ANALYSIS OF LICHENS UNDER ENVIRONMENTAL STRESS USING PYROLYSIS-GC-MS AND PYROLYSIS-GC-FID.

TANYA FRANCES MacGILLIVRAY

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ANALYSIS OF LICHENS UNDER ENVIRONMENTAL STRESS USING PYROLYSIS-GC-MS AND PYROLYSIS-GC-FID.

by

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ABSTRACT

Plants often react to various kinds of stress by changes in their organic composition as the result of defense or stress induced metabolism. This project investigated lichens as stress monitors, searching for changes in their chemistry under various environmental conditions. Qualitative analysis was carried out using Pyrolysis-Gas-Chromatography-Mass-Spectrometry following a micro-scale acetone extraction of dried and ground lichen samples. Only the newest growth of lichen was used. Semi-quantitative analysis was carried out using Pyrolysis-Gas-Chromatography with a Flame Ionization Detector.

Cladina mitis and *Cladina rangiferina* exposed to conditions of visible and/or ultraviolet light were received from Ontario. *Bryoria trichodes* and *Usnea dasypoga* were collected from sites of various ozone levels in New Brunswick. *Alectoria sarmentosa* was collected from sites of varying sulfur dioxide exposure in Newfoundland. Changes in the unique lichen phenolics and fatty acids were detected. Results suggest that unsaturated fatty acids may be useful indicators of ultraviolet, ozone and possibly sulfur dioxide stress. It seems some lichen phenolics such as salazinic acid, alectoronic acid and alectosarmentin, as well as possibly usnic acid and atranorin, may be used to detect these stresses and may be involved in defensive biochemical responses. Future work should focus on verifying which of these changes are specific to a given stress.

ACKNOWLEDGMENTS

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Dr. Moire Wadleigh is acknowledged for her help in suggesting sites for the sulfur dioxide study to correspond to data previously collected by her student Anna Nicholle Evans. Dr. Peter Scott is thanked for his verification of the identities of the lichens collected from the field. Many thanks are due to Dr. Jack Elix of the Australian National University for his generous assistance in providing several samples of lichen products used as references. I would like to thank my lab-mates for their help and encouragement. Funding from the School of Graduate Studies, the Department of Chemistry and the Natural Science and Engineering Research Council (NSERC) is gratefully acknowledged.

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LIST OF ABBREVIATIONS AND SYMBOLS

'	minutes (used in G.P.S. coordinates)
"	seconds (used in G.P.S. coordinates)
12:0	dodecanoic acid
14:0	tetradecanoic acid
16:0	hexadecanoic acid
18:0	octadecanoic acid
18:1	octadecenoic acid
18:2	octadecadienoic acid
18:3	octadecatrienoic acid
ABS	absorbance
A. sarmentosa	*Alectoria sarmentosa*
avg	average
B. trichodes	*Bryoria trichodes*
cdate	date of monitor collection
CFS:MR	Canadian Forestry Service: Maritimes Region
C. mitis	*Cladina mitis*
cont.	control
C. rangiferina	*Cladina rangiferina*
ctime	time of monitor collection

Da	Daltons
DB-1	100% dimethylsiloxane column film
DB-5	95% dimethyl-(5%diphenyl)-siloxane column film
DNA	deoxyribonucleic acid
deg	degrees
E	East
edate	start date of monitor exposure
etime	start time of monitor exposure
exp	experimental
eV	electron volts
FID	flame ionization detector
GC	gas chromatograph
G.P.S.	global positioning system
HPLC	high performance liquid chromatography
id	internal diameter
IAP	index of air pollution
MS	mass spectrometry
MW	molecular weight
N	North
NSERC	Natural Science and Engineering Research Council

ppb	parts per billion
ppm	parts per million
ppmh	parts per million hours
psi	pounds per square inch
Py	pyrolysis
^{32}S	sulfur of atomic weight 32
^{34}S	sulfur of atomic weight 34
TIC	total ion chromatogram
TMAH	tetramethylammonium hydroxide
U. dasypoga	*Usnea dasypoga*
UHP	ultra high purity
USP	United States Pharmacopeia
UV	ultraviolet
UV-A	ultraviolet class A
UV-B	ultraviolet class B
UV-C	ultraviolet class C
µV	microvolt
VOC	volatile organic compounds
wt/vol	weight per volume

CHAPTER 1

INTRODUCTION

1.1 Plant Stress

Plants under stress will show visible signs such as a change in color (clorosis) or tissue death (necrosis) (1, 2, 3). Plant stressors include air pollutants (1), drought (4), infection (4), and ultraviolet (UV) light (5, 6, 7). Scientists have been able to observe results of stress on the cellular level (3, 8). Less obvious results of stress are thought to occur on the molecular or chemical level before the visible symptoms appear (3). These less salient markers have become a subject of interest in recent years as they may be used as early indicators of stress before damage occurs (3).

1.2 Lichen Stress

Lichens are often used as stress monitors for air pollutants due to their high sensitivity (1, 2, 3, 8) and are often absent from industrial and urban areas of poor air quality (1, 3, 8). Lichens have been used in studies to map the effects of such stresses as ozone and sulfur dioxide, such as that shown in Figure 1.1 (1, 9), an example of a map created using an index of atmospheric purity or IAP based on the variety and extent of lichen coverage (1). Studies have also examined lichen response to UV light (5, 7), heavy metals (1, 3, 8, 10) and fluoride deposition (3, 8, 11).

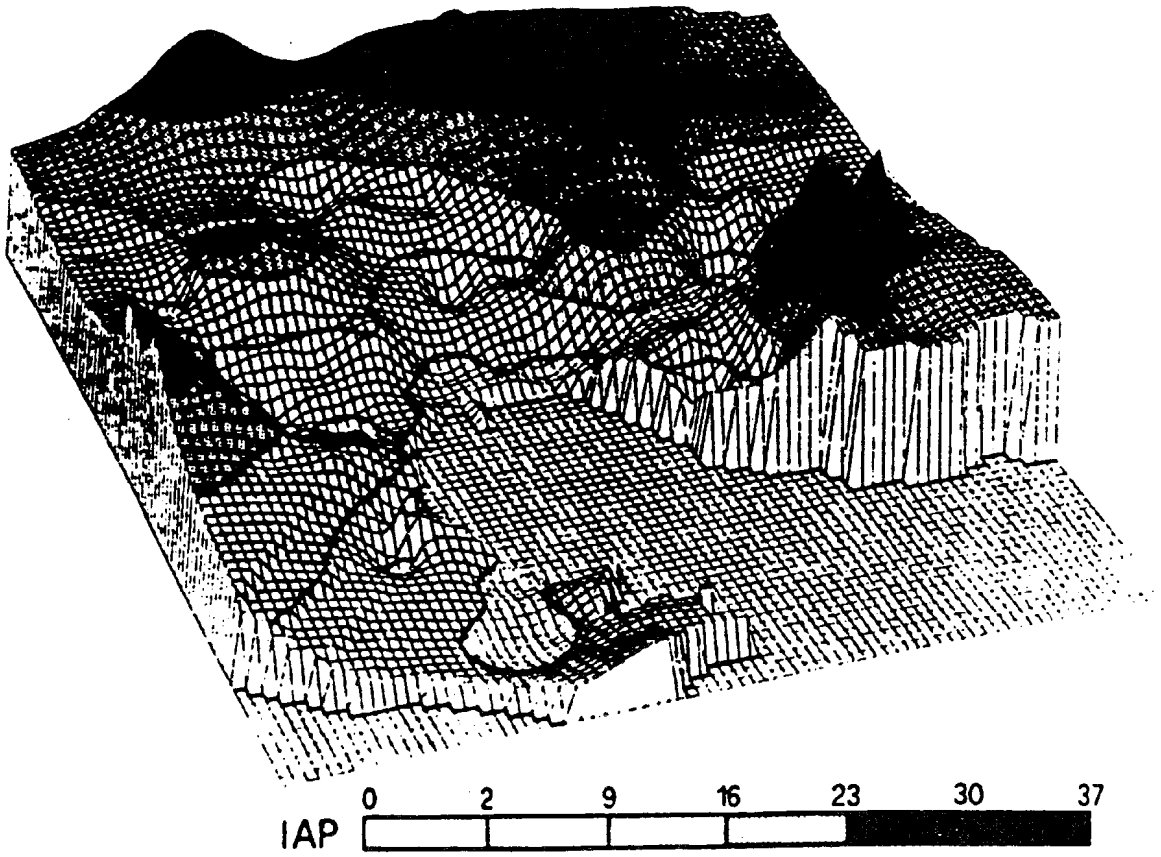


Figure 1.1 Three dimensional IAP (index of air pollution) map from a lichen survey. ***The z-axis is the IAP axis*** **(1).**

Lichens are very sensitive to pollutants for several reasons. Since they lack roots, they depend on the atmosphere for most of their nutrients including moisture (1, 2, 3). Also, they lack the waxy cuticle and adjustable pores or stomata of higher plants which would limit their exposure to the atmosphere (2, 3). As a result, they have greater exposure to pollutants (2, 3). Also, lichens have few methods of getting rid of adsorbed

pollutants so accumulation often occurs (1). This build in concentration is very harmful producing a response either in the form of defense (1, 12, 13) or as the result of damage to sensitive biochemicals (2, 13, 14).

1.3 Lichen Biology

Many different species of lichens occur in nature, inhabiting a wide variety of environments (1, 15). Species have evolved to survive in arctic to tropical temperatures, from extreme low level shores to high altitudes, and they can live on substrates such as soil, trees, rock and in some cases even glass (1). They can survive almost anywhere as long as they are moist enough for metabolic activity for a long enough period in the year to produce growth (1). Their lifetimes can extend from tens to hundreds of years (1). They grow in three body or thallus forms: crustose, which live entirely embedded in substrate surfaces; foliose, which are flat and leafy in appearance; and fruticose, which grow in bush-like clumps (1, 2, 3).

A lichen is considered to be a single symbiotic organism consisting of algal and fungal partners, as seen in Figure 1.2 taken from Richardson's "Pollution monitoring with lichens" (1). The algal cells use the fungal tissue for support and protection while they produce simple carbohydrates by photosynthesis (1). The fungal cells use these simple molecules for cell maintenance as well as further synthesis (1).

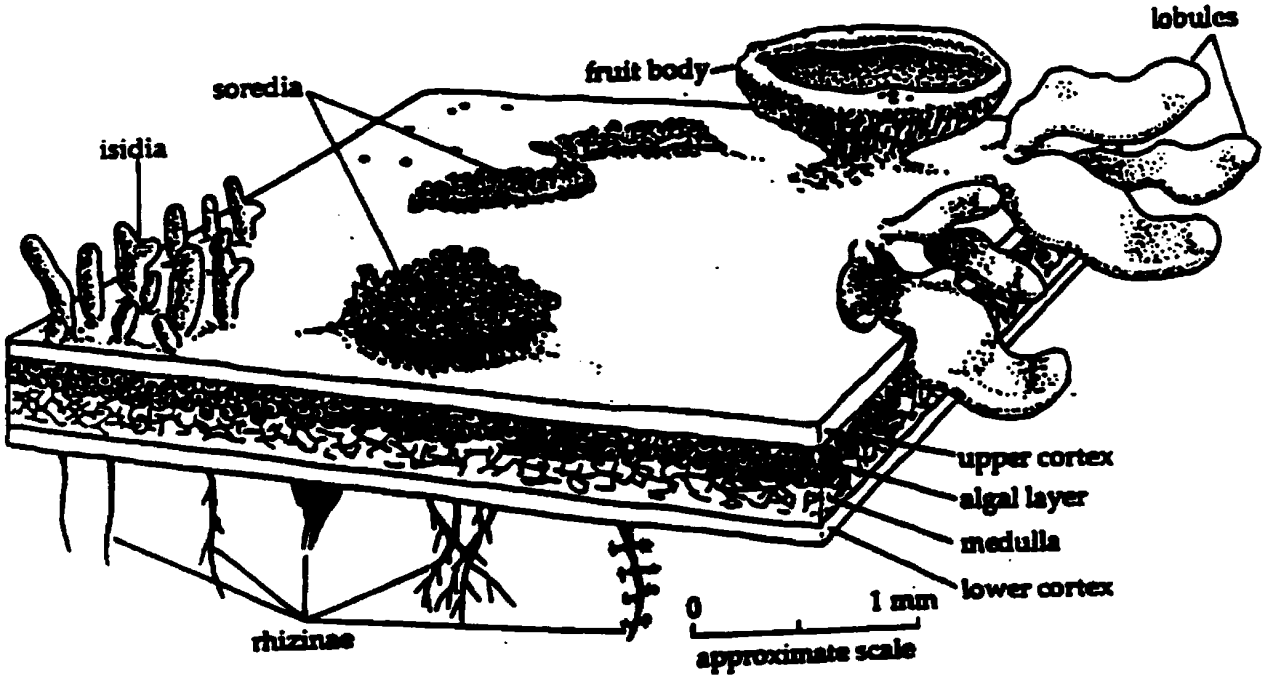


Figure 1.2 The structure of a generalized foliose lichen (1).

1.4 Lichen Acids

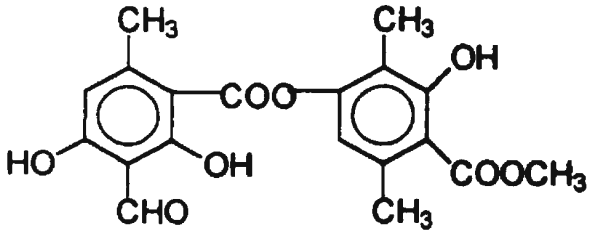
One area of synthesis unique to lichens is the production of the so-called "lichen acids" or "phenolics" (1, 16, 17). Neither the algal nor fungal components can produce these compounds on their own (1). There are several classes of lichen acids distinguished by structural characteristics (1). Most contain at least one carboxylic acid group, hence the term "acid" (1). Generally they contain a benzene ring attached to another carbon

ring by an ester and/or ether link (17). They vary greatly and a single lichen species may contain a number of lichen acids (4, 16). Examples of some of the main classes are shown in Figure 1.3 (16,17).

The purpose behind the production of the lichen acids has been investigated (4, 18, 19). The fact that they exist for the most part in crystal form in the extracellular space, as seen in Figure 1.4 (7), suggests they are not available for or vital to the internal cell functions (16, 17, 19). Certain classes have been found to have antibiotic activity (4, 18, 19, 20) and some of these have been adapted for human use (4). Other acids have antioxidant activity (19) or are proposed to act as light filters (4). Still others have anti-feedant (4, 20) or alelopathic effects which preferentially promote the lichen's growth and reproduction at the expense of other organisms (4).

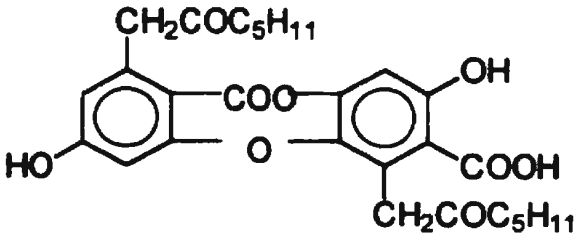
Since most lichen acids seem to function in lichen defense or self-promotion (18, 20, 21), it seems likely that they may offer a method to study lichen chemical response to stress (4). It is believed that a given stressor will cause an increase in the production of a corresponding defensive acid or that normal acid levels will be reduced as they react or decompose in response to the stress (4, 13, 22). A correlation has already been found between phenolic acid levels and UV-B exposure (7).

Depsides



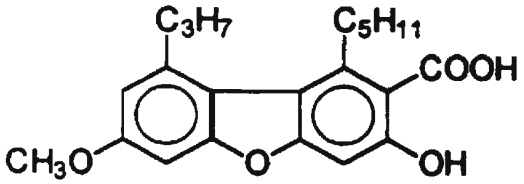
(eg.)Atranorin

Depsidones

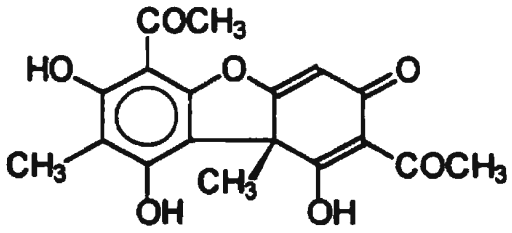


(eg.) Alectoronic Acid

Dibenzofurans



(eg.)Didymic acid



Usnic Acid

Figure 1.3 Examples of lichen acid classes: ***a depside (atranorin), a depsidone (alectoronic acid), a dibenzofurane (didymic acid) and usnic acid.***

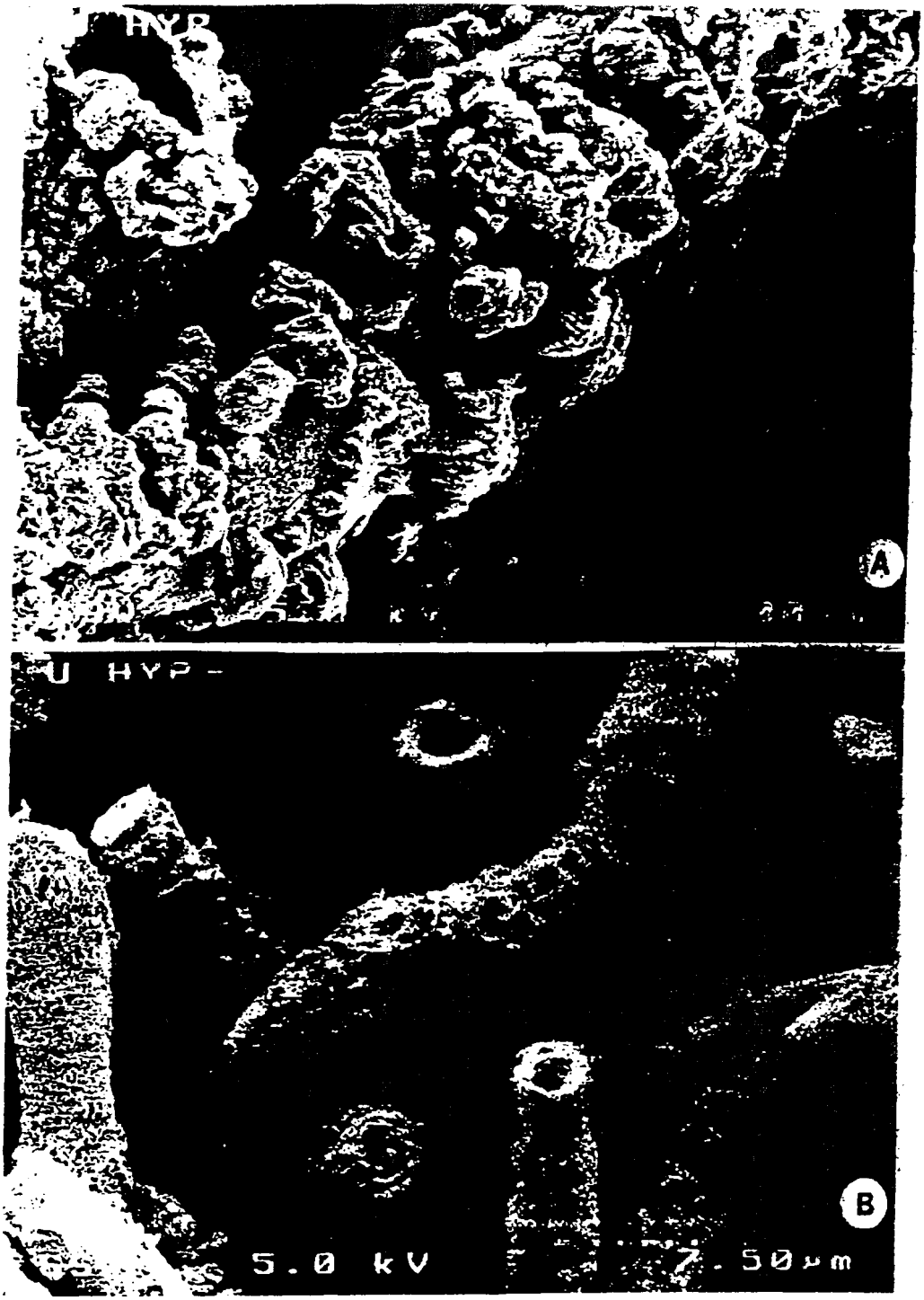


Figure 1.4 FESEM micrographs of medullary hyphae (A) with surface deposits before an acetone extraction and (B) without the deposits after an acetone extraction.(7)

1.5 Lichens and Ultraviolet Light

Ultraviolet radiation is divided into three groups, UV-A (320-400 nm), UV-B (320-290 nm) and UV-C (200-290 nm) (5, 7, 23). The latter is the most energetic and therefore has the greatest potential for harm but it is absorbed completely by the upper atmosphere (5, 7). Ultraviolet-B promotes carcinogenic and mutagenic activity, but is absorbed for the most part by stratospheric ozone (5, 7). UV-A is considered to be somewhat less harmful but is not absorbed much by the atmosphere (5). Thinning of the protective ozone layer has become a matter of concern (5, 7). It has spurred much research into potential effects of and defenses against the resulting increase in UV-B exposure (5). Arctic and Antarctic lichens have been proposed as good subjects to study the increase in UV-B exposure as they exist in the areas where the most drastic ozone depletion occurs (5).

The most devastating effects of UV-B exposure are the formation of dimers, replacements and other changes in the cellular DNA (5). Most organisms have devised ways to repair some of these effects and some have methods to avoid these problems in the first place (5). Lichens are thought to contain compounds, which act as UV filters (5, 7). Specifically, some of the lichen acids absorb strongly in the UV region with maxima in the UV-B band (5). In particular, those with a carbonyl ortho-hydroxyl chromophore unit will exhibit this behavior (24). Examples of such compounds are atranorin and usnic acid (5). Previous UV studies by Dr. D. Fahselt have shown a negative correlation

between lichen phenolic levels and UV-B exposure (7). Some of these phenolic lichen acids occur in the upper cortex above the photosynthetic layer (4). *Cladina mitis* and *Cladina rangiferina*, two lichen species which are otherwise very similar chemically (16, 25), are distinguished by the presence of atranorin in one and usnic acid in the other. This kind of substitution of atranorin for usnic acid is common to several genera of lichens suggesting they may perform similar functions (5), likely as UV absorbers (4).

1.6 Lichens and Ozone

Ozone in the stratosphere is beneficial as a UV absorber (3, 5). However, when it is introduced at the tropospheric or ground level it becomes a problem (8, 26). It is produced at ground level when nitrogen oxides accumulate from pollution sources such as car exhaust (3). Nitrogen dioxide exists in equilibrium with nitrogen oxide and oxygen radicals (3). Volatile organic compounds (VOC's) such as synthetic solvents and naturally occurring terpenes upset this equilibrium by reacting with nitrogen oxide leaving an accumulation of oxygen radicals which will react to form ozone (2, 3). The greater the concentrations of nitrogen oxides and VOC's, the greater the amount of ozone formed (2, 3).

Studies have shown that ozone has detrimental effects on crops (3), forests (2, 3), and lichens (1, 3). Ozone will decompose in a living organism to produce hydrogen peroxide, hydroxide ions, and various oxygen radicals (3, 12, 27). These products will

oxidize organic compounds such as saturated lipids, unsaturated fatty acids, and sulfhydryl moieties in proteins (27). One of the results of this is a disruption of membrane permeability and eventually lipids appear in the cytoplasm, photosynthesis rates drop, and the protoplasmic membranes disintegrate (27).

The oxidation of membrane lipids is thought to be one of the most important mechanisms of ozone injury (27). Once the membranes of the chloroplast are disrupted the precise arrangement of light gathering molecules will no longer function, causing the halt of photosynthesis (3, 5). Thus the membrane lipids such as fatty acids are likely to show ozone stress (27). Protective responses may occur in the form of antioxidants, likely as lichen acids (27). The depsidones, which make up the second most common group of the lichen phenolics (17), are known to show antioxidant activity (19).

1.7 Lichens and Sulfur dioxide

Sulfur dioxide is an air pollutant produced primarily by the burning of fossil fuels (3). Although stricter controls have decreased the levels of SO_2 in many countries, it is still very common and is considered to be the main cause of lichen death due to air pollutants (1). Sulfur dioxide can change to a number of toxic forms and so can have several kinds of detrimental effects (3, 27, 28). In lichens, SO_2 can be absorbed as the gas or it can react with water to form sulfurous acid (H_2SO_3) and bisulfite ions (HSO_3^-) depending on pH (27). It may oxidize to form sulfuric acid (H_2SO_4) and sulfate ions

(SO_4^{-2}) (27).

The aqueous forms of sulfur dioxide seem to be the most toxic, especially sulfurous acid which is notably less corrosive than sulfuric acid suggesting the effects are not strictly due to acidity (28). The bisulfite ions produced from sulfurous acid can participate in both reduction and oxidation reactions (29). Thus biosulfite has the potential to interfere with the electron transport system in photosynthesis (29) and may explain why photosynthesis can be affected before chlorophyll degradation is observed (30).

Typical results of sulfur dioxide exposure include chlorophyll deterioration, reduced photosynthesis, membrane damage, enzyme damage, and K^+ ions leakage (2, 3, 10, 29) as well as decreased lipid levels (3). One effect is the localization of lichens to trees with bark that has higher buffering capacity, due primarily to increased acidity (10). Therefore potential indicators of damage include membrane components, lipids, saccharides, and possibly lichen acids. Antioxidants might be useful in reducing the sulfur forms to relatively harmless hydrogen sulfide (H_2S) (3).

1.8 Pyrolysis and Thermochemolysis

Pyrolysis is a degradative analytical technique (31, 32) which uses heat to break a large molecule into smaller fragments or "pyrolysates" for easier identification and study (31). The pyrolyzer is often directly interfaced to a gas chromatograph (see Figure 1.5)

(31). A mass sensitive detector may be used with this technique as well (31, 32). Pyrolysis is often used for the analysis of very small samples (<mg level) (31). The temperatures used generally fall in the range of 600 to 800°C, where lower temperatures produce larger more characteristic fragments (31). Rapid heating (~1 second) using a filament pyrolysis apparatus yields volatile products (31). This allows complex, nonvolatile samples to be chromatographically identification (31). This method of sample introduction has been used successfully in the study of ozone stress on

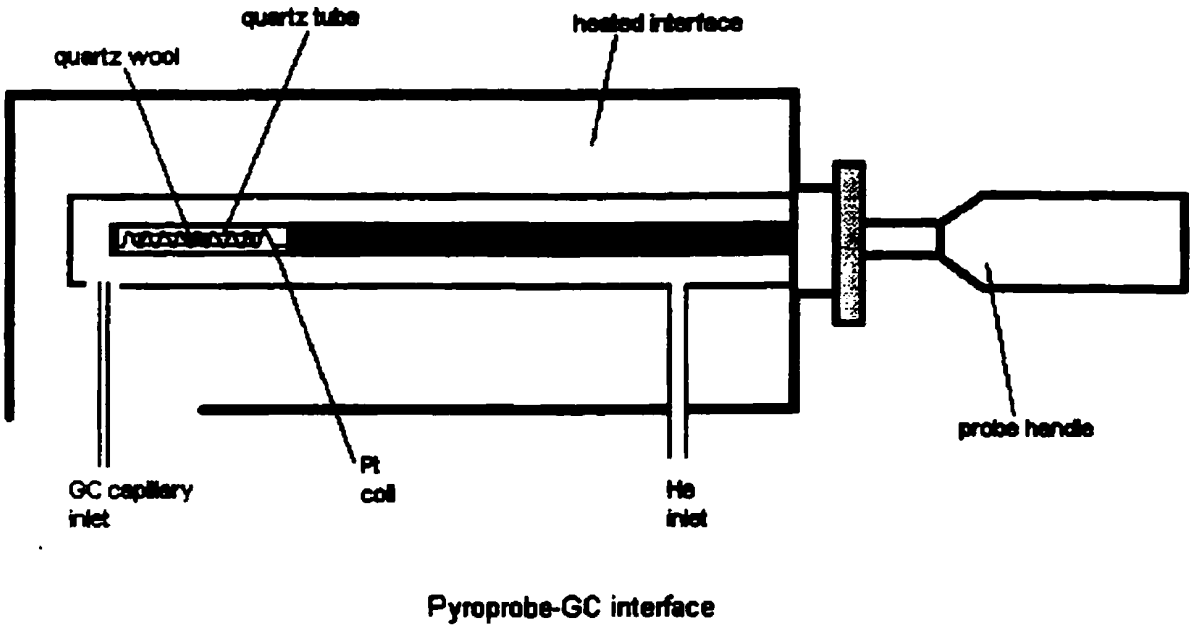


Figure 1.5 Pyroprobe-gas chromatography interface.

white pine where the needles of the trees were analyzed (22). It is therefore a logical choice for the analysis of lichen tissue. Some work has used pyrolysis-gas

chromatography to analyze lichen thalli but the focus was on the identification of saccharides, proteins and lipids rather than lichen phenolics (33).

Thermochemolysis is pyrolysis in the presence of a chemical reaction and also has been called thermally-assisted-chemolysis (34). Commonly this involves a derivatization reaction used to modify organic analytes (35). Methylation reactions are used to reduce the polarity of the pyrolysis fragments, resulting in faster elution from a GC column (34). Tetramethylammonium hydroxide is the reagent used to replace acidic protons on carboxylic acids and phenols with methyl groups (35). This is achieved in a two step endothermic mechanism as shown in Figure 1.6 (35). The resulting products will have lower boiling points and will elute more quickly from a GC column.

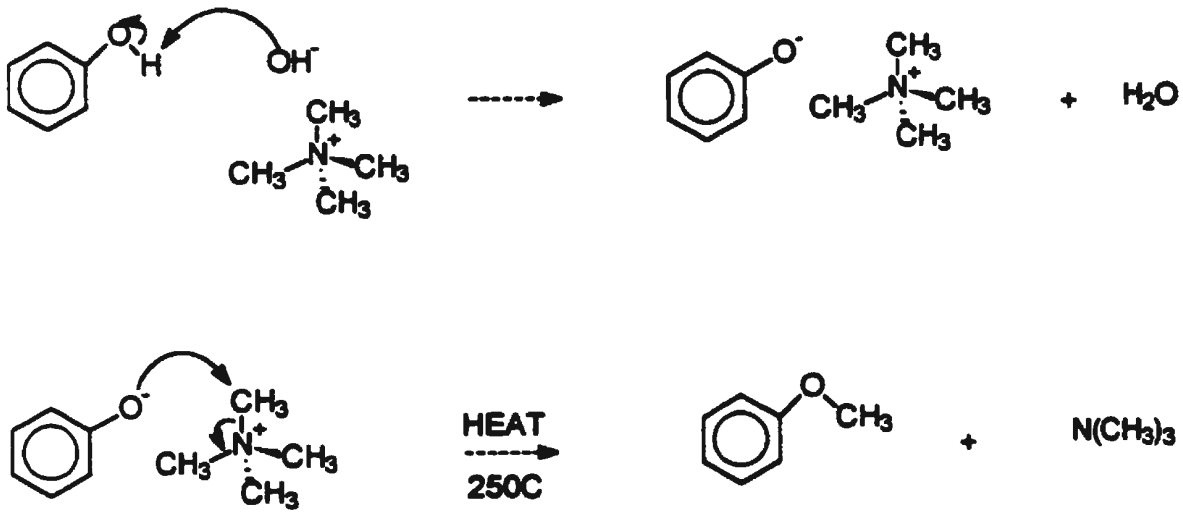


Figure 1.6 Mechanism of methylation using TMAH.

In this study, the objective was to test if chemical changes could be identified in stressed lichens using pyrolysis gas chromatography-mass spectrometry. Changes were semi-quantified using pyrolysis gas chromatography with flame ionization detection. The method of pyrolysis was based loosely on that used in Zhang's pine study (22) but was developed to suit lichen physiology and chemistry. The greater complexity of lichen chemistry necessitated focussing the study either on more polar polysaccharides or less polar components such as the lichen phenolics, fatty acids and sterols. Including solvent extraction in the sample pretreatment allowed the removal of these less polar components for analysis. Thermochemolysis using tetramethylammonium hydroxide was used to enable elution of the resulting pyrolysis products.

CHAPTER 2

EXPERIMENTAL

2.1 Materials

Reference compounds were used for pyrolysis product identification in GC-MS analysis wherever possible. They included usnic acid (99%), atranorin, arabitol (99%), octadecadienoic acid (99%) stigmasterol (95%) and lichenan, all from Sigma. Many lichen phenolics are not commercially available. Small samples (~1 mg) of psoromic acid, fumarprotocetraric acid, squamatic acid, barbatic acid, physodic acid, thamnolic acid, salazinic acid and alectoronic acid were kindly provided by Dr. J. Elix of the Australian National University upon request.

Color tests used to identify the field collected lichens used 0.45 M potassium hydroxide (Fisher) and 6% sodium hypochlorite (Javex bleach) according to Hale's "Guide to the Lichens" (36). The gas chromatograph used UHP grade helium (Air Liquide). The flame ionization detector used USP grade air (Vital Aire) and UHP grade hydrogen (Air Liquide). The acetone extracts were dried under a flow of UHP nitrogen (Air Liquide). Solvent grade acetone was used for the extractions. Tetramethylammonium hydroxide pentahydrate (Aldrich) was used to make a 10% wt/vol solution in HPLC grade methanol (Sigma).

2.2 Sample Collection and Pretreatment

Five species of fruticose lichens were collected for use in three studies. In each study the lichen species was chosen based on suitability, availability and past research. The UV study used *Cladina mitis* and *Cladina rangiferina* both of which were being used by Dr. Dianne Fahselt for studies of the effects of UV on total phenolic levels. Both species contain possible UV filtering compounds.(16) The sulfur dioxide study used *Alectoria sarmentosa* which had been used in a previous study of sulfur isotopes and levels in the same sampling areas by Dr. Moire Wadleigh. It was hoped that the data could be correlated between the two studies. *Alectoria sarmentosa* was to be used in the ozone study as well, but this species did not grow well in the ozone areas sampled. Instead, other more abundant "old man's beard" species *Usnea dasypoga* and *Bryoria trichodes* were collected. Their ability to survive in areas of apparent stress suggested they were more likely candidates for defensive response.

In study I, *Cladina mitis* and *Cladina rangiferina* were collected in Ontario by Dr. D. Fahselt and Mike Begora of the University of Western Ontario. The lichens were subjected to four conditions of light. These included a control condition where lichens sampled from natural light outdoors were dried and stored in the dark. Under such conditions the lichen components remain unchanged for up to a year (Dr. Fahselt, personal communication). Under the next set of conditions, lichens were exposed to three

days of visible light (400-700 nm) from a fluorescent light source. Under the third set of conditions, lichens were exposed to three days of visible and UV-A (320-700 nm) light created using the visible light source as well as a phosphorescent UV light source with a mylar film used to exclude UV wavelengths below 320 nm. Under the fourth set of conditions, lichens were exposed to three days of visible, UV-A and UV-B light (290-700 nm) produced using the same light sources with a cellulose acetate film to exclude UV-C wavelengths.

Three lichen thalli from each set of conditions were received. The lichens were analyzed separately and the results averaged for each condition. New growth on each lichen, consisting of the two most recent branches, was separated from the rest and ground to a fine powder using a small quartz mortar and pestle. The most recent lichen growth of fruticose lichens contains more lichen acids and is more likely to be a site of stress and response (4).

In the next two studies, lichens were sampled from field locations in New Brunswick and Newfoundland, shown in Figure 2.1. In study II, two species of fruticose lichens were collected from the New Brunswick. They were sampled at five sites along a transect extending from Point Lepreau inland toward Fredericton (see Figure 2.2). These sites have been used by the Canadian Forestry Service: Maritimes Region (CFS:MR) in studying the effects of low level ozone (Dr. Cox, personal communication). These sites have various levels of ground level ozone exposure. Ozone is a problem in southern New

Brunswick because the pollutant precursors arrive from the United States (37). The lichens were collected from fir trees.

A passive ozone monitor, supplied by Dr. R. Cox and John Malcolm of the CFS:MR, was left to obtain current ozone readings for correlation with the changes in lichen composition. The monitor plates contain paper impregnated with a blue dye, indigo, which changes to yellow isatin upon oxidation by ozone (38, 39). The ozone exposure is found by extracting isatin and measuring the absorbance using a spectrometer set at a wavelength of 408nm (38). The molar amount of isatin is used to find the molar ozone exposure (38). Field blanks and a calibration formula are used to find the ozone exposure in parts per million hours (38). The time of exposure is then used to give the average ozone level in parts per million or parts per billion (38). This analysis was carried out at the Canadian Forestry Service.

The lichens were collected July 16, 1998. The ozone monitors were left from July 16 to August 6, 1998. Those plates were collected and replaced with fresh plates from August 6 to August 20, 1998. This was necessary as the plates were only to be exposed for about three weeks (~ 60 ppmh) (38). At two sites blanks were also left in the field. These were plates that were sealed in parafilm. Once the ozone levels were measured these blanks were subtracted. The measured ozone levels reflect the relative summer levels for the sites chosen, and should reflect site differences for the year.

Atlantic Canada

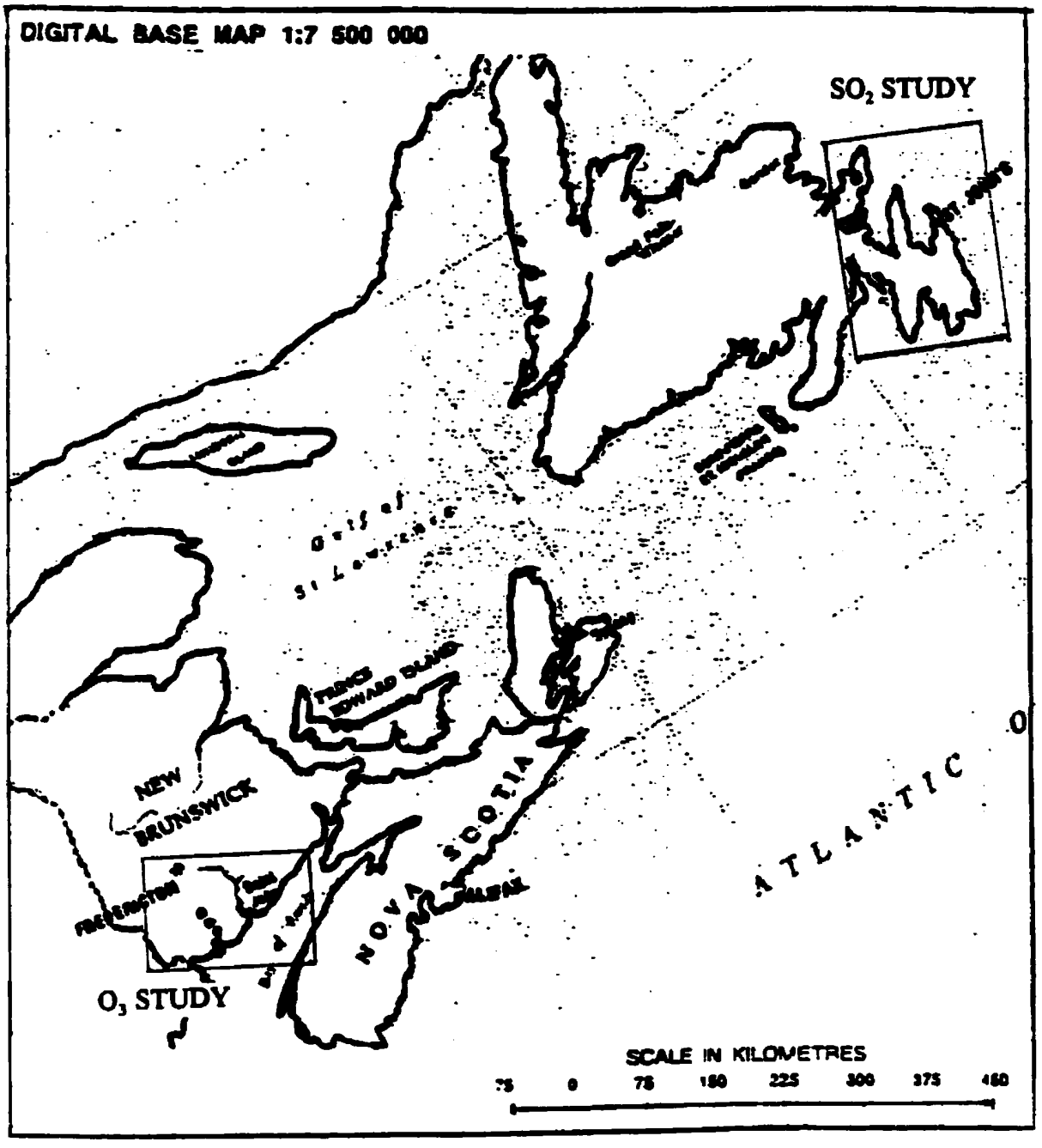


Figure 2.1 Map of general field sampling areas.

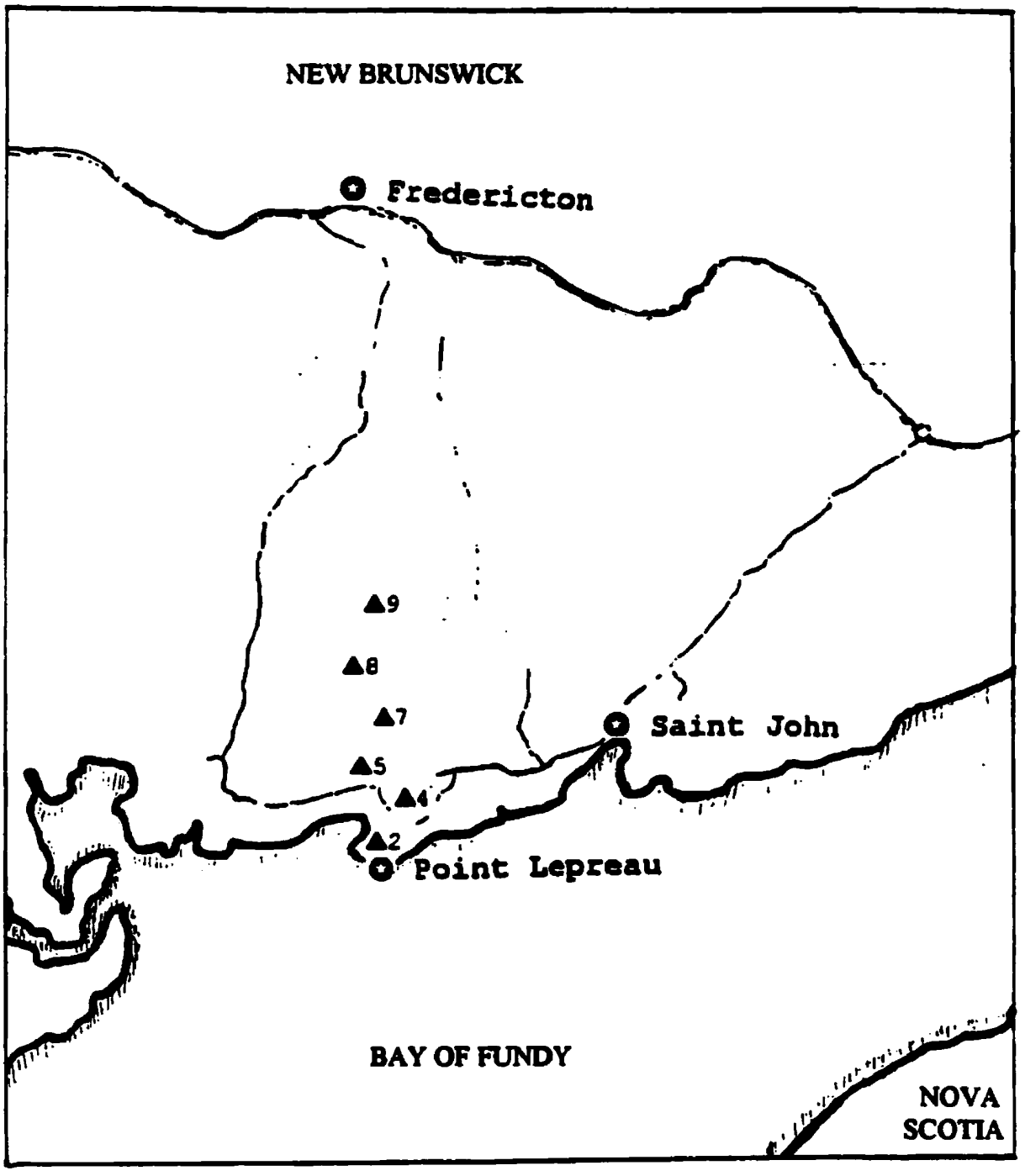


Figure 2.2 Map of ozone sampling sites in southern New Brunswick.

The lichens were cleaned and separated according to physical characteristics. Identification was based on Hale's taxonomic guide (17) using the appearance and color changes in response to potassium hydroxide (0.45 M) and (6%) sodium hypochlorite solutions (17). The identities were confirmed by Dr. P. Scott of the Memorial University on Newfoundland biology department. The two most abundant species which were found at all of the sample sites were *Usnea dasypoga* (or *Usnea filipendula*) and *Bryoria trichodes* (or *Alectoria canadensis*) (40). The alternate names reflect changes in accepted lichen taxonomy (40).

The lichens were cleaned of extraneous material and dried for 24 hours in a forced air oven at 35°C ± 5°C. Once dehydrated the lichens will cease metabolic activity until rehydrated (1). The most recent growth represented by the last 2.5 ± 0.5 cm of the hair-like thalli was ground to a fine powder using a quartz mortar and pestle. According to availability, approximately equal portions were mixed from two to four individual lichen thalli collected at each site. The resulting mixed sample for each site was analyzed in triplicate.

In study III a fruticose lichen, *Alectoria sarmentosa*, was collected from five sites in Newfoundland shown in Figure 2.3. The sites were chosen to correspond to those used in a study by Nicholle Evans and Dr. M. Wadleigh of the Memorial University of Newfoundland Earth Sciences department (41). Higher average sulfur concentrations were measured near the Come by Chance oil refinery as seen in Figure 2.4 (41). Also,

the sulfur isotope ratio, $\delta^{34}S$ (^{34}S / ^{32}S), decreased to values characteristic of anthropogenic or pollutant sulfur at sites closer to the oil refinery (41). Their study also used *Alectoria sarmentosa* (41). The sites used in this study included two near Come by Chance, close to the North Atlantic Oil Refinery (C2 and C6). Two sites were chosen on Random Island (R2 and R3) and one site was near Bonavista (B4). Another site near Bonavista (B3) was examined but did not contain any of the lichens growing on the same substrate used at the other sites, namely fir trees. The sites were chosen in these areas because the prevailing winds are from the southwest, carrying sulfur dioxide emissions from the refinery toward the northeast (42).

The lichens, collected on August 11 to 12, 1998, were cleaned and dried over 24 hours in the forced air oven at 35°C ± 5°C. The newest growth, 2.5 ± 0.5 cm, of two to four lichen thalli from each site were ground together to a fine powder using a quartz mortar and pestle. The resulting mixed sample for each site was analyzed in triplicate.

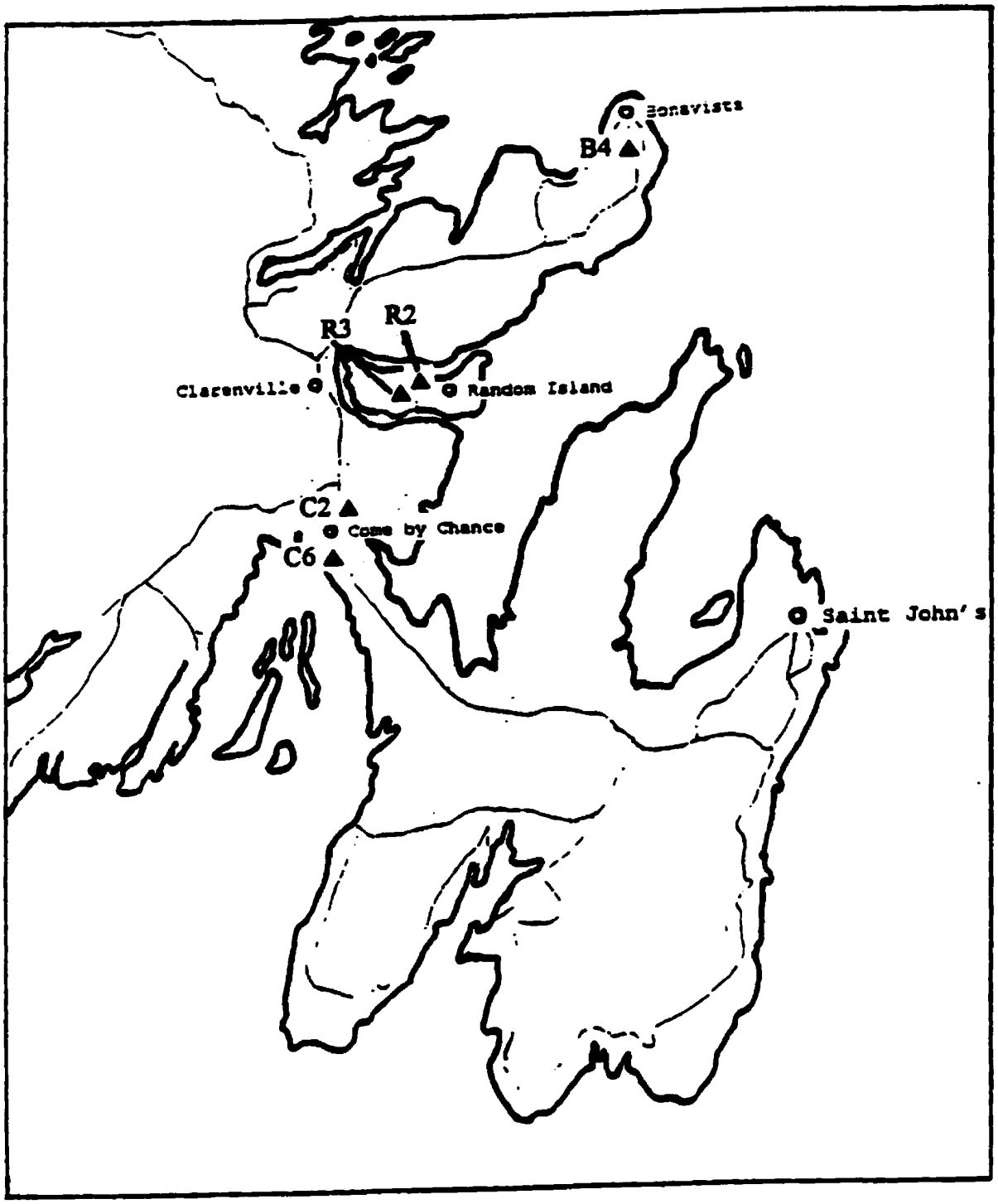


Figure 2.3 Map of sulfur dioxide sampling sites in Newfoundland.

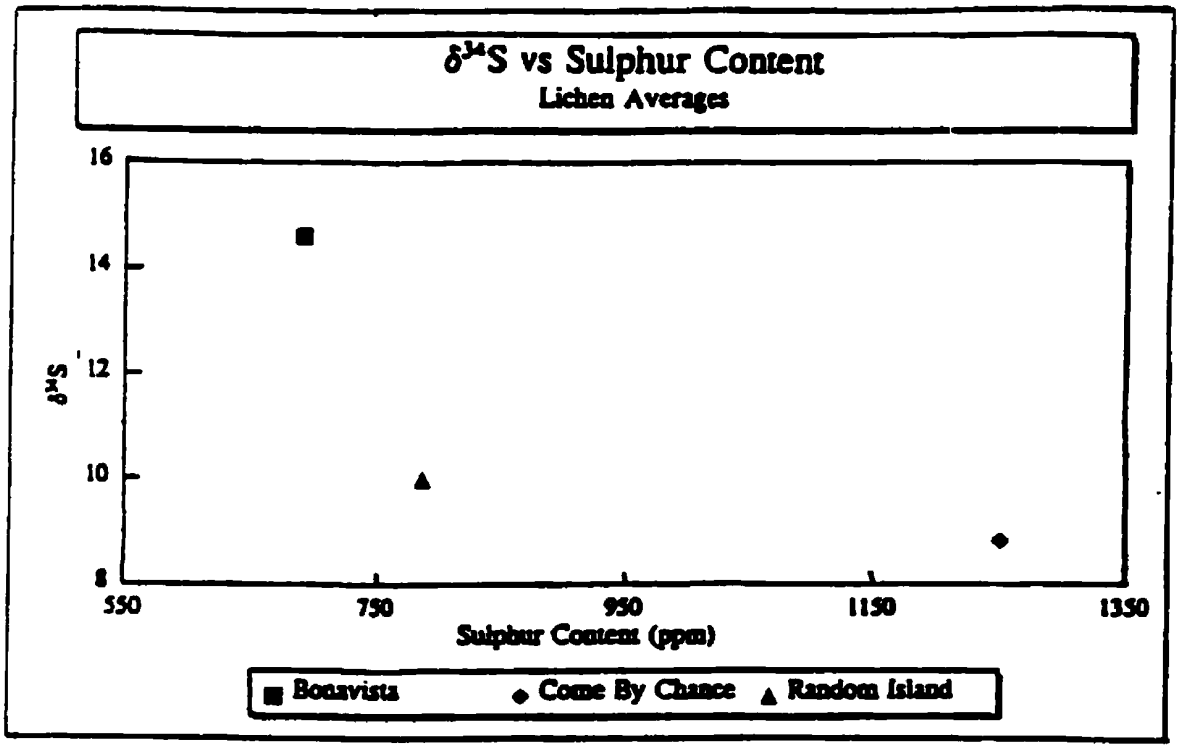


Figure 2.4 Sulfate and sulfur isotope results from a previous study using A. sarmentosa (41).

2.3 Lichen Analysis
2.3.1 Lichen Treatment

Qualitative and semi-quantitative analyses were carried out to look for changes in lichen composition. For both qualitative and semi-quantitative analyses, the pyrolysis method was the same. The development of this method is discussed in chapter three. Various conditions including types of columns, direct pyrolysis versus thermochemolysis, reaction times, sample weights and solvent extractions were investigated. In the optimal method, a 2.00 (± 0.01 mg) sample of lichen powder was weighed using a small piece of a Fisher weigh paper, on a Perkin Elmer AD-2Z Autobalance. The powder was then transferred to a small screw-cap vial and 200 µl of acetone was added. The mixture was sonicated for two minutes using an ultrasonic cleaner (Mettler Electronics Corporation). The resulting slurry was filtered through a quartz wool plug in a Pasteur pipette. The vial was washed with an additional 200 µl of acetone, which was also filtered.

The filtrate was collected in another screw-cap vial and was dried under a nitrogen stream. The residue was re-dissolved in 30 µl of acetone using two minutes of sonication. A 10 µl portion of this extract was transferred to a quartz wool plug in a quartz tube, which had been cleaned in a Bunsen burner flame. Once the solvent had evaporated, 10 µl of a 10% TMAH solution in methanol was added. This was left to react for 40 minutes in order to go to completion. The sample was then ready to be introduced into the GC using a 120 Pyroprobe from Chemical Data Systems

Incorporated.

The quartz tube was inserted into the probe's platinum filament. The probe was then inserted into the GC interface which was kept at 250°C. After a 20 second equilibration time, the sample was pyrolyzed at 450°C for 10 seconds. The column used was a DB-5 capillary column from Chromatographic Specialties Incorporated with a length of ~ 30 m, id of 0.25 mm, and a film thickness of 0.25 μm. The film consisted of 5% phenyl and 95% methyl groups on the siloxane backbone.

2.3.2 Gas Chromatography-Mass Spectrometry

For qualitative analysis, a Hewlett-Packard 5890 Series II gas chromatograph was used interfaced to a Hewlett-Packard 5971 Series mass sensitive detector. Both instruments were controlled using HPChem software, which also collected the mass spectral data.

The GC injector temperature was 300°C, and the oven temperature was held at 60°C for five minutes before ramping up to 285°C at 8°C/min. The final temperature was held for 6 minutes. This temperature program was also used for the GC-FID. The GC column pressure was 10.5 psi and the injector split was 20 ml/min of helium carrier gas.

The mass detector was tuned daily using the Autotune program. Ionization occurred by electron impact using electrons with 70 eV of energy. The detector was a quadrupole used in scan mode from m/z of 35 to 650. The interface between the GC and

detector was maintained at 290°C. There was an initial 5 minute hold time during which no data was collected to avoid swamping of the detector by excess TMAH's breakdown products, namely trimethylamine. The split flow of the carrier was carefully vented into a hood.

2.3.3 Gas Chromatography-Flame Ionization Detector

For semi-quantitative analysis, a Varian model 3700 GC with an internal flame ionization detector was used. An FID is better for semi-quantitative and quantitative analysis because it has higher sensitivity, has a large linear response range, has low noise and is more reproducible (43). The production of a signal from a mass spectrometer depends on the ionization of the analytes, a process which is not terribly reproducible (43). Generally, a mass selective detector requires the use of internal standards for good quantitative data, but this is difficult to do when analyzing complex mixtures (43). Data was collected using a Hewlett-Packard 3395 integrator, the parameters for which are listed below.

Table 2.1 Integrator parameters used for monitoring FID signal.

Zero	=	0, -3.8
Attenuation	=	4
Chart speed	=	0.6 cm/minute
Rejection area	=	1000 (1/8 μV-seconds)
Threshold level	=	4
Peak width	=	0.4 minutes

The GC temperature program was the same as that used for the GC-MS. The helium make-up flow was 32 ml/min, hydrogen flow was 28 ml/min, and the air flow was 270 ml/min. The helium split was 12 ml/min, the column pressure was 20 psi, and the detector and injector temperature temperatures were kept at 300°C.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Method Development

Originally, it was hoped that a simpler method of direct pyrolysis of intact lichen powder could be used. However, several changes had to be made before a suitably resolved chromatogram could be obtained. Lichens contain a number of different compounds, some from the mycobiont (fungus), some from the photobiont (algae), and some produced through cooperation of the two (4, 17). Such complex samples are often studied with pyrolysis-chromatography using a fingerprint approach (31). However, clear chromatographic peak resolution without interferences is still necessary.

Initial attempts at direct lichen pyrolysis were carried out on the control or least stressed samples of all five species. Ground dried lichen was used in an attempt to monitor as many components as possible. The results were similar for all species under each method so those of *Cladina mitis* are shown as examples. In Figure 3.1, part A shows the result from ~1.0 x 10^2 µg of the *C. mitis* control sample pyrolyzed at 700°C, separated on a DB-1 column (100% methyl siloxane film). There are few recognizable peaks, fewer still that are sharp and all seem to overlap. In addition there is a poor baseline.

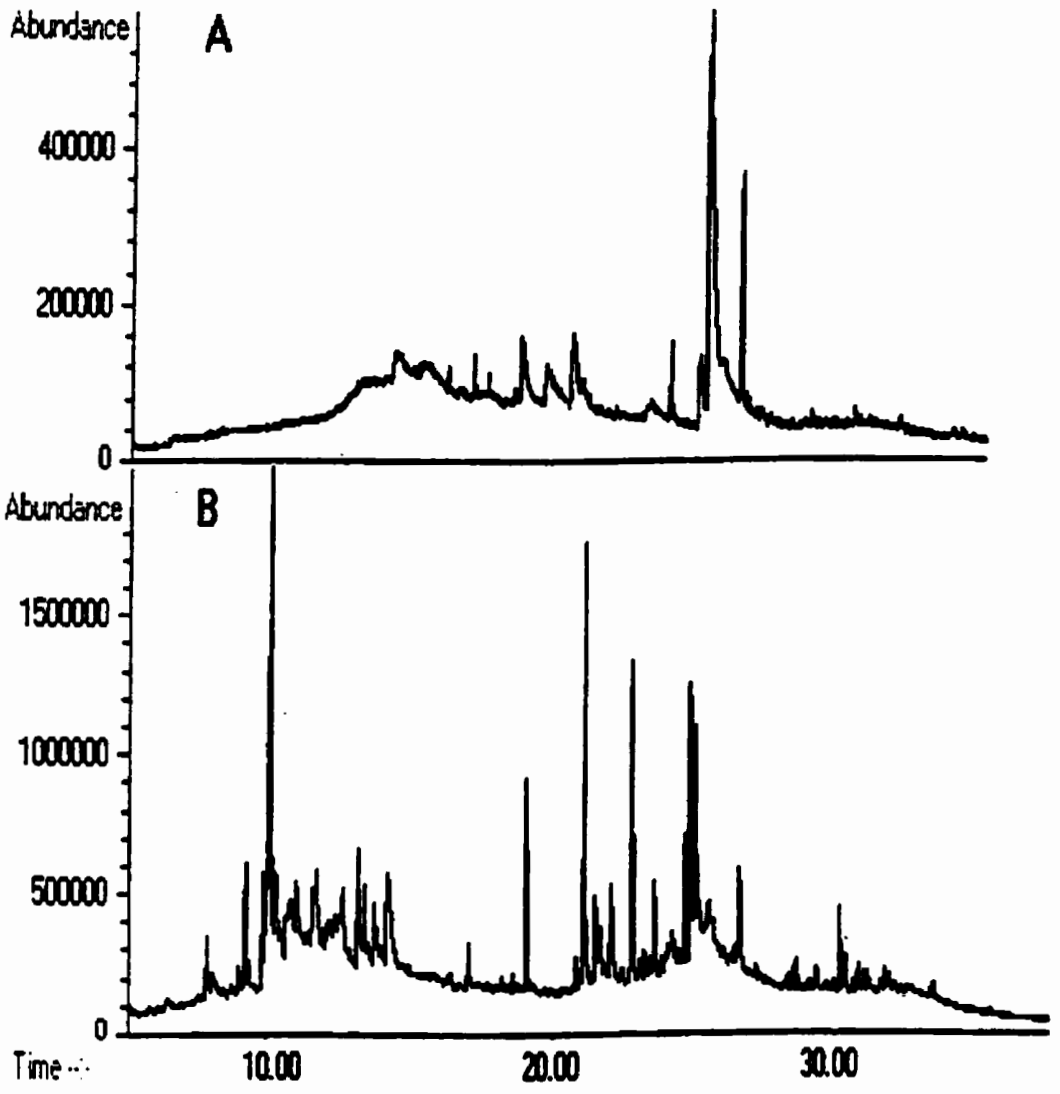


Figure 3.1 Method development I: (A) Direct pyrolysis and (B) thermochemolysis of *C. mitis* lichen powder at 700°C chromatographed on a DB-1 column.

Thermochemolysis was attempted using TMAH as a methylating reagent. This was intended to decrease the sample polarity to yield better peak resolution and faster elution times. In Figure 3.1, part B shows the resulting chromatogram. The peaks are a little better resolved and more are visible but

there is still a great deal of overlap and a poor baseline.

At this point a more polar column was chosen. The DB-5 column gave much better resolution for the most part, as seen in Figure 3.2, part A. However, there is still an interference problem in two main areas around retention times of ~15 and ~30 minutes. Mass spectra revealed the cause to be mono- and di-saccharides, respectively. As the lichens carry out photosynthesis, they are expected to contain large amounts of such carbohydrates. However, these compounds have several hydroxyl groups, which are somewhat susceptible to methylation by TMAH (44). Several products of varying degrees of methylation can result, each of which have several possible isomers. Thus, several peaks of similar mass spectra and slightly different polarities are produced from original mono- and di-saccaharides. These peaks cover a range of retention times and interfere with other compound identifications including the lichen acids.

The methylation was comparable with 10% and 25% TMAH solutions so the lower concentration was used to avoid excessive corrosive damage to the GC column. A reaction time of 20 minutes with TMAH solution was used initially but the reproducibility of the chromatograms was poor. A time of 40 minutes gave better

reproducibility.

Since the methylation reaction is endothermic (35) it was hoped that lowering the pyrolysis temperature would decrease the number and intensity of the saccharide peaks by decreasing the degree of hydroxyl methylation. The result seen in Figure 3.2, part B, shows a reduction in the intensity of these peaks but they still interfered with other compounds.

At this point it was decided to focus on the less polar components including the lichen acids to look for defensive responses to stress. A solvent extraction could remove them from the more polar saccharides. Acetone was the most successful solvent used though methanol and the TMAH solution were also tried. The extraction was carried out on ~2 mg of lichen powder using 200 µl of acetone with methylation occurring afterward, as detailed in the experimental section. The resulting chromatogram seen in Figure 3.2, part C shows a flat baseline with sharp, well resolved peaks.

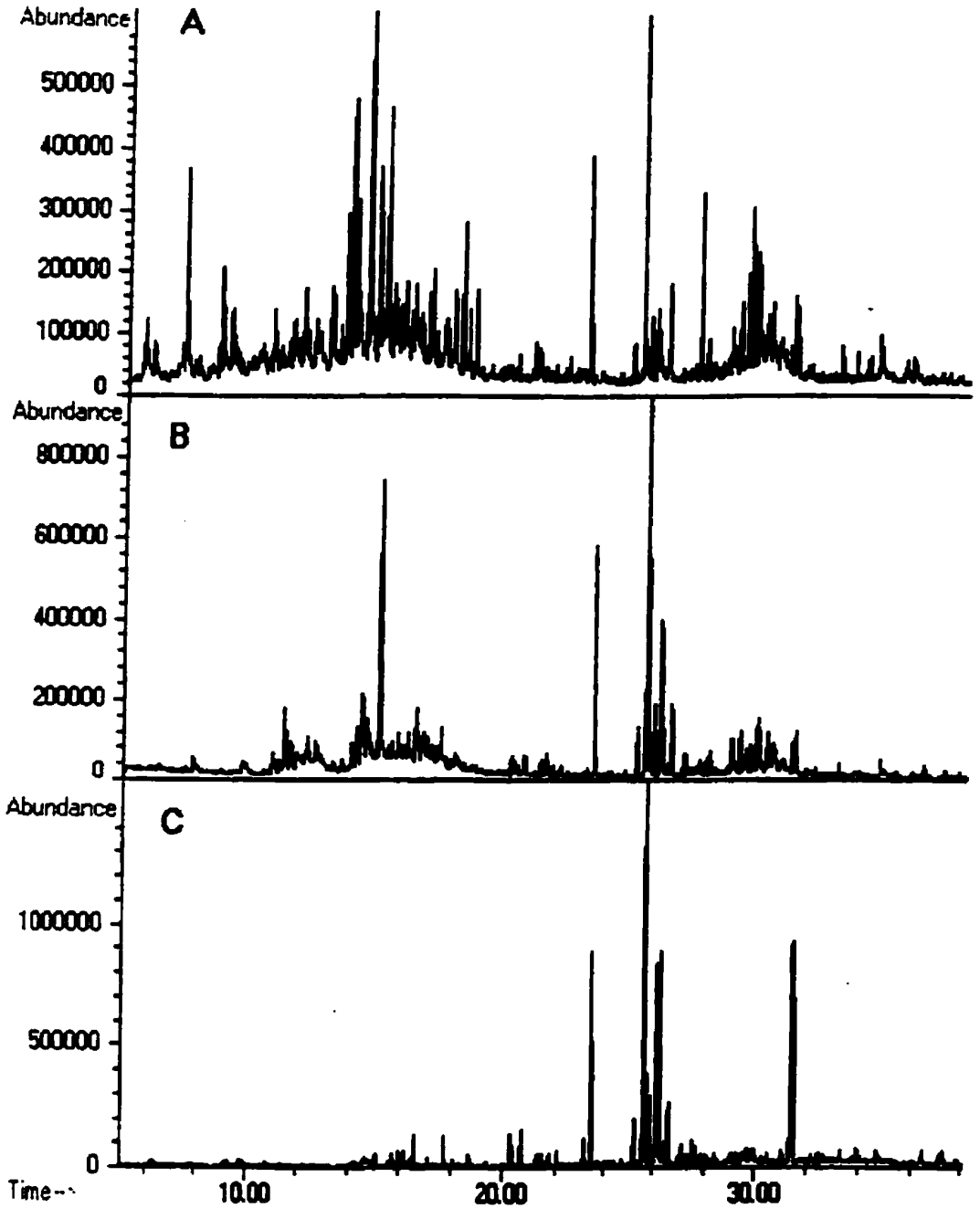


Figure 3.2 Method development II. (A) Thermochemolysis on DB-5 column at 700°C, (B) thermochemolysis on DB-5 column at 350 °C, (C) thermochemolysis of acetone extract on a DB-5 column at 450°C all using *C.mitis*.

3.2 Qualitative Results Using GC-MS

Chromatograms of each of the five species sampled in this project are shown in Figure 3.3. They reveal distinct differences even between the closely related *Cladina* species. These chemical profiles may be of interest to taxonomists who often use secondary compounds to distinguish closely related species (1, 4, 16, 17).

The mass spectra show that lichen acetone extracts contain three main types of components. The most intense peaks often are fatty acids detected as the methyl esters. Presumably they come from base hydrolysis followed by methylation of fatty acids released from neutral membrane lipids (45, 46, 47). The relatively high intensity of these fatty acids reflects their high susceptibility to the methylating agent (35). Therefore, they may not be the most abundant compounds in the extracts.

The lichen acids represent a large number of peaks in the extracts. Because lichen acids are mostly large, structurally complex molecules, a large variety of compounds are formed during pyrolysis which appear across a range of retention times. For some of the smaller lichen acids, single ring fragments, intact parent molecules and intact parent molecules with extra methyl groups can be detected, as seen with atranorin in Figure 3.4. The larger fragments are often the most characteristic, though there are exceptions. For example, fumarprotocetraric acid gives a fragment unique among the lichen acids which is relatively small, as seen in Figure 3.5. It originates from the four carbon alkene/carboxylic acid functional group.

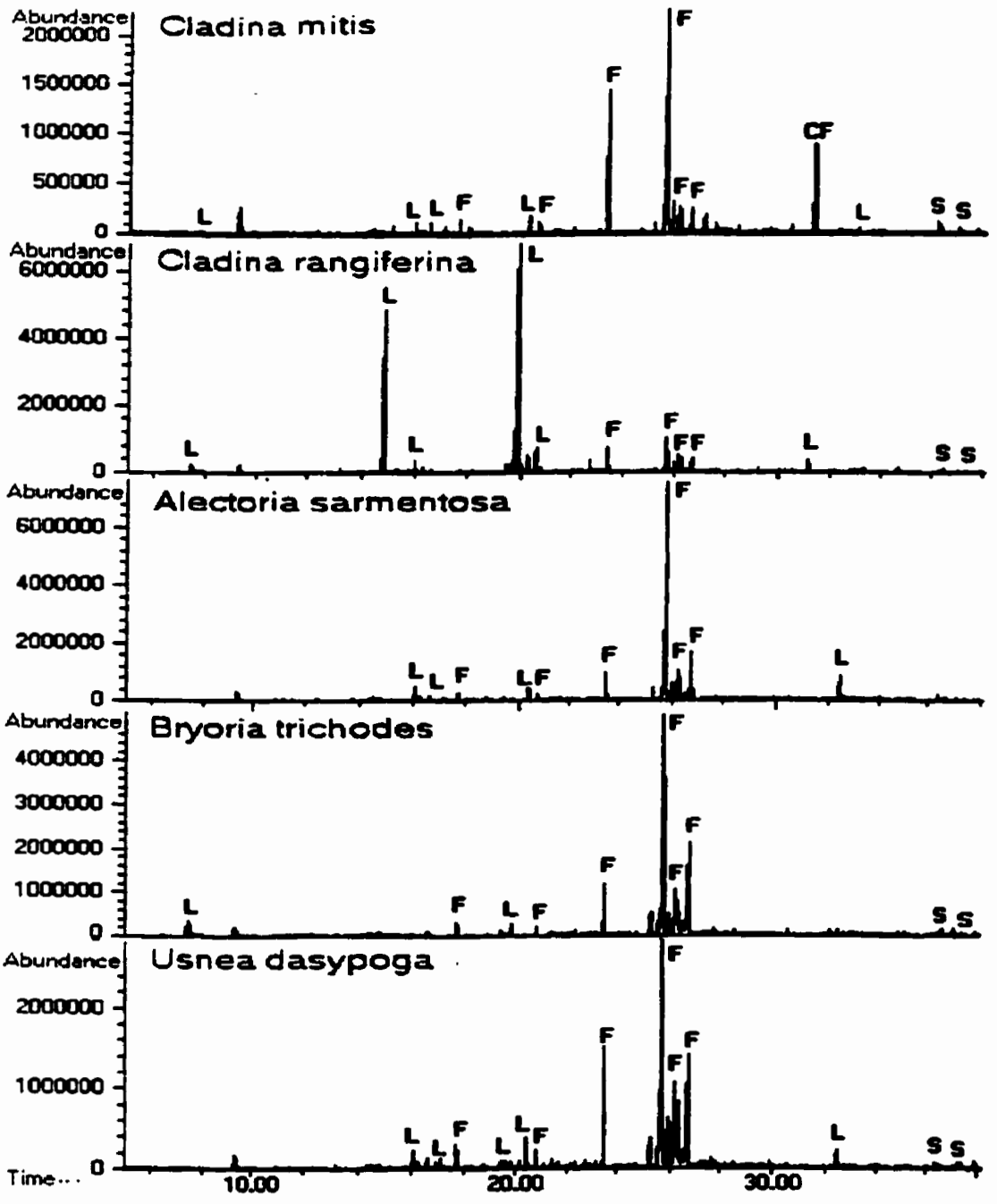


Figure 3.3 TMAH-Pyrolysis-GC-MS total ion chromatograms of the acetone extracts of five speceis of lichens. ***(L = pyrolysis products of lichen acids, F = those of fatty acids, CF = those of complex fatty acids, S = those of sterols).***

A third component group from the lichen extracts which has been identified is the sterols. Stigmasterol in particular has appeared in several species, giving low intensity peaks at high retention times. Sterols, including stigmasterol (48) have also been found in lichens in lipid analyses (46, 48, 49).

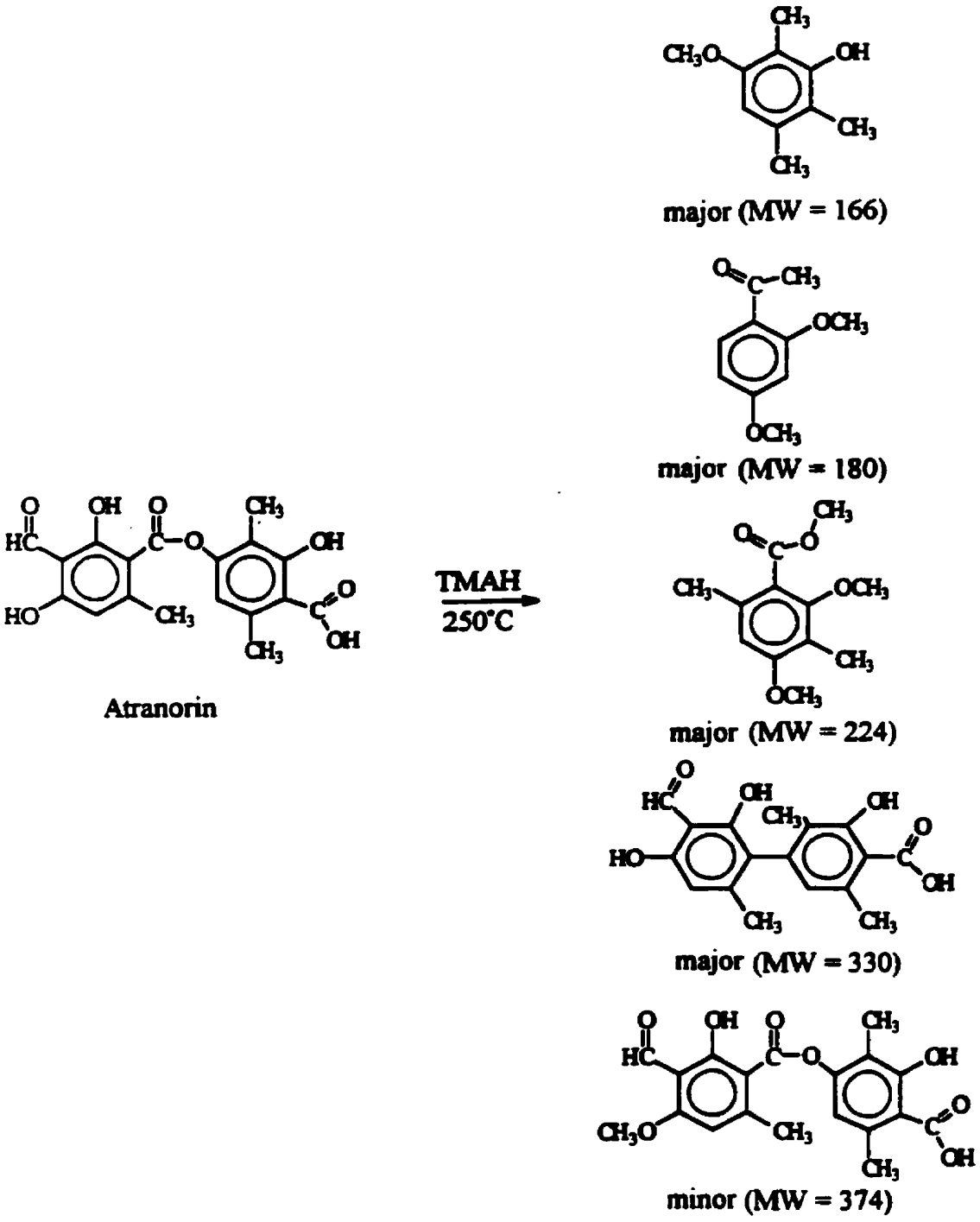


Figure 3.4 Some thermochemolysis products of atranorin.

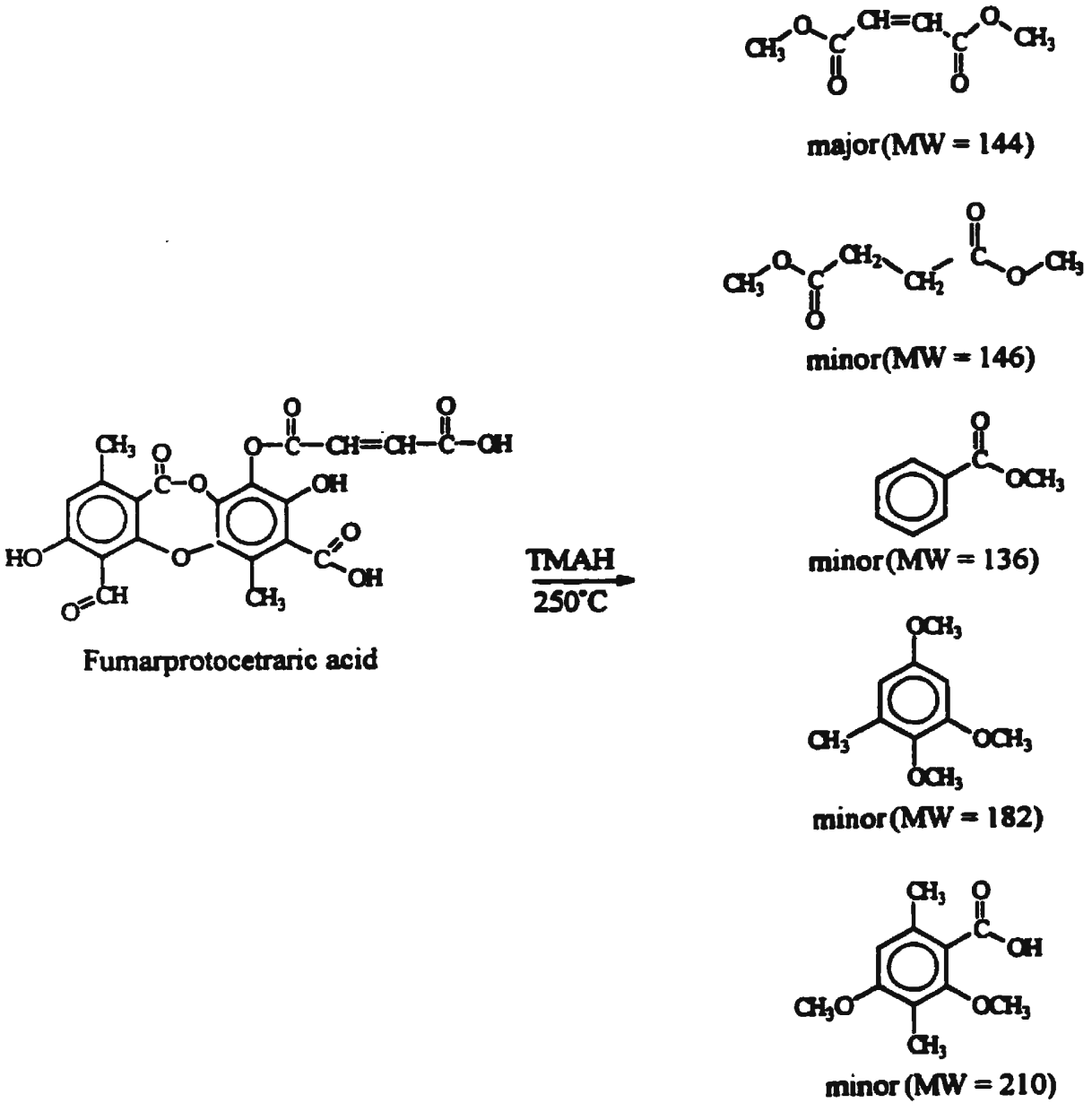


Figure 3.5 Some thermochemolysis products of fumarprotocetraric acid.

3.2.1 *Cladina rangiferina*

The chemical profile of the acetone extract of *Cladina rangiferina* is shown in Figure 3.6. As well as fatty acids, it contained atranorin, fumarprotocetraric acid and stigmasterol pyrolysis products. Their original structures are shown in Figure 3.7. Judging from the intensity of some distinctive peaks, there seems to be a great deal of atranorin present. This compound is one which absorbs strongly in the UV-B range making it a possible light filter (5). Its depside character also indicates that it may have slight antioxidant activity common to members of this chemical group (19). Fumarprotocetraric acid, a depsidone, is more likely to have stronger antioxidant activity (19).

Stigmasterol appears to be one of the common sterols for lichens (48). It was also found in a number of other species, but in each case only in minor amounts. Several fatty acids were detected as well. They are commonly found in lichens (45, 46, 47). In particular hexadecanoic acid (16:0), octadecanoic acid (18:0), octadecenoic acid (18:1) and octadecadienoic acid (18:2) were detected in *C. rangiferina.* Several peaks of octadecadienoic acid methyl ester are seen due to the isomerization of the double bonds caused by strongly basic TMAH (50).

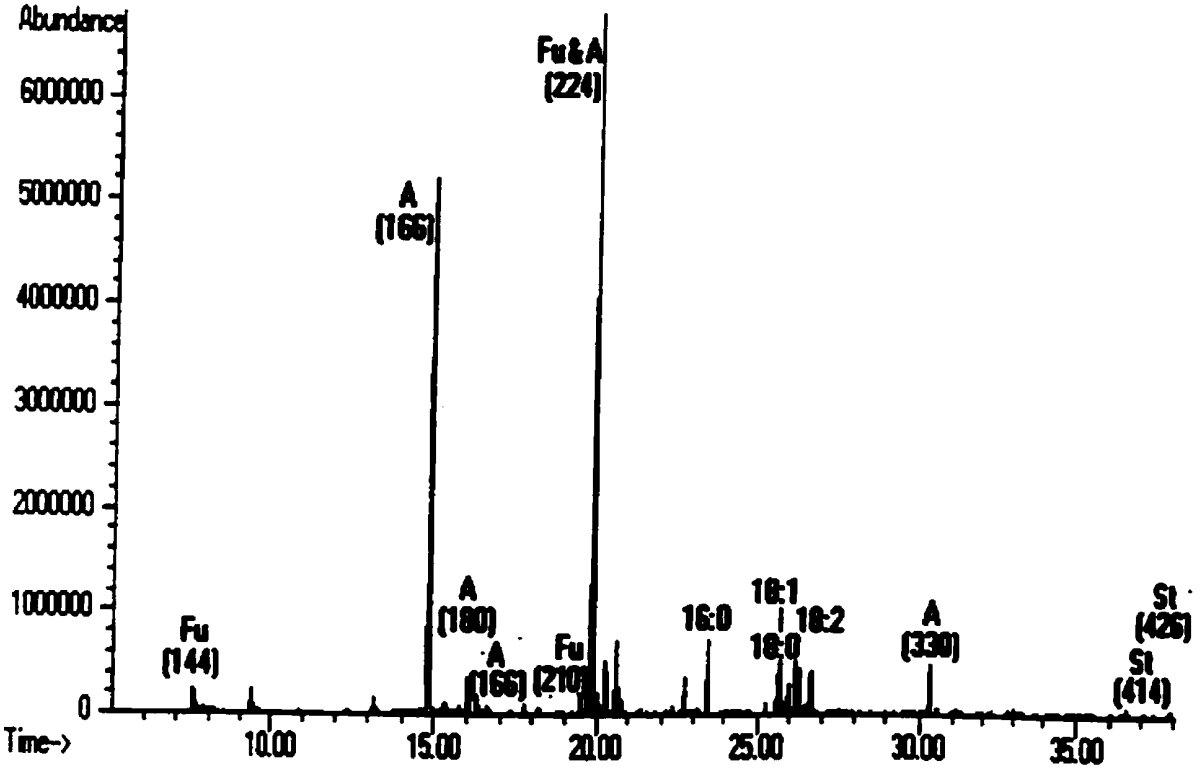
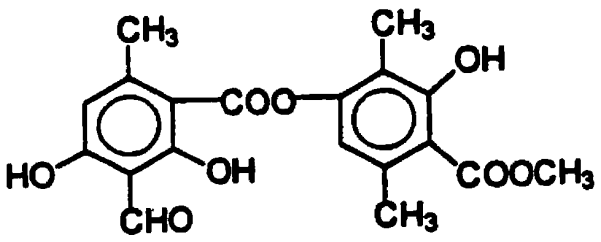
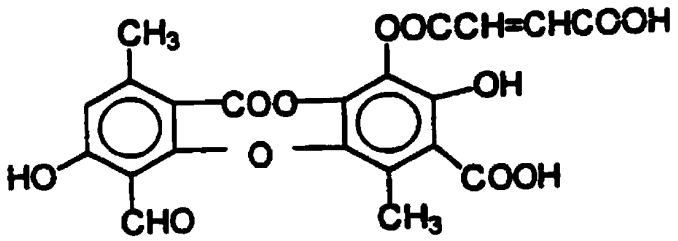


Figure 3.6 GC-MS total ion chromatogram of the acetone extract of *Cladina rangiferina*. *([ ] denotes the molecular weight of the TMAH thermochemolysis product. Thermochemolysis products are represented by Fu = fumarprotocetraric acid, A = atranorin, St = stigmasterol, 16:0 etc. = methyl esters of fatty acids.)*

Atranorin



Fumarprotocetraric acid



Stigmasterol

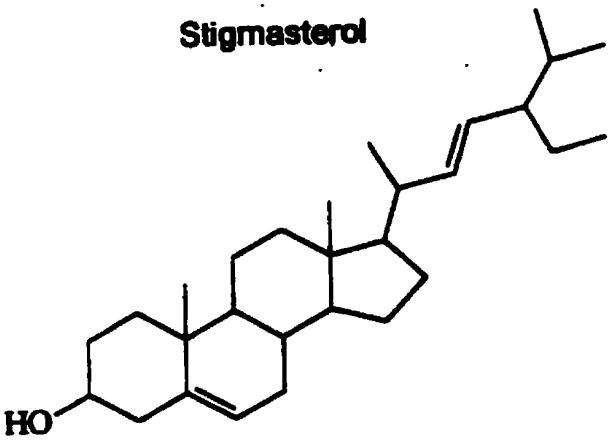


Figure 3.7 Structures of atranorin, fumarprotocetraric acid and stigmasterol.

3.2.2 *Cladina mitis*

The chemical profile of the acetone extract of *Cladina mitis*, seen in Figure 3.8, revealed that this species contained fumarprotocetraric acid as well as usnic acid, a dibenzofuran-like compound often considered to exist in a class of its own (5, 17). Usnic acid, shown in Figure 3.9, is known to absorb strongly in the UV-B region and it is thought to be a light filter (5). It appears to replace atranorin in closely related species of several genera of lichens (5). Usnic acid is also known to have antibiotic activity (17).

C. mitis also contained stigmasterol and a number of saturated (12:0, 14:0, 16:0, 18:0) and unsaturated (18:1, 18:2) fatty acids. Many of these were also found in *C. rangiferina*. A notable distinction was the presence of the complex fatty acid rangiformic acid, shown in Figure 3.9. It is found as an extracellular deposit with the lichen phenolics (4, 17). These compounds resemble others found in non-lichenized fungi (17).

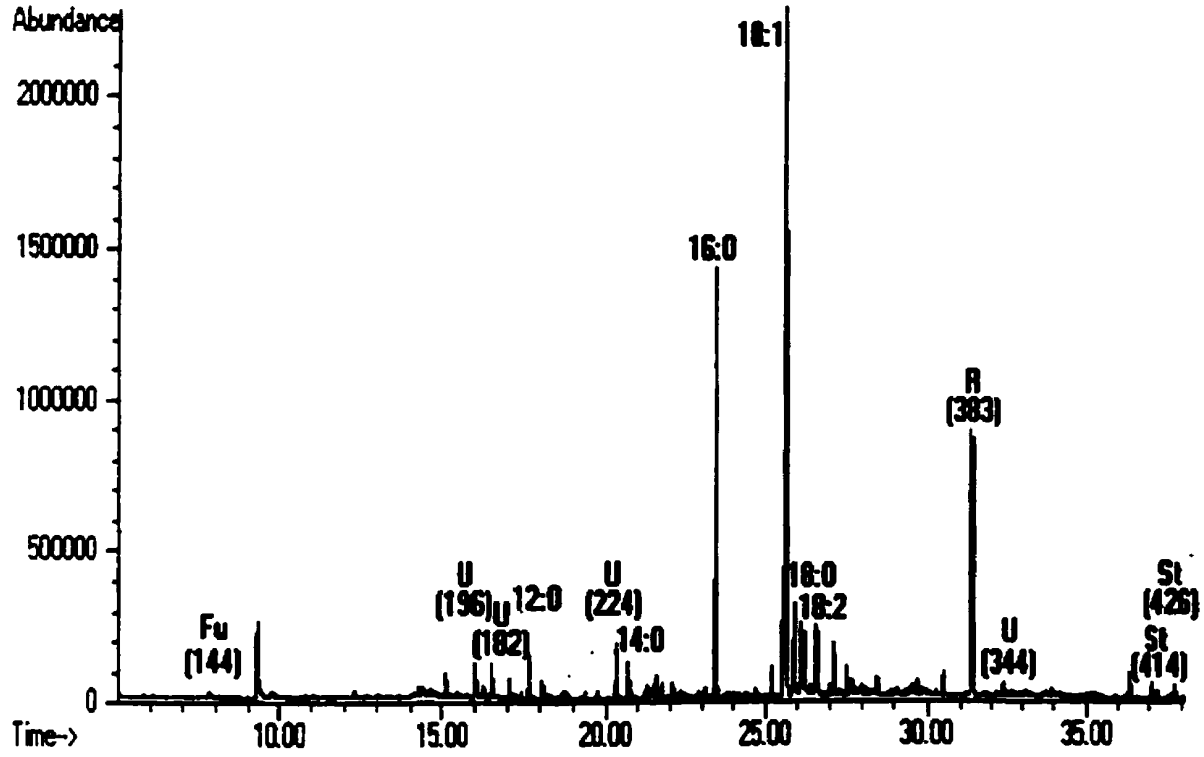
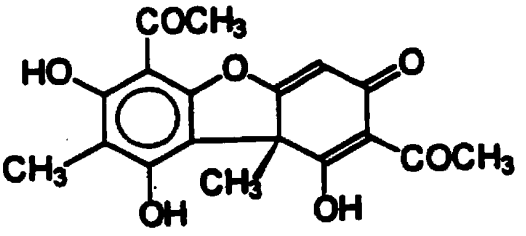
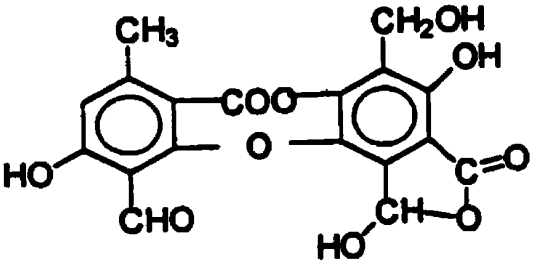


Figure 3.8 GC-MS total ion chromatogram of the acetone extract of *Cladina mitis*. *([ ] denotes the molecular weight of the TMAH thermochemolysis product. Thermochemolysis products are represented by Fu = fumarprotocetraric acid, U = usnic acid, R = rangiformic acid, St = stigmasterol, 16:0 etc. = methyl esters of fatty acids.)*

Usnic Acid



Salazinic Acid



Rangiformic acid

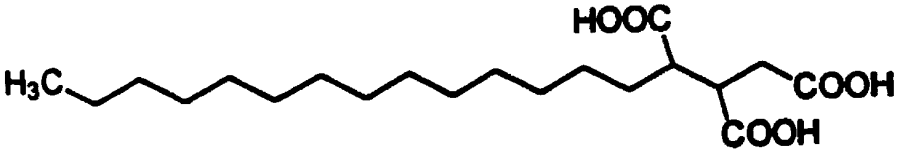


Figure 3.9 Structures of usnic acid, rangiformic acid, and salazinic acid.

3.2.3 *Usnea dasypoga*

The chemical profile of the acetone extract of *Usnea dasypoga* is shown in Figure 3.10. It contained the usual fatty acids found in the other species (16:0, 18:0, 18:1, 18:2) as well as a few others (12:0, 14:0, 18:3). It also contained stigmasterol, which was found in some of the other species. The phenolic components detected were usnic acid and salazinic acid, a depsidone shown in Figure 3.9. This is likely to have some antioxidant activity (19) and so will be of interest in the semi-quantitative analysis.

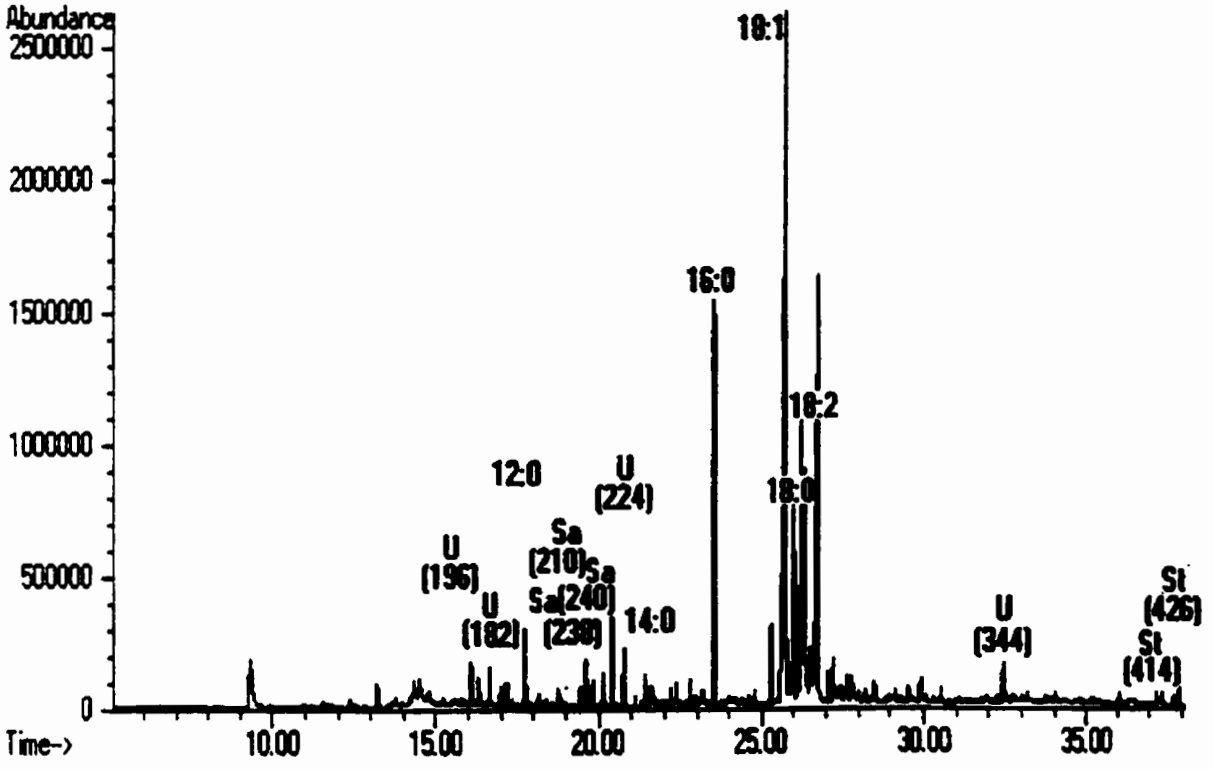


Figure 3.10 GC-MS total ion chromatogram of the acetone extract of *Usnea dasypoga*. (*[ ] denotes the molecular weight of the TMAH thermochemolysis product. Thermochemolysis products are represented by Sa = salazinic acid, U = usnic acid, St = stigmasterol, 16:0 etc. = methyl esters of fatty acids.)*

3.2.4 *Bryoria trichodes*

The chemical profile of the acetone extract of *Bryoria trichodes* is shown in Figure 3.11. This species was found to contain several fatty acids (12:0, 14:0, 16:0, 18:0, 18:1, 18:2) as well as stigmasterol, much like the other species studied. It also contained fumarprotocetraric acid and a sterol-like compound of molecular weight 410, possibly a stigmastadienone. The mass spectrum of the compound is shown in Figure 3.12.

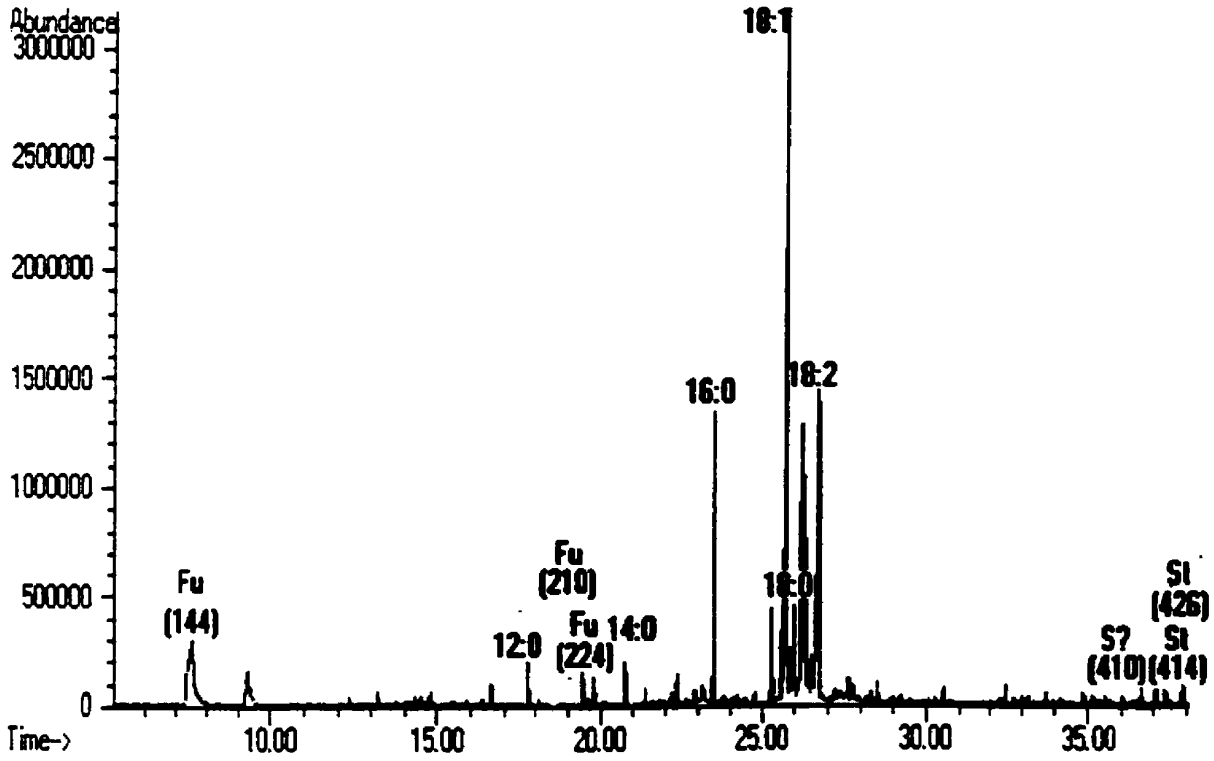


Figure 3.11 **GC-MS total ion chromatogram of the acetone extract of *Bryoria trichodes*.** ***([ ] denotes the molecular weight of the TMAH thermochemolysis product. Thermochemolysis products are represented by Fu = fumarprotocetraric acid, St = stigmasterol, S? = unknown sterol, 16:0 etc. = methyl esters of fatty acids.)***

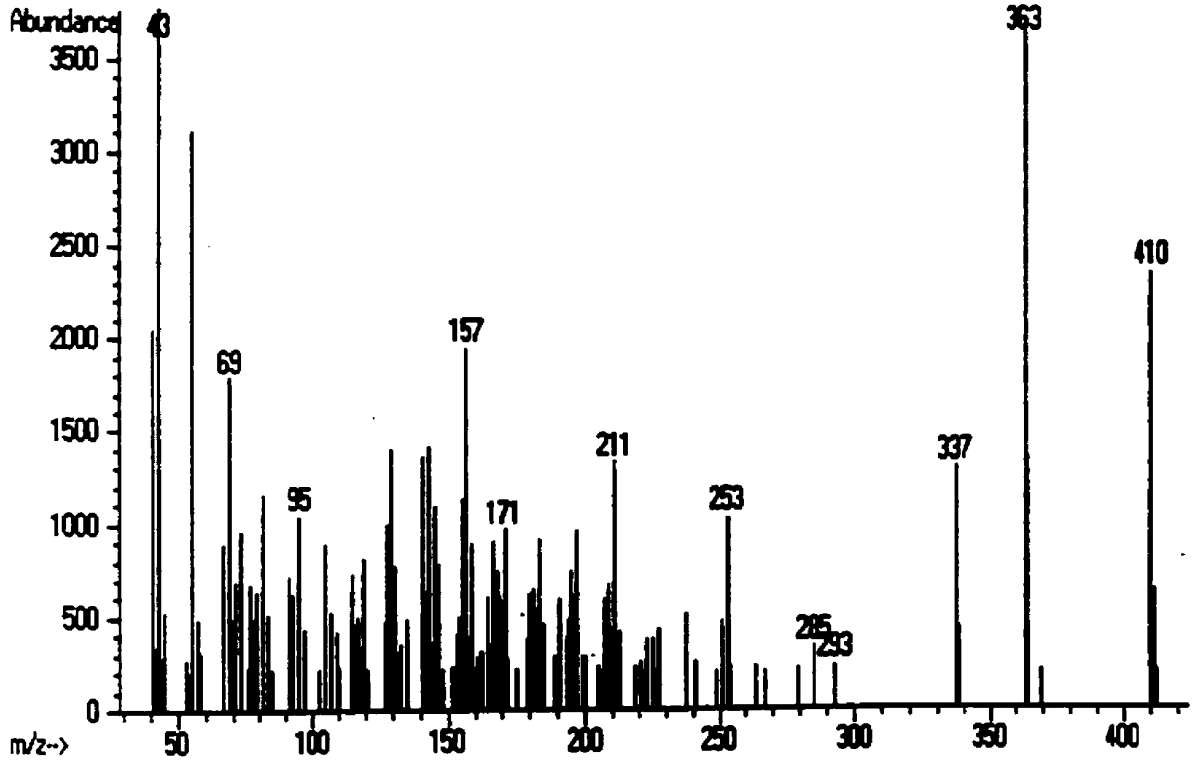


Figure 3.12 Mass spectrum of the thermochemolysis products of an unknown sterol in *B. trichodes*.

3.2.5 *Alectoria sarmentosa*

The chemical profile of the acetone extract of *Alectoria sarmentosa* is shown in Figure 3.13. This species was found to contain the same fatty acids found in *B. trichodes*. Also, there were several phenolic components including alectoronic acid and/or collatolic acid. These compounds differ only in the presence of a hydroxyl versus a methoxyl group, seen in Figure 3.14. They were not distinguished by this method due to the methylation step, so they were grouped together and were referred to as alectoronic acid. The final hold time was extended by five minutes to fully detect their high molecular weight pyrolysates.

Also present was usnic acid and another depside or depsidone that has not been fully identified. The mass spectra of two co-occurring thermochemolysis fragment peaks (As[310] and As[328]) of molecular weights 310 and 328 shown in Figure 3.15 exhibit patterns consistent with a typical lichen phenolic. Also, the apparent losses of methyl $(M-15)^{\cdot}$ and methoxy $(M-31)^{\cdot}$ groups in their mass spectra and the retention times of the As[310] and As[328] peaks support this hypothesis.

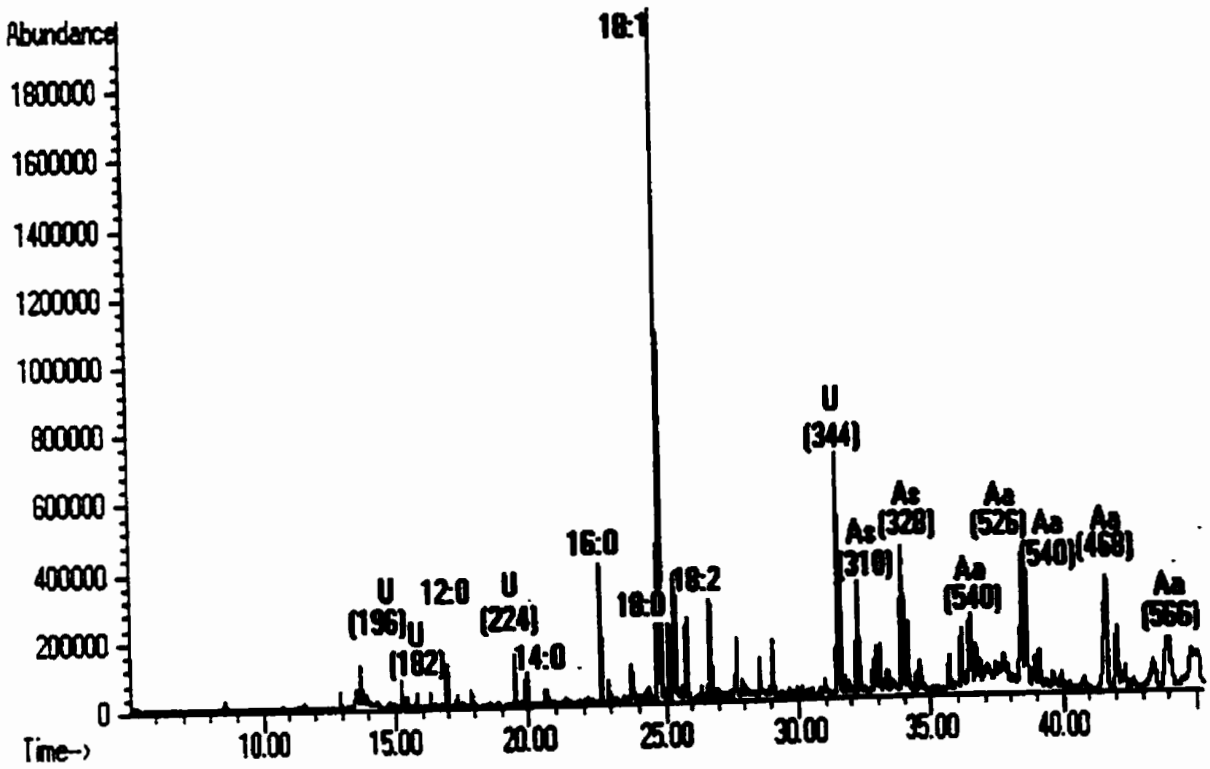


Figure 3.13 GC-MS total ion chromatogram of the acetone extract of *Alectoria sarmentosa*. ([ ] denotes the molecular weight of the TMAH thermochemolysis product. Thermochemolysis products are represented by U = usnic acid, Aa = alectoronic acid, As = alectosarmentin, 16:0 etc. = methyl esters of fatty acids.)

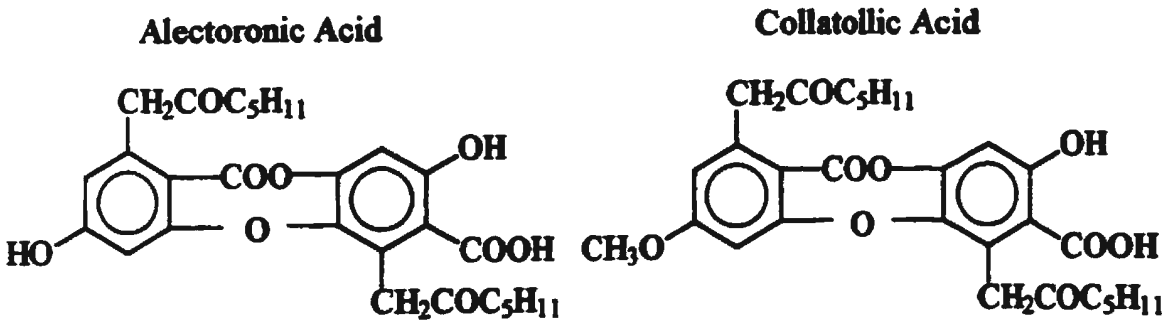


Figure 3.14 Structures of alectoronic acid and collatolic acid.

Several possibilities for the origin of these two fragments were found in the literature including squamatic acid, barbatic acid and physodic acid (16, 25, 51, 52). Samples of these acids were tested but none gave the desired pyrolysates. The structures of these lichen acids are shown in Figure 3.16. Alectosarmentin (Figure 3.17) has been detected in *A. sarmentosa* recently (52). It is a dibenzofuranoid lactol, the structure of which has only been identified in the last few years (52). It is possible that the 310 and 328 MW peaks are products from this compound. Methylation at the three hydroxyl groups would produce a 328 MW product easily (see Figure 3.17). A 310 MW product is more difficult to explain without knowing more about the thermochemolysis behavior of such compounds.

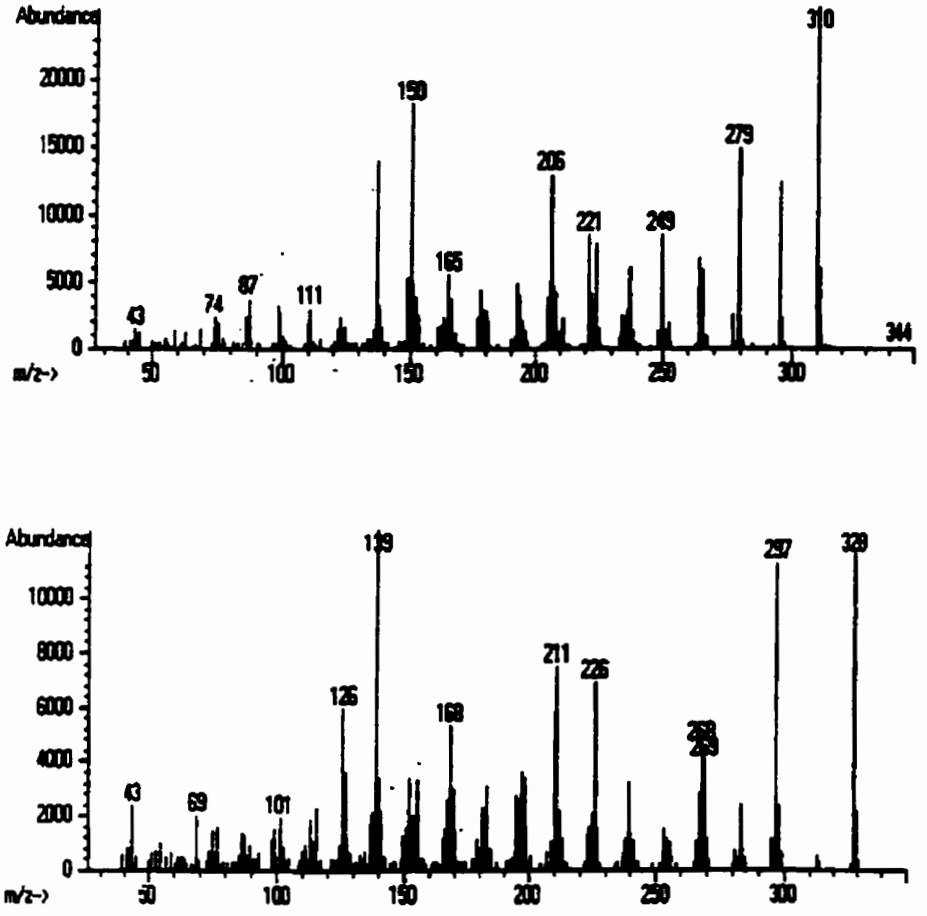
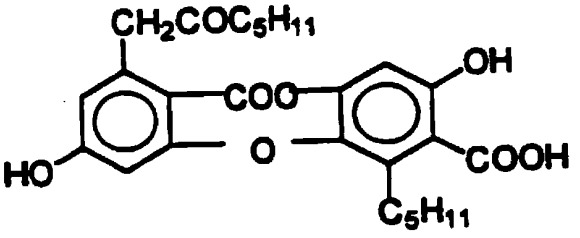
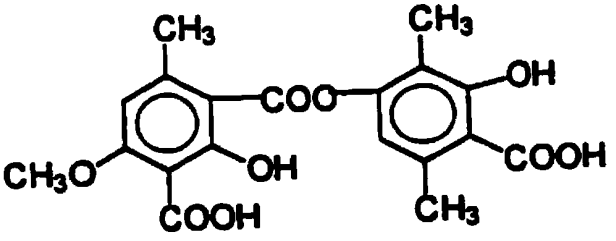


Figure 3.15 Mass spectra of the unknown thermochemolysis products (with MW 310 and 328) from *A. sarmentosa*.

Physodic Acid



Squamatic Acid



Barbatic Acid

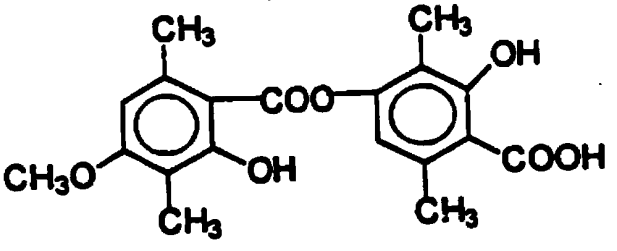


Figure 3.16 Structures of squamatic, barbatic and physodic acids (17).

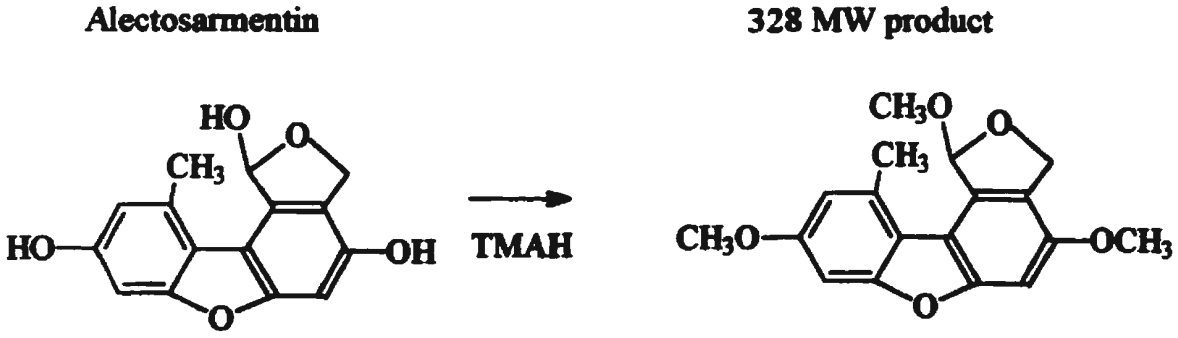


Figure 3.17 Alectosarmentin and the possible structure of the 328 MW product whose mass spectrum is shown in Figure 3.15.

Table 3.1 summarizes the main components detected via thermochemolysis fragment markers in each species, including a few compounds which have yet to be fully identified. Predictions have been made of the probable identifications from peak patterns, mass spectra and from published lists of the known components of some lichens (16, 25, 51).

Table 3.1 Chemical constituents of five lichen species' determined by TMAH-Pyrolysis-GC-MS.

Chemical	*Cladina rangiferina*	*Cladina mitis*	*Usnea dasypoga*	*Alectoria sarmentosa*	*Bryoria trichodes*
Lichen acids: alectoronic/collatolic acid				X	
atranorin	X				
fumarprotocetraric acid	X	X			X
salazinic acid			X		
usnic acid		X	X	X	
alectosarmentin (?)				X	
Higher fatty acids: rangiformic acid		X			
Sterols: stigmasterol	X	X	X		X
Fatty acids: 12:0		X	X	X	X
14:0		X	X	X	X
16:0	X	X	X	X	X
18:0	X	X	X	X	X
18:1	X	X	X	X	X
18:2	X	X	X	X	X
18:3			X		

3.3 Semi-quantitative Results Using GC-FID

3.3.1 Ultraviolet Study

3.3.1.1 *Cladina rangiferina*

In *Cladina rangiferina* the first compound of interest was atranorin. Figure 3.18 shows the trend of atranorin levels between the four light exposure conditions. The peak areas of the thermochemolysis markers of atranorin (ie. in Figure 3.6 A[180], A[166], and A[330]) were averaged for the three analyzed lichens. These were summed to give an overall measure of atranorin in each condition. Similarly, the error bars in the graph are the summed standard deviations from the markers. This method of data analysis is used throughout the semi-quantitative analysis of all species studied.

There is a slight increase in the visible condition, probably due to increased photosynthesis or replacement of atranorin that was degraded under the natural light conditions represented by the control. Under the visible with UV-A (or mylar) condition there is a slight decrease and this was also seen for the acetate condition, which included UV-B as well as visible and UV-A. This seems to indicate degradation of atranorin by ultraviolet light.

This trend is very interesting because of atranorin's strong absorbance of UV and proposed light filtering role (5). However, the standard deviations are quite large, leading one to conclude that the differences are less than significant. This may be due to

experimental factors as it also occurs with the other components, as well as with the other species in the study. In particular, the use of a three day exposure time may not be sufficient to cause significant quantitative changes in the lichen chemistry of these species. It was used because past work has shown significant qualitative changes after similar exposure times (M. Begora, personal communication). Also, the interthalline or individual differences in lichens are known to be an important factor (53).

The use of only three thalli, each of which were only analyzed once, may not be the best method for lichen studies. A larger sample set and/or replicate analyses from each thallus would provide more data allowing for better estimates of the true mean, leading to smaller standard deviations and therefore more significant results.

Within the same species the only other components that show any consistent trend were the fatty acids. The unsaturated fatty acids (in Figure 3.6, peaks 18:1 and 18:2) show a distinct trend across the four exposure conditions seen in Figure 3.19. Lichens exposed to visible light only show less fatty acids, either due to less stress or due to a decrease in photosynthesis that can occur in the presence of excessive visible light (5). Lichens exposed to visible plus UV-A light and those exposed to visible plus UV-A and UV-B light, showed a slight increase in unsaturated fatty acids. This may indicate increased production of lipids to bolster or repair membranes that have been damaged by UV-induced radical mechanisms (5). Again the results are less than significant.

The saturated fatty acids, seen as the methyl esters, (in Figure 3.6, peaks 16:0 and

18:0) showed a similar trend here as well, but in other species they are less reliable indicators of stress. This is because there are other compounds which will produce the same saturated fatty acid methyl esters as thermochemolysis products. The complex fatty acids, such as rangiformic acid will produce 16:0 and 18:0 saturated fatty acids as pyrolysates. The behavior of such peaks with contributions from different sources is difficult to interpret. As a result, only the unsaturated fatty acid markers were used. The other components of *C. rangiferina* showed no consistent trends in their levels across the exposure conditions.

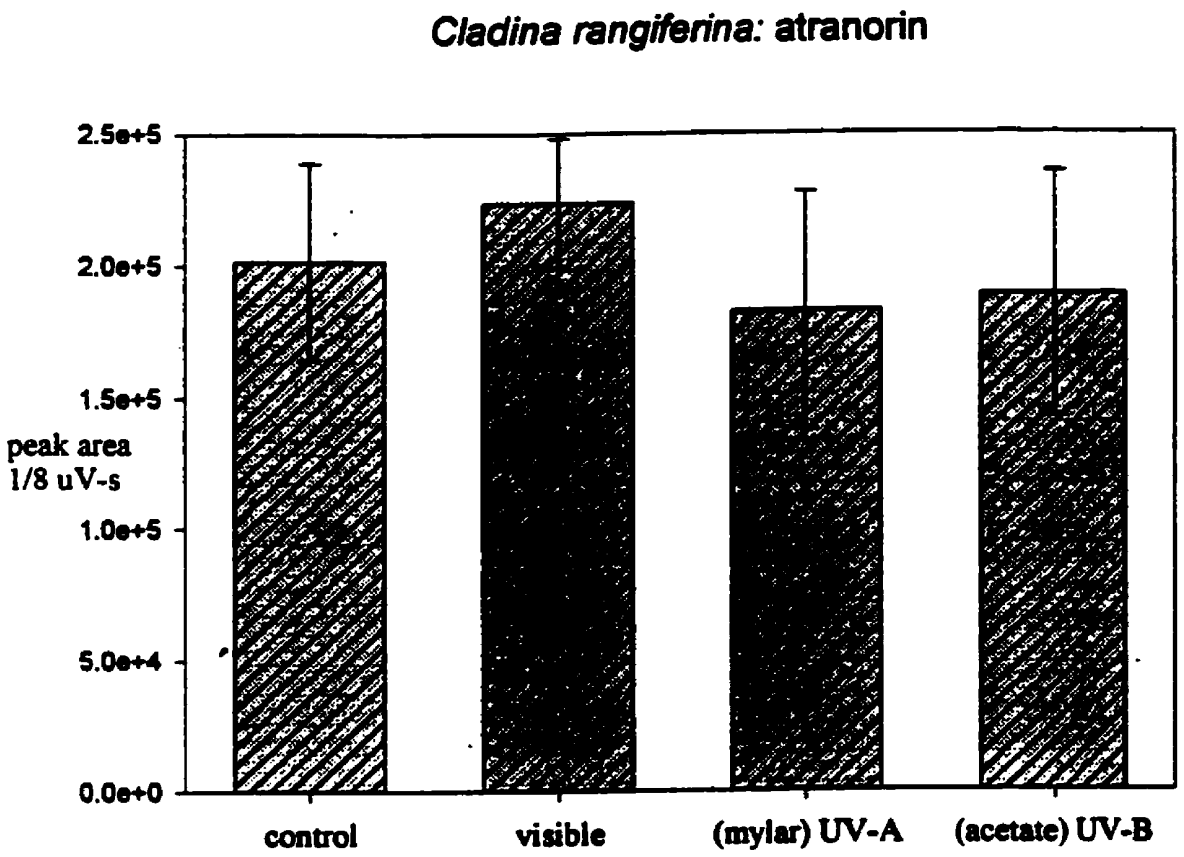


Figure 3.18 Relative content of atranorin in *C. rangiferina* exposed to various conditions of light.

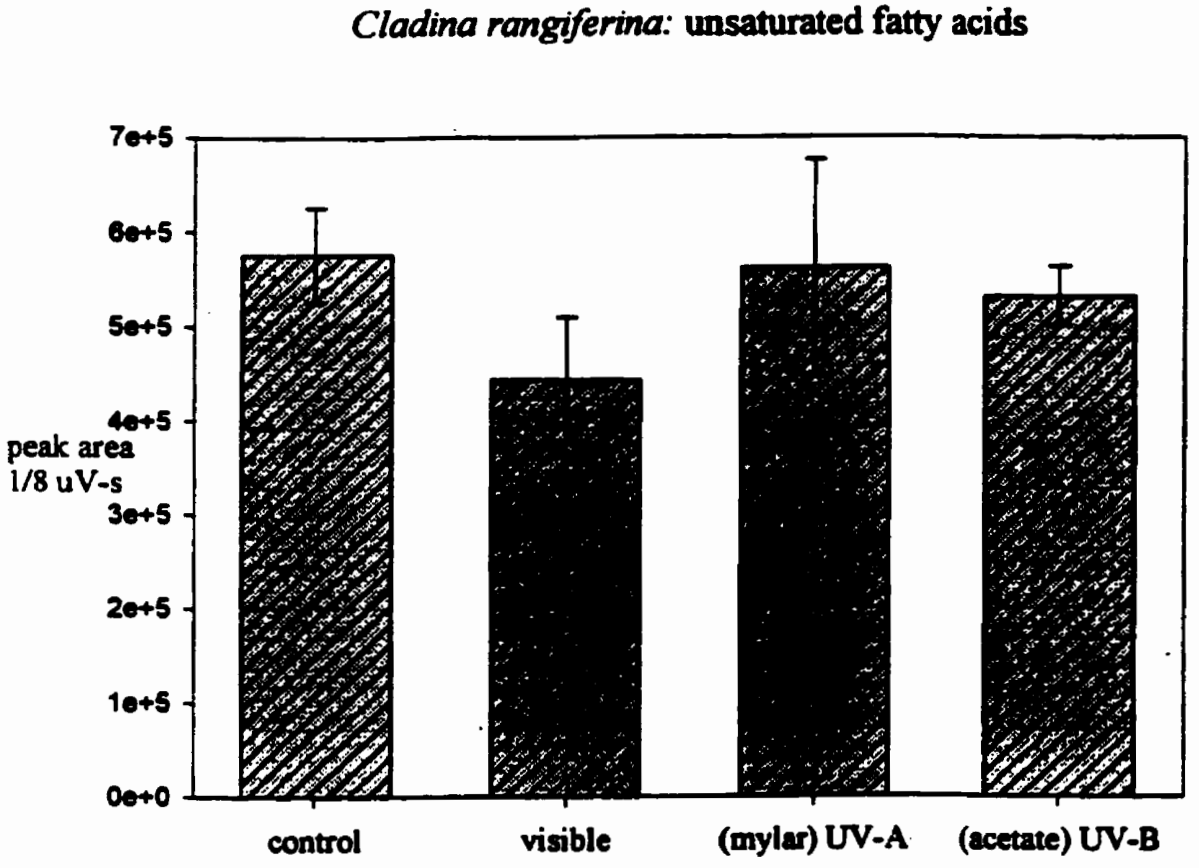


Figure 3.19 Relative content of unsaturated fatty acids in *C. rangiferina* exposed to various conditions of light.

3.3.1.2 *Cladina mitis*

The first compound of interest in *Cladina mitis* was usnic acid. Figure 3.20 shows the trend of the relative content of this acid across the light exposure conditions used. Like atranorin in *C. rangiferina*, the amount of usnic acid shows an increase under visible light alone. This is likely due to increased photosynthesis or less UV stress compared to the natural control condition. There is a slight decrease in the presence of UV-A, seen in the mylar condition. This may be due to degradation of usnic acid upon UV exposure. In the presence of UV-B there is a slight increase, possibly indicating an increase of usnic acid production triggered by UV-B (7). This would be a defensive response.

Again, the standard deviations are relatively large and overlap, leading to less than significant results. However, it is interesting to note that the trend resembles that seen with atranorin in *C. rangiferina*. Usnic acid is another UV absorber with strong absorbance through the whole range of UV-B (5). These two compounds often seem to replace one another in closely related species' suggesting they perform similar roles as light filters (5). Usnic acid also absorbs visible light differently resulting in a characteristic yellow color, whereas most lichen phenolics including atranorin are colorless (4, 17).

The trend for rangiformic acid content is shown in Figure 3.21. This trend is very similar to that seen with usnic acid. In the visible condition, there is slightly more

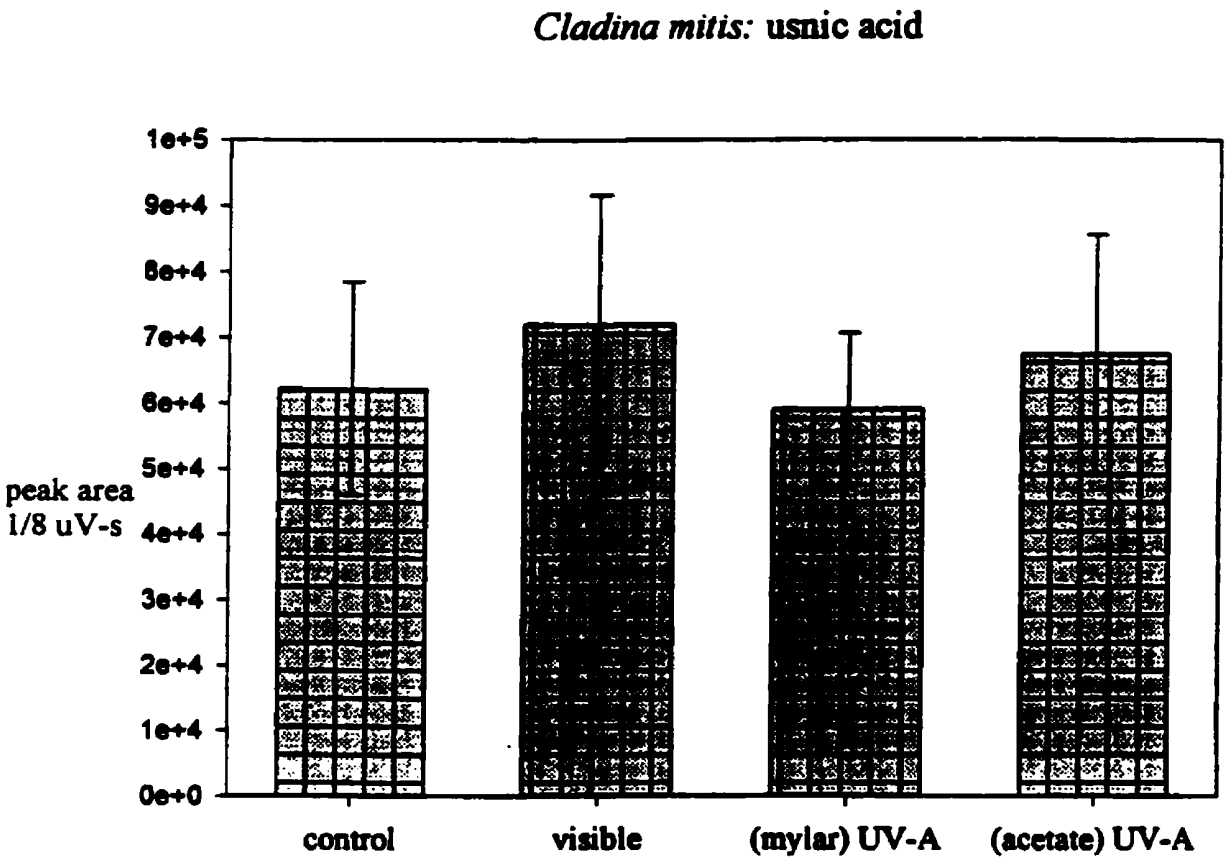


Figure 3.20 Relative content of usnic acid in *C. mitis* exposed to various conditions of light.

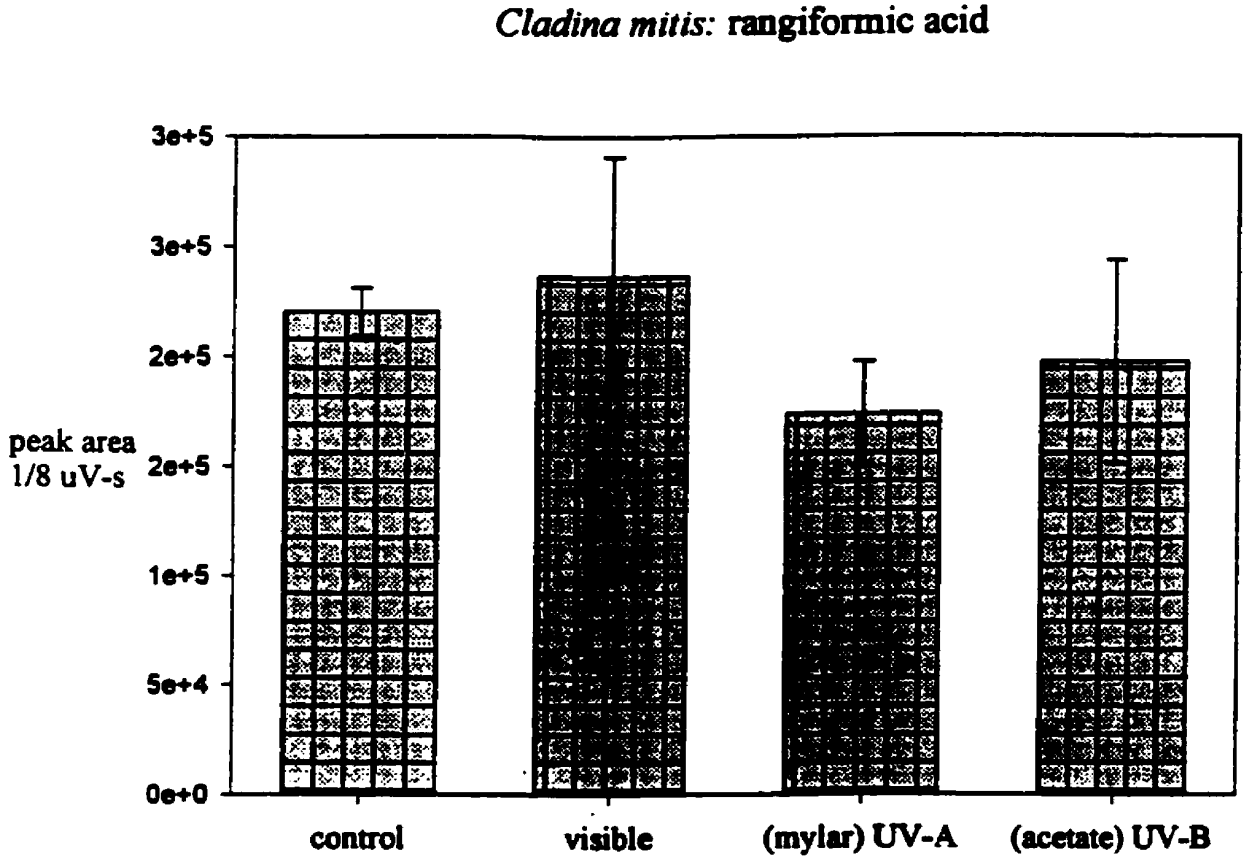


Figure 3.21 Relative content of rangiformic acid in *C. mitis* exposed to various conditions of light.

rangiformic acid but it decreases in the presence of UV-A. When UV-B is introduced there is a slight increase. This is likely due to increased photosynthesis from visible light, stress degradation from UV-A, and a stress-induced increased in production from UV-B.

In the same species, the unsaturated fatty acids also show a distinct trend. It is shown in Figure 3.22 where one can see a steady increase in fatty acids up to and including the UV-A condition, with a slight decrease once UV-B is introduced. The increase in fatty acids may first reflect increased photosynthesis and finally stress in the UV-A condition, where damaged membranes are repaired or built up by the production of more lipids. The decrease seen in the UV-B condition seems to coincide with the slight increases found in usnic and rangiformic acids, suggesting a reduction in stress in the presence of these defensive compounds.

The other components of the *C. mitis* extracts did not show distinct trends. Those discussed above are still less than significant, as were those from *C. rangiferina*. They have large error bars which tend to overlap, leading one to question the validity of the trends seen. However, larger sample sets, replicate analyses, and longer exposure times may yet yield significant results from these species.

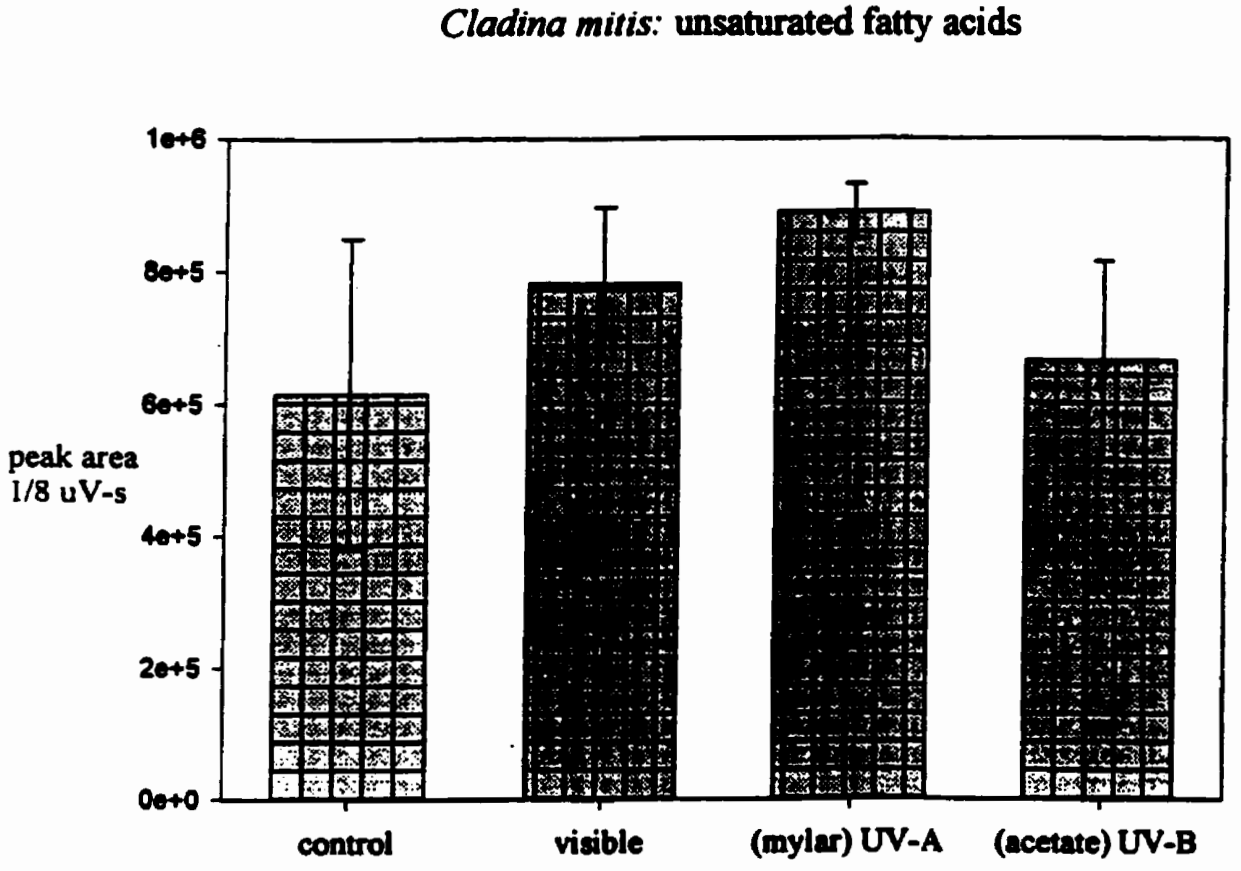


Figure 3.22 Relative content of unsaturated fatty acids in *C. mitis* exposed to various conditions of light.

3.3.2 Ozone Study

Usnea dasypoga was analyzed further using the semi-quantitative method (GC-FID). Two main components of the acetone extracts showed significant changes between sample sites of varying ozone levels. Figure 3.23 shows the trend seen with salazinic acid content. The ozone levels measured from the passive monitors are indicated along the x-axis with the sample site number. There was a significant drop between the lowest ozone level site and those between 21-26 ppb. At the highest ozone level (29 ppb) the salazinic acid was significantly increased.

The initial drop seemed to be due to degradation by increased ozone exposure. Salazinic acid is a depsidone and is likely to have antioxidant activity (19). Thus, it is not surprising to see a reduction in this compound under oxidative stress. The increase seen at the highest ozone level seemed to indicate a stress-induced production of the acid, likely a defensive response. Further studies are necessary to support this hypothesis, though other studies have suggested that the production of lichen phenolics like usnic acid may be induced by stressful UV-B exposure (4, 54).

The unsaturated fatty acids showed a distinctly different pattern, seen in Figure 3.24. At low ozone levels these fatty acids were relatively low. However, as the ozone levels increased so do the fatty acids. At the highest ozone level, the unsaturated fatty acids showed a significant decrease.

This initial increase was likely due to increased lipid production used to repair or maintain the membranes that were under attack (27). At the highest ozone level the decrease in fatty acids coincided with the increase in salazinic acid. This suggests that a there is a relationship between the two, possibly that the induced production of salazinic acid had decreased the amount of membrane stress. Again, this is just a hypothesis and further studies are required particularly confirming that the relative ozone levels are true and reproducible.

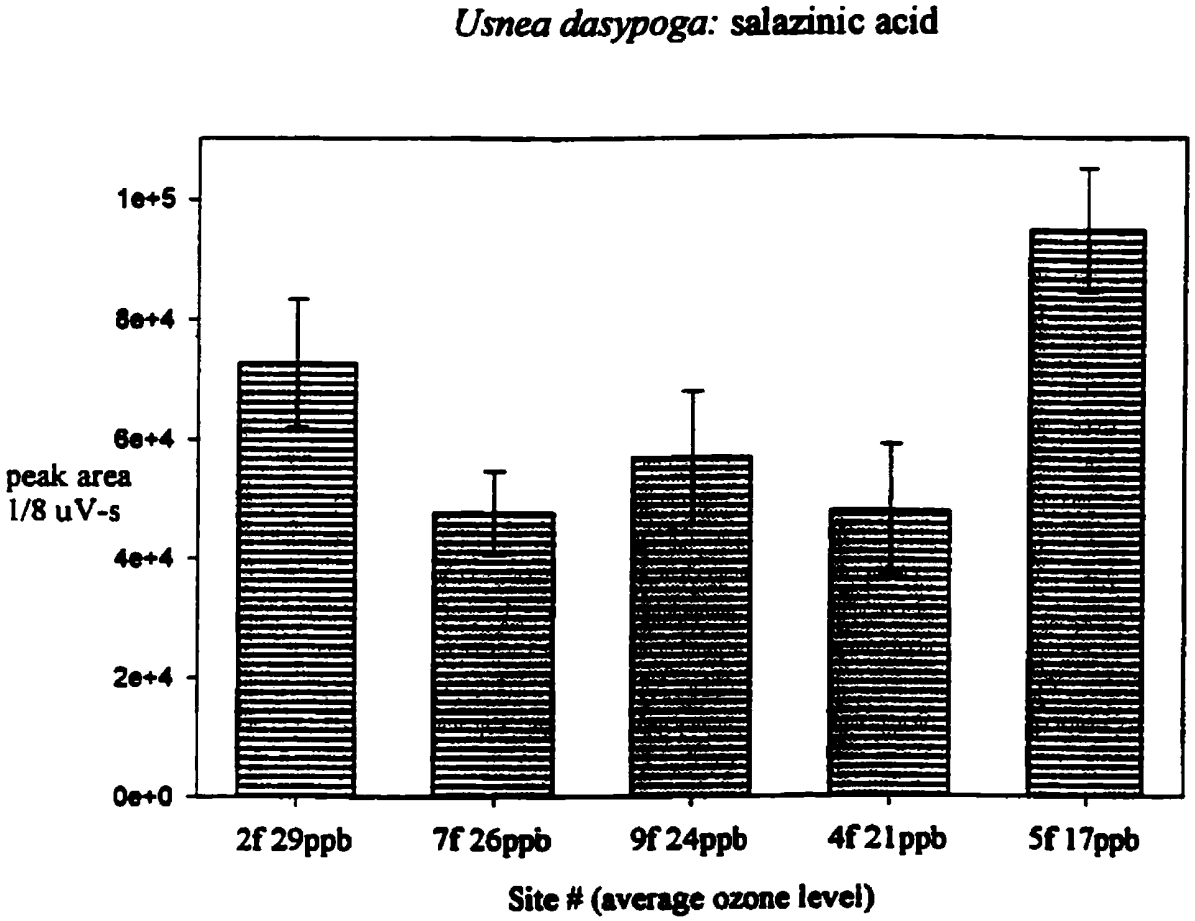


Figure 3.23 Relative content of salazinic acid in *U. dasypoga* from various sites of ozone exposure.

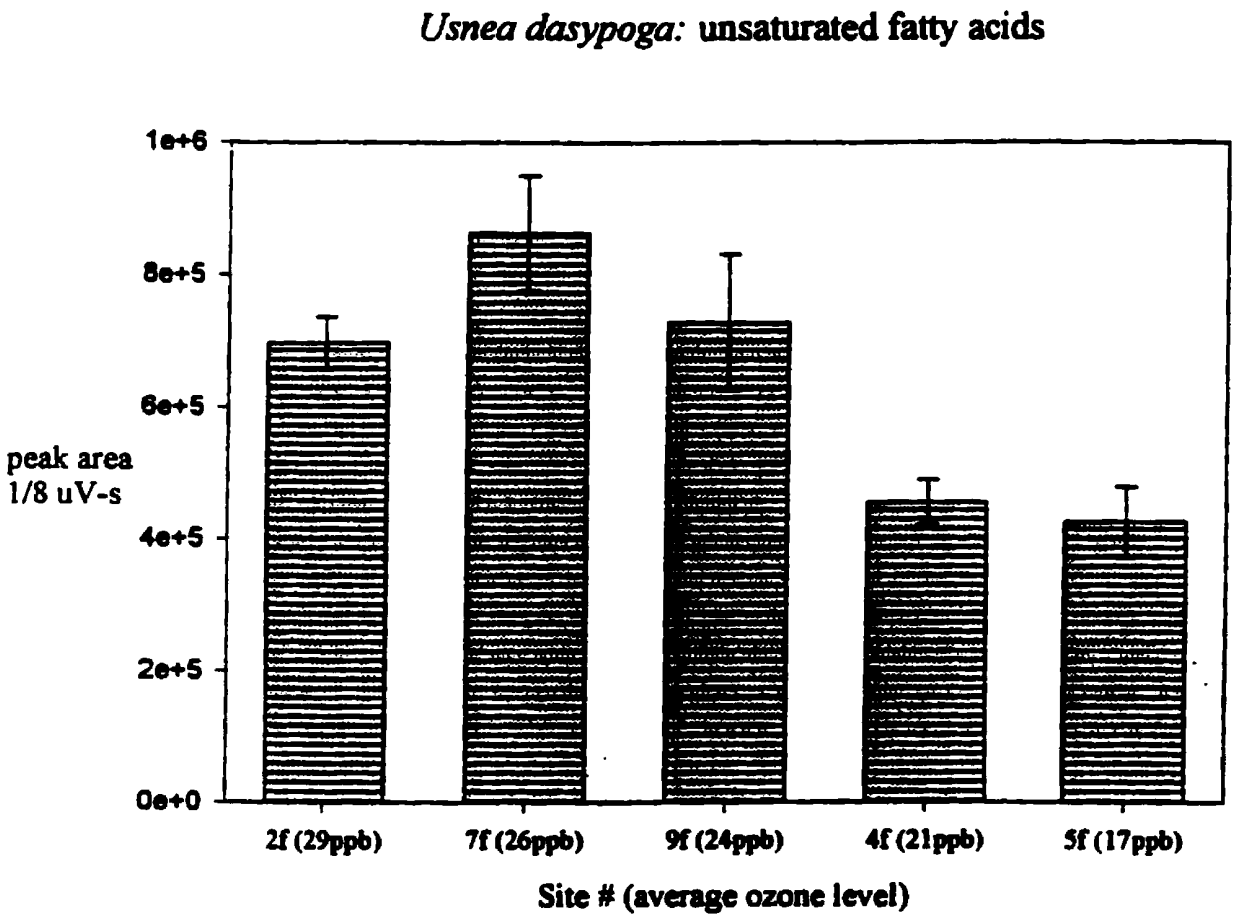


Figure 3.24 Relative content of unsaturated fatty acids in *U. dasypoga* from various sites of ozone exposure.

3.3.3 Sulfur Dioxide Study

Alectoria sarmentosa showed changes in three main components of its acetone extracts. Alectoronic acid's pattern of occurrence at the various sample sites is shown in Figure 3.25. There was little change between the four sites at Come by Chance and Random Island, but there was a significant decrease at the Bonavista site. This suggested a response to some kind of environmental change but it does not appear to correspond to sulfur dioxide and its likely source, the oil refinery.

The unsaturated fatty acids showed a distinct pattern seen in Figure 3.26. At the two sites nearest Come by Chance where the oil refinery was located, the acids had similarly low levels. At the Random Island site R2 there were significantly more fatty acids. At the other Random Island and Bonavista sites, there were lower levels compared to R2, but they were still significantly higher than those seen at the Come by Chance sites.

It is difficult to explain these patterns. There were significant differences seen between sites but they do not seem to be related to their relative location to the refinery (SO_2 source). There are other several factors at work in the environment such as humidity, pollutants from nearby roads, and light exposure for which no account has been made. The presence of additional factors is often a problem with field studies, hence much care must be taken in selecting sites that are as similar as possible with the exception of the factor of interest. However, the observations made at this time can be

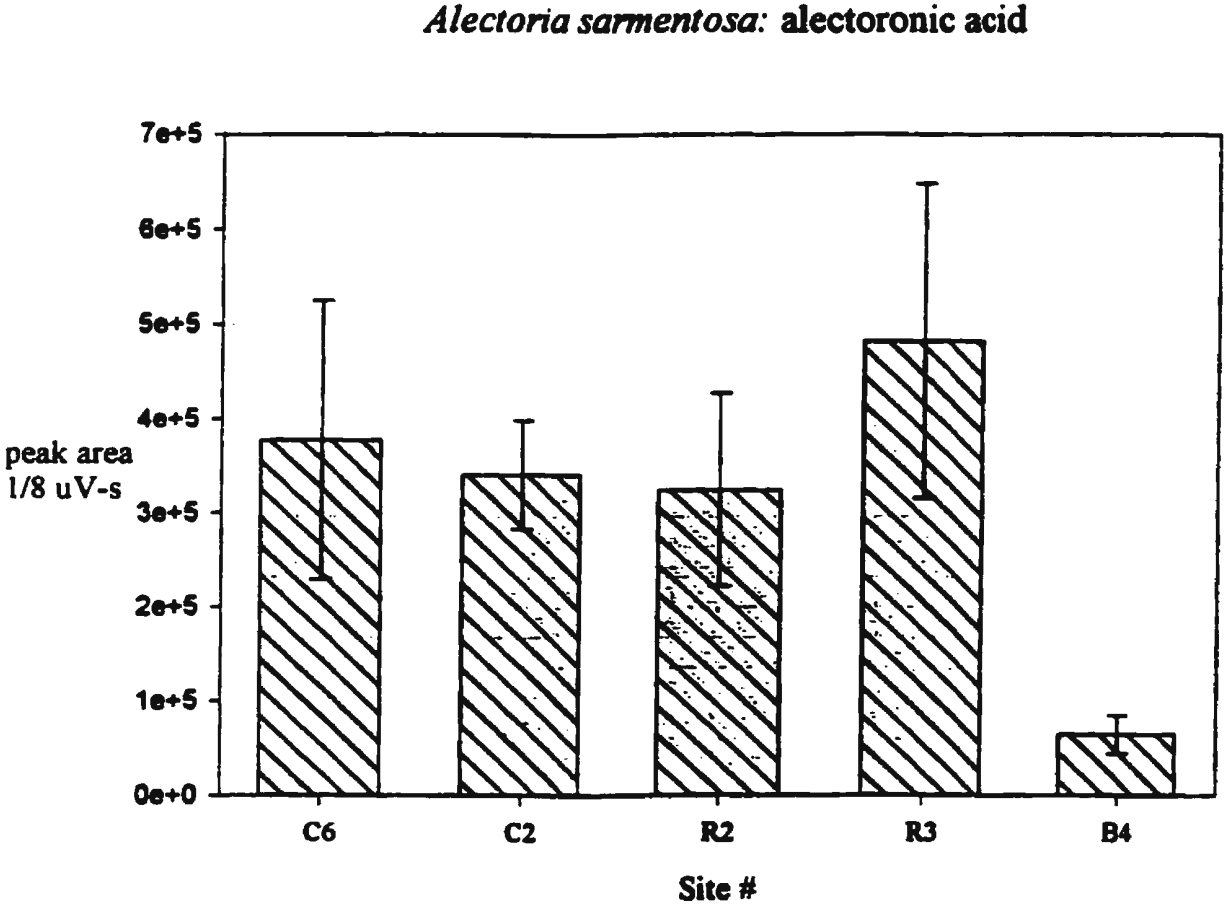


Figure 3.25 Relative content of alectoronic acid in *A. sarmentosa* from various sites of sulfur dioxide exposure.

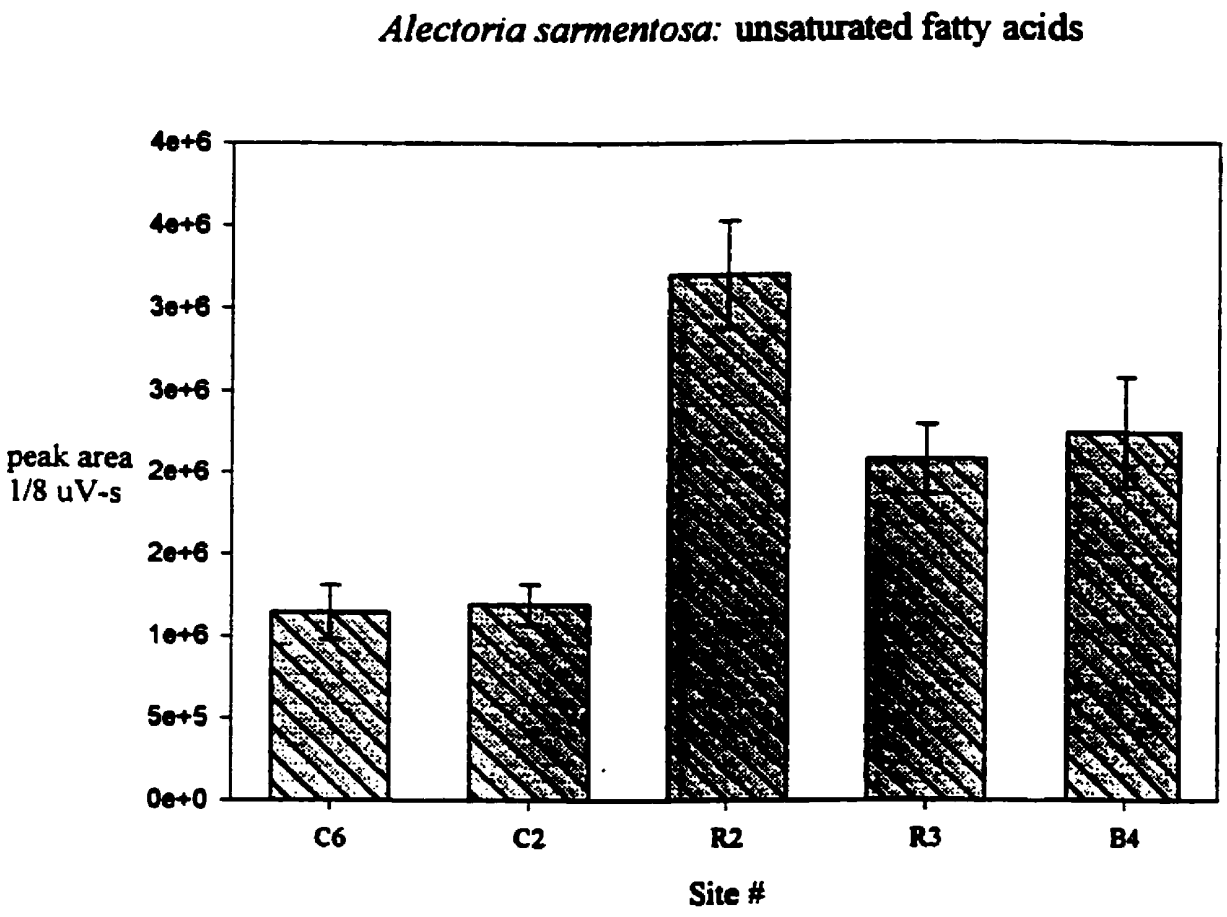


Figure 3.26 Relative content of unsaturated fatty acids in *A. sarmentosa* from various sites of sulfur dioxide exposure.

used as a basis for further work.

The final thermochemolysis markers of interest have not been fully identified yet, as discussed earlier (section 3.2.5). The pattern is shown in Figure 3.27. There is a great deal of the markers present at the first site (C6) which was located southward and therefore essentially upwind of the oil refinery (42). The lichens were large and relatively healthy in appearance at this site. The next site (C2) was the closest one downwind, and it shows a drastic decrease in this marker. The lichens and other vegetation as well as the air quality felt by the researchers themselves, were much poorer at this site. At the Random Island and Bonavista sites, the marker levels were increased from the minimum seen at site C2.

The first downwind site seemed to show intense degradation of the marker possibly due to the presence of sulfur dioxide stress. The increase seen at the more remote sites seemed to indicate some recovery from this stress. The marker has been tentatively identified as a lichen phenolic, possibly alectosarmentin as discussed in section 3.2.5.

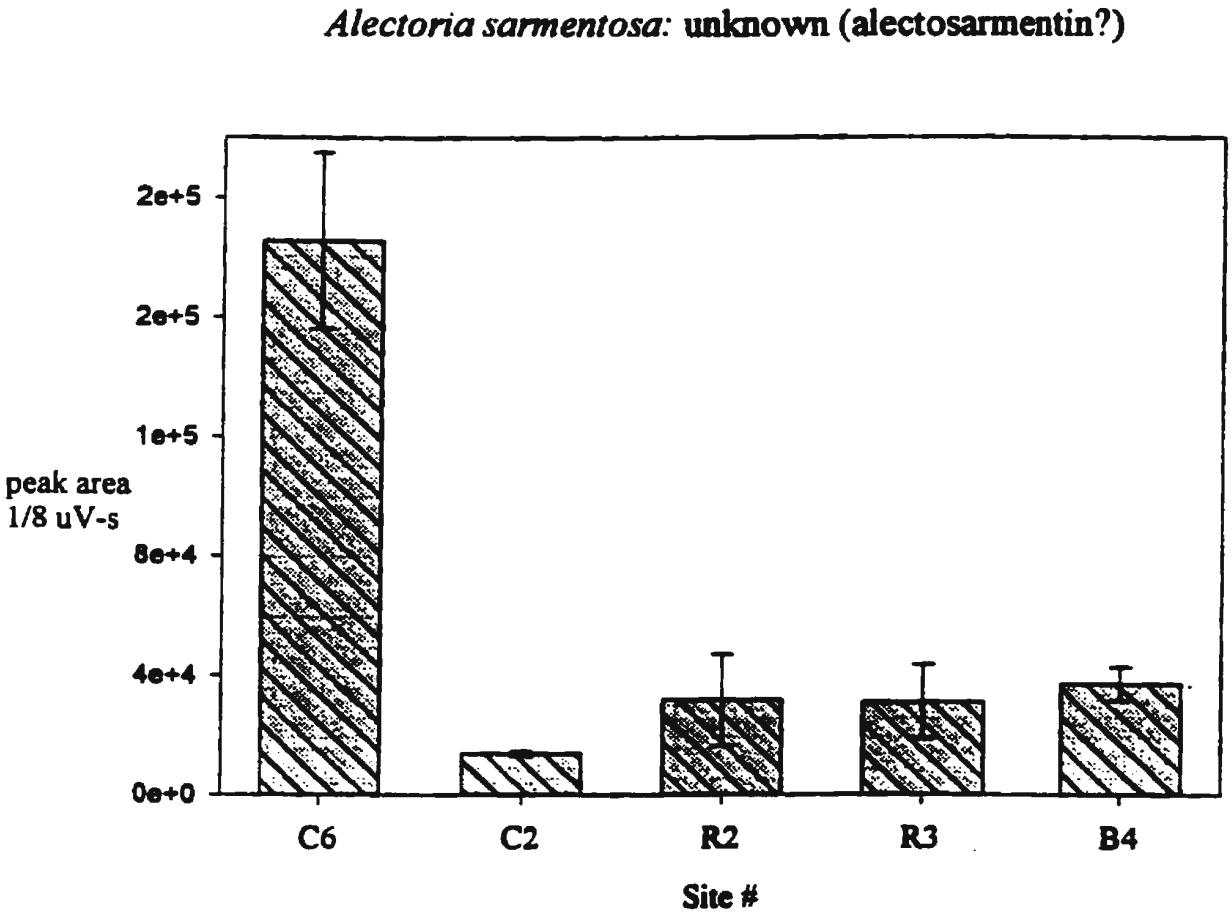


Figure 3.27 Relative content of unknown lichen phenolic (possibly alectosarmentin) in *A. sarmentosa* from sites of various sulfur dioxide exposure.

CHAPTER 4

CONCLUSIONS

In conclusion, TMAH thermochemolysis, combined with gas chromatography using a mass spectrometer, is a suitable technique for the qualitative analysis of lichen acetone extracts. The resulting GC-MS total ion chromatogram (TIC) profiles may be of use to taxonomists for distinguishing new and/or closely related species of lichens based on their phenolic components (17). This method allows for the detection and identification of the lichen phenolics, complex fatty acids, simple fatty acids, and some sterols based on characteristic pyrolysates determined from reference compounds.

When this method is modified by the use of a flame ionization detector, it becomes suitable for semi-quantitative analysis. This can be useful for bio-monitoring studies as different components show distinctly different trends under different conditions. Given the high sensitivity of lichens (1) this could eventually provide a system of early detection of environmental stress.

From the ultraviolet study it is difficult to draw any strong conclusions due to problems with the significance of the results. Interesting trends suggest that usnic acid, rangiformic acid, and the unsaturated fatty acids may be compounds of particular interest in *Cladina mitis*. Atranorin and the unsaturated fatty acids seem to be compounds of interest in *Cladina rangiferina*. The trends exhibited by these compounds seem to be of a

stronger and less random nature than those of the other components of these lichens.

From the ozone study more significant results allow for the identification of salazinic acid as an indicator of a defense response in *Usnea dasypoga*. The unsaturated fatty acids seem to be good indicators of apparent ozone stress in this species. The salazinic acid and unsaturated fatty acids present in the lichens exposed to ozone stress seem to show an inverse relationship with one another.

From the sulfur dioxide study, several components of *Alectoria sarmentosa* showed significantly different levels at different sample sites. Alectoronic acid and the unsaturated fatty acids do not seem to be responding to proximity to the refinery. The compound tentatively identified as alectosarmentin does seem to be influenced greatly in lichen samples sampled very close and downwind of the oil refinery.

There are several areas in which further work can be done. More significant results might be obtained from UV exposure of the *Cladina* species' if larger sample sets, replicate analyses, and longer exposure times were used. Other species might also be used.

For the ozone study, analysis of the second lichen *Bryoria trichodes* may reveal whether fumarprotocetraric acid, another depsidone, plays a similar defensive role against ozone damage as salazinic acid seemed to do in *Usnea dasypoga*. Also, lab experiments using more direct ozone level control might be useful to verify our findings as well as to determine what levels of ozone are required to cause the responses seen in salazinic acid

and the unsaturated fatty acids. Another approach to field sampling would be to sample lichens in areas where there are visible signs of ozone damage to tree stands such as white pine.

Lab experiments with controlled sulfur dioxide exposures would be helpful for the third study. This would determine whether any or all of the compounds changing in the field samples of *Alectoria sarmentosa* were doing so in response to this stress or another such as predation, infection by bacteria or fungus, or drought (4). Also, an attempt might be made to obtain a sample of alectosarmentin to verify whether it is the source of the 310 and 328 MW unknowns.

Once the stress indicators have been identified fully in each case, selected ion monitoring (SIM) of suitable markers may be used with Py-GC-MS. The resulting selectivity would allow the exclusion of the acetone extraction step, saving time and effort. Preliminary work monitoring the 328 fragment in *A. sarmentosa* has shown higher levels in the C6 sample compared to the C2 sample, as was found with the acetone extraction method.

Future work might involve sampling lichens from different substrates to see if they affect the stress responses. Also, investigating one species of lichen under each stress study might allow the researcher to determine whether unique responses occur to each stress or if they are the same regardless of the kind of stress. Eventually this method may be useful for long-term field studies of air quality and light stress.

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APPENDICES

Appendix 1 Site Descriptions: Ozone Study in New Brunswick

Site 2 Point Lepreau

Ozone plate numbers: set 1 P729, P727

set 2 P896, P895

G.P.S. coordinates (Global Positioning System): N 45 deg 03' 806"

E 66 deg 27' 303"

Species' sampled: Usnea dasypoga

Bryoria trichodes

Directions:

Start from the Hugh John Flemming Building which houses the Canadian Forestry Service. Take a right at the lights at the main driveway and turn right at the first exit. Take Exit 293 onto Route 7. Take Exit 97 onto Route 1 towards St. Stephen and Lorneville. Take Exit 73 onto Route 790 towards Lepreau, Maces Bay and Dipper Harbor. Continue down to the Point Lepreau Power Plant. You must call ahead to get through security, speak to Curtis Nason in Health Physics (659-6490). Once through the security gate, continue on paved road past the plant. Take the second left onto a dirt road and continue around the back of the storage buildings and plant. Turn right onto the lighthouse road, also a dirt road. Stop where the nature trail starts, on the left side of the road within sight of the lighthouse. This is 4.6 km from the point where Route 790 is first crossed after the Lepreau and Maces Bay turn off. The monitor and sampled lichens

were located on the right side of the trail, in the woods directed back towards the dirt road at the first turn of the trail. There was a tree banded in orange tape ~ 10 m north of the monitor.

Site 4 Chance Harbor

Ozone plate numbers: set 1 P725, P721 active monitors

P735, P728 blanks (wrapped in parafilm)

set 2 P884, P885 active monitors

P888, P886 blanks (wrapped in parafilm)

G.P.S. coordinates: N 45 deg 05' 745"

E 66 deg 26' 266"

Species' sampled: Usnea dasypoga

Bryoria trichodes

Directions:

Take the first right out from the Point Lepreau plant security gate on "Old Ridge Road". Turn right at the stop sign ~ 0.5 km from the gate. You should pass a graveyard ~ 1.7 km form the gate. Take a left at the Y-shaped intersection towards Chance Harbor, ~ 2.7 km from the gate. Take another left onto a dirt road 0.4 km from the Y-shaped intersection, just before the bridge. There is a small bridge on the dirt road which must be crossed. Continue on the dirt road keeping to the right until past some small shacks. There is a pit

where a car can be parked. The sample site is located about halfway up the hill on the left towards the hydro-line tower. A small fir was banded with orange paint at the base of the hill next to a dead tree trunk. The monitor was left ~ 10 m south of the tape-banded tree, and lichens were collected in the same spot.

Site 5 Provincial Park Trail

Ozone plate numbers: set 1 P707, P730

set 2 P887, P902

G.P.S. coordinates: N 45 deg 10' 250"

E 66 deg 26' 440"

Species' sampled: Usnea dasypoga

Bryoria trichodes

Directions:

From the Point Lepreau plant security gate, take the right onto Old Ridge Road heading back towards the Petromart and Route 790. There is a provincial park skidoo/all-terrain trail on the right side of the road about 9.4 km from the Point Lepreau Gate. On the left side of the road is a dirt road at about the same place where one can park. Walk along the trail across the small bridge and past the point where the trail crosses the large power lines. A juniper is marked on the right side of the trial with orange paint. About 10 m north is a fir where the ozone monitors were left and lichens were sampled.

Site 7 Rural Road A

Ozone plate numbers: set 1 P715, P703

set 2 P899, P900

G.P.S. coordinates: N 45 deg 15' 738"

E 66 deg 29' 542"

Species' sampled: Usnea dasypoga

Bryoria trichodes

Directions:

Continue North along Route 790 (from Site 5) to the stop sign 0.5 km past the Lepreau, Maces Bay, Dipper Harbor turning from Route 1. Take a left at the stop sign onto a dirt road. Go over a bridge 6.6 km from the stop sign. Stop where there is a sandy clearing on the left side of the road, 10.6 km from the stop sign. A tree at the edge of the pit is marked with orange paint. The ozone monitor and lichens were located about 30 m from the road, ~ 20 m of the sandy clearing and ~ 10 m in the trees.

Site 8 Rural Road B

Ozone plate numbers: set 1 P710, P739 active monitors

P726, P736 blanks (wrapped in parafilm)

set 2 P893, P892 active monitors

P894, P897 blanks (wrapped in parafilm)

G.P.S. coordinates: N 45 deg 21' 310"

E 66 deg 29' 767"

Species' sampled: Usnea dasypoga

Bryoria trichodes

Directions:

From Site 7, continue along the same dirt road. Cross a bridge 5.8 km from Site 7 (16.4 km from stop sign). Take a right 9.3 km from Site 7 (20.9 km from the stop sign). Cross another bridge 12.9 km from Site 7(24.5 km from the stop sign). Take a left at the fork located 15.6 km from Site 7 (27.2 km from the stop sign). Stop where cutting occurs and where a white pine 'skeleton' is seen on the right side of the road. A birch tree can be seen further up hill hanging out over the road from the left side. This is 18.5 km from Site 7 (or 30.1 km from the stop sign). There is a track at the edge on the right side of the road. A tree was marked with orange tape on the left of the track at the edge of the woods. There are a series of 3 tape-marked trees leading to a wide tree banded with tape. This is ~ 10 m North of the location of the monitor and the sampling site.

Site 9 Rural Road C

Ozone plate numbers: set 1 P738, P731

set 2 P901, P873

G.P.S. coordinates: N 45 deg 28' 470"

E 66 deg 28' 113"

Species' sampled: Usnea dasypoga

Bryoria trichodes

Directions:

From Site 8, continue until a fork occurs 4.7 km from Site 8. Take the left branch and continue 6.9 km until another turning. Take the right branch. Stop where there is a small place to park on the right which leads up to a trail, ~ 14.6 km from Site 8. This is the last high hill along this dirt road. A tree at the edge of the trail on the right side was marked with paint. It was located near a tire several meters along the trail. The monitor and lichens were located a few meters away.

Appendix 2 Site Descriptions: Sulfur dioxide Study in Newfoundland

Site C6 (CBC 006)

Species sampled: Alectoria sarmentosa

Directions:

From Clarenville, head on Route 1. Take a left off Route 1 onto "Refinery Road" toward the refinery owned by North Atlantic Refining Limited. At about 0.8 km from Route 1 take a left onto a dirt road. Stop 0.7 km along the dirt road. There is a pond on the left side (due East of the sample site). The lichens were sampled from the woods on the right side of the road.

Site C2 (2-CBC)

Species sampled: Alectoria sarmentosa

Directions:

From Route 1 take the left at the Come by Chance turnoff. Stop where the crest of a small hill crosses a dirt/gravel path which was the old railway, ~ 1.2 km from turnoff. Walk along the path ~ 100 m and turn left towards the trees. This is ~ 50 m before a pond. The trees where the lichens were sampled are ~ 50 m in from the path.

Site R3 (3-RI)

Species sampled: Alectoria sarmentosa

Directions:

On Random Island, take Route 231 until the Brookside Take-out is reached, just before Hickmans Harbour. The stream nearby flows from Hickmans Harbour Big Pond into Tween Bridge Pond and eventually Hickmans Harbour. Take a left onto the dirt road. Stop where a trail goes to the stream on the left and continues up the hill on the right side of the road, ~ 100 m from the highway. Walk up the trail. The trees at the crest of the hill were sampled.

Site R2 (2-RI)

Species sampled: Alectoria sarmentosa

Directions:

The site is near the Hickmans Harbour Big Pond. Continue on the dirt road from Site R3 for about 2 km. Pass one dirt road that goes to the waters edge on the right. Stop at the second road to the water. The trees nearby were sampled.

Site B3 (3-B)

Species sampled: Alectoria sarmentosa

Directions:

The site is located along highway 230 on the Bonavista peninsula. Before reaching the town of Bonavista, stop where a dirt road appears on the left of Route 230. This is just

past the Bonavista Hotel (with the "O Happy Sight" sign) and just before Route 230 crosses the old railway tracks. Sampling was done on the right side of the highway, about 75 m from the road along a rough trail in the woods (likely a moose trail).

Site B4 (4-B)

Species sampled: Alectoria sarmentosa

Directions:

The site is located ~ 500 m from Route 230 along a gravel cart-track. The cart-track appears just around the place where the old railway tracks cross the highway. Walk along the track, passing a pond and power lines on the left side. Samples were collected in the woods on the left side, at the top of a small hill, just where the track curves right.

Appendix 3 Raw Data From Ozone Monitor Plates

Plates used in Tanya MacGillivray's lichen study, 1998

	exposed		collected			
Site#	date	time	date	time	plate	exp/cont
2	7/16/98	11:55	8/6/98	10:55	P729	E
2	7/16/98	11:55	8/6/98	10:55	P727	E
4	7/16/98	13:00	8/6/98	11:41	P735	C
4	7/16/98	13:00	8/6/98	11:41	P728	C
4	7/16/98	13:00	8/6/98	11:41	P721	E
4	7/16/98	13:00	8/6/98	11:41	P725	E
5	7/16/98	15:16	8/6/98	12:50	P730	E
5	7/16/98	15:16	8/6/98	12:50	P707	E
7	7/16/98	16:50	8/6/98	13:15	P715	E
7	7/16/98	16:50	8/6/98	13:15	P703	E
8	7/16/98	18:25	8/6/98	14:30	P726	C
8	7/16/98	18:25	8/6/98	14:30	P736	C
8	7/16/98	18:25	8/6/98	14:30	P710	E
8	7/16/98	18:25	8/6/98	14:30	P739	E
9	7/16/98	19:55	8/6/98	15:30	P738	E
9	7/16/98	19:55	8/6/98	15:30	P731	E
2	8/6/98	10:55	8/19/98	10:52	P896	E
2	8/6/98	10:55	8/19/98	10:52	P895	E
4	8/6/98	11:41	8/19/98	11:15	P888	C
4	8/6/98	11:41	8/19/98	11:15	P886	C
4	8/6/98	11:41	8/19/98	11:15	P884	E
4	8/6/98	11:41	8/19/98	11:15	P885	E
5	8/6/98	12:50	8/19/98	11:41	P887	E
5	8/6/98	12:50	8/19/98	11:41	P902	E
7	8/6/98	13:15	8/19/98	13:15	P899	E
7	8/6/98	13:15	8/19/98	13:15	P900	E
8	8/6/98	14:30	8/19/98	13:45	P894	C
8	8/6/98	14:30	8/19/98	13:45	P897	C
8	8/6/98	14:30	8/19/98	13:45	P893	E
8	8/6/98	14:30	8/19/98	13:45	P892	E
9	8/6/98	15:30	8/19/98	15:15	P901	E
9	8/6/98	15:30	8/19/98	15:15	P873	E

data from Lepreau analyser:

edat	etime	cdat	ctime	avgppb	hrs
7/16/98	19:55	8/6/98	15:30	34.9	499.6
8/6/98	10:55	8/19/98	10:52	31.8	312.0

** based on 98 field calibration: ppmh=avg.abs / .021 corrected.

plate	exp/cont	ABS	average	blanks	ppmh**	hours	avg. ppb
P729	E	0.363	0.332	0.045	15.79	503.0	31.4
P727	E	0.390					
P735	C	0.042					
P728	C	0.056					
P721	E	0.283	0.234		11.14	502.7	22.2
P725	E	0.275					
P730	E	0.265	0.198		9.43	501.6	18.8
P707	E	0.221					
P715	E	0.307	0.261		12.43	500.4	24.8
P703	E	0.305					
P726	C	0.037					
P736	C	0.044					
P710	E	0.378	0.314		14.95	500.1	29.9
P739	E	0.340					
P738	E	0.246	0.226		10.76	499.6	21.5
P731	E	0.296					
P896	E	0.294	0.176	0.077	8.38	312.0	26.9
P895	E	0.212					
P888	C	0.069					
P886	C	0.095					
P884	E	0.210	0.132		6.29	311.6	20.2
P885	E	0.208					
P887	E	0.180	0.096		4.57	310.9	14.7
P902	E	0.166					
P899	E	0.265	0.171		8.14	312.0	26.1
P900	E	0.231					
P894	C	0.074					
P897	C	0.071					
P893	E	0.208	0.158		7.52	311.3	24.2
P892	E	0.262					
P901	E	0.266	0.177		8.40	311.7	27.0
P873	E	0.241					

Appendix 4 Summary of Ozone Data

	Ozone Concentration		(ppb)
Site #	July 16-Aug. 6	Aug. 6- Aug. 19	overall average
2	31.4	26.9	29.1
4	22.2	20.2	21.2
5	18.8	14.7	16.8
7	24.8	26.1	25.5
8	29.9	24.2	27.0
9	21.5	27.0	24.3
Lepreau Analyzer	34.9	31.8	33.4