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Discrimination and classification of tobacco wastes by identification and quantification of polyphenols with LC–MS/MS

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Abstract: The chemical composition of polyphenols in tobacco waste was identified by HPLC-PDA–ESI/MS/MS and the contents of chlorogenic acids and rutin in 10 varieties of tobacco wastes were determined by HPLC–UV. The relationships between the contents of active polyphenols and the varieties of tobacco wastes were interpreted by hierarchical cluster analysis (HCA) and principal component analysis (PCA). The results showed that 15 polyphenols were identified in a methanolic extract of dried tobacco waste. The tobacco wastes were characterized by high levels of chlorogenic acids (3-CQA, 5-CQA, and 4-CQA) and rutin; their ranges in the 10 tobacco varieties were 0.116––0.196, 0.686–1.781, 0.094–0.192, and 0.413–0.998 %, respectively. According to multivariate statistics models, two active compound variables can be considered important for the discrimination of the varieties of tobacco wastes: chlorogenic acids and rutin. Consequently, samples of 10 tobacco varieties were characterized into three groups by HCA based on the PCA pattern. In conclusion, tobacco waste could be used as a new pharmaceutical material for the production of natural chlorogenic acids and rutin in the ethnopharmacological industry.

Keywords: tobacco (*Nicotiana tabacum* L.) waste; HPLC–PDA–ESI/MS/MS; chlorogenic acids; rutin; multivariate statistical analysis.

INTRODUCTION

Solid tobacco waste is classified as an agro-industrial waste. The total global tobacco (*Nicotiana tabacum* L.) waste production in the year 2005 was more than 1.25 million metric tons. In China, 460 million kg of tobacco waste per year are generated at various stages of the post-harvest processing of tobacco and during

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the manufacture of tobacco products.[1] Tobacco waste has no immediate use and cigarette companies have to pay for its disposal. The majority of the waste is destroyed by burning. Indeed, the disposal of this waste is a serious problem because tobacco waste is toxic due to the presence of nicotine;[2] thus, governments worldwide must enforce legislation for the controlled disposal of tobacco waste in order to avoid harmful effects to the environment. As a type of high organic biomass, tobacco wastes have potential applications for soil amendment and the production of tailored organic fertilizer[3] and desulfurization adsorbents.[4]

Currently, the chemical compositions of tobacco leaves and wastes have attracted a considerable amount of attention throughout the world,[5–8] and tobacco waste is considered to be a good source of a large number of bioactive substances, such as chlorogenic acids and rutin.[1] Chlorogonic acid is a member of the caffeoylquinic acids (CQAs) family.[9] The members of this family possess a wide range of biological properties, such as antibacterial, antioxidant, hepatocyte protective, antimutagenic, inhibitory of HIV-1 RT and active against the human herpes simplex virus, adenoviruses, SARS and AIV (H5N1).[10–15] Similarly, rutin is a number of the phytochemical group of compounds the “protective” properties of which include antioxidant, antimicrobial, anticancer, and cardiovascular-protective activities.[16,17] Therefore, considering that chlorogenic acid and rutin are highly valuable natural polyphenol compounds used as medical and industrial materials in China, the identification and quantification of these main polyphenols in tobacco waste is of great importance for its large scale application in the ethnopharmacological industry.

LC–MS was used previously to characterize cinnamoylamino acid conjugates, to discriminate between individual isomers of mono-acyl and di-acyl chlorogenic acids[18–21] and to characterize flavonoids,[22] In this study, ion trap HPLC--PDA–ESI/MS/MS and HPLC-UV methods were developed for the qualitative profile and quantitative determination, respectively, of polyphenols in tobacco waste. These methods were applied to the qualitative profiling of polyphenols in tobacco waste and the determination of the contents of chlorogenic acids and rutin in waste from 10 tobacco varieties.

The study revealed that the correct classification of the variety of the tobacco waste is a new problem in controlling the quality of tobacco waste. This is due to the fact that the unique combination of tobacco variety and cultivation zone results in different chemical compositions within the tobacco waste. Unfortunately, little attention has previously been paid to this question. Therefore, in an attempt to evaluate the relationships between the polyphenols within a given tobacco waste and the tobacco varieties in the waste source, multivariate analysis methods (MAM) are certainly necessary. MAM was successfully applied to determine the source of biomass from different geographical locations.[23–26] Representative methods of MAM are hierarchical cluster analysis (HCA) and principal

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component analysis (PCA). In this study, the HCA and PCA methods were developed to evaluate the tobacco waste resources to guide the high added value utilization of all varieties of tobacco wastes. The overall similarity of the active polyphenols contained in tobacco wastes among the varieties was calculated with the HCA method on the basis of the PCA pattern.

The objective of this work was the evaluation of tobacco waste as a potential ethnopharmacological resource for the production of chlorogenic acids and rutin, in order to develop a new bioprocess for the synthetic utilization of tobacco waste.[27] Processing tobacco wastes could solve the environmental problems derived from the disposal of large amounts of tobacco waste. Moreover, obtaining stabilized tobacco wastes as natural ethnopharmacological materials could be advantageous to the traditional Chinese medicine (TCM) industry in China. This would not only improve the uses available for tobacco wastes, but would also provide a new source of natural and functional polyphenols.

EXPERIMENTAL

Samples and reagents

The wastes of 10 tobacco samples were collected from different countries and regions of the world in 2007 and authenticated as the wastes of *Nicotiana tabacum* L. by Prof. Ding-qiang Lu (College of Life Science and Pharmaceutical Engineering, Nanjing University of Technology). All samples were dried at 60 °C using a herb disintegrator (Qinzhou Sanyang Package Equipment Co., Ltd.) and then sieved (60 mesh).

HPLC-grade methanol, acetic acid, and acetonitrile were purchased from TEDIA Co. (Fairfield, OH, USA). Chlorogenic acid (5-caffeoylquinic acid, 5-CQA), caffeic acid (CA), scopolin, and rutin were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (NICPBP), Beijing, China. Neochlorogenic acid (3-caffeoylquinic acid, 3-CQA), cryptochlorogenic acid (4-caffeoylquinic acid, 4-CQA), and nicotine were purchased from Chengdu Biopurify Phytochemicals Ltd., Chengdu, China. Isoquercitrin, luteolin-7-rutinoside, and quinic acid (QA) were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA).

Sample preparation

Tobacco waste samples (0.15 kg) were extracted in 70 % v/v aqueous methanol (0.50 L), using ultrasonic waves for 30 min at a time, for a total of 2 h.[27] The bulk extracts were diluted with 70 % v/v aqueous methanol to form a solution of 1.0 mg/mL. Solutions of pure compounds were prepared at a concentration of 1.0 mg/mL in methanol. The sample solutions were filtered through a 0.45 μm filter and 10 μL of each was injected for analysis.

Equipment

HPLC-PDA–MS was performed on a Waters system (Millipore Corp., Milford, MA, USA). The LC equipment comprised a Waters 2695 Separations Module, an autosampler with a 50 μL loop and a Waters 2996 photodiode array detector with a light-pipe flow cell (recorded at 328, 320, 280, and 254 nm, and scanning from 200 to 400 nm). This was interfaced with a mass spectrometer fitted with Micromass® Quattro micro™ API source and ESCi™ Multi-Mode Ionization Plus ESI source.

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HPLC–UV was performed using LB-5 pump (Beijing Satellite Manufactory, Beijing, China) with a UV detector (Shimadzu Seisakusho Ltd., Kyoto, Japan) and N-2000 workstation (Hangzhou Mingtong S & T Ltd., Hangzhou, China).

HPLC-PDA–ESI-MS/MS

The HPLC-PDA–ESI-MS/MS experiments were performed according to the literature.[14,27] The HPLC separation was performed on an Alltima C_{18} (250 mm×4.6 mm×5 μm) column (Alltech, Deerfield, IL, USA). The mobile phase contained solvents A and B, where A was water/acetonitrile/acetic acid (97.5:2:0.5 v/v/v) and B was acetonitrile/acetic acid (99.5:0.5 v/v). The gradient profile was as follows: 2 % B for 5 min; 2 to 5 % B in 5 min; 5 to 10 % B in 20 min; 10 to 35 % B in 3n 5 min; 35 to 100 % B in 15 min. Then the composition was re-established by going from 100 to 2 % B over 5 min and maintained at 2 % B for 5 min. The wavelength range of the PDA detection was from 200 to 400 nm. The flow rate was 1.0 mL min^{-1} for HPLC and PDA detection, with the column kept at 40 °C. A splitter was connected between the PDA and MS detectors, which reduced the flow rate to 0.20 mL min^{-1} for MS detection. The electrospray ESI-MS/MS was operated in the negative ion mode with scanning range of *m/z* 100–800. The capillary voltage was 3.0 kV, the cone voltage 30.0 kV and the ion source temperature was 120 °C. High purity nitrogen (99.9 %) at a flow rate of 500 L h^{-1} and at 350 °C was used as a dry gas to evaporate the solvent. Nitrogen was also used as the nebulizer gas at 50 psi.

Determination of chlorogenic acids in tobacco wastes

The HPLC-UV experiments were performed according to the literature.[14,27] The HPLC separation was performed on an Alltima C_{18} (250 mm×4.6 mm×5 μm) column (Alltech, Deerfield, IL, USA). The mobile phase consisted of acetonitrile/ammonium acetate buffer (pH 4.5) (5:95 v/v). The flow-rate was 1.0 mL min^{-1} and the UV detection was realized at 327 nm with the column kept at 30 °C.

Determination of rutin in tobacco wastes

The HPLC separation was performed on an Alltima C_{18} (250 mm×4.6 mm×5 μm) column (Alltech, Deerfield, IL, USA). The mobile phase consisted of methanol/acetic acid solution (pH 4.5) (50:50 v/v). The flow-rate was 1.0 mL min^{-1} HPLC and the UV detection was realized at 360 nm with the column kept at 30 °C.

Multivariate statistical analysis

Hierarchical cluster analysis and principal component analysis were employed to analyze the relationships between the contents of polyphenols (chlorogenic acids and rutin) and waste from the tobacco varieties. Multivariate methods were applied to the mean values of three replicates from each determination result. The initial variable values were standardized, the mean centered and autoscaled to variance prior to analysis to avoid any effects of the scale of the units with which they were measured.

Principal component analysis, PCA, was used to reduce the initial data from linear combinations of the original variables. PCA breaks down the matrix of the initial data, **X**, to express them as a least-square model.[28] PCA is a data compression method based on correlation amongst variables. The aim of PCA is to group correlated variables and replace them with new sets called principal components (PCs). PCs are completely uncorrelated and are built as simple linear combinations of the original variables. PCs contain most of the data set variability but in a much lower dimensional space. When redundancy is removed, only the first few principal components are required to describe the information contained in the ori-

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ginal data set.[24,29] The principal component loadings of the data were analyzed after application of Varimax normalized rotation of the PCs coordinate system. The score plots of the first PC may be used to investigate the interrelationships among the objects, as they allow the observation of clusters of objects. The interrelationships among the variables may also be studied through the respective loading plots.[30]

Hierarchical cluster analysis, HCA, is a technique used for classifying objects that have been characterized by the values of a set of variables into different groups. The clusters are formed by grouping objects according to similarity, and the results are presented in the form of dendrograms, which allow the distances between objects to be visualized. The between-groups linkage or the unweighted pair group method with arithmetic mean (UPGMA) technique, which defines the distance between two clusters as the average of all the pairs of distances between elements of both clusters, was adopted.[24,31,32] Similarities and dissimilarities were quantified by Square Euclidean distance measurements. The data analysis was realized using the SPSS v.13.0 statistical package (SPSS, 2005).

RESULTS AND DISCUSSION

Identification of the chemical constituents in tobacco wastes by HPLC–PAD-ESI/MS/MS

In the methanolic extracts of the dried tobacco wastes, compounds **1** (quinic acid), **2** (nicotine), **3** (3-CQA), **4** (scopolin), **5** (5-CQA), **6** (4-CQA), **7** (caffeic acid), **11** (rutin), **12** (isoquercitrin) and **13** (luteolin-7-rutinoside) were identified by comparing their retention times and UV and MS spectra with those of standard compounds. Moreover, the structures of all compounds in the methanolic extracts of dried tobacco wastes were determined by MS SCAN and MS MRM modes and by comparing the UV and MS spectrum data with those reported in literature.[18,19,33–36] The 15 polyphenols identified from the methanolic extracts of the dried tobacco waste are given in Table I and the structures of the polyphenols identified from the tobacco wastes, with the IUPAC numbering system, are shown in Fig. 1.

TABLE I. Polyphenols identified in the methanolic extract of dried tobacco wastes

No.	τ_R min	Molecular weight	Parent ion MS *m*/*z* $[M–H]^-$	Daughter ion MS/MS *m*/*z*	λ_{max} / nm (UV)	Identification[a]
1	3.2	192	191.3	127.3	254	Quinic acid
2	6.7	162	161.3	103.7	259, 282	Nicotine
3	21.5	354	353.3	191.3, 179.3, 173.3, 135.3	240, 298 *sh*,328	3-*O*-Caffeoyl-quinic acid (3-CQA)
4	31.4	192	191.3	179.3	254, 294 *sh*, 342	Scopolin
5	32.5	354	353.3	191.3, 179.3	240, 298 *sh*, 328	5-*O*-Caffeoyl-quinic acid (5-CQA)
6	33.2	354	353.3	191.3, 179.3, 173.3, 135.3	240, 298 *sh*, 328	4-*O*-Caffeoyl-quinic acid (4-CQA)

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TABLE I. Continued

No.	τ_R min	Molecular weight	Parent ion MS *m/z* $[M–H]^-$	Daughter ion MS/MS *m/z*	λ_{max} / nm (UV)	Identification[a]
7	37.5	180	179.4	135.3	217, 240, 298 *sh*, 325	Caffeic acid
8	40.8	338	337.3	191.4, 173.3	240, 298 *sh*, 314	5-*O*-*p*-Coumaroylquinic acid (5-*p*CoQA)
9	42.9	368	367.7	191.4, 173.4	240, 298*sh*, 324	4-*O*-Feruloylquinic acid (4-FQA)
10	43.5	338	337.3	191.4, 163.3	240, 298 *sh*, 314	3-*O*-*p*-Coumaroylquinic acid (3-*p*CoQA)
11	48.5	610	609.3	301.3, 271.3, 179.3, 151.3	256, 298 *sh*, 360	Rutin
12	50.5	464	463.3	271.3, 217.3, 179.3, 151.3	256, 298 *sh*, 362	Isoquercetin
13	51.7	594	593.4	285.3	255, 298 *sh*, 340	Luteolin-7-rutinoside
14	52.8	516	515.3	353.3, 335.3, 191.3, 179.3	240, 298 *sh*, 330	1,5-Di-*O*-caffeoylquinic acid (1,5-diCQA)
15	54.5	516	515.3	353.3, 191.3, 179.3, 135.3	240, 298 *sh*, 330	3,5-Di-*O*-caffeoylquinic acid (3,5-diCQA)

[a]IUPAC numbering system for all CQAs

The HPLC profiles of the 70 % methanol extract of tobacco waste detected with negative ion mode ESI-MS TIC and UV at 320 nm are shown in Fig. 2.

Characterization of caffeoylquinic acids (MW 354)

Typically, as can be seen from Fig. 2, three peaks (**3**, **5**, and **6**) were found in the total ion chromatogram (TIC) detected by the negative ion ESI-MS scan mode and UV at 320 nm. The largest peak (**5**) was identified as chlorogenic acid (5--*O*-caffeoylquinic acid) by comparison with the standard compound. The chromatographic profiles of 70 % methanolic extracts of tobacco wastes detected by the negative ion ESI-MS multiple reaction monitoring (MRM) mode are shown in Fig. 3 as MS MRM ESI-TIC, MS MRM, *m/z* 353.3 → 173.3 and MS, *m/z* 353.3 → 191.3. Peak **6** was clearly identified as 4-*O*-caffeoylquinic acid by its MS MRM (*m/z* 353.3 → 173.3) (Figs. 3A and 3B), because the MS^2 base peak of 4-CQA is *m/z* 173.3. Although the MS^2 base peak of 3-CQA and 5-CQA are both *m/z* 191.3, there was a difference in the secondary ion at *m/z* 179.5. Therefore, the smallest peak (3) was identified as 3-*O*-caffeoylquinic acid by its MS MRM (*m/z* 353.3 → 191.3) (Figs. 3A and 3C) and by comparing the UV and MS spectra data with those reported in literature.[18,19,33,34]

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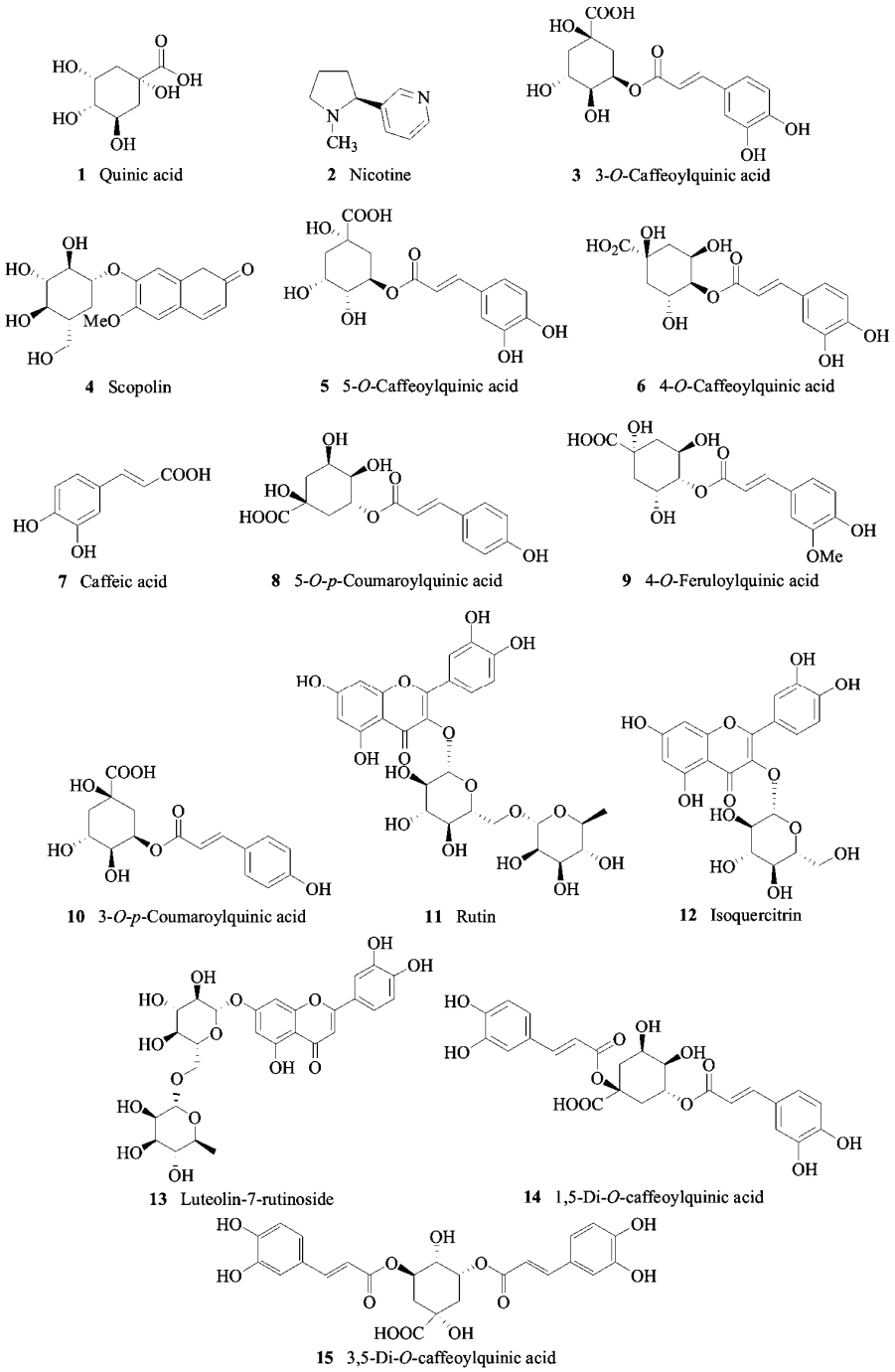

Fig. 1. Structure of the polyphenols identified in tobacco waste determined by HPLC-PDA–ESI/MS/MS.

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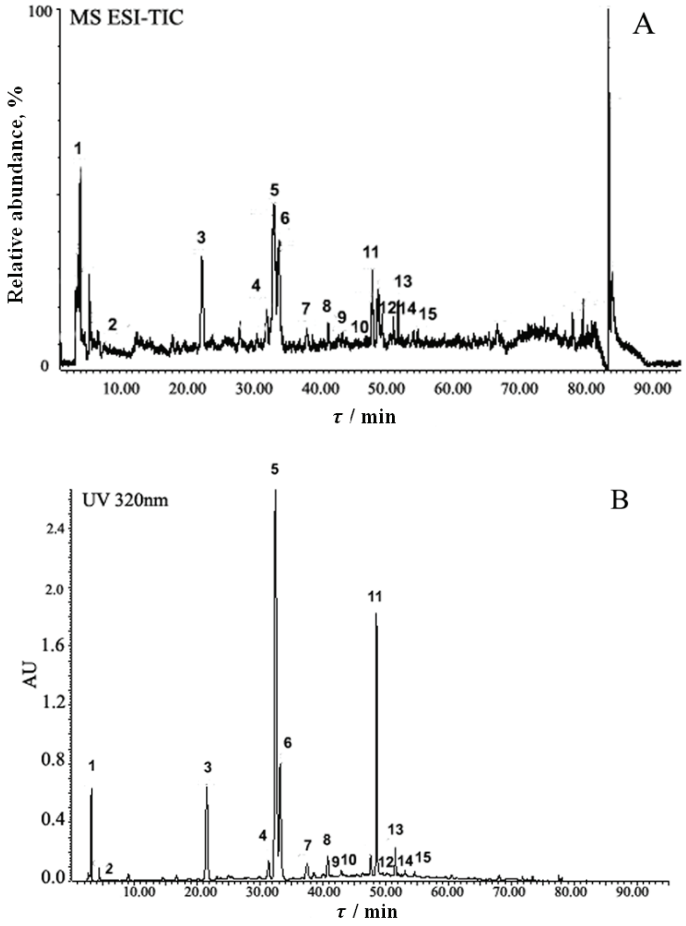

Fig. 2. Chromatographic profiles of a 70 % methanolic extract of tobacco wastes: MS SCAN ESI-TIC (A) and UV at 320 nm (B).

Characterization of p-coumaroylquinic acids (MW 338)

As can be seen from Fig. 2, two peaks (**8**, **10**) were found in the TIC detected by the negative ion ESI-MS scan mode and UV at 320 nm. The chromatographic profiles of 70 % methanolic extracts of the tobacco wastes are shown in Fig. 4 as MS MRM, *m/z* 337.3 → 191.4, MS MRM, *m/z* 367.3 → 173.3 and MS MRM *m/z* 515.3 → 353.3. Based on the MS^2 base peak *m/z* 191.4, the larger peak (8) and the smaller peak (10) were easily identified as 5-*O*-*p*-coumaroylquinic acid (5-*p*CoQA) and 3-*O*-*p*-coumaroylquinic acid (3-*p*CoQA), respectively, by their MS MRM (*m/z* 337.3 → 191.4, Fig. 4A) and by comparing the UV and MS^2 spectral data with those reported in the literature.[18,19,34,35]

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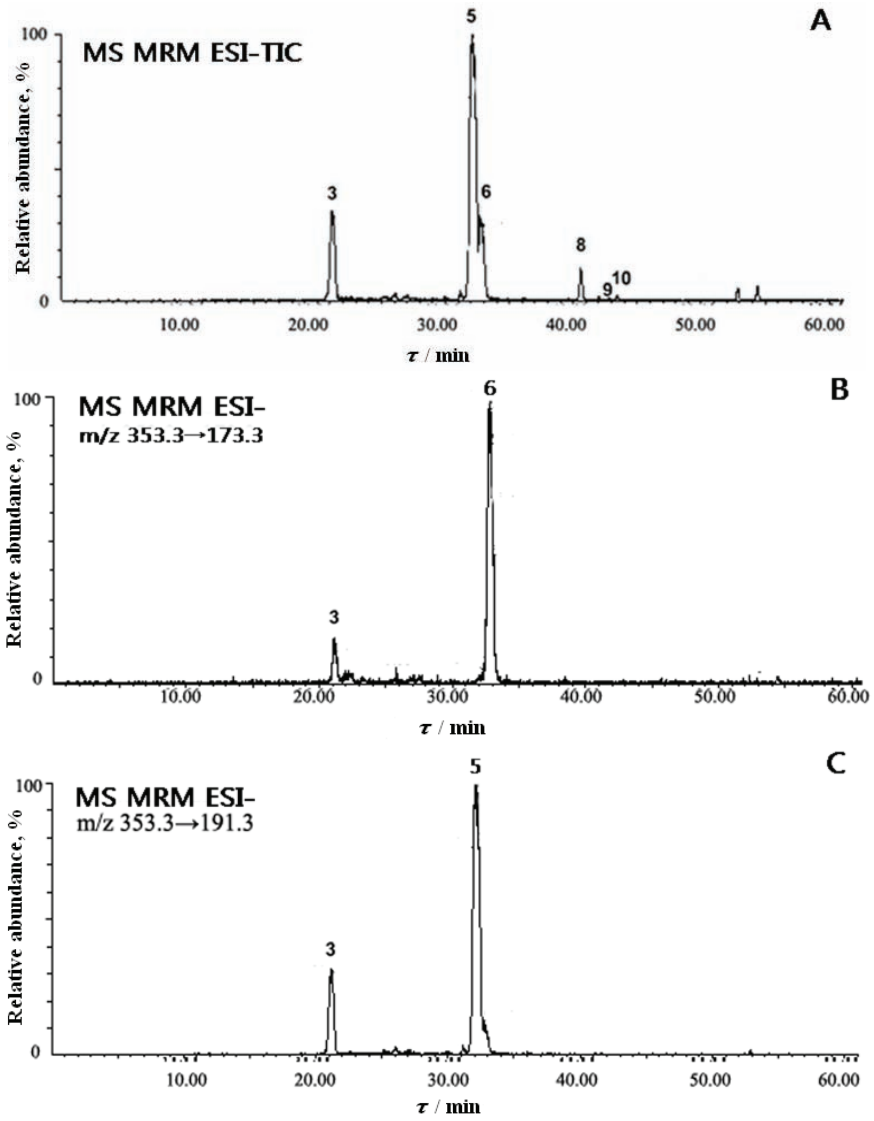

Fig. 3. Chromatographic profiles of a 70 % methanolic extract of tobacco wastes: A) MS MRM ESI-TIC; B) MS MRM, *m/z* 353.3 → 173.3; C) MS MRM, *m/z* 353.3 → 191.3.

Characterization of feruloylquinic acids (MW 368)

Based on the MS^2 base peak *m/z* 173.3, a peak (**9**) was found in the TIC, detected by negative ion ESI-MS and UV at 320 nm (Fig. 2), which was identified as 4-*O*-feruloylquinic acid (4-FQA) by its MS scan and MRM (*m/z* 367.3 → → 173.3) (Fig. 4B) spectral data and by comparing the UV and MS spectra data with those reported in the literature.[18,37] However, as can be seen from Fig. 4B,

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the MRM (*m/z* 367.3 → 173.3) data also showed that a very small peak in the UV 320 nm chromatographic profiles (retention time: 42.02 min) had *m/z* 367.3 and *m/z* 173.3 ions and had the split pathway of *m/z* 367.3 → 173.3, as well. Thus, it may be a derivative of 4-FQA.

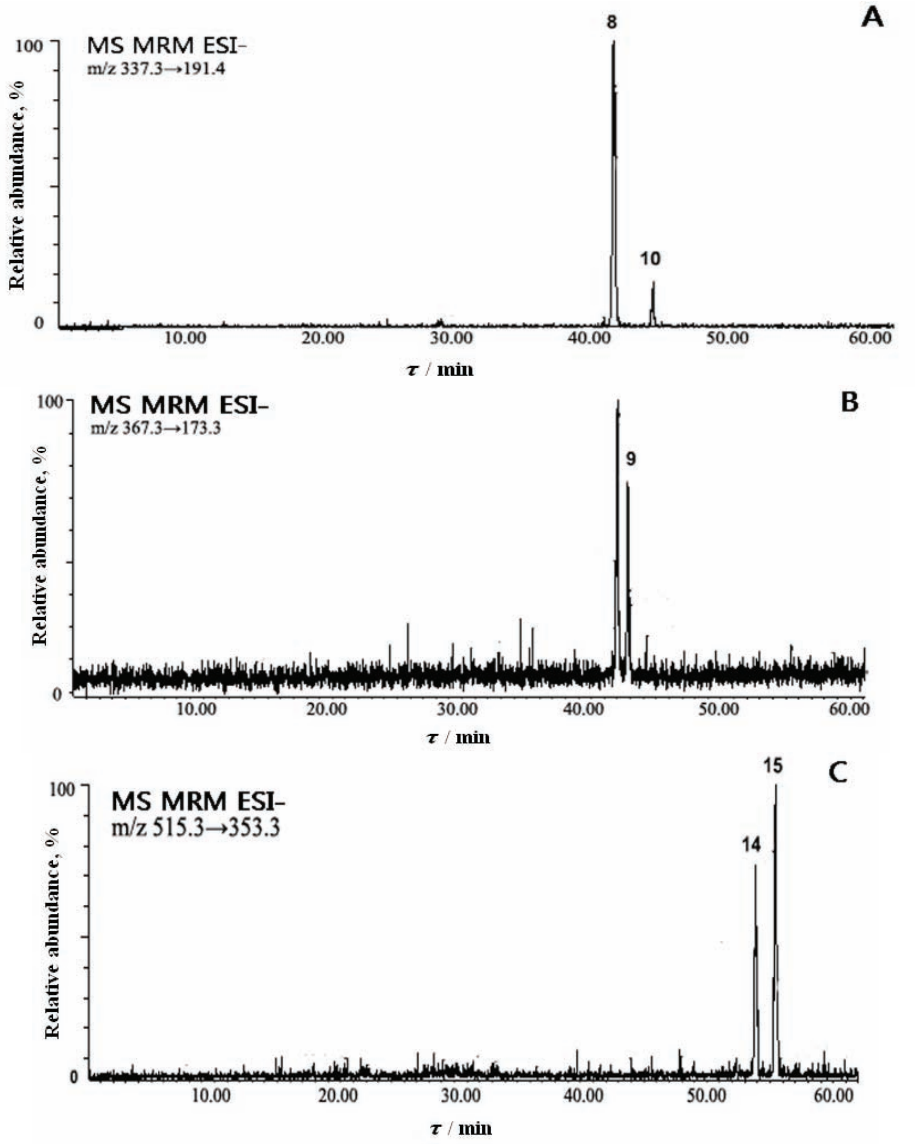

Fig. 4. Chromatographic profiles of a 70 % methanolic extract of tobacco wastes: A) MS MRM, *m/z* 337.3 → 191.4; B) MS MRM, *m/z* 367.3 → 173.3; C) MS MRM, *m/z* 515.3 → 353.3.

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Characterization of di-caffeoylquinic acids (MW 516)

Typically, two peaks (14 and 15) were found in the TIC detected by the negative ion ESI-MS scan mode and UV at 320 nm (Fig. 4C).

Comparing the UV and MS spectra data with those reported in literature,[9,18] as can be seen from Fig. 4C, based on the MS^2 base peak *m/z* 353.3, peaks 14 and 15 were identified as 1,5-di-*O*-caffeoylquinic acid (1,5-diCQA) and 3,5-di--*O*-caffeoylquinic acid (3,5-diCQA) by their parent ion mode (*m/z* 515.3), daughter ion mode (*m/z* 353.3), and MS MRM (*m/z* 515.3 → 353.3, *m/z* 353.3 → 173.3 and *m/z* 353.3 → 191.3).

Characterization of quinic acid, nicotine, scopolin, caffeic acid, rutin, isoquercitrin and luteolin-7-rutinoside

Seven peaks (1, 2, 4, 7, 11, 12 and 13) were found in the TIC detected by the negative ion ESI-MS SCAN mode and UV at 320 nm. Comparing the UV and MS spectra data with those reported in CA, peaks 1 (quinic acid), 2 (nicotine), 4 (scopolin), 7 (caffeic acid), 11 (rutin), 12 (isoquercitrin), and 13 (luteolin-7-rutinoside) were identified by comparing their retention times and UV and MS spectra with those of standard compounds.

Contents of chlorogenic acids and rutin in tobacco wastes by HPLC–UV

Calibration curves were obtained by plotting peak areas *vs*. six different concentrations of the standard solutions. The calibration curve equations were: neochlorogenic acid (3-CQA) $y = 6.196\times10^7x - 4.453\times10^4$ ($r^2 = 0.9997$); chlorogenic acid (5-CQA) $y = 5.841\times10^7x - 4.384\times10^4$ ($r^2 = 0.9996$); cryptochlorogenic acid (4-CQA) $y = 5.068\times10^7x - 4.025\times10^4$ ($r^2 = 0.9996$)[27] and rutin $y = 3.001\times10^7x - 1.100\times10^5$ ($r^2 = 0.9997$). The developed method was successfully applied to the simultaneous determination of 3-CQA, 5-CQA, 4-CQA, and rutin in 10 samples of tobacco waste that were obtained from various countries and regions in the world. The results of the determinations are given in Table II.

From the results presented in Table II, it was found that the contents of 3-CQA, 5-CQA, 4-CQA, chlorogenic acids, and rutin varied greatly among the different samples. In the majority of cases, the contents of 3-CQA, 5-CQA, 4-CQA and rutin in the 10 samples were within the ranges 0.116–0.196, 0.686–1.781, 0.094–0.192 and 0.413–0.998 %, respectively. Chlorogenic acids were the main components in the tobacco wastes, with total contents varying from 0.897 to 2.130 % in the 10 samples; an almost 2.4-fold variation. Similar variation could also be found for the contents of rutin in the tobacco wastes. The reasons for these variations in their contents might be the difference in the origin of the plant, the effect of environment, and/or other factors, such as the season at the time of collection, the drying process used, storage conditions, *etc*.

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Evaluation of tobacco waste resource by HCA on the basis of PCA pattern

As variations in the contents of polyphenols may influence the quality and potency of tobacco wastes, it was necessary to develop an effective statistic analysis method to evaluate the quality of tobacco wastes. Thus, for the application of tobacco wastes as a potential ethnopharmacological resource in the production of chlorogenic acids and rutin, the overall similarity of chlorogenic acids and rutin contents among the varieties was calculated using the HCA method based on the PCA pattern.

TABLE II. Contents of neochlorogenic acid (3-CQA), chlorogenic acid (5-CQA), cryptochlorogenic acid (4-CQA), chlorogenic acids and rutin in 10 tobacco wastes (n = 3)

No.	Source	Content of active compounds, %[a]				
		3-CQA (x_1)	5-CQA (x_2)	4-CQA (x_3)	Chlorogenic acids[b] (x_4)	Rutin (x_5)
1	Jin'an Songyun 87BSL Kunming (B1L and B2L mixed) (2005)	0.150±0.015	0.870±0.048	0.111±0.014	1.132±0.075	0.998±0.065
2	Jin'an Songyun 87CSL Kunming (C1L and C2L mixed) (2005)	0.157±0.007	0.704±0.044	0.129±0.008	0.990±0.058	0.719±0.036
3	Zimbabwe B10A (2004)	0.116±0.007	0.686±0.052	0.094±0.011	0.897±0.069	0.655±0.057
4	Jin'an Songyun 87MZL Kunming (C4L, X2L and X3L mixed) (2005)	0.157±0.006	0.709±0.032	0.140±0.004	1.006±0.042	0.725±0.070
5	Nanrun Hongda B2F (2004)	0.182±0.003	1.483±0.033	0.158±0.003	1.823±0.038	0.848±0.069
6	Wei said Artemis K326 C1L (2002)	0.141±0.003	0.908±0.027	0.123±0.006	1.172±0.035	0.413±0.051
7	Nanrun Hongda X2F (2004)	0.196±0.009	1.114±0.068	0.192±0.014	1.502±0.090	0.537±0.052
8	Zimbabwe TL40 (2004)	0.154±0.002	0.950±0.033	0.157±0.008	1.261±0.042	0.614±0.077
9	Nanrun Hongda C2F (2004)	0.190±0.008	1.781±0.084	0.159±0.021	2.130±0.108	0.807±0.096
10	Jiangsu Tobacco Company Nanjing Branch (2007)	0.156±0.003	0.699±0.015	0.151±0.002	1.006±0.018	0.464±0.024

[a]Data are expressed as mean ±*SD* of three experiments; [b]data are the sum of contents of 3-CQA, 5-CQA and 4-CQA

The data was pre-processed using auto-scale and incremental linkage methods for data from tobacco wastes samples.[23] The goal of PCA is to group cor-

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related variables and replace them with new sets called PCs. PCs contain most of the data set variability, but in a much lower dimensional space.[29] The first principal component, PC_1, is defined as the direction of maximum variance of the entire data set. PC_2 is the direction that describes the maximum variance in the subspace orthogonal to PC_1. The subsequent components are taken orthogonally and describe the maximum remaining variance.[23–26] In the present study, the PCA score plots for the contents of chlorogenic acids and rutin in tobacco wastes using auto-scale pre-processing are shown in Fig. 5.

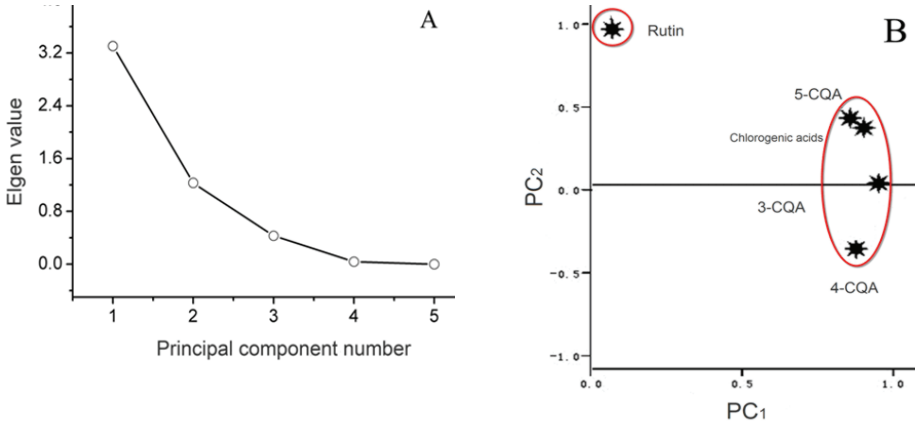

Fig. 5. PCA results for the contents of chlorogenic acids and rutin in different varieties of tobacco wastes. A) PCA score plots; B) the contribution of content parameters to PC_1 and PC_2.

As can be seen from Fig. 5, for data of the contents of chlorogenic acids and rutin in tobacco wastes, two PCs describe 90.661 % of the data, and three PCs describe 99.264 % of the data. A clear differentiation between the tobacco waste samples from different locations was observed. PC_1 and PC_2 conserved 90.661 % of the total variance of the original data according to the following equations:

$$PC_1 = 0.935x_1 + 0.918x_2 + 0.786x_3 + 0.951x_4 + 0.250x_5 \quad (1)$$

$$PC_2 = -0.174x_1 + 0.230x_2 - 0.547x_3 + 0.163x_4 + 0.906x_5 \quad (2)$$

where x_1–x_5 are the content of 3-CQA, 5-CQA, 4-CQA, chlorogenic acids and rutin given in Table II, respectively.

Equation (1) indicates the significant importance of the contributions of x_1–x_4 to PC_1, while Eq. (2) indicates that the contribution of x_5 to PC_2 is much greater than the other ones. According to the multivariate statistics models, two phenolic compound variables can be considered important for discriminating between varieties of tobacco wastes: chlorogenic acids and rutin. Therefore, data regarding the varieties of tobacco wastes can be analyzed by PC_1 and PC_2, and the developed PCA method is suitable for reducing overlapping and unnecessary

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data, in order to evaluate the relationships between the contents of polyphenols and the varieties of tobacco wastes.

The main objective of HCA is to display data in natural clusters, showing patterns in two-dimensional space.[22] Similarities and dissimilarities between the contents of polyphenols and the varieties of tobacco wastes as the results of either direct cluster analysis or HCA based on the PCA pattern are shown in Figs. 6A and 6B, respectively.

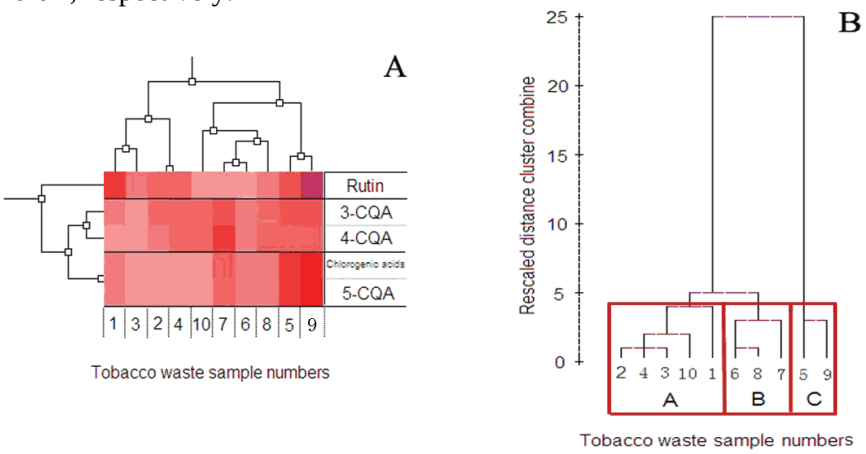

Fig. 6. Similarities and dissimilarities between the contents of the main polyphenols (chlorogenic acids and rutin) and different varieties of tobacco wastes, resulting from cluster analysis: A) direct cluster analysis; B) HCA based on the PCA pattern.

The dendrogram with tobacco wastes from different sources, shown in Fig. 6, is qualitative in nature and permits visualization of clusters and correlations amongst samples. Based on the contents of chlorogenic acids and rutin, see Fig. 6A, it is difficult to classify tobacco waste samples using the direct cluster analysis method. In HCA, the Euclidean distances among samples or variables are transformed into similarity indices. A small distance corresponds to a large index and means a large similarity.[29–31] As can be seen from Fig. 6B, three distinct clusters represent the sources of the tobacco wastes samples; that is, group A contains sample 2, 4, 3, 10 and 1, group B contains sample 6, 8, and 7, and group C contains samples 5 and 9. Therefore, HCA analysis showed that it is possible to differentiate between phenolic contents and the sources of samples with different geographical origins, and of different varieties: the 10 varieties of tobacco wastes samples are clearly separated into three groups. In a general way, the developed HCA method based on the PCA pattern is suitable for grouping overlapping varieties of tobacco waste resources depending on the contents of polyphenols. The results obtained using the HCA method can provide valuable information to guide high added value usage of chlorogenic acids and rutin in varieties of tobacco wastes throughout the world.

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CONCLUSIONS

In this paper, the identification of 15 polyphenols in tobacco wastes is reported. It was also demonstrated that tobacco wastes have a potential application as ethnopharmacological materials in the pharmacological industry, due to the unusual dominance of chlorogenic acids (chlorogenic acid, neochlorogenic acid and cryptochlorogenic acid) and rutin contained within the waste. Moreover, according to multivariate statistics models, using the HCA method based on the PCA pattern, the chlorogenic acid and rutin contents of tobacco waste can be considered important for discriminating between different varieties of tobacco wastes. Ten varieties of tobacco were characterized into three groups using this method. In conclusion, tobacco wastes containing natural chlorogenic acids and rutin could be advantageous for industrial applications and multivariate methods could be useful for the analysis and interpretation of a large number of tobacco waste resources in the world. In further studies, the heterogeneity of tobacco wastes must be regulated and problems concerning the collection, as well as conveyance of the biomass, must be resolved for the practical utilization of tobacco wastes.

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И З В О Д

РАЗЛИКОВАЊЕ И КЛАСИФИКАЦИЈА ДУВАНСКОГ ОТПАДА ОДРЕЂИВАЊЕМ ПОЛИФЕНОЛА МЕТОДОМ LC–MS/MS

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Хемијски састав полифенола у дуванском отпаду је идентификован методом HPLC--PDA–ESI/MS/MS, садржај хлорогенских киселина и рутина у десет врста дуванског отпада је одређен методом HPLC–UV и однос између садржаја полифенола и врсте дуванског отпада је анализиран хијерархијском кластерском анализом (HCA) и анализом основних компоненти (PCA). Петнаест полифенола је идентификовано у метанолном екстракту сувог дуванског отпада. Измерене су велике концентрације хлорогенских киселина (3-CQA, 5-CQA и 4-CQA) и рутина, у опсегу 0,116–0,196, 0,686–1,781, 0,094–0,192 и 0,413–0,998 %. Према мултиваријантном статистичком моделу, две променљиве се могу сматрати важним у разликовању врсте дуванског отпада: хлорогенске киселине и рутин. Десет врста отпада је класификовано у три групе кластерском анализом на основу садржаја основних компоненти. Користећи ове податке, дувански отпад може наћи примену као фармацеутски материјал за изоловање природних хлорогенских киселина и рутина у етнофармаколошкој индустрији.

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