Digestion Efficiency during Alkaline Persulfate Oxidation for Determination of Total Phosphorus Content of Biological Samples

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Abstract

Quantifying total phosphorus contents of organisms can elucidate their physiological condition and the nutrient cycles of ecosystems. Simple, brief, and safe persulfate oxidation methods have been used for total P determination, but oxidizing solutions of different compositions and volumes have been used. Two certified reference materials were used to evaluate digestion efficiencies of different solutions for this study. Although the phosphorus recoveries were low (<90%) without NaOH, phosphorus recoveries using the solution with 4% K$_2$S$_2$O$_8$ and 0.15 M NaOH were complete. Results demonstrated that digestion efficiency depends on the K$_2$S$_2$O$_8$ concentration and on the pH condition. Moreover, the phosphorus recoveries were achieved at >4 mL/mg solution/material ratios for both standard materials. Therefore, the author recommends using >4 mL of the 4% K$_2$S$_2$O$_8$ solution with 0.15 M NaOH for sample materials of <1 mg to quantify the total phosphorus of biological samples.
Introduction

Phosphorus (P), an essential element for all living organisms, is found in the body mainly in nucleic acids and phospholipids. The P contents in organism bodies can vary depending not only on the organism metabolism\(^\text{1-4}\) but also on the dissolved P concentrations in aquatic environments and P contents of food resources.\(^\text{5-9}\) Therefore, measuring the P content of an organism body is important for elucidating their physiological and growth conditions on an individual scale\(^\text{10}\) and material and nutrient cycles at an ecosystem scale.\(^\text{3,11,12}\)

To measure the P contents of organisms, conversion of total P in the organisms to phosphate (PO\(_4^{3-}\)) is usually important. For that conversion, persulfate (K\(_2\)S\(_2\)O\(_8\)) oxidation (PO) method has been the most widely used among the various methods,\(^\text{11,13,14}\) such as high-temperature dry combustion,\(^\text{14-16}\) acid digestion using H\(_2\)SO\(_4\) and/or HNO\(_3\),\(^\text{17}\) and microwave digestion,\(^\text{18,19}\) possibly because of the method’s simplicity. In the PO method, biological samples are digested in oxidizing solution including K\(_2\)S\(_2\)O\(_8\) solution at autoclave temperatures.

In the PO method, digestion efficiency is affected by the K\(_2\)S\(_2\)O\(_8\) concentration, the pH condition, the volumes (i.e., solution/material ratio), and autoclaving duration.\(^\text{20}\) According to some earlier studies using P-containing compounds, effects of K\(_2\)S\(_2\)O\(_8\) concentrations and pH conditions on compound digestion differ according to the compound.\(^\text{21}\) Because organism bodies contain complex and various forms of P, evaluating suitable PO conditions for P content determination in organisms’ body is important. To date, to ascertain the total P contents of organism samples using the PO method, different compositions of oxidizing solutions and autoclaving durations have been used. For instance, Urabe\(^\text{11}\) used a 1% K\(_2\)S\(_2\)O\(_8\) solution and a 2 h autoclaving
duration for zooplankton. Suzumura\textsuperscript{22} used a 3\% K$_2$S$_2$O$_8$ solution and a 30 min autoclaving duration for net-collected plankton and cultured phytoplankton. Gibson \textit{et al.}\textsuperscript{13} used a 3\% K$_2$S$_2$O$_8$ with a 0.15 M NaOH solution and a 2.5 h autoclaving duration for zooplankton. However, detailed evaluations have rarely been made of the solution/material ratio.\textsuperscript{22} For this study, the author evaluated the most suitable K$_2$S$_2$O$_8$ concentrations, pH conditions, and solution/material ratios for PO method to find the P contents of biological samples.

\textbf{Experimental}

\textit{Standards}

The PO methods were tested using two commercially available organism standards (certified reference materials) for elemental analysis: powdered squid tissue (\textit{Heterololigo bleekeri} soft tissue; P = 1.25 wt.\%) and powdered plant leaf (\textit{Camellia sinensis} leaf; P = 0.339 wt\%). These standards were purchased from the National Metrology Institute of Japan (NMIJ CRM 7406-a and NMIJ CRM 7505-a). These standards, which are animal and plant substances with certified P values, are used to evaluate differences of digestion efficiencies among animal and plant samples.

\textit{Reagents}

All solution preparations were conducted using purified water (MQ-water) prepared using a Milli-Q Gradient System (Millipore Corp.). All solutions were of analytical grade.

As described earlier, various methods using PO method have been reported for finding the total P contents of natural samples, such as water and suspended particles,
sediments, and organisms. For this study, various persulfate solutions were tested. For those, K$_2$S$_2$O$_8$ (for N and P analysis; Fujifilm Wako Pure Chemical Corp., Japan) was dissolved in MQ water (MQ-PO solution) to produce concentrations of 1%, 2%, 3%, and 4% (w/v). However, the K$_2$S$_2$O$_8$ was dissolved in 0.15 M NaOH solution (alkaline-PO solution). The K$_2$S$_2$O$_8$ concentrations were therefore 1%, 2%, 3%, and 4% (w/v).

**Procedures**

Experiment 1: Approximately 1 mg (0.8–1.3 mg) of the two standard materials (powdered squid tissue and plant leaf) were weighed accurately using an electric balance (XP6V; Mettler Toledo, USA). For small amounts of powdered material, static electricity causes the sample to adhere to the tube wall. Therefore, the weighed material was placed in a pre-burned glass cup, which was made a 200 µL-glass insert (5183-2090; Agilent Technologies Japan Ltd., Japan) cut to 5 mm height. The cup including the material was placed in a pre-burned screw-capped tube (TST-SCR13-100; Iwaki, Japan). To this screw-capped tube, 2 mL of each PO solution was added before the tubes were capped tightly with Teflon-lined caps. The sample was sonicated for 30 min and was heated at 120°C for 30 min using an autoclave. After cooling, the solution was made up to 50 mL using the volumetric flask. Phosphorus liberated by the process is in the form of phosphate. The solution for phosphate was analyzed using standard colorimetric methods on a spectrophotometer (UVmini-1240; Shimadzu Corp., Japan). Blank P values from each PO solution were also analyzed. This evaluated blank P value includes procedural and solution blanks. After all presented P concentrations were blank-subtracted, the P recovery (%) of the standards was calculated.

Using a pH meter (LAQUA twin; Horiba Scientific, Japan), the pH values of the
PO solutions were measured before and after autoclaving.

The respective P recoveries of the squid tissue and plant leaf were examined using simple regression analysis to evaluate the statistical significance \((p < 0.05)\) of the differences observed.

Experiment 2: The amount of PO-digestible sample was tested per constant volume of solution. Approximately 0.2–5 mg of the two standard materials were weighed and placed in a test tube as described above. To the test tube, 4 mL of 4% \(\text{K}_2\text{S}_2\text{O}_8\) alkaline-PO solution, which was the highest recovery obtained in Experiment 1 (see Results), was added. The remainder of the procedure was performed identically to the explanation presented above.

Carbon and nitrogen contents of the squid tissue and plant leaf were measured using an EA/IRMS (Delta V Plus; Thermo Fisher Scientific Inc., Germany).

**Results and Discussion**

In PO method, the compositions and volumes of the oxidizing solution and the autoclaving duration are crucially important for digestion efficiency.\(^{20}\) For this study, 2–4 mL of volume and 30 min of the autoclaving duration was used for a small-scale and simple PO method. This study first investigated relations between the digestion efficiency and the compositions and volumes of the solutions. Subsequently, author propose the most suitable conditions of the PO method for biological samples.

**Blank P values**

Table 1 presents blank P values derived from oxidizing solutions and procedures used for the PO methods used for this study. The PO method produced low blank P values
(0.4–1.5 nmol-P/mL-solution) among all oxidizing solutions. The blank P values were higher than that reported by Suzumura\textsuperscript{22} (0.004 nmol-P/mL-solution) and lower than those reported by Monaghan and Ruttenberg\textsuperscript{25} (7.14–12.24 nmol-P/mL-solution). Monaghan and Ruttenberg\textsuperscript{25} demonstrated the necessity of purifying the persulfate to reduce the blank. However, the blank P values did not correlate with the K\textsubscript{2}S\textsubscript{2}O\textsubscript{8} concentration, indicating that the blank P values do not originate from the K\textsubscript{2}S\textsubscript{2}O\textsubscript{8} reagent. Therefore, the blank P value might be derived from procedures during the PO method. Moreover, the blank P value in this study was 10\textsuperscript{2}–10\textsuperscript{3} lower than the P derived from the 1 mg of squid tissue and plant leaf. Therefore, author infer that the blank P values used for this study do not interfere with the total P measurement.

\textit{MQ-PO Solutions}

Increasing the K\textsubscript{2}S\textsubscript{2}O\textsubscript{8} concentration caused a decrease in final pH (1.4 for 1\% MQ-PO solution to 1.1 for 4\% MQ-PO solution) and an increase in P recovery (Table 1). The P recovery of the squid tissue increased concomitantly with increasing K\textsubscript{2}S\textsubscript{2}O\textsubscript{8} concentration (simple regression analysis; \( p < 0.01 \)) from 76\% for the 1\% MQ-PO solution to 87\% for the 3 and 4\% MQ-PO solutions. However, P recovery with the 4\% MQ-PO solution was similar to that for a 3\% MQ-PO solution. This result indicates that increasing the K\textsubscript{2}S\textsubscript{2}O\textsubscript{8} concentration is insufficient to achieve complete P recovery. The P recovery of plant leaf did not vary with increasing K\textsubscript{2}S\textsubscript{2}O\textsubscript{8} concentration (\( p > 0.05 \)) from 81\% for the 1\% MQ-PO solution to 88\% for the 4\% MQ-PO solution. Because the P recovery of the plant leaf was maximal at 4\% K\textsubscript{2}S\textsubscript{2}O\textsubscript{8} concentration, a further increase in K\textsubscript{2}S\textsubscript{2}O\textsubscript{8} concentration might engender higher recovery. However, considering the solubility of K\textsubscript{2}S\textsubscript{2}O\textsubscript{8} (5.2 g/100 mL at 20°C), it might be difficult to increase the concentration further.
For this study, complete P recovery could not be achieved even with a 4% MQ-PO solution. Some organic P compounds are digested under initially alkaline conditions.\textsuperscript{26,27} Actually, Ormaza-González and Statham\textsuperscript{27} demonstrated that some phosphomonoesters (4-nitrophenyl phosphate, glycerophosphate, and glucose-6-phosphate) were more digested by alkaline PO (initial pH = ca. 9.7 to final pH = 4–5) than by acidic PO method. Therefore, an initial alkaline pH might be important for the digestion of biological samples containing such P compounds, which are digested favorably in an alkaline condition. Among all MQ-PO solutions in this study, the pH values before and after autoclaving were acidic, which might be the cause of the incomplete P recoveries.

\textit{Alkaline-PO Solutions}

Increasing the K$_2$S$_2$O$_8$ concentration increased P recovery both of the squid tissue and plant leaf (Table 1; $p < 0.05$ for squid and $p < 0.05$ for leaf), as well as the MQ-PO solution. The 4% alkaline-PO solution achieved nearly complete P recovery for both the squid tissue (96%) and the plant leaf (99%). Compared with the MQ-PO solution at the same K$_2$S$_2$O$_8$ concentration, the P recovery was improved, suggesting that the P recovery is not influenced solely by the K$_2$S$_2$O$_8$ concentration. That result probably reflects differences in the digestion efficiency of P-containing compounds under the respective pH conditions.

Langner and Hendrix\textsuperscript{28} studied P recovery from Orchard Leaves (NIST SRM 1571). Actually, P recoveries of 80–89% were obtained using the PO solution with 1.35–3.32% K$_2$S$_2$O$_8$ and 0.15–0.37 M NaOH. They reported that the low P recovery suggests that phosphorus bonds resistant to K$_2$S$_2$O$_8$ were present. Their presence requires more drastic digestion conditions such as sulfuric acid and nitric acid digestion to oxidize organic material and release phosphorus. However, the estimated final pH
yielded by the composition of the PO solution in the study is probably alkaline. That finding indicates that the acidic final pH might be sufficient to digest the phosphorus bonds resistant to K$_2$S$_2$O$_8$.

Inorganic P compounds, such as metaphosphate and polyphosphate, and nucleotides are digested under acidic conditions. An alkaline PO method (initial pH = ca. 9.7 to final pH = 4–5) reported by Ormaza-González and Statham$^{27}$ yielded lower P recoveries from polyphosphates (70–85%) than those obtained using acidic PO method (ca. >95%). An alkaline PO method (initial pH = 9.0 to final pH = 8.2–8.5$^{29}$) reported by Labry et al.$^{21}$ showed low P recoveries from polyphosphates (3–47%) and nucleotides (ca. 30%). An alkaline PO method (initial pH = 12.57 to final pH = 2.12) described by Ebina et al.$^{30}$ achieved nearly complete P recovery from polyphosphates (>97%) and nucleotides (>98%). Both polyphosphates and nucleotides contain P–O–P bonds in their structures. Therefore, strong acidic conditions (pH < 2.12) are likely to be necessary to digest compounds with the P–O–P bond.

For the alkaline-PO solutions addressed in this study, increasing the K$_2$S$_2$O$_8$ concentration caused a decrease in the final pH (12.6 for 1% alkaline-PO solution to 1.7 for 4% alkaline-PO solution; Table 1). The final pH values of 3 and 4% alkaline-PO solutions were strongly acidic. Therefore, complete P recovery apparently requires that the initial pH be alkaline and that the final pH be sufficiently acidic. The 4% alkaline-PO solution with 0.15 M NaOH used for this study fulfills these conditions. It might have achieved complete P recovery.

**Optimal solution/material ratio**

To ascertain the optimal amount of alkaline-PO solution for P recovery from the organism body, the author analyzed the standard materials (squid tissue and plant leaf)
using various ratios of solution/material (mL/mg) in the alkaline PO. The author used approximately 0.2–5 mg of the materials for 4 mL of 4% alkaline-PO solution, which were consistent with approximately 0.8–20 mL/mg of the ratio. Using the alkaline PO method, P recoveries of the squid tissues and plant leaf increased concomitantly with the increase of the solution/material ratio. At ratios less than 1, the P recoveries (77% for the squid tissue and 87% for the plant leaf; Fig. 1) were lower than 90% in both materials, possibly because of insufficient oxidant. The alkaline PO method yielded >95% P recovery at four of the ratios for the squid tissue and two for the plant leaf. The P recovery did not decrease when the solution/material ratio was increased further.

In this study, the minimum solution/material ratio showed that complete P recovery was lower for the plant leaf than it was for the squid tissue (Fig. 1). The incomplete P recovery at the low solution/material ratio might be attributable to insufficient oxidant derived from the oxidizing solution. The increased amounts of organic matter might consume all the available oxidant. Measured carbon and nitrogen contents of the standard materials used for this study are presented in Table 2. The increasing amount of the carbon was likely to have influenced the digestion efficiency (Fig. 2a). However, the solution/carbon ratio (mL/µg-C) did not correlate with the P recovery, which suggests that the amount of carbon alone cannot explain the difference in digestion efficiency between the squid tissue and plant leaf. However, the solution/nitrogen ratio (mL/µg-N) correlated strongly with the P recovery, suggesting that the amount of nitrogen might also be effective for quantification of the digestion efficiency (Fig. 2b). Nitrogen is oxidized first in the alkaline condition. As the digestion proceeds, the resulting low pH permits digestion of phosphorus-containing compounds. Therefore, the digestion efficiency of P can be ascertained from the amount of nitrogen in the initial alkaline condition. Further study is necessary to verify
this hypothesis.

**Conclusions**

For this study, the author examined the suitable composition for ascertaining the total P content in biological standard materials. The blank P values were sufficiently low relative to P derived from the squid tissue and plant leaf. To achieve complete P recovery from the squid tissue and plant leaf, the initial pH of alkaline and strongly acidic final pH might be important for digesting various P compounds. Results suggest that 4% K₂S₂O₈ with 0.15 M NaOH solution meets these conditions best. Amounts of carbon and nitrogen in the sample are effective for evaluating the digestion efficiency. Therefore, the author concluded that the use of >4 mL of 4% K₂S₂O₈ with 0.15 M NaOH solution for biological samples of <1 mg is the most suitable means of ascertaining the total P contents.

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Table 1 Analytical results of initial and final pH values, reagent blanks, and P recoveries from the squid tissue and plant leaf. Values in brackets are standard deviation (n = 3).

| Solvent | K$_2$S$_2$O$_8$ concentration (% w/v) | pH | Blank P value (nmol/mL) | Recovery (%) |
|---------|--------------------------------------|----|-------------------------|--------------|
| MQ      | 1                                    | 4.2 | 1.4                     | 1.5 (0.0) | 76 (0) | 81 (6) |
|         | 2                                    | 4.0 | 1.2                     | 0.7 (0.4) | 80 (1) | 80 (7) |
|         | 3                                    | 3.8 | 1.2                     | 0.4 (0.5) | 87 (1) | 81 (1) |
|         | 4                                    | 3.6 | 1.1                     | 1.5 (0.7) | 87 (1) | 88 (8) |
| 0.15M NaOH | 1                                | 12.9 | 12.6                | 0.9 (0.1) | 84 (1) | 88 (3) |
|         | 2                                    | 12.9 | 8.9                    | 0.8 (0.2) | 90 (0) | 82 (4) |
|         | 3                                    | 12.9 | 2.0                    | 0.3 (0.1) | 90 (1) | 96 (4) |
|         | 4                                    | 13.0 | 1.7                    | 0.4 (0.1) | 96 (3) | 99 (1) |
Table 2  Analytical results of carbon and nitrogen contents of two standard materials.

|               | C (wt.%) | N (wt.%) |
|---------------|----------|----------|
| Squid tissue  | 45.3     | 13.0     |
| Plant leaf    | 46.6     | 3.6      |
Figure Captions

Figure 1. P recoveries (%) at various solution/material ratios (mL/mg). Variation of the ratios was achieved by varying the sample weight (0.2–5 mg) per 4 mL of the 4% alkaline-PO solution.

Figure 2. P recoveries (%) at various solution/carbon (a) and solution/carbon nitrogen (b) ratios using 4% alkaline-PO solution. The amounts of carbon and nitrogen were calculated from carbon and nitrogen contents (wt.%) of the squid tissue and plant leaf determined using EA-IRMS. Dashed lines represent regression lines in the low range of P recoveries.
Fig. 1

Figure 1 (Onishi)
Figure 2 (Onishi)

(a) P recovery (%) vs. Solution/carbon (mL/µg-C)

(b) P recovery (%) vs. Solution/nitrogen (mL/µg-N)

$r^2 = 0.3074$

$r^2 = 0.8746$

Fig. 2