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Heat-Induced Changes of Plastid Pigments in Tobacco Laminas from Different Stalk Positions During Flue-Curing Period

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This investigation studied the evolution of plastid pigments in tobacco laminas from different stalk positions during flue-curing period with different temperatures. Chlorophyll and carotenoid content, as well as moisture of tobacco laminas, were monitored during the flue-curing period. Data obtained indicated that the majority of plastid pigments degradation occurred during the yellowing stage under relatively lower temperature (36 to 44 ºC). Meanwhile, degradation of individual plastid pigment was found to be inhomogeneous during the flue-curing period and the declines of pigment contents varied from 96.7 % for violaxanthin to 60.6 % for β-carotene. The rate of total chlorophylls degradation being more marked than that of total carotenoids and which was confirmed by the evolution of the ratio of their contents. Different stalk positions also contributed to the degradation content difference in tobacco laminas, with bottom laminas to be the most obvious, followed by top and middle laminas, respectively. While at the end of the flue-curing period, the content of any pigment in top laminas was almost the same as in middle laminas, both higher than that in bottom laminas. It is also observed that, during the flue-curing period, the moisture content in tobacco laminas and the degradation contents of pigments showed a positive correlation. In general, all obtained results demonstrate that the evolution of moisture and plastid pigments contents in tobacco laminas directly depend on the designed flue-curing temperature conditions and a fine control of temperature would help to modulate the degradation of pigments with the aim of improving the quality of flue-cured tobacco laminas.

Key Words: Pigments, Tobacco, Flue-curing, Degradation.

INTRODUCTION

Changes of plastid pigments in leaves may be induced by many environmental stresses, including light, ozone and heat[1,2]. For tobacco laminas, the major changes of plastid pigments occur during growth, senescence and flue-curing periods, which have a dramatic impact on the cured tobacco quality. Also, during different periods,

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the changes of plastid pigments are mainly induced by different environmental stress. Understanding the main influence factors of the degradation of plastid pigments during different stages would make it convenient to obtain a desirable product by a fine control of conditions.

Tobacco plastid pigments mainly consist of chlorophyll a, chlorophyll b, neoxanthin, violaxanthin, lutein and β-carotene[3]. The content and composition of pigments in tobacco laminas vary with tobacco types, varieties, growth and development stages and curing methods[4]. In tobacco, the degradation of the plastid pigments may lead to the formation of many components which can enhance the aroma of tobacco product[5]. Enzell[6] reported that approximately 80 aroma constituents in tobacco derived from the oxidative degradation of carotenoids. Moreover, many results had demonstrated that only about one third of aroma compounds in cigarette smoke derive directly from tobacco and other aroma constituents mainly produced from transformation of other substances such as carotenoid and polyphenol during curing and storage[7-9]. Therefore, apart from contributing to the appearance colour of tobacco, the plastid pigments in tobacco also play important roles on internal quality of tobaccos. Thus, considering the importance of carotenoids degradation to the formation of flavour components, it is of great significance to study the degradation of plastid pigments in tobacco laminas during flue-curing period. Furthermore, pigments contents in flue-cured tobacco laminas can be used as useful and more informative indicator of internal quality of cigarette materials[3]. Furthermore, colour quality of flue-cured tobaccos can also assist to assess their economic value in tobacco market[9]. In addition, carotenoids in tobaccos have some negative effects on smokers by forming carcinogenic polynuclear aromatic hydrocarbons (PAHs) through pyrolysis during cigarette smoking[10].

Before 1990s, considerable efforts had been devoted to the changes of plastid pigments in tobacco laminas during senescence and curing periods. Gopalam and Gopalachari[11], for example, examined the changes of chlorophyll, total xanthophylls and total carotenes in Indian flue-cured tobacco during maturation and curing periods. Harold and Kasperbauer[12] investigated several aspects of plastid pigment changes during senescence and curing of American flue-cured tobacco and Burley tobacco, respectively. Their results, however, did not always lead to the same conclusions concerning various factors. So far, there is lack of comprehensive information about a detailed description of heat-induced changes of plastid pigments during flue-curing in flue-cured tobaccos, especially Chinese tobaccos. Furthermore, the changes of plastid pigments performed on tobacco laminas from different stalk positions have also been less studied. Therefore, in this paper, we not only focused on describing the degradation of plastid pigments with the change of temperature during different stages of flue-curing period, but also compared the distribution pattern of plastid pigments in different stalk positions at each stage of flue-curing period. Meanwhile, the relation between the maintenance of moisture content and the declines of pigments contents in tobacco laminas during different stages of

flue-curing period was also investigated. Present results provide useful information for manufacturing high-quality tobacco and improving the flue-curing rules for tobacco laminas from different stalk positions.

EXPERIMENTAL

The tobacco samples were obtained from Yunnan Academy of Tobacco Science (Kunming, China). β-Carotene, β-apo-8'-carotenal (internal standard), triethylamine (TEA), butylated hydroxytoluene (BHT) were purchased from Sigma (St. Louis, MO, USA). Lutein and violaxanthin standards were prepared from spinach. Analytical grade chemicals and solvents, including potassium hydroxide, anhydrous sodium sulfate, sodium chloride, neutral alumina, methanol, petroleum ether (b.p. 30-60 °C), acetone, absolute ethanol and hexane were obtained from Shanghai Chemical Reagents Company (Shanghai, China). Methanol, acetonitrile, methylene chloride were of HPLC grade purchased from Fisher Chemical Company.

Preparation of tobacco samples: Honghuadajinyuan (abbreviate Hongda) is a major tobacco variety with good aroma quality grown in Yunnan, China. Hongda tobacco laminas from different stalk positions of the same plantlet are as follows: bottom laminas (4-6 tablets), middle laminas (10-12 tablets), upper laminas (15-18 tablets). All tobacco laminas designated for flue-curing were complete maturation and tobacco laminas from the same stalk position were harvested at the same time. The flue-curing experiment was carried out at the experimental base of Yunnan Academy of Tobacco Science (Kunming, China) in August, 2007. During flue-curing, laminas were placed in curing chamber for 120 h and different temperature was imposed at different time stages. Lamina samples were withdrawn at 4 °C intervals during flue-curing. After midribs were removed from the leaf, the laminas were weighted. Then the laminas were freeze-dried and reweighed to determine moisture content. The levels of pigments shown in this paper based on the pigment contents in 1 g dry weight of tobacco samples.

Analysis of plastid pigments in tobacco laminas: A spectrophotometric method was used to determine chlorophyll a and chlorophyll b in tobacco laminas. Lutein and violaxanthin standards were prepared from spinach as described by Kimura[13]. A modification procedure described by Lakshminarayana[14] was used for the analyses of violaxanthin, lutein and β-carotene. For the analyses of carotenoids, 0.5 g ground tobacco powder was mixed with 25 mL cold acetone containing 0.1 % BHT as antioxidant in a 100 mL conical flask which was wrapped with tinfoil and then 100 μg β-apo-8'-carotenal was added as the internal standard. After being vigorously shaken on a vibrator for 2.5 h, the mixture was filtered and the residue was washed with 10 mL cold acetone for 2-3 times. The filtrate was combined and dried with 10 g anhydrous sodium sulfate. The extract was concentrated with a rotary evaporator at a temperature *ca.* 40 °C and then the residue was dissolved in 1 mL hexane. Subsequently, the extract was filtered through a 0.45 μm nylon membrane filter and analyzed directly by HPLC. Sample handling and extraction were carried out under dim yellow light to minimize photo-isomerization and oxidation of carotenoids.

The HPLC chromatographic separations were performed on an Agilent HP-1100 series (Wilmington, DE, USA) with ChemStation software, an automated sample-injection system (model G1313A), quaternary pump (model G1354A), four channel in-line vacuum degasser (model G1322A) and a photodiode-array detector (model G1315A). A Zorbax SB- C_{18} (4.6 mm × 150 mm, i.d., 5 μm) was used for carotenoids analysis. The mobile phase was composed of acetonitrile:methanol: methylene chloride with the following gradient elution: 0-4 min (92:8:0) - 10 min (75:20:15) - 20 min (60:20:20) - 5 min (92:8:0). The eluent contained 0.05 % triethylamine as solvent modifier and 0.1 % BHT as antioxidant. The flow rate was 1.0 mL/min and the injection volume was 10 μL. The column was set at 25 ºC. All the chromatograms were monitored at 450 nm. Each carotenoid was identified by the order of elution and their relative retention times in comparison with standard samples. Quantitative analysis of carotenoids was carried out by the internal standard method. The reliability of the methods for determining pigments was confirmed by the analysis of repeatability and recovery. The results showed good repeatability with the relative standard deviations (RSD) ranging from 3.5 to 4.7 % for chlorophylls and 2.3 to 3.7 % for carotenoids and the recovery of pigments ranging from 91.8 to 100.8 %. All analytical experiments were performed in duplicate. The results were averaged for analytical data of two samples.

RESULTS AND DISCUSSION

The flue-curing procedure of tobaccos aims to form the thin and yellow tobaccos that are helpful to improve their aroma[15]. The flue-curing period consists of three essential stages: yellowing, leaf drying and stem drying[16]. The changes of plastid pigment contents can be regarded as one of the indexes to regulate the flue-curing conditions influence on tobacco quality. Tables 1 and 2 show individual plastid pigment distribution pattern in tobacco laminas from different stalk positions during the whole flue-curing period and corresponding temperature changing patterns during different stages of flue-curing period are shown in Fig. 1.

In yellowing stage with flue-curing time 60 h, *i.e.* the first stage with relative low temperature (the initial temperature 28 ºC and maximum 44 ºC, Fig. 1) of flue-curing period, an obvious decrease of plastid pigment content was found, which also reflects the obvious colour change of tobacco laminas. It should be noted that the significant decreases of chlorophyll contents occurred between 36 and 44 ºC (Table-1), while the degradation rate of carotenoids had been accelerating with the increasing temperature from 40 to 48 ºC and this degradation trend continued to the next stage (Table-2). The reason is mainly ascribed to activity of chlorophyllase, lipoxygenase and peroxidase, which were involved in chlorophyll and carotenoid degradation, respectively. The chlorophyllase mainly catalyzes the first step of chlorophyll breakdown by hydrolysis of chlorophyll into chlorophyllide and phytol, which are subsequently converted to pheophorbide. While lipoxygenase and peroxidase mostly participate in the degradation of carotenoid *via* hydroperoxides formation from lipid oxidation. These changes were in agreement with other works on thermal

TABLE-1
CONTENTS OF CHLOROPHYLLS IN TOBACCO LAMINAS FROM DIFFERENT STALK POSITIONS DURING FLUE-CURING PERIOD (μg/g)

Temp. (ºC)	Chlorophyll a			Chlorophyll b			Total chlorophylls		
	Top	Middle	Bottom	Top	Middle	Bottom	Top	Middle	Bottom
28	1.232	1.025	0.985	0.528	0.435	0.415	1.760	1.460	1.400
32	1.012	0.906	0.814	0.452	0.407	0.358	1.464	1.313	1.172
36	0.798	0.757	0.746	0.394	0.357	0.368	1.192	1.114	0.814
40	0.487	0.547	0.348	0.281	0.282	0.165	0.768	0.829	0.513
44	0.293	0.339	0.224	0.159	0.184	0.110	0.452	0.523	0.334
48	0.151	0.196	0.132	0.089	0.106	0.063	0.240	0.302	0.195
52	0.133	0.145	0.107	0.082	0.081	0.054	0.215	0.226	0.161
56	0.142	0.158	0.120	0.092	0.090	0.065	0.234	0.248	0.185
60	0.127	0.136	0.102	0.086	0.082	0.052	0.213	0.218	0.154
64	0.126	0.130	0.099	0.082	0.080	0.049	0.208	0.210	0.148
68	0.118	0.124	0.094	0.082	0.078	0.044	0.200	0.202	0.138

TABLE-2
CONTENTS OF CAROTENOIDS IN TOBACCO LAMINAS FROM DIFFERENT STALK POSITIONS DURING FLUE-CURING PERIOD (μg/g)

Tmep. (ºC)	Violaxanthin			Lutein			β-Carotene			Total carotenoids		
	Top	Middle	Bottom	Top	Middle	Bottom	Top	Middle	Bottom	Top	Middle	Bottom
28	0.081	0.073	0.062	0.182	0.164	0.150	0.142	0.127	0.117	0.405	0.364	0.329
32	0.072	0.068	0.054	0.164	0.154	0.135	0.135	0.122	0.111	0.371	0.344	0.300
36	0.065	0.059	0.046	0.150	0.142	0.118	0.127	0.119	0.107	0.347	0.320	0.271
40	0.053	0.051	0.036	0.126	0.126	0.090	0.116	0.105	0.085	0.295	0.282	0.213
44	0.032	0.040	0.027	0.100	0.098	0.082	0.108	0.094	0.080	0.240	0.232	0.189
48	0.019	0.022	0.017	0.054	0.066	0.045	0.060	0.073	0.048	0.132	0.161	0.110
52	0.011	0.016	0.012	0.049	0.047	0.038	0.048	0.056	0.033	0.108	0.119	0.083
56	0.007	0.012	0.009	0.047	0.047	0.034	0.048	0.055	0.036	0.102	0.114	0.079
60	0.005	0.007	0.004	0.046	0.046	0.032	0.048	0.054	0.036	0.099	0.107	0.072
64	0.004	0.005	0.002	0.045	0.045	0.030	0.046	0.053	0.034	0.095	0.103	0.066
68	0.004	0.003	0.002	0.043	0.044	0.030	0.044	0.050	0.032	0.091	0.094	0.065

processing of other plants such as enzymatic oxidation of carotenoids[17]. As for all enzymes in general, their activity could enhance with increasing temperature in their active temperature ranges, while for different varieties of enzymes, the active temperature also differ. This fact can explain why the obvious degradation of chlorophyll and carotenoid occurred in different ranges of temperature, as well as their different degradation extents. At the end of this stage, the largest degradation extent for chlorophyll a (77.2 %) was found in bottom laminas and only 23.9 % decline for β-carotene in top laminas. The degradation extents of the other three plastid pigments lay between chlorophyll a and β-carotene. The degradation extent of chlorophylls was much larger than that of carotenoids that indicated the tobacco laminas began to present yellow colour.

Leaf drying stage covers the next 36 h with a mean temperature of 50 ºC (maximum 56 ºC, Fig. 1). During this stage, it was noteworthy that two kinds of chlorophylls' contents firstly fluctuated down and then increased a little and gradually reached the initial value of this stage (Table-1). The chlorophyll again increased may be attributed to the release of chlorophyll-protein complexes, which was decomposed owing to cell death with increasing temperature and newly formed free chlorophylls also further degraded. Although carotenoids didn't exhibit such re-increasing case, no statistically significant changes were observed for the degradation of lutein and β-carotene over 52 ºC. However, the degradation of violaxanthin was still continuing and its content was below 0.010 mg/g at the end of leaf drying stage (Table-2). These results may in part reflect the relative stabilities of individual carotenoid to temperature. During this relative high temperature range, individual pigment content changed little except violaxanthin, which was probably caused by the inactivation of enzymes or the moisture content in tobacco laminas didn't maintain the metabolic activity of pigments with increasing temperature.

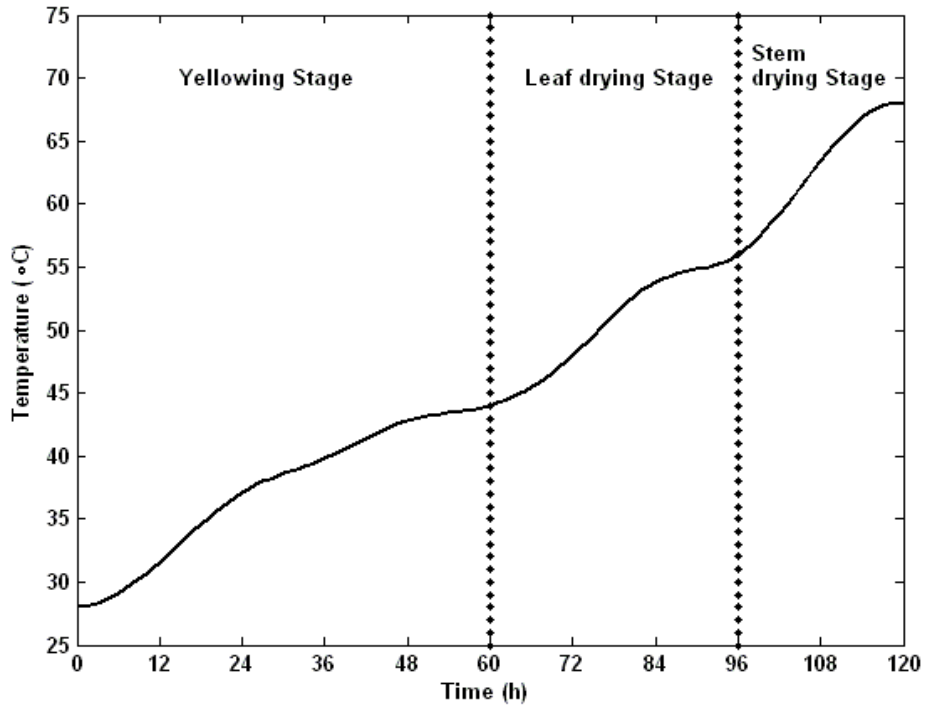

Fig. 1. Temperature-time profile applied during flue-curing period of tobacco laminas

Stem drying stage was the last stage of flue-curing process and the highest temperature was up to 68 ºC. At this stage, the degradation of chlorophyll and carotenoid nearly stopped and their contents present invariability (Tables 1 and 2).

At the end of flue-curing period, the diminution of total chlorophylls ranged from 87 to 91 %. As for chlorophyll a and chlorophyll b, although their degradation behaviour was similar, the difference also found in the degradation contents (Table-1). The diminution of chlorophyll a was larger than that of chlorophyll b, which may be attributed to a higher oxidation of chlorophyll a than that of chlorophyll b under these temperatures. With regard to carotenoids, Court and Hendel[18] reported that the higher oxygenation degree of carotenoids would help to their degradation during tobacco flue-curing. This phenomenon was also observed in our work, in which highly oxygenated xanthophylls, violaxanthin, exhibited almost completely degradation (95-96.7 %), whereas, less oxygenated lutein and β-carotene only decreased 73-80 and 60-73 %, respectively. Because of the differences of the curing conditions or genetic characteristics of the tobacco cultivars, the results in present case was somewhat disagree with the early studies[18]. The different change between chlorophyll and carotenoid content was reflected by the change of tobacco colour which was converted from green to yellow or orange. This fact is clearly confirmed by the evolution of carotenoid to chlrophyll ratio (car/chl), shown in Fig. 2. The tendency of this ratio during yellowing stage is to rise, while during the leaf drying stage it shows a decreasing trend because of the fluctuation of chlorophyll content, to the last stage, it tends to be stable and the value of which is much higher than that before flue-curing.

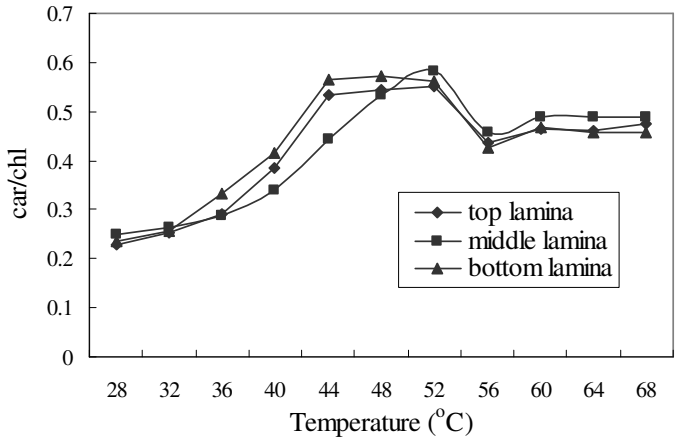

Fig. 2. Evolution of total carotenoids to total chlorophylls content ratio (car/chl)

With regard to the changes of pigments in tobacco laminas from different stalk positions, although heat-induced degradation of pigments in all stalk positions occurred at the same temperature conditions during flue-curing period. It is also found many differences in the degradation extent of any pigment in three stalk positions. Before flue-curing, the contents of major chlorophylls and carotenoids in different stalk positions exhibit a decreased tendency from top to bottom laminas. Different

growth conditions such as sunshine, temperature and nutrient could contribute to this variation. During flue-curing period, the degradation behaviour of total chlorophylls in bottom and top laminas was similar and the declines contents also close to 90 %, both were larger than that in middle laminas (86.2 %). As for total carotenoids, the degradation in bottom laminas was most obviously (80.2 %) and then were top and middle laminas (77.5 and 74.2 %, respectively). Interestingly, with regard to the absolute declines of pigments contents, the largest value was found in top laminas (1.560 μg/g for total chlorophylls and 0.314 μg/g for total carotenoids), while there was no great variation of those between middle and bottom laminas. Thus, at the end of flue-curing period, it was found that the distribution pattern for any pigment changed little in top and middle laminas, which were a little higher than that distributed in bottom laminas (Tables 1 and 2). The reason for this variation can be described to the differences in enzymes contents and activity, as well as their chemical reaction environments in different stalk positions' tobacco laminas, which mainly influenced the degradation contents of pigments[19]. Plastid pigments in top and bottom laminas were mainly degraded during yellowing stage, while for middle laminas, this process continued to the beginning of leaf drying stage, which may related to the different dehydration rates in different stalk positions' laminas. In addition, the change tendency of the ratio between total carotenoids and chlorophylls in different stalk positions also reflected these differences and it is also found no matter before or after flue-curing, the ratio in different stalk positions has little difference (Fig. 2).

Generally, the moisture content of tobacco lamina may be one of the better indicators of cell viability. The degradation of pigments can't occur without moisture, which in tobacco laminas varied with the changes of relative humidity and temperature during flue-curing[12,20]. Low temperature slightly affected the moisture content, which presented more rapid decrease with temperature increasing (Fig. 3). Meanwhile, the maintenance of moisture content could be responsible for the catabolism of the cell organelles, which also lead to the degradation of pigments during flue-curing period. Cell organelles in tobacco laminas which contain relative high moisture content would have vigorous metabolism. Therefore, during yellowing stage, the pigments degraded rapidly while moisture content changed little, then, to the leaf drying stage, moisture content changed rapidly while the pigments decreased more slowly and during the stem drying stage. When the temperature up to 68 ºC, the laminas only contained approximately 4 % moisture content and the degradation of pigments even stopped (Fig. 3). In general, the results may, in part, reflect that the maintenance of moisture content and the declines of pigments contents present a certain positive correlation during flue-curing period of tobacco laminas. With regard to the dehydration rates in different stalk positions' laminas, it was worthy to mention that the greatest loss of moisture content in top and bottom laminas occurred during the leaf drying stage, while for that in middle laminas, which changed gradually during the first two stages. The stem drying stage, moisture content was similar in

all stalk positions' laminas. Therefore, these changes of moisture content further interpreted why there was different duration for pigments degradation in tobacco laminas from different stalk positions.

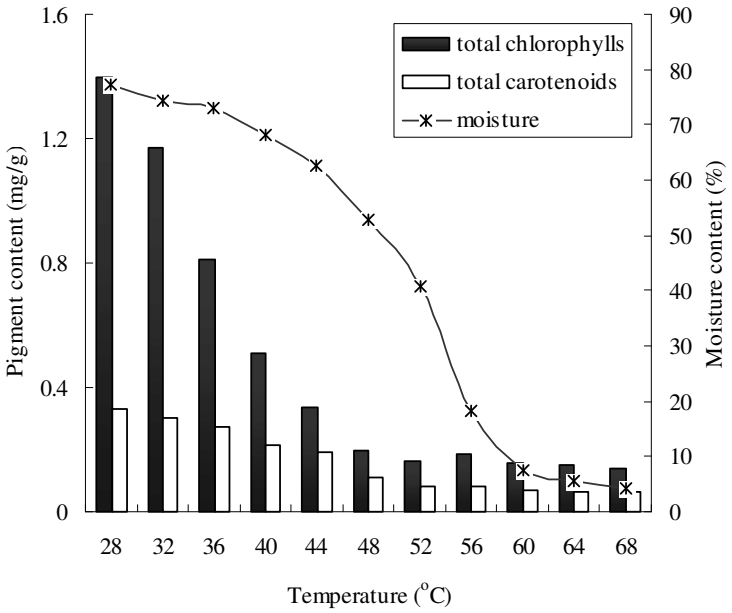

Fig. 3. Evolution of moisture and plastid pigment contents during flue-curing period of tobacco laminas

Conclusion

In conclusion, present results show that the degradation of plastid pigments in tobacco laminas during flue-curing is mainly induced by heat. Besides, different degradation patterns of plastid pigments contents in different stages also indicated different sensitive of individual pigment to temperature. During flue-curing, the dramatic degradation of plastid pigments occurred in the yellowing stage with the temperature from 36 to 44 ºC. After the flue-curing period, the largest decline of 96.7 % for violaxanthin reflected its higher sensitivity to temperature than any other pigments. As for changes of pigments occurred in different stalk positions, those in bottom laminas were most obvious, followed by that in top and middle laminas during the imposed flue-curing conditions. Based on these results, different flue-curing conditions should be designed to flue-cured tobacco laminas from different stalk positions and control of temperature is the most important factor to be optimized to modulate the degradation of plastid pigments. Therefore, performing this kind of temperature model during flue-curing for tobacco laminas from different stalk positions would lead to different pigments retention which can satisfy various

needs of flue-cured tobaccos for cigarette manufacture. In addition, our conclusions might shed light on the research on flue-curing characteristics of tobacco laminas from different stalk positions and the comprehensive evaluation of the relationship between chemical composition and tobacco quality and safety, as well as the manufacture of high flavour and less harmful cigarettes. Further studies designed to provide more information about other conditions, such as light, humidity and flue-curing methods which can also influence the degradation of pigments, are currently in progress.

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