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Multiple dual-mode centrifugal partition chromatography as an efficient method for the purification of a mycosporine from a crude methanolic extract of *Lichina pygmaea*

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ABSTRACT

Centrifugal partition chromatography method was applied to the separation and purification of a crude methanolic extract of a cyanobacterial lichen, *Lichina pygmaea*. A multiple dual-mode was used to separate two compounds of interest, namely mycosporine-serinol and a glutamic acid derivative. These compounds are described here for the first time in a lichen. Their structures were identified by UV, IR, ESI-MS, ^{1}H NMR, ^{13}C NMR, and 2D NMR.

1. Introduction

As many lichens resist to extreme conditions of UV-radiation and temperature, they are supposed to contain UV screening compounds to protect themselves against damages caused by solar radiation. *Lichina pygmaea* (Lightf.) C. Agardh, a black marine lichen, consists in a symbiosis between a fungus (*Ascomycota* phylum) and a cyanobacterium (*Calothrix* genus). It is known to contain primary metabolites, polyols and sugars as mannitol, mannosido-mannitol, glycerol, fructose, glucose, saccharose, galactose, glycogen, arabinose and possibly volemitol and xylose, but also polyosides and a galactomannan polysaccharide [1–4]. Besides lipophilic pigments given as xanthophyll carotenoids, phycocyanin, phycoerythrin and chlorophyll A [5], hydrophilic compound like mycosporine-glycine has recently been described in this lichen as an antioxidant [6]. Focusing on mycosporine compounds, we aimed at overcoming the usual lack of selectivity, adsorption and tailing problems often occurring with the purification processes of such compounds.

Discovered in the 60s, mycosporines and MAAs (Mycosporine-like Amino Acids) are water soluble and low molecular weight molecules, that absorb ultraviolet radiation between 310 and 365 nm with a molar extinction coefficient between 28 000 and 50 000 $M^{-1} \cdot cm^{-1}$ [7,8]. They are accumulated by a wide range of prokaryotic and eukaryotic microorganisms, marine algae, corals and other marine life forms, as secondary metabolites. Their function as solar radiation filters to prevent from damages caused by ultraviolet radiations is now well established, as their concentration is closely correlated to UV radiation fluctuations [9–11]. However, their implication in different mechanisms such as oxidative stress, salt stress, dessication, thermal stress, fungal reproduction or intracellular nitrogen reservoir has also been suggested [12].

Extraction of these compounds is usually operated with solvents as methanol and water in various proportions and their purification is often costly and time-consuming. Therefore, as commercially available standards are lacking, most of the characterization of mycosporines and MAAs has been based on the comparison of retention times and absorption spectra to data given in the literature [13]. When isolated, purification of these structures proceeds with different techniques, the most employed ones involving purification on Dowex 50W (elution with water and dilute HCl) or preparative reversed-phase high-performance liquid chromatography (HPLC) with UV detection [7]. These techniques are often combined in order to obtain the compound with sufficient purity [14–16].

So we applied a liquid–liquid chromatography technique, namely centrifugal partition chromatography (CPC), for the separation and the purification of mycosporines. CPC is a development

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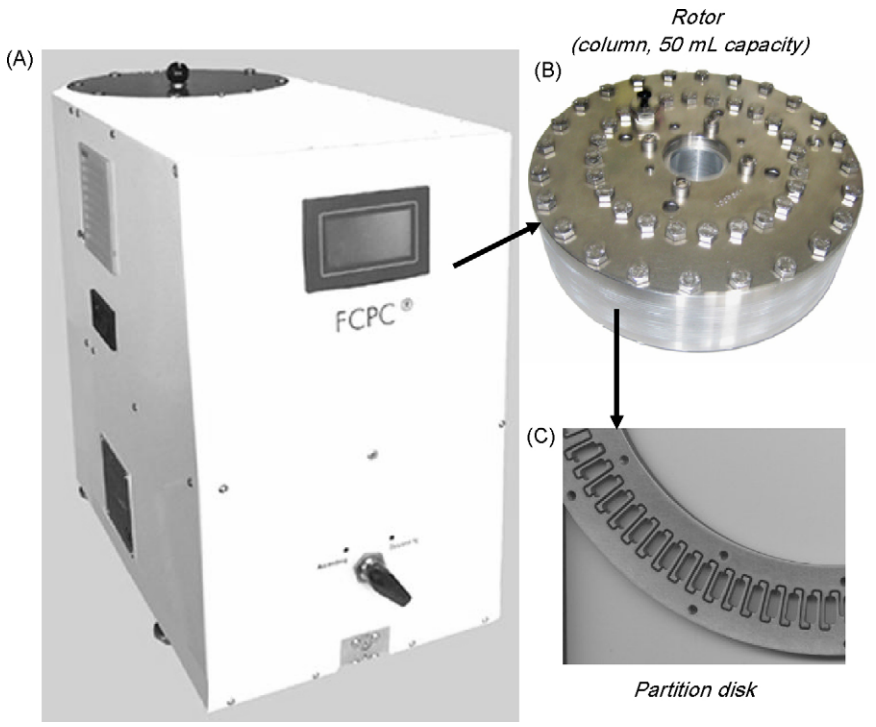

Fig. 1. Kromaton Technologies FCPC 50 mL apparatus (A), the rotor (B) and part of a partition disk (C). Note the connecting ducts centered on the bottom and the top of each cell. The upper and lower cell walls consist of the interdisk Teflon gaskets.

of countercurrent chromatography (CCC), invented in the late 60s by Ito et al., based on the partition between two immiscible phases of a solvent system [17,18]. CPC represents one of the few technical solutions to the challenge of maintaining a phase stationary while another is pumped through it [19]. It involves partition cells radially engraved in a disk connected to each other by capillary ducts (see Fig. 1). The constant centrifugal force field resulting from the disk rotation causes decantation in each cell, retaining one of the phases against the flow of the other, thus allowing a continuous process. CPC can be used either in a normal mode with a polar stationary phase, but also in an inverse mode with an apolar stationary phase. This technique also allows fractionation to be carried out in a normal-phase mode followed by a reversed-phase mode (or vice-versa), by a switching valve between descending and ascending modes, called the "dual" mode. CPC allows the use of a wide range of biphasic systems, and provides important benefits for natural compound purification, such as no sample loss on solid support and high recovery. In this paper, the selectivity problem encountered with the selected biphasic system was avoided by an efficient use of the multiple dual-mode (MDM) strategy as described by Delannay et al. [20]. It required iterative dual-mode steps, during the same run, with only one sample injection, to isolate a mycosporine and another compound from a crude methanolic extract of *L. pygmaea*. This work was partially presented as a poster communication [21] and detailed herein.

2. Experimental

2.1. Reagents

Solvents such as acetic acid (AcOH), acetonitrile (ACN), chloroform ($CHCl_3$), ethyl acetate (AcOEt) and methanol (MeOH) purchased from Carlo Erba Reactifs (Val de Reuil, France), and *n*-butanol (*n*-BuOH), dichloromethane (CH_2Cl_2) and diethylether (Et_2O) purchased from VWR (Fontenay sous bois, France) used for CPC and TLC analyses were of analytical grade.

Trifluoroacetic acid (TFA) was purchased from Sigma–Aldrich (Lyon, France) and triethylamine (TEA) from Acros Organics (Halluin, France). Acetonitrile used for HPLC analyses was of HPLC grade and purchased from Carlo Erba Reactifs (Val de Reuil, France). Water was of ultrapure quality.

2.2. Preparation of crude extract

Lichen material was collected on the west coast of France, on the rocky shore of Dinard (48°38.09′N, 02°08.15′W), in December 2007. It was sorted out, washed and dried under ambient atmosphere. After macroscopic and microscopic observations, comparing to a reference sample of the "Des Abbayes" herbarium, it was identified as *L. pygmaea* (Lightf.) C. Agardh and a voucher specimen is kept in the laboratory with the reference JB/07/98.

Extraction by three successive solvents was performed on 270 g of the dried lichen, namely ethyl acetate then methanol and methanol 50% in water. Separation and analyses were conducted on the methanolic extract of *L. pygmaea* (meLp), as we characterized the presence of the mycosporine in it by HPLC, using a diode array detector and by comparison with a standard kindly provided by Pr Karsten (University of Rostock, Germany).

2.3. FCPC apparatus

The separations were performed on a FCPC® C 50 Kromaton Technologies apparatus (Angers, France) using a rotor made of 800 cells for a 57 mL total volume (measured volume). This apparatus was able to rotate from 800 to 2000 rpm, producing a centrifugal acceleration in the partition cell of $84 \times g$ at 1000 rpm and $335 \times g$ at 2000 rpm. It was able to support up to 75 bars (1000 psi). The solvents were pumped by a HPLC pump 422 from Kontron instruments (Montigny le Bretonneux, France). The samples were introduced into the CPC column via a 6-port medium pressure injection valve (Upchurch Scientific, CIL Cluzeau, Ste Foy la Grande, France) equipped with a 5 mL sample loop. Fractions of 2 mL were

collected by a mini-collector MC30 (Köhler Technische Produkte, Neulussheim, Germany). Experiments were conducted at room temperature.

2.4. Solvent system screening

Different solvent systems were prepared and evaluated using thin layer chromatography (TLC). For each, 500 μL of both phases of the solvent system were mixed, and a small amount of dried meLp sample was dissolved in this two phases mixture. Then, 10 μL of each phase was applicated on a TLC plate by an Automatic TLC Sampler 3 (ATS 3, CAMAG, Muttenz, Switzerland), which provided the application of an exact volume with an exact width by band-wise spray-on. The plate was revealed in order to visualize polar compounds (the TLC system described below). Then, the solvent mixture was chosen according to the most equilibrated repartition of each compound of the meLp sample between each phase of the solvent system.

2.5. CPC experimental conditions

The separation was performed with the system composed of *n*-butanol, acetic acid and water (4:1:5, v/v/v), in the isocratic mode. The rotor was first filled with the lower phase of the solvent system, as the stationary phase (see Fig. 2). The apparatus was rotated at 1800 rpm and the upper mobile phase of the solvent mixture was then pumped into the inlet of the column (rotor) at a flow rate of 4 mL/min in the ascending mode. 500 mg of the meLp sample was

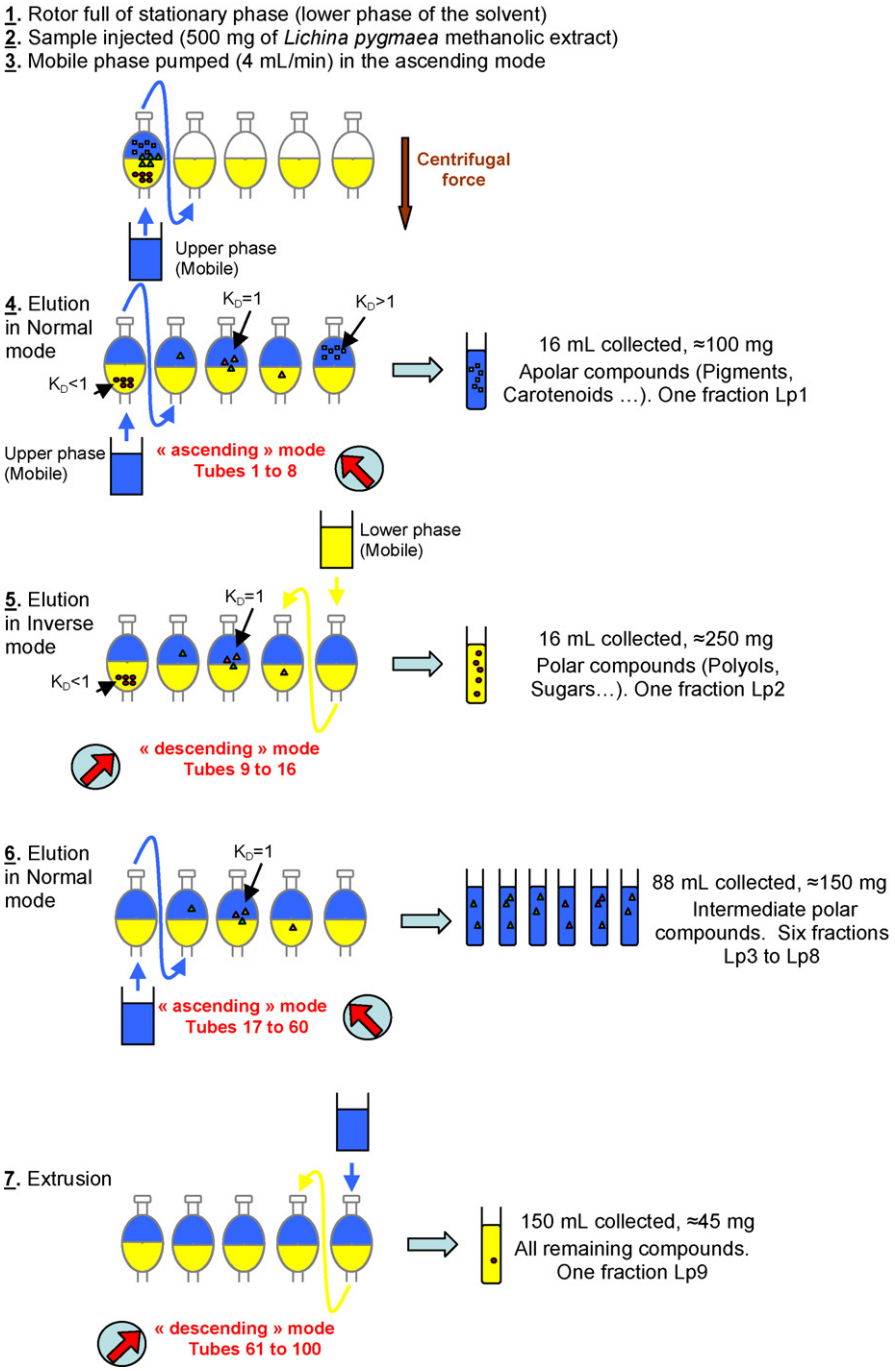

Fig. 2. Scheme representing the operating conditions for the *Lichina pygmaea* methanolic extract purification using the "multiple-dual-mode" CPC.

diluted in a mixture of 2 mL of the upper phase and 2 mL of the lower phase. It was loaded in the 5 mL injection-loop, and injected in the column in a "sandwich" mode, i.e. at the same time than the mobile phase. The back pressure was 25 bars. The stationary phase retention at the end of the separation represented 52% of the column volume (57 mL). The content of the outgoing organic phase was offline monitored by TLC analysis.

Elution first occurred in the ascending mode (normal-phase mode): the rotor was filled with the lower polar phase of the solvent mixture, and the pumped mobile phase is the apolar upper phase. After collecting eight tubes each containing 2 mL, the switching valve is turned to the descending mode and the mobile phase pumped is the lower one this time. Then, after collecting again eight tubes of 2 mL, we switched again to the ascending mode (upper mobile phase). The separation proceeded that way during the next 44 tubes of 2 mL. Extrusion was performed after the 60th tube: the upper phase was pumped in the descending mode to eject the totality of the lower phase out of the rotor.

2.6. HPLC analyses

All the fractions obtained with the CPC separation were analysed by HPLC. They were performed on a C18 column (Equisorb, ODS2, 5 μm, 250 mm × 4.6 mm, CIL Cluzeau, Ste Foy la Grande). Each fraction was diluted in acetonitrile/water (10:90) at concentration of 1 mg/mL and after passing through a 0.2-μm membrane filter, 20 μL was injected into the column, using a gradient elution with a flow rate of 1 mL/min. An initial isocratic hold until 10 min with 90% of 0.1% acetic acid in water (solvent A) and 10% of acetonitrile (solvent B) was followed by a gradient ranging from 10% to 100% of acetonitrile between 10 and 90 min. The mobile phase composed of 100% acetonitrile is maintained for 10 min, in order to wash the column. Peak detection was carried out online using a diode array detector (HPLC 540 DAD, Kontron instruments, Montigny le Bretonneux, France) at 310 and 254 nm, and absorption spectra (210–400 nm) were recorded each second directly on the HPLC-separated peaks.

2.7. TLC analyses

The compounds of each fraction were separated on a TLC plate (Merck, Darmstadt, Germany). The developing solvent used was $CHCl_3$/MeOH/H_2O (6:4:1, v/v/v). Detection of compounds was performed under UV lamps at 365, 312 and 254 nm, but also by spraying them with sulfuric *p*-anisaldehyde (and heating) or 2,4-dinitrophenylhydrazine.

2.8. Isolation and identification

After CPC separation, we obtained nine fractions (Lp1–Lp9) some of which were already enriched in some compounds of interest. Subsequent purification and isolation were performed to identify the compound structures.

2.8.1. Isolation from Lp6 fraction of compound **1**

After a first wash of the Lp6 fraction (1040 mg) in a separating funnel by dichloromethane (from water), the aqueous layer enriched in compound **1** was purified on a C-18 column (C-18 Hydro Chromabond, Macherey-Nagel, Düren, Germany) using water/acetonitrile (90:10) as the mobile phase. 121 mg of pure compound **1** was finally made to precipitate in acetonitrile.

2.8.2. Isolation from Lp8 fraction of compound **2**

Compound **2** was purified on a C-18 column (C-18 Hydro Chromabond, Macherey-Nagel, Düren, Germany) using water/acetonitrile (gradient ranging from 0% acetonitrile to 100%) as the mobile phase from 568 mg of Lp8 fraction. 25 mg of compound **2** was finally isolated by semi-preparative HPLC on a Hypersil® BDS C-18 column (250 mm × 10 mm, 5 μ, Thermo electron corporation, Runcorn, UK) using acetic acid 0.1% as the mobile phase.

2.8.3. Identification of compounds **1** and **2**

Compound **1** was identified by MP, IR, ^{1}H NMR, ^{13}C NMR, 2D NMR, MS, and $[\alpha]_D^{20}$. Compound **2** was identified by ^{1}H NMR, ^{13}C NMR, MS and UV spectra.

MP was measured on a Kofler hot bench. FTIR spectra were run on a PerkinElmer 16 PC spectrometer. ^{1}H NMR, ^{13}C NMR data, at 500 and 125 MHz, respectively, COSY and CH experiments were recorded on a Bruker DMX 500 WB NMR spectrometer, using D_2O and DMSO-d_6 for compound **1**. ^{1}H NMR and ^{13}C NMR data were recorded at 270 and 67.5 MHz, respectively, on a JEOL GSX 270 WB, using D_2O and pyridine-d_5 for compound **2**. Electrospray ionization (ESI) high-resolution mass spectrometric (HRMS) measurements for exact mass determination were performed on a Micromass ZAB-SpecTOF mass spectrometer at the Centre Régional de Mesures Physiques de l'Ouest. UV spectra were performed on a UVIKON 931 spectrophotometer. $[\alpha]_D^{20}$ was recorded on a PerkinElmer Polarimeter model 341.

NMR data of compound **1** are reported in Table 1. Chemical shifts in D_2O for this compound are reported here for the first time, and values in DMSO-d_6 are consistent with the reference describing this compound [22]. Melting point has been measured at 160–162 °C

Table 1
NMR data for compound **1** (^{1}H 500 MHz, ^{13}C 125 MHz).

Position	^{1}H (D_2O) *J* (Hz)	^{1}H (DMSO-d_6) *J* (Hz)	^{13}C (D_2O/DSS)	^{13}C (DMSO-d_6/TMS)
3	2.08 (2H, m)	1.90 (2H, m)	28.7	27.0
4	2.41 (2H, m)	2.33 (2H, m)	32.1	29.9
1″	3.68 (3H, s)	3.68 (3H, s)	58.4	55.3
2	3.70 (1H, m)	3.23 (1H, t, *J* = 6.4)	56.6	53.5
2″	3.76 (3H, s)	3.79 (3H, s)	58.5	55.5
5′	6.43 (1H, dd, *J* = 8.7, 2.5)	6.39 (1H, dd, *J* = 8.6, 2.6)	107.6	104.1
3′	6.55 (1H, d, *J* = 2.5)	6.53 (1H, d, *J* = 2.6)	102.1	99.0
6′	6.71 (1H, d, *J* = 8.7)	6.62 (1H, d, *J* = 8.6)	116.8	112.2
1			176.3	169.6
5			176.9	171.2
1′			132.8	132.0
2′			157.0	153.3
4′			151.2	147.3
1′-NH		6.59 (1H, s)		
1-OH 2-NH_2 5-NH		9.99 (br s), 3.78, 3.69		

Table 2
Stationary phase retention on FCPC 50 apparatus.

	Solvent = BuOH/AcOH/H_2O (4:1:5)					
Rotor speed (rpm)	1800	2000	1800	2000	1800	2000
Flow rate (mL/min)	6	6	4	4	2	2
Ascending mode ($\%_R$)	49	51	52	49	44	51
Descending mode ($\%_R$)	46	44	47	42	51	46

and other physicochemical properties are in accordance with the Ref. [22].

NMR data of compound **2** were compared to NMR values described for the expected mycosporine-glycine [16,23]. However in D_2O, the characteristic singlet corresponding to the two protons H-9 [16,23] was not observed, but a five-proton signal between 3.6 and 3.8 ppm. UV spectrum gave a maximal absorption wavelength at 309 nm, like mycosporine-glycine, but ESI-MS spectrum gave a positive ion $[M+H]^+$ at *m/z* 262. High-resolution mass spectrum indicated the molecular formula $C_{11}H_{19}NO_6$, and pyridine-d_5 NMR confirmed that compound **2** was mycosporine-serinol, as described by Favre-Bonvin et al. [24].

3. Results and discussion

3.1. Solvent system selection

TLC plates were informative for the repartition of water-soluble compounds between the two phases of each solvent system. The more is the number of compounds in common between the upper and the lower phase, the better is the partition. We essentially focused on two plots, one corresponding to a compound absorbing at 254 nm, and giving a positive reaction with sulfuric *p*-anisaldehyde, and the other one absorbing at 312 nm (like the mycosporine previously described in *L. pygmaea*). Thus, BuOH/AcOH/H_2O (4:1:5), was selected as the solvent system for CPC separation. It can be noticed that this system is acidic with pH 3–4 and pH 4–5 for lower and upper layers, respectively.

3.2. Choice of operating conditions

3.2.1. Determination of suitable rotor speed and flow rate

Different procedures with the CPC apparatus were experimented in order to determine the conditions that provided the best retention percentage. In a first time, working in an ascending mode, the upper-layer was chosen as the mobile phase, and two rotor speeds (1800 and 2000 rpm) along with three different flow rates (2, 4 and 6 mL/min) were tested. In a second time, the same procedure was conducted in the descending mode, with the lower-layer as the mobile phase. Retention percentage was calculated with the following formula:

$$\%_R = \frac{Vr - Ve}{Vr} \times 100$$

$\%_R$: retention percentage; Vr: rotor volume and Ve: ejected stationary phase volume.

The results are reported in Table 2. The best retention value was obtained in the ascending mode with a rotor speed of 1800 rpm and a flow rate of 4 mL/min. It is worth to notice that the same conditions in the descending mode do not provide the best retention value but a suitable one for a multiple-dual-mode. A slight bleeding phenomenon, namely a little loss of the stationary phase, could occur but it was found very weak.

Table 3
HPLC analyses of the *Lichina pygmaea* (Lp) fractions obtained from FCPC treatment.

Fraction	Rt for **1** (254 nm)	Rt for **2** (310 nm)	Integration (mAbs min)
Lp1			
Lp2		2.85 min	169.05
Lp3			
Lp4			
Lp5			
Lp6	14.55 min		25.34
Lp7		2.65 min	16.89
Lp8		2.87 min	352.76
Lp9		2.74 min	108.37

Rt: retention time.

3.2.2. Retention time (Rt) determination

The CPC eluted solvents were collected each 30 s (2 mL/tube) and checked with TLC. Retention times (±30 s) of each compound could then be estimated. A first CPC experiment in the "ascending" mode failed, as the two polar compounds we wanted to isolate were co-eluted between 4 and 12 min (see Fig. 3). However, compound **1** was observed to be eluted during 8 min while compound **2** was eluted during 26 min. In a second experiment, the ascending elution mode was turned to descending mode just before the anticipated 4 min elution time. By this way, compound **2** appeared soon after the switch at Rt 5.5 min and compound **1** at Rt 8 min. So a first isolation of compound **2** was achieved with only one dual-mode.

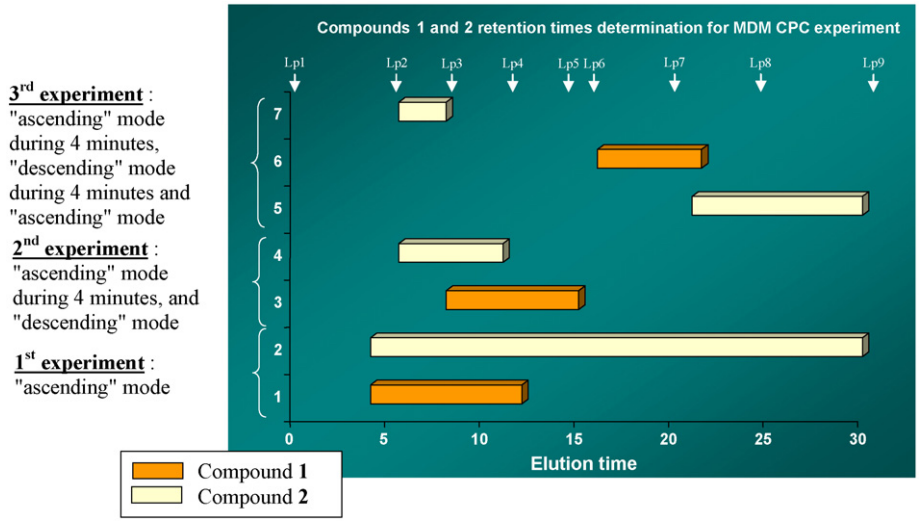

Fig. 3. Compounds **1** and **2** retention times according to FCPC experimental conditions.

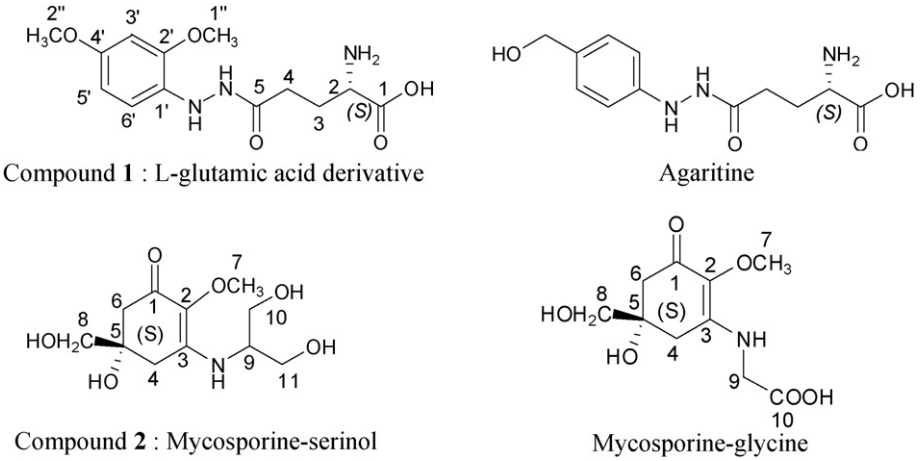

Fig. 4. Chemical structures of isolated compounds from *Lichina pygmaea*, agaritine (isolated from *Agaricus bisporus*) [27] and mycosporine-glycine.

3.2.3. Multiple-dual-mode experiment

As compound **1** elution was delayed to Rt 8 min, a second switch to the ascending mode at T_0 + 8 min allowed to obtain pure compound **1** during 5.5 min (Rt 16 min) of elution followed by pure compound **2** during 9 min, with a very little overlap (30 sec maximum) of the two compounds. By this multiple-dual-mode, where three elution modes were successively used in the same experiment (see Fig. 3), compound **1**, in less quantity, was bound to stay in the column while compound **2** was eluted. Therefore, compounds **1** and **2** were separated in two enriched fractions from a crude methanolic extract in one row. This experiment was repeated 24 times on 500 mg of the meLp each time with a good repeatability, to obtain 1.04 g of compound **1** enriched fraction (Lp6) and 0.68 g of compound **2** enriched fraction (Lp8).

3.3. Fraction analyses

From a first crude methanolic extract containing more than 20 compounds, nine fractions were obtained through CPC. Lp1 composed of the first nine tubes essentially contained apolar compounds. Through TLC behaviour and literature [5], these compounds appear to be terpenoids, carotenoids and chlorophyll pigments, to be further investigated. In fraction Lp2, composed of tubes 10–17, we have to manage with very polar compounds, such as polyols like mannitol or volemitol or sugars like fructose, glucose, saccharose and arabinose, already described in *L. pygmaea* [1–3]. Fractions Lp3 to Lp8 contain intermediate polar compounds, they are enriched in compounds having a good repartition between the two phases of the solvent system. Lp9 corresponds to the extrusion of the phases out of the column and is composed of all the remaining compounds.

According to TLC and HPLC analyses (see Table 3), fraction Lp6 contains compound **1** and Lp2, Lp7, Lp8 and Lp9 contain compound **2**, Lp8 being the purest and most concentrated fraction. It can be noted that compound **1** is isolated on its cationic form, as the solvent system is acidic and the pH of the molecule about 6. After final purification from these two fractions (Lp6 and Lp8) we obtained compounds **1** and **2** in a good purity and identified them as a L-glutamic acid derivative and mycosporine-serinol, respectively (see Fig. 4 and Section 2.8.3). The L-glutamic acid derivative **1**, has already been described once in cultures of *Penicillium* as a myeloperoxydase inhibitor [22] and is described here for the first time in a lichen. The mycosporine identified here do not correspond to the one described in *L. pygmaea* by De la Coba Luque et al. [6]. Mycosporine-glycine can be easily confused with mycosporine-serinol through HPLC analyses, as they have the same UV spectrum profile. Therefore, the presence of mycosporine-serinol and the absence of mycosporine-glycine in the crude extract of our *L. pygmaea* sample were confirmed by LC–MS (data not shown).

4. Conclusion

It is the first time CPC is used for the study of secondary metabolites of *L. pygmaea* and for the purification of mycosporines. It revealed to be useful for the direct treatment of a complex methanolic extract. Two compounds including a mycosporine were then readily purified through HPLC. The structural analysis showed compound **1** to correspond to a glutamic acid derivative and compound **2** to mycosporine-serinol, differing from the mycosporine-glycine previously reported in this lichen [6]. These two compounds are described here for the first time in a lichen and they appear to be produced by the fungus while previously isolated from *Ascomycota* and *Basidiomycota* [22,24].

As mycosporines and MAAs have been found in different organisms from prokaryotic to eukaryotic systems [25] including different fungi and also cyanobacteria (putative MAAs in *Calothrix* species [26]), the biogenetic pathway to mycosporine-serinol in *L. pygmaea* remains unclear. By the same way, γ-glutamyl-phenyl hydrazines like compound **2** have already been described in fungi. Agaritine, isolated from the *Agaricus bisporus* (Basidiomycota) has a similar structure, just differing through the nature of the aromatic ring substituents [27]. As the glutamyl residue has a central position in fungal nitrogen metabolism, it can be supposed that this compound is synthesized by the fungus. The cyanobiont provides nitrogen to the mycobiont by transformation of atmospheric nitrogen fixed in heterocysts as ammonia via a nitrogenase. As glutamine synthetase is highly depressed in the cyanobiont compared to free-living cyanobacterium *Calothrix* in *Lichina confinis* (very similar species to *L. pygmaea*) [28], it can be supposed that the glutamine metabolism occurs in the mycobiont.

This lichen symbiosis seems to allow a high nitrogen metabolism as nitrogen content in *L. pygmaea* has been evaluated to correspond to 6.49% of dry weight [29]. Such secondary metabolites are not frequently encountered in most of the lichen species, especially when the photobiont is a green algae (90% of lichens).

These compounds will now be evaluated for biological activities as antioxidant and cytotoxic.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2009.05.040.

References

[1] G. Pueyo, Rev. Bryol. Lichénol. 32 (1963) 279.
[2] G. Pueyo, Bryologist 66 (1963) 74.
[3] G. De Lestang Laisné, Rev. Bryol. Lichénol. 34 (1966) 346.
[4] A. Prieto, J.A. Leal, M. Bernabé, D.L. Hawksworth, Mycol. Res. 112 (2008) 381.
[5] M. Quillet, Bull. Lab. Maritime Dinard. 47 (1962) 68.
[6] F. De La Coba Luque, J. Aguilera Arjona, F. Lopez Figueroa. International Patent WO2007026036. 08.03.2007 (Universidad De Malaga, Spain).
[7] W.M. Bandaranayake, Nat. Prod. Rep. 15 (1998) 159.
[8] W.C. Dunlap, J.M. Shick, J. Phycol. 34 (1998) 418.
[9] F. Garcia-Pichel, C.E. Wingard, R.W. Castenholz, Appl. Environ. Microbiol. 59 (1993) 170.
[10] A. Torres, M. Hochberg, I. Pergament, R. Smoum, V. Niddam, V.M. Dembitsky, M. Temina, I. Dor, O. Lev, M. Srebnik, C.D. Enk, Eur. J. Biochem. 271 (2004) 780.
[11] F. Garcia-Pichel, R.W. Castenholz, Appl. Environ. Microbiol. 59 (1993) 163.
[12] A. Oren, N. Gunde-Cimerman, FEMS Microbiol. Lett. 269 (2007) 1.
[13] T. Rezanka, M. Temina, A.G. Tolstikov, V.M. Dembitsky, Folia Microbiol. (Prague, Czech Repub.) 49 (2004) 339.
[14] H.J. Suh, H.W. Lee, J. Jung, Photochem. Photobiol. 78 (2003) 109.
[15] H. Young, V.J. Patterson, Phytochemistry 21 (1982) 1075.
[16] W.M. Bandaranayake, J.E. Bemis, D.J. Bourne, Comp. Biochem. Physiol. C 115 (1996) 281.
[17] Y. Ito, R.L. Bowman, Science 167 (1970) 281.
[18] A. Berthod (Ed.), Countercurrent Chromatography—The Support-Free Liquid Stationary Phase (Wilson & Wilson's Comprehensive Analytical Chemistry), vol. 38, Elsevier, Amsterdam, 2002.
[19] A.P. Foucault, Centrifugal Partition Chromatography, in: Chromatographic Science Series, vol. 68, Marcel Dekker, New York, 1995.
[20] E. Delannay, A. Toribio, L. Boudesocque, J.M. Nuzillard, M. Zeches-Hanrot, E. Dardennes, G. Le Dour, J. Sapi, J.H. Renault, J. Chromatogr. A 1127 (2006) 45.
[21] C. Roullier, M. Chollet-Krugler, A. Bernard, J. Boustie, Planta Med. 74 (2008) PC81.
[22] T. Tanimoto, I. Tanaka, M. Nakajima. International Patent JP2001131133. 05.15.2001 (Sankyo Co. Ltd., Japan).
[23] S. Ito, Y. Hirata, Tetrahedron Lett. 28 (1977) 2429.
[24] J. Favre-Bonvin, N. Arpin, C. Brevard, Can. J. Chem. 54 (1976) 1105.
[25] R.P. Sinha, S.P. Singh, D.P. Hader, J. Photochem. Photobiol. B 89 (2007) 29.
[26] S. Brenowitz, R.W. Castenholz, FEMS Microbiol. Ecol. 24 (1997) 343.
[27] B. Levenberg, J. Biol. Chem. 239 (1964) 2267.
[28] S. Janson, A.N. Rai, B. Bergman, New Phytol. 124 (1993) 149.
[29] K.A. Kershaw, Physiological Ecology of Lichens, Cambridge University Press, Cambridge, 1985.