

FORENSIC ANALYSIS OF CITES-PROTECTED *DALBERGIA* TIMBER FROM THE AMERICAS

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ABSTRACT

Species identification of logs, planks, and veneers is difficult because they lack the traditional descriptors such as leaves and flowers. An additional challenge is that many transnational shipments have unreliable geographic provenance. Therefore, frequently the lowest taxonomic determination is genus, which allows unscrupulous importers to evade the endangered species laws. In this study we explore whether analysis of wood using a Direct Analysis in Real Time (DART) Time-Of-Flight Mass Spectrometer (TOFMS) can assist in making unequivocal species determinations of *Dalbergia*. DART TOFMS spectra were collected from the heartwood of eight species of *Dalbergia* and six other look-alike species. In all, fourteen species comprising of 318 specimens were analyzed and the species chemical profiles were examined by statistical analysis. *Dalbergia nigra* (CITES Appendix I) was differentiated from *D. spruceana*; *D. stevensonii* (Appendix II) was distinguished from *D. tucurensis* (Appendix III), and all the look-alike timbers could be readily distinguished. Surprisingly, *D. retusa* (Appendix III) could not be differentiated from *D. granadillo*, and we postulate that they are synonymous. We conclude that DART TOFMS spectra are useful in making species identifications of American *Dalbergia* species, and could be a valuable tool for the traditional wood anatomist.

Keywords: Wood identification, illegal logging, DART, TOFMS.

INTRODUCTION

The increased demand for rosewood (*Dalbergia* spp.) has augmented the illegal logging of these taxa. Enforcement of illegal importation is challenged by the fact that law enforcement officers are often uncertain of the geographic provenance of seized logs and the timber lacks the traditional morphological characters (leaves, flowers, etc.) used to make species determinations. In order to identify the species of timbers, planks, and veneers, we demonstrate the use of a specific mass spectrometer method that allows for species level identification of *Dalbergia* spp. from the Americas. This

technique will assist wood anatomists to make unequivocal species determination and by extension help reduce illegal timber trade.

The genus *Dalbergia* of the Leguminosae (subfamily Papilionoideae) (Germplasm Resources Information Network; Lewis & Schrire 2003) is a complex genus composed of some 250 species distributed throughout the tropics (Klitgaard & Lavin 2005; Mabberley 2008). It was first described in the late 1700s by Linnaeus (1781), and to this day many of the original *Dalbergia* species descriptions (such as Pittier 1922) are vague, brief, and rely on classical botanical diagnostic features such as flowers, leaves, etc. Recent phylogenetic work on the family, particularly the “dalbergoid” legumes (Lavin *et al.* 2001) is a good example of the challenges *Dalbergia* presents in this phylogenetic analysis. Three of the five *Dalbergia* specimens used were identified only as *Dalbergia* spp. despite the genus’s pantropical distribution, exemplifying the difficulty of accurate species identifications.

The Forest Products Laboratory web-based “Common Names Database of World Timbers” (2014) lists 60 species that are known by the common name of rosewood. Of these, 16 belong to the genus *Dalbergia*. Rosewoods have characteristic colors and textures that make them highly desirable. They were used historically as a luxury wood to decorate European palaces and are still used to manufacture fine furniture and cabinetry. Rosewoods are also highly regarded for use in making musical instruments due to their acoustic qualities (Flynn & Holder 2001; Jenkins *et al.* 2002). In September of 2014, 20 cubic meters of *Dalbergia stevensonii* was selling for USD 79,000 (Alibaba.com) making it one of the costliest woods on the market. Of the 58 species of *Dalbergia* listed by the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES), five are from the Americas, one is from Africa, one is from Indochina, and 43 species are from Madagascar (DuPuy *et al.* 2002). Only *Dalbergia nigra* (Vell.) Allem. *ex* Benth. (Brazilian rosewood) is listed under CITES Appendix I and is therefore banned from international commercial trade (CITES 2014).

The Forensics Laboratory of the U.S. Fish and Wildlife Service and the Center for Wood Anatomy Research of the Forest Products Laboratory regularly receive requests to identify species of imported wood suspected of being in violation of CITES. In order to help ensure compliance with timber importation regulations, these federal agencies conduct research into methods that can be used to separate restricted species from similar but unrestricted species. For example, Miller and Wiemann (2006) used physical and fluorescent properties to separate two important Brazilian species, *Dalbergia nigra*, restricted by CITES Appendix I, from *Dalbergia spruceana* Benth., which has no restrictions. Lancaster and Espinoza (2012a) used entirely different methods, based on mass spectrometry, to distinguish *Dalbergia nigra* from other species of *Dalbergia*. Carvalho (1997) wrote a synopsis of the Brazilian species of *Dalbergia* but did not include wood anatomical descriptions.

Wood anatomical characters have been the traditional means of identifying samples of timber or finished wood. However, wood anatomy has limitations, and in some instances identification is only possible to genus or a group of species. For example, Gasson *et al.* (2010) studied seven tropical American species of *Dalbergia*, including *D. nigra*,

and determined that all seven shared 15 diagnostic characters. Multivariate analysis of quantitative anatomical characters (*e.g.*, vessel diameter and frequency, ray width, height and frequency) was only able to separate the species into two groups. Group 1 included *D. nigra*, *D. spruceana*, *D. tucurensis* Donn.Sm. and *D. stevensonii* Standl.; group 2 included *D. miscalobium* Benth., *D. cearensis* Ducke and *D. granadillo* Pittier. To aid in the identification of protected tree species, Gasson *et al.* (2011) published light micrographs of all CITES-listed tree taxa, and Gasson (2011) discussed the precision to which these species can be identified using wood anatomy, but the reality is that many of these species are very difficult to identify using wood anatomy alone.

The addition of physical characteristics, such as density, can improve the ability to distinguish between otherwise similar-appearing species. Of the species in group 1 above, *D. nigra* and *D. spruceana* grow in Brazil, and *D. tucurensis* and *D. stevensonii* grow in Central America. If provenance is known, the two Brazilian species can be separated by density (Miller & Wiemann 2006), as can the two Central American species (Wiemann & Ruffinatto 2012).

Fluorescence characteristics are sometimes helpful in separating similar species. Guzmán *et al.* (2008) tabulated the fluorescence spectra of surface, water extract and ethanol extract of 92 Mexican genera, including three species of *Dalbergia*. In their study, none of the *Dalbergia* species exhibited surface fluorescence, one of them exhibited only water extract fluorescence, and two of them exhibited only ethanol extract fluorescence. Richter *et al.* (1996), on the other hand, found faint surface fluorescence in *D. funera* Standl. from Mexico but not in eight other species, and Wiemann and Ruffinatto (2012) found surface fluorescence in some specimens of *D. stevensonii* but not in others. Water and ethanol extract fluorescences separated *D. nigra* from *D. spruceana* (Miller & Wiemann 2006) but could not separate *D. tucurensis* from *D. stevensonii* (Wiemann & Ruffinatto 2012). Chemistry may help to separate species, as demonstrated by Kite *et al.* (2010) who isolated a neoflavonoid which appears to be unique to *D. nigra* in the 15 species of *Dalbergia* they studied.

When the geographic source of an unidentified species is known, wood anatomy and physical properties can often be sufficient to give a reliable identification (Miller *et al.* 2002). But the international timber trade is global, and the incentives for providing false information are substantial, so provenance is often unknown or dubious.

Direct analysis in real time (DART) time-of-flight mass spectrometry (TOFMS) may hold a solution to the problem of overlapping anatomical character states and uncertain provenance of wood and timber. DART is a novel way of creating ions, which are subsequently detected and analyzed in a mass spectrometer. DART ionization relies on helium atoms that are moved to an excited state whereby, through a complex reaction, these ionized molecules in turn transfer protons to the chemical compounds encountered in the wood sample, giving a characteristic protonated signal ($M+H$) for each molecule encountered. When installed on a TOFMS, DART provides high accuracy mass measurements. A detailed description of the DART ionization mechanisms has been thoroughly reported by Cody (2013), Cody and Dane (2010) and Cody *et al.* (2005). In practice, a sliver of wood is placed between the DART and the TOFMS inlet for about 6 to 10 seconds, and the protonated stream removes the molecules that are met on the

wood surface. These molecules are then drawn into the mass spectrometer. The ease of use cannot be overstated, and it is routine for a lab to be able to analyze more than 100 wood samples per day where each specimen needs no prior sample preparation.

Other advantages of DART are that it is used in an ambient (open air) environment, it does not require difficult sample preparation, it needs no radioactive components, and it gives instant results (Cody *et al.* 2005; Harris *et al.* 2011; Cody 2013). It has been used to separate two species of *Quercus* (Cody *et al.* 2012), ten American species of *Dalbergia* (Lancaster & Espinoza 2012a), and the presence of *Aquilaria* commercial products (Lancaster & Espinoza 2012b). Recently it was reported that using DART TOFMS distinguished wild from cultivated populations of *Aquilaria* (Espinoza *et al.* 2014). Because the technique is new, it is still unclear what its limitations might be. Here we report the application of using the DART TOFMS methodology to the challenging problem of identifying *Dalbergia* wood samples.

MATERIALS AND METHODS

Specimens

Eight species of *Dalbergia* from South and Central America and six species of timbers that resemble rosewood were collected from curated xylarium collections and/or commercial wood sources (Table 1). The species were chosen because of their CITES protection, their similarity in appearance to a protected species, or their commercial importance. In total, 318 specimens representing 14 species were obtained. Table 1 shows the source of the specimens, which included the USDA Forest Product Laboratory (MAD, SJR), USDA Animal and Plant Health Inspection Service (APHIS), Oregon State University Xylarium (OSU), Laboratorio de Productos Florestais, Brasília, Brazil (LPF), La Xiloteca del Instituto de Biología, UNAM, Mexico City, México (XIB), Eissenbrand Inc. Exotic Hardwoods, Torrance, CA, USA (EIEH), Cook Woods, Klamath Falls, OR, USA (CW), Gilmer Wood Co., Portland, OR, USA (GW), Carlton McLendon Inc., Atlanta, GA, USA (CMI), PFC Shanty Navarro Hurtado, Brazilian Federal Police (SNH), Bell Forest Products Inc. (BFPI), and the Botany collection at the University of South Carolina (USC). Commercially obtained specimens were verified by either anatomical analysis or statistical consensus results with known curated specimens.

Anatomy methods

The process used for preparing the wood samples followed the method described by Barajas-Morales *et al.* (1997). Xylarium samples were prepared in one-centimeter blocks for the anatomical studies and determination of specific gravity. The wooden blocks were softened in a boiling solution of 6% ethylenediamine for 3–4 hours (Kukachka 1977). The blocks were then cut with a sliding microtome into radial, tangential and transversal sections of 15 to 25 μm thickness. The sectioned samples were stained with safranin 1, and dehydrated in various alcohol solutions and the resulting samples were mounted on glass slides using synthetic resin. To measure vessels and fibers, the samples were macerated by placing 2–3 cm long wood chips in Jeffrey's solution for 24 hours.

Table 1. Specimens studied and their source.

Source abbreviations described under Specimens in Materials and Methods. Geographic provenance from South and Central Africa.

Species	CITES	n	Country of origin	Source (n)
South America:				
<i>Dalbergia nigra</i> (Vell.) Benth.	App I	24	Brazil	LPF (2) EIEH (17) CW (5)
<i>Dalbergia spruceana</i> (Benth.) Benth.		17	Brazil	GW
<i>Dalbergia cearensis</i> Ducke		20	Brazil	EIEH
<i>Dalbergia decipularis</i> Rizzini & A.Mattos		19	Brazil	EIEH
<i>Machaerium scleroxylon</i> Tul.		20	Bolivia	EIEH
<i>Swartzia tomentosa</i> DC.		19	Brazil	EIEH
<i>Phoebe porosa</i> (Nees & Mart.) Mez		20	Brazil	EIEH
<i>Caesalpinia echinata</i> Lam.	App II	18	Brazil 1 Unknown	EIEH (1) MAD, SJR (4) CMI (10) APHIS (1) USC (1) SNH (1)
Central America:				
<i>Dalbergia retusa</i> Hemsl.	App II	34	20 Mexico 8 Panama 2 Nicaragua 1 Costa Rica 2 C. America 1 unknown)	OSU (1) MAD, SJR (19) (13) XIB (1) EIEH (19)
<i>Dalbergia granadillo</i> Pittier	App II	11	8 Mexico 1 Guatemala 1 Nicaragua 1 unknown	MAD, SJR (5) XIB (5) CW (1)
<i>Dalbergia stevensonii</i> Standl.	App II	30	C. America	MAD, SJR (14) XIB (2) EIEH (14)
<i>Dalbergia tucurensis</i> Donn.Sm.	App III	54	C. America	BFPI (16) CMI (10) CW (18) MAD, SJR (9) XIB (1)
<i>Caesalpinia platyloba</i> S.Watson		12	Mexico	XIB (2) EIEH (10)
<i>Platymiscium yucatanum</i> Standl.		20	Mexico	XIB (6) CMI (14)

Specific gravity was measured by drying the samples at 105 °C for 24 hours, weighing the dried samples and then measuring their dry volumes using the water displacement method of Olesen (1971). Twenty-five measurements were made for measurable structures, indicating the average, maximum, minimum and deviation standard for each, in accordance with the IAWA Committee (1989 [Wheeler *et al.*]).

Chemistry methods

All of the samples were analyzed directly by DART-TOFMS by holding a wood sliver in the gas stream with no further sample preparation. A mass calibration standard of poly(ethylene glycol) 600 (Ultra, Kingstown, RI, USA) was run between samples. 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, USA) in positive ion mode. The DART source parameters were: electrode 1 voltage, 150 V; electrode 2 voltage, 250 V; and gas heater temperature at 450 °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, 2300 V. Spectra were obtained over the mass range of m/z 60 to 1000 at 1 scan per second. The helium flow rate for the DART source was 2.0 mL per second. The resolving power of the mass spectrometer, as stated by the manufacturer, was 2.0 mDa.

TSSPro3 (Shrader Analytical Labs, Detroit, MI, USA) data processing software was used to export the text files of the mass-calibrated, centroided mass spectra for molecular formula determination and further analysis. Heat maps and statistical analysis of the data sets were conducted using the Mass Mountaineer software (RBC Software, Peabody, MA, USA). The classification algorithms of Mass Mountaineer were used to calculate the principal components of each data set. Kernel Discriminant Analysis (KDA) was performed using selected diagnostic ions. For all models, a tolerance of 5 mDa was applied. To assess model accuracy, leave-one-out cross-validation (LOOCV) was employed. The LOOCV is based on the distance from the cluster mean of each sample that is omitted. 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. In short, LOOCV is a metric of how well the model performs. Mass Mountaineer software is also capable of assigning an estimate of probability to an unclassified (*e.g.*, unknown) spectrum.

Linear discriminant analysis (LDA), a supervised learning algorithm, relies on *a priori* assignment of a class membership in order to achieve the greatest separation between classes in a training set. Kernel Discriminant Analysis (KDA) is an extension of LDA that maps features into a higher-dimensional space by using a typically nonlinear function. This allows points that cannot be linearly separated in a two-dimensional space to be separated in higher dimensions. Estimated probabilities are based on Z scores (distance divided by standard deviation) based on a normal distribution (Baudat & Anouar 2000).

Unsupervised cluster analysis was done with Gene cluster 3.0 (De Hoon 2002) to give a hierarchical cluster analysis of arrays using correlation (uncentered) similarity metric. A dendrogram was produced using Java TreeView (Saldanha 2004).

RESULTS

Anatomy

Most American *Dalbergia* have been described elsewhere and therefore this paper will not review the existing literature. *Dalbergia granadillo* lacks a detailed description, but it is sympatric with *D. retusa* and the two species exhibit a high similarity in appearance. In both cases the sapwood is creamy to yellowish brown, slightly reddish near the heartwood and frequently grayish near the bark. The heartwood is reddish brown, abundantly streaked with irregular blackish-brown to black lines; wet or freshly cut wood has a characteristic grassy smell, and a fleeting spicy taste; it exhibits a dull luster that gives it a waxy appearance; texture is medium, with interlocked grain; it is extremely hard and heavy with a specific gravity of 1.1. Growth rings are present but inconspicuous, delimited by thick-walled fibers and sometimes marginal parenchyma. The vessels are circular in outline, 150–300 μm in diameter, solitary and in radial groups of 2–5, and fewer than two per mm^2 ; their lumina contain abundant brown to black gum. Perforations are simple. Intervessel pits and ray-vessel pits are alternate, vestured, and 10–12 μm in diameter. Axial parenchyma is diffuse-in-aggregates, in uniseriate or biseriate bands, vasicentric, aliform, confluent, and marginal. The fusiform parenchyma cells are two per strand, often containing crystals. Rays are 11 per mm , composed entirely of procumbent cells, mostly uniseriate but occasionally biseriate, with dark red deposits. Ray height is 110–300 μm . Fibers are libriform, 20–21 μm in diameter with cell walls 3 μm thick cell walls, 900–1300 μm in length, and frequently contain brown deposits. All elements are storied.

Chemistry results and statistical analysis

South America Dalbergia spp. and look-alike species

DART TOFMS data produces a graphical spectrum that shows the molecules present in a specimen. An example based on two species is shown in Figure 1, where it compares *Dalbergia tucurensis* with *Caesalpinia echinata* Lam., a look-alike species from Brazil. Each peak corresponds to a molecule detected in the wood sample.

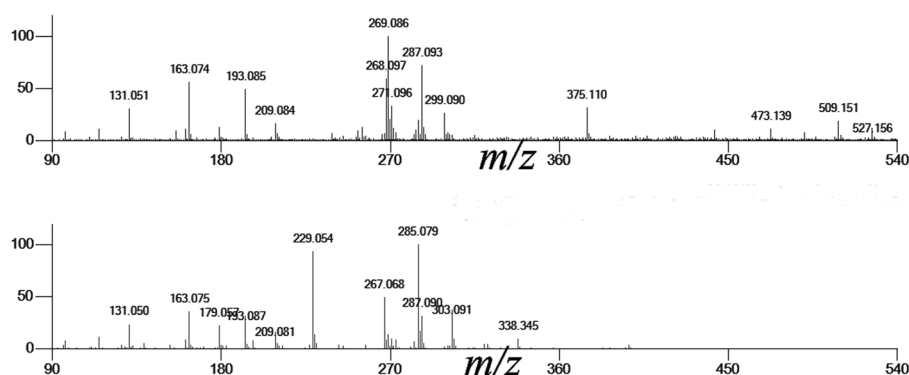


Figure 1. Examples of DART TOFMS spectrum profiles of *Dalbergia tucurensis* (top) and *Caesalpinia echinata* (bottom).

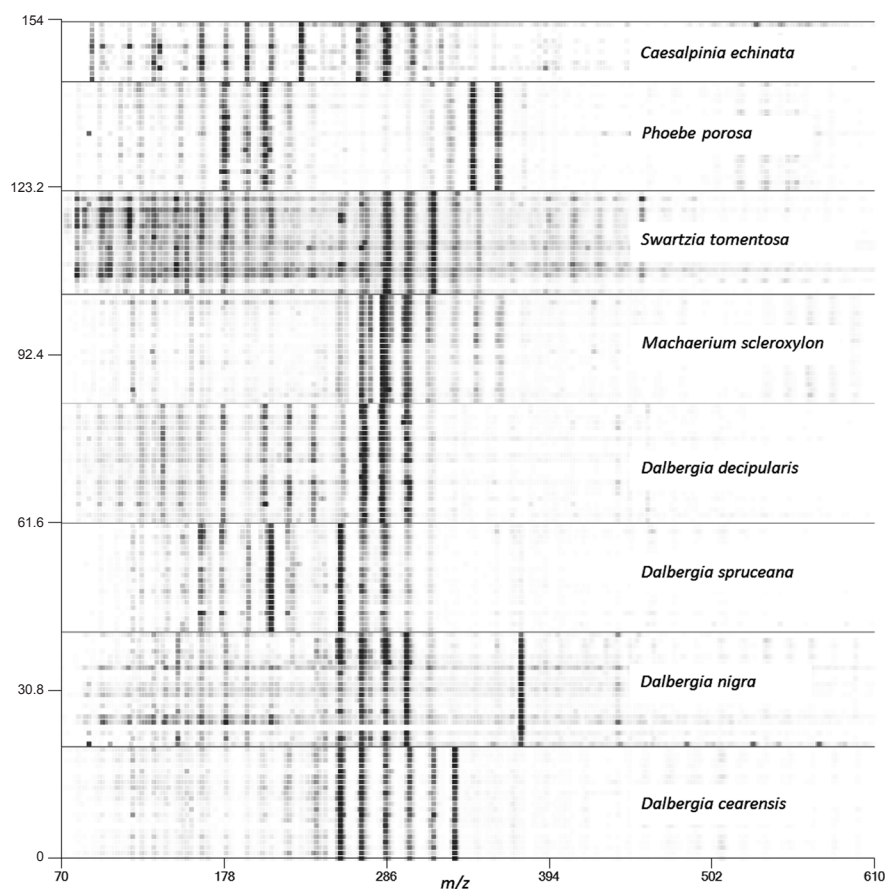


Figure 2. Heat map of eight South American species. The x axis is the molecular mass of compounds detected and the y axis is the sample number.

Figure 2 is an example of a heat-map which is a graphical representation of the spectral results of a large dataset for eight species from South America. The x axis is the molecular mass for the molecules present and the y axis is the number of each sample. The intensity of the color in a column is correlated to the quantity of the molecule in a spectrum; an intense color indicates a higher concentration of the molecule and conversely a hint of color indicates trace concentration. Figure 2 demonstrates that all the species analyzed have distinct chemical profiles. An advantage of displaying the data in a heat-map is that molecules that are diagnostic to a particular species are immediately apparent. For example, Figure 2 demonstrates that only *D. nigra* shows the presence of caviunin (m/z 375.111) so the presence of caviunin can be used as a diagnostic compound. It should be stressed that the heat-map is only a graphical representation of the raw mass spectra of the samples and is not a statistical procedure.

The mass spectra of the South America samples were also analyzed by Kernel discriminant analysis and the statistical graph is shown in Figure 3. This supervised

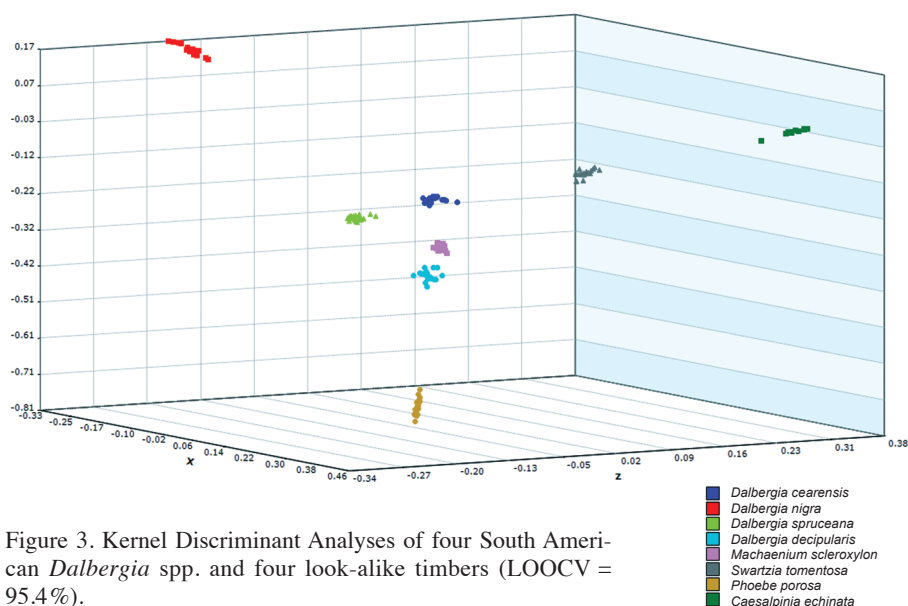


Figure 3. Kernel Discriminant Analyses of four South American *Dalbergia* spp. and four look-alike timbers (LOOCV = 95.4%).

classification analysis demonstrates that these eight species can be differentiated. Clear separation was shown with 95.4% classification accuracy using leave-one-out-cross validation (LOOCV). The LOOCV is based on the distance from the cluster mean of each sample that is omitted. Therefore the heat-map and the KDA analysis suggest that spectral data collected on the DART TOFMS could be helpful when either the anatomical diagnostic features are confounding (as in *D. nigra* vs *D. spruceana*) or are non-existent (as in the case of wood veneers).

Central America *Dalbergia* spp. and look-alike species

Figure 4 is the heat-map for Mexican and Central American species. An examination of this figure shows that the chemical profiles of the Mexican and Central American species are not as distinct as those from the reference samples from South America. *Dalbergia stevensonii* and *D. tucurensis* have very similar chemical profiles, but they can be distinguished because the molecules at m/z 375.111 (tentatively assigned to caviunin/isocaviunin^H C₁₉H₁₈O₈) are unique to *D. tucurensis* and those at m/z 315.086 (tentatively assigned to onogenin^H C₁₇H₁₄O₆) are unique to *D. stevensonii*. Figure 4 also shows that *D. granadillo* and *D. retusa* appear to have nearly identical chemical profiles and cannot be differentiated using the heat-map.

The mass spectra of the Central America *Dalbergia* spp. were also analyzed by KDA and the statistical graph is shown in Figure 5. This supervised classification analysis shows that the six species produced only five clusters and that *D. granadillo* cannot be differentiated from *D. retusa*.

LOOCV for the KDA analysis of Figure 5 was only 88%, because *D. granadillo* and *D. retusa* form a single cluster. If these two species are combined into a single class the analyses increase the LOOCV to 98% (graph not shown).

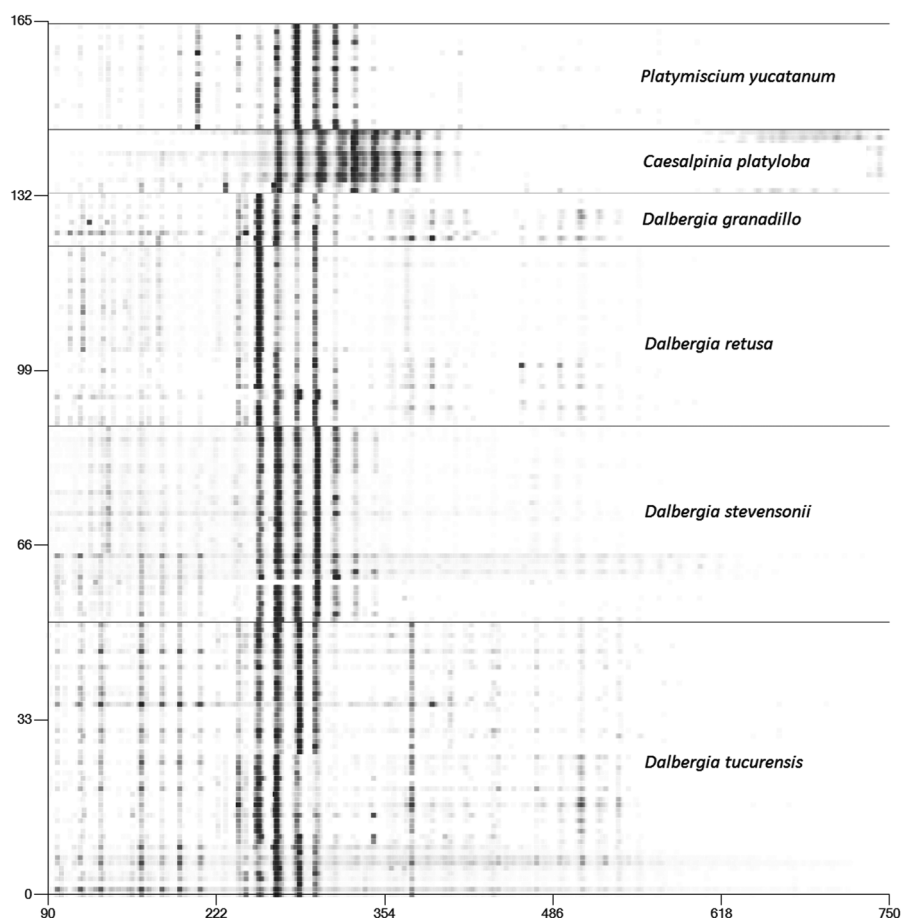


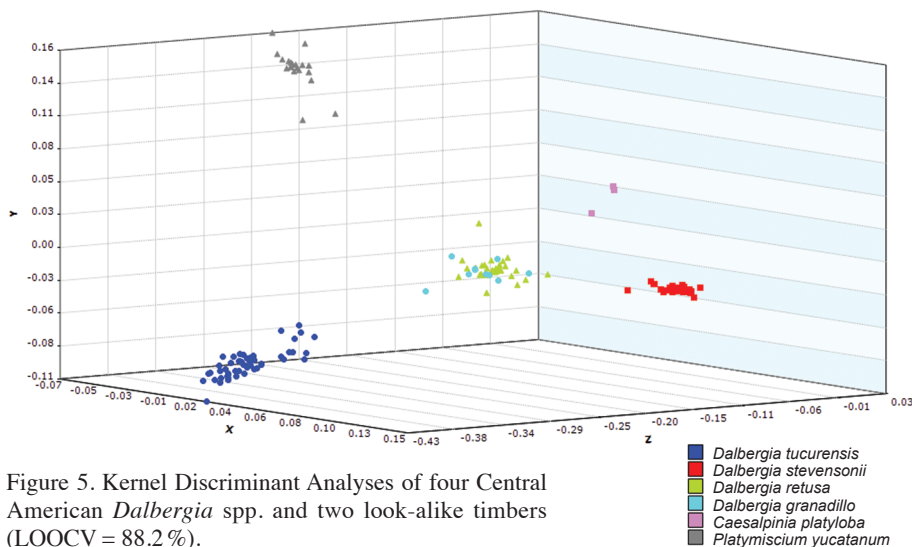
Figure 4. Heat map for six Central American species. The x axis is the molecular mass of compounds detected and the y axis is the sample number.

Because the previous analysis could not differentiate *D. granadillo* and *D. retusa* we conducted a cluster analysis of the data using Cluster 3.0, and Java Tree view. Unlike KDA, this analysis is not supervised and was performed using an uncentered correlation of 436 variables of the spectra data.

Figure 6 shows the dendrogram of the analysis. The dotted lines in this figure are the *D. granadillo* specimens. The dendrogram shows that the *D. granadillo* groups with the *D. retusa* samples and that these two species cannot be differentiated.

DISCUSSION

The historical demand for *Dalbergia* wood has not abated, and the prices commanded for its purchase are higher than ever before. Only one species, *D. nigra*, is entirely banned from international trade while many other species require easily acquired or



no CITES permits. Since the trade is in logs and timber, the traditional morphological characters of these taxa (flowers and foliage) are absent, and all identification of the specimens must be done on the wood itself. Traditionally, all wood classification has been done using anatomy characters.

Anatomical descriptions for some of the species listed in Table 1 are well documented in InsideWood (Wheeler 2011). Because their anatomy is so similar, separation of *D. nigra* from *D. spruceana* and *D. retusa* from *D. granadillo* have caused difficulties in CITES enforcement. Although *D. nigra* (CITES Appendix I) cannot be separated from *D. spruceana* (unrestricted by CITES) using wood anatomical characters, DART TOFMS analysis can assist in distinguishing the species. Additionally, the inclusion of look-alike species does not present any confounding problems.

Of the Central America species, the challenges for a wood anatomist are to distinguish *D. stevensonii* from *D. tucurensis* and *D. retusa* from *D. granadillo*. We have shown that DART TOFMS analysis can assist in distinguishing *D. stevensonii* from *D. tucurensis*. However, the heat-map graph, KDA, and cluster analysis failed to separate *D. retusa* from *D. granadillo*.

When Pittier (1922) originally described the new species of *D. granadillo* he stated: "*The specimens at hand are hardly satisfactory for a description, but they belong to a section heretofore not known to be represented in Mexico and differ from the other Middle America species of the group in the shape, consistence and induments of the leaflets, and in the shape and appearance of the pods. It is consequently pretty safe to consider them as corresponding to a type specifically distinct.*" [bold-face added].

Pittier's 1922 descriptions also included *D. retusa* and *D. hypoleuca* Pittier, species today considered to be synonymous and defined by the single name *D. retusa*. Current wood anatomy databases such as InsideWood (InsideWood 2004; Wheeler 2011), and the CITESwoodID software (Richter *et al.* 2008; Richter *et al.* 2014), cannot separate *D. granadillo* from *D. retusa*. A challenge in the study of *D. granadillo* is that worldwide there are only 10 known curated reference wood samples.

After reviewing the literature, the equivocal anatomy descriptions, the absence of reference specimens, and the results of our chemical analyses, we believe that *D. granadillo* is most likely yet another taxon synonymous with *D. retusa*. Further collections of these two species for morphological and molecular analysis are needed to confirm or refute this claim.

In conclusion, we found that DART TOFMS spectra were useful in making species separations of American *Dalbergia* spp. and look-alike timber species, and could be a useful tool for the traditional wood anatomist. However, given that there are 250 species (Mabberley 2008), we question whether DART can separate all of them. We assumed that the inability to differentiate *D. retusa* from *D. granadillo* is due to synonymy, but it is also possible that they are distinct species whose spectra are not separable. Many more samples from many more species must be tested to resolve this problem.

LEGAL NOTE

The findings and conclusions in this article are those of the authors and do not necessarily represent the views of the U.S. Fish and Wildlife Service.

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Accepted: 9 February 2015