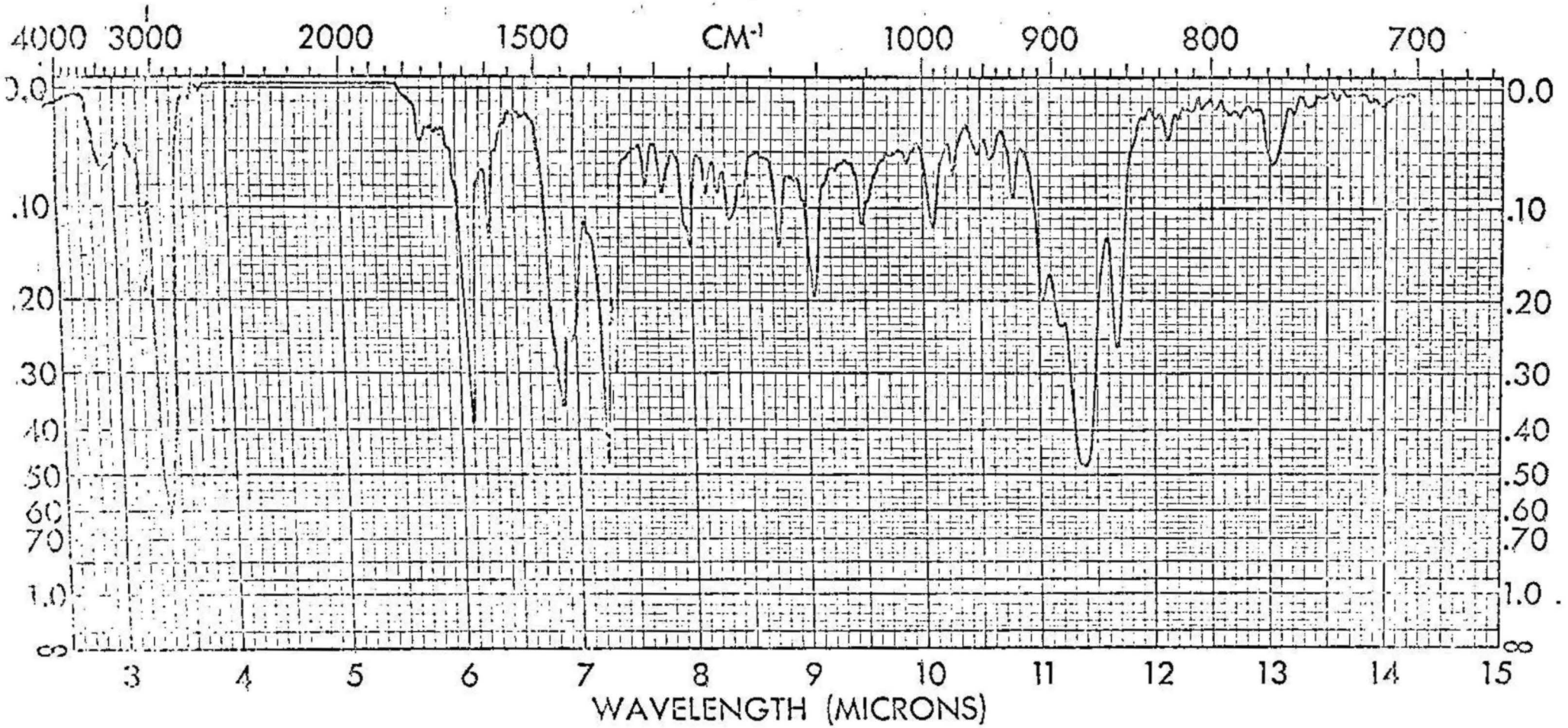


Fig. 28. IR Spectrum of Peak 17 (β-Pinene) of Essential Oil of Anemopsis.

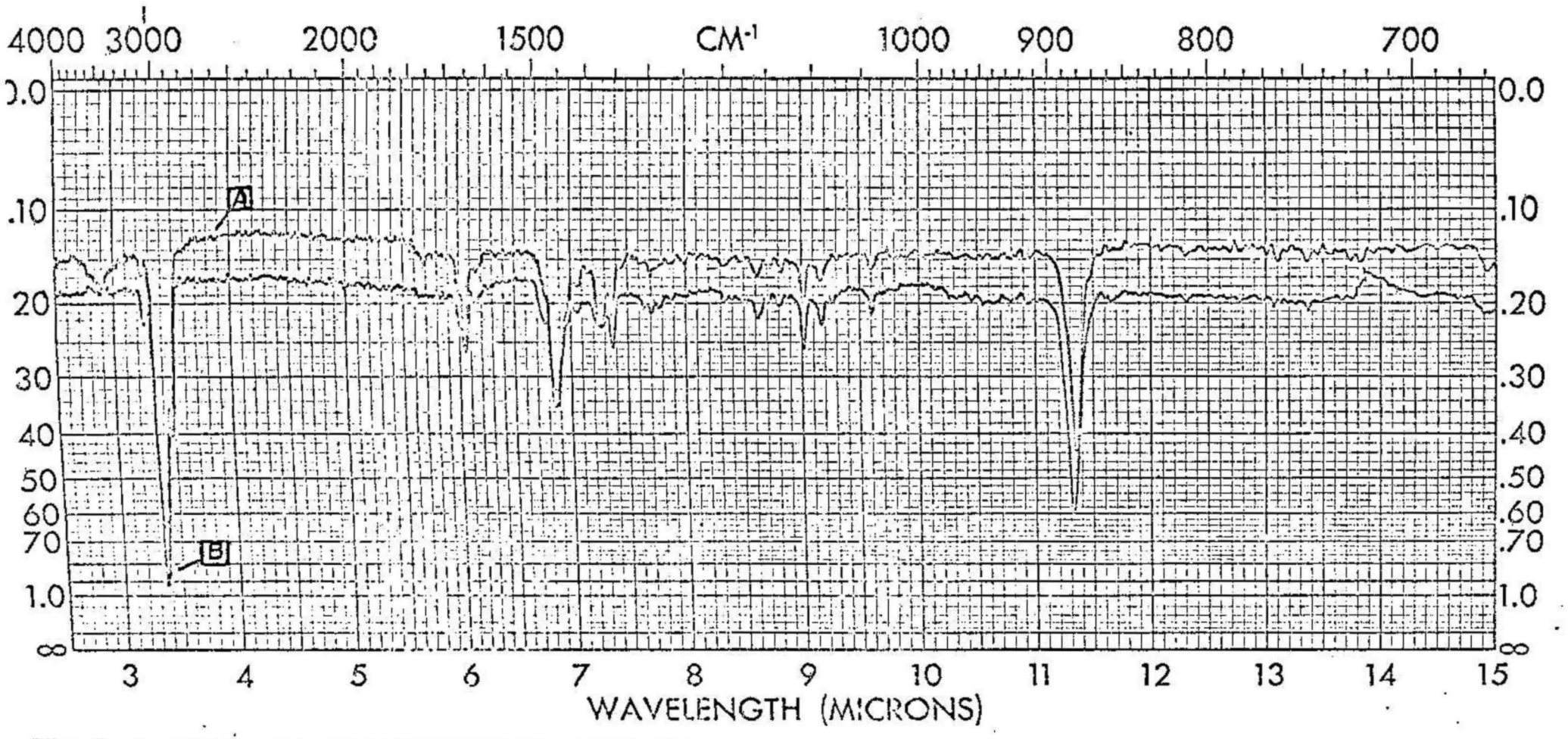


Fig. 29. IR Spectra of peak 18 (A) of Essential Oil of Anemopsis and of Camphene (B).

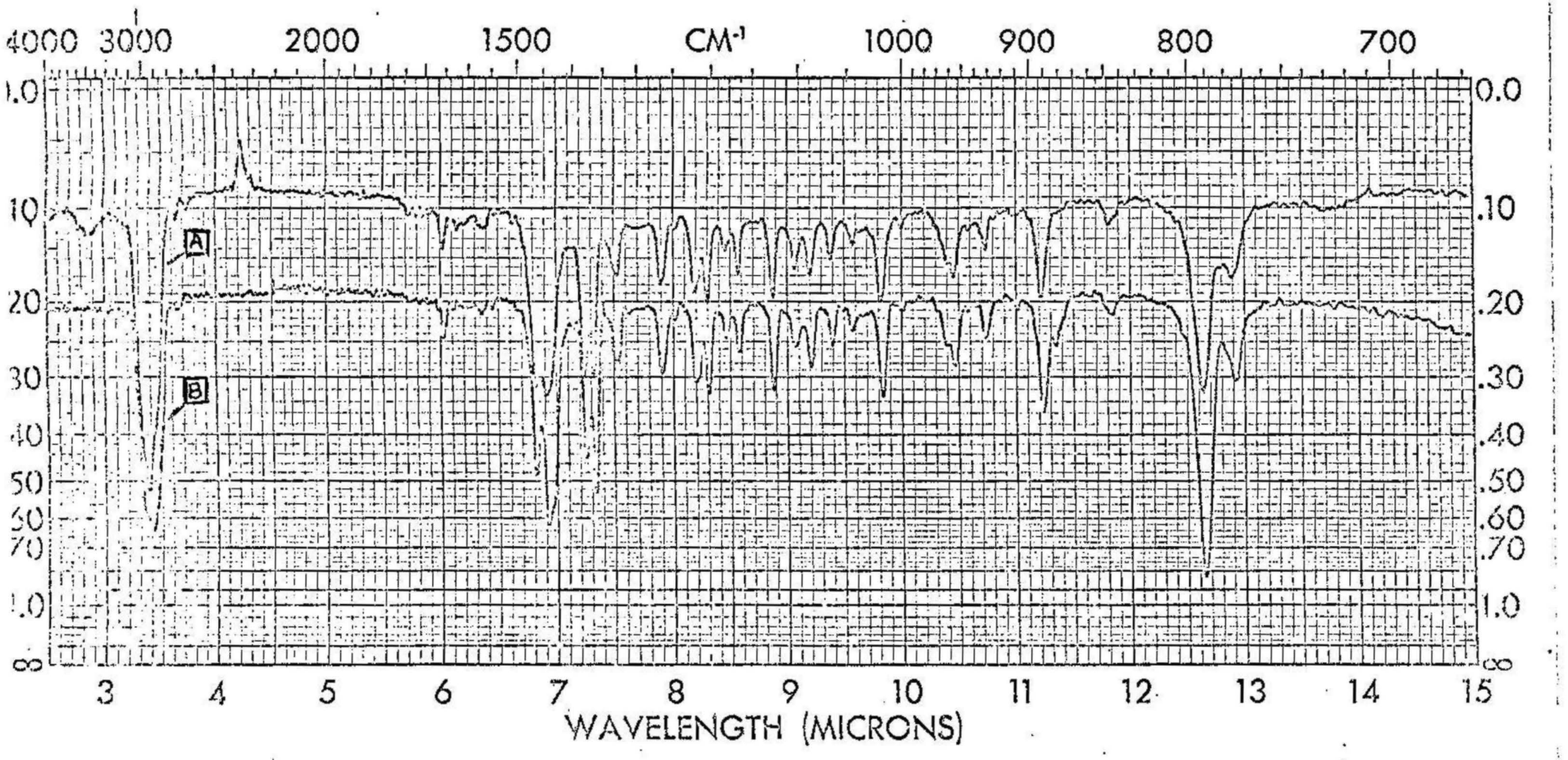


Fig. 30. IR Spectra of Peak 19 (A) of Essential Oil of Anemopsis and of α-Pinene (B).

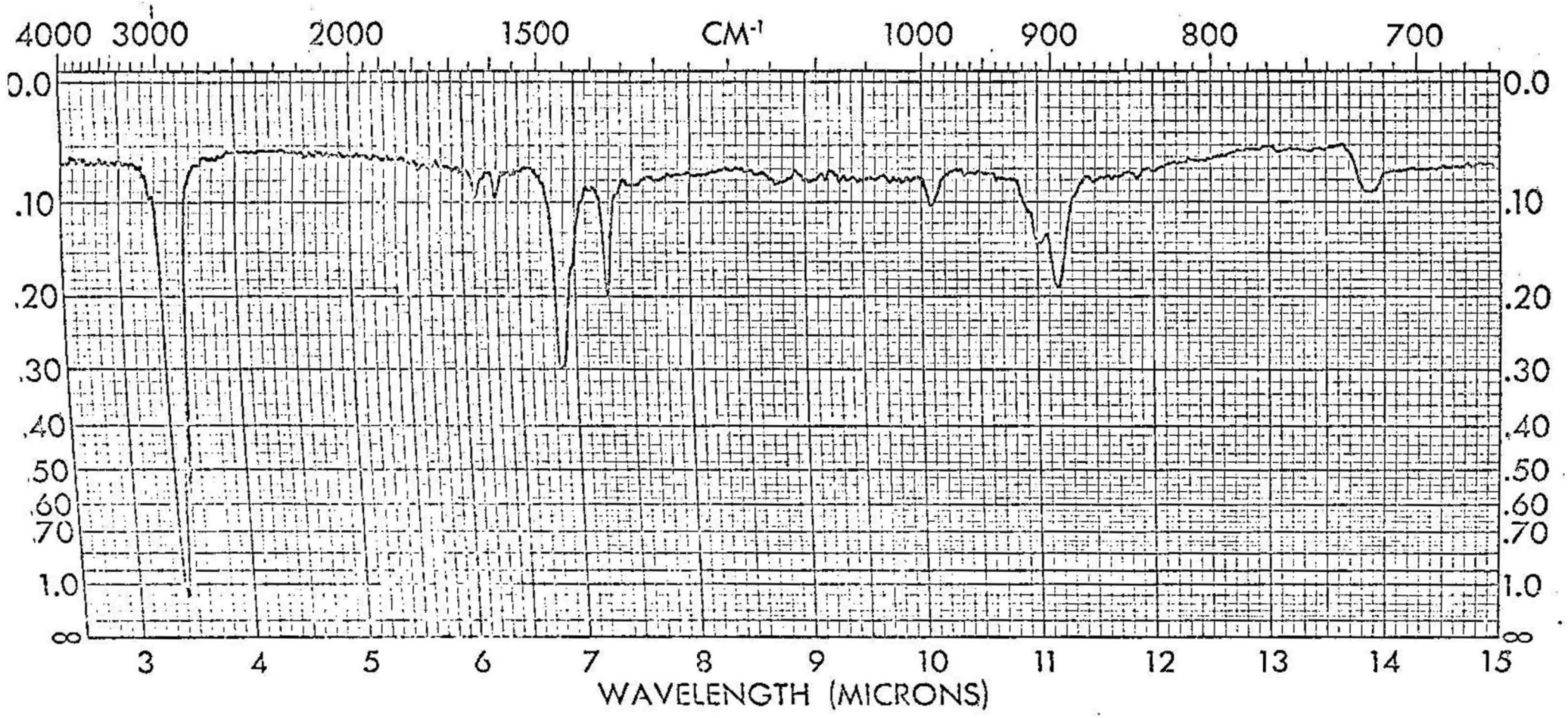


Fig. 31. IR Spectrum of the Total Fraction under Peaks 20 - 25 of Essential Oil of Anemopsis.

Table VI. Quantitative Data on the Constituents in the Essential Oil of Anemopsis.

Peak	compound	Integration counts	% Conc. v/v
1	Thymol	5140	13.38
2	Methyleugenol	21950	57.1
3	Piperitone	3090	8.0
4	Unsat. Ketone	700	1.8
5	Unknown	270	0.7
6A	Sec. Cycl. alcohol	>1200	1.8
6B	Esdragol		1.3
7	Cycl. Ketone	130	0.34
8	Cycl. Ester	600	1.53
9	Thymolmethylether	1140	2.96
10A	Unknown	90	0.23
10B	Linalool	120	0.30
11	Unknown	-	-
12	Unknown	-	-
13	p-Cymene	420	1.1
14	1:8-Cineole	520	1.35
15	d-Limonene	360	0.93
17	Pinene	150	0.40
18	Camphene	170	0.44
19	Pinene	560	1.44
20-25	Hydrocarbons	1800	4.6

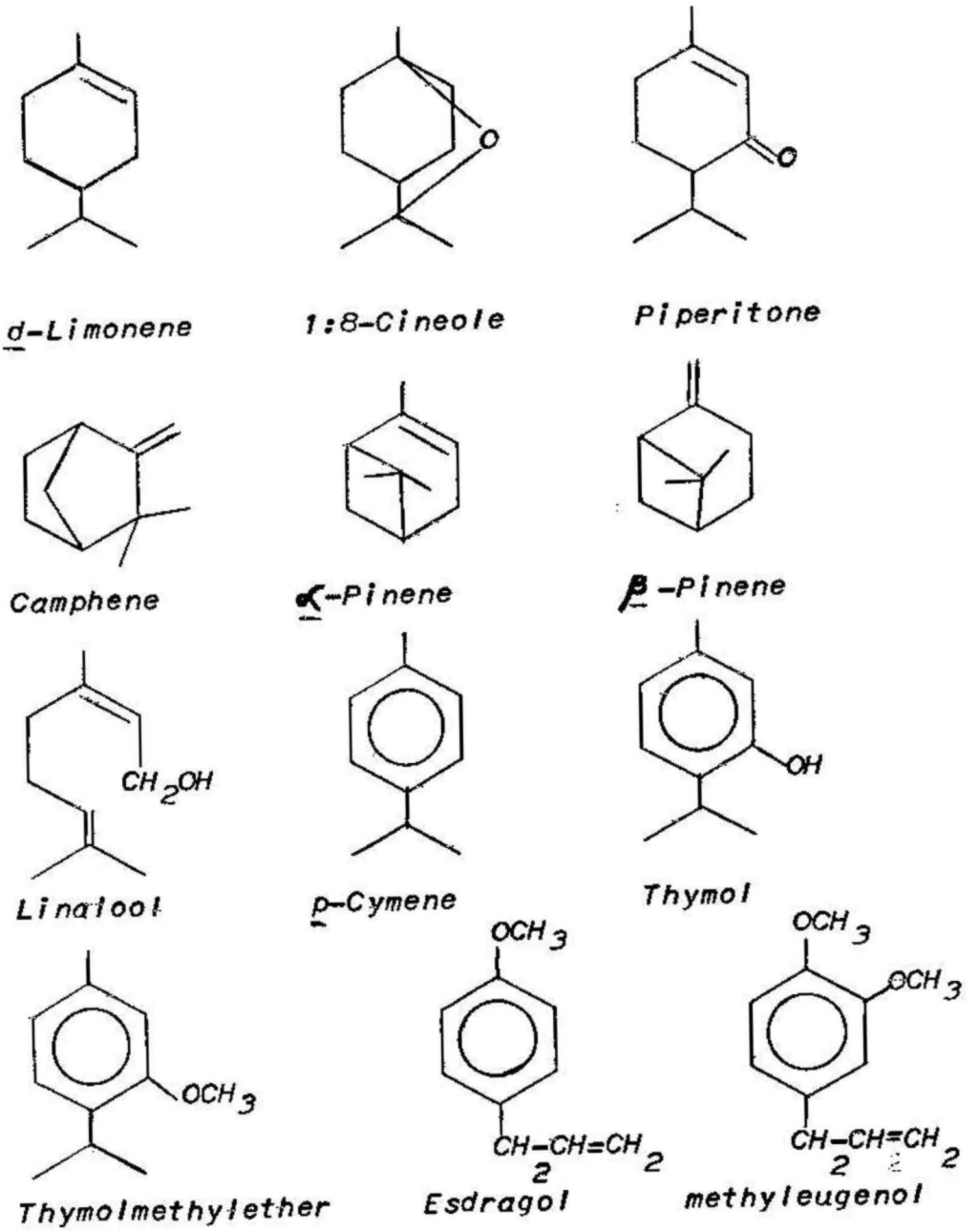


Fig. 32. Structures of Compounds isolated from Essential Oil of Anemopsis

CHAPTER IV

SUMMARY

The essential oil, hydrodistilled from the roots and rhizomes of Anemopsis californica (Nutt.) Hook and Arn. (family Saurauraceae), was analyzed by gas chromatography on two stationary phases: Reoplex-400 and Carbowax-20,. The retention data and quantitative analysis were carried out using the isotherml mode of gas chromatography. In order to identify the various constituents, the peaks eluting out of the chromatogram were condensed individually so as to yield a number of peak fractions. The resolution and collection of the fractions were facilitated by the use of isothermal-linear PTGC.

Although retention data were useful in giving a clue to the identity of the essential oil constituents, the major emphasis was placed on spectral analysis of the condensed fractions. Infrared spectroscopy was the principle method employed. The comparison of the IR spectra obtained under the same conditions of the constituents and of authentic GC-pure compounds made it possible to identify the following compounds in the essential oil: esdragol, thymolmethylether, linalool, p-cymene, 1:8-cineole, d-limonene, β-pinene, camphene and

α-pinene. Esdragol and thymolmethylether were also identified on the basis of their NMR spectra. On the basis of IR spectra alone, although confirmatory data are lacking at present, the following compounds have been tentatively identified: two ketones, a sec. diameric alcohol, an ester (principally formate) and six hydrocarbons.

BIBLIOGRAPHY

BIBLIOGRAPHY

1. Schneider, A., "Pharmacol Plants and Their Culture". California State Board of Forestry Bulletin 2, Superintendent of State Printing, Sacramento, 1912, pp 1270.

2. Horton, W. J., and Paul, E. G., J. Am. Chem. Soc., 79 2264 (1957).

3. Childs, R. F. and Cole, J. R., J. Pharm. Sci., 54 789 (1965).

4. Acharya, R. N., M.S. Thesis, "Essential Oil of Anemopsis californica", University of the Pacific, Stockton, California, 1967.

5. Acharya, R. N., and Chaubal, M. G., J. Pharm. Sci. 57, 1020 (1968).

6. Kulka, K., Perfum. Essent. Oil Record, 53, 147 (1962).

7. Guenther, E., "The Essential Oils", Vol I.-IV, D. Van Nostrad Co., New York, 1948.

8. Nigam, I. C. and Levi, L., Can. J. Chem., 46, 1944 (1968).

9. Brochmann-Hansen, E., J. Pharm. Sci., 51, 1017 (1962).

10. Phillips, C.,"Gas Chromatography", Butterworths Scientific Publications, London, 1956.

11. Keulemans, A. I. M., "Gas Chromatography", 2 nd Ed., Reinhold Publishing Corporation, New York, 1960.

12. Langer, S. H., and Sheehan, R. J., "Theory and Principles of Chosing and Designing Selective Stationary Phases", in Purnell, J. H., Ed., "Progress in Gas Chromatography", Interscience Publishers, New York, 1968.

13. Burchfield, H. P. and Storrs, E. E., "Biochemical Applications of Gas Chromatography", Academic Press, New York, 1962, p. 371.

14. Martin, A. J. P. and James, A. Y., Analyst, 77, 915 (1952).

15. Von Rudloff, E., Can. J. Chem., 38, 631 (1960).

16. Von Rudloff, E., Phytochemistry, 1, 195 (1962).

17. Von Rudloff, E., Can. J. Chem., 41, 1737 (1963).

18. Von Rudloff, E., Can. J. Chem., 41, 2876 (1963).

19. Nigam, M. C., Nigam, I. C., Handa, K. L. and Levi, L., J. Pharm. Sci., 54, 799 (1965).

20. Nigam, I. C. and Levi, L., Perfum. Essent. Oil Record, 54, 814 (1965).

21. Nigam, M. C., Handa, K. L., Nigam, I. C. and Levi, L., Can. J. Chem., 43, 521 (1965).

22. Nigam, M. C., Nigam, I. C. and Levi, L., Can. J. Chem., 43, 3373 (1965).

23. Purnell, J., "Gas Chromatography", J. Willey & Sons, Inc., New York, 1962.

24. Griffith, J., James, D. H. and Phillips, C.S., Analyst, 77, 897 (1952)

25. Purnell, J., Ed. "Progress in Gas Chromatography", Interscience Publishers, New York, 1968.

26. Dal Nogare, S. and Juvet, R. S. Jr., "Gas-Liquid Chromatography", Interscience Publishers, New York, 1966.

27. Crippen, R. C. and Smith, C. E., J. Gas Chromatog., 3, 37 (1965).

28. Kroman, H. S. and Bender, S. R., J. Chromatog., 13, 242 (1964).

29. Nigam, I. C., Sahasrabudhe, M., McConnell, D. T. W., Barlet, J. C. and Levi, L., Perfum. Essent. Oil Record, 53, 614 (1962).

30. Giuffrida, L., J. Assoc. Offic. Agri. Chemists, 48, 345 (1965).

31. Thomas, P. J. and Dwyer, J. L., J. Chromatog., 13 366 (1964).

32. Edwards, R. A. and Fagerson, R. S., Anal. Chem., 37, 1630 (1965).

33. Teranishi, R., Flath, R. A., Mon, T. R. and Stevens, K. L., J. Gas Chromatog., 3, 206 (1965).

34. Freeman, S. K., Ed. "Interpretive Spectroscopy", Reinhold Publishing Corporation, New York, 1965.

35. Gill, J. M., Bauman, F. and Hartman, C. H., Research Notes, Fall, 1966, Varian Aerograph Inc., Walnut Creek, California.

36. Gill, J. M. and McNair, H. M., Research Notes, Fall, 1965, Wilkens Instrument & Research Inc., Walnut Creek, California.

37. Brinner, E., Fliszar, S. and Ricca, M., Helv. chim. Acta , 42, 749 (1959).

38. Mitzner, B. M., Theimer, E. T. and Freeman, S. K., Appl. Spectr., 19, 169 (1965).

39. Bohrman, H. W. and Youngken, H. W. Jr., Phytochemistry, 7, 1415 (1968).

40. Scheuler, P. J. and Webster, R. H., Perfum. Essent. Oil Record, 55, 723 (1964).