

# Simultaneous Determination of Organic Acids and Nitrate in Xylem Saps of the Hyperaccumulator *Alyssum Murale* by RP-HPLC after Solid-Phase Extraction with Nanosized Hydroxyapatite

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## Abstract

An analytical approach based on solid-phase extraction (SPE) with nanosized hydroxyapatite and reversed-phase high performance liquid chromatography (RP-HPLC) for the simultaneous determination of organic acids (oxalic, malic, malonic, citric, and fumaric) and nitrate in xylem saps of the hyperaccumulator *Alyssum murale* is presented in this study. The optimum experimental conditions for the separation and determination of organic acids and nitrate are studied. The interference from nitrate on oxalic acid in RP-HPLC is eliminated by SPE with nanosized hydroxyapatite, and the simultaneous determination of organic acids and nitrate is achieved by RP-HPLC at the optimum chromatographic conditions. The accuracy of the method is confirmed with an average recovery ranging between 95.2% and 99.8%, the relative standard deviations (RSD) are less than 2.0%. This method is successfully applied to determine the organic acids and nitrate in xylem saps of the hyperaccumulator *Alyssum murale*.

## Introduction

Low molecular weight organic acids containing one or more carboxyl and hydroxyl groups are widely distributed in many plants. Analysis of these organic acids has become increasingly important because they are involved in various physiological activities of plants. Some organic acids such as citric, malic, succinic, and oxalic acids play a key role in the Krebs cycle which is the central energy-yielding cycle of the cell (1). Many of them are believed to be significant for the cation transport (either nutrient or toxic) in xylem vessels (2). In addition, low molecular weight organic acids are one of the most concerned chelators in continuous phytoextraction, which is an environment-friendly and cost-effective technology to remediate the heavy metal-polluted soils and waters by hyperaccumulators

(3). It is generally considered that low molecular weight organic acids contribute not only to metal detoxification but also to metal acquisition, transport, and shoot accumulation (3,4). For example, citric acid has been reported to form complexes with Ni<sup>2+</sup> in xylem of the hyperaccumulator *Alyssum murale* (5) and has been suggested to contribute to Zn<sup>2+</sup> accumulation and tolerance (6). Oxalic acid in the xylem saps may have great importance during the long distance transportation and accumulation of heavy metals like cadmium (7–9) and uranium (10). The success in clarification of these functions depends on the accurate determination of organic acids in xylem saps of hyperaccumulator plants.

*Alyssum murale*, a herbaceous perennial (Brassicaceae family) native to Mediterranean serpentine soils, has been identified as a Ni hyperaccumulator plant with the capacity to accumulate Co from Co-contaminated soils (11–13). The choice of *Alyssum murale* is made because of its importance in phytoremediation and because of its ability to exudate xylem fluid under slight negative pressure. Therefore, determining the concentrations of low molecular weight organic acids and nitrate in xylem saps may be very useful for understanding the mechanisms of metal hyperaccumulation for *Alyssum murale*.

For the simultaneous determination of organic acids in plant samples, many separation methods have been developed, including gas chromatography (GC), ion chromatography (IC), and reversed-phase high performance liquid chromatography (RP-HPLC). None of these methods are without limitations. As low molecular weight organic acids are generally non-volatile, their determinations with GC require a derivatisation step, which is often quite tedious, time-consuming and retards the reproducibility of the analysis (14). IC can be used to determine both the organic and inorganic anions, however, high concentrations of sulfate anions have a severe interference on the determination of oxalic acid (15). Ion exclusion column HPLC has also been used for the separation of organic acids in root exudates and cell saps of buckwheat, after the samples are purified by passing through a cation-exchange resin column

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followed by an anion-exchange resin column, but results are still unsatisfying since the complex pretreatments will unavoidably lead to the loss of organic acids (16,17). Currently, RP-HPLC is widely used for the determination of low molecular weight organic acids in plant parts and foods because of the simplicity, rapidity, and stability of the method (18–21). However, oxalic acid in the xylem sap samples cannot be detected directly due to the relatively low resolution between nitrate and oxalate peak, as well as the high concentrations of nitrate ions in xylem saps, which have significant ultraviolet absorbency at 210 nm, gives an unretained peak, which is large enough to mask the first-eluting oxalic acid (21–24). Despite the fact that the type of eluent, organic modifier, flow rate, column temperature, and ion pair (IP) reagent are taken into consideration (22), the results are still unsatisfactory. Therefore, in order to achieve accurate and reliable results, an efficient method to eliminate the interference from nitrate on oxalic acid is needed.

Recently, SPE is widely used for the separation and clean-up of analytical samples (25). Compared with traditional extraction technologies, it is simple, fast, cost-effective, less polluting to the environment and can easily be automated (26). Numerous substances have been used in this technology, such as organic chelate resin (27), C<sub>18</sub>-bonded silica gel (28), and nanosized materials (29,30). Recently, nanosized materials are new solid materials made of nanoparticles that gained importance in recent years for their special properties. One of the unique properties of nanosized materials is that most of the atoms are on the surface. The surface atoms are unsaturated and can therefore bind with other atoms, and possess highly chemical activity (26,31). Nanosized hydroxyapatite [Ca<sub>10</sub>(PO<sub>4</sub>)<sub>6</sub>(OH)<sub>2</sub>, HAP] powders exhibit greater surface area and adsorption property, compared to bulk materials. It has been successfully applied for the adsorption of phenol from aqueous solution (32); however, there are few reports of the separation of low molecular weight organic acids and inorganic acid with nanosized hydroxyapatite.

In this work, we developed an HPLC method with a new pretreatment for the simultaneous determination low molecular weight organic acids and nitrate in xylem saps of the hyperaccumulator *Alyssum murale*. The interference from nitrate on oxalic acid was avoided by SPE with nanosized hydroxyapatite. Organic acids (oxalic, malic, malonic, citric, and fumaric) and nitrate were determined simultaneously by RP-HPLC combined with a UV-vis detector. Application of the method is suitable for determination of organic acids and nitrate in xylem sap samples. As we know, few studies on the determination of organic acids and nitrate in xylem saps using SPE purification with nanosized hydroxyapatite have been reported.

## Experimental

### Reagents and standard solutions

All reagents used were of analytical-reagent grade. Oxalic, malic, malonic, citric acid, and fumaric acids were obtained

from Sinopharm Group Chemical Reagent Co., Ltd (Shanghai, China), and the other reagents were purchased from Nanjing Chemical Reagent Company (Nanjing, China). Deionized water from a Millipore Milli-Q (Billerica, MA) system was used for all solutions, dilution and the mobile phase.

Stock standard solutions were prepared by dissolving organic acids and nitric acid in deionized water and were stored in 4°C refrigerator. Analytical standard solutions were prepared from these stock solutions by serial dilution.

### Preparation and characterization of nanosized hydroxyapatite materials

The nanosized hydroxyapatite materials used in the SPE procedure were prepared by the thermal decomposition of precursors. This method was described in detail in our previous work (33). Appropriate amounts of Ca(NO<sub>3</sub>)<sub>2</sub> × 4H<sub>2</sub>O, (NH<sub>4</sub>)<sub>2</sub>-EDTA, and NH<sub>4</sub>H<sub>2</sub>PO<sub>4</sub> aqueous solutions were mixed together at a molar ratio of EDTA:Ca:P = 2.667:1.667:1, and stirred until a homogeneous solution was formed for ~5 min. The final pH of the solution was controlled to 5–6 by the addition of ammonia solution. After slowly evaporating the solvent, the precursors were obtained and then were dried at 80°C. The dried precursors were calcined at 800°C for 4 h in air to obtain nanosized hydroxyapatite materials.

The phase purity and crystallinity of the as-synthesized samples were determined by powder X-ray diffraction (XRD) using Cu Kα (λ = 1.5405 Å) radiation on a Rigaku D/max-III B X-ray powder diffractometer (Tokyo, Japan). The morphology and size of the synthesized nanosized hydroxyapatite were characterized by a Hitachi Model H-7650 transmission electron microscope (TEM) (Tokyo, Japan).

### Plant material and collection of plant xylem sap samples

Seeds of *Alyssum murale* were sown in trays containing a mixture of vermiculite and perlite moistened with deionized water. After germination in the dark (about 1 week), vermiculite and perlite were washed from the roots, and the seedlings were cultivated in hydroculture in a green house with supplementary light to provide a 16 h photoperiod and with the temperature controlled at 25°C. The hydroculture solutions contained the following composition: 1 mmol/L KNO<sub>3</sub>, 1.5 mmol/L Ca(NO<sub>3</sub>)<sub>2</sub>, 0.5 mmol/L MgSO<sub>4</sub>, 20 μmol/L KH<sub>2</sub>PO<sub>4</sub>, 1 μmol/L H<sub>3</sub>BO<sub>3</sub>, 0.7 μmol/L MnSO<sub>4</sub>, 0.5 μmol/L ZnSO<sub>4</sub>, 0.01 μmol/L (NH<sub>4</sub>)<sub>6</sub>Mo<sub>7</sub>O<sub>24</sub>. Nutrient solutions were adjusted to pH 6.1 with 1 mmol/L 2-(N-morpholino)-ethane sulfonic acid (MES) and 0.5 mmol/L NaOH (34). 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 Wei et al. (15). 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 h and then stored at -70°C after filtration on a 0.45-μm filter.

### Instrumentation and chromatographic conditions

The HPLC analyses were carried out on a LC-20AT Shimadzu liquid chromatograph equipped with an SPD-20A Shimadzu UV-vis detector (Kyoto, Japan). A reversed-phase C18 (Shim-pack VP-ODS 250 × 4.6 mm, 5 μm particle size) column connected with a C18 (Shim-pack GVP-ODS 10 × 4.6 mm, 5 μm particle size) guard column were used. The mobile phase was a buffer solution containing 50 mmol/L (NH<sub>4</sub>)<sub>2</sub>HPO<sub>4</sub> adjusted to pH 2.5 with H<sub>3</sub>PO<sub>4</sub> and was filtered through a 0.45-μm membrane filter supplied by XinYa Corporation (Shanghai, China). This mobile phase must be prepared fresh daily. 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 25°C. The optimum wavelength for determination was 210 nm with a sensitivity of 0.02 absorbance unit, full scale. The injection volume was 20 μL and each sample was injected in triplicate. The determination of acids was done in peak area mode. The detector signal was recorded on a HW-2000 chromatographic workstation.

materials were sphere-like particles with almost no amorphous constituents and weakly aggregated. The grain sizes of the samples were distributed in the range of 40–60 nm. This TEM observation on the shape of particles agreed with our XRD

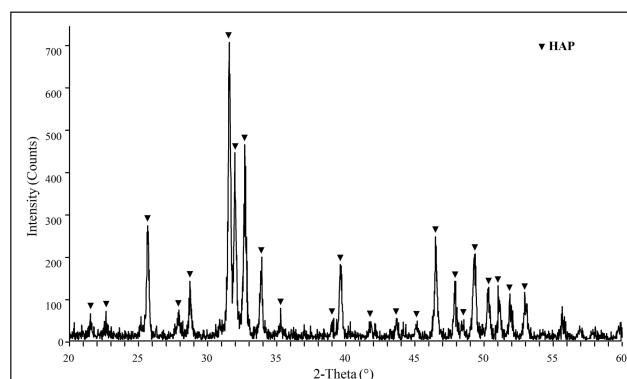


Figure 1. The XRD patterns of the synthetic nanosized hydroxyapatite samples.

### Analytical procedures

Organic acids and nitrate in the xylem samples were identified and determined by RP-HPLC after SPE with nanosized hydroxyapatite. First, the xylem samples were diluted with distilled water in a ratio of 1:5, and the pH was adjusted to approximately 2.5 using 0.1 mol/L H<sub>2</sub>SO<sub>4</sub>, then 0.1 g nanosized hydroxyapatite was shaken with xylem sap samples at room temperature. After 60 min, the suspensions were centrifuged at 3000 rpm for 10 min and filtered through a 0.45-μm filter, the filtrates were used for the RP-HPLC analysis. Finally, the organic acid adsorbed by nanosized hydroxyapatite was eluted with 2.0 mL 0.1 mol/L (NH<sub>4</sub>)<sub>2</sub>HPO<sub>4</sub> and determined by RP-HPLC at the optimum chromatographic conditions.

Table I. Analytical Characteristics of the Present RP-HPLC Method.

Organic acids and nitrate	Regression equation	Coefficient (r <sup>2</sup> )	Detection Limit (mg/L)	Quantification limit (mg/L)	Intraday RSD* (% , n = 5)	Interday RSD* (% , n = 5)
Nitrate	y = 8.1372x + 0.4262	0.9996	0.0012	0.0064	—*	—*
Oxalic	y = 0.2264x - 0.3928	0.9990	0.042	0.069	1.5	2.3
Malic	y = 0.0193x - 0.0433	0.9990	0.221	0.712	1.2	2.2
Malonic	y = 0.0133x - 0.012	0.9990	0.214	0.533	1.3	3.1
Citric	y = 0.0157x - 0.0346	0.9991	0.418	0.876	2.4	4.6
Fumaric	y = 1.2862x + 0.0399	0.9991	0.0022	0.019	2.0	3.0

\* The intraday and interday precisions (RSD %) were evaluated in the absence of nitrate due to the low resolution between nitrate and oxalic acid.

### Results

#### Characteristics of the nanosized hydroxyapatite

The XRD pattern of synthetic hydroxyapatite is shown in Figure 1. The prepared samples were in good agreement with the Powder Diffraction File [Ca<sub>10</sub>(PO<sub>4</sub>)<sub>6</sub>(OH)<sub>2</sub>, PDF Card No. 09-0432], and no characteristic peaks of impurities, such as calcium hydroxide and calcium phosphates, were observed, meaning that pure and well crystallized samples were obtained under the present experimental conditions. By applying the Scherrer formula to the full width at half maximum of the diffraction peaks (35), we could calculate the average particle sizes of the synthetic hydroxyapatite as 56.4 nm.

Figure 2 showed the TEM micrograph of the synthetic hydroxyapatite samples. It indicated that the nanosized

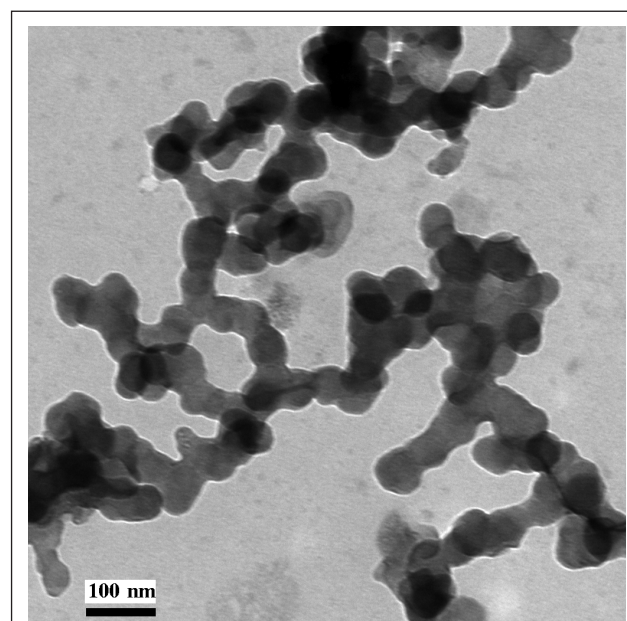


Figure 2. The TEM micrograph of the synthetic nanosized hydroxyapatite samples.

studies. The synthetic nanosized hydroxyapatite particles were used as the SPE sorbents.

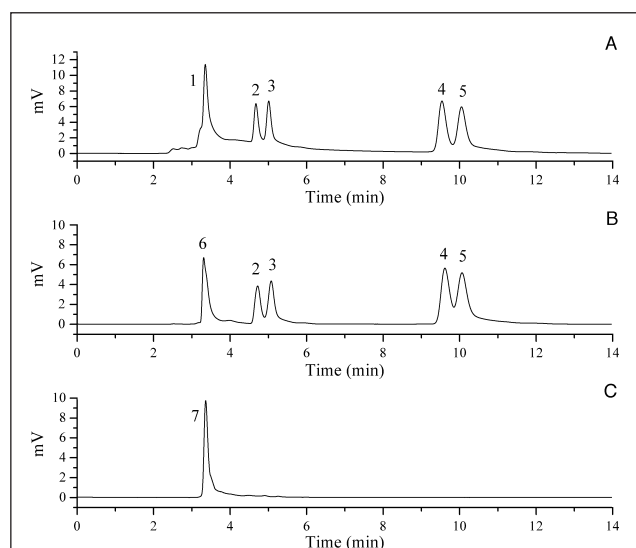
### Chromatographic analysis of mixed standards

The organic acids and nitrate were identified by comparison of their retention time with those of standards. An external calibration method with freshly prepared mixed standard solutions was used for quantitative determination of the acids. Figure 3A shows the chromatogram of a mixed standard solution of five organic acids (oxalic, malic, malonic, citric, and fumaric acids) and nitrate being separated by this reversed-phase high performance liquid chromatographic method. However, oxalic acid could not be separated due to the presence of nitrate.

In this study, a SPE procedure was developed for the further separation of oxalic acid and nitrate. It was the first time nanosized hydroxyapatite had been used to separate organic acids and nitrate in xylem saps. The chromatogram of mixed standard solution after SPE with nanosized hydroxyapatite is shown in Figure 3B, and the nitrate peak was identifiable. Figure 3C shows the chromatogram of oxalic acid desorbed from nanosized hydroxyapatite. From the results previously mentioned, it was concluded that the interference from nitrate on oxalic acid in RP-HPLC could be avoided effectively by the SPE procedure. Therefore, organic acids and nitrate were simultaneously determined by RP-HPLC. The proposed SPE procedure could be successfully applied to the xylem sap samples of *Alyssum murale*.

### Chromatographic analytical characteristics

Optimized chromatographic conditions were set and the following analytical characteristics were evaluated: detection



**Figure 3.** RP-HPLC chromatogram of a standard mixture of five organic acids and nitrate, (A); the chromatogram of mixed standard solution after solid-phase extraction with nanosized hydroxyapatite, (B); the chromatogram of oxalic acid desorbed from nanosized hydroxyapatite (C). Peaks: 1 = oxalic acid and nitrate, 2 = malic acid, 3 = malonic acid, 4 = citric acid, 5 = fumaric acid, 6 = nitrate, and 7 = oxalic acid. The concentrations of oxalic, malic, malonic, citric, fumaric acid, and nitrate in the standard mixture are 10.0, 50.0, 50.0, 100.0, 0.5, and 0.1 mg/L, respectively.

and quantitation limits, calibration curves, precision, and recovery.

### Detection and quantitation 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  $s_b + 10s$  (36). Table I shows the detection and quantitation limits of organic acids analyzed by RP-HPLC. The detection limits ranged from 0.0012 mg/L for nitrate ion to 0.418 mg/L for citric acid and the quantification limits ranged from 0.0064 mg/L for nitrate ion to 0.876 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 I 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 malonic acids, 600 mg/L for citric acid, 10 mg/L for fumaric acid and nitrate.

### Precision

With the standard addition method, the precision was measured using *Alyssum murale* 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.

**Table II. Recoveries of Standards Added to Xylem Sap Samples and of Standard Solutions after Solid-Phase Extraction Procedure ( $n = 3$ ).**

Organic acids and nitrate	Recoveries (%) (mean) $\pm$ SD		Recoveries (%) (mean) $\pm$ SD	
	Standard solutions	RSD (%)	Spiked samples	RSD (%)
Nitrate	100.04 $\pm$ 0.078	0.08	99.8 $\pm$ 0.058	1.96
Oxalic	99.6 $\pm$ 0.72	0.14	95.6 $\pm$ 1.15	1.66
Malic	102.1 $\pm$ 0.092	0.19	96.9 $\pm$ 0.98	1.04
Malonic	100.2 $\pm$ 0.13	0.21	97.0 $\pm$ 2.02	1.68
Citric	98.7 $\pm$ 1.21	0.30	95.2 $\pm$ 3.93	1.54
Fumaric	99.4 $\pm$ 0.99	0.10	96.4 $\pm$ 2.91	1.49

**Table III. Concentrations of Nitrate and Organic Acids in Xylem Saps of the Hyperaccumulator *Alyssum murale*.**

Organic acids and nitrate	Concentration (mg/L)
Nitrate	610.4
Oxalic	2.677
Malic	21.33
Malonic	30.61
Citric	5.726
Fumaric	ND*

\* Not detectable.

Intraday precision (RSD %) of the method was calculated by parallel analysis of the above samples in five replicate experiments during one day. Interday precision (RSD %) was calculated by parallel analysis in five replicate experiments for five consecutive days. The results showed that the intraday RSD values were 1.2%–2.4% and interday RSD values were 2.2%–4.6%. The results are shown in Table I.

#### Recovery

To establish the efficiency of the organic acids SPE, the method was applied to a mixture of standard solutions. The results are shown in Table II. This procedure was also performed on a mixture of organic acids added to the xylem sap samples. Table II shows the recoveries of these organic acids after applying the SPE method to xylem sap samples.

#### Analysis of organic acids and nitrate in the xylem saps of *Alyssum murale*

Organic acids and nitrate in the xylem saps of *Alyssum murale* have been determined according to the steps described in the analytical procedures. Figure 4 shows the representative chromatogram of xylem sap of *Alyssum murale*. It indicated that malic, malonic, and citric acids in xylem saps could be separated and identified in one injection by RP-HPLC. Oxalic acid could not be detected due to the relatively low resolution between nitrate and oxalate peak, as well as the high concentrations of nitrate ions in xylem saps (21–24). After the application of SPE, oxalic acid in the xylem saps was successfully determined by RP-HPLC, and Figure 5 shows the chromatogram of oxalic acid in the xylem saps of *Alyssum murale* after the elimination of nitrate by the present method. The analytical results were shown in Table III.

## Discussion

#### Optimization of SPE conditions

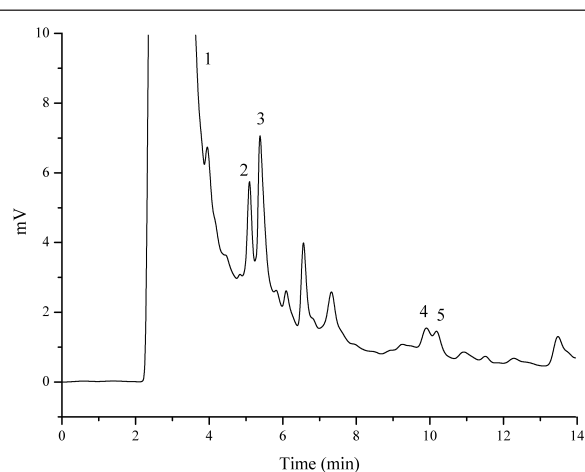
In this work, our particular interest focused on the development of an effective method to eliminate the interference from nitrate on oxalic acid in RP-HPLC, therefore, it was essential to investigate the adsorption and desorption behavior of organic acids and nitrate on nanosized hydroxyapatite.

#### Optimization of adsorption

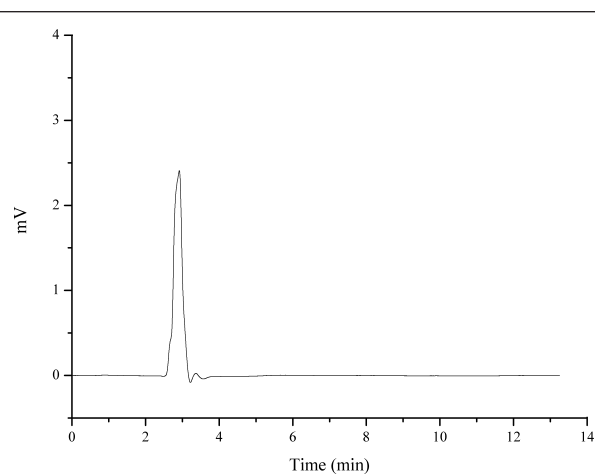
The influences of different parameters such as solution pH, contact time, and the amount of nanosized hydroxyapatite sorbents on the adsorption of organic acids and nitrate were studied and discussed. Figure 6 shows the effect of pH on the adsorption of organic acids and nitrate by nanosized hydroxyapatite. It could be seen that the adsorption percent of oxalic acid decreased continuously with increasing pH, oxalic acid was adsorbed poorly at pH > 8.0. A quantitative adsorption (> 98%) was found in the pH range of 2.0–4.0, after 60 min of contact time at the initial pH 2.0–3.0, oxalic acid in the solution could be adsorbed completely. For other organic acids, such as malic acid, malonic acid, citric acid, fumaric acid and nitrate, no quantitative adsorption was observed.

The results could be explained as follows: nanosized hydroxyapatite was a polar inorganic material, which had relatively high affinity to polar organic molecules (37–39). The ability of an organic acid adsorbed by hydroxyapatite depended on its polarity and the coordination ability with surface calcium ions. Highly polar oxalic acid could be adsorbed by nanosized hydroxyapatite through strong coordination with surface calcium ions. But the other relatively low polar organic acids (malic, malonic, citric, and fumaric acids) would be repulsed by the inorganic nanosized hydroxyapatite, and the affinity of these organic acids to calcium was lower than oxalic acid (40).

Effects of contact time, and the amount of nanosized hydroxyapatite sorbents on the adsorption of oxalic acid were studied and the results are shown in Figure 7A. The results revealed an adsorption took place in two distinct steps. The first step (first 10 min) involved a rapid oxalic acid adsorption and quantitatively predominant. The second one (10–60 min) exhibited a subsequent adsorption continued until equilibrium was reached. It was slow and quantitatively insignificant step. Therefore, a shaking time of 60 min was considered to be suit-



**Figure 4.** The representative RP-HPLC chromatogram of xylem sap samples of *Alyssum murale*. 1 = oxalic acid and nitrate, 2 = malic acid, 3 = malonic acid, 4 = citric acid, 5 = fumaric acid.



**Figure 5.** The chromatogram of oxalic acid in the xylem sap samples of *Alyssum murale* after the interference of nitrate is eliminated.

able for the SPE of oxalic acid. The adsorption of oxalic acid as a function of the amount of nanosized hydroxyapatite was given in Figure 7B. The adsorbent weight was varied from 0.02 to 1.0 g/10 mL and equilibrated for 120 min. The experimental results revealed that adsorption percent increased up to the optimum weight of 0.1 g for oxalic acid beyond which the adsorption percent did not change with the adsorbent weight.

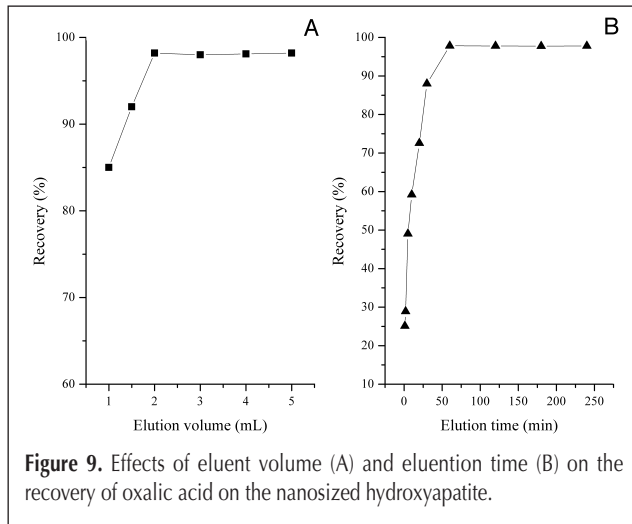
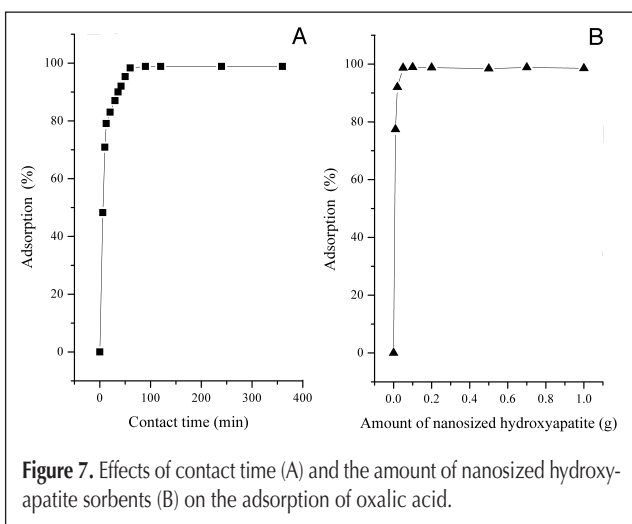
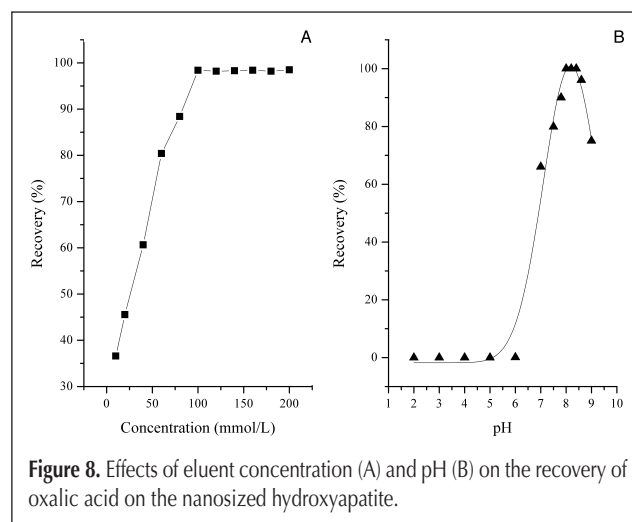
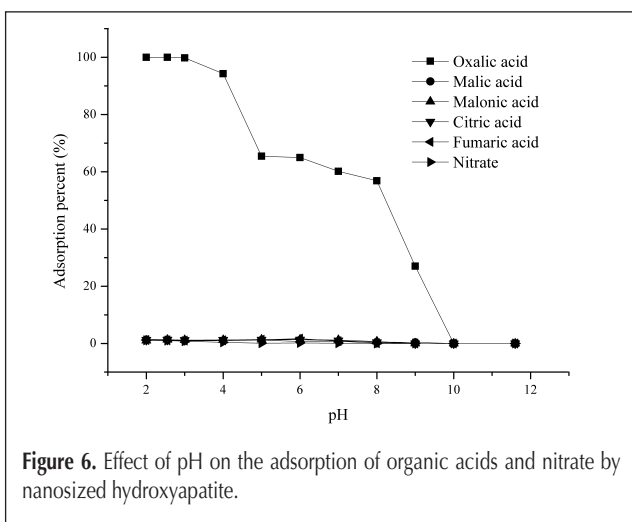
Therefore, it was concluded that the proposed SPE procedure was effective for the separation of oxalic acid from mixed nitrate and organic acids solutions, due to its good selectivity to oxalic acid.

#### Optimization of desorption

In order to recover the oxalic acid adsorbed by the nanosized hydroxyapatite fast and quantitatively, the  $(\text{NH}_4)_2\text{HPO}_4$  solution was used as eluent (22–24). The desorption of oxalic acid by  $(\text{NH}_4)_2\text{HPO}_4$  solution as functions of  $(\text{NH}_4)_2\text{HPO}_4$  concentration and pH were given in Figure 8A and 8B. It was clear that the recovery increased sharply with  $(\text{NH}_4)_2\text{HPO}_4$  concentration and reached to 98% at 0.1 mol/L of the eluent. Therefore, 0.1 mol/L  $(\text{NH}_4)_2\text{HPO}_4$  was chosen for desorption of oxalic acid from the loaded nanosized hydroxyapatite. As shown in Figure

8B, oxalic acid 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. Thus, the effects of eluent volume and elution time on the recovery of oxalic acid were studied by keeping the  $(\text{NH}_4)_2\text{HPO}_4$  concentration of 0.1 mol/L and pH 8.0. It was found that with 2.0 mL of eluent contain 0.1 mol/L  $(\text{NH}_4)_2\text{HPO}_4$  (pH= 8.0) was sufficient to elute oxalic acid quantitatively, and the complete desorption of oxalic acid could be achieved within 60 min. The results were shown in Figure 9A and 9B.

When the standard mixture of organic acids and nitrate were subjected to the SPE procedure, the interference from nitrate to oxalic acid was avoided effectively due to the selective adsorption of oxalic acid by nanosized hydroxyapatite, and then the adsorbed oxalic acid could be desorbed and recovered quantitatively by 0.1 mol/L  $(\text{NH}_4)_2\text{HPO}_4$ . Therefore, the simultaneous determination of organic acids and nitrate could be achieved. The results are shown in Figure 3. Based on the aforementioned results, it could be seen that the application of SPE with nanosized hydroxyapatite provided an effective method for elimination of the interference from nitrate on oxalic acid in RP-HPLC.



### Evaluation of enrichment factor

It was generally considered that the enrichment factor of biological samples was not as high as other samples due to the low volume of the samples. In the present paper, the enrichment factor of the xylem sap samples was 1–5. Meanwhile, different volumes of sample solutions containing 1.0 mg oxalic acid were used for the evaluation of enrichment factor, and the enrichment factor could reach 25 with satisfying results (recovery > 97%).

### Optimization of chromatographic conditions

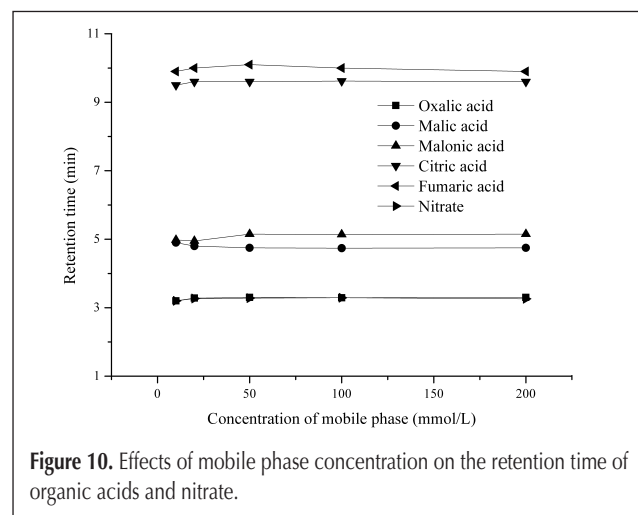
The key work of this study was to achieve a simultaneous determination of organic acids and nitrate in xylem saps of the hyperaccumulator *Alyssum murale* by using RP-HPLC. In order to achieve the best separation, a series of experiments were performed.

### Influence of the column temperature

The column was controlled by a thermostat to several different temperatures and the best results were obtained at a temperature of 25°C.

### Influence of mobile phase

The phosphate buffer solution was widely used as the mobile phase for the determination of organic acids (20,22,24,39). Different concentrations of the phosphate buffer solution had direct effects on the buffer capability. Furthermore, the concentrations could affect the forms of organic acids in the mobile phase and the ion intensity, consequently the separation effect of the chromatographic column. In the experiment, by keeping the pH of the mobile phase at 2.5, the effect of the mobile phase concentration on the separation of organic acids were investigated at an  $(\text{NH}_4)_2\text{HPO}_4$  concentration range from 10 to 200 mmol/L. As could be seen in Figure 10, malic acid and malonic acid could not be separated at low concentrations of  $(\text{NH}_4)_2\text{HPO}_4$ , the retention time of the organic acids changed less significantly when the concentration of  $(\text{NH}_4)_2\text{HPO}_4$  changed from 50 mmol/L to 200 mmol/L, however, high concentrations of phosphate buffer solution had deleterious effects on HPLC pump and column, finally 50 mmol/L  $(\text{NH}_4)_2\text{HPO}_4$  in mobile phase was selected.

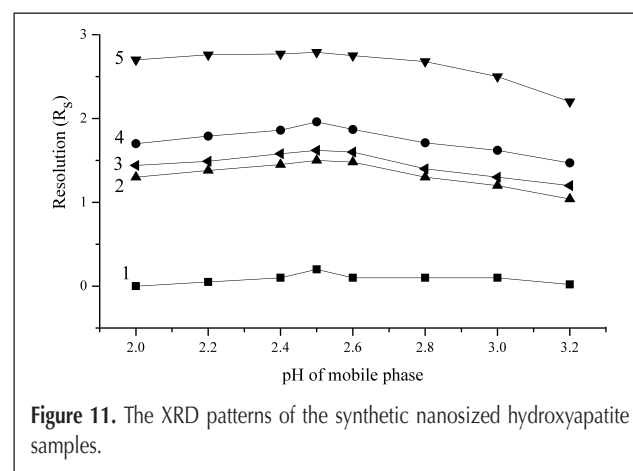


### Influence of mobile phase pH and flow-rate

Several mobile phase flow-rates (0.5–1.5 mL/min) were tested and finally a flow-rate of 1.0 mL/min were selected. The effect of the pH value of the mobile phase on the resolution ( $R_s$ ) of the separated organic acids and nitrate were examined. Because the chromatographic column might be deteriorated at extremely low pH conditions, the influence of mobile phase was tested at pH > 2.0. Figure 11 showed the effect of mobile phase pH on the resolution when pH was changed from 2.0 to 3.2. It was demonstrated that pH of the mobile phase could greatly affect the resolution of organic acids, the resolution increased as the increase of pH value, but when the pH value of the mobile phase was more than 2.6 the resolution between malic acid and malonic acid, malonic acid and citric acid, citric acid and fumaric acid were reversely decreased. It was generally considered that the part of the carboxyl group in the organic acid molecules could dissociate into carboxylate anions at high pH values, thus, a low mobile phase pH was chosen to ensure that the analyzed organic acids were in their fully protonated state and to minimize electrostatic interactions with the stationary phase (41), similar studies have been conducted in other papers (20,42). The experiment indicated that the suitable pH value was 2.5. Thus, the pH value of 2.5 was applied to the present study. Similarly, several wavelengths from 200 to 240 nm were tested, and finally the optimum wavelength for determination was 210 nm.

### Organic acids and nitrate concentrations in the xylem saps of *Alyssum murale*

The xylem sap samples of the hyperaccumulator *Alyssum murale* were subjected to the proposed SPE procedure, and then organic acids and nitrate in xylem saps were determined simultaneously by RP-HPLC at optimized chromatographic conditions. 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. This process was repeated three times with each sample. The results are shown in Table III, and chromatograms are shown in Figure 4 and 5. It is not possible to compare our results those of other workers because it is the first time that this study has been carried out to determine organic acids and nitrate in xylem saps of the hyperaccumulator *Alyssum murale* by RP-HPLC.



## Conclusion

The simultaneous analysis of organic acids and nitrate in xylem sap samples by RP-HPLC is a difficult task due to the interference from nitrate ions on oxalic acid. The proposed method based on SPE and RP-HPLC was successfully applied to avoid the interference and determine the organic acids and nitrate simultaneously in xylem saps of the hyperaccumulator *Alyssum murale*. SPE with nanosized hydroxyapatite has proved to be a useful way for eliminating the interference from nitrate on oxalic acid in xylem sap samples. RP-HPLC separation was excellent with a retention time of less than 12 minutes. The accuracy of this method was tested and obtained average recoveries ranging between 95.2% and 99.8%, the relative standard deviations were less than 2.0%. Thus, the developed method was proficient in the simultaneous determination of organic acids and nitrate in xylem sap samples.

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