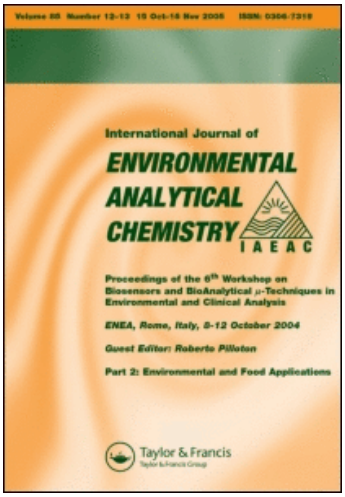

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Determination of phosphate in freshwaters by flow injection with immobilized enzyme and chemiluminescence detection

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Determination of phosphate in freshwaters by flow injection with immobilized enzyme and chemiluminescence detection

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A flow injection method for the determination of phosphate in freshwaters is described using immobilized pyruvate oxidase based on luminol chemiluminescence (CL) detection. Hydrogen peroxide released by the enzymatic reaction of pyruvate in the presence of phosphate is coupled with luminol in an alkaline medium generated CL response using cobalt(II) as a catalyst. The calibration graph was linear over the range of $2–10 \times 10^{-6}$ M phosphate ($r^2 = 0.9985$) with relative standard deviation ($n = 5$) 1.8–3.8%. The detection limit was 2×10^{-7} M with a sample throughput of $60\,h^{-1}$. The common interferences were removed off-line by treating water samples with iminodiacetate chelating resin. The method was applied to freshwater samples and the results obtained [$1.05–1.58 \times 10^{-6}$ M ($0.10 \pm 0.02–0.15 \pm 0.01$ mg PO_4^{3-} L^{-1})] were in reasonable agreement with the results obtained using the molybdenum blue method with spectrophotometric detection [$1.47–1.89 \times 10^{-6}$ M ($0.14 \pm 0.03–0.18 \pm 0.02$ mg PO_4^{3-} L^{-1})]. The immobilized pyruvate oxidase exhibited good operational activity at 30°C for a week and storage stability at 4°C over a period of three months.

Keywords: Chemiluminescence; Flow injection analysis; Phosphate; Immobilized pyruvate oxidase; Hydrogen peroxide; Freshwaters

1. Introduction

Phosphorus occurs in water in various concentrations both in dissolved or particulate forms and as inorganic or organically bound species. Total phosphorus concentrations in water can vary from less than $0.01\,mg\,L^{-1}$ to over $1.0\,mg\,L^{-1}$ in heavily polluted rivers [1]. The inorganic forms of phosphorus comprise orthophosphate (largely PO_4^{3-}) and pyrophosphate ($P_2O_7^{4-}$) as well as their cyclic polymers (metaphosphates) and linear polymers (polyphosphates), which together are referred to as condensed phosphates. Condensed phosphates, both dissolved and particulate, are generally resistant to hydrolysis in natural waters [2, 3]; however, it may be converted to dissolved orthophosphate by mild acid hydrolysis at about 100°C.

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Organic phosphorus compounds are typically phosphate esters although phosphonates in which carbon is bonded directly to phosphorus are also of interest because of their occurrence in marine invertebrates, bacteria and phytoplankton. Specific compound groups include [4] nucleic acids, phospholipids, inositol phosphates, phosphoamides, phosphoproteins, sugar phosphates, phosphorus containing pesticides and organic condensed phosphates. Analytically, organic phosphorus is considered as the fraction that is converted to orthophosphate after oxidative destruction of organic matter. The severity of the oxidation procedure depends on the nature and, to a lesser extent, the amount of the organic phosphorus-containing compounds [5]. Phosphorus in aquatic systems may originate from natural sources such as the mineralization of algae and the dissolution of phosphate minerals from anthropogenic point source changes of sewage and industrial effluents and from diffuse inputs from grazing and agricultural land [6].

Various methods have been reported for the determination of inorganic and organic phosphates in environmental and biological samples using different detection systems [7–10]. Phosphorus has been determined by photometric measurement of 12-phosphomolybdenum blue species in the presence of mild reducing agent [11]. The use of chemiluminescence for the determination of orthophosphate species with greater sensitivity and selectivity has been achieved based on oxidation of luminol with molybdophosphoric heteropoly acid. The detection limit (3 s of the blank) was $0.03\,\mu g\,P\,L^{-1}$ with relative standard deviation ($n=4$) in the range 1.2–4.7% and a sample throughput of $180\,h^{-1}$ [12]. The use of ion chromatography for the determination of As(V), Ge(IV), P(V) and Si(IV) has been reported based on FI–CL detection [13]. An anion exchange column (low-capacity IonPac AS4; 250×4.0 mm i.d.) was employed for chromatographic separation and a long reaction coil length ($10\,m\times0.5$ mm i.d.) was used for the formation of heteropoly acid maintained at 80°C with detection limit of 10, 50, 1.0 and $10\,\mu g\,L^{-1}$ respectively.

A number of FI–CL methods for phosphate have been reported based on immobilized enzymes, biosensors and solid phase chromatographic separation in biological and environmental matrices. Kawasaki *et al.* [14] reported the use of purine nucleotide phosphorylase, xanthine oxidase and urate oxidase, immobilized on controlled porosity glass beads for phosphate determination. Hydrogen peroxide released by the enzymatic reactions of phosphate and inosine was detected by the luminol–peroxidase system with limit of detection 0.5 pmol phosphorus in 50 μL of blood serum. Pettersson [15] reported the inhibition of alkaline phosphatase hydrolysis of substrates such as 4-methylumbelliferyl phosphate as the basis for the determination of phosphate. As little as $0.1\,\mu g\,P\,L^{-1}$ could be detected in natural waters by measuring the decrease in fluorescence of the product of enzymatic hydrolysis. Ikebukuro *et al.* [16] reported a biosensor system for phosphate determination. Hydrogen peroxide generated by pyruvate oxidase is coupled with luminol catalysed by an immobilized peroxidase. The limit of detection was 74 nM with a sample throughput of $20\,h^{-1}$. The pyruvate oxidase immobilized column was stable over a period of two weeks. Nakamura *et al.* [17] reported the use of pyruvate oxidase immobilized physically onto *N*-hydroxysuccinicacidimido beads with out cross-linking agent for the determination of phosphate based on automated FI–CL detection system. The detection limit for phosphate was 96 nM with a relative standard deviation of 2.3% ($n=5$) at 25 ± 0.1°C and a sample throughput $30\,h^{-1}$. Again, the lifetime of the enzyme column was only two weeks. A solid-phase based chemiluminescence optical sensor has been reported for trace

orthophosphate in waters exploiting multisyringe flow injection analysis. The limit of detection was $4\,\mu g\,P\,L^{-1}$ ($1.23 \times 10^{-7}\,M$) with a sampling rate of $11\,h^{-1}$ [18]. Different flow analysis techniques for phosphorus determination has been overviewed elsewhere [19].

In the present work pyruvate oxidase is covalently bound to derivatized controlled pore glass beads by glutaraldehyde immobilization procedure and a flow injection method is developed for the determination of phosphate in freshwater samples. The limit of detection (2 s) was $2 \times 10^{-7}\,M$ with a sample throughput of $60\,h^{-1}$ and immobilized enzyme column stable for at least three months. The method is based on enzymatic reaction followed by luminol chemiluminescence detection. The sequence of reactions is:

$$CH_3COCOO^- + O_2 + HPO_4^{2-} + 2H^+ \xrightarrow[TPP,\,MgCl_2,\,FAD]{\text{pyruvate oxidase}} CH_3COOPO_3H^- + CO_2 + H_2O_2$$

$$\text{luminol} + 2H_2O_2 + OH^- \xrightarrow{Co(II)} \text{light} + \text{3-aminophthalate} + N_2 + 3H_2O$$

2. Experimental

2.1 *Materials and methods*

Pyruvate oxidase (pyruvate: oxygen oxidoreductase (phosphorylating); EC 1.2.3.3, from Aerococcus sp., 100 units), thiamine pyrophosphate (TPP), flavin adenine dinucleotide (FAD, disodium salt), magnesium chloride hexahydrate, glutaraldehyde, controlled pore glass (CPG, 200–400 mesh; pore diameter 117 Å), luminol, 3-aminopropyltriethoxysilane, pyruvic acid (PA, sodium salt) and chelating resin (Chelex 100, sodium form, 50–100 mesh) were obtained from Sigma Chemical Co., St. Louis, MO, USA. All other chemicals were analytical grade (E. Merck, Darmstadt, Germany). FAD stock solution was prepared in citrate buffer (0.02 M, pH 4.5) stored at 4°C and used when required. Solutions of PA, TPP, $MgCl_2 \cdot 6H_2O$ were prepared by dissolving the required amount of compounds in deionized water and diluted up to the mark. Luminol and cobalt(II) stock solutions (10 mM) were prepared by dissolving the required amount of luminol (5-amino-2,3-dihydrophthalazine-1,4-dione) in 50 mL of carbonate buffer (0.1 M, pH 10.5) followed by sonication for 30 min and cobalt(II) nitrate in 50 mL of deionized water. Subsequent standards of each were prepared by serial dilution of the stock solutions with carbonate buffer (0.2 M, pH 10.5). Stock solutions ($100\,mg\,L^{-1}$) of Zn(II), Cu(II), Ni(II), Mn(II), Co(II), Cr(III) and Fe(III) were prepared from atomic absorption standards (Spectrosol, BDH, UK) in 0.02 M HNO_3 and various working solutions were prepared from these stock solutions for interference studies.

2.2 *Enzyme immobilization*

Pyruvate oxidase (100 units) was immobilized on 0.5 g of derivatized CPG by cross-linking with glutaraldehyde, following the procedures reported previously [20, 21]. The immobilization was carried out by incubating the derivatized glass beads overnight at 4°C with the enzyme dissolved in 0.5 mL of phosphate buffer (100 mM, pH 6.0). After immobilization, the aqueous phase was measured for protein content according

to the reported method [22] to evaluate the yield of the immobilization procedure. The immobilized enzyme was packed in a glass column (2.5×40 mm) plugged with glasswool at both ends and washed with citrate buffer (0.02 M, pH 4.5, containing reaction mixture) and used as needed. The immobilized column was utilized for about 100 h without any appreciable change in their activity and the enzymatic activity was completely preserved after three months storage in citrate buffer (0.02 M, pH 4.5, containing reaction mixture) at 4°C.

2.3 *Instrumentation and procedures*

The flow injection chemiluminescence manifold used for this work is shown in figure 1. A peristaltic pump (Minipuls 3, Gilson, 4 channels, Switzerland) was used to propel the citrate buffer (0.02 M, pH 4.5, containing reaction mixture, i.e., PA, $MgCl_2 \cdot 6H_2O$, TPP and FAD) and luminol (1×10^{-4} M prepared in carbonate buffer (0.2 M, pH 10.5), containing Co(II) of 1×10^{-5} M) streams at a flow rate of 1.0 mL min^{-1}. A rotary injection valve (Rheodyne 5020, Anachem, Luton, UK) was used to inject phosphate standards (225 µL) into reaction mixture stream to pass through the immobilized pyruvate oxidase reactor inserted in this channel to oxidize pyruvate in the presence of phosphate to produce hydrogen peroxide and was merged at a T-piece with the luminol stream. The merged stream was allowed to travel 3.0 cm before passing a quartz glass spiral flow cell (1.1 mm i.d., 130 µL internal volume) placed directly in front of an end window photomultiplier tube (PMT, Thorn EMI, 9798QA, Electron Tubes, Ruislip, UK) and an aluminum foil was placed behind the coil to reflect light on the photo cathode. The PMT, glass coil and T-piece were enclosed in a light tight housing [12] enabling the CL reaction to be monitored. The PMT was attached with a power supply (Electron Tubes, PM20SN, UK) and an amplifier of 15 V from an independent power supply (BBH Power Products, UK). The detector output was recorded using a chart recorder (Kipp & Zonen Delft BV, Holland). The immobilized enzyme packed glass column was thermostated using a circulating water bath (*Clifton*, Nickel Electro Ltd. England) at 30°C when in operation.

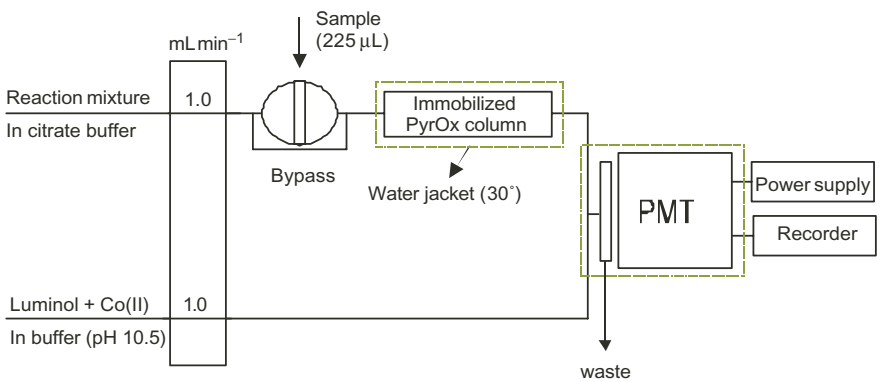

Figure 1. Flow injection chemiluminescence (FI–CL) manifold for the determination of phosphate in freshwaters using immobilized pyruvate oxidase column.

3. Results and discussion

3.1 *Yield of the cross-linking method*

In the cross-linking procedure more than 80% of the enzyme incubated with alkylaminated beads was covalently bound to the glass. About 5–15% of the protein incubated with glutaraldehyde derivatized glass beads were detected in the supernatant after the reaction. The immobilized enzyme packed in the glass column was used for about a 3 month period (stored at 4°C) without any appreciable change in its activity.

3.2 *Optimization of the FI manifold*

The experimental conditions for the formation of the hydrogen peroxide using pyruvate oxidase packed column and its subsequent oxidation of luminol in basic medium were optimized for determination of phosphate. The key parameters optimized were citrate buffer, reaction mixture concentrations (PA, $MgCl_2$, TPP and FAD), flow rate, sample volume, temperature (table 1), carbonate buffer, luminol and cobalt(II) (figure 2). All these studies were performed with a 1×10^{-5} M phosphate solution. A univariate approach was adopted in order to understand the effect of each variable on immobilized pyruvate oxidase/enzymatic reaction and system response.

The effect of pH optimum on the activity of pyruvate oxidase immobilized was studied using a reaction mixture prepared in citrate buffer (0.02 M) of different pH values (3.5–6.0) as a carrier stream. Maximum hydrogen peroxide produced enzymatically was observed at pH 4.5 and therefore was used for all further investigations, while Nakamura *et al*. [17] used *N*-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) buffer with a pH of 7 (0.02 M). For soluble pyruvate oxidase the pH optimum is 5.7 [23]. The effect of reaction mixture concentrations on immobilized pyruvate oxidase including PA, $MgCl_2$, TPP and FAD were studied over the range $2–10 \times 10^{-4}$ M, $2–10 \times 10^{-2}$ M, $2–10 \times 10^{-2}$ M and 5–25 nM respectively prepared in citrate buffer (0.02 M, pH 4.5). As shown in table 1, pyruvic acid, 4×10^{-4} M, magnesium chloride, 6×10^{-2} M, thiamine pyrophosphate, 6×10^{-2} M and flavin adenine dinucleotide, 10 nM were the rate limiting concentrations and are used for subsequent studies.

The effect of flow rate on the CL intensity was investigated in terms of sensitivity, speed, and reagent consumption (table 1). A flow rate of 1.0 mL min^{-1} for both channels gave the maximum CL response (0.6–1.4 mL min^{-1}) with steady base line

Table 1. Effect of reagents concentrations and key physical parameters on the determination of phosphate (1×10^{-5} M) using immobilized pyruvate oxidase based on FI–CL manifold.

Parameter	Range	Optimized
Citrate buffer/pH	3.5–6	4.5
Pyruvic acid/M	$2–10 \times 10^{-4}$	4×10^{-4}
$MgCl_2$/M	$2–10 \times 10^{-2}$	6×10^{-2}
TPP/M	$2–10 \times 10^{-2}$	6×10^{-2}
FAD/nM	5–25	10
Flow rate/mL min^{-1}	0.6–1.4	1.0
Sample volume/μL	90–405	225
Temperature/°C	23–60	50

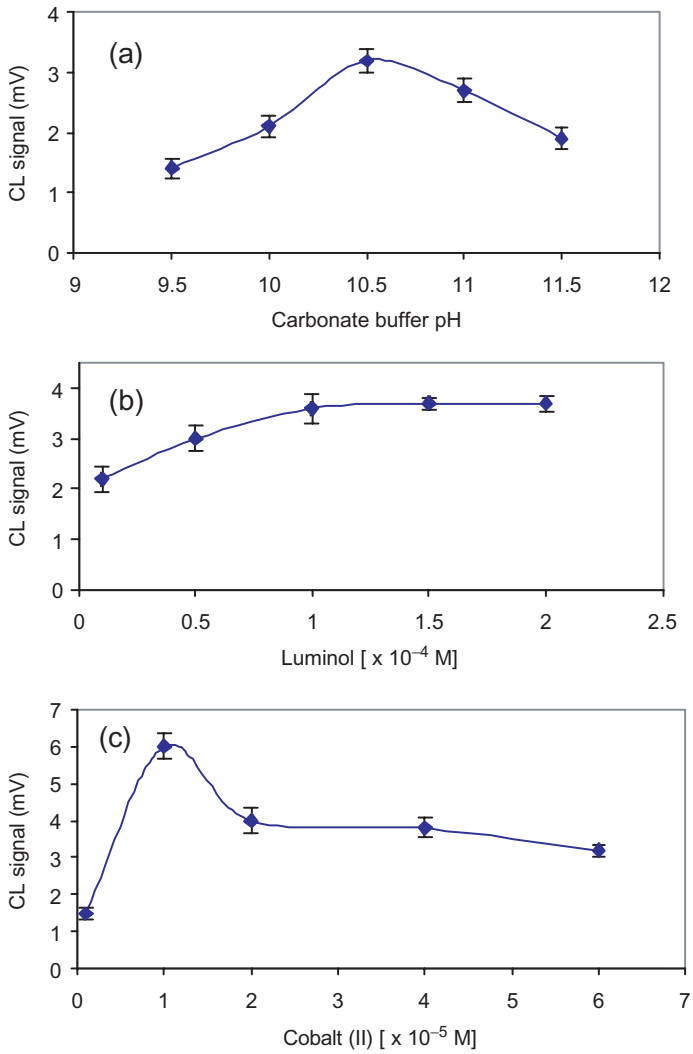

Figure 2. Variation of CL intensity with; (a) pH of carbonate buffer (0.2 M); and concentrations of (b) luminol and (c) cobalt(II).

and reproducible peak height and was used subsequently. Similarly, sample injection volume (225 μL) gave highest CL response and was used for economy of sample consumption and less dispersion. The effect of temperature on the activity of pyruvate oxidase packed column was studied from 23–60°C by flowing water through the water jacket around the enzyme column using a circulating water bath. CL response increased with temperature up to 50°C. However, the column was maintained at 30°C to protect the enzyme from denaturation and to increase the lifetime of the enzyme column.

The efficiency of luminol–CL is particularly dependent on the reaction pH and in the proposed FI–CL system the effect of carbonate buffer (0.2 M) was investigated in the range 9.5–11.5. Maximum CL response was observed at pH 10.5, shown in figure 2(a). The influence of chemiluminescent reagent (luminol) and

catalyst (cobalt(II)) concentrations was studied over the range $0.1–2 \times 10^{-4}$ M and $0.1–6 \times 10^{-5}$ M respectively. CL response increased up to 1×10^{-4} M luminol, above which no appreciable increase in intensity was observed due to saturation (figure 2b). Cobalt(II) was used as a catalyst and the optimum concentration was found at 1×10^{-5} M (figure 2c) and therefore was used for all further experiments.

3.3 *Calibration graph and limit of detection*

A linear calibration graph of CL response *vs.* [phosphate] over the range $2–10 \times 10^{-6}$ M was obtained with a correlation coefficient of 0.9985 ($n = 5$) and regression equation was $y = 0.458x + 0.4185$ [$y =$ CL response (mV), $x =$ concentration (M)]. The relative standard deviation was 1.8–3.8% ($n = 4$) over the range studied with a limit of detection (2 s) 2×10^{-7} M phosphate and a sample throughput of 60 h^{-1}.

3.4 *Interferences*

The effect of diverse ions present in water on the determination of phosphate (1×10^{-5} M) was studied in the range 0.1–250 mg L^{-1}. Figure 3 shows that sodium, potassium, calcium, zinc and nickel, had negligible effect while cobalt(II) (0.1 mg L^{-1}) and iron(III) (0.5 mg L^{-1}), enhanced CL response due to their action as catalysts for luminol oxidation in the presence of molecular oxygen [24, 25]. Manganese(II) (0.5 mg L^{-1}) has a suppressive effect on both the CL signal blank and on phosphate response as reported previously [26]. Copper(II) (0.5 mg L^{-1}) and chromium(III) (0.5 mg L^{-1}) had also a suppressive effect on phosphate response. The effect of all of these cations was eliminated by stirring the water sample (25 mL) with iminodiacetate chelating resin (Chelax 100, sodium form, 50–100 mesh; Sigma, 2.0 g) for 10 min and then injected to FI–CL system for phosphate determination. Sulfate (250 mg L^{-1}) has negligible effect while nitrate (10 mg L^{-1}) suppressed phosphate response during the enzymatic reaction.

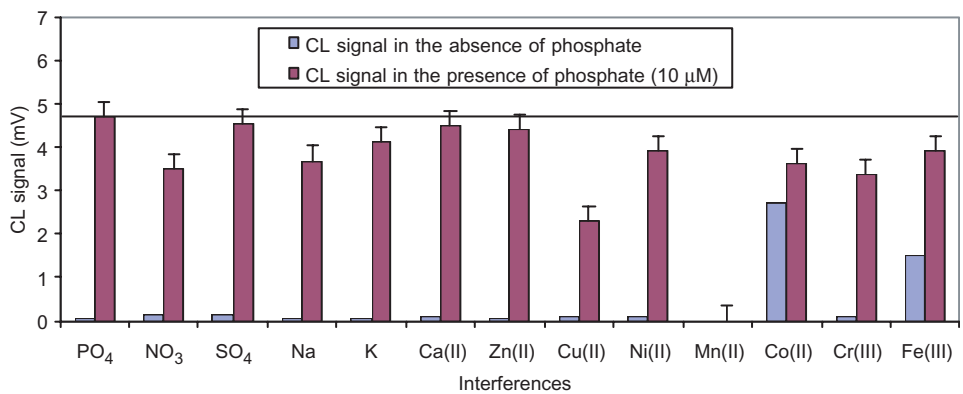

Figure 3. Effect of anions (NO_3^- 10 mg L^{-1}, SO_4^{2-} 250 mg L^{-1}) and cations (Na(I) 50 mg L^{-1}, K(I) 20 mg L^{-1}, Ca(II) 75 mg L^{-1}, Zn(II) 0.5 mg L^{-1}, Cu(II) 0.5 mg L^{-1}, Ni(II) 0.5 mg L^{-1}, Mn(II) 0.5 mg L^{-1}, Co(II) 0.1 mg L^{-1}, Cr(III) 0.5 mg L^{-1} and Fe(III) 0.5 mg L^{-1}) on luminol CL oxidation and PO_4^{3-} (1×10^{-5} M) determination using immobilized pyruvate oxidase. The dashed line shows the response for phosphate in the absence of any interfering species.

Table 2. FI–CL measurement of phosphate in five freshwater samples and comparison with spectrophotometric (molybdenum blue) reference method.

Sample	pH	Conductivity (mS)	Proposed method ($\times 10^{-6}$ M PO_4^{3-})	Reference method* ($\times 10^{-6}$ M PO_4^{3-})
1	7.3	1.1	1.26 (0.12 ± 0.03 mg L^{-1})	1.68 (0.16 ± 0.04 mg L^{-1})
2	7.6	0.8	1.58 (0.15 ± 0.01 mg L^{-1})	1.89 (0.18 ± 0.02 mg L^{-1})
3	7.1	1.5	1.05 (0.10 ± 0.02 mg L^{-1})	1.47 (0.14 ± 0.03 mg L^{-1})
4	7.5	1.8	1.16 (0.11 ± 0.04 mg L^{-1})	1.68 (0.16 ± 0.05 mg L^{-1})
5	7.3	1.6	1.37 (0.13 ± 0.02 mg L^{-1})	1.78 (0.17 ± 0.04 mg L^{-1})

*Molybdenum blue method with spectrophotometric detection. Uncertainties represent the 95% confidence interval for each sample ($n = 4$).

3.5 *Application to water samples*

The proposed method was applied to the determination of phosphate in freshwater samples. Samples were collected from various locations of Quetta valley, Balochistan, Pakistan in acid washed (10% v/v HCl) high density polyethylene (HDPE) bottles. After collection, samples were filtered through a cellulose membrane filter (cellulose acetate, pore size 0.45 μm, 47 mm diameter, Whatman, Maidstone, UK), kept refrigerated in the dark at 4°C and analysed within 12 h of sampling. The results obtained for the five samples [range $1.05–1.58 \times 10^{-6}$ M PO_4^{3-} ($0.10 \pm 0.02–0.15 \pm 0.01$ mg PO_4^{3-} L^{-1})] and results from reference method (spectrophotometric detection of molybdenum blue using ascorbic acid reduction [27] are shown in table 2. The results from the two methods are in reasonable agreement but the spectrophotometric method has a small positive bias, probably due to interference from arsenate [12].

4. Conclusions

The proposed flow-injection chemiluminescent method is simple with a limit of detection of 2×10^{-7} M phosphate using immobilized pyruvate oxidase enzyme. The method is rapid with sampling rate of 60 h^{-1} and the lifetime of the immobilized column on CPG was a three-months period (stored at 4°C) without any appreciable change in its activity compared with the reported methods [16, 17]. The common interfering cations present in waters were removed off line by treating with chelating resin. The method was applied to freshwater samples collected from Quetta valley and the results obtained were in reasonable agreement with the molybdenum blue method [27].

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