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RESEARCH ARTICLE

Precise Spectrophotometric Method for measurement of Peroxiredoxin activity in Biological Samples

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ABSTRACT:

Herein, we describe a simple spectrophotometric method for the measurement of peroxiredoxin activity and demonstrate reproducibility, accuracy, and precision. In these experiments, peroxiredoxin activity was measured by incubating enzyme samples with phosphate buffer solution containing suitable concentrations of the substrates 1,4-dithio-DL-threitol (DTT) and hydrogen peroxide. Titanium sulfate was added to stop enzyme reactions, and subsequent reactions with residual hydrogen peroxide produced pertitanic acid, which was spectrophotometrically measured at 405 nm. Advantages of this method are including the elimination of catalase interference and allowing application of this method to all types of biological tissues. The peroxiredoxin assay is simple and can be completed with few additions. The method is precise, with coefficients of variation of 2.93% within runs and 5.4% between runs. Data from the present peroxiredoxin assay were strongly correlated with those from the ferrous oxidation–xylenol orange (FOX) method (r = 0.9835).

KEYWORDS: Peroxiredoxin, pertitanic acid, FOX reagent, dithiothreitol, H_2O_2.

1. INTRODUCTION:

Peroxiredoxins were originally identified as antioxidants and were recently recognized as key biochemical redox regulators. Peroxiredoxins are thiol dependent peroxidases that lack heme and selenium and are expressed in most organisms [1]. As in other mammalian antioxidant systems, enzymes of the peroxiredoxin family ameliorate oxidative stress by metabolizing hydroperoxides [2]. As unique redox enzymes, peroxiredoxins initially interact directly with H_2O_2 via sulfhydryl groups on conserved peroxidatic cysteine residues (C_p). Subsequent conversion of C_p to cysteine sulfenic acid (C_r) leads to reactions with C_r residues of other peroxiredoxins and the formation of disulfide bonded homodimers [3].

Six isoforms of peroxiredoxin (PRX1–PRX6) have been identified in mammals [4], and while PRX 6 has one cysteine (Cys) residue, PRXs 1–5 have two cysteine (Cys) residues. These residues are essential for peroxiredoxin activity and participate in redox cycling reactions [5]. In comparison with selenium dependent-glutathione peroxidases, peroxiredoxins do not require metal ions [6], but reduce all types of organic hydroperoxides e.g., cumene hydroperoxide and inorganic hydroperoxides e.g., hydrogen peroxide [7].

Recent investigations of PRX enzymes have been motivated by the discovery of their wide ranging organic and inorganic hydroperoxide substrates and high catalytic rate constant of approximately 10^7 M^{-1} s^{-1} [8]. However, at 1 mM H_2O_2, many eukaryotic PRXs are inactivated, and the half-life of human PRX2 is just 20 s under these conditions [9]. These observations indicate the need for sensitive design and implementation of kinetic peroxiredoxin assays.

Nelson and Parsonage [10] used four differing methods to measure PRX activities with peroxide substrates. The first of these was performed using NADPH-coupled reactions with thioredoxin and thioredoxin reductase. The second assay was performed by directly monitoring

thioredoxin oxidation, the third depended on competition with horseradish peroxidase, and the fourth protocol was performed according to FOX assay of peroxide consumption. However, because all of these assays require pure enzyme, their clinical applications are limited. Moreover, although PRXs reduce peroxides in the presence of either NADH or NADPH under physiological conditions, glutathione peroxidase and catalase also reduce H_2O_2, thus hampering specific assessments of peroxiredoxin mediated reductions in H_2O_2 levels.

Nelson and Parsonage [10] indicated that the presence of catalase enzyme hampered measurements of PRX activities in all of the methods. Because the only solution to this problem involves the purification of peroxiredoxin enzymes [10], these methods are not applicable to biological tissues. To address these issues, we developed the present peroxiredoxin assay using sodium azide to exclude interference from catalase enzyme activity [11,12] in samples containing PRX enzymes, and achieved highly specific determinations of PRX activities. Glutathione peroxidase does not affect the hydrogen peroxide concentration due to the absence of a significant concentrations of it is substrate (glutathione) in the reaction solutions. The glutathione peroxidase enzyme needs two substrates to complete it is enzymatic activity (glutathione and hydrogen peroxide). The absence of one of them leads to the termination of its enzymatic activity. Therefore, there is no interfering between the activities of peroxiredoxin and glutathione peroxidase.

2. MATERIAL AND METHODS:

2.1. Chemicals:

Sorbitol ($C_6H_{14}O_6$), ammonium ferrous sulfate; titanium tetrachloride ($TiCl_4$), xylenol orange dye (3,3′-bis[N, N-bis(carboxymethyl) aminomethyl]-*o*-cresolsulfonephthalein tetrasodium salt), sodium azide, 1,4-dithio-DL-threitol (DTT), and hydrogen peroxide (H_2O_2) were purchased from commercial suppliers and were of analytical grade.

2.2. Instrument:

Measurement of the end product pertitanic acid was performed using a Shimadzu 1800 spectrophotometer.

2.3. Reagents and solutions:

Ferrous oxidation−xylenol orange (FOX) Reagent A was prepared by dissolving 0.98 g of ammonium ferrous sulfate (25 mM) in 100 ml of 2.5 M sulfuric acid (H_2SO_4), and the solution maintained stability after three weeks at 4°C. FOX Reagent B was prepared by dissolving 1.8217 g of sorbitol (100 mM) and 0.0085 g of xylenol orange (125 μM) in 100 ml of distilled water, and the solution attained stability after 1 week at 4°C. Practical FOX reagent was prepared fresh daily by adding 1 volume of FOX reagent A to 100 volumes of FOX reagent B. Titanium reagent was prepared by dissolving 0.1% (w/v) $TiCl_4$ in 20% (v/v) H_2SO_4 (Caution: adding $TiCl_4$ to sulfuric acid will release chlorine gas). Phosphate buffer (50 mM; pH 7.0) was prepared in distilled water as a 1:1.5 mixture of KH_2PO_4 (6.81 g/L) and $Na_2HPO_4.2H_2O$ (8.90 g/L) solutions [13]. Hydrogen peroxide (H_2O_2) (2.1 mM) was prepared daily in 50-mmol/L phosphate buffer and were standardized daily using a molar extinction coefficient of 43.6 M^{-1} cm^{-1} at 240 nm [13]. 1,4-dithio-DL-threitol (DTT) (2.1 mM) was prepared by dissolving 0.0323 g of DTT in 100 ml of phosphate buffer. Sodium azide (10 mM) was prepared by dissolving 0.06501g of NaN_3 in 100 ml of distilled water. The human Prx2 was purified as described by Lim *et al.* [14]. Briefly, three millilitres of blood was drawn by peripheral venous puncture and moved into suitable heparinized tube. The sample was centrifuged after 10 min at 400xg for 10 min, followed by separation and disposal of the buffy coat and plasma. The next step included washing 500 µl of the remaining red blood cell sediment three times with 5 ml of 0.9% NaCl solution, with centrifugation at 400xg for 10 min after each wash. Erythrocytes were lysed in Tris-HCl buffer (pH 7.6, 50 mM) containing 1 mM DTT and 0.5 mM EDTA, and after centrifugation, the lysates were treated with 30% ammonium sulfate and were then dialyzed against lysis buffer. Supernatants were then loaded into HiPrep 16/10 DEAE FF columns, and hemoglobin was removed by additional washing of the column with buffer. Finally, bound proteins were eluted using a linear NaCl gradient of 0–250 mM, and PRDX activity were adjusted according to FOX assay of enzyme activity [10]. The final specific activity reaches to 6.3 $U.mg^{-1}$ protein.

2.4. Tissues preparation:

The current study was used male albino mice and male albino rats that obtained from the animal house at the College of Science, University of Babylon, Iraq. Immediately before assessment of tissue peroxiredoxin activities, animals were sacrificed and liver and kidney tissues were surgically excised. Kidneys and livers were directly washed in 0.9% (w/v) NaCl solution to remove contaminating blood and were then homogenized using a glass homogenizer and ice cold 1.15% (w/v) potassium chloride solution. Homogenate solutions were then filtered and diluted (at a ratio of 1:500) with 0.05 M phosphate buffer for analyses of peroxiredoxin activity, which were completed immediately.

2.5. Procedures:

2.5.1. FOX assay procedure:

PRX activity was measured by monitoring ferrous ion oxidation in the presence of xylenol orange dye as described by Nelson and Parsonage [10] with slight modifications. In these assays, H_2O_2 was used to

selectively oxidize ferrous ions, and ferric ion concentrations were then determined using xylenol orange dye. Peroxiredoxin activity was measured by incubating enzyme samples in phosphate buffer containing the substrates 1,4-dithio-DL-threitol (DTT) and H_2O_2 for 3 min. Subsequently, 50-µl aliquots of reaction mixtures were mixed and incubated with 950 mL of the FOX reagent at room temperature, and changes in absorbance relative to those of the reagent blank were measured at 560 nm (Table 1).

2.5.2. Novel titanium sulfate assay:

Peroxiredoxin activity was determined by incubating enzyme samples in phosphate buffer solution containing 1,4-dithio-DL-threitol (DTT) and H_2O_2 (Table 2). Enzyme reactions were stopped by additions of titanium sulfate, which reacted with residual hydrogen peroxide to produce pertitanic acid. Pertitanic acid concentrations were then determined at 405 nm.

Table 1. The steps of the procedure used for assessing the activity of peroxiredoxin.

Reagents	**Test**	**Standard (STD)**	**Blank**
Phosphate buffer	600 µL	700 µL	900 µL
Sodium azide	100 µL	100 µL	100 µL
DTT	200 µL	200 µL	------
Sample containing peroxiredoxin enzyme (Serum, RBC, and homogeneous tissues)	100 µL	-------	------
The reaction is started with the addition of 200 µL of 2.1 mM H_2O_2, yielding 350 µmol/L initial concentration, followed by vigorous mixing. The assay done in an adjusted water bath at 37°C. All reagents kept at 37°C.			
H_2O_2	200 µL	200 µL	200 µL
Mix the contents of the tube by vortexing and incubate at 37°C for 3 min; transfer 50 µl aliquots from each tube in dry test tubes.			
Practical FOX reagent	950 µL	950 µL	950 µL
The tubes were incubated at room temperature for 30 min. Change in absorbance was measured against the reagent blank at 560 nm.			

Table 2. The steps of the procedure used for assessing the activity of peroxiredoxin.

Reagents	**Test**	**Standard (STD)**	**Blank**
Phosphate buffer	600 µL	700 µL	900 µL
Sodium azide	100 µL	100 µL	100 µL
DTT	200 µL	200 µL	------
Sample containing peroxiredoxin enzyme (Serum, RBC, and homogeneous tissues)	100 µL	-------	------
The reaction is started with the addition of 200 µL of 2.1 mM H_2O_2, yielding 350 µmol/L initial concentration, followed by vigorous mixing. The assay done in an adjusted water bath at 37°C. All reagents kept at 37°C.			
H_2O_2	200 µL	200 µL	200 µL
Mix the tubes with vortex, incubate at 37 °C for 3 min, after that, add:			
Titanium reagent	2 ml	2 ml	2 ml
After that, the tubes were kept at room temperature for fifteen minute. The test tubes should be centrifuged to remove the precipitate. Changes in absorbance were measured at 405 nm against the reagent blank.			

2.6. Calculation:

$$\text{The residual hydrogen peroxide in test tube} = \frac{\text{A.test}}{\text{A.STD}} \times \textit{Conc.of STD}$$

$$\text{Peroxiredoxin activity (µmol of } H_2O_2 \text{ utilized/min)} = \frac{\text{Conc. of } H_2O_2 \text{ in STD - Conc. of } H_2O_2 \text{ in test}}{\text{time (3 min)}} * \textit{Dilution Factor}$$

3. RESULTS AND DISCUSSION:

Peroxiredoxin reactions were stopped by the addition of titanium tetrachloride in sulfuric acid, which denatures peroxiredoxin. Unreacted hydrogen peroxide was then reacted with the titanium to produce the pertitanic acid, which has absorbance maxima at 405 nm. Colorimetric determinations revealed a single peak of pertitanic acid at 405 nm (Fig. 1), and absorbance was proportional to residual H_2O_2 after completion of enzyme reaction. To elucidate the linear rate of enzyme activity, we obtained the straight line by plotting the absorbance reading of the formed pertitanic acid at 405 nm against a range of hydrogen peroxide concentrations (as shown in Fig. 2).

The present novel assay of peroxiredoxin activity is subject to interference by various sugars, amino acids, and proteins in biological samples. Thus, to determine the interference of such organic compounds, we determined peroxiredoxin activities in 10 volumetric flasks containing various sugars, amino acids, and proteins (Table 3) at final concentrations of 0.5 mM, and H_2O_2 at 0.5 mM in 50 mM phosphate buffer (pH 7.4). Assay recovery rates were calculated for each of the potential interfering biological contaminants (Table 3), and indicated that the present method is robust in the presence of all potential confounders at equimolar concentrations to that of H_2O_2.

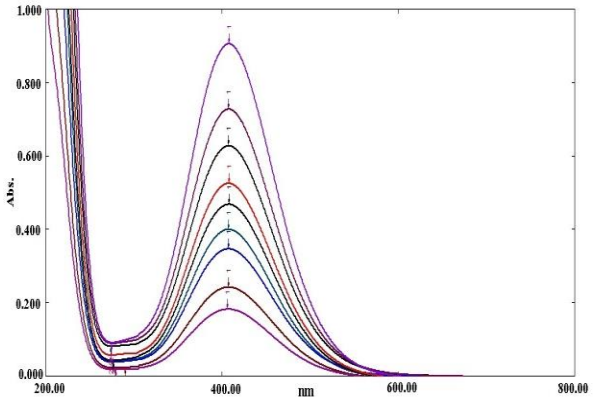

Fig 1. Absorption spectra of the colorimetric end product pertitanic acid.

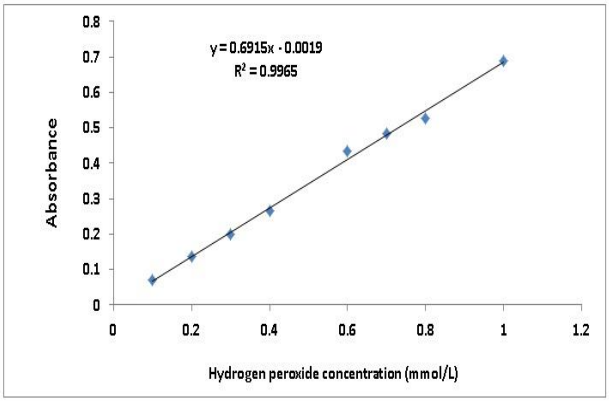

Fig. 2. The regression straight line that obtained by plotting the absorbance reading of the formed pertitanic acid at 405 nm against a range of hydrogen peroxide concentrations.

Table 3. Results of the effects of various interferences on peroxiredoxin activity.

Chemical interferences	Concentration of supposed interference µmol/L	Added hydrogen peroxide concentration µmol/L	Found hydrogen peroxide concentration µmol/L	Recovery %
-----	------	500	500	-----
Glucose	500	500	490	98
Fructose	500	500	492	98.4
Xylose	500	500	495	99
Albumin	5	500	490	98
Casein	5	500	505	101
Ascorbic acid (vitamin C)	500	500	495	99
Thiamine (vitamin B1)	500	500	492	98.4
Histidine	500	500	505	101
Lysine	500	500	508	101.6
Arginine	500	500	508	101.6

To ensure accuracy, the present peroxiredoxin assay was performed in the presence of the listed chemicals. Reaction mixtures contained 1-ml aliquots of 500-U/l peroxiredoxin with 9-ml aliquots of interfering chemicals in 50-mM phosphate buffer (pH 7.4). Total peroxiredoxin activity was adjusted to 50 U/l using the FOX method as described by Nelson and Parsonage [10]. Relative percentage errors associated with each interfering biological contaminant are presented in Table 4.

Table 4. The effects of several types of interference on the peroxiredoxin activity, the results represent the mean of three measurements of any interference.

Supposed chemical interferences	Concentration of supposed chemical interferences	Added peroxiredoxin U/l	Found peroxiredoxin U/l	Relative error (%)
-----	------	50	50	0
Glucose	500 µmol/l	50	49	2
Fructose	500 µmol/l	50	49	2
Xylose	500 µmol/l	50	48	4
Albumin	5 g/l	50	51	−2
Casein	5 g/l	50	51	−2
Ascorbic acid (vitamin C)	500 µmol/l	50	47.5	5
Thiamine (vitamin B1)	500 µmol/l	50	48	4
Histidine	500 µmol/l	50	51	2
Lysine	500 µmol/l	50	51	−2
Arginine	500 µmol/l	50	49	2

The present novel method was validated by correlating determinations of peroxiredoxin activity with those of the FOX method [10] in matched samples. These analyses indicated high precision (Table 5) and a strong correlation (Table 6) between the methods.

Table 5. Precision of the current procedure (titanium sulfate). All measurements were done at optimum conditions of enzymatic reaction (temperature = 37 °C, time of reaction = 3 min, volume of reaction solution = 1200 µL, and concentration of substrates = 350 µmol/L).

	n	Mean (±SD) µmol of H2O2 utilized/min	CV %
Within-run	25	105 ± 3.5	2.93 %
Between-run	25	102± 5.5	5.4 %

Table 6. Statistical comparison between the peroxiredoxin activities that measured by FOX method and titanium sulfate method (U.L^{-1}).

No. of measures	25
Mean of peroxiredoxin, which measured with fox's Method	109
Mean of peroxiredoxin, which measured with the present method	106
Mean of peroxiredoxin, which measured with both methods	107.5
Regression coefficient B	0.9877
Regression coefficient A	0.0123
Correlation coefficient	0.9835

Peroxiredoxin activities of liver and kidney tissue homogenates from male albino mice were determined using the titanium sulfate method. Liver homogenates had expectedly high peroxiredoxin activities (Fig.3). In agreement, liver peroxiredoxin activities were previously used to evaluate oxidative stress [15, 16]. Moreover, Bae et al., were discovered that peroxiredoxin protects against alcohol-induced oxidative injury in mouse liver [17]. It is accepted that expression level of Prx was involved in inflammatory regulation by immune stimuli; also, it is protect TGF-β induced fibrosis by inhibiting stat 3 activation in rat kidney interstitial fibroblast cells [18]. In addition, Ahn SH, et al., were identified several proteins interacting with Prx V in mouse kidney under hypoxic condition to induce renal oxidative stress [19]. The present comparisons of the titanium sulfate and FOX methods (Fig.3) with tissue homogenates showed compatibility of the methods.

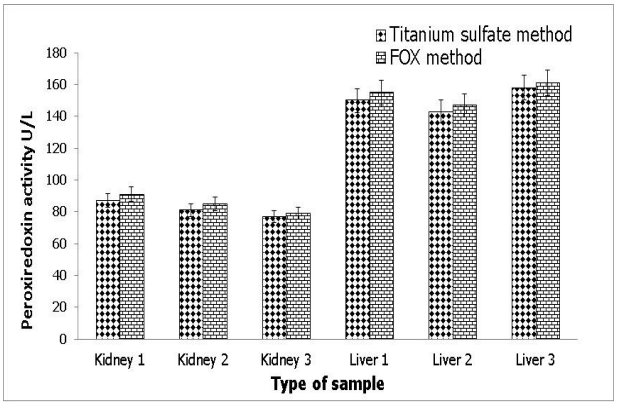

Fig.3. The peroxiredoxin activity of some tissue homogenates (1:500 diluted homogenate) calculated with the titanium sulfate method in comparison to that with the FOX method. Data are presented as (mean ± SD) (number of measurements =3).

The FOX method is accurate, precise and highly credible. The only aspect that the FOX method did not take into account is the presence of catalase enzyme in biological sample. To address these issues, we developed the present peroxiredoxin assay using sodium azide to exclude interference from catalase enzyme activity in samples containing PRX enzymes, and achieved highly specific determinations of PRX activities. The results summarized in Tables (5 & 6) show that the linearity of the FOX method reaches about 1100 U.L^{-1}. The limit of quantification (LOQ) and limit of detection (LOD) of the method based on FOX reagent for peroxiredoxin assessment were found to be 2 and 6 U mL^{-1}, respectively. On the other hand, the linearity of the titanium sulfate method reaches about 900 U.L^{-1}. The limit of quantification (LOQ) and limit of detection (LOD) of the method based on titanium sulfate reagent for peroxiredoxin assessment were found to be 4 and 12 U mL^{-1}, respectively.

To study the potential impacts of EDTA that might change the peroxiredoxin activity, five volumetric flask were prepared by mixing 2 ml of H_2O_2 with known concentration (3 mmol/L) and 3 ml of a series concentrations of EDTA dissolved in phosphate buffer (50 mM, pH 7.4). The final volume completed to 10 ml with titanium sulfate reagent. Changes in absorbance were measured at 405 nm against the reagent blank. Fig. 4 proofs that EDTA not interfered with titanium sulfate method.

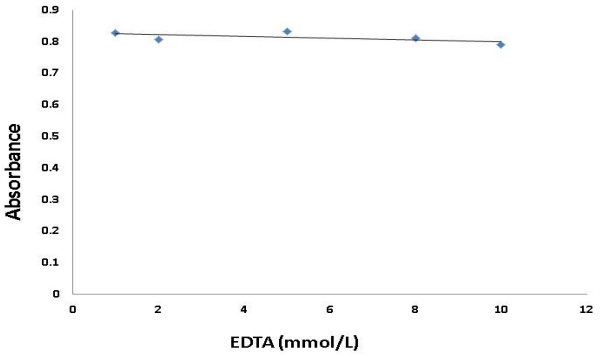

Fig 4. The correlation between EDTA concentration and absorbance of pertitanic acid.

The optimal hydrogen peroxide concentration was calculated using a practical experiment. Peroxiredoxin activity was calculated using several concentrations of hydrogen peroxide. The results have shown in fig. 5 demonstrate that the best concentration is 350 µmol/L. In previous study, Pascual et al., [20] were proved that susceptibility of Prx2 to hyperoxidation and concomitant inactivation is started at 500 µM H_2O_2.

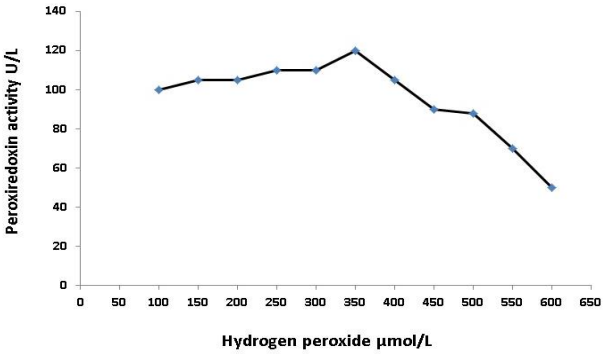

Fig 5. The correlation between hydrogen peroxide concentration and practical activity.

An ideal reaction time of 3 min was preferred in the present method because this corresponded to the time when the absorbance plateau was achieved, reflecting optimal hydrogen peroxide dissociation by the activity of the peroxiredoxin enzyme (as shown in fig 6).

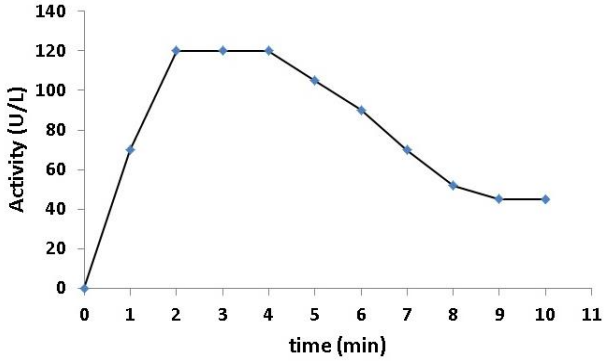

Fig. 6. The peroxiredoxin activity over a different time periods.

3.1. Optimization of peroxotitanium complex formation:

Recently, chemometric tools have been commonly utilized to the optimization of bio analytical procedures, reflecting their benefits such as a reduction in the number of experiments that require be performed resulting in lower chemical reagent consumption and significantly less laboratory work. Additionally these methods support the improvement of mathematical models that permit to assess of the relevance as well as statistical significance of the factor effects being considered. Finally, chemometric tools have been commonly utilized to estimate the interaction effects between the experimental factors [21]. To optimize the formation of the pertitanic acid in peroxiredoxin activity assays, we applied the response surface methodology (RSM) using a Box–Behnken design as an index of precision of the assay.

Box and collaborators have been developed response surface methodology [22]. This method was initiated from the graphical perspective created after fitness of the mathematical model, and its use has been commonly approved in texts on chemometrics. RSM involves a group of statistical and mathematical procedures that are utilized the fit of empirical models to the experimental data achieved in relation to experimental design. To complete RSM objective, square or linear polynomial functions are utilized to designate the system studied and, subsequently, to explore (displacing and modeling) experimental situations until its optimization [23].

Reaction mixtures were adjusted to volumes of 3.2 mL and were incubated for 5 min. Optimal enzyme reactions were achieved with 15 U/l peroxiredoxin, and independent variables included concentrations of DDT, H_2O_2, and titanium reagent (Table 7). Absorbance of the peroxotitanium complex was identified as a dependent variable and was related to peroxiredoxin assay sensitivity. Analyses of RSM were performed using Design Expert 11 software. Relationships between dependent and independent variables were defined using the following second order polynomial [Eq. (1)]:

$$Y = \beta_o + \sum \beta_i X_i + \sum \beta ii X_i^2 + \sum \beta_{ij} X_i X_j + \varepsilon,$$

Where β0, βi, βii, and βij are intercept, linear, quadratic, and interaction coefficients, respectively, and ε denotes the residual H_2O_2. X_i and X_j are evaluated parameters, i.e. DDT and H_2O_2 concentration respectively.

Table 7. Box-Behnken design was to optimize the peroxotitanium complex formation as index to the precision of the peroxiredoxin activity assay. The independent variables were included the concentrations of DDT, H_2O_2 and titanium reagent. The dependent variable was comprised the absorbance reading of the formed peroxotitanium complex.

Run [a]	DDT µmol/L	H_2O_2 µmol/L	Titanium reagent µmol/L	Absorbance of the peroxotitanium complex [b]
1	350	325	1500	0.19
2	350	375	1500	0.2
3	325	350	1500	0.194
4	325	375	2000	0.205
5	350	350	2000	0.195
6	350	350	2000	0.195
7	375	325	2000	0.19
8	350	350	2000	0.195
9	375	350	2500	0.198
10	350	375	2500	0.2
11	375	350	1500	0.18
12	325	350	2500	0.18
13	375	375	2000	0.2
14	350	325	2500	0.18
15	325	325	2	0.19

[a] The Box-Behnken design was used to generate this a random experiments.

[b] the absorbance reading of the formed peroxotitanium complex, associated with the peroxiredoxin assay sensitivity.

The Box–Behnken design statistical tool has been used previously used to achieve ideal conditions for analytical procedures [21, 24]. The present Box–Behnken analysis was performed with three central points to optimize DDT/H_2O_2/Titanium reagent concentrations in assessments of peroxiredoxin activity (Table 7), and the resulting quadratic model was significantly associated with peroxotitanium complex absorbance in ANOVA regression analyses (R^2 = 0.95; $P < 0.001$). In the absence of significant lack-of-fit ($P > 0.05$), peroxotitanium complex formation was precisely predicted using the quadratic model, and high H_2O_2 concentrations increased absorbance values of the peroxotitanium complex in association with the peroxiredoxin assay sensitivity and titanium reagent concentrations (Fig. 7A). Finally, the quadratic regression model indicated optimal concentrations of DDT, H_2O_2, and titanium reagent of 325 and 325 µmol L^{-1}, and 2.0 mM, respectively (Fig. 7B).

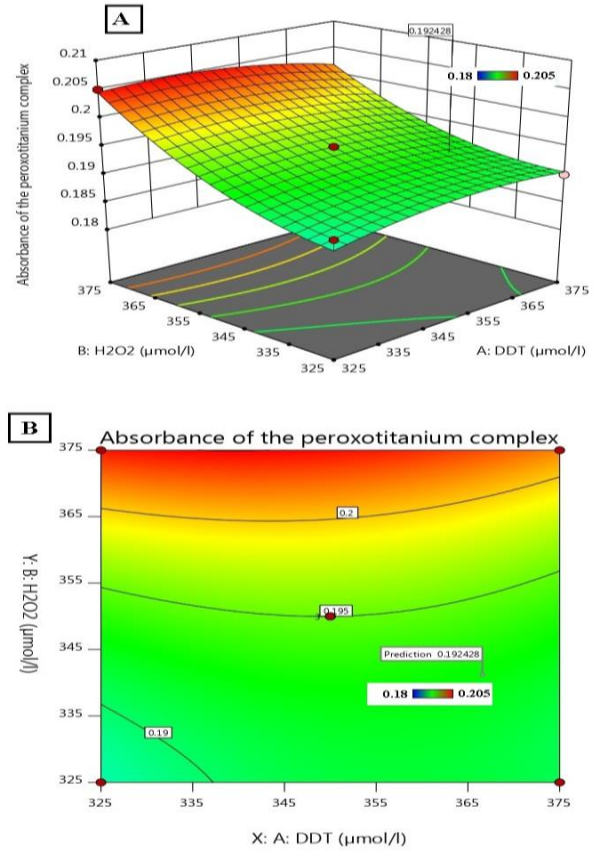

Figure 7: Surface (A) and contour (B) plots of optimized peroxotitanium complex formation for peroxiredoxin assays were generated using the response surface methodology (RSM). The contour plot shows optimal concentrations of H_2O_2, DDT, and titanium reagent, at a predicted peroxotitanium complex absorbance value of 0.192.

CONCLUSIONS:

Herein, we present a simple protocol for the measurement of peroxiredoxin activities that can be used in the presence of catalases. Moreover, we used titanium sulfate as a sensitive probe for hydrogen peroxide, and showed accurate determinations of peroxiredoxin activities at low peroxide concentrations that do not inactivate peroxiredoxins.

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Competing financial interests:

The author declares no competing financial interests

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