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An efficient protocol for quantifying catalase activity in biological samples

Mahmoud Hussein Hadwan[1*], Abdulsamie Hassan Alta'ee[1,2], Rawa M. Mohammed[3], Asad M. Hadwan[4,5], Hawraa Saad Al-Kawaz[6] and Zainab Abbas Al Talebi[1]

Abstract

Background Catalase is an important enzyme that helps protect cells against oxidative stress. The current protocol presents a reliable method for measuring catalase (CAT) enzyme activity in biological systems using the CUPRAC-CAT method.

Methods In the CUPRAC-CAT method, the component of the enzymatic reaction was incubated before adding the $Cu(Nc)_2^{2+}$ reagent. The unreacted substrates reduced the Cu(II)-the neocuproine complex, resulting in the highly colored Cu(I)-neocuproine product, which could be detected spectrophotometrically at 450 nm. The negative correlation between catalase activity and the absorbance of the Cu(I)-neocuproine complex was examined. To assess the optimization of Cu(I)-neocuproine complex production, response surface methodology (RSM) was employed, specifically utilizing the Box–Behnken design (BBD). Additionally, the reliability of the newly developed protocol was confirmed through Bland–Altman analysis of catalase activity in paired samples, employing the peroxovanadate method.

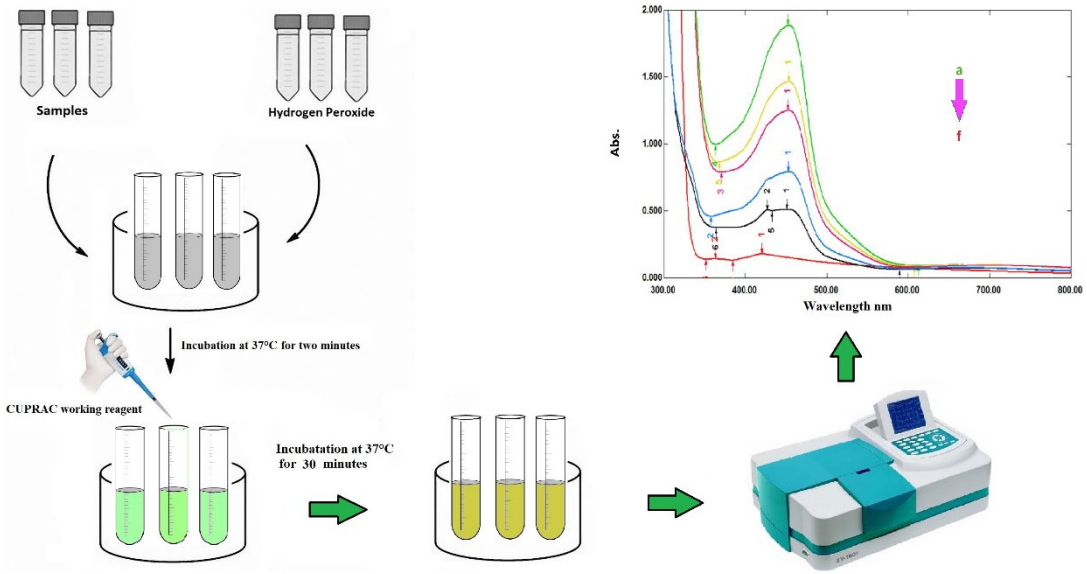
Results The novel method is just as accurate as the established standard; the correlation between the two methods was 0.99. The CUPRAC-CAT method is stable, sensitive, linear, reproducible, accurate, and selective and can be used for quantifying oxidative stress while measuring catalase activity in liver tissue homogenates.

Conclusions This study has demonstrated a straightforward and dependable protocol for evaluating catalase activity. The protocol is free from interference and can be easily employed in scientific research, ensuring a high level of accuracy and precision. The CUPRAC-CAT method is an effective technique to monitor bacterial contamination. This method provides quick and reliable results that can help ensure food safety and prevent or address bacterial contamination.

Keywords Catalase activity, Enzymology, Spectrophotometry, CUPRAC, Bland Altman

*Correspondence:
Mahmoud Hussein Hadwan
mahmoudhadwan@gmail.com
Full list of author information is available at the end of the article

Graphical abstract



Background

Catalase (EC1.11.1.6) is an enzyme in plants, animals, and aerobic microbes (Baker et al. 2023). It is a tetramer composed of a polypeptide chain with over 500 amino acids, containing four porphyrin heme groups. Catalase exhibits two distinct activities: a “peroxidase activity” reaction at low peroxide concentrations and a "catalase activity" reaction at high peroxide concentrations, similar to peroxidases. It has a remarkable turnover rate of over 10^7 s^{-1}, making it one of the fastest enzymatic reactions in biological systems (Baker et al. 2023; Hamza and Hadwan 2020).

Various methods have been used to assess CAT activity in cell lysates. One familiar method is spectrophotometry, which measures changes in H_2O_2 concentration by monitoring absorbance at 240 nm. However, this method has two limitations: high H_2O_2 concentrations can inhibit enzyme activity, and UV light can be absorbed by proteins and DNA, making it unsuitable for samples containing these materials (Mukhtar et al. 2022; Fu et al. 2020). The second type of assay involves measuring oxygen release using Clark-type electrodes (Szczepanczyk et al. 2023). These assays are commonly performed in single-cell analytical devices as kinetic assays. A simple kinetic microassay has been developed for measuring catalase activity, which allows for the simultaneous assessment of multiple samples in a short period. This makes it an excellent choice for applications requiring high-throughput screening and numerous parallel tests (Li and Schellhorn 2007).

In an additional protocol, the catalase activity is measured using a kinetic model that employs pyrocatechol (PC) and iso-nicotinic acid hydrazide (INH) to indicate unreacted hydrogen peroxide. The assay assumes that catalase oxidizes H_2O_2, generating quinone from the oxidation of PC, which combines with oxidized INH to produce a red chromogenic product with maximum absorbance at 490 nm (Shivakumar et al. 2011). Another method for assessing catalase activity in biological tissues has been developed. This method uses the catalytic properties of molybdenum to oxidize pyrogallol red (PGR) through unreacted H_2O_2. The reduction in PGR’s absorbance over time is directly proportional to the concentration of H_2O_2 and, therefore, to the catalase activity (Farman and Hadwan 2021).

A catalase activity method was described by Iwase et al. (2013). The enzyme produces oxygen bubbles captured by Triton X-100, forming foam. The height of the foam can be measured to determine the catalase activity. A calibration plot using defined catalase activity units yielded the best linear fit over 20–300 U. An additional method for determining catalase activity in erythrocyte lysates was introduced using a coupled-enzyme protocol that detects NADH absorbance at 340 nm. The method involves the peroxidic interaction of ethanol, hydrogen peroxide, and catalase, producing

NADH. Glucose oxidation generates hydrogen peroxide as an in situ substrate. Catalase utilizes hydrogen peroxide to convert ethanol to acetaldehyde, which is further oxidized to acetate by aldehyde dehydrogenase while generating NADH. The COBAS centrifugal analyzer is used to monitor the production rate of NADH at 340 nm. The reaction is linear until it reaches 800 U/L (Torrellas et al. 2020).

A method for assessing catalase activity involves mixing the enzyme and substrate in a reaction test tube attached to a manometer (Mukherjee et al. 2022). The volume of oxygen produced from the breakdown of hydrogen peroxide is measured while a motor-driven stirrer stirs the reagents, allowing for continuous monitoring of oxygen evolution. Another method involves a kinetic approach based on the peroxidation of ethanol through hydrogen peroxide. This produces acetaldehyde and water, followed by quick oxidation of acetaldehyde into acetic acid and NADPH by adding an excess of $NADP^+$ and aldehyde dehydrogenase. A COBAS centrifugal analyzer is used at 340 nm to monitor the rate of NADPH production. The reaction remains linear until 800 U/L (Yasmineh et al. 1992; Li et al. 2020).

Despite the availability of numerous protocols for estimating catalase activity, spectrophotometric methods remain the preferred option among chemists. Spectrophotometric protocols utilize reagents that create colored complexes which absorb visible light. Some examples include the carbonato cobaltate (III) complex (Hadwan 2018), the peroxovanadate complex nm (Hadwan and Kadhum 2018), and the pertitanic acid formation that absorbs light at 405 nm (Hadwan and Khabt 2018). A CUPRAC reagent utilizing an optical sensor was created by Bekdeşer et al. (2014). The reagent incorporates a copper(II)-neocuproine (Cu(II)-Nc) complex, which is fixed onto a Nafion cation-exchanger film. When the reagent interacts with hydrogen peroxide (H_2O_2), it produces a brightly colored Cu(I)-Nc chelate. The changes in absorbance at 450 nm are then measured to quantify the reaction.

This essay describes a simple method to measure CAT activity using the CUPRAC reagent. The samples were incubated in a phosphate buffer with hydrogen peroxide, and the CUPRAC reagent evaluated the unreacted substrate. The unreacted H_2O_2 creates a colorful Cu(I)-neocuproine product by reducing the Cu(II)-neocuproine complex. The maximum wavelength of this complex is 450 nm. A decrease in the absorbance is linked to catalase activity. This method is precise, efficient, reliable, interference-free, and easy to use in laboratory experiments. It is also suitable for clinical diagnosis.

Methods

Ammonium acetate ($NH_4CH_3CO_2$), copper(II) chloride ($CuCl_2.2H_2O$), calcium chloride ($CaCl_2$), hydrochloric acid (HCl), dipotassium phosphate (K_2HPO_4), hydrogen peroxide (H_2O_2, 30%), sodium dihydrogen phosphate dihydrate ($NaH_2PO_4.\ 2H_2O$), sodium hydroxide (NaOH), disodium hydrogen phosphate dihydrate ($Na_2HPO_4.2H_2O$), trichloroacetic acid (Cl_3CCOOH), and sodium nitrate ($NaNO_3$) were purchased from Thomas Baker (Chemicals) Pvt. Ltd.

Neocuproine (2,9-dimethyl-1,10-phenanthroline) and bovine serum albumin were obtained from Sigma-Aldrich.

Animals

Albino rats and mice were obtained from the animal house at the University of Babylon, Iraq. These animals were kept in well-ventilated cages with carefully regulated lighting and humidity. They were provided regular food and water per the WSAVA Animal Welfare Recommendations (Monteiro et al. 2023). Once the animals were killed, their liver tissues were surgically collected and washed with a 0.9% NaCl solution (w/v) to eliminate contaminants and blood. The liver tissues were then homogenized in 1.15% (w/v) cold KCl, and the resulting mixture was filtered. After filtration, the mixture was diluted with a 0.05 M phosphate buffer solution at a ratio of 1:500. This diluted sample was considered suitable for measuring CAT enzyme activity. Albino rats and mice are frequently used in catalase assessment studies due to their widespread availability, familiarity with researchers, ease of handling, and ethical considerations. Their use allows for consistent results, efficient experimental procedures, and adherence to ethical guidelines. However, it is worth noting that other animal species or cell cultures may also be used depending on research requirements and available resources.

Reagents preparation

To prepare a pH 7.4, 50 mM phosphate buffer, two solutions were made. Solution (a) involved dissolving 6.81 g of KH_2PO_4 in 1 L of distilled water (DW), while solution (b) involved dissolving 8.90 g of $Na_2HPO_4.2H_2O$ in 1 L of DW. The two solutions were mixed in a 1:1.5 ratio to prepare a freshly prepared phosphate buffer. A solution of hydrogen peroxide (H_2O_2) with a concentration of 5 mM is prepared daily in a 50 mM phosphate buffer solution with a pH of 7.4. The final concentration is achieved using a molar extinction coefficient of 43.6 M^{-1} cm^{-1} at a wavelength of 240 nm. A solution of Copper(II) chloride (10^{-2} M) was prepared by dissolving 0.4262 g of $CuCl_2{\cdot}2H_2O$ in 250 ml of distilled water. To create a pH 7.0, 1.816 M ammonium acetate buffer (NH_4Ac), 35 g of

NH_4Ac was dissolved in 250 mL of distilled water (D.W.). A solution of Neocuproine (Nc) was prepared by dissolving 208.26 mg of Nc in 100 ml of 96% ethanol.

A fresh reagent called CUPRAC, consisting of Cu(II), Nc, and NH4Ac in a 1:1:1 (v/v/v) ratio, was used for the experiment.

Instrument

The enzymatic assessment measurements were performed using the Shimadzu 1800 spectrophotometer.

Procedure

The detailed procedure is shown in Table 1.

After 30 min, the absorbance was measured at 450 nm and compared to the blank.

Calculation

To calculate catalase activity, the following equation is utilized:

$$\text{Catalase Actiity of test } kU = \frac{2.303}{t} \times \left[\log \frac{S^{o}}{S-M}\right] \times \frac{V_t}{V_s}$$

t: time, S^{o}: absorbance of the standard tube, S: absorbance of the test tube, M: absorbance of the control, V_t: total volume of the test tube, V_s: sample volume.

Optimization of the current method

Response surface methodology (RSM) was utilized with Box–Behnken Design (BBD) to optimize the CUPRAC-CAT method. The design expert software 13 (Otieno et al. 2023) was applied to estimate the statistical parameters and design the CUPRAC-CAT method for optimal results. Preparing the CAT standard solution involved dissolving 5 mg of CAT powder (HI Media chemicals; India) in 100 mL phosphate buffer (50 mM, pH 7.0) to prepare a catalase solution (300 U L^{-1}). The final activity was adjusted with the peroxovanadate method (Hadwan and Kadhum 2018). The independent variables were the neocuproine and hydrogen peroxide concentration and the incubation time (Table 2). On the other hand, the catalase activity of the present spectrophotometric method served as the dependent variable.

The enzyme activity was assessed by determining the rate constant (k) of a first-order reaction mathematical equation. In order to establish the relationship between the dependent and independent variables through mathematical modeling, a second-order polynomial equation (Eq. (1)) was employed:

$$Y = \beta 0 + \sum \beta iXi + \sum \beta iiXi^2 + \sum \beta ijXiXj + \varepsilon \quad (1)$$

where Y represents the response variable, X_i denotes the independent variables, and β_0, β_i, β_{ii}, and β_{ij} represent the intercept, linear, quadratic, and interaction coefficients, respectively. The term ε accounts for random error.

Signal stability

The study investigated the stability of the Cu(I)-neocuproine complex's orange color at 450 nm over time intervals ranging from 15 min to one week.

Table 1 The procedure of the CUPRAC-CAT method

Reagents	Test	Control	STD	Blank
Sample	250 µL	250 µL	–	–
Distilled water	–	2000 µL	250 µL	2250 µL
H_2O_2	2000 µL	–	2000 µL	–
The solution was vortexed and incubated at 37 °C for 2 min. Then, the reaction was terminated with 250 µl of 10% TCA. The final solution was mixed well and centrifuged for 15 min at 3000 xg. A 50 µL of supernatant was removed in a clean tube. Finally, the working solution was added				
Working reagent	3 mL	3 mL	3 mL	3 mL

Table 2 The results of the Box–Behnken design application. The Box–Behnken design was utilized to investigate the effects of independent factors on catalase activity, which included neocuproine concentration, hydrogen peroxide concentration, and incubation time. The dependent variable, catalase activity, was assessed with the CUPRAC-CAT method. The results were obtained by averaging three replicates

Run	Hydrogen peroxide concentration (mM)	Neocuproine concentration (mM)	Incubation time (min)	Catalase activity Katal/L
1	5.5	10	3.5	302
2	8	15	1	260
3	5.5	18.4	3.5	301
4	8	15	6	281
5	3	15	1	189
6	5.5	10	3.5	300
7	5.5	10	3.5	305
8	8	5	6	270
9	3	5	6	238
10	1.3	10	3.5	292
11	3	5	1	240
12	5.5	1.6	3.5	270
13	5.5	10	7.7	245
14	5.5	10	3.5	288
15	8	5	1	270
16	9.7	10	3.5	318
17	3	15	6	282

Linearity and sensitivity

The current assay was evaluated using a range of catalase activities from 0.01 to 8.0 kat. mL^{-1}. The sensitivity and linearity of the CUPRAC-CAT method were measured by comparing it with the peroxovanadate method (Hadwan and Kadhum 2018) using an online program for comparison of analytical methods and assessment of bias (Bahar et al. 2017). The sensitivity of the CUPRAC-CAT method was determined by calculating the limits of detection (LOD) and quantitation (LOQ) (Alanazi et al. 2024).

Matrix effect and interference of antioxidants

The matrix effect refers to the effect of sample components other than the analyte on an analytical assay. For instance, the presence of glutathione, uric acid, and other antioxidants in biological samples can potentially interfere with the assay. However, such interference can be mitigated by using a control test tube. Fortunately, eliminating matrix effect interference on catalase activity is a relatively simple process. A control test tube was incorporated into the assay design to counteract any interference caused by antioxidant biomolecules present in the catalase-containing sample. The absorbance of the sample is a combination of unreacted substrates and sample interferences. However, the absorbance of the control only reflects the presence of interference compounds. Any substance that could affect the precision was eliminated to ensure the accuracy of the modified method. This was achieved by subtracting the absorbance of the control from that of the sample. This subtraction ensured that the remaining absorbance solely represented the unreacted substrates, guaranteeing a measurement free from any interfering factors.

Accuracy, selectivity, and reproducibility

Analyzing catalase activity samples aimed to investigate potential biochemical interferences in CAT activity measurements. To achieve this, four biomolecule cocktails were prepared and used to evaluate potential sources of interference in CAT activity. Each cocktail consisted of a known level of CAT activity (330 U/mL) and 0.1 mL of the prepared biomolecules dissolved in a phosphate buffer (25 mM; pH 7.2). The final CAT activity was standardized to 300 U/l using the peroxovanadate method (Hadwan and Kadhum 2018). The composition of the experimental sample tubes is presented in Table 3.

We tested the current method's reliability by performing intra- and inter-day reproducibility tests on various biological samples. We used the relative standard deviation (RSD) to demonstrate the results. To remove any blood or contaminants, the rat livers were washed with a 0.9% (w/v) sodium chloride solution after surgical extraction from the male albino rats and mice. To remove any blood or contaminants, the rats' livers were washed with a sodium chloride solution (0.9%, w/v) before surgically removing them. Subsequently, the liver tissues were homogenized in a potassium chloride solution (1.15%, w/v) and kept at 10°C, and the resulting sample was subjected to filtration. To prepare a suitable source for catalase (CAT) activity, the filtered sample was diluted at 1:500 with a phosphate buffer solution (0.05 M, pH 7.4).

Table 3 The sensitivity and precision of the CUPRAC-CAT method have been demonstrated by examining possibly interfering biomolecules

n	Added CAT activity Katal/L	Added biomolecules
1	297	–
2	297	4 mM of glucose, ribose, lactose, and fructose
3	297	1 mM of serine, methionine, valine aspartic acid, cysteine, and asparagine
4	297	1% BSA

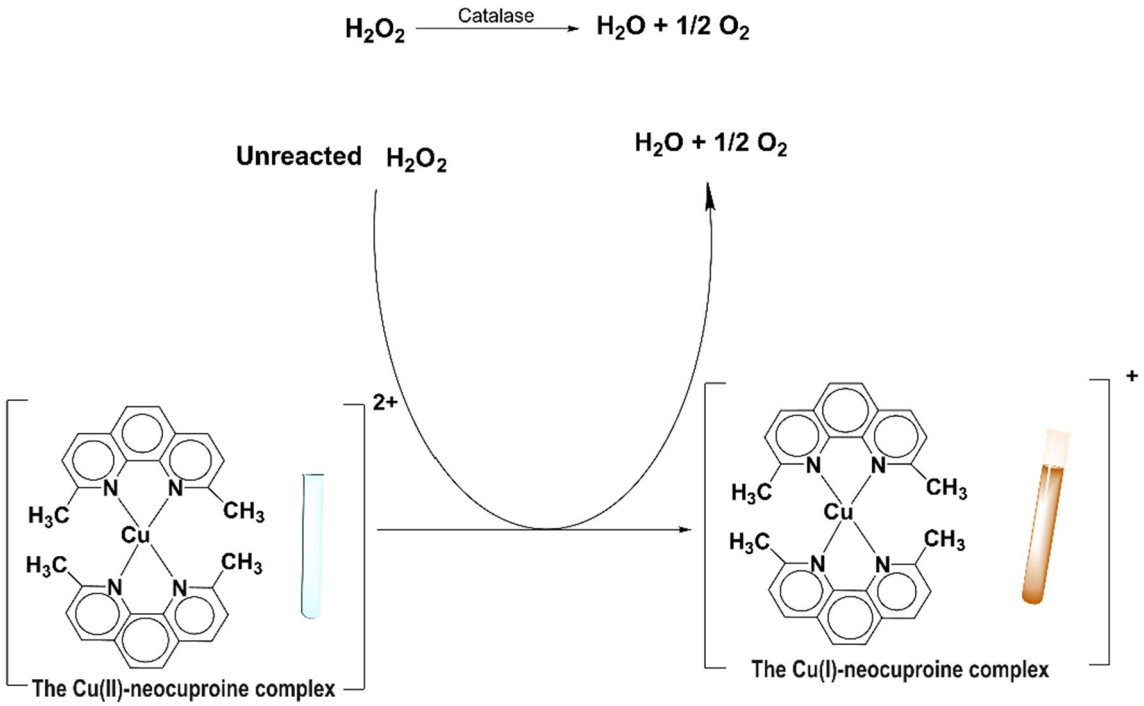
Validation

The efficiency of the CUPRAC-CAT method was verified by comparing it to the peroxovanadate method (Hadwan and Kadhum 2018). This was done through Bland–Altman analysis and Passing-Bablok regression (Sudová et al. 2024). The statistical analysis was performed through an online program designed for estimating bias and comparing analytical methods (Bahar et al. 2017).

Results

The CUPRAC-CAT assay for measuring CAT activity is a unique and innovative method

This study presents a novel protocol called the CUPRAC-CAT method for measuring catalase (CAT) activity. The method utilizes the cupric neocuproine complex ($Cu(Nc)_2^{2+}$) as an oxidizing probe with chromogenic properties (Ayaz et al. 2022) for assessing antioxidant status in biological samples, the CUPRAC method has been applied to evaluate oxidant and antioxidant enzymes in various studies (Azeez and Hadwan, 2023; Ahmed et al. 2021; Obeid and Hadwan 2021). In the context of CAT activity measurement, the enzyme samples are combined with specific concentrations of hydrogen peroxide, followed by adding the CUPRAC reagent ($Cu(Nc)_2^{2+}$) to estimate the remaining unreacted hydrogen peroxide. Scheme 1 illustrates the reduction in the Cu(II)-neocuproine complex to the Cu(I)-neocuproine complex, which is measured via spectrophotometry at a



Scheme 1 The general reaction of the CUPRAC-CAT method involves using hydrogen peroxide as a substrate for CAT. The enzymatic reaction of catalase produces oxygen (O_2), and water (H_2O). In the presence of the Cu(II)-neocuproine complex, the reaction with the unreacted hydrogen peroxide (H_2O_2) results in the formation of a stable yellow-orange Cu(I)-neocuproine complex. The Cu(II)-neocuproine complex has a light blue color. The Cu(I)-neocuproine complex has a yellow color

wavelength of 450 nm (CUPRAC method). The decrease in absorbance of the Cu(I)-neocuproine product indicates CAT enzyme activity. This complex is formed due to the CAT reaction and exhibits a single peak at 450 nm. The absorbance observed corresponds to the undissociated H_2O_2, as depicted in Fig. 1.

Optimization of the CUPRAC-CAT assay

Box–Behnken Design (BBD) was applied as a function of response surface methodology (RSM) to achieve optimal conditions of the CUPRAC-CAT method. Following reference (Haque et al. 2023), BBD is a highly efficient testing instrument that requires optimizing three key factors—peroxide concentration, neocuproine concentration, and incubation time—to achieve the ideal catalase activity. The CUPRAC-CAT method's regression model is located in Table 4. It was established by analyzing the variance (ANOVA) of the RSM. Based on the resulting F-value of 5.7, it can be concluded that the model is significant. However, the lack-of-fitness F-value of 8.5 indicates that the model is significant compared to its corresponding p value. The ANOVA analysis of the CUPRAC-CAT method also revealed that the suggested model's three independent variables had a significant and fitting relationship. To analyze the interactions between the variables, contour graphs and three-dimensional (3D) graphs were utilized in the BBD. The response plot in Fig. 2a–f illustrates the interactions between the factors—peroxide concentration, neocuproine concentration, and incubation time. These figures demonstrate significant curvature and suggest a good fit for the model.

After conducting experiments, we discovered that the best concentrations for maximizing catalase activity were 5.5 mmol L^{-1} for H_2O_2 and 10 mmol L^{-1} for neocuproine, with an incubation time of 3.5 min. Our results showed that the activity level peaked at 300 ± 2 U/L, exactly as predicted. This demonstrates that our RSM analysis was a trustworthy predictor of real laboratory conditions.

Based on the Model F-value of 5.70, it can be concluded that the model is significant. The chance of an F-value this large resulting from noise is only 1.59%.

Signal stability

The present study observed that the orange chelate complex retains high stability at room temperature. Based on our measurements, the CUPRAC complex displayed a notably stable absorbance at 450 nm for over 48 h at a temperature of 25 °C.

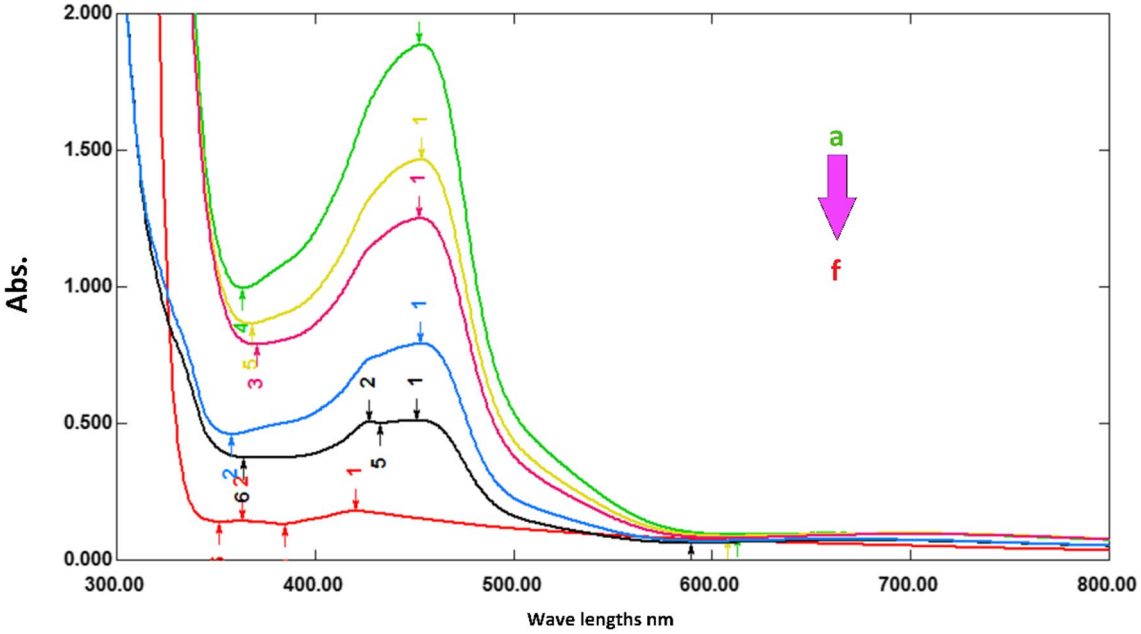


Fig. 1 The CAT activity enzyme showed an inverse correlation with the intensity of the Cu(I)-neocuproine complex. A solution with an appropriate concentration of H_2O_2 was used to reduce $(Cu(Nc)_2^{2+})$ and produce a colored Cu(I)-neocuproine complex $(Cu(Nc)_2^{+})$. The resulting complex was analyzed, and absorption spectra (**a**–**f**) were obtained for various H_2O_2 concentrations. The spectra (**a**–**f**) are a 5 mM, b 4 mM, c 3 mM, d 1 mM, e 0.5 mM, and f 0.1 mM

Table 4 The ANOVA values for the experimental variables used in the CUPRAC-CAT assay

Source	Sum of squares	*df*	Mean square	*F*-value	*p*-value	
Model	14499.22	9	1611.02	5.70	0.0159	Significant
A-Hydrogen peroxide concentration (mM)	2261.92	1	2261.92	8.00	0.0255	
B-Neocuproine Concentration (mM)	155.62	1	155.62	0.5505	0.4823	
C-Incubation time (min)	2485.42	1	2485.42	8.79	0.0210	
Residual	1978.90	7	282.70			
Lack of fit	1812.15	4	453.04	8.15	0.0581	Not significant
Pure error	166.75	3	55.58			
Cor total	16,478.12	16				

Sensitivity and linearity

Based on the data in Fig. 3, the CUPRAC-CAT assay was linear within 0.05–7.5 U/mL of CAT enzyme activity, with a Pearson's r value of 0.999. The assay exhibited great sensitivity, with low LOD (0.022 U/mL) and LOQ (0.09 U/mL) values. These results demonstrate that the CUPRAC-CAT method is highly sensitive. In terms of linearity, the method performed similarly to the peroxovanadate method.

Reproducibility, accuracy, and selectivity of the CUPRAC-CAT method

Bekdeşer et al. (2014) used a CUPRAC sensor composed of Cu(II)-neocuproine (Cu(II)-Nc) complex immobilized on a Nafion film to measure catalase activity. The sensor reacts with unreacted H_2O_2 and produces a colored Cu(I)-Nc chelate, which is then measured at 450 nm. The present protocol has numerous advantages over the optical sensor described by Bekdeşer et al. Firstly, the current method requires an incubation time of only 2 min, while the Bekdeşer method requires 30 min. Secondly, the CUPRAC sensor measured the unreacted hydrogen peroxide in a continuous enzymatic reaction. However, there is competition between the CUPRAC sensor and catalase to react with hydrogen peroxide. The catalase enzyme has the largest turnover number of enzymes, with reported values up to 4×10^7 s^{-1}. In contrast, the

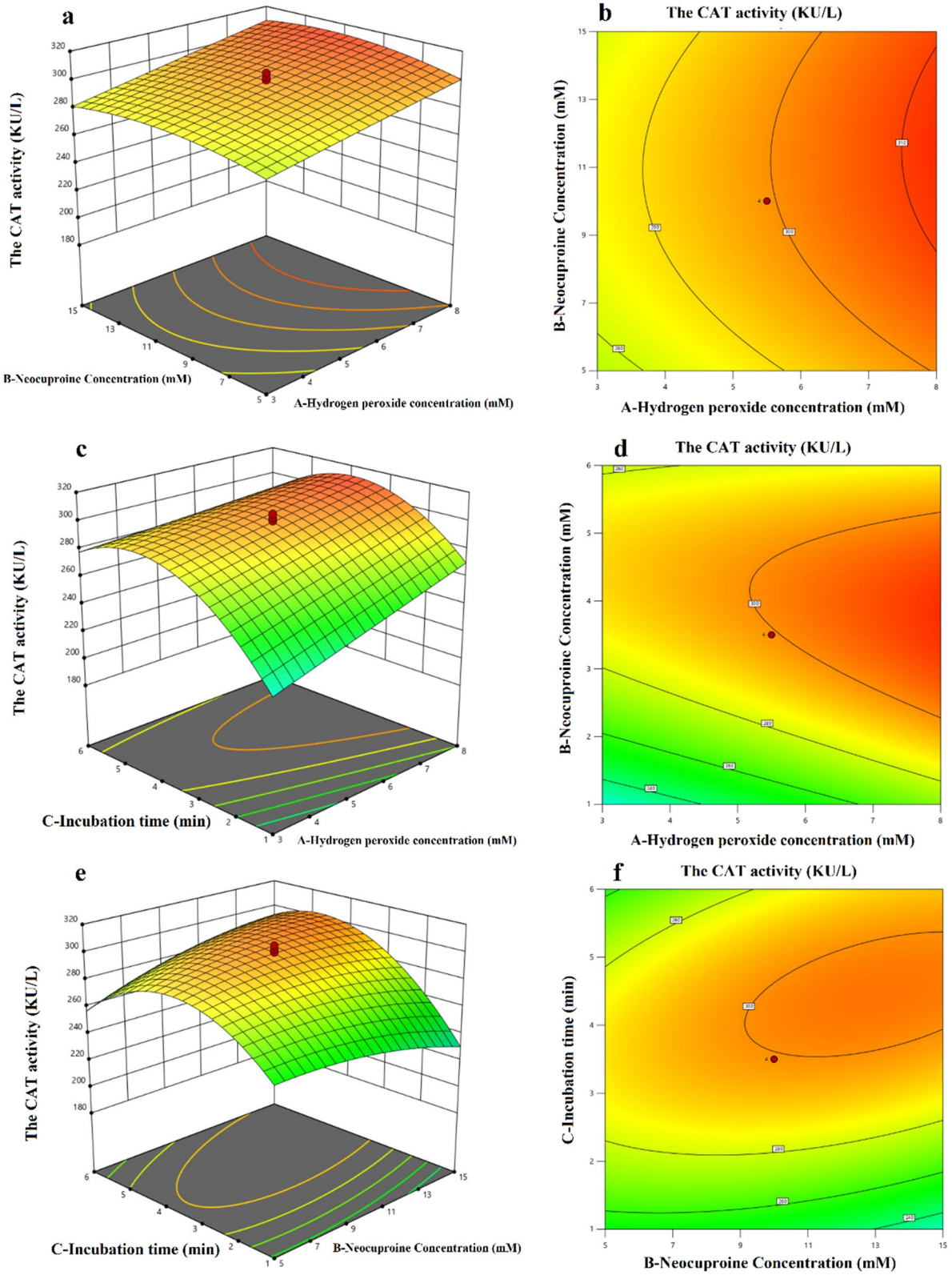


Fig. 2 The graphs display contour and 3D surface plots, demonstrating the relationship between peroxide concentration, neocuproine concentration, and incubation time. The value for each measurement was calculated as the average of three repeated measurements

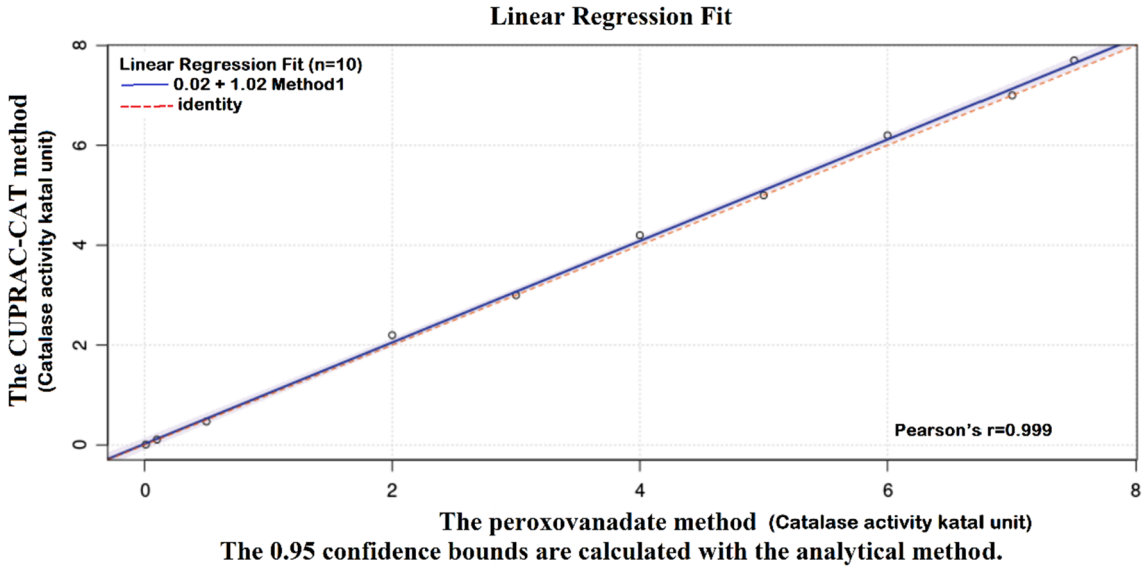


Fig. 3 A comparison between the CUPRAC-CAT and peroxovanadate methods. A series of CAT concentrations were studied to determine the linearity of the method. This was achieved by drawing a straight line between the CUPRAC-CAT and peroxovanadate methods

CUPRAC sensor requires 30 min to react efficiently with hydrogen peroxide. To solve this problem, the current protocol utilized trichloroacetic acid to stop the catalase enzymatic reaction. This makes the newly developed method more efficient and faster. We conducted tests to evaluate the potential interferences of different biomolecules such as amino acids, proteins, and sugars, in the CUPRAC-CAT method for measuring catalase (CAT) activity. Table 5 presents the assay-relative error for each mixture of potential interfering biomolecules, indicating that the proposed procedure can accurately determine CAT activity even in the presence of various types of interfering chemicals.

The current method employs a two-pronged approach to eliminate interferences. Firstly, it involves diluting CAT enzyme-containing biological samples before starting the protocol. It is crucial to perform the dilution step when using the CUPRAC method as it is extremely sensitive. Secondly, the procedure addresses potential interference from biomolecules such as amino acids, proteins, sugars, and vitamins by utilizing a correction factor through a control test. During the procedure, the absorbance of a sample comprises the combination of unreacted hydrogen peroxide and interference from biomolecules present in the CAT-containing sample. However, the absorbance observed in the control is solely attributed to the presence of interfering chemicals in the sample. By subtracting the absorbance of the control from that of the sample, we successfully eliminated any potential chemical interferences that could impact the absorbance of the CUPRAC end product. Consequently, the remaining absorbance solely originates from the unreacted hydrogen peroxide.

In enzymology, employing a control to address interferences is common when the substrate or product of an enzymatic reaction possesses functional groups that are shared with interfering biomolecules. One notable example is using a control in the assessment protocol

Table 5 The correlation between biological molecules that cause interference and the relative error percentage in measuring CAT activity using the CUPRAC-CAT protocol

Test tube no	Added biomolecules	Added catalase activity (katal/L)	Found catalase activity (katal/L)	Relative error (%)
1	–	297	297	–
2	4 mM of glucose, ribose, lactose, and fructose	297	292	1.684
3	1 mM of serine, methionine, valine aspartic acid, cysteine, and asparagine	297	305	2.694
4	1% BSA	297	304	2.357

of 5'-nucleotidase activity to eliminate the interference caused by alkaline phosphatase (Das Neves et al. 2023; Qian et al. 2023).

Application

We analyzed catalase activity in liver tissue homogenates from rats and mice using the CUPRAC-CAT assay. The results aligned with expectations and are presented in Table 6. Moreover, the intra-day and inter-day RSD% values of the CUPRAC-CAT assay were acceptable, ranging from 3.49 to 3.86% and 3.8 to 4.4%, respectively (Table 5).

Assessing the catalase activity can effectively measure the liver's ability to alleviate oxidative stress. Numerous systematic studies have evaluated the oxidant/antioxidant balance by analyzing catalase activity in laboratory animal livers (Hadwan and Kadhum 2018; Bekdeşer et al. 2014).

Additional application

Contamination of vegetables by aerobic bacteria poses serious threats to food safety and human health. This study explores using a sensitive catalase test as a viable and efficient protocol to monitor aerobic bacterial contamination in vegetables. The catalase test is a quick and cost-effective means of assessing the microbiological quality of vegetables, ensuring the safety of consumers, and enabling appropriate interventions by measuring the catalase activity of bacterial isolates from vegetable samples (Adelere and Lateef 2023). The manuscript discusses the rapid catalase test's importance, usefulness, and potential impact on food safety practices.

To explore the potential applications of the CUPRAC-CAT method, we investigated the catalase (CAT) activity in lysates obtained from five different bacterial laboratory strains. Our study aimed to quantify CAT enzyme activity in bacteria, and interestingly, the results showed that the CUPRAC-CAT method yielded results comparable to those of the peroxovanadate method. The summarized findings in Table 7 demonstrate that Staphylococcus aureus exhibited higher catalase enzyme activity among the tested bacterial species than the other strains.

The fast CAT test provides results within minutes, allowing for swift decision-making regarding the safety and quality of vegetable products. This rapid turnaround time enables timely interventions and minimizes the risk of contaminated vegetables reaching consumers. Catalase activity is positively correlated with the concentration of aerobic bacteria, allowing for accurate prediction of contamination levels. However, the assay's sensitivity can be influenced by the specific microbial culture involved and diminished by non-microbial catalase in food samples.

To overcome the potential interference from vegetable-derived catalase, it is important to incorporate a control vegetable sample in the assay protocol. By comparing the CAT activity levels in the control samples (representing vegetable-CAT only) with the experimental samples (representing vegetable plus bacterial CAT), it becomes possible to differentiate between catalase activity contributed by vegetables and that originating from bacterial contamination. A significant increase in CAT activity in the contaminated samples compared to the control samples suggests the presence of additional CAT activity stemming from bacterial contamination. This difference in activity indicates that the presence of bacteria impacts the overall measured catalase activity.

This comparative analysis is important because it can distinguish between the CAT activity of vegetables and bacterial contamination. Any significant variations in CAT activity levels within the experimental samples can be identified by establishing a baseline using control samples. Such variations can provide evidence of bacterial catalase activity.

Table 7 Comparison of CAT Activity in different bacterial strains using the peroxovanadate and CUPRAC methods

Name of bacteria	Peroxovanadate method (Katal Unit)	CUPRAC method (Katal Unit)
Staphylococcus aureus	14.7 ± 0.27	15.2 ± 0.31
pseudomonas aeruginosa	10.9 ± 0.12	10.4 ± 0.15
Escherichia coli	8.7 ± 0.09	8.2 ± 0.12
Klebsiella pneumonia	10.7 ± 0.15	10.8 ± 0.18
Enterococcus faecalis	0.00	0.00

Table 6 Comparative analysis of catalase activity in male albino mice and rats using the peroxovanadate and CUPRAC-CAT methods: assessment of intra-day and inter-day examination

Samples	The catalase activity, expressed in Katal per milliliter (Katal. mL^{-1}), is measured for homogeneous tissue							
	The peroxovanadate method				The CUPRAC-CAT method			
	Intra-day ± SD	RSD%	Inter-day ± SD	SD%	Intra-day ± SD	RSD%	Inter-day ± SD	SD%
Male Albino Mice	4.51 ± 0.18	3.99	4.85 ± 0.17	3.5	4.62 ± 0.17	3.67	4.7 ± 0.18	3.82
Male Albino Rats	5.85 ± 0.21	3.61	5.95 ± 0.12	2.01	5.77 ± 0.15	2.59	5.82 ± 0.17	2.92

The test is economical, requiring minimal resources and equipment. It can be easily integrated into routine quality control practices, making it accessible to small-scale and large-scale vegetable producers. Also, it allows for repeated testing of the same sample, facilitating thorough monitoring and evaluation of bacterial contamination. Implementing the quick catalase test as a routine monitoring tool can greatly enhance food safety practices in the vegetable industry. It enables early detection of bacterial contamination, facilitating prompt interventions such as enhanced cleaning and sanitation measures, targeted control strategies, and removing affected batches from the supply chain. The test also provides valuable data for quality control assessments, enabling producers to identify potential contamination sources and implement preventive measures to mitigate future risks.

Validation

The effectiveness of the current method was examined and confirmed through a Bland–Altman analysis. As assessed by the current assay, CAT activity was compared to the activity determined by the pervanadate method (Hadwan and Kadhum 2018) using identical CAT samples. The Bland–Altman plot, depicted in Fig. 4, illustrates the mean relative bias and other variations between the CUPRAC-CAT and peroxovanadate methods. The Pearson r value of > 0.98 between the CUPRAC-CAT method and the peroxovanadate findings for various samples indicates a significant relationship between the two methods.

Discussion

This essay introduces the CUPRAC-CAT method, a novel and efficient approach for quantifying catalase (CAT) enzyme activity in biological samples. This method is particularly valuable for assessing oxidative stress and monitoring bacterial contamination in various biological samples, including liver tissue homogenates, bacterial isolates, and vegetable samples. The CUPRAC-CAT method utilizes the cupric neocuproine complex ($Cu(Nc)_2^{2+}$) as a chromogenic oxidizing probe to measure CAT activity. Extensive validation and comparison with established standard methods have demonstrated the stability, sensitivity, linearity, reproducibility, accuracy, and selectivity of the CUPRAC-CAT method. Furthermore, the essay discusses the potential applications of the CUPRAC-CAT method in food safety practices, emphasizing its significance in ensuring consumer safety and quality control in the vegetable industry.

The essay provides a comprehensive overview of the CUPRAC-CAT method, covering various aspects such as its application, validation, optimization, and comparison with existing methods. This discussion highlights the significance and implications of the CUPRAC-CAT method in enzymology, food safety, and quality control. The CUPRAC-CAT method was optimized using response surface methodology (RSM) and the Box–Behnken Design (BBD) approach. This optimization aimed to determine the optimal levels of peroxide, neocuproine, and incubation time for measuring CAT activity. The results indicated that the working solution of the CUPRAC-CAT method is highly efficient in measuring

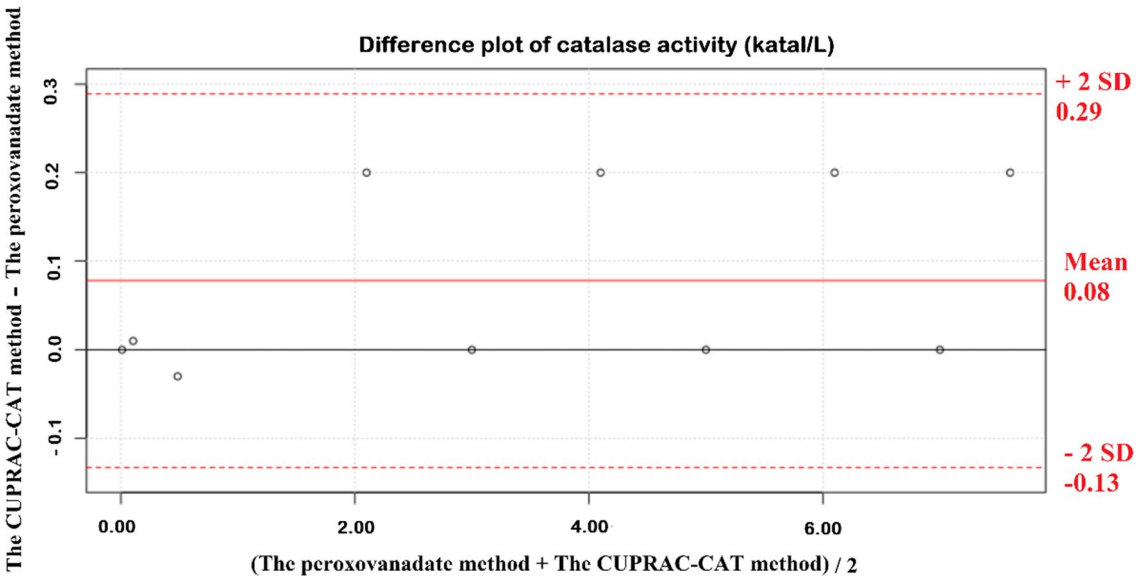


Fig. 4 Comparative analysis of catalase activity measured by CUPRAC-CAT and peroxovanadate methods Using Bland–Altman plot: assessment of average relative bias

CAT activity at low substrate concentrations due to its remarkable sensitivity to hydrogen peroxide. The regression model for the CUPRAC-CAT method, presented in Table 4, was developed by conducting a variance analysis (ANOVA) of the response surface methodology (RSM). The obtained *F*-value of 5.7 indicates the significance of the model. However, the lack-of-fitness *F*-value of 8.5 suggests that the model's significance should be evaluated concerning its corresponding *p*-value. Additionally, the ANOVA analysis demonstrated that the three independent variables in the proposed model exhibited a significant and appropriate relationship. Based on experimental results, the optimal concentrations for maximizing catalase activity were 5.5 mmol L^{-1} for H_2O_2 and 10 mmol L^{-1} for neocuproine, with an incubation time of 3.5 min. The activity level peaked at 300 ± 2 U/L, exactly as predicted. This demonstrates that the RSM analysis reliably predicted real laboratory conditions.

The results demonstrated that the CAT activity measured using the CUPRAC-CAT method aligned with expectations, and the method exhibited acceptable intra-day and inter-day relative standard deviation (RSD%) values, indicating its reliability and reproducibility. Furthermore, the manuscript compared CAT activity using the CUPRAC-CAT method to values obtained from the peroxovanadate method, further validating the accuracy and reliability of the novel protocol. The correlation between the two methods was highly significant, with a Pearson *r* value of > 0.98, affirming the robustness of the CUPRAC-CAT method in measuring CAT activity in diverse biological samples.

The essay also highlights the potential utility of the CUPRAC-CAT method in monitoring bacterial contamination in vegetable samples. The quick catalase test provided by the CUPRAC-CAT method offers prompt and reliable results, supporting food safety measures and enabling swift actions to mitigate contamination risks. The method's rapid turnaround time, cost-effectiveness, and minimal resource requirements make it an ideal tool for routine quality control practices in the vegetable industry. Implementing the CUPRAC-CAT method as a routine monitoring tool can greatly enhance food safety practices, enabling early detection of bacterial contamination, facilitating prompt interventions, and providing valuable data for quality control assessments.

Furthermore, the essay thoroughly compares the CUPRAC-CAT method with established standard methods, such as the peroxovanadate method. The Bland–Altman analysis and correlation studies demonstrated the equivalence of the CUPRAC-CAT method to the peroxovanadate method in measuring CAT activity. The manuscript also presents a Bland–Altman plot comparing catalase activity measured with the CUPRAC-CAT and peroxovanadate methodologies, further validating the efficacy and reliability of the CUPRAC-CAT method. The high correlation between the two methods indicates the potential for the CUPRAC-CAT method to replace or complement existing standard methods for measuring CAT activity in biological samples.

Advantages

The CUPRAC-CAT method demonstrates a notable level of sensitivity, enabling the detection of even low levels of CAT activity. This characteristic renders it well-suited for assessing CAT activity in a wide range of sample types, such as biological fluids, cells, and tissues. The assay specifically measures CAT activity and remains unaffected by the presence of other antioxidant enzymes or molecules within the sample. This level of specificity ensures an accurate evaluation of CAT activity without interference from other components. The versatility of the CUPRAC-CAT method allows for its application to diverse sample types, including plant extracts, food samples, and biological samples. Moreover, the simplicity of the assay and its compatibility with standard laboratory equipment make it easily executable without the need for specialized instrumentation, thereby making it accessible to researchers across various settings.

Disadvantages

The CUPRAC-CAT method requires specific reaction conditions, such as pH, temperature, and time, to ensure precise and reliable results. Any deviations from the optimal conditions can affect the accuracy and reproducibility of the assay. Additionally, the CUPRAC-CAT method may not be suitable for measuring extremely high or low CAT activities due to its limited dynamic range. Researchers must ensure that the catalase activity level falls within the assay's linear range to obtain accurate quantification results.

Conclusions

The essay describes a new method to quantify catalase activity in biological samples. The method is discussed regarding its application, validation, optimization, and implications in food safety and quality control. The essay highlights the importance of the CUPRAC-CAT method in enzymology and its potential impact on food safety practices. Due to its robustness, reliability, and efficiency, the CUPRAC-CAT method is a valuable tool for researchers, food industry professionals, and quality control practitioners. It can be used to assess oxidative stress and monitor bacterial contamination in various biological samples.

Abbreviations

ANOVA	Analysis of the variance
BBD	Box–Behnken design
CAT	Catalase
INH	Iso-nicotinic acid hydrazide
LOD	Limits of detection
LOQ	Limits of quantitation
RSM	Response surface methodology

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Author contributions
All authors contributed to the study's conception and design. MHH, AMH, and ZAA prepared material, collected data, and analyzed it. MHH wrote the first draft of the manuscript. AHA, HSA, RMM, and AMH corrected and approved the final manuscript. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
The authors declare that all data supporting the findings of this study can be found within the paper. Additional data supporting the findings of this study are available from the corresponding author upon request.

Declarations

Ethics approval and consent to participate
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Consent for publication
The authors declare that they agreed with the content that all gave explicit consent to submit. They obtained consent from the responsible authorities at the institute where the work was carried out before submission.

Competing interests
The authors declare that they have no competing interests.

Declaration of generative AI and AI-assisted technologies in the writing process
While preparing this work, the authors used [Chat Gpt 3.5] to [correct the lignoceric errors]. After using this tool, the authors reviewed and edited the content as needed and took full responsibility for the publication's content.

Author details
[1]Chemistry Department, College of Science, University of Babylon, Hilla City, Babylon Governorate 51002, Iraq. [2]Chemistry Department, College of Medicine, University of Babylon, Hilla City, Babylon Governorate 51002, Iraq. [3]Department of Medical Physics, University of Al-Mustaqbal, Hilla City, Babylon Governorate 51001, Iraq. [4]Faculty of Natural Sciences, University of Tabriz, Tabriz, Iran. [5]Al-Manara College for Medical Sciences, Al-Amarah City, Iraq. [6]Department of Pathological Analysis, College of Science, Al-Qasim Green University, Al-Qasim City 51013, Iraq.

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