

## Article

# Structural Characterization and Stability Evaluation of Melanin from *Liquidambar formosana* Hance Leaves: A Potential Natural Pigment for Food Applications

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## Abstract

In this study, purified *Liquidambar formosana* Hance melanin (P-LHM) was structurally characterized and evaluated for stability. The yield of P-LHM was approximately 3.47%. Analytical results revealed P-LHM is a melanin-rich complex where eumelanin and pheomelanin polymers are intimately associated with specific flavonoids, phenolic acids, and terpenoids. The condensation molecular formula of P-LHM might be  $([C_{24}H_{29}NO_9]_n)$ . The stability evaluation showed that under specific conditions (natural light, darkness, pH = 7–11, 25–100 °C, Na<sup>+</sup>, Al<sup>3+</sup>, Fe<sup>2+</sup> solution, and low concentrations of reducing agents), the retention rate of P-LHM was >90%. Given its excellent stability, LHM may be used as a new type of food ingredient in the processing of black-colored foods. Meanwhile, the rich phenolic compounds provide a theoretical basis for the development of functional colorants, enhancing the functional value of food colorants.

**Keywords:** *Liquidambar formosana* Hance; melanin; structural characterization; stability

## 1. Introduction

*Liquidambar formosana* Hance is a deciduous broad-leaved tree belonging to *Hamamelidaceae* R. Br. It is widely distributed in southern regions of China (south of the Qinling Mountains and Huaihe River) and has strong environmental adaptability and significant ecological value [1]. Its leaves are rich in active ingredients, such as amino acids, polysaccharides, flavonoids [2], polyphenols, and terpenoids [3], which have high potential economic value. The leaves of *Liquidambar formosana* Hance are used to dispel wind and dampness and to treat dysentery, enteritis, indigestion and stomachache [4]. With increasing demand for natural pigments, *Liquidambar formosana* Hance leaves have attracted much attention as a kind of natural plant melanin resource with biological activity.

Melanin is a heterogeneous polymer [5] of dark brown to black phenolic or quinonoid intermediates formed via oxidative polymerization of phenolic or indolic compounds [6]. It is usually interconnected with proteins in living organisms and is insoluble in water, acids, and organic solvents. Classified by origin, melanins can be divided into animal melanin, plant melanin, and microbial melanin [7]. According to the structural differences in pigment monomer subunits, they are further categorized into eumelanin, pheomelanin, and allomelanin [8]. Eumelanin is a heterogeneous polymer consisting of 5,6-dihydroxyindole and



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5,6-dihydroxyindole-2-carboxylic acid. Similarly, pheomelanin originates from the common precursor dopaquinone. In its biosynthesis pathway, L-dopa combines with cysteine to form cysteinyl-dopa (CD isomer), which subsequently undergoes oxidation, cyclization, and rearrangement to yield 1,4-benzothiazine derivatives [9]. Allomelanins represent a distinct class of nitrogen-free heterogeneous pigments widely distributed among fungi and plants. At present, the structural characterization of the first two melanins has been confirmed to a certain extent, but that of the third remains unclear. Current research indicates that melanin has anti-oxidation [10], antibacterial [11], anti-inflammatory, anti-tumor, metal chelation [12], thermoregulation [13], and radiation protective properties [14], and it is widely used in food, cosmetics [15], bioelectronics [16], biomedical applications [17], material science, agriculture, electronics and bioelectronics, and other fields [18]. Existing studies on melanin primarily focus on extraction and purification technology, physical and chemical properties, and evaluation of single active ingredients. Several scholars have conducted preliminary research on the structure of melanin. For example, Islam et al. [19] isolated a plant-type eumelanin from cultured tobacco BY-2 cells. Xie et al. [20] demonstrated that red and yellow varieties of *Stropharia rugosoannulata* contain eumelanin and pheomelanin. Huang et al. [21] studied *Mesona chinensis* and identified its skeleton structure as  $[C_7H_8N_4O_2]$ . Li et al. [22] extracted extracellular melanin from *Ascosphaera apis* and obtained the molecular formula  $(C_{10}H_6O_4N_2)$ .

As a food plant resource [23], the leaves of *Liquidambar formosana* Hance contain melanin. The melanin from *Liquidambar formosana* Hance (LHM) can serve as a safe and stable natural pigment [24], providing a rich range of tones from brown to deep black for baked goods, pastries, beverages, and condiments, replacing synthetic pigments and caramel color, while also aligning with the trend of clean labeling. However, industrial applications remain constrained due to instability and degradation during extraction, influenced by environmental factors and the internal structure of LHM. Therefore, structural characterization and stability studies of LHM are critical for enhancing its utilization.

In this study, the crude extract of melanin from *Liquidambar formosana* Hance (C-LHM) was separated and purified by alcohol precipitation and macroporous adsorption resins. Then, by comprehensively performing ultraviolet-visible absorption spectroscopy (UV-Vis), gel permeation chromatography (GPC), Fourier transform infrared spectroscopy (FT-IR), elemental analysis (EA), thermogravimetric analysis (TGA), high-performance liquid chromatography (HPLC), nuclear magnetic resonance (NMR) and ultra performance liquid chromatography/tandem mass spectrometry (UPLC-MS/MS) techniques, the structural characterization and material composition of P-LHM were studied for the first time. Additionally, stability was systematically evaluated under thermal, photolytic, and pH conditions, oxidizing and reducing agents, and the presence of metal ions. These findings provide a theoretical basis for the further study of LHM and offer valuable insights for the commercial application of LHM in the food field.

## 2. Materials and Methods

### 2.1. Materials and Sample Preparations

The fresh *Liquidambar formosana* Hance leaves used in this study were sourced from Panguniang Food Co., Ltd. (Wuzhishan, China). First, 1 kg of *Liquidambar formosana* Hance leaves was homogenized with deionized water at a 1:10 ratio (*w/v*, leaves to water) for 1 min using a mechanical crusher to form a uniform mixture. The pH value of the solid liquid system was adjusted to pH = 11 with 1% sodium hydroxide solution, and the solution was extracted at room temperature for 48 h, and then in a water bath at 80 °C for 3 h. The C-LHM was obtained by squeezing extracted *Liquidambar* leaves through three layers of gauze, centrifugation (Thermo, Shanghai, China, Pico21, 4000 r/min, 30 min),

and vacuum rotary evaporation (Jiangsu Changzhou Machinery Equipment Co., Ltd., Changzhou, China, FS400, 60 °C, 45 r/min).

## 2.2. Isolation and Purification

AB-8 (AR, Beijing Solab Technology Co., Ltd., Beijing, China) resin was a weakly polar adsorbent. Melanin and its main phenolic and flavonoid associated components contain benzene rings, hydroxyl groups and other structures, which were combined with resins through hydrophobic interaction and hydrogen bonds. The static adsorption experiment showed that the saturated adsorption capacity of AB-8 resin for C-LHM was approximately 53 mg/g of wet resin. AB-8 macroporous adsorption resin was used to separate and purify LHM. The pretreated AB-8 resin was wet-packed into a chromatography column (a 16 mm × 200 mm glass chromatography column, Shanghai Qite Analytical Instrument Co., Ltd., Shanghai, China). The sample, at a concentration of 10 mg/mL, was loaded in a volume of 2.0–2.3 bed volumes (BV) at a flow rate of 2 BV/h.

The ethanol solution demonstrated strong polarity and hydrogen bonding capacity, which could effectively and competitively disrupt the interaction forces between the pigment molecules and the resin. Meanwhile, its moderate hydrophobicity assisted in eluting the hydrophobic components. Through pre-experimental optimization, the 60% ethanol solution ensured a high elution rate while avoiding the co-elution of excessive polar impurities that resulted from the use of higher ethanol concentrations. After adsorption equilibrium, the bound melanin was eluted using 60% ethanol (*v/v*) as the eluent at a flow rate of 2 BV/h. The eluate was concentrated via rotary evaporation under reduced pressure at 55 °C and subsequently freeze-dried to obtain the P-LHM.

## 2.3. UV-Vis Spectrum

The ultraviolet spectroscopy spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, MA, USA, Evolution Pro) was employed to perform full-wavelength scanning of the LHM solution. The P-LHM was reconstituted as a test solution at a mass concentration of 0.1 mg/mL (*w/v*). Absorbance values were measured across a 190–400 nm wavelength range, and an UV-Vis curve graph was plotted.

## 2.4. GPC Analysis

The relative molecular mass of C-LHM and P-LHM was determined by Agilent PL-GPC50 (Agilent Technologies, Inc., Church Stretton, UK). The operation was carried out under the conditions of a column temperature of 30 °C, injection volume of 1 µL, sample concentration of 0.10 mg/mL, and double-distilled water as the mobile phase.

## 2.5. FT-IR Analysis

FT-IR spectra of samples were analyzed by Fourier transform infrared spectroscopy (Thermo Fisher Scientific, Nicolet IS50). KBr was dried in an oven (Shanghai Jinghong Experimental Equipment Co., Ltd., Shanghai, China, DHG-9053A) at 105 °C until constant weight was achieved to eliminate residual moisture. The sample was mixed with KBr at a ratio of 1:100 (*w/w*), ground evenly near an infrared lamp, and then an appropriate amount of the powder was placed in a tableting mold for tableting. Using KBr as the measurement background, a Fourier transform infrared spectrometer was used at a wavelength range of 400–4000 cm<sup>−1</sup> to measure the sample [25].

## 2.6. Elemental Analysis

An organic element analyzer (Thermo Fisher Scientific, Inc., Waltham, MA, USA, Thermo Fisher flash 200) was used to determine the C, H, N, and S contents of C-LHM and P-LHM.

## 2.7. TGA Analysis

The thermal decomposition behavior of LHM was analyzed using a TA Discovery TGA 550 (TA Instruments, New Castle, DE, USA) thermogravimetric analyzer. Experimental parameters were configured as follows: samples (5–10 mg) were weighed and subjected to heating from 50 °C to 800 °C under a high-purity nitrogen atmosphere at a flow rate of 100 mL/min, with a controlled heating rate of 5 °C/min.

## 2.8. HPLC Analysis

LHM was analyzed using an UltiMate 3000 high-performance liquid chromatography (HPLC) system (Thermo Fisher Scientific, Inc., Waltham, MA, USA) equipped with a UV detector. The sample concentration was 0.5 mg/mL. The area normalization method was used to estimate the relative content of each component. Separation was carried out on an Agilent ZORBAX SB-C18 column (4.6 × 330 mm), which was kept at 30 °C. The mobile phase consisted of two components: acetonitrile (A) and 0.1% formic acid (B). The gradient elution program was set as follows: 80–90% B from 0 to 10 min, followed by 70–80% B from 10 to 35 min. The detection wavelength was 200 nm, the flow rate was 1 mL/min, and the injection volume was 10 µL [26].

The inter-batch precision of the relative peak area percentages of the three main chromatographic peaks (#4, #11, #19) in P-LHM was investigated for three consecutive days ( $n = 3$ ), and the RSD values were 1.2%, 1.8%, and 1.5% respectively, indicating good repeatability of the method.

## 2.9. NMR Analysis

The sample was completely dissolved after adequate oscillation and ultrasonic treatment, and no insoluble precipitate was observed. The proton nuclear magnetic resonance ( $^1\text{H}$ NMR) spectra were acquired on a Bruker AVANCE III 400 MHz (Bruker Corporation, Rheinstetten, Germany) spectrometer, with deuterated chloroform ( $\text{CDCl}_3$ ) serving as the internal reference. Additionally, the  $^{13}\text{C}$ NMR spectra were obtained using a JNM-ECZ400S/L1 spectrometer (JEOL Ltd., Akishima, Tokyo, Japan), with all chemical shifts reported in  $\delta$  (ppm) units.

## 2.10. UPLC-MS/MS Analysis

The data acquisition instrument system mainly includes Ultra Performance Liquid Chromatography (UPLC, ExionLC AD) and tandem mass spectrometry (MS/MS) (SCIEX, Framingham, MA, USA). The experimental parameters were configured as follows: UPLC separation was performed using an Agilent SB-C18 column (dimensions: 2.1 mm × 100 mm, particle size: 1.8 µm). The mobile phase consisted of two components: solvent A (ultrapure water containing 0.1% formic acid) and solvent B (acetonitrile with 0.1% formic acid). The gradient elution protocol began with 95% A and 5% B, transitioning linearly over 9 min to 5% A and 95% B, which was maintained for 1 min. The initial composition (95% A, 5% B) was then restored in 1.1 min and held for 2.9 min. The flow rate was fixed at 0.35 mL/min, the column temperature was maintained at 40 °C, and the injection volume was 2 µL [21].

This research institute adopted the Plant Extensive Targeted Metabolome Database V4.5 (Wuhan Maiwei Metabolic Biotechnology Co., Ltd., Wuhan, China). No standard substances were used during the testing process. The mass accuracy of the precursor ion annotation was maintained at  $\pm 5$  ppm. For MS/MS fragments, a tolerance of  $\pm 10$  ppm was applied. The obtained MS/MS spectra were matched with the reference spectra in the database for compound labeling.

Broad-target qualitative analysis was conducted at three levels: Level 1: the secondary mass spectrometry of the sample substance (all fragment ions of the substance), RT, and the matching score of the database substance are 0.7 points or above; Level 2: the secondary mass spectrometry of the sample substance, RT and the matching score of the database substance are 0.5–0.7 points; Level 3: the sample substances Q1, Q3, RT, DP and CE are verified to be consistent with the substances in the database.

### 2.11. Stability Analysis

The effects of light, pH, temperature, metal ions and oxidant and reducing agents on the stability of P-LHM were assessed by the absorbance method. The experiment was repeated three times on each group, and the melanin retention rate was calculated according to the following formula:

$$R (\%) = 100 A/A_0, \quad (1)$$

where  $A$  represents the absorbance value of the P-LHM solution after a certain period of time, and  $A_0$  represents the initial absorbance of the P-LHM solution.

#### 2.11.1. Lighting

P-LHM solution was prepared using NaOH (0.1 mol/L). Then, 10 mL was accurately measured and placed in a centrifugal tube, then exposed to UV light (1.5 W/m<sup>2</sup> @ 340 nm), natural light, or darkness. The absorbance values of the samples at 197 nm were measured on days 0, 1, 2, 3, 4 and 5. The melanin retention rate was calculated, and the change curves of the retention rate of P-LHM over time were drawn.

#### 2.11.2. pH

P-LHM solutions (0.1 mg/mL) with pH = 3, 5, 7, 9, and 11 were prepared. Hydrochloric acid and sodium hydroxide were used at a concentration of 1 M each to adjust the pH of the system. Then, 10 mL of the solution was accurately measured and placed in a centrifugal tube at room temperature. The absorbance values of the samples at 197 nm were measured at the 0, 2, 4, 6, 8, 10 and 12 h. The melanin retention rate was calculated, and the change curves of the retention rate of P-LHM over time were drawn.

#### 2.11.3. Temperature

P-LHM solution was prepared using NaOH (0.1 mol/L). Then, 10 mL was accurately measured and placed in a centrifugal tube at 25, 50, 75 and 100 °C for 12 h. The absorbance values of the samples at 197 nm were measured respectively at the 0, 2, 4, 6, 8, 10 and 12 h. The melanin retention rate was calculated, and the change curves of the retention rate of P-LHM over time were drawn.

#### 2.11.4. Metal Ions

P-LHM solution (0.1 mg/mL) containing 0.01 mol/L of each metal ion (NaCl, CaCl<sub>2</sub>, MgCl<sub>2</sub>, CuCl<sub>2</sub>, FeCl<sub>2</sub>, AlCl<sub>3</sub>, and Fe<sub>2</sub>(SO)<sub>4</sub>) was prepared. Then, 10 mL was accurately measured and placed in a centrifugal tube. The absorbance values of the samples at 197 nm were measured on days 0, 1, 2, 3, 4 and 5. The melanin retention rate was calculated, and the change curves of the retention rate of P-LHM over time were drawn.

#### 2.11.5. Oxidizing Agent and Reducing Agent

Different concentrations of oxidizing agent (H<sub>2</sub>O<sub>2</sub>) and reducing agent (Na<sub>2</sub>SO<sub>3</sub>) were added to 0.1 mg/mL of LHM solution, resulting in final concentrations of oxidizing reducing agent of 0.5%, 1.5%, 2.0% and 3.5%. The absorbance values of the samples at

197 nm were measured on days 0, 1, 2, 3, 4 and 5. The melanin retention rate was calculated, and the change curves of the retention rate of P-LHM over time were drawn.

### 2.12. Data Statistics and Chart Drawing

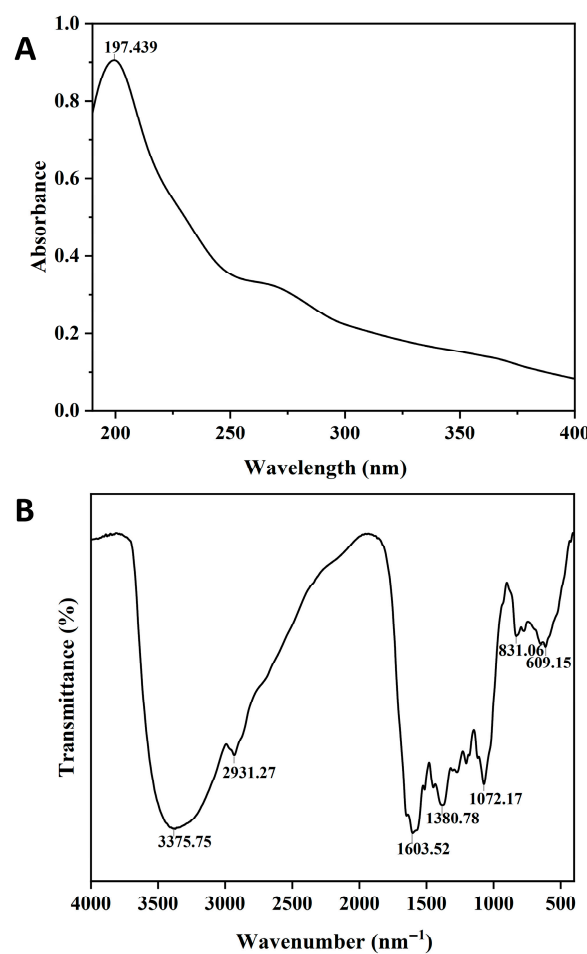
SPSS 20.0 was used for one-way analysis of variance (ANOVA) ( $p < 0.05$ ) and post hoc analysis. Origin 2021 and Mestre Nova 11.0.0 were used to draw related graphics. All experiments were repeated three times.

## 3. Results and Discussion

Based on fresh leaves, the yield of C-LHM was approximately 9.3%, while the P-LHM was about 3.47% (based on C-LHM).

### 3.1. UV-Vis

As shown in Figure 1A, the UV-Vis spectrum of LHM shows strong absorption in the ultraviolet region, and the absorbance value gradually decreases with the increase in wavelength. This is due to changes in the complex conjugated structure of melanin, which is consistent with the typical absorption curve of melanin. The maximum absorbance value of LHM appeared at 197.439 nm, which is similar to the melanin pigment isolated from Indian squid *Uroteuthis duvaucelii* [27]. The difference between the two might be due to different sources of melanin and slight differences in structure [28].



**Figure 1.** Spectral characterization of melanin from *Liquidambar formosana* Hance. (A) UV-Vis absorption spectrum; (B) FT-IR spectrum.



### 3.2. GPC and FT-IR

As shown in Table 1, the GPC analysis revealed that the proportion of P-LHM with a molecular weight greater than  $5.0 \times 10^3$  Da was 22.33%, representing an increase of 19.35% compared to that of C-LHM. By comparison, it can be seen that the molecular weight of the components in the purified P-LHM increased, which is consistent with the typical high-molecular-weight characteristics of melanin.

**Table 1.** The molecular weight distribution of C-LHM and P-LHM in GPC analysis.

| Sample | Molecular Weight Range (Da) | Percentage (%) | Mn     | Mw      |
|--------|-----------------------------|----------------|--------|---------|
| C-LHM  | >5000                       | 2.98           | 14,208 | 673,434 |
|        | 5000–3000                   | 1.05           | 3774   | 3866    |
|        | 3000–2000                   | 1.54           | 2387   | 2423    |
|        | 2000–1000                   | 6.06           | 1319   | 1372    |
|        | <1000                       | 88.36          | 325    | 395     |
| P-LHM  | >5000                       | 22.33          | 12,533 | 32,667  |
|        | 5000~3000                   | 4.36           | 3821   | 3914    |
|        | 3000~2000                   | 4.47           | 2421   | 2458    |
|        | 2000~1000                   | 11.44          | 1348   | 1405    |
|        | <1000                       | 57.39          | 381    | 456     |

As shown in Figure 1B, melanin had characteristic absorption in the  $3600\text{--}3000\text{ cm}^{-1}$ ,  $1650\text{--}1600\text{ cm}^{-1}$  and  $1500\text{--}1400\text{ cm}^{-1}$  ranges of FT-IR. Signals observed between  $3600$  and  $2800\text{ cm}^{-1}$  are attributed to stretching vibrations of amine, amide, carboxylic acid, phenolic, and aromatic amino functional groups. The absorption peak at  $2930\text{ cm}^{-1}$  corresponds to the stretching vibration of aliphatic alkyl C-H bonds [29]. The absorption was strong at  $1600\text{ cm}^{-1}$ , which was caused by the electron absorption of light by the chromophore of eumelanin [30]. The  $1500\text{--}1400\text{ cm}^{-1}$  region is characteristic of aliphatic C-H deformation in melanin. The diagnostic absorption feature of melanin manifested as a peak centered at  $1400\text{ cm}^{-1}$  ( $\text{CH}_2\text{-CH}_3$ ). The absorption at  $1200\text{ cm}^{-1}$  likely originates from C=O stretching in -COOH groups and phenolic C-O stretching. The peak at  $1072\text{ cm}^{-1}$  indicates in-plane C-H deformation of aliphatic structures, which is also a characteristic feature of melanin. Peaks observed in the  $950\text{--}800\text{ cm}^{-1}$  range are associated with out-of-plane bending vibrations of aromatic C-H groups. Absorption bands below  $609\text{ cm}^{-1}$  are attributed to alkene substitution patterns in melanin.

### 3.3. EA

As shown in Table 2, C:N was approximately 49:1, indicating the presence of aliphatic groups in the pigment [31], which is consistent with the FT-IR results. Since the content of S elements before and after purification was similar, the presence of S element could not be ignored. According to the classification method of melanin, it contained 0.199% S, indicating that the LHM contained eumelanin [32]. In addition, the N element content was 0.915%. The synthesis pathways of eumelanin and pheomelanin are similar and might involve cystine and cysteine [33], while typical allomelanin does not contain N element [34], indicating that LHM contains no allomelanin and very little pheomelanin. This result is consistent with those of Zhang et al.'s [35] studies indicating that oyster mushrooms do not contain allomelanin. In summary, it is preliminarily speculated that LHM is composed of eumelanin and pheomelanin.

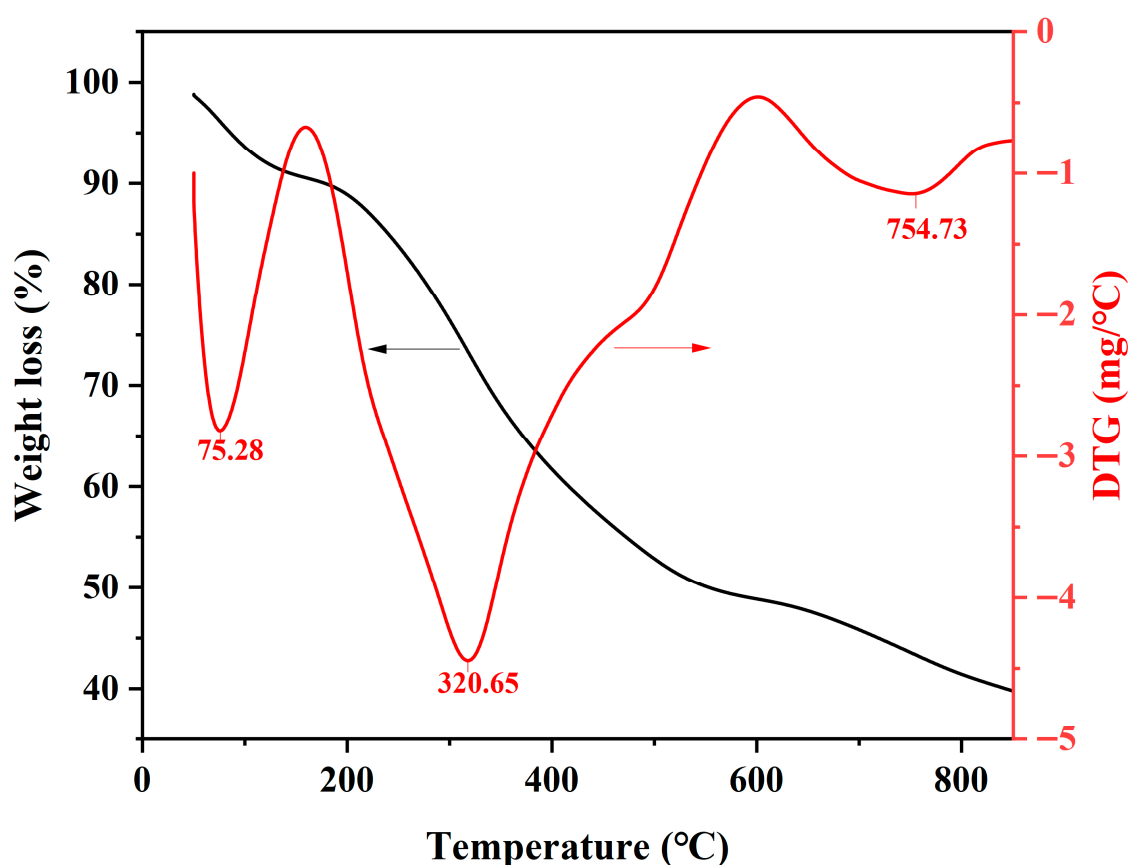
**Table 2.** Elemental composition of the melanin in *Liquidambar formosana* Hance.

| Sample | N (%) | C (%) | H (%) | S (%) | O (%) |
|--------|-------|-------|-------|-------|-------|
| C-LHM  | 0.99  | 34.40 | 4.73  | 0.19  | 59.69 |
| P-LHM  | 0.92  | 43.94 | 4.61  | 0.20  | 50.33 |

Note: The content of oxygen element was calculated using the equation:  $O\% = 100\% - N\% - C\% - H\% - S\%$ .

### 3.4. TGA

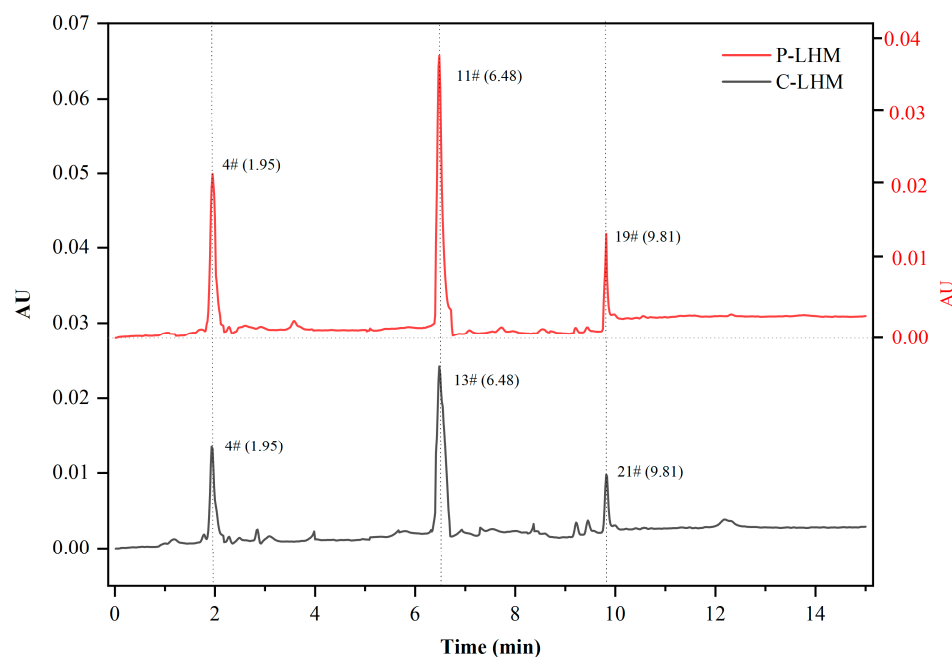
As shown in Figure 2, the TGA profile revealed three distinct thermal degradation stages: The first mass loss peak at 77.43 °C is predominantly attributed to the evaporation of weakly bound water [30]. The second mass loss event at 319.72 °C corresponds primarily to CO<sub>2</sub> evolution, resulting in 72.86% residual mass after this stage. The third degradation phase observed at 754.73 °C is characteristic of decarboxylation processes, with final residue reaching 43.36% at 850 °C. It has been reported that the decomposition temperature of the aliphatic component of melanin is <400 °C, while the aromatic component decomposes at >400 °C. Therefore, it is speculated that LHM contains aliphatic and aromatic components. These results are consistent with the FT-IR results.

**Figure 2.** TGA of melanin in *Liquidambar formosana* Hance.

### 3.5. HPLC

As shown in Figure 3, C-LHM had the largest peak area for 4# (1.95 min), 13# (6.48 min), and 21# (9.81 min), accounting for 14.82%, 26.88%, and 8.17% of the total peak area, respectively. P-LHM was mainly composed of three parts. The peaks area of 4# (1.95 min), 11# (6.48 min), and 19# (9.81 min) accounted for 21.56%, 45.12% and 10.13% of the total peak area, respectively. In summary, the content of total impurities decreased after purification, and the three main components of P-LHM increased from 49.87% to 76.81%.



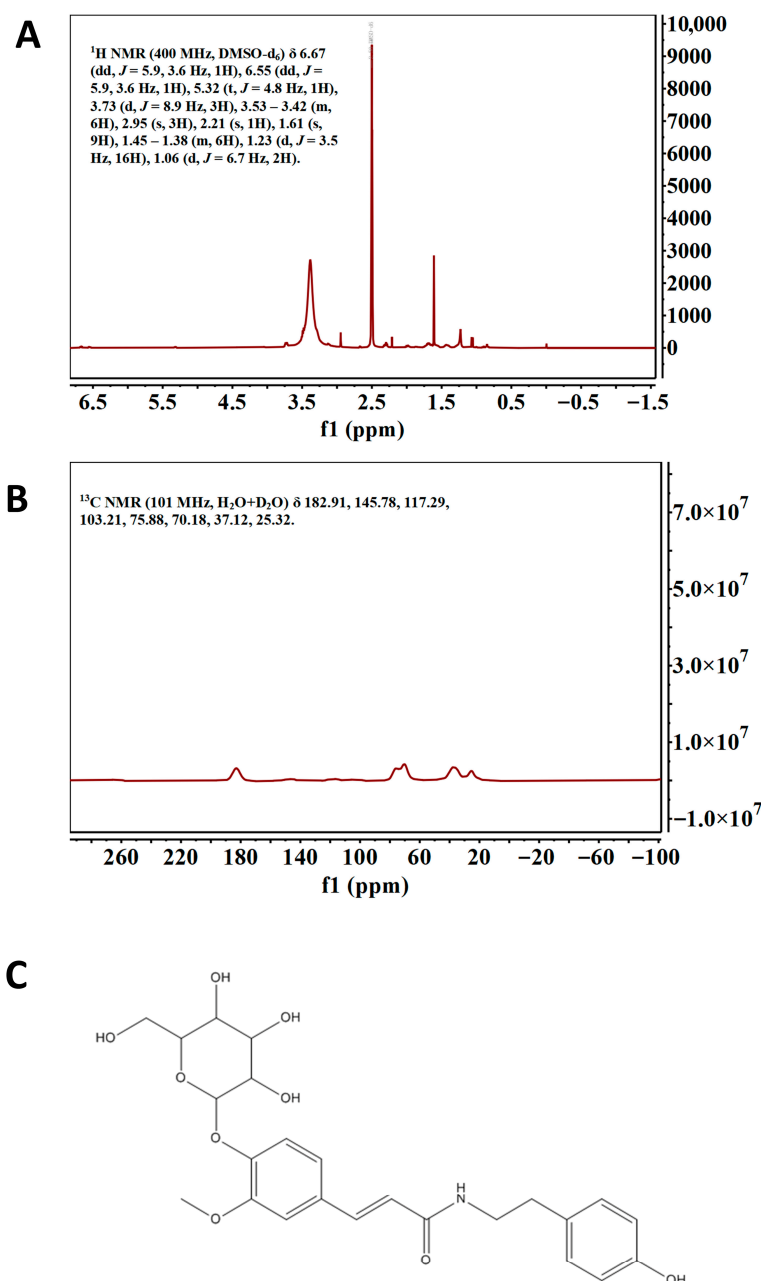


**Figure 3.** HPLC chromatography analysis of crude *Liquidambar formosana* Hance melanin (C-LHM) and purified *Liquidambar formosana* Hance melanin (P-LHM).

### 3.6. NMR

As shown in Figure 4A,B, the data for P-LHM were as follows:  $^1\text{H}$ NMR (400 MHz,  $\text{DMSO-d}_6$ )  $\delta$  6.67 (dd,  $J = 5.9, 3.6$  Hz, 1H), 6.55 (dd,  $J = 5.9, 3.6$  Hz, 1H), 5.32 (t,  $J = 4.8$  Hz, 1H), 3.73 (d,  $J = 8.9$  Hz, 3H), 3.53–3.42 (m, 6H), 2.95 (s, 3H), 2.21 (s, 1H), 1.61 (s, 9H), 1.45–1.38 (m, 6H), 1.23 (d,  $J = 3.5$  Hz, 16H), 1.06 (d,  $J = 6.7$  Hz, 2H);  $^{13}\text{C}$ NMR (101 MHz,  $\text{H}_2\text{O} + \text{D}_2\text{O}$ )  $\delta$  182.91, 145.78, 117.29, 103.21, 75.88, 70.18, 37.12, 25.32.

The  $\delta$  1.06 bimodal signal in  $^1\text{H}$ NMR is derived from a methylene group near the chiral center. The  $\delta$  37.12 signal in  $^{13}\text{C}$ NMR corresponds to a methylene group adjacent to oxygen or nitrogen. The  $\delta$  2.95 signal in  $^1\text{H}$ NMR is a unimodal signal corresponding to  $\delta$  37.12 in  $^{13}\text{C}$ NMR and is assigned to a methylene group in amides or quaternary ammonium salts. The  $\delta$  25.32 signal in  $^{13}\text{C}$ NMR corresponds to long-chain alkyl or tert-butyl groups, which, together with  $\delta$  1.61 (9H) and 1.23 (16H) in  $^1\text{H}$ NMR, suggest the presence of multiple tert-butyl or long-chain alkane structures. The  $\delta$  5.32 triple peak in  $^1\text{H}$ NMR corresponds to the end matrix of the glycoside, and the  $\delta$  103.21 signal in  $^{13}\text{C}$ NMR is a typical end carbon, which further supports the presence of a glycoside structure. The double peaks of  $\delta$  3.73 in  $^1\text{H}$ NMR indicate the presence of methoxy [36]. The polyether chain signals ( $\delta$  3.53–3.42, 6H) correspond to  $\delta$  70.18 and 75.88 in  $^{13}\text{C}$ NMR, representing oxygen-linked structures on the sugar ring. The  $\delta$  6.67 and 6.55 signals in  $^1\text{H}$ NMR are ortho-di-substituted aromatic rings and conjugated olefin structures, which could be attributed to  $\text{CH}_3$ ,  $\text{CH}_2$ , and aromatic protons [37]. These are supported by the  $\delta$  145.78 and 117.29 signals in  $^{13}\text{C}$ NMR, with 145.78 possibly representing quaternary carbon and 117.29 representing tertiary carbon. The  $\delta$  182.9 signal in the  $^{13}\text{C}$ NMR spectrum corresponds to the carbonyl group of the melanoidin quinone moiety [38,39]. The carboxylic acid ionizes to carboxylic acid group, and the resulting carbonyl group exhibits a higher chemical shift, which aligns with the value of  $\delta$  182.91 in  $^{13}\text{C}$ NMR. Combined with other analysis results, we infer that the condensation molecular formula of melanin might be  $([\text{C}_{24}\text{H}_{29}\text{NO}_9]_n)$ . A schematic diagram of potential repeating units in the LHM complex is shown in Figure 4C.

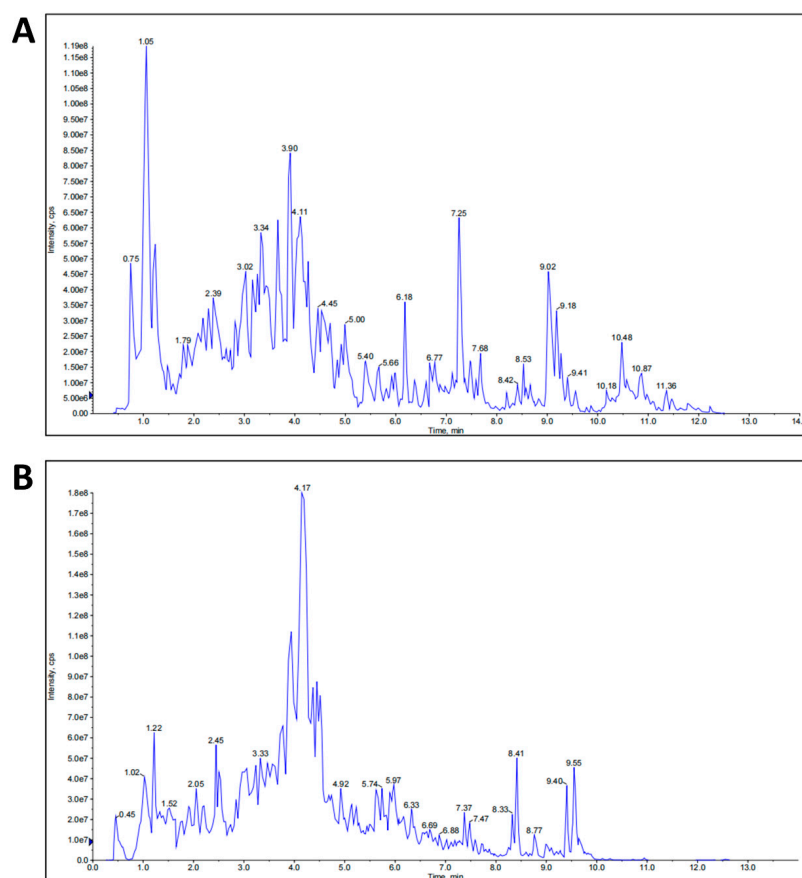


**Figure 4.** NMR spectrogram of the melanin in *Liquidambar formosana* Hance. (A) <sup>1</sup>H NMR, (B) <sup>13</sup>C NMR. (C) Schematic diagram of potential repeating units in the LHM complex.

### 3.7. UPLC-MS/MS

Figure 5 shows the total ion current diagram of UPLC-MS/MS analysis of LHM mixed samples. The scientific notation in the figure follows the instrument output format (8e3 represents  $8 \times 10^3$ ). In MRM mode, the chromatographic peak areas of representative compounds with high abundance in each category (such as quercetin 3-O-xyloside, cryptochlorogenic acid, and ursolic acid) were monitored for three consecutive days ( $n = 3$ ). The inter-batch RSD was consistently under 5% (the specific range was between 2.1% and 4.7%), indicating good repeatability of the method. As shown in Table 3, it was observed that the main types of substances in C-LHM include: flavonoids (14.95%), phenolic acids (13.22%), terpenoids (12.08%), and alkaloids (11.66%). For P-LHM, the main substance types include: flavonoids (24.58%), phenolic acids (19.20%), and terpenoids (16.24%). By comparison, it is evident that after purification, the contents of alkaloids, lipids, amino acids, lignans, coumarins and organic acids decreased, while the contents of flavonoids, phenolic acids

and terpenoids increased significantly. Therefore, it can be inferred that LHM is mainly composed of flavonoids, phenolic acids and terpenoids.



**Figure 5.** UPLC-MS/MS total ion current diagram of the melanin in *Liquidambar formosana* Hance. (A) Negative ion model; (B) positive ion model. The blue triangle symbol on the vertical axis indicates the exact position of the coordinate scale.

**Table 3.** The main types and content of *Liquidambar formosana* leaf melanin analyzed by UPLC-MS/MS.

| Sample | Class I                     | Number of Items | Percentage (%) |
|--------|-----------------------------|-----------------|----------------|
| C-LHM  | Flavonoids                  | 354             | 14.95          |
|        | Phenolic acids              | 313             | 13.22          |
|        | Terpenoids                  | 286             | 12.08          |
|        | Alkaloids                   | 276             | 11.66          |
|        | Lipids                      | 183             | 7.73           |
|        | Amino acids and derivatives | 179             | 7.56           |
|        | Lignans and coumarins       | 175             | 7.39           |
|        | Organic acids               | 108             | 4.56           |
| P-LHM  | Flavonoids                  | 233             | 24.58          |
|        | Phenolic acids              | 182             | 19.20          |
|        | Terpenoids                  | 154             | 16.24          |
|        | Alkaloids                   | 79              | 8.33           |
|        | Lipids                      | 22              | 2.32           |
|        | Amino acids and derivatives | 39              | 4.11           |
|        | Lignans and coumarins       | 52              | 5.49           |
|        | Organic acids               | 37              | 3.90           |

The percentages of each major compound category in Table 4 represent the proportions of total quantified compounds within that category. These small molecules are the main coexisting components within the melanin complex, rather than intrinsic structural compo-

nents of melanin itself. As shown in Table 4, the main flavonoid substances in LHM include quercetin and its glycoside derivatives and kaempferol and its glycoside derivatives, among others. Among these, the contents of quercetin-3-O-xyloside and kaempferol-3-O-arabinside were more than 3%. The main phenolic acids, including benzoylglycerol 3-glucuronide, cryptochlorogenic acid, homogentianic acid, and 4-O-Feruloylquinic acid, all exceeded 4%. The main terpenoids were ursolic acid and its derivatives, and the contents of 7-Deoxygelsemide, asiatic acid, and madasiatic acid were more than 3%.

**Table 4.** The main substances of melanin in *Liquidambar formosana* Hance.

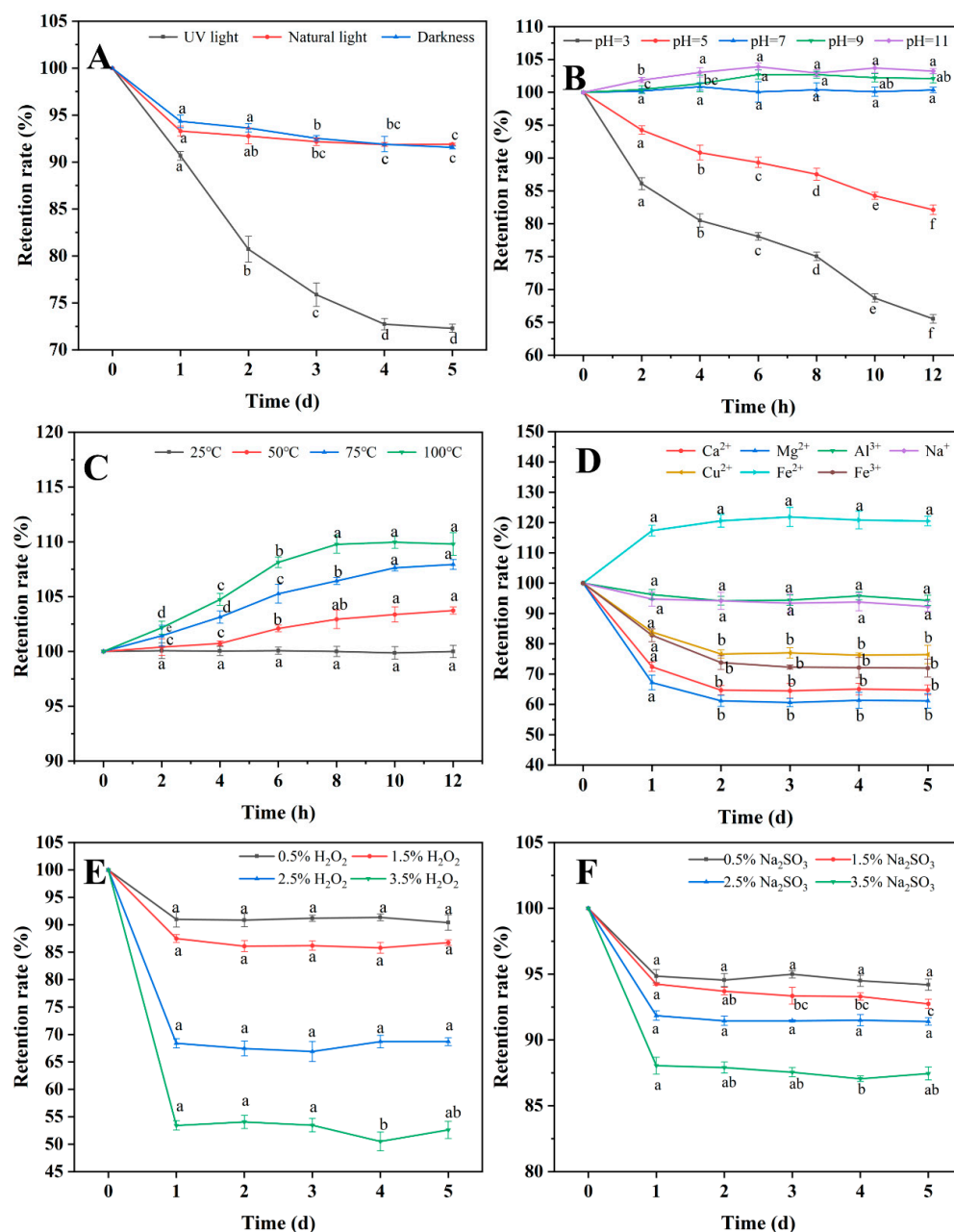
| Class I        | Molecular Formula                               | Level | Molecular Weight | Compound Name                                                 | Percentage (%) |
|----------------|-------------------------------------------------|-------|------------------|---------------------------------------------------------------|----------------|
| Flavonoids     | C <sub>20</sub> H <sub>18</sub> O <sub>11</sub> | 1     | 434.0849         | Quercetin-3-O-xyloside (Reynoutrin)                           | 3.42           |
|                | C <sub>20</sub> H <sub>18</sub> O <sub>10</sub> | 1     | 418.0900         | Kaempferol-3-O-arabinside                                     | 3.24           |
|                | C <sub>22</sub> H <sub>22</sub> O <sub>12</sub> | 1     | 478.1111         | Isorhamnetin-3-O-Glucoside                                    | 2.99           |
|                | C <sub>22</sub> H <sub>24</sub> O <sub>11</sub> | 3     | 464.1319         | Hesperetin-5-O-glucoside                                      | 2.87           |
|                | C <sub>21</sub> H <sub>20</sub> O <sub>12</sub> | 1     | 464.0955         | Quercetin-4'-O-glucoside (Spiraeoside)                        | 2.87           |
|                | C <sub>21</sub> H <sub>20</sub> O <sub>12</sub> | 1     | 464.0955         | Quercetin-7-O-glucoside                                       | 2.84           |
|                | C <sub>21</sub> H <sub>20</sub> O <sub>11</sub> | 2     | 448.1006         | Luteolin-3'-O-glucoside                                       | 2.82           |
|                | C <sub>16</sub> H <sub>14</sub> O <sub>6</sub>  | 3     | 302.0790         | Dihydrokaempferide                                            | 2.72           |
|                | C <sub>22</sub> H <sub>22</sub> O <sub>12</sub> | 1     | 478.1111         | 6-C-Methylquercetin-3-O-glucoside                             | 2.35           |
|                | C <sub>22</sub> H <sub>24</sub> O <sub>11</sub> | 3     | 464.1313         | Homoeriodictyol 7-O-glucoside                                 | 1.95           |
|                | C <sub>21</sub> H <sub>22</sub> O <sub>11</sub> | 3     | 450.1162         | Dihydrokaempferol-3-O-glucoside                               | 1.39           |
|                | C <sub>20</sub> H <sub>18</sub> O <sub>10</sub> | 1     | 418.0900         | Kaempferol-3-O-arabinside (Juglanin)                          | 1.11           |
| Phenolic acids | C <sub>16</sub> H <sub>20</sub> O <sub>10</sub> | 2     | 372.1056         | Benzoylglycerol 3-glucuronide                                 | 6.65           |
|                | C <sub>16</sub> H <sub>18</sub> O <sub>9</sub>  | 1     | 354.0951         | Cryptochlorogenic acid (4-O-Caffeoylquinic acid)              | 4.54           |
|                | C <sub>8</sub> H <sub>8</sub> O <sub>4</sub>    | 3     | 168.0423         | Homogentisic acid                                             | 4.26           |
|                | C <sub>17</sub> H <sub>20</sub> O <sub>9</sub>  | 1     | 368.1107         | 4-O-Feruloylquinic acid                                       | 4.01           |
|                | C <sub>8</sub> H <sub>8</sub> O <sub>4</sub>    | 3     | 168.0423         | 3,4-Dihydroxybenzeneacetic acid                               | 3.97           |
|                | C <sub>16</sub> H <sub>22</sub> O <sub>4</sub>  | 1     | 278.1518         | Monooctyl Phthalate                                           | 3.21           |
|                | C <sub>12</sub> H <sub>12</sub> O <sub>9</sub>  | 3     | 300.0481         | Vnilyltartaric acid                                           | 2.99           |
|                | C <sub>16</sub> H <sub>18</sub> O <sub>8</sub>  | 3     | 338.1002         | 4-O-p-Coumaroylquinic acid                                    | 2.95           |
|                | C <sub>9</sub> H <sub>8</sub> O <sub>4</sub>    | 3     | 180.0423         | Caffeic acid                                                  | 2.88           |
|                | C <sub>16</sub> H <sub>22</sub> O <sub>4</sub>  | 1     | 278.1518         | Diisobutyl phthalate                                          | 2.84           |
|                | C <sub>9</sub> H <sub>10</sub> O <sub>2</sub>   | 1     | 150.0681         | Hydrocinnamic acid                                            | 2.44           |
| Terpenoids     | C <sub>10</sub> H <sub>12</sub> O <sub>4</sub>  | 3     | 196.0736         | 7-Deoxygelsemide                                              | 4.81           |
|                | C <sub>30</sub> H <sub>48</sub> O <sub>5</sub>  | 1     | 488.3502         | Asiatic acid                                                  | 4.22           |
|                | C <sub>30</sub> H <sub>48</sub> O <sub>5</sub>  | 1     | 488.3502         | Madasiatic acid                                               | 4.08           |
|                | C <sub>15</sub> H <sub>18</sub> O <sub>3</sub>  | 1     | 246.1256         | Leucodin                                                      | 2.85           |
|                | C <sub>12</sub> H <sub>18</sub> O <sub>2</sub>  | 1     | 194.1307         | 5-hydroxy-1,4a-dimethyl-3,4,5,6,7,8-hexahydronaphthalen-2-one | 2.60           |
|                | C <sub>30</sub> H <sub>48</sub> O <sub>4</sub>  | 1     | 472.3553         | Pomolic acid                                                  | 2.53           |
|                | C <sub>30</sub> H <sub>48</sub> O <sub>4</sub>  | 1     | 472.3553         | 2,3-Dihydroxy-12-ursen-28-oic acid                            | 2.51           |
|                | C <sub>24</sub> H <sub>30</sub> O <sub>12</sub> | 3     | 510.1737         | 6-O-Trans-Caffeoyl Ajugol                                     | 2.35           |
|                | C <sub>21</sub> H <sub>36</sub> O <sub>11</sub> | 3     | 464.2258         | Rhodiolide F                                                  | 2.06           |
|                | C <sub>16</sub> H <sub>26</sub> O <sub>7</sub>  | 2     | 330.1679         | Schizonepetoside A                                            | 1.79           |
|                | C <sub>30</sub> H <sub>46</sub> O <sub>5</sub>  | 2     | 486.3345         | 2,19-Dihydroxy-3-oxours-12-en-28-oic acid                     | 1.53           |
|                | C <sub>12</sub> H <sub>10</sub> O <sub>4</sub>  | 3     | 218.0579         | Baldrinal                                                     | 1.32           |
|                | C <sub>30</sub> H <sub>48</sub> O <sub>4</sub>  | 1     | 472.3553         | 2-Hydroxyursolic acid                                         | 1.26           |

### 3.8. Stability Analysis

Given that melanin itself is a naturally heterogeneous polymer mixture, the concept of “purity” differs from that of small-molecule compounds. After reacting, its chemical composition changes, generating new polymers. However, these newly generated products are also part of the melanin material family and are generally considered more stable and more mature forms (similar polymerization processes occur during many natural melanin maturation processes). The newly generated compounds are not typically considered “impurities” or “degradation impurities” but rather lead to the evolution of material functional properties. Therefore, from the perspective of functional stability, the retention rate of absorbance (even enhancement) actually indicates that the material can transform into a more stable state with potentially better performance.

### 3.8.1. Effect of Light on the Stability of Melanin in P-LHM

As can be seen in Figure 6A, the melanin retention rate dropped sharply to 72.06% from the 1st to the 4th day under UV light, indicating that UV light has a strong degradation effect on melanin. It is speculated that melanin has a certain absorption capacity for UV light, leading to the degradation of its internal groups [40]. The retention rate of P-LHM was almost unchanged under natural light and darkness conditions. Therefore, it is concluded that P-LHM is stable under natural light and darkness conditions, and UV light has a certain effect on its stability. Therefore, when using P-LHM in food processing, it is advisable to avoid using UV light for sterilization, as this can significantly ( $p < 0.05$ ) reduce color fading caused by intense ultraviolet light.



**Figure 6.** The stability of melanin in *Liquidambar formosana* Hance under different factors. (A) Lighting, (B) pH, (C) temperature analysis, (D) metal ions, (E) oxidizing, and (F) reducing agents. Different letters indicate significant differences ( $p < 0.05$ ).

### 3.8.2. Effect of pH on the Stability of Melanin in P-LHM

As can be seen in Figure 6B, at pH = 3, melanin in LHM may undergo protonation in the conjugated system of pigment molecules, disrupting electron delocalization and resulting in an unstable state. The retention rate was almost unchanged at pH = 7. The retention rate increased slightly in the range of pH 9–11, which might be due to the deprotonation of acidic groups of pigment molecules under alkaline conditions, which enhanced the light absorption ability at specific wavelengths. It is also possible that alkaline conditions promote the oxidation or polymerization of pigment molecules, resulting in increases in chromophore and the conjugation degree, which stabilizes P-LHM [41]. This requires subsequent structural analysis of the products of the reaction system for confirmation. Therefore, it is concluded that P-LHM is stable under neutral and alkaline conditions, and its color is easy to change under acidic conditions. Stability under neutral and alkaline conditions means that P-LHM has the potential to be applied to baked goods, pastries, rice products, plant-based products, and so on.

### 3.8.3. Effect of Temperature on the Stability of Melanin in P-LHM

As can be seen in Figure 6C, between 0 and 10 h, with the increase in temperature, the retention rate of P-LHM showed an increasing trend. This might be due to the enhancement of oxidative polymerization of pigment molecules under high-temperature conditions and the repolymerization of thermal degradation products. The absorbance of the sample at a wavelength of 197 nm increased at 50 °C, 75 °C and 100 °C in the later stage of the reaction. This might be due to the formation of new compounds with larger conjugated structures and stronger molar absorption coefficients in the system. This led to a retention rate exceeding 100% after calculation. At 25 °C, the melanin retention rate of P-LHM was almost unchanged and in a stable state. Therefore, within a certain range, the retention rate of P-LHM showed an upward trend with the increase in temperature and the passage of time, and then tended to stabilize, which is consistent with the observed color changes. Compared to melanin from walnut flower [42], P-LHM exhibited stronger thermal stability at high temperatures. These results provide a theoretical basis for the subsequent application of P-LHM.

### 3.8.4. Effect of Metal Ions on the Stability of Melanin in P-LHM

As can be seen in Figure 6D, the retention rate of P-LHM was stable in Na<sup>+</sup> solution. This is because Na<sup>+</sup> is an inert alkali metal ion with low charge density, making it difficult to form stable coordination bonds with functional groups in melanin, such as phenolic hydroxyl and carboxyl groups. Therefore, it had no significant effect on the pigment structure, and the retention rate was stable. Al<sup>3+</sup> usually has a coordination number of 6, which may form a single layer of passivation structures on the pigment surface. This inhibits further oxidation and degradation, maintaining stability [43]. Ca<sup>2+</sup> and Mg<sup>2+</sup> are alkaline earth metal ions (with medium charge density). They might form ionic bridges with multiple carboxyl or phenolic hydroxyl groups in melanin, causing the pigment molecules to cross-link and aggregate into insoluble complexes. The concentration of free pigments in the solution decreased, leading to a reduction in the retention rate. Experimentally, it was found that some turbidity occurred in the Ca<sup>2+</sup> and Mg<sup>2+</sup> solution systems. The melanin extracted by Wang et al. [44] from *Osmanthus fragrans* seeds also showed a similar reaction. Cu<sup>2+</sup> and Fe<sup>3+</sup> are transition metal ions that catalyze the oxidative degradation of phenolic units in melanin, resulting in the breakdown of chromophore and a decrease in retention. Fe<sup>2+</sup> has reducing properties. As a catalyst for P-LHM, it improves the retention rate of P-LHM, which is similar to *Guizotia abyssinica* [45]. To summarize, P-LHM had a higher pigment retention rate in Na<sup>+</sup>, Al<sup>3+</sup>, and Fe<sup>2+</sup> solutions, maintaining a relatively stable state.



However,  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{Cu}^{2+}$ , and  $\text{Fe}^{3+}$  reduced the retention rate of P-LHM. Compared to water-soluble squid ink melanin [46], P-LHM did not precipitate when in contact with metal ions ( $\text{Fe}^{3+}$ ,  $\text{Fe}^{2+}$ ,  $\text{Al}^{3+}$ , and  $\text{Cu}^{2+}$ ), making it more suitable for use as a food coloring agent in food processing.

### 3.8.5. Effect of Oxidizing and Reducing Agents on the Stability of Melanin in P-LHM

As can be seen in Figure 6E, the retention rate of P-LHM in  $\text{H}_2\text{O}_2$  solution systems with different concentrations decreased rapidly within the first day. Meanwhile, the retention rate decreased more significantly with the increase in concentration. When the concentration of  $\text{H}_2\text{O}_2$  was 3.5%, the melanin retention rate was only 52.15% on the 5th day, and the pigment was nearly colorless. This indicates that P-LHM could play a certain antioxidant role and has the potential to be added to food as an antioxidant.

$\text{Na}_2\text{SO}_3$  is an efficient, mild, water-soluble, food-grade, and widely used classic reducing agent, commonly applied in industrial bleaching and preservation. It can effectively disrupt the conjugated structure of most natural pigments without being so aggressive as to cause unpredictable, complex side reactions (such as severe oxidation or polymerization). It is very suitable for studying the stability and reaction characteristics of plant pigments. As can be seen in Figure 6F, the retention rate of P-LHM decreased with the increase in  $\text{Na}_2\text{SO}_3$  concentration. When the  $\text{Na}_2\text{SO}_3$  concentration was 0.5–2.5%, the retention rate of P-LHM remained above 90% after 5 days. In the solution system with 3.5%  $\text{Na}_2\text{SO}_3$  concentration, the retention rate dropped to 87.65% after 5 days, and the solution turned dark brown.  $\text{Na}_2\text{SO}_3$  as a reducing agent destroys the chromophore (mainly the conjugated double bond system) in the black pigment molecules of P-LHM, thus causing the color to fade and the retention rate to decrease. The results indicate that a low concentration of  $\text{Na}_2\text{SO}_3$  solution had little effect on the stability of P-LHM, but a high concentration of  $\text{Na}_2\text{SO}_3$  solution affected its stability. Therefore, in food processing, strong reducing agents should be avoided in combination to prevent redox reactions that may affect color.

## 4. Conclusions

In this study, melanin was purified by the alcohol precipitation method and macroporous adsorption resin, and its structure and stability were studied in detail. The results show that LHM had a strong absorption capacity at a wavelength of 197 nm and displayed a typical characteristic absorption peak. Additionally, P-LHM is a melanin-rich complex, where eumelanin and pheomelanin polymers are intimately associated with specific flavonoids, phenolic acids, and terpenoids. Its condensation molecular formula might be  $([\text{C}_{24}\text{H}_{29}\text{NO}_9]_n)$ . Evaluation of P-LHM's stability showed that under specific conditions (natural light, darkness,  $\text{pH} = 7\text{--}11$ ,  $25\text{--}100\text{ }^\circ\text{C}$ ,  $\text{Na}^+$ ,  $\text{Al}^{3+}$ ,  $\text{Fe}^{2+}$  solution, and low concentrations of reducing agents), the retention rate of P-LHM was  $>90\%$ .

Based on its outstanding stability, LHM has the potential to be used as a food ingredient in the processing of black-colored foods, such as baking, steamed food, and beverage sterilization. This could alleviate pressure on existing melanin resources. However, it should be noted that these potential applications have not been verified in actual food matrices, which remains a key objective for subsequent research.

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