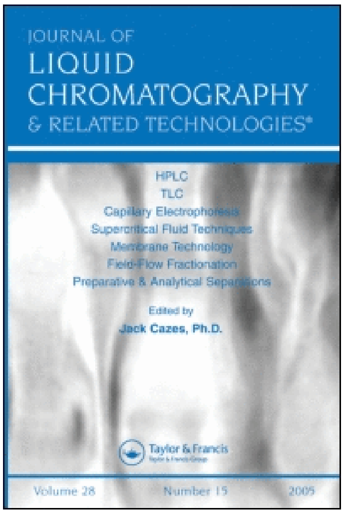

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SIMULTANEOUS EXTRACTION AND DETERMINATION OF ORGANIC ACIDS IN XYLEM SAPS BY RP-HPLC AFTER SOLID-PHASE EXTRACTION WITH SMALLER PARTICLE-SIZED HYDROXYAPATITE NANOCRYSTALS

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☐ *A simple and reliable RP-HPLC method based on solid-phase extraction with smaller particle sized hydroxyapatite nanocrystals was developed for the simultaneous extraction and determination of oxalic, malic, and citric acids in xylem saps of pokeweed. The smaller particle sized hydroxyapatite nanocrystals exhibited greater surface area and more active sites on the surface, consequently, higher adsorption ability to organic acids, which was proved to be an effective solid-phase extractor for the simultaneous extraction of organic acids in xylem saps. Oxalic, malic, and citric acids were determined by RP-HPLC at the optimum chromatographic conditions after the solid-phase extraction procedure. The accuracy of the method was confirmed with an average recovery ranging between 94.2% and 97.9%, the relative standard deviations (RSD) were less than 2.0%. This method was successfully applied to determine the organic acids in xylem saps of pokeweed.*

Keywords hydroxyapatite nanocrystals, RP-HPLC, simultaneous extraction, smaller particle sized, solid-phase extraction, xylem sap

INTRODUCTION

Reversed-phase high performance liquid chromatography (RP-HPLC) has been widely used for the determination of organic acids in plant samples such as tissues, leaves, roots, and xylem saps because of the simplicity, rapidity, and stability of the method.[1,2] However, the presence of small inorganic anions in xylem saps such as nitrate ions, which have significant UV absorbency at 210 nm, gives an unretained peak, which is

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large enough to mask the first-eluting oxalic acid.[3–5] Optimization of the separation, which took into consideration the type of eluent and organic modifier, flow rate, and column temperature, could not improve the separation of oxalic acid.[6,7] In a previous work,[1] we developed a method to eliminate the interference from nitrate ions on oxalic acid in RP-HPLC by solid-phase extraction with nanocrystalline hydroxyapatite [HAP, $Ca_{10}(PO_4)_6(OH)_2$], where xylem oxalic acid could be adsorbed selectively by HAP rather than nitrate, malic, or citric acid, and then the retained oxalic acid was eluted and determined by RP-HPLC without interference from nitrate ions. It was considered that nanocrystalline HAP was a polar inorganic material, which had relatively high affinity for polar organic molecules.[8] The ability of an organic acid adsorbed by HAP depended on its polarity and the coordination ability to surface calcium ions. Oxalic acid could be selectively adsorbed by nanocrystalline HAP due to its higher polarity and stronger coordination ability to calcium ions on the HAP surface, but the polarity of malic and citric acids were relatively low and had weaker coordination ability to calcium; they would be repulsed by the inorganic nanocrystalline HAP. Thus, the interference from nitrate ions on oxalic acid could be avoided effectively. The aforementioned method has been successfully applied to determine oxalic, malic, and citric acids in xylem saps of *Alyssum murale* by RP-HPLC,[2] which included analyses of malic and citric acids in the supernatant after solid-phase extraction with HAP as well as oxalic acid in the eluent.

However, it should be pointed out that the nanocrystalline HAP used in the previous work was prepared in our laboratory by a sol-gel method,[1,9] in order to obtain phase pure HAP, the precursors must be calcined at a temperature above 750°C, and this process promoted an increase in the particle size and a decreasing of the surface area. The obtained nanocrystalline HAP had a sphere-like shape with mean size in the range 50–60 nm.[9] By contrast, in the case of the chemical precipitation method, where the temperature did not exceed 100°C, the smaller particle sized HAP nanocrystals ($<30\,nm$) with greater surface area could be synthesized. Moreover, it was generally accepted that the adsorption ability of HAP was controlled by the surface area, which showed a direct dependence on the particle size of HAP nanocrystals. Da Rocha et al.[10] have also indicated that the drop in the adsorption capacity of HAP for cadmium could be explained by a reduction in its surface area, which was induced by the increase of the particle size. In another work,[11] we revealed the size-dependent defluoridation properties of nanocrystalline HAP prepared by the sol-gel method, and the smaller the particle size, the better the adsorption efficiency; meanwhile, it was found that the smaller particle sized HAP nanocrystals synthesized by a chemical precipitation method exhibited higher adsorption ability than that of sol-gel method.[12] It might concluded that the decrease in particle size of HAP nanocrystals led to an increase in surface area, accompanied by a concomitant rise in the quantity of active sites on the surface, which would cause a remarkable enhancement of the adsorption ability. In this study, it is expected that the smaller particle sized HAP nanocrystals synthesized by the chemical precipitation would overcome the repulsion to relatively low polarity organic acids and adsorb oxalic, malic, and citric acids simultaneously due to the presence of more active sites on the surface. Therefore, oxalic, malic, and citric acids in xylem saps could be extracted and determined by RP-HPLC simultaneously.

The objective of this study is to improve our previous work by using smaller particle sized HAP nanocrystals as the solid-phase extractor, with the purpose of developing a simple and reliable RP-HPLC method for the simultaneous extraction and determination of organic acids in plant xylem saps.

EXPERIMENTAL

Reagents and Standard Solutions

Analytical standard-grade oxalic, malic, and citric acids were obtained from Sigma (St. Louis, MO, USA). Diammonium hydrogen phosphate, orthophosphoric acid, and all other regents used were of analytical-reagent grade and supplied by Nanjing Chemical Reagent Company (Nanjing, China). Water was purified by passage through a Compact Milli-RO and Milli-Q water system from Millipore (Milford, MA, USA), and was used for all solutions, dilution, and the mobile phase.

Stock standard solutions were prepared by dissolving organic acids in Milli-Q water; these solutions remained stable for several weeks if kept refrigerated at 4°C. Working standard solutions were prepared daily by dilution of stock solutions with Milli-Q water. All solutions used were filtered through a 0.45 μm membrane filter from Millipore to remove any impurity.

Preparation and Characterization of HAP Nanocrystals

The smaller particle sized HAP nanocrystals used in this study were synthesized by a chemical precipitation method,[12] which was considered to be an effective route to obtain HAP nanocrystals with lower crystallinity, smaller size, and greater surface area.[13] The procedure employed for the synthesis of the smaller particle sized HAP nanocrystals was as follows. First, 0.5 M $Ca(NO_3)_2$ solutions and 0.3 M $(NH_4)_2HPO_4$ solutions were obtained by dissolving $Ca(NO_3)_2 \cdot 4H_2O$ and $(NH_4)_2HPO_4$ in distilled water, respectively, and the pH of both solutions was adjusted to 11.0 by adding ammonia solution. The reactant molar ratio of Ca/P was kept at 1.667. The solution of $(NH_4)_2HPO_4$ was dropwise added into the $Ca(NO_3)_2$ solution, and the white suspension was obtained. During the addition, the pH of the suspension was maintained at 11.0 using ammonia solution. After the complete addition, the suspension was further stirred for 24 hr and centrifuged at 4000 rpm for 10 min. Then the precipitated HAP was washed with deionized water until the pH was 7.0. The resultant powders were dried at 80°C for 24 hr, and then calcined at 100°C for 1 hr to obtain HAP nanocrystals.

The phase purity and crystallinity of the as-synthesized samples were determined by powder X-ray diffraction (XRD) using Cu Kα ($\lambda = 1.5405$ Å) radiation on a Rigaku D/max-IIIB X-ray powder diffractometer. The morphology and size of the synthesized HAP nanocrystals were characterized by a Hitachi Model H-7650 transmission electron microscope (TEM).

Plant Material and Collection of Plant Xylem Sap Samples

The choice of pokeweed (*Phytolacca americana L.*) was made because of its ability to exudate xylem fluid under slight negative pressure. Furthermore, it could be used for the phytoextraction of manganese and rare earth elements from contaminated soils.[14–16] Seeds of pokeweed were treated with concentrated sulfuric acid for 15 min to promote germination,[17] then they were sown in sand in a plastic basin. After germination in the dark (about 1 week), the seedlings were cultivated in hydroculture in a green house with supplementary light to provide a 16-hr photoperiod and with the temperature controlled at 25°C day/20°C night and a relative humidity (RH) of 70–75%. Hoagland's nutrient solution was used,[15] which consists of 5 mmol/L $Ca(NO_3)_2 \cdot 4H_2O$, 5 mmol/L KNO_3, 2 mmol/L $MgSO_4 \cdot 4H_2O$, 1 mmol/L KH_2PO_4, 0.1 mmol/L Fe-EDTA, 46 µmol/L H_3BO_3, 5 µmol/L $MnCl_2 \cdot 4H_2O$, 0.32 µmol/L $CuSO_4 \cdot 5H_2O$, 0.76 µmol/L $ZnSO_4 \cdot 7H_2O$, 0.5 µmol/L Na_2MoO4. Nutrient solutions were adjusted to pH 6.0 with 1 mmol/L 2-(N-morpholino)-ethane sulfonic acid (MES) and 0.5 mmol/L NaOH. The hydroculture solutions were aerated continuously and replaced every 3 days to prevent nutrient depletion and pH change. Xylem saps were collected from 6 to 8 week old plants, according to the method described by Morita et al.[18] The plants were cut with a sterilized razor blade just below the cotyledon internode, and then the decapitated stumps were fitted with a test tube (10 mL) maintaining negative pressure by means of a hand operated vacuum pump. During the suction procedure, the test tubes used to collect the saps were kept on ice. The saps were collected within 2 hr and then stored at −20°C after filtration on a 0.45 µm Millipore filter.

Instrumentation and Chromatographic Conditions

The chromatographic analyses were carried out with a Shimadzu LC-20A HPLC system (Shimadzu, Japan) consisting of a binary LC-20AT HPLC pump, a Model 7725 injection valve with a 20 μL loop, and an SPD-20A UV-Vis detector. Data processing was performed with a HW-2000 chromatography workstation (Nanjing Qianpu Software, China).

Chromatographic separations were performed on a reversed-phase C_{18} column (250 mm × 4.6 mm, 5 μm, Shim-pack VP-ODS column). The mobile phase was 5.0 g/L $(NH_4)_2HPO_4$ adjusted to pH 2.5 with H_3PO_4 and filtered through a 0.45 μm Millipore filter (Milford, MA, USA). The separation was carried out by isocratic elution with a flow rate of 1.0 mL/min, and the column temperature was maintained constant at 30°C. The UV detector was set at 210 nm with a sensitivity of 0.02 absorbance units, full scale. Quantitation was based on the peak area measurement.

Analytical Procedures

The oxalic, malic, and citric acids in xylem saps were extracted by solid-phase extraction with the smaller particle sized HAP nanocrystals according to the method described in our previous work.[1] Briefly, a 0.1 g HAP nanocrystals was shaken with an appropriate volume of the xylem sap samples at room temperature, after contact for 60 min, the suspensions were centrifuged at 4000 rpm for 10 min and filtered through a 0.45 μm Millipore filter. Afterwards, organic acids adsorbed by the HAP nanocrystals were eluted with 100 mmol/L $(NH_4)_2HPO_4$ solution. And the analytes in the eluent were determined by RP-HPLC at the optimum chromatographic conditions.

RESULTS AND DISCUSSION

Characteristics of HAP Nanocrystals

The XRD pattern of the as-synthesized HAP nanocrystals was shown in Figure 1. It was in good agreement with the reference pattern of pure hydroxyapatite (JCPDS no. 09-0432). The wide peaks indicated that HAP with a very small size and a low degree of crystallinity had been obtained, which was known to be a typical characteristic of calcium phosphate synthesized by the wet method. Figure 2 shows the TEM micrograph of the synthetic HAP samples. It indicates that the particle size of the HAP nanocrystals was less than 30 nm, which is consistent with the XRD data

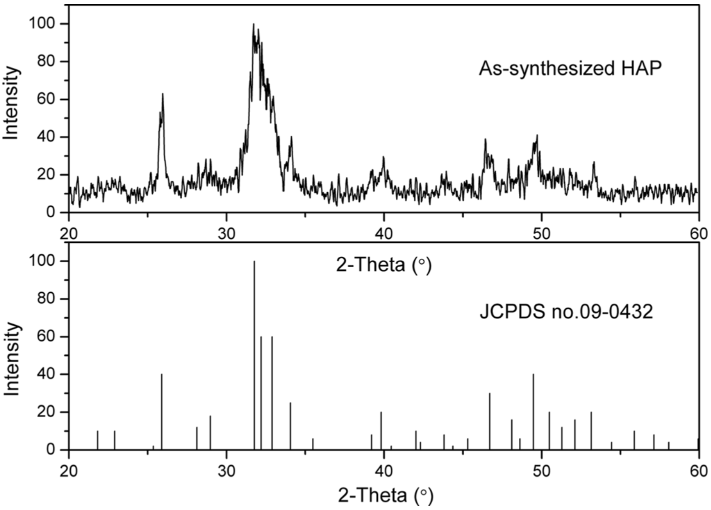

FIGURE 1 XRD patterns of the as-synthesized HAP nanocrystals and the reference pattern of pure hydroxyapatite (JCPDS no. 09-0432).

and previous literature.[12,13] The as-synthesized smaller particle sized HAP nanocrystals were used as adsorbents to extract organic acids from xylem sap samples.

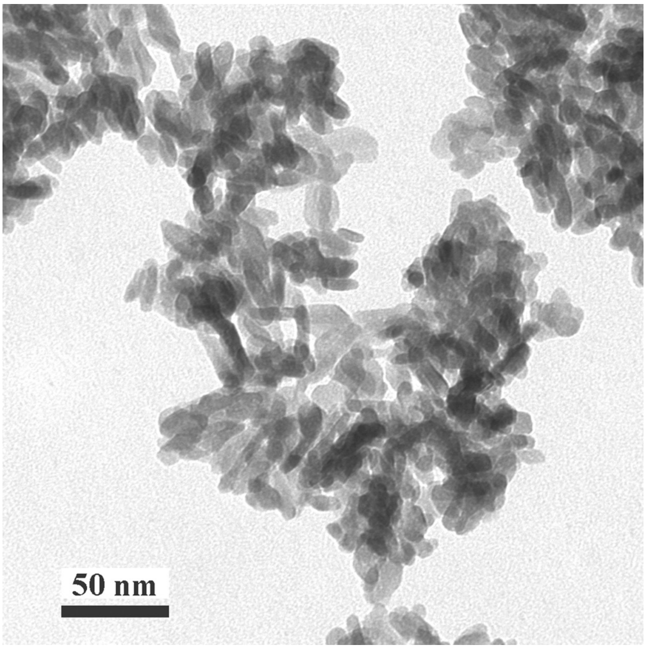

FIGURE 2 The TEM micrograph of the synthetic HAP nanocrystals.

Optimization of Solid-Phase Extraction Conditions

In this work, our particular interest focused on the development of an efficacious method to extract oxalic, malic, and citric acids simultaneously by solid-phase extraction with smaller particle sized HAP nanocrystals; therefore, it was essential to investigate the adsorption and desorption behavior of organic acids on HAP nanocrystals.

Adsorption Studies

The effects of several parameters, such as pH, contact time, and the amount of adsorbents on the adsorption of organic acids were studied. The pH value played an important role with respect to the adsorption of different organic acids on the surface of HAP nanocrystals, and in this study the pH level was investigated for each organic acid. As shown in Figure 3, the adsorption percent of organic acids decreased continuously with increasing pH, which was in good agreement with our previous work.[1] A quantitative adsorption of oxalic acid by the smaller particle sized HAP nanocrystals was found in the pH range of 2.0–5.0, while malic and citric acids could be adsorbed quantitatively at pH 2.0–4.0 in the present study. However, it was previously reported that malic and citric acids could not be adsorbed by nanocrystalline HAP and the adsorption of oxalic acid was found in a very narrow pH range of 2.0–3.0. The relatively large size of HAP and the repulsion to the relatively low polarity organic acids

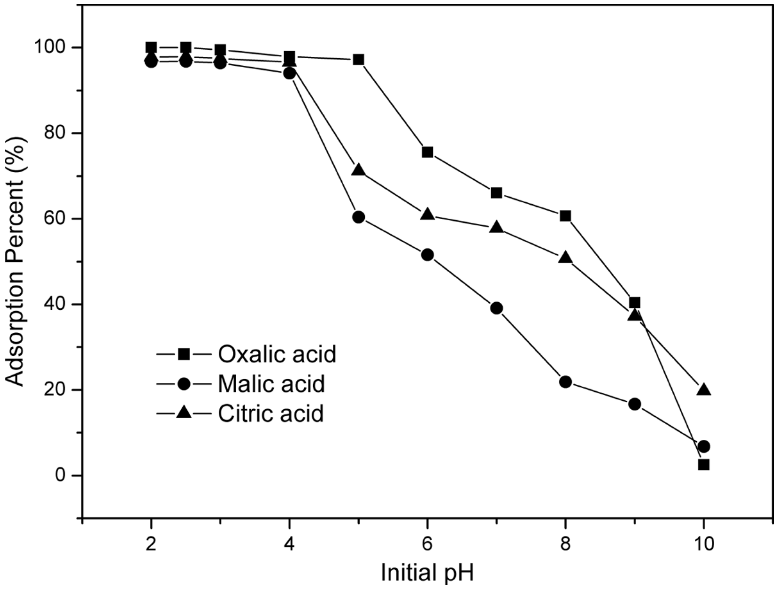

FIGURE 3 Effect of pH on the adsorption of the studied organic acids on HAP nanocrystals.

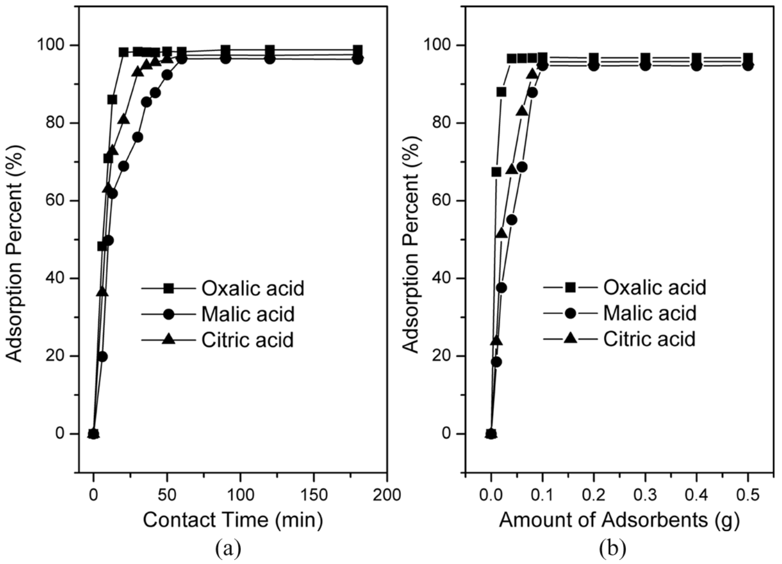

FIGURE 4 Effect of contact time (a) and the amount of adsorbents (b) on the adsorption of organic acids.

might be responsible for the low adsorption ability to malic and citric acids. Figure 4a and 4b shows the effects of contact time and the amount of adsorbents on the adsorption of organic acids, respectively. It indicates that organic acids could be adsorbed quantitatively by the smaller particle sized HAP nanocrystals within 60 min, and 0.1 g HAP nanocrystals were sufficient for the adsorption of organic acids. These results meant that oxalic, malic, and citric acids could be extracted simultaneously by the smaller particle sized HAP nanocrystals. It could be, therefore, deduced that the smaller particle sized HAP nanocrystals had a higher ability to adsorb organic acids. As reported by our group and other authors,[10,12] the decrease in particle size of HAP nanocrystals led to an increase in surface area, accompanied by a concomitant rise in the quantity of active sites on the surface, which would cause a remarkable enhancement of the adsorption ability. As a result, the repulsion of the polar inorganic HAP to relatively low polarity organic acids would be overcome by the higher adsorption ability of the smaller particle sized HAP nanocrystals. Thus, the simultaneously extraction of oxalic, malic, and citric acids could be achieved by the smaller particle sized HAP nanocrystals in the present study.

Desorption Studies

In order to recover the organic acids adsorbed by the smaller particle sized HAP nanocrystals quantitatively, the $(NH_4)_2HPO_4$ solution was used as an eluent.[1] The desorption of organic acids by $(NH_4)_2HPO_4$ solution

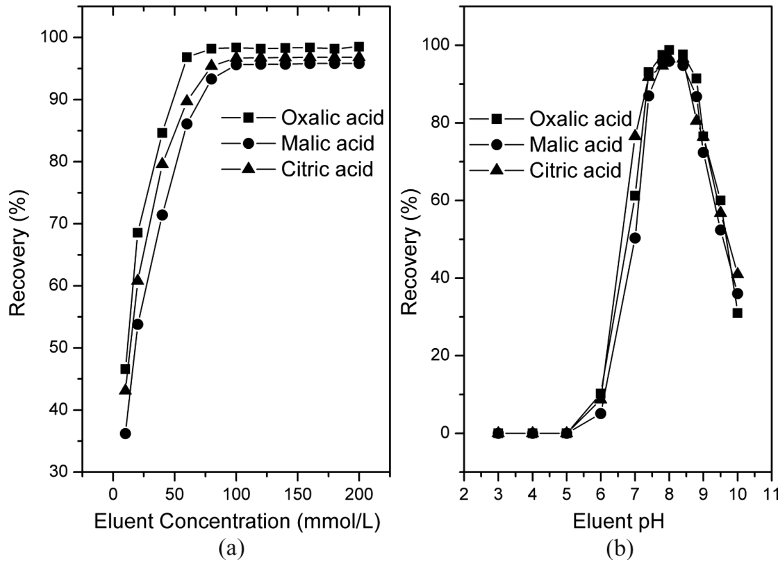

FIGURE 5 Effects of eluent concentration (a) and pH (b) on the recovery of organic acids.

as functions of eluent concentration and pH are given in Figure 5a and 5b, respectively. It was demonstrated that the recovery of organic acids increased sharply with the eluent concentration and reached 98% at 100 mmol/L; therefore, a 100 mmol/L $(NH_4)_2HPO_4$ solution was chosen for desorption of organic acids from the smaller particle sized HAP nanocrystals. As shown in Figure 5b, organic acids could not be desorbed at $pH < 6.0$, the recovery increased sharply above pH 6.0, and had a complete desorption at pH 8.0. In addition, organic acids could be desorbed quantitatively within 120 min according to the preliminary experiments. Thus, the smaller particle sized HAP nanocrystals loaded with organic acids were treated with 100 mmol/L $(NH_4)_2HPO_4$ solution at pH 8.0 for 120 min in order to recover the organic acids.

Optimization of Chromatographic Conditions

Chromatographic Separations

First of all, working with standard solutions, oxalic, malic, and citric acids were separated and identified by the present chromatographic method. Organic acid peaks were identified by comparing their retention times in the sample solution with that of the standard solution, and the concentrations were quantified using calibration curves. Figure 6 shows the chromatogram of a mixed standard solution with three organic acids (oxalic, malic, and citric acid) separated and identified by this method. The typical chromatogram of the xylem sap of pokeweed is shown in

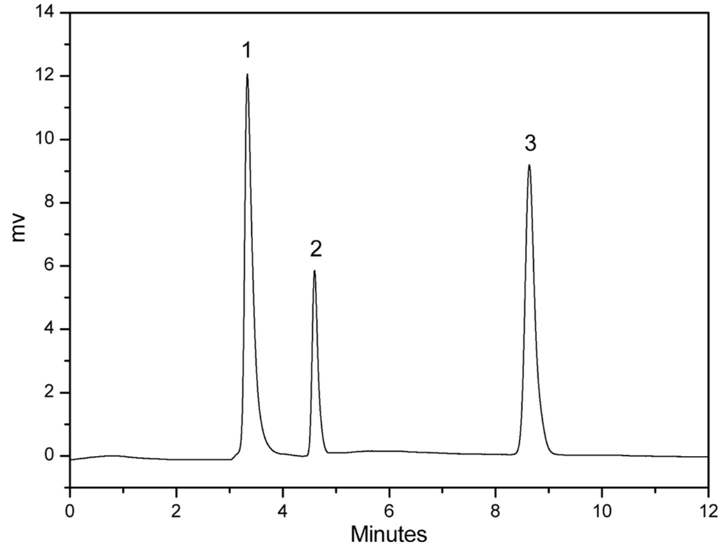

FIGURE 6 RP-HPLC chromatogram of a standard mixture of three organic acids. Peaks: 1 = oxalic acid, 2 = malic acid, 3 = citric acid.

Figure 7a. We could see the determination of oxalic acid was impossible due to the presence of nitrate ions in high concentrations in xylem saps. In our previous work,[1] the interference from nitrate on oxalic acid could be avoided by solid-phase extraction with HAP nanocrystals. However, the relatively large sized HAP could only adsorb oxalic acid due to its lower adsorption ability, so the determination of organic acids in xylem saps had to include analyses of malic and citric acids in the supernatant after solid-phase extraction with HAP as well as oxalic acid in the eluent,[2] which would increase the time demand for separation and financial cost compared to simultaneous extraction and determination of organic acids. In this study the smaller particle sized HAP nanocrystals were employed as solid-phase extractor, and the oxalic, malic, and citric acids in xylem saps of pokeweed were simultaneously extracted and determined by RP-HPLC. As can be seen in Figure 7b and 7c, the proposed method was suitable for the simultaneous determination of organic acids in xylem saps.

Influence of the Column Temperature

The column was thermo stated at several different temperatures. The best results were obtained at a temperature of 30°C.

Influence of Mobile Phase

Solutions of $(NH_4)_2HPO_4$ buffered with H_3PO_4, recommended in the literature[3,19] for determination of organic acids were used. Moreover, the

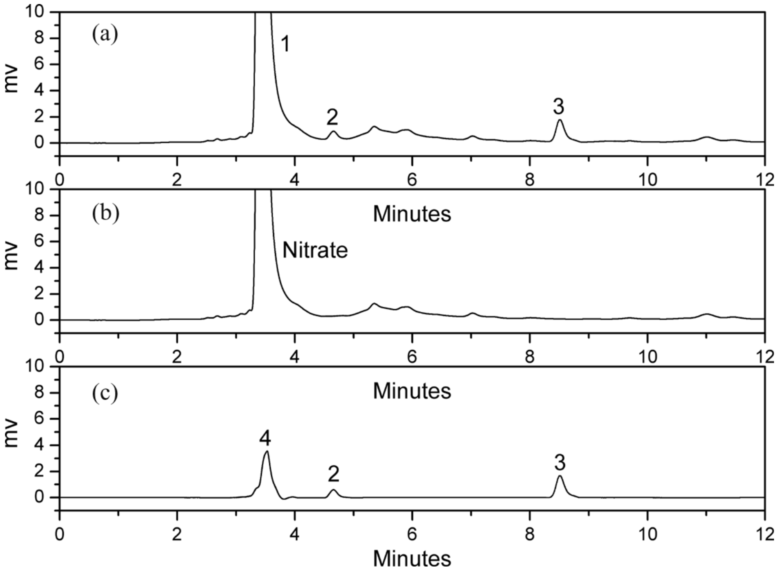

FIGURE 7 (a) The representative RP-HPLC chromatogram of xylem sap sample of pokeweed. 1 = oxalic acid and nitrate, 2 = malic acid, 3 = citric acid, 4 = oxalic acid. (b) The chromatogram of xylem sap sample after solid-phase extraction with HAP nanocrystals. (c) The chromatogram of organic acids in xylem saps of pokeweed extracted by the proposed solid-phase extraction procedure.

concentrations could affect the forms of organic acids in the mobile phase and the ion intensity and, consequently, the separation performance of the chromatographic column. Several concentrations of $(NH_4)_2HPO_4$ were tested and, finally, 5.0 g $(NH_4)_2HPO_4$ in the mobile phase was selected.

Influence of Mobile Phase pH and Flow Rate

Several mobile phase flow rates (0.5–1.5 mL/min) and pH values between 2.0 and 3.0 were tested, and a pH 2.5 and a flow rate of 1.0 mL/min were selected. Several wavelengths from 200 to 240 nm were tested and, finally, the optimum wavelength for determination was 210 nm.

Chromatographic Analytical Characteristics

Optimized chromatographic conditions were set and the following analytical characteristics were evaluated: detection and quantification limits, calibration curves, precision, and recovery.

Detection and Quantification Limits

The detection limit was calculated as $s_b + 3s$, where s_b was the average signal of 10 blank injections and s was the standard deviation. The quantification limit was calculated as $s_b + 10s$.[20] Table 1 shows the

TABLE 1 Analytical Characteristics of the Present RP-HPLC Method

Organic Acids	Regression Equation	Coefficient (r^2)	Detection Limit (mg/L)	Quantification Limit (mg/L)	Intraday RSD (%, n = 5)	Interday RSD (%, n = 5)
Oxalic	$Y = 13760x + 10312$	0.9992	0.042	0.069	1.5	2.3
Malic	$y = 1008.3x - 1853.4$	0.9994	0.221	0.712	1.3	2.1
Citric	$y = 1308.5x - 928.3$	1	0.417	0.816	2.5	4.7

detection and quantification limits of organic acids analyzed by RP-HPLC. The detection limits ranged from 0.042 mg/L for oxalic acid to 0.417 mg/L for citric acid and the quantification limits ranged from 0.069 mg/L for oxalic acid to 0.816 mg/L for citric acid.

Calibration Curves

Calibration curves were determined for six different concentrations of standard solutions for each organic acid analyzed. Each calibration sample was injected in triplicate. Calibration graphs for each compound were obtained by plotting concentration against peak area and applying the least squares method. Table 1 shows the parameters and correlation coefficients of the calibration plots. Each plot was linear over a wide interval from the detection limit to at least 200 mg/L for oxalic acid, 400 mg/L for malic, and 600 mg/L for citric acid.

Precision

With the standard addition method, the precision was measured using pokeweed xylem sap samples spiked with organic acid standard solutions in the operating range with high (5.0–10.0 mg/L) and low (0.5–2.0 mg/L) concentrations. Intra-day precision (RSD%) of the method was calculated by parallel analysis of the aforementioned samples in five replicate experiments during one day. Inter-day precision (RSD%) was calculated by parallel analysis in five replicate experiments for five consecutive days. The

TABLE 2 Recoveries of Standards Added to Xylem Sap Samples and of Standard Solutions After Solid-phase Extraction Procedure (n = 3)

	Standard Solutions		Spiked Samples	
Organic Acids	Recoveries (%) (mean) ± SD	RSD (%)	Recoveries (%) (mean) ± SD	RSD (%)
Oxalic	99.6 ± 0.72	0.14	95.8 ± 1.15	1.68
Malic	102.1 ± 0.092	0.19	97.9 ± 0.98	1.23
Citric	98.7 ± 1.21	0.30	94.2 ± 3.93	1.84

TABLE 3 Concentrations of Organic Acids in Xylem Saps of Pokeweed

Organic Acids	Concentration (mg/L)
Oxalic	3.26
Malic	5.53
Citric	9.12

results showed that the intra-day RSD values were 1.3%–2.5% and inter-day RSD values were 2.1%–4.7%. The results are shown in Table 1.

Recovery

To establish the efficiency of the organic acids extracted by solid-phase extraction with the smaller particle sized HAP nanocrystals, the method was applied to a mixture of standard solutions. The results are shown in Table 2. This procedure was also performed on a mixture of organic acids added to the xylem sap samples. Table 2 shows the recoveries of these organic acids after applying the solid-phase extraction method to xylem sap samples.

Analysis of Organic Acids in the Xylem Saps of Pokeweed

The xylem sap samples of pokeweed were subjected to the proposed solid-phase extraction procedure with the smaller particle sized HAP nanocrystals, and then oxalic, malic, and citric acids in xylem saps were determined simultaneously by RP-HPLC at the optimized chromatographic conditions. Figure 7c shows the chromatogram of oxalic, malic, and citric acids in xylem saps of pokeweed determined by the present method. The analytical results are shown in Table 3. Compared to the previous work, the proposed method allowed detection and quantification of oxalic, malic, and citric acids in xylem sap samples in one injection.

CONCLUSIONS

A simple and reliable RP-HPLC method was developed and validated for the simultaneous extraction and determination of oxalic, malic, and citric acids in xylem saps of pokeweed after solid-phase extraction with smaller particle sized HAP nanocrystals. The previous work was improved and organic acids in xylem sap samples could be extracted simultaneously by the smaller particle sized HAP nanocrystal, due to its higher adsorption ability compared to the relatively large size HAP. The proposed method fulfilled all the requirements to be identified as a reliable and feasible method, showing good linearity, precision, accuracy, and selectivity. Therefore, this established method is useful for simultaneous quantitative analysis of oxalic, malic, and citric acids in xylem saps of pokeweed and could be adapted to other plant samples.

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