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An Online Solid Phase Extraction Method for the Determination of Ultratrace Level Phosphate in Water with a High Performance Liquid Chromatograph

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Abstract

Phosphorous monitoring is important for eutrophication control in aquatic ecosystems, but ultratrace level concentrations may not be detected by conventional analytical methods. A method for measuring ultratrace level phosphate by online solid-phase extraction combined with HPLC was developed. A short column (50 mm) packed with octadecylsilane (ODS) was used for extraction of phosphoantimonylmolybdenum blue and dodecyltrimethylammonium hydrophobic ion-pair complexes. The ion-pair complexes entrapped on the ODS column were eluted with CH_3CN/H_2O (35/65; flow rate, 1.0 ml min^{-1}) and monitored by an ultraviolet/visible spectrophotometer (λ=872 nm). Phosphate concentration was determined from the peak area of the ion pair. The limit of detection for orthophosphate was 0.15 μg PO_4 l^{-1} and the dynamic range was 0.15–100 μg PO_4 l^{-1}. Although our method was susceptible to silicate interference, it could be corrected by a proposed interference correction equation.

Key words: Phosphate detection; Ultratrace detection; Ion pair;
dodecyltrimethylammonium ion

Introduction

Phosphorous is a limiting factor for primary production in aquatic ecosystems because it is an essential nutrient for photosynthetic organisms. Agricultural runoff and discharged wastewater contain excess phosphate (PO_4) that causes eutrophication in closed water bodies, leading to undesirable growth of algae and toxic microbes such as *Microcystis* (Lürling and Roessink, 2006), anaerobic conditions at the bottom of the water bodies, and progression of organic pollution. Monitoring of ultratrace level phosphate (<2 μg PO_4 l^{-1}) including its distribution, transport, and chemical and biological transformations, is an important aspect of eutrophication control and of aquatic ecosystem modeling (Asaoka and Yamamoto, 2011; Arhonditsis and Brett, 2005; Luff and Moll, 2004; Tsiaras et al., 2014; Wei et al., 2004; Zhang and Chi, 2002). Because of biological uptake, phosphate in surface water frequently goes undetected by conventional analytical methods even in eutrophic water bodies (Wei et al., 2004). In oligotrophic water bodies, phosphate is often present at concentrations less than 1 μg l^{-1}, below the limit of detection (LOD) of standard methods for measuring phosphate concentration (APHA, 1999).

Phosphate analysis is commonly performed with a phosphoantimonylmolybdenum blue (PAMB) method (APHA, 1999) originally described by Murphy and Riley (1962). In our proposed method, ammonium molybdate and potassium antimonyl tartrate react in acid medium with orthophosphate to form a heteropoly acid, or phosphomolybdic acid. The phosphomolybdic acid is reduced to intensely colored phosphoantimonylmolybdenum blue (PAMB) by ascorbic acid (APHA, 1999). Because PAMB is an anionic species, it can form an ion pair with an organic cation such as quaternary ammonium ions (Kiso et al., 2002).

Other methods for detecting trace phosphate are solvent extraction of PAMB; complex formation of PAMB with cationic dyes such as rhodamine (Frank et al., 2006;

Nasu and Minami, 1989; Taniai et al., 2003), crystal violet (Fogg et al., 1977), or malachite green (Susanto et al., 1995a,; 1995b); and flow-injection analysis (APHA, 1999; Dinz et al. 2004; Estela and Cerdà, 2005; Mesquita et al., 2011; Ribeiro et al., 2013). Ultratrace levels of phosphate have also been detected by flow-injection analysis systems combined with PAMB-complex formation with a cationic dye (Li et al., 2005; Motomizu and Li, 2005; Susanto et al., 1995; Zhang and Chi, 2002; Yaqoob et al., 2004;). These systems required specially designed sophisticated instruments, and the reagent solutions were not stable (Li et al., 2005).

PAMB paired with quaternary ammonium salts can be extracted using a solid phase extraction technique (Liang et al, 2007; Ma et al., 2008). We developed a simple method for the detection of ultratrace phosphate by solid-phase extraction with HPLC. This method incorporates PAMB, an ionic species that forms hydrophobic ion–pair complexes with organic cation. We previously used PAMB and quaternary ammonium ion-pair formation to develop a spot test based on color-band formation in a mini column to detect PO_4 at concentrations of 3–18 mg l^{-1} (Kiso et al., 2002). In this study, an online solid-phase extraction unit equipped with an HPLC system was used to concentrate PAMB–quaternary ammonium ion pairs, which were detected by an ultraviolet/visible (UV/Vis) spectrophotometer after elution with an organic solvent.

In this study, the conditions for color development, ion-pair formation, extraction, and elution of the ion-pair were optimized to detect dissolved ultratrace level phosphate with a modified HPLC system.

2. Experimental

2.1 Reagents and solutions

All reagents were of analytical grade or the highest grade available. All reagent solutions were prepared with ultrapure water (18.2 MΩ cm^{-1}, Milli-Q Gradient A10;

70 Millipore, Billerica, MA, USA) and stored in polypropylene or
71 poly(tetrafluoroethylene) bottles.

72 The stock solution of phosphate (1000 mg PO_4 l^{-1}) was prepared with KH_2PO_4
73 (Sigma-Aldrich, St. Louis, MO, USA), and the standard solutions were prepared by
74 dilution of the stock solution at concentrations of 0.1–100 μg PO_4 l^{-1}.

75 The mixed color reagent solution was prepared according to the US Standard
76 Method (APHA, 1999) with reagents used as received from Nacalai Tesque (Kyoto,
77 Japan): 5 ml of 2.743 g l^{-1} $K(SbO)C_4H_4O_6 \cdot 1/2H_2O$ solution, 15 ml of 40 g l^{-1}
78 $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ solution, and 30 ml of 0.1 mol l^{-1} *L*-ascorbic acid solution were
79 added to 50 ml of 2.5 mol l^{-1} H_2SO_4 solution. Dodecyltrimethylammonium (DTMA)
80 bromide was dissolved in methanol at 0.1% w/v. HPLC grade acetonitrile (CH_3CN) was
81 used as the solvent for the solid-phase extraction.

82 Some researchers have reported the effects of interference ions such as Fe, Cu, Mn,
83 Al, Zn, Pd and Ca on phosphate detection with using PAMB. Cu^{2+} caused a positive
84 interference on phosphate determination over 10 mg l^{-1} (Galhardo et al., 2000).
85 However, this concentration level is obviously low in natural and seawater samples. It
86 was also reported that silica and arsenic reacted with ammonium molybdate (Murphy
87 and Riley, 1962). Therefore, we tested the interference with co-ionic species for PO_4^{3-}
88 detection using the following solutions: arsenate (As) (III) solution prepared with
89 $NaAsO_2$, As(V) solution with $Na_2HAsO_4 \cdot 7H_2O$, and Si solution with an analytical
90 standard of Si for atomic absorbance.

91

92 ***2.2 Color development and ion-pair formation***

93 To form PAMB, 2 ml of the mixed color reagent solution was added to 50 ml of
94 the standard solution containing 10 μg PO_4 l^{-1}. The reaction was conducted for 10 min at
95 room temperature (approximately 20°C). Thereafter, 1.5 mL of DTMA solution was

added to the PAMB solution to form the PAMB-DTMA ion-pair complexes. This reaction was allowed to proceed under ultrasonication in an ice bath for 15 min.

2.3 Online solid-phase extraction-HPLC system

We combined an online solid-phase extraction unit with an HPLC system consisting of two pumps (880-PU and PU-2080 plus, JASCO), a sample injector (Rheodyne Model 7725i) equipped with a sample loop of 5 ml, a 6-way valve (Rheodyne Model 7000) equipped with a short column (4.6 mm i.d. × 50 mm long) packed with octadecylsilane (ODS; ULTRON VX-ODS, Shinwa Chemical Industry, Kyoto, Japan), and a UV/Vis detector (UV-2070 plus, JASCO). Chromatogram data were processed with JASCO-BORWIN/HSS-2000 programs. The system diagram and the operational procedure are shown in **Fig. 1**.

2.4 Online solid-phase extraction protocol

The solid-phase extraction of the PAMB-DTMA ion pairs was influenced by sample-loading and column-washing conditions, mobile-phase composition, and the flow rate of the mobile phase. On the basis of our examination of these variables, the following procedure was employed.

The solution containing PAMB-DTMA ion-pair complexes was injected into the sample loop (5 ml), then the solution was introduced into the ODS column with water at a 2.0 ml min^{-1} flow rate by pump 1 (880-PU), followed by washing with 7 ml of water at a 2.0 ml min^{-1} flow rate. The flow channel of the 6-way valve was changed and the entrapped ion-pair was eluted with CH_3CN/H_2O (35/65) at a 1.0 ml min^{-1} flow rate by pump 2 (PU-2080 plus). Then the eluent was monitored by the UV/Vis detector (UV-2070 plus) at λ=872 nm. The 6-way valve was returned, and the ODS column was washed with water for approximately 3 min to condition the column.

2.5 Interference with co-ionic species

The solutions containing As(III), As(V), and Si were added to the standard solution containing 5 μg PO_4 l^{-1} at the following concentrations: 5 μg As(III) l^{-1}; 1, 3, and 5 μg As(V) l^{-1}, and 5, 100, 2000, and 5000 μg Si l^{-1}. These solutions were also employed for the analytical procedure described above.

2.6 Recommended online solid-phase extraction protocol to determine natural samples.

2.6.1 Reagents and solutions

The composition of the mixed color reagent solution was slightly modified to alleviate interference with silicate and diluent effect attributed to the addition of the mixed color reagent solution to samples. Five ml of 3.5 g l^{-1} $K(SbO)C_4H_4O_6 \cdot 1/2H_2O$ solution, 15 ml of 52 g l^{-1} $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ solution, and 0.8 g of diluted *l*-ascorbic acid (1:9= *l*-ascorbic acid:NaCl) were added to 50 ml of 2.5 mol l^{-1} H_2SO_4 solution. A 2.5 ml portion of this mixed color reagent solution was added to 25 mL samples, consistent with the final color reagent concentrations based on the US Standard Method (APHA, 1999). Dodecyltrimethylammonium (DTMA) bromide was dissolved in methanol at 0.1% w/v.

2.6.2 Color development and ion-pair formation

The normal protocol for the ion-pair formation was as follows: The mixed color reagent solution (2.5 mL) was added to 25 ml of the sample solution. The sample solution was kept at 50^{o}C for 15 min to form PAMB. Thereafter, 0.75 ml of 0.1% w/v DTMA solution was added to the PAMB solution and the reaction mixture was agitated under ultrasonication in an ice bath for 15 min to form PAMB-DTMA complexes.

148 The optimum temperature for PAMB formation was examined under the following
149 conditions: standard solution of 1 mg PO_4 l^{-1}, temperature in the range of 25 to 70^oC,
150 the reaction time of 15 min, and absorbance measurement with a UV/Vis
151 spectrophotometer (V-530; JASCO, Tokyo, Japan) using a 50 mm cell at λ=880 nm.
152 The dosage of 0.1% w/v DTMA solution, which influences both the PAMB-DTMA
153 ion-pair formation and efficiency of the online solid phase extraction, was examined by
154 addition of 0-0.15 ml of 0.1% w/v DTMA solution to 25 mL of PAMB solution
155 prepared with 10 μg PO_4 l^{-1} standard solution.
156 The reaction for PAMB-DTMA ion-pair complex formation was allowed to proceed
157 under ultrasonication in an ice bath for 15 min. The peak area measurement of the
158 PAMB-DTMA ion-pair complex was followed by the modified online solid phase
159 extraction protocol to be described later (2.6.3).
160 To alleviate silicate interference on PO_4 l^{-1} measurement, the optimization of the final
161 H_2SO_4 concentration in the PAMB solution was examined in the range of 0.28 to 0.56 g
162 l^{-1}. Color development and PAMB-DTMA ion-pair complex formation were conducted
163 as described earlier. The PAMB-DTMA complex was measured by the modified online
164 solid phase extraction protocol to be described below (2.6.3).
165
166 ***2.6.3 Recommended online solid-phase extraction protocol***
167 To improve efficiency of washing and conditioning of the ODS column, the mobile
168 phase loading condition was slightly modified. The solution containing the PAMB-
169 DTMA ion-pair complexes was injected into the sample loop (5 ml), then the solution
170 was introduced into the ODS column with water at a 2.0 ml min^{-1} of flow rate by pump
171 1 (880-PU), followed by washing with 7 ml of water at 2.0 ml min^{-1} of flow rate. Then,
172 the entrapped PAMB-DTMA ion-pair complexes were eluted using pump 2 (PU-2080
173 plus) by turning the 6-way valve, where the elusion was conducted by gradient elution

174 mode when a gradient unit (LG-2080-02 : JASCO) was located between a degassor
175 (DG-2080-53 : JASCO) and pump 2.
176 The mobile phase of CH_3CN/H_2O (30/70) was loaded at 1 mL min^{-1} before eluting
177 the PAMB-DTMA ion-pair (0-1.5 min). Thereafter the PAMB-DTMA ion-pair was
178 eluted completely by CH_3CN/H_2O (35/65) at a flow rate of 1.0 ml min^{-1} from 1.5 to 5.5
179 min. Thereafter, 100% of CH_3CN was loaded at a flow rate of 2 mL min^{-1} for 5 min for
180 column cleaning, and subsequently CH_3CN/H_2O (35/65) was loaded at 1.0 ml min^{-1}
181 flow rate for 3 min and pure water was loaded at 1.0 ml min^{-1} for column conditioning.
182 The eluent was monitored by the UV/Vis detector (UV-2070 plus) at λ=872 nm.
183
184 ***2.6.4 Effect of interference with silicate on PO_4 detection by the recommended online***
185 ***solid-phase extraction method***
186 The effects of the interference with silicate and salinity on PO_4 analyses by the
187 recommended online solid-phase extraction method was examined because silicate may
188 compete with PAMB formation, and high salinity may influence the observed
189 absorbance for common absorptiometry due to reflection.
190 Silicate interference was examined with silicate standard solution (0 to 20 mg Si l^{-1})
191 prepared by dilution of a 1000 mg l^{-1} of a purchased silicate standard solution
192 (analytical grade, Wako Pure Chemical). The prepared silicate solution was employed
193 following the same protocol including the color development, ion-pair formation and
194 online solid-phase extraction (see 2.6.2 and 2.6.3).
195 The effects of salinity were examined with the standard solutions of 0-10 μg PO_4 l^{-1}
196 containing 3.5% NaCl. Phosphate detection was conducted using the same procedure
197 described in 2.6.2 and 2.6.3.
198
199 ***2.6.5 Natural sample analyses with the recommended online solid-phase extraction***

method

Two types of natural samples were collected from the Sea of Enshu and Kozenji-gawa River as shown in **Fig. 2**. Latitude and longitude of the sampling station of Kozenji-gawa river was 35°04'02.1"N and 136°04'01.8"E, respectively, and those of the Sea of Enshu are shown in **Table S1**. The Sea of Enshu is facing the Pacific Ocean along the coast of Shizuoka Prefecture, Japan. The surface seawater samples at 1 m depth and middle layer (sampling depth shown in **Table S1**) were collected by a Bandon water sampler on September 10th 2011. The collected samples were immediately filtered with a glass fiber filter (0.6 μm, Advantec) and transported to the laboratory in a container that was kept dark and cool. The samples were stored at -40^{o}C before analyses.

Kozenji-gawa River is located in Shiga prefecture, Japan, flowing through an intermountain area surrounded by coniferous forests. The sampling station is located in headwater of Kozenji-gawa River within 20 ha catchment. The sampling was conducted every week from November 3rd, 2008 to April 26th, 2010. The collected stream water samples were filtered with a nuclepore filter (0.4 μm; Whatman) and transported to the laboratory in a container that was kept dark and cool. The sample was stored at -40^{o}C before analyses.

The stored samples (both sea water and river water samples) were analyzed by the same protocol for color development, ion-pair formation and online solid-phase extraction as described in 2.6.2 and 2.6.3.

3. Results and Discussion

3.1 Mobile phase for extraction

The mobile-phase compositions for elution of the retained ion pair on the ODS column were examined with 30%–100% aqueous CH_3CN. The chromatograms for 100 μg PO_4 l^{-1} standard solution showed two main peaks, peak A and peak B (**Fig. 3)**. The

maximum absorbance of peak B was observed at 872 nm (**Fig. 4**), indicating that peak-B reflected the PAMB-DTMA ion-pair complex for which the spectra were obtained by the UV/Vis spectrophotometer (V-530). Peak A showed a broad spectrum that likely reflected the remaining reagents such as free DTMA.

Peak-B was separated from peak A at a CH_3CN content <40%. At CH_3CN=40%, however, a very small peak was detected just before peak B. At CH_3CN=30%, peak B broadened, and the peak intensity decreased markedly. Therefore, CH_3CN=35% was employed for optimum mobile-phase composition.

The area of peak B did not vary flow rates of 0.5 and 1.0 ml min^{-1}. Although the peak intensity at 0.5 ml min^{-1} was lower (approximately 70%) than that at 1.0 ml min^{-1}, the symmetry factors of peak B were 1.23 and 1.54 for 0.5 and 1.0 ml min^{-1}, respectively. In the range of 1.0–2.0 ml min^{-1}, the peak area decreased with increased flow rate, owing to significant tailing of the peak. Therefore, a flow rate of 1.0 ml min^{-1} was employed for the following experiments.

3.2 Sample loading and column washing

The flow rate for sample loading into the ODS column may have affected the extraction efficiency. Flow rate varied in the range of 1.0–4.0 ml min^{-1} with the standard solution containing 10 μg PO_4 l^{-1}, but the areas of peak A and peak B were not influenced by the flow rate. On the basis of these results, we employed a flow rate of 2 ml min^{-1} in the following experiments.

Because peak A was caused by the reagents remaining in the column, the column was washed with water to decrease peak A. The washing volume affected the chromatograms at a flow rate kept at 2 ml min^{-1}. The intensity of peak A decreased with increased washing volume, and peak A disappeared at washing volumes >6 ml (**Fig. 5**).

In our previous work on a spot test for color-band determination of phosphate (Kiso

et al., 2002), we employed benzylcetyldimethylammonium (BCDMA) as a quaternary ammonium ion. For the current method, however, when BCDMA was employed for the online extraction process, a large area of a blank sample was observed. Low water solubility of BCDMA may have prevented effective removal of the residual BCDMA by the washing process. When DTMA was used, the peak area of the blank sample decreased effectively; thus DTMA was a more suitable quaternary ammonium ion.

3.3 Interference

Because the molybdenum blue method is susceptible to interference from Si and As(V), we investigated the interfering effects of As(V), As(III), and Si for the standard solution of 5 μg PO_4 l^{-1}. The effects on the peak areas of these species are summarized in **Table 1**. No significant interference was seen from As(III) at 5 μg As l^{-1} or from Si at <2000 μg Si l^{-1}. Interference from As(V) gave error rates of +23.9% with 5 μg As l^{-1} and +8.5% at 3 μg As l^{-1} of As(V). This interference was attributable to formation of arsenoantimonylmolybdenum blue–DTMA ion pairs that eluted at a retention time close to that of the PAMB-DTMA ion pairs (**Fig. 6a)**. These different ion pairs were separated, however, under the mobile phase conditions of 0.5 ml min^{-1} (**Fig. 6b)**. Considering that the intensity of peak B decreased at 0.5 ml min^{-1}, more efficient elution conditions such as a gradient elution mode may be necessary for simultaneous detection of PO_4^{3-} and As(V) at ultratrace levels.

3.4 Recommended online solid-phase extraction protocol to determine natural samples.

We hereby propose the recommended online solid-phase extraction protocol to detect ultra trace level phosphate in natural samples by slightly modifying the online solid-phase extraction protocol described above.

278

279 ***3.4.1 Color development and ion-pair formation***

280 The optimum reaction temperature for color development was determined on the
281 basis of the intensity of absorbance at 880 nm. The absorbance of PAMB increased
282 asymptotically with increase of the reaction temperature and reached plateau level in the
283 range of over 50^{o}C. Therefore, the color development reaction was conducted at 50^{o}C in
284 the analysis that followed (**Fig. S1**).

285 The effect of DTMA dosage on the solid phase extraction of the PAMB-DTMA ion-
286 pair was evaluated under the following conditions: 0–1.5 ml of the DTMA solution and
287 25 ml of 10 μg PO_4 l^{-1} PAMB solution. The peak area of the PAMB-DTMA ion-pair was
288 measured with the modified online solid-phase extraction protocol. The peak area
289 reached the maximum when 0.75 mL of the DTMA solution was added to 25 ml of the
290 PAMB solution (**Fig. S2**). The peak area decreased rapidly when the DTMA solution
291 dosage increased more than 1 ml, and this was caused by the precipitation of ion-pair
292 complex.

293 Silicate reacted with ammonium molybdate (Murphy and Riley, 1962). This reaction
294 is influenced by H_2SO_4 concentration. Therefore, the final concentration of H_2SO_4 in the
295 PAMB solution was changed into the range of 0.28 to 0.56 g l^{-1} (**Fig. 7**). In the case
296 when H_2SO_4 concentration was more than 0.42 g l^{-1}, both the peak areas of PO_4 (10 μg
297 PO_4 l^{-1}) solution and PO_4 (10 μg PO_4 l^{-1}) + silicate mixed solution (10 mg Si l^{-1})
298 decreased. When lower than 0.35 g l^{-1} H_2SO_4 concentration, however, the peak area of
299 the PO_4 + silicate mixed solution was obviously larger than that of the PO_4 solution. The
300 interference with silicate was unavoidable, therefore 0.35 g l^{-1} of the final concentration
301 of H_2SO_4 in the PAMB solution was employed in this work.

302

303 ***3.4.2 Calibration curve with the recommended online solid-phase extraction method***

The calibration curve for phosphate (0–100 μg PO_4 l^{-1}) is shown in **Fig. 8**. The reproducibility of the proposed method was examined for the ultratrace level standard solutions. The average peak areas (0.2–100 μg PO_4 l^{-1}) and the RSDs (n=3) are 0.64-8.26%. Based on the RSD of the blank sample, the LOD (S/N ratio=3) was evaluated to be 0.15-μg PO_4 l^{-1} (0.05 μg P l^{-1}). The LOD obtained in this study is similar to the lowest levels reported for all other methods (Ma et al., 2008; Li, et al., 2005; Liang et al, 2007; Susanto et al., 1995b; Wu and Ruzicka, 2001; Yaqoob, et al., 2004; Zhang and Chi, 2002).

The dynamic range of this recommended method was 0.15–100-μg PO_4 l^{-1} (0.05~33-μg P l^{-1}) and is extremely wide in comparison with the other analytical methods for ultratrace level phosphate (Frank et al., 2006; Li et al., 2005; Mesquita et al., 2011; Susanto et al., 1995; Wu and Ruzicka, 2001; Yaqoob, et al., 2004; Zhang and Chi, 2002).

Although manual sample preparation processes were required for the color development and the ion-pair formation, the proposed method has the following advantages: when sample absorbance was lower than the detection limit in conventional PAMB methods, the PAMB solutions can be analyzed using the proposed method only after the addition of the DTMA solution.

3.4.3 Effect of salinity and silicate on PO_4 analyses with the recommended online solid-phase extraction method

The effect of salinity on PO_4 detection using the modified online solid-phase extraction method was examined on the basis of the calibration curves for the PO_4 (0-10 μg PO_4 l^{-1}) standard solutions with and without distilled ion exchange water and 3.5% NaCl. The difference between both calibration curves was very small as shown in **Fig. S3**: the differences of the peak area were in the range of -1.2 to 1.7%. The results

revealed that the effect of salt concentration in sea water samples can be negligible.

The calibration curve for silicate (0-20 mg l^{-1}) obtained by the modified online solid-phase extraction method is shown in **Fig. S4**. Silicate caused positive error in the quantification of trace level phosphate, although the effect of silicate can be negligible for phosphate detection in the concentration range measured by common absorptiometry. When both peak areas for phosphate and silicate are measured under same conditions, the effect of silicate on phosphate detection can be evaluated as a correction equation. Figure 9 shows the correction equation. For example, the peak area for 5 mg l^{-1} silicate corresponds to that for 2.1 μg PO_4 l^{-1}. Therefore, when PO_4 concentration in samples was much lower than that of silicate, PO_4 concentration should be corrected by eliminating interference with silicate.

3.4.4 Determination of PO_4 concentration in natural samples with the recommended online solid-phase extraction method

The concentrations of PO_4 in surface seawater collected from the Pacific Ocean was 1.35 to 8.42 μg PO_4 l^{-1} and 1.39 to 8.50 μg PO_4 l^{-1} under rising tide and ebbing tide conditions, respectively (**Table 2**). The phosphate concentrations of the middle layer were 1.25 to 3.74 μg PO_4 l^{-1} (rising tide) and 0.41 to 1.98 μg PO_4 l^{-1} (ebbing tide). To verify the effect of salinity on the detected PO_4 in seawater, the standard addition method was conducted for the sample of the middle layer (rising tide) at St. 7 (**Fig. S5**). The salinity of the sample was 3.4% and the detected PO_4 concentration was 1.71 μg PO_4 l^{-1}. On the other hand, the PO_4 concentration by the standard addition method was 1.29 μg PO_4 l^{-1}. Both values are quite similar, and it was revealed that the recommended method was not influenced by salinity.

The Kozenji river samples were measured using the recommended online solid-phase extraction method **(Fig. 10)**. Observed PO_4 concentration was overestimated because

the samples contained silicate in the range of 6.04 to 9.54 mg l^{-1}. Therefore, PO_4 concentration was corrected by using the interference correction equation shown in **Fig. 9**. The corrected PO_4 concentrations in Kozenji river were in the range from <0.15 to 8.92 μg PO_4 l^{-1}. The recommended online solid-phase extraction method could determine ultratrace or trace level PO_4 effectively compared to the conventional method.

4. Conclusion

This study demonstrated the effectiveness of an online solid-phase extraction method for the detection of ultratrace level phosphate. Under the recommended conditions, the LOD was 0.15 μg PO_4 l^{-1} with the dynamic range of 0.15–100 μg PO_4 l^{-1}. The proposed method involves manual procedures for color development and ion-pair formation, but requires only an HPLC system. In addition, it was indicated that the proposed method was not influenced by salinity and that silicate interference can be eliminated by using the interference correction equation.

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Figure captions

Fig. 1 Diagram of the online extraction system and operational procedure

Fig. 2 Sampling stations on the Sea of Enshu and Kozenji-gawa River

Fig. 3 Effect of the mobile phase composition on chromatograms

Sample: 100 μg PO_4 l^{-1}; Sample loading rate: 2.0 ml min^{-1}; Washing volume: 5 ml ;

Flow rate of the mobile-phase: 1.0 ml min^{-1}

Fig. 4 Absorption spectrum of peak-A and peak-B

Fig. 5 Effect of the volume for column washing on the chromatogram

Sample: 10 μg-PO_4 l^{-1}; Sample loading rate: 2.0 ml min^{-1}; mobile phase:

CH_3CN/H_2O=35/65: flow rate of he mobile phase: 1.0 ml min^{-1}

Fig. 6 Separation of PO_4^{3-} and As(V)

Phosphate concentration: 5 μg PO_4 l^{-1}; Arsenate concentration: 5 μg-As(III) l^{-1}

(a) Mobile-phase: CH_3CN/H_2O=35/65 at 1.0 ml min^{-1}; λ=872 nm

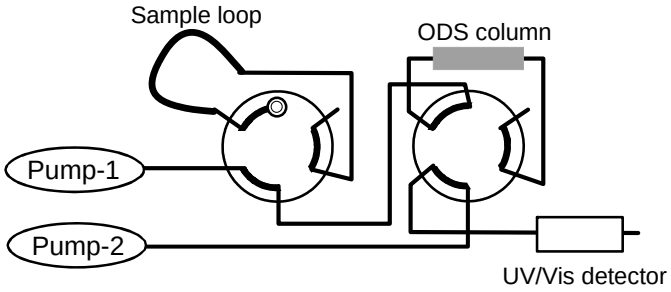
(b) Mobile-phase: CH_3CN (100%) at 0.5 ml min^{-1}; λ=872 nm

Fig.7 Effect of H_2SO_4 final concentration in PAMB solution on the peak area of PO_4 or PO_4 and Si mixed solution

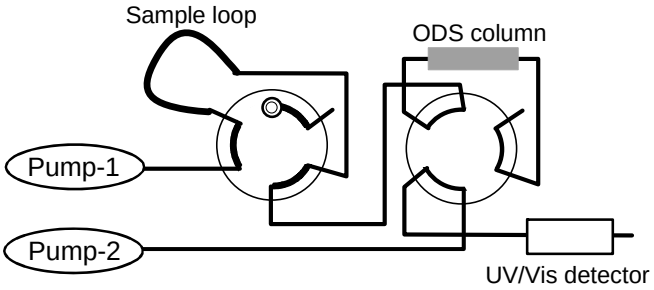
Fig. 8 Calibration curves for PO_4 using modified online solid phase extraction method.

Fig. 9 Compensation equation calculated from the peak areas of silicate and trace level phosphate

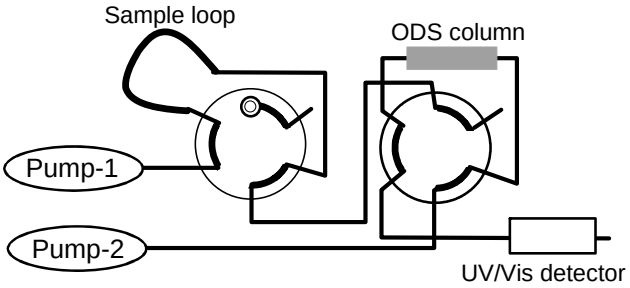
Fig. 10 Concentration of PO_4 in Kozenji-gawa River water



(A) Sample injection and column conditioning



(B) Sample loading to ODS column



(C) Elution and detection

Fig. 1

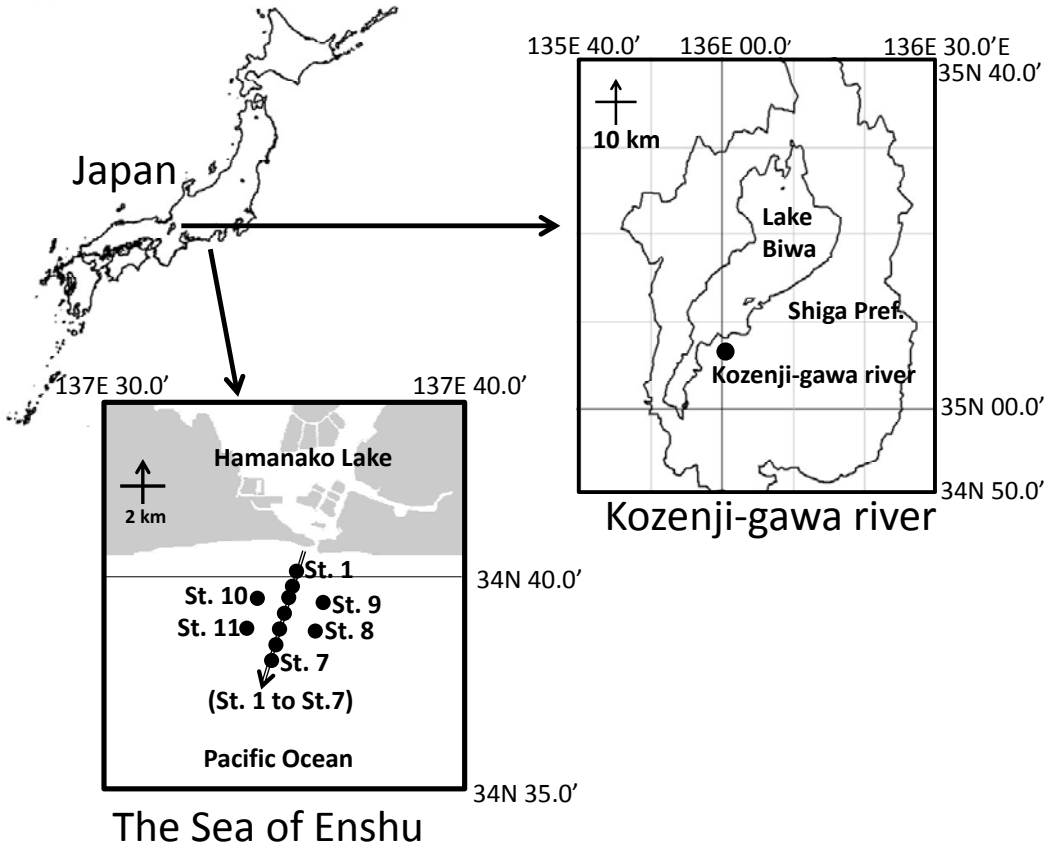

Japan
135E 40.0′
136E 00.0′
136E 30.0′E
35N 40.0′
10 km
Lake
Biwa
Shiga Pref.
Kozenji-gawa river
35N 00.0′
34N 50.0′
Kozenji-gawa river
137E 30.0′
137E 40.0′
Hamanako Lake
2 km
St. 1
St. 10
St. 9
St. 11
St. 8
St. 7
(St. 1 to St.7)
Pacific Ocean
34N 40.0′
34N 35.0′
The Sea of Enshu

Fig. 2

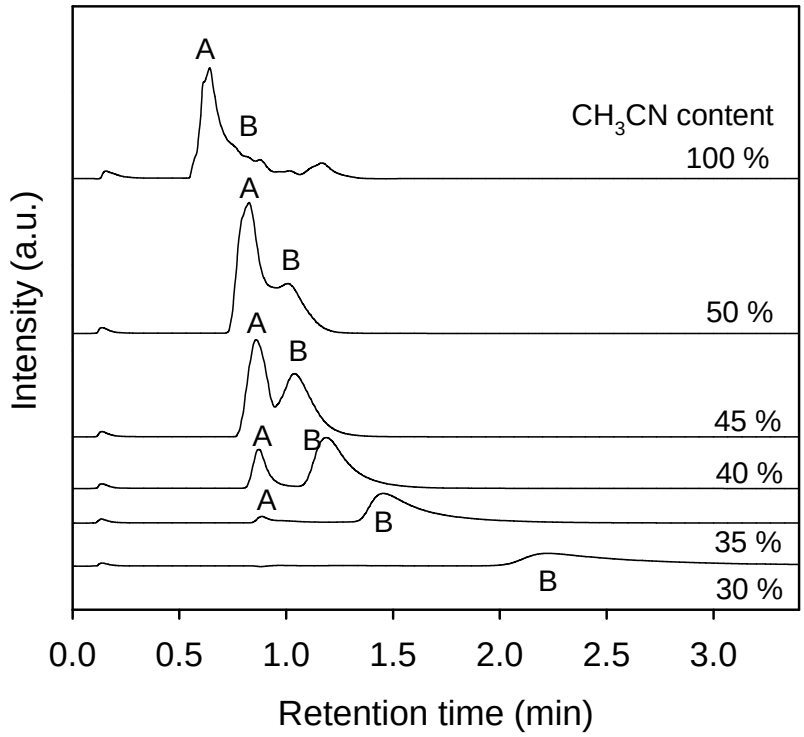

A
B
CH3CN content
100 %
A
B
50 %
A
B
45 %
A
B
40 %
A
B
35 %
B
30 %
Intensity (a.u.)
0.0
0.5
1.0
1.5
2.0
2.5
3.0
Retention time (min)

Fig. 3

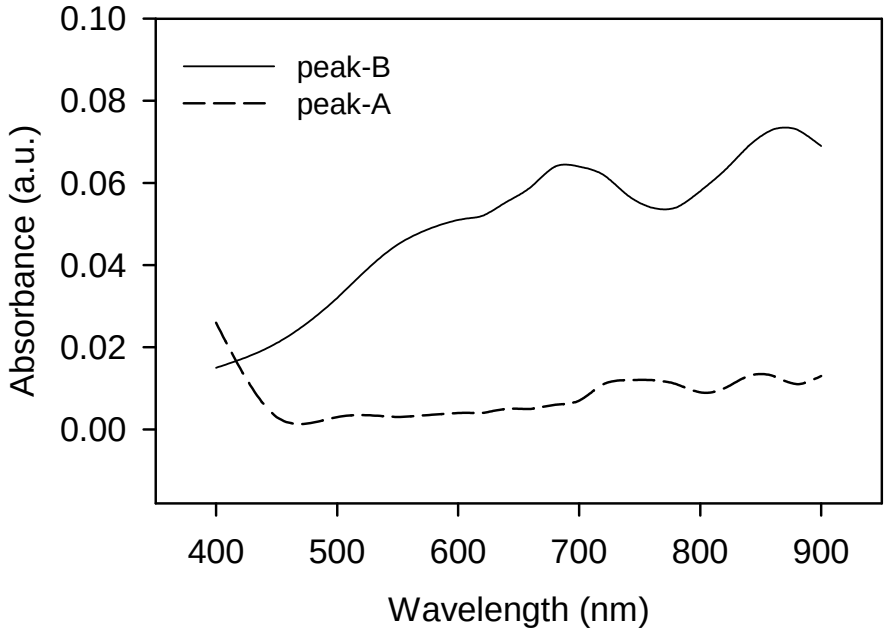

0.10
0.08
0.06
0.04
0.02
0.00
Absorbance (a.u.)
peak-B
peak-A
400
500
600
700
800
900
Wavelength (nm)

Fig. 4

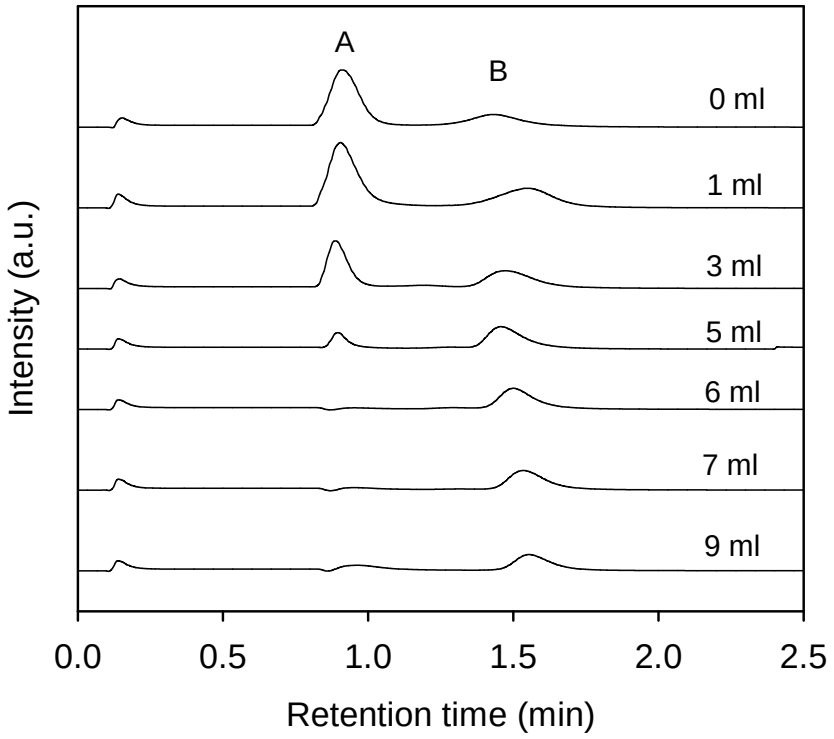

A
B
0 ml
1 ml
3 ml
5 ml
6 ml
7 ml
9 ml
Intensity (a.u.)
0.0
0.5
1.0
1.5
2.0
2.5
Retention time (min)

Fig. 5

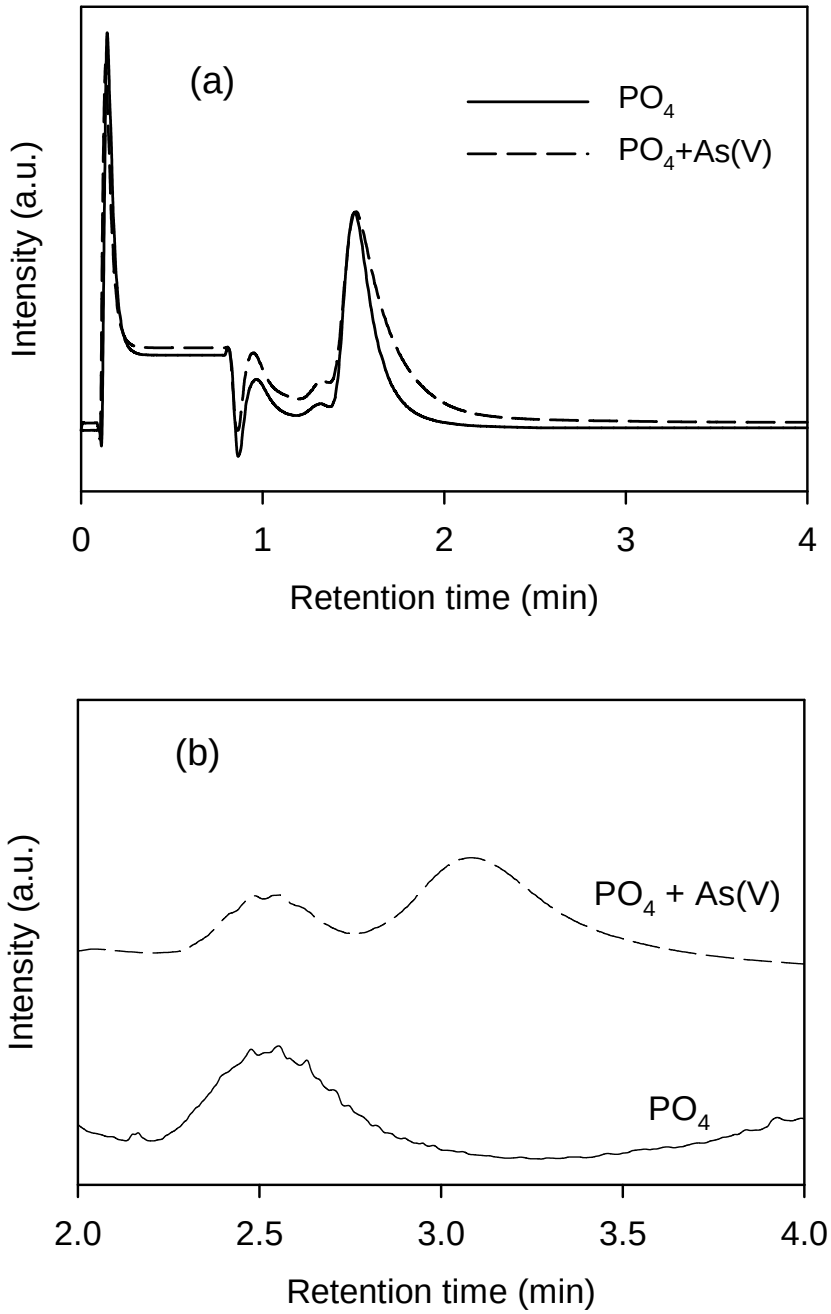

(a)
PO4
PO4+As(V)
Intensity (a.u.)
0
1
2
3
4
Retention time (min)
(b)
PO4 + As(V)
PO4
Intensity (a.u.)
2.0
2.5
3.0
3.5
4.0
Retention time (min)

Fig. 6

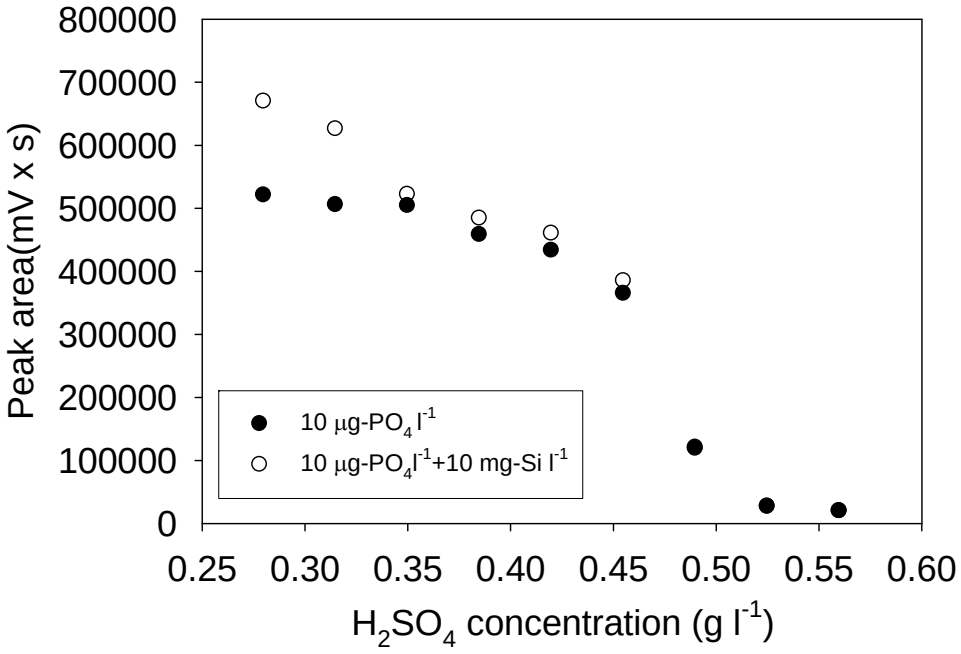

800000
700000
600000
500000
400000
300000
200000
100000
0
Peak area(mV x s)
0.25
0.30
0.35
0.40
0.45
0.50
0.55
0.60
H2SO4 concentration (g l-1)
10 µg-PO4 l-1
10 µg-PO4 l-1+10 mg-Si l-1

Fig.7

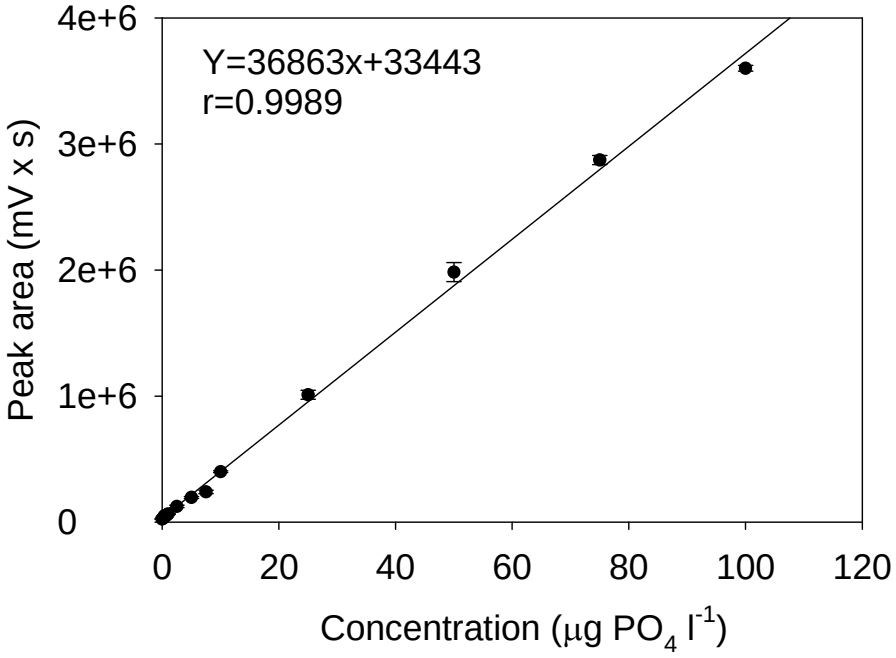

Y=36863x+33443
r=0.9989
Peak area (mV x s)
4e+6
3e+6
2e+6
1e+6
0
0
20
40
60
80
100
120
Concentration (μg PO4 l-1)

Fig. 8

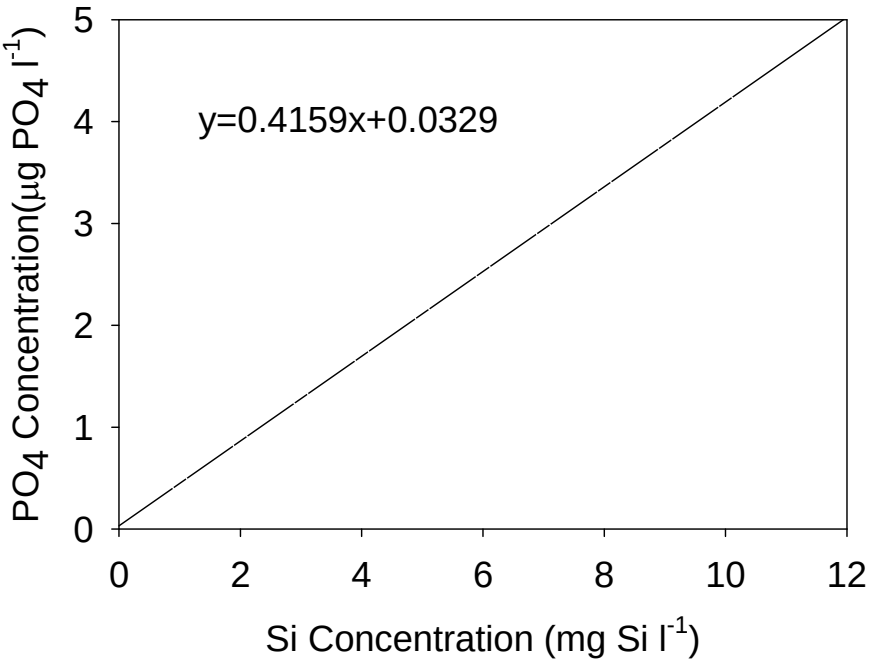

y=0.4159x+0.0329
PO4 Concentration(µg PO4 l-1)
Si Concentration (mg Si l-1)
0
1
2
3
4
5
0
2
4
6
8
10
12

Fig. 9

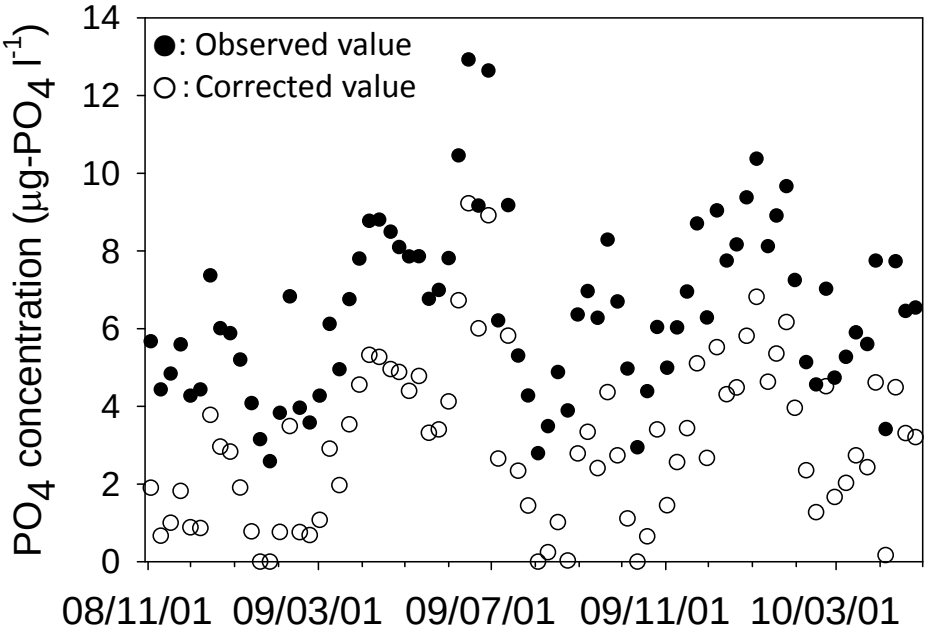

●: Observed value
○: Corrected value
PO4 concentration (μg-PO4 l-1)
14
12
10
8
6
4
2
0
08/11/01
09/03/01
09/07/01
09/11/01
10/03/01

Fig. 10

Table 1 Interference from chemical species on phosphate detection

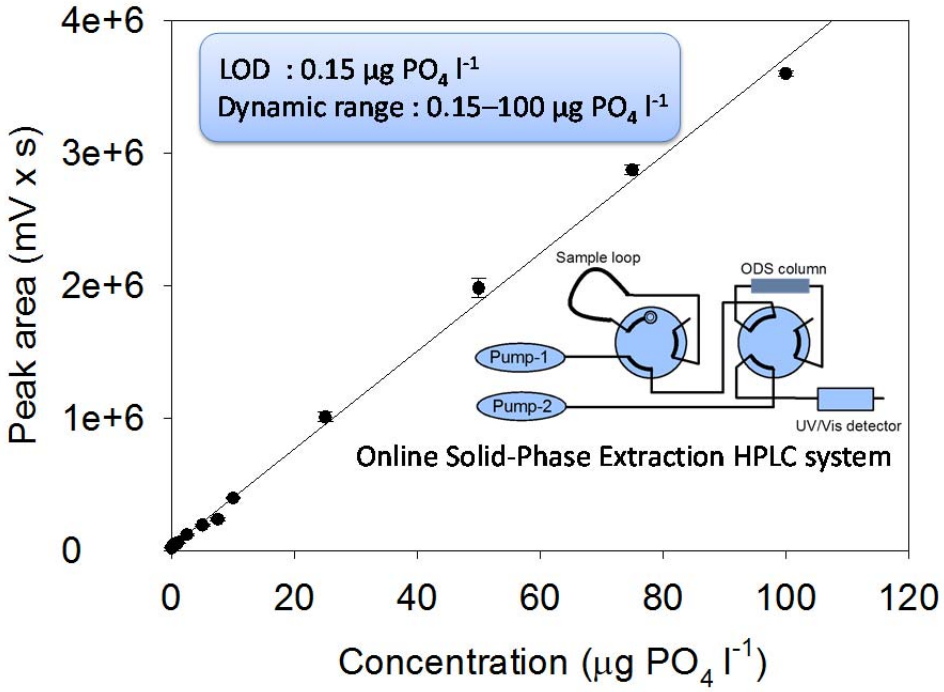
Phosphate concentration: 5 μg PO_4 l^{-1}

Species	Concentration	Error (%)
As(V)	1 μg l^{-1}	+2.7
	3 μg l^{-1}	+8.5
	5 μg l^{-1}	+23.9
As（III）	5 μg l^{-1}	+3.6
Si	5 μg l^{-1}	-1.5
	100 μg l^{-1}	+1.1
	2000 μg l^{-1}	＋10.3
	5000 μg l^{-1}	＋27.1

Table 2 Concentration of PO_4 in seawater collected from the Sea of Eshu (μg PO_4 l^{-1})

	Surface later		Middle layer	
Station	Rising tide	Ebbing tide	Rising tide	Ebbing tide
St.1	2.51	3.22	2.71	0.51
St.2	5.68	1.46	2.59	0.41
St.3	5.59	8.5	3.15	0.47
St.4	4.45	4.84	1.37	0.48
St.5	3.62	2.17	2.7	0.8
St.6	3.43	3.23	1.25	0.78
St.7	8.42	1.39	1.71	0.83
St.8	5.95	1.73	3.74	0.82
St.9	4.05	3.15	3.02	1.98
St.10	1.35	2.05	2.46	1.07
St.11	1.71	1.66	1.44	0.92

Graphical Abstract



Supplemental material

An Online Solid Phase Extraction Method for the Determination of Ultratrace Level Phosphate in Water with a High Performance Liquid Chromatograph

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Table S1 Latitude and Longitude of sampling stations in the Sea of Enshu

Stations	Latitude	Longitude	Sampling depth (m)
St.1	34°40'14“N	137°35'42“E	1 and 5
St.2	34°40'01“N	137°35'36“E	1 and 10
St.3	34°39'45“N	137°35'29“E	1 and 15
St.4	34°39'22“N	137°35'18“E	1 and 15
St.5	34°38'59“N	137°35'08“E	1 and 15
St.6	34°38'23“N	137°34'51“E	1 and 15
St.7	34°37'31“N	137°34'27“E	1 and 15
St.8	34°38'59“N	137°36'15“E	1 and 15
St.9	34°39'45“N	137°36'33“E	1 and 15
St.10	34°39'45“N	137°34'23“E	1 and 15
St.11	34°38'59“N	137°34'01“E	1 and 15

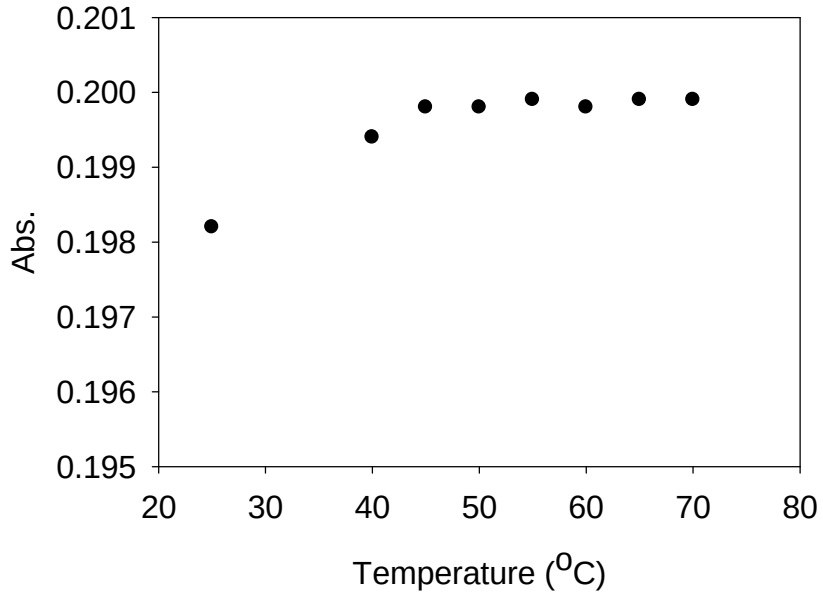

Fig. S1 Effect of reaction temperature on PAMB absorbance

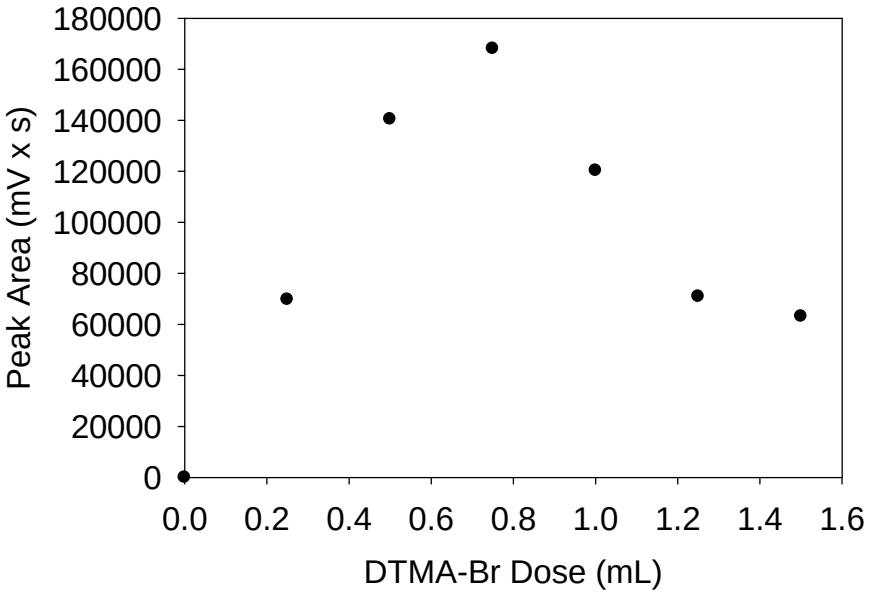

Fig. S2 Effect of DTMA dose on the peak area of the PAMB-DTMA ion-pair complexes using the modified online solid phase method

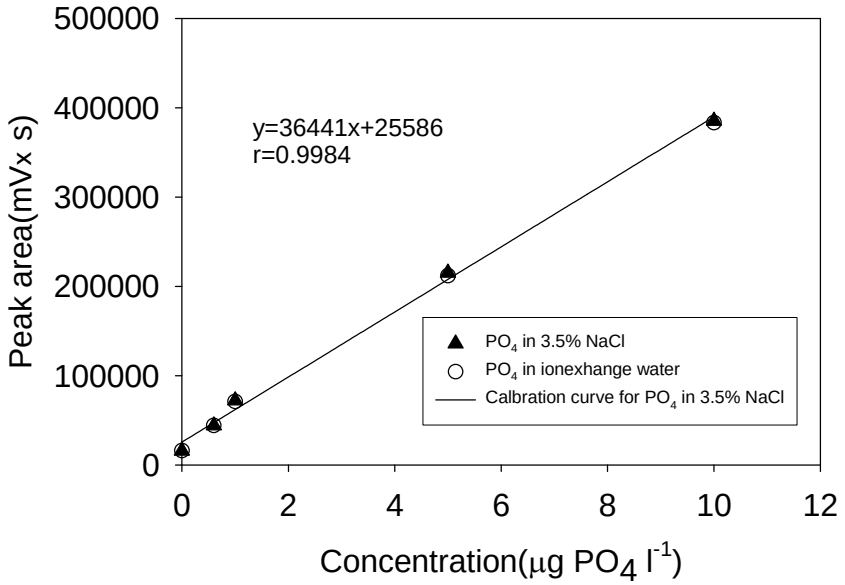

Fig. S3 Calibration curve for PO_4 in ion exchange water or 3.5% NaCl solution

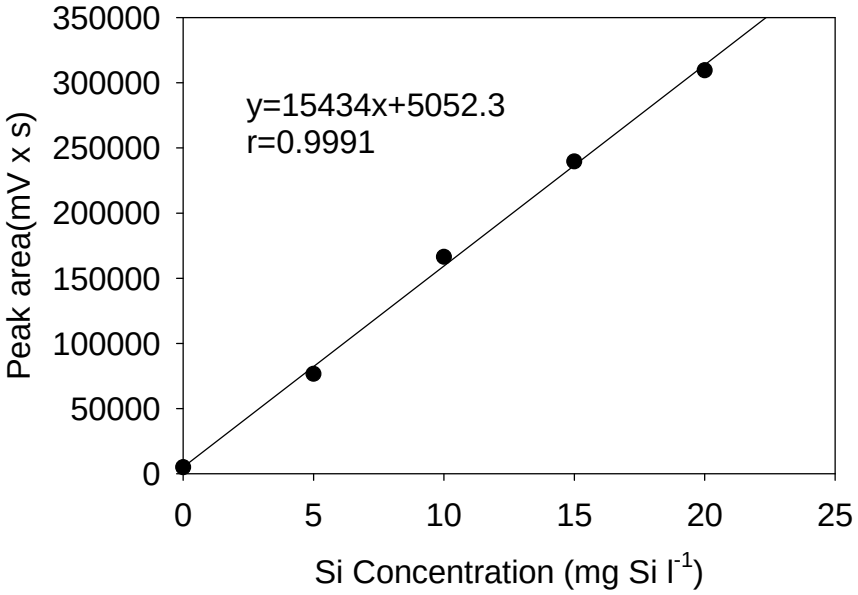

Fig. S4 Calibration curves for silicate obtained by the modified online solid phase extraction method

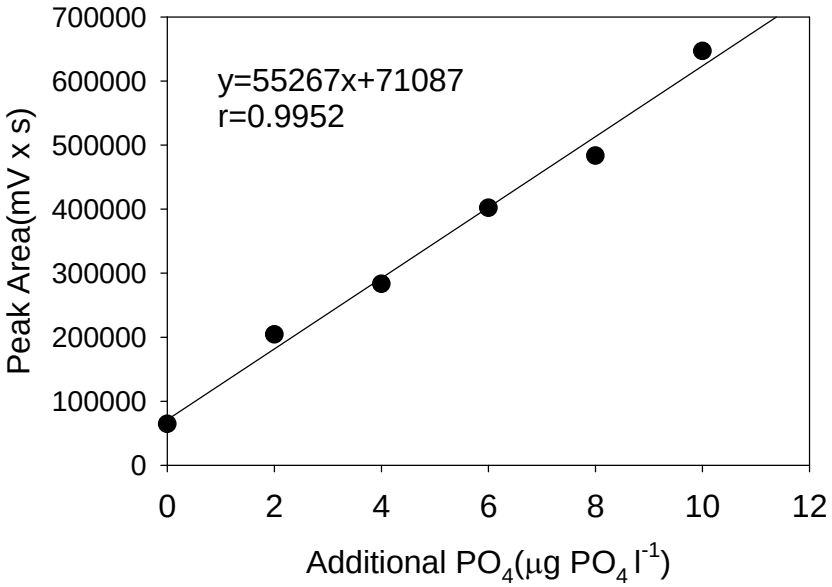

Fig. S5 Concentration of PO_4 in seawater collected from station 7 in the Sea of Enshu determined by standard addition method using the modified solid phase extraction method