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Research article

Nanolevel of detection of ascorbic acid using horse-radish peroxidase inhibition assay

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ABSTRACT

Ascorbic acid plays a significant role in regulation of various bodily functions with high concentrations in immune cells and being involved in connective tissue maintenance. Commonly it is detected through various colorimetric methods. In this study, we propose a one-step simple method based on the inhibitory activity of ascorbic acid on horseradish peroxidase and hydrogen peroxide. The detection is observed by colorimetric changes to TMB (3,3′,5,5′ tetramethylbenzidine). The enzyme inhibition unit was optimized with a high level of linearity ($r^2 = 0.9999$) and the level of detection and level of quantification were found to be 1.35 nM and 4.08 nM, respectively with higher sensitive compared to the HPLC method (11 μM). Both intra and inter-assays showed high correlations at different AA concentrations. ($r^2 > 0.9999$). Similar results were also observed for vitamin C tablets, ascorbate salts, fruits, and market products ($r^2 = 0.999$). There was negligible effect of interference by citric acid, lactic acid, tartaric acids, and glucose with high recoveries (>98%) at 1 mg/mL to 0.0078 mg/mL concentration ranges. The recovery error (RE%) was found to be less than 10%. Our detection method is distinguished by its simplicity, nano-level of detection, reproducibility, and potential application and adaptability as a point-of-use test.

1. Introduction

Ascorbic acid (AA) commonly known as vitamin C, is a derivative of glucose that is involved in several hydroxylation reactions in our body [1]. Vitamin C can reduce the oxidizing ability of peroxidase substrates by scavenging free radicals which in turn boosts the immune system and regulates the cellular redox metabolism [2]. Ascorbic acid promotes the synthesis of collagen and

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mucopolysaccharide, increases the compactness of blood capillaries, reduces permeability and fragility, and increases body resistance [3,4]. In its absence, hematopoietic dysfunction, anemia, increased permeability of the microvascular wall, increased fragility, and bleeding due to easy rupture of the blood vessels, are caused while in severe cases, bleeding from muscles and internal organs, which are clinically commonly referred to as scurvy is caused [4]. Being an electron donor, AA serves as an important quality indicator of foods and drinks due to its low-molecular-weight antioxidant property [5]. Owing to many studies that have presented the advantage of topical application of AA for skin-lightning, anti-aging and photoprotection purposes, AA has been widely used in the cosmetic industry [6–9]. The skin-lightning effect of AA can be attributed to its reduction in melanin synthesis through the suppression of tyrosine's catalytic activity [10]. Additionally, the antioxidant activity of AA helps to prevent many non-communicable diseases such as cancer, and physiological ones associated with lifestyle or age related diseases such as hypertension, muscular degeneration, neurodegeneration, cataracts, etc. [11,12].

Given the importance of AA to humans, a series of new assays have been developed in recent years, and many improvements have been made to the classical methods [1]. Recently, rapid, low-cost as well as reliable assays were developed by the combination of electrochemical-based techniques combined with micro- and nano-sized electrode materials. These electrochemical microsensors allow real-time monitoring capability, high sensitivity and selectivity of target biomolecules. Such development irrespective of its interdisciplinary involvement spanning multiple research fields such as material science, nanotechnology, and electrochemistry, high operation technology with complicated steps while at the same time being costly. Furthermore, the presence of a variety of low- and high-molecular weight compounds such as the macromolecules as well as polyphenols, pesticides, pigments, acid and bases may produce interfering signals that may affect the high performance of these sensor-based techniques resulting in false positives or negatives [13,14].

Recently, there has been the development of instrument-free point of care microfluidic device. The device involves two color channels with a connector that determines the results based on the ratio of distances [15]. Furthermore, cerium-doped strontium-based metal organic frameworks (Ce-SrMOFs) and Ce-BPyDC has been studied to provide peroxidase-like nanozyme activity for total oxidant capacity detection in body [16,17]. Additionally, there has been work on carbon nanodots and paper-based assay, where the former lowered detection limit while maintaining an increase in sensitivity as well as selectivity while the latter decreased the cost of production [18,19]. AA was detected using TMB with other types of oxidizing agents with oxidase-like and/or peroxidase-like activity such as such as Ce-BPyDC, Co–Fe Prussian blue analogue nanocube (Co–Fe PBA NC), Cu-imidazole-2-carboxaldehyde (Cu-ICA) and Fe–Mn bimetallic nanozyme (FeMnzyme) [17,20–22].

In our current approach, we designed a method of detection of AA by inhibiting the horseradish peroxidase redox cycle where the color development of the reaction is inhibited either by competing and/or inhibiting the charge transfer cycle (CTC) of TMB and hence the reduction of colorless TMB into blue color end product is inhibited. In this reaction, TMB acts as an electron donor and converts compound I into compound II and finally back the enzyme into its original state by utilizing another hydrogen ion and turning colorless TMB into a blue color solution [23–26]. Since the reaction is carried out by peroxidase, the relative activity of it can be calculated by the rate of oxidized co-substrate formed [27,28]. The presence of AA as a strong electron donor, the catalytic reaction can easily block the involvement of TMB by donating electrons to complete the reaction cycle while itself being oxidized to colorless dehydroascorbic acid [29,30]. The detection of the colorimetric signal is further enhanced and stabilized using a selected stopping solution like sulphuric acid. The presence and quantity of AA in various samples can easily be determined using the spectrophotometric method by measuring the inhibition of colorimetric intensities followed by the calculation of the percent (%) of inhibition of HRP in the Redox cycle against the concentration of AA. In our proposed method, we have extracted the enzyme horseradish peroxidase (HRP), from the root of horse radish, purified, and used following standardization [31]. We also formulated equations for the measurement of enzyme inhibition unit (IU), correlates with the concentrations of AA, and constructed a calibration curve for its application of detection of AA from fruits and vitamin C products commonly available in the market.

2. Experimental procedures

2.1. Preparation of horseradish peroxidase

Horseradish peroxidase (HRP) enzyme was extracted from *Raphanus raphanistrum*. The crude fluid was extracted and centrifuged at 10000 rpm (6720 g) for 10 min. The supernatant was collected and filtered through a 0.2 μm syringe filter (Corning, USA). The resultant solution containing HRP was allocated into a 0.5 mL vial and preserved at -20 °C until further use. The purity of HRP was determined using Genscript SurePage™ (GenDScript Biotech, USA) protocol against reference HRP (Cat. N0. P8375-2KU, Sigma-Aldrich, USA) while the band patterns were recorded using ImageQuant™ LAS 500 (GE, USA). The selected regions were quantified by using NIH ImageJ software (NIH, USA).

2.2. Enzymatic performance of purified HRP

Myeloperoxidase (MPO) (Sigma-Aldrich, USA) and reference horseradish peroxidase. were used to observe the performance of purified HRP. HRP was diluted in 96 well-plate at different concentrations of 1:500, 1:750, 1:1000, 1:1250, 1:1500, 1:1750, and 1:2000. Chromogen solution, 3,3′,5,5′-tetramethylbenzidine (TMB) (Sigma-Aldrich, USA), was added to observe the color intensity. Diluted HRP was tested and confirmed at a defined OD value of 2.5–2.8.

2.3. Development and optimization of HRP inhibition assay protocol

2.3.1. HRP inhibition assay was developed as a one-step procedure

The assay protocol was optimized by dissolving 100 mg of ascorbic acid (AA) (Sigma-Aldrich, USA) in 1 mL of double distilled water (ddH_2O). The stock solution was diluted to a concentration of 10 mg/mL in phosphate buffer saline (PBS) solution and stored as master stock. The working solution was prepared by further dilution of the master stock into 1.0 mg/mL in PBS. Further dilutions were carried out ranging from 100 μg/reaction to 1.0 μg/reaction and seeded as triplicates in 96 well-plate. HRP [50 μL laboratory extracted-1:1250 and/or ref-1:100 k (0.1 μg/reaction)] was added, followed by 50 μL of TMB + H_2O_2 (Sigma-Aldrich, USA). The plate was incubated in the dark at room temperature for 10 min. Finally, 50 μL of sulphuric acid (1.5 mM) (Merck-Millipore, USA) was added to stop the color development and the plate was read at 450 nm using a microplate reader (Thermofisher, USA). Three blanks (PBS and TMB) and three enzymes (HRP, PBS) were kept as controls. The limit of detection (LOD) and the limit of quantification (LOQ) were obtained by testing for IU at ranges from 10 μg/mL to 0.25 μg/mL.

2.3.2. Calculation of percent of inhibition, inhibition unit, and concentration of ascorbic acid

The percent inhibition of HRP by AA was calculated using the formula mentioned:

$$\%\ \boldsymbol{of\ Inhibition} = \mathbf{100} - \left[\left(\frac{(\boldsymbol{ODs} - \boldsymbol{ODp})}{(\boldsymbol{ODe} - \boldsymbol{ODp})}\right) \times \mathbf{100}\right] \tag{A.1}$$

ODs is the value of the optimal density of the sample, while ODp is the value of the optimal density of the plate and ODe is the value of the optimal density of the enzyme.

The least amount of AA (μg/mL) inhibiting >99% of the reaction is considered as one inhibition unit (1 IU). Following the calculation of inhibition unit (IU), AA concentration was calculated using the following formula:

$$\frac{\mathbf{1}\boldsymbol{IU} \times \boldsymbol{PI}}{\mathbf{100}} \boldsymbol{x\ df} = \boldsymbol{mg} \Big/ \boldsymbol{mL} \tag{A.2}$$

Where 1 *IU* is as defined above, *PI* is the percent of inhibition and *df* is the dilution factor of the test sample.

2.3.3. Chromatographic assay (HPLC)

The analysis of ascorbic acid was performed using a reversed-phase column packed with octadecyl silane bound to 5 μm porous silica particles (Thermo Scientific, Lithuania), with dimensions of 150 mm × 4.6 mm. A calibration curve was prepared in the concentration range of 100 μg/mL to 10 μg/mL diluted in the mobile phase. The mobile phase consisted of a mixture of MilliQ Water, methanol, and glacial acetic acid in a ratio of 69:30:1 by volume. The pH of the buffer was adjusted using 0.0 1 N HCl. The analytes were scanned spectrophotometrically at a wavelength of 254 nm using a Shimadzu SPD-40 spectrophotometer, and the data obtained were processed using LabSolutions CS software (Shimadzu, Kyoto, Japan). The intermediate precision was checked by repeating the tests on five different days in triplicates.

2.3.4. Stability of ascorbic acid

The stability of AA at laboratory lighting was conducted using concentrations of AA ranging from 100 μg/mL to 10 μg/mL under two conditions such as exposing plates to laboratory light and in dark (fully covered with aluminum foil). Two experiments were carried out at 2-h intervals according to the aforementioned protocol and the obtained results were calculated as per the formula mentioned in A1 & A2).

2.4. Construction of ascorbic acid calibration curve

100 mg/mL (5.6 mM) concentration of ascorbic acid master stock was prepared using ddH_2O and stored at 4 °C in the dark until further use. The master stock was further diluted ranging from 100 μg/mL to 0.78 μg/mL (0.56 μM–0.0044 μM) in phosphate buffer saline (PBS) solution and HRP inhibition assay was conducted with both laboratory and reference HRPs according to the aforementioned protocol in triplicates. The percent of inhibition and enzyme inhibition units were calculated according to formulas A1 & A2. The calibration curve was constructed by plotting enzyme (HRP) inhibition units (IUs) against concentrations of standard ascorbic acid.

2.5. Limits of detection and quantitation assay

For the determination of LOD and LOQ, ascorbic acid solutions were subjected to serial dilutions until a signal/noise ratio of ≈3.3 for LOD and ≈10 for LOQ was obtained. The standard error and slope generated by calibration curve using 19 samples were used for the LOD and LOQ are measured using ICH guidelines (2021) [32].

$$LOD = (Standard\ error\ X\ 3.3)/slope \tag{A.3}$$

$$LOD = (Standard\ error\ X\ 10)/slope \tag{A.4}$$

2.6. Validation of the proposed method

The validity of the developed method was tested according to the standard procedures (ICH Guidelines, 2021 [32]. The linearity and ranges were studied by preparing calibration curves which were constructed in triplicates at nineteen (19) concentrations of standard AA. All statistical analysis and linearity of calibration graphs were prepared using Microsoft Excel 2019.

2.6.1. Precision studies (intra and inter-assay)

The precision and accuracy of the proposed method were determined by analyzing ten (n = 10) different concentrations of the AA in triplicate using ICH guidelines (2021) [32]. Repeatability of precision studies was conducted by analyses of ten (n = 10) different concentrations of AA in triplicates on the same day. Accuracy was determined for both intra- and inter-day variations by analyzing the same concentration of samples in triplicates.

2.6.2. Interference and recovery studies

Possible interference with ascorbic acid by citric acid, lactic acid, tartaric acid, and glucose, was measured at different concentrations. 16 IU–0.5 IU of ascorbic acid was mixed with 1 mg/mL of citric acid, lactic acid, tartaric acid, and glucose. Testing was carried out using the HRP inhibition assay using the aforementioned protocol. Relative error was measured using the formula:

$$RE\ (\%) = \left(\frac{Cf - Ct}{Ct} \right) \times 100 \tag{A.5}$$

Where *RE* is the relative error, Cf is the observed value of percent inhibition and *Ct* is the true percent of inhibition of standard ascorbic acid. Non-inference substance was decided if the relative error obtained was less than 10%.

2.7. Application of the method

2.7.1. Determination of ascorbic acid in vitamin C effervescence tablet

Tablets were weighed and finely powdered and an amount of powder equivalent to 100 mg of ascorbic acid was weighed and dissolved in ddH_2O. For the sachet of vitamin C, powder from the sachet weight equivalent to 100 mg was used. The suspensions were sonicated for 10 min, with any remaining residues being removed by filtration. The clear solutions obtained were then appropriately diluted with PBS and a master stock solution of concentration 10 mg/mL was prepared. A working solution of 1 mg/mL was prepared from the master solution and finally used as a range from 100 μg/reaction to 10 μg/reaction. Standard ascorbic acid was used as a control.

2.7.2. Determination of ascorbate salts

Ascorbate salts (Sodium, glucoside, and ether; MySkinRecipes, Thailand) were diluted in PBS with concentration ranging from 16 to 0.25 enzyme units equivalent to 1 mg/mL to 0.0156 mg/mL and was tested using the aforementioned assay protocol. A standard ascorbic acid using identical concentrations was used as a control.

Further to identify inhibitory effects of other components in ascorbate salt (ascorbate glucoside) we spiked varying concentrations of ascorbic acid (10 μg to 1 μg) with the constant amount of one of the salts (ascorbate glucoside, 10 μg).

2.7.3. Recovery of ascorbic acid from spiked effervescence vitamin C tablet

A standard AA solution of different concentrations ranging from 32 to 0.25 enzyme units (IU) equivalent to 2 mg/mL to 0.0156 mg/mL was spiked with an equal concentration of vitamin C tablet (according to the manufacturer's claim) and experimented according to the developed protocol. Results were analyzed in terms of rate of recovery and percent of recovery error using formulas mentioned below:

$$\text{Recovery rate}\ (\%) = \left(\frac{(\text{Qrec} - \text{Qpre})}{\text{Qadd}} \right) \times 100 \tag{A.6}$$

where Q_{rec} represents mg of AA recovered, Q_{pre} represents quantity of AA present in vitamin C tablet and Q_{add} represents mg added ascorbic acid into equal concentrations of vitamin C tablet

$$\text{Recovery error}\ (\%) = \left(\frac{\text{Cqf} - \text{Cqt}}{\text{Cqt}} \right) \times 100 \tag{A.7}$$

where *Cqf* is the quantity of recovered AA (mg/mL) and *Cqt* is the quantity of standard AA is added.

2.7.4. Determination of ascorbic acid in fruits

Fruits (orange, mangosteen, grapes, and tomatoes) were crushed, collected fluid (1 mL) and each preparation was filtrated through 0.2 μm membrane filters fitted to a syringe. For the assay, the filtrate solution was further diluted in two-fold dilution in PBS and conducted the assay forehead mentioned protocol. Standard ascorbic acid ranging at concentrations from 32 to 0.25 enzyme units equivalent to 2 mg/mL to 0.0156 mg/mL was used as a reference.

2.8. Statistical analysis

The linear association between exposure and outcomes was assessed using a simple linear regression model. Additionally, the r^2 between the two assays on different days was examined. For data analysis and figure preparation, we utilized GraphPad Prism 8.3.2, STATA (version 15), and Python-3.11 within Jupyter Notebook.

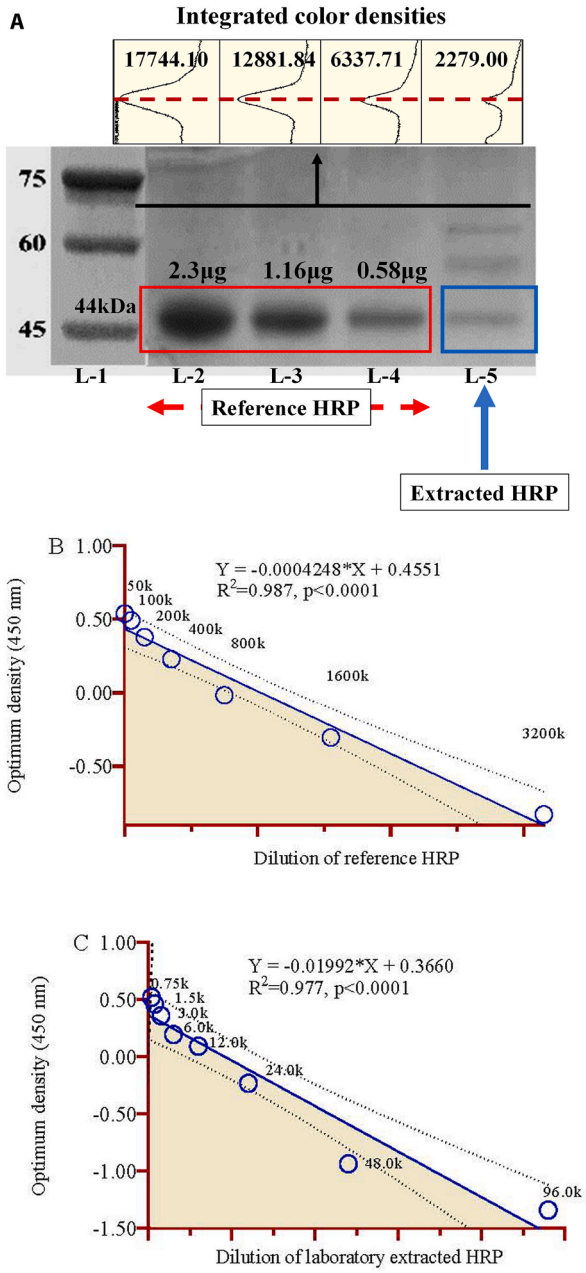

Figure-1. Molecular characterization of laboratory-extracted HRP using (A) SDS-PAGE analysis and its comparison with reference HRP. [L-1; protein marker (75 kDa–45 kDa), L-2-L4; reference HPR at different concentrations and L-5; laboratory extracted HRP] (B) The optimal density of HRP was further evaluated at different dilutions and 1:1250 dilution was used for the development of ascorbic acid-based HRP inhibition ($r^2 >$ 0.987). (C) Comparative analysis of optical density of horseradish peroxidase with myeloperoxidase ($r^2 = 0.977$). Linear regression was used to estimate the regression coefficient and p-value.

2.9. Samples preparation

After the weighing of the tablets, they were finely powdered and an amount equivalent to 100 mg of ascorbic acid was dissolved in ddH_2O. The suspensions were sonicated for 10 min followed by filtration to remove solid residues. Finally, the filtered solutions were then diluted in with PBS as required for the experiment.

3. Results and discussion

3.1. Extraction, purification, and enzymatic performance of HRP

The extracted fluid from the root of locally available radish following purification, the presence of HRP was determined by SDS-PAGE analysis whereby a band at 44 kDa was confirmed and compared with reference HRP (Figure-1(A)) which was previously reported [31]. The presence of an additional two bands on top of 44 kDa in laboratory-extracted HRP could be contaminants of the crude extract from the root of horseradish. The quantitative analysis of the selected band in SDS-PAGE was conducted by NIH- ImageJ software program and compared with reference HRP [33]. The biological activity (peroxidase) of the extracted HRP was confirmed in the presence of H_2O_2+TMB solution and compared with myeloperoxidase MPO) which showed a high level of OD values at 450 nm wavelength (>3.0 at 1:500 dilution; $r^2 > 0.987$) (Figure-1(B) and Figure-1(C)). The enzymatic activity of extracted horseradish peroxidase is supported by previous reports [2,27]. The working dilution of the extracted HRP was determined by further titration and 1:1250 dilution of master stock of HRP (OD value > 2.5–2.8), Similarly, the reference HPR was also optimized and 1:100 k (500 ng/reaction) dilution was found to be optimum (OD > 2.7–2.9) for the comparative analysis of the assay. The concentration and/or dilutions of both HRPs were validated in three separate runs using 54 replicates (n = 54) (SD = 0.119; SE = 0.134; *p-value*<0.0001) and were used for the optimization and development of the assay.

3.2. Development and optimization of the method

3.2.1. Colorimetric method

The assay protocol was developed using 12 concentrations of standard AA ranging from 100 μg/mL - 2.5 μg/mL against both

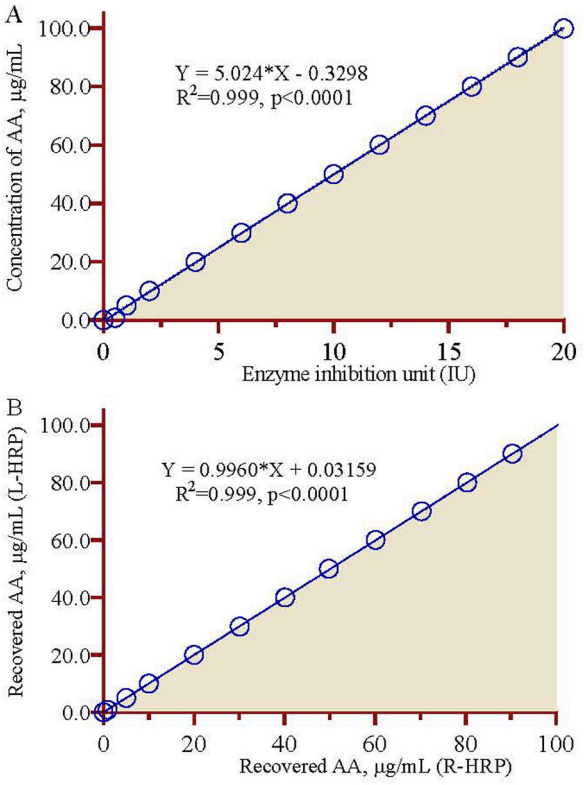

Figure-2. The establishment of the assay protocol involved two main steps. (A), a linear association was established for the assay protocol using 12 concentrations of AA ranging from 100 μg/mL to 2.5 μg/mL, yielding a high correlation coefficient ($r^2 = 0.999$; $P < 0.0001$). (B), a comparative analysis was conducted to assess AA recovery using both laboratory HRP (L-HRP) and reference HRP (*R*-HRP), with a similarly high correlation coefficient ($r^2 = 0.999$; $P < 0.0001$). The regression lines were fitted using a linear regression model, and the p-value was estimated.

laboratory and reference HRPs according to the aforementioned protocol. Results obtained following the experiment were calculated in terms of percent inhibition using (formulas A.1 & A.2) and a linear curve was generated taking the concentration of ascorbic acid as an abscissa and the enzyme inhibition unit (IU) as an ordinate and as shown in Figure-2(A). A strong linear relation is obtained within a range of 2.5 μg/reaction to 100 μg/reaction, and a linear equation is Y = 0.996x+0.0315, wherein Y is the value enzyme inhibition unit (IU) and x is the concentration of AA, and the unit is μg/reaction; a high level of linear correlation coefficient was also achieved (r^2 = 0.999). The comparative performance was analysied against the reference HRP and is presented in Figure-2(B).

3.2.2. Determination of one enzyme inhibition unit (1 IU)

The enzyme inhibition unit *(*1 IU*)* was determined using low concentration of standard AA ranging from 10 μg/reaction to 1 μg/reaction according to the aforementioned protocol and 1 IU was established at a concentration of 3 μg/reaction with strong linearity *(*r^2 = 0.9999*)* and is shown in Figure-3. The IU was further validated using 50 replicates in two separate assays using both laboratory and reference HRPs and showed good consistency in terms of concentration versus IU *(*SD = 0.015; SE = 0.238; *p-value*<0.0001*)*.

3.2.3. Chromatographic method (HPLC)

The protocol was further optimized using HPLC using standard AA ranging from 80 to 10 μg/mL and a calibration curve was constructed and the result is shown in Figure-4(A). A consistent and identical retention time (3.533 min) was observed against all concentrations of standard AA detected by HPLC (100 μg/mL – 40 μg/mL) and a high level of linear correlation coefficient was also achieved in terms of peaks and concentration of AA and is presented in Figure-4(B) (r^2 = 0.987; SD = 0.0597; *p-value*<0.0001). The sharp and symmetrical peaks with minimal interferences from equipment noise are supported by previous reports [34,35]. Results obtained from the HPLC analysis of standard AA supported the accuracy of the protocol of the current method of detection of AA by HPR inhibition assay (Figure-4(C)).

3.3. Construction of calibration curve based on the optimized assay protocol

Linearity and ranges were studied by preparing calibration curves, which were constructed in triplicates at 19 concentrations (100 μg/reaction to 0.78 μg/reaction) of standard AA using laboratory-extracted HRP. The assay was carried out in triplicates according to the aforementioned protocol and the colorimetric spectra of each set of concentration at the excitation wavelength of 450 nm were measured, enzyme inhibition units were calculated based on the percent inhibition of the reaction and shown in Figure-5. The linearity of the coefficient of correlation between the concentration of AA and IU was found to be tightly correlated (r^2 = 0.9999; SE = 0.00282; *p-value*<0.0001*)*.

3.4. Determination of limit of detection and limit of quantification

For the experimental protocol described previously, the linearity data were computed presented in Figure-5. The regression equation derived was y = 0.32x+0.0001 with r^2 of 0.9999, where x represents the concentration of AA in μM/reaction, and y represents the IUs. The LOD and the LOQ were found to be 1.35 nM and 4.08 nM, respectively. To the best of our knowledge, the detection level of ascorbic acid in terms of LOD and LOQ by this method is more sensitive than published reports and is presented in Table-1 [36–43].

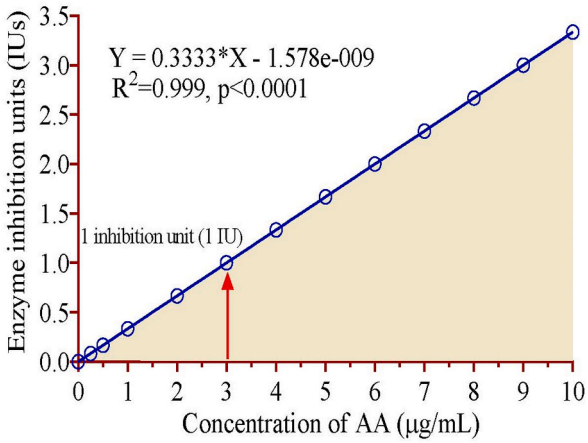

Figure-3. The determination of enzyme inhibition units (IU) involves setting one IU at a concentration of 3 μg/mL of standard AA, with r^2 of 0.999. The lowest concentration of AA, at which 100% inhibition was observed, was statistically significant (*p-value*<0.001). The linear regression model was employed to fit the regression line.

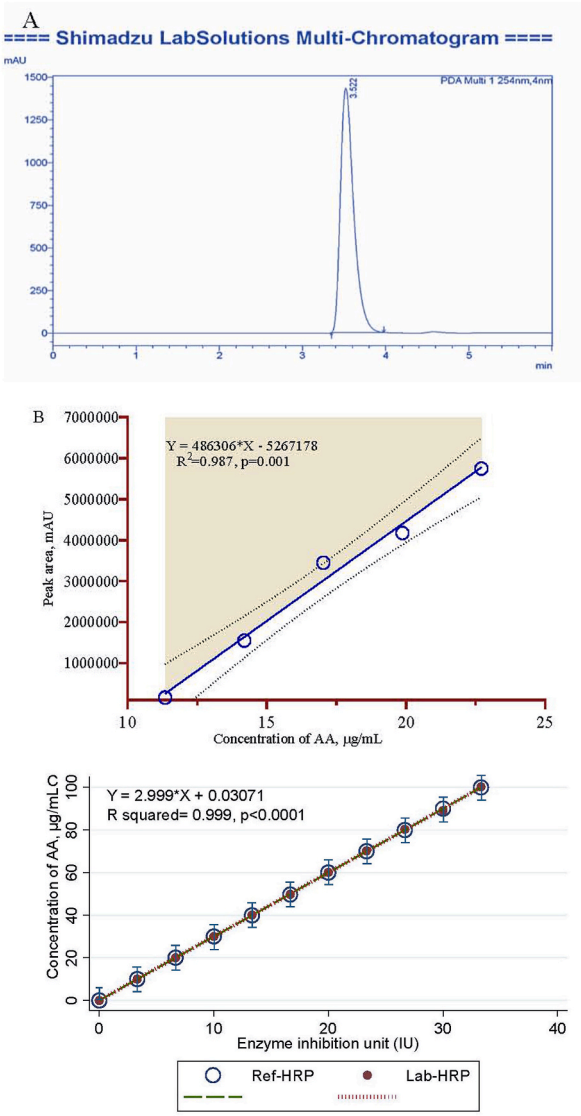

Figure-4. A chromatogram of standard ascorbic acid by HPLC analysis; (A) The chromatogram exhibits clear, sharp, and singular identical peaks with an identical retention time of 3.533 min. (B) The calibration curve, constructed based on HPLC peaks and concentrations of ascorbic acid, indicates that the last peak with a retention time of 3.533 min corresponds to a concentration of 11 µM/mL ($r^2 = 0.987$, *p-value* = 0.001). (C) The proposed method successfully detects standard ascorbic acid at a concentration of 10 µg/mL (0.056 µM) with a high correlation coefficient ($r^2 = 0.999$, *p-value*<0.0001). Linear regression was utilized to estimate the regression coefficient and p-value.

3.5. Validations of the developed AA detection method

3.5.1. Stability of the proposed method following exposure to laboratory lighting

For the experimental protocol described previously, the effect of photodegradation of AA on the proposed method was conducted and compared under two conditions: laboratory lighting and darkness for 4 h. The degradation rate of AA was calculated using the formula mentioned in experimental procedures at 2-h intervals and there is no apparent degradation was observed even after 4 h of treatment as presented in Figure-6(A)&(B). This finding strongly suggested that the detection of AA by the proposed method will not be affected at 4 h of exposure to laboratory conditions as our technique is a stepped 15-min test.

3.5.2. The intra- and inter-assays

For the experimental protocol described previously, linear plots were constructed using calculated values of IU against the concentrations of AA which showed excellent correlation coefficient ($r^2 = 0.99$, 95% CI = 0.99, 1.00) of enzyme inhibition units against concentrations of AA, and intermediate precision was checked by repeating the experiments day apart in triplicates. The repeatability, intermediate precision, and accuracy were evaluated by the recovery studies and are presented in Figure-7(A) and Figure-7(B). The R.

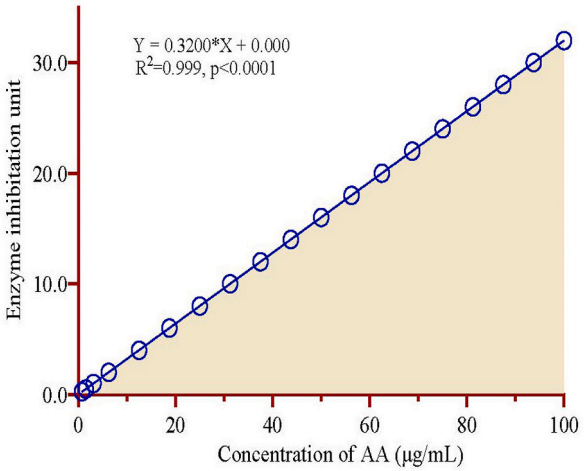

Figure-5. The Calibration Curve, generated with 19 varied concentrations of standard ascorbic acid (ranging from 100 µg/mL to 0.78 µg/mL), demonstrated exceptional linearity ($r^2 = 0.9999$) in relation to enzyme inhibition units. The slope of the curve was calculated as 0.320, and the statistical significance was confirmed with a *p-value* of less than 0.001. The regression line was fitted using the linear regression model.

Table-1
Comparative analysis of analytical assays for the detection of Ascorbic Acid by colorimetric methods (AA) (Adapted from Di et al. [36]).

Catalysts	Linear range (µM)	LOD (µM)	LOQ[a] (µM)	Refs
Ag^+/TMB	0.2–10	0.05	0.151515	[36]
CQDs/TMB/H_2O_2	10–70	3.26	9.878788	[37]
MoS_2/NC/TMB/H_2O_2	0.2–80	0.12	0.363636	[39]
ClO/TMB	1–70	0.58	1.757576	[41]
MIL-68	3–40	0.256	0.775758	[42]
Co-POP/TMB	20–400	1.6	4.848485	[43]
HRP/TMB/H_2O_2/OD	0.056–0.00442	0.00135	0.004091	Our method

[a] LOQ was calculated by multiplication of LOD with 3.03 (LOQ = LOD × 3.03).

S.D. values, for the two systems, on intra-day and inter-day experiments were found to be within the acceptable limits of less than 1.0% (0.157%) indicating that the methods were sufficiently precise. Besides, good recoveries of ascorbic acid were obtained at each concentration (within 100 ± 0.02), suggesting the accuracy of the proposed methods. The proposed assay protocol was validated using high concentrations of standard AA ranging from 1 mg/mL to 8.0 mg/mL and obtained results showed good linearity ($r^2 = 0.999$) in terms of concentrations and enzyme inhibition units and is presented in Figure-7(C). This finding indicated that the proposed method can be equally applicable to samples with higher concentrations.

3.5.3. Interference and recovery study

3.5.3.1. Vitamin C tablets. For the experimental protocol described previously, results of the analysis of the recovery of ascorbic acid in the presence of interference substances commonly incorporated with commercial products like effervescence vitamin C tablets are presented in Table-2. The average values of recovery ranged from 97.7% to 104.2% with recovery errors well below the acceptance rate (Table-2) and are in conformance with the requirements of the AOAC about the level of recovery of an analytical method [34].

3.5.3.2. Interference study with acids commonly found in citrus fruits. Possible interference with ascorbic acid by citric acid, lactic acid, tartaric acid, and glucose, was measured at different concentrations, 16 IU–0.5 IU (1 mg/mL of ascorbic acid was mixed with 1 mg/mL of citric acid, lactic acid, tartaric acid, and glucose). The experiment result is shown in Figure-8 and shows the recovery error well below the acceptance rate (Recovery rate >10 is considered as positive interference). During the evaluation of the methods for adaptability and accuracy, it was noted that all the approaches exhibited a significant level of agreement, with r^2 of 0.999 when compared to Standard-AA. Notably, the C + A method demonstrated the lowest mean squared error (MSE) at 0.000001, indicating superior model performance. Therefore, it can be concluded that the C + A method, along with other approaches, stands out as the most effective method to measure ascorbic acid (Figure-8).

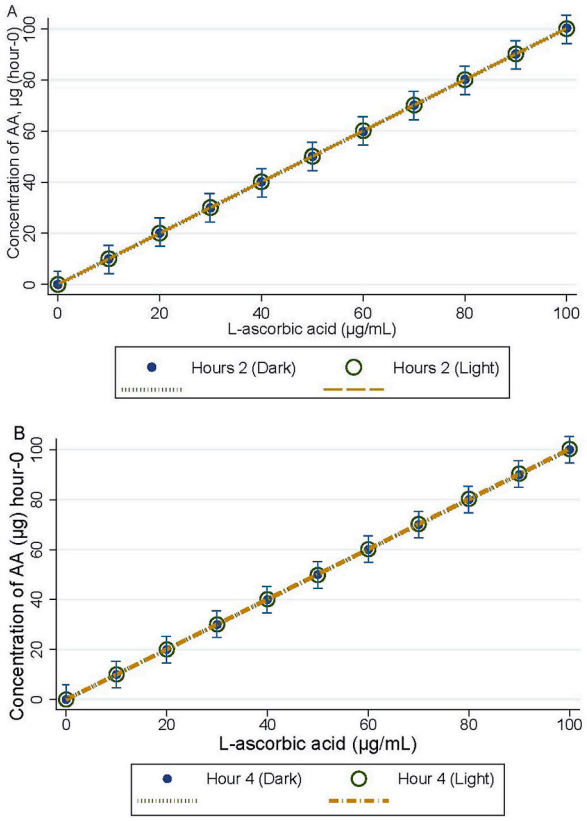

Figure-6. The method's stability was assessed under different lighting conditions in a laboratory setting. Two setups were employed: (A) exposure to laboratory lighting and (B) darkness achieved by covering with aluminium foil. After a 4-h study, no significant changes were observed. For the exposed-to-light setup (light), the r^2 was 0.999 with a standard error of 0.00165, while the foiled condition (dark) had an r^2 of 0.9999 with a standard error of 0.00747. The regression line was established using the linear regression model.

3.6. Analytical application of the proposed method

3.6.1. Detection of AA from vitamin C tablets

To check the applicability of the methods in the determination of ascorbic acid, the technique was used to analyze commercial pharmaceutical formulations such as vitamin C tablets (effervescence, sachet, and chewing), based on the experimental protocol described previously. Ascorbic acid detected from the commercial tablets was compared with standard concentrations of AA. The details analysis is presented in Table-3 with good recovery (98%–100.4%) and low level of detection error (>10%). HPLC analysis of vitamin C tablet also showed a single peak of AA with a retention time around 3.535 min which is identical with the standard AA and is shown in Figure-9(A)&(B). This observation indicated that our new method will be highly applicable in monitoring the quantity and quality of products in the market.

3.6.2. Detection of AA from ascorbate salts

For the experimental protocol described previously, three ascorbate salts (sodium (SAA), glucoside (GAA), and ether (EAA)) were tested along with standard AA and are presented in Figure-9(C). Out of three salts tested, only sodium ascorbate showed strong linearity with standard AA ($r^2 = 0.9999$; SE = 0.0077; *p-value* = 4.41E-13) while the new method failed to detect the presence of L-ascorbic acid in other two salts. These can be attributed to the formation of dehydroascorbic acid which could in-turn undergo hydrolysis resulting in ring-opening. This ultimately results in the irreversible loss of the activity of ascorbic acid and its reducing property [44].

Furthermore, upon spiking varying concentration of ascorbic acid with a constant concentration of ascorbate glucoside (10 µg), we observe that there is a strong correlation of coefficient ($r^2 = 0.998$) between spiked and control ascorbic acid at all concentrations (10 µg–1.0 µg) (Supplementary Figure-1).

3.6.3. Detection of AA from fresh fruits

For the experimental protocol described previously, the developed AA detection method was also used for the detection of AA from fresh fruits available in the local market such as oranges, grapes, mangosteen, and tomatoes. 1 mL filtrate (0.22 µm) of juice from each

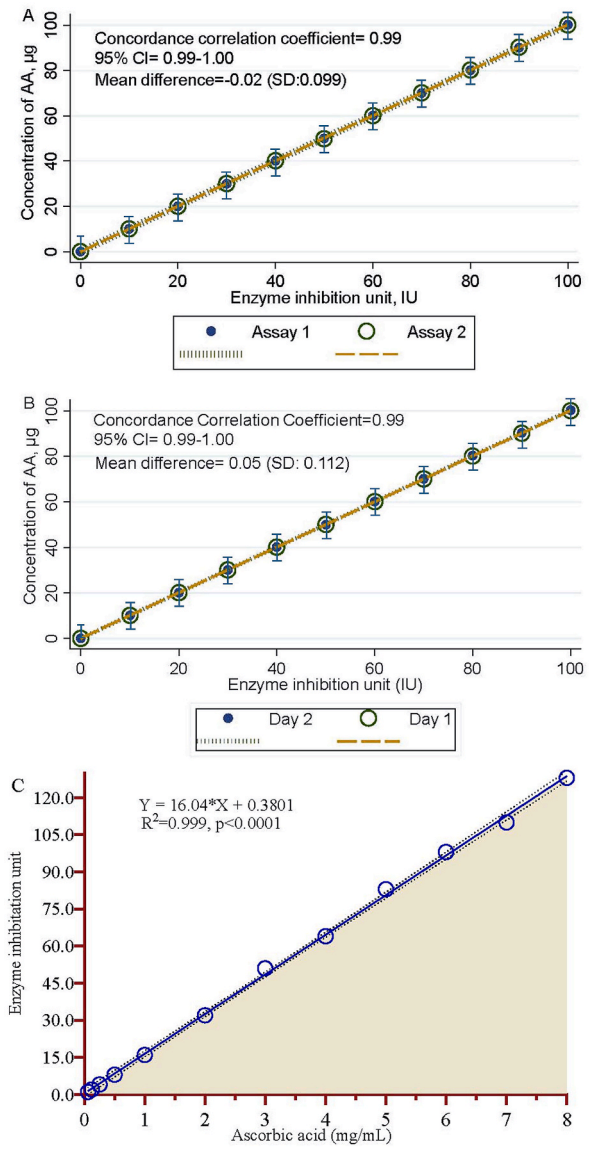

Figure-7. Validation of assay protocol and comparison with reference HRP. (A) In the intra-assay, utilizing 10 different concentrations of ascorbic acid (ranging from 100 µg to 10 µg per reaction), the r^2 was 0.999, with a 95% confidence interval (CI) of 0.99–1.00, and a mean difference of 0.02. (B) In the inter-assay, conducted a day apart, the CCC was again 0.999, with a 95% CI of 0.99–1.00, and a mean difference of 0.05. This robust CCC value signifies a high level of agreement between intra- and inter-assay developments. (C) The calibration curve was validated using high concentrations of ascorbic acid (n = 8), demonstrating a high correlation coefficient (r^2 = 0.999) concerning the concentrations of ascorbic acid and enzyme inhibition units.

specimen was diluted in PBS (1:10) and tested using the protocol mentioned. A standard AA solution (1 mg/mL) was also tested as test control during the assay and resultant OD values were converted into percent inhibition of reaction according to formula-1 and the concentration was calculated according to formula-2 and is presented in Table-4. The presence of ascorbic acid in one of the fruit samples (orange) was analyzed by HLPC and a single and identical peak of retention time was 3.662 min which is identical to the standard ascorbic acid (Supplementary Figure-2). The obtained concentration of ascorbic acid from tested fruits is closely related to reports previously published [44–52].

4. Conclusion

In our current study, we present the concept of measuring the percent (%) of inhibition of reaction for the detection of AA in the presence of chromogen substrate TMB which produced a deep blue color during the enzymatic degradation of hydrogen peroxide by HRP. In our technique, AA either competes and/or inhibits the involvement of TMB and/or HRP which is oxidized during hydrolysis of

Table-2
The degree of recovery of ascorbic acid following spiking with Vitamin C effervescence tablet.

Quantity of AA in Vitamin C Tablet (mg/ml)	Standard Ascorbic acid added (mg/ml)	Total amount of AA in spiked sample (mg/ml)	Recovered AA obtained following assay (mg/ml)	Percent (%) of recovery against each spiked sample (*)	Percent (%) of recovery error against each spiked sample (#)
2.000	2.000	4.000	3.957 (±0.024)	97.84	−1.08
1.000	1.000	2.000	1.999 (±0.006)	99.85	−0.07
0.500	0.500	1.000	0.998 (±0.001)	99.63	−0.19
0.250	0.250	0.500	0.499 (±0.000)	99.40	−0.30
0.125	0.125	0.250	0.249 (±0.000)	99.55	−0.22
0.063	0.063	0.125	0.125 (±0.000)	99.63	−0.19
0.031	0.031	0.063	0.062 (±0.006)	98.42	−0.79
0.016	0.016	0.031	0.033 (±0.010)	108.00	4.00

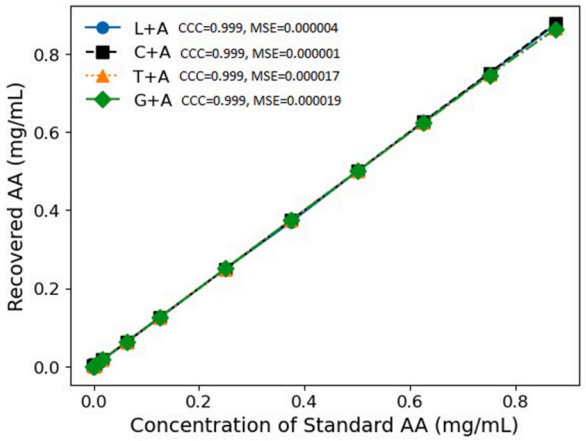

Figure-8. Evaluation of the methods for adaptability and accuracy. Correlation coefficient was measured to compare the two continuous observation and the estimate showed that all the approaches exhibited a significant level of agreement, with a correlation coefficient of 0.999 when compared to Standard-AA.

Table-3
Detection of ascorbic acid from marketed pharmaceutical products and their comparison with standard ascorbic acid (mean ± SD, n = 3).

	Estimated ascorbic acid (μg/sample)										
	100	90	80	70	60	50	40	30	20	10	R^2
Standard AA[a]	100.44 (±0.22)	90.40 (±0.2)	80.28 (±0.14)	70.20 (±0.1)	60.22 (±0.11)	49.92 (±0.04)	40.13 (±0.07)	29.97 (±0.01)	20.03 (±0.01)	10.01 (±0.00)	0.9999
C-vit (E)[b]	99.82 (±0.09)	89.96 (±0.02)	79.97 (±0.01)	69.75 (±0.12)	60.00 (±0.00)	49.99 (±0.00)	40.02 (±0.01)	30.01 (±0.00)	20.02 (±0.01)	10.02 (±0.01)	0.999
C-vit (S)[c]	99.84 (±0.08)	89.67 (±0.17)	79.79 (±0.11)	69.45 (±0.27)	59.92 (±0.04)	49.93 (±0.04)	39.92 (±0.04)	30.00 (±0.00)	20.02 (±0.01)	10.01 (±0.01)	0.999
C-vit (CT)[d]	98.70 (±0.65)	88.91 (±0.55)	78.83 (±0.58)	62.44 (±3.78)	59.40 (±0.30)	49.60 (±0.20)	39.77 (±0.12)	29.94 (±0.03)	19.98 (±0.01)	9.99 (±0.00)	0.9962

Percent (%) of detection of ascorbic acid/sample											Detection Error
Standard AA[a]	100.44	100.44	100.35	100.28	100.37	99.85	100.33	99.91	100.15	100.06	(-0.085 – 0.439)
C-vit (E)[b]	99.82	99.95	99.96	99.65	100.00	99.99	100.05	100.02	100.12	100.16	(-0.01 – 0.163)
C-vit (S)[c]	99.84	99.63	99.74	99.22	99.87	99.86	99.80	100.01	100.11	100.14	(-0.13 – 0.14)
C-vit (CT)[d]	98.68	98.77	99.52	87.90	98.99	99.19	99.42	99.79	99.91	99.91	(-09.00 – 10.79)

[a] Ascorbic acid.
[b] Effervescence tablet.
[c] Granule in sachet.
[d] Chewing tablet.

H_2O_2 by HRP, and positive competition and/or inhibition was recorded as lack of blue color development compared to wells without inhibition. The reaction was stopped, and the degree of inhibition can easily be measured by spectrophotometer at 450 nm wavelength.

The inhibition unit of the enzyme by AA linearly correlated from 100 μg/mL to 0.78 μg/mL or 1.35 nM–4.08 nM, respectively. The rate of inhibition of oxidized co-substrate by AA served as a measure for the relative activity of peroxidase and determination of the

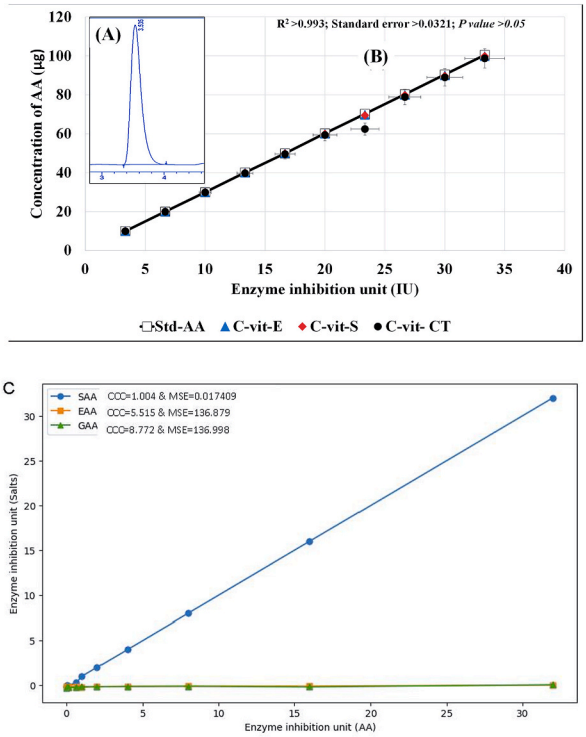

Figure-9. Detection of AA from commercial products. (A) Chromatogram of ascorbic acid from pharmaceutical products (vitamin-C tablet) which shows a single peak which is identical to the standard ascorbic acid in terms of retention time (3.535 min). (B) Linearity of regression analysis showed a very strong correlation of coefficient among three products with standard ascorbic acid ($r^2 > 0.999$). (C) Detection of AA from ascorbate salts and comparative analysis against standard AA. A good linearity is observed between standard AA and sodium ascorbate while no AA was detected in other two salts [SAA (sodium ascorbate); EAA (ester ascorbate); GAA (ascorbyl glucoside)].

Table-4
The amount of AA detected from fresh fruit juice using the AA detection method. (Quantity expressed in mg/100 mL).

Name of fruits	Obtained concentration of AA (mg/100 ml)	Reference quantity of AA (mg/100 ml)	Reference(s)
Orange	55.32	29.0–82.5	[37,38]
Grapes (37 pcs)	57.91	22.0–64.0	[39,40]
Mangosteen	5.91	2.00	[41,42]
Tomato	35.24	26–49	[43]

concentration of AA using the formula of % of inhibition which showed very good correlation ($r^2 > 0.999$) with acceptable ranges of SD (±0.016) and SE (±0.005) values. The performance of our proposed methodology was evaluated in vitamin C supplements and natural orange juice. Our method was able to determine AA in samples representative of those found in real world. Recoveries values against different interference substances showed excellent linearity ($r^2 > 0.999$) with minimum recovery errors (<10%).

Though our study shows promising results, one of the main limitations is that we did not test for ascorbate peroxidase (APEX) inhibition which may have been present in our extracted sample. It has been previously presented that both the HRP and APEX have almost identical function though their post-translational modifications are different whereby the former requires glycosylation [53]. Additionally, we have not tested for and identified the additional compounds that may be in the fruit juices or effervescent tablets and may affect the reaction. However, since in both cases inhibition activity was observed in the presence of ascorbic acid, henceforth we did not include it within the scope of this study.

In summary, our newly developed HRP inhibition is a one-step method, rapid, and simple with minimum reagents and could play a breakthrough in the detection of AA from aqueous solution at the field level of application. Furthermore, the developed test is easily reproducible in various laboratory settings.

Ethical statement

The study does not involve experimentation on any human or animal, nor specimens obtained from them.

Data availability statement

Data will be made available on request.

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CRediT authorship contribution statement

Bijon Kumar Sil: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Mohd Raeed Jamiruddin:** Writing – review & editing, Writing – original draft, Visualization, Validation, Resources, Methodology, Investigation, Formal analysis, Data curation. **Md Ahsanul Haq:** Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Formal analysis, Data curation. **Nattanit Aekwattanaphol:** Writing – review & editing, Writing – original draft, Resources, Methodology, Investigation. **Prem Ananth K:** Writing – review & editing, Writing – original draft, Resources, Methodology, Investigation. **Limbadri Salendra:** Writing – review & editing, Writing – original draft, Resources, Methodology. **Himanshu Paliwal:** Writing – review & editing, Writing – original draft, Resources, Methodology. **Pijush Kumar Paul:** Writing – review & editing, Writing – original draft, Resources, Methodology. **Wilaiporn Buatong:** Writing – review & editing, Writing – original draft, Resources, Methodology. **Teerapol Srichana:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Teerapol Srichana has patent #10202302127Y pending to Intellectual Property Office of Singapore. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e30715.

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