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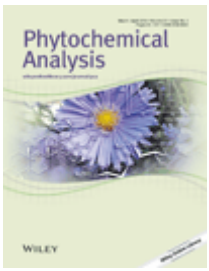

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Short title (< 70 char): **Chemical Profiling of Lichen Extracts using the Van Krevelen Diagram**

Short abstract (< 80 words):

Because most metabolomics tools are designed for LC-MS, it is often difficult to obtain structural information when straying from this gold standard. The Van Krevelen diagram is adapted for this purpose. Therefore, a new classification method is benchmarked against the classification tool ClassyFire, and various extracts from four lichens are analyzed with high resolution mass spectrometry (HRMS). Lichen metabolites are efficiently classified and the obtained results are consistent with the extraction protocols. This approach provides structural insight in lichen metabolites from HRMS.

ABSTRACT

Introduction: In recent years, LC-MS has become the gold standard for metabolomic studies. Indeed, liquid chromatography is relatively easy to couple with the soft electrospray ionization. As a consequence, many tools have been developed for the structural annotation of tandem mass spectra. However, it is sometimes difficult to do Data Dependent Acquisition (DDA), especially when developing new methods that stray from the classical LC-MS workflow.

Objective: An old tool from petroleomics that has recently gained popularity in metabolomics, the Van Krevelen (VK) diagram, is adapted for an overview of the molecular diversity profile in lichens through HR-MS.

Methods: A new method is benchmarked against the state-of-the-art classification tool ClassyFire using a database containing most known lichen metabolites ($n \approx 2{,}000$). Four lichens known for their contrasted chemical composition were selected, and extractions with apolar, aprotic polar and protic polar solvents were performed to cover a wide range of polarities. Extracts were analyzed with Direct Infusion ElectroSpray Ionization Mass Spectrometry (DI-ESI-MS) and Atmospheric Solids Analysis Probe Mass Spectrometry (ASAP-MS) techniques to be compared with the chemical composition described in the literature.

Results: The most common lichen metabolites were efficiently classified, with more than 90% of the molecules in some classes being matched with ClassyFire. Results from this method are consistent with the various extraction protocols in the present case study.

Conclusion: This approach is a rapid and efficient tool to gain structural insight regarding lichen metabolites analyzed by high resolution mass spectrometry without relying on DDA by LC-MS/MS analysis. It may notably be of use during the development phase of novel MS-based

55 metabolomic approaches.

56

57 Keywords: Lichens, Metabolomics, Structural Classification, Extract Comparison

INTRODUCTION

In recent years, significant advances in mass spectrometry have led to an increase in the measurement precision, sensitivity and frequency of acquisition of tandem mass spectra. These different parameters, combined with the extensive separation capabilities of liquid chromatography, have made LC-MS the gold standard in metabolomics.[1,2] It is indeed a preferred analytical technique because it provides an insight into the composition of an extract in a form that could then be used by the industry.[3] However, this also implies (i) that a time consuming extraction step must first be carried out and (ii) that a certain number of compounds will not be detected because they will be retained on the column during separation. The extraction step determines, through the choice of solvent, the fraction of the metabolome that can be studied.[4] To obtain a more comprehensive insight into the chemical composition of an organism, it becomes necessary to perform various extraction steps, which further increases the workload. Therefore, it is interesting to develop new tools for a comprehensive understanding of this composition by directly analyzing the biological material. Natural products (NPs), which are the first historical source of drugs and remain an important subject of study for the pharmaceutical industry today,[5] are of special concern.

With the establishment of LC-MS as a standard for metabolomics, many biocomputing tools have been developed and combined to facilitate the processing and interpretation of the massive amounts of data generated.[6] Most, if not all, of these tools have been created to process data containing tandem mass spectra, which are commonly acquired in a Data Dependent Acquisition (DDA).[7] It is possible to isolate features characterized by a dataset each comprising one m/z value, one retention time, and one tandem mass spectrum, which can be linked together by scores or shared patterns, notably on the GNPS platform.[8–10] Finally, these tandem mass spectra allow to propose identifications of a substantial number of molecules for each sample by comparing

them with experimental[11,12] or *in silico* databases.[13] This structural annotation can allow a hierarchical classification of the molecules composing an extract, in particular thanks to the ClassyFire tool.[14]

The use of tandem mass spectra, while being the strength of these tools, is also their weakness. For instance, when developing new mass spectrometry tools, it is not always possible to proceed *ab initio* to such an acquisition associating mass and tandem mass spectra. Data Independent Acquisition (DIA) is also possible and fragments all ions present in a wide range of *m/z* regardless of intensity. However, it then becomes difficult to interpret the resulting multiplexed spectra, and the *ad hoc* algorithms require substantial computation time, which is not suitable for a rapid screening.[15] As an alternative, the option was to develop a tool providing structural insight directly from the molecular formula (MF), which can readily be computed from high resolution MS data.[16] Indeed, an old tool from petroleomics – the Van Krevelen (VK) diagram[17] – was previously used to estimate the structural class associated with a MF through element ratios, notably for studying the composition of some alcohols.[18,19] To avoid the computational burden of MF determination, a local database search can be performed to determine element ratios for Van Krevelen analysis,[20] however in this approach the analysis ignores masses absent from the database, which can be problematic in a compound discovery context. Furthermore, a database search can sometimes generate mismatches because of cation adducts or the presence of 'atypical' atoms – e.g. chlorinated metabolites, often described in lichens[21] – so, direct MF computation was chosen. For users wishing to try this kind of Van Krevelen-based approach, a Python package (PyKrev) has recently been published and offers a number of processing options, including a classification function – but not tailored for phytochemicals.[22]

Lichens, as organisms constituted of a fungus living in a symbiotic relationship with an alga

and/or a cyanobacterium,[23] possess an original chemistry comprising varied and unique compounds[21] and are thus well suited for the development and assessment of such a tool. They are also an interesting source of bioactive compounds,[24,25] and have been shown to be the ancestors of major fungal lineages (e.g. *Penicillium spp., Aspergillus spp.*).[26]

This paper is the first part of a study aiming to establish a novel concept using Ambient Mass Spectrometry for NP research, focused on four lichen species: *Evernia prunastri* (L.) Ach., *Lichina pygmaea* (Lightf.) C. Agardh., *Parmelia saxatilis* (L.) Ach. and *Roccella fuciformis* (L.) DC. The lichens were selected for their chemical diversity and an extensive list of the metabolites described in these species is provided as **Table S1**. In this paper a method capable of obtaining large structural insights into the composition of lichen extracts is presented without being reliant on tandem mass spectra.

EXPERIMENTAL

General Procedures.

All solvents and reagents used in this study were HPLC grade (Sigma-Aldrich) and were used without further purification. In order to avoid any contamination by plastic compounds, the use of glass containers was preferred and the use of colored plastics was strictly prohibited. Eppendorf micropipetting systems were used for metering the extract solutions. For experiments requiring the thermal desorption of extracts, samples were deposited on Marienfeld melting point capillaries.

Lichen Material.

This study was conducted on herbarium specimens. They were collected in: Cressensac, France (N 45°0’19321” O 1°30’51.191”), August 2006 for *E. prunastri*; Roscoff, France (N 48°43'31.458" O 3°58'8.651"), June 2016 for *L. pygmaea*; Girona, Spain (N 42°27’ 53.399” O 1°47’23.816”), July

2017 for *P. saxatilis*; and Saint-Coulomb, France (N 48°41'29.299" O 1°56'45.855"), May 2003 for *R. fuciformis*. Voucher specimens are kept in the herbarium of the University of Rennes, France under the respective reference codes: JB/06/51, JB/16/204, JB/17/213 and JB/03/02.

Extraction Protocol.

The dried weights were chosen for each extraction so as to extract a sufficient quantity of material for all analyses (**Figure S1**). For each lichen, 2 extracts were obtained with methanol and acetone, and 2 extracts were from a successive extraction using cyclohexane, acetone then methanol (CAM) and using cyclohexane, acetone then water (CAW). CAM and CAW extracts were recombined *pro rata* to the extracted quantities. The selected quantities were 2 g for both the single-step 'Acetone' and the multiple-step 'CAM' extracts, 500 mg for the 'Methanol' extract and 4 g for the 'CAW' extract. All extractions were performed using an Accelerated Speed Extractor Büchi SpeedExtractor E-914 after dispersion of ground lichens in celite (1:1 ratio, m/m). A 2 g sand bed made of Büchi quartz sand of 0.3-0.9 mm granulometry was used to avoid clogging of the sintered part of the extraction cartridges. Each extraction step consisted of 3 maceration cycles of 10 min in 40 °C solvent, with a discharge time of 2 min and a degassing between every cycle.

HR-MS Analyses.

Direct infusion electrospray mass spectrometry (DI-ESI-MS) analyses were acquired on a Q-Exactive mass spectrometer (Thermo Scientific, Bremen, Germany) equipped with a Thermo HESI-II ion source. DI-ESI-MS analyses were performed on 15 µg/mL extract solutions using two ionization solvents, acetone and methanol. Acquisition time was set to 2 min (230 scans). Optimized parameters included a sheath gas set at 20 a.u., a transfer capillary voltage of 2800 V and an S-lens RF level of 50 a.u., the other parameters were set to default. The Thermo Scientific

Q-Exactive™ was calibrated with Pierce LTQ Velos ESI Positive and Negative Ion calibration solutions and lock-mass calibration was applied on palmitic acid during acquisition (deprotonated molecule $[M–H]^-$ *m/z* 255.2330 and protonated molecule $[M+H]^+$ *m/z* 257.2475). Experiments were performed in a single batch with analytical triplicates. The system was rinsed with the working solvent up to noise level between each sample. No loss of sensitivity was observed when rerunning samples as control.

Atmospheric Solids Analysis Probe Mass Spectrometry (ASAP-MS) analyses were acquired on a Maxis 4G spectrometer (Bruker Daltonics, Bremen, Germany) equipped with an APCI ion source and a direct insertion probe (DIP). The extracts were deposited on Marienfeld melting point capillaries as homogeneous solutions and dried with nitrogen before insertion in the APCI source. Preliminary tests having shown a major thermolability of some compounds, analyses were performed with a temperature ramp-up (50 °C every 15 sec) over 2 min. The temperature range covered was therefore 100-400 °C with 120 scans in the range *m/z* 100-2000. Source parameters were optimized as following with a nebulization gas (nitrogen) pressure of 2.5 bars and a dry gas at 2.5 $L.min^{-1}$. Voltages were set to: 500 V for the transfer capillary, -500 V for the end plate offset, with a 2000 V charging voltage and a 4000 V working voltage. An ion-cooler of 175 V_{p-p} was applied on the pusher. The Corona discharge was set to 500 nA for positive ionization and 2000 nA for negative ionization. External calibration of the spectrometer was done with PEG 600 in positive mode and PEG diacid 600 in negative mode. Lock-mass calibration was applied on palmitic acid by post-processing with R-script.[27] During the batch two replicates were performed to obtain a total of 8 acquisitions on the merged data for interpretation. Experiments were performed as a single batch with a thermal desorption to noise level between each run, no loss of sensitivity was observed on controls.

Bioinformatic Data Processing.

Thermo Scientific *.RAW files were converted to *.mzXML using readw 4.3.1 and Bruker *.d folders were converted to *.mzXML using Bruker CompassXport 3.0.6.9 from the command line. The obtained files were processed with R-script and Microsoft Visual Basic, following the Seven Golden Rules of MF determination,[16] every step was controlled manually while encoding the scripts, and exported in the form of *.csv files.

Peak lists were extracted from the *.mzXML files using R packages `xcms`,[28–30] `MSnbase`,[31] and `CAMERA`,[32] and automated instrument detection was performed with the `readMzXmlData`[33] package. The peak picking parameters were optimized for each instrument. Isotopic contributions calculation and clustering followed by deisotoping were performed with a 3 ppm tolerance using element restrictions depending on *m/z* as described in the Seven Golden Rules. For this purpose the mass ranges were refined according to the compounds referenced in the online Dictionary of Natural Products (DNP) v26.2[34] (**Table 1**). After an in-depth study of the mass spectra, it appeared that isotopic contributions could not be seen for monoisotopic ions of less than 0.01 % relative intensity, so this value was set as a threshold for the calculation of isotopic clusters.

The molecular formulae were then calculated by querying the online platform ChemCalc[35] with R-script, taking into account the calculated isotopic patterns, allowing an error of 4 carbons and 2 nitrogens. $[M+Na]^+$ adducts were further accounted for, when generating the formulae for ESI-MS. When no isotope pattern was detected, **Table 1** features were considered. However, sulfur and chlorine were not considered unless detected. The absolute mass range error was set to 1.0 milli-unified atomic mass (mu) for orbitrap data (DI-ESI-MS) and 1.7 mu for Q-TOF data (ASAP-MS). Based on the Double Bond Equivalent (DBE) the script filtered out the formulae corresponding to radical ions for ESI-MS. The results were coherent with manual MF

194 determination of known compounds and yielded most often a single formula, though the script was
195 able to calculate the five best formulae.

196 The different elemental ratios were then calculated with Visual Basic in order to filter the
197 formulae according to the Seven Golden Rules to remove the potentially incoherent ones. An
198 adduct correction was applied according to the formulae and DBE with the following considered
199 adducts: ASAP-MS: $M^{+\bullet}$, $[M+H]^+$, $M^{-\bullet}$, $[M-H]^-$ and ESI-MS: $[M+H]^+$, $[M+Na]^+$, $[M-H]^-$,
200 $[M+Cl]^-$. The corresponding exact molecular masses were subsequently calculated.

201 Chemical classes associated with the Van Krevelen diagram were retrieved from the
202 literature and double-checked against an in-house database of lichen compounds, the complete
203 projet is available[12] (see **Technical validation**). The *Van Krevelen coordinates i.e. the H/C and*
204 *O/C ratios* were then imported in R-script and merged in a single matrix that can be filtered by all
205 experimental variables. A rectangle approximation of the chemical groups presented **Figure 1** was
206 applied to tag the detected compounds, which were filtered for plotting in the form of a histogram
207 (relative proportion within a method) stacked with a heatmap (absolute count of detected features).
208 The plots were created with R package `plot3D`[36] and were made interactive with `plot3Drgl`.
209 [37] Although the presented plots were constructed with the merged data of all lichens, the provided
210 scripts allow for species filtering (e.g. **Figure S2**). The Van Krevelen coordinates were also used
211 to plot the Van Krevelen diagrams using R-script.

212 The entire processed dataset is available at https://osf.io/6pyuq/
213 (dataset_solvents_comp_publi1.tsv) along with sample files for Q-Orbitrap and Q-TOF data in the
214 *.mzXML format. The scripts are deposited at
215 https://github.com/siollivier/directacquisitionproject.

Technical validation.

Database establishment. An in-house database of lichen compounds was compiled from the literature. The total number of structures contained in this compiled database is about 2,000 entries[12]. The database will be provided by the authors upon reasonable request.

The structural information was translated into InChIKey, a particularly popular format in chemoinformatics, because it is standardized and of fixed length, allowing easy data processing (example: mycosporine serinol, VVTDHOIRNPCGTH-NSHDSACASA-N).

A detailed classification of the lichen compounds was then obtained by submitting these InChIKeys to ClassyFire,[14] a tool to obtain a hierarchical classification of compounds, through the `classyfireR` package.[38] This structure-based bioinformatics approach allows for a comprehensive result through the establishment of a chemical taxonomy—in hierarchical order: chemical kingdom, superclass, class, subclass, and additional levels 5-9.

Evaluation of the method against a state-of-the-art classification tool. The accuracy of the classification established by **Figure 1** was examined in comparison to this database of lichen compounds. Within the database, compounds were selected according to different levels of their ClassyFire classification, which was compared to the classification obtained with Van Krevelen.

The same level of precision cannot be expected from a classification based only on the MF. However, depending on the structure of the compounds, it is possible to expect a result in one or more classes (more or less specific) of this diagram. Thus, some areas of the diagram (i.e. benzenoids or prenyl derivatives) can be considered as areas that extend more specific ones. During the evaluation of the protocol, the compounds were considered as correctly classified if their Van Krevelen annotation was in accordance with the expected matches between ClassyFire and Van

Krevelen that are shown in **Table 2**.

RESULTS AND DISCUSSION

Determination and validation of the *Van Krevelen chemical groups*.

First, a search was conducted in the literature to identify the positioning of the defined chemical groups on the Van Krevelen diagram.[18,19,39–41] These bibliographic results were then refined according to a database organized with ClassyFire and the final ranges are specified in **Figure 1**. The database used to validate these annotations mostly contains compounds of MW < 500 Da, including mainly, in terms of Classyfire classes, depsides and depsidones (> 20 %), as illustrated in **Figure 2**.

In order to guarantee the quality of the following results, it is first necessary to evaluate the accuracy of the classification on datasets not acquired in DDA. The proportion of correctly classified molecules in each class is an effective way to perform this validation.

However, two biases to this estimation should be considered: (i) the classification being based on H/C and O/C ratios, classes containing a large number of heteroatoms (N, S, *etc*.) such as amino acids are more likely to be incorrectly classified. Others have addressed this issue by including various elemental ratios, but this promising approach has yet to achieve the same level of specificity for classification;[42] (ii) some ClassyFire classifications include very structurally diverse derivatives within the same class, particularly for compounds with a lipid or carbohydrate function.

The proportions of molecules correctly classified in each of the classes considered are presented in **Figure 3**. As expected, amino acids and analogues, fatty acyls and carbohydrates have a lesser overlap between the two classifications, with respectively 56%, 59% and 68% of

compounds being correctly classified.

On the other hand, all the other structural classes considered have an overlap of more than 70%. For 4 of the 10 structural types considered, the similarity between the two classifications is even higher than 90%. These are benzenoid derivatives, chromones, depsides and depsidones as well as prenol lipids, which include terpenes. These chemical families are among the most frequently reported in lichens, as illustrated in the recently published tandem mass spectra database of the most common lichen compounds.[12]

The encouraging results of the method provided by processing non-experimental data gave us the incentive to proceed to a practical evaluation. In the following case study, this method is used to obtain a rapid evaluation of the diversity of compounds detected by multiple ionization techniques, presenting a comparison of results from two DI-ESI-MS conditions vs ASAP-MS.

Case Study: Evaluation of the Metabolic Diversity of Lichen Extracts.

In order to cover a large panel of structures from lichen extracts, four lichen samples known to have very distinct metabolite profiles were chosen (the number of compound in the ClassyFire classes are given in **Table S2** for each lichen). Protocols used for each lichen species (two single-step and two three-step ones) were established to have a comparison with a simple extraction generally used in chemotaxonomic studies in lichenology and more exhaustive extractions (pooling successive extracts from polar to apolar ones) (**Figure S1**). These extracts were analyzed with DI-ESI-MS and ASAP-MS to compare the performance of these techniques to reveal lichen compounds and evaluate their distribution in structural families (**Figure S2**).

The DI-ESI-MS experiments were conducted with two ionization solvents, acetone and methanol since this aspect of the experiment heavily impacts the compound detection. Indeed, with

positive ion mode data, Principal Component Analysis (PCA) shows that the two ionization solvents form distinct groups on the PC2 component (**Figure S3**). PC1 clearly differentiates DI-ESI-MS from ASAP-MS analysis and post-treated data with an adduct correction resulted in a slightly better clustering of samples with regard to raw data. While the positive ion mode detects a huge number of compounds when compared to the negative ion mode, the Van Krevelen diagram provides a broad and clear overview of the chemical diversity of the diverse chemical families in lichens. The complementarity of these three methods is also revealed through the distinct zones mostly covered by these 3 MS conditions on the Van Krevelen diagram with all discriminant coordinates (**Figure S4**).

In other words, a single detection mode is not sufficient to cover the metabolic diversity of lichen compounds and each technique is more or less suitable for a class of compounds to be revealed. This assumption was verified using a bioinformatic quantification of the number of molecules ionized in various samples obtained with different extraction protocols: depending on their MF, a structural class was assigned to the compounds according to the cartography presented **Figure 1**.

With regard to the relative performance of the extraction protocols compared in this study the quantitative and the qualitative aspects are considered. Comparing extractions with a single solvent, yields are higher when a polar protic solvent (i.e. methanol) is used instead of a polar aprotic solvent (i.e. acetone). The Cyclohexane-Acetone-Methanol (CAM) and Cyclohexane-Acetone-Water (CAW) extractions gave yields in the same range compared to a single methanol extraction (**Figure S1**).

Considering the performance of the MS techniques used to reveal the composition of extracts, the results obtained with the two multi-step solvents were analyzed, which are considered

to be the more exhaustive. In **Figure 4** ASAP-MS allowed the detection of more compounds than ESI-MS in negative mode (average number of ions: 424/run for ASAP-MS vs. 116/run for ESI-MS in acetone), but fewer in positive mode (average: 482/run for ASAP-MS vs. 1434/run for ESI-MS in acetone). Furthermore, these results are coherent with what could be expected when considering the protic solvent selected, and the most appropriate extraction and ionization methods for each class of compounds are summarized in **Table 3**. In addition, the comparison with the in-house database[12] exhibits differences for Fatty acyls or Amino-acids, as examples, with higher frequencies in the experimental data than in the database.

Regarding the extraction protocols.

The successive extraction steps method appears to be better suited than the methanol extraction to gather terpenes and prenol derivatives (respectively 2.0 and 1.5 times more, in positive ASAP-MS), while 1.3 times more carbohydrates were extracted with water (negative ESI-MS in methanol).

A comparison with the single-step extractions (**Figure S5**) shows that an acetone extraction would appear as a good choice for studies targeting polyphenols & derivatives or condensed aromatic structures as well as terpenes. On the other hand, as methanol has the closest extraction profile to that of the successive solvent extraction and comparable yields, it seems to be a good compromise. However as expected with a protic solvent fewer terpenes are extracted with methanol.

Regarding the MS methods.

Compared to ESI-MS, ASAP-MS favors the ionization of terpenes (relative frequency within an ionization method up to r.f. 20 %) and unsaturated hydrocarbons (r.f. 10 %) in positive mode, and

benzenoids (r.f. 35 %) in negative mode. However, ASAP-MS appears less favorable for polyphenols & derivatives but, interestingly, quite favorable for condensed aromatic compounds (up to r.f. 20% in some conditions, see **Figure 4d**).

This piece of data also shows differences between the two DI-ESI-MS ionization solvents that may be of use for targeted studies: in negative mode, carbohydrates and fatty acyls' ionization is superior in methanol (15 vs. 10 % and 20 vs. 10 % r.f. respectively) whereas that of polyphenols & derivatives and condensed aromatic compounds is better in acetone (40 vs. 25 % and 15 vs. 10 % respectively). Differences are less marked in positive ionization, except for the condensed aromatic structures that are better ionized in methanol (> 15 vs. 10 %). Such compounds are common occurrences in lichens (e.g. depsides for polyphenols & derivatives and xanthones for condensed aromatic compounds).[21,43]

Furthermore, in positive mode there appears to be a similar ionization profile for benzenoids in all ionization methods (**Figure 4**, **Figure S5**), questioning the complementarity of the methods in this aspect. A referral to the Van Krevelen diagram however reveals that the ionized compounds have some discriminant elemental ratios with each method (Figure 5). VK discriminant coordinates are clearly found in low H/C ratio for ASAP-MS and in high H/C ratio for DI-ESI-MS in Methanol, whereas DI-ESI-MS in Acetone as few discriminant coordinates, showing a complementarity within the ionization modes. A referral to the Van Krevelen diagram however reveals that some elemental ratios are common to 2 or 3 ionization modes (**Figure 6**). Moreover, an overall higher number of occurrences in acetone extract is clearly observed, with a specificity in high H/C ratio– once again showing the usefulness of the Van Krevelen diagram for natural products metabolomics. To illustrate the potential of the use of Ambient MS for validation or new identification of Natural Product, in comparison with the in-house database, classical expected compounds but also new

coordinates are found in DI-ESI-MS and ASAP-MS as proofs of principle (**Figures S6-S10**).

Overall, the interest of the Van Krevelen diagram was demonstrated for the structural classification of metabolites (without the need to acquire tandem mass spectra data), and its applicability was shown with real-life cases by studying the chemical composition of lichens.

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TABLES

Table 1. Element restriction ranges established from the online Dictionary of Natural Products v26.2.

Mass range [Da]	C max	H max	N max	O max	S max	Cl max
< 200	15	30	8	7	6	4
< 400	30	58	10	14	12	7
< 600	42	86	13	21	12	8
< 800	56	108	16	25	20	10
< 1000	66	126	25	37	20	11
< 1500	100	182	26	44	20	11

476 **Table 2.** Matches between ClassyFire and the Van Krevelen diagram for the most common lichen
477 metabolites. The matches were determined to fit properly with the usual classification of lichen
478 compounds by Huneck and Yoshimura.[21]

ClassyFire Classification (level)	Expected Area(s) in the Van Krevelen Diagram
Amino Acids and analogues (subclass)	Amino acids; Nucleic acids
Anthraquinones (subclass)	Condensed aromatic compounds
Benzene derivatives (class)	***Polyphenols and derivatives***; Benzenoids
Carbohydrates and conjugates (subclass)	Carbohydrates
Chromones (level 5)	***Polyphenols and derivatives***; Benzenoids
Depsides and depsidones (class)	***Polyphenols and derivatives***; Benzenoids
Dibenzofurans (subclass)	Condensed aromatic compounds; Benzenoids
Fatty acyls (class)	Fatty acyls; Prenol derivatives
Prenol lipids (class)	Terpenes; Prenol derivatives; Unsaturated hydrocarbons
Xanthones (level 7)	Condensed aromatic compounds

479

480 **Table 3.** An overview of the most efficient parameters in this study for the extraction and MS
481 analysis of lichen metabolites, with regards to the number of compounds ionized and the amount
482 of other metabolites that may hinder the interpretation for each structural class.

Class	Extraction	Ionization	MS Polarity
Carbohydrates	CAW	ESI in methanol	NEG
Amino Acids	Methanol	ESI in acetone	POS
Nucleic Acids	CAW	ESI in acetone	NEG
Prenol Derivatives	CAM	ASAP (APCI)	POS
Fatty Acyls	Methanol	ESI in methanol	NEG
Unsaturated Hydrocarbons	CAM	ASAP (APCI)	POS

Terpenes	CAM or Acetone	ASAP (APCI)	POS
Benzenoids	Acetone	ESI in acetone	POS
Polyphenols and derivatives	Acetone	ESI in acetone	NEG
Condensed Aromatic Compounds	Acetone	ESI in methanol	POS

FIGURE LEGENDS

Figure 1. Identification of the chemical groups on the Van Krevelen diagram.

Figure 2. (a) The distribution of the molecular weights of the compounds contained in the validation database (LDB-Lit, solid line) shows a good overlap with the experimental data (dotted line); (b) The Classyfire classes of the compounds in the LDB-Lit illustrate the diversity of the molecules used for technical validation.

Figure 3. Proportions of molecules in the database accurately classified by the Van Krevelen diagram (in accordance with **Table 2**).

Figure 4. Evaluation of the chemical diversity for the merged data of the three-step extractions: 'Cyclohexane-Acetone-Methanol' in positive mode (a) and negative mode (b), and 'Cyclohexane-Acetone-Water' in positive mode (c) and negative mode (d). Both the relative frequency for each technique (histogram, vertical axis) and the absolute number of detected ions (heatmap) are presented. The relative frequency represents the proportion of a chemical class within a given ionization method. The database content is proposed in comparison to show the existing potential in new identifications.

Figure 5. Extracted Van Krevelen diagram for benzenoids i.e. in the (O/C 0.2-0.4; H/C 0.8-1.8) range, in all three ionization methods for the acetone (a) and Cyclohexane-Acetone-Methanol extracts (b). The size of the circle, represented by a coefficient of expansion (cex), is proportional to the number of occurrences of the (O/C, H/C) coordinates without correction factor (100 gives a cex of 100). The colors (green, blue and red, alpha of 0.4) indicate respectively discriminant VK coordinates for DI-ESI-MS in Methanol, ASAP-MS and DI-ESI-MS in Acetone ionization mode.

Figure 6. Extracted Van Krevelen diagram for benzenoids i.e. in the (O/C 0.2-0.4; H/C 0.8-1.8) range, in all three ionization methods for the acetone (a) and Cyclohexane-Acetone-Methanol extracts (b). The size of the circle is proportional to the number of occurrences of the (O/C, H/C) coordinates with a correction factor of 0.1 (100 gives a cex of 10). Non-discriminant VK coordinates for a single ionization mode are plotted in violet.

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Author Notes

SO, PJ, FLD, JB and NLY designed the experiments. SO and FLD selected the lichen material, which was identified by JB. DO optimized the ASE protocol; SO, PJ and FL carried out the experiments. SO and NLY encoded the R-scripts and Visual Basic macro used for data processing. DO compiled the database for validation, DO and SO curated the database. All authors contributed to the redaction of the manuscript. None of the authors declare any competing financial interest.

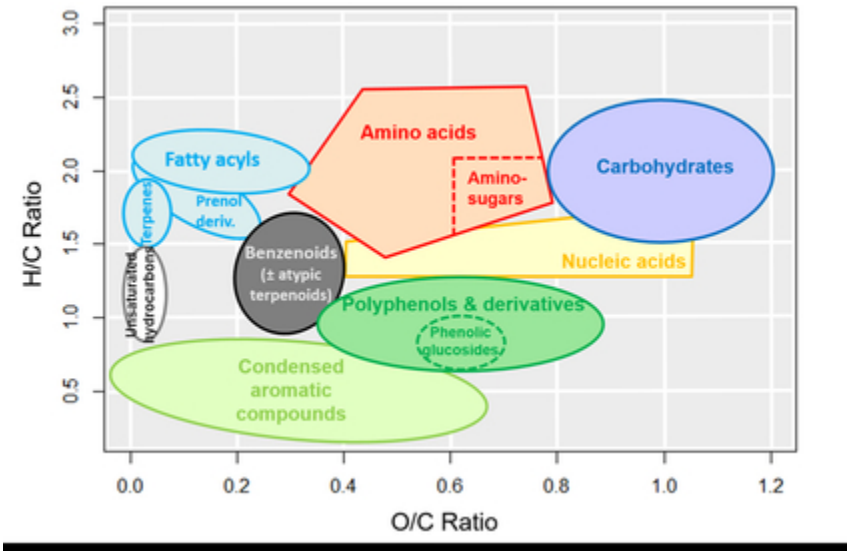

Figure 1. Identification of the chemical groups on the Van Krevelen diagram.

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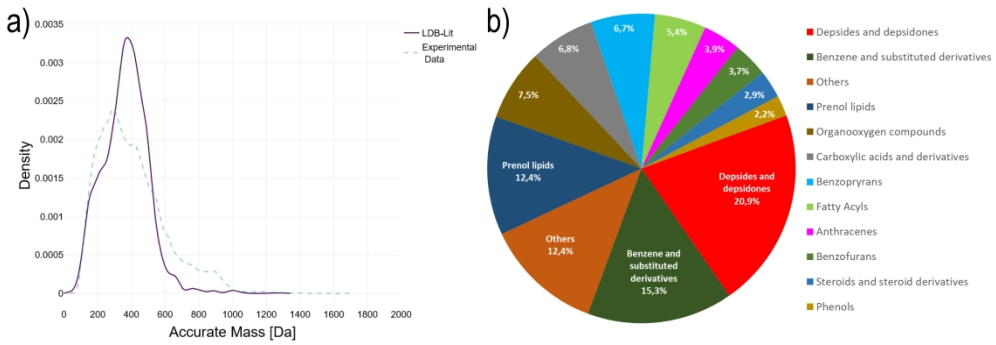

Figure 2. (a) The distribution of the molecular weights of the compounds contained in the validation database (LDB-Lit, solid line) shows a good overlap with the experimental data (dotted line); (b) The Classyfire classes of the compounds in the LDB-Lit illustrate the diversity of the molecules used for technical validation.

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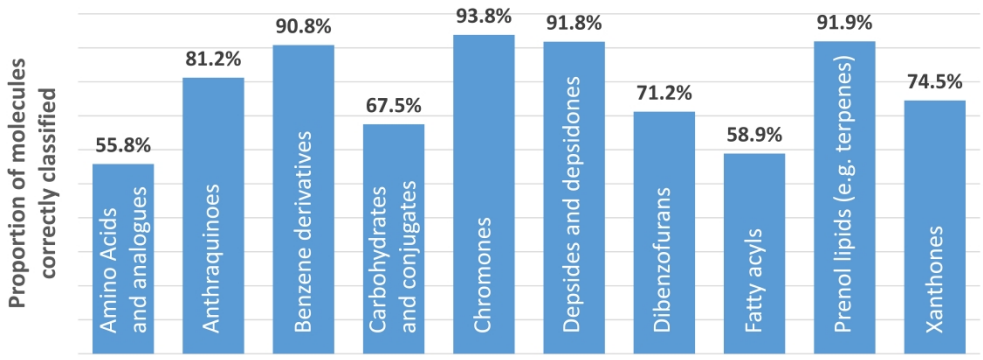

Figure 3. Proportions of molecules in the database accurately classified by the Van Krevelen diagram (in accordance with Table 2).

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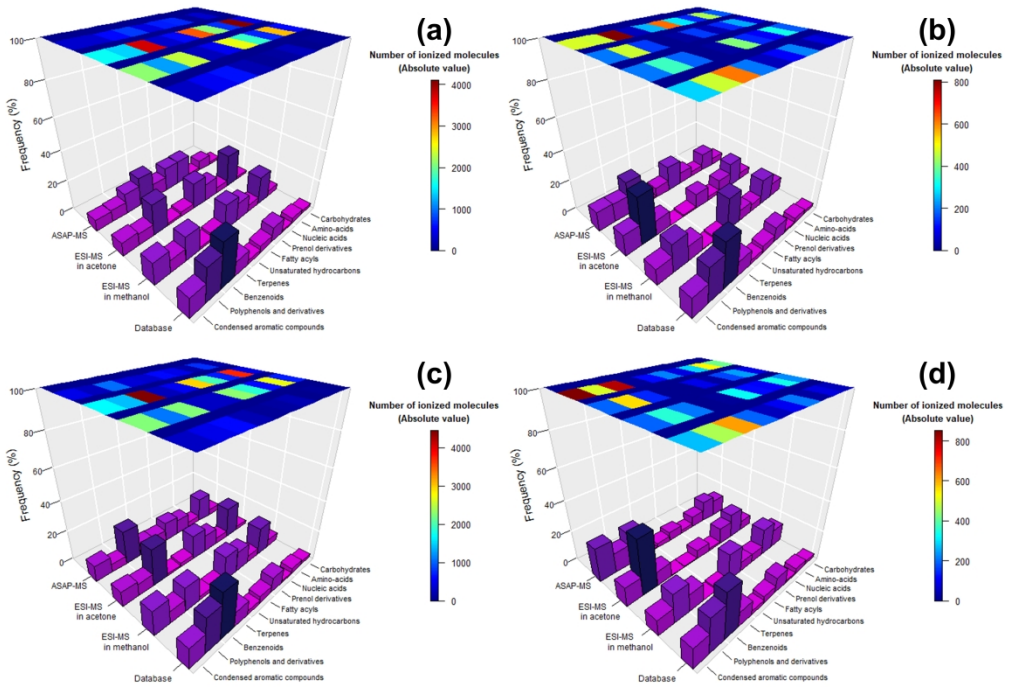

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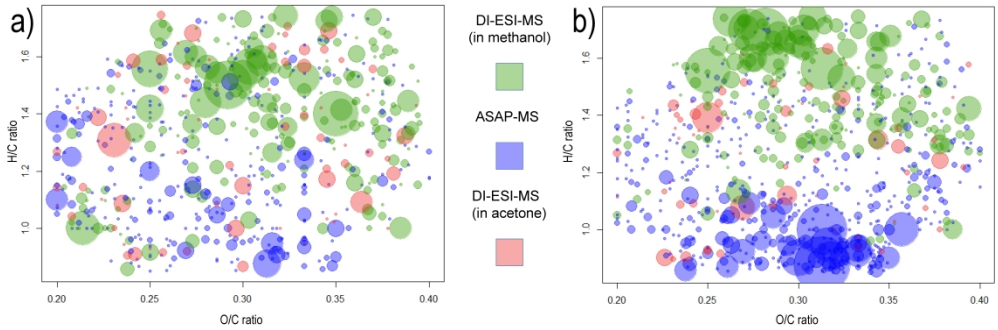

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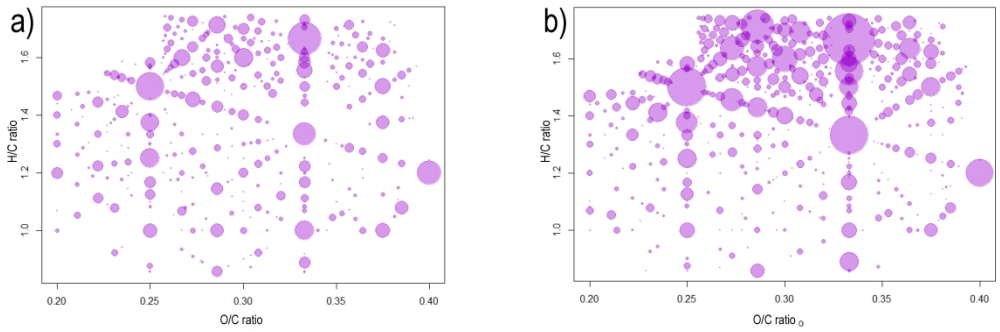

Figure 6. Extracted Van Krevelen diagram for benzenoids i.e. in the (O/C 0.2-0.4; H/C 0.8-1.8) range, in all three ionization methods for the acetone (a) and Cyclohexane-Acetone-Methanol extracts (b). The size of the circle is proportional to the number of occurrences of the (O/C, H/C) coordinates with a correction factor of 0.1 (100 gives a cex of 10). Non-discriminant VK coordinates for a single ionization mode are plotted in violet.

950x328mm (200 x 200 DPI)