THE ANALYSIS OF ORGANIC CONSTITUENTS IN LEAVES BY PYROLYSIS-GAS CHROMATOGRAPHY AND ITS APPLICATION TO SELECTED ENVIRONMENTAL EFFECTS ON PLANTS

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The Analysis of Organic Constituents in Leaves by Pyrolysis-Gas Chromatography and Its Application to Selected Environmental Effects on Plants

by

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ABSTRACT

Forest decline has caused great concern in North America. In recent years, the relationship between environmental stress and the concentration of certain organic compounds in plant leaves, e.g., carbohydrates, phenolics and tannins, has been recognized. Traditional methods for the determination of organic constituents in leaves are very difficult as well as time-consuming. As an alternative method of analysis, Pyrolysis-Gas Chromatography-Mass Spectrometry (PY-GC-MS) can be a rapid and comprehensive method to profile the chemical composition of biomaterials. Furthermore, enhanced structural information of biomolecules containing acidic functional groups has been obtained by a modification of direct pyrolysis -- simultaneous pyrolysis methylation (SPM). Initial investigations were focused on the characterization of important classes of biomarkers (e.g., tannins, phenolics) in leaves, and, optimization of sample preparation procedures and pyrolysis conditions. Finally, the pyrolytic techniques were examined as a tool to quantify the compositional changes in leaves of trees which have been subjected to different environment stresses. Results will be reported for three field applications 1) the effect of thinning on balsam fir trees 2) the effect of fertilization on kalmia plant 3) and the effect of ozone on white pine trees. It was found that certain pyrolysates of carbohydrates, tannins and of a dihydrochalcone where good indicators of fertilization and water stress. Two unique pyrolysate bio-markers were discovered in white pine needles as indicators of ozone fumigation levels and ozone damage.

DEDICATION

This thesis is dedicated to my husband ChengYong Yang and my parents, Xiandao Zhang and Ming Jiang.

GLOSSARY

CI	Chemical Ionization
C.V.	Coefficient of Variation
EI	Electron Impact Ionization
FAB	Fast Atom Bombardment
FID	Flame Ionization Detector
FD	Field Desorption
FI	Field Ionization
FIMS	Field Ionization Mass Spectrometry
GC	Gas Chromatography
GC-MS	Gas Chromatography-Mass Spectrometry
HPLC	High Performance Liquid Chromatography
m/z	Mass to Charge Ratio
Methylute	Trimethylanilinium Hydroxide
MS	Mass Spectrometry
NMR	Nuclear Magnetic Resonance Spectroscopy
^{1}H NMR	Proton Nuclear Magnetic Resonance Spectroscopy
^{13}C NMR	Carbon Nuclear Magnetic Resonance Spectroscopy
PY	Pyrolysis
PY-GC	Pyrolysis-Gas Chromatography

PY-MS	Pyrolysis-Mass Spectrometry
RT	Retention Time
SPM	Simultaneous Pyrolysis Methylation
PC	Paper Chromatography
TIC	Total Ion Chromatogram
TLC	Thin Layer Chromatography
TMAH	Tetramethylammonium Hydroxide
UV	Ultraviolet

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CHAPTER 1

INTRODUCTION

1.1. Overview of Organic Constituents in Plant Leaves

1.1.1. Introduction to Phytochemistry

Phytochemistry is a science between natural product organic chemistry and plant biochemistry. As described by Harborne (1), "Phytochemistry is concerned with the enormous variety of organic compounds that are elaborated and accumulated by plants, and deals with the chemical structures of these compounds, their biosynthesis, turnover and metabolism, their natural distribution and their biological functions." This science of Phytochemistry has shown considerable growth in recent years.

The most important difference between plant and animal metabolism is a plants' ability to convert carbon dioxide and water to carbohydrates, and inorganic nitrogen to organic nitrogen. In the plant metabolism, in addition to carbohydrates and amino acids, a large variety of other organic compounds take part in metabolic reactions, and are called metabolites. Some metabolites are ubiquitous among plants, while others are confined in only certain species or in certain parts of plants. Nevertheless, all the compounds are believed to play certain roles in plant metabolism.

Most metabolites are derived from the five main biosynthetic routes in plants, namely sugar metabolism, the acetate-malonate pathway, the acetate-mevalonate pathway, the shikimic acid pathway, and amino acid metabolism (2) (Figure 1.1).

The chemical compounds in plants can be characterized in different ways. Usually, classification is based on biosynthetic origin and the presence of certain functional groups. Thus, the organic compounds in leaves can be divided into phenolics, terpenoids, steroids, organic acids, lipids, carbohydrates, amino acids and proteins, nucleic acids and derivatives, alkaloids, and porphyrins (1). Several major constituents in leaves, which are phenolics, lipids, and carbohydrates, will be described further.

1.1.2. Plant Phenolics

Phenolics include a wide range of plant compounds possessing an aromatic ring with one or more hydroxyl groups. Much interest has been drawn to plant phenolics for the last 200 years due to their potential for revealing the systematic and evolutionary relationships of plants, and also to their biological functions in plant defense systems against herbivores and phytopathogens. The major groups of phenolics are listed in Table 1.1 (3). Limited by the scope of this project, only three groups, phenolic acids, tannins, and lignin, which are often viewed as important bioindicators of environmental change, are described in more detail.

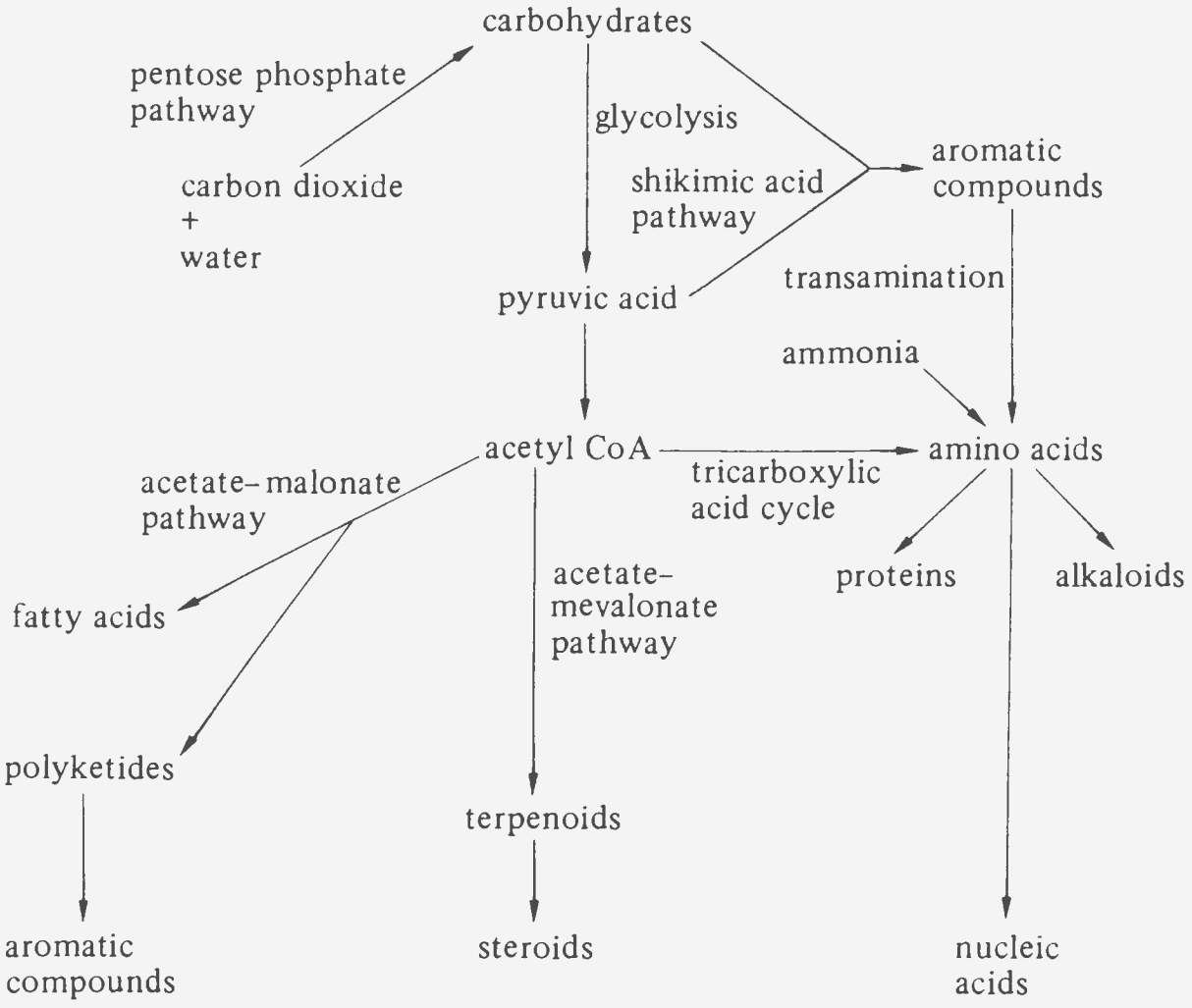
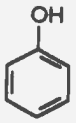
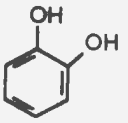
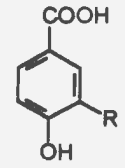
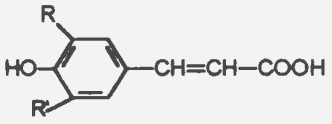
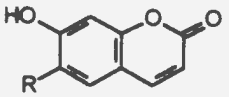
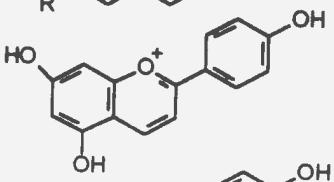
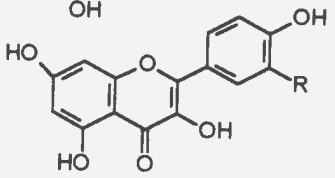
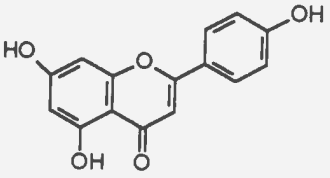
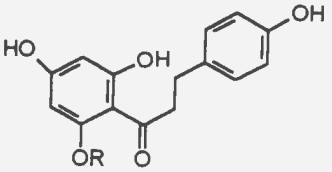
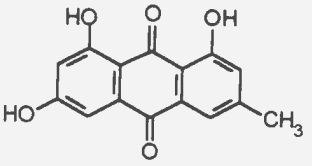
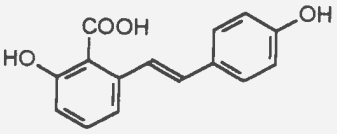


Figure 1.1 Biosynthetic Pathways in Plants

Table 1.1 The Major Classes of Plant Phenolics

Names	Examples
Phenols and phenolic acids	 Phenol  Catechol
	 R=OH, protocatechuic acid R=H, *p*-hydroxybenzoic acid $R=OCH_3$, vanillic acid
	 R=R'=H; *p*–coumaric acid R=OH,R'=H; caffeic acid $R=OCH_3$, R'=H; ferulic acid $R=R'=OCH_3$; sinapic acid
Coumarins	 R=H, umbelliferone R=OH, aesculetin $R=OCH_3$, scopoletin
Anthocyanins	 Pelargonidin
Flavonols	 R=H, kaempferol R=OH, quercetin
Flavones	 Apigenium
Flavanoids	 R=H, phloretin R=O-glucose, phloridzin
Xanthones	 Emodin
Stibenes	 Lunularic acid
Tannins	
Lignin	

Phenolic Acids

Two families of phenolic acids are commonly found in leaves, a range of substituted benzoic acid derivatives (C_6-C_1) and those derived from cinnamic acids (C_6-C_3) which are very widespread. Both types of phenolic acids usually occur in conjugated or esterified forms (1, 3).

Among the benzoic acids, *p*-hydroxybenzoic, vanillic and syringic acids occur in vast amounts as combined forms in lignin which may be released by alkaline hydrolysis. In addition, protocatechuic acid is similarly widespread but the corresponding tri-hydroxy acid, gallic acid, is only found as a constituent of tannins or as its dimer ellagic acid, which is the dilactone of hexahydroxydiphenic acid (see Figure 1.2).

Only four cinnamic acids, *p*-coumaric, caffeic, ferulic and sinapic acids, are well known in leaves and among these acids, *p*-coumaric is by far the commonest. The natural occurring cinnamic acids are the more stable trans isomers. However, the isomers are easily interconvertible under the action of light.

Phenolic acids also form a large range of phenolic glycosides in leaves by connecting to sugars through sugar-aglycone linkages, usually β-linked. Rhamnosides and arabinosides are the exceptions where the aglycones are α-linked.

Tannins

Tannins have been known as preservatives of animal skins for a long time. Because of their ability to combine with proteins and other polymers such as carbohydrates, alkaloids and gelatin, tannins can transform raw animal skins into valuable leather (4). This ability also gives tannins very important roles to play in plant defense systems, microbial decomposition and other plant physiological processes, although questions about their roles still exist (5, 6).

Tannins are very common in leaves. They can be classified into two major groups: hydrolysable and condensed tannins. Hydrolysable tannins are esters of sugars, usually glucose with either gallic acid or hexahydroxydiphenic acid (Figure 1.2 a). The condensed tannins are polyflavanoids which consist of chains of flavan-3-ol units. The most common is the polymer of catechin or its isomer epicatechin (7) (Figure 1.2 b).

Lignin

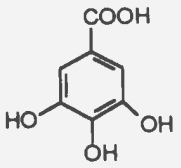
Vascular plants synthesize lignin in the lignifying tissues to provide rigidity to the cell walls. Lignin represents the second most abundant natural product after cellulose in the cell walls of higher plants. It is a highly branched and polyphenolic molecule of complex structure formed by polymerization of substituted cinnamyl alcohols (Figure 1.3).

One of the most serious obstacles in the chemistry of lignin as a macromolecule is its insolubility and its association with polysaccharides in plant cell walls. Because of lignin's resistant nature, it is difficult to ascertain if lignin fractions extracted by various techniques represent anything like native lignin (8, 9).

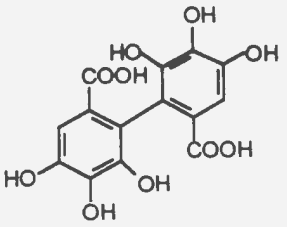
1.1.3. Carbohydrates

Carbohydrates are the first complex organic compounds formed in the plant by photosynthesis. They serve as a major source of respiratory energy and provide a means of storing (as starch) and transporting(as sucrose) energy. They are also the building blocks of cell walls (cellulose). In addition, sugars have other important biological functions in the form of plant glycosides and nucleotide sugars (1, 10).

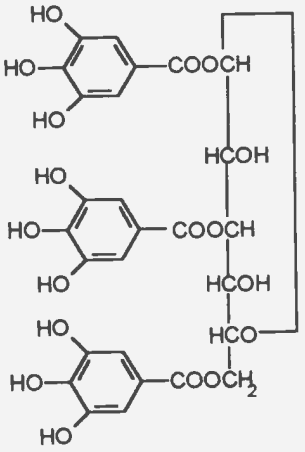
Carbohydrates can be divided into three groups on the basis of their molecular sizes. They are simple monosaccharides (e.g. glucose, fructose) and their derivatives, oligosaccharides formed by condensation of two to eight monosaccharides (e.g. sucrose), and polysaccharides (e.g. cellulose, starch), which are composed of even more monosaccharide units (1, 10). The major carbohydrates in leaves are displayed in Figure 1.4.



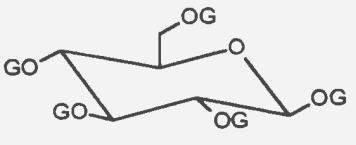
Gallic acid



Hexahydroxydiphenic acid

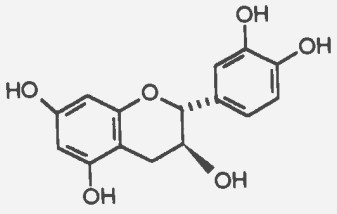


Tannic acid

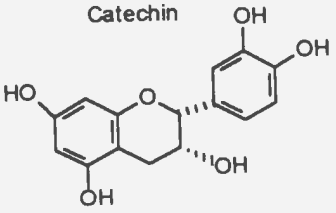


G= Gallate group

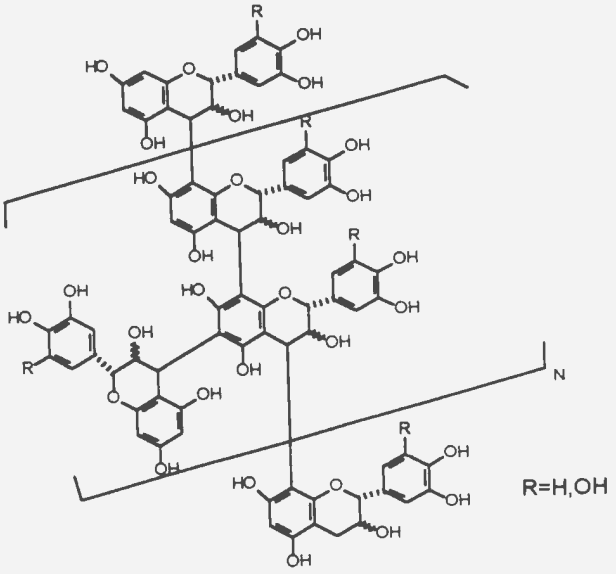
Figure 1.2 (a) Structure of Hydrolysable Tannins



Catechin



Epicatechin



Proanthocyanidin

(b) Structure of Condensed Tannins

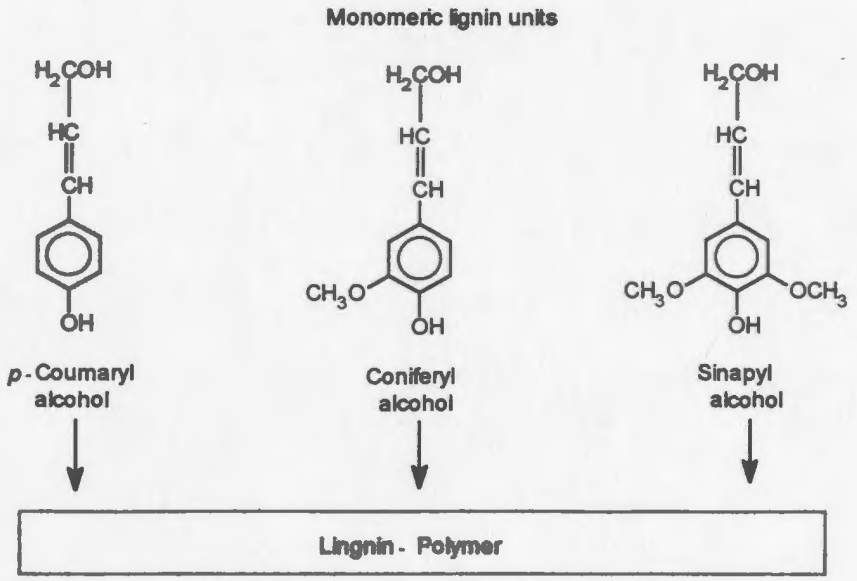


Figure 1.3 Formation of Lignin

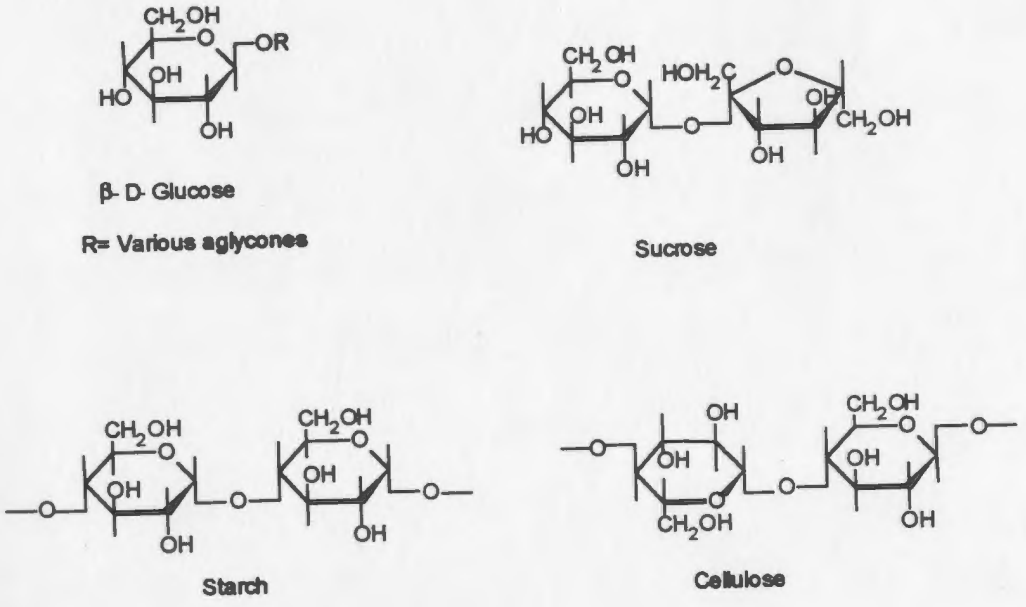


Figure 1.4 The Major Carbohydrates in Plants

1.1.4. Fatty Acids

Lipids are one of the major organic groups in plant leaves, and they carry out multiple functions. Some are used for energy storage, but a larger fraction of lipids occur as structural components in cell membranes. They are also present in the cuticular layers that protect cells and prevent excessive water loss. Based on their chemical structures, lipids can be divided to fatty acids, glycerides, phospholipids, glycolipids, and waxes (fatty acid esters).

Among the lipids, fatty acids and their esters are the major group found in leaves. Although all aliphatic carboxylic acids may be described as fatty acids, the term is usually restricted to the longer chain members of the series which are practically insoluble in water but soluble in organic solvents. Free fatty acids or their salts are much less common than their esters. Most fatty acids found in nature have an unbranched carbon chain consisting of an even number of carbon atoms. The common fatty acids in plants are either saturated or unsaturated compounds of C16- or C18-chain length (Table 1.2). Palmitic acid, the primary metabolite of the acetate-malonate pathway (Figure 1.1), is the major saturated fatty acid of the leaf lipids. Unsaturated acids, such as oleic, linoleic, and linolenic acids are widespread in leaves (1, 2, 10).

Table 1.2: Typical Fatty Acids in Plants

Carbon chain	Trivial name	Formula
	Saturated acids	
C_{14}	Myristic	$CH_3(CH_2)_{12}CO_2H$
C_{16}	Palmitic	$CH_3(CH_2)_{14}CO_2H$
C_{18}	Stearic	$CH_3(CH_2)_{16}CO_2H$
C_{20}	Arachidic	$CH_3(CH_2)_{18}CO_2H$
	Unsaturated acids	
C_{16}	Palmitoleic	$CH_3(CH_2)_5CH=C(CH_2)_7CO_2H$
C_{18}	Oleic	$CH_3(CH_2)_7$ $CH=CH(CH_2)_7CO_2H$
C_{18}	Linoleic	$CH_3(CH_2)_4CH=CHCH_2CH=CH$ $(CH_2)_7CO_2H$
C_{18}	Linolenic	$CH_3CH_2CH=CHCH_2CH=CH$ $CH_2CH=CH(CH_2)_7CO_2H$

1.2. Environmental Effects on the Organic Composition of Plant Leaves

Plants in the natural environment are subject to many environmental stresses during their annual growth cycles. These environment stresses or factors can be divided into five major groups described in Table 1.3 (11).

Table 1.3 Classification of Environmental Factors

Climatic factors	temperature, light intensity, moisture and seasonal effects
Edaphic factors	macronutrients (N, P, K, S, Mg, Ca); microbes, micronutrients (Cu, B, Mn) in soil.
Unnatural pollutants	O_3, industrial gases and gasoline fumes
Animals	symbiosis, herbivores
Competition from other plants	competition for nutrients,light and moisture

Biochemical response of plants to environmental stresses involves changes in biochemical compositions and metabolism of cells. These changes help maximize the utilization of the internal and external resources of plants, and minimize the damaging effect of environmental stresses on plants (12).

Environmental stresses are sensed primarily by leaves since many regulatory systems of plants, e.g., carbon allocation system, are present in the leaves. The changes of concentration of organic constituents in leaves, to some extent, may reflect plants' responses to various stresses. The environmental effects on the levels of secondary metabolites have received high attention because of their important functions in plant defense systems (12).

Forest decline has become a worldwide phenomenon. Atmospheric pollutants,

as well as other environmental factors, such as nutrient deficient soil and drought, are believed to be mainly responsible for the decline. These environmental factors have caused great concern and their impact on the organic compositions in leaves have been studied extensively (13).

Gaseous Pollutants

Gaseous pollutants in ambient air are considered a prime suspect for forest damage. Pollutants, including ozone, sulphur dioxides, hydrogen peroxide, fluorides, and nitrogen oxides, can impair plant health either singly or in combination (14, 15).

Ozone is a major pollutant in many parts of the world. It is known that ozone oxidizes the sulhydryl groups of proteins, and attacks biological membranes by peroxidation of unsaturated lipids (16, 17). Alteration of fatty acids has been observed in a number of studies (18-20). The membrane breakdown may result in increased polyphenoloxidase activity, thus affecting tannins and total phenolic content (15). Early observations showed that ozone enhanced the levels of anthocyanin pigment in *Rumex* leaves (21), and of caffeic acid in bean leaves (22). Recently, increased tannin content was observed in loblolly pine leaves fumigated by ozone (23). Evidence of lignin damage by ozone was detected in beech leaves (92). In another study, changes in carbohydrate concentrations were shown in shortleaf pine needles exposed to ozone (24).

Sulphur dioxide is another major pollutant, especially in developing countries. It has been demonstrated that sulphur dioxide can significantly reduce the levels of various lipids in pine needles (25). Exposure to sulphur dioxide usually leads to an increase in soluble sugars (25), possibly due to a breakdown of polysaccharides. In *Pinus banksiana* needles, it has been found that sulphur dioxide decreased the content of two major organic acids, namely quinic and shikimic acid, and increased the content of syringic acid (26). Also observed was the decrease of tannin content in Japanese cedar exposed to sulphur dioxide (27, 28).

Nutrient Stress

The most important nutrients that are required by plants from soil are nitrogen, potassium and phosphorus. It is proposed that where light is non-limiting, but plants are nitrogen stressed, protein metabolism demands little carbon from carbohydrates, allowing excess carbon-based secondary metabolites to be produced (29). An inverse relationship between total phenolic and nitrogen in the soil was observed in the leaves of several plants (30-33). It was shown that potassium fertilization reduced the phenol and carbohydrate contents in chili pepper leaves (34). A number of publications reported that the deficiency in nitrogen and other major nutrients generally increased the production of secondary metabolites (29, 35, 36). It is indicated that the chemical defense system of plants is enhanced under the conditions of nutrient stress.

Water stress

Climate factors inducing drought are harmful to plants. Plants have various responses to water-deficient stress. The initial response of a plant includes a change in the plant's internal water status. This is followed by increased concentrations of certain hormones in leaves such as abscisic acid, and auxins which reflect the changes which have occurred in at least some of the processes in which they take part (37). Proline accumulation is common in plants under water stress (38). It was reported that the total soluble sugars increased and soluble protein decreased in the leaves of alfalfa plants subjected to water stress (38).

1.3. Traditional Methods for Analyzing Organic Constituents of Plants

1.3.1. Preparation of Plant Materials

In order to characterize the chemical structures of organic compounds, and understand their biosynthesis and biological functions in plants, especially in leaves, analytical methods should be established to quantify the large number of organic compounds. Generally, before identification, different constituent extraction, isolation, and purification of various groups of compounds must be conducted (1).

Ideally, phytochemical analysis requires fresh plant material, but sometimes rapid sample preparation is not possible, and dried plants have to used. To avoid chemical

change, it is important that the drying operation is carried out under controlled conditions. When choosing a method for extraction and isolation, the properties of compounds being isolated and the compositions of plant materials must be taken into consideration (1).

The separation and purification of plant constituents are mainly carried out using one or more chromatographic techniques. The chromatographic fractions are further identified by ultraviolet (UV), nuclear magnetic resonance (NMR) or mass spectroscopic (MS) measurements (39). A brief description of these techniques will be given below.

1.3.2. Chromatographic Techniques

Plant constituents can be separated by chromatographic techniques, including paper chromatography (PC), thin layer chromatography (TLC) gas chromatography (GC) and high performance liquid chromatography (HPLC). The suitable range of compounds to which these chromatographic techniques can be applied and their advantages are summarized in Table 1.4 (1, 33).

Table 1.4 Chromatographic Techniques

Techniques	Suitable compounds	Advantages
PC	carbohydrates, amino acids, nucleic acids, organic acids and phenolic compounds	simple, RT is reproducible
TLC	all lipid soluble components, i.e., lipids, steroids, carotenoids, simple quinones, chlorophylls	fast, simple, versatile
GC	volatile compounds; fatty acids, mono-and sesquiterpenes, hydrocarbons, and sulphur compounds; derivatized non-volatile compounds	high resolution reproducible sensitive
HPLC	non-volatile compounds as well as many of those listed above	versatile, reproducible, fast

Although PC and TLC are simple and are the essential techniques for the separation of various organic compounds in plants, they do not always produce compounds pure enough for further analysis. GC and HPLC do not suffer from these drawbacks. When combined with mass spectrometry or U.V. detection (diode array), GC and HPLC can provide both quantitative and qualitative data in a single operation (40). However, the use of GC is limited to volatile compounds. Non-volatile and thermally unstable compounds require chemical derivatization. A large variety of derivatization techniques have been developed to enhance the volatility and thermal stability of organic compounds, as well as to improve GC resolution and to increase the

stability of the molecular ions in EI mass spectra (41-43).

Derivatization processes usually involve tedious work and often leads to multiple substitution. In many cases, HPLC does not require sample derivatization and has therefore become a very important and versatile method for the study of natural involatile compounds. Further advantages include the availability of both analytical and preparative modes. When phenolic acids and flavonoids are investigated, chromatography is usually carried using a reversed phase mode. Polar flavonoid aglycones and flavonoid glycosides have also been successfully analyzed by reversed phase liquid chromatography on octadecylsilyl-bonded columns with methanol-water solvent (44). A similar reversed phase column and a mobile phase of acidified water - methanol gradients were employed in the separation of phenolic compounds in spruce trees (14). The disadvantage of HPLC relative to PC and TLC is the requirement of higher cost equipment and more expensive detectors, such as fluorescence detectors.

1.3.3. Spectrophotometric Assay

Spectrophotometric assay (using visible or UV) is usually employed to do quantitative analysis in ecological studies of plants. Several methods have been established for the analysis of total phenolic and tannins.

The Folin-Dentis (45) and Hagerman-Butler (4) methods are used for quantifying total phenolics. The former is based on a redox reaction between the phosphomolybdic/phosphotungstic acid reagent and phenolics to produce a colour change. The latter makes use of the complexation reactions of phenolics with iron.

Two methods are available to measure condensed tannins; one through the formation of coloured complexes with vanillin (46) and the other by measuring anthocyanidin production through acid hydrolysis (47).

Hydrolysable tannins can be quantified by nitrous acid (48) or iodate methods (6). These two methods are based on the reaction of hexahydroxydiphenic acid with nitrous acid, and the reaction of gallotannin with potassium iodate, respectively.

The above spectrophotometric methods have been studied for their relative merits (49-51). It was found that the techniques for total phenolics measurement correlate well. However, there is considerable discrepancy between the methods examined for condensed tannins. Futhermore, the methods for measuring hydrolysable tannins can not be used with confidence when they are compared with chromatographic analysis.

The spectrophotometric techniques available all have drawbacks and are subject

to many errors (51). For example, it is difficult to relate the results of total phenolic content of plants which may have evolved specific adaption to single phenolic compounds or groups of compounds.

1.3.4. Mass Spectrometry

The development of mass spectrometry (MS) in recent decades has revolutionized biochemical research. Only micrograms of sample is needed for this technique. Both molecular weight and ion fragmentation patterns which characterize a particular compound can be obtained from a simple sample injection. MS is usually combined with GC (GC-MS) and HPLC (HPLC-MS). The GC-MS technique has wide application in biochemistry research. The extreme sensitivity and selectivity of this method make it particularly advantageous for the analysis of plant phenolics with molecular weight less than 300 (52). Compounds in a complex sample can be positively identified by comparing the obtained mass spectra with that of authentic compounds, or, more tentatively, with a mass spectral library. When authentic compounds are not available, careful interpretation of the mass spectra is needed.

A great deal of progress has been made in recent years in the area of instrumentation for HPLC-MS, notably in the area of interfaces and ionization sources. years. Different HPLC interfaces and ionization sources are available. The technique has

proved very successful for the analysis of high moleculat weight analytes. For example, the structure of the complicated anthocyanidins in plant tissues was characterized using on-line HPLC/electrospray mass spectrometry collision-induced dissociation (53).

In many cases, electron-impact mass spectrometry (EIMS) of underivatized phenolics and especially their glycosides do not produce measurable molecular ion peaks. This has been circumvented by field-desorption (FD), chemical ionization (CI) and fast atom bombardment (FAB) mass spectrometry. These "soft" ionization techniques are capable of analyzing fragile or non- volatile organic compounds. For example, FAB-mass spectrometry can detect the molecular ions of plant phenolic glycosides whose O-glycosidic moiety are usually lost in EI mode (54).

1.3.5. Nuclear Magnetic Resonance Spectroscopy

During the last two decades, very rapid progress has been made in nuclear magnetic resonance spectroscopy (NMR) in both instrumentation, with the introduction of high magnetic fields and computerised Fourier Transform (FT) spectrometers, and the theory, with the development of one-dimensional and two-dimensional pulse sequences. Early NMR data of organic compounds were obtained with proton NMR (^{1}H NMR) by measuring the magnetic moments of their hydrogen atoms. Recently advances in FT-NMR has led to very rapid development of Carbon-13 NMR (^{13}C NMR) which

characterizes the carbon skeleton of the molecule.

^{13}C NMR is essentially complementary to ^{1}H NMR and the combination of the two techniques provides a very powerful means of structural elucidation of organic compounds in plants. A large number of papers about NMR characterization of plant compounds have been published. For example, the structure of complex polyphenols and flavone glycosides are elucidated by ^{1}H NMR (55) and ^{13}C NMR (56, 57). Usually pure samples are required for identification. NMR has also been applied to quantitative analysis in plants. The ^{13}C signal strengths have been used to determine the lignin and tannin contents of plant materials (58), where a spin locking pulse sequence was used to simplify the NMR spectrum.

1.4. Introduction to Analytical Pyrolysis

Pyrolysis (PY) is the thermal degradation of materials in an inert atmosphere. It can be divided to two categories; applied and analytical pyrolysis. Applied pyrolysis concerns with the production of commercially useful materials by means of pyrolysis. Analytical pyrolysis is the technique that characterizes materials or chemical processes by the instrumental analysis of the pyrolysates (59, 60).

The strategies in analytical pyrolysis can be classified into two major groups, the

fingerprinting mode and the identification mode. Fingerprinting of the chemical composition of materials can be achieved by pyrolysis-mass spectrometry (PY-MS), while identification of individual pyrolysis products is obtained by pyrolysis-gas chromatography mass spectrometry (PY-GC-MS). PY-MS is sometimes carried with field ionization (FI) or field desorption (FD) techniques (61). These soft ionization techniques produce almost exclusively molecular ions of thermal degradation products of plant materials. The integrated PY-FI mass spectra have been successfully applied to characterization of different samples (125).

Although PY-MS has been proven very powerful, it is very expensive and subject to several problems. The identification of mass peaks is complicated by the presence of isobaric compounds and isomeric molecular structures. Furthermore, it is not easy to relate the mass peak distribution of pyrolysis products in a spectrum to the structure of the polymer analyzed. Fortunately, detailed studies on the identified ions of the pyrolysates by PY-GC-MS can help in understanding data obtained from PY-MS experiments. For this reason, PY-MS and PY-GC-MS are often combined as powerful complementary techniques in the chemical analysis of complex samples (125).

The PY-GC and PY-GC-MS techniques allow for the rapid volatilization, separation and identification of pyrolysis products. In qualitative analysis, such as the matching of plant samples, the total pyrograms from unknown samples are usually

compared with those of authentic samples by "fingerprint" matching.

In order to achieve quantitative results, reproducibility of pyrolysis is essential. Several parameters should be carefully controlled, among which sample size, temperature, and gas velocity used are the most important ones (62, 63). As the sample size increases, the probability of secondary reactions increases since heat transfer is slower in large samples. The mode and temperature characteristics of pyrolysis steps also affect pyrolysis. Usually, samples of relatively high volatility are pyrolysed with low ambient temperature and a short temperature rise time. In PY-GC, a high gas velocity within the pyrolyzer is required in order to ensure fast removal of pyrolysates and to prevent high boiling products from condensing in pyrolysis zone. However, the carrier gas should be controlled so as not to affect the temperature-time profile of pyrolysis.

Due to the variety of pyrolysis instrumentation, inter-laboratory reproducibility is still a problem for both PY-GC and PY-MS, although pyrolysis can be highly reproducible in a single lab with strictly controlled conditions. If all the steps are tightly controlled, and similar pyrolyzers are used, inter-laboratory reproducibility can be achieved (64).

A notable feature of analytical pyrolysis is its versatility in various applications.

PY-MS and PY-GC-MS have been applied to the analysis of synthetic polymers, biopolymers, fossil fuels, soils and sediments, many of which are not amenable to chromatographic or chemical analytical techniques without initial degradation and fractionation steps (45, 65-68). Thermal degradation products reflect, to a large extent, the original structure of the pyrolysed polymeric material. Under carefully selected and clearly defined pyrolysis conditions, quantitative results can also be achieved (69).

1.5. Pyrolysis-Derivatization-Gas Chromatography

Although PY-GC is considered a reliable and reproducible technique for the identification and structure elucidation of macromolecules, polar pyrolysis products are usually difficult to measure due to their low volatility, resulting in condensation soon after the pyrolysis zone. As well, due to their chemical nature, carboxylic acids and phenolic acids tend to decarboxylate under the high temperature required for pyrolysis.

The chemistry and applications of thermal methylation or ("on column" methylation) of biological and medical samples by direct injection of an aqueous solution of the quaternary N-methyl ammonium salt of organic acids into the hot injection port of a gas chromatograph have been reviewed by Kossa (70). This technique is very simple and can prevent decarboxylation of carboxylic acids or phenolic acids. The thermal methylation process involves two sequential reactions; 1)

formation of quaternary N-methyl ammonium salt by deprotonation of the acid with a strongly basic quaternary N-methyl ammonium hydroxide, and 2)thermal decomposition of the salt, upon injection into the hot injector of the chromatograph to yield a tertiary amine byproduct and the methyl derivatives of the acid. Both carboxylic acids (pk_a 4-5) and phenols (pk_a 9-12) can be deprotonated quantitatively in the liquid phase and subsequently methylated upon heating.

Challinor (71) modified this technique to on-line PY-GC using tetramethylammonium hydroxide (TMAH) as a derivatizing reagent and defined the technique as simultaneous pyrolysis methylation (SPM). It was demonstrated that the polybasic acids, polyhydric alcohols, complex esters in drug formulations, natural waxes, kerogen and proteins can be identified by SPM (72). Recently, SPM was reported to be very useful in analyzing diterpenoid carboxylic acids in natural resins and resinates, which have been proven very difficult to analyze by other means (73, 74). By this technique, resin acid methyl esters do not undergo significant thermal degradation or isomerization. In-source pyrolytic methylation mass spectrometry using TMAH has also been successfully applied to the analysis of *p*-coumaric acid and lignin in plant materials, allowing discrimination between phenolic acids and phenolic alcohols (84).

Despite the great utility of this technique, it also has shortcomings (70). Under the conditions of high alkalinity and high temperature that are employed, certain base-

and heat-sensitive pyrolysates can be lost. Some unusual N-containing side-products were observed when both hydroxy and carbonyl-containing methyl esters undergo side reactions with the N-containing methylating reagent (39).

1.6. Pyrolysis of Plant Leaves

Direct chromatography is not feasible for many organic polymers in plants, such as tannins, lignin and polysaccharides. Pyrolysis breaks down polymer molecules into smaller, volatile, more easily identified fragments. Plant materials such as grass, wood, pollens, forest litter and plant leaves have been successfully characterized using a variety of analytical pyrolysis methods and MS ionization techniques (75-78). In our study, emphasis is placed on the pyrolysis of leaf constituents.

Pyrolysis products are usually derived from the thermal degradation of the main constituents of leaves such as carbohydrates, lignin, lipids and proteins (79-83). Primary building blocks of these biopolymers form pyrolysis products characteristic of different sources of leaves.

Under pyrolysis conditions, phenolic acids usually decarboxylate (84, 85). *P*-coumaric acid and ferulic acid pyrolyse to their decarboxylated fragments vinylphenol and vinylguaiacol, respectively.

Previous pyrolytic studies have demonstrated that condensed tannins (86) and other polyphenols, such as chlorogenic acids, fragment to catechol upon pyrolysis (87). Lignin can also produce a small amount of catechol (86). Consequently, extraction of the group of compounds to be analyzed from the leaf samples has to be conducted before using catechol as a diagnostic fragment of a certain polyphenol for quantification by pyrolysis.

Pyrolysis of lignin leads primarily to the cleavage of certain C-C and C-O bonds between the aromatic and aliphatic chains or within the aliphatic chains (9, 78, 88). Most of the fragments are from *p*-hydroxyphenyl, guaiacyl, and syringyl moieties. The relative ratios of these moieties reflect the lignin type in the leaves. In addition to monomeric lignin fragments, dimeric lignin fragments have also been observed by PY-FIMS (89).

Carbohydrate composition and structure are considerably more difficult to identify by pyrolysis because of their facile fragmentation and dehydration to nondiagnostic low molecular weight products. The initial pyrolytic reactions occur by heterolytic cleavage of the O-glycosidic bonds. Through dehydration reactions, due to loss of water, hexose units primarily form typical pyrolysis products such as anhydrohexoses, dianhydrohexoses and levoglucosenone (90). Similarly, anhydropentose pyrolysates can also be produced from pentose units of hemicellulose. Secondary

pyrolytic reactions can yield furan- and pyran-type products and smaller acyclic compounds dehydrated from primary pyrolysis products (91).

Some thermally-stable compounds such as 10-nonacosanol, monoterpenes, diterpene acids, α-tocopherol, ergosterol, β-sitosterol, kaempferol and flavon glycosides have been observed as pyrolysis products of intact leaves by PY-FIMS (61, 79, 82).

1.7. Pyrolytic Studies and Environmental Stress

Forest decline is a multi-factor problem causing multiple changes within the plants. The multivariability requires analytical methods which are rapid and provide maximal and universal information about the composition of the plant material. Analytical pyrolysis may meet this criteria.

PY-FIMS in combination with pattern recognition techniques can supply a valuable approach for detecting early damage to trees by environmental stress. In extensive studies by Schulten, the abundance of antioxidant α-tocopherol was found to decrease dramatically in the PY-FI mass spectra of the ozone fumigated beech leaves (92). In spruce needles, the ergosterol signal from PY-MS was chosen as a biochemical indicator for fungi attack (93) where higher ergosterol concentrations were observed in spruce needles in environments favouring fungi growth. The amount of proline as

measured by its MS signal was shown to be an indicator of water stress (93). The abundance from proline was shown to have an inverse relationship with the absolute water content of the leaves.

In spite of the feasibility of PY-FIMS in environmental stress studies, the high cost of the instrument restricts its application. So far, no pyrolytic studies in environmental stress and its effects on leaf composition by the PY-GC have been reported.

1.8. Objectives of This Study

Plants produce a large number of discrete organic compounds. The concentration of some of these organic compounds can be affected by environmental factors or stresses. Forest damage has caused great concern in North America and Europe. Knowledge of the relationship between various environmental stresses and specific indicator compounds in plant can supply valuable information in forestry practices, such as forest restoration and in monitoring the health of our forest (94). However, the present methods for analyzing the organics in plants in ecological studies, e.g., tannins, are not only difficult and laborious, but also often inaccurate. The wide application of analytical pyrolysis in biomaterials suggests that PY-GC may have great potential in rapid characterization of organic compounds in leaf samples. The objective of this study

is therefore to establish PY-GC-MS as a useful means for monitoring changes in the concentrations of certain leaf constituents, if any, as the result of certain controlled environmental changes and stresses on plants. The present research is focused on the following three areas of study.

1.Establish a proper analytical procedure for the pyrolysis of leaves. This includes testing for suitable sample preparation and the pyrolysis techniques, and optimising the conditions of pyrolysis and chromatography. Two pyrolysis techniques, direct pyrolysis and simultaneous pyrolysis methylation (SPM), are investigated and compared in this study.

2. Characterize distinctive pyrolysis products of the major organic constituents in leaves, i.e., phenolic acids, tannins, carbohydrates, lignin, and fatty acids by PY-GC-MS. A range of leaf species were chosen which had significant differences in composition, particularly with respect to polyphenolic compounds.

3. Examine whether PY-GC can be utilized for measuring any compositional changes in leaves subjected to certain environmental stresses. Both direct pyrolysis and SPM are carried out to explore compositional changes in leaves. Field studies include the analysis of leaves a) from tree stands which were thinned and unthinned. b) from trees subjected to ozone fumigation, and, c) from plants subjected to nutrient stress.

CHAPTER 2

EXPERIMENTAL

2.1. Materials

2.1.1. Chemicals

Tetramethylammonium hydroxide (TMAH), tannic acid, gallic acid, catechin, epicatechin, *p*-coumaric acid, quinic acid, shikimic acid, arbutin, kaempferol, quercetin, α-phenylglucoside, β-phenylglucoside, phloretin, and cellulose were purchased from Sigma Chemicals (St.Louis, Mo.). Trimethylanilinium hydroxide (Methylute) was purchased from Pierce (Rockford, Il.).

2.1.2. Leaf Samples

Oak Leaves

Northern red oak *(Quercus rubra)* leaves were donated by Dr. Caroline Preston, Forestry Canada, Pacific Region. They were air-dried and ground to a fine powder.

Tannin Extracts

The tannin extracts of oak *(Quercus rubra)* leaves, balsam fir *(Abies balsamea)* needles and kalmia *(Kalmia angustifolia* L.*)* leaves were also from Dr. Caroline Preston. The procedure for tannin extraction is described elsewhere (95).

Nutrient Stressed Kalmia Leaves

Leaves from kalmia *(Kalmia angustifolia* L.*)* plants were supplied by Dr.Brian Titus, Forestry Canada, Newfoundland and Labrador Region. Kalmia plants were dug up in the field in central Newfoundland in Oct., 1990 and replanted in a green house in St.John's later. The automated controls in the green house were set as 16 hours of light per day with daytime and night temperatures of 25°C and 20°C respectively, and a relative humidity of 65% to imitate summer growing conditions. Fertilization treatments in solution were repeated five times, each consisting of N (as ammonium nitrate), P (as super triple P) and K (as potash), applied at a rate equivalent to the field application rates of 150, 60, and 100 kg/ha of N, P, K respectively. Plant leaves from 12 plants were harvested on 20 Feb., 1992. Both current and second year leaves were clipped and separated as two (pooled) groups of leaves for analysis .

Methanol-Water Extract and Methanol-Water Extracted Residue of Kalmia Leaves

Fresh kalmia leaves were homogenized in deionized water (1:2, w/w) and then extracted with methanol-water (4:1, v/v), and centrifuged. The supernatant was filtered, concentrated by a rotary evaporator and freezed-dried (methanol-water extract). The pellet from centrifugation was combined with the filtration residue of the supernatant and evaporated to remove methanol. A small amount of deionized water was then added, and the sample was freeze-dried (methanol-water extracted residue).

Thinned/Unthinned Balsam Fir Leaves

Leaves from thinned/unthinned balsam fir *(Abies balsamea)* which had previously been analysed for nitrogen content as part of a moose browse study (129) were obtained from Dr. B Titus. The thinning reduced stem density from 3000 stems/ha (unthinned) to about 200 stems/ha (thinned). The leaves from balsam fir trees were air-dried and ground to a fine powder. Five gm of needles were collected from each tree; from two trees which were classified as unthinned (U) and from five other trees classified as thinned (T) .

Ozone Fumigated White Pine Leaves

Needles from white pine fumigated with increasing ozone dosages were obtained from Dr. Roger Cox, Forestry Canada, Maritimes Region. Selected white pine *(Pinus strobus)* were approximately 50 feet tall. Four branch chambers were placed over selected branches at the top of the canopy of each tree. Figure 2.1 shows the chamber for branch ozone fumigation system. Air of controlled ozone concentration was delivered up the tree from a trailer on the ground through a gas supply system. On each tree, four treatments were applied to the chambers covering the branches. One chamber delivered charcoal filtered (C.F.) air and three delivered simulated ozone "events" with peak concentrations of 80, 120, and 160 ppb, respectively. Ozone fumigations were started on 28 July, 1992. Both current year and second year needles were collected on 26 August, 1992. The accumulated ozone dosages were calculated from ozone concentration and the exposure time of trees. The needles were air-dried and ground .

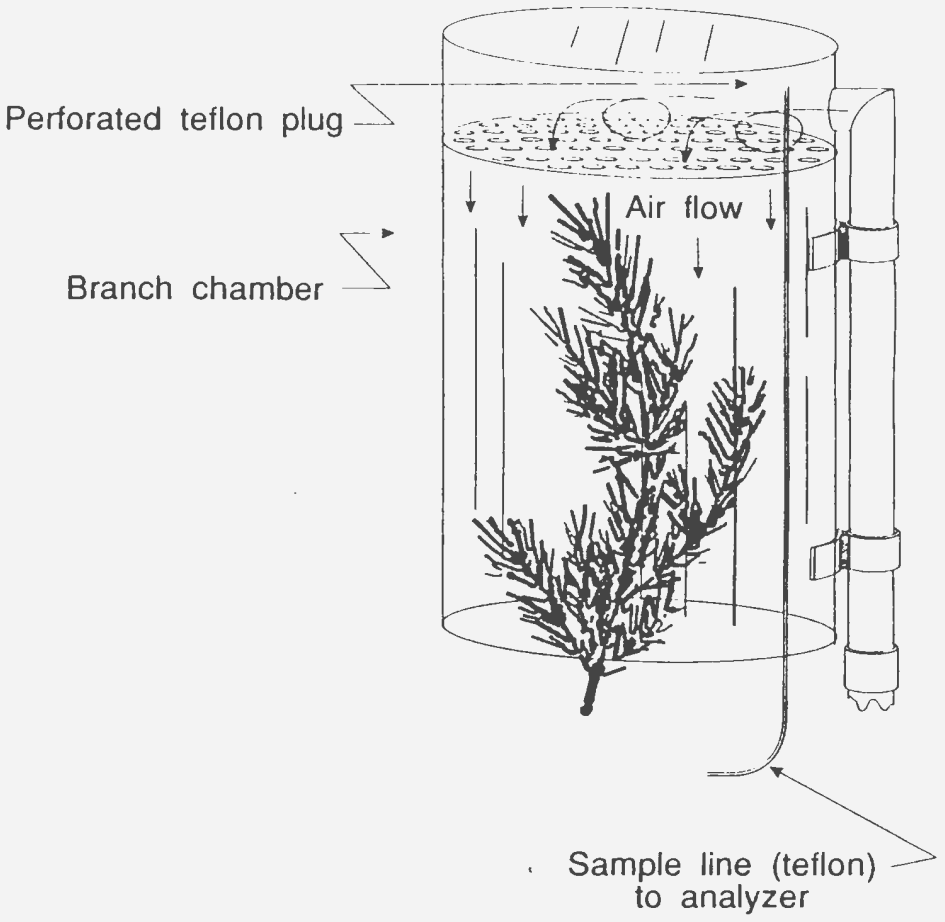


Figure 2.1 Schematic of the Chamber Used for Branch Ozone Fumigation

2.2. Sample Pretreatment for Pyrolysis

The samples were prepared for pyrolysis in two different ways. In one method, the fresh leaves were air-dried, then ground to a fine powder. In the other method, the fresh leaves were homogenized in a minimum amount of water, then freeze-dried. The pyrograms of leaves prepared by both methods did not show any significant difference in pyrolytic analysis. The method of grinding after air drying was adopted for all samples due to its simplicity.

2.3. CDS Pyroprobe 120

The Pyroprobe 120 (Chemical Data Systems, Oxford, Pa.) consists of a probe fitted with a platinum coil. The sample was placed in a quartz tube or a small quartz boat, and then inserted into the centre of the coil. The coil was heated resistively to a preset temperature and pyrolysates were swept by the GC carrier gas at a flow of 13 ml min^{-1} from the pyrolysis zone into the interface zone and finally into the GC injection port. The interface zone was maintained at 250°C for direct pyrolysis, and at 200°C for SPM. The pyroprobe was attached to the GC injection port by a 3 cm long stainless steel Universal Needle Assembly, supplied by Chemical Data Systems.

2.4. Pyrolysis Methods

2.4.1. Direct Pyrolysis

About 20 μg for chemical standards, or 400 μg ± 20 μg for leaf samples was used for pyrolysis. Each sample was weighed into a small precleaned quartz-tube using a Perkin Elmer Autobalance, model AD-2Z. All samples were pyrolysed at 500°C for 20 seconds. Maximal heating rate (ramp off) was used.

2.4.2. Simultaneous Methylation Pyrolysis (SPM)

Sample sizes for SPM were about 10 μg for the chemical standards, about 50 μg for the tannin extracts, and 200 μg ± 10 μg for other leaf samples. Each sample was weighed into a small pre-cleaned quartz-boat using the autobalance. A small piece of quartz wool was placed in the centre of the boat to hold both the sample and the reagent within the centre of the boat. A 10 μl aliquot of methylating reagent was added. Two methylating reagents, tetramethylamonium hydroxide (25% w/v in deionized water) and trimethylanilinium hydroxide (0.2 M in methanol) were investigated. The loaded boat was inserted in platinum coil and pyrolysed at 400°C for 20 seconds, using maximal heating rate.

2.5. Pyrolysis-Gas Chromatography

The CDS Pyroprobe 120 was attached to the injection port on a Varian 3700 gas chromatograph equipped with a flame ionization detector. The injection port and detector were maintained at 280°C. For direct pyrolysis experiment, a Cp-sil8 capillary column (25 m x 0.25 mm, 1.2 μm film thickness; 95% dimethyl, 5% phenyl; Chrompack, Middelburg, The Netherlands) was used. For simultaneous pyrolysis methylation, a BP 1 column (20 m x 0.22 mm, 0.25 μm film thickness; dimethyl siloxane; SGE, Austin, Te.) was used. The temperature program settings were as follows: 100°C, held for 2 minutes, increasing at 5°C per min until 270°C, then held for 10 minutes. The split flow was 12 ml/min with column flow rate of approximately 1.2 ml/min.

The chromatograms were recorded using a Spectra-Physics SP 4290 integrator via a Labnet interface. Chromatographic data was stored on a Tandy 1200HD personal computer, and controlled using a Spectra-Physics Winner data software system (San Jose, Ca.).

2.6 Pyrolysis-Gas Chromatography-Mass Spectrometry

2.6.1. Electron Impact Ionization

The CDS Pyroprobe 120 was interfaced to the injection port of a Hewlett Packard Model 5790A GC-MSD equipped with a HP 5970A workstation. The injection port and MS inlet were maintained at 280° C. The columns, oven temperature program, and flow rates were the same as those described in section 2.5. The electron impact ionization voltage was 70 eV. The mass spectrometer was scanned from 40 to 550 a.m.u. at 0.9 s per decade.

2.6.2. Chemical Ionization

The CDS Pyroprobe 120 was attached a Finnigan MAT 6000 GC-MS equipped with a finnigan workstation. The conditions of chromatographic separation were identical to those described in section 2.5. The column outlet was led directly into the ion source of the quadruple mass spectrometer equipped with a CI volume source. The temperature of the source was maintained at 200° C. The CI reagent gas , isobutane, was admitted into the source until the pressure was approximately 0.4 torr. The ionization voltage was set at 110 eV and the mass spectrometer was scanned from 80 to 450 a.m.u. at a scan rate of 1 scan/sec.

CHAPTER 3

PYROLYSIS-GAS CHROMATOGRAPHIC ANALYSIS OF LEAVES

3.1. Taxonomic Information on Plant Leaves Used in the Study

The selection of plant leaves for analysis is based on achieving the objectives of our study. First, the pyrolysis products of leaves from a shrub plant, kalmia, from two angiosperm trees, balsam fir and white pine, and, from a gymnosperm tree, oak, were compared, and key pyrolysates were identified. Second, kalmia, balsam fir and white pine plants were also involved in the environmental stress studies. The chemical compositions in these plant leaves and plants' responses to environmental stresses are related to their taxonomy. Therefore, a brief summary of their taxonomy is given first.

Kalmia (*Kalmia angustifolia L.)* , also called lambkill or sheep-laurel, is an evergreen shrub in the heath family (Ericaceae). The leaves are poisonous to cattle, sheep, goats and horses. Generally, it grows to 1-3 feet, on poor lands of eastern regions of North America (96). Condensed tannins are abundant in the Ericaceae family (97, 98). In addition, previous investigations have demonstrated the presence of dihydrochalcone (e.g. phloretin, phloridzin) and grayanotoxin compounds in kalmia leaves (99-101).

Balsam fir *(Abies balsamea)*, growing in many places, is an evergreen tree of the pine family. The leaves are solitary needle-like. The tree is usually 40-80 feet high (102). Terpenes are abundant in balsam fir leaves (103). Information about the organic composition of balsam fir leaves so far is very limited.

Northern red oak (*Quercus rubra*) is a deciduous tree around 60-100 feet tall in beech family. It is a valuable hard wood timber tree (104). The high tannin content of oak leaves has been of interest for a long time. The condensed tannins are very low compared to the hydrolysable tannins. It has been reported that condensed tannins disappear in the leaves in some seasons of a year. Tannins may play very important roles in plant defense systems (5, 105).

White pine (*Pinus strobus*), also a valuable timber tree, is an evergreen tree about 60-100 feet tall in pine family. The leaves are needle like, soft, usually in bundles of 5 needles of 3 to 5 inches long (102). The lipids in pine needles were reported as high as 28 % (106).

3.2. Direct Pyrolysis of Plant Leaves

3.2.1. Optimizing Pyrolysis Conditions

Several chromatographic columns were examined in order to properly resolve the pyrolysates of leaves from direct pyrolysis. The columns included a Chrompack Cp-sil8 column (25 m x 0.25 mm, 1.2 μm film thickness; 95% dimethyl, 5% phenyl), a J&W DB 17 column (20 m x 0.179 mm, 0.3 μm film thickness; 50% phenyl; Chromatographic Specialties, Brockwille, ONT.), and a SGE BP 1 column (20 m x 0.22 mm, 0.25 μm film thickness; dimethyl siloxane). Of these three columns examined, the non-polar BP 1 was unsatisfactory due to short retention times and poor resolution. The polar DB 17 column was unsuitable because of its inability to resolve several chromatographic peaks near the 1,6-anhydroglucopyranose peak, an important pyrolysate of leaves. The slightly polar CP-sil8 column was found to be most suitable for the separation of the unusually wide range of pyrolysates obtained from pyrolysis of leaves.

A high pyrolysis temperature, i.e. >800 ℃, was found to be unsuitable and only a few low molecular weight fragments were detected by GC. On the other hand, at much lower temperatures i.e. <400℃, it was observed that a large portion of leaf residue remained in the pyrolysis tube. When pyrolysis was carried out at the temperature between 450℃-600℃, anhydrosugars and phenolic fragments which are

very important pyrolysates gave good yields. This pyrolysis temperature range using the Pyroprobe system was also selected as an optimum range in a number of PY-GC studies for the analysis of biomaterials (90, 107, 108). The quartz-tube selected should fit into the platinum coil snugly for efficient heat transfer. Efforts were made to use the same tube and similar sample weights when analyzing each set of leaf samples. The highest pyrolysis yields were obtained with the maximum temperature ramp speed of the pyrolyzer.

3.2.2. Pyrolysis of Tannin Standards

Catechin is the major monomeric residue of condensed tannins (Figure 1.2). When pyrolysed, it degrades primarily to catechol, **15**[1] (86). Tannic acid, the polymer of hydrolysable tannins (Figure 1.2) yields the major pyrolysis product 1,2,3-benzenetriol (pyrogallol), **26**, as a result of decarboxylation when subjected to direct pyrolysis. The pyrograms of catechol and tannic acid by direct pyrolysis are shown in Figure 3.1 a, b. These pyrolysis products are small fragments of the initial structures and do not readily reflect the original structures of tannins.

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[1] boldface numbers refer to identification numbers of compounds in Table 3.1

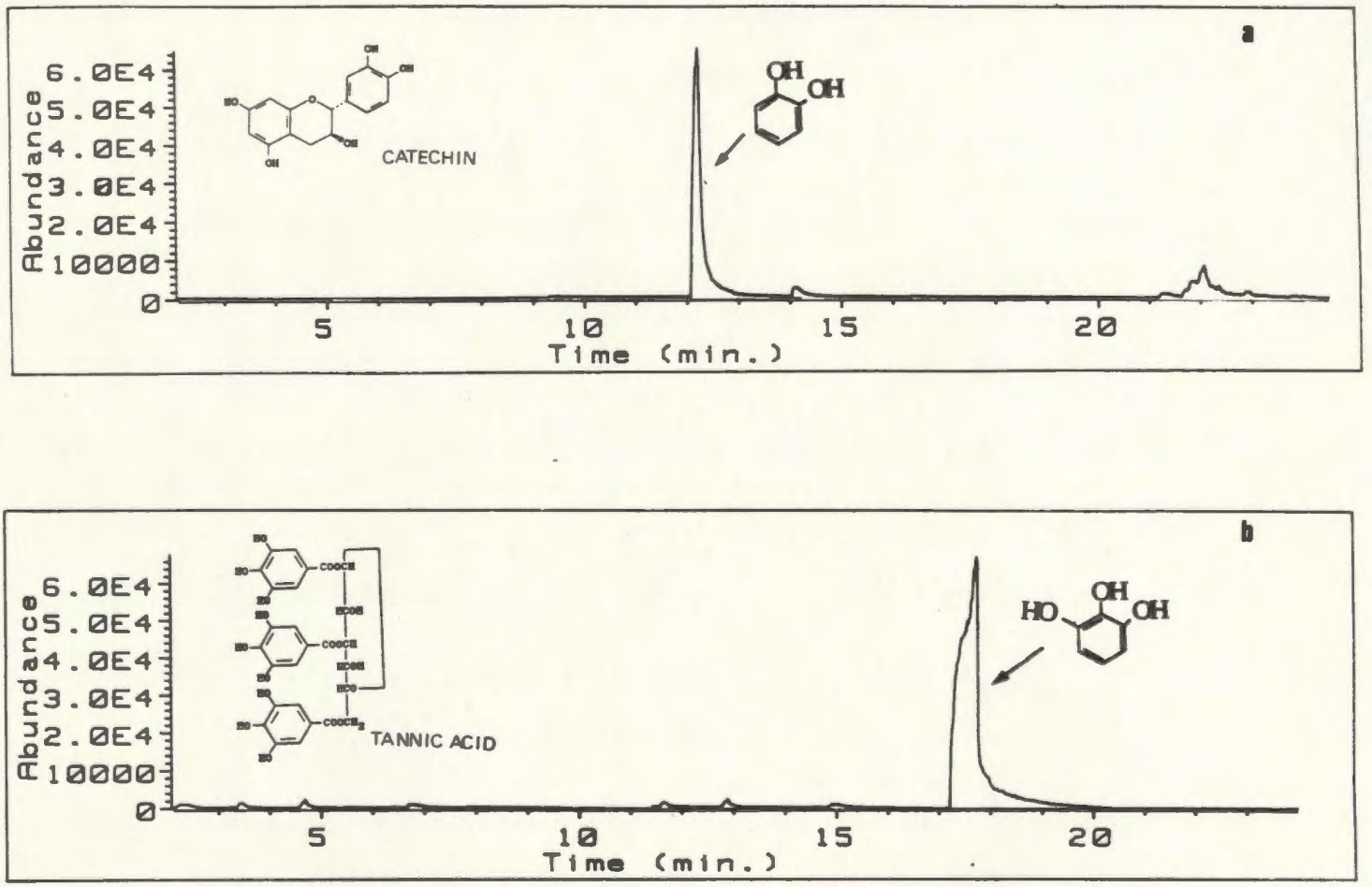


Figure 3.1 TIC Pyrograms (Direct Pyrolysis) of (a) Catechin and (b) Tannic Acid

3.2.3. Pyrolysis of Kalmia Leaves

Direct pyrolysis was applied to the analysis of kalmia leaves. The pyrogram of kalmia leaves (second year) is presented in Figure 3.2 and the identified pyrolysis products are listed in Table 3.1. Identification of most compounds were provided by comparing their EI mass spectra with those in the mass spectral library (109) and/or with those previously reported in the literature.

A large number of pyrolysates from phenolic compounds and carbohydrates were identified. More complex phenolic compounds (e.g. lignin, tannins, etc.) are likely pyrolysed to produce a mixture of relatively simple phenols which are the result of thermal cleavage of ether and certain C-C linkages. Most of these phenolic products retain their substitution patterns. Thus polyphenolic constituents in leaves from *p*-hydroxyphenyl, guaiacyl and syringyl moieties can be characterized. In kalmia leaves, most phenolic fragments identified have *p*-hyroxyphenyl groups, i.e. compounds **35, 36, 39**, suggesting a predominance of *p*-hydroxyphenyl-containing polyphenolics. These pyrolysates are unique to kalmia and are not observed in leaves from oak and balsam fir. On the other hand, common lignin pyrolysates such as compounds **23, 30, 40**, and **43**, present in oak and fir pyrograms, are noticeably absent in kalmia leaves. Kalmia, being a shrub, as opposed to tree has a much different lignin (polyphenolic) composition and its pyrogram clearly reflects this.

Catechol, **15**, observed as a major pyrolysate of catechin (section 3.2.2) is the one of the dominant peaks in the pyrogram of kalmia. This is consistent with the fact that Kalmia is known to contain condensed tannins.. As expected, pyrogallol, **26**, the major pyrolysis product of hydrolyzable tannins (tannic acid), was absent.

A number of carbohydrate pyrolysates were identified in the pyrogram of kalmia leaves. 1,6-anhydroglycopyranose, **31**, is a major product and its broad shape is characteristic of the chromatographic peaks of anhydrosugars using non-polar columns (107). This common anhydrosugar can be formed from O-glycosides and/or from polymeric carbohydrates such as starch and cellulose. Other anhydrohexoses, **27, 34,** and an anhydropentose, **14**, were also observed in the kalmia pyrogram originating from other types of sugars. Pyran- and furan-type fragments, **1, 10, 13, 22,** are other common pyrolysis products of carbohydrates (90).

Direct pyrolysis of biomaterials usually leads to decarboxylation of phenolic acids and fatty acids. Although the mass spectra of some peaks in the pyrograms resemble that of decarboxylated fatty acids (i.e. hydrocarbons), molecular weight information of these compounds were not available, making their identification difficult.

3.2.4. Pyrolysis of Balsam Fir Leaves

The pyrogram which is typical of balsam fir leaves is illustrated in Figure 3.3 and identification of its pyrolysis products are listed in Table 3.1.

A major feature of the pyrogram is the presence of guaiacyl-containing pyrolysates. As with kalmia leaves, syringyl-containing pyrolysates are absent, indicating that lignin in balsam fir leaves are structurally distinct. Catechol, **15**, is present, but at lower level than in kalmia leaves. This indicate that fir leaves may have condensed tannins. Pyrogallol was not found in the pyrogram.

The abundant carbohydrate-derived fragments are much lower in molecular weight, i.e., furan-type compounds, **1**, **2**, **5**, **18** and pyran-type compounds, **12**, **13** whereas the intact pyrolysis product, **31**, 1,6-anhydroglucopyranose was produced at relatively lower level than that from kalmia. This result may reflect major differences in nature of the glucose-containing compounds in conifer leaves versus shrubs and deciduous trees.

3.2.5. Pyrolysis of Oak Leaves

The pyrogram of oak leaves is shown in Figure 3.4 and its identified pyrolysates are listed in Table 3.1. A large number of phenolic products having alkyl, methoxy, vinyl, alcohol and keto side groups were identified and overall pyrogram is far more complicated than that obtained from kalmia and balsam fir leaves. The *p*-hyroxyphenyl, guaiacyl and syringyl fragments were all detected in the pyrograms. This suggests that lignin in oak leaves is composed of all three basic units and therefore structurally different from that in kalmia and balsam fir leaves. This is in agreement with reports indicating that syringyl components are usually present in angiosperm trees, not in gymnosperm trees (88, 110).

Both catechol, **15**, and pyrogallol, **26**, are observed as major pyrolysis products, thus indicating that oak leaves may have both hydrolysable and condensed tannins. As will be seen in the SPM pyrogram of oak leaves (section 3.3), condensed tannin pyrolysates are almost absent. This shows that direct pyrolysis is not always capable of determining tannin structures. It has been reported that lignin in leaves can contain small amount of catechol residues (86).

Although several phenolic pyrolysis products are identified, it is very difficult to determine whether these small phenolic fragments originated from simple phenolics

or from phenolic polymers, such as tannins and lignin. It has been reported that 4-vinylphenol, **17**, can be derived primarily from *p*-coumaric acid residues, which are very common constituents in leaves (75). But It should be noted that **17**, can also come from *p*-hydroxyphenyl residues of lignin as well (75).

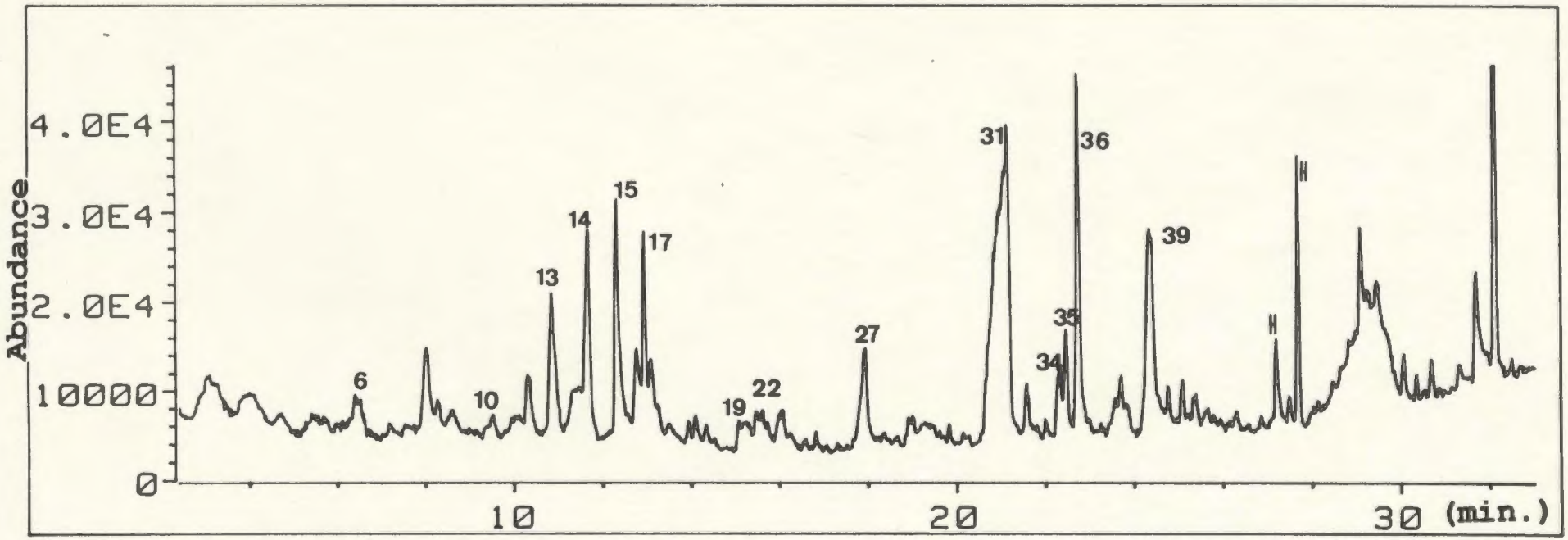


Figure 3.2 TIC Pyrogram (Direct Pyrolysis) of Kalmia Leaves

H: unidentified hydrocarbons

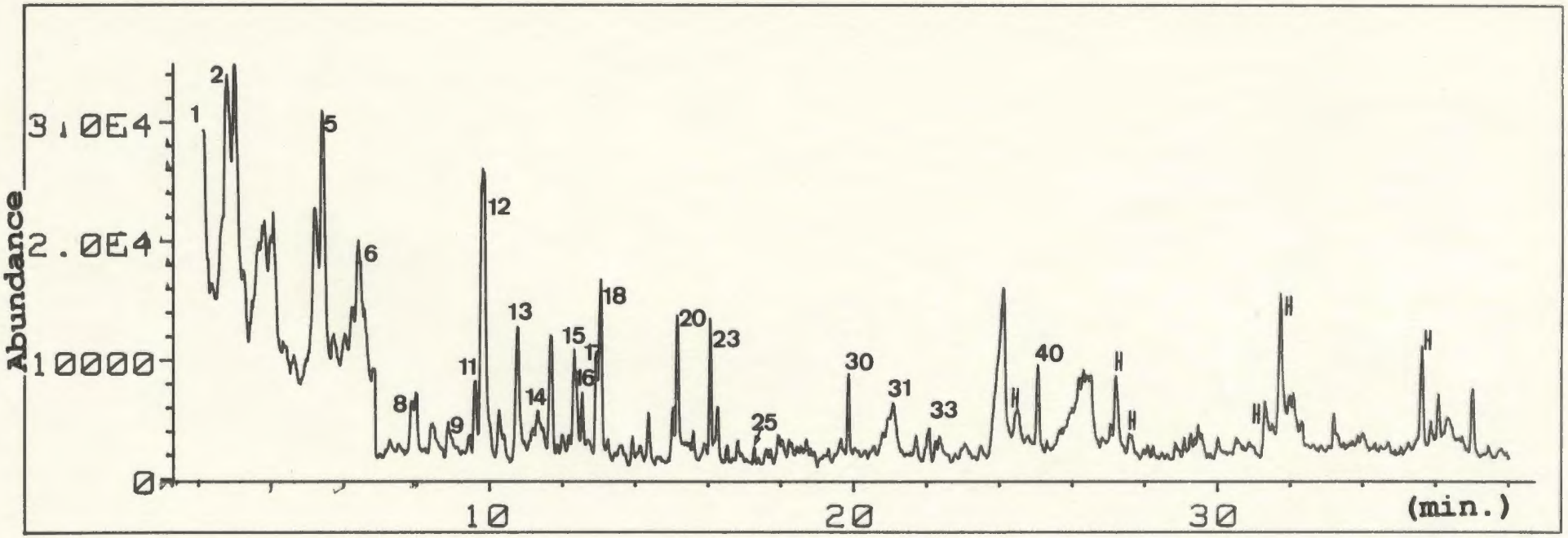


Figure 3.3 TIC Pyrogram (Direct Pyrolysis) of Balsam Fir Leaves

H: unidentified hydrocarbons

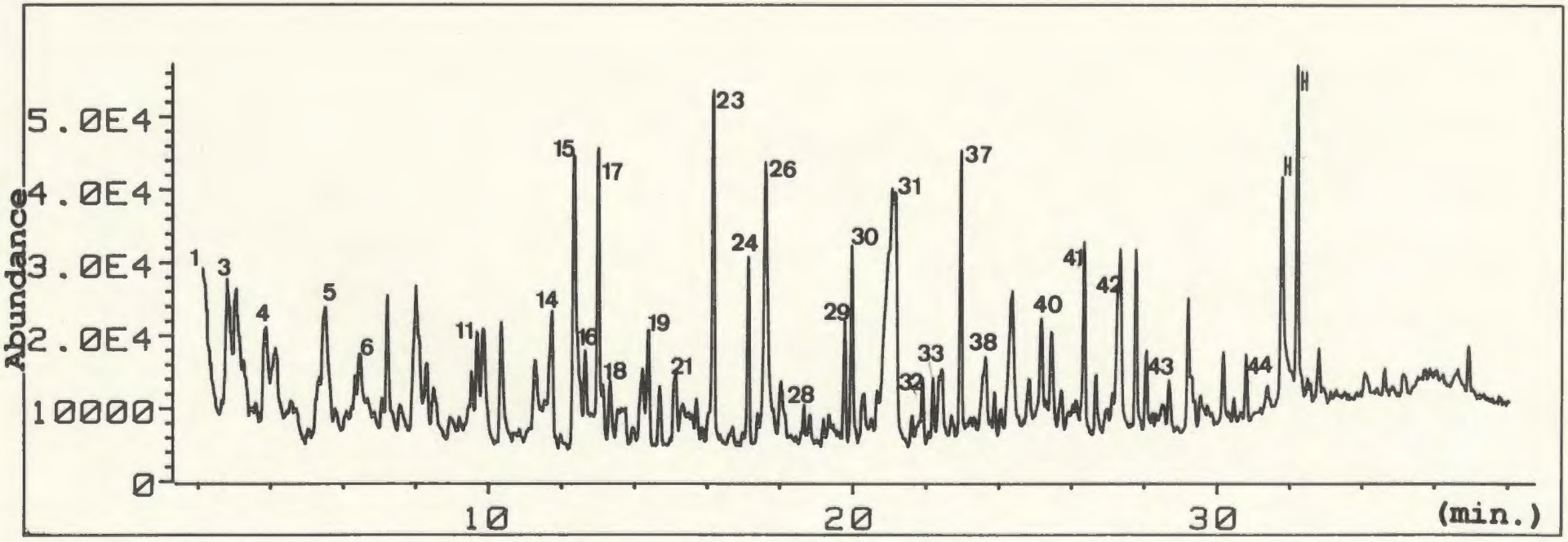


Figure 3.4 TIC Pyrogram (Direct Pyrolysis) of Oak Leaves

H: unidentified hydrocarbons

Table 3.1: Identification of Pyrolysis Products in the Pyrograms (Direct Pyrolysis) of Leaves

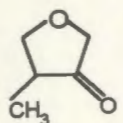
No.	Identification[a]	Reference	Mass	RT	Leaf Species[b]		
					Kalmia	Balsam fir	Oak
1	4-methyltetrahydrofuran-3-one	(75)	96	2.13	Y	Y	Y
2	2-hydromethylfuran	(75)	98	2.77	N	Y	N
3	4-penten-2-one, 3-methyl	(109)	96	2.83	N	N	Y
4	furfural	(75)	96	3.89	N	N	Y
5	2,3-dihydro-5-methylfuran-2-one	(75,109)	98	5.48	N	Y	Y
6	phenol	(75,109)	124	6.43	Y	Y	Y
7	4-hydroxy-5,6-dihydro-2H-pyran-2-one	(75,109)	114	7.21	N	N	Y
8	2-hydroxy-3-methyl-2-cyclopenten-1-one	(75)	112	7.89	N	Y	N
9	methylphenol	(75,109)	108	8.94	N	Y	N
10	2-furoic acid methyl ester	(75)	128	9.51	Y	N	N
11	guaiacol	(75)	124	9.66	N	Y	Y
12	3-hydroxy-2-methyl-4H-pyran-4-one	(75)	126	9.81	N	Y	N
13	3,5-dihydroxy-2-methyl-5,6-dihydro-4H-pyran-4-one	(75)	144	10.79	Y	Y	N
14	1,4-anhydro-pentopyranose	(75)	132	11.61	Y	Y	Y
15	catechol	(75,109)	110	12.35	Y	Y	Y

16	4-methylguaiacol	(75, 109)	138	12.62	N	Y	Y
17	4-vinyl phenol	(75,109)	120	13.02	Y	Y	Y
18	5-hydroxymethyl-2-furaldehyde	(75,90)	126	13.35	N	Y	Y
19	3-methoxycatechol	(75)	140	14.70	Y	N	Y
20	4-ethyl-2-methylguaiacol	(75)	152	15.04	N	Y	N
21	4-methylcatechol	(75,109)	124	15.10	Y	N	Y
22	1,4-dideoxy-D-glycero-1-eno-pyranos-3-ulose	(75,109)	144	15.66	Y	N	N
23	4-vinylguaiacol	(75)	150	16.14	N	Y	Y
24	2,6-dimethoxyphenol	(75)	154	17.14	N	N	Y
25	eugenol	(75)	164	17.21	N	Y	N
26	pyrogallol	(75)	126	17.65	N	N	Y
27	1,6-anhydropyranose	(90)	162	17.84	Y	N	N
28	vanillin	(75)	152	18.67	N	N	Y
29	2,6-dimethoxy-4-methylphenol	(75)	168	19.76	N	N	Y
30	trans isoeugenol	(75)	164	19.97	N	Y	Y
31	1,6-anhydroglucopyranose	(90)	162	21.00	Y	Y	Y
32	4-ethyl-2,6-dimethoxyphenol	(75)	182	21.90	N	N	Y
33	guaiacylacetone	(75)	180	22.12	N	Y	Y
34	1,4-anhydrogalactopyranose	(63, 109)	162	22.31	Y	N	N
35	4-hydroxyphenyl-2-butanone	(109)	164	22.52	Y	N	N
36	4-hydroxy-α-methyl benzenepropenol	(109)	166	22.76	Y	N	N

37	2,6-dimethoxy-4-vinylphenol	(75)	180	22.83	N	N	Y
38	propiovanilione	(75)	180	23.64	N	N	Y
39	4-hydroxy-benzene acetic acid methyl ester	(109)	166	24.44	Y	N	N
40	dihydroconiferyl alcohol	(75)	182	25.15	N	Y	Y
41	2,6-dimethoxy-4-(2-propenyl)-phenol	(75)	194	26.38	N	N	Y
42	trans coniferaldehyde	(75)	178	27.31	N	N	Y
43	syriagylacetone	(75)	210	28.05	N	N	Y
44	dihydrosinapyl alcohol	(75)	212	30.78	N	N	Y

[a] Structures of products listed on the next page

[b] Y: observed
N: not observed



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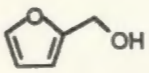

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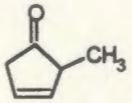

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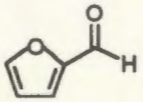

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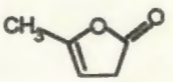

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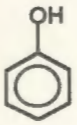

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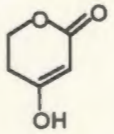

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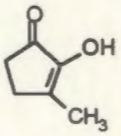

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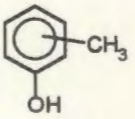

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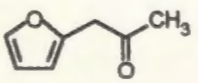

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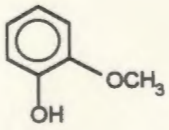

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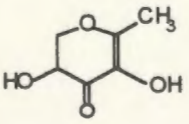

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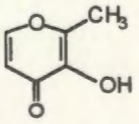

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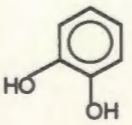

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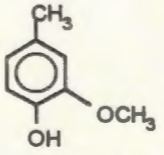

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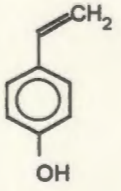

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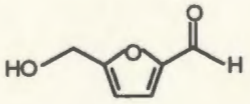

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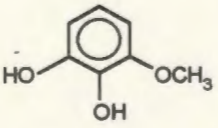

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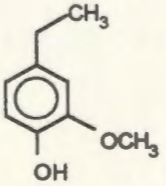

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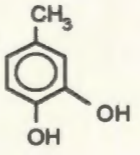

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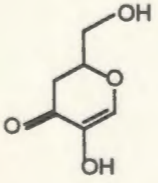

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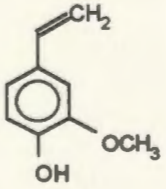

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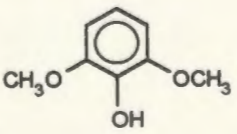

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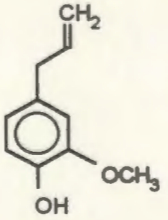

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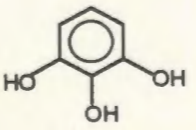

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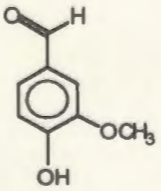

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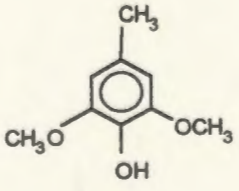

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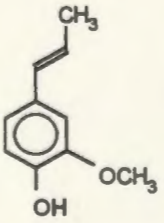

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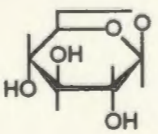

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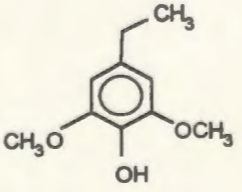

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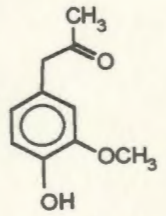

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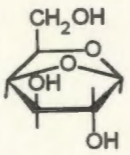

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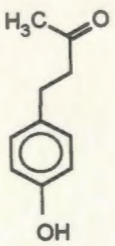

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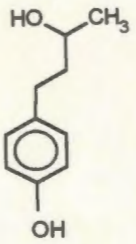

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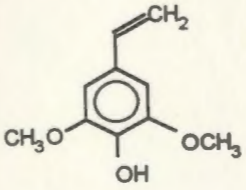

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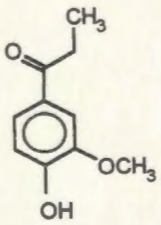

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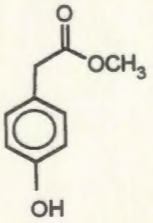

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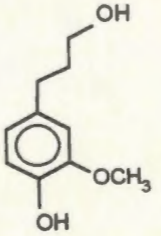

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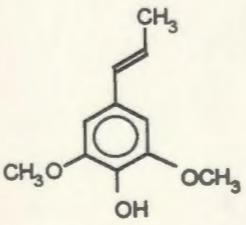

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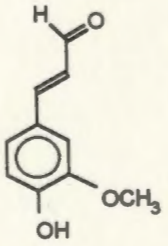

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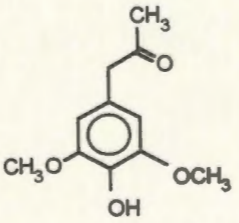

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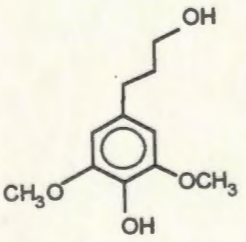

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3.3. Simultaneous Pyrolysis Methylation of Leaves

Although the technique of direct pyrolysis generated some distinctive and unique pyrolysates which may be correlated to specific compound in leaves, (e.g. anhydrosugars), it is not always able to generate distinctive products of the phenolic and/or carboxylic acid-containing compounds (e.g. fatty acids, tannins). This is because of the non-volatile nature of many of the acidic functional group-compounds and generated pyrolysates. This results in further fragmentation, particularly decarboxylation to low molecular weight, non-distinctive products. Furthermore, a significant fraction of the semi-volatile acidic pyrolysates will be condensed in the transfer zone before reaching the GC column. To overcome this problem, the technique of simultaneous pyrolysis methylation has been investigated whereby a methylating reagent combined with thermal fragmentation is used in order to generate more volatile and higher molecular weight pyrolysates. The methylation of phenolic and carboxylic acid functional groups is a very important step in analysis.

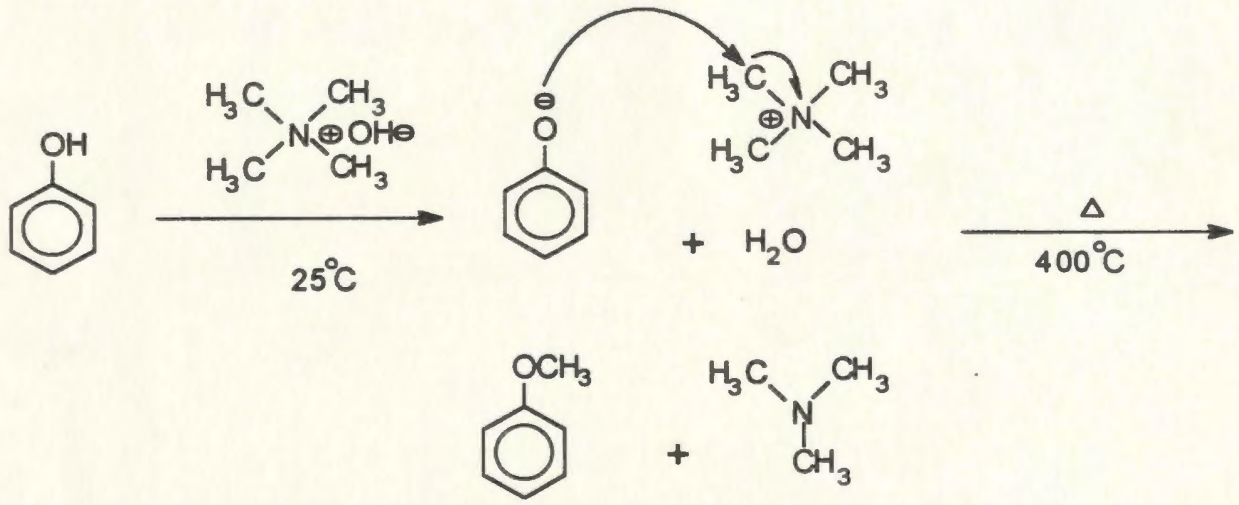
3.3.1. Optimizing Pyrolysis Conditions

Trimethylanilinium hydroxide (Methylute) and tetramethylammonium hydroxide (TMAH) were investigated as methylating reagents for simultaneous pyrolysis methylation. The phenolic acid polymer tannic acid was used as the representative

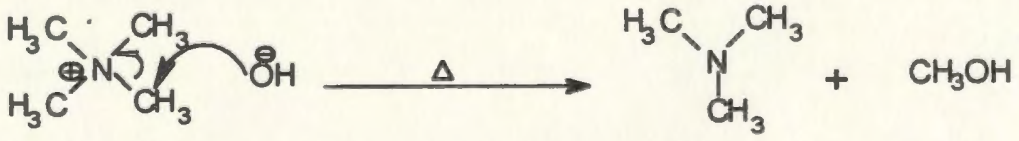
analyte. When Methylute in methanol (0.2 M) was added and pyrolysed along with the sample, the resulting pyrogram was masked by an enormous reagent peak and contained several unmethylated phenolics and decarboxylated products. Obviously, the methylating efficiency of Methylute was poor under the pyrolysis conditions used. In comparison, when aqueous TMAH (25% w/w) was used for SPM, decarboxylation was avoided, and a fully methylated gallic acid (i.e. 3,4,5-trimethyoxybenzoic acid, methyl ester, **61**) was generated in high yield (see section 3.3.2). A few low molecular weight phenolic acid standards (i.e., 4-hydroxylbenzoic acid and *p*-coumaric acid) were also tested under the high temperature required for pyrolysis. TMAH again fully methylated the acidic analytes in high yields.

The chemical reaction of TMAH with a phenol to produce phenol methyl ether is illustrated below (Equation 1). The excess TMAH that was present in the sample was also thermally decomposed upon pyrolysis to yield a tertiary amine and methanol (Equation 2) (111, 112). These reagent products eluted quickly from the column without interfering with the chromatographic analysis of leaf pyrolysates.

Equation 1



Equation 2



Several chromatographic columns ranging in polarity were examined for the separation of pyrolysis products of leaf samples generated by SPM. The columns included a SGE BP 1 column (20 m x 0.22 mm, 0.25 μm film thickness; dimethyl siloxane), a J&W DB 5 column (30 m x 0.25 mm, 0.5 μm film thickness; 5% phenyl), a J&W DB 17 column (20 m x 0.179 mm, 0.3 μm film thickness; 50% phenyl), and a J&W DB 1701 column (30 m x 0.25 mm, 1.0 μm film thickness; 14% cyanopropyl phenyl). The non-polar BP 1 column was found to chromatographically resolve the majority of the pyrolysis products, while allowing for the elution of higher molecular weight compounds at high column temperature due to the column's non-polar stationary phase.

Although higher yields of some monomeric phenolic acids were achieved as pyrolysis temperatures increased, certain high molecular weight model compounds, e.g. phloretin (see section 3.3.2.), fragmented extensively when the pyrolysis temperature was above 500 °C. The main objective of analytical pyrolysis is to obtain structural information from intact high molecular weight pyrolysates, thus the pyrolysis temperature was examined in SPM experiments. It was found that a lower temperature of 400 °C was suitable in generating more abundant higher molecular weight products, i.e., fully methylated phloretin, **75**.

Samples were weighed into the centre of the small quartz-boat before the

methylating reagent TMAH was added. A small piece of quartz-wool was placed in the centre of the boat beforehand to hold both the sample and the reagent. This contained the reaction within a small and more reproducible pyrolysis zone. It was also important that the same quartz boat was used all through the sample set.

3.3.2 Pyrolysis Products of Standards

A wide range of standards were examined by SPM which reflect the diverse chemical structures of organics found in leaves. These included tannins, phenolic acids, phenolic glucosides, carbohydrates and structural polymers.

Tannin Standards and Tannin Extracts

Direct pyrolysis of tannin standards, i.e. tannic acid and catechin (section 3.2.2.) resulted in the production of pyrogallol and catechol, respectively. Since catechol and pyrogallol could be pyrolysates from other phenolics and lignin, they cannot be used as diagnostic fragments of tannins in leaves.

When tannic acid and catechin were pyrolysed with TMAH reagent, their pyrograms were completely different from that by direct pyrolysis. The pyrolysis products were successfully methylated and yields were greatly increased. As was expected, decarboxylation was avoided in the case of tannic acid and 3,4,5-

trimethoxybenzoic acid methyl ester, **61**, was the only pyrolysis product of tannic acid (Figure 3.5 b).

However, in the case of catechin (Figure 3.5 a), several fragments were formed from pyrolysis possibly because the catechin molecule is thermally fragile. Table 3.2 gives the mass spectra data of the major catechin fragments. The fragmentation mechanism of catechin is proposed in Figure 3.6. Compounds t1 and t2 are fragments from A ring of catechin. Compounds t3 and t4 are the fragments of B ring. They are likely to be isomers since they have similar mass spectra. Interestingly, two late eluting products, t5 and t6, were observed but molecular weight information on these could not be obtained even under CI-MS. It is believed that these compounds might be related to partly methylated and/or fully methylated derivatives of intact catechin. Epicatechin is another common monomeric unit of condensed tannins. It is postulated that under the basic conditions of SPM, catechin epimerizes and rearranges to a common pyrolysate composition including epicatechin. This is demonstrated in Figure 3.7 (113). Although no large and intact pyrolysis product was produced frcm catechin, the results however did give a number of pyrolysis fragments, which together, is a unique series of compounds structurally related to catechin.

In order to investigate the pyrolysis behaviour of native tannins in leaves, the tannin extracts of kalmia, balsam fir and oak leaves were analyzed by SPM. Their

pyrograms are shown in the Figure 3.8 a, b, c. The results show clearly that the technique of SPM can characterize the chemical nature of native tannins. The six characteristic fragments of catechin were present in all three pyrograms. All three leaf species are expected to contain catechin-containing tannins. The 3,4,5-trimethoxybenzoic acid methyl ester, representing the hydrolysable tannin product was not found in the kalmia tannin extract. On the other hand, hydrolysable tannins in oak leaves are dominant in the pyrogram (Figure 3.8 c). The other peaks in the pyrograms are probably, due to some simple phenolic compounds extracted along with tannins. It is known that most tannins in tannin extracts of leaves are polymeric in nature. These results from SPM of tannin extract thus suggest that polymeric tannins fragment to monomeric tannins before being methylated.

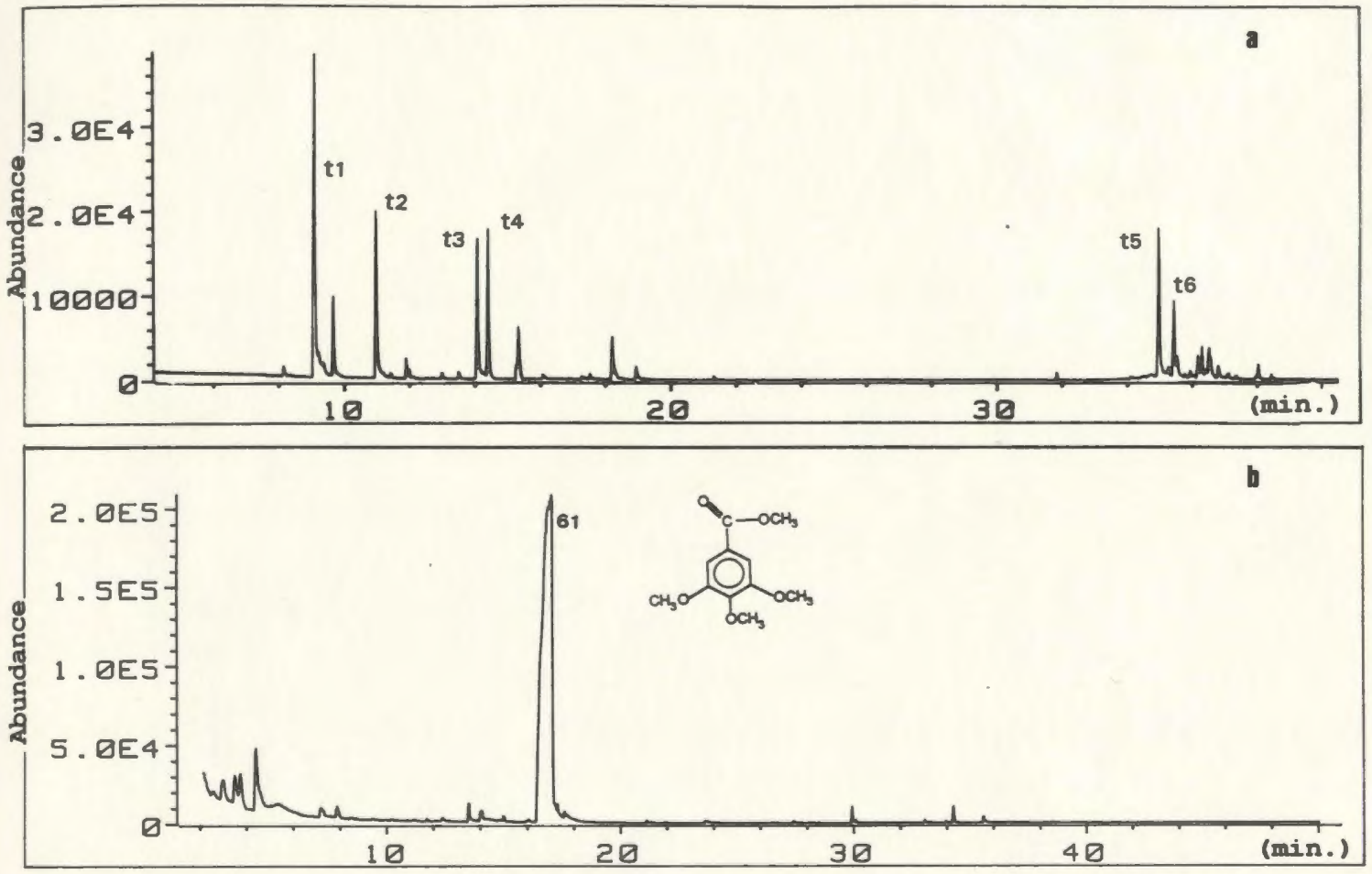


Figure 3.5 TIC Pyrograms (SPM) of (a) Catechin and (b) Tannic Acid

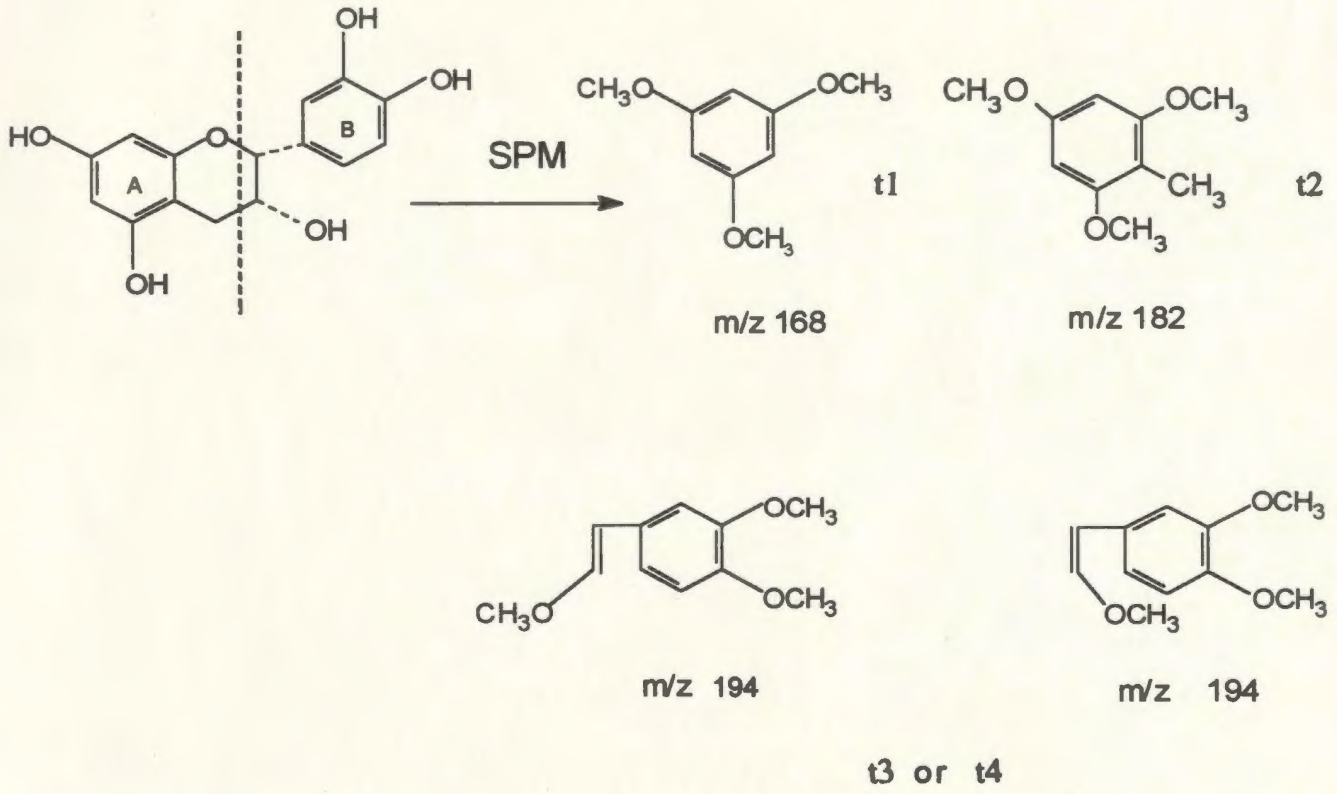


Figure 3.6 Proposed Fragmentation Pathway of Catechin under SPM Conditions

Table 3.2 EI Mass Spectral Data of Major Pyrolysis Products of Catechin Standard

Fragment (peak#)*	m/z [abundance]			
t1(**50**)	168 [100]	139 [90]	125 [25]	109 [26]
t2(**51**)	182 [100]	181 [28]	153 [28]	167 [25]
t3(**58**)	194 [100]	179 [80]	151 [48]	
t4(**59**)	194 [100]	179 [80]	151 [48]	
t5(**76**)	181 [100]	165 [20]		
t6(77)	195 [100]	165 [10]		

* as listed in Table 3.3

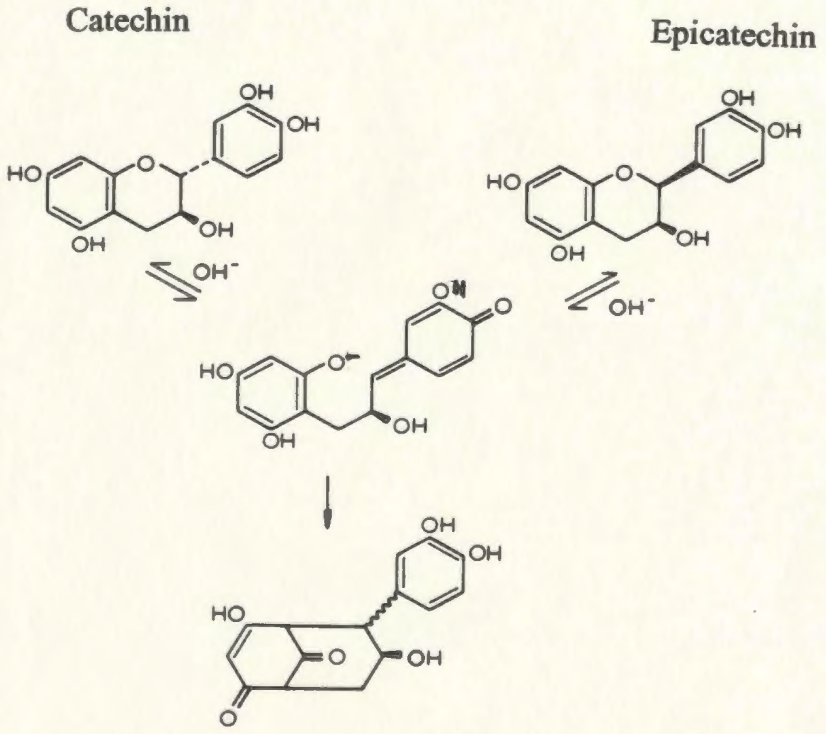


Figure 3.7 Epimerisation and Re-arrangement of Catechin in Alkaline Solution

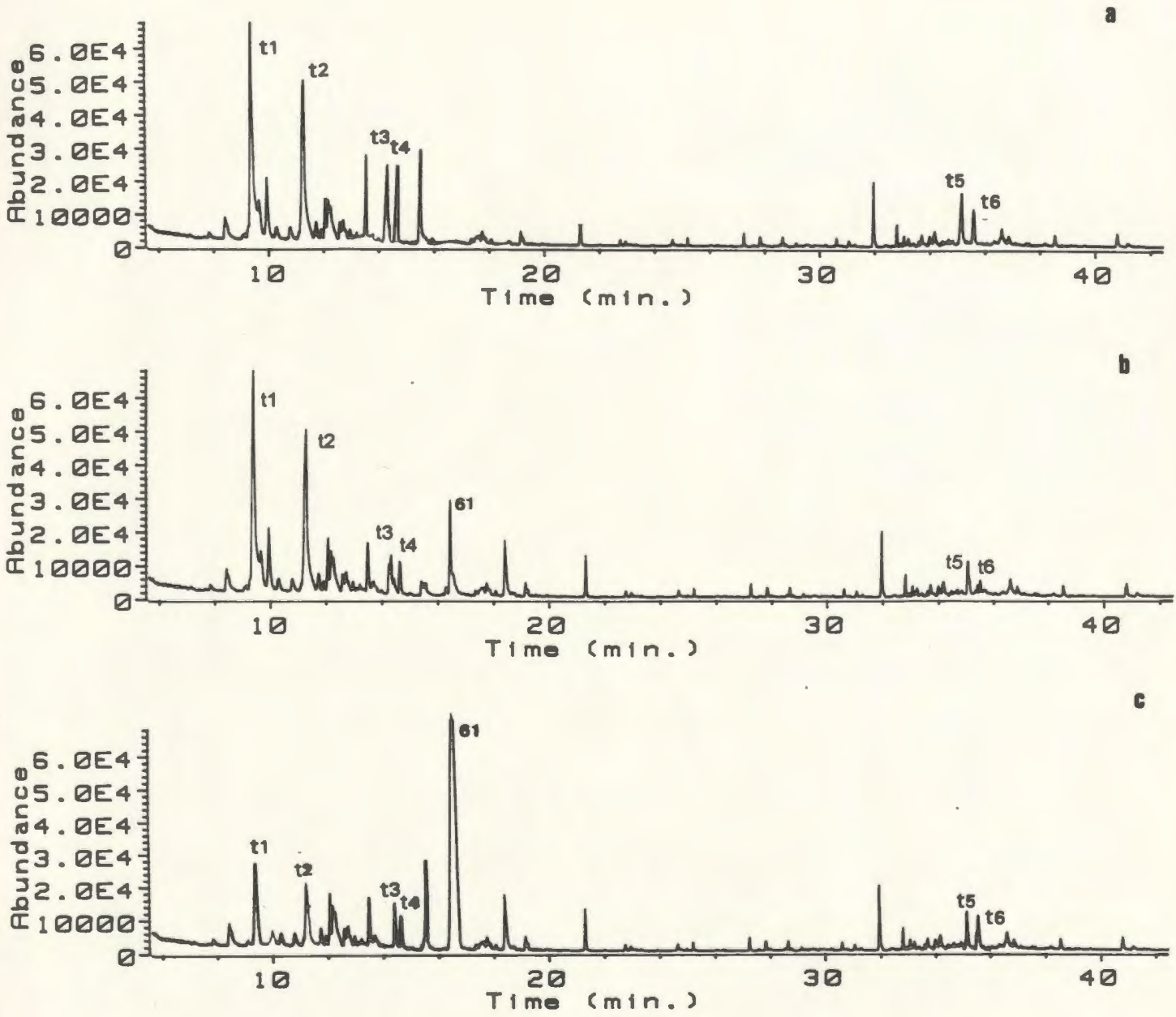


Figure 3.8 TIC Pyrograms (SPM) of Tannin Extracts of (a) Kalmia Leaves, (b) Balsam Fir Leaves and (c) Oak Leaves

Shikimic acid

Shikimic acid is the metabolic precursor of various phenolic compounds in leaves. It was also examined because of its non-phenolic acid structure. When pyrolysed by SPM, two H_2O molecules were readily lost and the product stabilized as an aromatic compound. The methylation of the remaining phenolic and the carboxylic acid groups gave rise to a distinctive product 3-methoxybenzoic acid methyl ester, **46** as illustrated in Figure 3.9 a, b. Compound **46** should be a unique product for shikimic acid because its SPM precursor, 3-hydroxy benzoic acid, is fortunately not be found in leaves.

Cellulose

Cellulose is the major component of cell walls and comprises of a linear chain of (1→4)-β-D-glucose units. When cellulose was pyrolysed with TMAH, the major pyrolysis peak was identified as permethylated glucopyranoside (Figure 3.10 a, b). The small peaks were attributed to partially methylated glucose. This is a very unusual but exciting result of SPM experiments, because it indicates that under the conditions used hydroxyl groups of sugars can be methylated. No other study has reported this for sugars.

Phloretin

Phloretin is a dihydrochalcone compound in the flavonoid group, which is rare in nature. Both phloretin and its glycosides (phloridzin) have been reported in kalmia

plants (99-101). Phloridzin is of special interest to pharmacologists due to its ability to produce glucosuria in man (114). The pyrogram of phloretin is given in Figure 3.11 a. The product at RT 34.34 min with the base peak ion of m/z 195 and a parent mass ion at m/z 330 is due to fully methylated phloretin structure, **75** (Figure 3.11 b). This compound can also easily fragment at high temperature to benzenepropanoic acid methyl ester (**53**)

Arbutin

Arbutin, or hydroquinone β-D-glucopyranoside, is present in many species of leaves. It's SPM pyrogram is given in Figure 3.12 a. Unexpectedly, fully methylated arbutin is observed and elutes at 26.99 min. Its EI mass spectrum (Figure 3.12 b) shows the parent ion m/z 342. Again, as with the SPM of cellulose, the hydroxyl groups of the sugar are methylated. In contrast to cellulose, the yield of permethylated glucose moieties of arbutin is very high indicating that better methylation can be achieved when the carbohydrates are of low molecular weight and readily soluble along with the methylating reagent. The known pyrolysis product of phenyl glycosides, that of 1,6-anhydroglycopyranose, is also present in the pyrogram as its permethylated derivative, **49**.

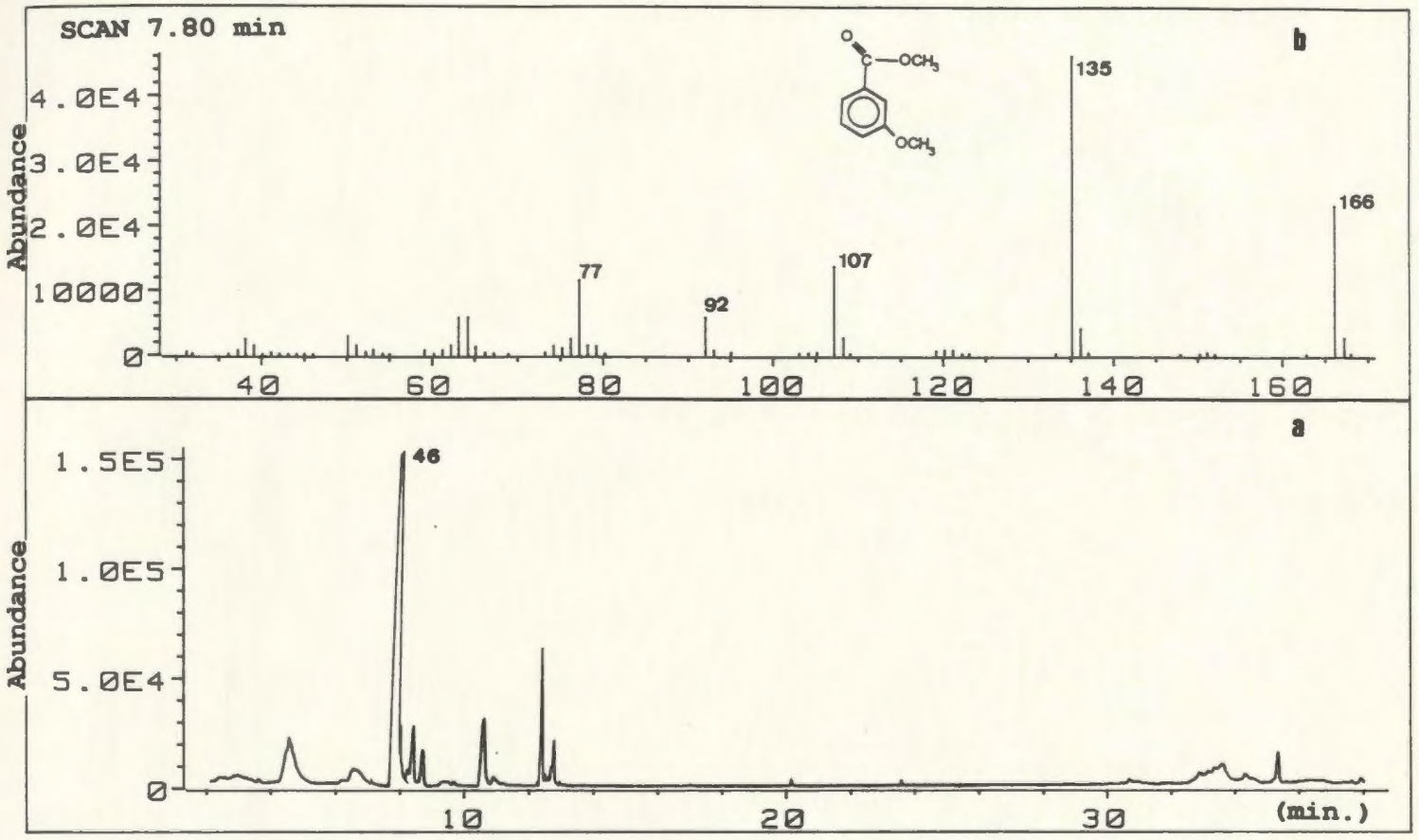


Figure 3.9 (a) TIC Pyrogram (SPM) of Shikimic Acid and (b) Mass Spectrum (EI) of Its Major Pyrolysis Product, **46**

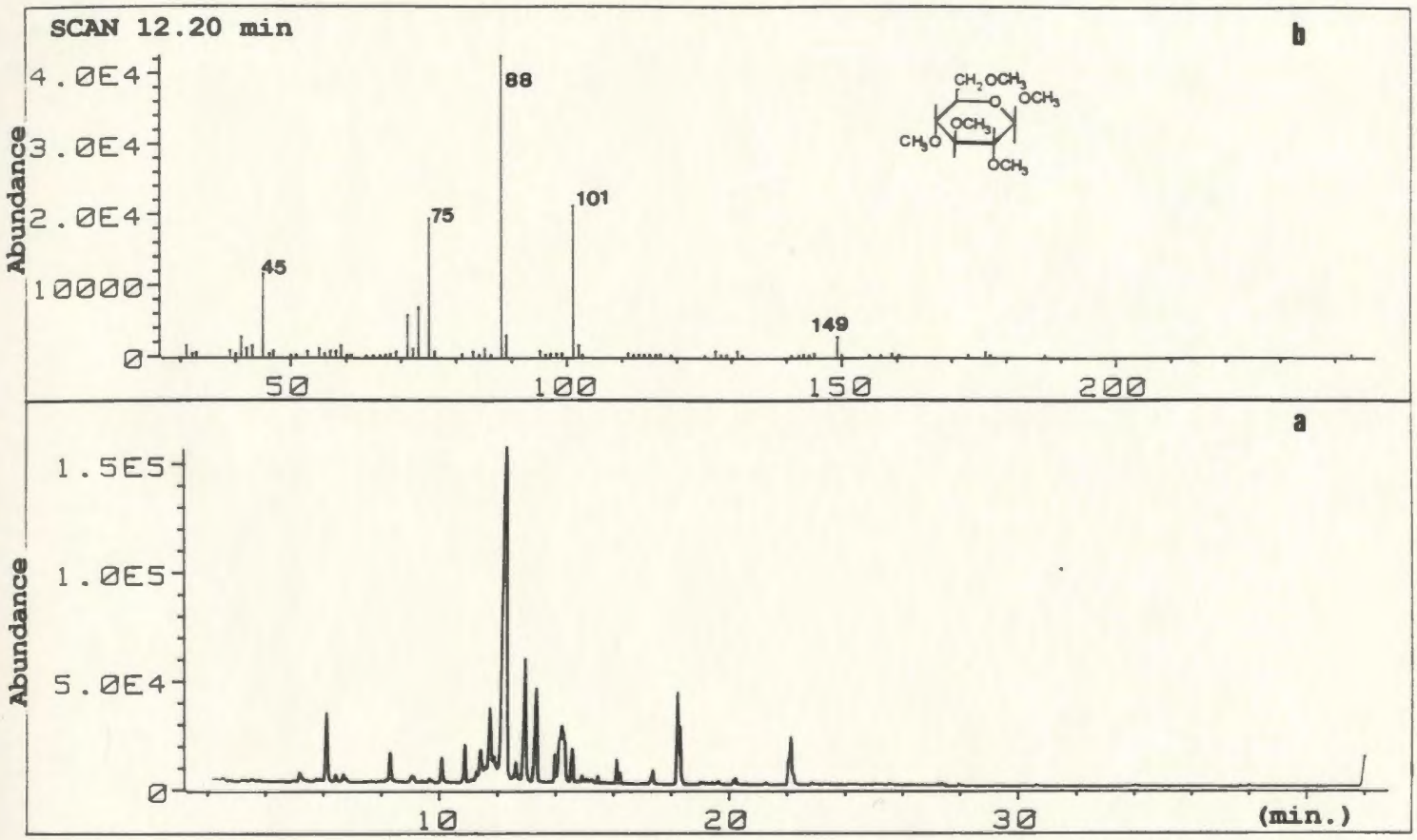


Figure 3.10 (a) TIC Pyrogram (SPM) of Cellulose and (b) Mass Spectrum (EI) of Permethylated Glucopyranoside

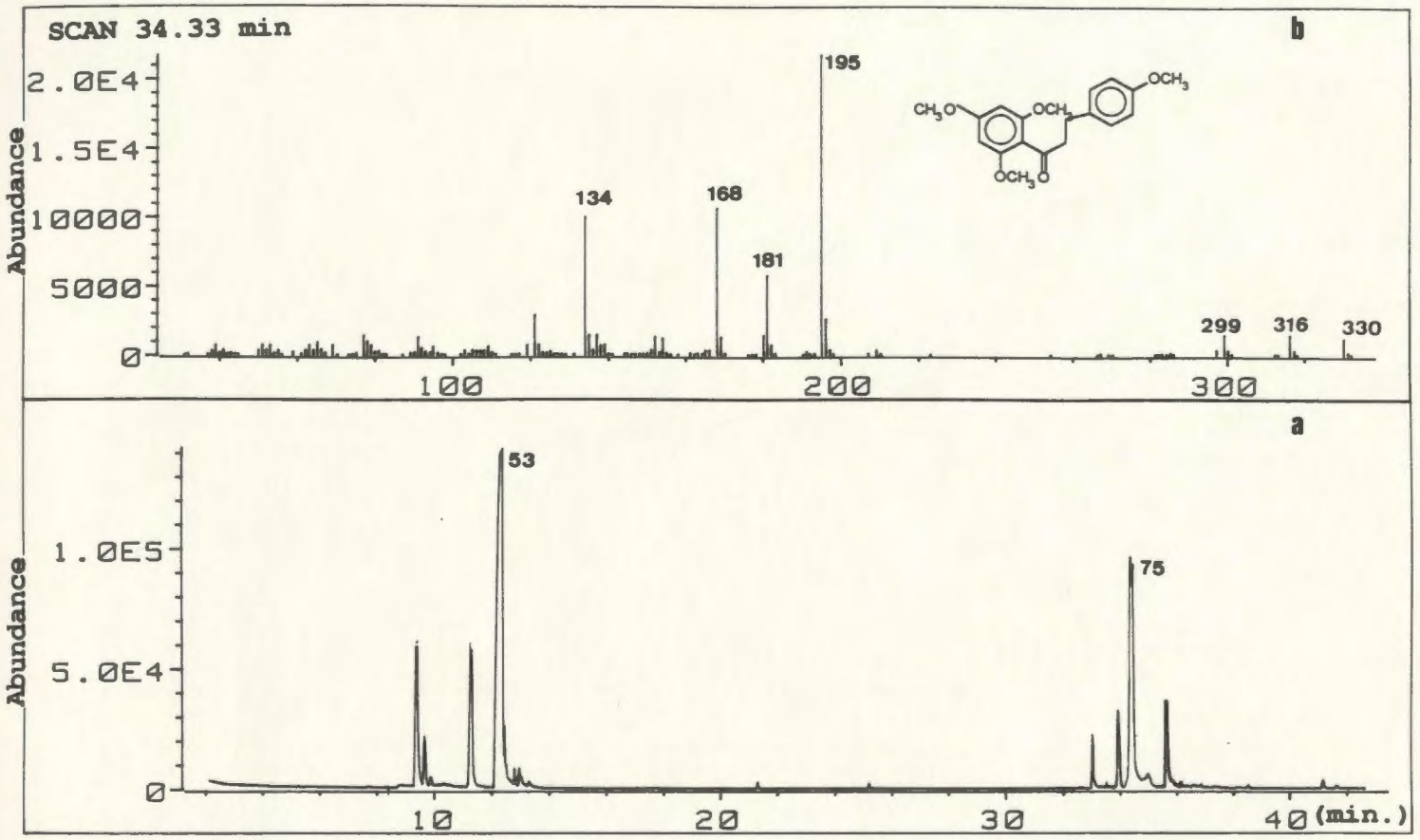


Figure 3.11 (a) TIC Pyrogram (SPM) of Phloretin and (b) Mass spectrum (EI) of Permethylated Phloretin, **75**

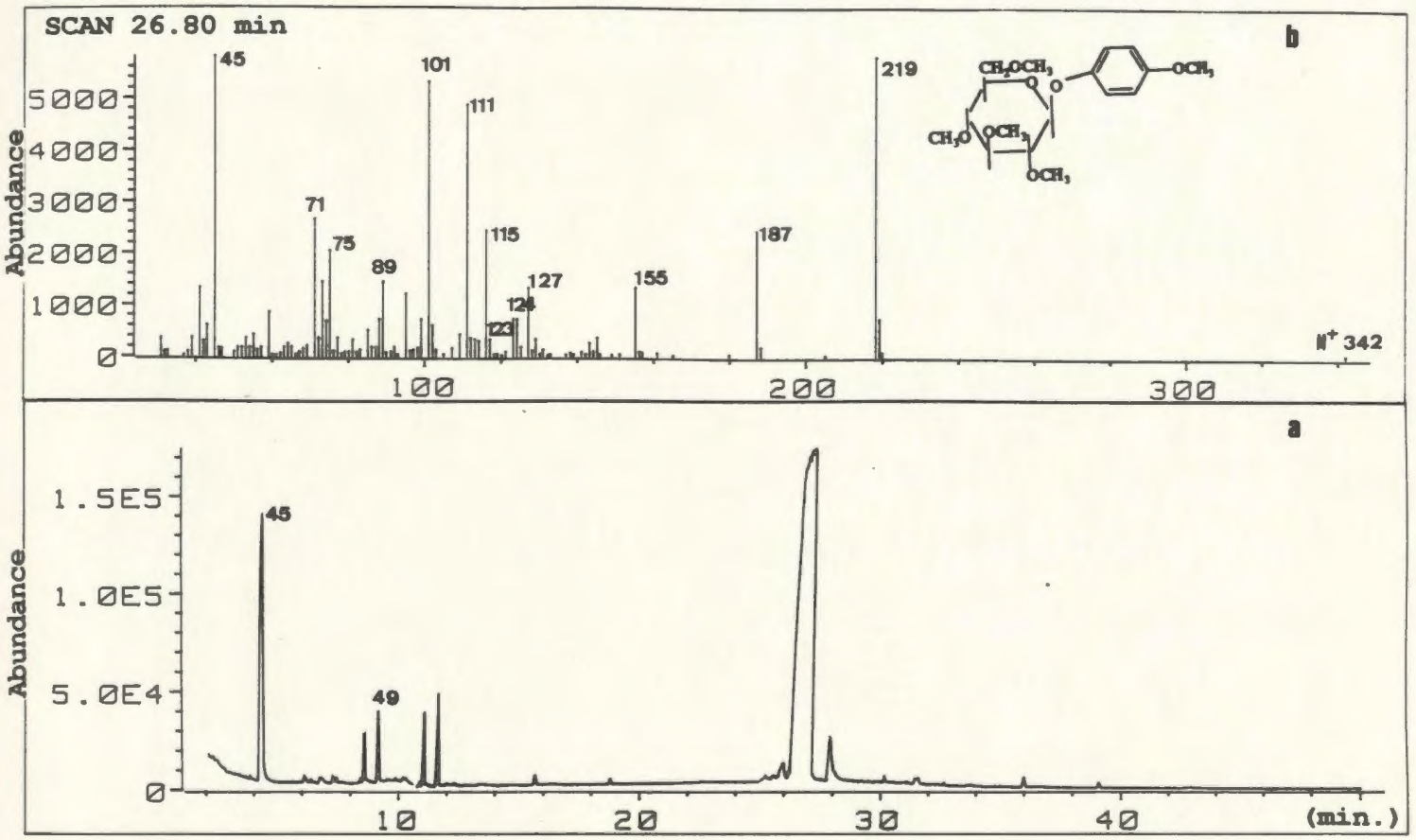


Figure 3.12 (a) TIC Pyrogram (SPM) of Arbutin and (b) Tentative Mass Spectrum (EI) of Permethylated Arbutin

3.3.3. Pyrolysis of Kalmia Leaves

Kalmia leaves were pyrolysed using TMAH methylating reagent. Figure 3.13 a shows the resulting pyrogram. The same kalmia leaves were subsequently extracted with methanol-water and the extract was also analyzed by SPM (Figure 3.13 b). It was noted that all pyrolysates in the pyrogram of the extract could also be identified in the corresponding intact leaves. An important result of the methanol-water extraction pyrogram indicates that many of the pyrolysates observed from intact leaves arise from the extractable fraction of the leaf. As was seen with water soluble phenolic standards (section 3.3.2.), high yields of methylated products are obtained when part of the constituents of the leaf are solubilized in the TMAH reagent. The identification of the products whose peaks are numbered are listed in Table 3.3.

Several methylated phenols and phenolic acids were identified, although methylated phenols were small in abundance. This is in contrast to the direct pyrolysis of kalmia (Figure 3.2) where phenolic products were very abundant as the result of thermal decarboxylation of phenolic acids. What is also noticeable in the SPM pyrogram of kalmia is the complete lack of identifiable lignin pyrolysates. Lignin, being insoluble, may not be as amenable to SPM conditions as are the lower molecular weight phenolics.

A number of methylated products of simple phenolic acids were identified. Among them, 3,4-dimethoxy benzoic acid methyl ester, **55,** is a product of the methylation of protocatechuic acid or vanillic acid. Product 3-(4-Methoxyphenyl)-2-propenoic acid, methyl ester, **60,** is from coumaric acid. The corresponding 3-(3,4-dimethoxyphenyl)-propenoic acid, methyl ester, **63,** is from either caffeic acid or ferulic acid (Table 1.1). As will be noted, these methylated phenolic acids have also been observed in the pyrograms of other leaves examined.

All SPM pyrolysis products of catechin and condensed tannin extract of kalmia leaves (**47, 51, 58, 59, 76, 77**) were identified in the pyrogram of the intact kalmia leaf. Hydrolysable tannins are absent in kalmia leaves. This has been proven by Dr.Preston's unpublished NMR results (118). Hydrolysable tannins usually occur in higher plants (35).

There is a significant amount of fatty acids in kalmia leaves. Numerous methylated fatty acid derivatives have been identified, among them are lauric acid (C12), **54**, myristic acid (C14), **62**, palmitic acid (C16), **64**, stearic acid (C18), **68**, arachidic acid (C20), **73**, and behenic acid (C22), **74**. As a primary metabolite of the acetate-malonate pathway (Section 1.1.3.), palmitic acid presented an abundant peak in all the pyrograms of leaves investigated. In addition, two small peaks, **69** and **67**, with molecular weights 294 and 296 respectively, are methylation products of the

corresponding unsaturated fatty acids, linoleic and oleic acid. The spectra of these methylated derivatives of fatty acids have well-defined parent ions.

The fragment ion of m/z 74, produced by γ-hydrogen transfer via a McLafferty rearrangement, is characteristic of the spectra of methyl ester derivatives of fatty acids bearing no C-2 substituent. This ion usually carries a substantial fraction of the total ion current and can thus be used for the detection of low levels of fatty acid methyl esters in such a series by selected ion mode. Mass spectra of straight chain esters all exhibit the same basic pattern, differing only in certain mass values associated with chain length (116, 119). Figure 3.14 is the extracted ion chromatogram of kalmia leaves for m/z 74, indicative of peaks derived from fatty acids.

The methylation of carbohydrates is usually very complicated, producing a large number of methylated isomers from a large range of sugars which may be found in leaves (e.g., hexoses, pentoses, fructose etc.). Surprisingly, in the pyrogram of kalmia leaves as well as other leaf species, no chromatographic peak can be attributed to permethylated methylglucopyranoside, the major pyrolysis product of cellulose (section 3.3.2). In fact, another pyrolysis product, permethylated 1,6-anhydroglucopyranose, **49**, is observed. Phenolic glucosides and other O-glucosides are common in leaves, and the anhydrosugar **49** is likely produced upon pyrolysis of these. There are likely a number of other methylated sugar derivatives in the pyrogram, as yet unidentified. Mass spectra

of methylated carbohydrates are very similar, and generally, molecular ions are not recognizable in the EI mass spectra. The ions of highest abundance from methylated sugars, are usually those at m/z 101, 88, 75 and 45 (115). A number of pyrolysate peaks having these corresponding m/z ions have been found in kalmia leaves.

Several phenolic O-glycoside compounds can be detected in the SPM pyrograms of kalmia leaves. The major peak, **70** at 27.20 min has an almost identical mass spectrum (Figure 3.16 a) as that of permethylated phenyl glucosides, but the retention times are different, permethylated phenyl-glucosides eluting several minutes earlier. No pyrolysate observed could be assigned to methylated arbutin, although its retention time is very close to the unknown peak, **70**. Their mass spectra are similar and the main difference noted is the variation in the ratio of abundance of fragment ions m/z 187 and 219. For peak **70**, the most abundant fragment ion is m/e 187 while in arbutin m/z 219 is most abundant. Since the permethylated phenolic glucosides are not very stable under both CI and EI conditions, no molecular weight information is available. The mass fragment at m/z 219 is the result of loss of C-1 aglycone. It is very low in the mass spectrum of permethylated methyl glucoside, but significantly increased in the phenyl O-glycosides (120). Thus m/z 219 can be used as the characteristic peak of O-phenyl glycosides-type compounds present in leaves. The extracted ion chromatogram of m/z 219 of kalmia leaves is shown in Figure 3.15.

Another large and unknown peak, **71**, eluting at 29.99 min, shows a molecular weight of 330 by CI-MS. This peak is very dominant in the pyrogram of the extract residue of kalmia leaves and may be attributed to a pyrolysate of unextractable lignin. The mass spectrum of **71** is shown in Figure 3.16 b.

Permethylated phloretin, **75**, with the molecular weight 330, and its major fragment benzene propanoic acid, 4-methoxy methyl ester, **53**, were identified in the SPM pyrogram of kalmia. These two products were absent in the pyrograms of balsam fir, oak and white pine leaves.

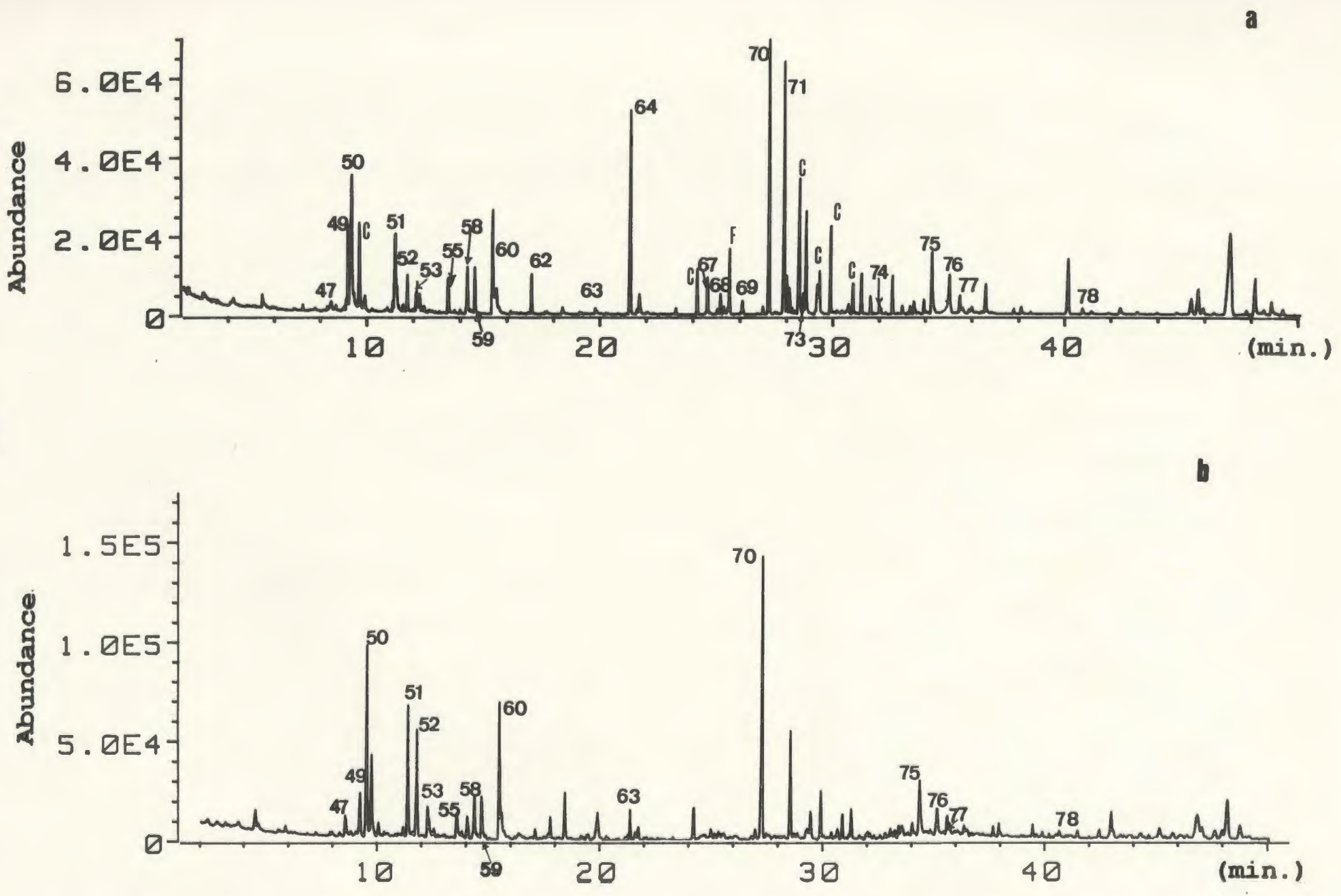


Figure 3.13 TIC Pyrogram (SPM) of (a) Kalmia Leaves and (b) Methanol-water Extract of Kalmia Leaves (Second Year)

C: carbohydrate origin F: fatty acid origin

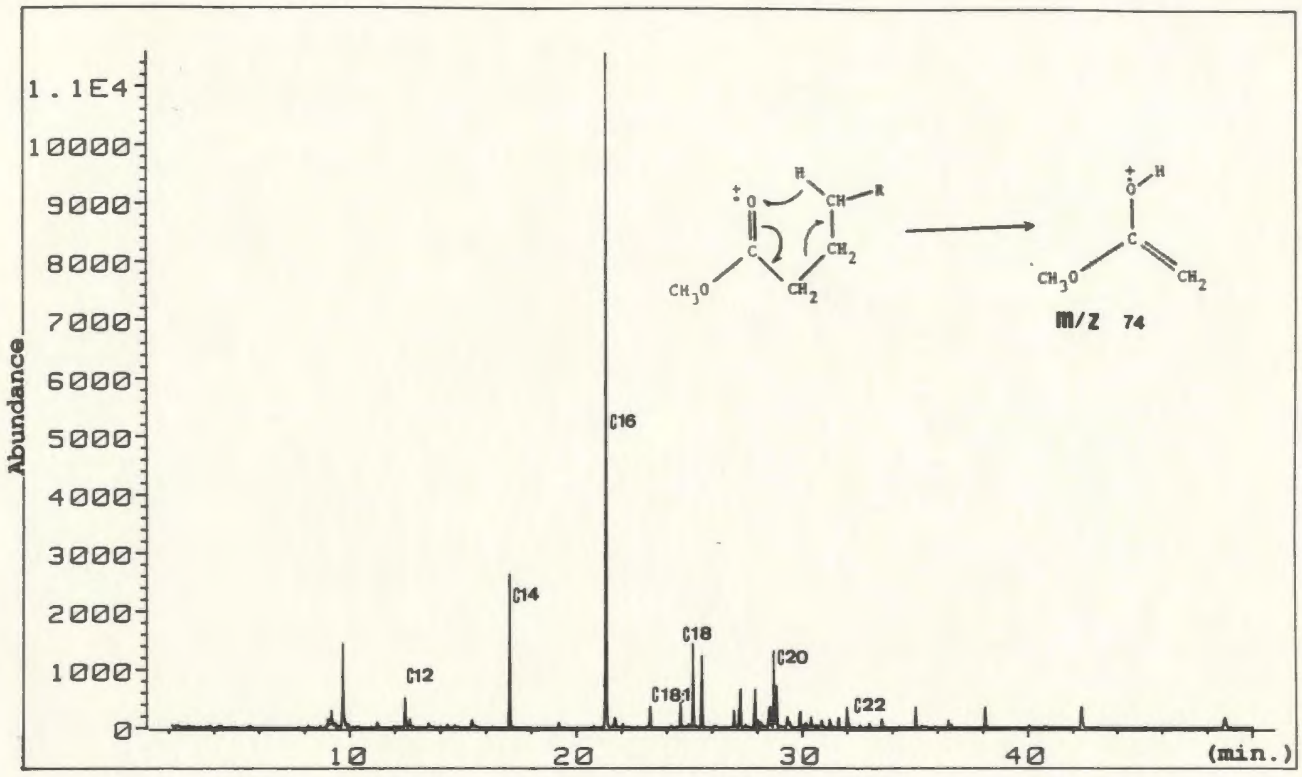


Figure 3.14 Extracted Ion Chromatogram of m/z 74 of Kalmia Leaf Pyrogram (SPM)

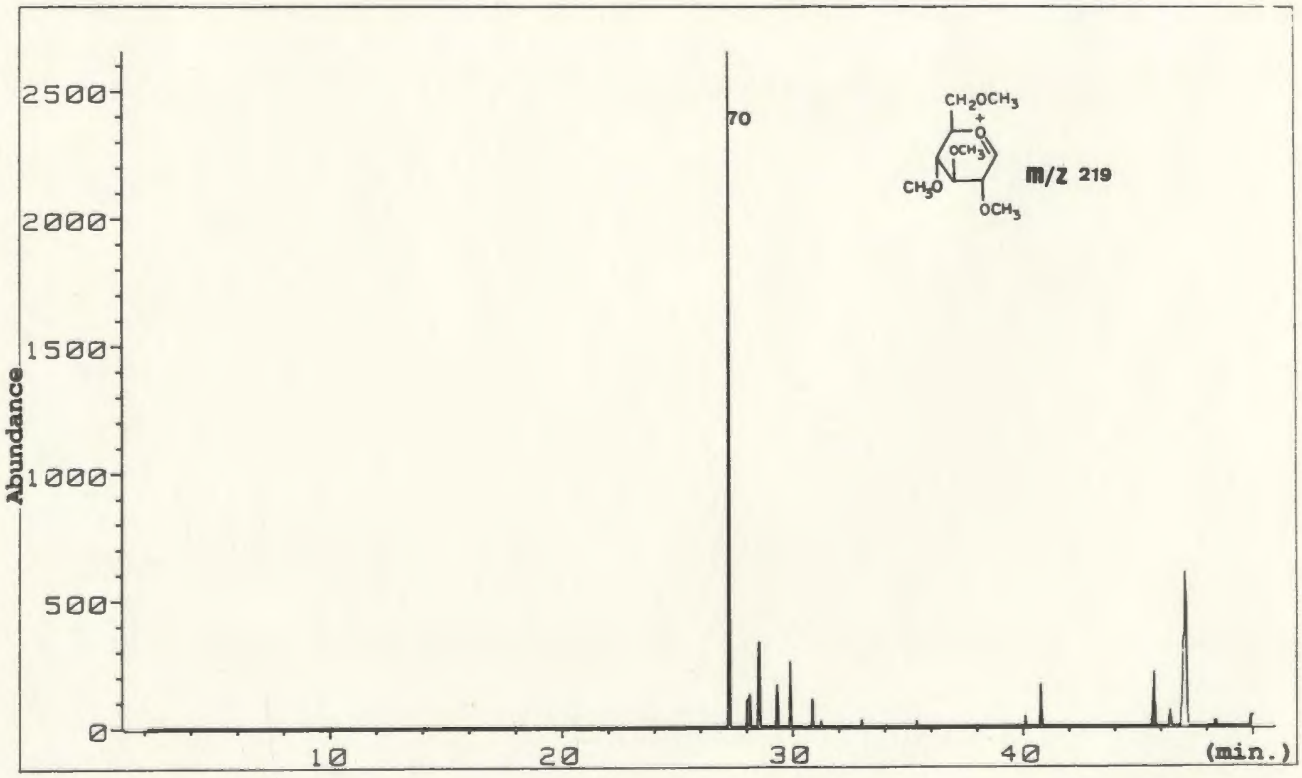


Figure 3.15 Extracted Ion Chromatogram of m/z 219 of Kalmia Leaf Pyrogram (SPM)

3.3.4. Pyrolysis of Balsam Fir Leaves

The SPM pyrogram of balsam fir leaves is shown in Figure 3.17 and the identification of pyrolysis products which are numbered is listed in Table 3.3. The pyrogram of balsam fir leaves is similar to other leaves having common pyrolysis products. The distinctive features of SPM pyrolysis which are different in balsam fir are as follows; the pyrolysate corresponding to hydrolysable tannins, **61**, and condensed tannins, **50, 51, 58, 59** are at very low abundance; an abundant peak, **72**, with the molecular ion at m/z 340 (measured by CI), has not been identified (mass spectrum is given in Figure 3.16 c). Peaks having fatty acid-like spectra are abundant, the major one being **65**; many seem not to be strait chain saturated fatty acids. Authenticated compounds are needed for unambiguous identification. Peak **66** with molecular weight 284, is attributed to C17 fatty acid, odd carbon number fatty acids being unusual in plant leaves. Numerous peaks having sugar-like mass spectra were observed between RT 9-13 min.

3.3.5. Pyrolysis of Oak Leaves

The SPM pyrogram of oak leaves is given in Figure 3.18 and peak identification is listed in Table 3.3. The methylated pyrolysis products from phenolic acids and from condensed tannins are low in abundance. The very abundant product, 3, 4, 5-

trimethoxybenzoic acid methyl ester, **61**, indicates a high content of hydrolysable tannins in oak leaves (105). Most tannins in oak leaves are in the hydrolysable tannin class, while condensed tannins only constitute a small percentage of total tannins. Intact kaempferol, **78**, and quercetin derivatives, **79**, are identified in oak leaves, but in small quantity. The molecular ions of the pyrolysis products **78** and **79**, m/z 342 and 358, correspond to permethylated kempferol and partially methylated quercetin, respectively. Using standards, it was found that a large portion of these two flavanols fragmented to smaller compounds.

Like kalmia leaves, SPM of oak leaves also produce the unknown product, **71**, with molecular weight 330 (measured by CI) in large yield.

3.3.6. The Pyrolysis of White Pine Needles

The SPM pyrogram of white pine needles is shown in Figure 3.19. Although many of pyrolysis products are similar to those obtained from balsam fir, the yields are much greater. Similar to the leaves of balsam fir , white pine needles contain only small amounts of hydrolysable and condensed tannins. An abundant product, 3-methoxy benzoic acid methyl ester, **46**, is present and is believed to be derived from shikimic acid (section 3.3.2.). The pyrogram has another noticeably large peak, **55**, identified as 3,4-dimethoxy benzoic acid methyl ester, a common pyrolysis product of leaves. From

retention time 24 min to 30 min, a unusual group of compounds having similar fatty acid-like spectra were observed, but were not identified. The unknown peak, **72**, identified in balsam fir leaves, is also present in the pyrogram of white pine needles. As in the case of balsam fir leaves, white pine needles gave a number of pyrolysis products with sugar-like mass spectra in the range of RT 9 min to 13 min.

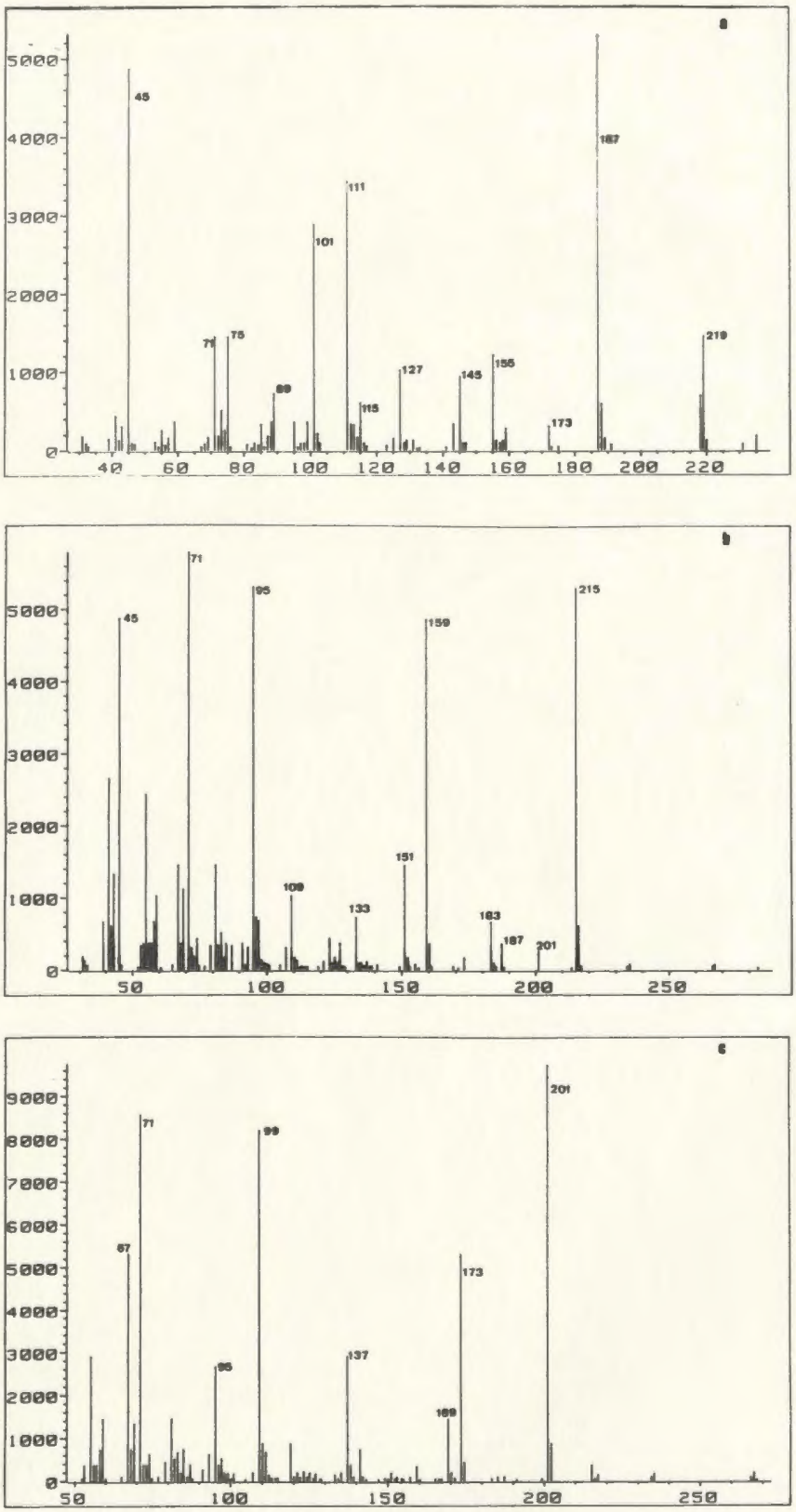


Figure 3.16 Mass Spectra (EI) of Unidentified Peaks, (a) **70**, (b) **71** and (c) **72** Observed in Leaf Pyrograms (SPM)

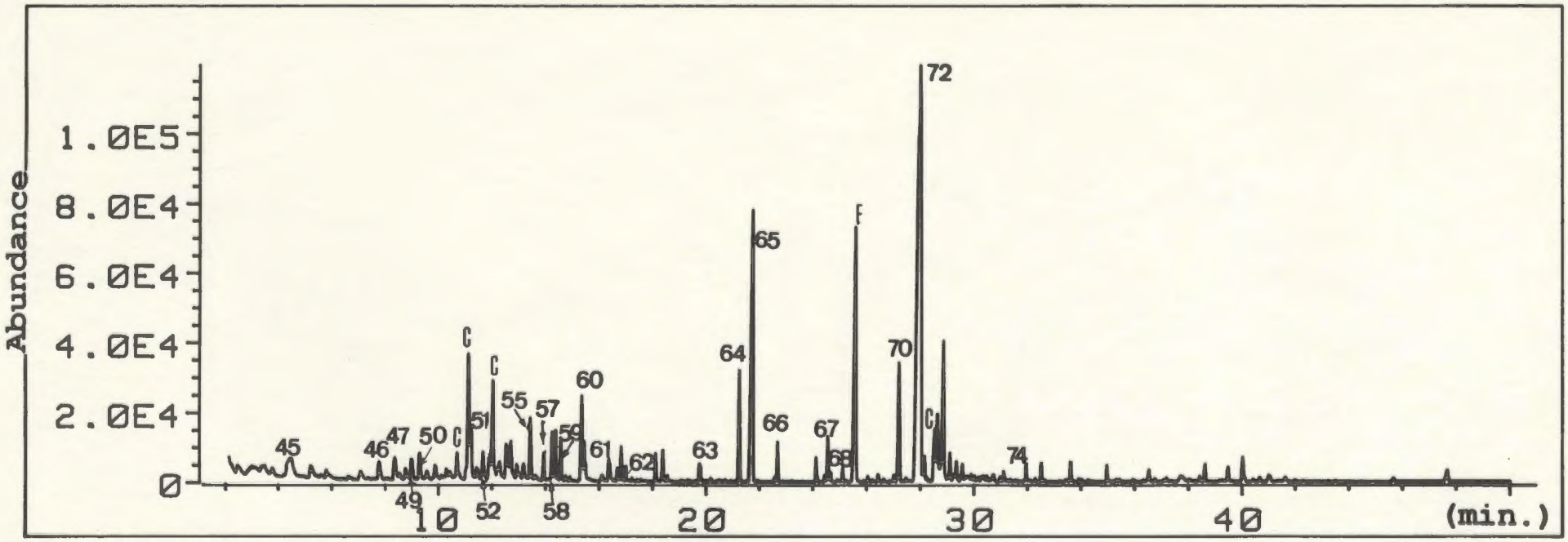


Figure 3.17 TIC Pyrogram (SPM) of Balsam Fir Leaves

C: carbohydrate origin F: fatty acid origin

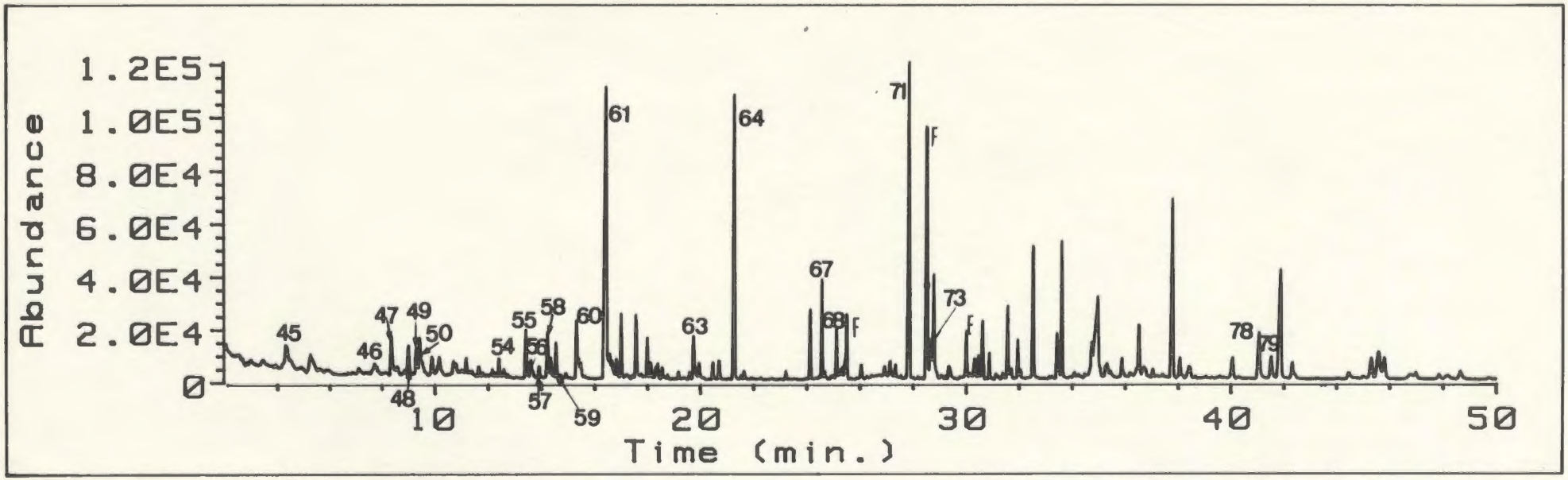


Figure 3.18 TIC Pyrogram (SPM) of Oak Leaves

C: carbohydrate origin F: fatty acid origin

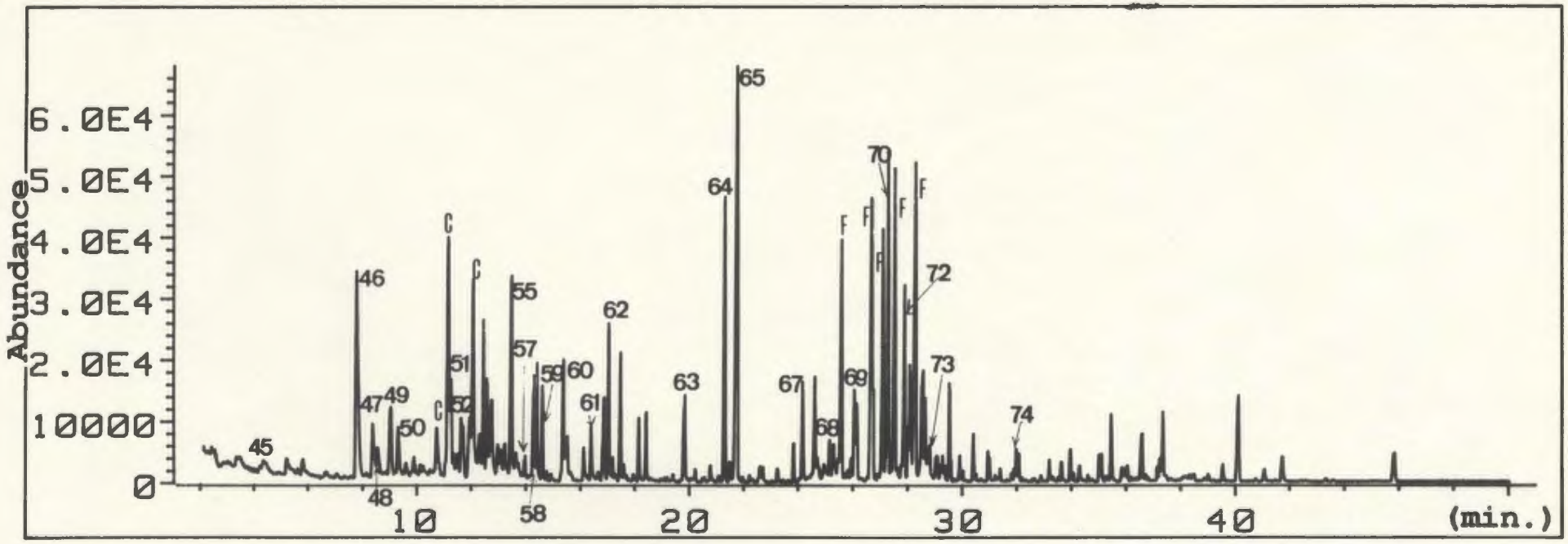


Figure 3.19 TIC Pyrogram (SPM) of White Pine Needles

C: carbohydrate origin F: fatty acid origin

Table 3.3: Identification of Pyrolysis Products in Pyrograms (SPM) of Leaves

No.	Identification[a]	Reference[b]	Mass	RT	Leaf species[c]				origin[d]
					Kalmia	balsam fir	white pine	oak	
45	1,4-dimethoxybenzene	(109)	138	4.39	N	Y	Y	Y	P
46	3-methoxybenzoic acid methyl ester	(109),S	166	7.80	N	Y	Y	N	P
47	1,2,3-trimethoxybenzene	(109)	168	8.31	Y	Y	Y	Y	P
48	4-methoxybenzoic acid, methyl ester	(109),S	166	8.68	N	Y	Y	Y	P
49	permethylated 1,6-anhydroglucopyranose	121	232	9.09	Y	Y	Y	Y	C
50	1,3,5-trimethoxybenzene (t1)	(109),S	168	9.82	Y	Y	Y	Y	T
51	t2		182	11.47	Y	Y	Y	Y	T
52	methylated fructose derivative	S	?	11.74	Y	Y	Y	Y	C
53	4-methoxybenzenepropanoic acid, methyl ester	(109),S	194	11.90	Y	N	N	N	P
54	methyl laurate	(116,119)	214	12.44	Y	N	Y	Y	F
55	3,4-dimethoxybenzoic acid methyl ester	(109)	196	13.46	Y	Y	Y	Y	P

56	3,4,5-trimethoxybenzaldehyde	(109)	196	13.58	N	N	N	Y	P
57	quinic acid ester	S	216	14.03	N	Y	Y	Y	P
58	t3		194	14.61	Y	Y	Y	Y	T
59	t4		194	15.20	Y	Y	Y	Y	T
60	2-propenoic acid,3-(4-methoxyphenyl)-methyl ester	(109),S	192	15.75	Y	Y	Y	Y	P
61	3,4,5- trimethoxybenzoic acid methyl ester	(109)	226	16.31	N	Y	Y	Y	T
62	methyl myristate	(116,119)	242	17.04	Y	Y	Y	Y	F
63	2-propenoic acid, 3-(3,4-dimethoxyphenyl)-methyl ester	(109),S	222	19.95	Y	Y	Y	Y	F
64	methyl palmitate	(116,119)	270	21.32	Y	Y	Y	Y	F
65	?		272	21.71	N	Y	N	Y	F
66	methyl margarate	(116,119)	284	22.72	N	Y	N	N	F
67	methyl oleate	(116,119)	296	24.61	Y	Y	Y	Y	F
68	methyl stearate	(116,119)	298	25.17	Y	Y	Y	Y	F
69	methyl linoleate	(116,119)	294	26.09	Y	N	Y	Y	F
70	a phenolic glycoside?	(115)	?	27.20	Y	Y	Y	N	C
71	?		330	27.91	Y	N	N	Y	L?
72	?		340	27.96	N	Y	Y	N	?

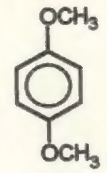
73	methyl arachidate	(116,119)	326	28.73	Y	N	Y	Y	F
74	methyl behenate	(116,119)	354	31.98	Y	Y	Y	N	F
75	methylated phloretin	S	330	34.34	Y	N	N	N	P
76	t5		?	35.10	Y	Y	Y	Y	T
77	t6		?	35.31	Y	Y	N	Y	T
78	methylated kaempferol	S	342	41.02	Y	N	N	Y	P
79	methylated quercetin	S	358	41.74	N	N	N	Y	P

[a] Structures of products listed on the next page

[b] S: peaks are identified by methylation of standard compounds

[c] Y: observed
N: not observed

[d] P: simple phenol and phenolic acid origin
T: tannin origin
C: carbohydrate origin
F: fatty acid origin
L: lignin origin



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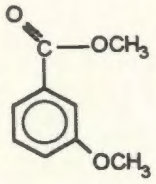

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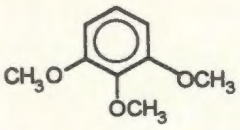

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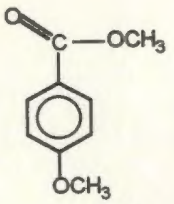

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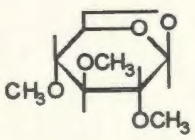

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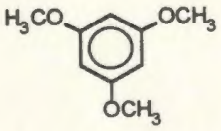

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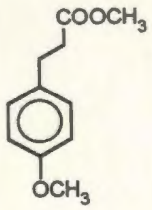
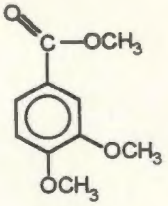


53

$C_{11}H_{23}COOCH_3$

54



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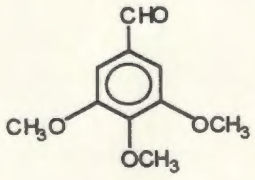

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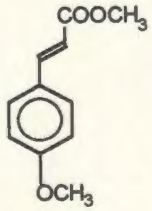

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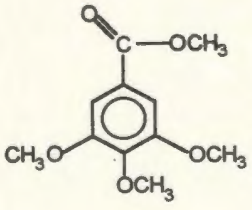
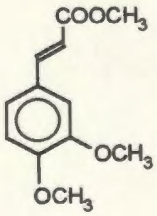


61

$C_{13}H_{27}COOCH_3$

62



63

$C_{15}H_{31}COOCH_3$

64

$C_{15}H_{33}COOCH_3$

66

$C_{17}H_{33}COOCH_3$

67

$C_{17}H_{35}COOCH_3$

68

$C_{17}H_{31}COOCH_3$

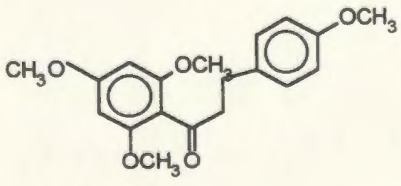
69

$C_{19}H_{39}COOCH_3$

73

$C_{21}H_{43}COOCH_3$

74



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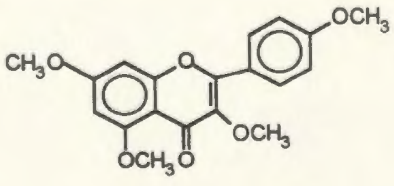

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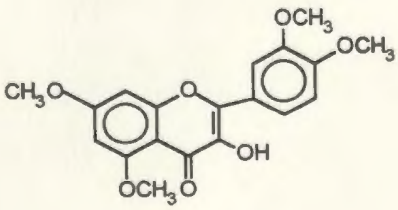

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CHAPTER 4

SELECTIVE STUDIES OF THE EFFECTS OF ENVIRONMENTAL STRESS ON THE CHEMICAL COMPOSITION OF PLANT LEAVES

4.1 Introduction

In the previous Chapter, PY-GC-MS has been shown to be a useful method to characterize various organic constituents of leaves. The pyrolysis products of leaves either by direct pyrolysis or by SPM give complementary information about the carbohydrates, simple phenolic acids, tannins and fatty acids in leaves. The analytical steps are straight forward and rapid. Optimum procedures of sample analysis have been established. Under strictly controlled conditions, PY-GC shows very good reproducibility.

Environmental change can cause multiple changes in concentration of organic constituents of leaves. PY-GC should have the advantage over conventional methods in its capability to measure a wider range of compositional changes in leaves at the same time since the whole leaf is analyzed. In addition, PY-GC doesn't require laborious sample pretreatment. The method is relatively cheap, and easy to operate. In contrast, traditional methods of composition measurement are more time-consuming and subject to many errors. Flame ionization (FID) and mass spectrometer detection are

equally sensitive for the measurement of pyrolysis products. However, FID is more reproducible and more common than mass spectrometric detection, therefore FID was chosen in studies involving the measurement of compositional changes. Two techniques, direct pyrolysis and SPM, were applied to application studies. Quantitative measurements involved calculating peak area of pyrolysis products divided by sample weights. these values were then used for calculating the differences in abundances of pyrolysates among pyrograms. The important assumption that yield of an individual product was proportionally related to mass of leaf sample pyrolysed was experiment ally proven for representative samples.

The applications of PY-GC in environmental studies described in this chapter are not systematically chosen but, rather, are field studies concerned with forestry-related issues in Atlantic Canada.

4.2. Application of Direct Pyrolysis Technique

4.2.1. The Effect of Forest Thinning on the Composition of Balsam Fir Leaves

The series of leaves from balsam fir trees subjected to thinning were supplied by Forestry Canada. An important measurement, the nitrogen content in balsam fir leaves, was determined by Dr.Brian Titus. It was found that the average nitrogen content in leaves from thinned balsam fir was higher than that in unthinned trees and

that nitrogen content also varied among the two sets of treatments (Table 4.1).

Initially two balsam fir leaf samples, one from unthinned stands and one from thinned stands were analyzed. Results from direct pyrolysis indicated that the relative amount of 1,6-anhydroglucopyranose, **31** in unthinned leaves is higher than that in thinned leaves. Further analysis of the sets of leaves showed a clear inverse relationship between the amount of nitrogen in the leaves and the relative amount of 1,6-anhydroglucopyranose pyrolysate (Table 4.1). Further replications of analysis (individual 0.5 mg samples from the same pooled tree leaves) showed that reproducibility of peak area was very satisfactory, where the coefficient of variation (C.V.) of triplicate results was less than 4%. It has shown that 1,6-anhydroglucopyranose is a distinctive product of glucose-containing carbohydrates. The results thus indicate that the concentration of glucose-containing carbohydrates in balsam fir leaves does change and this relative change can be measured by PY-GC. The reason for the adaptive change of balsam fir (thinned versus unthinned) in terms of carbohydrate content is discussed in section 4.4.

Table 4.1 Relative Yield of 1,6-Anhydroglucopyranose Pyrolysate of Leaves from Thinned and Unthinned Balsam Fir

Sample number	U-37	U-35	T-29	T-19	T-1	T-11	T-7
Sample type	unthinned	unthinned	thinned	thinned	thinned	thinned	thinned
N % [1]	0.66	0.83	0.99	1.2	1.4	1.6	1.9
Area/µg*	38.8	37.8	35.0	30.5	29.3	24.8	18.2

* Peak area of **31 was divided by the sample weight; triplicate analysis C.V. 3- 4%.**

[1] determined by the Analytical Services Laboratory, Newfoundland Region (129).

4.2.2. The Effect of Nitrogen Fertilization on the Composition of Kalmia Leaves

Finding chemical indicators in kalmia leaves that can reflect the fertility of soil is part of the Green Plan project in cooperation with Dr. Brian Titus, Forestry Canada.

Preliminary experiments involving the direct pyrolysis of kalmia leaves were carried out. Again 1,6-anhydroglucopyranose, **31**, showed differences in concentration as the result of the nutrient stress. The data in Table 4.2 shows that nutrient stress on kalmia plant increased 1,6-anhydroglucopyranose by one fold in current year leaves, and by 50% in second year leaves. The same magnitude of increase by nutrient stress was also demonstrated in the methanol-water extract of second year leaves, possibly

indicating that the differences in the content of glucose-containing compounds was represented in the water -extractable components of the leaf.

Table 4.2 Relative Yield of 1,6-Anhydroglucopyranose Pyrolysate of Leaves from Fertilized and Unfertilized Kalmia Plants

Kalmia leaf	Peak area of **31**/μg of sample*		
	Second year		Current year
	Whole leaf	CH_3OH-H_2O extract	Whole leaf
Fertilized	99.5	94.5	40.3
Unfertilized	159	158	93.8

* for triplicate analysis, C.V. 3-4%

4.3. Application of Simultaneous Pyrolysis Methylation Technique

4.3.1. The Effect of Nitrogen Fertilization on The Composition of Kalmia Leaves

In order to adequately examine the changes of other constituents such as tannins and phenolic acids in kalmia leaves, they were analyzed by the SPM method. The

pyrograms representing current year and second year kalmia leaves are displayed in Figure 4.1. It is quite evident that the SPM pyrogram of current year kalmia leaves is significantly different from that of second year leaves. Among the polyphenolic compounds, phloretin, **75**, a dihydrochalcone compound, occurs in both ages of kalmia leaves, but is surprisingly high in the current year leaves. As well, compound **57**, tentatively identified as methylated mono-dehydrated quinic acid is almost absent in the second year kalmia leaves, but present in current year leaves.

The changes of condensed tannin content as measured by tannin pyrolysates observed in the pyrograms are demonstrated in Figure 4.2. The relative amounts of condensed tannin fragments increased by at least one fold in current year leaves of unfertilized kalmia plants when compared to fertilized plants. The increase of condensed tannin fragments in second year leaves of unfertilized kalmia is much smaller, only about 10%.

In addition to the condensed tannin fragments, other pyrolysis products can reflect the effect of nutrient stress and composition change (Table 4.3 and Table 4.4). Among these compounds, methylated phloretin, **75**, appears to be a useful stress indicator. There is a significantly higher amount of **75** in unfertilized leaves than in fertilized leaves for both ages as measured by SPM.

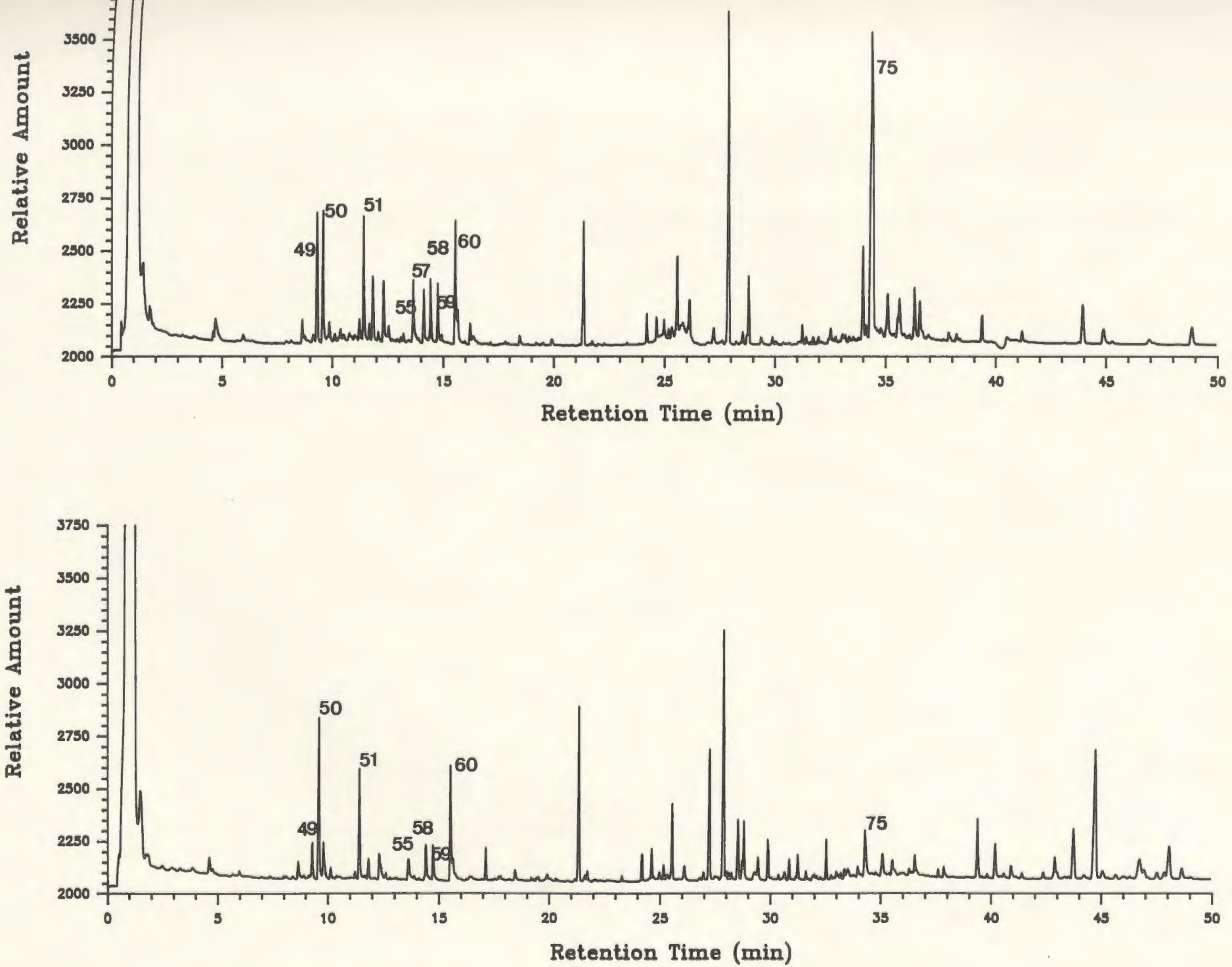

Figure 4.1 Comparison of Pyrograms (SPM) of Current and Second Year Kalmia Leaves

Another possible indicator, unsaturated C18 fatty acid methyl ester (methyl oleate), **68**, decreased under nutrient stress by 37% in current year leaves, and by 54% in second year leaves. The amount of permethylated 1,6-anhydroglucose, **49**, almost doubled in current year leaves of unfertilized versus fertilized plants. This change is similar to that of the 1,6-anhydroglucose indicator observed by direct pyrolysis. In the second year leaves, compound **49** was too small to measure any difference between samples.

Some unidentified chromatographic peaks also showed responses to nutrient stress. In current year leaves, the level of the peak at RT 11.96 min increased 66% when plants were subjected to nutrient stress. In second year nutrient stressed leaves, the levels of the peak at RT 31.42 min and the peak at RT 43.73 min increased 26% and 20% respectively, all due to nutrient stress. Further identification of these peaks requires authentic standards.

Table 4.3 Relative Yields of Key Pyrolysates (SPM) between Fertilized and Unfertilized Kalmia Leaves (Current year)

Pyrolysis Product[a]	**49**	C.V.[b]	**67**	C.V.[b]	**75**	C.V.[b]
Fertilized Area/μg	8.3	9.0%	14.1	10%	95.3	7.1%
Unfertilized Area/μg	81.7	3.4%	8.8	10%	276	9.0%

[a] **49**, permethylated 1,6-anhydrosugar; **67**, methyl oleate; **75**, methylated phloretin,

[b] Based on triplicate results

Table 4.4 Relative Yields of Key Pyrolysates (SPM) between Fertilized and Unfertilized Kalmia Leaves (Second year)

Pyrolysis product[a]	**67**	C.V.[b]	**75**	C.V.[b]
Fertilized Area/μg	20.0	1.5%	11.7	11%
Unfertilized Area/μg	11.4	7.5%	22.5	10%

[a] **67**, methyl Oleate; **75**, methylated phloretin

[b] Based on triplicate results

It should be mentioned that a number of simple phenolic acid derivatives, such as 3,4-dimethoxybenzoic methyl ester, **55**, and 4-methoxyphenyl-2-propenoic acid methyl ester, **60**, did not show changes in concentration in response to nutrient stress. Further study is required on other plants to examine whether simple phenolic acids can be excluded as nutrient stress indicators. It will be shown in the next section that phenolic acids can play a role in indicating stress of an atmospheric pollutant, ozone.

A discussion of why and how nutrient stress can affect the composition of leaf components is given in section 4.4.

The linear dynamic range was determined for methylated phloretin and condensed tannin pyrolysates in current year leaves of unfertilized kalmia plants (Figure 4.3). Good linearity was obtained when the sample weights were less than 500 μg.

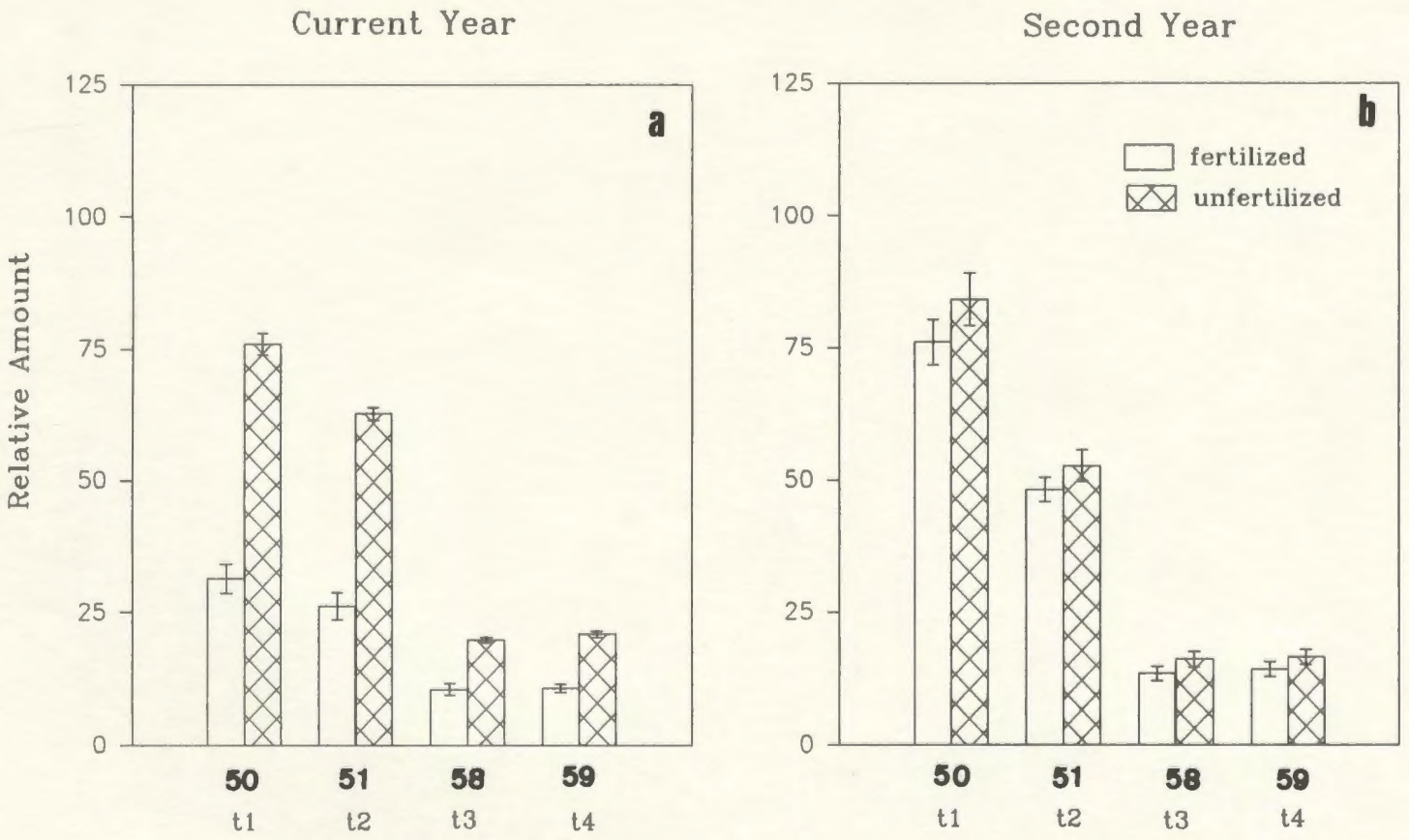

Figure 4.2 Relative Amount of Condensed Tannin Pyrolysates (SPM) from Fertilized and Unfertilized Kalmia Leaves

(a) Current Year and (b) Second Year

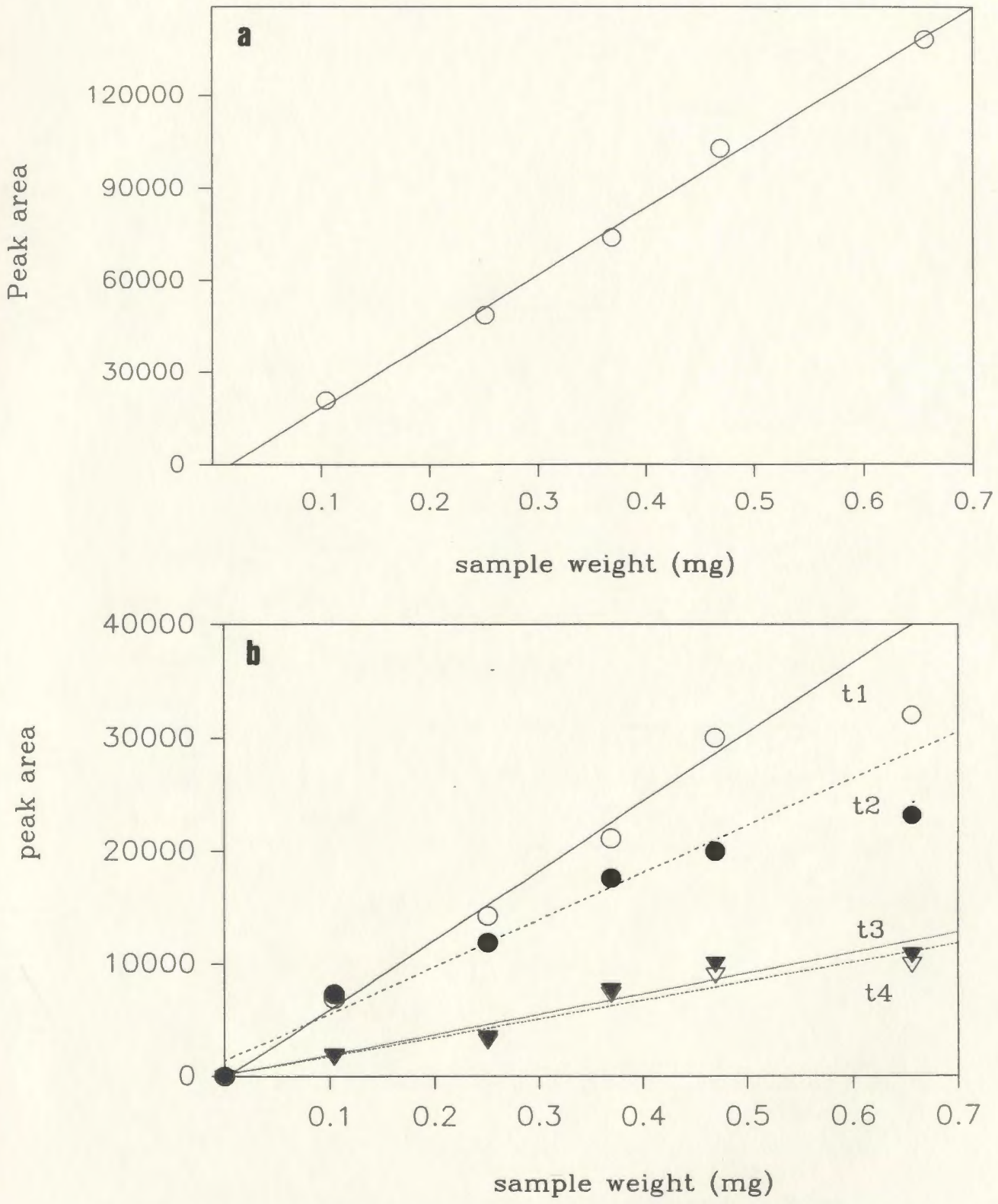

Figure 4.3 Linear Dynamic Range for Yields of (a) Phloretin and (b) Condensed Tannin Pyrolysates from Unfertilized Kalmia Leaves (Current Year)

4.3.2. The Effect of Ozone Fumigation on the Composition of White Pine Needles

In collaboration with Dr. Roger Cox, Forestry Canada, the effect of ozone fumigation on the compositional changes in white pine needles was investigated by the SPM method.

Both second year and current year white pine needles were analyzed. A comparison of the pyrograms of current year and second year white pine needles showed that they differ only in concentration, not in the nature of pyrolysis products (Figure 4.4). Notably, the concentrations of 3-methoxy benzoic acid methyl ester, **46**, and 3,4-methoxy benzoic acid methyl ester, **55**, in the current year needles are at least twice as what they are in the second year needles. Compound **46** has been found to be the major SPM product of shikimic acid (section 3.3.2.). Compound **55** can be produced either from methylated protocatechuic acid or vanillic acid present in the leaf.

It was very interesting to discover it that the relative amounts of these two compounds in the second year white pine needle pyrograms decreased as the ozone dosage on the tree leaves increased. Table 4.5 and Figure 4.4 show an inverse relationship between ozone dosage fumigated on the needles and the relative amounts of **46** and **55**. This result is only from one set of experiments, that of the leaf sample

of tree 4. Similar trends were observed from other trees subjected to the same fumigation treatment. Compared with the clear difference in the pyrograms of second year needles with ozone treatment, virtually no difference was observed among that of control samples and current year needles fumigated with ozone. This result therefore suggests that current year needles may have some resistance to ozone fumigation. A further discussion on this topic can be found in section 4.4.

A linear dynamic range was obtained when sample weights were between 130 μg to 350 μg of white pine leaf samples for the two pyrolysis product indicators, **46** and **55** (Figure 4.6).

The white pine needles from trees grown outside fumigating chambers were also analyzed. The pyrogram is comparable to that obtained from chamber 5 (lowest ozone dosage). Therefore, the use of a chamber did not show any effects on the compounds investigated.

Tannin concentrations and their resulting pyrolysis products were too low in white pine needles to measure. If required, extraction of tannins would have to be done in order to determine their concentration changes in response to ozone fumigation or other environment factors.

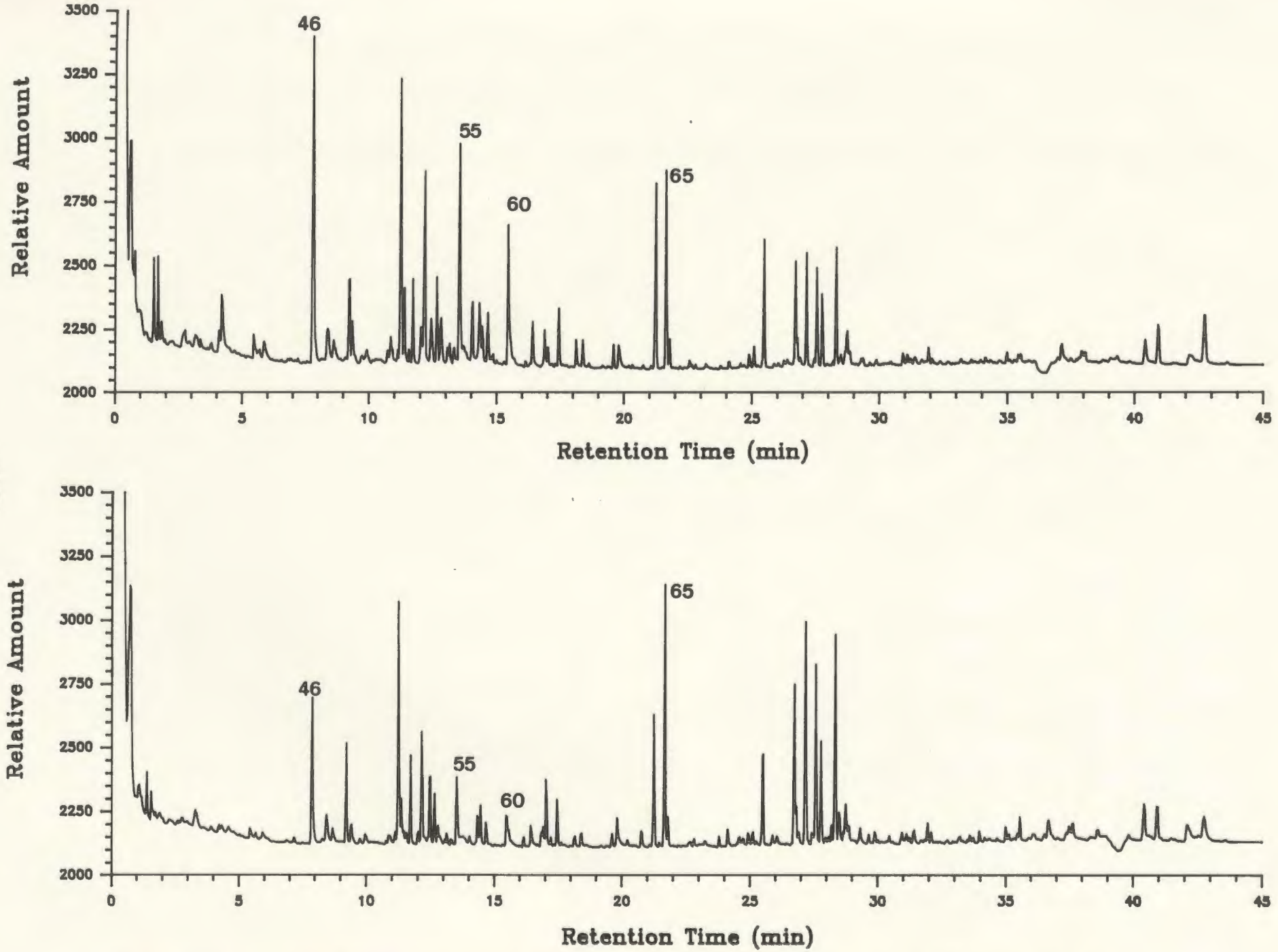

Figure 4.4 Comparison Between Pyrograms (SPM) Between Current and Second Year White Pine Needles

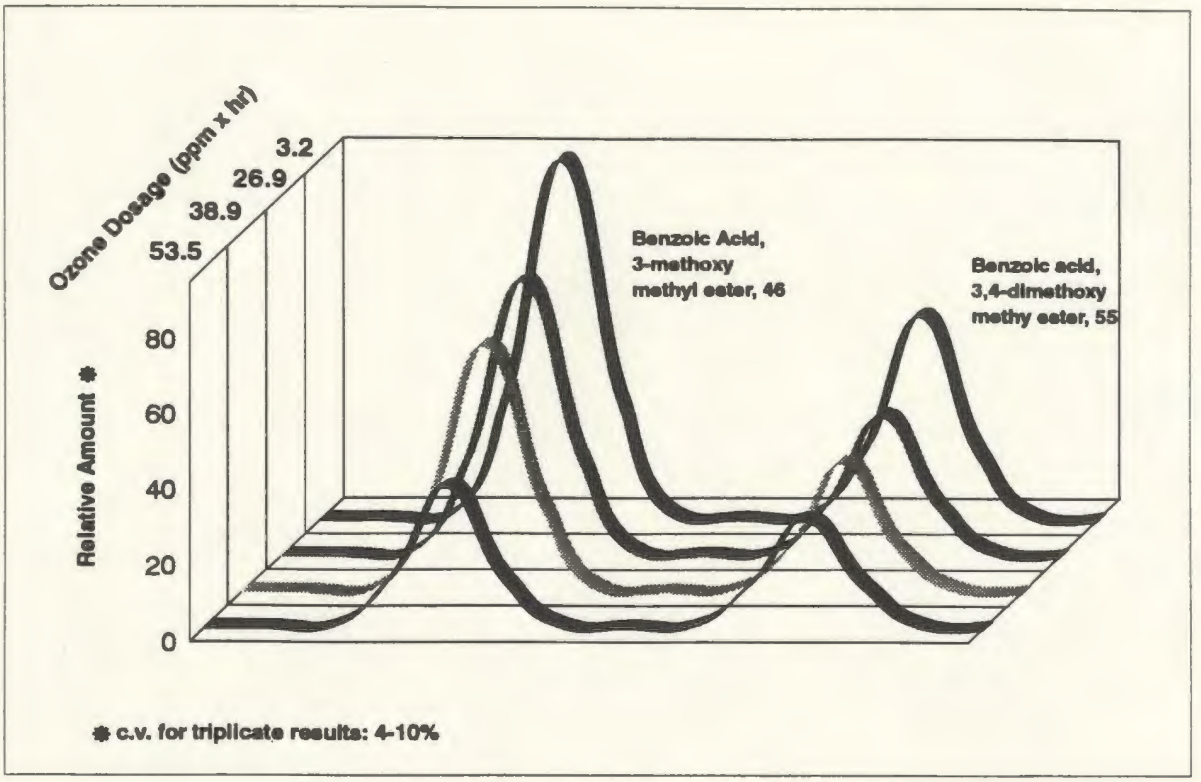

Figure 4.5 Relative Yields With Respect to Pyrolysates **46** and **55** from White Pine Needles (Second Year,Tree 4) Fumigated by Ozone

Table 4.5 Relative Yields of Key Pyrolysates from White Pine Needles (Second Year, Tree 4) Fumigated by Ozone

No.	Chamber 5	Chamber 1	Chamber 2	Chamber 3
Ozone dosage (ppmhr)	3.20	26.9	38.9	53.5
Peak area of **46**/ μg of sample)	95.2	72.8	65.2	37.1
C.V.*	8.4%	6.0%	9.8%	9.7%
Peak area of **55**/μg of sample	54.2	36.9	34.1	28.3
C.V.*	3.7%	9.7%	4.7%	7.9%

* Based on triplicate analysis

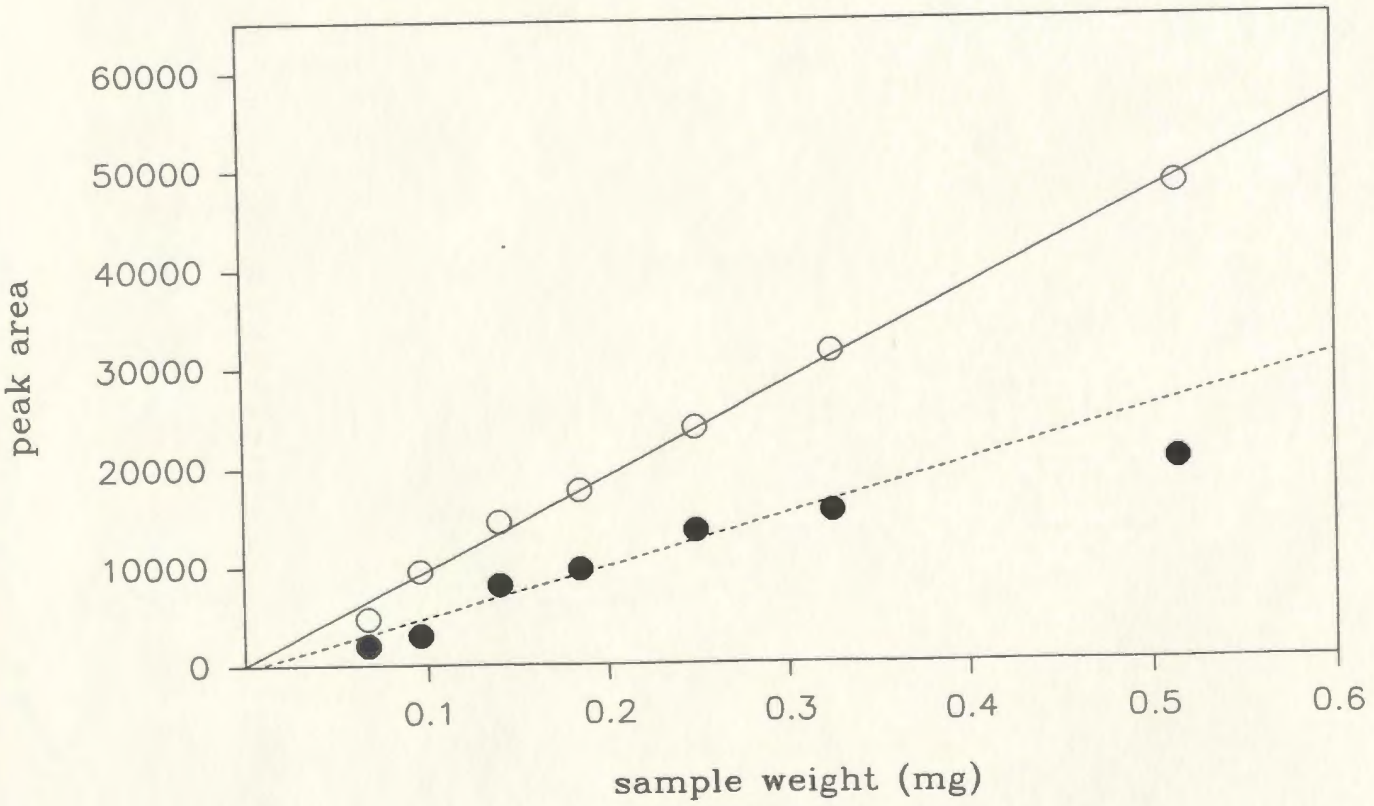

Figure 4.6 Linear Dynamic Range for Yields of Pyrolysis Products, 3-Methoxy Benzoic Acid Methyl Ester, **46** (○) , and 3,4-Dimethoxy Benzoic Methyl Ester, **55**, (●) from Second Year White Pine Needles

In an initial attempt to minimize error from variations in sample weight, and/or pyrolysis yields, an internal standard, 2-hydroxybenzoic acid, was added into the samples before they were pyrolysed. However, the amount of leaf sample and internal standard was too small to handle without causing additive errors. Further efforts were focused on searching for a pyrolysis product in the pyrogram of white pine needles which did not change in peak intensity among the various leaf samples. Based on this criteria, pyrolysate **65** was selected as an internal reference compound. The area ratios of "indicator" compound **46** and compound **55** to compound **65** were calculated (Table 4.5). It was found that the values of these ratios have similar relationships with ozone dosage as did the peak areas/μg (Table 4.6). But reproducibility was much improved. The average C.V. of triplicate analysis was reduced to 3.6% from 7.5%.

Table 4.6 RelativeYields of Key Pyrolysates in White Pine Needles (second year, tree 4) Fumigated by Ozone. Use of an Internal Pyrolysate Standard

	Chamber 5	Chamber 1	Chamber 2	Chamber 3
Ozone dosage (ppmhr)	3.2	26.9	38.9	53.5
Peak area ratio **46/65**	0.612	0.558	0.462	0.281
C.V.*	2.3%	5.5%	3.7%	3.5%
Peak area ratio **55/65**	0.294	0.241	0.235	0.170
C.V.*	2.2%	4.1%	1.1%	6.6%

* Based on triplicate analysis

4.4 Discussion

4.4.1. Kalmia Plants and Fertilization

Kalmia plants can affect forest restoration by competing effectively for nutrients, and the "Kalmia Problem" has drawn much attention in Atlantic Canada and Forestry Canada. *Kalmia angustifolia* (Ericaceae) usually co-exists with spruce and other commercially important trees. This shrub plant flourishes after land is burnt or clear cut and can become the dominant species, particularly in nutrient-poor heathland. Once this plant is established, it is very difficult to eradicate (122). It has been shown that kalmia can impede spruce seed germination and can check the growth of spruce. Several studies have pointed to kalmia's secondary metabolites for this inhibitory effect. First, toxic compounds such as the procyanidins, catechin and epicatechin have been found in very high concentration in ericaceous plants (97, 98). Secondly, allopathy, soil acidification and changes in humus decomposition all occur around kalmia dominant locations, due to the release of secondary metabolites from kalmia leaf litter (123-125). However, it has been noted that spruce can grow adequately among kalmia on nutrient-rich sites, although poorly on nutrient-poor sites. It was therefore hypothesized that kalmia might produce more secondary metabolites in nutrient-stressed conditions as a means of dominating the growth site.

The above hypothesis concerning nutrient conditions and the content of secondary

metabolites have been supported in the present study. Without fertilization, kalmia leaves were found to contain higher amounts of condensed tannins and dihydrochalcone compounds (e.g. phloretin) than when fertilized (Figure 4.2 , Table 4.3 and 4.4.). This is supported by other studies, which showed that increases of tannins and total phenolics was the result of nutrient stress in several other plants (30, 31, 33, 36). In kalmia leaves, dihydrochalcone compounds (e.g. phloretin) appears to be especially sensitive to nutrient stress. The dihydrochalcone compounds react with proteins, and thus can interact with enzymes and interfere with the biological processes in plant cells (99). It has also been shown that phloretin is a potent inhibitor of plant growth (114). Besides, both phloretin and its glycoside (phloridzin) have exhibited a high deterrence to herbivores (126). Like phloretin, tannins also play a very important role in plant defense system. Therefore, the high contents of condensed tannins and dihydrochalcone compounds in kalmia leaves as observed in this study may contribute to the inhibitory effects of kalmia on the growth of black spruce. The increase of these compounds in a nutrient-stressed environment may help kalmia plants compete with other plants and create a better defense against herbivores.

Also observed in this study is an increase in carbohydrate content in kalmia leaves under nutrient-stressed conditions (Table 4.2), concomitant with the increase of tannin and phloretin content (Table 4.2). Changes in carbohydrate content in response to nutrient conditions have also been reported in other studies. Lower amount of

carbohydrates due to nitrogen fertilization was shown in tobacco leaves (32). Potassium fertilization can also lead to the decrease of both reducing and non-reducing sugars in chili pepper leaves (34). Although the direct pyrolysis used for carbohydrate analysis in this study does not measure the concentrations of individual carbohydrate groups (e.g. starch, glycoside, cellulose), this method provides a rapid, economic, and simple means of assessing changes in carbohydrate content in plant leaves.

An explanation of the observed relationship between nutrient stress and the increase of tannins, phloretin and carbohydrates is provided by Bryant's carbon/nutrient balance theory (127). Under nutrient-stressed conditions, nutrients containing N, P, and K in plant leaves can be limiting, while carbon is readily available through photosynthesis. Although a reduction in nutrient concentration in soil reduces photosynthetic rates, nutrient stress affects growth much more strongly so that carbohydrates and carbon-based secondary metabolites accumulate in the leaf. Since young leaves are more vulnerable to herbivores than old leaves, they have adapted to be more sensitive to nutrient stress by producing more secondary metabolites for self-defense than old leaves under nutrient-stressed conditions. This explains why more dramatic changes in secondary metabolites were observed in current year kalmia leaves than in second year leaves.

The relationship between nutrient availability and of secondary metabolites such

as tannins and phloretin has important implications in forest restoration. This study has provided a basis for using fertilization as a useful means of forest restoration. Economic constraints usually require that specific sites of suitable soil quality be chosen for maximal benefit. Since the site soil quality is mainly determined by nutrient content, it can be estimated by measuring the amount of tannins and phloretin in kalmia growing on the site.

4.4.2 Thinning of Tree Stands and Balsam Fir

Preliminary experiments related to nutrient availability have also been done on the leaves of balsam fir trees under a specific environmental change, thinning. Balsam fir trees from unthinned stands are thought to be subjected to nutrient stress and to a less extent water stress due to high competition among trees. The term for this condition is that they are limited by "source availability" (128). Hormer found that leaves from trees in dense, closed-canopy stands had significantly lower nitrogen, astringency, and lignin contents and higher cellulose content than that in the open canopy stands. In that study, the density of balsam fir stems in close canopy stands is about twice as high as in the open canopy stand (128). As well, significantly higher concentrations of minerals and crude fats occurred in balsam fir leaves from thinned compared to unthinned stands (129). Our results showed a decrease of carbohydrate contents as a result of thinning. As in the case of kalmia leaves, this observation can also be explained by the carbon-

nutrient balance theory. The increase of source availability in thinned stands leads to the increase of plant growth, which in turn consumes more carbon, thus causing the decrease in carbohydrates in the leaves.

4.4.3. The Effect of Ozone Fumigation on White Pine Needles

An inverse relationship between ozone dosage fumigation and the amount of shikimic acid and phenolic acid compounds in second year white pine needles has been demonstrated in this study (Fig 4.4, Table 4.5). This result indicates that the shikimic acid pathway has been affected by ozone fumigation. The shikimic acid pathway is a basic and important process in higher plants, because it is the source of aromatic amino acids and numerous secondary metabolites (3) (Figure 4.7). Consequently, disruption of the shikimic acid pathway may lead to physiological disorders in plants.

Shikimic acid and phenolic acid compounds may have decreased as a result of decreased vigour in ozone-stressed white pine needles of second year growth. Ozone caused visible damage in second year white pine needles. Visible injury and the decrease in productivity have been reported in reference to air pollution in a number of studies of other plants (15). Loehle proposed that the allocation of carbon to defensive compounds in the leaves would be reduced as a result of decreased vigour in pollution-stressed trees (117). It has been reported that Japanese Cedar polluted by

sulphur dioxide contains reduced levels of glucose, shikimic acid, and total phenolics (27, 28). This result was attributed to the inhibitory effect of sulphur dioxide on photosynthetic activity. It is highly possible that ozone, a major air-pollutant, can also reduce the amount of secondary metabolites for self-defense by decreasing plants' vigour and photosynthetic activity.

Alternatively, ozone may affect shikimic acid and other metabolic pathways in plant by damaging plant cell membranes. It is known that ozone oxidizes the unsaturated lipids and the sulfhydryl groups of protein in plant cell membranes (19). Under physiological conditions ozone is converted into hydroxyl radicals, which attack other metabolites and enzymes inside the cells, thus changing the organic composition and metabolism of plants (15). To counteract the damaging effect of ozone, plant cells can motivate production of antioxidants such as vitamin E (α-tocopherol) to consume ozone (92). In general, phenolic compounds can also act as antioxidants (130, 131). Shikimic acid and protocatechuic acid both have reducing ability (132). They might also be consumed as antioxidants in reaction with ozone. The chemical nature of their oxidized products have not been investigated in the present study.

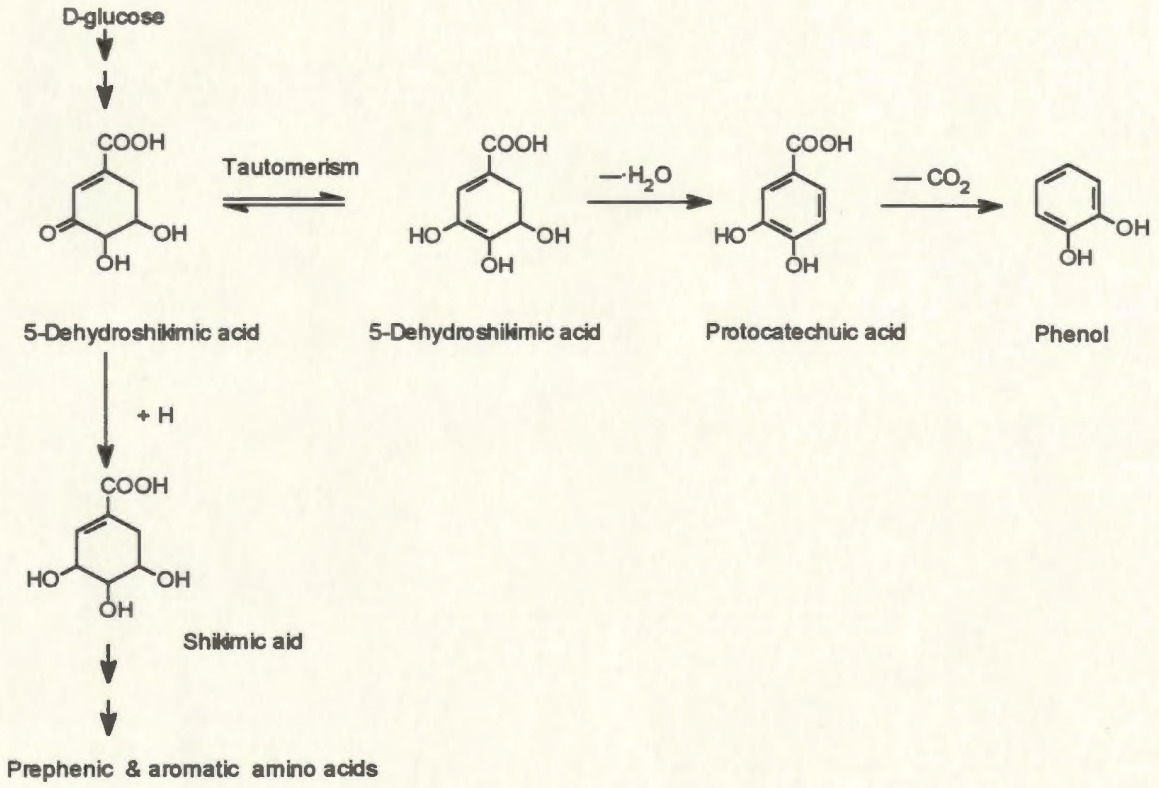

Figure 4.7 Shikimic Pathway

CHAPTER 5

CONCLUSIONS

Analytical pyrolysis has been demonstrated in this study to be not only a useful tool for screening organic constituents in plant leaves, but also a very promising technique for ecological studies. Two complementary pyrolysis techniques were examined; direct pyrolysis and simultaneous pyrolysis methylation. The two techniques can be used for characterizing leaves from different species of plants, and, for differentiating the age of the foliage from the same plant.

Direct pyrolysis resulted in the formation of small pyrolysis fragments from biopolymers such as lignin and carbohydrates, and from smaller molecules such as phenolic and fatty acids. Nevertheless, the substitution patterns of lignin monomers could be identified in the pyrolysis fragments. Moreover, the distinctive anhydrosugars were stable pyrolysis products and the major anhydrosugars formed from plant leaves, 1,6-anhydroglucopyranose showed good reproducibility in measurements (C.V. 5% or less). However, the direct pyrolysis of phenolic acids and tannin constituents of the leaf did not afford distinctive products. This is because some small phenolic pyrolysis fragments can be derived from both free phenolic compounds and from phenolic polymers such as tannins and lignin. It is thus difficult to relate such fragments to tannins or phenolic acids as important chemical markers in ecological studies.

The optimization and application of another technique, simultaneous pyrolysis methylation (SPM), were investigated in this study. Tetramethylammonium hydroxide (TMAH) has been proven to be a good methylating reagent for on-line pyrolysis methylation. The acidic constituents and pyrolysates of leaves were successfully methylated under pyrolysis temperature conditions. Larger and more polar products were observed in the pyrograms as their methylated derivatives. More importantly, carboxylic acids and phenolic acids did not suffer decarboxylation as was observed in the method of direct pyrolysis. A number of methylated derivatives of phenolic acids, polyphenolics and fatty acids present in leaves were identified. However, some methylated pyrolysates still remain unidentified due to an insufficient mass spectral library of methylated biomolecules and methylated standards. Further structural investigation of these peaks may reveal more information on carbohydrates and lignin compositions in leaves. A promising result of our study revealed that aliphatic hydroxy groups of carbohydrates can be methylated and that intact permethylated O-glycosides could be obtained. The methylation of free carbohydrates under basic conditions is very complicated and thus many of methylated pyrolysis products from leaf carbohydrates have not been identified. It is suggested that further research in this area be undertaken.

Tannins are the group of polyphenolic compounds that have very important physiological functions. Most conventional methods available for tannin analysis are laborious, difficult and are subject to poor quantitative analysis. The technique of SPM

can supply a valuable approach for measuring both hydrolysable and condensed tannins in leaves. Hydrolysable tannins produced one unique pyrolysis product. Although condensed tannins were pyrolysed into several fragments, the pyrolysis process is reproducible and a feasible pathway of the formation of these unique tannin fragments is proposed. The fact that we can distinguish among tannins directly from a very complex bio-materials is a very powerful tool.

From the pyrolysate profile of leaves, comparisons were made on the distribution of condensed tannins and hydrolysable tannins among different plants. The leaves of a shrub plant, kalmia is known to have a high content of condensed tannins, while hydrolysable tannins are absent. In the leaves of oak, hydrolysable tannins are present in large quantity, while the concentration of condensed tannins are comparatively low. The leaves of balsam fir and white pine trees contain only small amount of both condensed tannins and hydrolysable tannins. If tannin content is low, in order to accurately analyze them, tannins should be simply extracted from the leaves and pyrolysed. Another approach would be to use mass spectrometric detection in the selective ion mode (more sensitive), and selectively detect the distinctive tannin ion fragments of the pyrolysates of intact leaves.

Analytical pyrolysis-gas chromatography was applied to a number of controlled environmental stress studies. Ozone fumigation, nutrient stress, and forest thinning were

selected as the stress factors on plants which were expected to cause the compositional changes in leaves. It was shown that the contents of pyrolysates from carbohydrates, tannins and dihydrochalcone compounds in leaves reflect the source availability of plants. Two unique chemical bio-markers in white pine needles were also discovered and they can be used to indicate the ozone damage to the trees. Although these field results are only preliminary, they may provide important information on the health of forest, on the forest restoration, and, aid in understanding some of the bio-physiological process in plants.

Reproducibility in pyrolytic analysis of leaves is a key point when the compositional changes in different environments have to be measured. Both FID and MS give comparable sensitivity and stability. Once the pyrolysates have been properly identified by MS, compositional differences in leaf sample can be measured by the less expensive FID. Reproducibility in analysis is reasonable if the same quartz tube or boat is used for a series of samples. However, the pyrolysis temperature may not be reproducible all the time and inconsistency in sample handling can lead to errors in analysis. In addition, compositional differences in leaves caused by environment stress are sometimes so small that better reproducibility is required. The addition of internal standards would overcome some of these difficulties. However, the suitability and handling of an internal standard is not very amenable to analytical pyrolysis. Thus attention was focused on finding a pyrolysate peak in the pyrogram of leaf samples as

an internal reference. The pyrolysis product chosen as reference peak should not change in concentration and have the similar pyrolysis behaviour as the compounds of interest. Choosing a reference constituent in leaves needs a thorough understanding of the pyrolysis pathways of intact biomaterials. Initial efforts in finding a suitable internal reference proved very promising.

In summary, PY-GC-(MS) is a rapid and convenient method for profiling the organic compositions in leaves. As a very important qualitative and quantitative tool for analyzing biomaterials, this technique can be further improved in terms of its reproducibility. Further application of this method will provide more positive results that have ecological significance in environmental stress studies.

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