Grading of Agarwood Based on Their Chemical Profiles Using GC-MS Incorporating Chemometric Approaches

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Agarwood is one of the valuable natural products important for fragrance industry and medicinal applications. The material is valuable and has a long-standing history of applications in perfumery, incense making, traditional medicine, and for spiritual rituals. The quality of agarwood is classically evaluated based on physical characteristics. There is lack of a standardized quality grading system to objectively assess the quality of agarwood. This poses significant challenges to verify the authenticity, quality, and consistency of this valuable natural product. This study extracted essential oils from two samples of agarwood (SS and KPY) for analysis using Gas Chromatography Mass Spectrometry (GCMS). Principal Component Analysis (PCA) and variable selection approaches were applied to examine the chemical profiles of the agarwood samples. Results showed that the chemical profiles of the two groups of agarwood were differentiable. The agarwood indicators were identified based on the criteria that a compound is significant, consistently found in replicates of extracts, and supported by literatures. A total of 11 compounds are recommended as the quality indicators of agarwood: allo-khusiol, chlorodifluoroacetate-isolongifolol, agarospirol, α-kessyl acetate, β-dihydroagarofuran, selina-3,11-dien-9-ol, α-epi-7-epi-5-eudesmol, α-agarofuran, 4-phenyl-2-butanone, alloaromadendrene, and eremophila-1(10). The findings of this study provide a set of criteria for objective evaluation of essential oil from agarwood.

Keywords: Essential Oil; Aquilaria genus; Hydrodistillation; GCMS analysis; quality indicators

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The *Aquilaria* genus of the Thymelaeaeoae family is renowned for producing heartwood impregnated with resin known as agarwood. The material is valuable and has a long-standing history of applications in perfumery, incense making, traditional medicine, and for spiritual rituals [1]. A total of 15 species of *Aquilaria* are found in Asia, including Sumatra, India, Vietnam, Burma, Laos, Cambodia, Malaysia, the Philippines, and Papua New Guinea [2]. The species recorded in Malaysia include *Aquilaria malaccensis*, *Aquilaria rostrata*, *Aquilaria hirta*, and *Aquilaria beccariana*.

Agarwood is the resin produced by *Aquilaria* trees in response to physical, biological or chemical injuries. As the lesion worsens due to pathogenic infection, the resin continues to collect in the wood tissues. The wood becomes denser and darker after resin deposition [3]. A notable rise in demand for agarwood has led to over-exploitation of *Aquilaria* species, causing them to be depleted from the natural habitats. The Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) lists agarwood trees as critically endangered species [4].

Agarwood has classically been graded according to its physical characteristics such as resin concentration, color, aroma, shape, and density [5]. The water sinking technique is commonly used for assessment of the quality of agarwood. According to Yang et al. [6], agarwood of high quality will sink in water due to its high resin content, hence it is referred to as “sinking scent" or Chen Xiang in Mandarin.

Typically, agarwood is classified according to its origin, prior to evaluation based on fragrance, color, density, size, and shape. The price of agarwood relies on the buyer’s perception and market forces rather than a set of objective criteria. Ismail et al. [7] revealed that the lack of a standardized quality grading system poses significant challenges to verify the authenticity, quality, and consistency of agarwood. This further creates challenges for producers, traders, and buyers to make well-informed decisions regarding their purchase, usage, and value. Without a standard grading system, there is a risk of adulteration and mislabeling of agarwood products in the market. Therefore, it is crucial to establish the criteria for determination of the quality of agarwood. This study aimed to determine the chemical profiles of essential oils from two agarwood samples, to employ chemometric techniques for classification of agarwood, and to identify compounds for assessment of agarwood quality.

Figure 1. Two agarwood samples from Borneo

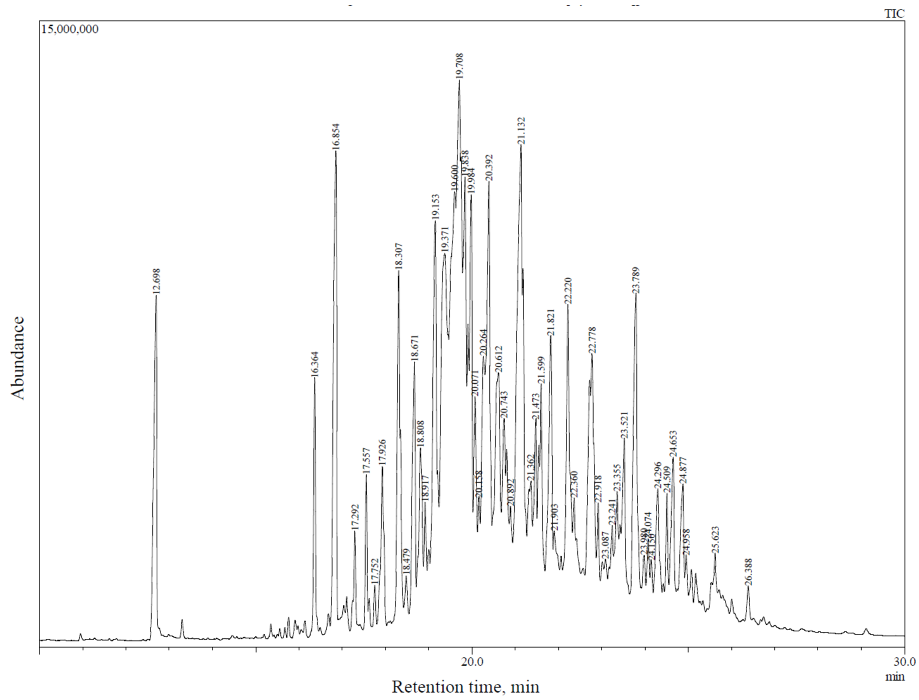
EXPERIMENTAL

Samples

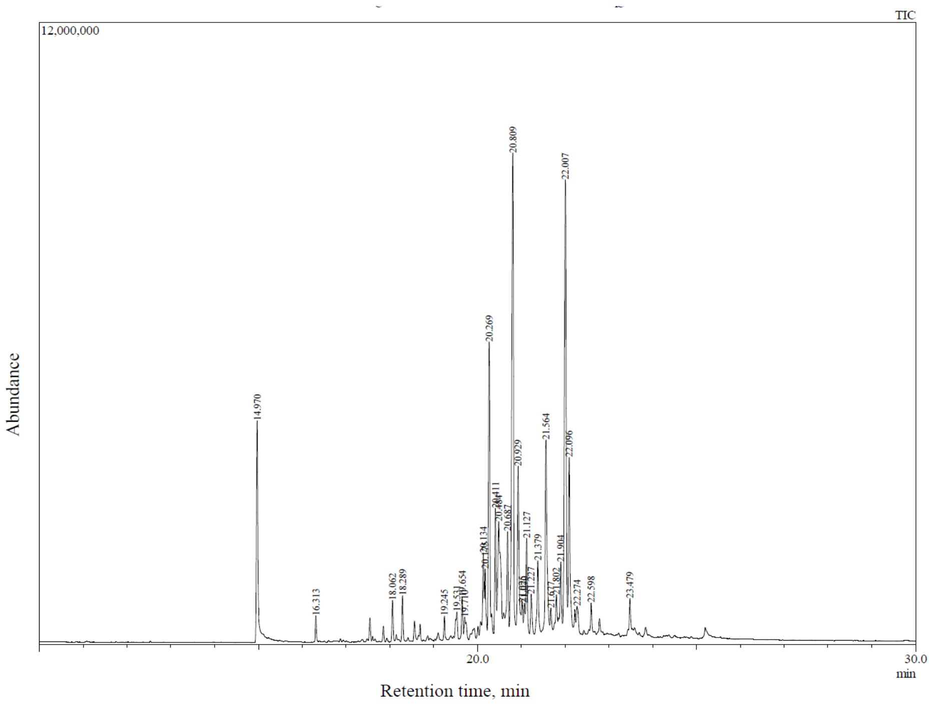
Two samples of wild agarwood, labelled as KPY and SS, were donated by a local agarwood collector (Figure 1). The samples were weighed and cut into small pieces using a nail clipper. The agarwood pieces were soaked in water for three days at a ratio of 1:12 (w/v) in a round-bottom flask prior extraction.

Essential Oil Extraction

The essential oil was extracted using the hydro-distillation technique. A round-bottom flask containing 20 g of agarwood pieces in water was attached to a Clevenger apparatus. The sample was boiled at 100°C for 8 hrs. The water and essential oil mixture was condensed and collected in the vessel of the Clevenger apparatus. Pentane (min. 99%) was added to the collecting vessel where the essential oil was trapped to minimize the loss of volatile organic compounds. The extraction was repeated to collect a second fraction of essential oil. Upon completion of the extraction process, the pentane-agarwood mixture was collected in a vial and purged with nitrogen. The essential oil was then dissolved in hexane and 1 μL of the sample was injected into the Gas Chromatography-Mass Spectrometer (GCMS) using splitless mode. Each sample was subjected to the aforementioned extraction procedure thrice, and each extraction was repeated to yield a second fraction. The extracts were subjected to analysis using GCMS in triplicates (2 samples × 3 extractions × 2 fractions × 3 replicates = 36 chromatograms).



KPY



SS

Figure 2. Representative chromatograms of two agarwood samples, SS and KPY.

Gas Chromatography-Mass Spectrometry (GC-MS)

The GCMS (Shimadzu, QP2010 Plus) equipped with BPX-5 capillary column (30 m × 0.25 mm ID × 0.25 µm film thickness) was used to analyze the essential oils. The program was set at 50°C initially for 5 min and gradually increased to 250°C for 10 min at 10°C/min. A flow rate of 1.0 mL/min of pure helium was used as the carrier gas and the total program time was 35 min. The method is according to Samling et al. with slight modifications. The mass spectral library of the National Institute of Standards and Technology (NIST 17) was used to determine the chemical compounds present [8].

Statistical Analysis

The compounds detected in all chromatograms were organized into a peak table with rows representing samples and columns denoting variables. The peak table was standardized and subjected to Principal Component Analysis (PCA). Score and loading plots were produced to examine the clustering of samples. T-statistic was employed to determine the significant compounds differentiating two groups of samples. The t-value for each variable was calculated based on the following equation. Variables with the highest absolute values of t are the most significant variables, whilst the signs indicate which class they represent as a marker. The statistical analysis was performed using Matlab R2013b.

$$t_m = \frac{\bar{x}_{mA} - \bar{x}_{mB}}{S_m\sqrt{\frac{1}{n_A} + \frac{1}{n_B}}}$$

$\bar{x}_{mA}, \bar{x}_{mB}$ mean of each variable calculated for groups 1 and 2

S_m pooled standard deviation for each variable over the two classes

$$S_m = \sqrt{\frac{(n_A - 1)s_{mA}^2 + (n_B - 1)s_{mB}^2}{n_A + n_B - 2}}$$

n_A , n_B the number of samples in groups 1 and 2

RESULTS AND DISCUSSION

Exploratory Analysis

The chromatograms of the essential oils of SS and KPY are illustrated in Figure 2. A total of 242 and 118 compounds were identified in KPY and SS, respectively. The compounds were organized into a peak table of dimension (36 × 318). The chemical profiles demonstrated some variations between samples and replicates, which is not unexpected. Essential oil is a complex mixture of chemical compounds that could differ according to parts where agarwood is formed, geographical locations, species and etc. Gao et al. [9] reviewed the compounds present in 4 species of agarwood, summarizing a total of 367 compounds mainly from the groups of agarofurans, agarospiranes, eudesmanes, erepmophilanes, guaianes, candinanes, prezizanes, and chromones.

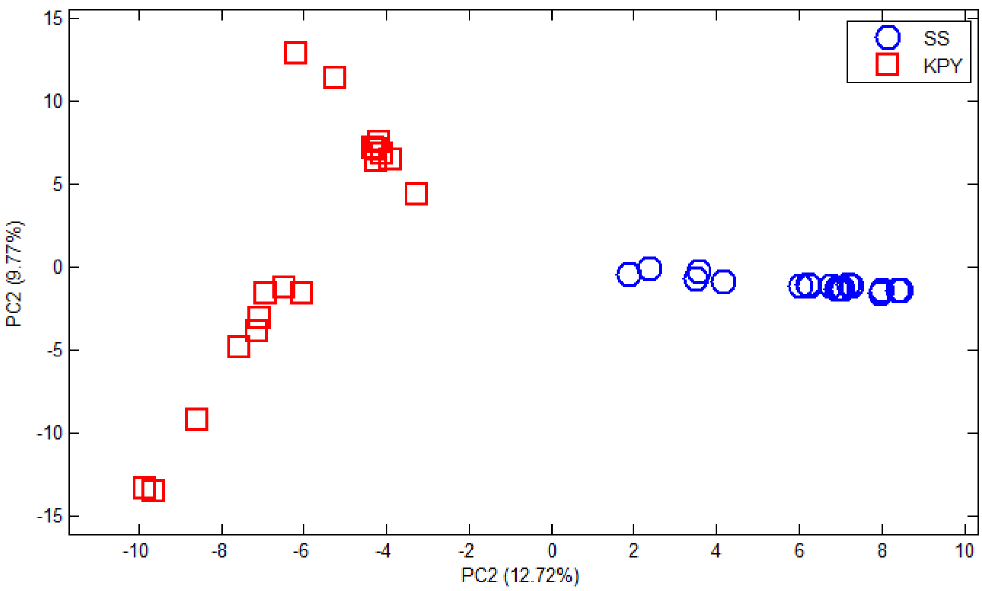


Figure 3. Score plot of PC2 versus PC1 based on chemical profiles.

The peak table was subjected to PCA, yielding the score plot of PC2 versus PC1 (Figure 3). The total variance explained by the first two PCs was 22.49%. A total of 30 compounds were consistently identified in the two samples and their abundances are summarized in Table 1. These compounds are mainly sesquiterpenoids, sesquiterpenes, and mono-terpenoids. According to Abd Majid et al. [9], the quality of agarwood depends primarily on the content of oxygenated sesquiterpenes (sesquiterpenoids). A high-grade agarwood consists of comparatively higher sesquiterpenoids. Eeremophilane is also identified as a marker for grading of agarwood oil according to Yang et al. [6], whilst Yu et al. [11] suggests 2-(2-phenylethyl) chromone for classification of high-grade wild Chi-Nan.

Chemical Compositions

The common compounds detected were evaluated according to literatures. Agarospirol, α-agarofuran and β-dihydroagarofuran are typical characteristics of agarwood where their presence is corroborated by [12] and [13]. These compounds contribute to woody scent of agarwood. Ismail et al. [14] reported the predominant presence of 4-phenyl-2-butanone (a derivative of 2-(2-phenylethyl) chromone), the highest in abundance among other compounds, in high-quality agarwood oils. In this study, both KPY and SS demonstrated considerable percentages of 4-phenyl-2-butanone at 5.48% and 7.33%, respectively. Aristolene was also detected in both KPY and SS samples in almost every replicate of extraction with their average abundance registered at 4.64% and 3.67%, respectively. Aristolene was reported in essential oils from wild agarwood of Hainan, Guanxi, and Guandong; it is regarded as a quality indicator [15]. α-Kessyl acetate was detected in both samples with a higher percentage identified in KPY (2.02%) compared to SS (0.74%). This compound is similarly identified in profound abundance in one of the highest quality Kynam agarwood in Asia [16]. Agarospirol on the other hand exhibited a higher percentage in SS, in contrary to the presence of α-kessyl acetate. Aristolene, agarospirol, and cubenol are identified as the major compounds in the essential oil of agarwood where pharmacological potentials and genotoxicity are demonstrated [17]. The higher amounts of aristolene and α-kessyl acetate in KPY support that it is potentially a higher-grade agarwood than SS.

The percentage of eremophila-1(10) [2-((2*R*,8*R*,8a*S*)-8,8a-dimethyl-1,2,3,4,6,7,8,8a-octahydro-naphthalen-2-yl)propan-2-ol] in KPY (6.38 %) was comparatively higher than that in SS (4.07%). Eremophila-1(10) is an important compound reported in high-grade agarwood collected by a Malaysian agarwood supplier, Kedaik Agarwood Sdn. Bhd. [18]. The presence of 1,1,4,7-tetramethyldecahydro-1H-cyclopropa[e]azulene-4,7-diol, commonly known as alloaromadendrene, was detected at comparable amounts in both samples (KPY: 3.51% and SS: 2.11%). This compound has also been found in medium grade agarwood. Carissone [(4a*S*,7*R*)-7-(2-Hydroxypropan-2-yl)-1,4a-dimethyl-4,4a,5,6,7,8-hexahydronaphthalen-2(3H)-one] and γ-eudesmol [1,2,3,4,4a,5,6,7-octahydro-2-naphthalenemethanol] are eudesmane-type of sesquiterpenes detected in both samples (KPY: Carissone 0.68% and γ-eudesmol 2.35%; SS Carissone 0.32% and γ-eudesmol 0.43%). Eudesmane sesquiterpenes are often absent in low-quality agarwood [7]. These sesquiterpenes of eudesmane class play a crucial role in unique fragrance and therapeutic properties associated with high-quality agarwood. The presence of these compounds can be used to differentiate agarwood of different qualities; a greater abundance of carissone and γ-eudesmol in KPY again points to that KPY is of higher grade than SS. Globulol was detected in both agarwood samples. This compound was detected in fungal-inoculated agarwood reported by [19]. The presence of 1-[2-(2,2,6-trimethyl-bicyclo[4.1.0]hept-1-yl)-ethyl]-vinyl ester acetic acid was comparable in both samples, whilst (2,4a,5,8a-tetramethyl-1,2,3,4,4a,7,8,8a-octahydro-1-naphthalenyl) acetate ester was more prominent in KPY (1.91%) than SS (2.39%). The presence of (2,4a,5,8a-tetramethyl-1,2,3,4,4a,7,8,8a-octahydro-1-naphthalenyl) acetate ester was similarly revealed in Laos agarwood [16]. Ahmaed et al. [18] further revealed the exclusive presence of longifolenaldehyde in high-quality agarwood chips. The sample of KPY exhibited a higher percentage of longifolenaldehyde (16.11%) than SS (1.34%), inferring that KPY is more superior in quality. As for 8-oxo-9H-cyclo-isolongifolene, it is scarcely reported in the literatures, however, it is found in the essential oil of *Jatropha ribifolia* roots, known as purging nut [20].

Compounds uniquely identified in KPY include thymol (4.45%), selina-3,11-dien-9-ol (0.43%), α-vetivone (2.00%), italicene ether (2.62%) and crypto-meridiol (3.00%). Thymol (8.3843% ± 7.7453) was revealed in a study on thirty-year-old *Aquilaria sinensis* trees in Guandong province, China with five wounded signs on the trunks. The agarwood is regarded of high quality considering its age and its origin in the wild [21]. α-Vetivone [(4*R*-cis)-4,4a,5,6,7,8-hexahydro-4,4a-dimethyl-6-(1-methylethylidene)-2(3H)-naphthalenone] was detected exclusively in a 4-year-old wild *Aquilaria sinensis* at 6.36% in Hainan province, China. The compound was undetected in the control and fungal-inoculated agarwood of the same age [22]. This implies that α-vetivone can be potentially used to differentiate wild and cultivated agarwood. Both cryptomeridiol and italicene ether are compounds commonly found in the essential oil of agarwood. Italicene ether [(1*R*,3a*R*,5a*R*,9a*S*)-1,4,4,7-tetramethyl-1,2,3,3a,4,5a,8,9-octahydrocyclopenta[c]benzo-furan] has been reported in agarwood from Vietnam, Indonesia, Malaysia, Myanmar and Cambodia [23], whilst selina-3,11-dien-9-ol is regarded as one of the key markers for high-quality agarwood oil in Japan [24]. Li et al. [25] ascertained the presence of crytomeridiol as part of new sesquiterpernoids bearing methyl ester group of agarwood collected in Thailand. The unique presence of quality markers including thymol, selina-3,11-dien-9-ol, and α-vetivone indicates that KPY is of higher quality than SS, offering superior aroma, potency, and market value.

Table 1. Abundance of compounds commonly identified in two agarwood samples, SS and KPY.

No.	Retention time (min)	Compounds	Abundance (%) SS	Abundance (%) KPY
1.	7.27	Benzaldehyde	0.72 ± 0.19	0.09 ± 0.02
2.	12.00	Acetophenone	0.83 ± 0.14	0.30 ± 0.02
3.	12.65	4-phenyl-2-butanone	7.33 ± 4.50	5.48 ± 2.96
4.	17.28	β-Dihydroagarofuran	0.70 ± 0.33	4.33 ± 3.00
5.	17.97	α-Agarofuran	0.45 ± 0.22	2.29 ± 2.66
6.	18.28	diepicedrene-1-oxide	0.77 ± 0.24	8.55 ± 5.85
7.	18.32	α-Santalol	1.14 ± 0.00	0.71 ± 0.24
8.	18.82	(2a*S*,3a*R*,5a*S*,9b*R*)-2a,5a,9-Trimethyl-2a,4,5,5a,6,7,8,9b-octahydro-2H-naphtho[1,2-b]oxireno[2,3-c]furan	0.62 ± 0.00	0.76 ± 0.28
9.	19.03	(1a*R*,3a*S*,7*S*,7a*S*,7b*R*)-1,1,3a,7-Tetramethyldecahydro-1H-cyclopropa[a]naphthalen-7-ol	1.45 ± 0.94	0.30 ± 0.00
10.	19.11	1,2,3,4,4a,5,6,7-octahydro-2-Naphthalenemethanol	0.43 ± 0.24	2.35 ± 1.70
11.	20.06	6-Isopropenyl-4,8a-dimethyl-1,2,3,5,6,7,8,8a-octahydro-naphthalen-2-ol	3.36 ± 1.71	0.57 ± 0.11
12.	20.14	1,1,4,7-Tetramethyldecahydro-1H-cyclopropa[e]azulene-4,7-diol	2.11 ± 1.11	3.51 ± 3.74
13.	20.25	Agarospirol	7.98 ±1.43	4.09 ± 1.98
14.	20.28	(2,4a,5,8a-tetramethyl-1,2,3,4,4a,7,8,8a-octahydro-1-naphthalenyl) acetate ester	2.39 ± 1.12	1.91 ± 0.98
15.	20.31	2-((4a*S*,8*R*,8a*R*)-4a,8-Dimethyl-3,4,4a,5,6,7,8,8a-octahydronaphthalen-2-yl)propan-2-ol	0.41 ± 0.16	2.41 ± 1.81
16.	20.40	Aristolene	3.47 ± 0.98	4.64 ± 1.19
17.	20.47	2-((2*R*,8*R*,8a*S*)-8,8a-Dimethyl-1,2,3,4,6,7,8,8a-octahydronaphthalen-2-yl)propan-2-ol	4.07 ± 1.85	6.38 ± 3.49
18.	20.95	Thunbergol	1.02 ± 0.05	1.30 ± 1.03
19.	21.06	Longifolenaldehyde	1.34 ± 1.28	16.11 ± 9.06
20.	21.12	1-[2-(2,2,6-trimethyl-bicyclo[4.1.0]hept-1-yl)-ethyl]-vinyl ester acetic acid	2.15 ± 0.28	4.81 ± 0.84
21.	21.80	Cubenol	0.85 ± 0.30	1.45 ± 0.28
22.	22.27	Longifolene	1.13 ± 0.88	0.77 ± 0.32
23.	22.58	Isoaromadendrene epoxide	0.58 ± 0.36	0.49 ± 0.00
24.	22.59	Globulol	1.56 ± 1.05	2.16 ± 1.33
25.	22.60	α-Kessyl acetate	0.74 ± 1.15	2.02 ± 1.12
26.	22.86	Valerenic acid	0.17 ± 0.05	0.23 ± 0.00
27.	23.47	n-Hexadecanoic acid	0.98 ± 0.46	1.18 ± 0.29
28.	23.48	(7a-Isopropenyl-4,5-dimethyloctahydroinden-4-yl)methanol	0.52 ± 0.09	1.07 ± 0.75
29.	23.53	Dibutyl phthalate	0.17 ± 0.00	0.78 ± 0.00
30.	23.82	(4a*S*,7*R*)-7-(2-Hydroxypropan-2-yl)-1,4a-dimethyl-4,4a,5,6,7,8-hexahydronaphthalen-2(3H)-one	0.32 ± 0.13	0.68 ± 0.29

Compounds characteristically found in SS include allo-khusiol [(3*S*,3a*S*,6*R*,7*S*,8a*S*)-3,7,8,8-tetramethyloctahydro-1H-3a,6-methanoazulen-7-ol], chlorodifluoroacetate-isolongifolol, 8-oxo-9H-cycloisolongifolene, 2-((2*R*,4a*R*)-4a,8-dimethyl-1,2,3,4,4a,5,6,7-octahydronaphthalen-2-yl)prop-2-1en-1-ol, and (1*R*,4a*R*,7*R*,8a*R*)-7-(2-hydroxypropan-2-yl)-1,4a-dimethyldecahydronaphthalen-1-ol. Allo-khusiol (16.11%) and chlorodifluoroacetate-isolongifolol (12.84%) represent 28.95% of the chemical constituents in SS. Allo-khusiol was reported in the incense of high-grade agarwood from Endau-Rompin Forest Reserve Forest [26]. In addition, isolongifolol (15.1 - 20.9%) and allo-khusiol (14.4 - 18.5%) were further found in the essential oil of agarwood from Sinharaja Reserve Forest, Sri Lanka [27]. These two compounds are fragrant materials of high value, suggesting that SS is also of high-quality grade.

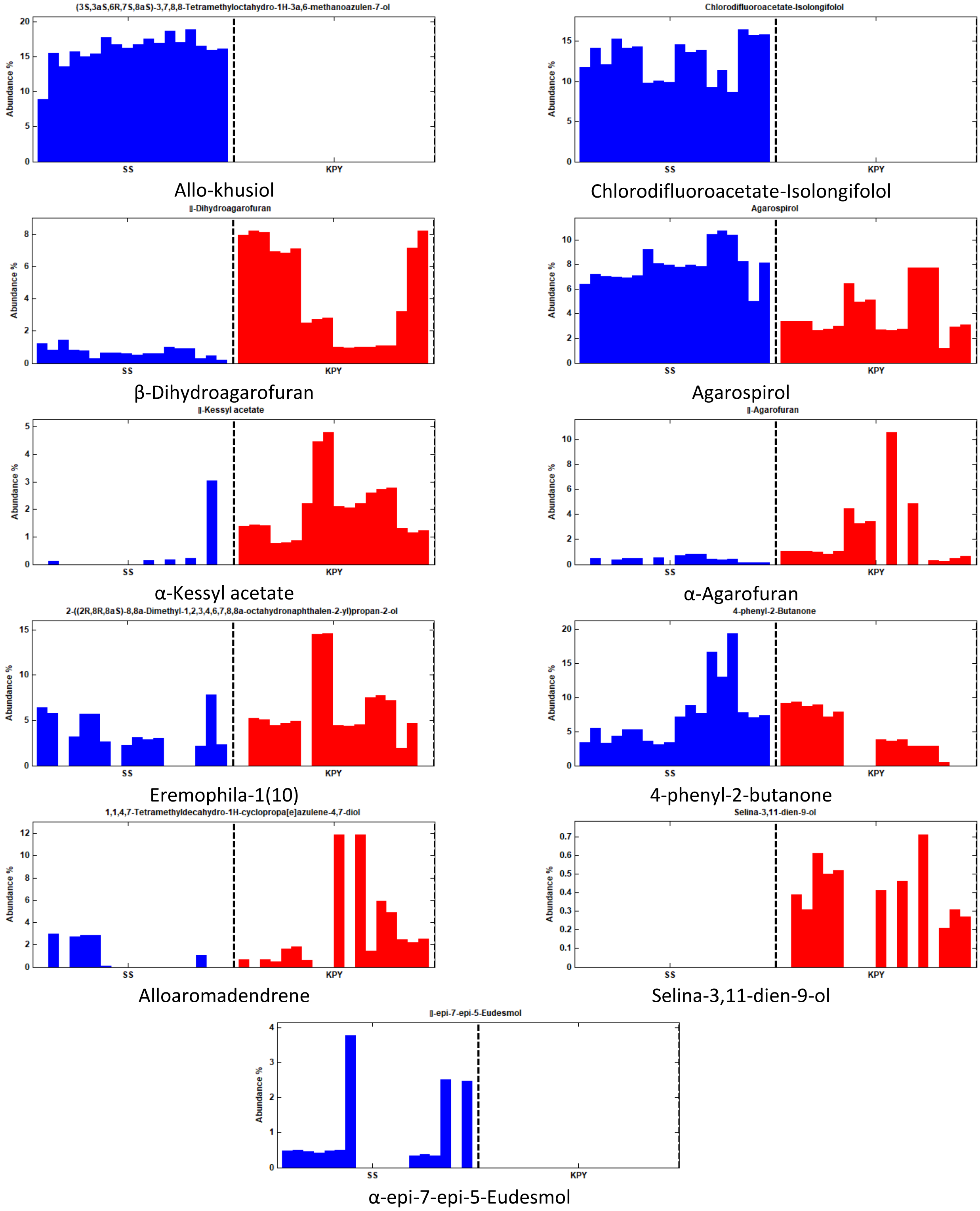


Figure 4. Abundance of compounds of quality indicators.

Variable Selection for Grading of Agarwood

The peak table was subjected to t-test for variable selection. Compounds of the top 40% significant variables that are consistently present in 60% of one or both groups of samples were identified. The indicators were chosen from the list based on the criteria that the compound is significant, consistently present, and supported in literatures as a quality marker. A total of 11 compounds were identified meeting the above criteria and their abundance is illustrated in Figure 4. The distribution of indicator compounds in the essential oils of KPY and SS suggest that both samples are good-quality agarwood. Presently, there is no established guidelines for grading of essential oil from agarwood, nevertheless, Liu et al. [27] reported that high-quality agarwood exhibits high alcohol extractive content.

i. (3*S*,3a*S*,6*R*,7*S*,8a*S*)-3,7,8,8-Tetramethyloctahydro-1H-3a,6-methanoazulen-7-ol(allo-khusiol)
ii. Chlorodifluoroacetate-Isolongifolol
iii. Agarospirol
iv. α-Kessyl acetate
v. β-Dihydroagarofuran
vi. Selina-3,11-dien-9-ol
vii. α-epi-7-epi-5-Eudesmol
viii. α-Agarofuran
ix. 4-phenyl-2-butanone
x. 1,1,4,7-Tetramethyldecahydro-1H-cyclopropa[e]azulene-4,7-diol (alloaromadendrene)
xi. 2-((2*R*,8*R*,8a*S*)-8,8a-Dimethyl-1,2,3,4,6,7,8,8a-octahydronaphthalen-2-yl)propan-2-ol' (eremophila-1(10))

CONCLUSION

The chemical profiles of essential oils from the two agarwood samples, SS and KPY, demonstrated considerable variations. The compounds identified differ according to samples and replicates, corroborating the variability. For grading of agarwood quality, the chemical compositions were subjected to variable selection. The quality indicators were determined based on the criteria that: 1) a compound is identified as a significant discriminatory variable; 2) the compound is consistently found in the replicates; and 3) the compound is supported in literatures. A total of 11 compounds were determined as the quality indicators for grading of agarwood in this study. Considering the above criteria, both SS and KPY are considered good-quality agarwood.

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