

# **Ascorbic Acid as a Reductant for Extraction of Iron-Bound Phosphorus in Soil Samples: A Method Comparison Study**

Jia-Zhong Zhang<sup>1</sup> and Nathan Timothy Lanning<sup>2</sup>

<sup>1</sup>National Oceanic and Atmospheric Administration/Atlantic Oceanographic and Meteorological Laboratory, Miami, FL, United States

<sup>2</sup>Texas A&M University, Department of Oceanography, College Station, TX, United States

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## **Abstract**

Sequential extraction is commonly used to identify and quantify different forms of phosphorus (P) associated with particulate samples. Iron-bound P is an important fraction of total particulate phosphorus because iron (Fe) is ubiquitous in natural environments. Three reductant solutions, i.e., NaOH, dithionite and ascorbic acid, have been used to extract solid phase reactive iron and associated phosphorus from sediments and soils. This study compares the efficiencies of three different methods in extracting Fe and Fe-bound P and evaluates the potential and limitation of each method. Based on the results of this comparative study it is recommended that the ascorbic acid reduction method be used for extraction of Fe-bound P in particulate samples, such as soil and sediment.

**Key words** soil phosphorus, iron-bound phosphorus, ascorbic acid, sequential extraction, fractionation, analytical method

## **Introduction**

Phosphorus (P) is a limiting nutrient in many terrestrial and aquatic ecosystems (Elser et al., 2007; Zhang and Chi, 2002; Zhang and Huang, 2007). In these systems, phosphorus occurs dominantly in particulate phases as a result of sparingly low solubility of phosphorus-bearing minerals and high affinity of dissolved inorganic and organic phosphorus species to adsorb on solid surfaces (Huang and Zhang, 2010; 2011; Zhang and Huang, 2011; Flower et al., 2016). Consequently, analysis of phosphorus in solid phase is essential in agriculture, geoscience and environmental studies (Cross and Schlesinger, 1995). Sequential chemical extraction is a commonly used method to identify and quantify different forms of phosphorus associated with particulate samples. Iron-bound P is an important fraction of total particulate phosphorus pools

because iron (Fe) is ubiquitous in natural environments. Various methods have been used to extract Fe-bound P but few studies have compared different methods in order to evaluate their performance (Levy and Schlesinger, 1999).

Sequential extraction of soil phosphorus was originally developed by Chang and Jackson in 1957 (Chang and Jackson, 1957). Many modifications have been published since then with the Hedley method being the most popular procedure utilized in present agriculture communities to fractionate different forms of phosphorus in soil samples (Hedley et al., 1982; Tiessen and Moir, 2008). In this method, a 0.1 M NaOH solution is used to extract Fe-bound P. The NaOH is not a reductant but a strong alkaline solution. Its mechanism of dissolution of Fe(III) in the solid phase is to form high order Fe(III)–hydroxo complex,  $\text{Fe}(\text{OH})_4^-$  (Cornell and Schewertmann, 1996). In marine and freshwater systems, the Ruttenberg method is commonly used to fractionate different forms of P in sediment, dust and suspended particulate samples (Ruttenberg, 1992; Zhang et al, 2004; 2010). In this method, a mixed solution of dithionite and bicarbonate (BD) is used to target Fe-bound P. Unlike the NaOH solution, dithionite is a strong reductant and was considered more specific to the solid Fe(III) phase (Lucotte and Anglejan, 1985). The processes of dithionite reduction of solid phase Fe(III) to dissolved Fe(II) at neutral pH resembles the iron oxides reduction occurring in anoxic or suboxic conditions within natural soil and sediment environments. However, decomposition of dithionite produces elemental sulfur precipitation that interferes with subsequent phosphate determination by the molybdenum blue spectrophotometric method (Ruttenberg, 1992; Zhang et al., 2004; 2010). Solvent extraction and co-precipitation have been used to circumvent the problem but the resulting procedure becomes labor-intensive and time-consuming (Ruttenberg, 1992; Huerta-Diaz et al., 2005).

Levy and Schlesinger made a comparison of these two commonly used methods with 16 soil samples (Levy and Schlesinger, 1999). They found that Fe-bound P in the two methods was poorly correlated, in contrast to a good correlation found in calcium-bound P fraction (Levy and Schlesinger, 1999).

In addition to dithionite and NaOH, ascorbic acid has been used to extract solid phase Fe in soil and sediments (Kostka and Luther, 1994; Reyes and Torrent, 1997; Anschutz et al., 1998; Anschutz and Deborde, 2016). Ascorbic acid solution at neutral pH has been found to be a selective extractant for reactive iron oxide because it is moderate reductant but not a strong complexing anion (Reyes and Torrent, 1997). The objective of this study is to evaluate the kinetics of ascorbic acid reduction of iron oxides and release of iron-bound P in soils through the comparison of extraction efficiency of NaOH, dithionite and ascorbic acid on 7 soil samples collected in New England forests. Based on the results of this comparative study, a recommendation will be made for an optimal method to extract Fe-bound P in soil and sediment samples.

## **Materials and Methods**

### ***Sampling and analysis***

Soil samples were collected from forests throughout the New England region, one from New Haven, Connecticut, one from near Springfield, Massachusetts and five from New York state spanning from near Albany to Buffalo. The detailed locations of sampling site are listed in Table 1. Samples were collected from soil under natural vegetation in undisturbed ecosystem. Roots and other woody material were removed from samples before drying. All samples were freeze dried and passed through a 2-mm sieve.

All chemicals used in this study were of reagent grade. Ascorbic acid reduction solution was prepared by dissolving 58.8 g tri-sodium citrate di-hydrate ( $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot 2\text{H}_2\text{O}$ , MW 294.10) and 8.5 g ascorbic acid ( $\text{C}_6\text{H}_8\text{O}_6$ , MW 176.12) in 1 L de-oxygenated water. This solution was buffered at pH 6 and prepared freshly daily to minimize the loss of its reducing power by air oxidation. Bicarbonate-dithionite (BD) reduction solution was prepared by dissolving 9.2 g sodium bicarbonate ( $\text{NaHCO}_3$ , MW=84.01) and 19.2 g sodium dithionite ( $\text{Na}_2\text{S}_2\text{O}_4$ , MW 174.11) in 1 L deionized water followed by adjusting the pH to 7 (Zhang et al, 2010). Citrate was not used in the BD solution as discussed previously (Zhang et al, 2004). The sodium hydroxide (0.1 M) solution used in the Hedley method was made by dissolving 4 g NaOH in 1 L deionized water.

A 0.5 g unground soil sample, each in replicate, was mixed with 50 mL of different extraction solution (ascorbic acid, dithionite and NaOH) in a 60 mL polypropylene centrifuge tube. The tubes were shaken at 25°C in a VMR temperature-controlled shaking incubator for a predetermined time. The sample slurry was centrifuged at 3000 g for 10 minutes before the supernatant was filtered through a 0.45  $\mu\text{m}$  membrane. The filtrate was used to determine phosphate and total dissolved iron concentration by a Hewlett Packard 8453 UV-visible spectrophotometer. Prior to sample analysis, pH of extract solution was adjusted to ~6 with either a HCl or NaOH solution. This procedure removes the buffer capacity of extract solution in order to facilitate subsequent phosphorus molybdenum blue color development at an optimal pH (Zhang et al., 1999). For dithionite extract, two aliquots of filtrate were separately collected for phosphate and total iron analysis. Samples for total iron analysis were performed immediately after collection with a spectrophotometric method. Since dithionite interferes with phosphate analysis, the containers with phosphate sample were opened to air for 72 hours, allowing oxygen

to oxidize the excess dithionite to sulfate. This procedure circumvents the precipitation of elemental sulfur from decomposition of dithionite (Zhang et al., 2004).

Dissolved phosphate in the extract was determined by its reaction with molybdate at pH 1 in the presence of antimony potassium tartrate acting as a catalyst to form a phosphomolybdate complex that was subsequently reduced by ascorbic acid to form phosphomolybdenum blue (Zhang and Chi, 2002). A stock antimony potassium tartrate solution was prepared by dissolving 3 g of antimony potassium tartrate ( $\text{K}(\text{SbO})\text{C}_4\text{H}_4\text{O}_6 \cdot 1/2\text{H}_2\text{O}$ , MW 333.94) in 1 L of deionized water. Stock ammonium molybdate solution was prepared by dissolving 2.3 g of ammonium molybdate in 192 mL of a 2.5 M  $\text{H}_2\text{SO}_4$  solution before adding 50 mL of stock antimony potassium tartrate solution and bringing it up to 1 L with deionized water and mix. A mixed single color reagent was prepared daily by first dissolving 0.53 g ascorbic acid in 30 mL of deionized water and then mixed with 70 mL stock ammonium molybdate solution. For phosphate sample analysis, 1 mL of the mixed reagent was added to each 5 mL sample (sample: reagent = 5:1). After 15 minutes for color development, absorbance was measured at 890 nm wavelength on the Hewlett Packard 8453 spectrophotometer. Method linearity was observed between 0  $\mu\text{M}$  - 10  $\mu\text{M}$  P when a 1 cm cuvette was used.

Total dissolved iron in the extract was first reduced to dissolved Fe(II) by ascorbic acid and then determined by its reaction with FerroZine at pH 5.5 forming a color complex (Stookey, 1970; Zhang et al., 2001). Ascorbic acid solution was prepared by dissolving 7 g ascorbic acid in 100 mL of deionized water. A 0.1 M FerroZine solution was prepared by dissolving 0.511 g FerroZine ( $\text{C}_{20}\text{H}_{13}\text{N}_4\text{NaO}_6\text{S}_2 \cdot x\text{H}_2\text{O}$ , MW 492.46) in 100 mL of deionized water. Ammonium acetate buffer solution was prepared by dissolving 5 g of ammonium acetate in 100 mL of deionized water and then adjusting the pH to 5.5 with drop-wise addition of HCl solution. To

analyze iron samples, 0.1 mL of ascorbic acid was added to 5 mL of sample and mixed; waiting 1 minute to allow complete reduction of Fe(III) to Fe(II). Then 0.1 mL of FerroZine solution and 0.1 mL ammonium acetate buffer solution were added to the sample and mixed. After 2 minutes, the absorbance was measured at 562 nm. The method linearity was observed between 0  $\mu\text{M}$  to 40  $\mu\text{M}$  Fe when a 1 cm cuvette was used.

## **Results and Discussion**

### ***Kinetics of reduction of Fe oxides by ascorbic acid and release of Fe-bound P in soils***

Kinetics of ascorbic acid reduction of iron oxides in seven soil samples is shown in Figure 1. Seven soil samples showed similar kinetics. Dissolved iron extracted from the ascorbic acid solution increased with reaction time of the soil sample with ascorbic acid, reaching a maximum concentration after 16 hours of extraction. The maximum iron extracted after 16 hours ranged from 32.68 to 71.98  $\mu\text{mol Fe g}^{-1}$  in the samples tested.

Similar kinetics results were observed in the release of iron-bound P from ascorbic acid extraction (see Figure 2). Iron-bound P extracted from ascorbic acid solution increased with reaction time of soil sample with ascorbic acid, also reaching a maximum concentration after 16 hours of extraction time. The maximum iron-bound P ranged from 2.32 to 6.66  $\mu\text{mol P g}^{-1}$  in the seven samples.

Analysis of time series data reveals almost a constant mole ratio of Fe to P in the extract of a given soil sample during the course of extraction; however, the Fe to P mole ratio varied from sample to sample, spanning from a minimum ratio of  $10.84 \pm 0.47$  in sample 6 to a maximum ratio of  $14.42 \pm 0.29$  in sample 3. The averaged mole ratio of Fe to P from seven samples tested was  $12.62 \pm 1.21$ . Based on these results, the extraction time of 16-hour in ascorbic acid solution at pH 6 was selected for extraction of iron-bound P in soil samples.

### ***Fe reduction in different extracting solutions***

Soil samples digested by high temperature combustion (550°C) followed by 1 M HCl extraction for 16 hours produced the highest Fe content, ranging from 33.5  $\mu\text{mol Fe g}^{-1}$  to 73.6  $\mu\text{mol Fe g}^{-1}$  for the 7 soil samples tested. The high temperature combustion method is used as a reference method in this study to estimate the relative recovery of other methods. Extraction with NaOH, dithionite and ascorbic acid solutions produced similar dissolved Fe contents from the soil samples (Table 1). This indicates three solutions are efficient for extracting reactive Fe from soil. Among them, ascorbic acid produced almost the same Fe content as high temperature combustion method with an average relative recovery of 99.9%. NaOH extract results in Fe content in a range of 31.4-71.7  $\mu\text{mol Fe g}^{-1}$ , having an average relative recovery of 96.2%. Dithionite extract results in Fe content of 28.6-70.1  $\mu\text{mol Fe g}^{-1}$ , an average relative recovery of 90.0%.

### ***Fe-bound P from three extracting solutions***

In contrast to the similar Fe results from three extracts, the Fe-bound P contents measured from three extracts were significantly different. The NaOH extract results in Fe-bound P content of soil samples ranged from 0.65-3.20  $\mu\text{mol P g}^{-1}$ . Dithionite extraction produced Fe-bound P content ranging from 0.88-6.05  $\mu\text{mol P g}^{-1}$ . Ascorbate acid extraction produced Fe-bound P content ranging from 0.90-6.48  $\mu\text{mol P g}^{-1}$ . As shown in Table 1, ascorbic acid and BD extraction produced similar Fe-bound P for all soil samples tested. However, the results of NaOH extraction are quite different from either BD or ascorbate extractions. With exception of sample 1, all other samples extracted by NaOH resulted in a lower Fe-bound P content than that from dithionite or ascorbic acid extractions (Table 1).



## **Conclusions and Recommendations**

While three extracting solutions yielded similar dissolved iron contents from seven soil samples tested, the recovery of Fe-bound P varied between different extracting solutions. A good agreement in Fe-bound P was found between dithionite and ascorbic acid methods. Although the dithionite method has been shown to have a good recovery for Fe and Fe-bound P, it suffers interference from dithionite induced elemental sulfur precipitation. The ascorbic acid method is recommended as an optimal method for extraction of Fe-bound P for soil and sediment samples because it provides excellent recovery and is an interference-free method for subsequent P determination by the molybdenum-blue colorimetric method.

In the recommended procedure, a fresh extracting solution should be prepared daily by dissolving 58.8 g tri-sodium citrate di-hydrate and 8.5 g ascorbic acid in 1 L de-oxygenated water. A 0.5 g soil or sediment sample is mixed with 50 mL of extracting solution in a 60 mL polypropylene centrifuge tube. The tubes are shaken for 16 hours at 25°C. The sample slurry is centrifuged at 3000 g for 10 minutes before the supernatant is filtered through a 0.45  $\mu\text{m}$  membrane. The filtrate is used to determine the concentrations of phosphate and total dissolved iron by a UV-visible spectrophotometer.

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**Table 1.** Locations of soil sampling site in New England, USA.

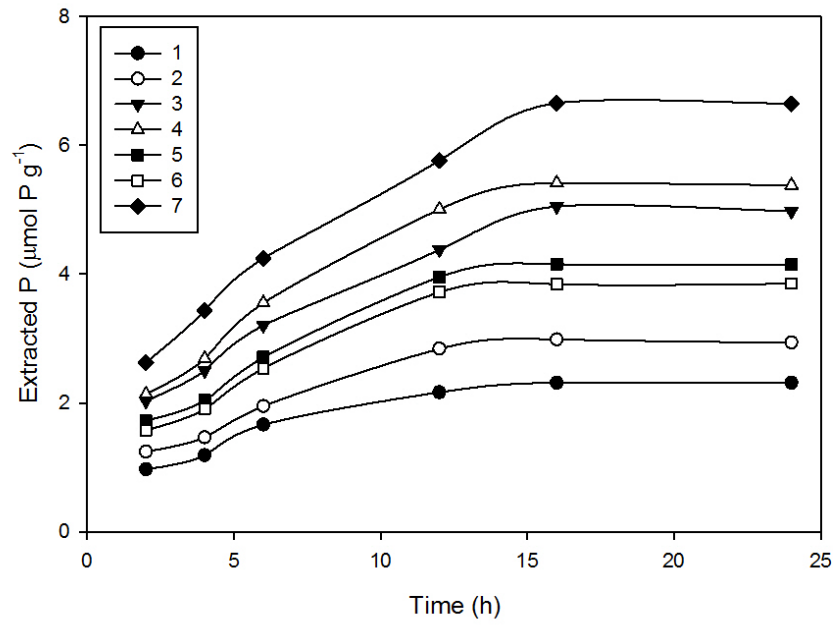
Station	Latitude	Longitude
1	41 17' 20.50" N	72 15' 57.30" W
2	42 11' 43.60" N	72 55' 50.00" W
3	42 53' 45.95" N	74 5' 39.13" W
4	43 4' 40.77" N	75 6' 16.93" W
5	43 5' 40.69" N	76 19' 6.01" W
6	43 1' 45.59" N	77 54' 19.97" W
7	42 48' 8.36" N	78 46' 47.37" W

**Table 2.** Results of method comparison for extraction of Fe and Fe-bound P in 7 soil samples.

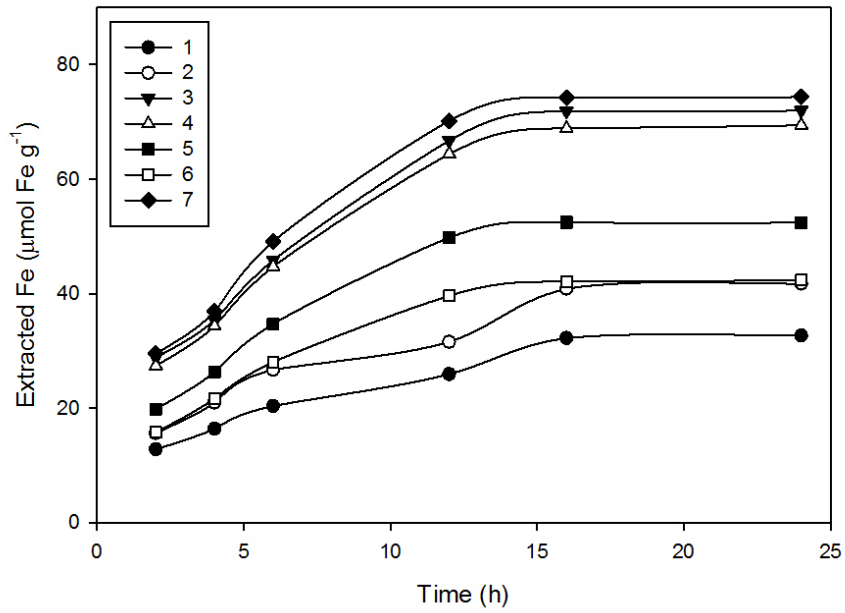
Sample Number	Total Fe measured ( $\mu\text{mol Fe g}^{-1}$ )			
	Combustion	NaOH	Ascorbic Acid	BD
1	33.54	31.41	32.23	28.62
2	41.82	40.20	40.87	35.16
3	69.25	74.64	71.86	67.07
4	66.53	63.00	68.89	60.15
5	52.39	50.21	52.47	47.64
6	43.24	38.17	42.12	37.63
7	73.57	71.67	74.25	70.05

Sample Number	Combustion-TP*	Fe-bound P measured ( $\mu\text{mol P g}^{-1}$ )		
		NaOH	Ascorbic Acid	BD
1	14.22	1.23	0.90	0.88
2	13.66	1.41	2.28	1.92
3	17.43	0.08	3.16	3.13
4	22.69	2.00	4.22	4.25
5	12.78	0.43	3.94	3.70
6	15.05	0.73	2.89	2.71
7	18.79	1.81	6.48	6.05

\*This combustion method measured total P instead of Fe-bound P, and the latter was extracted using NaOH, ascorbic acid or BD.



**Figure 1.** Ascorbic acid extracted Fe-bound P measured in seven soil samples as a function of time (hour) used in the extraction procedure.



**Figure 2.** Ascorbic acid extracted Fe measured in seven soil samples as a function of time (hour) used in the extraction procedure.