Supercritical Extraction Technique of Agarwood Essential Oil Induced by Plant Hormones

Zhihong Pan,[a] Xian Wang,[b] Qingde Li,[c,*] Jichang Li,[d] and Haikun Wang [e]

Samples of agilawood (agarwood), which were studied in this work, were produced in Zhongshan City, Guangdong Province, China. To enhance incense production, a specific concentration of plant hormone is employed for induction. The extraction technology of agilawood essential oil was explored using supercritical carbon dioxide fluid, which exhibited a more pronounced induction effect. The pressure, temperature, and flow rate, respectively, were 8, 16, and 24 MPa; 35, 45, and 55 °C; and 20, 30, and 40 L/h. A Box-Behnken analysis was adopted for experimental data, which involved 33 experiments. The data were fitted with the equation $Y = 2.18 + 0.1312X_1 - 0.025X_2 + 0.1236X_3 - 0.0025X_1X_2 - 0.0125X_2X_3 + 0.0175X_{12} + 0.035X_{22} - 0.1275X_{32}$. Hence, the optimal process parameters in the supercritical extraction of agarwood essential oil were as follows: the pressure, temperature, and flow rate of 24 MPa, 35 °C, and 33 L/h, respectively. An analysis was conducted with the statistical analysis software Design-Expert 11, which indicated that the extraction yield of agarwood essential oil by supercritical carbon dioxide was mainly affected by the pressure and flow rate. The yield was proportional to the pressure and flow, and inversely proportional to the temperature.

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Keywords: Agarwood essential oil; Supercritical extraction; Box-Behnken regression analysis; Design-Expert 11

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INTRODUCTION

The *Aquilaria sinensis* tree holds medicinal significance and is officially recognized in China's Pharmacopoeia as the agilawood tree, which is also known as agarwood. However, due to the declining availability of wild agilawood resources and the increasing market demand, there has been rapid progress in the development of artificial cultivation technology for agilawood and artificial incense production. Nevertheless, the industry still faces challenges related to low incense production and yield.

In the field of wood discipline, a novel incense production method using plant hormone induction has been introduced, surpassing the traditional non-mechanical and microorganism induction methods. This cutting-edge technique has garnered significant scientific attention. Specifically, the *Aquilaria sinensis* tree is induced to produce agilawood through the application of plant hormones. Through observation, the cumulative formation process of the main compounds involved in incense production from the

Aquilaria sinensis tree has been studied, and the chemical composition of agilawood produced via plant hormone induction has been analyzed. Additionally, extensive research has been conducted on the systematic extraction of supercritical agilawood essential oil.

With the increasingly serious environmental issues, green industry is gradually becoming the focus for development of industrial processes, so non-polluting solvents that are harmless to humans have replaced previous organic solvents (Chen *et al.* 2022). At present, new alternative solvents are usually adopted in the supercritical fluid technology, such as water and carbon oxide. Supercritical carbon dioxide has been found useful for the extraction of natural products, and its solubility is related to its density, pressure, and temperature.

Supercritical fluid technology could reduce damage to the environment, as it is non-toxic and harmless. It could reduce the waste discharge to a certain extent. It is often used for the isolation and purification of materials, preparation of micro and nano particles, preparation of special functional products, wafer surface cleaning, replacing the previous organic solvent, food processing, extraction, and other technical fields (Dahham *et al.* 2015; Xiao *et al.* 2021). Hence, supercritical fluid technology was selected for preparing agarwood essential oil. The extraction process is carried out under high pressure, so it has higher requirements of pressure resistance for the whole tubing system of the device. The microcomputer automatic monitoring is being used in the production process, which could greatly improve the safety and reliability of the system and decrease the operation costs. Plant essential oil is extensively applied in different fields, and its application is preferred due to the advantages of safety and other factors (Hashim *et al.* 2014; Hidayat *et al.* 2021; Wigati *et al.* 2022). Essential oil could be divided into the categories of single essential oil and compound essential oil, and is mainly composed of terpenes, aldehydes, esters, alcohols, and other chemical components. The refining and preparation of essential oil mainly refers to the extraction and production of a part of the plant (such as a seed, petal, leaf, bark, *etc.*). Different essential oils have different effects due to different chemical components (Hashim *et al.* 2021; Kao *et al.* 2021; Zanan *et al.* 2022). The common preparation methods of plant essential oil include water evaporation, pressing, freezing compression, immersion, solvent extraction, and supercritical fluid extraction (Yoswathana 2013; Dahham *et al.* 2015; Arumugham *et al.* 2021). There are no relevant reports on essential oil extraction with supercritical carbon dioxide. In this study, the authors used supercritical carbon dioxide to extract agarwood essential oil.

EXPERIMENTAL

Materials

Aquilaria sinensis is a member of the *Aquilaria* genus within the Thymelaeaceae family, and it is an exclusive tree used for incense production in China. It is primarily found in regions such as Hainan, Guangdong, Guangxi, Fujian, Taiwan, and Yunnan.

The experimental materials for this study were derived from the *Aquilaria sinensis* trees at the Innovation Training Base of Postgraduates located in Guangdong Zhongshan Southwest Forestry University. These selected trees are situated within the geographical coordinates of northern latitude 22° 11’ to 22° 47’ and eastern longitude 113° 09’ to 113° 46’. The trees grow at an altitude of 11 meters and have a tree age of 7 years. The diameter at breast height for these trees ranges from 7.6 to 10.8 centimeters.

Experimental Methods

Hormone induction reagent processing

For this experiment, 18 *Aquilaria sinensis* trees were carefully selected as the test subjects and divided into three distinct processing groups, namely Group A, Group B, and Group C. The hormone induction reagent used for each group varied as follows: Group A: The trees in this group were treated individually with methyl jasmonate. Group B: The trees in this group underwent a treatment where methyl jasmonate and ethephon were mixed in a volume ratio of 1:1. Group C: The trees in this group were treated individually with ethephon. In all three groups, different concentrations of the reagent were used, specifically 0% (control group), 1%, 2%, and 5%. Each group consisted of 6 trees, and the specific processing method applied to each tree is illustrated in Fig. 1. To determine the dosage of the reagent for coating, the size required for the application was standardized to 4 cm in length, 4 cm in width, and 3 mm in thickness. A slid-walk microtome was used to slice the sample, and then the slices were observed with Nikon 80i biological microscope. The slices were photographed with Nikon 80i built-in software to measure the area of dark secondary metabolites. Table 1 outlines the corresponding reagent preparation based on the dosage calculations, and a thickness gauge, conforming to the specified size, was employed for uniform coating during the experiment.

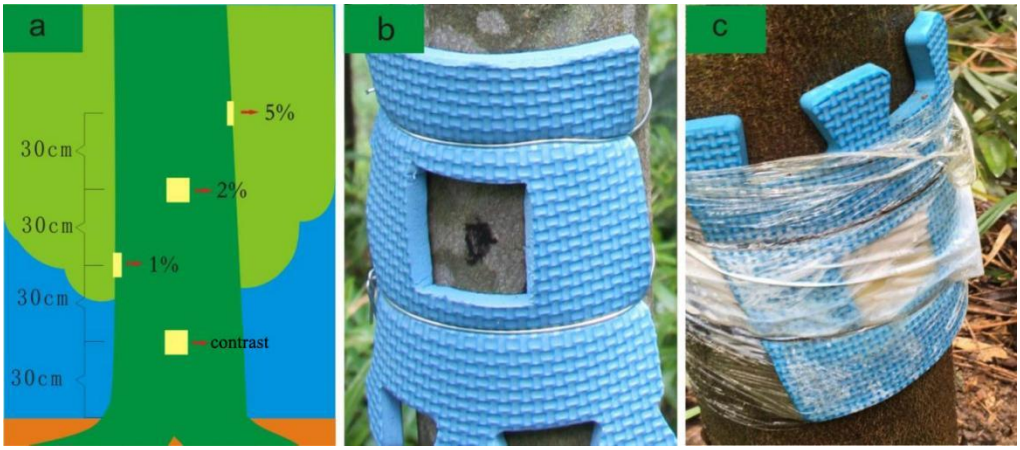

Fig. 1. Sketch map of chemical treatment (a) and picture of treatment in the scene (b) (c)

(1) On May 1, 2020, the experiment commenced by selecting a relatively flat area on the surface of each tree trunk to apply the corresponding reagent using a thickness gauge. The reagent was applied at intervals of more than 20 cm between two adjacent treatments, and each application was staggered transversely by approximately 90°. The starting treatment position was approximately 30 cm above the ground. The 18 tree trunks (No. 1-18) were subjected to different concentrations of reagents, including 0% (control group), 1%, 2%, and 5%, arranged from the lowest to the highest concentration. Specifically, tree trunks No. 1-6 were treated with 0%, 1%, 2%, and 5% Me-JA (methyl jasmonate) from lowest to highest concentration, while tree trunks No. 7-12 received the mixed reagent (1:1 volume ratio of Me-JA and Et (ethephon)) from lowest to highest concentration. Tree trunks No. 13-18 were treated with 0%, 1%, 2%, and 5% Et reagent (ethephon) from lowest to highest concentration. The lower part of each tree trunk was used as the control group and treated only with lanolin. The processing parts were not subjected to repeated irritation during the experiment.

Table 1. Calculation of Chemical Quality in Every Group

Sample tree No.	Treatment reagent	Concentration	MeJA (g)	1.3 times MeJA(g)	*4	ET (g)	1.3 times ET(g)	*4	Lanolin (g)	1.3 times Lanolin (g)	*4	Gross weight (g)	1.2	1.3 times total weight (g)
1-2	Me-JA	1%	0.048	0.0624	0.2496	0	0	0	4.752	6.1776	24.7104	4.8	5.76	6.24
3- 4	Me-JA	2%	0.096	0.1248	0.4992	0	0	0	4.704	6.1152	24.4608	4.8	5.76	6.24
5- 6	Me-JA	5%	0.24	0.312	1.248	0	0	0	4.56	5.928	23.712	4.8	5.76	6.24
7- 8	Me-JA&ET	1%	0.024	0.0312		0.024	0.0312	0.1248	4.752	6.1776	24.7104	4.8	5.76	6.24
9- 10	Me-JA&ET	2%	0.048	0.0624	0.2496	0.048	0.0624	0.2496	4.704	6.1152	24.4608	4.8	5.76	6.24
11- 12	Me-JA&ET	5%	0.12	0.156	0.624	0.12	0.156	0.624	4.56	5.928	23.712	4.8	5.76	6.24
13- 14	ET	1%	0	0	0	0.048	0.0624	0.2496	4.752	6.1776	24.7104	4.8	5.76	6.24
15- 16	ET	2%	0	0	0	0.096	0.1248	0.4992	4.704	6.1152	24.4608	4.8	5.76	6.24
17- 18	ET	5%	0	0	0	0.24	0.312	1.248	4.56	5.928	23.712	4.8	5.76	6.24
1- 18	Lanolin (control)	0%	0	0	0	0	0	0	4.8	6.24	24.96	4.8	5.76	6.24

The higher part of each tree trunk, treated with 1%, 2%, and 5% reagent, constituted the experimental group. For tree trunks with even numbers (No. 2, 4, 6, 8, 10, 12, 14, 16, and 18), the reagent was replaced, and the original reagent was replaced with the same concentration about 60 days and 120 days after the initial processing.

(2) On June 25, 2020, the reagent on the tree trunks in the experimental group with even numbers was gently scraped off to prevent damaging the surface of the Aquilaria sinensis. Subsequently, the same concentration, composition, and size of reagent was reapplied to the same parts. Specifically, for tree trunks No. 2, 4, and 6, the 1%, 2%, and 5% Me-JA reagent at higher positions was replaced. For tree trunks No. 8, 10, and 12, the 1%, 2%, and 5% Me-JA and Et reagent at higher positions was replaced. Finally, for tree trunks No. 14, 16, and 18, the 1%, 2%, and 5% Et reagent at higher positions was replaced.

(3) On August 20, 2020, the same reagent replacement steps were repeated as done previously in the experiment.

Supercritical extraction method

The supercritical extraction equipment (SC5+1, Deyang Sichuang Technology Co., Ltd., Sichuan, China) was selected for extraction of agarwood essential oil under the supercritical carbon dioxide (Ma *et al.* 2021). The device mainly is composed by a CO_2 cylinder, a refrigeration device, a temperature control system, a safety protection device, a carrier tank, a storage tank, a purifier, a mixer, a heat exchanger, a plunger pump, an extraction cylinder, a separator, and a mass flowmeter.

The experimental procedure consisted of three parts: 1. Crushing the raw material of agilawood wood; 2. Supercritical fluid extraction; 3. Calculating the essential oil yield.

Fig. 2. The experimental procedure for extraction of agarwood essential oil

The experimental procedure is shown in Fig. 2. The ultrasonic vibrator (JP-070S, Kunshan Yinghua Electronic Equipment Co., Ltd., Jiangsu, China) was selected for impurity separation and extraction of the agilawood sample (Yuanyi Agilawoods Co., Ltd., Zhongshan, China). After preparing 60-mesh agilawood powder, it was placed in the dry extraction device for supercritical fluid extraction. Then, the pressure reducing valve of carbon dioxide cylinder was turned on (Xinwang Gas Co., Ltd., Wenzhou, China), with the purity > 99.5%. Thereafter, the centrifugal air compressor (CHORUS90, Denair Energy-saving Technology Co., Ltd., Shanghai, China) was used to compress carbon dioxide and push it forward with agilawood (Waluyo *et al.* 2021; Wigati *et al.* 2022). Finally, the system was heated by the temperature controller vacuum oven (HJ-ZK-2, Huajie Oven Manufacturing Co., Ltd., Jiangsu, China), to increase the temperature of carbon oxide. When the critical pressure was reached, the heating was continued while maintaining the pressure, to reach the critical temperature and state. During this process, the UV-Visible detector (The agilawood molecule was scanned and analyzed using a Cary50, Spectrophotometer/G27-200035, Agilent Technologies Australia Pty Ltd., Mulgrave, Australia) to obtain an accurate critical value, while maintaining the time and flow rate to obtain the extracted agarwood essential oil. Finally, the extracted agarwood

essential oil was weighed with the electronic balance (AND/HR-200, Mettler-Toledo (Switzerland) GmbH, Greifensee, Switzerland) to determine the essential oil yield.

The Box-Behnken method in the Design-Expert 11 (Stat-Ease Inc., Minneapolis, MN, USA) software was selected for experimental design and data processing. An orthogonal experimental design method was used to study the multiple factors and levels. Based on the orthogonality, some representative points were selected from the comprehensive test to perform the test in Design-Expert 11, and these points are featured as the "uniform and distributed, neat and comparable properties".

The agarwood essential oil extraction protocol was designed with Design-Expert 11 for the orthogonal test, and the test was controlled by the single factor to further process data. The experimental time was 2 h for supercritical fluid extraction and the sample of agilawood was in powder form, passing through 60-mesh sieve. In this study, 60 g sieved powder was weighed, the pressure, temperature and flow rate were set to 8, 16, and 24 MPa; 35, 45, and 55 °C; and 20, 30, and 40 L/h, respectively, for the numerical cross-over experiment. Then, the amount and purity of agarwood essential oil were compared to obtain the optimal extraction value of agarwood essential oil.

Table 2. All-factor Level

Coding and Level	Pressure (kPa)	Temperature (°C)	Flow Rate (L/h)
-1	8	35	20
0	16	45	30
1	24	55	40

RESULTS AND DISCUSSION

Analysis of Anatomic Construction

The anatomic construction of *Aquilaria sinensis*, in the absence of incense production (lanolin processing sample), was examined. The cell structure of *Aquilaria sinensis* includes the presence of phloem, and the parenchymatous tissue contains a significant amount of starch, which serves as a distinguishing feature. The healthy wood of *Aquilaria sinensis*, associated with incense production, exhibits slight apparent growth profiling and displays a slightly yellowish-white color, serving as macro features. When observed under a magnifier, the fresh wood has a subtle glossiness, emits a mild fragrance, and becomes sweet upon drying. Additionally, it features slightly smaller tube holes.

In the radial section of the wood, ray stripes are evident, and the texture appears straight, representing the microscopic feature of the healthy wood of *Aquilaria sinensis* without incense production, as depicted in Fig. 3. The cross-section of the guide tube may take the form of circular, oval, or elliptical shapes. As for the diffuse-porous woods, solitary pores and short-diameter multiple pores (2~4), and pore clusters are occasionally observed, with simple perforation in circular and oval shapes. The pit-type column lies between the pipes, and there is an absence of tylose and vegetable glue in the guide pipe. Axial parenchyma is limited, and oil cells are not present. The wood fiber wall is thin and contains numerous bordered pits. The wood ray does not show a storied arrangement and primarily focuses on the linear ray, ranging in height from 1 to 21 cells or more. Occasionally, multi-column rays with a width of 2 cells may also be observed. The ray tissue exhibits the special-shaped III-type and few heterotype III.

The presence of gums in the ray cells is limited, and there are no crystals, oil cells, or mucous cells seen. The wood includes a significant amount of phloem, which is visible to the naked eye, appearing in an island-like pattern and being evenly distributed in the basic tissues. The tree's skin covers the secondary phloem, cortex, and periderm, with the secondary included phloem encompassing the bast fiber and phloem parenchyma cells.

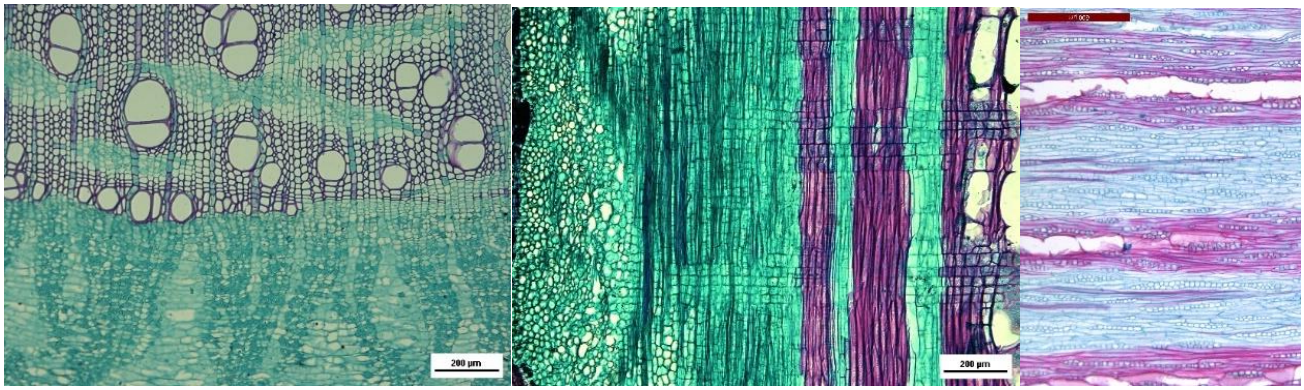

Fig. 3. The anatomy pictures of healthy *A. sinensis*
Note: Cross section on the left, radial section in the middle, tangential section on the right.

The anatomical structure analysis of hormone-treated and lanolin-controlled processing samples indicates that no apparent dark secondary metabolites were formed in the hormone control processing samples. However, *Aquilaria sinensis* is capable of forming dark secondary metabolites through two types of hormone treatments. The type and structure of cells did not exhibit significant changes, as shown in Fig. 3.

After applying methyl jasmonate on the surface of the *Aquilaria sinensis* tree, the following cells generated dark secondary metabolites after 180 days: phellem layer cells, phloem ray cells, bast thin-walled cells, and certain cambium cells. When ethephon was applied on the surface of the *Aquilaria sinensis* tree, the phellem layer cells and phloem ray cells generated dark secondary metabolites after 180 days. When a mixed reagent of methyl jasmonate and ethephon was applied on the surface of the *Aquilaria sinensis* tree, the following cells generated dark secondary metabolites after 180 days: phellem layer cells, phloem ray cells, bast thin-walled cells, cambial cells, ray cells, included phloem cells, and guide pipes. These results indicate that the mixture of reagents induced *Aquilaria sinensis* to generate a wider range of cells with dark secondary metabolites.

The position of the dark secondary metabolites in the cells was characterized using Photoshop and Image-Pro Plus, and the area of dark secondary metabolites was measured in the 1 mm chordwise width. The different concentrations of reagent processing include non-replacement of the reagent (no repetition) and replacement of the reagent (repeated). The area of dark secondary metabolites generated in the 1% methyl jasmonate and 1% ethephon induction experimental group showed few sporadic occurrences. When 2% methyl jasmonate and 2% ethephon were used for induction, the area of dark secondary metabolites apparently increased compared to the induction with 1% methyl jasmonate and 1% ethephon. However, when using 5% methyl jasmonate and 5% ethephon for induction, the area of dark secondary metabolites formed apparently decreased compared to the induction with 2% methyl jasmonate and 2% ethephon. It is important to note that no apparent dark secondary metabolites were generated in the samples of the control group.

The area of dark secondary metabolites in the *Aquilaria sinensis* tree, induced by the reagent mixture of 1%, 2%, and 5%, was evidently larger than that formed through the separate induction with the same concentration of hormones. This demonstrates that the reagent mixture was more effective in inducing the generation of dark secondary metabolites.

Throughout all concentrations and reagent treatment conditions, it was found that replacing the induction reagent every 60 days during the induction period significantly increased the area of dark secondary metabolites in the *Aquilaria sinensis* tree compared to using the same concentration of reagent without replacement. This improvement in the substance generation area of dark secondary metabolites enhanced the overall induction effect.

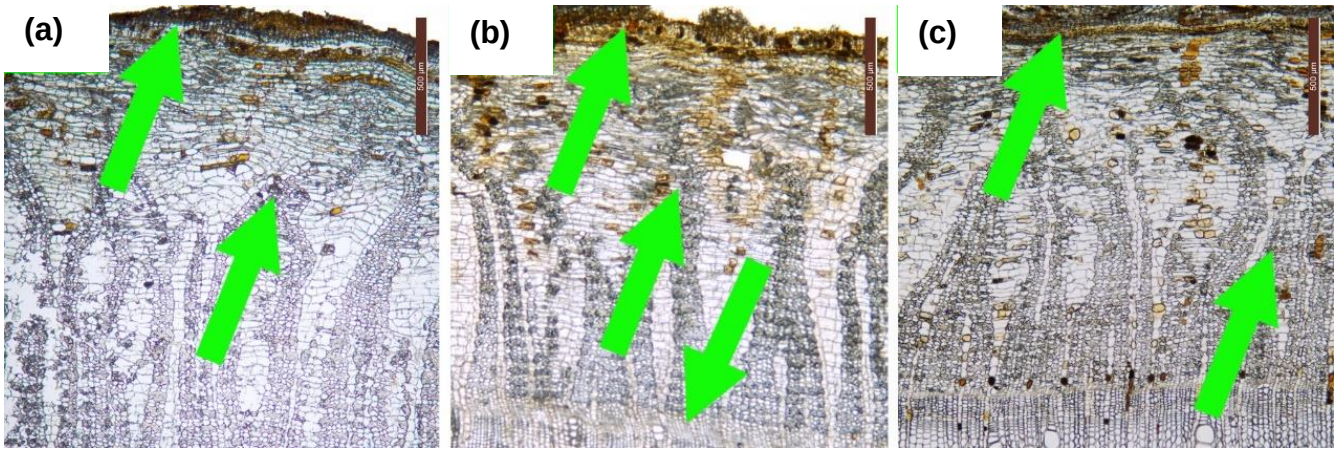

Fig. 4. The position of cells that undergoing generation of resin **(a)** The surface of A. sinensis coated with methyl jasmonate; **(b)** the surface of A. sinensis coated with the mixed agent including methyl jasmonate and ethephon; **(c)** the surface of A. sinensis coated with ethephon

The combination of 1% methyl jasmonate and ethephon in the reagent mixture led to the production of numerous dark secondary metabolites. In contrast, separate induction using 1% methyl jasmonate and 1% ethephon did not generate any dark secondary metabolites individually. This indicates that methyl jasmonate and ethephon play an evident cooperative role in inducing the production of dark secondary metabolites. When the concentration of the reagent mixture was increased to 2%, or the reagent was replaced every 60 days, the area of dark secondary metabolites substantially increased. Consequently, among all the processing schemes, the reagent mixture of methyl jasmonate and ethephon, with a 2% concentration and replacement every 60 days during the induction period, was considered to be the superior induction scheme for incense production in *Aquilaria sinensis*.

Experimental Scheme of Supercritical Fluid Extraction

In the total-factor experiment, the supercritical extraction time was 2 h, and the sample of agilawood powder was passed through a 60-mesh sieve. Briefly, 60 g sieved powder was weighed, and the pressure (X_1), temperature (X_2), and flow rate (X_3) were selected. The detailed experimental plan and factor levels are shown in Tables 3 and 2, respectively.

Table 3. Total Factor Experimental Data 3^3

Test Serial Number	Pressure (kPa)	Temperature (°C)	Flow Rate (L/h)	Yield (W%, g/g)
1	8	35	20	1.32
2	8	35	30	1.98
3	8	35	40	1.89
4	8	45	20	0.9
5	8	45	30	1.71
6	8	45	40	1.71
7	8	55	20	0.96
8	8	55	30	1.65
9	8	55	40	1.68
10	16	35	20	1.35
11	16	35	30	2.1
12	16	35	40	2.19
13	16	45	20	1.5
14	16	45	30	2.04
15	16	45	40	2.1
16	16	55	20	1.41
17	16	55	30	2.13
18	16	55	40	2.1
19	24	35	20	1.68
20	24	35	30	2.7
21	24	35	40	2.91
22	24	45	20	1.77
23	24	45	30	2.22
24	24	45	40	2.52
25	24	55	20	1.56
26	24	55	30	2.4
27	24	55	40	2.61

Experimental Data Processing

Significance analysis

The significant test indicates that a hypothesis is established for the parameters of population (random variable) or population distribution form. Then, the sample information is adopted to judge whether the hypothesis (alternative hypothesis) is reasonable, namely, whether there is significant difference between the population truth and the original hypothesis. The significant test is adopted to judge whether there is a difference between the experimental processing group and the control group, or two processing effects, and whether the difference is significant (Xu *et al.* 2017; Gwee *et al.* 2020; Sivaramakrishnan and Incharoensakdi 2020; Hashim *et al.* 2021). Experimental data in Table 4 was analyzed with the statistical analysis software Design-Expert 11. Taking the yield of agilawood as the variable, and the temperature, pressure and flow rate as the fixed factors, the P-value was evaluated. The F-value is the significant test of regression equation, and the P-value is the significant value. When $0.01 < P < 0.05$, it indicates that the factor is significant. $P < 0.01$ indicates that the factor is extremely significant.

The significance test was carried out for regression equation fitting model of yield, and the results are shown in Table 4. As shown in the table, the regression model corresponding to yield regression equation ($P < 0.0001$) was extremely significant. The determination coefficient of Box-Behnken model was $R^2 = 0.99840$, the prediction determination coefficient was $R_{pre}^2 = 0.7444$, and the adjusted determination coefficient

was R_{adj}^2 = 0.9635, which is close to 1, which indicated that the model fitting is relatively accurate.

The regression equation coefficient significance test results showed that the primary item X_1 (P < 0.0001) in the yield regression equation was extremely significant, X_2 (P < 0.05) was significant, X_3 (P < 0.0001) was extremely significant, the interaction items $X_1 X_2$, $X_1 X_3$, and $X_2 X_3$ were not significant, the quadratic term X_1^2 and X_2^2 were not significant, and X_3^2 was extremely significant. According to the P-value, it could be concluded that the order of influence of softening parameters on bending properties was: pressure (X_1) > flow rate (X_3) > temperature (X_2). The yield was directly proportional to the pressure and flow and was inversely proportional to the temperature.

The analysis results are shown in Table 4.

Table 4. Significance Analysis Table

Variance Sources	Quadratic Sum	Degree of Freedom	Average Variance	F-Value	P-Value	Significance Level
Regression model	0.3434	9	0.0382	47.92	< 0.0001	Extremely significant
X1	0.1378	1	0.1378	173.04	< 0.0001	Extremely significant
X2	0.0050	1	0.0050	6.28	0.0407	Significant
X3	0.1275	1	0.1275	160.11	< 0.0001	Extremely significant
X2 X3	0.0000	1	0.0000	0.0314	0.8644	
X1 X2	0.0000	1	0.0000	0.0000	1.0000	
X1 X3	0.0006	1	0.0006	0.7848	0.4051	
X1²	0.0013	1	0.0013	1.62	0.2439	
X2²	0.0052	1	0.0052	6.48	0.0384	
X3²	0.0684	1	0.0684	85.94	< 0.0001	
Residual	0.0056	7	0.0008			
Lack-of-fit value	0.0056	3	0.0019			
Pure error	0.0000	4	0.0000			
Total	0.3490	16	R2 = 0.99840 Radj 2 = 0.9635 Rpre2 = 0.7444			

* R2 is the square of related coefficient. R is directly the correlation coefficient of the dependent variable and independent variable in the linear equation of one variable, while the multivariate is the complex correlation coefficient.

According to the variance analysis of Design-Expert 11, the fitted equation of the yield Y is given by formula 1.

$$Y = 2.18 + 0.1312X_1 - 0.025X_2 + 0.1236X_3 - 0.0025X_1X_2 - 0.0125X_2X_3 + 0.0175X_{12} + 0.035X_{22} - 0.1275X_{32} \quad \text{(Formula 1)}$$

Design-Expert 11 was selected to substitute the formula 1 into the plot of reaction surface and contour, as shown in Figs. 5 and 13. The X_1, X_2, and X_3 refer to the pressure (unit: MPa), temperature (unit °C), and flow rate (unit L/h). The factor level is shown in Table 2, where -1, 0, and 1 are adopted as the codes of the low, middle, and high levels. The maximum yield corresponding to each response surface is shown in Table 5.

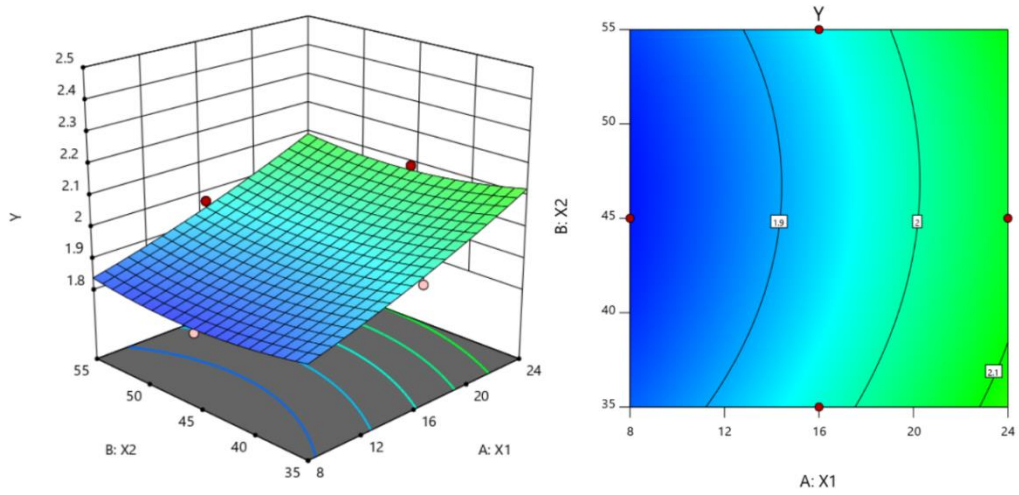

Fig. 5. X3 (flow rate) level-1, pressure, and temperature corresponding to the yield curve diagram and contour map

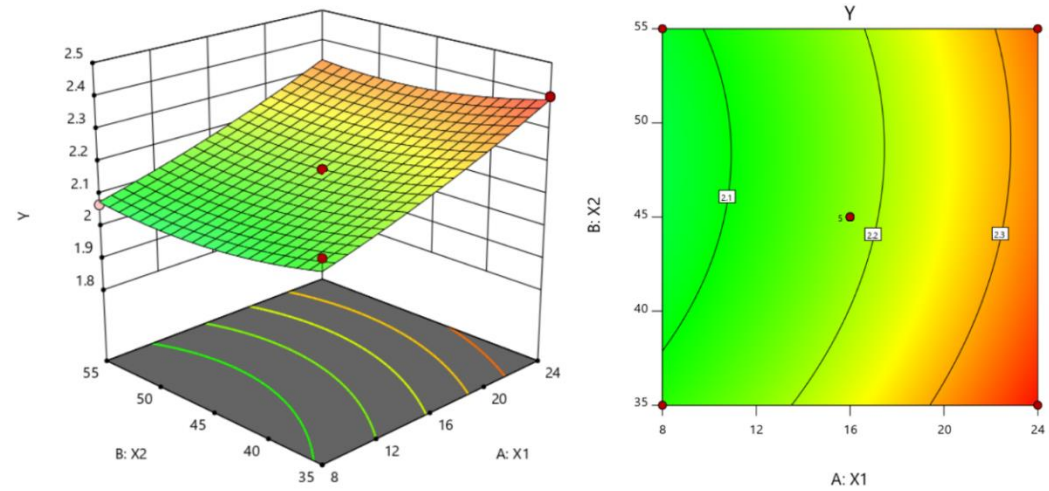

Fig. 6. X3 (flow rate) level 0, pressure, and temperature corresponding to yield curve diagram and contour map

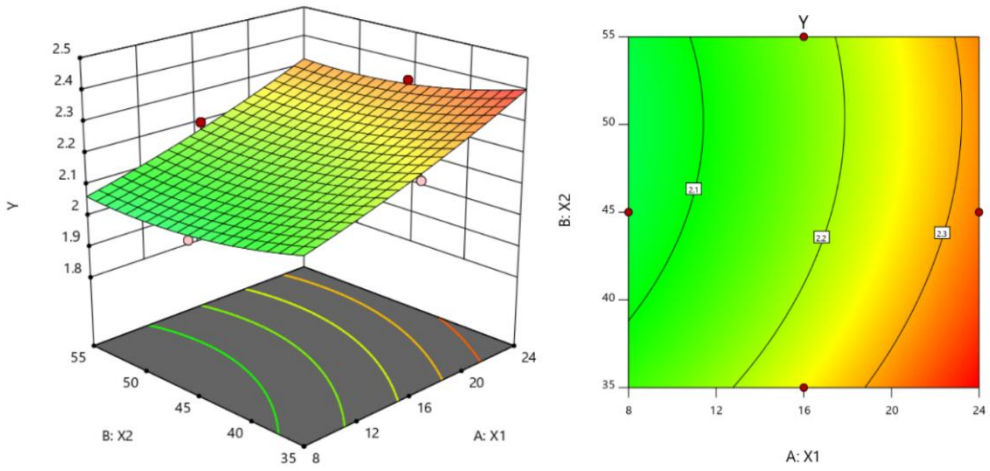

Fig. 7. X3 (flow rate) level 1, pressure, and temperature corresponding to yield curve diagram and contour map

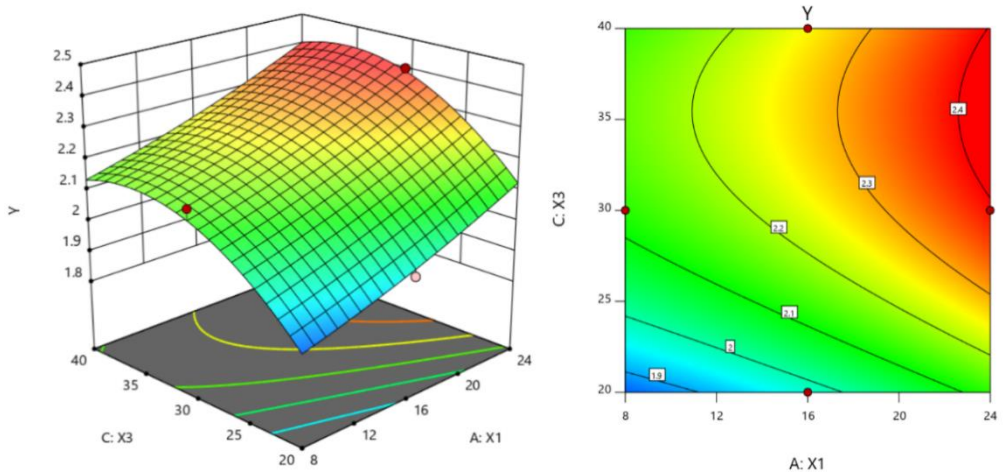

Fig. 8. X2 (flow rate) level -1, pressure, and temperature corresponding to yield curve diagram and contour map

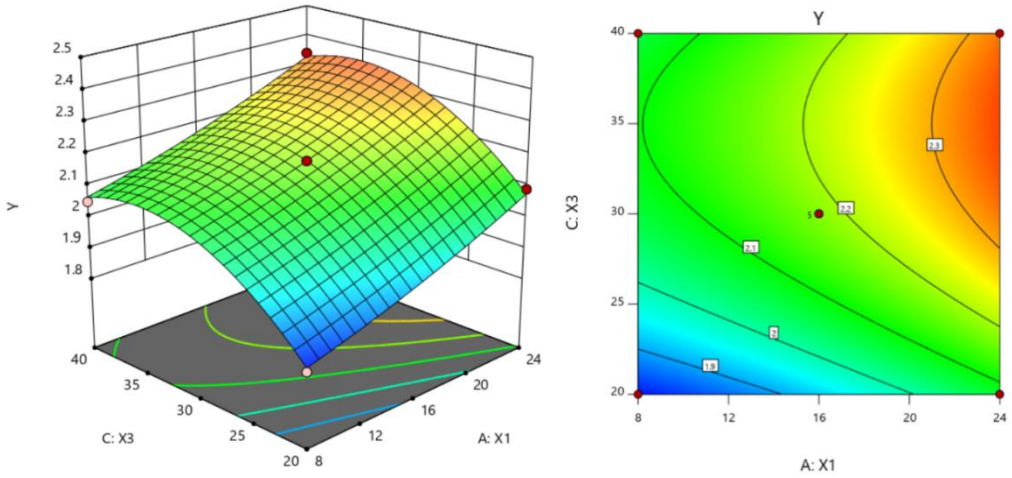

Fig. 9. X2 (flow rate) level 0, pressure, and temperature corresponding to yield curve diagram and contour map

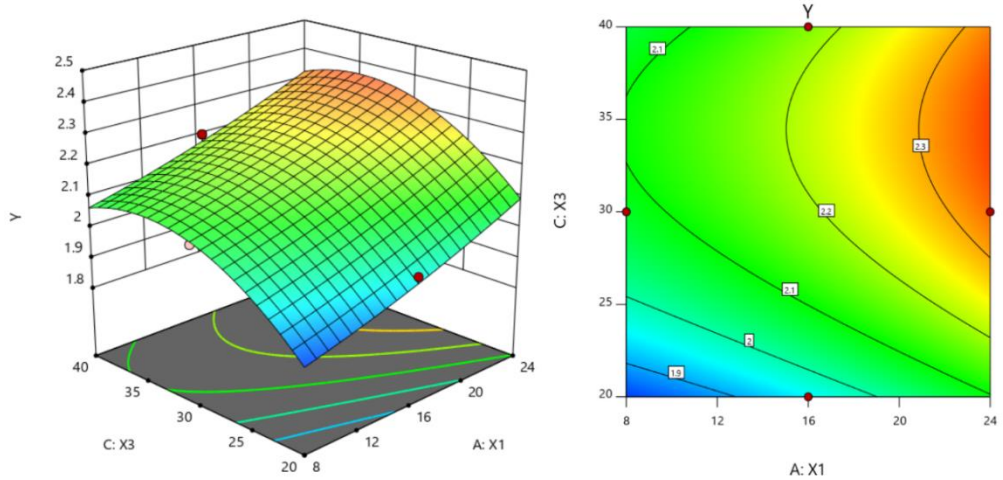

Fig. 10. X2 (flow rate) level 1, pressure, and temperature corresponding to yield curve diagram and contour map

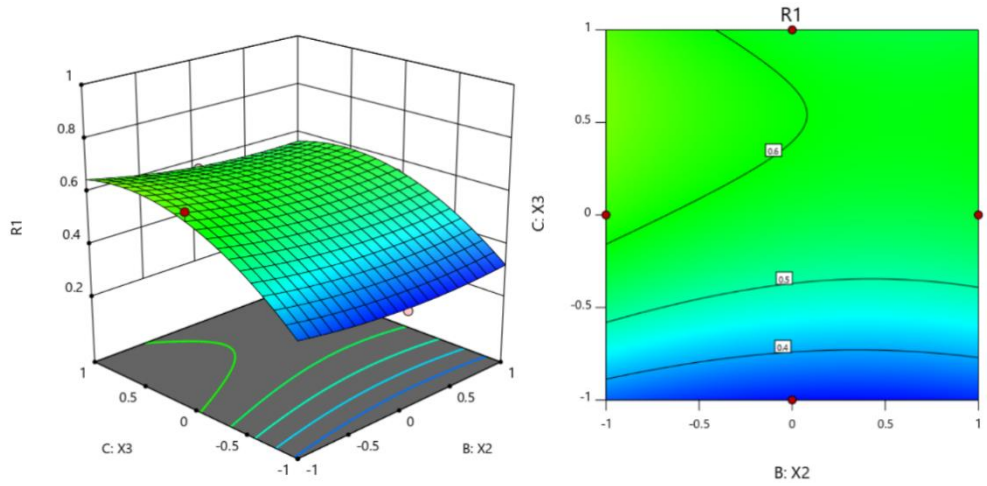

Fig. 11. X1 (flow rate) level -1, pressure, and temperature corresponding to yield curve diagram and contour map

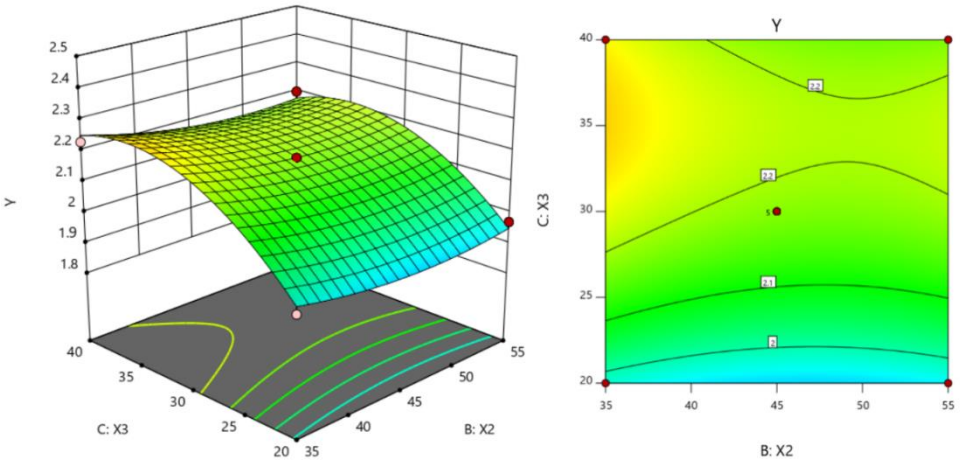

Fig. 12. X1 (flow rate) level 0, pressure, and temperature corresponding to yield curve diagram and contour map

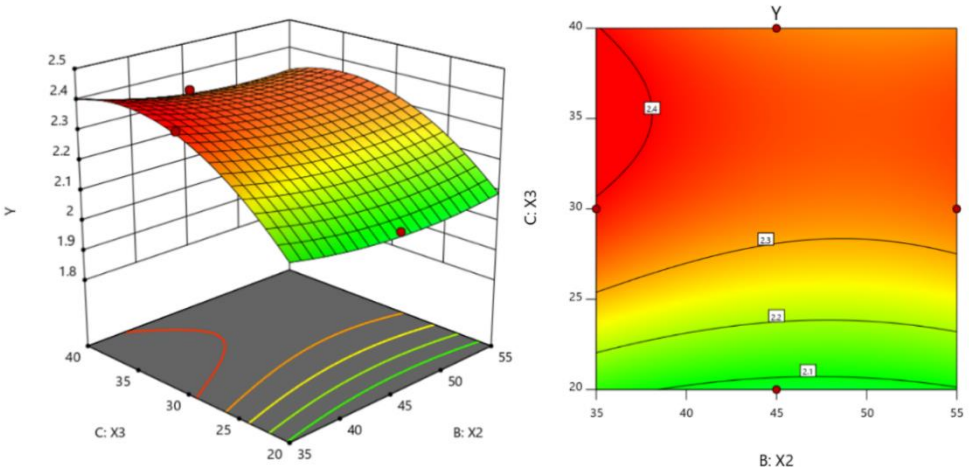

Fig. 13. X1 (flow rate) level 1, pressure, and temperature corresponding to yield curve diagram and contour map

Comprehensive parameter optimization and verification

The yield of the agarwood essential oil is the ultimate goal of the extraction of agilawood. Taking the pressure (X1), temperature (X2), and flow rate (X3) as the design factors, and the highest yield as the optimization objective according to the results, the optimized process parameters could be obtained through the Design Expert 11 software: pressure of 23.7701 MPa, temperature of 35.4653 °C, flow rate of 33.3034 L/h, expected yield of 2.41369%. Modified optimal process parameters: the pressure, temperature, and flow rate were 24 MPa, 35 °C, and 33 L/h, respectively.

Table 5. The Maximum Yield of the Response Surface Map

No.	Conditions	Yield Maximum (W%，g/g)
Fig. 5	X3 = -1	2.1250
Fig. 6	X3 = 0	2.3838
Fig. 7	X3 = 1	2.3975
Fig. 8	X2 = -1	2.4066
Fig. 9	X2 = 0	2.3600
Fig. 10	X2 = 1	2.3590
Fig. 11	X1 = -1	2.1699
Fig. 12	X1 = 0	2.2782
Fig. 13	X1 = 1	2.4020

* The maximum yield is calculated by the software Design-Export 11

Table 6 shows that the extraction yield of the agarwood essential oil was 2.41% according to the modified optimal softening conditions. The average deviation was -0.35%, which indicates that the predicting results of the regression model in the Box-Behnken method was close to the actual value. The predicted accuracy was high.

Table 6. Response Surface Optimization Result Verification

No.	X1 (MPa)	X2 (°C)	X3 (L/h)	Y (%)	Deviation (%)
Predicted value	24	35	33	2.414	---
Verification group 1	24	35	33	2.361	-1.87%
Verification group 2	24	35	33	2.411	2.12%
Verification group 3	24	35	33	2.38	-1.29%
Average group	24	35	33	2.384	-0.35%

Analysis of Agarwood Essential Oil Obtained by Supercritical Extraction by GC-MS

GC-MS (Trace 1300 ISQ 7000)

Analysis conditions of the gas chromatograph were as follows: Chromatographic column: TG-5MS column length: 30 m, inner diameter: 0.25 mm, film thickness: 0.25 μm; Injection port temperature: 280 ℃; Shunting mode: Splitless; Flow rate of carrier gas: 1.2

mL/min; Carrier gas: He; Temperature programming: 40 ℃ (5min) 5 ℃/min 180 ℃ 8 ℃/min 250 ℃ (1min) 10 ℃/min 290 ℃ (10min) 10 ℃/min 320 ℃ (30min); Injection volume: 1uL.

Mass spectrum condition: Temperature of ion source: 280 ℃; Ionization method: EI; Ionizing energy: 70eV.

Table 7. Test Results

Serial Number	Peak name	Retention time	Chemical formula	CAS	Relative Peak Area
1	Ethyl Alcohol	2.87	C_2H_6O	64-17-5	9.06
2	Oxalic acid	2.982	$C_2H_2O_4$	144-62-7	7.65
3	glycolic acid	3.154	$C_2H_4O_3$	79-14-1	2.47
4	Acetic acid	3.793	$C_2H_4O_2$	64-19-7	29.55
5	Methyl acetate	3.869	$C_3H_6O_2$	79-20-9	1.08
6	Ethylene glycol acetate formate	6.322	$C_5H_8O_4$		0.13
7	2,2-dimethoxyethanol	6.803	$C_4H_{10}O_3$	30934-97-5	0.14
8	benzaldehyde	11.638	$C_7H_6O_1$	100-52-7	0.21
9	6,7-Dimethyl-1,2,3,5,8,8a-hexahydronaphthalene	20.466	$C_{12}H_{18}$	107914-92-1	0.18
10	4-phenylbutan-2-one	20.775	$C_{10}H_{12}O$	2550-26-7	0.52
11	2,3,5-Trimethylfuran	22.468	$C_7H_{10}O$	10504-04-8	0.05
12	Phenol, 5-ethenyl-2-methoxy-	22.706	$C_9H_{10}O_2$	621-58-9	0.03
13	Benzene, 1-(1,2-dimethyl-3-methylenecyclopentyl)-4-methyl-, cis-	22.792	$C_{15}H_20$	54808-83-2	0.05
14	Ethyl 3-phenylpropanoate	23.522	$C_{11}H_{14}O_2$	2021-28-5	0.03
15	1,3-Diacetin	23.821	$C_7H_{12}O_5$		0.22
16	3-Ethylphenol, n-propyl ether	24.698	$C_{11}H_{16}O$		0.16
17	4,6-Di-tert-butyl-2-methylphenol	26.137	$C_{15}H_{24}O$	616-55-7	0.15
18	Tricyclo[5.2.2.0(1,6)]undecan-3-ol, 2-methylene-6,8,8-trimethyl-	26.887	$C_{15}H_{24}O$		1.70
19	β-Guaiene	27.019	$C_{15}H_{24}$	88-84-6	0.03
20	Tetracyclo[6.2.1.1(3,6).0(2,7)]dodec-4-ene, 11-isopropylidene-	27.095	$C_{15}H_20$		0.01
21	(-)-Nootkatene	27.176	$C_{15}H_{22}$	5090-61-9	0.03
22	Valencene	27.257	$C_{15}H_{24}$	4630-07-3	0.02
23	Cycloisolongifolene, 8,9-dehydro-	27.307	$C_{15}H_{22}$		0.01
24	4-Epi-cis-Dihydroagarofuran	27.454	$C_{15}H_{26O}$		0.07
25	Anisylacetone	27.536	$C_{11}H_{14}O_2$	104-20-1	0.12
26	ethyl 2,4-dihydroxy-6-methylbenzoate	27.662	$C_{10}H_{12}O_4$	2524-37-0	0.00
27	α,α-Diethyl-o-methoxybenzyl alcohol	27.764	$C_{12}H_{18}O_2$	53847-40-8	0.01
28	(-)-Italicene ether	27.87	$C_{15}H_{24}O_1$	104188-25-2	0.07
29	Isolongifolene, 4,5,9,10-dehydro-	28.012	$C_{15}H_20$	156747-45-4	0.04
30	2(3H)-Benzofuranone, 3a,4,5,6-tetrahydro-3a,6,6-trimethyl-	28.159	$C_{11}H_{16}O_2$	16778-26-0	0.02
31	α-Costol	28.22	$C_{15}H_{24}O_1$	65018-15-7	0.04
32	1,4-Ethanoquinoxaline, 2,3-dihydro-	28.341	$C_{10}H_{12}O_2$	7140-45-6	0.01
33	Tricyclo[6.3.0.0(1,5)]undec-2-en-4-one, 5,9-dimethyl-	28.529	$C_{13}H_{18}O$		0.12
34	Isolongifolene, 4,5-dehydro-	28.579	$C_{15}H_{24}O$		0.38
35	α-Costol	28.615	$C_{15}H_{24}O$	5956/12/7	0.05
36	α-Vetivone	28.939	$C_{15}H_{22}O$	15764-04-2	0.24
37	cis-Eudesm-6-en-11-ol	29.061	$C_{15}H_{26}O$	194607-96-0	0.15
38	Isolongifolene, 4,5,9,10-dehydro-	29.157	$C_{15}H_20$	156747-45-4	0.01
39	Benzene, 1-(1,2-dimethyl-3-methylenecyclopentyl)-4-methyl-, cis-	29.233	$C_{15}H_20$	54808-83-2	0.08
40	6S,10R-Dimethylbicyclo[4.4.0]decan-1-en3-one	29.345	$C_{12}H_{18}O$	69460-62-4	0.06

41	Costol	29.517	$C_{15}H_{24}O$	515-20-8	0.24
42	Cycloisolongifolene, 8,9-dehydro-	29.573	$C_{15}H_{22}$		0.18
43	Hydroxygrass extract	29.613	$C_{15}H_{22}O$	4176-16-3	0.03
44	Valerenol	29.705	$C_{15}H_{24}O$	101628-22-2	0.05
45	Arctiol	29.725	$C_{15}H_{26}O_2$	36061-11-7	0.14
46	Aromadendrene oxide-(2)	29.841	$C_{15}H_{24}O$		0.07
47	trans-Valerenyl acetate	29.983	$C_{17}H_{26}O_2$	101527-74-6	0.04
48	Nootkaton-11,12-epoxide	30.019	$C_{15}H_{22}O_2$		0.02
49	3,5,11-Eudesmatriene	30.227	$C_{15}H_{22}$	193615-07-5	0.12
50	(+)-gamma-Eudesmol	30.485	$C_{15}H_{26}O$	117066-77-0	0.81
51	β-Guaiene	30.941	$C_{15}H_{24}$	88-84-6	0.51
52	(-)-GUAIOL	31.478	$C_{15}H_{26}O$	489-86-1	2.23
53	Diepicedrene-1-oxide	31.676	$C_{15}H_{24}O$		0.15
54	6,7-Dimethyl-1,2,3,5,8,8a-hexahydronaphthalene	31.965	$C_{12}H_{18}$	107914-92-1	0.85
55	Cycloisolongifolene, 8,9-dehydro-	32.026	$C_{15}H_{22}$		0.12
56	Acetic acid, 3-hydroxy-6-isopropenyl-4,8a-dimethyl-1,2,3,5,6,7,8,8a-octahydronaphthalen-2-yl ester	32.081			0.02
57	Acetic acid, (1,2,3,4,5,6,7,8-octahydro-3,8,8-trimethylnaphth-2-yl)methyl ester	32.269	$C_{16}H_{26}O_2$	314773-27-8	0.41
58	1(2H)-Naphthalenone, 3,4,4a,5,6,7-hexahydro-4a,5-dimethyl-3-(1-methylethenyl)-, [3S-(3α,4aα,5α)]-	32.39	$C_{15}H_{22}O$	562-23-2	0.18
59	Tricyclo[5.2.2.0(1,6)]undecan-3-ol, 2-methylene-6,8,8-trimethyl-	32.461	$C_{15}H_{24}O$		0.07
60	(E)-2,6-Dimethoxy-4-(prop-1-en-1-yl)phenol	32.532	$C_{11}H_{14}O_3$	20675-95-0	0.01
61	2aS,3aR,5aS,9bR)-2a,5a,9-Trimethyl-2a,4,5,5a,6,7,8,9b-octahydro-2H-naphtho[1,2-b]oxireno[2,3-c]furan	32.598	$C_{15}H_{22}O_2$	352457-43-3	0.04
62	6-(1-Hydroxymethylvinyl)-4,8a-dimethyl-3,5,6,7,8,8a-hexahydro-1H-naphthalen-2-one	32.791	$C_{15}H_{22}O_2$		0.23
63	(R)-1,5,8-Trimethyl-6,7,8,9-tetrahydronaphtho[2,1-b]furan	33.064	$C_{15}H_{18}O$	59462-26-9	0.81
64	(E)-2-((8R,8aS)-8,8a-Dimethyl-3,4,6,7,8,8a-hexahydronaphthalen-2(1H)-ylidene)propanal	33.206	$C_{15}H_{22}O$	137695-18-2	0.30
65	Khusimyl methyl ether	33.384	$C_{16}H_{26}O$	300349-20-6	0.07
66	Isoaromadendrene epoxide	33.728	$C_{15}H_{24}O$		0.53
67	trans-Valerenyl acetate	33.804	$C_{17}H_{26}O_2$	101527-74-6	0.14
68	Widdrenal	33.845	$C_{15}H_{22}O$	470-41-7	0.04
69	Agarotetrol	34.002	$C_{17}H_{18}O_6$	69809-22-9	0.26
70	6-Isopropenyl-4,8a-dimethyl-1,2,3,5,6,7,8,8a-octahydronaphthalene-2,3-diol	34.205	$C_{15}H_{24}O_2$	1005284-62-7	1.18
71	α -Cyperone	34.671	$C_{15}H_{22}O$	473-08-5	0.58
72	2aS,3aR,5aS,9bR)-2a,5a,9-Trimethyl-2a,4,5,5a,6,7,8,9b-octahydro-2H-naphtho[1,2-b]oxireno[2,3-c]furan	35.021	$C_{15}H_{22}O_2$	352457-43-3	0.68
73	4-(3,3-Dimethyl-but-1-ynyl)-4-hydroxy-2,6,6-trimethylcyclohex-2-enone	35.097	$C_{15}H_{22}O_2$	930090-10-1	0.08
74	Aromadendrene oxide-(2)	35.659	$C_{15}H_{24}O$		8.62
75	6-(1-Hydroxymethylvinyl)-4,8a-dimethyl-3,5,6,7,8,8a-hexahydro-1H-naphthalen-2-one	36.171	$C_{15}H_{22}O_2$		0.73
76	Alloaromadendrene oxide-(2)	36.805	$C_{15}H_{24}O$		5.27
77	Acetic acid, 3-hydroxy-6-isopropenyl-4,8a-dimethyl-1,2,3,5,6,7,8,8a-octahydronaphthalen-2-yl ester	37.377	$C_{17}H_{26}O_3$		1.24

78	5,8-Dihydroxy-4a-methyl-4,4a,4b,5,6,7,8,8a,9,10-decahydro-2(3H)-phenanthrenone	37.737	$C_{17}H_{26}O_3$		0.40
79	Hydroxyvalerenic acid	38.087	$C_{15}H_{22}O_3$	1619-16-5	3.44
80	carissone	38.289	$C_{15}H_{24}O_2$	473-10-9	1.17
81	4-(2-Isopropyl-5-methylphenyl)-3-methylbutyric acid	38.842	$C_{15}H_{22}O_2$	22291-58-3	2.63
82	Azatadine maleate	39.511	$C_{20}H_{22}N_2$	3964-81-6	0.05
83	cis-13-Octadecenoic acid	39.866	$C_{18}H_{34}O_2$	13126-39-1	0.72
84	stearic acid	40.109	$C_{18}H_{36}O_2$	1957/11/4	0.83
85	Allocryptopine	40.19	$C_{21}H_{23}NO_5$	485-91-6	0.27
86	2-(2-phenylethyl)chromen-4-one	42.252	$C_{17}H_{14}O_2$	61828-53-3	1.56
87	Octan-2-yl palmitate	43.469	$C_{24}H_{48}O_2$	55194-81-5	0.15
88	Phthalic acid, di(2-propylpentyl) ester	44.092	$C_{24}H_{38}O_4$		0.42
89	6-Methoxy-2-phenethyl-4H-chromen-4-one	45.197	$C_{18}H_{16}O_3$	84294-89-3	2.65
90	erythro-8-(1,2-Dibromo-2-phenylethyl)-3,7-dihydro-1,3,7-trimethyl-1H-purine-2,6-dione	45.567	$C_{16}H_{16}Br_2N_4O_2$	99765-14-7	0.21
91	Ethyl [5-hydroxy-1-(6-methoxy-4-methyl-3-quinolinyl)-3-methyl-1H-pyrazol-4-yl]acetate #	45.83	$C_{19}H_{21}N_3O_4$		0.95
92	(Z)-docos-13-enamide	46.378	$C_{22}H_{43}NO$	112-84-5	0.12
93	l-Proline, n-heptafluorobutyryl-, isobutyl ester	46.484	$C_{13}H_{16}F_7NO_3$		0.19
94	(E,E,E,E)-Squalene	46.621	$C_{30}H_{50}$	111-02-4	0.08
95	4'-(n-Propyl)oxy-4-methoxy-2'-methylchalcone (isomer 1)	47.138	$C_{20}H_{22}O_3$		0.26
96	6,7-dimethoxy-2-(2-phenylethyl)chromen-4-one	47.645	$C_{19}H_{18}O_4$	84294-87-1	1.30
97	Quinazolin-4(3H)-one, 2-(4-methoxybenzylthio)-3-methyl-	47.933	$C_{17}H_{16}N_2O_2S$	90852-47-4	0.12
98	24-Norursa-3,9(11),12-triene	48.582	$C_{29}H_{44}$	930591-91-6	0.15
99	6,7-dimethoxy-2-(2-(4-methoxyphenyl)ethyl)chromone	50.381	$C_{20}H_{20}O_5$	117596-92-6	0.19
100	stigmasterol	51.795	$C_{29}H_{48}O$	83-48-7	0.14

The agilawood essential oil was obtained through supercritical extraction, and its GC-MS analysis was performed to authenticate 100 volatile components. The majority of these components exhibited the sesquiterpenes structure, and the relevant components are listed in Table 7. The analysis and detection results reveal that the agarotetrol content in the agilawood essential oil obtained from artificial incense production *via* induction of the reagent mixture hormone reached 0.26%, significantly surpassing the requirement of not being lower than 0.1%, as specified in the Chinese Pharmacopoeia (2020). This indicates that the artificial agilawood obtained through the induction of the reagent mixture hormone used in the project is a simple and efficient operation.

The induction method greatly enhances the incense production velocity of *Aquilaria sinensis*, reducing the production cycle of agilawood, and yields a remarkable incense production effect. Moreover, it effectively addresses the imbalance between supply and demand for agilawood, leading to essential economic and social benefits.

CONCLUSIONS

1. Among all the processing schemes, the reagent mixture of methyl jasmonate and ethephon was shown to be the most effective inductive agent. The concentration of this inductive agent was set at 2%, and during the induction period, the agent was replaced every 60 days. This particular processing scheme can be regarded as the superior induction method for incense production in *Aquilaria sinensis*.
2. The water-bath supercritical fluid equipment was adopted to extract the agarwood essential oil, and the supercritical extraction time was 2 h. The sample of agilawood was in powder form, passing through a 60-mesh sieve. In the experiment, 60 g sieved powder was weighed, and the pressure, temperature, and flow rate were set to 8, 16, and 24 MPa; 35, 45, and 55 °C; and 20, 30, and 40 L/h, respectively, for a 3^3 total-factor experiment. The experimental data were used in the Box-Behnken analysis. The equation was obtained by fitting $Y = 2.18 + 0.1312X_1 - 0.025X_2 + 0.1236X_3 - 0.0025X_1X_2 - 0.0125X_2X_3 + 0.0175X_{12} + 0.035X_{22} - 0.1275X_{32}$.
3. With the Design Expert 11 software, the optimal process parameters were obtained in the supercritical fluid extraction of agarwood essential oil: the pressure, temperature, and flow rate were 24 MPa, 35 °C, and 33 L/h. The yield of agarwood essential oil extracted with the supercritical carbon dioxide was mainly affected by the pressure and flow rate. The yield was directly proportional to the pressure and flow, and inversely proportional to the temperature.
4. The fitting research indicated that the extraction yield was 2.41% according to the modified optimal softening conditions. The average deviation was -0.35%. It indicated that the predicting results of the regression model in the Box-Behnken method was close to the actual value. The predicted accuracy was high.
5. This study analyzed the optimal process parameters of water-bath supercritical fluid equipment in the extraction of agarwood essential oil to provide new theoretical and practical support for the extraction of agarwood essential oil. It is conducive to reduce the loss ratio of extraction and improve the utilization rate for the extraction of agarwood essential oil.
6. The gas chromatography – mass spectrometry (GC-MS) data of agilawood essential oil obtained via supercritical fluid extraction were analyzed to authenticate 100 volatile components. Most of the components had sesquiterpene structure. The analysis and detection results show that agarotetrol contents in agilawood essential oil of artificial incense production obtained via induction of reagent mixture reached 0.26%, which is far higher than the requirements of no less than 0.1% as specified in Chinese Pharmacopoeia (2020).

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Declaration of Conflicting Interests

The author(s) declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Author Contributions

Zhihong Pan, Xian Wang, and Qingde Li conceived and designed the experiment. Jichang Li and Haikun Wang analysed and discussed the data. Zhihong Pan and Xian Wang wrote the manuscript, Qingde Li, Jichang Li, and Haikun Wang made revisions.

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