A simple metabolite profiling approach reveals critical biomolecular linkages in fragrant agarwood oil production from *Aquilaria malaccensis* – a traditional agro-based industry in North East India

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Agarwood production is a highly profitable agro-based industry in the Southeast of Asia, with a worldwide market of US$ 6–8 billion. *Aquilaria* spp. are woody plants which are the source of fragrant resins produced by the plant due to fungal infection. Mechanism of agarwood production is not well understood, but agarwood oil is in great demand in perfume industry and traditional medicine. Traders judge the quality of the resinous agarwood without any standard procedure. The oil is extracted from the agarwood following a step of fermentation. In the present study, we determined the profile of metabolites in the extracts of raw and fermented agarwood as well as three commercial grades of agarwood oil from Assam, by gas chromatography-mass spectrometry-based metabolomics analysis coupled with multivariate statistical analysis. The importance of fermentation in agarwood oil production was noted, as three agarwood aromatics, viz. 10S, 11S-Himachala-3(12), 4-diene, agarospirol and i-propyl 12-methyltetradecanoate were formed during fermentation of agarwood. The study also confirms that metabolite profiling can be a simple and effective method to distinguish grades of agarwood oil and their commercial products.

Keywords: Agarwood, aromatic oils, fermentation, multivariate statistics.

AGARWOOD, also called aloeswood, eaglewood, *agaru*, *gaharu* is a resinous material found in *Aquilaria* spp. (family: Thymelaeaceae) in Southeast Asia, which is formed by a complex plant–microbial interaction[1]. Agarwood oil is a valuable component of perfumes, incense and is also used in traditional medicine as digestive, sedative, analgesic, antiemetic, aphrodisiac, sedative, carminative and antimicrobial[2,3]. Agarwood is regarded as the most expensive wood in the world and its market demand is increasing rapidly with a current global market of US$ 6–8 billion making it one of the most profitable agro-based industries. The price of agarwood and its oil ranges from US$ 9,700 to 32,000/kg, based on quality[4]. Agarwood production is confined within a geographical area starting from the North East of India through continental Southeast Asia and the Indo-Malaysian archipelago up to Papua New Guinea, including countries like China, India, Vietnam, Indonesia, Malaysia and Thailand[5].

Formation of resinous agarwood from which perfume compounds are recovered is a rare event in nature which is poorly understood. Resin-impregnated agarwood is distilled to recover fragrant oils that are predominantly made up of sesquiterpenes and chromones. Chemical diversity of agarwood oils from different geographical locations has been studied to some extent[6–8]. Assam, is considered to be the cradle of agarwood aromatics with ancient traditions of agarwood production which is thriving and growing. However, there has been no proper scientific study to understand the intricacies of these traditional practices in relation to agarwood production. The few existing reports are only confined to (GC-MS) based chemical cataloguing of resinous and non-resinous wood[9,10]. In fact, the steps in traditional oil extraction involve interlinked biochemical processes that are yet to be documented and studied (Figure 1). In this context the fermentation of agarwood chips prior to distillation is of particular relevance. Moreover, there is no standard procedure to assess the quality of agarwood and its oils, which often leads to under or overestimation. For any improvement in the process of oil production and its evaluation, a systematic study is required. In the present study we simulated the oil production process in the laboratory based on the traditional knowledge prevalent in production units in rural Assam and the application of metabolomics-based techniques to understand the fate of the metabolites during these steps. GC-MS profiling coupled with multivariate statistics was employed to study the metabolites of agarwood, fermentation water, various grades of oil and their commercial products. To the best of our knowledge there have been no other studies on

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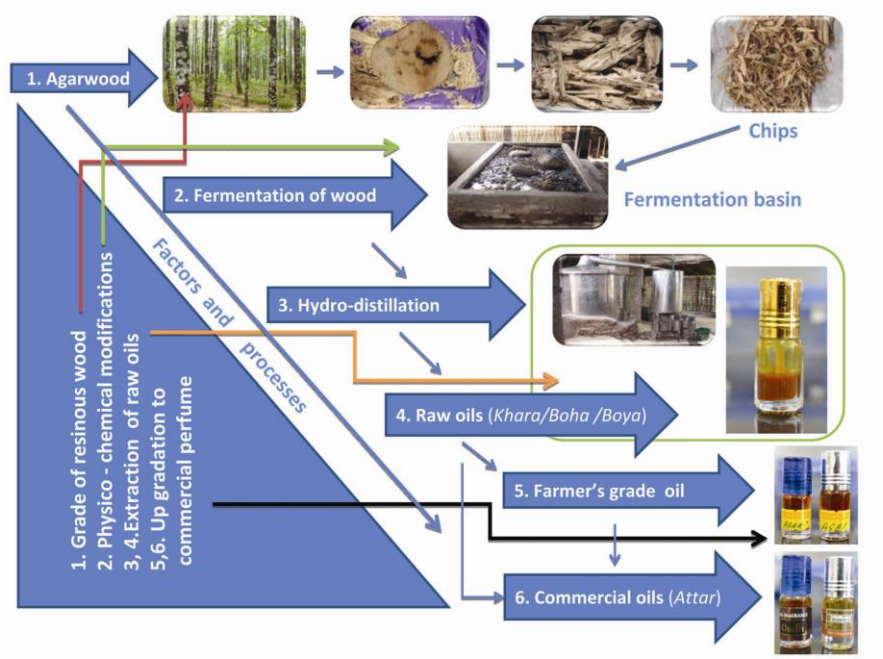


Figure 1. Steps involved in traditional production of fragrant oil from resin impregnated agarwood tissue in Assam.

non-targetted metabolic profiling of Assam (Indian) agarwood oil taking cues from age-old agro-based traditions of production.

Materials and methods

Collection of samples

Agarwood samples were collected from four different locations of Assam, namely Janji (Jorhat; 26°52′N, 94°27′E), Hojai (Nagaon; 26°0′18″N, 92°51′E), Namti (Sivasagar; 26°51′N, 94°37′E) and Nahorani (Golaghat; 26°13′N, 93°52′E). These locations were identified based on the presence of local agarwood procurement, processing and trading units. The physical distances separating these locations ranged from 20 km (Janji–Namti) up to 200 km (Hojai–Namti). Details are available in Supplementary Figure S1 online.

Wood tissue

Aquilaria malaccensis wood tissues, both resin impregnated (R) and non-resinous (W) were collected separately from individual agarwood plants 10–12 years of age. The resin-impregnated tissues were recovered from plants sourced for oil processing in distilleries. Non-resinous tissue was obtained from healthy plants of similar age in homestead plantations of the same locality. Resin-impregnated wood appeared dark in colour and was heavier in contrast to non-resinous wood, which was pale white in colour and lighter in weight.

Crude and commercial agarwood oils

Agarwood oil of three grades, locally called *Khara*, *Boha* and *Boya* were collected from a distillation unit in Nahorani village (district Golaghat). *Khara* is the first distillate of a batch and is of the highest grade followed by *Boha* and *Boya*. Also, two different commercially available agarwood-based perfumes (*agar attar*) were purchased from the local market. The first was a farmer's grade *attar* (two samples) sourced from local perfumers in Hojai (Nagaon, Assam), while the other (two samples) was cheaper commercial *attar* (Table 1).

Fermentation of agarwood tissue by soaking in water

In Assam, agarwood tissue is traditionally subjected to soaking in water basins for a period of 45–90 days prior to distillation (Figure 1). An experiment simulating the fermentation process practised in the distilleries was carried out in the laboratory. Dried resin impregnated agarwood chips and non-resinous wood chips were powdered in the laboratory and soaked separately in water for a period of 45 days in a 100 ml beaker and incubated at room temperature. GC-MS analysis of the *n*-hexane extract of the fermented samples was performed along with the agarwood oils and unfermented resinous and non-resinous agarwood tissues.

Metabolite profile

GC-MS-based metabolite profiles were generated from *n*-hexane extracts of the resin impregnated and non-resinous agarwood tissue, oils and fermentation samples.

Table 1. Agarwood oil-based perfumes purchased from commercial *Attar* dealers of Assam

Name and type	Source	Price (INR) per 3.0 ml	Description	Code[a]
Dum agar attar	Hojai (Nagaon), Assam	150.00	Farmer's grade oil (attar); third distillate of local dhum[b] wood	H1
Oudh attar	Hojai (Nagaon), Assam	100.00	Farmer's grade oil from local wood (attar)	H2
Oudh attar	S. R. Fragrance (Thane), Maharashtra	30.00	Commercial oil (attar)	L1
Rooh-al-oudh	Al-Taiba Perfumery (Kolkata), West Bengal	30.00	Commercial oil (attar)	L2

[a]Codes H and L stand for higher and lower quality of oils depending upon the details given by the manufacturer/trader and the price of the oils.
[b]A particular grade of wood used in agarwood oil production in India.

Solvent extraction

Considering that the samples were diverse and small in quantity, a simple and reproducible extraction strategy capable of maximizing the representation of biomolecules was adopted. For this, resinous and non-resinous wood samples were powdered and 250 mg of the powdered wood tissue was extracted with 5.0 ml *n*-hexane thrice by replenishing solvent during extraction every 24 h. In case of laboratory fermentation, the wood and water mixture was similarly extracted together with *n*-hexane thrice for a total of 72 h. The agarwood oil samples were also extracted in a similar method with *n*-hexane by dissolving 250 mg oil in 5.0 ml solvent. Extractions were performed by transferring each hexane fraction into a glass vial that was tightly sealed and stored at 4°C till analysis.

Gas chromatography and mass spectroscopy

The extracted samples were redissolved in spectroscopy-grade *n*-hexane and filtered through 0.2 μm filter for GC-MS analysis performed in a Perkin Elmer Clarus 680/600C unit fitted with Elite 5 MS column (length: 30 m, ID: 0.25 mm, film thickness: 0.25 μm). The oven programme started at 70°C for 2 min and ramped at 20°C/min up to 140°C and without holding, again ramped at 5°C/min to 290°C and then held for 5 min. Next 1.0 μl sample was injected at 280°C using He as carrier gas with a solvent delay of 8 min. Split ratio was 10 : 1. The mass spectrometer (Clarus 600C; single quad) was operated in the electron ionization (EI) mode at 70 eV with a source temperature of 200°C and a continuous scan from *m*/*z* 50 to 600. The peaks were identified by matching the mass spectra with the National Institute of Standards and Technology (NIST) library, USA.

Representation by Venn diagram

Chemical profiles of samples obtained from GC-MS analysis followed by NIST library search were classified based on their identities and represented as Venn diagram using the multiple dataset analysis feature of the Vennture software[11].

Statistical analysis

In all the experiments, treatments were replicated thrice and individual experiments were repeated once prior to analysis of the resulting data. The mean values of the data were used for subsequent statistical analyses performed using SPSS 18.0.0 statistical package for the chemometric data[12]. Two popular multivariate statistical methods, viz. hierarchical cluster analysis (HCA) using agglomerative statistics (clustered by between group linkage and measured by squared Euclidean distances) and principal components analysis (PCA; where principal components are extracted based on eigenvalue (value >1) and rotated in varimax (for orthogonal plotting)) were performed on the datasets to assess the degree of relatedness between the samples to analyse the possible presence of biomolecular linkages[13]. All discernible peaks with reference to their retention time were subjected to pre-processing with 0 and 1 designating presence (1) or absence (0) of a particular peak at a given retention time (± 0.05 min) from the total ion chromatogram (TIC) for the given sample. Subsequently, the data were fed into the SPSS 18.0.0 software (SPSS Inc., Chicago, Illinois, USA) and analysed for classification and dimension reduction features leading to dendrograms and orthogonal plots for HCA and PCA respectively.

Results and discussion

Classifying agarwood tissue and oils

GC-MS profiles of the three grades of agarwood oil were different (Figure 2 *a–c*). Multivariate statistical analysis (HCA and PCA) was performed with the GC-MS data by considering all discernible peaks in the TIC. In the HCA dendrogram, four individual clusters were combined to form two super clusters where the oils (*Boha* and *Boya* oils) grouped closely followed by *Khara* oil at a rescaled distance of 6 and resinous wood clustered at a distance of 17 from *Khara* oil. Non-resinous wood appeared as a monoclade at a distance of 18 units from the oils (Figure 2 *d*). In PCA two components were extracted and the plot showed grouping among the three oil grades (Figure 2 *e*). NIST library search was performed for the major peaks

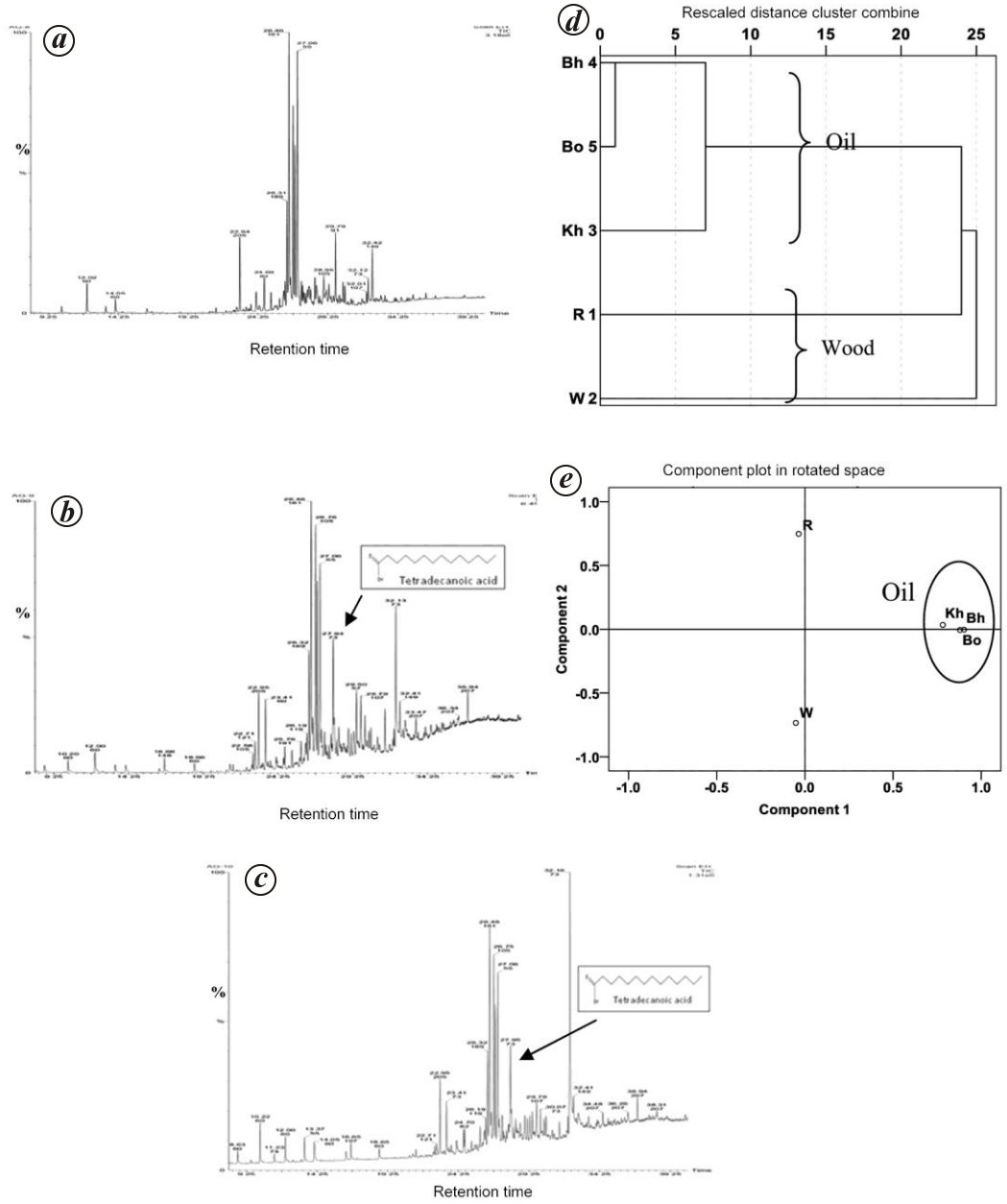


Figure 2. Difference in biochemical profiles of different grades of raw agarwood oil and tissue. Total ion chromatographs of three different grades of raw agarwood oil in Assam, viz. (***a***) *Khara* (Kh), (***b***) *Boha* (Bh) and (***c***) *Boya* (Bo), mentioned in decreasing order of commercial value are compared. (Note: tetradecanoic acid is a major aromatic component of *Boha* and *Boya* oils.) Dendrogram of hierarchical cluster analysis (***d***) and orthogonal plot of principal components analysis (***e***) for three different grades of raw agarwood oil and two distinct wood types (R, resinous; W, non-resinous) are also shown.

which identified a total of 50 different compounds in all the three oil samples. Among these, 11, 16 and 2 compounds were unique to *Khara*, *Boha* and *Boya* respectively, and 12 compounds were common in all the three oils (Table 2 and Figure 3). Chemical difference in the composition of agarwood oils can explain their physical and olfactory distinctness, which has a significant role in grading of the oils[14]. In the HCA dendrogram, *Boha* and *Boya* oils showed closer relatedness compared to *Khara* oil. A similar observation was noted in chemical classification of the oils, where totally 17 major compounds were common between *Boha* and *Boya* compared to 15 between *Khara* and *Boha* and 13 between *Khara* and *Boya* (Figure 3). It was observed that resinous wood clustered along with the oils, although at a significant distance. In the PCA plot the three grades of oil appeared to group together, whereas a weak correlation was observed between the oils and wood samples. In the sequence of events in nature, resinous wood is formed by resin deposition on non-resinous wood from which the oils are then

Table 2. Qualitative chemical composition of three grades of raw agarwood oils of Assam depicted by their major components

Compound	Molecular weight	Retention time	Compound registry no.[a]	Relevance to fragrance industry	Major component of oil grade[b]		
					Kh	Bh	Bo
Methyl beta-L-arabinopyranoside, methyl	164	8.62	ChemSpider ID: 92301	NA	–	+	+
Hexanoic acid	116	10.2	CAS no.142-62-1	Caproic acid; esters in fragrance industry	+	+	+
2-Methylheptanoic acid	144	11.23	CAS no. 1188-02-9	Flavour agent	–	–	+
Heptanoic acid	130	12	CAS no.111-14-8	Esters in fragrance industry	+	+	+
Cyclohexanecarboxylic acid	128	13.3	CAS no. 98-89-5	Shikimic acid	+	+	+
Octanoic acid	144	14.05	CAS no. 124-07-2	Esters in fragrance industry	+	+	+
Phenol-4-ethyl-	122	14.52	CAS no. 123-07-9	Fragrance industry	+	–	–
Nonanoic acid	158	16.29	CAS no. 112-05-0	Esters in fragrance industry	+	+	+
2-Methyl-3,5-dinitrophenyl-beta-phenylpropionate	330	16.63	PubChem CID 570487	NA	+	–	–
1,3-Cyclopentadiene,5,5-dimethyl-1-propyl-	136	16.64	PubChem CID 00572124	Floral volatile	–	–	+
N-decanoic acid	172	18.65	CAS no. 334-48-5	Flavour agent for food	+	+	+
Hentriacontane	436	19.77	CAS no. 630-04-6	Wax	–	+	–
5,6-Decadien-3-yne-5,7-diethyl-	190	20.99	PubChem CID 595131	NA	+	–	–
Undecanoic acid	186	21.03	CAS no.112-37-8	Fragrance industry	–	+	–
1,4-Methanocycloocta[D]pyridazine, 1,4,4A,5,6,9,10,10A-octahydro-11,11-11-dimethyl-, (1-alpha., 4-alpha, 4A-alpha, 10A-alpha)	204	21.24	CAS No. 88637-37-0	Diphenhydramine, Benadryl	–	+	–
Azulene,1,2,3,4,5,6,7,8-octahydro-1,4-dimethyl-7-(1-methylethenyl)-, [1S-(1-alpha,7-alpha, 8A beta)]-	204	21.23	CAS no. 3691-11-0	δ-Guaiene; fragrance industry	+	–	+
Azulene,1,2,3,3A,4,5,6,7-octahydro-1,4-dimethyl-7-(1-methylethenyl)-[1R-1-alpha,3A-beta,4-alpha,7-beta)]	204	21.95	CAS no. 22567-17-5	γ-Gurjunene; fragrance industry	+	–	–
Naphthalene,1,2,4A,5,6,8A-hexahydro-4,7-dimethyl-1-(1-methylethyl)-	204	22.58	CAS no. 483-75-0	Cadinene; fragrance industry	–	+	–
2-Butanone,4-(4-methoxyphenyl)-	178	22.71	CAS no. 104-20-1	Fragrance industry	–	+	–
2-(4A,8-dimethyl-1,2,3,4,4A,5,6,7-octahydro-naphthalen-2-yl)-prop-2-en-1-	220	22.94	PubChem CID 579791	Fragrance industry	+	+	+
5-(7A-isopropenyl-4,5-dimethyl-octahydroinden-4-yl)-3-methyl-penta-2,4-dien-1-ol	288	22.95	PubChem CID 5373093	Essential oil component	–	+	–
Dodecanoic acid	200	23.41	CAS no. 143-07-7	Lauric acid	–	+	+
Decahydro-8A-ethyl-1,1,4A,6-tetramethylnaphthalene	222	23.75	PubChem CID 579596	Essential oil component	+	+	–
2,4,4-Trimethyl-3-hydroxymethyl-5A-(3-methyl-but-2-enyl)-cyclohexene	222	24.11	PubChrm CID 550281	Essential oil component	+	–	–
Diethyl phthalate	222	24.62	CAS no. 84-66-2	Solvent/binder for fragrances; toxic	–	+	–
1-Formyl-2,2-dimethyl-3-cis-(2-methyl-but-2-enyl)-6-methylidene-cyclohexane	220	24.69	PubChem CID 5365987	Essential oil component	+	–	–
1,4-Dimethyladamantane [1-alpha, 3-beta, 4-beta, 5-alpha, 7-beta]-	164	25.17	PubChem CID 590907	Volatile organic compound	+	–	–
1,3A-Ethano(1H)inden-4-ol,octahydro-2,2,4,7A-tetramethyl-	222	25.18	CAS no. 62511-51-7	Essential oil component	–	+	–

(*Contd*)

Table 2. (*Contd*)

Compound	Molecular weight	Retention time	Compound registry no.[a]	Relevance to fragrance industry	Major component of oil grade[b]		
					Kh	Bh	Bo
4A,7-Methano-4AH-naphth[1,8A-B] oxirene,octahydro-4,4,8,8-tetramethyl-	220	25.79	CAS no. 67999-56-8	Essential oil component; antimicrobial	–	+	–
2-Naphthalenemethanol,1,2,3,4,4A,5,6,7-octahydro-alpha, alpha,4A,8-tetramethyl-, (2R-cis)-	222	26.3	CAS no. 1209-71-8	γ-Eudesmol; fragrance industry	+	+	+
Hinesol	222	26.45	CAS no. 23811-08-7	Fragrance industry	+	–	–
10S,11S-himachala-3(12),4-diene	204	26.75	CAS no. 60909-28-6	Fragrance industry	+	+	+
Agarospirol	222	26.87	CAS no. 1460-73-7	Fragrance industry	+	+	+
Naphthalene,1,2,3,4,4A,5,6,8A-octahydro-7-methyl-4-methylene-1-(1-methylethyl)-, (1-alpha,4A-beta,8A-alpha)-	204	26.88	CAS no. 39029-41-9	Fragrance industry	–	+	+
2*R*-Acetoxymethyl-1,3,3-trimethyl-4T-(3-methyl-2-buten-1-yl)-1T-cyclohexanol	282	27.06	PubChem CID 550401	Fragrance industry	+	+	+
Tetradecanoic acid	228	27.94	CAS no. 544-63-8	Myristic acid; fragrance industry	–	+	+
3,7,11-Trimethyl-3-hydroxy-6,10-dodecadien-1-yl acetate	282	28.96	PubChem CID 5366047	Essential oil component	–	+	–
2(1H)-Naphthalenone,3,4,6,7,8,8A-hexahydro-1,8A-dimethyl-7-(1-methylethenyl)-, (1R,7R,8AR)-	218	29.14	CAS no. 799813-25-5	Essential oil component	–	+	–
1-propyl-12-methyltetradecanoate	284	29.49	CAS no. 110-27-0	Isopropyl myristate; fragrance industry	–	+	–
Androstan-17-one, 3-ethyl-3-hydroxy-(5-alpha)-	318	29.78	PubChem CID 561925	Essential oil component; medicinal	+	–	–
Trichothec-9-en-8-one,12,13-epoxy-3,7,15-trihydroxy-, (3-alpha, 7-alpha)	296	29.79	CAS no. 51481-10-8	Fragrance industry	–	+	–
12-Bromododecanoic acid	278	30.05	Pubchem CID 175468	NA	–	+	–
2(3H)-Naphthalenone,4,4A,5,6,7,8-hexahydro-4,4A-dimethyl-6-(1-methylethenyl)-, [4R-(4-alpha, 4A-alpha, 6-beta)]-	218	30.33	CAS no. 4674-50-4	Nootkatone; essential oil component; fragrance industry	+	+	–
2(3H)-Naphthalenone, 4,4A,5,6,7,8-hexahydro-4A,5-dimethyl-3-(1-methylethylidene)-, (4AR-cis)-	218	30.46	PubMed CID 612721	Essential oil component; fragrance industry	+	+	–
Tetradecanoic acid,10,13-dimethyl-methyl ester	270	31.4	CAS no. 267650-23-7	Essential oil component; medicinal	–	+	–
2,6-Diisopropylnaphthalene	212	32.01	CAS no. 24157-81-1	Essential oil component	+	–	–
N-hexadecanoic acid	256	32.12	CAS no. 57-10-3	Essential oil component; fragrance industry	+	+	+
Dibutyl phthalate	278	32.4	CAS no. 84-74-2	Solvent/binder for fragrances; toxic	–	+	–
Methyl-3,9,11-guaiatrien-12-oate	246	32.41	PubChem CID 577319	NA	+	–	–
Methyl-12,15-octadecadienoate	294	36.94	ChemSpider ID: 20121591	NA	–	+	+

[a]Compound registry numbers are taken from Chemical Abstract Service (CAS), American Chemical Society, USA; PubChem Compound, National Institutes of Health, USA and ChemSpider, Royal Society of Chemistry, UK. [b]Oil grades. Kh, Khara; Bh, Boha; Bo, Boya. NA, Not available.

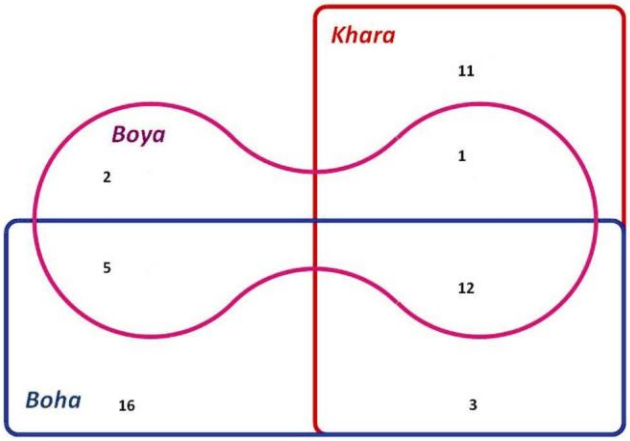


Figure 3. Venn diagram showing chemical relatedness of the three grades of raw agarwood oil, viz. *Khara*, *Boha* and *Boya* as revealed by their major chemical components (see Table 2).

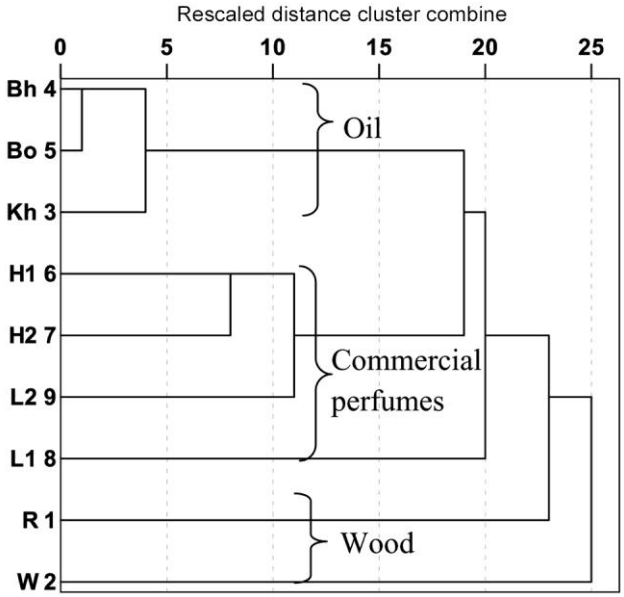


Figure 4. Contrast in biochemical profiles of commercially available perfume with different grades of agarwood oil. Hierarchical cluster analysis comparing three different grades of raw oil (*Khara* (Kh), *Boha* (Bh) and *Boya* (Bo)) and wood tissue (R, resinous and W, non-resinous) with a set of good (H) and inferior (L) quality commercial perfumes available in the local market.

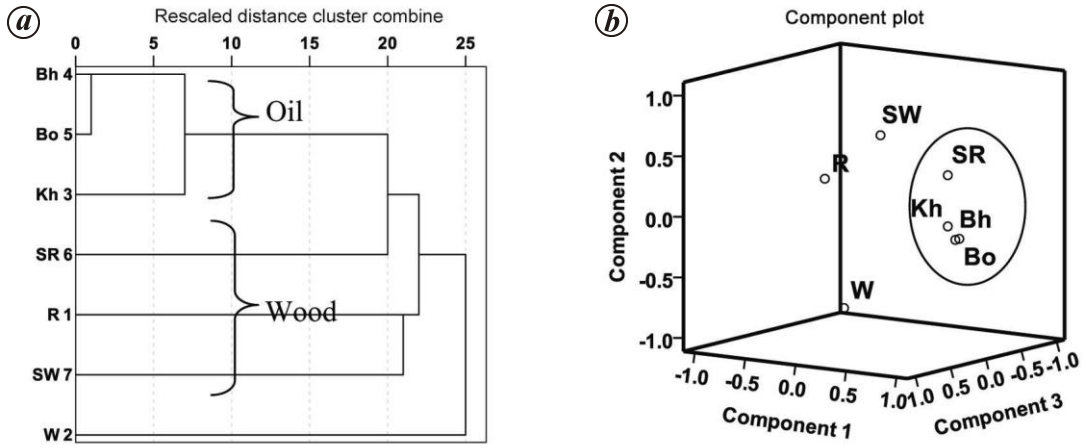
extracted by hydrodistillation after a fermentation step. Therefore, different degrees of chemical relatedness are expected to exist in different types of sample, which is reflected in their GC-MS profiles. Both HCA and PCA, therefore, can complement each other when applied to GC-MS-based metabolite profiling to differentiate agarwood oil grades effectively. *Khara*, *Boha* and *Boya* oils are the trade standard nomenclature for crude agarwood oil produced in traditional distilleries of Assam. At present, the grading is done based upon physical attributes of colour and odour. PCA can be applied to identify the critical components of a chemical mixture such as a particular grade of oil[15]. Considering only the major components of Khara, Boha and Boya oil (Table 2), simple PCA was able to show that by selectively removing single, groups or all the 11 distinct components of the superior Assam *Khara* oil from the analyses, the critical component(s) conferring the special aroma may be identified from the complex mixture (details of the study are available in Supplementary Figure S2 online). Geographical origin of agarwood oil is an important criterion in the international perfume market because distinct aromatic characters are attributed to region/country of origin of the oil. Few reports exist on classification of commercial grades of agarwood oil from Malaysia, Thailand and Bangladesh using different chemical profiling approaches[6–8]. With regard to Indian (Assam) agarwood, available reports are confined to chemical cataloguing of infected/resinous wood to be compared with uninfected tissue[9,10]. The fact that all discernible GC-MS peaks present in the TIC were considered for scoring during statistical analysis, a more intense representation of the chemical composition of the sample analysed was possible.

This was further validated when chemical profiles of commercial agarwood perfume (*agar attar*) samples were compared with the local grades of crude agarwood oil (*Khara*, *Boha* and *Boya*) and resinous and non-resinous wood tissue (Table 1). From the dendrogram (Figure 4), it is clear that differences exist between the crude and commercial oil samples as they clustered separately more than 15 rescaled distance units. However, it was interesting to note that the higher and more expensive farmer's grade perfumes of local origin (Hojai, Nagaon) showed proximity to the crude oils (*Khara*, *Boha* and *Boya*). The cheaper grades were found to cluster separately, where *Rooh-al-oudh* a cheap commercially sold *attar* was a significant outlier. Resinous wood weakly clustered with the oils, whereas non-resinous wood remained an outlier.

When it is challenging to classify, group or distinguish between commercial agarwood compounds, grades and products, a simple GC-MS-based multivariate statistical approach as reported here can be effectively applied. Considering the fact that adulterated and fake agarwood products are often pushed into the market, metabolite profile-based fingerprints (such as for different grades of raw Assam agarwood oil) can be useful for the perfume industry.

Fermentation of agarwood in water basins

Multivariate analyses of all the peaks of the GC-MS chromatograms of the laboratory-simulated fermentation of resinous and non-resinous wood were done with oils and unfermented resinous and non-resinous wood. After agglomerative HCA cluster combination, the seven different cases were represented in the dendrogram with the



c

	RT[a]	Sample types analysed[b]							Compound
		R	W	Kh	Bh	Bo	SR	SW	
1	26.75	–	–	+	+	+	+	–	10*S*,11*S*-Himachala-3(12),4-diene
2	26.87	–	–	+	+	+	+	–	Agarospirol
3	29.49	–	–	+	+	+	+	–	I-Propyl-12-methyltetradecanoate

[a]RT, Retention time (all discernible peaks considered); [b]R, Resinous wood; W, Non-resinous wood; Kh, *Khara* oil; Bh, *Boha* oil; Bo, *Boya* oil; SR, Resinous wood subjected to fermentation (1.5 months); SW, Non-resinous wood subjected to fermentation (1.5 months)

Figure 5. Understanding biochemical linkages during fermentation of agarwood. Hierarchical cluster analysis (***a***) and principal components analysis (***b***) of GC-MS data to compare chemical profiles of the fermented resinous (SR) and non-resinous (SW) wood soaked in water for 1.5 months with unfermented resinous (R) and non-resinous (W) wood and three grades of raw agarwood oil (*Khara* (Kh), *Boha* (Bh) and *Boya* (Bo)). Markings indicate the observed grouping. (***c***) The compounds formed during fermentation are tabulated.

crude agarwood oils clustering as before within a rescaled distance of 1–7 followed by fermented resinous, fermented non-resinous and unfermented resinous wood at distances of 13, 14 and 15 rescaled units respectively, from the oil samples (Figure 5 *a*). The non-resinous wood sample was a significant outlier. PCA plot for the same dataset reflected the grouping pattern of the oil samples followed by fermented resinous and non-resinous wood (Figure 5 *b*).

A comparison of the chromatographs revealed that three major peaks present in the fermented resinous wood were also present in the oils. They were 10S, 11S-Himachala-3(12), 4-diene, agarospirol and i-propyl-12-methyltetradecanoate, none of which interestingly is present in the unfermented resinous wood tissue (Figure 5 *c*). Agarospirol has been reported from agarwood oil but less commonly from agarwood tissue[2,3,8]. Another study reported that oxo-agarospirol could be detected in agarwood infected with fungi only at a later stage[16]. In the same study, it was hypothesized that stress compounds produced initially by plants in response to fungal infection were metabolized by fungi into fragrant molecules like oxo-agarospirol. Physical changes in the wood owing to prolonged soaking result in swelling that leads to softening of cell walls. The cellulose microfibrills in the cell walls become weak as stronger hydrogen bonds are formed between water and cellulose than between cellulose molecules[17]. This increases the surface area of activity within the wood tissue, particularly the sites of the resinous deposits which become freely accessible for microbial action. In the perfume industry the period of soaking of agarwood chips in water is carefully optimized as it is considered as a critical determinant of oil quality[18]. The present study shows evidence of possible correlation between the traditional fermentation step and the aromatic property of the fragrant agarwood oil.

Conclusion

A simplified metabolite profiling approach was found to be effective in studying the biomolecular linkages in the production process of agarwood oils. The metabolite profiles of resinous, non-resinous, crude oils and commercial products were distinguished. Here we have reported the role of fermentation in agarwood oil production. The aromatic compounds found in the agarwood oil such as 10S, 11S-Himachala-3(12), 4-diene, agarospirol and i-propyl-12-methyltetradecanoate were formed during the fermentation of agarwood. These findings will help improve the agarwood oil production process and may also be adopted for similar products which are formed due to plant–microbial interactions.

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