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Analytical Methods

ARTICLE

Wood Chemotaxonomy via ESI-MS profiles of phytochemical markers: The challenging case of African versus Brazilian Mahogany woods

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The harvest of the Brazilian Mahogany (\textit{Swietenia macrophylla}) is a main cause of the Brazilian Amazon deforestation and has been therefore prohibited. African Mahogany (\textit{Khaya ivorensis}) was then introduced for Amazon reforestation and the commercialization of such wood is legal, thus creating a challenging problem of wood certification. Herein we report that a wood chemotaxonomic method based on distinct profiles of phytochemical markers is able to promptly characterize both the native and foreign Mahogany species. This challenging task has been performed via a simple, fast and unambiguous methodology using direct electrospray ionization mass spectrometry (ESI-MS) analysis of a simple methanolic extract of a tiny wood chip. Typical limonoids such as khivorin, khayanolide A and mexicanolide for the African mahogany and phragmalin-type limonoids for the native Brazilian species, as well as distinct polyphenols such as catechin derivatives and cinchonain form the characteristic phytochemical markers pools for both species. This rapid methodology could be used therefore to monitor legal and illegal mahogany tree harvesting, and hence to control Amazon deforestation. It could also be applied to create a wood certification program for African and Brazilian mahogany trees, as well as for wood certification in general.

Introduction

Mahogany (\textit{Swietenia macrophylla}) - also known as "green gold" - is probably one of the most precious wood species from the Brazilian Amazon. Due to the superior aesthetics, physical characteristics and ease of woodworking, Mahogany has been used to produce noble and luxury furniture items.\textsuperscript{1}

During the 1990’s, millions of cubic meters of native Mahogany were removed from the Amazon forest,\textsuperscript{2} and this devastation is listed among the main causes of the dramatic Brazilian Amazon forest deforestation. Consequently, mahogany was included in 2002 in Appendix II of the Convention on International Trade in Endangered Species (CITES), which established strict regulation of international trade of an endangered species.\textsuperscript{3} Due to its endangered status and importance in the global market, Mahogany is the focus of many efforts towards its conservation, harvesting and regeneration.\textsuperscript{4}

In 2003, the Brazilian government prohibited the harvesting of Mahogany trees.\textsuperscript{5} Even though several legal actions are in place to counter illegal logging and the subsequent trade, there is however a lack of effective mechanisms to identify the origin of timber and wood products. To solve this problem, the Brazilian forest certification program (Cerflor) was established in 2002, and has been developed by the National Institute of Metrology, Quality and Technology (INMETRO).\textsuperscript{6}

\textit{Khaya ivorensis}, which occurs on the West Coast of Africa from Sierra Leone to Cabinda, is also a famous African Mahogany species. Due to its high-quality timber and its high resistance to drill pointer (\textit{Hypsipyla grandella}), the major pest of Brazilian mahogany (\textit{S. macrophylla}), the African mahogany has been increasingly used for Amazon reforestation. This tree specimen was found to grow about 30% faster than Brazilian mahogany. Currently, it is estimated that there are over one million African mahogany trees planted in Brazil and at investments are increasing in its culture.\textsuperscript{7}

Brazilian and African Mahogany species belong to the same \textit{Meliaceae} family and \textit{Swietenioidae} subfamily differing only in genera but most importantly in their pools of phytochemicals. The African Mahogany belongs to the \textit{Khaya} genus whereas the Brazilian Mahogany belongs to the \textit{Swietenia genus}.\textsuperscript{8} The \textit{Meliaceae} family is characterized by the presence of limonoids with a large range of biological activities.\textsuperscript{9,10,11} The \textit{Khaya} genus is closely related to the \textit{Swietenia} genus, but is known to exhibit unique phytochemical markers. For instance, several limonoid classes, such as khivorins, angolenates, mexicanolides and fissionolides have been isolated from
different parts of K. ivorensis\textsuperscript{12,13,14} whereas S. macrophylla shows mainly phragamalin-class limonoids.\textsuperscript{15,16,17,18} The configuration at C-6 of mexicanolides, phragmals and khyanolides from Khaya is also of the 6S configuration whereas those from Swietenia species are 6R. These metabolic differences indicate that their chemotaxonomic differentiation is feasible.

Direct infusion mass spectrometry (MS) using electrospray ionization (ESI-MS) has been widely applied for rapid, direct and effective fingerprinting characterization of complex mixtures including those of extracts of natural products.\textsuperscript{19,20,21,22,23,24} Recently, both ESI-MS as well as ambient\textsuperscript{25} Venturi easy ambient sonic-spray ionization MS (V-EASI-MS)\textsuperscript{26} fingerprinting have been applied to characterize typical phytochemical markers\textsuperscript{16,17} which were found to be unique to the Brazilian mahogany and absent in other typical of very similar morphology but quite contrasting Brazilian wood families.\textsuperscript{27} Herein, direct ESI-MS fingerprinting of a simple methanolic extract obtained from a tinny wood chip in which pools of phytochemical markers are detected was tested in a much challenging task: to promptly and effectively differentiate woods from Brazilian and African mahogany trees which belong to different species but to the same tree family.

Experimental

Wood samples

Samples of certified African Mahogany (K. ivorensis) were donated by the Brazilian Agricultural Research Corporation (EMBRAPA – Oriental Amazon). The African mahogany was raised in the city of Belem, in Para State in Brazil; Wood pieces of certified Brazilian Mahogany (S. macrophylla) were donated by a local lumberyard. We ensure that no Brazilian Mahogany tree was harvest to conduct this work.

Sample preparation

An extract of the wood sample was prepared “in situ” before analysis. The most external layers were discarded to avoid the sampling of oxidized compounds or even some possible contamination. For Brazilian mahogany (BM), the samples were randomly collected from wood pieces and mixed just before extraction. For the African Mahogany (AM), the samples were collected via the whole tree stem cross section radius, from sampling points spaced by 3 cm from each other (Scheme 1). After the collection, the wood samples were cut into small pieces (ca. 0.5 mm of diameter) and 10 µL of methanol (HPLC Grade, Tedia, Brazil) was added for each 1 mg of wood precisely weighed. Samples were vortexed for 2 min and then centrifuged for 5 min in a microtube centrifuge. Methanolic extracts were then diluted (1:100 v/v) in methanol with 0.1% of ammonium hydroxide for ESI(-)-MS. For ESI(+)-MS, 2 µL of a sodium chloride 0.1 mmol-L\textsuperscript{-1} aqueous solution was added at the final solution to favor the formation of sodium adducts.

Scheme 1. Sampling scheme for African Mahogany wood. The samples were collected in selected parts throughout the whole ratio of the tree stem cross section.

ESI-MS and ESI-MS/MS analysis

ESI-MS and ESI-MS/MS data were acquired in both the negative and positive ion modes using a QTOF (Micromass, Manchester, UK) mass spectrometer. The operation conditions were as follows: 3.0 kV capillary voltage, 100 °C source temperature, desolvation temperature of 100 °C, sampling cone voltage of 30 V and extraction voltage of 3.0 V. The diluted methanolic extract was directly injected into the ESI source by using an automatic injection pump (Harvard Apparatus) with a continuous flow of 10 µL·min\textsuperscript{-1}. The full scan ESI-MS were acquired in the range of m/z 50 to 2000 and the total time for acquisition of each spectrum was set at 2 min, at an acquisition rate of 1 scan per second. The ESI-MS/MS were obtained via collision-induced dissociation (CID) and acquired from m/z 50 to m/z values lightly above that of the ion under study. Argon was used as collision gas, with collision energies varying from 10 to 40 eV, optimized for each ion. Spectra were processed using the Masslynx 4.0 software (Waters, Manchester, UK). The TOF analyzer was daily calibrated with a 0.1% (v/v) phosphoric acid solution in acetonitrile/water 1:1 (v/v). The same solution was used for internal lock-mass calibration in ESI-MS acquisition.

For an unambiguous molecular attribution, FT-ICR-MS analysis was performed in a Thermo Scientific 7.2 T electrospray ionization Fourier transform ion cyclotron resonance mass spectrometer (Thermo Scientific, Bremen, Germany). A scan range of m/z 200–1000 was used, and 100 microscans were summed in each acquisition. The average resolving power (Ro) was 400,000 at m/z 400. Time-domain data (ICR signal or transient signal) were acquired for 700 ms. microscans were co-added using Xcalibur version 2.0 (Thermo Scientific).

Results and Discussion

ESI-MS QTOF fingerprinting

Woods are mainly composed of cellulose, hemicellulose and lignin \textsuperscript{28} and such composition is known to vary as a function of several parameters such as tree part, geographic origin and environmental conditions.\textsuperscript{29} The most detailed identification of trees has been commonly achieved not based on these major constituents but on the analysis of the minor constituents in an approach known as chemotaxonomy.\textsuperscript{30,31} Such minor relatively low MW constituents, known as extractives (around 4-10%), can be
obtained from the wood sample via extraction with water or organic solvents such as methanol.\textsuperscript{32, 33}

The composition of the methanolic extracts of Brazilian and African mahogany wood were therefore investigated using high resolution ESI-MS. Figure 1 shows the ESI(+)-QTOF spectra of the methanolic extracts for both African (AM) and Brazilian mahogany (BM). Figure 1a is a representative spectrum for an extract obtained from a pool of wood fragments collected from all the sampling points of the AM stem cross-section (Scheme 1), whereas Figures 1b-d shows the spectra of three different BM samples.

![Figure 1. ESI(+) -MS of the methanolic extracts for (a) AM and (b-d) three BM samples from three different trees.](image)

Note that the differences between the phytochemical markers detected in the mass spectra for the AM and BM samples are truly remarkable. All the 3 BM samples display a set of very abundant ions in the \( m/z \) 700 to 900 range (Figs 1b,c,d) whereas in the AM spectrum (Fig. 1a) no ions of significant abundances are detected in this \( m/z \) range. Both trees belonging to the same family are quite morphologically similar hence they are hard to distinguish via visual inspection, but their different genus strong impacts the chemical profile of secondary metabolites obtained via simple and rapid methanolic extraction.

The phytochemicals present in the methanolic extracts were also investigated via ESI(-)-QTOF. As Figure 2 shows, the spectra for BM and AM samples are quite similar which a set of common ions such as those of \( m/z \) 289, 577 and 865, but again differentiation is properly attained via the presence of a very abundant and unique marker ion of \( m/z \) 451 which unambiguously characterizes the BM samples. This ion is barely detected in the AM extracts.

![Figure 2. ESI(-)-MS of the methanolic extracts for (a) AM and (b-d) BM samples from three different trees.](image)

**FT-ICR-MS analysis with molecular formula attribution**

The secondary metabolites identified as phytochemical ion markers for both AM and BM samples may belong to several classes of natural products such as flavonoids, terpenes, phenols, alkaloids, sterols, waxes, fats, tannins, sugars, carotenoids, polyphenols, and limonoids.\textsuperscript{34, 35} These molecules play important roles in the plant metabolism\textsuperscript{36} and important phytochemicals have been identified in mahogany trees using different analytical tools.\textsuperscript{37} These extractives are complex mixtures of several isobaric species and, of course, isomeric species which cannot be separated by MS. To obtain unambiguous molecular formulas for these marker ions via accurate (< 1 ppm) mass measurements, FT-ICR-MS analysis of the extracts with ultra-high resolution and accuracy was performed (Tables 1-3).

Table 1 summarizes the attributions for the ESI(+)-FT-ICR-MS ions from BM samples. Note that the presence of phragmalin-type limonoids in the BM extracts has been indicated by ESI(+)-MS analysis\textsuperscript{27} and by other classical phytochemical approaches.\textsuperscript{15, 16, 18, 38.}
Table 1. Molecular formula and DBE (Double Bond Equivalents) of [M + Na]⁺ ions attributed to markers phytochemicals via ESI(+)−FT-ICR-MS analysis in the methanolic extracts of BM samples.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Theoretical m/z by FTICR MS</th>
<th>Error (ppm)</th>
<th>Molecular Formula</th>
<th>Possible components or isomers</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>735.26</td>
<td>735.26</td>
<td>-0.51</td>
<td>C₃₀H₁₇O₁₀Na⁺</td>
<td>(3) Swietenenigrin J</td>
<td>[18]</td>
</tr>
<tr>
<td>795.28</td>
<td>795.28</td>
<td>-0.51</td>
<td>C₃₀H₁₆O₁₀Na⁺</td>
<td>(2) Swietenin D</td>
<td>[17]</td>
</tr>
<tr>
<td>827.30</td>
<td>827.30</td>
<td>0.00</td>
<td>C₃₀H₁₅O₁₀Na⁺</td>
<td></td>
<td></td>
</tr>
<tr>
<td>867.39</td>
<td>867.39</td>
<td>-1.05</td>
<td>C₃₀H₁₄O₁₀Na⁺</td>
<td>(8) Swietenenol D</td>
<td>[17]</td>
</tr>
<tr>
<td>837.29</td>
<td>837.29</td>
<td>17</td>
<td>C₃₀H₁₄O₁₁Na⁺</td>
<td></td>
<td></td>
</tr>
<tr>
<td>877.05</td>
<td>877.05</td>
<td>0.52</td>
<td>C₃₀H₁₃O₁¹Na⁺</td>
<td>(4) Swietenenol C</td>
<td>[17]</td>
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<td>869.31</td>
<td>869.31</td>
<td>15</td>
<td>C₃₀H₁₂O₁₁Na⁺</td>
<td>(5) 2-Acetoxyswietenenolide</td>
<td></td>
</tr>
<tr>
<td>877.31</td>
<td>877.31</td>
<td>0.53</td>
<td>C₃₀H₁₂O₁¹Na⁺</td>
<td></td>
<td>[17]</td>
</tr>
<tr>
<td>883.33</td>
<td>883.33</td>
<td>15</td>
<td>C₃₀H₁₁O₁¹Na⁺</td>
<td>(6) Swietenin I</td>
<td>[17]</td>
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<tr>
<td>853.14</td>
<td>853.14</td>
<td>0.50</td>
<td>C₃₀H₁⁰O₁¹Na⁺</td>
<td></td>
<td>[17]</td>
</tr>
<tr>
<td>911.33</td>
<td>911.33</td>
<td>0.51</td>
<td>C₃₀H₉O₁¹Na⁺</td>
<td>(7) Swietenin K</td>
<td>[17]</td>
</tr>
<tr>
<td>479</td>
<td>479</td>
<td>0.36</td>
<td>C₃₀H₈O₁¹Na⁺</td>
<td>Diacetoxyiswietenolide D</td>
<td></td>
</tr>
<tr>
<td>927.35</td>
<td>927.35</td>
<td>0.24</td>
<td>C₃₀H₇O₁¹Na⁺</td>
<td>(9) Swietenin M</td>
<td>[17]</td>
</tr>
</tbody>
</table>

Via ESI(−)−MS and based on common classes found in wood extracts, we postulate that mainly organic acids and polyphenols are detected, as indicated by Table 2. The major marker ion of m/z 451, which is unique in the BM extracts could be attributed to cinchonain IA/IB. Note that this molecule has also been reported in other types of tree woods such as Phyllocladus trichomanoides, Rhizoma Smilacis glabrae and Trichilia catigua, but this is the first report of this biomarker as the main polyphenol in Swietenia macrophylla.

Table 2. Molecular formula and DBE attributed to markers ions via ESI(−)-FT-ICR-MS analysis in the methanolic extracts of BM samples.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Theoretical m/z by FTICR MS</th>
<th>Error (ppm)</th>
<th>Molecular Formula</th>
<th>Possible compound name and isomer</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>289.07</td>
<td>289.07</td>
<td>0.82</td>
<td>C₁₂H₇O₂⁺</td>
<td>(+) Catechin</td>
<td>[46]</td>
</tr>
<tr>
<td>341.06</td>
<td>341.06</td>
<td>1.00</td>
<td>C₁₂H₇O₂⁺</td>
<td>Epicatechin</td>
<td></td>
</tr>
<tr>
<td>451.10</td>
<td>451.10</td>
<td>1.22</td>
<td>C₁₂H₇O₂⁺</td>
<td>Cinchonain fragment</td>
<td></td>
</tr>
<tr>
<td>577.13</td>
<td>577.13</td>
<td>1.49</td>
<td>C₁₂H₇O₂⁺</td>
<td>Procyanidin dimer</td>
<td></td>
</tr>
</tbody>
</table>

Tables 3 and 4 summarizes the ion attributions for the AM extract, whereas Figure 3 shows the chemical structures and numbers (according to Tables 1 and 3) of the most important limonoids identified in both AM and BM extracts.

Note in Table 3 that khayanolides and seneganolides-type limonoids are attributed as the major limonoids detected for the AM sample, a finding that agrees with the known chemotaxonomy of such genus. Error! Indicador não definido.

Table 4 also show polyphenols such as (−)-epicatechin/(+)-catechines as the major constituents attributed in the ESI(−) spectrum of the AM sample. Note that a series of ions were attributed to polymeric epicatechin detected in their deprotonated forms [M−H]⁻, e.g. as catechin dimers of m/z 577, trimers of m/z 865 and tetramers of m/z 1135, which are known as proanthocyanidins. This same series of polymeric tannins was also identified in the BM samples (Table 2).

Table 3. Molecular formula of [M + Na]⁺ ions attributed to markers phytochemicals via ESI(+)−FT-ICR-MS analysis in the methanolic extracts of AM samples.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Theoretical m/z by FTICR MS</th>
<th>Error (ppm)</th>
<th>Molecular Formula</th>
<th>Possible compound name and isomer</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>401.20</td>
<td>401.20</td>
<td>-0.35</td>
<td>C₂₂H₂₄O₇Na⁺</td>
<td>(10) Minaldolide</td>
<td>[13]</td>
</tr>
<tr>
<td>493.21</td>
<td>493.21</td>
<td>-0.33</td>
<td>C₂₂H₂₄O₇Na⁺</td>
<td>(11) Methyl angolensate</td>
<td>[10, 14]</td>
</tr>
<tr>
<td>509.23</td>
<td>509.23</td>
<td>-0.36</td>
<td>C₂₂H₂₄O₇Na⁺</td>
<td>(12) Methyl-6-hydroxyangolensate</td>
<td>[10, 14]</td>
</tr>
<tr>
<td>535.23</td>
<td>535.23</td>
<td>-0.42</td>
<td>C₂₂H₂₄O₇Na⁺</td>
<td>(13) Fisetinolide</td>
<td>[46]</td>
</tr>
<tr>
<td>539.18</td>
<td>539.18</td>
<td>-0.46</td>
<td>C₂₂H₂₄O₇Na⁺</td>
<td>(14) 1-O-deacetylkhayanolide E</td>
<td>[14]</td>
</tr>
<tr>
<td>541.20</td>
<td>541.20</td>
<td>-0.46</td>
<td>C₂₂H₂₄O₇Na⁺</td>
<td>(15) Khayalactol</td>
<td>[14]</td>
</tr>
<tr>
<td>551.22</td>
<td>551.22</td>
<td>-0.23</td>
<td>C₂₂H₂₄O₇Na⁺</td>
<td>(16a) 3-Acetylswieteninolide A</td>
<td>[13, 10]</td>
</tr>
<tr>
<td>555.26</td>
<td>555.26</td>
<td>-0.69</td>
<td>C₂₂H₂₄O₇Na⁺</td>
<td>(16b) 2-Hydroxyangolenside or</td>
<td></td>
</tr>
<tr>
<td>583.21</td>
<td>583.21</td>
<td>-0.59</td>
<td>C₂₂H₂₄O₇Na⁺</td>
<td>(16c) 3-O-deglycosyl-3-O-</td>
<td></td>
</tr>
<tr>
<td>609.26</td>
<td>609.26</td>
<td>-0.99</td>
<td>C₂₂H₂₄O₇Na⁺</td>
<td>acetylswietenine</td>
<td></td>
</tr>
</tbody>
</table>

Table 4. Formulas and DBE attributed to markers ions via ESI(−)-FT-ICR-MS analysis in the methanolic extracts of AM samples.
The very unique BM anion of $m/z$ 451 (Figure 4c) dissociates to a very abundant fragment ion of $m/z$ 341 likely due to the loss of one catecol moiety from the cinchonain structure. The $[M + Na]^+$ ion of $m/z$ 911, which is the most abundant ion in the ESI(+) mass spectrum of the BM extract (Figure 1a-c), dissociates as expected from its proposed structure mostly through the neutral loss of acetic acid (60 Da) to form the fragment ion of $m/z$ 851 (Figure 4d).  

Spatial distribution of phytochemicals in African Mahogany

To investigate whether different parts of a tree would provide different pools of phytochemical markers detected by ESI-MS, the variation of ESI(+) mass spectra of the methanolic extracts as a function of the stem cross section of the AM tree was monitored (Figures 5 and 6). Samples were collected from points separated by 3 cm and numbered from P1 (central point) to P8 (most external point) and bark, as detailed in Experimental Section and Scheme 1. Samples were therefore collected from the major parts of the tree, including the pith (P1), the primary and secondary xylem (P2 to P4), cambium (P5), phloem (P6 and P7), P8 (phloem inner bark) and external bark.

Figure 5 shows very similar ESI(+) mass profiles except for P7 (Fig. 5d), with an abundant and unique ion of $m/z$ 365 and most particularly for the bark (Fig. 5e), with a predominant and unique ion of $m/z$ 509. Even though the ion of $m/z$ 365 is the base peak in P7, it cannot be considered a trustable phytochemical marker to of AM, because it is not present in all the collected samples throughout the tree radius.
The ESI(-)-MS profiles (Figure 6) show an interesting trend, that is, the relative abundances of the epicatechin polymer ions, that is of the dimer (m/z 577), trimer (m/z 865) and tetramer (m/z 1153) increases as a function of tree radius, and this trend can be clearly seen, for instance in Figure 7, for the ion of m/z 865. This finding seems to agree with the knowledge that polymerization of tannins increases with tree aging.46 Another important aspect is again the uniqueness of the bark spectrum (Fig. 6e) similarly to what was observed for ESI(+) -MS. Indeed, it has been reported that the amount and variability of secondary metabolites is much higher in the bark.29 Samples from the bark should therefore be avoided when using phytomarkers of Mahogany samples for chemotaxonomy differentiation.

Conclusions

A set of well characterized phytochemical markers that can be used to differentiate both BM and AM was detected by ESI-MS. Although more accurate MS instrumentation was used in this study, the methodology should work as well in simpler mass spectrometers, such as for quadrupoles, or even portable mass spectrometers with miniaturized ion traps allowing field screening of illegal trees harvesting.

ESI-MS in both the negative and positive ion modes of a methanolic extract of a tiny piece of a wood sample has been therefore demonstrated to provide a rapid and efficient way to differentiate wood. The differentiation of the African and Brazilian mahogany samples has demonstrated that the methodology is selective enough to differentiate woods even when belonging to the same family. The concern that too distinct pools of phytochemical markers would be detected...
from different parts of the tree has also been eliminated since quite similar and characteristics profiles were obtained excepted from the bark region. We propose that this prompt and unmistakable chemotaxonomic differentiation involving simple and rapid analyses can be useful not only to investigate legal x illegal exploration of mahogany in Brazil, but could be expanded to other wood chemotaxonomic differentiation cases via both laboratory as well as field analysis.

Acknowledgements

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Notes and references

Wood Chemotaxonomy via ESI-MS

187x135mm (300 x 300 DPI)