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Determination of Polyphenols in Tobaccos by High Performance Liquid Chromatography with Matrix Solid-Phase Dispersion Extraction and Clean-Up

FENGXIA LIANG* and ZHENYU ZHANG
Physical Testing and Chemical Analysis Department, Anhui Provincial Centre for Disease Control and Prevention, Hefei, Anhui, P.R. China
Tel: (86)(551)2863265; E-mail: lfx@ahcdc.com.cn

A simple method for the determination of polyphenols in tobaccos using matrix solid-phase dispersion (MSPD) and high performance liquid chromatography was developed. The dry ground tobaccos and octadecyl bonded silica (C_{18}) adsorbent were ground with a pestle to produce a homogenous material for MSPD column. Eluted with methanol (0.003 mol/L) and aqueous hydrochloric acid (80:20, v:v), the interfering components such as grease and wax were retained in the C_{18} adsorbent and the extract was clean enough for direct injection into HPLC. The mobile phase of methanol-trifluoroacetic acid (0.03 %) (pH = 2.5) was used for gradient elution. A 345 nm was selected as detection wavelength. The recoveries of tobacco polyphenols were from 94.0 to 97.5 % and the relative standard deviations were in the range of 3.62-6.31 %. The new method was compared with a conventional method based on the use of refluxing extraction followed by solid-phase extraction clean-up. The consumption of less sample preparation time and fewer toxic solvents with better clean-up efficiency than refluxing extraction SPE clean-up technique indicates the suitability of this method.

Key Words: Matrix solid-phase dispersion, Polyphenols, Tobaccos.

INTRODUCTION

Polyphenols are widely distributed among plants. They are of great importance in the nutritional, organoleptic and commercial properties of agricultural foodstuffs through their contributions to their sensory properties such as colour, bitterness and flavour[1,2]. Owing to their antioxidant activity and their function as free radical scavengers, polyphenols appear to protect against cardiovascular disease and have potential anticarcinogenis properties[3,4]. Due to these beneficial effects, research on separation and determination of polyphenols seems attractive.

Analysis of polyphenols in tobaccos usually includes extraction with methanol or mixtures of it with water, clean-up by Soxhlet extraction usually with hexane or solid-phase extraction (SPE)[5,6]. The extract was then analyzed by liquid chromatography coupled with UV-visible detection. These classical sample preparation techniques need two steps: (a) extraction and (b) clean-up. These two steps consume much time and solvent, moreover, they are laborious. Consequently, there has been an increasing demand of methods that simultaneously extract and purify the sample in single step such as matrix solid-phase dispersion (MSPD). In MSPD, the sample is blended together with a suitable sorbent to generate a material which has a unique chromatographic character that may selectively elute a single compound or several classes of compounds[7,8]. This method simplifies the extraction and clean-up steps, reduces sample manipulation and has been widely used in the analysis of drugs and pollutants in foods[9].

The aim of this work is to develop a MSPD extraction method that allows simultaneously extraction and clean-up of polyphenols in single step followed by direct HPLC analysis. Its comparison to refluxing extraction method followed by solid-phase extraction clean-up has also been studied. The results indicate that proposed method is suitable for the determination of polyphenols in tobaccos.

EXPERIMENTAL

Standards of chlorogenic acid, scopolin, rutin and quercitrin were purchased from Sigma-Aldrich (Sigma). Methanol was of HPLC-grade. Octadecyl bonded silica (100-200 mesh) was obtained from Supelco Company (USA). Ultra-pure water was obtained from a water purification system.

Matrix solid-phase dispersion extraction: 0.1 g of dry ground tobaccos were weighed and blended with 0.4 g C_{18} adsorbent by grinding with a pestle for 5 min to produce a homogenous mixture for MSPD column. Then the column was eluted with 10 mL methanol-aqueous hydrochloric acid (0.003 mol/L) (80:20, v:v). The strongly remained non-polar interfering components were retained in the column and separated from the target compounds. In order to verify if all the polyphenols were eluted out by 10 mL of methanol-aqueous hydrochloric acid (0.003 mol/L) (80:20, v:v), a stronger elution solvent, tetrahydro furan (THF) was used to elute the column. The methanol-aqueous hydrochloric acid (0.003 mol/L) and THF extracts were collected, respectively and filtered through a 0.45 μm nylon membrane for liquid chromatography determination.

Refluxing extraction and SPE clean-up: 0.1 g of tobacco sample was refluxed in a boiling water bath with 22.5 mL of methanol-aqueous

hydrochloric acid (0.003 mol/L) (80:20, v:v) for 0.5 h. The extract was diluted to 25 mL with methanol-aqueous hydrochloric acid (0.003 mol/L). A C_{18} cartridge was conditioned with methanol and followed by water without allowing the cartridge to dry out. 5 mL of the extract was passed through the cartridge and the last 2 mL was collected and filtered through 0.45 μm membrane for subsequent HPLC analysis.

Ultraviolet spectroscopy: 10 mL of the extract of MSPD extraction was diluted to 25 mL with methanol-aqueous hydrochloric acid (0.003 mol/L). Then, the extract was measured by UV-2401PC spectrophotometer (Shimadzu, Japan) in the range of 210-500 nm. The extract of refluxing extraction-SPE clean-up was measured using the same method.

HPLC analysis: The extract was analyzed using Agilent (Palo Alto, CA, USA) 1100 liquid chromatography equipped with a GB11A dual solvent pump, a GBBA autosampler, a G1316 thermal column compartment, a G1315A photo-diode array detector and Agilent 3D chemstation software Rev.6.0. An ODS-2Hypersil column (250 mm × 4.6 mm i.d., 5 μm particle size) was used throughout this study. The composition of the mobile phase was 0.03 % trifluoroacetic acid (pH = 2.5) (A) and methanol (B) at a flow rate of 1 mL/min. The gradient elution conditions were: 0-15 min, 90-20 % A; 15-20 min, 20-90% A; The system temperature was maintained at 25 ºC. The injection volume was 10 μL and was injected with an autosampler. A 345 nm was selected as the detection wavelength.

RESULTS AND DISCUSSION

Optimization of MSPD extraction: Aqueous methanol is often selected as extraction solvent because of its property of co-dissolution with the polyphenols[10-12]. Fig. 1 shows the extraction yields using different extraction solvents. It is noticed that 10 mL of MeOH-aqueous hydrochloric acid (0.003 mol/L) (80:20, v:v) would elute all the target compounds while MeOH-aqueous hydrochloric acid (0.003 mol/L) (60:40, v:v), MeOH-water (80:20, v:v), MeOH required more solvents to elute all the polyphenols. Therefore, MeOH-aqueous hydrochloric acid (0.003 mol/L) (80:20, v:v) was selected as the optimal agent. Polyphenols are easy to ionize, hence hydrochloric acid was added to the methanol to prevent ionization and make them easily eluted by methanol.

In order to verify if all the target polyphenols were eluted by 10 mL of MeOH-aqueous hydrochloric acid (0.003 mol/L) (80:20, v:v), a stronger solvent THF was further employed to elute the column and no more polyphenols could be detected in the chromatogram. It demonstrated that all the polyphenols were eluted by 10 mL of MeOH-aqueous hydrochloric acid (0.003 mol/L) (80:20, v:v).

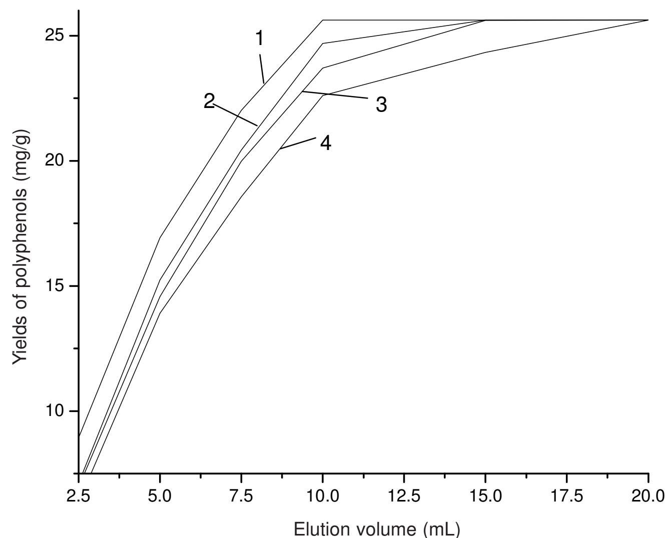

Fig. 1. Yields of polyphenols using different elution solvents
Elution solvents: (1) MeOH-aqueous hydrochloric acid (0.003 mol/L) (80:20, v:v), (2) MeOH-aqueous hydrochloric acid (0.003 mol/L) (60:40, v:v), (3) MeOH-water (80:20, v:v), (4) MeOH

Comparison of MSPD extraction with refluxing extraction-SPE cleanup: When polyphenols were extracted using the conventional method, some of the non-polar compounds such as grease and wax were simultaneously extracted as well. The non-polar compounds can contaminate the chromatographic column for they can't be eluted from the column by the mobile phase and thus a clean-up step was necessary after extraction procedures. In present experiment, C_{18} column as MSPD sorbent was used. C_{18} not only acts as adsorption separation material but also plays an important part in disrupting and dispersing the sample due to its good mechanical strength. After the grinding procedure, the gross architecture of tobacco sample was disrupted and then, polyphenols and some of the non-polar interfering compounds such as grease and wax in tobaccos were simultaneously absorbed into C_{18} sorbent. In the elution step, the polyphenols were eluted out by aqueous methanol while the non-polar interfering components were not simultaneously eluted out by methanol because C_{18} has strong retentive character for non-polar compounds[13]. Thus the polyphenols were extracted and the interfering components were separated from target polyphenols in the elution step. Unlike the conventional method, extraction and clean-up was achieved in one step in MSPD.

MSPD extraction method was compared with refluxing extraction-SPE clean-up method in the aspects of extraction and clean-up efficiency. From

Table-1, one can noticed that the extraction efficiency of MSPD is comparable to refluxing extraction-SPE clean-up. The run time for MSPD (0.5 h) is shorter than refluxing extraction-SPE clean-up (1 h) and also the volume of the solvent used has been reduced from 25 to 10 mL.

TABLE-1
COMPARISON OF MSPD EXTRACTION WITH REFLUXING EXTRACTION-SPE CLEAN-UP (AVERAGE ± SD) (mg/g)

Extraction method	MSPD	Refluxing extraction-SPE clean-up
Chlorogenic acid	14.76 ± 0.55	13.56 ± 0.18
Scopolin	0.19 ± 0.01	0.19 ± 0.01
Rutin	10.24 ± 0.13	10.57 ± 0.12
Quercitrin	0.44 ± 0.02	0.42 ± 0.01
Solvent (mL)	10	25
Sample preparation time (min)	30	60

The non-polar interfering components couldn't be eluted from the chromatographic column by the mobile phase. In order to compare the clean-up efficiency of the two methods, the ultraviolet spectroscopy of the extracts of MSPD and refluxing extraction-SPE clean-up were measured. The two methods have similar absorbency in 340 nm (Fig. 2) which was mainly the absorbance of polyphenols. This result was confirmed by the extraction efficiency. The range of 210-260 nm, in which the absorbency of the extract

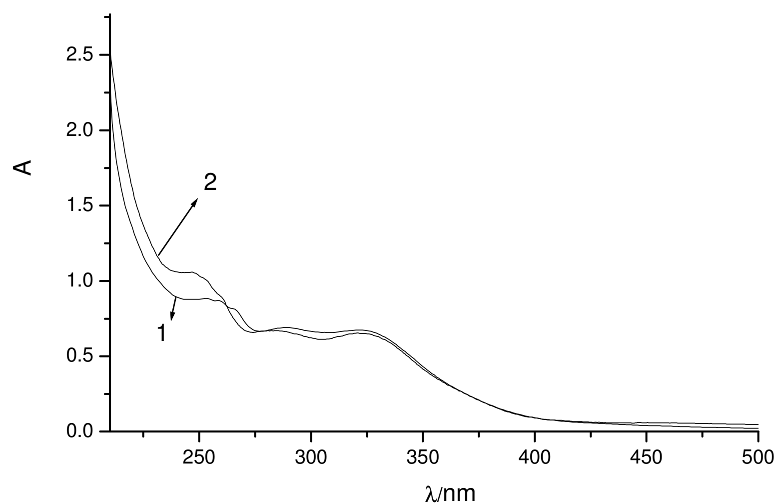

Fig. 2. Ultraviolet spectroscopy of extracts of MSPD (1); refluxing extraction-SPE clean-up (2)

of refluxing extraction-SPE clean-up was larger than MSPD, was mainly because of the absorbance of non-polar compounds such as grease and wax. The larger the absorbency from 210 to 260 nm, the dirtier its extract was. Thus, it is concluded that MSPD extraction proves better clean-up efficiency.

HPLC separation and detection: Polyphenols are easy to ionize in neutral solution and severe peak tailing can occur if the neutral solution was used as HPLC mobile phase. Methanol and phosphate buffer solution was often selected as the mobile phase. It is found in present experiment that phosphate buffer solution may form deposition with Ca^{2+} in the tobacco extract which may clog the chromatographic column. However, trifluoroacetic acid aqueous solution has no such negative effects. If the pH of the mobile phase was lower than 2, the separation systems would be damaged. Consequently, aqueous trifluoroacetic acid solution (pH 2.5) and methanol were finally chosen. For the polyphenols can't be separated completely by isocratic elution, the gradient elution with methanol and trifluoroacetic acid solutions was employed. The gradient elution was as follows: A (trifluoroacetic acid solution, pH 2.5) and B (methanol) for 0 min (A 90 % + B 10 %), 15 min (A 20 % + B 80 %), 20 min (A 90 % + B 10 %). Polyphenols were separated completely under this condition and the chromatograms of standard and tobacco sample are shown in Fig. 3.

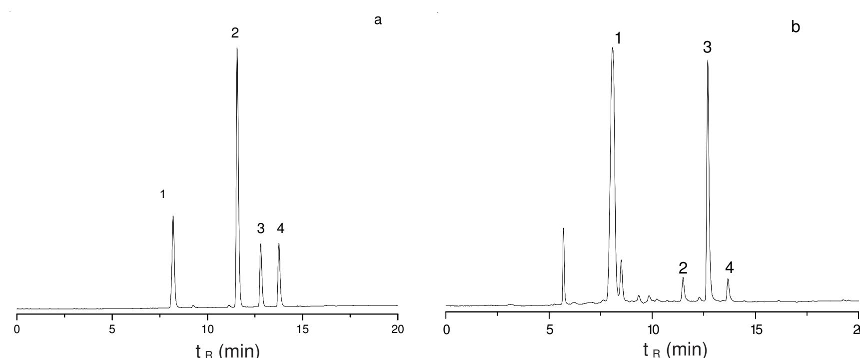

Fig. 3. Chromatograms of standard (a) and tobacco sample using MSPD extraction (b) 1, chlorogenic acid 2, scopolin 3, rutin 4, quercitrin

The identification of the compounds was based on the comparison of the retention times with authentic reference compounds. The concentration of polyphenols was determined by external standard methods. The calibration curve of the peak area (y) *versus* the concentration (x, μg/mL) was linear; y = 5.197x + 16.632, R = 0.9995 (n = 5) from 5-200 μg/mL for chlorogenic

acid; y = 3.257x + 1.442, R = 0.9999 (n = 5) from 5-200 μg/mL for rutin; y = 13.090x + 20.202, R = 0.9994 (n = 5) from 0.5-20 μg/mL for scopolin; y = 3.023x + 0.071, R = 0.9998 (n = 5) from 0.5-20 μg/mL for quercitrin. The concentrations of polyphenols in our analyzed solution were in the middle of the linear range.

Reproductivity and recovery: The reproducitivity of MSPD procedure was assessed by performing extractions on the same sample for five times. The relative standard deviations are shown in Table-2. The recovery of polyphenols was measured by spiking standard solution of polyphenols in the tobacco sample. The recoveries are in the range from 94.0 to 97.5 %.

TABLE-2
RECOVERY TEST OF MSPD (n=5)

Polyphenols	Original (mg/g)	Added (mg/g)	Found (mg/g)	Recovery (R/%)	RSD (S_r/%)
Chlorogenic acid	14.76	8.00	22.76	96.0	4.20
Scopolin	0.19	0.24	0.43	95.8	6.31
Rutin	10.24	8.00	18.24	94.0	3.62
Quercitrin	0.44	0.80	1.24	97.5	4.88

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