A PHYTOCHEMICAL INVESTIGATION OF *Marrubium vulgare* L.

a

thesis

presented by

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PREFACE

ABSTRACT

A phytochemical investigation of *Marrubium vulgare* L. has been made. The volatile oil, obtained by distillation, was found to be composed of at least seventy-two components of which forty have been identified. The composition of this oil has been compared with that of the volatile oil obtained from *M. peregrinum* L. The presence of the diterpene marrubiin has been established and the effects of drying on its final concentration in the commercial herb examined. In addition to a comparison of nodal differences, ontogenetic variations in oil composition and marrubiin content have been studied. The relevance of oil composition in the chemotaxonomy of the family Labiatae has been discussed.

STATEMENT OF AUTHOR'S EDUCATIONAL AND RESEARCH EXPERIENCE

The author graduated with a B.Sc. (Hons.) from the Faculty of Agriculture, Cairo University, in June 1962. In 1963 he was offered a post as a demonstrator in the Department of Botany of the same Faculty and commenced research under the supervision of Professor Dr. A. Zaher and Dr. M.K. Fouad, being awarded the Degree of M.Sc. in April 1968 for a thesis entitled "Morphological and histological studies of *Vicia faba* L." He was then appointed as an assistant lecturer and registered for a Ph.D. degree in the same Department, passing all the courses required for the partial fulfilment of this degree. In September 1971, on being offered a place in the Department of Pharmacy, Victoria University of Manchester, he commenced research as a full-time postgraduate student. The results of this research form the thesis which is now presented.

DECLARATION

None of the work referred to in this thesis has been submitted in support of an application for another degree or qualification of this or any other University or other institute of learning.

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INTRODUCTION

1. ORIGIN AND SCOPE

The dried leaves and flowering tops of *Marrubium vulgare* L., Labiatae have been used for many years as a popular domestic remedy for coughs, colds and pulmonary affections (1,2). The pharmacological properties of the plant have been attributed both to the marrubiin content of the herb and to the presence of volatile oil (3). Although several investigations have been made regarding the chemistry and pharmacology of marrubiin (4-28) little has been reported concerning the volatile oil. Brieskorn et al.(29), and Bartarelli (21) determined the volatile oil content of the herb, indicating that it was extremely small, but little information was given about the composition of the oil or the chemistry of its components. Perhaps the low yield of oil presented a major problem when using traditional methods of analysis.

With the advent of sophisticated and sensitive techniques, for example, gas chromatography, nuclear magnetic resonance and mass spectroscopy, the study of natural products has become easier. The low oil content of this herb should no longer be the major obstacle in the examination of its composition. It is the aim of this investigation to examine the composition of the volatile oil of *Marrubium vulgare* L. and to identify the compounds present. With this knowledge, the pharmacological properties attributed to the volatile oil may be evaluated.

It is also aimed in this investigation to follow the formation of marrubiin in plants throughout the growing season, since conflicting evidence occurs about its origin. It has been suggested that marrubiin is an artifact arising from the volatile oil during the drying of the plants (30). This suggestion was supported when it was reported (25) that a new diterpenoid compound, named premarrubiin, had been isolated from *Marrubium vulgare* L.

Premarrubiin was considered to be the major diterpene in the fresh plant, marrubiin being formed from it due to the conditions experienced during the isolation procedures. However, Breccia and Badiello (24) have studied the biosynthesis of marrubiin during the flowering period of *Marrubium vulgare* L. using labelled precursors. They concluded that marrubiin was present in the living plants at the time of flowering and it had a half-life of about twenty-four hours. Although Popa (26) is reported to have studied the accumulation dynamics of marrubiin as a function of plant development, the results of this investigation are not available.

In stating the constituents found in *Marrubium vulgare* L., the literature (2,3,21) gives the impression that the diterpene lactone marrubiin is in no way connected with the volatile oil. Unlike the volatile oil, marrubiin is not steam volatile, but it is still a terpenoid compound and as such should be considered as part of the total product of terpenoid biosynthesis (31,32). To investigate in what way, if any, marrubiin formation is related to the formation of steam volatile terpenoid compounds in the plant, the chemical composition of the volatile oil has been determined at different times throughout the growing season. On comparing the results of this investigation with those obtained for marrubiin formation, any general trends and links in the production of the various groups of terpenoid compounds should emerge.

On commencing these investigations, a general survey of the literature revealed that plants of certain genera of the family Labiatae had a high volatile oil content, others had a low volatile oil content, whilst in the remainder, volatile oil was absent. This feature had also been observed by El-Gazzar and Watson (33) who used it to support a suggestion made by Bentham (34) that the family could be divided into two main groups. Iridoids,

which are considered to be monoterpene lactones (32,35), were also found in plants of this family (36-38) mainly in the genera in which volatile oil was absent. It was also interesting to note that iridoids occurred frequently in plants of the Scrophulariaceae (37,39,40) of which certain genera had the quadrangular stems and opposite leaves normally associated with the Labiatae (41). Therefore, it appeared that it was not just the presence or absence of volatile oil which was important when considering the chemotaxonomy of the Labiatae, but also the types of terpenoid compound present and their distribution in plants of the family. A more detailed knowledge of the terpenoid constituents of *Marrubium vulgare* L., and, if possible other species of the genus, would provide further information which could be useful in chemotaxonomic studies. Whilst this work has been in progress, Kooiman (42) has investigated the occurrence of iridoid glycosides in the Labiatae. The importance of his results and the results of these investigations will be considered in the Discussion.

2. GENERAL INTRODUCTION

Marrubium vulgare L. is a plant of the Labiatae, which is a family of world wide distribution, whose centre is chiefly in the Mediterranean region, where it forms a major part of the vegetation. The family has some 200 genera and 3200 species and is economically important as a source of volatile oils (essential oils) which are used either in perfumery or as flavours in the pharmaceutical and food industries (43). Guenther (44) listed some 18 genera and 70 species of the Labiatae whose volatile oils are used in this way, the more important being Peppermint oil from *Mentha piperita* L., Japanese mint oil from *Mentha arvensis* L., Basil oil from *Ocimum* spp., Lavender oil from *Lavandula officinalis* Chaix., Origanum oil from *Origanum* spp. and Rosemary oil from *Rosmarinus* spp.

The secretory structures afford a valuable character for the identification of the genera and the species. The occurrence together of diverse types of clothing trichomes and characteristic short-stalked glands with a uni- or multi-cellular head is characteristic of the family Labiatae.

The secretory structures of the family Labiatae in general are represented by external glands which secrete the volatile oils. They have a stalk and a head region, the stalk may be uni-cellular or multi-cellular, and may even have several rows of cells whilst the head, which is the secretory part, may also be uni-cellular or multi-cellular. The secreted oil accumulates beneath a cuticle. The following forms of glandular trichomes occur in the Labiatae:

(a) Unicellular headed trichomes having either a short stalk of one to three cells, or a longer bicellular stalk composed of a long basal cell and a short neck-cell, or a still longer stalk, similarly uniseriate and provided with a short neck-cell at the apex.

(b) Bicellular headed trichomes which in some cases have quite a short stalk of either one or two cells, or a longer bicellular stalk in which the upper cell is developed as a neck cell.

(c) Trichomes with a head consisting of four cells. These glandular trichomes are very widely distributed and invariably have short stalks. They may either be the only type of trichome found in a species, or they may be accompanied by other glandular trichomes having a greater number of cells in the head. The first of these alternatives applies to several species of which one is *Marrubium leonuroides* Desr.

(d) Trichomes with a head consisting of eight cells, and mostly having a very short stalk. In surface view, they may have a rather scale-like appearance (labiatous glands).

(e) Trichomes with a head consisting of a minimum of sixteen cells.

Non-glandular trichomes are very varied and afford valuable characters for differentiation. The normal form of trichome is multi-cellular and uni-seriate, unicellular trichomes being rare in the Labiatae. The uni-seriate trichomes vary in length, sometimes only consisting of two cells but more often of a greater number, their walls being either smooth or

provided with cuticular thickenings. The basal cell is commonly spherical and swollen, or seated on a pedestal composed of several epidermal cells. Another form of uniseriate bicellular trichome occurs which has a short basal cell and a long terminal one.

Different types of branched trichomes occur in the Labiatae. In the genus *Marrubium* branched protective trichomes occur. These consist of a central multicellular uniseriate trichome surrounded by two to four unicellular trichomes, all of which are fused at the base. *Ballota hirsuta* can be distinguished from *Marrubium vulgare* L. by the presence of branched trichomes of which the central branch terminates in a glandular head of one to four cells (45-48).

The genus *Marrubium* contains some 35 species (41) of which *Marrubium vulgare* L. is distributed as a common weed in waste places (49). In the British Isles, plants are mainly confined to the southern half of England, but they do extend into some parts of Scotland. It is rare in Ireland (50).

Marrubium vulgare L. is one of 30-40 medicinal species distributed through Europe, North Africa, extra tropical Asia and the U.S.A. (51). The plant is hardy, easily grown, flourishing best in a dry poor soil. Plants can be propagated from seeds sown in the spring, cuttings, or by dividing the rhizome which is the most usual method. If raised from seeds, the seedlings should be planted out in rows, with a space of about nine inches or more between each plant. No further cultivation will be needed other than weeding. Plants do not flower until they are two years old (52).

Marrubium vulgare L. has a number of synonyms: Horehound, White horehound, Common horehound, Common white horehound, Hoarhound, Horehowne, Horone hound bene, Marrube, Marvel and Mawroll (49,52 and 53). Horehound will be the one used in the present work. The Romans esteemed Horehound for its medicinal properties, and the generic name *Marrubium* is said to be derived from Maria urbs, an ancient town of Italy. An alternative derivation of the name is from the Hebrew marrob which means a bitter juice. The Egyptian Priests called this plant "Seeds of Horus" "Bull's blood" or "Eye of the star" (52).

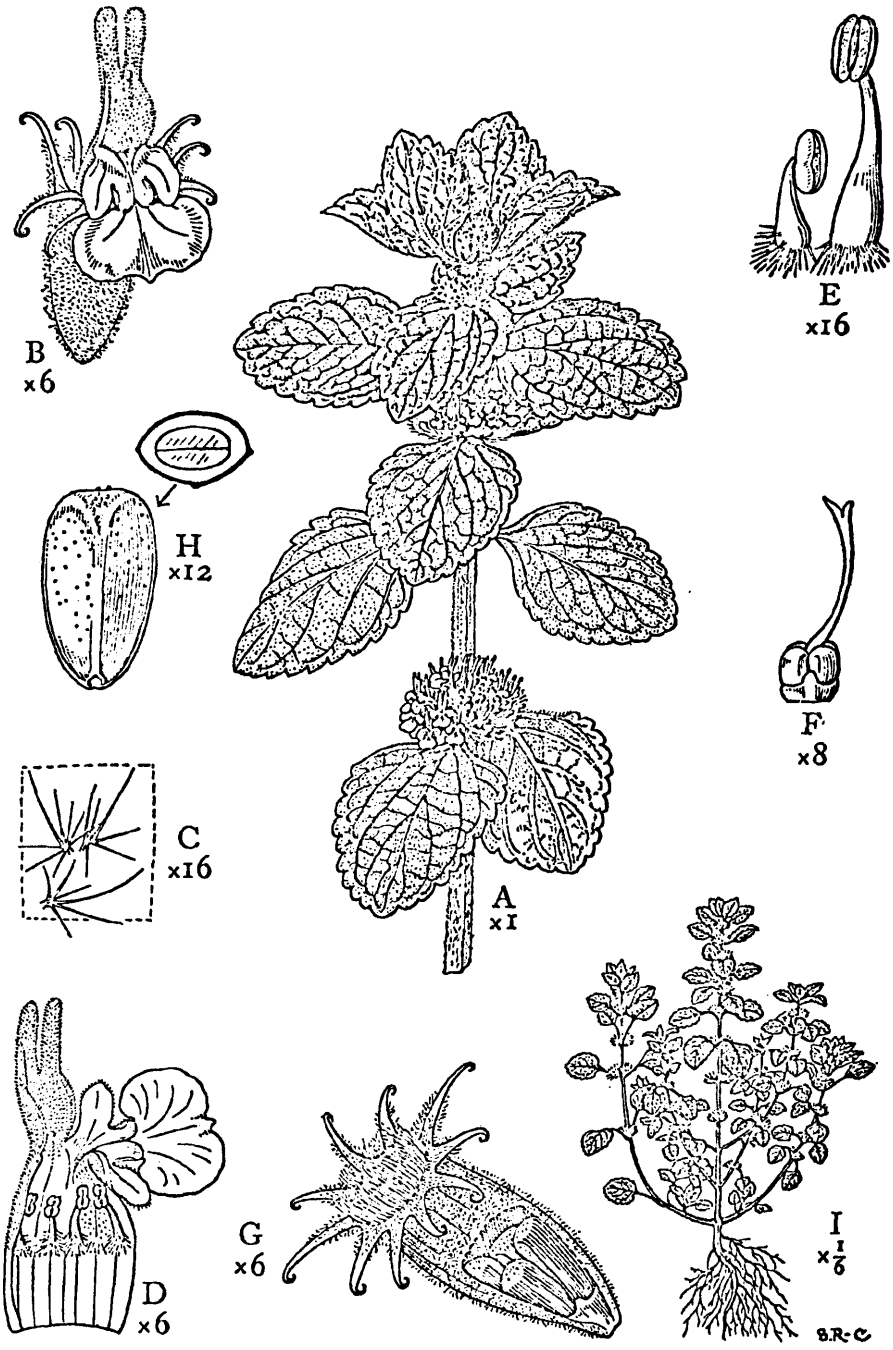


FIGURE 1: *Marrubium vulgare* L.

A, upper part of a flowering stem; B, flower; C, hairs from the calyx - similar on all parts of the plant; D, corolla spread out, and the stamens; E, stamens; F, gynoecium; G, fruiting-calyx - partly cut away to show the nutlets; H, nutlet and transverse section of same; I, plant. Corolla white; calyx-teeth ochre-yellow. Whole plant white-hoary; leaves more or less green depending on denseness of the covering of hairs.

3. THE GENERIC AND SPECIFIC CHARACTERS OF *Marrubium vulgare* L.

Marrubium vulgare L. is illustrated in Figure 1, (54). The following description has been compiled from several sources (2,41,43,46,49-53,55-63).

Horehound is a perennial herb, 30-60cm. in height, with a short stout rhizome and branched stems. Stem erect, rather thick, quadrangular, much branched from the base and pubescent, especially when young. Leaves petiolate. In transverse section the petiole is as shown in Figure 2 (46).

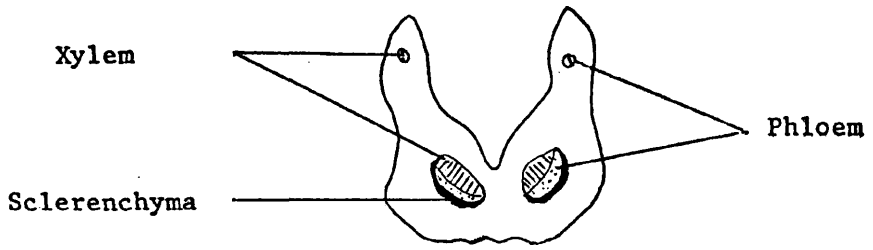


FIGURE 2: Petiole of *Marrubium vulgare* L., X26.

Leaves are much wrinkled, having a white-woolly abaxial surface and green adaxial surface or being somewhat tomentose on both surfaces. Leaves roundish-ovate or rhombic-ovate in shape, the margin being strongly and irregularly crenate-serrate, except at the cuneate or truncate base.

Flowers exist in dense whorls or clusters in the axils of the leaves, each cluster being broader than high. Bracts are not differentiated having linear bracteoles. Flowers small. Calyx tubular, woolly, with ten ascending-spreading subulate or spinulose teeth which are hooked at the tip.

Calyx teeth alternate short and long. Corolla white, 1.5cm. in length, the basal tubular part being included in the calyx. Upper lip of the corolla erect, strapshaped, much longer than the lower, entire, or bifid at the apex, the division not reaching halfway down the lip. The lower lip spreading, three-cleft with a broad middle lobe. Stamens four, included within the corolla tube. All anthers have two lobes. Ovary deeply four-lobed. Style two-lobed. Fruits in form of nutlets smooth and rounded at the top.

Horehound flowers from June to September. The plants are mainly pollinated by bees or self-pollinated, being homogamous or weakly protandrous. Small female flowers occur. The plant has a bitter taste and a curious musky smell which is diminished by drying and lost on storage.

The main diagnostic features of *Marrubium vulgare* L. are the dense, woolly hairs and the ten hooked calyx teeth. Other species which have occasionally been substituted for Horehound may be differentiated using these characters. *M. peregrinum* L. has much smaller, hardly tomentose, elongated lanceolate leaves having the same colour on both surfaces. The flowers are formed in whorls each containing six to ten flowers and the calyx has five straight teeth. *M. candidissimum* L. has five nearly straight calyx teeth and is also less bitter and aromatic. Black Horehound, *Ballota nigra* L. has dark green leaves with rough, not woolly hairs. It also has a disagreeable odour.

4. CHEMISTRY OF *Marrubium vulgare* L.

The isolation of the following substances from Horehound has been reported:

(a) Marrubiin (4-27). (b) Volatile oil (21,29). (c) Alkaloids, 0.3% betonicine, 0.2% choline and 0.001% unidentified alkaloids (21,64-66). (d) Hydrocarbons (67). (e) Sterol (18,21). (f) Glucosides (21). (g) Ursolic acid, 0.12% (21,29) and gallic acid (21). (h) Tannic acid, 2.6-2.9% (21,66). (i) Resinous and waxy materials (21,68). (j) Mucilaginous and pectic substances (21). (k) Vitamin C (21,66). (l) Iron (21). (m) Potassium nitrate, 2.8-3.0% (21).

Marrubiin:

Previous investigations dealing with Horehound have been confined mainly to the isolation, structure elucidation and biosynthesis of the bitter principle marrubiin, as this was reported to be the major constituent of the plant. Marrubiin was first isolated in 1842 (4). Different samples of the herb produced widely different amounts of marrubiin according to the season of harvesting, conditions of drying and length of storage. The marrubiin content of Horehound varied from 0 to 1% (6,9-11 and 15). However, it has been suggested recently that marrubiin may not be originally present in the plant (25).

The structure of marrubiin has long been established. In 1908 Gordin (5) considered marrubiin to be a lactone $C_{21}H_{28}O_4$, m.p. 154.5-155.5°C which on hydrolysis yielded marrubic acid $C_{21}H_{30}O_5$, m.p. 173-174°C, which in turn was capable of forming an ethyl ester m.p. 87°C. McCrea (6)

described marrubiin as a glucoside, whilst Hollis et al.(7) concluded that marrubiin was a hydroxyditerpene of the manoyl oxide type having the formula $C_{20}H_{28}O_4$. Lawson and Eustice (8) found that analytical results for marrubiin and its derivatives favoured the formula $C_{20}H_{28}O_4$ rather than $C_{21}H_{28}O_4$ as suggested by Gordin (5). After a series of investigations begun in 1949, Cocker et al. (11) considered marrubiin to be a furan derivative of the formula $C_{20}H_{28}O_4$. In the ultra violet (u.v.) region, marrubiin showed maxima at 2080, 2120 and 2160 A^{o} which were in substantial agreement with those expected for a substituted furan. Hardy and Rigby (12) concluded that marrubiin was a γ-lactone, as the infra red (i.r.) absorption maximum was at 1780 cm^{-1} in carbon disulphide. The stereochemistry of the diterpene lactone marrubiin has been the subject of much controversy, but as a result of the evidence advanced (13,14,17,20,22 and 27) the following formula is generally accepted:

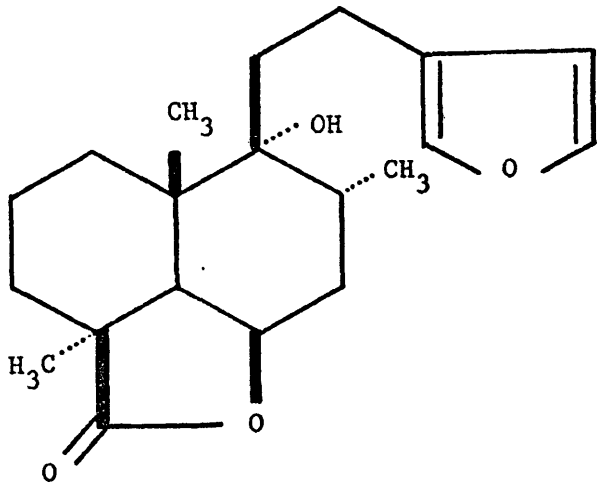


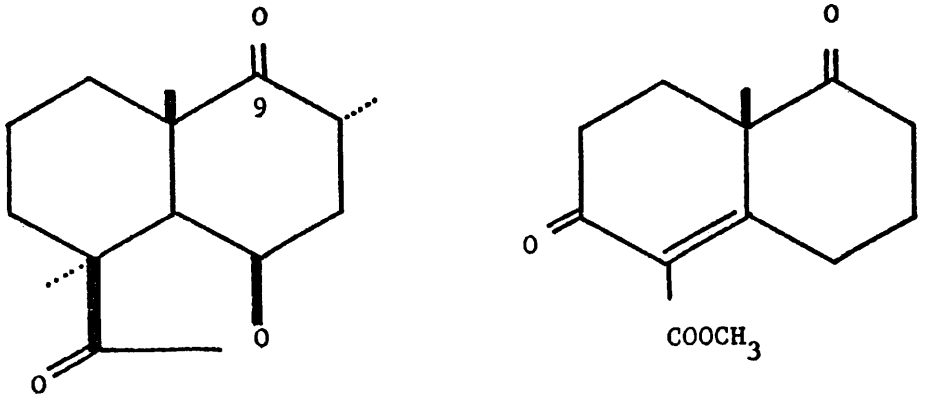
FIGURE 3: Marrubiin, $C_{20}H_{28}O_4$.

The kinetics of the incorporation into marrubiin of succinic acid, mevalol acetone and acetic acid labelled with ^{14}C was studied in flowering Horehound plants (19). Data obtained from the degradation of the marrubiin synthesized, suggested that at least three carbon atoms of succinic acid were involved in the synthesis of marrubiin. The evidence suggested that succinic acid was incorporated into marrubiin by prior conversion to isoprenic units which contained only one of the carboxyl groups of succinic acid. Acetic acid and mevalol acetone were also incorporated into marrubiin.

Breccia and Badiello (24) used the following labelled precursors to study the biosynthesis of marrubiin: (2-14C) mevalonate, (1-14C) acetate, (2-14C) pyruvate, (2,3-14C) succinate, (1,4-14C) succinate, (5-14C) keto-glutarate and (1,5-14C) citrate. The labelled compounds were supplied in solution to the flowering plants cut at the base of the stem. The incorporation results indicated that marrubiin underwent a rapid turnover with a biological half-life of about 24 hours. Labelled marrubiin was formed 10-100 times more actively when (2-14C) mevalonate was used than with other precursors. The results suggested that, with the exception of mevalonate, all the precursors were degraded to acetate units through the Kreb's cycle before their utilization. Some samples of marrubiin were degraded chemically to the corresponding keto derivative in order to determine the distribution of radioactivity between the decahydronaphthalenic and furanic parts. The results were in good agreement with the distribution of the radioactivity in the molecule as expected from the terpenoid structure of marrubiin.

The role of Horehound cellular fractions in the biosynthesis of marrubiin has also been investigated. (2-14C) acetate, (2-14C) mevalonate and (23-14C) succinate were used as precursors. Using a cell homogenate freed from mitchondria, the highest incorporation was obtained with acetate and mevalonate while succinate was incorporated only in the presence of the crude homogenate (23).

Mangoni et al. (28) suggested that the keto lactone (I), Figure 4, easily obtained by the degradation of marrubiin, could be an intermediate for the synthesis of marrubiin since in (I) most of the structural features of marrubiin were present as well as a keto group at the 9-position, which could be used to introduce the side chain bearing the furan ring. The synthesis of marrubiin has been achieved starting from this keto lactone which was prepared stereoselectively from the known keto ester II.



I II

FIGURE 4: Keto lactone (I) and keto ester (II).

Volatile oil:

Little was known about the composition of the volatile oil obtained from Horehound. Both Brieskorn et al. (29) and Bartarelli (21) stated that the herb contained 0.06% $^{v}/_{w}$ of volatile oil of undetermined composition. The presence of an unidentified sesquiterpene ($C_{15}H_{22}O_2$, b.p. 200-5°C) was reported by Nicholas (18) and Bartarelli (21).

5. PHARMACOLOGICAL PROPERTIES ATTRIBUTED TO *Marrubium vulgare* L.

Horehound is a well known old domestic remedy for coughs and other pectoral complaints. It has been known to medical science for many centuries. Hippocrates, who was born about 450 B.C., included Horehound in his list of 'simples' and since then it has been in constant demand (69).

Several remarkable properties have been attributed to Horehound preparations and the following list was compiled from Culpepper's Herbal (70):

(a) A remedy for shortness of breath or asthma. (b) Curing coughs. (c) An expectorant. (d) Expelling the afterbirth. (e) An antidote for poisons. (f) An analgesic in earache and pains of the side. (g) Clearing obstructions of the liver and spleen. (h) An anthelmintic. (i) Healing ulcers. (j) Relieving itching.

Watt and Bradwijk compiled the following survey of the medicinal uses of Horehound (71). In South Africa the Europeans use an infusion of Horehound in febrile conditions, especially in typhoid fever. In many parts of the world the infusion has been used as a domestic remedy for bronchitis producing profuse expectoration. It is stated to be a tonic and, in large doses it acts as a purgative. In the U.S.A. the herb has been used for colds, in dyspepsia, as a stimulant tonic, purgative and as a vermifuge, while in Australia the leaves are a cough remedy.

The parts of Horehound which are used consist of the dried leaves and flowering tops of the plant (2). The plants should be gathered when in flower. It may be used in the dry state but it is certainly less active than when fresh (50).

Horehound herb has been prepared in several ways for use as a medicine: (a) As an infusion in water (2,50,58,59,70,71). (b) As a syrup (2,41,60). (c) As a candy (52,70). (d) As a powder (2,52,70). (e) As an ointment (52,70).

It was included in the following pharmacopoeias:
(a) Greek (1868). (b) Portuguese (1876). (c) Russian (1880). (d) Spanish (1884). (e) French (1895). (f) U.S.A. (1905). (g) Mexican (1925). (h) German (1941). (i) British (1949). (j) Austrian (1960). (k) Hungarian (1970).

Although no longer an official substance of the British Pharmaceutical Codex (72), Horehound is still widely used in the British Isles in herbal and patent medicines (73).

The pharmacological properties of marrubiin and its derivatives have been reviewed and are briefly described as follows:

(a) Gastro-intestinal properties

Krejči and Zadina (74) made a direct determination of the amounts of bile and pancreatic juice secreted into the duodenum of rats and found that secretion was materially enhanced by the administration of marrubic acid and its sodium salt, even though the action was not long sustained. Marrubiin did not stimulate choleresis.

By comparison, spray dried extracts of powdered leaves and flowers showed hypocholeretic and antispasmodic properties (75). The extract, which was non-toxic to mice by intraperitoneal injection at 1.2g./kg. of body weight, was toxic at 4g./kg. Tablets containing 0.25g. of the powder proved to be useful in the prevention of migraine caused by digestive troubles, postprandial flushing and in the treatment of diarrhoea.

An aqueous extract of the leaves and flowers of Horehound had an antagonistic effect towards hydroxytryptamine when tested in vitro on guinea pig ileum and the uterus of rat in oestrus. In vivo, in mice the extract also had an antagonistic effect toward the peripheral and central effects of hydroxytryptamine. The extract relaxed the sphincter of Oddi in dogs, decreasing the amplitude and frequency of contractions (76).

(b) Abortive properties

Kchouk and Chadli (77) carried out experiments with gravid guinea pigs, rats and mice, using a decoction of Horehound in order to determine its abortive effects. This investigation was initiated when a six months pregnant woman had aborted after the ingestion of 100ml. of a decoction of Horehound. From their results, this product appeared to show a certain abortive activity in rats, its action on guinea pigs and mice was less convincing.

(c) Anticancer properties

In a literature survey of plants which had been used in the treatment of cancer, Hartwell (78) gave some 30 references to the use of Horehound. No experimental evidence was presented to support its successful use as an anticancer.

(d) Antifungal and antibacterial properties

The intramuscular injection of 80mg. of magnesium marrubyl marrubinate protected hens from subsequent inoculation of *Bacillus anthracis*. In a concentration of 1:500 it had no effect on the growth of *B. anthracis* in vitro (79).

In slightly acid media magnesium marrubinate and magnesium marrubyl marrubinate decreased alcoholic fermentation by yeast, the marrubinate being about half as active as the more complex compound. The mechanism of action was not clear, but part of the effect appeared to be due to cell damage caused by the formation of fine needle crystals of marrubinic (marrubic) acid within the yeast cells (80).

The addition of Horehound extracts to Raulin and Sabouraud media used for growth of *Rhizopus nigricans* at levels from 1:1000 to 1:500,000 caused definite retardation of growth at the lower dilutions, while stimulation was noted with the higher dilutions. Neither the formation of zygospores nor the germination of the spores was affected (81).

6. CHEMOTAXONOMY OF THE FAMILY LABIATAE

With the exception of some minor modifications, the system used for the classification of the family Labiatae is still the same as that established some 140 years ago (34). Morphological characters, which can vary even in a given genus, are used in classical systems of plant taxonomy, those of the flower, particularly the androecium and gynoecium, being the most important. This is true for the family Labiatae, and unless the reproductive organs are attached to a sample, it becomes difficult, sometimes impossible, to make a full identification of the plant. With the development of rapid and accurate methods of screening plants for particular chemicals, chemotaxonomy is being accepted as a possible alternative method of plant classification (82).

Erdtman (83) defined chemotaxonomy as the study of the distribution of characteristic chemical constituents found in related, or supposedly related, plants. Von Rudloff (82) mentioned that the secondary products, or the so-called plant extractives, are most suitable for chemotaxonomic investigations and that groups of chemically, or biosynthetically related compounds give more useful data than do single compounds.

For the purpose of classification, Erdtman (84) considered that a knowledge of the genesis of plant constituents is just as important as a knowledge of their structures. The reason is simply that identical compounds may be synthesized in different ways in different plants, being therefore analogous but not homologous in the biological sense of the terms. Our knowledge concerning the exact course of the biological synthesis of natural products remains quite limited. Biochemical studies of fundamental importance have revealed the existence of several rather general biosynthetic pathways such as the well known acetate-malonate-fatty acid, the shikimic-prephenic acid, and the mevalonic acid pathways.

The results of several investigations, both morphological and chemical, have suggested that the family Labiatae could be divided into two main groups, this suggestion being made first by Bentham as early as 1848 (34). Wunderlich (85) showed that the family was composed of two types of plant, one type having tricolpate binucleate pollen grains while the other type was characterized by hexacolpate trinucleate pollen grains. The concept of two groups was emphasized by the differentiation patterns of seed development and of mature seed anatomy. El-Gazzar and Watson (33,86,87) added further evidence in favour of this suggestion based on computational analysis of extensive comparative anatomical and morphological observations. Again two groups were recognised within the family, each group containing the same taxa as the groups recorded by Wunderlich. They found additional evidence to support this division, the trinucleate group being rich in volatile oil, the binucleate group with the exception of *Pogostemon* and *Prostanthera* being poor (less than 0.1% volatile oil). They also noted that the first group was susceptible to the rust *Puccinia menthae*, whilst, with the exception of certain species of *Ajuga* and *Mellitis*, records for rust attack on plants of the second group were lacking.

Novitskaya and Krishtopa (88) stated that the seeds of plants from the trinucleate group had a high content of linolenic acid in their fixed oils, while the other group had a low content. More recently, the results of investigations made by Kooiman (42) have added further evidence. By testing plants of the family for the presence of iridoid glycosides, he found that they were absent in the trinucleate group, while a large number of plants from the other group had these compounds. The trinucleate group includes the tribes Ocimoideae, Satureieae (Elsholtzieae, Menthoideae, Thymeae, Melisseae), Monardeae and Nepeteae, while the binucleate group includes the tribes Stachydeae (Scutellarieae, Melitteae, Marrubieae, Lamieae), Prasieae, Prostanthereae and Ajugoideae (87).

The weight of evidence in favour of dividing the family into two groups is heavy, but certain anomalies occur when volatile oil and iridoid glycoside content are used as the basis for this division. The use of more detailed information such as the types of terpenoid compound present, the relative amounts of each type or even the biosynthetic capability of a plant, might help to overcome these difficulties. As stated earlier, Erdtman (84) considered that a knowledge of the genesis of plant constituents was important for the purpose of plant classification, therefore a schematic diagram which shows the inter-relationship of the biosynthetic pathways of the various terpenoid compounds, including the iridoids, is given in Figure 5. Further subdivision of the two major groups may be possible using this type of biochemical data.

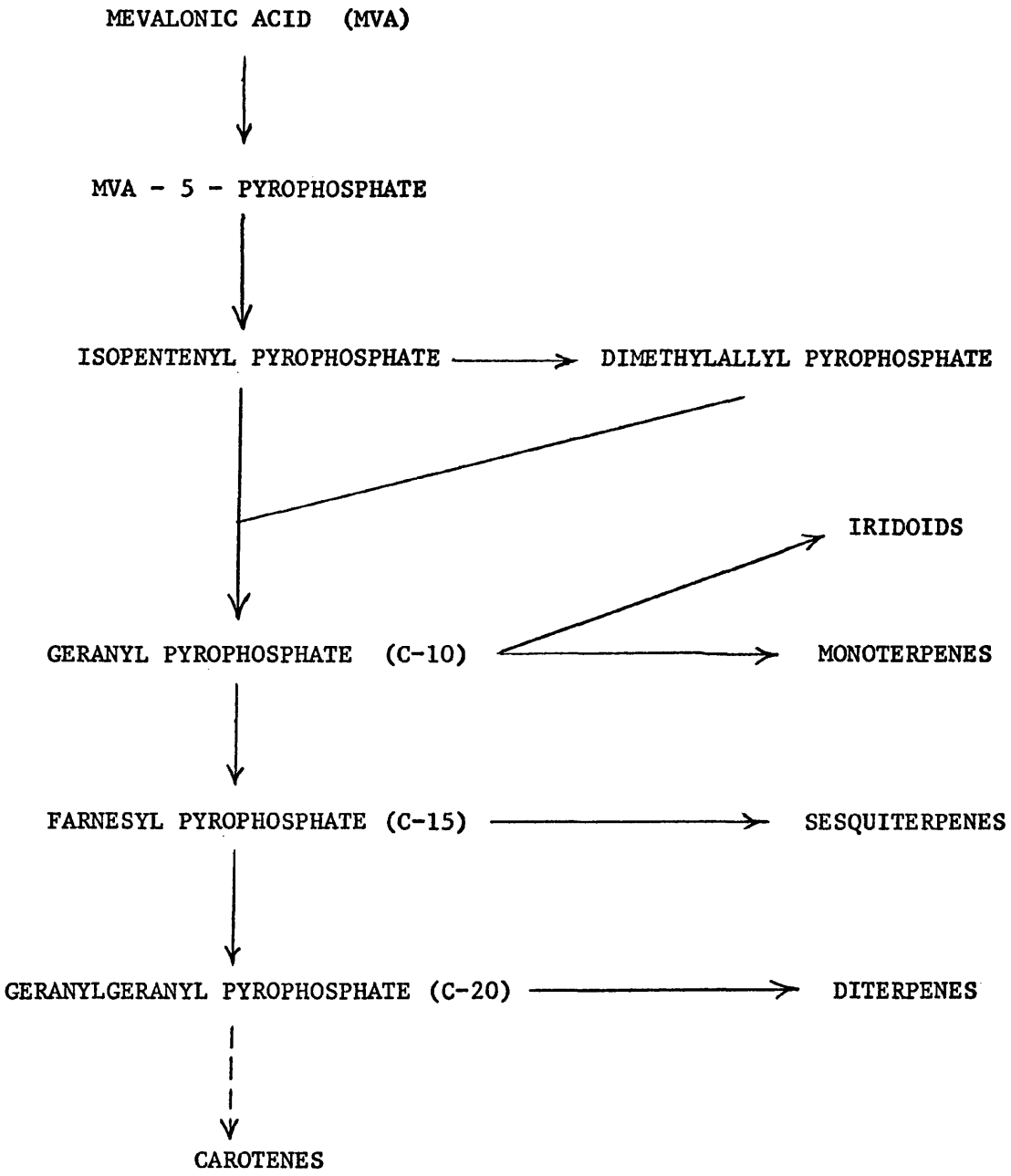


FIGURE 5: Diagram of the conversion of mevalonic acid into terpenoid compounds (31,32).

EXPERIMENTAL

1. SOURCE OF PLANT MATERIAL

Horehound herb is available commercially, and was purchased from Brome and Schimmer Ltd., England. Since the herb is frequently subject to adulteration (2), authentication of the commercial material was carried out.

(a) Macroscopical examination

As the commercial herb was supplied in a broken condition, it was difficult to compare its macroscopical characters with the official descriptions. The calyx and quadrangular stem were the only characters to remain intact, and these were examined and compared with the calyx and stem of a herbarium specimen of Horehound. This was part of the Pharmacy Museum collection, Pharmacy Department, University of Manchester.

During these examinations of the first sample, large quantities of seeds were found. Hanna and Kozlowski (89) had suggested that the minimum germination capability of Horehound seeds was 80%, viability being retained in full up to two years after harvesting. Therefore, these seeds were sown and cultivated along with a sample of Horehound seeds obtained from the Royal Botanical Gardens, Kew. This enabled a full macroscopical comparison to be made between flowering plants grown from seeds taken from the commercial sample of herb, flowering plants grown from authentic seeds, the herbarium specimen and standard descriptions of the flora.

Figure 6 shows a flowering plant grown from seed taken from the commercial sample of herb and Figure 7 shows in greater detail, the flowers of the same plant. The characters of these plants agree with the standard description given in the Introduction. The commercial herb was also examined for any foreign matter. None was present, therefore the herb was proved to be authentic.

FIGURE 6: A flowering plant of *Marrubium vulgare* L. grown from seed taken from the commercial sample of herb.

FIGURE 7: Flowers of *Marrubium vulgare* L. grown from seed taken from the commercial sample of herb.

(b) Microscopical examination

In view of the difficulties associated with making a full routine macroscopical examination on each batch of Horehound herb, a comparative study was made of the microscopical structure of the herbarium specimen and the commercial sample. The examination was confined to the epidermal characters of the leaf fragments and the structure of the petiole in transverse section, since the vascular structure of the petiole of the family Labiatae is of taxonomic interest (46).

The material was prepared for examination by placing it overnight between two water saturated filter papers. The leaf fragments and the petiole after sectioning, were mounted for examination in either chloral hydrate or phloroglucin and hydrochloric acid (90).

For further authentication, materials were collected for a full microscopical study to investigate the secretory structures of the commercial sample of Horehound. They were taken at the flowering stage from the plants grown in the greenhouse. Specimens were taken from different plant organs; the middle of the stem, the lamina, the petiole through its distal end, and different parts of the inflorescence at its median portion.

Materials were killed and fixed for at least 24 hours in F.A.A. (10 ml. formalin, 5 ml. glacial acetic acid and 85 ml. ethyl alcohol 70%). After fixation, materials were washed in 50% ethyl alcohol and dehydrated in a tertiary butyl alcohol series (91), before being embedded in paraffin wax (melting point 54.5^{o}C). Sections which were cut on a Cambridge microtome to a thickness of 12μ were stained with crystal violet/erythrosin (92) before mounting in Canada balsam.

On examination of the prepared sections, it was found that trichomes of the following types covered the whole epidermal surface:

(i) Non-glandular trichomes:

Three types of non-glandular trichome with lignified walls were present.

(a) Unicellular trichomes (Figure 8,A).

(b) Branched multicellular trichomes with a series of two to four unicellular branches (Figure 8,B).

(c) Branched multicellular trichomes with a series of two to six unicellular branches and one or two central multicellular branches (Figure 8,C). These trichomes were seated on a pedestal composed of several epidermal cells. This type of trichome represented the main covering trichome of Horehound and covered densely the whole epidermis.

(ii) Glandular trichomes:

Two types of glandular trichome were found.

(a) Labiatous glands having short or sometimes long stalks with a head of eight cells which had a rather scale-like appearance in surface view (Figure 8,D). These glands had a golden colour due to the presence of the volatile oil. Laminae and floral parts were covered by numerous labiatous glands while stems and petioles had a smaller number.

(b) Glandular trichomes mostly having short stalks and a head composed of four cells with radial vertical walls placed at right angles to the surface of the organ bearing the trichome (Figure 8,E).

In addition to the glandular trichomes, another type of secretory structure was observed in the midrib region. The presence of resin ducts confirmed the findings that Horehound secreted resinous materials (21,68). Nectaries occurred in the flowers on the basal parts of the stamens. They had a dense cytoplasm and were elongated like palisade cells.

Two large median vascular bundles accompanied by a few small ones were found in the petiole. Horehound petiole represented in the slide collection of Kew had the same structure (46).

The microscopical characters of the herbarium sample and the commercial sample of Horehound were found to be identical. They agreed with the descriptions given for this material in the British Pharmaceutical Codex, 1949 (2) and the Hungarian Pharmacopoeia, 1970 (63).

The macroscopical and microscopical characters of each new batch of material were examined to see that they conformed to the previously mentioned descriptions.

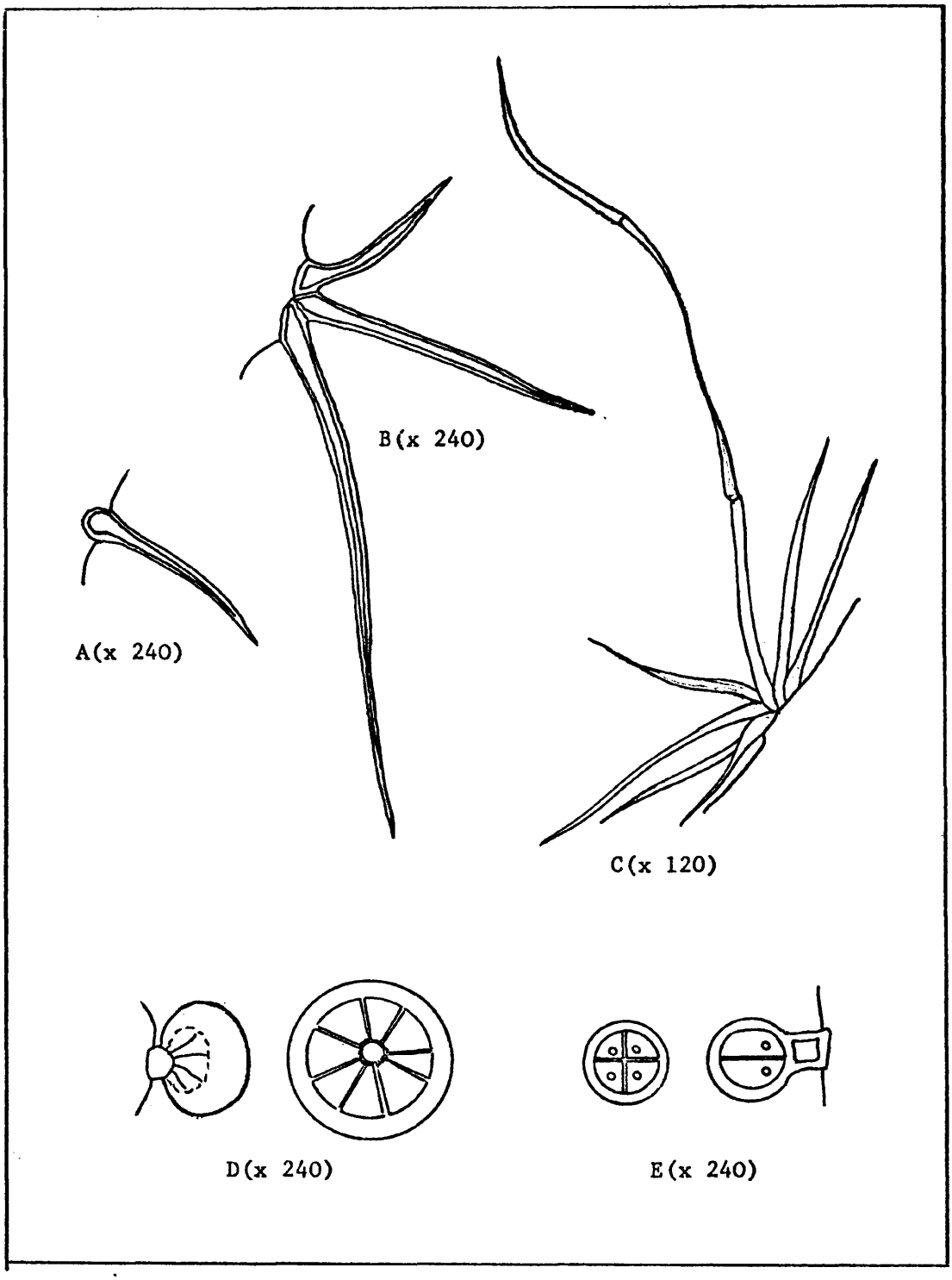


FIGURE 8: The trichomes of *Marrubium vulgare* L.

A: Unicellular trichome. B: Branched trichome having unicellular branches. C: Branched trichome with multicellular central branch. D: Labiatous gland.
E: Glandular trichome with a head consisting of four cells.

2. DETERMINATION OF THE MOISTURE CONTENT

Five samples of commercial herb, each of 10g., were weighed into flat bottomed glass dishes. Each sample was dried to constant weight in an oven at 110°C. The moisture content, expressed as the mean of five determinations, was found to be 8.2%

3. DETERMINATION OF THE VOLATILE OIL CONTENT

A modified method of that given in the British Pharmacopoeia, 1968 (93) was adopted to determine the volatile oil content. A hundred grammes of herb were mixed with one litre of distilled water in a spherical three-litre flask. An electrothermal heating mantle was used as the heat source, the distilling vapours being condensed by means of a double surface condenser which was open to the atmosphere. The distillate was collected using a water estimator of the heavy entrainer type (Quickfit) as the receiver. This enabled the aqueous portion of the distillate to be automatically separated from the oil and returned to the distilling flask. Distillation was carried out until there was no further increase in the volume of volatile oil. The time of distillation was four hours.

In replicate distillations, the volume of volatile oil distilled was found to be 0.05ml., therefore the percentage of oil in the sample was 0.05% $^{v}/_{w}$.

4. EXTRACTION OF THE VOLATILE OIL

The methods available for the commercial extraction of volatile oils are listed by Guenther (94) as follows:

(a) Distillation of volatile oil using:

i. Water distillation, in this method the plant material is in direct contact with boiling water in the still.

ii. Water and steam distillation, where the plant material is raised above the level of the water and wet steam of low pressure is passed through the material.

iii. Steam distillation, where live steam at high pressure is passed through the plant material.

(b) Extraction:

i. Extraction with cold fat (enfleurage).

ii. Extraction with hot fat (maceration).

iii. Extraction with volatile solvents.

The use of enfleurage and maceration is limited to flower oils with delicate perfumes, whilst solvent extraction, using petroleum ether as the solvent, has been used for other plant parts. Studies by Sticher and Flück (95) on the isolation of the volatile oil from *Mentha piperita* L.,

Labiatae, suggested that there was no qualitative difference between samples obtained either by the direct isolation from the oil glands with glass capillaries, distillation or solvent extraction.

Although solvent extraction reduces the risk of artifact formation which is often encountered when high temperatures are used in steam distillation (35), if used with Horehound herb the hydrocarbons from the waxy cuticle (67) would also be removed. Therefore, the volatile oil would still have to be separated from the waxy product if solvent extraction was used. As the direct isolation of the volatile oil from the oil glands was not a practical method for obtaining large quantities of oil, one of the distillation methods had to be chosen.

On a small scale, water distillation or water and steam distillation offer the advantage of simplicity. Diffusion conditions are good with water distillation, but in some cases a relatively low yield of oil is obtained due to the hydrolysis of esters and the retention of water soluble and high boiling point components by the residual water in the still.

To investigate the effect of these distillation methods on the composition of Horehound oil, samples of the oil were obtained using water distillation and water and steam distillation. Water distillation was carried out as follows: One kilo of the herb was placed in a Quickfit spherical flanged flask of ten litres capacity. This had a wide neck which eased the loading and unloading of the plant material. Eight litres of distilled water were placed in the flask which was supported by a dexion stand. An electrothermal heating mantle (size ten litres) was used as the heat source. The distilling vapours were condensed using a double surface condenser, the distillate being collected in a water estimator of the heavy entrainer type. After four hours, there was no further increase in the volume of volatile oil, therefore distillation was discontinued. The oil was removed from the receiver by means of a Pasteur pipette and placed

in a small specimen tube which contained anhydrous sodium sulphate. After one hour, this tube was centrifuged in order to obtain a clear sample of the oil.

For water and steam distillation, the apparatus was modified by placing a perforated metal tray within the flask, thus raising the herb well above the surface of the water. No other changes were made to the method.

Samples of 0.4 µl of the volatile oils obtained were subjected to examination by gas liquid chromatography (g.l.c.). Figures 9 and 10 proved that apart from a slight quantitative difference in the low boiling point hydrocarbon fraction, there was no difference in the qualitative composition of the oils. The method of water distillation was chosen for use in this investigation as it was easier to perform.

Since on distillation the yield of oil was very small, ether was added to the oil in the receiver to aid its collection. The ethereal solution was removed from the surface of the water by means of a Pasteur pipette and was dried for 18 hours over anhydrous sodium sulphate before removing the ether under reduced pressure at room temperature. The oil obtained was stored in the dark at a temperature of $0^{o}C$ until required for analysis.

To determine whether the composition of the oil was affected by extraction with ether, a 0.4 µl. sample of the oil collected in this way was subjected to g.l.c. analysis (Figure 11). Comparing Figures 9 and 11, apart from a trace of ether in the latter, no qualitative differences were found. Extraction of the oil with ether improved the yield of some components, since the peak areas of the components detected up to a retention time of 12 minutes were larger in this sample of oil.

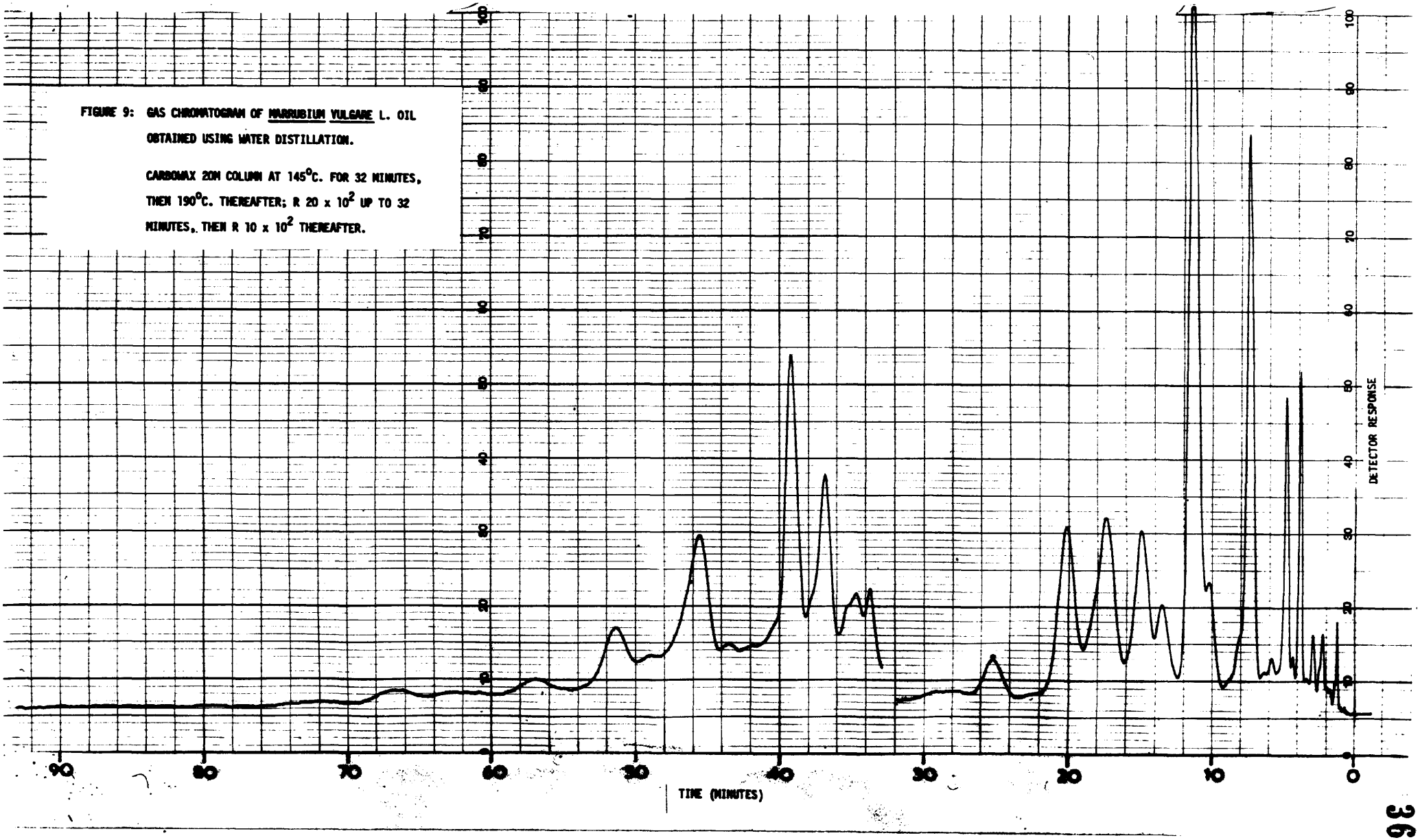


FIGURE 9: GAS CHROMATOGRAM OF MARRUBIUM VULGARE L. OIL OBTAINED USING WATER DISTILLATION.

CARBOWAX 20M COLUMN AT 145°C. FOR 32 MINUTES, THEN 190°C. THEREAFTER; R 20 x 10^2 UP TO 32 MINUTES, THEN R 10 x 10^2 THEREAFTER.

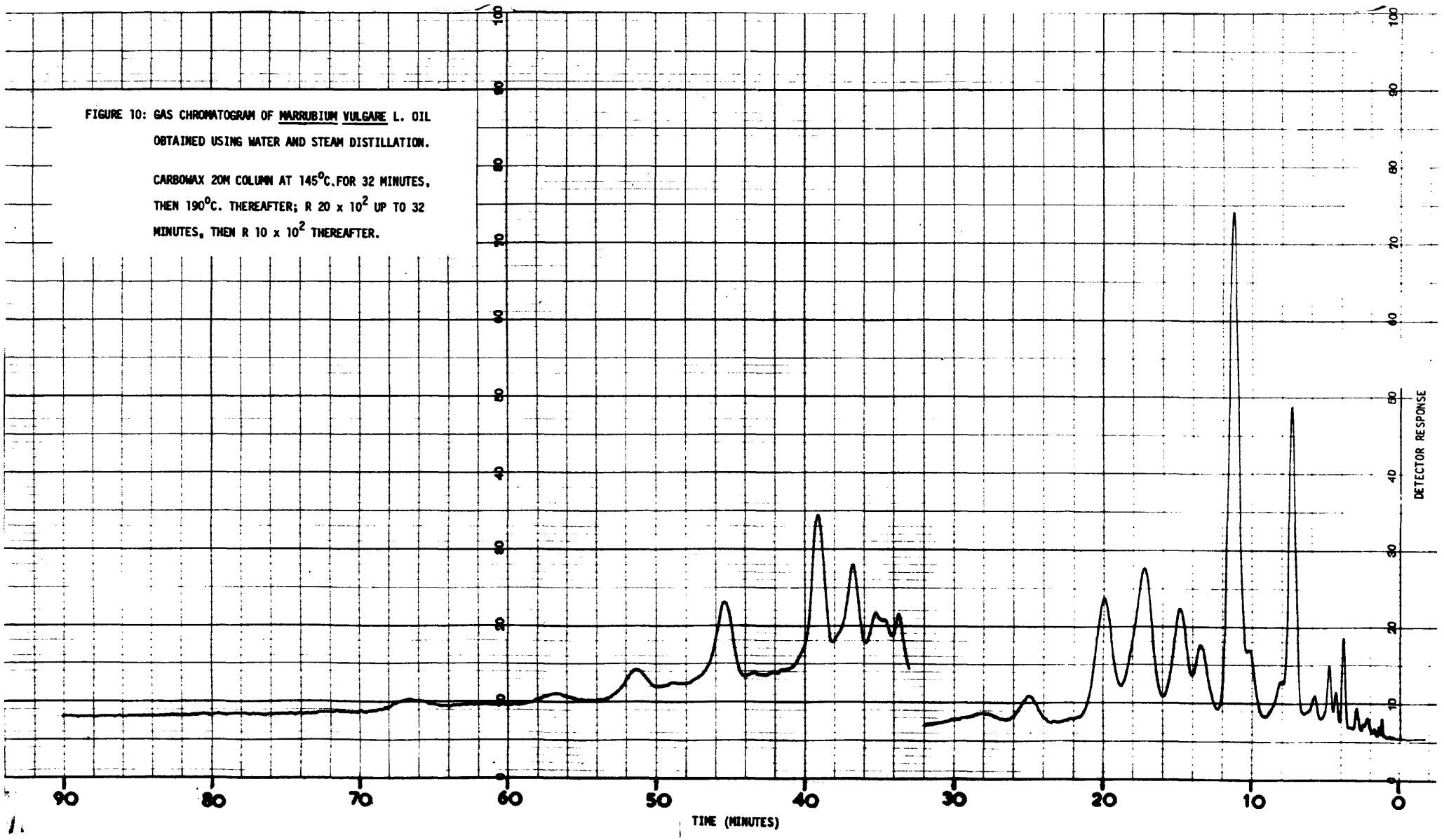


FIGURE 10: GAS CHROMATOGRAM OF MARRUBIUM VULGARE L. OIL OBTAINED USING WATER AND STEAM DISTILLATION.

CARBOWAX 20M COLUMN AT 145°C.FOR 32 MINUTES, THEN 190°C. THEREAFTER; R 20 x 10^2 UP TO 32 MINUTES, THEN R 10 x 10^2 THEREAFTER.

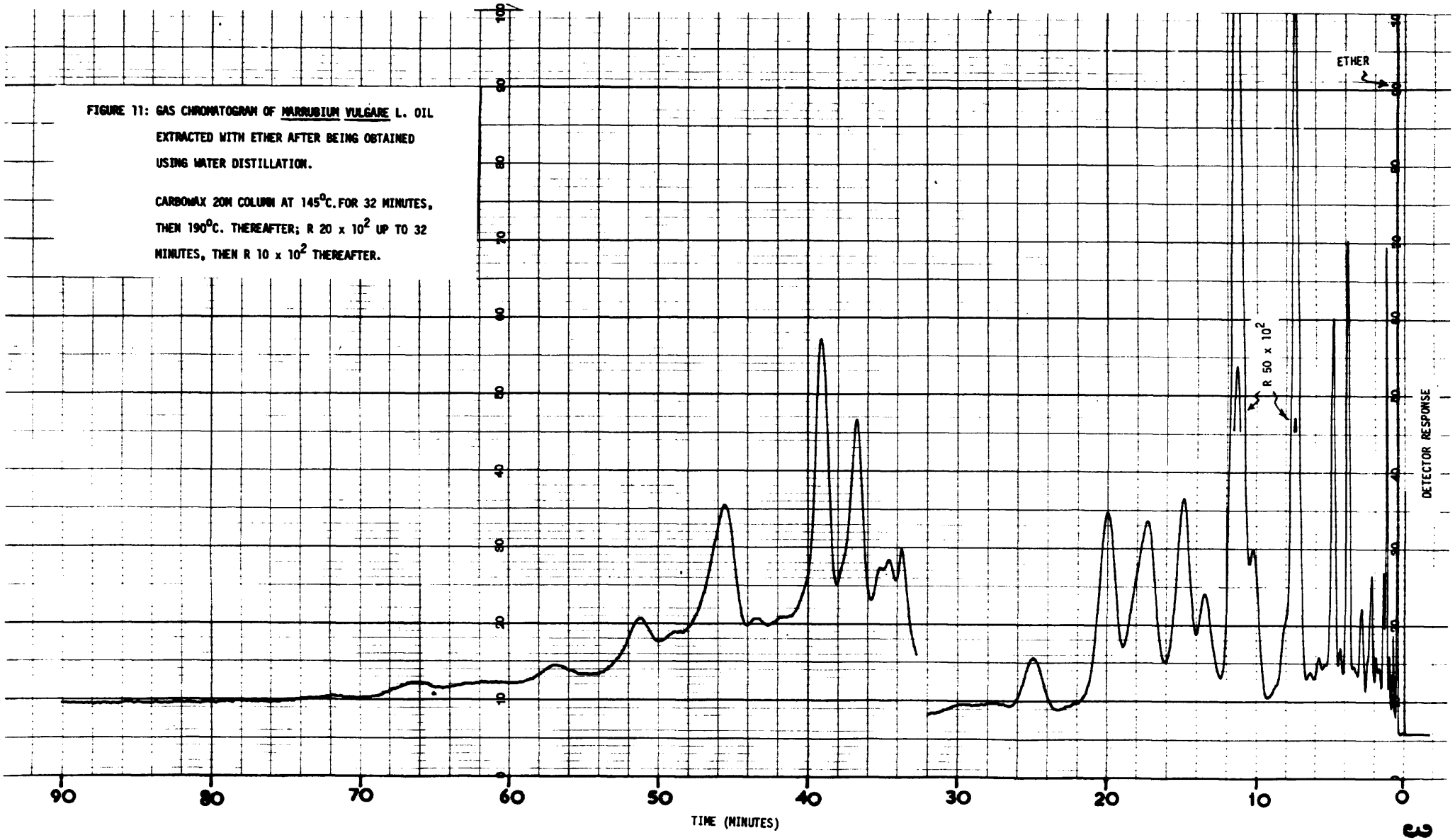


FIGURE 11: GAS CHROMATOGRAM OF MARRUBIUM VULGARE L. OIL EXTRACTED WITH ETHER AFTER BEING OBTAINED USING WATER DISTILLATION.

CARBOWAX 20M COLUMN AT 145°C. FOR 32 MINUTES, THEN 190°C. THEREAFTER; R 20 x 10^2 UP TO 32 MINUTES, THEN R 10 x 10^2 THEREAFTER.

5. PHYSICAL PROPERTIES OF THE VOLATILE OIL

The volatile oil is a yellow or somewhat greenish yellow mobile liquid, having an aromatic musky smell. The refractive index was measured using an Abbe 60 Refractometer, Degree Scale model (Bellingham and Stanley, Ltd., England); $n_D^{20^o}$ 1.3129. There was insufficient oil available to determine accurately the specific rotation and the density which is less than one.

6. THIN LAYER CHROMATOGRAPHY OF THE VOLATILE OIL

The first accounts of the use of t.l.c. techniques were published in 1938 (96). Stahl (97) reported that in the period from 1938 to 1962 there were sixty references dealing with the separation of volatile oils and terpene derivatives using t.l.c. In a later survey (98) he found that the number of these publications had reached three hundred and thirty-four. For the analysis of volatile oils, t.l.c. can be used with advantage in combination with g.l.c., and it is even possible to use t.l.c. as the only separation technique. The following are examples of its use in the family Labiatae; *Lavandula angustifolia* (99), *Marjorana hortensis* (100), *Melissa officinalis* (101), *Mentha* spp. (95,102), and *Thymus* spp. (103). Thin layer chromatography has therefore been used as one of the techniques of analysis in the current investigation.

Silica gel is the most widely used absorbent for the separation of volatile oils (35). Plates were prepared using Shandon t.l.c. equipment (104). Silica gel was mixed with distilled water (25g. to 50ml.) and spread as layers of 250 μ or 500 μ thickness according to whether they were to be used qualitatively or preparatively. The plates were activated by heating at 110°C for 30 minutes, and then stored in a desiccator cabinet

until required. Materials to be investigated were applied as solutions in ethanol using a micropipette. Chromatographic tanks were lined with filter paper to ensure that the atmosphere was fully saturated with solvent vapours. The solvent front was allowed to travel 15 cm. before the plates were removed from the tanks.

Solvent systems which have been used previously for the separation of terpenoid compounds include: benzene: acetone, 95:5 (105), benzene:ethyl acetate, 95:5 (95), benzene:chloroform, 1:1 (106), benzene (107), benzene: hexane, 1:1 (108), hexane:ethyl acetate, 85:15 (109) and hexane (110). The suitability of each of these systems for the separation of Horehound oil was examined. It was found in general that the more polar the solvent system, the better the separation obtained. However, no one solvent system gave complete separation of all components of the oil. Since of the solvent systems examined, benzene:ethyl acetate, 95:5 gave the best separation, it was used for the greater part of the following investigations, although in certain cases, hexane has also been used. Figure 12 shows the separation of the oil using both the solvent systems used in this investigation.

The suitability of the following reagents was tested for the general detection of separated components:

A. Vanillin - sulphuric acid: 3g. vanillin was dissolved in 100ml. absolute ethanol and 0.5ml. sulphuric acid was then added to the solution. After spraying, plates were heated at 110°C for 5 minutes (97).

B. A 1% solution of vanillin in concentrated sulphuric acid. After spraying, the plates were heated at 110°C for 5 minutes (106).

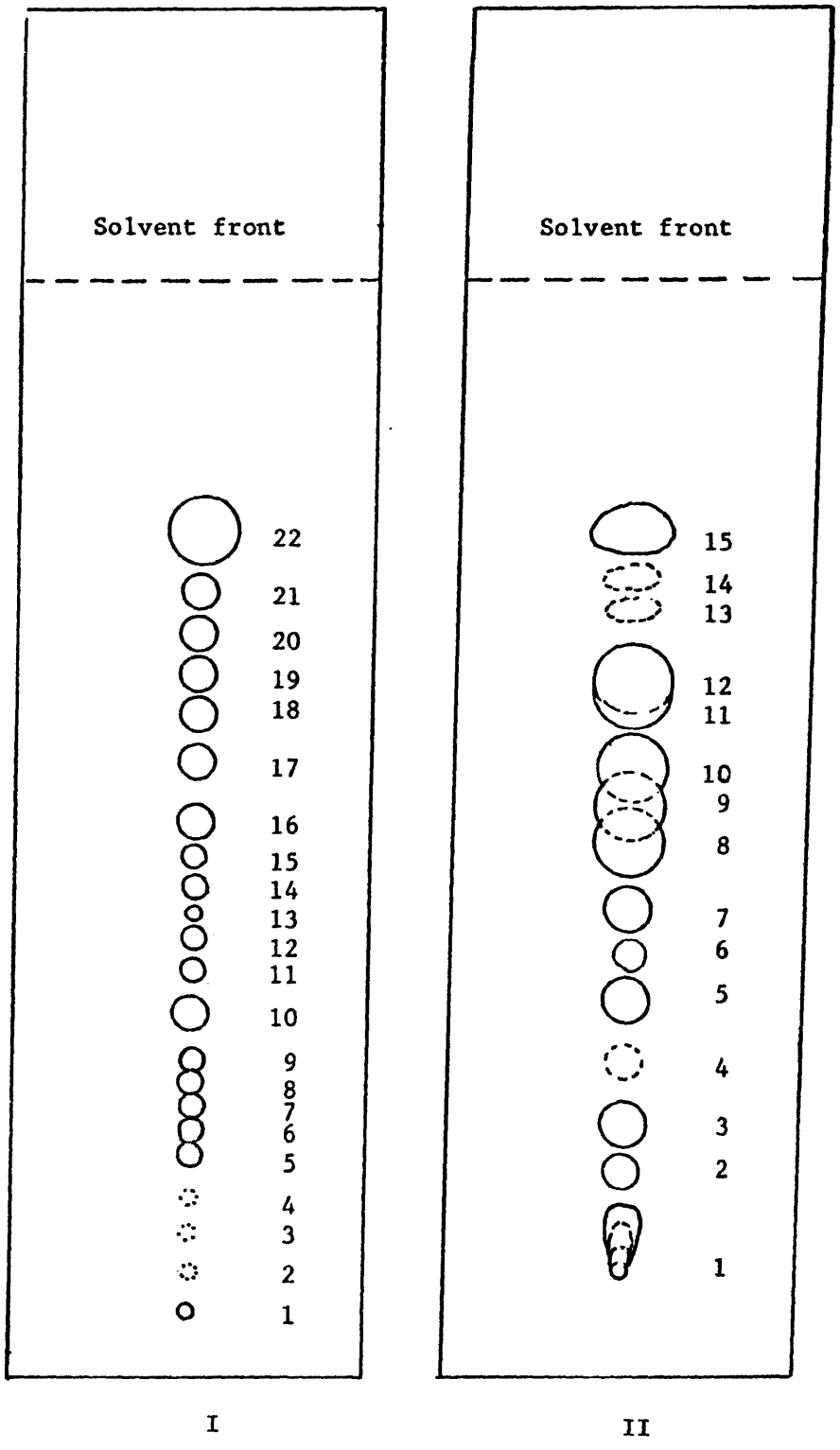


FIGURE 12: Chromatoplates of *Marrubium vulgare* L. oil using solvent systems, I. Benzene:ethyl acetate, 95:5 and II. Hexane.

C. Anisaldehyde - sulphuric acid: 5ml. anisaldehyde in 50ml. glacial acetic acid with addition of 1ml. of sulphuric acid. After spraying, the plates were heated at 110°C for 5 minutes (97).

In use B caused general discolouration to the adsorbent layer, whilst C gave faint responses. Spray reagent A was the most satisfactory of the three, therefore it was used to detect the separated components of Horehound oil.

The R_f values of the separated components of the oil using benzene: ethyl acetate, 95:5 and hexane were determined and are given, expressed as the mean of twelve runs, in Table 1. The pattern of t.l.c. separation indicates that the composition of the oil is complex.

The technique used by Betts (106) in the identification of the components found in oils extracted from the umbelliferous fruits was also tried in an attempt to obtain more information about the components of Horehound oil. The results of this investigation are summarized in Table 2.

In an attempt to improve the separation of the components of the oil the use of two-dimensional t.l.c. was examined. Tyihák and Held (111) reported that this technique has been neglected in the analysis of volatile oils, only four investigations having been reported in which this method was used. After chromatography of the oil with benzene:ethyl acetate in one direction, a second run was carried out in hexane after rotating the plates through 90°. Figure 13 indicates that this method improved the separation. The large spot (number 22) produced in the benzene:ethyl acetate run was separated into a further seven spots in the second run with hexane. None of the other spots were separated further by hexane.

TABLE 1

R_f values of the components of *Marrubium vulgare* L. oil separated by, I. benzene:ethyl acetate, 95:5 and II. hexane.

Benzene:ethyl acetate, 95:5		Hexane	
Spot number	R_f value	Spot number	R_f value
1	0.00	1	0.04
2	0.04	2	0.10
3	0.08	3	0.15
4	0.12	4	0.21
5	0.17	5	0.27
6	0.20	6	0.31
7	0.23	7	0.36
8	0.25	8	0.42
9	0.27	9	0.47
10	0.32	10	0.50
11	0.40	11	0.57
12	0.42	12	0.59
13	0.43	13	0.65
14	0.44	14	0.68
15	0.46	15	0.73
16	0.47		
17	0.52		
18	0.57		
19	0.62		
20	0.67		
21	0.71		
22	0.79		

TABLE 2

Different characters of the separated spots of *Marrubium vulgare* L. oil using solvent system of benzene:ethyl acetate, 95:5 and the visualization technique suggested by Betts (106).

Spot number	After running		After bromination		2,4-Dintro-phenylhyd-razine	Vanillin/ H_2SO_4
	Daylight	Ultra violet	Daylight	Ultra violet		
1	Pink	Purple	Yellow	Yellow		Faint purple
2	Yellow	Yellow	"	"		Faint purple
3			"	"		Faint purple
4			"	"		Turquoise
5			"	"		Turquoise
6			"	Purple		Grey
7		Purple	"	"		Purple
8		"	"	"	Orange	Blue
9			"	Yellow		Turquoise
10			"	Purple	"	Black
11		"	"	"	"	Green yellow
12		"				Purple
13					"	Purple
14	Pink	"	"	"	"	Green yellow
15						Pink
16		"				Blue green
17						Faint purple
18					"	Turquoise
19					"	Blue
20						Grey
21	Pink	"	"	"		Pink
22	"	"	"	"	"	Purple violet

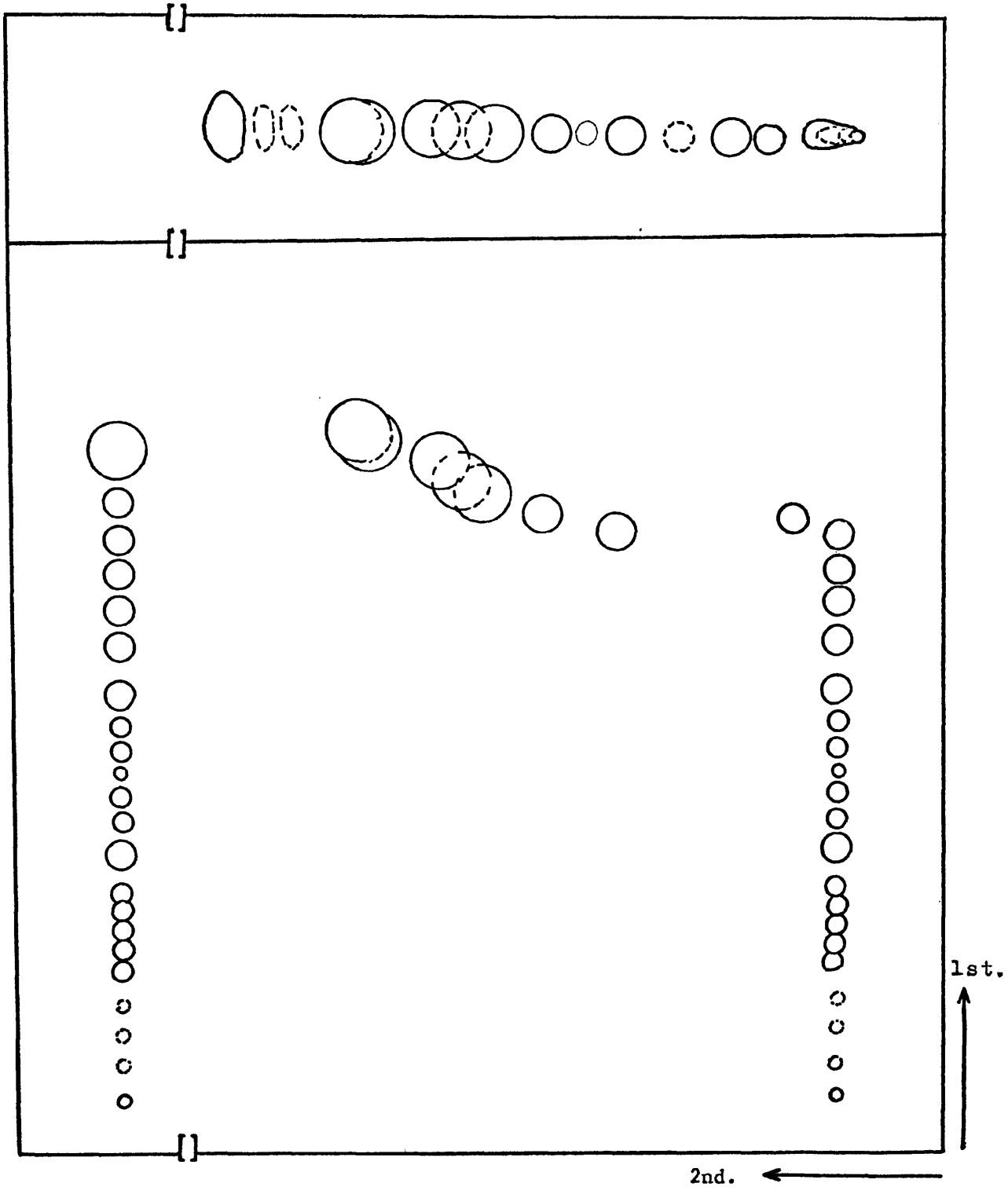


FIGURE 13: One- and two- dimensional chromatoplate of *Marrubium vulgare* L. oil.
Solvents: benzene:ethyl acetate, 95:5 in the first run and hexane in the second run.

Although two-dimensional t.l.c. was useful in improving the separation of the oil components, it was not a practical technique for preparative separations, where band streaking of the oil on a base line was required to obtain a sufficient loading of material.

Another technique which might have been useful to increase the degree of separation was continuous elution, especially for spots 5 to 9 which had very similar R_f values. A Shandon chromatank for continuous elution chromatography was used, with benzene:ethyl acetate, 95:5 as the solvent system. Running times of 1.5, 2.0 and 2.5 hours were tried, Figure 14 showing the separation achieved in a continuous run of 2 hours. Although the separation of spots 5 to 9 was improved, the improvement was not sufficient for this technique to be used preparatively to obtain pure compounds for further analysis.

Another modification, to separate terpenes according to the number of double bonds, involved t.l.c. on silica gel plates spread as a slurry with 2.5% aqueous silver nitrate instead of with water (35). The oil was subjected to this technique, but no improvement in separation was observed.

Vágujfalvi and Tyihak (107) screened fifty species of forty-five genera in twenty-seven plant families for volatile oil components. From their experiments they gave a suggested pattern of behaviour for certain chemical groups. Figure 15 shows the application of these results to Horehound oil, but in the absence of R_f values it is difficult to place too much reliance on any resulting suggestions, although they do perhaps give a rough guide to the possible chemical groups.

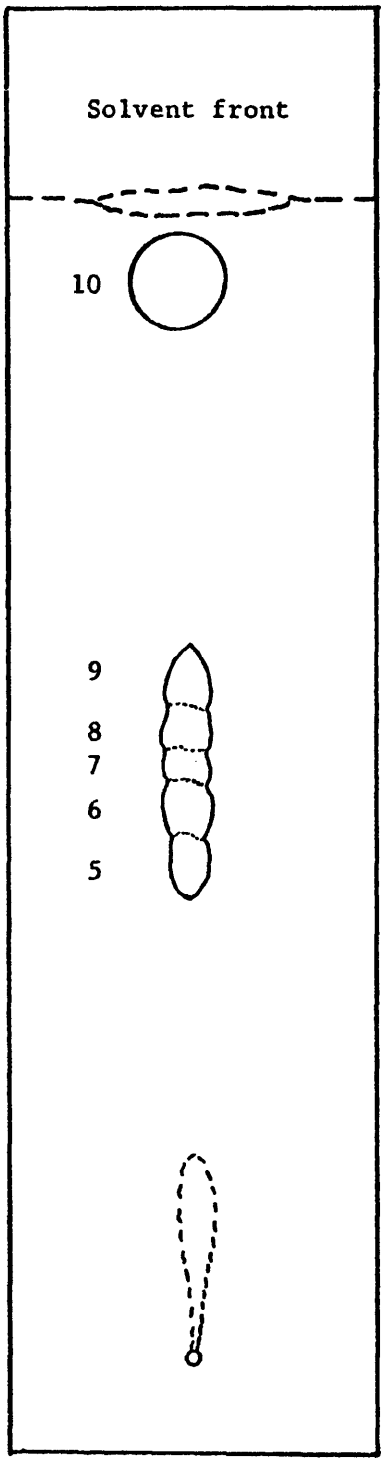


FIGURE 14: Chromatogram of *Marrubium vulgare* L. oil using a continuous elution technique for a period of two hours. Solvent: benzene:ethyl acetate, 95:5.

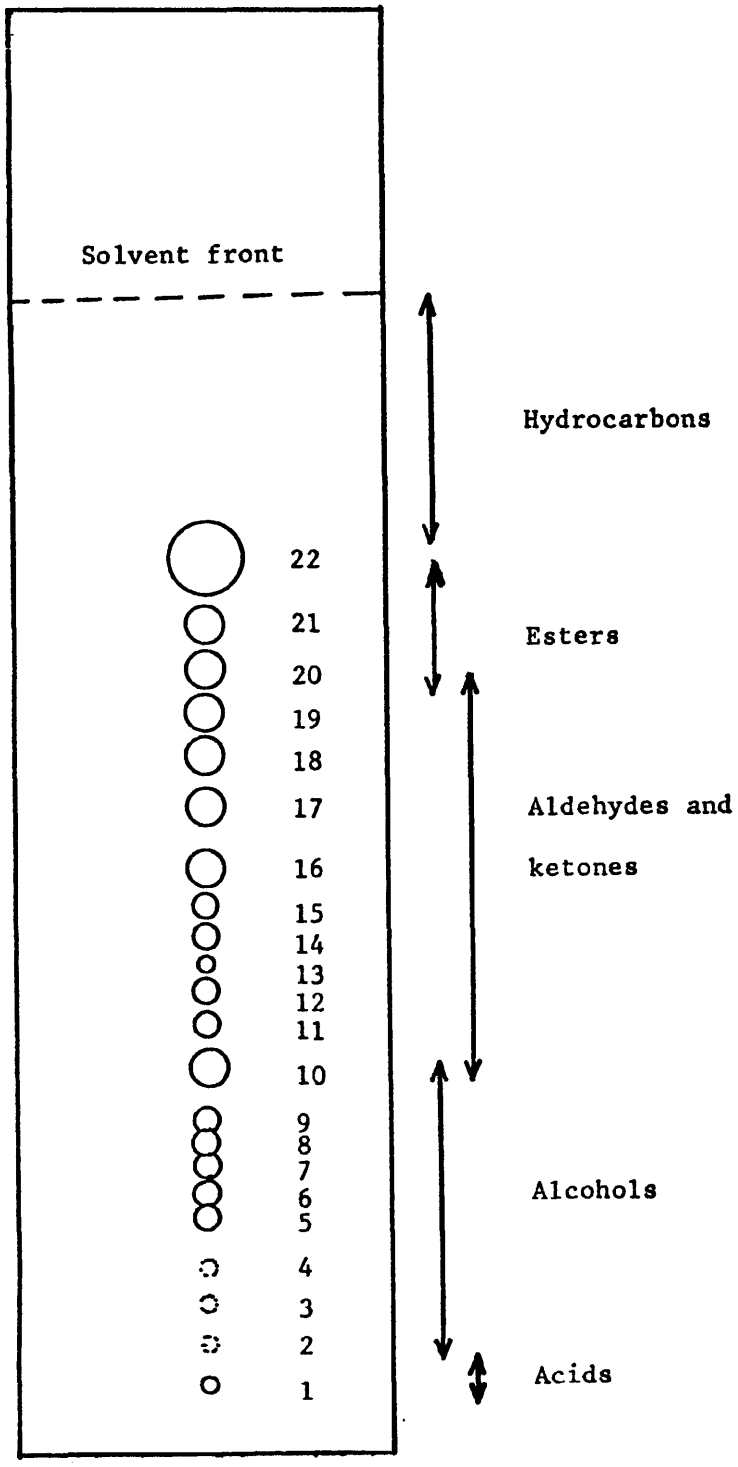


FIGURE 15: The t.l.c. separation patterns of different chemical groups, as suggested by Vágujfalvi and Tyihak (107), applied to a typical t.l.c. separation of the volatile oil of *Marrubium vulgare* L.

The different t.l.c. analyses of Horehound oil proved that it had a complex composition and any separated spot might be composed of more than one component. As a technique for the complete separation of the oil components, t.l.c. could not be used alone, but it was useful as a complimentary method of analysis.

7. GAS LIQUID CHROMATOGRAPHY OF THE VOLATILE OIL

Gas chromatography is an excellent technique for the separation, characterization and quantitative analysis of volatile oils. The volatile oils of the following plants of the Labiatae are some of the tremendous number of materials which have been studied using this technique: *Salvia lavandulaefolia* Vahl. (112), *Thymus* spp. (113), *Mentha* spp. (114,115, 116) and *Lavandula* spp. (117).

The choice of columns (solid support and liquid phase) and optimum operating conditions for the g.l.c. of a given material are still more or less subject to trial and error. The solid support Chromosorb P is mechanically strong, with little dust formation and may be coated with 30-40% of a liquid stationary phase. Because of its surface activity, the separation of polar compounds involves considerable tailing and if used with this type of compound, a method should be applied to eliminate the adsorptive effect of the support (118). Chromosorb P (Johns-Manville) was chosen as solid support in this investigation because of its mechanical strength. About two hundred liquids applicable as stationary phases are commercially available, a wide variety of them having been employed for the separation of volatile oils. Breckler and Betts (119,120) observed the general patterns of the relative retention times (r.r.t.) of known

volatile oil components to linalol, related to changes in column temperature and stationary phase. They used stationary phases of non-polar dimethyl polysiloxane (SE30), the increasingly polar diethylene glycol succinate (DEGS) and the strongly polar Carbowax 20M at column temperatures of 160,175,190,205 and 220°C. From their experiments, they suggested that if the behaviour patterns of an unknown volatile oil component on these three columns at different temperatures were studied, it would be possible to predict to which of the following five chemical groups it might belong: terpenoid ester, terpenoid hydrocarbon, terpenoid alcohol, substituted aromatic and terpenoid carbonyl. As it was intended to use this technique in the current investigation, the same stationary phases were chosen; Carbowax 20M (Wilkens), DEGS and SE30 (Phase Sep). Three columns, each having a stationary phase concentration of 15%, were prepared as follows:

For each column, 10g. Chromosorb P (non-acid washed) were weighed and placed in a flat-bottomed glass dish before being dried in a hot air oven at 200°C for a period of four hours. The support was then allowed to cool in a desiccator. A 0.2% $^{w}/_{v}$ solution of polyvinyl pyrrolidone P.V.P. (BDH) in dry methanol (25ml.) was added to the dried support with gentle stirring, the methanol being removed by placing the support in warm oven at 60°C for four hours. P.V.P. was added to the Chromosorb P to deactivate its surface activity. A solution of the liquid phase, prepared with the aid of gentle heat, was added to the solid support and stirred gently. (Carbowax 20M was dissolved in methanol, DEGS in acetone and SE30 in toluene). Solvents were removed by heating in an oven at 60°C for six hours. The support, impregnated with liquid phase, was sieved through a British Standard 60 mesh sieve, the material retained by the sieve being discarded. Before packing, the internal surface of a

six feet long, 1/8th inch outer diameter stainless steel column was cleaned using acetone. After drying at 150°C for several hours, the internal surface was then coated with epikote resin 1004 (Shell) to prevent any possible interaction between the column and the separated components of the oil. The inlet end of the column was plugged with glass wool and the appropriate packing material was fed in at the opposite end of the column using a vacuum. The coated support was added a little at a time, the column being gently vibrated until no further material could be added. It had been calculated that 4.0 to 4.5g. of packing material would be required to fill the columns to the right packing density. The amount of packing material used was in agreement with this calculated value. All three columns were conditioned at a temperature of 200°C before use.

A Perkin-Elmer F11 gas chromatograph with a flame ionization detector was used for all qualitative analyses. Since a temperature programming unit was not available, the following time/temperature conditions were used in routine work.

Column	Conditions
Carbowax 20M	145°C for 32 minutes, then 190°C for 58 minutes
DEGS	130°C for 20 minutes, then 190°C for 70 minutes
SE30	160°C for 45 minutes, then 190°C for 45 minutes

The other conditions were as follows:

Carrier gas	Nitrogen, flow rate 30ml/minute
Injector temperature	200°C
Detector temperature	240°C

Typical chromatograms of the oil using these columns are shown in Figures 11, 16 and 17. The significance of the numbers used in Figures 16 and 17 is explained later in Tables 6 and 7.

For the preparative work, an Aerograph Model 705 gas chromatograph was used in conjunction with a ten feet, $^{3}/_{8}$th inch outer diameter stainless steel column which was packed with Chromosorb P as support and 20% Carbowax 20M as liquid phase. The column was prepared using the method described for the qualitative columns. It had been calculated that 63-73g.of packing material would be required to fill the preparative column to the right packing density; the actual amount of support used was 70g. Nitrogen was used as carrier gas. At first the recommended flow rate of 26ml./minute was maintained at the detector,(121) but as the split ratio was 1:27, this gave a flow rate of 700ml/minute at the collector. In practice this was found to be far too high to enable efficient condensation and recovery of the separated compounds. Therefore,the carrier gas flow rate was reduced to 5ml./minute at the detector and 100ml./minute at the collector, which still gave a satisfactory split ratio of 1:20. This modification improved the yields of the separated components. Temperature conditions were as follows: column : 145°C for 100 minutes, then 190°C for 120 minutes, injector : 200°C, collector : 200°C and detector : 240°C.

Fractions of oil which had been collected after a preparative g.l.c. separation of a sample of the oil, were checked for purity by means of t.l.c. Even those fractions which were expected to consist of only one component were found to contain at least two or three. Therefore, complete separation and collection of pure components could not be achieved by preparative g.l.c. alone.

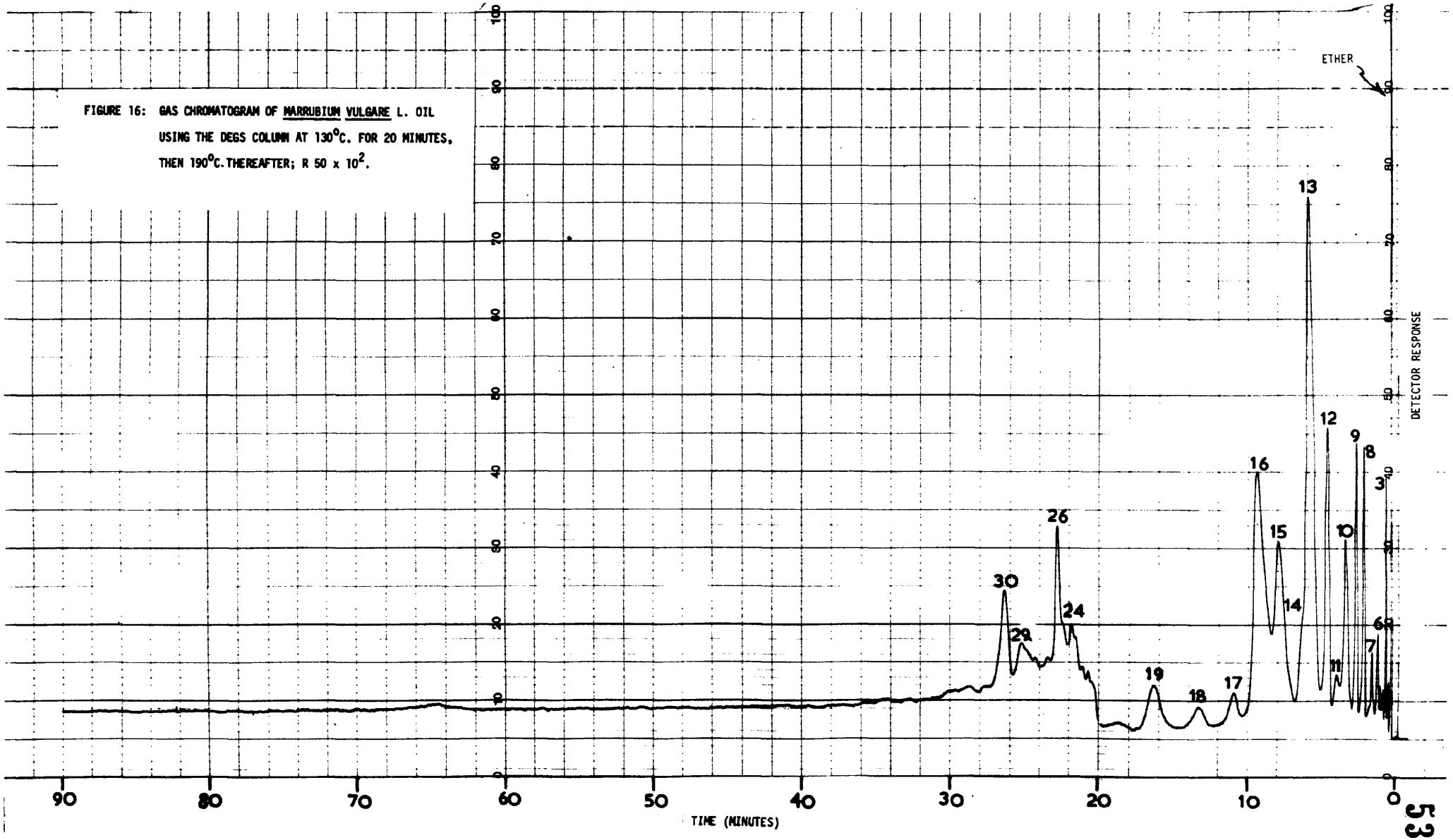


FIGURE 16: GAS CHROMATOGRAM OF MARRUBIUM VULGARE L. OIL USING THE DEGS COLUMN AT 130°C. FOR 20 MINUTES, THEN 190°C. THEREAFTER; R 50 x 10^2.

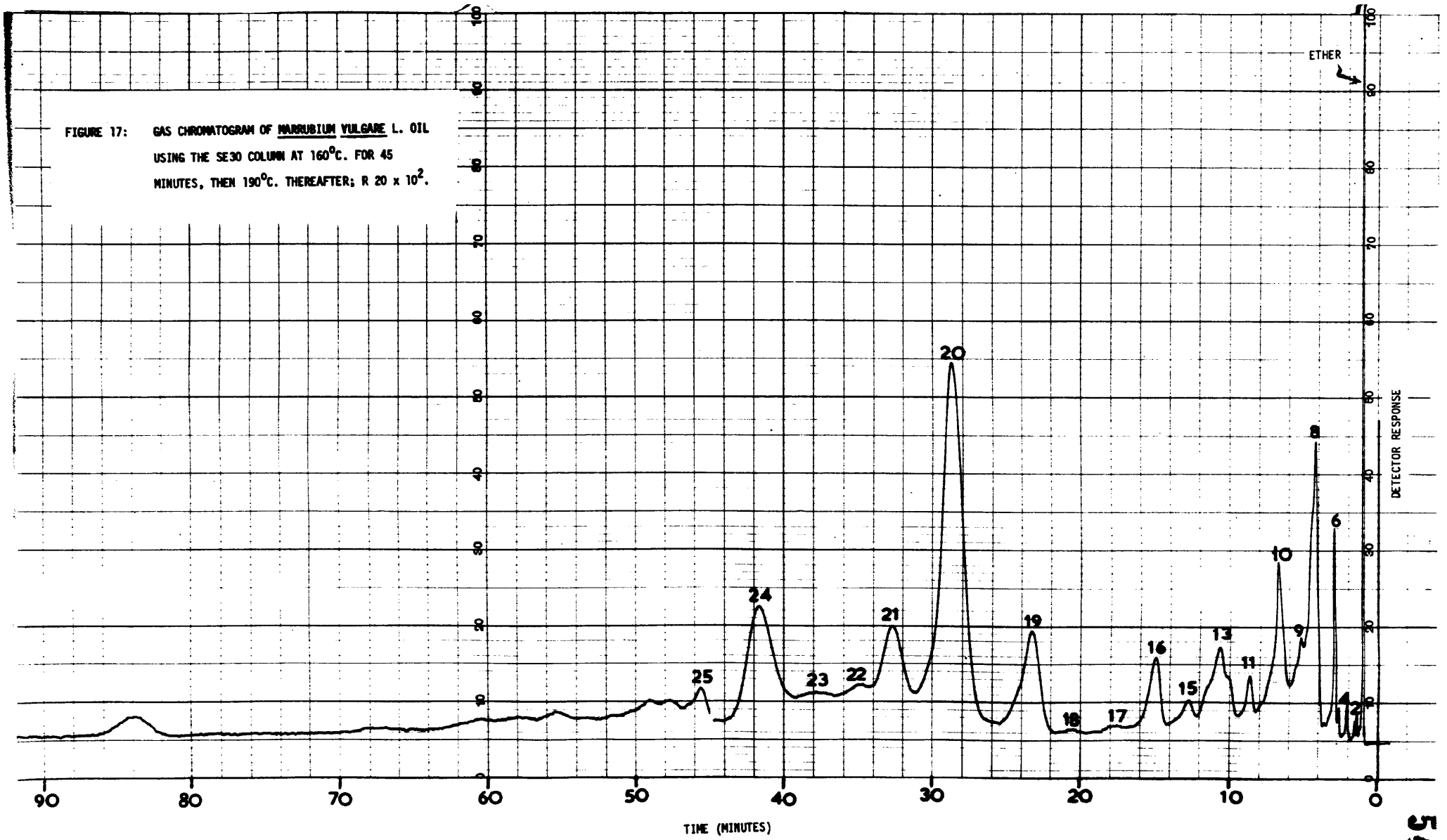


FIGURE 17: GAS CHROMATOGRAM OF MARRUBIUM VULGARE L. OIL USING THE SE30 COLUMN AT 160°C. FOR 45 MINUTES, THEN 190°C. THEREAFTER; R 20 x 10^2.

Other workers (114,116,122) had found it advantageous to fractionate volatile oils using column chromatography prior to preparative g.l.c. separation. Using this technique, oils were separated readily into a hydrocarbon fraction and oxygenated terpene fraction by elution with solvents of different polarity. Each fraction could be then further separated, thus facilitating the isolation of pure components. Prior to further preparative g.l.c. samples of oil were prefractionated as follows:

Silica gel (180g.) for chromatography, 100-200 mesh (Fisons), was mixed with n-hexane (Analar) and packed into a 40cm. long glass column. A three gramme sample of Horehound oil was dissolved in n-hexane (Analar) and carefully applied to the column before being eluted with two litres of n-hexane (Analar). The n-hexane fraction, which contained the hydrocarbon compounds, was removed prior to further elution of the column with 600ml. of dry methanol. The methanol fraction contained the oxygenated components of the oil. Solvents were removed under vacuum at a temperature of 40°C using a Büchi Rotavapor 'R'. The weight of the fraction of the oil eluted by the n-hexane was 0.94g., while that eluted by methanol was 2.1g. A sample of the fraction eluted with n-hexane was examined using i.r. spectroscopy which confirmed the absence of any carbonyl ($1700-1750cm^{-1}$), hydroxyl ($3300-3600cm^{-1}$) or ring oxygen ($1160-1250cm^{-1}$) containing compounds. Both fractions were subjected to g.l.c. examination. Figures 18 and 19 show a typical chromatogram of each fraction using the Carbowax 20M column.

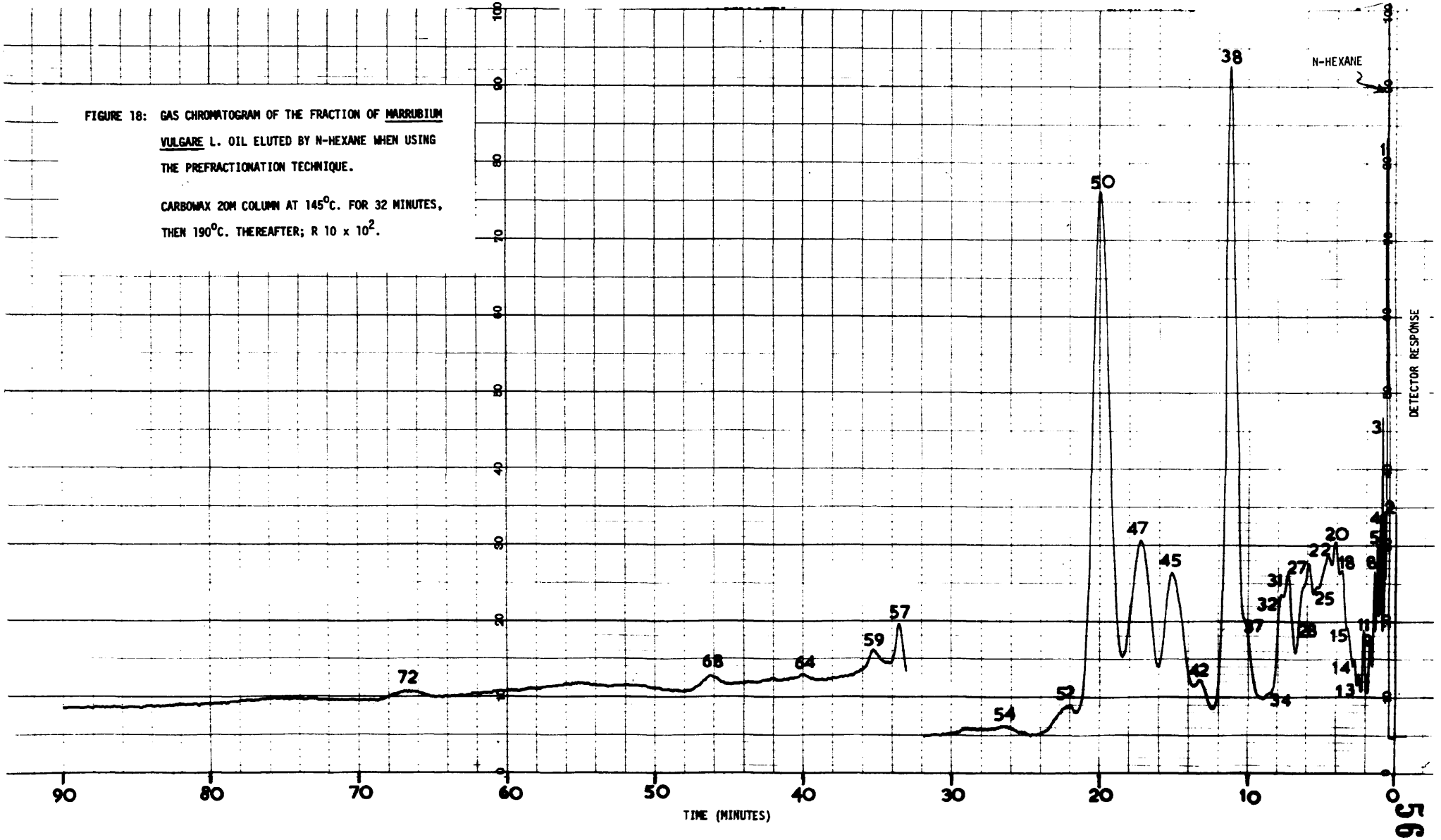


FIGURE 18: GAS CHROMATOGRAM OF THE FRACTION OF MARRUBIUM VULGARE L. OIL ELUTED BY N-HEXANE WHEN USING THE PREFRACTIONATION TECHNIQUE.

CARBOWAX 20M COLUMN AT 145°C. FOR 32 MINUTES, THEN 190°C. THEREAFTER; R 10 x 10^2.

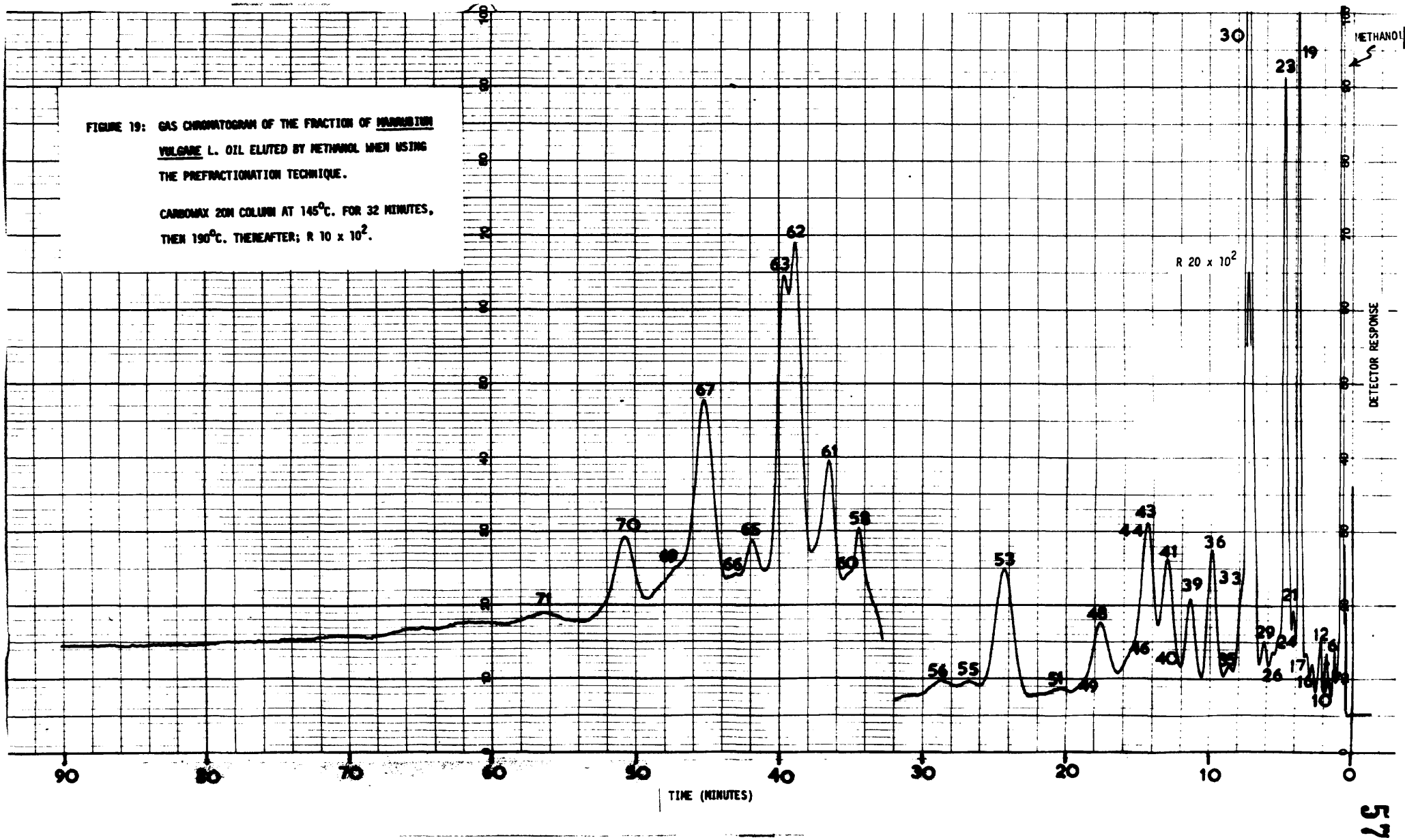


FIGURE 19: GAS CHROMATOGRAM OF THE FRACTION OF MARRUBIUM VULGARE L. OIL ELUTED BY METHANOL WHEN USING THE PREFRACTIONATION TECHNIQUE.

CARBOWAX 20M COLUMN AT 145°C. FOR 32 MINUTES, THEN 190°C. THEREAFTER; R 10 x 10^2.

Each fraction was then separated as follows. Aliquots (200 µl) were repeatedly injected using a Hamilton syringe of 500 µl. capacity. Each major peak and group of trace components were collected separately in small U-shaped tubes which were cooled in a large thermal flask containing solid carbon dioxide and acetone. The purity of each compound collected was tested by means of qualitative t.l.c. and g.l.c. Components which proved to be pure were then subjected to further analytical techniques.

Most of the major components were collected in a pure condition, but some samples on purity testing were still found to contain two components. Before such components could be subjected to analysis, further separation had to be carried out. This was achieved by using preparative t.l.c. on silica gel G layers of 500 µ thickness and a solvent system of benzene:ethyl acetate, 95:5. After development, a narrow side portion of the plates was sprayed with vanillin-sulphuric acid reagent and heated at 110°C for five minutes. The required areas of adsorbent layer were then scraped off the surface of the plate, the components being removed from the adsorbent by elution with ether. After removing the ether, the components were subjected to further analysis.

As a result of g.l.c. analyses on different columns it was found that Horehound oil was composed of seventy-two components. For ease of reference, the components were designated 1-72 according to their retention times when using the Carbowax 20M column (Figures 18 and 19). These retention times and the retention times of the components relative to peak 30 (later found to be linalol) are given in Table 3.

TABLE 3

Retention times and relative retention times of the components of *Marrubium vulgare* L. oil to linalol using the Carbowax 20M column

Component	r.t. (min.)	r.r.t./ linalol	Component	r.t. (min.)	r.r.t./ linalol	Component	r.t. (min.)	r.r.t./ linalol
1	0.60	0.08	25	5.40	0.74	49	18.10	2.48
2	0.70	0.10	26	5.80	0.79	50	20.10	2.76
3	0.90	0.12	27	6.00	0.82	51	21.40	2.93
4	1.10	0.15	28	6.40	0.88	52	22.40	3.07
5	1.25	0.17	29	6.50	0.89	53	25.40	3.48
6	1.30	0.18	30	7.30	1.00	54	26.80	3.67
7	1.40	0.19	31	7.30	1.00	55	27.30	3.74
8	1.55	0.21	32	7.90	1.08	56	30.40	4.17
9	1.75	0.24	33	8.00	1.10	57	33.90	4.64
10	1.90	0.26	34	8.80	1.21	58	34.80	4.77
11	2.10	0.29	35	8.90	1.22	59	35.70	4.89
12	2.35	0.32	36	10.00	1.37	60	35.70	4.89
13	2.40	0.33	37	10.30	1.41	61	36.60	5.01
14	2.80	0.38	38	11.30	1.55	62	39.00	5.34
15	2.90	0.40	39	11.50	1.57	63	39.70	5.44
16	3.00	0.41	40	12.80	1.75	64	40.00	5.48
17	3.40	0.47	41	13.10	1.79	65	42.00	5.75
18	3.70	0.51	42	13.40	1.84	66	43.20	5.92
19	3.80	0.52	43	14.30	1.96	67	45.30	6.21
20	4.10	0.56	44	14.75	2.02	68	46.40	6.36
21	4.20	0.58	45	15.30	2.10	69	47.50	6.51
22	4.70	0.64	46	15.40	2.11	70	51.00	6.99
23	4.80	0.66	47	17.20	2.35	71	56.60	7.75
24	5.30	0.73	48	17.80	2.43	72	71.20	9.75

8. RELATIVE RETENTION TIMES OF THE VOLATILE OIL COMPONENTS TO LINALOL ON COLUMNS OF DIFFERING POLARITY OVER A TEMPERATURE RANGE

Breckler and Betts (119,120) suggested that it was possible to predict the chemical groups to which unknown volatile oil components belonged from their relative retention times to linalol on columns of differing polarity over a temperature range. As the higher temperature used in this range, 220°C, was above the optimum operating temperature for the Carbowax 20M and DEGS liquid phases, it was decided to use the lower range, 130-190°C, for the routine work in this investigation. To ensure that this change in temperature range did not alter the trends observed previously, as many of the reference standards as possible were re-examined. The general patterns of the following five chemical groups, A. terpenoid hydrocarbons (α-pinene, β-pinene, limonene and cineole), B. terpenoid esters (linalyl acetate and terpinyl acetate), C. terpenoid alcohols (geraniol, citronellol and menthol), D. substituted aromatics (anethole and safrole) and E. terpenoid carbonyls (camphor, carvone and citronellal) are shown in Figures 20, 21 and 22 and the data are given in Table 18 (Appendix).

Certain fractions and pure components of the oil, which had been collected by preparative g.l.c., were examined using this method, Table 19 (Appendix). The relative retention times of each component of the oil to linalol on the three columns were plotted against temperature. The general patterns of behaviour of the known chemical groups (Figures 20, 21 and 22) which had been plotted on to tracing paper were placed over the plotted behaviour of each component of the oil. As an example, the behaviours of components 38 and 53 are shown in Figure 23. From these comparisons, predictions have been made of the identity of certain components of the oil and these are given in Table 4.

It was not possible to predict the chemical groups to which components 31,38,44,45,47,50 and 52 might belong because their relative retention times to linalol when using the SE30 column were very much greater than those of the compounds from the five chemical groups previously investigated. On subsequent analysis, it was found that components 31,38,44 and 50 were respectively γ-caryophyllene, β-caryophyllene, α-humulene and δ-cadinene. Sesquiterpene hydrocarbons occur in a large number of volatile oils but Breckler and Betts omitted this group of compounds from their investigations and predictions, possibly because of the difficulty of obtaining commercial samples. Therefore, to remedy this omission, the general patterns of behaviour of these four compounds were also plotted on Figures 20,21 and 22 to represent the chemical group sesquiterpene hydrocarbons. As components 45,47 and 52 exhibited similar patterns of behaviour, they were also considered to be sesquiterpene hydrocarbons.

9. THE INTER-RELATIONSHIP OF THIN LAYER CHROMATOGRAPHIC AND GAS LIQUID CHROMATOGRAPHIC DATA

In order to be able to inter-relate data obtained from both t.l.c. and g.l.c. separations the following comparison was made. Components collected by preparative g.l.c. were subjected to t.l.c. examination using the solvent systems benzene:ethyl acetate, 95:5 and hexane. The information obtained is shown in Table 5.

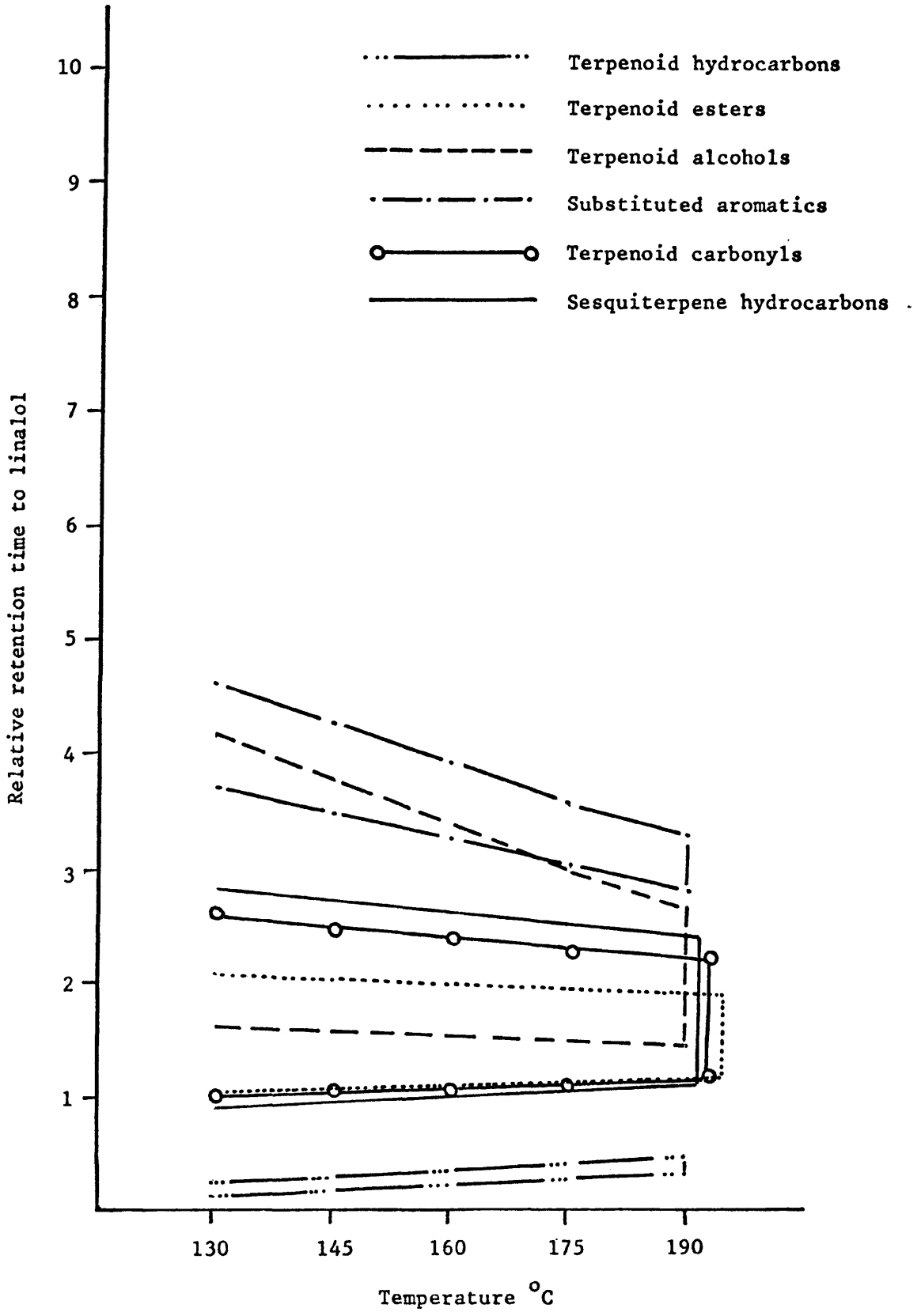


FIGURE 20: Relative retention times of certain chemical groups to linalol on the Carbowax 20M column using a range of temperatures.

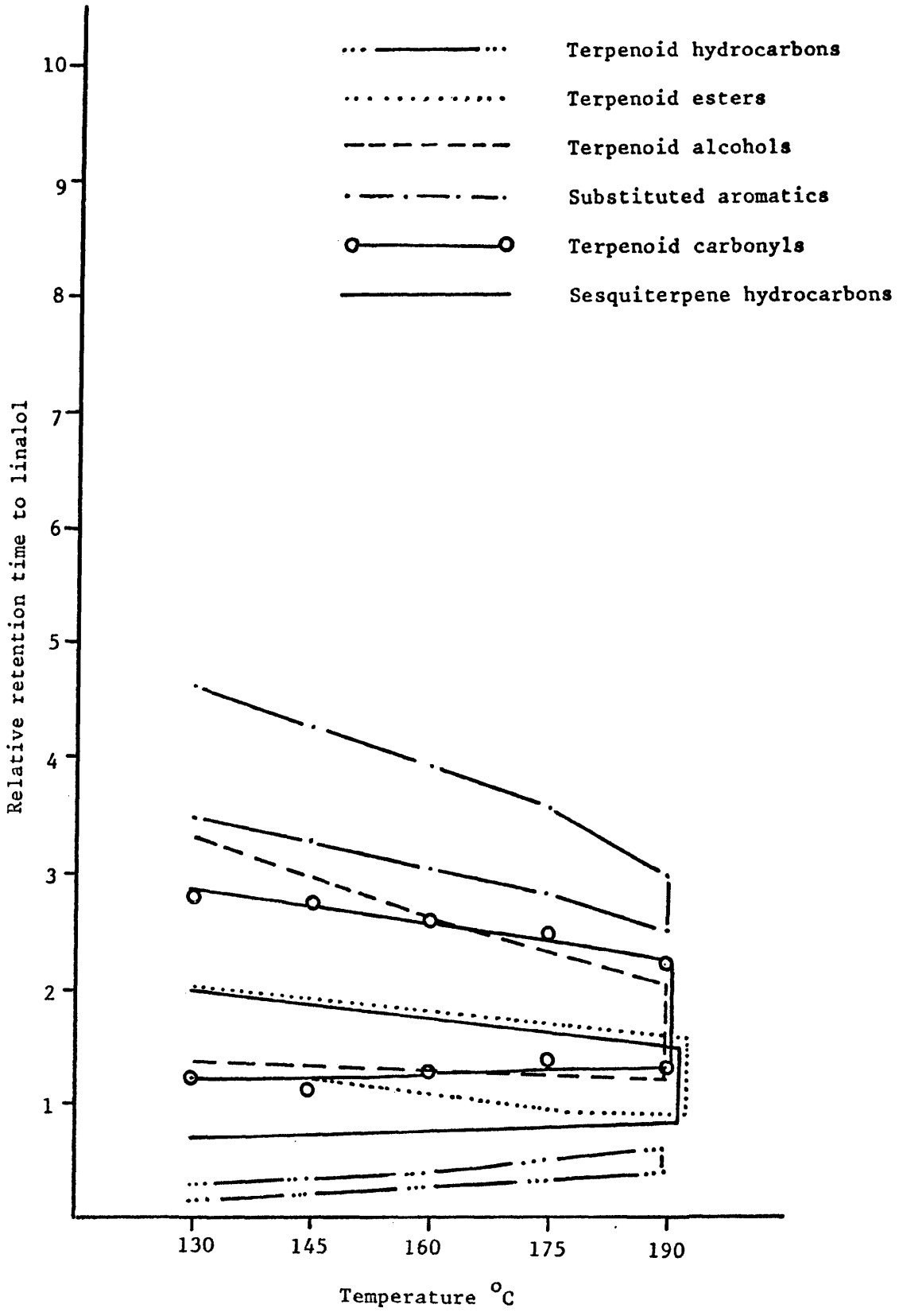


FIGURE 21: Relative retention times of certain chemical groups to linalol on the DEGS column using a range of temperatures.

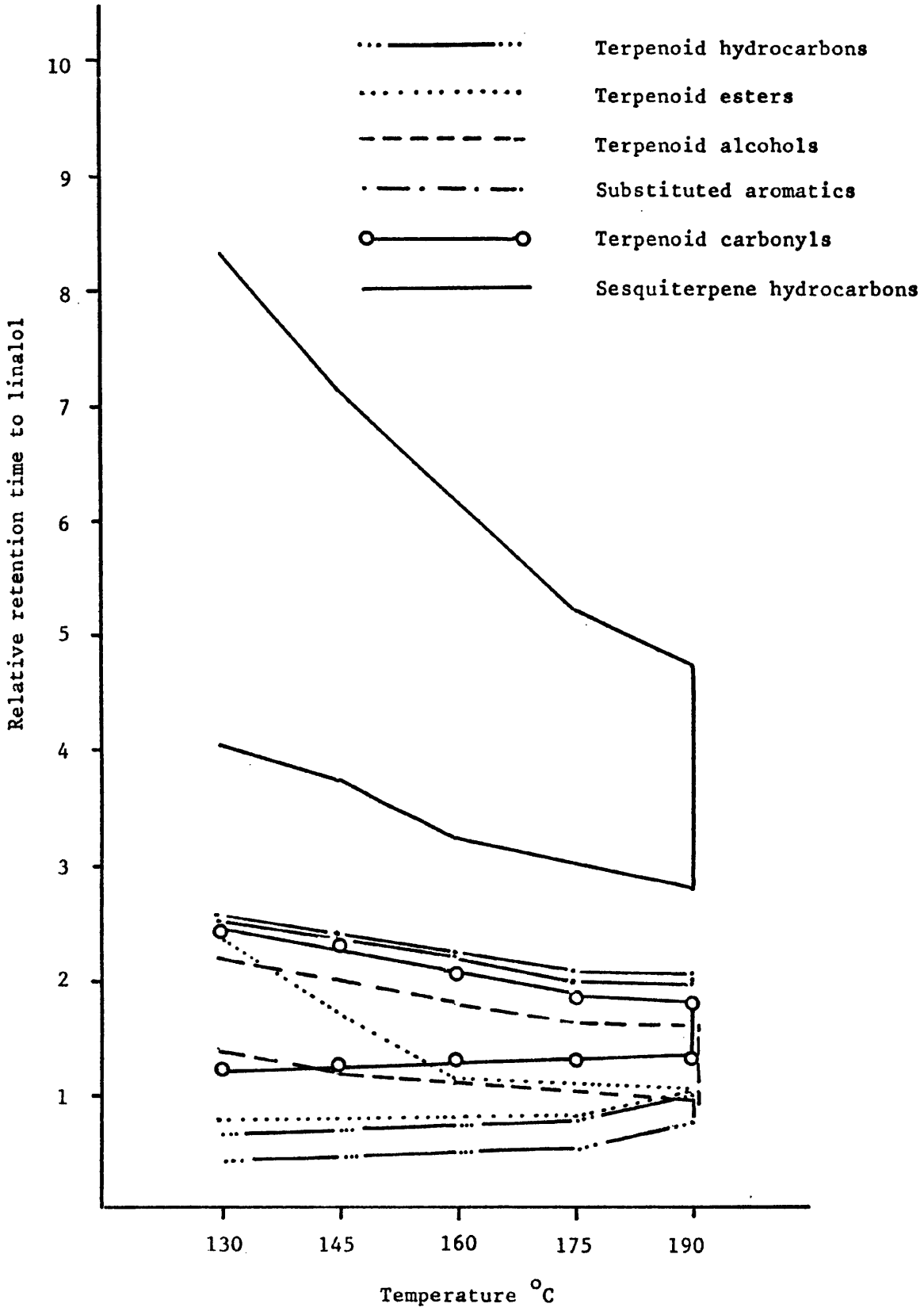


FIGURE 22: Relative retention times of certain chemical groups to linalol on the SE30 column using a range of temperatures.

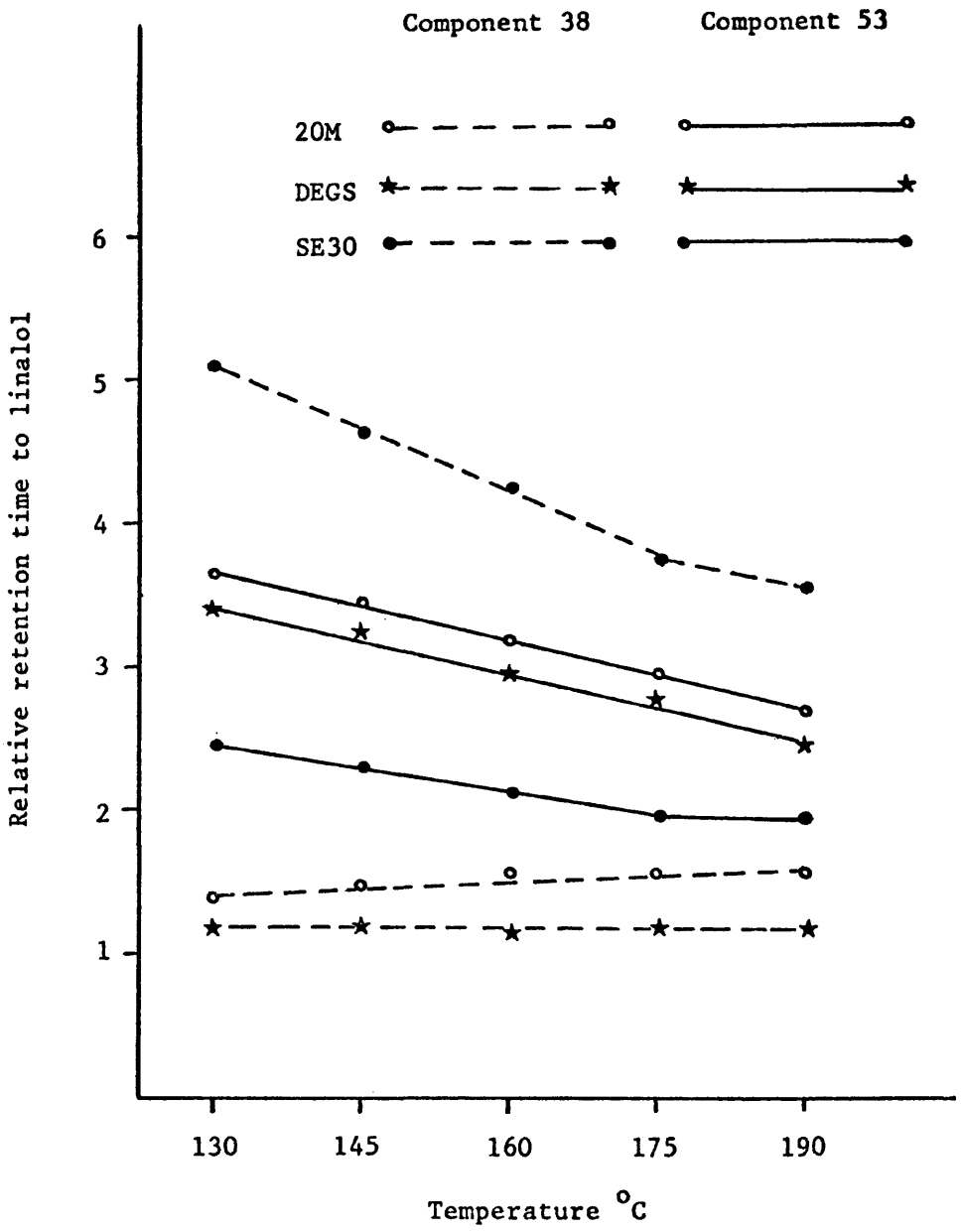


FIGURE 23: Relative retention times of components 38 and 53 to linalol on the three columns using a range of temperatures.

TABLE 4

The predictions of the chemical groups to which certain components of *Marrubium vulgare* L. oil might belong from their relative retention times to linalol on given stationary phases over a range of temperatures (130 to 190°C)

A: Terpenoid hydrocarbons. B: Terpenoid esters.
C: Terpenoid alcohols. D: Substituted aromatics.
E: Terpenoid carbonyls.

Component	Suggested chemical groups			Identity
	20M	DEGS	SE30	
5	A	A	A	A
8	A	A	A	A
9	A	A	A	A
11	A	A	A,B	A
12	A	A	A,B	A
13	A	A	A,B	A
14	A	A	A,B	A
15	A	A	A,B	A
18	A	A	A,B,C	A
26	-	B,E	C,E	E
31	-	-	-	-
33	B,E	B,E	B,C,E	B or E
35	B,E	B,C,E	C,E	E
36	B,E	B,E	B	B
38	B,C,E	B,E	-	-
39	B,C,E	B,C,E	C,E	C or E
41	B,C,E	C,E	C,D,E	C or E
43	B,C,E	B,C,E	C,E	C or E
44	B,C,E	B,E	-	-
45	B,C,E	B,E	-	-
46	B,C,E	B,C,E	C,E	C or E
47	C,E	B,C,E	-	-
48	C,E	C,E	C,E	C or E
49	C	C,E	E	C or E
50	C	B,C,E	-	-
51	C	C	B,C	C
52	C	C,E	-	-
53	C,D	D	D	D
55	C,D	C	B,C,E	C
56	D	D	D	D

TABLE 5

The inter relationship of t.l.c. and g.l.c. data of *Marrubium vulgare* L. oil (solvents: I. benzene:ethyl acetate, 95:5, II. hexane; column: Carbowax 20M)

Solvent	Spot number (t.l.c.)	Component number (g.l.c.)
I	1	19
	2	-
	3	13
	4	15
	5	40,43,55
	6	71
	7	51
	8	18,23,46,58
	9	30,39
	10	14,62,63
	11	12
	12	8
	13	41
	14	48
	15	11,60,61
	16	67
	17	-
	18	29
	19	26
	20	36
	21	53
	22	5,9,31,38,44,45,47,50,52
II	8	44
	11	38
	12	45,47,50,52
	15	31

10. IDENTIFICATION OF THE VOLATILE OIL COMPONENTS

From the g.l.c. analyses, the volatile oil was known to be a complex mixture containing seventy-two components. Only thirteen of these could be isolated from the oil as pure compounds using preparative chromatographic techniques because, (a) the volume of volatile oil available for separation was small, (b) some of the components were only present in extremely small or trace quantities and (c) the resolution of others was poor. Therefore, the techniques which could be used for the further analysis of the separated materials depended on sample purity and amount available. A list of the techniques used and the materials to which these were applied is given below.

Techniques	Components
Nuclear magnetic resonance spectroscopy	38, 50.
Mass spectroscopy	38,50,53.
Ultra violet spectroscopy	38,50,53.
Infra red spectroscopy	19,23,30,38,39,43,44,45 47,48,50,52,53,62/63, 65,67/69,70.
Thin layer chromatography and Gas liquid chromatography	5,8,9,11-15,18,19,23,26,29-31, 33,35,36,38-41,43-53,55 56,58,60-63,67,71.

The nuclear magnetic resonance (n.m.r.) spectra were determined in carbon tetrachloride solutions on a Perkin Elmer n.m.r. spectrometer model R10 operating at 60MHz, tetramethyl silane was used as an internal standard, peak areas were measured using an integrator.

Mass spectra (m.s.) were recorded on an A.E.I. MS 9 mass spectrometer using acetone (Analar) as solvent.

Qualitative ultra violet (u.v.) absorption spectra were recorded on a Perkin Elmer model 137 spectrophotometer using ethanol as solvent, in all cases there was insufficient material for quantitative u.v. analysis.

The infra red (i.r.) spectra were recorded on a Perkin Elmer model 237 spectrophotometer using sodium chloride discs. The separated compounds were examined as liquids or, if solid compounds, as pastes prepared by mixing the solid compounds with liquid paraffin. For compounds available only in micro-amounts, a technique similar to the one described by Hunter (123) was adopted, in which the sample was confined between two lines etched 5mm apart across the surface of one of the discs. A solution of the sample in ether was deposited between the etched lines using a Hamilton microlitre syringe. When the ether had evaporated the second disc was placed carefully on top of the etched disc.

Results from the preliminary t.l.c. and g.l.c. analyses were used as an aid to compound identification; see Figure 15, Tables 3, 4 and 5 and Tables 19 and 21 (Appendix). On identification, if an authentic pure sample of the compound was available, further comparative t.l.c. and g.l.c. analyses were performed. As a final check for identification, a sample of the oil was enriched with the reference material and subjected to t.l.c. and g.l.c. separations, the resulting chromatoplates and chromatograms being examined for enhancement of the appropriate spot or peak.

The following two solvent systems were used in the t.l.c. investigations:
Solvent system I: Benzene : ethyl acetate, 95:5.
Solvent system II: Hexane.

The g.l.c. separations were performed using the following columns: (1) Carbowax 20 M, (2) DEGS and (3) SE30 at the appropriate temperature conditions.

The results of these analyses are given below:

Components 1 - 18:

Using the Carbowax 20 M column at a temperature of 145°C the first 3.7 minutes of the run were recorded on the chromatogram as a series of congested peaks. The prefractionation of the volatile oil on the Silica gel column separated these peaks into two groups, oxygenated and non-oxygenated compounds. Using preparative g.l.c., this fraction of the volatile oil could not be resolved sufficiently to collect its components individually; therefore, after prefractionation these components were collected either in the group of oxygenated compounds or the group of non-oxygenated compounds. Since the total amount of these compounds was small, each group was investigated as a unit using g.l.c. and t.l.c. techniques, components being located on the different g.l.c. columns by their relative peak areas. Chart speeds were increased in order to aid resolution.

The investigations indicated that this fraction of the volatile oil contained eighteen components, although some were present only as traces. The g.l.c. behaviour of these eighteen components suggested that they were monoterpene hydrocarbons, therefore, where available, reference monoterpene hydrocarbons which might be expected to occur within this group were examined using the same techniques. By comparing the g.l.c. and t.l.c. behaviour of the unknown components with that of the reference standards, nine of the eighteen components could be identified. Using solvent system I, the R_f values were 0.79, 0.42, 0.79, 0.46, 0.40, 0.67, 0.32, 0.62 and 0.25 for components 5,8,9,11,12,13,14,15 and 18 respectively. All remained on the starting line when solvent system II was used. The relative retention times of these components to linalol were compared with those of the reference standards given in Table 20 (Appendix). The

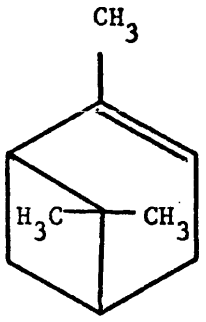
identified components are listed below and their structures are shown in Figure 24.

Component number	Identity
1-4	Unidentified non-oxygenated hydrocarbons
5	∝- Pinene
6,7	Unidentified oxygenated hydrocarbons
8	Camphene
9	β-Pinene
10	Unidentified oxygenated hydrocarbon
11	Limonene
12	Cineole
13	β-Phellandrene
14	ρ-Cymene
15	Terpinolene
16,17	Unidentified oxygenated hydrocarbons
18	Allo-ocimene

Component 19:

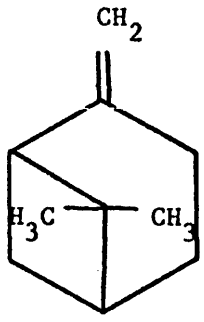
Figure 25 (A and B) shows the i.r. spectrum of this component indicating that it contains a hydroxyl group (3350cm^{-1}). On consulting published chromatographic data for the alcoholic compounds previously found in volatile oils (95,115), 3-octanol was found to have similar characters. A reference sample of 3-octanol (Koch-Light) was found to exhibit t.l.c., g.l.c. and i.r. spectroscopic characters which were identical to those of component 19. Therefore, component 19 was proved to be 3-octanol, an aliphatic alcohol (124), molecular weight (M.W.) 130, $C_8H_{18}O$;

$$C_2H_5 - \underset{\underset{OH}{|}}{\overset{\overset{H}{|}}{C}} - C_5H_{11}$$



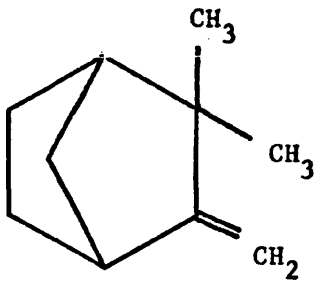
α - Pinene

$C_{10}H_{16}$, M.W.136



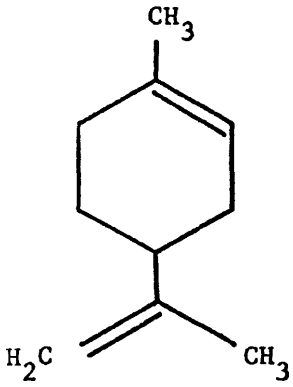
β- Pinene

$C_{10}H_{16}$, M.W.136



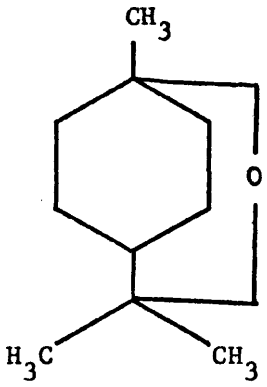
Camphene*

$C_{10}H_{16}$, M.W.136



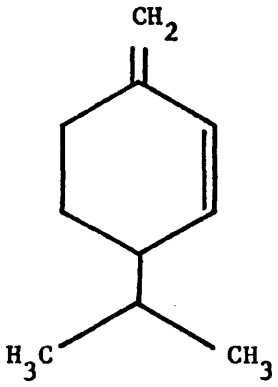
Limonene

$C_{10}H_{16}$, M.W.136



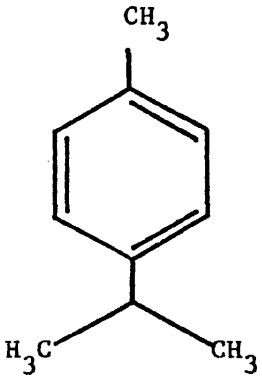
Cineole

$C_{10}H_{16}O$, M.W.154



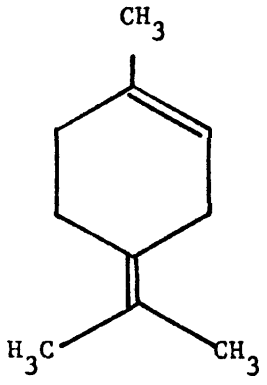
β-Phellandrene

$C_{10}H_{16}$, M.W.136



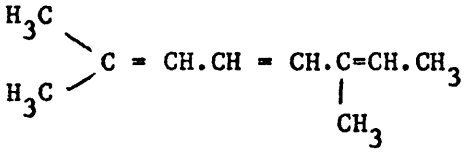
p - Cymene

$C_{10}H_{14}$, M.W.134



Terpinolene

$C_{10}H_{16}$, M.W.136



Allo-ocimene

$C_{10}H_{16}$, M.W.136

FIGURE 24: Components found in the simple monoterpene fraction of *Marrubium vulgare* L. oil (124,125*)

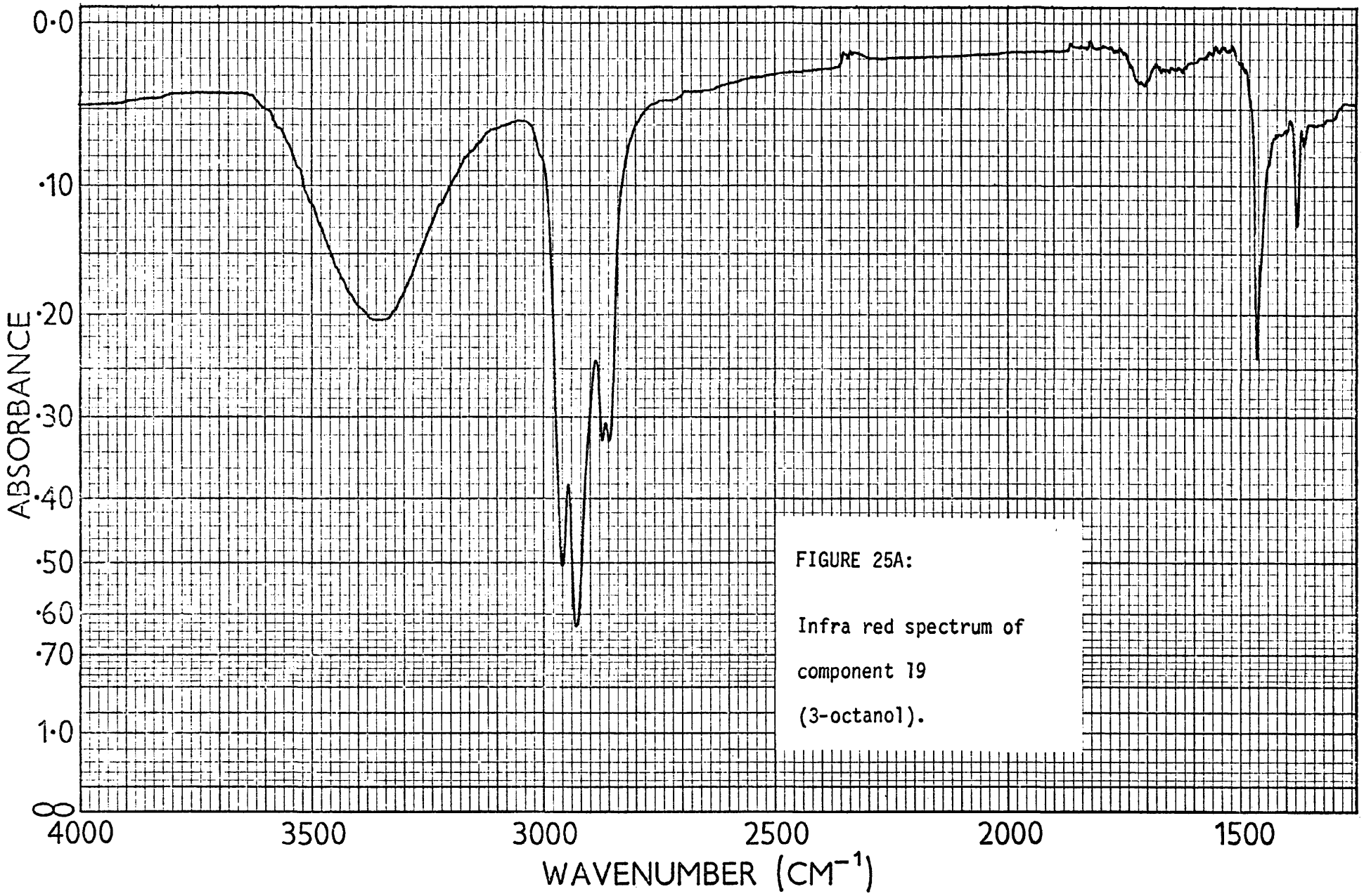


FIGURE 25A:

Infra red spectrum of component 19 (3-octanol).

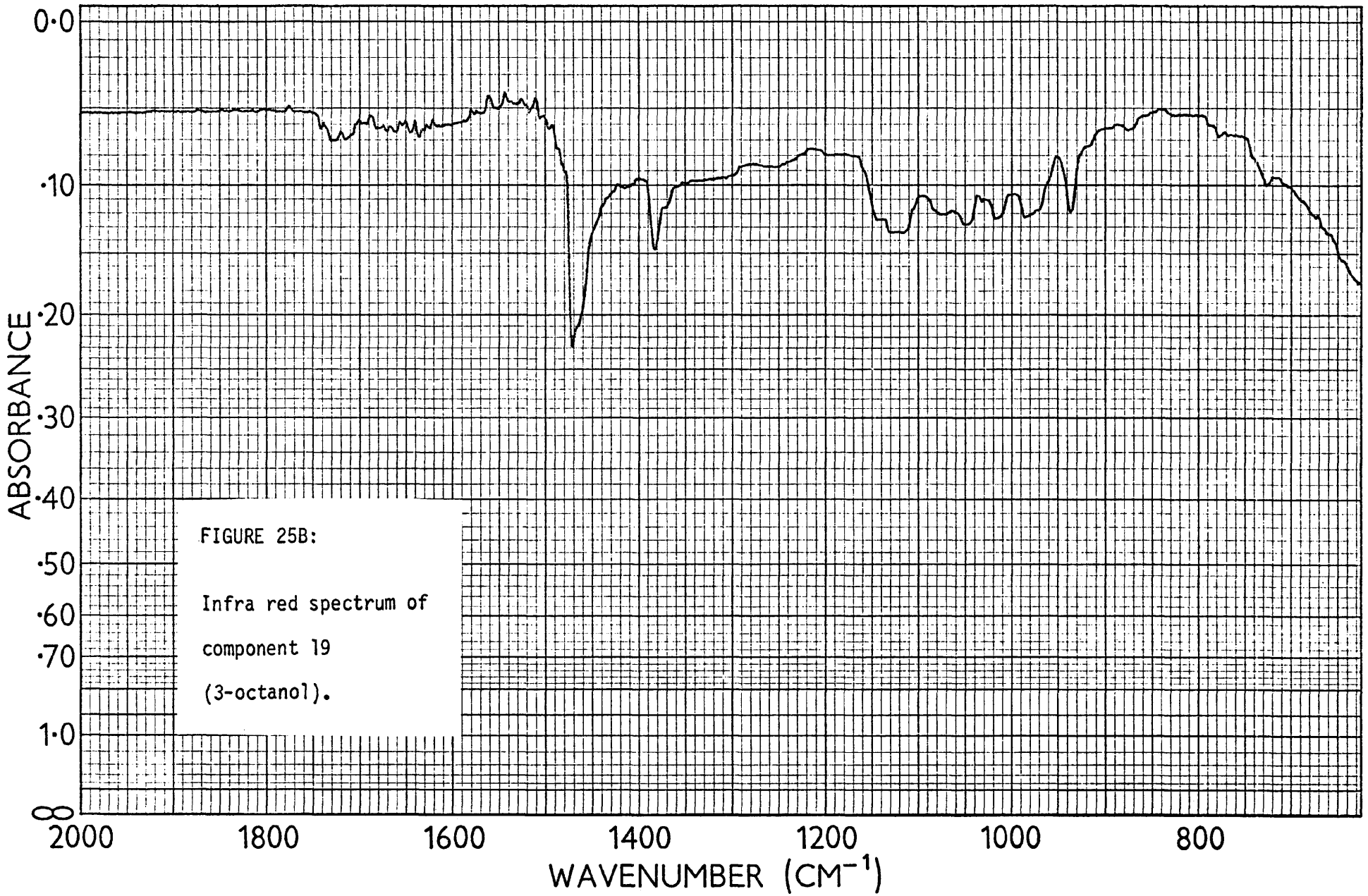


FIGURE 25B:

Infra red spectrum of component 19 (3-octanol).

Components 20-22:

Components 20, 21 and 22 occurred in the volatile oil in extremely small quantities. Components 20 and 22 were non-oxygenated compounds since they were eluted with n-hexane during the prefractionation technique, while component 21 was eluted with methanol indicating that it might be an oxygenated compound. Insufficient data were obtained to enable the identification of these three compounds.

Component 23:

This component had an R_f value of 0.25 using solvent system I. The i.r. spectrum Figure 26 (A and B) shows the following significant bands: 3360 (a hydroxyl group), 3080 (an olefinic hydrogen), 2860-3000, 1470, 1380, 990 and 920 cm^{-1}.

The t.l.c., g.l.c. and i.r. spectroscopic characters of component 23 proved that it was an alcoholic compound, possibly 1-octen-3-ol, on consultation of data from similar investigations (114,115). An authentic pure sample of 1-octen-3-ol showed t.l.c., g.l.c. and i.r. behaviour which was identical to that of component 23. Therefore, component 23 was 1-octen-3-ol an aliphatic alcohol (124), M.W. 128.

$$C_8H_{16}O,\ CH_3.(CH_2)_4.\underset{\underset{OH}{|}}{CH}.\ CH = CH_2.$$

Components 24-29:

This group of components represented a very small portion of the volatile oil. It was possible to identify components 26 and 29 from their chromatographic behaviour, but not the others which were found only in trace amounts. Components 24,26 and 29 were oxygenated compounds since they were found in the fraction of the oil eluted with methanol when using the prefractionation technique. Components 25,27 and 28 were non-oxygenated compounds since they were found in the oil fraction eluted by n-hexane.

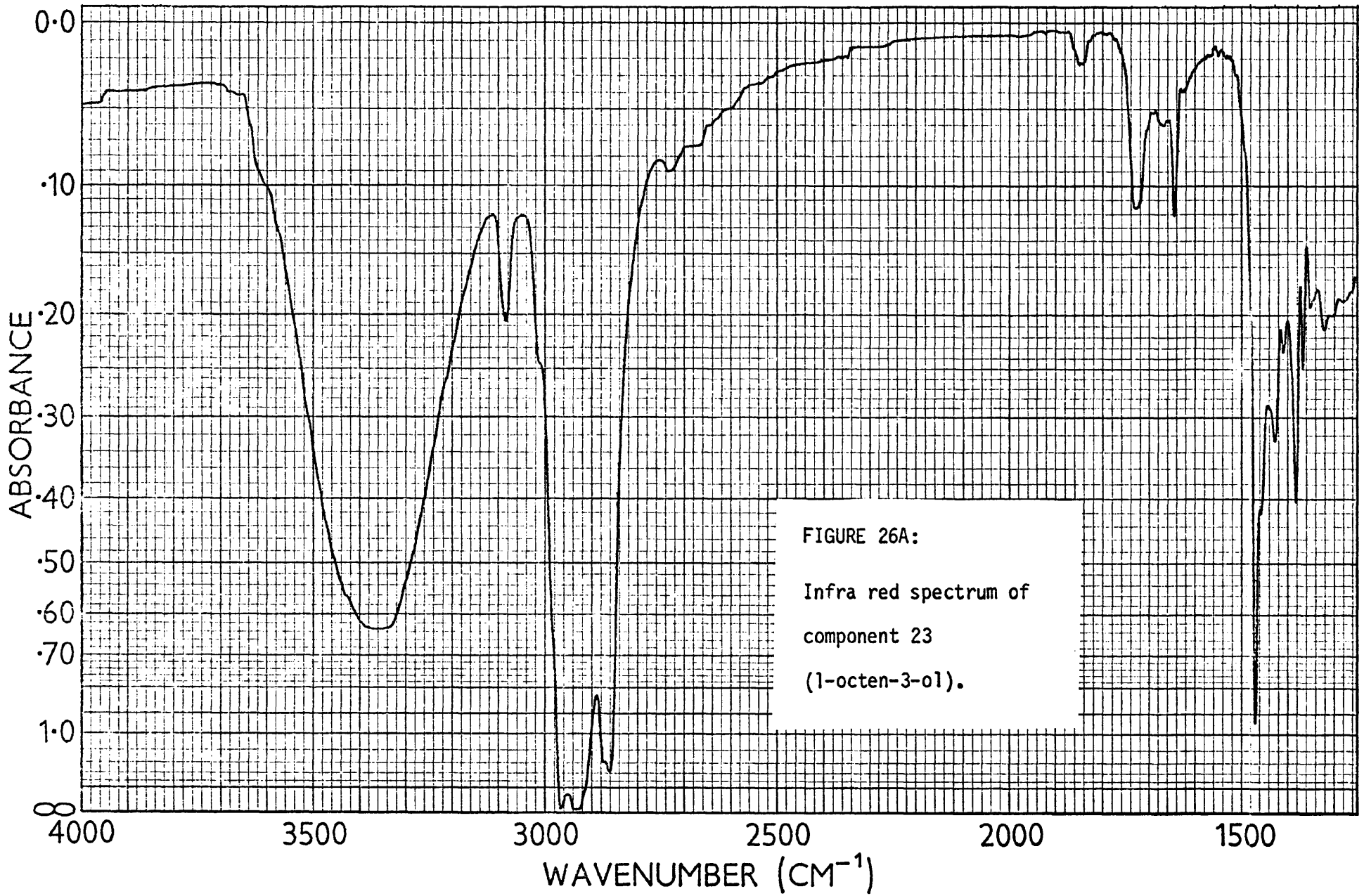


FIGURE 26A:

Infra red spectrum of component 23 (1-octen-3-ol).

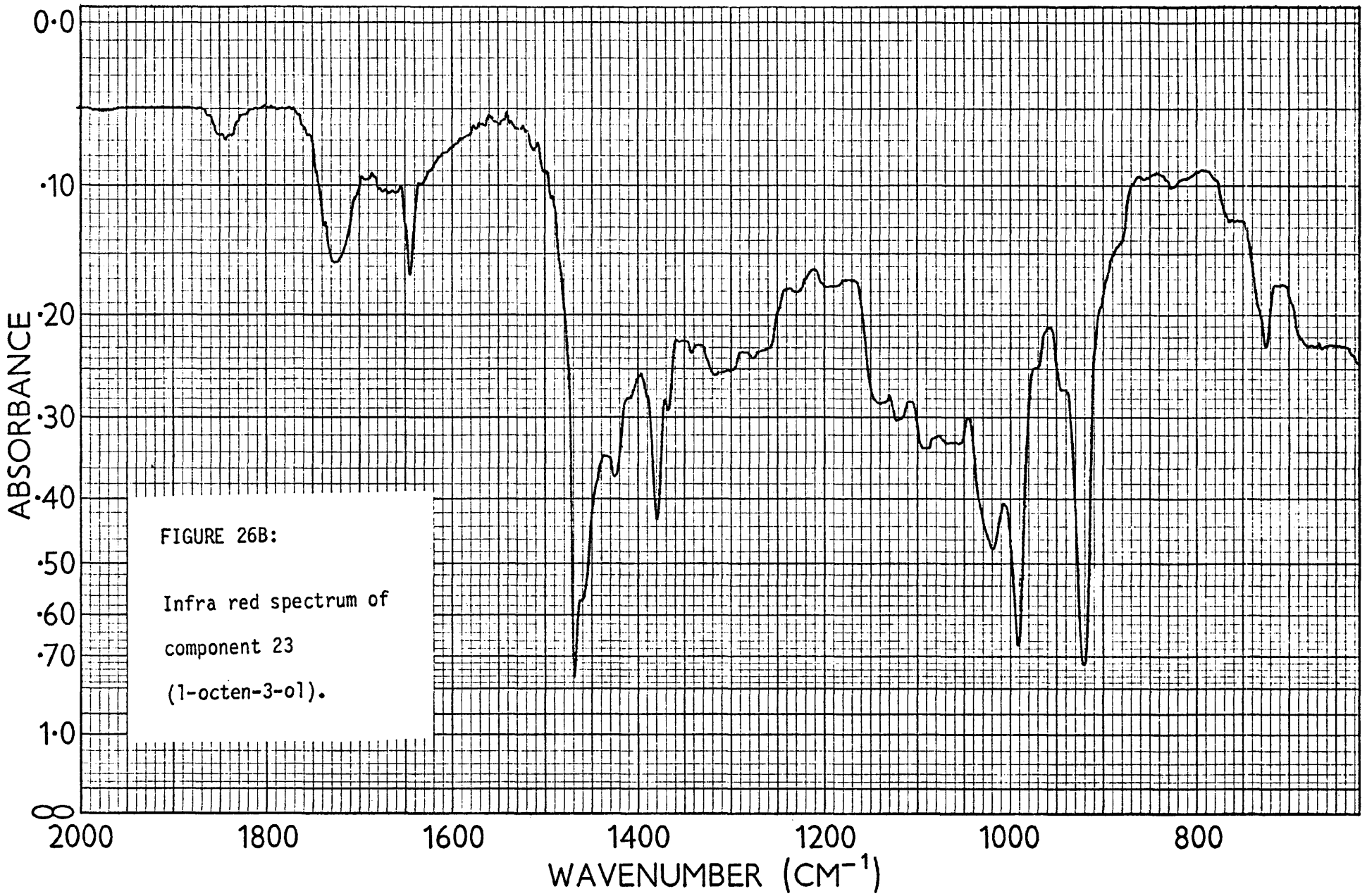
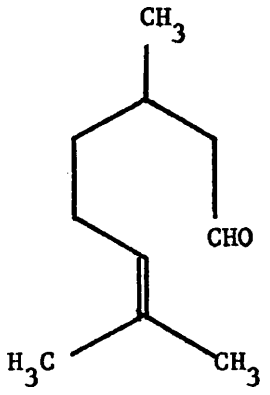


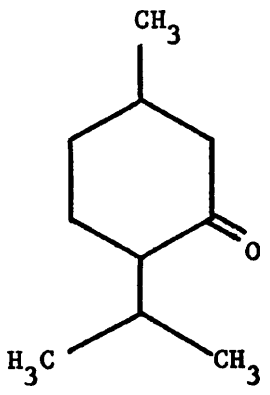
FIGURE 26B:

Infra red spectrum of component 23 (1-octen-3-ol).

Using solvent system I, the R_f values were 0.62 and 0.57 for components 26 and 29 respectively. Their g.l.c. behaviour suggested that both components might be carbonyl compounds. The t.l.c. and g.l.c. behaviours of components 26 and 29 were compared with those of carbonyl compounds known to occur in volatile oils (114,115,126). These comparisons suggested that component 26 was citronellal and component 29 was menthone. Data obtained from pure samples of these two compounds proved that component 26 was citronellal, an aliphatic aldehyde, M.W. 154 and component 29 was menthone, a cyclic terpene ketone, M.W.154, Figure 27 (125).



Citronellal, $C_{10}H_{18}O$.



Menthone, $C_{10}H_{18}O$.

FIGURE 27

Component 30:

This component had an R_f value of 0.27 using solvent system I indicating that it might be an alcohol. The g.l.c. behaviour suggested that this component was linalol, an aliphatic alcohol, M.W.154, Figure 28 (127).

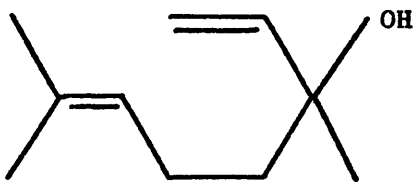


FIGURE 28: Linalol, $C_{10}H_{18}O$.

The i.r. spectrum Figure 29 (A and B) showed significant bands at 3400, 3090, 2970, 2930, 2860, 1645, 1450, 1415, 1380, 1115, 995, 920 and 835 cm^{-1}. It was recorded (127) that linalol exhibits the following principle peaks: ca 3450 and 1115 cm^{-1} (a hydroxyl group), 1642, 1412, 995 and 920 cm^{-1} (a terminal vinyl). On the comparison of an authentic pure sample of linalol with component 30, the t.l.c., g.l.c. and i.r. spectroscopic behaviours of both were found to be identical. Therefore, component 30 was proved to be linalol.

Component 31:

This component, which occurred in the oil only in small amounts, had the following R_f values: solvent system I: 0.79, solvent system II: 0.73 indicating that it might be a hydrocarbon. The relative retention times of this component to linalol suggested that it did not belong to any of the chemical groups examined by Breckler and Betts (119,120).

As will be mentioned later, β-caryophyllene was found to be the main component of Horehound oil. Since in many volatile oils, e.g. clove oil, β-caryophyllene occurs together with its α- and γ-isomers (124,125), it seemed likely that one or both of these would be present in this oil.

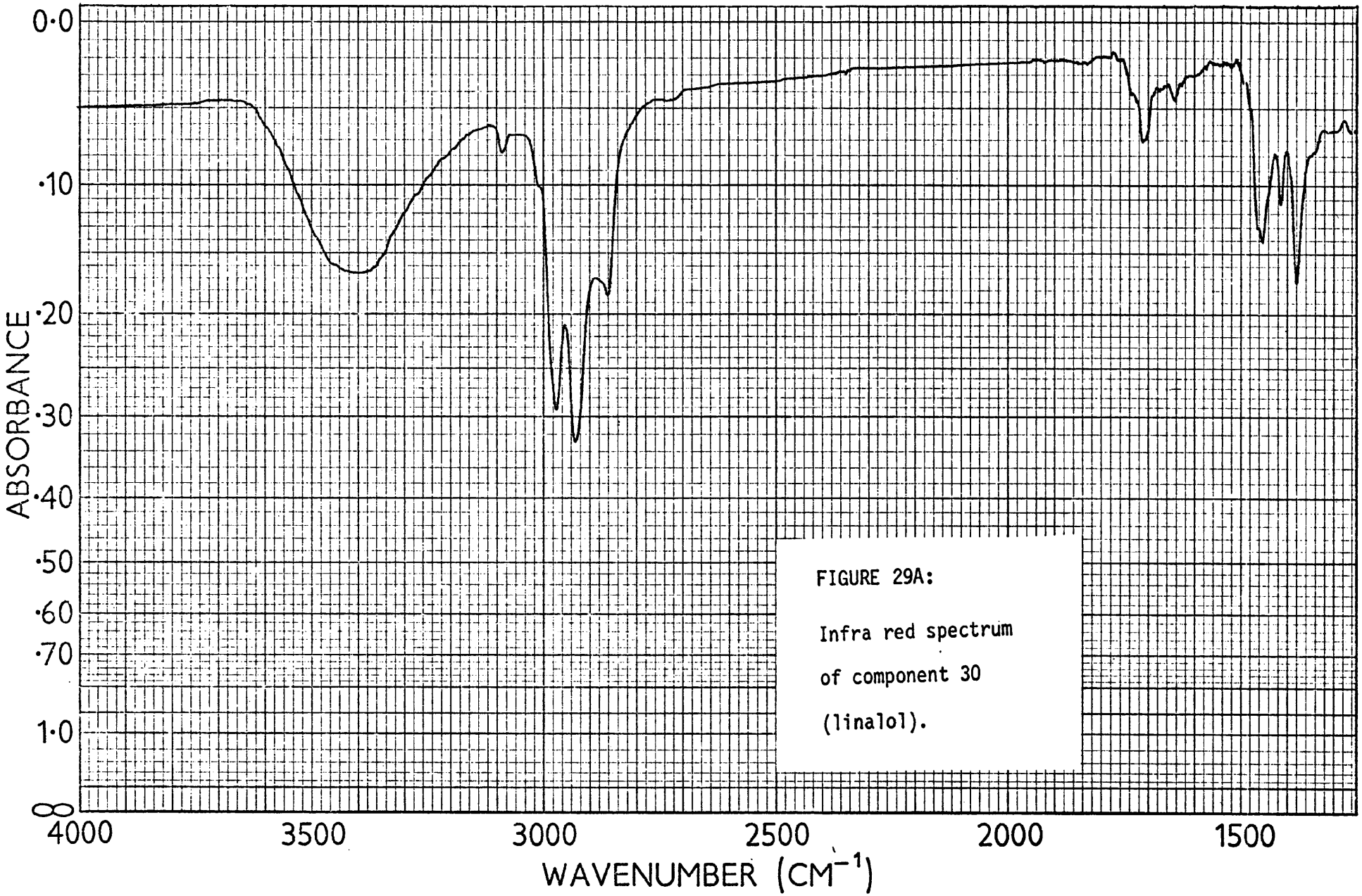


FIGURE 29A:

Infra red spectrum of component 30 (linalol).

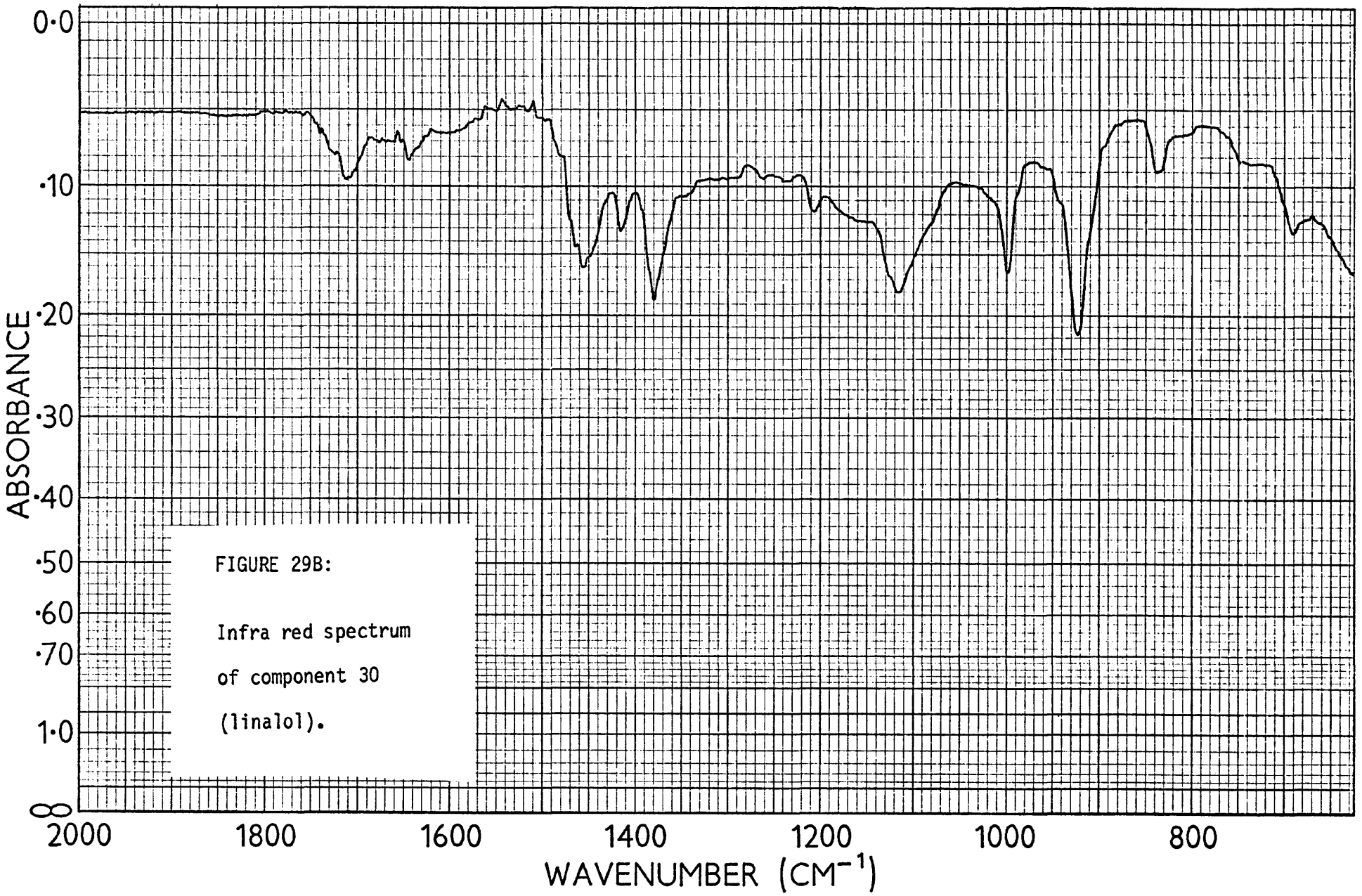


FIGURE 29B:

Infra red spectrum of component 30 (linalol).

The chromatographic data of pure samples of each isomer were compared with the data of sesquiterpenoid compounds found in this oil. These t.l.c. and g.l.c. investigations proved that the characters of component 31 and γ-caryophyllene were identical. Therefore, component 31 was γ-caryophyllene a bicyclic sesquiterpene hydrocarbon, M.W. 204, Figure 30 (125).

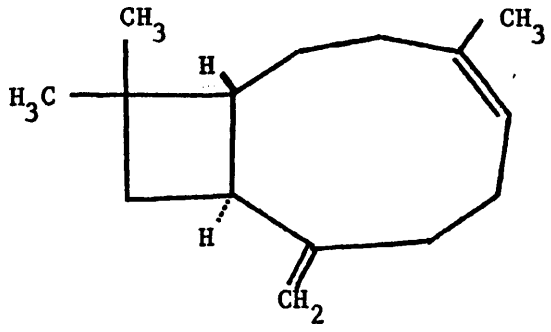


FIGURE 30: γ-Caryophyllene, $C_{15}H_{24}$.

Component 32:

This component was eluted in a trace amount as part of the n-hexane fraction in the prefractionation technique, indicating that it was a non-oxygenated compound. It was not possible to identify this compound further.

Component 33:

The relative retention times of this component to linalol suggested that it might be a terpenoid carbonyl compound. On consulting data from similar investi-gations (117,128) camphor showed the same behaviour. The g.l.c. data obtained using a sample of camphor B.P. was found to be identical with data obtained for component 33. Therefore, component 33 was camphor, a cyclic terpene ketone, M.W. 152, Figure 31 (125).

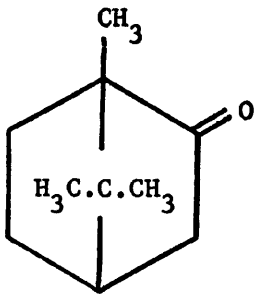


FIGURE 31: Camphor, $C_{10}H_{15}O$.

Component 34:

This component was only present in the oil in trace amounts occurring in the non-oxygenated fraction when using the prefractionation technique. No further information was available for this component.

Component 35:

The relative retention times of this component to linalol suggested that it might be a terpenoid carbonyl compound. On consulting data from similar investigations (129) carvomenthone showed the same behaviour. The g.l.c. data obtained from a pure sample of carvomenthone was found to be identical with data obtained for component 35. Therefore, it was concluded that component 35 was carvomenthone a cyclic terpene ketone, M.W. 154, Figure 32 (124).

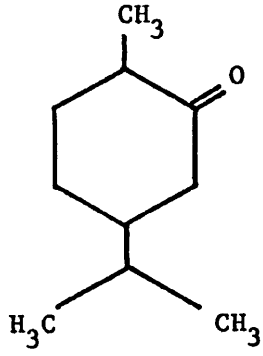


FIGURE 32: Carvomenthone, $C_{10}H_{18}O$.

Component 36 :

This component exhibited the following chromatographic characters. The R_f value using solvent system I was 0.67 indicating that it could be an ester. The relative retention times of this component to linalol also suggested that it might be a terpenoid ester. On consulting the literature (126,128), bornyl acetate was found to give similar chromatographic data. The t.l.c. and g.l.c. behaviour of a pure sample of bornyl acetate (Aldrich) was compared with that of component 36. This comparison proved that component 36 was bornyl acetate a terpenoid ester, M.W. 196, $C_{12}H_{20}O_2$, $CH_3COOC_{10}H_{17}$(125).

Component 37:

This component was a non-oxygenated compound since during the pre-fractionation technique it was eluted by the n-hexane. No further information was available for this component.

Component 38:

This component was considered to be the major component as will be shown later. It had the following R_f values: solvent system I: 0.79, solvent system II: 0.57 indicating that it could be a hydrocarbon. The relative retention times of this component to linalol (Figure 23) suggested that it did not belong to any of the chemical groups previously examined (119,120).

The following physical data for component 38 were recorded. The n.m.r. spectrum (Figure 33) showed 1-proton singlets at 8.4 and 9.0 T, a multiplet range at 7.3-8.2 T, a 2-proton doublet at 5.12 T and a broad resonance at 4.5-5.0 T. The low resolution mass spectrum (Figure 34) showed significant ions at 41 (the base peak) , 43, 48, 50-52, 64,

67-71, 79-85, 91-95, 105-109, 119-121, 123, 133,135, 147, 148, 161, 189 and 204 (M^+). The u.v. spectrum (Figure 35) showed $\lambda_{max.}$ 225 nm. The i.r. spectrum (Figure 36) gave significant bands at 3060, 2940, 2910, 2840, 1670, 1630, 1445, 1380, 1365, 1270, 1180, 1030, 985, 880 (the strongest band in the fingerprint region) and 870 cm^{-1}.

The n.m.r. spectrum showed unsplit 3-proton singlets at 9 and 8.4 T indicating the presence of 2 independent methyl groups ($-CH_3$). The chemical shift of the former signal suggested a methyl group attached to saturated carbon which must be tertiary since the peak was not split; i.e. $CH_3 - \overset{|}{\underset{|}{C}} -$. The chemical shift of the 8.4 T signal indicated a methyl group attached to a double bond; i.e. $CH_3 - \underset{|}{C} = C\diagup\diagdown$. The 2-proton doublet centred at 5.12 T exhibited a fine splitting ($J = 0.5\ H_2$) indicative of a gem disubstituted double bond; i.e. $CH_2 = C\diagup\diagdown$, the chemical shift confirmed this conclusion. The broad resonance 4.5 - 5.0 T could be a hydroxyl group (an exchange reaction was required to confirm this) or it could be due to an additional olefinic proton. However, no hydroxyl stretching was shown in the i.r. spectrum. The mass spectrum showed a molecular ion of 204 which was consistent with the n.m.r. data. The peak at 189 m/e (M-15) could be due to loss of a methyl group. The u.v. spectrum indicated no significant chromphore, and since no peak was present at $\lambda_{max.}$ 260nm., component 38 was not an aromatic compound. The i.r. spectrum showed intense peaks at 2940, 2910, 2840 and 1445 cm^{-1} indicating a large amount of C-H stretching; i.e. component 38 was a highly saturated compound, while the sharp peak at 3060 cm^{-1} suggested the presence of olefinic hydrogen. The intense peak at 880 cm^{-1} indicated a gem disubstituted olefinic structure; i.e. $CH_2 = C\diagup\diagdown$ which was substantiated by the presence of strong peak at 1630 cm^{-1}. The less intense peak at 1670 cm^{-1} was an indication of a trisubstituted olefine structure; i.e. $\overset{C}{\underset{C}{\diagup\diagdown}}C = C\overset{H}{\underset{C}{\diagup\diagdown}}$.

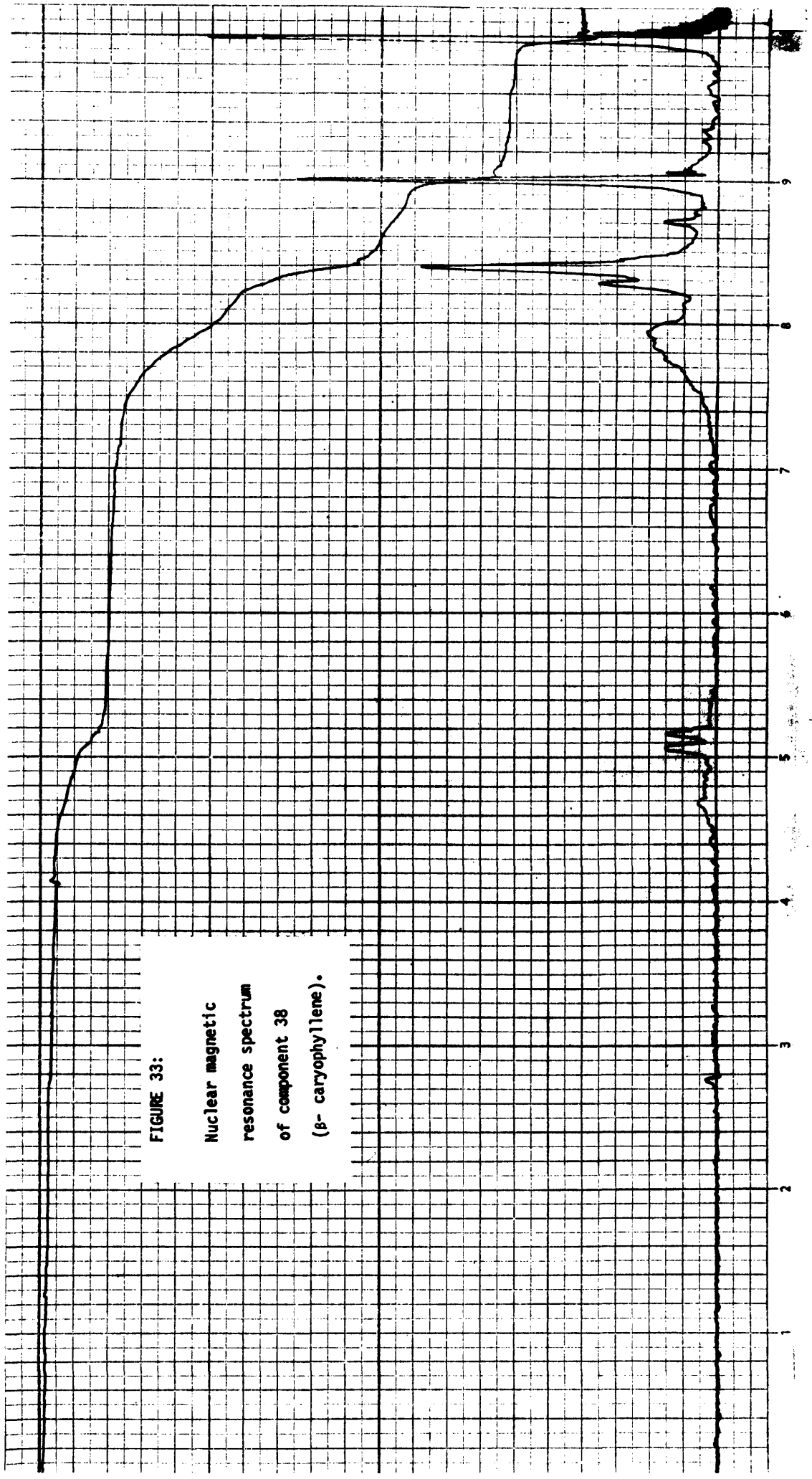


FIGURE 33: Nuclear magnetic resonance spectrum of component 38 (β- caryophyllene).

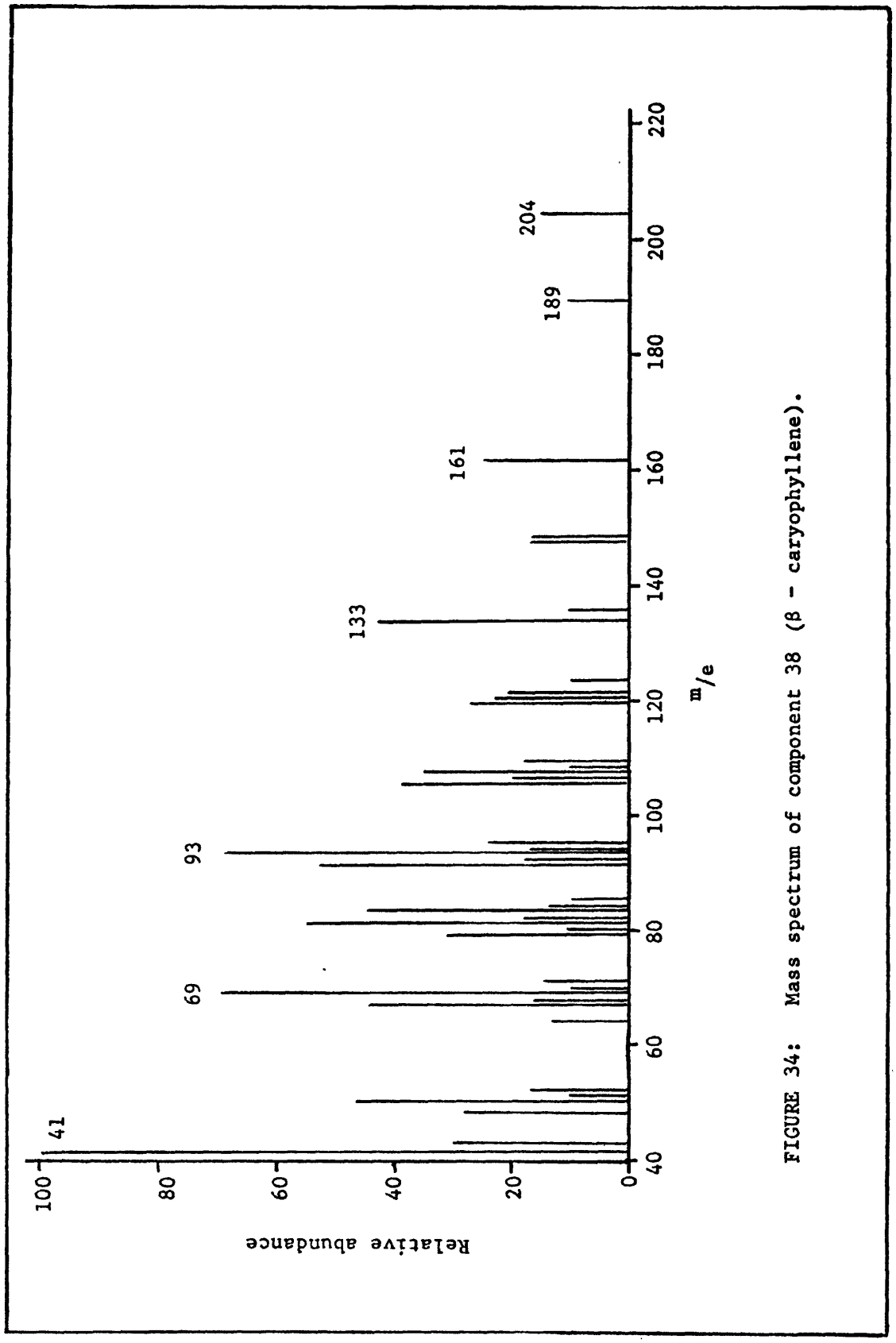


FIGURE 34: Mass spectrum of component 38 (β - caryophyllene).

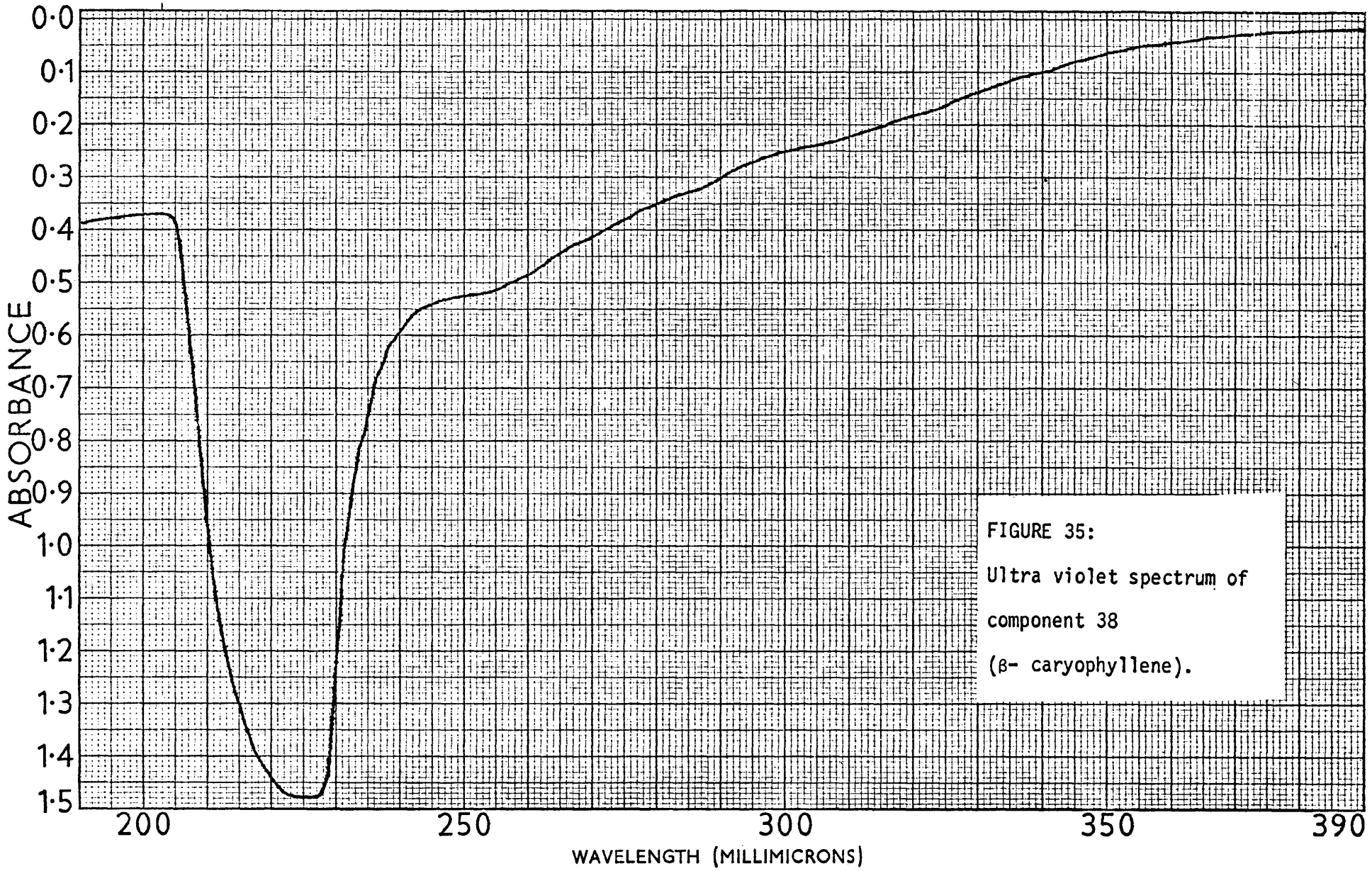


FIGURE 35:

Ultra violet spectrum of component 38 (β- caryophyllene).

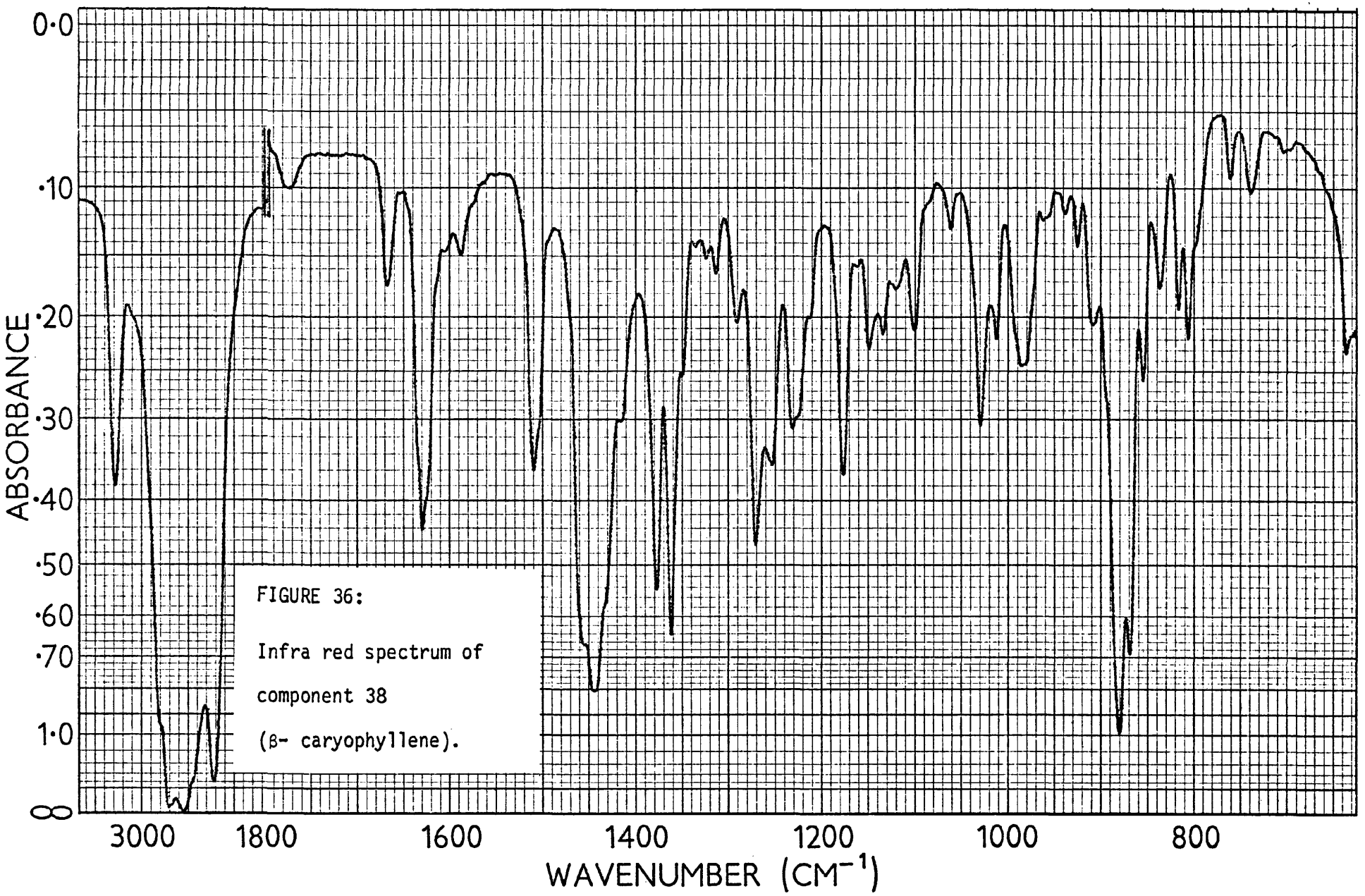
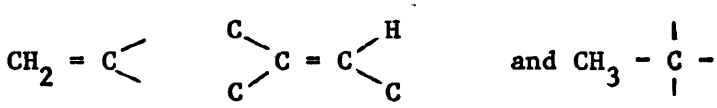


FIGURE 36:

Infra red spectrum of component 38 (β- caryophyllene).

From the above evidence, it was concluded that component 38 had the empirical formula $C_{15}H_{24}$ (a sesquiterpene hydrocarbon). The suggestions made from the t.l.c. data that component 38 might be a hydrocarbon were substantiated by the spectroscopic data which also suggested the presence of the following structural groups:



The data obtained for component 38 were then compared with data published for the sesquiterpene hydrocarbons and were found to agree with the following characters given for β-caryophyllene, a bicyclic sesquiterpene hydrocarbon, Figure 37 (125).

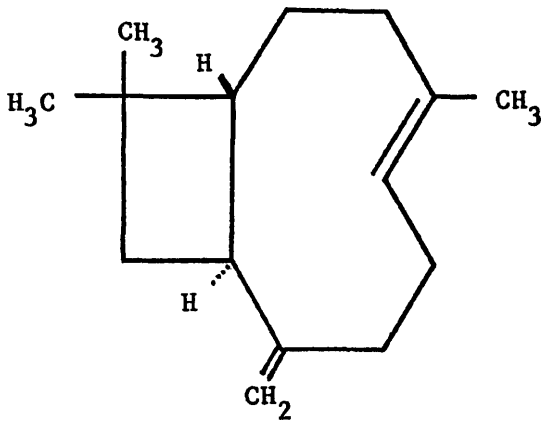


FIGURE 37: β-Caryophyllene, $C_{15}H_{24}$.

The T value of 8.4 in the n.m.r. spectrum was characteristic of the trans configuration of the endocyclic double bond in the 9-membered ring of β-caryophyllene, since the value would be 8.3 T for the cis isomer γ-caryophyllene (130). The mass spectrum (131) and the i.r. spectrum (132,133) of β-caryophyllene were identical to those given by component 38.

It was also indicated that in the i.r. spectrum the five strongest bands in 1250-300 cm^{-1} region in a decreasing order of intensity should be 883, 873, 1181, 542 and 985 cm^{-1}. The bands at 1365 and 1381 cm^{-1} were due to the gem-dimethyl group on the 4-membered ring of β-caryophyllene (134). The u.v. spectrum of β-caryophyllene should show an absorption maximum at 215 n.m. and no absorption in the 250-280 n.m. region (135).

β-Caryophyllene (Koch-Light) was purified using preparative g.l.c. and spectroscopic characters of both this purified sample and component 38 were identical, proving that component 38 was β-caryophyllene.

Component 39:

This component had an R_f value of 0.27 using solvent system I indicating that it could be an alcohol. The relative retention times of this component to linalol suggested that it might be either a terpenoid alcohol or a carbonyl compound. However, as a negative response was obtained to 2,4-dinitrophenylhydrazine (2,4-DNPH) when this component was separated using t.l.c., it was concluded that the first suggestion was more reliable (an alcohol).

This finding was confirmed when this component was examined by i.r. spectroscopy, Figure 38(A and B), since the following significant bands were recorded: 3370 (a hydroxyl group), 2960, 2930, 2860, 1470, 1380, 1050 and 1030 cm^{-1}. The relative retention times of this component to linalol were identical to those of menthol which had been used as a standard compound in earlier investigations, Table 18 (Appendix). When the t.l.c., g.l.c. and i.r. spectroscopic data for this component were compared with those of a sample of menthol. B.P., they were also found to be identical.

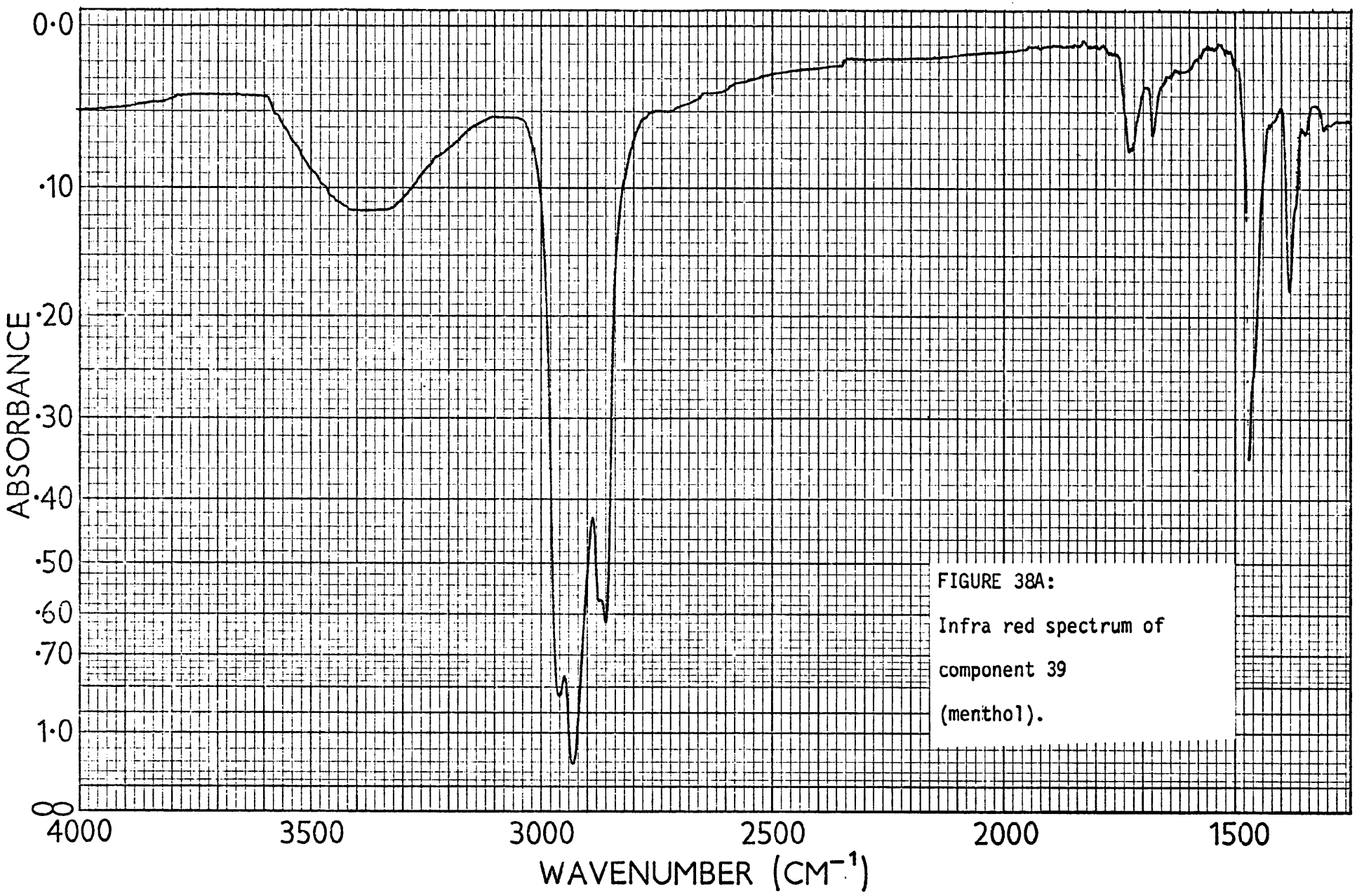


FIGURE 38A:

Infra red spectrum of component 39 (menthol).

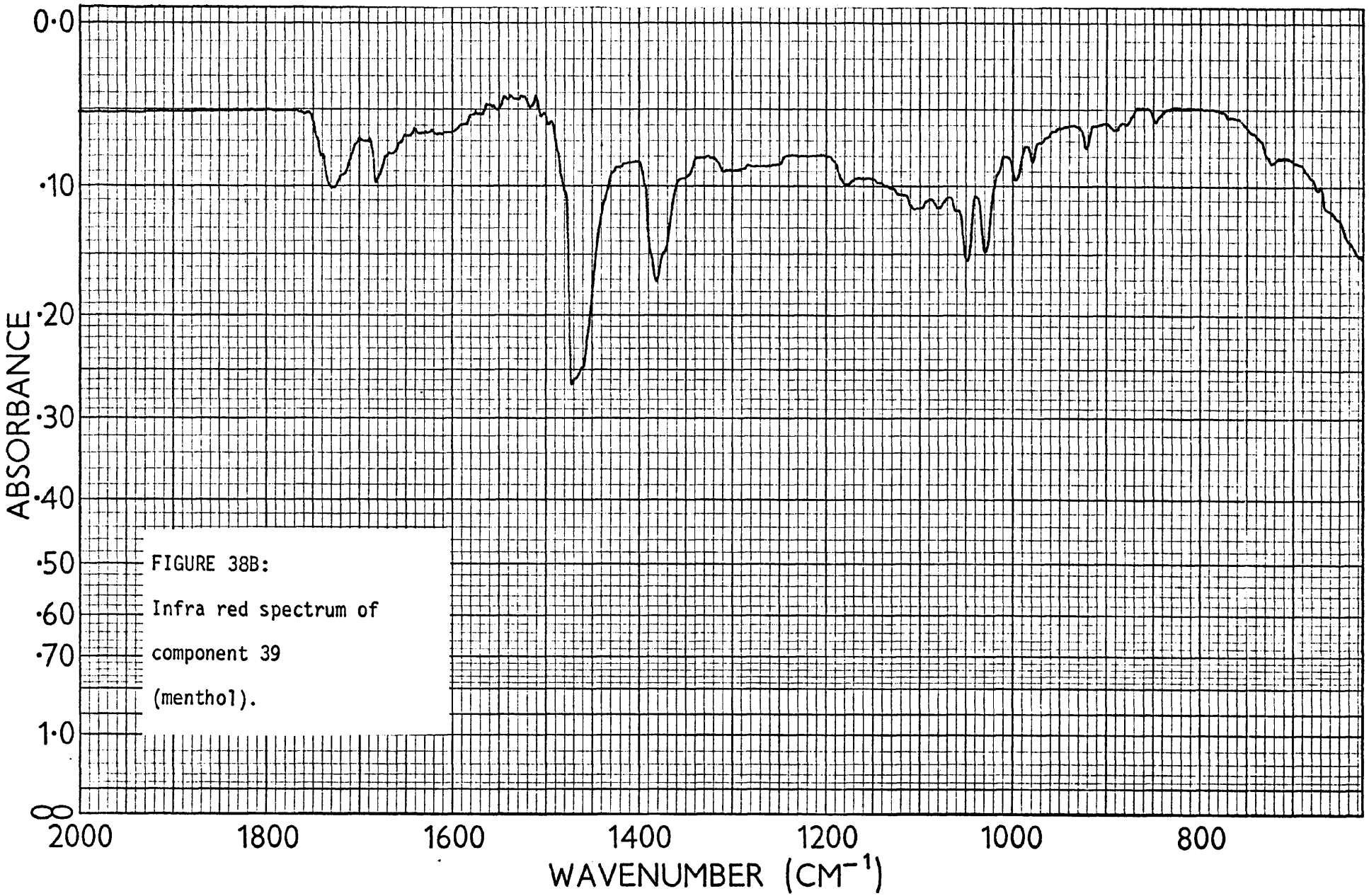


FIGURE 38B:

Infra red spectrum of component 39 (menthol).

Therefore, it was proved that component 39 was menthol, a cyclic terpene alcohol, M.W. 156, Figure 39(124).

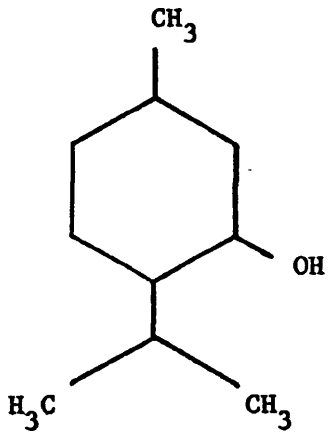


FIGURE 39: Menthol, $C_{10}H_{20}O$.

Components 40-42:

The retention times of these components were almost identical when separated using the Carbowax 20M column. Components 40 and 41 were eluted with methanol using the prefractionation technique indicating that they were oxygenated compounds.

Component 40 had an R_f value of 0.17 using solvent system I. On consulting the literature (136), estragole (methyl chavicol) was found to show similar chromatographic data. The t.l.c. and g.l.c. behaviour of a pure sample of estragole (Emanuel) was compared with that of component 40. This comparison proved that component 40 was estragole, an ether, M.W. 148, Figure 40 (124).

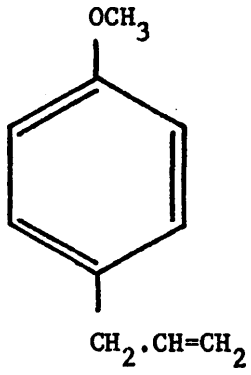


FIGURE 40: Estragole, $C_{10}H_{12}O$.

Component 41 had an R_f value of 0.43 using solvent system I indicating that it could be an aldehyde or a ketone. The relative retention times of this component to linalol suggested that it might be a terpenoid alcohol or a carbonyl compound. However, the positive response of this component to 2,4-DNPH confirmed the second suggestion (a carbonyl compound). Since pulegone is a ketone having a similar chromatographic behaviour (114-116), a pure sample (Aldrich) was compared with this component. The t.l.c. and g.l.c. investigations of both proved that component 41 was pulegone a cyclic terpene ketone, M.W. 152, Figure 41 (124).

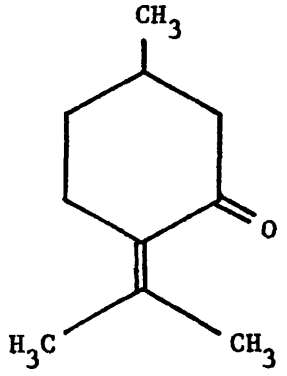


FIGURE 41: Pulegone, $C_{10}H_{16}O$.

Component 42 which was only present in the oil in trace amounts, was eluted with the n-hexane fraction indicating that it was a non-oxygenated compound. The preliminary investigations did not provide any further information about this component. However, when observing the changes which occurred in the chemical composition of the volatile oil during the growing season, further data were obtained which suggested that this component was a sesquiterpene hydrocarbon.

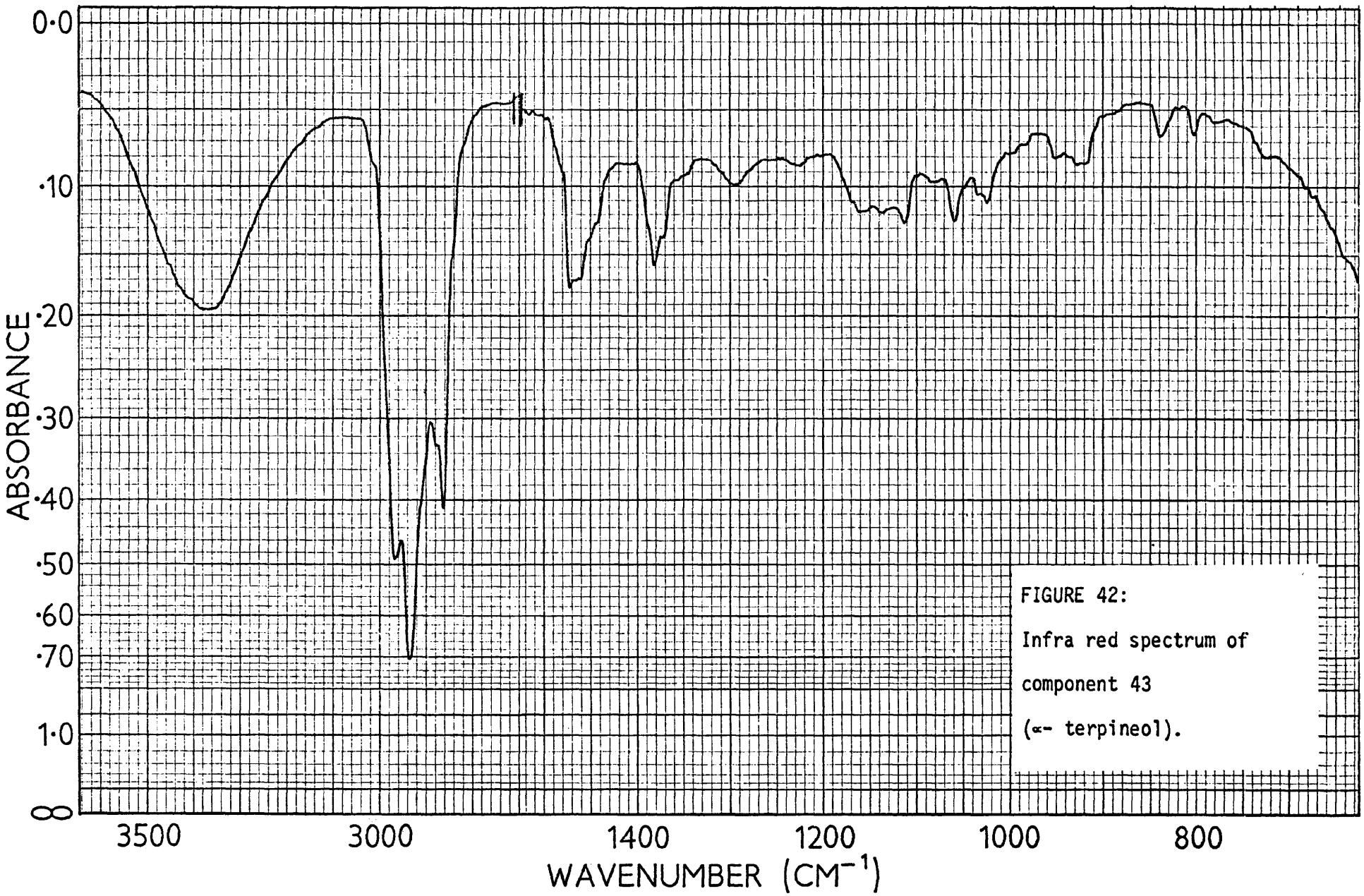


FIGURE 42:

Infra red spectrum of component 43 (α- terpineol).

Component 43:

This component was eluted as part of methanol fraction during the pre-fractionation technique indicating that it might be an oxygenated compound. Since this component and component 44 were collected as one fraction using preparative g.l.c., preparative t.l.c. was used to finally separate them. Their purity was checked before further analysis was carried out.

Component 43 had an R_f value of 0.17 using solvent system I indicating it might be an alcohol. The relative retention times of this component to linalol suggested that it could be either a terpenoid alcohol or a carbonyl compound. However, the i.r. spectrum (Figure 42) confirmed the first suggestion (a terpenoid alcohol), since the following significant bands were recorded: 3370 (a hydroxyl group), 2970, 2930, 2860, 1470, 1380, 1295, 1115, 1060, 1025, 920, 840 and 800 cm^{-1}. ∝-Terpineol is an alcoholic compound commonly found in volatile oils (124) and it was known to have a similar chromatographic behaviour to that of component 43 (114,115,128). Therefore, data obtained by examining a pure sample of a ∝-terpineol was compared with t.l.c., g.l.c. and spectroscopic data of component 43. As both sets of data were identical, it was concluded that component 43 was ∝-terpineol a cyclic terpene alcohol, M.W. 154, Figure 43(124).

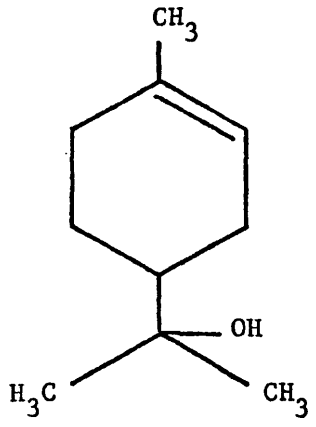


FIGURE 43: ∝-Terpineol, $C_{10}H_{18}O$.

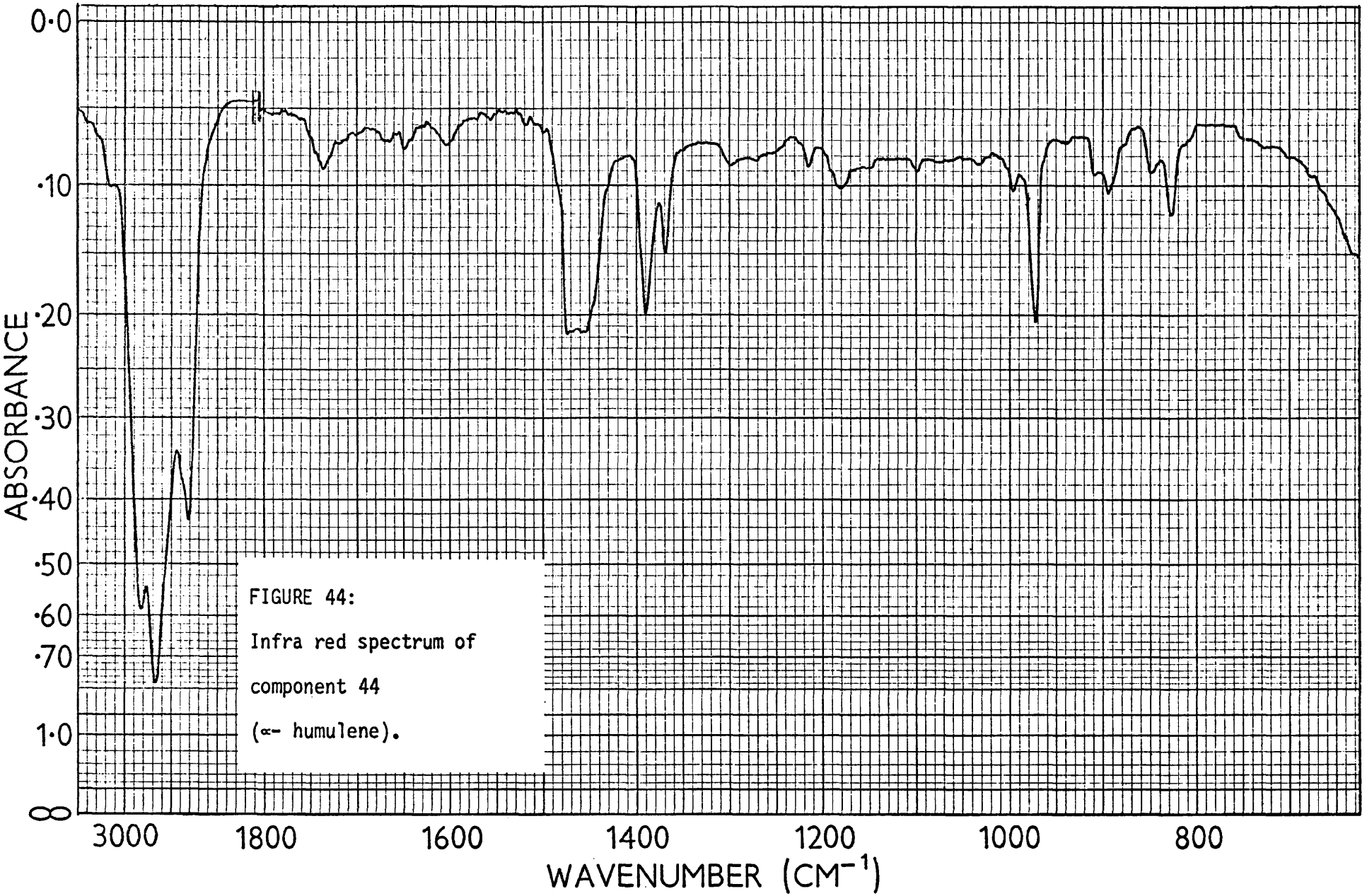


FIGURE 44:

Infra red spectrum of component 44 (∝- humulene).

Component 44:

This component, after purification using preparative t.l.c., showed the following chromatographic data. The R_f values: solvent system I: 0.79, solvent system II: 0.42. This suggested that this component might be a hydrocarbon. The relative retention times of this component to linalol suggested that it did not belong to any of the chemical groups previously examined (119,120). The i.r. spectrum (Figure 44) shows significant bands at 3000-2860, 1460, 1390, 1370, 1180, 970, 900 and 825 cm^{-1}.

The t.l.c. behaviour of this component suggested that it might be a hydrocarbon, the i.r. spectrum did not rule out this suggestion. Since the presence of β- and γ-caryophyllene has already been established, the presence of α-humulene might also be expected (124,125). A pure sample of α-humulene was compared with component 44 using t.l.c. and g.l.c. techniques. The data obtained for the two compounds were identical. The i.r. spectrum of this component was also identical to that published for α-humulene (133,137,138). Therefore, component 44 proved to be α-humulene (formerly known as α-caryophyllene) a monocyclic sesquiterpene hydrocarbon, M.W. 204, Figure 45 (125).

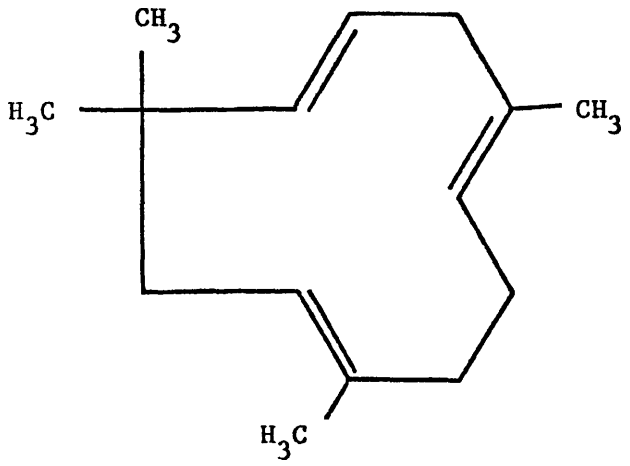


FIGURE 45: α-Humulene, $C_{15}H_{24}$.

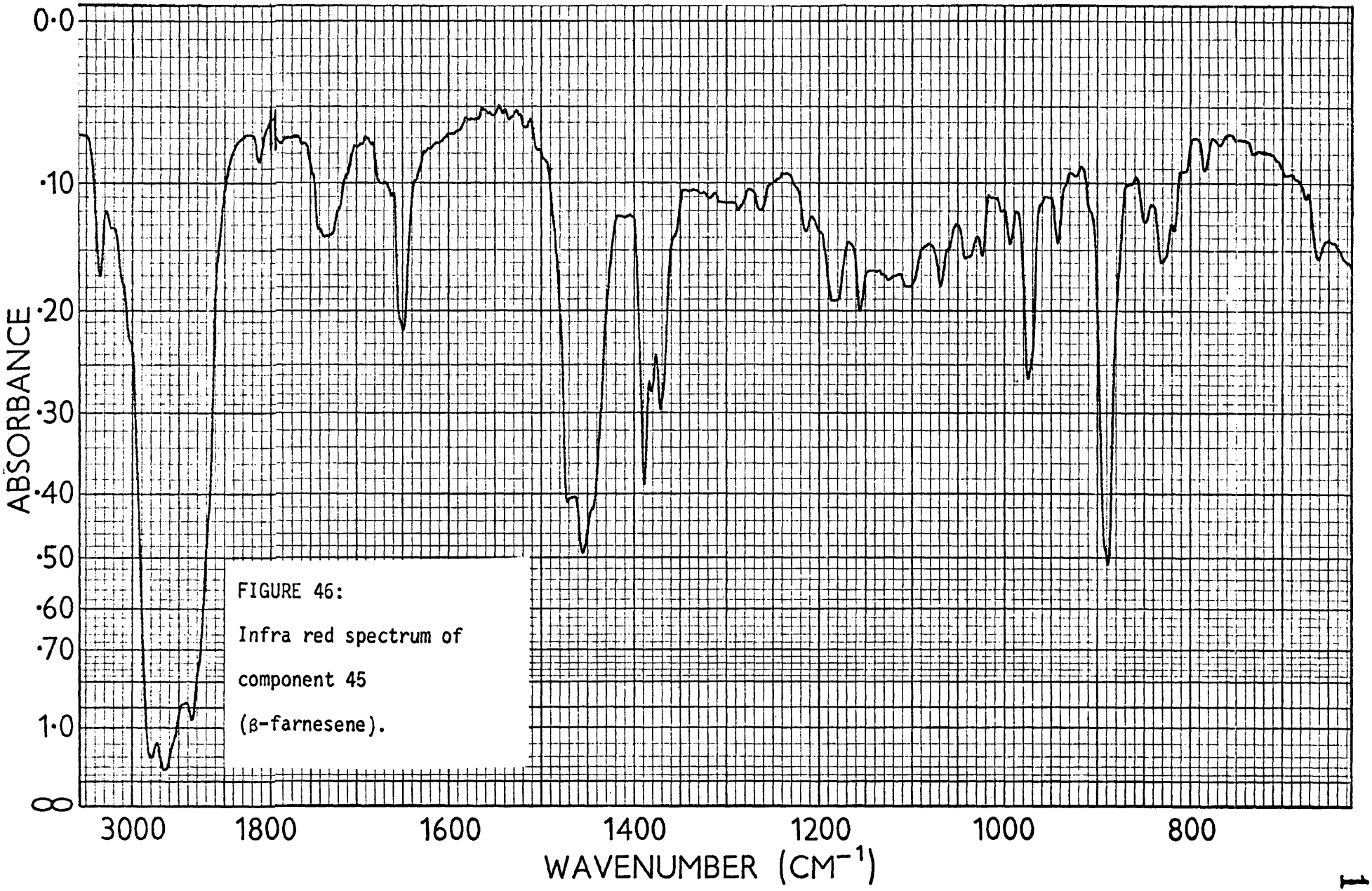


FIGURE 46:

Infra red spectrum of component 45 (β-farnesene).

Component 45:

This component had the following R_f values: solvent system I: 0.79, solvent system II 0.59 indicating it might be a hydrocarbon. The relative retention times of this component to linalol suggested that it might be a sesquiterpene hydrocarbon. The i.r. spectrum of this component (Figure 46) gave the following significant bands: 3070, 2960, 2930, 2870, 1650, 1455, 1390, 1380, 1370, 1185, 1155, 1105, 1070, 975, 889 and 830 cm^{-1}.

The t.l.c. and g.l.c. investigations suggested that component 45 was a sesquiterpene hydrocarbon. This suggestion was not disproved by the i.r. data which when compared with the i.r. spectra given by Wenninger et al. (133), was found to be identical to that of β-farnesene, having the following strong bands in decreasing order of intensity in the region of 1250-300 cm^{-1}: 889, 987, 825 and 1106 cm^{-1}. From the above evidence it was concluded that component 45 was β-farnesene an acyclic sesquiterpene hydrocarbon, M.W. 204, Figure 47 (133).

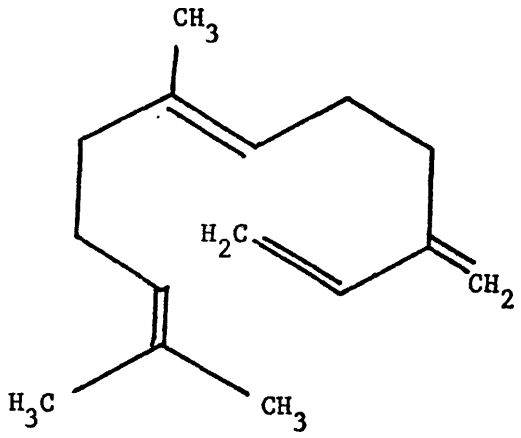


FIGURE 47: β-Farnesene, $C_{15}H_{24}$.

Component 46:

This component had an R_f value of 0.25 using solvent system I, indicating that it might be an alcohol. The relative retention times of this component to linalol suggested that it might be either a terpenoid alcohol or a terpenoid carbonyl. However, the negative response shown for this component to 2,4-DNPH made the first suggestion (an alcohol) more reliable. When the published data for similar investigations were consulted (128,129,136), borneol was found to show similar characters. Therefore, a pure sample of borneol (Koch-Light) was compared with component 46 using t.l.c. and g.l.c. analysis. The data obtained from both compounds were identical, proving that component 46 was borneol, a cyclic terpene alcohol, M.W. 154, Figure 48 (125).

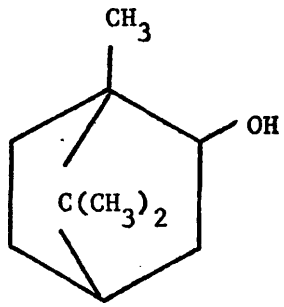


FIGURE 48: Borneol, $C_{10}H_{18}O$.

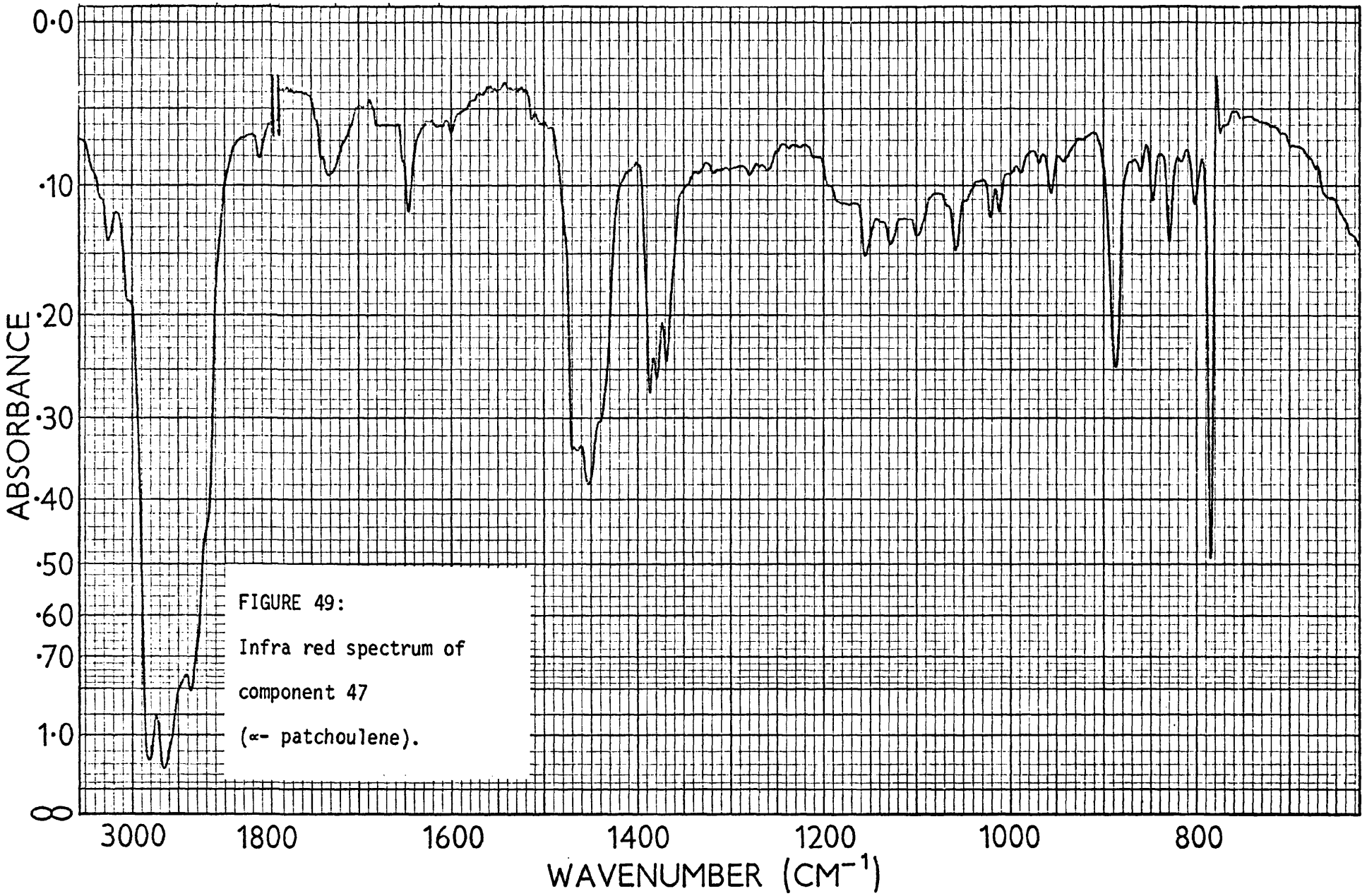


FIGURE 49:

Infra red spectrum of component 47 (∝- patchoulene).

Component 47:

This component had R_f values of 0.79 and 0.59 using solvent systems I and II respectively, indicating that it might be a hydrocarbon. The relative retention times of this component to linalol suggested that it might be a sesquiterpene hydrocarbon. The i.r. spectrum of this component (Figure 49) showed significant bands at 3050, 2960, 2930, 2870, 1645, 1450, 1385, 1380, 1370, 1155, 1055, 1020, 1010, 990, 955, 885 and 785 cm^{-1}.

Since the data obtained indicated that component 47 might be a sesquiterpene hydrocarbon, the i.r. spectrum of this component was compared with those given by Wenninger et al. (133). It was concluded that component 47 was ∝-patchoulene a tricyclic sesquiterpene hydrocarbon, M.W. 204, Figure 50 (133), since both spectra had the following strong bands in decreasing order of intensity in the region 1250-300 cm^{-1}; 787, 882, 987 and 1140 cm^{-1}.

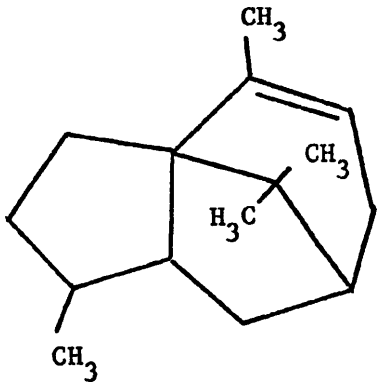


FIGURE 50: ∝-Patchoulene, $C_{15}H_{24}$.

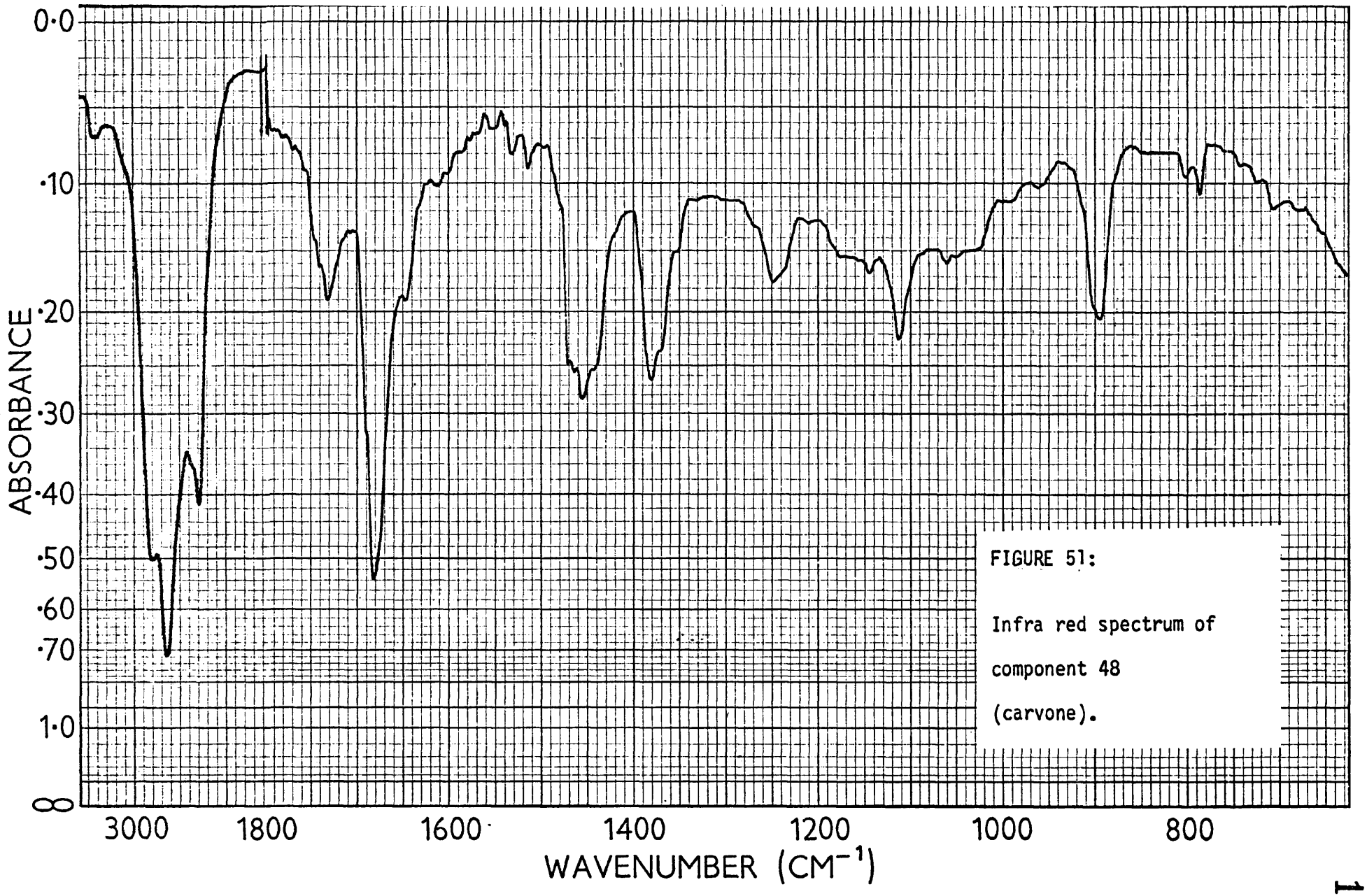


FIGURE 51:

Infra red spectrum of component 48 (carvone).

Component 48:

This component had an R_f value of 0.44 using solvent system I indicating that it might be an aldehyde or a ketone. The relative retention times of this component to linalol suggested that it might be either a terpenoid alcohol or a carbonyl compound. The i.r. spectrum (Figure 51) showed significant bands at 3080, 2915, 2860, 1680 (a carbonyl compound), 1450, 1380, 1250, 1115 and 895 cm^{-1}. These data suggested that component 48 might be a carbonyl compound. When published data for similar investigations (116,129) were consulted, carvone was found to show similar characters. A pure sample of carvone (Koch-Light) was subjected to t.l.c., g.l.c. and i.r. analyses, the data obtained being identical to those of component 48. Therefore, component 48 was carvone a cyclic terpene ketone, M.W. 150, Figure 52 (124).

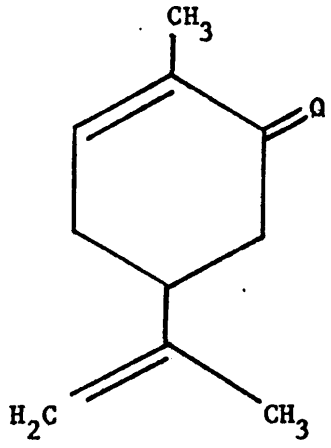


FIGURE 52: Carvone, $C_{10}H_{14}O$.

Component 49:

This component was eluted with methanol during the prefractionation technique indicating that it might be an oxygenated compound. The relative retention times of this component to linalol suggested that it might be either a terpenoid alcohol or a carbonyl compound. On comparison with published data from similar investigations (114-116), piperitone, a cyclic terpene ketone, M.W. 152, Figure 53 (124) was found to have similar characters. The g.l.c. data of an authentic pure sample of piperitone were compared with those of component 49 and found to be identical. Therefore, it was concluded that component 49 was piperitone.

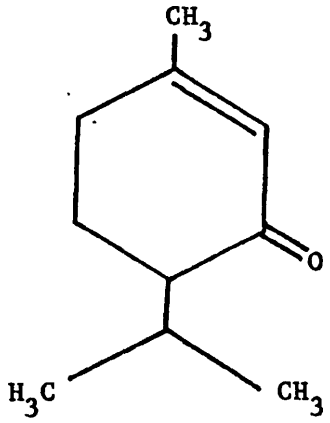


FIGURE 53: Piperitone, $C_{10}H_{16}O$.

Component 50:

This component had the following R_f values: solvent system I: 0.79, solvent system II: 0.59 indicating that it might be a hydrocarbon. The relative retention times of this component to linalol suggested that it did not belong to any of the chemical groups previously examined (119,120).

The following physical data of component 50 were recorded.

The n.m.r. spectrum (Figure 54) gave signals at 4.7, 8.1, 8.4, 9.0 and 9.1 τ. The low resolution mass spectrum (Figure 55) showed significant ions at 41-43, 53-57, 65, 67-71, 77-85, 91-97, 105-110, 115-123, 129-135, 141-143, 145-149, 156-161 (the base peak), 162,163,175-178,189,190 and 202-204 (M^+). The u.v. spectrum of this component (Figure 56) exhibited $\lambda_{max.}$ 220 nm. and since no peaks were found at $\lambda_{max.}$ 260 nm. this component was not an aromatic compound. The i.r. spectrum (Figure 57) showed significant bands at 3000-2800 (an intense band indicating a large amount of C-H stretching; i.e. this component was a highly saturated compound), 1470, 1450, 1390, 1380, 1370, 1240, 1180, 1115, 890, 875 and 835 cm^{-1}.

The t.l.c. data suggested that component 50 might be a hydrocarbon. The mass spectrum had a molecular ion of 204 indicating that it could be a sesquiterpene hydrocarbon. The broad multiplet at 4.7 τ, although it was not integrated, could be due to an olefinic proton ($\overset{H}{>}C = C<$).

These data were compared with those published for the sesquiterpene hydrocarbons and were found to agree with the following characters, given for δ-cadinene, a bicyclic sesquiterpene hydrocarbon, M.W. 204, Figure 58 (139).

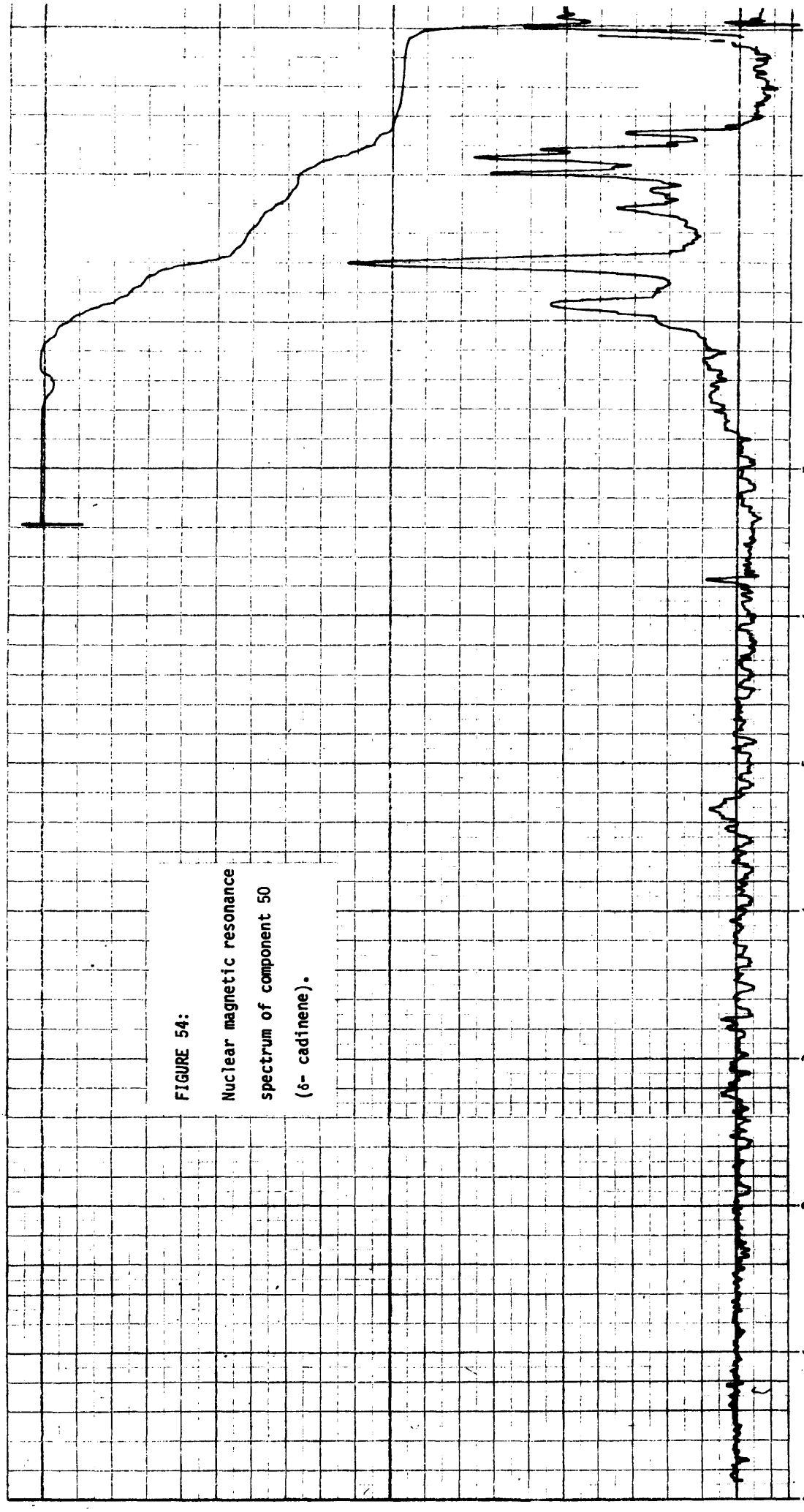


FIGURE 54:

Nuclear magnetic resonance spectrum of component 50 (δ- cadinene).

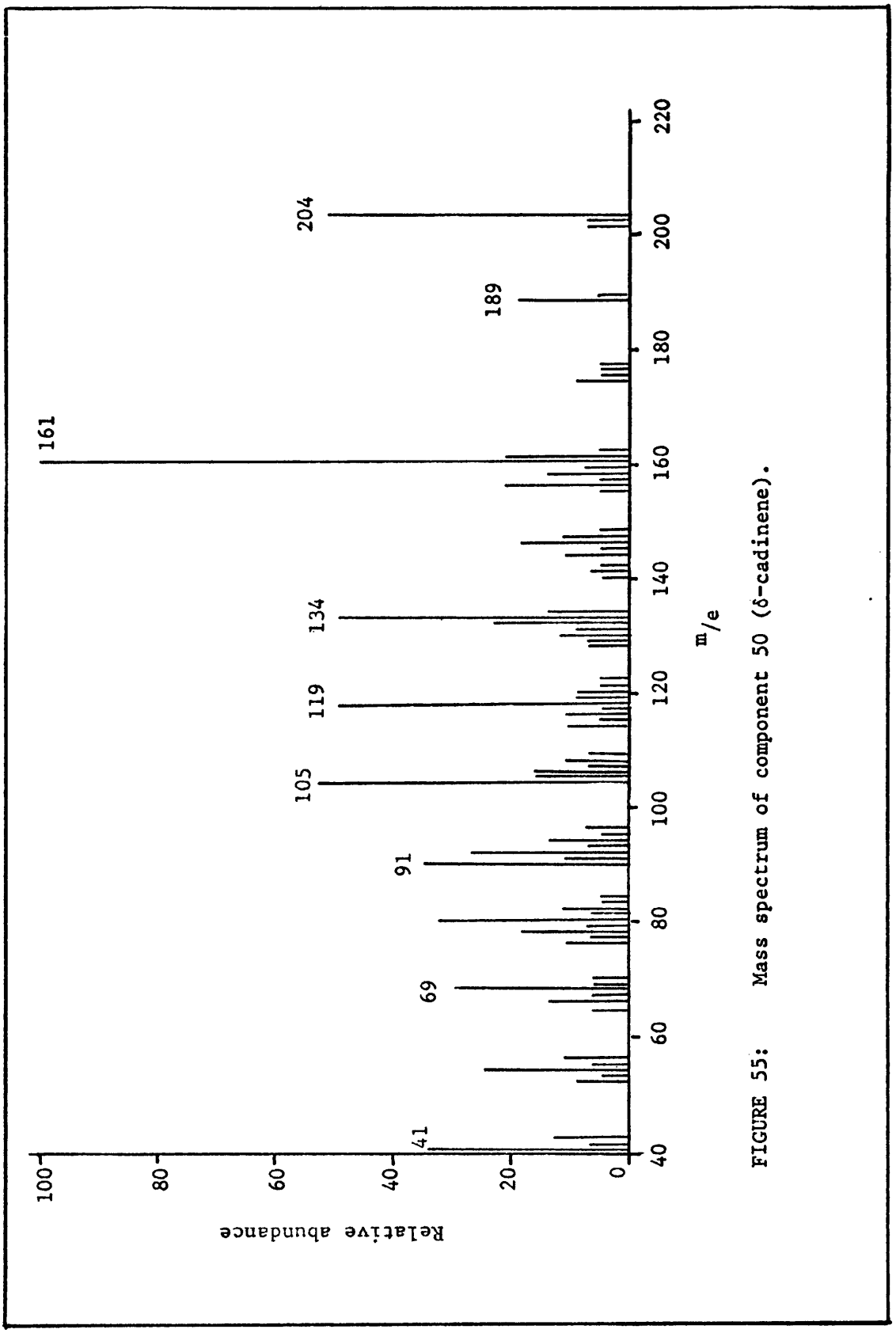


FIGURE 55: Mass spectrum of component 50 (δ-cadinene).

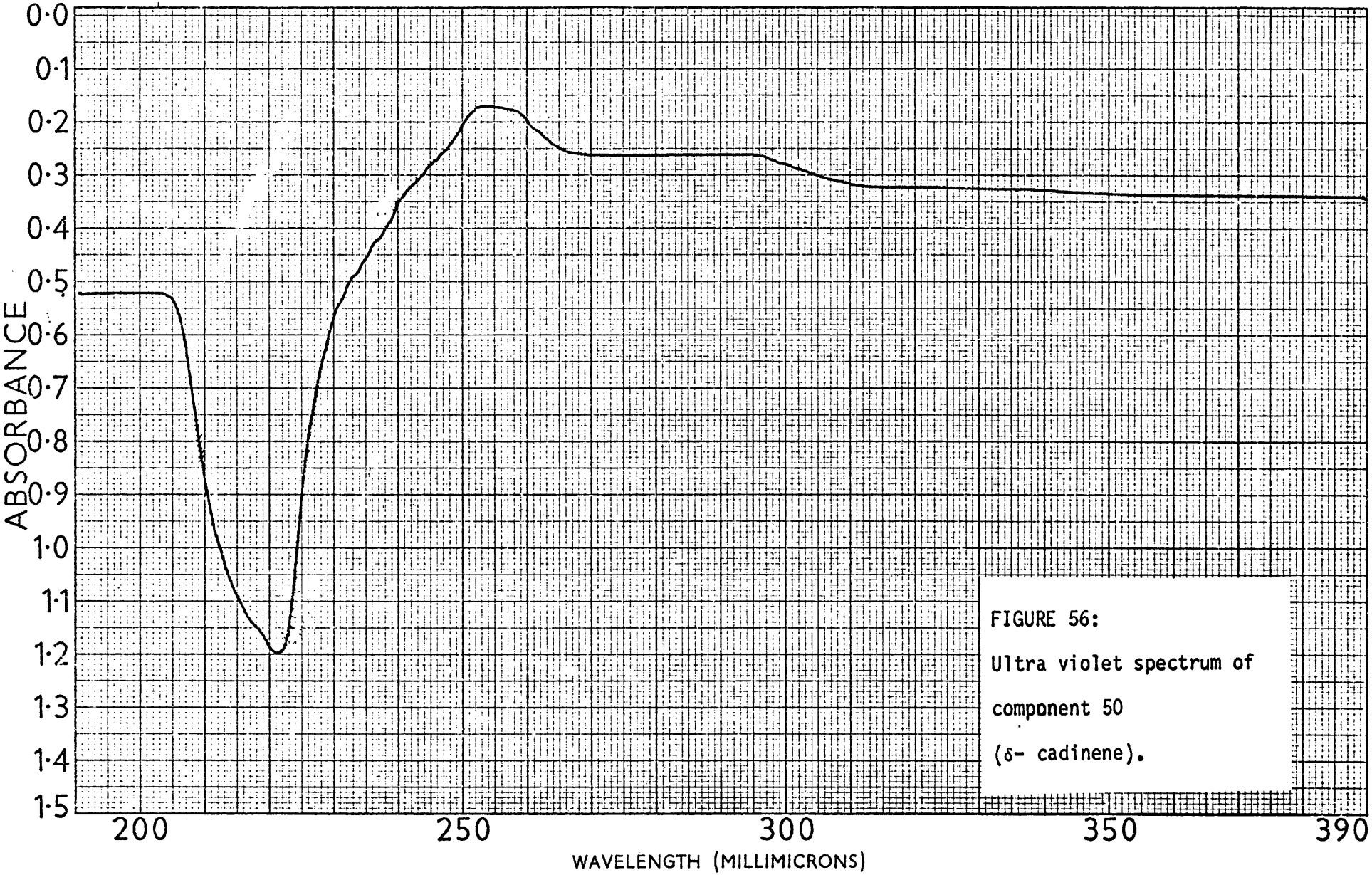


FIGURE 56:
Ultra violet spectrum of component 50 (δ- cadinene).

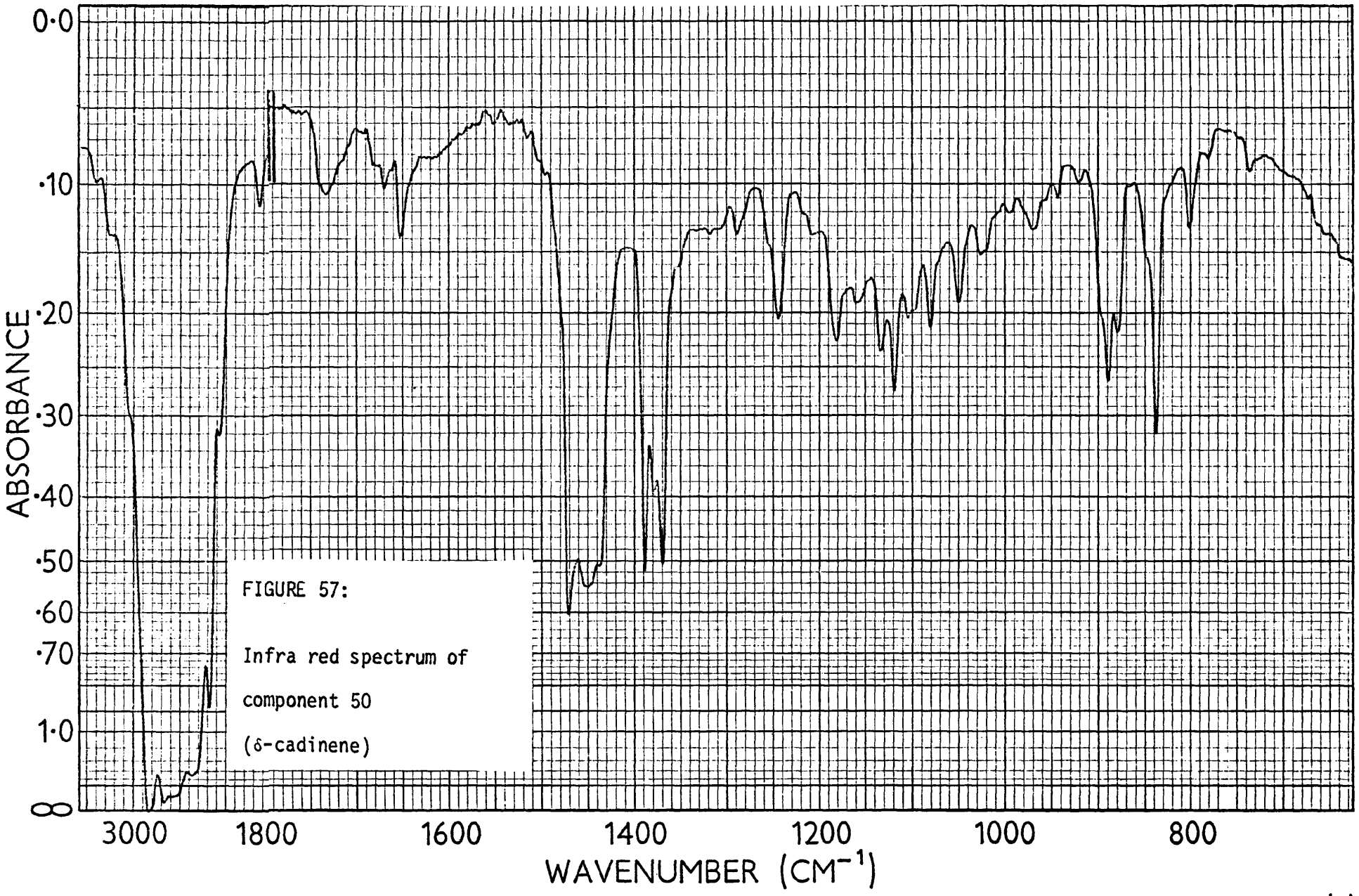


FIGURE 57:

Infra red spectrum of component 50 (δ-cadinene)

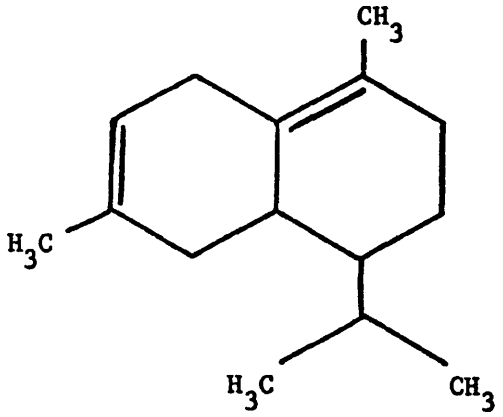


FIGURE 58: δ-Cadinene, $C_{15}H_{24}$.

The following signals were found in the n.m.r. spectrum of δ-cadinene (140):9.21 τ (3H, doublet), 9.05 τ (3H, doublet), 8.39 τ (6H, broad singlet), 8.08 τ (4H, multiplet), 4.67 τ (1H, broad multiplet). The mass spectrum of δ-cadinene (131,139) was identical with that of component 50. The fragmentation sequence of δ-cadinene was interpreted as shown in Figure 59 (139). The i.r. spectrum of δ-cadinene (133,141) agreed with that of component 50. It was stated (133) that δ-cadinene should have the following strong bands in the 1250-300 cm^{-1} region arranged in a decreasing order of intensity 832, 1239, 1113, 872 and 1178 cm^{-1}.

From the above evidence component 50 proved to be δ-cadinene.

Component 51:

This component had an R_f value of 0.23 using solvent system I indicating that it might be an alcohol. The relative retention times of this component to linalol suggested that it might be either a terpenoid alcohol or a carbonyl compound. However, a negative response was shown by this component to 2,4-DNPH, which made the first suggestion (an alcohol) more reliable. On consulting data from similar investigations (136) nerol was

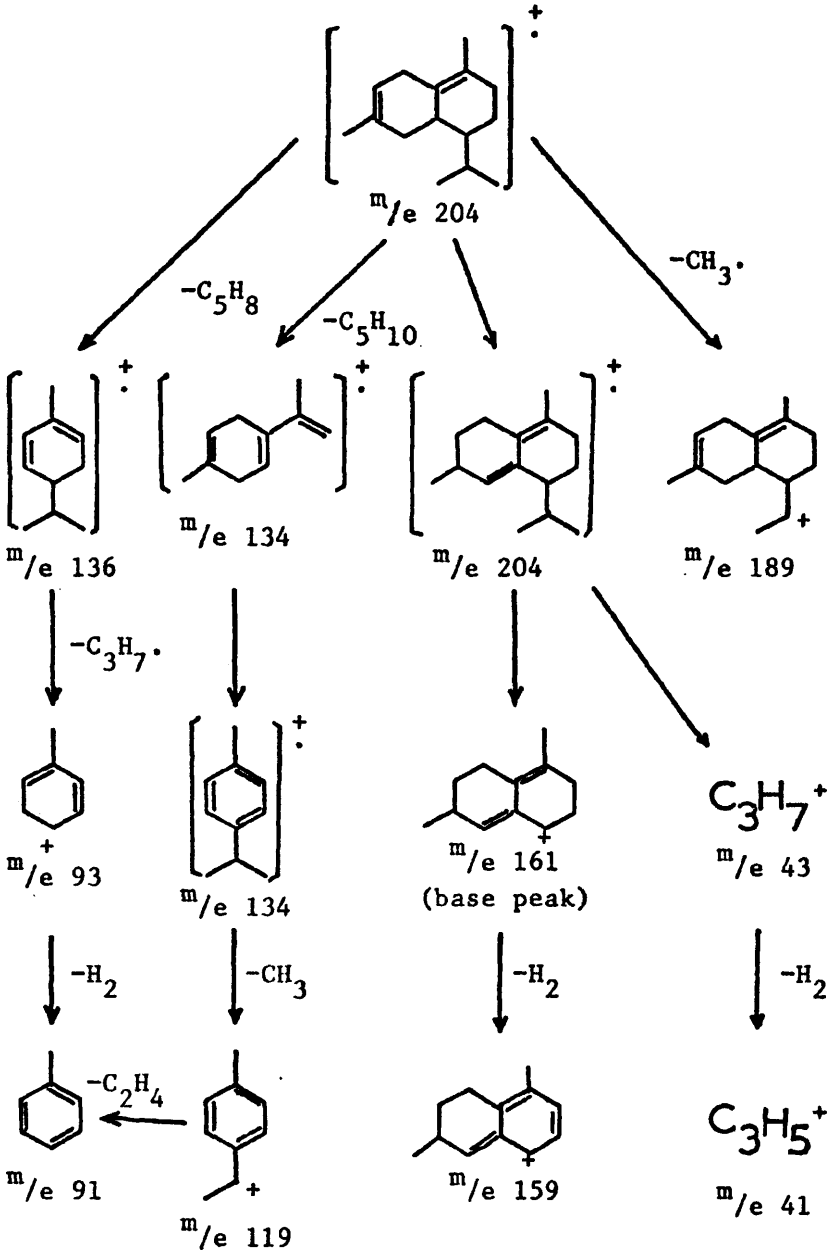


FIGURE 59: Fragmentation sequence of δ-cadinene (139).

found to show the same chromatographic characters. A pure authentic sample of nerol was subjected to t.l.c. and g.l.c. analyses, the data obtained being identical to that of component 51. Therefore, it was concluded that component 51 was nerol, an aliphatic alcohol, M.W. 154, Figure 60 (125).

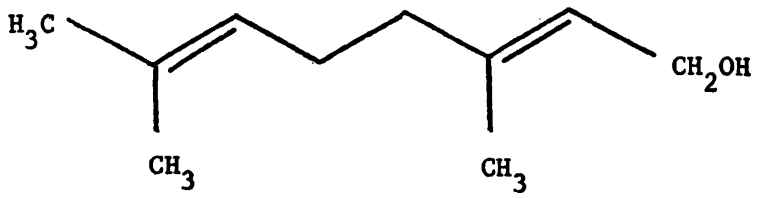


FIGURE 60: Nerol, $C_{10}H_{18}O$.

Component 52:

This component showed the following data. The R_f values: solvent system I: 0.79, solvent system II: 0.59 indicating that it might be a hydrocarbon. The relative retention times of this component to linalol suggested that it might be a sesquiterpene hydrocarbon. The i.r. spectrum (Figure 61) showed significant bands at 2960, 2930, 2870, 1460, 1390, 1380, 1370, 1180, 1140, 1065, 945, 885 and 810 cm^{-1}.

Since these data suggested that component 52 could be a sesquiterpene hydrocarbon, the strongest band of its i.r. spectrum in the 1250-300 cm^{-1} region (810 cm^{-1}) was compared with data given by Wenninger et al. (133). This comparison suggested that component 52 was calamenene, a bicyclic sesquiterpene hydrocarbon, M.W. 202, Figure 62 (133).

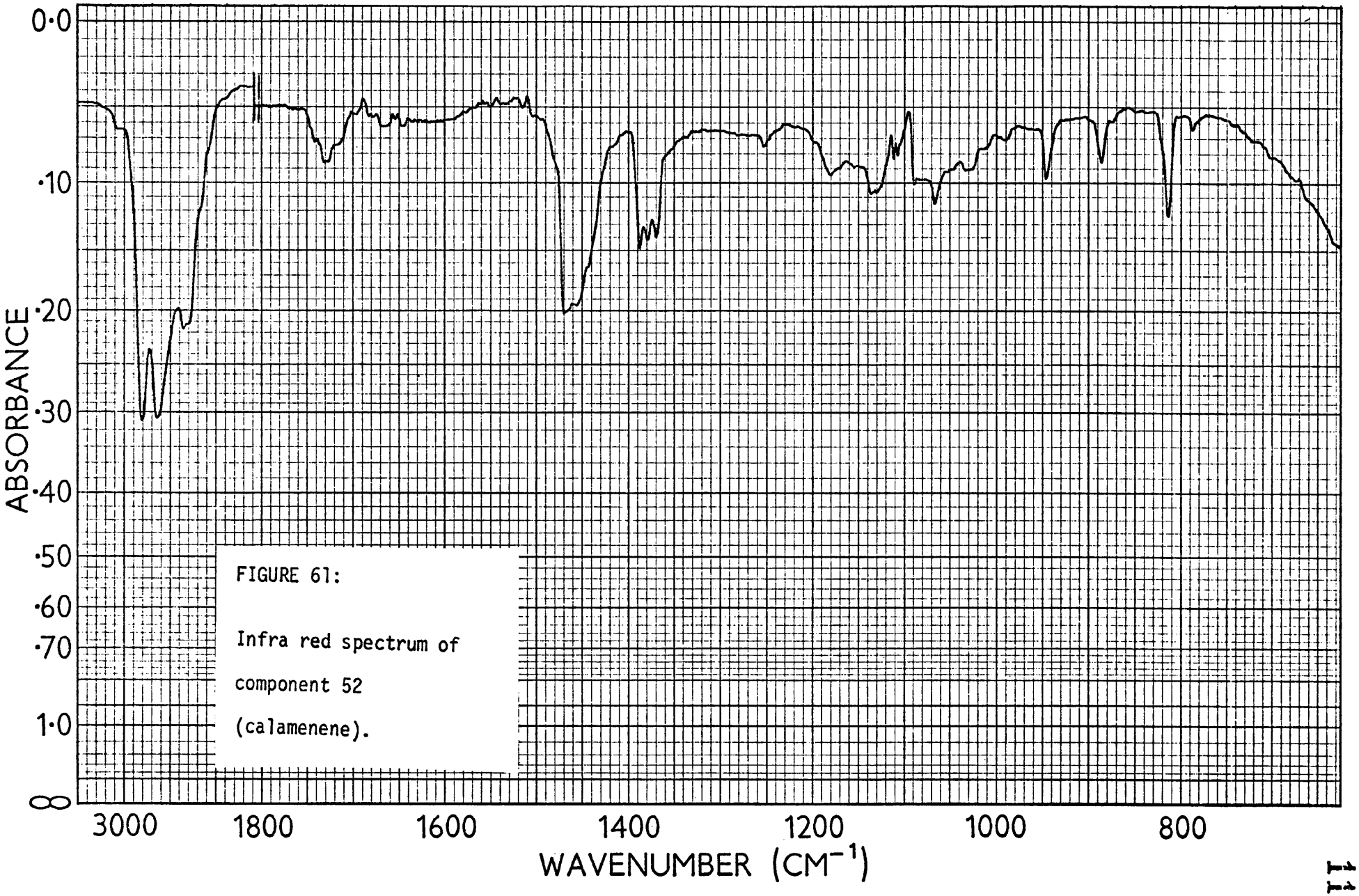


FIGURE 61:

Infra red spectrum of component 52 (calamenene).

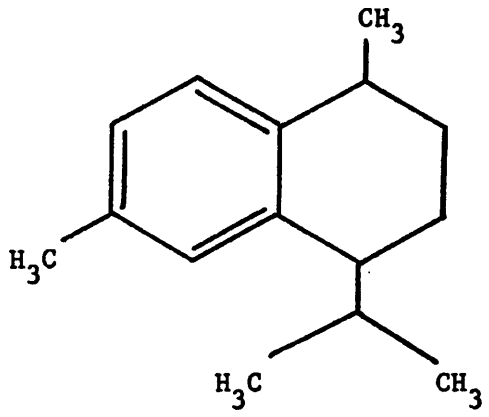


FIGURE 62: Calamenene, $C_{15}H_{22}$.

Component 53:

This component had the following R_f values: solvent system I: 0.71, solvent system II: 0.10. The relative retention times of this component to linalol (Figure 23) not only suggested that it might be a substituted aromatic compound but it was also identical to anethole which had been used as a standard compound in earlier investigations, Table 18 (Appendix).

The following physical data for component 53 were recorded. The low resolution mass spectrum (Figure 63) showed significant ions at 41 (the base peak) 42, 43, 49-51, 53, 55-59, 67-70, 77-81, 91, 93-97, 115-117, 119-124, 131, 133-137, 147 and 148 (M^+). The u.v. spectrum (Figure 64) exhibited λmax. 215 and 260 and λinfl. 280-315 nm. The i.r. spectrum (Figure 65) showed significant peaks at 3005-2800, 1610, 1575, 1510, 1450, 1280, 1245, 1170, 1035, 960, 835 and 780 cm^{-1}.

The suggestion that this component was an aromatic compound was supported by the peak in the u.v. spectrum at λmax. 260 nm. and the four characteristic absorption peaks at 1610, 1575, 1510 and 1450 cm^{-1} in the i.r. spectrum. The mass spectrum showed a molecular ion of 148 which would be expected if component 53 was anethole.

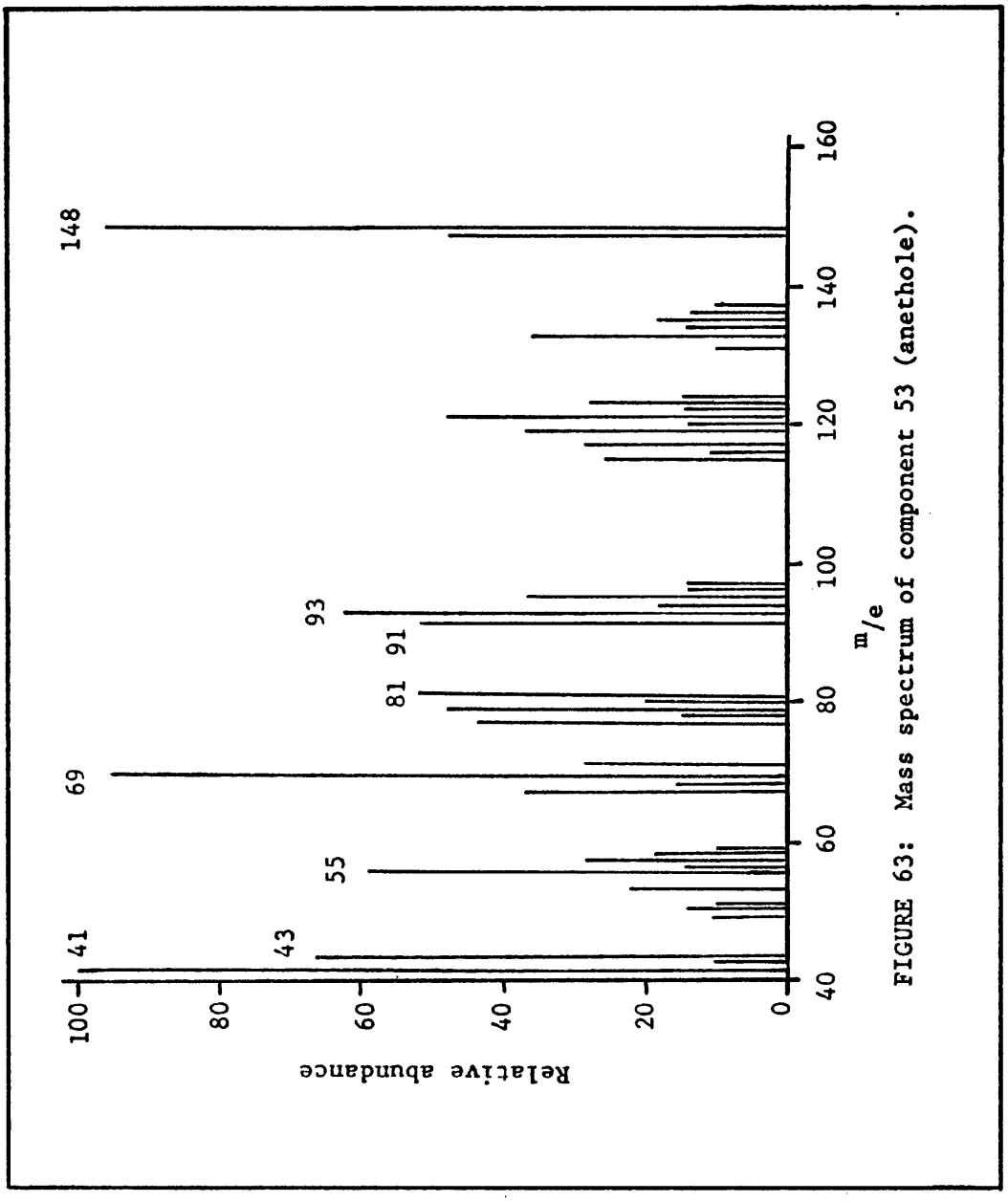


FIGURE 63: Mass spectrum of component 53 (anethole).

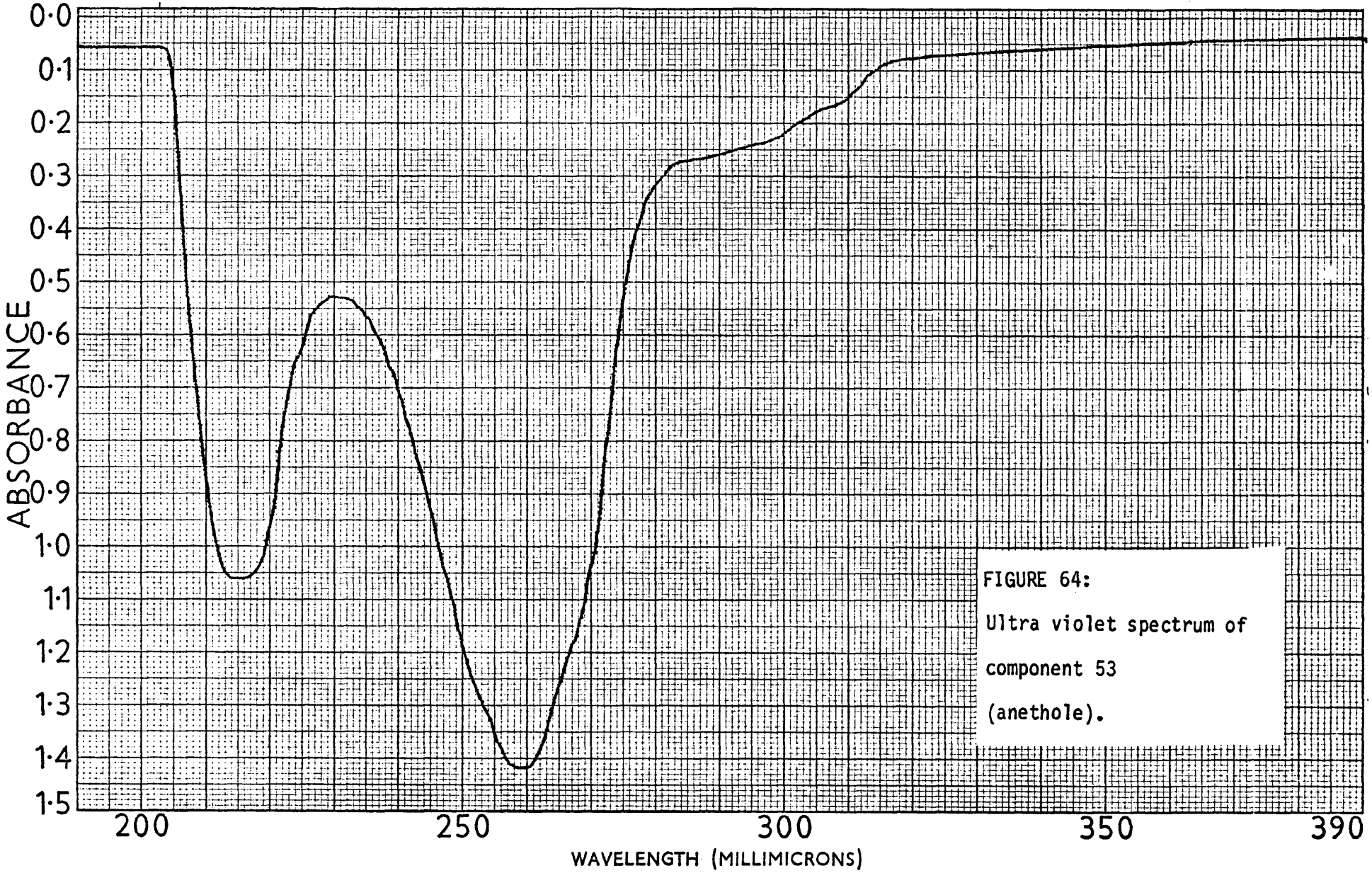


FIGURE 64:

Ultra violet spectrum of component 53 (anethole).

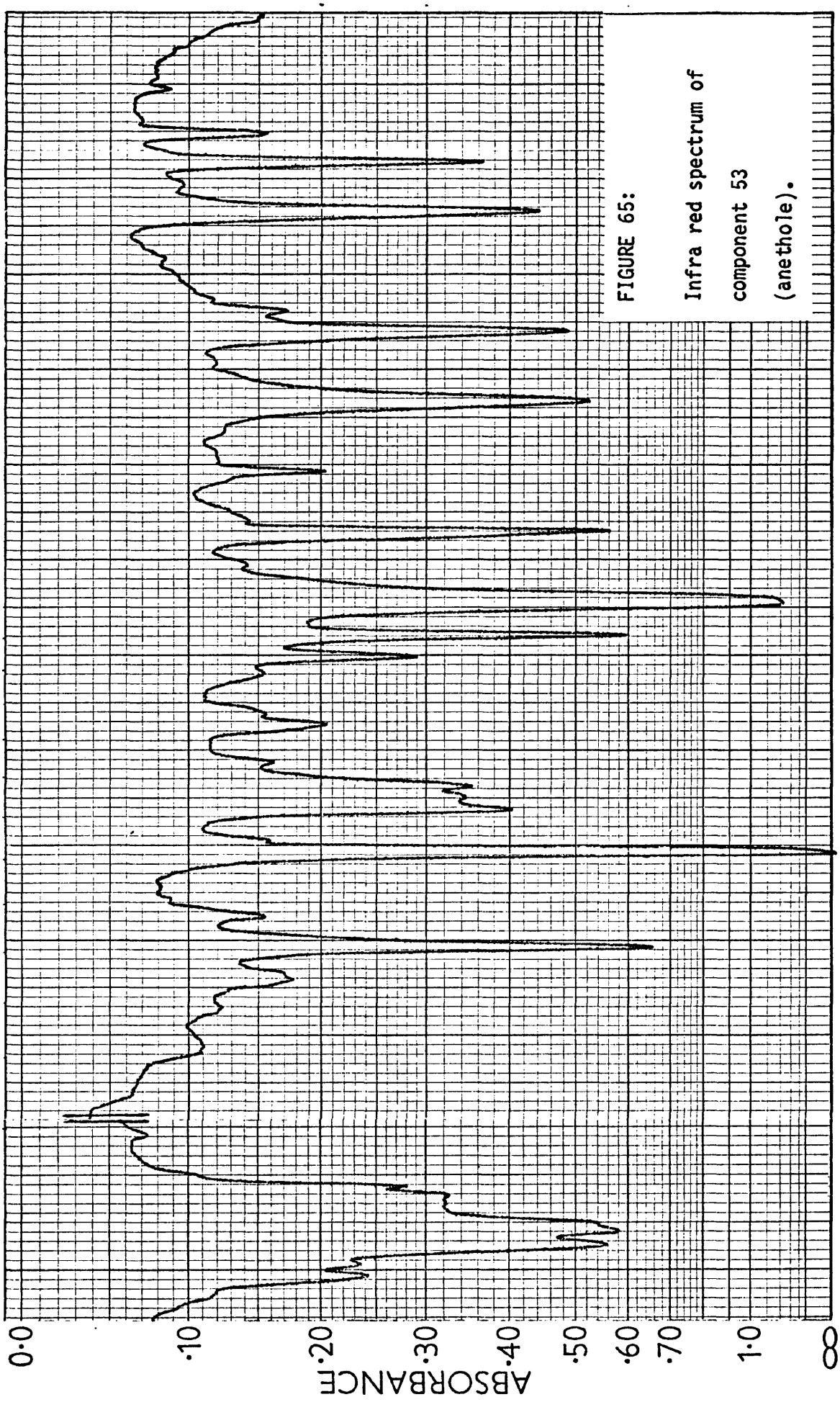


FIGURE 65:

Infra red spectrum of component 53 (anethole).

Literature data for anethole indicated that the u.v. spectrum showed $\lambda_{max.}$ 258, $\lambda_{min.}$ 228 and $\lambda_{infl.}$ 290 nm. (142) and the i.r. spectrum had a strong absorption at 961 cm^{-1} due to the propenyl group ($-CH=CH-CH_3$), while the three peaks at 1255, 1173 and 1040 were due to the methoxyl group ($-OCH_3$) (143).

The physical data of component 53 not only agreed with the published data but were identical also in all respects when compared with the t.l.c., g.l.c. and spectroscopic data of pure anethole (BDH).

From the above evidence component 53 was found to be anethole, an ether, M.W. 148, Figure 66 (124).

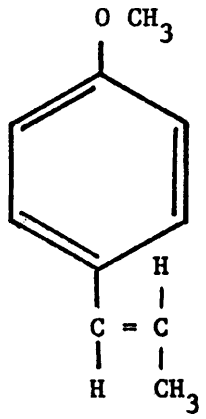


FIGURE 66: Anethole, $C_{10}H_{12}O$.

Components 54-61:

This group of components showed a poor resolution when subjected to preparative g.l.c. separation, therefore they were treated as two groups, namely oxygenated and non-oxygenated components. The latter group contained components 54, 57 and 59, but as they were only found in the oil in trace quantities, no further analysis was possible. Components 55, 56

58, 60 and 61 were oxygenated compounds since they were eluted with methanol during the prefractionation technique.

Component 55 had an R_f value of 0.17 using solvent system I, indicating that it might be an alcohol. The relative retention times of this component to linalol suggested that it might be a terpenoid alcohol. Geraniol is an alcoholic compound commonly found in volatile oils (124) and it was known to have similar chromatographic behaviour to that of component 55 (126, 136). Therefore, data obtained by examining a pure sample of geraniol (Koch-Light) were compared with the t.l.c. and g.l.c. data of component 55. As both sets of data were identical, it was concluded that component 55 was geraniol, an aliphatic alcohol, M.W. 154, Figure 67 (125).

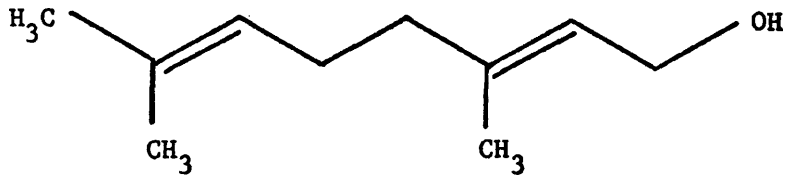


FIGURE 67: Geraniol, $C_{10}H_{18}O$.

The relative retention times of component 56 to linalol suggested that it might be a substituted aromatic. When the g.l.c. data were compared with those of safrole (H & W), used in earlier investigations, Table 18 (Appendix), they were found to be identical. It was concluded that component 56 was safrole an ether, M.W. 162, Figure 68 (125).

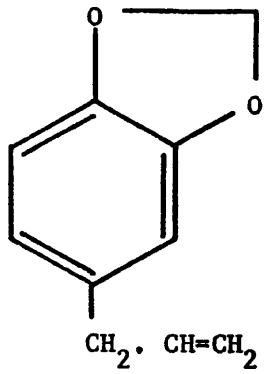


FIGURE 68: Safrole, $C_{10}H_{10}O_2$.

Component 58 had an R_f value of 0.25 using solvent system I. On consulting chromatographic data from similar investigations (114) piperitenone showed similar chromatographic characters to those of component 58. Therefore, an authentic pure sample of piperitenone was subjected to t.l.c. and g.l.c. analysis and the data obtained were compared with those of component 58. They were found to be identical, therefore, component 58 was concluded to be piperitenone, a cyclic terpene ketone, M.W. 150, Figure 69 (124).

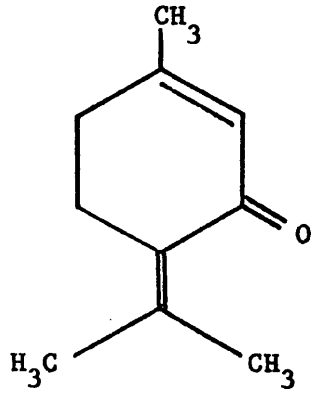


FIGURE 69: Piperitenone, $C_{10}H_{14}O$.

Component 60 had an R_f value of 0.46, using solvent system I. When piperitenone is present in a volatile oil, piperitenone oxide is often also present (144). Therefore, the t.l.c. and g.l.c. behaviour of a pure sample of piperitenone oxide was compared with the behaviour of component 60 and found to be identical. It was concluded that component 60 was piperitenone oxide.

Component 61 had an R_f value of 0.46 using solvent system I. Treibs (145) suggested that when caryophyllene is present in a volatile oil, the presence of caryophyllene oxide might also be expected. Since caryophyllene was found in Horehound oil, a pure sample of caryophyllene oxide was subjected to t.l.c. and g.l.c. analysis. It was found that the chromatographic data given by component 61 were identical to those of caryophyllene oxide. Therefore, it was concluded that component 61 was caryophyllene oxide (epoxy-dihydrocaryophyllene), an oxide, M.W. 220, $C_{15}H_{24}O$ (124).

Components 62/63:

These components were eluted with methanol in the prefractionation technique, but they were not separated using preparative g.l.c., being collected as one fraction. This fraction was composed of a yellow liquid, component 62, and fine white crystalline needles of low melting point, component 63. As they showed similar R_f values using solvent systems I, II and even when the more polar methanol was used as the mobile phase, it was difficult to separate them using preparative t.l.c. However, some needle crystals of component 63 were picked carefully from this fraction and although contaminated with component 62, they were subjected to i.r. spectroscopic analysis. The i.r. spectrum showed a pronounced band at 3360 cm^{-1} indicating the presence of a hydroxyl group and a less pronounced band at 1720 cm^{-1} indicating the presence of a carbonyl group. It is

suggested from these data that component 63 contains a hydroxyl group whilst the presence of the carbonyl group is due to contamination by component 62. In the absence of any further data it was concluded that component 62 might be a carbonyl compound while component 63 might be an alcoholic compound.

Components 64-66:

Component 64 was eluted by n-hexane when the oil was prefractionated, indicating that it was a non-oxygenated compound, while components 65 and 66 were eluted by methanol, hence they might be oxygenated compounds. Since all three were found in the oil as traces it was not possible to determine their identity.

Components 67-69:

Components 67 and 69 were considered to be oxygenated compounds, since they were eluted with methanol when the oil was prefractionated, while component 68, which was only present in the oil in trace amount , was a non-oxygenated compound. On using preparative g.l.c., components 67 and 69 were collected as one fraction. As they had similar R_f values using solvent system I and II, it was not possible to separate them by preparative t.l.c. Component 67 represented the main portion of this fraction while component 69 occurred only in trace amount . On consulting data from similar investigations (115) eugenol showed a similar chromatographic behaviour to that of component 67. The i.r. spectrum of this fraction was similar to that obtained for pure eugenol (BDH) apart from an extra peak at 1720 cm^{-1} (a carbonyl group) which might be due to contamination by component 69. The following significant bands were recorded: 3460, 2850-3090, 1720, 1640, 1615, 1515, 1470, 1455, 1435, 1370, 1270, 1235, 1210, 1150, 1120, 1035, 995, 910, 850, 815 and 745 cm-1. Further t.l.c. and

g.l.c. analyses again indicated that component 67 was eugenol. This conclusion was supported further by placing a drop of this fraction on a slide, adding a drop of 3% aqueous solution of sodium hydroxide saturated with sodium bromide and covering with a cover glass. Needle and pear-like crystals arranged in rosette-like bunches were formed. These are characteristic of sodium eugenolate (146).

From the above evidence, it was concluded that component 69 was a carbonyl compound and that component 67 was eugenol, a phenol, M.W. 164, Figure 70 (124).

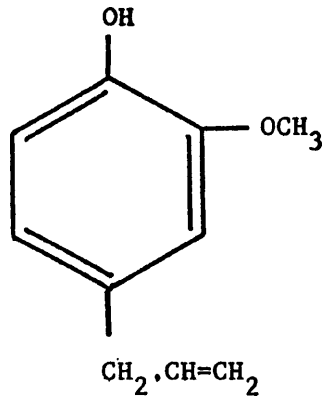


FIGURE 70: Eugenol, $C_{10}H_{12}O_2$.

Component 70:

This component was part of the methanol fraction when the oil was prefractionated, indicating that it might be an oxygenated compound. The i.r. spectrum showed a significant peak at 1735 cm^{-1} indicating that it contained a carbonyl group. As only a small amount was collected, it was not possible to identify it further.

Component 71:

This component was eluted with methanol indicating that it might be an oxygenated compound. It had an R_f value of 0.20 using solvent system I suggesting that it could be an alcohol. Using the SE30 column (160°C) this component was detected after 28 minutes as a broad unresolved peak indicating that the boiling point of this component might be in the region of 160°C. When the column temperature was increased to 190°C a sharp peak of retention time 13.2 minutes was given. On consulting data from similar investigations (126) farnesol showed a similar behaviour to that of component 71. Pure farnesol was subjected to t.l.c. and g.l.c. analysis and was found to have identical characters to those of component 71. Therefore, it was concluded that component 71 was farnesol, an acyclic sesquiterpene alcohol, M.W. 222, Figure 71 (125).

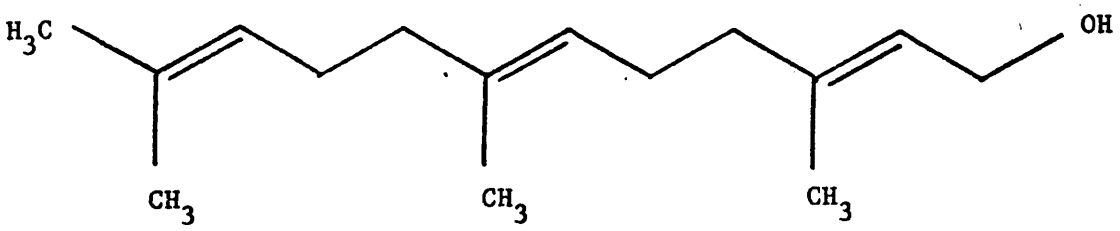


FIGURE 71: Farnesol $C_{15}H_{26}O$.

Component 72:

This component was eluted with the non-oxygenated part of the oil during the prefractionation technique. Since it was only present in the oil in trace amount , no further analysis was possible.

These investigations proved that the oil is a complex mixture of at least seventy-two components. It was possible to identify forty of these components, their identities being listed below.

5. α-pinene, 8. camphene, 9. β-pinene, 11. limonene, 12. cineole, 13. β-phellandrene, 14. p-cymene, 15. terpinolene, 18. allo-ocimene, 19. 3-octanol, 23. 1-octen-3-ol, 26. citronellal, 29. menthone, 30. linalol, 31. γ-caryophyllene, 33. camphor, 35. carvomenthone, 36. bornyl acetate, 38. β-caryophyllene, 39. menthol, 40. estragole, 41. pulegone, 43. α-terpineol, 44. α-humulene, 45. β-farnesene, 46. borneol, 47. α-patchoulene, 48. carvone, 49. piperitone, 50. δ-cadinene, 51. nerol, 52. calamenene, 53. anethole, 55. geraniol, 56. safrole, 58. piperitenone, 60. piperitenone oxide, 61. caryophyllene oxide, 67. eugenol, 71. farnesol.

The peaks produced by these components on the chromatograms obtained when using the DEGS and SE30 columns (Figures 16 and 17) are identified in Tables 6 and 7.

TABLE 6

Name of the components of *Marrubium vulgare* L. oil found in the peaks of the chromatogram obtained using the DEGS column

Peak number	Name of components
3	α-pinene
4	β-pinene, camphene
5	limonene
6	cineole, β-phellandrene
7	p-cymene, terpinolene
8	3-octanol
9	1-octen-3-ol
10	γ-caryophyllene
11	citronellal, menthone
12	allo-ocimene, linalol
13	camphor, β-caryophyllene
14	carvomenthone, bornyl acetate, menthol
15	α-humulene, β-farnesene, borneol, α-patchoulene
16	estragole, pulegone, α-terpineol, δ-cadinene, calamenene
18	carvone, piperitone, nerol
19	anethole, geraniol
21	safrole
22	piperitenone
26	piperitenone oxide, caryophyllene oxide, farnesol
30	eugenol

TABLE 7

Name of the components of *Marrubium vulgare* L. oil found in the peaks of the chromatogram obtained using the SE30 column

Peak number	Name of components
6	α-pinene
7	camphene
8	β-pinene, 3-octanol, 1-octen-3-ol
9	limonene, cineol, β-phellandrene, p-cymene
10	terpinolene, linalol, nerol, geraniol
11	citronellal, menthone, camphor
12	estragole, menthol
13	carvomenthone, borneol
14	allo-ocimene, α-terpineol
15	pulegone, carvone, piperitone
16	bornyl acetate, anethole, safrole
17	piperitenone oxide
18	piperitenone
19	γ-caryophyllene, eugenol
20	β-caryophyllene, caryophyllene oxide, farnesol
21	α-humulene
22	β-farnesene
23	α-patchoulene
24	δ-cadinene
25	calamenene

11. QUANTITATIVE ANALYSIS OF THE VOLATILE OIL

The quantitative analysis of the volatile oil was carried out using a recorder fitted with a disc integrator, model DM246 (Disc Instruments Ltd.) in conjunction with the Perkin Elmer F11 gas chromatograph. Reference materials were available for only seven of the components which gave separate peaks on g.l.c. analysis of the volatile oil. These components were α-pinene, 3-octanol, 1-octen-3-ol, linalol, β-caryophyllene, anethole and eugenol. Using a Hamilton repeat dispenser syringe, five solutions of differing concentration were prepared for each reference material; the solvent used was absolute ethanol. The range of concentrations selected for a given material was such that the values obtained for peak areas gave a spread around the peak area value obtained for the same material on analysis of the oil. Having selected the appropriate analytical column, a microlitre Hamilton syringe fitted with a "Reprojector" adjusted to deliver 0.4 µl., was used to inject each solution and the volatile oil. Each determination was carried out in duplicate. Calibration curves were prepared by plotting the number of traverses under a given peak against solution concentration. Figure 72 shows a typical calibration curve obtained for anethole. The results of the quantitative determinations of the materials listed above are given in Table 8, see also Table 22 (Appendix).

It was not possible to prepare calibration curves for the other compounds found in the oil. In an attempt to obtain an estimate of the amounts of these materials, their peak areas expressed as a percentage of the total peak area are given in Table 9. These figures have not been adjusted to take into account the "effective carbon response" of each component (147), therefore these values are not absolute.

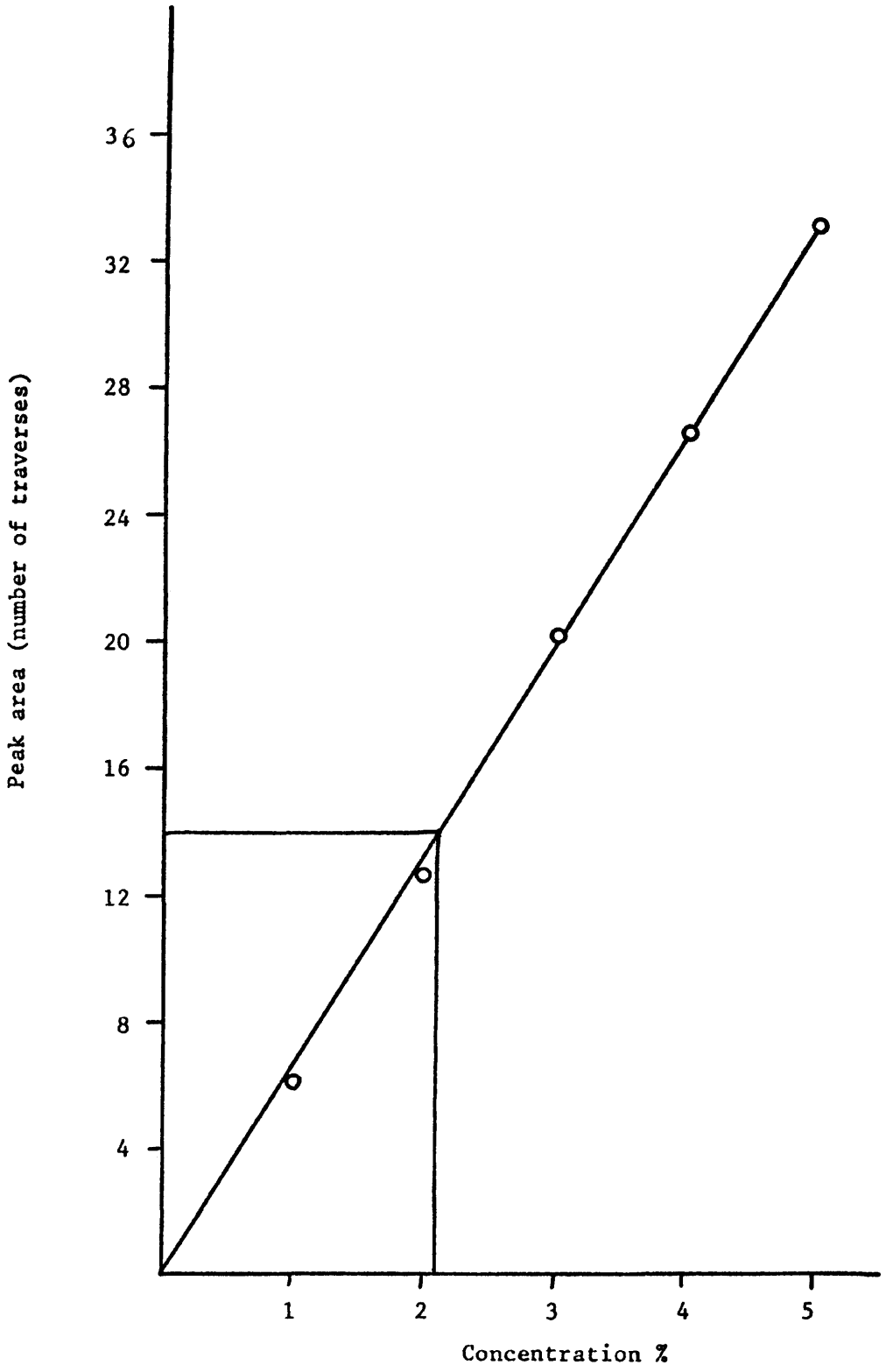


FIGURE 72: Calibration curve used to calculate percentage of anethole in *Marrubium vulgare* L. oil.

TABLE 8

Results of the quantititative analysis of *Marrubium vulgare* L. oil, calculated by means of calibration curves

Component	% in the volatile oil
∝-Pinene	0.57
3-Octanol	2.15
1-Octen-3-ol	2.60
Linalol	3.30
β-Caryophyllene	6.60
Anethole	2.10
Eugenol	6.50

TABLE 9

The peak area of each component of *Marrubium vulgare* L. oil, expressed as a percentage of the total peak area

	Components	%		Components	%
1-4	Traces	0.47	40	Estragole	
5	α-Pinene	0.79	41	Pulegone	2.77
6,7	Traces		42	Traces	
8	Camphene	0.34	43	α-Terpineol	1.78
9	β-Pinene		44	α-Humulene	4.55
10	Traces		45	β-Farnesene	2.57
11	Limonene	0.28	46	Borneol	
12,13	Cineole, β-Phellandrene	0.77	47	α-Patchoulene	2.57
14,15	ρ-Cymene, Terpinolene	0.75	48	Carvone	4.06
16,17	Traces		49	Piperitone	
18	Allo-ocimene	0.14	50	δ-Cadinene	6.73
19	3-Octanol	2.40	51	Nerol	
20-22	Traces	0.40	52	Calamenene	2.18
23	1-Octen-3-ol	2.64	53	Anethole	2.77
24,25	Traces		54	Traces	0.59
26	Citronellal	1.50	55	Geraniol	
27,28	Traces		56	Safrole	
29	Menthone		57	Traces	3.36
30	Linalol	3.44	58	Piperitenone	
31	γ-Caryophyllene	3.68	59	Traces	
32	Traces		60	Piperitenone oxide	2.77
33	Camphor	1.19	61	Epoxy Dihydrocaryophyllene	3.96
34	Traces		62/63	Carbonyl/Alcoholic comp.	4.95
35	Carvomenthone	0.40	64-66	Unidentified	5.54
36	Bornyl acetate	1.98	67	Eugenol	6.53
37	Traces		68-70	Unidentified	5.34
38	β-Caryophyllene	9.50	71	Farnesol	2.75
39	Menthol	3.56	72	Traces	

100

12. CHANGES IN THE CHEMICAL COMPOSITION OF THE VOLATILE OIL DURING THE GROWING SEASON

In order to investigate seasonal variations in the chemical composition of the volatile oil, plants, grown from seeds sown in September, were cultivated in a greenhouse at a minimum temperature of 18°C. As only a limited number of plants could be grown, insufficient material was available for regular distillations using the method previously given. Betts (148) used a microdistillation technique to obtain carvone from small numbers of Caraway and Dill fruits. This method has been adapted to obtain volatile oil from small quantities of fresh Horehound leaves.

Micro-distillations were carried out using the apparatus shown in Figure 73. A leaf sample of between 0.5 and 5g. fresh weight was co-distilled with 160ml. of distilled water until 5ml. of water remained in the distilling flask. The aqueous condensate was passed directly into a 100ml. separating funnel which contained 5ml. of ether and 5ml. of distilled water. Water was drained periodically from the separator into the draining flask. When distillation was complete (about four hours), the condenser was washed out into the separating funnel with 2ml. of ether. The funnel contents were shaken gently and allowed to separate, the aqueous phase being discarded. A further 2ml. of ether were added to the water in the draining flask. After shaking and separation, this ether was added to the ether in the funnel which was dried overnight using anhydrous sodium sulphate, then evaporated under vacuum at a temperature of 25°C using a Büchi Rotavapor 'R'. Prior to g.l.c. separation, 100µl. of ether were added to the volatile oil residue using a Hamilton repeat dispenser syringe. One microlitre of this solution was taken for analysis.

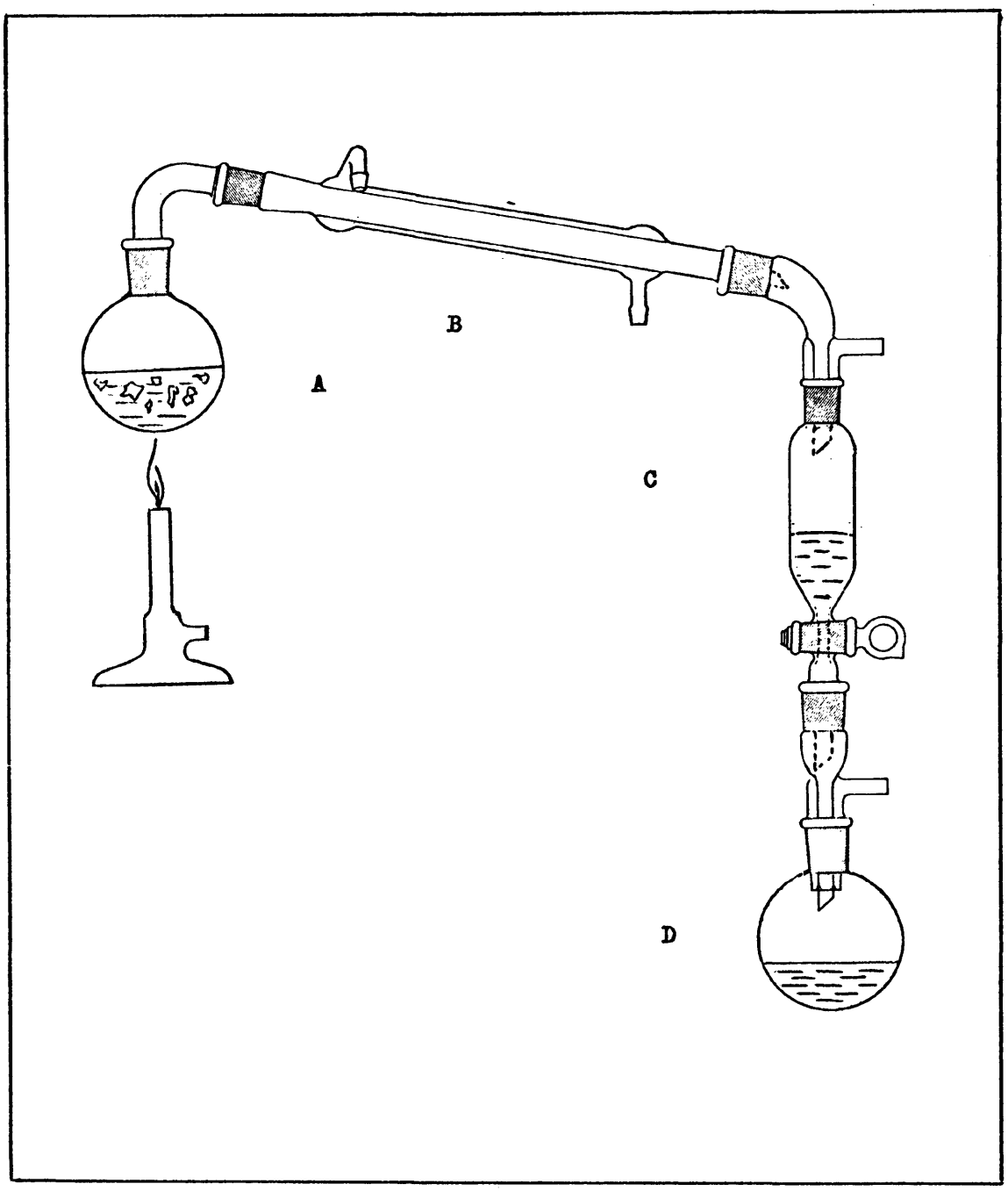


FIGURE 73: Apparatus used for micro-distillation of *Marrubium vulgare* L. oil.

A. Distilling flask. B. Condenser.

C. Separating funnel. D. Draining flask.

To follow any changes in the chemical composition of the volatile oil during plant development, basal pairs of leaves were taken from the plants throughout the growing period. The time intervals between the collection of samples was determined by the development of further leaf pairs on the stems, material being taken from several plants every time a new pair of leaves unfolded. This random sample was treated by the method previously described.

From the resulting chromatograms, the area of each measurable peak, calculated as peak height times width at half height, was determined. In order to compare the results obtained from different samples, each value was then made relative to linalol. These figures are given in Table 10. Also included in this table are the values obtained for linalol in each sample, standardized to an equivalent sample weight of 0.5g. The changes in the chemical composition of the volatile oil are shown in Figure 74 (A and B).

Although not shown in Figure 74, it was noted that component 42, which only occurred as a trace amount in the volatile oil distilled from the dry herb, appeared as one of the major components of the oil distilled from plants which were 297 days old (Figure 77). The determination of the relative retention times of this component to linalol on different columns at different temperatures was possible, now that the peak could be located easily. The results suggested that component 42 was a sesquiterpene.

These investigations showed that the chemical composition of the oil differed at different stages of plant growth, but the differences were mainly quantitative.

TABLE 10

Relative areas of the peaks of certain components of *Marrubium vulgare* L. oil to linalol during the growing period

Components	Plant age in days									
	47	55	78	131	165	187	222	235	257	297
1-Octen-3-ol	0.41	0.50	0.73	0.54	0.28	0.44	0.37	0.28	0.19	0.11
β-Caryophyllene	2.15	1.28	1.50	1.98	1.79	2.04	3.47	2.34	2.77	3.55
Pulegone	1.45	0.58	0.79	1.40	1.10	1.93	1.60	1.39	1.92	1.66
Piperitone	5.10	1.17	2.44	5.89	1.26	5.79	3.79	2.64	3.90	2.00
δ-Cadinene	1.20	0.95	0.57	1.30	0.22	1.12	0.53	0.33	0.46	-
Geraniol	9.00	1.45	3.90	11.96	1.70	10.64	6.30	4.28	5.95	1.24
Piperitenone oxide	11.55	0.94	4.88	8.73	0.91	8.06	9.08	5.86	6.18	1.50
62/64	15.90	2.90	6.99	9.95	2.60	11.42	12.44	10.82	7.95	2.04
Eugenol	16.65	2.89	6.93	20.98	4.69	19.62	12.50	6.97	7.14	1.50
Farnesol	16.38	2.42	6.67	23.03	2.59	20.30	13.11	7.73	2.81	2.07
72	16.00	2.22	6.40	21.28	1.40	20.11	11.26	7.51	8.35	1.96
Linalol (absolute)	0.80	1.80	2.46	3.36	1.88	0.67	0.18	0.23	1.50	1.40

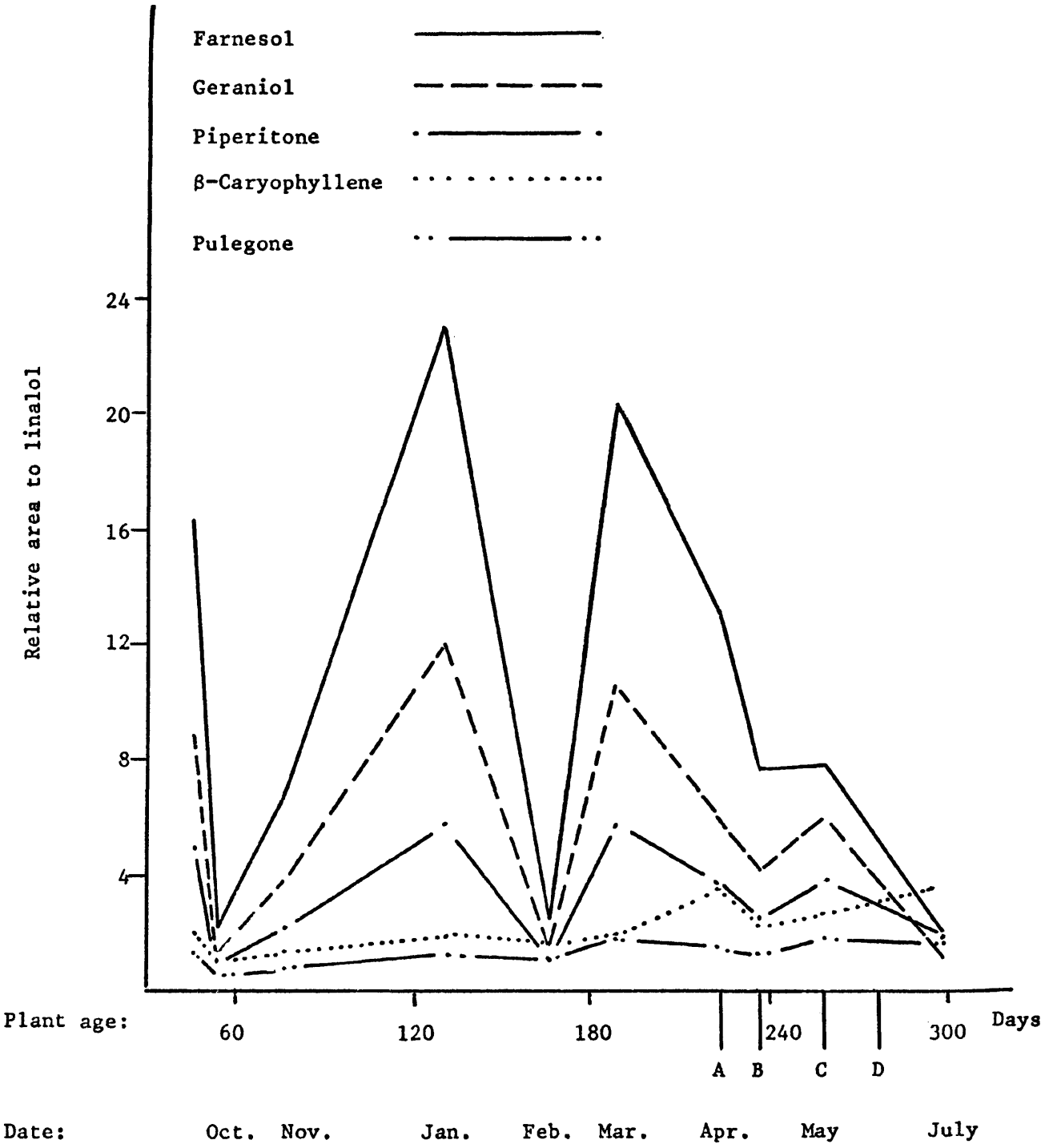


FIGURE 74A: Changes in the area of the peaks of certain components of *Marrubium vulgare* L. oil relative to linalol during the growing period.

A. Marrubiin formation.

B. First flowers opened.

C. Full bloom.

D. Seed formation.

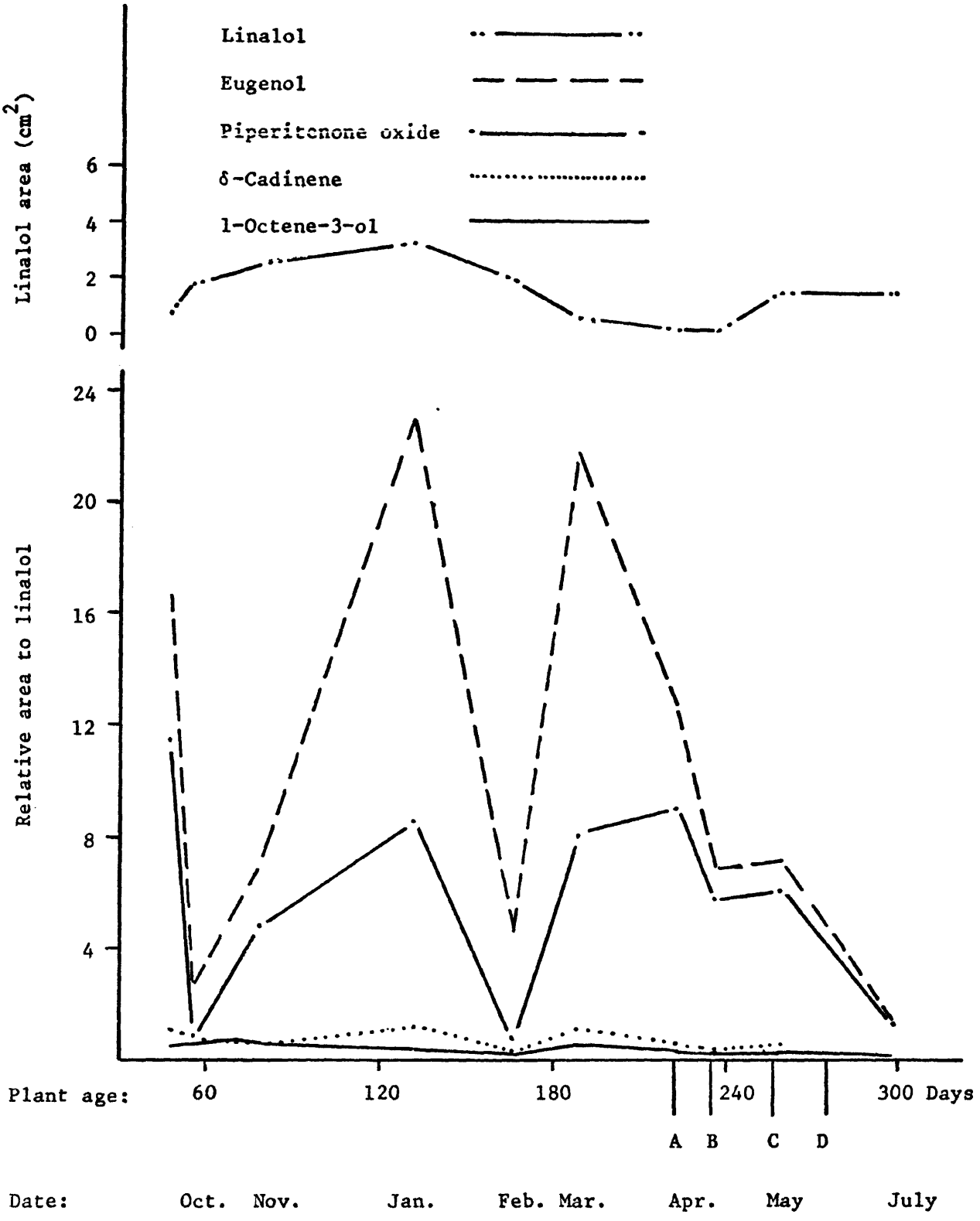


FIGURE 74B: Changes in the area of the peaks of certain components of *Marrubium vulgare* L. oil relative to linalol and the absolute areas of the linalol peaks during the growing period.

A. Marrubiin formation.

B. First flowers opened.

C. Full bloom.

D. Seed formation.

13. COMPARISON OF THE VOLATILE OILS OBTAINED FROM LEAVES AT DIFFERENT NODES

At certain times during the growing period, leaves of different absolute ages were taken on the same day from the same sample of plants. Each sample was distilled using the micro-distillation method and the resulting oils were subjected to g.l.c. analysis using the method described in section twelve. The area of each measurable peak on the chromatogram was determined and made relative to linalol. The results are shown in Table 11 and Figure 75. Also included in Table 11 are the values obtained for linalol in each sample, standardized to an equivalent sample weight of 0.5g.

From these results it can be seen that oil distilled from a given pair of leaves differed in its quantitative composition from the oil obtained from leaves removed at another node. The same overall trends in oil composition changes were observed in the various leaf pairs, any change in these trends being governed by plant vigour and maturity.

TABLE 11

Comparison of the composition of *Marrubium vulgare* L. oil obtained from leaves at different nodes, as shown by differences in the peak areas of certain components relative to linalol

Components	Plant age in days, and node number counting from plant tip									
	165		187		222		235		257	
	5	6	7	8	9	10	11	12	1	12
23 1-Octen-3-ol	0.39	0.28	0.29	0.44	0.51	0.37	0.31	0.28	0.15	0.19
38 β-Caryophyllene	1.75	1.79	1.80	2.04	3.62	3.47	2.18	2.34	4.54	2.77
41 Pulegone	0.98	1.10	1.65	1.93	2.80	1.60	1.45	1.39	1.65	1.92
49 Piperitone	1.36	1.26	3.61	5.79	5.88	3.79	2.08	2.64	1.29	3.90
50 δ-Cadinene	0.38	0.22	0.59	1.12	0.95	0.53	0.21	0.33	-	0.46
55 Geraniol	1.99	1.70	5.12	10.64	9.95	6.30	2.17	4.28	1.31	5.95
60 Piperitenone oxide	0.76	0.91	4.90	8.06	11.82	9.08	1.54	5.86	3.92	6.18
62/64	4.32	2.60	7.94	11.42	17.23	12.44	7.96	10.82	2.92	7.95
67 Eugenol	4.33	4.69	8.78	19.62	18.45	12.50	3.08	6.97	-	7.14
71 Farnesol	3.58	2.59	9.76	20.30	19.73	13.11	3.94	7.73	-	2.81
72 Unknown	3.76	1.40	8.20	20.11	18.58	11.26	3.85	7.51	-	8.35
30 Linalol (absolute)	3.80	1.88	0.38	0.67	0.15	0.18	0.14	0.23	1.85	1.50

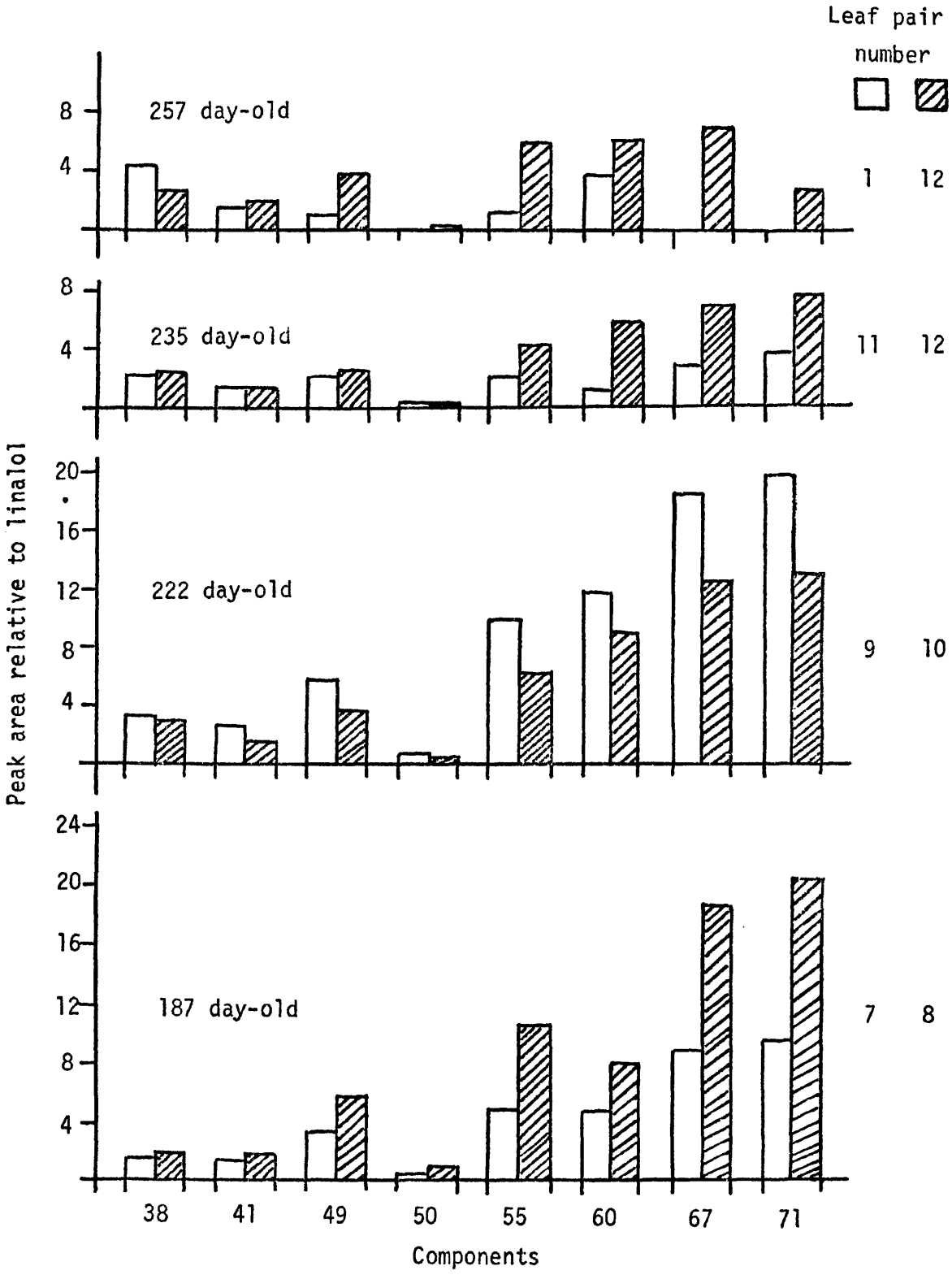


FIGURE 75: Diagramatic representation of changes in the composition of *Marrubium vulgare* L. oils obtained from leaves at different nodes.

14. COMPARISON OF THE CHEMICAL COMPOSITION OF THE VOLATILE OILS OBTAINED FROM *Marrubium vulgare* L. and *M. peregrinum* L.

Differences in the chemical composition of the volatile oils distilled from plants of *Marrubium peregrinum* L. grown in the greenhouse from seeds obtained from the Royal Botanical Gardens and plants of *M. vulgare* L. were determined. After flowering and seed formation (July) a five gramme sample obtained from several plants of each species was distilled using the micro-distillation technique, the volatile oils being subjected to g.l.c. analysis using the Carbowax 20M and SE30 columns. Gas chromatograms of the volatile oil of the two species under investigation are shown in Figures 76 and 77.

A comparison of the two chromatograms indicated that at this stage of plant development, differences in the composition of the oils were quantitative not qualitative. For example, the oil distilled from *M. peregrinum* L. contained larger amounts of β-caryophyllene and ∝-humulene, whereas the oil distilled from *M. vulgare* L. contained larger amounts of epoxy dihydrocaryophyllene, piperitenone, component 42 and components 62/63; all other components being present to the same degree.

Von Rudloff (82) found from the systematic analysis of the leaf oils of North American conifers that differences in the oil composition obtained from individual species were quantitative. He suggested that any qualitative differences might lead to correlations at higher taxonomic levels. The results of this investigation would appear to be in agreement with this suggestion.

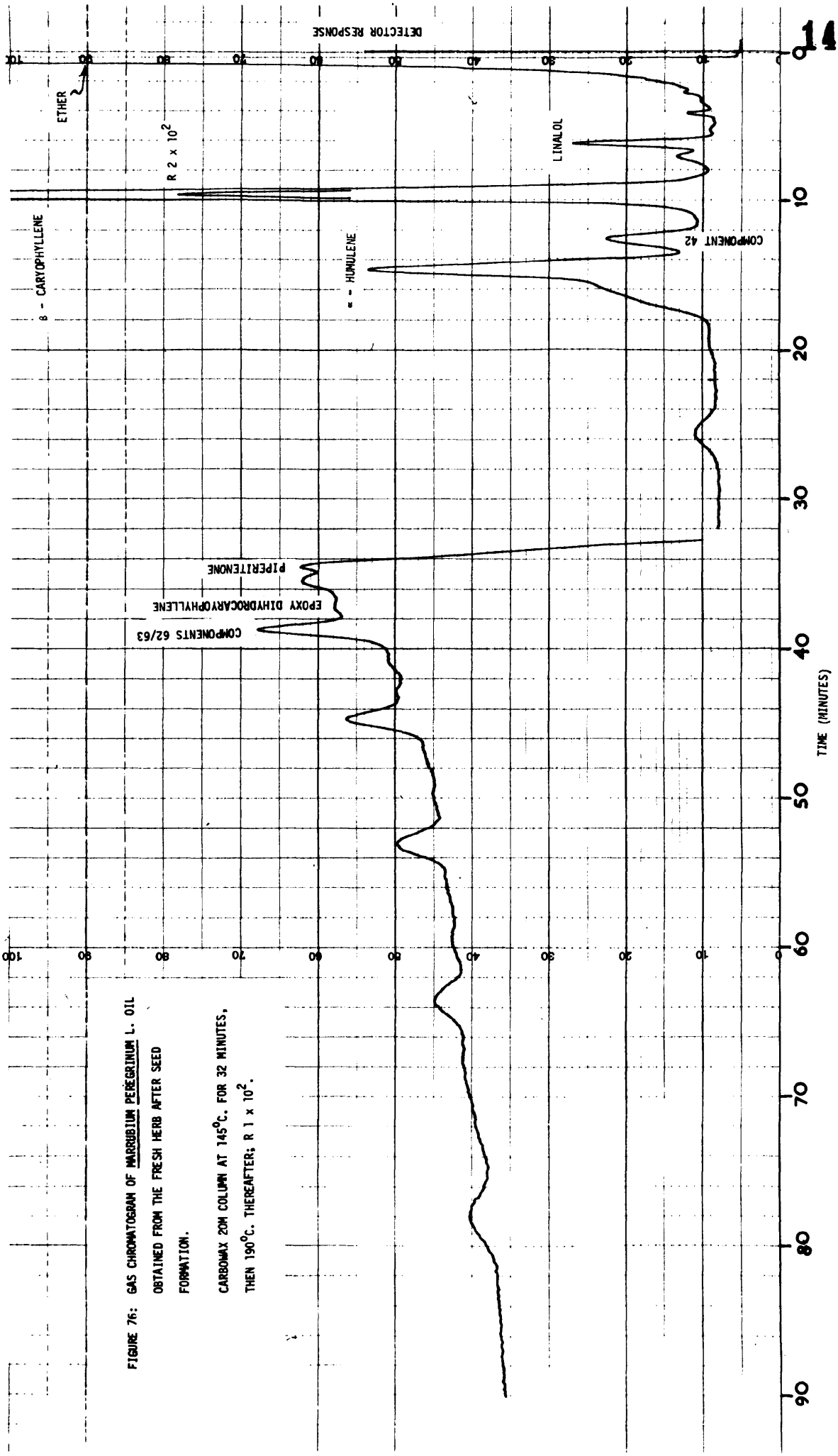


FIGURE 76: GAS CHROMATOGRAM OF MARRUBIUM PEREGRINUM L. OIL OBTAINED FROM THE FRESH HERB AFTER SEED FORMATION.

CARBOWAX 20M COLUMN AT 145°C. FOR 32 MINUTES, THEN 190°C. THEREAFTER; R 1 x 10^2.

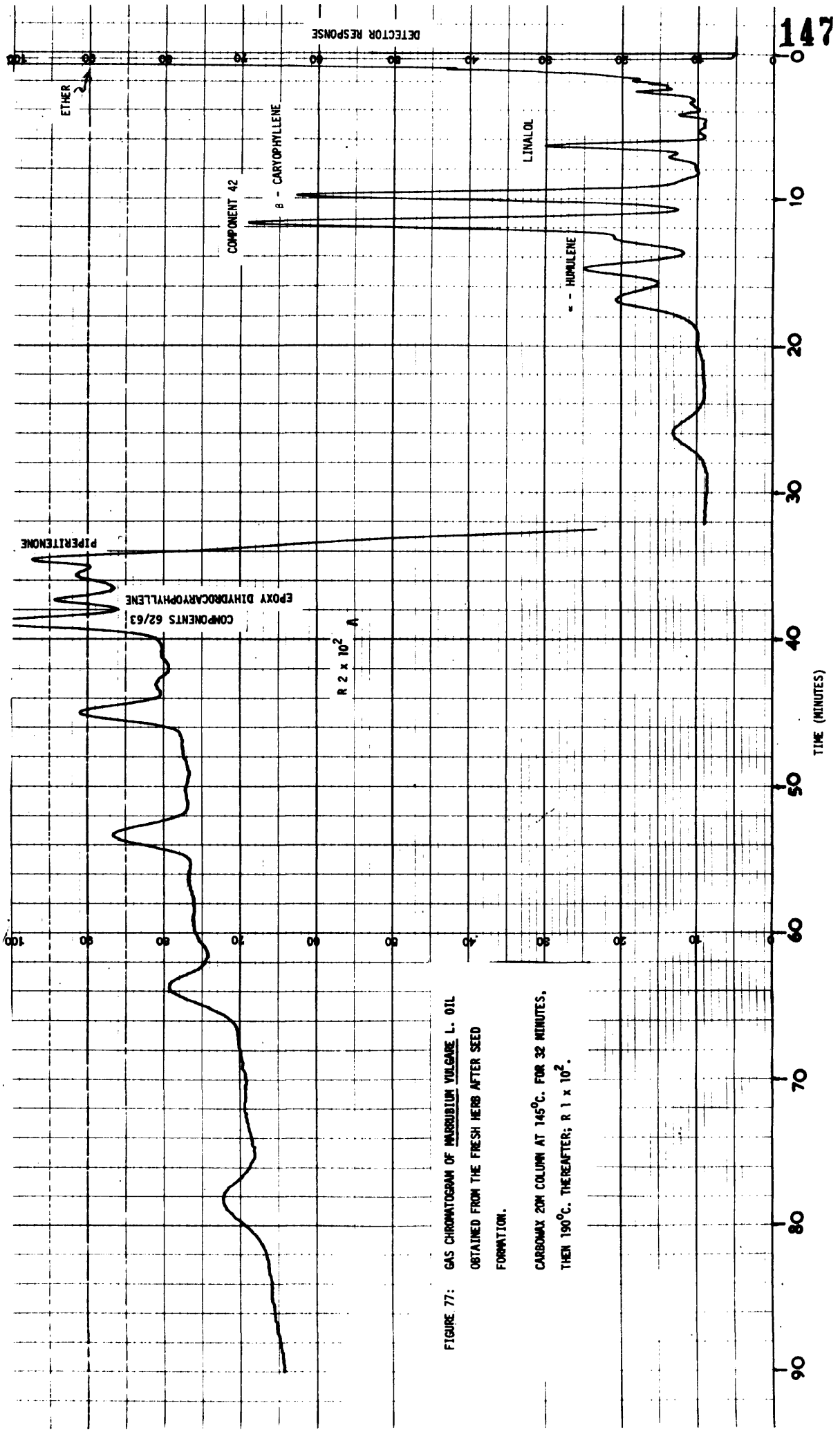


FIGURE 77: GAS CHROMATOGRAM OF MARRUBIUM VULGARE L. OIL OBTAINED FROM THE FRESH HERB AFTER SEED FORMATION.

CARBOWAX 20M COLUMN AT 145°C. FOR 32 MINUTES, THEN 190°C. THEREAFTER; R 1 x 10^2.

15. PRESENCE AND CHANGES IN THE CONTENT OF MARRUBIIN

The presence and content of marrubiin in Horehound plants were evaluated during the growing season in an attempt to investigate whether any relationship might occur between marrubiin formation and volatile oil formation. It had been suggested that marrubiin was an artifact arising from the volatile oil during the drying of Horehound (30). Henderson and McCrindle (25) put forward evidence to support this suggestion, concluding that marrubiin was an artifact formed from a diterpenoid material which they named premarrubiin.

Using the method of Henderson and McCrindle (25) a preliminary experiment was carried out to isolate either premarrubiin or marrubiin in order to determine which compound should be assayed in this investigation. Horehound herb (50g.) was shaken with 600ml. cold acetone in a flask for 30 minutes. The extract was filtered and the acetone was evaporated at 40°C using a Büchi Rotavapor 'R' with vacuum applied. The residue (0.7g) was examined by t.l.c. for the presence of premarrubiin and marrubiin, solvent system: ethyl acetate: light petroleum (40-60°C) 3:7, the separated compounds being visualized by means of Ehrlich's reagent (1% p-dimethyl aminobenzaldehyde in ethyl alcohol and concentrated hydrochloric acid 2:1). Three Ehrlich active spots were detected having R_f values of 0.40 (the major spot), 0.22 and 0.17. Henderson and McCrindle (25) stated that the major Ehrlich positive spot was produced by premarrubiin (However, on later analysis, the i.r. spectrum of the compound recovered from this spot, Figure 80, proved that it was a keto lactone and not premarrubiin). In an attempt to convert the premarrubiin in the extract, if present, to marrubiin, a small amount of the residue was mixed with chloroform and another portion was refluxed with ethanol for three hours (25). The resulting solutions were tested as before by t.l.c. No marked difference

was observed in the results indicating that if premarrubiin was present in the extract, it was not converted to marrubiin as had been suggested. The information given by Henderson and McCrindle (25) was insufficient to enable a repeat of their experiments and it was not possible to isolate premarrubiin.

A second attempt to isolate marrubiin was made using the method of Cocker et al. (11). Powdered Horehound (20g.) was extracted in a Soxhlet apparatus with 200ml. of acetone for 20 hours. The acetone was then concentrated to about 15ml. by evaporation at 40°C with vacuum applied using a Büchi Rotavapor 'R'. The concentrated solution was filtered and the filtrate was evaporated to remove the remaining acetone. Four millilitres of ether were added to the residue, and the whole allowed to stand overnight. The ether, which dissolved any waxes and chlorophyll, was filtered off leaving a residue of impure marrubiin. This residue was dissolved in 5% alcoholic sodium hydroxide (1ml.), boiled for 10 minutes and poured into a large excess of water (400ml.). The mixture was centrifuged and the precipitated material was washed with ether then filtered. The purified marrubiin was dried by leaving it in a descicator overnight.

The identity of this material was checked by comparison of its t.l.c. behaviour with that of a reference sample of pure marrubiin using ethyl acetate:petroleum ether (40-60°C) 3:7 as solvent and then visualizing by means of Ehrlich's reagent. Both samples gave a similar Ehrlich active spot with an R_f value of 0.22.

The isolated marrubiin, in the form of a paraffin paste was also examined by i.r. spectroscopy. The spectrum is shown in Figure 78 (A and B). The principle peaks 3460, 2880, 1740, 1560, 1505, 1470, 1380,

1270, 1240, 1200, 1155, 1100, 1066, 1050, 1010, 985, 880, 815, 768 and 753 cm^{-1} were in substantial agreement with those in the published data (11). This confirmed that the material isolated was marrubiin.

Since the amount of fresh plant material available for this investigation was limited, several small scale extraction procedures were tested to find a reproducible method to assay the marrubiin present in the growing plants. The following method of extraction and assay proved to be satisfactory.

A sample of 0.5-5g. of fresh leaves was mixed with 40-80ml. acetone and refluxed for five hours to ensure complete extraction of the marrubiin. Anhydrous sodium sulphate was added to the solution and the whole allowed to stand overnight. The solution was then filtered and the residue washed twice with acetone. These washings were added to the filtrate, the combined solutions being evaporated at 40°C under vacuum until no further acetone was removed. Evaporation was then continued at 60°C for a further 30 minutes to remove any traces of residual water. A fixed volume of acetone 500µl. measured by means of a Hamilton syringe, was added to the residue prior to t.l.c. investigations. Five microlitres of the resulting solution were subjected to quantitative t.l.c. analysis. Replicate applications of the solution were made on four different plates, a standard solution of pure marrubiin being applied as a marker. The solvent system was ethyl acetate: light petroleum (40-60°C) 3:7, and separated compounds were visualized using Ehrlich's reagent. Using a light box, the outline of each marrubiin spot was traced five times onto tracing paper. The area of each spot was determined using one centimetre graph paper.

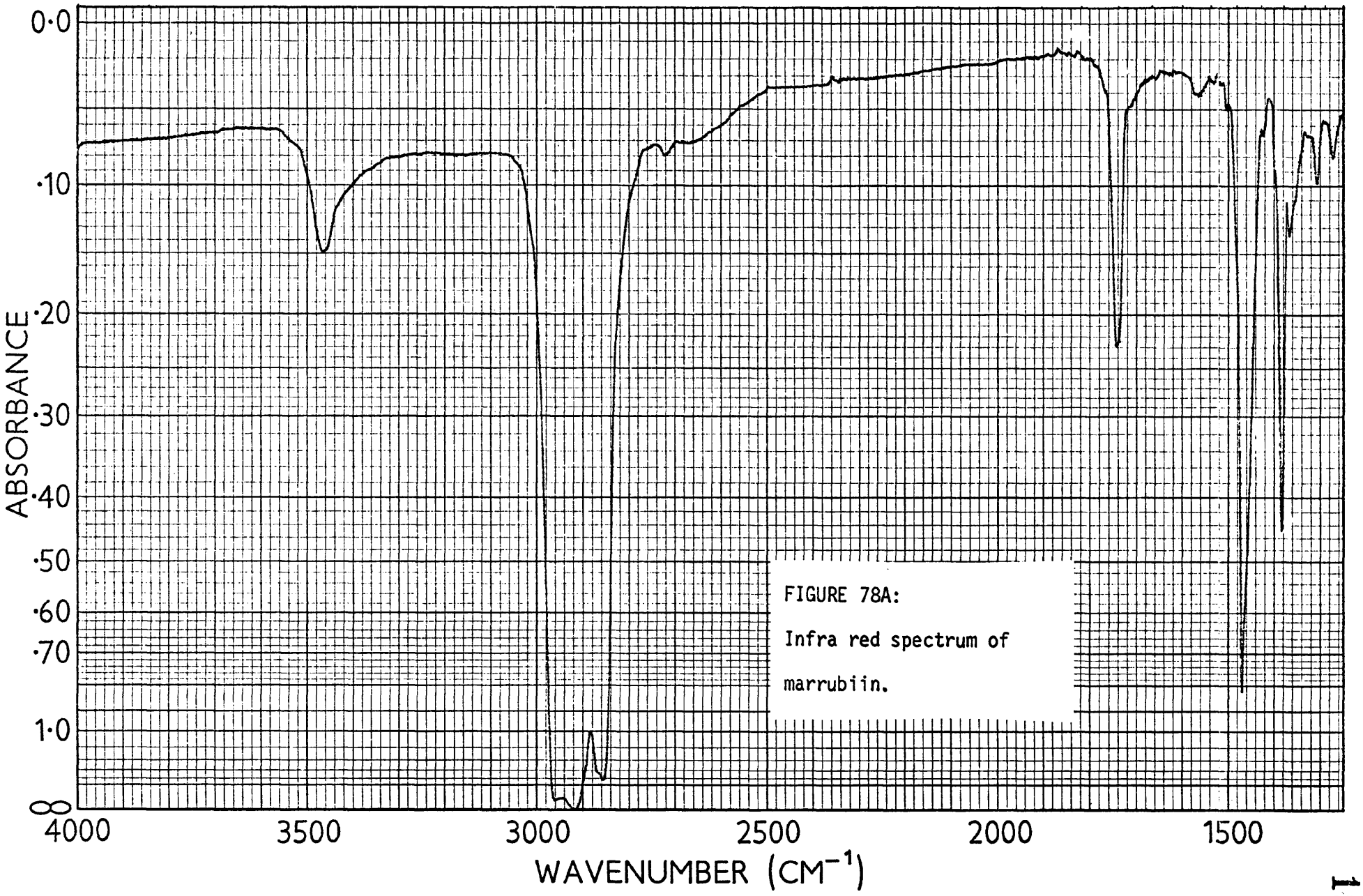


FIGURE 78A:

Infra red spectrum of marrubiin.

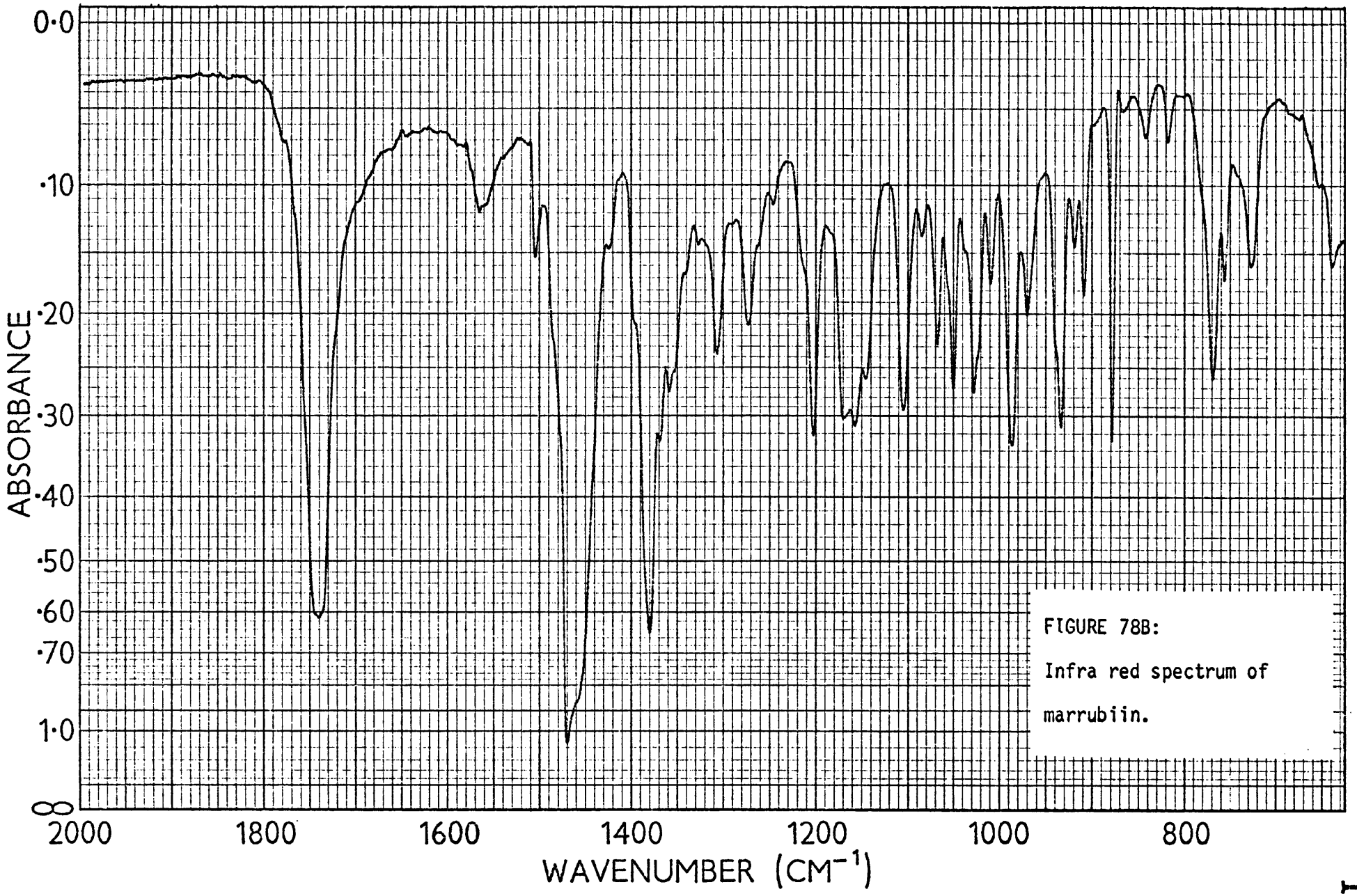


FIGURE 78B:

Infra red spectrum of marrubiin.

To determine the amount of marrubiin in the plants under investigation a calibration curve was prepared. Marrubiin (10mg.) was dissolved in acetone and made up to 1 ml. using a volumetric flask. This was used as the stock solution. After performing a t.l.c. investigation to determine the range of dilutions that could be detected, eight solutions of marrubiin were prepared by mixing 50µl. of the stock solution (500µg. of marrubiin) with nil, 50, 150, 350, 750, 1550, 3150 and 6350µl acetone. This gave standard solutions containing 50,25,12.5, 6.3, 3.1, 1.6, 0.8 and 0.4µg. of marrubiin respectively in each 5µl. Using a Hamilton repeat dispenser syringe, 5µl. of each of the eight solutions were applied to a 20 x 20 cm. plate. This was repeated ten times. The plates were developed in ethyl acetate: petroleum ether (40-60^{o}C) 3:7 and the resulting spots were detected by Ehrlich's reagent. Each spot was measured by tracing its outline five times onto tracing paper using a light box. The area of each spot was determined using one centimetre graph paper. An analysis of variance (149) of the results proved that there was no significant difference between the areas produced by equal volumes of the same solution on the ten different plates used (Tables 12 and 13). The calibration curve was constructed by plotting the logarithm of the weights of marrubiin applied against resulting mean spot areas. This was linear over the range of 0.4-10µg. marrubiin (Figure 79).

To investigate the reproducibility of the application technique on the same as well as different plates, five spots of a standard solution of 12.5µg. marrubiin in 5µl. were applied to each of two plates (20 x 20 cm.) which were then treated as before. An analysis of variance (Tables 14 and 15) proved that the method of application was reproducible.

TABLE 12

Area measurements of spots produced by marrubiin solutions of different concentrations

Amount of marrubiin	Spot area (mm) on different chromatoplates										Average
µg	1	2	3	4	5	6	7	8	9	10	
50.0	86.0	88.8	84.4	83.0	65.4	78.5	83.8	84.6	57.4	73.4	78.5
25.0	57.0	46.6	72.2	68.0	49.4	67.4	58.6	57.0	54.0	56.6	58.7
12.5	42.4	45.0	44.0	40.2	43.8	44.0	42.6	57.0	39.2	42.2	44.0
6.3	38.4	33.8	34.6	37.8	34.6	37.0	35.8	28.4	31.8	31.6	34.4
3.1	31.2	25.2	25.2	31.0	23.4	30.2	31.0	24.6	23.2	22.6	26.8
1.6	24.2	19.6	21.6	24.4	15.4	22.6	19.2	24.0	18.6	19.8	20.9
0.8	19.0	13.4	20.0	17.4	14.6	15.6	10.8	18.0	12.0	12.4	15.3
0.4	12.0	8.2	11.0	11.8	9.0	11.4	8.0	11.0	9.8	7.6	10.0

TABLE 13

Analysis of variance of area measurements of spots produced by marrubiin solutions of different concentrations.

Source of variation	d.f.	S.S.	M.S.	F	L.S.D.
Spot area	9	718.07	79.79	2.19	n.s.
Amount of marrubiin	7	38076.03	5439.43	149.27**	5.3 cm^2
Residual	63	2295.68	36.44		
Total	79	41089.78			

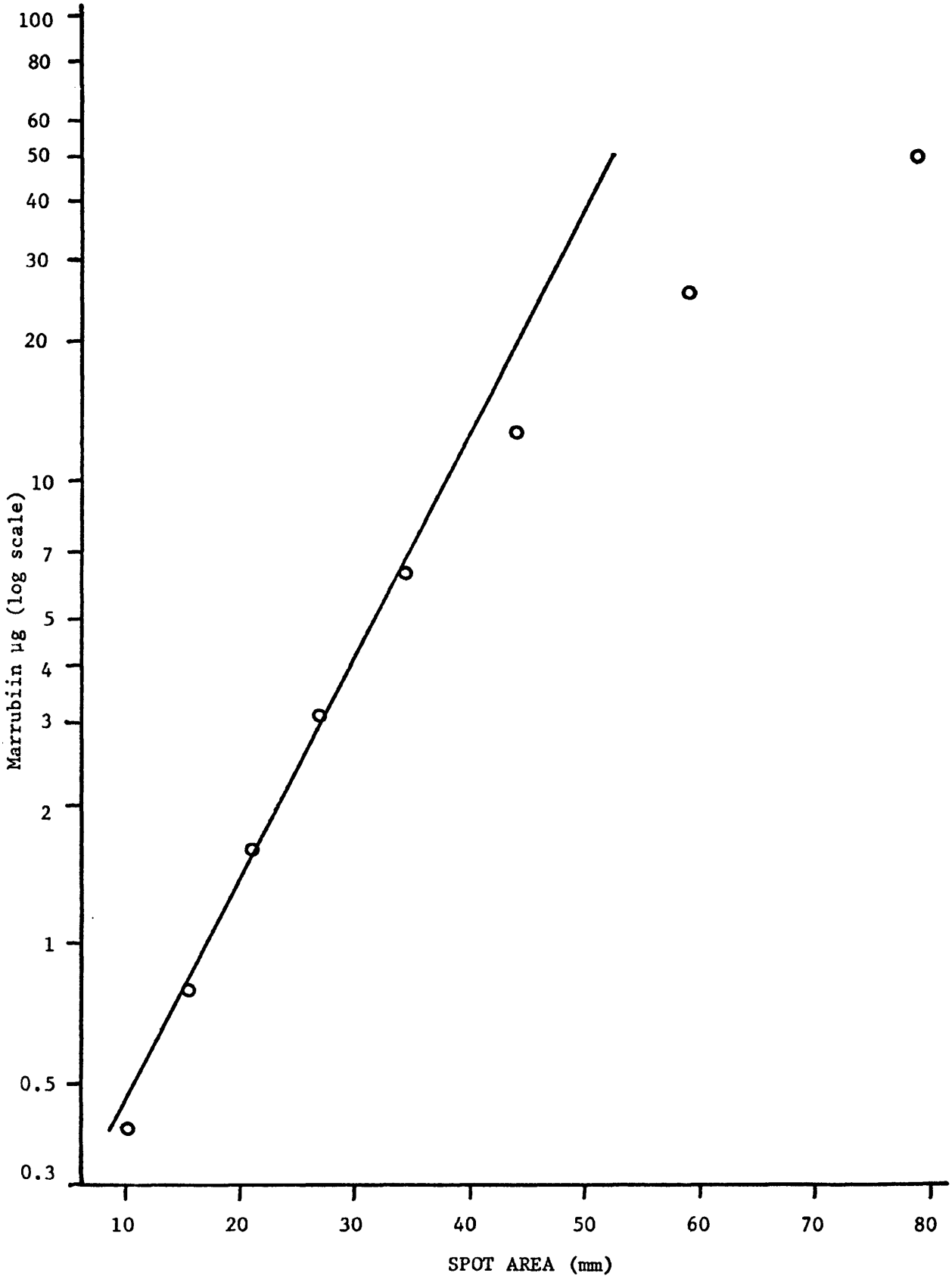


FIGURE 79: Calibration curve used to determine the amount of marrubiin present in samples of *Marrubium vulgare* L.

TABLE 14

Spot area measurements made to test the reproducibility of the t.l.c. application procedure on the same and different plates.

Injections	Spot area (mm)		Average
	Plate I	Plate II	
1	45.2	43.0	44.1
2	42.4	42.0	42.2
3	44.4	36.6	40.5
4	41.0	44.4	42.7
5	41.6	36.8	39.2
Average	42.9	40.6	41.7

TABLE 15

Analysis of variance of the reproducibility of the t.l.c. application procedure on the same and differe nt plates

Source of variation	d.f.	S.S.	M.S.	F	
Different plates	1	29.38	29.38	3.24	n.s.
Same plate	4	13.92	3.48	0.38	n.s.
Residual	4	36.30	9.08		
Total	9	79.60			

To investigate the marrubiin content during the development of the plants throughout the growing season, a sampling system similar to that adopted with the volatile oil was followed. The most mature leaves (the basal pairs) were collected up to seed formation at intervals determined by the development of further leaf pairs on the stems, samples being taken from several plants every time a new pair of leaves unfolded. Their marrubiin content was determined using the method previously described. After seed formation, a random sample of leaves, which represented fresh herb material, was taken and assayed. For comparison, the commercial herb was also assayed. The results of this investigation are shown in Table 16.

Marrubiin was first detected as traces when the plants were 131 days old. However, it was not detected in a measurable amount until plants were 222 days old, which was just before the onset of flowering; and it reached a maximum of 1.03% when the seeds were formed (297 day-old plants). This was a greater value than that given by the sample of the commercial herb (0.0004% calculated as fresh weight).

A comparison of the marrubiin content of leaves at different nodes was made during the growing period. Leaves of different absolute ages were taken on the same day from the same sample of plants. Each sample was assayed for its marrubiin content using the method mentioned before. From the results (Table 17), it was found that each leaf pair contained a different concentration of marrubiin. As with volatile oil formation, this again suggested that the biosynthetic activity of each leaf pair was independent of other leaf pairs on the same stem, but was influenced by plant vigour and maturity.

TABLE 16

Changes in marrubiin content; results of assays carried out during the growing period of *Marrubium vulgare* L. (calculated per 100g. fresh weight)

Plant age (days)	Sample weight (g.)	Mean spot area ($mm.^2$)	Marrubiin	
			µg./5µl.	mg./100g.
47	0.5	-	-	-
55	0.5	-	-	-
78	0.5	-	-	-
131	0.5	-	-	Traces
165	2.0	-	-	-
187	2.0	-	-	-
222	4.0	36.0	8.8	22.0
235*	5.0	37.2	9.4	18.8
257	2.0	33.5	6.2	31.0
297**	5.0	31.8	5.2	1030.0
Dry herb	5.0	36.6	0.9	1.8***

* First flowers opened.

** Fresh herb after seed formation, 50ml. acetone was added to this extract.

*** Calculated per 100g. of air dried material.

TABLE 17

Comparison of the marrubiin content of leaves at different nodes (calculated per 100g. fresh weight)

Plant age (days)	Sample weight (g.)	Node number*	Marrubiin (mg.)
222	4.0	9	11.2
222	4.0	10	22.0
235	5.0	11	19.6
235	5.0	12	18.8
257	0.5	1	44.0
257	2.0	6	11.0
257	2.0	12	31.0

* Numbered from the plant tip.

Marrubium vulgare L. PLANTS

Leaf material was collected when the plants were known to be devoid of marrubiin (187 days old). A sample of 15g. was well mixed and divided into three equal portions which were treated as follows. One sample was assayed immediately for marrubiin using the method previously described, the second sample was dried in a hot air oven at 105°C for five hours prior to assay, and the third sample was allowed to air dry for thirty days prior to assay.

Marrubiin was not detected in any of the three samples although an Ehrlich positive compound of R_f 0.40 was detected in the air dried sample.

When the plants were known to contain marrubiin (222 days old) a further sample of leaf material (10g.) was taken, mixed well and divided into two equal portions which were treated as follows. One sample was assayed immediately for marrubiin using the method described and the second sample was allowed to air dry for thirty days prior to assay.

Although marrubiin was detected in the sample which was assayed immediately (22mg./100g. fresh weight of leaf material), it was not detected after air drying. However, the Ehrlich positive compound of R_f 0.40 which was not detected in the first sample, was found after the sample had been air dried. This spot was also detected as a major compound in the commercial herb. Preparative t.l.c. was carried out using an extract of the commercial herb to collect a sample of this unknown compound. Figure 80 (A and B) shows the i.r. spectrum of this compound which had no hydroxyl group (absence of significant peaks at 3300-3600 cm^{-1}) c.f. marrubiin. It might however be a lactone (1780 cm^{-1}) with a ketonic group (1715 cm^{-1}). The i.r. spectrum of this compound was compared with that of the keto lactone found by Mangoni (28,150). Both spectra were similar, indicating that they could be the same compound.

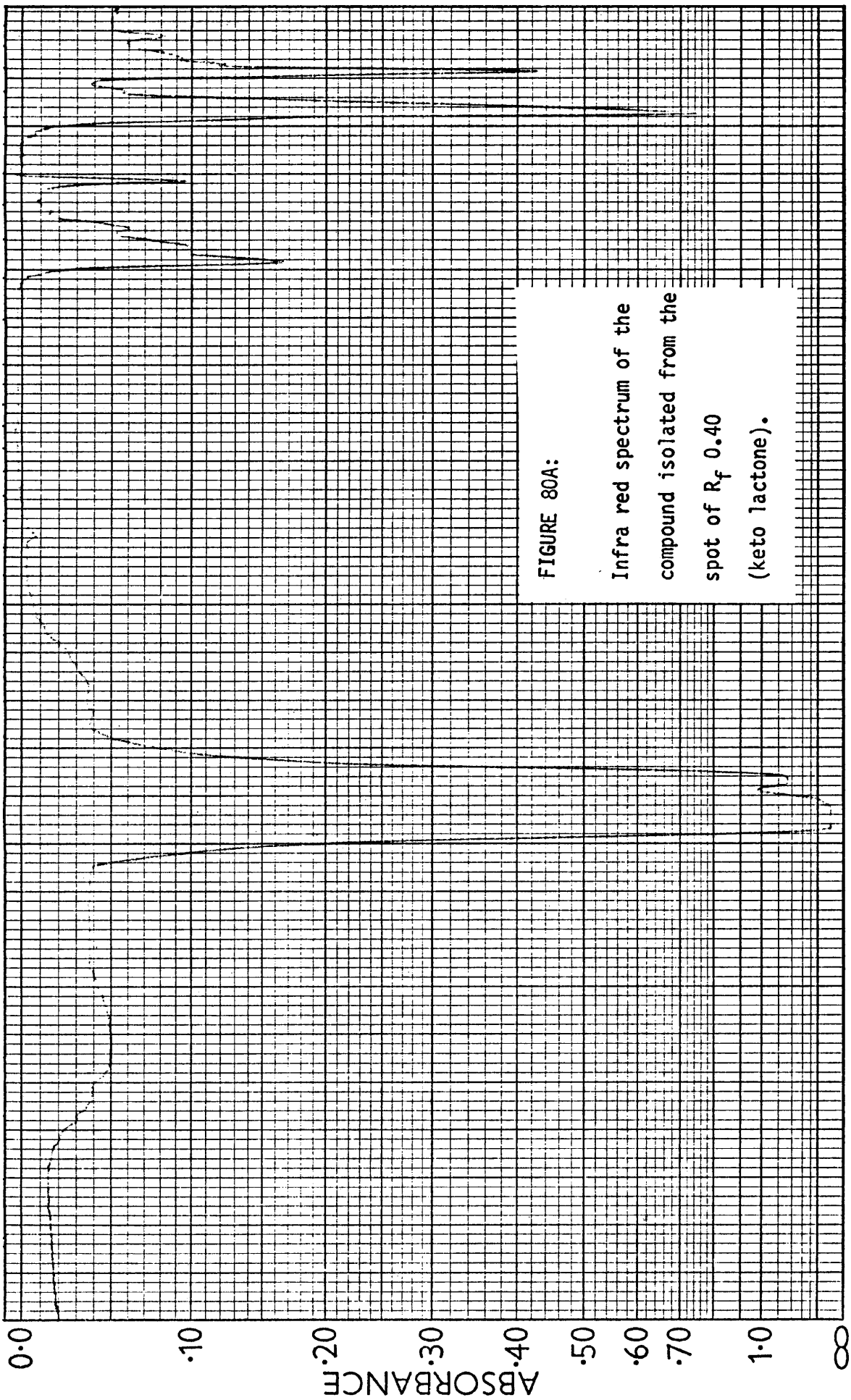


FIGURE 80A:

Infra red spectrum of the compound isolated from the spot of R_f 0.40 (keto lactone).

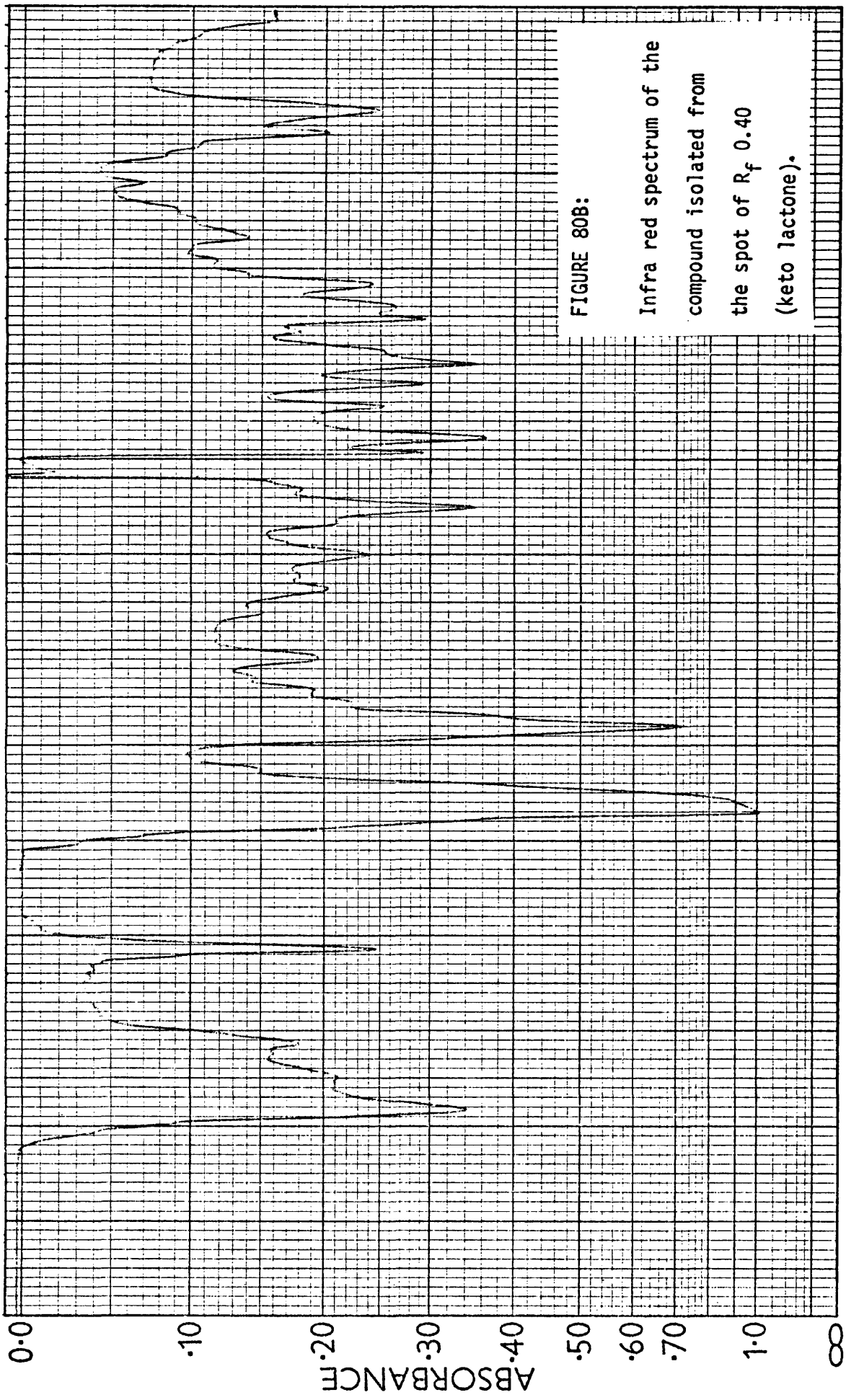


FIGURE 80B:

Infra red spectrum of the compound isolated from the spot of R_f 0.40 (keto lactone).

17. INVESTIGATION OF *Marrubium vulgare* L. FOR THE PRESENCE OF IRIDOIDS

When screening the Labiatae for the presence of iridoids, Kooiman (42) only tested the fruits of *Marrubium vulgare* L., the results of these tests being negative. To determine whether iridoid glycosides were present in the vegetative parts, the following tests were performed on fresh and dry samples of the herb. Fresh material (2g.) was shredded with scissors and dropped into 10ml. boiling water (to inactivate enzymes) and extracted for three minutes. A trace of calcium carbonate was previously added to the water to prevent acidic conditions which may result in glycoside hydrolysis. Dry herb (0.5g.) was boiled with 10ml. distilled water for three minutes. After filtration and cooling, the aqueous extract of each sample was shaken with an equal volume of ether to remove interfering substances. After separation from the ether, 1ml. of each aqueous extract was warmed for one minute with 5ml. of Trim and Hill reagent (Glacial acetic acid - 10vols. -, 0.2% $CuSO_4.5H_2O$-1vol.-, conc.HCL-0.5vol.-). No green-blue or blue colours were produced by either of the samples, indicating the absence of iridoids (36). Neither fresh nor dry Horehound herb was found to contain iridoids.

18. MATERIALS AND PREPARATION OF GLASSWARE

Unless otherwise stated, all reagents and solvents were of standard laboratory reagent grade.

All general glass apparatus was cleaned by overnight immersion in chromic acid followed by a thorough rinsing in distilled water. It was then dried for five hours at 110°C in a hot air oven.

The qualitative Hamilton syringes were cleaned thoroughly using organic solvents (ether or ethanol), while the preparative Hamilton syringe was cleaned by overnight immersion in Decon 90 solution (Medical Pharmaceutical Developments Limited, Brighton) followed by a thorough rinsing in distilled water and final washing in ethanol.

DISCUSSION

DISCUSSION

Harborne (35) stated "that complex mixtures in volatile oils tend to be the rule rather than the exception. While there may be ten or fifteen easily detectable components, there may be many other terpenoids present in trace amounts." The volatile oil obtained from *Marrubium vulgare* L. by means of water distillation, is no exception. Even using modern sensitive techniques of analysis, the identification of all the components present has not been easy. Although the low oil content has not been the major obstacle to compound identification, it has still limited the amounts of pure components which could be obtained by preparative methods of separation. The complexity of the volatile oil and the number of components which were present in trace amounts have proved to be the major problems. It was not possible to obtain a complete separation of most components using g.l.c. and when possible, the amounts obtained were insufficient for detailed analysis. A prefractionation technique using column chromatography prior to preparative g.l.c. largely overcame the problem of oil complexity by separating the components present into hydrocarbons and oxygenated terpenes. In cases where these combined techniques failed to produce adequate separation, preparative t.l.c. had to be used. In spite of these difficulties, the volatile oil was found to contain at least seventy-two components of which forty have been identified. The identity of these components and their concentration in the volatile oil are given in Table 9.

Of the forty components identified in this investigation, none has shown the characters of the unidentified sesquiterpene previously isolated from Horehound herb by Nicholas (18) and Bartarelli (21). This compound was the only sesquiterpene to be investigated by these workers, which

suggested that it might be the major component of the volatile oil. It is unlikely that any of the unidentified components isolated in the current investigation could be this compound as they only occur in trace amounts. The extraction methods used by Nicholas and Bartarelli, in which the herb was boiled for long periods with acetone or ethanol, were not specific for volatile oils, and although it might be expected that any sesquiterpenes which were isolated might be part of the volatile oil, this is not necessarily so. The conditions of extraction were such, that the compound they extracted could possibly be an artifact.

After collection, the individual components of Horehound oil were examined using the method of compound classification suggested by Breckler and Betts (119,120). They investigated the merits of using relative retention time observations on three different types of stationary phase at a series of different temperatures and came to the conclusion that volatile oil components could be divided into five chemical groups depending on their relative responses. At the outset, it was realized that this method of compound classification had certain limitations. In their studies, Breckler and Betts used a small number of standard compounds to cover the limits of each of the chemical groups which were investigated. Even so, overlapping of the groups occurs (Figures 20,21 and 22). The degree to which the various groups overlap would be increased if more standard compounds had been used, thus making compound classification more difficult. This problem might be overcome if trends of behaviour are also considered.

Breckler and Betts used a temperature range of 160 to 220^{o}C. Since the upper limit of this range is higher than the recommended working temperature for Carbowax 20M and DEGS columns (118,151), a lower temperature range, 130 to 190^{o}C, was used in the current investigation. On a re-

examination of some of the standard compounds, it was found that this lowering of the temperature range did not alter the behaviour patterns which had been observed by these workers. However, some of the components which are present in Horehound oil, were not resolved using this new temperature range. For satisfactory separation, the lowest temperature which could be used with these compounds was found to be 190°C.

In practice, this method of compound classification has been useful in the preliminary identification of the separated components. On examination of the results obtained, the identity of some of the components could be determined directly, as their datum was identical to that of one of the standard compounds. With other components, the evidence suggested that they belonged to one particular chemical group, thus narrowing the field for further investigation. Such correct conclusions could be reached for the low boiling point hydrocarbons such as camphene and β-phellandrene, which have a discrete range of retention times to linalol, and compounds like substituted aromatics, for example, estragole, whose relative retention times change markedly on stationary phases of different polarity.

The behaviour patterns of some compounds were such that on classification two possible chemical groups emerged. Even when this occurred, the component, on subsequent analysis, was found to belong to one of these groups and not to any of the others. For example, using this method, it was suggested that component 41 was either a terpenoid alcohol or a terpenoid carbonyl. Further analysis proved that the component was pulegone which is a terpenoid carbonyl.

Betts (120) intended to use this method in the identification of the components found in the volatile oil obtained from *Eucalyptus* leaves. Although Eucalyptus oil and other volatile oils are known to contain sesquiterpene hydrocarbons (152), Breckler and Betts did not examine the behaviour of this type of compound. Therefore, data on sesquiterpene hydrocarbons are not included in their results. This omission is hard to justify but it may be explained by the fact that the preparation and authentication of sesquiterpene hydrocarbons are delicate and difficult operations and that such compounds are not readily available commercially. In the current investigation, sesquiterpene hydrocarbons have been studied as a group and their behaviour was found to be characteristic. When their relative retention times to linalol were determined using a column with a non-polar stationary phase (SE30), all the investigated sesquiterpene hydrocarbons showed a range of retention times which were significantly different from the other chemical groups (Figure 22).

The volatile oil of *Marrubium vulgare* L. was found to be composed of 35% sesquiterpenes (8 components), 15% terpenoid alcohols (8 components), 13% substituted aromatics (4 components), 10% terpenoid carbonyls (8 components), 7% terpenoid oxides (2 components), 3% simple monoterpenes (9 components), 2% terpenoid esters (1 component) and 15% unidentified compounds (32 components). Even if these figures are adjusted to take into account the effective carbon response of the different compounds, as suggested by Ackman (147), the sesquiterpene hydrocarbons were found to be the largest chemical group within Horehound oil.

The composition of volatile oils obtained from plants of the family Labiatae differs with different plants and although the same groups of compounds may be present, the ratio of their respective concentrations varies. Some oils are composed mainly of monoterpenoid compounds, the

oil obtained from *Mentha piperita* L. (115) being composed of 84% oxygenated monoterpenoid compounds, 7% pre-monoterpene hydrocarbons and 5.5% sesquiterpene hydrocarbons, whilst the oils obtained from *Pogostemon patchouli*, Patchouly oil, (153) and *Marrubium vulgare* L. contain mainly complex sesquiterpenoid compounds. As well as differences in chemical groups, the concentration of individual compounds can vary. The major component of Horehound oil, β-caryophyllene, is a compound which is distributed widely through the family Labiatae being present in the following genera (44): *Nepeta, Rosmarinus, Thymus, Mentha, Salvia, Perilla, Origanum* and *Lavandula.* In each it constitutes a different percentage of the total concentration. This suggests that the potential of a plant to produce different types of compounds differs according to its genus.

The gastro-intestinal (74-76), abortive (77), antifungal and antibacterial properties (79-81) of Horehound herb have been attributed to the presence of marrubiin, and the alkaloids present are stated to be responsible for the cardiac activity (66). The herb has also been used in the treatment of pulmonary affections, in febrile conditions, as a purgative and as a vermifuge but there are no reports to indicate which compounds might be involved.

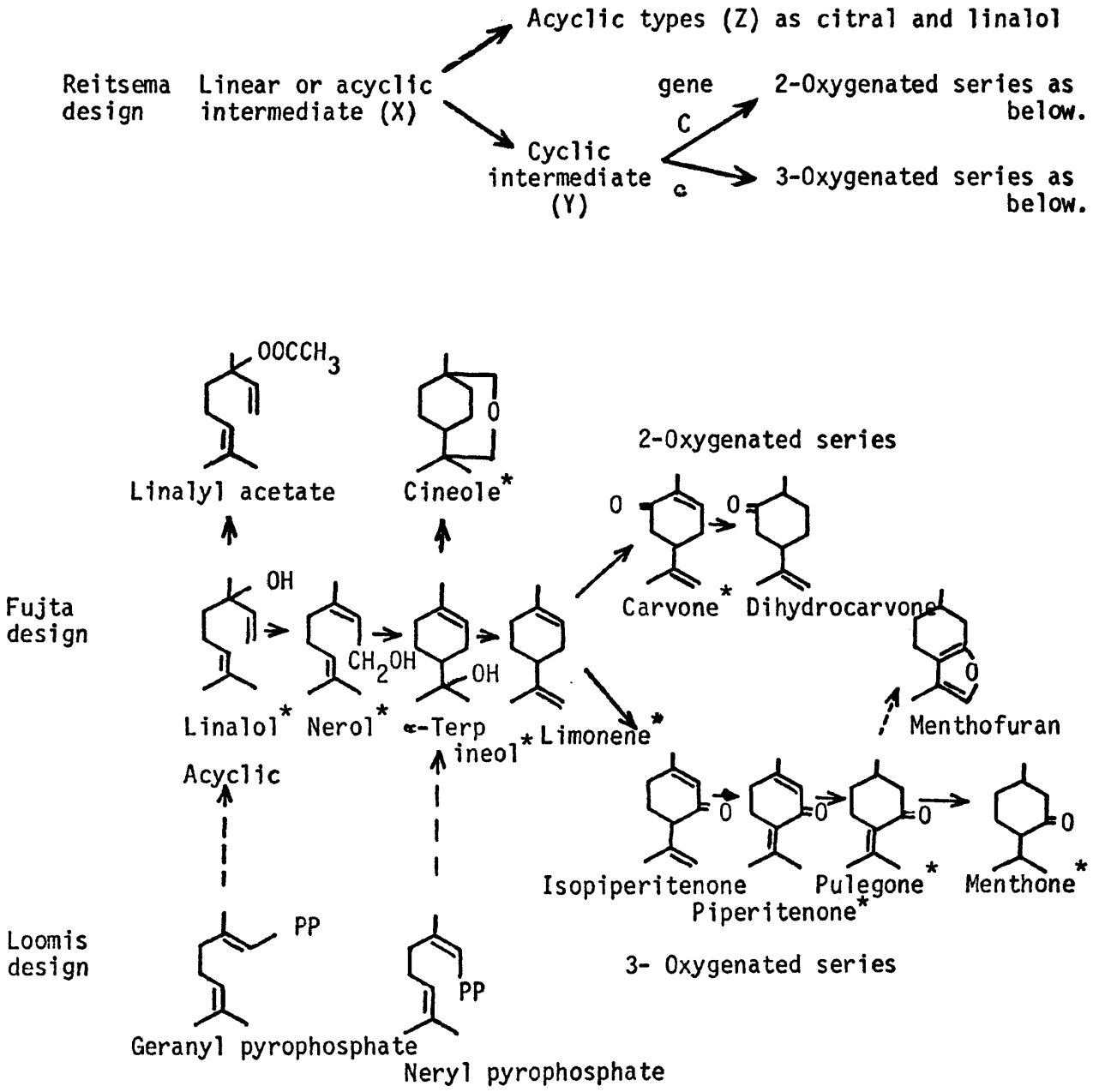
One of the aims of this investigation was to evaluate the pharmacological properties of the volatile oil obtained from Horehound, when its composition was known. Of the oil components which have been identified, none has proved to be a new compound. Two of the components which have been isolated and identified are stated to have the following properties (72): eugenol (6.6% of the oil) acts as an analgesic and anethole (2.1% of the oil) is considered to be an expectorant compound. As those components which have still to be identified are present only in trace amounts, it seems unlikely that they are individually responsible for any pharmacolo-

gical action. It has been suggested that volatile oils (154,155,156) and marine sesquiterpenoid compounds (157) may have antimicrobial activity. Therefore, the successful use of the herb in the treatment of pulmonary affections and as a vermifuge, may be due to the presence of the volatile oil. It is difficult to conclude from the evidence of this investigation whether the volatile oil has any pharmacological properties, and if it does, whether they can be attributed to the oil as a whole or to any of its individual components.

Murray and Lincoln (158) correlated the different biochemical relationships which had been proposed by different authors for the principle monoterpenes of Peppermint oil. These are shown in Figure 81A which gives an abbreviated diagram of the synthesis of these components and illustrates the differences between the three designs postulated for the origin of cyclic from acyclic compounds. As shown in Figure 81B, Burbott and Loomis (159) proposed a similar scheme but included some extra compounds. These biochemical schemes could also explain the relationships which occur between eleven of the components found in Horehound oil. This suggests that these biosynthetic pathways are also present in *Marrubium vulgare* L.

Hefendehl (160) reported that the composition of Peppermint oil was very variable, the absolute age of the leaf and the moment of collection being the main causes of variability in fresh leaves. As the age of the leaves increased, the amount of hydrogenated compounds in the oil, for instance menthol rather than menthone, also increased. In studies to examine the seasonal variations which took place in the composition of the oil of *Mentha arvensis* var. *glabrata* (114), it was found that significant changes in the quantitative composition occurred only in very young plants. The maximum yield of oil was obtained at the start of the flowering period, and there was practically no variation in composition when oils were obtained from plants grown in different localities.

(A)



(B)

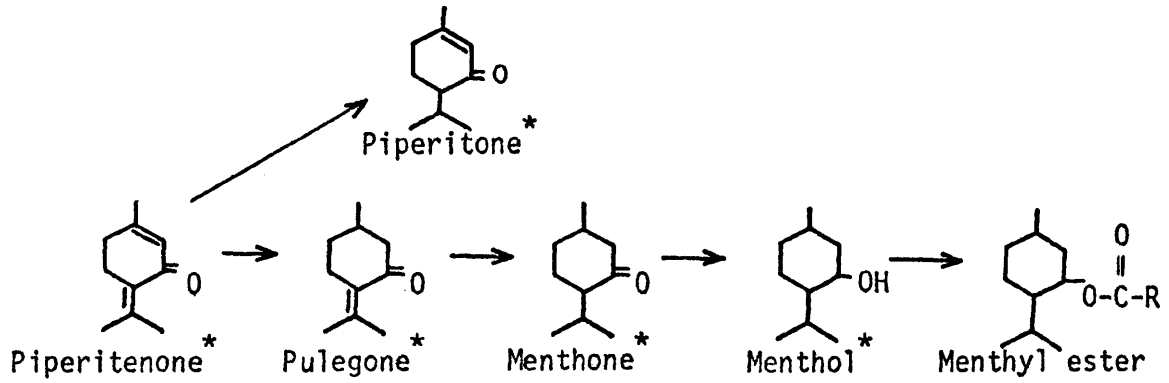


FIGURE 81: Biochemical relationships of monoterpenes in *Mentha* spp (158,159).
*: Compounds present in the volatile oil of *Marrubium vulgare* L.

In the current investigation, experiments have been carried out to study changes which might occur in the chemical composition of Horehound oil during the growing season. To enable comparisons to be made between the composition of volatile oils obtained from leaf pairs of different ages, the concentration of each component was made relative to linalol. Although it was realized that linalol could be a dynamic constituent, the use of this method in conjunction with an estimate of the absolute linalol content, should overcome the problem of changes in oil content which could occur from sample to sample. Some components, for example geraniol, farnesol, eugenol and piperitenone oxide were present in relatively larger quantities in the fresh plant, especially in the early stages of the growing period. The presence of large amounts of geraniol and farnesol could be due to their role as precursors in the biosynthesis of many of the components found in the oil. The dynamic nature of the components in the volatile oil, is shown by component 42 which was first detected when plants were 235 days old. This compound, thought to be a sesquiterpene, then increased in amount until it became the major component in plants of 297 days old (Figure 77). In the dry herb, it was present in trace amounts only. This turnover of component 42, even during this late period of growth, suggests that the sesquiterpenes which accumulate in the oil at this stage, are not necessarily end products.

The increases in individual components occurred during active periods of growth prior to stem branching, 131 days old, and flowering, 187 days old (Figure 74). In the early stages of growth, monoterpenoid compounds were the major components present in the oil, an increase in sesquiterpene hydrocarbon concentration taking place towards the flowering period. It was also noted that the accum-ulation of the diterpene marrubiin coincided with increases in sesquiterpene content. This seems to suggest that the

plant's potential to produce different compounds during the growing season is linked to the maturity of the plant and changes in its morphology. In the young plant, simpler monoterpenes are the major products of synthesis, whilst during and after flowering the more complex sesquiterpenes and diterpenes are favoured.

From the results of kinetic studies using radioactive carbon dioxide ($^{14}CO_2$), Burbott and Loomis (161) suggested that the monoterpenes of *Mentha piperita* L. were subject to metabolic turnover. Peppermint cuttings rapidly incorporated the label into the monoterpenes but it was then lost without any corresponding change in the amount of monoterpenes present. When Peppermint plants were grown in a controlled environment and analyzed at intervals, leaf pair by leaf pair, there was a steady increase in monoterpenes until the time of floral initiation, followed by a rapid decrease. They suggested that monoterpenes might serve as substrates for energy metabolism in the secretory cells after other stored substrates have been depleted. Further studies by Loomis et al. (159, 162-164) investigated the metabolic turnover of labelled monoterpenes, sesquiterpenes and triterpenes. From the differential utilization of labelled precursors they concluded that the monoterpenes, the sesquiterpenes and even the triterpenes were produced at different sites in the plant. The site at which the formation and metabolism of monoterpenes occurred, was not readily accessible either to carbon substrates or to oxygen. It seemed likely that only certain metabolites, such as sucrose, could reach the secretory cells, and the mevalonic acid required for monoterpene synthesis must be produced within these cells. Such a situation would make the cells of the oil gland extremely dependent on the types and amounts of substrates available in adjacent cells.

These findings could explain the trends observed in *Marrubium vulgare* L. Seasonal variations in oil composition could be brought about by alterations to the reaction rate of each synthesis at the individual sites, or a change in turnover rate might be responsible for the accumulation or depletion of a particular compound. Alternatively, fluctuations in oil composition may be linked to substrate availability. Whether one, two or all three mechanisms are involved, they would seem to be governed by changes in plant maturity.

The suggestion made by Loomis and his co-workers that different sites of synthesis are involved in the production of different constituents of the volatile oil, is at variance with the findings of Sticker and Flück (95). They stated that there was no qualitative difference between samples of *Mentha piperita* L. oil obtained by distillation, solvent extraction or by direct isolation from the oil glands by means of glass capillaries. As this last method removes oil from one specific site of synthesis, it would be expected to have a composition which was different from that of the other two, according to the criteria mentioned previously.

As the quantity of plant material available for investigation was limited and the amount of oil present was small, it was not possible to determine the oil content of the plants at different times during the growing season. However, by using a standard method of micro-distillation and identical conditions of g.l.c. analysis, variations in the amounts of the individual components present in the oil have been observed. Although absolute values of oil content cannot be obtained from these data, they can be used to predict changes in oil content. Since the total amount of the individual components is greatest in the younger leaves, it is suggested that the volatile oil content is highest at this stage. The quantity of the volatile oil present in the plant fluctuates throughout the growing period, two peaks

being noted (Figure 74). The first increase occurred when plants were 131 days old, which was at the time when there was considerable elongation of the plant stem and a general increase in plant vigour. The second peak occurred prior to flowering when plants were between 187 and 222 days old, coinciding with the start of marrubiin accumulation. However, unlike the marrubiin which continued to accumulate, the volatile oil content then dropped markedly, no further increases being detected.

The marrubiin content and the composition of volatile oils of leaves from different nodes were investigated. It was found that the volatile oil distilled from a given pair of leaves only differed in its quantitative composition from the volatile oil obtained from leaves removed at another node. Also, each pair of leaves had a different marrubiin content, and at full bloom, the younger leaves had the highest concentration. From these results, it is suggested that the biosynthetic activity of each leaf pair is independent of other leaf pairs on the same plant, but all are influenced by plant vigour and maturity. It is also concluded that for any analysis of volatile oil or determination of marrubiin content, the stage of plant growth should be stated.

As previously stated, much evidence has been recorded in respect of the occurrence of marrubiin in the plant. Several suggestions have been made; 1. that marrubiin is an artifact arising from the volatile oil (30), 2. that it is an artifact arising from a diterpenoid material named premarrubiin (25) and 3. that it is present in the plant during the flowering period (24). The marrubiin content of plants is said to vary from 0 to 1% according to the season of harvesting, the conditions of drying and the length of storage.

In the present work, regardless of the extraction technique used, the presence of premarrubiin in the fresh or dried herb has not been established. It did not make any difference whether the herb was shaken with cold acetone for thirty minutes (the mild conditions suggested for premarrubiin extraction) or whether the herb was continuously extracted for twenty hours with hot acetone in a Soxhlet apparatus, marrubiin and a keto lactone were the only compounds to be isolated.

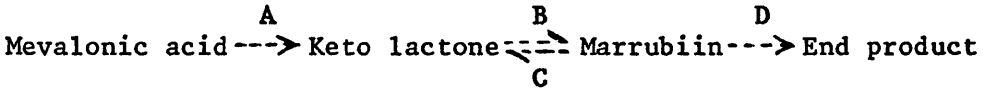
Marrubiin was first detected in trace amounts in growing plants which were 131 days old. It then disappeared, before it was again detected, this time in a considerable quantity, during the active period of growth prior to flowering (222 days old). The concentration dropped slightly as the first flowers developed, then increased again during the full bloom stage, reaching its maximum concentration (1.03%) after the formation of the seeds. By feeding labelled precursors to Horehound plants at the flowering stage, Breccia and Badiello (24) found that marrubiin had a half life of about twenty-four hours, indicating that it was an intermediate compound in some non-defined biosynthetic pathway whose end products were not identified. The increase in marrubiin content which was observed in the current investigation after plants had flowered, suggests that as the plant becomes older, this turnover rate decreases until no more marrubiin is metabolised. This leads to an accumulation of this metabolite. If marrubiin has an extremely short half life in young plants, it would explain why this compound was not detected in the early stages of growth.

The effects of drying on marrubiin formation in the herb have also been investigated. A sample of fresh leaves was taken from plants which were 187 days old, and on extraction was found to be devoid of marrubiin or related compounds. An identical sample was immediately oven dried and a further sample was allowed to air dry. No marrubiin was detected in either sample after drying, although a keto lactone was present in the air dried

material. As volatile oil was present at this stage of plant growth, marrubiin cannot be an artifact arising from the volatile oil during drying. When marrubiin was known to be present in the growing plants (at 222 days) a further sample was allowed to air dry before extraction. The marrubiin content was found to have decreased markedly on drying, only trace amounts now being present. However, a keto lactone (R_f 0.40), which was not present in the sample before drying, was now the major Ehrlich positive spot.

These experiments proved that marrubiin is not an artifact which arises either from premarrubiin (25) or the volatile oil (30) during the drying of the herb. In fact, on air drying, the marrubiin content of the herb decreases, with a corresponding increase in the keto lactone content.

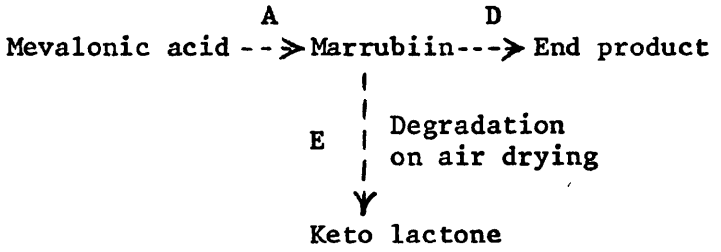
Mangoni et al. (28) suggested that a keto lactone (Figure 4), easily obtained by the degradation of marrubiin, could be an intermediate in marrubiin synthesis. Starting with this compound, they were able to synthesise marrubiin, but they do not state whether they considered this keto lactone to be an intermediate in the plant. The evidence suggests that the Ehrlich positive compound (R_f 0.40) isolated in the present work was in fact the keto lactone studied by Mangoni et al. If this compound is an intermediate in marrubiin biosynthesis, the following series of reactions may be proposed to explain what happens in the plant during growth and on drying.



Before flowering, conversions A, B and D proceed at equal rates to produce an unidentified end product, the keto lactone and marrubiin not being detected because they have a rapid turnover. The air drying of leaf samples collected at this stage favoured reactions A and C, as the keto lactone was detected after such treatment. On rapid oven drying, neither the keto

lactone nor marrubiin accumulated. After flowering, stages A and B continue at an equal rate but reaction D ceases, leading to an accumulation of marrubiin. During the air drying of samples collected at this stage, conversions A and C again take place, with a subsequent accumulation of the keto lactone.

Alternatively, the keto lactone is not an intermediate in the biosynthesis of marrubiin but merely a degradation product formed during air drying. This seems a more likely explanation.



If conversion E is enzyme controlled and marrubiin is thermo-stable, rapid oven drying may prevent the formation of the keto lactone.

Although there is no evidence to show how the marrubiin content of the herb changes on prolonged storage, the above results indicate that time of harvesting and conditions of drying affect its concentration in the commercial herb. The Hungarian Pharmacopoeia (63) states that Horehound herb should be used before flowering, the young soft-stalked aerial shoots being preferred. Clapham (41) recommended that it be used when in flower and although it could be used after drying, it was certainly less active than when fresh. In the former case no marrubiin would be present in the plant, in the latter case, although present, it would not be at its maximum concentration. If pharmacological activity is dependent on marrubiin content, Horehound herb should be collected after seed formation and either used when fresh or if stored, dried by a method which prevents keto lactone formation.

When considering the suitability of flavonoids for use in chemotaxonomic studies, Harborne (165) stated that they possessed the following necessary requirements: (a) chemical complexity and structural variation, (b) physiological stability, (c) widespread distribution and (d) easy and rapid identification. Von Rudloff (82) suggested that as terpenes also fulfilled these criteria, they might also be used in a similar way. He also stated that qualitative differences might lead to correlations at higher taxonomic levels whereas quantitative differences might be characteristic of individual species. On comparing the compositions of oils obtained from different genera of the Labiatae qualitative differences were observed. For example, Lawrence et al. (115) recorded the presence of ninety-nine components in the volatile oil of *Mentha piperita* L. and when these are compared with the components which have been identified in the volatile oil of *Marrubium vulgare* L., it can be seen that some are common to both oils whilst others are present only in one. At the present time, anethole, borneol, bornyl acetate, camphor, carvone, farnesol, geraniol and α-patchoulene are found only in Horehound oil, menthofuran, menthyl acetate and thymol being found only in Peppermint oil. As far as individual species in the genus *Marrubium* are concerned, a study of the chemical composition of the volatile oils obtained from samples of *M. vulgare* L. and *M. peregrinum* L. revealed that they differed quantitatively but not qualitatively. It had been hoped to study the composition of volatile oils obtained from further species of this genus, but plant materials and seeds were not available. However, this limited evidence would seem to support the proposition made by von Rudloff.

The original system devised for the classification of the family Labiatae was established by Bentham (34) who suggested that it could be divided into two groups as already outlined in section six of the Introduction. The criteria used by Bentham as the basis of this division are

not clearly known, but using this system, nearly all of the plants which are commercially important as producers of volatile oils are included in one group. Over the years, information has been accumulating to support this division of the family into two groups. This evidence includes, the morphology of the pollen grains (85), the patterns of seed development and mature seed anatomy (85), the susceptibility to rust (86), the volatile oil content (33), the linolenic acid content of the fixed oils in the seeds (88) and the presence of iridoid glycosides (42).

The last three studies represent the first attempts to classify members of the family Labiatae on a chemical basis. El-Gazzar and Watson (33) attempted to subdivide the family according to whether plants had a high oil content (group I) or a low oil content (group II). Using these criteria, good correlation was found between the existing traditional classification and the proposed chemical classification. However, when problems arose, rather than using differences in the chemical composition of the volatile oils, these authors reverted to morphological characters to resolve the anomaly. When using oil content, they found that the genera *Pogostemon* and *Prostanthera* were placed in group I where previously they would have been classified with plants which were found in group II. Conversely, the genera *Prunella* and *Cleonia* now fell in group II instead of group I, as they might have expected. The morphology of the stamens was used to transfer these genera back to their original groups (86). Kooiman (42) considered that the presence of iridoid glycosides should be used in conjunction with low oil content as a criterion for classification, but this still did not resolve the problem posed by the genus *Pogostemon*.

To establish a more reliable system of chemotaxonomy, it is suggested that all the groups of compounds which are produced by related biosynthetic pathways should be considered. Therefore, in the case of the Labiatae, all the following types of compound, not just one, should be used in any system

of chemical classification; iridoids, monoterpenes, sesquiterpenes, diterpenes and other related compounds (Figure 5). In this way the more complex as well as the simple compounds would be used in the classification of the family. This ideal was held by Ponsinet et al.(166) who stated that more chemotaxonomic significance could be attached to compounds as they became more distant from the simple direct pathway. Diterpenes have not received any attention in this connection, but their presence has been recorded in the following genera: *Marrubium* (18,21,167), *Sideritis* (168), *Stachys* (169), *Leonotis* (170), *Teucrium* (171), *Rosmarinus* (172) *Lavandula* (173) and *Plectranthus* (174). A knowledge of all the types of terpenoid compounds which are present plus information on the quantity of each, gives an indication of the "biosynthetic capability" of a plant. It is felt that this criterion could be used to advantage in chemotaxonomy.

To illustrate how the idea of "biosynthetic capability" might be used in the classification of the Labiatae, the following is given as an example. It was noted that the quantitative nature of the oil composition, especially in terms of groups of compounds rather than individuals, differed from genus to genus. The volatile oil of *Mentha piperita* L. (115) although high in content, is composed mainly of simpler monoterpenoid compounds (84%). The situation in *Lavandula officinalis* Chaix. (117), which also has a high oil content, is similar, the oil being composed mainly of the relatively simple compounds of linalol (31%) and linalyl acetate (38%). Although the presence of a diterpene has been recorded, the amounts are small. On the other hand, the volatile oil of *Marrubium vulgare* L., as has been shown by this investigation, although low in content is composed mainly of the more complex sesquiterpenoid compounds (35%). In addition, large amounts of the diterpene marrubiin are present in this plant. Unlike *Marrubium vulgare* L., *Pogostemon patchouli* has a high oil content, but it still has

the same capability to produce more complex compounds (153), the oil being composed mainly of the sesquiterpenes, patchouly alcohol, α-guaiene, α-bulnesene and β-patchoulene (total 79%). According to these data it is more reasonable to group the genera of *Pogostemon* and *Marrubium* together based on their potential to produce more complex compounds. Thus by considering their "biosynthetic capabilities", *Pogostemon* would be classified into group II without having to resort to morphological characters. Although only one species from each genus has been used in this example, further studies along these lines could prove to be rewarding.

As advances have been made in the techniques available for plant analysis, interest in chemotaxonomy has increased. However, in addition to the variations which have been observed in volatile oil composition during the growing season, differences can occur due to artifact production during extraction and analysis. Therefore, it may be difficult to reach correct conclusions if data, already recorded by various workers who used different and less sensitive techniques are compared with more recent results. To obtain the reliable information which is required to establish chemotaxonomy on a sound basis, standard techniques should be used.

Only the role of terpenoid compounds in the chemotaxonomy of the family Labiatae, has been considered in this discussion. The presence of other plant constituents for example alkaloids, should not be overlooked. These latter compounds are reported to be present not only in *Marrubium vulgare* L. (21, 64-66) but also in *Ajuga australis* R.Br., *Hyssopus officinalis* L. and *Salvia polystachys* Drt. (175), information which could be extremely important in the further classification of the family. Until detailed information is available about all the constituents which are present in each species of the family, it is concluded that traditional taxonomy and chemotaxonomy should be used as complimentary methods of classification.

APPENDIX

TABLE 18

The relative retention times of reference compounds to linalol on given stationary phases using a range of temperatures

Stationary phase	Column temperature °C				
	130	145	160	175	190
	α-Pinene (A)				
20M	0.14	0.17	0.20	0.27	0.35
DEGS	0.17	0.19	0.27	0.36	0.45
SE30	0.45	0.50	0.54	0.56	0.78
	β-Pinene (A)				
20M	0.17	0.24	0.29	0.36	0.42
DEGS	0.19	0.27	0.32	0.42	0.51
SE30	0.56	0.63	0.65	0.68	1.19
	Limonene (A)				
20M	0.25	0.29	0.35	0.42	0.50
DEGS	0.26	0.32	0.39	0.48	0.55
SE30	0.69	0.74	0.76	0.78	0.97
	Cineole (A)				
20M	0.26	0.32	0.37	0.45	0.52
DEGS	0.30	0.38	0.43	0.55	0.63
SE30	0.69	0.76	0.78	0.82	1.03
	Linalyl acetate (B)				
SE30	0.76	0.80	0.81	0.82	0.97
DEGS	1.20	1.18	1.11	0.93	0.94
20M	1.12	1.11	1.13	1.09	1.10
	Terpinyl acetate (B)				
SE30	2.41	1.72	1.12	1.00	1.06
DEGS	1.98	1.90	1.79	1.70	1.63
20M	2.11	2.06	2.03	1.91	1.87

(cont.)

Table 18 (cont.)

Stationary phase	Column temperature °C				
	130	145	160	175	190
	Geraniol (C)				
SE30	1.40	1.19	1.10	1.07	0.91
DEGS	3.30	3.00	2.61	2.40	2.06
20M	4.16	3.74	3.32	2.93	2.65
	Citronellol (C), (H&W)				
SE30	2.19	2.02	1.80	1.67	1.64
DEGS	2.23	2.05	1.86	1.75	1.63
20M	2.85	2.62	2.42	2.18	2.00
	Menthol (C)				
DEGS	1.35	1.35	1.32	1.33	1.25
SE30	1.62	1.54	1.50	1.43	1.45
20M	1.59	1.57	1.55	1.48	1.48
	Anethole (D)				
SE30	2.50	2.35	2.20	2.00	1.95
DEGS	3.48	3.30	3.00	2.85	2.50
20M	3.70	3.48	3.26	3.02	2.77
	Safrole (D)				
SE30	2.51	2.39	2.25	2.03	2.05
DEGS	4.50	4.20	3.79	3.50	3.00
20M	4.59	4.17	3.94	3.55	3.32
	Camphor (E)				
20M	1.00	1.08	1.16	1.18	1.23
SE30	1.22	1.26	1.28	1.27	1.31
DEGS	1.18	1.10	1.29	1.38	1.32
	Carvone (E)				
SE30	2.02	1.96	1.85	1.77	1.77
20M	2.46	2.43	2.39	2.27	2.23
DEGS	2.82	2.75	2.57	2.50	2.25

(cont.)

Table 18 (cont.)

Stationary	Column temperature °C				
phase	130	145	160	175	190
	Citral (E)				
SE30	2.42	2.28	2.05	1.87	1.82
20M	2.60	2.35	2.28	2.09	2.03
DEGS	2.80	2.63	2.39	2.28	2.00

TABLE 19

The relative retention times of some oil components of *Marrubium vulgare* L. to linalol on given stationary phases using a range of temperatures

Component number	Stationary phase	Column temperature °C				
		130	145	160	175	190
5	20M	0.14	0.17	0.20	0.27	0.35
	DEGS	0.17	0.19	0.27	0.36	0.45
	SE30	0.45	0.50	0.54	0.56	0.78
8	20M	0.16	0.21	0.24	0.33	0.38
	DEGS	0.19	0.24	0.27	0.42	0.51
	SE30	0.50	0.55	0.60	0.64	0.91
9	20M	0.17	0.24	0.29	0.36	0.42
	DEGS	0.19	0.27	0.32	0.42	0.51
	SE30	0.56	0.63	0.65	0.68	1.19
11	20M	0.25	0.29	0.35	0.42	0.50
	DEGS	0.26	0.32	0.39	0.48	0.55
	SE30	0.69	0.74	0.76	0.78	0.97
12	20M	0.26	0.32	0.37	0.45	0.52
	DEGS	0.30	0.38	0.43	0.55	0.63
	SE30	0.69	0.76	0.78	0.82	1.03
13	20M	0.26	0.33	0.37	0.60	0.50
	DEGS	0.28	0.35	0.41	0.52	0.59
	SE30	0.69	0.74	0.78	0.82	1.00
14	20M	0.32	0.38	0.43	0.51	0.56
	DEGS	0.34	0.41	0.48	0.55	0.65
	SE30	0.66	0.71	0.76	0.78	0.94
15	20M	0.34	0.40	0.45	0.52	0.58
	DEGS	0.34	0.38	0.48	0.55	0.63
	SE30	0.92	0.96	0.97	0.96	1.19
18	20M	0.47	0.51	0.55	0.61	0.69
	DEGS	0.88	0.86	0.90	0.96	1.06
	SE30	1.16	1.29	1.24	1.14	1.31

(cont.)

Table 19 (cont.)

Component number	Stationary phase	Column temperature °C				
		130	145	160	175	190
19	20M	0.47	0.52	0.55	0.59	0.65
	DEGS	0.48	0.53	0.57	0.65	0.69
	SE30	0.60	0.67	0.73	0.77	0.82
21	20M	0.53	0.58	0.63	0.68	0.74
	DEGS	0.60	0.65	0.68	0.70	0.75
	SE30	0.93	0.96	1.00	1.00	0.91
23	SE30	0.55	0.57	0.63	0.67	0.73
	DEGS	0.60	0.65	0.68	0.70	0.75
	20M	0.62	0.66	0.68	0.68	0.74
26	20M	0.80	0.86	0.85	0.94	0.96
	DEGS	0.89	0.92	1.18	1.24	1.25
	SE30	1.31	1.31	1.29	1.29	1.47
29	20M	0.80	0.86	0.92	1.00	1.04
	DEGS	0.89	0.97	1.00	1.06	1.06
	SE30	1.28	1.30	1.29	1.28	1.47
30	SE30	1.00	1.00	1.00	1.00	1.00
	DEGS	1.00	1.00	1.00	1.00	1.00
	20M	1.00	1.00	1.00	1.00	1.00
31	DEGS	0.70	0.73	0.79	0.85	0.87
	20M	0.91	1.00	1.05	1.09	1.13
	SE30	4.09	3.77	3.35	3.00	2.82
33	20M	1.00	1.08	1.16	1.18	1.23
	SE30	1.22	1.26	1.28	1.27	1.31
	DEGS	1.18	1.10	1.29	1.38	1.32
35	20M	1.20	1.28	1.31	1.38	1.40
	DEGS	1.36	1.41	1.43	1.42	1.41
	SE30	1.62	1.63	1.56	1.56	1.75
36	20M	1.33	1.37	1.38	1.44	1.40
	DEGS	1.36	1.41	1.41	1.36	1.35
	SE30	2.53	2.46	2.28	2.14	2.38

(cont.)

Table 19 (cont.)

Component number	Stationary phase	Column temperature °C				
		130	145	160	175	190
38	DEGS	1.25	1.25	1.21	1.25	1.19
	20M	1.46	1.55	1.60	1.59	1.61
	SE30	5.14	4.70	4.30	3.80	3.59
39	DEGS	1.35	1.35	1.32	1.33	1.25
	SE30	1.62	1.54	1.50	1.43	1.45
	20M	1.59	1.57	1.55	1.48	1.48
40	SE30	1.56	1.54	1.47	1.42	1.63
	20M	1.82	1.75	1.77	1.75	1.73
	DEGS	1.87	1.79	1.77	1.70	1.61
41	20M	1.82	1.79	1.79	1.88	1.83
	DEGS	1.94	1.90	1.86	1.79	1.76
	SE30	2.01	1.98	1.85	1.80	2.03
43	SE30	1.70	1.64	1.57	1.52	1.72
	DEGS	1.98	1.90	1.82	1.73	1.61
	20M	2.05	1.96	1.90	1.88	1.77
44	DEGS	1.60	1.55	1.54	1.50	1.44
	20M	1.95	2.02	2.05	1.98	1.97
	SE30	6.00	5.40	4.90	4.27	3.98
45	DEGS	1.51	1.46	1.45	1.36	1.02
	20M	2.04	2.10	1.94	1.97	1.94
	SE30	-	5.83	5.06	4.40	4.59
46	SE30	1.59	1.54	1.50	1.44	1.69
	DEGS	1.84	1.87	1.82	1.82	1.80
	20M	2.09	2.11	2.02	2.00	1.94
47	DEGS	1.70	1.64	1.63	1.60	1.54
	20M	2.35	2.35	2.34	2.27	2.23
	SE30	6.81	6.10	5.35	4.80	4.41
48	SE30	2.02	1.96	1.85	1.77	1.77
	20M	2.46	2.43	2.39	2.27	2.23
	DEGS	2.82	2.75	2.57	2.50	2.25

(cont.)

Table 19 (cont.)

Component number	Stationary phase	Column temperature °C				
		130	145	160	175	190
49	SE30	2.22	2.14	2.00	1.90	2.16
	20M	2.52	2.48	2.42	2.44	2.33
	DEGS	2.83	2.60	2.64	2.42	2.27
50	DEGS	1.97	1.85	1.70	1.63	1.50
	20M	2.81	2.76	2.68	2.52	2.39
	SE30	8.33	7.15	6.20	5.25	4.75
51	SE30	1.40	1.38	1.18	1.02	1.06
	DEGS	2.87	2.71	2.41	2.12	1.53
	20M	3.29	2.93	2.75	2.59	2.31
52	DEGS	2.15	2.00	1.95	1.70	1.69
	20M	3.04	3.07	2.83	2.81	2.58
	SE30	-	-	-	5.52	5.63
53	SE30	2.50	2.35	2.20	2.00	1.95
	DEGS	3.48	3.30	3.00	2.85	2.50
	20M	3.70	3.48	3.26	3.02	2.77
55	SE30	1.40	1.19	1.10	1.07	0.91
	DEGS	3.30	3.00	2.61	2.40	2.06
	20M	4.16	3.74	3.32	2.93	2.65
56	SE30	2.51	2.39	2.25	2.03	2.05
	DEGS	4.50	4.20	3.79	3.50	3.00
	20M	4.59	4.17	3.94	3.55	3.32

TABLE 20

The relative retention times of some reference standards to linalol on given stationary phases at various temperatures

Compound	Stationary phase	Column temperature °C				
		130	145	160	175	190
α-Pinene (H & W)	20M	0.14	0.17	0.20	0.27	0.35
	DEGS	0.17	0.19	0.27	0.36	0.45
	SE30	0.45	0.50	0.54	0.56	0.78
Camphene (Emanuel)	20M	0.16	0.21	0.24	0.33	0.38
	DEGS	0.19	0.24	0.27	0.42	0.51
	SE30	0.50	0.55	0.60	0.64	0.91
β-Pinene (Koch-Light)	20M	0.17	0.24	0.29	0.36	0.42
	DEGS	0.19	0.27	0.32	0.42	0.51
	SE30	0.56	0.63	0.65	0.68	1.19
Limonene (BDH)	20M	0.25	0.29	0.35	0.42	0.50
	DEGS	0.26	0.32	0.39	0.48	0.55
	SE30	0.69	0.74	0.76	0.78	0.97
Cineole (Fisons)	20M	0.26	0.32	0.37	0.45	0.52
	DEGS	0.30	0.38	0.43	0.55	0.63
	SE30	0.69	0.76	0.78	0.82	1.03
β-Phellandrene	20M	0.26	0.31	0.37	0.60	0.50
	DEGS	0.28	0.35	0.41	0.52	0.59
	SE30	0.69	0.74	0.78	0.82	1.00
p-Cymene (BDH)	20M	0.32	0.38	0.43	0.51	0.56
	DEGS	0.34	0.41	0.48	0.55	0.65
	SE30	0.66	0.71	0.76	0.78	0.94
Terpinolene	20M	0.34	0.40	0.45	0.52	0.58
	DEGS	0.34	0.38	0.48	0.55	0.63
	SE30	0.92	0.96	0.97	0.96	1.19
Allo-ocimene	20M	0.47	0.53	0.55	0.61	0.69
	DEGS	1.04	0.86	2.91	3.58	2.39
	SE30	1.16	1.29	1.24	1.14	1.31

TABLE 21

The retention times of some oil components of *Marrubium vulgare* L. on given columns using the appropriate temperature conditions*

Components	Retention time (minutes)		
	Carbowax 20M	DEGS	SE30
58	34.8	21.6	20.6
60	35.7	23.2	18.0
61	36.6	23.2	29.2
62	39.0	23.2	15.2
63	39.7	23.2	10.8
67	45.3	26.9	23.6
71	56.6	23.2	28.0

* Carbowax 20M column: 145°C for 32 minutes then 190°C thereafter.
DEGS column: 130°C for 20 minutes then 190°C thereafter.
SE30 column: 160°C for 45 minutes then 190°C thereafter.

TABLE 22

Data used for the construction of calibration curves used in the quantitative analysis of the volatile oil: Number of traverses produced in response to different concentrations of reference compounds

Conc. %	Number of traverses						
	α-Pinene	3-Octanol	1-Octen-3-ol	Linalol	β-Caryophyllene	Anethole	Eugenol
0.25	1.90						
0.50	3.70						
1.00	6.50	3.20	5.15			6.08	
2.00	9.78	11.40	10.88	14.80		12.73	10.78
3.00		16.35	14.40			20.28	
4.00	11.08	18.20	16.25	21.95	32.58	26.60	23.75
5.00		19.05	17.18			33.20	
6.00				27.33			30.65
8.00				30.25	53.35		37.33
10.00				32.55			40.60
12.00					64.65		
16.00					71.00		

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