ABSTRACT

A brief history of taxonomic advances in the Filicopsida (ferns) is presented. Biochemical studies of the ferns and the application of n-alkane composition to taxonomic problems in the Plant Kingdom are also included. A procedure for the isolation and identification of n-alkanes in the hexane extract of tropical tree ferns is reviewed. Sample cleanup is accomplished by saponification of the crude extract followed by silver nitrate-impregnated silica gel chromatography. Infrared spectroscopy and state-of-the-art gas chromatography are used to present confirmatory evidence of sample cleanup. The identification of n-alkanes in the hydrocarbon and unsaponifiable fractions is achieved by co-retention with known n-alkanes in hexane washings of Parafilm. A graphical presentation of the n-alkane composition is used to illustrate differences in the seven species studied. A more comprehensive study of the Cyatheaceae and the Dicksoniaceae is suggested to reveal chemotaxonomic applications.

THE ISOLATION AND IDENTIFICATION OF NORMAL ALKANES IN SEVEN SPECIES OF CYATHEACEAE AND DICKSONIACEAE

A Thesis

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Master of Science

by

David Lynn Heavner

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CHAPTER I

THE INTRODUCTION

The taxonomy and phylogeny of the ferns, the Filicopsida, has been in a state of flux for the last two centuries. Fortunately, improvements have been realized within the past fifty years because of a contributory effort by many taxonomists, morphologists, cytologists, paleobiologists, and, more recently, chemists. This effort is due in part to a realistic evaluation of the problems associated with the classification and phylogeny of the ferns. Not all of these problems are taxonomic in nature; the geographic diversity of the ferns, historical inaccuracies, political and personal differences, and economic pressures have all contributed in some ways to further compound the problem. The discussion that follows illustrates some of the difficulties related to fern taxonomy.

Most fern species are already extinct, and many living species are in danger of extinction due to a lack of responsible conservation policies in developing nations where many ferns are found. A few countries, such as Costa Rica, have the foresight to protect their tropical rain forests; others, such as India, have denuded thousands of square miles of virgin forest area that can never be

replaced.[1,2]

Over ninety percent of the extant species of ferns are found in tropical or hard-to-reach areas. Until recently, most interested scientists were unable to study the ferns in situ, and the number of herbarium species available to those involved in tackling fern taxonomy was limited. With modern travel this situation has improved.

Generally, the classification and phylogeny of the ferns has been subjective and, in many cases, artificial. Taxonomists interested in constructing a natural system based on true genetic relationships must be objective in their review of historical and current literature.

Taxonomists have been divided historically into two schools of thought: the splitters and the lumpers. As a result, there are still disagreements as to the number of large groups (orders and families) belonging to the Filicopsida. Since the ferns are relatively simple plants, the number of characteristics available for taxonomic and phyletic purposes is limited. The development of new characters, possibly chemical ones, is necessary to aid existing morphological and cytological techniques.

This discussion is complete by no means, but it does serve to illustrate the complexity of the problems on a grand scale. Although a comprehensive study of the

Filicopsida is not within the scope of this paper, a more limited review of two families, the Cyatheaceae and the Dicksoniaceae, is possible. These two families of tree ferns represent about 1000 species found in the tropics of the Old World and the Americas. Some taxonomists prefer to lump these two families into one larger group while others maintain that separate family status is more appropriate. Three monotypic genera, Lophosoria, Metaxya, and Thyrsopteris, are presently included in the Cyatheaceae and Dicksoniaceae, but recent cytological evidence places these genera into individual monotypic families.[3,4] The fourth monotypic genus, Cystodium, is usually included in the Dicksoniaceae. The delineation of the taxa in these two families would provide valuable information on the evolutionary development of the ferns (Figure 1). Unfortunately, classical taxonomic methods have failed to eliminate many questions concerning the taxonomy and phylogeny of the Filicopsida. The introduction of new taxonomic characters may be justified.

Within the past 20 to 30 years the search for new taxonomic characters has led to the involvement of chemists and to the development of a new branch of science, chemotaxonomy. Biochemical studies in the Plant Kingdom have demonstrated that the presence, absence, and distribution of certain secondary metabolic by-products may be useful

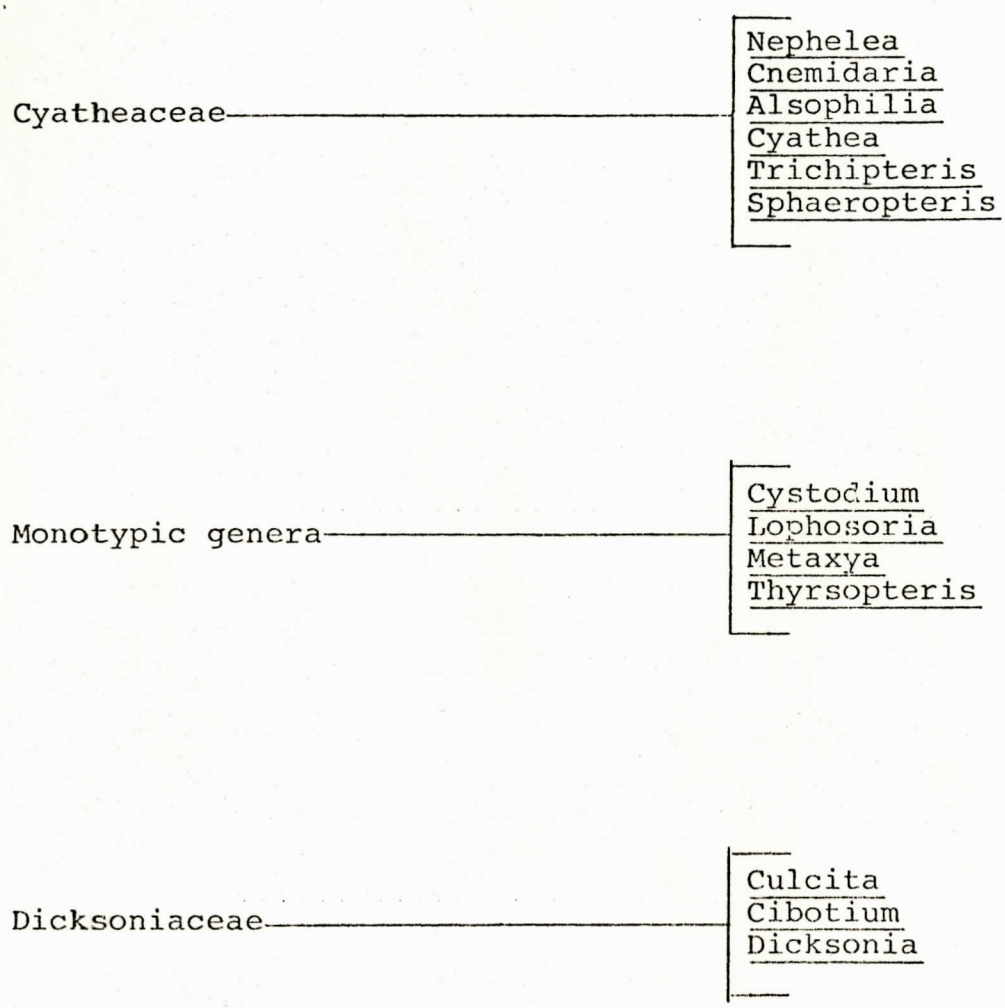

Figure 1. Classification of Cyatheaceae and Dicksoniaceae

in taxonomy where morphological or cytological characters have failed to eliminate questionable classifications. The advancement of chemotaxonomy as a useful tool has not endangered the position of more classical taxonomic methods; instead it has added another character for comparison studies. The continued improvement of analytical instrumentation for the identification of natural products has been phenomenal and may be compared to the development of the microscope for morphology and cytology. With this improvement the identification of chemical characters has become more accurate and routine.

The selection of chemical substances for taxonomic studies is valid since a plant's ability to synthesize compounds is determined by genetic characters just as any morphological or cytological feature is hereditary in nature. The selection of these compounds for chemotaxonomic studies is based on several considerations.[5] First, the substances should be secondary metabolites; they should not be involved in the primary metabolic pathways of the plant. Second, they should be impervious to environmental and analytical factors. Finally, the recovery of these constituents should be relatively simple and quantitatively reproducible. Fortunately, the waxy cuticle of plants appears to be a convenient reservoir of many secondary by-products and is an ideal area for study.

The normal alkanes, major constituents of the plant cuticle, prove to be suitable substances for chemotaxonomy since they fit the criteria previously mentioned. The purpose of this study is to develop an analytical method for the isolation and identification of normal alkanes in the fern cuticle. Seven species of tree ferns in the Cyatheaceae and the Dicksoniaceae are used for this purpose with the hope that their normal alkane distribution patterns may suggest more inclusive studies in the future. Classical extraction techniques, infrared spectroscopy, and state-of-the-art gas chromatography are used to attain this end.

CHAPTER II

THE HISTORICAL REVIEW

In April of 1972, the world leaders of fern systematics gathered in London for a Symposium on the Phylogeny and Classification of the Filicopsida. The purpose of this meeting was to review the historical advancement of fern taxonomy, to discuss specific taxonomic problems in the Filicopsida, and to outline directions for future studies. At that time, a detailed historical outline of the development of fern taxonomy was presented by R. E. G. Pichi Sermolli.[6] This presentation was one of the most comprehensive in recent times and is the basis for the historical review that follows. Only those events that resulted in positive contributions to fern taxonomy and phylogeny are included in this discussion.

Classical Fern Systematics

Andrew Cesalpino[7] in 1583 broke from the tradition of classifying plants according to their utilitarian nature and attempted a systematic approach to botany based on anatomical characteristics of the foliage and flowers of plants. In this study Cesalpino included a large group of plants possessing a conspicuous absence of seeds and flowers. He was the first to actually identify this group

known as the Cryptograms. Most researchers in the next century studied morphological characteristics of the ferns and did little to actually promote any new classification systems.

With the publication of his Species Plantarum and Genera Plantarum in the mid-1700's, Linnaeus[8,9] introduced the binomial system of nomenclature that is the backbone of systematic botany. This system was an artificial one and did not include any criteria for classification based on natural relationships. The early classifications of plants were artificial in that they were designed to serve practical purposes. The natural systems based on real relationships are more common today and are based on studies of genetically controlled characteristics such as morphological, cytological, and chemical features. Still, Linnaeus' contributions were outstanding as he succeeded in classifying 14 genera and 182 species of ferns.

During the same period, Wiggers[10], in his Primitiae Florae Holsaticae, proposed a division of the Filicopsida into three smaller groups, introducing a hierarchial system of classification to the ferns. In 1802, Mirabel[11] further subdivided the pteridophytes into four orders that he called families. Three of these groups of pteridophytes are still recognized today.

The first book devoted entirely to fern systematics

was Swartz's [12] Synopsis Filicum in 1806. Although organization of higher fern groups was poor, the delineation of genera was advanced for its time. De Candolle[13] in his Théorie élémentaire de la Botanique (1813) proposed the elevation of ferns to their present taxonomic level based on the presence of a vascular system. In 1827 Kaulfauss[14], an advanced pteridologist, suggested a familial classification scheme that included most of the fern families recognized today. His system was the first to recognize Cyatheaceae as a family.

The next few years were highlighted by the introduction of fossil record studies and natural classification systems into fern systematics. Brongniart[15], in his Histoires Végétaux Fossiles, was the first to recognize the importance of fossil records. Endicher[16] extended Brongniart's work to include living ferns; this study was the first to include fossil and living plants in the same classification system. Presl[17] advanced the cause even further with the publication of Testamen Pteridographiae in 1836. Presl was one of the first pteridologists to observe natural rather than artificial groups of plants. He identified more characters for comparison and as a result, his system included natural orders, families, and genera with relatively few species in each.

W. J. Hooker's[18,19,20] ideas concerning classification

were quite contrary to those of Presl. Hooker, in his three publications, Genera Filicum, Species Filicum, and Synopsis Filicum, suggested a lumping of genera artificially while Presl preferred splitting genera into larger natural groups.

One of the most comprehensive works in the mid-1800's was Fée's[21] Mémoires sur la Famille des Fougeres. In this series of eleven volumes, Fée recognized 188 genera and greatly improved classifications on the familial level. Much of his work was based on Presl's earlier publication. The first book to actually deal with the phylogeny of the ferns was L'évolution du Règne Végétale. Les Cryptogames by DeSaporta and Marion.[22] Published about twenty years after Darwin's theory of evolution, this important work again expressed the necessity of including fossil records for taxonomy.

Index Filicum by Christensen[23] did not change the accepted familial classifications of the day, but it did include valuable information for research on the generic and specific level. In the 1920's Bower[24] published The Ferns, a progressive study on the phylogeny of the ferns. "His book is an endless source of information," according to Sermolli, "and probably no other pteridologist contributed, in such a large measure as Bower, to our knowledge

of the ferns."[25]

In 1929 Copeland[26] published a revised version of The Oriental Genera of Polypodiaceae in which he attempted to classify these ferns according to their phylogenetic standing. Apparently he was torn between the practice of splitting and lumping as he recognized the validity of both viewpoints. In 1947 Copeland[27] published Genera Filicum in which he lumped all living ferns into 3 orders and 20 families (Figure 2). Although this book is one of the best available on fern taxonomy, some of his colleagues considered his familial classifications defective.

R. E. G. Pichi Sermolli[28] in 1958 proposed the only major recent revision of any taxonomic importance. His detailed classification system included 7 subclasses, 24 orders, and 55 families (Figure 3).

The remainder of the century has been devoted primarily to the advancement of cytotaxonomy and phytochemistry. Manton's[29] book, Problems of Cytology and Evolution in the Pteridophyta, led to increased interest in cytology and cytotaxonomy. Following Manton's lead, Mehra[30] published a phylogenetic system based on cytological characters in 1961. In 1977 Love et al[4] published a book entitled Cytochemical Atlas of the Pteridophyta that uses cytological characters for their classification purposes.

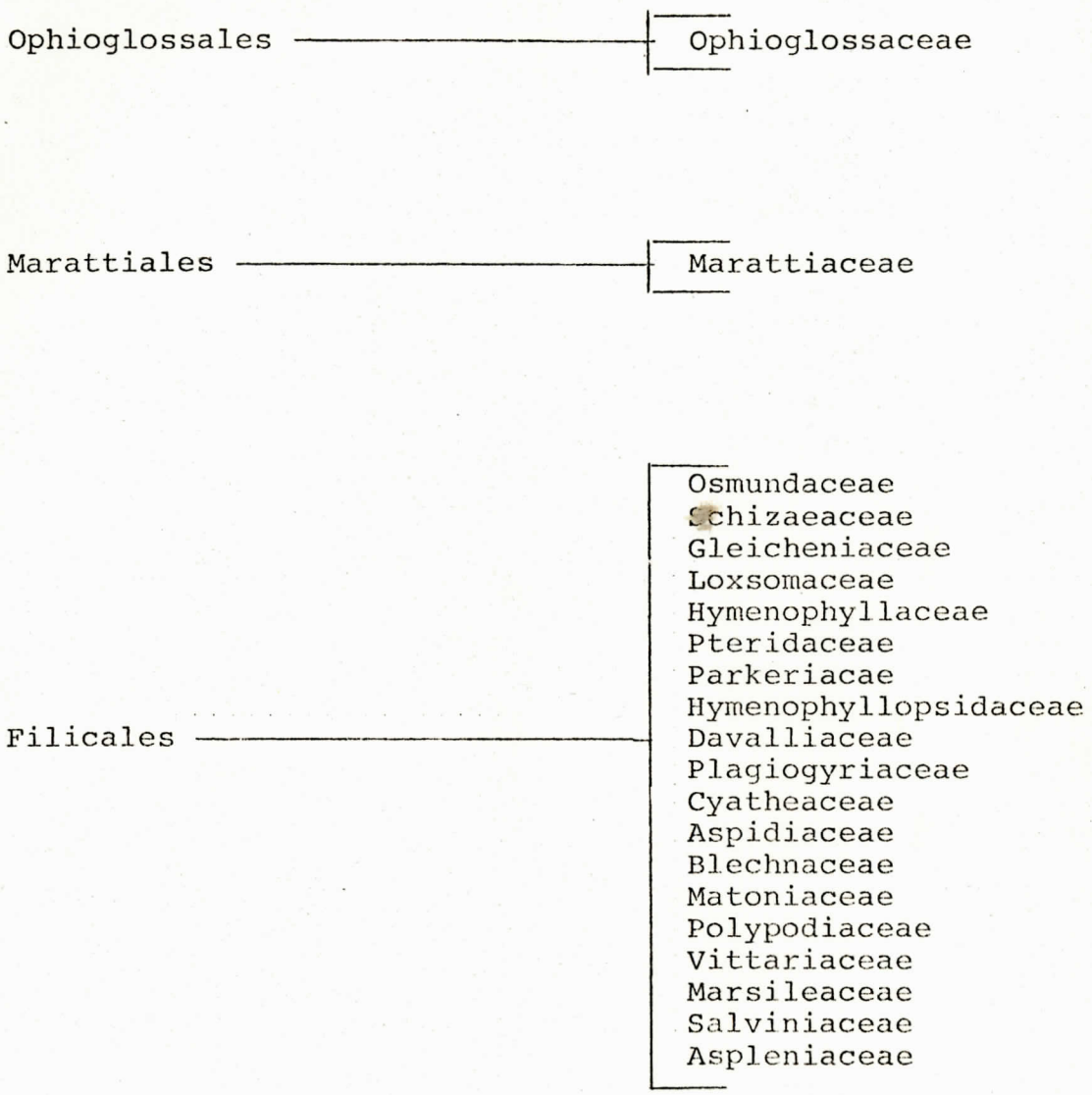

Figure 2. Classification of the Filicopsida as proposed by Copeland in 1947*

*E. B. Copeland. "Genera Filicum, the genera of ferns," Chronica Botanica, Waltham, Mass., (1947)

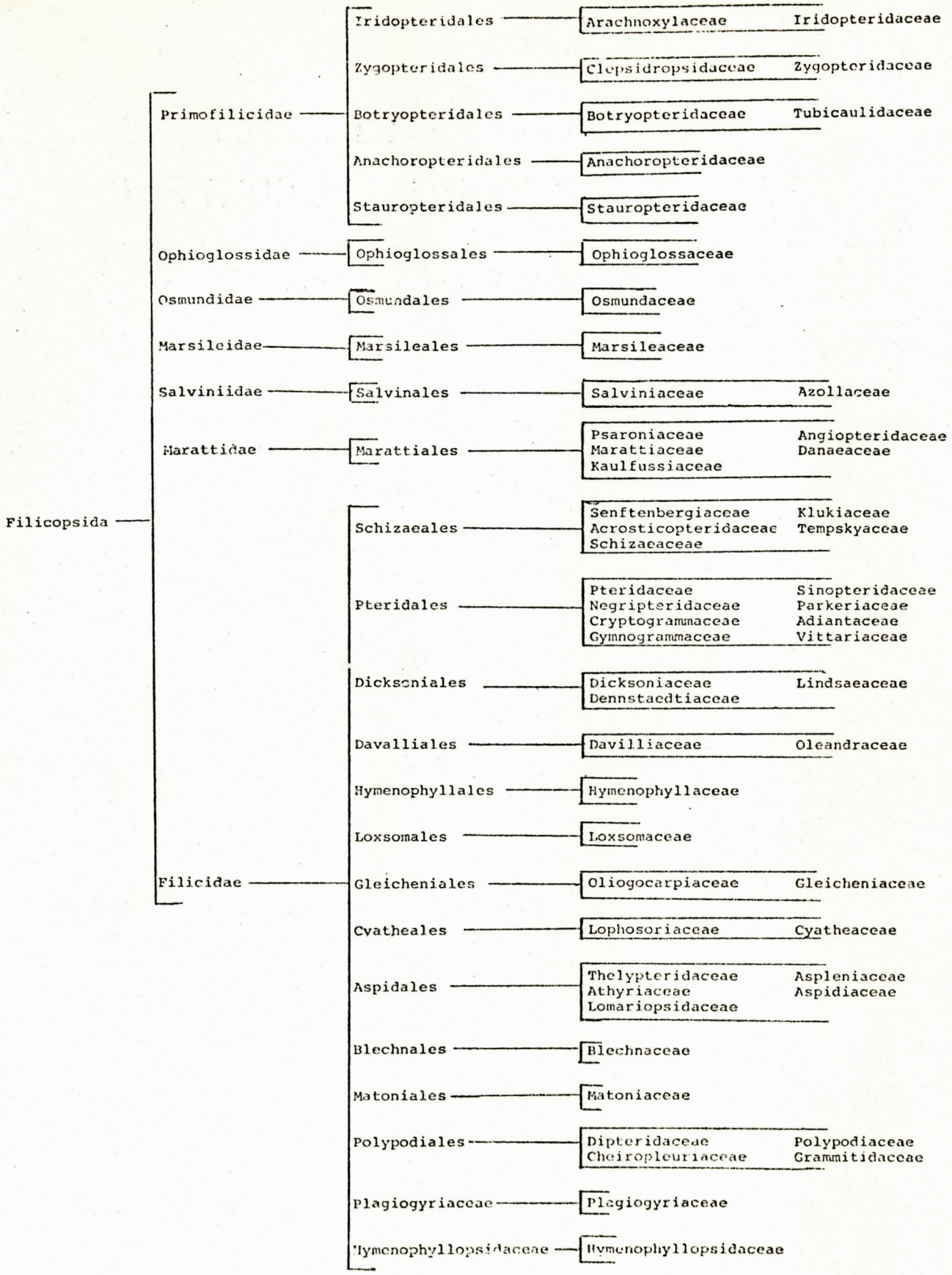

Figure 3. Classification of the Filicopsida as proposed by Pichi Sermolli in 1958.*

*Pichi Sermolli, R.E.G., Uppsala Univ., Arrskrift, 6, 70 (1958).

Phytochemical Investigations in the Filicopsida

With the exception of the Polypodiaceae, reports of biochemical surveys in the Filicopsida are rare before 1967. Since that time, several comprehensive studies have been initiated to identify secondary constituents demonstrating chemotaxonomic possibilities. A review of these studies is included in the following discussion.

Cyanogenic compounds have been identified in approximately 30 species, but, as yet, no taxonomic correlations have been shown. The ferns are known to contain numerous free protein amino acids and non-protein amino acids. One study has indicated the ability to differentiate Asplenium and Athyrium species based on ornithine and glutamic acid derivatives.[31] Unfortunately most amino acid studies in the Filicopsida demonstrate a wide variation of amino acids, even in closely related species.

Encouraging, but often contradictory, taxonomic results have been reported in the identification of fern cell wall hydrolysates. A survey of mannose concentration in the cell wall of Coniferae, Cycadales, and Magnoliidae by Cronquist[32] indicated that concentrations greater than three percent suggest a more primitive phylogenetic standing. To relate this characteristic to the ferns, a study of 109 species of New Zealand ferns was initiated, but the results were conflicting.[33] The two most primitive

orders of Filicopsida, the Ophioglossales and the Marattiales, were found to have mannose concentrations less than three percent, opposite the expected result. Within the order, Filicales, the more primitive families and subfamilies of Polypodiaceae did show high mannose concentrations agreeing with results obtained in the earlier study.

Another class of compounds studied in the Filicopsida is the hydroxy-aromatic acids. One study of over 130 species did not uncover any correlation with taxonomic standing.[34,35] A survey by Cooper-Driver and Swain[36] of 45 species in Adiantum did demonstrate four distinct groups based on their hydroxybenzoic acids and cinnamic acids distribution pattern; these patterns did not correlate with any previous classical groupings.

One of the best phytochemical studies to date deals with the identification of acylphloroglucinol derivatives in Dryopteris species.[37] About 20 of these compounds have been identified in Dryopteris species with an absence of these derivatives in other species studied. Since six species of Dryopteris do not contain the acylphloroglucinol derivatives, there is speculation that some of these species have been identified incorrectly. Within the Dryopteris several chemical races have been delineated, based on the qualitative and quantitative patterns.[38,39]

The identification of flavonoid compounds in ferns has been widely reported in the literature. One of the most comprehensive surveys was conducted by Voirin[40] where 142 species of Filicopsida covering all the orders and families were studied. On the basis of leucocyanidin concentration, Voirin claimed that the Ophioglossales and the Marattiales are more advanced than the Filicales and the Salvinales; this arrangement is contrary to accepted classification systems. A re-investigation of Voirin's work by Cooper-Driver and Swain[41] questioned his analytical work. Another study by Smith and Levin[42] on Asplenum species showed that hybrid species produce flavonoids identifiable with two-dimensional paper chromatography. An additive effect was observed when chromatograms of the individual parents were compared to a chromatogram of their hybrid offspring. Finally, a flavonoid study by Lloyd[43] in 1971 supported his earlier cytological and morphological results that three genera of ferns, Onoclea, Onocleopsis, and Matteucia are more closely related to Aspidiaceae than to Blechnaceae.

Relatively few studies of hydrocarbons in the Filicopsida are recorded in the literature; only one of these reports the normal alkane distribution patterns. In that study by Bottari et al[44], one tree fern is included, Cyathea manniana. These workers reported no alkanes in

the leaf and trunk portions of the fern. Bottari also isolated triterpenes, ecdysones, and sterols; most of the isolated triterpenoids were derivatives of hopane, fernane, filicane, and adianane. According to Bottari:

> However on present data, it appears that the triterpenoid hydrocarbon content can hardly be used as a general chemotaxonomic criterion of classification. It is doubtful that a wider study of the same secondary constituents would alter this conclusion. The fact that plants collected in different places, or in the same places, or in the same place at different times, may show different ratios of the same triterpene derivatives . . . could also be another factor which renders substances unsuitable for a general chemotaxonomic classification.

Normal Alkane Studies in the Plant Kingdom

Although the application of normal alkane distribution patterns to fern chemotaxonomy has found little favor, this information has been used to resolve some problems in other taxa of the Plant Kingdom. Before the introduction of gas chromatography most studies of the plant cuticle were performed by fractional crystallization, melting point determinations, and x-ray diffraction, as the work of Chibnall et al[45] demonstrates. This type of analysis was cumbersome and often led to erroneous results. Plant cuticle studies were discouraged as a result of these analytical difficulties. The development of gas chromatography and chemotaxonomy helped to renew interest

in the plant cuticle studies. The review that follows is limited to the analysis and chemotaxonomic implications of one fraction of the plant cuticle, the alkanes.

G. Eglinton[46] in 1961 was the first worker to mention the normal alkane distribution patterns in relation to chemotaxonomy. Eglinton was optimistic in his reasoning:

> The employment of leaf waxes as an advantageous taxonomic criteria is suggested by the following considerations: (a) The universality of the occurence of these waxy coatings; (b) The already observed species variation in the wax composition; (c) The reported lack of seasonal variation in the wax composition. This may be due to the fact that the wax is extracellular and is almost certainly an end-product insulated from the regular essential metabolic functions of the plant; (d) The simplicity of sampling; (e) The present-day availability of the precise and rapid micro-analytical tools of gas-liquid chromatography, mass spectrometry, and infrared spectrometry.

Eglinton and his colleagues selected a group of closely related genera of the Crassulaceae descended from a common ancestor in the Canary Islands. This situation was not unlike that encountered with many species in the Galapagos Islands. Eglinton's results showed a normal alkane range from C_{25} to C_{35} with odd-numbered alkanes showing a greater abundance than even-numbered alkanes. Some species even possessed a high isoalkane concentration. Unfortunately his results were not sufficiently discriminating. Species within genera appeared to have similar patterns, but differences between related genera were not

striking. In his conclusion, however, Eglinton suggests that some possibilities of a taxonomic relationship in the hydrocarbon patterns do exist.

In a later study, Eglinton[47] observed the n-alkane patterns in 12 families and 21 genera of New Zealand plants chosen primarily for their pharmacological interest. Again, the plants contained distribution patterns with the odd-numbered carbon atoms, C_{27}-C_{33}, predominating; iso-alkanes were present in trace amounts. He found that the shortest and longest n-alkane chains differed by about ten carbon atoms and that a simple distribution curve with a single maximum was observed when odd- and even-numbered alkanes were considered separately in a plot of percent alkane versus carbon number. He found almost superimposable alkane patterns between varieties of species, but he also noticed clear-cut differences within genera and families.

Reports within the last five years have been more optimistic. In a study of aquatic plants, Nishimoto[48] found that the n-alkane composition of submerged and floating leaved plants was distributed in a narrow range with a single maximum on the high molecular weight side. In contrast, the n-alkane composition of algae was distributed in a wide range with a maximum on the low molecular weight side.

In 1975 Scora[49] found a n-alkane range from C_{25} to C_{35} with odd-numbered alkanes predominating in Persea and Beilschmiedia species. The patterns demonstrated marked interspecific differences. For example, P. indica and P. donell-smithii, two phyletically distinct species, possessed individually distinct n-alkane patterns. Parents of hybrid varieties could also be identified by their n-alkane composition.

Shropshire[50] analyzed the leaves of Photinia glabra, Photinia serrulata, Photinia fraseri, Heteromeles arbutifolia, Cotoneaster pannosa, and Eriobotrya japonica for the presence of normal alkanes. Again a ten-carbon distribution range was observed from C_{23} to C_{33}. He concluded that the composition patterns demonstrated generic differences supporting the separate placement of Heteromeles.

A study by Stocker[51] in 1977 included the normal alcohols as well as the normal alkanes. The predominant alkanes were C_{29} and C_{31}; the main alcohols were C_{30} and C_{32}. Stocker concluded that the subspecies, Erythocoffea and Pachycoffea, of Coffea species were distinguishable by their alkane patterns.

The possible use of the n-alkanes as taxonomic characters in the Labiatae was suggested by Yaghmai[52] in 1979. He observed qualitative and quantitative differences of n-alkanes in Rosmarinus officinalis and Marrubium

vulgare. Aside from the typical n-alkanes, he found three other homologous series of branched alkanes: 2-methyl (iso-), 3-methyl (anteiso-), and a 3,9-dimethyl series.

One of the most unusual reports with respect to the existence of additional homologous series is found in a study by Severson[53] in 1978. The hexane-soluble fraction of several strains of flue-cured tobacco was analyzed for its hydrocarbon content. As expected, the predominant hydrocarbon was a normal alkane, $n\text{-}C_{31}$, with 20 percent of the total. The hydrocarbon fraction also included an anteiso-alkane, $a\text{-}C_{31}$, with 14 percent of the total and an iso-alkane, $i\text{-}C_{31}$, with 13 percent of the total. A comparison of the total alkane content of conventionally-grown flue-cured tobacco versus close-grown flue-cured plants also proved interesting. Although the total alkane content in the cuticle of close-grown tobacco was about 50 percent less than that in the conventional tobacco, the actual percent of the individual alkanes did not vary appreciably. This result is encouraging and tends to alleviate some doubt as to the effect of geography and environmental factors on the alkane distribution pattern of the cuticle.

A study by Corrigan[54] on 12 species of Galium and Asperula confirmed that geography and environmental factors have little effect on the alkane distribution patterns.

The alkane content of various parts of the plants did demonstrate differences, however. Thus, it is important to note the plant organs sampled before drawing any taxonomic conclusions.

CHAPTER III

THE EXPERIMENTAL METHOD

Sample Collection and Preparation

The fern samples used in this study were collected at various locations, dried in the field, and identified by competent botanists (Table I). Voucher specimens of each fern are located in the herbarium in the Appalachian State University Department of Chemistry. The dried fronds were macerated in a Waring blender at low speed for five to ten minutes and were weighed before hexane extraction (Table II). Refer to Figure 4 for a flow diagram of the extraction process.

Extraction Procedure

The macerated fronds were transferred from the blender to individual 500-ml Erlenmeyer flasks where hexane (Pesticide Grade Hexanes, Fisher Scientific Company) was added to cover the fern material. The volume of hexane used was 30 to 80 ml depending upon the compactibility of the frond material in each flask. After the addition of hexane, the flasks were stoppered, placed on an automatic shaker for several minutes, and stored away from the light. After a 24-hr extraction period, the hexane was decanted into a narrow-mouth, screw-cap, amber bottle, and a new

TABLE I

CYATHEACEAE AND DICKSONIACEAE SPECIES STUDIED

Fern	Collection Date; Voucher Number	Collected By	Identified By	Location
Dicksonia berteroana (Cholla) Hook	9 Jan 1976 76-1	R. W. Soeder	R. A. White, Duke University	Mas Atierra, Chile trail to Selkirk Mirador, elev. 400 m (Robinson Crusoe Islands)
Alsophilia australis (R. Br.) Tryon	8 May 1974 74-9	R. Coveny (#5466) Royal Botanic Gardens, Sydney, Australia	M. Tindall, Royal Botanic Gardens Sydney, Australia	Springwood, New South Wales Australia, Sassafras gully, elev. 300 m
Cnemidaria horrida (L.) Presl	24 Dec 1973 73-125	R. W. Soeder	G. R. Proctor, Inst. of Jamaica, Kingston, Jamaica	Near Bath, Jamaica, Cornpuss Gap Road
Nephelea grevilleana (Mart.) Tryon	24 Dec 1973 73-124	R. W. Soeder	G. R. Proctor, Inst. of Jamaica, Kingston, Jamaica	Near Bath, Jamaica, Cornpuss Gap Road
Cyathea divergens Kze.	20 Jun 1972 72-54	R. W. Soeder	R. Tryon, Gray Herb., Harvard Univ., Cambridge, Massachusetts	El Palme, Costa Rica 17 km south on Pan American Highway
Sphaeropteris brunei (Christ) Tryon	21 Jun 1972 72-62	R. W. Soeder	R. Tryon, Gray Herb., Harvard Univ., Cambridge, Massachusetts	El Palme, Costa Rica 40 km south on Pan American Highway
Trichipteris stipularis (Christ)	21 Jun 1972 72-56	R. W. Soeder	R. Tryon, Gray Herb., Harvard Univ., Cambridge, Massachusetts	El Palme, Costa Rica 30 km south on Pan American Highway

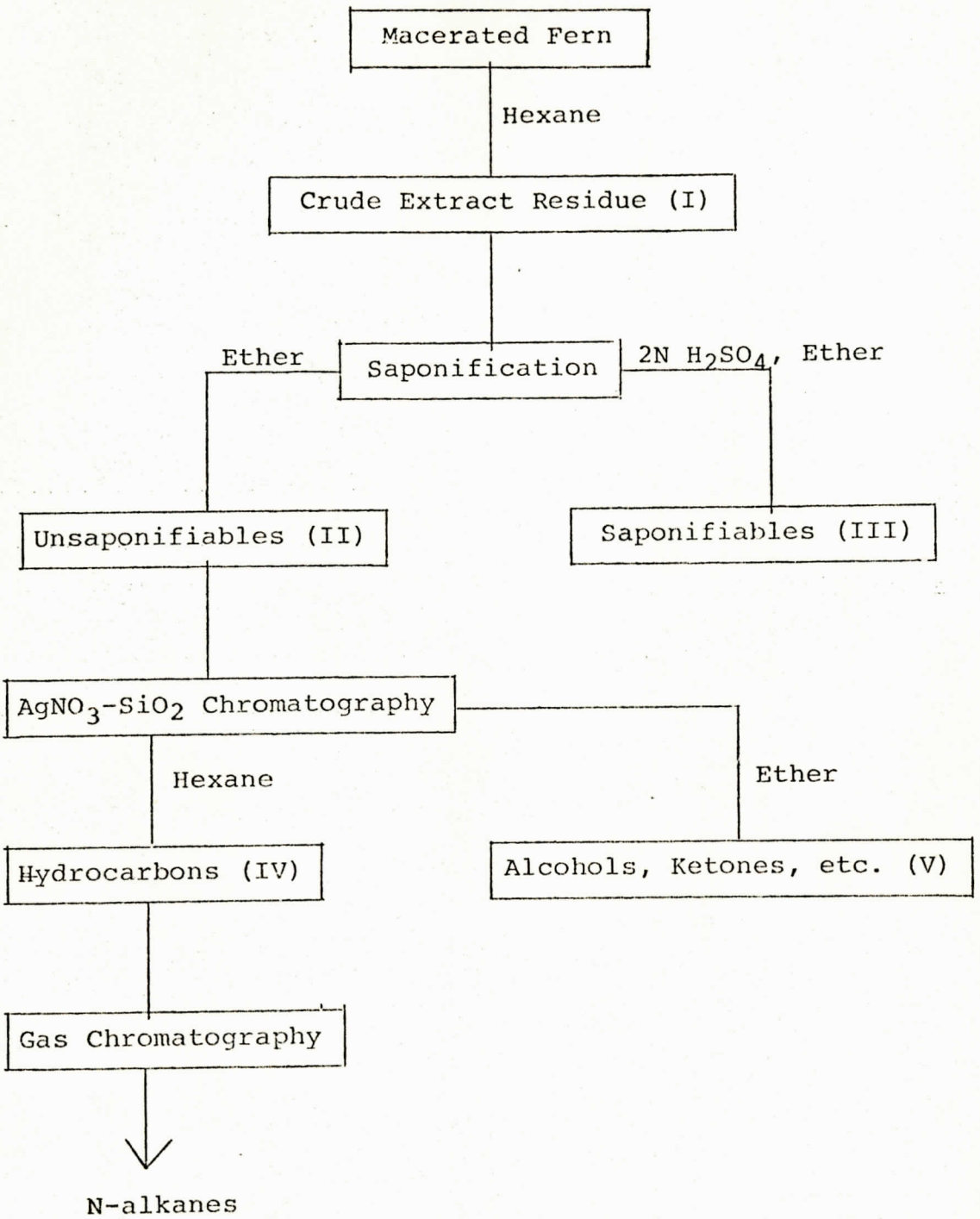

Figure 4: Flow diagram of extraction procedure for the isolation of n-alkanes

volume of hexane was added to the flask to repeat the extraction process. After seven 24-hr extraction periods, the combined extracts were vacuum-filtered through Whatman #4 filter paper. A simple distillation apparatus was used to reduce sample volume to 25 ml. After concentration, the samples were transferred to 50-ml Erlenmeyer flasks and stored at 0° C for five days. During this period, a solid material deposited in the bottom of the flask. This material was removed by vacuum filtration through a cooled (0° C) Gooch crucible. The hexane filtrate was allowed to evaporate in a porcelain dish for recovery of the crude extract (I).

Saponification Procedure

The saponification reagent used in the sample clean-up was prepared with 17.56 g of NaOH, 200 ml of 95% ethanol, and 20 ml of distilled water. Saponification of the crude extract (I) appeared to be complete after a reflux period of four to five hours with 25 ml of the saponification reagent. The basic reaction mixture was transferred to a 250-ml separatory funnel, diluted with 30 ml of distilled water, and vigorously shaken with three 30-ml portions of diethyl ehter. The combined ether extracts were concentrated and transferred to a porcelain dish for evaporation and recovery of the unsaponifiable fraction (II). The

remaining reaction mixture was acidified drop-wise with 2N H_2SO_4 followed by extraction with three 30-ml portions of diethyl ether. The combined ether extracts containing the saponifiable fraction (III) were stored at 0° C in 250-ml flasks.

Column Chromatography

The silver nitrate-impregnated silica gel used as the adsorbent for column chromatography was prepared by mixing 600 g of SiO_2 (Chrom. Grade Type I, 60-200 mesh, Sigma Chemical Company), 90 g of $AgNO_3$ (Reagent Grade, A.C.S., Reagents, Incorporated), and 700 ml of distilled water in a one-liter round-bottom flask. The water was removed by evaporation on a rotary evaporator at 55-60° C for three hours. Activation and removal of residual water was acccomplished by oven-drying at 155° C for 17 hr. The chromatography apparatus consisted of a glass column, one cm (o.d.) by 57 cm in length, fitted with a solvent reservoir and a ground-glass stopcock. The column was dry-packed with $AgNO_3$-SiO_2 to within one inch of the solvent reservoir. Hexane was allowed to percolate slowly through the adsorbent until the solvent began to elute from the column end.

The unsaponifiable fraction (II) was loaded on the column, and 100 ml of hexane were added to the solvent

reservoir. An elution rate of 15 to 30 drops per minute was maintained as the hexane was collected in a 250-ml Florence flask. The hexane was removed by distillation and evaporation to yield the hydrocarbon fraction (IV). Following hexane elution, 100 ml of diethyl ether were added to the solvent reservoir. Again, the elution rate was set at 15 to 30 drops per minute as the ether containing the more polar fraction (V) was collected in a 250-ml Florence flask. This fraction was stored at 0° C.

Gas Chromatography of the Unsaponifiable and Hydrocarbon Fractions

The gas chromatograph used for this analysis was a Hewlett-Packard 5880A model equipped with a flame ionization detector. Calibration of the instrument was performed by injection of hexane washings of Parafilm (American Can Co.). Retention times of the n-alkanes were recorded and used to identify the alkane constituents of the seven fern samples studied. Conditions for analysis of these fractions were as follows:

Column: Methyl silicone (SP-2100), 50 m, 0.2 mm i.d., fused silica glass capillary, deactivated with Carbowax 20M.

Injector temperature: 300° C.

Detector temperature: 320° C.

Column temperature: Programmed, 60-280° C @ 3° C. isothermal hold for 90 min.

Carrier gas: Hydrogen @ 25 p.s.i., linear flow velocity of 30 cm sec^{-1} at 280° C.

Flame gases: Air at 400 ml min^{-1}, Hydrogen at 30 ml min^{-1}.

Chart speed: 0.2 cm min^{-1}.

Range and Attenuation: 2↑1 to 2↑3.

Injection volume: 1 microliter.

Injection type: splitless purged 0.25 min after injection.

Purge flow: 70 ml min^{-1}.

Septum purge flow: 6 ml min^{-1}.

Infrared Spectra of Fractions

All infrared spectra in this study were recorded on a Perkin-Elmer Model 137 Infracord. Sample fractions were placed between two sodium chloride plates and scanned on the slow setting.

CHAPTER IV

RESULTS AND DISCUSSION

Infrared Spectra

The infrared spectra recorded on fractions I-V in the extraction process provide qualitative and semi-quantitative information on the presence of functional groups and compound types in each fraction (Figures 5-9). In reviewing these spectra it is important to note that the hexane-soluble fraction of these ferns contains cytoplasmic as well as cuticle constituents; therefore, the infrared spectra may suggest the presence of substances other than those expected from cuticle-specific extraction techniques (Table III).

Fraction I, the crude extract, contains all substances present in the hexane-soluble portion of the fern material. The strong, wide band at 1725 cm^{-1} is indicative of carbonyl groups found in esters, ketones, and organic acids normally expected in hexane-soluble extracts of the cuticle. The wide band at 3500 cm^{-1} suggests hydrogen bonding exhibited by the hydroxyl group of alcohols. Since alcohols and acids of leaf waxes are normally found as esters, the hydrogen bonding here seems to indicate the presence of either free alcohols or free acids. The remaining bands at 2950 cm^{-1}, 1460 cm^{-1}, and 1370 cm^{-1} are

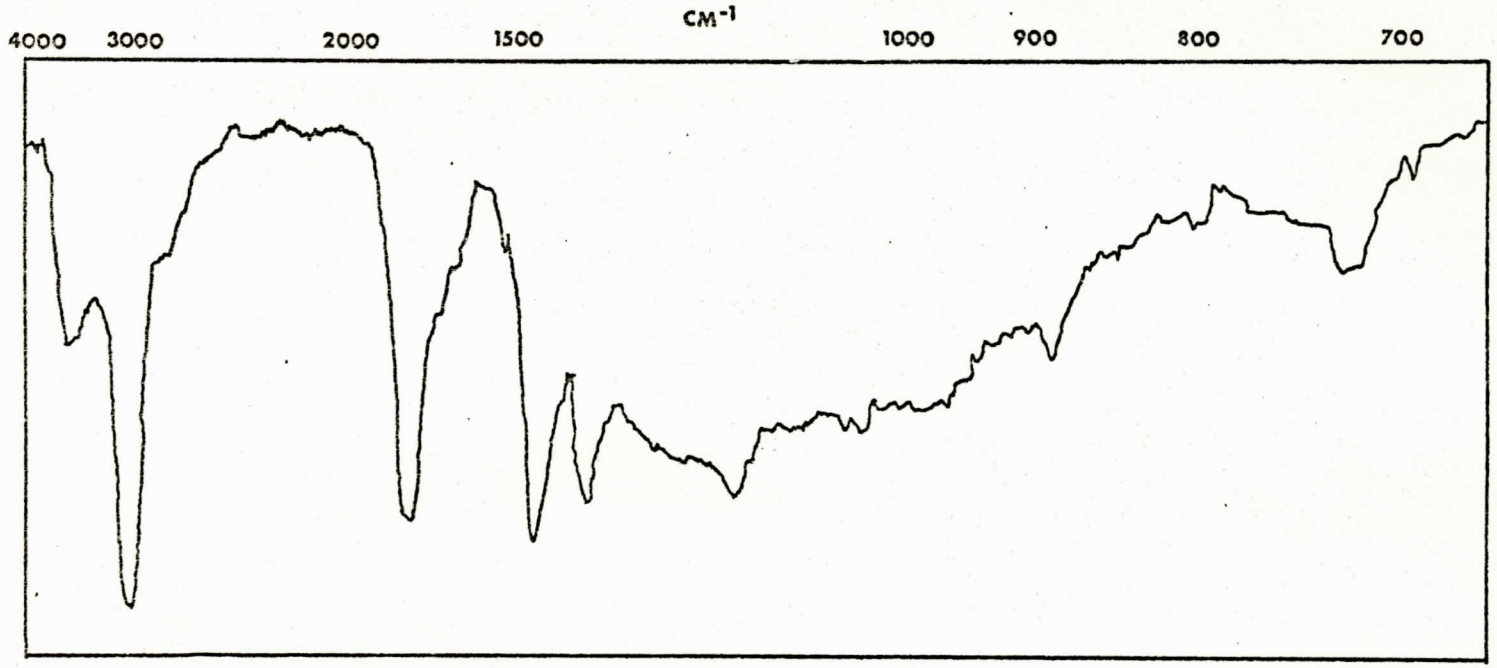

Figure 5. Infrared spectrum of the crude extract (I) of Trichipteris stipularis.

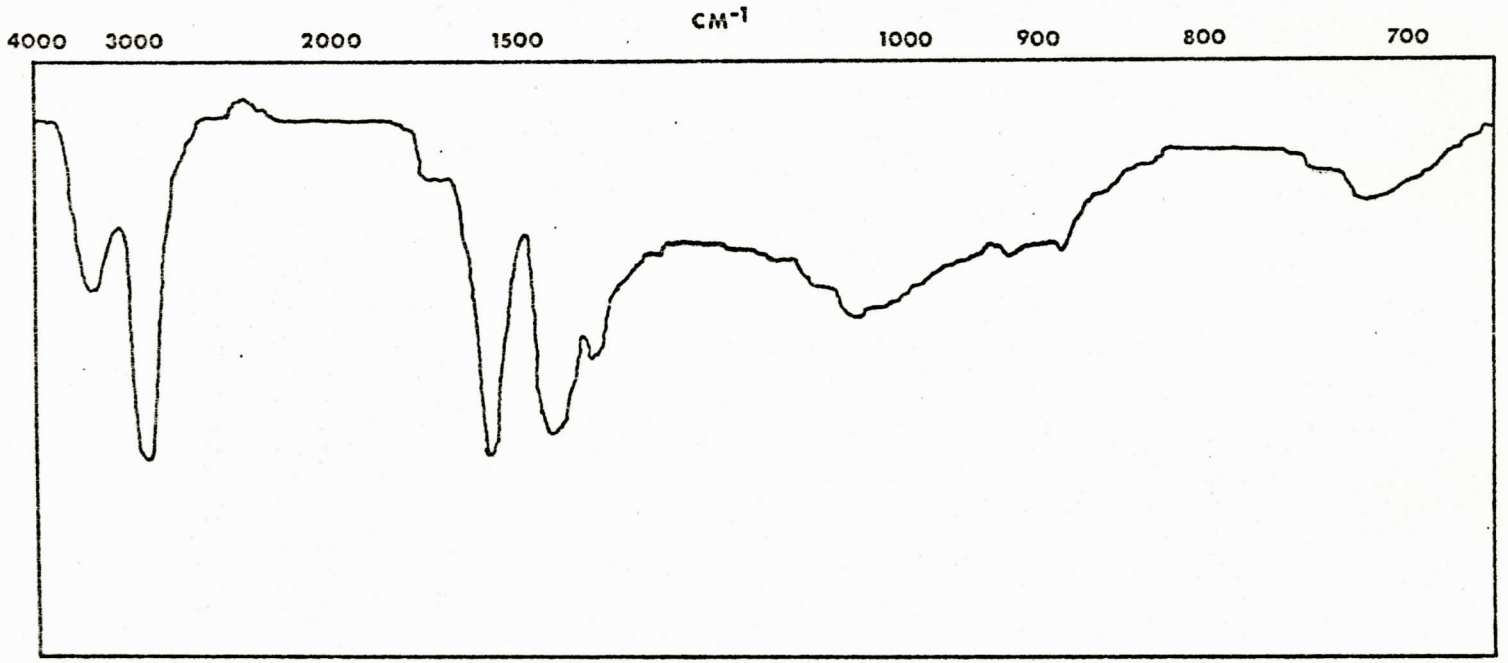

Figure 6. Infrared Spectrum of the Unsaponifiable fraction (II) of Trichipteris stipularis.

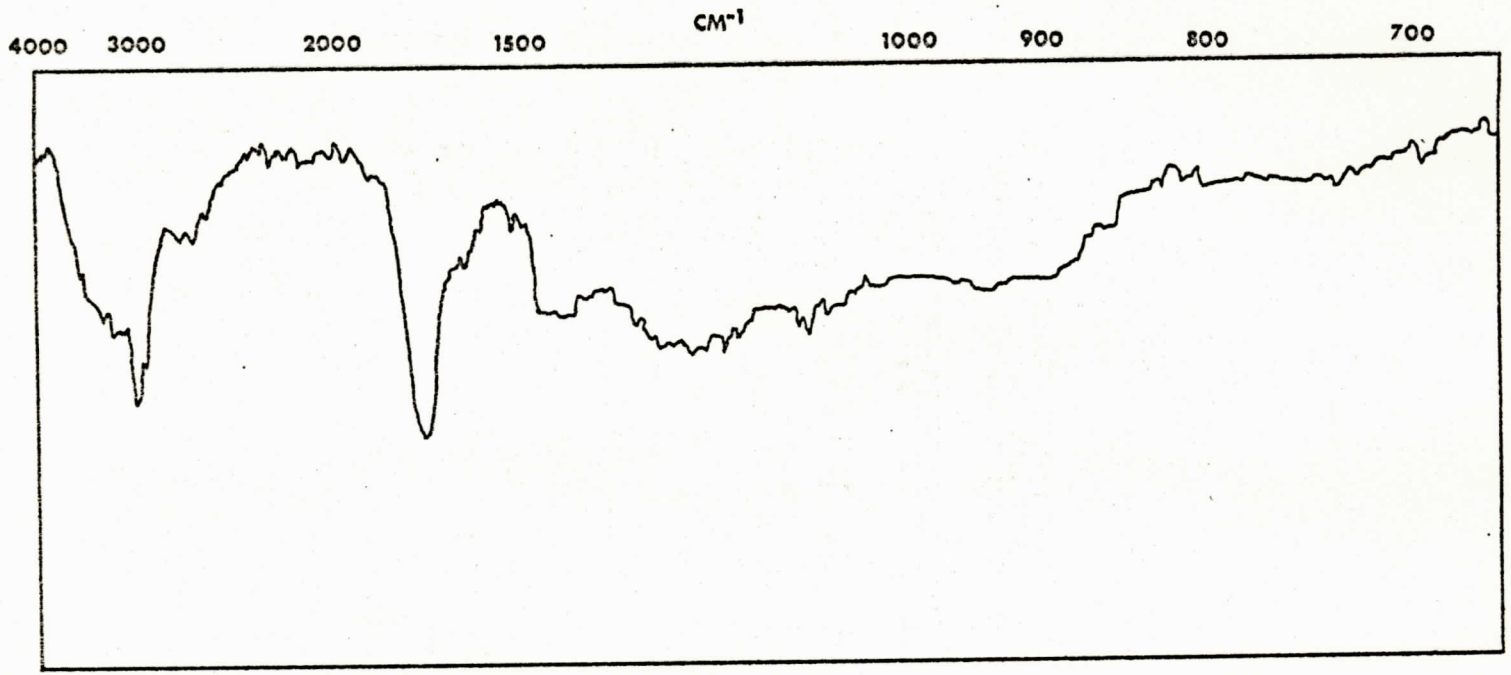

Figure 7. Infrared spectrum of the saponifiable fraction (III) of Nephelea grevilleana.

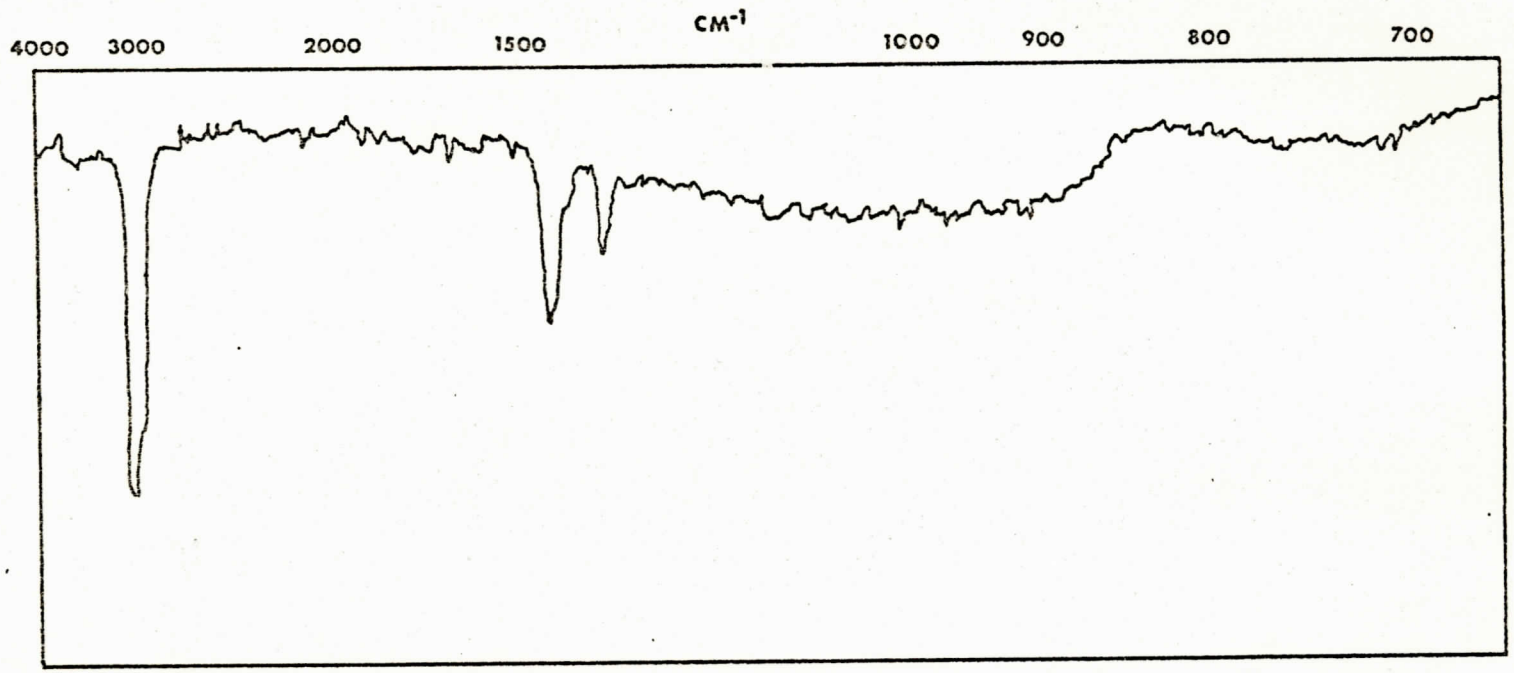

Figure 8. Infrared spectrum of the hydrocarbon fraction (IV) of Trichipteris stipularis.

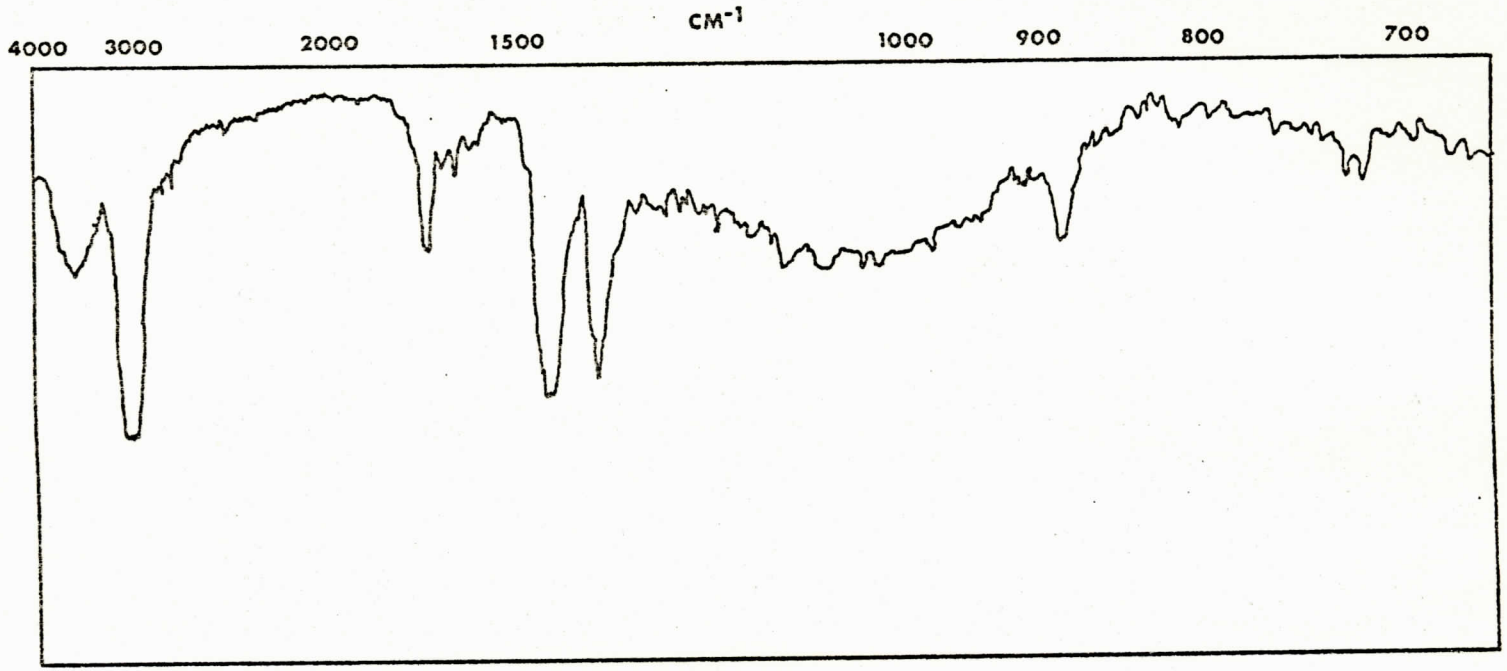

Figure 9. Infrared spectrum of the base-neutral fraction (V) of Trichipteris stipularis.

TABLE III

MAJOR CONSTITUENTS OF LEAF WAXES*

Type	Range	Frequency
Alkanes	Normal: odd C_{21}-C_{37}	Common
	Normal: even C_{20}-C_{34}	Common
	Branched: C_{27}-C_{33}	Infrequent
Alcohols (usually as esters)	Primary: even C_{22}-C_{32}	Common
	Primary: odd C_{25}-C_{31}	Infrequent
	Secondary: odd C_{21}-C_{33}	Common
	Diols & Ketols	Rare
	Terpene Alcohols	Infrequent
Aldehydes (as polymers)	Normal: C_{24}-C_{34}	Rare
Ketones	Di-n-alkyl	Rare
Acids (Usually as esters)	Normal: even C_{14}-C_{34}	Common
	Normal: odd C_{15}-C_{33}	?
	Keto Acids	Rare
	Dibasic Acids	Rare
Esters	Between n-acids and primary and secondary alcohols	Common

*T. Swain (ed.), "Chemical Plant Taxonomy," Academic Press, London and New York, 1963, p. 191.

due to the stretching and bending of methyl and methylene groups.

Fraction II, the unsaponifiable fraction, should contain all hydrocarbons,bases,and other neutral compounds. The wide band at 3400 cm^{-1} is expected from the hydrogen bonding by hydroxyl groups of alcohols. There is an absence of a strong carbonyl absorption at the expected 1700 cm^{-1} region. Instead, this band appears to be shifted to a longer wavelength region at 1560 cm^{-1}. The methyl and methylene stretching and bending are noted at 2950 cm^{-1}, 1440 cm^{-1}, and 1360 cm^{-1}.

Interpretation of the spectrum of fraction III is relatively straight-forward; this fraction should contain acids from the expected esters in the plant cuticle. The wide band from 3500 cm^{-1} to 2500 cm^{-1} strongly indicates hydrogen bonding of a carboxylic acid group, and the strong, wide absorption at 1725 cm^{-1} indicates the carbonyl group. The typical methyl and methylene bands are masked slightly by the acid group absorptions.

Fraction IV, the saturated hydrocarbons, from hexane elution through the $AgNO_3$-SiO_2 column demonstrates the absence of any functional groups. The stretching and bending absorption bands predominate at 2950 cm^{-1}, 1460 cm^{-1}, and 1370 cm^{-1}. The absence of bands in the fingerprint region, 1000 cm^{-1} to 650 cm^{-1}, illustrates the

ability of the $AgNO_3$-SiO_2 to selectively retard the elution of any unsaturated or aromatic compounds. The saturated hydrocarbons are permitted to elute with hexane, leaving the double-bonded species adsorbed in a weak charge transfer complex with the silver ion.

Diethyl ether elution yields fraction V. This fraction should contain alcohols, ketones, aldehydes, and other basic and neutral compounds originally in the unsaponifiable portion of the extract. The carbonyl absorption band at 1725 cm^{-1} is present although much weaker than in previous spectra. The typical hydroxyl group absorption at 3500 cm^{-1} is seen as a broadened band typical of intermolecular hydrogen bonding.

Fused Silica Glass Capillary Columns

Less than five years after the introduction of gas-liquid chromatography in 1952 by James and Martin[55], M. J. E. Golay[56] suggested the use of capillary columns, but difficulties with drawing equipment and liquid phase coating techniques delayed the development of high resolution chromatography until the late 1960's. Since that time, refinements in instrumentation and column technology have permitted the expanded use of these columns in gas chromatography.

The conversion of methods to capillary chromatography has improved the analysis of high molecular weight hydrocarbons in natural product chemistry and petroleum research, but many problems still exist: (1) Rigorous and often time-consuming cleanup procedures are required before chromatographic analysis; (2) Temperatures required to completely elute these compounds often exceed the capabilities of the instrument and the upper limit of the liquid phase coating; (3) Usually several hours of analysis time are required to satisfactorily resolve these mixtures; (4) Since most separations of high molecualr weight hydrocarbons are based on boiling point differences, the resolution with capillary columns is still inadequate to separate the numerous closely related compounds; (5) Injector temperatures required for volatilization of these mixtures often lead to septum ghost peaks and undesirable artifacts; (6) Most laboratories without GC-MS systems are ill-equipped to identify the immense number of compounds resolved with capillary systems; (7) Until recently many commercial integrators did not have the capacity to handle the hundreds of peaks found in these samples. Manual integration techniques are too time-consuming and inaccurate for capillary work.

With the introduction in 1979 of fused silica columns and a new commercial instrument, the Hewlett-Packard 5880A,

many of these problems were eliminated.[57] The development of fused silica columns grew out of the fiber optics industry with modifications to the optic strand drawing equipment. Fused silica is an amorphous glass, SiO_2, that is prepared by the burning of SiH_4 or $SiCl_4$ in the presence of O_2. This process results in a much lower metallic content than is found in soda lime or borosilicate glass columns. Metal oxides in the synthetic silica are normally present in concentrations less than one part per million. A polyimide coating is applied to the outside of the column to increase its mechanical durability in day-to-day use. Since the fused silica columns are inherently straight the need for column end-straightening equipment is eliminated. Connections to the detector and injector are accomplished with high temperature rubber O-rings or graphite ferrules.

The major advantages of fused silica columns are increased inertness, efficiency, and higher maximum temperature limits. For example, most 50-meter fused silica columns exhibit over 250,000 theoretical plates with helium as a carrier gas. Using hydrogen as a carrier gas will nearly double the number of available theoretical plates, reduce the analysis time, and reduce the column head pressure required to maintain the optimum linear flow velocity. Hydrogen is the carrier gas of choice (particularly in temperature programmed runs) because of its

"flatter" curve in a HETP versus linear velocity plot, but the dangers involved have always limited its use (Figure 10). Fortunately the Hewlett-Packard instrument is designed with the possible use of hydrogen in mind. The inlet carrier gas is flow-controlled with a back pressure regulator used to set the column head pressure.[58] In the event of column breakage during normal use, only a small volume of hydrogen could leak into the oven, thus reducing any danger of hydrogen ignition that would exist with normal column head pressures.

To evaluate this instrument and the fused silica column for hydrocarbon analyses, a standard for n-alkane retention times was prepared from Parafilm, a commercial sealing material.[59] Identification of the n-alkanes in hexane washings of this material was accomplished by spiking the homologous series with a known n-alkane, tricosane. A chromatogram of this standard demonstrates the ability of fused silica columns, with hydrogen as a carrier gas, to resolve the homologous series of normal alkanes (Figure 11). The small peaks between the normal alkanes are most likely iso-and anteiso-alkanes, but positive identification has not been made. One of the major disadvantages of conventional capillary columns has always been that of low sample capacity; fused silica capillary columns are no exception. The noticeable fronting of major peaks in the

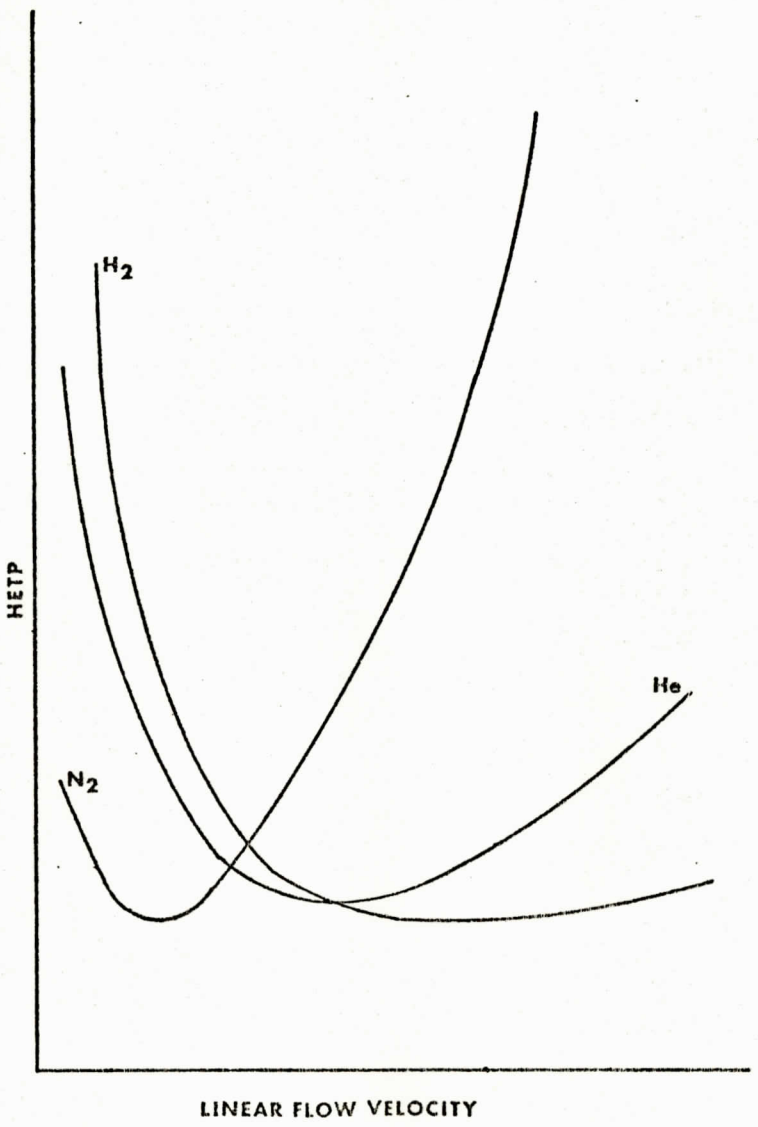

Figure 10. Graph of HETP vs. linear flow velocity illustrating the increased efficiency obtained with hydrogen as a carrier gas.*

*Pooney, T. A., Industrial Research/Development, Oct. (1978).

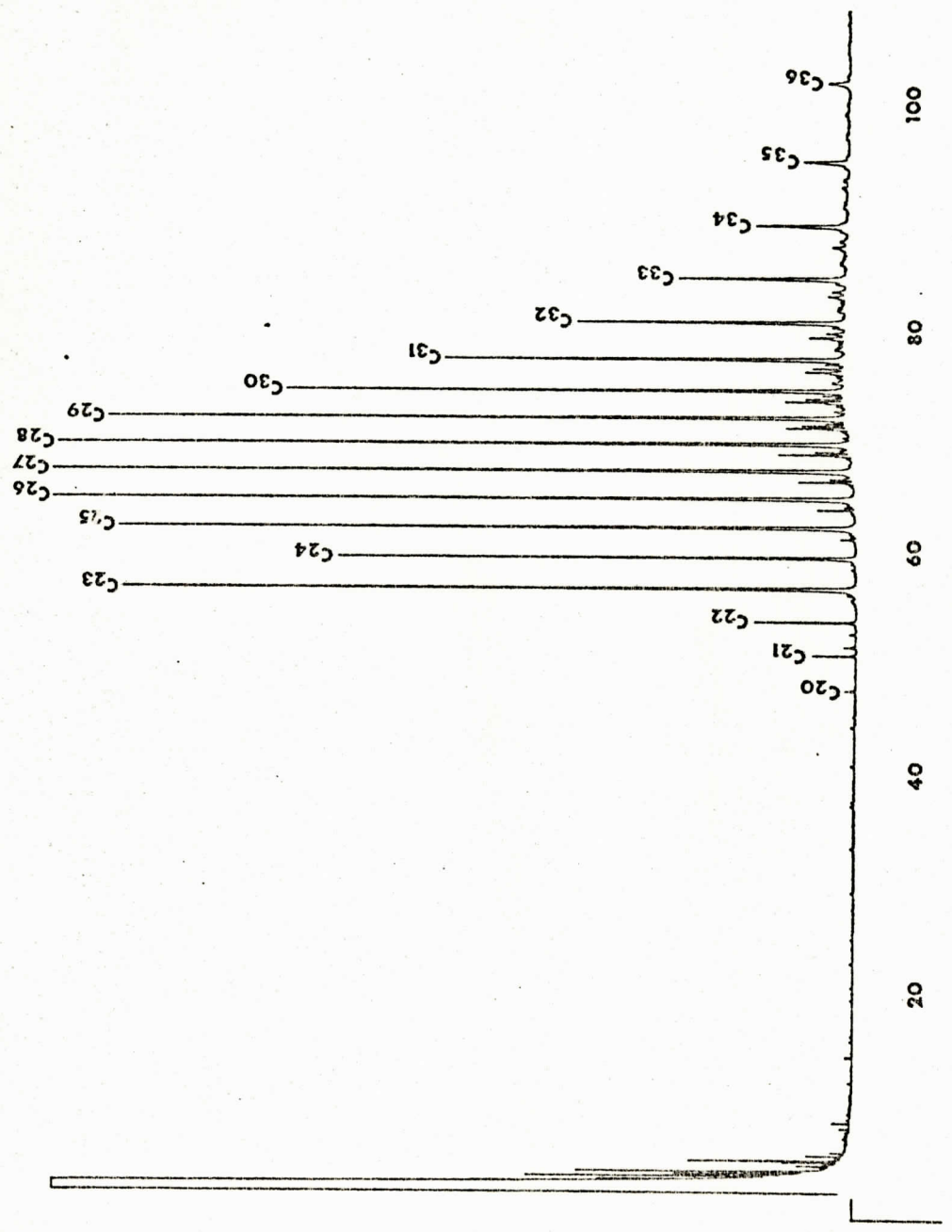

Figure 11. Chromatogram of the normal alkane homologous series standard prepared from Parafilm. Tricosane, C_{23}, was used as a marker for identification.

standard chromatogram are indicative of sample overload. Usually each individual component will demonstrate overloading above the five to ten nanogram per component level.

Chromatograms of the Unsaponifiable and Hydrocarbon Fractions

Identification of the normal alkanes in the seven species of Cyatheaceae and Dicksoniaceae is based on co-retention times of the Parafilm homologous series. Only those alkanes in the C_{21} to C_{34} range are positively identified although trace amounts of other n-alkanes may be present. The minimum area reject mode of the HP 5880A data system was set at a threshold level high enough to eliminate integration of noise peaks. Therefore, some n-alkanes seen on the chromatogram were not integrated or included in the data analysis.

The chromatogram of the unsaponifiable fraction of Nephelea grevilleana demonstrates the presence of an homologous series eluting earlier than the first identified normal alkane (Figure 12). Since normal alcohols are also expected in the unsaponifiable fraction this may be an homologous series of n-alcohols; however, the presence of a lower homologous series of n-alkanes cannot be eliminated as a possibility. Alcohols analyzed on borosilicate or soda lime glass capillary columns with a methyl silicone liquid phase would most likely exhibit a marked tailing

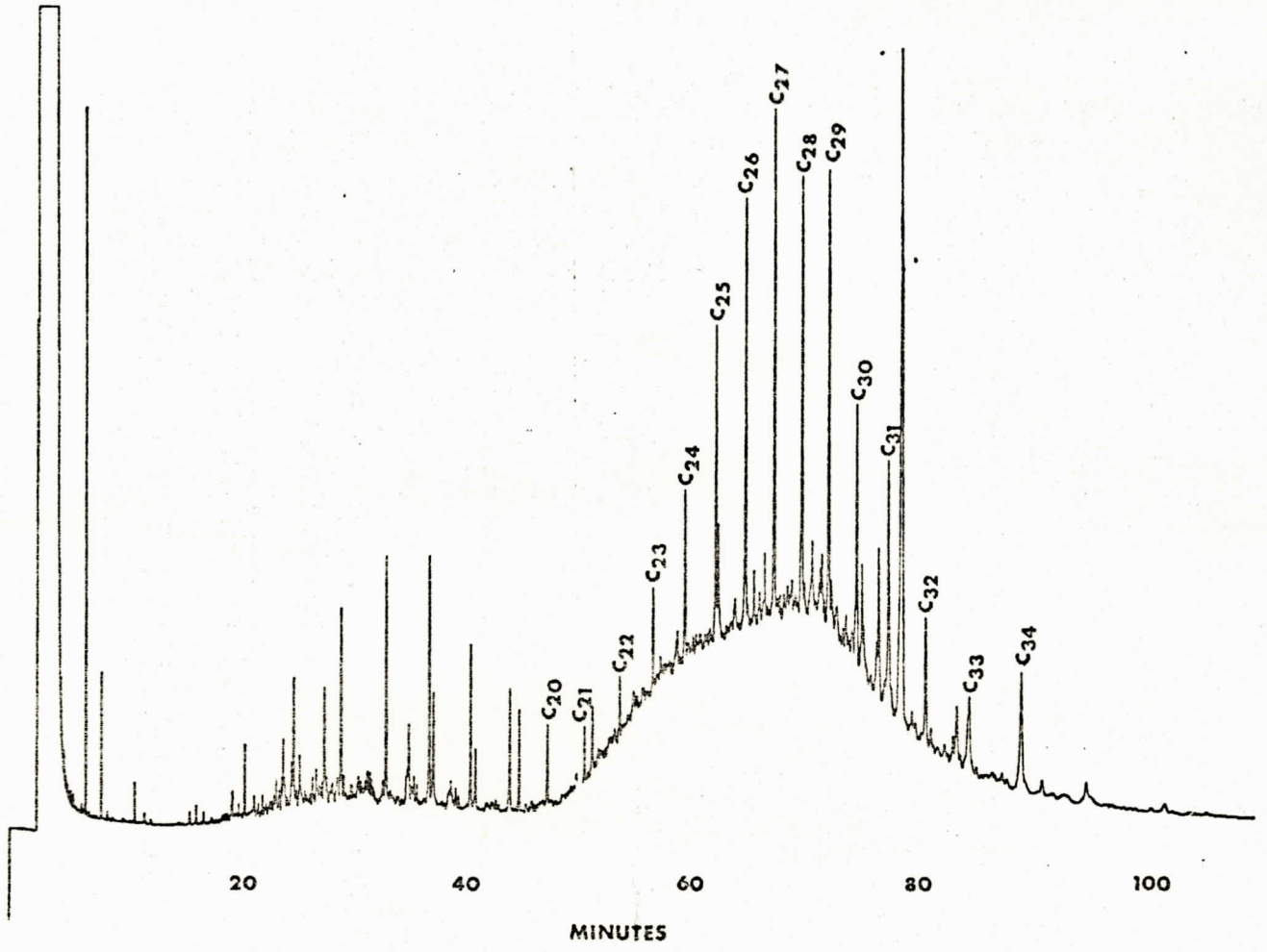

Figure 12. Chromatogram of the unsaponifiable fraction of Nephelea grevilleana.

effect, but due to the relative inertness of the fused silica column, tailing is negligible with alcohols. There is not a predominance of any n-alkane in this fern species, but the distribution maximum is found at C_{27} (Table IV). The major peak between C_{31} and C_{32} constitutes 16 per cent of the total chromatogram area. Although positive identification has not been established, this compound may be a high molecular weight di-n-alkyl-ketone or other ketone such as 10-nonacosanone. This ketone has been identified in another tree fern, the monotypic Lophosoria quadripinnata, by Soeder and Hodgin.[60]

The chromatogram of the unsaponifiable fraction of Sphaeropteris brunei indicates the presence of at least two other homologues, possibly normal alcohols (Figure 13). The normal alkane homologous series in this species appears to be a minor one. The predominant n-alkane constituent is even-numbered, C_{22}; the predominant odd-numbered n-alkane is C_{27} (Table IV).

The chromatogram of the hydrocarbon fraction of Trichipteris stipularis shows that the major constituents are the normal alkanes (Figure 14). This is another example of the effectiveness of the adsorption chromatography in the extraction procedure. The distribution pattern shows a predominance of odd-numbered normal alkanes with the even-numbered normal alkanes subordinate (Table

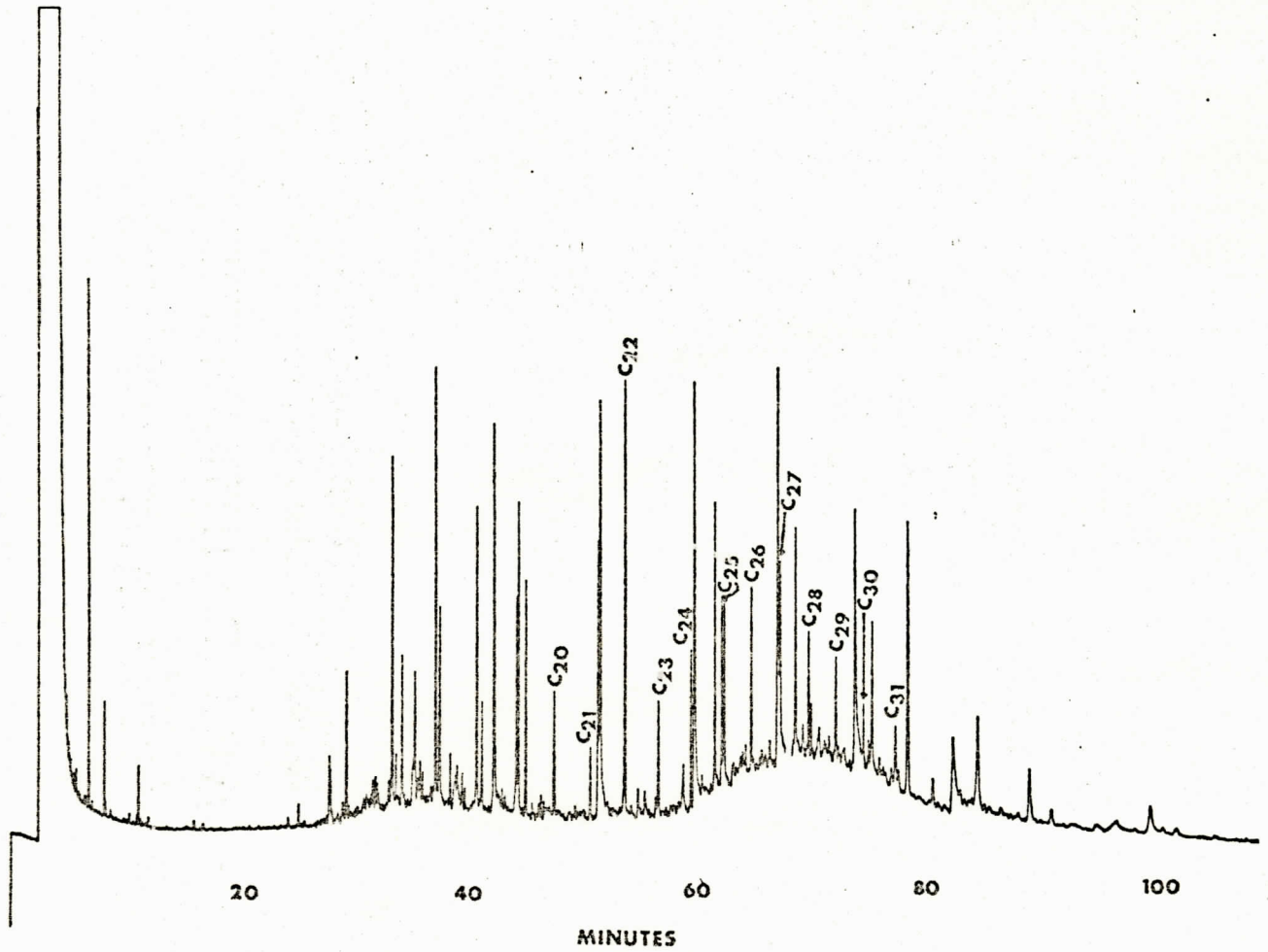

Figure 13. Chromatogram of the unsaponifiable fraction of Sphaeropteris brunei.

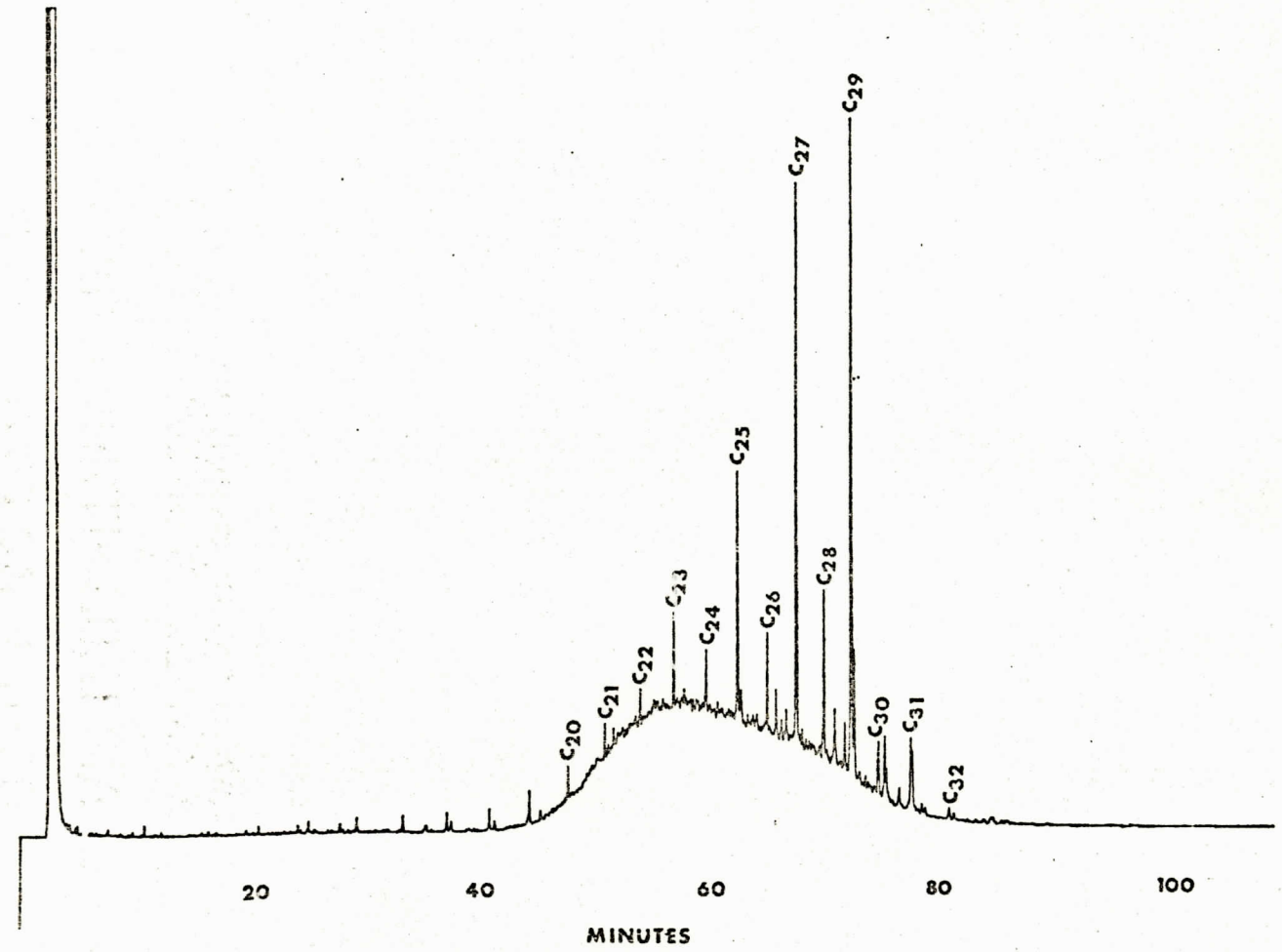

Figure 14. Chromatogram of the hydrocarbon fraction of Trichipteris stipularis.

IV). The distribution maximum for the odd-numbered alkanes is C_{29}.

The chromatogram of the hydrocarbon fraction of Alsophilia australis is marked by the predominance of the n-alkane, C_{29}, representing 55 per cent of the total alkanes (Figure 15). The distribution pattern for the even-numbered n-alkanes demonstrates a maximum at C_{28} (Table IV).

The pattern of Cyathea divergens shows an almost even distribution of n-alkanes from C_{21} to C_{32} (Figure 16). The odd-numbered maximum is at C_{29} with the even-numbered maximum at C_{24} (Table IV).

The hydrocarbon fraction of Cnemidaria horrida was spiked with tricosane for identification in an earlier packed column GLC study (Figure 17). The pronounced fronting and tailing of this component illustrates the low sample capacity of the fused silica columns. The maximum for the odd-numbered distribution is at C_{27}; the even-numbered maximum is at C_{24} (Table IV).

The chromatogram of the unsaponifiable fraction of Dicksonia berteroana also demonstrates the presence of an homologous series other than the normal alkanes (Figure 18). The major peak found in N. grevilleana also appears to be present in this chromatogram representing 8.8 per cent of the total area. The odd-numbered normal alkane distribution exhibits a maximum at C_{27}, and the even-numbered

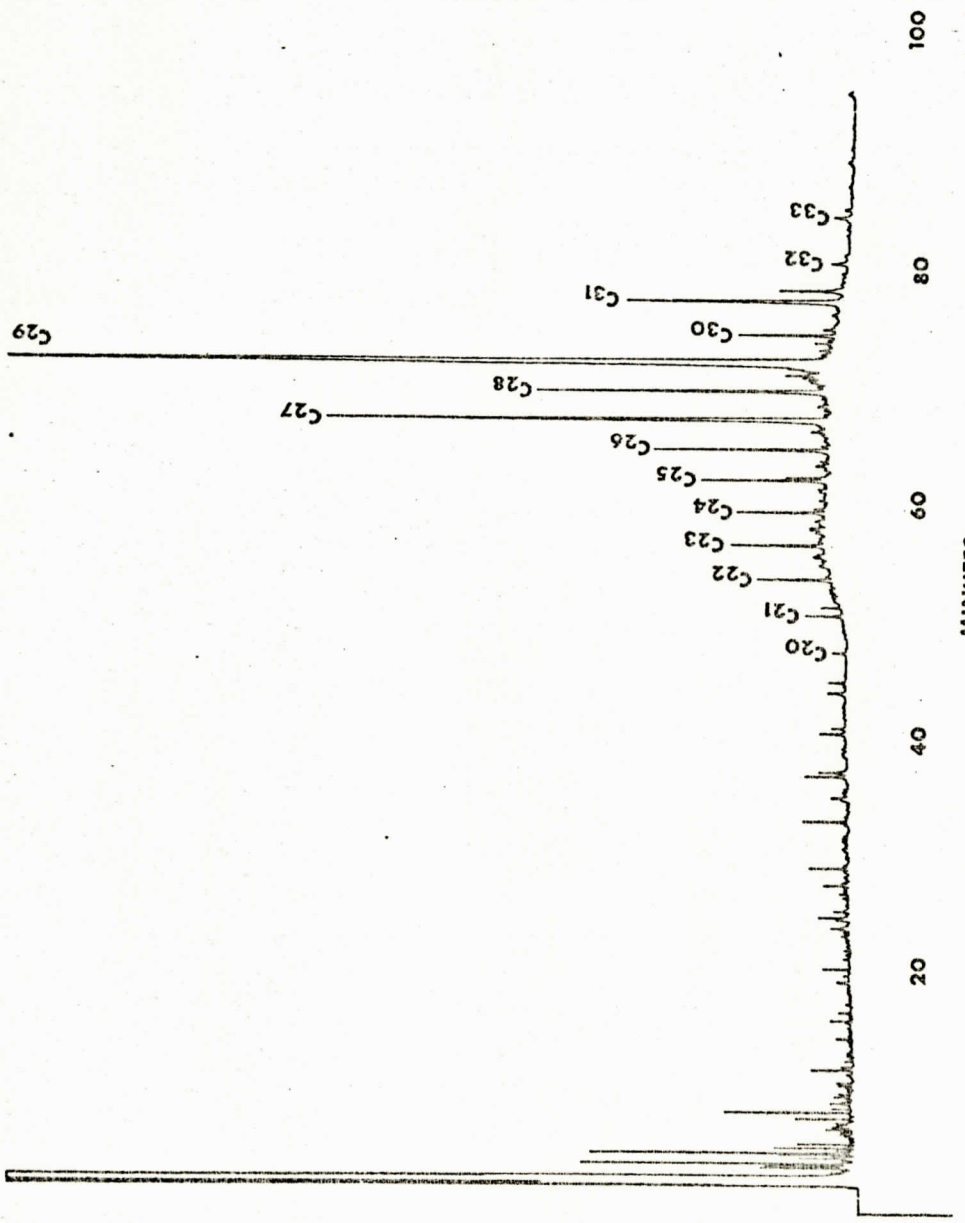

Figure 15. Chromatogram of the hydrocarbon fraction of Alsophilia australis.

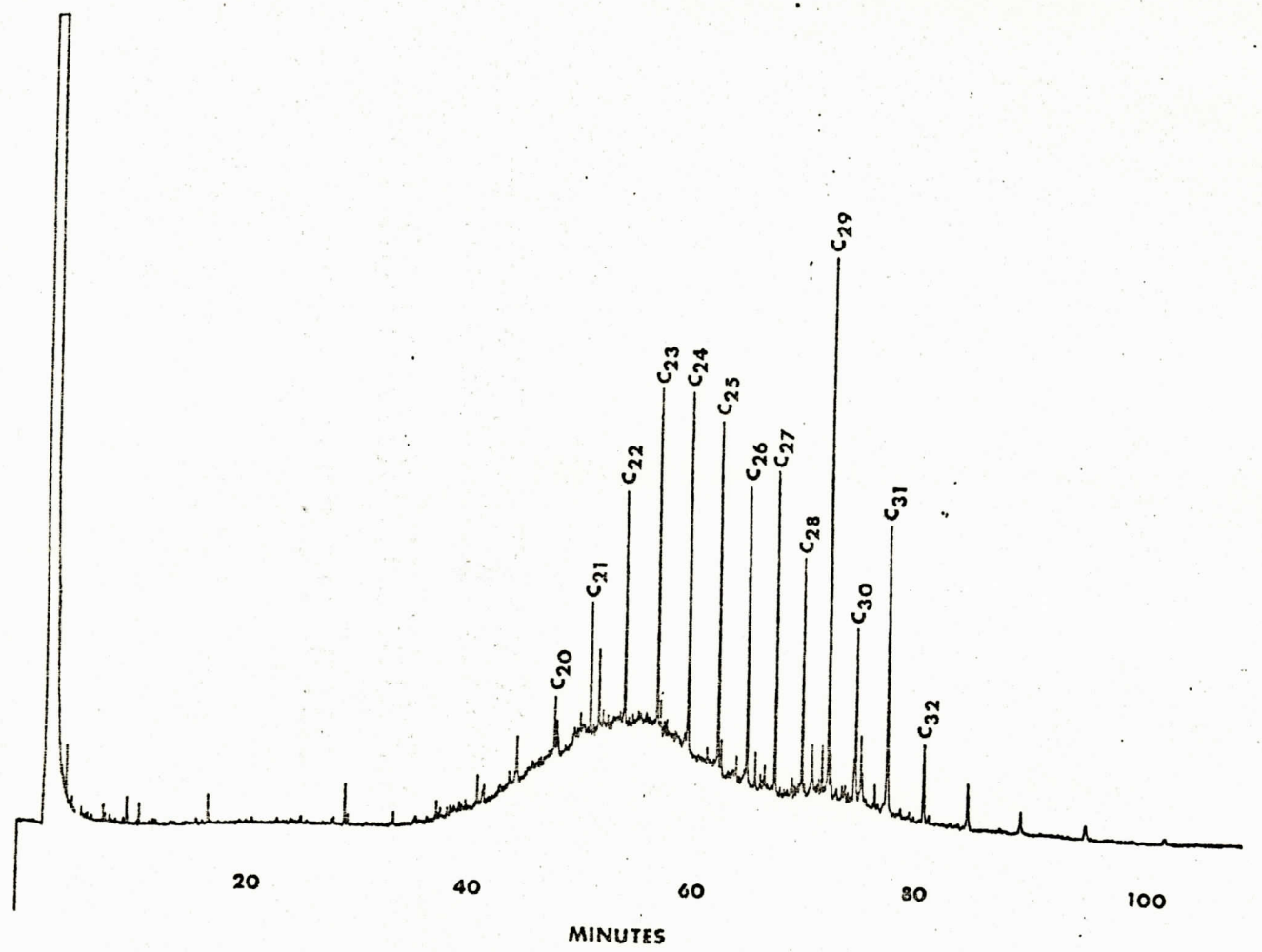

Figure 16. Chromatogram of the hydrocarbon fraction of Cyathea divergens.

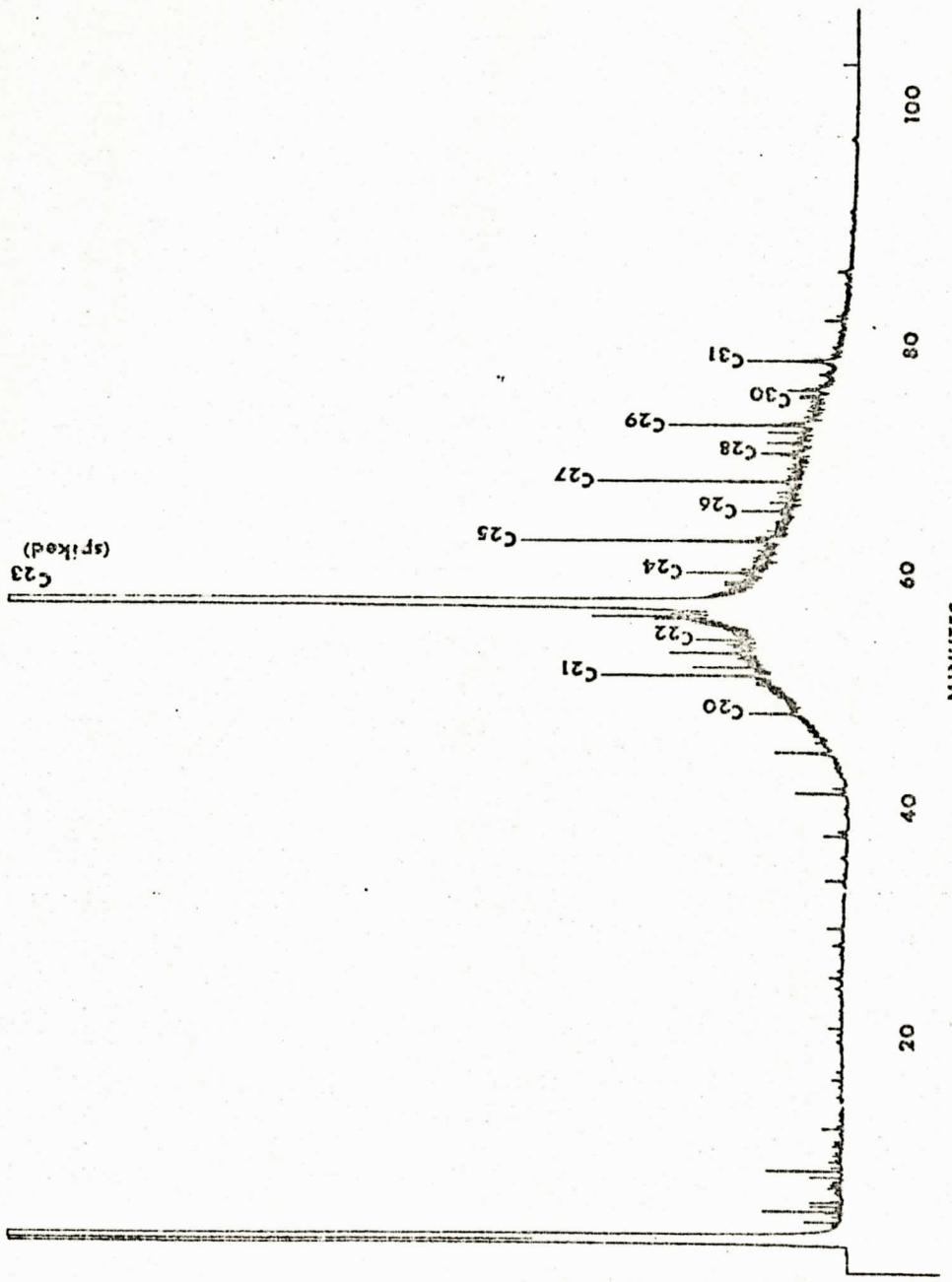

Figure 17: Chromatogram of the hydrocarbon fraction of Cnemidaria horrida.

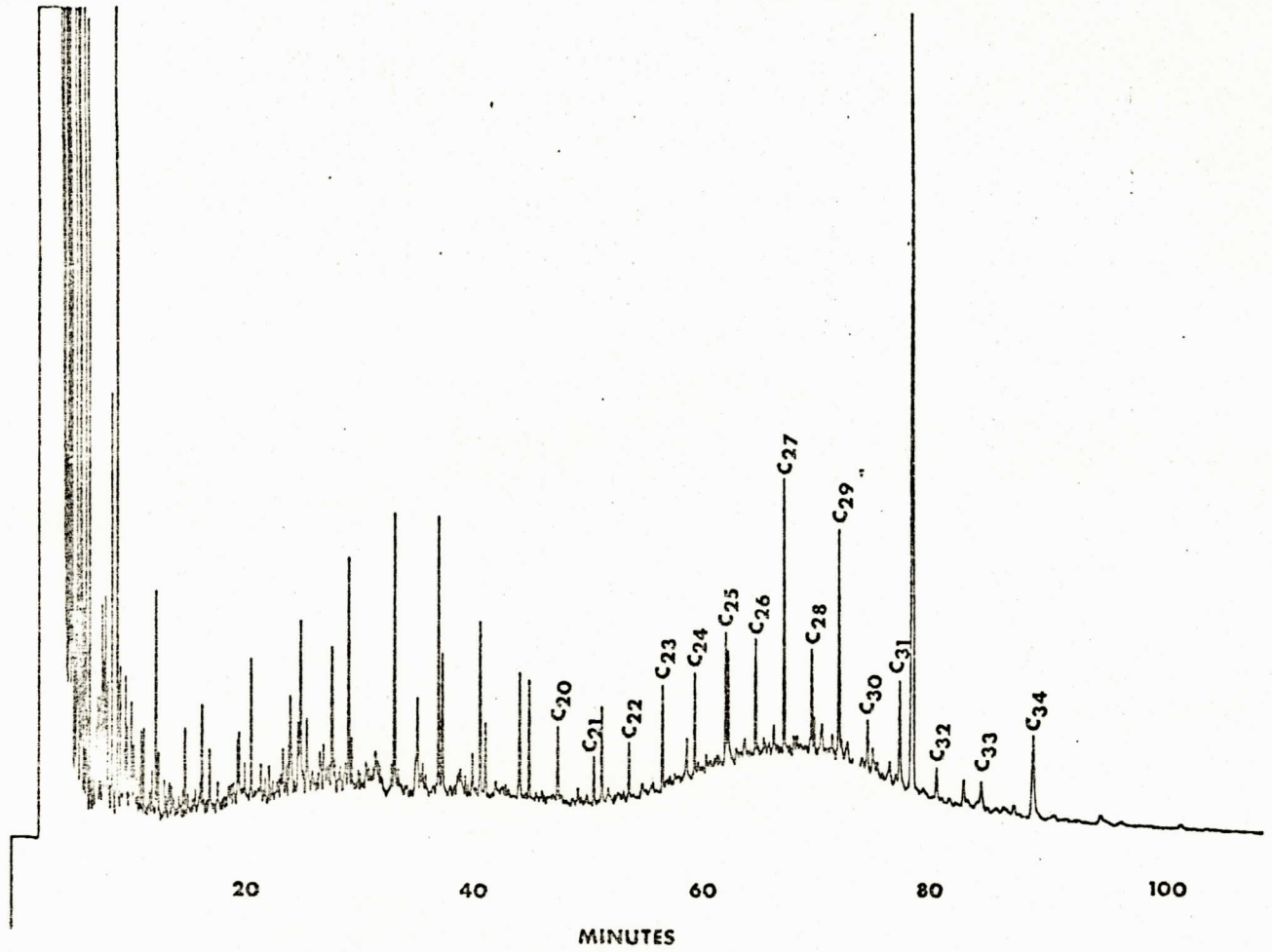

Figure 18. Chromatogram of the unsaponifiable fraction of Dicksonia berteroana.

pattern exhibits a maximum at C_{34} (Table IV).

A graphical representation of Table IV is presented in Figure 19. This clearly illustrates the distribution patterns of the seven species studied. The species demonstrate marked differences in their n-alkane distribution patterns; however, due to the limited nature of this study, these differences should not be used to draw any conclusions concerning their taxonomic standing.

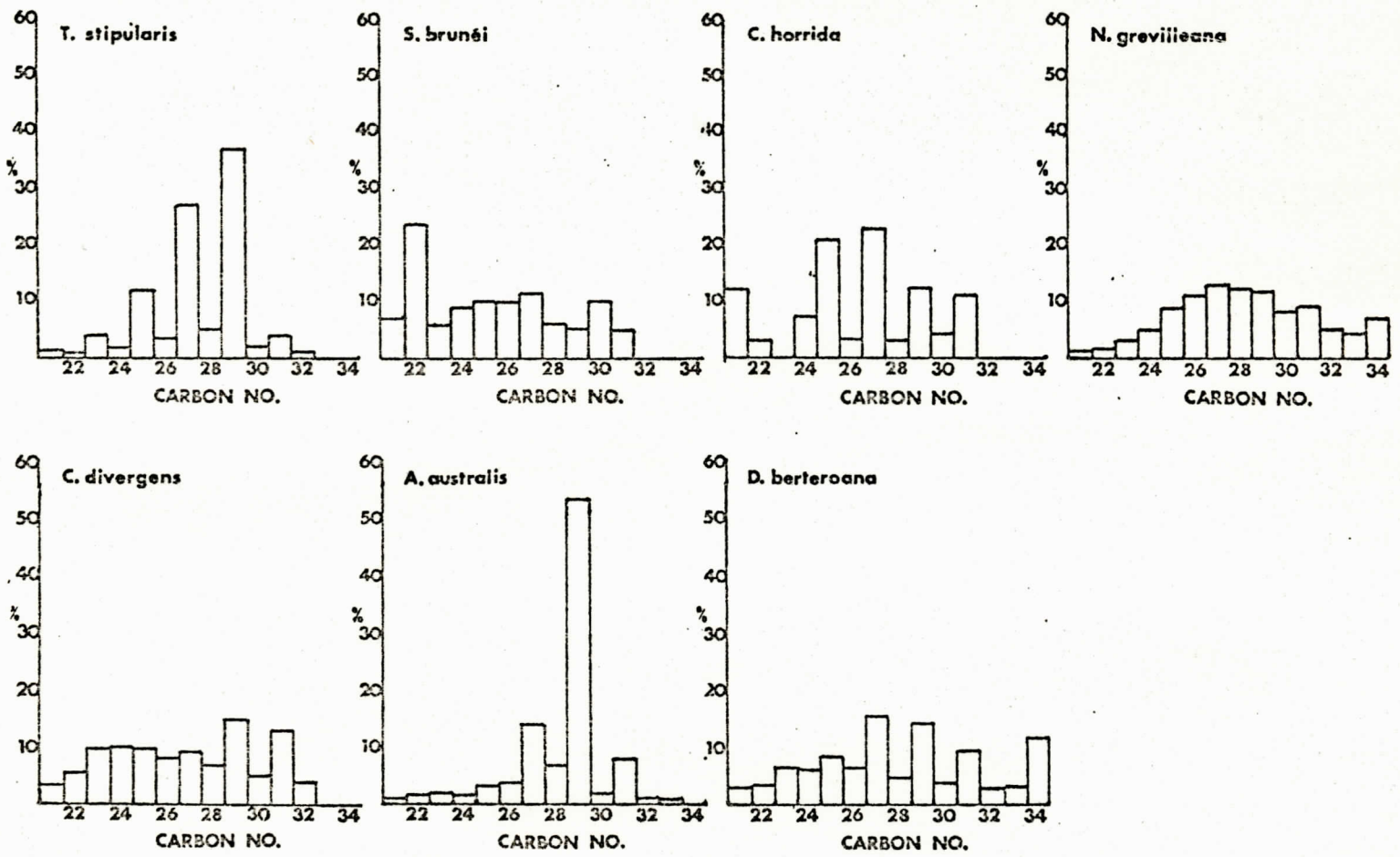

Figure 19. Normal alkane distribution in Cyatheaceae and Dicksoniaceae species studied. The percent of each n-alkane is calculated as the percent of the total n-alkane fraction assuming unitary detector response.

CHAPTER V

THE CONCLUSION

Recent phytochemical studies in the Plant Kingdom demonstrate the successful application of normal alkane distribution patterns to taxonomic problems. Although this study is limited to only seven species in the Cyatheaceae and Dicksoniaceae, qualitative differences in their leaf wax composition are obvious. The patterns appear to be significantly distinct, but because of the limited nature of this study, the usefulness of the normal alkanes as taxonomic characters remains uncertain.

Infrared spectra of each fraction confirm the efficiency of saponification and silver nitrate-silica gel column chromatography. The use of fused silica columns with hydrogen as a carrier gas proves to be effective in resolving the unsaponifiable and hydrocarbon fractions encountered. The elimination of the time-consuming column chromatography step is possible, thereby reducing the number of samples required for each fern.

Further studies should include the analysis of the saponifiable fraction of fatty acids and the identification of the additional homologues in the unsaponifiable fraction. A GC-MS survey of the Cyatheaceae and the Dicksoniaceae would be desirable for positive identification of components.

A more comprehensive survey of the ferns is suggested. If possible, this study should include at least three species in each genera of the Cyatheaceae and the Dicksoniaceae; the monotypic Lophosoria quadripinnata, Metaxya rostrata, Thyrsopteris elegans, and Cystodium sorbifolium should be analyzed as well. Hopefully the results of this expanded survey will sufficiently indicate the true value of normal alkanes in fern chemotaxonomy.

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