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Statistics aided optimization for high-performance liquid chromatographic analysis of organic acids in tobacco

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Abstract

HPLC analysis for organic acids in tobacco was optimized with the aid of statistical experimental design, a central composite face-centered design. In the design, only thirteen HPLC analyses were needed for identifying two optimal separation parameters. A Bio-Rad Aminex HPX-87H column was used for the analyses. An optimal separation for seven acids in tobacco was found at a temperature of 57°C and a mobile phase of 0.032 N sulfuric acid solution, or at a temperature of 70°C and a mobile phase of 0.024 N sulfuric acid solution, with a flow rate of 0.6 ml min^{-1}. © 1999 Elsevier Science B.V. All rights reserved.

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1. Introduction

Organic acids are present in both green tobacco plants and cured leaves, and the major acids in tobacco are citric, malic, oxalic and acetic [1]. Their concentrations and distribution vary with tobacco types, stalk positions and amount of NO_3^- in the fertilizer [2]. Organic acids are actively involved in the TCA cycle and other metabolic mechanisms in green tobacco plants [1]. Non-volatile carboxylic acids, especially malic, citric, oxalic and maleic, have a negative impact on smoking taste, while volatile organic acids, such as formic, acetic, propionic, and isobutyric, are important contributors to flavor and aroma [3–5]. It is a common interest to determine organic acids in tobacco accurately.

Early chromatographic determinations of organic acids included partition chromatography on silica gel columns with gradient elution [6], gas chromatography of the trimethylsilyl derivatives [7], and an anion exchange separation of organic acid mixture [8]. These methods, however, had low recovery of the acids, incomplete separation and long analysis time. More recently many analytical procedures have been developed for analyses of organic acids in food products [9,10]. However, they cannot be directly applied to determine acids in tobacco because of complexity of its matrix. An analytical procedure was reported for determining water-soluble anions in tobacco and related solid matrices [11]. Only three organic acids including malate, hydrogen tartrate, and oxalate could be determined. A quantitative method was developed to analyze some tobacco anions by eluent suppressed anion exchange chromatography [12]. The method had limitations in its precision and accuracy.

A high-capacity cation-exchange resin in the H^+

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form is commonly used in ion-exclusion chromatography for separating organic and inorganic weak acids [13]. An aqueous solution of a strong acid such as sulfuric acid is generally used as a mobile phase for the separation. The sample acids are suppressed and they are entirely in their molecular forms. The elution of organic acids is in approximately the order of ascending pKa value, but other variables such as additional hydrogen bonding and adsorption may modify the elution order slightly [14]. The Bio-Rad Aminex HPX-87H column is a widely used column of this type [15]. With this column we often encounter difficulties in the separation of oxalic acid from the front peak (the extraction solvent), phosphoric acid from citric acid, and malic acid from other interference in tobacco. The present study intended to optimize HPLC separation of seven acids with the aid of experimental design.

2. Experimental

2.1. Instrumentation

A Waters' HPLC system was employed in the experiment. The system consisted of a 600E multisolvent delivery pump with a column heater, a 717 plus autosampler, and a 410 differential refractometer. An Aminex HPX-87H column (300 mm×7.8 mm I.D.) and a guard column (30 mm×4.6 mm I.D., Bio-Rad, CA, USA) were packed with sulfonated divinylbenzene–styrene copolymer resin of 9 μm particle size. The data system consisted of an 80486-CPU personal computer, Waters' Millennium Chromatography Manager software and an ink-jet printer.

2.2. Main parameters influencing separation

In liquid chromatography, a fundamental relationship of resolution R_s for a pair of adjacent peaks can be expressed as

$$R_s = \frac{1}{4}\sqrt{N}(\alpha - 1)\left(\frac{k'}{1+k'}\right)$$

where N is theoretical plate number, α is selectivity, and k' is capacity factor. The equation shows that the peak resolution can be improved by increasing α, N and/or k' value. Since the stationary phase of the column is selected as a fixed parameter, the most powerful parameters for increasing the resolution value may be the solvent strengths of the mobile phase and separation temperatures. These two parameters were included in the experimental design. The flow rate of the mobile phase is another minor parameter influencing the resolutions and it was examined separately.

2.3. Experimental design

A central composite face-centered design (CCF) was employed and generated by the RS/Discover software (BBN Software Products, Massachusetts, USA). Two factors included in the design were separation temperatures ranging from 25°C to 70°C and solvent strengths from 0.008 N to 0.032 N sulfuric acid solutions. The resultant experiment of the design is shown in Table 1. The complete CCF experiment with two factors required only a total of 13 HPLC analyses, which consisted of 9 individual analyses plus 4 replicates.

2.4. Standard solution and sample preparations

The standard organic acid solution contained 0.056% oxalic acid (dihydrate), 0.05% citric acid (monohydrate), 0.10% malic acid, 0.04% lactic acid (lithium salt, 98%), 0.015% pyroglutamic acid,

Table 1
The resultant experiment of a central composite face-centered design

Run	Temperature	Solvent strength
1	48°C	0.020 N
2	25°C	0.032 N
3	48°C	0.008 N
4	48°C	0.020 N
5	70°C	0.020 N
6	48°C	0.020 N
7	48°C	0.020 N
8	70°C	0.032 N
9	70°C	0.008 N
10	48°C	0.020 N
11	25°C	0.008 N
12	25°C	0.020 N
13	48°C	0.032 N

0.03% monosodium phosphate (monohydrate), 0.10% acetic acid and 0.18% sulfuric acid. All the chemicals were purchased from Sigma Chemical Company (Missouri, USA).

For sample preparation, approximately 2.5 g ground dry tobacco was weighed into a 125-ml Erlenmeyer flask and 100 ml 1 N H_2SO_4 extraction solution added. The sample was extracted with a magnetic stir bar on a Lab-line multi-magnestir for 3 h. The extract was then filtered through a Whatman #1 filter paper (125 mm in diameter) and the first 20 ml filtrate collected. The sample was then cleaned up with a Sep-Pak C_{18} cartridge (500 mg, Waters) and a BondElut SCX cartridge (500 mg, Varian Associates, California, USA) in combination with a Baker spe-12G vacuum manifold. The C_{18} cartridge was attached to the top of the SCX cartridge using an adapter. The cartridge assembly was conditioned with 3 ml methanol followed by 5 ml distilled water. Approximately 6 ml of the filtrate was loaded to the wet cartridge assembly and passed through it into a test tube under vacuum. The first 3 ml eluent was discarded and the next 2 ml eluent collected. The sample was filtered through a 0.45-μm GHP Acrodisc (Gelman Sciences, Michigan, USA) into a 1-ml vial and loaded onto the autosampler. A 20-μl aliquot of the standard solution or the sample was injected for HPLC analysis.

2.5. HPLC analysis of organic acids

HPLC analyses were carried out according to the random sequence of the experimental design generated by the computer software. Each of 13 analyses was made after the HPLC system was equilibrated approximately 2 h at a specified temperature and a solvent strength. The analyses were performed at a flow rate of 0.6 ml min^{-1} using the standard organic acid solution described above. The retention times with respect to temperatures and solvent strengths were plotted. From the figure, separation temperatures and solvent strengths of maximum resolution of the poorest resolved peak-pairs were identified for

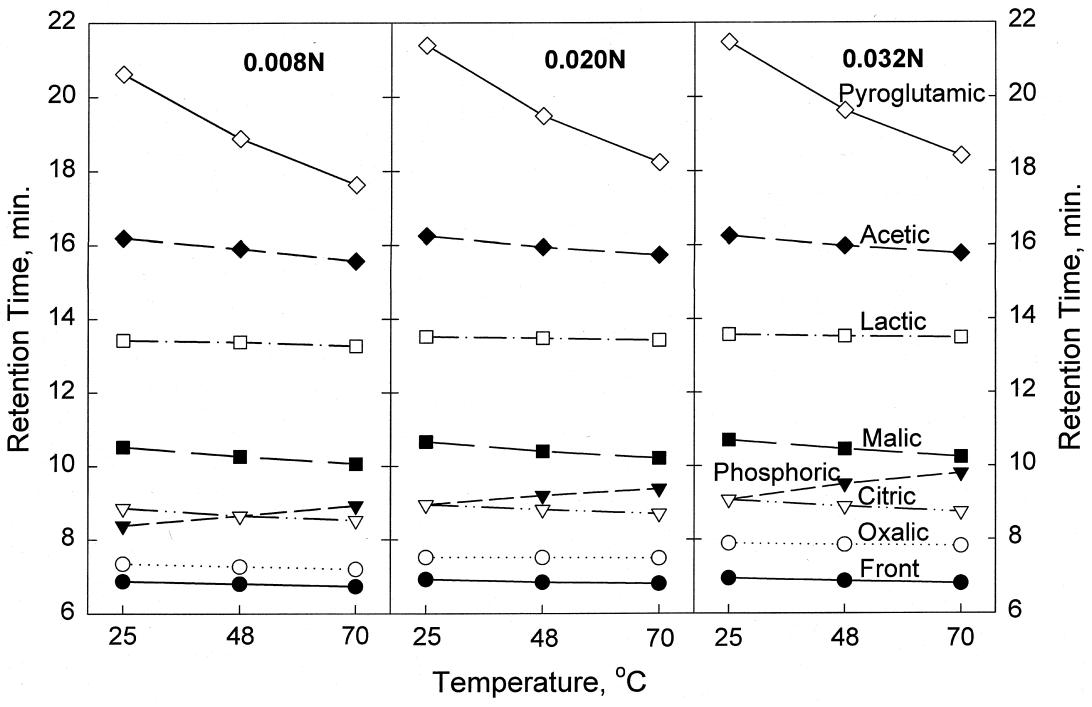

Fig. 1. Effects of separation temperatures and solvent strengths on the retention times and resolutions of organic acids.

fine-tuning. The optimized separation parameters were then validated by tobacco samples.

3. Results and discussion

3.1. Effect on retention time and resolution

An increase in the separation temperature increased the retention time of phosphoric acid, but reduced those of all the other components at all the solvent strengths (Fig. 1). The degree of temperature effect on the retention times varied with individual components and the greatest effect was found on those of phosphoric and pyroglutamic acids. The opposite responses of phosphoric acid from other organic acids may be associated with changes in pK values at increased temperatures. When the temperature is raised from 0°C to 50°C, the pK value increases in phosphoric acid, decreases in citric acid and remains unchanged in acetic and lactic acids [16]. An increase in the pK value of phosphoric acid at a higher temperature resulted in a longer retention time [14].

At all three separation temperatures the retention times of all acid peaks including the front peak increased with increases in the acid concentrations in the mobile phase (Fig. 1). Changes in the solvent strengths had a slightly greater effect on the retention times of oxalic, phosphoric and pyroglutamic acids

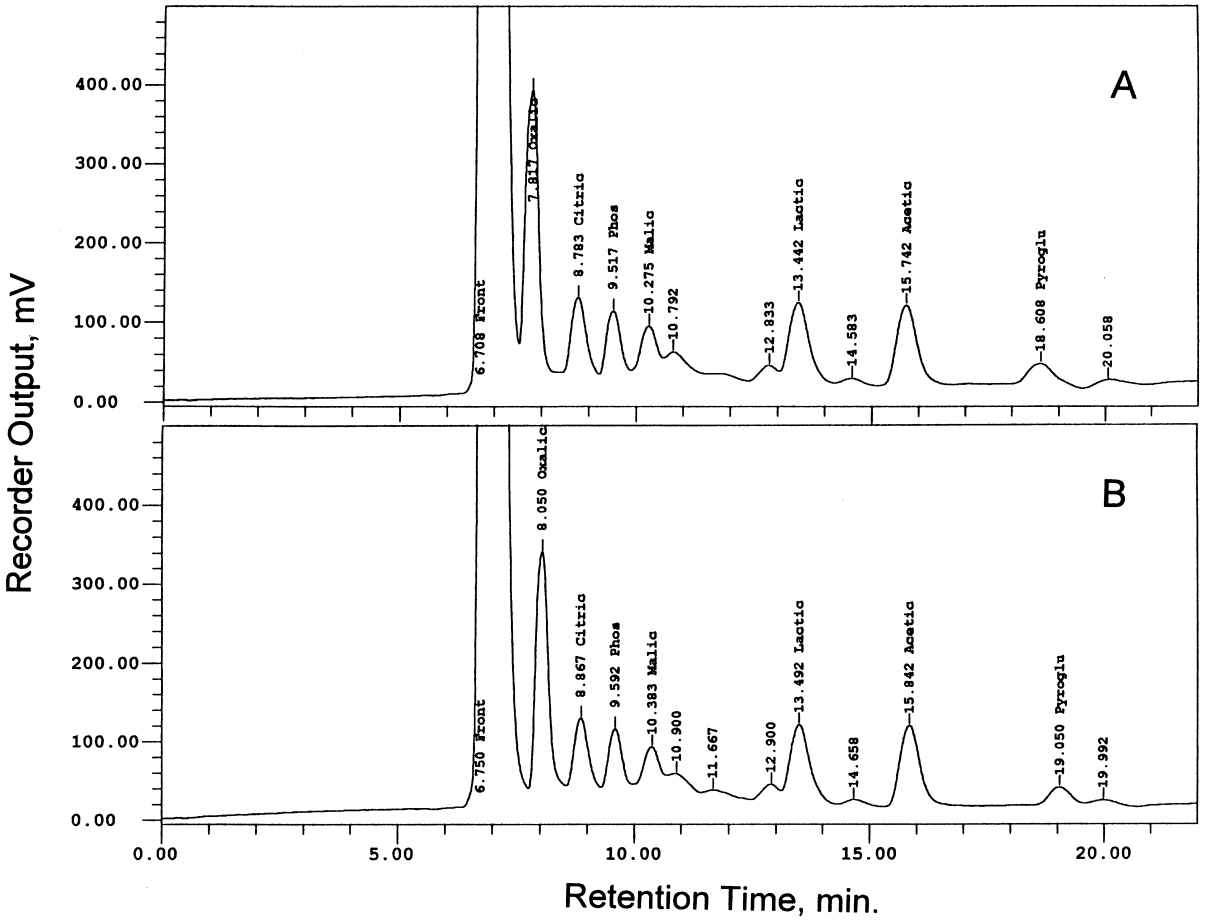

Fig. 2. Optimized chromatograms of organic acids in tobacco run at 70°C and 0.024 N sulfuric acid mobile phase (Panel A), and at 57°C and 0.032 N sulfuric acid mobile phase (Panel B).

Table 2
Effect of flow rates on the resolutions of three pairs of peaks at 70°C and 0.024 N

Flow rate	Resolution		
(ml/min)	Front and oxalic	Citric and phosphoric	Phosphoric and malic
0.6	1.212	1.038	1.165
0.7	1.162	1.021	1.110
0.8	1.159	0.925	1.014

than on those of the other acids. Compared to the separation temperature, the solvent strength had less effect on the retention times of all acids.

At 0.020 N and 0.032 N levels, the resolutions between the front and oxalic peaks and between the citric and phosphoric peaks increased as the temperatures increased from 25°C to 70°C (Fig. 1). At the same temperature an increase in the acid level resulted in increased resolution between the front and oxalic peaks. Therefore, a good separation could be obtained at a higher temperature and a higher acid level.

3.2. Optimal temperature, solvent strength and flow rate

A cursory examination of Fig. 1 indicates two separations with maximum resolution of the poorest resolved pairs of peaks at 70°C and 0.020 N plus or at near 58°C and 0.032 N. From here the parameters were fine-tuned and the optimal separations achieved. Fig. 2 shows two optimized chromatograms of organic acids in tobacco separated at a temperature of 70°C with a mobile phase of 0.024 N sulfuric acid solution (Panel A), or at a temperature

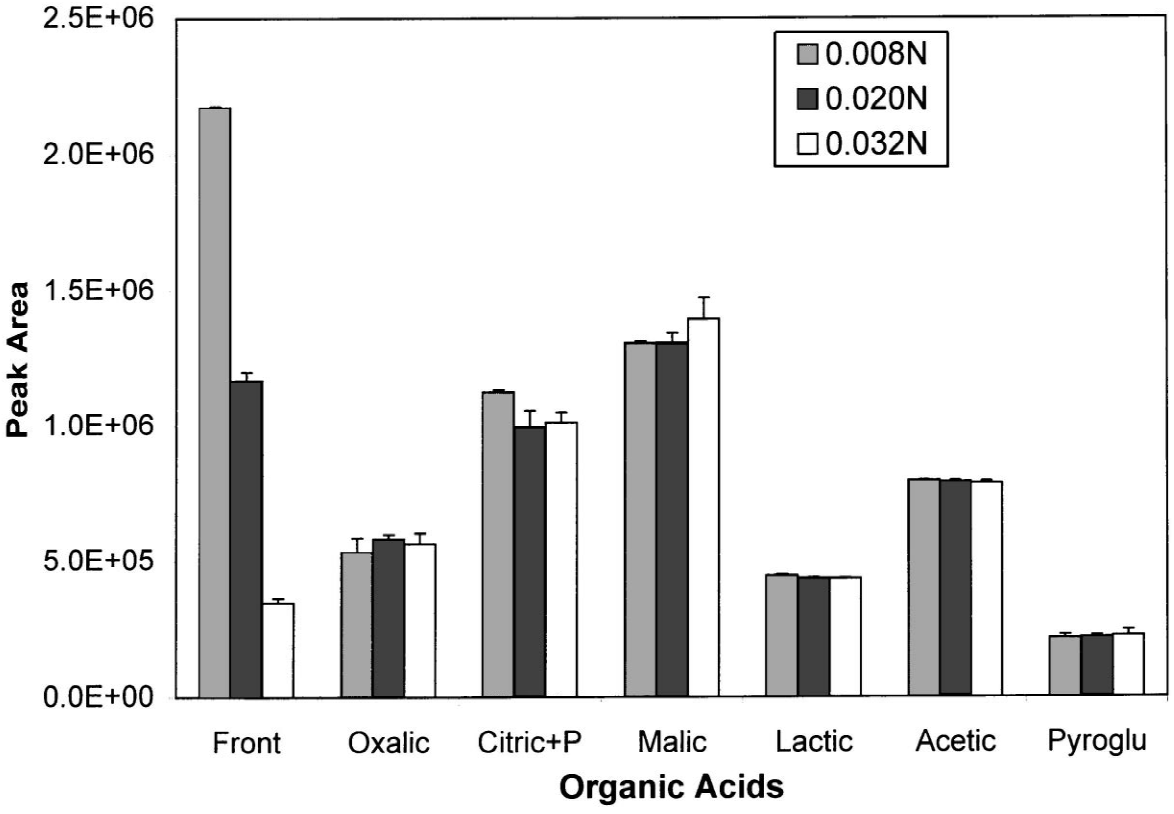

Fig. 3. Effect of the solvent strengths on the peak areas of organic acids. The peak areas were the average values with standard deviations from three separation temperatures. Because citric and phosphoric acid peaks (citric + P) merged in many analyses, the areas of two peaks were added together.

of 57°C with a mobile phase of 0.032 N sulfuric acid solution (Panel B).

Increasing flow rate from 0.6 ml min^{-1} to 0.8 ml min^{-1} shortened the retention times of all the components and reduced analytical time from 22 minutes to 16 minutes, but deteriorated the peak resolutions slightly (Table 2).

3.3. Effect on peak areas

Although increasing the acid concentration in the mobile phase resulted in a high background, it had a slight or no effect on the peak areas of the acids except the front peak (Fig. 3). The average area of the front peak decreased by 84% as the acid concentration increased from 0.008 N to 0.032 N. This indicated that the increased resolution between the front and oxalic peaks was partly due to the decreased area of the front peak at the high acid concentration in the mobile phase. Therefore, an increase in the acid concentration allowed us to obtain a baseline separation between the front and oxalic peaks.

4. Conclusions

The central composite face-centered design could be used for optimizing analytical parameters and speeding up HPLC method development. Optimal separation parameters for organic acids were found at 57°C and 0.032 N or at 70°C and 0.024 N. Higher sulfuric acid concentration in the mobile phase reduced the area of the front peak substantially, but had a slight or no effect on the peak areas of the other acids. At the flow rates between 0.6 and 0.8 ml min^{-1}, a better resolution was achieved at a slower mobile phase flow rate. A UV detector may be needed for analyzing tobacco samples with high sugars since interference from sugars can be reduced significantly.

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