

Study on the Determination of Polyphenols in Tobacco by HPLC Coupled with ESI-MS After Solid-Phase Extraction

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Abstract

A high-performance liquid chromatography method coupled with electrospray ionization-mass spectrometry for the determination of polyphenols in tobacco is studied. The polyphenols are extracted from a tobacco sample by being refluxed in a boiling water bath with 80% methanol and purified by solid-phase extraction with a C₁₈ cartridge. The chlorogenic acid, rutin, scopoletin, caffeic acid, scopolin, and other polyphenols are satisfactorily separated on a Nova-Pak C₁₈ chromatographic column (3.9 × 150 mm) with methanol and 0.05 mol/L potassium dihydrogen phosphate buffer solution gradient elution as mobile phase at a flow rate of 0.5 mL/min. Each of the polyphenols is monitored by photodiode array detector at its maximum wavelength: chlorogenic acid, 326.1 nm; rutin, 354.8 nm; scopoletin, 344.0 nm; caffeic acid, 323.7 nm; and scopolin, 365.2 nm. The limits of detection are: 100 ng/mL for chlorogenic acid, 125 ng/mL for rutin, 60 ng/mL for scopoletin, 50 ng/mL for caffeic acid, and 100 ng/mL for scopolin. The key polyphenols in tobacco are identified by comparing the retention time, the UV-spectrum, and the mass spectra with those of the standards. The recovery of tobacco polyphenols is 94–105%, and the relative standard deviations are 1.28–1.49%. This method is successfully applied to qualitatively and quantitatively analyze the polyphenols in tobacco with good results.

Introduction

Polyphenols are important components in tobacco. There is a close relationship between polyphenols and the quality of tobacco. The polyphenols in tobacco greatly affect the odor and taste of smoking (1–5). Therefore, the determination of polyphenols in tobacco and cigarettes is important. For the determination of polyphenols, the main techniques are spectrophotometry (5–9), gas chromatography (GC), and high-performance liquid

chromatography (HPLC) (4,5,9–11). Spectrophotometry is simple and does not require expensive or complicated test equipment, but this technique can allow only the determination of the total polyphenols. GC and HPLC have the advantage that the various polyphenols can be simultaneously determined. Because of their nonvolatility, polyphenols need derivatization before determination by GC (4,5). The sample preparation process is complex and has a low recovery (4,5). HPLC is the most effective method for this analysis. For the traditional HPLC method, the tobacco sample needs to be grease removed by soxhlet extraction for 2.5 h with nonpolar solvents, such as *n*-hexane or mineral ether (4,5,11). This procedure may lead to the loss of polyphenols and result in a low recovery.

Both GC and HPLC methods utilize peak retention time for qualitative analysis. Sometimes the use of retention time is not so reliable, and the components can not be identified without standards. In order to get a more convenient procedure and more reliable results, we developed a new HPLC method in which the tobacco extract can be purified by solid-phase extraction (SPE) with a C₁₈ cartridge. Some of polyphenols without standards (such as isomers of chlorogenic acid and kaempferol-3-rutinoside) were identified by UV spectrum and electrospray ionization (ESI)-mass spectrometry (MS) spectrum. SPE can greatly shorten the sample preparation time and reduce the loss of polyphenols. This method was applied to the quantitative analysis and identification of polyphenols in tobacco with good results.

Experimental

Chemicals and instruments

HPLC-grade methanol, potassium dihydrogen phosphates, chlorogenic acid, rutin, scopoletin, caffeic acid, and scopolin standard samples were obtained from the Fluka Corporation (Buchs, Switzerland).

The HPLC-UV system used consisted of a Waters (Milford, MA) 2690 Alliance separation module and a Waters 996 photodiode

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array detector (PAD). The HPLC–MS system consisted of Micromass (Wythenshawe, U.K.) Quattro ESI–MS–MS detector and a PerkinElmer (Norwalk, CT) LC-480 binary pump.

The chromatographic column used was a Waters Nova-Pak C₁₈ chromatographic column (3.9 × 150 mm), and the SPE cartridge used was a Waters Sep-Pak C₁₈ cartridge (1 cc/30 mg, 30 μm).

Sample preparation

The polyphenols were extracted from the tobacco sample by being refluxed in a boiling water bath with 45 mL of 80% methanol for 30 min. After cooling, the solution was filtered, transferred into a 50-mL volumetric flask, and diluted to volume with 80% methanol. Then, 5 mL of the solution was passed through the C₁₈ cartridge at the flow rate of 10 mL/min. The last 2 mL was collected and filtered with 0.45 μm of filters for subsequent HPLC analysis.

HPLC–UV and HPLC–MS method

The HPLC–UV analysis was conducted on a Waters 2690 Alliance separation module equipped with a 996 photodiode array detector. The HPLC–MS analysis was conducted on a Perkin-Elmer LC-480 binary pump and Micromass Quattro-I ESI–MS–MS detector. For HPLC–UV, the polyphenols were separated on a Waters Nova-Pak C₁₈ (3.9 × 150 mm) column with methanol and 0.05 mol/L potassium dihydrogen phosphate buffer solution gradient elution as the mobile phase at flow rate of 0.5 mL/min. The composition of the mobile phase was: A (methanol) and B (0.05 mol/L potassium dihydrogen phosphate buffer solution) for 0 min (A 10% + B 90%), 15 min (A 80% + B 20%), 20 min (A 80% + B 20%), and 25 min (A 10% + B 90%) in linear ramp at a flow rate of 0.5 mL/min. The injection volume was 10 μL, and was injected with an autosampler. A tridimensional chromatogram was recorded from 210–400 nm with a photodiode array detector.

For HPLC–MS, a Nova-Pak C₁₈ column (3.9 × 150 mm, 5 μm) (Waters) was used in our development work. The composition of the mobile phase was: A (methanol) and B (water containing 0.2% formic acid) for 0 min (A 10% + B 90%), 15 min (A 80% + B 20%), 20 min (A 80% + B 20%), and 25 min (A 10% + B 90%) in linear ramp at a flow rate of 0.5 mL/min. The mobile phase was delivered by a PerkinElmer binary pump. A splitter, constructed from a Valco low-dead volume tee (Valco Instruments, Houston, TX) and appropriate length of 0.17-mm-i.d. polyetheretherketone tubing (Waters) to achieve the desired split ratio, was used to reduce the liquid chromatographic (LC) effluent to 0.1–0.2 mL/min for the ESI–LC–MS interface. Flow injections were made by means of a Rheodyne Model 7125 injector for all MS scanning mode experiments. Volumes of 10 μL of extract were injected. A Quattro (Micromass) triple-quadrupole tandem MS equipped with an ESI ion source was used. Data acquisition, data processing, and instrument control were performed using Microsoft Windows NT (v4.0)-based Masslynx software (Micromass) on a Pentium II (Digital Equipment, Maynard, MA) computer. The following operating parameters were used: source temperature, 100°C; nebulizing gas, 150 L/h; drying gas, 380 L/h; and ESI capillary voltage, +2.8 kV. The scan ranges were *m/z* 100–700 in single MS mode and *m/z* 100–420 in the MS–MS mode, both at a scan rate of 3 s/scan. The skimmer lens (between the sample cone and the skimmer) voltage was set 5 V higher than the cone voltage. The

cone voltage was set at 25 V.

Daughter ion mode was performed using the following parent-to-fragment transition: *m/z* 120–400 for caffeoylquinic acid. A dwell time of 0.25 s, an interchannel delay of 0.02 s, and a span of 0.1 Da were used. The collision gas (argon) pressure was 2.7 × 10⁻³ mbar. The collision voltage was 30 V.

Results and Discussion

Optimization of HPLC parameters

Polyphenols in tobacco can be separated on a reversed-phase column with methanol and potassium dihydrogen phosphate buffer solution as the mobile phase. A Waters Nova-Pak C₁₈ column (3.9 × 150 mm) was selected for this experiment. Methanol and 0.05 mol/L potassium dihydrogen phosphate buffer solution was selected as the mobile phase. Polyphenols cannot be separated completely by isocratic elution with a different proportion of a methanol and buffer solution mixture. Therefore, the

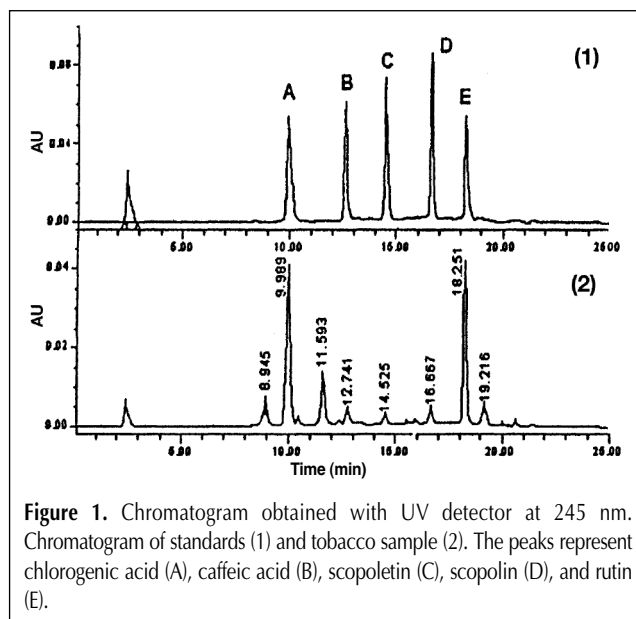


Figure 1. Chromatogram obtained with UV detector at 245 nm. Chromatogram of standards (1) and tobacco sample (2). The peaks represent chlorogenic acid (A), caffeic acid (B), scopoletin (C), scopolin (D), and rutin (E).

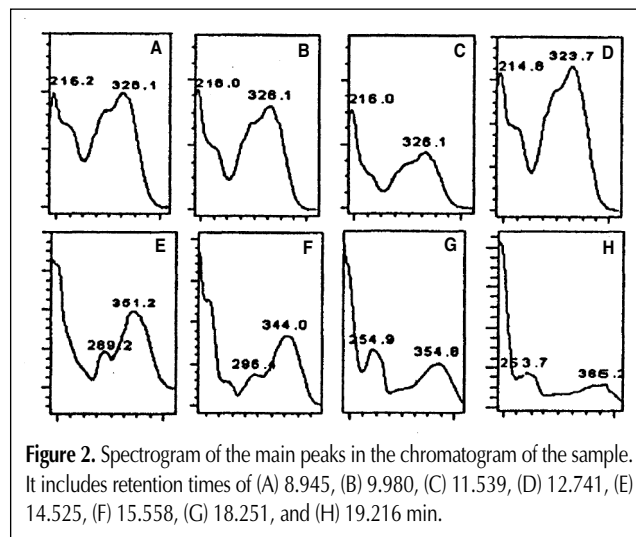


Figure 2. Spectrogram of the main peaks in the chromatogram of the sample. It includes retention times of (A) 8.945, (B) 9.980, (C) 11.539, (D) 12.741, (E) 14.525, (F) 15.558, (G) 18.251, and (H) 19.216 min.

gradient elution with methanol and 0.05 mol/L potassium dihydrogen phosphate buffer solution as mobile phase was selected. The proper composition of mobile phase was selected as the following: A (methanol) and B (0.05 mol/L potassium dihydrogen phosphate buffer solution) for 0 min (A 10% + B 90%), 15 min (A 80% + B 20%), 20 min (A 80% + B 20%), and 25 min (A 10% + B 90%) in linear ramp at the flow rate of 0.5 mL/min. Under this condition, the polyphenols were separated completely. The purity of every peak was identified with PAD, and there was no overlap in peaks. A typical chromatogram of polyphenols standards and tobacco sample of photodiode array detector at 345 nm is shown in Figure 1, and the UV spectra of the key peaks are shown in Figure 2.

Identification of peaks by comparing retention time and UV spectra with standards

In Figure 1, the five peaks [A, 3-caffoylquinic acid (chlorogenic acid); B, caffeic acid; C, scopoletin; D, scopolin; and E, rutin] were identified by comparing retention times and UV spectra with the standards. No standards were obtained for the other three key peaks (8.945-, 11.593-, and 19.216-min peak). The nonconfirmation was obtained only by comparing the spectra against the library. The 8.945- and 11.593-min peaks had identical UV spectra

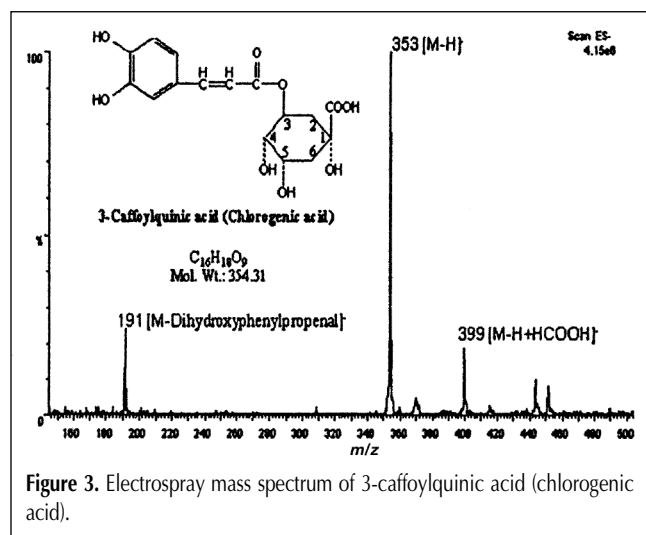


Figure 3. Electrospray mass spectrum of 3-caffoylquinic acid (chlorogenic acid).

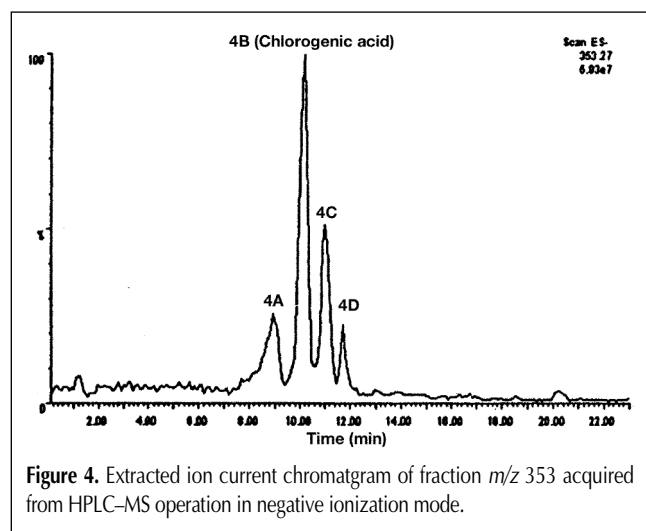


Figure 4. Extracted ion current chromatogram of fraction m/z 353 acquired from HPLC-MS operation in negative ionization mode.

with chlorogenic acid. They were probably other isomers of caffoylquinic acid. The 19.216-min peak had a similar UV spectrum as rutin. It was probably a flavone. To get more information, ESI-tandem-MS technology was used.

Characterization of isomers of caffoylquinic acid by ESI-MS-MS spectrometer

Although electrospray is considered to be a mild ionization method and generally produces quasi-molecular ions with little

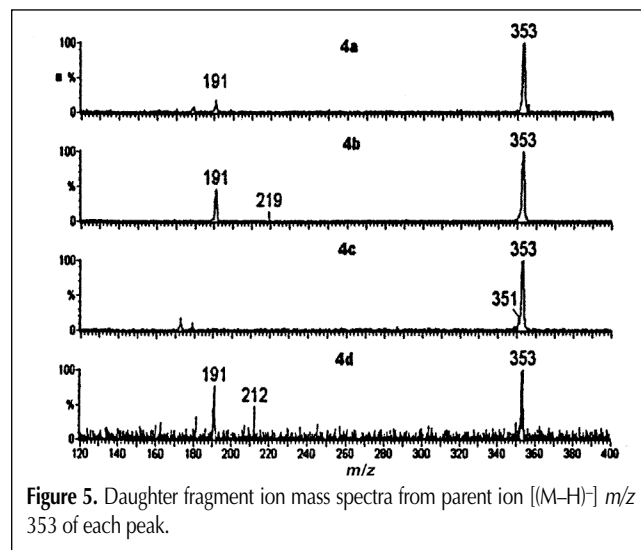


Figure 5. Daughter fragment ion mass spectra from parent ion $[(M-H)^-]$ m/z 353 of each peak.

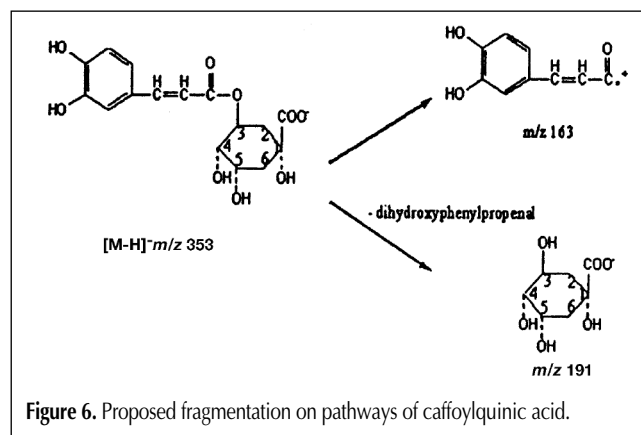


Figure 6. Proposed fragmentation on pathways of caffoylquinic acid.

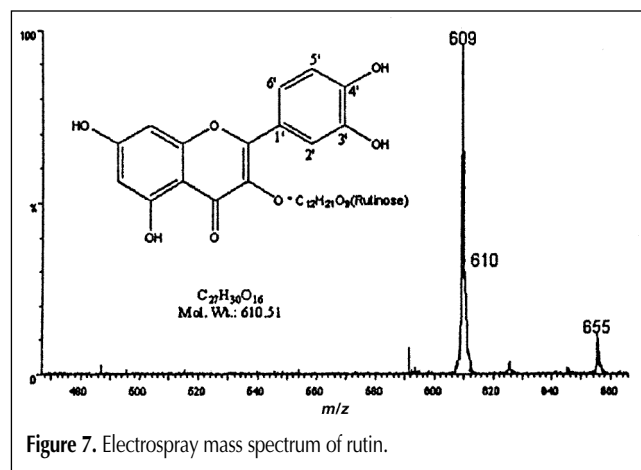


Figure 7. Electrospray mass spectrum of rutin.

fragmentation, it is well-known that by varying the potential differences between the sampling cone and the skimmer in the ESI ion source, collision-induced dissociation (CID) of these ions from the analyte will occur. As in our case, if the skimmer voltage is maintained constant, this potential difference will be directly proportional to the cone voltage. The production of these structurally characteristic fragments at high cone voltage can be very helpful for identification purposes when a single mass analyzer is used.

Caffoylquinic acid has three isomers: 3-caffoylquinic acid (chlorogenic acid), 4-caffoylquinic acid, and 5-caffoylquinic acid (1). The chlorogenic acid was identified by comparing the retention time and UV spectrum with standard. To identify the other

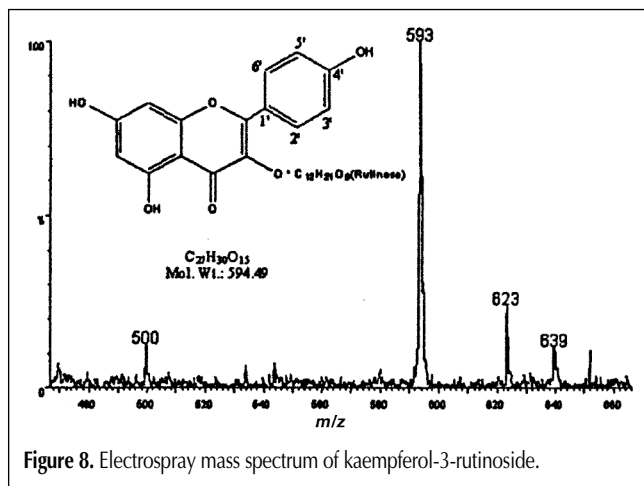


Figure 8. Electrospray mass spectrum of kaempferol-3-rutinoside.

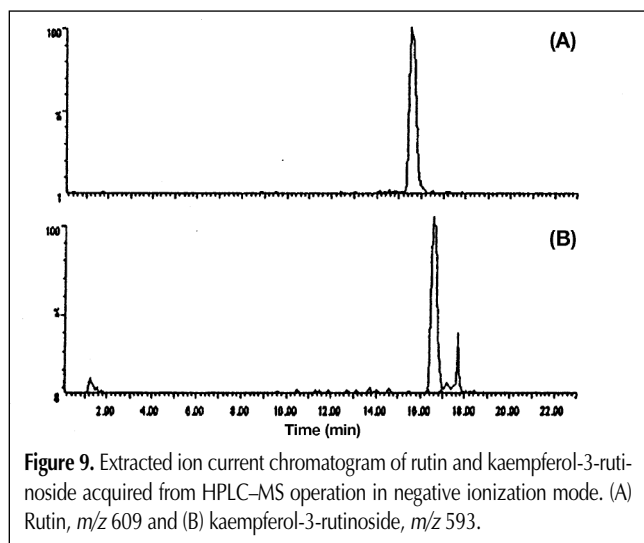


Figure 9. Extracted ion current chromatogram of rutin and kaempferol-3-rutinoside acquired from HPLC-MS operation in negative ionization mode. (A) Rutin, m/z 609 and (B) kaempferol-3-rutinoside, m/z 593.

two isomers of caffoylquinic acid, ESI-tandem-MS was used. The electrospray mass spectrum of chlorogenic acid was shown in Figure 3. By ESI negative acquisition mode, caffoylquinic acid produced a quasi-molecular ion $[M-H]^-$ at m/z 353, low abundance fragment ions resulting from the addition of formic acid ($[M-H + HCOOH]^-$ at m/z 399), and a loss of dihydroxyphenylpropenal (at m/z 191). An extracted ion current chromatogram of m/z 353 fragment acquired by HPLC-MS operation in negative ionization mode was shown in Figure 4. Four peaks [4A, 4B (chlorogenic acid), 4C, and 4D] were observed clearly in this chromatogram. Each peak has a similar fragment ion mass spectrum. Protonated molecules of caffoylquinic acid were subjected to CID in the MS-MS mode. In this case, (as shown in Figure 5)—with moderate collision energy (30 eV)—the fragment resulting from the loss of dihydroxyphenylpropenal (m/z 191) was fragment ions. The proposed fragmentation pathways were presented in Figure 6. We suggested that the m/z 353 ion in the mass spectrum of caffoylquinic acid be derived from consecutive fragmentation at m/z 191 from protonated molecule. Our data indicated that the m/z 353 ion ($[M-H]^-$) was only observed in the parent scan of m/z 191, suggesting that the latter ion be formed through a simple loss of dihydroxyphenylpropenal. Peak 4C did not produce the fragment ion from the parent ion at m/z 191. Therefore, we concluded that 4A and 4D were the isomers of caffoylquinic acid. According to the peak sequence reported by the literature (11) we can conclude that 4A is 5-caffoylquinic acid and 4D is 4-caffoylquinic acid in extracted ion current chromatograms of m/z 353 fragment.

Characterization of kaempferol-3-rutinoside by ESI-MS

Another key flavone in tobacco is kaempferol-3-rutinoside (1,2), but we could not purchase the commercial standard sample. The rutin and kaempferol-3-rutinoside are homologous. The difference between rutin and kaempferol-3-rutinoside is the hydroxide in 3' position carbon (Figures 7 and 8). The molecule weight of kaempferol-3-rutinoside is 594. To identify the kaempferol-3-rutinoside, an extracted ion current chromatogram of m/z 593 fragment acquired by HPLC-MS operation in negative ionization mode was shown in Figure 9B. A peak was observed clearly in this chromatogram. The mass spectrum of the peak was shown in Figure 8. It produces the protonated molecular ion at m/z 593, low abundance fragment ions resulting from the addition of formic acid ($[M-H+HCOOH]^-$ at m/z 639). By comparing the extracted ion current chromatogram of rutin (m/z 609, Figure 9A) and the mass spectrum of rutin (Figure 8), we can conclude that the peak (Figure 9B) is kaempferol-3-rutinoside.

Regression equation, coefficient, and detection limit

The regression equation of polyphenols for quantitative analysis was established based on the standards samples of 100, 20, 4.0, 0.8, and 0.16 mg/mL injected and its peak areas. To get maximum sensitivity, PAD monitoring was performed at the maximum wavelength of each polyphenols: chlorogenic acid, 326.1 nm; rutin, 354.8 nm; scopoletin, 344.0 nm; caffeic acid, 323.7 nm; and scopolin, 365.2 nm. The limit of detection was calculated by the ratio of signal to noise ($s/n = 3$). The

Table I. The Regression Equation, Coefficient, and Detection Limit

Components	Regression equation	Coefficient (r)	Detection limit (ng/mL)
Chlorogenic acid	$C(\text{mg/mL}) = 2.220 \times 10^{-7} A - 0.00368$	0.9996	100
Rutin	$C(\text{mg/mL}) = 3.226 \times 10^{-7} A + 0.00325$	0.9998	125
Scopolin	$C(\text{mg/mL}) = 1.942 \times 10^{-7} A + 0.00121$	0.9998	60
Caffeic acid	$C(\text{mg/mL}) = 1.854 \times 10^{-7} A + 0.00236$	0.9995	50
Scopoletin	$C(\text{mg/mL}) = 2.125 \times 10^{-7} A + 0.00165$	0.9996	100

results are shown in Table I. The standards of 5-caffeoylquinic acid, 4-caffeoylquinic acid, and kaempferol-3-rutinoside were not available. For this reason, 5-caffeoylquinic acid and 4-caffeoylquinic acid were quantitated by the calibration with chlorogenic acid and kaempferol-3-rutinoside was quantitated by calibration with rutin.

Reproducibility and recovery

The reproductivity of this method was examined five times for the determination of the same tobacco sample. The determination results of standard deviations are shown in Table II, and the recoveries were obtained by adding 5 µg/mL of polyphenol standards in tobacco samples. The results are shown in Table II.

Sample preparation

For the determination of polyphenols in tobacco, the polyphenols were extracted from the sample by being refluxed in a boiling water bath with 80% methanol. By this procedure, some of the nonpolar compounds (such as leaf pigment, grease, wax, and others) were simultaneously extracted into this solution as well. The nonpolar compounds can contaminate the chromatographic column because they cannot be eluted from the column by the mobile phase of polyphenols separation. Therefore, the nonpolar compounds would be removed from the sample solution before being injected into the column. For the traditional sample preparation, the tobacco sample needs to be removed from the nonpolar compounds by soxhlet extraction for 2.5 h with nonpolar solvents (such as *n*-hexane or mineral ether). This procedure may result in the loss of polyphenols, a low recovery, and long time requirements. To improve this, we developed a new method that included the purification of the sample solution by SPE with a Waters Sep-Pak C₁₈ cartridge. The polyphenols can be extracted from the tobacco sample with 80% of methanol directly, and the methanol solution was passed through the cartridge at a flow rate of 10 mL/min. The nonpolar compounds can be retained when the sample solution passes through the cartridge, but the polyphenols can not. This procedure can remove the nonpolar compounds from sample solution quickly; it requires only 10 min. Twenty samples can be prepared simultaneously by the Waters

extraction manifold for SPE cartridges. In addition to saving time, this method can reduce the loss of polyphenols and achieve a better recovery and precision. A comparison between a traditional procedure and this method is shown in Table III.

Conclusion

Compared with the traditional HPLC method for polyphenols, this method uses SPE to purify samples with a C₁₈ cartridge. This greatly reduces the sample preparation time and achieves high recovery. It is more reliable to characterize the polyphenols by online HPLC–ESI–MS–MS. This method can be applied to the qualitative and quantitative analysis of polyphenols in tobacco.

Acknowledgement

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Table II. Method Precision and Recovery

Components	Added (mg)	Found (mg)	Recovery (%)	RSD% (n = 5)
Chlorogenic acid	10.0	9.4	94	1.36
Rutin	10.0	10.3	103	1.49
Scopolin	10.0	9.7	97	1.31
Caffeic acid	10.0	9.6	96	1.28
Scopoletin	10.0	10.5	105	1.47

Table III. The Comparison of This Work with Traditional Procedure

Method	Time needed	RSD% (n = 5)	Recovery (%)
This method	10 min	1.28–1.49	94–105
Traditional method	2.5 h	2.36–3.95	88–92