ISSN 0101-2061 Food Science and Technology

DOI: http://dx.doi.org/10.1590/1678-457X.36216

Validation of spectrophotometric microplate methods for polyphenol oxidase and peroxidase activities analysis in fruits and vegetables

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Abstract

Enzymes polyphenol oxidase (PPO) and peroxidase (POD) play important roles in the processing of fruits and vegetables, since they can produce undesirable changes in color, texture and flavor. Classical methods of activity assessment are based on cuvette spectrophotometric readings. This work aims to propose, to validate and to test microplate spectrophotometric methods. Samples of apple juice and lyophilized enzymes from mushroom and horseradish were analyzed by the cuvette and microplate methods and it was possible to validate the microplate assays with satisfactory results regarding linearity, repeatability, accuracy along with quantitation and detection limits. The proposed microplate methods proved to be reliable and reproducible as the classical methods besides having the advantages of allowing simultaneous analysis and requiring a reduced amount of samples and reactants, which can beneficial to the study of enzyme inactivation in the processing of fruits and vegetables.

Keywords: microplate; polyphenol oxidase; peroxidase; enzymatic assay; apple juice.

Practical Application: Rapid assessment of POD/PPO activities in fruits and vegetables with reduced amounts of reactants.

1 Introduction

Monitoring the activity of enzymes polyphenol oxidase (PPO, E.C.1.14.18.1) and peroxidase (POD, E.C.1.11.1.7) is an important control point for harvesting, storing and processing of fruits and vegetables. These enzymes oxidize a wide variety of aromatic compounds and thermal processing is usually applied to reduce activity in food products, consequently avoiding color and off-flavor development, especially in fruit juices (Hammer, 1993; Robinson, 1993; Ramírez & Whitaker, 2002; Adams & Brown, 2007; Terefe et al., 2014). Effective methods for detecting enzymatic activity are necessary to ensure food quality, flavor and sensorial properties.

There are several methods to determine the activity of POD based on different hydrogen donors, such as guaiacol, pyrogallol, ABTS (2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt), 4-methoxyl-α-naphthol and phenol plus amino-antipyrine (Yuan & Jiang, 2002). Among them, ABTS is a widely used substrate in the spectrophotometric determination of POD activity because the method is sensitive and the chromogenic products are stable (Pütter & Becker, 1983; Yuan & Jiang, 2002).

Since the products of PPO activity (quinones and quinone polymers) are colored, spectrophotometric assays are frequently adopted to assess enzymatic activity by the rate of product formation; however, polarographic methods can also be used to measure the decrease of oxygen concentration. Numerous substrates can be used with spectrophotometric methods, such as pyrocatechol, 4-methylcatechol or 3,4-dihydroxy-L-phenylalanine (Hammer, 1993; Mayer & Harel, 1993; Ramírez & Whitaker, 2002). The selection of the appropriate substrate is also important, seeing that the colored compounds formed from the oxidation products have their maximum absorption at different wavelengths (Vámos-Vigyázó & Haard, 1981).

The cuvette spectrophotometric assays have been a standard practice for determining PPO and POD activities in food due to their simplicity and convenience. Albeit reliable, these enzymatic activity methods for food materials are time consuming and require intensive labor, limiting the number of samples that can be analyzed daily. Lately, methods have been developed utilizing spectrophotometry by microplates, because it may have the same sensitivity, requires smaller amounts of sample and reactants and is less time consuming, since several tests can be run simultaneously in a spectrophotometer with a plate reader (Murshed et al., 2008; Revanna et al., 2013; Ortiz et al., 2014; Bobo-García et al., 2015). Consequently, the objective of this work was to propose and to validate a reliable microplate assay for determining PPO and POD activities in fruits and vegetables. The methods were developed based on the classical cuvette assays and were tested with samples of apple juice and standardized enzymes solutions from mushroom and horseradish.

2 Materials and methods

2.1 Chemicals

Monopotassium phosphate (KH_2PO_4), sodium phosphate dibasic heptahydrate ($Na_2HPO_47H_2O$) and ascorbic acid analytical grade were purchased from Synth (São Paulo, Brazil). Hydrogen peroxide solution (100 V) was obtained from Lafan (Várzea

Received 15 Dec., 2016
Accepted 03 May, 2017
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Paulista, Brazil). Lyophilized peroxidase (POD) from horseradish type VI (P6782), lyophilized polyphenol oxidase from mushroom type Tyrosinase (T3824), 2,2′-Azino-bis(3-ethylbenzothiazolin e-6-sulfonic acid) diammonium salt (ABTS) and pyrocatechin were purchased from Sigma-Aldrich (St Louis, USA).

2.2 Samples and preparation

For validating the microplate methods and comparing them with classical assays, stock solutions of POD and PPO were prepared by using the commercial enzymes. The POD stock solution (0.50 mg mL^{-1}) was obtained by diluting the lyophilized peroxidase in 10 mL of 67 mM phosphate buffer pH 6.0 (59 mM KH_2PO_4 and 8 mM $Na_2HPO_4 \cdot 7H_2O$).

The PPO stock solution (0.43 mg mL^{-1}) was obtained by diluting the lyophilized polyphenol oxidase in 10 mL of 50 mM phosphate buffer (pH 6.5), containing 35 mM KH_2PO_4 and 15 mM $Na_2HPO_4 \cdot 7H_2O$. The stock solutions of POD and PPO were then diluted in buffer to obtain standard solutions with different enzymatic activities. The concentrations were 0.00330, 0.00550, 0.00825, 0.0138, 0.0193, 0.0248 and 0.0303 μg mL^{-1} for the seven POD standard solutions and 0.0433, 0.0866, 0.195, 0.303, 0.390, 0.498 and 0.585 μg mL^{-1} for the seven PPO standard solutions.

To test the use of the microplate methods in the analysis of fruit juices, the POD and PPO activities of apple juice were determined. Apples (*Malus domestica* Borkh, cv Fuji Suprema) were obtained from an orchard located in the State of Santa Catarina (Southern Brazil). The selected fruits were squeezed with a domestic juice maker SS-1080 (Fun Kitchen, USA) and an ascorbic acid solution (0.2 g L^{-1}) was blended with the fresh juice to retard browning. Part of the fresh juice was sterilized in a vertical autoclave 103 (Fabber-Primar, São Paulo, Brazil) in pressure condition of 2 atm for 20 min, denominated 'sterilized juice'. Subsequently, both juices were centrifuged at 2,000 rpm for 15 min at room temperature using a TD-66 centrifuge (Tominaga Works, Tokyo, Japan) and the supernatants were analyzed immediately for determining the enzymatic activity. It was confirmed that PPO and POD activities could not be detected in the sterilized juice (data not shown). Later, the fresh and sterilized juices were blended to obtain seven different values of enzymatic activity (1:0, 9:1, 2:1, 1:1, 1:2, 1:9, 0:1 – ratio of fresh: sterilized juice).

All the apple juice samples, PPO solution from mushroom and POD solution from horseradish were analyzed for PPO and POD activity by the cuvette and microplate methods described herein.

2.3 Spectrophotometric cuvette assays

The fruit or vegetable extract to be used as a sample for PPO and POD activity assays is to be obtained according to the literature (Vámos-Vigyázó & Haard, 1981). The classical method of Zemel et al. (1990) was considered for measuring the PPO activity. At first, 1.5 mL of 50 mM phosphate buffer (pH 6.5) and 0.5 mL of the sample were added to a 10 mm path length polyacrylic cuvette, and then incubated for 1 min at 25 °C. After incubation, 1.0 mL of a 50 mM pyrocatechin solution (freshly prepared) was added and mixed. For quantification, absorbance increase at 420 nm was recorded every 10 s at 25 °C for 3 min, using a SpectraMax plus 384 spectrophotometer (Molecular Devices, Sunnyvale, USA). This UV/VIS spectrophotometer is compatible with cuvettes and 96- 384-well microplates and uses a monochromator with a wavelength accuracy of ±1.0 nm from 190 to 1000 nm. The reference value (0.000 absorbance) was determined by using a blank solution containing phosphate buffer and pyrocatechin.

The classical protocol for determining POD activity was carried out according to Pütter & Becker (1983). First, 1.5 mL of 67 mM phosphate buffer pH 6.0 and 0.5 mL of the sample were pipetted into a 10 mm path length polyacrylic cuvette, and incubated at 25 °C for 1 min. To start the reaction, 0.2 mL of a 1.7 mM ABTS solution and 0.2 mL of 0.8 mM hydrogen peroxide solution were added and mixed. The formation of ABTS radical at 25 °C was monitored every 20 s for 5 min at 405 nm, using a SpectraMax plus 384 spectrophotometer (Molecular Devices, Sunnyvale, USA). The reference value (0.000 absorbance) was determined by using phosphate buffer as sample for the assay.

For the cuvette methods, the absorbance was plotted against time and the slope was determined by linear regression. One unit of enzymatic activity (U) was defined as an absorbance increase of 0.001 per minute under the assay conditions.

2.4 Spectrophotometric microplate assays

In order to adapt the classical cuvette methods to the microplate reader, the volumes of solutions and samples needed to be reduced. The proportionality was preserved and the total volume of the mixture was reduced from 3.0 mL to 200 μL for the PPO assay and from 2.4 mL to 201 μL for the POD assay. A flat bottom polystyrene microplate with 96 wells (Greiner Bio-one, Frickenhausen, Germany) was used. The microplate was incubated at 25 °C for 10 min before use. Absorbance readings were made at 25 °C with a SpectraMax plus 384 spectrophotometer (Molecular Devices, Sunnyvale, USA).

For the PPO microplate assay, 100 μL of 50 mM phosphate buffer (pH 6.5) and 33 μL of the sample were added to a microplate well. The mixture was then incubated at 25 °C for 1 min. A freshly prepared solution of pyrocatechin (50 mM, 67 μL) was added and the absorbance at 420 nm was monitored every 10 s for 3 min. The reference value for absorbance was set by using the phosphate buffer as a sample for the assay in another well (blank).

For the POD microplate assay, 125 μL of 67 mM phosphate buffer (pH 6.0) and 42 μL of the sample were added to a microplate well. The mixture was then incubated at 25 °C for 1 min; then, 17 μL of 1.7 mM ABTS solution and 17 μL of 0.8 mM hydrogen peroxide solution were added. Absorbance at 405 nm was monitored every 20 s for 5 min. The reference value for absorbance was set by using the phosphate buffer as a sample for the assay (blank).

For the microplate methods, the absorbance readings were first corrected as explained in the next section, then plotted against time; the slope was determined by linear regression. One unit

of enzymatic activity (U) was defined as an absorbance increase of 0.001 per minute under the assay conditions.

2.5 Path length correction

The absorbance readings obtained with the microplate methods need to be corrected to correspond to the readings obtained from the cuvette methods because of differences in the light path length. The absorbance (*Abs*) of a dilute mixture is directly proportional to the concentration (*C*), the light path length (*b*) and the molar absorptivity (ε), according to Lambert-Beer's Law: $Abs = \varepsilon \cdot b \cdot C$. In a cuvette, the light horizontally travels a standard path of 10 mm. However, in a microplate well, the light travels vertically and the thickness of the liquid layer depends on the mixture volume, on the meniscus shape and on the geometry of the well (Lampinen et al., 2012).

According to Lampinen et al. (2012), the liquid path length in a microplate well can be determined from absorption readings of the water present in the mixture at 975 nm. The wavelength of 900 nm was used as a reference to eliminate the background absorbance because of the low absorption of water at this wavelength. The correction factor for path length can thus be defined as in Equation 1:

$$K = \frac{Abs_{975}^{C} - Abs_{900}^{C}}{Abs_{975}^{M} - Abs_{900}^{M}} \quad (1)$$

where *Abs* is the absorbance and superscripts *C* and *M* correspond to cuvette and microplate well readings, respectively. Consequently, microplate absorbance readings Abs^{M} need to be corrected as in Equation 2:

$$Abs = K \cdot Abs^{M} \quad (2)$$

to correspond to a cuvette reading with a 10 mm path length.

The correction factor was determined separately for the PPO and POD assays. A 10 mm path length polyacrylic cuvette and a flat bottom polystyrene microplate with 96 wells (Greiner Bio-one, Frickenhausen, Germany) were used. Cuvette and microplate readings were made at 975 and 900 nm, 25 °C, for the corresponding assay buffers in the total volume of the mixture (PPO: 50 mM phosphate buffer pH 6.5 with 3.0 mL in cuvette and 200 µL in well; POD: 67 mM phosphate buffer pH 6.0 with 2.4 mL in cuvette and 201 µL in well). For determining *K*, the numbers of replicates were six and eight for the cuvette and microplate readings, respectively.

2.6 Microplate methods validation and testing

The standard solutions of commercial POD and PPO were used as samples to validate the microplate methods, following the guidelines from the International Conference on Harmonization guidelines (ICH Expert Working Group, 2005). Seven concentrations of the horseradish POD (from 0.003 to 0.03 µg mL^{-1}) and seven concentration of mushroom PPO (from 0.04 to 0.6 µg mL^{-1}) were analyzed. Performance characteristics, such as linearity, detection limit (LOD), quantitation limit (LOQ), precision (repeatability) and accuracy, were taken into account for the validation.

Calibration curves of the standard solutions were obtained in enzyme concentration ranges corresponding to the activity levels usually observed in fruit and vegetables. Three independent analytical curves were obtained from seven concentrations of POD and PPO, which were prepared in triplicate from the stock solutions. The calibration curves were made by plotting the enzymatic activity (U) against the enzyme concentration (µg mL^{-1}). For PPO and POD assays, the linearity was evaluated by linear regression (least squares).

The LOD and LOQ were based on the standard deviation of the response and the average slope of three curves. LOD was calculated as $3.3 \cdot \sigma / S$ and LOQ was calculated as $10 \cdot \sigma / S$, where σ is the standard deviation of the response and *S* is the slope of the linear regression (ICH Expert Working Group, 2005).

The precision (repeatability) was evaluated with three concentrations of POD and PPO standard solutions analyzed during the same day and under the same experimental conditions in eight replicates. The precision was expressed as a relative standard deviation (*RSD* %) between the replicate measurements.

Accuracy was evaluated as the percentage recovery of known amounts of POD and PPO standards solutions added to the apple juice by using three activity levels in eight replicates.

In order to test the microplate methods, the POD and PPO activities were determined in apple juice. The supernatants of the centrifuged fresh apple juice and centrifuged sterilized apple juice were blended in seven different proportions (1:0, 9:1, 2:1, 1:1, 1:2, 1:9, 0:1 – ratio of fresh: sterilized juice) to obtain different values of enzymatic activities, which were evaluated by cuvette and microplate methods.

2.7 Statistical analysis

All the samples were analyzed in triplicate for the cuvette methods and in eight replicates for the microplate methods. Differences between the results of the microplate and cuvette methods were compared by *F*-test and *t*-test using software Statistica 12 (StatSoft, Tulsa, USA). The methods were considered statistically different with 95% confidence, when the *P*-value was < 0.05 for *F*-test and *t*-test.

3 Results and discussion

3.1 Comparison between cuvette and microplate methods

The path length correction factors obtained were $K = 1.80 \pm 0.06$ for the POD assay and $K = 1.76 \pm 0.04$ for the PPO assay (Equation 1) and the absorbance readings obtained by microplate methods were corrected (Equation 2). If the objective of the activity measurement is to obtain the residual activity (processed/unprocessed), then the determination of the correction factor is not necessary.

The enzymatic activity results of the standard POD and PPO solutions from the cuvette and microplate methods are presented in Figure 1, while the POD and PPO activities in the apple juice extracts are shown in Figure 2. For all the samples, the parity charts present a good agreement between microplate and cuvette results for the PPO and POD assays.

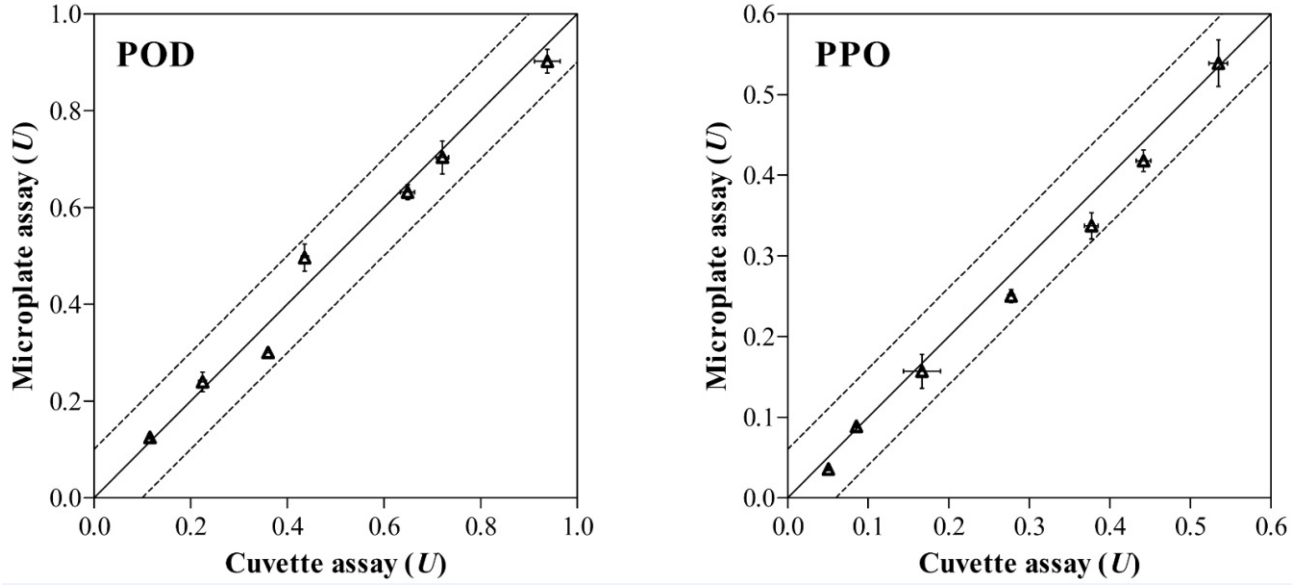

Figure 1. Comparison between cuvette and microplate measurements of the standard solutions: POD activity assay (– = ± 0.1) and PPO activity assay (– = ± 0.06).

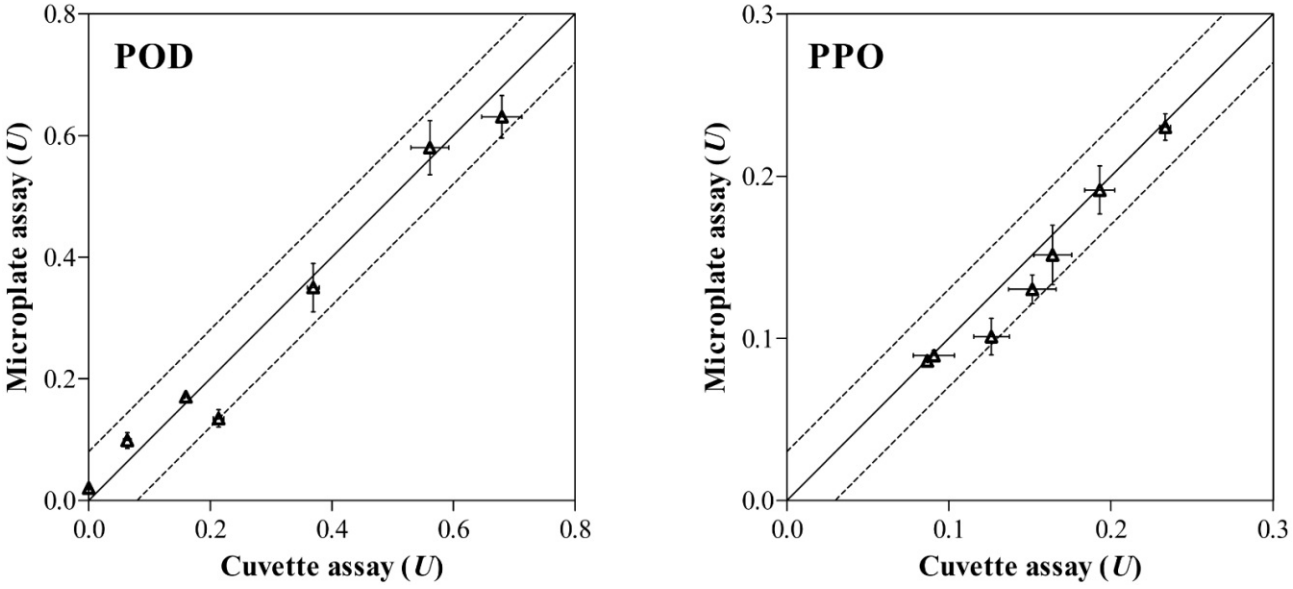

Figure 2. Comparison between cuvette and microplate measurements of apple juice: POD activity assay (– = ± 0.08) and PPO activity assay (– = ± 0.03).

The agreements between the cuvette and microplate methods were explored by statistical analysis in seven different concentration/dilution levels of apple juice, POD from horseradish and PPO from mushroom. For all the concentrations evaluated, the *P*-values for parameter *F* of *F*-test were above 0.05 indicating that the standard deviation between the cuvette and microplate measurements was not statistically significant at a 95% confidence level. All *P*-values for parameter *t* of the *t*-test were not lower than 0.05, which indicates that there is not a statistical difference between the means of the two assays at a 95% confidence level. These results confirm the interchangeability between the cuvette and the microplate techniques for POD and PPO assays in the concentration range studied.

The application of these protocols is of particular interest for studying enzyme inactivation kinetics in the processing of fruits and vegetables, in which a large number of samples must be handled. The microplate assays can measure 24 samples in quadruplicate by using 5 min of the spectrophotometer, instead of eight hours for the classical spectrophotometric assay. Besides, it is noteworthy that the amount of sample required for the enzymatic microplate assay is 12 to 15 times smaller than that required for the cuvette method. The scaled down assays presented here have advantages for kinetic studies by allowing multiple simultaneous assays, in contrast with the classical methods. The ease of adaptation to the microplate techniques and the advantages over the cuvette assay allow routine use for

determining POD and PPO activities in extracts from juice and vegetables.

3.2 Validation characteristics

The analytical measurements of chemical substances or microbiological organisms require validation procedures in order to evaluate the reliability and applicability of the method (ICH Expert Working Group, 2005). The validation characteristics of microplate assays for quantifying POD and PPO activities were determined in terms of linearity, LOD, LOQ, precision and accuracy; the results are summarized in Table 1.

Within a given range, the linearity of the microplate methods was evaluated for the ability of relating the measured signal with the concentration of a reference standard (analyte) (ICH Expert Working Group, 2005). The calibration curves for POD and PPO activities could be linearly fitted with no interception parameter, since no activity is expected in pure buffer, in the range of 3.3 to 30.3 ng mL^{-1} for the POD assay (Figure 3A) and 43 to 585 ng mL^{-1} for the PPO assay (Figure 3B). For statistically verifying linearity, the correlation coefficient (r) and the coefficient of determination (r^2) were calculated and are presented in Table 1. The linearity results were satisfactory to the studied concentration ranges, showing a consistent proportionality between enzyme concentration and enzyme activity.

According to Table 1, the smallest amount of horseradish POD that can have its activity detected, but not necessarily quantitated in the standard solution, is 0.870 ng mL^{-1}. For the mushroom PPO, this lower bound is 5.82 ng mL^{-1}. The smallest amount of enzyme that can have its activity measured reliably is 2.64 ng mL^{-1} for POD and 17.6 ng mL^{-1} for PPO. The low values of LOD and LOQ for the POD and PPO assays indicate high sensitivity of the proposed microplate methods (Horwitz, 2003; ICH Expert Working Group, 2005).

The repeatability refers to the precision of results under the same operating conditions over a short interval of time (ICH Expert Working Group, 2005). The proposed assays had a good degree of agreement among results, since the *RSD* values provided are lower than 15% (Table 1) indicating the high precision of the methods.

Table 1. Validation of proposed microplate methods for determining enzymatic activities of POD and PPO in the standard solutions.

Parameters	POD assay		PPO assay	
Linear regression*	r^2	0.988	r^2	0.995
	r	0.994	r	0.998
	slope	19.0 U (μg mL^{-1}) $^{-1}$	slope	0.420 U (μg mL^{-1}) $^{-1}$
LOD		0.870 ng mL^{-1}		5.82 ng mL^{-1}
LOQ		2.64 ng mL^{-1}		17.6 ng mL^{-1}
Repeatability (n = 8) RSD %	2.7 ng mL^{-1}	5.1%	152 ng mL^{-1}	5.3%
	5.0 ng mL^{-1}	3.7%	271 ng mL^{-1}	4.0%
	6.5 ng mL^{-1}	3.5%	338 ng mL^{-1}	4.0%
Accuracy (n = 8) %	1.3 ng mL^{-1}	103%	60 ng mL^{-1}	96%
	2.4 ng mL^{-1}	100%	85 ng mL^{-1}	103%
	6.2 ng mL^{-1}	101%	108 ng mL^{-1}	103%

*average of three calibration curves.

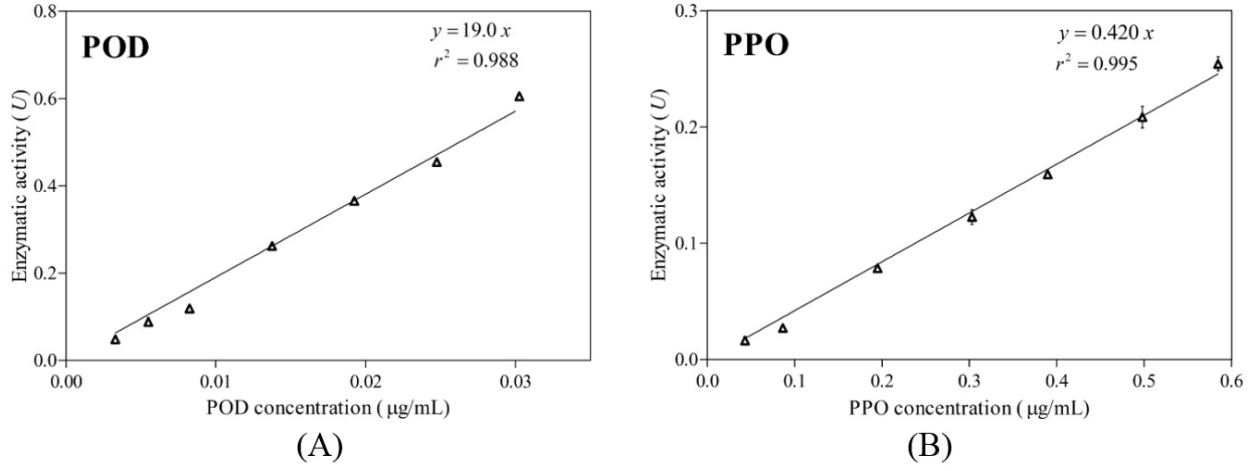

Figure 3. Calibration curves for quantifying POD activity (A) and PPO activity (B) in the standard solutions. Means of triplicates with error bars indicating standard deviation.

Accuracy can be expressed as the percent of recovery and may be estimated by a known added amount of analyte in the sample using a minimum of three concentrations levels (ICH Expert Working Group, 2005). The percent of recovery was considered satisfactory for both enzymes since the recovery remained between the limits of 96-103% for three concentration levels of POD and PPO in the standard solutions.

4 Conclusion

The proposed microplate methods for PPO and POD activity determination were validated and the results were satisfactory regarding linearity, repeatability, accuracy and quantitation along with detection limits. The response of the microplate reader assay is similar to the cuvette method for both enzymes with a good correlation between methods. The advantages of the microplate assay are the reduced amounts of sample and reactants required (reduction of 92%) besides the possibility of running several tests simultaneously in the spectrophotometer, with a time reduction from 8 h to 5 min to read, for instance, 24 samples in quadruplicate. This protocol can be beneficial to study enzyme inactivation kinetics in the processing of fruit and vegetables, in which a large number of samples must be handled. Currently, non-thermal technologies are under study to determine optimal processing conditions for enzymatic inactivation with minimal impact on quality attributes (Cullen et al., 2012), thus, the use of microplate techniques will be very beneficial.

Acknowledgements

The authors acknowledge the financial supports from FAPESP - São Paulo Research Foundation (grants 2014/17534-0, 2014/06026-4 and 2013/07914-8) and CNPq - National Council for Scientific and Technological Development (grant 459177/2014-1). The authors are thankful to Prof. Adalberto Pessoa Junior from the Faculty of Pharmaceutical Sciences at University of São Paulo for providing the research laboratory facilities.

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