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Major Carotenoids in Tobacco Laminas: Identification and Quantification by HPLC with Photodiode Array Detection

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The carotenoid compositions in various tobacco laminas are determined by high-performance liquid chromatography (HPLC) with the photodiode array detection (DAD). The carotenoids are extracted from powdered tobacco laminas by cold acetone containing 0.1 % butylated hydroxytoluene (BHT). A mobile phase of CH_3CN-H_2O (90:10, v/v) (**A**) and $CH_3COOC_2H_5$ (100 %) (**B**) with following gradient elution is developed: 100 % **A** in the beginning, maintained for 20 min, decrease to 50 % **A** in 50 min, maintain for 5 min and return to 100 % A in 1 h. A total of 11 carotenoids including some isomers in tobacco laminas are resolved within 55 min by using a ZorBAX SB-C_{18} column with the flow-rate at 1.0 mL/min and detection at 445 nm. The limits of detection (LODs) of carotenoids varies from 6.0 to 14.0 ng/mL. The relative standard deviations (RSD) are from 2.69 to 3.63 % and the recovery ranges from 89.6 to 96.3 %. The method is successfully applied for the quantification of carotenoids in green, fresh flue-cured and aging tobacco laminas and the analysis results show that total carotenoids in tobacco laminas range from 49.66 to 820.1 μg/g. Lutein and β-carotene are the most representative carotenoids in flue-cured and aging tobacco laminas because other carotenoids degrade greatly during flue-curing and aging periods. Furthermore, it worth to mention that zeaxanthin is not found in green tobacco laminas while presented in fresh flue-cured and aging tobacco laminas, luteoxanthin as a new carotenoid is first tentatively identified in the present work and α-carotene is not found in any significant amount in all those tobacco laminas.

Key Words: Carotenoids, Tobacco, HPLC, Quantification, Identification.

INTRODUCTION

Tobacco, a nightshade plant which grows in many countries, has drawn widespread attention of many researchers. In tobacco, carotenoids, as the essential chemical composition, have many functions as following: firstly, tobacco's various colours

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are largely due to the presence of diverse carotenoids, which can also contribute to recognize different classes of tobaccos and may directly influence the selling price of tobacco product; secondly, carotenoids not only play many important roles in the photosynthesis of plant chloroplast but also the precursors of the plant hormone abscisic acid (ABA)[1]. Many researchers had demonstrated that carotenoids are the precursors of many aroma components which can affect the internal quality of tobacco[2]. Thus, studies on the relationship between different tobacco laminas and carotenoids' distribution could be helpful for research on quality of tobacco and aroma substances in cigarette smoke.

Several procedures have been described for the determination of carotenoids in tobacco laminas, such as paper chromatography, thin-layer chromatography, column chromatography and low pressure liquid chromatography. But many of these procedures were quite time-consuming and involve extensive sample manipulating, which can result in the formation of artifacts and most of them were published in 1970s[3,4]. Now, reversed-phase HPLC has been regarded as a preferred method for the separation, identification and quantification of carotenoids in many plants because of its improved separation efficiency[5,6]. Especially with the use of photodiode array detection (DAD), which can measure simultaneously at a wide range of wavelengths and scan the wavelength spectra of any chromatographic peak. Although some analysis of carotenoids in different tobacco laminas has been done using HPLC with a C_{18} column, some authors also reported inadequate carotenoids profile or lack in resolving geometrical isomers. For instance, six carotenoids that include neoxanthin, violaxanthin, lutein, zeaxanthin, β-cryptoxanthin and β-carotene were isolated by Court[7], while in some other studies four carotenoids (neoxanthin, violaxanthin, lutein and β-carotene) were determined[8,9], Yang *et al.*[10] have determined only two carotenoids namely lutein and β-carotene. Furthermore, none of the above studies have reported full spectrum of carotenoids and their isomers. Due to unavailability of comprehensive information of carotenoids and their isomers profile in different tobacco laminas, this study was undertaken to develop an appropriate HPLC method to determine a relative complete array of carotenoids including their isomers in tobacco laminas and provide useful information for the evaluation of tobacco appearance and internal quality.

EXPERIMENTAL

β-Carotene and α-carotene mixture standard (2:1, 95 %), zeaxanthin (98 %), β-apo-8'-carotenal (internal standard, 96 %), *tert*-butyl-hydroxytoluene (BHT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Analytical grade chemicals and solvents, including potassium hydroxide, anhydrous sodium sulfate, sodium chloride, methanol, petroleum ether (bp 30-60 °C), acetone, absolute ethanol were obtained from Shanghai Chemical Reagents Company (Shanghai, China). Methanol, acetonitrile, *n*-hexane, chloroform, ethanol, methylene chloride, ethyl acetate and triethylamine were of HPLC grade purchased from Merck (Darmstadt, Germany).

Sample preparation: All tobacco samples were obtained from Yunnan Academy of Tobacco Science (Kunming, China) and University of Science and Technology of China. The green tobacco laminas were cleaned and washed with deionized water, after separating the midrib from the laminas, cut into pieces, quickly freeze-dried and ground into fine powder. The freshly flue-cured and aging tobacco laminas were dried for 3 h at 40 ºC and then ground into fine powder. Spinach, used for preparation of neoxanthin, violaxanthin and lutein standards, was obtained from a local supermarket, washed with deionized water, cut into pieces, freeze-dried and ground into fine powder. Moisture contents of all tobacco laminas were determined before carotenoids extraction. The levels of carotenoids shown in this paper referred to the carotenoids contents in 1 g dry weight of tobacco laminas.

Instrumentation: The carotenoids separation was performed on a Agilent 1100 series HPLC system (Agilent Technologies Inc., Palo Alto, CA, USA) equipped with a 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). All data were acquired and processed by Agilent Chemstation (Rev.A.08.03) software. The Heidolph rotary evaporator (model 4001) was from ITS Science (China) Co. Ltd. The nitrogen evaporator system (model N-EVAP) and the baking oven were from LWL Development LTD. (Shanghai, China). The freeze-dryer (model DTY-1SL) was from Beijing DETIANYOU Technology Development Co., Ltd. (Beijing, China). The spectrophotometer (model CE3021) was from Cecil Co. (Cambridge, UK). The Mili-Q-185 Plus water purification system (Millipore, Bedford, USA) was used to prepare deionized water (0.22 μm).

Method

Carotenoids extraction: 0.5 g ground tobacco was mixed with 25 mL cold acetone containing 0.1 % BHT as antioxidant in a 100 mL Erlenmeyer flask which was wrapped with tinfoil and added 50 μg β-apo-8'-carotenal as internal standard. After vigorously shaking on a vibrator for 1 h, the sample was filtrated directly through Whatmann No. 1 filter paper and the residue was repeatedly extracted three times in order to extract carotenoids completely. Then, the filtrate was combined and dried with 15 g anhydrous sodium sulfate. The extract was evaporated to dryness in a rotary evaporator at a temperature not exceeding 35 ºC and redissolved in about 1 mL acetonitrile-methanol-ethyl acetate (4:3:3, v/v/v). After filtrating through a 0.45 μm nylon membrane filter, 10 μL of the extract was injected for HPLC analysis. Sample handling and extraction were carried out at 4 ºC, under dim yellow light and nitrogen gas was flushed into vials to minimize photo-isomerization and oxidation of carotenoids. In addition, after extraction, the sample was immediately subject to HPLC analysis to avoid the carotenoids degradation in acetone solvent.

Preparation of lutein, violaxanthin and neoxanthin standards: Because of absence of commercial standards of lutein, violaxanthin and neoxanthin, they were prepared from spinach, using a method described by Kimura[11]. Initially, 15 g freeze-

dried spinach powder was mixed with 90 mL cold acetone in a 250 mL volumetric flask and shaken for 1 h, then the extract was filtrated, partitioned to petroleum ether, saponified overnight, in the dark, at room temperature under N_2 stream, using 10 % methanolic KOH. After which, the saponified extract was washed with saturated saline and distilled water to remove alkali. The upper layer was dried with anhydrous sodium sulfate and concentrated to 2-3 mL, which was poured into a column (30 cm × 20 mm i.d.) containing 30 g neutral alumina (activate at 110 ºC for 3 h). Anhydrous sodium sulfate was poured into the column to form a layer of 1cm above the adsorbent. A solvent system of acetone and petroleum ether with a proportion of 85:15 and 60:40 (v/v) was used to elute lutein and violaxanthin, respectively and 100 % acetone was used to elute neoxanthin. All the isolated standards were further confirmed by the method of TLC[12]. The purity of the isolated standards was verified by HPLC according to the method of Kimura[11]. Once the desired purity was verified, the concentrations of the pure standards were determined by a spectrophotometer with the absorbance measured at 446 nm for lutein, 440 nm for violaxanthin and 438 nm for neoxanthin, respectively. The concentrations were calculated, using the following formula[13]:

$$\text{Concentration (g mL}^{-1}) = \frac{E}{E_{1\,cm}^{1\%} \times 1000}$$

where E is the absorbance and $E_{1\,cm}^{1\%}$ is the extinction coefficient (lutein 2250, violaxanthin 2250 and neoxanthin 2243). The final concentrations of lutein, violaxanthin and neoxanthin were calculated to be 192.0, 42.6 and 54.8 μg/mL, respectively and the purity calculated as the percentage of the carotenoids peak area relative to total area was 90-95 % for neoxanthin, 93-96 % for violaxanthin and 94-98 % for lutein, respectively.

Preparation of standard solutions: The stock solutions of β-apo-8'-carotenal (IS) (5 mg), zeaxanthin (1 mg), α-carotene and β-carotene (6 mg) were prepared by dissolving them using chloroform and *n*-hexane mixture (1:9, v/v) in a 10 mL of volumetric flask and made them to a final concentration of 500, 100, 200 and 400 μg/mL, respectively. A final fixed concentration of β-apo-8'-carotenal (IS, 50 μg/mL) was added to the five different concentrations of zeaxanthin (1.0, 5.0, 10.0, 15.0, 25.0 μg/mL), α-carotene (1.0, 10.0, 20.0, 35.0, 50.0 μg/mL) and β-carotene (2.0, 20.0, 40.0, 60.0, 100.0 μg/mL). The isolated standards of lutein, violaxanthin and neoxanthin were concentrated and resolved in ethanol. The standard solutions of lutein (1.92, 19.2, 57.6, 96.0, 192.0 μg/mL), neoxanthin (0.86, 8.6, 17.2, 25.8, 42.6 μg/mL) and violaxanthin (1.1, 14.5, 27.9, 41.3, 54.8 μg/mL) were prepared separately by dilution of each stock solution which was isolated from spinach and β-apo-8'-carotenal (IS, 50 μg/mL) was also added to each standard solutions. All standard solutions were stored in air-tight screw topped brown bottles under nitrogen at -20 ºC and BHT was added at 0.1 % to avoid degradation and oxidation.

HPLC analysis of carotenoids in tobacco laminas: For the qualitative and quantitative analysis, carotenoids separations were carried out on a Zorbax SB-C_{18} column (250 mm × 4.6 mm i.d, 5 μm). The appropriate mobile phase consisted of CH_3CN:H_2O (90:10, v/v) (**A**) which contained 0.1 % butylated hydroxytoluene (BHT) as an antioxidant and 0.1 % triethylamine (TEA) as a solvent modifier and $CH_3COOC_2H_5$ (100 %) (**B**) with the following gradient elution: 100 % **A** in the beginning, maintained for 20 min, decreased to 50 % **A** in 50 min and maintained for 5 min, returned to 100 % **A** in 1 h with the flow rate at 1.0 mL/min. The column was equilibrated for 15 min at the beginning before each injection. The injection volume was 10 μL and column temperature was set at 23 ºC. Chromatograms were processed at 445 nm and the spectrum from 300 to 800 nm was recorded and stored.

RESULTS AND DISCUSSION

Optimization of chromatographic conditions: In order to achieve an effective separation of the carotenoids of interest in tobacco laminas, a range of mobile phase conditions was evaluated, which included different sets of solvent systems with various proportions. The first set was consisted of ternary mixtures of acetonitrile, methanol and methylene chloride which was widely used on monomeric or polymeric reversed-phase C_{18} column by many authors[14-17]. When this mobile phase was initially used in present study, only non-polar carotenoids such as α-carotene and β-carotene were achieved good separation. As to polar carotenoids, especially for lutein and zeaxanthin, resolution of them was extremely difficult because they have the same empirical formula and almost identical structures, as well as their very close polarity[18]. Although Nelis *et al.*[16] investigated that lutein and zeaxanthin were separated using a mobile phase consisting of acetonitrile:methanol:methylene chloride (70:20:10) on a Zorbax ODS C_{18} column. In present experiment, baseline resolution of them was not achieved by using this mobile phase with various isocratic and gradient elution, which was in agreement with Heinonen's study[17]. At the same time, the resolution of neoxanthin and violaxanthin also was poor by using this mobile phase on this kind of C_{18} column. For this reason we performed further work to select a new mobile phase.

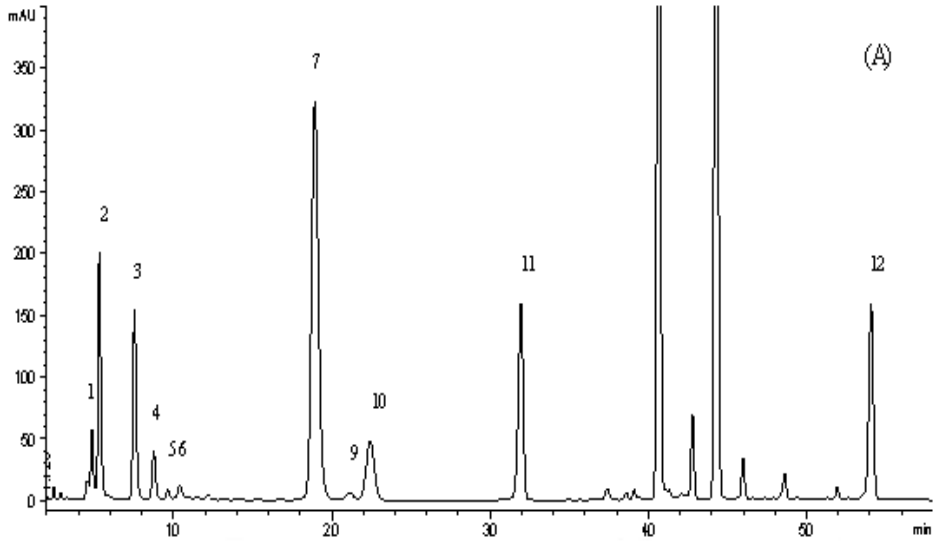
Subsequently, in order to achieve a good separation of lutein and zeaxanthin, the second set of mobile phase designed to introduce a range of final percentages of water (0-10 %) in acetonitrile in combination with ethyl acetate. Methanol was removed because it could lead to higher absorption in mobile phase. It was reported that the chlorinated solvent was associated with carotenoid losses, so we chose ethyl acetate to replace methylene chloride. As a result of this modification, it was observed that a complete separation of lutein and zeaxanthin was achieved under whether only CH_3CN or CH_3CN-H_2O system. Meanwhile, in order to improve the separation of early eluting xanthophylls (neoxanthin and violaxanthin), we used 100 % CH_3CN-H_2O system (90:10, v/v) at the beginning for the separation of polar carotenoids and then gradually in the combination of 0 to 50 % ethyl acetate for the

resolution of non-polar carotenoids. In addition, to ensure the maximum column recovery of carotenoids under investigation as possible, 0.05 % triethylamine was added in mobile phases as solvent modifiers, as well as antioxidant BHT, which was also included in the mobile phase at the final concentration of 0.1 % to avoid degradation of carotenoids during the chromatographic analysis.

Column temperature has also been reported that it could influence the carotenoids retention times and column selectivity. Changes in column temperature could cause significant changes in the charomatographic response of carotenoids[19]. Therefore, several column temperatures ranged from 18 to 35 ºC also investigated in present work to achieve a greater retention and good separation. It was observed that the best separation was achieved at 23 ºC and which eventually was chosen as optimal in combination with other HPLC conditions, under which all carotenoids of interest in tobacco laminas achieved good separation.

Although diode array detector allowed several wavelengths of interest, several authors also chose a more selective wavelength according to special samples as far as colour concerned[20]. For example, 486 nm was selected for the determination of carotenoids in Valencia orange juice, because the absorption in the red-orange region of the visible spectrum was maximum at this wavelength[21]. In present case, various wavelengths also investigated and 445 nm was chosen to the final monitor wavelength, under which all carotenoids of tobacco laminas were separated from each other and had the greatest absorption.

Therefore, using the above optimal chromatographic conditions, zeaxanthin and lutein showed well separated peaks on the chromatography and baseline resolution was also achieved with reasonable retention times and symmetrical peaks for other carotenoids in tobacco laminas (Fig. 1).



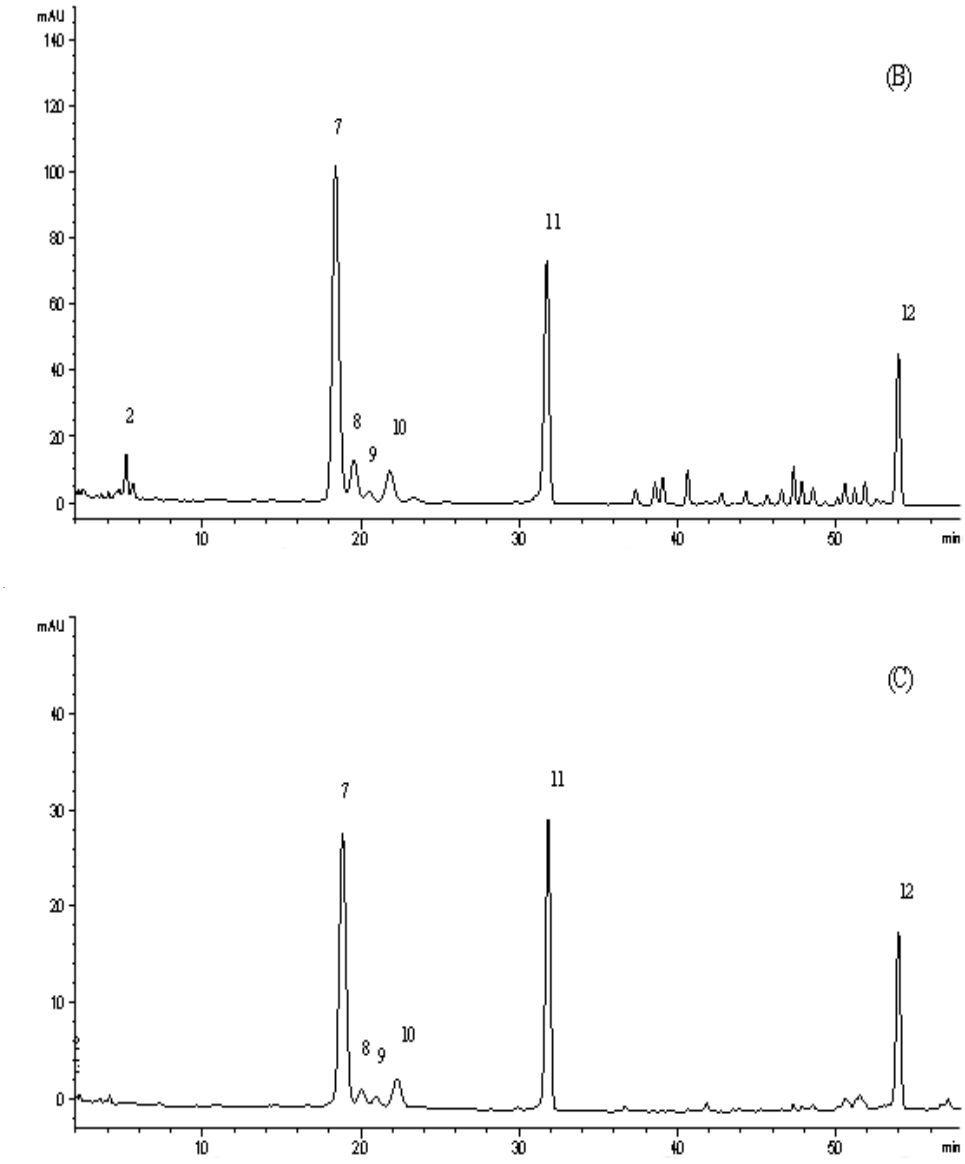


Fig. 1. HPLC profiles of carotenoids in: (A) green tobacco laminas; (B) fresh flue-cured tobacco laminas and (C) aging tobacco laminas. Chromatographic separation on a Zorbax SB-C_{18} column (150 mm × 4.6 mm i.d, 5 μm), conditions: flow rate 1 mL min^{-1}, UV absorbance detection at 445 nm; mobile phase: acetonitrile-H_2O (90:10, v/v) (A) and ethyl acetate (100 %) (B); 0-20 min (100 % A) -50 min (50 % A) - 55 min (50 % A) - 60 min (100 % A). Peaks identification: (1) neoxanthin a; (2) neoxanthin b; (3) violaxanthin; (4) luteoxanthin; (5) *cis*-luteoxanthin; (6) *cis*-violaxanthin; (7) lutein; (8) zeaxanthin; (9) *cis*-lutein; (10) *cis*-lutein; (11) β-apo-8'-carotenal; (12) β-carotene

Validation of the chromatographic method

Linearity, limit of detection (LOD) and limit of quantification (LOQ): The limit of detection and limit of quantification were calculated from the equation of the calibration curve according to the method which was described by Dias *et al.*[22]. The results are shown in Table-1. It was observed that good linearity in calibration concentration range which was adequate for the carotenoids determination in tobacco samples, as well as the results of LODs and LOQs also confirmed that the method was very sensitive for the carotenoids study.

TABLE-1
LINEARITY, LODS AND LOQS OF CAROTENOIDS USING THE PRESENT METHOD

Carotenoids	Linear range (μg/mL)	Calibration curve	Correlation coefficient	LOD (ng/mL)	LOQ (ng/mL)
Neoxanthin	0.86-42.6	Y = 0.3557X-0.0259	0.9972	9.0	29.0
Violaxanthin	1.10-54.8	Y = 0.3469X+0.0292	0.9976	14.0	46.0
Lutein	1.92-192.0	Y = 0.6702X+0.0397	0.9987	8.0	25.0
Zeaxanthin	1.00-25.0	Y = 0.1741X+0.0104	0.9984	11.0	32.0
α-Carotene	1.00-50.0	Y = 0.2151x-0.0142	0.9992	7.0	23.0
β-Carotene	2.00-100.0	Y = 0.4109X+0.0323	0.9996	6.0	19.0

Precision (RSD) and recovery (accuracy): For the repeatability analysis, carotenoids in tobacco laminas were determined 7 times under the controlled conditions. Relative standard deviations (RSD, %) for neoxanthin, violaxanthin, lutein, zeaxanthin and β-carotene were 3.24, 3.63, 2.75, 3.02 and 2.69, respectively.

To study the recovery, three concentrations of each carotenoid standard were added to the tobacco sample for extraction. The ratios of the content of each carotenoid to those in tobacco laminas were *ca.* 0.5, 1.0, 2.0. After HPLC analysis, the recovery of each carotenoid was calculated based on the ratio of the concentration obtained by HPLC to the concentration of each carotenoid standard initially added to the sample. Finally, it was found that recoveries of each carotenoid were all between 89.6 and 96.3 %. Due to absence of commercial standards of *cis* isomers and their extinction coefficients were similar to those of their parent *trans* forms, so the present recoveries and RSDs for *cis* isomer of carotenoids were considered equivalent to those *trans* forms.

Qualitative and quantitative analysis of carotenoids in tobacco laminas: The chromatograms (Fig. 1) were corresponded to green, fresh flue-cured and aging tobacco laminas extract. All 12 carotenoids including internal standard β-apo-8'-carotenal in tobacco laminas were resolved within 55 min (Fig. 1). Carotenoids were initially identified by their retention times and absorption spectra including the wavelength of maximum absorption and spectral fine structure, with comparison to available authentic standards. The chromatographic data and spectral characteristics of authentic standards (Table-2) compared with those of unknown peaks in Fig. 1, thus, peak 2, 3, 7, 8, 11, 12 in tobacco laminas were successfully identified

TABLE-2
POSITIVE AND TENTATIVE IDENTIFICATION OF ALL-*trans* AND *cis* FORMS OF CAROTENOIDS IN TOBACCO LAMINAS

Peak	Carotenoids	RT (min)	λ (nm)								%III/II		Q-ratio	
			Observed				Reported				Obs.[a]	Rep.	Obs.[a]	Rep.
1	Neoxanthin a	4.739	–	418	440	468	–	419	441	470[b]	66.4	63.3[b]	–	–
2	Neoxanthin b	5.214	–	414	438	466	–	418	441	470[b]	85.2	81.6[b]	–	–
3	Violaxanthin	7.408	–	417	440	470	–	414	442	472[b]	88.6	90.0[b]	–	–
4	Luteoxanthin	8.626	–	400	422	450	–	400	424	450[b]	98.2	97.9[b]	–	–
5	*cis*-Luteoxanthin	9.515	–	408	433	464	–	408	432	462[c]	46.5	–	–	–
6	*cis*-Violaxanthin	10.274	330	412	434	464	328	414	435	463[d]	54.2[d]	61.0	0.17	0.18[d]
7	Lutein	18.996	–	420	446	474	–	426	447	474[b]	55.3	50.0[b]	–	–
8	Zeaxanthin	20.199	–	428	454	480	–	428	452	481[b]	27.0	28.0[b]	–	–
9	*cis*-Lutein	21.230	330	420	443	470	332	416	440	470[e]	42.7	–	0.13	0.13[e]
10	*cis*-Lutein	22.563	332	420	442	468	332	421	446	–[e]	43.8	–	0.26	0.16[e]
11	β-apo-8'-carotenal	32.235	–	–	460	–	–	–	–	–	–	464[e]	–	–
12	β-Carotene	54.135	–	429	454	478		429	454	479[b]	32.7	26.1[b]	–	–

[a]A gradient mobile phase of acetonitrile-H_2O (90:10,v/v) and ethyl acetate (from 100:0, v/v to 50:50, v/v) was used. [b]A mobile phase of acetonitrile-methanol (75:25), MTBE and H_2O (from 95:0:5, v/v/v to 26:47:0, v/v/v) was used by Lee[28]. [c]A mobile phase of n-hexane and acetone (from 100:0, v/v to 60:40, v/v) was used by Mercadante *et al.*[29]. [d]A mobile phase of methanol and MTBE (from 95:5, v/v to 50:50, v/v) was used by Vera de Rosso *et al.*[27]. [e]A mobile phase of methanol-acetonitrile- H_2O (84:14:2) and methylene chloride (from 100:0, v/v to 55:45, v/v) was used by Chen[30].

as all-*trans*-neoxanthin, all-*trans*-violaxanthin, all-*trans*-lutein, all-*trans*-zeaxanthin, β-apo-8'-carotenal and all-*trans*-β-carotene, respectively.

Generally, because of lack of some commercial standards of carotenoids, especially of *cis* isomers, peaks were usually identified by means of published criteria, for example, the absorption maxima, % III/II (the percentage of the quotient between band III and band II (normally λ_{max})) and Q-ratios (the quotient between the *cis* peak band and band II (normally λ_{max})) were used to identify the carotenoids and their isomers[23]. Typically, carotenoids always exhibit the typical three-peak absorption, which is the most distinguishing feature differentiate from other compounds (chlorophylls *i.e.*) on the HPLC chromatogram. For carotenoids' *cis* isomers, it has been reported that it can give the compound a slightly lighter colour and bring both a hypsochromic shift (normally between 2 and 6 nm at lower wavelengths) and a hydrochromic effect (decrease in absorbance due to the appearance of a "*cis*" peak *ca.* 142 nm below the λ_{max} of the *trans*-isomer)[24]. The information of absorption, % III/II and Q-ratios obtained from the absorption spectra for individual carotenoid in the current HPLC method, along with the previously published results are shown in Table-2. The obtained results mostly agree with the early reports, a little difference may attribute to that they were measured in different solvent systems[25].

Based on the criteria described above, for those carotenoids peaks which had no authentic standards comparison, peak 1 was tentatively identified as neoxanthin a, because it presents a similar spectrum to violaxanthin with a reduced fine structure[24]. Peak 6 was tentatively assigned to *cis*-violaxanthin due to the hypsochromic shift of 6 nm relative to its *trans*-isomer (peak 3) and that agreed with the previous

study[24]. Peak 9 and 10 were tentatively identified as lutein isomers based on the spectra characteristics and Q-ratios as reported in the literature (Table-2) and which further confirmed according to the criteria described by Chen[26] that all-*trans*-lutein standard was illuminated at 25 ºC for 24 h would generate its isomers. Peak 4 and 5 were tentatively identified as luteoxanthin and its isomers because a hypsochromic shift of 20 nm occurred when compared to violaxanthin, which also in accordance with the results obtained in previous studies[27,28].

The quantification of carotenoids was based on the calibration curves using β-apo-8'-carotenal as internal standard. Because of absence of standards of *cis* forms of carotenoids, the *cis* isomers were quantified according to the calibration curves of their corresponding all-*trans*-isomers, while luteoxanthin and its isomer quantified based on the standard curve of violaxanthin.

Application of carotenoids profiling to different tobacco samples: The quantitative distribution of carotenoids in green, freshly flue-cured and aging tobacco laminas are detailed in Tables 3 and 4. Fig. 1 shows the HPLC chromatograms of carotenoids in three representative kinds of tobacco samples. The differences in carotenoids contents and profiles in these three kinds of tobacco samples can be seen clearly except a common point that α-carotene was not found in all tobacco samples. The green tobacco laminas contained the most profiles of carotenoids in greatest quantity except zeaxanthin which was too little to achieve its LOD (Table-4 and Fig. 1A). As depicted in Table-3, in green tobacco laminas from different stalk positions, the major carotenoid was lutein representing approximately 49-55 % of the total carotenoids, followed by β-carotene (~23-25 %), *cis*- and *trans*-neoxanthin (~10-12 %), *cis*- and *trans*-violaxanthin (~9-12 %) and *cis*- and *trans*-luteoxanthin (~3-5 %). Moreover, it was noteworthy to mention that luteoxanthin and its *cis*-isomer were observed in tobacco samples for the first time in present study. Likewise, evaluation of carotenoids in the freshly cured tobacco laminas revealed some significant losses. It is obvious that violaxanthin and luteoxanthin including their isomers disappear, while the peak of zeaxanthin appear on the HPLC chromatograms (Fig. 1B). This may be interpreted that tobacco laminas contained violaxanthin de-epoxidase and hence was enable to convert violaxanthin to zeaxanthin under heat treatment[29]. Nevertheless, heating did not change the general profile of lutein and β-carotene, indicating that they were more stable compared to violaxanthin. The result obtained in present experiments was also found in spinach by Bunea[30]. Meanwhile, *cis*-isomers of lutein were found to be a little increase which may form from the conversion of *trans*-isomer during the heat treating process. In the same manner, carotenoids profiles in aging tobacco laminas with different varieties were also investigated (Fig. 1C and Table-4). In these tobacco samples, compared the carotenoids profiles of freshly flue-cured tobacco laminas, neoxanthin was further disappeared and the content of other carotenoids decreased significantly.

Furthermore, from the results of Table-4, it was observed that carotenoids contents in green tobacco laminas varied in different stalk positions, which exhibited a decreased

TABLE-3
CAROTENOIDS CONTENT IN GREEN AND FRESH FLUE-CURED TOBACCO LAMINAS FROM DIFFERENT STALK POSITIONS (μg/g)

Peak number	Carotenoids	Green			Fresh flue-cured		
		Top	Middle	Bottom	Top	Middle	Bottom
1	Neoxanthin a	12.11	11.46	5.95	–	–	–
2	Neoxanthin b	79.42	71.43	56.84	52.43	46.27	30.47
3	Violaxanthin	75.82	66.71	47.32	–	–	–
4	Luteoxanthin	34.86	20.86	18.94	–	–	–
5	*cis*-Luteoxanthin	3.52	2.88	1.85	–	–	–
6	*cis*-Violaxanthin	11.96	10.23	8.08	–	–	–
7	Lutein	372.50	308.50	274.70	272.40	258.10	178.90
8	Zeaxanthin	–	–	–	27.81	22.02	19.52
9	*cis*-Lutein	6.85	5.19	4.56	7.18	6.48	6.02
10	*cis*-Lutein	33.65	32.84	25.33	39.45	34.67	–
12	β-Carotene	189.40	168.70	136.50	156.40	144.80	114.20
	Total carotenoids	820.10	698.80	580.10	555.70	512.30	349.10

– Not detected.

TABLE-4
CAROTENOIDS CONTENT IN DIFFERENT TYPES OF AGING TOBACCO LAMINAS (μg/g)

Peak number	Carotenoids	Flue-cured tobacco		Oriental tobacco		Burley tobacco	
		A	B	A	B	A	B
1	Neoxanthin a	–	–	–	–	–	–
2	Neoxanthin b	–	–	–	–	–	–
3	Violaxanthin	–	–	–	–	–	–
4	Luteoxanthin	–	–	–	–	–	–
5	*cis*-Luteoxanthin	–	–	–	–	–	–
6	*cis*-Violaxanthin	–	–	–	–	–	–
7	Lutein	72.70	57.05	26.92	23.91	42.17	41.62
8	Zeaxanthin	10.52	8.63	7.39	4.21	6.49	8.02
9	*cis*-Lutein	5.24	4.28	–	–	–	–
10	*cis*-Lutein	15.50	8.11	6.12	5.74	9.25	8.16
12	β-Carotene	57.52	42.50	18.14	15.80	23.16	26.15
	Total-carotenoids	161.5	120.6	58.57	49.66	81.07	83.95

– Not detected.

tendency from top to bottom laminas. Different growth conditions such as sunshine, temperature and nutrient could contribute to this variation. For the carotenoids in fresh flue-cured tobacco laminas, a significant decrease also found in comparing to that in green tobacco laminas, which could attribute to the heat-induced carotenoids degradation. For the content of carotenoids in six aging tobacco laminas, including flue-cured, burley and oriental tobacco which were the main material in the manufacture of commercial cigarettes, it is found that the carotenoids contents decreased in the order of flue-cured tobacco, burley tobacco and oriental tobacco and which

also present the lowest carotenoids contents compared to other two representative kinds of tobacco sample. Therefore, these results suggested that the major carotenoids degradation occurred during the curing process with continuous decomposition during aging period. Such degradation of carotenoids may provide important implications for the formation of flavour compounds in cigarette manufacture.

Conclusion

In this paper, a HPLC method was developed as a reliable method to determine an array of carotenoids in tobacco samples. Using the described concise method, 11 carotenoids including their *cis* isomers in tobacco laminas has been characterized from their spectral data and retention times obtained with authentic standards or literature values. Furthermore, in present study, not only lutein and zeaxanthin, as well as some carotenoids isomers were resolved, but also a new carotenoid (luteoxanthin) was tentatively identified in tobacco samples for the first time. Meanwhile, it is confirmed that α-carotene was not found in all investigated tobacco samples. Apparently, the HPLC method proposed in this study provided a better resolution power in analyzing carotenoids of tobacco samples compared to reported literatures. Besides, separation and quantification of carotenoids contained in three representative kinds of tobacco laminas (green, fresh flue-cured and aging) by the described method showed a clear differentiation between those of different samples. The variation in carotenoids contents and profiles indicated that carotenoids degradation in tobacco laminas not only occurred during the flue-curing process but also continued to the aging period. Basically, further studies are designed to extend this methodology for determination of carotenoids in some representative commercial products of tobaccos, as well as in some food samples.

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