CONVENTION ON INTERNATIONAL TRADE IN ENDANGERED SPECIES
OF WILD FAUNA AND FLORA

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Interpretation and implementation of the Convention

Species trade and conservation

Trees

Malagasy ebonies (*Diospyros* spp.) and Malagasy rosewoods (*Dalbergia* spp.) (Decision 16.152)

ANALYSIS OF SELECT *DALBERGIA* AND TRADE TIMBER USING DIRECT ANALYSIS IN REAL TIME AND TIME-OF-FIGHT MASS SPECTROMETRY FOR CITES ENFORCEMENT

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Research Article

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Analysis of select *Dalbergia* and trade timber using direct analysis in real time and time-of-flight mass spectrometry for CITES enforcement

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RATIONALE: International trade of several *Dalbergia* wood species is regulated by The Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES). In order to supplement morphological identification of these species, a rapid chemical method of analysis was developed.
METHODS: Using Direct Analysis in Real Time (DART) ionization coupled with Time-of-Flight (TOF) Mass Spectrometry (MS), selected *Dalbergia* and common trade species were analyzed. Each of the 13 wood species was classified using principal component analysis and linear discriminant analysis (LDA). These statistical data clusters served as reliable anchors for species identification of unknowns.
RESULTS: Analysis of 20 or more samples from the 13 species studied in this research indicates that the DART-TOFMS results are reproducible. Statistical analysis of the most abundant ions gave good classifications that were useful for identifying unknown wood samples.
CONCLUSIONS: DART-TOFMS and LDA analysis of 13 species of selected timber samples and the statistical classification allowed for the correct assignment of unknown wood samples. This method is rapid and can be useful when anatomical identification is difficult but needed in order to support CITES enforcement. Published 2012. This article is a US Government work and is in the public domain in the USA.

Fourteen species of the Leguminosae family are listed by the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES).[1] *Dalbergia nigra*, Brazilian rosewood, is the only Leguminosae species listed under Appendix I of the CITES and it is banned from international commercial trade.[1] Along with many other *Dalbergia* species, Brazilian rosewood has been used for hundreds of years in furniture and cabinetry and it is highly regarded for use in musical instruments due to its acoustic qualities.[2,3]

As much as 80% of logging in the Amazon is illegal and illegal trade may account for over 10% of the global timber trade.[4–6] In addition to denying revenue to some countries, illegal trade makes sustainable management of timber stocks difficult.[6]

The traditional identification of wood products relied on diagnostic anatomical features, either macroscopic or microscopic.[7–9] However, morphological examination of wood anatomy is insufficient for distinguishing *D. nigra* from other closely related *Dalbergia* taxa seen in commercial trade.[8–10] The anatomical characteristics of *D. nigra* and *D. spruceana* (Amazon rosewood), the latter of which is not protected by CITES, are too similar for reliable morphological species identification.[9]

Three species of trees, *D. nigra*, *Swartzia tomentosa*, and *Aniba roseodora*, are commonly called 'rosewood', which is a source of confusion on import and export permits. *Aniba roseodora* is commonly called rosewood or pau rosa.[11] Another source of confusion is that illegal import shipments of *D. nigra* are often falsely declared as imbuia (*Phoebe porosa*) or pau ferro (*Machaerium scleroxylon*) due to similarities in the appearance of the wood.[12–15]

Dalbergia and *Machaerium* are part of a common *Dalbergia* clade.[16] Their close relationship is corroborated by the similar distribution of neoflavanoids in their woods.[17] The *Dalbergia* clade is characterized by the presence of related neoflavonoids, including dalbergiquinols, dalbergions, neoflavenes, and the dalbergins.[17]

Dalbergia melanoxylon, African blackwood, is known for its use in high quality musical instruments and carvings.[3] International trade in its timber has been stable, and there is no immediate threat to its sustainability; however, future supplies are difficult to predict due to lack of inventory data and the unknown annual harvest of its timber.[3,18] It is the most expensive timber exported from Tanzania, costing 18 000 USD per cubic meter.[3] In recent years, conservation groups such as SoundWood and the African Blackwood Conservation Project have designated African blackwood as a key species for conservation efforts.[19,20]

The current research examined 13 species of Leguminosae (Table 1), 10 of which were *Dalbergia*, using Direct Analysis in Real Time Time-of-Flight Mass Spectrometry (DART-TOFMS). Two species of commercial *Dalbergia* were not included in this study due to the lack of specimens: *D. tucurensis* and *D. miscobolobium*. DART-MS uses an ambient atmospheric ionization source that provides rapid analysis and requires minimal or no sample preparation.[21,22] The principal ionization mechanisms for DART-TOFMS have been thoroughly discussed.[21,23] Cody *et al*. demonstrated the use of DART-TOFMS and linear discriminant analysis to differentiate and identify oak

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Table 1. Wood samples examined

Species	Origin	Commercial source	Verified	n
Dalbergia nigra	Brazil	Eisenbrand Inc. Exotic Hardwoods, Torrance, CA, USA	*Dalbergia cf. nigra* (A. Wiedenhoeft)	1
			Could not confirm *Dalbergia nigra*(A. Wiedenhoeft) Validated by DART	15
		Cook Woods, Klamath Falls, OR, USA	*	5
		Laboratorio de Productos Florestais, Serviço Florestal Brasileiro, Brasília, Brazil	*Dalbergia nigra* (V. T. Rauber Coradin)	2
Dalbergia spruceana	Brazil	Gilmer Wood Co., Portland, OR, USA	*Dalbergia cf. spruceana* (by A. Wiedenhoeft)	20
Dalbergia retusa	Mexico	Eisenbrand Inc. Exotic Hardwoods, Torrance, CA, USA	*Dalbergia retusa* (A. Wiedenhoeft)	17
			*	3
Dalbergia cearensis	Brazil	Eisenbrand Inc. Exotic Hardwoods, Torrance, CA, USA	*Dalbergia ceareansis* (A. Wiedenhoeft)	18
			*	2
Dalbergia latifolia	Malaysia & India	Eisenbrand Inc. Exotic Hardwoods, Torrance, CA, USA	*Dalbergia cf. latifolia* (A. Wiedenhoeft)	35
			*	5
Dalbergia melanoxylon	Tanzania	Eisenbrand Inc. Exotic Hardwoods, Torrance, CA, USA	*Dalbergia melanoxylon* (A. Wiedenhoeft)	18
			*	2
Dalbergia frutescens (decipularis)	Brazil	Eisenbrand Inc. Exotic Hardwoods, Torrance, CA, USA	*Dalbergia decipularis*(A. Wiedenhoeft)	18
			*	2
Dalbergia stevensonii	Honduras	Eisenbrand Inc. Exotic Hardwoods, Torrance, CA, USA	*Dalbergia cf.stevensonii* (A. Wiedenhoeft)	18
			*	2
Dalbergia baronii	Madagascar	Gilmer Wood Co., Portland, OR, USA	*Dalbergia* sp. (A. Wiedenhoeft)	7
			*	3
Dalbergia madagascariensis	Madagascar	Eisenbrand Inc. Exotic Hardwoods, Torrance, CA, USA	*Dalbergia cf. madagascariensis* (A. Wiedenhoeft)	15
			*	2
Machaerium scleroxylon	Bolivia	Eisenbrand Inc. Exotic Hardwoods, Torrance, CA, USA	*Machaerium scleroxylon* (A. Wiedenhoeft)	18
			*	2
Swartzia tomentosa	Unknown	Eisenbrand Inc. Exotic Hardwoods, Torrance, CA, USA	*Swartzia* sp. (A. Wiedenhoeft)	18
			*	2
Phoebe porosa	Brazil	Eisenbrand Inc. Exotic Hardwoods, Torrance, CA, USA	*Phoebe porosa* (A. Wiedenhoeft)	18
			*	2

*Species identification was not confirmed using morphology. DART-TOFMS and LDA classification agreed with the seller's assertion.

Table 2. Tentative assignments for peaks found in *Dalbergia*, *Machaerium*, *Swarztia*

Name	Formula	Mass (*m/z*)
Unidentified compound		209.12 $[M^{+\bullet}]$ or $[M+H]^+$
Dalbergione I[29]	$C_{16}H_{14}O_3$	254.0943 $[M]^+$
Dalbergione I[29]	$C_{16}H_{14}O_3$	255.0943 $[M+H]^+$
Dalbergin[32]	$C_{16}H_{13}O_4$	269.0813 $[M+H]^+$
Dihydroxymethoxyflavone	$C_{16}H_{12}O_5$	284.0684 $[M^{+\bullet}]$
Dihydroxymethoxyflavone	$C_{16}H_{12}O_5$	285.0771 $[M+H]^+$
Melanettin[25]	$C_{16}H_{13}O_5$	285.0762 $[M+H]^+$
3,4-Dimethoxydalbergione[15]	$C_{17}H_{17}O_4$	285.1126 $[M+H]^+$
Dalnigrin[25]	$C_{17}H_{15}O_5$	299.0919 $[M+H]^+$
Kuhlmannin[25]	$C_{17}H_{15}O_5$	299.0919 $[M+H]^+$
(2S)-Canadenatenin E**	$C_{17}H_{17}O_5$	302.1154 $[M+H]^+$
Melannein**[25]	$C_{17}H_{15}O_6$	315.0869 $[M+H]^+$
Dihydroxytrimethoxyflavone**	$C_{18}H_{16}O_7$	345.0974 $[M+H]^+$
Caviunin**[25,33]	$C_{19}H_{19}O_8$	375.1080 $[M+H]^+$

**Not present in mass spectrum of every species.

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species.[24] The objective of our study was to determine if that technique could be used to discriminate among 13 species of timber that have conservation and commercial value.

EXPERIMENTAL

Material

The wood samples used in this study were purchased from various vendors listed in Table 1. Species verification of 236 of the 270 wood samples was carried out by Dr Alex C. Wiedenhoeft (Center for Wood Anatomy Research, Forest Products Laboratory, U.S. Forest Service, Madison, WI, USA). Two reference standards of *D. nigra* were also obtained from Dr Vera Teresinha Rauber Coradin (Laboratorio de Productos Florestais, Serviço Florestal Brasileiro, Brasília, Brazil). In order to test the DART-TOFMS and linear discriminant analysis (LDA) method, 30 wood samples were not submitted for species verification and these samples were treated as unknowns. These samples are denoted with an asterisk in Table 1.

Methods

Mass spectra were acquired using a DART-SVP ion source (IonSense, Saugus, MA, USA) coupled to a JEOL AccuTOF time-of-flight mass spectrometer (JEOL USA, Peabody, MA,

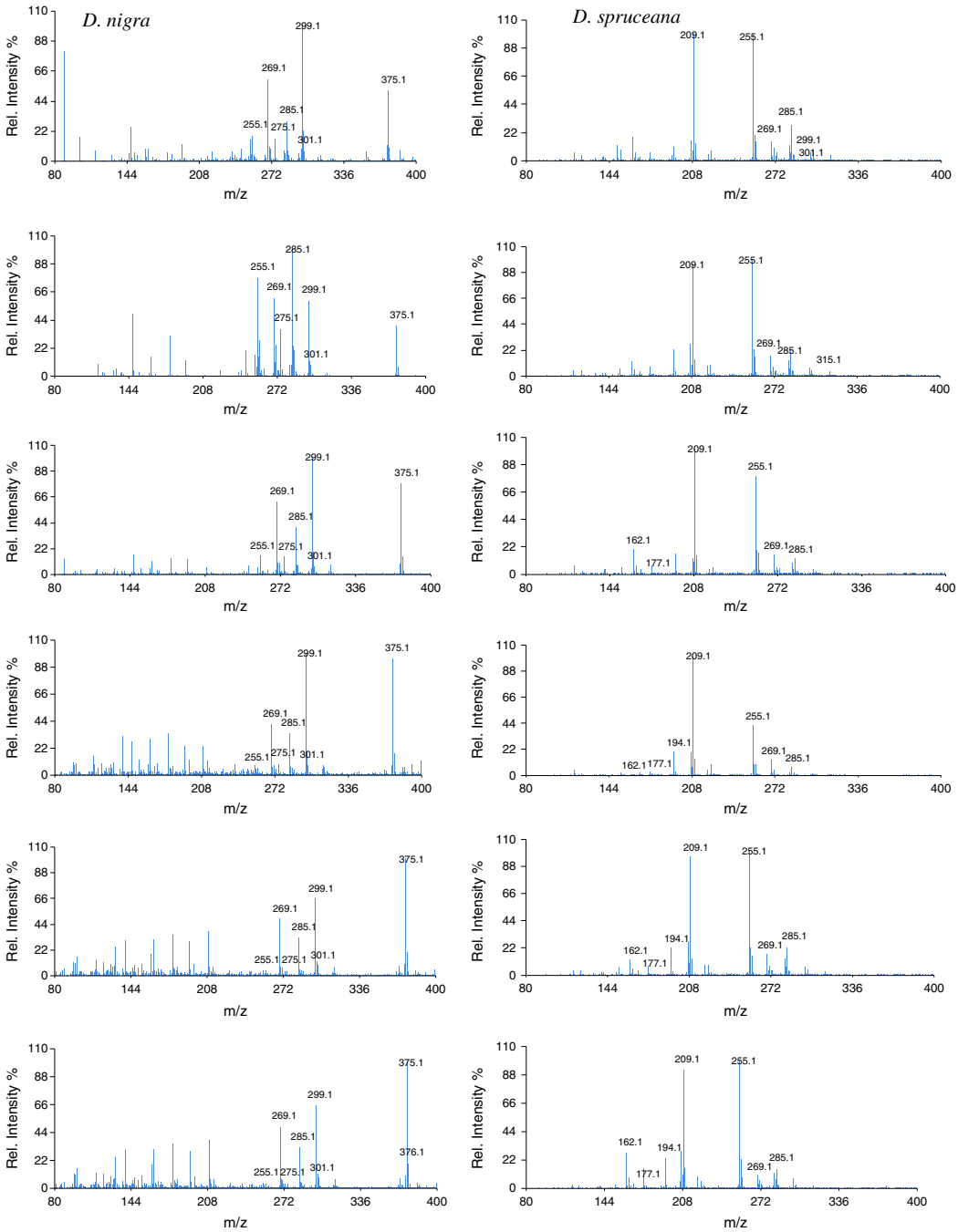

Figure 1. Mass spectra for six samples each of *D. nigra* and *D. spruceana* acquired using DART-TOFMS.

USA) in positive ion mode. The DART source parameters were: needle voltage, 3.5 kV; electrode 1 voltage, 150 V; electrode 2 voltage, 250 V; and gas heater temperature, 350 °C. The mass spectrometer settings included: ring lens voltage, 5 V; orifice 1 voltage, 20 V; orifice 2, 5 V; cone temperature, 120 °C; peaks voltage, 600 V; bias, 28 V; focus voltage, –120 V; reflectron voltage, 870 V; pusher voltage, 778 V; pulling voltage, –778 V; suppression voltage, 0.00 V; flight tube voltage, –7000 V; and detector voltage, 2000 V. Spectra were obtained over the mass range of *m/z* 50 to 1100 at 1 scan per second. The helium flow rate for the DART source was 2.0 mL s^{-1}. The resolving power of the mass spectrometer, as stated by the manufacturer, was ± 2.0 milli *m/z* units.

Slivers of wood no wider than 4 mm were cut from each block and placed in the DART helium gas stream for 6 s. A mass calibration standard of poly(ethylene glycol) 600 (Ultra, Kingstown, RI, USA) was run between each sample.

In order to compare the mass spectra obtained with DART-TOFMS with those obtained using electrospray ionization mass spectrometry (ESI-MS),[25] slivers of wood from *D. nigra* and *D. spruceana* were extracted in methanol for 48 h. The methanol solutions were analyzed using DART-TOFMS by sampling the extract with a melting point tube. To determine if the distribution of chemical compounds was homogenous throughout the heartwood of *D. nigra*, a cross section of heartwood (7.5 cm radius) was sampled every centimeter (with half-centimeter increment at 7.5 cm) from the core to the cambium for a total of nine samples.

TSSPro3 (Shrader Analytical Labs, Detroit, MI, USA) data processing software was used to export the text files of the mass-calibrated, centroided mass spectra into elemental composition and classification software Mass Spec Tools II (RBC Software, Peabody, MA, USA). Principal components

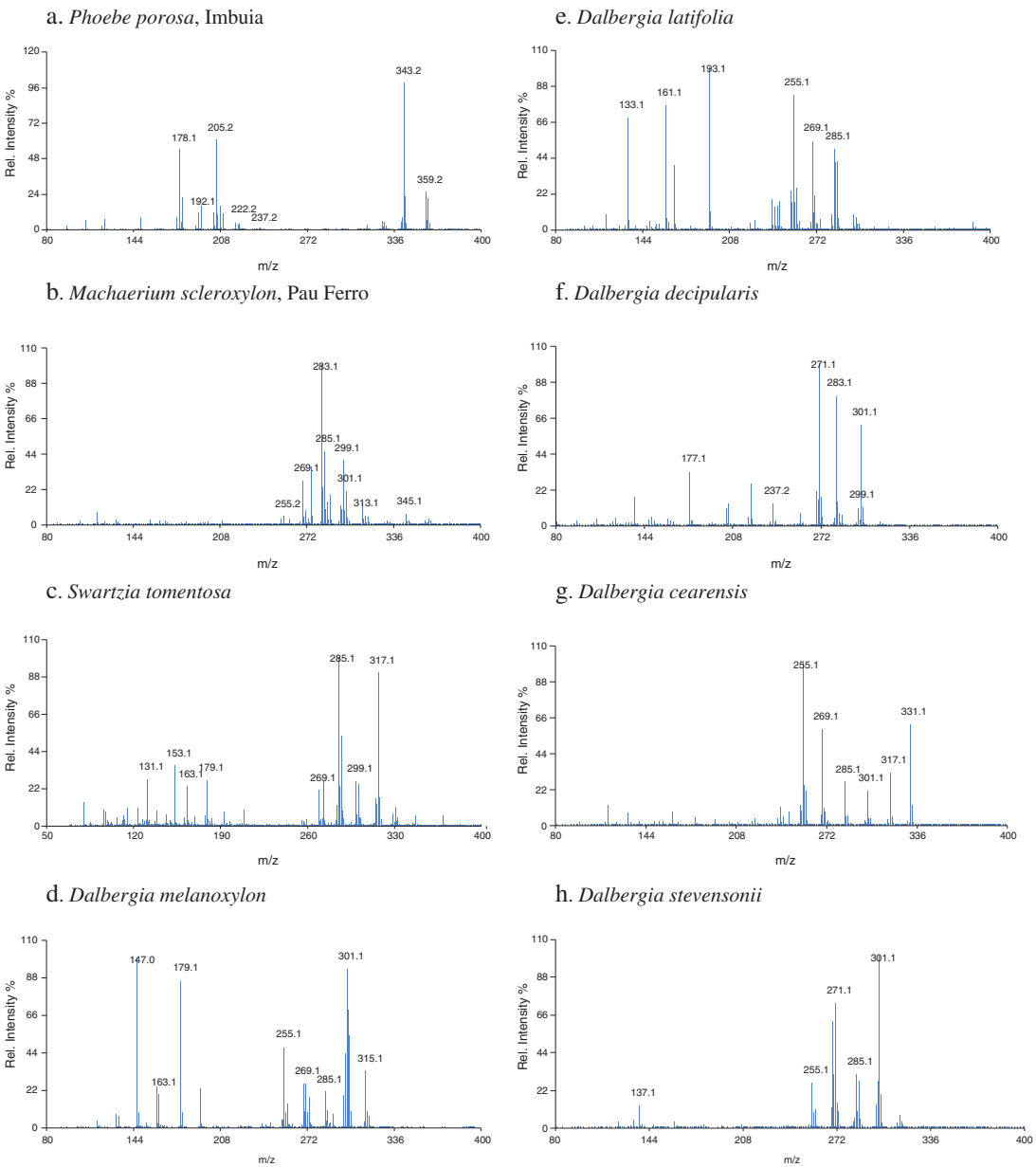

Figure 2. A typical mass spectrum from each of 11 wood species.

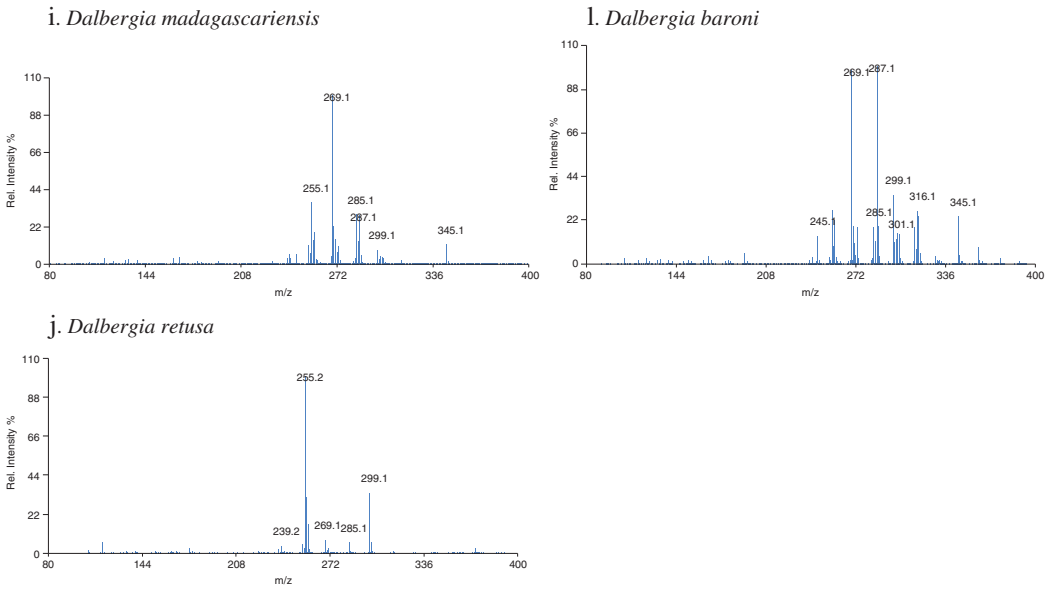

Figure 2. (Continued)

were calculated by using the correlation matrix. The ions selected for the statistical classification of wood species had abundances of at least 20% relative to the most abundant ion in the mass spectrum (20% threshold).

RESULTS AND DISCUSSION

The DART source usually produces $[M+H]^+$ or $[M^{+\bullet}]$ ions, although some molecules may produce both.[28] As in APCI, if an analyte molecule has a higher proton affinity than water clusters, it will be protonated to form the $[M+H]^+$ ion. $[M^{+\bullet}]$ ions are formed through Penning ionization in which a metastable atom or molecule transfers energy to the analyte molecule, which loses an electron.[27,28] Table 2 shows examples of both types of ions.

Species validation of *D. nigra*

Morphological differentiation of *D. nigra* from *D. spruceana* is difficult when the origin of the timber is known (i.e., NE Brazil), but problematic when the source of the wood is unknown.[9] Wood anatomists have relied on fluorescence techniques and the presence of diagnostic compounds to validate wood.[9,25] In this study, we only had three known reference samples (see Table 1). In order to validate the identity of the 15 suspected *D. nigra* samples, the fluorescence test of Miller and Wiemann was conducted.[9] All 15 samples showed fluorescence similar to that of *D. nigra* and dissimilar to that of D. *spruceana* (results not shown).

The three known standards of D. *nigra* were then analyzed using DART-TOFMS and it was found that a typical spectrum was dominated by ions at *m/z* 375.11, 299.09, 269.08, and 285.02 (Table 2). The 15 suspected samples of *D. nigra* exhibited spectra that were very different from that of *D. spruceana* (see below and also Fig. 1).

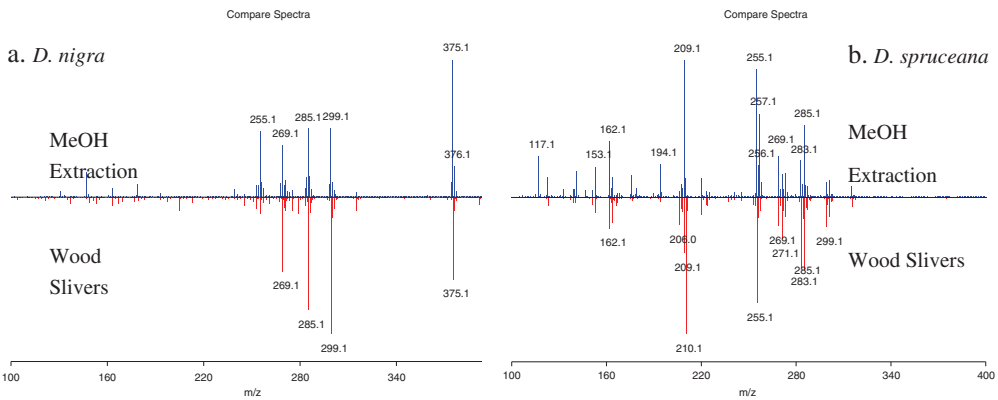

Figure 3. Relative intensity vs. *m/z* value of ions detected in methanol extracts of wood slivers (above the horizontal line) vs. unextracted wood slivers (below the line): (a) *Dalbergia nigra* and (b) *Dalbergia spruceana*.

The last validation step was to conduct a principal component analysis (PCA) using the reference standards (eight layers of *D. nigra* heartwood plus five other confirmed *D. nigra* samples) to create a training set (n = 13) that was compared against *D. spruceana* (n = 20). In this analysis, the 15 unconfirmed *D. nigra* samples listed in Table 1 were treated as unknowns. The PCA results classified all 15 samples that could not be confirmed morphologically with the *D. nigra* cluster and provided a strong inference that these samples were indeed *D. nigra* as purported by the seller, and were thus validated for the purposes of this study.

Reproducibility

In order to demonstrate the reproducibility of the DART-TOF mass spectra, the spectra of 18 samples of *D. nigra* and 20 samples of *D. spruceana* were compared (Fig. 1 shows six spectra of each). There were minor relative ion abundance variations in the spectra from each species, but the overall spectral pattern for each species was consistent. The reproducibility seen with *D. nigra* spectra was also observed for the other species tested. A representative positive-ion mass spectrum for the remaining 11 species analyzed is shown in Fig. 2.

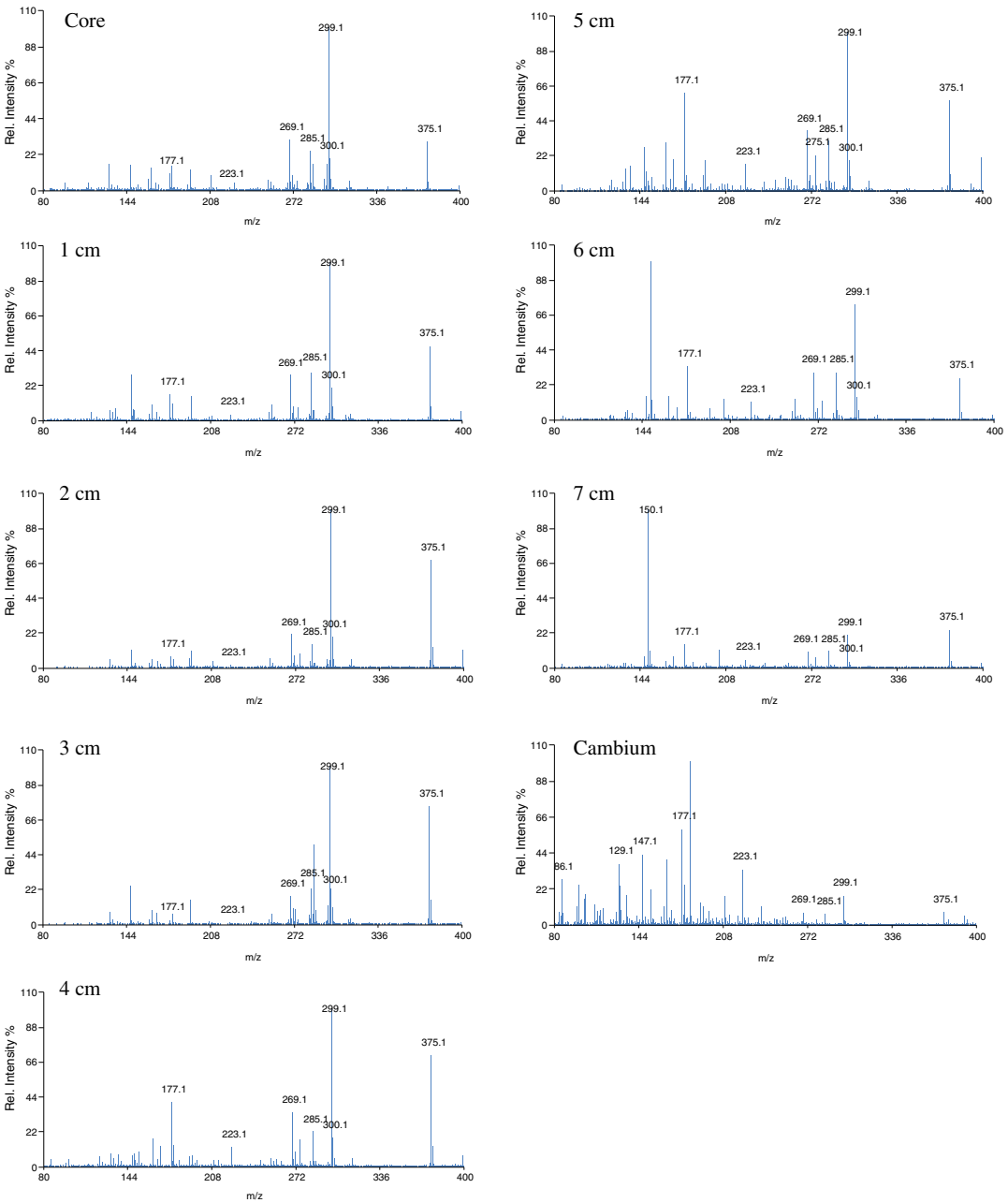

Figure 4. Mass spectra acquired for wood slivers from a cross section of a *D. nigra* trunk selected at 1-cm intervals from the core outward 7 cm. A mass spectrum for a cambium sample is also shown.

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Methanol extraction vs. wood sliver analysis

In order to compare the mass spectra obtained with DART-TOFMS with those obtained using ESI-MS,[25] two slivers of wood from *D. nigra* and *D. spruceana* were extracted in methanol for 48 h. The methanol solutions were analyzed using DART-TOFMS by sampling the extract with a melting point tube while the wood slivers were placed directly in the DART helium gas stream.

The mass spectrum of the methanol-extracted samples and the direct analysis of the wood slivers of *D. nigra* and *D. spruceana* are shown in Fig. 3. Most of the ions observed in the mass spectra (Figs. 3(a) and 3(b)) for the methanol extracts (above the line) and the wood slivers (below the line) were the same, while their relative abundances showed differences. These results validate that the data obtained by direct analysis of the wood slivers of *Dalbergia* spp. is similar to that of the more time-consuming methanol extraction approach.[25]

Distribution of compounds in *D. nigra*

The mass spectra from incremental (radial) analysis of a cross section of a *D. nigra* tree trunk are shown in Fig. 4. The data show that the sample spectra from the core to the last exterior growth ring of the heartwood (7 cm) are similar. The analysis of the cambium layer showed similar ions to those observed in the heartwood sample, but at different intensities.

Differentiation of *D. spruceana* and *D. nigra*

Miller and Wiemann suggested that *D. nigra* and *D. spruceana* could be differentiated using a series of traits including water and ethanol fluorescence of heartwood extracts.[9] However, the *D. nigra* and the Mexican *Dalbergia* species *D. granadillo* and *D. stevensonii* have similar fluorescent properties, which render fluorescence techniques inconclusive when the provenance of the timber is unknown.[26] Kite *et al.* used liquid chromatography-ultraviolet (LC-UV)-ESI high-resolution mass spectrometry to analyze methanol extracts of *D. nigra* heartwood and found a novel compound, dalnigrin, which is unique to *D. nigra*.[25]

When using DART-TOFMS, the spectral differences between *D. spruceana* and *D. nigra* were pronounced. The diagnostic compound dalnigrin found exclusively in *D. nigra* could not be differentiated from kuhlmannin without chromatographic separation because these isomers have the same *m/z* value of 299.0914 for their $[M+H]^+$ ions.[25] Tentative assignments of the ions detected are listed in Table 2. The predominant ions

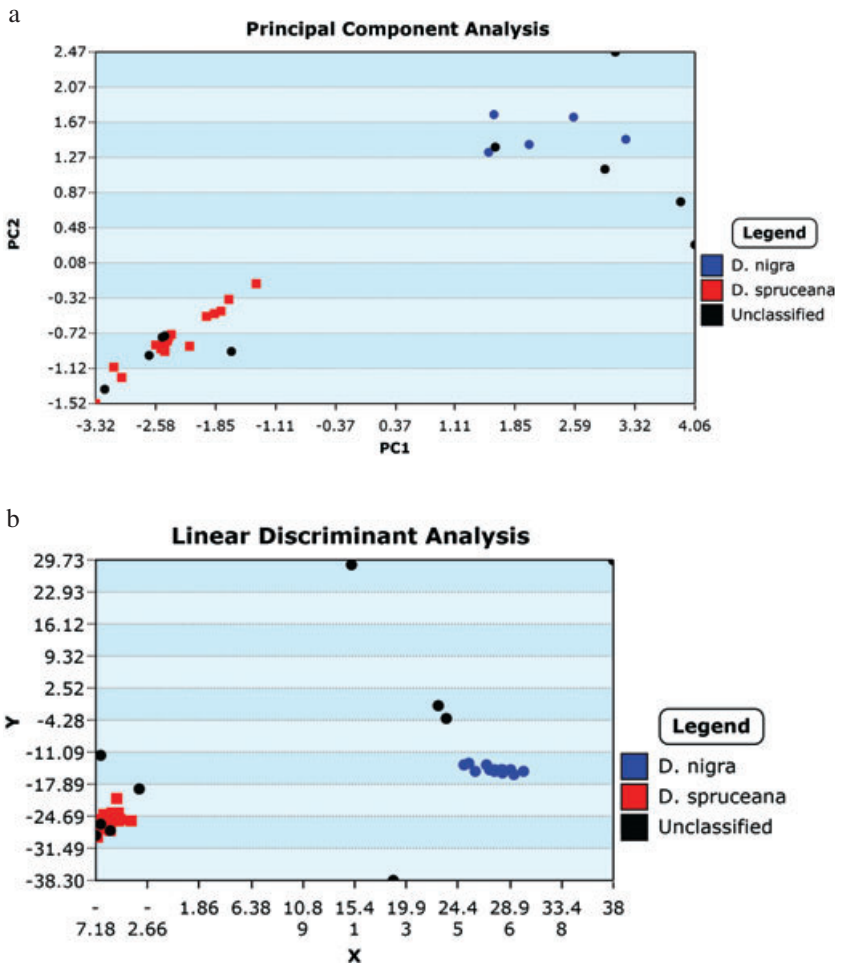

Figure 5. (a) Principal component analysis of *D. nigra* vs. *D. spruceana*. The cluster of points on the left corresponds to *D. nigra*, and the cluster of points on the right corresponds to *D. spruceana*. (b) Linear discriminant analysis yielded a cluster of points on the right for *D. nigra* and on the left for *D. spruceana*.

detected in *D. spruceana* were found at *m/z* 209.12 (unidentified compound) and 255.10 (dalbergione I). In *D. nigra*, the main ions detected include caviunin (*m/z* 375.11), dalbergin (*m/z* 269.08), and melanettin (*m/z* 285.02). These peaks do not represent the full range of possible compounds detected, but rather demonstrate that our data are consistent with compounds identified by others.[15,25,29,32]

One of the advantages of using DART-TOFMS, especially for complex samples, is the high specificity provided by the exact masses measured for the ions. Two ions at a nominal mass of *m/z* 285 were found for different compounds. Melanettin or dihydroxymethoxyflavone (*m/z* 285.0758) from *D. nigra* and 3,4-dimethoxydalbergione (*m/z* 285.1126) from *Machaerium* species could be readily distinguished.

Principal component analysis (PCA) and linear discriminant analysis (LDA) were employed to compare the spectra of these two species. The training sets consisted of 18 samples of *D. nigra* and 20 samples of D. *spruceana*; the ions selected for the statistical analysis were those with ion abundances of at least 20% relative to the base peak in the spectrum. The error tolerance was set at 0.005 u of *m/z* value of each ion. Figure 5 shows the graphical representation of the PCA and LDA analysis. The three principal components accounted for 76.61% of the variance (Fig. 5(a)). Clear separation was shown with 100% classification accuracy for PCA and 91.24% classification accuracy for LDA using leave-one-out cross-validation (LOOCV). The LOOCV is based on the distance from the cluster mean of each sample that is omitted.[24] Essentially, each sample is successively omitted from the training set and placed as an unknown, thus subjecting each sample for comparison against the entire training set. As an additional validation, five unverified samples from each species were excluded from the training set and used as unknowns. All ten were correctly classified with PCA and LDA (Fig. 5).

Except for the ions listed in Table 2, elemental compositions of the ions, whether they are precursor or product ions, and identifications of the compounds that produced the ions are not known. This knowledge is not required for statistical classification schemes. The power of LDA lies in the fact that one can analyze an unknown sample and compare its results against a population, and not only against a single reference standard or diagnostic ion. Analyses by DART-TOFMS provide numerous ions that are used in the LDA. Therefore, the statistical data clusters created by LDA can serve as reliable anchors for species identification, even when all the ions detected are not characterized.

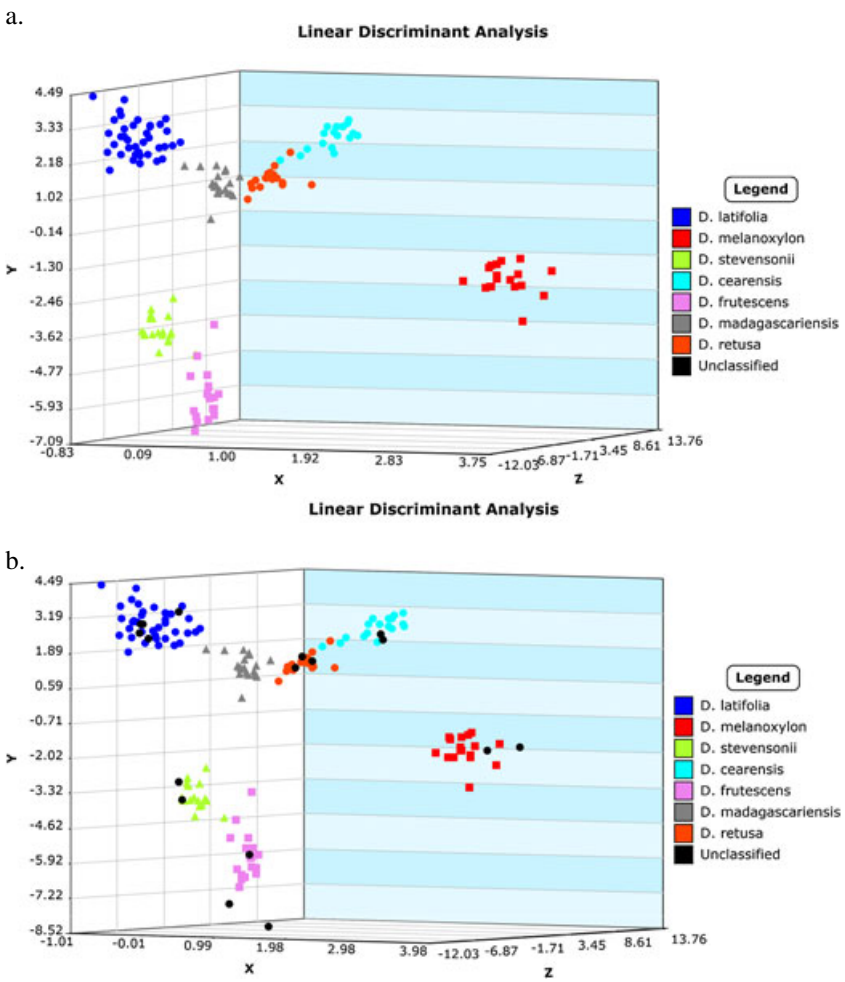

Figure 6. Linear discriminant analysis of eight *Dalbergia* species: (a) LDA of all samples and (b) LDA of samples with retained species as unknowns.

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Table 3. Tentative assignments for *Phoebe porosa*[30]

Name	Formula	Mass (*m/z*)
Cadinene	$C_{15}H_{24}$	205.1856 $[M+H]^+$
Carqueyl acetate	$C_{12}H_{16}O_2$	210.1494 $[M+NH_4]^+$
Oreodaphnol	$C_{15}H_{24}O_2$	237.1888 $[M+H]^+$
Porosadienone	$C_{15}H_{23}O$	219.1752 $[M+H]^+$

Assignments of other *Dalbergia* spp.

Braga de Oliveira *et al*. studied the occurrence of neoflavanoids and isoflavanoids in *Dalbergia* and *Machaerium* species and suggested that these natural products are useful chemotaxonomic pointers.[17] Because the taxonomic family Fabaceae includes *Dalbergia*, *Machaerium*, and *Swartzia*, we have tentatively inferred that some of the key ions detected in *Machaerium* and *Swartzia* (such as dalbergin at *m/z* 269) are from the same compounds as were found in *Dalbergia* (Table 2).[14,31]

The elemental compositions determined from the exact masses of other predominant ions observed for samples of *D. retusa*, *D. cearensis*, *D. melanoxylon*, *D. decipularis*, *D. stevensonii*, *D. madagascariensis*, *D. latifolia*, and *D. baronii* are listed in Table 2. Although there is minor intra-species variation, the differences between species are pronounced. Using LDA, the separation of these other *Dalbergia* species is feasible, as shown in Fig. 6(a). Leave-one-out cross-validation of the LDA gave a 91.72% correct classification [due to the small sample size of *D. baronii* (n = 10), it was not included in LDA or PCA analysis].

In order to corroborate the accuracy of the DART-TOFMS and LDA approach, 16 wood samples were retained, and their species were not verified by a wood anatomist. These samples were analyzed, but their spectra were not used in the training set to create the classification functions of LDA. When these samples were classified by LDA, 15 of them were correctly identified as their suspected taxa (Fig. 6(b)). The misclassification involved a sample of *D. stevenonsii* incorrectly classified as *D. madagascariensis*.

Analysis of woods commonly seen in trade

Because the timbers of *Swartzia tomentosa*, *Phoebe porosa* (imbuia), and *Machaerium scleroxylon* (pau ferro) are commonly seen in trade, and because there is a fear that *D. nigra* could be illegally imported with deliberate mislabeling of shipments, we also analyzed wood from these species to determine if they could be differentiated from each other and from *D. nigra*. Tentative assignments of the compounds detected in *Phoebe porosa* are listed in Table 3.[30] Figure 7 shows the results of the LDA and demonstrates that these taxa can be differentiated: LDA provided 96.15% classification accuracy. Two samples from each species whose taxa were not verified were treated as unknowns. All eight samples were correctly classified by the LDA analysis.

CONCLUSIONS

Analysis of 20 or more samples from the 13 species studied in this research established that DART-TOFMS provided reproducible mass spectra that are useful for species identification. Statistical analysis of the most abundant ions provided accurate classifications of unknown wood samples in over 90% of the cases.

The analysis of wood samples that were extracted in methanol gave results that are comparable with those obtained by the direct analysis of dry wood slivers. Given that there is essentially no sample preparation, and the ease of operating the DART-TOFMS instrument, many samples could be analyzed rapidly, and their spectra could be compared statistically using LDA with those of known reference populations of timber, rather than with a single reference standard or diagnostic ion. Therefore, the power of this method lies in the fact that hundreds of samples can be run quickly, and their spectra can be used to create species populations of data. These data clusters then serve as reliable anchors for species identification, although not all the compounds detected are characterized. Wood anatomists who are challenged with the difficult morphological identification, D. *nigra* vs. *D. spruceana*, could use this tool to assist in their analyses.

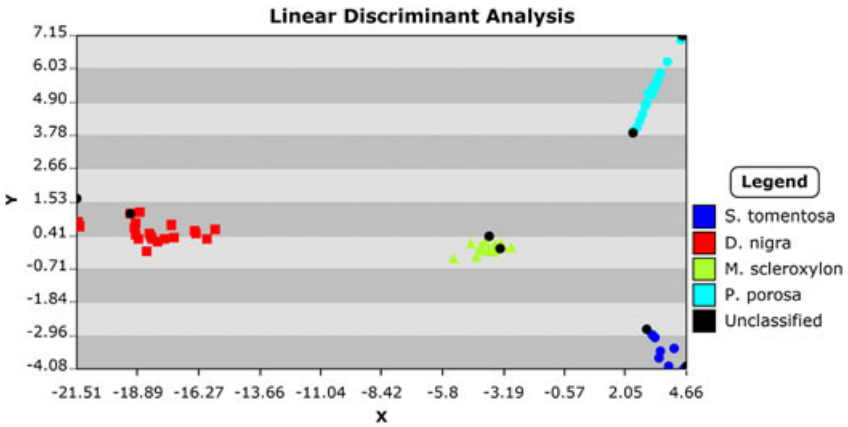

Figure 7. Linear discriminant analysis of commonly traded *Swartzia tomentosa*, *Phoebe porosa* (imbuia), *Machaerium scleroxylon* (pau ferro), and *Dalbergia nigra*.

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