ANALYSIS OF LICHEN PHENOLICS AS ENVIRONMENTAL STRESS INDICATORS BY LIQUID CHROMATOGRAPHY COUPLED TO MASS SPECTROMETRIC AND UV-VIS DIODE ARRAY DETECTORS

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by

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ABSTRACT

Lichens that are under environmental stress undergo changes in their metabolism and biochemistry. The most interesting compounds that are involved in this response are the lichen phenolics. Changes in the relative amounts of these compounds might indicate differing levels of environmental stress, such as ultraviolet light exposure and ground level ozone. Semi-quantitative analysis of dried and ground lichen samples was performed on an Agilent 1100 series LC-MS using reversed phase liquid chromatography with ESI-MS and UV diode array detection.

Cladina mitis and *Cladina rangiferina* exposed to differing exposures of ultraviolet light were obtained from Ontario. *Usnea dasypoga* and *Bryoria trichodes* were collected from different sites with varying ground level ozone exposure in New Brunswick. Changes in the relative amounts of lichen phenolics such as usnic acid, atranorin and fumarprotocetraric acid were of interest as these compounds have demonstrated ultraviolet absorption in the UV-B wavelengths and/or antioxidant properties. These compounds may be involved in a defensive response of the lichen to environmental stress.

A method of analysis was developed that requires neither a chemical derivatization step nor a pre-concentration step. This method improvement simplifies the analytical method, shortens analysis times and removes the necessity for the use of toxic reagents. These improvements increase the viability of this method for use as a future pollution monitor in rural and undeveloped areas.

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LIST OF ABBREVIATIONS AND SYMBOLS

‘	minutes (used in G.P.S. coordinates)
“	seconds (used in G.P.S. coordinates)
API-ES-MS	atmospheric pressure ionization-electrospray mass spectrometry
AU	units of absorbance
B. trichodes	*Bryoria trichodes*
C. mitis	*Cladonia mitis*
C. rangiferina	*Cladonia rangiferina*
DAD	diode array detector
ESI	Electrospray ionization
eV	electron volts
FIA	flow injection analysis
FID	flame ionization detector
GC	gas chromatography
GPS	global positioning system
HPLC	high performance liquid chromatography
m/z	mass to charge ratio
NO_X	oxides of nitrogen
NSERC	Natural Sciences and Engineering Research Council of Canada

LC-MS	liquid chromatography-mass spectrometry
ppb	parts per billion
s	shoulder
TIC	total ion current
TLC	thin layer chromatography
TMAS	tetramethylammonium hydroxide
U. dasypoga	*Usnea dasypoga*
UV	ultraviolet
UV-A	ultraviolet class A
UV-B	ultraviolet class B
UV-C	ultraviolet class C

CHAPTER 1

INTRODUCTION

1.1 Lichens and Environmental Monitoring

Lichens have been used for decades as monitors of the health of ecosystems (1, 2, 3, 4). This can be done by observing the quality of live lichen, (5, 6) or by cataloguing all the lichen species in an area and tracking the numbers of species over time (1, 7). A reduction in the health of the lichen or downward trend in biodiversity might indicate that a particular ecosystem was under some environmental stress (1). An increase in airborne pollution may affect the abilities of some more sensitive lichens to compete with more hardy varieties, which would lead to a decrease in biodiversity of lichens in that area (1). A different approach to using lichens as environmental monitors involves quantitatively analysing for pollutants, like lead, (8) uranium, (9) fluoride (10, 11, 12) and zinc (13), which have been absorbed into the porous lichen tissue (or thallus). Lichens are long-lived organisms that are very porous and tend to absorb pollutants that have been deposited on them, either from rainfall or from the atmosphere. Lichen are also sensitive to gaseous pollutants like SO_2, NO_2, and O_3, due to their large surface area and lack of defences that are common to the higher plants (waxy cuticle, stomata, etc.) (1). Studies have also shown that lichen are sensitive to increased harmful ultraviolet rays, (14, 15, 16) conditions like those that can be found in Antarctica, where maximum thinning of upper level ozone has been observed. Another study found that by determining the maximum tolerable concentration of SO_2 for different lichen species, and then mapping

areas where each lichen was found to be thriving, it was possible to create a map of SO_2 concentrations (1). Each of these studies employ lichens as passive environmental monitors. Our goal in this project was to determine whether the phenolic compounds produced by the lichen and believed to be linked to defence (1, 17, 18, 19) could be analysed quickly by LC-MS so that these responses could be used as a quantitative passive biomonitor for environmental stressors such as ground level ozone and increased levels of harmful ultraviolet radiation.

1.1.1 Biology and Biochemistry of Lichens

Lichen are a group of organisms that exist as a symbiotic relationship between an algae and a fungus (1). The fungus (or mycobiont) provides a fibrous structure of hyphae for the algae (or photobiont) to grow on, and the algae provides sugars from photosynthesis as nourishment for the fungus (1). The fungal and algal partners can be separated and can be cultured separately, however, since they have evolved together and have specialized to exist as members of the symbiotic relationship, neither symbiont will flourish alone (20). The body of a lichen is referred to as the thallus, (plural thalli). Lichen are subdivided into three different forms, crustose, foliose and fruticose (1, 5, 10). Crustose lichens grow embedded in the outer layers of a substrate, with little exposed tissue (e.g. lichen that grow on rocks that cannot be scraped off with a knife). The second type of lichen is foliose, which has structures that seem to resemble leaves or petals, attached at a centre point and radiating outwards, with the "petals" also attached to the surface of the substrate, usually parallel to the substrate. Fruticose lichens are stem-like

or hair-like in that they have structures that are only attached at a single point. An example of fruticose lichen is the type of lichen known as "Old Man's Beard" which hangs from trees in hair-like strands. All the lichens used in this study are fruticose lichens. *Cladonia mitis* and *C. rangiferina* are reindeer lichens that grow on the ground, while *Bryoria trichodes* and *Usnea dasypoga* are "Old Man's Beard" types. The mechanisms that lichens use for defence are not as well understood as the defences of the higher plants. Higher green plants have a waxy outer cuticle which allows the leaves to shed deposited pollutants better, and pores (called stomata) that can be closed to restrict the flow of airborne pollutants to the inside of the leaf (5, 10). Many lichen produce complex phenolics and it is hypothesized that these phenolics may play a role in protecting the lichen from environmental damage. These lichen phenolics consist of depsides, depsidones and dibenzofurans (21, 22) where the depsides consist of two or three orcinol or β-orcinol moieties linked by an ester bond (a depside bond), the depsidones are similar with the addition of an oxygen bridging bond that completes the characteristic 7 membered ring and the dibenzofurans whose skeleton matches that of their namesake. The lichen phenolics are not produced in significant quantities by either the mycobiont or the photobiont if they are allowed to grow separately (1). These compounds are found as crystalline extracellular deposits so it is hypothesized that they are not necessary for internal cell metabolism (23).

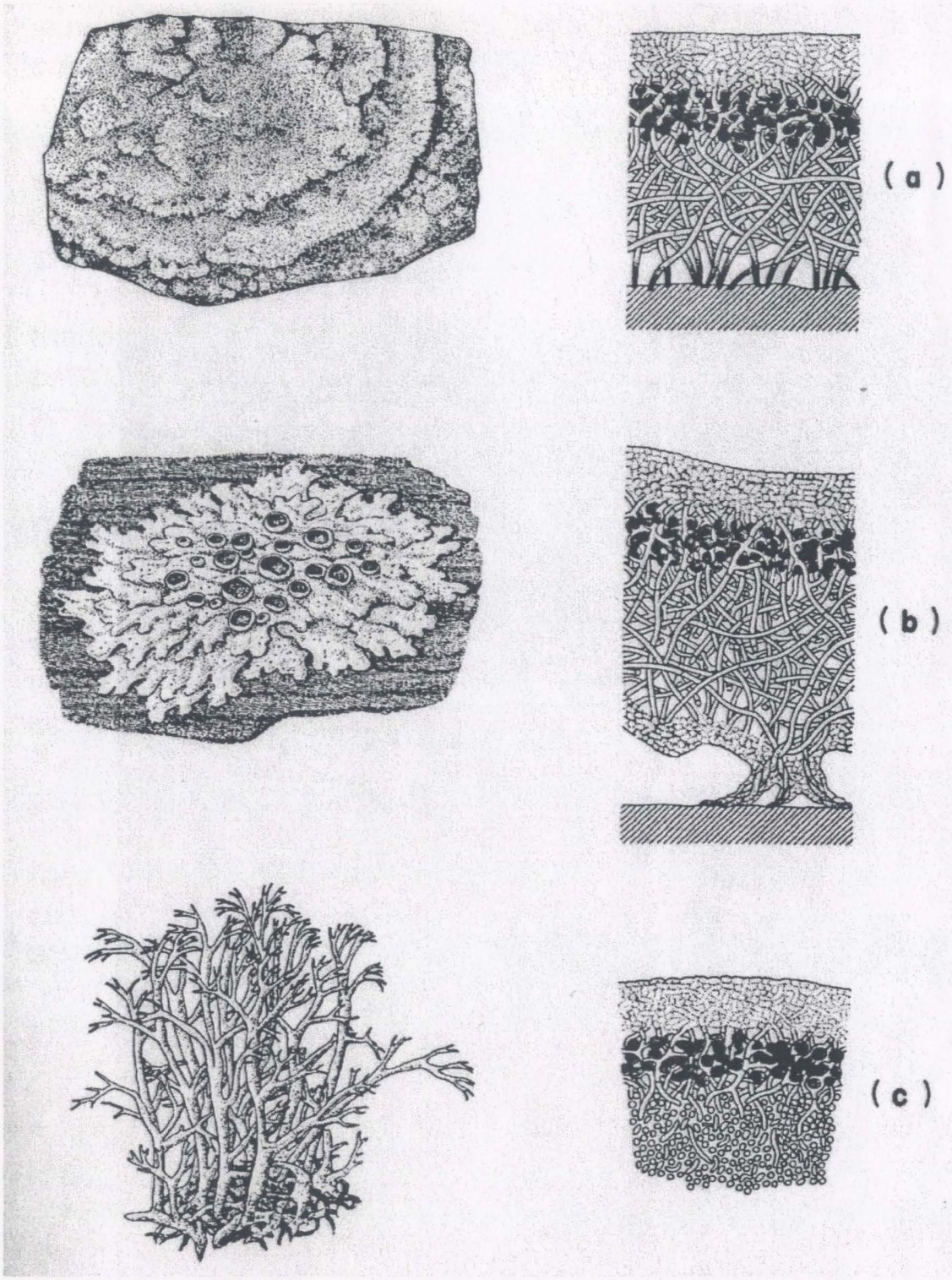


Figure 1.1 Growth forms of the lichen thallus a) crustose b) foliose c) fruticose (1)

1.1.2 Use of Lichen as Environmental Monitors

Most lichen can only be found in pristine areas where there is little to no air pollution, (1, 10) however, there are a few species, such as *Xanthoria* that thrive in more polluted urbanized areas (1). Surveys can be used to map the ranges of various lichens with different tolerances to airborne pollution. The data can then be used to produce a geographical representation of mean levels of air pollution (1). Over time, changing concentrations of pollutants can be mapped, and by using data about prevalent wind directions and speeds, the sources of this pollution can be identified (1).

If the goal of the study is to observe the effects of pollution in a small area, then a different technique can be employed. This technique involves monitoring how many different species of lichen are present in an area, and observing how that number changes with time. Areas with increasing levels of pollution may experience a loss of biodiversity over time, as the more sensitive lichens are no longer competitive (1). Comparing relative population amounts of lichens that are sensitive to pollution to lichen populations that are relatively hardy can shed insight into the changing levels of pollution in an area. Transplantation of mature lichen into polluted areas can discern the physical or chemical effects of high levels of airborne pollution on a sensitive lichen species so that such effects can be used in areas where there has been a rise in pollutant concentrations (1, 24, 25).

Figure 1.2 Reindeer lichen in Howley, Newfoundland. (Photograph taken by the author.)

One chemical technique involves extracting pollutants that have been deposited or precipitated on the lichen by prevailing winds or rainfall from the lichen thallus and developing a geographical representation of the concentrations of airborne pollutants in the area sampled (1). The porous structure of the lichen provides a large surface area for adsorption and absorption. Heavy metals such as uranium, (9) zinc, (13) and lead (8) and non-metals like fluoride (10, 11, 12) have been studied in this fashion. This method is useful in the study of analytes that form deposits on the lichen surface, or that are adsorbed into the lichen structure, however the type of pollutants that are of interest to this study are not persistent and cannot be extracted from the lichen so a different methodology is necessary.

It is has been proposed that lichen phenolics may be a form of defence against such stressors as harmful levels of ultraviolet radiation (23, 26, 27, 28) and oxidizing ground-level ozone (19, 28). If that is the case, there may be an increase in the production of these lichen phenolics in areas with higher levels of stressors, or there may be changes in the lichen phenolics at the molecular level, in the form of photolysis or oxidative damage (29, 30). If these lichen phenolics are analysed quantitatively, a link between concentrations of phenolics in the lichen thallus and degrees of environmental stress could be found.

1.1.3 Atmospheric Stressors

As has already been discussed, lichens are long lived organisms (1) that share many qualities with the more complex plants, but that are generally thought to be lacking

in sophisticated defence mechanisms against the effects of airborne pollution and harmful UV rays (1). Some of the main constituents of gaseous airborne pollution include ground level ozone, and SO_2. Heavy metals can also be deposited on the lichen by prevailing winds and by contaminated rainfall.

Ground level ozone is produced by a photochemical reaction between volatile organic compounds and oxides of nitrogen (Knox) (5, 10), high concentrations can be harmful to people, livestock and crops (5, 10). Not to be confused with the stratospheric ozone layer which filters out the higher energy UV rays, ground level ozone is formed mainly as a result of burning fossil fuels and chemical manufacturing processes (10). Ozone decomposes in a living organism to produce hydrogen peroxide (H_2O_2) hydroperoxide (HO_2^-) and hydroxide (OH^-) anions and hydroxyl ($OH\cdot$) and oxygen ($O\cdot$) radicals, which in turn cause oxidative damage to membrane lipids, fatty acids and proteins (10, 31, 32). This causes a disruption of membrane integrity which causes lipids to leak into the cytoplasm (32). Photosynthesis ceases and protoplasmic membranes disintegrate. Oxidation of membrane lipids is believed to be one of the most important mechanisms in ozone injury (32). Lichen phenolic acids that have antioxidant properties would combat this injury, and some of the lichen phenolics have been demonstrated to possess antioxidant activity (33).

The damaging effects of ultraviolet radiation increase as wavelengths get shorter, so the spectrum of UV light is separated into three classifications: UV-A (320-400 nm), UV-B (320-290 nm) and UV-C (200-290 nm) (2, 34). UV-C is the most energetic type of UV radiation, however it is completely absorbed in the stratosphere (2). UV-B radiation

is therefore the most energetic type that reaches the surface of the earth, though in low intensity as most is absorbed by ozone in the stratosphere (2). Thinning of the protective ozone layer is allowing higher intensities of UV-B radiation to reach the surface of the earth (2). UV-B radiation promotes carcinogenic and mutagenic activity, and rising levels are a cause for concern (2). UV-B radiation can cause formation of dimers, replacements and other changes to cellular DNA. Contrary to expectations, the latest evidence suggests that high levels of UV light passing through the ozone hole in the Antarctic are having little effect on photosynthesis (16). Antarctic mosses and liverworts increase production of sunscreen pigments to block out harmful UV rays (16). Many lichen phenolics have an absorption maximum in the UV-B range, (2) as can be seen in Table 1.1, so it is possible that these compounds form a type of defence for the lichen. Studies have shown a negative correlation between amounts of lichen phenolics and cumulative amounts of UV-B exposure (2, 28).

	λ_{MAX}
Atranorin	210, 252, 262s, 320
Usnic acid	234, 282
Fumarprotocetraric acid	212, 240, 318

Table 1.1: Absorbance maxima of three lichen phenolics that have been suggested as part of a defense mechanism against UV radiation. (39)

1.1.4 Lichen phenolics

The lichen acids, more aptly named lichen phenolics, are of particular interest to chemists (1, 17, 20). These lichen phenolics are produced by the symbiotic relationship in the lichen, though some may be produced in small quantities in the mycobiont (35).

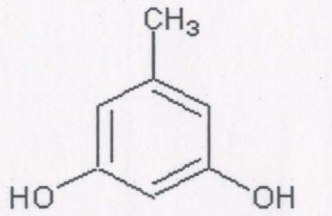
There are several types of lichen phenolics differentiated by their structural characteristics (1). Many of these phenolics are substituted depsides, depsidones and dibenzofurans, most containing at least one carboxylic acid moiety (20, 21). The lichen phenolic is considered to have two different portions for the purposes of naming, an "acid" piece and an "alcohol" piece, that is determined by their position relative to the ester linkage between them. (In a typical esterification reaction one reagent is an alcohol and the other is a carboxylic acid). The orcinol (5-methylbenzene-1,5-diol) depsides have a carbon-carbon bond in an *ortho-* position to the ester bond, while the *β*-orcinol (2,5-dimethylbenzene-1,3-diol) depsides have carbon-carbon bonds in both the *ortho-* and *meta-* positions, with the *meta-* carbon-carbon bond flanked by two carbon-oxygen bonds (see Figure 1.4) (21). Depsidones differ in that the two aromatic moieties are joined by an ether bond as well as an ester bond, thus forming a 7-membered heterocyclic ring. The lichens that were in this study contain β-orcinol depsides, depsidones or dibenzofurans. Each lichen species may contain one or more lichen phenolics (22, 29). Some lichen phenolics are also found in small quantities in the fungi that comprise the mycobiont of a lichen, though not in the high amounts that are seen in lichens themselves (35).

It is thought that the production of lichen phenolics must be necessary for the survival of the organism as it would consume energy to produce these substances that could be best utilized elsewhere, however the specific purpose for the production of these phenolics is unknown. It has been hypothesized that lichen phenolics may provide a form of UV defence, (29) defence from oxidative stress, (33) anti-feedant (preventing consumption by insects or animals) (18, 29) or alelopathic (antagonizing organism that

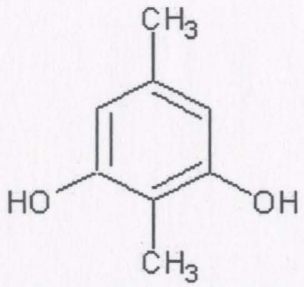
would compete for the same location) (29) action. Many lichen phenolics have been shown to exhibit these defensive activities that could be self-promoting to the lichen.

If these lichen acids are actually a form of defence, then it can by hypothesized that stressors affecting the lichen would cause one of two measurable responses in the levels of lichen phenolics present: either the lichen will produce more of the required phenolic in an effort to counteract the effects of higher amounts of stress, or the lichen phenolics will react with the stressor to neutralize its harmful effects, and be decomposed to a different, possibly less active form in the process of protecting the lichen (29, 30).

Three lichen phenolics are highlighted due to their abundance in the lichens studied. Atranorin (Figure 1.4, B) has been shown to absorb UV-B radiation (26), usnic acid (Figure 1.6) is a UV-B absorber (26) and has also demonstrated antioxidant properties, and fumarprotocetraric acid (Figure 1.5, E) is a depsidone and depsidones have been shown to have antioxidant properties (33).



5-methylbenzene-1,3-diol



2,5-dimethylbenzene-1,3-diol

Figure 1.3: Orcinol (5-methylbenzene-1,3-diol) and β-orcinol (2,5-dimethylbenzene-1,3-diol) form the structural basis for separating the depsides and depsidones into groups.

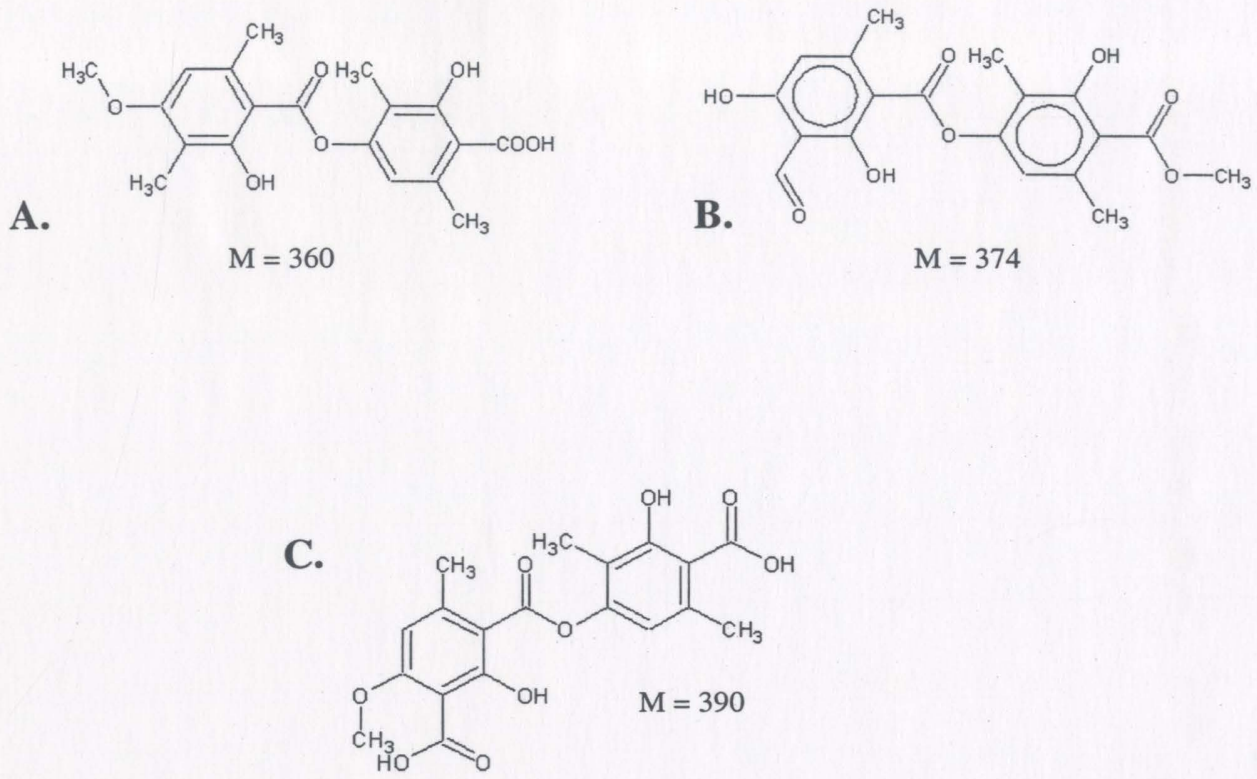


Figure 1.4: Three typical β-orcinol depsides; A: barbatic acid, B: atranorin and C: squamatic acid.

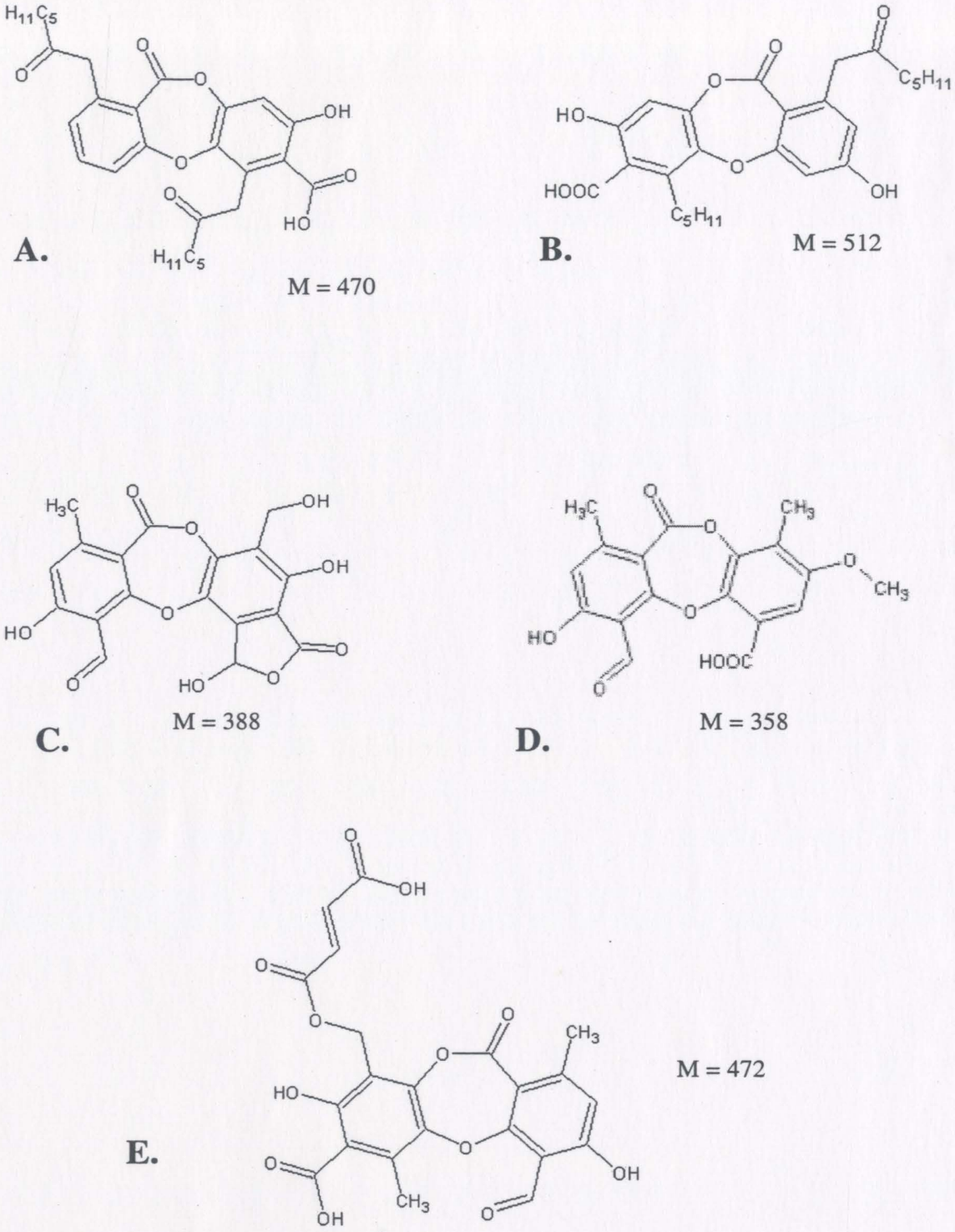


Figure 1.5: Two typical orcinol depsidones; A: physodic acid, B: alectoronic acid and three typical β-orcinol depsidones; C: salazinic acid, D: psoromic acid and E: fumarprotocetraric acid.

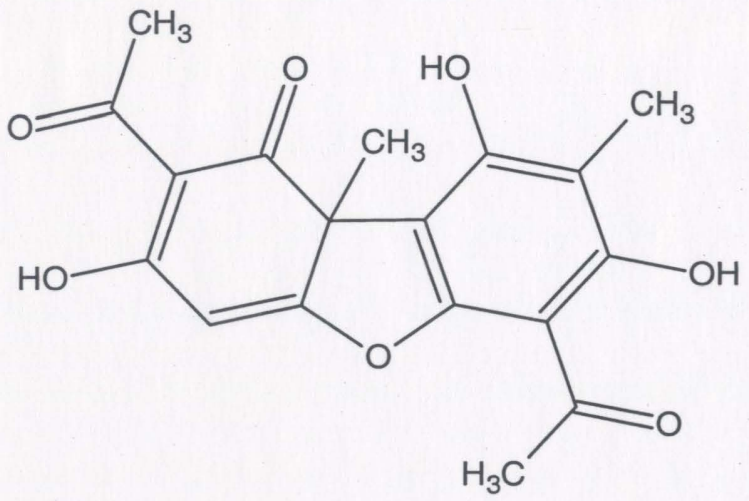


Figure 1.6: Structure of usnic acid, a dibenzofuran derivative that is found in many lichen species.

1.2 Lichen Analysis

Different methods have been employed to study lichen phenolics, including thin-layer chromatography (TLC) (36), thin layer densitometry (37), high performance liquid chromatography (38, 39) and pyrolysis gas chromatography-mass spectrometry (28).

TLC has been popular for analysis of lichen phenolics, and is by far the most widely used, due to its ease of use and low cost. Depsides and depsidones absorb UV light, so detection of these compounds on the TLC plates doesn't require treatment (such as being converted to coloured complexes) to reveal the chromatogram. Solvent systems for separation of the different lichen phenolics are well documented in the literature due to the popularity of the technique (40). A closely related technique called TLC UV-densitometry involves quantitatively measuring the density dependant UV absorbance or fluorescence of a spot on a TLC plate. This technique offers some advantages over HPLC; less time is required to develop a method for optimal separation and multiple samples can be analysed concurrently (37). Unfortunately some compounds have been shown to exhibit photolability under the conditions of the densitometric analysis (37) and so it is not an ideal method.

High-performance liquid chromatography coupled with a photodiode array detector (DAD) allows collection of all the data that would be available from a traditional HPLC, including retention times and some inferences that can be made on peak purity based on the shapes of the individual peaks, as well as the complete UV-vis spectra of the corresponding peaks as recorded by the DAD. Since the UV spectra of many of the secondary lichen metabolites are well documented (42), this data can serve to help

identify lichen phenolics in lichen extracts. Many of the orcinol and β-orcinol depsides and depsidones have been shown to co-elute (53) and in these cases a more selective type of analysis is required for identification. Mass spectrometry is a more selective technique and combined with retention times and UV spectra it can be applied to identify lichen phenolics for which standards are not readily available.

Most lichen acids are not commercially available and in many cases synthesizing standards is not an option. In addition, there seemed to be a niche for a method for analysing very small samples of lichen, without the requirement for a chemical derivatization step. With such a method, the last few millimetres of growth on fruticose lichen could be collected and analysed. Many small samples could be collected by a single researcher, their locations identified with a GPS and recorded. This data would provide a "snapshot" map of the pollution levels in an area for a single day, or for an entire season, depending on the amount of lichen that is analyzed and the speed of its growth.

High performance liquid chromatography with UV-vis diode array detection coupled to an electrospray ionization mass spectrometer is well suited to this analysis. Most lichen acids have an absorption maximum in the ultraviolet spectrum, and the DAD is equipped to record the full absorption spectrum of each chromatographic peak (41). The UV spectra of many lichen phenolics are documented so this is an aid to identification (42). The diode array detector and the mass spectrometric detector are both very sensitive, allowing for detection of very small concentrations of lichen phenolics.

Electrospray ionization is a soft form of ionization, therefore unfragmented $[M-H]^-$ peaks are expected in the negative ion scan (43).

1.2.1 Electrospray mass spectrometry

Electrospray ionization mass spectrometry is well suited to direct sample introduction from a high-performance liquid chromatograph, since the eluting solvent can easily be removed in the mass spectrometer in the first drying stage (43, 44). Electrospray is considered to be a soft ionization technique that can produce ions from polar, non-volatile and thermally labile compounds (43, 44, 45). A liquid sample is introduced to the spectrometer via a stainless steel needle that is maintained at a potential difference of -25 keV with respect to a cylindrical electrode that surrounds the needle. This induces a fine spray of charged liquid particles. The charged droplets pass through a desolvating capillary and as the solvent is removed the droplets reduce in size until the electrostatic repulsion between the charged analyte molecules cause the analyte to desolvate and become charged molecules in the gas phase (46). (Figure 1.9) This process is considered to be a very "soft" form of ionization (compared to electron impact ionization, for example) so there is very little fragmentation of the ions that are formed. If fragment ions are desirable a potential difference (known as the fragmentation voltage) between the end of the capillary and the skimmer can ionize the nitrogen drying gas and this gas can collide with the analyte and increase fragmentation in a process called Collision Induced Dissociation. The analyte ions may also become multiply charged (45) and this can be very useful for analyzing compounds with high molecular weights; the multiple charges

lower the m/z ratio below the upper limit of the detector, allowing molecules that are usually to large to be detected to be analyzed. This method can be used for both negatively and positively charged species. The majority of ions that are formed in the positive mode are M^+ and the ions that are formed in negative scan mode are primarily $[M-H]^-$. There are advantages and disadvantages of each type of scan when analysing for lichen phenolics; a positive scan will show low abundances since ionization of a carboxylic acid favours the formation of negative ions while a negative scan shows much higher abundances accompanied by interference peaks from salt adducts (45), predominantly Na^+ and K^+, and clusters (46). Formation of these clusters ($[M-H+CO_2]$, $[M-H+H_2O]$ etc.) is favoured in the presence of formic acid in negative mode (47).

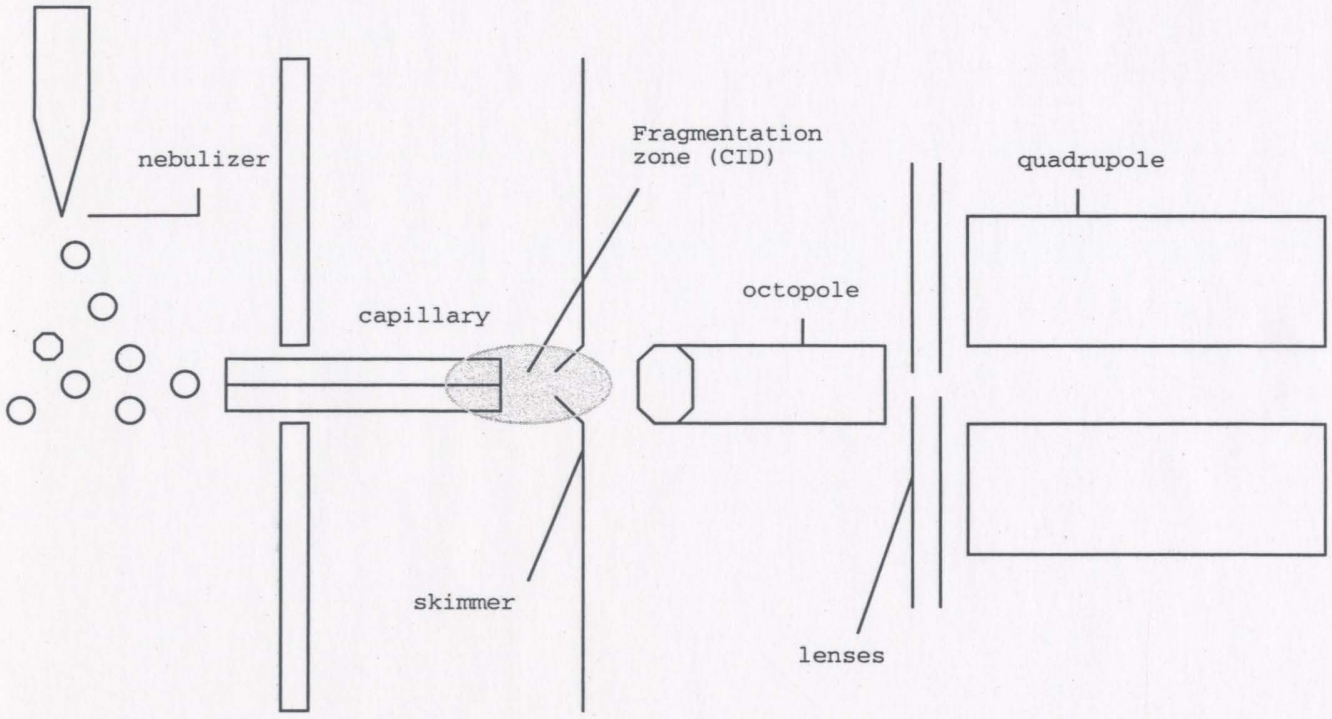


Figure 1.7: Schematic of an electrospray mass spectrometer.

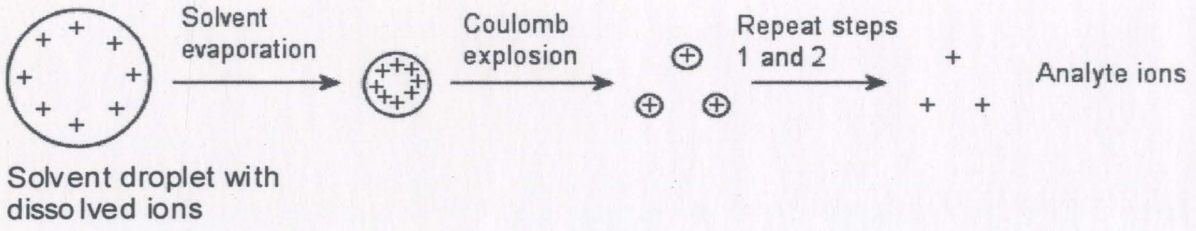


Figure 1.8: Diagram of the mechanism of desolvation within an electrospray mass spectrometer to produce gas phase ions.

CHAPTER 2

EXPERIMENTAL

2.1 Materials

Atranorin (a mixture of 50% atranorin and 50% chloroatranorin as delivered by the manufacturer) and usnic acid (99%) were obtained from Sigma-Aldritch. Dr. Jack Elix of the Australian National University provided lichen phenolics that are not commercially available, including fumarprotocetraric acid, rangiformic acid, salazinic acid, psoromic acid, squamatic acid, barbatic acid, physodic acid and alectoronic acid.

Solvents used included nanopure water, ACS grade concentrated formic acid, ACS grade concentrated phosphoric acid and HPLC grade methanol. The drying gas for the mass spectrometer was nitrogen gas in the form of liquid nitrogen that was produced in the liquid nitrogen plant in the Memorial University physics department.

All samples were passed through 13 mm 0.45 micron Fisherbrand syringe filters to prepare samples for injection into the LC-MS.

2.2 Sample Collection and Preparation

A complete description of the sampling methods and maps to the sites where the lichens were collected have been published in earlier works (28, 50) An abridged description of sampling methods will be included here. The UV study used lichens *Cladonia mitis* and *Cladonia rangiferina* that were provided by Dr. Dianne Fahselt who had used these species in studies on the effect of UV light on total phenolic levels. (26, 48)

The lichens were exposed to four different lighting conditions; visible light (400-700nm), visible light plus UV-A (320-700), visible plus UV-A and UV-B (290-700) and a control set that was stored in the dark, but otherwise treated in the same fashion. The visible light samples were exposed to a fluorescent light source. The visible plus UV-A samples were irradiated by the visible source and by a phosphorescent source equipped with a mylar filter that excludes wavelengths shorter than 320 nm. The sources used for the visible, UV-A and UV-B exposed lichen were the same, with the mylar filter exchanged for a cellulose one that excludes UV-C wavelengths. All the lichen samples, save the control, were exposed for three days. All the lichens were then dried overnight in an oven, which renders them metabolically inactive (50), and stored in the dark. The most recent growth was clipped from three thalli and pulverized in a ball mill. This was stored at room temperature, in the dark, in sealed vials. This process was performed in Dr. Fahselt's laboratory. The lichen used in the ozone related study were collected by the Atlantic Forestry Service, and prepared by Tanya MacGillivray in Dr. Helleur's

laboratory. (28, 50) The lichen were cleaned of debris, and the newest growth on three thalli was collected and ground into a fine powder in an agate mortar and pestle. Passive ozone monitors were analyzed by the Atlantic Forestry Service. (50)

2.3 Lichen Analysis

2.3.1 Extraction of Lichen Phenolics

In preparation for analysis, the lichen phenolics were extracted from control lichen material. Initially, acetone was investigated as a suitable solvent but it presented one difficulty; a large solvent peak at the beginning of the UV diode array chromatogram since acetone absorbs at 254 nm. Extraction with acetone, drying under nitrogen gas and dissolution in methanol was attempted, however; once the extracts were dried, dissolution in a comparable amount of methanol was not possible. Finally, extraction of the lichen phenolics in methanol was attempted and the quantity and number of phenolics detected was comparable with those detected in acetone extraction. Previous research suggested that the carboxylic moieties on the lichen phenolics would form methyl esters if extraction was performed in methanol (51) but no evidence of this phenomenon was observed.

Initially, extractions were performed with ~10 mg of lichen powder which required filtration of the extract through a glass wool plug in a Pasteur pipette before filtration through a 15 mm diameter 0.45 micron regenerated cellulose syringe filter could take place. (Filtration of small particles is necessary to prevent clogging of the guard column) The glass wool filters were exchanged for 0.45 micron syringe filters due to their ease of use. Experiments were performed to determine the smallest sample size of lichen powder that could be reliably weighed on a Perkin Elmer AD-2Z Autobalance microbalance, extracted, and analyzed. The final amount was determined to be ~2 mg,

with the limiting factor being the mass which is large enough to be easily weighed on the microbalance, yet small enough to not clog the 0.45 micron syringe filters.

The final procedure for extraction of lichen sample and filtration of extract is as follows: ~2 mg of lichen powder was weighed out on a Perkin Elmer AD-2Z Autobalance by difference. The weighing vessels used were aluminum pans that were originally intended for performing differential scanning calorimetry measurements. The lichen powder was transferred to a 1.5 mL amber glass vial with volume graduations. Addition of 500 μL of methanol was followed by 10 minutes agitation in an ultrasonic cleaner (Mettler Electronics Corporation). This was allowed to settle for 5 minutes, and the supernatant was filtered through a 0.45 micron syringe filter. A second aliquot of 500 μL was added to the lichen sample, and sonicated for a further 10 minutes. The resultant slurry was filtered through the same syringe filter as before, 500 μL of methanol was added to the lichen sample vial to rinse it, the final volume was also filtered, and all three volumes are combined to reach a final volume of 1.5 mL. It was confirmed by LC-MS that a third extraction did not yield a significant amount of the lichen phenolics hence the lichen phenolics were exhaustively extracted.

2.3.2 Flow Injection Analysis with Diode-Array and ESI

One type of analysis that can be performed on an LC-MS by bypassing the chromatography column is flow injection analysis (FIA). This process involves connecting the autoinjector directly to the inlet of the mass spectrometer. In this way many small injections of a solution can be performed while incrementally changing a

single parameter. There is no chromatographic separation, so this method is most useful when solutions of standards are analyzed, as a mixture of compounds will have overlapping masses in the mass spectrum. FIA was utilized for optimizing the parameters of the mass spectrometer. This was particularly useful in determining the optimal fragmentation voltage that would deliver the maximum number of $[M-H]^-$ molecular ions to the detector while minimizing fragmentation. Extracted ion chromatograms of the $[M-H]^-$ ions of the selected lichen acids were used to determine the optimal voltage for multiple lichen phenolics, as would be encountered in a lichen extract. Injection volume was 5 μL, with the fragmenting voltage ranging from 25 eV to 400 eV in steps of 25 eV.

Flow Injection Analysis was also utilized when evaluating atmospheric pressure chemical ionization (APCI) as a possible method for quantifying lichen acids. This method resulted in excessive fragmentation with minimal molecular ions for all parameters tested and the technique was not used.

2.3.3 High Performance Liquid Chromatography-Electrospray Ionization Mass Spectrometry

For HPLC-ESI-MS analyses, an Agilent 1100 series liquid chromatograph-mass sensitive detector equipped with a diode-array UV-visible detector was used. The equipment was controlled with HP LC-MSD Chemstation software, which was also used to collect and analyze the data.

The solvents used were **A:** 0.1M aqueous formic acid and **B:** methanol with an initial condition of 30% methanol, increasing linearly to 100% over 30 minutes,

remaining at 100% methanol for 5 minutes, then returning to initial conditions over one minute at the completion of the analysis. The method that was compatible with the HPLC-DAD and electrospray mass spectrometry was developed from the method suggested by Huneck, Feige and Lumbsch (38) where the water-methanol-*o*-H_3PO_4 (70:30:1) solvent system was similar to that suggested by C. F. Culberson for the HPLC (52). The substitution of formic acid for the phosphoric acid was necessary as the manufacturer's specifications state that the steel fittings of the mass spectrometer are not compatible with phosphoric acid. An Eclipse XDB-C18, 5μm, 4.6 × 250 mm reversed phase column fitted with a guard column was used in the separation of the polyphenolics. Initially the solvent flow rate was 0.7 $mL \cdot min^{-1}$, during the course of this research the flow rate was increased to 1.0 $mL \cdot min^{-1}$ to reduce analysis times.

The diode array was set to collect chromatographic data at 254 nm, over a bandwidth of 16 nm,. The slit width was 1 nm. UV-vis absorbance spectra were collected for each peak from 210 nm to 400 nm.

The fragmenting voltage of the mass spectrometer was set at 70 eV. The drying gas rate was 12 L per minute with a nebulizer pressure of 45 PSI. (It should be noted that these pressures are not the recommended pressures from the manufacturer, but somewhat lower; the liquid nitrogen storage Dewar that was used as a source for nitrogen used as a drying gas could not provide pressures higher than the ones reported above.) Drying gas temperature was held at 350°C. Ions were collected over a mass range of 100-1000 mass units in scan mode.

2.4 Treatment of Data

As mentioned earlier, the lichen in both the UV study and the ozone study were sampled in such a way that the samples would be representative of the population of lichens available in each area. Only the newest growth on each lichen thallus was used, ensuring that the lichen tissue sampled was all the same age, and would have had the same environmental conditions before the tests were carried out. The samples from each experimental condition were combined to further homogenize the lichen material.

It was decided to use the DAD to quantify the lichen phenolics found in the extracts as some fragmentation and adduct formation were seen in some mass spectra of fumarprotocetraric acid and usnic acid. The peak areas were normalized to the mass of lichen powder that was extracted. Two replicate extractions were performed on lichen powder from each experimental condition, and each of these two extracts were analyzed on the LC-DAD-MS three times to reduce error in the mean values. The mean and standard deviations of these values were used draw the conclusions found in this work. An analysis of variance was performed and negated the null hypothesis in each case, confirming that the results were significantly different.

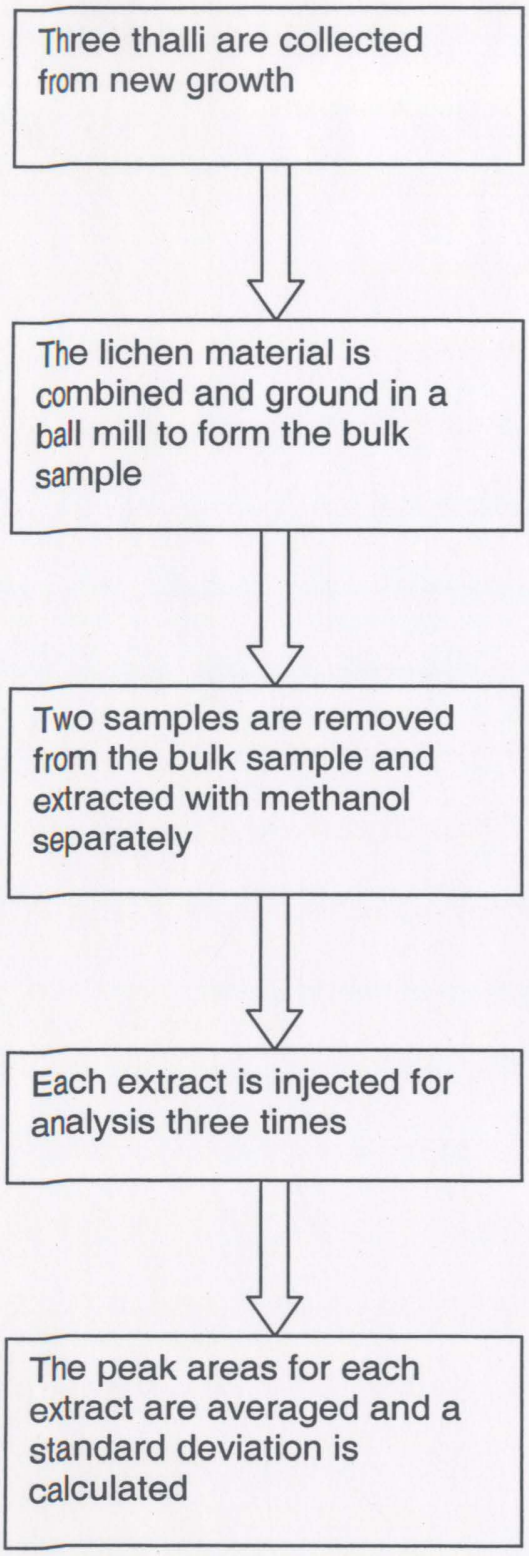


Figure 2.1: Flowchart describing the analysis procedure.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Method Development

The first goal of this project was to develop a method for analyzing lichen phenolics in an LC-ESI-MS equipped mass spectrometer with a diode array. Methods existed for analysis by LC-DAD that used a reversed phase column and with a mobile phase that combined methanol and dilute 0.1% v/v aqueous phosphoric acid in isocratic elution, a fixed gradient or changing gradients. The acid is required to ensure that the lichen phenolics do not elute with the solvent peak. These methods could not be adapted directly to LC-ESI-MS as the stainless steel fittings in the mass spectrometer are not compatible with phosphoric acid. Formic acid was chosen since it is compatible with the internal components of the mass spectrometer, because it has a pK_a of 3.75 and because it is volatile and should not leave any residue in the mass spectrometer inlet. (The mass spectrometer is particularly sensitive to the formation of salt adducts as well as to a build-up of non-volatiles on the inlet.) A pK_a of 3.75 is also convenient, as it assures that at no time will the eluent have a pH less than 2, that being the lower operating limit for the column. This is a precautionary measure to avoid damaging the column. Chromatograms using the method as proposed by Huneck, Feige, and Lumbsch (38) and the new method using formic acid were performed to compare the two. It was found that the run time could be considerably decreased if the flow rate was increased to 1.0 $mL \cdot min^{-1}$, instead of 0.7 $mL \cdot min^{-1}$. Also, the concentration of formic acid in the aqueous component ofthe

mobile phase was increased from 0.01 M to 0.1 M. (The equivalent pH of dilute formic acid to phosphoric acid at 0.1% v/v concentration.) This gave an aqueous phase with a pH of 3.2, which allowed for a better separation of the late-eluting compounds atranorin and usnic acid.

Flow injection analysis (FIA) was performed on the major constituents of the lichen phenolic mixtures (atranorin, usnic acid (Figure 3.1) and fumarprotocetraric acid) to determine the optimal settings for the control parameters of the electrospray mass spectrometer. This involved injecting small samples of a solution (5 μL with a concentration of about 100 μg per mL) sequentially and changing one parameter incrementally. The diode array response does not change because each injection contains the same volume of the same solution. The extracted ion chromatogram of the molecular ion of each lichen phenolic was then used to find the optimal setting for a mixture of phenolics. The parameters that were optimized in this way were the ionization voltage, and the drying gas temperature. The ionization voltage of 70 eV was chosen as it was shown to give the maximum abundances of all three standards while minimizing fragmentation as well as formation of clusters and adducts. The drying gas flow rate was not optimized as 1 $mL \cdot min^{-1}$ is the upper limit for liquid flow rate that can be accommodated by the mass spectrometer and thus the drying gas flow rate must then be set at the highest acceptable level for optimal performance. Due to limitations with the N_2 gas source (a large volume liquid nitrogen storage Dewar) the maximum flow rate was 11 $L \cdot min^{-1}$ instead of 13 $L \cdot min^{-1}$ as recommended by the manufacturer.

For this study the sample preparation was tailored in such a way that a small sample size could be used, allowing for multiple analyses of a single lichen thallus. The LC-MS is a sensitive detector for lichen phenolics, so the lower limit of the sample size was found to be the mass which was reproducible on the Perkin Elmer AD-2Z Autobalance. The ground lichen powder is very fine and susceptible to the effects of static electricity, making a reproducible mass below 100 μg difficult to obtain. An average sample mass of 2 mg was chosen as it was sufficiently large to minimize the effects of static electricity, and small enough to resist overloading the 0.5 μm cellulose syringe filter as to block it.

This sample preparation method contains no chemical derivatization step (50), as the lichen phenolics absorb strongly in the ultraviolet spectrum, also, they are not strongly attracted to the polar reversed phase column unless the pH is below their pKa.

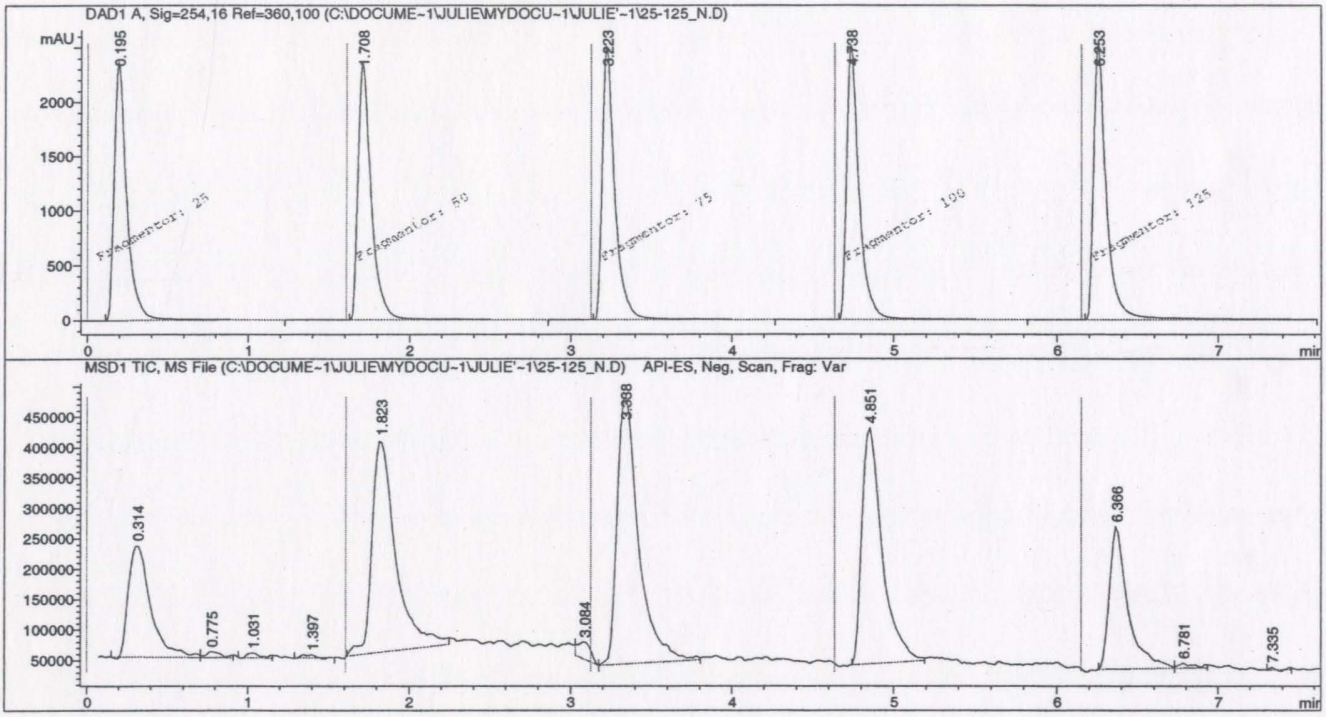


Figure 3.1 Diode array absorbances at 254 nm and total ion current for flow injection analysis to determine the optimal fragmentation voltage for usnic acid.

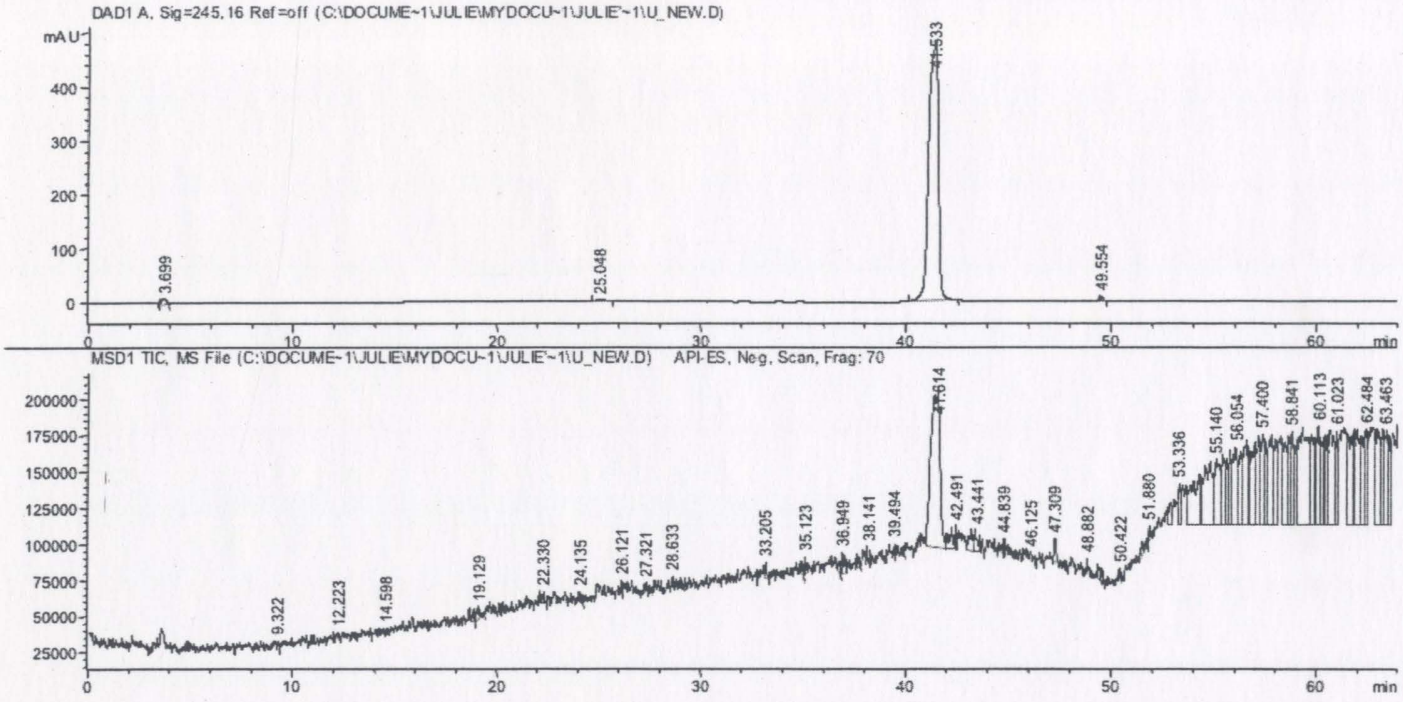
3.2 Qualitative Lichen Analysis

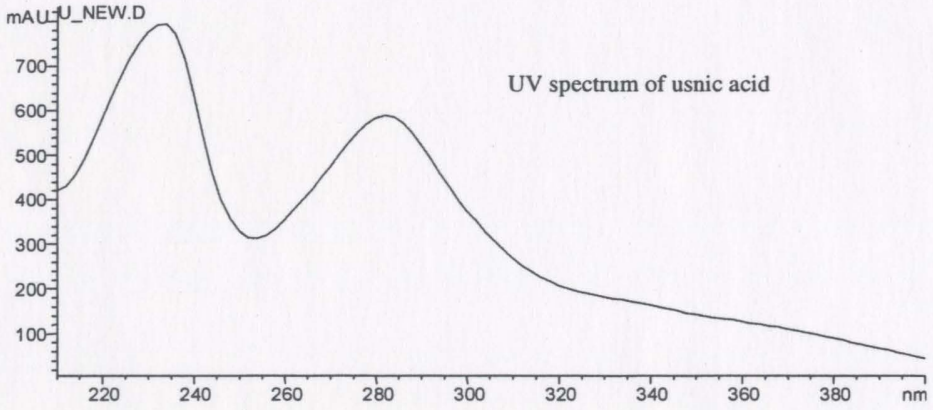
Qualitative analyses of the extracts of the four lichen species that were sampled in this study were performed using all of the capabilities of the LC-MS instrument; liquid chromatography coupled with a diode array detector and ESI as well as flow injection analysis. Specific lichen phenolics can be identified by their UV spectra as well as by mass spectrometric analysis. Flow injection analysis allows for analysis of previously purified lichen phenolics for the purpose of investigating the behaviour of lichen phenolics in the environment of the ESI mass spectrometer.

Diode array detection allows for identification and quantification of probable lichen phenolics as the majority of lichen acids have UV chromophores and absorb to some degree in the UV range, with rangiformic acid being a notable exception. The absorption spectra of a wide range of lichen phenolics are known, and absorption maxima can form a basis for identifying lichen phenolics. Since even closely related lichen species may have lichen phenolic profiles that are different, which lichen phenolics are present, and their relative intensities can form a "fingerprint" of the species. Taxonomists often use secondary compound profiles, records of the phenolics that are common to a species, and their relative amounts, to identify closely related species.

With ESI operating in negative scan mode, one can expect to find a pseudo-molecular ion peak at $[M-H]^-$, however, in addition to this peak, some of the lichen phenolics show fragmentation peaks, as well as adducts and clusters. Sodium adducts are a well known phenomenon in ESI-MS, and care is usually taken to insure that no sodium

is being introduced into the sample. Here sodium was already a component of the lichen tissue, and could not be avoided. Usnic acid exhibits a peak at 709.1 m/z that could be the $[2M-2H+Na]^-$ ion. Calculations for the carbon isotope ratio indicate that this peak does contain twice the number of carbon atoms as the $[M-H]^-$ peak of usnic acid. FIA was performed for atranorin, usnic acid and fumarprotocetraric acid to determine an optimal fragmentor voltage that would give the maximum ion abundance with the least interferences, and it was noted that higher fragmentor voltages gave a higher ratio of the 709 m/z peak to the $[M-H]^-$ peak of usnic acid. Fragmentor voltages ranging from 25 eV increasing to 125 eV in 25 eV intervals were tried. Another phenomenon that has been documented is the formation of clusters in aqueous solutions containing formic acid. (47) Fragmentation is rare at the voltages that were employed in this study (70V), however fragmentation of fumarprotocetraric acid into fumaric acid and protocetraric acid was observed. Also, some fragments were observed where a depside (such as atranorin) ester bond between the two aromatic rings was cleaved, but this was anticipated and fragmentation was kept to a minimum by using low fragmenting voltages.





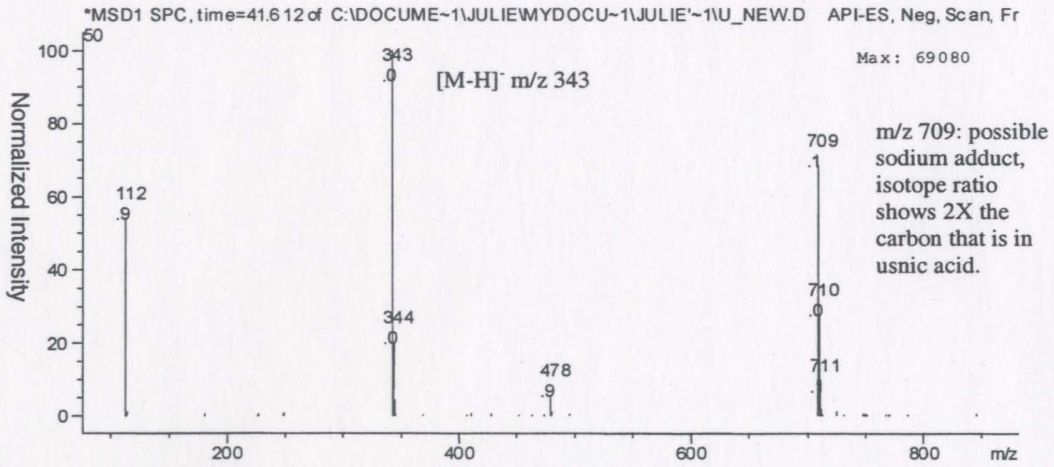


Figure 3.2 Analysis of a standard solution of usnic acid. DAD absorbance measured at 254nm, TIC, full UV spectrum and mass spectrum of usnic acid.

3.2.1 *Cladina mitis*

The combined chromatograms of the methanol extract of *C. mitis* indicated the presence of fumarprotocetraric acid and usnic acid. Usnic acid absorbs strongly in the UV-B range and is considered to be a potential light filter. Related species of several closely related lichens contain usnic acid instead of atranorin which lends credence to the idea that they perform the same light-blocking role. An aliphatic lichen acid, rangiformic acid was also detected in an extracted ion chromatogram, though it doesn't absorb at 254 nm, and doesn't show up in the chromatogram produced using UV detection at 254 nm. Lichen phenolics exist as extracellular deposits within the lichen, and though rangiformic acid resembles a fatty acid, and could be expected to be found in lichen membranes, it is found in these same extracellular deposits, indicating that it may serve a similar purpose in the lichen.

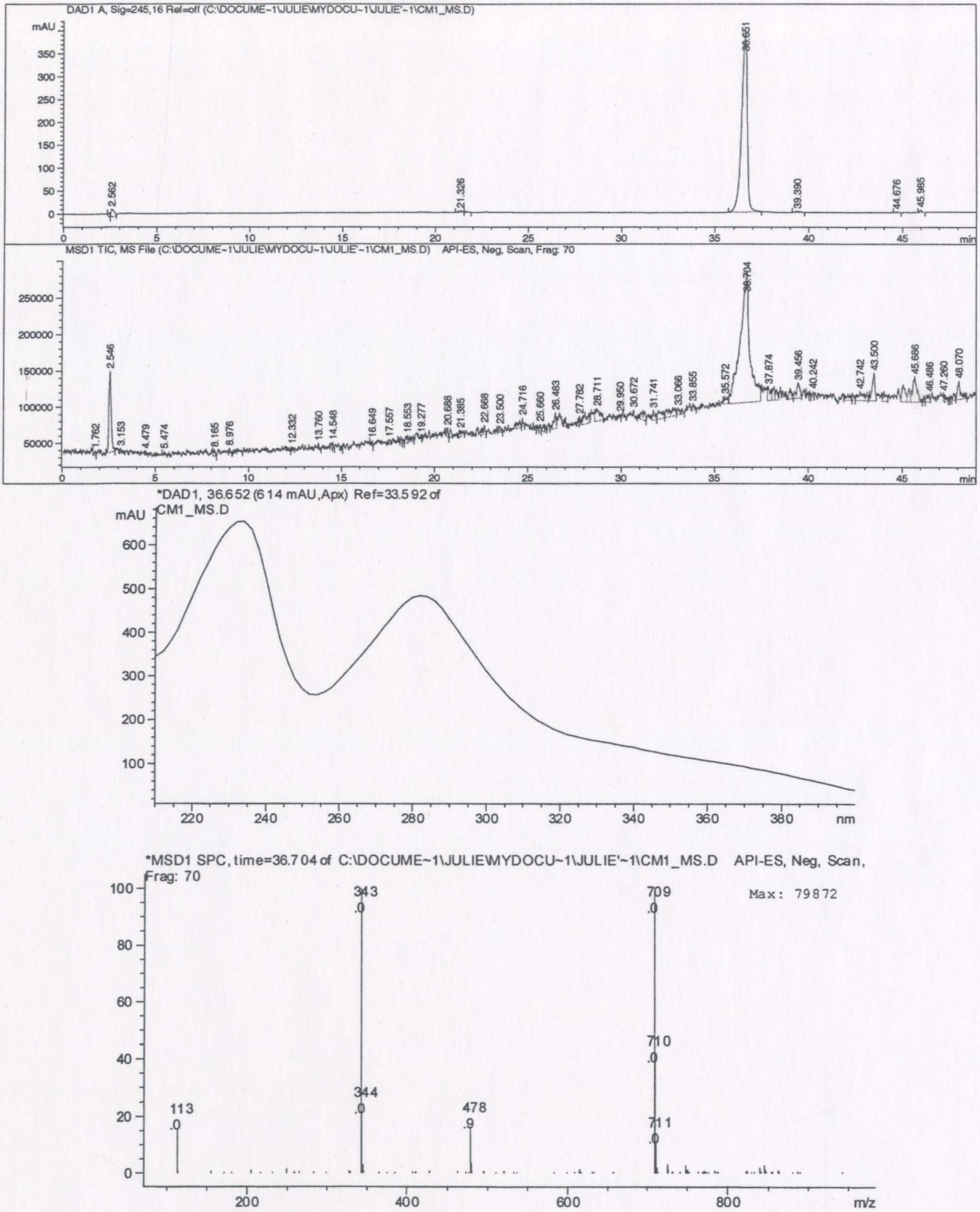
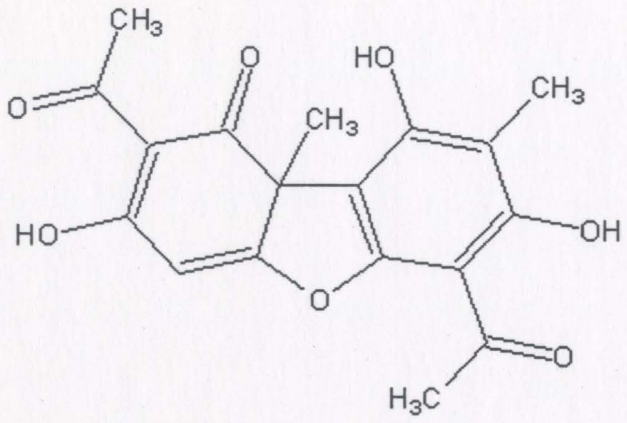


Figure 3.3: Analysis of the methanol extract of *C. mitis*; Chromatogram at 254 nm, TIC, UV spectrum and mass spectrum of the 36.6 minute peak matches the UV spectrum and mass spectrum of usnic acid.

Usnic acid



Rangiformic acid

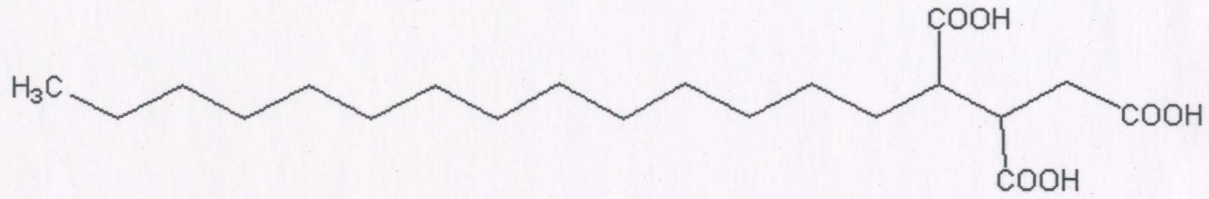


Figure 3.4 Structures of usnic and rangiformic acid.

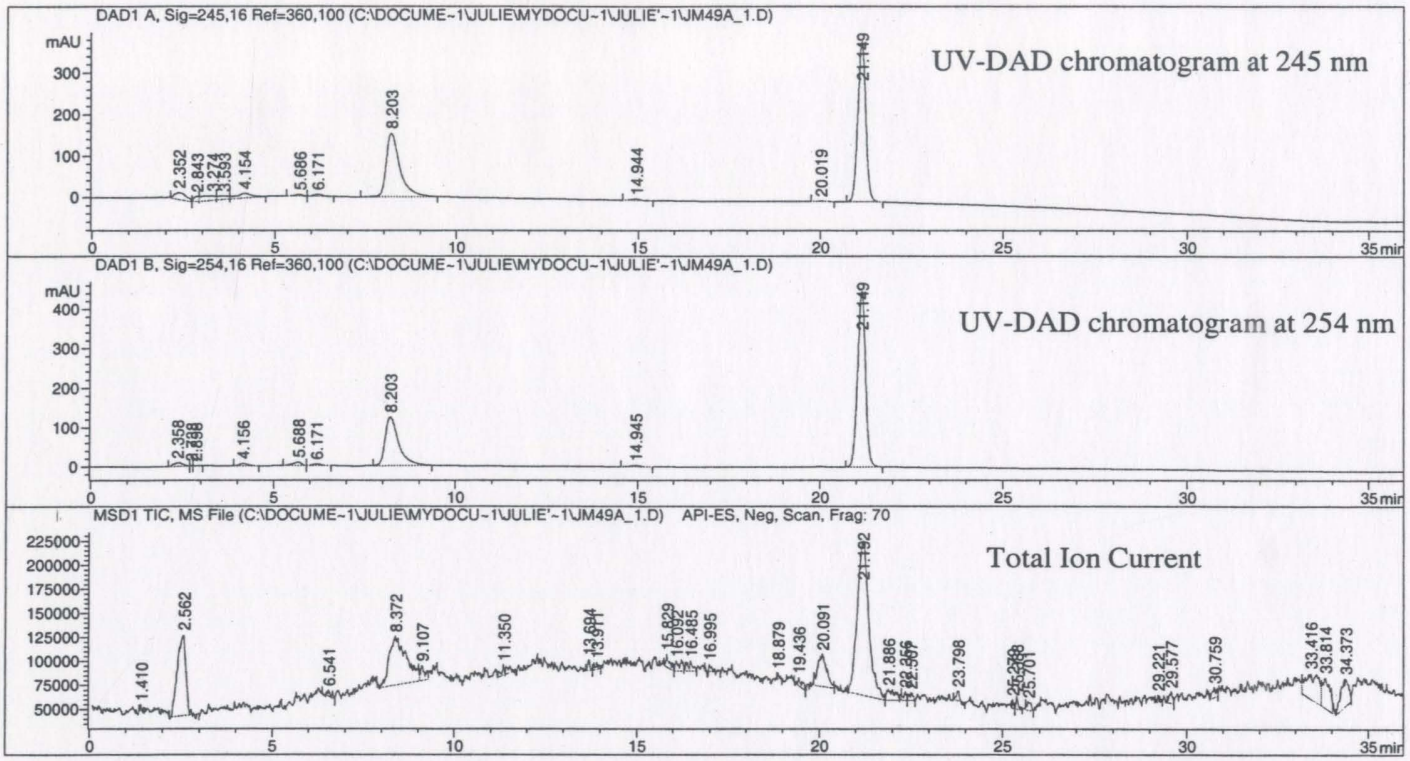


Figure 3.5: Analysis of the methanol extract of *C. mitis*: DAD chromatograms at 245nm, 254nm and Total Ion Current.

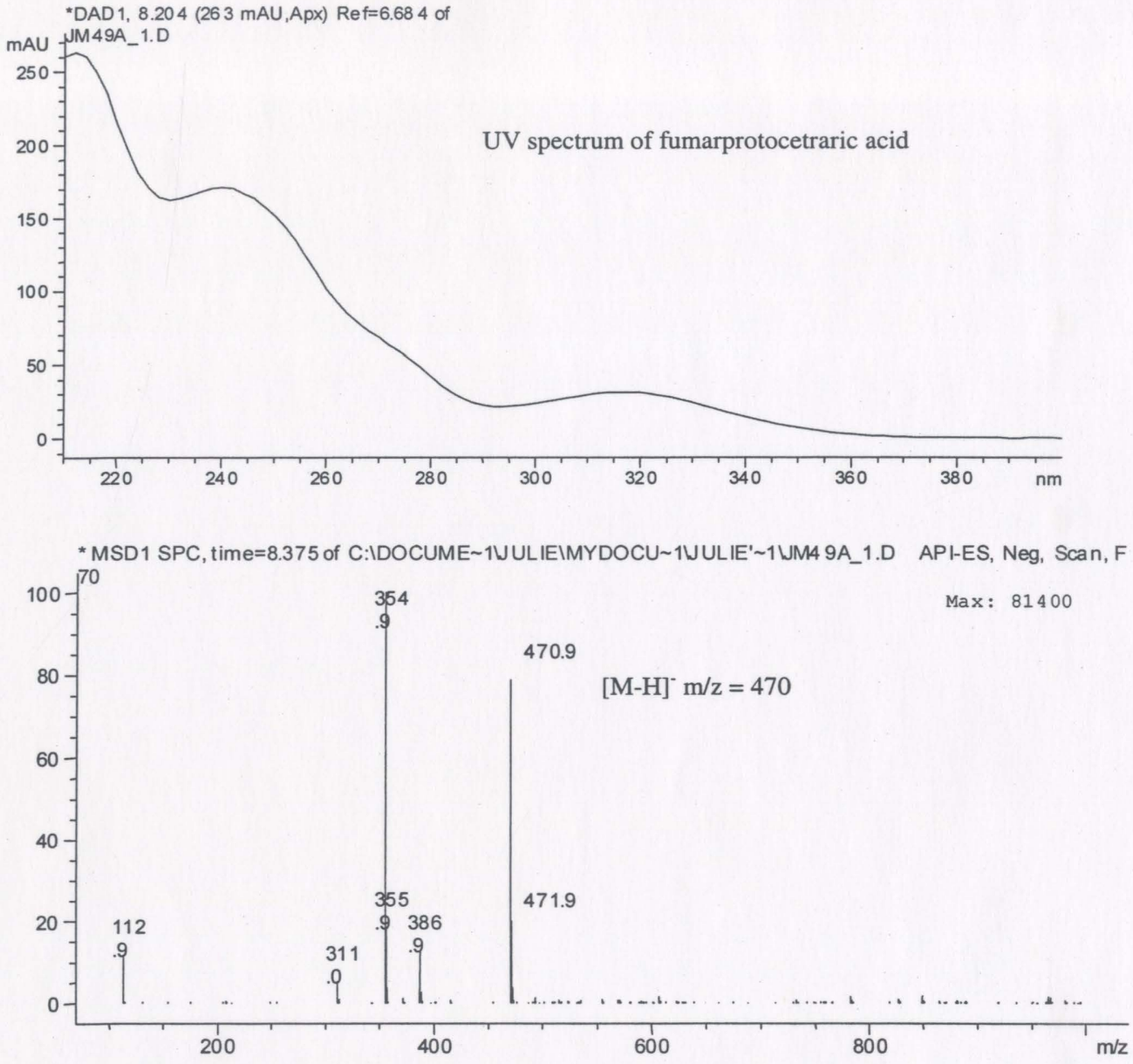
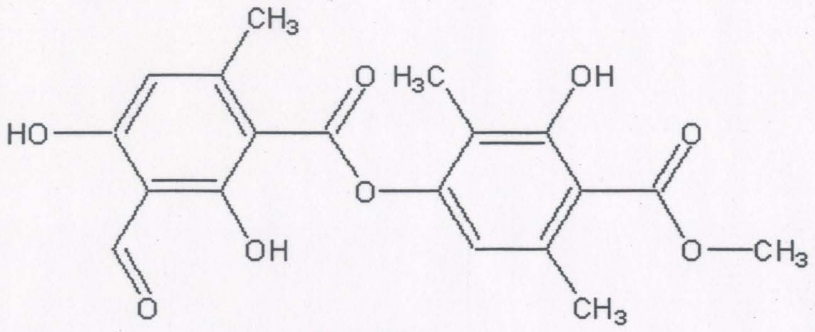


Figure 3.6: UV spectrum and mass spectrum of fumarprotocetraric peak at 8.20 minutes as seen in Figure 3.5.

3.2.2 *Cladina rangiferina*

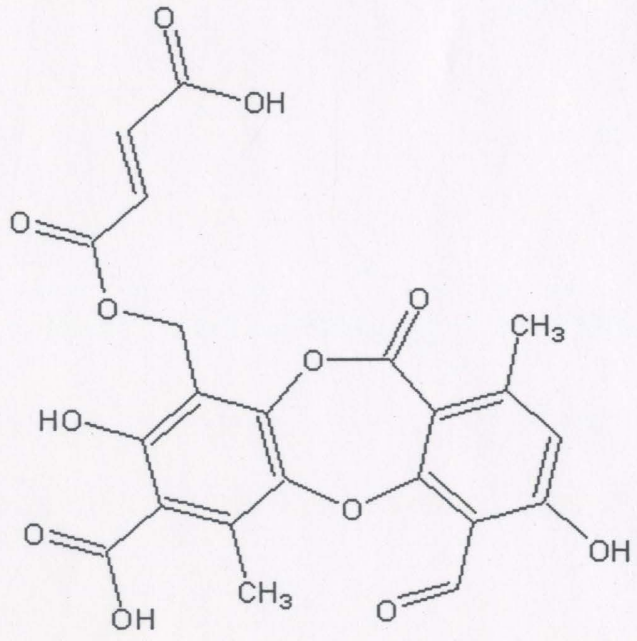
The chromatogram of the methanol extract of *C. rangiferina* (Figure 3.8) indicates the presence of fumarprotocetraric acid and significant quantities of atranorin (Figure 3.9). Fumarprotocetraric acid was identified by mass spectrum and UV spectrum in the qualitative study, however, it was below the limit of quantitation in the quantitative study. Atranorin was abundant in the extracted sample, and can be identified both by its characteristic ultraviolet absorption spectrum and by the peak in the total ion chromatogram. Atranorin absorbs strongly in the ultraviolet, especially in the UV-B range, and is considered to be a light filter for the lichen, to protect it from the damaging influences of UV radiation. (26)

Atranorin



M = 374

Fumarprotocetraric acid



M = 472

Figure 3.7: Structures of atranorin and fumarprotocetraric acid

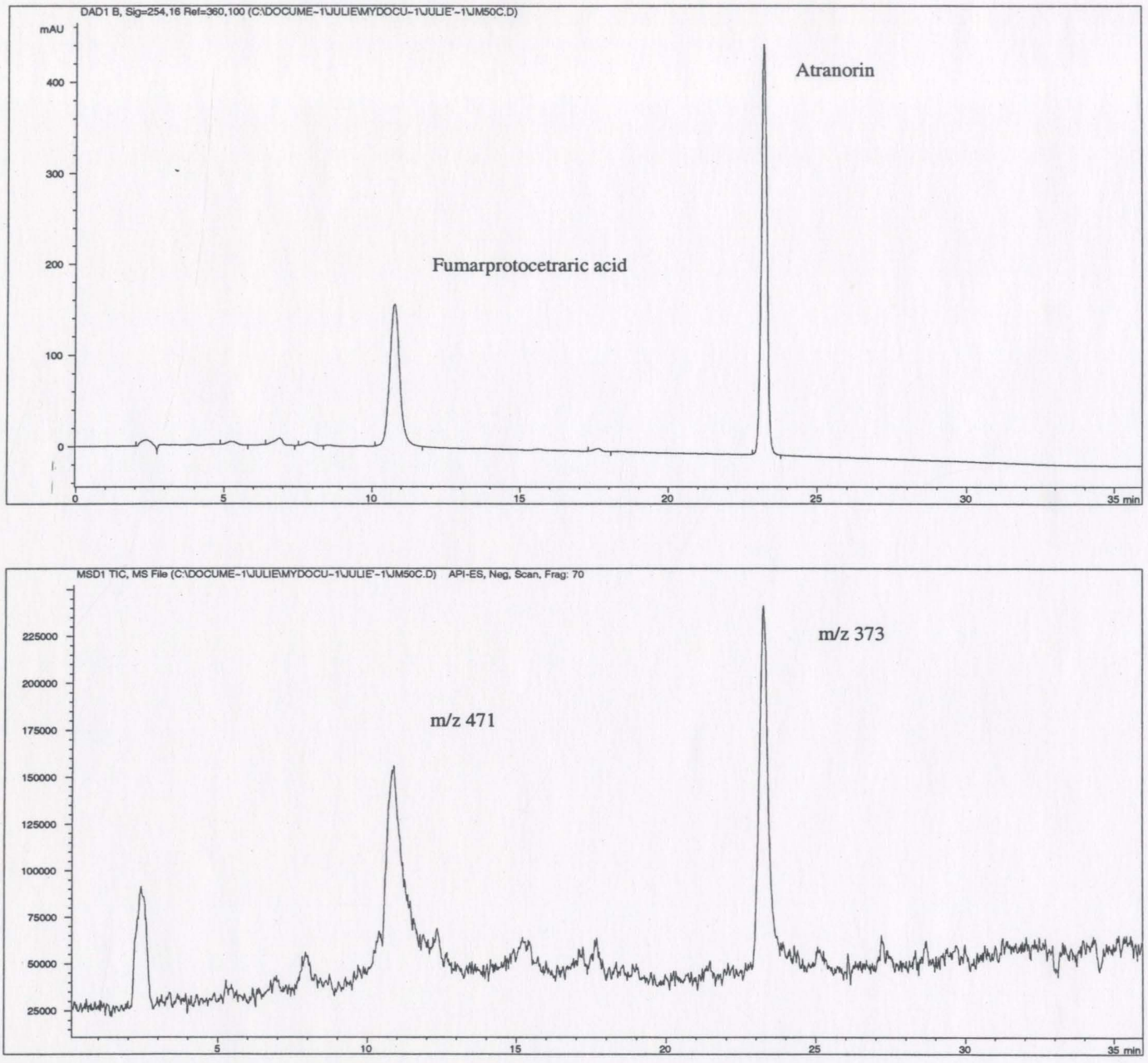


Figure 3.8: Liquid chromatogram and total ion current for the extract of *C. rangiferina*

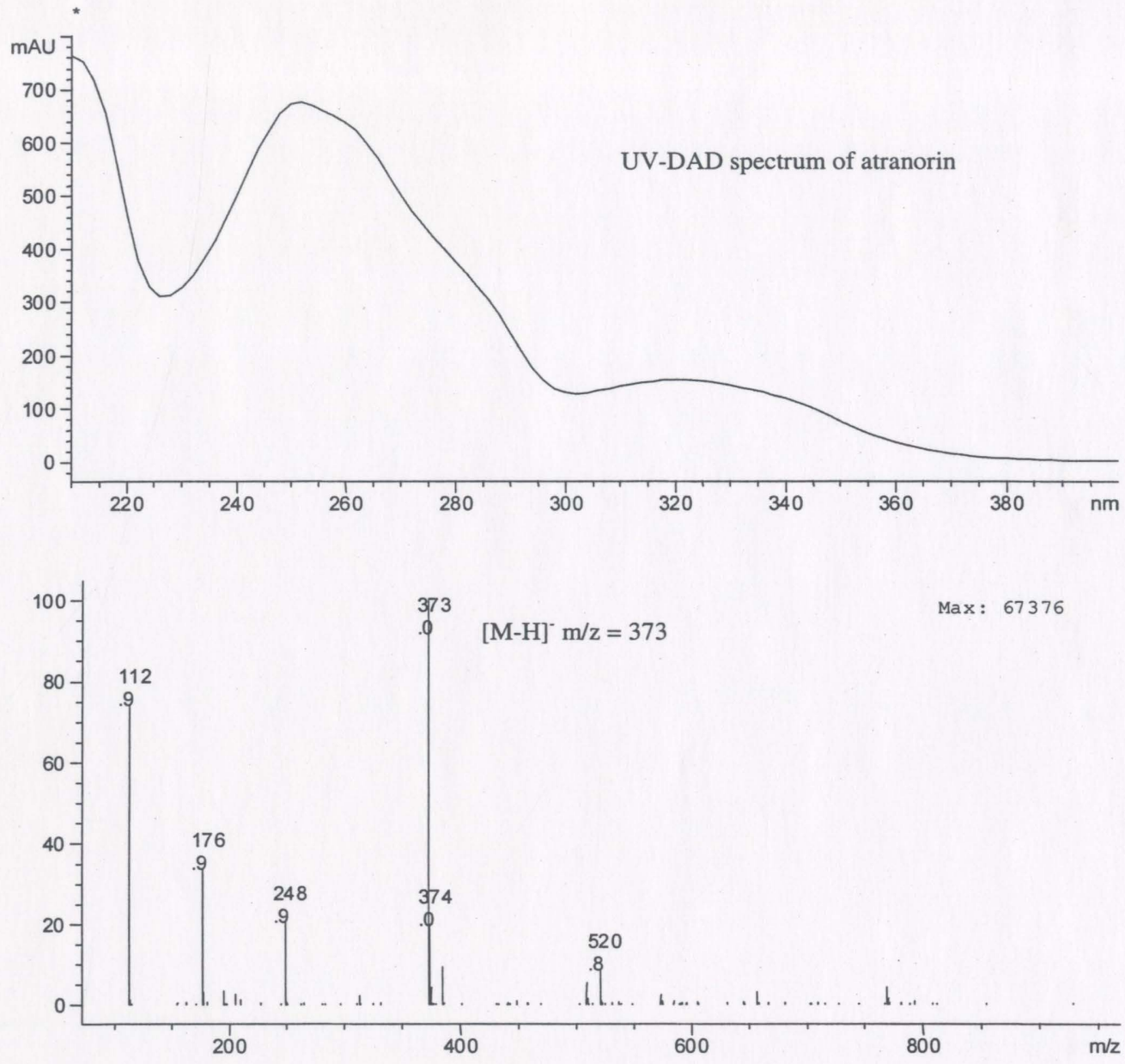


Figure 3.9: UV-DAD and mass spectra of atranorin from a methanol extract of *C. rangiferina*.

3.2.3 *Usnea dasypoga*

Salazinic acid and usnic acid compose the phenolic component of the methanol extract of *U. dasypoga* as seen in Figure 3.9. Both of these compounds absorb in the UV-B range, and could absorb harmful ultraviolet rays before they can interact with the lichen thalli. Salazinic acid may also act as an antioxidant. (33)

Figure 3.10: Structure of salazinic acid

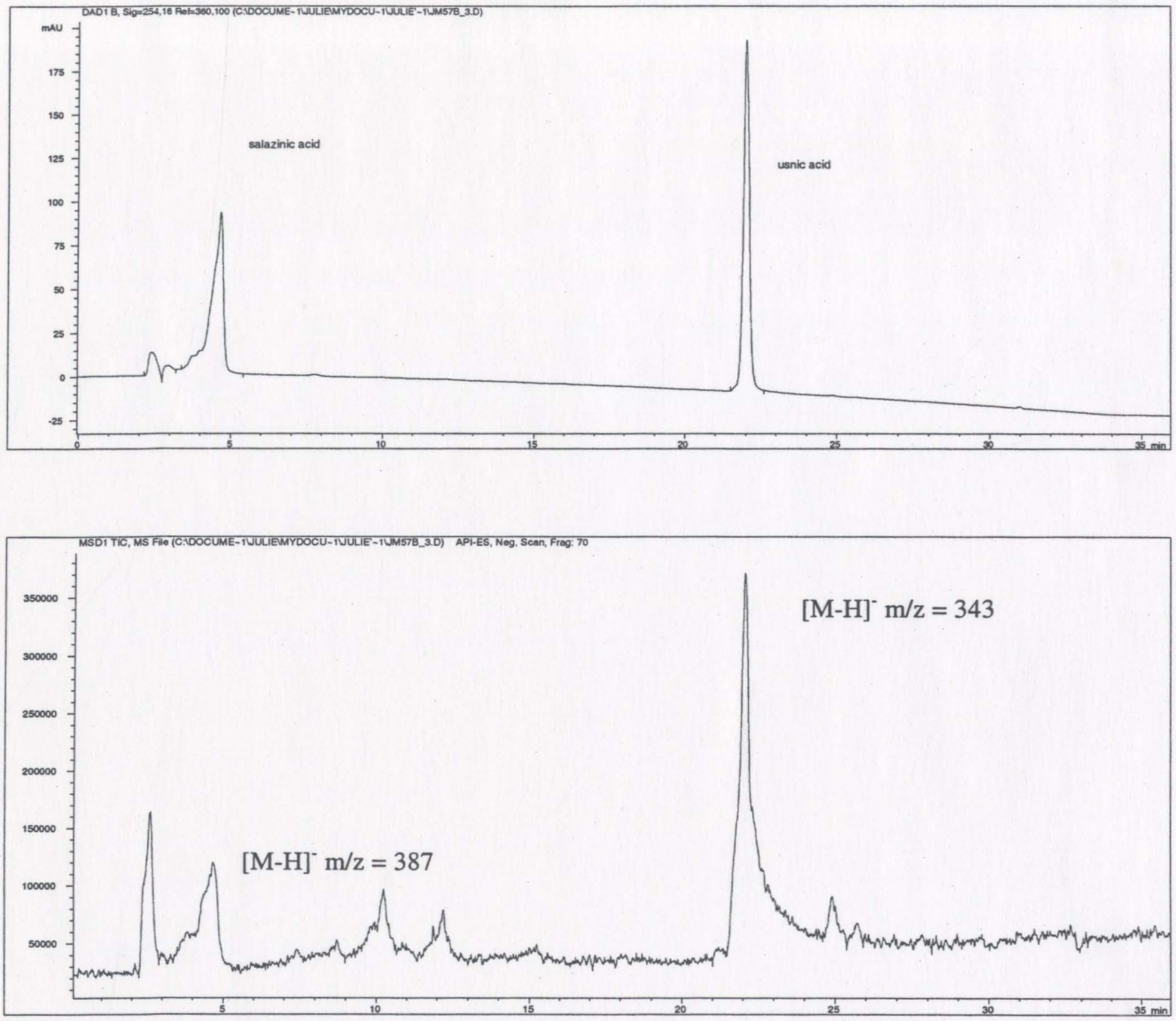


Figure 3.11: Liquid chromatogram and total ion current for the extract of *U. dasypoga*

3.2.4 *Bryoria trichodes*

The main phenolic component of the extract of *B. trichodes* is fumarprotocetraric acid. Fumarprotocetraric acid absorbs UV-B radiation, and has also been shown to demonstrate antioxidant ability (33). It is possible that this compound serves a dual role as environmental protection in this lichen as antioxidant and UV filter.

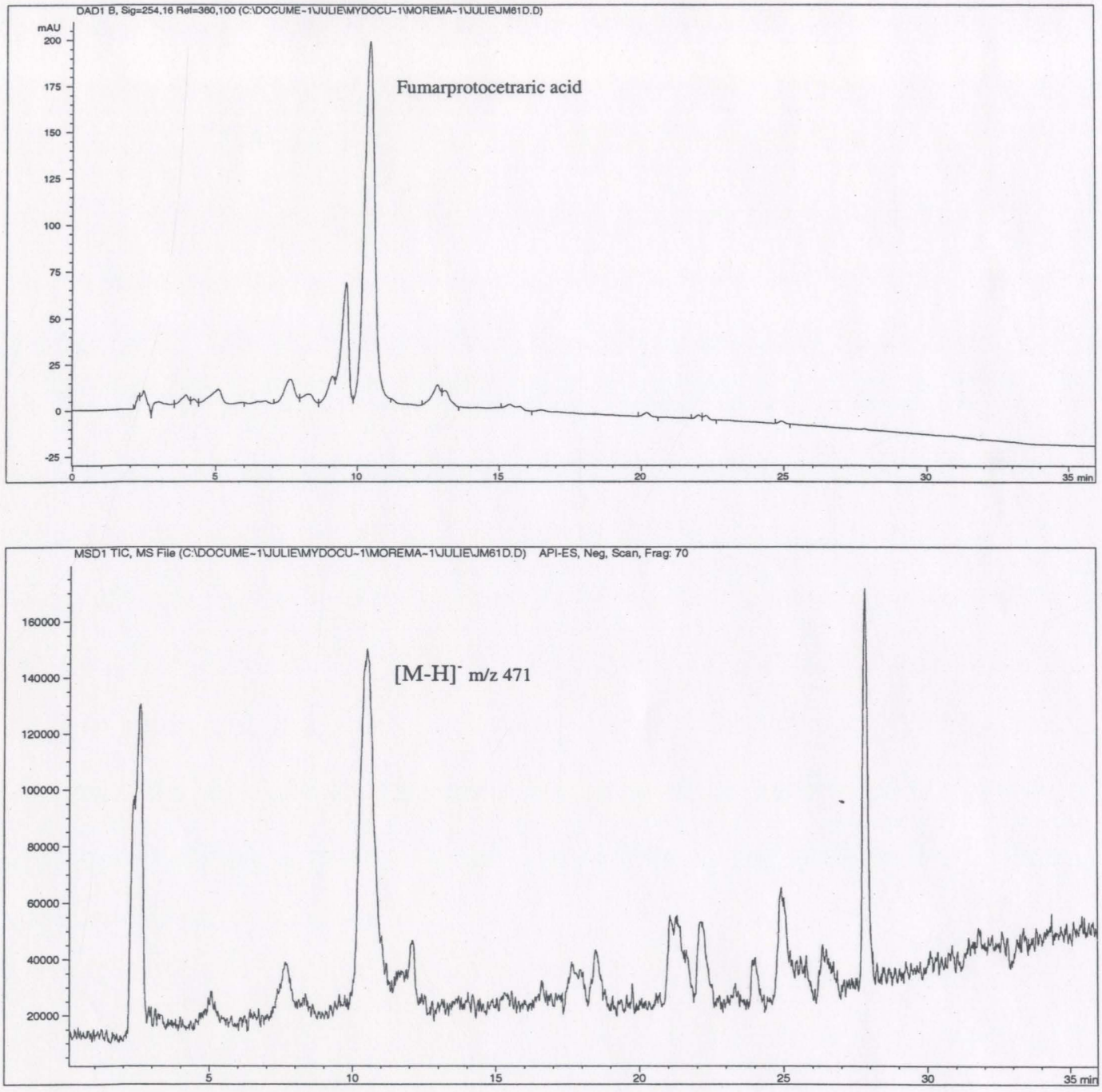


Figure 3.12: Liquid chromatogram and total ion current for *B. trichodes*

3.3 Lichen Acid Analysis and Environmental Stressors

It has been assumed for some time that the role of lichen phenolics is as a form of defence for the lichen. These compounds require a large investment of energy that the lichen might be better served by using somewhere else, unless these compounds serve as an evolutionary advantage to the lichen. It stands to reason that the lichen wouldn't produce these compounds if they weren't beneficial in some way to the lichen as a species. Lichen phenolics exist as extracellular deposits, so are not considered to be available as intermediates or catalysts for any metabolic pathways. (17) Most of the lichen phenolics absorb UV radiation in the UV-A and UV-B ranges, and many fluoresce in the UV or in the visible spectrum. Some of the phenolics also exhibit antioxidant and antifeedant properties. If these compounds are responsible for protecting the lichen from these environmental stressors, then it stands to reason that there will be measurable changes in the levels of these compounds in the lichen thalli, and that these changes are detectable in extracts of the thalli by using liquid chromatography with a UV-vis diode array detector and with ESI-MS.

3.3.1 Ultraviolet study

3.3.1.1 *C. mitis*

The main phenolic of interest in the methanol extracts of *C. mitis* is usnic acid. It has been shown to be both a possible UV absorber, as well as an antioxidant. Semi-quantitative results for the analysis of the relative amounts of this phenolic in response to exposure to differing intensities of UV radiation can be seen in Figure 3.13. These results seem to suggest that exposure to increasing energies of UV radiation cause a depletion of usnic acid in the lichen. This would seem to suggest that even visible light has sufficient energy to deplete usnic acid in the lichen although UV-A has a greater effect, and UV-A plus UV-B exposure has the most pronounced impact on the amount of usnic acid in the lichen. If usnic acid is photolysed or undergoes rearrangement (54) after absorption of ultraviolet light, it might stand to reason that more of an effect will be observed with more energetic wavelengths of light. It is also possible, however, that a longer excitation wavelength could elicit a reaction where a shorter, more energetic excitation wavelength would not if the absorption wavelength is quantized.

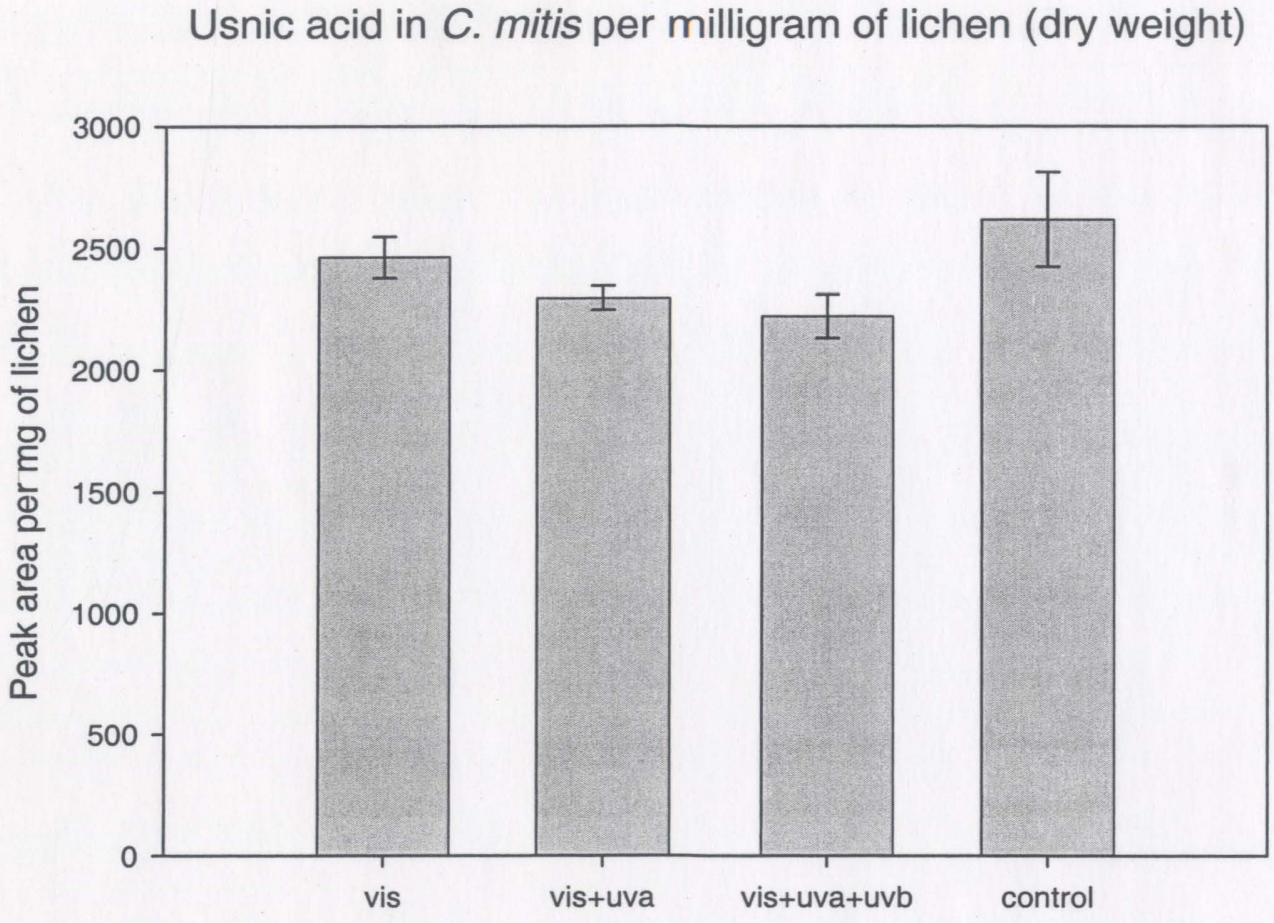


Figure 3.13 Relative content of usnic acid in *C. mitis* exposed to various light conditions

3.3.1.2 *C. rangiferina*

The relative amounts of atranorin in *C. rangiferina* can be seen in Figure 3.14. It appears that with increasing energies of UV radiation there is more atranorin in the lichen thallus. This seems to suggest that more atranorin is synthesized with increasing intensities of radiation. If atranorin does perform the role of UV blocker in the lichen, then a biological response that produces higher levels of this UV blocking agent in response to higher levels of radiation would be advantageous to the lichen. The control sample that was collected in the wild but kept in the dark isn't significantly different from the irradiated samples in this case, this may indicate that the control may have endured some light stress in the laboratory, either during sample handling, or as an extract. Precautions such as using brown glass containers were used to reduce the effect that light would have on the extracted samples.

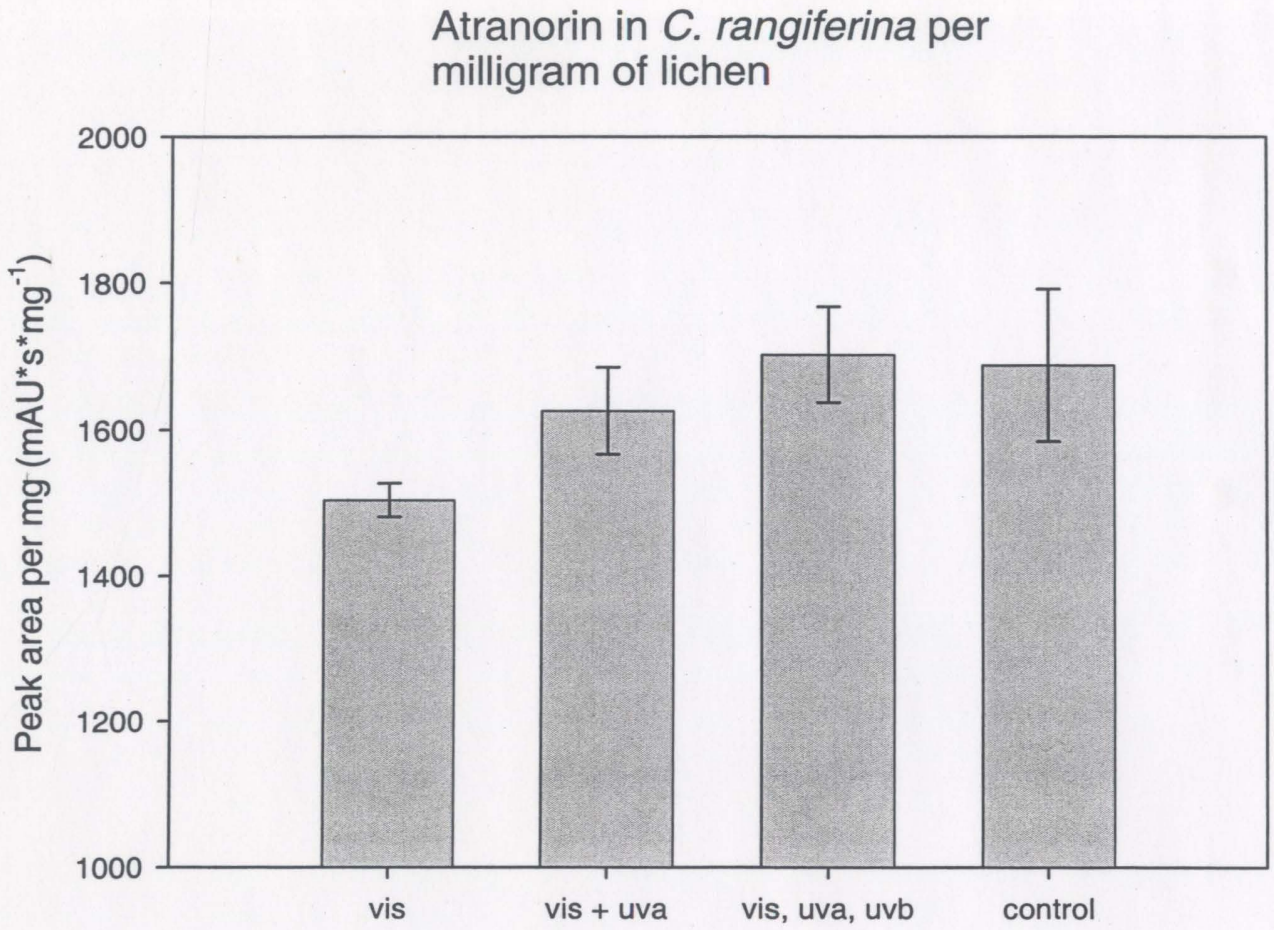


Figure 3.14 Relative levels of atranorin in *C. rangiferina* exposed to various light conditions.

3.3.2 Ground Level Ozone Study

3.3.2.1 *U. dasypoga*

Relative amounts of usnic acid in *U. dasypoga* from 5 sites in New Brunswick can be seen in Figure 3.15. It appears that usnic acid levels drop with increasing levels of ground level ozone. This suggests that either the lichen's ability to produce usnic acid is being compromised by oxidative damage from ground level ozone, or that usnic acid is reacted with the ground level ozone as an anti-oxidant and is chemically altered during the process. Fahselt and Begora (36) found that lichens that were irradiated with ultraviolet radiation showed a decrease in usnic acid but no increase of usnic acid constituents, possibly the oxidative products are similarly volatile.

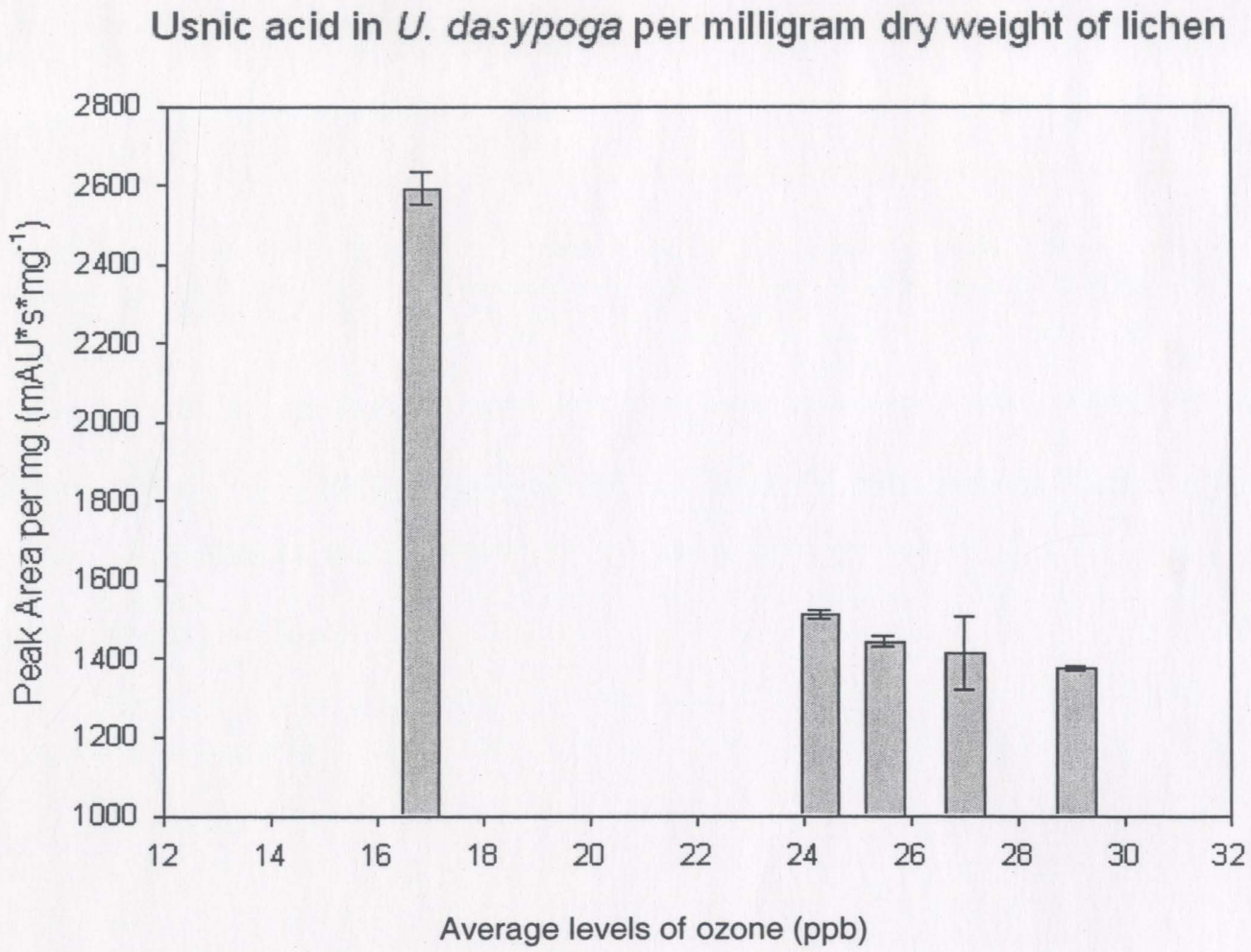


Figure 3.15 Relative levels of usnic acid in *U. dasypoga* from various sites of ozone exposure

3.3.2.2 B. trichodes

The relative amounts of fumarprotocetraric acid in lichen from five sites with differing ozone exposures can be seen in Figure 3.14. There does not seem to be any significant correlation, however, it is suspected that since fumarprotocetraric acid also absorbs UV light, more than one mechanism may be at play in this instance. The lichens were sampled at 5 sites on a transect extending inland between Point Lepreau, NB and Fredericton, NB. All 5 sites were rural sites, but they have various levels of ground level ozone. Microclimate effects near each of the sites may contribute to unpredictable environmental influences.

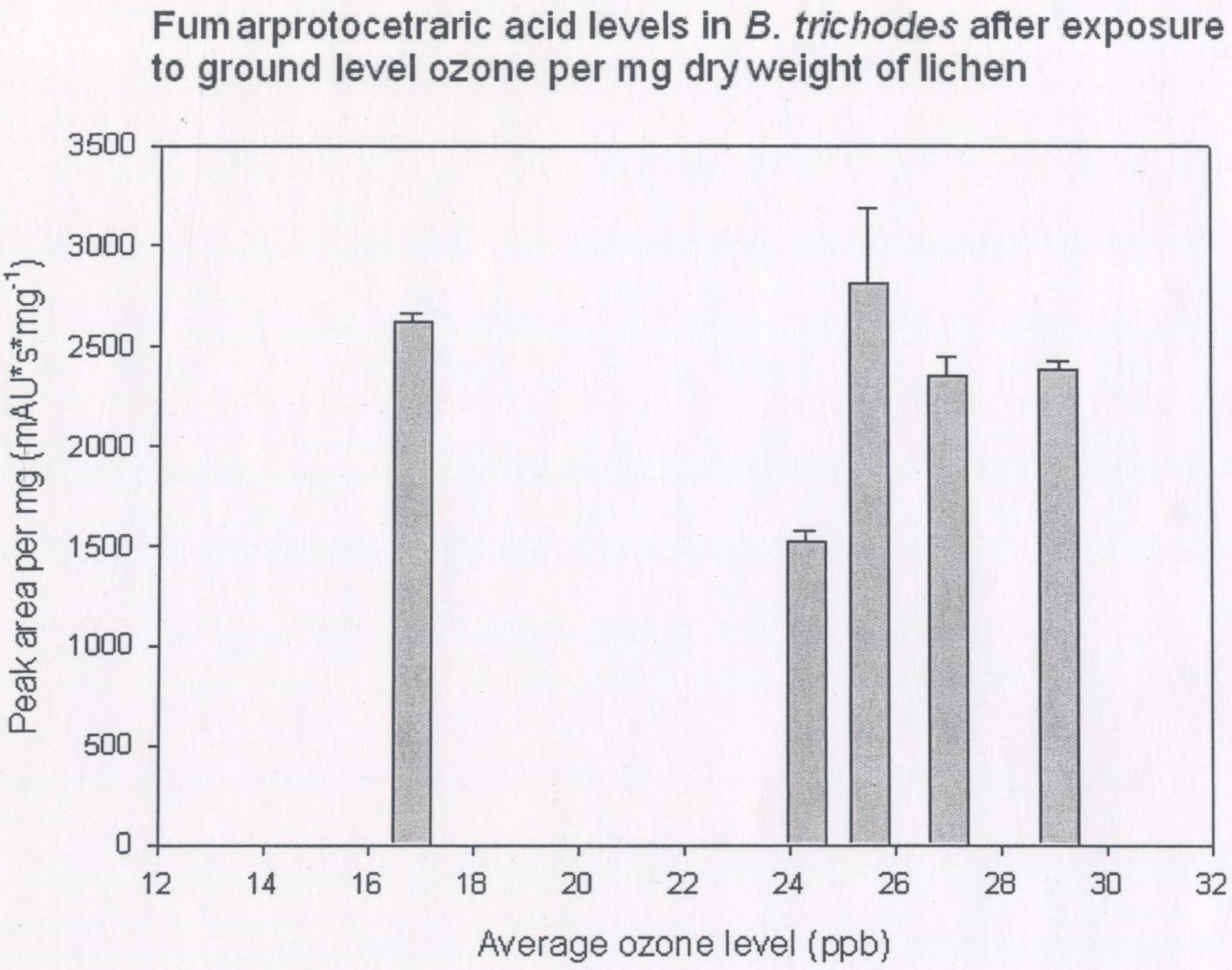


Figure 3.16 Relative levels of fumarprotocetraric acid in *B. trichodes* from various sites of ozone exposure.

CHAPTER 4

CONCLUSIONS

In conclusion, liquid chromatography coupled with electrospray mass spectrometry is a good technique for the qualitative and semi-quantitative analysis of lichen acids. Quick, easy sample preparation as well as automated sample injection allow for the possibility of mapping the changes in lichen phenolic production over a large area. The small (~2 mg) sample sizes that are required would allow a single researcher to collect many samples over a wide area. The type of data required from the analysis can be customized by adjusting the operating parameters of the LC-MS.

In negative scan mode the analysis is more sensitive due to the prevalence of negative ions from ionized carboxylic acid moieties, however, there are also adducts and clusters formed that must be taken into account. The same clusters and adducts are less prominent in the positive ionization mode; unfortunately this mode is less sensitive as M^+ ions of lichen phenolics are not as readily formed in due to the acidic nature of the compounds. The UV-vis diode array detector is sufficient for the semi-quantitative analysis of UV absorbing lichen phenolics. The mass spectral data complements the chromatographic data, and UV spectra of specific chromatographic peaks allow for identification of lichen phenolics. This is particularly useful when pure samples of the lichen phenolic in question are not readily available, (e.g. rangiformic acid and fumarprotocetraric acid) or when trying to classify a newly discovered lichen species.

The ultraviolet study offers some insight into the function of lichen phenolic compounds in lichens, in particular the conditions under which lichen phenolics are formed. It appears that amounts of atranorin in *C. rangiferina* increase with increasing ultraviolet exposure, however, levels of usnic acid in *C. mitis* have been observed to decrease under the same conditions. It is possible that usnic acid undergoes photolysis by levels of light, particularly UV-A and UV-B are of a higher intensity than natural sun light. It is possible to detect what could be photolytic fragments in the extracts of *C. mitis* that are exposed to artificial ultraviolet light (36). The lichens that were used in the UV study were irradiated over two days with higher intensities of light than would be encountered in their natural environment. This process may have lead to breakdown of usnic acid in the lichen that would have not naturally have occurred, or which may have occurred to a lesser degree. Fahselt and Begora (36) compared the effects of irradiating an extract of a usnic acid containing lichen with the same exposure to the live lichen tissue. Compounds believed to be the result of the photolysis of usnic acid were found in the irradiated extract, but not in the extract of irradiated lichen. The compounds that result from photolysis may be utilized by the lichen in some way. The increase in atranorin levels in *C. rangiferina* with increasing levels of UV exposure may be explained in two ways. One possible explanation is that the increased levels of radiation stimulate a higher rate of photosynthesis, and this increase in the metabolism of the lichen is accompanied by an increase in the rate of production of lichen phenolic compounds. It is also feasible that the production of atranorin is a defensive response to stress. Another possibility is that visible light and UV-A radiation could stimulate the

production of lichen phenolic compounds due to increased photosynthesis while UV-B, having a higher energy, could cause a decrease in these same compounds through photolysis. Many photolytic reactions are quantized; perhaps UV-B radiation contains the wavelength that has the appropriate energy to cause a change in the lichen phenolic molecule.

Several compounds in the terrestrial ozone study show promise as possible indicators of environmental oxidative stress in *U. dasypoga* and *B. trichodes* including salazinic acid, fumarprotocetraric acid and usnic acid. These compounds have previously been found to have antioxidant activity in laboratory studies (33). There is a marked decrease in usnic acid levels in *U. dasypoga* with exposure to increasing levels of ground level ozone. If the usnic acid reacted with ozone or oxygen free radicals as an antioxidant, it is probable that the usnic acid was cleaved into smaller fragment compounds. Fumarprotocetraric acid and salazinic acid have both shown antioxidant properties in laboratory testing, though our measurements of these compounds in *B. trichodes* were inconclusive. These results may indicate that these compounds are protection for the lichen for more than one type of stressor, which may be why there is no direct correlation with the measured amounts of ground level ozone at each site.

Electron micrograph studies show that the lichen phenolics exist as crystalline deposits on the surface of the lichen thallus (23). It seems to be feasible that crystals of these UV absorbing compounds would also absorb the UV light, and the crystals are in an optimal position on the surface of the lichen thallus to act as a protective barrier. What is

not as clear is whether these lichen phenolic crystals can perform as antioxidants by reacting with ozone in the solid state.

In regard to future work in the field of lichen phenolics, there are a number of avenues of research that bear further investigation. Analyses of viable lichens that have been exposed to varied natural levels of ultraviolet light for extended periods would better demonstrate the defensive responses. Healthy lichen from an unpolluted environment that are transplanted and exposed to controlled levels of ozone in a fumigation setup would be informative as well. As lichen phenolics exist as extracellular crystalline deposits, some experiments could be performed on the purified crystals to identify degradation products of ultraviolet and oxidative stress. Monitoring these degradation products of compounds that exhibit both UV blocking and antioxidant properties would help in identifying and quantifying environmental stressors for individual species. In a natural environment, the lichen would be exposed to multiple types of stressors. It is likely that harmful UV light and oxidizing ground level ozone are present in many lichen habitats at the same time, and it would be beneficial to be able to distinguish whether the changes in the levels of their lichen phenolics is due to one or the other or both stressors in concert.

Lichens have proven to be excellent monitors of the health of an ecosystem; even small changes in air quality can change the distribution of species in a particular area. Through the scope of this study, some relationships between the levels of certain lichen phenolics and exposure of the lichen to external stressors such as excess ground level ozone and harmful levels of UV light have been observed. There seem to be at least two

separate mechanisms at work: an increase in the rate of lichen phenolics in response to stress, or an increase in light available for photosynthesis, causing an accumulation of phenolic in the lichen thallus, and a decrease in the available amount of lichen phenolics through photolysis, oxidative cleavage or another method that causes decomposition of the phenolics in the thallus. It is entirely feasible that both of these mechanisms are occurring simultaneously. When more is known about how the combination of effects from different environmental stressors act on the lichen phenolics, the ability to closely monitor the relative health of an ecosystem as it changes over time, especially the changes that are related to industry and climate change will become a reality.

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APPENDICES

Appendix 1. Sampling Locations for Ozone Study in New Brunswick

Site #	Location	GPS coordinates		Lichens Collected	
2	Point Lepreau	N 45 deg 03' 806"	E 66 deg 27' 303"	*U. dasypoga*	*B. trichodes*
4	Chance Harbour	N 45 deg 05' 745"	E 66 deg 26' 266"	*U. dasypoga*	*B. trichodes*
5	Provincial Park Trail	N 45 deg 10' 250"	E 66 deg 26' 440"	*B. trichodes*	
7	Rural Road A	N 45 deg 15' 738"	E 66 deg 29' 542"	*U. dasypoga*	*B. trichodes*
8	Rural Road B	N 45 deg 21' 310"	E 66 deg 29' 767"	*U. dasypoga*	*B. trichodes*
9	Rural Road C	N 45 deg 28' 470"	E 66 deg 28' 113"	*U. dasypoga*	*B. trichodes*

Appendix 2. Summary of Ozone Data (50)

	Ozone Concentration		Overall average
Site #	July 16-Aug. 6	Aug. 6-Aug. 19	(ppb)
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

Appendix 3: Map of Sampling Locations in New Brunswick (50)