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Multivariate statistical analysis of tobacco of different origin, grade and variety according to polyphenols and organic acids

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ABSTRACT

The aim of this work was to determine the concentration of polyphenols, organic acids in tobacco of different areas, grades and varieties by ultra-performance liquid chromatography tandem mass spectrometry (UPLC/MS/MS) and to achieve statistical classification by principal component analysis (PCA) and linear discriminant analysis (LDA). The obtained results revealed that tobacco of different varieties can be correctly classified according to the contents of polyphenols or organic acid. The results of PCA showed that different grades and geographic regions cannot completely be discriminated using polyphenols or organic acid as independent variables. However, there were marked differences in special class from the same type or grade tobacco. At the same time, the results of LDA also showed that the samples were correctly classified at 100% for different varieties of tobacco, but only 55.3% and 60% for different grades and areas, respectively. These results demonstrated that the composition of polyphenols and organic acids can be used as the useful variables to characterize the type and the special class or grade of tobacco.

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1. Introduction

Polyphenols and organic acids are widely present in tobacco plant and production, and play an important role in the process of physiological metabolism of tobacco [1,2]. Polyphenols are important components of tobacco, due to their contribution to sensory properties—color, flavor and bitterness [3] and also to antioxidant properties [4]. Organic acids and their derivatives are the main flavor components of tobacco [5], in which non-volatile acids can regulate the pH value of tobacco which improves the smoking taste and then indirectly affects the tobacco aroma [6]. Because of their importance, considerable efforts have been made to investigate and determine polyphenols and organic acids in tobacco and their products by different instrumental procedures [7–11].

Although chemometrics have been widely applied in the field of food and wine [12–14], several articles have been published on the application of chemometrics to characterize volatile compounds of tobacco of different areas or to construct fingerprints of tobacco [15,16]. The applications of a multivariate analysis technique to get an overall view of the differences among tobacco of different grades, areas and varieties have not been published. For this reason, the goal of our work was to determine the contents of polyphenols and organic acid in tobacco and to differentiate their grades, areas and varieties using multivariate analysis. We analyzed different tobacco samples of nine grades, eight geographic regions and four varieties to investigate the differences among them and to get a clear view which class of compounds is appropriate to characterize the tobacco.

2. Materials and methods

2.1. Reagents and chemicals

All polyphenol standards (rutin, chlorogenic acid, caffeic acid, esculetin, scopoletin, quercitrin, pyrocatechol, kaempferol, kaempferol-3-rutinoside (KF-3-R), 5-o-caffoylquinic acid (5-O-CA), 4-o-caffoylquinic acid (4-O-CA), and aesculin) were purchased from Sigma-Aldrich (Shanghai, China). Malic acid, oxalic acid, citric acid, lactic acid, succinic acid were purchased from China Chemical Reagent Co., Ltd. Stock solutions of polyphenols were prepared in 80% methanol/water (v/v, adjusted to PH2) at a concentration of 1 mg mL^{-1} and stock solutions of organic acids were prepared with pure water (acidified to pH 2 with HCl) at a concentration of 10 mg mL^{-1}. All of the standard solutions were stored at 4 °C, and further diluted to appropriate concentration for experiments.

Methanol (HPLC grade) was obtained from Dikma (USA). Analytical grade acetic acid and hydrochloric acid were purchased from China Chemical Reagent Co., Ltd. Ultra pure water was prepared with the Milli-Q system Millipore (Bedford, MA, USA). All solvents were filtered through 0.22 μm nylon membranes and degassed prior to use. The solid phase extraction (SPE) cartridge used was Sep-Pak C18 (50 mg mL^{-1}) from Waters (Milford Massachusetts, Ireland).

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They were sequentially conditioned with 3 mL of methanol and 3 mL of deionized water.

2.2. Tobacco samples collection and treatment

One hundred and thirty three ($n=133$) tobacco samples were sourced from nine different tobacco grades ($n=38$, Yb, Yc, Yx, Kb, Kc, Kx, Hb, Hc and Hx), four varieties of samples ($n=30$, flue-cured tobacco, sun-cured tobacco, burley and oriental tobacco), and eight regions ($n=35$, Jiangxi and Hunan provinces (central-south of China), Yunnan province (southwest of China), Fujian province (east of China), Heilongjiang province (northeast of China), Henan province (central-north of China), USA, Zimbabwe and Brazil) supplied by Hongta group. Y, K and H are the abbreviation for "Yun 85, 87", "K326", "Hongda", which are classes of flue-cured tobacco, respectively. Letter b, c and x represent the upside, middle and under parts of tobacco leaf.

20 g of tobacco samples was milled to a 60 mesh powder (selected by sieve), and then was conditioned at 22 °C and 60% relative humidity for at least 24 h prior to extract. 0.1 g of tobacco sample was accurately weighted and transferred into a 250 mL glass conical flask, then 10 mL 80% methanol/water (v/v, pH 2) was added, ultrasonic extraction of 30 min at 40 W power. 3 mL supernatant was passed through the pre-conditioned SPE cartridge, followed by directly collecting the extract and then filtered with 0.22 μm nylon membranes for UPLC/MS/MS analysis. The solid phase extraction was performed with the vacuum station.

2.3. UPLC/MS/MS analysis of polyphenols and organic acid

A Waters Acquity UPLC system (with a binary solvent delivery system and an autosampler Waters, Milford, MA, USA) coupled with an AB/MDS Sciex API3200 mass spectrometer was utilized for sample analysis. Waters Acquity BEH C18 column (100 mm × 2.1 mm i.d., 1.7 μm particle size) was used for analyte separation. UPLC conditions were column oven, 45 °C; injection volume, 2 μL; flow rate, 0.4 mL min^{-1}. Mobile phase: A, methanol; B, 0.3% (v/v) acetic acid in water. UPLC separation was achieved using a gradient elution as follows: 0–10 min, 15–70%A, curve 5; 10.1–11 min, 100%A; 11.1–12 min return to initial conditions to equilibration of the column.

Mass detection conditions were as follows: ionization mode, negative electrospray ionization source (ESI); ion spray voltage, −3.5 kV; ion source temperature, 450 °C; curtain gas, 10 psi. High-purity nitrogen (>99.999%) was used as curtain and auxiliary gas. Compound-dependent parameters were optimized using flow injection analysis. Data acquisition was carried out using different retention time window; dwell time of 100 ms was used. For each analyte, two ion transition pairs were used in a multiple reaction mode. MRM transitions and compound-dependent parameters are summarized in Table 1.

2.4. Statistical analysis

To ascertain the significance of differences of polyphenols and organic acids concentration among different grades, areas and varieties of tobacco, the data were statistically evaluated by analysis of variance (ANOVA). Principal component analysis (PCA) was used for reducing the dimensionality of the data to a small number of components, to examine the possible grouping of samples according to the grade, variety and origin. Linear discriminant analysis (LDA) is a supervised classification technique based on the determination of linear discriminant functions, which maximize the ratio of between class variance and minimize the ratio of within class variance [17]. PCA and LDA were separately performed on data expressing polyphenols, organic acid levels in tobacco, in order to classify the tobacco samples. PCA was performed separately for each chemical parameter studied (polyphenols and organic acid profiles) and also for the global data. LDA was performed for the global data. PCA and LDA were performed using Minitab statistical software.

3. Results and discussion

3.1. Optimization of UPLC–MS/MS Method

As organic acids are the strong polar compounds and are easy-ionizing, most of the organic acids exist in the form of ions in aqueous solution. However, ion in the column is almost not reserved. Most of the work our predecessors did in the determination of organic acids and polyphenols, usually need a special column [18,19] or special mobile phase of liquid chromatography (LC) and usually need the buffer salt [20,21]. The objective of this work was to develop a rapid method for determining the polyphenols and organic acid in tobacco simultaneously with UPLC/MS/MS. UPLC showed many advantages, including reduced run time, less solvent consumption an increased peak capacities [21]. Although some analytes cannot be separated fully, the MS detector with Multiple Reaction Monitoring (MRM) scan mode has the strong anti-interference ability. It is easy to achieve the qualitative and quantitative analysis through extracting their specific ion-pairs even in compounds with the same retention time. By optimizing different concentrations of acetic acid and the ratio of methanol in mobile phase, as well as the column temperature, investigating the retention capacity of chromatographic column for organic acids and polyphenols, the above-mentioned chromatography experiment conditions were chosen as the optimum conditions. Finally, all test compounds under the optimum conditions were separated in less than 6 min.

3.2. Method validation and quality parameters

With the aim of verifying that the SPE-UPLC–MS/MS developed method was suitable for quantitative determination of polyphenols and organic acids in tobacco, method quality parameters were estimated (Table 2). All the polyphenols and organic acid calibration curves have correlation coefficients (r) higher than 0.9910. The limits of detection (LOD) and limits of quantification (LOQ) of the overall method were calculated as the concentration giving a signal-to-noise ratio of three ($S/N=3$) and ten ($S/N=10$), respectively. These limits were estimated by injecting 5 μL of diluted standard solutions. The values of LODs were in the range of 0.01–35.00 mg kg^{-1} while the LOQs were in the range of 0.05–50 mg $kg^{-1}$for test compounds (see Table 2).

Precision, accuracy and the recovery of target compounds were assessed by analyzing spiked tobacco samples containing known concentrations of the investigated analytes (10 ng mL^{-1} or 100 ng mL^{-1}) and extracted by the described SPE method. The intra-day precision was measured on a single day using six replicates of each spiked matrices under the same conditions (same analyte, apparatus, regents and short interval of time) whereas inter-day precision was calculated during six consequent days using also three replicates of each matrices spiked at the same concentration. The intra-day and inter-day precisions were lower than 7.4% and 10.7% for all the analytes (Table 2). All results were within the acceptable range and did not exceed 15% therefore we concluded that the method is accurate and precise. All of these values of recovery were satisfactory for most of the target compounds (≥80.3%) (Table 2) and indicated good method accuracy and repeatability.

3.3. Multivariate statistical analysis

Under these conditions, the developed analytical method was used to measure polyphenols and organic acids in tobacco samples. All of collected tobacco samples (including 4 varieties, 9 grades and 8 areas) were analyzed. The differences among these samples were emphasized by the PCA according to the variety, grade and origin.

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