An Enzyme-based Spectrophotometric Method for the determination of Phenolic Compound (2-Methoxyphenol) Using Peroxidase From *Ipomea batata*

ABSTRACT

Phenolic compounds and their derivatives are considered priority pollutants because they are harmful to living organisms even at low concentrations. Due to their toxicity and persistence in the environment, many efforts are been made to develop simple and effective methods for their determination. This study describes a peroxidase-based method for the quantitative determination of a named phenolic compound. The method is based on the oxido-reductase activity of peroxidase in a H_2O_2/2-Methoxyphenol system. Calibration curve of peroxidase activity (absorbance) against the phenol concentration forms the basis for its quantitative estimation. Factors influencing the reaction were evaluated and optimized; Optimum pH and temperature were 6 and 40°C respectively while optimum reaction time was 6 minutes. The calibration curve for the analyte was linear (R^2 =0.998) within the concentration range of 0.01 – 5 mM. Repeatability of analysis was 3.8% RSD for 7 replicate measurements. Recovery tests for the analyte in water samples gave values between 85.33 – 112%.

Keywords: Peroxidase, Hydrogen peroxide, 2-Methoxy phenol, Guaiacol, Enzyme, Method development

1. INTRODUCTION

Phenolic compounds are a class of organic compounds with hydroxyl group(s) directly bonded to one or more aromatic rings. The first member of chemicals belonging to this category of compounds is called phenol, also known as carbolic acid, benzophenol or hydroxybenzene with the chemical formula of C_6H_5OH. All other members of the group are derivatives of this compound. Phenolic compounds and derivatives are widely distributed in the environment mainly in water bodies [1]. They are mostly introduced into the aquatic environment as wastes from paper manufacturing, agriculture, pharmaceuticals, petrochemical industry, coal processing and municipal wastes [2]. Upon entry into water bodies, the compounds have the tendency of undergoing transformations into other moieties that can even be more harmful than the original compounds. This transformation is normally due to their interaction with physical, chemical and biological factors in the water [3].

Phenolic compounds in the environment are of major concern because of their toxicity and persistent nature. They are considered as priority pollutants and appear in the list of dangerous substances of the US Environmental Protection Agency (USEPA) [4]. This enlistment is due to the fact that the chemicals are noted to be toxic and have severe short- and long-term effects on humans and animals [5]. Long-term exposure to phenolic derivatives has been associated with toxic effects in bone marrow [6], cardiac depression, blood changes, as well as kidney and liver damage [7], malfunctioning of the biochemical system and reproductive impairment in domestic animals [8]. In addition, some phenolic compounds have been identified as carcinogenic and capable of suppressing the immune system [7,9].

Analytical methods commonly used for the determination of phenolic compounds include gas chromatography (GC), high-performance liquid chromatography (HPLC) and capillary electrophoresis. Though these methods are convenient and popular, they have some drawbacks such as long analysis time, expensive equipments, use of large amounts of chemical reagents and require well trained qualified personnel. Enzyme-based approaches can offer advantageous alternatives because of their high sensitivity, low cost and simplicity of operation. They can also be made into portable sensing systems that can be used for field monitoring.

Several enzymatic systems have been reported for the determination of phenols and their derivatives; the enzymes commonly employed are laccases, tyrosinases and peroxidases. The reported methods showed good selectivity, sensitivity, reproducibility and simplicity of application. In this work, an enzyme assay for the determination of phenolic compound (2-methoxy phenol) in water using peroxidase from sweet potato (*Ipomea batata*) has been investigated. *Ipomea batata* is known to be a rich source of peroxidase enzyme. Several studies have extracted and characterised the enzyme from sweet potato however, there are few reports of its use for analytical applications. This study is a novel attempt at exploring the potential of *Ipomea batata* peroxidase for use in the determination of a phenolic compound.

2. MATERIAL AND METHODS

2.1 Materials

Sodium Phosphate buffer solutions with various pH values were prepared by mixing standard stock solutions of 0.1 M Na_2HPO_4 and 0.1 M NaH_2PO_4 (both from BDH chemicals) and adjusting the pH with HCl or NaOH. Guaiacol (2-methoxy phenol) was purchased from LOBA Chemie, and H_2O_2 was from Merck Chemicals. Sweet potato (*Ipomea batata*) tubers were obtained from a local market in Wukari, Nigeria.

2.2 Enzyme extraction

Comment [JS1]: % recovery
% LoD
Stability of the enzyme (range temp, pressure, light if any)

A piece of potato tuber was washed, peeled and diced into tiny pieces. Twenty grams (20g) of the diced potato was homogenized thoroughly in 100 mL extraction buffer (sodium phosphate buffer, 0.1M, pH 7.0). The homogenate was filtered through four layers of cheese cloth and the filtrate was centrifuged at 4,000 rpm for 30 minutes (C2041, Centurion centrifuge). The supernatant was collected as crude peroxidase extract and stored at 4°C.

2.3 Enzyme Assay

Peroxidase (POD) activity was assayed spectrophotometrically at 470 nm using guaiacol as a phenolic substrate with hydrogen peroxide. The assay cocktail contained 1 mL of phosphate buffer solution (0.1 M, pH 7), 1 mL 0.5% H_2O_2, 1 mL of 0.5 mM Guaiacol and 1mL of the enzyme extract. The mixture was rapidly transferred into a 1-cm path length cuvette and the absorbance at 470 nm was recorded against a blank continuously for 5 min (at intervals of 1 min) using ultraviolet-visible spectrophotometer (T60 U). The blank sample contained the same solution mixture without the enzyme extract [10].

2.4 Optimization of Experimental Conditions

2.4.1 Optimization of substrate concentration

The activity of POD as a function of substrate concentration was investigated and optimized. The reaction mixture contained 1 mL phosphate buffer (0.1M pH 7), 1 mL of 0.5% H_2O_2 (v/v), 0.1 mL guaiacol (0.01 – 10 mM) and 1 ML of extraction buffer. The absorbance of the reaction was recorded against a blank at intervals of 1 minute for 5 minutes

2.4.2 Optimization of pH

Using optimized substrate concentration obtained in 2.4.1, the optimum pH value for the assay was determined by evaluating enzyme activity at different pH levels (5, 6, 7 8 and 9). Peroxidase activity was assayed as stated earlier (2.3)

2.4.3 Optimization of Temperature

POD activity was determined at 30, 40, 50, 60 and 70°C. The substrate, buffer solutions and enzyme extract were incubated separately for 5 min at the various temperatures before mixing. Assay activity was obtained as previously described.

2.4.4 Optimization of Reaction Time

The effect of time was evaluated by taking measurements of the peroxidase assay activity at intervals of 1 minute for a period of 10 minutes.

2.5 Determination of phenolic compound (2-Methoxyphenol)

2.5.1 Preparation of Calibration Curve

Standards of 2-Methoxyphenol were prepared at 0.1 – 10 Mm concentration and subjected to peroxidase assay as described in 2.3. A graph of absorbance against phenolic compound concentration forms the basis for quantitative determination of the compound.

2.5.2 Application to Real Water Samples

To assess application of this method, determination of 2-methoxy phenol was tested on environmental samples collected from three streams within Wukari, Nigeria. Known quantities of the phenolic compound were used to spike the samples to concentrations of 1.5, 3.5 and 7.5mM/L. The samples were left overnight to equilibrate before analysis.

2.5.3 Method validation

The method was validated according to the International Conference on Harmonization (ICH) guidelines [11]. The parameters validated were linearity, precision, accuracy, limits of detection and quantification. For Linearity, the calibration graphs were obtained by plotting the absorbance of standards against the concentration of the phenolic compound under consideration. Data derived from the regression line provided mathematical estimates of the linearity. The intraday precision was assessed by analyzing seven replicates of three different concentrations within a day; precision was taken as relative standard deviation (%RSD) of the measurements. To evaluate the method accuracy, recovery test were performed on spiked sample solutions. The accuracy of the assay was determined by by calculating the percent recovery (%R) of the analyte. LOD and LOQ were calculated by using the equations: LOD = 3.3 x σ/ S; LOQ = 10 x σ/ S, where σ is the standard deviation of y-intercepts of regression lines and S is the slope of the calibration curve.

3. RESULTS AND DISCUSSION

3.1 Method Principle

The proposed method for phenol determination presented in this study is based on the oxidoreductase activity of peroxidase in a H_2O_2/Guaiacol system according to the equation:

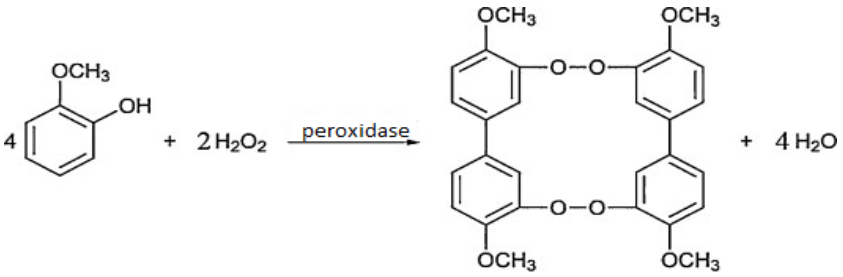

Figure 1. Peroxidase-catalyzed oxidation of guaiacol to tetraguaiacol

In enzymatic analytical methods, the biological enzyme is used to convert a specific target into another product which gives a measurable analytical signal and hence a means for quantitative estimation. The current study involves the conversion of guaiacol (2- methoxy phenol) to tetraguaiacol by peroxidase. In the above reaction, peroxidase enzyme first reduces hydrogen peroxide to generate reactive oxygen species. The released oxygen then binds instantly with a guaiacol to form tetraguaiacol which is brownish in colour. The formation of tetraguaiacol was measured spectrophotometrically and quantitatively related to the concentration of the reacting phenol. Many phenolic compound have been shown to undergo similar reactions in the presence of peroxidases; they include m-cresol, p-cresol, o-cresol, anisole, resorcinol, catechol, pyrogallol, hydroquinone, phoreguicinol among others [12]. These phenolic derivatives were shown to be converted to their respective quinones in the presence of peroxidases. In general, little selectivity is shown by the enzyme peroxidase with respect to phenols therefore the enzymatic method presented in this study can be adapted for a large number of phenolic compounds.

3.2 Optimization of Experimental Conditions

3.2.1 Effect of Substrate Concentration

The effect of various concentrations the substrate (Guaiacol) was evaluated in order to establish the best concentration for optimal assay performance. The results obtained are presented in Figure 2.

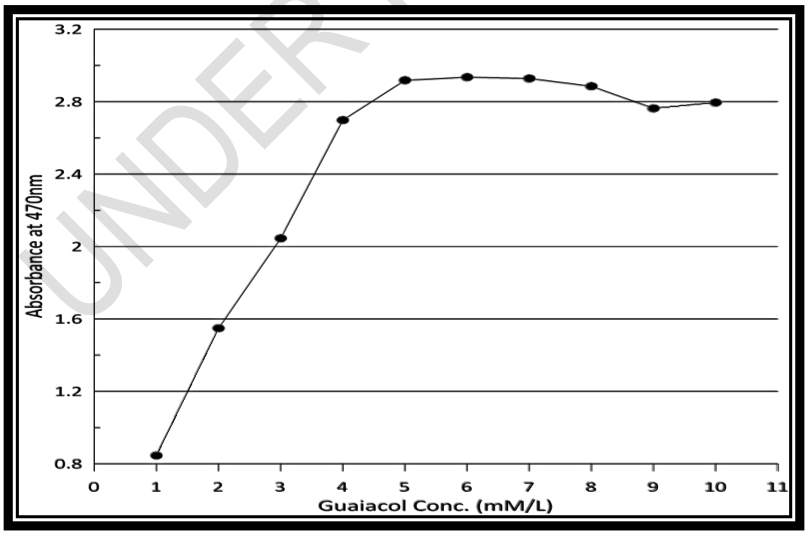

Figure 2. Effect of Substrate concentration on the Conversion of guaiacol to tetraguaiacol

Our results showed significant increase in product formation with increasing substrate concentration from 1- 5mM. From 6mM concentration, no significant increase in activity was observed therefore, the concentration (5 mM guaiacol) was selected as the best for the assay. Our findings here show a classical Mm~~-~~ichealis–Menten type behaviour which explains how the rate of an enzyme-catalysed reaction depends on the concentration of the enzyme and its substrate. According to the model, the rate of reaction initially increases rapidly in a linear fashion as substrate concentration increases. The rate then plateaus, and further increase in the substrate concentration has no effect on the reaction velocity as all enzyme active sites are already saturated with the substrate

3.1.2 Effect of Reaction Time

The effect of reaction time on guaiacol oxidation is presented in Figure 2. The assays were carried out at different reaction times (1 - 10 minutes), whilst keeping other conditions constant. With the increase in reaction time, the conversion of guiacol to tetraguaiacol increases until a peak was reached at 7 minutes. From 8 minutes, the absorbance of the assay starts declining thus suggesting a favourable back reaction at that time. It is a known fact that most enzyme-catalyzed reactions are highly reversible therefore at long incubation times, the tendency exists for the backward reaction to be favoured hence resulting in a declined or false response. Appropriate reaction time should be long enough to permit a significant amount of product formation but not too long in order to avoid a decline in activity that comes with high product formation. Optimum time of 5 minutes was selected as the best for the current study. At this reaction time, 97.88% of the highest obtainable activity was achieved therefore it is considered sufficient.

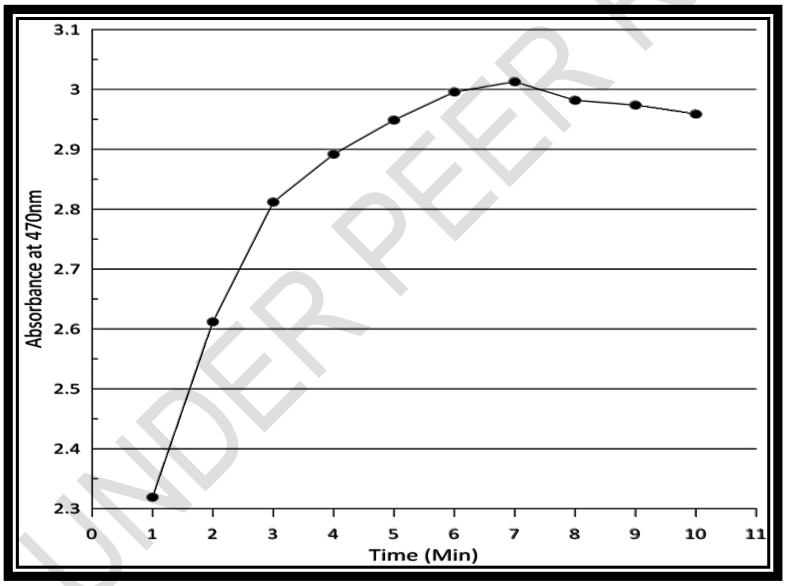

Figure 3. Optimisation of Reaction Time for conversion of Guaiacol to Tetraguaiacol

3.1.2 Effect of Assay pH

The activity of POD (formation of tetraguaiacol) was measured at different pH values ranging from 5 - 10. As shown in Figure 4, the highest activity for the assay was observed when the reaction was performed at pH 6. At pH 7, a slight drop in activity (5%) was noted. However, from pH 8 to 10, a notably high decline in assay activity was observed. It is well known that the pH has a strong influence in enzyme catalysis; too high or too low pH can cause enzyme deactivation due to

denaturation of the proteins. In general, most plants enzymes show maximum enzyme activity at or near neutral pH. Different optimum pH values for PODs obtained from various sources have been reported as follows: pH 6.0 - 8.5 for kiwifruit POD using p-phenyllene diamine as substrate [13] pH 6.0 for spring cabbage POD using guaiacol as substrate [14] and pH of 6 for hot pepper using guaiacol as substrate [15]. The optimum pH value we report in this study falls within the range of values that have been reported for PODs from other plant sources

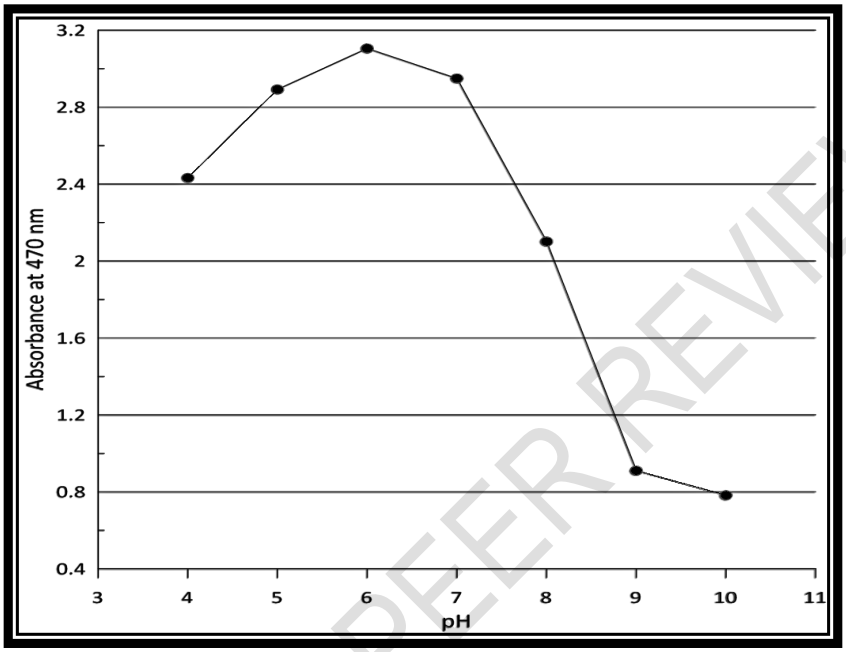

Figure 4: Optimisation of pH for Conversion of Guaiacol to Tetraguaiacol

3.1.2 Effect of Temperature

The Temperature dependence for the oxidation of guaiacol to tetraguaiacol is presented in Figure 5. Optimal temperature for the assay was found to be 40 °C with an absorbance of 3.142. Other temperature values tested yielded significantly lower activities, with the lowest activity being 1.845 absorbance obtained at a temperature of 70 °C. The assay activity increased when the temperature was increased from 30 to 40°C, further increase in temperature resulted in decreased activity probably due to thermal denaturation of the enzyme at higher temperatures. This situation is similar with previously reported studies on oxidation of guaiacol using PODs from other plant sources; it was found that the highest activity of POD from hot pepper (*Capsicum annuum L.)* was obtained at 40°C [13]. Similarly, POD from rosemary (*Rosmarinus officinalis* L.) leaves also showed highest activity at 40 °C [14]. In general, the temperature at which PODs show maximum activity has been reported to be in the range of 30°C to 60°C. Although the optimum temperature obtained in this study was 40°C, the ambient temperature which was 33±3°C was used throughout the investigation; this was chosen as a compromise between enzyme activity and its stability.

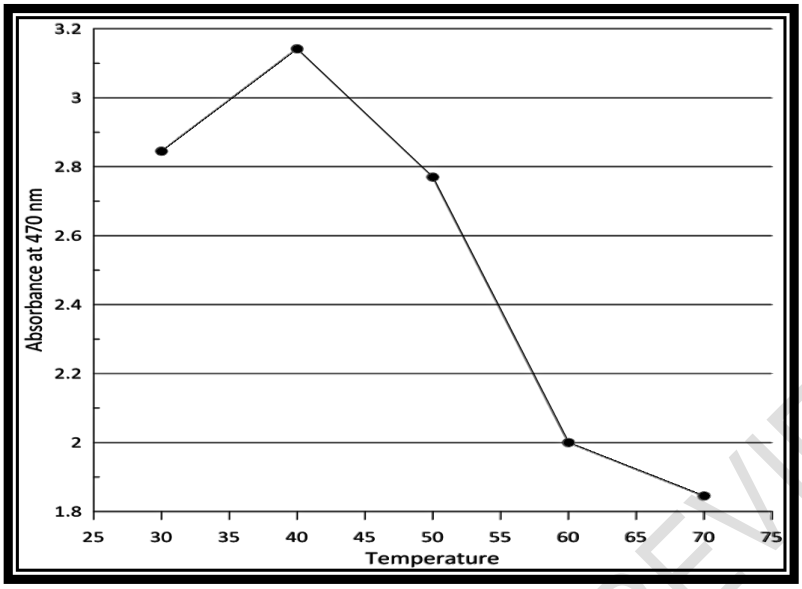

Figure 5. Optimisation of Temperature for Conversion of Guaiacol to Tetraguaiacol

3.2 Determination of Phenolic compound (2-Methoxyphenol)

The analytical curve for 2-Methoxyphenol was constructed using five concentrations (1 - 5mM). POD assays on the solutions were performed using pre-determined optimum experimental conditions. Table 1 shows the analytical parameters obtained from the calibration curve. A good slope with intercept near zero was established with a correlation coefficient of 0.998. The detection and quantification limits were 0.018 and 0.06 mM respectively. Inter-day precision determined using three concentration levels (n=7) showed that the method provided high levels of precision when used by the same analyst on the same day with RSD values ranging from 2.4 to 3.8%. For the concentration levels considered in this study, the recommended precision as a function of analyte concentration is in the range of 1.3 to 5.3 [15]. The values obtained in this study therefore indicate good repeatability.

Table 1. Analytical and Method Validation Parameters

Parameter	**Value**
Linear Range	0.1 - 5mM
Regression Equation	y = 0.2214x + 1.2215
Correlation coefficient	0.998
Limit of Detection (LOD)	0.018mM
Limit of Quantitation (LOQ)	0.06mM
Precision (n=7)	3.8 % RSD
Accuracy (%Recovery)	85–112%

Accuracy evaluation via recovery studies was performed in triplicate on surface water samples samples using three concentration levels. The results obtained (Table 2) showed a range of 85 to 112% recoveries. The range for acceptable mean recovery expands as the concentration of the analyte decreases and the target mean recovery for analyte concentrations ranging from 100 ppb to 100% is in the range of 80 -110% [14]. With the exception of one sample (stream B,3.5), the values recorded in this study were all within this limits recommended by AOAC [15] . We can see that the phenolic content obtained from this assay is higher in stream B than in A. This finding suggests higher pollution by phenolic compounds in the environment around stream B or possible discharge of polluted waste water into the stream.

Table 2. Determination of Method accuracy as % Recovery of 2-methoxy phenol rom Environmental Samples Spiked with Different levels of Standard Concentration (n=3)

Sample	Added (mM/L)	Recovered (mM/L)	Recovery (%)
Stream A	1.5	1.28	85.33 ± 1.12
	3.5	3.37	96.28 ± 2.96
	7.5	6.95	92.66 ± 2.25
Stream B	1.5	1.62	108 ± 1.84
	3.5	3.92	112 ± 2.68
	7.5	7.64	101.86 ± 1.44

4. CONCLUSION

This study evaluated a peroxidase based enzymatic method for the determination of 2- methoxy phenol in water samples. The optimized experimental conditions for the assay performance were obtained and practical applicability of the method was demonstrated by the good recoveries obtained for the investigated analyte. The presented procedure is simple and can be adopted by low resource laboratories. As the method is based on the oxidation of phenols via peroxidase catalytic activity this makes it lacking in selectivity because a wide number of phenolic compounds can undergo oxidation under the same reaction conditions. What this means is that the method cannot be selectively applied for the determination of just a particular phenol, however it can be useful for the estimation of total phenolic compounds in aqueous samples. With further improvements and validation this method can be extended to the analysis of phenolic compounds in other sample types.

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