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Development and optimization of organic acid analysis in tobacco with ion chromatography and suppressed conductivity detection

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Abstract

With the aid of a central composite face-centered design, an ion chromatographic method was developed and optimized for analyzing organic acids in tobacco. A Dionex-100 ion chromatograph with an ion suppressor and a conductivity detector, and a Bio-Rad Aminex HPX-87H column were employed. Only 13 analyses were required to optimize two factors: column temperature and eluent strength. Two sets of optimal conditions for separating nine acids were found: 1.8 m*M* HFBA eluent and 42 °C column temperature, and 0.8 m*M* HFBA eluent and 50 °C column temperature. The flow-rate was 0.6 ml min^{-1} and the analysis time was 18 min or less. A sample preparation procedure included extraction of 2 g ground tobacco with 100 ml of 5 m*M* sulfuric acid solution for 3 h, filtration of the extract, and dilution of the filtrate 10-fold with deionized water. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Tobacco; Conductivity detection; Central composite face-centered design; Organic acids

1. Introduction

Organic acids are major components present in both green tobacco plants and barn-cured tobacco. The major carboxylic acids in tobacco include citric, malic, oxalic and malonic, and in total consist of 5–18% of barn-cured tobacco weight, depending on tobacco types [1]. Carbohydrates and organic acids together with cultivars, cultural practices, and curing are of great importance in differentiating major tobacco types [2]. Organic acids are known to affect leaf quality [3]. Because of their importance, considerable effort has been made to quantify organic acids in tobacco and its products with different instrumental procedures [4–7].

Organic acids are commonly separated by an ion-exclusion column in a high-performance liquid chromatograph and detected by either an ultraviolet (UV) detector or a refractive index (RI) detector. In ion-exclusion chromatography (IEC), a high capacity sulfonated styrene divinylbenzene resin in the H^+ form is typically used and an eluent usually contains a strong or weak acid. An acidic eluent suppresses the dissociation of organic acids so that their retention on the resin matrix increases. The elution of organic acids is approximately in the order of ascending pK_a value, but other variables, for instance, additional hydrogen bonding and adsorption, and hydrophobicity of the acids, may modify the elution order slightly [8,9]. Small molecules, such as

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soluble sugars, propylene glycol, have retention times similar to those of citric, malonic and malic on an ion-exclusion column. Levels of reducing sugars such as dextrose can be as high as 22% in flue-cured tobacco [1] and a considerable amount of propylene glycol is added as a humectant in cigarettes. Those compounds tend to interfere with the separation of the above organic acids. Since an RI detector has poor selectivity and low sensitivity, it is difficult to quantify organic acids in flue-cured tobacco with high reducing sugars [4]. Some organic acids have very weak absorbance at the low end of the UV spectral region and the UV detection at this region is very sensitive to other unknown interfering organic compounds in tobacco [10,11]. Sample clean-up procedures are required in both RI and UV detection [4,11].

For detection of organic acids, a conductivity detector would provide greater sensitivity than a UV or RI detector and eliminate the interferences from soluble sugars and humectants in tobacco and its products that appear in both UV and RI detectors. Many studies have recently been conducted on organic acid analysis by ion chromatography (IC) with conductivity detection. Uses of various weak acids (e.g. succinic acid), aromatic acids (e.g. phthalic acid), and sugars with alcohols (e.g. sucrose with methanol) as eluents have been examined for separating aliphatic carboxylic acids by IEC with non-suppressed conductivity detection [12–14]. Those aqueous eluents provide a reasonable separation and highly sensitive detection only for some carboxylic acids. Post-column buffering after IEC was employed to promote dissociation of organic acids and enhance sensitivity of conductivity detection [10]. One of the drawbacks is that the system requires a second pump. Organic acids in fresh or fermented cucumbers were separated successfully with an ion exclusion column and suppressed conductivity detection [15]. However, the above methods cannot be applied directly to determine organic acids in tobacco because it has a different composition of organic acids and a complex matrix. In this study, we have employed a statistical design to develop and optimize an IC procedure of organic acid analysis with suppressed conductivity detection for tobacco samples.

2. Experimental

2.1. Instrumentation

The instrumental equipment consisted of a Dionex (CA, USA) DX-100 integrated ion chromatography system with an attached conductivity detector and a 25-μl loop, a Dionex anion-ICE micromembrane suppressor (AMMS-ICE II), a Dionex pressurizable eluent reservoir pressured with helium, a Dionex pressurizable reservoir pressured with helium for delivery of suppressor solution, a Dionex automated sampler, a Bio-Rad (CA, USA) Aminex HPX-87H column (300 mm×7.8 mm I.D.), a Bio-Rad guard column (30 mm×4.6 mm I.D.), and a Precision Scientific (IL, USA) water bath for heating the analytical column. The analytical column was packed with sulfonated styrene divinylbenzene resin in the H^+ form (9 μm particle size). Dionex's AI-450 chromatography software and a personal computer were used for instrument control, data collection, and data analysis.

2.2. Operating conditions

Eluents containing sulfuric, hydrochloric and heptafluorobutyric (HFBA) acids were tested for efficiency of separation. It was found that all three acids with the same normality had very similar capability to separate organic acids, but different background conductivities. Sulfuric acid had the highest background conductivity while HFBA had the lowest. Therefore, the latter was used in all the experiments. Two factors, eluent strength and column temperature, were optimized. The flow-rate was set at 0.6 ml min^{-1}. The AMMS-ICE II suppressor was connected between the analytical column and the detector. A 2 ml min^{-1} counter-current flow of the suppressor solution, containing 5 m*M* tetrabutylammonium hydroxide, passed through the suppressor to reduce the background conductivity of the eluent. The conductivity detector was set at 30 μS full-scale.

2.3. Standard solution and sample preparation

The chemicals for the standard solutions were

purchased from Sigma (MO, USA). Deionized water was obtained with a water purification system from Millipore and the specific resistance of water was close to 18 MΩ cm. The highest concentration of the standard mixture, dissolved in 5 m*M* sulfuric acid solution, was 0.3 m*M* phosphoric, 0.5 m*M* citric, 0.2 m*M* malonic, 0.7 m*M* malic, 0.2 m*M* succinic, 0.8 m*M* lactic, 0.5 m*M* formic, 2.7 m*M* acetic and 0.3 m*M* pyroglutamic. The lowest concentration of the standard mixture was a 10-fold dilution of the highest. The standard mixture with 50% of the highest was used to optimize the separation of organic acids.

For the preparation of tobacco samples, approximately 2 g ground dry tobacco was weighed into a 125-ml Erlenmeyer flask, and 100 ml of an appropriate solution added. After adding a stirring bar, the flask was placed on a Lab-Line Multi-Magnestir stirrer for extraction. Optimal conditions of an extraction time and an extraction solution were determined and the details described in Section 3.2. The extract was filtered through a Gelman Acrodisc 25-mm syringe filter with 0.45-μm GHP membrane (MI, USA). The filtrate was diluted 10-fold with deionized water and a 25-μl aliquot of the diluted solution injected into the IC for analysis.

2.4. Experimental design

A central composite face-centered (CCF) design, which is a cubic response surface methodology design, was used for optimizing the separation factors. The factor end-points define the vertices of the cube and the axial points are in the middle of all the cube's faces. The resulting design has good prediction variance inside the volume of the cube. In the design, the column temperature ranged from 25 to 50 °C with a middle point of 37.5 °C and the eluent strength varied from 0.4 to 2.2 m*M* HFBA solutions with a middle point of 1.3 m*M* HFBA. The complete CCF experiment with two factors required only nine individual analyses plus four replicates (Table 1). The experimental design for the studies on the recovery of organic acids, sample extraction time, and the acid concentration of an extraction solution was a randomized complete block design with three replications.

Table 1
The resulting experiment of a central composite face-centered design for two factors

Analysis	Column temperature (°C)	HFBA in eluent (m*M*)
1	37.5	1.3
2	25.0	2.2
3	37.5	0.4
4	37.5	1.3
5	50.0	1.3
6	37.5	1.3
7	37.5	1.3
8	50.0	2.2
9	50.0	0.4
10	37.5	1.3
11	25.0	0.4
12	25.0	1.3
13	37.5	2.2

3. Results and discussion

3.1. Optimization of separation

3.1.1. Effect on retention time

The column temperature and the eluent strength have been shown to be two major factors influencing the separation of organic acids with IEC and refractive index detection [4]. By applying a CCF design, optimal analytical conditions were obtained with only a few HPLC analyses. In this study, the same CCF design was employed to identify optimal column temperature and eluent strength for IEC and suppressed conductivity detection. Phosphoric, citric, malonic, malic, succinic, lactic, formic, acetic, and pyroglutamic acids were found to be present in tobacco samples and all of them were included in the optimization procedure. Oxalic acid, a predominant compound in tobacco, could not be separated from the front peak within the eluent acid concentrations used in the present study.

Effects of column temperatures and eluent strengths on the retention times of organic acids are shown in Fig. 1. At all three temperatures, the retention times of all the organic acids increased as the eluent acid concentration increased from 0.4 to 2.2 m*M*. The magnitude of the increases was in the following descending order: pyroglutamic> malonic>formic>malic>citric, lactic, succinic>

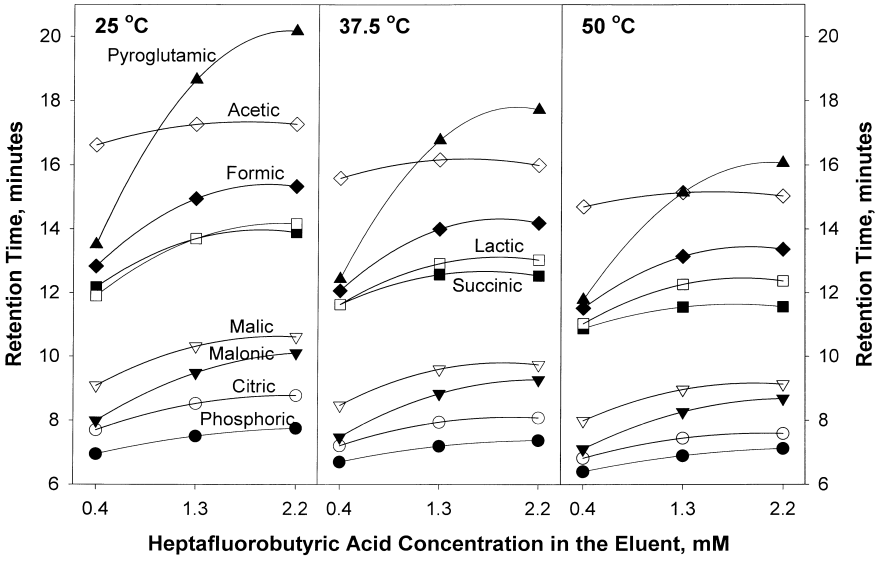

Fig. 1. The effect of column temperature and eluent strength on the retention times of organic acids.

acetic>phosphoric. The retention times at all three eluent strengths decreased when the column temperature increased from 25 to 50 °C and the magnitude of the decreases was in the following descending order: pyroglutamic>succinic>malic, malonic, citric, acetic>lactic>phosphoric. The responses of the retention times of all the acids to the changes in two factors were not linear, which was somewhat different from those at higher acid concentrations of the eluent [4]. Three points were fitted into a second-order polynomial (Fig. 1).

It can be seen from Fig. 1 that the resolutions between three pairs of peaks (malonic and malic, succinic and lactic, and acetic and pyroglutamic) were critical for choosing a column temperature and eluent strength to be fine-tuned. At 25 °C, all the acids except lactic and succinic were separated very well. At 37.5 and 50 °C, lactic and succinic started to be separated at two high acid concentrations and the other six acids tended to be separated better at the high acid concentrations. Since the retention time of pyroglutamic acid varied greatly in the response to the changes in the two factors, two sets of conditions for separating all the organic acids of interest between 37.5 and 50 °C were observed: one around 0.8 m*M* HFBA and 48 °C, and the other around 1.8 m*M* HFBA and 44 °C. Starting from those points, the factors were fine-tuned and chromatograms with fine-tuned parameters shown in Fig. 2. It can be seen from Fig. 2 that an optimal separation was obtained with an eluent of 1.8 m*M* HFBA solution and a column temperature of 42 °C or with an eluent of 0.8 m*M* HFBA solution and a column temperature of 50 °C. Most acids were separated at the baseline under both the conditions.

3.1.2. Effect on peak area

Due to partial peak merging at various column temperatures and eluent strengths, many peaks could not be integrated. The peak areas of the two optimal conditions were compared and the ratio of the peak areas of their corresponding acids at 1.8 m*M* HFBA and 42 °C to 0.8 m*M* HFBA and 50 °C shown in Table 2. The peak areas of only malonic and malic were slightly lower in the former than in the latter. This suggests that the sensitivity would be higher at a higher eluent acid concentration and a lower column temperature than at a lower eluent acid concentration and a higher column temperature. Therefore, the former condition would be preferable.

3.2. Extraction of organic acids

An aqueous solution of sulfuric acid is often used

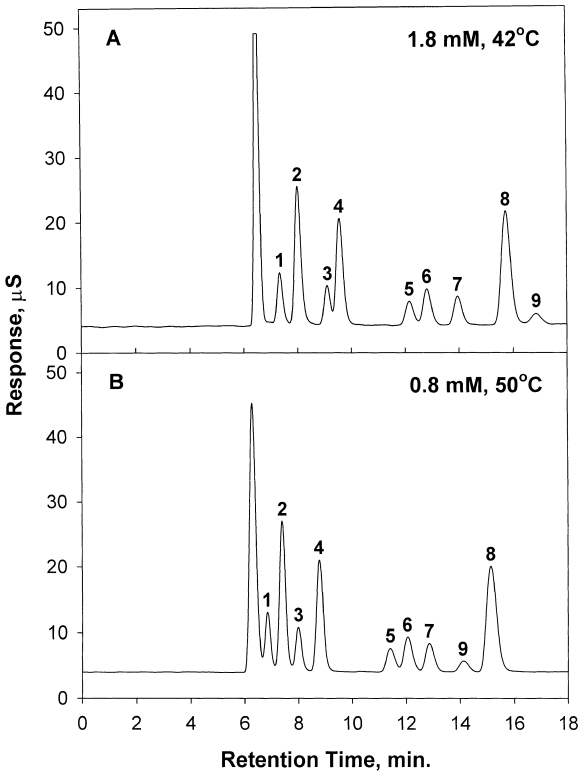

Fig. 2. Chromatograms of a standard mixture run at the optimal condition of 1.8 m*M* HFBA and 42 °C (Panel A) or 0.8 m*M* HFBA and 50 °C (Panel B). The acids were phosphoric (1), citric (2), malonic (3), malic (4), succinic (5), lactic (6), formic (7), acetic (8), and pyroglutamic (9).

to extract organic acids in plants. However, a high sulfuric acid concentration would increase the size of the front peak, thereby affecting the separation and integration of phosphoric and citric peaks in an IC system. An attempt was made to identify suitable extraction conditions for the current IC procedure. All the following treatments were replicated three times. In an extraction solution study, five concentrations of sulfuric acid solutions were 0 (DI water), 5, 50, 250, and 500 m*M*, and tobacco samples were extracted for 3 h after adding the solutions. Overall, the DI water and 5 m*M* sulfuric acid solution appeared to be the best for the extraction of all the acids (Fig. 3A). Since an acidic extract could be kept longer than a water extract, the 5 m*M* acid solution was selected.

Table 2
The ratio of the peak areas run at 1.8 m*M* HFBA and 42 °C to those run at 0.8 m*M* HFBA and 50 °C

Acid	Ratio of peak areas
Phosphoric	1.18
Citric	1.01
Malonic	0.93
Malic	0.99
Succinic	1.07
Lactic	1.08
Formic	1.04
Acetic	1.00
Pyroglutamic	1.06

Data were calculated from Fig. 2.

It is unclear whether the organic acids extracted with the 5 m*M* sulfuric solution include both free and bound molecules. Since similar acid levels were found in both the water extract and 5 m*M* sulfuric acid extract (Fig. 3A), it is suggested that only free acids were extracted. In our separate studies, however, it was found that the phosphoric acid level in tobacco samples with pH greater than 6.5 was five times higher in a 5 m*M* sulfuric acid extract than in a water extract (data not shown), indicating bound phosphate became free and was extracted. Nevertheless, the dilute acid at the 5 m*M* level was necessary to obtain consistent results.

In a sample extraction time study, the five time periods were 0.5, 1, 2, 4, and 16 h, and the extraction solution was 5 m*M* sulfuric acid solution. The concentrations of succinic, lactic and acetic acids were significantly higher ($P<0.05$) when the samples were extracted for 2 h or more than for 1 h or less, whereas, those of the other acids were similar at almost all five extraction periods (Fig. 3B). Apparently, it would be sufficient to extract samples for 2–4 h.

3.3. Linearity of calibration curves, reproducibility, LOD and recovery

A calibration curve for each acid was calculated by regressing the peak area against the corresponding acid concentration from five standards in triplicate. The calibration curve for each acid was linear and the determination coefficients ranged from 0.999 to 1.000 (Table 3). The reproducibility, calculated from five injections of 50% of the highest standard mixture and expressed as relative standard deviation,

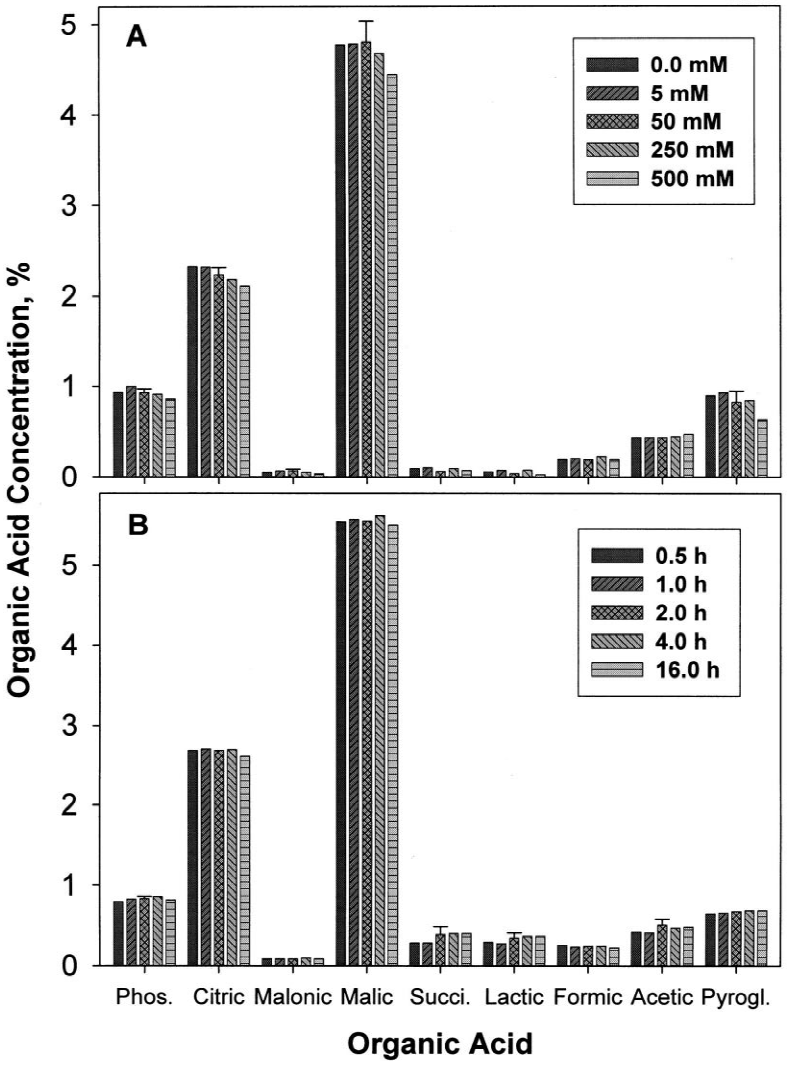

Fig. 3. Effects of sulfuric acid concentrations in the extraction solutions (Panel A) and extraction time periods (Panel B) on the extractability of organic acids in tobacco samples. The bar on the top of the middle column is the value of least significant difference at the 0.05 significance level.

ranged from 0.5 to 2.3%. The limits of detection (signal-to-noise ratio=3) were obtained from the calibration curves and their values were between 5 and 30 μM (Table 3).

The recovery study was conducted by adding 5 ml of 5 mM H_2SO_4 solution (containing 7.0 mg phosphoric, 24.8 mg citric, 6.2 mg malonic, 25.0 mg malic, 6.0 mg succinic, 18.2 mg lactic, 5.8 mg formic, 40.1 mg acetic, and 10.1 mg pyroglutamic) to dry ground tobacco (2 g) with three replications. The samples were mixed completely and set on the laboratory bench overnight. The samples were then extracted with 100 ml of 5 mM H_2SO_4 solution for 3 h. After filtration and dilution, the aliquots were injected. The recovery of each compound was calculated from dividing the determined amount by the

Table 3
Analytical characteristics at 1.8 m*M* HFBA and 42 °C

Acid	t_R (min)	r^2 of calib. curve	RSD (%) (n=5)	LOD μ*M*	Recovery (%) (x±SD)
Phosphoric	7.34	0.999	0.7	11.2	104±4
Citric	8.01	1.000	0.5	4.6	100±5
Malonic	9.10	0.999	2.1	6.4	97±5
Malic	9.56	1.000	0.5	8.1	99±4
Succinic	12.15	0.999	1.3	7.6	100±6
Lactic	12.81	1.000	1.2	21.9	102±4
Formic	13.95	1.000	1.0	15.6	104±4
Acetic	15.73	1.000	0.5	29.7	102±3
Pyroglutamic	16.85	0.999	2.3	16.7	99±2

added amount and multiplying by 100%. The average recoveries of nine acids, expressed as the mean of three replications±SD, varied from 97 to 104% with a standard deviation ranging from 2 to 6% (Table 3).

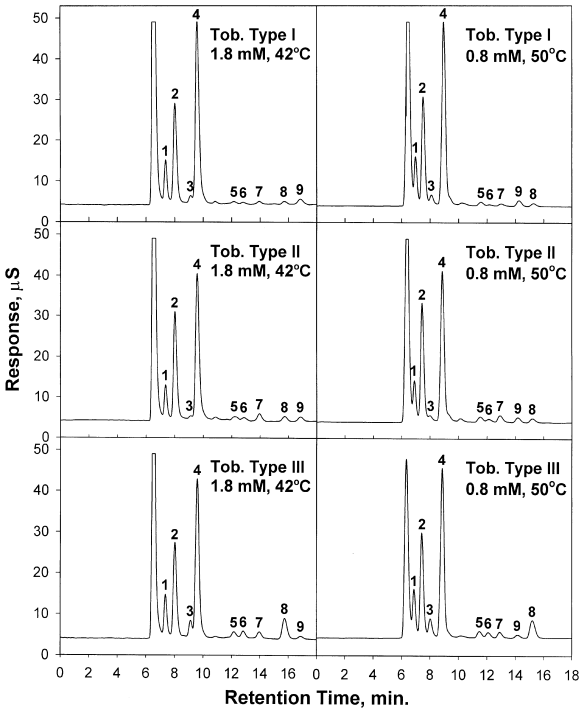

Fig. 4. Chromatograms of three types of tobacco samples run at 1.8 m*M* HFBA and 42 °C (left column), and at 0.8 m*M* HFBA and 50 °C (right column). The acids were phosphoric (1), citric (2), malonic (3), malic (4), succinic (5), lactic (6), formic (7), acetic (8), and pyroglutamic (9).

3.4. Analysis of organic acids in three types of tobacco

The current method was used to determine the organic acids in three types of tobacco. The samples were extracted with 5.0 m*M* sulfuric acid solution for 3 h. The extracts were filtered through Gelman filters. The filtrates were diluted 10-fold with deionized water before injection. Typical chromatograms are shown on the left column in Fig. 4 operated at 1.8 m*M* HFBA and 42 °C and on the right column at 0.8 m*M* HFBA and 50 °C. Superb separation for all the organic acids of interest was obtained.

The organic acids in Tobacco Type III were analyzed with two sets of the optimal conditions and the results shown in Table 4 for comparison. Analysis of variance indicated that the concentrations of organic acids obtained with the two conditions were not statistically different at the 0.05 significance level. Therefore, both sets of analytical conditions were suitable.

4. Conclusions

An IC method with suppressed conductivity detection for analyzing organic acids in tobacco was developed and optimized with the aid of a CCF design. The optimal conditions were found at 1.8 m*M* HFBA and 42 °C, or 0.8 m*M* HFBA and 50 °C.

Table 4
Comparison of organic acids in Tobacco Type III determined by the two optimal conditions

Acid	1.8 m*M* and 42 °C (%)	0.8 m*M* and 50 °C (%)
Phosphoric	0.78±0.02	0.76±0.01
Citric	2.64±0.07	2.69±0.02
Malonic	0.39±0.01	0.40±0.01
Malic	5.94±0.13	6.10±0.08
Succinic	0.24±0.01	0.27±0.02
Lactic	0.54±0.02	0.58±0.02
Formic	0.21±0.01	0.21±0.01
Acetic	1.05±0.02	1.01±0.01
Pyroglutamic	0.42±0.03	0.45±0.01

Analysis of variance indicated that there was no statistical difference in the acid concentrations between the two optimal conditions at the 0.05 significance level.

For higher sensitivity, the former condition was preferable. The standard mixture should be dissolved in 1.0 m*M* sulfuric acid solution in the former and in 0.5 m*M* sulfuric acid solution in the latter to prevent a negative peak. The flow-rate was 0.6 ml min^{-1} and a typical analysis was completed in less than 18 min. The sample preparation was minimal. Dry ground tobacco samples (2 g) were mixed with 100 ml of 5 m*M* sulfuric acid solution in a 125-ml Erlenmeyer flask and extracted with a magnetic stirrer for 3 h. The extract was filtered with a Gelman filter and diluted 10-fold with DI water before injection. The IC method not only reduces the cost and time of sample preparation, but also extends the lifespan of the analytical column [15]. In order to maintain the resolution of the analytical column, the guard column needs to be changed after analyzing approximately 200 samples.

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