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OF WILD FAUNA AND FLORA

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Interpretation and implementation of the Convention

Species trade and conservation

Trees

Agarwood-producing taxa (*Aquilaria* spp. and *Gyrinops* spp.)
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EVALUATING AGARWOOD PRODUCTS FOR 2-(2-PHENYLETHYL) CHROMONES USING DIRECT ANALYSIS IN REAL TIME TIME-OF-FIGHT MASS SPECTROMETRY

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Evaluating agarwood products for 2-(2-phenylethyl)chromones using direct analysis in real time time-of-flight mass spectrometry

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RATIONALE: Agarwood is the resinous material harvested from threatened *Aquilaria* species. We investigated how many protonated 2-(2-phenylethyl)chromone ions were sufficient to make an accurate identification of agarwood. Analysis of 125 reference samples was carried out by direct analysis in real time time-of-flight mass spectrometry (DART-TOFMS). The identification criteria developed were applied to commercial samples.
METHODS: We developed a technique that uses DART-TOFMS to detect 2-(2-phenylethyl)chromones. Additionally, we developed a set of criteria to infer the presence of *Aquilaria* in commercial samples of wood chips, sawdust, incense and liquids. Additionally, we examined other fragrant woods to determine if they contained a chemical profile that could be falsely identified as agarwood.
RESULTS: Analysis of reference and commercial samples (n = 151) established that DART-TOFMS provides reproducible mass spectra that are useful for inferring the genus of suspected agarwood samples. We identified 17 ions which were useful for authenticating agarwood. Comparison of the number of chromone ions detected by direct analyses of dry wood chips versus eluent analysis of methanol-extracted wood showed that results were similar. Lastly, analysis of 25 scented woods of other species did not give false positive results.
CONCLUSIONS: Reliable criteria for inferring agarwood include the presence of diagnostic ions, *m/z* 319.118 or 349.129, in addition to ten or more ions characteristic of 2-(2-phenylethyl)chromones. Wood anatomists challenged with difficult morphological identifications can use this tool to assist in their analyses. Published 2012. This article is a US Government work and is in the public domain in the USA.

Agarwood is the commercial name given to the fragrant resinous heartwood obtained from certain trees of the genus *Aquilaria* (Malvales: Thymelaeaceae). Since agarwood is used by many ethnic cultures, it is also known by other trade names such as eaglewood, agar, ghara, gaharu and kalambak in Malaysia, kanankoh and jinkoh in Japan, oudh, and aloeswood.[1,2] It is widely accepted that the dark resinous material of *Aquilaria* is created as a response to some form of injury to the tree such as cutting, moth infestation, fire, bacteria, and chemical wounding.[2–5] Trade in agarwood has intensified in recent years due to demand and commercial value. Prices for agarwood products range greatly and differ for the type of material. Essential oils can sell from USD 100/kg for low quality to USD 30 000/kg for high quality.[1,2] Agarwood commercial products are commonly seen in the form of perfumes, oils, wood chips, incense sticks, incense resin balls, incense sawdust, and traditional medicines.

Aquilaria species are found throughout southeast Asia and Indonesia.[6] Different regions and cultures prefer certain characteristics in harvested resin. Not all species, however, produce resin. Paoli *et al*. reported historical ranges in natural forests from 0 to 10% of trees containing resin. The primary source of agarwood is *Aquilaria malaccensis*. *A. malaccensis* is renowned for its fragrance and is the highest commercially exploited non-timber forest product.[6,7]

All *Aquilaria* spp. are listed in Appendix II of the Convention on the International Trade in Endangered Species of Flora and Fauna (CITES) meaning that trade in these species is not prohibited, but is regulated.[8] Of the nineteen species of *Aquilaria*, the International Union for Conservation of Nature (IUCN) Red List of Threatened Species lists one species as critically endangered (*A. crassna*), one species as threatened (*A. rostrata*) and seven species as vulnerable (*A. banaensae*, *A. beccariana*, *A. cimingiana*, *A. hirta*, *A. malaccensis*, *A. microcarpa*, and *A. sinensis*).[9] Comptom and Ishihara reported in TRAFFIC International that poor forest management of *Aquilaria* trees has been caused by the transition from ethnic merchants who controlled the felling method to more opportunistic and destructive harvesting methods.[10]

Chemical analyses of agarwood extracts and essential oils show a very complex matrix containing agarofurans, cadinanes, eudesmanes, valencanes and eremophilanes, guaianes, prezizanes, vetispiranes, 2-(2-phenylethyl)chromones, tetrahydro-2-(2-phenylethyl)chromones, and many other simple volatile aromatic compounds.[2] The most abundant compounds in agarwood have been found to be 2-(2-phenylethyl)chromone derivatives (41%) and other sesquiterpenes (52%).[4] Naef[2] reported the presence of 39 2-(2-phenylethyl)chromones from various *Aquilaria* species, of which 16 compounds are unique to agarwood: the highly oxidized 5,6,7,8-tetrahydro-2-

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(2-phenylethyl)chromones. These highly oxidized chromones have not been detected in healthy (non-injured) *Aquilaria* trees and appear to be unique to agarwood.[2,4]

Since the trade of agarwood involves commercial products that have been modified such that there are no anatomical features for species identification, we decided to investigate if the highly oxidized chromones could be used to characterize the taxonomic source of illegal products being imported in to the USA in contravention of CITES permit requirements. The samples analyzed in this research included *Aquilaria* voucher samples, intact wood chips, as well as commercial products of wood chips, sawdust, incense sticks, incense resin balls, perfumes, and sprays. Since many of the commercial products had been adulterated by the addition of scented volatile oils, a simple extraction procedure was developed to remove extraneous contaminants. All samples were analyzed using direct analysis in real time time-of-flight mass spectrometry (DART-TOFMS). DART-MS uses an ambient atmospheric ionization source that provides rapid analysis and requires minimal sample preparation. Cody *et al*. have thoroughly discussed the principal ionization mechanisms for DART-TOFMS.[11]

EXPERIMENTAL

Material

Five *Aquilaria* reference samples were obtained from Dr. Alex C. Wiedenhoeft of the Center for Wood Anatomy Research, Forest Products Laboratory, U.S. Forest Service, Madison, WI, USA (Table 1). Twenty (20) plantation-cultivated agarwood (*Aquilaria crassna*) reference samples from both Vietnam and Thailand as well as 20 non-cultivated samples of mixed geographical origin agarwood (*Aquilaria* spp.) reference samples were obtained from Dr. Robert A. Blanchette of the Department of Plant Pathology, University of Minnesota, St. Paul, Minnesota, USA (Table 1). In order to investigate if the distribution of 2-(2-phenylethyl)chromones was homogenous in commercial wood shipments, three different seizures (identified as *Aquilaria* spp. by inspectors of U.S. Customs and Border Protection) were selected for subsampling with 20 samples from each seizure analyzed (n = 60). Commercial samples suspected to contain agarwood (n = 151) were obtained from the Office of Law Enforcement of the U.S. Fish and Wildlife Service and consisted of an assortment of wood chips, sawdust, various incense preparations, and liquids. Additionally, 25 other fragrant woods were analyzed. The wood samples either had been previously validated[12] or were part of the 'Wood Species from Amazon, Manaus, Amazonia Brazil' reference collection. The sandalwood samples were purchased from a local vendor and were not validated. In total, 276 samples were analyzed for the presence of ions that are characteristic of 2-(2-phenylethyl)chromones.

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, 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 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. 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, is 6000 (FWHM) for protonated reserpine at nominal *m/z* 609.

Aquilaria reference standards were sonicated in methanol for 8 min and then left to stand for 2 days at room temperature in order to extract the chromones. The methanol eluent was analyzed by coating a capillary tube (1.5–1.8 × 90 mm) followed by DART-TOFMS analysis. A mass calibration standard of poly(ethylene glycol) 600 (Ultra, Kingstown, RI, USA) was run between each sample. All commercial samples (chips, sawdust, and various incenses) were prepared differently from the reference samples because these products are typically coated with scented oils. Approximately 1 × 0.3 cm of the commercial samples was sonicated in methanol for 8 min. The methanol was then decanted and the sample was washed with distilled water until the supernatant was clear. The samples were then sonicated again in methanol for 8 min and then left to stand for 2 days at room temperature in order to extract the chromones. The methanol eluent was analyzed by coating a capillary tube followed by DART-TOFMS analysis. Perfumes and liquids were analyzed without prior sample preparation by coating a capillary tube into the solution followed by DART-TOFMS analysis.

Sixty-eight (68) *A. crassna* reference samples were analyzed both directly and by the methanol extraction process described, in order to determine if direct analysis of heartwood by DART-TOFMS yields the same chromone profile as the spectra of methanol-extracted heartwood.

Nineteen 2-(2-phenylethyl)chromones reported by Naef[2] were used to construct a search library and these compounds are listed in Table 2. Table 2 also contains the simple naming convention used by Naef and their corresponding CAS names.

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). Mass Spec Tools II was then used to search individual spectra for the presence of the protonated diagnostic molecules 67-105[2], with 1% intensity threshold and 10 mmu tolerance.

RESULTS AND DISCUSSION

DART-TOFMS is a technique that is notable because it provides reproducible mass data, it can analyze solid samples without the need for chemical extractions, and it provides high mass accuracy. Positive-ion DART produces mass spectra that are dominated by peaks that represent protonated molecules. Each peak typically corresponds to a single protonated compound or set of isomers. Therefore, reliance on a single ion to make taxonomic inference has high risks due to the potential of other compounds present that could coincidentally have the same molecular weight. Consequently it becomes critical to determine how many ions are sufficient to make a genus or species determination in complex

Table 1. *Aquilaria* reference samples used in this study, number of target chromone ions detected and presence (+) or absence (−) of key ions whose masses are consistent with diagnostic chromones

Specimen	Genus	Species	Country	No. of target ions present	319.118 *(m/z)* (Compounds 88, 91, 92, 93)	349.129 *(m/z)* (Compounds 89, 90, 96, 97)
SJRw 12907	*Aquilaria*	*crassna*	–	13	+	+
SJRw 49539	*Aquilaria*	*malaccensis*	–	9	+	+
MADw 35916	*Aquilaria*	*sinensis*	China	11	+	–
SJRw 26747	*Aquilaria*	*sinensis*	–	7	–	+
SJRw 13948	*Aquilaria*	sp.	–	16	+	+
T1	*Aquilaria*	*crassna*	Thailand	16	+	+
T2				17	+	+
T3				16	+	+
T4				17	+	+
T5				17	+	+
T6				17	+	+
T7				14	+	+
T8				17	+	+
T9				17	+	+
T10				17	+	+
T11				16	+	+
T12				15	+	+
T13				16	+	+
T14				12	+	+
T15				13	+	+
T16				16	+	+
T17				17	+	+
T18				13	+	+
T19				17	+	+
T20				16	+	+
V1	*Aquilaria*	*crassna*	Vietnam	13	+	+
V2				14	+	+
V3				16	+	+
V4				14	+	+
V5				14	+	+
V6				15	+	+
V7				14	+	+
V8				17	+	+
V9				15	+	+
V10				15	+	+
V11				15	+	+
V12				15	+	+
V13				14	+	+
V14				16	+	+
V15				13	+	+
V16				13	+	+
V17				14	+	+
V18				11	+	+
V19				14	+	+
V20				14	+	+
T1	*Aquilaria*	spp.	Thailand,	14	+	+
T2			Vietnam,	12	+	+
T3			Cambodia	13	+	+
T4				11	+	+
T5				11	+	+
T6				13	+	+
T7				14	+	+
T8				12	+	+
T9				17	+	+
T10				12	+	+
T11				15	+	+
T12				14	+	+
T13				13	+	+

(*Continues*)

Table 1. (Continued)

Specimen	Genus	Species	Country	No. of target ions present	319.118 *(m/z)* (Compounds 88, 91, 92, 93)	349.129 *(m/z)* (Compounds 89, 90, 96, 97)
T14				13	+	+
T15				15	+	+
T16				11	+	+
T17				16	+	+
T18				15	+	+
T19				12	+	+
T20				17	+	+

Table 2. 2-(2-Phenylethyl)chromones reported in agarwood extracts (Naef[2]). Because of isomeric configurations only 19 masses can be identified by DART-TOFMS

Compound	Chromone type	Predicted (*m/z*)	Formula
67	2-(2-phenylethyl)chromone	251.107	$C_{17}H_{15}O_2$ $[M+H]^+$
68	7-hydroxy-2-(2-phenylethyl)chromone	267.102	$C_{17}H_{15}O_3$ $[M+H]^+$
69	6-hydroxy-2-(2-phenylethyl)chromone		
70	6-methoxy-2-(2-phenylethyl)chromone	281.118	$C_{18}H_{17}O_3$ $[M+H]^+$
71	2-[2-(4-methoxyphenyl)ethyl]chromone		
72	6-hydroxy-2-[2-(4′-hydoxyphenyl)ethyl]chromone	283.097	$C_{17}H_{15}O_4$ $[M+H]^+$
73	6-hydroxy-2-[2-(2′-hydoxyphenyl)ethyl]chromone		
74	6,8-dihydroxy-2-(2-phenylethyl)chromone		
75	5,8-dihydroxy-2-(2-phenylethyl)chromone		
86	6-hydroxy-2-[(2*R*)-7′-hydroxy-2-phenylethyl]chromone		
103	oxidoagarochromone A		
76	5-hydroxy-6-[2-(3′-methoxyphenyl)ethyl]chromone	297.113	$C_{18}H_{17}O_4$ $[M+H]^+$
77	6,7-dimethox-methoxy-2-(2-phenylethyl)chromone		
78	6-hydroxy-2-(2-(4′-methoxyphenyl)ethyl)chromone 6-hydroxy-7-methoxy-2-(2-phenylethyl)chromone		
79	6-methoxy-2-[2-(4′-methoxyphenyl)ethyl]chromone	311.128	$C_{19}H_{19}O_4$ $[M+H]^+$
80	6-methoxy-2-[2-(3′-methoxyphenyl)ethyl]chromone		
81	6,7-dimethoxy-2-(2-phenylethyl)chromone		
82	5,8-dihydroxy-2-[2-(4′-methoxyphenyl)ethyl]chromone	313.108	$C_{18}H_{17}O_5$ $[M+H]^+$
104	oxidoagarochromone B		
83	6-methoxy-2-[2-(3-methoxy-4-hydroxyphenyl)ethyl]chromone	327.123	$C_{19}H_{19}O_5$ $[M+H]^+$
84	6,7-dimethoxy-2-[2-(4′-methoxyphenyl)ethyl]chromone	341.139	$C_{20}H_{21}O_5$ $[M+H]^+$
85	7,8-dimethoxy-2-[2-(3′-acetoxyphenyl)ethyl]chromone	369.134	$C_{21}H_{21}O_6$ $[M+H]^+$
87	5,6,7,8-tetrahydro-6β,7β-dihydroxy-2-(2-phenylethyl)chromone	287.128	$C_{17}H_{19}O_4$ $[M+H]^+$
88	4 *H*-1-benzopyran-4-one,5,6,7-tris(acetyloxy)-2-[2-[2-(acetyloxy)phenyl]-ethyl]-5,6,7,8-tetrahydro-[5*S*-(5α,6β,7α)]-agarotetrol	319.118	$C_{17}H_{19}O_6$ $[M+H]^+$
91	isoagarotetrol		
92	5,6,7,8-tetrahydro-5β,6β,7α,8β-tetrahydroxy-2-(2-		
93	phenylethyl)chromone		
89	(5*S* *,6*R* *,7*S* *)-5,6,7-trihydroxy-2-(3-hydroxy-4-methoxyphenethyl)-5,6,7,8-tetrahydro-4 *H*-chromen-4-one	349.129	$C_{18}H_{21}O_7$ $[M+H]^+$
90	(5*S* *,6*R* *,7*R* *)-5,6,7-trihydroxy-2-(3-hydroxy-4-methoxyphenethyl)-5,6,7,8-tetrahydro-4 *H*-chromen-4-one		
96	4 *H*-1-benzopyran-4-one,5,6,7,8-tetrakis(acetyloxy)-5,6,7,8-tetrahydro-2-[2-(4-methoxyphenyl)ethyl]-, [5S-(5a,6b,7b,8a)]-		
97	4 *H*-1-benzopyran-4-one,5,6,7,8-tetrahydro-5,6,7,8-tetrahydroxy-2-[2-(4-methoxyphenyl)ethyl]-,[5S-(5α, 6β,7α,8β)]-		
94	4 *H*-1-benzopyran-4-one,5,6,7,8-tetrahydro-5,6,7,8-tetrahydroxy-2-[2-(2-hydroxyphenyl)ethyl]-,[5*S*-(5a,6β,7a,8β)]-	335.113	$C_{17}H_{19}O_7$ $[M+H]^+$
95	5α,6β,7β,8α-tetrahydroxy-2-[2-(2-hydroxyphenyl)ethyl]5,6,7,8-tetrahydrochrome		

(*Continues*)

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Table 2. (Continued)

Compound	Chromone type	Predicted (*m/z*)	Formula
100A	4 *H*-1-benzopyran-4-one,5,6,7,8-tetrahydro-5,6,7,8-tetrahydroxy-2-(2-hydroxy-2-phenylethyl)-,[5*S*-[2(*S**),5α,6β,7α,8β]]-		
100B	4 *H*-1-benzopyran-4-one,5,6,7,8-tetrahydro-5,6,7,8-tetrahydroxy-2-(2-hydroxy-2-phenylethyl)-,[5S-[2(*R**),5α,6β,7α,8β]]-		
98	5,6,7,8-tetrahydroxy-2-(3-hydroxy-4-methoxyphenethyl)-5,6,7,8-tetrahydro-4 *H*-chromen-4-one	365.124	$C_{18}H_{21}O_8$ $[M+H]^+$
99	5α,6β,7β-trihydroxy-8α-methoxy-2-(2-phenylethyl)-5,6,7,8-tetrahydrochromone	333.134	$C_{18}H_{21}O_6$ $[M+H]^+$
101	4 *H*-1-benzopyran-4-one,8-chloro-5,6,7,8-tetrahydro-5,6,7-trihydroxy-2-(2-phenylethyl)-,(5*R*,6*R*,7*R*,8*S*)-rel-(+)-	337.084	$C_{17}H_{18}ClO_5$ $[M+H]^+$
102	8-chloro-5,6,7-trihydroxy-2-(3-hydroxy-4-methoxyphenethyl)-5,6,7,8-tetrahydro-4 *H*-chromen-4-one	382.082	$C_{18}H_{19}ClO_7$ $[M+H]^+$
105	oxidoagarochromone C	329.102	$C_{18}H_{17}O_6$ $[M+H]^+$

materials such as wood. Naef[2] reported 39 chromones that have been identified in *Aquilaria* spp., but due to isomeric configurations only 19 unique masses could be characterized with DART-TOFMS. Therefore, a key question of this research was to determine how many of the 19 unique masses reported by Naef were sufficient to determine if a wood sample, or a commercial preparation (sawdust, incense, perfume, etc.), contains agarwood, especially when commercial samples are sometimes adulterated with scented oils.[2] An advantageous circumstance that limits the scope of research is that the context for the analysis are fragrant wood samples, or scented commercial products that are manufactured for their pleasing smell and therefore our research excluded the analysis of unscented wood samples.

Reference samples

Figure 1 shows a typical spectrum of an *Aquilaria* reference standard. The number of target 2-(2-phenylethyl)chromone ions detected in the agarwood reference samples ranged between 6 and 17, with an average (mode) of 15 ions (Table 1). It is meaningful to remark that 119 of the 125 reference samples (95%) contained both ions consistent with protonated 5,6,7,8-tetrahydro-2-(2-phenylethyl)chromones (*m/z* 319.118 and/or *m/z* 349.129**)**, the highly oxidized agarwood chromones that to date have only been reported in infected *Aquilaria* spp. samples. The remaining six references samples contained at least one of these ions.

Batch analysis of three *Aquilaria* spp. seizures

Analysis of the three batches of commercial wood chips (n = 60) showed that the average (mean) number of target chromone ions present per sample was fourteen ions with a range of eight to sixteen ions (see Table 3). This data shows that although the distribution of 2-(2-phenylethyl)chromones is heterogeneous throughout the heartwood, the majority of the samples had fifteen (mode) or more compounds.

Table 4 displays the frequency the 2-(2-phenylethyl)chromone ions were detected in the *Aquilaria* reference standards and the *Aquilaria* spp. batch testing. Of the potential 19 unique masses reported in the literature, only two ions (compounds 85 and 102) occurred less than 5% of the time. The remaining 17 ions were therefore used for making taxonomic determinations (see Table 4). Table 4 shows the ion assignment of the chromone compound(s), and the frequency the ion was detected.

Table 3. Number of 2-(2-phenylethyl)chromone compounds detected in commercial wood chip batches

	n	Mode	Mean	Range
Batch 1	20	15	14	11–16
Batch 2	20	15	13	8–15
Batch 3	20	14	14	10–16

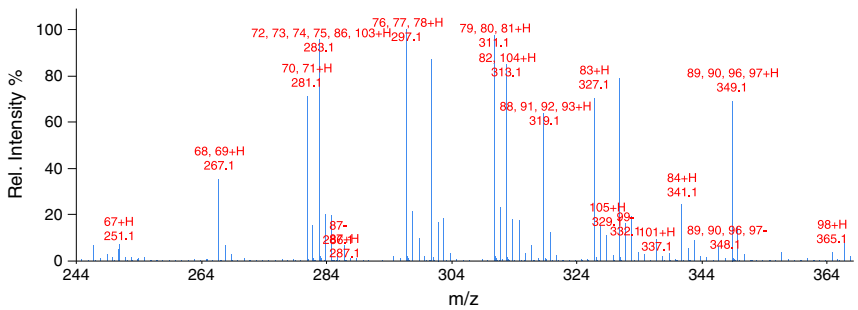

Figure 1. Mass spectrum of *Aquilaria crassna* from Thailand showing 17 masses consistent with target chromones.

Table 4. Frequency of target chromone ions detected in 65 *Aquilaria* reference samples and 60 samples of seized wood chips. Asterisk denotes highly oxidized chromones

Compound	Percent frequency	Protonated (*m/z*)	Formula
101	50.4%	337.084	$C_{17}H_{18}ClO_5$ $[M+H]^+$
67	54.4%	251.107	$C_{17}H_{15}O_2$ $[M+H]^+$
98	56.0%	365.124	$C_{18}H_{21}O_8$ $[M+H]^+$
105*	66.4%	329.102	$C_{18}H_{17}O_6$ $[M+H]^+$
94, 95, 100A, 100B*	75.2%	335.113	$C_{17}H_{19}O_7$ $[M+H]^+$
82, 104*	75.2%	313.108	$C_{18}H_{17}O_5$ $[M+H]^+$
99*	84.0%	333.134	$C_{18}H_{21}O_6$ $[M+H]^+$
87*	85.6%	287.128	$C_{17}H_{19}O_4$ $[M+H]^+$
70, 71	90.4%	281.118	$C_{18}H_{17}O_3$ $[M+H]^+$
84	91.2%	341.139	$C_{20}H_{21}O_5$ $[M+H]^+$
68, 69	92.0%	267.102	$C_{17}H_{15}O_4$ $[M+H]^+$
79, 80, 81	94.4%	311.128	$C_{19}H_{19}O_4$ $[M+H]^+$
89, 90, 96, 97*	96.0%	349.129	$C_{18}H_{21}O_7$ $[M+H]^+$
83	96.0%	327.123	$C_{19}H_{19}O_5$ $[M+H]^+$
72, 73, 74, 75, 86, 103	96.8%	283.097	$C_{17}H_{15}O_4$ $[M+H]^+$
76, 77, 78	97.6%	297.113	$C_{18}H_{17}O_4$ $[M+H]^+$
88, 91, 92, 93*	99.2%	319.118	$C_{17}H_{19}O_6$ $[M+H]^+$

The data shows that the ion at *m/z* 349.129, which is present about 96% of the time, is consistent with the diagnostic, highly oxidized agarwood chromones designated as compounds 89, 90, 96, and 97. Additionally, the data shows that the ion at *m/z* 319.118, which occurs over 99%, is consistent with the diagnostic, highly oxidized agarwood chromone compounds 88, 91, 92, and 93.

These results provide empirical support that positive identification of *Aquilaria* should rely on two criteria: (1) the presence of a highly oxidized agarwood chromone (*m/z* 319.118 and/or *m/z* 349.129) plus (2) the occurrence of ten or more of the other target chromone ions. The first criterion is based on the assumptions that the highly oxidized agarwood chromones are specific to *Aquilaria* spp. The second criterion is based on the minimum average of ions in the reference sample set (see Table 3).

Analysis of commercial sample

Results from the analysis of the 151 commercial products suspected of containing agarwood are summarized in Table 5. In the analysis of the commercial samples where adulteration with scented oils is common, we applied the criteria that a agarwood positive sample had to have: (1) at least one of the highly oxidized chromone diagnostic ions (*m/z* 319.118 and/or *m/z* 349.129), plus (2) ten or more of the ions that are characteristic of the 2-(2-phenylethyl)chromones (see Table 4).

Table 5. Summary of results of 151 commercial products

	n	Positive	Percent positive
Incense: resin and sticks	56	3	5%
Liquid spray	6	2	33%
Medicinal pills	1	0	0%
Perfume	18	0	0%
Sawdust	15	8	53%
Wood chips	55	42	76%

Analysis of direct vs. methanol-extracted samples

The comparison of 68 directly analyzed heartwood reference samples and 60 methanol-extracted heartwood samples demonstrated that DART-TOFMS yields similar chromone profiles for both methods of analysis. The average number of target chromone ions for the mixed-origin reference samples via direct analysis was 13 target ions and for the methanol extraction the average was 13.5 target ions. The cultivated Vietnam samples had averages of 14 target ions for direct analysis and 14.3 target ions for methanol extraction. The cultivated Thailand samples had the greatest difference with averages of 13 target ions for direct analysis and 15.8 target ions for the methanol extraction. Though the averages were not exactly the same, all of the reference samples contained the *m/z* 349.129 and *m/z* 319.118 ions. Both methods satisfied the criteria for inferring the presence of agarwood, though methanol extractions have slightly higher sensitivity towards chromones. This was consistent with previous findings of Lancaster and Espinoza which demonstrated that the direct analysis of *Dalbergia nigra* and *Dalbergia spruceana* heartwood by DART-TOFMS yields the same chemical profile as the spectra of methanol extracted heartwood.[12]

Analysis of other fragrant woods

Analysis was conducted on 25 other scented woods to investigate if these samples could give false positive results. Results of the analysis are shown in Table 6. When the criteria for inferring the presence of *Aquilaria* were applied, none of the samples could be confused with agarwood because they either (1) lacked the diagnostic ions (*m/z* 319.118 or *m/z* 349.129) and/or (2) had fewer than ten target ions present.

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Table 6. Number of target ions detected in other fragrant woods, and presence (+) or absence (−) of key ions whose masses are consistent with agarwood

Samples	No. of target ions present	319.118 *(m/z)* (Compounds 88, 91, 92, 93)	349.129 *(m/z)* (Compounds 89, 90, 96, 97)
Abies magnifica	0	−	−
Boswellia spp. bark (Frankincense)	1	−	−
Boswellia spp. resin (Frankincense)	0	−	−
Caesalpinia echinata	2	−	−
Caesalpinia platyloba	0	−	−
Caryocar spp.	7	+	−
Cedrela lilloi	0	−	−
Dalbergia baroni	2	−	−
Dalbergia decipularis	1	−	−
Dalbergia maritima	3	+	−
Dalbergia spruceana	0	+	−
Emmotum nitens	0	−	−
Iranthera sp.	3	−	−
Licaria rigida	1	−	−
Machaerium scleroxlyon	4	−	−
Phoebe porosa	1	−	−
Pterocarpus indicus	1	−	−
Pterocarpus soyauxii	1	−	−
Schefflera morototoni	1	−	−
Scleronema micranthum	2	−	−
Swartzia tomentosa	2	−	−
Swietenia macrophylla	5	−	+
Swietenia mahagoni	0	−	−
Santalum spicatum (treated)	2	−	−
Santalum spicatum	0	−	−

CONCLUSIONS

Chromones are benzopyran derivatives and are part of many major biological metabolites such as flavanones and flavanols. It is not surprising that many wood species could contain some of the target ions that are characteristic of the 2-(2-phenylethyl)chromones because of isomeric forms or fragmentation of larger biomolecules. However, in this study we found that a reliable criteria for inferring *Aquilaria* was the presence of the diagnostic ions of *m/z* 319.118 or *m/z* 349.129 plus the presence of ten or more of the target ions that are characteristic of the 2-(2-phenylethyl)chromones (see Table 4).

The diagnostic ions of *m/z* 319.118 and *m/z* 349.129 are consistent with the highly oxidized chromones (5,6,7,8-tetrahydro-2-(2-phenylethyl)chromones) and appear to be unique to agarwood.[2,4] Additionally, the presence of ten or more ions characteristic of the 2-(2-phenylethyl)chromones is an added assurance of certainty. There have been reports of grasses that produce 2-(2-phenylethyl)chromones such as *Bothriochloa ischaemum* which contains two compounds, *Eremophila georgei* which contains one compound, and *Imperata cylindrical* which contains four compounds, but, because of their nature, none of these samples could be confused with agarwood products.[2,13,14]

Analysis of 276 samples studied in this research established that DART-TOFMS provided reproducible mass spectral data that are useful for inferring the taxonomic source of agarwood samples. The presence of key ions that are characteristic of 2-(2-phenylethyl)chromones provided accurate identification to genus (*Aquilaria*) in the commercial samples when the criterion described was invoked. Wood anatomists who are challenged with difficult morphological identification resulting from the lack of diagnostic features can use this tool to assist in their analyses.

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