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Differentiation of Mistletoes on the Basis of Geographical Origin Using Capillary Gas Chromatography and Multivariate Analysis

by

Michael M. Looney

A dissertation presented to the
Graduate Faculty of Middle Tennessee State University
in partial fulfillment of the requirements
for the degree of Doctor of Arts

December, 1996

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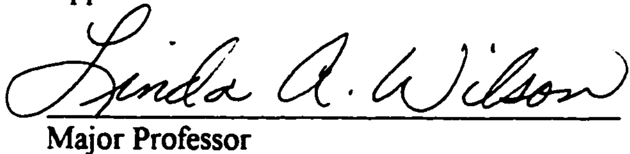

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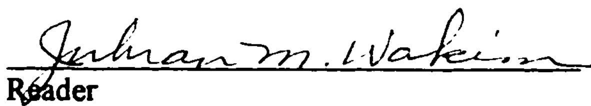

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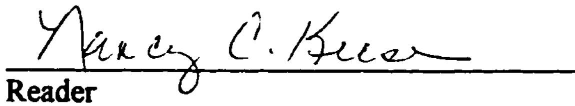

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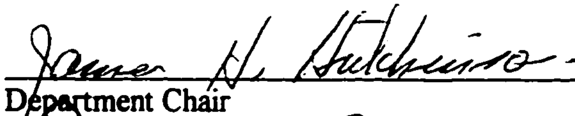

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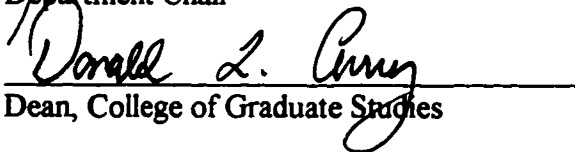

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ABSTRACT

Differentiation of Mistletoes on the

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Gas Chromatography and Multivariate Analysis

By Michael M. Looney

Multivariate statistical analysis of gas chromatographic data has been applied to the differentiation of species of mistletoe based on their geographic origin. Mistletoe plants were collected from 26 locations in Texas and 13 locations in Tennessee, Alabama, Georgia, and Florida. Hexane extractions were analyzed by split injection capillary gas chromatography. The column used was a Hewlett Packard capillary column Ultra 1, 25-meters long with an I. D. of 0.2 millimeters and a film thickness of 0.11 micrometers. The oven was programmed from 100°C to 315°C at a rate of 7.5°C per minute and then held at 315°C for 30 minutes.

When the normalized chromatographic data were subjected to principal component analysis and hierarchical cluster analysis, two main clusters were seen. One contained only Texas samples and the other contained southeastern United States samples plus several Texas samples, possibly due to overlapping ranges of the species and/or seasonal variation. Texas samples were also evaluated separately. Two clusters were observed:

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one consisted of samples from north Texas and the other from south Texas. Some overlap of samples did occur probably due to an extended growth range of some species and/or subspecies.

The effects of different hosts and seasonal variation were evaluated using similarity indices. Samples were collected from four different hosts in and around Kerrville, Texas. In some cases, there was as much variation between samples from the same type of host as there were between hosts. Samples were collected from the same mistletoe plant monthly for nine consecutive months. Notable seasonal variations were detected, with the greatest difference occurring between the months of November and April.

This method appears to have value as a chemotaxonomic technique for the differentiation of mistletoes, but further study is warranted. Compounds that are independent of host, season, gender, and method of collection must be carefully selected for use in speciation of mistletoe.

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CHAPTER 1

INTRODUCTION

Interest in mistletoe has resurfaced recently due to the use of the alkaloid extracts in medicine. Since the 1920's these extracts have been used in the treatment of human neoplasia (1). Most early studies were not well documented and thus much controversy existed on the use of these extracts. In more recent studies West and Feng (2) showed that the alkaloid rubrine C caused muscle contraction in mice and rabbits and also lowered the blood pressure in cats. Graziano, et al. (3) isolated tyramine from mistletoe and determined that it had a hypertensive effect. Ellington (4) isolated five proteins from mistletoe and showed that four had a depressor effect and one a pressor effect in anesthetized dogs. Khwaja, et al. (1) showed that alkaloids extracted from mistletoe inhibited the growth of leukemia cells in mice and humans.

Mistletoes are found in one of four biological families; Misodendraceae, Loranthaceae, Viscaceae, or Eremolepidaceae. These families are members of the order Santalales which includes self-nourishing plants that appear to grow like trees. The members of this family are hemiparisitic (with chlorophyll) or parasitic (without chlorophyll) and attach to the roots or stems of other plants. Gill and Hawksworth (5) indicated a single family status of mistletoes (Loranthaceae), but Kuijt (6) and Cronquist (7) put forward the four family classification and found that they include over a hundred genera and thousands of species. Most of the mistletoes are evergreen hemiparasites

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growing on the trunks and branches of trees. As hemiparasites, mistletoes establish connections to the host plant from which they derive water and nutrients, but not carbon.

Historically, mistletoes have been used as objects in religious and supernatural ceremonies. The British and Europeans were especially enchanted with mistletoe growing on trees in the winter. These mostly agrarian populations put great significance on the greenery in an otherwise stark landscape. The Europeans gave mistletoe its mystical status, which has spread across the world and into many cultures. The Europeans also performed much of the early scientific studies on mistletoe. H. G. Bull, in 1864, wrote about the properties of mistletoe:

> The real properties of the plant itself are those of a slight tonic. The leaves and shoots have an astringent and rather bitter taste and strong extracts made from them are nauseous, bitterish and sub-austere. The berries are reputed to act as a purgative and are even now sometimes given to sheep for this purpose (8).

According to Calder (8), the term mistletoe comes from an Anglo-Saxon word "Misteltan". "Mistel" means dung, and "Tan" signifies twig. In other words the dung twig. This term graphically describes one of the mechanisms by which mistletoe are spread. Birds feed on mistletoe berries and spread the seeds through their droppings.

Some of the early beliefs about mistletoe centered around the plant having special significance. Oak trees were considered to have special powers and many religious ceremonies were held in oak groves. Mistletoe does not parasitize oak trees very readily, and when one was found the "mistletoe oak" (9) became the focus of the ceremonies. Calder (8) reported other beliefs about mistletoe which include associations with

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Christianity, power of fertility, ornamentation at Christmas time, and a cure for sterility and epilepsy.

It is commonly believed that the term mistletoe connotes parasitic behavior on branches of trees and shrubs, but not all mistletoes show this behavior. There are at least three genera of Loranthaceae which are trees or shrubs with root systems. These are found almost exclusively in Australia, they derive their mineral and water supply by establishing underground connections with the root systems of the host (10).

For those mistletoes that are parasitic, the dependence on a host plant is critical. Removal from the host results in death. The water and nutrient conducting xylem (11) of the host is of utmost importance for survival. The mistletoes tap into the xylem of the host in order to obtain the nutrients. Harris (12) reported that the osmotic concentration of the tissue of mistletoes is higher than that of the host on which they live. Lamont (13) studied the nutrient connection between the host and parasite and found that haustoria (massive absorptive organs which are root-like branches embedded in the host) provide the mechanism for passage of the nutrients from the host. Haustoria work in much the same way as any root system and thus rob the host of nutrients. Panvini and Eickmeier (14) found that the mineral concentrations in the mistletoe were one to three times higher than in the host and water use efficiency was about the same as the host. They noted some mixed results in the area of water use efficiency. Lamont and Southall (15) found similar results for mineral differences between the host and the mistletoe. The combined effect of water and nutrient parasitism leads to the following effects on a host: reduced

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growth rate, poor fruit and seed crops, malformation of woody tissues, sparse foliage, reduced crown, predisposition to disease, and premature death.

Seed dispersal is also critical in the life-cycle of mistletoes. As mentioned earlier, seed dispersal is carried out mostly by birds. Calder (8) studied host specificity and the activity of birds. He found that the relationship between birds and host was important in the survival of a mistletoe species. The color, texture, and form of the mistletoe is usually similar to the host and when the host has leaves, during most of the year, the mistletoe goes unnoticed. Birds eat the berries of the mistletoe, carry them to neighboring trees of the same species as the original host, and deposit the seeds in the new host. Dowsett-Lemaire (16) studied the relationship between tinkerbirds and mistletoes growing in south-central Africa. It was determined that the bird and mistletoe cohabitate the same range. Reid (17) studied the coevolution of mistletoes and birds in Australia. It was found that some birds are entirely dependent on mistletoe fruit, while others are only partially dependent. No coevolution evidence was determined, but some highly specific regional mutualism was found. Other minor mechanisms of dispersal include the spread by mammals and gravity, but these can not be compared to the activity of birds.

Of the four families of mistletoe that exist, there are two families that are very common: Loranthaceae and Viscaceae. Barlow and Wiens (18, 19) studied the differences between these two families and found that the differences center around floral structure and chromosomal count. They found that the Loranthaceae have large, conspicuous brightly colored flowers and the Viscaceae have small, simply constructed inconspicuous

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flowers. They also found that the number of chromosomes in the Loranthaceae varied from 8 to 12, whereas in the Viscaceae the number was relatively constant at 14.

Barlow (20) studied the biogeography of mistletoes and found that they exist throughout the world, but are concentrated in the paratropical regions of East Asia, Central America, South America, the United States, and Africa. The Loranthaceae are essentially found in the southern hemisphere and the Viscaceae are found in the northern hemisphere. Thus, Viscaceae is the family found in the United States.

The family Viscaceae consists of seven genera and about 400 species. The genus, *Phoradendron*, is confined to the New World and consists of some 170 species. Seven species are found in the United States (20). Wiens (21) studied the taxonomy and distribution of this genus. His work is considered the definitive work in this area. This genus is divided regionally into species; *serotinum*, found in the eastern United States and *tomentosum*, found in the central to western United States, comprise the greatest proportion of samples. *P. tomentosum* is further divided into two subspecies *tomentosum* growing in central Texas from Mexico to Oklahoma and *macrophyllum* growing from West Texas to California. The range of *P. serotinum* is from eastern Texas through the southeastern United States (22) up to Ohio through New Jersey (23). *Phoradendron* is thus identified as American mistletoe. The two mistletoe species that are the subject of this study are *P. tomentosum* and *P. serotinum*. Recently (24), a name change has been proposed for *serotinum*. The new species name, if accepted, would be *leucarpum*. *Arceuthobium* is another genus that is found in North America which has a growing range in Texas. This genus consists of some 24 species all of which are easily distinguishable

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from the *Phoradendron* species due to their size and shape. They are many times called "dwarf mistletoe" (20).

Chemotaxonomy is a very powerful tool that has been used for many years to distinguish species of biological samples. Chemotaxonomy is a hybrid subject encompassing chemistry, a highly specific and exact science, and systematics, which is more of an art. Chemical methods in taxonomy are used to record the presence or absence of various compounds, and to some extent, concentrations of these compounds. The taxonomist usually decides which are important characters for classification or interpretation and which are not. Large classes of compounds which are being used for taxonomic purposes are high molecular weight molecules, such as proteins, (25, 26) and relatively low molecular weight compounds, such as alkaloids (27) and terpenes (28). Chromatographic methods are usually used, since they are relatively fast, accurate, and inexpensive.

Takhtajan (29) indicates that chemistry has been utilized for plant classification since the early part of the 19th century, when the chemistry of natural products and the elucidation of their structure and biosynthesis began. It has grown, since that time, into a highly useful method for differentiating species: plant, animal, and insect. Cronquist (30) points out a number of problems associated with the application of chemical data to taxonomy. These problems are that chemical data are relatively hard to get, chemical analysis tends to consume the sample in the process of obtaining the chemical information, and chemical data does not lend itself to easily recognized patterns. Most taxonomists look for an easily observed character that lends itself to some pattern and they do not like

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to destroy their samples, but rather to preserve them for later analysis. There is then a built-in controversy between the traditional taxonomist and the chemotaxonomist.

Morphology has traditionally been the major source of information on which taxonomic schemes are based. It provides a great deal of observed differences among individuals, and lends itself readily to use by taxonomists. Some morphological observations include size measurements and floral structure (31). In the case of mistletoe the morphological characteristics are: leaf size and shape, internode length, shape of the flower, pubescence, and fruit size (21). The taxonomist then looks for patterns in these observations in order to classify the sample as a particular species. Most chemical approaches to taxonomy help the taxonomist to decide between alternative classifications already put forward. Chemotaxonomy will probably never replace comparative morphology as the primary source of taxonomic criteria for differentiating species, but it has become an important additional tool in identification.

In order to classify biological species, the taxonomist observes taxonomic characters. Heywood (32) defined a taxonomic character as "any attribute referring to form, structure, physiology, or behavior which is considered separately from the whole organism for a particular purpose such as comparison, identification, or interpretation." Defined in this way, chemical data could be used in the same way as traditional morphological features.

The current taxonomy of mistletoe leaves much to be desired. At the family and genus level the classifications are clear, but at the subgenera level it is an entirely different matter. Trelease, in 1916, performed the first comprehensive taxonomic study of

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mistletoe. To some, Trelease is what is known as a taxonomic "splitter". A "splitter" is one who looks for the slightest of variations in taxonomic markers leading to more species being identified and named than probably exist. Trelease once stated:

> "...in a monographic assemblage such as is here offered no lasting harm can come from the most radical segregation of forms possible on morphologic and geographic considerations..." (21).

Wiens (21), in 1964, reclassified many of the species of Trelease using a much more conservative approach. Data were available in the study that went beyond gross morphology. Cytology, physiology, and genetics of the genus *Phoradendron* was investigated. Even with this comprehensive work a great number of species were still classified throughout the world. Kellogg (33) offers the following observations on the difficulty of studying the taxonomy of *Phoradendron*:

1) any classification of herbaria samples is almost impossible due to their deterioration over time,

2) there are between 100 and 150 classified species, and

3) leaf shape varies within a particular species.

These facts lead to the observation that even highly trained taxonomists have a difficult task of differentiating species of mistletoe thus chemotaxonomy may be important in the future.

Flake and Turner (34) indicate that gas chromatography can be applied to taxonomic studies since almost every major plant group can be identified using volatile compounds. They reported the use of terpene molecules to identify species and races of juniper collected over a 500,000 square mile area from Texas to Washington DC. The

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characterization was done using a weighted variance of the quantitative distribution of 37 terpene compounds which they called a "terpenoid affinity profile". This profile classified all species with the same accuracy as traditional methods of taxonomy.

Gas chromatography is the analytical method that will be used in the current project. It is a method for separating components of mixtures of volatile compounds. The technique has been used in a variety of applications since 1952. In that year Martin and James (35) used gas-liquid chromatography to separate a mixture of fatty acids.

A gas chromatograph consists primarily of an injection port, carrier gas, column, detector, and data system. The components of the mixture are carried through the column by the carrier gas and are separated by their differing affinities for the stationary phase in the column and the mobile phase or carrier gas. The main component of the instrument is the column. The original columns consisted of four to twelve foot lengths with outside diameters ranging from one-eighth to one-quarter inch. They were packed with a granular material which was coated with the stationary phase. These columns did not provide a very good resolution for closely related compounds such as a mixture of hydrocarbons. Today, capillary gas chromatography is used when the analysis of a complex mixture is desired. A capillary column is an open tube about 25 meters in length with a small bore, about 0.25 to 0.50 millimeters. The inside wall of the tubing is coated with a thin film of a high boiling point liquid called the stationary phase. The use of a capillary column gives a chromatogram with a good deal of resolution even when a mixture of many compounds is analyzed. A small sample is injected into the column and a carrier gas, usually helium, carries the components of the mixture through the column. The length of time that each

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component is retained by the column is called the retention time. The retention time is characteristic of the extent of absorption of the component in the stationary phase and the flow rate of the carrier gas. When two or more components are present, each usually behaves independently of the others. The components are separated based on their varying affinities for the stationary phase. When the components are eluted at the end of the column, they will appear one after another in the gas stream.

To detect the components as they leave the column, a detector is used and many types are available. A typical detector is the flame ionization detector which mixes the eluted gas with hydrogen and air and the mixture is burned. As the sample is burned, ions and electrons are formed and detected by a decrease in resistance and peaks are recorded by the data system and the chromatogram is produced.

This chromatogram is unique to, and characteristic of, the mixture being studied. The data that are obtained from the gas chromatogram include retention time, which is characteristic of the component, and peak area and peak height, which are proportional to the amount of the component. Each mixture gives a pattern of these variables that is known as a "fingerprint". When comparing more than one mixture, the process is known as profile analysis. Profile analysis allows the researcher to differentiate among similar samples. If the exact chemical composition of the components of the mixture is desired, it is then necessary to identify the individual compounds using other techniques such as mass spectrometry.

There are countless applications of gas chromatography and profile analysis in the areas of biology and chemotaxonomy. Many foods have been analyzed using flavor and

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fragrance compounds (36, 37, 38, 39). As an example, Biggers, et al. (37) studied varieties of coffee. They found that *Coffea aribica* and *Coffea canephora* could be differentiated and blends of these coffees could be analyzed. Bade, et al. (40) used gas chromatography of leaf oils in the analysis of oranges and grapefruit. They found, based on 17 volatile components, that the volatile oil profile could be used as a taxonomic character.

Steltenhamp and Casazza (41) and Mookherjee and Trenkle (42) used the analysis of carbonyl compounds in the identification of the essential oils in *Lavandula* (lavender) plants. They found that the hybrid plant, lavendin, produces a hardier plant. However, this plant produced an oil that was much harsher than true lavender. Thomas and Egger (43, 44) similarly analyzed camomile plants. They determined the presence of novel ketones and esters in these plants. Brehm and Alston (45) and Harney and Grant (46) profiled the genera *Baptisia* and *Lotus*, members of the pea family, to determine various species using phenolic compounds. In these genera, the chromatographic analyses allowed an accurate identification of species. Von Rudloff, and co-workers analyzed terpenes in the volatile oils of *Tanacetum vulgare* (tansy) (47) and *Juniperus sabina*, (juniper) (48, 49, 50, 51, 52) for characterization of species. They found that terpenes are an excellent group of compounds to use in these differentiations. They used visual comparison of the chromatograms and were able to distinguish among five species of juniper based on 26 peaks and percentage composition of these peaks when an internal standard was added. They also compared the natural oils of these plants with those commercially produced and found no significant differences. Greinwald, et al. (53)

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identified the presence of alkaloid compounds in ten species of pea. Some taxonomic significance was noted using the gas chromatographic profiles of the flower extracts.

Profile analysis has been used in differentiating species of many insects. Lavine, and co-workers (54, 55, 56) have differentiated the European bee and the Africanized bee based on the concentration of cuticular hydrocarbons. They found that the concentration levels of only a few hydrocarbons are necessary to differentiate the species. Brill, et al. (57, 58) also used cuticular hydrocarbons to differentiate between colonies of fire ants. As with Lavine, they used only seven hydrocarbon peaks to differentiate among species. Lavine and Carlson (59) differentiated the *Helicaverpa zea* moth from the *Helicaverpa armigera* moth. They also found that only a few hydrocarbons were necessary to show differences in species. Cuticular hydrocarbon patterns have recently been used to differentiate dampwood termites (60) and Malaysian fruit flies (61). The field of insect chemotaxonomy by gas chromatography and profile analysis seems to be increasing in popularity. In each of these studies the technique of multivariate analysis was used to analyze the complex chromatographic data that was obtained.

Stevens, et al. (62) suggest that the components of epicuticular waxes of leaves are attractive taxonomic markers because of their universal presence. The use of leaf material as the source of these compounds has become important due to the complexity of the profile which can serve as a taxonomic fingerprint (63). The leaf extracts generally consist of long-chain alkanes, alcohols, fatty acids, fatty acid methyl esters, aldehydes, wax esters and triterpenes. Most findings concerning the composition of the leaf extracts may be summarized as follows: compounds with carbon numbers less than C_{25} and more than C_{34}

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are not present to any appreciable amount. Waldron, et al. (64) found that the compounds belong overwhelmingly to the n- and branched series of compounds with the n-series usually in greater amount. Eglinton, et al. (63) determined that the content of odd carbon-numbered alkanes is greater than the content of even carbon-numbered alkanes by a factor of more than ten. Carruthers and Johnstone (65) determined that some tobacco species contain high proportions of branched alkanes rather than the expected n-alkane content. Jarolimek, et al. (66) also found a number of branched alkanes present in three species of plants totally unrelated to each other. Douglas and Eglinton (67) found that the shorter chain length compounds, below C_{29}, show a much smaller ratio of odd to even carbon-numbered compounds. Thus, most plants, mistletoes included, show a dominance of the odd-numbered n-compounds centered around C_{29} and C_{31}.

The principal requirement for a taxonomic character is that it should be specific for a species. Eglinton, et al. (63) found that leaf hydrocarbon patterns were species specific. They studied four genera which contain a total of 35 species of the family *Sempervivoidceae* (houseleeks, herbs) which are endemic to the Canary Islands. Similarities were found in the patterns of the C_{31} and C_{33} hydrocarbons, but enough differences in concentration were found to differentiate the species.

Maffei (68) found that leaf wax n-alkanes and iso-alkanes could be used to differentiate several species of *Lamiaceae* (mint). The percentage of each alkane C_{25} to C_{35} for 58 samples was calculated and multivariate analysis techniques were used to distinguish the between families and genera. Zygadlo, et al. (69) used 17 alkanes and 14 fatty acids, extracted from leaves, and analyzed by gas chromatography and cluster

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analysis to differentiate species of *Condalia*. They were able to place samples from 14 populations into two species specific groups. Rojas, et al. (70) used gas chromatographic patterns of fatty acid methyl esters to classify peach palms to different races. They used principal component analysis (PCA), hierarchical cluster analysis (HCA), k-nearest neighbor (KNN), and soft independent modeling of class analogy (SIMCA) to distinguish groups using the fatty acid patterns. They were able to place peach palm samples into two races. Bohm, et al. (71) used flavonoids extracted from leaves of *Holocarpha* (tarweeds) to distinguish four species. Greenway, et al. (72) used PCA on gas chromatography-mass spectroscopy data of phenolic compounds to differentiate *Leuce* (poplar) species. They also determined that hybrids of species show patterns intermediate between the parental plants. Saitoh, et al. (73) studied the alkaloid content of sixty tobacco species, they found that all species contained the alkaloids, but the amounts varied with the species.

To use a chemical profile of the leaf extracts of a given species as a taxonomic character, the pattern should be independent of the season, the location, and the age of the individual specimen. Lavine and Carlson (59) mentioned above, also collected their moth samples form Florida, Mississippi, and South Africa and found the profiles to be the same irrespective of location. Eglinton and Hamilton (74) collected *Aeonium urbicum* (herbs) samples from four locations in four different months. Immature leaves, adult leaves, and dead leaves were collected and analyzed. They found that the C_{31} to C_{34} hydrocarbon pattern varied little and they concluded that the hydrocarbon profile could be used for a species without regard to season, location, or age. Bahr and Thakur (75) differentiated two species of *Ficus* (figs): *F. hispida* and *F. infectoria*. They also found that samples

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collected during different months contained all the hydrocarbons in the range C_{25} to C_{34}, but the relative abundance was quite different. Thus it seems that results are inconclusive regarding the season. They noted that perhaps leaf waxes were not a good taxonomic character considering the variation in the profile based on season.

The question of host specificity comes to mind when dealing with parasitic behavior. Hemmerly, and co-workers (76, 77, 78, 79, 80, 81) have found that the mistletoe species *P. serotinum* prefer particular hosts, such as *Celtis laevigata* (elm) and *Carya ovata* (walnut) but the parasite is found on almost every species of tree in the southeastern United States. *P. tomentosum* also grows well on a particular host, but again grows abundantly on all species of trees in Texas. Dossaji, et al. (82) reported that *P. tomentosum* grows on three different hosts in Texas: *Ulmus crassifolia* (elm); *Prosopis glandulosa* (mesquite); and *Celtis laevigata* (mulberry). They utilized paper chromatography of the leaf extracts to determine that the distributional pattern of flavone C-glycosides was uniform and independent of the host tree.

Mistletoe has received very little attention in the area of taxonomy or chemotaxonomy in the past 30 years. May (31) used paper chromatography as an aid in differentiating ecotypes of *P. tomentosum*. Tilney and Lubke (83) also used paper chromatography of phenolic compounds to differentiate 12 species of Loranthaceae growing in South Africa. Crawford and Hawksworth (84) studied the flavonoid patterns of 36 of the 38 known species of dwarf mistletoes using paper chromatography. Thirty-two New World species and four of the six Old World species were examined. They found, in most instances, that the patterns were consistent with known taxonomical

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classification. Gedalovich-Shedlitzky, et al. (85) used gas chromatographic profiles of the derivatized sugars of the polysaccharides extracted from the viscin mucilage to distinguish among three genera of mistletoe. They found that the extracts may be a useful taxonomic character.

The goal of the present study is to determine whether gas chromatographic profiles of solvent extracts of mistletoe leaves can be used to group the mistletoe samples by species based on geographical origin. The study is also being conducted to determine whether more than one species of mistletoe grows in Texas. The effects of seasonal variation and different host plants upon the chromatographic profile will also be investigated. This study is limited to mistletoe growing in Texas and the southeastern United States and is not intended to be inclusive of all mistletoe species growing elsewhere.

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CHAPTER 2

METHODS AND MATERIALS

Sample Collection

Mistletoe samples were collected from twenty-six locations in south, central, near east, north, and west Texas (Figure 1). In addition, eighteen samples were collected from thirteen locations in Alabama, Georgia, Florida, and Tennessee (Figure 2). According to Wiens (21), the samples collected in Texas, ***Phoradendron tomentosum***, should be a different species than the samples collected in the Southeast, ***Phoradendron serotinum***. Other Texas samples were collected from Iowa Park, Kerrville, and Falfurrias (Figure 3) for the study of the effects of seasonal variation and different hosts.

Extraction Method

Undamaged leaves collected from individual plants were allowed to dry, at room temperature, in open containers for a minimum of fourteen days. After drying, approximately three grams of leaves were crumpled into small pieces, placed in labeled 50-milliliter Erlenmeyer flasks, and extracted in 20 milliliters of chromatography grade hexane (OmniSolv HX0297-1 EM Science) for 96 hours. The extracts were removed from the leaves, placed in labeled, 20 milliliter vials, and allowed to evaporate under a hood for 24 hours. The dry samples were dissolved in three milliliters of hexane and filtered using a one milliliter syringe and 25 mm diameter PTFE membrane with 0.2 μm pore size microfilter disks (Supelco ISO-DISC P-252). The samples were stored in two-dram glass

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Figure 1. Mistletoe collection sites in Texas T1,2 = San Antonio, T3 = Beeville, T4 = Laredo, T5 = George West, T6 = Devine, T7,8 = Falfurrias, T9 = Hondo, T10 = Stockdale, T11 = Kenedy, T12 = Burnet, T13,14 = Austin, T15 = Kerrville, T16 = Georgetown, T17 = Fischer, T18 = Boerne, T19 = San Marcos, T20 = Terlinqua, T21 = Lajitas, T22 = Sonora, T23 = Mineral Wells, T24 = Lampasas, T25 = Iowa Park, T26 = Marble Falls, T27 = Hamilton, T28 = Marathon, T29 = Bastrop, T30 = Giddings, T31 = Brenham

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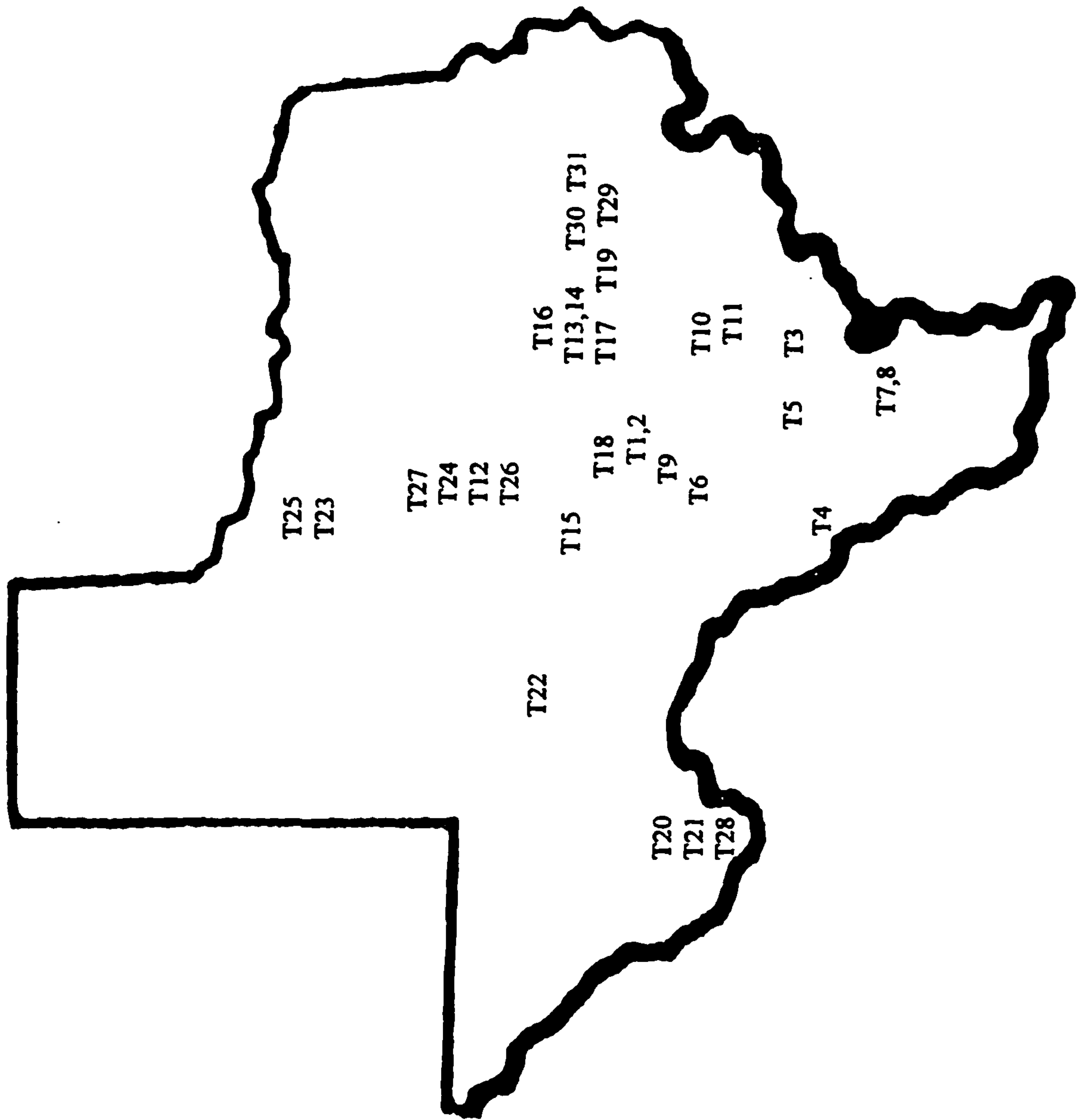

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Figure 2. Mistletoe collection sites in the Southeast S1 = Tennessee, I-24E, Mile 124; S2 = Tennessee, I-24E, Mile 125; S3 = Tennessee, Monteagle; S4 = Tennessee, I-24E, Mile 143; S5 = Tennessee, I-24E, Mile 144; S13 = Tennessee, I-24E, Mile 112; S6 = Georgia, I-59S, Mile 10; S7 = I-59S, Mile 11; S8 = I-59S, Mile 9; S9,12 = Alabama, I-59S, Mile 215; S10 = Alabama, Collensville; S11,18 = Alabama, I-59N, Mile 176; S14,15,16,17 = Florida, Ponce de Leon

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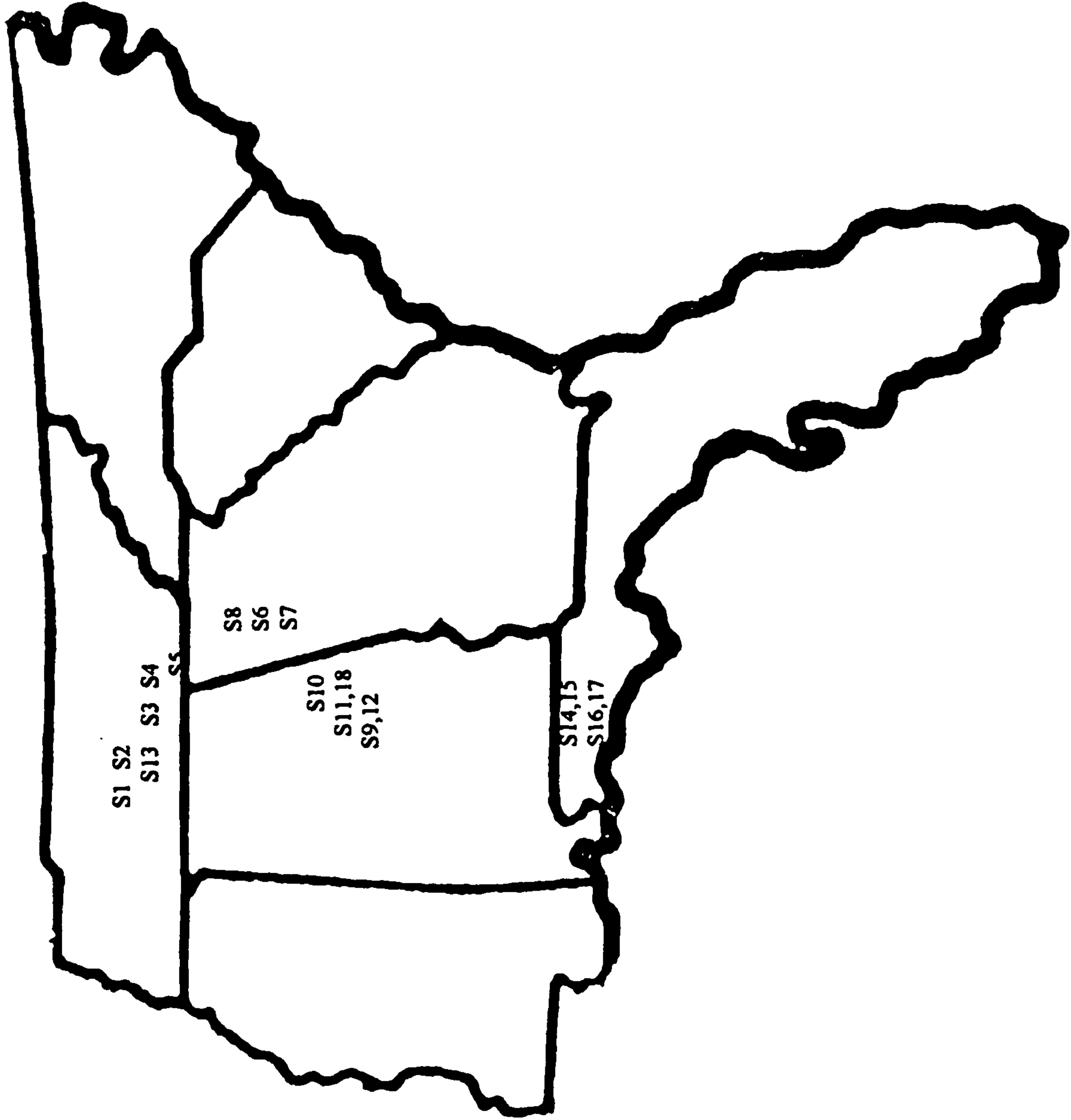

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Figure 3. Locations of other samples collected in Texas I1-I10 = Iowa Park; K1-K5, t1-t9, H1-H8 = Kerrville; F1-F10 = Falfurrias. These samples were used for the study of the effects of seasonal variation and different hosts.

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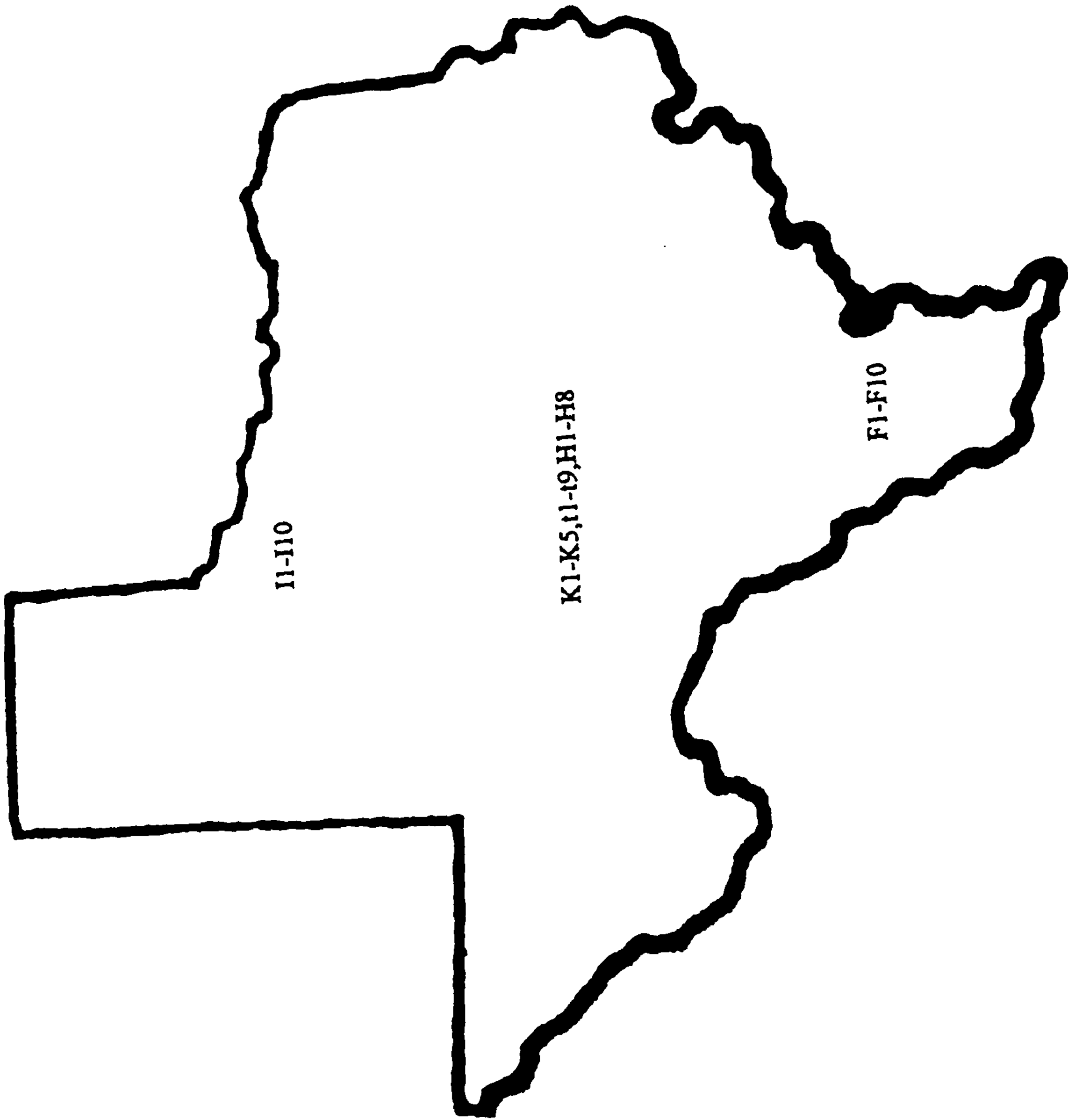

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vials with foil lined screw caps in a refrigerator at 8°C until analyzed. Prior to analysis, the samples were transferred to amber autosampler vials 32 millimeter x 11 millimeter (HP5181-3376) fitted with aluminum septum seals (HP5061-3370) crimped in place using a Wheaton crimper (W224301).

Sample Analysis

The samples were analyzed using a Hewlett Packard (HP5890II) capillary gas chromatograph which was fitted with a "split/splitless" capillary injector operated in the split mode, a flame ionization detector, and an automatic sampler (HP7673). The column of choice was a Hewlett Packard capillary column Ultra 1, 25 meters x 0.2 millimeter I.D. and 0.11 micrometer film thickness. Helium was used as the carrier gas with a head pressure of 76 pounds per square inch (psi) and a flow rate of 0.4 milliliter/minute (mL/min). The detector make-up gas was nitrogen with a head pressure of 40 psi and a flow rate of 30 mL/min through the detector. The flame gases were air and hydrogen with head pressures of 40 psi and 18 psi respectively and flow rates 400 mL/min and 30 mL/min respectively. The initial oven temperature was set at 100°C which was increased to 315°C at a rate of 7.5°C per minute. The oven remained at 315°C for 30 minutes for a total run time of 58.7 minutes. The injector temperature was 330°C and the detector temperature was 325°C.

Instrument control, data collection, and analyses were performed using a Hewlett Packard Series II Chemstation (HP3365) software loaded on a Hewlett Packard Vectra

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486/33U computer where the data was stored. The chromatograms were printed on a Hewlett Packard Model LaserJet 4 printer.

Initially, a method was written to control the gas chromatograph. All the parameters mentioned above were written into the method. A sequence was also written to control the automatic sampler. Prior to running samples, the method and sequence were loaded into the computer's memory and the sequence was run.

Computer Analysis of Data

The chromatograms were printed using the raw data from retention time zero minutes to 58.7 minutes with a relative minimum peak area of 1000. These chromatograms contained as many as 96 peaks with a good deal of overlap in the peaks. Most of the retention time data appeared between ten minutes and 30 minutes, therefore this range was chosen for further examination. Using the Chemstation, the chromatograms were reprinted between retention times 10 minutes and 30 minutes (Figure 4). If an overlap still remained, further narrowing of the retention time windows around the overlap was done in order to differentiate the peaks. This processing capability allows for comparison of samples without having to re-analyze them.

These data were subjected to a computerized pattern recognition program called Ein*Sight (version 3.0) (Infometrix, Seattle, WA). This program was loaded onto a Packard-Bell Statesman 486 microcomputer. The results were printed on a Hewlett Packard DeskJet 600C printer. The data were furthered analyzed by Microsoft Excel spreadsheet software loaded on the same computer system. Two types of data analyses

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Figure 4. A comparison of chromatograms of a mistletoe sample showing (a) the original and (b) the first enlargement emphasizing the 10 to 30 minute range.

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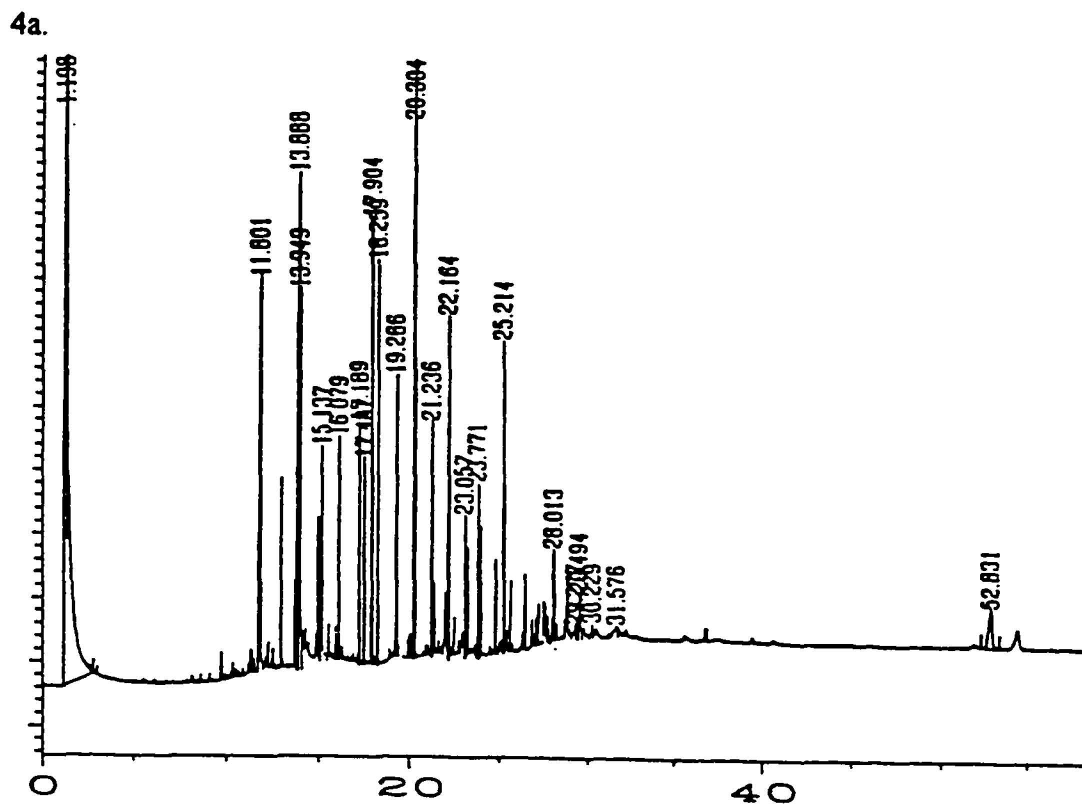
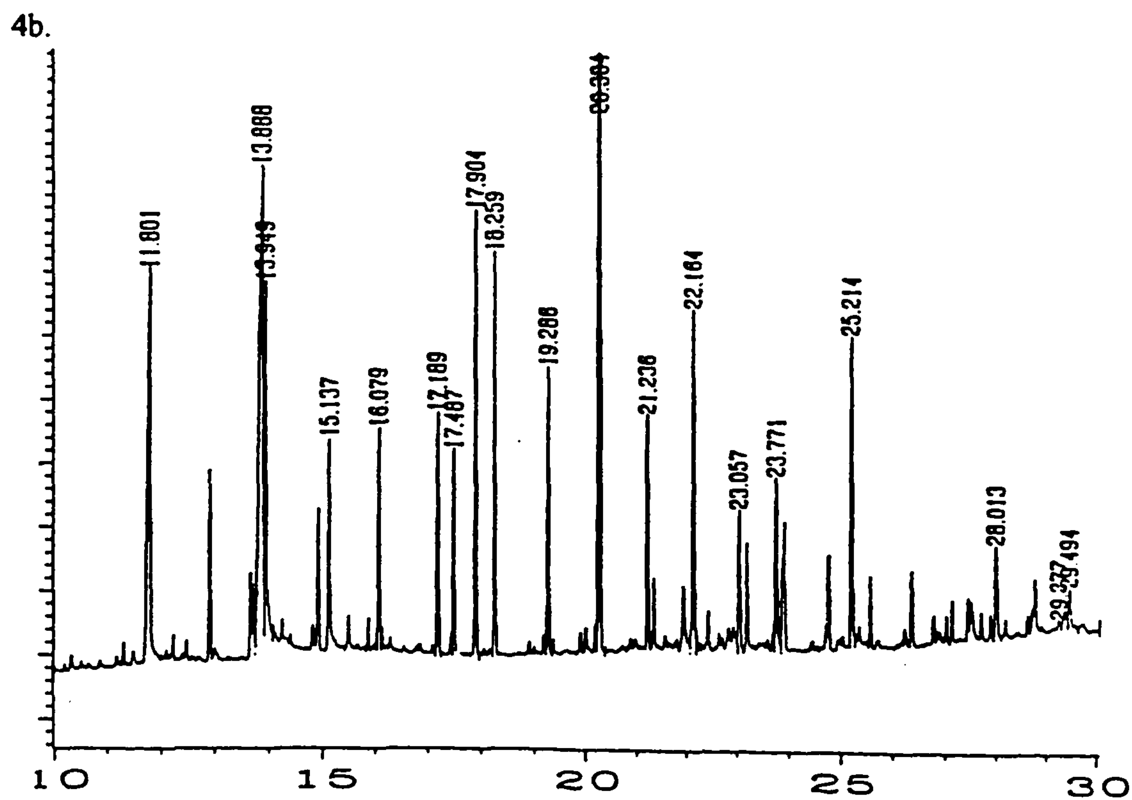
4a.



4b.



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were performed: principal component analysis (PCA) and hierarchical cluster analysis (HCA). When one has a set of samples with N variables, each sample can be represented as a point in N-dimensional space. Since humans have difficulty in visualizing data in more than three dimensions, pattern recognition analysis is a method by which these multivariate data can be visualized.

Hierarchical cluster analysis is a method by which distances between points in this N-dimensional space are calculated. The samples that are closer together are more similar than those that are farther apart. A dendrogram, a tree-shaped map, is developed from these distance calculations by linking samples and clusters of samples as a function of distance. Many linkage strategies are used which are mathematical formulas for calculating the distance matrices in various ways. Basically, each sample is considered a cluster of one. Then the two most similar samples are linked. Once this new cluster has been linked, it is linked to another cluster and this combination defines a third cluster. The distances between all existing clusters are computed, and the smallest distance is again searched and another cluster is created. Continuing this process links all the samples at some level of similarity. A dendrogram is produced from this linking procedure. The branches of the dendrogram have lengths that are proportional to the distances between the connected clusters. Similarity units are used to represent the distances in the dendrogram with the most similar samples assigned a value of one and the most dissimilar samples are assigned a value of zero.

Principal component analysis is a method of pattern recognition in which the N-dimensional data is reduced to two or three dimensions. Linear combinations of the

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variables generate new axes that are orthogonal (uncorrelated) to one another and yet retain most of the variance of the original data set. This produces a plot that yields the greatest amount of information possible in any two dimensional plot. The new axes that are created are prioritized based on the variance that they account for which occurs in the original data set. The greatest amount of variance is generally described by the first principal component followed by the second principal component, and so on. Eigenvector analysis is the mathematical model that forms the basis for PCA. In the current study, the normalized peak areas form the original data set. The transformed data set is a series of eigenvectors that represents the same relationships between samples that were present in the original data set. These eigenvectors or principal components represent the significant information in the data, their order represents their importance in describing the original data set. The algorithm that is used in Ein*Sight to calculate the principal components is the non-iterative partial least squares method. Other methods are available depending on the software that is used.

It is recommended that the data be preprocessed as the initial step in HCA and PCA. Ein*Sight offers four types of preprocessing; mean-centering, variance scaling, range scaling, and autoscaling. The method of choice is usually determined by trial and error. The clustering techniques available in HCA are single link, complete link, centroid, incremental sum of squares, median, group average, and Lance and Williams flexible.

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CHAPTER 3

RESULTS AND DISCUSSION

Texas Mistletoes Compared With Southeastern Mistletoes

The Texas samples used in this part of the study were collected at the locations indicated in Figure 1 from October 1994 through March 1995 with most being collected during October and January. The mistletoe samples from the southeastern United States were collected at the locations indicated in Figure 2 during late February and early March 1994.

Visual comparison of chromatograms of mistletoes growing in Texas and those growing in the southeastern United States show some differences as illustrated in Figure 5. The chromatograms of the southeastern samples were all very similar while those of the Texas samples showed more variation. Some distinct differences do exist at retention times of 13.7, 15.1, 21.3, 22.9, and 23.8 minutes but they are not consistent across all chromatograms. The subtle differences in peak area are difficult to compare when one is comparing several chromatograms. Therefore the data were subjected to pattern recognition analysis. The peak area data were placed in a spreadsheet and the mean and standard deviation was calculated for each peak. Those peaks showing the greatest difference in mean value and the least difference in standard deviation when comparing each group were chosen for pattern recognition. This resulted in 12 chromatogram peaks with the following retention times: 13.7, 14.9, 15.1, 21.3, 21.4, 22.9, 23.1, 23.8, 25.4, 25.5, 25.6, and 25.7 minutes being chosen for analysis. Some of these peaks appeared as

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Figure 5. A comparison of chromatograms (a) from Texas and (b) from the southeast.

Note the differences at retention times of 13.7, 15.1, 21.3, 22.9, and 23.1 minutes which are indicated with the symbol ∇.

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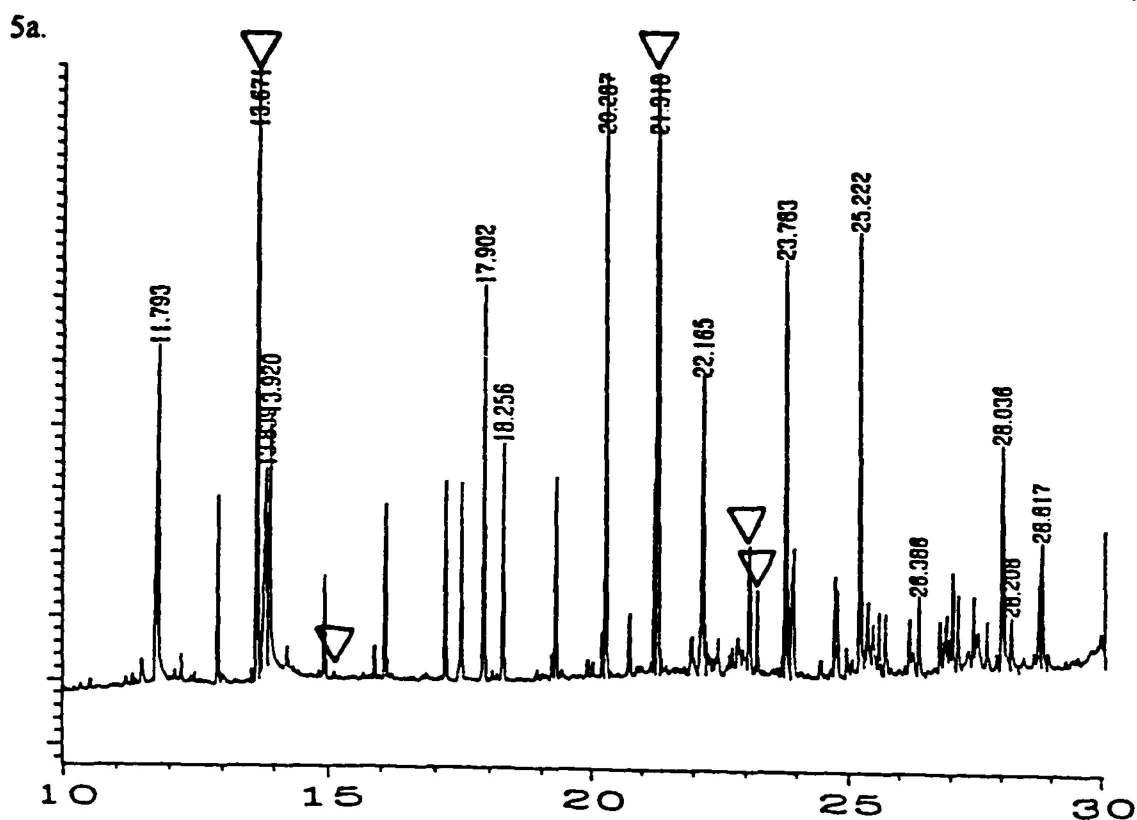
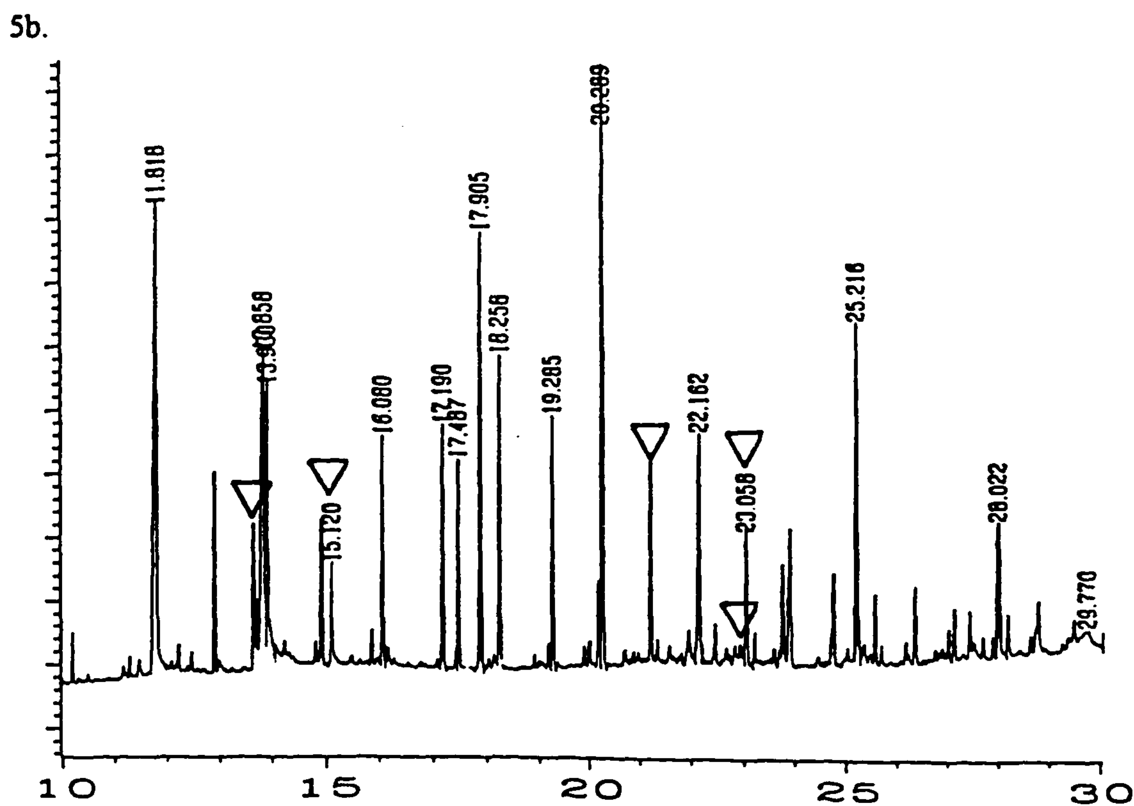
5a.



5b.



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major peaks in all the chromatograms and some appeared in one or the other. Each chromatogram contained a major peak at about 17.9 minutes. This peak was chosen for use as a retention time internal standard since it appeared at an intermediate retention time. To compensate for slight differences in conditions and to ensure the identity of the peaks, the relative retention time (RRT) for all peaks was calculated based on a RRT of 10.00 for the peak at 17.9 minutes. The data were further treated by normalizing each peak area to the total area of all peaks chosen for analysis. This was done in order to eliminate any differences in sample concentration or in size of sample injected.

Data for 49 mistletoe samples (31 collected in Texas and 18 collected in the southeast) were subjected to PCA and HCA. The preprocessing technique chosen for HCA with this data set was mean centering (subtract the average from the values of each variable). The clustering technique chosen was incremental sum of squares. A dendrogram (Figure 6) was constructed using this data revealing that at a similarity level of 0.279 two clusters are present. The top cluster consists of 25 samples, all 18 of the southeastern samples appear in this cluster with seven Texas samples. The bottom cluster consists of 24 samples, all from Texas. Of the 31 Texas samples, 74% were clustered together in the bottom cluster.

According to Levine, et al. (86), when comparing complex chromatographic data, one should attempt to reduce the number of descriptors (peaks) in order to minimize misclassification of samples due to chance alone. A ratio of number of patterns (chromatograms) to descriptors should be greater than four for each class in order to reduce chance misclassification to less than one percent.

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Figure 6. A hierarchical cluster analysis dendrogram produced by Ein*Sight for the data set of Texas and southeast mistletoe samples using 12 peaks. The preprocessing technique was mean centering and the clustering technique was incremental sum of squares.

Note the presence of two clusters. Cluster one consists of 25 samples, 18 from the southeast and seven from Texas. Cluster two consists of 24 samples, all from Texas.

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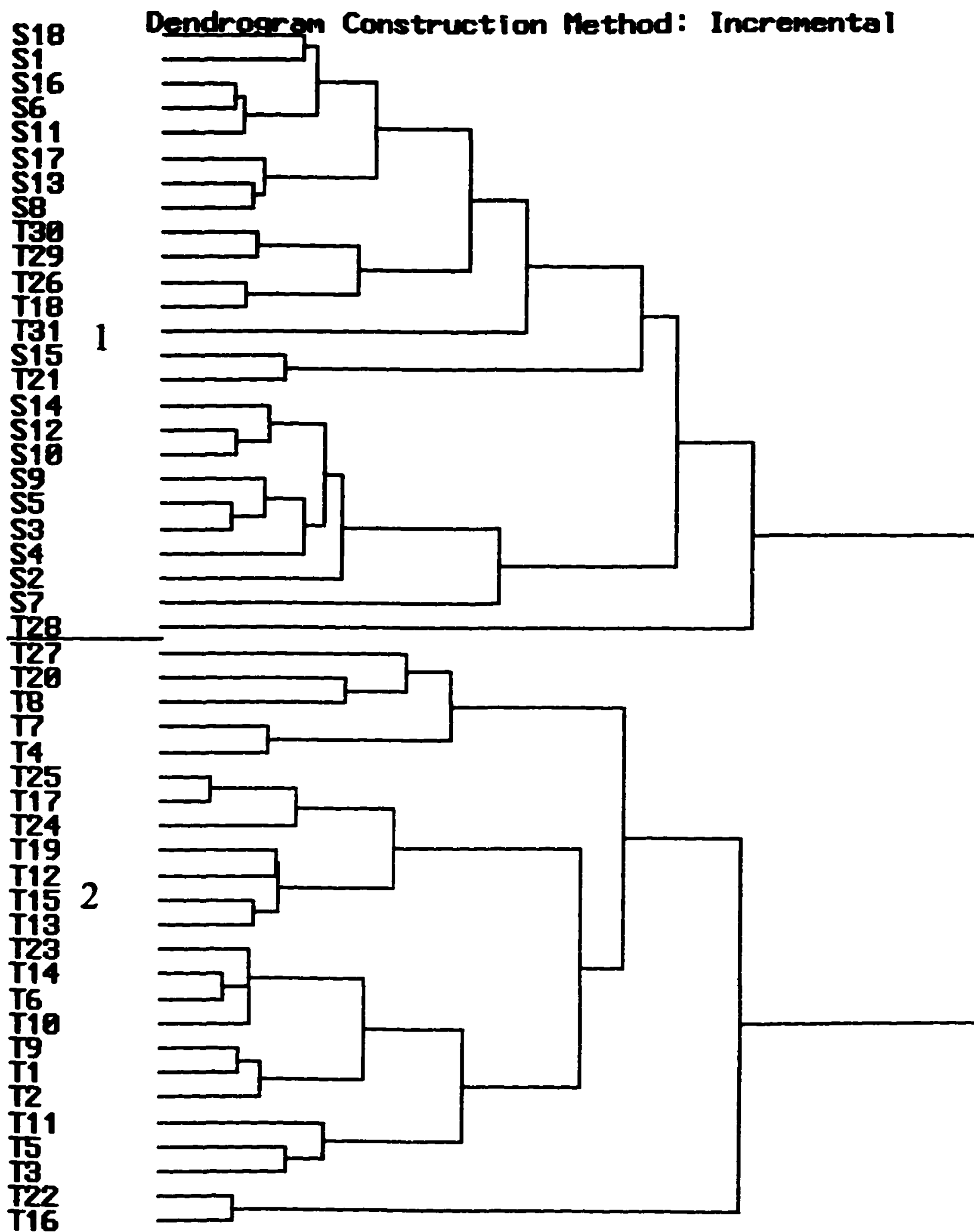

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In an attempt to improve the level of similarity of the clusters and reduce chance misclassification, the number of peaks was reduced to seven. These seven peaks were well resolved and consistent across samples. The peaks selected had retention times of 13.7, 14.9, 15.1, 21.3, 22.9, 23.1, and 23.8 minutes. Table I contains the RRT and normalized peak area for the Texas samples and the same data for the southeastern samples are contained in Table II.

A new dendrogram (Figure 7) was constructed using this smaller set of data revealing that at a similarity level of 0.653 three clusters are present. The top cluster consists of 22 samples, again all 18 southeastern samples appear in this cluster along with four Texas samples. The middle cluster consists of five samples, all from Texas. These five samples form a cluster with the southeastern samples at a similarity level of 0.588. The third cluster consists of 22 samples, all from Texas. This second dendrogram shows much better similarity levels for the clusters than the first and is also more consistent with the geographical origin of the samples.

The cluster of five Texas samples consists of samples T22, T28, T29, T30, and T31. Three of these samples T29, T30, and T31 are the easternmost samples collected in Texas and could be *P. serotinum*. Samples T22 and T28 are two of the westernmost samples collected. Of the four Texas samples that were classified among the southeastern samples, T20 and T21 were the other western Texas samples. The remaining Texas samples that were clustered with the southeastern samples, T3 and T11, were the most southeastern of the Texas samples.

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TABLE I
Relative Retention Times and Normalized Peak Areas of Texas Samples

RRT: Sample	7.63	8.34	8.45	11.90	12.81	12.88	13.28
T1	17.6	5.3	0.0	50.2	1.7	9.3	15.9
T2	23.8	5.7	0.0	41.2	1.4	7.3	20.4
T3	38.8	6.0	0.0	21.5	1.9	11.8	20.0
T4	11.8	5.9	0.9	50.4	1.5	8.3	21.2
T5	11.8	2.2	0.2	69.2	1.1	4.8	10.7
T6	21.9	5.6	0.0	54.7	1.6	7.9	8.2
T7	19.4	5.6	1.3	57.0	1.0	7.2	8.6
T8	11.2	6.3	0.0	61.2	1.9	9.0	10.4
T9	28.8	5.6	0.0	38.4	2.1	8.8	16.3
T10	26.4	3.3	0.0	49.1	2.0	5.9	13.4
T11	53.4	14.6	0.0	0.0	2.8	14.8	14.3
T12	6.5	2.5	5.0	52.4	0.9	5.6	27.0
T13	11.4	4.1	0.0	65.2	1.0	5.7	12.6
T14	12.6	4.5	0.4	66.4	1.0	5.4	9.6
T15	6.0	4.7	0.5	65.2	1.6	5.9	16.1
T16	1.7	2.4	0.1	62.2	1.9	5.8	25.9
T17	1.7	6.6	2.5	25.6	4.6	12.2	46.8
T18	9.0	11.7	0.0	35.2	2.0	13.1	29.1
T19	0.7	3.1	0.0	44.9	3.7	4.0	43.5
T20	11.1	27.0	0.0	5.1	3.3	24.6	28.9
T21	3.6	41.6	0.0	0.0	6.4	35.7	12.7
T22	3.4	5.6	1.0	0.0	1.6	11.6	76.9
T23	10.3	3.5	0.4	50.1	1.9	5.5	28.2
T24	2.8	7.4	1.1	62.2	7.5	19.0	0.0
T25	2.7	11.1	4.5	57.2	6.0	18.6	0.0
T26	12.0	5.9	1.0	38.1	1.6	11.3	30.1
T27	3.2	4.0	6.4	73.9	3.4	9.1	0.0
T28	6.5	8.0	0.0	1.3	4.4	15.6	64.2
T29	2.7	16.4	0.0	2.4	3.7	18.9	55.8
T30	11.0	10.7	1.1	4.6	3.1	15.0	54.5
T31	7.2	16.9	0.0	5.2	0.0	18.6	52.1

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TABLE II

Relative Retention Times and Normalized Peak Areas of Southeastern Samples

RRT: Sample	7.63	8.34	8.45	11.90	12.81	12.88	13.28
S1	23.9	15.5	2.7	1.3	5.6	27.4	23.6
S2	0.9	18.2	23.7	2.7	5.9	27.1	21.4
S3	10.1	14.4	28.2	2.1	3.7	18.5	23.0
S4	12.4	9.7	25.5	0.0	3.3	14.3	34.8
S5	14.2	11.5	20.3	2.4	3.3	17.9	30.3
S6	8.0	19.1	0.0	2.6	3.5	27.3	39.5
S7	6.5	16.7	44.6	0.7	3.4	20.7	7.4
S8	6.3	8.2	40.1	2.5	1.7	10.8	30.5
S9	19.8	18.8	17.5	0.0	3.9	22.2	17.6
S10	5.5	16.4	25.1	5.4	3.6	24.6	19.4
S11	14.8	14.5	0.0	8.6	3.3	25.2	33.6
S12	12.4	15.1	28.8	3.2	3.4	17.9	19.2
S13	11.0	18.4	0.0	3.3	3.4	25.5	38.4
S14	5.2	13.4	18.1	3.7	5.7	18.5	35.3
S15	3.8	33.5	0.0	0.0	7.2	35.2	20.2
S16	18.3	15.4	6.5	3.5	3.8	25.4	27.1
S17	17.9	11.5	12.5	5.7	2.7	18.6	31.2
S18	3.1	16.7	0.0	6.6	5.0	25.0	43.6

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Figure 7. A hierarchical cluster analysis dendrogram produced by Ein*Sight for the data set of Texas and southeast mistletoe samples using seven peaks. The preprocessing technique was mean centering and the clustering technique was incremental sum of squares.

Note the presence of three clusters. Cluster one consists of 22 samples, again all 18 southeastern samples and four from Texas. Cluster two consists of five Texas samples. Cluster three consists of 22 samples, all from Texas.

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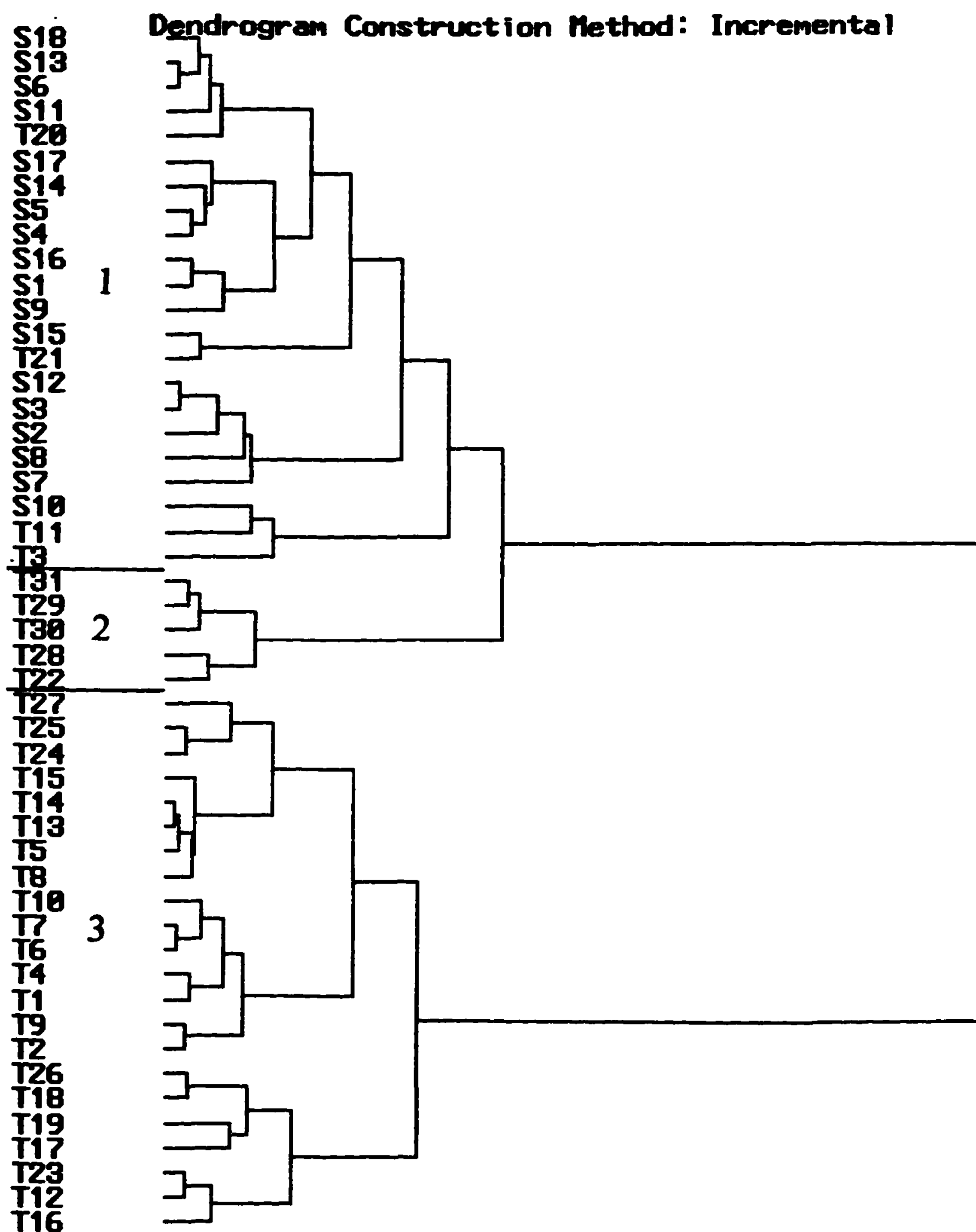

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For the PCA the preprocessing technique was range scaling (subtract the minimum, divide by the range). In the PCA plot (Figure 8) principal components one and two preserve 81% of the variance contained in this smaller data set. Eight Texas samples are plotted with the southeastern samples, thus 74% of the Texas samples cluster together. All of the southeastern samples cluster together in the PCA plot as they did in the dendrogram. The one Texas sample that was grouped with the southeastern samples in the HCA dendrogram that was not grouped with these samples in the principal component plot was sample T3. This sample was collected from southeast Texas and no apparent reason for its anomalous behavior is evident.

Clustering of the samples on the basis of geographical origin was observed. Perhaps the southeastern clustering can be attributed to a single species of mistletoe (*P. serotinum*) growing in this geographic location (21). Clustering of the Texas samples by geographic origin was also evident. Studies on the distribution of mistletoes growing in Texas (21, 22, 31) have shown that three taxa are present. May (22) indicates that *P. serotinum* occurs in eastern Texas, *P. tomentosum tomentosum* grows well in the central corridor of Texas from Mexico to Oklahoma, and *P. tomentosum macrophyllum* extends from the Edwards plateau to California. With this overlap in taxa it is possible that the Texas samples included three different taxa. Since the Texas samples were collected from October through March, it is also possible that seasonal variation had a major impact on the clustering. This aspect is investigated in the later part of this study.

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Figure 8. A principal component plot produced by Ein*Sight for the data set of Texas and southeast mistletoe samples using seven peaks. The preprocessing technique was range scaling.

Note the samples are represented by a number preceded by a T for the Texas samples and an S for the southeast samples. Eight Texas samples are clustered with the southeastern samples. With the exception of one of these samples, T3, they are the same samples that clustered with the southeastern samples in the dendrogram shown in Figure 7.

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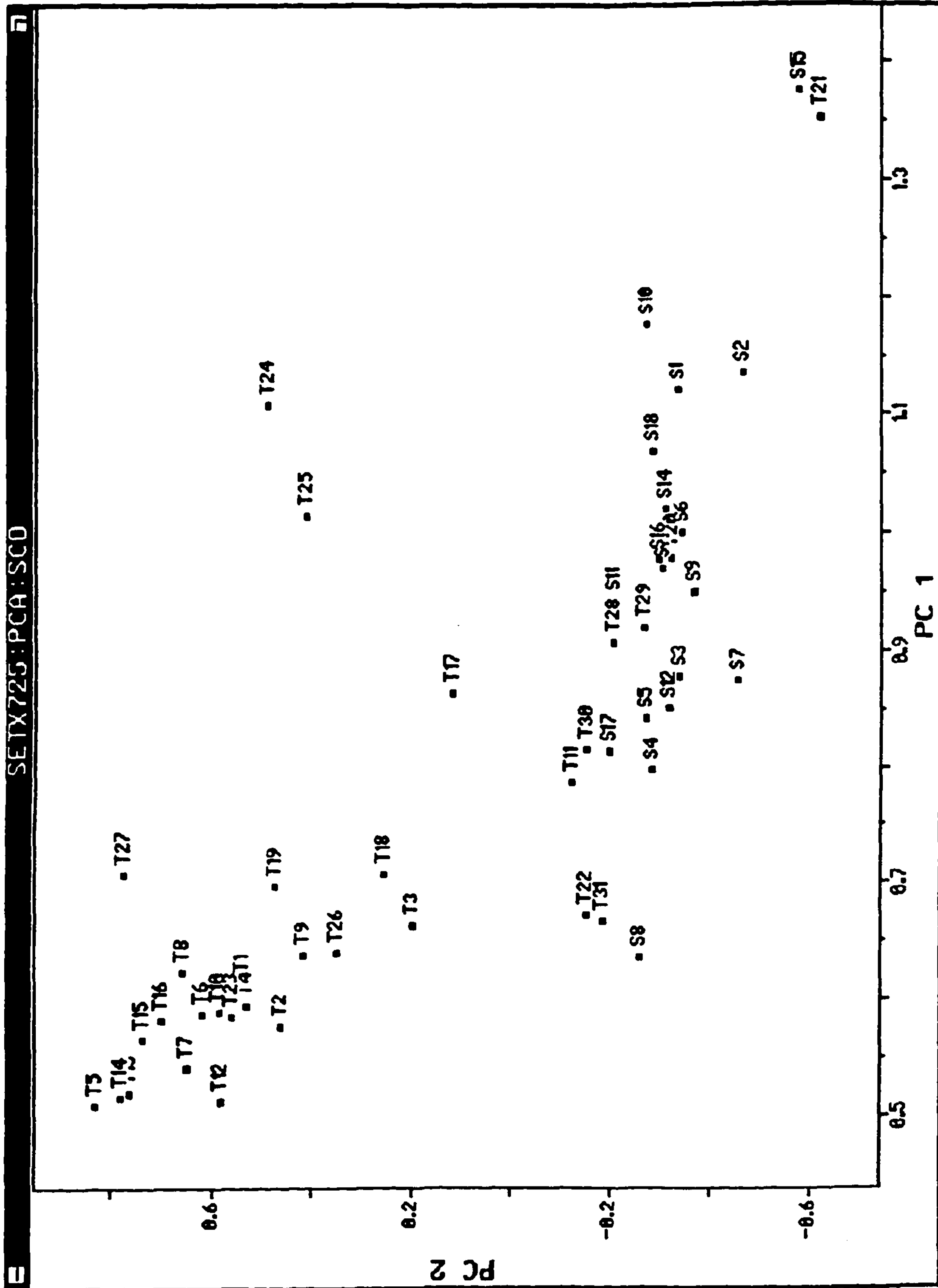

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Differentiation of Texas Samples Based on Geographic Origin

During the first three weeks of March, 1996, ten samples from different plants of mistletoe were collected from mesquite hosts in and around Iowa Park, Texas (North Texas) and ten samples were collected in and around Falfurrias, Texas (South Texas) as shown in Figure 3. Figure 9 shows typical chromatograms for samples from these two geographic regions. As with the Texas and southeastern samples, the peak area data were entered into a spreadsheet and the mean and standard deviation were calculated. The peaks showing the greatest difference in means and the least standard deviation when comparing each group were selected for further analysis. Sixteen peaks were chosen to be subjected to pattern recognition having retention times of 11.8, 13.8, 15.2, 15.5, 16.0, 16.3, 20.2, 20.3, 21.3, 21.4, 23.8, 25.2, 26.8, 27.1, 28.1, and 28.3 minutes. The RRT for each of these peaks was calculated based on the peak at 23.8 minutes which was a major peak in all chromatograms. The peak areas were normalized to the total area of all peaks chosen for analysis.

In constructing the HCA dendrogram for this data set, the preprocessing technique was mean centering and incremental sum of squares clustering was used. The dendrogram (Figure 10) indicates a similarity level of 0.201 for two clusters of samples. Six of the ten Falfurrias samples appear in the top cluster and seven of the ten Iowa Park samples appear in the bottom cluster. In order to improve the similarity level of the clusters and the pattern to descriptor ratio, the number of peaks was reduced to four. Table III contains the RRT and normalized peak areas for these four peaks.

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Figure 9. A comparison of chromatograms (a) from Iowa Park, Texas (North Texas) and (b) from Falfurrias, Texas (South Texas).

Note the greatest differences occur at retention times in the ranges of 15.1 to 18.2 minutes and 26.4 to 28.8 minutes. Other specific retention times of 20.2, 21.3, 22.1, 23.8, and 28.0 minutes should be noted. These retention times are indicated with the symbol ∇.

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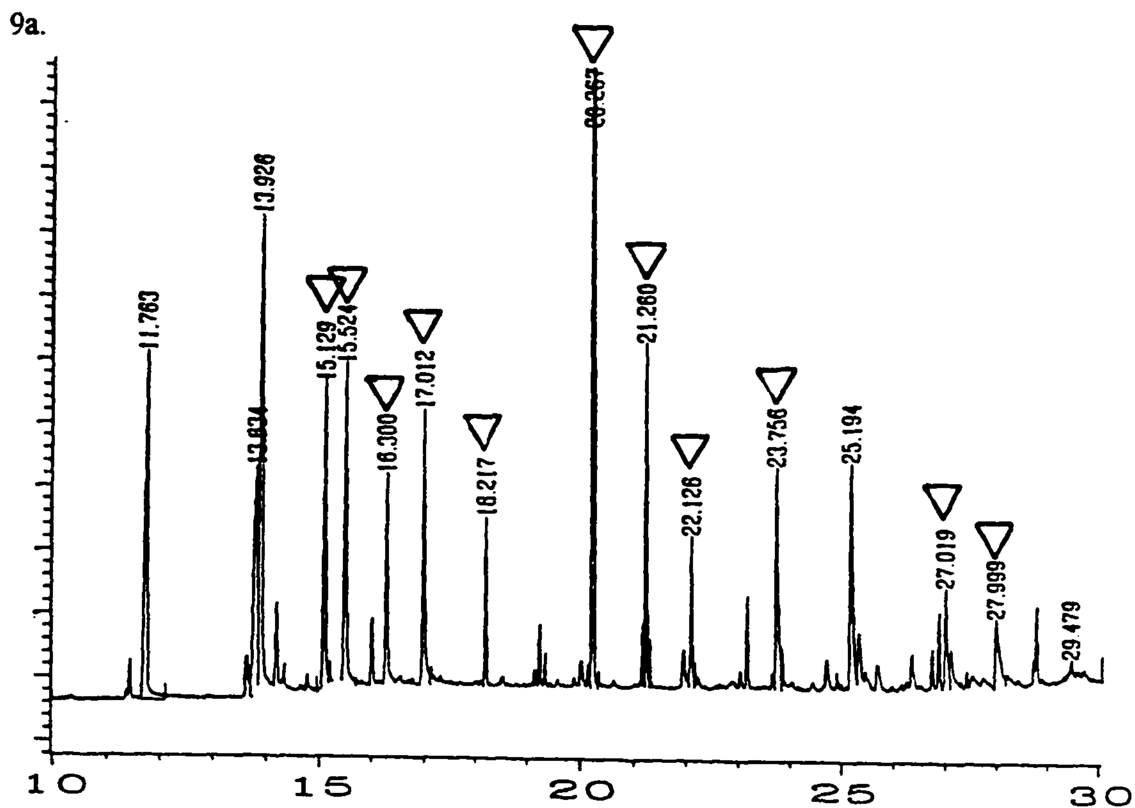
9a.



9b.

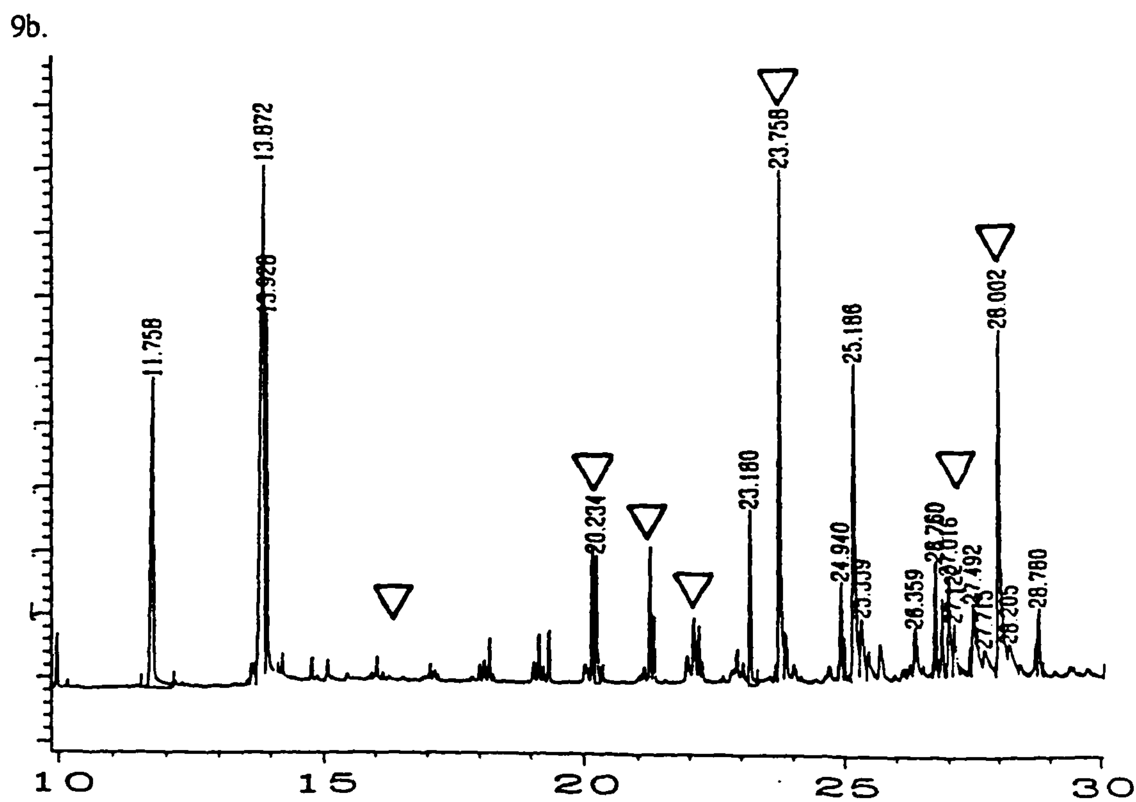


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Figure 10. A hierarchical cluster analysis dendrogram produced by Ein*Sight for the data set of 16 peaks for Iowa Park, Texas and Falfurrias, Texas mistletoe samples. The preprocessing technique was mean centering and the clustering technique was incremental sum of squares.

Note the presence of two clusters. The first cluster consists of nine samples, six from Falfurrias and three from Iowa Park. The second cluster consists of 11 samples, seven from Iowa Park and four from Falfurrias.

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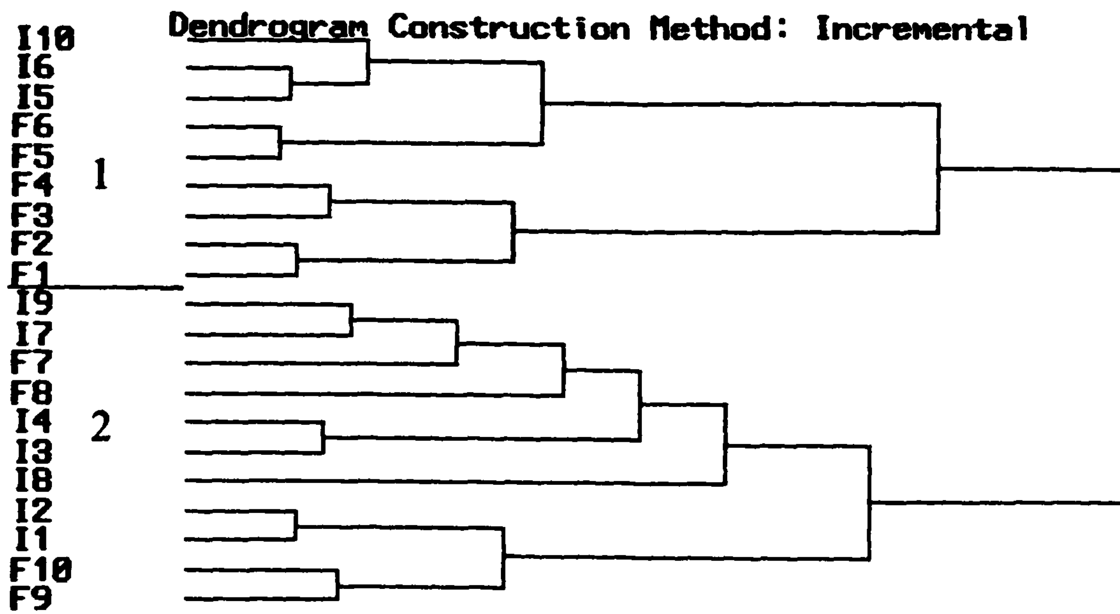

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TABLE III
Relative Retention Times and Normalized Peak Areas of Iowa Park (North Texas) and Falfurrias (South Texas) Samples

RRT: Sample	4.95	5.84	10.00	10.60
I1	9.4	11.4	38.4	40.8
I2	9.3	10.0	39.0	41.7
I3	7.5	4.5	48.5	39.5
I4	11.2	7.0	37.5	44.3
I5	14.6	9.8	38.0	37.7
I6	12.5	6.0	44.0	37.4
I7	10.5	8.9	45.9	34.8
I8	10.5	5.7	57.3	26.4
I9	10.1	6.8	38.4	45.0
I10	22.1	11.7	38.1	28.1
F1	31.8	28.5	17.6	22.1
F2	25.7	36.4	17.9	20.1
F3	19.5	42.8	18.4	19.3
F4	21.2	38.0	20.5	20.4
F5	15.7	26.7	30.9	26.7
F6	15.0	44.8	23.4	16.8
F7	15.1	43.2	22.8	18.9
F8	11.6	48.6	24.6	15.2
F9	21.7	12.5	29.3	36.5
F10	19.8	31.9	20.9	27.4

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A new dendrogram was constructed (Figure 11) that shows a similarity level of 0.746 for two clusters. All of the Iowa Park (North Texas) samples cluster together and nine of the ten Falfurrias (South Texas) samples cluster. The one Falfurrias sample that clusters with the Iowa Park samples could indicate a broad distribution of the subspecies growing in Texas.

In the PCA plot (Figure 12), variance scaling (divide by the standard deviation) was used. Again tight clustering of the Iowa Park samples is evident, sample F9 from Falfurrias is grouped with these samples, but all other Falfurrias samples are clustered together. Ninety-eight percent of the original variance of this smaller data set is accounted for in the first two principal components.

It is evident that these samples can also be differentiated on the basis of geographical origin based on four peaks. In order to say that these two groups of samples represent different species or subspecies, one would require a detailed taxonomical study which is beyond the scope of this project. May (31) studied *P. tomentosum tomentosum* growing in Texas and claimed that two ecotypes exist based on geographical origin. These ecotypes or races adapt to a different set of habitat conditions but it is not unreasonable to expect some overlap to occur in their growth range.

Host Comparison

To determine the effects of different hosts on the mistletoe characteristics, eight samples were collected from four different hosts in Kerrville, Texas on January 14, 1996. The hosts compared were Spanish oak, elm, mulberry, and mesquite. Fourteen peaks were chosen for this analysis with the following retention times; 11.7, 13.8, 15.1, 15.5,

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Figure 11. A hierarchical cluster analysis dendrogram produced by Ein*Sight for the smaller data set of four peaks for Iowa Park, Texas and Falfurrias, Texas mistletoe samples. The preprocessing technique was mean centering and the clustering technique was incremental sum of squares.

Note the presence of two clusters. The first cluster consists of 11 samples, all ten of the Iowa Park samples appear along with one Falfurrias sample. The second cluster consists of the remaining nine samples from Falfurrias.

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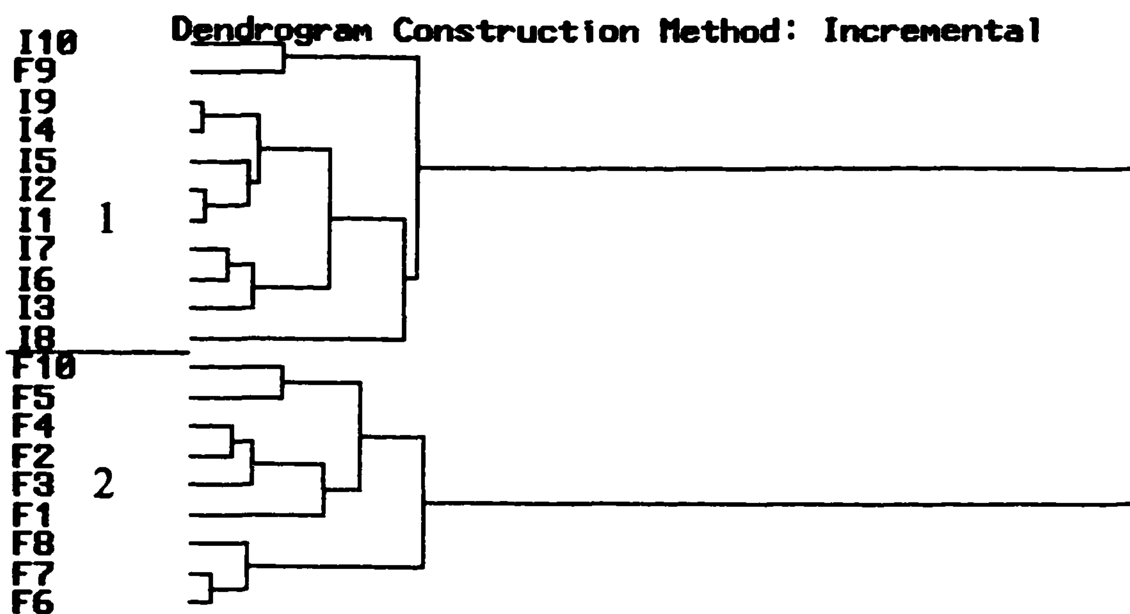

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Figure 12. A principal component plot produced by Ein*Sight for the smaller data set of four peaks for Iowa Park, Texas and Falfurrias, Texas mistletoe samples. The preprocessing technique was variance scaling.

Note the samples are represented by a number preceded by an I for the Iowa Park samples and an F for the Falfurrias samples. Note that with the exception of F9 clustering with the Iowa Park samples, there is good clustering of samples from the two geographic regions.

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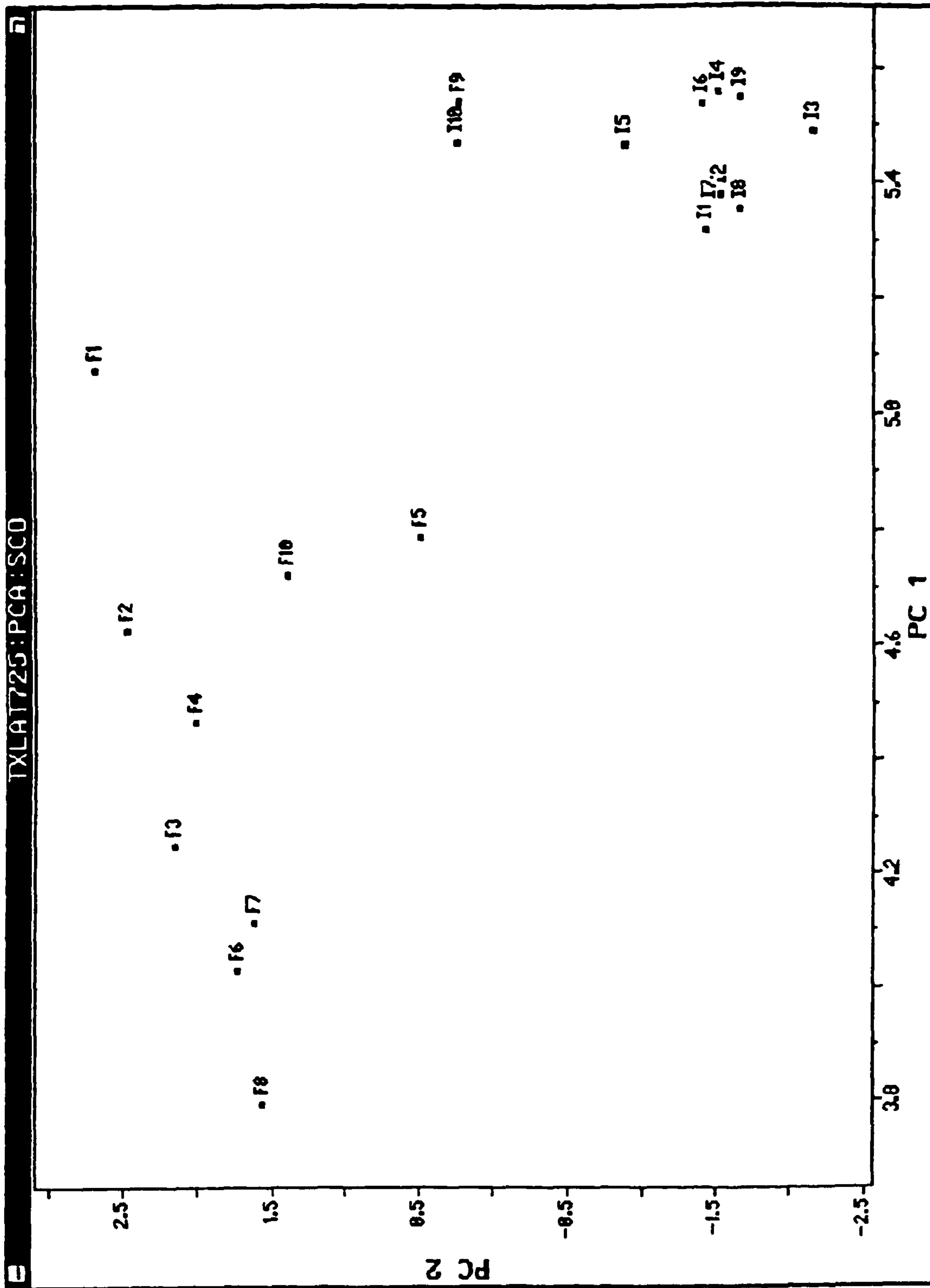

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16.0, 20.2, 21.2, 22.1, 23.1, 23.8, 25.1, 27.9, 28.2, and 28.7 minutes. These peaks were chosen because they were relatively well resolved and were readily identifiable in all chromatograms. The relative retention times were calculated based on the peak at 23.8 minutes. The host identity, relative retention times and normalized peak areas are shown in Table IV.

In order to compare the samples mathematically, a similarity index was calculated using the following formula (87):

$$SI = \frac{\sum X \bullet \sum Y}{\left(\sum X^2 \bullet \sum Y^2\right)^{1/2}}$$

where X is the normalized peak area for a sample at a particular retention time, Y is the normalized peak area for the standard (expected) at the same retention time, and SI is the similarity index. Each host sample was considered the expected and all others were compared against it. The results of the similarity index calculations are shown in Table V. When a perfect match is obtained the similarity index has a value of 1.000 such is the case when each host is compared against itself. A complete mismatch would result in a similarity index of 0.000. In some cases the difference between different hosts is less than the difference between samples from the same host. Hosts one and two were both Spanish oak trees and show the highest similarity, 0.915, within a particular host pair. Host four, elm, and host five, mulberry, show the greatest similarity between hosts. Thus the data are inconclusive and further study is warranted.

In an attempt to improve the similarity indices the number of peaks was reduced to ten. These ten peaks were chosen because they showed the least difference in standard

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TABLE IV

Relative Retention Times and Normalized Peak Areas for Host Comparison Using 14 Peaks

Host	RRT: Sample	4.93	5.83	6.39	6.54	6.75	8.52	8.95	9.31	9.75	10.00	10.61	11.79	11.88	12.11
Spanish Oak	H1	9.4	48.2	7.6	4.0	1.1	4.3	2.4	0.9	1.6	8.1	9.2	0.8	0.2	2.2
Spanish Oak	H2	6.3	28.7	8.9	4.6	0.9	11.5	8.6	2.6	2.7	13.5	9.3	0.5	0.5	1.5
Elm	H3	7.4	4.9	0.1	0.1	0.2	10.0	4.9	3.0	1.7	14.5	18.5	26.3	5.6	2.9
Elm	H4	9.2	37.7	0.6	1.4	0.3	5.6	8.3	2.1	1.4	14.8	11.5	4.8	0.6	1.8
Mulberry	H5	9.4	34.7	5.7	4.2	1.9	4.7	7.6	2.1	0.8	8.2	13.9	4.5	0.1	2.1
Mulberry	H6	9.1	13.9	3.5	2.1	0.5	7.1	16.1	3.4	2.1	16.0	17.7	3.2	0.1	5.4
Mesquite	H7	8.5	7.7	2.8	1.2	0.7	19.8	9.2	5.3	2.7	16.4	17.3	2.4	0.2	5.8
Mesquite	H8	9.1	13.0	9.1	3.9	1.2	1.4	19.1	1.9	4.7	13.8	1.6	1.5	0.1	3.6

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TABLE V
Comparison of Similarity Indices for Four Different Hosts Using 14 Peaks

Host	Expected Sample	H1	H2	H3	H4	H5	H6	H7	H8
Spanish Oak	H1	1.000	0.915	0.342	0.952	0.966	0.648	0.491	0.624
Spanish Oak	H2	0.915	1.000	0.505	0.945	0.948	0.840	0.757	0.795
Elm	H3	0.342	0.505	1.000	0.533	0.520	0.708	0.730	0.447
Elm	H4	0.952	0.945	0.533	1.000	0.977	0.807	0.655	0.725
Mulberry	H5	0.966	0.948	0.520	0.977	1.000	0.803	0.641	0.717
Mulberry	H6	0.648	0.840	0.708	0.807	0.803	1.000	0.898	0.843
Mesquite	H7	0.491	0.757	0.730	0.655	0.641	0.898	1.000	0.651
Mesquite	H8	0.624	0.795	0.447	0.725	0.717	0.843	0.651	1.000

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deviation in their normalized peak areas. Tables VI and VII show the data for this reduced number of peaks. With one exception (H3), all hosts compare at least at the 0.9 level of similarity. H1 and H2 compare at a 0.937 level, H3 and H4 compare at a 0.652 level, H5 and H6 compare at a 0.950 level, and H7 and H8 compare at a 0.975 level. Note that when H5 is used as the expected value in the similarity index formula, the similarities range between 0.787 and 0.962. The most similar comparison, in this set, being between H1 and H5, that is comparing a Spanish oak host to a mulberry host. Again, in some cases the difference between different hosts is less than the difference between samples from the same host. From this one might conclude that a careful selection of peaks would minimize any small effect due to different hosts. This is consistent with what was found by Dossaji, et al. (82). They determined that the distributional pattern of *C*-glycosides was uniform in *P. tomentosum tomentosum* irrespective of the host. In the current study we were looking at a mixture of long-chain alkanes, alcohols, fatty acids, fatty acid methyl esters, aldehydes, wax esters, and triterpenes (63).

Seasonal Variation

In order to study the effects of seasonal variation on the mistletoe chromatograms, samples were collected in Kerrville, Texas on the first Sunday of each month September 1995 through May 1996 from the same mistletoe plant growing on a mesquite host. The months of collection and sample identification are indicated as follows; September (t1), October (t2), November (t3), December (t4), January (t5), February (t6), March (t7), April (t8), and May (t9). Four samples were also collected on the same day in January

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TABLE VI

Relative Retention Times and Normalized Peak Areas for Host Comparison Using 10 Peaks

Host	RRT: Sample	4.93	6.54	6.75	9.31	9.75	10.00	10.61	11.79	11.88	12.11
Spanish Oak	H1	25.1	10.6	2.9	2.5	4.2	21.7	24.5	2.1	0.5	6.0
Spanish Oak	H2	14.9	10.9	2.2	6.2	6.4	31.8	21.9	1.1	1.2	3.5
Elm	H3	9.2	0.1	0.2	3.7	2.1	18.1	23.1	32.8	6.9	3.6
Elm	H4	19.2	2.9	0.5	4.4	2.9	30.9	24.1	1.1	1.2	3.7
Mulberry	H5	19.9	9.0	4.1	4.5	1.8	17.3	29.5	9.5	0.1	4.4
Mulberry	H6	15.3	3.5	0.8	5.8	3.5	26.8	29.8	5.3	0.1	9.1
Mesquite	H7	14.1	2.0	1.1	8.8	4.5	27.1	28.6	3.9	0.3	9.7
Mesquite	H8	15.9	6.7	2.2	3.3	8.2	24.0	30.7	2.7	0.2	6.3

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TABLE VII
Comparison of Similarity Indices for Four Different Hosts Using 10 Peaks

Host	Expected Sample	H1	H2	H3	H4	H5	H6	H7	H8
Spanish Oak	H1	1.000	0.937	0.655	0.949	0.962	0.940	0.922	0.958
Spanish Oak	H2	0.937	1.000	0.652	0.973	0.894	0.949	0.947	0.954
Elm	H3	0.655	0.652	1.000	0.677	0.787	0.755	0.733	0.709
Elm	H4	0.949	0.973	0.677	1.000	0.911	0.971	0.967	0.959
Mulberry	H5	0.962	0.894	0.787	0.911	1.000	0.950	0.928	0.957
Mulberry	H6	0.940	0.949	0.755	0.971	0.950	1.000	0.996	0.984
Mesquite	H7	0.922	0.947	0.733	0.967	0.928	0.996	1.000	0.975
Mesquite	H8	0.958	0.954	0.709	0.959	0.957	0.984	0.975	1.000

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1996 for comparison. Initially, 14 peaks were chosen for this analysis. The retention times that were compared are the same as those that were compared in the host study; 11.7, 13.8, 15.1, 15.5, 16.0, 20.2, 21.2, 22.1, 23.1, 23.8, 25.1, 27.9, 28.2, and 28.7 minutes. The relative retention times were calculated based on the peak at 23.8 minutes and the peak areas were normalized in the usual manner. Similarity indices were calculated using the same equation used in the host study and are shown in Table VIII. The seasonal variation similarity indices are quite different with the best matches occurring in adjacent months and the most mismatches occurring in distant months.

The number of chromatogram peaks was reduced to seven by selecting the peaks with the greatest standard deviation of the normalized peak areas. The month of collection, relative retention time, and normalized peak areas are shown in Table IX along with the mean and standard deviation of each peak area. For comparison the four samples collected in January are shown in Table X. The means were somewhat different for the peaks at the relative retention times of 5.83, 8.95, 11.79, and 11.88 minutes. The standard deviations for six of the seven peaks in the seasonal variation samples were higher than those for the samples collected in January and significantly higher in four of the peaks. The similarity indices were again calculated on this reduced number of peaks and are shown in Table XI. The similarity indices for this reduced number of peaks is almost identical to the data for the larger number of peaks, with the greatest similarity occurring between adjacent months and the least occurring between distant months. Similarity indices were also calculated for the four samples collected in January and the results are shown in Table XII. The similarity indices for all of the January samples is 0.95 and

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TABLE VIII

Comparison of Similarity Indices for Seasonal Variation Using 14 Peaks

Month	Expected Sample	t1	t2	t3	t4	t5	t6	t7	t8	t9
September	t1	1.000	0.983	0.868	0.502	0.399	0.564	0.298	0.247	0.346
October	t2	0.983	1.000	0.851	0.510	0.415	0.585	0.299	0.244	0.336
November	t3	0.868	0.851	1.000	0.752	0.625	0.839	0.469	0.481	0.580
December	t4	0.502	0.510	0.752	1.000	0.965	0.919	0.881	0.918	0.951
January	t5	0.399	0.416	0.625	0.965	1.000	0.840	0.964	0.967	0.974
February	t6	0.564	0.585	0.839	0.919	0.840	1.000	0.725	0.716	0.810
March	t7	0.298	0.299	0.469	0.881	0.964	0.725	1.000	0.958	0.964
April	t8	0.249	0.244	0.481	0.918	0.967	0.716	0.958	1.000	0.972
May	t9	0.346	0.336	0.580	0.951	0.974	0.810	0.964	0.972	1.000

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TABLE IX
Relative Retention Times and Peak Areas for Seasonal Variation Using Seven Peaks

Month	RRT: / Sample	4.93	5.83	8.95	10.00	10.61	11.79	11.88
September	t1	0.1	0.1	53.1	22.0	12.2	11.2	1.4
October	t2	4.1	1.3	58.3	16.5	14.1	3.6	1.6
November	t3	8.6	4.8	29.0	26.3	24.9	4.8	1.7
December	t4	17.9	29.7	13.1	21.9	15.5	0.8	0.1
January	t5	12.9	40.3	11.7	16.0	16.4	1.0	0.1
February	t6	21.9	16.3	14.1	15.0	23.3	4.1	1.5
March	t7	9.1	44.6	8.2	10.3	10.7	8.0	1.4
April	t8	11.6	49.0	4.0	22.2	10.1	0.3	0.7
May	t9	12.6	36.2	6.4	19.1	10.6	4.4	1.4
Mean		11.0	24.7	22.0	18.8	15.3	4.2	1.1
Standard Deviation		6.6	19.4	20.4	4.8	5.5	3.6	0.6

TABLE X
Relative Retention Times and Peak Areas for Four Samples Collected in January

	RRT: / Sample	4.93	5.83	8.95	10.00	10.61	11.79	11.88
	K1	14.4	12.6	5.8	22.5	16.1	23.2	5.5
	K2	15.3	12.7	6.4	23.2	19.2	18.7	4.5
	K3	12.0	6.9	12.5	16.2	20.2	24.3	8.0
	K4	14.6	8.6	10.3	15.4	20.2	23.9	7.1
Mean		14.1	10.2	8.8	19.3	18.9	22.5	6.3
Standard Deviation		1.4	2.9	3.2	4.1	1.9	2.6	1.6

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TABLE XI

Comparison of Similarity Indices for Seasonal Variation Using Seven Peaks

Month	Expected / Sample	t1	t2	t3	t4	t5	t6	t7	t8	t9
September	t1	1.000	0.983	0.869	0.504	0.402	0.568	0.302	0.249	0.353
October	t2	0.983	1.000	0.851	0.510	0.416	0.587	0.301	0.244	0.341
November	t3	0.869	0.851	1.000	0.752	0.625	0.843	0.472	0.482	0.588
December	t4	0.504	0.510	0.752	1.000	0.965	0.921	0.884	0.919	0.961
January	t5	0.402	0.416	0.625	0.965	1.000	0.840	0.967	0.969	0.982
February	t6	0.568	0.587	0.843	0.921	0.840	1.000	0.723	0.720	0.810
March	t7	0.302	0.301	0.472	0.884	0.967	0.723	1.000	0.964	0.964
April	t8	0.249	0.244	0.482	0.919	0.969	0.720	0.964	1.000	0.986
May	t9	0.353	0.341	0.588	0.961	0.982	0.810	0.964	0.986	1.000

TABLE XII

Comparison of Similarity Indices for Four Samples Collected in January

Expected / Sample	K1	K2	K4	K5
K1	1.000	0.991	0.957	0.969
K2	0.991	1.000	0.948	0.962
K4	0.957	0.948	1.000	0.995
K5	0.969	0.962	0.995	1.000

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higher. This indicates that there is much greater variation in the samples collected from one plant in different seasons than in the samples collected from different plants at the same time.

Based on these data, it appears that the season of collection of the mistletoe samples has a dramatic effect on the chromatographic pattern. This could indicate a set of peaks that should be avoided in analyses in which the season of collection is not known. Tocher, et al. (88) studied the seasonal variation in triglycerides in dwarf mistletoes. They found that the proportion of these compounds changed drastically from June to October which is consistent with the results of the current study.

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CHAPTER 4

CONCLUSIONS

Mistletoe is a more complex biological specimen to study than was first thought when the current project was initiated. The growth ranges of mistletoes are very broad and ecotypes have been shown to exist in complex populations of species with the possibility of hybridization occurring. Thus detailed taxonomy should be considered in any future study with mistletoe.

In the current study, capillary gas chromatography was the method of choice for the analysis of samples. This method coupled with profile analysis and multivariate statistics helped to differentiate the mistletoe samples. There have been many studies using these techniques on biological samples but no direct references to mistletoe have been found in the current literature.

The southeastern United States sample chromatograms showed the most consistency probably due to the fact that one species of mistletoe grows in this geographic area and they were collected over a one to three week time period. The Texas samples showed more variation in their chromatograms possibly due in part to more than one species growing in Texas and perhaps because they were collected over a longer period of time. Samples of all three species were probably collected. On the basis of hierarchical cluster analysis (HCA), the Texas samples and southeastern samples could be differentiated from one another, separating into two major clusters and one minor cluster.

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All of the southeastern samples were clustered together along with four samples from Texas. A cluster of five Texas samples was evident and the remaining 71% of the Texas samples were clustered, showing that possibly more than one species was collected in Texas. The principal component analysis (PCA) plot also showed good grouping of the two sample sets. Based on geographical origin of the samples, the preliminary analysis indicates that species can be differentiated using this technique, however, the effect of seasonal variation can not be overlooked at this point.

When samples from the extremes of North Texas and South Texas were compared, separation into two groups was noted using HCA and PCA. All of the North Texas samples grouped together and nine out of ten of the South Texas samples appeared together. It again appears that good separation can be achieved based on geography but further study is necessary to determine if different species or subspecies grow in these two areas of Texas. It also appears that the ranges of the species and subspecies growing in Texas overlap to some extent and this overlap may be a complicating factor in this type of research. Seasonal variation did not play a role in this clustering due to the short period of time of sample collection.

No significant difference was noted with the samples collected from four different hosts; Spanish oak, elm, mulberry, and mesquite trees when peaks were carefully selected. Similarity indices were calculated for each host compared with all others. The lowest similarity that was noted, 0.68, was for the two elm trees sampled. In some cases, there was as much variation between samples from the same type of host as there was between different hosts. All other comparisons were at a similarity level of at least 0.94.

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The samples for the seasonal variation study show a wide variation in similarity index, mean, and standard deviation. The samples collected over the nine-month period September 1995 through May 1996 showed close similarities in adjacent months but wide variation in distant months. These differences indicate that season of sample collection affects the chemical profile and is a variable that must be controlled in future studies.

This method appears to have value as a chemotaxonomic technique for the differentiation of mistletoes but further study is warranted. Compounds that are independent of host, seasonal variation, gender, and method of collection must be carefully selected for analysis.

The results of this project have answered some questions about mistletoes. Other questions have arisen during the project which are beyond the scope of the original hypothesis. In the coming years, research will be continued by students at Schreiner College in conjunction with students at Middle Tennessee State University to answer some of these questions. Some future studies might include: alternate extraction methods; more comprehensive host and time-of-year analyses; determination of the effect of length of time from sample collection to leaf extraction and effect of method of drying samples; determination of the geographical dispersion of species and hybridization of these species, including traditional taxonomy coupled with chemotaxonomy; effect of climatic conditions; gender related differences; evaluation of which groups of compounds are most useful in speciating mistletoe such as fatty acids, hydrocarbons, wax esters, or other compounds, which are independent of season, host, or gender and collection methods.

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GLOSSARY

Chemotaxonomy The use of chemical evidence (of both primary and secondary metabolism) in taxonomy.

Cuticle A nonliving layer secreted by and overlying the epidermis, e.g. the layer of cutin on the outside of some plant cell walls, especially the shoot epidermis where it forms a continuous layer which, with the epicuticle, has relatively low permeability to water and gases.

Deciduous Plants which shed leaves habitually before a cold period.

Dioecious Having the sexes in separate individuals.

Ecotype A form or variety of any species possessing special inherited characteristics enabling it to succeed in a particular habitat.

Epicuticle In plants, the layer of waxes including long chain (C_{20}) alkanes, alcohols, acids, and esters on the surface of the cuticle.

Family A group of similar genera of taxonomic rank below order and above genus; in plants, the names usually end in -aceae.

Genus A taxonomic rank of closely related forms, which is further subdivided into species and therefore below family and above species.

Haustorium An outgrowth from a parasite which penetrates a tissue or cell of its host and acts as an organ for absorbing nutrients.

Hemiparasite A plant capable of photosynthesis but dependent on another plant for water and mineral nutrients, for example mistletoe.

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Internode The distance between two successive nodes of a stem.

Mistletoe A hemiparasitic shrub found growing on various deciduous trees and having yellowish green leaves, inconspicuous flowers, and glutinous white berries.

Morphology The study of the structure and forms of organisms, as opposed to the study of their functions.

Order Taxonomic rank below class and above family; for plant, the names usually end in -oles.

Parasite An organism which lives in or on another organism and derives substances from it without rendering it any service in return.

Phoradendron serotinum The taxonomic classification of mistletoe growing in the southeastern United States.

Phoradendron tomentosum The taxonomic classification of mistletoe growing in Texas.

Pubescence A covering of fine hairs or down.

Species A group of individuals that (1) actually or potentially interbreed with each other but not with other such groups, (2) show continuous morphological variation within the group but which is distinct from other such groups. Taxonomically, species are grouped into genera and divided into subspecies and varieties or, horticulturally, into cultivars.

Taxonomy The science of classification as applied to living organisms, including study of means of formation of species.

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Xylem The vascular tissue with the prime function of water transport; it consists of tracheids and vessels and associated parenchyma and fibers.

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