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1 **Efficiency and selectivity of ionic liquids in microwave-assisted extraction of major**
2 **lichen phenolic compounds: a scalable process with recycling of ionic liquids.**

3
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13
14 **Abstract**

15 Introduction: *Pseudevernia furfuracea*, a lichen used classically for cosmetic applications,
16 contains interesting metabolites possessing antimicrobial and anti-inflammatory or
17 antioxidant properties.

18 Objectives: Ionic liquids combined to microwave-assisted extraction (IL-MAE) was
19 successfully applied for metabolites extraction from *P. furfuracea*.

20 Materials and methods: Three imidazolium and pyridinium-based ionic liquids: 1,3-
21 dimethylimidazolium methylsulphate [C₁C₁Im][MeSO₄], 1-ethyl-3-methylimidazolium
22 ethylsulphate [C₂C₁Im][EtSO₄], and *N*-ethylpyridinium ethylsulphate [C₂Py][EtSO₄] were
23 assessed for this process. The efficiency of the extraction method was evaluated using thin
24 layer chromatography (TLC) coupled to a Camag® spectrophotodensitometer and using
25 HPLC analysis.

1 Results: ILs under MAE showed extraction time-economy (15 min versus 24 h for
2 conventional heating) and high selectivity in extracting the targeted metabolites: atranorin
3 (AT), methyl- β -orcinol carboxylate (MOC), fumarprotocetraric acid (Fum. Ac.), and
4 physodic acid (Phys. Ac.) despite the increased degradation of AT under MAE. We showed a
5 tunable selectivity of ionic liquids (IL) towards extracting metabolites by changing anion or
6 cation due to the modification of the interaction between the IL and the metabolites. While
7 [C₂Py][EtSO₄] was the most efficient IL and could extract all the targeted metabolites,
8 [C₂C₁Im][EtSO₄] was the most selective. It fully extracted AT and partially Fum. Ac..
9 Moreover, the lichen prepared by mixing procedure provided AT and Fum. Ac. more than
10 milled one. A 100 times scale-up extraction was successfully performed on mixed samples
11 with full IL recycling after back extraction.

12 Conclusion: IL-MAE is reliable for lichen metabolites extraction. The method is
13 reproducible, scalable, with possible IL recycling, opening the door for potential industrial
14 applications.

15
16 **Keywords:** *Pseudevernia furfuracea*; Lichens; Ionic liquid; Phenolic compounds,
17 Microwave-assisted extraction (MAE); Pilot-scale, recycling of ionic liquids.

18 19 1. INTRODUCTION

20
21 Lichens are a large group of symbiotic organisms with around 17,000 reported species.¹
22 These organisms, which are able to live in extreme conditions, produce different types of
23 phenolic specialized metabolites including monophenolic compounds, depsides, depsidones,
24 and diphenyl ethers for the most lichen-specific metabolites.² Lichens are employed in
25 various fields of application including perfumery, cosmetics, and for medicinal purposes^{3,4}
26 owing to the interesting biological activities of their metabolites including antitumor, anti-

1 inflammatory, antiviral, antibacterial or antioxidant (for review see the literature^{5,6,7,8}).

2 Extracting these metabolites in their pure form is of great interest. A potential candidate class

3 of solvents to fulfill this task is the ionic liquids (ILs), attracting a growing interest in the

4 extraction process for the isolation and/or purification of various bioactive compounds from

5 biomass matrices⁹ or plants^{10,11}. ILs are solvents of interest for extraction processes due to

6 their unique properties such as low vapour pressure, non-flammability, solvents miscibility,

7 and good extractability of various compounds.^{9,11,12} Some disadvantages have been

8 nevertheless revealed such as the potential environmental impact of some toxic ILs and the

9 relatively high cost of large scale industrial applications. It is thus necessary to consider low

10 or non-toxic ionic liquids and ensure their recovery at the end of the extraction process¹² to

11 reduce the economical and ecological costs. Thus, ionic liquids combined to microwave-

12 assisted extraction (IL-MAE) was applied herein to *Pseudevernia furfuracea* (L.) Zopf, a

13 lichen of high interest due to its richness in active phenolic compounds and its application in

14 the perfume industry (1900 tons/year).^{13,14} Various extracts derived from this lichen already

15 exhibited significant antimicrobial, anti-inflammatory, anticancer and antioxidant

16 activities.^{15,16,17} The major isolated metabolites from *P. furfuracea* are two depsides (atranorin

17 (AT) and chloratranorin) and some depsidones (physodic acid (Phys. Ac.) or olivetoric acid

18 according to the chemotype). These latter metabolites (AT or Phys. Ac.) are known for their

19 antimicrobial^{18,19} and anti-inflammatory²⁰ or antioxidant²¹ properties. We have already studied

20 the influence of sample preparation method (mixing or milling of lichens) on the efficiency of

21 MAE using acetone to extract AT from *P. furfuracea* var. *furfuracea*.¹⁴ We have also

22 investigated the influence of the anion chain length on the extraction of norstictic acid from

23 *Pertusaria Pseudocorallina* using ionic liquid-based on imidazolium cation combined with

24 microwave irradiations.²² An additional study was recently performed on *Stereocaulon*

25 *glareosum* confirming the ability of imidazolium cations to interact with lichen metabolites.²³

1 In this work, we explore various parameters influencing the IL-MAE of four metabolites
2 from *P. furfuracea* var. *furfuracea* (Fig. 1) including the method used to prepare the samples
3 and the structure of ILs. Three different ILs bearing inorganic anions and pyridinium- or
4 imidazolium-derived aromatic cations were assessed (Table 1). We explore the influence of
5 these structural modifications of the ionic liquids on the extraction efficiency of the major
6 secondary metabolites from *P. furfuracea*. Additionally, we report the successful 100 times
7 scale up of the process with complete recycling of the ionic liquid.

8 < Fig. 1 >

9 < Table 1 >

10

11 **2. EXPERIMENTAL**

12 **2.1 Materials**

13 All the commercial products were used as received without further purification. *n*-
14 hexane, acetone, and tetrahydrofuran (THF) were purchased from Carlo Erba (Milano, Italy);
15 toluene, diethyl ether, and formic acid from Prolabo (Paris, France). 1-Methylimidazole,
16 pyridine, diethylsulfate, dimethylsulfate, chlorobutane, and
17 bis((trifluoromethyl)sulfonyl)imide lithium salt from Fluka (Illkirch, France). Lichen
18 metabolites standards were provided from the library of the CO₂Int team (University of
19 Rennes 1).

20 *Pseudevernia furfuracea*, known as Oak moss, was collected from cedar in Morocco
21 (Voucher JB/02/10) specimen. The lichen was identified as *P. furfuracea* var. *furfuracea*²
22 according to macroscopic, microscopic, and chemical thalline reactions using potassium
23 hydroxide (K⁺), sodium hypochlorite (C⁻), and with comparison to the literature. After
24 cleaning and identification by chemical tests, the lichen was air-dried and stored in a closed
25 glass at room temperature.

1

2 **2.2 Apparatus**

3 A CEM Explorer® 24 samples with a programmable heating power (from 0 to 300
4 W) and irradiation time was used for MAE experiments. Small scale experiments were
5 carried out in 10 mL closed glass vessels. For the scale-up process, a single-mode
6 Synthrowave® 1000 Prolabo system was used with a 800 mL quartz vessel at an operating
7 frequency of 2450 MHz, with a maximal output power of 300 W and associated to a stirring
8 blade.

9 HPLC system equipped with a Kontron 325 pump and a diode array detector (DAD), an
10 analytical C₁₈ Spherisorb column (250 x 4.6 mm, 5 µm) and with a 20 µL external loop for
11 injection was used.

12 TLC plates (Merk silica gel 60F254) were prepared with Camag® automatic sampler
13 III (ATS3) and the metabolites used were quantified with Camag® TLC scanner II.

14

15 **2.3 Synthesis of ILs**

16 The structures of ILs are reported in Table 1. [C₁C₁Im][MeSO₄] [C₂C₁Im][EtSO₄], and
17 [C₂Py][EtSO₄] were synthesized following a previously reported procedure.^{24,25} The ILs were
18 dried at 70°C under vacuum during 12 hours and their structure was verified by ¹H NMR (see
19 SI, Fig.S1).

20 **1,3-Dimethylimidazolium methylsulphate** [C₁C₁Im][MeSO₄]

21 ¹H NMR (300 MHz, D₂O) δ: 8.50 (s, 1H, NCHN), 7.28 (d, 2H, ³J = 3 Hz, NCHCHN), 3.75
22 (s, 6H, NCH₃), 3.61 (s, 3H, OCH₃).

23 **1-Ethyl-3-methylimidazolium ethylsulphate** [C₂C₁Im][EtSO₄]

1 ^1H NMR (300 MHz, CDCl_3) δ : 9.23 (s, 1H, NCHN), 7.42 (d, 2H, $^3J = 3$ Hz, NCHCHN), 4.13
2 (q, 2H, $^3J = 7.35$ Hz, NCH_2), 3.93 (q, 2H, $^3J = 7.05$ Hz, OCH_2), 3.82 (s, 3H, NCH_3), 1.37 (t,
3 $^3J = 7.5$ Hz, OCH_2CH_3), 1.08 (t, 3H, $^3J = 7.0$ Hz, NCH_2CH_3).

4 ***N*-Ethylpyridinium ethylsulphate** [C_2Py][EtSO_4]

5 ^1H NMR (300 MHz, CDCl_3) δ : 9.15 (d, 2H, $^3J = 6$ Hz, CHN), 8.42 (t, 1H, $^3J = 7.6$ Hz,
6 NCHCHCH), 7.99 (t, 2H, $^3J = 7$ Hz, NCHCH), 4.71 (q, 2H, $^3J = 7$ Hz, NCH_2), 3.92 (q, 2H,
7 $^3J = 7$ Hz, OCH_2), 1.55 (t, 3H, $^3J = 7$ Hz, OCH_2CH_3), 1.10 (t, 3H, $^3J = 7$ Hz, NCH_2CH_3).

8

9 **2.4 Lichen sample preparation**

10 *P. furfuracea* was collected from Montpellier. After cleaning and identification according to
11 chemical thalline reactions, a voucher specimen (reference JB/02/10) was deposited in the
12 herbarium of CORInt TEAM, Rennes, France.

13 The grinding of the lichen samples was performed following a described method¹⁴ either with
14 blender to obtain mixed samples or with planetary milled system to obtain milled samples.

15

16 **2.5 MAE procedure**

17 IL-MAE process was performed using the CEM Explorer®, 24. 250 mg of milled or mixed
18 *Pseudevernia furfuracea* were added to 5 mL of IL or acetone and irradiated under
19 microwave for 15 min at 75°C and 20 W maximum. These quantities refer to the optimal
20 conditions, previously published by our group²². After irradiation, the samples were cooled
21 down to 50 °C. The obtained extracts were filtered through cotton and separated from the
22 entire lichen residue by 10 min centrifugation at 4500 rpm using a Jouan E96 centrifuge. The
23 acetone extracts were dried under *vacuum*.

1 A total extraction of metabolites was carried out in the same conditions using successively *n*-
2 hexane, diethyl ether, acetone and THF. This procedure was repeated three times.

3 **2.6 Conventional extraction method**

4 Briefly, 250 mg samples were mixed with 5 mL of IL and heated at 75°C for 24 h under
5 stirring (900 rpm) using a Carrousel Radley®. The samples were cooled down to 50°C. The
6 resulting extracts were filtered through cotton and separated from the entire lichen residue by
7 10 min centrifugation at 4500 rpm.

8 **2.7 Recovery of compounds, analysis and quantification**

9 **2.7.1 Recovery**

10 After the extraction process, the metabolites were isolated from the filtered extracts (2 mL)
11 by three times extraction using water (1 mL) and diethyl ether (1 mL). The organic fractions
12 were collected and the solvent was removed under *vacuum*. The final aqueous extracts were
13 analyzed by HPLC using the method detailed in 2.11 and the dried organic extract was
14 dissolved in acetone (1 mg/mL) and then analyzed by TLC coupled to a
15 spectrophotodensitometer following the procedure described in 2.7.2. All procedures were
16 repeated three times to determine the repeatability of the results.

17 **2.7.2 Quantification using a spectrophotodensitometer Camag®**

18 A standard solution of each metabolite was prepared in acetone (0.05-0.3 mg/mL). The
19 standard solution was used for the preparation of 5 point on calibration curve from 0.2 to 1.2
20 µg. The linear range was from 0.01 to 0.6 mg/mL. The correlation coefficient of the
21 calibration curve for all metabolites was 0.99. The crude extract was dissolved in acetone at 1
22 mg/mL and analyzed following a previously reported procedure.¹⁴ The standard solutions (4
23 µL) and the samples (4 µL) were spotted on TLC in the form of band with 4 mm of width and
24 positioned from the bottom of TLC plates (20 × 10 cm) at 15 mm by a Camag® microliter

1 syringe. TLC plates were eluted in two mobile phases (*n*-hexane/ethyl acetate/formic acid
2 100/30/5 or toluene/ethyl acetate/formic acid 70/25/5). Their retention factor (R_f) was in *n*-
3 hexane/ethyl acetate/formic acid 100/30/5 as follows: Fum. Ac. 0.24, Phys. Ac. 0.35, MOC:
4 0.52, AT: 0.65. The quantification was performed in absorbance/reflectance mode of a
5 Camag® TLC Scanner II under ultraviolet (UV) light at 238, 252, 263 or 267 nm for Fum.
6 Ac., AT, Phys. Ac. or MOC (see Fig. S2). The extraction efficiency of the compounds in
7 samples was defined by the determination of the extraction yields as follows:

$$8 \quad \text{yield (\%)} = \frac{\text{mass of the compound in extract}}{\text{mass of the lichen material}} \times 100$$

9 The ratio in the total yield of the compounds was defined as follows:

$$10 \quad \text{ratio in the total yield (\%)} = \frac{\text{mass of the compound in extract}}{\text{sum of mass of the compound in extract}} \times 100$$

11 The higher mass of compound in sample was determined in accordance to the best extraction
12 yield obtained using either IL, acetone or for total extraction.

13 **2.8 Physicochemical measurements**

14 Gas adsorption measurements were performed using a previously described process.¹⁴ The
15 contact angle between lichen sample and IL was measured using a Digidrop GBX Contact
16 Angle Meter with video-camera system and Windrop software. After deposition of a drop on
17 lichen sample using 4 μL droplet, two measurements were performed for each sample at
18 room temperature on the left and right sides of droplets.

19 **2.9 Scale-up of extraction**

20 In an 800 mL reactor, 500 mL of [C₂Py][EtSO₄] were added over 25 g of mixed *P.*
21 *furfuracea*, the mixture was irradiated using Synthewave® 1000 (75°C, 20 min). The extracts
22 were collected by filtration over cotton and high speed centrifugation (10000 rpm, 15 min)
23

1 using a Centrikon T-1170 centrifuge. The metabolites were isolated following 5 cycles of
2 liquid-liquid extraction (5 x H₂O/diethyl ether: 200 mL/200 mL, (v/v)) and evaporation of the
3 organic phase under vacuum for further analyses

4

5 **2.10 . Recycling of ILs**

6 A supplementary back-extraction process of ILs was performed on the aqueous phase
7 obtained after filtration to recover the organic compounds remained in this phase. Briefly, 3
8 mL of aqueous mixture of [C₂Py][EtSO₄] and all organic compounds were eluted on silica gel
9 chromatography using ethyl acetate (as mobile phase) to collect the remaining compounds.
10 Subsequently, methanol was used to recover the IL. The solvents were then removed under
11 *vacuum* and the collected products were analyzed by HPLC.

12 Another experiment was carried out on a mixture of ILs and metabolites obtained after the
13 filtration step using SPE column such as Chromafix® C18 ec or Strata™ Phenyl and THF as
14 mobile phase. For these experiments, 0.5 mL mixture of ILs and metabolites were deposited
15 on the column and eluted with 2.5 mL THF. After centrifugation for 5 min, ILs and THF
16 containing metabolites were separated. THF was then removed under *vacuum* and the
17 collected products were analyzed by HPLC.

18 **2.11 HPLC analyses**

19 HPLC analyses were performed at room temperature on the aqueous phase of the hydrophilic
20 ILs after filtration using 0.45 µm Nylon membranes. The mobile phase consisted of solvent A
21 (0.2 % acetic acid aqueous solution) and solvent B (MeOH) with a 0.8 mL.min⁻¹ flow rate
22 either used with a 60:40 ratio (eluant 1) or with a gradient elution (eluant 2) as follows: from
23 0 to 10 min: 90/10 (A/B); from 10 to 15 min: from 90/10 to 10/90 (A/B); from 15 to 30 min:
24 10/90 (A/B), from 30 to 35 min: from 10/90 to 90/10 (A/B); from 35 to 45 min: 90/10 (A/B)
25 .and . The UV detection wavelengths used were 239, 254, 270, 312 or 330 nm. The volume

1 of each injection volume was fixed to 20 μ L. Lichen metabolite identification was carried out
2 by comparing their retention time with corresponding peaks in the standard solution.

3 **2.12 Cytotoxicity assay**

4 Cytotoxic properties of ILs and acetone were determined with a standard tetrazolium assay.²⁶
5 B16 cells were seeded at 15 000 cells/mL and HaCaT cells were seeded at 17 000 cells/mL in
6 RPMI1640 medium with 5% fetal calf serum (FCS) at day 0 in well-plate. Cell lines were
7 incubated at 37 °C in an atmosphere of 5% carbon dioxide (CO₂) during 24 h. After this time,
8 ILs or acetone were added at 100 μ mol/L. Cell growth and viability were measured after 48 h
9 of incubation at 540 nm, after adding 10 μ L of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-
10 diphenyltetrazolium bromide), incubation at 37°C for 3 hours, and dissolution of crystals
11 with 150 μ L of dimethyl sulfoxide. Each experiment was repeated three times.

12

13 **Results and discussion**

14 **3.1 Selectivity of ILs in MAE**

15 We highlighted previously the interest of the aromatic imidazolium cations in interacting with
16 π -systems such as depsidones.²² Moreover, the interest of these cations to extract various
17 lichen metabolites was recently confirmed.²³ We have also reported an optimization study²⁷
18 using six imidazolium and pyridinium-based ILs for MAE from *P. furfuracea*. Microwave
19 irradiation is generally more efficient to extract the the targeted lichen metabolites (Fig. 1)
20 than conventional heating. The optimal time and temperature for MAE were defined to be 75
21 °C and 15 min, leading to a considerable time economy compared to 24 h for conventional
22 heating.²⁷ The shorter heating period allows a sufficient contact time without subjecting the
23 lichen metabolites into degradation . Additionally, we have found that a temperature above
24 90°C leads to metabolites degradation.

1 We report herein the MAE efficiencies of metabolites from *P. furfuracea* using three
2 hydrophilic^{27,28} ILs [C₁C₁Im][MeSO₄], [C₂C₁Im][EtSO₄], and [C₂Py][EtSO₄] (Table 1) and
3 compare them to acetone, the conventional flammable organic solvent used for lichen
4 metabolites extraction.² Noteworthy, few studies reported the use of pyridinium-based ILs for
5 the extraction of natural products. A recent study exhibited for example the highest ability of
6 [EPy]Br to extract inorganic and organic iodine compounds from *Laminaria*.²⁹

7 According to the results depicted in Fig. 2, each ionic liquid presents an affinity toward
8 specific metabolites whereas acetone extracted two metabolites among the four targeted ones:
9 AT (with a high rate of 0.91%) and Phys. Ac (0.67%). Concerning the MOC extraction, the
10 highest extracted quantity was achieved using [C₂C₁Im][EtSO₄] as extraction solvent (0.31%)
11 (Fig. 2). This amount decreased by around 40 % (0.31% vs 0.21% and 0.18%) for
12 [C₁C₁Im][MeSO₄] and [C₂Py][EtSO₄] and around 85% for acetone (0.04%) (Table 2).
13 Interestingly, a total extraction of MOC using four successive solvents (see section 2.5) was
14 as efficient as [C₂C₁Im][EtSO₄] (Table 2). In contrast, AT extraction with all the tested ILs
15 was less effective compared to those with acetone or total extraction due to its easier
16 degradation under ILs. Indeed, the low stability of AT metabolite under various extraction
17 conditions is well known³⁰. However, the extraction of Phys. Ac., Fum. Ac. and AT was
18 efficient using [C₂Py][EtSO₄] with 0.66%, 0.16% and 0.26%, respectively (Fig. 2). These
19 extractions were 9 to 5 times lower with [C₁C₁Im][MeSO₄] (Fig. 2) and they were not
20 possible or very weak (for Fum. Ac.) using [C₂C₁Im][EtSO₄]. Moreover [C₂Py][EtSO₄] was
21 as effective as acetone for Phys. Ac and exhibited a higher efficiency for Fum. Ac. (Table 2).
22 These features can be attributed to the stronger interactions with targeted analytes using
23 pyridinium cation including π -stacking, hydrogen bonding and ionic interactions as already
24 described.³¹ A simple modification of the anion-cation association can thus modify the IL
25 physicochemical properties. Consequently, two ILs with different structures but similar or
26 close polarity give rise to distinct results when used as extraction solvents.³² Modifying the IL

1 structure affected the interactions between anion and cations of these salts on the one hand,
2 and between the salts and metabolites which changed the lichen metabolites extraction
3 potential of IL(s) on the other hand.

4 Moreover, we have evaluated the impact of these ILs and acetone at 100 $\mu\text{mol/L}$ on the cell
5 viability of B16 (a murine melanoma cell line) and HaCaT (a human keratinocyte cell line)
6 (see SI, Fig.S3). The three ILs exhibited no toxicity (around 99% cell viability for the two
7 cell lines after IL treatment) while acetone showed 21% inhibition of viability of HaCaT
8 cells.

9

10 < Fig. 2>

11 < Table 2>

12 **2.7 Impact of lichen sample preparation**

13 We demonstrated previously the importance of lichen preparation on the AT extraction
14 efficiency using acetone in favor to the mixing of lichens with blender process.¹⁴ The impact
15 of lichen sample preparation method (milled or mixed) on the IL-MAE efficiency of
16 metabolites is assessed, in this study, using the two imidazolium-based IL $[\text{C}_1\text{C}_1\text{Im}][\text{MeSO}_4]$
17 and $[\text{C}_2\text{C}_1\text{Im}][\text{EtSO}_4]$ (Fig. 3) which exhibited the least ability to extract AT. This latter
18 metabolite was slightly better extracted using $[\text{C}_1\text{C}_1\text{Im}][\text{MeSO}_4]$ from mixed lichens (0.074
19 %) than from milled lichens (0.048%), whereas, it was not extracted using $[\text{C}_2\text{C}_1\text{Im}][\text{EtSO}_4]$.
20 In contrast, Fum. Ac. was more efficiently extracted from mixed sample using both
21 $[\text{C}_1\text{C}_1\text{Im}][\text{MeSO}_4]$ and $[\text{C}_2\text{C}_1\text{Im}][\text{EtSO}_4]$. The extraction rate of Fum. Ac. using
22 $[\text{C}_1\text{C}_1\text{Im}][\text{MeSO}_4]$ increased from 0.044% for the milled lichen to 0.170% for the mixed one.
23 Upon using $[\text{C}_2\text{C}_1\text{Im}][\text{EtSO}_4]$, the rates increased from 0.03% to 0.073%, respectively. By
24 contrast, an opposite trend was observed for the extraction rate of MOC with these two ILs
25 from milled and mixed samples. A decrease from 0.21% to 0.045% was calculated for

1 [C₁C₁Im][MeSO₄] and from 0.31% to 0.11% for [C₂C₁Im][EtSO₄], respectively. Finally
2 using the less selective [C₁C₁Im][MeSO₄], a relatively small increase of extraction rate was
3 observed for Phys. Ac. Using the milling process (0.136%) vs the mixing process (0.100%).

4 < Fig. 3 >

5 These results confirm that the mixing process yielded less fine powders than the milling
6 process but with larger specific surface area and pore volume¹⁴ which are more favourable for
7 AT and Fum. Ac. extraction, regardless of the solvents used (acetone or ILs). The extraction
8 rate of Phys. Ac. was nevertheless less impacted by the grinding process.

9

10 **2.8 Scale-up of the process and IL back-extraction procedure**

11 The detailed process for the 100 times scale-up, described in Fig. 4, was performed on 25 g of
12 mixed lichen sample keeping the same liquid/solid ratio used for the small scale. An
13 additional step (step 2) was incorporated to achieve a better performance of the process: a
14 manual stirring before MAE to ensure a better homogenization of solution. The other steps
15 are similar to those used for small-scale extraction: filtration on cotton at 50 °C to avoid
16 clogging on filters due to the viscosity of IL (step 4), centrifugation to separate the different
17 phases (step 5), and liquid extraction using an organic phase non miscible with IL in order to
18 concentrate extracted metabolites (step 6) before quantification using a Camag®
19 spectrophotodensitometer.

20 < Fig. 4 >

21 In order to verify the good repeatability between the small-scale extraction and scaling-up on
22 mixed sample which highly impacts Fum. Ac. extraction, we have first chosen the most
23 selective imidazolium-based IL [C₂C₁Im][EtSO₄]. This IL could only extract MOC and Fum.
24 Ac.. The same trends were observed for the scale-up compared to the small-scale. Extraction

1 rates of 0.31% (vs 0.28%) and 0.21% (vs 0.18%) for MOC and Fum. Ac. were recorded.
2 Noteworthy, no traces of AT were detected in any of the cases.

3 We have then extended this process on mixed sample using the most efficient pyridinium-
4 based IL, [C₂Py][EtSO₄]. Contact angles measured between this hydrophilic IL on milled or
5 mixed lichens were identical (102°) indicating a very light wettability of lichens using this IL,
6 regardless of the grinding method used. We have thus hypothesized that, in accordance to
7 these latter results and to those reported on surface area in 3.2 part, the extraction from mixed
8 lichen using this IL would be more efficient.

9 As already observed in section 3.3, the mixing process was more favourable for AT and Fum.
10 Ac. extraction than milled process with an extraction rate of 0.85% for AT and of 0.75% for
11 Fum. Ac. The extraction efficiency for MOC and Phys. Ac. were quite similar between the
12 small-scale and the scale-up process, regardless of the grinding process used. These results
13 display the interest of this pyridium-based IL for the extraction of the major metabolites from
14 *P. furfuracea*.

15 In both cases of scale-up, the aqueous phases obtained after liquid-liquid extraction using
16 diethyl ether were analyzed by HPLC in order to determine the eventual possibility of back-
17 extraction of ILs used during the process. Some lichen metabolites were detected at different
18 wavelengths highlighting the non-total recovery of the lichen extracted metabolites after
19 liquid-liquid extraction (Fig. 5a and Fig S4). After a supplementary step of purification on
20 silica gel chromatography using ethyl acetate for the elution of metabolites then methanol for
21 the elution of ILs, we have confirmed the absence of metabolites in the back-extracted IL.
22 This procedure which corresponds to a conventional method to separate lichen metabolites
23 simultaneously allows the purification of lichen metabolites.

24 To decrease the solvent consumption used, particularly during the back-extraction, we have
25 successfully performed this separation directly on a mixture of IL and metabolites after the

1 filtration step using a SPE column as Chromafix® C18 ec or Strata™ Phenyl and
2 tetrahydrofuran as solvent. HPLC analysis exhibited a high purity of ILs (Fig. 5b). With these
3 latter experiments, we succeeded to scale-up the process and we finally proved the full
4 recycling of ionic liquid to be used for further studies. Further experiments are in progress in
5 order to improve the extraction of lichens using more eco-friendly solvents.

6 < Fig. 5 >

7

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11

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15

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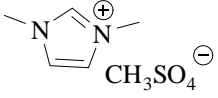
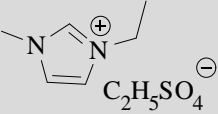
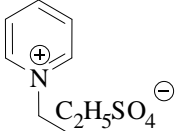
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1 **Table 1.** Assessed ionic liquids.

Ionic liquid (IL)	Label	Yield (%)	Melting point (°C)
	[C ₁ C ₁ Im][MeSO ₄]	100	35.8 ^a
	[C ₂ C ₁ Im][EtSO ₄]	98	< 65.0 ^a
	[C ₂ Py][EtSO ₄]	100	unknown

2 ^a www.chemicalbook.com

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4 **Table 2.** Comparative study of extraction efficiency of lichen metabolites using different
5 solvents expressed as ratio in total yield (%)

Compound	Ratio in total yield (%)				Total extraction
	[C ₁ C ₁ Im][MeSO ₄]	[C ₂ C ₁ Im][EtSO ₄]	[C ₂ Py][EtSO ₄]	Acetone	
MOC	66	100	58	12.5	66
AT	47.5	0	31	78	100
Fum. Ac.	28	2	100	0.6	11
Phys. Ac.	20	0	98	100	33

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7

1 **Figures**

2

3 **Fig. 1.** Targeted lichen metabolites

4 **Fig. 2.** Extraction rate (%) of targeted metabolites from milled lichens using MAE with
5 [C₁C₁Im][MeSO₄], [C₂C₁Im][EtSO₄] and [C₂Py][EtSO₄] and acetone.

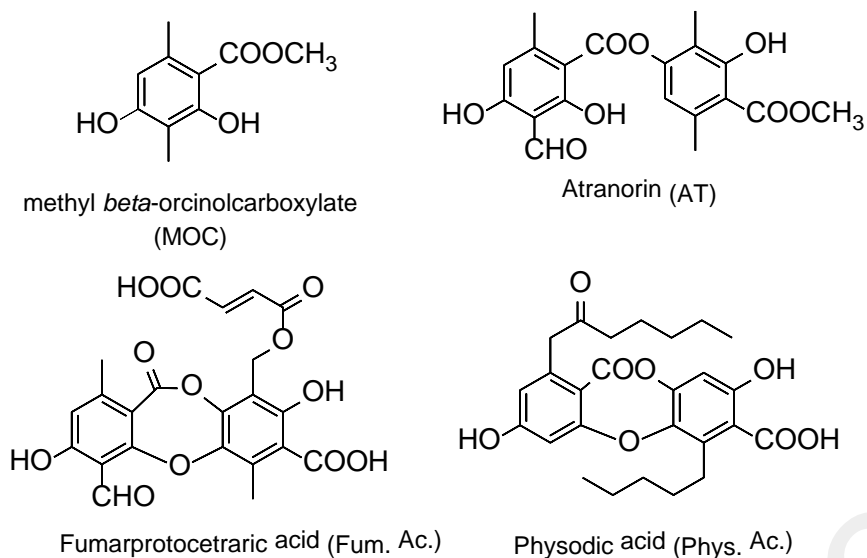
6 **Fig. 3.** Extraction rate (%) of targeted metabolites using MAE combined to
7 [C₁C₁Im][MeSO₄] and [C₂C₁Im][EtSO₄] on milled or mixed samples

8 **Fig. 4.** Scale-up process performed on 25 g mixed sample with 500 mL ILs

9 **Fig. 5** HPLC chromatograms of extract of IL [C₂Py][EtSO₄] and metabolites a) before and b)
10 after purification with small phase column (Chromafix® C18ec) at 254 nm (eluant 2); AT:
11 28.88 min.

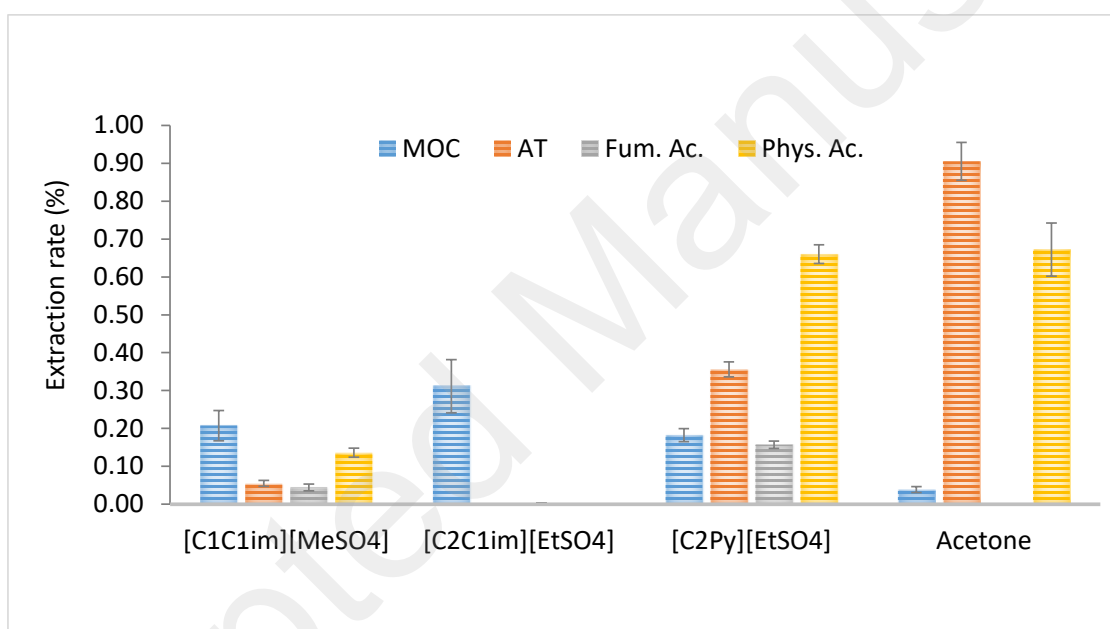
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Fig. 1. Targeted lichen metabolite



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Fig. 2. Extraction rate (%) of targeted metabolites from milled lichens using MAE with [C₁C₁Im][MeSO₄], [C₂C₁Im][EtSO₄], [C₂Py][EtSO₄] and acetone.

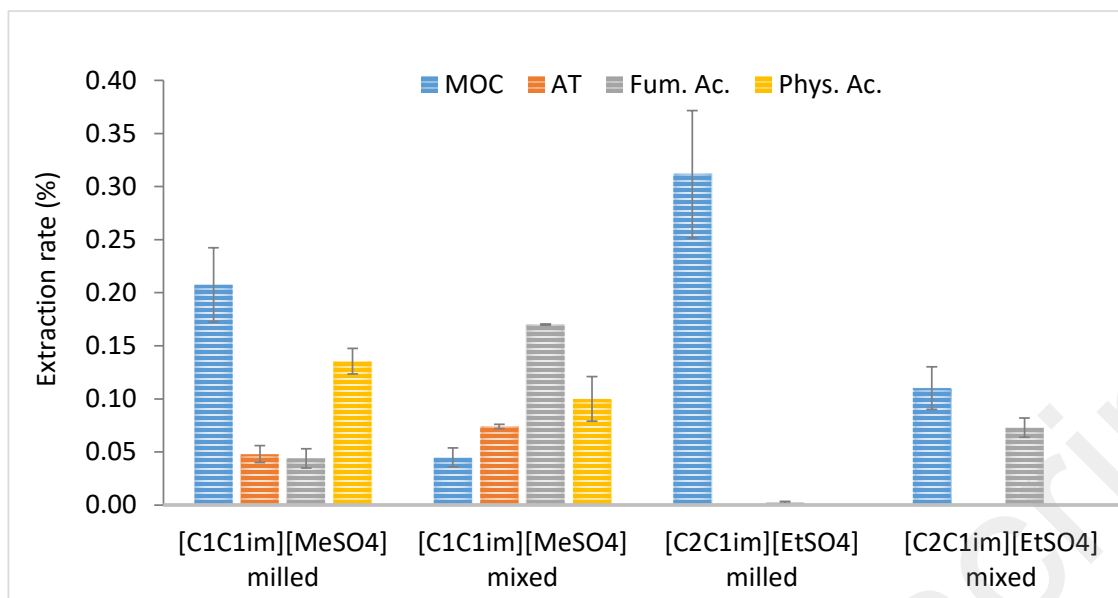


Fig. 3. Extraction rate (%) of targeted metabolites using MAE combined to $[C_1C_1Im][MeSO_4]$ and $[C_2C_1Im][EtSO_4]$ on milled or mixed samples

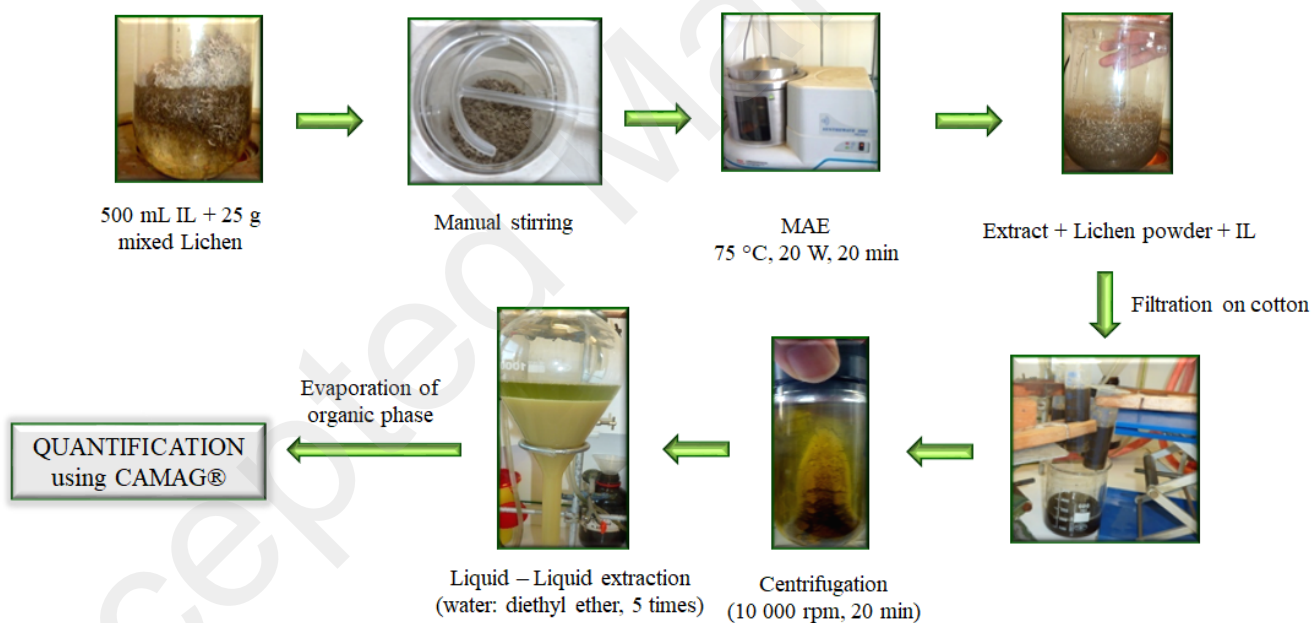
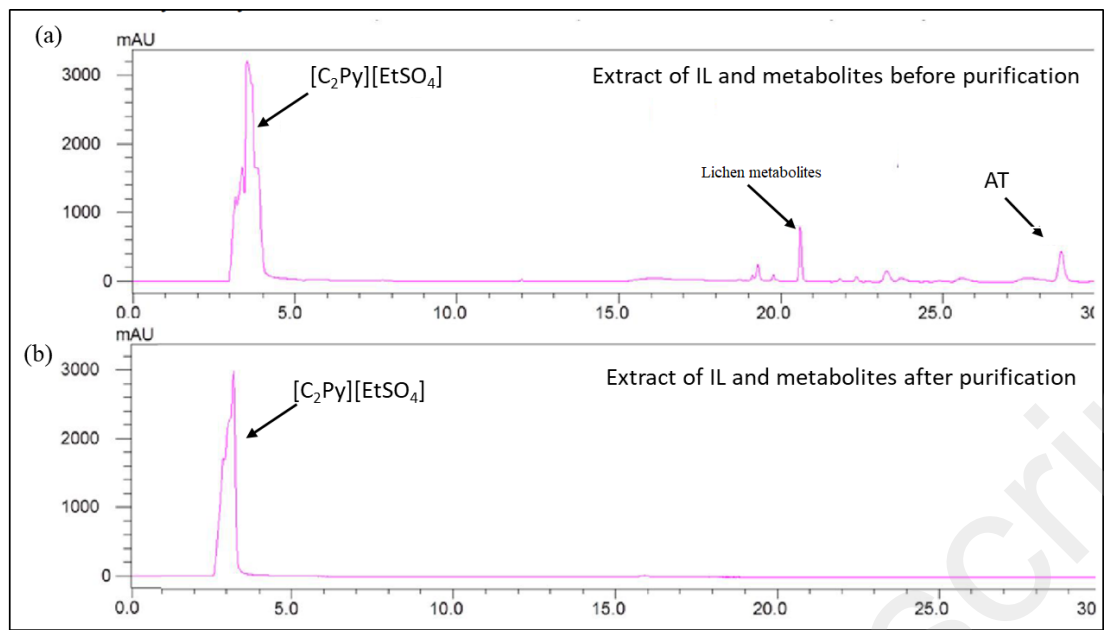


Fig. 4. Scale-up process performed on 25 g mixed sample with 500 mL ILs



1

2 **Fig. 5** HPLC chromatograms of extract of IL $[C_2Py][EtSO_4]$ and metabolites a) before and b)
3 after purification with small phase column (Chromafix® C18ec) at 254 nm (eluant 2); AT:
4 28.88 min.

5