New Phytologist

Research

Methods

Opportunities in phytochemistry, ecophysiology and wood research via laser ablation direct analysis in real time imaging-mass spectrometry

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Received: *15 July 2021*
Accepted: *11 November 2021*

New Phytologist (2021)
doi: 10.1111/nph.17893

Key words: endangered species, laser ablation direct analysis in real time imaging-mass spectrometry (LADI-MS), metabolite spatial distribution mapping, small-molecule profiling, wood analysis.

Summary

- Analysis of wood transects in a manner that preserves the spatial distribution of the metabolites present is highly desirable to among other things: (1) facilitate ecophysiology studies that reveal the association between chemical make-up and environmental factors or climatic events over time; and (2) investigate the mechanisms of the synthesis and trafficking of small molecules within specialised tissues. While a variety of techniques could be applied to achieve these goals, most remain challenging and impractical.
- Laser ablation direct analysis in real time imaging–mass spectrometry (LADI-MS) was successfully used to survey the chemical profile of wood, while also preserving the small-molecule spatial distributions. The tree species *Entandrophragma candollei* Harms, *Millettia laurentii* DeWild., *Pericopsis elata* (Harms) Meeuwen, *Dalbergia nigra* (Vell.) Benth. and *Dalbergia normandii* Bosser & R.Rabev were analysed.
- Several compounds were associated with anatomical features. A greater diversity was detected in the vessels and parenchyma compared with the fibres. Analysis of single vessels revealed that the chemical fingerprint used for timber identification is mainly determined by vessel content.
- Laser ablation direct analysis in real time imaging–mass spectrometry offers unprecedented opportunities to investigate the distribution of metabolites within wood samples, while circumventing the issues associated with previous methods. This technique opens up new vistas for the discovery of small-molecule biomarkers that are linked to environmental events.

Introduction

Trees and the wood derived from them are one of the most important raw materials on Earth (Niemz & Sondereggar, 2017). Wood makes up the largest part of forest biomass and the global estimate of carbon stored in these lignified tissues is upwards of 400 petagrams (Chave *et al.*, 2009; Beeckman, 2016). The importance of wood lies in its many and varied uses. On the one hand, its tensile strength and mechanical properties make it an optimal substance from which building materials, furnishings, musical instruments and paper products can be made. On the other hand, its hydrocarbon-rich attributes make it a quality biofuel. Beyond these uses, the tree tissue itself can be seen as an important reservoir of industrial and medicinal chemicals and food products (e.g. natural rubber derived from *Hevea brasiliensis* (Willd. ex A.Juss.) Müll.Arg.; drugs such as paclitaxel derived from *Taxus brevifolia* Nutt.; maple syrup harvested from *Acer saccharum* Marschall; and tree resins derived from species such as several within the Pinaceae family). This same tissue can provide a faithful historical record of natural and anthropogenic phenomena that have occurred over the life of the tree, the study of which falls under the discipline of dendrochronology. Because it is a valued commodity, there has been unrelenting pressure on numerous timber species that has led, in some cases, to their near extinction. Even though several regulatory mechanisms exist for their protection (e.g. convention on international trade in endangered species (CITES), the European Union timber regulation (EUTR), and the US Lacy Act), the illegal timber trade remains the most profitable natural resource crime (May, 2017).

It is well established that the species identity of wood defines its physical and chemical properties, which in turn specifies the uses for which it is best suited. For this reason, depending on what the wood is to be used for, there is an immense range of analytical techniques, many of which are dated, but a subset of which are state-of-the-art, that have been brought to bear for its analysis. The invention of the microscope revealed the fine structure of wood and offered the first explanations of its mechanical properties (Kisser *et al.*, 1967).

For example, the discipline of dendrochronology has for a long time relied on manual measurement of growth rings for the estimation of ring chronologies. However, recent innovations such as densitometry have shown the untapped potential of applying X-ray computerised tomography (CT) scanning to tree ring research (Van den Bulcke *et al.*, 2019). Since the observation was made that wood traits can provide insight into a tree's ecophysiology and how it responds to a changing environment (Poorter *et al.*, 2010), assessments of ecophysiology have been made through analysis of wood anatomical images of relatively small areas of woody surfaces, or complete pith-to-bark transects using microscopy. Pattern recognition and machine learning approaches have also recently been shown to have utility in accomplishing this task (De Mil *et al.*, 2018).

A significant breakthrough in wood science was the discovery of the connection between the chemistry of the lignified cell wall and its structure, and this finding has spurred interest in the molecular constituents of wood. However, the chemical make-up of wood is inherently difficult to study. This is because the attributes of the wood matrix are not easily accommodated by the available sampling techniques, particularly if the desire is to glean information about the spatial distributions of molecular constituents within wood transects. Because a significantly large surface area would need to be surveyed to glean information about recurring structural patterns from which associations between molecular composition and anatomical features can be extracted, analysis approaches must be able to accommodate fairly large samples. Furthermore, they must permit interrogation of chemical information at the hard woody surface, preferably without sample wetting, as this might distort the spatial distributions of molecules by solubilising them and causing them to travel to locations different from the tissues or organelles with which they are specifically associated. These requirements have limited the number of chemical analysis methods that have been applied to wood. Near infrared spectroscopy (NIR), which is useful for probing bulk material and requires little to no sample preparation, has been used to characterise wood chemical and physical properties (Schwanninger *et al.*, 2004; Tsuchikawa, 2007; Defoirdt *et al.*, 2017). Because the observed spectra represent the average of the contributions of all the chemical components detected, NIR generally cannot be used to extract information on the specific molecules that are present. Several mass spectrometry (MS) techniques have been applied for timber identification (Kite *et al.*, 2010; Cabral *et al.*, 2012), with direct analysis in real time-time of flight MS (DART-TOFMS) (Cody *et al.*, 2005) being the most well developed and widely used (McClure *et al.*, 2015; Musah *et al.*, 2015; Deklerck *et al.*, 2019). While MS in particular provides access to information on small molecules such as metabolites, current approaches for their detection usually require that the tissue be ground and extracted, which precludes the possibility of determining the spatial distributions of molecules in the tissue. Nevertheless, the application of mass spectrometric techniques opened new vistas in the areas of tree systems biology, metabolomics and ecophysiology. Furthermore, MS has facilitated exploration of the high chemical and structural diversity of the more than 200 000 compounds discovered to date (Dixon & Strack, 2003; Holopainen *et al.*, 2018), with the most important falling into the terpenoid, alkaloid and phenolic compound classes (Holopainen *et al.*, 2018). Several studies have reported the characterisation and isolation of such molecules within wood species (Kamnaing *et al.*, 1999; Schreiner *et al.*, 2018).

There are several reasons why it would be highly desirable to be able to analyse wood transects in a manner that preserves the spatial distributions of the small molecules present. One is the impact that the information gleaned would have on the ability to develop optimal protocols for the isolation of useful natural products. If the locales within the tissue where such compounds are concentrated are known, then more efficient protocols that are tailored to extract the compounds can be devised. A second is the potential it has for addressing the challenge of illegal trade. DART-TOFMS for timber identification shows the metabolite profile within one small region of the timber. However, by preserving the spatial distributions of these molecules, the radial variability within a tree can be preserved and used to facilitate timber identification. Third, it could be of great benefit to ecophysiology studies to enable interrogation of tree response to environmental conditions by registering the change in molecular profile in response to various stimuli. Fourth, it has the potential to be of tremendous assistance in addressing longstanding questions in the field of dendrochronology. For example, the science of utilising apparent tree rings to determine the exact year in which they were formed, and in turn associating this information with the study of climate and atmospheric conditions during different periods in history, is well developed. However, the potential absence of visible tree rings in tropical species makes this approach difficult for tropical woods. But if there are chemical markers within the wood that can be associated with climate, and these can be discovered by chemical scanning across transects, this approach could be a powerful means to 'read' the 'chemical rings', so to speak, particularly in those cases in which there are no visible rings present. The determination of metabolites and chemical markers in the wood could then be associated with climatic events or even act as a proxy in climate reconstruction. Lisec *et al.* (2006) have already proposed that metabolite profiling has important applications in diagnostic characterisation of different genetic and environmental conditions and can also aid in understanding the complex changes apparent under such circumstances. Finch and co-workers ascribe the success of geographical provenancing of timber using DART-TOFMS to be a consequence of the presence of climate responsive molecules whose abundance in the wood is regulated by local environmental conditions (Finch *et al.*, 2017). However, the ability to conduct

investigations of the types outlined above has been hampered by the limitations imposed by the absence of technologies that can be readily directed towards interrogation of the complex wood matrix.

While there is a wide variety of MS techniques that in principle could be applied to the study of wood, most remain impractical. Matrix-assisted laser desorption ionisation (MALDI) MS, secondary ion MS (SIMS), liquid extraction surface analysis (LESA), laser ablation inductively-coupled plasma (LA-ICP)-MS, desorption electrospray ionisation (DESI), nano-DESI and laser ablation electrospray ionisation (LAESI) have also been successfully used for imaging-MS (Fowble *et al.*, 2017). These techniques each require a specific sample pretreatment step that could impact the amount of useable data that can be acquired. As Fowble and colleagues indicate, successful experiments usually involve significant and often time-consuming method development, and this in turn defines the sample types that can be analysed (Fowble *et al.*, 2017). Recently, a new imaging-MS technique that combines a laser ablation system with a TOF mass analyser interfaced with a DART ion source (termed laser ablation direct analysis in real time imaging-MS (LADI-MS)) was presented, and it was shown to have utility in imaging the tissue distributions of small molecules in both biological and nonbiological matrices under ambient conditions (Fowble *et al.*, 2017, 2019). The LADI-MS approach exhibits attributes that could potentially overcome several of the bottlenecks associated with surveying the chemical profile of wood in a manner that preserves the spatial distributions of the small molecules present. Among its attractive features are that little to no sample preparation steps are required; the analysis is performed under ambient conditions at atmospheric pressure, thereby reducing the loss of small molecules under the high vacuum conditions required by other methods; the absence of the need for solvent; the ability to analyse large and irregularly shaped samples; and the ability to detect a broad range of molecules spanning the dielectric constant spectrum (Fowble *et al.*, 2017).

Here, we present the results of analysis by LADI-MS of samples of *Entandrophragma candollei* Harms (named Kosipo), *Millettia laurentii* De Wild. (named Wenge), *Pericopsis elata* (Harms) Meeuwen (named Afrormosia), *Dalbergia nigra* (Vell.) Benth. and *Dalbergia normandii* Bosser & R.Rabev, which are all of economic and forensic importance. The results illustrate and give access to information on: (1) the relationship between chemical make-up and certain wood anatomical patterns; (2) the potential for using small-molecule spatial distributions in wood for timber identification; and (3) the distribution of metabolites within wood transects.

Materials and Methods

Laser ablation direct analysis in real time imaging–mass spectrometry set-up and sample scanning

The set-up of the LADI-MS analysis was exactly as described previously (Fowble *et al.*, 2017). The wood cross-sections were placed in the system's airtight 800 ml sample chamber, which is mounted on a motorised, *x,y* moveable stage. Briefly, ion images were acquired using an Elemental Scientific Laser NWR213 laser imaging system (ESI, Bozeman, MT, USA) coupled with a DART-SVP ion source (IonSense, Saugus, MA, USA) and Jeol AccuTOF mass spectrometer (Jeol USA, Peabody, MA, USA). Laser parameters were optimised for each sample to achieve the strongest MS signal (Table 1). The scan speed used was defined by the spot size. Briefly, it was found that a scan speed of 5 $\mu m\ s^{-1}$ less than the spot size was optimal to ensure, on the one hand, that the laser did not remain at the same spot for too long while, on the other hand, enabling it to move slowly enough for sufficient ablation of the surface to occur. For the acquisition of mass spectra, the spectrum recording interval was set at 0.6 s per scan. Spectra in the mass range *m/z* 60–900 were acquired in positive-ion mode at 350°C or 500°C. The ambient conditions set for the analyses included an orifice 1 voltage of 20 V and orifice 2 and ring lens voltages of 5 V each. The ion guide voltage was set to 600 V to allow for the analysis of ions over *m/z* 60. The mass spectrometer has a resolving power of 6000 FWHM. The rate of helium flow for the DART-SVP ion source was 2.0 $l\ min^{-1}$. Calibration was achieved using polyethylene glycol (PEG) as a reference standard. The dimensions of the area to be surveyed were selected and the sample surface was ablated from left to right one line scan at a time at a constant velocity. At the end of each row there was a wash-out delay which served to prevent carry over of ions. TssPro3 software (Shrader Software Solutions, Grosse Pointe, MI, USA) was used for peak calibration and centroiding. Reconstructed ion chromatograms were created for each of the ions of interest, exported to Microsoft Excel files, changed to the Agilent file format and then imported into Iolite

Table 1 Optimised laser parameters resulting in the strongest MS signals for the samples analysed.

	Species				
Laser parameter	*Entandrophragma candollei*	*Millettia laurentii*	*Pericopsis elata*	*Dalbergia nigra*	*Dalbergia normandii*
Fluence	*c.* 12.2 $J\ cm^{-2}$	*c.* 8.3 $J\ cm^{-2}$	*c.* 9.6 $J\ cm^{-2}$	*c.* 2.0 $J\ cm^{-2}$	*c.* 2.0 $J\ cm^{-2}$
Frequency	20 Hz	20 Hz	20 Hz	20 Hz	20 Hz
Spot size	70 × 70 μm^2	50 × 50 μm^2	50 × 50 μm^2	80 × 80 μm^2	80 × 80 μm^2
Scan speed	65 $\mu m\ s^{-1}$	45 $\mu m\ s^{-1}$	45 $\mu m\ s^{-1}$	75 $\mu m\ s^{-1}$	75 $\mu m\ s^{-1}$
He flow rate	650 $ml\ min^{-1}$	750 $ml\ min^{-1}$	650 $ml\ min^{-1}$	450 $ml\ min^{-1}$	450 $ml\ min^{-1}$
Warm-up time	5 s	5 s	5 s	5 s	2 s
Wash-out time	5 s	2 s	5 s	5 s	2 s

imaging software (The University of Melbourne, Australia) (https://iolite.xyz). This software permits the coupling of the data file from the mass spectrometer to the text file created by the laser system and allows the creation of the ion images. For some samples, the line scan data were exported and heatmaps were created in RSTUDIO (RTeam, Boston, MA, USA) instead of the IOLITE software. It was discovered that optimal imaging results were obtained for very smooth surfaces, as this resulted in enhanced visualisation of anatomical features. Therefore, the roughness of the wood surface was addressed by first using a lower grain size, and then the smoothness of the surface was gradually enhanced by further sanding to the following increasing grain sizes: 24, 40, 80, 120, 150, 240, 400, 600, 800 and 1200.

Thermal desorption coupled with gas chromatography–mass spectrometry

Slivers from *P. elata*, *E. candollei* and *M. laurentii* were analysed using a 7890B gas chromatogram and a 5977B mass spectrometer (Agilent, Santa Clara, CA, USA) coupled with a Gerstel multipurpose sampler (MPS) thermal desorption unit (TDU) and cooling inlet system (CIS) (Gerstel, Linthicum, MD, USA). Slivers of wood were shaved from the sample using a razor blade and placed in microvials which were housed within TDU tubes, and desorption was conducted in splitless mode. The TDU had an initial temperature of 40°C that was increased at 100°C min^{-1} to 175°C and held for 5 min. The analytes were cryogenically trapped in the CIS on a liner packed with glass wool at a temperature of −120°C. This temperature was then increased at 12°C min^{-1} to 275°C and held for 3 min to transfer the analytes to the DB-5MS Ultra Inert column (30 m, 0.25 mm ID, 0.25 µm) (Agilent). The initial oven temperature was 40°C and was held for 4 min before increasing at 15°C min^{-1} to 300°C, where it was held for 2 min. The helium flow rate was 0.85078 ml min^{-1}. The mass spectrometer had an ion source temperature of 230°C and spectra were collected in the *m/z* range 30–600. Mass spectral analysis was performed using MASSHUNTER Qualitative Analysis Software (Agilent) and the 2017 National Institute of Standards and Technology (NIST) Library Database (NIST MS SEARCH 2.3) was used to tentatively identify compounds.

Wood species

Entandrophragma candollei, *Millettia laurentii* and *Pericopsis elata*: The authenticated stem disc samples (9.68 × 5.73 cm; 7.07 × 5.90 cm; and 7.30 × 4.04 cm, respectively) for the analysed species were acquired from the Tervuren wood collection (Royal Museum for Central Africa, Tervuren, Belgium, sample collection numbers: Tw56452, Tw57828 and Tw60955, respectively). The growth ring numbers that are indicated on the samples were assigned before the present study. For visible light images, the sanded samples were imaged using a flatbed scanner (SilverFast SE, Plus 8; LaserSoft Imaging AG, Kiel, Germany) at 1200 dpi, and stored as 48-bit colour images. The scanned area for *M. laurentii* included both sapwood and heartwood to determine whether there was a difference in metabolite presence and distribution between the two.

Dalbergia nigra and *Dalbergia normandii*: The stem disc samples (6.76 × 2.69 cm and 7.74 × 3.53 cm, respectively) were acquired from the United States Fish and Wildlife Forensics Laboratory (Ashland, OR, USA). Hyperspectral images were collected using a Video Spectral Comparator (VSC 8000; Forester + Freeman, Worcestershire, UK). The *D. nigra* images were collected at a wavelength of 645 nm and magnification of 4.8. The integration was set to 31 ms, the iris was set at 76% and the brightness was 50. The *D. normandii* images were collected at a wavelength of 725 nm and magnification of 3.82. The integration was 27 ms, the iris was set at 50% and the brightness was 50. The corresponding LADI-MS-derived ion image was then superimposed on the hyperspectral image.

Results

Association of anatomical structures to small-molecule chemistry – investigation of *E. candollei*, *M. laurentii* and *P. elata*

One of the ways in which knowledge of the spatial distribution of small molecules within wood tissue could advance understanding of tree physiology, is in its potential to reveal compartmentalisation of metabolites, because this information would hint at specialised functions that these molecules may have within various microenvironments. There are several well characterised anatomical features within wood. Examples include vessels that serve as a conduit for the transport of water and nutrients, fibres that are responsible for mechanical support, and the parenchyma, whose main function is storage. By and large, these structural features are visually apparent either unaided or facilitated by light microscopy. However, little information is known about the chemical phenotypes that are associated with these functionally distinct structures. In initiating an investigation into the possible association between anatomical features and the presence of tissue-specific metabolites, it would be most helpful to consider notable species about which considerable information has already been gathered. *E. candollei*, *M. laurentii* and *P. elata* are species whose wood anatomy is well characterised by virtue of their economic importance and their threatened status. The distribution of *E. candollei* ranges from Guinea to the Democratic Republic of the Congo (DRC) and in the south to Angola. The species is listed as vulnerable on the International Union for Conservation of Nature (IUCN) red list, although the latest assessment is from 1998 and should be updated (Hawthorne, 1998). *Millettia laurentii* is endemic to a limited area in Central Africa, from the east of Cameroon, Equatorial Guinea and Gabon to the western parts of the Central African Republic (CAR) and the DRC (Saha Tchinda *et al.*, 2018). The IUCN red list classifies the species as endangered. However, as with *E. candollei*, this assessment was made in 1998 (African Regional Workshop (Conservation & Sustainable Management of Trees Z, July 1996), 1998). *Pericopsis elata* has been observed from Côte d'Ivoire in the east, to the CAR and DRC, and the species has also been introduced to

western Uganda (Anglaaere, 2008). It is listed as endangered on the IUCN red list, with the latest assessment having been made in 2020 (Hills, 2020). More importantly, it has been classified as CITES Appendix II since 1992. All three species are important on the African continent both locally and in international trade, and are highly prized for their timber, especially *P. elata*.

Photographs of the transects for *E. candollei*, *M. laurentii* and *P. elata* are shown in Figs 1–3 respectively. In each case, the vessels, which appear as circles or ovals, are readily apparent in the magnified images. Also observed are the fibres, axial parenchyma and radial parenchyma (the wood rays). The areas surveyed by LADI-MS were 0.33 × 1.16 cm, 0.39 × 0.55 cm and 0.16 × 1.23 cm for *E. candollei*, *M. laurentii* and *P. elata*, respectively. Because the analyses were performed under soft ionisation conditions (orifice 1 = 20 V), the observed peaks represent unfragmented protonated precursors of the detected ions. Using a relative abundance threshold cutoff of 1%, 92, 431 and 147 ions were detected for *E. candollei*, *M. laurentii* and *P. elata*, respectively. Of the ion images representative of the spatial distributions of detected high-resolution masses, a subset were found to align with anatomical features that were also observable visually by microscopy imaging. These are listed in Table 2 and shown in Figs 1–3 for each species. The high mass accuracy results revealed the molecular formulas of the compounds whose identities were subsequently determined by thermal desorption-gas chromatography–MS (TD-GC-MS) analysis (listed in Table 2). Supporting Information Fig. S1 shows the chromatograms obtained from analysis by TD-GC-MS of *E. candollei*, *M. laurentii* and *P. elata*. Figs S2–S4 show the comparisons of the electron ionisation mass spectral fragmentation patterns of detected compounds to those of the compound matches in the NIST mass spectral library, rendered as head-to-tail plots.

To our knowledge, the identified molecules listed in Table 2 are reported for the first time as being detected in the species analysed. Both glycerin and benzyl alcohol were identified in all three wood species analysed by TD-GC-MS. In the LADI-MS images, a high ion abundance is indicated by orange and a low ion abundance is indicated in purple (Figs 1–3). Therefore, the

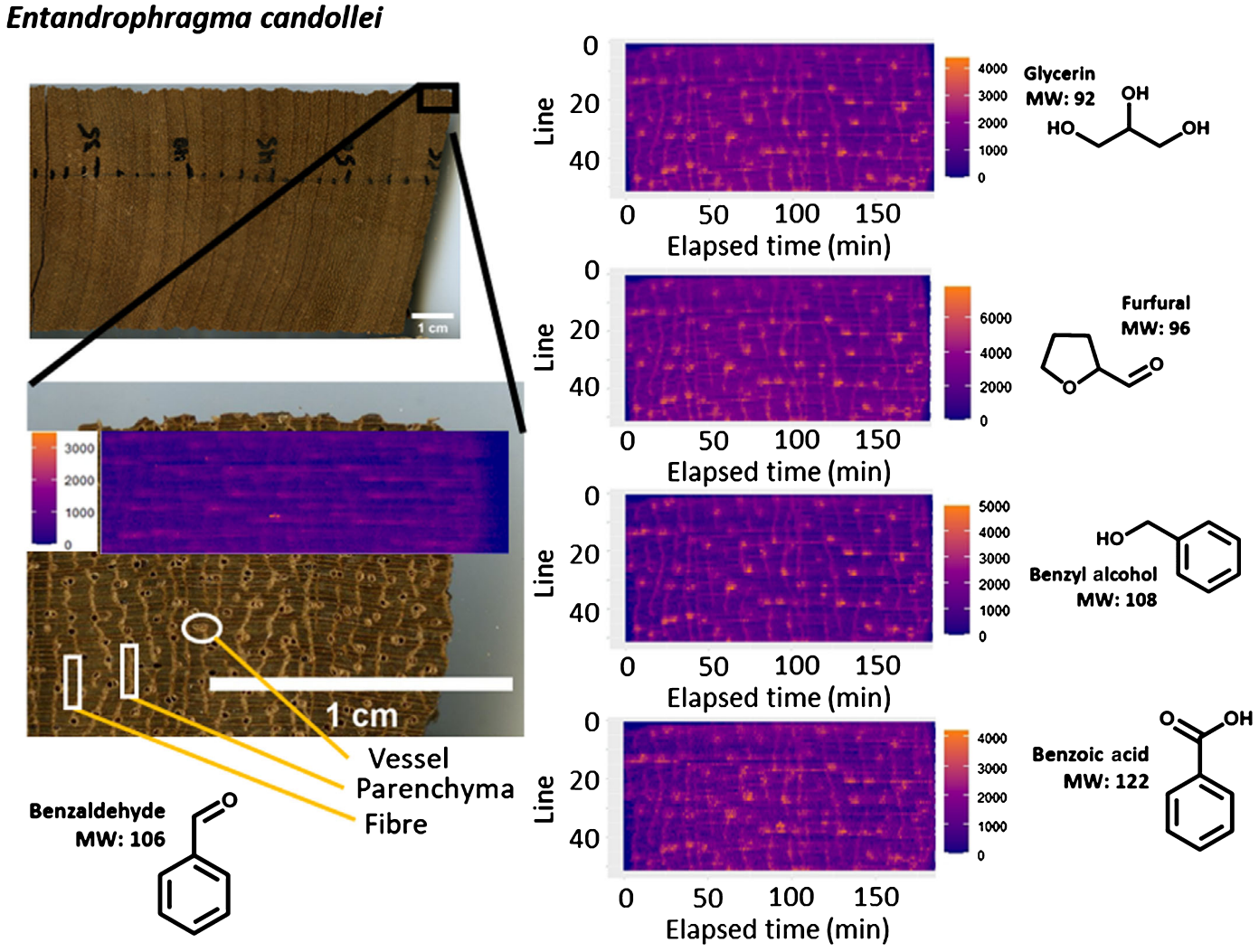

Fig. 1 Wood transects for *Entandrophragma candollei* overlayed with the laser ablation direct analysis in real time imaging–mass spectrometry ion images of detected high-resolution masses which align with anatomical features. The confirmed chemical structures corresponding to the ion images are shown.

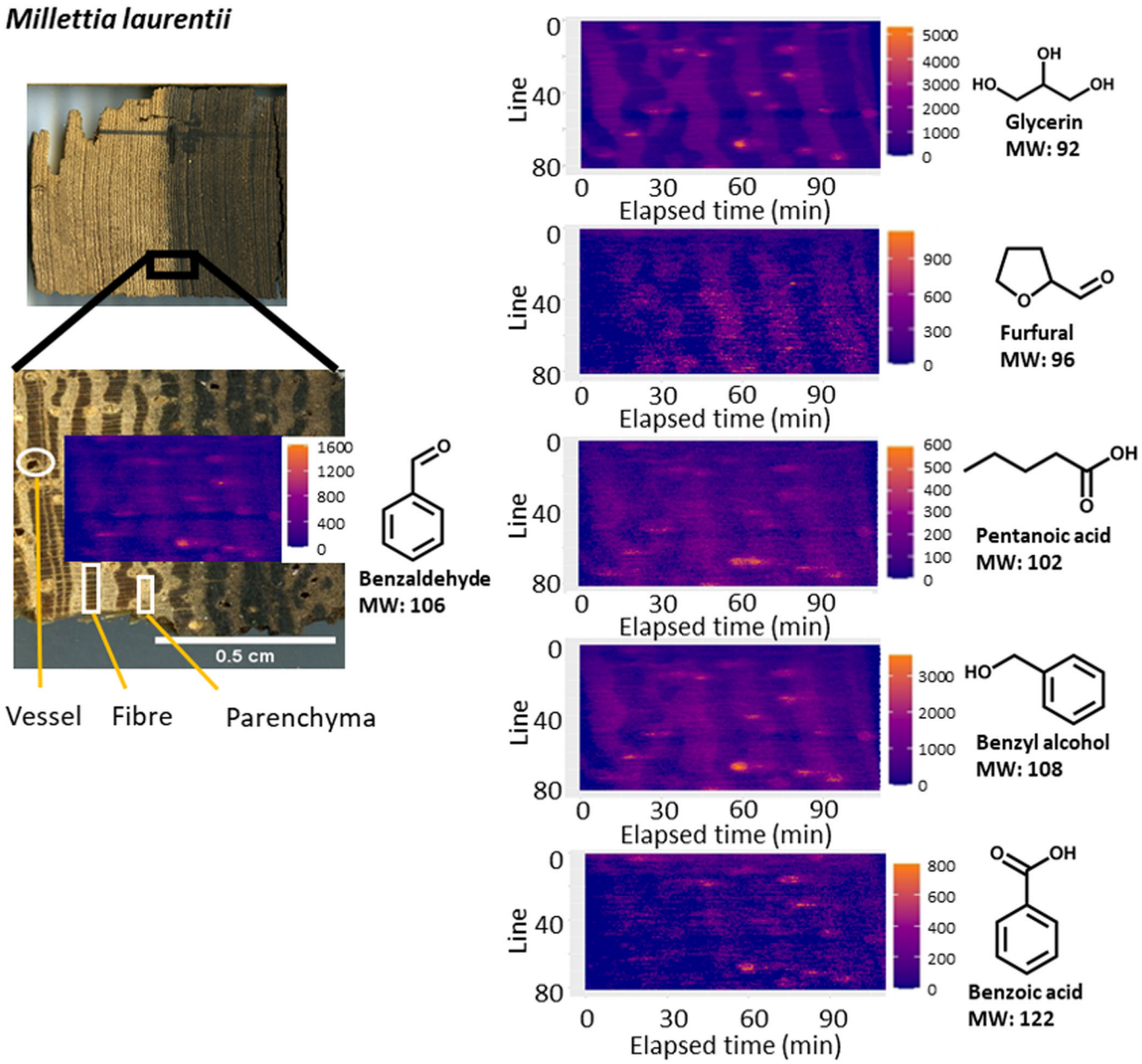

Fig. 2 Wood transects for *Millettia laurentii* overlayed with the laser ablation direct analysis in real time imaging–mass spectrometry ion images of detected high-resolution masses which align with anatomical features. The confirmed chemical structures corresponding to the ion images are shown.

ion images reveal that glycerin and benzyl alcohol were localised in the vessels and axial parenchyma and were not present in the fibres. While glycerin is more commonly associated with animal cells, it is also found in plant oils such as shea butter and coconut oil and has been detected in *Arabidopsis thaliana* (L.) Heynh. (Fiehn *et al.*, 2000; Nikiforova *et al.*, 2005), *Pimpinella anisum* L. (Fujimatu *et al.*, 2003) and *Pycnandra acuminata* (Pierre ex Baill.) Swenson & Munzinger (Callahan *et al.*, 2008). It has also been found in the latex of *Pycnandra acuminata* (Callahan *et al.*, 2008). Benzyl alcohol is a well known natural product that occurs in numerous plant species including coffee (i.e. volatiles) (Holscher & Steinhart, 1995), *Dianthus caryophyllus* L. (Schade *et al.*, 2001), *Hesperis matronalis* L. (Majetic *et al.*, 2007), *Nymphaea lasiophylla* Mart. & Zucc., *N. lingulata* Wiersema and *N. rudgeana* G.Mey. (Maia *et al.*, 2014), *Silene latifolia* Poir. (Junker *et al.*, 2018) and *Prunus avium* (L.) L. (Cozzolino *et al.*, 2019). It has also been found in *Petiveria alliacea* L. (Kubec & Musah, 2001).

Newly reported to be present in both the *E. candollei* and *M. laurentii* samples were furfural, benzaldehyde and benzoic acid. The presence of furfural has been shown in pine needle extracts and it appeared to have antifungal activity against the fungus *Alternaria mali* (Jung *et al.*, 2007). Benzaldehyde occurs in *Dianthus caryophyllus* L. (Schade *et al.*, 2001) and *Hesperis matronalis* L. (Majetic *et al.*, 2007). The compound was also extracted from the leaves of *Prunus persica* (L.) Batsch (Verma

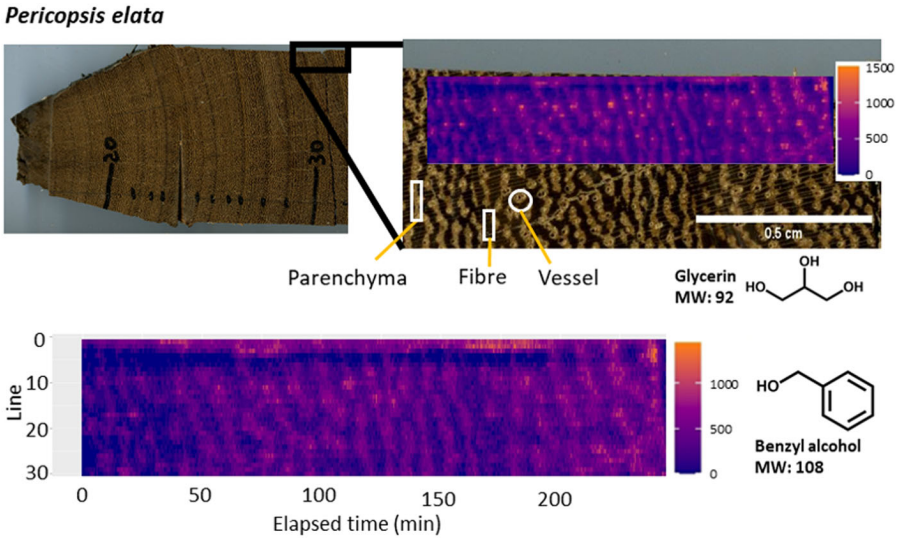

Fig. 3 Wood transects for *Pericopsis elata* overlayed with the laser ablation direct analysis in real time imaging–mass spectrometry ion images of detected high-resolution masses which align with anatomical features. The confirmed chemical structures corresponding to the ion images are shown.

Table 2 Ions detected by LADI-MS and whose spatial distributions aligned with vessel and parenchymal anatomical features in the transects of the indicated species.

				Species[a]		
$M+H^+$ (observed)	$M+H^+$ (calculated)	Corresponding formula	Compound identity[b]	*Entandrophragma candollei*	*Millettia laurentii*	*Pericopsis elata*
93.056	93.0552	$C_3H_8O_3+H^+$	Glycerin	+	+	+
97.030	97.0290	$C_5H_4O_2+H^+$	Furfural	+	+	–
103.076	103.0759	$C_5H_{10}O_2+H^+$	Pentanoic acid	–	+	–
107.050	107.0497	$C_7H_6O+H^+$	Benzaldehyde	+	+	–
109.065	109.0653	$C_7H_8O+H^+$	Benzyl alcohol	+	+	+
123.045	123.0446	$C_7H_6O_2+H^+$	Benzoic acid	+	+	–

[a]The symbols (+) and (–) indicate the presence and absence of the indicated compound, respectively.
[b]Established by thermal desorption-gas chromatography–mass spectrometry.

et al., 2017). Benzoic acid is a known chemical constituent in plants, as it and its volatile derivatives play a large role in plant pollination, growth, defence and communication (Dudareva *et al.*, 2006). Benzoic acid has been extracted from black galls on *Populus tremuloides* Michx (Pausler *et al.*, 1995). These black galls appeared to play a role in protection against heartwood rot. Benzaldehyde, benzoic acid and furfural show a similar pattern for *E. candollei* and *M. laurentii*, although the pattern for *M. laurentii* for benzaldehyde and furfural is weaker compared with benzyl alcohol.

The *M. laurentii* image permits comparison of sapwood and heartwood segments. For most species, the heartwood is darker in colour compared with the sapwood. Once sapwood transforms to heartwood, the living sapwood cells die and the resulting discoloration is due to the production and secretion of substances formed in the parenchyma cells (Thomas, 1977). When heartwood is formed, metabolic activity in the parenchyma cells produces metabolites such as isoflavones and terpenes from carbohydrates (Thomas, 1977). Afterwards, these metabolites diffuse into the adjacent cells. They can make up as much as 20% of the wood of tropical species (Pettersen, 1984). Major compound classes represented are: terpenes, fatty acids, aromatic compounds and volatile oils (Thomas, 1977). They are known to play a role in the natural durability of the timber (i.e. its resistance to biological decay) (Sehlstedt-Persson & Karlsson, 2010). The sapwood also contains living parenchyma cells in contrast with the nonliving heartwood. Fig. 2 shows that benzoic acid and furfural have a lower intensity in the sapwood compared with the heartwood, indicating that the relative distributions of benzoic acid and furfural may serve as a chemical means by which to distinguish the two areas, particularly in species for which visually apparent colour differences between the sapwood and heartwood are absent. It is important to note that there is a significant variation in the distribution of the metabolites throughout the wood and that some metabolites can also be found in the sapwood, although the heartwood usually contains the largest amounts (Thomas, 1977). The intensity of the other molecules appears to be consistent across the gradient, indicating that they appear in both the sapwood and the heartwood.

Pentanoic acid has previously been reported in *Valeriana officinalis* L. (Puri & Hall, 1999), *Durio zibethinus* L. (Li *et al.*, 2012) and *Coffea arabica* (de Melo Pereira *et al.*, 2019), but is reported

in *M. laurentii* for the first time here. The LADI-MS images generated from this ion show the anatomical pattern of the wood, even though the intensity within vessels varies. This finding aligns with previous reports that organic acids tend to accumulate in the cell vacuoles of plants (Puri & Hall, 1999).

Targeted vessel analysis and timber identification – investigation of *Dalbergia nigra*

In the area of forensic identification of wood, the most widely used technique for species determination is wood anatomy analysis. However, timber identification by metabolite profiling has become increasingly important (Sisco & Forbes, 2021), even though little information is known about the full range of the metabolites detected by the most important of the techniques used, which is DART-TOFMS. The metabolite profiling approach to wood identification involves the screening of the unknown wood sample against a database of species-specific DART mass spectra, with statistical analysis tools used to select the highest species matches from within the database. Afterwards a multivariate classification algorithm allocates the unknown spectra to the right species. Therefore, it does not rely on knowledge of the actual compounds represented in the mass spectra, as the match is based on recognition of species-specific patterns. The success of this approach is a consequence of the fact that there is a subset of masses that define species identity, even though the molecules to which the masses correspond may not themselves be known. In principle, LADI-MS could be used to investigate where within the wood tissue the markers associated with the *m/z* values that are most important for species identification reside. A preliminary investigation of this question was conducted using *Dalbergia nigra*. This species, which is also known as Brazilian rosewood, is endemic to Brazil and is endangered. It is a CITES Appendix I species and therefore its international trade is prohibited except when the purpose of importation is not commercial. Nevertheless, it tends to be harvested illegally for use in the fashioning of furniture and musical instruments. The *Dalbergia* genus is comprised of more than 250 species (Hung *et al.*, 2020), and while wood anatomy can be readily used to identify a given timber as a member of the genus, it can be much more difficult to use this technique to distinguish between species within the genus. The ability to do so is important because, while all the species in the genus are CITES regulated, the penalties associated with illegal trade differ depending upon the specific CITES designation. In this regard, species matching by metabolome profiling based on the species-specific DART mass spectra has proven identification potential (Lancaster & Espinoza, 2012; McClure *et al.*, 2015). This observation implies that there are specific molecules that are important in enabling the ability to distinguish one species from another. Examples of such molecules that have been detected in *Dalbergia* species include dalbergichromine, dalbergin, caviunin, and the constitutional isomers afformosin and dalnigrin (Afendi *et al.*, 2012). LADI-MS might have utility in providing information on where within the wood these biomarkers, which are most important in enabling distinctions to be made between species in the *Dalbergia* genus, are localised. This was explored using a *D. nigra* transect.

Fig. 4 shows representative LADI-MS spectra observed in the analysis of the content of a *D. nigra* vessel vs the tissue surrounding it, in the form of a head-to-tail plot in which the spectrum of the surrounding tissue appears in blue (upper), and that of the vessel appears in red (lower). To acquire the spectra, a charge-coupled device camera associated with the laser unit was used to fire the laser at areas of interest. Comparison of the spectra indicates that, while the profile of masses observed in the lower mass range (i.e. below *m/z* 250) are very similar, a number of masses appear at higher *m/z* values in the spectrum of the vessel content that are absent in the spectrum of the surrounding tissue. Among these are nominal *m/z* 255, 269 and 299, whose high-resolution masses correspond to $C_{16}H_{14}O_3 + H^+$, $C_{16}H_{12}O_4 + H^+$, and $C_{17}H_{14}O_5 + H^+$. These masses have been tentatively identified as dalbergichromene, dalbergin and afformosin/dalnigrin respectively. These results support the premise that the metabolite fingerprint on which DART-TOFMS timber identifications are based is mainly determined by the vessel content of the species in question.

Metabolite distribution – Interrogation of *Dalbergia* species

The goal in the experiments, the results of which are described here, was to determine the distribution of compounds within pith-to-bark transects of the *Dalbergia* species. LADI-MS-derived metabolite distribution profiles within *D. nigra* are presented in Fig. 5. The sample was analysed using a laser spot size of 80 μm^2, thereby yielding a spatial resolution of 155 × 80 μm^2. A hyperspectral image of the transect, taken at a wavelength of 645 nm,

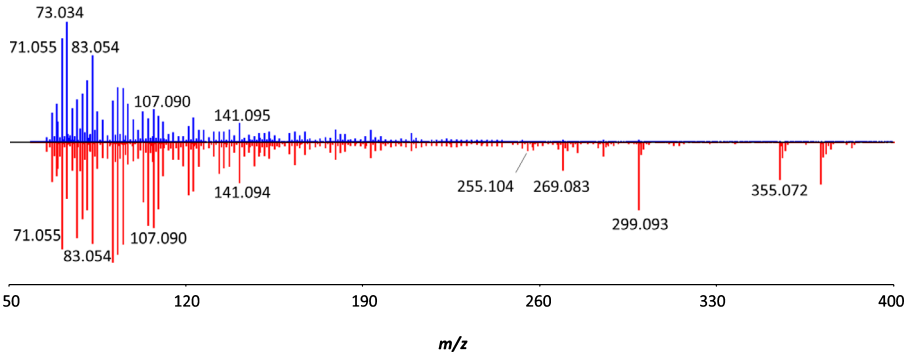

Fig. 4 Representative laser ablation direct analysis in real time imaging–mass spectrometry spectra of *Dalbergia nigra* wood tissue and vessel content. Upper (blue): spectrum of wood tissue surrounding *D. nigra* vessels. Lower (red): representative spectrum of the content of a vessel.

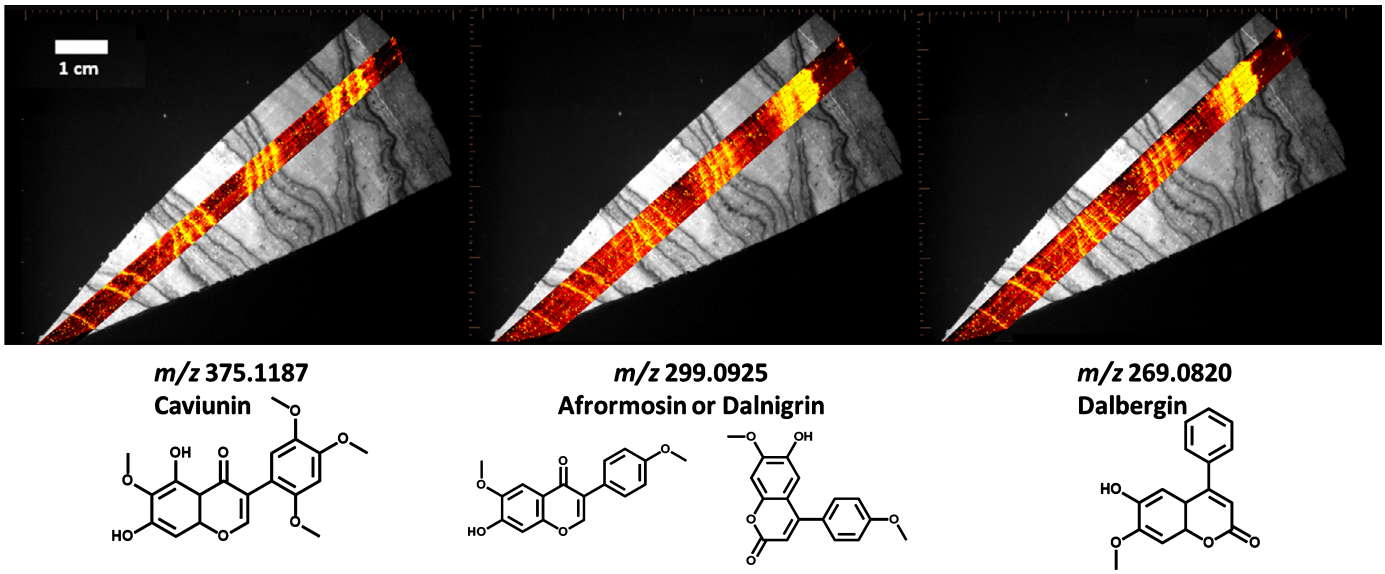

Fig. 5 *Dalbergia nigra* stem disc showing the presence of various molecules (black, not present; red, low presence; orange, medium presence; yellow, high presence). The molecules are: caviunin, afformosin and/or its constitutional isomer dalnigrin, and dalbergin.

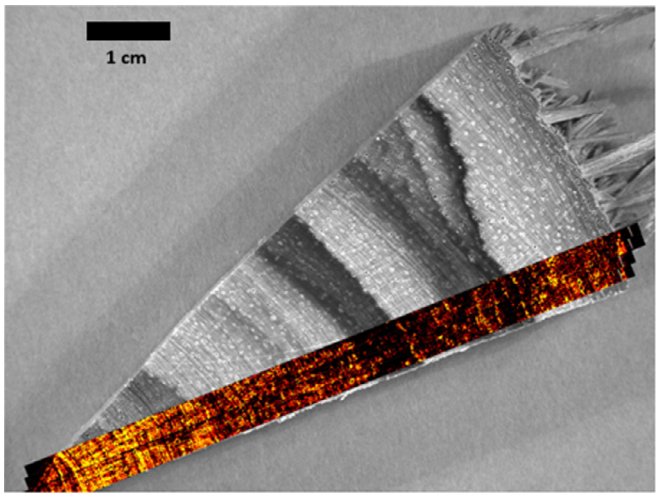

Fig. 6 Stem disc piece from *Dalbergia normandii* showing the distribution of sitosterol-OH (black, not present; red, low presence; orange, medium presence; yellow, high presence).

is shown in Fig. 5, and reveals features that are not visually apparent in ambient light. Colour-overlaid ion images of masses consistent with caviunin (listed in KNApSacK as being present in *Dalbergia* spp.; Afendi *et al.*, 2012), afrormosin/dalnigrin and dalbergin are shown. In all three cases, the high intensity areas (indicated in yellow) were found to align with the nonperiodical markings typical of this genus. Caviunin has been shown to be the diagnostic ion that enables discrimination of *D. nigra* from other *Dalbergia* species (Espinoza *et al.*, 2015; Wiemann & Espinoza, 2017). The distribution of sitosterol-OH in *D. normandii* is shown in Fig. 6. There are conflicting reports on the importance and relevance of this steroid, which has been detected in various tropical species (Kilic & Niemz, 2012). On the one hand, its presence has been linked to increased mass-loss caused by fungal activity. On the other hand, its presence has been associated with increased natural durability (Deklerck *et al.*, 2020). Therefore, for the latter observation, its presence could provide information on wood quality. Revelation of the presence and distribution of molecules such as sitosterol, which may impact the commercial value of wood, could be very beneficial to the timber industry and timber grading in particular.

Discussion

The chemical make-up of wood and in particular, the spatial distributions of the molecules present, is inherently difficult to study, as the attributes of the wood matrix are not easily accommodated by the available sampling and imaging techniques. LADI-MS provides a way to analyse wood transects that preserves the spatial distributions of the small molecules present and enables interrogation of the association between observed metabolites and anatomical structures, as is illustrated for *E. candollei*, *M. laurentii* and *P. elata*. In this method, the laser is used to ablate and desorb the sample from the surface, and the DART-TOFMS is used to ionise and detect the signals of the molecules present. For this reason, the optical properties of the molecules themselves do not influence the signal being detected, because the output of the experiments are mass spectra. The laser parameters provide opportunities to refine the mass spectral interrogation of anatomical features that are specific to a given sample. For example, in the analysis of *D. nigra* and *D. normandii*, a laser spot size of 80 μm^2, which was slightly smaller than the diameter of the vessels observed in these samples, was used. As one of the parameters that can be modified, the laser spot size can be decreased to *c.* 4 μm^2 so that the spatial distributions of small molecules within structures that are of the same order of magnitude or higher can be mapped. However, one of the trade-offs of reducing the laser spot size is the diminution of

the mass spectral signal, as a consequence of the reduction in the surface area being ablated. This challenge can be overcome to some extent by optimisation of other laser parameters so that the MS signal can be enhanced. These include the fluence and helium flow rate. To generate the ion images, multiple rows of a user-defined length of up to 10 cm are scanned and subsequently combined together to generate the full image of the ablated area.

Multiple samples of the same species (i.e. different individuals) were analysed in this study to assess the variability between the results for samples of the same species grown in different geographic regions. From these experiments, it was observed that, while chemical striations of the type seen in this work for *Dalbergia* species biomarkers appeared, they were not always associated with the visible markings in the figured heartwood. Therefore, while it is established that the presence of various diagnostic biomarkers can be used for the forensic identification of wood using DART-TOFMS, it remains unclear whether additional species information can be inferred from the chemical pattern map, particularly as these patterns may vary as a consequence of events within the life of a tree (e.g. disease, environmental stress, climatic events, etc.) and perhaps even by genotypic variations within species. A more systematic investigation of the association of the presence of particular compounds and environmental factors is the subject of ongoing investigations.

Forensic analysis of timber by DART-TOFMS is now a well established method for the identification of illegally traded species (Espinoza *et al.*, 2015; Deklerck *et al.*, 2019). The success of this approach is a consequence of the fact that timber species exhibit markedly reliable DART-TOFMS-derived fingerprint profiles, the statistical analysis of which enables accurate prediction of species identity. These distinct chemical profiles are, in turn, a consequence of biomarkers that are unique to a species or genus. What LADI-MS interrogation of vessel content revealed is that the metabolome fingerprint that is the basis of species identification by DART-TOFMS is determined primarily by vessel content. Therefore, several metabolites that are diagnostic for *Dalbergia*, such as caviunin and afrormosin/dalnigrin, were shown to be localised in the vessels in contrast with the surrounding tissue. Furthermore, the revelation of the spatial distributions of small molecules such as caviunin, afrormosin/dalnigrin, dalbergin and β-sitosterol along pith-to-bark transects offers the ability to develop optimal protocols for the isolation of useful natural products. Ultimately, the revelation of the spatial distributions of the important identifying molecules and the visualisation of their radial variability within transects has the potential to be used for timber identification. Interestingly, within the *Dalbergia* samples are faint nonperiodical markings whose appearance is enhanced with hyperspectral imaging. These striations, commonly referred to as 'figured heartwood' are characteristic of the genus. LADI-MS analyses revealed that the markings are associated with high levels, relative to the surrounding tissue, of a number of *Dalbergia*-specific metabolites including caviunin, dalnigrin/afrormosin or dalbergin (Fig. 5).

As mentioned previously, analysis using NIR is another approach that has been successfully applied to the forensic identification of wood. It is a noninvasive method that uses wood NIR spectral characteristics and pattern recognition algorithms to provide species information. The method described here is distinguished from the forensic application of NIR spectroscopy for wood in a number of ways. While NIR provides a composite signal based on an averaging of the spectroscopic responses of individual components present in wood, LADI-MS gives specific molecular profile information that reveals the presence of distinct molecular markers that are associated with anatomical features. Therefore, while NIR spectral analysis can rapidly provide pattern signatures that can be used for the identification of timber in a forensics context, LADI-MS opens up new vistas in the study of phytochemistry, ecophysiology and wood anatomy research by enabling the pursuit of molecular and mechanistic studies.

The observation of furfural, benzoic acid and benzaldehyde deserves special comment. While all three chemicals are known natural products that are constitutively present in some plant species, it has also been demonstrated that their presence can be a consequence of the breakdown of precursor compounds during sample processing. For example, furfural can be derived from xylose during tissue preparation using steam treatment (Walker *et al.*, 2018), and benzoic acid and benzaldehyde have been detected following various sample processing methods (Kota *et al.*, 2004; Wang *et al.*, 2014). However, while it is known that these compounds can be artefacts of the analysis and sample treatment approaches used for their detection, we do not believe them to be artefacts of the laser ablation process used in this work, because we were able to detect them by the independent method of TD-GC-MS in the absence of laser ablation.

While proof of principle of the power of the LADI-MS approach for the mapping within wood tissues of the locales of small molecules was demonstrated, a number of constraints of the method should be highlighted. It would be ideal if pith-to-bark transects of indefinite length could be surveyed in a single analysis. Although the current method will accommodate lengths of up to 10 cm, larger samples will require segmented runs, the results of which are then stitched together. This is cumbersome and may sometimes be impractical because it will require that transects > 10 cm in length be cut into smaller segments that can be accommodated by the sample chamber. Second, depending on parameters such as laser spot size, scan speed and level of spatial resolution desired, analysis times could be quite lengthy. For example, a smaller spot size will result in greater resolution but longer analysis times in order to observe particular anatomical features. A third constraint is imposed by the DART ionisation mechanism. Because analytes are ionised by proton transfer from protonated water clusters generated from the interaction of metastable helium with atmospheric water, only analytes with a proton affinity greater than that of water are detected. Furthermore, the greater the proton affinity of an analyte, the stronger its signal. This creates the potential for analytes with low proton affinities, but which are present in larger relative concentrations, to give relatively weak MS signals, while those with high proton affinities (e.g. amines) but which are present at lower concentrations would yield intense signals. In summary, the relative intensities observed in the mass spectrum at a particular coordinate of the area surveyed cannot in and of themselves be used to draw inferences about the relative

amounts of the detected compounds. Therefore, an alternative method would need to be used to extract information on the exact relative amounts of compounds of interest.

The LADI-MS approach will facilitate future research studies into tree responses to environmental conditions by registering the changes in molecular profiles that occurred in reaction to various stimuli. Tree rings can be viewed as archives that can reveal climate information from the distant past. This prominent feature of pith-to-bark transects is primarily observed in temperate species in which ring formation is associated with climate. However, tropical trees, in general, do not exhibit this characteristic, thereby making tree ring dating and other information about climate harder to extract. If the climatic event timeline is registered within the transect in an alternative form, for example in terms of specific chemical profiles, and if these chemical marker distributions could be mapped within the tissue, it would provide a powerful means by which to extract climate information from tropical trees. One way to detect the presence of such biomarkers is to determine whether there are molecules that are specifically found within tree rings. If such molecules are found, their spatial distributions within the transects of tree species for which there are no visually apparent rings can be determined. This would facilitate investigation of climate through detection of 'chemical rings'. Therefore, the results presented here show the potential for LADI-MS analysis of wood transects to serve as a means by which to access the chemical information embedded in wood, in a manner that may reveal climate event timelines. This possibility is the subject of ongoing investigations in our laboratories.

Acknowledgements

The financial support of the National Science Foundation (grant nos. 1429329 and 1710221) to RAM and the Belgian American Education Foundation (Gustave Boël – Sofina Belgian American Educational Foundation Fellowship) to VD is gratefully acknowledged. Thanks are also extended to Aiden Espinoza for preparation of the *D. nigra* and *D. normandii* samples and Stijn Willen for the preparation of the *E. candollei*, *M. laurentii* and *P. elata* samples for LADI-MS analysis.

Author contributions

RAM conceived the project and supervised its implementation. EOE and HB provided samples and expert guidance. VD, KLF and AMC performed experiments. Elements of this work appear in the Ph.D. thesis of KLF. RAM wrote the manuscript. VD and AMC contributed to writing the manuscript.

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Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Generated thermal desorption-gas chromatography–mass spectrometry chromatograms for the three samples analysed, *Entandrophragma candollei*, *Millettia laurentii* and *Pericopsis elata*.

Fig. S2 Mirror plots for the subset of compounds listed in Table 2 (in the body of the article) identified to be present in *E. candollei*.

Fig. S3 Mirror plots for the subset of compounds listed in Table 2 (in the body of the article) identified to be present in *M. laurentii*.

Fig. S4 Mirror plots for the subset of compounds listed in Table 2 (in the body of the article) identified to be present in *P. elata*.

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