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Photolability of Secondary Compounds in Some Lichen Species

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Abstract

Ultraviolet light is known to alter thallus concentrations of lichen phenolics, thus attempts were made to assess photostability. Lichen products extracted from 14 lichen species were applied to thin-layer chromatography (TLC) plates, exposed to high intensity UV-A/B and developed in suitable solvents. Banding patterns of extracts containing gyrophoric acid, lecanoric acid and tenuiorin were not altered by UV, but patterns of species containing usnic acid, atranorin, barbatic acid and didymic acid changed visibly. Thin-layer chromatography, gas chromatography and mass spectroscopy showed that usnic acid is broken down by UV into photoproducts with molecular weights lower than usnic acid itself. However, banding patterns of extracts from intact thalli exposed to UV were not visibly different from unexposed controls for any of the four species tested, *Cladonia uncialis*, *Cladonia rangiferina*, *Peltigera aphthosa* and *Umbilicaria mammulata*. Nevertheless, quantitative analysis of *Cladonia uncialis* showed consistently lower-than-control amounts of usnic acid in irradiated subsamples of all whole thalli tested, suggesting that degradation probably occurred. Live air-dried material consistently maintained control levels of phenolics after irradiation, but hydrated subsamples tended to show decreased concentrations of usnic acid.

Keywords: Lichens, photostability, UV, degradation, secondary products, usnic acid

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1. Introduction

Most ultraviolet (UV) light that reaches the earth's surface is UV-A (320–400 nm) but some UV-B (280–320 nm) from solar emissions also penetrates stratospheric ozone. The flux of UV-B is minimal, but it is increasing slowly due to depletion of the ozone layer. Several reviews have considered the survival value of lichen products (Rundel, 1978; Lawrey, 1986; Fahselt, 1994; Elix, 1996) and one possibility is that, due to their ability to absorb light in the ultraviolet (UV) range (Hale, 1956; Rao et al., 1967; Huneck and Yoshimura, 1996), they are implicated in the exclusion or procuration of radiant energy. For example, phenolics may provide a chemical defence against harmful UV (Quilhot et al., 1992; Fahselt, 1993; Fahselt, 1994; Quilhot et al., 1994; Swanson et al., 1996; Swanson and Fahselt, 1997). Lichens are capable of responding to environment both morphologically and physiologically (Kappen, 1988), and irradiance may be one factor eliciting response. Surely lichens growing in open or unshaded conditions, particularly more long-lived species (Jahns and Ott, 1997), would be vulnerable to UV, if unable to accomodate in some way.

Ultraviolet light can excite electrons and produce a variety of effects on organic molecules. An excited electron may return to ground by re-emission of light energy as fluorescence. It may also cross to some other electronic state, transfer energy to another atom, add to a different molecule, or be further excited by absorbing a second quantum (Cox and Kemp, 1971). It is well known that proteins and nucleic acids may be damaged by UV-C and UV-B (e.g., Spikes, 1989), but the possibility that UV alters low molecular weight molecules, such as lichen phenolics, has seldom been considered. If secondary products of lichens are necessary to thallus functioning, there could be significant energetic consequences should they prove to be photolabile.

Previous growth chamber studies involved exposure of lichens simultaneously to visible and supplementary ultraviolet (UV-A and UV-B). These showed that levels of secondary products were higher in UV-treated samples than in unirradiated controls when UV-B was removed by filtration, and lower when UV-B was included rather than excluded (Swanson and Fahselt, 1997; BeGora and Fahselt, 2001). Even in the presence of UV-B, however, concentrations were no less than controls. One intention of this paper was to examine under controlled conditions the effects of UV separately from visible light on the chromatographic patterns of extracts from 14 lichen species. Other objectives were to use mass spectroscopy to assess the nature of UV damage on pure usnic acid, as large quantities of this compound were available commercially, and subsequently to evaluate whether this compound was afforded protection from degradation when located within a thallus. Effects on whole thalli were also tested in three lichens not containing usnic

acid. A last objective was to assess whether the amount of usnic acid remaining after irradiation depended on metabolic activity and in addition, because conglutinate lichen materials are more translucent when wet (Honegger, 1998), to determine whether UV-alteration was more pronounced in hydrated than in dry thallus samples.

2. Materials and Methods

Peltigera aphthosa (L.) Willd., *Stereocaulon saxatile* Magn., *Cladonia cristatella* Tuck., *Cladonia uncialis* (L.) Wigg. and *Cladonia rangiferina* (L.) Harm., *Umbilicaria deusta* (L.) Baumg. and *Xanthoparmelia cumberlandia* (Gyeln.) Hale were collected from soil or rock surfaces near Gravenhurst, Ontario, Canada (44°53.7' N, 79°23.8' W) and *U. mammulata* (Ach.) Tuck. was obtained nearby from the face of a vertical granite boulder (44°53.5' N, 79°24.2' W). All species were collected on July 30, 1998 and *C. uncialis* was re-collected on May 8, 2000 for quantification experiments. *Flavoparmelia caperata* (L.) Hale, *Punctelia borreri* (Sm.) Krog and *P. rudecta* (Ach.) Krog, collected May 18, 2000 from Concession VI (Part Lot 30) Grey County (44°19' N, 80°56' W), Ontario, Canada from dead branches in a hemlock/cedar stand. *Physcia adscendens* (Fr.) H. Olivier and *Parmelia sulcata* Taylor were obtained from dead branches at the edge of a lowland forest in Bruce County (44°10' N, 81°19' W), Ontario, Canada on May 18, 2000, while *Parmotrema perforatum* (Jacq.) A. Massal. was collected on February 25, 2000 in McClellanville, South Carolina, USA (33°05' N, 07°92' W) from branches of live oak. Species were identified or confirmed by P.Y. Wong (Canadian Museum of Nature). Lichen substances were determined using standardized TLC (Culberson and Kristinsson, 1970; Culberson, 1972; 1974) and voucher specimens were deposited in the University of Western Ontario lichen herbarium.

For all species a minimum of 3 g of material was gathered for phenolic extraction. Excess debris (e.g. mosses, leaves, substrate) was removed from samples using forceps and a small paintbrush. All samples were dried to constant weight at 30°C for a minimum of 48 hours prior to weighing out samples for phenolic extraction.

UV effects on TLC patterns of extracted lichen products

To remove phenolics for testing of photolability under UV light, three 25 mg samples from each of the fourteen species were placed in acetone-rinsed, 1.7 ml graduated microcentrifuge tubes (VWR Scientific) for extraction. Acetone (0.50 ml) was then added and each of the tubes tightly capped. After 3–5 minutes the acetone solution was transferred to a similarly-prepared microcentrifuge

tube in a Temp-Blok Module Heater (37°C) and allowed to evaporate. Acetone extraction was repeated three more times and all four extracts from one sample were combined and evaporated in the same tube.

The dried residue from each acetone extraction was re-dissolved in 0.50 ml of acetone and the tube immediately sealed to minimize concentration changes due to evaporation. Twenty µl from each of the re-dissolved extractions was spotted in 5 µl aliquots on 10 × 20 cm pre-coated Merck silica gel 60 F-254 thin-layer plates. Between successive applications the microcentrifuge tube was tightly re-capped.

After spotting 3 replicates for a given species, the silica plate was placed under UV-A/B using an Electronic Multiwave™ transilluminator (Canberra-Packard Canada Ltd.) equipped with 4–8 W (NEC T5 F8BL) black light lamps (peak 328 nm, range 300–400 nm, exposure ~9.2 kJm^{-2}). Then another series of applications of the same replicate extracts was spotted on the same plate and the plate was re-exposed to UV-A/B for another 20 minutes. Spotting and re-exposure of the plate was repeated again, so that each of the extracts was exposed to UV for 20, 40 and 60 min. Finally, a fourth set of applications was spotted on the same plate but not exposed, and this served as a control. To assess UV effects on commercial usnic acid, as opposed to usnic acid in thallus extracts, three 2 µl aliquots of a 5 mg ml^{-1} solution of (+)-usnic acid (Sigma U 7876) were applied to a TLC plate and exposed to UV for the same periods of time as phenolics extracted from thalli. The prolonged exposure of lichens to solar radiation in nature could not be duplicated under experimental conditions, but partial compensation was made by employing a source of UV irradiance that was more intense than in the field.

Extracts from each lichen species were spotted on a separate plate and plates were developed in the standard solvent (Culberson and Kristinsson, 1970; Culberson, 1972; White and James, 1985) providing the best separation (Table 1). Plates were allowed to air dry for 3–4 hours before examining under the transilluminator for evidence of change in normal chromatographic patterns. Images were captured using a digital camera and stored electronically.

Assessing the possibility of UV-induced degradation

Commercial pure usnic acid dissolved in acetone was applied to the origin of preparative TLC plates (10 × 20 cm) and the plates were exposed to UV-A/B for 40 min or 5 hr. The plates were developed in toluene:acetic acid (100:15 v/v) (Culberson and Kristinsson, 1970), air-dried for 3 hr and examined qualitatively under UV (BeGora and Fahselt, 2000). Prominent fluorescing bands appearing after 40 min were eluted in toluene. The eluate was centrifuged at 16,000 g to precipitate suspended silica particles, and the

Table 1. The standard solvent found to provide best separation of components in extracts from each lichen species tested.
Solvent compositions are: (A) toluene:dioxane:acetic acid (90:22.5:2.5 v/v/v), (B) hexane:methyl-t-butyl ether:formic acid (60:45:10 v/v/v), (C) toluene:acetic acid (100:15 v/v), and (G) toluene:ethyl acetate:formic acid (139:83:8 v/v/v) (Culberson and Kristinsson, 1970; White and James, 1985).

Lichen species	Solvent
Cladonia cristatella	A
Cladonia rangiferina	B
Cladonia uncialis	C, A
Flavoparmelia caperata	B
Parmelia sulcata	B
Parmotrema perforatum	A
Peltigera aphthosa	A
Physcia adscendens	A
Punctelia borreri	A
Punctelia rudecta	C
Stereocaulon saxatile	C
Umbilicaria deusta	C
Umbilicaria mammulata	G
Xanthoparmelia cumberlandia	A

supernatant decanted for analysis by gas chromatography mass-spectrometry (GC-MS) using a Varian 3400cx coupled to a Varian Saturn 4D MS equipped with a 30 cm × 0.25 mm I.D. DB-5MS phase column with a 0.25 micron film thickness (J&W Scientific 122-5532). The column temperature was initially 90°C and increased to 250°C at a rate of 20°C min^{-1}. The run time was 30 min. In an attempt to determine the chemical structure of photo-products, a computer library search was performed on the resulting mass spectra of the major compounds isolated.

TLC patterns of lichen products from UV-irradiated thalli

Intact thalli of *Cladonia uncialis*, a lichen containing usnic acid, were exposed to UV-A/B to determine whether this compound would be susceptible to breakdown *in situ*. Intact thalli of three other lichens, *Peltigera aphthosa*, *Umbilicaria mammulata*, and *Cladonia rangiferina*, were similarly treated. For *Cladonia uncialis* and *C. rangiferina*, podetia within a 10 × 10 cm area constituted one sample. For the foliose lichens, *Umbilicaria mammulata* and

Peltigera aphthosa, a thallus with a diameter or breadth of approximately 5–10 cm was considered a single sample.

Three air-dried samples of each species were exposed to UV-A/B on the transilluminator for one hour. These were then dried to constant weight at 30°C and a 25 mg portion of each sample from each of the four species was extracted in a microcentrifuge tube and re-dissolved in 0.50 ml acetone, as described above. From each extract 20 µl was applied to a TLC plate prior to development in the most effective of the standard solvents (Table 1).

Usnic acid concentrations after UV-exposure in relation to vitality and hydration

Ten samples of *Cladonia uncialis* were collected randomly from within a 3 × 3 m area of a lichen mat to quantify the effect of high intensity UV on secondary constituents in intact lichens. Within one week of collection each sample was separated into six subsamples, five each approx. 50 mg and one approx. 250 mg. One of the 50 mg subsamples was set aside as a control and the remaining four were exposed to UV under different circumstances. Half were killed to assess whether the capacity for metabolic response affected the degree of UV damage to secondary products. Killing was achieved by placing twenty subsamples in a Toshiba microwave oven (ERX-1600C, 1.3 kW, 120 V, 60 Hz) at full power for two 30-sec periods with an interval between of 2 min. The interval was necessary to prevent formation of thermal degradation of phenolics which was detectable with TLC after longer exposure times. A negative tetrazolium staining test (Moore, 1973) was considered to indicate lack of metabolic activity.

Half of the live and half of the heat-killed subsamples were wetted and compared to control subsamples to determine if the hydration of thalli affected the extent of UV damage to secondary products. Wetting was accomplished by submersion in distilled water for 15 min prior to UV-A/B exposure under the transilluminator. Wetted thalli initially contained 250–350% water by weight and were maintained in a moist condition with a fine spray of distilled water every 20 min during the preparation procedure. The water content in air-dried subsamples, including controls, at the outset of the experiment was approximately 5–10% by weight.

Subsamples of *C. uncialis* prepared in the four ways just mentioned were held under ambient light in the laboratory for two hours, during which time wetted samples were allowed to undergo re-saturation respiration, and then all were exposed to high intensity UV-A/B for 2 hr. During irradiation wetted subsamples were lightly sprayed with distilled water every 30 min. All subsamples were oven-dried at 30°C to constant weight and 25 mg of each of the

10 replicates within a given treatment was weighed and placed in acetone-rinsed microcentrifuge tubes for phenolic extraction.

Following extraction, separate 10 × 20 cm TLC plates were spotted with 16 µl, in 4 µl aliquots, of acetone-dissolved dried residues from each of the 10 subsamples of each treatment. The plates were then developed in toluene:acetic acid (100:15 v/v) (Culberson and Kristinsson, 1970). Densitometric analysis of TLC-separated extracts from UV-exposed and control lichens was performed following the methods of BeGora and Fahselt (2000).

The above experiment was repeated by dividing, treating and exposing another 10 samples of *Cladonia uncialis*. Extracts of each sample were applied to plates in 5 µl aliquots to a total of 20 µl, and the plates developed in toluene:dioxane:acetic acid (90:22.5:2.5 v/v/v). To permit statistical analysis of both experiments it was necessary to construct a standard curve relating integrated density values (IDV) expressed as pixels by the transilluminator to arbitrary absolute units of usnic acid and then convert observed IDV's to absolute amounts. One-way analysis of variance (ANOVA) and Tukey's comparison test (MINITAB 1996) were performed to compare substance concentrations in subsamples irradiated under different conditions.

Production of a standard curve for usnic acid

Homogeneous lichen material for the standard curve was prepared by combining approx. 250 mg from each of the 10 sample thalli of *C. uncialis* and grinding in liquid nitrogen. Then six oven-dried 0.1 g samples of homogenized thallus material were placed in microcentrifuge tubes for phenolic extraction. Phenolics were removed using four acetone washes, each of 1.00 ml. All washes were combined, evaporated to dryness, and re-dissolved in 1.00 ml of acetone. Dilution series were prepared yielding solutions that corresponded to 5, 10, 20, 30, 40 and 60 mg thallus ml^{-1} acetone. Two replicates of 20 µl from all 6 solutions were spotted on a single 10 × 20 cm TLC plate; the plates were developed and the IDV for usnic acid recorded for each concentration. The relationship established permitted IDV's obtained from known amounts of treated thalli to be converted into absolute units (compound mg^{-1} thallus ml^{-1} acetone).

3. Results

The secondary products found in each of 14 lichen species are presented in Table 2. For five species, *Peltigera aphthosa*, *Punctelia borreri*, *Punctelia rudecta*, *Umbilicaria deusta* and *Umbilicaria mammulata*, thin-layer chromatographic banding patterns of extracted compounds exposed to high

Table 2. Major compounds present in extracts from different lichen species and an indication of whether differences in TLC banding patterns were induced by exposure to UV-A/B.

Species	UV-induced differences in TLC banding patterns?	Compounds identified
Cladonia cristatella	Yes	Didymic acid, barbatic acid
Cladonia rangiferina	Yes	Atranorin, fumarprotocetraric acid
Cladonia uncialis	Yes	Usnic acid
Flavoparmelia caperata	Yes	Atranorin, protocetraric acid, usnic acid, caperatic acid
Parmelia sulcata	Yes	Atranorin, salazinic acid
Parmotrema perforatum	Yes	Atranorin, connorstictic acid, norstictic acid
Peltigera aphthosa	No	Tenuiorin, methyl gyrophorate, methyl lecanorate
Physcia adscendens	Yes	Atranorin
Punctelia borreri	No	Gyrophoric acid
Punctelia rudecta	No	Lecanoric acid
Stereocaulon saxatile	Yes	Atranorin, lobaric acid
Umbilicaria deusta	No	Gyrophoric acid
Umbilicaria mammulata	No	Gyrophoric acid
Xanthoparmelia cumberlandia	Yes	Norstictic acid, stictic acid, constictic acid, usnic acid

intensity UV-A/B were not perceptibly different from those of un-irradiated controls (Table 2). Extracts from all remaining species, including *Cladonia cristatella, C. rangiferina, Flavoparmelia caperata, Parmelia sulcata, Parmotrema perforatum, Physcia adscendens, Stereocaulon saxatile, Xanthoparmelia cumberlandia* and *C. uncialis*, differed from controls in that fluorescing substances accumulated at the origins, the amounts appearing to increase over time (e.g., Figs. 1a, 1b, 2a). In addition, chromatograms of irradiated extracts from *C. uncialis* revealed two fluorescing bands (Fig. 2a) that were soluble in toluene:acetic acid (100:15 v/v). Commercial usnic acid exposed to UV (Fig. 2b) showed fluorescing bands with the same mobility as those in extracts from *C. uncialis*.

Irradiation of commercial usnic acid heavily streaked on TLC plates, followed by separation twice in toluene:acetic acid (100:15 v/v), provided sufficient photoproducts for examination by gas chromatography and mass spectroscopy (GC-MS), and thus permitted damage assessment. Three main fluorescent bands were formed within the first 15 min of UV exposure and

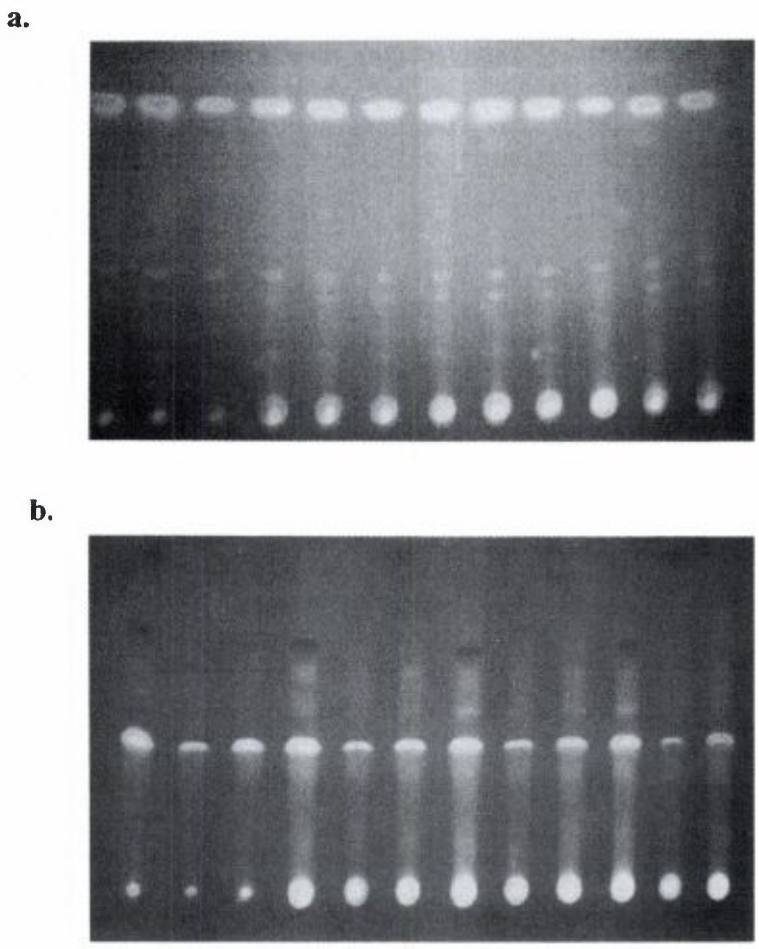

Figure 1. Chromatographic banding patterns on TLC plates exposed on a transilluminator to UV-A/B for 0, 20, 40 and 60 min, n = 3. (a) Extracts from *Cladonia rangiferina* developed in hexane:methyl t-butyl ether:formic acid (60:45:10 v/v/v); (b) extracts of *Cladonia cristatella* developed in toluene:dioxane:acetic acid (990:22.5:2.5 v/v/v).

increased in intensity over time. By 40 min these were more prominent (Fig. 3a), and by 5 hr yet others appeared (Fig. 3b). Analysis was focused on the three earlier-formed bands. The insoluble material at the origin and each of the two well-separated soluble bands were eluted in acetone, but GC revealed several peaks in the former which could not be resolved. Thus, mass spectra were only determined for the other two bands each including one peak, at $R_t \cong 10$ min.

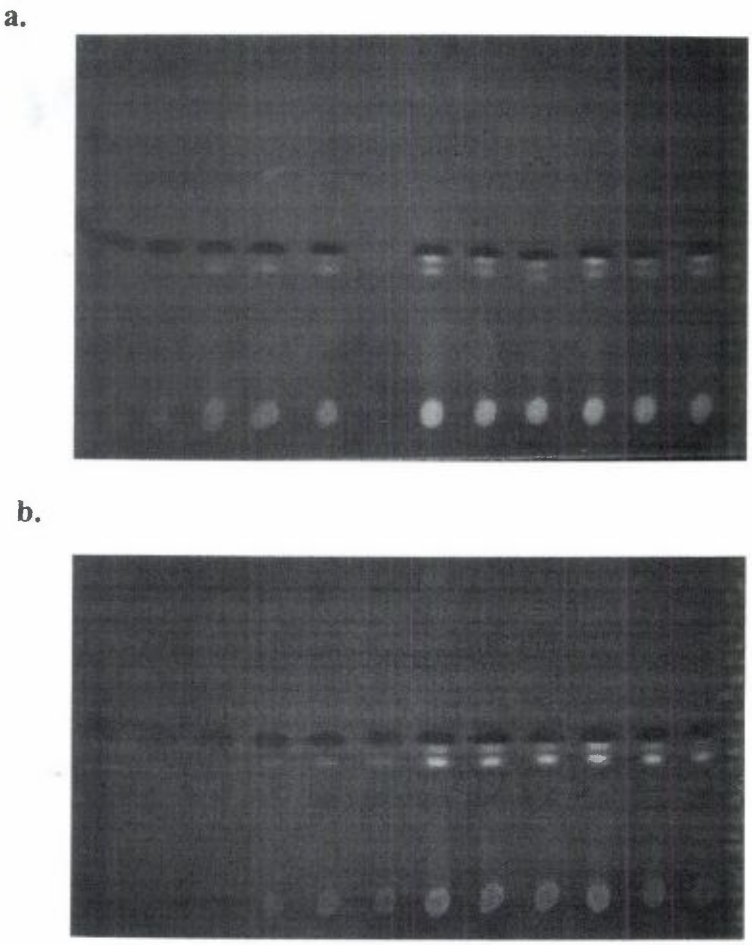

Figure 2. Chromatographic banding patterns of substances applied to TLC plates before exposure on a transilluminator to UV-A/B for 0, 20, 40 and 60 min and development in toluene:acetic acid (100:15 v/v), n = 3. (a) Acetone extract of *Cladonia uncialis* containing usnic acid, (b) commercial usnic acid.

Compounds eluted from these mobile bands had similar mass spectra, that is, mass ions with molecular weights 299 and 298 in bands 1 and 2, respectively (Fig. 4). Smaller fragments of the two also corresponded closely. However, no matches were found by comparing either with known substances in the MS library.

Irradiation of intact thalli that were subsequently extracted for lichen substances produced no visible changes in chromatographic patterns in any of

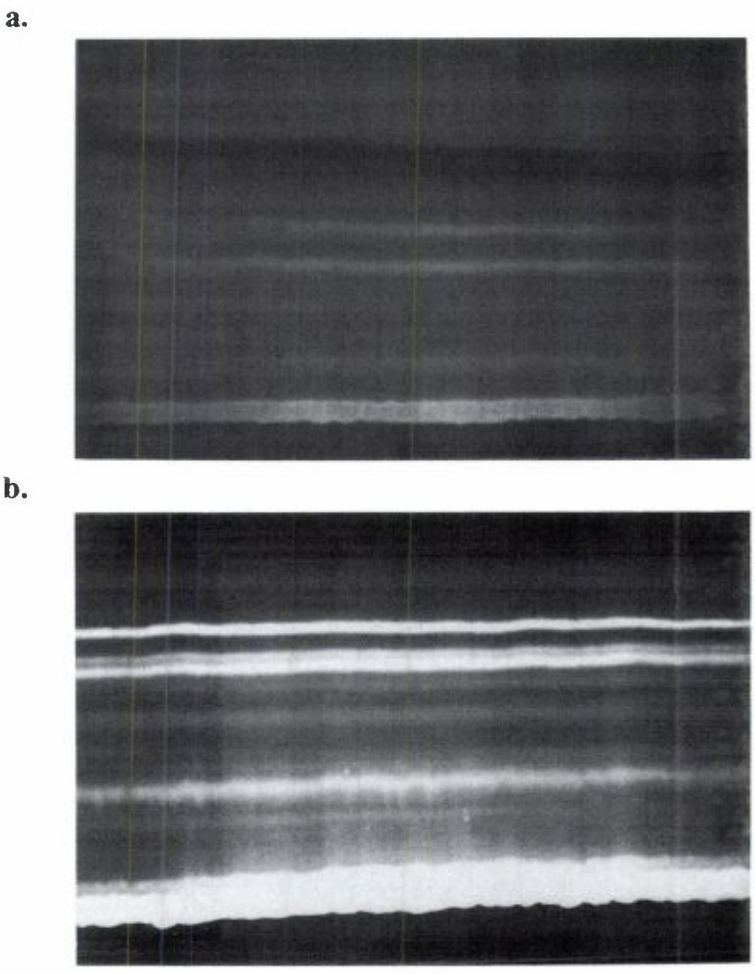

Figure 3. Chromatographic separation of pure usnic acid streaked on preparative TLC plates and exposed to UV-A/B prior to development twice in toluene:acetic acid (100:15 v/v). (a) Exposed 40 min; (b) exposed 5 hr.

the four species tested, even after 60 min of irradiation. Patterns formed by compounds of two species, *Umbilicaria mammulata* and *Peltigera aphthosa*, were thus the same regardless of whether compounds were exposed in situ or after isolation. In contrast, chromatographic responses of *Cladonia rangiferina* and *C. uncialis* were dependent on whether lichen products were irradiated before or after extraction from the thallus. There was no visible change in phenolics patterns in these species if intact thallic were exposed.

In all 10 samples subdivided for UV-exposure under different conditions usnic

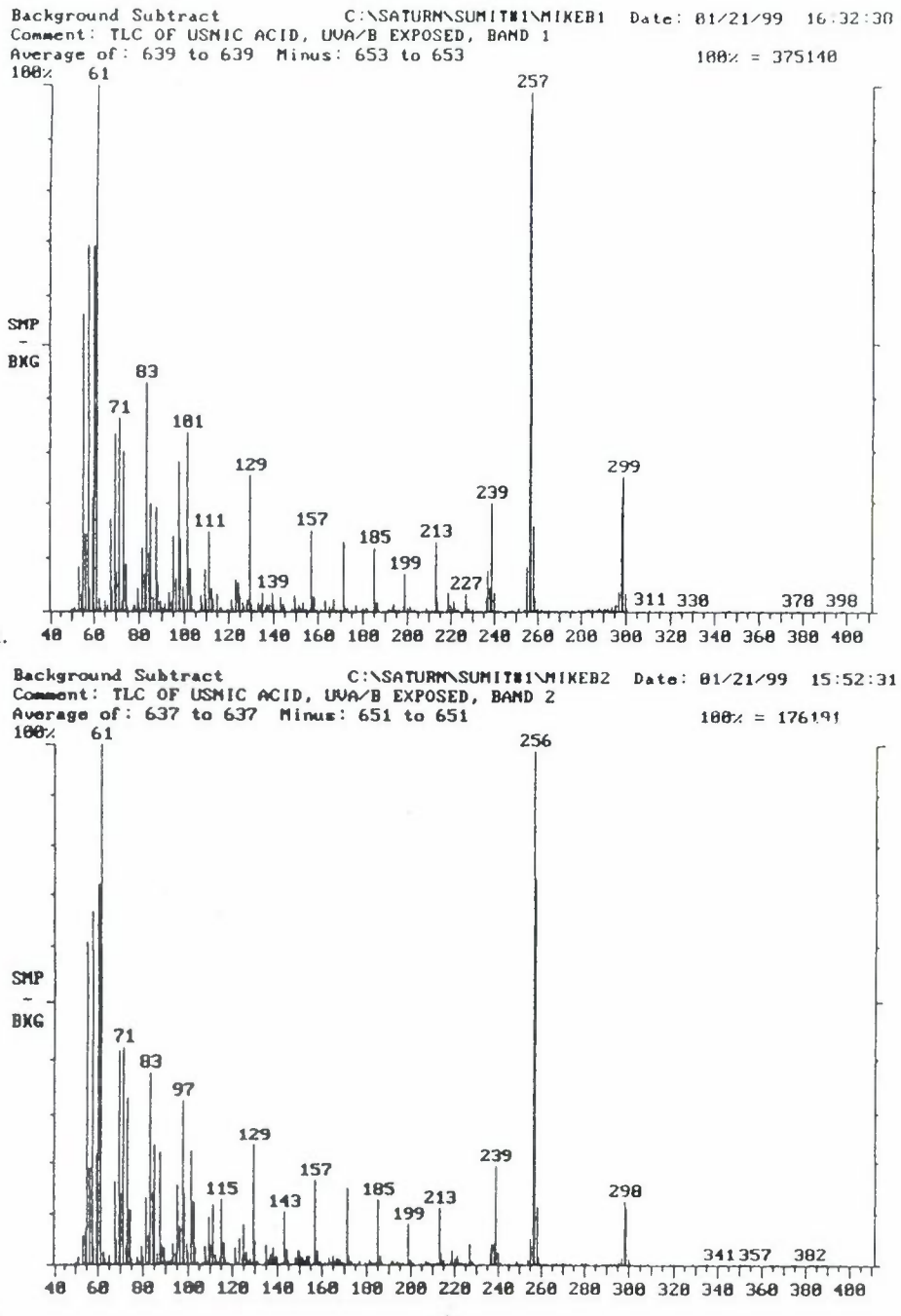

Figure 4. Mass spectra of substances with retention time (R_t) ~10 min in gas chromatography separation of mobile fluorescent products formed by UV irradiation of commercial usnic acid. (a) R_t ~10 peak from band 1; (b) R_t ~10 peak from band 2.

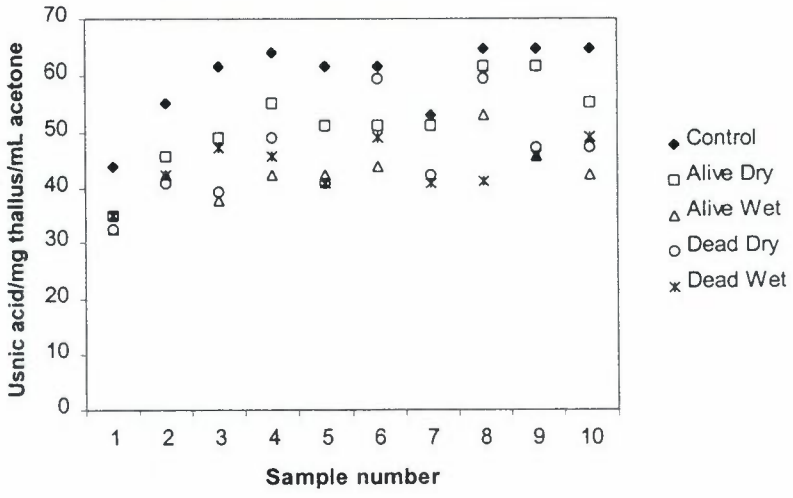

Figure 5. Usnic acid concentrations (compound mg^{-1} thallus ml^{-1} thallus) in subsamples from each of ten samples of *Cladina uncialis* exposed to UV-A/B for two hr. Four of the subsamples were subjected to a different UV treatment, while the control subsample received no supplemental UV.

acid concentrations were highest in the control and lower in all of the corresponding irradiated subsamples (Fig. 5). In nine of the 10 irradiated samples, the live and dry subsample contained the highest amounts of usnic acid, and the treatments most frequently with lowest concentration were wetted, and either killed or live (Fig. 5). Differences among treatments were shown by ANOVA ($df = 4$, $F = 10.18$, $P<0.02$) to be significant.

Tukey's comparison test ($\alpha = 0.05$) demonstrated that usnic acid concentration in irradiated live dry samples was not significantly lower than in controls (Fig. 6a), but that the levels of extractable usnic acid in all other categories of subsamples were significantly reduced due to UV exposure. Amounts accumulated in killed subsamples were significantly less than controls, regardless of hydration, and both categories of wetted subsamples contained significantly lower quantities. Thus, of the four categories of irradiated subsamples, three had significantly reduced amounts of usnic acid.

Analysis of variance of the repeated experiment also indicated significant differences among treatments ($df = 4$, $F = 3.86$, $P<0.02$). The standard errors were larger (Fig. 6b), but proportional decreases in usnic acid concentration were greater and the direction of trends was similar to the first trial. Mean levels of usnic acid were significantly lower than controls in three of the four irradiation

treatments, and Tukey's test showed the reduction was statistically significant in killed thallus portions that were hydrated as well as in live thalli.

4. Discussion

Controlled experiments are not directly translatable into field phenomena, but may provide insight into particular aspects of lichen photochemistry and ecology. For example, a UV transilluminator permits the effects of UV irradiance on lichen products to be isolated from effects of visible or photosynthetically active radiation (PAR).

Stability of isolated lichen substances

The conclusion of Hidalgo et al. (1992) and Quilhot et al. (1994) that lichen compounds are photostable was based on the low photoconsumption quantum yields of various dissolved lichen metabolites at pH values much more alkaline or acidic than biological solutions. Our observation that TLC banding patterns of *in situ* substances exposed to UV were not changed appreciably for five of 14 tested lichens supports this conclusion (Table 2). Nevertheless, the observed changes in chromatographic patterns following UV-exposure of other lichen extracts indicated that secondary substances probably differ from one another in regard to photostability. This is consistent with the observation that, when TLC-separated spots are UV-scanned densitometrically, it takes longer to obtain a stable reading with some than others (BeGora and Fahselt, 2000).

That usnic acid generated a number of new bands when irradiated, including two prominent fluorescing bands that appeared within the first 20 min of irradiation, showed clearly that this compound is susceptible to photodegradation. The accumulation of insoluble fluorescent substances at the origin after UV-exposure was further evidence of photolability (Fig. 2). Insoluble irradiation products were also found in several lichens containing no usnic acid. For example, only one of the three *C. cristatella* samples shown in Fig. 1 contained sufficient usnic acid to be detected by TLC, but fluorescent material appeared at the origin of each one, so perhaps barbatic acid and/or didymic acid were also susceptible to damage by UV and yield fluorescent photoproducts which did not migrate in the solvents used.

While TLC provides an indication of some photochemical modification of lichen substances, it seems to underestimate the extent of change. For example, thallus concentrations of phenolics in *Umbilicaria* (Swanson and Fahselt, 1997) are significantly decreased by UV-B irradiation, yet lichen extracts containing tridepsides in the present study showed no evidence in TLC chromatographic

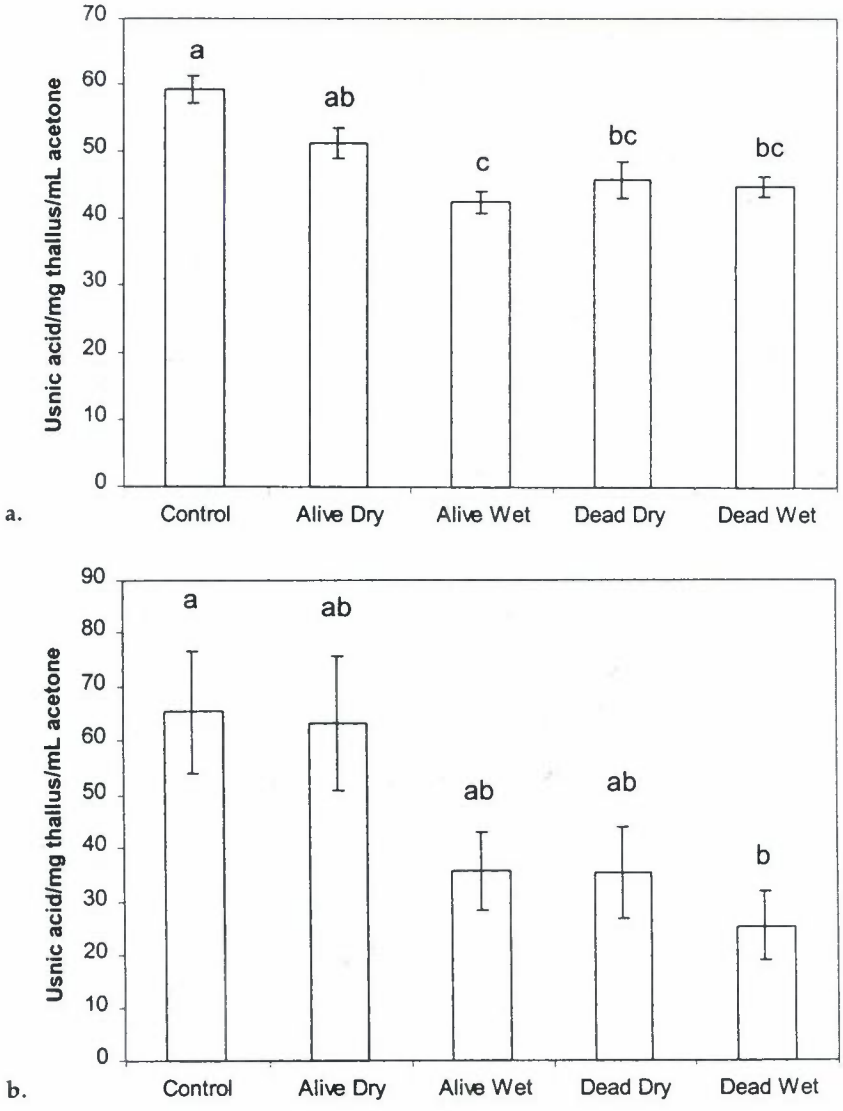

Figure 6. Mean usnic acid concentrations (compound mg^{-1} thallus ml^{-1} thallus) in UV-exposed and control subsamples of *Cladina uncialis*, n = 10. Exposed subsamples were irradiated under four different conditions. Vertical bars represent standard error, and the same letter above bars indicates they were not significantly different according to Tukey's multiple comparison test.

patterns of instability. Perhaps photoproducts of these compounds cannot be detected under the bands of UV used.

Molecular modification of usnic acid?

Ultraviolet irradiation thus appears to have the potential for modifying lichen chemistry, and GC-MS indicated that degradation can occur. Spectra obtained from the two fluorescent bands that migrated in the solvent used were very similar, and due to limitations of the instrumentation, could have been interpreted as the same compound. However, since they were well separated by TLC it is more probable they were isomers with different solubilities. But since neither corresponded to anything in the MS library, and were not assignable to any other feasible or stable structure, their identity remains unknown. In any case the mass of each was clearly less than the molecular mass, 344, of usnic acid (Culberson, 1969), confirming that the two bands represented breakdown products. The increasing number of bands produced during the course of UV exposure may have been the result not only of degradation of usnic acid itself, but also of earlier-formed photoproducts. The UV used in these experiments included wavelengths in both the UV-A and UV-B range, but previous growth chamber experiments with whole thalli showed that reduction in amounts of both atranorin and usnic acid (BeGora and Fahselt, 2001) is produced by the higher energy UV-B band.

Photolysis of usnic acid was previously studied by Takani and Takahashi (1985) who proposed that, under nitrogen in the presence of a nucleophile such as tetrahydrofuran-methanol, usnic acid is ultimately converted to usnic acid methoxide. However, it is unlikely that such a reaction could take place in an oxidizing atmosphere and therefore it would not explain the degradation observed in our experiments.

Stability of lichen products within the thallus structure

An explanation is required for the fact that some lichen compounds showed chromatographic evidence of instability when exposed to UV as extracts, but not as constituents of an intact lichen. Even the most conspicuous fluorescent photoproducts of pure usnic acid did not appear after whole thalli of *C. uncialis* had been irradiated for 60 min. However, because quantification showed that decreases after UV exposure were in some cases statistically significant, it appears that phenolics must have been degraded. The resulting photoproducts were perhaps not detected due to irreversible binding on cell walls or matrix material. Thus, it appears that TLC, while capable of revealing some photochemical change, does not indicate all, because it showed no evidence that usnic acid in whole thalli, or tridepsides exposed after extraction, had been altered.

If usnic acid is naturally degraded by UV, lower concentrations might be expected in lichens exposed to high intensity irradiance. Photolability is not,

however, inconsistent with the higher amounts reported in sunny locations (Rundel, 1969). This observation could be explained by selection for the high-usnic acid variants within a population or by physiological response to irradiance.

Levels of usnic acid in relation to vitality and wetting

Thallus concentrations of any lichen product are undoubtedly affected by the rate of synthesis as well as by possible loss due to photochemical or other type of degradation. Incorporation of labeled carbon into usnic acid takes place at various times of the year but mainly during the winter months (Taguchi et al., 1969), so phenolic content is expected to partly reflect seasonal patterns of photosynthetic carbon fixation.

Of the four categories of UV-exposed *C. uncialis*, those most similar to unirradiated controls in both quantification trials were living and irradiated while dry. However, because mean concentrations of usnic acid in *C. uncialis* were not significantly higher in live subsamples than in killed, with the same moisture content, it would appear that momentary metabolic competence was not particularly important to the maintenance of phenolic content. However, synthesis is presumably necessary during some period of the year, to achieve normal levels.

Thallus hydration seems to have influenced usnic acid concentrations because levels in UV-exposed wet material were generally lower than in air-dried controls. This was always the case with dead subsamples and, in the first experiment, with live ones as well. In the second experiment, the mean concentration in wet live subsamples was well below that in dry, but the difference was not statistically significant. Lower phenolic concentrations in wet material would be consistent with increased transmission of irradiance, and such a result was expected because conglutinate materials in the cell wall and matrix system of lichens are more transparent when hydrated (Honegger, 1998) and probably permit increased penetration of UV as well as visible light. Some loss of usnic acid, however, would probably not be totally debilitating as lichens usually encounter less UV-B when wet than when dry, due to the presence of heavy cloud cover in rainy periods.

Regardless of whether visible light is provided experimentally, exposure to UV-B along with UV-A produces lower phenolic concentrations than UV-A alone (Swanson and Fahselt, 1997; BeGora and Fahselt, 2001). However, damage seems to be ameliorated if white light is available. That is to say, the present UV treatments which involved no visible light, all reduced concentrations of usnic acid in *C. uncialis*, but when this species is treated with UV in conjunction with white light, concentrations never decrease below control

levels (BeGora and Fahselt, 2001). In fact, high intensity UV-A with visible light increases concentrations of both usnic acid in *C. uncialis* and atranorin in *C. rangiferina* above control levels (BeGora and Fahselt, 2001), and also promotes phenolic accumulation in *Umbilicaria* (Swanson and Fahselt, 1997). A band within the visible range may have a stimulatory effect similar to that of UV-A.

5. Conclusions

The effects of UV differ depending on the lichen substances involved, but photodegradation can occur and may be more extensive than shown by changes in TLC banding patterns. More information is obviously needed to understand the photostability and function of a wider range of lichen products.

Absorption of ultraviolet by a cortical substance such as usnic acid, and deployment to produce photochemical change, probably means less radiation available to damage proteins and nucleic acids. Furthermore, it is understandable if, in the course absorbing radiant energy, lichen products are modified structurally. However, even after irradiation with high-intensity UV, lichen substances remain in a thallus, consistent with the observation that phenolics are detectable in most field-collected samples. Although usnic acid tends to be more readily degraded in hydrated thalli, this probably does not disadvantage a lichen seriously. Visible light may ameliorate photodegradative effects of UV in nature and, presumably, synthesis in certain seasons of the year would eventually compensate for any loss.

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