Chromatographic fingerprint analysis of metabolites in natural and artificial agarwood using gas chromatography–mass spectrometry combined with chemometric methods

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ARTICLE INFO

ABSTRACT

Agarwood is a resinous material formed in wounded *Aquilaria sinensis* in China, which is widely used as an effective traditional Chinese medicine (TCM). This study is aimed to use gas chromatography–mass spectrometry combined with chemometric methods to create reliable criteria for accurate identification of natural agarwood and artificial agarwood, as well as for quality evaluation of artificial agarwood. Natural agarwood and artificial agarwood (stimulated by formic acid or formic acid plus fungal inoculation) were used as standards and controls for the gas chromatography–mass spectrometry (GC–MS) and multivariate analysis. The identification criteria developed were applied to commercial agarwood. A reliable criteria including correlation coefficient of GC–MS fingerprint of natural agarwood and 22 markers of metabolism in natural and artificial agarwood was constructed. Compared with chemically stimulated agarwood (formic acid) and in terms of the 22 markers, artificial agarwood obtained by formic acid stimulation and fungal inoculation were much closer to natural agarwood. The study demonstrates that the chemical components of artificial agarwood obtained by comprehensive stimulated method (formic acid plus fungal inoculation) are much closer to the natural agarwood than those obtained by chemically stimulated method (formic acid), as times goes by. A reliable criteria containing correlation coefficient of GC–MS fingerprint of natural agarwood and 22 metabolism markers can be used to evaluate the quality of the agarwood. As an application case, three samples were identified as natural agarwood from the 25 commercial agarwood by using the evaluation method.

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1. Introduction

Aquilaria trees (Thymelaeaceae), a kind of large evergreen trees, distributed in south and south-east Asia, such as Bangladesh, Bhutan, India, Indonesia, Iran, Malaysia, Myanmar, Philippines, Singapore, Thailand, and China [1–4], is the main plant species for the production of agarwood (also called Chen-Xiang, agar, aloeswood, eaglewood, gaharu, or kalamabak, depending on the region). Agarwood is the most highly valuable resinous wood used as a digestive, sedative and antiemetic drug, and is also popular as incense and perfume in the world market [5–9]. Agarwood may form in the stem, branch or root of *Aquilaria* and *Gyrinops* trees, naturally infected by a variety of fungi including *Aspergillus* spp., *Botryodyplodia* spp., *Diplodia* spp., *Fusarium bulbiferum*, *F. laterium*, *F. oxysporum*, *F. solani*, *Penicillium* spp. and *Pythium* spp. [10–13] and naturally wounded by wind, lightning strikes, the gnawing of ants or insects. But these natural processes always develop very slowly over decades [14–16]. Owing to demand and commercial value, trade in agarwood has intensified in recent years, and wild *Aquilaria* forests have been destroyed in almost all countries.

For the protection of wild *Aquilaria* resources and their sustainable use, all *Aquilaria* spp. have been listed in Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) [17]. Comptom and Ishihara reported in TRAFFIC International that poor forest management of *Aquilaria* trees has been caused by the transition from ethnic merchants who controlled the felling method to more opportunistic and destructive harvesting methods [18]. And some countries such as China,

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India, Vietnam, Indonesia, Malaysia and Thailand have drawn much attention in *Aquilaria* cultivation to produce agarwood.

Aquilaria sinensis (Lour.) Gilg, the main plant resource in China for agarwood, is chiefly distributed in South China [19]. More than 2000 acres of *A. sinensis* trees are now widely cultivated in Hainan, Fujian, Guangxi, and Guangdong provinces, more than one-fourth of which are over 5 years older and can be used for agarwood induction. The common agarwood-inducing methods now used in China and Southeast Asian countries include trunk wounding by a knife cut and hammering nails into tree trunks. A formic acid or formic acid plus fungal infected combined method has also been developed recently [20–22].

The sesquiterpenes and phenylethyl chromone derivatives are the main compounds in agarwood [16,23–27]. Meanwhile, some studies have been carried out to compare the quality of artificial and natural agarwood. Tamuli and Bhuiyan studied the quality of agarwood (*A. agallocha* Roxb.) formed through fungus infection by gas chromatography–mass spectrometry (GC–MS) [28,29]. Yu et al. identified lignum *Aquilariae resinatum* Qinan from four species by FTIR spectrum [30]. Commercial agarwood and similar products have been evaluated by characteristics, extract, and color reaction [31]. Three Chinese agarwood (*A. sinensis*) samples produced by the methods of nail insetting, holing, and trunk breaking have been evaluated through GC–MS [32]. Lancaster et al. evaluated the quality of agarwood products for 2-(2-phenylethyl) chromones using direct analysis in real time/time-of-flight mass spectrometry [33]. Espinoza et al. distinguishing wild from cultivated agarwood (*Aquilaria* spp.) using direct analysis in real time and time of-flight mass spectrometry [34].

In this study, artificial method of formic acid or formic acid plus fungal inoculation (*Fusarium* sp., *Hypocrea jecorina*, *Botrysphaeria rhodina*, and *Nigrospora oryzae*, were isolated and identified from *A. sinensis*) was selected to stimulate *A. sinensis* tree. The qualities of these artificial agarwood together with natural agarwood were tested and evaluated. A chromatographic fingerprint method (GC–MS) was used to assess the quality of natural and artificial agarwood. XCMS data set was produced by XCMS package [29] working on R-gui. Principal component analysis (PCA) [35,36] and orthogonal partial least-squares discriminant analysis (OPLS-DA) [37,38] were employed to find potential markers, and automated mass spectral deconvolution and identification system (AMDIS) [39] was then used to identify the chemical markers. An accurate classification model for various agarwood samples was thus established.

2. Experimental

2.1. Materials and chemicals

Sixty-two agarwood samples (Table 1) were selected and analyzed. Samples were analyzed in triplicate. Ten batches of natural agarwoods were collected from wild *A. sinensis* plant by Xinyi City Rare Aloes Development Co., Ltd (Guangdong Province, China), and identified as *A. sinensis* by Prof. Yan (College of Traditional Medicine, Guangdong Pharmaceutical University).

A method of chemical stimulation or chemical stimulation plus fungal inoculation was used to induce the formation of resinous in 5-year-old *A. sinensis* trees, which were planted around a farm of Xinyi suburban district of Guangdong province, China. *Fusarium* sp. A2, *Nigrospora oryzae* A8, *Botrysphaeria rhodina* A13, and *Hypocrea jecorina* M71 [40] were selected to inoculate *A. sinensis* trees. The fungal strains were isolated from *A. sinensis* (Xinyi, Guangdong Province, China) [40] and preserved at the Guangdong Provincial Key Laboratory of Microbiol Culture Collection and Application, Guangdong Institute of Microbiology. The trees were

Fig. 1. A wood sample collected from trunk of *A. sinensis* based on formic acid stimulation combined with *Botrysphaeria rhodina* A13 inoculation (F1, Table 1) (A). The dark brown resins of artificial agarwood were further sectioned based on color characteristics (B).

approximately 3–4 m high, larger than 10 cm in diameter, and distances between every two trees were 50–70 cm. A drilling device was used to make holes with 0.5 cm in diameter and 4–5 cm depth in the trunks of trees at a height of 1 m. 1% formic acid or 1% formic acid following by fungal liquid fermentation product was injected slowly into the xylem part of the tree, which can stimulate the tree to produce resinous [21]. The agarwood was then harvested with not less than 12 months after agarwood induction. The dark brown resins of artificial agarwood was collected (Fig. 1A and B).

Twenty-five batches of commercial agarwood were purchased from Qingping medicinal herbs market (Guangdong Province, China), and denoted by S in Table 1.

Chloroform (purity > 99.0%) were purchased from Guangzhou Chemical Reagent Factory (Guangdong Province, China). Alkane standards (C_{10}–C_{31}) were purchased from AccuStandard Inc. (USA).

2.2. Sample preparation

All samples were dried at room temperature, subsequently cut into small pieces, and then filtered by 40-mesh sieves. The powder samples of agarwood (0.5 g) were extracted with chloroform (10 mL, 24 h) at room temperature. The solvent was evaporated by water bath (80 °C) to obtain viscous semi solid masses, which were then reconstitute to 2 mL of chloroform and stored in a dark and air-tight sealed vial at 4 °C and then 1 μl of each sample was used for GC–MS analysis.

2.3. Apparatus and chromatographic conditions

GC–MS analysis were performed using a GCMS QP-2010E (Shimadzu) and equipped with a Rtx-5MS (Restek Corp. Bellefonte, USA) capillary fused silica column (30 m × 0.25 mm I.D. × 0.25 μm film thickness). The oven temperature program initiated at 90 °C,