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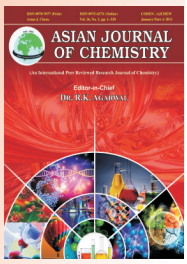

Development of Method to Determine C6-C3 Phenolic Acids in Tobacco (*Nicotiana tabacum*) by HPLC

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A method to simultaneous determination of four C6-C3 phenolic acids in tobacco by high performance liquid chromatography (HPLC) was established. Four C6-C3 phenolic acids were well separated in 40 min. The proposed method had high sensitivity, accuracy and repeatability with good linear relationship ($R^2 \geq 0.9992$) when the concentration of phenolic acids was between 1.113 and 102.332 μg/mL. This method was applied to tobacco samples. The results showed that the content of four C6-C3 phenolic acids was significantly different in tobacco at different regions and growth stages, and the contents of *p*-coumaric acid, ferulic acid and sinapic acid in tobacco were influenced by the ecological conditions more than growth stages. This might be one of the reasons that the tobacco leaves in different regions had different quality.

Keywords: Tobacco, HPLC, C6-C3 phenolic acids.

INTRODUCTION

In recent years, phenolic acids have attracted widespread attention as potential antioxidant activity, anticancer effects, healthcare functions for chronic diseases and their ubiquity in a wide range[1-4]. There are two types of main skeleton of phenolic compounds found in nature: (1) C6-C1 phenolic compounds. The basic skeleton is benzoic acid, for example, protocatechuic acid, vanillic acid, syringic acid, gallic acid, *etc*. (2) C6-C3 phenolic compounds. They are mainly present in the form of cinnamic acid derivatives, such as caffeic acid, ferulic acid, sinapic acid and *p*-coumaric acid, *etc*. It usually exists in the form of conjugates which connected together with quinic acid or glucose[5].

The C6-C3 phenolic compounds which comprised of phenolic hydroxyl and carboxyl compounds were the intermediate products of phenylpropanoid metabolic pathway and the metabolite of lignin biosynthesis in tobacco. C6-C3 phenolic compounds were closely related to adversity resistance and physiological activity of tobacco. Therefore, the determination of C6-C3 phenolic acids in tobacco is important for judging the quality and flavor of tobacco.

Determination of phenolic compounds by high performance liquid chromatography (HPLC) has been reported in plant, such as fruits[6,7], vegetables[8,9], honey[10-12], medicinal plants[13], food crops[14,15] and herbs[16]. However, it took 40-50 min or even 70-90 min to separate the main phenolic compounds including *p*-coumaric acid, sinapic acid and ferulic acid with poor separation effects[7,18,19]. To the best of our knowledge, there were no systematic reports in the literature regarding the determination of C6-C3 phenolic compounds (*p*-coumaric acid, ferulic acid and sinapic acid) in tobacco. In the present study, we set out to investigate the method for determination of the C6-C3 phenolic compounds in tobacco with a short analysis time by HPLC.

EXPERIMENTAL

The reference substances including caffeic acid, *p*-coumaric acid, ferulic acid and sinapic acid were reagent grade with purity ≥ 98 % and purchased from Sigma-Aldrich (St. Louis, MO, USA). The internal substance cinnamic acid was reagent grade with purity ≥ 98 % and purchased from Beijing Century Aoke Biotechnology Reagent. (Beijing, China). Acetonitrile, acetic acid and other reagents were HPLC grade and purchased from Tedia Co. (Tedia, USA).

Collection and pretreatment of tobacco sample: The 11th leaves (from the bottom to top) of tobacco (*Nicotiana tabacum* L. K 326) were collected from five regions (Heilongjiang, Shanxi, Shandong, Guizhou and Chongqing) at five growth stages (every 10 days after flowering until one day before harvest), respectively. Immediately after the sampling, the leaves were frozen with liquid nitrogen. Then all samples were dried with vacuum freeze-drying until constant weight (no water) and ground to powder for the analysis of C6-C3 phenolic compounds.

Free phenolic acids extraction: Accurately 0.6 g of tobacco sample was weighted into a flask and 200 µL of 100 µg/mL cinnamic acid (internal standard) was also added. 20 mL extraction solvent were added and then ultrasound extraction was used (100 Hz, room temperature). At last, the extraction solution was filtered with 0.22 µm nylon membrane. The different extraction reagent and time were as shown in Table-1.

TABLE-1
EXTRACTION CONDITIONS OF FREE PHENOLIC ACIDS

Extraction solvent	Ultrasound extraction time (h)
70 % methanol	0.5
	1.0
	2.0
80 % methanol	0.5
	1.0
	2.0
100 % methanol	0.5
	1.0
	2.0

Bound phenolic acids extraction: Accurately 0.6 g of tobacco sample was weighted into a flask and 200 µL of 100 µg/mL cinnamic acid (internal standard) was also added. The sample was hydrolyzed by 40 mL of different concentration of NaOH solutions (1, 2 or 4 mol/L) with 4 h oscillation and then centrifuged 15 min at 4 °C with 10000 rpm. The supernatant was transferred into another flask and the pH was adjusted to 2 using 6 mol/L HCl. After extracted three times with 20 mL of ethyl acetate and ethyl ether with volume ratio 1:1, the organic phases were combined. The organic layer was dried by anhydrous sodium sulfate and concentrated at 50 °C using rotary evaporator until organic phase was evaporated to dryness. Finally, the residue was diluted with 2 mL acetonitrile and the solution was filtered with 0.22 µm nylon membrane.

Instruments and chromatographic conditions: The fresh tobacco were dried by a PiloFD-4.3V freeze dryer (SIM International Group Co. Ltd., Newark, USA). The centrifugation was carried out using a 5804R refrigerated centrifuge (Eppendorf, Hamburg, Germany). Phenolic acids was analyzed by HPLC (Waters Corporation, Milford, MA, USA) equipped with a C_{18} ODS_2 column (4.6 mm × 250 mm, 5 µm; waters corporation, ireland). The mobile phases were 2 % acetic acid aqueous solution (A) and 2 % acetic acid acetonitrile solution (B). The program was as follows: linear gradient from 95 % A/5 % B to 70 % A/30 % B, 0-40 min; linear gradient from 70 % A/30 % B to 95 % A/5 % B, 40-50 min. The column temperature was 35 °C and the flow rate was 1.0 mL min^{-1}. The detection wavelength was 310 nm and the injection volume was 4 µL.

The C6-C3 phenolic acid compounds were qualitatively detected by the comparisons of the reference samples in terms of chromatographic retention time and it was quantitatively detected by the calibration curves with the internal standard methods.

RESULTS AND DISCUSSION

Optimization of wavelength: In order to obtain the maximum absorption wavelength of each compound, the full wavelength was scanned at 190-400 nm. The results showed that there were two maximum absorption regions which existed in the range of 230-235 and 320-330 nm. 310 nm was selected as the detection wavelength due to the high degree of separation and signal-to-noise ratio obtained.

Optimization of extraction solvents: Both free and bound phenolic acid compounds in tobacco samples were analyzed. The free C6-C3 phenolic acid compounds were extracted with different concentration of methanol. The results showed that the free C6-C3 phenolic acid compounds wasn't detected in the solution extracted by 70 % methanol, 80 % methanol solution and 100 % methanol for 0.5, 1.0 and 2.0 h, respectively and the result was also validated with UPLC-Xevo QTof MS. Since there is no free phenolic acid was found in tobacco, we mainly focus on the method of bound phenolic acid in the following study.

Based on previous studies of bound phenolic acids in other crops[9,15,20], 1, 2 and 4 mol/L NaOH solutions were used as extraction solvents. As shown in Table-2, the phenolic acid contents were highest in 4 mol/L NaOH solution and 4 mol/L NaOH was further applied to analysis of tobacco samples.

TABLE-2
CONTENT OF BOUND C6-C3 PHENOLIC ACIDS IN TOBACCO WITH DIFFERENT EXTRACTION SOLVENTS (µg/g)

Compounds	NaOH (1 mol/L)	NaOH (2 mol/L)	NaOH (4 mol/L)
Caffeic acid	1.15	1.23	1.24
p-Coumaric acid	22.03	24.08	24.76
Ferulic acid	10.56	12.76	12.98
Sinapic acid	7.15	8.54	9.03

In present study, free C6-C3 phenolic acids weren't detected in tobacco leaves, but the bound C6-C3 phenolic acids could be released under strong alkali condition. In order to prevent ionization of carboxyl and phenolic hydroxyl groups the solutions A (2 % acetic acid solution)-B (2 % acetic acid acetonitrile solution) were selected as mobile phase to achieve the four kinds of C6-C3 phenolic acids well separated within 40 min. In previous studies, caffeic acid, *p*-coumaric acid, ferulic acid and sinapic acid were separated at 31-80 min with RP-C18 (LiChroCART, 250 × 4 mm, 5 µm) for separation and solvent A (0.02 M phosphate buþer, pH 2.15) and solvent B (methanol: 0. 02M phosphate buþer, pH 2.15, 40:60 v/v) for elution[17] and caffeic acid, *p*-coumaric acid and ferulic acid were separated at 57 min using C18 (Phenomenex, 150 × 4.6 mm; 5 µm) with mobile phase in (A) 0.1 % formic acid and (B) methanol[9]. Though caffeic acid and *p*-coumaric acid were separated at 20-28 min with using inertsil C18 ODS-3 (150 × 4.0 mm, 3 µm; GL Sciences, Inc., Japan) and mobile phase was consisted of 50 mM H_3PO_4, pH 2.5 (A) and acetonitrile (B), however, peaks of ferulic acid and sinapic acid were completely overlaid together[7]. Compare with previous studies[7,9,17,20], it was reduced the separation time and separated well of the four C6-C3 phenolic acids in our research.

Meanwhile, the extraction conditions in light of the major features of C6-C3 phenolic acids in tobacco were optimized. During the experimental process, it was found that the accuracy and precise could be improved, when the two problems of

filtering blockage and emulsification in extraction were processed with the low-temperature high-speed centrifugation.

Linearity and limit of detection: The linearity of the method was evaluated by the regression equation of C6-C3 phenolic acid with the concentration of the reference substance as the X-coordinate and the peak area/internal standard peak area as Y-coordinate (Table-3). The results showed that a good linearity can be found when the concentration in the range of 1.113-102.332 µg/mL with all correlation coefficient higher or equal to 0.9992. The limit of detection was calculated with signal-to-noise ratio S/N = 3.

TABLE-3
REGRESSION EQUATIONS OF C6-C3 PHENOLIC ACIDS

Compounds	Regression equation	R^2	Concentration range (µg/mL)	Limit of detection (ng/mL)
Caffeic acid	y=0.033 x - 0.0165	0.9994	1.113-56.236	21.4
p-Coumaric acid	y=0.056 x - 0.0268	0.9992	1.047-102.332	37.2
Ferulic acid	y=0.0327 x - 0.0064	0.9995	1.110-56.620	30.4
Sinapic acid	y=0.0211 x - 0.0167	0.9992	1.067-53.881	25.5

Repeatability and stability test: The repeatability of proposed methods was evaluated by using 6 replicates for the same batch. The relative standard deviation (RSD) of bound phenolic acids including caffeic acid, *p*-coumaric acid, ferulic acid and sinapic acid were 3.21, 4.76, 3.73 and 4.52 %, respectively. Furthermore, the same samples were reanalyzed for the stability at 0, 2, 4, 8, 12 and 24 h after extracted respectively and the RSD of caffeic acid, *p*-coumaric acid, ferulic acid and sinapic acid were 0.16, 0.31, 0.42 and 0.35 %, respectively. The results demonstrated that the proposed method had high repeatability and stability. A typical standard and sample chromatogram were shown in Fig. 1.

Recovery: Accurately 0.6000 mg of powder tobacco samples was weighed and mixed with reference substances caffeic acid, *p*-coumaric acid, ferulic acid and sinapic acid according to the experimental method in above, respectively. Then, the recovery was calculated according to the amount of standard samples and the quantity of samples before and after the addition of reference substances. The results showed that the recovery was 97.12, 97.32, 96.16 and 96.73 %, respectively.

Applications: The tobacco leaves from five provinces of China (Heilongjiang, Shandong, Shanxi, Guizhou and Chongqing) in 2013 were sampled at different growth stages and the phenolic acids were determined with newly established method. The results showed that the content of caffeic acid in fresh tobacco was very low (Table-4) and caffeic acid was only detected in samples from Heilongjiang at 10 days and 20 days after flowering and samples from Chongqing at 10 days after flowering, with the content ranged from 0.44 to 2.07 µg/g. In the other hand, analysis of variance (Table-5) showed that except caffeic acid the influence of region, growth stage and their interaction on contents of *p*-coumaric acid, ferulic acid and sinapic acids were highly significant.

Our study indicated that the contents of *p*-coumaric acid, ferulic acid and sinapic acid in flue-cured tobacco leaves had significant differences among different regions and growth stages. These results were consistent with previous report in which different growth stages and environments could influence the quantity and types of the metabolism in cell[21].

Conclusion

Considering overall experiment costs, separation time, accuracy and stability, the method which was consisted of 4 mol/L NaOH as extraction solvent and A (2 % acetic acid solution)-B (2 % acetic acid in acetonitrile solution) as mobile phase was optimized to separate caffeic acid, *p*-coumaric acid, ferulic acid and sinapic acid. Its high sensitivity, accuracy and repeatability could be employed to determination of contents of C6-C3 phenolic acids in flue-cured and fresh tobacco.

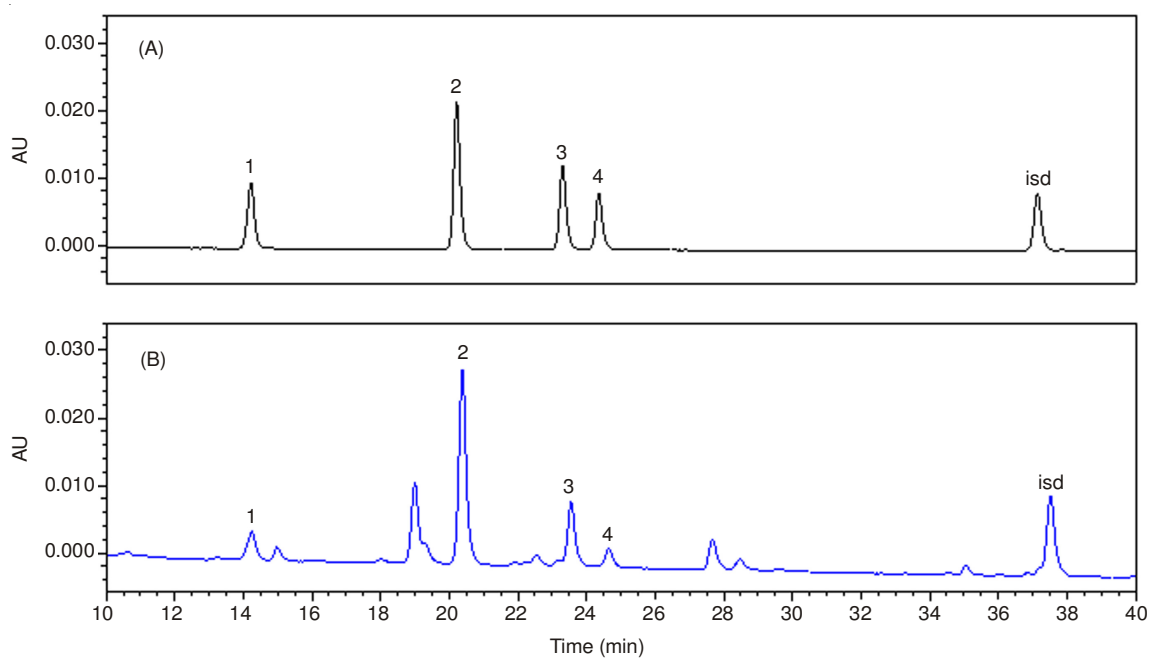

Fig. 1. HPLC chromatogram of standard samples (A) and tobacco samples (B) (1) caffeic acid; (2) *p*-coumaric acid; (3) ferulic acid; (4) sinapic acid; isd:internal standard (cinnamic acid)

TABLE-4
CONTENT OF BOUND C6-C3 PHENOLIC ACIDS IN TOBACCO AT DIFFERENT GROWTH STAGES (DRY WEIGHT, μg/g)

Regions	Growth stage	Caffeic acid	*p*-Coumaric acid	Ferulic acid	Sinapic acid
Chongqing	10 Days after flowering	1.44 ± 0.23	41.46 ± 4.44	14.82 ± 5.10	9.77 ± 2.24
	20 Days after flowering	N	43.68 ± 8.77	15.49 ± 2.44	12.56 ± 2.02
	30 Days after flowering	N	29.44 ± 2.15	16.86 ± 1.95	13.31 ± 1.36
	1 Day before harvest	N	32.25 ± 5.75	14.81 ± 4.49	17.08 ± 3.74
Heilongjiang	10 Days after flowering	1.02 ± 0.02	93.80 ± 16.37	22.95 ± 6.42	12.80 ± 2.53
	20 Days after flowering	2.07 ± 0.93	26.98 ± 3.56	25.25 ± 5.36	12.74 ± 4.05
	30 Days after flowering	N	19.83 ± 2.94	18.34 ± 6.96	24.49 ± 4.07
	40 Days after flowering	N	20.22 ± 1.56	14.13 ± 3.18	33.50 ± 5.95
	1 Day before harvest	N	19.24 ± 2.34	17.87 ± 2.59	37.76 ± 6.63
Shandong	10 Days after flowering	N	26.31 ± 3.24	22.85 ± 5.65	6.59 ± 1.33
	20 Days after flowering	N	30.77 ± 3.90	14.96 ± 2.14	6.09 ± 0.61
	30 Days after flowering	N	20.31 ± 1.28	16.13 ± 1.13	6.62 ± 0.26
	40 Days after flowering	N	16.22 ± 0.98	11.55 ± 0.94	15.34 ± 1.71
Shanxi	1 Day before harvest	N	15.21 ± 3.60	16.03 ± 5.77	12.54 ± 2.43
	10 Days after flowering	N	63.26 ± 6.50	20.08 ± 4.44	11.09 ± 1.99
	20 Days after flowering	N	45.39 ± 5.91	14.44 ± 5.23	13.89 ± 4.52
	30 Days after flowering	N	59.76 ± 8.76	30.72 ± 14.19	23.68 ± 5.48
	40 Days after flowering	N	45.87 ± 4.75	24.76 ± 1.31	21.62 ± 5.34
	1 Day before harvest	N	45.91 ± 4.73	24.78 ± 1.25	21.64 ± 5.40
Guizhou	10 Days after flowering	N	57.96 ± 5.01	13.01 ± 2.42	5.98 ± 1.00
	20 Days after flowering	N	52.39 ± 5.81	20.93 ± 1.80	7.74 ± 0.85
	30 Days after flowering	N	41.15 ± 3.25	15.77 ± 2.66	14.48 ± 1.96
	1 Day before harvest	N	45.93 ± 6.56	18.36 ± 1.45	29.91 ± 3.55

N means not detected

TABLE-5
VARIANCE ANALYSIS OF BOUND C6-C3 PHENOLIC ACIDS IN TOBACCO AT DIFFERENT GROWTH STAGES AND REGIONS

Origin	Dependent variable	Sum of squares	Degree of freedom	Mean square	F	Significance
Region	*p*-Coumaric acid	16178.48	4	4044.62	118.68	0.000
	Ferulic acid	1178.22	4	294.55	12.71	0.000
	Sinapic acid	3475.90	4	868.98	70.15	0.000
Growth stage	*p*-Coumaric acid	13189.29	4	3297.32	96.75	0.000
	Ferulic acid	214.65	4	53.66	2.32	0.000
	Sinapic acid	4801.06	4	1200.27	96.89	0.000
Region × growth stage	*p*-Coumaric acid	16826.24	14	1201.87	35.27	0.000
	Ferulic acid	1766.04	14	126.15	5.45	0.000
	Sinapic acid	1851.28	14	132.23	10.67	0.000

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