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Analytical Method Optimization of H_2O_2 Derivatization Reaction Time for HPLC Analysis under Tropical Laboratory Conditions

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ABSTRACT

Purpose of the study: This study aims to evaluate the suitability of the commonly reported 2-hour derivatization time for hydrogen peroxide (H_2O_2) determination using HPLC-PDA by assessing the formation behavior of triphenylphosphine oxide (TPPO) under tropical laboratory conditions.

Methodology: The study employed an HPLC–PDA system (Shimadzu LC-20AD) with a reversed-phase C18 column (Waters X-Bridge, 4.6 × 150 mm, 5 µm). Derivatization of H_2O_2 with triphenylphosphine (Sigma-Aldrich) was performed using acetonitrile (Merck). The time-dependent kinetics of TPPO formation were evaluated based on chromatographic peak area monitoring. Data were processed using integrated instrument software.

Main Findings: The TPPO peak area increased progressively with reaction time and approached a plateau at approximately 60 min, indicating near-complete derivatization under the studied conditions. No substantial increase in analytical response was observed at longer reaction times. Repeatability evaluation at 60 min showed acceptable analytical precision with %RSD below 2%, demonstrating that a shorter derivatization time was adequate for routine analysis.

Novelty/Originality of this study: This study provides a context-specific evaluation of derivatization time as part of analytical method optimization in HPLC systems under tropical laboratory conditions. The findings demonstrate the importance of verifying derivatization parameters under specific working environments to improve analytical efficiency and ensure reliable chromatographic performance.

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1. INTRODUCTION

Hydrogen peroxide (H_2O_2) does not possess a significant UV-absorbing chromophore [1], making its direct determination by conventional HPLC–UV detection challenging [2]. To overcome this limitation, derivatization is commonly employed in analytical chemistry to convert non-detectable analytes into detectable forms [3]. In the determination of H_2O_2 by high-performance liquid chromatography (HPLC), derivatization with triphenylphosphine (TPP) to form triphenylphosphine oxide (TPPO) represents an established indirect analytical approach [4], [5]. The reaction involves the oxidation of TPP by H_2O_2, resulting in the formation of TPPO [6], a more polar and UV-active product due to the presence of the phosphoryl (P=O) group [7] (**Figure 1**). The formation of TPPO enables quantification of H_2O_2 through monitoring of a stable UV-absorbing product [8], thereby improving analytical sensitivity and specificity [9].

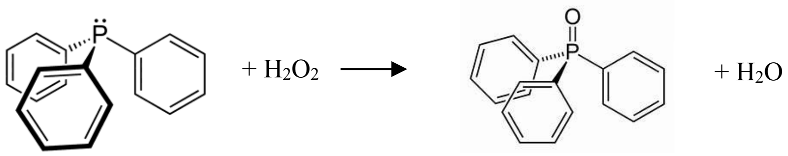

Figure 1. Oxidation of TPP by H_2O_2 to form TPPO and water.

Previous analytical methods utilizing TPP derivatization for H_2O_2 determination have reported different reaction times under varying experimental conditions. A reaction time of approximately 2 hours has been reported under one set of analytical conditions [4], whereas a shorter reaction time of 40 min has been reported under different experimental conditions [5]. These variations indicate that derivatization time is not a fixed parameter and may depend on factors such as chromatographic conditions, reaction environment, solvent composition and experimental setup [10].

In derivatization-based analytical methods, reaction time is a critical parameter that directly affects analytical accuracy and efficiency [11]. Insufficient reaction time may lead to incomplete conversion and potential underestimation of analyte concentration [12], whereas excessively long reaction times reduce analytical efficiency and sample throughput [13].

From a chemical kinetics perspective, the rate of TPPO formation is influenced by several factors governing the oxidation reaction between TPP and H_2O_2. Temperature plays an important role according to the Arrhenius principle, where increasing temperature enhances molecular motion and collision frequency [14], thereby accelerating the reaction rate [15]. Solvent polarity may influence the stability of reactants, intermediates, and transition states during the oxidation process [16]. In addition, reagent concentration affects the probability of effective molecular collisions [17], while activation energy governs the minimum energy required for TPPO formation [18]. Variations in these parameters may alter the time required to achieve complete derivatization and stable TPPO formation under different analytical conditions.

Environmental temperature is one of the experimental factors that may influence derivatization kinetics [19]. Many analytical methods reported in the literature are developed under laboratory conditions typical of temperate regions, whereas laboratories located in tropical regions generally operate at higher ambient temperatures [20], commonly ranging from approximately 22–33 °C [21] depending on geographical and environmental conditions. These differences make evaluation of derivatization conditions under tropical laboratory environments scientifically relevant.

Despite the widespread application of TPP derivatization for H_2O_2 determination, systematic evaluation of derivatization time under specific laboratory conditions remains limited. In many analytical procedures, reaction time is adopted directly from previously reported methods without verification of whether the same conditions remain appropriate under different experimental environments. This may affect analytical efficiency and reproducibility, particularly in derivatization-based methods where reaction completion is essential for accurate quantification. Therefore, reassessment of derivatization time is important not only to improve analytical performance, but also to establish experimentally justified analytical conditions adapted to practical laboratory environments. The present study offers a context-specific evaluation of TPPO formation kinetics under tropical laboratory conditions, providing insight into the optimization of derivatization parameters in HPLC analytical systems.

Based on these considerations, this study aims to evaluate the suitability of the commonly applied 2-hour derivatization time for H_2O_2 determination using HPLC–PDA under tropical laboratory conditions. The formation of TPPO was monitored as a function of reaction time to determine whether the literature-reported incubation period remains appropriate under the studied analytical conditions.

2. RESEARCH METHOD

2.1. Research Design

This study employed a time-dependent experimental approach to evaluate the formation kinetics of TPPO during the derivatization reaction between H_2O_2 and TPP. Derivatization time was treated as the independent variable, while the chromatographic peak area of TPPO obtained by HPLC–PDA analysis was used as the analytical response to monitor reaction progression. All other chromatographic parameters were maintained constant based on previously validated conditions [22]. The experiment was conducted under typical tropical laboratory conditions at an ambient temperature of approximately 32.6 °C, without specific temperature control, in order to reflect routine analytical practice and to evaluate the suitability of the literature-reported derivatization time.

2.2. Materials and Reagents

Hydrogen peroxide solution (30%, analytical grade, Merck), acetonitrile (HPLC grade, Merck), and triphenylphosphine (analytical grade, Sigma-Aldrich) were used as received without further purification. Purified water was produced in-house using a Merck water purification system.

2.3. Instrumentation and Chromatographic Conditions

Chromatographic analysis was performed using a high-performance liquid chromatography system equipped with a photodiode array detector (HPLC–PDA, Shimadzu LC-20AD) with a dual-pump configuration. Separation was carried out on a reversed-phase C18 column (Waters XBridge, 4.6 mm × 150 mm, 5 µm), which was selected to provide adequate retention and separation of the relatively nonpolar TPPO derivatization product.

The mobile phase was applied using a gradient elution system, with its composition detailed in **Table 1**. The flow rate (1.0 mL min^{-1}), injection volume (10 µL), column temperature (40 °C), and detection wavelength (222 nm) were adopted from the previously validated method [22]. The detection wavelength corresponded to the maximum absorption wavelength (λ_max) of TPPO under the studied analytical conditions. These chromatographic parameters were maintained constant throughout the study to ensure consistent analytical performance and to isolate the effect of derivatization time.

Table 1. Gradient Elution Program of the Mobile Phase

Time (min)	Acetonitrile (% v/v)	Water (% v/v)
0.01	50	50
5.50	50	50
6.50	100	0
9.00	100	0
10.00	50	50
20.00	50	50
20.01	Stop	

2.4. Derivatization Procedure

The evaluation of derivatization reaction time was performed using a H_2O_2 standard solution at a concentration of 100 µg mL^{-1}. A 1000 µg mL^{-1} H_2O_2 stock solution was prepared by diluting 0.167 g of a 30% H_2O_2 solution to 50 mL with ultrapure water in a volumetric flask. The working standard solution (100 µg mL^{-1}) was obtained by appropriate dilution of the stock solution using a diluent consisting of acetonitrile and water (65:35, v/v). The concentrations of H_2O_2 and TPP, as well as the derivatization procedure, were adopted from a previously validated method [22] to ensure adequate formation of TPPO within the optimal detection range of the HPLC–PDA system.

A TPP stock solution (0.01 M) was prepared by dissolving 65.5 mg of triphenylphosphine in acetonitrile and diluting to 25 mL in an amber volumetric flask. An aliquot of 1 mL of the TPP stock solution was transferred into a light-protected 10 mL centrifuge tube, followed by the addition of 5 mL acetonitrile and 3 mL ultrapure water. The mixture was vortex-mixed for 1 min. Subsequently, 1 mL of the H_2O_2 standard solution (100 µg mL^{-1}) was added and vortexed again to initiate the derivatization reaction.

The reaction time was defined as the interval between the addition of H_2O_2 to the TPP solution and the injection into the HPLC system. The initial reaction time (0 min) corresponds to immediate injection following mixing and sample preparation. At this stage, the reaction mixture was filtered through a 0.45 µm membrane filter, and the filtrate was transferred into an HPLC vial for analysis. The same filtered reaction solution was subsequently used for all injections. The progress of the derivatization reaction was monitored by injecting the solution at nominal reaction times of 0, 20, 40, 60, 80, 100, and 120 min without further sample preparation. The time intervals were defined based on the chromatographic run time (20 min per injection) and therefore represent approximate reaction durations under practical laboratory conditions.

2.5. Data Acquisition and Evaluation of Reaction Time

Reaction progress was monitored by measuring the TPPO peak area at each selected time interval. Chromatographic data were acquired using the instrument’s integrated software system. The relationship between TPPO peak area and reaction time was evaluated descriptively and by regression analysis to assess the time-dependent formation trend of TPPO and to determine the progression of the derivatization reaction toward completion. A reaction time was considered adequate when the TPPO peak area showed no further significant increase with increasing reaction time, indicating that the reaction had approached completion.

Since the derivatization reaction between TPP and H_2O_2 proceeded continuously throughout the analytical sequence, independent replicate preparations at identical nominal reaction times were not performed during monitoring of reaction progression. Therefore, the reported peak area values represent sequential measurements of the same reaction system under practical laboratory conditions.

Analytical repeatability was evaluated at the reaction time identified from the reaction time evaluation study. A separate derivatization mixture was freshly prepared under the same experimental conditions and allowed to react for the selected reaction time prior to HPLC injection. Repeated injections (n = 6) were subsequently performed, and the repeatability of the analytical response was evaluated based on the relative standard deviation (%RSD) of the TPPO peak area, with an acceptance criterion of less than 2% according to commonly accepted HPLC analytical method validation guidelines [23]. The literature-reported derivatization time of approximately 2 hours [4] was used as a reference point for comparison.

3. RESULTS AND DISCUSSION

3.1. Effect of Reaction Time on TPPO Formation

The effect of derivatization time on the formation of TPPO was evaluated by monitoring the chromatographic peak area at nominal reaction times of 0, 20, 40, 60, 80, 100, and 120 min. The corresponding numerical values are summarized in Table 2, while the relationship between reaction time and TPPO peak area is illustrated in Figure 2.

Table 2. TPPO Peak Area at Different Reaction Times

Time (min)	Peak Area
0	1281826
20	4035740
40	4269909
60	4376125
80	4400794
100	4397128
120	4407549

Regression analysis demonstrated a positive time-dependent trend in TPPO formation during the derivatization process ($R^2 = 0.8062$). The TPPO peak area increased progressively with reaction time and gradually approached a plateau phase, indicating that the derivatization reaction was approaching completion. The moderate coefficient of determination likely reflects the non-linear progression of TPPO formation, characterized by a marked increase during the early stage of the reaction (0–60 min) followed by gradual stabilization of the analytical response at later reaction times. This behavior is consistent with reaction kinetics principles, where the oxidation reaction between TPP and H_2O_2 proceeds rapidly while reactant concentrations remain relatively high [24], leading to frequent effective molecular collisions and active progression of the derivatization process [25].

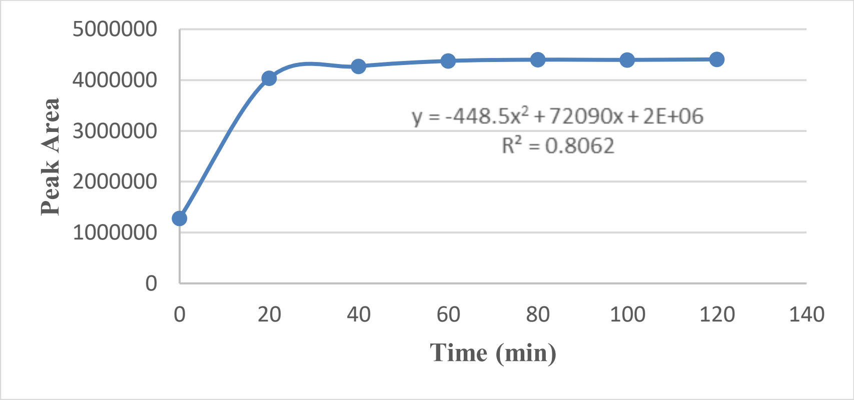

Figure 2. Time-Dependent Formation of TPPO during Derivatization of H_2O_2 with TPP

Beyond approximately 60 min, the increase in TPPO peak area became less pronounced, and the analytical response gradually stabilized. The relatively comparable peak area values observed at 80, 100, and 120 min suggest that the conversion of TPP into TPPO had approached a near-steady condition under the studied experimental conditions. This plateau behavior indicates that additional reaction time produced only minimal further increase in TPPO formation.

Since the derivatization reaction between TPP and H_2O_2 proceeded continuously throughout the analytical sequence, independent replicate preparations at identical nominal reaction times were not performed. Separate preparations could introduce additional variability due to ongoing TPPO formation during waiting time prior to injection. Therefore, reaction progress was monitored sequentially using a single derivatization mixture under consistent analytical conditions. Consequently, the reported peak area values represent sequential measurements of the same reaction system and should be interpreted as approximate reaction durations under practical laboratory conditions.

3.2. Evaluation of Reaction Time

Previous studies have reported a derivatization time of approximately 2 hours as a standard preparative condition for the reaction between TPP and H_2O_2 [4]. In such methods, the reaction time was generally applied as a fixed parameter without detailed evaluation of reaction progress. In contrast, the results of the present study indicate that TPPO formation approached completion within a shorter reaction times. The TPPO peak area progressively increased during the early reaction stage and became relatively stable after approximately 60 min, suggesting that the derivatization reaction had proceeded sufficiently under the studied conditions.

Repeatability assessment performed at 60 min demonstrated acceptable analytical precision, while only minimal changes in TPPO response were observed at longer reaction times. These findings indicate that a derivatization time of approximately 60 min is practically adequate for H_2O_2 determination under the applied analytical conditions.

A shorter derivatization time of 40 min has also been reported under different analytical conditions [5], further supporting the view that derivatization time is not a universal parameter. It should be noted that the derivatization reaction occurs prior to chromatographic analysis [26]; therefore, variations in reaction time cannot be attributed to differences in chromatographic column type. Instead, such variations are more likely associated with differences in reaction conditions [27] , including solvent composition [28], reagent concentration [29], and overall experimental setup. These factors can influence reaction kinetics and contribute to differences in the time required to reach a stable analytical response [30].

In the present study, the evaluation was performed using a standard solution to isolate the effect of reaction time on TPPO formation without interference from complex sample matrices. This approach allows a clearer interpretation of reaction progression and minimizes external variability. The findings highlight the importance of experimentally verifying derivatization time under specific working conditions rather than directly adopting literature-reported values. This consideration is particularly relevant for laboratories operating in tropical environments, where reaction kinetics may differ from those reported under temperate conditions [31], thereby reinforcing the need for context-specific method evaluation.

The experiment was conducted under practical tropical laboratory conditions at an ambient temperature of approximately 32.6 °C. However, temperature was not continuously controlled throughout the analysis. Therefore, the observed reaction behavior reflects practical laboratory conditions rather than a fully controlled kinetic study. Further investigations under controlled temperature conditions may provide more detailed insight into the influence of environmental temperature on TPPO formation kinetics.

Table 3. Comparison of Derivatization Conditions Reported in Previous Studies and the Present Work

Sample type	Reaction time	Derivatization Conditions	HPLC Conditions	Main Contribution	Reference
Hair Cosmetics Product	~120 min	TPP derivatization at 25 °C	C18 HPLC-UV	Fixed derivatization time applied without kinetic evaluation	[4]
Adulterated milk	40 min	TPP derivatization at 25 °C	C8 HPLC-PDA	Shorter derivatization time reported under different analytical conditions	[5]
Standard H_2O_2 solution	~60 min adequate	TPP derivatization under tropical laboratory conditions	C18 HPLC-PDA	Time-dependent evaluation of TPPO formation and reassessment of derivatization time under practical laboratory conditions	Present study

As summarized in Table 3, previous studies generally applied derivatization time as a fixed analytical parameter under specific experimental conditions. In contrast, the present study systematically evaluated the time-dependent formation of TPPO under tropical laboratory conditions to assess the suitability of literature-reported reaction times in practical analytical environments. This comparison highlights the contribution of the present work toward context-specific optimization of derivatization parameters in HPLC-based analytical methods.

3.3. Stability of TPPO Response

To evaluate the repeatability and stability of the analytical response at the selected practical derivatization time, a separate derivatization mixture was prepared and allowed to react for 60 min under the same experimental conditions. Repeated injections (n = 6) were subsequently performed using the same derivatized solution. The results showed that the TPPO peak area remained consistent across consecutive injections, with a relative standard deviation (RSD) of 0.093% (n = 6), which is below the commonly accepted limit of 2% for repeatability in HPLC analytical method validation [23]. The individual peak area values are summarized in **Table 4**.

Table 4. Repeatability of TPPO Peak Area at 60 min Reaction Time

Injection	Peak Area
1	4397435
2	4394424
3	4389573
4	4386377
5	4388543
6	4392838
Mean	4391532
SD	4105
%RSD	0.093

The low %RSD value indicates good repeatability of the chromatographic system and demonstrates that the analytical response had become sufficiently stable at 60 min for routine analytical application. These findings support the conclusion that a derivatization time of approximately 60 min provides adequate TPPO formation with acceptable analytical precision under the studied laboratory conditions.

3.4. Implications for Analytical Practice

From an analytical perspective, reducing the derivatization time from 2 hours to approximately 60 min offers a significant advantage in terms of laboratory efficiency and sample throughput. This improvement is particularly relevant for routine HPLC analysis, where shorter derivatization time can enhance analytical workflow and reduce total analysis time without compromising analytical reliability.

The present findings further demonstrate that reassessment of derivatization conditions under practical laboratory environments can improve analytical workflow efficiency while maintaining reliable chromatographic performance. Since derivatization efficiency may be influenced by reaction conditions, verification of reaction time is essential to ensure optimal TPPO formation and consistent analytical response. In addition, the time-dependent monitoring of TPPO formation provides a practical illustration of the relationship between reaction kinetics and analytical response in derivatization-based chromatographic methods.

4. CONCLUSION

The derivatization reaction time of H_2O_2 with triphenylphosphine was successfully evaluated under tropical laboratory conditions using HPLC–PDA analysis. The results demonstrated that TPPO formation reached a practically adequate level within approximately 60 min, while repeated injections at this reaction time showed acceptable analytical repeatability. These findings indicate that derivatization time should be experimentally verified under specific laboratory conditions rather than directly adopted from literature-reported methods. The present study contributes to the context-specific optimization of derivatization parameters in HPLC-based analytical methods. Further studies are recommended to evaluate the applicability of the optimized reaction time in complex sample matrices and under controlled temperature conditions.

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AUTHOR CONTRIBUTIONS

Conceptualization, D.; Methodology, D.; Software, D.; Validation, D.; Formal Analysis, D. and H.; Investigation, D.; Resources, D.; Data Curation, D..; Writing – Original Draft Preparation, D.; Writing – Review & Editing, H.S. and A.R.; Visualization, D.; Supervision, K., P.S., and S.; Project Administration, D.; Funding Acquisition, D. and H.

CONFLICTS OF INTEREST

The authors confirm that there are no conflict of interest regarding the publication of this research.

USE OF ARTIFICIAL INTELLIGENCE (AI)-ASSISTED TECHNOLOGY

Not applicable.

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