Symbiosis, 30 (2001) 301–315
Balaban, Philadelphia/Rehovot

Acetone Rinsing – A Method for Testing Ecological and Physiological Roles of Secondary Compounds in Living Lichens

KNUT ASBJØRN SOLHAUG and YNGVAR GAUSLAA*
Department of Biology and Nature Conservation, The Agricultural University of Norway, P.O. Box 5014, N-1432 Ås, Norway, Fax. +47-64948502
E-mail. yngvar.gauslaa@ibn.nlh.no

Received February 28, 2001; Accepted April 23, 2001

Abstract

Acetone can extract secondary compounds from many air-dry lichens without affecting their viability. More polar solvents were highly detrimental to photosystem II (PSII), whereas less polar solvents failed to extract the compounds. Acetone tolerance, assessed by chlorophyll fluorescence after a 48 hrs recovery subsequent to the acetone submersion, varied between the 12 studied species. *Peltigera aphthosa* suffered already after 30 minutes, whereas the vitality of *Lasallia pustulata* did not start to decline until after 250 hrs submersion. Lichens with *Coccomyxa*, *Dictyochloropsis* and *Nostoc* photobionts were more acetone-susceptible than the five tested *Trebouxia* lichens. Whereas secondary compounds could be completely extracted within <1 hr in *Xanthoria parietina*, a 200 hrs extraction was needed for *L. pustulata* and *Xanthoparmelia conspersa*. Secondary compounds of *P. aphthosa*, *Nephroma arcticum* and *Lobaria pulmonaria* could not be extracted before the viability was severely affected. Accordingly, *Trebouxia* lichens appeared to be most suited for testing ecological and physiological roles of secondary compounds.

Keywords: Lichen photobionts, secondary compounds, acetone tolerance, extraction methods, chemical ecology

*The author to whom correspondence should be sent.

0334-5114/2001/$05.50

1. Introduction

A high tolerance to concentrated acetone appears to be a common characteristic of desiccated poikilohydric organisms of various taxonomic groups, including also ferns and bryophytes (K.A. Solhaug and Y. Gauslaa, unpublished data) and dried seeds (Milborrow, 1963; Tao and Khan, 1974). Laboratory experiments as well as long-term field transplantation studies (Solhaug and Gauslaa, 1996) have shown that both the photobiont and the mycobiont of the air-dry lichen *Xanthoria parietina* survive repeated acetone rinsing. Since acetone can efficiently extract several water insoluble secondary compounds in lichens (e.g. Huneck and Yoshimura, 1996), apparently without adverse effects, acetone rinsing allows testing of the various assumed roles of secondary compounds (e.g. Fahselt, 1994; Elix, 1996). Secondary compounds in lichens are produced by the mycobiont (Culberson, 1969; Culberson et al., 1977), but are often assumed to influence the symbiosis between the two bionts. Until now, acetone rinsing has been applied to study the function of some secondary compounds on high irradiance protection (Solhaug and Gauslaa, 1996), water relations (Lange et al., 1997; Souza-Egipsy et al., 2000) and chemical defense (Lawrey, 2000). We have additional recent, not yet published results showing that acetone-rinsed lichens actually resynthesize secondary compounds under certain environmental conditions. This amazing possibility to study functional roles could be caused by the extracellular location of secondary compounds as crystals attached to the outside of medullary hyphae (e.g. Fahselt and Alstrup, 1997) and photobionts (e.g. Honegger and Peter, 1994), which means that the acetone does not need to enter the cells in order to extract the compounds.

So far, acetone tolerance has only been tested for a few lichen species. Recent preliminary experiments showed that extended extractions were required for a complete extraction of some lichens, and too long acetone-exposures were found to be detrimental. Therefore, in order to apply acetone rinsing as a method for studying the roles of various secondary compounds in a variety of lichen species, more knowledge is needed concerning: (1) species-specific tolerances of prolonged acetone rinsing, and (2) time requirements for an efficient extraction. A main objective of this paper was to gather such knowledge by screening a number of lichens originating from various substrates (epiphytic, terricolous, saxicolous) and habitats (shaded and sun-exposed). Since the susceptibility could vary between different photobionts, lichens inhabited by various photobiont genera were selected. A final objective was to apply other solvents to lichens in order to see whether these could replace acetone in cases where acetone tolerance or extraction rate was low.

2. Methods

Acetone tolerance

Twelve lichen species, inhabited by five different photobiont genera, from habitats ranging from open, sun-exposed rock to shaded forest were collected in various locations in southern Norway (Table 1), in order to assess their tolerance to extended acetone rinsing. Their content of secondary compounds according to Krog et al. (1994) is included in Table 1. Air-dry lichens were brought to the laboratory, dried further at room temperature for 24 hrs before being stored at –20°C until the start of the experiment in end of March 2000. Collected thalli of each species were then cut in 3–4 cm^2 pieces, and mixed. Ten samples of each species were randomly selected as controls. All remaining thalli of each species were placed in a glass container with a lid, covered by acetone at time zero and left at room temperature. After 0.5, 1, 2, 4, 8, 15.1, 32.5, 61.5, 123, 246, 409, 635 and 1015 hrs, respectively, 10 randomly selected samples of each lichen species were removed, and left under a ventilator for about 12 hrs to let all the remaining acetone evaporate. Afterwards, samples from all exposure durations and controls were moistened by gently spraying distilled water, and thereafter kept hydrated for 48 hrs at 18°C and 3 μmol m^{-2} s^{-1} incandescent light in order to recover from reversible damages.

Photobiont viability was assessed subsequent to recovery by measuring F_V/F_M. Reductions of F_V/F_M caused by down regulation of photosystem II (PSII), state transitions or milder forms of photoinhibition, will have recovered after 9 hrs of darkness (Quick and Stitt, 1989; Ögren, 1994). Therefore, our F_V/F_M measurements assessed only long-lasting damage to PSII. Chlorophyll *a* fluorescence induction curves were recorded with a portable fluorometer (Plant Efficiency Analyser, Hansatech, King's Lynn, Norfolk, UK). Samples were dark pre-adapted for 15 min. Fluorescence induction curves of 5 s duration were recorded at one randomly selected position (measuring area 0.126 cm^2) of each thallus sample during an irradiance of 1500 or 3000 μmol m^{-2} s^{-1} from light emitting diodes. 1500 μmol m^{-2} s^{-1} was used for lichens with the photobiont genera *Dictyochloropsis* and *Coccomyxa*, since a higher irradiance would have caused overscale errors due to high fluorescence yield; 3000 μmol m^{-2} s^{-1} was used for lichens with *Nostoc* and *Trebouxia* photobionts.

Acetone extraction experiments

Seven of the lichen populations in Table 1 were selected in order to study whether their secondary compounds could be completely extracted before the viability declined. All lichens were not tested because of extremely low acetone

Table 1. Names, photobiont, secondary compounds, and details on habitats and location of studied lichen species.

Lichen species	Photobiont	Habitat	Collection date	Locality	UTM-grid	Secondary compounds[6)]
Peltigera canina (L.) Willd.	*Nostoc*	Terricolous, open, N-facing rock, 40 m	20/3 2000	Akershus, Ås, Kinn	NM 987174*	No compounds
Degelia plumbea (Degel.) P.M. Jørg. & James	*Nostoc*	Epiphytic, in open forest	3/6 1996	Sør-Trøndelag, Åfjord, Kringsvatnet	NR 6075**	No compounds
Lobaria amplissima (Scop.) Forssell	*Dictyochloropsis reticulata*[3)]	Epiphytic, in open forest	3/6 1996	Sør-Trøndelag, Åfjord, Kringsvatnet	NR 6075**	± Scrobiculin
L. pulmonaria (L.) Hoffm.	*Dictyochloropsis reticulata*[3)]	Epiphytic, shaded forest, 40 m	23/5 1999	Vest-Agder, Kristiansand, Selåsen	MK 484434*	Stictic acid, norstictic acid (+)
L. virens (With.) J.R. Laundon	*Dictyochloropsis reticulata*[3)]	Epiphytic, heavily shaded stem, 10 m	7/6 1999	Vest-Agder, Kristiansand, Greppestøl	MK 426493*	± Scrobiculin
Nephroma arcticum (L.) Torss.	*Coccomyxa*	Terricolous, in open, subalpine stand, 960 m	1/8 1999	Buskerud, Hol, Osjøen	NM 5994**	Nephroarctin, phenarctin, methyl gyrophorate, zeorin, ± usnic acid
Peltigera aphthosa (L.) Willd.	*Coccomyxa*	Terricolous, open N-facing rock, 40 m	20/3 2000	Akershus, Ås, Kinn	MM 987174*	Tenuiorin, methyl gyrophorate, gyrophoric acid (+), terpenoids

Table 1. Continued.

Lichen species	Photobiont	Habitat	Collection date	Locality	UTM-grid	Secondary compounds[6)]
Neofuscelia pulla (Ach.) Essl.	*Trebouxia* cf. *gigantea*[4)]	Saxicolous, sun-exposed, SW-facing rock, 3 m	7/6 1999	Vest-Agder, Kristiansand, Justvik	MK 434510*	(1) stenosporic acid (2) divaricatic acid (3) glomelliferic acid, perlatolic acid. All: ± gyrophoric acid
Xanthoparmelia conspersa (Ach.) Hale	*Trebouxia gigantea*[2)]	Saxicolous, sun-exposed, SW-facing rock, 3 m	7/6 1999	Vest-Agder, Kristiansand, Justvik	MK 434510*	Usnic acid, stictic acid, norstictic acid
Xanthoria parietina (L.) Th. Fr.	*Trebouxia arboricola*[1)]	Epiphytic, sun-exposed stem, 100 m	16/3 2000	Akershus, Ås, Østby	PM 004166*	Parietin
Lasallia pustulata (L.) Mérat	*Trebouxia* cf. *jamesii*[5)]	Saxicolous, sun-exposed, W-facing rock, 3 m	23/5 1999	Vest-Agder, Kristiansand, Kongshavn	MK 414510*	Gyrophoric acid
Umbilicaria spodochroa (Hoffm.) DC.	*Trebouxia* cf. *jamesii*[5)]	Saxicolous, sun-exposed, SW-facing rock, 20 m	6/6 1999	Vest-Agder, Kristiansand, Odderøya	MK 414437*	Gyrophoric acid

[1)]Beck et al. (1998), [2)]Friedl (1989), [3)]Tschermak-Woess (1995), [4)]Friedl et al. (2000), [5)]Thomas Friedl (personal communication), [6)]Krog et al. (1994) with the following abbreviations: ±: present or absent, (+) present in small quantities. Grids: * WGS84, ** ED50.

tolerance (*Peltigera aphthosa*), insufficient lichen material (*Lobaria amplissima, L. virens*), or lack of secondary compounds (*Degelia plumbea, Peltigera canina*) (Table 1). Ten small pieces, 40–100 mg dry matter, of each species were selected and dried overnight at room temperature in a desiccator before dry mass was determined.

Each single thallus piece (n=10) of every species was put in one glass vial and 5 ml 100% acetone was added. After the first extraction period, the acetone extract was removed and placed in an empty vial. Immediately afterwards a new 5 ml acetone was added to the same lichen sample (second extraction), and the acetone was transferred to a second vial subsequent to the second extraction period. This procedure was continued until either a satisfactory extraction had been obtained, or until the total accumulated extraction had reached a time when F_V/F_M was likely to decline according to the tolerance experiment. Accumulated extraction durations followed a logarithmic scale. The first acetone was added at time zero (t_0). Successive extracts were harvested at t_1 to t_{11}: 0.083, 0.25, 0.6, 1.25, 2.6, 5.3, 10.6, 21.3, 45, 95 and 178 hrs after the start, each extraction duration was therefore t_n-$t_{(n-1)}$. The extracts were left to evaporate completely before each evaporated extract was redissolved in 5 ml ethanol. Some extracts were diluted because of a poorer solubility in ethanol compared to acetone, or because of high absorbances.

Absorbance spectra were measured with a Shimadsu UV-2101PC spectrophotometer for the wavelength range 250–550 nm. For species with more than one distinct absorbance peak, the peak at the longest wavelength was selected for measuring the absorbance. These wavelengths were selected since our concern has been high irradiance and UV stress in lichens (Gauslaa and Solhaug, 1999, 2000) and protection against damaging solar radiation (Gauslaa and Solhaug, 2001). Secondary compounds have been assumed to protect against excess radiation, and no solar radiation below 280 nm penetrates the atmosphere. The wavelengths selected for measurements were: *Lasallia pustulata*: 303 nm, *Lobaria pulmonaria*: 311 nm, *Neofuscelia pulla*: 306 nm, *Nephroma arcticum*: 314 nm, *Parmelia sulcata*: 310 nm, *Umbilicaria spodochroa*: 302 nm, *Xanthoparmelia conspersa*: 310 nm and *Xanthoria parietina*: 436 nm. *Xanthoparmelia conspersa* showed somewhat complicated pattern with a shoulder at about 310 nm at the two first extractions. Thereafter, a peak at 310 nm was distinct at the 6 following extractions, but disappeared at the 3 last extractions. For species with gradually disappearing peaks at late extractions, the peak wavelength at earlier extractions was selected for measurements. All absorbances were calculated for 1 g dry matter of lichen material in 5 ml acetone and 1 cm light pathway in the cuvette.

Extraction experiments with other solvents

A few lichens (*Xanthoria parietina, Physcia tenella* and *Parmelia sulcata* collected at the *X. parietina* locality in Table 1) and winter hardened, air-dry fronds of the evergreen fern *Polypodium vulgare* were immersed (n=10) in the following solvents: ethanol, methanol, acetone, chloroform, n-hexane and petroleum ether (boiling range 100–140°C), for 4 times 30 minutes. After extraction, they were left in a fume hood for about 25 hrs to let remaining solvents evaporate. Then samples from all treatments including controls were moistened by gently spraying with distilled water and thereafter subjected to the same recovery protocol as mentioned above before measuring F_V/F_M.

3. Results

Acetone tolerance

The lichens containing the photobionts *Coccomyxa* and *Nostoc* (Table 1) were the most sensitive to acetone rinsing, being killed or severely affected after 8 hrs (Fig. 1). The *Coccomyxa* photobiont from *P. aphthosa* was significantly affected already at the first harvest after 30 minutes (P=0.0058, Mann-Whitney rank sum test), and F_V/F_M was reduced from 0.781±0.003 (mean±SE) to 0.418±0.084 within one hour (P<0.0001). The photobiont *Dictyochloropsis reticulata* (Table 1) appeared more resistant than *Nostoc* and *Coccomyxa*, F_V/F_M being normal (*L. pulmonaria*) or slightly reduced (*L. amplissima* and *L. virens*) after 8 hrs acetone rinsing, and the PSII was fully destroyed (*L. virens*) or heavily damaged after 60 hrs (Fig. 1). *Trebouxia* was clearly the most acetone-resistant photobiont, remaining fairly healthy after 60 (*X. parietina, N. pulla*) to 250 hrs (*U. spodochroa*), and not killed before 250 (*X. parietina*) to 1000 hrs (*L. pustulata, U. spodochroa, X. conspersa*). Standard errors were small in healthy and severely damaged specimens, while the variation was larger at an intermediate damage level (Fig. 1), indicating variability in resistance. For each of the three most resistant species (*X. conspersa, L. pustulata, U. spodochroa*), all individual samples started to decline at about the same time (data not shown). The more sensitive species showed lowered F_V/F_M-values in some thalli much earlier than in other thalli, e.g. in *N. arcticum* where the F_V/F_M-value of the most sensitive replicate fell already after 1/2 hr, whereas the most resistant replicate stayed constant until 8 hrs.

The five tested *Trebouxia* lichens were from the most sun-exposed habitats (Table 1). Therefore, photobiont genus, sun- and desiccation exposure were somewhat confounded factors. Nevertheless, acetone tolerance appeared to be

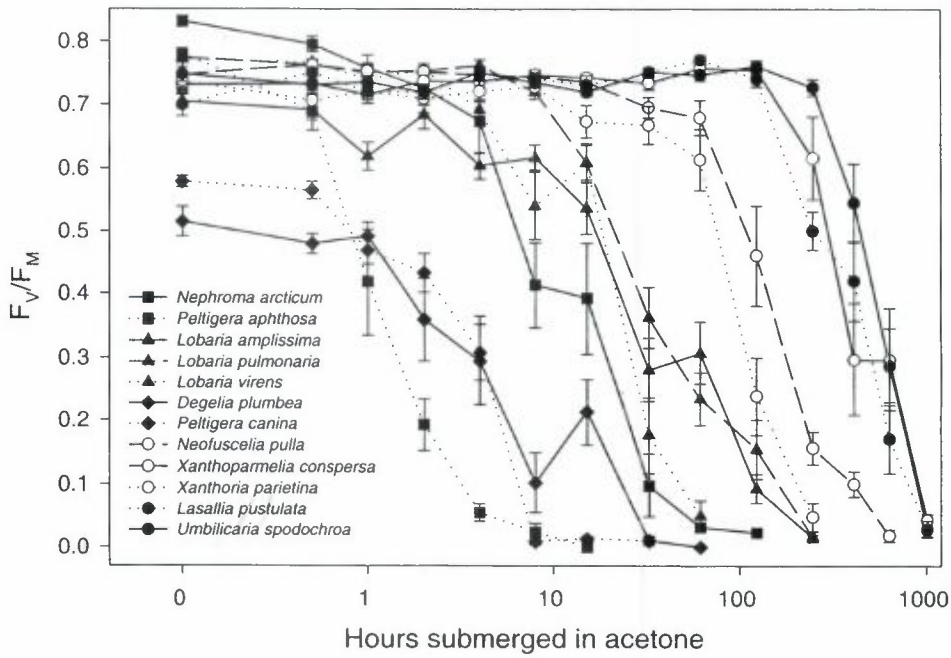

Figure 1. Viability of 12 lichen species, measured as PSII efficiency (F_V/F_M), after submersion in 100% acetone for various durations. Symbols show mean F_V/F_M-values (after 48 hrs recovery) and vertical bars show standard errors (n=10) when larger than symbols. Note the logarithmic scale at the abscissa from duration of 30 minutes and longer. The logarithmic scale is broken between 0 hrs and 30 minutes, 0 hrs reflects controls not exposed to acetone.

more related to photobiont type than to environmental conditions. For instance, the relatively resistant *L. pulmonaria* was collected from a considerably more shaded environment than any of the more susceptible lichens containing *Nostoc* or *Coccomyxa* as the main photobiont (Table 1).

Extraction efficiency of acetone

The extraction efficiency varied between species. Whereas 200 hours were needed for *L. pustulata* to reach satisfactory extraction yields, 2.5 hrs were enough for *X. parietina*, *U. spodochroa* and *N. pulla* (Fig. 2). The amount of extracted secondary compounds decreased with time for *X. parietina*, *U. spodochroa*, *N. pulla* and *X. conspersa*, with *X. parietina*, *U. spodochroa* and *N. pulla* being the fastest and *X. conspersa* being the slowest extractable lichen (Fig. 2a). Whereas the relative absorbance in extracts of *X. parietina* was

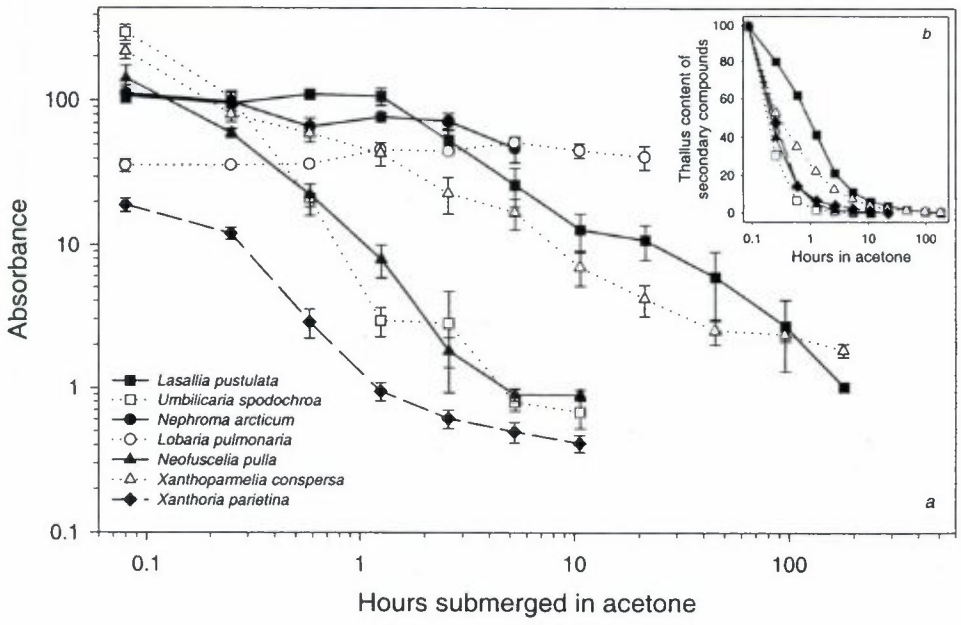

Figure 2. a. Mean absorbances of successive acetone extracts of intact, desiccated thalli of 7 lichen species taken at a logarithmic time scale. The time passed since the previous extraction represents the extraction duration. Absorbances are calculated for 1 g DM lichen in 5 ml acetone and 1 cm light pathway. Vertical bars show standard errors (n=10) when larger than symbols. b. Thallus content of secondary compounds. Fig. 2b shows the same data as Fig. 2a, but calculated as the relative content of secondary compounds in the lichen thalli prior to each extraction event. The total extracted amount is set to 100. *Nephroma arcticum* and *Lobaria pulmonaria* are omitted due to incomplete extraction of these species.

reduced to 5% of start values within 1 hour and to 2% after 10 hours, the corresponding reduction for *X. conspersa* was 20% after 1 hour, 3% after 10 hours, and still 1% after 100 hours. Comparing extractions (Fig. 2a) with tolerance limits (Fig. 1) we can conclude that secondary compounds can be extracted from all these species without severe reduction in F_V/F_M. This was not the case for *L. pulmonaria* and *N. arcticum*. For *L. pulmonaria* even a slight increase in absorbance was observed (Fig. 2a) until its tolerance limit (Fig. 1) was exceeded. The absorbance after 5 and 10 hours were 144 and 127% of the first extracts, with hardly any decrease at the final extraction period (Fig. 2a). For *N. arcticum*, the situation was even worse.

Lasallia pustulata needed a very long time to be extracted. The absorbance did not start decreasing until the 5th extraction (Fig. 2a), and 100 hours were

needed to reduce the absorbance to 2.5% of start values. However, since this species ranked among the most acetone-resistant species (Fig. 1), acetone rinsing could safely extract secondary compounds.

Some extraction modifications were tried in order to improve the extraction rate from the slowly extractable *L. pustulata*. These were (1) increasing the extraction temperature from the standard room temperature to 40°C, (2) continuous shaking during extraction, and (3) simultaneous shaking and ultrasound. However, the outcome of all these treatments applying four subsequent 30-minute acetone extractions, were similar to results of the standard acetone rinsing at room temperature with no shaking (data not shown).

Experiments with other solvents

Ethanol and methanol killed desiccated *X. parietina* thalli rapidly and completely, whereas acetone, chloroform, petroleum ether, n-hexane did not reduce F_V/F_M significantly (Fig. 3). Identical results were found for *Physcia tenella*, and for air-dry fronds of the evergreen fern *Polypodium vulgare* (data not shown).

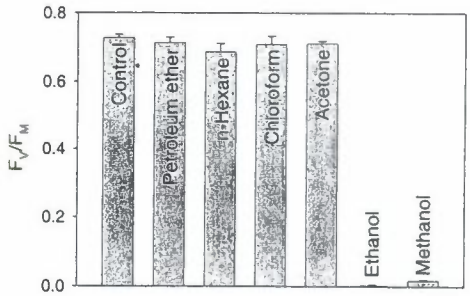

Fig. 3.

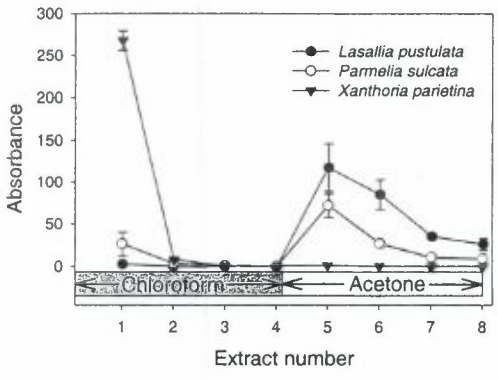

Fig. 4.

Figure 3. Viability of *Xanthoria parietina*, measured as PSII efficiency (F_V/F_M), after being submerged air-dry in various solvents 4 times for 30 minutes. Mean F_V/F_M-values (after 48 hrs recovery) and standard errors of mean (n=10) are shown. Solvents are ranked by increasing polarity according to Stahl (1967).

Figure 4. The relative absorbance of four successive chloroform extracts followed by four successive acetone extracts for three lichen species. Each extraction lasted for 30 minutes. Symbols show mean absorbances and vertical bars show standard errors (n=3) when larger than symbols.

Whereas ethanol and methanol were very destructive, petroleum ether and n-hexane failed to extract secondary compounds from tested lichens (data not shown). Chloroform was as efficient as acetone in extracting parietin from *X. parietina* (Fig. 4). However, chloroform did not extract any compounds from *L. pustulata*, and only small amounts from *Parmelia sulcata* (Fig. 4). Therefore, no means were found to improve the extraction yield from living lichens compared to simple acetone extractions.

4. Discussion

Survival in acetone

We have focussed on the viability of the photobiont by measuring damage as a persistent depression in PSII efficiency subsequent to a recovery period. The mycobionts, when seen at the levels of suborders, families and genera, are strongly selective towards their photobiont (Rambold et al., 1998). At the time when the PSII was destroyed, i.e. with F_V/F_M-values <0.1, the thallus appeared severely affected judging from an unpleasant smell and sometimes a modified, softer texture and changed colour. The odour became distinct immediately after moistening, and persisted until the time of measurements 48 hrs later, whereas the colour changed after a few hours. Since the mycobiont represents the quantitative important component of most lichens, the changes in texture and smell suggest that the mycobiont died at about the time when the photobiont was killed. A killed mycobiont could possibly release detrimental signals to the photobiont, and vice versa.

In this study, various lichens exhibited different levels of acetone tolerance (Fig. 1). The reason for these species-specific susceptibilities is not known. Lange (1953) studied the drought resistance of the mycobiont of some species in Fig. 1, and ranked the measured species by increasing level of tolerance: *Lobaria pulmonaria* < *Peltigera aphthosa* = *Nephroma arcticum* < *Lasallia pustulata*. Therefore, since acetone extracts residual water from submersed air-dry thalli, the high acetone tolerance of species like *L. pustulata* from sun-exposed and dry habitats could be reflecting a high drought resistance.

The applied solvents in Fig. 3 vary in polarity (Stahl, 1967). Ethanol and methanol with a high polarity, kill the lichens very fast, whereas the somewhat less polar acetone is minimally destructive (Fig. 3). The other, considerably less polar solvents, could therefore possibly be less harmful than acetone. However, for the tested lichens they are less efficient extracting agents, and therefore less useful. Other extraction techniques like grinding and boiling (Huneck and Yoshimura, 1996) were not tried, because of obvious detrimental effects.

Extraction efficiency

The tested lichen species also showed different time requirements for a complete extraction of secondary compounds (Fig. 2a). There was no simple relationship between acetone tolerance and extraction efficiency. Earlier extractions showed that 4 times 5 minutes were sufficient for *X. parietina* (Solhaug and Gauslaa, 1996), but so short extractions were inefficient for e.g. *U. spodochroa* (unpublished data). Preliminary studies on other species than *X. parietina* showed that extraction yield increased with extraction time of each repeated extraction, suggesting that a high concentration of secondary compounds in the acetone has not been a major cause for the delayed extractions. To facilitate a comparison between species and visualize the total extraction efficiency, the insert (Fig. 2b) shows the relative thallus content of secondary compounds as calculated by setting the sum of all successive absorbances to 100 for each species. *Nephroma arcticum* and *L. pulmonaria* were omitted in Fig. 2b because of high absorbances in late extracts. Since the relative absorbances asymptotically approached zero (Fig. 2b), the assumption of nearly 100% extraction yield could not be too far from reality.

The anatomical and physiological reasons for the different tolerance limits and extraction efficiencies are not obvious. Secondary compounds are deposited as extracellular surficial crystals outside the hyphal wall, as reviewed by Fahselt (1994). However, the location and enclosure of the crystal-covered hyphae could vary between the lichen species. The easily extractable parietin in *X. parietina* is situated in the upper part of the cortex and around the apical cells of paraphyses in apothecia, whereas all other tested lichen species (Fig. 2a) have secondary compounds in the medullary layer. Therefore, a complete extraction of parietin within 20 minutes (Solhaug and Gauslaa, 1996) could be a consequence of its location on the upper surface. Furthermore, the upper cortex of *X. parietina* is more porous when dry (Souza-Egipsy et al., 2000).

A comparison between the acetone-resistant *X. conspersa* and the relatively susceptible *L. pulmonaria* is interesting, because of their identical secondary medullary compounds, stictic and norstictic acid (Tab. 1). Although *X. conspersa* additionally contains the cortical compound usnic acid, the extraction yield starts declining much earlier as compared to *L. pulmonaria* (Fig. 2). Such contrasting extraction kinetics might be due to differing thallus anatomies or differences in the location/size of secondary-compound crystals in these two lichen mycobionts, which are not closely related (e.g. Tehler, 1996).

Similar explanations could be given for the strikingly slower extraction rate in *L. pustulata* compared to *U. spodochroa* (Fig. 2), since gyrophoric acid is the only known secondary compound in Norwegian material of these two Umbilicariaceae-species (Krog et al., 1994). Several anatomical differences between the two species have been reported (Valladares, 1994a, b; Valladares

et al., 1998). For instance, *U. spodochroa* has a much thinner upper and lower cortex than *L. pustulata* (Valladares et al., 1996), although the latter has more coarse cortical fissures (Valladares, 1994b). A heavy accumulation of crystallized secondary substances has been observed in an electron microscope study of *L. pustulata* (Valladares and Ascaso, 1994).

Also the two Parmelioid species *N. pulla* and *X. conspersa* have different extraction rates (Fig. 2). However, both genera have a pored epicortex and similar upper cortices (Elix, 1993), and the two species have a seemingly similar thallus anatomy (Galløe, 1948). Therefore, their different extraction rates could possibly reflect their different secondary chemistry (Table 1). In conclusion, contrasting extraction rates could result from both different thallus anatomy and/or different secondary chemistry.

Practical considerations

The practical implications of reported results are important: whereas some lichens are killed by acetone before secondary compounds are completely extracted (*L. pulmonaria, N. arcticum, P. aphthosa*), prolonged acetone exposures can successfully be applied for other species. *Trebouxia* lichens appear especially resistant. Lichens with a rich accumulation of secondary compounds offer unique opportunities for testing ecological and physiological roles of secondary metabolites, since many compounds can be removed from intact, living lichens. Acetone rinsing also allows studies of their induction and production in intact lichens, as already found for *X. parietina* (Solhaug and Gauslaa, 1996). However, both acetone resistance and extractability of compounds should always be carefully checked for new species to ensure that the compounds are sufficiently extracted and that the viability is not affected. Acetone rinsing is less useful in several of the lichens with *Nostoc* as the main photobiont, partly because of their apparently low resistance to acetone submersion, but also because many of the lichens with *Nostoc* as the photobiont are deficient in lichen compounds, e.g. Krog et al. (1994).

Our results open a new and interesting potential application of acetone-rinsing for studying biont interactions in tripartite lichens, i.e. lichens with green algae as the main photobiont and *Nostoc* located in internal or external cephalodia. For instance, assuming that the cephalodia in *L. pulmonaria* is inhabited by a *Nostoc* with a similar susceptibility to acetone as the *Nostoc* in *P. canina*, an eight-hours acetone rinsing could probably fully destroy the *L. pulmonaria* cyanobacteria without affecting the green algal photobiont (Fig. 1). If so, the ecological importance of cyanobacteria in tripartite lichens could be tested experimentally.

Acknowledgements

The research was funded by the Research Council of Norway. We thank Prof. Dr. Thomas Friedl (Universität Göttingen), Andreas Beck (LMU München), Michael Lakatos (Universität Kaiserslautern) for their information concerning photobiont identity of studied lichen species, Prof. Dr. Wolfgang Bilger for thoughtful comments on the manuscript, and Annie Aasen for recording many of the numerous absorbance spectra.

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