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Rapid and Sensitive Analysis of Eight Polyphenols in Tobacco by Rapid Resolution Liquid Chromatogarphy

Fuwei Xie[1,2], Ajuan Yu[1], Dengke Hou[1], Huimin Liu[2], Li Ding[2], Shusheng Zhang[1*]
[1]*Chemistry Department of Zhengzhou University, Zhengzhou, China*
[2]*Zhengzhou Tobacco Research Institute of CNTC, Zhengzhou, China*
E-mail: *zsszz@126.com
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Abstract

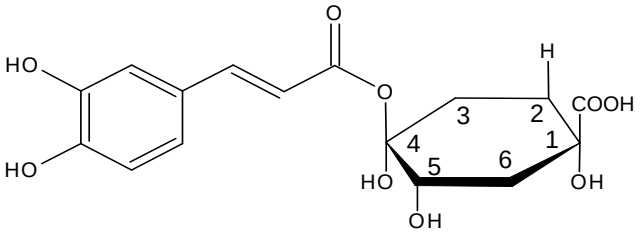
A rapid resolution liquid chromatographic (RRLC) method for the determination of eight polyphenols in tobacco was developed. Polyphenols were extracted from tobacco samples by methanol/ water in an ultrasonic bath, then subjected to clean up by solid phase extraction. The separation was performed on a 50 × 4.6 mm, 1.8 μm ZORBAX Eclipse XDB-C18 column. Compared with conventional HPLC method, the analysis time for eight polyphenols by RRLC method was reduced from 20 to 5 min without sacrificing resolution, and the sensitivity was improved. This method appears simple, accurate and precious. The relative standard deviations (RSD) of overall analysis procedure for eight tobacco polyphenols were less than 2% with the recoveries ranging from 94% to 107%. This method could be applied to the rapid determination of major polyphenols in tobacco with satisfactory results.

Keywords: Solid Phase Extraction, RRLC, Tobacco, Polyphenols

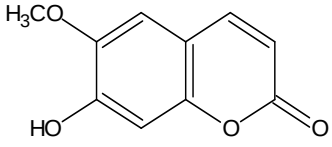
1. Introduction

Polyphenols, including tannic, cumarine, flavonid, and derivatives of simple phenols, are secondary metabolities in tobacco plant. They play important roles on the growth of tobacco and the quality of tobacco leaf [1-3]. The research results showed that in flue-cued tobacco leaves chlorogenic acid, scopoletin and rutin are the major polyphenols [4], and their combustion could generate phenolic compounds considered as carcinogens [5]. Therefore, it can pose serious harm on consumers' health during smoking. To understand the polyphenol content in tobacco, polyphenol transfer during smoking and the relationship between polyphenol and smokers' health, it is necessary to develop a practical method for the determinations of polyphenols in tobacco.

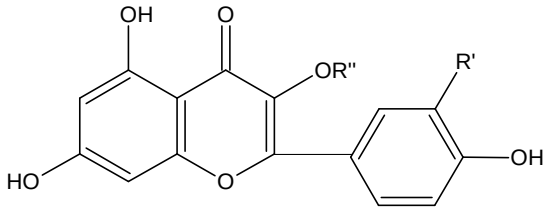
To date, several analytical methods for the analysis of polyphenols in tobacco have been reported by means of spectrophotometry [6], gas chromatography (GC) or GC-MS [7], high-performance liquid chromatography (HPLC) with chemiluminescence detection [8], ultraviolet detection (UVD) [9,10] and MS detection [11], and capillary electrophoresis (CE) method [12]. Among these methods, spectrophotometry is only used for the determination of the total polyphenols, GC and HPLC are the most powerful. However, GC cannot be used directly to determine polyphenols due to their poor volatility, high polarity and/or thermal instability. It needs time-consuming derivation. In contrast GC, HPLC is more effective and appropriate for the separation and determination of polyphenols. In order to obtain satisfactory separation of about 5 - 10 polyphenols by the conventional HPLC, the longer retention or analysis times between 20 - 30 min are required. Obviously, it cannot satisfy the requirement for the rapid analysis of high sample throughput. To solve this problem, various strategies aimed at increasing the speed and performance of chromatographic separation can be considered. Currently, the smaller stationary phase particles (<2.0 μm), new bridging structure of stationary phase and high-pressure systems are commercially available. Using this new rapid resolution liquid chromatography (RRLC) technique, higher linear velocity, faster run time, higher sensitivity and resolution are achieved [13-15]. Thus, the aims of the present work are 1) to develop a practical extraction and clean-up procedure prior to the analysis of eight polyphenols (**Figure 1**) in tobacco; 2) to develop a rapid and sensitive method to the analysis of eight polyphenols; and 3) to determine the polyphenols in actual tobacco samples by the developed RRLC.



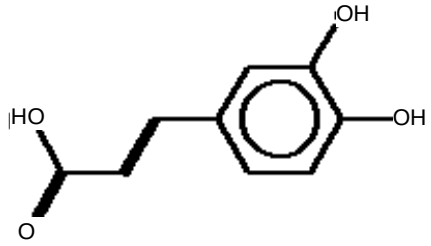
3-O-caffeoyl-d-quinic acid
Chlorogenic acid (3-O-caffeoyl-d-quinic acid)
Neo Chlorogenic acid (5-O-caffeoyl-d-quinic acid)



Scopoletin



Rutin	R' = OH	R" = Rutinosyl
Quercitrin	R' = OH	R" = d-Glucosyl
Kaempferol-3-rutinoside	R' = H	R" = l-Rhamnosido-d-Glucosyl R



Caffeic acid

Figure 1. Structures for the eight polyphenols.

2. Experimental

2.1. Reagents and Solution

All solvent (HPLC grade) were purchased from J&T Baker (Phillipsburg, NJ, USA), include methanol, acetonitrile and Formic acid. The certified standards used in this study were 5-O-caffeoylquinic acid (NG, 98%), chlorogenic acid (CG, 98%), 4-o-caffeoyl-quinic acid (YG, 98%), caffeic acid (CA, 98%), scopoletin (SP, 98%), rutin(RT, 98%), kaempferol-3-rutinoside (KR, 98%) and quercitrin(QT, 98%), respectively. They are obtained from Sigma-Aldrich, USA.

Stock solutions of polyphenols were prepared at 1000 μg/ml in methanol and stored under refrigeration at 4˚C in the dark. Quantification of samples was made using calibration curves of the eight polyphenols at the final concentration of 0.5, 1.0, 10, 30, 60 and 120 μg/mL in the solution of methanol-water (9:1, v/v). Each determination was performed in triplicate.

2.2. Apparatus and RRLC Conditions

Agilent 1200 RRLC (Agilent, USA) with a binary pump, a degasser, autosampler and a DAD UV detector; Agilent 1200 High Performance Liquid Chromatographiy (HPLC) (Agilent, USA) with a binary pump, a degasser, Autosampler and a DAD detector, All the operations and the data acquiring were controlled by a Agilent chemstation software.

The separation of eight polyphenols was optimized and performed on an Agilent Eclipse XDB-C_{18} RRLC column, 50 × 4.6 mm id (1.8 μm pore size, Agilent, USA). The mobile phase consisted of (A) 0.1% formic acid aqueous solution and (B) acetonitrile. The solvent program was initially 2 min isocratic with 90% A and 10% B, then from 2 to 5 min linear gradient to 30% B, finally at 6.5 min linear gradient to 10% B and re-equilibrium for additional 1.5 min for subsequent analysis. The flow rate was 2.0 mL/min. The detection wavelength was set at 340 nm. The injection volume was 5 μL. The column temperature was set at 30˚C. Thus, the total time for one-run chromatographic separation was not more than 8 min.

2.3. Tobacco Sample Pretrement

The tobacco samples were prepared in our laboratory as follow. One hundred grams of the fluecured tobacco leaves (without peduncle) were chopped and crushed to produce the tobacco powder at 40 mesh. 0.25 g of tobacco powder was extracted with ultrasonic extraction for 30 min in 40 mL of methanol-water (70:30, v/v) solution and centrifuged at 10,000 rpm for 10 min. Afterwards, 5 mL solution of centrifuge was loaded onto a Waters Sep-Park-C_{18} cartridge (500 mg) previously conditioned with 10 mL methanol and 10 mL deionized water, respectively. The first 3 mL eluates were discarded, the following 2 mL eluates were collected and filtered through a 0.22 μm membrane. The tobacco extract was directly analyzed by RRLC.

3. Results and Discussion

3.1. Optimization of RRLC Conditions

The separation of eight polyphenols was initially performed on a ZORBAX Eclipse XDB-C_{18} column (150 × 4.6 mm id, 5 μm) by conventional HPLC. Although they were achieved the batter separation under the optimized HPLC conditions, the analysis time was more than 20 min (**Figure 2(a)**). It is not suitable for the throughput analysis. Thus, a new RRLC separation system was chosen. Using the Eclipse XDB-C_{18} RRLC column (50 × 4.6 mm i.d., 1.8 μm), different mobile phase, elution program, flow rate and column temperature were investigated. For example, at flow rate 1.0 mL/min and column temperature 20˚C, The longest retention time was less than 6 min, however SP and RT can not be resolved (**Figure 2(b)**). By increasing the flow rate from 1 to 2 mL/min and column temperature from 20˚C to 30˚C, eight polyphenols were separated in baseline within 5 min (**Figure 3(a)**). More importantly, the analysis was faster 3 times than conventional HPLC without sacrificing resolution. micellar liquid chromatography (MLC) also can separate and quantify 3 tobacco polyphenols (chlorogenic acid, rutin and scopoletin) in less than 10 min, but the theoretical plate of chlorogenic acid in tobacco sample using RRLC (2116 plates) was much higher than that using MLC (576 plates).

3.2. Choice of Extraction and Cleanup Method

To extract polyphenols from tobacco leaf powder, the following three methods were compared using 40 mL methanol/ water (70:30, v/v) as extraction solvent for 0.2500 g tobacco leaves: 1) refluxed at 60˚C for 40 min; 2) ultrasonic extraction under 50 Hz for 40 min; and 3)

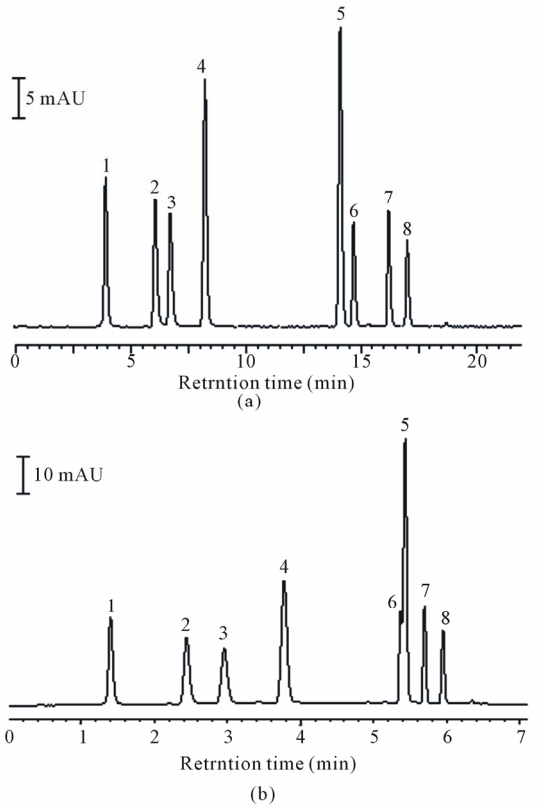


Figure 2. Separations of eight polyphenols by (a) HPLC and (b) RRLC at 20˚C. (a) HPLC column: Eclipse XDB-C18 150 × 4.6 mm i.d., 5 μm; Mobile A: 0.1% formic acid /H_2O; B: cetonitrile; Flow rate: 1.0 mL/min; Temperature: 20˚C. (b) RRLC column: Eclipse XDB-C18 50 × 4.6 mm i.d., 1.8 μm; Mobile A: 0.1% formic acid/H_2O; B: acetonitrile; Flow rate: 1.0 mL/min; Temperature: 20˚C; Peaks: 1, 5-O-caffioylquinic acid; 2, chlorogenic acid; 3, 4-o-caffioylquinic acid; 4, caffeic acid; 5, scopoletin; 6, rutin; 7, kaempferol-3-rutinoside; 8, quercitrin.

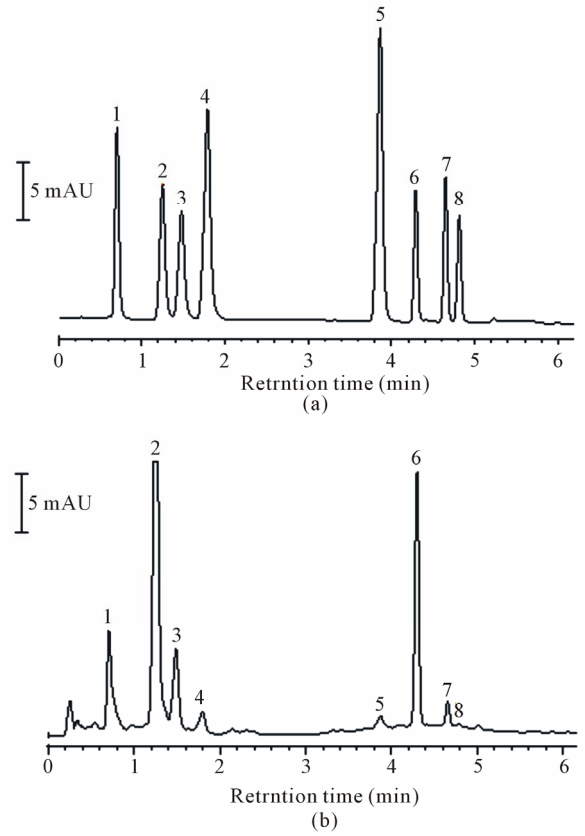


Figure 3. Chromatograms for (a) polyphenol standards and (b) tobacco samples by RRLC RRLC column: Eclipse XDB-C18 50 × 4.6 mm i.d., 1.8 μm; Mobile A: 0.1% formic acid/ H_2O; B: acetonitrile; Gradient: 0 min (10% B) ~ 2.0 min (15% B ~ 5.0 min (30% B) ~ 6.5 min (50% B) ~ 8.0 min (10% B); Flow rate: 2.0 mL/min; Temperature: 30˚C. Peaks: 1, 5-o-caffioylquinic acid; 2, chlorogenic acid; 3, 4-O-caf-fioylquinic acid; 4, caffeic acid; 5, scopoletin; 6, rutin; 7, kaempferol-3-rutinoside; 8, quercitrin.

mechanical shaking extraction under 15 cycles per minute for 40 min. The results indicated that the extraction efficiency of refluxed extraction and ultrasonic extraction (Recoveries of eight polyphenols >90%) were better than mechanical shaking extraction (Recovery of Scopoletin <80%). Because ultrasonic extraction is simpler than two others, it was chosen for the polyphenols extraction in this paper.

SPE has been proven to be an effective tool for selectively removing interferences, enabling sensitive, selective and robust analysis. There are some weak polar materials in tobacco extract which can not be removed completely from C_{18} column by mobile phase, such as fatty substance, wax, pigment, and so on. Moreover, the smaller stationary phase particles make it easier plug for RRLC than HPLC. So it is necessary to clean up the extract before RRLC analysis. In this paper, the solution of extraction was clean up with a Waters Sep-Park C_{18} cartridge, by which the weak polar material was retained, and the ployphenol fractions were collected for RRLC analysis.

3.3. Linearity of Calibration and Limit of Detection for Eight Ployphenols

Using the optimized RRLC condition, the linear ranges of the UV response at 340 nm were observed over the concentration range from 0.5 to 120 μg/mL for eight ployphenols. The regressions between peak area (y) and concentration (x, μg/ml) yielded the linear equations as decribed in **Table 1**.

The limit of detection (LOD) was evaluated by calculating a signal to noise ratio of 3 (S/N = 3). The result was summarized in **Table 1**.

From **Table 1**, it can be seen that the LODs by RRLC were lower than those by HPLC, which enhanced the detection sensitivity for eight ployphenols.

3.4. Method Validation

A series of samples analysis were performed to validate the performance of the method. The accuracy was assessed with recovery assay by adding eight standard polyphenols to sample at low and high levels. The recovery was calculated by comparing the found mount of standards to those of added. The precision was evaluated from replicated determinations (n = 5) performed on the different day for same samples. The recoveries of eight polyphenols ranged from 94% to 107% with the relative standard deviation (RSD) less than 2.0%.

3.5. Application to Real Tobacco Samples

The proposed RRLC method was used for routine analysis of four tobacco samples from different areas. A typical chromatogram was displayed in **Figure 3(b)** and the results obtained were summarized in **Table 2**. The results are consistent with those determined by Zhang [16] and

Table 1. Regression equation and the LODs.

Compound	Regression equation	Correlation coefficient	LOD/RRLC (ng/mL)	LOD/HPLC (ng/mL)
NG	y = 1.9951x – 0.5391	R^2 = 0.9994	59.4	128.2
CG	y = 1.8874x – 0.9993	R^2 = 0.9996	105.3	211.2
YG	y = 1.7039x – 0.6706	R^2 = 0.9999	103.5	178.6
CA	y = 3.5595x – 0.9966	R^2 = 0.9991	51.3	69.4
SP	y = 4.2813x + 0.1804	R^2 = 0.9995	30.3	43.0
RT	y = 1.2785x + 0.2096	R^2 = 0.9995	66.7	93.8
KR	y = 1.3913x + 0.3109	R^2 = 0.9995	58.8	84.7
QT	y = 1.0483x + 0.1352	R^2 = 0.9995	84.5	121.0

Table 2. The determination results of eight polyphenols in tobacco samples.

Compounds	Flue-cured tobacco (Yunnan)	Burley tobacco (Hubei)	Oriental tobacco (Yunnan)	Zimbabwel
NG	0.247%	0.008%	0.156%	0.203%
CG	1.522%	0.024%	0.666%	1.156%
YG	0.361%	0.013%	0.289%	0.263%
CA	0.016%	0.007%	0.016%	0.025%
SP	0.017%	0.005%	0.008%	0.032%
RT	0.779%	0.024%	0.424%	0.075%
KR	0.060%	0.004%	0.080%	0.049%
QT	0.022%	unfound	0.014%	0.020%

Li [17]. The method demonstrated that it is usable and applicable to the rapid and sensitive determination of polyphenols in tobacco.

4. Conclusions

In this paper, a simple, fast, sensitive and reproducible RRLC analytical method for for eight polyphenols in the tobacco samples was developed by coupling with a practical sample pretreatment. With ultrasonic extraction and Solid-phase extraction (SPE), higher recoveries were obtained with lower matrix interfering for RRLC separation of eight polyphenols. The developed method was suitable for high throughput analysis of the tobacco samples.

5. Acknowledgements

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