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Chemotaxonomic discrimination of lichens species using infrared chalcogenide fibre optic sensor: a useful tool for on-field biosourcing.

M. Le Corvec,^{ab} C. Boussard-Plédel,^c F. Charpentier,^b N. Fatih,^b B. Le Dare,^d F. Massart,^d F. Rojas,^c H. Tariel,^b O. Loréal,^e B. Bureau,^c J. Boustie,^d O. Sire^a and F. Lohézic – Le Dévéhat^{d,*}

Abstract: Many analytical methods are known to discriminate natural products based on the set of chemical compounds they contain. Here, we evaluated the ability of the Fibre Evanescent Wave Spectroscopy (FEWS) to discriminate lichen natural extracts. Ten species of lichens including four chlorolichens (three *Cladonia species* and *Stereocaulon scutelligerum*), three tripartite lichens (*Stereocaulon species*) and three cyanolichens (*Lichina pygmaea*, *Collema cristatum*, *Peltigera membranacea*) belonging to six genera and six families were studied. One macroscopic cyanobacterium (*Nostoc sp.*), corresponding to the photobiont partner of *Peltigera* and *Collema* cyanolichens, was included for comparison. MIR spectra were acquired from lichens organic extracts between 3800 – 950 cm^{-1} using a chalcogenide infrared fibre optical device. Principal Component Analysis (PCA) and PCA-LDA (PCA-Linear Discriminant Analysis) based on the concept of variable clustering has been applied to absorbance MIR second derivative spectra for classification of the 11 species. The method succeeded in separating all lichen species and in identifying the major compounds. Moreover the signature of the sample was specific of each lichen suggesting that minor compounds played a role. Our data showed that FEWS optical sensor profiling is a rapid, efficient and convenient tool for metabolic profiling and suggest that it can be used: i) in chemotaxonomic approaches, ii) in processes for certification requirements in active compounds availability, and iii) in identifying new biosourcings in so far non investigated lichen species.

1. Introduction

Lichens like corals and mycorrhiza are symbiotic organisms including a photosynthetic partner and saprophytic fungi mostly belonging to Ascomycetes. When the photosymbiont is a green alga, the lichen is called chlorolichen while it is called cyanolichen when they accommodate cyanobacteria. An additional photosymbiont to a primary one indicates tripartite lichen. This mutualistic exchange allowed them to live in extreme environments (Antarctic, desert,...) thanks to the biosynthesis via acetyl polymalonyl, shikimate and mevalonate pathways of various families of compounds such as depsides, depsidones, dibenzofurans, aliphatic acids, anthraquinones, pulvinic acids, mycosporines, xanthenes and terpenoids¹. In most cases, the cyanolichens contain hydrophilic compounds (mycosporines and mycosporines-like amino acids) while the phenolic metabolites resulting from the polymalonyl pathway are more specific of tripartite and chlorolichens. These polyphenolic compounds (depsides, depsidones, dibenzofurans) exhibited very interesting biological activities^{2;3}. In the last century, the dibenzofuran usnic acid has been largely studied for various antimicrobial properties⁴. More recently, some new biological targets revealed the potentialities of lichen compounds: some depside (perlatolic acid) and depsidone (physodic acid) showed anti-inflammatory activities by inhibiting mPGEs1 (microsomal prostaglandin E synthase-1)^{5;6}, atranorin and derivatives (depsides) inhibited the hepatitis C virus⁷; protolichesterinic acid was patented for its depigmenting activities⁸, lobaric acid and derivatives inhibited PTP1B (Tyrosine-protein phosphatase non-receptor type 1) suggesting a role in type 2 diabetes⁹. Until now, 1054 lichen compounds have been characterized and new ones are regularly discovered^{1;10}. So, it is of interest to develop new methods in aim of profiling lichens for their chemical composition. In this objective, the potential of numerous methods has been evaluated on lichens like LC-MS¹¹, LDI-MS¹², Raman spectroscopy¹³⁻¹⁵. Here, we propose a method based on mid infrared (MIR) spectroscopy. Infrared spectroscopy is commonly used for identification of molecular functional groups. The mid-infrared spectrum ranges from 4000 to 400 cm⁻¹ (2.5µm to 25 µm) which embed most of the fundamental vibrations of the molecular functional groups which is useful to fingerprint molecules¹⁷. As lichens are bioindicators^{18;19}, MIR spectral analyses have been performed to estimate air pollution. Indeed, lichens exposed to ammonia, nitric acid vapour or sulphuric acid displayed

new IR absorption bands which were assigned to the *de novo* synthesis of new compounds within the lichen thallus^{20;21}. Moreover, some assigned marker IR bands have been used to monitor environmental stresses (UV-exposure, desiccation, low temperatures)^{13;14}. Here, chalcogenide glass optical fibres, with a transparency in the 4000 to 850 cm⁻¹ domain, are used to collect MIR spectra of lichens organic extracts. The principle of the fibre evanescent wave spectroscopy (FEWS) is based on the general concept of ATR but the use of a fibre permits to increase the number of total internal reflections²² and their hydrophobic nature makes them less sensitive to water which often screens the contribution of molecules of interest. This technology has been successfully applied to analyse food contamination²³, for the monitoring of bacterial biofilms²⁴, for the study of biological complex samples like serum^{25;26} and more recently FEWS was demonstrated to be useful for quick identification of septic arthritis by studying synovial fluid²⁷.

We propose here to use infrared FEWS combined with adequate statistical analysis to fingerprint lichen samples representative of vegetal and fungi materials. We demonstrate that this method can allow for a rapid identification of known compounds present in a mixture and helping thereby the discovery of new biosourcings. Moreover, as natural compounds are usually characterized by their chemical signature, this technique can be helpful for quality control by providing an analytical validation of raw materials.

2. Materials and methods

2.1. Lichen samples collection

Ten lichens and one cyanobacterium were collected in France but also in the Reunion Island and Italy (Table 1, Figure 2S-supplementary data). Four contained chlorococcoid algae (*Stereocaulon scutelligerum*, *Cladonia convoluta*, *C. rangiferina*, *C. portentosa*) and three contained only cyanobacteria, two of these belonging to the *Nostoc* genus (*Collema cristatum* var. *marginale*, *Peltigera membranacea*) and one to *Calothrix* (*Lichina pygmaea*). The three remaining lichen species (*Stereocaulon evolutum*, *S. vesuvianum*, *S. vulcani*) contained chlorococcoid algae and additional cyanobacteria in cephalodia. One macroscopic cyanobacterium (*Nostoc* sp.), corresponding to the photobiont partner of *Peltigera* and *Collema* cyanolichens, was included for comparison. After identification by Harrie Sipman for *Stereocaulon vulcani* and *S. scutelligerum* and the

others by Claude Roux, the specimens set was deposited at the Herbarium of the Department of Pharmacognosy and Mycology of Rennes with reference numbers (Table 1).

2.2. Chemicals and reagents

All chemicals (acetonitrile, chloroform, methanol, tetrahydrofuran, toluene, ethyl acetate, formic acid) were of analytical reagent grade and were purchased from Sigma (St Quentin-Fallavier, France). Authentic atranorin, lobaric acid and stictic acid, (+)-usnic acid were previously isolated from *Stereocaulon halei*²⁸ and *Usnea articulata*²⁹, respectively. Norstictic acid, fumarprotocetraric acid and perlatolic acid were available in the laboratory under the codes JB/A/036, JB/A/003a, JB/A/130, respectively.

2.3. Extraction of lichen samples

Ten replicates of whole thalli (250 mg each) were powdered for each species and 6 mL of a mixture chloroform/methanol (2/1) were added in each tube. Extraction was performed using a Heidolph Synthesis® apparatus during 2 hours at room temperature to avoid any methanolysis reaction under stirring (1,000 rpm). This operation was repeated three times and filtrates were pooled to yield the organic extracts which were then evaporated, weighed and stored under nitrogen. For each sample the weight ratio of secondary metabolites is calculated as the ratio between the dried organic extract and the lichen powder.

2.4. Preparation of sample solutions

All dried organic samples were re-dissolved in chloroform/methanol (1/1) to reach a concentration of 100 mg.mL⁻¹, centrifuged (3,000 rpm for 5 minutes). The supernatant was evaporated and weighed to allow the preparation of organic solutions in chloroform/methanol (1/1) at 10 mg.mL⁻¹.

2.5. Chalcogenide infrared optical fibres

The MIR spectra were collected using a Fourier Transform Infrared Spectrophotometer (Bruker T27) coupled with a chalcogenide optical fibre (Figure 1). The principle of the measurement is based on the evanescent wave spectroscopy. The light emitted by the black body source of the spectrometer is injected at the input of the fibre and collected at the output by a cooled MCT detector. The sensing part of the fibre in contact with the organic solution is shaped in a looping head having a diameter equal to 2 mm. This design permits to increase the sensitivity of the sensor by multiplying the number of reflexions in the fibre¹⁷. The reference spectrum is collected against air. Then, 10 µL of solution are deposited on the loop of the fibre and the signal is recorded after 2 min. Every single beam spectrum is the average of 100 scans collected within about 2 min. Between two measurements, the fibre loop is cleaned by immersion for 10s in an ultrasonic

bath filled with water in which 2 drops of RBS-25™ (Carl Roth, Karlsruhe, Germany) have been added. Then, the fibre is rinsed two times with distilled water and dried with an absorbing paper. The signal amplitude is checked before each new measurement

2.6 Treatments and analysis of Infrared spectra

For each sample, ten measurements were achieved corresponding to each replicate. The spectra of two pure compounds, atranorin and stictic acid were also recorded. MIR spectra were pre-processed and analysed in the 3800-950 cm⁻¹ frequency domain, where most of the biomolecules show up. Raw spectra were pre-processed using Extended Multiplicative Signal Correction (EMSC) for baseline correction and normalization. Second derivatives of spectra were also calculated, smoothed using a 13-points Savitzky-Golay smoothing algorithm, and normalized by vector normalization over the whole spectral range. Second derivatives were obtained after elimination of the contribution of environmental CO₂ by generation of a straight-line from 2800 to 1800 cm⁻¹. Quality test was applied to eliminate spectra that exhibit low S/N ratio.

2.7. Reproducibility

The reproducibility of the optical sensors was assessed by achieving thirty measurements of a unique organic sample of *Stereocaulon scutelligerum* (10 mg.mL⁻¹), which were recorded with two distinct fibres.

2.8. Statistical analysis of Infrared spectra

Two methods were used for spectra analysis. Firstly, a Principal Component Analysis (PCA) (unsupervised method) was used to extract the important information from the data matrix and to feature the pattern of similarity between the observations and the variables by displaying them as a factorial map³⁰. Secondly, a supervised method, linear discriminant analysis (PCA-LDA), was used to discriminate species. LDA was used to classify the lichens species, but this method requires fewer spectral variables than samples per group. So it was necessary to reduce the dimension of the spectral data set by previously performing a PCA. The population of lichens was split in two sub-groups, *i.e.* *calibration* (7 to 8 spectra of each species) for model construction and *validation* (1 or 2 spectra of each species) to evaluate the performance of the model.

2.9. High performance thin layer chromatography (HPTLC)

Two µL of the organic samples (10 mg.mL⁻¹) were spotted using an automated sample applicator, CAMAG ATS-4 on HPTLC plates

(Merck silica gel 60F254) and a densitometric UV scanner CAMAG Scanner type 3 was used for plate analysis (CAMAG, Muttenz, Switzerland). Both instruments were controlled by the Wincats software V1.4.3. After migration in the solvent (toluene/ethyl acetate/formic acid (139/83/8)), plates were scanned at 6 wavelengths 220, 260, 280, 310, 340 and 370 nm. Then, they were revealed by specific reagents as anisaldehyde-H₂SO₄ to dye the lichen's secondary metabolites.

3. Results and discussion

3.1. Metabolite profiling of the lichen samples

A representative panel of the three symbiotic forms found among lichens (and so of the metabolites they contain) was selected for the study: four chlorolichens, three cyanolichens and three tripartite species. A macroscopic cyanobacterium was also included in this panel as representative of the cyanobiont of the cyanolichens *Peltigera membranacea* and *Collema cristatum*. The dried organic extracts of these eleven species allowed calculation of an extraction yield which reflects the amount of secondary metabolites. The combination of organic solvents was also chosen to extract a large variety of secondary metabolites. The extraction weight ratio varied between 2 and 12 %. The best and the lowest extraction weight ratio were observed with the cyanolichens *Peltigera membranacea* (12 %), *Collema cristatum* and *Lichina pygmaea* (2%) (Figure 1S-supplementary data). No correlation between the content in secondary metabolites and the nature of the photobiont present in the thalli was observed.

These organic extracts were spotted on TLC plates and exhibited a large panel of secondary metabolites as revealed under UV then after spraying the plates with the sulphuric anisaldehyde reagent (Figure 2). Standards available in the lab (atranorin, fumarprotocetraric acid, norstictic acid, perlatolic acid, stictic acid, usnic acid) were compared to the lichen extracts and confirmed their presence for *Cladonia species*^{12; 31; 32} and *Stereocaulon species*³³. Some lichens exhibited similar metabolic profiles like *S. vulcani* and *S. vesuvianum* whose major compounds were atranorin and stictic acid (Figure 3). The three cyanolichens and the cyanobacterium showed also similar profiles.

Some distinct species produce same metabolites. *Cladonia convoluta* and *Cladonia portentosa* both synthesize usnic acid, *C. convoluta* and *C. rangiferina* have in common fumarprotocetraric acid while all *Stereocaulon* species synthesize atranorin as a major compound (Figure 2 and Figure 3). If *C. convoluta* was easy to discriminate from both other *Cladonia species* through their contrasted morphological appearance (Figure 2S-supplementary data); this was not the case for two *Stereocaulon species*, *S. vulcani* and *S. vesuvianum* (Figure 4). Moreover, for these latter species, HPTLC-UV profile of secondary metabolites is not fully conclusive to differentiate them (Figure 2).

3.2. FT-IR spectral data

The MIR spectra of the lichen extracts (10 replicates for each species) and two pure metabolites (stictic acid and atranorin) were acquired at the same concentration (10 mg.mL⁻¹). Analyses were performed in the 3200-2800 cm⁻¹ and 1800-950 cm⁻¹ domains. As shown in Figure 5a, which displayed the spectra of three species, the 3200-2800 domain is dominated by three peaks at 2958, 2923 and 2852 cm⁻¹ which correspond to $\nu_{\text{as}}(\text{CH}_2)$, $\nu_{\text{s}}(\text{CH}_2)$ and $\nu_{\text{as}}(\text{CH}_2)$, respectively. In opposite, the 1800-950 cm⁻¹ domain, exhibited large differences between species. Numerous absorption bands show up in this frequency domain which concerns all biochemical classes: lipids, proteins and sugars. To enhance the separation of overlapping peaks³⁴, second derivative spectroscopy was applied to increase the spectral resolution (Figure 5b).

3.3. Reproducibility

Spectra exhibiting low S/N ratio (two and four for fibre 1 and 2, respectively) were discarded.

A PCA was processed in the 3200-2800, 1800-950 cm⁻¹ spectral range. Statistical tests in the two first principal components, which represented 87% of the total variance, revealed no difference ($p < 0.05$) between the fibres (Figure 3S-supplementary DATA).

3.4. Distinction between species

Due to the complexity of spectral data reflecting the sample's molecular composition, multivariate analysis was used to discriminate the lichen samples. At first, PCA was used to explore the structure of second derivative spectra. The PCA scores plot from the 1800 – 950 cm⁻¹ infrared region (Figure 6) showed the low variance of replicates. An efficient inter species discrimination is attained from the two first components (PC1 and PC2), which features up to 49% of the total variance. This observation highlights that the inter-species variability is larger than the variability inside species clusters. The PC1 best discriminates *Cladonia convoluta* and *Cladonia portentosa* from all other investigated species whereas PC2 discriminates cyanolichens except *Peltigera membranacea*. Nevertheless, the cyanolichens and *Nostoc* were clearly separated from the chlorolichens and tripartite lichens. None of the 7 first PCs allows for isolating chlorolichens from the tripartite lichens suggesting that, despite different morphological typologies, they share similar secondary metabolites panels.

Then, PCA-LDA was used to classify the species. The classification model was set up from 80 % of the samples (calibration). The remaining 20% (validation) were submitted to prediction in order to evaluate the model's performance. The best results were obtained from the 1800-950 cm⁻¹ domain with 4 components. The model allowed a classification performance of 100% (Table 1S-supplementary data). In validation, the classificatory discriminant analysis indicated correct classification except for *Collema cristatum* spectra which were classified in the *Nostoc* group (Table 2S-supplementary data). This result was consistent with what is observed in Figure 6. Indeed the cyanolichens *Collema cristatum* and *Lichina pygmaea* and the cyanobacterium *Nostoc* exhibited close

PCA scores suggesting that the metabolites extracted from these three species are similar. Thus, these results demonstrate that MIR spectra can discriminate lichens species within families and genera. Our results well match those reported by Alcantara³⁵ who compared the FTIR spectral data of 11 lichen species and discriminate them at a species level. Nevertheless, their analyses were performed on lichen powders using KBr pellets. As a consequence, their analyses were mainly based on carbohydrates signals, specifically on polyols and monosaccharides which amounts are much higher than the secondary metabolites of interest on which our analyses are based on.

3.5. Major compounds identification from MIR spectra

In order to identify the major compounds present in the lichen extracts from MIR spectra (1800-950 cm^{-1}), three major secondary metabolites usnic acid, atranorin and stictic acid were selected and compared to the spectra of four lichen extracts *i.e.* *Cladonia portentosa*, *Cladonia convoluta*, *Stereocaulon vesuvianum* and *Stereocaulon vulcani*. As discussed previously, Figure 6 showed that *Cladonia convoluta* and *Cladonia portentosa* exhibited high PC1 scores. This indicated that this PC reflected a prominent variability between the extracted metabolites. By HPTLC, usnic acid was identified as the main metabolite extracted from these two species (Figure 2). The PC1 main loadings were located at 1627, 1369, 1288, 1187, 1143, 1118, 1068 and 1037 cm^{-1} (Figure 7a). These peaks fitted well the main usnic acid spectral peaks assigned in a spectral bank³⁷. Moreover, Liao et al¹⁶ used the peak at 1191 cm^{-1} as a marker of usnic acid in lichens for imaging technology. From this result, it is suggested that the PC1 largely matches the signature of usnic acid. To evidence other metabolites, a PCA was achieved on the 1800-950 cm^{-1} domain with the remainder lichens to exclude the variability assigned to high usnic acid contents, hence by excluding *Cladonia convoluta* and *Cladonia portentosa* samples. Once the factorial map was established (Figure 8), the MIR spectra of atranorin and stictic acid, both main metabolites revealed by HPTLC in *S. vesuvianum* and *S. vulcani* (Figure 2), were spotted on the map. PC1 allowed discriminating *S. vulcani* from other lichen samples and the PC1 scores of second metabolites along the PC1 axis showed that stictic acid was located close to *S. vulcani* while PCA score of atranorin was very close to *S. vesuvianum*. This result strongly suggests that the main metabolite extracted from *S. vulcani* was stictic acid and that the PC1 primarily features the spectral signature of stictic acid in lichens extracts as evidenced from the corresponding second derivative spectra (Figure 7b). The main metabolites extracted from *S. vesuvianum* are atranorin and stictic acid but the PCA scores plot (Figure 8) and the derivative spectra suggested that *S. vesuvianum* infrared spectra was dominated by atranorin (Figure 7c). It is important to note that none of the first PCs discriminated atranorin however it is worth noticing that some spectral domains differed between metabolites and the species so suggesting that distinct compounds were also synthesized by these two species. Thus, even if *S. vulcani* and *S. vesuvianum* exhibit close HPTC-UV profiles, infrared analyses

permitted to distinguish them. Hence MIR spectroscopy appears to be more discriminant than planar chromatography (Figure 2).

4. Conclusion

In conclusion, our results suggest that the feasibility of profiling lichen samples by IR-FEWS is assessed. This is critical since chemotaxonomic of lichens is particular complex and very few scientists are trained for such discriminations. Indeed, the major compounds (the so-called secondary metabolites) can be easily identified with this technology. Moreover, as major secondary metabolites are critical for lichen species identification, this method can differentiate them quickly and ambiguities in species identification can be reliably resolved. Thus, IR-FEWS can be considered as a predictable chemotaxonomic tool. This first assay on fungal and algal material with lichen sample is conclusive and need to be tested on other natural organisms like plant materials. Through this, IR-FEWS could be used in various domains such as food processing to check the identity of raw material particularly since the technology is available now "on the field". Such a technology could be of great interest to solve a current paradox that is an ever increasing effort to screen organisms for the quest of new bio-active molecules concomitant with a somehow rapid decrease in taxonomy experts who sole can reliably identify complex species like lichens. Hence, biochemical profiling is critical as an unsupervised tool fitted to on-field rapidly screen of known biosourcings for their contents of molecules of interest or unreferenced organisms for their potential to synthesize a particular bioactive molecule. The design apparatus has now evolved in order to provider convenient disposable optical sensors which avoids sample cross-contamination and allows unbiased baseline correction. All together, these unique technical specifications (large spectral window, fast data collection, unbiased spectral reference, accompanying statistical data treatment, equipment portability) provide a unique global solution to conveniently screen new species, new biomolecules which are hardly needed by ever growing green biotechnologies.

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Abbreviations

ATR : Attenuated total reflection , EMSC : Extended Multiplicative Signal Correction, FEWS : Fibre Evanescent Wave Spectroscopy, FTIR: Fourier transform infrared spectroscopy , HPTLC : High Performance Thin Layer Chromatography, IR : infrared, LC-MS: Liquid Chromatography coupled to Mass Spectrometry, MIR: Middle Infrared, PC : , PCA-LDA: Principal Component Analysis and Linear Discriminant Analysis.

Keywords:

Lichen, natural product, vibrational spectroscopy, FEWS, Mid Infrared, chalcogenide fibre and sensor

Table 1 Systematic list, sampling location, time collection and reference numbers of the lichen species / *Nostoc sp.* used for the study.

	Lichen	Order	Family	Sampling site / altitude/ collection data	Voucher code
Chlorolichen	<i>Cladonia convoluta</i> (Lam.)Cout.	Lecanorales	Cladoniaceae	Cressensac, Lot (France), 260 m, September 2012	JB/12/148
Chlorolichen	<i>Cladonia portentosa</i> (Dufour)Coem.	Lecanorales	Cladoniaceae	Cressensac, Lot (France), 260 m, November 2005	JB/05/54
Chlorolichen	<i>Cladonia rangiferina</i> (L.)Weber x.F.H.Wigg	Lecanorales	Cladoniaceae	Lac Del Palm, Pyrénées (France), 1500 m, August 2002	JB/02/64
Chlorolichen	<i>Stereocaulon scutelligerum</i> Th.Fr	Lecanorales	Stereocaulaceae	Langevin valley, Reunion island, 1000m high, March 2011	JB/11/123
Tripartite lichen	<i>Stereocaulon evolutum</i> Graewe	Lecanorales	Stereocaulaceae	Commana, Brittany, (France), 260 m high, July 2010	JB/10/121
Tripartite lichen	<i>Stereocaulon vesuvianum</i> Pers	Lecanorales	Stereocaulaceae	Etna, Sicilia island (Italia), 1350 m high, August 2001	JB/02/01
Tripartite lichen	<i>Stereocaulon vulcani</i> (Bory)Ach.	Lecanorales	Stereocaulaceae	Langevin valley, Reunion island, 1000 m high, March 2011	JB/11/122
Cyanolichen	<i>Collema cristatum var.marginale</i> (L.)Weber x.F.H.Wigg	Peltigerales	Collemataceae	Cressensac, Lot (France), 260 m, October 2004	JB/04/31
Cyanolichen	<i>Lichina pygmaea</i> (Lightf.)C.Agardh.	Lichinales	Lichinaceae	Pointe de la Garde, Dinard (France), level of sea, December 2007	JB/07/98
Cyanolichen	<i>Peltigera membranacea</i> (Ach.)Nyl.	Peltigerales	Peltigeraceae	Les Glières, Savoie (France), 1200 m, August 2011	JB/11/130
Cyanobacterium	<i>Nostoc sp.</i>	Cyanobacteria		Betahon, Morbihan (France), level of sea, January 2012	JB/13/166

Legends figures

Figure 1: Schematic representation of the experimental set-up used for recording the mid-infrared spectra

Figure 2: TLC profile of the eleven organic samples in toluene/ethyl acetate/formic acid (139/83/8) after spraying sulfuric-anisaldehyde and heating. Major secondary metabolites have been identified with comparison with standards (At : atranorin; Fu: fumarprotocetraric acid; Lob: lobaric acid ; Nor: norstictic acid ; Per: perlatolic acid; St : stictic acid ; Us : usnic acid).

*Means that the lichens are belonging to the cyanolichens, ° = tripartite lichens, + = chlorolichens.

Figure 3: Structure of the major compounds of *S. vulcani*, *S. vesuvianum*, *Cladonia portentosa* and *Cladonia convoluta*.

Figure 4: Morphology of *Stereocaulon vesuvianum* (a) and *Stereocaulon vulcani* (b)

Figure 5: a)EMSC corrected Infrared spectra in absorbance and b)second derivative for 3 species. *Lichina pygmaea* (black), *Cladonia convoluta* (dark grey) and *S. evolutum* (light grey).

Figure 6: PCA scores plot of second derivative spectra in the 1800-950cm⁻¹ domain of the different lichens species

Figure 7: a) Second derivative spectra of *Cladonia convoluta* (black), *Cladonia portentosa* (dark grey), and PC1 loadings (light grey); b) Second derivative spectra of *S. vulcani* (black), Stictic acid (dark grey), and PC1 loadings (light grey); c) Second derivative spectra of *S. vesuvianum* (black), and Atranorin (dark grey).

Figure 8: PCA scores plot of second derivative spectra in the 1800-950cm⁻¹ domain of the different lichens species without *Cladonia convoluta* and *Cladonia portentosa*.

Figure 1S: Extraction yields for the lichen samples. 250 mg of ground lichen species were extracted with 6 mL of a mixture chloroform/methanol (2/1) during 2 hours at room temperature under stirring. This operation was repeated three times and filtrates were combined to give the organic extracts which were then evaporated to be weighed and stored under nitrogen. The extraction yield represents the ratio between the weight of the extract and the weight of the lichen powder.

Figure 2S: Morphology of the eleven species included in the study

Figure 3S: PCA scores plot of second derivative spectra in the 1800-950cm⁻¹ domain for the interfibre variability evaluation. The barycentre was represented with cross.

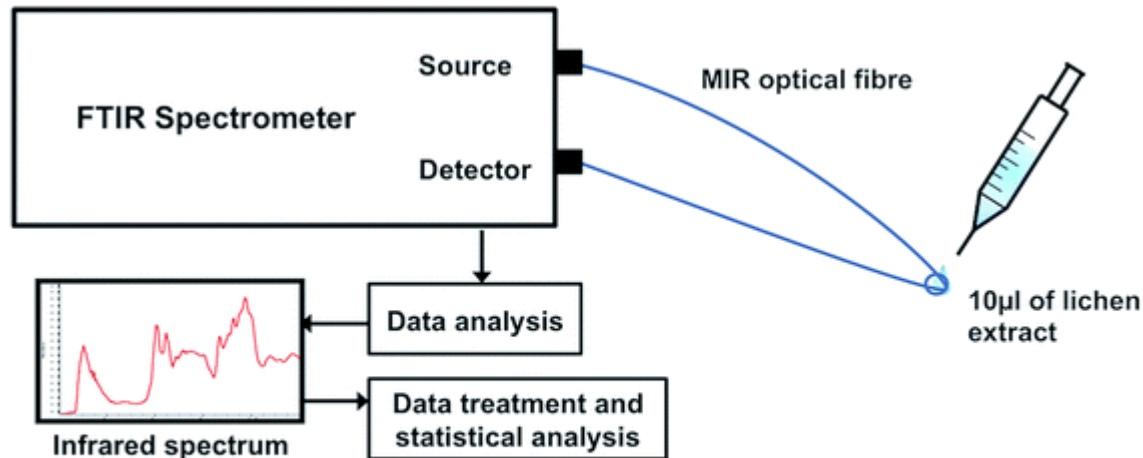


Fig. 1 Schematic representation of the experimental set-up used for recording the mid-infrared spectra.

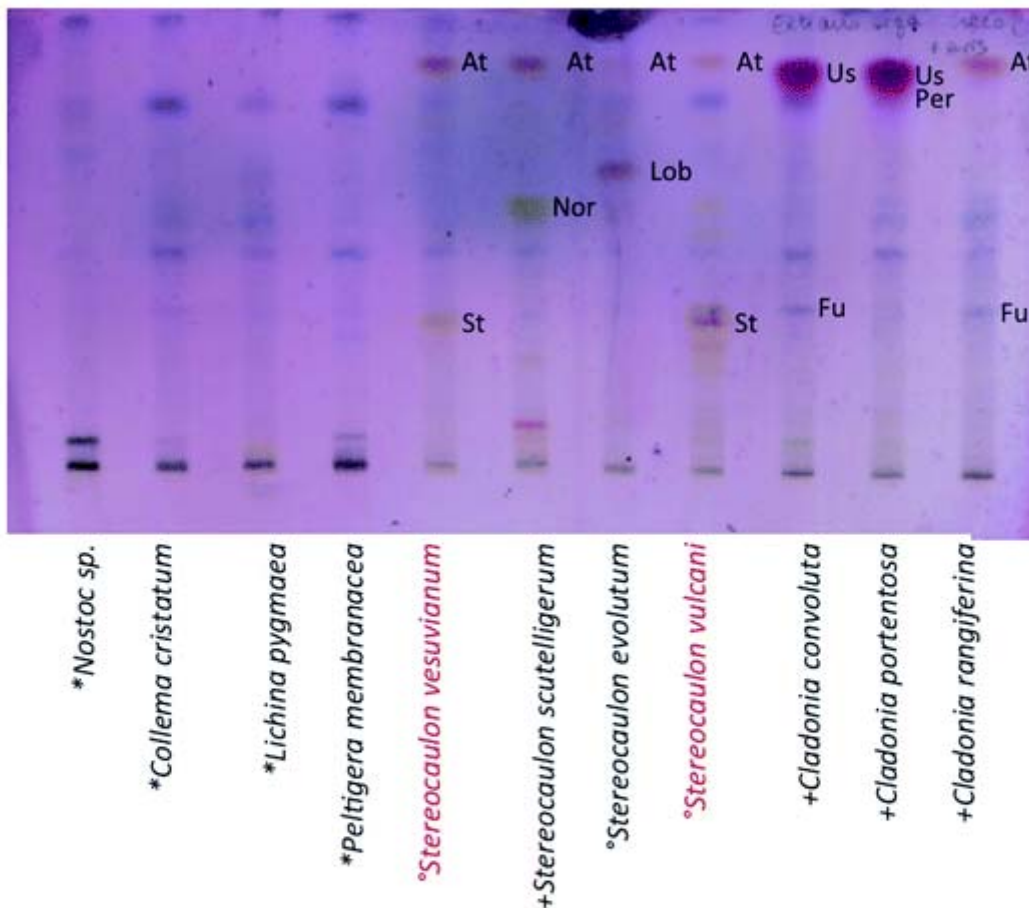


Fig. 2 TLC profile of the eleven organic samples in toluene/ethyl acetate/formic acid (139/83/8) after spraying sulfuric–anisaldehyde and heating. Major secondary metabolites have been identified with comparison with standards (At: atranorin; Fu: fumarprotocetraric acid; Lob: lobaric acid; Nor: norstictic acid; Per: perlatolic acid; St: stictic acid; Us: usnic acid). *Means that the lichens are belonging to the cyanolichens, ° = tripartite lichens, + = chlorolichens.

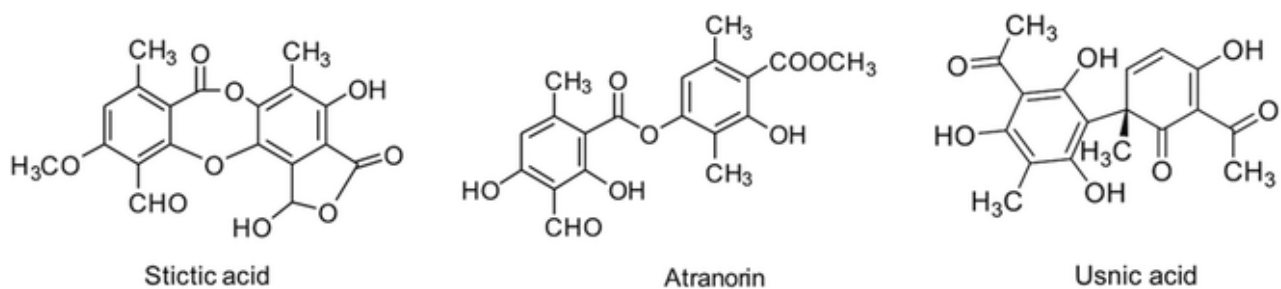


Fig. 3 Structure of the major compounds of *S. vulcani*, *S. vesuvianum*, *Cladonia portentosa* and *Cladonia convoluta*.

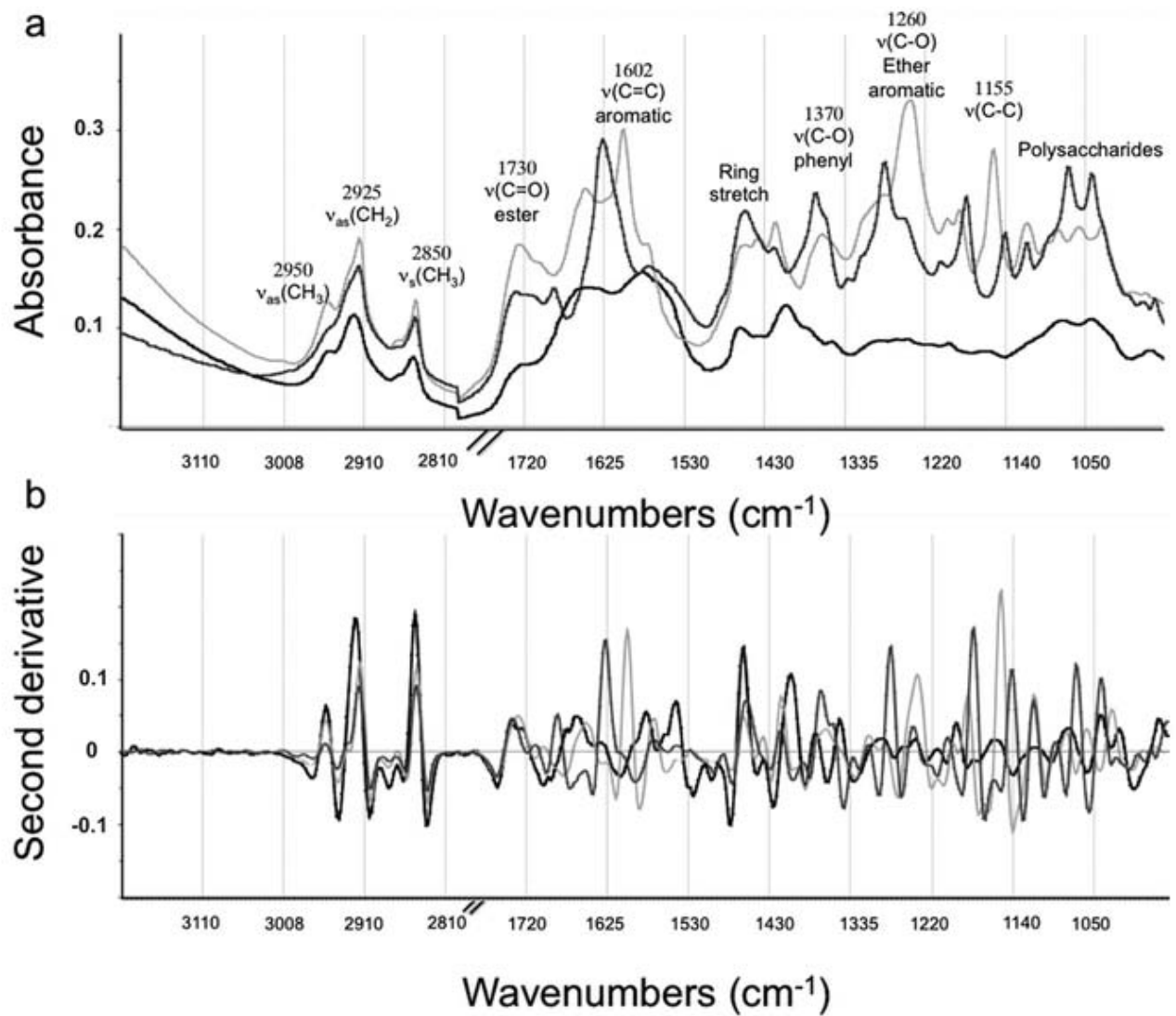


Fig. 4 (a) EMSC corrected infrared spectra in absorbance and (b) second derivative for 3 species. *Lichina pygmaea* (black), *Cladonia convoluta* (dark grey) and *S. evolutum* (light grey).

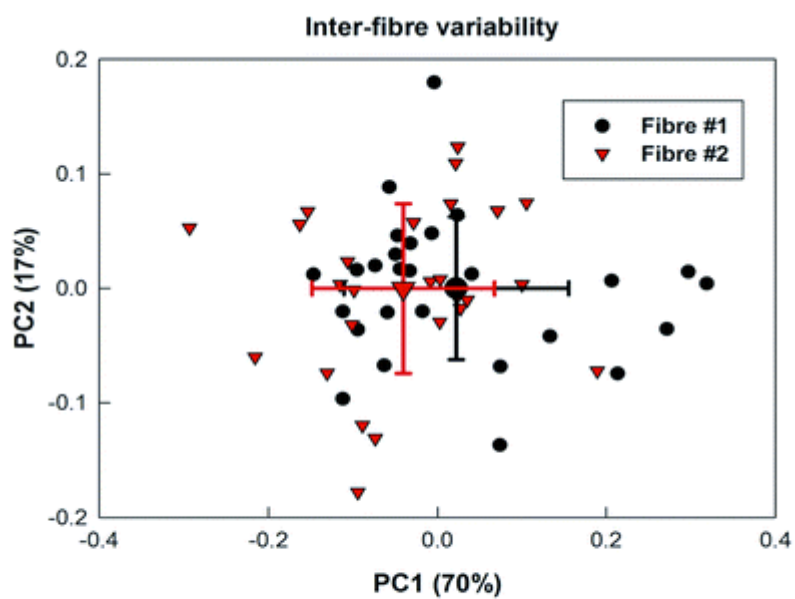


Fig. 5 PCA scores plot of second derivative spectra in the 1800–950 cm^{-1} domain for the interfibre variability evaluation. The barycentre was represented with cross.
