*Lazaroa 11: 141-148 (1989)*

# Estimation of chlorophyll degradation into phaeophytin in *Anaptychia ciliaris* as a method to detect air pollution

ESTEBAN MANRIQUE *, FERNANDO L. REDONDO *, ESTELA SERIÑA ** & JESÚS IZCO ***

**Resumen:** Manrique, E., Redondo, F. L., Seriña, E. & J. Izco.: *Estimación de la degradación de clorofila a feofitina en Anaptychia ciliaris como método para detectar la contaminación del aire. Lazaroa 11: 141-148 (1989).*

Se estudia la influencia de los siguientes factores sobre el valor del cociente de feofitinización DO 435 nm/DO 415 nm en el liquen *Anaptychia ciliaris* (L.) Körber: edad del liquen, concentración final de pigmentos, trasplante de los líquenes a áreas distintas sobre sustratos diferentes o similares y el valor de pH del sustrato original o el adoptado tras el trasplante. Se discute la utilización de *A. ciliaris* como un bioindicador de la presencia de factores acidificantes en la atmósfera o en el agua de lluvia que provoquen la feofitinización de las clorofilas.

**Abstract:** Manrique, E., Redondo, F. L., Seriña, E. & J. Izco. *Estimation of chlorophyll degradation into phaeophytin in Anaptychia ciliaris as a method to detect air pollution. Lazaroa 11: 141-148 (1989).*

The influence of the following factors on the ratio of the phaeophytination index (OD 435 nm/OD 415 nm), in *Anaptychia ciliaris* (L.) Köber is studied: the age of the lichen thallus, the final concentration of the pigments, the transplantation to different areas on similar or distinct substrates, and the pH value of the original or adopted substrate. The use of *A. ciliaris* as a bioindicator of acidic air pollution is discussed.

* Departamento de Biología Vegetal II. Facultad de Farmacia. Universidad Complutense. 28040 Madrid.

** Departamento de Biología Vegetal I. Facultad de Ciencias Biológicas. Universidad Complutense. 28040 Madrid.

*** Departamento de Biología Vegetal. Facultad de Farmacia. E-15706. Santiago de Compostela.

## INTRODUCTION

Lichens as air pollution bioindicators have been used for many years, since Nylander observations (in FERRY & al., 1973). Since then a great number of different qualitative or quantitative methods have been used, based on the study of the relative abundance of lichens around urban or industrial areas, or on investigations of the variability of various physiological parameters in lichen specimens belonging to one or a few species. Among these kind of works, some measure the changes caused by some air pollutants on the photosynthetic pigments of the algal symbiont. Many scientists have studied the relationship between chlorophyll phaeophytination and sulfur dioxide (RAO & LEBLANC, 1966; MOSS, 1967a; NASH, 1973; PUCKETT & al., 1973; KAUPPI, 1980; SILBERSTEIN & KELLER, 1986). These methods detect changes in the absorption spectrum of chlorophyll when transforms into phaeophytin (VERNON, 1960; MOSS, 1967b).

For the spectrophotometric measures, different methods for pigment extracts, have been used traditionally: 80 or 90 % acetone, methanol or ethanol in water or pyridine. Nevertheless, these extraction methods, when applied in lichens, present some problems, mainly when they include acetone, methanol or ethanol, because these chemicals show a high sensitivity to pH changes. These pH variations may be an important artefact when measuring chlorophyll-phaeophytin conversion in acidic pH. For this reason it is useful to add alcaline substances (as carbonates) into the extracts (BROWN & HOOKERooker, 1977). The use of pyridine also prevents the conversion of chlorophyll to phaeophytin caused by the acidification during the extraction process. SCHOAF & LIUM (1976), HISCOX & ISRAELSTAM (1979) and BURNISON (1980) used dimethylsulfoxide (DMSO) for pigment extraction for phytoplankton. This solvent not only behaves as a proton acceptor (alkaline), but it can extract pigments from undamaged cells, avoiding maceration. SPEZIALE & al. (1984) compared the extraction properties of DMSO, N,N-dimethylformamide and acetone. They emphasized the advantages of DMSO in the chlorophyll extraction processes. RONEN & GALUN (1984) proposed the DMSO as the more efficient solvent system to extract chlorophyll from the lichen *Ramalina duriaei* (De Not.) Jatta. The same authors indicated that the changes observed in the optical densities of the lichen pigment extracts, measured as the ratio OD 435 nm/415 nm, may be a good index to estimate the degradation of chlorophyll to phaeopigments. GARTY & al. (1985) using this method in transplants of *R. duriaei* in Israel, found a close correlation between phaeophytination by sulphur dioxide and heavy metals in the atmosphere.

In this work we discuss the use of the DMSO in pigment extraction from the lichen *Anaptychia ciliaris* and the Phaeophytination index (OD 435/OD415 nm) to detect acidic pollution in Spanish woodlands. This is

a preliminary work proving that the use of *Anaptychia ciliaris* is convenient for air monitoring.

## MATERIAL AND METHODS

The present study has been performed with the fruticose lichen *Anaptychia ciliaris,* because among other characteristics it does not contain acidic lichen substances. The absence of acidic lichen substances has been confirmed by thin layer chromatography and HPLC techniques in a very large number of specimens. *A. ciliaris* is very easy to identify and can be found growing on different substrates such as bark of *Quercus rotundifolia, Q. pyrenaica, Q. faginea, Ulmus sp., Pinus sylvestris, P. nigra* and *Juniperus oxycedrus,* and on calcareous and acidic rocks.

The lichen material was collected in several non-contaminated localities (control localities): Selas (Guadalajara, Spain), on *Quercus faginea;* Montejo de la Sierra (Madrid, Spain), on *Q. pyrenaica,* and Monte de El Pardo (Madrid, Spain), on *Q. rotundifolia* (map 1), substrates with very similar physical and chemical characteristics (bark pH between 5.6 and 6.7, mea-sured with the method proposed by JOHNSEN & SOCHTING 1973).

The specimens were analyzed within the first five days after collection. All the samples were air-dried and those that were not used were stored in the dark at −20° C.

For the extraction of the chlorophyll pigments we have followed the method described by RONEN & GALUN (1984). About 20 mg from each sample, aphotecia avoided, were inmmersed in 5 ml of DMSO, and remained in dark at 65° C for 40 minutes. The extraction was completed after this time because the lichen material was entirely transparent. The extract was diluted by adding 5 ml of DMSO, and the optical densities (OD) of the diluted extracts were measured at 435 nm, using DMSO as the blank. The turbidity of the extract was verified at 750 and 415 nm. This value usually was around zero, but in case it exceeded 0.001 OD units, the extract was passed through a Millipore AP 2001300 filter.

The degree of phaeophytination was calculated according to RONEN & GALUN (1984). These authors estimate that in the case of minimal chlorophyll degradation into phaeopigments, the quotient OD 435 nm/OD 415 nm is between 1.40 and 1.45. To know the maximal degree of degradation, 60 µl of an 1N HCl solution was added to the control extracts, and remained in the dark for 10 minutes. In these conditions the quotient reaches its minimal value, between 0.56 and 0.65. More acid and more time in the dark do not increase the chlorophyll degradation of the extracts. In parallel the same quotient was measured in two vascular plants, *Lolium perenne* and *Dactylis glomerata.*

Since the analyced specimens, although of identical weight, do not contain the same pigment concentration, we weighted 5, 10, 20, 30, 40 and 50

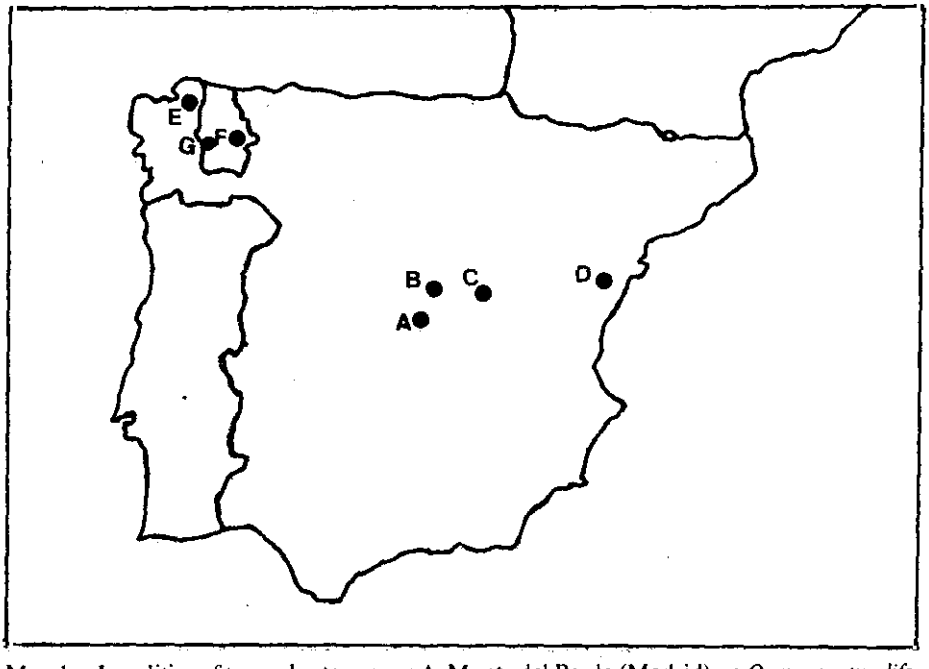
mg of *A. ciliaris* with the purpose of checking the effect of the final pigment concentration on the value of the quotient. This allowed us to avoid the problem of differences in age, algal distribution and shape of the specimens.

All the values obtained for the quotient are the average of at least three measures.

The specimens of *A. ciliaris* used as transplantats were analyzed within the first 48 hours after collection and inmediately transplanted to trees with similar or different bark than that of origin.

With the purpose of measuring the pH of the original and new substrates of *A. ciliaris*, pieces of the bark were processed by the method described by JOHNSEN & SOCHTING (1973).

In order to know what effect transplantation could cause to the transplanted lichen, three samples of *A. ciliaris* were allocated on the bark of each substrate, pines (*P. radiata, P. pinaster*) and oaks (*Quercus robur*), acidic and subneutral conditions respectively, from locality B (in which *Lobaria pulmonaria* is common) to an area that presumably is not polluted (localities E, F, and G). The value of the OD 435 nm/OD 415 nm of the trans-




Map 1.—Localities of transplants source: A, Monte del Pardo (Madrid) on *Quercus rotundifolia;* B, Montejo de la Sierra (Madrid) on *Q. pyrenaica;* C, Selas (Guadalajara) on *Q. faginea,* and localities where *Anaptychia ciliaris* was allocated to detect the effects of $SO_2$ fumigations; D, Morella (Castellón) on several substrates (see the text), and transplantation effects; E, Fontau (La Coruña); F, Abrairas (Lugo) on *Pinus pinaster,* and G, Guitiriz (Lugo) on *Quercus robur.*

plants was calculated before and after transplantation. The samples of lichen, once the quotient value of them was known, were transplantated inmediately the ist of July 1986 and checked during the next nine months in August, September and October 1986 and February and March 1987, when were allocated on pine barks, and only in March 1987, for those allocated on oak bark.

The transplantation were performed as follows. The samples of the lichen *A. ciliaris* were carefully removed from their substrates (the bark of *Quercus faginea, Q. pyrenaico* and *Q. rotundifolia*). After measuring the quotient O.D. 435 nm/O.D. 415 nm, the samples were transplanted to the new substrate, three duplicates on each tree, and fastened over the bark with a network of nylon at 2,5 meters over the ground. The lichens were transplanted on *Pinus radiata, P. pinaster* and *Quercus robur* barks.

At the end, and with the purpose of knowing the effect of the pH and the buffering capacity of the substrate in prevention of the chlorophyll degradation caused by acidification in the nearby medium, we measured the value of the quotient OD 435 nm/OD 415 nm in specimens of *A. ciliaris* that were found growing on different substrates in a confined area (fig. 1D). All of them have been subjected to a $SO_2$ fumigation of 62 $\mu g/m^3$, percentile 98, as monitored by ENDESA (1986), for almost five years.

## RESULTS AND DISCUSSION

The average of the quotient OD 435 nm/OD 415 nm calculated for 13 samples from each control locality was 1.40 ± 0.03. The maximal value of chlorophyll degradation, after adding 1N HCl, for the rate OD 435 nm/OD 415 nm was 0.66 ± 0.04 for the same samples. Similar values were obtained for the same parameter calculated in *Lolium perenne* and *Dactylis glomerata,* 1.36 ± 0.05 and 1.38 ± 0.03 for the maximal values, respectively. The size of the sample (age) and the final pigment concentration do not seem to interfere with the value of the quotient, at least over 20 mg of sample (table 1), and no so much for 5 and 10 mg. This could tell us that it

Table 1

Influence of the final pigment concentration on the value of the quotient OD 435 nm/OD 415 nm

| Dry weight (mg) | OD 750 nm | Quotient |
|---|---|---|
| 5 | 0.002 | 1.26 |
| 10 | 0.001 | 1.27 |
| 20 | 0.002 | 1.33 |
| 30 | 0.005 | 1.32 |
| 40 | 0.008 | 1.38 |
| 0 | 0.010 | 1.37 |

is possible to use samples of *A. ciliaris* with different ages or sizes, that is, with different pigment concentrations per weight unit. This is very important because it could allow us to know what the state of health of the lichens is in a locality which is presumed to have been subjected to air pollution, and in which this lichen presents a very dissimilar growth. Also, when the lichen is not very abundant in the polluted area to be studied, it is possible to make a transplantation of it from unpolluted localities, in which *A. ciliaris* grows luxuriantly, to the presumably contaminated locality, on different species of trees. The effect of transplantation and the different pH of the adopted substrate on the quotient is shown in table 2. In

Table 2

Evolution of the quotient OD 435 nm/OD 415 nm in three samples of *Anaptychia ciliaris* transplanted from oak (locality B) to pine and oak trees of localities E, F and G (see fig. 1)

| Time | Locality | | |
|---|---|---|---|
| | E (pine) | F (pine) | G (oak) |
| August | 1.41 ± 0.01 | 1.38 ± 0.05 | — |
| September | 1.27 ± 0.11 | 1.33 ± 0.10 | — |
| October | 1.43 ± 0.07 | 1.40 ± 0.05 | — |
| February | 1.31 ± 0.09 | 1.38 ± 0.05 | — |
| March | 1.36 ± 0.09 | 1.40 ± 0.07 | 1.34 ± 0.08 |

Table 3

Different pH values calculated for several bark trees
(Each value is the average of ten samples)

| | Minimal | Average | Maximal |
|---|---|---|---|
| *Pinus radiata* | 3.4 | 3.6 | 3.9 |
| *Pinus pinaster* | 3.4 | 3.7 | 4.0 |
| *Pinus sylvestris* | 3.8 | 4.1 | 4.2 |
| *Quercus pyrenaica* | 5.5 | 5.8 | 6.1 |
| *Quercus robur* | 5.9 | 6.0 | 6.2 |
| *Quercus faginea* | 5.2 | 5.7 | 6.0 |
| *Juniperus oxycedrus* | 4.2 | 4.7 | 4.9 |

this table we can see that the value of the OD 435 nm/OD 415 nm decreases between the moment of transplantation and the second month, but it recovers the initial values after the third month, and remains constant for nine months, on conifers (pines) as well as on decidous trees (oaks). The pH values of different barks are presented in table 3. The conifers present the more acidic conditions to the lichen.

Some authors have pointed out the idea that in the corticolous lichens, the pH of the bark and the buffering capacity may be two important factors that can have a significant influence on the lichen response to acidic pollution (BADDELEY & al., 1971; HILL, 1971; TÜRK & WIRTH, 1975). If so, the

lichens growing on acidic substrates or substrates with a very low buffering capacity would be the first to demonstrate the symptoms of injury in a polluted atmosphere.

In table 4 we present the phaeophytination quotient of samples of *A. ciliaris* that were growing naturally on different substrates in an area subjected to a $SO_2$ fumigation of 62 µgm-3, percentile 98 (ENDESA, 1986). The samples collected on pine and juniper barks were those which

Table 4

Substrate influence on the quotient OD 435 nm/OD 415 nm calculated for *Anaptychia ciliaris* submitted to a $SO_2$ fumigation of 62 µg m-3 (percentile 98)

| Substrate | Quotient | pH |
|---|---|---|
| *Pinus sylvestris-P. nigra* | 0.78 ± 0.01 | 4.1 |
| *Juniperis oxycedrus* | 0.83 ± 0.08 | 4.7 |
| *Quercus rotundifolia-Q. faginea* | 1.00 ± 0.05 | 5.7 |
| *Calcareous rock* | 1.20 ± 0.05 | 9.1 |
| Control locality (*Quercus faginea*) | 1.30 ± 0.03 | 5.7 |

showed the smallest values of the quotient. This value increases with the pH of the substrate. The specimens less affected by the $SO_2$ presents in the atmosphere were those which were growing on calcareous rocks. RONEN & al. (1985) stated that lichens are able to continue their photosynthetic activities without severe damage up to a degradation of 10 % of the chlorophyll. The same authors estimate that this degradation corresponds to a value of the quotient OD 435 nm/OD 415 nm of 1.28 in the lichen *Ramalina duriaei* (De Not.) Jatta.

Gaseous $SO_2$ and other gases (NOx) dissolve rapidly in water causing a drop in the pH of the resulting solution. These gases therefore can enter into the lichen thallus with the rain water or directly dissolve in the lichen water. In this way, the acidic contamination can affect the lichen pigments and cause degradation of the chlorophyll into phaeophytin.

We think that the results here discussed, allow us to use the method proposed by RONEN & GALUN (1984) to estimate the pigment degradation in lichens submitted to acidic air pollution. We also think that it is possible to use *Anaptychia ciliaris* as a good bioindicator of the kind of air pollution discussed above, not only to know the steady-state of the lichen pigments and therefore the environmental quality of the studied area but also, by means of transplantation from unpolluted areas, to monitor the decline of the lichen vitality since the moment of the transplantation.

*Acknowledgements:* We wish to thank ENDESA (Empresa Nacional de Electricidad) and CAICYT (Comisión Asesora de Investigación Científica y Técnica, project number 2954/83) for supporting this research.

## REFERENCES

Baddeley, M. S. Ferry, B. W. & Finegan, E. J. —1971— A new method of measuring lichen respiration: Response of selected species to temperature pH and sulphur dioxide — Lichenologist 5: 18-25.

Brown, D. H. & Hooker, T. N. —1977— The significance of acidic lichen substances in the stimation of chlorophyll and phaeophytin in lichens — New Phytol. 78: 617-624.

Burnison, B. K. —1980— Modified dimethyl sulfoxide (DMSO) extraction of chlorophyll a and b from algae using dimethyl sulfoxide — Limnol. Oceanogr. 21: 926-928.

Empresa Nacional De Electricidad (ENDESA) —1986— Control del medio ambiente central térmica de Teruel — Zaragoza, España.

Ferry, B. W. Baddeley, M. S. & Hawksworth, D. L. —1973— Air pollution and lichens — The athlone Press of the University of London.

Garty, J., Ronen, R. & Galun, M. —Correlation between chlorophyll degradation and the amount of some elements in the lichen Ramalina duriaei (De Not.) Jatta — Environmental & Experimental Bot. 25 (1): 67-74.

Hill, D. J. —Experimental study of the effect of sulphite on lichens with reference to atmospheric pollution — New Phytol. 70: 831-836.

Hiscox, J. B. & Israelstam, G. F. —1979— A method for the extraction of chlorophyll from leaf tissue without maceration — Canad. J. Bot. 57: 1332-1334.

Johnsen, I. & Sochting, U. —1973— Influence of air pollution on the epiphytic lichen vegetation and bark properties of deciduous trees in the Copenhagen area — Oikos, 24: 344-351.

Kauppi, M. —Fluorescence microscopy and microfluorimetry for the estimation of pollution damage in lichens — Ann. Bot. Fenn. 17: 163-173.

Moss, B. —1967a— A spectrophotometric method for the estimation of percentage degradation of chlorophylls to phaeo-pigments in extracts of algae. Limnol. Oceanogr. 12: 335-340.

Moos, B. —1967b— A note on the estimation of chlorophyll a in fresh water algal communities — Limmol. Oceanogr. 12: 340.

Nash, T. H. (III) —1973— Sensitivity of lichens to sulphur dioxide — Bryologist, 76: 33-339.

Puckett, K. J., Nieboer, E. Flora, W. P. & Richardson, D. H. S. —1973— Sulphur dioxide: Its effects on photosynthetic 14C fixation in lichens and suggested mechanisms of phytotoxicity — New Phytol. 72: 141-154.

Rad D. N. & Leblanc, F. —1966— Effects of sulphur dioxide on the lichen algae with special reference to chlorophyll — Bryologist 69: 69-75.

Ronen, R. & Galun, M. —1984— Pigment extraction from lichens with dimethyl sulfoxide (DMSO) and estimation of chlorophyll degradation — Environmental & Experimental Bot. 24: 239-245.

Ronen, R. Canaanil, O. Garty, J., Cahen, D. Malkini, S. & Galun, M. —1985— Photosynthetic parameters in Ramalina duriaei, in vivo, studied by photoacoustics — In Brown, D. H. (ed.): Lichen physiology and cell biology, pp. 9-22, Plenum Press.

Schoaf, W. T. & Lium, B. W. —1976— Improved extraction of chlorophyll a and b from algae using dimethyl sulfoxide — Limnol. Oceanogr. 21: 926-928, 1976.

Silberstein, L., & Keller, P. —1986— A comparison between the response of Ramalina duriaei and Xanthoria parietina to bisulfite exposure — Progress and Problems in Lichenology in the eighties. International Symposium. Westfälische Wilhems-Universität. Münster. Lecture and posters Abstracts.

Spezieale, B. J. Scheiner, S. P. Giamatteo, P. A. & Schindler, J. E. —1984— Comparison of N,N-Dimethyl formamide, Dimethyl sulfoxide, and Acetone for extraction of phytoplankton chlorophyll — Can. J. Fish. & Aquatic Sci. 41 (10): 1519-1522.

Türk, R. & Wirth, V. —1975— The pH dependence of $SO_2$ damage to lichens — Oecol. Berlin 19: 285-291.

Vernon, L. P. —1960— Spectrophotometric determination of chlorophylls and phaeophytins in plant extracts». Anal. Chem. 32: 1144-1150, 1960.