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Identification of Agarwood (*Aquilaria malaccensis*) Chips Incense Smoke and Headspace Volatile Compounds by GC-MS.EI.Q.TOF, SPME

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The new hybrid technique which combines gas chromatography and quadruple time of flight mass spectrometry (GC.MS.EI/Q.TOF) were used for the first time in screening and identification of multiple compounds of agarwood chipwood incense smoke and volatile headspace. To explore chemical constituent of *Aquilaria malaccensis* which result in the generation of a complex array of secondary metabolites, we described gas chromatography method, coupled to accurate mass time-of-flight mass spectrometry (Q.TOFMS.EI) for the untargeted and comprehensive chemical profiling of chipwood from naturally infected *A. quilaria malaccensis* trees. Chemical components of agarwood were extracted by applying a new technique; trapping smoke from burning incense and volatile headspace. The SPME.HS technique, solid phase microextraction (SPME) was employed and gas chromatography (GC.MS.EI/Q.TOF) were used as identification tools for the analysis. Divinylbenzene-carboxen-polydimethyl siloxane (DVB-CAR-PDMS) 50/30 µm fibre was used to extract, and analyze volatile compounds. In total, more than 550 peaks were detected, of which tentative identification of 28 of these compounds was reported, representing the major compounds that are mentioned in the literature of the oil studies. The total ion chromatogram detected compounds are distributed among a wide range over the chemical families. Furthermore, the feasibility of this methodology was achieved in grading agarwood chipwood by correlating the obtained chemical profiles of incense smoke and volatile headspace with the extracted oil profile from agarwood, is mentioned in detail; by the way, the major compounds found was represented by β-agarofuran, dihydro 1.27%, γ-cadinene 3.06%, α-bulnesene 3.68%, cadina-1(10),6,8-triene 3.96%, alloaromadendrene 2.09%, cadalene 3.93%, longifolene 2.43% and δ-cadinene 5.01%.

This work was one of the very few works that provided information about agarwood chipwood of *A. malaccensis* which represented a valuable data. Furthermore, the study revealed that the characterization of compounds from agarwood chipwood was highly possible through incense smoke and volatile compounds.

Key words: Agarwood; incense smoke; volatile headspace; solid phase microextraction GC-FID; GC-MS; Q.T.O.F; SPME

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Agarwood is a fragrant wood obtained from *Aquilaria* species (Thymelaeaceae) [1, 2, 3, 4]. Agarwood nominated by different way in different cultures (Chen xiang, gaharu, eaglewood, jinkoh, Kanankoh, Kyara, Qi-Nan) [1, 2, 4, 5]. There are nineteen species recognized in the taxonomical classification of genus thymelaeaceae up to date; the family is distributed from India to Burma,

Vietnam, Laos, Cambodia, and Malaysia; to Sumatra, Borneo, and Philippines [5, 4]. Not more than 10% of the trees in the natural forest of these countries contain agarwood and that refer to only eight species from the thymelaeaceae which can produce agarwood [6]. The deposits of the resin content of these trees only can be estimated by expert specialist after cutting down the tree and then quality of the deposit resin can be estimated [5]. The mechanism by which the resin is formed is still a mystery; many theory has been developed to explain the source of the resin formed in the heart of the wood which is known as agarwood. The famous assumption of these theories are thus the resin might be formed when the plant are injured by insects, physical cuts, bacterial infections or chemical stimulation [6]. The different part of the plants from *Aquilaria* species (thymelaeaceae) has been used in traditional medicine. It is used as drugs in folk remedies for treatment of fractures; as a sedative, analgesic and digestive, for contusion, and as treatment for cardiatonic, carminative, coughs and high fever as reported by Neaf [5]; furthermore as anaphylaxis at the same time [3, 4, 5, 7, 8, 9]. The classic way applied to grade agarwood chipwood was depending on human panel, like shape, odour, weight and resin content. No classification methods based on chemical constituent are available [1, 3, 4, 10]. Furthermore, all the reported methods are focused on the chemical constituent of the essential oil from agarwood. After the extraction process, chipwood was not taken to be classified according to its chemical constituents. Wong *et al.* [19] stated that the agarwood oil analysis revealed a wide range of chemical constituents belonging to the various family of chemical grouping, the majority among these families or group are sesquiterpenes alcohol, sesquiterpenes hydrocarbons, sesquiterpenes ketones, benzyl ketones and sesquiterpenes diol [16, 19]. In addition, Yamada [20] indicate that higher molecular weight terpenoids can be spread in the smoke compared to normal atmosphere because of the burning temperature. If the temperature was higher, more compounds arise from the burning and spread in the smoke [20]. Burning incense also produce acetaldehyde and formaldehyde because most materials produce aldehydes and ketones during combustion [16, 21, 22, 23]; this is to state that many of the chemical compounds that has been detected in the oil are not available in the volatile incense headspace and that due to the lower temperature of the extraction by SPME.

The rapid and simple technique of sample preparation SPME which was developed to be solvent-free, fast and applicable in various methods extractions has been used in this study to extract and analyse of agarwood chemical compouds that can be found in the incence smoke and volatile headspace [3, 4, 11, 12]. The method utilizes only a very small volume of sample compared to others [13, 14]. A 50/30 µm divinylbenzene-carboxen-polydivinylmethylsiloxane (DVB-CAR-PDMS) fibre was selected for extraction of a volatile compound of agarwood incense in this study. The SPME coupled with gas chromatography (GC) with FID and MS.EI/QTOF detector has been chosen in the determination and characterization of agarwood incense smoke and headspace volatile compounds, respectively.

The GC.MS.EI/Q.TOF basic principle is to determine an ion's mass-to-charge ratio (m/z) based on how long it takes to travel down the instrument's 'flight tube' to reach the detector. TOF MS come in a variety of configurations, including a single-stage time-of-flight mass spectrometer, a multi-stage TOF/TOF, and even a hybrid quadrupole-TOF (Q-TOF) instrument; the latter two enable MS/MS applications. Usually, time-of-flight mass spectrometers contain two detectors: a linear detector at the end of the flight tube, and 'reflector' detector, which captures ions that were redirected back towards the source by a 'reflection.' The reflection serves two purposes: correcting for dissimilarity in initial kinetic energy of identically sized molecules and extending the flight path, and improving resolution.

The GC.MS.EI/Q.TOF is a hybrid technique between GC and quadruple time of flight mass spectrometry GC-MS -Q.TOF. The GC.MS-Q.TOF involves the separation in one dimensional with standard chromatography column [13,15]. Recording full-spectral information using GC quadrupole MS (GC-MS) in scan mode can be successfully be used in identification of a large range of compounds in a sample, but it does not provide the required sensitivity for the identification of trace-level flavour compounds in agarwood by the same mean, selected ion

monitoring (SIM) conditions give the required sensitivity for trace level identification, but the main advantage its preclude the spectral information gathering. All the two negative sides above has been resolved by Q.TOF that provides the full spectral information of scan mode, but the sensitivity of SIM operation. Due to the high-resolution, sensitivity analysis of organic volatile compounds by time of flight gas chromatography GC-MS. EI -Q.TOF gain high peak capacity obtained with the combination of the high resolution. The method has been used for essential oils separations. Even the conventional GC methods of separation with the modern capillary column are offering high resolution with peak capacities but it failed to separate the sophisticated mixture of natural products complex [13]. This matter is resolved by the introduction of the new technique GC-MS. EI-GC chromatography coupled with quadruple time of flight mass spectroscopy GC-Q-TOF.

EXPERIMENTAL

A. malaccensis (agarwood) Sample

Three different samples of agarwood chips (high grade of *A. Malaccensis)* were collected from Kedaik Agarwood Sdn. Bhd., a well-known Malaysian agarwood supplier.

C7-C20 n-alkanes

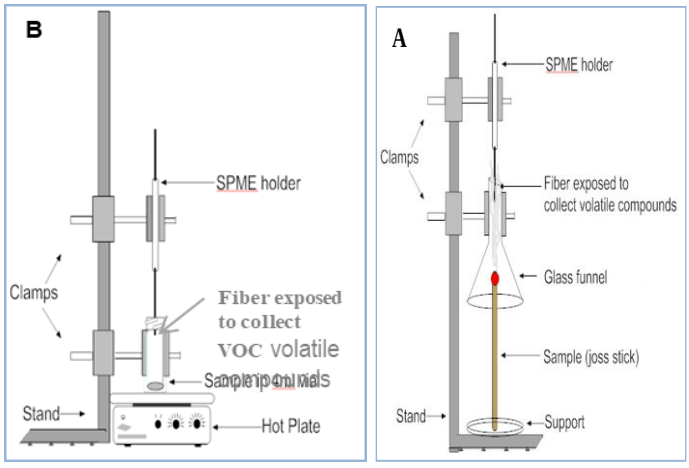
C7-C20 n-alkanes were supplied by Tokyo Chemical Industry Co., Ltd. (Toshima, Kita-ku, Tokyo).

Instrumentation

GC-FID. Chemical analyses were performed by gas chromatography-flame ionization detector (GC-FID) Agilent 7890B (Agilent technology, USA), equipped with DB-1 (100% dimethylpolysiloxane) capillary column, 30 m × 0.25 mm ID × 0.25 µm film thickness. The splitless mode was used with narrow SPME inlet liner at 220ºC injector temperature, carrier gas helium at 1.2 ml/min and 250ºC for both detectors temperature [3].

GC-MS.EI.Q.TOF. Sample separation was conducted by Agilent 7890A system in coupling with 7200 series quadrupole time of flight mass spectrometer (Agilent technology, USA), equipped with DB-1 (100% dimethylpolysiloxane) capillary column, 30 m × 0.25 mm ID × 0.25 µm film thickness. The MS transfer line temperature was 250ºC, carrier gas was helium (purity 99.999%) at flow rate 1.4 ml. min ¯1, oven temperature was programmed at 60ºC initially (held for 0.5 min) at 3ºC min to 100º C (hold for 3 min) then to 200ºC at 5ºC/min (hold for 3 min) and finally to 250ºC at 5ºC/min, injection temperature 200ºC, injection volume 1 µl, in spilitless mode. The Q.TOF system was used under total ion chromatogram (TIC) mode; temperature of ion source was 250ºC, ionization voltage was fixed at constant ionization energy of 70 eV. The mass range was 40-500 Dalton. Data acquisition and processing were done by Agilent Mass Hunter.

The SPME. Sampling apparatus was equipped with a 50/30 µm divinylbenzene-carboxen-polydivinylmethylsiloxane (DVB-CAR-PDMS) purchased from Supelco Inc., Bellefonte, PA, USA. This fibre was used to extract volatile compounds emitted from incense smoke and headspace volatile of agarwood, respectively. That SPME fibre was pre-conditioned at gas chromatography injection port using SPME narrow inlet for 30 min at temperature 200ºC before collecting the volatile compounds.



Sample collection.

Figure 1. SPME apparatus setup for sampling by (a) incense smoke and (b) volatile headspace [4].

Data handling. Data acquisition and processing was performed by Agilent MSD

ChemStation version 2.0 and Mass Hunter version B.06.00 Agilent technology; spectrum searching was performed with the National Institute of Standard and Technology 11 MS spectrum library.

RESULTS AND DISCUSSION

A total of 28 ions were identified in the sample of agarwood chipwood of *A. malaccensis* from Malaysia through the GC-MS.Q.TOF. The total Ion chromatogram detected compounds are distributed among wide range over the chemical families including monoterpenes and sesquiterpenes, hydrocarbons, oxygenated mono-terpenes and sesquiterpenes, norterpenoids, diterpenoids, short chain glycols, carboxylic acids and others. Furthermore, the study revealed that characterization of compounds from agarwood chipwood is highly possible through volatile incense sample. Compounds toluene, benzaldehyde and naphthalene appear in trace amounts in the results and has been reported by Ishihara *et al.* [16] and further conformed by Pripdeevech *et al.* [18], and others [4, 16, 17]. These are pyrolysis compounds among other compounds, especially in the smoke sample, the presence of these compound in the volatile headspace are rarely monitored when the analysis carried by normal GC or GC-MS so their presence in the incense volatile are less and that can be due to the increased temperature which has been applied during the burning of sample or oil extraction process which is usually by hydro-distillation. Some of the sesquiterpenes are also pyrolysis products from the resin as α-gurgujene, eremophila-1(10), 7(11)-dien-2-one and β-gurjunene among others compounds. These findings are also confirmed [16, 17]. Many of these pyrolysed form are also reported in his study for the oil of agarwood [3, 4, 16, 17, 18]. Beside that 2-butanone, 4-phenyl-β-agarofuran, dihydro-guaia-1(10), 11-diene are also presented in our study and has been confirmed by Ishihara's reports of agarwood smoke constituent [3, 4, 16]. Valencene-α-gurgujene-α-bulnesene also founded in this study and confirmed by Wong *et al*. [19]. In this works we confirmed also the presence of alpha–muurolen-gurjunene-spathulenol- (-)-aristo-lene- γ-gurjunene which has been confirmed in another paper reported by Nor Azah *et al.* [15]. Chemometric study of selected agarwood oils by GC–mass spectrometry which revealed agarwood oils showed concentrated volatile aromatic compounds mainly produced by the distillation of agarwood (*Aquilaria* sp.). At least 43 chemical compounds were identified including the above compounds [3, 4, 15]. From a report [25], we can notice the similarity in the compound identified during characterization of the chemical constituents of agarwood oils from Malaysia by comprehensive two-dimensional gas chromato-graphy time-of-flight mass spectrometry, and compounds that has been found in this research. We can confirm the presence of compounds α-cadinene–alpha-muurolen-aloaromadendrene-longifolene-δ-adinene. Also Tajuddin *et al.* [25] reported in 2010 the presence of the same compounds in our results. By the same time Naef [5] also confirmed the presence of compounds β-agarofuran and dihydro-α-bulnesene - benzalde-hyde - naphthalene [3, 4, 5, 24, 25].

In this study compounds benzaldehyde-2-butanone, 4-phenyl-α-cadinene-alpha, muurolen-β-agarofuran, dihydro-α-gurgujene-β-gurjunene-β-guaiene-spathulenol-valencene-γ-cadinene-bulnesene γ-gurjunene-δ-cadinene-α-copaene were founded in the samples under investigation and also confirmed by Ismail *et al.* [26] in the analysis of high quality agarwood oil chemical compounds by mean of SPME/GC-MS [3, 4, 26]. This strengthened our study that the high grade oil chemical constituents and the high grade chipwood of agarwood contained a lot of common compound in between. While compound β-patchoulene - α-cadinene - α-gurgujene - valencene - γ-cadinene - bulnesene - γ-gurjunene - alloaromadendrene - longifolene, were confirmed in reports on the enhancement of phytochemical production through *in vitro* polyploidization of agarwood-producing species, *A. malaccensis* [4, 27]. Compounds 2-butanone, 4-phenyl- β-agarofuran, dihydro - α-gurgujene - β-gurjunene- δ-cadinene in this work were confirmed in another research by Wong *et al.* [19]. They studied the metabolic profile of *A. malaccensis* by comprehensive two- dimensional GC, the work reveals the presence of compounds mentioned above [3, 4, 19]. While compounds cadina-1(10),6,8-triene - cadalene - cadin-1,3,5-trien-5-ol, which presented in the samples are multi-functional cadinene derivatives, unfortunately they are not reported in agarwood oil before, by the same time no available data handling the chipwood chemical constituents since Isihara *et al.* [16, 17], while compound eremophila-1(10),7(11)-

Table 1. Chemical compounds found in *A. malaccens* is sample 1 high grade by GC-MSEI.Q.TOF.l.

No	Compound label	RT	D. B. mass	Compound name	ID	Area %
1	Toluene	2.824	92.1384	Toluene	MS.Q.TO	0.22
2	Benzaldehyde	6.419	106.07545	Benzaldehyde	MS.Q.TOF/RI	0.29
3	Naphthalene	14.432	128.06014	Naphthalene	MS.Q.TO	0.18
4	2-butanone, 4-phenyl-	16.092	148.2017	2-Butanone, 4-phenyl-	MS.Q.TOF/RI	0.61
5	4,7-Methanoazulene, 1,2,3,4,5,6,7,8-octahydro-1,4,9,9-tetramethyl-, [1S-(1.alpha.,4.alpha.,7.alpha.)]-	26.108	204.3511	β-patchoulene	MS.Q.TOF/RI	0.28
6	α- cadinene;	33.329	204.1878	α- cadinene;	MS.Q.TOF	0.37
7	Alpha.-muurolen	26.863	204.3511	alpha.-Muurolen	MS.Q.TOF	
8	2H-3,9a-methano-1-benzoxepin, octahydro-2,2,5a,9-tetramethyl-, [3R-(3.alpha.,5a.alpha.,9.alpha.,9a.alpha.)]-	28.113	222.3663	β-Agarofuran, Dihydro	MS.Q.TOF/RI	1.27
9	1H-Cycloprop[3]azulene, 1a,2,3,4,4a,5,6,7b-octahydro-1,1,4,7-tetramethyl-, [1aR-(1a.alpha.,4.alpha.,4a.beta.,7b.alpha.)]-	28.279	66.04596	α-Gurgujene	MS.Q.TOF/RI	0.16
10	1H-cyclopropa[3]naphthalene, 1a,2,3,5,6,7,7a,7b-octahydro-1,1,7,7a-tetramethyl-, [1aR-(1a.alpha.,7.alpha.,7a.alpha.,7b.alpha.)]	28.407	78.04583	β-Gurjunene;	MS.Q.TOF/RI	0.2
11	Isolongifolene	30.14	202.3352	Isolongifolene	MS.Q.TOF	0.2
12	Beta.-guaiene	30.226	90.04515	.beta.-guaiene	MS.Q.TOF/RI	0.29
13	1H-cycloprop[e]azulen-7-ol, decahydro-1,1,7-trimethyl-4-methylene-, [1ar-(1a.alpha.,4a.alpha.,7.beta.,7a.beta.,7b.alpha.)]-	30.355	54.04608	Spathulenol	MS.Q.TOF/RI	0.34

14	γ-patchoulene	31.505	204.3511	γ-patchoulene	MS.Q.TOF/RI	0.2
15	(-)-aristolene	32.729	132.09166	(-)-Aristolene	MS.Q.TOF/RI	0.49
16	Naphthalene, 1,2,3,5,6,7,8,8a-octahydro-1,8a-dimethyl-7-(1-methylethenyl)-, [1R-(1.alpha.,7.beta.,8a.alpha.)]-	33.321	54.04605	Valencene	MS.Q.TOF/RI	0.87
17	Naphthalene, 1,2,3,4,4a,5,6,8a-octahydro-7-methyl-4-methylene-1-(1-methylethyl)-, (1.alpha.,4a.beta.,8a.alpha.)-	32.848	204.3511	γ- cadinene	MS.Q.TOF/RI	3.06
18	3H-3a,7-methanoazulene, 2,4,5,6,7,8-hexahydro-1,4,9,9-tetramethyl-, [3aR-(3a.alpha.,4.beta.,7.alpha.)]-	33.028	204.3511	Cyperene	MS.Q.TOF/RI	0.25
19	Azulene, 1,2,3,5,6,7,8,8a-octahydro-1,4-dimethyl-7-(1-methylethenyl)-, [1S-(1.alpha.,7.alpha.,8a.beta.)]-	33.58	66.04602	Guaia-1(10),11-diene; α-bulnesene	MS.Q.TOF/RI	3.68
20	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.)]-	34.286	66.04596	γ-gurjunene	MS.Q.TOF/RI	0.57
21	Cadina-1(10),6,8-triene	34.906	202.3352	Cadina-1(10),6,8-triene	MS.Q.TOF	3.96
22	Eremophila-1(10),7(11)-dien-2-one	35.113	203.1765	Eremophila-1(10),7(11)-dien-2-one	MS.Q.TOF	0.14
23	Alloaromadendrene	35.391	50.01479	Alloaromadendrene	MS.Q.TOF/RI	2.09
24	Naphthalene, 1,6-dimethyl-4-(1-methylethyl)-	35.649	198.3034	Cadalene	MS.Q.TOF	3.93
25	Longifolene-	36.006	54.046	Longifolene	MS.Q.TOF/RI	2.43
26	1-naphthalenol, 5,6,7,8-tetrahydro-2,5-dimethyl-8-(1-methylethyl)-	37.134	218.3346	Cadin-1,3,5-trien-5-ol	MS.Q.TOF	0.18
27	Naphthalene, 1,2,3,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-, (1S-*cis*)-	37.159	204.18280	δ- cadinene	MS.Q.TOF	5.01
28	α.-copaene	37.388		α.-copaene	MS.Q.TOF	0.87

dien-2-one is eremophilane, highly conjugated ketones. Eremophilane compound is famous in agarwood oils and has been reported in most of the studies done. Compound eremophila-1(10),7(11)-dien-2- one was also not mentioned in the literature before [4].

CONCLUSIONS AND OUTLOOK

In this study GC-MS.EI/Q.TOF and GC-FID were used successfully for the identifications of chemical compounds in volatile incense of high grade agarwood chipwood. Compared to the results obtained in agarwood incense with the data from the previous studies, there was a similarity between major groups, viz., sesquiterpene and oxygenated sesquiterpene in both of them. The data obtained confirmed the eligibility and feasibility of the developed method here for the quality identification of agarwood chipwood so it could be used as quality tools before further extraction process.

The GCMS.GCFID seems to be the universal method of analysis of agarwood chemical constituent. The results from GCMS.EI/Q.TOF indicated that the complexity of agarwood could be used as a power tool to resolve and overcome such complex profiles. SPME could be the key in resolving the aromatic profile of agarwood since it showed great capacity and accuracy in its analysis. Major agarwood compounds from different species consisting of different chemical groups such as monoterpene and sesquiterpenic hydrocarbons, oxygenated monoterpenes and sesquiterpenes (comprised of ketone, aldehyde, oxide, alcohols, lactone, keto-alcohol and diol), norterpenoids, diterpenoids, short chain glycols, carboxylic acids and others but at the same time each family has certain features of compound which could be considered as a signature of that species to make differential profile from other species of chromone.

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