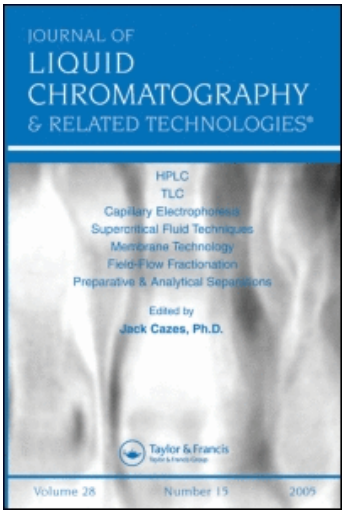

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OPTIMIZATION OF A REVERSED-PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY SEPARATION USING AN ION-PAIR REAGENT FOR THE DETERMINATION OF CARBOXYLIC ACIDS IN PLANT MATERIALS

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ABSTRACT

A method was developed to separate a number of mono-, di- and tri- carboxylic acids present in plant materials, using a C_{18} column and a simple eluent containing a phosphate buffer and tetra-n-butylammonium phosphate, an ion-pairing agent. The effects of the ion-pairing agent, pH, presence or absence of organic modifier and the concentration of the buffer, on the chromatographic response function (CRF), were investigated using a two level factorial experimental design. The results indicated that a number of main, two and three factor effects were important. The largest effect was due to the interaction of pH with ion-pairing concentration and was further investigated using univariate experiments. The mobile phase developed consisted of 10 mM tetra-n-butylammonium phosphate and 20

mM potassium dihydrogen orthophosphate at pH 3.3. Formic, glutamic, quinic, succinic, malic and tartaric acids were extracted from 3 different grass ecotypes, the extracts were cleaned-up using a C_{18} Sep-Pak® cartridge and then identified using the developed procedure.

INTRODUCTION

The analysis of short chain carboxylic acids such as citric, malic and tartaric has been widely reported in the literature because of their significance in the food and beverage industry,[1-4] medicine[5] and the transport of metals in plants.[6] These acids have also been shown to be important in the regulation of metals within metal tolerant plants, which are able to grow on soils containing elevated levels of metals.[7] A number of different biochemical and physiological adaptations have been postulated to account for this metal tolerance. For instance, one of the suggested zinc-tolerance mechanisms involves cellular chelation by malic acid, followed by transport to the vacuole for storage.[8] This mechanism has been investigated in various plant species by growing the plants in metal doped nutrient solutions followed by analysis of the organic acids present.[8-10] Methods used for the analysis of the acids in tolerant plants include titration,[8] enzymatic analysis[9] and gas chromatography,[10] all of which have a number of drawbacks. The first two methods are used for single acids only and the latter involves a derivatization step, which can lead to low and/or variable recoveries and multiple peaks.[5]

The determination of carboxylic acids by high performance liquid chromatography (HPLC) does not require their derivatization and has been reviewed by Schwarzenbach,[11] who identified four approaches: ion exchange, ion exclusion, ion-pair and ion suppression. The Krebs cycle acids have been separated by ion exclusion chromatography using a cation exchange column and dilute hydrochloric acid as the eluent.[12] Ion suppression was used to determine the organic acids in apple and cranberry juice, using two C_{18} columns in tandem and a potassium dihydrogen phosphate pH 2.4 buffer, with UV detection at 214 nm.[2] The analysis of the tomato xylem organic acids (malic, malonic, maleic, citric and fumaric acids) by ion suppression chromatography has been reported.[6] An ion-pairing method was used to separate citric acid cycle intermediates found in orange juice, urine and rat liver mitochondria. The method used a C_{18} column (150 x 4.6 mm i.d., 5 μm) and an eluent composed of 20 mM tetrabutylammonium hydroxide and 20 mM sodium sulphate at pH 7.0.[13]

The objective of HPLC optimization is the achievement of the desired separation with a minimum of time, effort and quantity of reagents.[14,15] There are a number of efficient optimization methods, which can be divided into two approaches.

The simultaneous approach (used in the present work) collects data according to a pre-defined experimental design, and aims to model chromatographic parameters by interpolation or extrapolation. Sequential approaches are based on search algorithms, where the response from previous separations are used to direct the search.

The ion-suppression and ion-exclusion methods described above, rely on the use of eluents with pH less than 2.5, which lead to a reduction in column life time. Ion-pair separations are carried out with a pH greater than the pKa values of the acids of interest and is usually in the range 3 to 7, hence deterioration of the column is reduced. The present work describes experiments carried out to develop a separation that could be used to simultaneously determine a number of acids present in plant material, without the need for derivatization, or the use of a mobile phase pH < 2.5.

MATERIALS AND METHODS

Reagents and Solvents

Tetra-n-butylammonium phosphate (Reagent grade), potassium dihydrogen orthophosphate (Analar grade) and acetonitrile (HPLC grade), all from BDH (Poole, UK). 1000 mg mL^{-1} stock standards of each of the following acids; quinic, malic, tartaric, succinic, citric, glutamic, maleic, glutaric, oxalic and fumaric (BDH, Poole, UK) were prepared in deionized water and stored frozen. The necessary mixtures and single acid standards were prepared from the stock solutions as necessary.

Chromatography Eluents

The eluents described in Table I were prepared in deionized water and filtered through a 0.45 μm pore size type HA filter (Millipore, Bedford, MA). The pH was adjusted using phosphoric acid or 2 M sodium hydroxide solution (BDH, Poole, UK), acetonitrile was added when necessary. The eluents were pumped through the columns for one hour prior to injection of the analyte.

Table 1

Mobile Phase Compositions and Responses For the Factorial Experiments

Expt. No.	Modifier % v/v		TBAP mM		pH		Buffer mM		Response CRF
	Level	Value	Level	Value	Level	Value	Level	Value	
1	--	0	--	1	--	3	--	20	43
2	--	0	+	10	--	3	--	20	31
3	--	0	--	1	+	5	--	20	20
4	--	0	--	1	--	3	+	200	23
5	--	0	--	1	+	5	+	200	0
6	--	0	+	10	--	3	+	200	26
7	--	0	+	10	+	5	--	20	18
8	--	0	+	10	+	5	+	200	38
9	+	5	--	1	--	3	--	20	21
10	+	5	+	10	--	3	--	20	23
11	+	5	--	1	+	5	--	20	21
12	+	5	--	1	--	3	+	200	18
13	+	5	--	1	+	5	+	200	7
14	+	5	+	10	--	3	+	200	10
15	+	5	+	10	+	5	--	20	21
16	+	5	+	10	+	5	+	200	21

TBAP = tetra-n-butylammonium phosphate concentration; Buffer = potassium dihydrogen ortho - phosphate concentration, Modifier = concentration of acetonitrile and CRF = chormatographic response function.

The time for an unretained peak to elute from the column (t_0) was determined as the first baseline disturbance after injection of deionized water. In the factorial experiments with a flow rate of 0.8 mL min^{-1}, t_0 was 2.8 minutes.

Equipment

A model 6000 Waters pump (Waters Assoc., Milford, MA) and a Rheodyne sample injector (Rheodyne model 7125, Contati, CA) fitted with a 20 μL sample loop. Detection at 210 nm was facillitated using a Pye-Unicam LC3 UV detector.

Two columns were used, in series, for the factorial experiments, namely a Waters μBondapak ODS 300 x 4.6 mm i.d., 5μm (Waters Assoc., Milford, MA) and an Alltech ODS 250 x 4.6 mm i.d., 10μm. The second column was removed for the univariate experiments.

Ion-Suppression Method

The starting point for this work was the Association of Official Analytical Chemists method developed to determine citric and malic acids found in apple and cranberry juice cocktail.[2] The method uses two C_{18} columns in tandem, a mobile phase consisting of a phosphate buffer at pH 2.4 and UV detection at 214nm. This approach was applied to the separation of a standard mixture of carboxylic acids, containing, 200 mg L^{-1} succinic, malic, tartaric, citric, quinic and 100 mg L^{-1} fumaric in deionized water. Peak identification was carried out by comparison of retention times with standards of the same concentration.

Factorial Experiment

Factorial experimental design and experimentation has been comprehensively described by a number of authors including: Box *et al*,[17] Morgan *et al.*[18] and Sundberg.[19] In the present work a two level full factorial experimental design was used to establish which eluent components have an influence on the separation of the acids, and to determine their interdependence. In such a design each variable or factor is investigated at two levels denoted by + or - (see Fig. 1). With four factors, 2^4 experiments (16 in total) have to be performed to calculate the magnitude of effects due to the factors themselves, and interactions between factors. The numerical values for the effect of one variable are obtained by subtracting the response at the minus level from another experiment at the plus level. The magnitude of the mean effect of one variable is then equal to the mean of these response differences. The interaction effect between two variables is calculated as the difference between those response differences where the variable is at a high level (+) and those where the same variable is at a low level (-). A tabular method has been developed to calculate these values.[16]

The factors chosen for investigation were: the concentration of phosphate buffer, eluent pH, concentration of ion-pairing reagent and inclusion of organic modifier. These factors and the values to use for the upper and lower levels of each were established from the literature.[11-13]

The practical limitations to the levels used were: (1) that the buffer concentration be sufficiently high to maintain a constant pH during the chromatographic run, and (2) the pH be less than 5.5 so that no problems with high mobile phase absorption at 210 nm would be encountered. The experiments described in Table 1 were used to analyse a standard mixture randomly and in triplicate.

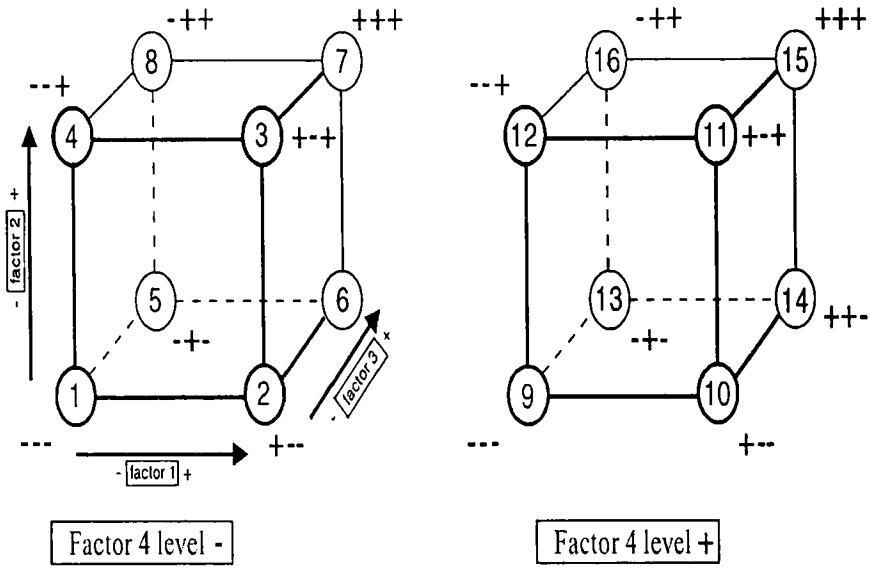

Figure 1. Schematic representation of a full 2-level factorial design for 4 variables. The number in each circle represents the experiment number.

Measurement of Response

A necessary condition for successful experimental optimization is a means of ordering the experiments numerically in relation to the quality of the separation. The choice of quality criteria is problematic because the separation requirements are often unclear and difficult to express quantitatively. The chromatographic response function (CRF) was first proposed by Morgan and Deming[20] as a measure of separation quality. Berridge[14] extended the concept to include more information concerning the quality and time taken for the separation. In the present work the approach of Berridge is used to measure the quality of each chromatogram and it is this measure that is optimized. The specific form used is given in Eqn. 1.

$$CRF = \sum_{i=1}^{L} R_i + L^x - a\,|T_m - T_L| - b(T_0 - T_1) \qquad (1)$$

R_i is the resolution between the peaks and limited to a maximum value of 2. L is the total number of peaks appearing in the chromatogram and is weighted

with an exponent x. The difference between an acceptable analysis time, T_m, and the time for the last peak to elute, T_L, is weighted by a factor a. The final term reduces the CRF value if the first peak, T_1, elutes prior to a specified minimum time T_0, and b is another weighting factor.

In the present work the weighting factors were set at a = 0.5, b = 0.5 and x = 2.0 because the maximum number of resolved peaks was the goal of the experiment, whereas analysis time was only a secondary consideration.

Extraction and Clean Up of the Carboxylic Acids

The extraction of the short chain carboxylic acids from the shoots of different species of grass was achieved using the method of Philips and Jennings[21] with slight modifications, such that the final evaporation under reduced pressure was carried out twice to reduce the carry over of formic acid.

A clean-up procedure was developed using a C_{18} solid phase extraction cartridge (Sep-Pak® Waters Assoc., Milford, MA) as follows: 20 mL extract shaken for 10 minutes, filtered through Whatman 542 paper (Whatman International Ltd., Maidstone, UK) and passed through the Sep-Pak®. The cartridge was pre-wetted with 2-3 mL methanol, flushed with 5 mL deionized water, 4 mL of extract was then passed through the column and the eluent retained.

This was repeated until all of the 20 mL extract had been passed through the column. The collected isolates were pooled, evaporated to dryness at 50 °C under reduced pressure and redissolved in 10 mL of the mobile phase.

RESULTS AND DISCUSSION

Initial Screening Experiments

The separation achieved for standards of malic, citric and fumaric acids was the same as that reported by Coppola and Starr.[2] However, when six acids representative of those found in plant material were analysed, co-elution of a number of components of the mixture occurred. This is shown in Fig. 2. Further experimentation[22] indicated that with the inclusion of an ion-pairing agent resolution of the analytes may be possible.

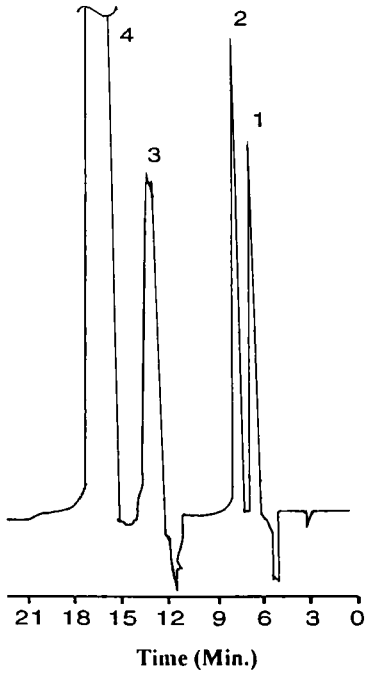

Figure 2. Separation of malic, citric, tartaric, quinic, succinic, and fumaric acids using the conditions of Coppola and Starr.[2] Eluent: 200 mM potassium dihydrogen orthophosphate, pH 2.4, with phosphoric acid. Column: Waters μBondapak ODS followed by Anachem ODS. Detection: UV at 210 nm. Flow rate: 1.0 mL $min.^{-1}$. Elution order: 1 = tartaric and quinic; 2 = malic; 3 = citric and succinic; 4 = fumaric.

Factorial Experiments

The CRF values for each run were determined and the mean values, shown in Table 1, were used to calculate the main and interactive effects as described elsewhere.[16] A positive value indicates that the separation measured is better at the higher variable level, whereas a negative value indicates the opposite. It can be seen from Table 2, that the presence of acetonitrile as an organic modifier (A), the pH of the eluent (P) and the concentration of the buffer (B) have relatively large numeric values and are, thereby, of importance. All three of these values are negative, indicating that the separation is better at the lower level of these factors. The only positive value is for the concentration of ion-pairing agent.

Table 2

Calculated Variable Effects and Interactions of the Factors Showing the Variation in CRF Value Caused by the Change in Eluent Composition

Variable	Calculated Estimate
Main Effect	
Modifier (A)	-7.1
Concentration of TBAP (T)	4.4
pH (P)	-6.1
Buffer concentration (B)	-6.9
Two-Factor Effects	
A & T	-2.4
A & P	5.6
A & B	-0.6
T & P	8.1
T & B	7.4
P & B	3.4
Three-Factor Effects	
A & T & P	-3.1
A & T & B	-6.4
A & P & B	-2.9
T & P & B	6.1
Four-Factor Effects	
A & T & P & B	-0.1

The two and three factor interactions T.P, T.B, A.T.B and T.P.B give large values whereas the four factor effect is negligible, the largest factor effect was for the interaction between ion-pairing reagent and pH. Therefore, the effect of pH on the separation was investigated univariately, at constant buffer and ion-pairing concentration.

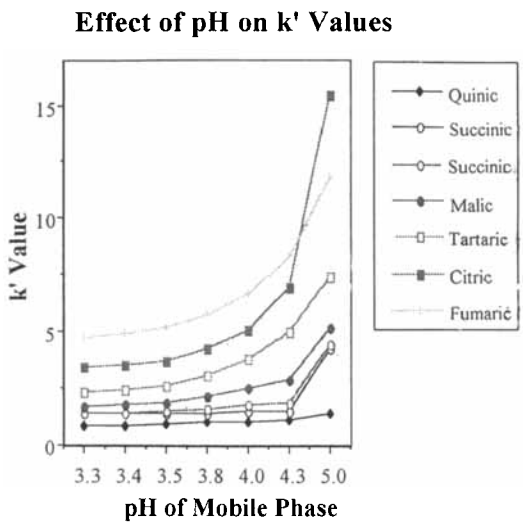

Figure 3. Effect of pH on the capacity factor, k′, of various carboxylic acids. The acids are identified in the legend.

Effect of pH on Separation

Initial screening experiments indicated that it was possible to remove the second column to improve the peak shape, without affecting the resolution.[22] Therefore, the univariate experiments were carried out using only the Waters μBondapak column.

The values and signs of the variable terms (Table 2) suggest that the most promising conditions at which to vary the pH would be: high level ion-pair reagent (10 mM), low level of buffer (20 mM) and absence of acetonitrile. The pH of this mobile phase was adjusted to 3.3, 3.4, 3.5, 3.8, 4.0, 4.3 and 5.0 using phosphoric acid or 2 M sodium hydroxide, depending on the desired pH. Each of these eluents was randomly chosen and the standard mixture of six acids was analyzed.

The results are summarized in Fig. 3, which shows the variation of capacity factor, k′, with pH. This graph can be interpreted by consideration of the pKa values for each acid. The largest change in k′ for every acid except quinic, occurs between pH 4.3 and 5.0. This is because the pKa_2 values are such that each acid looses a second proton between these pH values and so can

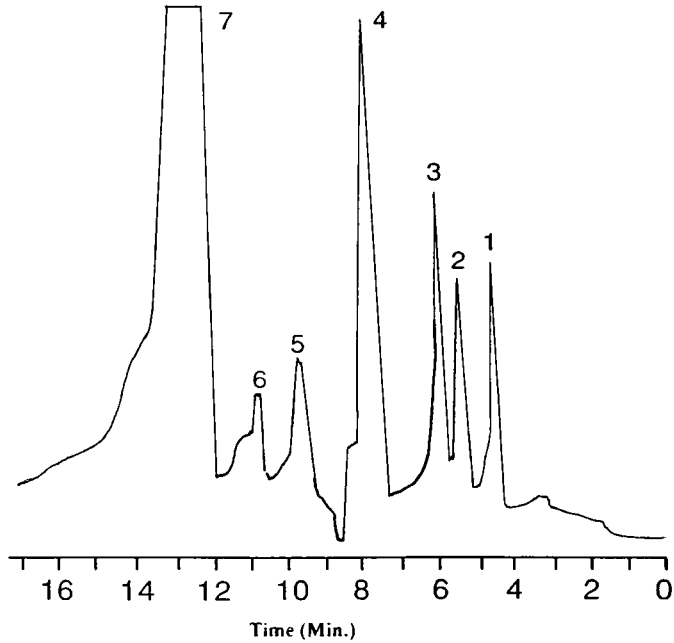

Figure 4. Separation of seven acids using the optimized conditions (all 200 mg L^{-1} except fumaric which was 100 mg mL^{-1}). Eluent: 10 mM tetra-n-butylammonium phosphate, 20 mM potassium dihydrogen orthophosphate, pH 3.3. Column: Waters μBondapak ODS. Detection: UV at 210 nm. Flow rate: 1.0 mL $min.^{-1}$. Elution order: 1 = quinic; 2 = succinic; 3 = malic; 4 = tartaric; 5 = citric; 6 = malonic; and 7 = fumaric.

interact more strongly with the ion-pair reagent. This interaction markedly increases their retention time. Two peaks were observed for succinic acid at pH's greater than 3.8, which could be because of the presence of multiply charged species (pKa_1 4.21 and pKa_2 5.64). Multiple peaks have been reported in ion-pair separations when inappropriate pH conditions are used and occurs because the variably ionized acid groups interact differently with the ion-pair reagent.[15] The lines for tartaric and fumaric are both similar in shape, which is a reflection of their virtually identical pKa values (pKa_1 3.04 and 3.03; pKa_2 4.37 and 4.37 respectively).

All six acids were separated satisfactorily after the optimization procedure, as shown in Fig. 4. This eluent composition was chosen for the analysis of plant extracts and represents a compromise, because the separation

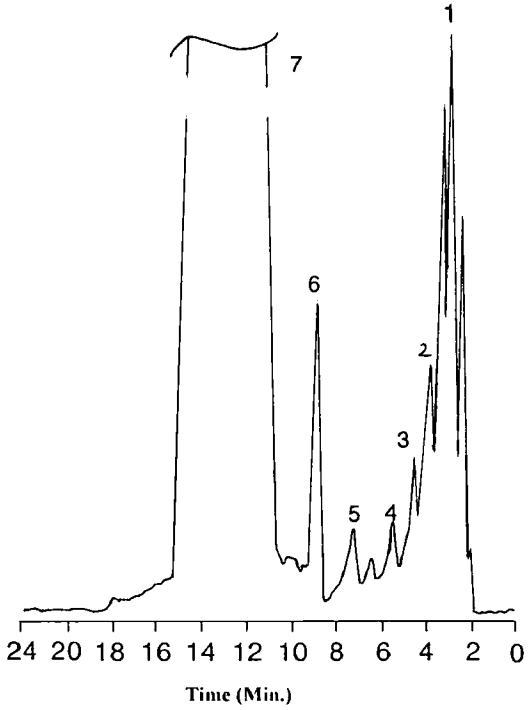

Figure 5. Separation of carboxylic acids extracted from copper-tolerant Parys *Agrostis tenuis*. Eluent: 10 mM tetra-n-butylammonium phosphate, 20 mM potassium dihdrogen orthophosphate, pH 3.3. Column: Waters μBondapak ODS. Detection: UV at 210 nm. Flow rate: 1.0 mL $min.^{-1}$ Elution order: 1 = formic; 2 = glutamic; 3 = quinic; 4 = succinic; 5 = malic; 6 = tartaric; and 7 = partially fumaric plus citric.

mechanism is a combination of ion suppression and ion-pairing depending on the pKa values for each acid. The use of a pH greater than the highest pKa value for the acids in the system, may have improved the separation, but was not possible due to the high absorption of this mobile phase at 210 nm.

A number of acid standards were run prior to the analysis of plant extracts, so as to determine retention times. Using the eluent developed above, glutamic, quinic, succinic, malic, tartaric, citric, malonic and fumaric acids could be separated. Maleic, glutaric and oxalic could be partially resolved from other acids.

Quantitation

Linear response was in the range 5.0 to 40.0 mg L^{-1} for each acid except fumaric,which was 2.5 to 20.0 mg L^{-1}. These were determined using standards made up in the mobile phase and containing 15.0 mg L^{-1} glutamic acid as internal standard. The curves for all the acids (except citric) had linear correlation coefficients between 0.990 and 0.950. The coefficient for citric acid was 0.850, which was due to peak splitting at concentrations greater than 10.0 mg L^{-1}.

Analysis of Plant Material

Copper tolerant Parys *Agrostis tenuis*, zinc/cadmium tolerant Merlin *Festuca rubra* and metal non-tolerant Cascade *Festuca rubra* were analyzed using the conditions described above and the chromatogram for *Agrostis tenuis* is shown in Figure 5. Co-injection of a number of different acid standards was used to identify the following acids as present in the extract: formic, glutamic, quinic, succinic, malic and tartaric. Elution of a large unidentified peak between 11 and 15 minutes interfered with the identification of citric and fumaric acids. Using the methodology outlined in this paper, we were able to show an increase in the concentration of malic acid in zinc-tolerant Merlin *Festuca rubra* on exposure to zinc but not copper, which did not occur to the same extent in a non-tolerant population.[23]

CONCLUSIONS

A simple separation procedure for the carboxylic acids present in plant material was investigated and optimized using factorial experiments. The optimized mobile phase consisted of 20 mM sodium dihydrogen orthophosphate and 10 mM tetra-n-butylammonium phosphate at pH 3.3 and could be used to separate glutamic, quinic, succinic, malic, tartaric, citric, malonic, and fumaric acids, which commonly occur in plant materials. This procedure could also be used for the analysis of carboxylic acids in other sample matrices.

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