Research Article

Fe_3O_4 Nano Particle Catalysed Spectrophotometric Method for the Quantification of Hydrogen Peroxide using Pyrocatechol and 3-Methyl-2 Benzothiazolinehydrazone Hydrochloride: Applications in Water, Vegetables and Milk samples

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Abstract

A simple, rapid and sensitive spectrophotometric method is described for the determination of hydrogen peroxide using pyrocatechol (PC) and 3-methyl-2 benzothiazolinehydrazone hydrochloride (MBTH) is presented. This model is based on the oxidation of PC by H_2O_2 in presence of Fe_3O_4 magnetic nanoparticles to form quinone, which couples with oxidized MBTH resulting in intense red chromogenic product having maximum absorbance at 510 nm. The optical density is directly proportional to hydrogen peroxide concentration and obeys Beer's law in the range 0.33 to 3.0 mmol L^{-1}. The molar absorptivity, sandel's sensitivity, detection limit and quantitation limit of the method were found to be 4.4×10^4 L mol^{-1} cm^{-1}, 0.002µg/mL/cm^2, 0.033 mmol L^{-1} and 0.09 mmol L^{-1} respectively. The results of analysis of the proposed method are compared favourably with those from a reference method.

Keywords: Nano particle, Pyro catechol, 3-Methyl-2 Benzothiazoline hydrazone Hydrochloride, Optical density

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Introduction

Hydrogen peroxide is a crucial part of atmospheric chemistry and biological systems. In the atmosphere, it is an oxidant which is produced from the combination of hydroperoxyl radicals ($HO_2^{\cdot}$) and their hydrated form [1]. The existence of H_2O_2, at extensively varying levels, has been reported in human and other animal aqueous and vitreous humors [2]. H_2O_2 is present in human body in kidney, urinary tract and bladder. In exhaled air of humans [3] and of rats contains hydrogen peroxide [4].

The existence of H_2O_2 in environment is one of world serious problem is, the death of aquatic animals and aquatic plants, by the high acidity of the rain results from the oxidation reaction of H_2O_2 [5]. Several literatures have explained high levels of H_2O_2 as being cytotoxic to a wide range of animal, plant and bacterial cells in culture. Due to the severe effects of H_2O_2 it is inevitable to quantify the level of H_2O_2 in environmental and biological samples. H_2O_2 is a substance highly used in food, pharmaceutical, clinical, environmental and other industries. Because of broad applicability and lack of simple methods for hydrogen peroxide, accurate analysis is needed. Moreover, it is the substance produced during many enzymatically catalysed reactions and it is the product of cell metabolism [6, 7].

Hydrogen peroxide is found in fresh fruit and vegetables. Eating fruits and vegetables raw assures that we get this hydrogen peroxide into our bodies, along with valuable enzymes. Mother's milk contains a good amount of hydrogen peroxide, and colostrums contain even more.

Concentration of H_2O_2 is significant parameter giving edge between harmless and harmful impact on the body. According to the Scientific Committee on Consumer Products, in tooth bleaching product, more than 0.1 % of H_2O_2, it may cause potential health problems and it causes the severe health problems if the concentration higher than 6 %. Exploration of the literatures reveals that, in natural water hydrogen peroxide has been found rather ubiquitously in a wide range of concentrations. The concentrations of H_2O_2 in ground water are as low as 50 µmol L^{-1} [8] has been reported. Concentration of hydrogen peroxide varies from about 10 to several hundred µmol L^{-1} in the surface oceans,

[9, 10]. Higher concentrations up to several μmol L^{-1} have been reported in lakes, estuaries and rivers, [11, 12]. The highest concentrations ranging from several μmol L^{-1} to tens of μmol L^{-1} in rain water, [13-15] have been obtained.

Determination of hydrogen peroxide is usually based on the production of colored peroxy compounds or on its oxidizing and reducing properties. The detailed literature survey shows that many analytical methods including titrimetric [16], fluorometric [17, 18], chemiluminescence [19], and electrochemical [20] these methods are either very expensive or less versatile. Determination of micro amount hydrogen peroxide with conventional titration method has been unable to meet the requirements, needing to establish some sensitive, rapid, simple method for determination of hydrogen peroxide. Monitoring method of hydrogen peroxide caused the great attention of analysts. Although chemiluminescence method, atomic absorption spectrometry *etc.* are proposed for the determination of hydrogen peroxide [21, 22] the price of instruments used is more expensive than that of spectrophotometer.

An exemplary method to quantify the H_2O_2 was made by spectrophotometry due to the rapidity, facile and inexpensive properties of it. For the determination of H_2O_2 high sensitivity and selectivity are required, hence spectrophotometric method is selected. Also due to the instability of the hydrogen peroxide, spectrophotometric method was used in this investigation. Several spectrophotometric methods are available for the assay of H_2O_2 but these methods includes the use of toxic reagents. To overcome all this we developed a novel, spectrophotometric method for the assay of H_2O_2. The developed method is successfully applied to different water samples and also the results obtained by the proposed method are compared with standard existing method shows the agreeing values.

The use of nanoparticles in recent years has been much interest in many branches of science and technology [23, 24]. Due to the unique chemical and physical properties of nanoparticles it is used in the various fields of Science and technology. Their high surface area to volume ratio provides more catalytic sites than bulk particles. In chemical studies, the role of these species is to catalyse reactions between reactants. In fact the nanoparticles play the nanocatalyst role in some reactions. It was observed that Fe_3O_4 magnetic nanoparticles possessed an intrinsic peroxidase-like activity [25]. Based on this, Fe_3O_4 MNPs is used as a new catalyst which as a potential application in the determination of hydrogen peroxide in water sample and in vegetable samples. Our data showed that this new method is efficient to determine H_2O_2 in water sample and in vegetable sample. The differences in rate of H_2O_2 decomposition are attributed to the specific surface area of iron oxide. Therefore, iron oxide NPs and nanowires with high specific surface area were employed to promote the production of intermediate oxidants from H_2O_2 decomposition. [26-30].

Methods

Apparatus

Systronics spectrophotometer model 106 with 1 cm matched glass cuvvets was used for measuring the absorbance. A pH-meter, EQUIP-TRONICS Model EQ-614 was employed for measuring pH.

Chemical Reagents and Their Preparation:

All the chemicals used in the assay were of analytical grade. MBTH and PC were purchased from Sigma-Aldrich and Merck, Germany respectively. MBTH (4.5 mM) and PC (18.0 mM) solutions were prepared by dissolving a requisite quantity in distilled water. Catalase (bovine liver, EC 1.11.1.7, 4326 units/mg) was purchased from Sigma-Aldrich, Germany and its solution was prepared by dissolving 10 mg in 5 mL of distilled water. It was used as a standard stock solution and stored at 4^{o}C. Further dilution was made with double distilled water when required. H_2O_2 (30%) was purchased from E Merck, Mumbai, India. The 2% v/v H_2O_2 stock solution was prepared daily and its concentration was standardized by titration with secondary standard potassium permanganate (Vogel, A.I. 2006). Tris buffer (0.5 M) of pH 9.8 was prepared by dissolving 1.514 g of tris (hydroxyl methyl) methyl amine [2-amino, 2-(hydroxyl methyl) propane-1-3-diol] in 25 mL using distilled water. Double-distilled water was used throughout the experiment. All solutions were preserved under temperature range of 0-10^{o}C.

Determination of H_2O_2 in water samples

Water samples (tap, lake, river, sea and rain) were collected without adding any additive in polyethylene bottles and subjected to analysis on same day. Water samples were filtered through a Whatman No. 41 filter paper, and then an aliquot of the filtrate was taken for analysis by the recommended method and reference method [31].

Synthesis of magnetite nanoparticles

Iron oxide nano particles were synthesised by mixing aqueous solutions of Ferric chloride and ferrous chloride in 2:1 molar ratio. The mixture was heated up to 50°C for 10 min. After heating, the solution was precipitated by adding 1.5 mol/L NaOH under vigorous mechanical stirring. This reaction mixture was gradually heated to 78°C and held at this temperature for 1 h under stirring and nitrogen protection. The particles were then separated by using a strong magnet and then were washed many times with double distilled water. The powder was then dried in hot air oven [32]

Experimental Protocol Conducted For the Quantification of Hydrogen Peroxide

The linearity for the assay of $H_2O_2$was determined in 3 mL of the solution containing 0.1545 mM MBTH, 0.6054 mM PC, and 0.1 ml of iron nanoparticles in 50 mM tris buffer at pH 9.8. The reaction was initiated at 30°C by adding 100 µL of varying concentrations of H_2O_2. The change in absorbance was continuously recorded at 510 nm. The absorbance was then plotted against the concentration of H_2O_2. The linearity of the graph was found between 0.33 – 3.0 mM H_2O_2.

Results and Discussions

Absorption Spectra

The formed reddish coloured complex was scanned in the wavelength range of 360- 700 nm against a corresponding reagent blank. The optimum wavelength of maximum absorption of the red coloured product was obtained at 510 nm. Optical density for the different concentration of H_2O_2 are shown in **Figure 1**

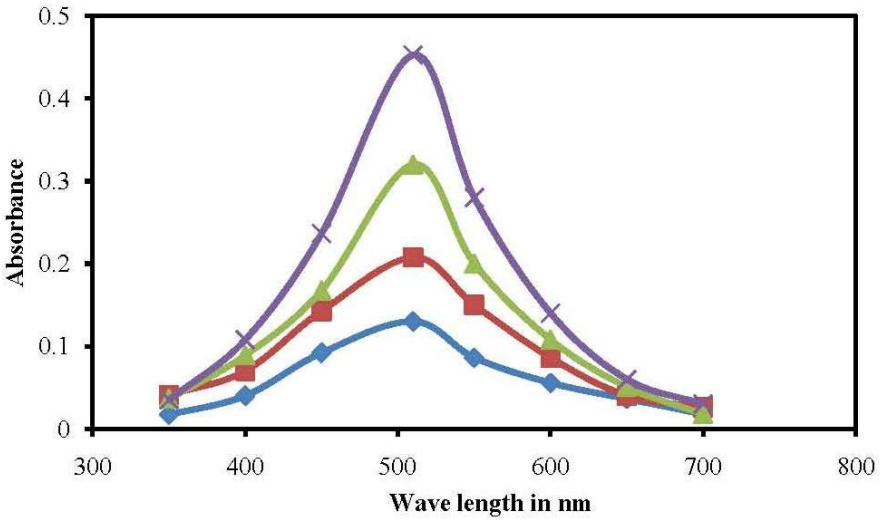


Figure 1 The absorption spectra of for different concentration of H_2O_2

Effect of MBTH and PC concentration

The effect of varying concentrations of MBTH and PC was studied and the results showed that the rate increased on increasing the concentration of MBTH from 0.1545 mM to 1.1 mM beyond which there is no effect of MBTH. Hence for all further assays MBTH concentration of 1.1 mM was selected. Similarly, the effect of PC concentration on the reaction rate was studied from 0.303 mM to 4.5 mM. The linearity was observed up to 3.0 mM, above this concentration there was no effect. Hence 3.0 mM was selected as the optimized concentration for all further analysis. The effect of MBTH and PC are shown in **Figure 2.**

Effect of Temperature on the Sensitivity of Peroxidase Assay

Temperature sensitivity was determined by pre-incubating 1.1 mM MBTH, 3.0 mM PC, 3.0 mM H_2O_2, and 0.1 mL nanoparticle in 50 mM tris buffer of pH 9.8 for 10 min at different temperatures (0-80 °C). The activity initially increased up to 30 °C and gradually decreased thereafter. 30 °C is taken as optimized temperature. The effect of temperature on the reaction is shown in below **Figure 3**.

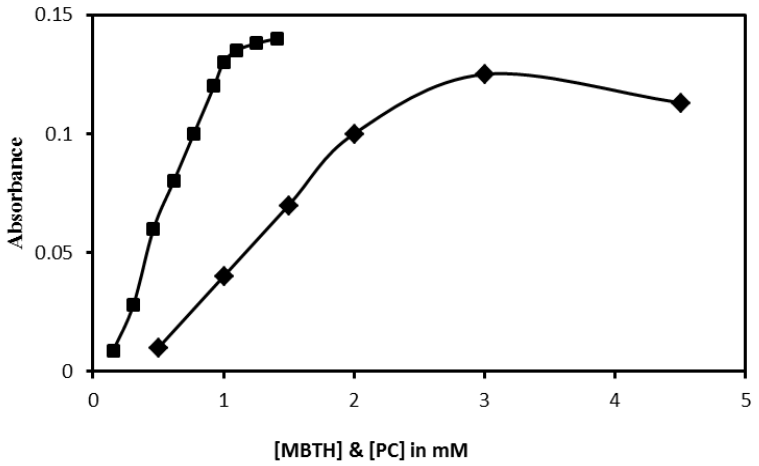


Figure 2 Effect of MBTH & PC on the reaction

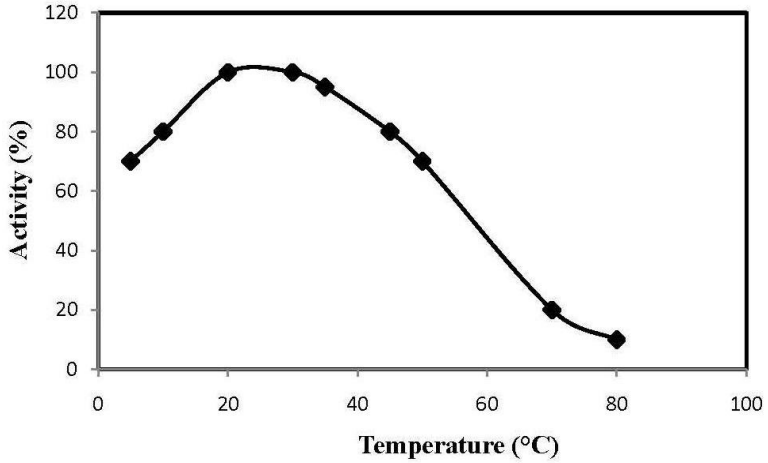


Figure 3 Effect of temperature on the reaction

Effect of pH and Concentration of Buffer Solutions

The pH of the medium had an important factor on the stability of the coloured product formed. We compared the stability of the product with KH_2PO_4/ K_2HPO_4, KH_2PO_4/NaOH, Na_3BO_3–NaOH, Tris- Hcl buffer, ammonia-ammonium chloride buffer and sodium acetate/ acetic acid buffer with pH controlled in the range 4.0 –10.5. The reaction shows maximum colour development in 50 mM tris buffer of pH 9.8. Hence, further studies were carried out at this pH (**Figure 4**).

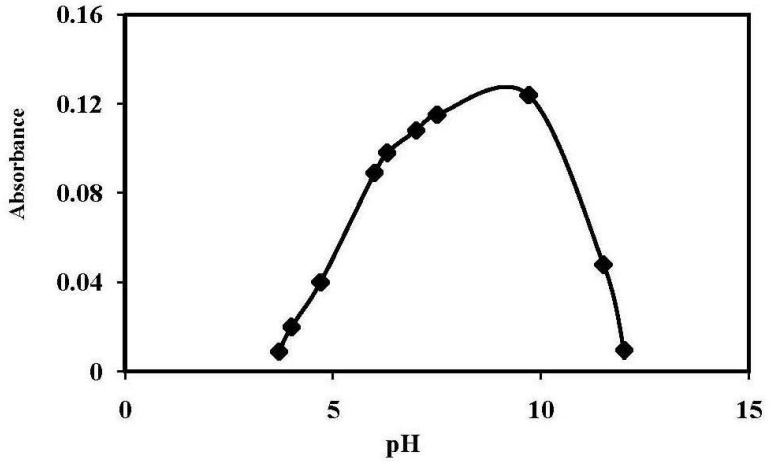


Figure 4 The effect of pH on the reaction

Recommended procedure for quantification of hydrogen peroxide

The linearity for the assay of $H_2O_2$was determined in 3 mL of the solution containing 0.1545 mM MBTH, 0.6054 mM PC, and 0.1 ml of iron nanoparticles in 50 mM tris buffer at pH 9.8. The reaction was initiated at 30°C by adding 100 µL of varying concentrations of H_2O_2. The change in absorbance was continuously recorded at 510 nm (**Figure 5**). The absorbance was then plotted against the concentration of H_2O_2. The linearity of the graph was found between 0.33 – 3.0 mM H_2O_2.

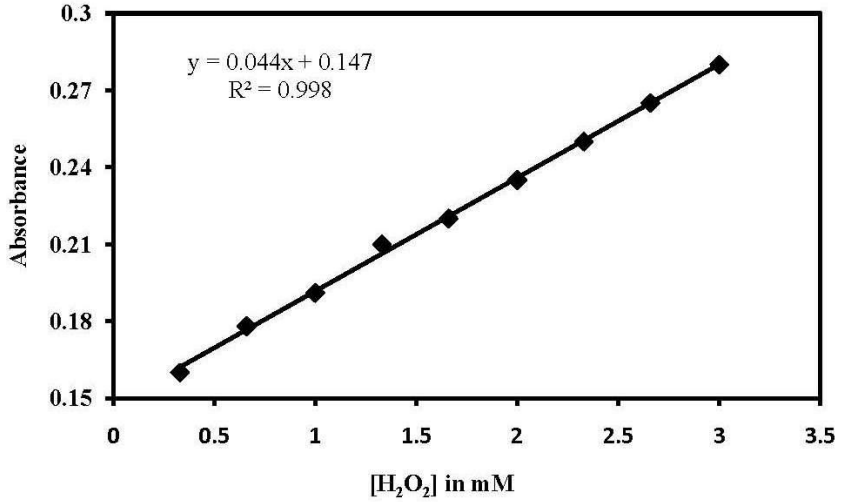
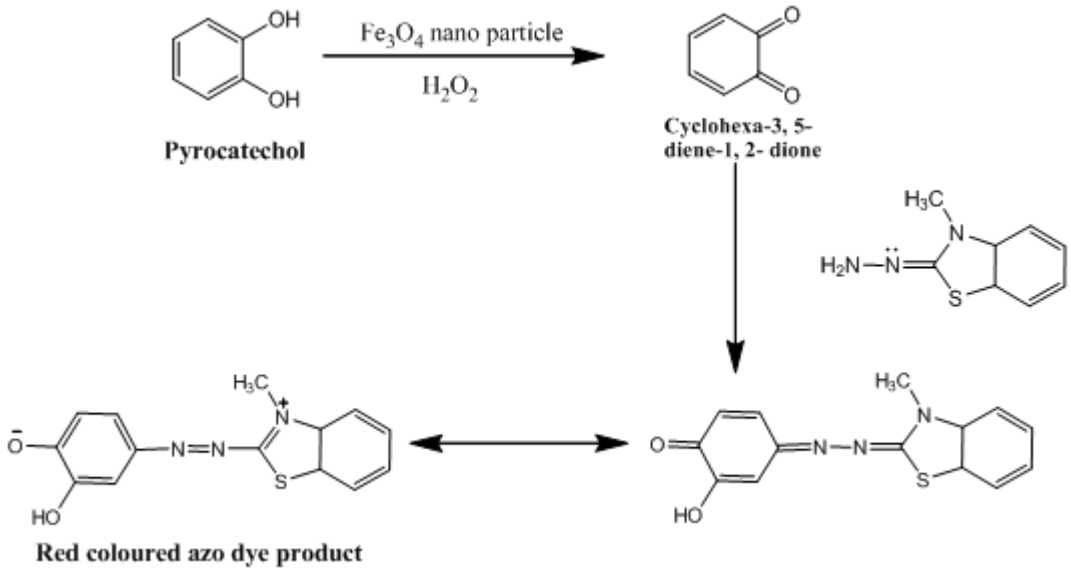


Figure 5 *Beer's law graph for quantification of H_2O_2*

Discussion of Proposed Reaction Pathway for the Enzyme Activity Response

A possible mechanism for the Fe_3O_4 magnetic nanoparticles catalysed reaction of MBTH and PC in presence of H_2O_2 involved the coupling of the reactants. Iron nanoparticles acts as a mimetics of peroxidase. In the proposed reaction, the process involves the double-displacement mechanism for the $H_2O_2$and electron donating phenolic compound. The nanoparticle is first oxidized by $H_2O_2$and then reduced by phenolic compounds. The phenolic compounds are converted to quinines [19]. In the presence of H_2O_2, MBTH undergoes peroxidation reaction to form MBTH radical cation [20], which gets coupled with quinone to form a red colored azo-dye product, showing a strong absorption at 510 nm. The Proposed reaction pathway for the formation of red coloured product is shown below.



Scheme Proposed reaction pathway for the formation of red coloured product

Analytical features

Under the optimum conditions, the calibration graph for the determination of H_2O_2 in the range of 0.3–3.0 mM has given a coefficient regression of 0.998. The molar absorptivity and Sandell's sensitivity were calculated and found to be 4.4×10^4 l mol^{-1} cm^{-1} and 0.002 µg mL^{-1} cm^{-1} respectively. The LOD and LOQ were found to be 0.033 and 0.09 mM, respectively. Six replicate determinations for 1.66 mM of H_2O_2 were taken to calculate the relative standard deviation which was found to be 0.23%

FTIR data interpretation

Fourier transform infrored spectroscopy (FTIR) spectra was performed to the dried sample of iron oxide nano particle using a FTIR – Shimadzu 8400 spectrophotometer in wavelength range of 3500-400 cm^{-1} with a resolution of 4 cm^{-1}. The dried sample was placed on a silicon substrate transparent to the infrared, and spectra were measured according to the transmittance method.

FTIR spectrum in the **Figure 6** shows that H-O-H bending vibration at about 1000-1600 cm^{-1}, typical of the H_2O molecule, is less intence. Additionally, the second absorption band, between 900-1000 cm^{-1}, corresponds to bendind vibration associated to the O-H bond. In the spectrum, the sample exhibits the peak at 600 cm^{-1} that is due to the streching vibration mode associated to the metal-oxygen absorption band (Fe- O bond in the crystal lattice of Fe_2O_3).

Scanning electron microscopy of the model was used for investigating shape and size of prepared powder **Figure 7** illustrates the SEM micrograph for the produced Fe_3O_4 magnetic nanoparticles, which has a spherical like particle shape with around less than 15 nm particle size. By this unique and fine size, it can be used as a mimetic of peroxidase.

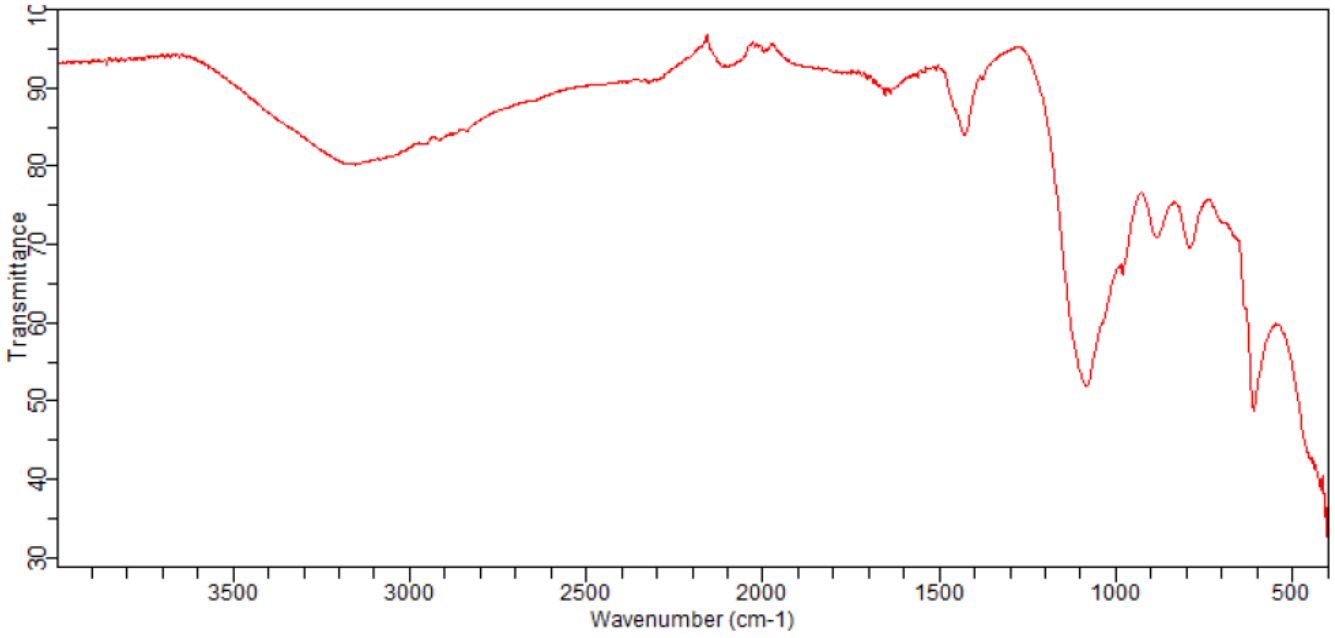


Figure 6 FTIR spectra of Iron nano particles

Application

The developed method was successfully applied to different vegetable, milk and water samples collected from different areas in and around Mysuru, which includes underground and surface water samples. The concentration of the sample was quantified by considering the standard absorbance. The results are compared with reference H_2O_2 assay method [31]. The results obtained by these two methods for the analysis of water, vegetable and milk samples are shown **Tables 1-3**.

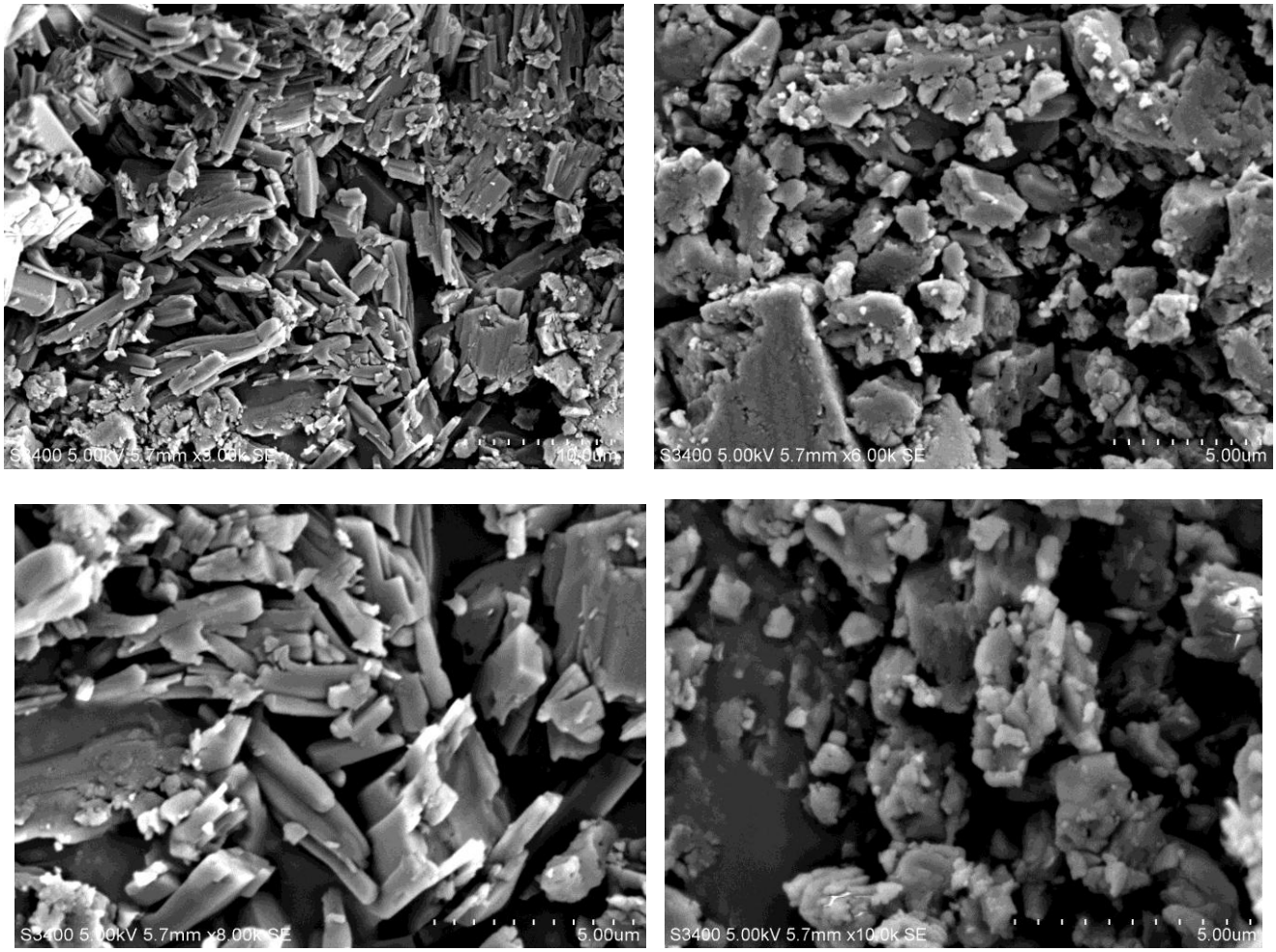


Figure 7 SEM of nano iron oxide powder

Table 1 Application of the recommended method for determination of H_2O_2 in various water samples

Sl. No	Sample	Proposed method [H_2O_2] in mMol	Standard method [H_2O_2] in mMol
1	Bangalore sample	0.42 ± 0.26	0.40 ± 0.22
2	Tumkur sample	0.35 ± 0.11	0.34 ± 0.35
3	Kukkarahalli lake water	0.34 ± 0.22	0.33 ± 0.28
4	JSS College tap water	0.33 ± 0.21	0.32 ± 0.18
5	Karanji lake water	0.42 ± 0.18	0.40 ± 0.22
6	Varuna Lake water	0.44 ± 0.17	0.42 ± 0.24
7	Edakallugudda	0.32± 0.10	0.30±0.14
8	Kochhi beach	0.81± 0.12	0.85±0.21
9	Mugur lake	0.88± 0.11	0.87±0.08
10	Mundrabad dam	0.50± 0.13	0.52±0.16
11	T. Narasipura	0.74± 0.17	0.75±0.26
12	Arsikere	0.59± 0.21	0.55±0.17

Mean ± Relative standard deviation (n = 5)

Table 2 Application of the recommended method for determination of H_2O_2 in milk samples.

Sl. No	Sample	Proposed method [H_2O_2] in mMol	Standard method [H_2O_2] in mMol
1	Cow	0.96± 0.25	0.88±0.27
2	Buffalo	1.20± 0.22	1.1±0.24
3	Goat	1.38± 0.19	1.40±0.26

[a]Mean of three replicate measurements

Table 3 Application of the recommended method for determination of H_2O_2 in various vegetable samples

Vegetable Samples	H_2O_2 in ppm		Added in ppm	Found by the proposed[a] method	Recovery
	Proposed method	Reference method			
Keera *Alternanthera sessilis)*	1.09	1.1	2	3.1	100.5
Menthya (Fenugreek greens)	1.55	1.49	2	3.61	103.0
Dantu (*Amaranthus dubius)*	2.42	2.38	2	4.39	98.5
Sapsige (*A. graveolens)*	1.03	0.99	2	4.09	104.5
Coriandur (*C. sativum)*	1.88	1.91	2	3.90	95.0
Palak (Spinacia oleracea)	2.00	2.01	2	4.02	101.0

[a]Mean of three replicate measurements

Conclusion

The developed method is simple, rapid, reliable, and highly sensitive for the assay of H_2O_2 in water samples which involves coupling of oxidized PC and MBTH using H_2O_2. Reagents used are easily available, economical, water-soluble, and the coupled product gets absorbed at a longer wavelength (500 nm) which enables to avoid the background interference caused by the biological constituents therefore this method is also applicable for the quantification of H_2O_2 in biological samples. The linearity of H_2O_2 in the range of 0.3–3.0 mM indicates the highly sensitivity of the method. The detection limit and quantification limit were found to be 0.033 and 0.09 mM, respectively hence this method can also applicable to quantification of H_2O_2 in water, vegetable and milk samples. Mugur lake water found to be more hydrogen peroxide content among the analysed sample, *Amaranthus dubius* commonly called as dantu contains more amounts of peroxide and among cow, buffalo and goat, goat milk is rich in peroxide content.

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