

Review

Application of Chromatographic Technology to Determine Aromatic Substances in Tobacco during Natural Fermentation: A Review

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Abstract: Flavor is an important index to evaluate the sensory quality of tobacco. The process of fermentation is a key step in the production of aromatic substances in tobacco leaves and an important factor in improving their quality. Worldwide, reams of research show that chromatographic technology plays an irreplaceable role in the tobacco aromatic chemistry. Nevertheless, the degradation mechanism of latent aromatic compounds and the formation mechanism of characteristic aromatic substances have not been fully and systematically elucidated. In this study, the latest progress of basic methods, techniques, and research results of the separation, analysis, and identification of aromatic substances in fermented tobacco leaves were reviewed, and the next research and application directions were prospected. It is expected to provide theoretical reference for the study of molecular mechanism of tobacco flavor, reveal the degradation mechanism of potential aroma compounds, and help improve the quality of tobacco.

Keywords: tobacco natural fermentation; chromatographic technology; aromatic substances; degradation and formation; flavor molecular mechanism



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1. Introduction

Natural fermentation of tobacco leaves is an important process in tobacco production. The quality of tobacco odor directly determines its economic value [1]. In the whole process of tobacco leaf production, the main technological processes include tobacco leaf curing, threshing and redrying, storing, fermenting, and rolling. After redrying, tobacco leaves often have certain quality defects, such as a heavy pungent odor, lack of strong taste, and inadequate flavor, which preclude their fulfillment of industrial cigarette production requirements [2]. To reduce these negative problems of tobacco leaves, it is often necessary to ferment them after redrying, which can decrease or eliminate noxious odors and lower harmful substances. Tobacco leaf fermentation helps improve the quality of tobacco leaves by changing their internal chemical composition under appropriate environmental conditions. After fermentation, the quality defects will be improved [3]. Moreover, appropriate addition of special tobacco flavors during tobacco fermenting can make tobacco leaves more fragrant and more effectively meet the needs of industrial production [4].

In 1858, British scholar Koller conducted the first study on the mechanism of tobacco leaf fermentation [5]. He contended that cigar smoke causes some carbohydrate degradation and transformation during storage. On this basis, Koller proposed the concept of fermentation. Later, other scholars suggested three related theories: oxidation, microbial action, and enzyme catalysis [6]. According to current research, the three approaches—alcohol oxidation, microbial action, and enzyme catalysis in tobacco leaves—interact with and complement each other. Because internal fermentation changes engender aromatic

substances, the implementation of tobacco fermentation technology at this stage is also based on these three concepts.

Although the chemical mechanism of tobacco fermentation remains unclear, it is known that tobacco leaf fermentation is a critical process for aroma production [7]. This process requires approximately 18 to 30 months. The important change at this stage is the conversion of potential aromatic substances into separate aroma substances. Aroma is an important substance that determines the quality of tobacco, including Maillard reaction products, carotenoids, cembranes, ladanums, glycosides, etc. In the past few decades, some organic compounds in food, food ingredients, and plant products have aroused considerable research interest. These chemical components are called latent aromatic substances because they do not have aroma—only through enzymatic reactions, oxidation, rearrangement, and cracking reactions under high temperature and high humidity conditions will they be degraded into aromatic substances. There are few volatile substances in tobacco leaves before fermentation [3]. Only when tobacco leaves undergo fermentation can a large number of changes in latent aromatic substances occur, so as to improve the quality of tobacco leaves. Usually, the concentration of volatile organic compounds in these substances is notably low; however, it has an important influence on the flavor style and quality [8].

In the new scientific field, chromatographic technology has been gradually developed and applied. Advanced chromatographic techniques can exert their inherent potential in various fields. For example, in the field of plant metabolomics, gas chromatography has high sensitivity for the analysis of volatile and semi-volatile substances. Metabolomics based on GC-MS has been reviewed by Ayodeji Adebo et al. [9] in recent years in the field of food fermentation, especially in cereal and bean fermented foods. Plants are rich sources of secondary metabolites with interesting biological activities. Since gas chromatography can quantitatively determine the substances existing at very low concentrations, it is often used as a means of analyzing metabolites. Al-Rubay et al. [10] reviewed the application of gas chromatography–mass spectrometry in the analysis of bioactive natural compounds in some plants. At the same time, other chromatography methods can detect some difficult volatile substances, such as amino acids, proteins, sugars, etc. Ibraimov et al. [11] reviewed the chromatographic analysis methods of tobacco products, and comprehensively summarized the analysis of tobacco products. Liquid chromatography has superiority for the analysis of non-volatile substances and macromolecular compounds. Peters et al. [12] developed a fast, reliable, and accurate toolbox based on stable isotope dilution analysis in combination with ultra-high-performance liquid chromatography–tandem mass spectrometry (SIDA-UHPLC-MS/MS) analysis that ensured a quick, easy, and precise quantification of the aroma compounds in mint. Wu et al. [13] developed a quantitative method for the determination of benzene methanethiol, which has a toasted aroma in Chinese sesame-flavored baijiu, by a derivatization reaction combined with LC-MS/MS.

Research on the tobacco fermenting mechanism was conducted in earlier decades, while studies on its analysis methods occurred relatively late. In 1944, Nagasaki et al. [14] identified and isolated certain phenolic compounds during tobacco fermentation. For example, in small amounts of volatile phenolic compounds, they found that the amount of volatile phenolic compounds in flue-cured tobacco decreased during the fermentation process. In the 1950s, the important aromatic components in flue-cured tobacco were isolated and identified by solvent extraction by Rowland et al. [15], such as neophytadiene and solanesol. Early tobacco research yielded only a small amount of aromatic components. In the 1960s, Stedman [16] systematically discussed the chemical composition of tobacco and flue gas and identified more than 100 kinds of acidic components in tobacco. This is the first systematic analysis of most tobacco aromatic components. In 1977, Wahlberg et al. [17,18] reported changes in volatile neutral acidic and alkaline components in flue-cured tobacco during its fermenting. In the same years, the acidic constituents of sun-cured Turkish tobacco were studied by Cunman et al. [19]. Of 93 acidic compounds investigated, 10 compounds were new in nature and 18 were new tobacco constituents. It is worth noting that

there are many substances such as acids, alcohols, and lipids in the components of tobacco leaves. In 1985, Sakaki et al. [20] reported changes in the components in the headspace of tobacco leaves during fermentation. The results showed that the volatile components, such as furfuryl alcohol, benzyl alcohol, and solanone, changed during the fermentation process. This finding can be used to judge the influence of fermentation on the quality of tobacco leaves. Li et al. [21] reported changes in the neutral aromatic components in flue-cured tobacco fermentation. In 2000, Xie et al. [22] identified 200 aromatic components in Burley tobacco by simultaneous distillation extraction and gas chromatography–mass spectrometry (GC-MS). Jin et al. [23] found that the relationship between the aromatic components and aromatic precursors of tobacco leaves can be divided into isoprenoids and norisoprenoids, alkaloids and its transformation products, phenylalanine, and lignin metabolites. In the early study of tobacco fermentation, the detection of aromatic substances in tobacco leaves was not complete because the technical means were not advanced.

There are many kinds of substances that form the unique tobacco flavor, and there are extremely complex interactions between various aromatic components. Different types and varieties of tobacco in different ecological environments and cultivation conditions will lead to different composition, content, and proportion of aromatic components. However, according to the content and distribution of some characteristic aromatic compounds, combined with the evaluation results, the flavor style and quality can be defined. Furthermore, the techniques of reducing cigarette tar and harmful materials can be accompanied by the loss of flavor, poor comfort, and other adverse conditions. Latent aromatic compounds have the characteristics of high stability and can release aromatic substances, which can have a stable aromatic compensation effect on low tar cigarettes, and are widely used in the tobacco flavoring process. Therefore, it is necessary to explore the degradation of potential aromatic compounds in tobacco leaves.

The production process of tobacco leaves is quite complex, and fermentation has a great impact on tobacco leaf. After fermentation, the potential aromatic substances in tobacco leaves will be transformed into aromatic substances, and the quality of tobacco leaves will change fundamentally [24]. When chromatographic technology is applied to the analysis of tobacco aromatic substances, the change can be found. On the other hand, in a specific period, when the changes in aromatic substances have no impact on the overall quality of tobacco leaves, chromatography technology can be used to judge the fermentation progress, reduce the time of the natural fermentation process, and improve efficiency. Through the fermentation process, the quality defects of raw tobacco can be reduced to a certain extent, such as excessive odor and high irritant components. At the same time, the fragrance of tobacco leaves can be improved. However, there is a lack of systematic research on the fermentation process of tobacco leaves. With this paper, we aim to provide corresponding analysis methods for different aromatic components in order to have predictive opinions on the best analysis methods of different potential aromatic substances, so as to provide constructive help for the systematic study of the tobacco fermentation process.

2. Pretreatment Methods of Tobacco Aromatic Substances

Common methods to separate aroma from tobacco include headspace distillation, simultaneous distillation, extraction, headspace separation, steam distillation, solvent extraction, etc. The headspace distillation method is to mix the tobacco leaf sample with water, heat it, boil it, and simultaneously introduce inert gas to it. Accordingly, the components that are azeotropic with water can be separated. Kim et al. [25] used a simple gas co-distillation device to separate essential oil from the tobacco leaves. The method involves co-distillation with water under a continuous flow of inert gas, followed by single solvent extraction of the distillate. The extract is analyzed by GC-MS. Li [21] extracted tobacco leaves with dichloromethane, and the neutral components extracted with dichloromethane were injected into N₂, azeotropically distilled with water. They were then co-distilled in top air to extract the volatile aroma substances and achieve separation. This author used

this method to extract 2 aldehydes, 13 ketones, and 3 alcohols in flue-cured tobacco from Henan and Yunnan to obtain a total of 18 important substances.

Simultaneous distillation and extraction (SDE) refers to the simultaneous distillation and extraction device that fully mixes the water phase and organic phase so that the aroma components and water azeotropically enter the organic phase. This is a common, simple, and quick method for extracting aroma substances [26]. The aroma components in tobacco were extracted by simultaneous distillation extraction and analyzed by GC-MS by Wu et al. [27]. Fifty-eight aroma components in tobacco samples were identified by an automated mass spectrometry deconvolution and identification system (AMDIS). The simultaneous distillation extraction process of volatile aroma components in flue-cured tobacco was optimized by single-factor experiments and response surface methodology (RSM) by Luo et al. [28], and then the qualitative and quantitative analysis of aroma components was carried out by gas chromatography–mass spectrometry. Using the single-factor experiments and RSM, it was possible to determine optimal operating conditions to obtain a high aroma extraction yield. The study provided the possibility for the tobacco industry to extract aroma components from tobacco with simultaneous distillation extraction.

The headspace separation method is to place tobacco leaves into a closed container and heat the container. After the inert gas is purged, the volatile substances are captured, thereby separating the substances. Matsukuraa et al. [29] used nitrogen as the carrier gas to separate the volatile substances. Then, they detected the volatile substances of flue-cured tobacco by GC-MS to determine the aroma substances with caramel flavor. These substances, such as 2-hydroxy-3-methyl-2-cyclopentenone, 3-hydroxy-2-methylpyran-4-one, and 4-hydroxy-2,5-dimethyl-3(2H)-furanone, are considered to play an important role in aroma substances. An ether trap analysis method for tobacco headspace components was proposed by Sakaki et al. [30]. Dried air is passed through cut tobacco and the swept headspace volatiles are absorbed in ice-cooled ether. The ether solution of headspace volatiles was analyzed, and 27 components were identified by GC and GC-MS. In south-western China, 97 aromatic compounds were extracted from tobacco by Wu et al. [31] using headspace analysis.

Furthermore, steam distillation is a traditional method to extract fine substances from plants. In 1972, Demole et al. [32,33] used this method to extract 81 aroma-related substances from burley tobacco, among which nine new aroma substances were related to tobacco quality. In 1976, 275 aroma components of flue-cured tobacco were identified by Lloyd et al. [34]. Although the steam distillation method is relatively stable, it requires numerous organic solvents for extraction, and the amount of treatment is relatively large.

Many kinds of extraction methods can be used when choosing an organic solvent for extraction. In one study, the headspace operation was conducted on the extraction solution to obtain the volatiles in tobacco leaves. On the other hand, aromatic substances were extracted with organic solvents and then separated by column chromatography with different eluants. Some were directly extracted from tobacco leaves by steam distillation, and volatile and semi-volatile substances were obtained [35].

The advantage of supercritical fluid extraction is to leverage the benefits of a fast speed and ultra-critical medium to quickly extract the chemical components in tobacco leaves. Fischer et al. [36] used supercritical fluid technology to extract nicotine from tobacco and optimize it. Solid phase microextraction (SPME) technology uses the stationary liquid in the device to absorb volatile aroma components, and then the material enters the gas chromatograph to achieve separation. Smetena et al. [37] analyzed tobacco alkaloids by SPME. Owing to the low volatility and ionic properties of alkaloids, the headspace sampling of dry or wet tobacco samples often requires more effort to improve the extraction efficiency.

Zhu et al. [38] established a chromatographic fingerprint method for tobacco flavor quality control. By comparing the performance of SDE, liquid–liquid extraction, and SPME, the fingerprint GC-MS analysis method of tobacco flavor was established. According to the results [36], SDE-GC-MS is an effective method for the rapid identification of volatile components in tobacco flavors. Twenty-eight fingerprint components were extracted.

Xiang et al. [39] established and verified a method suitable for qualitative and quantitative analysis of volatile flavor components in flue-cured tobacco, namely, headspace SPME gas chromatography time-of-flight mass spectrometry (HS-SPME-GC $\times$ GC-TOFMS). This method is simple, rapid, accurate, and environmentally friendly.

Dinga et al. [40] used the solvent extraction (SE) method to extract the neutral aroma components of tobacco and performed GC $\times$ GC-TOFMS detection. A total of 83 neutral aroma components were identified. Zhang et al. [41] established the dynamic changes in metabolites during tobacco growth and development. They established the method of derivatization GC-MS, and used the partial least squares test to analyze and screen the metabolites with significant differences. It was found that during the development of tobacco, the contents of metabolites related to glucose metabolism, amino acid metabolism, shikimic acid metabolism, and terpenoid metabolism increased at first and then decreased. Meanwhile, the contents of citric acid and fumaric acid, the intermediates of the tricarboxylic acid cycle, increased at first and then decreased.

Mohammed et al. [42] analyzed the volatile components of jujube varieties by SPME-GC-MS combined with multivariate data analysis. A total of 89 volatile compounds were identified, including 51% of fatty acids and 24.7% of phenylpropanoid derivatives. Wang et al. [43] used HS-SPME and GC-MS to extract volatile compounds from raisins. They used this method to analyze free and sugar-binding volatile compounds in three raisins with different aroma intensities, and they identified 91 compounds. Romero et al. [44] verified the method of determining the actual concentration of volatiles in virgin olive oil by solid-phase chromatography–mass spectrometry. In 1979, Forrest et al. [45] quantitatively analyzed the changes in carotenoid content in fresh tobacco leaves and cured tobacco leaves by resonance Raman spectroscopy. They found that the carotenoid content was greatly degraded during modulation. Furthermore, Court et al. [46] determined the concentrations of major fatty acids and nonvolatile organic acids in flue-cured tobacco by gas chromatography. SPME has been widely used in the extraction of aroma compounds from tobacco leaves. However, there are many studies on the extraction of aroma compounds from various plants. The extraction of other aroma compounds may provide a new way for the extraction of tobacco leaves.

In terms of different substance analyses, various pretreatment methods can be typically employed to optimize the analysis conditions and obtain different aroma substances. The methods used in the research and identification of these aromatic compounds have improved in recent decades. However, aromatic substances are essentially extracted by different pretreatment methods and then analyzed by chromatography. The qualitative and quantitative compositions of volatile or semi-volatile substances in tobacco can be determined using GC-MS methods. By using the LC-MS method, it is possible to determine the qualitative and quantitative composition of difficult to volatile or macromolecular compounds in tobacco. Combining different chromatographic methods can provide a more accurate evaluation of tobacco quality.

3. Important Aromatic Components in the Fermentation Process and Analytical Methods

The main aroma substances produced in the process of tobacco leaf fermentation are Maillard reaction products, carotenoids, cembranes, ladanums, and glycosides.

3.1. Maillard Reaction

The Maillard reaction, also known as “nonenzymatic browning”, is a complex reaction between reducing sugars and amino acids or proteins [47]. The Maillard reaction process [48] is shown in Figure 1. First, reducing sugars (such as glucose) causes a reaction with amino acids or proteins, and then form the Schiff bases, which are rearranged to form Amadori products. Owing to the influence of the pH value, the Amadori products will be degraded under different conditions. When the pH value is equal to or less than seven, the products are mainly subjected to 1, 2-enolization to form furfural. When

the pH value is higher than seven, the product will undergo a 2,3-enolization reaction, forming reducing ketones. Many cracking products will be produced, which are intermediates with high activity, and they can continue to react. Carbonyl groups can react with amino groups, so that the final product contains nitrogen. Carbonyl compounds react with amino acids to produce α -amino ketones and aldehydes. This is called the Strake reaction. In the continuous reaction, a series of sub-reactions will take place, including cyclization, dehydrogenation, rearrangement, isomerization, and condensation. Finally, brown nitrogen-containing polymer will be formed [49].

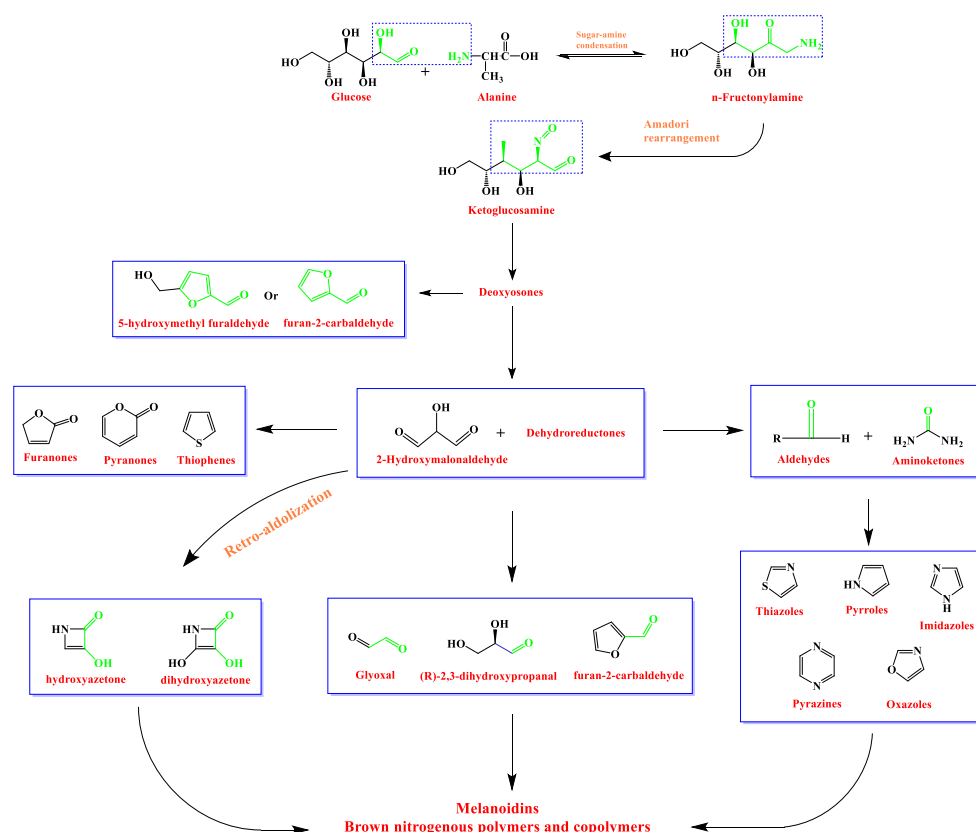


Figure 1. Maillard reaction process.

The commonly used methods to purify Maillard reaction products include thin layer chromatography, ultrafiltration, gel column chromatography, membrane dialysis, ultracentrifugation, SPE, electrophoresis, etc. Thin layer chromatography (TLC) is an important technology for rapid separation and qualitative analysis of small amounts of substances. It is often used for separation and semi-qualitative analysis of Maillard products. Lee et al. [50] reported glycerol cross-linked lens protein and identified the reaction products by TLC. Membrane dialysis is a method to remove unreacted raw materials and small molecular products from the reaction system, thereby obtaining Maillard products. Cellulose dialysis membrane was used to dialysis Maillard products by Ma et al. [51], and the products were analyzed by high-performance liquid chromatography (HPLC). The results showed that the dialysis membrane can completely remove small molecular substances; however, it engenders some losses from the large molecular substances.

Common characterization methods for the Maillard reaction include gas chromatography mass spectrometry, flow injection analysis, and liquid chromatography. Wang et al. [52] established an analytical method for simultaneous determination of five Amadori precursors in tobacco by HPLC-MS/MS. The results showed that this method was simple, rapid, sensitive, and specific for the analysis and detection of Amadori compounds in tobacco samples. Twenty kinds of free amino acids in flue-cured tobacco from different regions were analyzed by Li et al. [53] using pre-column derivatization-ultra performance

liquid chromatography–single quadrupole mass spectrometry. The amino acid content of flue-cured tobacco from different areas is obviously different. A new type of Pyro Vial was established by Mitsui et al. [54] for the study of Maillard reaction and the identification of Amadori intermediates. Jia et al. [55] established a LC-MS/MS method for simultaneous determination of six Amadori compounds in tobacco. Yuan et al. [56] established a method for simultaneous determination of Pro-Fru and Pro-Glu in tobacco samples by high-performance liquid chromatography tandem triple quadrupole mass spectrometry. Liu et al. [57] established a simple and rapid method for the simultaneous determination of ten Amadori compounds in tobacco by liquid chromatography tandem mass spectrometry. The results showed that Amadori is quickly transformed into Maillard reaction products. Sixteen free amino acids and six Amadori in tobacco were determined by HPLC-MS/MS by Zhang et al. [58]. This method is simple, rapid, and sensitive. Wang et al. [59] established a high-performance liquid chromatography–quadrupole time-of-flight mass spectrometry (HPLC-Q-TOF-MS) method for the simultaneous determination of 10 standard and 12 non-standard Amadori compounds in tobacco. The above methods used to determine Maillard products in tobacco leaves are list in Table 1.

Table 1. Methods for determining Maillard product in tobacco products.

Method	Detectable Component	Sample Preparation	Methodological Conditions	Reference
HPLC-QQQ-MS	Fru-Ala, Fru-Val, Fru-Pro, Fru-Phe, Fru-Trp	① Weigh 25 mg dried tobacco. ② Soak in 95 mL water. ③ Ultrasonic extraction in bath. ④ Add appropriate amount of methanol. ⑤ Centrifuge.	Column: X Bridge™ Amide (250 mm × 4.6 mm, 3.5 μm); Phase A: methanol; Phase B: water	[52]
GC-MS LC-MS	Volatile compounds, Semi-volatile compounds, Non-volatile compounds	① Weigh 1 g dried tobacco. ② Add 50 mL distilled water. ③ Supersonic simulation.	LC-MS: Column: Develosil ODS-SR-3; Mobile Phase A: 5 mM ammonium acetate; Mobile Phase B: acetonitrile GC-MS: HP-5 MS column (30 m × 250 m I.D., 0.25 μm df)	[54]
LC-MS/MS	Fru-Ala, Fru-Pro, Fru-Thr, Fru-Glu, Fru-Gly, Fru-Amb	① Weigh 2 g tobacco powder. ② Add 70% methanol aqueous solution. ③ Ultrasonic extraction. ④ Filtration. ⑤ Concentrated to dry by rotary evaporation at 30 °C under reduced pressure.	Column: Thermo Hypersil Gold (100 mm × 2.1 mm × 1.9 μm); Mobile Phase: methanol–water solution	[55]
HPLC-MS/MS	Pro-Fru, Pro-Glu	① Remove tobacco from flue-cured tobacco cigarette samples. ② Freeze-dried at −50 °C for 24 h. ③ Weigh 0.2 g samples. ④ Place in 200 mL volumetric flask. ⑤ Add 150 mL water. ⑥ Ultrasonic mixing 20 min. ⑦ Add water volume to scale.	Column: Agilent ZORBAX C18 (4.6 mm × 250 mm, 5 μm) temperature: 30 °C; Mobile Phase A: acetonitrile; Mobile Phase B: water	[56]

Table 1. Cont.

Method	Detectable Component	Sample Preparation	Methodological Conditions	Reference
LC-MS/MS	Glucosamine, Fru-Ala, Fru-Asn, Fru-Glu, Fru-Pro, Fru-Val, Fru-Lle, Fru-Leu, Fru-Phe, Fru-Trp	① 100 mg tobacco powder was weighed. ② 3 mL of a 30: 70 <i>v/v</i> methanol/water solution was added. ③ The solution was extracted by ultrasonic wave for 25 min. ④ Centrifuged at 10000 rpm for 10 min.	Column: Atlantis T3 (2.1 × 250 mm, 5 µm); Phase A: aqueous formic acid; Phase B: acetonitrile; Gradients: 0.00 min, 89.5% A; 0.10 min, 78% A; 10.00 min, 78% A; V = 600 mL/min	[57]
LC-MS/MS	Fru-Ala, Fru-Pro, Fru-Val, Fru-Leu, Fru-Phe, Fru-Trp	① Weigh 0.1 g tobacco samples in centrifuge tube. ② Add internal standard reserve liquid. ③ Ultrapure water; ultrasonic extraction 10 min, extraction 2 mL. ④ Centrifuged for 15 min, 0.22 µm microporous membrane.	Column: Acclaim Explosive E2 column (250 mm × 4.6 mm, 5 µm); Mobile Phase A: methanol; Mobile Phase B: 0.1% formic acid aqueous solution	[58]
HPLC-Q-TOF-MS	Fru-Ala, Fru-GABA, Fru-Arg, Fru-Asn, Fru-Asp, Fru-Cys, Fru-Gln, Fru-Glu, Fru-Gly, Fru-His, Fru-Leu, Fru-Lle, Fru-Lys, Fru-Met, Fru-Phe, Fru-Pro, Fru-Ser, Fru-Thr, Fru-Trp, Fru-Tyr, Fru-Val	① 1 g tobacco powder. ② Added 30 mL 30% methanol-water solution. ③ Ultrasonic extraction 25 min. ④ The supernatant was filtered through an organic filter membrane. ⑤ 50 µL was taken in a 10 mL volumetric flask, diluted with methanol; 1 mL was taken for HPLC-MS/MS analysis.	Column: Atlantis T3 liquid column; Mobile Phase: 0.2% formic acid aqueous solution (phase A) and acetonitrile (phase B); Gradient Elution Program: 0–0.1 min 89.5% A ~78.0% A, 0.1–10.0 min 78.0% A. The initial flow phase was used to balance two samples for 8 min.	[59]

It can be seen from previous studies that the main method for analyzing Amadori, an important product in Maillard reaction, is liquid chromatography. Perhaps because of the poor stability of Maillard reaction intermediates, no excessive substances were detected by LC-MS. By changing different pretreatment methods and optimizing chromatographic separation conditions and mass spectrometry detection parameters, a simple, rapid, sensitive, and efficient LC-MS detection method for Amadori can be obtained. Amadori compounds are important aroma precursors. Rapid and accurate determination of Amadori compounds is of great significance for Maillard reaction in tobacco processing.

3.2. Carotenoids

In 1831, the German chemist Wachenreder isolated a carbohydrate pigment from the root of carotene and named it carotenoid. Carotenoids generally consist of eight isoprene groups attached to the head and tail of compounds or oxidized derivatives, and their general structure is shown in Figure 2.

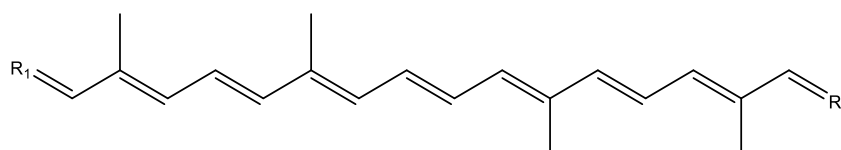


Figure 2. General formula of carotenoids.

Carotenoids are mainly composed of carbon and hydrogen, including α-hurotin, β-carotene, γ-carotene, and lycopene. Lutein is composed of carbon, hydrogen, and

oxygen and can form various oxygen-containing groups. Carotenoids are important aroma precursors in tobacco leaves. The metabolic enzyme catalytic pathway of carotenoids is shown in Figure 3. Firstly, dimethylallyl pyrophosphate (DMAPP) is catalyzed by geranylgeranyl diphosphate synthase (GGPPS) to generate geranylgeranyl diphosphate (GGPP). Then, GGPP is catalyzed by phytoene synthase (PSY), phytoene desaturase (PDS), ζ -carotenedesaturase (ZDS), ϵ -carotene isomerase, and cis-trans isomerase (Z-ISO, CRTISO) to form all trans-lycopene. All trans-lycopene is finally generated by the action of ϵ -cyclase (LCYE) and β -cyclase (LCYB) to form α -Carotene and β -Carotene, respectively.

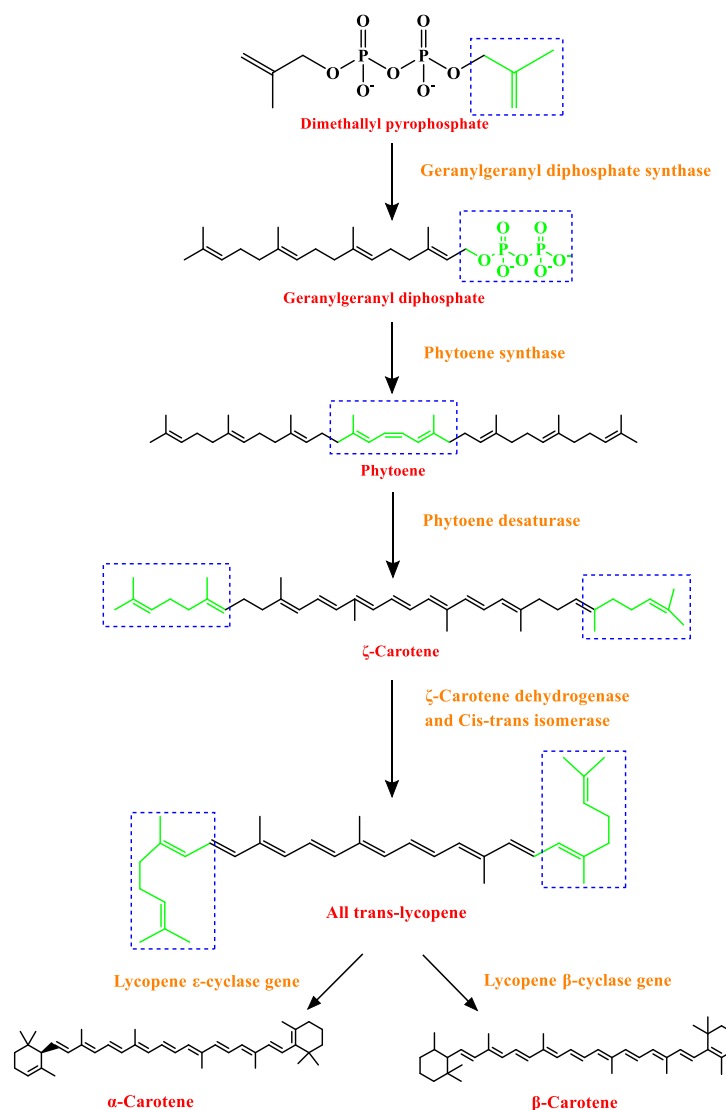


Figure 3. The metabolic processes of carotene.

Liquid chromatography is a common analytical and separation method for carotenoid separation. Britton et al. [60] first determined carotenoids by HPLC. Bijttebier et al. [61] compared the two methods (HPLC and UHPLC), and found that UHPLC technology can decrease the analysis time, decrease the peak width, and increase the chromatographic resolution. The application of UHPLC plays an important role in the analysis of imperatorin. For the determination of carotenoid content in tobacco, a rapid and accurate method was developed by Yang et al. [62], and the method has suitable linearity and reproducibility. The composition and content of carotenoids in tobacco leaves were detected by reversed-phase high-performance liquid chromatography (RP-HPLC) method [63]. Eleven carotenoids were identified in fresh tobacco leaves, including a new carotenoid—lutein and some isomers of carotenoids. Li et al. [64] established a method for the determination of imperatorin

in flue-cured tobacco by UHPLC. The mobile phase was acetonitrile–water–ethyl acetate, which greatly shortened the analysis time, saved solvent, reduced environmental pollution, and was suitable for batch sample analysis. The above methods used to determine carotenoids in tobacco leaves are list in Table 2.

Table 2. Methods for determining carotenoid in tobacco products.

Method	Detectable Component	Sample Preparation	Methodological Conditions	Reference
HPLC	β -carotene, lutein	① Twelve portions of 1.0 g tobacco leaf samples were weighed. ② Divided into two groups without shading and with black cloth shading. ③ Each group of samples was added with butylated hydroxytoluene (BHT) at 0, 0.01%, 0.05%, 0.10%, 0.15%, and 0.20%. ④ The samples were subjected to constant temperature oscillation and constant volume.	Column: Diamonsil-ODS-C18 column; Mobile Phase A: ethyl acetate ester; Mobile Phase B: 90% acetonitrile; Flow Rate: 0.8 mL/min	[62]
RP-HPLC-DAD	Neoxanthin, cis-neoxanthin, violaxanthin, luteoxanthin, cis-luteoxanthin, cis-violaxanthin, lutein, zeaxanthin, cis-lutein a, cis-lutein b, β -carotene	① 0.5 g smoke sample was accurately weighed and placed in a 100 mL conical flask with a grinding mouth, 25 mL acetone was added, and 50 μg internal standard was added. ② The sample was wrapped and sealed with aluminum foil for 2 h. ③ The oscillation extraction the filter residue was filtered quickly, washed with acetone, and the filtrate was combined.	Column: Zorbax SB C18 column (4.6 $\times$ 150 mm, 5 μ m); Flow Mobile Phase: gradient elution of acetonitrile, water, and ethyl acetate; Column Temperature: 25 $^{\circ}$ C; Flow Rate: 1.0 mL/min	[63]
UHPLC	Lutein, β -carotene	① 0.10 g sample was weighed and added with 2 mL internal standard solution. ② Filled with N₂ for exhaust. ③ Ultrasonic extraction. ④ Centrifugation.	Column: ACQUITY UPLC BEH C18 reversed-phase chromatography (100 mm $\times$ 2.1 mm, 1.7 μ m); Mobile Phase A: acetonitrile–water; Mobile Phase B: ethyl acetate	[64]

In the determination of carotenoids in tobacco leaves, carotenoids may be oxidized. After different pretreatment methods, the oxidation of carotenoids can be effectively prevented by liquid chromatography, which is suitable for the analysis and detection of large quantities of samples. Therefore, liquid chromatography is also a common method for the determination of carotenoids in tobacco leaves. With continuous research, some scholars began to try to use Raman spectroscopy to detect lutein and β -carotene in tobacco. This new method provides a new idea for us to detect substances in tobacco.

3.3. Cembranoids

Cembrane is an important diterpene compound that is mainly found in the seeds of herbs and tobacco flowers [65]. Cypressanes are typically degraded in the fermenting period of tobacco. The main substances include solanone, solanonefuran, and nordiketone. The degradation of cembrane compounds plays a crucial role in the aroma of tobacco. The rational utilization of the degradation pathways of cembrane compounds and understanding of degradation products are highly valuable to improving the quality of tobacco leaves. There are usually two mechanisms for the degradation of cembrane compounds in

tobacco. The first is single-line oxidation, which primarily occurs under the action of light or oxidant. The other is the enzymatic reaction, which mainly produces aroma compounds with different carbon chain lengths under the catalysis of different synthetases.

Cembranes have been studied since the 1960s. Roberts et al. [66] reported the substance for the first time. They used n-hexane to extract the surface of fresh burley tobacco leaves. The obtained extract was concentrated and separated by a chromatographic column to obtain α -2,7,11-cetriene-4,6-diol and β -2,7,11-cetriene-4,6-diol. Finally, the substance was characterized by nuclear magnetic resonance and infrared spectrometry (NMR and IR) classical analytical techniques. Nevertheless, owing to the limitations of the analytical methods, no absolute configuration was obtained. By 1975, Springer et al. [67] and Aasen et al. [68] used the X-ray single-crystal diffraction method and ozonation method to characterize the absolute configurations of the above cembrane compounds.

Different cembrane substances can be analyzed by various analytical methods. In the detection of tobacco volatile components, cembryltriene and its epoxy compounds are semivolatile substances and can be simultaneously detected. Chang et al. [69] analyzed cephrosotriene-4,6-diol in fresh burley tobacco by the butyl boric acid derivatization method. In 1990, Loughrin et al. [70] analyzed the headspace components of tobacco flowers, and GC-MS was used to analyze the volatile and semi-volatile aroma substances such as cephrosotriene. Peng et al. [71] compared the effects of different pretreatment methods of steam distillation, simultaneous distillation extraction, and headspace distillation extraction on the extraction of volatile and semi-volatile substances in tobacco. Owing to different pretreatment methods, GC-MS was used to measure three different results of cetriene. Shao et al. [72] studied the changes in main volatile aroma components in flue-cured tobacco at different levels of total nitrogen and protein by cluster analysis. The results showed that when the total nitrogen and protein content of flue-cured tobacco changed, the cembrane-like degradation products also changed significantly. The above methods used to determine cembranoids in tobacco leaves are list in Table 3.

Table 3. Methods for determining cembranoids in tobacco products.

Method	Detectable Component	Sample Preparation	Methodological Conditions	Reference
Solvent extraction	α -4,8,13-Duvatriene-1,3-diols, β -4,8,13-Duvatriene-1,3-diols	① The hexane was removed in vacuo and the residue was chromatographed on silicic acid. ② The silicic acid columns were eluted with hexane, with 10%, 25%, and 50% ether-hexane mixtures. ③ The duvatriediols were in fractions eluted with 50% ether-hexane.	NMR and IR	[66]
GC-MS	2-hexanol, (E) β -ocimene, hexanol, 4-methylhexanol, 6-methylheptanol, linalool, caryophyllene, humulene, benzyl alcohol, neophytadiene, caryophyllene epoxide, eugenol	① Flowers are put in a 5 L flask, and part of the flask is immersed in a 30 °C water bath. ② Connect the high-purity compressed air to the flask through the headspace device at a speed of 500 mL/min through a Teflon tube.	Column: Supelcowax10, capillary, (60 m $\times$ 0.32); GC Conditions: inlet temperature, 220 °C	[70]
1. Steam distillation/ solvent extraction 2. Simultaneous distillation and extraction 3. Headspace co-distillation	Aliphatic alcohols, aromatics, ionol derivatives, furfurals, ketols, terpenoids, furanones, pyranone, damascone, ionone derivatives, volatile fatty acids, semi-volatile fatty acids, anhydrides, esters aliphatics	① The dichloromethane solutions of volatile components obtained by three methods were extracted twice with 20 mL of a 5 wt % aqueous solution of sodium hydroxide and re-extracted twice with 10 mL dichloromethane to obtain the acidic fraction. ② The combined dichloromethane solution was extracted twice with 20 mL of a 5 wt % aqueous solution of hydrochloric acid and extracted twice with 10 mL dichloromethane to obtain the basic fraction.	Chromatographic separations were performed on a HP-5 MS column (30 m $\times$ 0.25 mm, 0.25 μ m)	[71]
GC-MS	Degradation products of aromatic amino acids, degradation products of cembrane-like compounds, neophytadiene, carotenoids	① The aroma components in tobacco leaves were extracted by steam synchronous distillation device, and the extract was extracted with dichloromethane.	Column: DB-5 (30 m $\times$ 0.25 mm $\times$ 0.25 μ m)	[72]

In the analysis of cembrane-like substances in tobacco leaves, the choice of analytical methods is more diverse, mainly because there are volatile and semi-volatile substances in the substance. However, we usually use GC-MS as a common means of analysis. Most volatile and semi-volatile substances can be detected by GC-MS.

3.4. Ladanum

Ladanum, also known as *Rosa roxburghii*, is a diterpenoid compound, mainly including fir alcohol, cedar alcohol, laibaidang diol, etc. In the curing and fermenting process of tobacco leaves, laibaidang can degrade to produce important aroma substances, such as norbornyl ether, lactone, dehydrolactone, γ -bicyclic isofarnesaldehyde, etc. However, this substance is not found in all tobacco leaves.

In the metabolic synthesis of diterpenoids in tobacco leaves, geranylgeranyl diphosphate (GGPP) generates 8- α -hydroxy-copalyl-pyrophosphate (8-OH-CPP) under the action of copalyl diphosphate synthase. Then, 8-OH-CPP is catalyzed by NtABS to generate Z-abienol and labdane-diol [73]. The degradation process of ladanum is shown in Figure 4.

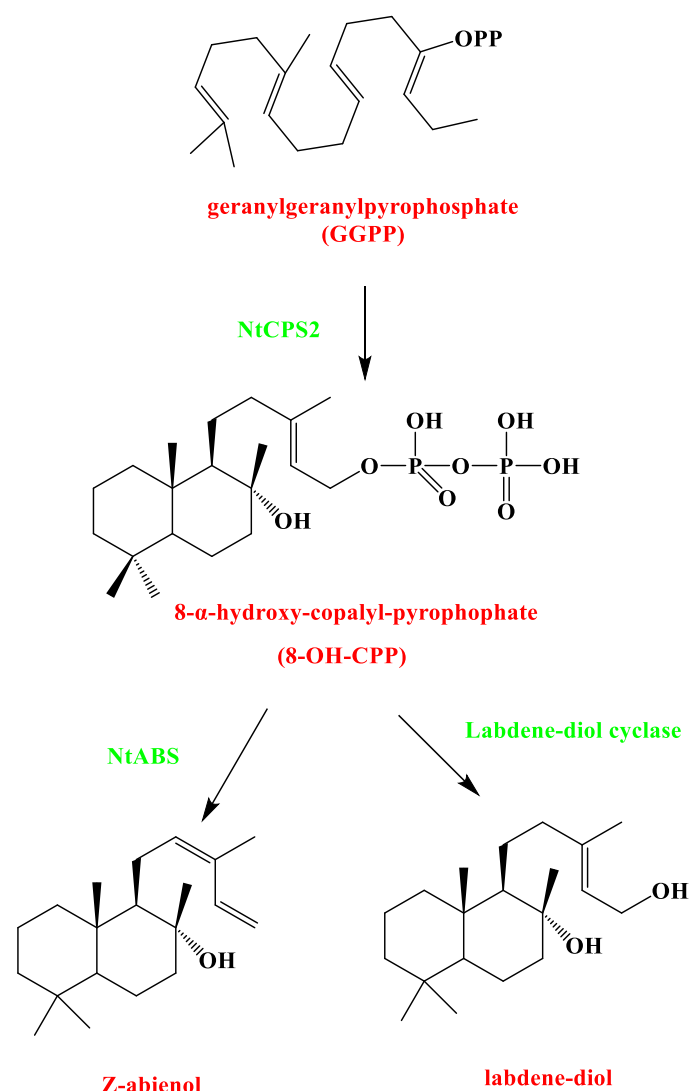


Figure 4. Metabolism process of diterpenoids from ladanum.

Laibaidangs and cembranes belong to diterpene compounds; nonetheless, the analysis of laibaidang substances is rarely undertaken. Guang et al. [74] used GC/GC-MS/GC-FTIR methods to analyze laibaidang. A total of 39 components were identified. Lei et al. [75] used a simultaneous distillation extraction method to extract the aromatic components

of laibaidang. GC-MS was used to identify more than 30 kinds of volatile components of domestic laibaidang and more than 20 kinds of imported laibaidang aromatic components.

3.5. Glycosides

Glycosides are important aroma precursors, being compounds produced by the dehydration of hydroxyl aminothio hydroxyl of monosaccharide or oligosaccharide and semi-aldol hydroxyl of another molecule. They widely exist in microorganisms and plants such as tobacco. The glycoside in tobacco can be divided into free and bound state. The aroma released by the glycoside cleavage inbound state plays an important role in improving tobacco aroma.

Gas chromatography and liquid chromatography are commonly used to detect glycoside substances. For enzymatic treatment of the tobacco glycosidic extract, 21 bound aroma compounds (aglycones) were identified by β -glucosidase hydrolysis by Cai et al. [76]. The analysis showed that the difference of tobacco types was reflected in the variety of their glycosides. Li et al. [77] analyzed and identified five glycosides in tobacco extracts by high-performance liquid chromatography–electrospray ionization mass spectrometry (HPLC-ESI-MS). Cai et al. [78] established an enzymatic gas chromatography–mass spectrometry method for the determination of three glycosides in tobacco and optimized the determination method by response surface methodology. Pang et al. [79] used high-performance liquid chromatography–atmospheric pressure chemical ionization mass spectrometry (HPLC-APCI-MS) to screen and identify the glycosides in tobacco leaves. The substances were characterized by MS/MS, MS, and electro-optical diode array (PDA) detection, and the contents of seven glycosides in tobacco leaves were detected. Yue et al. [80] used a response surface methodology to optimize ultrasonic-assisted extraction conditions, combined with liquid chromatography–tandem mass spectrometry (LC-MS/MS). Accordingly, they established a simple and efficient method for the absolute quantification of glycosides in tobacco and determined nine glycoside substances to verify it. Table 4 lists the above methods used to detect glycosides in tobacco leaves.

Table 4. Methods for determining glycosides in tobacco products.

Method	Detectable Component	Sample Preparation	Methodological Conditions	Reference
LC-MS	α -Ionol- β -D-glucopyranoside, 3-Oxo- α -ionol- β -D-glucopyranoside, 4-OH- α -ionol- β -D-glucopyranoside, 3-OH- β -damasenone- β -D-glucopyranoside, Lolidide- β -D-glucopyranoside	① Tobacco leaves were dried in an oven at 40 °C for 4 h and ground for later use.	Column: C18 (250 mm $\times$ 4.6 mm) Mobile Phase A: methanol; Mobile Phase B: water	[75]
GC-MS	Aliphatic alcohols, fatty acids, aromatic compounds, C13 norisoprenoids, terpenoids, polyphenols	① Glycosides were concentrated to dryness and re-dissolved in 15 mL of 0.2 M disodium hydrogen phosphate–citric acid buffer (pH = 5.6). ② Before adding the enzyme, the remaining volatiles were extracted with 20 mL of pentane–diethyl ether (1:1, v/v).	Column: HP-5 ms capillary (60 m $\times$ 0.25 mm i.d. $\times$ 0.25 mm); Toven = 60 °C (τ held = 1 min)–230 °C (τ held = 4 min), V = 2 °C/min	[76]
HPLC-APCI-MS	Scopolin, rutin, quercetin-3-glycoside	① The tobacco powder (0.5 g) was placed in a 10 mL capped flask. ② 5 mL of methanol was added. ③ The extraction was carried out for 10 min in an ultrasonic washer. ④ The extract was centrifuged for 2 min at 3000 rpm at room temperature. ⑤ The supernatant was transferred into a beaker.	Column: Hypersil C18 (5 μ m; 4.6 $\times$ 250 mm ²); Mobile Phase A: 1% mic acid water; Mobile Phase B: CAN	[77]
LC-MS/MS	Phenolic, glycosides, benzenoid, glycosides, sesquiterpene, glycosides	① The dried powders of flue-cured tobacco leaves (26.5 mg) were accurately weighed. ② Ultrasonic extraction with 5 mL of 72.4% methanol with 8.0 μ g/mL of IS in a 25 °C water bath for 51.4 min.	Column: BEH-C18, (100 mm $\times$ 2.1 mm I.D., 1.7 μ m); Mobile Phase A: water containing 0.1% formic acid	[78]

The continuous progress of analytical methods has thus played an important role in promoting the analysis of the tobacco leaf fermenting process and in accelerating comprehensive and systematic study of material degradation in this process.

4. Conclusions

The change in aromatic components in the tobacco leaf fermenting process can provide guidance for the improvement of tobacco leaf quality. Most previous research has focused on the study of single or single types of aromatic substances in tobacco leaves; the systematic study on the fermentation process of tobacco leaves lacks in-depth discussion. Therefore, in this paper, we assessed the research progress on chromatographic technology in tobacco aromatic substances analysis. The ongoing development of chromatographic technology provides solutions for the chemical diversity and metabolic information involved in the fermentation process. Summarizing the detection methods of different types of aroma components and forming standard detection schemes will help reduce the detection error. Through the in-depth study of fermentation mechanisms and regular degradation, changing relevant process conditions and shortening the fermentation cycle are conducive to the development of tobacco fermentation technology. The mechanism of tobacco leaf fermentation warrants further exploration to ensure that the substance change is more deeply understood.

5. Future Outlook

The formation and accumulation of aromatic substances is the main purpose of fermenting tobacco leaves. Chromatography and various extraction and separation methods are used to determine these components. Future research should strengthen the analysis of following aspects. First, most of the pretreatment methods of tobacco leaves are time-consuming, laborious, and expensive, so establishing a fast and convenient extraction method is an important problem to address. Moreover, the analysis of low concentrations of aromatic substances may be highly significant, but the simultaneous detection of multiple trace substances in the field of chromatographic technology remains a challenge. In addition, chromatographic analysis will produce ample data and spectrograms. Developing powerful and efficient data processing and analysis methods can greatly improve the accuracy of detection results.

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