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Separation and purification of chlorogenic acid from tobacco by-products by polyamide and silicagel column chromatography

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Methods for separation of chlorogenic acid (CA) from tobacco by-products were established. The liquid chromatography tandem mass spectrometry (LC-MS) method for identification and analysis of chlorogenic acid from tobacco materials has been developed. CA was isolated by polyamide and further purified by silicagel column chromatography. Results reveal that polyamide is suitable for separation of CA from tobacco extract. After one run treatment with polyamide, the content of chlorogenic acids was 40.3%. The product was further purified using silicagel column chromatography; the content of total chlorogenic acid was increased 2.29-fold from 40.3 to 92.2%. The LC-MS results showed that total chlorogenic acids were made up of four components: 5-CQA, 3-CQA, 4-CQA and cis-5-CQA. The content of 5-CQA was the most (78.2%) and other three chlorogenic acid derivatives were 1.9, 10.1 and 2.0%, respectively.

Key words: Tobacco by-products, chlorogenic acid, polyamide, silica gel column, LC-MS.

INTRODUCTION

Tobacco, an herbaceous plant, is one of the important economic crops in the world. The leaves of tobacco are the most essential material for cigarette production (Chen et al., 2013); but, more than 20% of the tobacco are discarded during production, and not used for other purposes (Zhang et al., 2012). Therefore, it is urgent to dispose the tobacco by-products. Chlorogenic acid, which is formed by esterification of caffeic acid and quinic acid, is the major phenolic compound in tobacco by-products. It is possessed of physiological effects such as anti-bacterial, antivirus and antioxidant (Sato et al., 2011; Zhao et al., 2010; Yun et al., 2012; Yuan et al., 2012;

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Abbreviations: CA, Chlorogenic acid; **LC-MS,** liquid chromatography tandem mass spectrometry; electrospray ionization (ESI).

Wang et al., 2009; Danino et al., 2009). It is feasible to utilize tobacco by-products as the material for separating chlorogenic acids. Generally, an ultrasonic method is used for the extraction of chlorogenic acid (Li et al., 2005; Mazvimba et al., 2012). Many materials, for example polyamide, macroporous resins (Wan et al., 2014; Zhang et al., 2008), silica gel column are used in separating and purifying chlorogenic acid. In recent years, polyamide has been widely used in separating plant effective components such as phenolic acids, flavonoids, quinones (Guo et al., 2011; Sun et al., 2013) and good results are obtained. Silica gel column chromatography is another material for separation according to different absorption capacity.

In the present study, we investigate the adsorption and desorption properties of chlorogenic acids on polyamide and developed an efficient method for the preparative separation of chlorogenic acids from tobacco by-products. In addition, chlorogenic acids in the final products were identified by liquid chromatography tandem mass spectrometry.

MATERIALS AND METHODS

Materials and reagents

Tobacco by-products provided by a company in Anhui province of China and were smash into 40 mesh by a pulverizer (selected by sieve), and then were kept at room temperature. The tobacco by-products extracts were prepared by ultrasonic-assisted procedure with 10-fold 95% ethanol (pH = 4.0) at 50°C for 1 h twice. The extracting solution was filtered and then condensed, stored at 4°C in the dark for the subsequent experiments. Ethanol, phosphoric acid (analytical grade) was purchased from Tingbest Co. (Nangjing, China), methanol (HPLC grade) was purchased from TEDIA Co. (USA), and all aqueous solutions were prepared with pure water produced by Milli-Q system (Bedford, MA, USA).

HPLC analysis

Quantitative analysis of the concentration of chlorogenic acid was carried out by HPLC on Agilent 1100 HPLC system composed of two quaternary pumps with a degasser, a thermostatted column compartment, a variable wavelength detector, autosampler and 1100 ChemStation software. Sample analysis was carried out on Ultimate C18 column (250 mm × 4.6 mm I.D., 5 µm) at a column temperature of 30°C. Mobile phase used was a mixture of 10 mmol/L phosphate buffer (pH4.0): methanol (25:75,v/v) at a rate of 1.0 mL/min. Injection volume was 10 µL and UV detection was at 326 nm. Each analysis was repeated for three times. All samples were filtered through 0.45 µm membrane before HPLC analysis. Standard curve method was used for quantification.

LC-MS analysis

HPLC–MS was conducted on an Agilent 6460 HPLC (Agilent, California, USA), coupled to negative electrospray ionization (ESI) tandem mass spectrometry (MS/MS) method. Chlorogenic acid separations were achieved on a C18 reverse phase column (100 × 2.1 mm; ZORBAX Eclipse Plus; Agilent, USA). The mobile phase, a solution of methanol and 0.1% aqueous formic acid (20:80,v/v), was set at a flow-rate of 200 µl/min. Mass spectra in the negative ion mode were operated under the following conditions: fragmenter voltage = 5 eV; voltage = 3500 V; nebulizer pressure = 45 psi; capillary temperature = 300°C; m/z range = 50 to 800.

The polyamide adsorption capacity for chlorogenic acid

Static adsorption test

1 g polyamide was put into a 150 mL conical flask and 20 mL of solution of tobacco extracts (1.15 mg/mL chlorogenic acid) was added. The flask was shaken on an incubation shaker (120 rpm) at 25°C for 8 h to reach adsorption equilibrium. The solution after adsorption was analyzed by HPLC, and then the ratio adsorption amount was calculated.

$$Qe = V_0 (C_0 - Ce)/W \quad (1)$$

Where, Qe is adsorption amount, which represents the mass of adsorbed on 1 g of polyamide resin at adsorption equilibrium; C_0 and Ce is the initial and equilibrium concentration of chlorogenic acid in the solutions, respectively; V_0 is the initial volume of solution added into the flask, and W is the weight of the polyamide resin.

Dynamic adsorption

Dynamic adsorption experiment was carried out on glass column (10 × 400 mm) wet-packed with polyamide at 25°C and the bed volume (BV) of the resin was 14 mL (equal to 3 g of dry resin). Sample solution containing certain concentration of chlorogenic acid (pH 4.0, adjusted by 2M H_3PO_4 solution) flowed through the glass column at different flow rates, and the chlorogenic acid in the eluent was determined by HPLC.

Dynamic desorption test

While adsorption of chlorogenic acid was confirmed, dynamic desorption test was carried out. A gradient program was adopted: the adsorbate-laden column was eluted with different concentration ethanol solution, and collected at 1 BV intervals. According to the monitoring of the concentration of chlorogenic acid in every interval, effect of ethanol concentration on the efficiency of elution had been investigated.

RESULTS AND DISCUSSION

Static adsorption on polyamide

Based on the HPLC analysis, the initial concentration of chlorogenic acid in the extraction solution was 4.68 mg/mL, and then diluted to a concentration of 1.15 mg/mL for static desorption test. The results of static adsorption were summarized in Table 1. As seen in Table 1, the polyamide adsorption capacity for chlorogenic acid was 9.78 mg/g.

Effect of the flow rate on dynamic adsorption

Sample solution containing same concentration of chlorogenic acid flowed through the glass column at

Table 1. Adsorption capacity of chlorogenic acid of tobaoo by-products on polyamide.

Initial mass of CA (mg)	Average equilibrim mass of CA (mg)	Mass of CA adsorbed (mg)	Qe (mg/g resin)
22.92	13.14	9.78	9.78

Table 2. Effect of different flow rate on chlorogenic acid adsorption.

Flow rate (mL/min)	Initial mass of CA (mg)	Content of CA in out flow liquid (mg)	Qe (mg/g resin)
0.15	22.92	8.38	14.54
0.32	22.92	7.52	15.40
0.75	22.92	5.27	17.65
1.5	22.92	5.83	17.09

Table 3. Effect of ethanol concentration on the efficiency of elution.

Fraction	Eluant (% ethanol)	Concentration (mg/mL)	Desorption ratio (%)
1	0	0.00	0.0
2	10	0.33	16.5
3	20	0.55	26.9
4	30	0.56	27.5
5	40	0.20	10.0
6	50	0.05	2.3
7	60	0.00	0.0

different flow rate to test dynamic adsorption. As shown in Table 2, the adsorption capacity increased with the increase of flow rate and reached the maximum adsorption capacity (17.65 mg/g) when the flow rate was 0.75 mL/min. The adsorption capacities decreased slightly at higher flow rate (1.5 mL/min) due to lack of sufficient time to adsorb by polyamide. Therefore, the flow rate was adjusted to 0.75 mL/min for all later experiments.

Effect of ethanol concentration dynamic desorption

Adding at the flow rate of 0.75 mL/min, the extract was absorbed on polyamide column for 30 min. Firstly, the column was eluted with 1 BV distilled water, then gradient eluted with 10, 20, 30, 40, 50 and 60% ethanol, and 7 elution fraction (1 BV for each) were collected for calculating the concentration and desorption ratio. As shown in Table 3, chlorogenic acid does not exist in fraction 1. In water solution, the chemical complexation between phenolic hydroxyl groups and the amide groups of the polyamide through the association of the hydrogen bond was so strong that chlorogenic acid is difficult to be eluted. Meanwhile, lots of impurities were removed with water, which was convenient for further isolation. Chlorogenic acid was mainly centered in five fractions (from 10 to 50% ethanol eluant), and the total desorption ratio was 83.2%. According to desorption ratios of the 5 fractions, 10, 40 and 50% ethanol were found to be not favorable for the eluant due to their low desorption ratios, but the difference between the desorption ratio of 30 and 20% ethanol is 0.6% only. As shown in Figure 1 to 2, the unknown peak, whose retention time was earlier than that of the peak represented chlorogenic acid (the highest peak), almost vanished in the 30% ethanol eluant. To decide the concentration of ethanol for further experiments, it was necessary to select 20 and 30% ethanol as eluant for dynamic desorption, respectively.

The dynamic desorption curve on polyamide was shown in Figure 3. By comparison, it can be seen that the shape of elution peak with 30% ethanol eluant is relatively concentrated and more symmetric than that of elution peak with 20% ethanol eluant. Approximately, 4 BV of desorption solution desorbed chlorogenic acid completely from polyamide resin. The two fractions of desorption solutions were combined and evaporated by rotary vaporization at 45°C under reduced pressure, then freeze-dried. The dried product was weighed and the contents, yields of chlorogenic acid were calculated (Table 4). Based on an overall consideration of various factors (the volume of eluant, yield and purity), 30%

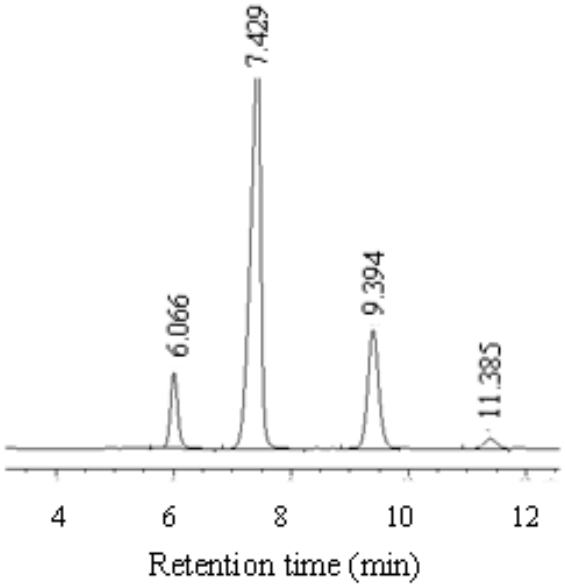

Figure 1. Chromatography of the 20% ethanol eluant (left).

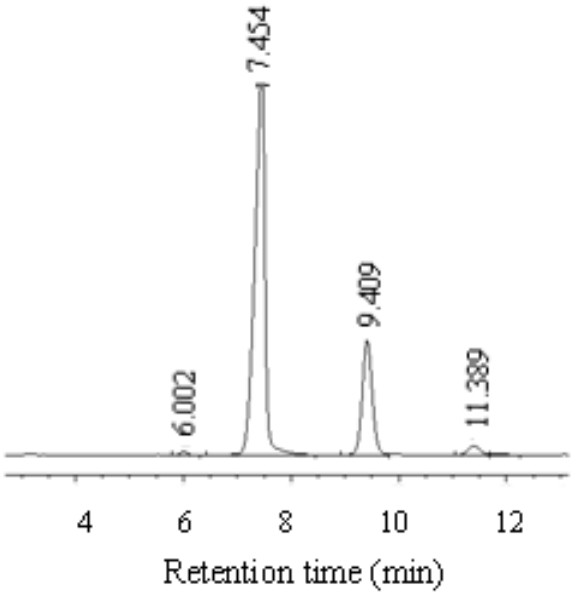

Figure 2. Chromatography of the 30% ethanol eluant (right).

ethanol was used as the eluant for further experiment.

Column chromatography

Polyamide (80 to 100 mesh) was packed in a glass column (10 mm × 40 mm i.d). Adjust the volume of the extract according to the volume of column packing.

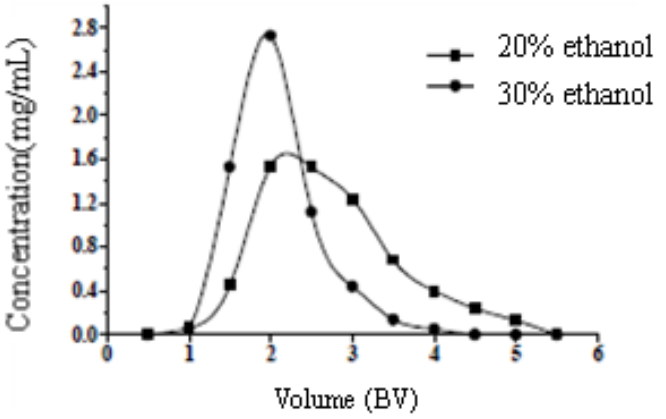

Figure 3. Elution curves of CA with two concentration of ethanol (left).

Chromatographic zone was set on top of the column packing to reach saturated adsorption equilibrium. The resin was first washed by pure water (1 BV) and then desorbed with 30% ethanol (5 BV). As shown in Figure 4, results of chromatography showed 3 bands significantly. A band moved the quickest, the color of the eluate is red, while the color of B band is dark, and C is yellow. The content of chlorogenic acid in the eluent of C band is 75.2%, while the content is 23.1% in B band, in which impurities took the most part. However, 23.1% of chlorogenic acid would be losing if the B band section was abandoned directly. This section was further purified by polyamide column with the same solvent system. This collecting eluate was merged with C band eluate then freeze-dried. The crude product was obtained and the content of chlorogenic acid was calculated. After treatment with polyamide twice, the content of chlorogenic acid reached 40.3% in the product, which is 5.7-fold as much as that in tobacco by-products.

Purification on silicagel column

The product after polyamide isolation was dissolved and then subjected to further chromatographic purification on silicagel column (300 × 25 mm id). The total content of chlorogenic acid after separation was 92.2%.

Quantitative determination

After detected by LC-MS in the negative ion mode, four peaks were separated on the total ion chromatogram (TIC) (Figure 5). Four quasi-molecular ions were 353.1[M-H]- and their peaks were rather strong in the mass spectrum.

Figure 6 shows the ion scan spectrum of this compound, with characteristic fragment ions at m/z191, 179,

Table 4. Effect of ethanol elution concentration on the product purity.

Sample	Eluant (% ethanol)	Yield (%)	Purity (%)
1	20	35.6	26.6
2	30	33.8	27.7

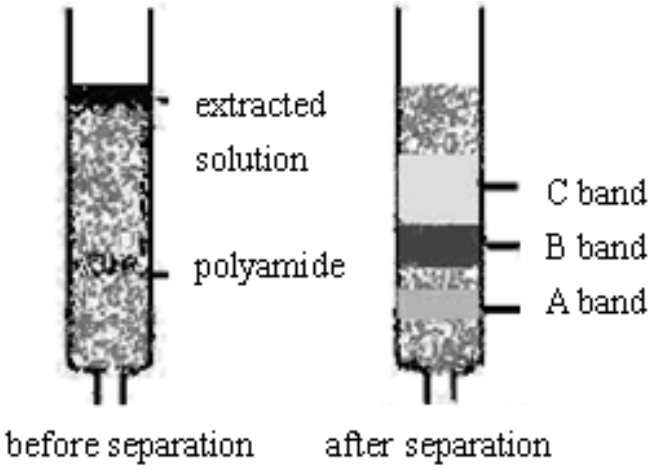

Figure 4. Column chromatography of polyamide (right).

173, 161 and 135. Ions at m/z 191 or 173 indicated fragments of the quinic moiety, and ions at m/z 179 or 161 indicated fragments of the caffeoyl moiety, and they were [quinic-H]-, [quinic-H-H_2O]-, [caffeic- H]-, [caffeic-H_2O-H]-, respectively. Ions at m/z135 indicated another fragment of caffeic acid. Except the difference of type among the fragments, the relative intensities of the fragment ions were quite different, which was related to the peak-heights. Along with relevant references (Zhang et al., 2013; Rodrigues and Bragagnolo, 2013; He et al., 2010), four peaks were identified as four isomers of caffeoylquinic acid [3- CQA (I), 5-CQA (II), 4-CQA (III) and cis-5- CQA (IV)], respectively. In the product, 5-CQA is the dominant compound, of which the content was 78.2% and other three chlorogenic acid isomers were 1.9% (3-CQA), 10.1% (4-CQA) and 2.0% (cis-5-CQA), respectively. IUPAC numbering system was used in this work for the structure of chlorogenic acids.

Conclusions

The separation and purification process of chlorogenic acid with polyamide and silicagel has been successfully developed in this study, and products with different purities (40.3, 92.2%) can be gained after different procedure. The LC-MS/MS system used in this work appeared to an excellent tool for identifying structures of different components in the high purity product, especially the isomers. The identification by LC-MS showed that the discarded tobacco leaves are rich in chlorogenic acid (5-CQA) as well as other isomers (3-CQA, 4-CQA, cis-5-CQA), which possess the similar pharmaceutical bioactivities. Thus, the tobacco by-products can be used as one of alterative materials for extracting chlorogenic acids. It is a great significance to explore the variety of materials and reduce the costs of large-scale separation process of chlorogenic acids.

Conflict of interests

The authors did not declare any conflict of interest.

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