Research Square

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Resolving the issue of non-specific substrate oxidation in nanozyme-assisted colorimetric assays

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Abstract

Nanoparticles exhibiting peroxidase-like activity (nanozymes) are emerging as promising alternatives to enzyme labels in colorimetric assays, including ELISA. Unlike natural peroxidases, nanozymes demonstrate distinct mechanisms for oxidizing chromogenic substrates such as 3,3',5,5'-tetramethylbenzidine (TMB), prompting the use of custom substrate formulations in contemporary research. However, analysis of the literature reveals that such compositions often yield abnormally high background signals due to non-specific oxidation of TMB, likely catalyzed by trace metal ions present in assay buffers. This phenomenon narrows the dynamic range, elevates limits of detection (LOD), and restricts the use of higher substrate concentrations that could otherwise enhance assay sensitivity. In this study, we identify the widespread use of sodium acetate buffer as a principal contributor to non-specific oxidation, owing to its limited chelating capacity. Statistical analysis demonstrates that lower pH, elevated TMB concentration, and, to a lesser extent, increased buffer molarity and hydrogen peroxide concentration are the most significant factors promoting non-specific reactions. Additional variables influencing background include reagent purity and organic co-solvent content. Employing platinum nanozymes as a model system, we propose two effective strategies to mitigate non-specific oxidation: (i) substitution of acetate buffer with citrate buffer, and (ii) supplementation of acetate buffer with chelating agents. These approaches reduce LOD by 3–12-fold while maintaining low background signals even under adverse conditions (high TMB concentration and low pH).

Introduction

The replacement of natural enzymes with artificial nanoscale analogues—commonly referred to as nanozymes—has emerged as a prominent trend in nanotechnology-driven analytical chemistry [1]. Most efforts have focused on mimicking metal-containing enzymes such as peroxidases, oxidases, catalases, superoxide dismutase, and laccases, given that many metal-based nanoparticles can replicate their catalytic functions, albeit often with lower activity [2]. Nevertheless, recent advances have yielded nanozymes with remarkable catalytic efficiency, in some cases rivaling or even surpassing their natural counterparts in analytical performance [3–5]. Beyond catalytic activity, nanozymes offer distinct advantages over enzymes, including enhanced stability under physical and chemical stress [6], resistance to common enzyme inhibitors [7, 8], and facile tunability of their properties [9]. These attributes make nanozymes attractive not only as robust labels in established assays under challenging conditions but also as platforms for developing novel analytical methodologies where traditional enzymes fall short [10].

The discovery that a wide range of metal-based—and even non-metallic—nanomaterials exhibit enzyme-like activities has spurred an explosion of research into nanozyme applications. Among these, peroxidase mimics are especially prevalent, owing to the central role of horseradish peroxidase (HRP) in clinical diagnostics [11]. HRP catalyzes the oxidation of diverse organic substrates in the presence of hydrogen peroxide, a reaction widely exploited for colorimetric, chemiluminescent, and fluorescent detection schemes [12]. Consequently, considerable research efforts are directed toward the rational

design and engineering of peroxidase-mimetic nanozymes with enhanced catalytic activity, aiming to improve the analytical performance of colorimetric assays, particularly by achieving lower limits of detection [9].

As researchers working extensively with both HRP and its nanoparticulate mimics, we have noted a recurring challenge in the literature: elevated non-specific background signals arising from substrate oxidation in the absence of nanozyme catalysts. The mechanism is based on Fenton- and Fenton-like reactions promoted by metal traces in the presence of hydrogen peroxide [13]. A representative example is paper [14] where color signal generated by nanozyme decreased by 25% after removal of metal traces from substrate solution using Chelex resin. This issue is well recognized in enzyme-based assays and underlies the high cost and stringent stabilization requirements of commercial ready-to-use substrate solutions [15]. However, for nanozyme-based systems—which necessitate even higher substrates concentration—the problem is often exacerbated. This leads sometimes to unexpected results, such as paper claiming light-dependent peroxidase-like activity of acetate buffer [16], which obviously stems from Fenton reactions produced by metal traces.

This paper aims to highlight and systematically analyze the issue of non-specific substrate oxidation in nanozyme-based colorimetric assays and to propose practical solutions. We focused on 3,3',5,5'-tetramethylbenzidine (TMB) substrate due to its popularity and high efficiency. The first part of the manuscript presents the results of a comprehensive literature analysis, demonstrating that non-specific oxidation is a widespread challenge that significantly hinders assay development and compromises analytical reliability. Subsequently, we conduct a systematic investigation into how the composition of the chromogenic solution influences the extent of non-specific oxidation, Finally, based on our findings, we propose simple and effective strategies to inhibit or eliminate this phenomenon, thereby enhancing the analytical performance of nanozyme-based assays.

Experimental

Materials

Platinum nanoparticles (200 ppm) stabilized with 14 kDa polyvinylpirrolidone were from M9 (Russia). Bovine serum albumin (BSA) was from Biolot (Russia). Biotinylated BSA was prepared as described in protocol [17]. Acetic acid, sodium citrate were from Vekton (Russia). Streptavidin was from ProspecBio (Israel). 3,3’,5,5’-tetramethylbenzidine (TMB) and aminotrimethylene phosphonic acid (ATMP) were from Macklin (China). Hydrogen peroxide and acetic acid were from JSC «LenReactiv» (Russia). Sodium hydroxide, citric acid, sodium acetate and 1-hydroxyethane-1,1-diyldiphosphonic acid (HEDP) were from Rushim (Russia). Dimethylformamide was from Ruskhim (Russia). Dimethylacetamide was from EKOS-1 (Russia). Ethanol was from Rusbio (Russia). DMSO was from Usolye-Siberian Chemical and Pharmaceutical Plant (Russia). Sulfuric acid was from SIGMATEC (Russia). Sodium acetate, biotin N-hydroxysuccinimide ester, sodium carbonate anhydrous and diethylenetriaminepentaacetic (DTPA) acid were from Sigma-Aldrich (USA). Sodium hydrogen phosphate and sodium salt of EDTA were from Dia-M

(Russia). Phosphate-buffered saline was from Rosmedbio (Russia). Tween-20 and glycerol were from ITW Reagents (USA). 96-well polystyrene plates (high binding), TCT cell culture 96-well plates were from Kirgen (China).

Instrumentation. The Multiskan Sky UV-Vis reader was from Thermo Scientific (USA). The VCX-130 ultrasonic processor was from Sonics & Materials (USA).

Literature analysis of non-specific TMB oxidation

Data regarding non-specific oxidation of 3,3',5,5'-tetramethylbenzidine (TMB) were manually extracted from the scientific literature, primarily from absorbance versus wavelength spectra presented for reaction media with and without nanoparticles. Such spectra are routinely reported to demonstrate the peroxidase-like activity of nanozymes.

In the majority of publications, precise absorbance values were not provided, with only graphical representations (spectra or histograms) available. In these instances, approximate absorbance values were estimated by visual assessment of the published figures. Occasionally, reported values corresponded to blank-subtracted absorbance. To standardize the dataset for plate-based assays—where blank absorbance is typically close to that of the buffer (0.04–0.06)—a value of 0.05 was added to all specified absorbance readings.

A significant limitation of the literature analysis was the frequent lack of detailed description of TMB solution composition, precluding rigorous quantitative comparison. Notably, the type and concentration of organic co-solvent were rarely specified, despite the use of unrealistically high TMB concentrations that, in the absence of a co-solvent, would lead to precipitation and heterogeneous reaction conditions. Furthermore, it was often unclear whether reported substrate concentrations referred to initial or final values. Even in cases where background signals appeared modest, the comparatively low catalytic activity of nanozymes relative to horseradish peroxidase (HRP) meant that such background could substantially interfere with assay performance and the accurate assessment of nanoparticle activity. Finally, reaction times were seldom reported, rendering direct comparison with the results of our regression analysis impractical.

Effect of reaction media on TMB non-specific oxidation

A set of 20 buffers was prepared using sodium acetate (Vekton, Russia) and acetic acid (Vekton, Russia) with pH values ranging from 3 to 5 and concentrations from 12.9 to 258 mmol/L. Prior to experiments, buffers were stored at + 3°C. Buffers were warmed in a water bath for 15 minutes to + 37°C before using. 96-well plates were also preheated on a thermoshaker for 15 minutes at + 37°C. TMB aliquots in the DMSO were stored at − 20°C and warmed for 10 minutes in a water bath at + 37°C before use.

To the preheated 96-well plate with flat bottom, 155 µL of buffer was added, followed by 20 µL of TMB solution and 25 µL of H_2O_2 solution. TMB and hydrogen peroxide solutions were introduced in order of increasing concentration using the same set of pipette tips for each reagent(tips were replaced only once per reagent). The entire well-filling procedure took about 10 minutes. Final concentrations were: TMB from 0.2 to 1.6 mmol/L, DMSO 10%, H_2O_2 from 1.37 to 1000 mmol/L, and sodium acetate buffer from 10 to 200 mmol/L. All plates were sealed with an adhesive film and incubated for 30 minutes on a thermoshaker at + 37°C and 350 rpm. Subsequently, 100 µL of 2 mol/L H_2SO_4 was added, and the absorbance at 450 nm (A_{450}) was measured using a spectrophotometer. All experiments were performed in triplicate (n = 3).

When performing regression analysis, data obtained with 10 mmol/L sodium acetate buffer at pH 3 were excluded from analysis due to its preparation requiring the addition of stronger acid, e.g., HCl (10 mmol/L acetic acid has pH ≈ 3.37).

The effect of an organic solvent on the non-specific signal

Sodium acetate buffers (12.9, 64.5, 129, and 258 mmol/L, pH 4) were stored in tubes at + 3°C. TMB solutions in organic solvents were prepared on the day of the experiment and kept in the dark at room temperature. Buffers and plates were preheated as described above.

To a 2 mL tube, 1550 µL of preheated buffer was added, followed by 50 µL of TMB solution in 100% DMF, DMA, or DMSO. To achieve the target final concentrations of the organic solvent in the reaction mixture (2.5%, 5%, or 10%), an additional 50 µL (for the 5% solvent condition) or 150 µL (for 10%) of the respective solvent was added. The final volume was adjusted to 1750 µL by adding 150 µL of deionized water (for 2.5%) or 100 µL of deionized water (for 5%). Solutions were mixed by vortexing.

The experiment with TMB in 95% ethanol was performed similarly, with the reagent volumes adjusted to achieve the target final concentrations of the organic solvent. To a 2 mL tube, 1550 µL of preheated buffer was added, followed by 53 µL of TMB solution in 95% ethanol and buffer were added. To obtain the desired final solvent concentration, an additional 52 µL of ethanol (for 5%) or 157 µL of ethanol (for 10%) was added. The final volume was adjusted to 1750 µL by adding 147 µL of deionized water (for 2.5%) or 95 µL of deionized water (for 5%). The solutions were mixed by vortexing.

Aliquots of 175 µL or 176 µL (for 10% ethanol) of the resulting solutions were transferred into the wells of a flat-bottom 96-well plate. Then, 25 µL of hydrogen peroxide solution (initial $[H_2O_2]$ = 296.32 mmol/L) was added to the wells, or 24 µL (initial $[H_2O_2]$ = 308.66 mmol/L) for the samples containing 10% ethanol.

The final concentrations in the wells were: H_2O_2 — 37.04 mmol/L and TMB — 0.8 mmol/L. All plates were sealed with an adhesive film and incubated for 30 min at + 37°C and 350 rpm in a thermoshaker. The

reaction was terminated by the addition of 100 µL of 2 mol/L H_2SO_4 to each well. Absorbance was measured at 450 nm using a spectrophotometer (A_{450}). All experiments were performed in triplicate.

Influence of the commercial source of reagents on the background signal

A set of 24 sodium acetate buffer combinations was prepared using two different sources of acetic acid and sodium hydroxide or three different sources of sodium acetate. Buffers were adjusted to pH 3, 4, or 5. The buffers were stored in tubes at + 3°C in a total volume of 10 mL each. Prior to the experiment, buffers and one plate were preheated as described above.

Aliquots of TMB in DMSO (8 mmol/L) were warmed in a water bath for 10 minutes. 155 µL of buffer was added to the wells of a flat-bottom 96-well plate. Then, 20 µL of TMB solution and 25 µL of H_2O_2 solution were added to the buffers. The plate was sealed with an adhesive film and incubated for 30 minutes on a thermoshaker at + 37°C and 350 rpm. The reaction was terminated by the addition of 100 µL of 2 mol/L H_2SO_4, and absorbance was measured at 450 nm (A_{450}). All experiments were performed in triplicate (n = 3).

Reduction of non-specific oxidation of TMB by using sodium citrate buffer

Sodium citrate buffers (12.9, 64.5, or 258 mmol/L with pH 3, 4, or 5) were prepared from citric acid and sodium citrate. A citrate-phosphate buffer was prepared from 100 mmol/L citric acid and 200 mmol/L sodium hydrogen phosphate [18]. All buffers were stored at + 3°C.

Prior to the experiment, buffers, plates, and TMB in DMSO aliquots (16 mmol/L and 4 mmol/L) were preheated as described above. Buffers were transferred to 8-channel reservoirs. Using a multichannel pipette, 155 µL of buffer was dispensed into the wells of a flat-bottom 96-well plate. Then, 20 µL of TMB solution (2664 mmol/L) and 25 µL of H_2O_2 solution (32.96 mmol/L) were added to the buffers. The final concentrations in the wells were as follows: TMB — 1.6 mmol/L or 0.4 mmol/L; DMSO — 10% (v/v); H_2O_2 — 333 mmol/L or 4.12 mmol/L; sodium citrate buffer — 10 mmol/L, 50 mmol/L or 200 mmol/L.

The plate was sealed with an adhesive film and incubated for 30 min on a thermoshaker at + 37°C and 350 rpm. The reaction was terminated by the addition of 100 µL of 2 mol/L H_2SO_4. Absorbance was measured at 450 nm. All experiments were performed in triplicate.

Inhibition of non-specific TMB oxidation by chelating agents

Solutions of DTPA, HEDP, EDTA, and ATMP were prepared at a concentration of 100 mmol/L and stored at + 3°C. Sodium acetate buffers containing chelating agents were prepared as follows: sodium acetate

buffers (14.32 mmol/L or 143.23 mmol/L, pH 3.5 or 4.0) were prepared from sodium acetate and acetic acid. To 12.5 mL of this buffer, a chelating agent was added. If the value deviated from the target (3.5 or 4.0), it was adjusted by adding sodium acetate or acetic acid of the same molarity as the buffer. The chelators were added in volumes of 3.1 µL, 31.3 µL, 312.5 µL, or 3125 µL, followed by the addition of deionized water (3121.9 µL, 3093.7 µL, 2812.5 µL, or 0 µL, respectively). Control buffers without chelators were prepared in parallel by adding 3125 µL of deionized water to 12.5 mL of buffer. The final volume was then adjusted to 25 mL with the appropriate sodium acetate buffer. The prepared buffers with chelating agents were stored in tubes at + 3°C.

Prior to the experiment, buffers, plates, and TMB aliquots (16 mmol/L) were preheated as described above. Separate solutions of TMB in DMSO and H_2O_2 were prepared and kept covered with foil.

To the wells of a flat-bottom 96-well plate, 160 µL of buffer was added using a multichannel pipette. Then, 20 µL of TMB solution and 20 µL of H_2O_2 solution (3333 mmol/L) were added to the buffers. The final concentrations in the wells were: TMB — 1.6 mmol/L; DMSO — 10% (v/v); and H_2O_2 — 333 mmol/L; sodium citrate buffer — 10 mmol/L or 100 mmol/L; chelators — 10 µmol/L, 100 µmol/L, 1000 µmol/L or 10000 µmol/L.

The plate was sealed with an adhesive film and incubated for 30 min on a thermoshaker at + 37°C and 350 rpm. The reaction was terminated by the addition of 100 µL of 2 mol/L H_2SO_4, and absorbance was measured at 450 nm using a spectrophotometer (A_{450}). All experiments were performed in triplicate (n = 3).

Conjugation of platinum nanoparticles with streptavidin

The conjugation procedure was adapted from the method reported by Jiang et al. [19]. The pH of the platinum nanoparticle suspension (200 ppm) was adjusted to 9 using 1 M NaOH. Subsequently, 100 µL of a 10 mg/mL streptavidin solution (prepared in deionized water) was added to 10 mL of the adjusted nanoparticle suspension. The mixture was incubated for 60 minutes at room temperature on a rotator (15 rpm) to facilitate adsorption of streptavidin onto the nanoparticle surface. Following incubation, bovine serum albumin (BSA) was added to a final concentration of 1% (w/v) to block non-specific binding sites, and the suspension was incubated for an additional 30 minutes under identical conditions. The nanoparticles were then purified by three successive centrifugation steps at 40,000 × g for 30 minutes at + 20°C. Each wash was performed with phosphate-buffered saline (PBS) containing 1% BSA. After each centrifugation cycle, the nanoparticle pellet was resuspended and subjected to brief probe sonication (3 mm probe, 60% amplification, 30 seconds) to ensure homogeneous dispersion. Finally, the purified conjugates (Pt/Str) were resuspended in an aqueous solution of 1% BSA and 20% glycerol (v/v), yielding a total volume of 4 mL. The working titer of the conjugate (1:250 dilution) was empirically determined in preliminary direct binding assays using biotinylated BSA (Bi-BSA) as the model analyte, based on the comparison of standard curves generated under varying dilutions.

Colorimetric assay with Pt/Str conjugates

For the immobilization of Bi-BSA, 100 µL of a Bi-BSA solution diluted in 0.1 mol/L sodium carbonate buffer (pH 9.5) was dispensed into each well of a 96-well microplate. The plates were incubated for 2 h at 350 rpm and + 37°C on a thermal shaker. Following incubation, the wells were washed three times with 350 µL of phosphate-buffered saline [20] containing 0.1% (v/v) Tween-20 (PBST). Subsequently, 300 µL of blocking solution (PBST supplemented with 1% BSA) was added, and the plates were incubated for 60 minutes at 350 rpm and + 37°C on a thermal shaker. After a further washing step, 100 µL of Pt/Str, diluted 1:250 in the blocking buffer, was added to each well. The plates were incubated for 60 minutes at 350 rpm and + 37°C, followed by washing. Next, 100 µL of freshly prepared TMB substrate solution was added to each well. The substrate consisted of either 0.1 mol/L (0.2 mol/L) sodium acetate or sodium citrate buffer (pH 4.0), with or without chelating agents, 10% (v/v) DMSO, TMB at concentrations ranging from 0.4 to 1.6 mmol/L, and H_2O_2 at concentrations from 10 to 1000 mmol/L (resulting concentrations are specified). The plates were incubated for 30 or 21 minutes at 350 rpm and + 37°C. The reaction was terminated by the addition of 100 µL of 2 mol/L H_2SO_4, and the absorbance at 450 nm was measured. In selected kinetic experiments, absorbance at 652 nm was recorded at 5-minute intervals to monitor reaction progress.

Statistical analysis

For the statistical analysis of literature data, the substrate concentrations and pH values for each acetate buffer system were compared between sets of papers reporting high and low levels of non-specific TMB oxidation using the Mann-Whitney test (GraphPad Prism 8, GraphPad Software, Inc., USA). Descriptive statistics for each dataset were also calculated and are provided in the Supplementary Information.

Standard curves were generated by fitting the calibration data to a four-parameter logistic (4PL) model (Eq. 1) using Origin 2020b (OriginLab Corporation, USA):

$$y = \mathrm{A}_2 + \frac{\mathrm{A}_1 - \mathrm{A}_2}{1 + \left(\frac{x}{x_0}\right)^p}$$

The lower asymptote (parameter A_1) was fixed to the mean absorbance of the zero calibrator. No weighting or Y^2 weighing was applied to the curve fit. The limit of detection (LOD) was calculated according to the recommendations of Ambruster and Pry [21]. First, the limit of blank (LoB) was determined:

$$\mathrm{LoB} = \mathrm{A}_{450} \text{ of zero calibrator} + 1.645 \times \text{ SD of zero calibrator}$$

The limit of detection (LOD) was determined as the concentration of Bi-BSA corresponding to the threshold absorbance value at 450 nm (A_{450}, T_{450}). T_{450} was calculated according to the following formula:

$$T_{450} = \mathrm{LoB} + 1.645 \times \mathrm{SD}$$

where LoB is the limit of blank and SD is the standard deviation of the A_{450} values for the lowest non-zero calibrator exceeding the LoB. The LOD was then back-calculated from the 4PL standard curve as the Bi-BSA concentration corresponding to an absorbance value equal to T_{450}.

The slope values were taken from the 4PL model (parameter p). Multiple regression analysis was performed using Minitab 18 (Minitab Inc., USA), while other statistical calculations were conducted in GraphPad Prism 8.

Results and discussion

Why does non-specific oxidation matters: literature analysis

Literature analysis (**Table S1**) identified over one hundred publications on nanozymes (the search was concluded at 110 papers) in which non-specific oxidation of TMB resulted in elevated background signals. For the purposes of this study, an absorbance threshold of ≥ 0.1 at 652 nm was defined as indicative of high background, corresponding to approximately 0.2 absorbance units at 450 nm following reaction termination with strong acid [22]; in one exceptional case, a value as low as 0.06 was included due to a comparable absorbance increase upon nanozyme addition. Analysis revealed that in nearly 38% of the 110 papers, non-specific oxidation was pronounced, with $A_{652} > 0.2$, translating to A_{450} values exceeding 0.35–0.40 (Fig. 1D).

For comparative purposes, a matched dataset of 103 papers reporting negligible non-specific oxidation ($A_{652} < 0.1$) was examined (**Table S2**). The two cohorts were compared based on buffer composition (type, pH), and substrate concentrations (Fig. 1A-C **and Tables S3-S5**). The key observations are summarized below, with their interpretation grounded in our experimental findings on the factors governing non-specific TMB oxidation, which are detailed in Section "*Key Factors Influencing Non-Specific Oxidation of TMB*".

As anticipated, acetate buffer was the most commonly employed reaction medium, followed by phosphate buffers (Fig. 1D,E). Notably, publications reporting lower background signals more frequently utilized alternative buffer systems, such as citrate, citrate-phosphate, or Tris-HCl.

A statistical comparison, restricted to studies using acetate-based systems, revealed no significant differences in pH or H_2O_2 concentrations between reports of high and low background. However, a significant disparity was observed in TMB concentration: papers reporting elevated non-specific oxidation used a markedly higher mean TMB concentration (1.354 mmol/L vs. 0.636 mmol/L; median: 0.500 mmol/L vs. 0.300 mmol/L). Our regression analysis (presented in Section "*Key Factors Influencing Non-Specific Oxidation of TMB*") substantiates that lower pH and higher TMB concentrations are the

primary predictors of strong non-specific oxidation in acetate buffers. Nevertheless, numerous studies achieving low background also employed high TMB levels at low pH, implicating additional, unspecified variables such as reaction time, temperature, and the concentration of any organic co-solvent.

It is important to acknowledge that many publications lack comprehensive reporting of reaction conditions. Consequently, this literature analysis remains primarily descriptive and precludes robust quantitative modeling of the individual factor effects.

In conclusion, the literature survey indicates that non-specific TMB oxidation is a relatively widespread, though not ubiquitous, issue in analytical applications of nanozymes. A rough estimate suggests that only 10–20% of all analyzed papers explicitly report elevated background levels; precise quantification is hindered by incomplete data reporting and the challenges of extracting numerical values from graphical representations. While many studies document control absorbance values in the range of 0.1–0.15 for TMB + H_2O_2 mixtures—values that may appear modest—the potential for assay interference remains significant. The following section provides a detailed rationale for why this phenomenon warrants careful consideration and mitigation in assay development.

Why non-specific oxidation matters: theoretical background

Non-specific oxidation of chromogenic substrates is a critical, yet often overlooked, factor that directly impacts the analytical performance and reliability of nanozyme-based colorimetric assays. Its significance can be understood from several key perspectives.

First, in colorimetric immunoassays, the background absorbance at 450 nm (A_{450}) in blank samples (lacking the target analyte) is typically maintained below 0.1 when using the TMB substrate system. Minimizing this background is a central objective of assay optimization, as it directly influences LOD and overall reliability. Spectrophotometric measurements are most reliable within a limited dynamic range (approximately 0–3 absorbance units). An increase in background absorbance consumes this valuable range, particularly at the lower end where linearity and precision are optimal. Furthermore, the variance of absorbance readings increases at higher values [23]. Consequently, elevated background narrows the dynamic range, raises the LOD (which is directly dependent on the variability of blank controls) [21], and undermines the primary goal of achieving ultra-low LODs in nanozyme-based assays.

This issue is exacerbated in nanozyme immunoassays. Unlike traditional enzyme labels, nanoparticle conjugates possess complex surfaces and a higher propensity for non-specific binding, making these interactions difficult to control [24]. Therefore, any additional source of background, such as non-specific substrate oxidation, is especially detrimental, particularly in point-of-care formats that rely on qualitative readouts.

Second, the background levels observed in many nanozyme-based assays significantly exceed those typical for commercial immunoassays, against which they are expected to compete. In ELISA

development, background absorbance is strictly controlled to ensure analytical rigor [25]. For practical implementation, nanozyme-based assays must comply with these established standards.

Third, recent literature advocates for increasing substrate concentrations to maximize signal intensity and improve LOD in nanozyme assays [26]. However, our experience with catalytic medium optimization indicates that such increases inevitably amplify non-specific Fenton reactions and elevate background signals [27]. Thus, as the field adopts these best practices, the issue of elevated background will become even more pronounced.

Fourth, non-specific oxidation interferes with the accurate measurement of nanozyme catalytic properties. A recent methodological advance recommends determining kinetic parameters (K_{cat}, K_m) using a matrix of high and low concentrations for both substrates, rather than the traditional single-fixed-concentration approach [28]. This improved method necessitates the use of substrate combinations that inherently promote non-specific oxidation, the intensity of which varies for each condition. To calculate the true catalytic activity of the nanozyme, this non-specific signal must be subtracted from the total absorbance. Therefore, methods to suppress non-specific oxidation are essential for accurate and simplified kinetic analysis.

Finally, a subset of nanozyme-based assays is designed for the direct quantification of H_2O_2 [29]. In these systems, any intrinsic signal generated by hydrogen peroxide itself—potentially concentration-dependent—can compromise assay accuracy.

In the following experiments, we identify the key factors influencing non-specific oxidation of TMB in the presence of H_2O_2 and propose effective strategies to mitigate this issue.

Key Factors Influencing Non-Specific Oxidation of TMB

To systematically investigate the impact of reaction conditions on non-specific oxidation, solutions of TMB in sodium acetate were prepared in 96-well plates. Following the addition of hydrogen peroxide, the mixtures were incubated for 30 minutes, after which the reaction was quenched with sulfuric acid and absorbance at 450 nm was recorded. The resulting dataset, comprising absorbance values and corresponding reaction parameters (pH, buffer molarity, TMB and H_2O_2 concentrations), was subjected to multivariate regression analysis. This statistical model was constructed to identify and quantify the key factors contributing to non-specific TMB oxidation and to elucidate their relative influence on the observed background signal.

Sodium acetate was selected as the reaction medium due to its widespread use in nanozyme research [30] and its recommendation in standard protocols for assessing nanozyme catalytic activity [31]. Non-specific oxidation is primarily driven by Fenton-type reactions, which are most efficient at acidic pH values where the solubility of transition metal cations is maximized. Accordingly, the pH range was set between 3.0 and 5.0 based on a literature analysis. Although acetate exhibits limited buffering capacity at pH 3.0–3.5, these conditions are frequently reported in the field and were therefore included for

comparison; strictly speaking, such solutions are more accurately described as sodium acetate solutions rather than buffers.

A range of buffer molarities was examined, as reducing ionic strength is expected to lower the concentration of trace metal impurities. Substrate concentrations were also varied to reflect the diversity reported in the literature and to account for nanomaterial-specific optima [26]. TMB concentrations spanned 0.2–1.6 mmol/L, while H_2O_2 levels ranged from 1.37 to 1000 mmol/L. Notably, nanozymes typically require higher H_2O_2 concentrations than HRP due to their elevated K_m values [26]. To prevent TMB aggregation, dimethyl sulfoxide (DMSO) was added at 10% (v/v), a concentration ensuring TMB solubility at higher pH where it is predominantly deprotonated.

Regression analysis of absorbance data revealed that under the tested conditions, non-specific oxidation could yield absorbance values significantly exceeding those appropriate for ELISA (Fig. 2, **Fig. S1-S5, and Eq. S1**). Both increased TMB concentration and lower pH significantly promoted non-specific oxidation, whereas buffer molarity and H_2O_2 concentration exhibited comparatively minor effects (Fig. 3). The limited influence of H_2O_2 is likely attributable to the fact that non-specific oxidation is predominantly governed by trace transition metals; for typical reagent grades, these impurities are minimal and H_2O_2 is present in large excess across all tested concentrations. Moreover, very high H_2O_2 levels may even scavenge hydroxyl radicals generated in Fenton reactions generating less reactive hydroperoxyl radicals ($HO_2^{\cdot}$) [32, 33]. As we show below (see Section "*Non-specific oxidation in nanozyme assay*"), this scavenging effect of H_2O_2 excess can be useful for nanozymes operating at extremely high H_2O_2 concentrations, as it decreases non-specific oxidation of TMB and background signal in immunoassay.

The effect of buffer molarity was unexpected, given prior experience with ultra-low ionic strength buffers (< 1 mmol/L) that exhibited negligible background even below pH 4 (not published data). An increase in ionic strength may suppress the rate of Fenton reactions, although this effect is strongly dependent on the nature of the electrolyte [34]. Additionally, acetate ions can act as scavengers for hydroxyl radicals, thereby competing with TMB and potentially influencing the extent of non-specific oxidation [35]. The interplay of these two factors likely accounts for the observed trends. Notably, in subsequent experiments involving alternative organic solvents (see Section "*Organic solvent*"), a clear correlation between buffer concentration and the degree of non-specific oxidation was established for solvents other than DMSO. Besides, in experiments with chelators lower non-specific signal was observed at lower ionic strength conditions (Section "*Chelators*"). Consequently, minimizing salt concentration in the reaction medium emerges as a beneficial strategy for reducing background signals.

Finally, considerable between-replicate variability in absorbance was noted, with certain wells exhibiting markedly higher signals despite stringent control of mixing and assay conditions. This inconsistency underscores the reproducibility challenges posed by excessive non-specific oxidation in assay development.

Organic solvent

Commercial TMB solutions for ELISA frequently incorporate a variety of organic solvents, whereas DMSO is predominantly employed in nanozyme-based assays. To evaluate the effect of solvent type and concentration on non-specific TMB oxidation, four organic solvents were compared: DMSO, dimethylacetamide (DMA), dimethylformamide (DMF), and ethanol. The rationale for this study was based on the potential of organic solvents to act as scavengers of hydroxyl radicals, which are central to the non-enzymatic oxidation pathway of TMB catalyzed by nanozymes [36].

Experiments were conducted under standardized conditions, with pH, TMB, and H_2O_2 concentrations set to the mean values across the experimental range. The results (Fig. 4) demonstrated that increasing solvent concentration either had no significant effect or, in certain cases, reduced non-specific oxidation. Notably, the use of solvents other than DMSO revealed a pronounced correlation between buffer molarity and the extent of non-specific TMB oxidation. At the highest tested buffer concentration (200 mmol/L), DMSO consistently yielded the lowest background signal among all solvents. Nevertheless, the overall choice of organic solvent has no significant effect on non-specific TMB oxidation.

Effect of reagent source and buffer preparation

The purity of reagents and the method of buffer preparation represent variables influencing non-specific oxidation. For instance, acetate buffer may be prepared either by mixing acetic acid with sodium acetate or by titrating acetic acid with NaOH; regardless of the approach, the concentration of trace metal impurities in the starting materials is the principal determinant of background signal.

To investigate this effect, acetate buffers at pH 3.0, 4.0, and 5.0 were prepared using acetic acid and bases (NaOH or sodium acetate) sourced from multiple commercial suppliers (Fig. 5). Two-way ANOVA demonstrated that the origin of acetic acid significantly ($p < 0.05$) affected non-specific absorbance at pH 3.0 and 4.0, with up to a 1.5–2-fold difference observed between certain suppliers. Additionally, variations were detected depending on the base employed for pH adjustment.

These findings underscore that reagents from different manufacturers can introduce substantial variability in non-specific signals. This factor must be considered when benchmarking results against literature data and during assay optimization to ensure reproducibility.

Chelators

As already mentioned, non-specific oxidation of TMB in ELISA is typically minimized. This is commonly achieved by using commercially available stabilized TMB solutions, which, when properly stored, can maintain low levels of non-specific oxidation for months. Such stabilization is usually accomplished by the addition of various stabilizers, among which chelating agents, such as EDTA or DTPA, are frequently employed [37]. Moreover, citrate buffer and citrate-phosphate buffers are more commonly used in ELISA experiments when custom TMB solutions are prepared. In general, literature analysis shows that chelators are not used in nanozyme assays. Among more than two hundred analyzed papers only one

(#103 in **Table S2**) reported addition of chelators in TMB solution. Moreover, the same paper reported an experiment with DTPA-free TMB solution (#53 in **Table S1**) despite a lower concentration of peroxide. Addition of DTPA prevented non-specific oxidation of TMB.

To demonstrate their efficiency, micromolar amounts of four commercially available chelators (EDTA, DTPA, HEDP, and ATMP), each bearing several carboxylic or phosphate groups, were added to acetate buffer prior to the preparation of TMB solutions. Reaction conditions were chosen to promote a high rate of non-specific oxidation of TMB. All tested chelating agents significantly reduced non-specific reactions even at concentrations as low as 10 μmol/L (Fig. 6).

Citrate buffers also facilitate stabilization of TMB solution due to their chelating ability (Fig. 7). However, for nanozymes, citrate buffer may be undesirable in certain cases, for example, because some nanozymes exhibit higher catalytic activity in acetate than in citrate [27]. In such cases, the application of chelators can be a viable solution. Previously, in the course of literature search we identified one paper (#96 in **Table S1**) in which a citrate-containing buffer did not prevent non-specific oxidation of TMB, in other cases this strategy was effective.

Non-specific oxidation in nanozyme assay

To demonstrate the efficiency of proposed strategies to decrease non-specific oxidation of TMB we used a model direct colorimetric assay of biotinylated BSA (Bi-BSA) using platinum nanozymes conjugated with streptavidin. First, we optimized concentration of substrates using citrate buffer to obtain conditions providing the highest signal intensity in immunoassay. Comparison of standard curves obtained at different times after addition of TMB solution showed that signal intensity and LOD dramatically increased with concentration of H_2O_2 (**Fig. S6-S8**). TMB concentration can also be maintained at a very high value limited only by its solubility. Therefore, we tested the effect of chelating agents on the background signal using maximum concentration of both substrates (TMB = 1.6 mmol/L, H_2O_2 = 100 and 1000 mmol/L). Specifically we prepared TMB solutions using 0.2 mol/L sodium acetate [31] and sodium citrate buffers, as well as sodium acetate buffer supplemented with 100 μmol/L of previously used chelators.

At 1000 mmol/L of H_2O_2 background signal for acetate buffer was higher than typical for ELISA (0.128), but still acceptable (**Fig. S9-S14**, Table 1). This relatively low background is explained by inhibition of Fenton reaction by excess of H_2O (Section "*Key Factors Influencing Non-Specific Oxidation of TMB*"). Nevertheless, chelators efficiently decreased signal in blank more than two-fold without compromising overall signal intensity. The citrate buffer kept background signal at similarly low level and additionally increased signal intensity being a more favorable reaction medium for platinum nanozymes as we showed in previous work [27]. Besides, chelating conditions provided 3.3-12.5-fold decrease of LOD and steeper slope of linear portion of standard curve. At 100 mmol/L of H_2O_2 background signal for acetate buffer was 0.346 (**Fig. S15-S20**, Table 2) which is higher than at 1000 mmol/L of H_2O_2 due to weaker

inhibitory effect of H_2O_2. Again, chelators and citrate buffer decreased LOD by 3.5–10 fold and absorbance of blank by 7-fold.

Table 1
Effect of buffer and chelators (100 µmol/L) on assay performance (TMB solution with 1000 mmol/L H_2O_2).

Buffer	LOD, pg/mL	A_{450} at 0 pg/mL	A_{450} at 10 ng/mL	Slope
Na-acetate	113.04	0.128 ± 0.013	2.521 ± 0.179	0.99 ± 0.02
Na-citrate	12.79	0.060 ± 0.001	3.552 ± 0.194	1.07 ± 0.02
Na-acetate + EDTA	34.77	0.060 ± 0.001	2.705 ± 0.183	1.26 ± 0.03
Na-acetate + HEDP	17.26	0.060 ± 0.001	2.690 ± 0.092	1.14 ± 0.07
Na-acetate + DTPA	14.07	0.064 ± 0.001	2.824 ± 0.130	1.16 ± 0.04
Na-acetate + ATMP	9.07	0.062 ± 0.001	2.936 ± 0.142	1.07 ± 0.06

Despite the efficiency of chelating agents they can decrease catalytic activity of nanozymes. For instance, EDTA can interfere with nanozymes POD-like activity [38]. Therefore, alternatives should be considered e.g. citrate-based buffer, or other chelating agents. Another option is to remove metal impurities from a buffer prior to assay, but in some cases this measure can be insufficient and non-specific signal will be significant [39].

Table 2
Effect of buffer and chelators (100 µmol/L) on assay properties (TMB solution with 100 mmol/L of H_2O_2)

Buffer	LOD, pg/mL	A_{450} at 0 pg/mL	A_{450} 300 ng/ml	Slope
Na-acetate	2055.28	0.346 ± 0.003	2.509 ± 0.154	1.14 ± 0.03
Na-citrate	203.83	0.050 ± 0.001	3.051 ± 0.127	1.10 ± 0,02
Na-acetate + EDTA	654.33	0.050 ± 0.001	1.662 ± 0.243	1.16 ± 0.02
Na-acetate + HEDP	614.10	0.049 ± 0.001	2.283 ± 0.226	1.02 ± 0.02
Na-acetate + DTPA	356.85	0.050 ± 0.002	2.065 ± 0.108	1.03 ± 0.01
Na-acetate + ATMP	337.73	0.049 ± 0.001	1.806 ± 0.157	1.15 ± 0.02

Conclusion

The literature analysis and experimental data presented in this work highlight the widespread issue of non-specific substrate oxidation in assays utilizing peroxidase-like nanozymes. This phenomenon can significantly compromise assay reliability and analytical performance. We propose straightforward and effective strategies to mitigate non-specific signals, which not only reduce background but can also

enhance the overall analytical characteristics of the assays. Beyond analytical applications, our findings are broadly applicable to the development and optimization of novel peroxidase-mimetic materials. In such studies, non-specific substrate oxidation poses a major challenge for the accurate determination of kinetic parameters and the quantitative assessment of nanozyme catalytic efficiency.

Declarations

Author contribution

AN: investigation, methodology, visualization, writing of original draft, editing, final manuscript; PK: conceptualization, experimental design, visualization, writing of the original draft, editing, final manuscript; MR: conceptualization, validation, supervision, resources, project administration, writing of original draft, editing, and final manuscript.

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Data availability

Data are available upon request to the corresponding author

Competing interests

The authors declare no competing interests.

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Figures

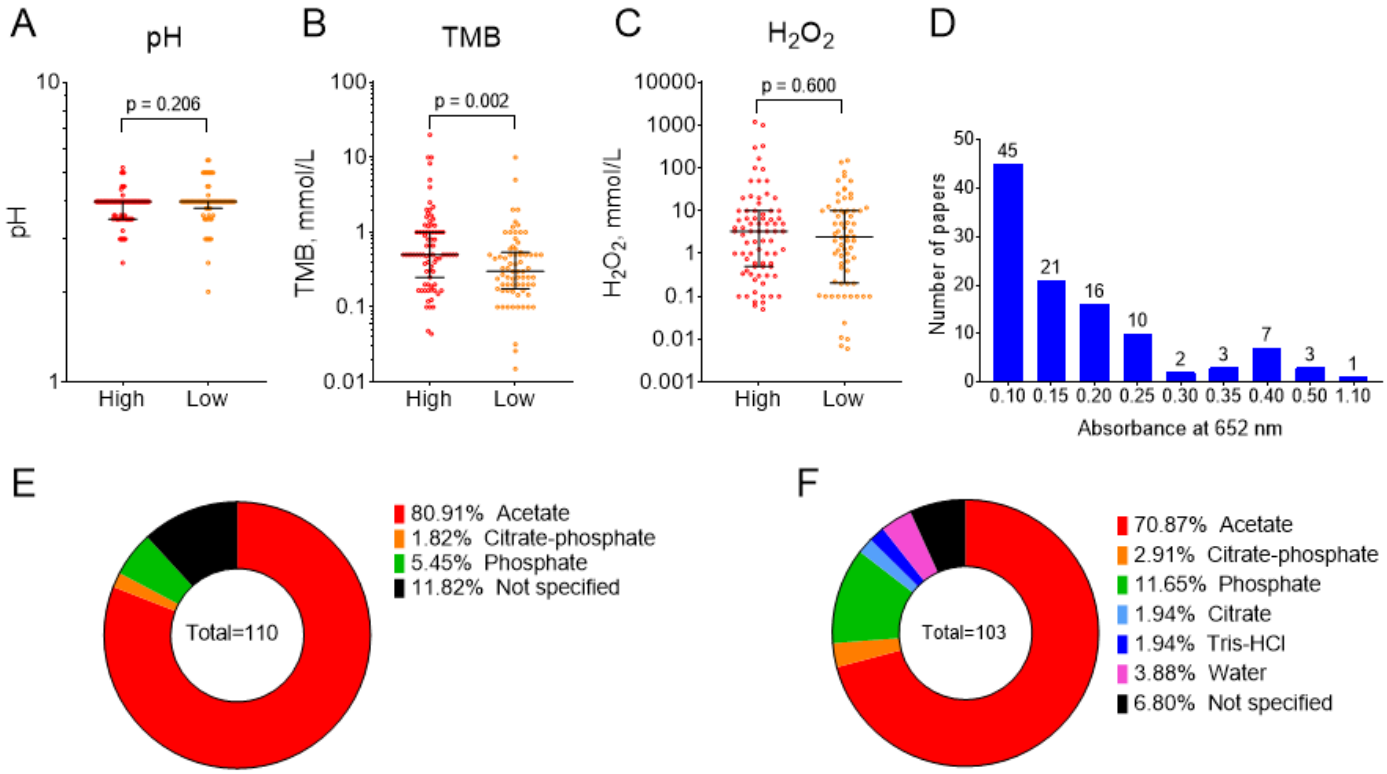


Figure 1

Results of literature analysis. **A–C** Comparison of the composition of the reaction medium (pH, TMB, and H_2O_2 concentrations) in publications reporting high and low levels of non-specific TMB oxidation. Data are presented as median and interquartile range; p values from the Mann–Whitney test are indicated. **D** Frequency distribution of observed absorbance values for TMB + H_2O_2 mixtures reported in publications with high levels of non-specific TMB oxidation (n = 110). **E**, **F** Distribution of buffer species used in the reaction medium in publications reporting high (**E)** and low (**F**) levels of non-specific TMB oxidation.

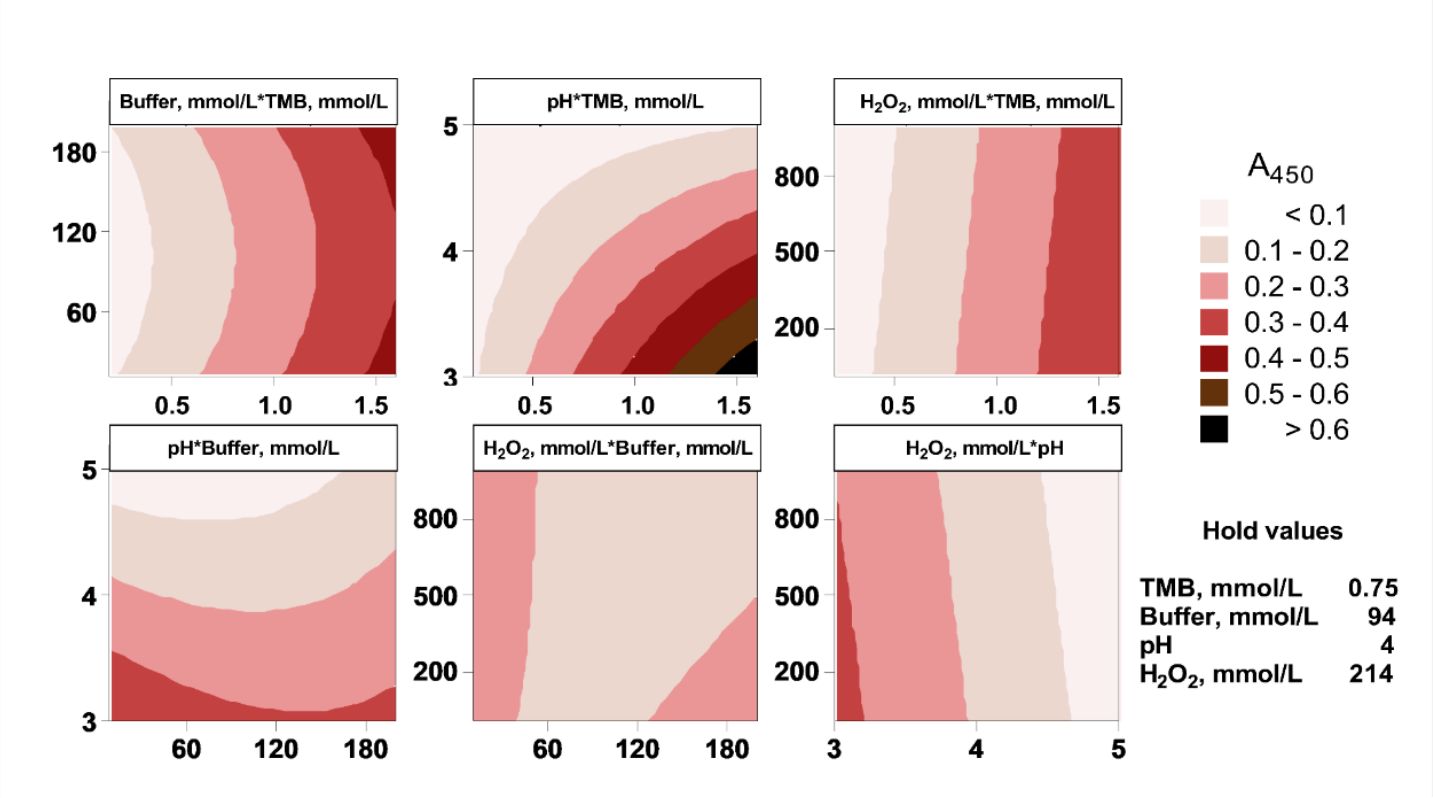


Figure 2

Contour plots of absorbance at 450 nm for various combinations of TMB and H_2O_2 reaction conditions. Non-specified factors were held constant at the values indicated in the legend.

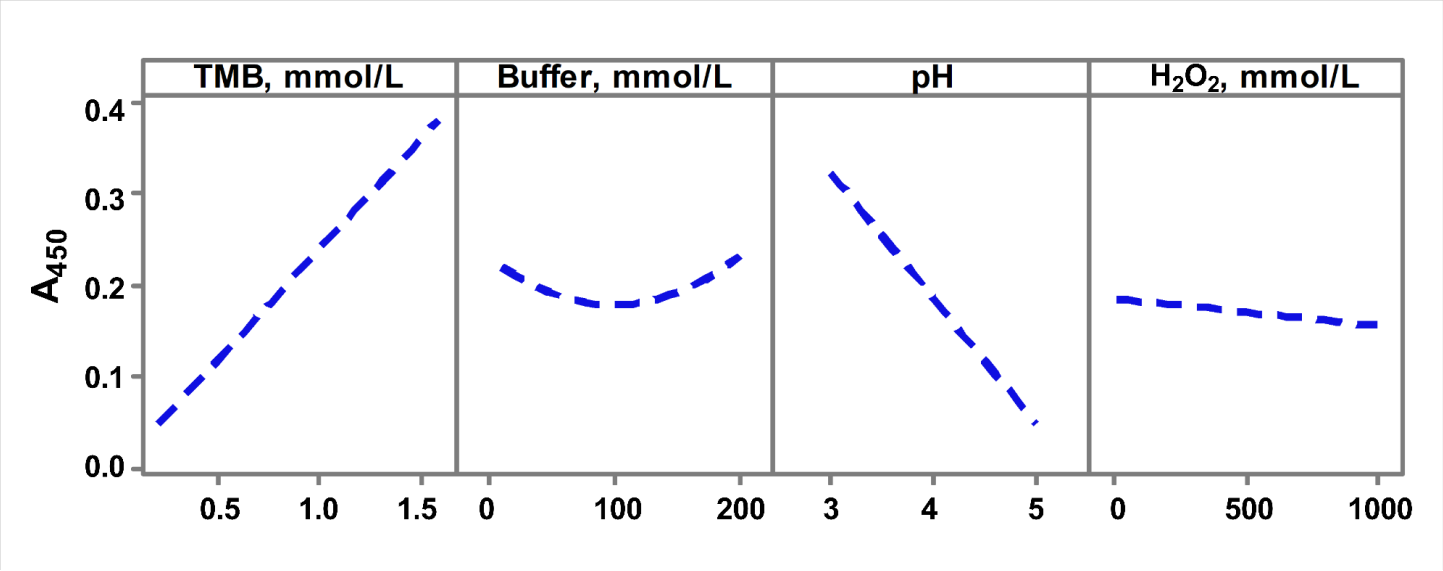


Figure 3

Main effects plots for absorbance at 450 nm. The plots illustrate the individual impact of each reaction parameter on the non-specific oxidation of TMB.

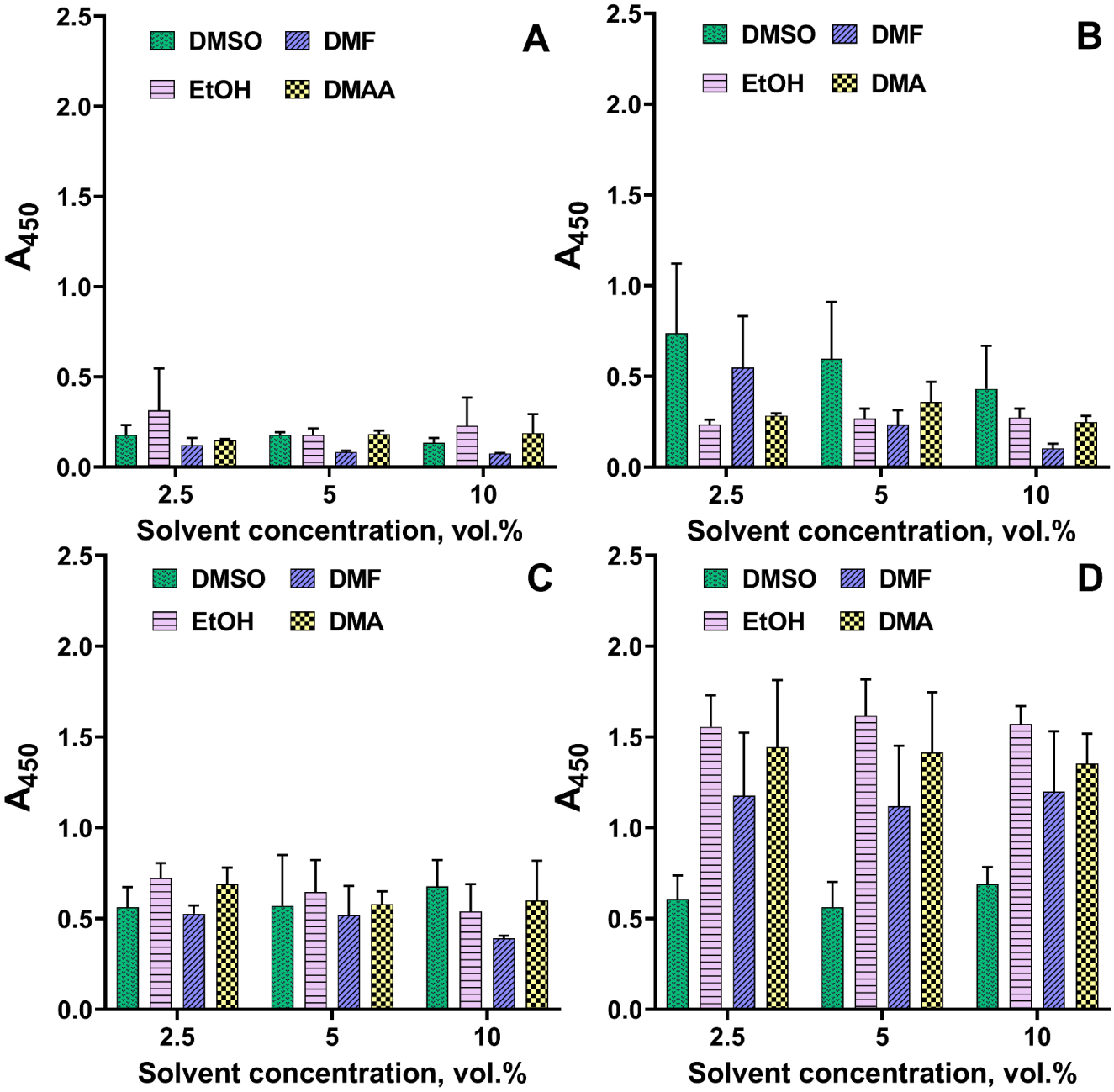


Figure 4

The effect of organic solvents on the non-specific oxidation of TMB. The influence of solvent type and concentration was tested in sodium acetate buffers of varying molarity: **A** 10 mmol/L, **B** 50 mmol/L, **C** 100 mmol/L, and **D** 200 mmol/L. Reaction conditions: pH 4.0, [H_2O_2] = 37.04 mmol/L, [TMB] = 0.8 mmol/L. Data are presented as the mean ± standard deviation (n = 3).

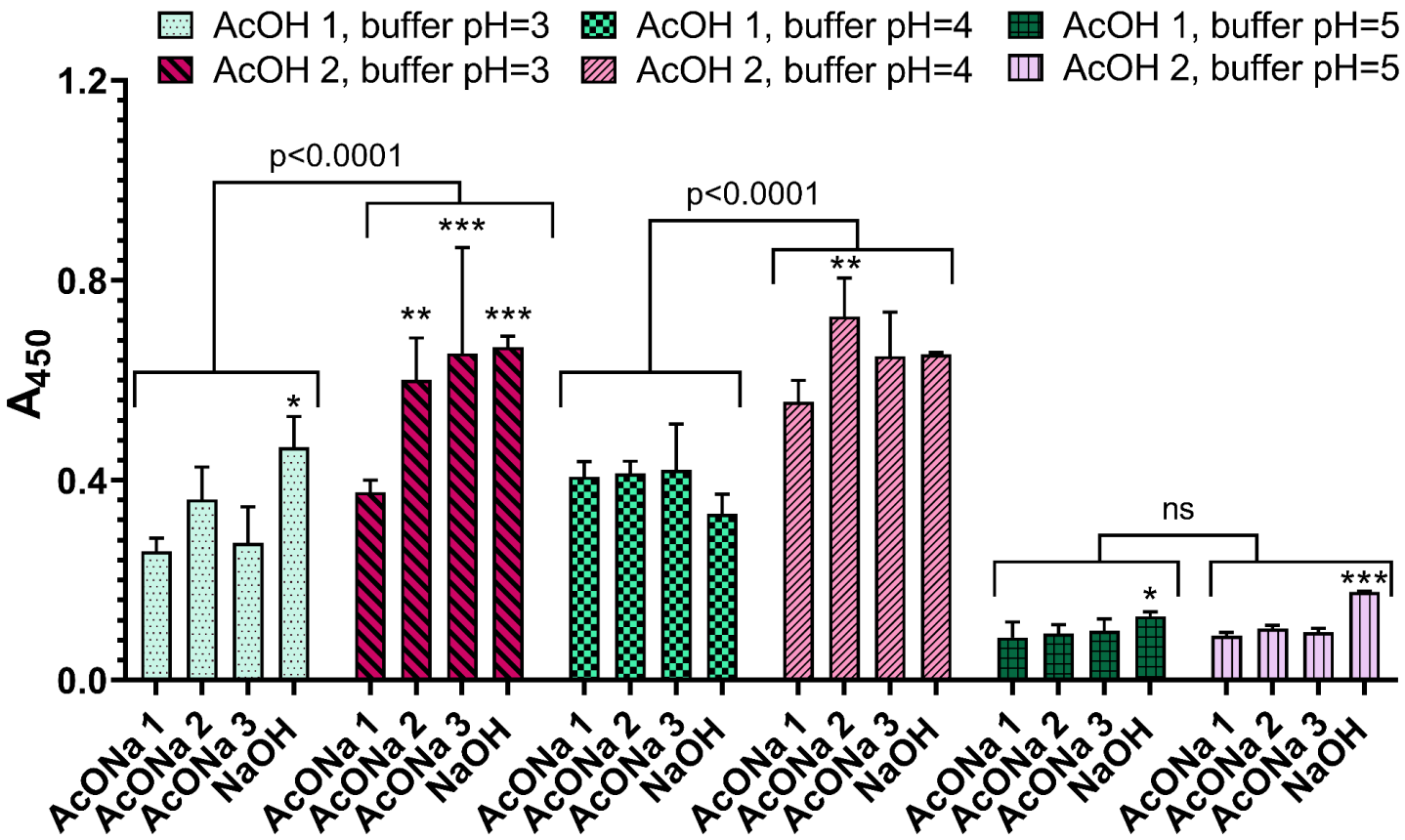


Figure 5

The effect of reagent source on non-specific TMB oxidation. The performance of sodium acetate (AcONa) and acetic acid (AcOH) from different commercial suppliers was compared. AcONa 1 was from Vekton, AcONa 2 from Reachem, and AcONa 3 from Sigma-Aldrich; AcOH 1 was from Vekton and AcOH 2 from LenReactiv. Statistical significance versus the Vekton sodium acetate (AcONa 1) is denoted as: $p < 0.05$ (□), $p < 0.01$ (□□), and $p < 0.001$ (□□□).Reaction conditions: 100 mmol/L buffers, $[H_2O_2]$ = 37.04 mmol/L, [TMB] = 0.8 mmol/L, 10% DMSO (v/v). Data are presented as the mean ± standard deviation (n = 3).

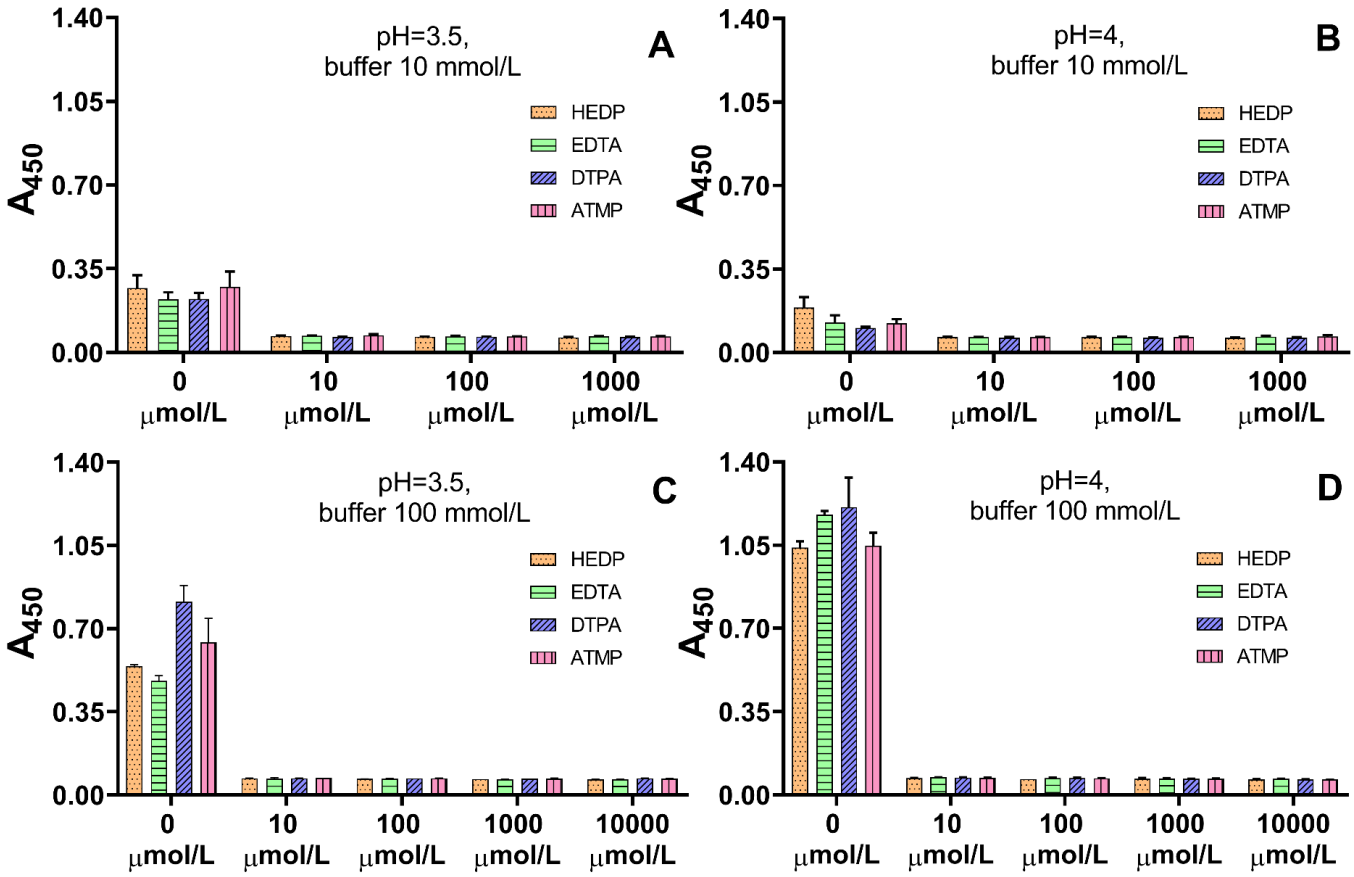


Figure 6

The effect of chelators on non-specific TMB oxidation. The ability of various chelating agents to suppress background oxidation was assessed in sodium acetate buffers of low and high ionic strength. **A, B** 10 mmol/L Na-acetate buffer; **C, D** 100 mmol/L Na-acetate buffer. Reaction conditions: [TMB] = 1.6 mmol/L, [H_2O_2] = 333 mmol/L, 10% DMSO (v/v). Data are presented as the mean ± standard deviation (n = 3).

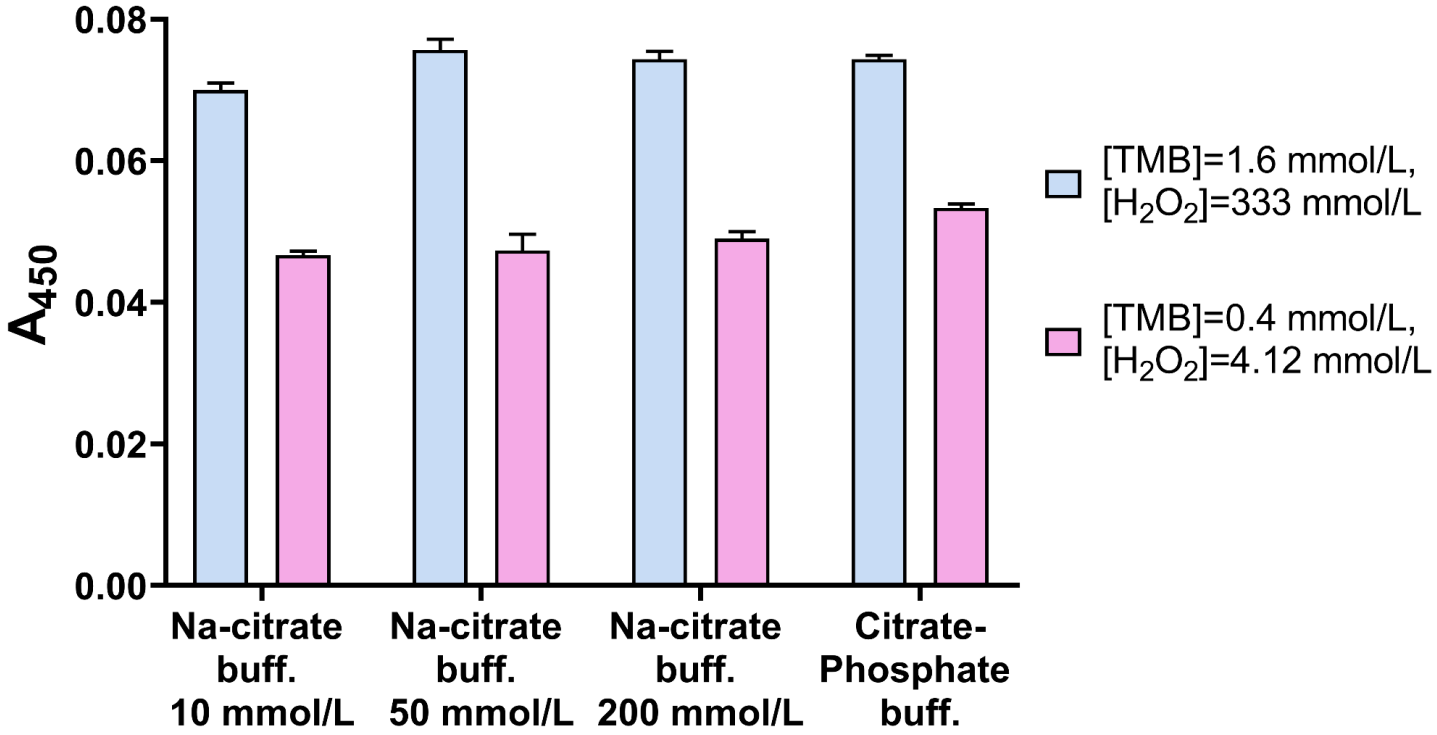


Figure 7

The effect of citrate-based buffers on non-specific TMB oxidation. Citrate and citrate-phosphate buffers were evaluated as alternative reaction media to mitigate background signal. Reaction conditions: pH 4.0, 10% DMSO (v/v). Data are presented as the mean ± standard deviation (n = 3).

Supplementary Files

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