



## A novel approach for authentication of shellac resin in the shellac-based edible coatings: Contain shellac or not in the fruit wax preservative coating

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### ABSTRACT

As an edible coating substrate, the detection of shellac resin has always been an intractable problem. In this paper, an authentication method of shellac resin in shellac-based edible coatings was established. Results showed that the authentication of shellac resin could be skillfully transformed as the identification of 13 targeted metabolites which were monomer compounds of shellac resin. The 13 targeted metabolites were further divided into 6 differential metabolites and 7 common metabolites with the metabonomic method and difference analysis of targeted metabolite contents. Then, four commercial soi-disant shellac-based coating solutions were selected to verify the feasibility of this method, and 7 common metabolites were detected in only one commercial sample, highly consistent with the results of shellac resin. All the above results indicated that the targeted metabolomics approach established in this study could provide a scientific basis for the qualitative authentication of shellac resin in the preservation coating.

### Introduction

Preservation is an important link in the post-harvest management of fruits and vegetables, which affects the supply, shelf life, and prices of agricultural products as well as food quality and safety (Miller, Silva, & Brandão, 2013). The coating is an essential method for preservation of fruits and vegetables; it reduces the negative impact on agricultural products by regulating the oxygen, carbon dioxide, ethylene, temperature, microorganism growth, and pressure, thus extending the shelf life (Luangtana-anan & Limmatvapirat, 2019). Considering the different physiological characteristics of fruits and vegetables, the development of preservation coatings, especially edible preservation coatings, with different functions has attracted a lot of interest (Y. Zhang et al., 2019; Yousuf, Qadri, & Srivastava, 2018).

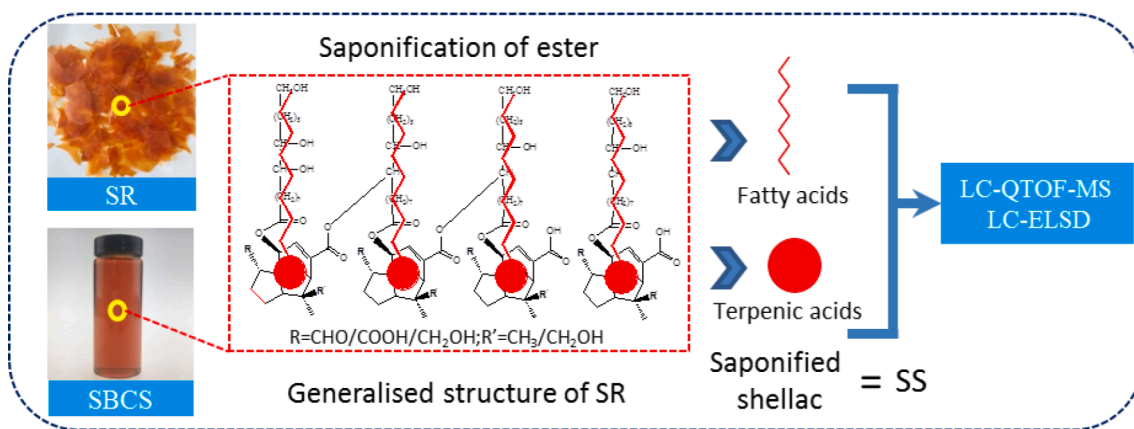
Shellac is a natural resin mixture secreted by scale insects (*Kerria lacca*) that consists of resin, pigment, and wax (Kun Li et al., 2016; A. R. Patel et al., 2013). It is mainly found in East, South, and Southeast Asian countries, including China, India, Thailand, Vietnam, and Myanmar

(Luangtana-anan et al., 2007). Shellac resin has excellent film-forming, water-resistant, and barrier properties and is widely used in coating and functional materials (Bellan, Pearsall, Crokek, & Langer, 2012; A. R. Patel et al., 2013). Moreover, being an animal-based resin, it has natural properties and good biocompatibility and biodegradability. FDA has approved its application in food and drug-related field (Patel et al., 2014), especially for fruit and vegetable preservation (Soradech, Nunthanid, Limmatvapirat, & Luangtana-anan, 2012). For example, Shellac has been widely used in extending the shelf lives of apples (Chauhan, Raju, Singh, & Bawa, 2011), bananas (Soradech, Nunthanid, Limmatvapirat, & Luangtana-anan, 2017), tomatoes (Chauhan et al., 2015), and pepper (Chitravathi, Chauhan, & Raju, 2016). Nevertheless, contamination and even fake shellac resin coating solutions have appeared due to high price fluctuations over recent years. For example, there have been several commercial disputes over whether a coating material contains shellac resin in China. However, the lack of appropriate detection methods also makes it impossible for consumers to authenticate various samples and prevents relevant authorities from reacting.

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**Scheme 1.** The schematic diagram of the saponified process for samples.

Our research group has been focused on examining the shellac-based functional packaging membrane such as leaf-mimetic membrane (Zhou et al., 2021), gas-permeation controllable packaging membrane (Ma et al., 2021b), and edible antibacterial film (Ma et al., 2021a). Shellac resin contains lactones and lactides composed of a variety of fatty acids and sesquiterpenes (Kai Li et al., 2020), so it has no fixed structure or composition, which makes it difficult to be detected. Furthermore, in the shellac-based coating formula, shellac resin is the substrate, combined with other types of polymers or auxiliary additives (Saber, Chockchaisawasdee, Golding, Scarlett, & Stathopoulos, 2017), which also challenges the detection.

Shellac resin is commonly detected with the gas chromatography-mass spectrometry method, which is essentially based on analyzing the monomer composition, namely fatty acids and sesquiterpenic acids in shellac resin (Coelho, Nanabala, Ménager, Commereuc, & Verney, 2012; Colombini, Bonaduce, & Gautier, 2003; Sutherland & del Río, 2014; Wang, Ishida, Ohtani, Tsuge, & Nakayama, 1999). However, gas chromatography requires the components to be vaporized within the detection temperature range, which may lead to information loss (Tamburini, Dyer, & Bonaduce, 2017). Some studies (Coelho et al., 2012; Tamburini et al., 2017) explored the detection of shellac resin with the LC-MS method, which further promoted a deep understanding of the chemical composition of shellac resin, and provided insight into the qualitative detection of shellac resin in shellac-based coatings for preservation. Yet, it still remains unclear whether shellac-based coating solution contains shellac resin. In fact, qualitative analysis of a natural resin with such a complex composition may be challenging to perform. The main reason is that there are too many components that make up this polyester resin, and there is no standard protocol or even a fixed structure. Moreover, due to the wide range of raw materials, the regional characteristics of the sample and the universality of the analysis method must be considered.

Metabolomics is a comprehensive analysis approach used for the qualitative or quantitative analysis of a large number of metabolites. Since its discovery (Nicholson, Lindon, & Holmes, 1999) *et al.*, it has been widely applied in a variety of disciplines, such as microbial metabolism study (Jin et al., 2019), drug development (Gou, Feng, Fan, Zhu, & Hu, 2017), and toxicity evaluation (Su, Wang, Bai, Chen, & Pei, 2019). The combination of metabolomics with modern instrumental analysis methods and chemometrics has greatly promoted its application in the food industry. For example, with the development of high-precision and high-resolution instruments and continuous improvement of databases, the targeted metabolomics approach has been widely used to authenticate the compounds in cordyceps sinensis (J. Zhang et al., 2015), fruit juice (J. Zhang et al., 2018), nuts (Klockmann, Reiner, Cain, & Fischer, 2017), and raw plant materials (Masson, Liberto, Brevard, Bicchi, & Rubiolo, 2014) as targeted metabolites.

The present paper explored a targeted metabolomic analysis

approach coupled with LC-TOF-MS/MS and LC-ELSD to authentic shellac resin in shellac-based coatings. To avoid biases, the compounds (fatty acids and terpenic acids) composing shellac resin monomers were chosen as the targeted metabolites. The difference analysis of the monomer composition of shellac resin from major manufacturing countries was performed to analyze commonly targeted metabolites as metabolomic biomarkers, develop a method to authenticate, and analyze shellac resin. We also aimed to prove the reliability of the method with the authentication analysis of commercial samples and control samples, thus providing scientific bases for the qualitative authentication of shellac resin in shellac-based coating products for fruit and vegetable preservation.

## Materials and methods

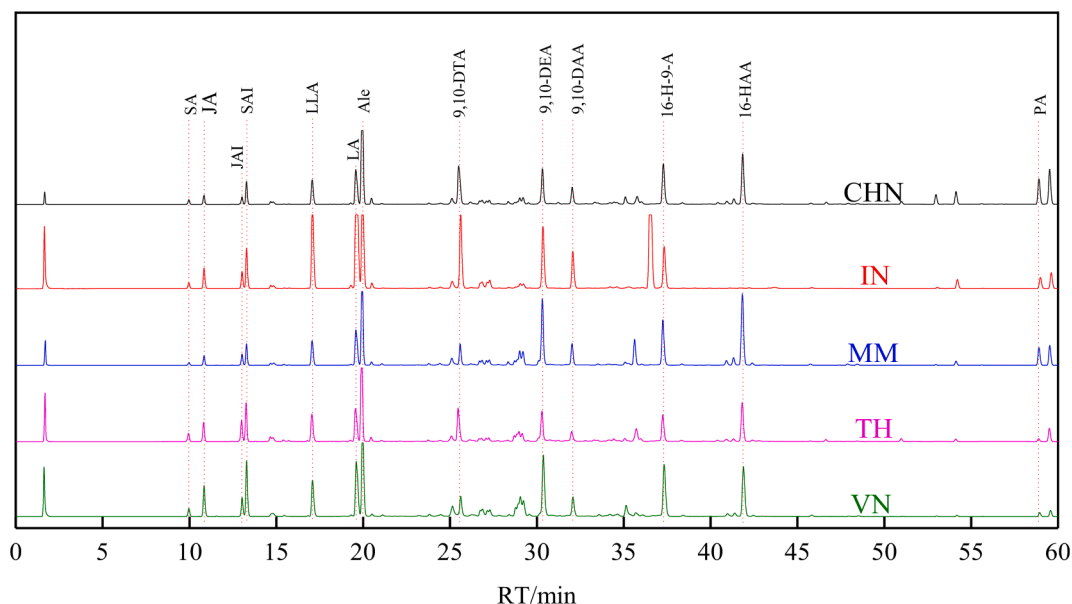
### Samples collection and treatment

All the chemical agents were analytical grade and purchased from Aladdin Co. Ltd (Shanghai, China). Aleuritic acid ( $P \geq 98\%$ ) was purchased from TCI Development Co., Ltd (Shanghai, China).

All samples were taken from commercial companies in China engaging in shellac product manufacturing (Table S1), which originated from major shellac manufacturing countries in East Asia, South Asia, and Southeast Asia, including China, India, Myanmar, Thailand, and Vietnam (Fig. S1). Non-Chinese shellac samples were raw materials imported by Chinese processing companies from the aforementioned countries. Due to the difficulty of sample collection, the sample size from different countries was not equal. Therefore, we chose to analyze all the collected samples of shellac so as to enhance the representativeness of each group of samples to the corresponding major producer of shellac. The sample of the quality control (QC) group was a mixture of the 5 samples (Table S1), with each sample accounting for 20% (w/w). Among shellac-based coating solutions (SBCS), G-1, G-2, G-3, and G-4 were commercial products in the Chinese market, which have been applied to preserve apples, citrus, and other fruits by coating. G-5 was a laboratory-made shellac-resin-based coating solution containing 9 wt% shellac resin.

### Pretreatment of shellac resin and shellac-based coating solution

The saponified process of shellac resin in coating liquid was performed according to the previously described approach (Nagappayya & Gaikar, 2010) and the main method is shown in Scheme 1. Samples (50 g) of shellac resin (SR) or shellac-based coating solution (SBCS) were put in a 250 mL round-bottom flask with a condenser, and the saponification process was implemented in 200 mL 25% (w/w) sodium hydroxide solution for 30 h at 90 °C. The saponified solution was salted out by saturated sodium chloride solution to remove the precipitate. Diatomite



**Fig. 1.** The chromatogram of SS from saponified shellac by LC-ELSD quantitative analysis method. CHN-China, IN-India, MM- Myanmar, TH-Thailand, VN-Vietnam.

was added to the salting-out solution, and the filtrate was obtained after filtration. The filtrate was acidized by 18% HCl solution to  $\text{pH} \approx 2.0$ , and the filter cake B (marked as SS) was obtained after washing, filtration, and drying. The product was dissolved in methanol for subsequent qualitative and quantitative analysis.

#### LC-QTOF-MS/MS analysis

The liquid chromatogram (Agilent Technologies, USA) was employed to separate saponified compounds from native shellac and bleached shellac with a Zorbax 5 SB-C18 (250 × 4.6 mm, 5  $\mu\text{m}$ ) column. The column temperature was 30 °C; the injection volume was 10  $\mu\text{L}$ , and the mobile phase was acetonitrile (A) and 0.1% formic acid-water (B). A gradient elution program was utilized for the separation and determination, A (5–100%) 60 min, and the flow rate was 1.0 mL/min. Mass spectrometric analysis was performed on a high-resolution mass spectrometer (6530 Accurate-Mass Q-TOF, Agilent, USA) with a capillary voltage of 3.5 kV. Under negative ionization modes, the mass spectra that were acquired ranged from  $m/z$  50–1700, containing 2 scans in roughly 3.0 s. The electrospray ionization (ESI) source temperature was set at 350 °C, the airflow of 540 L/h, collision energy (CE) was 10 V, and the collision energy range was  $\pm 1.0$  V. The MS and MS/MS spectra were analyzed by HPLC-MS MassLynx V4.1 software.

#### LC-ELSD quantitative analysis

Liquid chromatogram separation was performed on an Agilent 1260 LC system. The chromatographic separation condition was the same as that used in the qualitative analysis method by LC-QTOF-MS/MS. The optimum parameters of ELSD for evaporator temperature, drift tube temperature, and carrier gas (high purity nitrogen) flow rate were 70 °C, 60 °C, and 1.6 L/min, respectively. In order to eliminate the difference in retention time between LC-MS and LC-ELSD and facilitate the comparison and identification of compounds in the LC-ELSD chromatogram, aleuritic acid was chosen as the standard to correct the LC-ELSD chromatogram; it is referred to as ion chromatogram of LC-MS. All the data obtained by the LC-ELSD method was analyzed by SEMICA 14.0 software, Metabo Analyst data analysis website (<https://www.metaboanalyst.ca/>), and Origin 9.1 plotting software.

#### Targeted metabolite identification

Before metabolomic analysis, the first step was to gather information about a number of known biomarkers in shellac resin from the literature. Shellac resin is a natural hydrophobic biopolymer containing polyesters of hydroxy fatty acid and sesquiterpene acids (Tamburini et al., 2017; Wang et al., 1999). As shown in Table 2, 22 species compounds from shellac have been identified in the literature (Coelho et al., 2012; Colombini et al., 2003; Lu et al., 2014; Sutherland et al., 2014; Tamburini et al., 2017; Wang et al., 1999). In the present study, all compounds were identified based on mass accuracy, and spectral matching and their characteristic ions were compared with existing data (e.g., in Fig. S2, the LC-QTOF-MS/MS ion chromatograms, the peak of 303.31  $m/z$  in negative mode was conformed with the molecular weight of aleuritic acid (Ale) (Coelho et al., 2012; Colombini et al., 2003) and the MS/MS spectra, showed in supporting information, was consistent with the literature (Tamburini et al., 2017). Hence, the peak of 303.31  $m/z$  at the retention time of 20.408 min could be identified as aleuritic acid. The compounds composed of shellac, including 11 species of fatty acids and 11 species sesquiterpene acids, are listed in Table S2. All the identified compounds were validated when only the molecular weight and MS/MS characteristic ions were anastomosed with the experimental spectra simultaneously.

## Results and discussion

#### Identification of targeted metabolites in shellac resin

In terms of the structure, shellac resin contains lactones and lactides composed of chain-like and ring-like components (Kai Li et al., 2020; Kai Li et al., 2016). In terms of composition, shellac resin is a complex mixture composed of structurally similar compounds (Kun Li et al., 2016; Nagappayya et al., 2010; Sedaghat Doost et al., 2019). The structure and composition of shellac resin may be affected by the species of shellac insects and host plants, as well as climatic conditions (Tamburini et al., 2017). Therefore, authenticate and analyze differential or common metabolites within shellac resin as the targeted compound, especially those targeted compounds. To solve the above problems, the chain-like and ring-like compounds composing shellac resin monomers were screened for targeted metabolites. According to literature reports

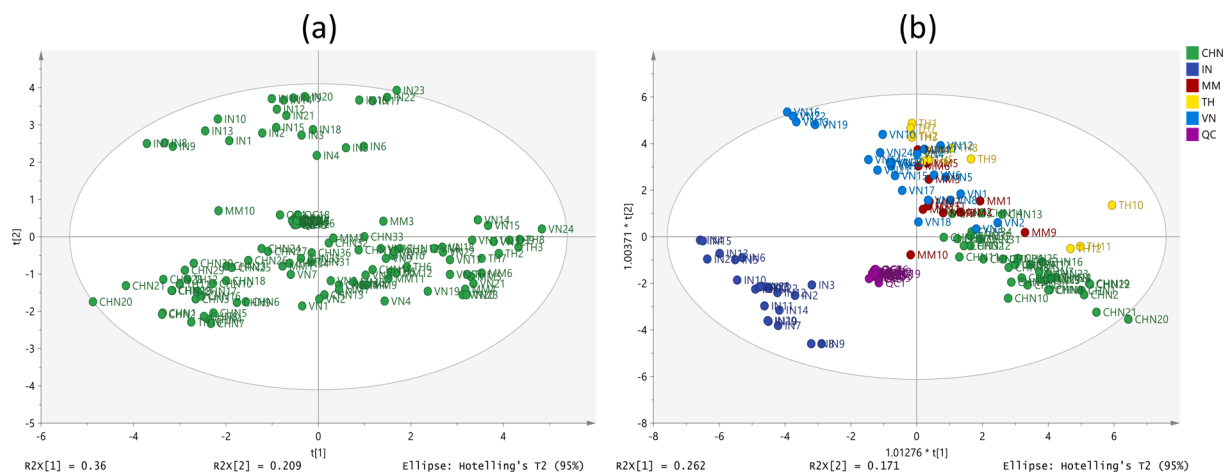


Fig. 2. The scores plot of PCA (a) analysis in unsupervised mode and PLS-DA (b) analysis in supervised mode, relative content data of targeted compounds.

(Coelho et al., 2012; Colombini et al., 2003; Lu et al., 2014; Sutherland et al., 2014; Tamburini et al., 2017; Wang et al., 1999), 22 compounds composing shellac resin monomers (Table S2), including 11 chain-like fatty acids and 11 ring-like terpenic acids, were found. Shellac resin samples originated from major shellac manufacturing countries in East Asia, South Asia, and Southeast Asia, including China, India, Myanmar, Thailand, and Vietnam. See Table S1 for the amount and distribution of samples.

Before detecting the monomer components of shellac resin, the shellac resin was saponified according to the method for preparing base hydrolysis for aleuritic acid (Nagappayya et al., 2010)(Scheme 1). The key point was to acidify the filtrate after saponification and detect targeted metabolic compounds with precipitates as the object. The function of the salting-out method was to remove most of the aleuritic acid, so as to prevent the sensitivity and accuracy of the detection of other compounds from interfering due to the excessive content of aleuritic acid alone (Fig. S3).

The saponified samples that were obtained using the method presented in Scheme 1 were marked as SS; the total ion flow diagram of LC-TOF-MS/MS is shown in Fig. S2. According to the molecular weight and ion fragment characteristics of SS and with reference to literature reports (Table S2), 17 compounds were screened out from the SS of shellac resin from different countries (Table S3). Due to the difference in ionization response of monomer compounds of different structures (Bonfiglio, King, Olah, & Merkle, 1999), the SS's mass spectrometry detector (Fig. S2) and ELSD detector (Fig. 1) showed significant differences in the intensity of their response to targeted metabolites. Given such differences, 4 compounds (laccishellolic acid, laccijalaric acid, butolic acid, and myristic acid, Table S3b) in LC-ELSD, whose detectors had insignificant response signals, were further removed, thus limiting the screening of targeted metabolites of shellac resin to 13 compounds (Table S3a). Furthermore, considering the comparative analysis of shellac resin from different countries intended to find commonly targeted metabolites that are representative of shellac resin, peak-area

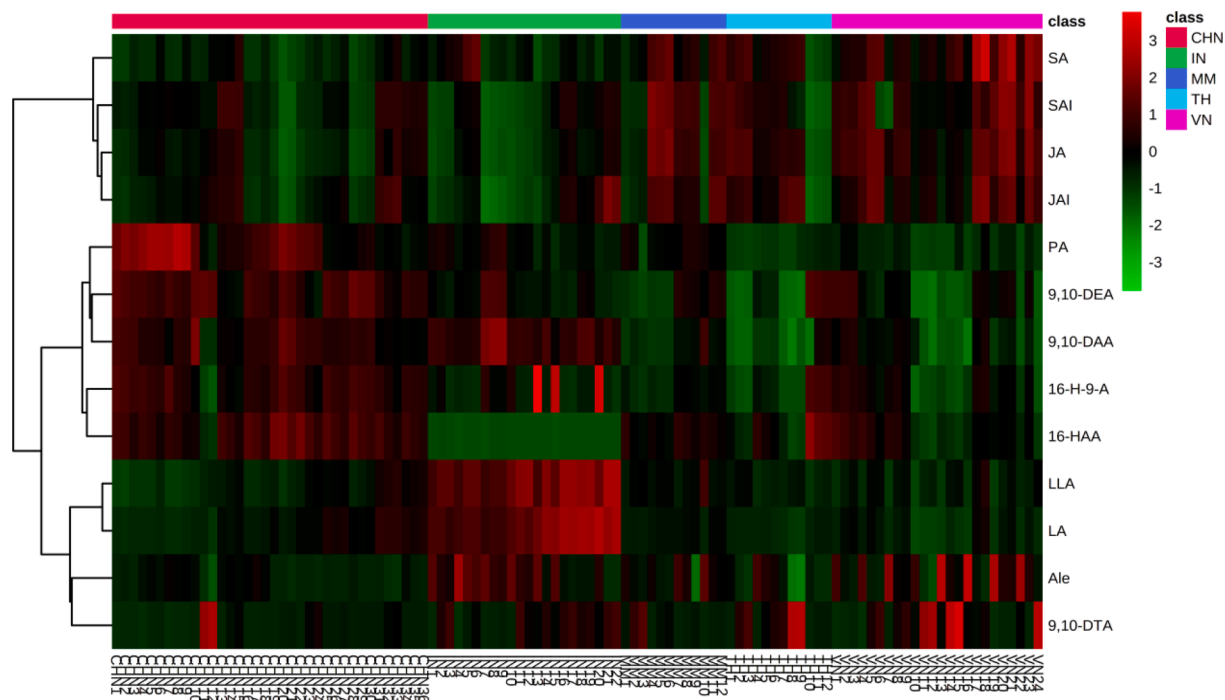


Fig. 3. Heat map representation of the SSR sample replicates. The analysis is based on the relative amount of the identified metabolites across all analyzed samples by the LC-ELSD method. The color-coding scale indicates the relative abundance within each metabolite: green, low abundance; red, high abundance.

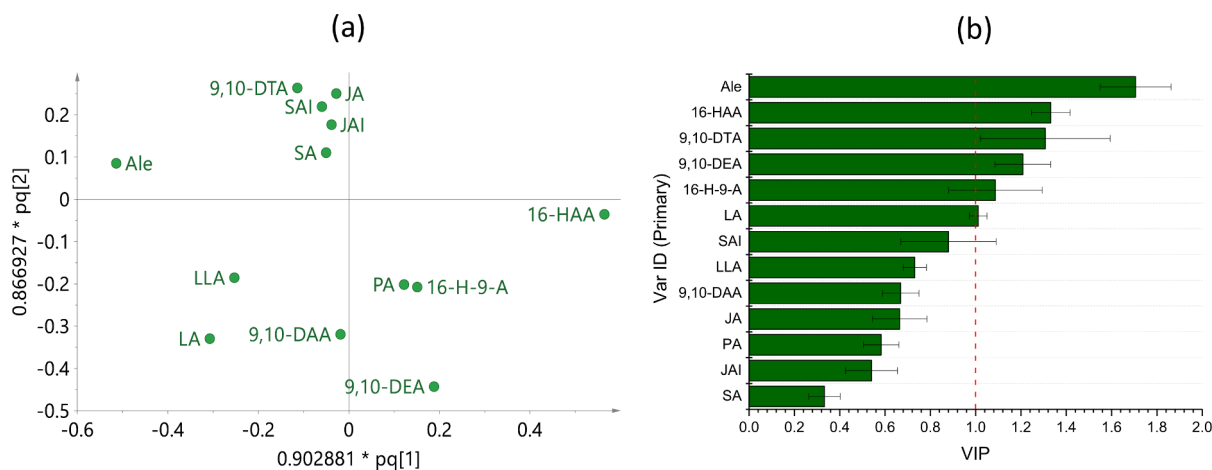


Fig. 4. Results of PLS-DA of SS obtained from different countries (CHN, IN, MM, TH, VN). Loading plot (a) and VIP scores (b).

normalized quantitative analysis of the monomer composition compounds of shellac resin was performed with the LC-ELSD method after authenticating the targeted metabolic compounds based on the molecular weight and fragment characteristics of the compounds in LC-TOF-MS/MS to prevent the aforesaid ionization mass spectrometry detector from suffering ion abundance difference caused by ionization response difference (Yüceer & Caner, 2020), and obtain more real and reliable metabolite content data of the compounds, so as to perform clustering analysis and difference analysis with the contents of targeted metabolites.

#### Establishment and analysis of targeted metabolomic model

##### Clustering analysis

To determine the clustering characteristics in the monomer composition of shellac resin from different countries, we first performed the unsupervised PCA analysis of the samples (Fig. 2a). Results showed that the first three principal components, PC1, PC2, and PC3, were responsible for 73.5% of the variance (Fig. S4), while six principal components were responsible for over 90% (92.2%) of the variance. These results indicated that the samples from different countries were dispersed, with insignificant clustering characteristics. Based on this, the supervised PLS-DA analysis was performed (Fig. 2b). Results showed that samples from the 5 countries could be divided into three clustering groups, namely, the China group, the India group, and the Myanmar, Thailand, and Vietnam group. Moreover, the samples showed obvious geographical clustering characteristics of East Asia, South Asia, and Southeast Asia. In the PLS-DA analysis model, the  $R^2_X$  was 0.967,  $R^2_Y$  was 0.514, and  $Q^2$  was 0.436. To verify the reliability of the model and compare the differences between targeted metabolites from different countries, a metabolomic analysis was performed on the targeted compounds.

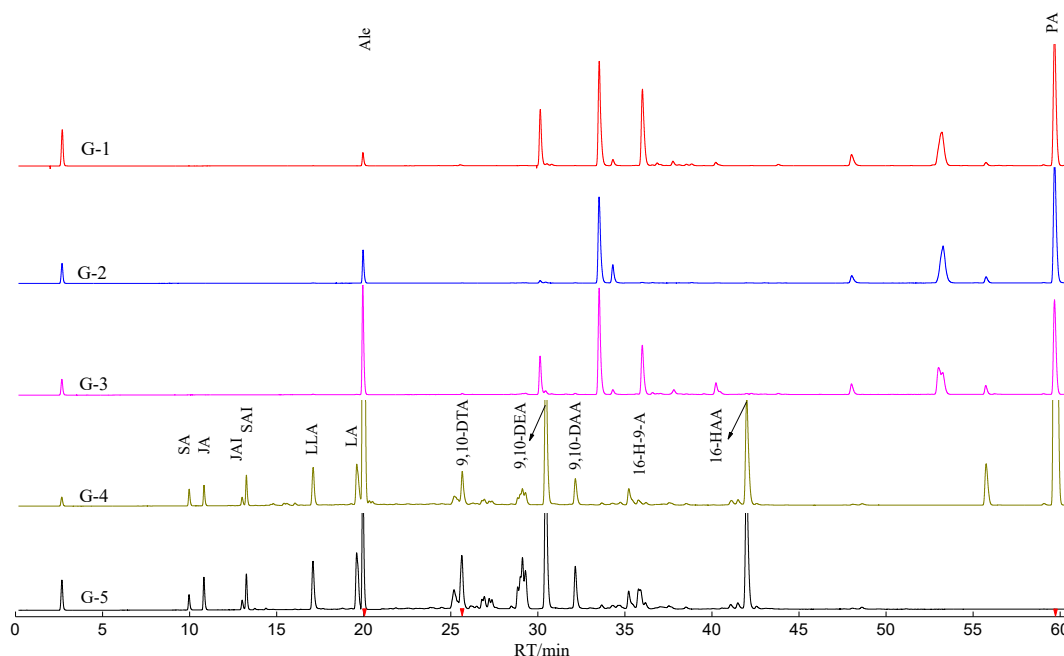
##### Targeted metabolomic analysis

To visualize the distribution of the 13 targeted metabolites in the shellac resin from China, India, Myanmar, Thailand, and Vietnam, the clustering heat map was made based on the compound contents shown in Fig. 3. Results showed that the contents and distribution of monomer compounds in shellac resin from the five countries were different. Shellac resin from China was rich in PA, 9,10-DEA, 9,10-DAA, 16-H-9-A, and 16-HAA; those from India were rich in LLA, LA, and Ale, and that from Myanmar, Thailand and Vietnam were rich in SA, SAJ, JA and JAI. Compared with the shellac resin from China and India, shellac resin from Myanmar, Thailand, and Vietnam were similar, as shown in the score plot (Fig. 2), indicating obvious geographical clustering characteristics of East Asia, South Asia, and Southeast Asia.

Coating solutions for fruit and vegetable preservation are

commercial products containing shellac as an optional formula. Driven by economic factors, shellac raw materials and their primary processing products (such as seedlac, flake shellac, and bleached shellac) are frequently circulating between different countries. In general, manufacturers of such coating solutions do not effectively distinguish their products by the source of shellac raw materials. In other words, the shellac resin raw materials in the coating solutions for fruit and vegetable preservation may come from any country where shellac is produced. Therefore, to make the qualitative detection method more reliable, metabolites need to be screened out from common metabolites from the five major shellac-manufacturing countries. Although the heat map visualized the content level and distribution of metabolites, no direct conclusion could be drawn on which metabolites could be used as the common metabolites of shellac resin from different countries and for the qualitative analysis of shellac resin. Therefore, the metabolomic difference analysis was adopted to further analyze the representation of different targeted metabolites in shellac resin from the five countries.

In the PLS-DA analysis model, the loading plot of the 13-targeted metabolites is shown in Fig. 4a. The closer the metabolite was to the center point, it was more common in 5 countries, while the farther it was from the center point, the greater the difference was in the 5 countries. Ale, 16-HAA, 9,10-DEA, and 9,10-DTA were the metabolites furthest away from the center points, which indicated that they were significantly different among the 5 countries. To quantitatively evaluate such a difference, the correlation was ranked by the VIP value of the metabolites (variables)(Fig. 4b). Generally, a metabolite with a VIP value greater than 1 could be deemed as a differential metabolite (Jandrić, Islam, Singh, & Cannavan, 2017; Xie et al., 2018). On the contrary, a metabolite with a VIP value below 1 could be deemed as a common metabolite. Therefore, shellac resin had 7 common metabolites (marked metabolites), which, if ranked by correlation from greatest to smallest, were SA, JAI, PA, JA, 9,10-DAA, LLA, and SAI, as well as 6 differential metabolites, which, if ranked by difference from greatest to smallest, were Ale, 16-HAA, 9,10-DTA, 9,10-DEA, 16-H-9-A, and LA. Comparison of the aforesaid 7 common metabolites revealed that only PA and 9,10-DAA were chain-like components, and the remaining 5 were all cyclic terpenes. In comparison, among the 6 differential metabolites, only LA was a ring-like component, and the remaining 5 were chain-like fatty acids. In terms of a component's marking of shellac resin, fatty acids had more extensive sources and rich structures. Therefore, cyclic terpenic acids have been suggested as a more suitable marker of shellac resin in the authentication analysis of shellac resin for coatings in fruit and vegetable preservation.



**Fig. 5.** The chromatogram of SS for commercial shellac-based coating solution (SBCS) products by HPLC-ELSD method. The SBCS, including G-1 to G-4, were commercial products and randomly purchased from markets in China. G-5 was prepared in the laboratory, and the content of shellac resin was 9 wt%.

#### Authentication and analysis of commercial SBCS for fruit and vegetable preservation

Based on the previously mentioned marker metabolites screened out with the metabolomic method, SBCS products of different brands claiming to contain shellac resin in their advertisements (G-1 to G-4, Fig. S5) were randomly bought from the Chinese market, after which a laboratory-made sample (G-5, the control sample) was made, and their SS were tested according to the method in 2.2 after they were pre-processed (Fig. S6). The results are shown in Fig. 5. Among commercially available coating products, only G-4 detected the 13-targeted metabolites authenticated in the paper, highly the same as the control sample (G-5). G-4 was a coating sample containing shellac resin as its formula component. In G-1, G-2, and G-3 samples, aleuritic acids of different contents were detected; nevertheless, no other marked metabolites in the three samples were detected except Ale and PA. As a fatty acid commonly contained in fats and waxes, PA may be introduced by other components in the formula, so it cannot be marked as a targeted metabolite alone. According to the published literature reports, Ale has not been reported in other substances except shellac resin. So theoretically, Ale may be a marked shellac metabolite. However, according to the aforesaid metabolomic authentication results (Fig. 4), when Ale was only used as the marked metabolite of shellac resin in coatings, its content greatly fluctuated, affecting the accuracy of its detection of it. Moreover, as a polyhydroxy fatty acid compound, Ale was a mature commercial product that could be added to the formula to escape detection, which was fully confirmed by the detection results of G-1, G-2, and G-3. None of the three samples contained any of the marker terpenic acid common metabolites in shellac resin, and only Ale and PA compounds could be added to the formula. Therefore, no shellac resin was found in G-1, G-2, and G-3 samples, while they were found in G-4 and G-5 samples. In conclusion, the targeted metabolomics method for screening marker metabolites was very reliable in the qualitative analysis of shellac resin in coatings for fruit and vegetable preservation. This method could provide a scientific basis for the qualitative detection and authentication analysis of whether an edible coating for fruit and vegetable preservation contains shellac resin.

#### Conclusion

Thirteen targeted metabolites were screened out from 22 monomer compounds constituting shellac resin with LC-QTOF-MS's molecular weight and ion fragment characteristics and LC-ELSD quantitative analysis. Moreover, the 13-targeted metabolites in shellac resin from the 5 countries were further divided into 6 differential metabolites and 7 common metabolites with the metabolomic method, supervised clustering analysis of samples, and difference analysis of targeted metabolite contents. Finally, the method's feasibility with commercially available coating solutions for fruit and vegetable preservation was verified by taking the common metabolites as the biomarkers. This method provides scientific bases for the authentication analysis of shellac resin in shellac-resin-based coating for preservation. Also, the establishment of this analysis method lays the foundation for the qualitative and quantitative detection of target components in the complex fresh-keeping wax coating in the future.

#### Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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#### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.fochx.2022.100349>.

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