The Lichenologist **37**(2): 181–189 (2005)
doi:10.1017/S0024282905014611 *Printed in the United Kingdom*

FT-Raman spectroscopy of the Christmas wreath lichen, *Cryptothecia rubrocincta* (Ehrenb.:Fr.) Thor

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Abstract: FT-Raman spectra have been obtained from the highly pigmented lichen *Cryptothecia rubrocincta* from a Brazilian vestigial rainforest habitat. Spectral signatures of the two main lichen substances, chiodectonic acid and confluentic acid, were identified in adjacent zones of the thallus. Of the characteristic zonal colours displayed by the thallus, the outer red zone contained chiodectonic acid and no calcium oxalate, and graded into by a pink zone with calcium oxalate monohydrate (whewellite) in association with chiodectonic acid, to the inside of which is a concentric white zone containing calcium oxalate dihydrate (weddellite); however, chemically differentiated sites (elliptical brown flecks with a major axis of *c.* 15 μm) in both the pink and red zones contained chiodectonic acid and calcium oxalate monohydrate. The role of Raman spectroscopy in the spatial identification of lichen substances in the thallial structures is demonstrated.

Key words: Brazil, calcium oxalate, chiodectonic acid, confluentic acid, *Cryptothecia rubrocincta*, FT-Raman spectroscopy, lichen

Introduction

Lichens are effective bioindicators of environmental change and their ability to colonize a wide range of substrata, including stone, brick, leaves and paint, indicates that they have the capability to adapt to a wide range of habitats. The creation of environmentally-friendly microniches through the production of key biochemicals is part of this successful survival strategy; in particular, highly-pigmented lichen thalli, such as *Cryptothecia rubrocincta*, may contain radiation-protectants, such as beta-carotene, rhizocarpic acid and parietin, which have vital roles in either repair mechanisms of DNA-damaged cells or for the absorption of UVB or UVC insolation from broad-band wavelengths in solar radiation (Cockell 1998; Cockell & Knowland 1999).

In the environmentally hostile Antarctic regions, those lichen species that have survived these extreme habitats are capable of producing biological protectants as strategies in reponse to the stress conditions of high UV-insolation, low temperatures and katabatic wind abrasion (Edwards *et al.* 1998). Despite this, recent studies of Antarctic lichens, such as species of *Xanthoria*, *Caloplaca* and *Acarospora*, indicate that along a transect from maritime Antarctica to the polar plateau the worsening climatic conditions are still too severe for the survival of epilithic species and only cyanobacterial endoliths and chasmoliths are found (Wynn-Williams & Edwards 2000*a*).

Experiments at Leonie Island in maritime Antarctica using artificially protected colonies of *Xanthoria elegans* have been undertaken (Edwards *et al.* 2004) from which it has been concluded that the production of the deep-orange coloured anthraquinone,

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parietin, is marginalized in favour of beta-carotene when UVB-radiation is filtered out using Perspex cloches; in the Raman spectra of this species, 22 Raman bands characteristic of beta-carotene and parietin have been identified (Edwards *et al.* 2003, 2004). The role of Raman spectroscopy for the non-destructive chemical analysis of lichen thalli and their encrustations is now well-established and novel information about the biodeteriorative capabilities and survival strategies of extremophiles have emerged from these studies (Wynn-Williams & Edwards 2001).

In the current work, specimens of a distinctive, zonally pigmented lichen, *Cryptothecia rubrocincta* (Fig. 1A), from vestigial rainforest sites in Brazil have been analysed non-destructively using FT-Raman spectroscopy and key spectral biomarker bands identified from our database. A particular advantage of the Raman microscopic study carried out here is the characterization of the chemical components of the multi-coloured specimens; these consisted of an outer red thallial zone, grading into a pink zone, both with small brown flecks (Fig. 1B), followed by a white crystalline zone. From this study, it is possible to derive some information about the protective chemicals produced by this lichen in a rainforest habitat and to relate these to a possible survival strategy adopted in this situation.

Material and Methods

Specimens

Several thalli of *Cryptothecia rubrocincta* (Ehrenb.:Fr.) Thor were collected from two sites in Brazil:

(i) thalli, *c.* 2.0 cm in diam. together with bark substratum, detached from trees on the perimeter (S-facing) of a vestigial rainforest, 650 m, Cantareira, Sao Paulo, leg. *Luiz F.C. de Oliveira*, March 2003 (hb. MRDS 112994).

(ii) single thallus, *c.* 5 cm diam. together with bark substratum, fragmented rainforest, 1240 m, Pinheiros, Santuario de Caraja, Minas Gerais, leg. *M.R.D.Seaward* 18 September 1997 (herb. MRDS 108177).

The lichen thalli form continuous, rather thick, circular patches, the older, central region covered with red, spherical to cylindrical isidia-like granules; from here outwards, three zones are differentiated, the first grey-green, the second white, and finally a bright red cottony rim (Fig. 1A). The red and green of this unmistakable subtropical to tropical woodland lichen give it a Christmas wreath look, hence its common North American name, the Christmas wreath lichen (Brodo *et al.* 2001).

According to Culberson (1969), the major lichen substances of *C. rubrocincta* (syn. *Herpothallon sanguineum* (Sw.) Tobl.; *Chiodecton sanguineum* (Sw.) Vain.) are chiodectonic acid and confluentic acid (=confluentinic acid in Culberson 1966), the chemical structures of which are shown in Figure 2. However, there is no information provided in the literature on the nature of the zonal chemistry.

Raman spectroscopy

FT-Raman spectra were obtained using a Bruker IFS 66/FRA 106 system with dedicated Raman microscope attachment and an Nd^{3+}/YAG laser operating at 1064 nm and an InGaAs liquid-nitrogen cooled detector. Individual spectral scans recorded at 4 cm^{-1} resolution over the wavenumber range 50–3500 cm^{-1} were accumulated for between 2000 and 4000 scans to improve the signal-to-noise ratio. In the macroscopic survey spectral mode the footprint at the specimen was 100 μm, which was reduced to about 20 μm using the microscope system with a $\times 40$ objective lens. Laser powers of *c.* 10 mW or less were used to minimize the risk of sample damage over the longer exposure times required for extended spectral data. Spectral wavenumbers are accurate to better than ± 1 cm^{-1} by internal laser calibration. Replicate spectra were obtained from at least three visually similar areas of the identified regions of interest in the lichen-substratal specimens.

Results and Discussion

Cryptothecia rubrocincta thalli are complex systems in which several distinctively coloured zones and structural regions can be identified visually (Fig. 1A); the spectroscopic requirement, therefore, was for a detailed Raman microscopic analysis following initial survey spectra designed to establish the spectral parameters necessary for the recording of data.

White crystalline zone

The spectra obtained from the white crystalline zone are typified by the example shown in Figure 3A; this shows two major bands at 1476 and 904 cm^{-1} with a third weaker band at about 500 cm^{-1}, which is compromised by background noise between

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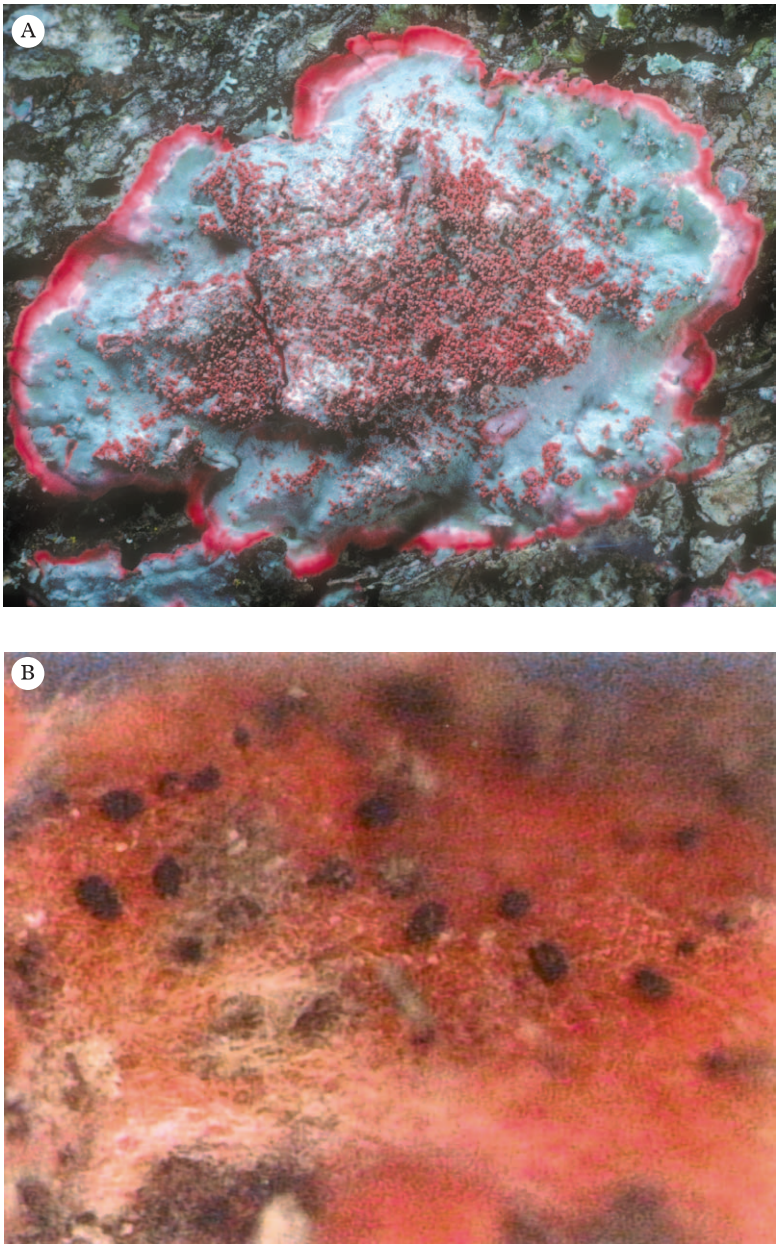

FIG. 1. *Cryptothecia rubrocincta*, the Christmas wreath lichen. A, the distinctive zonal colouration (see text for details); B, showing the red thallial zone (grading into pink sub-zone), with localized pigmented areas (brown flecks, *c.* 1·5 μm). Scale A: × 2.

300 and 800 cm^{-1}. These bands are characteristic of calcium oxalate dihydrate, weddellite, which has been found as a metabolic substance in several lichen systems and extremophiles growing on calcareous substrata (Edwards *et al.* 1992). It is of interest that, despite the calcium oxalate dihydrate being reported in the literature as

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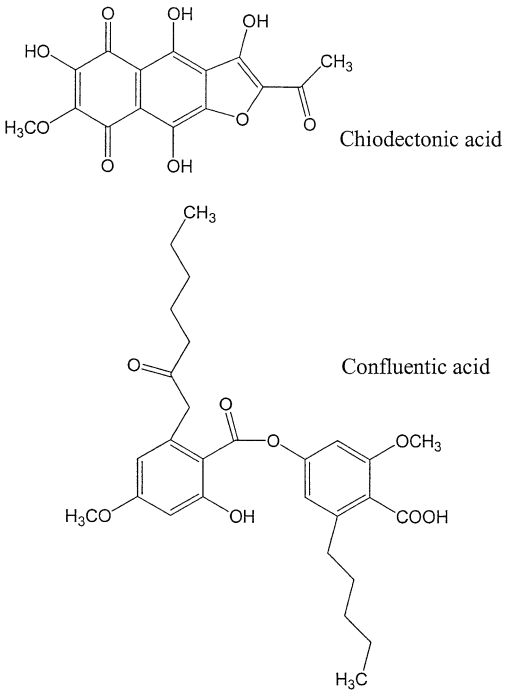

FIG. 2. Molecular structures of chiodectonic acid and confluentic acid, the two major lichen substances extracted from *Cryptothecia rubrocincta*.

metastable above temperatures of 5 °C, this material has nevertheless been noted previously in lichen systems as a product of biodeterioration in both Antarctic and temperate habitats (Wynn-Williams & Edwards 2000*a*, 2000*b*); it is reasonable to suppose that in the latter case calcium oxalate monohydrate, whewellite, the more stable of the two hydrates at elevated temperatures, would have been formed preferentially.

Several authors (Russell *et al.* 1998; Holder *et al.* 2000; Wynn-Williams & Edwards 2000*b*) have suggested that calcium oxalate production in lichen biodeterioration on calcareous substrata is not simply a waste product, namely the result of the reaction between oxalic acid produced by the mycobiont and calcium carbonate in the substratum or atmospherically-derived, but that a multi-purpose role is perceived for this material in the survival strategy: for example, the storage of water as a crystalline hydrate is essential for periods of drought in desiccated environments and the anti-herbivorous role for calcium oxalate has already been identified (Seaward *et al.* 1997). This scenario fits extremely well with the spectroscopic identification of calcium oxalate in weddellite and whewellite forms in lichen encrustations from a diverse range of habitats and locations; in some cases both forms are found together in significant amounts and in others one or other predominates.

Of relevance here is the availability of calcium for the formation of the calcium oxalate on a non-calcareous substratum, in this study represented by tree bark. The oxalic acid produced by the mycobiont is a final product of the metabolic process and is removed as insoluble, waste calcium oxalate through reaction with calcium ions; the provision of calcium in substrata such as limestone and marble also affords a mechanism for the hyphal penetration of the rock by the lichen. In this way, lichens such as *Dirina massiliensis* forma *sorediata* can effectively biodeteriorate calcareous substrata and incorporate up to 50% of their body mass as hydrated calcium oxalate; in one biodeterioration site we have calculated from Raman spectroscopic studies that over 1 kg of calcium carbonate substratum has been chemically converted into hydrated calcium oxalate for every m^2 of surface coverage (Seaward & Giacobini 1989) and we have detected spectroscopically signals of chemical alteration over some 10mm depth into the marble substratum.

In other cases (Prieto *et al.* 1999), the acquisition of calcium ions by oxalate complex formation in lichen thalli on non-calcareous substrata can be achieved by access to wind-borne material and from rainwater or snow melt run-off. Thus, we have identified spectroscopically the presence of calcium oxalate in lichen colonies growing on leaves in the canopy of a tropical rainforest (de Oliveira *et al.* 2002) and on brick, glass and granitic substrata (Edwards *et al.* 1997), none of which have a significant calcium content. In the present case, we clearly have another example of lichen colonization of a non-calcareous substratum

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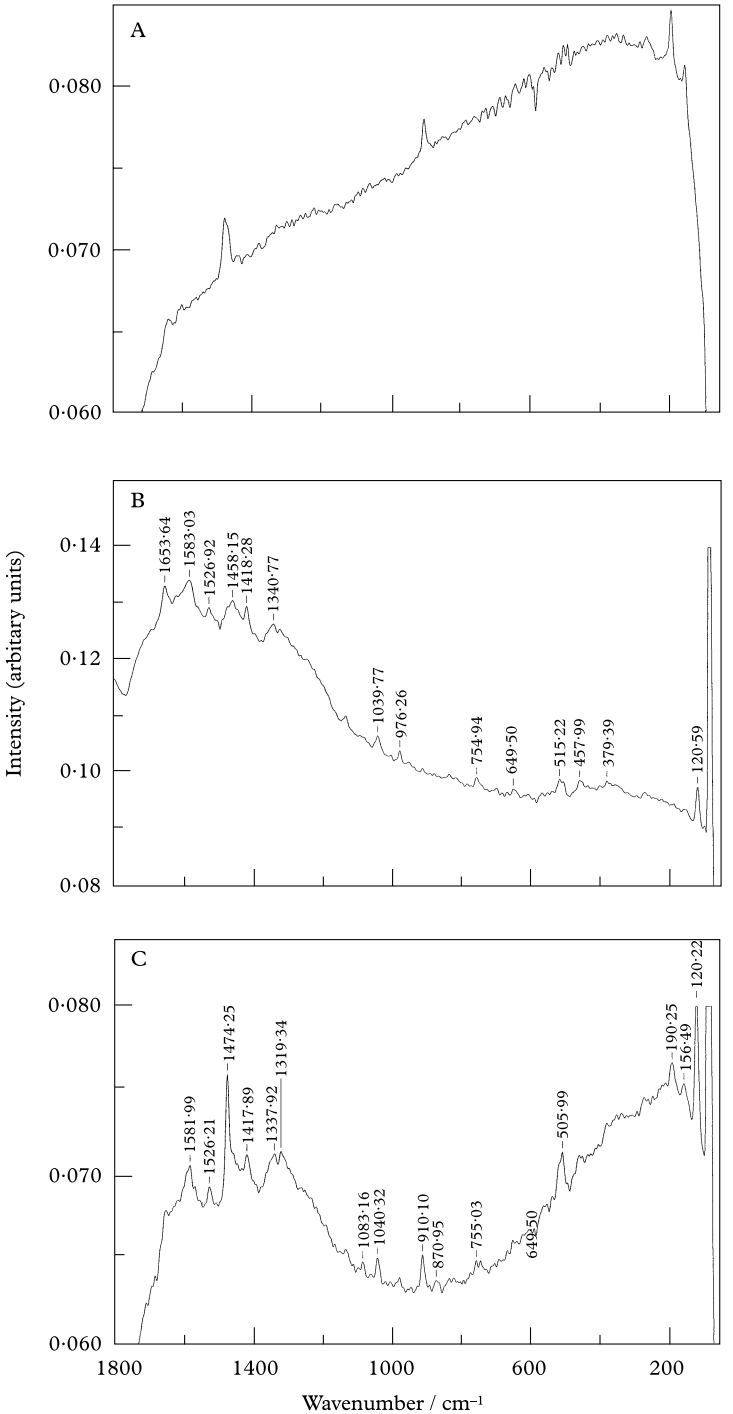

FIG. 3. FT-Raman microscope spectra of the coloured zones of *Cryptothecia rubrocincta*; 1064 nm excitation, × 40 lens objective, 4 cm^{-1} spectral resolution, 2000 spectral scans accumulated, wavenumber range 50–1800 cm^{-1}. A, white crystalline areas of the lichen encrustation; B, the red zone; C, the pink sub-zone to the inside of the red zone.

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in which the acquisition of calcium ions necessary for the formation of calcium oxalate is probably achieved from rain, bird excreta and airborne particles.

Red coloured zone

Under the microscope the visually uniform red zone can be seen to comprise two sub-zones since the dark red grades into a lighter red (pink). The dark red areas give the Raman spectrum shown in Figure 3B, in which features assignable to an aromatic quinone (1653 and 1584 cm^{-1}), beta-carotene (1526 and 1157 cm^{-1}) and chlorophyll (1320 cm^{-1}, broad) are observed. The quinone biomarker bands are highly relevant since chiodectonic acid (Fig. 2), identified in *C. rubrocincta* from wet chemical extractions, has an aromatic quinonoid structure. Key quinonoid spectral marker bands for parietin (and emodin) found in *Xanthoria parietina* have been similiarly characterized in the Raman spectrum (Edwards *et al.* 2004). Infrared spectral bands of chiodectonic acid are reported in Huneck and Yoshimura (1996), but the Raman spectrum has not been reported hitherto. With UV-absorption band maxima at 287, 510 and 538 nm (Culberson 1969), the chiodectonic acid is a deep-red coloured pigment and its biochemical function in the thalli could be reasonably ascribed to that of a radiation protectant; in combination with beta-carotene, which has an established role in cellular DNA repair following exposure of the organism to UV-radiation damage, such radiation protectants are often found in lichens and in extremophilic situations and are essential for survival. In a recent Raman spectroscopic investigation of colonies of *Xanthoria elegans* at the fringe of the ozone hole in maritime Antarctica (Edwards *et al.* 2004), we have noted the presence of the coloured anthraquinone parietin and beta-carotene which have a similar dualistic UV-protectant and DNA-repair role.

Weaker bands at 1628 cm^{-1} for the isolated C=C in the heterocyclic ring of chiodectonic acid, 1705 cm^{-1} for the ketonic C=O, 1039 cm^{-1} for the terminal C-OH and 976 cm^{-1} for the methyl rocking mode all provide further confirmation of the assignment of these features to chiodectonic acid in the red pigmented zone of this lichen.

In contrast, the lighter coloured, pinkish-red sub-zone, located concentrically to the inside of the red-pigmented zone (Fig. 1A), has a rather different Raman spectrum (Fig. 3C); here, the major features that we have assigned to chiodectonic acid are still present, but these are now superimposed on a broader background emission which accentuates the intensity of the 1584 cm^{-1} band. Likewise, the weaker bands at 1418, 1320, 1039, 976 and 755 cm^{-1} are still observable, but the strongest peak in this spectrum is a band at 1476 cm^{-1}, with supporting features at 904 and 505 cm^{-1} which are characteristic modes of calcium oxalate dihydrate. The beta-carotene bands at 1526 and 1157 cm^{-1} are still evident in this spectrum. It can be reasonably concluded therefore that the pink coloured sub-zone contains chiodectonic acid, beta-carotene and calcium oxalate dihydrate, the red and white intimate mixture of the chiodectonic acid and the calcium oxalate clearly giving rise to the characteristically lighter coloured sub-zone.

In previous studies of some lichen systems (Seaward *et al.* 1998), we have discerned that several mechanisms may be invoked for the disposal of waste calcium oxalate, one of which involves the localization of small 'pockets' of calcium oxalate away from the growing edge and this also seems to have occurred here. Another interesting chemical conclusion from Figure 3C is that the calcium oxalate dihydrate has not been converted into the monohydrate, unlike the situation we have noted in several other lichen-substratum systems, where the Raman spectrum shows the presence of both hydrates together.

Brown coloured flecks

Under the microscope, highly pigmented, locally differentiated areas (elliptical brown flecks), their major axis being *c.* 15 μm (Fig. 1B) are observed in both the red

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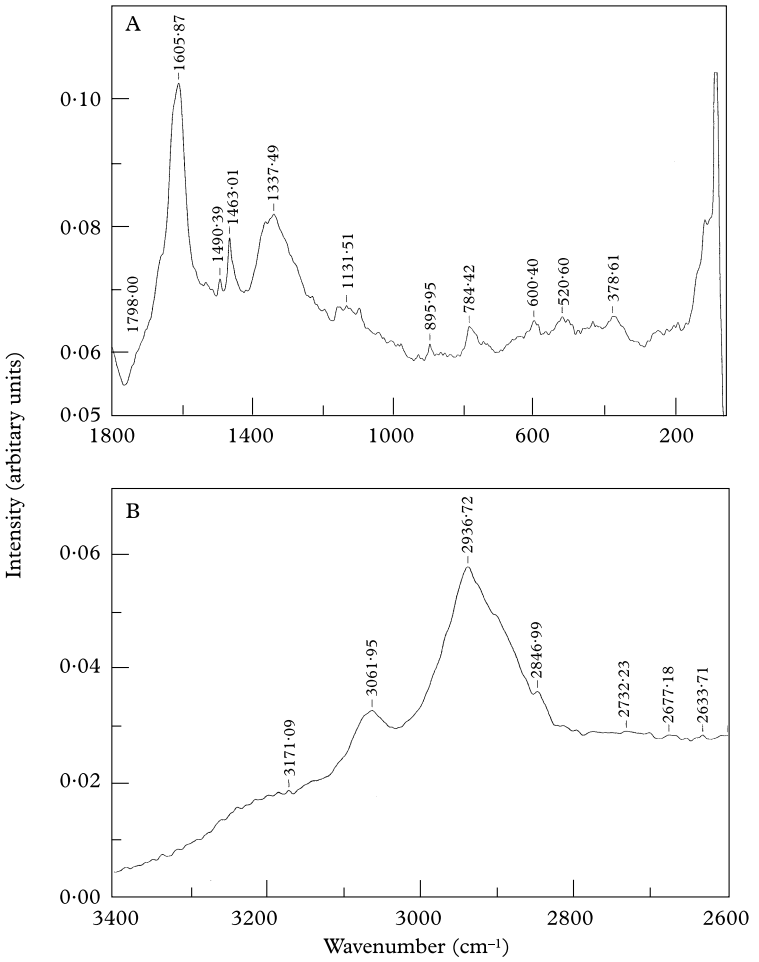

FIG. 4. FT-Raman microscope spectrum of localized brown flecks within the red zone of *Cryptothecia rubrocincta*; conditions as for Figure 3. A, wavenumber range 50–1800 cm^{-1}; B, wavenumber range 2600–3400 cm^{-1}.

and pink sub-zones of the thalli (Fig. 1A). Raman spectroscopical analyses with the capacity to determine the chemical nature of one of these microscopic brown areas are shown in Figure 4A & B for the wavenumber ranges 50–1800 cm^{-1} and 2600–3400 cm^{-1}, respectively. The spectrum shown in Figure 3B is very different from those in Figure 4. The strongest Raman band is now at 1605 cm^{-1} with weaker shoulders at about 1620 and 1670 cm^{-1}. Other bands occur at 1335 (broad), 1140, 1085, 950 and 785 cm^{-1}. These are all assignable to modes of confluentic acid (Fig. 2) and are also a good match for the published but unassigned (Culberson 1969; Huneck & Yoshimura 1996) infrared bands characteristic of this compound extracted from *C. rubrocincta*. Based on the molecular structure shown in Figure 2 for confluentic acid, carbonyl features at 1700, 1660 and 1605 cm^{-1} are all assignable to this molecule, with the strongest Raman band being expected for the aromatic ring quadrant stretching mode at 1605 cm^{-1}. Another infrared band of confluentic acid at 1335 cm^{-1} is close to the broad Raman band of chlorophyll at *c.* 1320 cm^{-1}. All the other Raman bands in Figure 4A are recorded in the infrared spectrum of con-

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fluentic acid extract (Culberson 1969). It is reasonable to conclude, therefore, that the brown flecks contain confluentic acid and that the features characteristic of chiodectonic acid are absent. Figure 4B confirms the aromatic nature of the material comprising the brown pigmented spectrum with an aromatic CH-stretching band at 3061 cm^{-1} and aliphatic CH modes at 2937, 2910 and 2845 cm^{-1}.

One of the most intriguing spectroscopic signatures from Figure 4A is the doublet at 1463, 1491 cm^{-1} and bands at 896 and 510 cm^{-1}, characteristic of calcium oxalate monohydrate; no evidence is seen in the Raman spectrum here for a mixture of weddelite and whewellite. Furthermore, the features of beta-carotene are absent.

Conclusions

In conclusion, the spectroscopic results from the present study of *Cryptothecia rubrocincta* thalli indicate the following:

(i) the white zone contains calcium oxalate dihydrate only,
(ii) the dark red zone contains chiodectonic acid, chlorophyll and beta-carotene, to the inside of which the pink sub-zone contains in addition significant amounts of calcium oxalate dihydrate,
(iii) the localized areas (elliptical brown flecks) within the red and pink zone contain confluentic acid and calcium oxalate monohydrate only, and
(iv) the Raman spectra generally indicate that calcium oxalate monohydrate and calcium oxalate dihydrate occur separately, and nowhere have we detected a mixture of these compounds.

Although the monohydrate of calcium oxalate is the more stable form in lichens of temperate habitats where the mean temperature is >5°C and where the moisture supply is plentiful, the significant presence of the metastable dihydrate is explained if we assume that the dihydrate is a primary formation product of the oxalic acid from the metabolic process with calcium ions and that ageing converts this into the more stable monohydrate. The association of the monohydrate with confluentic acid only in the localized brown flecks and not with the chiodectonic acid is highly indicative that these two lichen chemicals have different roles in the survival strategy of the species. It is reasonable to propose that in the early growing stages of the colony the thalli require the production of the UV-protectants chiodectonic acid and beta-carotene, which after the passage of time are then dispersed into the new growth areas, leaving behind isolated pockets of calcium oxalate dihydrate waste. With time, the metastable dihydrate is converted into the more stable monohydrate, which is now associated with confluentic acid; the role of confluentic acid in the brown flecks is not clear, but it would not be required for radiation protection, as confirmed by the deficiency of beta-carotene and chiodectonic acid in these areas.

The function of Raman spectroscopy to identify biochemicals spatially and non-destructively in the lichen, and where appropriate its encrustation, is seen to provide unique information about the location of these materials in the living organism and its substratum which is not available from the bulk wet chemical extraction procedures used hitherto. Although providing useful data about the molecular species in the system, wet chemical bulk extractions cannot give the spatial location of the sites of these chemicals in the organism. It is also noteworthy that the published chemical extraction results do not mention the presence of beta-carotene or of the hydrated calcium oxalates, both of which have clearly important roles in the biological survival strategy.

LFCO is grateful to the Conselho Nacional de Desenvolvimento Cientifico e Tecnologico (Brazil) for financial support during which this work was carried out. We are also grateful to Dennis Farwell for assistance in obtaining the FT-Raman spectra and to Stephen Sharnoff for permission to use Figure 1A.

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Accepted for publication 15 *November* 2004