Research Article

Phytochemical Analysis

Received: 5 March 2014, Revised: 3 June 2014, Accepted: 10 June 2014 Published online in Wiley Online Library

(wileyonlinelibrary.com) DOI 10.1002/pca.2532

Qualitative and Spatial Metabolite Profiling of Lichens by a LC–MS Approach Combined With Optimised Extraction

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Introduction – Lichens are self-sustaining partnerships comprising fungi as shape-forming partners for their enclosed symbiotic algae. They produce a tremendous diversity of metabolites (1050 metabolites described so far).
Objectives – A comparison of metabolic profiles in nine lichen species belonging to three genera (*Lichina*, *Collema* and *Roccella*) by using an optimised extraction protocol, determination of the fragmentation pathway and the *in situ* localisation for major compounds in *Roccella* species.
Methods – Chemical analysis was performed using a complementary study combining a Taguchi experimental design with qualitative analysis by high-performance liquid chromatography coupled with mass spectrometry techniques.
Results – Optimal conditions to obtain the best total extraction yield were determined as follows: mortar grinding to a fine powder, two successive extractions, solid:liquid ratio (2:60) and 700 rpm stirring. Qualitative analysis of the metabolite profiling of these nine species extracted with the optimised method was corroborated using MS and MS/MS approaches. Nine main compounds were identified: 1 β-orcinol, 2 orsellinic acid, 3 putative choline sulphate, 4 roccellic acid, 5 montagnetol, 6 lecanoric acid, 7 erythrin, 8 lepraric acid and 9 acetylportentol, and several other compounds were reported. Identification was performed using the *m/z* ratio, fragmentation pathway and/or after isolation by NMR analysis. The variation of the metabolite profile in differently organised parts of two *Roccella* species suggests a specific role of major compounds in developmental stages of this symbiotic association.
Conclusion – Metabolic profiles represent specific chemical species and depend on the extraction conditions, the kind of the photobiont partner and the *in situ* localisation of major compounds. Copyright © 2014 John Wiley & Sons, Ltd.

Supporting information can be found in the on-line version of this article.

Keywords: Localisation; metabolite profile; Taguchi experimental design; symbiotic organisms

Introduction

Lichens are symbioses comprising fungi and algae in self-sustaining partnerships in which each plays a well-defined role: fungi provide water, mineral substances, vitamins and physical protection for the photobiont; the photobiont releases photosynthates in the form of organic compounds to the fungal partner. This partnership leads to the production of a light-exposed and long-living morphological structure, the lichen thallus. The lichen thalli are outstanding by producing and accumulating a high diversity of specific secondary metabolites. This capacitiy comprises various biosynthetic routes, including the mevalonate, polyketide and/or shikimic acid pathways. Biological activities have been demonstrated for some of these metabolites, including anti-oxidant, cytotoxic and anti-microbial activities (Lauterwein *et al*., 1995; Ingolfsdottir, 2002; Lohézic-Le Dévéhat *et al*., 2007; Molnár and Farkas, 2010; Shukla *et al*., 2010; Shrestha and St. Clair, 2013).

Research in recent years has characterised abundant bacterial communities that are associated with lichens (Bates *et al*., 2011; Bjelland *et al*., 2011; Cardinale *et al*., 2012). The chemical composition of lichens may influence the abundance and the diversity of bacterial communities, or possibly regulate the biology of bacteria. Indeed, there is plethora of work showing anti-bacterial effects of lichen compounds by *in vitro* experiments, but the details of these effects are hardly known (Boustie and Grube, 2005). Usnic acid, a dominant secondary metabolite of many lichens, appears to inhibit bacterial biofilm production on polymer surfaces (http://aac.asm.org/content/48/11/4360). In the natural context, that is, on lichen surfaces, usnic acid may decrease bacterial diversity rather than their abundance (Grube *et al*., 2009). However, no work has been conducted with other lichen metabolites so far. This lack of knowledge can be resolved

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only by complete, precise and unambiguous analyses of lichen secondary metabolites, because lichens may produce a high number of closely related compounds that are poorly resolved and differentially quantified by routine analyses. Optimisation of procedures also requires refinement of extraction processes in order to obtain the most complete lichen metabolite profiles, for example using a Taguchi experimental design and HPLC analysis.

For optimisation of procedures we used *Roccella fuciformis* (L.) DC. Quantitative parameters were measured for total extraction yield, and percentage yield of its main compounds erythrin and lepraric acid. These two main extracellular secondary metabolites were isolated, purified and identified during a previous study (unpublished data) from *R. fuciformis*. A spatial profiling of the major compounds from *R. fuciformis* and *R. phycopsis* was performed in order to determine their putative role in the symbiotic association. On the other side, the optimal conditions were applied to maritime (*Roccella phycopsis*), marine (*Lichina confinis*, *L. pygmaea*), and inland (*Collema auriforme*, *C. cristatum*, *C. fuscovirens*, *Leptogium lichenoides*, *Synalissa symphoreae*) lichens to determine qualitative metabolite profiling by a LC–MS approach.

Experimental

Materials

Lichen materials were collected from France at Erquy (35) in April 2012 and from Austria at Kesselfallklamm near Graz in November 2012. Four species were collected from seashore rocks coast on Brittany (France) – *Roccella fuciformis* (L.) DC., 1805, *Roccella phycopsis* Ach., 1810, *Lichina confinis* (Lightf.) Agardh., 1821 and *Lichina pygmaea* (Müll.) Agardh – and five inland species were collected from rock surfaces (Austria) – *Collema auriforme* (With.) J.R. Laundon., 1984, *Collema cristatum* (L.) Weber ex F.H. Wigg., 1780, *Collema fuscovirens* (With.) J.R. Laundon, 1984, *Leptogium lichenoides* (L.) Zahlbr., 1924 and *Synalissa symphorea* (Ach.) Nyl., 1856. After cleaning, their identification was determined according to macroscopic, microscopic and chemical (thalline reactions using *para*-phenylenediamine (P), potassium hydroxide (K), sodium hypochlorite (C) or/and KC reagents) characters and by comparison with the literature. The lichens were air-dried, ground using various techniques (Table 1) and stored at room temperature in closed tubes.

Chemicals and reagents

Cyclohexane, diethyl ether, acetone, methanol, tetrahydrofuran and acetonitrile were purchased from Carlo Erba Reactifs (Val de Reuil, France) and/or from Sigma Aldrich (St Quentin Fallavier, France). For chromatographic analysis, HPLC and LC–MS grade water was obtained by an EasyPure (Barnstead, NH, USA) water purification system. Deuterated solvents were purchased from Euriso-top (Gif-sur-Yvette, France).

Erythrin, lepraric acid, roccellic acid and acetylportentol standards (Fig. 1) were isolated from *R. fuciformis* or *R. phycopsis* after purification procedures. A SPOT® flash liquid chromatography (Armen Instrument) was used for erythrin and lepraric acid. The stationary phase was a silica column pre-packed (SiO_2) (FSHP-1207-0025, 25 g, Biotage) and the mobile phase consisted of a gradient comprising dichloromethane:acetic acid (98:2) and ethyl acetate (100: 0 to 0: 100 in 180 min). The flow rate was at 10 mL/min. Roccellic acid and acetylportentol were obtained by precipitation and recrystallisation using acetic acid and methanol respectively.

Table 1. Assignment of factors and level setting in the orthogonal design L_9

Level	Factors			
	A: frinding systems	B: solvents[a]	C: solid: liquid ratio	D: stirring (rpm)
1	Mixer	1	2:15	0
2	Mortar	2	2:30	200
3	Ball grinding	4	2:60	700

[a]Solvents: 1, acetone; 2, acetone then methanol (two successive extractions); 4, cyclohexane, diether ethylic, acetone then methanol (four successive extractions).

Equipment

Grinding with a mixer was performed with a Brawn multimix MX32 at maximal power (III) for 2 min. Mortar grinding was undertaken after the mixer grinding until a fine powder was obtained. For the ball grinding, two systems were performed depending on the weight of lichens: Retsch® PM100 using corundum (Al_2SiO_3 polymorphous) with ten balls (5 of 10 mm diameter, 3 of 20 mm diameter and 2 of 30 mm diameter) and Retsch® MM400 in steel with one ball (25 mm diameter). In this study, we applied grinding for 20 min (except 20 min then 10 min after homogenisation for *R. fuciformis*) at 500 round per second (rps)/min with 1 min in each direction and 5 s pause between each direction for the first system. For the second system, applied to low weights, a frequency at 300/s was used for 30 s.

Extractions were performed with a Heidolph Synthesis 1®, with four programmable zones for temperature and stirring (0 at 1000 rpm). During this study, boiling temperature was applied for each solvent: 71°C for cyclohexane, 65°C for methanol, 56°C for acetone and 35°C for diethyl ether.

Experiments were conducted using HPLC with a Kontron 325 pump and a diode-array detector (DAD). The injector was fitted with a 20 μL external loop. The analytical column was a Waters Spherisorb® ODS2 reversed-phase C-18 (150 mm × 4.6 mm, 5 μm particle size).

The electrospray ionization (ESI)/MS^n spectra were obtained using a LCQ Deca ion trap mass spectrometer (Thermo Finnigan, Villebon sur Yvette, France) equipped with an ESI source as described in Parrot *et al*. (in preparation).

Sample preparation

Experimental plan. An amount of 0.2 g powder of *R. fuciformis* was placed into tubes. According to the orthogonal design L_9 (3^4) (Tables 1 and 2), the respective volumes and solvents were added. All extraction experiments were repeated three times and carried out to determine the effect of grinding (mixer, mortar and ball grinding), of solvents (one, two or four successive solvents), of solid:liquid ratio (2:15, 2:30, 2:60) and stirring rate (0 rpm, 200 rpm or 700 rpm) on extraction efficiency: 200 mg powder, 30 min of extraction and two successive extractions with the same solvent corresponded to the fixed parameters of the experimental plan.

After extraction time was complete, solutions were filtered at 0.45 μm, concentrated under vacuum, dissolved in adequate solvent for analysis by HPLC and LC–MS as described in the HPLC and MS analysis sections.

The total extraction yield was defined as follows:

$$\text{total yield}\ (\%) = (\text{total extract mass}/\text{mass of lichen}) \times 100$$

The extraction yield of the compounds (erythrin or lepraric acid) in samples was defined as follows:

$$\text{yield}\ (\%) = (\text{mass of compounds in extract}/\text{mass of lichen}) \times 100$$

The mass of compounds in the extract was determined by HPLC analysis using calibration curves.

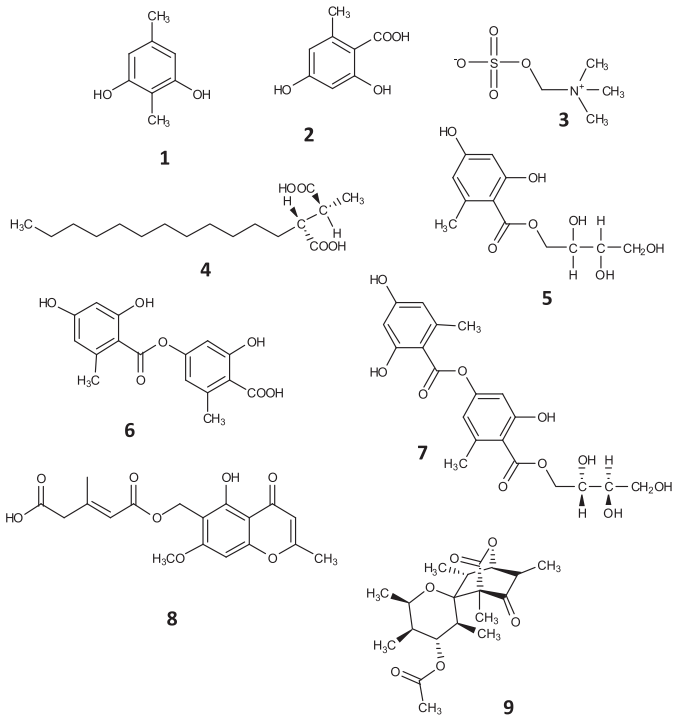

Figure 1. Chemical structure of β-orcinol (**1**), orsellinic acid (**2**), choline sulphate (CAS Number: 4858-96-2) (**3**), (+)-roccellic acid (**4**), montagnetol (**5**), lecanoric acid (**6**), (+)-erythrin (**7**), lepraric acid (**8**) and (+)-acetylportentol (**9**).

Table 2. Experimental conditions in the Taguchi experimental design, two additional experiments (10 and 11) and yields (%) of total extraction, erythrin and lepraric acid

Experiments	Factors[a]				Total extraction yield ± SD (%)	Erythrin yield ± SD (%)	Lepraric acid yield ± SD (%)
	A	B	C	D			
1	Mixer	1	2/15	0	13 ± 1.3	5.2 ± 1	5.2 ± 0.5
2	Mixer	2	2/30	200	22 ± 0.9	5.5 ± 0.8	4.8 ± 0.5
3	Mixer	4	2/60	700	21 ± 1.4	5.1 ± 0.2	4.3 ± 0.1
4	Mortar	1	2/30	700	20 ± 0.4	11.7 ± 0.7	4.9 ± 0.2
5	Mortar	2	2/60	0	27 ± 0.5	13.4 ± 0.8	5 ± 0.2
6	Mortar	4	2/15	200	20 ± 1.3	8.9 ± 0.8	4.4 ± 0.1
7	BB	1	2/60	200	17 ± 0.1	5.6 ± 0.3	5.1 ± 0.1
8	BB	2	2/15	700	22 ± 1.1	6.1 ± 0.7	4.5 ± 0.8
9	BB	4	2/30	0	20 ± 0.7	6.5 ± 0.1	5.3 ± 0.1
10	Mortar	2	2/60	700	29 ± 0.3	14.2 ± 0.2	8.1 ± 0.8
11	BB	1	2/30	0	15 ± 0.8	7.8 ± 0.9	10.7 ± 0.8

[a]A, grinding systems; B, solvents (1, 2 or 4 successive solvents); C, solid:liquid ratio; D, stirring rate.
BB, ball grinding.

Spatial profiling. Different parts (cortex, medulla, soralia) of *R. fuciformis* and *R. phycopsis* were separated from each other using a scalpel and extracted under the optimal conditions. After extraction time was complete, solutions were filtrated with cotton, dried under reduced pressure and dissolved in tetrahydrofuran:acetonitrile (50:50) for LC–ESI/MS/MS analysis.

Analysis by HPLC

Twenty microlitres of samples and standard solutions were injected on a C_{18}-column (150 mm × 4.6 mm, 5 μm). A HPLC gradient was applied: A (acetonitrile) and B (water). The following gradient was used at a flow rate of 1 mL/min: initial, 10% A; 1–20 min, 100% A linear and 3 min, 100% A; followed by washing and reconditioning the column during 7 min.

Twenty micrograms of samples dissolved in tetrahydrofuran and 2–14 μg standard solutions for each compound used for the preparation of six points calibration curves were injected. The elution was monitored at 254, 280, 312 and 365 nm. The two compounds were identified by comparison of their retention time, 10 min for erythrin and 12 min for lepraric acid, with those of the reference standards. The maximum wavelength of absorbance was observed for erythrin and lepraric acid at 280 nm and 254 nm respectively.

Qualitative approach

Mass spectrometry analysis. We performed LC–MS analysis using the method reported in Parrot *et al.* (in preparation), with either negative or positive ionisation mode for maritime species and cyanolichens

respectively. For HPLC, two gradient systems were applied: A (0.1% formic acid in water) and B (0.1% formic acid in acetonitrile). For *Roccella* species, the gradient described in Parrot *et al.* (in preparation) was applied (flow rate of 0.2 mL/min: initial, 20% B; 20–25 min, 80% B linear; 25–33 min, 100% B linear and 33–48 min 20% B linear; followed by washing and reconditioning the column) and the following gradient was applied for cyanolichens at a flow rate of 0.2 mL/min: initial, 5% B; 1–20 min, 95% B linear and 3 min, 5% B; followed by washing and reconditioning the column. Three micrograms and 150 ng were injected for the cyanolichens and maritime lichens respectively. The MS/MS parameters were optimised with erythrin for maritime lichens and with the mycosporine serinol for cyanolichens by direct infusion.

The MS^n spectra were recorded during the HPLC run using the following conditions: MS/MS analysis with starting collision-induced dissociation energy of 35 eV. The Xcalibur 1.0 software was used for data evaluation.

Taguchi experimental design

The Tagushi-based optimisation method allows optimisation with a minimum number of experiments. In this work, four experimental factors (grinding [A], solvent system [B], solid:liquid ratio [C] and stirring rate [D]) with three levels of setting were considered to be the dependent variables and are defined in Table 2. A complete evaluation of the impact of the chosen variables from three levels on the extraction efficiency requires 81 (3^4) experiments to be performed. A L_9 orthogonal array scheme was carried out (Table 3) with only nine runs required, as previously described in Bonny *et al.* (2009).

The HRMS measurements and NMR analysis

The HRMS measurements for exact mass determination were performed on a Micromass ZABSpecTOF spectrometer for ESI at the CRMPO (Centre Régional de Mesures Physiques de l'Ouest), Rennes 1. All spectra were recorded on a Bruker DMX 300 spectrometer (at 300 MHz (^{1}H) and 75 MHz (^{13}C)) and on a Bruker DMX 500 spectrometer (at 500 MHz (^{1}H) and 125 MHz (^{13}C)) using adequate deuterium solvents (dimethylsulphoxide (DMSO)-d_6, $CDCl_3$, C_3D_6CO and CD_3OD).

Optical rotation

Optical rotations were recorded with a Perkin Elmer 341 automatic polarimeter at 20°C at the sodium line; $[\alpha]_D$ measured at 20°C are given in 10^{-1} deg/cm^2/g.

The NMR and mass spectral data of compounds

Compounds **1** to **3** were identified according to their molecular mass and by comparison with data previously published (Huneck and Yoshimura, 1996).

Montagnetol (4). ^{1}H-NMR ($(CD_3)_2CO$, 300 MHz) data are comparable to published data (Basset *et al.*, 2010); high-resolution electron impact mass spectrometry (HREIMS): *m/z* 295.07930 $[M+Na]^+$ (calculated for $C_{12}H_{16}O_7Na$: 295.07937).

(+)-Roccellic acid (5). ^{1}H-NMR (DMSO-d_6, 300 MHz) and ^{13}C-NMR (DMSO-d_6, 75 MHz) data are comparable to published data (Huneck and Yoshimura, 1996); $[\alpha]_D$: +11 (c = 1, tetrahydrofuran (THF)); HREIMS: *m/z* 323.21980 $[M+Na]^+$ (calculated for $C_{17}H_{32}O_4Na$: 323.21983).

Lecanoric acid (6). ^{1}H-NMR ($(CD_3)_2CO$, 300 MHz) data are comparable to published data (Huneck and Yoshimura, 1996); HREIMS: *m/z* 317.06660 $[M-H]^-$ (calculated for $C_{16}H_{13}O_7$: 317.06668).

(+)-Erythrin (7). ^{1}H-NMR (DMSO-d_6, 300 MHz) and ^{13}C-NMR (DMSO-d_6, 75 MHz) data are comparable to published data (Huneck and Yoshimura, 1996); $[\alpha]_D$: +3 (c = 1, DMSO); HREIMS: *m/z* 445.1108 $[M+Na]^+$ (calculated for $C_{20}H_{22}O_{10}Na$: 445.11107).

Lepraric acid (8). ^{1}H-NMR ($CDCl_3$, 300 MHz) data are comparable to published data (Aberhart *et al.*, 1969; Huneck and Yoshimura, 1996); HREIMS: *m/z* 385.08990 $[M+Na]^+$ (calculated for $C_{18}H_{18}O_8Na$: 385.08994). ^{13}C-NMR ($CDCl_3$, 75 MHz): d ppm 18.93 (C-1), 20.47 (C-6'), 45.25 (C-4'), 54.32, 56.11 (C-9 and OMe), 89.61 (C-8), 105.04 (C-4a), 106.69 (C-3), 109.12 (C-6), 120.13 (C-2'), 149.89 (C-3'), 158.52 (C-8a), 160.82 (C-5), 164.09 (C-7), 166.12 (C-2), 166.76 (C-1'), 174.63 (C-5'), 182.36 (C-4).

(+)-Acetylportentol. ^{1}H-NMR (CD_3OD, 300 MHz) and ^{13}C-NMR (CD_3OD, 75 MHz) data are comparable to published data (Huneck and Yoshimura, 1996; Pettit *et al.*, 2004); $[\alpha]_D$: +40.3 (c = 1, CH_3OH); HREIMS: *m/z* 375.17820 $[M+Na]^+$ (calculated for $C_{19}H_{28}O_6Na$: 375.17836).

Statistical analysis

Statistical analyses (ANOVA, PCA) were performed using Matlab16 (MathWorks) and Excel 2007 (Microsoft) software for calibration curves, repeatability and detection limit. The *p*-values < 0.05 or < 0.01 are considered significant or highly significant.

Table 3. Summary of analysis of variance in the ANOVA model for each variable measured

Extraction yield system	Source	Sum of squares	Mean square	*F*-ratio	*p*-value
Total	Grinding method	47.34	47.34	25.74	0
	Number of solvents	205.64	205.64	111.80	0
	Solid:liquid ratio	58.99	58.99	32.07	0
	Stirring	7.26	7.26	3.95	0.0379
	Error	16.55	16.55		
	Total	335.79			
Erythrin	Grinding method	184.49	184.49	208.39	0
	Number of solvent	10.49	10.49	11.84	0
	Solid:liquid ratio	6.13	6.13	6.92	0.0059
	Stirring	16.78	16.78	18.96	0
	Error	7.97	7.97		
	Total	225.85			
Lepraric acid	Grinding method	0.21	0.21	0.80	0.466
	Number of solvents	0.89	0.89	3.40	0.056
	Solid:liquid ratio	0.50	0.50	1.94	0.173
	Stirring	1.86	1.86	7.13	0.005
	Error	2.34	2.34		
	Total	5.80			

Results and discussion

Method validation: calibration curves, repeatability and detection limit

A series of standard solutions of erythrin and lepraric acid within the range 2–14 μg were prepared to determine the linearity of this method. (The calibration lines of erythrin and lepraric acid standard solutions and the linear regression equation are available as online Supporting information.) The linear range was validated for the entire domain (2–14 μg) and the correlation coefficients of the calibration curves were 0.9919 and 0.9913. The limit of detection and quantification were determined as 0.79 and 2.08 μg for erythrin and 0.83 and 2.18 μg for lepraric acid (Amarouche, 2013). The repeatability of the determination by HPLC was also considered in order to determine the experimental errors (RSDr) and evaluated as 3.5% and 5.1% for erythrin and lepraric acid respectively.

Optimisation of extraction conditions

The aims of this study were to find the optimal extraction conditions to achieve optimal yields of lichen compounds and to apply them to the determination of the most complete chemical profiling of nine lichen species. The optimisation of extraction parameters was investigated with an experiment design: the Taguchi method (orthogonal design L_9 (3^4)). The main factors were chosen for their putative influence on the extraction efficiency: grinding (coarse to fine) and stirring rate (zero to high), which increase the surface contact with solvents; the polarity range of solvents used – nonpolar to polar (cyclohexane, diethyl ether, acetone then methanol), more polar (acetone then methanol) or moderate polar (acetone); and the solid:liquid ratio (2:15, 2:30 or 2:60) for determining the best ratio to avoid extraction saturation. The levels are shown in Table 1, and each experiment was replicated three times. The nine runs provided a statistical model to identify yields of the extraction process (Tables 2 and 3). The total extraction yield, erythrin yield and lepraric acid yield vary from 13 to 27%, 5.1 to 13.4% and from 4.3 to 5.3% (Table 2, entries 1–9). Additional runs 10 and 11, corresponding to the best combination for each variable for total extraction yield (run 10) and lepraric acid yield (run 11), showed an improvement in yield efficiency.

The main-effect plots (Fig. 2) were determined based on the data in Table 2 to analyse the influence of each factor (grinding, solvents, solid:liquid ratio and stirring rate) on total extraction yield, erythrin yield and lepraric acid yield. The value given corresponds to the mean of each value obtained for one parameter.

For the total extraction yield, Fig. 2 shows that the best conditions corresponded with the combination of mortar grinding, two successive extractions, a solid:liquid ratio (2:60) and 700 rpm for stirring. Indeed, taking into account each individual parameter (entries 1–9) the extraction yield was increased. The mortar grinding system increased the total extraction yield from 18.6 ± 4.9% (with mixer) or 19.6 ± 2.5% (with ball grinding) to 22.3 ± 4.0%, and two successive extractions with acetone then methanol increased this yield from 16.6 ± 3.5% (with acetone) or 20.3 ± 0.6% (with four successive solvents) to 23.7 ± 2.9%. The total extraction yield increased from 18.3 ± 4.7% to 21.7 ± 5.0% when the ratio varies from 2:15 to 2:60 and from 20 ± 7.0% to 21 ± 1.0% when the stirring increased from 0 to 700 rpm or from 200 to 700 rpm. Among the 11 runs in the orthogonal plan, the higher extraction yield was again obtained with the mortar grinding system and two successive extractions (acetone then methanol) (runs 5 and 10) (Table 2). Finally, the best combination for extraction yield (mortar grinding, acetone followed by methanol, solid:liquid ratio (2:60), 700 rpm] increased the efficiency until a 29 ± 0.3% total extraction yield (Table 2). However, the optimal conditions vary for selective extraction of the major compounds.

For erythrin yield, the mortar grinding was the best parameter. The yield increased from 5.3 ± 0.2% (mixer) or 6 ± 0.4% (ball grinding) to 11.3 ± 2.3%. The kind of solvents also influenced erythrin extraction efficiency, with yield increasing from 7.5 ± 3.6% to 8.3 ± 4.4% with two successive solvents but decreasing to 6.8 ± 1.9% with the use of four solvents. Erythrin yield strongly increased from 6.7 ± 1.9% to 8 ± 4.6% when the solid:liquid ratio varies from 2:15 to 2:60. The absence of stirring (0 rpm) allowed the best erythrin yield (8.4 ± 4.4% against 6.7 ± 4.7% or 7.6 ± 3.5% for 200 or 700 rpm). Combining the optimum for each variable (mortar grinding, acetone then methanol, solid:liquid ratio (2:60), 0 rpm; run 5) led to increase erythrin extraction efficiency (yield = 13.4 ± 0.8%; Table 2). We hypothesise that the ball grinding technique provoked thermal hydrolysis of erythrin.

Lepraric acid extraction efficiency was a little higher with the ball grinding system with a 4.9 ± 0.4% yield in comparison with

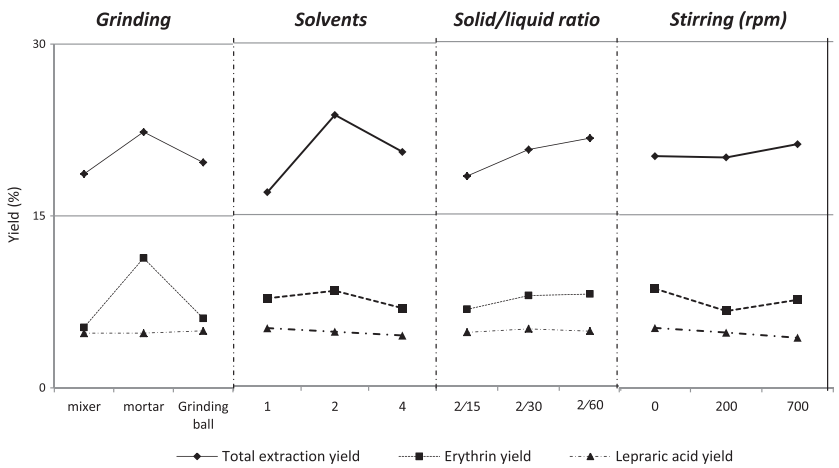

Figure 2. Main-effect plots during extraction optimisation.

mixer or mortar grinding, with yields of 4.7 ± 0.4% or 4.8 ± 0.3%, respectively (Fig. 2). The best efficiency for lepraric acid extraction was obtained with ball grinding, acetone, solid:liquid ratio (2:30), 0 rpm (yield = 10.7%; Table 2).

ANOVA was performed on the experimental results and provided a statistical model that reveals the relationships between the variables and their influence on the extraction yield. The *F*-test and the *p*-value showed that the four parameters (A, B, C and D) affect significantly ($p < 0.01$) the total extraction yield and erythrin yield from *R. fuciformis*. For lepraric acid yield, only the stirring parameter affects significantly the extraction yield (Table 3).

For a better understanding of the influence of extraction parameters we have implemented a spatial chemical profile study on *R. fuciformis* and *R. phycopsis*.

Spatial profiling metabolite

Direct localisation. Chemical reagents and their application in spot tests are traditionally used to facilitate the identification of lichens. Also the two *Roccella* species included here can be distinguished by the simple spot tests. Use of sodium hypochlorite (called 'C reagent') on cortex (for *R. phycopsis*) or on soralia (for *R. fuciformis*) produces a red colour, while the alcohol potash 10% (K reagent) caused a yellow colour to appear on the cortex for *R. fuciformis* (Fig. 3) (Smith *et al*., 2009).

Our chemical study isolated and identified various secondary metabolites: lepraric acid, erythrin and acetylportentol were the major compounds for *R. fuciformis*, while *R. phycopsis* provided erythrin and roccellic acid. Chemical reagents were performed on each of these compounds. Erythrin produced a red coloration using the C reagent, while a yellowish colour was obtained with the K reagent for lepraric acid. Acetylportentol and roccellic acid produced no colour change with the use of C or K reagents.

These initial observations, using spot tests, revealed a specific localisation of major compounds inside the thallus. Erythrin was localised in the soralia of *R. fuciformis* and in the cortex and medulla for *R. phycopsis*, whereas lepraric acid was specifically present in the cortex of *R. fuciformis*.

Indirect localisation. To confirm these latter observations, MS MS/MS and TLC analyses were performed on each different part of these lichens (cortex, medulla, soralia). For *R. fuciformis*, the MS spectra (Fig. 4) and TLC (data not shown) analyses showed that lepraric acid and acetylportentol (TLC analysis) are located

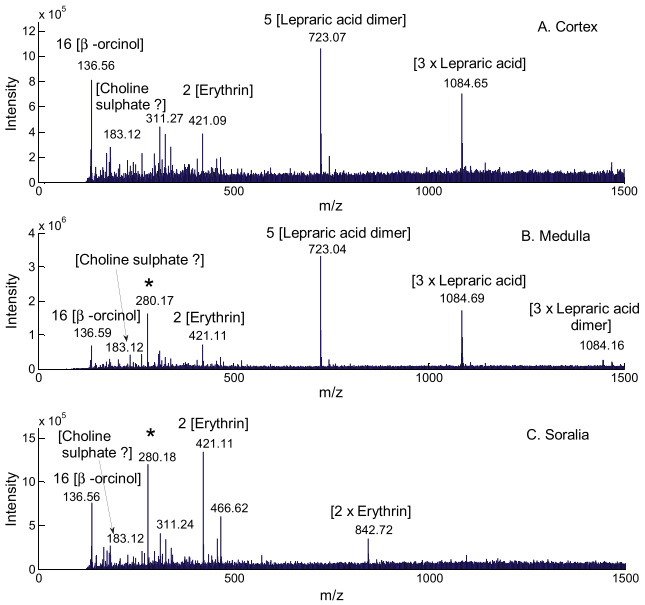

Figure 4. Mass spectrum (*m/z* $[M-H]^-$) of each part of *R. fuciformis*: (A) cortex, (B) medulla and (C) soralia. *The new unknown compound.

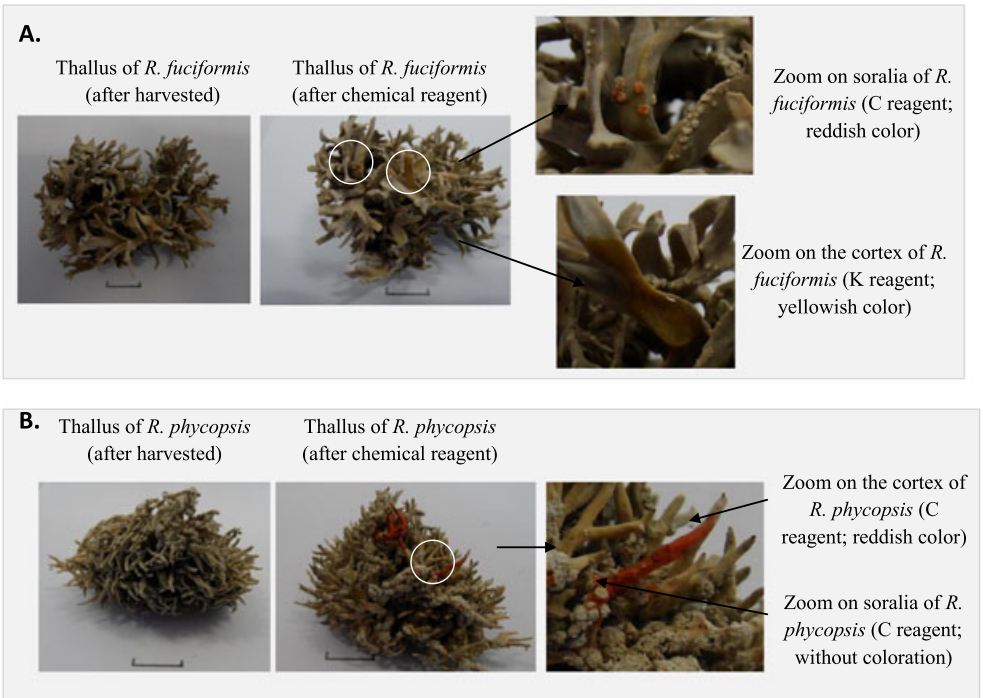

Figure 3. Pictures of (A) *R. fuciformis* and (B) *R. phycopsis* before and after application of chemical reagents (scale: 1 cm).

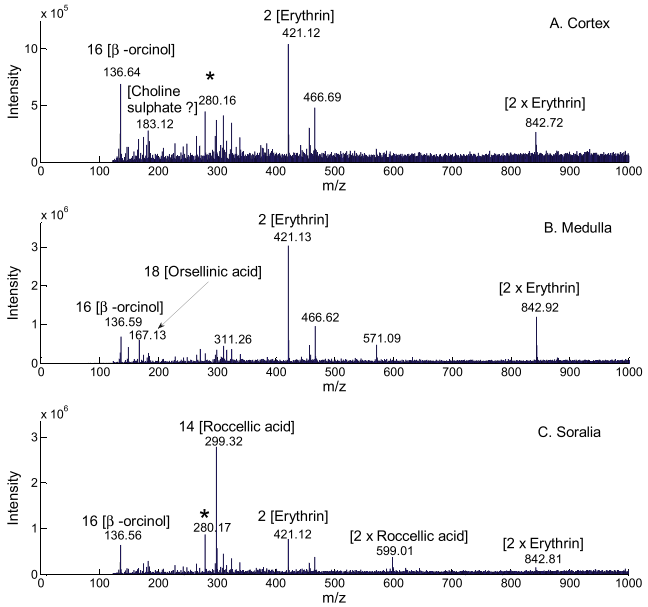

Figure 5. Mass spectrum (m/z $[M-H]^-$) of each part of *R. phycopsis*: (A) cortex, (B) medulla and (C) soralia. *The new unknown compound.

in the cortex and in the medulla, while erythrin was more specifically present in the soralia (Fig. 4). Their localisation in the external thalline parts of *R. fuciformis* meant that erythrin and lepraric acid were efficiently extracted without stirring, as indicated above in the optimisation of extraction conditions section. For *R. phycopsis*, these chemical profiles show a specific compartmentalisation of their major compounds. This compound (*m/z* $[M-H]^-$ 280 and MS/MS 211(50); 147(25)) has a specific localisation in the two Roccella species (in the cortex and soralia of *R. phycopsis* and in the medulla and soralia of *R. fuciformis*) (Figs. 4 and 5).

An unknown compound (*) was also observed on the MS spectra profiles from both *Roccella* species. This compound (*m/z* $[M-H]^-$ 280 and MS/MS 211(50); 147(25)) does not have a specific localisation in the thalli of *R. phycopsis*, but it was observed more specifically in the medulla and soralia of *R. fuciformis* (Figs 4 and 5). Thus a putative new compound was observed for these two *Roccella* species.

Application and qualitative analysis by MS

According to our objective to assess the most complete chemical profile, the optimal combination was selected to obtain the best total extraction yield and not the selective extraction of one compound. The best method (mortar grinding, acetone then methanol, solid liquid ratio (2:60), 700 rpm) of extraction was therefore applied to eight other lichens in order to determine qualitative metabolite profiles by MS and MS/MS analyses.

General profiles

Mass spectrometry has been used for the qualitative analysis of different marine, maritime and inland lichens using the extraction parameters defined in the Taguchi experimental design session. Various parameters, such as the geographical origin and the photobiont genus, were chosen for the selection of lichens to determine the efficiency of the method. Among these lichens, similarities and differences are present in the closely related species. The two maritime lichens (*R. fuciformis* and *R. phycopsis*) belong to the same genus and possess the same photobiont (*Trentepohlia* sp., *Chlorophyta*) and the same biogeographical distribution. The seven other lichens are cyanolichens: their photobiont corresponds to a cyanobacterial strain (*Calothrix* sp. for *Lichina* species, *Nostoc* sp. for *Collema* and *Leptogium* species and *Gloeocapsa* sp. for *Synalissa* species). These lichens represent two families of fungi: *Lichinaceae* (*L. confinis*, *L. pygmaea* and *S. symphorea*) and *Collemataceae* (*C. auriforme, C. cristatum, C. fuscovirens* and *Leptogium lichenoides*). The two *Lichina* species are marine lichens, whereas the others are found in inland habitats.

For maritime lichens (*R. fuciformis* and *R. phycopsis*) various metabolites have been described: nine secondary metabolites were reported for *R. fuciformis* (erythrin, lepraric acid, 5-hydroxy-7-methoxy-2-methyl-6-ethoxymethylchromone, acetylportentol, portentol, roccellic acid, picrorocellin, ethylorsellinate, choline sulphate) and five secondary metabolites for *R. phycopsis* (erythrin, roccellic acid, lecanoric acid, aspicillin, choline sulphate) (Aberhart *et al*., 1969; Culberson, 1970; Huneck, 1972; Smith *et al*., 2009).

For the *Roccella* species, nine compounds (**1–9**) were elucidated, and they were identified as **1** β-orcinol, **2** orsellinic acid, **3** putative choline sulphate, **4** roccellic acid, **5** montagnetol, **6** lecanoric acid, **7** erythrin, **8** lepraric acid and **9** acetylportentol by comparison with MS data and/or a certified standard, or by isolation and NMR identification. Various minor compounds could also be observed. Acetylportentol was not observed in the MS spectra due to its non-ionisation in the condition used (Fig. 6 and Table 4). Fragmentation pathways for five compounds (**4** to **8**) are proposed (Fig. 7). For compound **4**, three products ions were observed: at *m/z* $[M-H]^-$ 281, 255 and

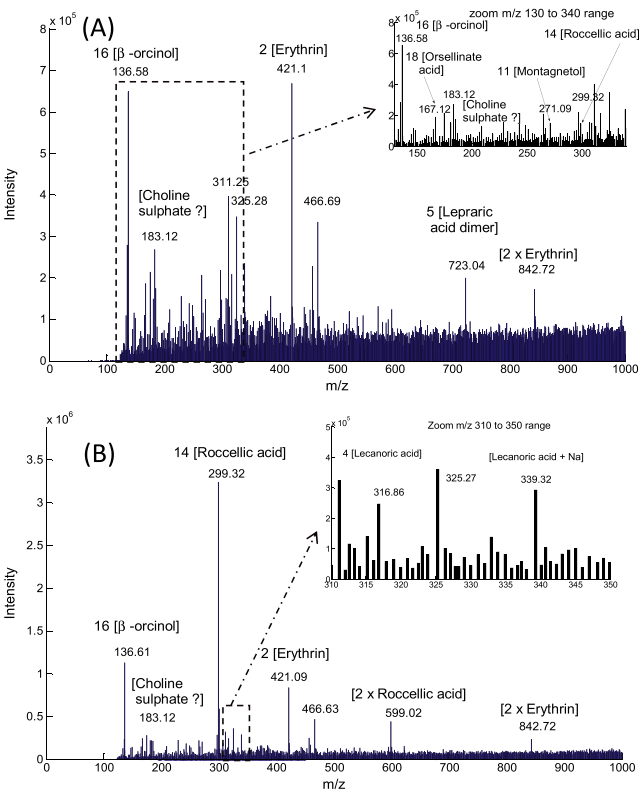

Figure 6. Mass spectrum (*m/z* $[M-H]^-$) of (A) *R. fuciformis* thallus and (B) *R. phycopsis* thallus.

Table 4. Retention times (t_R), UV absorptions (λ_{max}), molecular ions $[M-H]^-$, product ions (35 eV), percentage relative intensities % (RI%) of all compounds of *roccella* species

Number	t_R (min)	UV λ_{max} (nm)	$[M-H]^-$ *m/z*	Product ions (*m/z*) at 35 eV (IR%)	Identification[a]	Localisation	
						RF	*RP*
1	5.60	233, 257, 297	–	–	Unknown	+	+
2	9.03	233, 269, 302	421	167(100), 210(7.7), 269(13.4), 271(27), 324 (7.4), 352(13.4), 353 (8.6), 374(17.4), 377 (8.2), 419(12.5), 421(38), 439(13.1), 457(8.8)	Erythrin	+	+
3	11.65	234, 263, 290, 336	–	–	Unknown	+	–
4	12.40	233, 263, 280, 317, 336	317	167(100), 317(20.4)	Lecanoric acid	–	+
5	12.42	235, 260, 293, 336	723	504(15), 557(22), 596(76), 601(25), (619(25), 655(9.9), 696(100), 697(12), 741(43), 759(33)	Lepraric acid (dimer)	+	–
6	14.88	235, 263, 295, 311, 335	–	–	Unknown	–	+
7	14.92	235, 263, 304, 335	–	–	Unknown	+	–
8	18.92	235, 263, 280, 336	–	–	Unknown	+	+
9	20.47	235, 263, 289, 336, 362	–	–	Unknown	–	+
10	33.37	237, 263, 336	–	–	Unknown	+	+
11	3.36	–	271	123(4.9), 149(92.6), 167(100), 271(45.4)	Montagnetol	+	+
12	11.02	–	376	375(100), 376(22.4)	Unknown	+	–
13	15.24	–	251	182(0.4), 209(0.8), 251(100), 252(137)	Unknown	+	–
14	21.95	–	299	255(0.3), 281(0.8), 298(2.3), 299(100)	Roccellic acid	+	+
15	23.31	–	265	97(0.8), 166(0.7), 265(100), 266(4.4)	Unknown	+	–
16	–	–	136	–	B–orcinol	+	+
17	–	–	183	–	Choline sulphate	+	+
18	–	–	167	–	Orsellinic acid	+	+

[a]Identification by molecular mass (reported *m/z* $[M-H]^-$) and data published for *R. fuciformis* and *R. phycopsis*.
–, absence of metabolites; +, presence of metabolites; RF, *Roccella fuciformis*; RP, *Roccella phycopsis*.

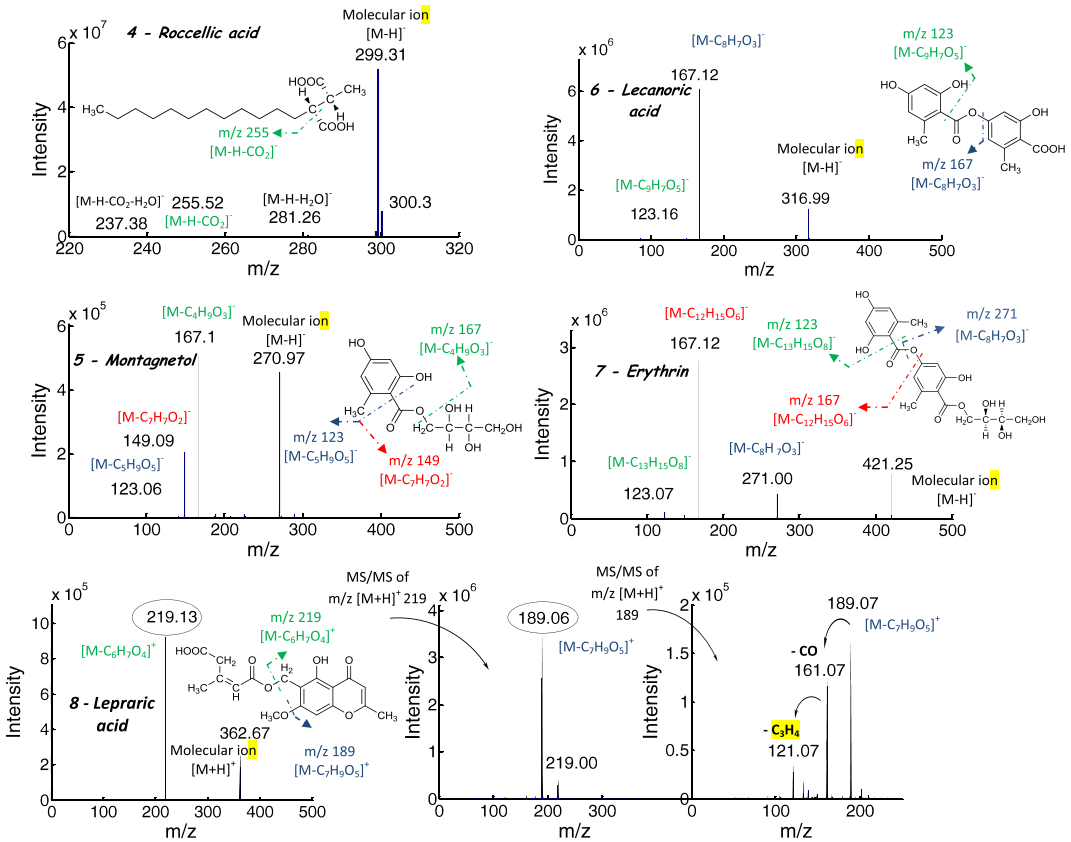

Figure 7. Fragmentation pathway of compounds **4** to **8**.

Table 5. Retention times (t_R), UV absorptions (λ_{max}), molecular ions $[M+H]^+$, product ions (35 eV), percentage relative intensities (RI%) of all compounds of cyanolichens species

Number	t_R (min)	UV λ_{max} (nm)	$[M+H]^+$ *m/z*	Product ions (*m/z*) at 35 eV (IR%)	Identification[a]	Localisation						
						CA	*CC*	*C*	*LC*	*LP*	*LL*	*SS*
1	1.95	309, 336, 379	–	–	Unknown	+	+	+	+	–	–	–
2	1.98	308, 336, 489	262	166(10), 182(15), 194(34), 216(100), 226(10), 230(22.4), 244(63)	Mycosporine serinol	–	–	–	+	+	+	+
3	3.32	265	–	–	Unknown	+	+	+	+	+	+	+
4	16.87	263, 288, 329	–	–	Unknown	–	–	–	–	–	+	–
5	19.02	263, 317, 377, 387, 440, 484	–	–	Unknown	+	+	+	+	–	–	–
6	20.27	263, 298, 334, 376, 387, 395, 492	–	–	Unknown	+	–	+	+	–	–	–
7	21.75	263, 336, 489	–	–	Unknown	–	–	–	–	–	+	–
8	23.28	262, 277	–	–	Unknown	+	+	+	+	+	+	+
9	26.12	263, 285, 336, 489	–	–	Unknown	–	–	–	–	–	–	+
10	4.73	–	145	55(1.4), 72(1.4), 73(100), 88(2.1), 99(1.7), 102(1.4), 127(5.5), 1128(6.9)	Unknown	+	+	+	+	+	+	+
11	8.74	–	188	169(0.9), 170(100), 184(0.5)	Unknown	–	+	–	–	–	–	+
12	9.75	–	240	108(77), 133(90), 195(8), 223(62), 240(100), 241(59)	Unknown	–	–	–	–	–	+	–
13	10.48	–	163	55(2), 89(14), 101(6), 107(100), 125(2), 142(1), 145(4)	Unknown	+	+	+	+	+	+	+
14	12.34	–	219	122(1.4), 161(1.4), 190(1), 203(100), 219(4)	Unknown	+	–	+	–	+	+	+
15	13.88	–	274	106(4), 256(7), 274(100), 275(12)	Unknown	+	–	–	–	–	–	–
16	14.51	–	343	107(1), 281(2), 240(100), 323(1)	Unknown	–	+	+	+	+	+	–
17	16.46	–	371	147(54.5), 241(74.3), 259(100), 283 (21.5), 325(33), 342(37.5), 355(48), 371(32), 386(22.5)	Unknown	+	+	+	+	+	–	+
18	17.02	–	375	115(5), 211(14), 319(42), 358(100), 359(11), 376(22)	Unknown	–	–	–	–	–	+	–
19	18.07	–	332	240(9), 331(2), 332(100)	Unknown	–	–	+	–	–	–	–
20	19.20	–	ND	–	Unknown	+	+	+	+	+	+	+
21	21.07	–	298	111(4), 153(5), 196(6), 245(18), 263(65.5), 280(100), 281(54), 298(18.5), 299(78)	Unknown	–	+	–	–	–	–	–
22	22.56	–	ND	–	Unknown	+	+	+	+	+	+	+
23	24.81		282	114(2.2), 123(3.4), 134(2), 153(4), 170(4), 177(5), 184(6.6), 247(67), 256(100), 297(3.3)	Unknown	+	+	+	+	+	+	+

[a]Identification by standard.

–, absence of metabolites; +, presence of metabolites; ND, not determined; CA, *Collema auriforme*; CC, *C. cristatum*; CF, *C. fuscovirens*; LC, *Lichina confinis*; LP, *L. pygmaea*; LL, *Leptogium lichenoïdes*; SS, *Synalissa symphorea*.

237 corresponding to the loss of CO_2, H_2O and CO_2 and H_2O group respectively. The MS/MS spectra of compounds **5–7** showed the same products ions at *m/z* $[M-H]^-$ 167 and 123, corresponding to orsellinic acid and the β-orcinol ring respectively. Moreover, compounds **5** and **7** presented, respectively, a product ion at *m/z* $[M-H]^-$ 149 and 271, corresponding to the loss of $C_7H_7O_2$ and $C_8H_7O_3$ respectively. So, these three compounds derived from the orcinol ring showed a similar fragmentation pathway. The MS/MS spectra in positive mode for compound **8** (*m/z* $[M+H]^+$ 363) showed four main products ions: *m/z* 219, 189, 161 and 121. The products ions *m/z* 219 and 189 correspond to a loss of $C_6H_7O_4$ and $CH_2=O$ groups. The two other product ions observed, *m/z* 161 and 121 correspond respectively to a loss of CO and C_3H_4 groups.

A low number of chemical compounds of the marine and inland cyanolichens (*Collema auriforme, C. cristatum, C. fuscovirens, Lichina confinis, L. pygmaea, Leptogium lichenoïdes* and *Synalissa symphorea*) have been reported. Only four secondary metabolites were mentioned: two aryl-hydrazides (L-glutamic acid 5-[(2,4-dimethoxphenyl)-hydrazide)] and pygmeine), aminocyclohexenone and mycosporine serinol for *L. pygmaea* (Roullier *et al*., 2009); collemin A, 3-acetic indole acid and mycosporine serinol for *C. cristatum* (Büdel *et al*., 1997; Torres *et al*., 2004; Temina *et al*., 2010).

A full mass scan (*m/z* 50–2000 range) data spectrum was acquired and is presented in Table 5 (see also the online Supporting information for UV and MS chromatograms). Several compounds were observed but only compound **2** (mycosporine serinol) was identified by comparison with available literature information. So, many lichen compounds remain to be isolated and elucidated from these cyanolichens species.

Comparison of chemical profiles. Roccella *fuciformis* and *R. phycopsis*, the two maritime lichens, showed similarities in their metabolite profiles. Indeed, four secondary metabolites known are common: β-orcinol, putative choline sulphate, roccellic acid and erythrin. The others are specific-species compounds such as lepraric acid for *R. fuciformis* and lecanoric acid for *R. phycopsis*. The MS approach for cyanolichens (marine and inland species) showed various similarities. Almost all secondary metabolites are produced by these seven cyanolichens. A similar chemical fingerprint has been observed for all these cyanolichens. Multivariate analysis (PCA) confirmed similarities between chemical profiling of these cyanolichens (e.g. one group was observed), while *R. fuciformis* was separated from *R. phycopsis* as two distinct groups were observed (data not shown).

Summary

Extraction optimisations of lichens by a Taguchi design combined with HPLC and implementation for nine different species by MS were performed. Several optimal extraction combinations have been shown through this study, including global and more specific approaches. The best system affording greater extraction efficiency was: mortar grinding, two successive solvents, solid:liquid ratio (2:60) and 700 rpm. Other parameters were selected for specific extraction of erythrin or lepraric acid, the major compounds of *R. fuciformis*. Nine compounds were identified and for the first time fragmentation pathways are proposed for five of them. The nine lichens studied were shown to possess close chemical profiles, depending on the nature of the photobiont partner (cyanobacteria or green algae).

A specific localisation of the major secondary metabolites from *R. fuciformis* and *R. phycopsis* was demonstrated here. The production sites and/or the accumulation of these metabolites are highly dependent on the entire lichen metabolism and this compartmentalisation of compounds may be explained by their potential activity and required functions. As lepraric acid and roccellic acid exert anti-bacterial activities (data not shown), we argue that their production will influence the associated bacterial community of their hosts. This aspect, however, remains to be tested (Parrot *et al*., in preparation). Even when the modulatory effect on bacterial communities has been demonstrated, further research would also be needed to investigate why differences exist in the localisation of compounds among species.

Acknowledgements

We gratefully acknowledge INSA Rennes for the ministerial grant for the PhD to Delphine Parrot. We are grateful to Professor Joel Boustie for the localisation of Brittany species, Isabelle Rouaud, Aurélie Bernard, Dr.Béatrice Legouin for technical help and Dr Sylvain Guyot (P2M2 platform, INRA, Rennes) for LC–MS analysis and Dr Philippe Jehan (CRMPO, Rennes) for MS analysis. We thank Professor Martin Grube for the identification of lichen samples and for the proofreading.

References

Aberhart DJ, Overton KH, Hunck S. 1969. Studies on lichens substances. Part LXII. Aromatic constituents of the lichen *Roccella fuciformis* DC. a revised structure for lepraric acid. *J Chem Soc* **8**: 704–707.

Amarouche S. 2013. Caractérisation d'une méthode de mesure: étape clé dans le processus de validation. *Tech l'ingénieur Qual au Lab* **16**: 1–18.

Basset JF, Leslie C, Hamprecht D, White AJP, Barrett AGM. 2010. Studies on the resorcylates: biomimetic total syntheses of (+)-montagnetol and (+)-erythrin. *Tetrahedron Lett* **51**: 783–785.

Bates ST, Cropsey GWG, Caporaso JG, Knight R, Fierer N. 2011. Bacterial communities associated with the lichen symbiosis. *Appl Environ Microbiol* **77**: 1309–14.

Bjelland T, Grube M, Hoem S, Jorgensen SL, Daae FL, Thorseth IH, Øvreås L. 2011. Microbial metacommunities in the lichen-rock habitat. *Environ Microbiol Rep* **3**: 434–442.

Bonny S, Hitti E, Boustie J, Bernard A, Tomasi S. 2009. Optimization of a microwave-assisted extraction of secondary metabolites from crustose lichens with quantitative spectrophotodensitometry analysis. *J Chromatogr A* **1216**: 7651–7656.

Boustie J, Grube M. 2005. Lichens – a promising source of bioactive secondary metabolites. *Plant Genet Resour* **3**: 273–28.

Büdel B, Karsten U, Garcia-Pichel F. 1997. Ultraviolet-absorbing scytonemin and mycosporine-like amino acid derivatives in exposed, rock-inhabiting cyanobacterial lichens. *Oecologia* **112**: 165–172.

Cardinale M, Steinová J, Rabensteiner J, Berg G, Grube M. 2012. age, sun and substrate: triggers of bacterial communities in lichens. *Environ Microbiol Rep* **4**: 23–28.

Culberson CF. 1970. Supplement to 'chemical and botanical guide to lichen products'. *Bryologist* **73**: 177–377.

Grube M, Cardinale M, De Castro J, Mu H, Berg G. 2009. Species-specific structural and functional diversity of bacterial communities in lichen symbioses. *Int. Soc. Microb. Ecol.* **3**: 1105–1115.

Huneck S. 1972. 6-hydroxymethyleugenitin, ein neues chromon aus *Roccella fuciformis*. *Phytochemistry* **11**: 1489–1490.

Huneck S, Yoshimura I. 1996. *Identification of Lichen Substances*. Springer-Verlag: Berlin; 498.

Ingolfsdottir K 2002. Usnic acid. *Phytochemistry* **61**: 729–736.

Lauterwein M, Oethinger M, Belsner K, Peters T, Marre R. 1995. *In vitro* activities of the lichen secondary metabolites vulpinic acid , (+)-usnic acid, and (−)-usnic acid against aerobic and anaerobic microorganisms. *Antimicrob Agents Chemother* **39**: 2541–2543.

Lohézic-Le Dévéhat F, Tomasi S, Elix JA, Bernard A, Rouaud I, Uriac P, Boustie J. 2007. Stictic acid derivatives from the lichen *Usnea articulata* and their antioxidant activities. *J Nat Prod* **70**: 1218–1220.

Molnár K, Farkas E. 2010. Current results on biological activities of lichen secondary metabolites: a review. *Z Naturforsch C* **65**: 157–173.
Parrot D, Jan S, Baert N, Guyot S, Tomasi S. in preparation. Comparative metabolite profiling and chemical study of *Ramalina siliquosa* complex using LC–ESI-MS/MS approach. *Phytochemistry* **89**: 114–124.
Pettit GR, Zhang Q, Pinilla V, Herald DL, Doubek DL, Duke JA. 2004. Isolation and structure of gustastatin from the Brazilian nut tree *Gustavia hexapetala*. *J Nat Prod* **67**: 983–985.
Roullier C, Chollet-Krugler M, Bernard A, Boustie J. 2009. Multiple dual-mode centrifugal partition chromatography as an efficient method for the purification of a mycosporine from a crude methanolic extract of *Lichina pygmaea*. *J Chromatogr B Analyt Technol Biomed Life Sci* **877**: 2067–2073.
Shrestha G, St. Clair LL. 2013. Lichens: a promising source of antibiotic and anticancer drugs. *Phytochem Rev* **12**: 229–244.
Shukla V, Joshi GP, Rawat MSM. 2010. Lichens as a potential natural source of bioactive compounds: a review. *Phytochem Rev* **9**: 303–314.
Smith C, Aptroot A, Coppins B, Fletcher A, Gilbert O, James PW, Wolseley PA. 2009. *The Lichens of Great Britain and Ireland*. The British Lichen Society: London; 1046.
Temina M, Levitsky DO, Dembitsky VM. 2010. Chemical constituents of the epiphytic and lithophilic lichens of the genus *Collema*. *Rec Nat Prod* **4**: 79–86.
Torres A, Hochberg M, Pergament I, Smoum R, Niddam V, Dembitsky VM, Temina M, Dor I, Lev O, Srebnik M, Enk CD. 2004. A new UV-B absorbing mycosporine with photo protective activity from the lichenized ascomycete *Collema cristatum*. *Eur J Biochem* **271**: 780–784.

Supporting Information

Supporting information can be found in the on-line version of this article.