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Chemotaxonomic Fingerprinting of Chilean Lichens Through Maldi and Electrospray Ionization Mass Spectrometry

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ABSTRACT

*The aim of this work was to study a fast, new, sensitive, and simple method for the chemotaxonomic classification of Chilean lichens (*Teloschistes chrysophthalmus, Ramalina farinacea, Usnea pusilla, Ramalina chilensis *and* Stereocaulon ramulosum*) using MALDI-TOF-MS and UPLC-ESI(-)-MS data. Lichens soluble proteins fingerprints were acquired by MALDI-TOF-MS and they were analyzed by chemometric (PCA). Lichens organic extracts fingerprints were obtained by UPLC-ESI(-)-MS. MALDI-TOF-MS associated with chemometric analysis was used to detect new m/z patterns of soluble proteins that were compared with Protein Data Bank of UnitPro. These data also permitted the satisfactory distinction among the families and species. UPLC-ESI(-)-MS fingerprints analyses of the organic extracts showed the presence of five major lichen compounds (atranorin, parietin, teloschistin, ramalinolic and usnic acids). In contrast to other techniques, MALDI-TOF-MS associated with chemometric analysis and UPLC-ESI(-)-MS provided a new, fast and sensitive method for chemotaxonomic characterization of lichens.*

Key words: Chilean lichens, chemotaxonomic, fingerprint, MALDI-TOF-MS, UPLC-ESI(-)-MS

INTRODUCTION

There are approximately 28.000 lichen species worldwide (Lucking et al. 2009) that synthesize a great variety and diversity of secondary metabolites (Hidalgo et al. 1994; Huneck et al. 1996; Fournet et al. 1997; Huneck 1999; Müller 2001; Stocker-Wörgötter 2008). Several secondary metabolites identified and characterized from lichens show important biological and antioxidant activities (Barnes 2000; Rancan et al. 2002; Boustie and Grube 2005; Oksanen 2006; Pereira et al. 2007; Russo et al. 2008; Schmeda-Hirschmann et al. 2008; Molnár and Farkas 2010; Shukla et al. 2010; Ghorbani et al. 2012; Manojlovic et al. 2012; Cuellar et al. 2013). Polysaccharides (as pectins) and lipids have also been detected in lichens (Olafsdottir and Ingólfsdottir 2001; Behera et al. 2005; Sassaki et al. 2005; Omarsdottir et al. 2007; Gielwanowska and Olech 2012). However, there is not much information reported about the presence of proteins in lichens (Fahselt 1980; Gorin and Iacomini 1985; Honegger and Bartnicki-Garcia 1991; Da Silva et al. 1993). For taxonomic determination of lichens, the content of secondary metabolites has been commonly used. These compounds are reasonably invariable within the

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same species and most of them are exclusive of these organisms (Quilhot et al. 1987; Eifler-Lima et al. 2000). Lichen chemotaxonomy identification is frequently carried out through the use of one or more techniques as color reactions, thin layer chromatography (TLC), fluorescence, gas chromatography-mass spectrometry (GC-MS), high performance liquid chromatography (HPLC), Nuclear Magnetic Resonance (NMR), infrared (IR), Matrix-Assisted Laser Desorption Ionization Mass Spectrometry (MALDI-MS) (Honda and Vilegas 1998; Carbonero et al. 2001; Alcantara et al. 2007; Rustichelli et al. 2008), etc. For all these techniques, the accurate delimitation of species is not obvious because the lichens are in constant evolution in response to environmental parameters (Clerc 1998; Leavitt et al. 2011; Lumbsch et al. 2011).

In recent years, MALDI has become a powerful tool due to its sensitive, ease in use and achievable mass range, allowing measurement of metabolites, lipids, peptides, proteins, etc on the same instrument (Krishnamurthy 1996; Vaidyanathan et al. 2002; Santos et al. 2004; Catharino et al. 2005; Nanni et al. 2007; Rustichelli et al. 2008; Gustafsson et al. 2011; Radebe et al. 2013). Analyses of samples without any pre-treatment generally give large amount of information, often with a high degree of similarity, which cannot be interpreted by visual examination. Then, chemometrics can be a useful tool to extract hidden information of data, in particular, Principal Component Analysis (PCA) (Belton et al. 1998; Weinmann et al. 2003; Peña-Méndez et al. 2005; Brandt and Ehmann 2010; Scotti et al. 2010; Ohmenhaeuser et al. 2013). The application of chemometric is increasing as a need to cover more areas of research (Pomerantsev and Rodionova 2010). The chemometric analyses have been used in food investigations, plant extracts and clinical chemistry (Howells et al. 1992; Defernez et al. 1995; Vogels et al. 1996; Ellis et al. 2007; Trygg et al. 2007; Lai et al. 2010; Aouidi et al. 2012). Up to now, a protocol of sample preparations for MALDI analysis of lichen proteins is not reported, as well as no content information about the type of proteins present in lichen species is available. This work was carried out to develop an alternative, simple and rapid method for the chemotaxonomy identification of lichen, which comprised protein and secondary metabolites analyses associated to morphological and reproductive characters description of Chilean lichens with the purpose to delimit taxonomically different species.

MATERIAL AND METHODS

Lichen Collection and Taxonomic Determination

Lichen samples of five species were collected from Region del Maule in Chile during July 2011 (Fig. 1), which included *Ramalina farinacea*, *Usnea pusilla*, *Ramalina chilensis* and *Stereocaulon ramulosum* from El Colorado (38º38"53' S, 71º14"54' W: 444 msnm) and *Teloschistes chrysophthalmus* from Talca (35º25"59' S, 71º40"00' W: 102 m). Vouchers of collected samples were deposited in the Lichen Herbarium of the Talca University and included latitude, longitude, altitude and substrate type (vouchers numbers 301, 302, 303, 304, 305, 306, 307, 308, 309 and 310).

Figure 1 - Studied lichen species. Ramalinaceae Family: A) *Ramalina farinacea* (L.) Ach., and B) *Ramalina chilensis* Bert. Ex Nyl. Teloschistaceae Family: C) *Teloschistes chrysophthalmus* (L.) Th. Fr. Parmeliaceae Family: D) *Usnea pusilla* (Räsänen) Räsänen. Stereocaulaceae Family: E) *Stereocaulon ramulosum* (Sw.) Rausch. The scale represents 2 cm.

Some morphological and reproductive characters and spot tests were used in the taxonomic determination through KOH (K); $Ca(OCl)_2$ (C); combination between K and C (KC) and *para*-phenylenediamine (Pd), according to Asahina and Shibata (1954) (Table 1).

Table 1 - Morphological, reproductive characters and spot tests of the studied lichen species.

Lichen	Growth form	Apothecia	Spores	Spot tests
B	fruticose	Lecanorine	hyaline, uniseptate	Medulla K-, C+, KC + yellow, Pd -
A	fruticose	absent, soredia	absent	Medulla K-, C+, KC + yellow, Pd -
E	composed	Biatorine	hyaline, spores 3-5 (7) celled	Medulla K-,KC-, C-, Cortex and medulla Pd+ first yellow, then red
C	fruticose	Lecanorine	hyaline, polarilocular	Thallus K+ purple, C-, KC-, Pd-
D	fruticose	Lecanorine	hyaline, simple	Medula K-, C+ yellow, KC-, Pd+ lightly yellow

A) *Ramalina farinacea* (L.) Ach., B) *Ramalina chilensis* Bert. Ex Nyl. C) *Teloschistes chrysophthalmus* (L.) Th. Fr. D) *Usnea pusilla* (Räsänen) Räsänen. E) *Stereocaulon ramulosum* (Sw.) Rausch.

Proteins and Organics Extractions

Extractions were carried out in an ultrasonic bath (Elmasonic One, Ultrasonic cleaning unit, Elma, Germany). The extractions were performed at constant temperature (24^{o}C) for 2 h. Soluble proteins of each lichen species were obtained using 250 mg of fresh thallus by maceration in double distilled water (1.0 mL). The aqueous extracts of proteins were centrifuged at 4000 rpm for 30 min. (Table Top centrifuge PLC-05, Germany); the supernatant was again centrifuged for 30 min. The total soluble proteins obtained were frozen in freezer and then lyophilized (Freezone 6 Freeze Dry System, Labconco, USA). Each lichen sample was successively extracted with diethyl ether, chloroform and acetone. The extracts were concentrated, weighed and kept in desiccators. The solvents used were obtained from Merck.

MALDI-TOF MS

A 1.0 μL aliquot of soluble proteins sample was hand-spotted on a target type Anchorchip small (400 μm) and dried by air. Then, 1.0 μL of matrix solution (cinapinic acid) was added on the top of the dried samples and allowed to dry at room temperature (24^{o}C).

All the experiments were carried out on an Autoflex Speed MALDI–TOF mass spectrometer (Bruker Daltonics Flex Control) equipped with a nitrogen laser (λ = 337 nm). The mass spectra were obtained in linear mode with an accelerating voltage of 20 kV. Mass range of 20–50 kDa and 30–210 kDa were selected. Each spectrum was automatically collected in the positive ion mode as an average of 300 laser shots (30 laser shots at 10 different spot positions). Laser energy was selected to produce the threshold for ion production. Each assay was performed in triplicate and six MALDI spectra were acquired for each lichen species (three spectra of 20-50 kDa and three spectra of 30-210 kDa). In total, ninety spectra were obtained.

Chemometric analysis

After processing of MALDI spectra, a data matrix for each lichen species was obtained. Due to the large number of data, a selection of a pattern of five relevant *m/z* values was performed. The average of 20 spectra obtained by MALDI-MS for each sample, normalized with the flex Control Analysis 3.3 software were exported to the statistical software R version 2.12.2 (The Foundation for Statistical Computing, Vienna, Austria) in order to perform the principal components (PCA).

LC Separation

Methanol of HPLC grade was purchased from Merck SA and acetic acid were used without previous purification. Deionized water was obtained from a Purelab classic (Elga, Labwater, UK). Chromatographic separations were performed on a Poroshell 120 C18 column (4.6 mm x 50 mm id, 2.7 μm; Agilent, USA) using an Ultimate 3000 UHPLC focused (Bruker Daltonics) equipped with a PDA 3000 detector. Isocratic solvents system, MeOH, H_2O, acetic acid (80:19.5:0.5, v/v) was used (Legaz and Vicente 1983). The column temperature was maintained at room temperature (18°C). The flow rate was 1.0 mL/min. The extracts were diluted at 1.0 mg/mL. Volume of injection was 20 μL. Detection was performed using both UV absorbance detector at 254 nm and ion trap mass detector. In order to test the reproducibility of the chromatography system, samples were injected on the UPLC column in triplicate.

UPLC-ESI(-)-MS

The mass spectrometric data were collected using an ion-trap AmaZon SL (Bruker, Germany) equipment. The column eluent entered the source

of the ESI-MS for continuous detection by MS. The ion trap scanned in the 100-850 *m/z* range and the maximum accumulation time for the ion trap was set at 200 ms. The optimum values of the ESI-MS parameters were: capillary voltage, -4.0 kV; drying gas temperature, 220°C; drying gas flow, 5.0 L/min; and nebulizing gas pressure of 8.0 psi. The instrument was controlled by trapControl 7.0 software from Bruker Daltonics and the data were processed by DataAnalysis 4.0 software from Bruker Daltonics.

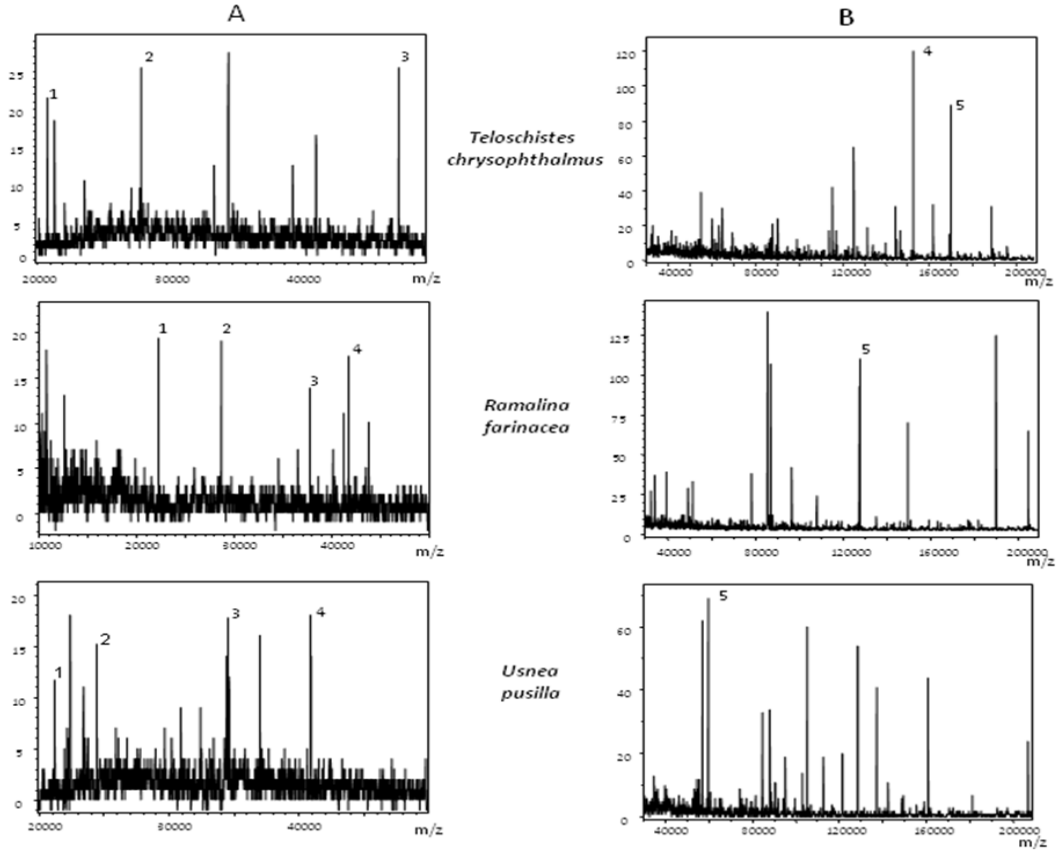
RESULTS

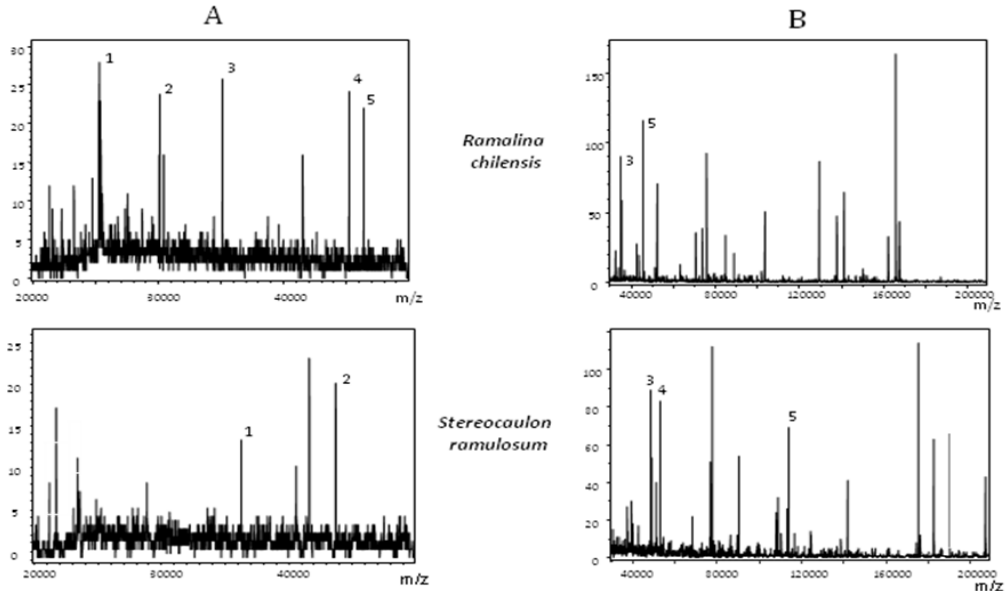
Taxonomic Determination

The representatives of *Ramalinacea*, *Teloschistaceae* and *Usneaceae* families presented lecanorine apothecia and shared the growth form and reproduction modality, except *R. farinacea* that was reproduced by soredia. *S. ramulosum* was characterized by presenting thallus and apothecia biatorine (Table 1). The spot tests for *Ramalina* species were chemically similar, but the KC and Pd tests for *U. pusilla* were different. *T. chrysophhalmus* and *S. ramulosum* presented distinct chemical reactions and also showed differences in the types of spores (Table 1).

MALDI-TOF-MS

In total, 15 samples (three of each species) of lichens were analyzed. Mass spectra fingerprints of lichen soluble proteins from the five Chilean lichen species showed a characteristic pattern for each one (Fig**.** 2). Under these conditions, reproducible mass spectra of lichens were obtained. In the mass spectra, the peaks of the high and major intensities were observed in the *m/z* region 30-210 kDa. The *m/z* pattern of soluble proteins of lichen species were compared with the Data Bank of UnitPro, which showed that these registers were not included in this Data Bank. Because of the high similarity of the mass spectra in each species, where the intensities of the individual peaks only varied a little, and where there was a large number data (*m/z* values), chemometric analysis was applied in an attempt to extract the underlying information contained in the spectra.





I: Lichens Protein profiles obtained in 20-50 kDa II: Lichens Proteins profiles obtained in 30- 210 kDa.

Figure 2 - Lichens Protein fingerprints obtained in 20-50 kDa and in 30-210 kDa.

Chemometric analysis

PCA was performed on the matrix data of lichens MALDI-TOF spectra. Due to the large number of data, a selection of a pattern of five relevant *m/z* values was performed. This analysis showed a PCA characteristic pattern of reproducibility for each studied lichen species. PCA for MALDI-TOF-MS data showed the separation of the lichens into families. *Parmeliaceae* and *Ramalinaceae* families were located on the upper first quadrant while *Teloschistaceae* family was located on the upper second quadrant. *Stereocaulaceae* was located on the bottom of the third quadrant (Fig. 3).

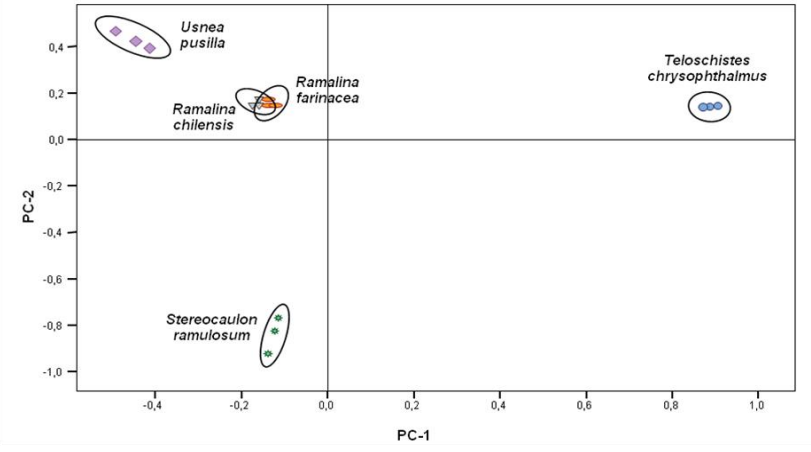


Figure 3 - PCA score plot of all lichens analyzed by MALDI-MS with lichen family distinction.

UPLC-ESI(-)-MS

UPLC chromatograms of lichen organic extracts were obtained in short analysis time with good resolution of the peaks. The lichen organic extracts fingerprints showed unique profiles and gave an idea of the number of major lichen compounds (Table 2 and Fig. 4). For example, Figure 5 showed that the acetone extract fingerprint of *U. pusilla* contained two main compounds (usnic acid and atranorin) identified by ESI(-)-MS/MS.

Table 2 - Detected Compounds by UPLC-ESI (-)-MS.

Lichen	Diethyl ether extract	Chloroformic extract	Acetonic extract
T. chrysophthalmus	Parietin *m/z* 283 Usnic acid *m/z* 343	Telochistin *m/z* 299	Telochistin *m/z* 299 Parietin *m/z* 283
R. chilensis	Usnic acid *m/z* 343 Ramalinolic acid *m/z* 431	Usnic acid *m/z* 343 Ramalinolic acid *m/z* 431	Usnic acid *m/z* 343 Ramalinolic acid *m/z* 431
U. pusilla	Usnic acid *m/z* 343 Atranorin *m/z* 373	Usnic acid *m/z* 343 Atranorin *m/z* 373	Usnic acid *m/z* 343 Atranorin *m/z* 373
R. farinacea	Usnic acid *m/z* 343 Protocetraric acid *m/z* 373	Usnic acid *m/z* 343 Protocetraric acid *m/z* 373	Usnic acid *m/z* 343 -
S. ramulosum	Atranorin *m/z* 373	-	Atranorin *m/z* 373

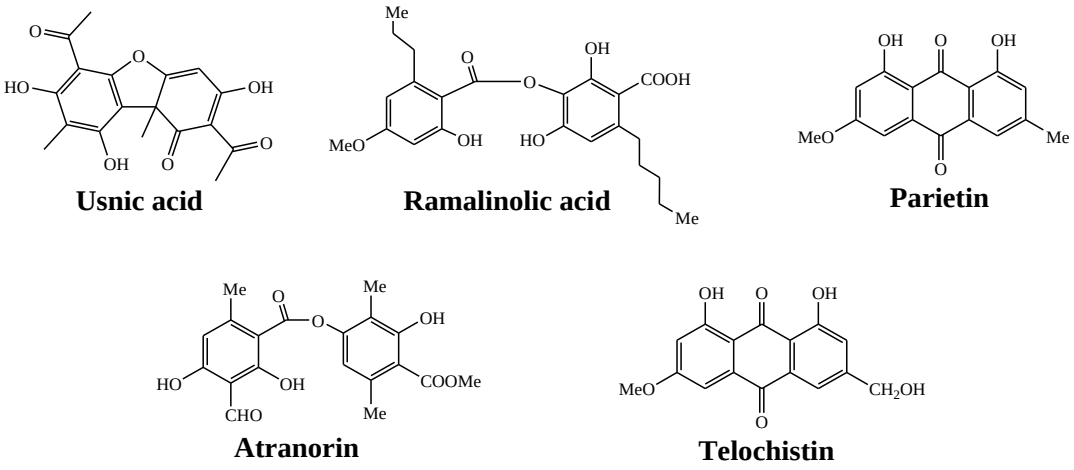


Figure 4 - Lichen compounds detected by UPLC-ESI (-)-MS.

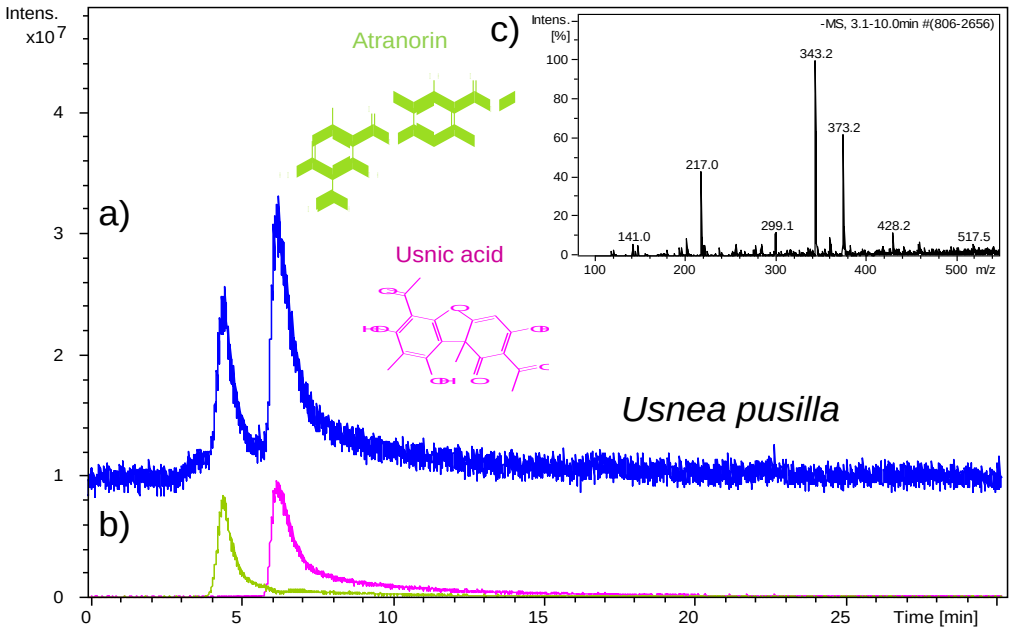


Figure 5 - Representative acetone extract fingerprint of *Usnea pusilla*. a) UHPLC-ESI-MS chromatogram showed two main compounds (usnic acid and atranorin) identified by ESI-(-)-MS/MS. b) Extracted ion chromatograms of *m/z* 343 (pink) and *m/z* 373 (green). c) Figure shows the ions for usnic acid (*m/z* 343) and atranorin (*m/z* 373).

DISCUSSION

There is a growing interest in phylogenetic studies and it sometimes is used as an advantageous tool in modern taxonomy but this to become reality, a huge database is required containing the signature sequences of all the species. This would need many years of research (Lucking 2009; Lumbsch et al. 2011). Hence, the chemotaxonomy classification is widely accepted as an important part of taxonomic studies (Honda and Vilegas 1998; Vitikainen 2001; Frisvad et al. 2008; Crespo et al. 2010; Tell et al. 2012; Lin 2013).

Figure 2 shows typical and distinguishable MALDI-MS in the positive ion mode of the extracts of each of the lichen species. As shown in Figure 2AI, the most characteristic and abundant ions were that of *m/z* 24.410, 27.987, and 47.627. An expansion of the *m/z* region, which had the main proteins of lichens (Fig. 2AII), showed two additional ions of *m/z* 152.787 and 169.955.

These ions were characteristic for *T. chrysophthalmus*. Figures 2BI and 2BII were representative for *R. farinacea* showing characteristic ions of *m/z* 22.050, 28.547, (37559, 41.281) and 127.341. *U. pusilla* presented four characteristic ions in the range of 20-40 kDa of *m/z* 21.245, 24.365, 34.512, and 40.471. Furthermore, several other ones between 80-120 kDa were characteristic for *U. pusilla*. When comparing *R. chilensis* (Fig. 2D) with *R. farinacea* (Fig. 2B), the ions of *m/z* 25.290, 30.074, 35.106, 45.187, and 46.353 were observed. Figure 2E showed the characteristic ions for *S. ramulosum* of *m/z* 36.257, 43.747, 49.268, 53.772 and 113.286. To test chemometrically, the distinctiveness of the MALDI-MS, PCA was employed. The PCA plot (Fig. 3), which covered 94% of the total data variance placed the samples into four well-defined groups comprised of the different species lichens.

CONCLUSIONS

Although the number of lichen species in this work was not very extensive, these preliminary results showed a new perspective of chemotaxonomic analysis in lichens. The PCA scores plot showed separation of the families, and *Ramalina* and *Usnea* species were located on the most positive side of PC2 axis, indicating a close relationship between these species. An overlapping of *Ramalina* species was observed, which showed that it belonged to the same family, whereas others species appeared in different quadrants, suggesting that the separation occurred due to each species containing different types of proteins. MALDI-TOF-MS together with chemometric analysis could be an excellent tool to obtain a fast method for the chemotaxonomic classification of lichens, which could be useful to identify the unknown lichen species.

UPLC-ESI(-)-MS of lichen organic extracts showed the main lichen compounds in the studied species. These results together with morphological, reproductive and protein data strengthened the new proposed method. Thus, this work presented preliminary results about of the protein content of lichen species and it envisaged in future to increases the data about the protein content for different lichen species.

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