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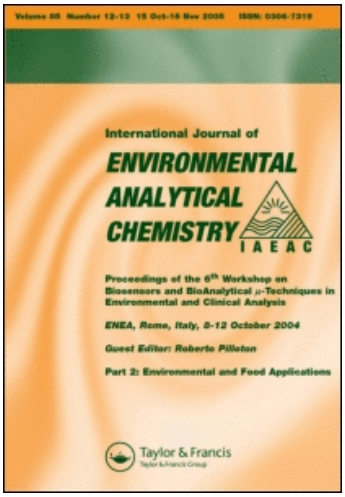

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Enzymatic flow-injection determination of phytase-hydrolysable phosphorus (PHP) in natural waters using immobilized 3-phytase

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Phytic acid, also known as *myo*-inositol hexakisphosphate, is a naturally occurring, non-toxic phosphoric ester that is abundant in seeds and plants as well as aquatic sediments and in soils. It is hydrolysed to a less phosphorylated form by phytases, which are naturally occurring phosphohydrolase enzymes. Because of their non-specificity, phytases also hydrolyse other phosphoester compounds, liberating bioavailable orthophosphate. A flow-injection (FI) method for the determination of phytase hydrolysable phosphorus (PHP) using immobilized phytase is proposed. The method is suitable for the determination of PHP in natural waters, a parameter that may provide a measure of potential long-term bioavailability of organic phosphorus in aquatic systems. Sample volume, temperature, and buffer composition for the immobilized phytase hydrolysis were all optimized. The effects of the potentially interfering ions Al^{3+}, Fe^{2+}, Fe^{3+}, Mn^{2+}, Cu^{2+}, Pb^{2+}, NO_2^-, NO_3^-, I^-, SO_4^{2-}, AsO_4^{3-}, SiO_4^{3-}, and EDTA and urea were also investigated. The method has a limit of detection of $5\,\mu g\,P\,L^{-1}$, and the optimized RSD was typically $<4\%$ ($n=4$). The spike recoveries of phytic acid as P were $\geq 95\%$. The optimized method was applied to the determination of phytase hydrolysable phosphorus in the Tamar Estuary, and the results obtained ($14–25\,\mu g\,P\,L^{-1}$) show that this fraction constitutes a significant portion of the total dissolved P pool in the estuary.

Keywords: Immobilized phytase; Controlled-pore glass; phytase hydrolysable phosphorus; Spectrophotometry; Phytic acid; Flow injection

1. Introduction

Phytic acid (*myo*-inositol hexakisphosphate) is a naturally occurring, non-toxic compound consisting of six phosphate ester groups bound to cyclohexanehexal (inositol). While there are several congeners of inositol hexakisphosphate, the

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myo-isomer (IP_6, phytic acid) is the most predominant [1], and it is widely distributed in both aquatic and terrestrial environments. Phytic acid is the major form of phosphate storage in seeds, cereals, and legumes, averaging >90% [2]. It is also a very important source of phosphate to the environment where it constitutes a substantial proportion of the total P in aquatic sediments and often >60% of the total extractable soil organic-P [3, 4].

Phytic acid is resistant to chemical hydrolysis, especially under alkaline conditions, with the maximum rate of hydrolysis occurring at ca. pH 4 [3] which decreases to a minimum in the pH range 0–1. It also forms insoluble phytate complexes with metal ions such as $Fe^{3+}$and Al^{3+}, and consequently phytic acid has long been considered to be immobile and refractory in soil and aquatic sediment systems. For example, phytic acid is not amenable to hydrolysis by exocellular alkaline phosphatase that is induced in algae by P-deficient conditions, and for this reason, it has also been considered to be biologically unavailable for plant and algal growth [5].

However, there is growing evidence to suggest that phytic acid, or cationic phytates, can occur in free soluble or colloidal forms in aquatic ecosystems, and that they may also be dephosphorylated by respiratory processes [6] or enzymatic hydrolysis by phytase produced by cyanobacteria [7]. Therefore, as part of the elucidation of the role and bioavailability of phytic acid in the aquatic biogeochemical cycle of phosphorus, it is desirable to determine the abundance of phytic acid in aquatic systems, and one approach involves the use of enzymatic hydrolysis with phytase. However, rather than give a selective measurement of phytic acid, this approach gives a response for *phytase hydrolysable phosphorus (PHP)*, which can be considered to be a class of organic phosphorus compounds which, while not as bioavailable as dissolved reactive phosphate (DRP) or alkaline phosphatase hydrolysable phosphate, are nonetheless potentially bioavailable.

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Phytases (*myo*-inositol hexakisphosphate phosphohydrolases) are enzymes that catalyse the hydrolysis of *myo*-inositol (1,2,3,4,5,6)-hexakisphosphate to less phosphorylated myo-inositol phosphates, free orthophosphate, and multivalent cations [8–10]. They occur in plants and some animals. Phytase from plant sources (EC. 3.1.3.26) first acts on the C_6 atom, while that from microbial sources (EC 3.1.3.8) acts on the C_3 atom [3, 9, 11]. They have a broad substrate specificity because of differences arising from the molecular characteristics of phytases purified from diverse sources. Phytase from *Aspergillum niger*, which also produces extracellular phosphomonoesterases, is the most common [10, 12, 13]. This phytase exhibits two activity maxima, the major one being at pH 2.5 with a second, less pronounced, maximum at pH 5.5 [14]. Some phytases can maintain their thermostability at 50–80°C without being denatured [10, 15].

A number of methods have been reported for the determination of phytic acid and assay of phytase activity. The majority are based on photometric detection of inorganic phosphate released by phytases at a defined temperature [13, 16]. A biosensor [17], HPLC [12, 18], and turbidimetric techniques [19] have also been reported. A number of methods have involved the use of phytase covalently bound onto various supports such as gelatine particles [20] or agarose beads [13]. Immobilization offers a better thermostability compared with the free enzyme because of the interaction of the support with the enzyme molecule, which yields a more rigid conformation. The support also improves and stabilizes enzyme activity through contact with water molecules in the

micro-environment [21] and enhances storage stability compared with that of the free enzyme [22].

Previous work by McKelvie *et al*. [13] involved the use of immobilized 3-phytase incorporated in a flow-injection system for the determination of phytase hydrolysable phosphorus (PHP), i.e. the orthophosphate produced by enzymatic hydrolysis. A limitation of this work was that only crude phytase was used, with the result that phytase hydrolysis efficiency was never greater than 60%, which necessitated a multiple regression calibration method in order to discriminate between dissolved reactive phosphorus in the sample and phosphate generated by enzymatic hydrolysis.

This article reports the application of FI to the determination of phytase hydrolysable phosphorus with purified phytase immobilized on CPG. The advantages of this improved method include near complete conversion efficiency, low limit of detection, and a high tolerance for potentially interfering ions.

2. Experimental

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2.1 *Reagents and standard solutions*

All reagents were of analytical grade and were prepared using ultra-pure water (UPW) with a resistivity of $18.2\,M\Omega\,cm^{-1}$, supplied by an Elga water purifier. All glassware and high-density polyethylene (HDPE) bottles used were washed with Neutracon® (Fisher Scientific) a phosphate-free detergent, rinsed at least three times with UPW, soaked in 10% (v/v) HCl overnight and finally rinsed three times with UPW [23]. Model organic phosphorus compounds obtained from the supplier indicated were all prepared in UPW to give stock solutions of 1 mM, which are stable for several months when stored at 4°C in the dark: phytic acid (MgK-phytate (95%), Ca-phytate (90%), Na-phytate (95%), all from Sigma), methyltriphenylphosphonium bromide (MTP, 98%, Fluka), adenosine-5′-triphosphate disodium salt (ATP, 98%, BDH), cocarboxylase (COC, 98%, Sigma), and penta-sodium triphosphate (STP, 98%, Fluka). The flow-injection system was calibrated with phosphate standards prepared in the range of 0.01–0.05 mg P L^{-1} from a 1000 mg P L^{-1} potassium monohydrogenphosphate (AR grade) stock solution.

Orthophosphate produced by hydrolysis in the immobilized phytase reactor was detected on-line in the flow-injection manifold (figure 1) by reaction with a reagent consisting of 0.0129 M ammonium molybdate and 0.0015 M potassium antimonyl tartrate in 0.9 M sulfuric acid. The phosphomolybdate produced by this reaction was reduced with 0.26 M ascorbic acid to produce phosphomolybdenum blue, which was detected spectrophotometrically at 790 nm. The absorbance was therefore the product of DRP + PHP. DRP was also determined separately using a calibration graph obtained by injecting standards of orthophosphate alone, and the absorbance due to PHP was determined by subtraction of the DRP absorbance from the total absorbance.

2.2 *Instrumentation and procedure*

The flow-injection enzymatic manifold for the determination of phytic acid is shown in figure 1. PTFE tubing (0.75 mm i.d.) was used for all manifold flow channels.

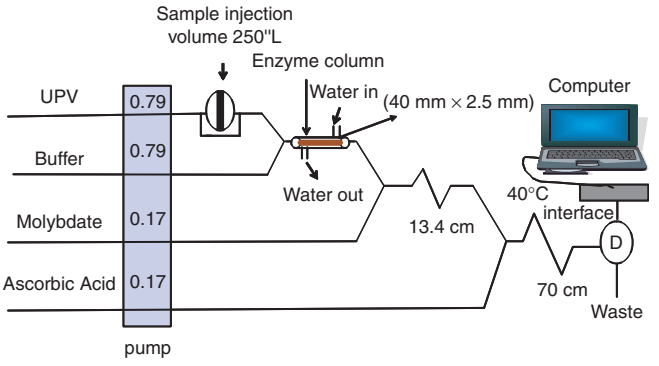

Figure 1. Flow-injection manifold incorporating immobilized phytase for the determination of phytase hydrolysable phosphorus (PHP) in natural waters.

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A peristaltic pump (Gilson) equipped with flow-rated PVC pump tubing was used to propel the UPW carrier and buffer (0.1 M acetate at pH 5.5 in 0.5 M NaCl solution) at 0.79 mL min^{-1}, and the ammonium molybdate and ascorbic acid reagents at 0.17 mL min^{-1} as shown. A rotary injection valve (Rheodyne 5020) was used to inject 250 µL of sample into the UPW carrier, which merged with a buffer stream before passing through the immobilized phytase reactor. The immobilized enzyme reactor was thermostatted at 40°C by flowing water through a glass water jacket. Orthophosphate produced by the hydrolysis of PHP was merged with the acidic molybdate stream to produce phosphomolybdate, which was subsequently reduced by the ascorbic acid stream at 40°C prior to detection in a spectrophotometer (Philips 5680 UV/VIS) fitted with a flow-through cell (Hellma 178.011) with an optical path length of 10 mm and an internal volume of 30 µL. The detector output was automatically recorded using a purpose written LabVIEW® programme.

2.3 *Immobilization procedure*

The immobilization method used was similar to that described by Parrado *et al.* [24] and Yaqoob *et al.* [25]. Dry controlled porosity glass, CPG (120–200 mesh, mean pore diameter 7.4 nm and 0.47 $cm^3 g^{-1}$ pore volume, with a surface area of 152.7 $m^2 g^{-1}$, Sigma) was weighed (1.0 g) and washed by boiling in 5% HNO_3 at 90°C for 60 min followed by rinsing with UPW several times to hydrate and clean the surface. The dry CPG was activated by treatment with 20 mL of a 10% aqueous solution of 3-aminopropyltriethoxysilane (98%, Sigma). A change in pH in the range 11.4–13.1 was observed. The pH was adjusted to 3.45 with HCl, followed by incubation in a water bath at 80°C for 2.5 h. The activated CPG was then filtered and washed several times with UPW. Derivatization of the CPG was carried out by weighing 0.5 g of the activated CPG and adding 2.5% glutaraldehyde (50% aqueous solution, photographic grade, Sigma) prepared in 0.1 M phosphate buffer at pH 7.0 and incubated for 2 h at room temperature, followed by washing with UPW.

Phytase (EC 3.1.3.8, crude form from *A. niger*, 2–5 units mg^{-1} solid, Sigma) was mixed with 10 mL of 0.1 M phosphate buffer (pH 6.0) at 4°C, followed by

centrifugation at 4°C for 20 min at 4500 rpm (Denley BR401 refrigerated centrifuge). Extraction of the enzyme was carried out with 60% ammonium sulfate for 30 min with stirring at 4°C. Ammonium sulfate precipitation is favoured by slow addition and stirring, and yields of 95% can be achieved [9]. The suspension was centrifuged at 4°C for 20 min at 4500 rpm and N_2 gas passed through the precipitate for 30 min and later dissolved in 0.5 mL of 0.1 M phosphate buffer at pH 6. This was then mixed with 0.5 g of the activated CPG and kept overnight at 4°C.

The immobilized phytase reactor was characterized according to the procedures recommended by the European Federation of Biology Working Party on Immobilized Biocatalysts [26]. The activity of the enzyme was measured before and after immobilization by the method of Engelen *et al.* [27] and was found to be >95%. The CPG-immobilized phytase was washed several times with 0.1 M acetate buffer at pH 5.5 in 0.5 M and 0.1 M NaCl, respectively, and stored at 4°C. The protein content of the enzyme was determined before and after immobilization, following the procedures reported by Lowry *et al.* [28]. More than 80% of the enzyme incubated with glutaraldehyde treated beads was covalently bound, and only 7.2% of the protein was detected in the aqueous phase. The immobilized enzyme in the packed glass column was stable for at least 4 months without any appreciable loss in activity [29].

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3. Results and discussion

The objective of this work was to develop an improved method for the determination of phytase hydrolysable phosphorus using an immobilized phytase reactor in conjunction with a flow-injection manifold for detection of the orthophosphate liberated by enzymatic hydrolysis, as shown in figure 1. The manifold was adapted from one previously used for the determination of phosphate in waters with a high arsenate concentration [29], by including a buffer stream to adjust the sample pH to the optimum for enzymatic hydrolysis. The following parameters were all investigated as part of the optimization of the sensitivity of the method; sample volume, temperature, buffer composition, and pH.

3.1 *Sample volume*

The volume of sample injected during analysis influences the detection limit, reproducibility, and sample throughput. The effect of incrementing sample volumes by 50 μL between 50 and 300 μL was investigated. A maximum absorbance was reached at 250 μL, and any increase in sample volume thereafter resulted in peak broadening. On this basis, an injection volume of 250 μL was selected as the optimum value.

3.2 *Effect of temperature*

Enzymatic reactions are strongly temperature-dependent [10, 30], and it is important to determine the optimal temperature for an enzyme after immobilization, as this may differ from that for an enzyme in the free, soluble form. As can be seen in figure 2, the optimum temperature, i.e. maximum activity in the immobilized enzyme reactor, was

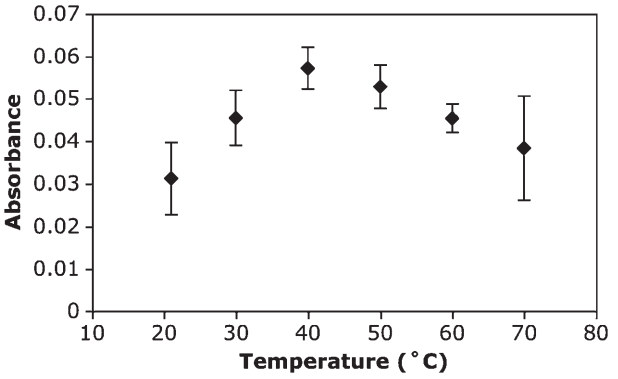

Figure 2. Effect of temperature on the efficiency of immobilized phytase. Error bars, $\pm 3\sigma$ ($n = 4$).

reached at 40°C, whereas for the free phytase, maximum activity occurred at 37°C. Therefore, the optimum temperature of 40°C was used in all subsequent work. This elevated temperature also favours the rapid reduction of phosphomolybdate to phosphomolybdenum blue.

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3.3 *Effect of carrier buffer*

The carrier buffer must maintain the specific pH at which the enzyme exhibits maximum activity. The maximum activity for phytase is reported to occur at pH 2.5 with a second optimum also reported at pH 5.5 when glycine buffer is used, while a single optimum at pH 5.5 is reported for acetate and succinate buffers [31]. For all subsequent work, an acetate buffer at pH of 5.5 was used for immobilized phytase measurements, because this condition is closer to that which will occur in natural waters. Acetate buffers at pH 5.5 showed no difference in conversion efficiency at 0.01, 0.05, and 0.1 M concentrations [29], and 0.1 M buffer was subsequently used to ensure that the optimum pH was maintained for samples with a high buffer capacity, such as marine waters.

3.4 *Substrate conversion*

Phytase from *A. niger* is known to hydrolyse a range of phosphomonoesters other than phytic acid, because the extracts may also contain other extracellular phosphomonoesterases [13, 32]. The results from an investigation of phytase selectivity are shown in figure 3, and indicate that almost complete enzymatic hydrolysis was achieved for MgK phytate. However, conversion efficiencies of only ca. 60% were found for Ca and Na phytate, and ca. 40% for cocarboxylase, ATP, and tripolyphosphate. The lower conversion observed for Ca and Na phytate and ATP is perhaps surprising, given that complete conversion has been reported to occur when hydrolysis was performed with soluble phytase [33]. However, the residence time of the injected sample in the immobilized phytase reactor is only some tens of seconds, and these differences may be due to some kinetic discrimination of the rates of enzymatic hydrolysis for the different

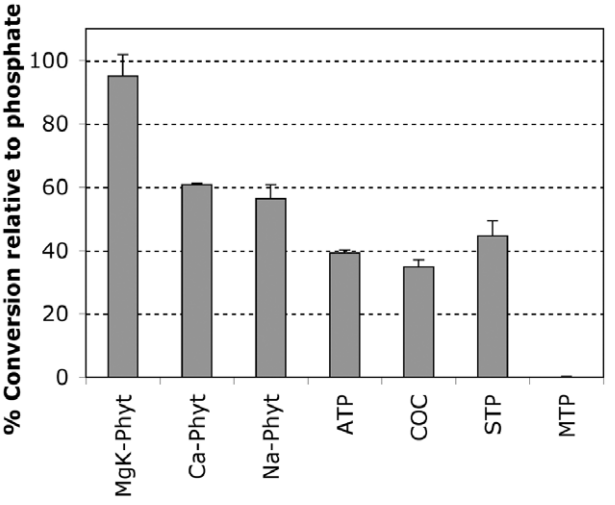

Figure 3. Percentage conversion relative to the phosphate signal of substrates using immobilised phytase buffered with 0.1 M acetate buffer at pH 5.5. Bar plots are mean percentage conversion $\pm 3\sigma$ ($n = 4$). Abbreviations: MgK-Phyt, phytic acid, magnesium potassium salt; Ca-Phyt, phytic acid, calcium salt; Na-Phyt, phytic acid, sodium salt; ATP, adenosine-5′-triphosphate disodium salt; COC, cocarboxylase; STP, sodium tripolyphosphate, MTP, methyltriphenylphosphonium bromide.

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metal phytates, or it may be due to the activating effect of Mg on the phytase, which has been reported previously [13].

What is notable from these results is the enhanced percentage conversion achieved for all forms of phytic acid salts, especially that for the MgK salt, compared with the earlier work of McKelvie *et al.* [13], who reported no better than 60% conversion. This improved conversion is probably a function of the purification of the enzyme prior to immobilization, and the use of CPG as the immobilization matrix.

The conversion efficiency of the optimized flow-injection system incorporating the immobilized phytase reactor was evaluated by injection of 10 and 20 $\mu g\,P\,L^{-1}$ as MgK phytate. Conversion efficiencies of 95% (RSD = 9%) and 96% (RSD = 11%) were obtained compared with the responses for orthophosphate at the same concentrations.

3.5 *Effects of cations and anions on the immobilized phytase*

While it was observed that Mg^{2+} had an activating effect on the immobilized phytase [13, 29], the potential for inhibition of phytase by metal ions such as copper, zinc, and manganese was also investigated. The cations tested for inhibition and their concentrations were Pb^{2+} 10 $mg\,L^{-1}$, Fe^{2+} and Fe^{3+} 1 $mg\,L^{-1}$, Cu^{2+} 1 $mg\,L^{-1}$, Al^{3+} 10 $mg\,L^{-1}$, Zn^{2+} 10 $mg\,L^{-1}$, and Mn^{2+} 10 $mg\,L^{-1}$, while the anions selected were NO_2^- 1.0 $mg\,L^{-1}$, NO_3^- 30 $mg\,L^{-1}$, I^- 1 $mg\,L^{-1}$, SO_4^{2-} 250 $mg\,L^{-1}$, AsO_4^{3-} 0.1 $mg\,L^{-1}$, SiO_4^{3-} 5 $mg\,L^{-1}$, EDTA 1 $mg\,L^{-1}$, and urea 1 $mg\,L^{-1}$, respectively. At these concentrations, all cations with the exception of Cu(II) had a pronounced inhibitory effect on the enzymatic hydrolysis of phytic acid (figure 4), which may be due to the strong chelation of these metals by phytic acid [34], deactivation of the enzyme

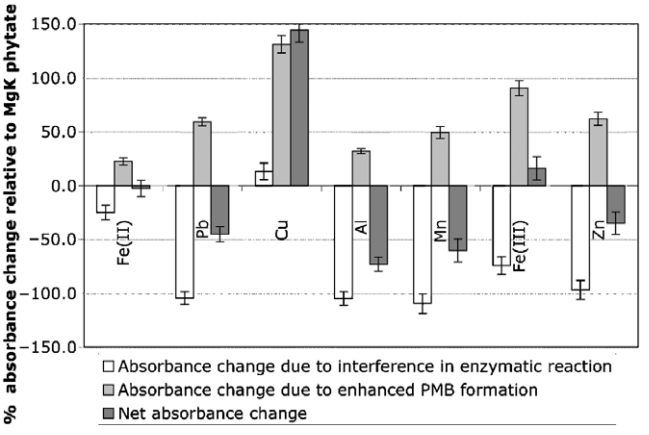

Figure 4. Interferences in the phytase hydrolytic reaction and in the formation of phosphomolybdenum blue (PMB) as a result of the addition of various cations. Bar plots are the mean percentage absorbance change due to interference $\pm 3\sigma$ ($n = 4$) relative to the signal obtained for MgK phytate alone.

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because of substitution for a metal cofactor ion [35], or a combination of both effects. The same metals also had a marked additive interference on the formation of phosphomolybdenum blue (figure 4).

However, it should be noted that the metal concentrations used in these inhibition studies are hugely in excess of the concentrations that might reasonably be expected in natural waters, and even those from impacted catchments like those of the Tamar River. For example, the Fe concentration used in inhibition tests was 5–50 times higher than that observed in the Tamar R, while for Cu, Pb, and Zn, the concentrations exceeded environmental concentrations by factors of 100–10,000 [36]. Consequently, metal inhibition of phytase activity is unlikely to cause inhibition in any but the most grossly metal-polluted samples.

Of the anions studied, arsenate and silicate were the major interferents at high concentrations due to their direct effect on the phosphate detection chemistry [37] (figure 5). All other anions had virtually negligible effect on both phosphomolybdenum blue (PMB) formation and phytase activity (figure 5), despite being present at relatively high concentrations, e.g. sulfate at 250 mg L^{-1}. Since a differential method is used in the analysis of real samples, i.e. an analysis performed with and without immobilized phytase reactor, the effect of these anions will normally be eliminated.

3.6 *Calculation of PHP concentration and analytical figures of merit*

The total concentration of DRP + PHP was determined using the manifold shown in figure 1 using a mixed calibration set consisting of 10–50 μg P L^{-1} for each of orthophosphate and MgK phytate. A calibration for DRP was also obtained by the injection of 10–50 μg P L^{-1} standards of orthophosphate alone, and the absorbance due to PHP was determined by subtraction. The figures of merit for detection of DRP and PHP were determined from these data. Limits of detection (LOD) for each calibration were calculated from the calibration regression plots by the method of

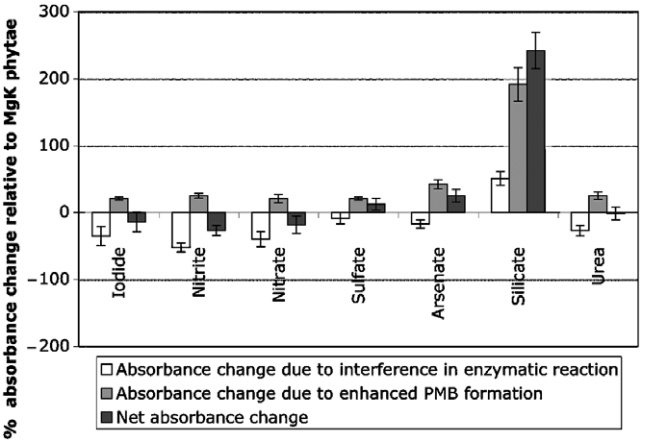

Figure 5. Interferences in the phytase hydrolytic reaction and in the formation of phosphomolybdenum blue (PMB) as a result of the addition of various anions. Bar plots are the mean % absorbance change due to interference $\pm 3\sigma (n = 4)$ relative to the signal obtained for MgK phytate alone.

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Table 1. Analytical figures of merit.

Parameter	Orthophosphate ($\mu g\,P\,L^{-1}$)	MgK phytate ($\mu g\,P\,L^{-1}$)
Calibration range ($\mu g\,P\,L^{-1}$)	10–50	10–50
Slope	1.293×10^{-4}	1.392×10^{-4}
Error in slope	8.4×10^{-6}	1.22×10^{-5}
Intercept	6.74×10^{-4}	7.96×10^{-4}
Error in intercept	2.54×10^{-4}	5.10×10^{-4}
Correlation coefficient (R)	0.9916	0.9973
Limit of detection ($\mu g\,P\,L^{-1}$)	8	5

Miller and Miller [38]. Figures of merit obtained from a typical calibration set are shown in table 1. There was no significant difference between the slopes for the (DRP + PHP) and DRP calibration data sets. The sample throughput was 30–40 samples per hour.

3.7 *Application to Tamar estuary samples*

Natural water samples were collected from different locations in the Tamar River estuary and were filtered *in situ* with a 0.45 μm membrane filter (Whatman, cellulose acetate) and analysed for phytase hydrolysable phosphorus using the manifold shown in figure 1, and the results obtained are shown in table 2. Before injection, the pH of the samples was adjusted to 5.5. The results (after subtraction of DRP to determine PHP) are shown in table 2 and compared with total dissolved phosphorus concentrations obtained by an acid peroxydisulfate autoclave method [39].

The results obtained show the distribution of phytase hydrolysable P (PHP) in the Tamar estuary at the time of sampling. The sampling points represent a wide range of estuarine conditions, with salinity, S, ranging from 0 (freshwater) to 25, and a range of potentially different phosphorus inputs. For example, there is considerable farming

Table 2. Results of phosphorus speciation for Tamar River estuarine samples.[a]

Sample location	pH	Salinity	TDP ($\mu g\,P\,L^{-1}$)	DRP ($\mu g\,P\,L^{-1}$)	DOP = (TDP − DRP) ($\mu g\,P\,L^{-1}$)	PHP ($\mu g\,P\,L^{-1}$)
Weir quay	8	25	42 ± 3	< LOD	38 ± 3	14 ± 0.7
Southward farm	7.8	18	38 ± 4	13 ± 0.3	25 ± 4	14 ± 0.6
Calstock	8	5	79 ± 4	20 ± 0.2	59 ± 4	17 ± 0.7
Rumleigh farm	8	0	77 ± 6	22 ± 0.4	55 ± 6	23 ± 0.7
Morwellham	8	0	58 ± 3	33 ± 1	25 ± 4	16 ± 0.3

[a]Mean concentration $\pm s_{\sigma-1}$ ($n = 4$). TDP: total dissolved phosphorus; DRP: dissolved reactive phosphorus; PHP: phytase hydrolysable phosphorus; DOP: dissolved organic phosphorus. The TDP was determined using an autoclave peroxydisulfate digestion technique [29, 39].

activity in the Southward Farm area while in the Rumleigh Farm area there are diffuse inputs from agriculture and a point source discharge into the estuary from a sewage treatment facility. These results show very clearly that the phytase hydrolysable phosphorus fraction comprises an appreciable proportion of both the total dissolved phosphorus and the dissolved organic phosphorus fractions (22–37% and 29–64%, respectively).

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4. Conclusions

The enzyme phytase can be effectively immobilized onto controlled porosity glass (CPG) functionalized with aminopropyltriethoxysilane and cross-linked with glutaraldehyde. The immobilized enzyme can be packed in a micro-column within a flow-injection manifold for the determination of phytase hydrolysable phosphorus in samples from the Tamar estuary.

The optimum temperature for the phytase after immobilization was 40°C as compared with 37°C in solution. After immobilization, the phytase showed optimum activity at both pH 2.5 and 5.5 in glycine buffer, and at pH 5.5 in acetate buffer. The immobilized phytase was stable for at least six months when stored at 4°C without any significant loss in activity.

The concentration of phytase hydrolysable P in samples from the Tamar estuary was in the range of 14–23 $\mu g\,P\,L^{-1}$ as compared with the dissolved organic phosphorus fraction (25–59 $\mu g\,P\,L^{-1}$) and the dissolved reactive phosphorus fraction (< LOD–33 $\mu g\,P\,L^{-1}$). The phytase hydrolysable P fraction was a significant component of the total dissolved P fraction (22–37%) and dissolved organic phosphorus (29–64%) in the Tamar River estuary at the time of this sampling.

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