Sequential Injection Lab-on-Valve System for the Determination of the Activity of Peroxidase in Vegetables

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Horseradish peroxidase (HRP) has been broadly used and investigated for many analytical purposes; it is an enzyme that catalyzes the reduction of hydrogen peroxide in the presence of a reducing compound. The objective of this work was to develop a methodology for the spectrophotometric determination of the activity of peroxidase in vegetable extracts using a flow method with a sequential injection lab-on-valve format. The developed system is based on the reaction between hydrogen peroxide (H_2O_2) and 2,2-azinobis(3-ethylbenzothiazoline-6)sulfonic acid (ABTS) catalyzed by the enzyme (HRP). The method presented a sample consumption of 15 μL per assay and a consumption of ABTS and H_2O_2 of 24 μg and 12 μg per assay, respectively. It was also possible to monitor online the thermal inactivation of peroxidase at different temperature ranges.

KEYWORDS: Sequential injection lab-on-valve; spectrophotometry; horseradish peroxidase; vegetables; thermal inactivation

INTRODUCTION

Peroxidase (E.C. 1.11.1.7) is an enzyme commonly found in vegetables that can catalyze a large number of reactions (*1*). These enzymes are widely distributed in the plant kingdom; however, the most studied is the one obtained from horseradish roots (horseradish peroxidase, HRP), which is also the most readily available commercially (*2*). The action of these enzymes can have a negative effect on the color and on the flavor of raw or processed food. They are involved in the enzymatic browning of vegetables, either separately or together with polyphenoloxidase (PPO). Peroxidase appears to be the most heat stable enzyme in plants, and since it is very resistant to thermal inactivation, it is widely used to evaluate the effectiveness of fruit and vegetable thermal blanching. The blanching process is a thermal procedure designed to inactivate the enzymes responsible for generation of off-flavors and off-odors. This procedure can be carried out by different methods, but water blanching is the most widely used technique for this purpose (*3*), and online monitoring of the blanching process is important to control the time of exposure and to achieve an efficient process. It is generally accepted that if the peroxidase originally present in the food is destroyed, it is quite unlikely that other enzyme systems have survived (*4, 5*). To minimize the quality deterioration of the products during frozen storage, it is necessary to reduce the activity of peroxidase (*6*). Nevertheless, the inactivation of peroxidase should not be complete, since there is evidence that the quality of the blanched and frozen products is better if there is some peroxidase activity left at the end of the blanching process. The complete absence of peroxidase activity is an indicator of overblanching (*7*).

The determination of the peroxidase activity has been described on the basis of colorimetric, chemiluminescence, electrochemical, or fluorimetric detection of the product formed from the peroxidase reducing substrate. The possible substrates include guaiacol, phenol, pyrogallol, *o*-tolidine, *o*-phenylendiamine cathecol, resorcinol, or *o*-dianisidine. One of the most frequently used substrates is guaiacol, but its reaction gives an undefined mixture of oxidation products, and the absolute molar absorptivity cannot be determined (*8, 9*). The high toxicity of these substrates is also a disadvantage. This limitation can be minimized by using flow based methods, which comprise lower reagent consumption. Furthermore, they permit the reduction of analysis costs, which is an important parameter in the development of new methodologies for bioassays (*10*). Flow injection methods (*11*) consist in the injection of a well-defined volume of sample solution into a carrier stream in a reproducible way; one or more reagents can be added downstream, and the formed reaction product is measured in a suitable flow through detector. For further reduction of reagent and sample consumption, a sequential injection system (*12*) can be used, where well-defined volumes of sample and reagents are sequentially aspirated to a holding coil and then propelled by reversed flow toward detection. Further miniaturization can be achieved by using sequential injection in a lab-on-valve format (SI-LOV). In this compact flow system, the detection cell is integrated on the top of the selection valve (*13, 14*). Thus, this flow method permits the implementation of bioassays at a microliter scale (*15*), with simple manipulation assuring robustness and repeatability (*12*). The SI-LOV system has been described as a useful tool in automating various biochemical methods (*16−18*).

The aim of this work was to develop a SI-LOV system for the determination of the peroxidase activity in vegetables. The

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determination is based on the reaction of hydrogen peroxide with 2,2-azinobis(3-ethylbenzothiazoline-6)sulfonic acid (ABTS) catalyzed by HRP (*19*). The oxidation of ABTS was followed by observing the increase in absorbance at 410 nm during 30 s. Taking advantage of the flow system versatility, the online monitoring of the thermal blanching process of an HRP enzymatic solution was also an aim of this study.

MATERIALS AND METHODS

Reagents and Solutions. All solutions were prepared from analytical grade reagents, and deionized water (conductivity < 0.1 μS/cm) was used throughout the work. A 100 mM potassium phosphate buffer (pH 6.0) (104873, Merck) and an enzyme diluent (pH 6.8), containing 40 mM potassium phosphate buffer, 0.25% (w/v) of bovine serum albumin (BSA, fraction V, Fluka), and 0.5% (v/v) of Triton X-100 (Sigma) were prepared. Potassium hydroxide (1.0 M, Merck) was used to adjust the pH of these solutions. For the preparation of the sample extracts, 100 mM potassium phosphate (pH 6.5) was prepared from monopotassium phosphate (Merck) and dipotassium phosphate (Merck). This buffer solution was kept cooled at 4 °C until use.

The daily working solutions of hydrogen peroxide were prepared by dilution from the stock solution (Perhydrol, 30% H_2O_2, *d* = 1.11, 107210, Merck). The ABTS stock solution was prepared by dissolving 10 mg (1 tablet) of ABTS (Sigma) in 25 mL of deionized water, and the daily working solution was prepared by additional dilution in potassium phosphate buffer (100 mM, pH 6.0).

An enzyme stock solution was prepared by resuspending 1.0 mg of lyophilized HRP (peroxidase from horseradish, EC 1.11.1.7, type VI, Sigma) in 1 mL of enzyme diluent. This stock solution was stable for at least one week when refrigerated. The working standard solutions were prepared daily by further dilution in the same buffer, in a concentration range between 0.5 and 2.0 mg/L.

Samples. Spinach (*Spinacia oleracea*), green beans (*Phaseolus vulgaris, L.*), and watercress (*Nasturtium officinale*) were purchased in a local market and stored at 4 °C until the preparation of the extracts. No vegetables were held longer than 72 h prior to processing. The extracts prepared were analyzed within one hour without other previous treatment than dilution.

Sample Extract. Preliminary study was performed to verify the occurrence of peroxidase and to establish the ratio between sample weight and the buffer solution volume for optimal reproducibility and linearity between enzyme concentrations and observed activity. This study was carried out using various green vegetables, such as green peppers (*Capsicum annuum*), collard greens (*Brassica oleracea*), rapini (*Brassica rapa*), watercress, spinach, and green beans. Initially, a portion of 5 g of sample was blended with 200 mL of buffer solution. When no initial activity was detected, the sample weight was increased to augment the ratio between sample weight and buffer solution volume. For the first three vegetables studied, the ratio was increased but no peroxidase activity was possible to detect. For the other vegetable samples, the ones used on this work, it was possible to extract peroxidase and the extraction parameters were established for each vegetable. For the extracts, 3 g of watercress, 25 g of spinach, and 30 g of green beans were used in 100 mL of buffer solution.

For the preparation of the sample extract, the vegetables were homogenized in 100 mL of buffer solution pH 6.5 at 4 °C, with a laboratory blender for 1 min. The slurry was filtered through four layers of cotton gauze and was centrifuged (Universal 320R, Hettich Zentrifugen, Germany) at 6080*g* during 20 min, to eliminate the turbidity of the homogenates. The supernatant was filtered through filter paper and kept on ice until analysis.

Apparatus. The SI-LOV system (FIAlab-3500, FIAlab Instruments, Medina, WA) presented in **Figure 1**, consisting of a bidirectional syringe pump (2500 μL of volume), a holding coil, a bidirectional variable speed peristaltic pump, and a lab-on-valve manifold mounted on the top of a six-port multiposition valve, was used. As a detection system, a USB 2000 Ocean Optics charge coupled device (CCD) array spectrophotometer equipped with fiber optics (id: 200 μm) and a DH-2000-BAL Mikropack UV/vis/NIR light source was used. FIAlab for windows 5.0 software on an Intel(R) Core (TM) 2 CPU Computer (1.86 GHz, 0.99 GB) was used for flow programming and data acquisition.
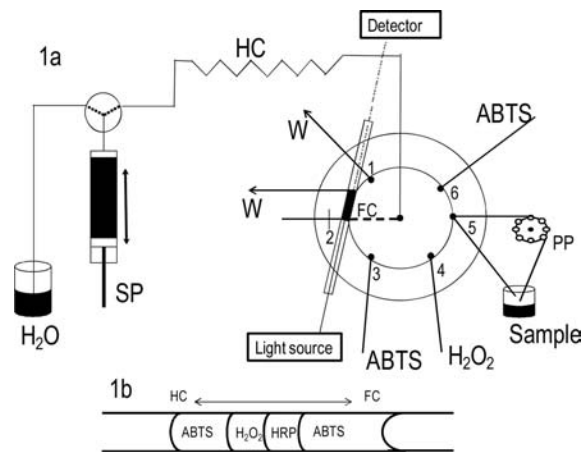

Figure 1. (**1a**) SI-LOV manifold for the determination of the peroxidase activity in vegetables extracts. SP, syringe pump; HC, holding coil; FC, flow cell; PP, peristaltic pump; ABTS, 160 mg/L; H_2O_2, hydrogen peroxide, 0.8 g/L; W, waste. (**1b**) Aspiration sequence.

Table 1. Flow Protocol Sequence of the Developed Method

step	description	volume (μL)	flow rate (μL/s)	selection valve position
A	aspirate carrier to SP	1000	150	
B	aspirate ABTS to HC	50	80	3
C	aspirate H_2O_2 to HC	15	25	4
D	aspirate sample to HC	15	25	5
E	aspirate ABTS to HC	100	80	6
F	reverse flow, reference scan	10	15	2
G	dispense HC content	110	15	2
H	stop flow, data acquisition			
I	system washing		150	2

A benchtop spectrophotometer, Thermo-Spectronic (Cambridge, U.K.) Heλios γ UV−vis, was also used during reference measurements.

Flow Procedure. The SI-LOV flow procedure is summarized in **Table 1**. The initial steps (A−E) consisted of the aspiration of carrier, reagents, and sample to the holding coil (in the order 1000 μL of carrier, 50 μL of ABTS, 15 μL of H_2O_2, 15 μL of standard, and 100 μL of ABTS). The following steps (F−I) consisted of reversing the flow and propelling the mixture toward detection for absorbance scanning (*t* = 30 s), followed by a final washing of the system.

The activity of peroxidase in the vegetable samples was calculated by interpolation on the calibration curves, plotting $\Delta A/\Delta t$ as a function of concentration of HRP. The calibration curve obtained under the optimized experimental conditions can be written as $\Delta A/\Delta t$ = 0.0041 ($\pm$0.0002) × [HRP] (mg/L) + 0.0016 ($\pm$0.0006).

Comparison Method. The accuracy of the results obtained in the analysis of the samples in the developed method was assessed by comparison with the method proposed by Sigma (*20*): 2.90 mL of 200 mg/L of ABTS was transferred into suitable cuvettes, where 50 μL of sample solution was added. Subsequently, 100 μL of 3.3 g/L of H_2O_2 solution was mixed and the increase in absorbance was registered at 405 nm for 2 min. The HRP activity was calculated by interpolation on the calibration curve obtained as the slope of the absorbance increase as a function of the enzyme concentration (mg/L). This calibration curve was established using the same standard solutions as in the developed flow procedure.

RESULTS AND DISCUSSION

Study of the Flow System. The physical parameters studied (such as the volumes of sample and reagents, the aspiration sequence, and the flow rates applied in this flow system) were reported in a previous work (*21*). The aspiration sequence consisted of 50 μL of ABTS, 15 μL of sample, 15 μL of enzyme, and 100 μL of ABTS. The flow rate of 30 μL/s was used to propel

Table 2. Means and Standard Deviations of the Results Obtained for All of the Samples Analyzed by the SI-LOV ($n = 5$) and the Comparison ($n = 3$) Methods, and Corresponding Relative Deviations (RD)

		watercress			spinach			green beans		
sample ID[a]		comp method (mg L^{-1})	SI-LOV method (mg L^{-1})	RD (%)	comp method (mg L^{-1})	SI-LOV method (mg L^{-1})	RD (%)	comp method (mg L^{-1})	SI-LOV method (mg L^{-1})	RD (%)
A	1	1.01 ± 0.13	0.93 ± 0.08	−8.0	6.93 ± 0.16	6.65 ± 0.27	−4.0	1.80 ± 0.09	1.84 ± 0.14	2.1
	2	0.95 ± 0.08	0.86 ± 0.10	−9.4	5.56 ± 0.25	4.56 ± 0.21	−18.0	1.96 ± 0.02	2.24 ± 0.00	14.4
	3	1.05 ± 0.10	1.39 ± 0.16	32.9	2.89 ± 0.11	3.57 ± 0.10	23.5	3.50 ± 0.52	3.96 ± 0.51	13.0
	4	1.08 ± 0.19	0.93 ± 0.08	−13.8	5.57 ± 0.62	6.25 ± 0.58	12.2	3.10 ± 0.38	2.99 ± 0.25	−3.5
	5	1.24 ± 0.08	1.46 ± 0.09	17.5	6.45 ± 0.70	4.31 ± 0.34	−33.2	2.06 ± 0.19	2.13 ± 0.26	3.7
	6	1.30 ± 0.21	1.80 ± 0.10	38.2	6.26 ± 1.55	5.63 ± 0.83	−10.1	1.08 ± 0.06	1.32 ± 0.11	21.8
	7	1.55 ± 0.18	2.32 ± 0.00	49.7	4.87 ± 0.59	5.63 ± 0.83	15.6	1.04 ± 0.13	1.36 ± 0.09	30.2
	8	1.84 ± 0.02	2.38 ± 0.23	29.6	4.13 ± 0.49	1.99 ± 0.07	−51.8	1.24 ± 0.09	1.47 ± 0.12	18.3
	9	1.66 ± 0.19	2.27 ± 0.12	36.5	4.28 ± 0.07	1.92 ± 0.08	−55.1	1.72 ± 0.44	1.80 ± 0.21	4.7
	10	1.83 ± 0.10	2.11 ± 0.35	15.1	3.95 ± 0.82	1.90 ± 0.10	−52.0	1.80 ± 0.02	2.30 ± 0.11	28.1
	11	1.41 ± 0.11	1.57 ± 0.12	11.5	5.11 ± 0.27	4.25 ± 0.29	−16.9	1.28 ± 0.21	1.50 ± 0.00	17.1
	12	2.33 ± 0.08	1.99 ± 0.14	−14.7	5.73 ± 1.20	2.11 ± 0.33	−63.1	1.41 ± 0.04	1.95 ± 0.11	38.6
B	1	0.51 ± 0.03	0.54 ± 0.10	5.7	3.31 ± 0.14	4.82 ± 0.13	45.6	1.19 ± 0.11	1.22 ± 0.00	2.8
	2	0.52 ± 0.04	0.39 ± 0.08	−24.2	3.40 ± 0.58	3.27 ± 0.27	−3.8	1.17 ± 0.04	1.51 ± 0.11	29.2
	3	0.73 ± 0.05	0.43 ± 0.13	−41.5	2.12 ± 0.37	1.79 ± 0.16	−15.6	1.62 ± 0.05	2.16 ± 0.18	33.6
	4	0.62 ± 0.08	0.43 ± 0.00	−31.1	5.28 ± 0.68	4.82 ± 0.20	−8.7	1.31 ± 0.30	2.08 ± 0.12	59.1
	5	0.65 ± 0.04	0.50 ± 0.10	−22.8	5.13 ± 0.72	4.91 ± 0.00	−4.3	1.19 ± 0.05	1.54 ± 0.13	29.0
	6	0.90 ± 0.11	0.64 ± 0.12	−28.1	4.80 ± 0.87	3.85 ± 0.36	−19.8	0.82 ± 0.05	1.00 ± 0.00	22.6
	7	1.11 ± 0.09	1.24 ± 0.00	12.2	2.76 ± 1.40	3.85 ± 0.95	39.5	0.65 ± 0.02	0.84 ± 0.09	30.0
	8	1.22 ± 0.08	1.51 ± 0.00	23.7	2.59 ± 0.21	1.22 ± 0.07	−52.8	0.93 ± 0.12	0.93 ± 0.00	0.8
	9	1.15 ± 0.06	1.46 ± 0.12	26.5	2.49 ± 0.31	1.37 ± 0.08	−45.2	0.90 ± 0.00	1.25 ± 0.00	38.6
	10	1.22 ± 0.09	1.30 ± 0.12	6.2	2.30 ± 0.10	1.11 ± 0.00	−51.7	1.04 ± 0.11	1.55 ± 0.11	48.9
	11	1.16 ± 0.07	1.03 ± 0.12	−11.3	3.18 ± 0.41	3.06 ± 0.65	−3.9	0.77 ± 0.02	1.00 ± 0.00	29.1
	12	1.47 ± 0.07	1.30 ± 0.12	−11.8	3.69 ± 1.44	1.74 ± 0.00	−52.9	1.03 ± 0.10	1.35 ± 0.14	30.6
C	1	0.38 ± 0.01	0.39 ± 0.08	4.1	3.08 ± 0.37	4.33 ± 0.00	40.6	0.86 ± 0.04	0.82 ± 0.00	−5.3
	2	0.30 ± 0.07	0.21 ± 0.08	−28.5	3.01 ± 0.32	2.17 ± 0.19	−27.9	0.98 ± 0.03	1.02 ± 0.00	3.8
	3	0.24 ± 0.25	0.25 ± 0.00	4.0	1.61 ± 0.22	1.83 ± 0.00	13.7	1.16 ± 0.08	1.43 ± 0.00	22.9
	4	0.34 ± 0.03	0.25 ± 0.00	−25.7	4.82 ± 0.27	3.75 ± 0.00	−22.2	1.20 ± 0.08	1.39 ± 0.12	16.1
	5	0.44 ± 0.05	0.32 ± 0.10	−26.7	5.03 ± 0.51	4.25 ± 0.28	−15.5	0.87 ± 0.07	1.03 ± 0.12	18.2
	6	0.77 ± 0.03	0.51 ± 0.00	−33.8	3.91 ± 0.23	4.48 ± 0.36	14.6	0.63 ± 0.06	0.65 ± 0.10	2.5
	7	0.84 ± 0.03	0.97 ± 0.00	16.1	3.29 ± 0.45	4.48 ± 0.18	36.2	0.50 ± 0.03	0.60 ± 0.00	19.7
	8	0.84 ± 0.07	0.97 ± 0.00	16.1	2.04 ± 0.13	0.84 ± 0.07	−58.9	0.66 ± 0.03	0.71 ± 0.00	7.0
	9	0.98 ± 0.08	0.97 ± 0.00	−0.6	1.58 ± 0.14	0.90 ± 0.00	−42.9	0.68 ± 0.06	0.75 ± 0.00	10.9
	10	0.87 ± 0.03	0.97 ± 0.00	11.2	1.50 ± 0.21	0.56 ± 0.00	−62.3	0.62 ± 0.03	0.90 ± 0.14	44.2
	11	0.82 ± 0.02	0.81 ± 0.15	−1.7	2.38 ± 0.21	1.74 ± 0.00	−27.0	0.60 ± 0.01	0.55 ± 0.11	−8.1
	12	1.02 ± 0.22	1.24 ± 0.00	22.1	1.76 ± 0.47	1.04 ± 0.11	−40.6	0.77 ± 0.01	0.80 ± 0.11	4.1

[a] A, sample extract analyzed without dilution. B, sample extract analyzed after dilution (dilution factor of 1.4). C, sample extract analyzed after dilution (dilution factor of 2).

the product of the reaction toward detection, and the flow was stopped after a preset time period for absorbance scanning.

With the physical conditions defined, the effect of the reagents concentration (H$_2$O$_2$, ABTS) on the analytical signal was studied with the aim to guarantee that they were not the limiting reagents in the reaction and that the activity detected was only dependent on the quantity of the enzyme present. These conditions are necessary for an initial rate based assessment of enzyme activity. Calibration curves up to 5 mg/L of HRP were performed. The concentration of H$_2$O$_2$ was studied in the range 0.7−1.2 g/L. When the concentration was increased from 0.7 to 0.8 g/L, the sensitivity improved about 12%. With a further increase of H$_2$O$_2$ concentration, it was possible to observe a slight decrease of the sensitivity; for this reason the concentration chosen was 0.8 g/L.

The concentration of ABTS was studied between 100 and 200 mg/L. The sensitivity of the method increased with increasing concentrations of ABTS. However, for concentrations higher than 160 mg/L, no linearity was attained; therefore, this concentration was chosen for further work.

Based on the comparison method, the stop time for absorbance scanning was initially established as 2 min. With the decrease of this time down to 30 s, no significant difference was obtained in the quality of the results. Therefore, to increase the determination rate, a 30 s stop period was used in the work.
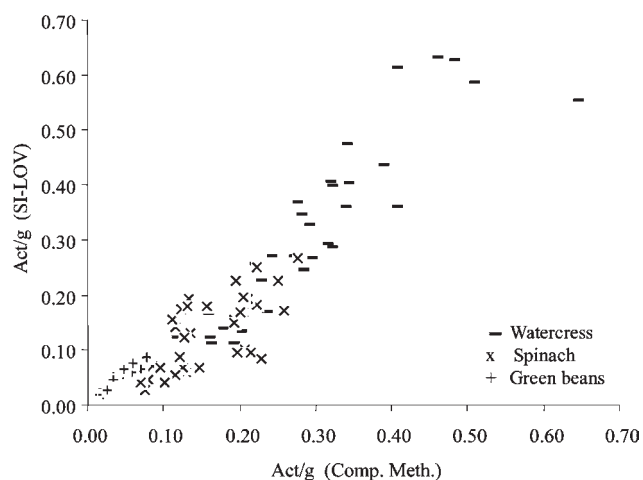
Figures of Merit of the Method. The performance of the developed method was evaluated in terms of reagent and sample consumption, determination rate, and application range. The method was also compared to the one chosen for its validation (*20*). The method presented a sample consumption of 15 μL per assay and consumption of ABTS and H$_2$O$_2$ of 24 μg and 12 μg, respectively, per assay. It was possible to achieve a linear range up to 2 mg/L with a throughput of one determination per minute, which corresponds to an increase of the determination rate of 50% compared to that of the comparison method. The limits of determination and quantification (*22*) obtained were 0.3 and 0.9 mg/L, respectively.

The operational stability of the developed system was calculated by performing the calibration procedure under identical physical and chemical conditions between and within different working days. A calibration curve [$\Delta A/\Delta t = 0.0041 \, (\pm 0.0002) \times$ [HRP] (mg/L) + 0.0016 ($\pm$0.0006)] with a RSD < 5% for the slope was obtained; the values in parentheses are the standard deviation values of the calibration curve parameters (5 standard solutions injected 5 times each), which were assessed during a period of two months ($n = 7$). The within day repeatability was evaluated at three different working days and was found to be better than 3.5% (assessed as the percentage of the standard error of the slope).

Table 3. Linear Relationships Obtained for the Analysis of Different Samples in the SI-LOV and the Comparison Methods, with Limits of the 95% Confidence Interval

sample[a]	sample dilution	linear relationship (n = 12)
watercress	A	$Act_{developed\ method} = 1.068\ (\pm0.574)\ Act_{comp\ method} + 0.132\ (\pm0.857)$
	B	$Act_{developed\ method} = 1.250\ (\pm0.408)\ Act_{comp\ method} - 0.275\ (\pm0.404)$
	C	$Act_{developed\ method} = 1.212\ (\pm0.289)\ Act_{comp\ method} - 0.135\ (\pm0.205)$
spinach	A	$Act_{developed\ method} = 0.914\ (\pm0.841)\ Act_{comp\ method} - 0.639\ (\pm4.431)$
	B	$Act_{developed\ method} = 1.002\ (\pm2.273)\ Act_{comp\ method} - 0.444\ (\pm0.635)$
	C	$Act_{developed\ method} = 1.077\ (\pm1.512)\ Act_{comp\ method} - 0.521\ (\pm0.491)$
green beans	A	$Act_{developed\ method} = 0.959\ (\pm0.180)\ Act_{comp\ method} + 0.313\ (\pm0.355)$
	B	$Act_{developed\ method} = 1.452\ (\pm0.478)\ Act_{comp\ method} - 0.157\ (\pm0.517)$
	C	$Act_{developed\ method} = 1.191\ (\pm0.316)\ Act_{comp\ method} - 0.060\ (\pm0.260)$

[a] A, sample extract analyzed without dilution; B, sample extract analyzed after dilution (dilution factor of 1.4); C, sample extract analyzed after dilution (dilution factor of 2).

Figure 2. Activity of peroxidase per fresh weight of the sample obtained by SI-LOV and by the comparison method.
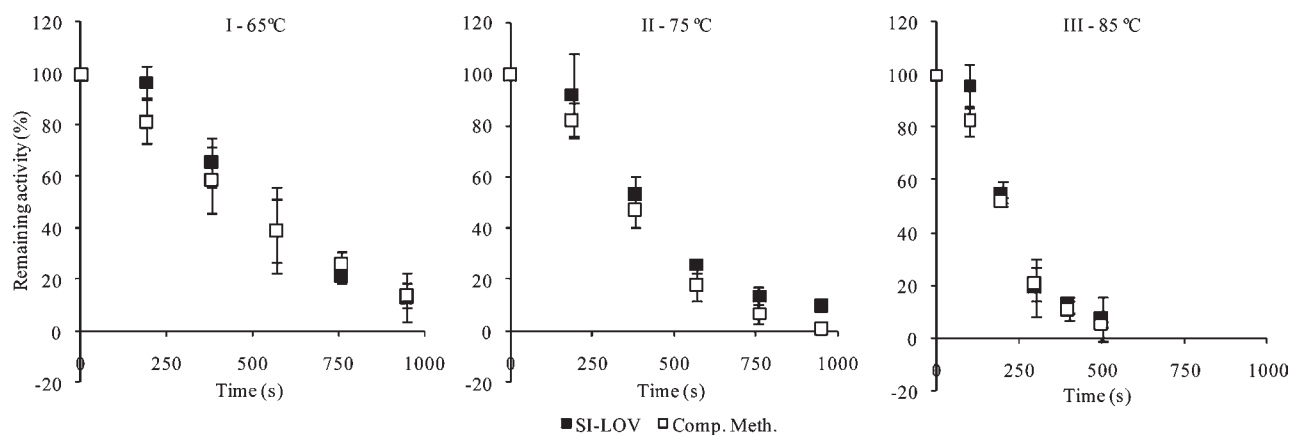
Sample Analysis. *Determination of the Activity of Peroxidase in Vegetables.* A total of 12 extracts for each sample were analyzed with and without dilution. This study was conducted to obtain an insight into the possible effect of the vegetable extract matrix on the spectrophotometric activity assay. In the case that no matrix effect can be identified, the activity values obtained should be independent of the dilution. For this reason, and to evaluate the accuracy of the developed method, the comparison method was also performed using the same sample extract. The results obtained in the sample analysis are summarized in **Table 2** and present a linear relationship [$Act_{developed\ method} = m\ Act_{comp\ method} + b$] described by the equations listed in **Table 3**, where m represents the slope and b corresponds to the origin of the regression line. In the case of a good agreement between the methods, m should not be significantly different from unity, and b should be close to zero. The values presented in parentheses are the limits of the 95% confidence intervals (22). It can be demonstrated that the results obtained for the developed method are in good agreement with those obtained for the comparison method.

The activity obtained in the developed and in the comparison method by fresh weight of sample (Act/g) is presented in **Figure 2**. It can be concluded that the activity obtained for all samples is characteristic of the sample and not dependent on the weight of sample used. It can be seen that green beans—the vegetables that are less susceptible to undesirable alterations in texture, flavors, and colors—are the vegetables that have lower peroxidase activity per weight of sample. On the other hand, watercress, which usually is a fresh product and has a short shelf life, has higher peroxidase activity per weight of sample.

When considering all the samples analyzed within one type of commodity and the activity values obtained for this commodity with different dilution rates, it is possible to conclude that the peroxidase concentrations in the diluted samples are not significantly different from the ones obtained without dilution. The F values calculated were as follows: 0.27 for watercress, 0.70 for spinach, and 1.3 for green beans; with a corresponding critical F value of 3.28. This result can be justified by the wide dispersion of the values itself within the studied commodities. However, if some of the individual samples are considered, a clear tendency can be verified, indicating an apparent matrix effect. This behavior is referred to as a frequent pitfall of spectrophotometric methods for enzyme activity assays in natural samples, resulting in the necessity of a strict control of the experimental conditions for batch assays. This also underlines the advantages of using

Figure 3. Variation of remaining activity (%) during a thermal inactivation process in solutions of HRP at different temperatures. Data are presented as the means of five independent experiments for the SI-LOV and three independent experiments for the comparison method; the error bars denote ± standard deviation.

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flow methods with inherent assay reproducibility for enzymatic determinations.

Thermal Inactivation of Peroxidase in Solutions. Taking advantage of the versatility of the system, it was possible to perform online the monitoring of the thermal inactivation of peroxidase, aiming also to provide a valuable tool for evaluation of the thermal blanching process effectiveness (*5, 23*).

The thermal inactivation of peroxidase enzyme in a solution of 2 mg/L was studied in the temperature range of 65−85 °C. The results obtained for the developed and for the comparison method are presented in **Figure 3**. The results of the developed method are in good agreement with the comparison method. It can also be concluded that, with the rise of the temperature from 65 to 75 °C, it is possible to reduce the procedure time at 200 s to achieve 20% of remaining activity. When the blanching temperature is 85 °C, it is possible to reduce the time of the process in a total of 470 s, to attain the 20% of remaining activity. A higher standard deviation is verified when the blanching is carried out at 65 °C (**Figure 3I**); this can be due to the fact that the enzyme is thermally stable up to 60 °C (*24, 25*) and the temperature of blanching is close to this limit.

In conclusion, the use of a lab-on-valve format with reduced injection volumes proved to be a useful tool for the development of the method for the determination of peroxidase in vegetables. The results obtained for the analysis of real samples are in good agreement with the results obtained for the comparison method, with lower sample and reagent consumptions. It was also possible to achieve a linear range up to 2 mg/L of HRP with a significant (50%) increase of the determination rate in comparison with the batch method. Using the same system, it was also possible to monitor online the thermal inactivation of the enzyme in solutions, which is a fundamental parameter for an efficient evaluation of the thermal blanching process.

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