Talanta 295 (2025) 128303

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Using a microfluidic paper-based analytical device and solid-phase extraction to determine phosphate concentration

Kaewta Danchana, Haruka Namba, Takashi Kaneta *

Department of Chemistry, Okayama University, 3-1-1 Tsushimanaka, Kita-ku, Okayama, 700-8530, Japan

ARTICLE INFO

Keywords:
Phosphate
Microfluidic paper-based analytical device
Solid-phase extraction
Anion exchanger
Molybdenum blue method

ABSTRACT

Phosphate is an essential nutrient, but in high concentrations it contributes to water pollution. Traditional methods for phosphate measurement, such as absorption spectrophotometry and ion chromatography, require expensive equipment and skilled operators. This study introduces a microfluidic paper-based analytical device (μPAD) that is designed to accomplish field-based, low-concentration phosphate measurements. This μPAD utilizes colorimetric detection based on the molybdenum blue method. Herein, we describe how the conditions were optimized in terms of design and sensitivity by adjusting reagent concentrations, paper thickness, and the time frames for sample introduction, and reaction. The operation consists of simply dipping the μPAD into a sample, capturing images in a home-made photo studio box, and processing the images with ImageJ software to measure RGB intensity. An additional preconcentration step involves solid-phase extraction with an anion exchange resin that achieves a 10-fold enrichment, which enables detection that ranges from 0.05 to 1 mg L^{-1} with a detection limit of 0.089 mg L^{-1} and a quantification limit of 0.269 mg L^{-1}. The replicated measurements showed good reproducibility both intraday and interday (five different days) as 4.7 % and 3.0 % of relative standard deviations, respectively. After storage in a refrigerator for as long as 26 days, this μPAD delivered stable and accurate results for real-world samples of natural water, soil, and toothpaste. The results produced using this system correlate well with those produced via spectrophotometry. This μPAD-based method is a cost-effective, portable, rapid, and simple approach that allows relatively unskilled operators to monitor phosphate concentrations in field applications.

1. Introduction

Phosphorus is one of the nutrients that is essential for the growth of plants and animals, and is generally found in the form of phosphate [1,2], which originates from agricultural runoff, animal waste, and fertilizers and is commonly present in lakes and streams. Excessive phosphorus released into bodies of water is a major source of pollution. High concentrations of phosphorus in water promote the rapid growth of plankton and aquatic plants, which are typical food sources for fish. If uncontrolled, however, such rapid growth accelerates eutrophication, which leads to a decrease in dissolved oxygen levels and a deterioration of water quality. Such conditions pose significant risks to human and animal health as well as to the environment [3].

For example, in 2011, Lake Erie in the United States demonstrated a toxic algal bloom crisis that was caused by a dramatic proliferation of blue-green algae, which produces toxins harmful to both humans and animals. This event significantly impacted communities and businesses that were dependent on the lake, and amounted to one of the most severe environmental crises of the decade. The crisis was primarily driven by high phosphorus input from agricultural activities, sewage, and industrial fertilizers that had accumulated over time. The decomposition of algae by bacteria further depleted oxygen levels, which created hypoxic conditions that produced areas referred to as "dead zones." Such oxygen-deprived areas are inhospitable to both fish and aquatic plants, and could lead to an ecosystem collapse. This example highlighted the critical importance of controlling phosphorus concentrations in rivers and lakes to preserve water quality and maintain ecological balance [4].

In recent years, several methods have been developed for the determination of phosphate concentration. These methods include spectrophotometry [5,6], ion chromatography [7,8], inductively coupled plasma optical emission spectroscopy (ICP-OES) [9,10], electrochemical methods [11–13], and fluorometry [14,15]. While these

This article is part of a special issue entitled: ICFIA 2024 published in Talanta.

* Corresponding author.
E-mail address: kaneta@okayama-u.ac.jp (T. Kaneta).

https://doi.org/10.1016/j.talanta.2025.128303
Received 28 December 2024; Received in revised form 28 April 2025; Accepted 8 May 2025
Available online 10 May 2025

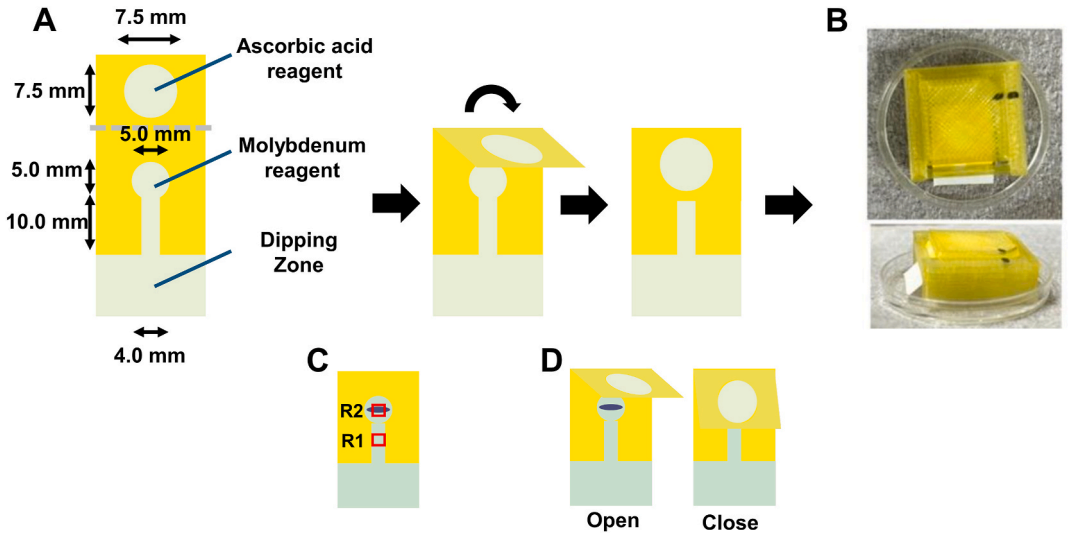

Fig. 1. A) The schematic diagram of the proposed μPAD; B) The μPAD in a 3D holder; C) ΔR calculation; and, D) Monitoring of color formation on the device setup. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

techniques are highly effective for phosphate analysis, they are often associated with significant drawbacks such as the high cost of sensitive equipment and the need for skilled operators to handle such complex instrumentation. Among these, spectrophotometry is considered a more affordable and accessible option. However, it has limitations that include a requirement of large quantities of reagents and the generation of substantial volumes of chemical waste.

The molybdenum blue method has become the standard colorimetric technique for determining orthophosphate concentrations in aqueous solutions. This method relies on the formation of a blue-colored phosphomolybdenum complex, which can be quantified spectrophotometrically. Initially described by Scheele in 1783 [16], the reaction involves molybdenum (VI) reacting with phosphate in an acidic medium to form molybdophosphoric acid. This compound is then reduced to produce a blue molybdenum-phosphate complex via the use of metals, ascorbic acid, or tin (II) chloride as reducing agents [17–19].

Conversely, microfluidic paper-based analytical devices (μPADs) are being promoted as accessible and practical alternatives to lab-based techniques, particularly in resource-limited settings or for on-site measurements because they are cost-effective, user-friendly, and adaptable to various applications depending on their designs. The concept of the μPADs was first introduced by the Whitesides' group in 2007 [20]. The μPADs have shown excellent characteristics such as low cost, ease of fabrication and operation, high disposability, and portability. Thus, μPADs have also been proposed as a promising tool for phosphate measurement.

Several studies have demonstrated the application of μPADs to phosphate measurements [21–29]. However, these devices typically require precise sample handling, which often involves the use of tools such as an autopipette [21] or micropipettes [22–24,27–29] in order to control the volume of sample introduction. Although electrochemical [23] and fluorometric [24] methods offer higher sensitivity than colorimetric approaches, they necessitate additional equipment such as potentiostats or fluorescence detectors. Moreover, some devices require premixing of reagents with a sample [28] or the addition of both a sample and reagents immediately before analysis [25], limiting their practicality as ready-to-use systems. Therefore, to enable the on-site analysis of phosphate in natural water samples, the following challenges must be addressed: (1) sample introduction without the need for a micropipette, (2) development of ready-to-use μPADs, and (3) achieving a detection limit below 1 mg L^{-1}, which corresponds to the regulatory standard for clear water.

Therefore, in the present study, we sought the development of a μPAD that could eliminate all issues, including the need for micropipettes, in determining phosphate concentrations in water samples. With this system, an operator simply dips the introduction zone of the μPAD into the sample solution. The introduction of a sample via simply dipping the μPAD into it has been demonstrated by both Komatsu et al. [30] and by our group [31,32]. The device then utilizes a colorimetric detection method that is based on the molybdenum blue assay. Upon reaction within the μPAD, phosphate forms a dark blue-colored product. However, until solid-phase extraction was developed for use as a pretreatment unit, the sensitivity of these μPADs was insufficient for detectable concentrations of phosphorus in real samples. This adjustment resulted in an approach that is straightforward, rapid, sensitive, and highly effective.

2. Experimental section

2.1. Materials and reagents

All chemicals used in this research were of analytical grade. Deionized water (DI water) with a resistivity of >18 MΩ cm was obtained using the Elix water purification system (Direct-Q UV3, Merck, Darmstadt, Germany) and was used to prepare all solutions. L-(+)-Ascorbic acid was obtained from Nacalai Tesque, Inc. Ammonium molybdate (VI) tetrahydrate was sourced from Sigma-Aldrich, while hydrochloric acid and dipotassium hydrogen phosphate were procured from Wako Pure Chemical Industries, Ltd. Ethylene glycol was supplied by Tokyo Chemical Industry Co., Ltd., and sulfuric acid was obtained from Kanto Chemical Co., Ltd. Nitric acid was provided by Junsei Chemical Co., Ltd., and potassium antimonyl tartrate was acquired from Katayama Chemical Industry Co., Ltd. The anion exchange resin used was Hypersep Spe™ SAX 100 mg (strong anion exchanger; column volume, 1 mL; bed weight, 100 mg; particle size, 40–60 μm) from Thermo Fisher Scientific, and was used for the sample pretreatment step.

Reagents were prepared under optimal conditions developed in a previous study [25]. The molybdenum reagent solution was prepared by mixing 0.126 M ammonium molybdate tetrahydrate and 6 mM antimony potassium tartrate, both in 6 M of sulfuric acid, at a ratio of 1:1. Ethylene glycol was added to the molybdenum reagent solution at a volume ratio of molybdenum reagent solution to ethylene glycol of 1:1.4 to stabilize the molybdenum reagent as reported in the literature [25]. The ascorbic acid reagent was prepared as a 1.0 M solution by dissolving ascorbic acid in DI water. A stock solution that contained 1000 mg L^{-1} PO_4^{3-} was prepared by dissolving sodium dihydrogen phosphate in DI

water.

2.2. μPAD fabrication

The μPAD was designed using Microsoft PowerPoint 2010, and the device dimensions are illustrated in Fig. 1. Yellow ink was chosen for printing the μPAD to enhance the visibility of the blue color produced by the expected reaction. The device was fabricated on filter paper (200 × 200 mm, Chromatography Paper 1CHR, Whatman™, GE Healthcare Life Sciences, UK) using a wax printer (ColorQube 8580 N, Xerox, CT, USA). Additional tests were conducted using other types of filter papers such as CHROMATOGRAPHY PAPER (3 MM CHR, 20.3 × 25.4 cm) and FILTER PAPER (ITEM 2, 485 × 560 mm), both from Whatman.

A 7.5 mm-diameter circular spot located on the top of the device serves as the ascorbic acid zone, and a 5.0 mm-diameter circular spot connects with the flow channel at the bottom of the device to serve as the molybdenum reagent zone. A schematic diagram of the device appears in Fig. 1A.

After printing the paper sheets that form the test devices, each was heated in an oven (ONW–300S, AS ONE, Tokyo, Japan) at 120 °C for 2 min to allow the wax ink to penetrate the backside of the paper. The heating process creates hydrophobic zones in the printed areas while leaving the unprinted areas hydrophilic. After completing the fabrication, we cut the test devices to the desired sizes and covered each backside with clear tape to prevent any leakage of liquid.

2.3. Measurement procedure

To prepare the test devices for use, 1.2 μL of 1.0 M ascorbic acid was applied to the upper zone and was allowed to dry. This process was repeated three times to insure sufficient amounts of ascorbic acid. Subsequently, 0.4 μL of molybdenum reagent was applied to each of the lower zones as indicated in Fig. 1A. The test devices were then folded along designated dotted lines to ensure proper overlap of the molybdenum reagent zones with the ascorbic acid reagent zones.

Once they were folded, the μPADs were placed in a custom holder fabricated using a 3D printer, as shown in Fig. 1B, to maintain firm contact between the molybdenum reagent zones and the ascorbic acid reagent zones (Movie_1, Supplementary data). Subsequently, the μPADs were dipped into a sample solution (2 mL) for 3 min, and upon removal the devices were given 5 min reaction periods to allow the formation of a dark blue product. A smartphone camera captured images of the devices. Each device was placed in a simple home-made photo studio box so that images could be taken under consistent lighting conditions.

The homemade photo studio box, which is similar to that used in our previous studies [31,33], consists of two white light-emitting diodes (LEDs) (maximum emitting wavelength = 448 nm, I.D. 3 mm), a voltage regulator (DROK, Japan), rechargeable Li–Po batteries (~8.4 V, 800 mAh, Keenstone Ltd.), and an on/off switch, all of which were purchased from Amazon, Japan. The rechargeable battery provided stable output voltage under control of the voltage regulator. The LEDs were connected in parallel and powered using the rechargeable batteries at a constant voltage of 2.70 V.

Absorbance measurements were performed using a UV-2400PC spectrophotometer at a wavelength of 650 nm. The molybdenum blue method was employed to compare the results of the μPADs in measuring real samples.

2.4. Data processing

After capturing the blue-colored images with a smartphone, the images were processed using ImageJ software (available for free at: https://imagej.net/ij/). The images obtained from the smartphone consisted of the three standard RGB (red, green, and blue) color channels. ImageJ software extracted the red-channel values, which could be visualized through the histogram option because blue color absorbs red light and reduces the light intensity in the red region of the reflected light. Consequently, as the phosphate concentration increased, the intensity of the blue color of the reaction also increased, which lowered the value of the red color. In the present study, the intensity is reported as ΔR, and is defined as the red intensity of R1 (the corrected area) minus the red intensity of R2 (the colored area), as shown in Fig. 1C. The ΔR values were measured for each device individually to minimize the influence of possible variations in the image-capture conditions such as unstable settings of the smartphone, the photographer's skill, and/or changes in ambient light. A higher ΔR value corresponds to a darker blue color, and indicates a high concentration of phosphate.

2.5. Sample preparation

Sample preparation involved manual filtration using syringe-mounted filters (pore size, 0.2 μm; cellulose acetate, DISMIC 13CP, Advantec, Tokyo, Japan). Water samples from the river and pond were filtered before measurement. A toothpaste extract was prepared by dissolving 0.7 g of toothpaste in 67.3 g of DI water. A turbid solution was obtained by shaking the mixture of toothpaste and water for 30 min, and then the turbid solution was filtered with the syringe filter, which resulted in a toothpaste extract. The toothpaste extract was diluted 100-fold prior to subsequent analysis. Soil samples were prepared by dissolving 20 g of soil in 50 mL of DI water. Similar to the toothpaste sample, a mixture of soil and water was shaken for 30 min, and the resultant turbid solution was filtered using the syringe filter to prepare the soil extract.

3. Results and discussion

The μPAD developed in the present study enables the determination of phosphate concentration through a straightforward process: the device is dipped directly into a sample, and the resultant color intensity is measured. The sample is introduced via capillary action, which allows phosphate to sequentially reach the molybdenum reagent zone and the ascorbic acid zone. This sequential mixing facilitates a stepwise measurement reaction that is enabled by the minimal effort of only dipping the device. Notably, a key advantage of the developed method is the elimination of additional equipment such as micropipettes for sample introduction.

3.1. Optimal thickness for the filter paper

The first parameter for optimizing the proposed μPAD was to optimize the thickness of the filter paper, which is the primary material of this device. We evaluated three thicknesses: 0.18, 0.26, and 0.34 mm. Values for both color intensity and linearity were assessed for each thickness. Phosphate samples with concentrations ranging from 0.5 to 12.5 mg L^{-1} were examined to find the optimal thickness. Since the fabrication process depends on the thickness of the filter paper, the conditions were adjusted accordingly, as summarized in Table S1 of the Supplementary data. The reagent volumes required to fill each spot were adjusted based on the paper thickness; thicker filter papers required larger volumes. Furthermore, increases in the thickness of the paper suppressed wax penetration, which in turn necessitated higher heating temperatures and longer heating times during fabrication.

In the present experiment, ascorbic acid was applied three times and the molybdenum reagent was applied once with different volumes depending on the thickness. Each layer was allowed to fully dry before proceeding to the measurement step. The results indicated that the thickness of the filter paper also influenced the color intensity. The devices fabricated with 0.26 and 0.34 mm filter papers produced darker blue coloration compared with those using 0.18 mm filter paper. Thinner filter papers allowed reagents to spread more uniformly across the center of the spot, whereas thicker papers accommodated greater reagent volumes, which resulted in reaction zones that were more fully

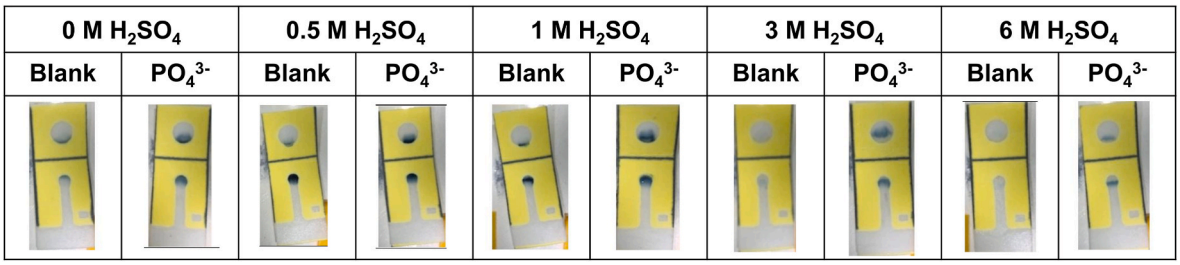

Fig. 2. The optimization of sulfuric acid using a 12.5 mg L^{-1} phosphate standard solution.

saturated. However, the best linearity was observed when using the 0.18 mm filter paper, which likely was due to a more homogeneous distribution of both the reagent and the sample on the paper and resulted in a more homogeneous color of the reaction.

3.2. Color development conditions

We also investigated the effects of the reaction conditions of the paper. The color development was monitored under two different conditions of the μPAD, which included both opened and closed states, as illustrated in Fig. 1D. After introducing a sample solution for 3 min, the device was removed from the dish and let stand for 5 min so that the reaction could proceed. After the reaction, the colored area was allowed to dry under either an opened or a closed state (Fig. 1D), because we speculated that the drying conditions would influence the color intensity.

The results demonstrated that devices left in the opened state exhibited better linearity, as indicated by the R^2 values. This outcome was attributed to the drying process — in the opened state, the device was uniformly exposed to air, ensuring complete drying at the time of measurement. By contrast, devices stored in the closed state dried unevenly, with some areas remaining wet while others only partially dried, which affected the intensity of the blue color. Additionally, in the closed state, prolonged contact between the ascorbic acid reagent zone and the molybdenum reagent zone caused unfavorable reactions in certain areas, which further impacted the results.

Based on these findings, we prioritized reproducibility over color intensity. Thus, as a consequence we selected the open state for drying and filter paper with a thickness of 0.18 mm as the optimal conditions for subsequent experiments.

3.3. Optimization of reagents

Molybdenum blue reactions proceed under strong acidic conditions. Thus, the molybdenum reagent solutions were prepared by dissolving ammonium molybdate tetrahydrate and potassium antimony tartrate in different acidic solutions that included sulfuric acid, nitric acid, and hydrochloric acid, in addition to DI water. The molybdenum reagent was insoluble in both nitric acid and hydrochloric acid and resulted in the formation of a white solid, whereas sulfuric acid and DI water completely dissolved the molybdenum reagent. Thus, only sulfuric acid or water was useable for preparing the molybdenum reagent.

According to the results in the preparation of the molybdenum reagent solution, the concentration of the sulfuric acid was also optimized by testing a range of concentrations: 0, 0.5, 1.0, 3.0, and 6.0 M. The μPADs were fabricated with molybdenum reagent solutions with different concentrations of sulfuric acid, and a 12.5-mg L^{-1} phosphate standard solution and DI water were measured as the test sample and blank sample, respectively, as shown in Fig. 2. Although the test sample showed a weak blue color at 0 M, so did the blank sample. When the concentration was increased to 0.5 and 1 M, even the blank sample generated an intense blue color. The intensity of the blue color in the blank sample gradually decreased, however, when the concentration of sulfuric acid was increased from 3 M to 6 M. Eventually, the blue color for the blank sample disappeared at a sulfuric acid concentration of 6 M, which was chosen as the optimal concentration of sulfuric acid.

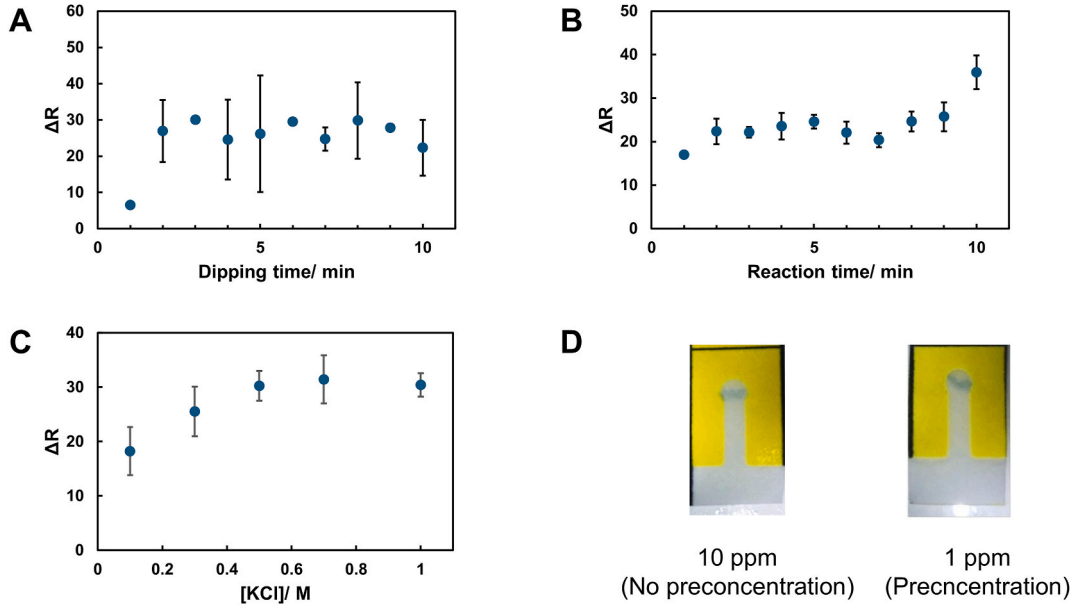

Fig. 3. A) Dipping-time study; B) Reaction-time study; C) The concentration of a KCl study using 10 mg L^{-1} of the phosphate standard; and, D) The images of the μPADs for 10 mg L^{-1} of phosphate without preconcentration and for 1 mg L^{-1} of phosphate with preconcentration.

3.4. Dipping and reaction times

The dipping time and the reaction time both influence the color intensity of the product; the dipping time determines the volume of the introduced sample, while the reaction time varies the color intensity in the molybdenum blue reaction. Maintaining a constant dipping time guarantees the introduction of a consistent volume of sample into the device. Therefore, we conducted a comparison of the color intensity across dipping and reaction times of 1–10 min.

The effects of the dipping and reaction times are shown in Fig. 3A and B. The ΔR value reached a plateau at a dipping time of longer than 2 min, as shown in Fig. 3A, whereas 3 min showed the most reproducible results. Note that a dipping time of 3 min corresponded to the time required to completely fill the flow channel and reach the two reaction zones. Therefore, we attribute the large standard deviations at 2 and 4 min to a supply of the sample solution into the μPADs that either was insufficient or in excess. As seen in Fig. 3A, the dipping times of 2 and 4 min showed large standard deviations. These results imply that the dipping time should be carefully controlled to 3 min to obtain reproducible data.

The reaction appeared to reach its peak at 2 min, as the ΔR values remained consistent from 2 to 9 min. While the ΔR value increased with a reaction time of 10 min, the optimal reaction time for reproducible results proved to be between 2 and 9 min. We set the dipping time and the reaction time to 3 and 5 min, respectively, based on the results shown in Fig. 3A and B.

3.5. Coupling with solid-phase extraction

According to the optimized conditions, we constructed a calibration curve for phosphate. The μPADs provided a linear relationship that ranged from 1 to 10 mg L^{-1} with a coefficient of determination of $R^2 = 0.989$. However, 1 mg L^{-1} of phosphate is too high for the measurement of actual samples because the standard value for water pollution is also less than 1 mg L^{-1}. Therefore, in order to measure actual samples with lower concentrations, we added a system of solid-phase extraction to concentrate the phosphate samples for the μPAD measurements. We used the small anion-exchange column shown in Fig. S1 of the Supplementary data and followed the procedure shown below.

1. Conditioning the ion exchange resin with DI water.
2. Passing 10 mL of the sample solution through the column with a syringe.
3. Passing 1 mL of a potassium chloride solution through the column with a syringe.
4. Washing the resin with 0.5 mL of potassium chloride solution three times.
5. Three washes of the resin with DI water to regenerate the anion exchanger.

In step 2, phosphate ions are selectively retained on the anion exchange resin while the other compounds pass through and are discarded. In step 3, chloride ions replace phosphate ions on the resin, which effectively elutes the phosphate ions along with the potassium chloride solution. This process results in a 10-fold preconcentration of the original sample solution [34]. The operation of solid-phase extraction is carried out by simply passing a sample solution and eluent through the column with a syringe, and the anion exchange column is also small, so it is fully compatible for on-site measurement. The column cartridge could be repeatedly used at least 100 times after being regenerated by washing successively with potassium chloride and water.

To efficiently collect phosphate, the concentration of potassium chloride (KCl) as the eluent was varied between 0.1 and 1 M, using a 10 mg L^{-1} phosphate solution as the standard. A 1-mL aliquot of a 10 mg L^{-1} phosphate solution was passed through the column, and then the phosphate was eluted with 1 mL of KCl at different concentrations. The results showed that 0.7 M KCl was the most effective concentration to elute the phosphate, as shown in Fig. 3C. Thus, 10 mL of a 1 mg L^{-1} phosphate solution was passed through the column, and the retained phosphate was eluted with 1 mL of 0.7 M KCl. When 1 mg L^{-1} of the phosphate solution was preconcentrated 10-fold, the effluent exhibited a blue color intensity comparable to that of a 10 mg L^{-1} phosphate sample solution without preconcentration, as shown in Fig. 3D. Therefore, we successfully coupled solid-phase extraction with the μPAD measurements and enhanced the detectability.

3.6. Analytical performance

A calibration curve for phosphate determination was constructed for concentrations ranging from 0.05 to 1 mg L^{-1} with preconcentration. The ΔR values were plotted against the concentration of phosphate, and the relationship showed good linearity with a coefficient of determination of $R^2 = 0.997$. The limits of detection (LOD) and quantification (LOQ) were calculated according to methods established in a previous study [35]. The LOD was determined to be 0.089 mg L^{-1}, while the LOQ was found to be 0.269 mg L^{-1}. These results demonstrate that the device is capable of detecting phosphate concentrations as low as approximately 0.3 mg L^{-1}, which effectively meets the objective of measuring concentrations lower than 1 mg L^{-1}. The LOD and LOQ obtained by a conventional spectrophotometer were calculated to be 0.032 and 0.096 mg L^{-1}, respectively. The LOD and LOQ of the μPADs were three-fold higher than those of the spectrophotometry.

Repeatability was tested by measuring a 0.25 mg L^{-1} phosphate sample 10 times. The relative standard deviation (RSD) was calculated for the ΔR values, yielding 4.7 % for intraday measurements and 3.0 % for interday measurements on five different days, which indicates a level of repeatability sufficient for practical applications.

The stability of the device was also evaluated by storing it in a plastic container wrapped in aluminum foil to shield it from light and keeping it in a refrigerator at 4 °C. Reagents were pre-deposited to the μPAD, and changes in color intensity were monitored over time. Large variations in ΔR values were observed within the first two days (0 day and the first day), but the performance of the device was rather stable after the second day. These findings indicate that the μPADs should be kept in a common refrigerator for 48 h after fabrication to yield reproducible results. Over a 20-day evaluation period, the device maintained stable performance with no significant degradation or discoloration observed in the reagent zones. However, after 27–30 days of storage, the reagent zones began to discolor, which rendered the device unsuitable for further measurements. Specifically, a light orange discoloration appeared in the ascorbic acid zone, while the molybdenum reagent zone turned blue even before exposure to a phosphate solution. The μPADs were useable for at least 25 days, whereas further improvement in the conditions for storage is needed for a longer lifetime of the μPADs.

Based on the literature, this study employed polyethylene glycol (PEG) to stabilize the molybdenum reagent. Alternative stabilizers could enhance the stability of the μPADs, as our previous research demonstrated that poly(vinyl alcohol) effectively stabilized hydrogen peroxide deposited on paper [36]. Similarly, Lewińska et al. reported that silica xerogel also stabilizes hydrogen peroxide on paper [37]. Consequently, other hydrophilic polymers and xerogels could also serve as potential stabilizers for the molybdenum reagent.

3.7. Application to real samples

Finally, we evaluated the optimized μPADs by measuring phosphate concentrations in real samples, which included river water, pond water, soil, and toothpaste. Calibration curves were constructed for both the μPAD and a spectrophotometer using phosphate standards ranging from 0.05 to 1.0 mg L^{-1}. The spectrophotometer directly measured the absorbance of the samples without preconcentration, whereas the μPADs required preconcentration with the anion exchange resin. The

Table 1
Application to real samples by the proposed μPAD compared with that by a spectrophotometer.

Samples	The proposed μPAD (mg L^{-1})	Spectrophotometer at 650 nm (mg L^{-1})
River water	0.28 ± 0.01	0.24 ± 0.09
Pond water	Not Detected	0.08 ± 0.00
Toothpaste extract	0.55 ± 0.15	0.72 ± 0.13
Soil extract	0.73 ± 0.07	1.04 ± 0.14

calibration curves are shown in Fig. S2 of the Supplementary data. Phosphate concentrations in the real samples — river water, pond water, soil, and toothpaste — were measured using both methods with triplicate measurements for each sample. It is important to note that the measured concentrations in this study represent only the soluble fraction of the analyte. This is because filtration may result in analyte loss due to adsorption onto the filter, and the preparation method may not fully extract the analyte from solid samples due to strong adsorption onto particles. All experiments were conducted on the same day, and the results were applied to the respective calibration curves to calculate phosphate concentrations. The results are summarized in Table 1, which compares the μPAD measurements with those from the spectrophotometer.

The pond-water samples exhibited a very low phosphate concentration that was undetectable by the μPAD. This result was consistent because the spectrophotometer determined the concentration of phosphate in the pond water sample to be 0.08 mg L^{-1}, which is lower than the LOD of the μPAD. By contrast, the phosphate concentrations in river water, toothpaste, and soil samples ranged from approximately 0.20 to 1.0 mg L^{-1}. For these samples, the results obtained using the μPAD closely matched those from the spectrophotometer, which confirmed the reliability and accuracy of the μPAD for phosphate measurement. A paired *t*-test for the results in Table 1 indicates no significant difference between the values of the μPAD and those of spectrophotometry ($P = 0.27$ at a significance level of 0.05). These findings highlight the potential of the μPAD for practical field applications, particularly in scenarios where rapid, cost-effective, and portable detection is required.

3.8. Comparison with other μPADs for phosphate determination

Numerous research studies have been published on paper-based analytical devices (PADs) for phosphate determination, which demonstrates their application across various sample types, including water, soil, and various environmental sources. Those studies are summarized in Table 2. The molybdenum blue method is a commonly used colorimetric approach for phosphate detection due to its sensitivity, reliability, and simplicity. Some studies have combined the molybdenum blue method with other dyes to enhance the performance of the colorimetric reagents. These approaches typically achieve a LOD of around 0.1 mg L^{-1}.

Conversely, the proposed method demonstrates a detection range of 0.05–1 mg L^{-1}, which makes it particularly suitable for low-concentration phosphate samples such as those from water sources. With refrigeration, the stability of the proposed method is limited to 26 days. While this is slightly shorter than the methods reported by both Racicot et al. (>35 weeks) and Richardson et al. (28 days), the timetable for the proposed method is sufficient for most practical applications.

This method employs simple and accessible tools such as wax-printed paper and anion-exchange resin that provide a portable and cost-effective alternative to more complex techniques such as either fluorescent sensors or electrochemical sensors. Furthermore, the performance of the proposed method has been validated using real-world samples such as pond water, river water, soil, and toothpaste, which demonstrates a versatility that is comparable to other methods reported in the literature.

4. Conclusions

A sensitive method to determine phosphate concentration was successfully designed using a μPAD coupled with solid-phase extraction. The optimal concentrations of reagents were determined for phosphate detection in real samples. A blue color developed immediately when the reagent solutions reacted with phosphate, which indicated a successful reaction. We were able to construct a calibration curve for the proposed μPAD to enable the determination of standard phosphate concentrations in the range of 0.05–1 mg L^{-1}. By optimizing the reagent concentrations and incorporating an anion exchange resin for sample preconcentration, the device achieved a reliable detection of phosphate at concentrations as low as 1 mg L^{-1}. Additionally, the device demonstrated stable performance over 26 days when stored in a normal refrigerator. The proposed method is applicable to on-site analysis because operations such as sample preconcentration, sample introduction, image capture, and data processing, all are achieved without the need for sophisticated instruments. The preconcentration requires only a column and a syringe. To introduce a sample, an operator simply dips the μPAD into the sample solution. Once the colorimetric reaction is complete, a home-made photo studio box is used to capture the images of the μPAD in the

Table 2
Comparison of the μ PAD for the determination of phosphate.

Method	Year	Principle	Linear Range	LOD	Sample	Shelf life	Ref.
PAD colorimetry	2012	Molybdenum blue method	0.2–10 ppm	0.05 ppm	water	Ambien 15 days	[21]
PAD colorimetry	2014	Molybdenum blue method	0.1–1 ppm, 1–10 ppm	0.05 ppm	soil	Ambient 15 days	[22]
Reagent less PAD	2016	Screen-printed electrochemical sensor - P–Mo complex	up to 300 uM	4 uM	water	Ambient 30 days	[23]
PAD fluorometry	2019	Fluorogenic phosphate-binding protein	2–64 ppb	1.1 ppb	Environmental sample	–	[24]
PAD colorimetry	2020	Molybdenum blue method	1–10 ppm	0.13 ppm	water	refrigerator at ≤4 °C for up to 35 weeks	[25]
PAD colorimetry	2021	Dye adsorption, Brilliant green and molybdenum blue method	13.6–0.27 mg L^{-1}	0.07 mg L^{-1}	Aqueous phase	–	[26]
PAD colorimetry	2021	Molybdate/antimony reagent	0–1000 ppm	3 ppm	water	Freezer more than 28 days, Ambient more than 1 week	[27]
Dip strip assay colorimetry	2021	Molybdenum blue method	0.1–25 ppm	0.134 ppm	water, sea water	4 months and expected to be 2 years at room Temp.	[28]
A epoxy resin screen-printed PAD	2022	Molybdenum blue method	0.5–40 mg L^{-1}	0.25 mg L^{-1}	Soil	–	[29]
Dip-type PAD with colorimetry and preconcentration	2025	Molybdenum blue method	0.05–1 mg L^{-1}	0.09 mg L^{-1}	Water, soil, toothpaste	Refrigerator for 26 days	This work

field. Data processing requires only simple software or applications that could extract the intensity of the red color. Although ImageJ software was used to process the data in this study via the use of a personal computer, smartphone applications will facilitate on-site analysis because of their excellent portability. Therefore, the current method should be an accessible tool for monitoring elevated levels of phosphate concentration, which could lead to environmental pollution from an otherwise crucial nutrient in natural water.

CRediT authorship contribution statement

Kaewta Danchana: Writing – original draft, Visualization, Supervision, Methodology, Investigation, Funding acquisition, Formal analysis. **Haruka Namba:** Visualization, Validation, Methodology, Investigation, Formal analysis. **Takashi Kaneta:** Writing – review & editing, Visualization, Supervision, Resources, Project administration, Methodology, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This work was supported by The Kurita Water and Environment Foundation (KWEF), Grant Number 23H035. TK acknowledges financial support from Kyoritsu Chemical-Check Lab. Corp.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.talanta.2025.128303.

Data availability

Data will be made available on request.

References

[1] E. D'Angelo, J. Crutchfield, M. Vandiviere, Rapid, sensitive, microscale determination of phosphate in water and soil, J. Environ. Qual. 30 (2001) 2206–2209, https://doi.org/10.2134/jeq2001.2206.
[2] X. Zhu, J. Ma, Recent advances in the determination of phosphate in environmental water samples: insights from practical perspectives, Trends Anal. Chem. 127 (2020) 1159082, https://doi.org/10.1016/j.trac.2020.115908.
[3] R.P. Mihajlović, V.M. Kaljević, M.P. Vukašinović, L.V. Mihajlović, I.D. Pantić, Spectrophotometric method for the determination of phosphorus in natural waters using the bismuth-phosphomolybdate complex, WaterSA 33 (2007) 513–518. https://hdl.handle.net/10520/EJC116456.
[4] A.M. Michalaka, E.J. Anderson, D. Beletsky, S. Boland, N.S. Bosch, T.B. Bridgeman, J.D. Chaffin, K. Cho, R. Confesor, I. Daloglu, J.V. DePinto, M.A. Evans, G. L. Fahnenstiel, L. He, J.C. Ho, L. Jenkins, T.H. Johengen, K.C. Kuo, E. LaPorte, X. Liu, M.R. McWilliams, M.R. Moore, D.J. Posselt, R.P. Richardsh, D. Scavia, A. L. Steiner, E. Verhamme, D.M. Wright, M.A. Zagorski, Record-setting algal bloom in Lake Erie caused by agricultural and meteorological trends consistent with expected future conditions, Proc. Natl. Acad. Sci. USA. 110 (2013) 6448–6452, https://doi.org/10.1073/pnas.1216006110.
[5] S. Oladeji, F. Adelowo, K. Odelade, Evaluation of phosphate level in water samples (Ogbomosho rivers) using UV-Visible spectrophotometric method, IJSRES 4 (2016) 102–108, https://doi.org/10.12983/ijsres-2016-p0102-0108.
[6] M.C.H. Vaughan, W.B. Bowden, J.B. Shanley, A. Vermilyea, B. Wemple, A. W. Schroth, Using in situ UV-Visible spectrophotometer sensors to quantify riverine phosphorus partitioning and concentration at a high frequency, Limnol Oceanogr. Methods 16 (2018) 840–855, https://doi.org/10.1002/lom3.10287.
[7] C. Xie, J. Xu, J. Tang, S.A. Baig, X. Xu, Comparison of phosphorus determination methods by ion chromatography and molybdenum blue methods, Commun. Soil Sci. Plant Anal. 44 (2013) 2535–2545, https://doi.org/10.1080/00103624.2013.811518.
[8] P.J. Antony, S. Karthikeyan, C.S.P. Iyer, Ion chromatographic separation and determination of phosphate and arsenate in water and hair, J. Chromatogr. B 767 (2002) 363–368, https://doi.org/10.1016/s0378-4347(01)00424-8.
[9] M.M.R. Mostert, The determination of phosphate in rocks and iron ores using inductively coupled plasma with optical emission spectroscopy (ICP-OES), Geochem. Explor. Environ. Anal. 19 (2019) 465–473, https://doi.org/10.1144/geochem2018-079.
[10] M.K. Jaffar, N.S.M. Sa'adan, L.S. Chua, Determination of available phosphorus in soil using ICP-OES and UV-Vis spectrophotometer: a comparison, Malays, J. Soil Sci. 28 (2024) 255–267.
[11] W.L. Cheng, J.W. Sue, W.C. Chen, J.L. Chang, J.M. Zen, Activated nickel platform for electrochemical sensing of phosphate, Anal. Chem. 82 (2010) 1157–1161, https://doi.org/10.1021/ac9025253.
[12] M.B. Arvas, H. Gürsu, M. Gençten, Y. Sahin, Electrochemical formation of molybdenum phosphate on a pencil graphite electrode and its potential application for the detection of phosphate ions, Anal. Methods 10 (2018) 4282–4291, https://doi.org/10.1039/C8AY01653D.
[13] S. Sun, Q. Chen, S. Sheth, G. Ran, Q. Song, Direct electrochemical sensing of phosphate in aqueous solutions based on phase transition of calcium phosphate, ACS Sens. 5 (2020) 541–548, https://doi.org/10.1021/acssensors.9b02435.
[14] Z. Zhang, H. Tao, Q. Cao, L. Li, S. Xu, Y. Li, Y. Liu, Ratiometric fluorescence sensor for sensitive detection of inorganic phosphate in environmental samples, Anal. Bioanal. Chem. 414 (2022) 3507–3515, https://doi.org/10.1007/s00216-022-03973-0.
[15] J.J. Tsai, Y.C. Zhan, Y.Y. Wu, Y.C. Chen, A facile switch-on fluorescence sensing method for detecting phosphates from complex aqueous samples, Sens. Actuators B-Chem. 380 (2023) 133298, https://doi.org/10.1016/j.snb.2023.133298.
[16] C.W. Scheele, S.F. Hermbsta, Sammtliche physische und chemische Werke [Wiesbaden], Martin Sändig 1 (1971) 185–200 (reprint: original 1793).
[17] E.A. Nagul, I.D. McKelvie, P. Worsfold, S.D. Kolev, The molybdenum blue reaction for the determination of orthophosphate revisited: opening the black box, Anal. Chim. Acta 890 (2015) 60–82, https://doi.org/10.1016/j.aca.2015.07.030.
[18] P.J. Worsfolda, L.J. Gimbert, U. Mankasingh, O.N. Omaka, G. Hanrahan, P.C.F. C. Gardolinski, P.M. Haygarth, B.L. Turner, M.J. Keith-Roach, I.D. McKelvie, Sampling, sample treatment and quality assurance issues for the determination of phosphorus species in natural waters and soils, Talanta 66 (2005) 273–293, https://doi.org/10.1016/j.talanta.2004.09.006.
[19] A. Müller, C. Serain, Soluble molybdenum blues "des Pudels Kern", Acc. Chem. Res. 33 (2000) 2–10, https://doi.org/10.1021/ar9601510.
[20] Y. Hashimoto, T. Kaneta, Chromatographic paper-based analytical devices using an oxidized paper substrate, Anal. Methods 11 (2) (2019) 179–184, https://doi.org/10.1039/C8AY02298D.
[21] B.M. Jayawardane, I.D. McKelvie, S.D. Kolev, A paper-based device for measurement of reactive phosphate in water, Talanta 100 (2012) 454–460, https://doi.org/10.1016/j.talanta.2012.08.021.
[22] B. Manori Jayawardane, W. Wongwilai, K. Grudpan, S.D. Kolev, M.W. Heaven, D. M. Nash, I.D. McKelvie, Evaluation and application of a paper-based device for the determination of reactive phosphate in soil solution, J. Environ. Qual. 43 (2014) 1081–1085, https://doi.org/10.2134/jeq2013.08.0336.
[23] S. Cinti, D. Talarico, G. Palleschi, D. Moscone, F. Arduini, Novel reagentless paper-based screen-printed electrochemical sensor to detect phosphate, Anal. Chim. Acta 919 (2016) 78–84, https://doi.org/10.1016/j.aca.2016.03.011.
[24] M. Sarwar, J. Leichner, G.M. Naja, C.Z. Li, Smart-phone, paper-based fluorescent sensor for ultra-low inorganic phosphate detection in environmental samples, Microsyst. Nanoeng. 5 (2019) 56, https://doi.org/10.1038/s41378-019-0096-8.
[25] J.M. Racicot, T.L. Mako, A. Olivelli, M. Levine, A paper-based device for ultrasensitive, colorimetric phosphate detection in seawater, Sensors (Basel) 20 (2020) 2766, https://doi.org/10.3390/s20102766.
[26] V. Choudhary, L. Philip, Stable paper-based colorimetric sensor for selective detection of phosphate ion in aqueous phase, Microchem. J. 171 (2021) 106809, https://doi.org/10.1016/j.microc.2021.106809.
[27] S. Richardson, A. Iles, J.M. Rotchell, T. Charlson, A. Hanson, M. Lorch, N. Pamme, Citizen-led sampling to monitor phosphate levels in freshwater environments using a simple paper microfluidic device, PLoS One 16 (2021) e0260102, https://doi.org/10.1371/journal.pone.0260102.
[28] H. Heidari-Bafroui, A. Charbaji, C. Anagnostopoulos, M. Faghri, A colorimetric dip strip assay for detection of low concentrations of phosphate in seawater, Sensors (Basel) 21 (2021) 3125, https://doi.org/10.3390/s21093125.
[29] T. Thongkam, K. Hemavibool, A simple epoxy resin screen-printed paper-based analytical device for detection of phosphate in soil, Anal. Methods 14 (2022) 1069–1076, https://doi.org/10.1039/D1AY02011K.
[30] T. Komatsu, R. Maeda, M. Maeki, A. Ishida, H. Tani, M. Tokeshi, Dip-type paper-based analytical device for straightforward quantitative detection without precise sample introduction, ACS Sens. 6 (2021) 1094–1102, https://doi.org/10.1021/acssensors.0c02367.
[31] S. Seetasang, T. Kaneta, Dip-and-read, organic solvent-compatible, paper-based analytical devices equipped with chromatographic separation for indole analysis in shrimp, ACS Sens. 7 (2022) 1194–1200, https://doi.org/10.1021/acssensors.2c00300.
[32] K. Danchana, H. Iwasaki, K. Ochiai, H. Namba, T. Kaneta, Determination of glutamate using paper-based microfluidic devices with colorimetric detection for food samples, Microchem. J. 179 (2022) 107513, https://doi.org/10.1016/j.microc.2022.107513.
[33] Y. Chen, K. Danchana, T. Kaneta, Comparison of protein immobilization methods with covalent bonding on paper for paper-based enzyme-linked immunosorbent assay, Anal. Bioanal. Chem. 416 (2024) 6679–6686, https://doi.org/10.1007/s00216-024-05575-4.
[34] O. Coskun, Separation techniques: chromatography, North Clin. Istanb. 3 (2016) 156–160, https://doi.org/10.14744/nci.2016.32757.

[35] A.C. Olivieri, Practical guidelines for reporting results in single- and multi-component analytical calibration: a tutorial, Anal. Chim. Acta 868 (2015) 10–22, https://doi.org/10.1016/j.aca.2015.01.017.
[36] T. Boonpoempoon 1, W. Wonsawat, T. Kaneta, Long-term stabilization of hydrogen peroxide by poly(vinyl alcohol) on paper-based analytical devices, Sci. Rep. 9 (2019) 12951, https://doi.org/10.1038/s41598-019-49393-6.
[37] I. Lewińska, P. Bącal, Ł. Tymecki, Hydrogen peroxide stabilization with silica xerogel for paper-based analytical devices and its application to phenolic compounds determination, Anal. Chim. Acta 1320 (2024) 343028, https://doi.org/10.1016/j.aca.2024.343028.