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Chemical Profiles of Incense Smoke Ingredients from Agarwood by Headspace Gas Chromatography-Tandem Mass Spectrometry

Wen-Yi Kao [1,2,†], Chien-Yun Hsiang [3,†], Shih-Ching Ho [2], Tin-Yun Ho [4,*] and Kung-Ta Lee [1,*]

1 Department of Biochemical Science and Technology, National Taiwan University, Taipei 10617, Taiwan; mbkwi@dcb.org.tw
2 Development Center for Biotechnology, New Taipei City 22180, Taiwan; scho@dcb.org.tw
3 Department of Microbiology, China Medical University, Taichung 40402, Taiwan; cyhsiang@mail.cmu.edu.tw
4 Graduate Institute of Chinese Medicine, China Medical University, Taichung 40402, Taiwan
* Correspondence: tyh@mail.cmu.edu.tw (T.-Y.H.); ktlee@ntu.edu.tw (K.-T.L.); Tel.: +886-422-053-366 (ext. 3302) (T.-Y.H.); +886-233-664-436 (K.-T.L.)
† These authors contributed equally to this work.

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Abstract: Agarwood, the resinous wood in the heartwood of *Aquilaria* trees, has been used as incense in traditional Chinese medicine for its sedative, aphrodisiac, carminative, and anti-emetic effects. Grading of agarwood is usually based on its physical properties. Therefore, it is important to develop analytic methods for judgment and grading of agarwood. Here, we created a headspace (HS) preheating system that is combined with gas chromatography-mass spectrometry (HS GC-MS) to analyze the chemical constituents in the incense smoke produced by agarwood. Incense smoke generated in the HS preheating system was injected directly to GC-MS for analysis. A total of 40 compounds were identified in the incense smoke produced by Kynam agarwood, the best agarwood in the world. About half of the compounds are aromatics and sesquiterpenes. By analyzing chemical constituents in the incense smoke produced by Vietnamese, Lao, and Cambodian varieties of agarwood, we found that butyl hexadecanoate, butyl octadecanoate, bis(2-ethylhexyl) 1,2-benzenedicarboxylate, and squalene were common in the aforementioned four varieties of agarwoods. 2-(2-Phenylethyl) chromone derivatives were identified only in the incense smoke produced by Kynam agarwood, and were the major ingredient (27.23%) in the same. In conclusion, this is the first study that analyzes chemical profiles of incense smoke produced by agarwood using HS GC-MS. Our data showed that 2-(2-phenylethyl) chromone derivatives could be used to assess quality of agarwoods. Moreover, HS GC/MS may be a useful tool for grading quality of agarwood.

Keywords: agarwood; headspace preheated system; gas chromatography-mass spectrometry; 2-(2-phenylethyl)chromone

1. Introduction

Agarwood, also called Gaharu, Chén-xīang, Jin-koh, Kyara, or Oud, is the resinous wood formed in the heartwood of *Aquilaria* trees. *Aquilaria* species, including *A. sinensis* (Lour.) Glig., *A. agallocha* Roxb., and *A. malaccensis* Lam., taxonomically belong to Thymelaeaceae and are found mainly in Southeast Asia, such as Vietnam, Indonesia, Laos, Cambodia, and Malaysia [1,2]. Agarwood and its oil are important and useful natural substances that have been used to produce valuable products. Agarwood has been used as incense in religious ceremonies for centuries as well as in traditional Chinese medicine for its sedative, aphrodisiac, carminative, and anti-emetic effects [3–9]. The value of agarwood depends on its quality. However, grading of agarwood tends to be discretionary and

subjective, because agarwood is usually graded based on its physical properties, such as color, density, resin formation, by sensory panels [1,2,10]. Kynam, also known as Kanankoh in Japan, is regarded to have the highest quality among different varieties of agarwood [11]. The high resin content, better water sinking quality, and darker color of Kynam enable it to produce a special and pleasant scent [1]. Kynam has been considered to be the noblest spiritual wood, both in ancient societies and in modern times, because of its scarcity. At present, the cost of Kynam agarwood is about 350 Euro per gram [12]. Kynam agarwood has a very complex scent, and even grading professionals cannot easily distinguish it from others. Therefore, it is important and essential to develop analytic methods for judgment and grading of agarwood.

Chemical constituents of agarwood have been studied by several research teams [1,3,13]. Previous studies identified chemical constituents of agarwood essential oils or organic solvent extracts with column chromatography or spectroscopic techniques [14,15]. Other studies prepared agarwood essential oils, hydrodistillates, or solvent extracts for gas chromatography (GC) or multidimensional GC analysis [16,17]. However, analysis of chemical constituents in agarwood solvent extracts is not suitable for differentiation of agarwood because agarwood is usually used to produce incense smoke. A few research studies analyzed chemical ingredients in the incense smoke produced by heated agarwood [11,18,19]. For example, Ishihara et al. [11] analyzed chemical constituents in the incense smoke that was trapped in the Tenax TA adsorbent resin, and extracted in diethyl ether for GC and GC-mass spectrometry (MS) analysis. They found 53 chemical compounds in the incense smoke produced by high quality (Kanankoh) and low quality (Jinkoh) varieties of Vietnamese agarwood, and most of them were sesquiterpenes and aromatics. Hung et al. [18] prepared an agarwood extract by heating a sealed vial that contained agarwood powder in a water bath at 90 °C for 30 min. The extract was absorbed in a solid-phase microextraction (SPME) needle with polydimethylsiloxane (PDMS) fiber for 30 min, and then desorbed for GC-MS analysis. They found six peaks that could be used for evaluating the quality or price of the most expensive agarwood. Zhou et al. [19] collected incense smoke produced by agarwood and incense sticks, absorbed the different kinds of collected incense smoke in glass fiber pads, and extracted substances from the glass fiber pads with dichloromethane for GC-MS analysis. Nevertheless, these studies analyzed chemical constituents in the incense smoke that was either absorbed in matrices or extracted in solvents.

Analysis of chemical constituents in the incense smoke produced by agarwood may be an approach to grading of agarwood. Most of past research studies determine chemical ingredients in agarwood solvent extracts [13–17]. However, analysis of chemical constituents in solvent extracts is not suitable in the case of agarwood because it is usually used to produce incense smoke. In this work, we used a headspace (HS) preheating system that is combined with GC-MS (HS GC-MS) to extract heavier sample matrices for GC analysis. Incense smoke produced by heated agarwood was directly analyzed with GC-MS. We could obtain more authentic chemical profiles of incense smoke produced by agarwood without solid-phase extraction. Moreover, by comparing chemical constituents in the incense smoke produced by different grades of agarwood, we found that HS GC/MS-MS could be used to grade quality of agarwood.

2. Results and Discussion

2.1. Morphological Observation of Various Grades of Agarwoods

Agarwood is traditionally graded based on its physical properties, such as resin content, water sinking quality, color, scent/aroma, as well as agarwood-inducing method, formation time, and place of origin [1,2]. Both Kynam and Vietnamese varieties of agarwood showed first-grade colors and had softer scents (Figure 1A,B). However, the Kynam variety fully sank in water, while the Vietnam variety merely partially submerged in water. Both the Lao and Cambodian varieties of agarwood fully floated up, showed second-grade colors, and had sweet scents (Figure 1C,D). However, the sweet scent of Cambodian agarwood was induced by insect infection.

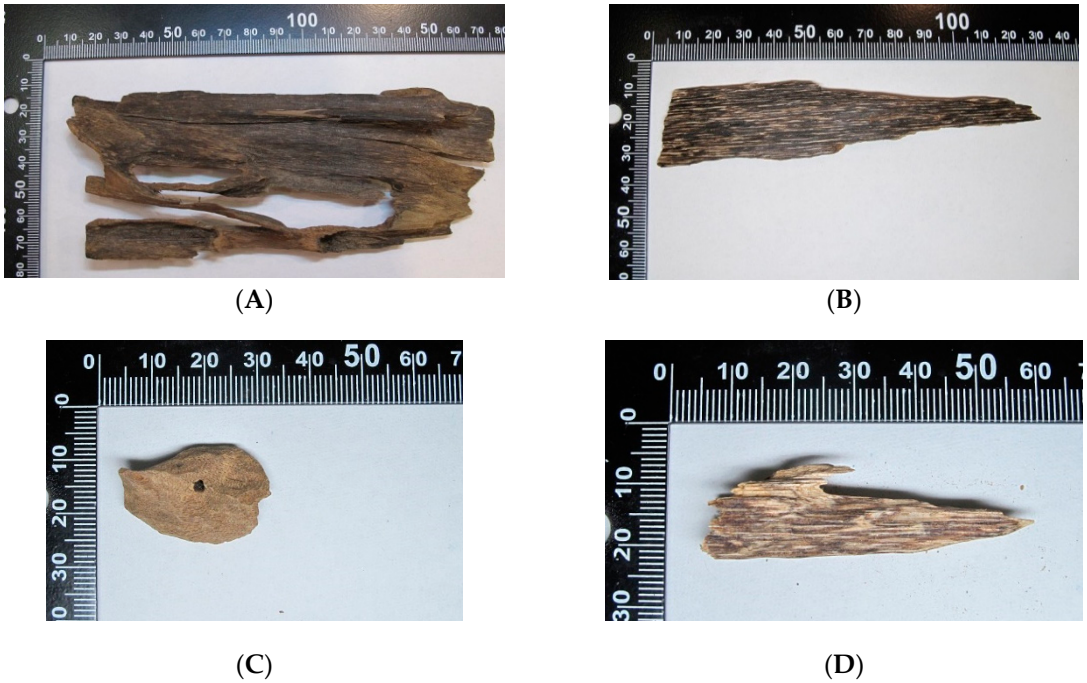


Figure 1. Morphological observation of Kynam (**A**), Vietnam (**B**), Liao (**C**), and Cambodian agarwoods (**D**).

2.2. HS GC-MS/MS Analysis

Chemical constituents of agarwood have been intensively studied by several research teams [1,3,13]. Different extraction methods have been developed to determine chemical composition of essential oils and related compounds from agarwood chips using GC, GC-MS, solid phase microextraction, GC-flame ionization detector, GC-olfactometry, or comprehensive two-dimensional gas chromatography (GC×GC) [20]. Most studies suggest that hydrodistillation is the method of choice for determining the essential oil content of agarwood [7]. Agarwood has only a slight scent at room temperature, and releases a pleasant aroma when heated. Burning incense is a common traditional practice in many families and most temples in Asia, for religious reasons and for its pleasant smell [1,2]. Incense smoke contains many kinds of fragrant sesquiterpenes and aromatics [11]. Without interphase mass transfer, how to obtain a large amount of incense smoke is an important parameter for efficient analysis. Therefore, we prepared the incense smoke of agarwood using a HS preheating system, and tested the incubation times and temperatures to obtain the maximum amount of analytes. Previous studies extracted agarwood oils through incubation at 40 °C for 10 min, or at 90 °C for 30 min [18]. In this study, we prepared the incense smoke of agarwood through incubation at 150 °C for 30 min, and injected the smoke into GC-MS/MS for chemical profiling analysis (Figure 2).

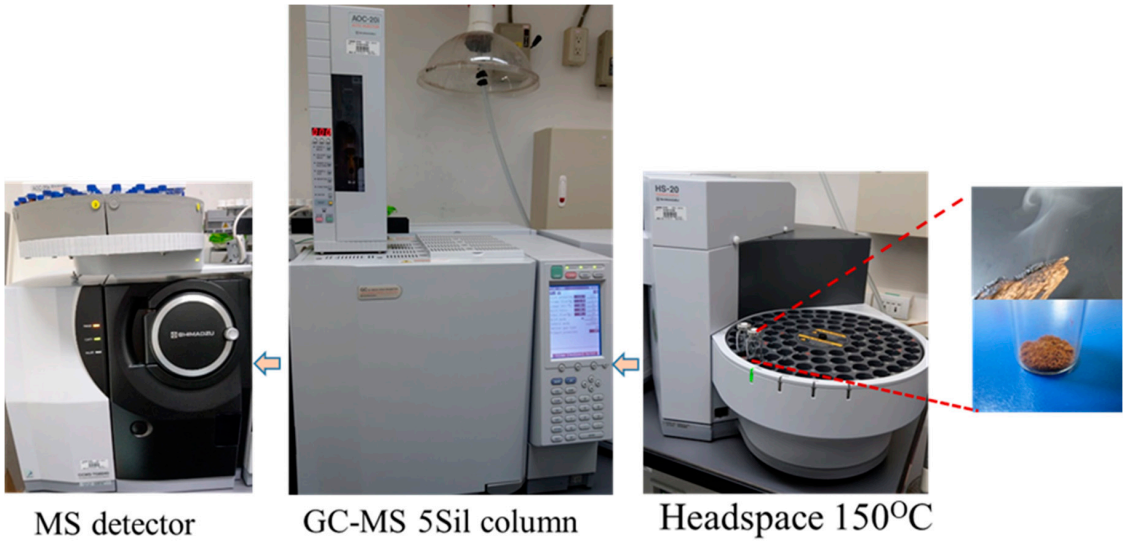


Figure 2. Experimental design and incense smoke detection process. Diagram shows that the incense smoke of agarwoods was generated by headspace preheated system and the smoke was injected directly to GC-MS for analysis.

2.3. Chemical Profiles of Kynam Agarwoods Using HS GC/MS-MS

Figure 3 and Figure S1 shows the GC chemical fingerprint of incense smoke produced by Kynam agarwood. The detection period was setup from 2.5 min to 65 min. Forty peaks were identified in the GC-MS/MS analysis, and the identified compounds are summarized in Table 1. As shown in Figure 4, these compounds belong to different kinds of chemical categories, including aromatics (33%), sesquiterpenes (38%), alkanes (8%), terpene (3%), triterpenes (3%), and others (18%). Since sesquiterpenes and triterpenes were mostly identified in agarwoods, other terpenes, except sesquiterpenes and triterpenes, were classified as "terpene" in this study. Moreover, the major component in the incense smoke of Kynam was 2-(2-phenylethyl) chromone (27.23%).

In 1993, Ishihara et al. [11] analyzed two kinds of agarwood incense smoke obtained with solvent extraction. Fifty components were identified in higher quality agarwood, while 36 components were identified in low quality agarwood. The incense smoke of both higher and low-quality agarwood was composed mainly of sesquiterpenes (53% and 61%, respectively) and aromatics (44% and 31%, respectively). The major components in higher quality agarwood were guaia-1(10), 11-dien-15-oic acid (9.92%), and 2-(2-phenylethyl) chromone (5.83%). Hung et al. [18] identified 17 components that could be used as markers for classification and differentiation of agarwood. 2-(2-Phenylethyl) chromone derivatives (flindersiachromone) were also identified in most expensive agarwoods.

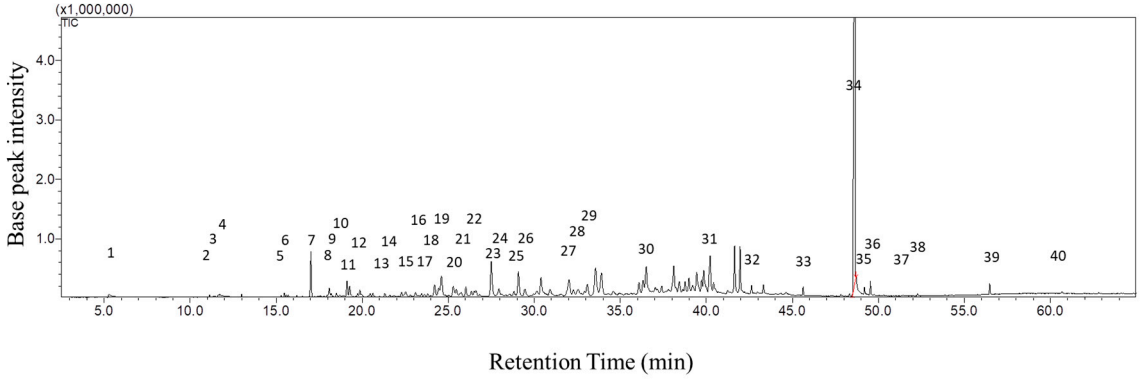


Figure 3. GC-MS chemical fingerprint of incense smoke from Kynam agarwood. Number indicates the identified compound, and the details of compounds are listed in Table 1.

Table 1. List of compounds identified in the incense smoke from Kynam agarwood by GC-MS/MS.

No.	Compound	LRI [a]	Retention Time (min)	Content [b] (%)	Detected *m*/*z*	Chemical Formula	Type [c]
1	Benzaldehyde	1581	5.296	0.17 ± 0.05	106	C_7H_6O	Ar
2	exo-7-(2-Propenyl)bicyclo [4.2.0]oct-1(2)-ene	1720	11.137	0.04 ± 0.02	148	$C_{11}H_{16}$	Other
3	4-Methoxybenzaldehyde	1731	11.617	0.14 ± 0.05	136	$C_8H_8O_2$	Ar
4	1,8-Nonadiene-3-yne, 2,8-dimethyl-7-methylene-	1734	11.717	0.04 ± 0.03	160	$C_{12}H_{16}$	Other
5	Cubenene	1818	15.273	0.03 ± 0.02	204	$C_{15}H_{24}$	S
6	α-Longipinene	1823	15.471	0.26 ± 0.12	204	$C_{15}H_{24}$	S
7	Tricyclo[5 2.2.0(1,6)]undecan-3-ol, 2-methylene-6,8,8-trimethyl-	1860	17.017	1.30 ± 0.42	220	$C_{15}H_{24}O$	Ar
8	Naphthalene	1885	18.086	0.18 ± 0.22	202	$C_{15}H_{22}$	Ar
9	δ-Guaiene	1888	18.196	0.13 ± 0.08	204	$C_{15}H_{24}$	S
10	Nootkatene	1895	18.502	0.12 ± 0.06	202	$C_{15}H_{22}$	Ar
11	Kessane	1910	19.119	0.39 ± 0.29	222	$C_{15}H_{26}O$	Ar
12	4a,5-Dimethyl-3-(prop-1-en-2 -yl)-1,2,3,4,4a,5,6,7-octahydronaphthalen-1-ol	1928	19.863	0.31 ± 0.07	220	$C_{15}H_{24}O$	Ar
13	*cis*-α-Santalol	1962	21.312	0.28 ± 0.09	220	$C_{15}H_{24}O$	S
14	β-Vetivenene	1969	21.593	0.10 ± 0.02	202	$C_{15}H_{22}$	T
15	γ-Eudesmol	1998	22.799	0.06 ± 0.06	222	$C_{15}H_{26}O$	S
16	10s,11s-Himachala-3(12),4-diene	2017	23.61	0.27 ± 0.12	204	$C_{15}H_{24}$	Ar
17	Agarospirol	2022	23.808	0.21 ± 0.04	222	$C_{15}H_{26}O$	S
18	β-Guaiene	2031	24.209	0.53 ± 0.24	204	$C_{15}H_{24}$	S
19	Cubenol	2041	24.606	1.24 ± 0.43	222	$C_{15}H_{26}O$	S
20	α-epi-7-epi-5-Eudesmol	2057	25.296	0.95 ± 0.28	222	$C_{15}H_{26}O$	S
21	Bulnesol	2074	26.021	0.26 ± 0.18	222	$C_{15}H_{26}O$	S
22	α-Tetralone	2089	26.624	0.53 ± 0.26	218	$C_{15}H_{22}O$	Ar
23	α-Kessyl acetate	2110	27.497	0.91 ± 1.31	280	$C_{17}H_{28}O_3$	Ar
24	(1*R*,7*S*,*E*)-7-Isopropyl-4,10-dimethylenecyclodec-5-enol	2120	27.941	0.33 ± 0.32	220	$C_{15}H_{24}O$	Ar
25	10-epi-Elemol	2141	28.822	0.19 ± 0.15	222	$C_{15}H_{26}O$	S
26	Ledol	2156	29.445	0.53 ± 0.10	222	$C_{15}H_{26}O$	S
27	Longiverbenone	2217	32.017	0.82 ± 0.74	218	$C_{15}H_{22}O$	Other
28	Cryptomeridiol	2230	32.552	1.95 ± 1.10	240	$C_{15}H_{28}O_2$	S
29	2a*S*,3a*R*,5a*S*,9b*R*)-2a,5a,9-Trimethyl-2a,4,5,5a,6,7,8,9b-octahydro-2*H*-naphtho[1–b]oxireno[2–c]furan	2243	33.081	0.40 ± 0.45	234	$C_{15}H_{22}O_2$	Ar
30	10-*epi*-γ-Eudesmol	2324	36.506	2.21 ± 0.19	222	$C_{15}H_{26}O$	S
31	Sandaracopimarinal	2413	40.221	2.19 ± 0.57	286	$C_{20}H_{30}O$	Ar
32	γ-Gurjunene	2470	42.629	0.52 ± 0.10	204	$C_{15}H_{24}$	S
33	Butyl hexadecanoate	2542	45.628	0.75 ± 0.46	312	$C_{20}H_{40}O_2$	Other

Table 1. *Cont.*

No.	Compound	LRI [a]	Retention Time (min)	Content [b] (%)	Detected *m/z*	Chemical Formula	Type [c]
34	2-(2-Phenylethyl)chromone	2613	48.64	26.19 ± 1.0	250	$C_{17}H_{14}O_2$	Other
35	Butyl octadecanoate	2635	49.538	1.13 ± 0.69	340	$C_{22}H_{44}O_2$	Other
36	1-Iodo-octacosane	2640	49.743	0.05 ± 0.03	520	$C_{28}H_{61}I$	Ak
37	1-Iodo-eicosane	2680	51.447	0.09 ± 0.06	408	$C_{20}H_{41}I$	Ak
38	Bis(2-ethylhexyl) 1,2-benzenedicarboxylate	2700	52.277	0.34 ± 0.38	390	$C_{24}H_{38}O_4$	Other
39	Squalene	2806	56.472	1.19 ± 1.30	410	$C_{30}H_{50}$	Tri
40	Nonacosane	2900	60.669	0.08 ± 0.05	408	$C_{29}H_{60}$	Ak

[a] LRI: Linear retention index as tested on SH-Rxi-5Sil column using the series of C8–40 *n*-alkanes; [b] Values are the mean ± standard error (n = 3); [c] Ak: alkane; Ar: aromatics; S: sesquiterpene; T: terpene; Tri: triterpene.

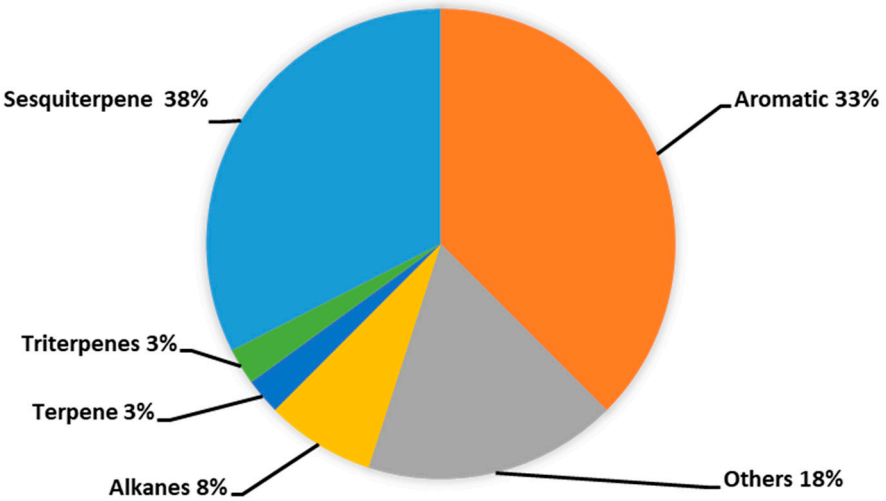


Figure 4. Pie chart showing chemical classification of 40 compounds identified in the incense smoke from Kynam agarwood.

A total of 132 constituents have been identified in solvent extracts from different varieties of agarwood in the past 5 decades, and they belong to sesquiterpenes (52%), 2-(2-phenylethyl) chromone derivatives (41%), and aromatics (1%) [3]. In this study, we found that aromatics and sesquiterpenes were the two major chemical categories in the incense smoke of Kynam agarwood as analyzed with HS GC-MS. The difference may result from the extraction procedure or varieties of agarwood.

Sesquiterpenes and 2-(2-phenylethyl) chromone derivatives were the two predominant constituents in agarwood [2]. As chromone derivatives could not be pyrolyzed [11], Espinoza et al. [20] used direct analysis with real time time-of-flight mass spectrometry (DART-TOFMS) to analyze 60 commercial agarwood chips without extraction, and identified the presence of key ions and the characteristics of 2-(2-phenylethyl) chromones. They found that 8–16 target chromone ions were present in each sample. These results showed that the highly oxidized agarwood chromones were specific to *Aquilaria* spp.

2.4. Chemical Profiles of Different Grades of Agarwoods Using HS GC/MS-MS

To further compare chemical constituents in the incense smoke produced by Vietnamese, Lao, and Cambodian varieties of agarwood with those in the same produced by the Kynam variety, we prepared the incense smoke using a HS preheating system, and the smoke was directly injected to GC for chemical identification. Lao agarwood shares similar physical properties with Cambodian

agarwood, and both varieties exhibited similar patterns in GC (Figure 5 and Figure S2). A total of 110 constituents were identified in the incense smoke produced by four varieties of agarwood, including 40 compounds in the Kynam variety, 25 compounds in the Vietnamese variety, 45 compounds in the Lao variety, and 31 compounds in the Cambodian variety. Four compounds were commonly identified in the four varieties of agarwood, while 28, 13, 26, and 19 compounds were specifically identified in the Kynam, Vietnamese, Lao, and Cambodian varieties of agarwood, respectively (Figure 5D and Table 2). Butyl hexadecanoate, butyl octadecanoate, bis(2-ethylhexyl) 1,2-benzenedicarboxylate, and aqualene were commonly found in the incense smoke produced by the four varieties of agarwood. Ishihara et al. [11] analyzed the smoke profiles of two different varieties of Vietnamese agarwood that were absorbed in Tenax TA, and found small amounts of benzaldehyde and 2-(2-phenylethyl) chromone were present in both varieties of Kynam (Kanakoh) agarwood. Ismail et al. [21] analyzed the chemical constituents of agarwood oil that was absorbed in PDMS and divinylbenzene-carboxen-PDMS, and found that 4-phenyl-2-butanone was one of the major compounds that contributed to the scent. 4-Methoxy-benzaldehyde was the first compound identified in the incense smoke of agarwood in this study, whereas it had not been identified in agarwood smoke or oils in other studies. These findings indicate that the identification of constituents in agarwood is affected by adsorbents. Therefore, more authentic chemical profiles of agarwood can be achieved with the use of incense smoke without matrix absorption.

Table 2. Compounds commonly or specifically present in the incense smoke of agarwoods.

Compound	Retention Time (min)	LRI	Detected *m/z*	Chemical Formula	Agarwoods [a]
Butyl hexadecanoate	45.628	2542	312	$C_{17}H_{40}O_2$	K, V, L, C
Butyl octadecanoate	49.538	2635	340	$C_{22}H_{44}O_2$	K, V, L, C
Bis(2-ethylhexyl) 1,2-benzenedicarboxylate	52.277	2700	390	$C_{24}H_{38}O_4$	K, V, L, C
Squalene	56.472	2800	410	$C_{30}H_{50}$	K, V, L, C
Benzaldehyde	5.296	1581	106	C_7H_6O	K
4-methoxy-Benzaldehyde	11.617	1731	136	$C_8H_8O_2$	K
1,8-Nonadien-3-yne, 2,8-dimethyl-7-methylene-	11.717	1734	160	$C_{12}H_{16}$	K
Cubenene	15.275	1818	204	$C_{15}H_{24}$	K
α-Longipinene	15.471	1823	204	$C_{15}H_{24}$	K
Tricyclo[5.2.2.0(1,6)]undecan-3-ol, 2-methylene-6,8,8-trimethyl-	17.017	1860	220	$C_{15}H_{24}O$	K
Naphthalene	18.086	1885	202	$C_{15}H_{22}$	K
δ-Guaiene	18.196	1888	204	$C_{15}H_{24}$	K
Nootkatene	18.502	1895	202	$C_{15}H_{22}$	K
4a,5-Dimethyl-3-(prop-1-en-2-yl)-1,2,3,4,4a,5,6,7-octahydronaphthalen-1-ol	19.863	1928	220	$C_{15}H_{24}O$	K
cis-α-Santalol	21.312	1962	220	$C_{15}H_{24}O$	K
β-Vetivenene	21.593	1969	202	$C_{15}H_{22}$	K
γ-Eudesmol	22.799	1998	222	$C_{15}H_{26}O$	K
10s,11s-Himachala-3(12),4-diene	23.610	2017	204	$C_{15}H_{24}$	K
β-Guaiene	24.209	2031	204	$C_{15}H_{24}$	K
Cubenol	24.606	2041	222	$C_{15}H_{26}O$	K
α-Tetralone	26.624	2089	218	$C_{15}H_{22}O$	K
α-Kessyl acetate	27.497	2110	280	$C_{17}H_{28}O_3$	K

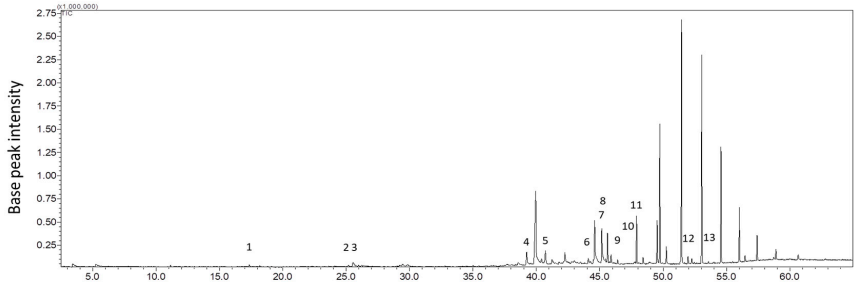
Table 2. *Cont.*

Compound	Retention Time (min)	LRI	Detected *m*/*z*	Chemical Formula	Agarwoods [a]
(1*R*,7*S*,*E*)-7-Isopropyl-4,10-dimethylenecyclodec-5-enol	27.941	2120	220	$C_{15}H_{24}O$	K
Ledol	29.445	2156	222	$C_{15}H_{26}O$	K
Longiverbenone	32.017	2217	218	$C_{15}H_{22}O$	K
2a*S*,3a*R*,5a*S*,9b*R*)-2a,5a,9-Trimethyl-2a,4,5,5a,6,7,8,9b-octahydro-2*H*-naphtho[1–b]oxireno[2–c]furan	33.081	2243	234	$C_{15}H_{22}O_2$	K
10-*epi*-γ-Eudesmol	36.506	2324	222	$C_{15}H_{26}O$	K
Sandaracopimarinal	40.221	2413	286	$C_{20}H_{30}O$	K
γ-Gurjunene	42.629	2470	204	$C_{15}H_{24}$	K
2-(2-Phenylethyl)chromone	48.640	2613	250	$C_{17}H_{14}O_2$	K
1-iodo-Triacontane	49.743	2640	548	$C_{22}H_{44}O_2$	K
Eicosane, 1-iodo-	51.447	2680	408	$C_{20}H_{41}I$	K
2-Isopropenyl-4a,8-dimethyl-1,2,3,4,4a,5,6,7-octahydronaphthalene	17.354	1868	204	$C_{15}H_{24}$	L
Dehydrodeoxybaimuxinol	19.844	1927	220	$C_{15}H_{24}O$	L
Rosifoliol	22.267	1985	222	$C_{15}H_{26}O$	L
1H-Cyclopropa[a]naphthalene, 1a,2,3,3a,4,5,6,7b-octahydro-1,1,3a,7-tetramethyl-, [1aR-(1aα,3aα,7bα)]-	23.598	2017	204	$C_{15}H_{24}$	L
Guaiol acetate	23.692	2019	222	$C_{15}H_{26}O$	L
Isovalencenol	24.782	2045	218	$C_{15}H_{22}O$	L
4-isopropenyl-1-methoxymethoxymethyl-cyclohexene	25.046	2051	196	$C_{12}H_{20}O_2$	L
trans-α-Bisabolene	25.447	2061	204	$C_{15}H_{24}$	L
β-Patchoulene	25.667	2066	204	$C_{15}H_{24}$	L
Androstan-17-one, 3-ethyl-3-hydroxy-, (5α)-	25.938	2072	318	$C_{21}H_{34}O_2$	L
Acetate, (2,4a,5,8a-tetramethyl-1,2,3,4,4a,7,8,8a-octahydro-1-naphthalenyl) ester	26.337	2082	250	$C_{16}H_{26}O_3$	L
Dehydrofukinone	27.707	2115	218	$C_{15}H_{22}O$	L
Neoisolongifolene, 8,9-epoxy-	33.235	2246	218	$C_{15}H_{22}O$	L
6-Isopropenyl-4,8a-dimethyl-1,2,3,5,6,7,8,8a-octahydro-naphthalen-2-ol	34.576	2278	220	$C_{15}H_{24}O$	L
(*S*)-*cis*-Verbenol	36.717	2329	152	$C_{10}H_{16}O$	L
Card-20(22)-enolide, 3,5,14,19-tetrahydroxy-, (3β,5β)-	38.348	2368	406	$C_{23}H_{34}O_6$	L
Espatulenol	39.248	2390	220	$C_{15}H_{24}O$	L
5-Isopropenyl-2-methylcyclopent-1-enecarboxaldehyde	39.504	2396	150	$C_{10}H_{14}O$	L
Levoverbenone	39.650	2399	150	$C_{10}H_{14}O$	L
Cyclobutene, 4,4-dimethyl-1-(2,7-octadienyl)-	39.908	2405	190	$C_{14}H_{22}$	L
Alloaromadendrene oxide-(1)	40.407	2417	220	$C_{15}H_{24}O$	L

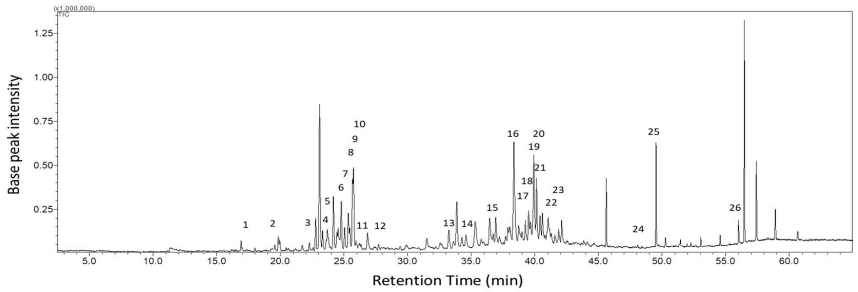
Table 2. *Cont.*

Compound	Retention Time (min)	LRI	Detected *m/z*	Chemical Formula	Agarwoods [a]
3-Oxatricyclo[20.8.0.0(7,16)]triaconta-1(22),7(16),9,13,23,29-hexaene	41.858	2452	406	$C_{29}H_{42}O$	L
Andrographolide	42.100	2458	350	$C_{20}H_{30}O_5$	L
Docosa-2,6,10,14,18-pentaen-22-al, 2,6,10,15,18-pentamethyl-, all-trans	48.062	2600	384	$C_{27}H_{44}O$	L
2,6,10-trimethyl-Tetradecane	49.731	2639	240	$C_{17}H_{36}$	L
Heptadecanal	51.965	2693	254	$C_{17}H_{34}O$	L
4,5-di-epi-Aristolochene	17.343	1868	204	$C_{15}H_{24}$	V
α-Costal	25.156	2054	218	$C_{15}H_{22}O$	V
6,7-Dimethyl-1,2,3,5,8,8a-hexahydronaphthalene	25.964	2073	162	$C_{12}H_{18}$	V
Dihydrocolumellarin	39.222	2389	234	$C_{15}H_{22}O_2$	V
Columellarin	40.694	2424	232	$C_{15}H_{20}O_2$	V
Isovelleral	44.081	2505	232	$C_{15}H_{20}O_2$	V
Octadecanoic acid	45.149	2530	284	$C_{18}H_{36}O_2$	V
1-Hexacosene	45.755	2545	364	$C_{26}H_{52}$	V
Pentadecanal-	46.405	2560	226	$C_{15}H_{30}O$	V
n-Pentadecanol	47.783	2593	228	$C_{15}H_{32}O$	V
2-Nonadecanone	48.071	2600	282	$C_{19}H_{38}O$	V
Dotriacontane, 1-iodo-	52.442	2704	576	$C_{32}H_{65}I$	V
Tetracosanal	53.568	2731	352	$C_{24}H_{48}O$	V
α-Ylangene	14.047	1789	204	$C_{15}H_{24}$	C
5,5-dimethyl-4-(3-oxobutyl)-Spiro[2.5]octane	15.489	1824	208	$C_{14}H_{24}O$	C
Diepicedrene-1-oxide	17.013	1860	220	$C_{15}H_{24}O$	C
7-*epi*-α-Cadinene	17.220	1865	204	$C_{15}H_{24}$	C
α-Guaiene	17.385	1869	204	$C_{15}H_{24}$	C
Alloaromadendrene oxide-(2)	19.874	1928	220	$C_{15}H_{24}O$	C
Diethyl phthalate	21.888	1976	222	$C_{12}H_{14}O_4$	C
Hinesol	23.982	2026	222	$C_{15}H_{26}O$	C
cis-Eudesm-6-en-11-ol	24.456	2037	222	$C_{15}H_{26}O$	C
Longipinane, (*E*)-	25.371	2059	206	$C_{15}H_{26}$	C
Pentadecanoic acid	35.990	2312	242	$C_{15}H_{30}O_2$	C
Palmitoleic acid	39.168	2388	254	$C_{16}H_{30}O_2$	C
Hexadecane	45.448	2537	226	$C_{16}H_{34}$	C
trans-Geranylgeraniol	48.077	2600	290	$C_{20}H_{34}O$	C
Tetradecanal	48.432	2608	212	$C_{14}H_{28}O$	C
Hexadecanal	50.261	2652	240	$C_{16}H_{32}O$	C
Octacosane	51.439	2680	394	$C_{28}H_{58}$	C
Oxirane, hexadecyl-	51.970	2693	268	$C_{18}H_{36}O$	C
Heptacosane	53.037	2718	380	$C_{27}H_{56}$	C

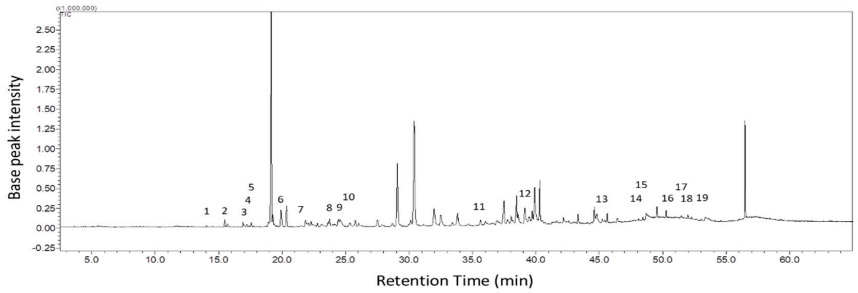
[a] K: Kynam; V: Vietnam; L: Liao; C: Cambodian.



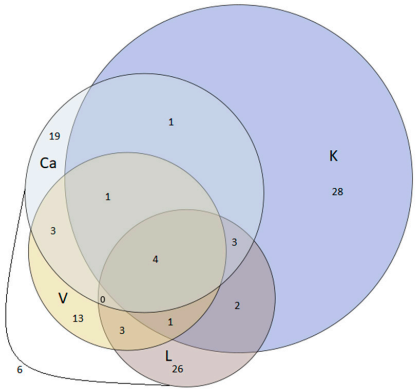
(**A**)



(**B**)



(**C**)



(**D**)

Figure 5. GC-MS chemical fingerprint of incense smoke from agarwoods. (**A**) Vietnam agarwood. (**B**) Liao agarwood. (**C**) Cambodian agarwood. Number indicates the compound specifically present in the agarwood. (**D**) Venn diagram of 110 compounds identified in the incense smoke from Kynam (K), Vietnam (V), Liao (L), and Cambodian (Ca) agarwoods.

2.5. Chemical Ingredients of Different Grades of Agarwoods

Sesquiterpenoids and 2-(2-phenylethyl) chromone derivatives are two predominant constituents in agarwood [1–3]. Sesquiterpenoids, the essential ingredients in luxury perfumes, have been identified in different varieties of agarwood. In addition to sesquiterpenes, 2-(2-phenylethyl) chromone derivative (flindersiachromone, 2-phenethyl-4*H*-chromen-4-one) is another key reference constituent in agarwood. 2-(2-Phenylethyl)chromone derivatives can only be extracted in solvents or supercritical CO_2, and are never found in hydrodistillates [22–24]. Substitute chromones in different grades of agarwood have also been studied. 2-(2-Phenylethyl) chromone derivatives can be detected in 76% of agarwood chips using direct analysis with DART-TOFMS without chemical extraction; however, DART-TOFMS is only used for distinguishing *Aquilaria* genus from others instead of agarwood grading [20]. One previous study showed that 2-(2-phenylethyl)chromone derivatives were found in incense smoke produced by Vietnamese agarwood, with a GC peak area of 5.83% in Kanankoh (high quality variety of agarwood) [11]. Another study also showed that 2-(2-phenylethyl)chromone derivatives were present only in expensive agarwood [15]. In this study, we found that 2-(2-phenylethyl) chromones were identified in the incense smoke of the Kynam variety (27.23%), but not in the same of Vietnamese, Lao, or Cambodian variety. So far, 39 different 2-(2-phenylethyl) chromone derivatives have been identified in different varieties of agarwood [20,25]. In line with these studies, our findings suggest that analysis of 2-(2-phenylethyl) chromone derivatives can be used to assess quality of agarwood. Moreover, the contents of 2-(2-phenylethyl) chromone derivative in triple duplicates of Kynam smoke were 25.18%, 27.23%, and 26.17% (Supplementary Materials Figure S1). Therefore, our data suggested that the HS GC/MS developed in this study can be a useful tool for grading quality of agarwood through direct analysis of incense smoke.

3. Materials and Methods

3.1. Reagents

All chemicals were purchased from Sigma (St. Louis, MO, USA), unless indicated.

3.2. Agarwood Materials

All natural agarwoods, including Kynam, Vietnam, Liao and Cambodia, were purchased from Chenglin Inc. (Yunlin, Taiwan). Agarwoods used in this study were identified and qualified by The International Tropical Timber Organization. The voucher specimen has been deposited in Development Center for Biotechnology. The agarwood chips were pulverized into a fine powder before use.

3.3. Sample Preparation

The incense smoke of agarwood was prepared using HS preheated system. Agarwood powder (50 mg) was weighted and placed in a 20 mL glass vial. The vial was sealed with Teflon, shaken, and incubated at 150 °C for 30 min in a headspace autosampler oven (Model HS-20, Shimadzu Corporation, Kyoto, Japan).

3.4. GC-MS/MS Analysis

Chemical constituents of incense smoke were analyzed by GS-MS/MS. Incense smoke (1 mL) was injected into GC-MS/MS (Model GC-2010 Plus and GCMS-TQ8040, Shimadzu Corporation, Kyoto, Japan). Temperature of sample line and transfer line was kept at 150 °C Ultra-high purity helium (99.99%) was used as the carrier gas at a flow rate of 5.1 mL/min. The chromatographic separation was conducted with SH-Rxi-5Sil MS capillary column (30 m × 0.25 mm inner diameter, 0.25 µm thickness, Shimadzu Corporation, Kyoto, Japan). The column temperature program was set as follows: 60 °C initial temperature and subsequently ramped to 140 °C at a rate of 6 °C/min, held at 140 °C for 5 min, ramped to 160 °C at a rate of 2 °C/min, held at 160 °C for 5 min, ramped to 280 °C at a rate

of 5 °C/min, held at 280 °C for 10 min. The GC-MS interface temperature was maintained at 250 °C. Electron ionization was used as the ionization method, and the ion source temperature was set at 230 °C. The mass spectra obtained with full scan and the mass ranged from m/z 45 to 550 were used for determining the unknown components.

Raw data were processed using GCMS solution Version 4.41 (Shimadzu Corporation, Kyoto, Japan). Mass spectral fragmentation patterns were compared with those stored in the NIST Mass Spectral Library (nist14), which is built up by using the pure substances and the mass spectra from literatures. In order to get the linear retention index values of the volatile compounds, a series of *n*-alkanes calibration standard (C8–C40) was run in the same condition (Figure S3). A Venn diagram was generated by eulerr program of R package [26].

4. Conclusions

So far, grading of agarwoods is flexible and subjective, because of the absence of standard grading system. Scent is the most important parameter for the quality assessment of agarwoods. In this work, we established a simple, automated, robust, and high-throughput method to analyze the incense smoke generated from a minimum amount of agarwood sample by HS GC-MS/MS. In comparison with the incense smoke constituents in Vietnam, Liao, and Cambodian agarwoods, we found that Kynam agarwood was composed of more complex constituents. Moreover, 2-(2-phenylethyl)chromone derivative was present in Kynam instead of Vietnam, Liao, and Cambodia agarwoods, suggesting that the content of 2-(2-phenylethyl)chromone derivative could be applied to grade the quality of agarwoods.

Supplementary Materials: The Supplementary Materials are available online.

Author Contributions: Methodology, W.-Y.K. and S.-C.H.; Supervision, T.-Y.H. and K.-T.L.; Writing—original draft, W.-Y.K. and C.-Y.H.; Writing—review & editing, C.-Y.H., T.-Y.H. and L.-T.L.

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Conflicts of Interest: The authors declare no conflict of interest.

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Sample Availability: Samples of the agarwoods are available from the authors.