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Determination of Organic Acids in Tobacco by Reversed Phase Liquid Chromatography

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A reversed phase liquid chromatography separation method with ultraviolet detection has been developed for the determination of organic acids in tobacco. The gradient mobile phase employed was 20 mM phosphate buffer (pH = 2.6) and acetonitrile at a flow-rate of 1.0 mL/min. All organic acids were eluted in less than 16 min. Target compounds were detected at 210 nm. Limits of detection (LOD) for the 11 acids ranged from 0.0007–1.419 mM and recoveries between 84.2–106.3% were found. Reproducibility (RSD) was better than 2.5% for retention times and between 0.42 and 1.78% for peak areas.

Key Words: Reversed phase liquid chromatography, Organic acid, Tobacco.

INTRODUCTION

Tobacco plants contain a number of organic acids, which are contributors to flavour and aroma, mainly including oxalic, tartaric, acetic, malonic, formic, maleic, malic, lactic, succinic and citric acid. The contents of these acids strongly affect not only the quality of tobacco, but also the balance of the flavour[1]. Meanwhile, the quantities and proportions of organic acids vary with the type of tobacco, ferment duration, aging process, the climate and soil environments, etc. Organic acids have been reported to be very useful in distinguishing different tobacco types[2, 3]. Therefore, reliable determination of organic acids in tobacco is considered to be essential for the classification of tobacco material according to its quality.

Tobacco is an extremely complex mixture of chemicals and analysis of its components is very difficult and laborious sample preparation is frequently required. Quantitative determination of organic acids can be achieved using different analytical methods. Gas chromatography using trimethylsilyl derivatives offers excellent resolution and high detector sensitivity for the measurement of organic acids[3–7]. However, complex extraction steps and tedious derivative process lead to low recovery and long analysis time inevitably. In recent years, CE methods have emerged as powerful tools for analysis of organic acids in tobacco[8], but a highly sensitive detector is necessary, which make it rather difficult for CE become a routine analysis technique. Although ion exchange HPLC has been employed in the determination of organic acids[9, 10], high column temperature, expensive ion-exchange column and poor resolution limit its development.

In this paper, a reversed-phase liquid chromatographic (RP-HPLC) method with UV detector was applied to analyze simultaneously various volatile and non-volatile organic acids, which play an important role in tobacco quality. Simple pretreatment, mild operation conditions, high sensitivity and satisfactory recovery indicate that RP-HPLC is suitable for routine analysis of organic acids in tobacco.

EXPERIMENTAL

Acetonitrile (HPLC-s gradient grade) and methanol (HPLC-s gradient grade) were obtained from Yuwang Industrial & Commercial Co. (Shandong, China). Other chemicals used were of analytical grade, purchased from Chemical Reagent Co. (Shanghai, China). Deionized water (produced with a Millipore Milli-Q water-purification system) was used to prepare all solutions. All standards and samples were filtered through 0.45 μm Millipore filter and the mobile phase solvents were degassed prior to use.

The HPLC apparatus used in this study consisted of a Waters liquid chromatograph equipped with an on-line degasser and a Waters 600 pump. Data were acquired and processed using the Waters Millenium chromatography software. The injection system used was a 20 μL sample loop. Detection was done by a Waters 2487 variable wavelength detector at a wavelength of 210 nm. A Sinnochrom ODS column (4.6 × 200 mm, particle size 5 μm) (Dalian Elite Analytical Instruments Co., Ltd., China) was used for reverse-phase separation.

Chromatographic procedure

The gradient mobile phase consisted of (A) 20 mM KH_2PO_4 adjusted to pH 2.6 with ortho-phosphoric acid, and (B) acetonitrile-water (3 : 2, v/v). The column was eluted with a flow rate of 1 mL/min, starting with 100% of Solvent A for the first 8 min, increasing linearly the percentage of Solvent B to 12% over the next 4 min, and holding at 12% B in A from 12–16 min. 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 column was conditioned with a 20% and then 100% (v/v) acetonitrile-water mobile solvent every fourth sample, in order to fully flush the column of hydrophobic compounds from previous injections. The injection volume is 10 μL. All experiments were performed at an ambient temperature of 25 ± 2°C.

Sample preparation

Tobacco leaves were dried in a drying oven at 60°C for 12 h, ground into powder and then sieved with a 0.25 mm boult. For analysis of organic acids, *ca.* 2 g tobacco powder was weighed into a 125 mL Erlenmeyer flask and 70 mL deionized water added. Extraction was carried out in an ultrasonic bath for 40 min at room temperature. The suspension was filtered under vacuum using a Buchner funnel, and the tobacco residue was rinsed a few times with 2–3 mL deionized water. Then the filtrate was transferred into a 100 mL volumetric flask.

The tobacco extraction was purified through a C_{18} cartridge (500 mg, Supelco, USA) that had been conditioned by washing with 3 mL of methanol and then with 5 mL of deionized water prior to use. After the extraction was loaded to the cartridge, the first 3 mL eluent was discarded and the next 2 mL eluent connected. A 10 μL aliquot of the eluent was injected for HPLC analysis. All values reported are based on peak area.

RESULTS AND DISCUSSION

Optimization of separation conditions

To establish the optimum conditions for the analysis of organic acids by HPLC, flow rate, pH of the buffer, percentage of organic solvent in the buffer have been considered and studied in the following section.

Table-1 illustrates the effect of flow-rate on the separation. Organic acids were eluted earlier with a faster flow-rate. There was no noticeable effect of the resolution varying the flow-rate from 0.5–1.2 mL/min. According to the table, the flow-rate was set at 1.0 mL/min to maintain suitable residence time of analytes.

TABLE-1
THE EFFECT OF FLOW-RATE ON THE RETENTION TIME OF ORGANIC ACIDS

Compound	Flow rate (mL/min)			
	0.5	0.8	1.0	1.2
Oxalic	5.07	3.24	2.55	2.17
Tartaric	6.20	3.95	3.07	2.64
Formic	6.64	4.22	3.31	2.82
Malic	8.71	5.51	4.25	3.67
Malonic	9.40	5.95	4.56	3.96
Lactic	10.60	6.68	5.22	4.47
Acetic	11.45	7.20	5.62	4.81
Maleic	16.54	10.34	7.74	6.81
Citric	20.16	12.48	9.19	8.11
Succinic	22.95	14.20	10.69	9.29
Propionic	27.54	19.44	15.30	13.05

Since pH affects dissociation of organic acids[11–13], a careful manipulation of mobile phase pH can sometimes assist in resolving peaks that are difficult to separate. Table-2 shows the effect of pH on the retention time of the acids. At pH 3.0, a co-elution of malic-malonic was observed. When pH was decreased to 2.3, poor resolution between tartaric-formic was inevitable. Best results were achieved with a pH value of 2.6.

TABLE-2
THE EFFECT OF pH ON THE RETENTION TIME OF ORGANIC ACIDS

Compound	pH		
	2.3	2.6	3.0
Oxalic	2.63	2.55	2.53
Tartaric	3.25	3.07	2.97
Formic	3.37	3.31	3.27
Malic	4.49	4.25	4.17
Malonic	5.08	4.56	4.32
Lactic	5.40	5.22	5.15
Acetic	5.73	5.62	5.66
Maleic	8.45	7.74	8.00
Citric	10.75	9.19	8.53
Succinic	11.24	10.69	10.92
Propionic	15.66	15.30	15.42

To accelerate elution of organic acids, instead of methanol, acetonitrile was used to be addition of organic solvents of buffer, because acetonitrile offered the lower background absorbance at 210 nm. The retention times of samples were dependent on the percentage of water in the mobile phase, increasing with diminishing acetonitrile concentration. It has been demonstrated that the presence of acetonitrile in the mobile phase would change the pKa values of organic acids[11]. Therefore, 20 mM KH_2PO_4 at pH 2.6 was used as the mobile phase for the first 8 min and a composition of 88% KH_2PO_4 and 12% acetonitrile was chosen for following gradient eluent.

Calibration curve, limits of detection and precision

The chromatogram of the standard mixture of organic acids under optimum separation conditions described above is shown in Fig. 1. The separation of analyzed compounds is excellent, even with very high concentrations. The separation of all analytes was achieved in less than 16 min.

A six-point calibration using three replicate injections was obtained. The linearity of assay between peak area *vs.* the concentration and the limits of detection for the 11 organic acids are presented in Table-3. The correlation coefficients determined for the 11 peaks were better than 0.99, indicating a high correlation between the two. For all of these compounds the peak areas varied linearly with the concentration over one order of magnitude range. The detection limits, calculated at a signal-to-noise ratio 3 for these acids, range from 0.0007 mM for maleic acid up to 1.419 mM for propionic acid.

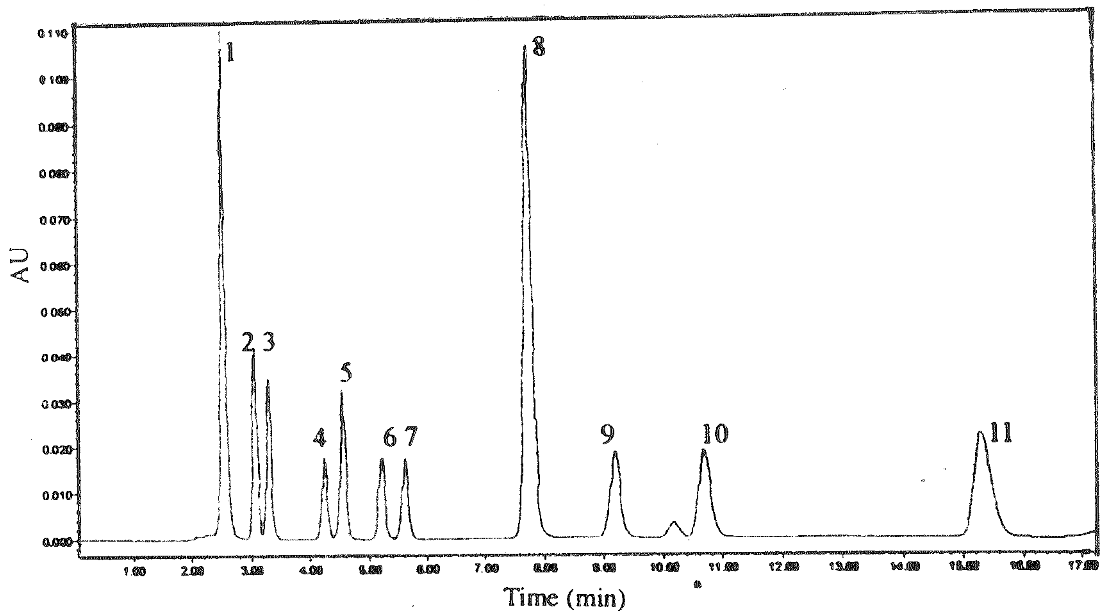

Fig. 1. Representative chromatogram of the organic acids from a standard mixture. Conditions as described under: Experimental peaks: 1 = oxalic (0.634 mM), 2 = tartaric (1.599 mM), 3 = formic (8.696 mM), 4 = malic (2.237 mM), 5 = malonic (3.843 mM), 6 = lactic (5.551 mM), 7 = acetic (6.661 mM), 8 = maleic (0.129 mM), 9 = citric (1.428 mM), 10 = succinic (6.774 mM), 11 = propionic (20.270 mM)

TABLE-3

QUANTITATIVE PARAMETERS OF ORGANIC ACIDS DETERMINED BY RP-HPLC

Compound	Retention time (min)	Calibration formula	Concentration range (mM)	Correlation coefficient (R^2)	Limit of detection (mM)
Oxalic acid	2.54	$y = 6.3740x + 0.1641$	0.127–3.804	0.9983	0.0060
Tartaric acid	3.08	$y = 1.9842x + 0.0516$	0.320–9.594	0.9984	0.0490
Formic acid	3.31	$y = 1.7794x + 0.0408$	1.739–52.176	0.9980	0.0750
Malic acid	4.28	$y = 1.0699x + 0.0323$	0.447–13.422	0.9975	0.2000
Malonic acid	4.61	$y = 2.0041x + 0.0586$	0.769–23.058	0.9972	0.1610
Lactic acid	5.26	$y = 1.2265x + 0.0348$	1.110–33.306	0.9983	0.4720
Acetic acid	5.67	$y = 1.1840x + 0.0294$	1.332–39.966	0.9988	0.5770
Maleic acid	7.86	$y = 10.0386x + 0.1943$	0.0258–0.774	0.9991	0.0007
Citric acid	9.30	$y = 1.9846x + 0.0533$	0.286–8.568	0.9983	0.1180
Succinic acid	10.91	$y = 2.3966x + 0.0438$	1.355–40.644	0.9995	0.5650
Propionic acid	15.52	$y = 4.2584x + 0.1100$	4.054–121.620	0.9987	1.4190

The precisions were expressed as relative standard deviation (RSD) of the eleven analytes based on six consecutive injections of the same mixed standard solution. The result was fairly good because the reproducibility (RSD) of retention times was found to range from 0.42–1.78% ($n = 6$), and the peak areas reproducibility (RSD) are typically less than 2.5% (Table-4). In addition, no differences were observed in the retention time and capacity factors of the analyzed organic acids during several days of operation. It was shown that the quantification and qualification of organic acids could be well done by this RP-HPLC method.

TABLE-4
RESULTS OF THE ANALYSIS OF PRECISION

Compound	Concentration (mM)	R.S.D. of retention time (%)	R.S.D. of peak area (%)
Oxalic acid	0.634	0.90	1.88
Tartaric acid	1.599	0.48	1.18
Formic acid	8.696	0.42	1.74
Malic acid	2.237	0.98	1.49
Malonic acid	3.843	0.69	2.14
Lactic acid	5.551	0.50	0.47
Acetic acid	6.661	0.56	1.08
Maleic acid	0.129	1.75	2.50
Citric acid	1.428	0.90	1.14
Succinic acid	6.774	1.78	0.57
Propionic acid	20.270	1.26	0.89

RSD: relative standard deviation (n = 6).

Application of tobacco samples: Eleven organic acids from tobacco samples were analyzed to demonstrate the practical applicability of the method. The chromatogram of tobacco samples is shown in Fig. 2. Peak identification was based on the retention time of the specified analytes and was confirmed by spiking authentic standard solutions to the sample extracts. As can be seen in Fig. 2, propionic acid was not detected. Other organic acids were separated without interference peaks.

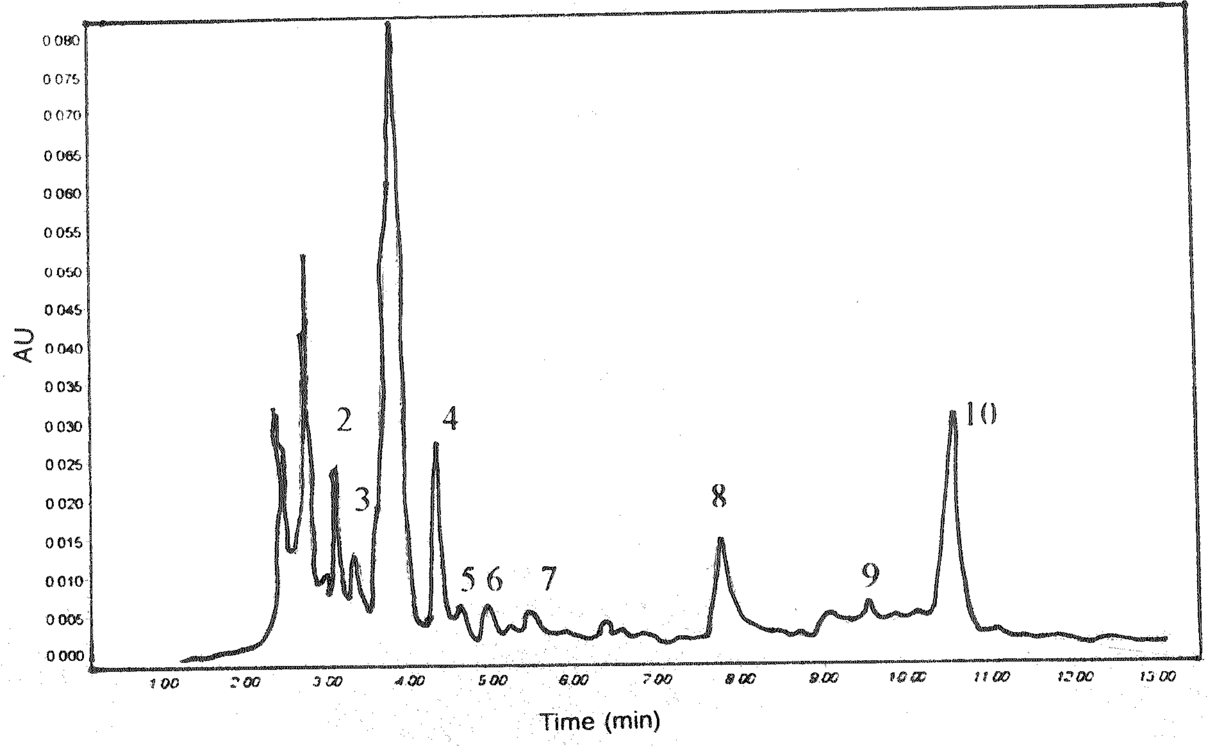

Fig. 2. Chromatograms of tobacco sample. Conditions and peak identification as in Fig. 1

In order to access the validity and applicability of the method, recovery was investigated by analyzing the mixture prepared by adding suitable amounts of standard mixture to tobacco samples with known contents of these target compounds and performing six replicate assays after addition. The recoveries of target compounds are listed in Table-5, ranging from 84.2–106.3%. The higher recoveries (> 100%) were found for the lower amounts of spiked standard. This was probably due to small amounts of co-eluting impurities in tobacco samples, which were more significant at the lower spike levels. These results indicate that the analysis of organic acids in tobacco can be well done by this RP-HPLC method.

TABLE-5
RECOVERY OF ORGANIC ACIDS FROM TOBACCO SAMPLES WITH KNOWN CONCENTRATIONS

Compound	Concentration (mM)			Recovery (%)	RSD (%)
	Initial	Added	Found		
Oxalic acid	0.276	0.317	0.604	103.5	2.4
Tartaric acid	1.448	0.799	2.216	96.1	0.6
Formic acid	7.056	4.348	11.106	93.1	0.5
Malic acid	4.439	1.118	5.432	88.8	1.3
Malonic acid	0.923	1.921	2.686	91.8	2.1
Lactic acid	2.458	2.776	4.883	87.4	3.5
Acetic acid	0.643	3.331	3.756	93.4	1.9
Maleic acid	0.028	0.064	0.096	106.3	5.8
Citric acid	0.638	0.714	1.239	84.2	3.1
Succinic acid	0.556	3.387	3.833	96.8	0.7
Propionic acid	0	10.135	10.274	101.4	0.2

RSD: relative standard deviation (n = 6)

Conclusions

A highly sensitive, reproducible and selective determination of organic acids of tobacco samples was performed using RP-HPLC with a UV detector. Compared with gas chromatographic methods and other currently available methods for the determination of organic acids in tobacco, the present RP-HPLC method appears to be more useful without complicated extraction or derivatization procedures and offer the most rapid, reliable and effective approach for routine analysis under optimum chromatographic conditions.

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