



**A New Substrate for Alkaline Phosphatase Based on  
Quercetin Pentaphosphate**

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3 **A New Substrate for Alkaline Phosphatase Based on Quercetin**  
4 **Pentaphosphate**  
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## Abstract

We describe the characterization and application of quercetin pentaphosphate (QPP), a new fluorimetric substrate for the detection of alkaline phosphatase (ALP) activity. QPP exhibits major absorbance peaks at 260/ 410 nm and a strong fluorescent at  $\lambda_{\text{ex}}/\lambda_{\text{em}} = 425/510$  nm at alkaline pH. The product of enzymatic reaction between QPP and ALP has a strong absorbance peak at 324 nm with no fluorescence activity at the investigated wavelengths. The product generated from the enzymatic reaction was found to be proportional to ALP activity and ALP activity was monitored by absorbance difference at 310 nm and 410 nm. The change in absorbance was found to be proportional to the ALP concentration with a linear detection range and a limit of detection of 0.01-16 U/L and 0.766 U L<sup>-1</sup>, respectively. The enzyme activity was also monitored by evaluating the change in fluorescence emission at 530 nm with a linear range of 0.01 – 8 U L<sup>-1</sup> and a detection limit of 0.062 U L<sup>-1</sup>. Further, the validity of the new substrate for ALP in conjugated form was tested using *Bacillus globigii* spores as model sample. A detection limit of 5998 spores/mL was obtained using QPP as a substrate. Unlike the parent compound, QPP substrate exhibits stability in solution for over three and half months and was stable under storage for over 12 months. The results obtained demonstrate the effectiveness of QPP for ALP and compares well with favorably with other fluorescent substrates such as Fluorescein, Alexa Fluor and Cy5.

**Key Words:** Quercetin pentaphosphate, alkaline phosphatase, substrate and fluorescence.

## 1. Introduction

Alkaline phosphatase (ALP) is a widely distributed non-specific phosphomonoesterase that functions through the formation of a covalent phosphoseryl intermediate. The enzyme also catalyzes phosphoryl transfer reactions, hydrolyzing a variety of orthophosphoric monoesters to various alcohols. Excess of ALP in the serum is normally associated with diseases of the liver and bones such as hepatitis, bone inflammation or softening and obstructive jaundice [1]. ALP is therefore one of the commonly assayed enzymes for routine clinical practice [2]. However, ALP activity studies are well known in the field of immunochemistry where it is actively used as a label in immunohistology and enzyme immunoassays. ALP has been extensively used for signal generation in enzyme linked Immunosorbent assay (ELISA).

ELISA, developed as alternative to radio-immunoassay, is a simple and versatile assay based on antibody–antigen reaction. ELISA utilizes enzymes attached to one of the reactants (conjugate) to allow quantitation after addition of a suitable substrate/chromogen [3]. Substrates are critical for detection and visualization steps in ELISA. Different factors determine the choice of a substrate, including the degree of sensitivity required, instrumentation used, safety issues and the type of enzyme. Most immunoassays use proteins conjugated to enzymes in order to generate a signal through the catalytic properties of the enzyme. Most commonly used enzymes are horseradish peroxidase (HRP) and alkaline phosphatase (ALP). ALP exhibits a slower catalytic rate compared to HRP but the reaction rates of ALP remain linear over long periods of time; therefore the sensitivity of ALP can be improved by allowing the reaction to proceed for a long time.

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3 Many substrates for ALP have been reported employing a variety of detection systems  
4 based on the hydrolysis activity of the enzyme on orthophosphoric monoesters [2, 4-8]. The  
5 broad substrate specificity of ALP accounts for the wide variety of assays utilizing the enzyme  
6 for substrate turnover. Currently, there is a large number of commercially available ALP  
7 conjugated immunoreagents mainly because of its high stability, long shelf life and high turnover  
8 rates [1].  
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18 Fluorimetric, spectrophotometric and electrochemical methods are common detection  
19 techniques for monitoring the activity of ALP enzymes. Based on these techniques many  
20 substrates for ALP have been reported. One of the most commonly used substrate is *p*-  
21 nitrophenyl phosphate (pNPP) for either colorimetric or spectrophotometric determination of  
22 ALP. Recently, various substrates for ALP assay utilizing fluorescence[2,9,10],  
23 phosphorescence[11], chemiluminescent[6] and electrochemical[1,4,5,8,12] methods have been  
24 reported. Electrochemical method is not widely used in immunoassays because of limited  
25 availability of suitable substrates that can be electrochemically activated in an enzymatic  
26 reaction. Further, in the case of ALP substrates the resulting phenolic products contribute to  
27 electrode fouling interfering with the inherent sensitivity of the sensor device.  
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43 In the current study we report the use of quercetin pentaphosphate (QPP) as a new  
44 substrate for alkaline phosphatase using both spectrophotometric, and fluorimetric methods. QPP  
45 was found to be stable for up to 1 year at 0-4 °C and the lifetime could be extended by storing at  
46 -20 °C. The products formed have a strong absorbance at 325 nm. QPP has strong emission at  
47 510 nm when excited at 425 nm. The product of enzymatic reaction has no fluorescence activity.  
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## 2. Materials and Methods

### 2.1. Materials and Solutions

Alkaline phosphatase,  $MgCl_2$  and diethanolamine (DEA) solution pH 9.8 were purchased from Sigma Aldrich, Milwaukee. Sodium hydroxide (NaOH (98.7%), sodium phosphate ( $Na_2HPO_4 \cdot 7H_2O$ ), sodium hydrogen phosphate monohydrate ( $NaH_2PO_4 \cdot H_2O$ ), sodium chloride (NaCl), sodium phosphate heptahydrate ( $NaH_2PO_4 \cdot 7H_2O$ ) and sodium hydrogen phosphate ( $NaH_2PO_4 \cdot H_2O$ ) were purchased from J.T. Baker, Phillipsburg, NJ. Both goat and rabbit anti-*Bacillus globigii* IgG (TC-7014-0002) were purchased from Tetracore Inc., Rockville MD while *Bacillus globigii* (*B. atrophaeus*) spore suspension (ATCC 9372; SUN-06) was purchased from NAMSA, Northwood, OH. Quercetin, N,N-diisopropylethylamine, Dimethylaminopyridine, Dibenzylphosphite were purchased from Sigma-Aldrich Company (St. Louis, MO) and were used as received. Anhydrous acetonitrile, ethyl acetate, hexane and methanol solvents were of analytical or high performance liquid chromatography (HPLC) grade. PBS (phosphate buffer saline) consisted of 0.045 M  $NaH_2HPO_4$ , 0.06 M  $Na_2HPO_4$ , 0.1 M NaCl and 0.004 M  $NaN_3$  in Nanopure water. PBST consisted of PBS with 0.05% (v/v) Tween 20. PBSTB consisted of PBST with 1% BSA (w/v). All the phosphate buffers were adjusted to PH 7.2 with concentrated NaOH. TBST buffer was prepared by 0.045M Trizma-HCl, 0.0055M Tris-base, 0.1M NaCl, 0.004M  $NaN_3$  and 0.05% (v/v) in Nanopure water. TBSTB consisted of TBST with 1% BSA. All Tris buffers were adjusted to pH 8.0 with concentrated HCl or NaOH. Carbonate buffer was made by titrating 320 mL of 0.1 M  $Na_2CO_3$  containing 0.004 M  $NaN_3$  with 0.1 M  $NaHCO_3$  to pH 9.6.

## 2.2. Instrumentation

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A multidetection microplate reader, Synergy HT RDR from BioTEK instruments was used for end point absorbance measurements. Fluorescence measurements were carried out on a RF-540 recording spectrofluorometer (Shimadzu, Kyoto, Japan) and processed with Panorama data analysis software. UV-VIS measurements were carried out using a Hewlett Packard diode array spectrophotometer (model HP-8553, Foster City, CA). This instrument was used to monitor absorbance changes during enzymatic hydrolysis of QPP. To monitor the synthetic reaction, TLC was performed on Baker Aluminum-backed silica gel 60/UV<sub>254</sub> plates. Companion Flash chromatography system (Teledyne Isco combiFlash) equipped with a pre-packed silica column was used for purification of the crude product before HPLC and LC-MS analysis.

HPLC analysis was performed on a Dionex Ultimate 3000 equipped with a photodiode array detector (PDA), Chromeleon software, and a reverse-phase C<sub>18</sub> Column (250×4.6 mm i.d., 5 μM, Zorbax-Rx-C<sub>18</sub>, Roxkland Technol., Newport, DE). The mobile phase compositions for HPLC analysis were either 1:1 methanol-water mixture or 40% acetonitrile in water in an isocratic flow. Samples were dissolved in acetonitrile for the QPP intermediate and water for QPP and the flow rate maintained at 0.5 mL min<sup>-1</sup> unless otherwise stated. Spectral analysis was set to scan from 210 to 600 nm.

LC-MS analyses was performed on HPLC system coupled with a photodiode array (PDA) detector and a Thermo Finnegan (San Jose, CA) LCQ Fleet mass spectrometer in sequence equipped with either APCI or ESI source, and Thermo Hypersil GOLD reverse-phase C<sub>18</sub> column (100 × 2.1.i.d., 3 μm). Bruker AM360 spectrometer operated by Tecmag NTNMR software was used for <sup>1</sup>H NMR analysis with CDCl<sub>3</sub> or DMSO-d<sub>6</sub> as solvent for the <sup>1</sup>H-NMR

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3 and  $^{13}\text{C}$ -NMR spectra analysis.  $^{31}\text{P}$ -NMR spectra were recorded at 121.5 MHz using deuterated  
4 dimethylsulfoxide (DMSO) as the solvent with respect to 85%  $\text{HPO}_3$  which was used as external  
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8 reference.  
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### 10 11 12 **2.3 Synthesis of 3,3',4',5,7 –penta-o-benzylphosphoquercetin (QPPI)**

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15 QPPI was synthesized according to a literature procedure with modifications [13].  
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17 Typically, a three-necked round bottomed flask was oven dried, fitted with septa, stirring bar,  
18 thermometer and  $\text{N}_2$  inlet. The flask was charged with quercetin (1 mmol) and anhydrous  
19 acetonitrile (30 mL). The mixture was stirred to dissolve the quercetin and then cooled to  $-10\text{ }^\circ\text{C}$   
20 by a salt ice-bath. Subsequently,  $\text{CCl}_4$  (3 mL) was added into the cooled solution of quercetin  
21 with stirring, followed by  $\text{N,N}$ -diisopropylethylamine (2 mL) and Dimethylaminopyridine  
22 (64.74 mg) consecutively. A minute later dibenzylphosphite (6 mL) was added drop wise to  
23 initiate the reaction. The reaction was monitored by TLC analysis.  
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34 After 45 min, 0.5 M  $\text{KH}_2\text{PO}_4$  (32 mL/100 mL  $\text{CH}_3\text{CN}$ ) was added and the mixture  
35 allowed to warm to room temperature. The mixture was then extracted with ethyl acetate.  
36 Resultant organic extracts were washed with water, saturated  $\text{NaCl}$ , dried over  $\text{Na}_2\text{SO}_4$ , filtered,  
37 evaporated under reduced pressure and the products were dried in vacuum and then subjected to  
38 spectrum analysis.  
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46 QPPI, clear yellow oil (796 mg, 57%),  $R_f$  (0.82), UV (Ethyl acetate); 257 nm and 305 nm  
47 (broad).  $^1\text{H}$ -NMR ( $\text{CDCl}_3$ , 360 MHz)  $\delta$  (ppm): 5.08-5.15 (m, 2-OH,  $\text{CH}_2$ ); 7.19 (s, 10-H, ); 7.23-  
48 7.41 (m, CH-Benzene, 40H):  $^{13}\text{C}$ NMR ( $\text{CDCl}_3$ , 360 MHz)  $\delta$  (ppm) 69.25-77.33 (C-Aliphatic),  
49 121.42-128.64 (C-aromatic), 155.67, 161.79 (C-Carbonyl).  $^{31}\text{P}$ NMR ( $\text{CDCl}_3$  Proton decoupled,  
50 121.5 mHz, Rel. to 85%  $\text{H}_3\text{PO}_4$ ) ; -4.35(s,1P), -5.61(s,1P), -5.78 (s,1P), -6.23 (s, 1P), -7..14  
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3 (s,1P):  $^{31}\text{P}$ NMR ( $\text{CDCl}_3$ , proton coupled, 121.5 MHz, Rel. to 85%  $\text{H}_3\text{PO}_4$ )  $\delta$  - (negative) ppm);  
4  
5 4.23-4.48 (m, 4H,  $-\text{CH}_2\text{Bn}$ ), 5.47-6.16 (m, 4H,  $-\text{CH}_2\text{Bn}$ ), 6.23-6.46 (m, 4H,  $-\text{CH}_2\text{Bn}$ ), 6.54-6.99  
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7 (m, 4H,  $-\text{CH}_2\text{Bn}$ ), 7.00-7.20 (m, 4H,  $\text{CH}_2\text{Bn}$ ). LC-MS: LC, Acetonitrile 40% in water ( $R_t$ , 6.81  
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9 min): MS (APCI-ion trap);  $m/z$  1603  $[\text{M}+\text{H}]^+$  1512, 1342, 1252, 1150, 1084, 980, 578, 556, 490,  
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11 368, 278.  
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## 18 2.4 Quercetin pentaphosphate (QPP)

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20 Catalytic hydrogenation experiments were carried out at ambient temperature under  
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22 atmospheric pressure of hydrogen following a previously reported procedure [14]. QPPI was  
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24 dissolved in 6 ml dichloromethane-methanol (1:1) mixture; followed by 10 mmol % of 5%  
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26 palladium on dry activated carbon. The reaction was allowed to proceed for  $\geq 1$  hour, and the  
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28 catalyst was removed by suction filtration. The resulting solution was evaporated under reduced  
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30 pressure, dried under vacuum, to afford the desired product as a yellow solid that was then  
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32 subjected to spectrum analysis.  
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37 QPP was obtained as amorphous yellow solid (324 mg, 99%), UV (DEA buffer); 266 nm,  
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39 and 410 nm;  $^1\text{H}$ NMR $^{31}$  (DMSO- $d_6$ , 300MHz, $\delta$ -ppm Rel. to  $\text{Me}_4\text{Si}$ ) 8.25 (broad *s*, -OH  
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41 (phosphate)), 6.56-7.47 (m, 5H, aromatic):  $^{31}\text{P}$ NMR (DMSO- $d_6$ , 121.5 MHz, Rel. to 85%  $\text{H}_3\text{PO}_4$ )  
42  
43 0.99 (*s*, 1P) 0.14 (*s*, 1P), -4.46 (*s*, 1P) , -4.63 (*s*, 1P), -5.68 (*s*, 1P): LC-MS: LC, Methanol-Water  
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45 ( $R_t$ , 4.76). MS;  $m/z$  702  $[\text{M}+\text{H}]^-$  , 668, 620, 540, 474, 460, 442, 380, 363, 327, 270, 230, 220,  
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## 2.5 ALP hydrolysis of QPP

For UV measurements, 160  $\mu\text{M}$  QPP was prepared in 1x DEA buffer (pH 9.8) containing 0.5 mM  $\text{MgCl}_2$  and used as a control. QPP was monitored for a period of 6 hrs for stability using DEA buffer as a blank. 6  $\mu\text{L}$  of 2 U L<sup>-1</sup> alkaline phosphatase prepared in DEA buffer was added to 1 mL of the QPP solution and UV measurements taken over a period of 6 hours. To characterize the products of the enzyme reaction the hydrolysis products were monitored by mass instrument for a period of 30 minutes. The mass spectrometer equipped with an ESI probe was operated either in positive or negative mode depending on the nature of the compounds.

Spectrophotometric endpoint determination of ALP activity was carried out a Synergy Multi plate Reader instrument. Different concentrations of the enzyme (2-16 U L<sup>-1</sup>) were incubated with a solution of QPP (125  $\mu\text{M}$ ) prepared in DEA buffer. The set up was carried out in a 24 well plate and monitored periodically for 2 hours taking intermittent measurements.

For fluorescence measurements QPP solutions of 15  $\mu\text{M}$  were prepared in DEA buffer. To these solutions ALP prepared in DEA buffer containing 0.5 M magnesium chloride was added at varying concentrations from 1- 6 units L<sup>-1</sup>. The fluorescence intensity was measured at  $\lambda_{\text{ex}}/\lambda_{\text{em}} = 425/530$  nm at different time intervals.

## 2.6 Assay of Avidin-ALP conjugate

Biotinylation was performed according to our previous report [15]. A 96 well Costar microtiter plate was used for immobilization of biomolecules. Each well used contained 100  $\mu\text{L}$  of the reagents. 5  $\mu\text{g mL}^{-1}$  of goat anti-BG prepared in 10 mM carbonate buffer pH 9.6 was

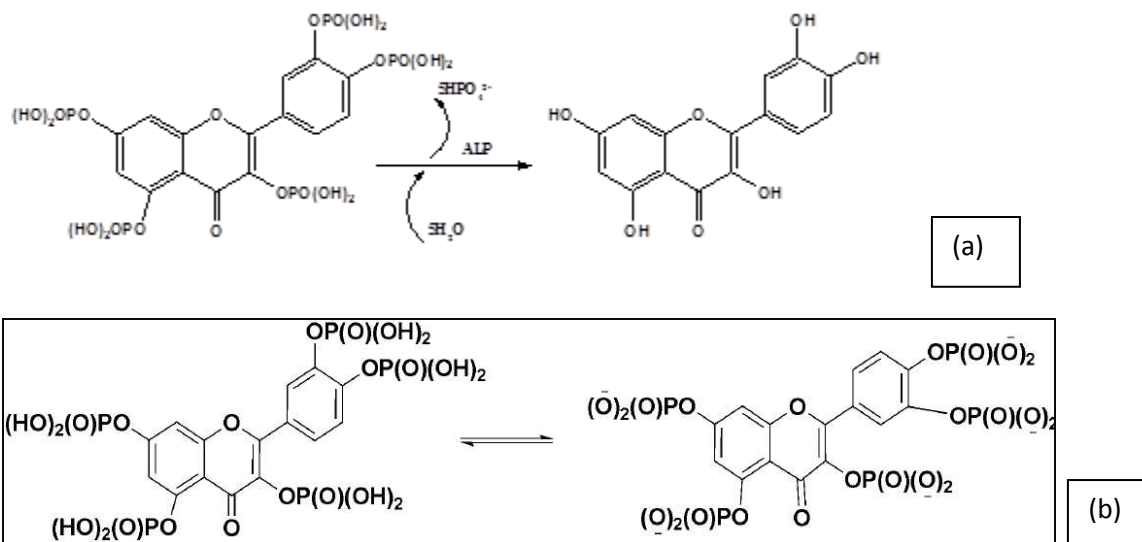
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3 immobilized onto the microtiter plate as the capture antibody and incubated overnight at 4°C.  
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5 The following day, the plate was rinsed several times with PBST buffer pH 7.2 and tapped dry.  
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7 The nonspecific binding was blocked by overnight incubation with blocking buffer (1 mg mL<sup>-1</sup>  
8 BSA in PBS) at 4°C. Again the plate was washed with PBST buffer and tapped dry. The plate  
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10 was coated with *Bacillus globigii* spore standards (0 – 16000 spores per mL) prepared in PBSTB  
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12 buffer pH 7.2 and incubated overnight at 4°C. The plate was again washed with PBST buffer and  
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14 incubated with the detecting antibody, rabbit anti-BG-biotin (5µg mL<sup>-1</sup>), for ~2 hours at room  
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16 temperature. The plate was washed with TBST buffer and tapped dry after which avidin-alkaline  
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18 phosphatase prepared in TBSTB buffer (dilution 1:20,000) was added. 125 µM of QPP prepared  
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20 in DEA buffer was added after 2 hours and absorbance readings obtained at 325 nm and 410 nm  
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22 at varying time intervals.  
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### 33 **3. Results and discussions**

#### 34 **3.1. Synthesis of QPP**

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37 The synthesis of QPP was carried out according to previously reported procedure for  
38 phosphorylation of phenols with some minor modifications [13, 14]. The presence of five  
39 phosphate groups could be detected by the appearance of five singlets in the <sup>31</sup>P NMR spectrum.  
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41 Characterization by <sup>1</sup>H-NMR and LC-MS as outlined in the experimental section further  
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43 confirmed the desired product. **Scheme 1** under Supplementary Information shows the proposed  
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45 synthetic process and enzymatic reactions.  
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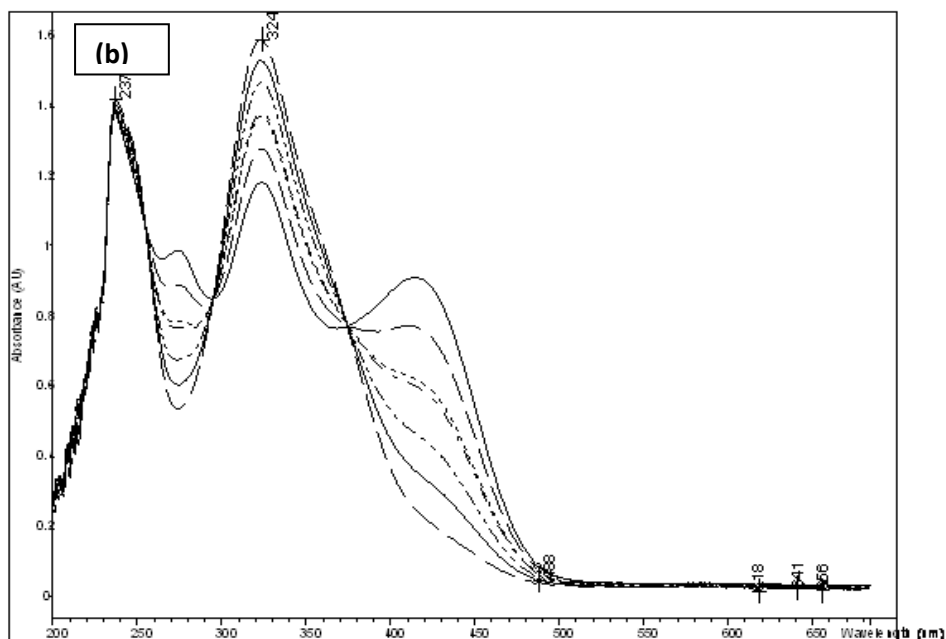
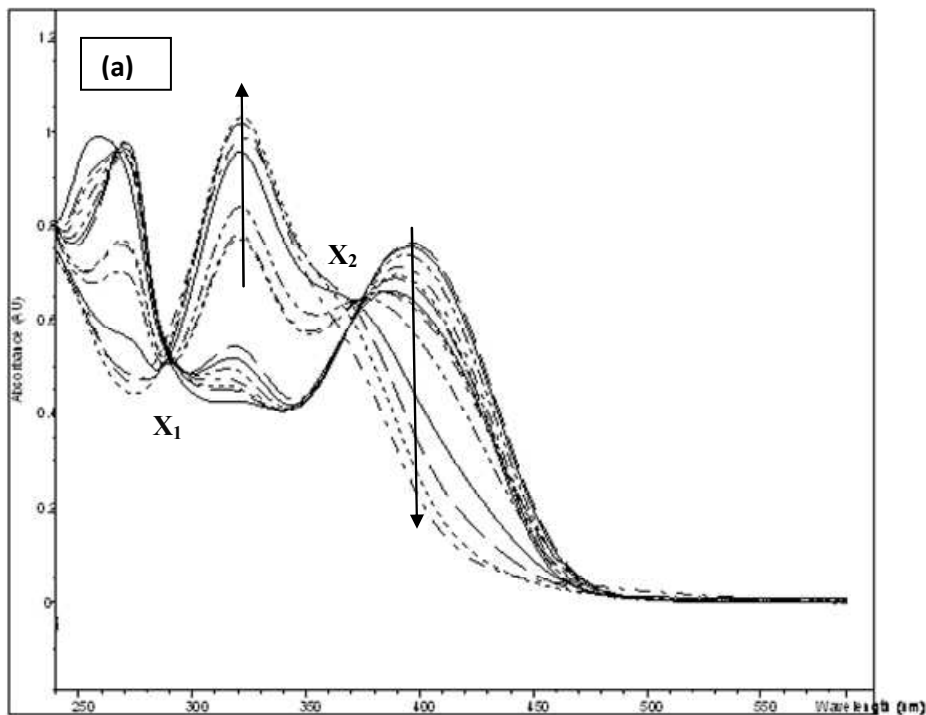
55 (a)



**Scheme S1.** (a) Proposed enzymatic conversion of QPP to quercetin, and (b) Deprotonation of QPP at high pH

### 3.2. Investigation of alkaline phosphatase activity on QPP

The UV-Vis spectrum of QPP was recorded before and after undergoing enzymatic hydrolysis. QPP in water possesses two characteristic absorption bands at 264 nm and 360 nm which are similar to those observed in quercetin at 256 and 372 nm. The band at 360 nm is associated with the cyanamoyl system (B+C ring) and the band at 254 nm corresponds to the absorption of the benzoyl moiety formed by the A+B ring. When QPP was exposed to DEA buffer pH 9.8 the peak at 360 nm shifted to ~400 nm, Figure 1(a). This is mainly attributed to deprotonation, a property which is also observed for most phenols exposed to basic media [16]. The absorption spectra and extinction coefficients of phosphates are influenced by the nature of the solvent, intra- and intermolecular hydrogen-bonding, steric effects, and the pH dependent formation of resonance forms with altered conjugation compared to the parent compounds.



**Figure 1.** UV-vis Spectra of (a) QPP in DEA buffer monitored for 6 hrs for stability and then exposed to ALP for 8 min. The arrows indicate the change in absorbance intensity. A hypsochromic shift is accompanied by a new emerging peak at 324 nm, and (b) QCR in DEA buffer monitored for 30 min showing similar features with those obtained for hydrolyzed QPP. A new emerging peak at 324 nm was observed for the enzymatic products of QPP.

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6 The observed isosbestic points,  $X_1$  and  $X_2$  are as a result of new absorbing species being  
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8 formed. Alkaline phosphatase is known to catalyze the reduction of phosphates to hydroxyl  
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10 groups. This property has been the basis for its utility in ELISA-based applications. For example;  
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12 para-nitrophenyl phosphate is converted to a para-nitrophenol in enzymatic assay. Our previous  
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14 work has utilized immunoassays that employ enzyme-labeled antibodies to provide detection  
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16 signals by hydrolysis of a phosphate-derived substrate [15]. Since QPP has five phosphate  
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18 moieties we believe the new emerging peak at 324 nm is due to hydrolysis of the phosphate ester  
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20 to phenolic form (quercetin) by ALP enzyme. These changes could also be confirmed by the  
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22 color change of QPP solution (prepared in Nanopure water) upon exposure to ALP. The activity  
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24 of ALP enzyme on QPP was monitored colorimetrically, over a period of 20 min. Immediately  
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26 after addition of ALP, the QPP solution color changed from faint yellow to light green, then faint  
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28 red **Figure S1**. The observed color change was attributed to possible de-protonation of QPP by  
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30 the high pH and the subsequent hydrolysis by ALP enzyme. These results compliment the  
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32 observation using UV-Vis spectroscopy and that indeed the conversion of QPP to quercetin  
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34 (QCR) occurred.

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42 Interestingly, no major quercetin peaks were observed upon exposing QPP to the enzyme.  
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44 Additionally, the products formed were highly soluble in aqueous media with no observable  
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46 precipitation. Generally, we would expect if quercetin was formed it would have characteristic  
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48 peaks at 383 nm and 257 nm with a shoulder at 272 nm. Further, since quercetin has limited  
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50 aqueous solubility it should precipitate out in aqueous media. We suspect that quercetin is  
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52 formed *in situ* and almost immediately oxidized under basic conditions forming soluble products  
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54 with the major species having an absorbance maximum at 324 nm. This new peak at 324 nm is  
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3 formed almost immediately after addition of ALP and progressively increases with time while  
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5 the peak at 410 nm decays rapidly and disappears less than 10 minutes later at the enzyme  
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7 concentrations conditions described in the experimental section.  
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11 To further confirm the results a sample of QCR (1 mM) was prepared in DEA buffer and  
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13 the UV-Vis investigated over time. **Figure 1(b)** shows the UV-Vis spectrum of QCR in DEA  
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15 buffer. The peak at 372 nm is shifted to 410 nm while the peak at 324 nm remained persistent  
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17 and increased with time. After 5 min the peaks at 410 nm and 257 nm disappears while the peak  
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19 at 324 nm increased. This behavior explains why characteristic absorbance peaks were not  
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21 visible in UV-Vis profile of QPP reacting with ALP. Unlike quercetin QPP is stable in basic  
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23 medium with the deprotonated resonance form being the major species, **Scheme 1 (b)**.  
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28 The activity of alkaline phosphatase on QPP was further monitored electrochemically using  
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30 Cyclic Voltammetry technique (CV) on a gold working electrode to verify if any redox peaks of  
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32 quercetin could be observed. However, since QPP is not electroactive and due to the slow  
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34 enzymatic action of ALP on the QPP no redox peaks were observed. Figure S2 depicts the redox  
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36 potentials of QPP's parent molecule quercetin.  
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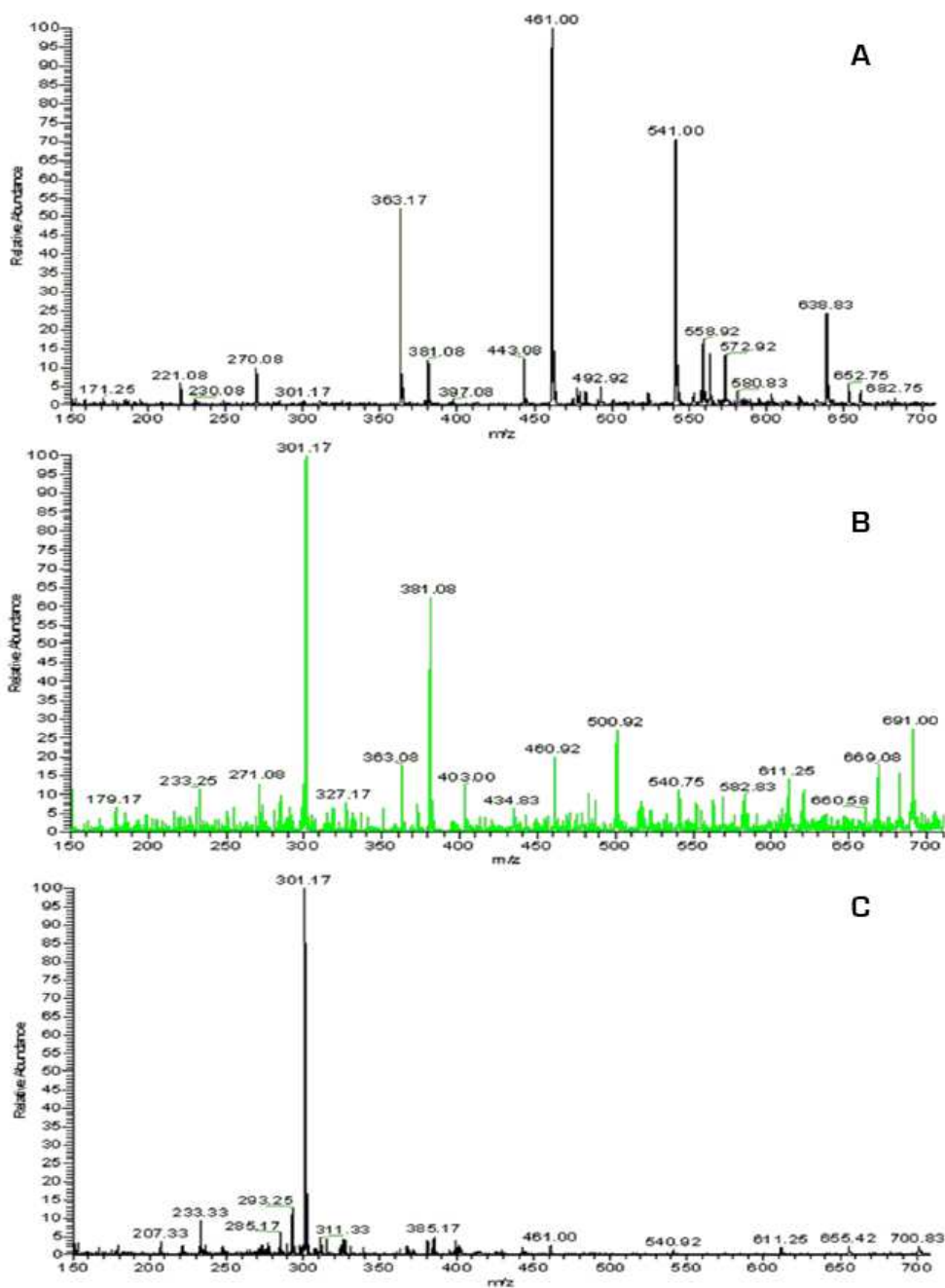
### 45 **3.3. ESI-MS Characterization of Enzymatic Products**

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47 Since UV-Vis and colorimetric assessment provided useful qualitative information, we  
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49 sought to characterize the products further and confirm that quercetin was the major product of  
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51 the hydrolysis reaction. Accordingly, tandem mass spectrometry with an ESI interface in  
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53 negative mode ionization was carried out to characterize the products and compare the extent of  
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55 conversion over time. **Figure 2A** shows the full mass scan of QPP before the addition of alkaline  
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3 phosphatase. Since no additives were used to preserve the composition of the product, the  
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5 molecular ion peak was not visible in the spectrum but there was evidence of tandem loss of five  
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7 phosphates groups. The ions at  $m/z$  638 corresponds to the loss of one phosphate group and a  
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9 water molecule;  $m/z$  541 corresponds to two phosphate groups;  $m/z$  461, three phosphate groups;  
10  
11  $m/z$  381 four phosphates and final peak at  $m/z$  301 corresponding to the loss of five phosphate  
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13 groups.  
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18 The ions at  $m/z$  363 and 443 correspond to the loss of water in addition to the respective  
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20 phosphate groups. When QPP was exposed to alkaline phosphatase, a significant enhancement of  
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22 peak intensity at  $m/z$  301 occurred almost immediately, **Figure 2B**. This fragment has been  
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24 associated with quercetin and is usually the molecular ion peak for negative mode ionization of  
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26 quercetin[17]. The parent peak at  $m/z$  691 is due to the deprotonation of QPP at high pH  
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28 consistent with the loss of ten protons. The enzymatic activity was monitored over time and the  
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30 phosphate peaks were observed to become less apparent. This was mainly attributed to the  
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32 activity of alkaline phosphatase enzyme on the phosphate groups and hence subsequent  
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34 dephosphorylation of QPP.  
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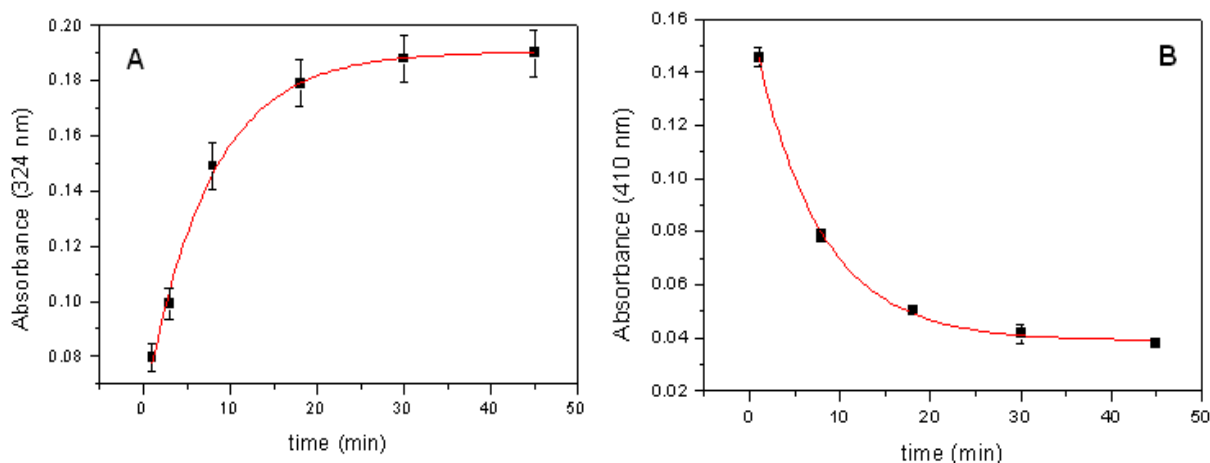


**Figure 2** (A) ESI-MS spectra of QPP in Methanol/water mixture. The ionization was carried out in negative mode (B) 5 minutes after exposure to alkaline phosphatase enzyme. The phosphate peaks began to disappear while quercetin molecular ion peak at  $m/z$  301 became dominant (C) 20 minutes after exposure to alkaline phosphatase enzyme. The phosphate peaks disappeared while quercetin molecular ion peak at  $m/z$  301 persisted.

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3 The molecular ion at  $m/z$  381 (one phosphate group) became more pronounced after  
4 addition of the enzyme indicating a sequential or rather a step wise dephosphorylation of QPP.  
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6 ESI-Mass analyses carried out after 20 min exposure to the enzyme showed a complete  
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8 transformation of QPP to quercetin evidenced by the most abundant ions at  $m/z$  301 and the  
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10 disappearance of the phosphate ion peaks, **Figure 2C**. These results further confirmed that AP  
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12 can act as a dephosphorylating agent and converts QPP to QCR which is further degraded in  
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14 alkaline pH.  
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### 22 **3.4. Spectrophotometric Determination of ALP activity**

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24 In order to explore the potential of QPP as ALP-enzyme substrate, different  
25 concentrations of ALP were introduced into plate wells containing a constant amount of QPP.  
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27 The experiments were carried out in triplicates using DEA as the blank. The control contained a  
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29 similar amount of QPP as the samples but lacked the enzyme. The activity of ALP on QPP was  
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31 monitored over time with constant amount of each reactant. This experiment was aimed at  
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33 optimizing the time and also determining the time range at which the enzyme activity is linear.  
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35 **Figure 3** shows a time course plot of QPP absorbance at 324 nm and 410 nm after incubation  
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37 with ALP for 45 minutes. The absorbance at 324 nm increased with time for the first 10 minutes  
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39 after which no significant change in absorbance was observed. These results indicate that the  
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41 enzyme catalyzed hydrolysis of QPP is a fast processes with most of the substrate being  
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43 converted to the product in minutes. The absorbance at 410 nm decreased rapidly within less  
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45 than 20 minutes and then levels off.  
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**Figure 3.** Change in absorbance at (A) 324nm and (B) 410nm with time. The enzyme concentration was fixed at 12 units L<sup>-1</sup>

From these results, 18 minutes was chosen for future ALP activity analysis because the sensitivity was found to be sufficient for practical application. A plot of the difference in absorbance between the two wavelengths (410 nm & 324 nm) against the concentration of the enzyme gave a linear change. **Fig. S3** shows a graphical representation of the results obtained after 18 minutes of reaction time. Using this method, the limit of detection was determined to be 76.6 mU L<sup>-1</sup> with a linear range of 0.01-16 U L<sup>-1</sup>.

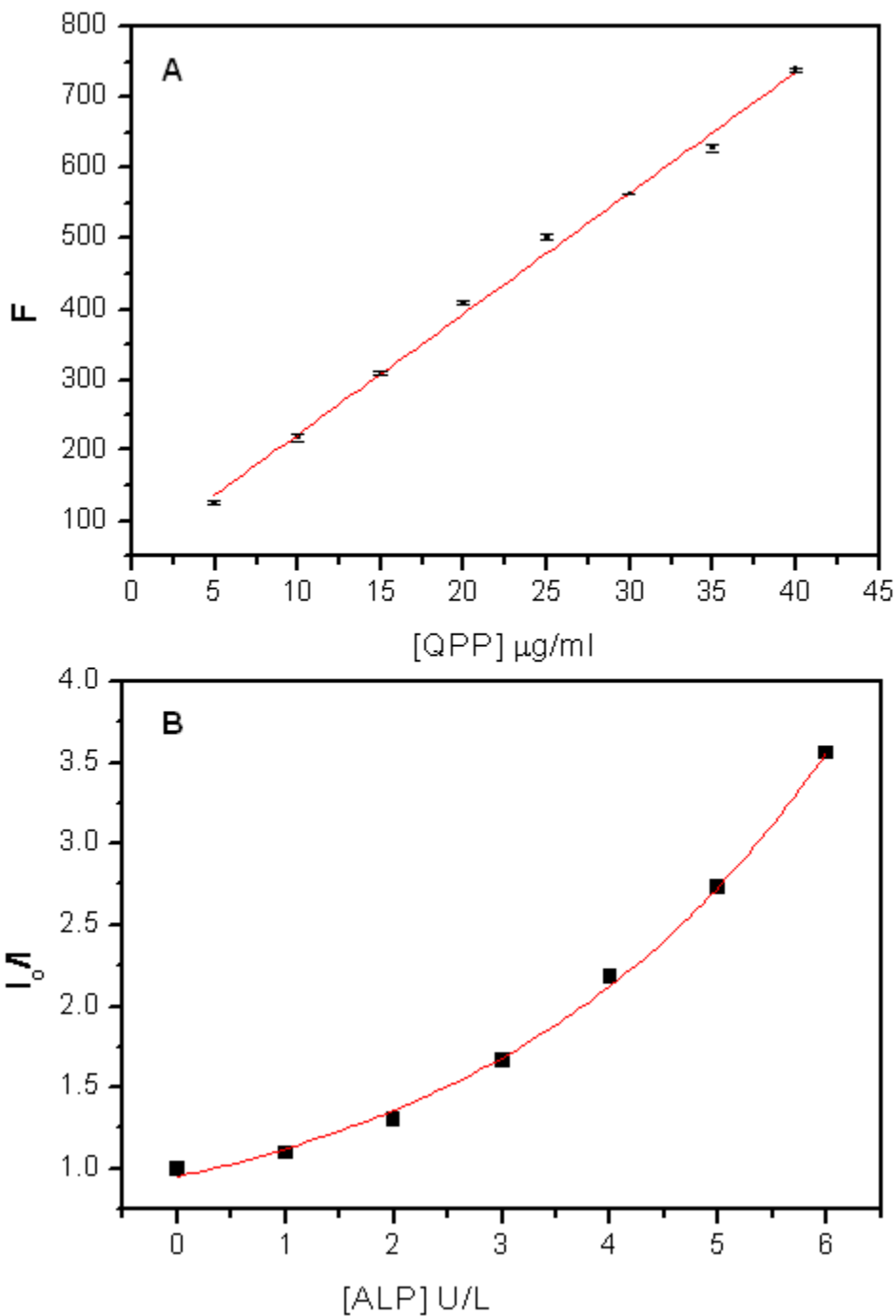
### 3.5. Fluorimetric Determination of ALP activity

Generally the sensitivity of fluorimetric method is higher than the sensitivity of spectrophotometric method, so we chose to extend the study to fluorimetric determination of ALP activity. When excited at 425 nm, QPP showed a characteristic emission maximum at 530 nm. Further studies using different concentrations of QPP revealed that the fluorescence intensity increased with increase in concentration of the analyte, **Fig. 4A**. Addition of a solution of

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3 alkaline phosphatase caused a decrease in the emission upon excitation at 425 nm. The decrease  
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5 in emission intensity was accompanied by a slight shift in the emission wavelength (530 nm)  
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7 attributed to enzyme-substrate complex. QPP concentration of 15 mM was adopted for future  
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9 experiment since it gave sufficient signal intensity with minimum enzyme requirements. The  
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11 fluorescence emission of QPP was observed to decay exponentially with time when incubated  
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13 with 20 U L<sup>-1</sup> of ALP (data not shown). The quenching of QPP native fluorescence can be  
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15 explained by Stern-Volmer plots according to equation 1.  
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$$\frac{F_0}{F} = 1 + k_q \tau_0 [Q] = 1 + k_{sv} [Q] \quad (\text{Eqn. 1})$$

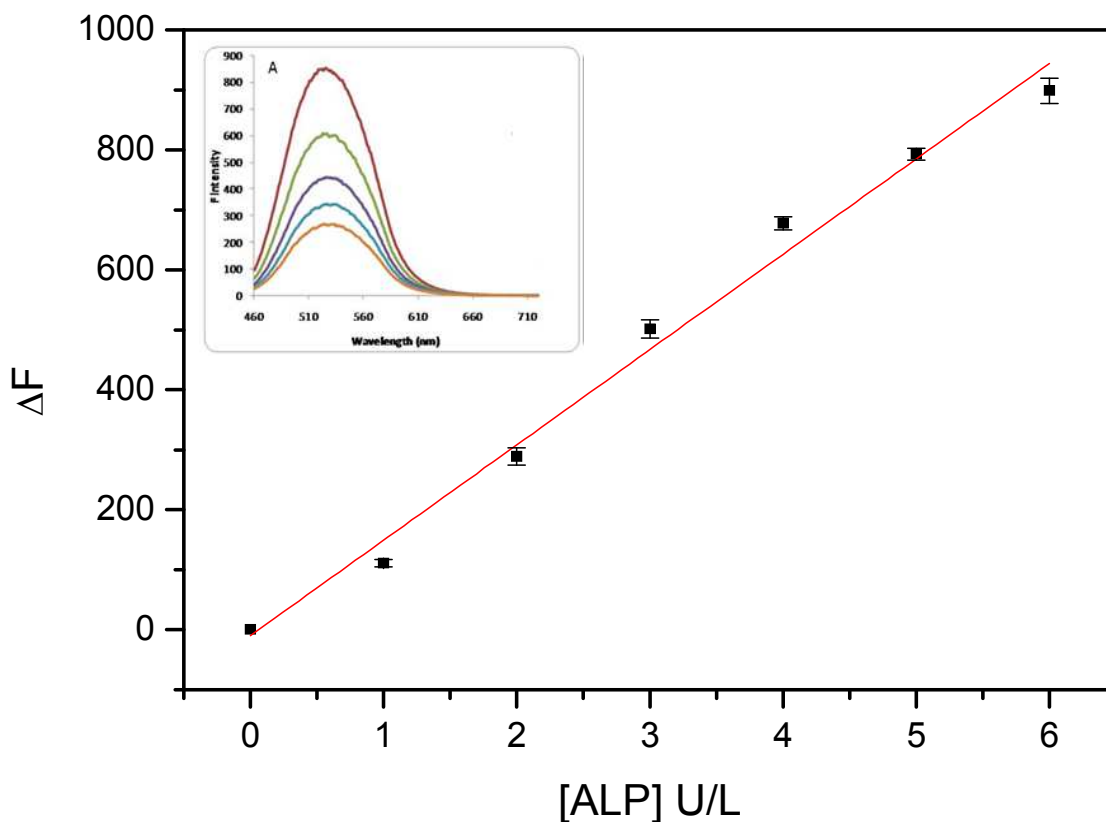
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24 Where  $k_q$  is the bimolecular quenching rate constant,  $\tau_0$  is the lifetime of the fluorophore  
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26 in the absence of the quencher, and  $k_{sv}$  is the Stern-Volmer quenching constant. For completely  
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28 dynamic or completely static quenching mode, a linear plot is observed when  $F_0/F$  is plotted  
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30 against  $[Q]$  [18]. An upward curvature, concave towards the y-axis is observed during instances  
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32 when collisions (dynamic) and complex formation (static) are involved [19]. From our  
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34 experiments, a plot of  $F_0/F$  versus the concentration of ALP (quencher in this case) showed an  
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36 upward curvature (**Fig. 4B**) characteristic of mixed static and dynamic quenching.  
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**Figure 4(A)** Fluorescence intensity change with increasing concentration of QPP (measured at  $\lambda_{ex}/\lambda_{em} = 425/510\text{nm}$ ).  $R^2=0.994$  **(B)** Stern-Volmer curve of QPP in the presence of ALP

The quenching effect of ALP on the fluorescence emission of QPP was evaluated with different concentrations of the enzyme. A series of solutions with different ALP concentrations

(2-6 U L<sup>-1</sup>) were investigated while holding constant the concentration of QPP (15 μM) after 20 minutes incubation time. From **Fig. 5**, it can be observed that QPP+ALP have a strong emission at ca. 530 nm. The emission maxima decreased with increase in concentration of ALP. The change in fluorescence ( $\Delta F$ ) was evaluated and plotted against the concentration of ALP. A linear response was observed with increasing concentration of ALP. A linear range of 0.01 – 8 U L<sup>-1</sup> was obtained with a detection limit of 0.062 U L<sup>-1</sup> calculated from the standard deviation of 5 blank measurements. Comparing with some existing methods, the discussed method presents high sensitivity for the determination of ALP activity.



**Figure 5.** Calibration curve for ALP determined by fluorimetric method after 20 min reaction time, with 15 μM QPP in DEA buffer. Inset: fluorescence emission spectrum of QPP after incubation with different concentrations of ALP (2-6 units/L) for 20 minutes (B).  $R=0.986$

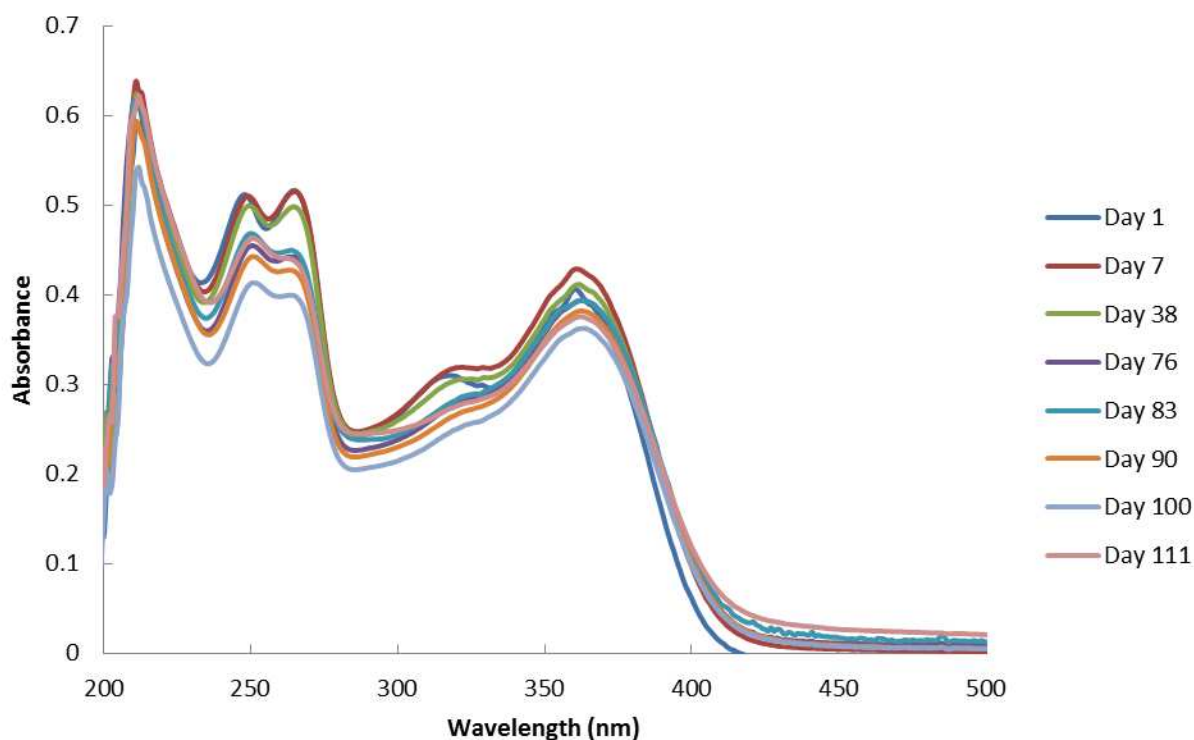
### 3.6. Validation using rabbit-IgG-ALP conjugate

In our long term plans we intend to use either spectrophotometric or fluorimetric enzyme activity measurements for the development of a simple immunoassay. In immunoassays the detector antibody-ALP conjugate activity is measured, thus we tested the validity of the new substrate for detecting anti-*Bacillus globigii* rabbit-IgG-ALP. *Bacillus globigii* (BG) (also referred to as *B.Subtilis* variant niger and recently classified as *Bacillus atrophaeus* [20]), is a spore forming nonpathogenic microorganism whose spores resemble those of *B. anthracis* in size, shape and surface morphology. For this reason BG spores have been widely used as non-pathogenic simulants for *B. anthracis* [15, 21-23]. *B. anthracis* is the causative agent of anthrax, a highly infectious pathogen previously weaponized for aerosol dispersion and food contamination in biological warfare [24]. Consequently, we chose BG for testing the validity of QPP as a substrate in immunoassays. The substrate dependency on rabbit-IgG-ALP concentration was determined by ELISA. A conventional sandwich ELISA was set up with QPP acting as the substrate for alkaline phosphatase. The detection wavelength was set at 325 nm and 410 nm, and absorbance endpoint measurements obtained after 2h. The absorbance changes were evaluated as the response and plotted against the concentration of *B*) spores. QPP substrate demonstrated a linear response with increasing concentration of the BG spores, **Figure S4**. The results demonstrate the effectiveness of the new substrate (QPP) also for ALP conjugates.

### 3.7 Stability Studies of QPP

The study clearly depicts that aqueous solution of QPP is very stable and this is clearly illustrated in the spectrum in Figure 6. Literature studies reveal that the parent Quercetin is very unstable

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3 and undergoes auto oxidation [14, 25]. From the stability test spectrum, it was clear that no  
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5 significant variation was observed since the absorption band of QPP was maintained at two  
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7 bands of 266 nm and 360 nm respectively which is in agreement with the literature [14]. It is  
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9 worth noting that QPP can be synthesized and then used as ALP substrate for a long time without  
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11 degradation.  
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47 Figure 6 UV-Vis Spectrum of stability study of 100  $\mu$ M of QPP in Phosphate buffer for about 3 and half  
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### 3.8 Comparison of QPP with Commonly Used ALP substrates

QPP substrate compares favorably with other substrates as shown in Table 1 below. Although its quantum yield is low, it provides strong sensitivity, excellent linearity and is very soluble in water.

**Table1: Comparison of QPP with Commonly Used ALP substrates**

Fluorophore	Ex (nm):	Em (nm):	MW	QY:	Advantages & Disadvantages	References
<b>QPP</b>	425	510	702	0.09	Offers ease of use, water soluble, strong sensitivity and excellent linearity, suitable for ELISA assays.	14
Ethidium Bromide	493	620	394	0.15	Less expensive. May be mutagenic.	31,33,37
Hydroxycoumarin	325	386	331	0.98	High sensitivity, water soluble. High intense fluorescence but lack covalent conjugation	28,29,34, 35
Lucifer Yellow	425	528	444	0.21	Favors dye coupling	31,37,38,39
Fluorescein	495	519	389	0.92	FITC, pH sensitive but less economical.	31,36
Cy5	625	670	792	0.28	Gives bright fluorescence. Less photostable	31,37
Alexsa Fluor 430	434	541	702	0.69	Intense fluorescent	31,40
Alexsa Fluor 555	555	565	1250	0.10	Excellent photostability	31,40
5-Bromo- 4-Chloro-3-Indolyl Phosphate (BCIP)	633	650	434		Not recommended for microwell (e.g., ELISA) or immunohistochemical applications. Light sensitive. Stable at least one year at 2-8 °C.	31,32
<b>Fast Red TR/Naphthol AS-MX and TR phosphate (4-Chloro-2-methylbenzenediazonium/ 3- Hydroxy-2-naphthoic acid 2,4-dimethylanilide phosphate) substrate</b>	560	635	371		Suitable for ELISA since it form a soluble end product. High resolution of the fluorescent product. Poor localization	30,31,41

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3 Abbreviations:

4 Ex (nm): Excitation wavelength in [nanometers](#) Em (nm): Emission wavelength in [nanometers](#)

5 MW: [Molecular weight](#) QY: [Quantum yield](#) QPP: Quercetin Pentaphosphate (this work)

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10 **4. Conclusion**

11 We have reported QPP as a new substrate for both spectrophotometric and fluorimetric  
12 determination of ALP activity. The linear range and detection limit for the determination of ALP  
13 using spectrophotometric method are 0.01-16 U L<sup>-1</sup> and 0.766 U L<sup>-1</sup>, respectively. Fluorimetric  
14 method afforded a higher sensitivity with a linear range of 0.01 – 8 U L<sup>-1</sup> and a detection limit of  
15 0.062 U L<sup>-1</sup>. We demonstrated the viability of the new substrate for determination of conjugated  
16 ALP with a detection limit of 5998 *bacillus globigii* spores mL<sup>-1</sup>. This detection limit is  
17 comparable to what was obtained using p-nitrophenyl phosphate (4269 spores mL<sup>-1</sup>) as a  
18 substrate for the same analyte using a similar method [15].  
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## Supplementary Material

Additional material of interest to the reader is available free of charge. The information includes, a pictogram showing changes of QPP solution before and after reacting with AP, changes in QPP absorbance and Rabbit anti-BG conjugated with ALP response of QPP substrate.

## Acknowledgement

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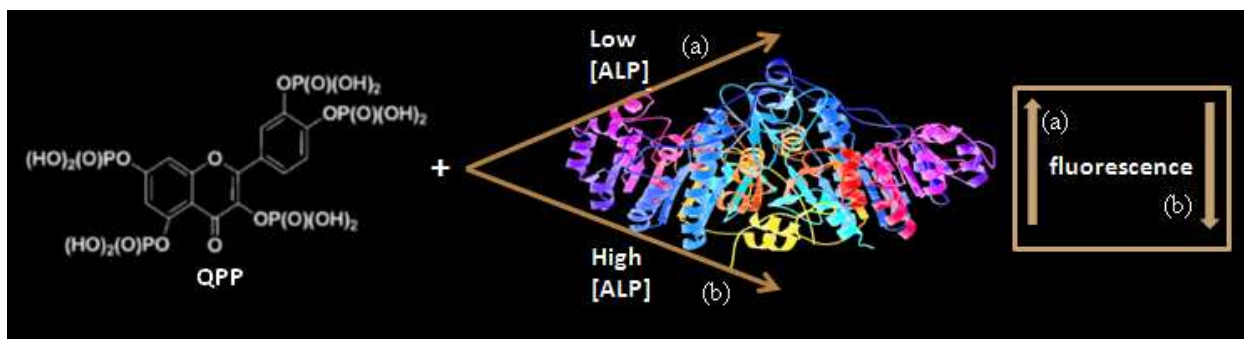
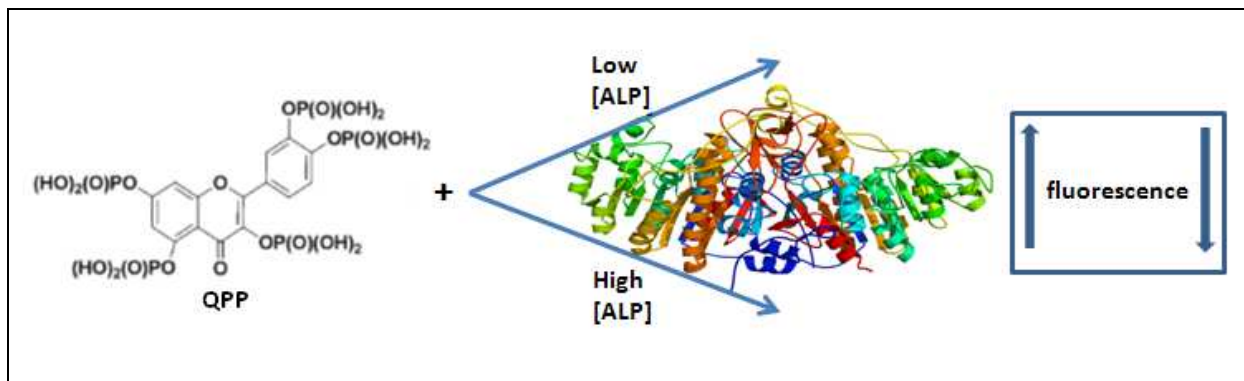
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