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Reversed Phase HPLC Studies on the Change of the Content of Organic Acids in Tobacco During Aging

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Tobacco leaf samples from different leaf positions, different aging time were extracted for the determination of organic acids. Organic acids in tobacco have been determined by RP-HPLC method combined with UV detection. The gradient mobile phase employed was 20 mM phosphate buffer (pH 2.6) and acetonitrile at a flow-rate of 1.0 mL/min. Recoveries for organic acids were in the range of 84.2 to 106.3 %. The relative standard deviation of the repeating of the migration time and peak area of five replicate analyses of 11 organic acids ranged from 0.42 to 1.78 % and from 0.47 to 2.5 %, respectively. The results show that organic acids concentrations varied with aging time and different leaf position.

Key Words: Reversed-phase liquid chromatography, Organic acids, Tobacco, Aging.

INTRODUCTION

Organic acids in tobacco are of profound significance, because they are contributors to flavour and aroma, which mainly include oxalic, tartaric, acetic, malonic, formic, maleic, malic, lactic, succinic and citric acids. The contents of these acids strongly affect not only the quality of tobacco, but also the balance of flavour[1]. Low content of organic acids leads to acrid taste, while high content results in sourish taste. During growth and development of tobacco, organic acids are considered as intermediates of carbohydrate metabolism and precursors of amino acid synthesis. The quantities and proportions of organic acids vary with tobacco type, ferment duration, aging process, climate, soil environment, *etc.* Organic acids act as a touchstone in distinguishing different tobacco types. Therefore, reliable determination of organic acids in tobacco is essential for classification of tobacco materials according to their quality.

There are several methods available for determining the content of organic acids. GC assays, as a well-established technique for the analysis of organic acids, allow an excellent separation of different organic acids

using trimethylsilyl derivatives[2-5], but the assays generally spend much longer time than HPLC assays. Although ion-exchange chromatography has been employed in the determination of organic acids[6,7], high column temperature, expensive ion-exchange column and poor resolution fetter its development. In recent years, capillary electrophoresis is becoming more and more important in the analysis of organic acids[8]. However, it is hindered by a low detection sensitivity arisen from the column diameter.

In the present studies, a fast and reliable method was developed to determine the presence and distribution of organic acids in different tobacco samples. Moreover, the variations of the content of organic acids using the method during the aging process to control tobacco quality were studied. For this reason, an analytical procedure has been developed and validated which permits: (i) the identification and quantification of organic acids present in tobacco; (ii) the comparison of these organic acids' profiles in different leaves position and at different aging stages.

EXPERIMENTAL

All chemicals were of analytical reagent grade, purchased from Chemical Reagent Co. (Shanghai, China), except acetonitrile and methanol, which were of HPLC grade (Yuwang Industrial & Commercial Co. Shandong, China). Deionized water was prepared using a Millipore Milli-Q water purification system.

All flue-cured tobacco samples studied were obtained in 2004 from Yunnan, China. To carry out aging, modulated tobacco leaves were placed in lightproof cartons from 2005 to 2007. Relative humidity was kept at 50-70 % in every rainy season in order to avoid mildewing. Samples were taken for analysis in autumn every year.

Extraction and sample preparation: The leaves were dried to constant weight at 60 °C in an oven, then powdered and passed through a sieve to obtain a homogeneous powder for the analysis. Tobacco powder (2 g) was extracted with deionized water (70 mL) for 40 min with sonication at room temperature. As tobacco is a very complex mixture containing thousands of constituents, further purification was necessary. Therefore, the crude extracts were filtered and the filtrate was purified as follows in order to eliminate pigment and other interfering compounds.

5 mL of filtrate was passed through a C_{18} cartridge from Waters (Milford, MA, USA) previously activated by flushing with 3 mL of methanol and then with 5 mL of deionized water. After the extraction was loaded to the cartridge, the first 3 mL eluent was discarded and the next 2 mL eluent was connected. The eluent was filtered on a 0.45 μm Millipore filter and submitted to HPLC analysis by injection of 10 μL.

HPLC analysis: A Waters (Milford, MA, USA) model 600 liquid chromatograph coupled to a model 2487 UV detector and equipped with a Sinnochrom (Dalian Elite Analytical Instruments Co., Ltd.) ODS column (200 × 4.6 mm, particle size 5 μm) was employed. Mobile phase A was 20 mM KH_2PO_4 adjusted to pH 2.6 with ortho-phosphoric acid and phase B was acetonitrile-water (3:2, v/v). A gradient programme was applied as follows: 0-8 min isocratic with 100 % A; 8-12 min linear increase to 12 % B; 12-16 min isocratic with 12 % B. Then the gradient was reversed to original conditions in 5 min and the column was allowed to equilibrate with solvent A for 20 min before the next injection. The flow rate during the analysis was 1.0 mL/min and peaks were detected at 210 nm. Identification was based on retention time and on-line spectral data in comparison with authentic standards. Establishing calibration curves for each compound determined, using the standards, performed quantification. The injection volume was 10 μL. All experiments were performed at ambient temperature of 25 ± 2 ºC. These parameters were kept constant throughout the analysis of samples. All mobile phases were filtered through a 0.45 μm Millipore filter and degassed under vacuum in an ultrasonic bath for 10 min before use.

RESULTS AND DISCUSSION

Validation was performed for RP-HPLC using the mobile phase gradient programme described above. The pH value and flow rate of mobile phase play an important role in achieving sufficient resolution, so they were tested to obtain high resolution. The pH value of 2.6 and flow rate of 1.0 mL/min were found to be optimal for the highest sensitivity and resolution. The relative standard deviation (RSD) of the repeatability of the migration time and peak area of five replicate analyses of 11 organic acids standard mixture ranged from 0.42 to 1.78 % and from 0.47 to 2.5 %, respectively. Recovery experiments were undertaken by injecting aliquots of a mixture of standard solutions of tobacco samples. Experiments were carried out in triplicate. Recoveries between 84.2 and 106.3 % indicated that the method described above was suitable for the determination of organic acids in the tobacco leaves.

The chromatograms of the standard mixture of organic acids and tobacco samples were shown in Fig. 1A and 1B, respectively under optimal separation conditions described above. The separation of analyzed compounds is excellent, even in very high concentrations. The separation of all analytes was achieved in less than 16 min. As shown in Fig. 1B, propionic acid was not detected. Other organic acids were separated without interference peaks.

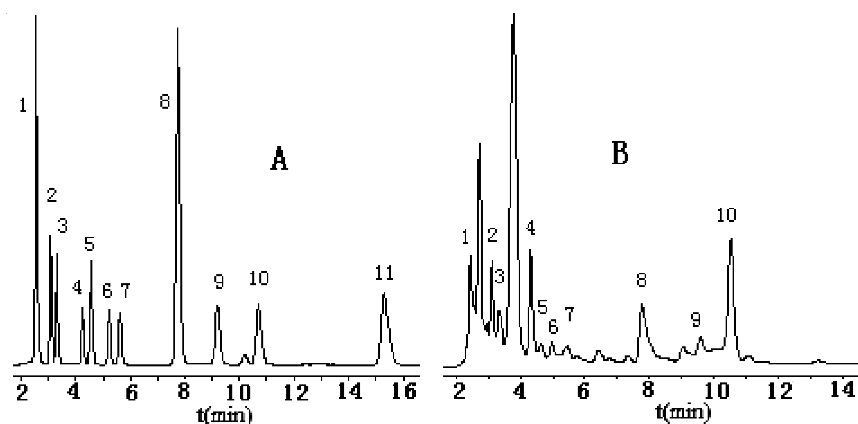

Fig. 1. HPLC chromatogram of (A) a standard mixture of organic acids, (B) a leaf extract of tobacco under the conditions described above. Key to peak identity: 1 = oxalic acid (0.634 mM), 2 = tartaric acid (1.599 mM), 3 = formic acid (8.696 mM), 4 = malic acid (2.237 mM), 5 = malonic acid (3.843 mM), 6 = lactic acid (5.551 mM), 7 = acetic acid (6.661 mM), 8 = maleic acid (0.129 mM), 9 = citric acid (1.428 mM), 10 = succinic acid (6.774 mM), 11 = propionic acid (20.270 mM)

The separation methods described above were applied to the analysis of organic acids in different tobacco samples and the results with respect to the content of organic acids are summarized in Table-1. In accordance with other studies, malic acid and oxalic acid were found to be the predominant organic acids in all of the samples and the content of maleic acid was the lowest[9]. Tobacco aging performed at ambient temperature is an indispensable step of improving tobacco quality and usability. In the course of aging, a lot of complicated chemical reactions related to organic acids were produced. At the beginning, the organic acids content in middle leaves was higher than upper and lower leaves and the content in lower leaves was the lowest. After three years of aging, as shown in Fig. 2, the organic acids content largely changed, but the content order in different position leaves was the same as before. This phenomenon is mainly due to the fact that lower leaves are situated at the bottom of the plant, where there is not ample sunshine, leading to fewer organic compounds synthesized and the soluble nutrition is transferred to the upside of the plant to guarantee the need of the growing-point, which results in the lowest content of organic acids in lower leaves. Whereas the middle leaves have higher degree of maturity and more soluble matter than the leaves from other positions, which leads to the highest content of organic acids. However, all organic acids didn't present the above order during the process of aging. Individual organic acid, such as oxalic acid, lactic acid and citric acid, presented

different variation. As shown in Table-1, for oxalic acid and citric acid, the content is the highest in upper leaves and the lowest in middle leaves. Further, the content of lactic acid is also the highest in upper leaves but the lowest in lower leaves.

TABLE-1
ANALYTICAL RESULTS OF ORGANIC ACIDS IN TOBACCO SAMPLES (mg/g)

Organic acids	Upper				Middle			
	2004	2005	2006	2007	2004	2005	2006	2007
Oxalic acid	15.18	17.29	18.76	16.92	9.18	14.08	14.12	12.54
Tartaric acid	4.19	6.39	7.88	7.50	6.80	10.87	15.23	15.47
Formic acid	2.91	4.67	4.89	4.97	4.56	7.12	6.96	6.66
Malic acid	49.17	54.71	61.35	47.99	63.68	70.88	71.81	69.84
Malonic acid	5.01	9.71	7.26	6.47	5.90	5.36	4.41	4.32
Lactic acid	12.67	14.55	15.84	14.85	11.18	12.55	12.68	11.64
Acetic acid	3.56	7.72	7.48	7.37	7.82	18.91	12.81	10.23
Maleic acid	0.07	0.08	0.24	0.28	0.18	0.28	0.31	0.41
Citric acid	7.93	8.95	12.66	11.70	7.51	8.27	11.72	10.08
Succinic acid	2.42	5.71	6.94	6.21	4.17	8.01	8.43	7.77
Total	103.11	129.78	143.31	124.26	120.98	156.33	158.48	148.96

Organic acids	Lower			
	2004	2005	2006	2007
Oxalic acid	12.78	16.27	14.37	13.50
Tartaric acid	5.31	7.78	9.97	6.63
Formic acid	3.15	4.93	4.15	3.12
Malic acid	38.44	41.82	41.56	37.50
Malonic acid	5.72	5.44	5.41	4.64
Lactic acid	8.23	10.72	11.82	9.77
Acetic acid	6.36	10.17	9.37	8.90
Maleic acid	0.02	0.07	0.06	0.05
Citric acid	7.83	8.79	10.35	8.21
Succinic acid	1.94	3.09	2.88	2.10
Total	89.78	109.08	109.94	94.42

As shown in Table-1 and Fig. 2, the total content of organic acids in different position leaves mostly increased at the beginning of aging. The possible reason was the decomposition of soluble carbohydrate (such as glucose and sucrose), decarboxylation of amino acids and degradation of other compounds led to produce a large amount of organic acids. But the increasing degree was different. For the upper leaves, the total content of organic acids increased from 103.11 to 129.78 mg/g during the first year (the increased rate is 25.87 %) and from 129.78 to 143.31 mg/g (the rate is 10.42 %) during the second year. The total content in middle and lower

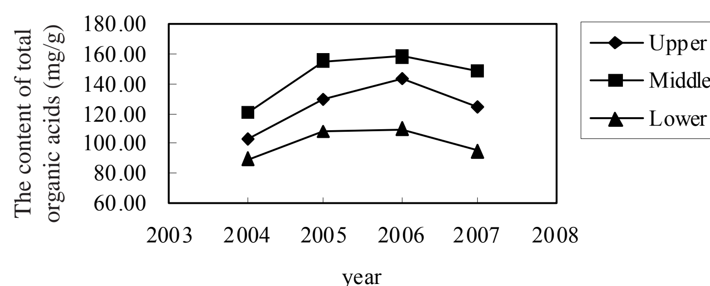

Fig. 2. Variation in the total amounts of organic acis during the process of aging

leaves increased by 29.22 and 21.49 % after one year, respectively. But only increased by 1.38 and 0.79 % during the second year. It is observed that the increasing degree of the content of organic acids is higher during the first year of aging and the increasing degree of volatile organic acids, such as formic acid and acetic acid (account for 75 % of the total volatile acids)[10], significantly exceeded non-volatile organic acids. For the different position leaves of the plant, increasing degree is the highest in middle leaves and the lowest in lower leaves. However, individual organic acid decreased during aging, on the contrary. For example, the content of malonic acid in middle and lower leaves both decreased during the aging time. In addition, at the third year of aging, the total content of organic acids in different position leaves all decreased more or less, which may be due to the decomposition and volatilization of the organic acids.

Conclusion

In conclusion, a sensitive and robust RP-HPLC method has been developed and validated for the detection and quantification of organic acids in tobacco leaf extracts. There are large differences in the contents of organic acids from different tobacco leaf position and different aging time. Malic acid and oxalic acid were the predominant organic acids in all of the samples and the content of maleic acid was the lowest. In addition, the total content of organic acids was the highest in middle leaves and lowest in lower leaves. The total content of organic acids from all tobacco leaf positions had increased at the beginning of aging and the increased degree was the highest in middle leaves and lowest in lower leaves. These differences have to be taken into account when tobacco leaf is selected for the commercial cigarette. During the aging time, the increase of volatile organic acids contributes to over-all leaf aroma and smoke flavour[11], whilst nonvolatile acids can regulate acid-base balance, reduce stimulation and improve smoking quality. Therefore, it is important to use the method described above to control the quality of the raw materials and aging process.

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