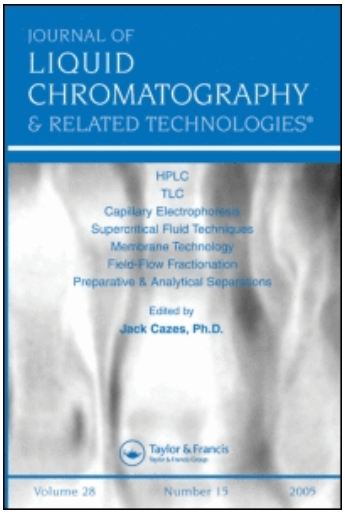

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Abstract: RP-HPLC analysis for low molecular weight organic acids in soil solution has been optimized. An Atlantis™ C_{18} column was used for the analyses. An optimal determination for eleven organic acids in soil solution was found at room temperature (25°C) and 220 nm detection wavelength, with a mobile phase of 10 mM KH_2PO_4–CH_3OH (95 : 5, pH 2.7), a flow rate of 0.8 mL/min and 10 μL sample size. The detection limits ranged 3.2–619 ng/mL, the coefficients of variation ranged 1.3–4.6%, and the recoveries ranged 95.6–106.3% for soil solution with standard addition on the optimal conditions proposed.

Keywords: Low molecular weight organic acid, Optimization, Soil solution

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INTRODUCTION

Low molecular weight organic acids are common in soils, especially in the immediate zone of the soil-root interface. In general, soil solution concentration of aliphatics ranges from less than 1 μM to 2 mM;[1–3] one extreme value of 6.7 mM succinic acid in soil solution from Norway spruce little layer has been published.[4] They play an important role in a number of soil processes, such as mobilization of plant nutrients and in the protection of plant roots when exposed to rhizotoxic concentrations of Al^{3+} in acid soils,[5,6] and affecting surface charges and electrokinetic properties of variable charge soil.[7,8] It has also been clearly demonstrated that organic acids have the potential to significantly enhance the rate of mineral weathering[9–11] and, thereby, be of importance for soil pedogenic processes.[12]

Various HPLC techniques have been used for the separation of low molecular weight acids in soil and litter extracts/solution.[1–4,13,14] These technique have low limits of detection, but, unsuitable capacity factors may cause problems, and interference from humic compounds, inorganic ions, such as Fe, Al, etc., in the solution might easily produce erroneous results. Ion-pair high performance liquid chromatographic (IP-HPLC) methods have achieved a better separation for organic acids than traditional reversed-phase high performance liquid chromatographic (RP-HPLC) methods. However, it is difficult to separate di- and tricaboxylic acids with similar retention time by an ion-pair reagent, and two or more separate optimum separation methods for di- and tricaboxylic acids will increase the time demand for their separation and financial cost.[13] The main advantage of RP-HPLC over IP-HPLC is in its easiness and rapidity of separation, and it is more suitable for a mass of sample analyses. However, the presence of small inorganic anions in the saps, such as nitrate ions, will affect the elution of some organic acids, e.g., malic acid.[14] Its separation and determination also depend on organic modifier, pH of the mobile phase, flow rate, column selected, and column temperature.[14–17] Loss of water from evaporation at high temperatures of the column will induce recoveries of organic acid more than 100%, and different column temperatures will increase the time demand for their separation.[1,16]

The aim of this study is to optimize reversed-phase high performance liquid chromatographic separation of eleven low molecular weight organic acids in soil with the Atlantis™ C_{18} column at room temperature (25°C).

EXPERIMENTAL

Apparatus and Reagents

The HPLC system consisted of a Waters 600 series, Waters 600 pump, 2487 UV-detector. An Atlantis™ C_{18} (4.6 mm × 250 mm × 5 μm, Waters

Company, USA) was used throughout. Data acquisition was performed using the Exceed 2000 Chromatograph Station (Zhejiang Science Instrument Co., Ltd).

A precise acidometer (pHS-3C, Shanghai REX Instrument Factory) was used for pH measurement; LG10-2.4A centrifuge (Beijing Medical Instruments Factory) and R-201 rotary evaporator (Senco Instruments Co., Ltd, Shanghai) for preparing the soil solution.

The oxalic acid, tartaric acid, methanoic acid, malic acid, lactic acid, acetic acid, citric acid, fumaric acid, propanoic acid, phosphoric acid, potassium dihydrogen phosphate, tetrahydrofuran, sodium hydroxide, sodium chloride, chlorhydric acid and ethyl acetate used were analytical reagents, unless otherwise stated. The propane diacid and butanedioic acid used were chromatographic standard reagents, and the methanol and acetonitrile used were chromatographic reagents.

Preparation of the Standard Sample

Oxalic acid (0.2500 g), tartaric acid, methanoic acid, malic acid, lactic acid, acetic acid, citric acid, fumaric acid, propanoic acid, proprane diacid and butanedioic acid were added into 250 mL volumetric bottles to get 11 stock solutions of 1 mg/mL by adding adequate bidistilled water, respectively. The standard solutions were acquired from the stock solutions by dilution.

All of the solutions used in the experiment were prepared by bidistilled water, except the chromatographic reagents. All of the samples and solutions of the mobile phase were filtered by the filtration membrane of 0.45 μm before entering the chromatographic column.

Experimental Design

In order to optimize RP-HPLC conditions for determination of the low molecular weight organic acids in soil solution, effects of concentration of mobile phase on separation, organic modifier in mobile phase on separation, pH of mobile phase on capacity factor, the flow rate of mobile phase on separation, and wavelength of detection were investigated, as shown in Table 1.

In the optimum condition proposed, the standard curve, linearity range, detection limit, and coefficient of variation of 11 kinds of low molecular weight organic acids, referred above by RP-HPLC, were obtained.

Preparation of the Soil Solution

Moist soil sample No. 1 and No. 2 (25.0 g, 20% w/w), which are a type of alumi-ortho acrisol, collected from subtropical China, were extracted with

Table 1. Experimental design

Factors	Concentration of KH_2PO_4 (mM)	Concentration of CH_3OH_3 (v/v %)	pH of mobile phase adjusted by H_3PO_4	Flow rate (mL/min)	Wavelength of detection (nm)	Sample size (μL)
Effect of concentration of mobile phase on separation	5, 10, 20	0	2.7	0.8	220	10
Effect of organic modifier on separation	10	0, 5, 10	2.7	0.8	220	10
Effect of pH of mobile phase on capacity factor	10	5	2.4, 2.7, 3.0, 3.3	0.8	220	10
The effect of the flow rate of mobile phase on separation	10	5	2.7	0.4, 0.6, 0.8, 1.0	220	10
Select the wavelength of detection	10	5	2.7	0.8	200, 220, 240	10

30 mL 0.1 M NaOH and saturated aq. NaCl for 20 h. The mixtures were centrifuged at 10000 rev/min for 30 min, followed by filtering the supernatant liquid, and then cooling in cold water. The supernatants were acidified to pH 2.5 with 1 M HCl to precipitate the humic substances. After standing for 16 h, the mixtures were filtered through the filtration membrane (0.45 μm). Organic acids were obtained by extracting the supernatant 3 times with 10 mL ethyl acetate for 5 min, evaporating the solvent to dryness in a rotary evaporator at 40°C, and redissolving the residue in 1 mL double-distilled water to get soil solutions with organic acids.

RESULTS AND DISCUSSION

Effect of Concentration of Mobile Phase on Separation

Because of the superior polarity of the acids, they can dissociate easily and will not be retained on the stationary phase if water is the main component of the mobile phase while being analyzed by RP-HPLC. In order to make organic acids exist in molecular state, an acid phase is usually used to restrain the dissociation and causes them to be retained on the column of an apolar bonded phase (ODS) to be separated. KH_2PO_4 was used for the mobile phase in the experiment.

Figure 1 showed that the capacity factor (k′) of each organic acid was highest in 10 mM KH_2PO_4. The K′ was slightly increased for oxalic acid,

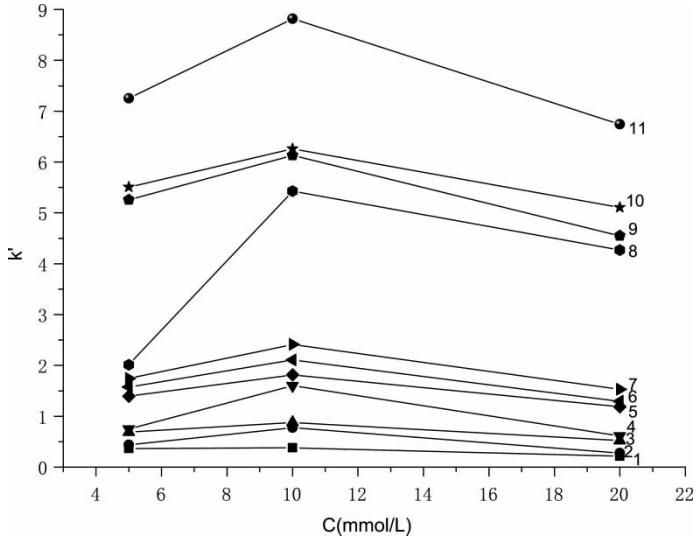

Figure 1. The effect of the concentration of mobile phase on capacity factor: 1. oxalic acid; 2. tartaric acid; 3. methanoic acid; 4. malic acid; 5. proprane diacid; 6. lactic acid; 7. acetic acid; 8. citric acid; 9. butanedioic acid; 10. fumaric acid; 11. propanoic acid.

tartaric acid, methanoic acid, malic acid, proprane diacid, lactic acid, and acetic acid, while it was significantly increased for citric acid, butanedioic acid, fumaric acid, and propanoic acid in 10 mM KH_2PO_4, compared with that in 5 mM and 20 mM KH_2PO_4. Therefore, 10 mM KH_2PO_4 is proposed as the mobile phase.

Since the K′s for oxalic acid, tartaric acid, and methanoic acid were still less than 1.0 in 10 mM KH_2PO_4 for the mobile phase, some interfering factors must be removed when the soil solution is prepared in advance to prevent from being interfaced by inorganic ions being interfaced with inorganic ions.

Effect of Organic Modifier in Mobile Phase on Separation

Since higher K for organic acids, e.g., propanoic and fumaric acids, means longer run times, methanol was preferentially selected as the organic modifier in the experiment due to its lower price, in order to get moderate K′ and shorten the retention time.

The retention time of the chromatographic separation is long, and fumaric acid cannot be separated with butanedioic acid effectively without methanol due to its serious tailing peak. After methanol was added into the mobile phase, the retention time of organic acid was significantly shortened and fumaric acid was separated with butanedioic acid successfully. The reversed sequence of peak times for butanedioic and fumaric acid (Table 2) was probably attributed to methanol decreasing the polarity of the mobile phase, and causing butanedioic acid with low polarity to elute ahead of fumaric acid.

The retention time of organic acid was significantly shortened in treatment with 5% methanol, compared with treatment without methanol,

Table 2. Effect of methanol on the retention time of chromatography

	Retention time (min)		
Organic acids	Without methanol	With 5% methanol	With 10% methanol
Oxalic acid	3.937	3.826	3.716
Tartaric acid	4.583	4.442	4.181
Methanoic acid	4.747	4.665	4.493
Malic acid	6.408	5.593	4.945
Proprane diacid	6.877	5.926	5.233
Lactic acid	7.528	6.475	5.659
Acetic acid	8.213	6.992	6.098
Citric acid	14.943	10.164	7.243
Butanedioic acid	16.743	11.650	8.442
Fumaric acid	16.449	12.967	9.766
Propanoic acid	22.538	15.973	12.477

Table 3. Effect of the content of methanol on the capacity factor of organic acid

Organic acids	With 5% methanol	With 10% methanol
Oxalic acid	0.2717	0.2484
Tartaric acid	0.4882	0.4172
Methanoic acid	0.5723	0.5111
Malic acid	0.8471	0.6654
Proprane diacid	0.9642	0.7619
Lactic acid	1.1463	0.9022
Acetic acid	1.3179	1.0491
Citric acid	2.1824	1.4338
Butanedioic acid	2.7109	1.8373
Fumaric acid	3.0389	2.2821
Propanoic acid	4.3402	3.1876

and it was slightly prolonged, compared with treatment with 10% methanol (Table 2). However, compared with the treatment in 5% methanol, the capacity factor was significantly decreased in the 10% methanol treatment (Table 3), and this probably increased difficulty in separation. So, 5% methanol is proposed as the organic modifier to add into the mobile phase.

Effect of pH of Mobile Phase on Capacity Factor

Figure 2 showed that the capacity factor (K′) of the organic acids and their degree of separation were decreased with increasing the pH. The increasing pH of the mobile phase will cause an increase in the degree of dissociation for the organic acids, and changes in retention of the alkyl group on the surface of ODS column. The order of peaks of butanedioic acid and fumaric acid were reversed and the selectivity of the column also changed at pH 3.0 and 3.3, compared with that at pH 2.4 and pH 2.7. However, degradation of the silanizing bonded-phase will increase with decreasing pH, therefore, pH 2.7 is proposed as optimal acidity of the mobile phase.

Effect of the Flow Rate of Mobile Phase on Separation

The retention time of organic acids was decreased with increasing flow rate (Table 4), and the experimental organic acids were successfully separated in 16 min at the flow rate of 0.8 mL/min. Among the experimental organic acids, oxalic acid was eluted first, and its retention time was decreased to 3.726 min at the flow rate of 0.8 mL/min from 7.274 min at the rate of

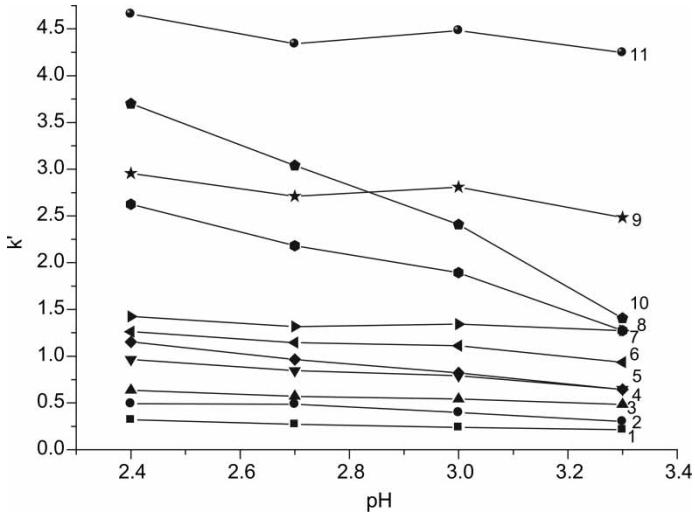

Figure 2. Effect of pH of mobile phase on capacity factor: 1. oxalic acid; 2. tartaric acid; 3. methanoic acid; 4. malic acid; 5. proprane diacid; 6. lactic acid; 7. acetic acid; 8. citric acid; 9. butanedioic acid; 10. fumaric acid; 11. propanoic acid.

0.4 mL/min. Propanoic acid was eluted last, and its retention time was decreased to 15.973 min at the flow rate of 0.8 mL/min, from 30.778 min at the rate of 0.4 mL/min. The retention time was slightly decreased when the flow rate increased to 1.0 mL/min from 0.8 mL/min. However, a higher

Table 4. Effect of the flow rate of mobile phase on the chromatographic retention time of organic acids

	Retention time (min)			
Organic acids	0.4 mL/min	0.6 mL/min	0.8 mL/min	1.0 mL/min
Oxalic acid	7.274	4.873	3.726	2.967
Tartaric acid	8.545	5.717	4.442	3.472
Methanoic acid	9.027	6.034	4.665	3.661
Malic acid	10.618	7.090	5.593	4.299
Proprane diacid	11.300	7.543	5.926	4.566
Lactic acid	12.322	8.235	6.475	4.991
Acetic acid	13.320	8.896	6.992	5.382
Citric acid	18.432	12.254	10.164	7.357
Butanedioic acid	21.294	14.186	11.650	8.512
Fumaric acid	23.406	15.556	12.967	9.312
Propanoic acid	30.778	20.506	15.973	12.256

flow rate of mobile phase will reduce the degree of separation of organic acids, and cause the reduction of a number of theoretical plates and the loss of silica gel in the column.[16] Therefore, the optimal flow rate proposed is 0.8 mL/min.

Effect of the Wavelength of Detection

The mixed standard sample of experimental organic acids, of which all concentrations were 50 μg/mL, was determined to study effect of the wave length of detection on the response of the chromatographic peak, and the results are listed in Table 5. Table 5 showed that the chromatographic peak was heightened with decreasing wavelength. There were smaller wide spread responses to the experimental organic acid and lower sensitivity under 240 nm, than under 200 nm and 220 nm. Moreover, there was more well proportioned peak value under 220 nm than under 200 nm; it is better for determination of various organic acids with well proportioned peak values. Therefore, 220 nm is proposed as the wave length of detection.

Calibration and Application

The qualitative determination by retention time and the quantitative determination by peak value, a satisfactory separation of 11 organic acids,

Table 5. Effect of the wavelength of detection on the response of chromatographic peak

	Chromatographic peak (mv)		
Organic acids	200 nm	220 nm	240 nm
Oxalic acid	340092	74885	11720
Tartaric acid	16651	12636	999
Methanoic acid	10139	6895	708
Malic acid	11340	6811	679
Proprane diacid	15621	6882	691
Lactic acid	5076	3431	359
Acetic acid	6614	3185	173
Citric acid	8334	5209	545
Butanedioic acid	4346	2178	135
Fumaric acid	672893	532074	81740
Propanoic acid	2760	1554	84

have been achieved by RP-HPLC in the optimal conditions proposed above. Figure 3 is the chromatogram of the mixing standard sample, which included 5 μg/mL fumaric acid, 10 μg/mL oxalic acid, 10 μg/mL tartaric acid, 10 μg/mL methanoic acid, 10 μg/mL malic acid, 50 μg/mL citric acid, 50 μg/mL butanedioic acid, 100 μg/mL propanoic acid, 100 μg/mL acetic acid, 100 μg/mL lactic acid, and 200 μg/mL proprane diacid.

Table 6 showed that the detection limit at a signal-to-noise ratio of 3 varied with different organic acids, and there was the highest detection limit with 619 ng/mL for propanoic acid and lowest detection limit with 3.2 ng/mL for fumaric acid. The correlation coefficient all was larger than 0.999, and the coefficient of variation ranged from 1.3% to 4.6%.

Soil solution samples prepared were determined by RP-HPLC on the optimal condition proposed. Oxalic, lactic, acetic, citric, and fumaric acid were determined in soil sample No. 1. Methanoic acid, proprane diacid, lactic, acetic, citric acid, fumaric and propanoic acid were determined in soil sample No. 2 (Table 7). The soil solution samples with standard addition showed that the recoveries ranged from 97.0–106.3% for soil sample No. 1 and 95.6–102.4% for soil sample No. 2.

The detection limit was improved significantly from that in previous researches by RP-HPLC,[13,14,16] IP-HPLC,[13] HPLC with ion exclusion column,[1] and GC/MS.[18] Compared with ion chromatography,[19] the detection limit for some organic acids, e.g. oxalic acid, was improved, and for some organic acids, e.g. citric acid, was not improved, but the run time was shortened significantly, indicating that it is more suitable for a mass of sample analyses.

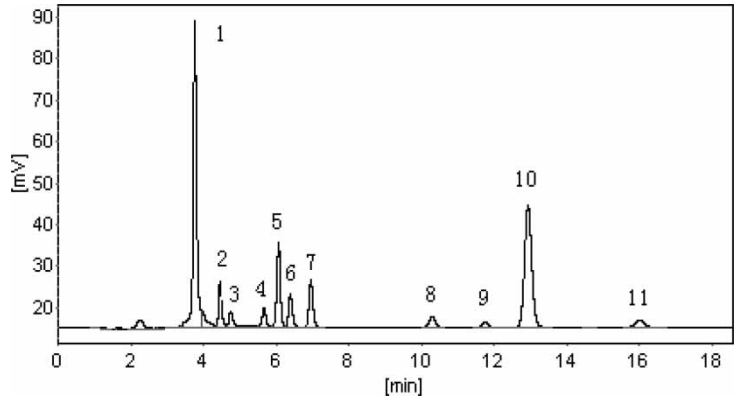

Figure 3. The chromatogram of mixing standard sample: 1. oxalic acid; 2. tartaric acid; 3. methanoic acid; 4. malic acid; 5. proprane diacid; 6. lactic acid; 7. acetic acid; 8. citric acid; 9. butanedioic acid; 10. fumaric acid; 11. propanoic acid.

Table 6. The linearity regression equation of various organic acids and their detection limit on the optimal condition proposed

Organic acids	Linearity range (μg/mL)	Regression equation	Correlation coefficient	Detection limit (ng/mL)	Coefficient of variation (%) (n = 10)
Oxalic acid	0.05 ~ 150	H = 1402.9C + 62.655	0.9999	4.5	4.6
Tartaric acid	1 ~ 80	H = 376.77C + 17.593	0.9998	96	3.1
Methanoic acid	5 ~ 350	H = 141.88C − 44.173	0.9996	563	1.3
Malic acid	5 ~ 400	H = 134.59C + 74.185	0.9990	87	1.9
Proprane diacid	1 ~ 300	H = 139.19C + 31.299	0.9999	257	4.6
Lactic acid	1 ~ 200	H = 66.275C + 55.142	0.9999	419	2.1
Acetic acid	1 ~ 800	H = 63.621C − 34.211	0.9999	432	3.2
Citric acid	1 ~ 200	H = 97.721C − 34.070	0.9991	330	2.5
Butanedioic acid	1 ~ 200	H = 40.419C + 26.070	0.9998	72	2.6
Fumaric acid	0.01 ~ 100	H = 9988.8C − 12.634	0.9999	3.2	3.0
Propanoic acid	10 ~ 1000	H = 28.724C + 39.768	0.9999	619	3.5

Note: H is the height of peak (mV), C is the concentration (μg/mL).

Table 7. Determination of soil solution samples

	Soil sample no. 1			Soil sample no. 2		
Organic acids	Soil solution (μg/mL)	Standard addition (μg/mL)	Recovery (%)	Soil solution (μg/mL)	Standard addition (μg/mL)	Recovery (%)
Oxalic acid	1.15	1.00	98.7	n.d.	1.00	96.0
Tartaric acid	n.d.	1.00	99.1	n.d.	1.00	97.1
Methanoic acid	tr	10.00	97.0	13.4	10.00	97.5
Malic acid	tr	1.00	97.7	tr	1.00	95.6
Proprane diacid	n.d.	10.00	100.5	1.30	10.00	99.2
Lactic acid	1.05	1.50	104.3	1.75	1.50	101.3
Acetic acid	2.00	2.00	106.3	13.5	15.00	102.4
Citric acid	4.50	5.00	99.0	0.45	0.50	98.4
Butanedioic acid	n.d.	10.00	101.0	n.d.	10.00	100.1
Fumaric acid	0.04	0.05	102.0	0.05	0.05	102.0
Propanoic acid	tr	2.00	101.0	0.61	2.0	99.8

Notes: n.d. stands for not detected; tr for trace amount.

CONCLUSION

RP-HPLC analysis for low molecular weight organic acids in soil solution has been optimized. An Atlantis^{TM} C_{18} column was used for the analyses. An optimal determination for eleven organic acids in soil solution was found at room temperature (25°C) and 220 nm detection wavelength, with a mobile phase of 10 mM KH_2PO_4–CH_3OH (95 : 5, pH 2.7), a flow rate of 0.8 mL/min and 10 μL sample size. The detection limit was improved significantly, and run time was shortened by the condition proposed with RP-HPLC. The advantage of the condition proposed was confirmed by determination of 11 kinds of low molecular weight organic acids in soil solution samples. The detection limits ranged from 3.2–619 ng/mL, the coefficients of variation ranged from 1.3–4.6%, and the recoveries ranged from 95.6–106.3% for soil solution with standard addition by the optimal conditions proposed.

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