

Optimized Assay for Hydrogen Peroxide Determination in Plant Tissue Using Potassium Iodide

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Abstract

Here, we present an optimization of colorimetric determination of hydrogen peroxide content in plants using potassium iodide. Our method is based on a one step buffer (extraction and reaction) for the determination of H₂O₂ in different plant tissues and overcomes interference of soluble antioxidant and color background. A particular attention is paid to buffer pH shown to be tissue dependent. With this inexpensive microplate method, it is possible to analyze 12 experimental samples in about 45 min all in triplicates, with blanks, controls and standard curve.

Keywords

Hydrogen Peroxide, Potassium Iodide, One Step, Microplate, Inexpensive

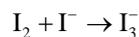
1. Introduction

Hydrogen peroxide (H₂O₂) is commonly produced in plants during normal physiological processes and in response to stress situations [1]. The quantitative determination of hydrogen peroxide is important in numerous studies since H₂O₂ is involved in oxidative cellular damages as well as in signalling processes [2] [3]. Owing to its importance, numerous methods and kits have been developed based on spectrophotometry, chemiluminescence [4], enzymatic method [5] and electrochemistry [6]. However, some of these methods are very expensive. Several years ago, an economic colorimetric method has been described for the determination of H₂O₂ [7] and it is widely used in the field of plant biology [8]-[10]. This method is based on potassium iodide (KI) oxidation by H₂O₂ in acidic medium according to the following equations:



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When hydrogen peroxide is added to colorless solution of potassium iodide (KI), the iodide ions (I^-) are slowly oxidized in iodine (I_2). In presence of iodide, iodine reacts to form triiodide (I_3^-) resulting in a yellowish solution. Therefore hydrogen peroxide could be quantified by spectrophotometric method by following I_3^- absorption. In the method described by Velikova *et al.* (2000), H_2O_2 determination relies on absorbance at 390 nm. However, the triiodide absorbs at 285 and 350 nm as presented in **Figure 1(a)**. Although the peak observed at 285 nm presents greater sensitivity, the peak at 350 nm has been used in this study. Indeed plant tissues show important absorption at 285 nm, which can interfere with the assay (**Figure 1(b)**, **Figure 1(c)**). Plant extracts have a colour background to take into account and contain molecules that interfere with H_2O_2 determination. **Figure 1(b)** and **Figure 1(c)** show no difference between the reaction spectra from tomato fruit and leaf extracts with KI or not. The purpose of this work was to optimize the iodometric assay for plant tissues.

Commonly, metabolite determination is made through two major steps: extraction of molecules from tissue and quantification of molecule of interest from this extract. However using the two-step protocol, H_2O_2 might be destroyed by ascorbic acid, which is an efficient H_2O_2 scavenger [11], during extraction step and may be underestimated or cannot be quantified at all (**Table 1(a)**). So, we proposed an alternative one-step method.

Table 1. (a) H_2O_2 concentration measured in absence and presence of 1 nmole ascorbic acid added before KI or together with KI; (b) Influence of the ratio of tomato tissue to buffer on hydrogen peroxide concentration. Statistical significance was determined with student t-test ($p < 0.05$) with “R” 2.12.2 statistical software (www.cran.rproject.org); (c) Hydrogen peroxide content in fruits and leaves of tomato plants treated or not with $HgCl_2$ using the optimized protocol. Statistical significance was determined by student t-test ($p < 0.05$) with “R” 2.12.2 statistical software (www.cran.rproject.org).

(a)

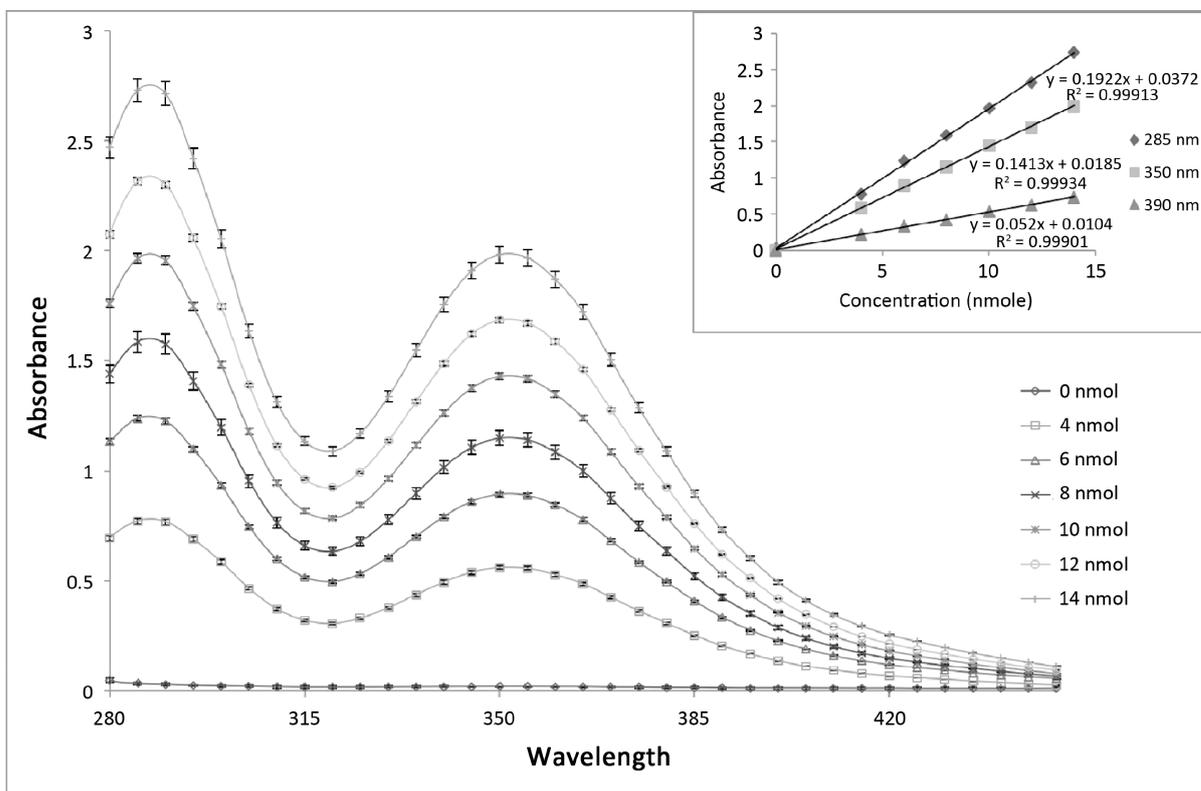
Expected H_2O_2 concentration (nmole)	Measured H_2O_2 concentration (nmole)	
	1 nmol ASA added before KI	1 nmol ASA added together with KI
0	0.00 ± 0.00	0.00 ± 0.01
1	0.00 ± 0.00	0.82 ± 0.04
2	0.00 ± 0.00	1.78 ± 0.13
3	0.32 ± 0.00	2.68 ± 0.13
4	1.46 ± 0.09	3.65 ± 0.03
5	2.43 ± 0.03	4.44 ± 0.06
6	3.33 ± 0.01	5.75 ± 0.10

(b)

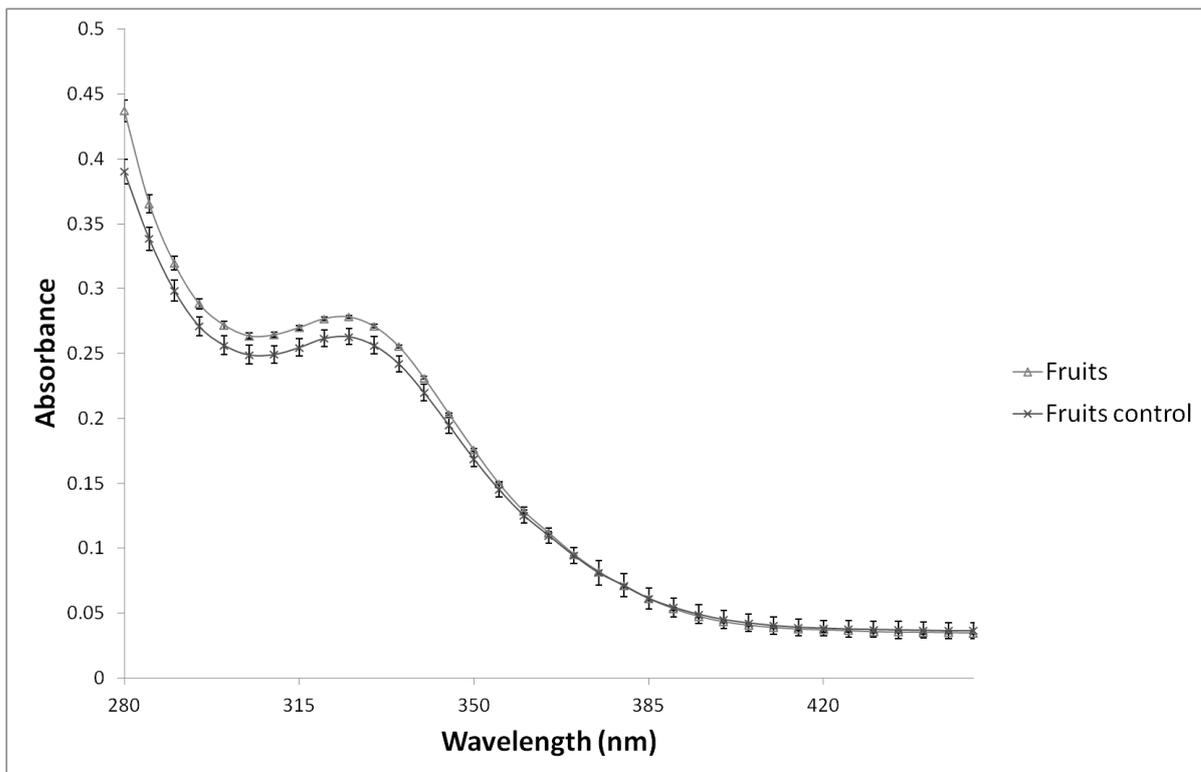
mg FW/ml	H_2O_2 (nmol/g)
50	491.20 ± 18.74 ^a
100	528.86 ± 0.61 ^a
150	612.10 ± 17.53 ^b
200	584.33 ± 12.45 ^a
250	377.96 ± 15.78 ^c

(c)

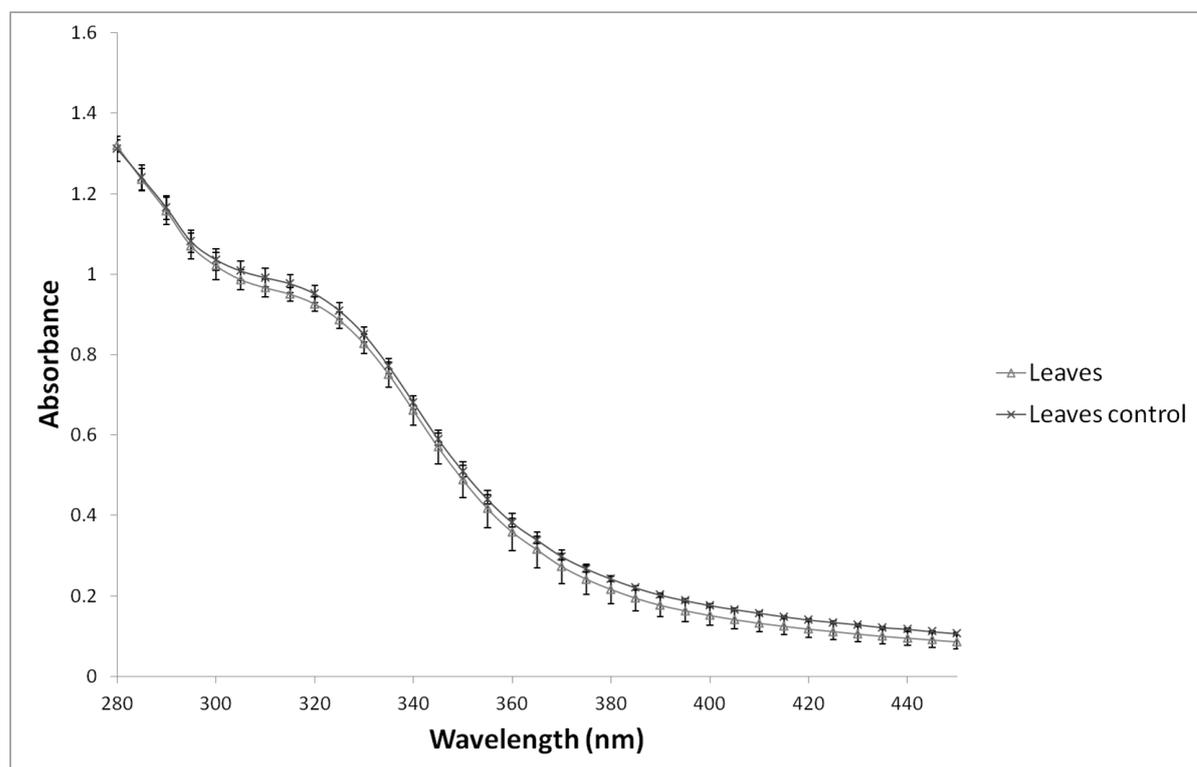
Organ	Treatment	H_2O_2 (nmol/gFW)
Fruits	Control	77.16 ± 9.07 ^a
	5 ppm $HgCl_2$	175.73 ± 5.79 ^b
Leaves	Control	472.57 ± 8.73 ^a
	5 ppm $HgCl_2$	836.97 ± 9.85 ^b



(a)



(b)



(c)

Figure 1. (a) Absorption spectra of I_3^- ions resulting from hydrogen peroxide (H_2O_2) reaction with potassium iodide (KI 1 M) using different H_2O_2 concentrations in 0.1% TCA. Inset shows calibration curve with slopes for hydrogen peroxide quantification measured at 350 and 390 nm at pH 7; (b) Absorption spectra of hydrogen peroxide (H_2O_2) reaction with potassium iodide (KI 1 M) in 0.1% TCA at pH 7 with Velikova *et al.* (2000) method. Fruit: Fruit homogenate in presence of KI. Fruit control: Fruit homogenate in absence of KI. Each value represents mean \pm standard deviation; (c) Absorption spectra of hydrogen peroxide (H_2O_2) reaction with potassium iodide (KI 1 M) in 0.1% TCA at pH 7 with Velikova *et al.* (2000) method. Leaves: Leaf homogenate in presence of KI. Leaves control: Leaf homogenate in absence of KI. Each value represents mean \pm standard deviation.

2. Materials and Methods

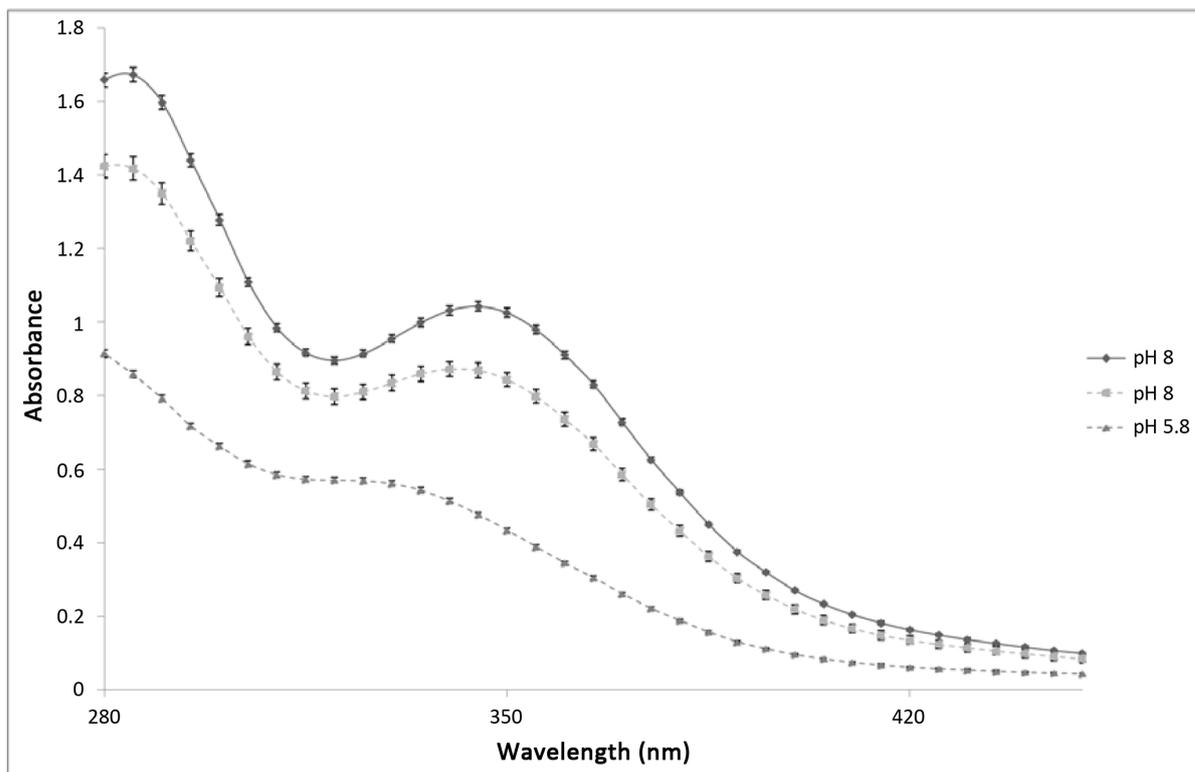
The protocol is as follows: tomato fruits were harvested, immediately frozen in liquid nitrogen, ground and the powder stored at -80°C until H_2O_2 determination assay. Frozen powder (150 mg) was directly homogenized with 1 ml of solution containing 0.25 ml Trichloroacetic acid (TCA) (0.1% (w:v)), 0.5 ml KI (1 M) and 0.25 ml potassium phosphate buffer (10 mM, pH adapted to studied tissue) at 4°C for 10 min (one-step buffer: extraction and colorimetric reaction combined). At the same time, for every sample, a control was prepared with H_2O instead of KI for tissue coloration background. Good care was taken to protect samples and solutions from light. The homogenate was centrifuged at $12,000 \times g$ for 15 min at 4°C . 200 μL of supernatant from each tube were placed in UV-microplate wells and left to incubate at room temperature ($20^\circ\text{C} - 22^\circ\text{C}$) for 20 min. Samples and blanks were analyzed in triplicate. A calibration curve obtained with H_2O_2 standard solutions prepared in 0.1% TCA was used for quantification (Figure 1(a)). The microplate reader used is a Power Wave HT microplate spectrophotometer from BioTek (France) equipped with an internal temperature incubator and shaker for kinetic and spectrum analysis. We used the KC4 data software to check the reader and to analyze reactions. To optimize the protocol, assays have been done by modifying pH of phosphate buffer. As shown in Figure 2(a), tomato fruit extracts exhibited maximum absorbance at 350 nm in 10 mM potassium phosphate buffer solution at pH 8. For tomato leaves the most efficient buffer for the reaction was at pH 5.8 (Figure 2(c)). The kinetic of the reaction was not modified by the modification of the buffer pH as shown in Figure 2(b) and Figure 2(d).

In addition, the influence of the ratio of tomato exocarp to “one-step buffer” was made in order to select the

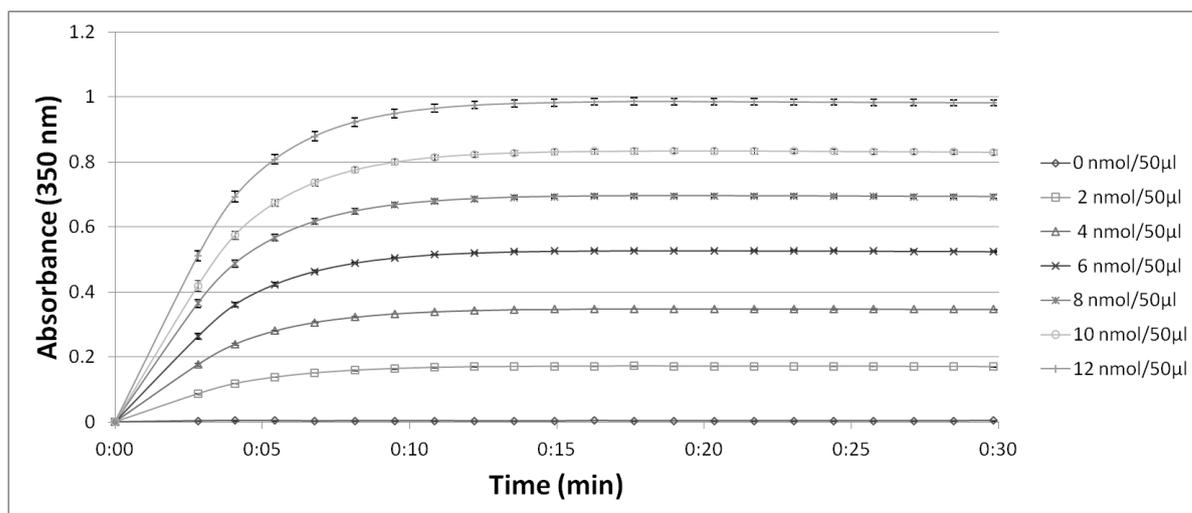
ratio that resulted in maximal H₂O₂ determination (Table 1(b)). The best ratio adapted to our sample was found to be between 150 and 200 mg for 1 ml of buffer. Recovery assays with 20 nmol/ml H₂O₂ made to complete the method revealed 100% recovery in 100, 150 and 200 mg. In order to confirm the efficiency of our assay, we compared the H₂O₂ contents in fruits (30 days after anthesis) and mature leaves from control plants and plants exposed to HgCl₂ (5 ppm) for 24 h, using the new protocol. The results are presented in Table 1(c).

3. Conclusion

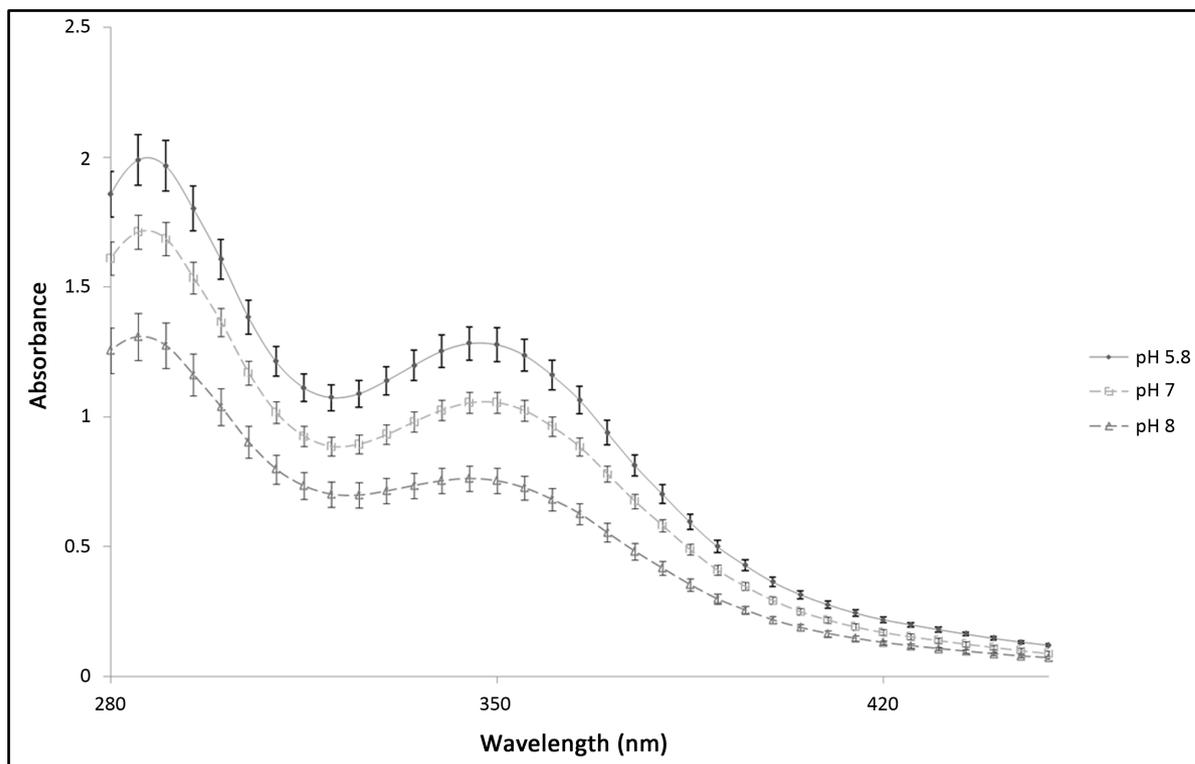
This optimized spectrophotometric method based on a one-step buffer (extraction and reaction) is suitable and



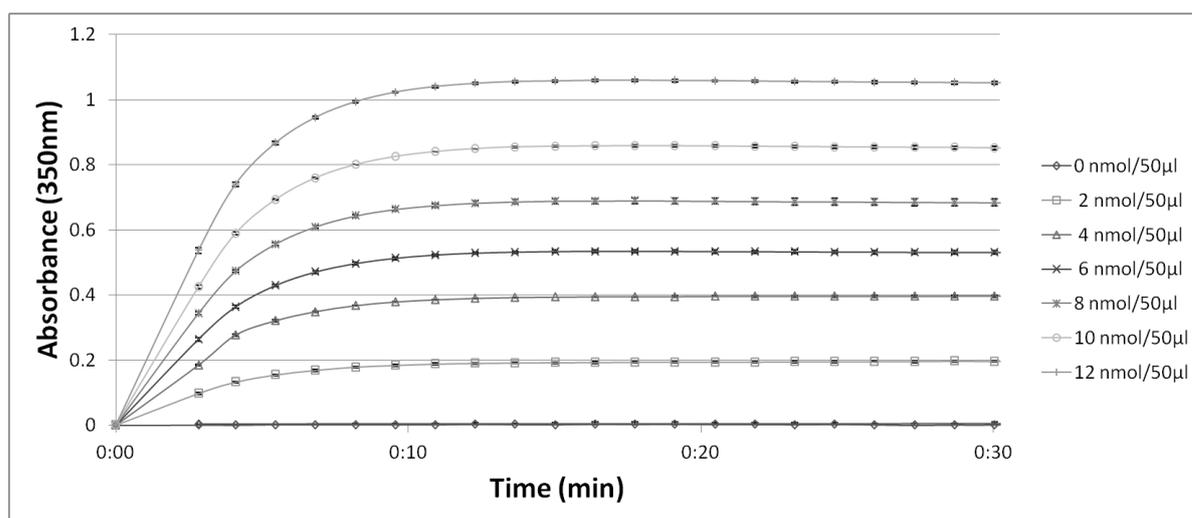
(a)



(b)



(c)



(d)

Figure 2. (a) Absorption spectra of hydrogen peroxide (H_2O_2) reaction with potassium iodide (KI 1 M) in fruits in 0.1% TCA at pH 5.8, pH 7 and pH 8. Highlighting better absorbance of fruit samples at pH 8 together with an absence of pH interference in background tissue coloration. Each value represents mean \pm standard deviation; (b) Reaction kinetic of reaction between hydrogen peroxide (H_2O_2) and potassium iodide (KI 1 M) at pH 8 showing end of reaction after 20 min; (c) Absorption spectra of hydrogen peroxide (H_2O_2) reaction with potassium iodide (KI 1 M) in leaves in 0.1% TCA at pH 5.8, pH 7 and pH 8. Highlighting better absorbance of leaf samples at pH 5.8 together with an absence of pH interference in background tissue coloration. Each value represents mean \pm standard deviation; (d) Reaction kinetic of reaction between hydrogen peroxide (H_2O_2) and potassium iodide (KI 1 M) at pH 5.8 showing end of reaction after 20 min.

reliable for the determination of H_2O_2 in different plant tissues. Indeed, this method allows overcoming interference of soluble antioxidant and color background. With this inexpensive microplate method, it is possible to

analyze 12 experimental samples in about 45 min all in triplicates, with blanks, controls and standard curve.

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