# PEROXIDASE AND POLYPHENOL OXIDASE INACTIVATION BY MICROWAVES IN SIMULATED SOLUTIONS

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**Abstract.** Green coconut water must be consumed soon after fruit opening because when in contact with oxygen, enzymes from coconut water such as peroxidase (POD) and polyphenol oxidase (PPO) begin reactions that modify typical food properties, causing nutritional value and color losses. To increase product shelf-life, commercial sterilization and pasteurization have been applied to inactivate these enzymes. Submitting the food to high temperatures causes sensory and nutritional losses. According to literature, microwave application to liquid foods has advantages because it promotes higher enzymatic inactivation rates at lower temperature and minimizes product quality losses. The aim of this work was to obtain knowledge on the behavior of these enzymes when submitted to microwave processing and verify possible influences of the major chemical constituents in coconut water on enzyme activities. Solutions (SIGMA standard enzymes) consisted of: POD/Water; POD/Water/Salts, POD/Water/Sugars, simulating the chemical constituents of coconut water, were submitted to a batch process in a microwave oven (CEM, Star System 2) at different temperature/time conditions. The same procedure was done for PPO. Results demonstrated that contact between salts and enzymes reduced the residual activities of POD and PPO. When the solution POD/Water/Salts was submitted to microwave energy, the enzyme activity was not detected. Other solutions also presented significant reductions in residual activity when submitted to microwave energy at temperatures from 70 to 90 ºC. These results can indicate an adequate choice temperature/time conditions to inactivate coconut water enzymes. The detection of which constituents of coconut water influence POD and PPO activity will supply useful information about the preservation of processed coconut water by microwaves.

**Keywords:** Microwaves, Inactivation of enzymes, Thermal processing.

## 1. Introduction

Green coconut water can be considered a natural isotonic drink, due to its minerals and sugars content and it also has a nice flavour. It has become a very popular drink in Brazil and can be found either *in natura* or processed. In order to avoid spoilage and enzymatic browning caused by peroxidase (POD) and polyphenol oxidase (PPO) when the product is exposed to air for a long time, coconut water can undergo different conservation processes such as UHT (Ultra High Temperature), conventional pasteurization, refrigeration and freezing (Campos et al., 1996; Abreu and Rosa, 2000; Duarte et al., 2002).

Microwave heating as an alternative method for liquid food pasteurization has gained better acceptance as it offers several advantages over the conventional method. This is because of the ability of microwave to heat products internally, greater penetration depth and faster heating rates that would potentially improve retention of thermolabile constituents in the food (Nikdel et al. 1993; Heddleson and Doores, 1994; Tajchakavit and Ramaswamy, 1997; Deng et al., 2003; Gerard and Roberts, 2004).

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Time and temperature are two important parameters that significantly affect thermal treatment. The lethality of microorganisms and inactivation of enzymes are related to temperature and time of exposure. Because of this the measurement of temperature and time are critical factors in thermal process control.

The inactivation of enzymes by microwave energy is by thermal destruction similar to conventional heating mechanisms. However, a problem that has often been encountered is the occurrence of temperature profiles within a product. The measurement of temperature profiles during microwave heating is more difficult than other traditional heating methods. In general, monitoring temperatures within microwave systems is conducted using fragile and expensive fiber optic probes (Deng et al., 2003; Gerard and Roberts, 2004).

PPO and POD are widely detected in many fruits and vegetables and are closely linked to enzymatic color changes with consequent loss of sensorial properties and nutritional quality (Robinson, 1991; Duarte et al., 2002).

Different names have been associated with PPO including tyrosinase, cresolase, cathecolase and phenolase and generally reflect the ability of this enzyme to utilize many different phenolic compounds as substrates. POD is a group of enzymes that catalyse oxidation-reactions. They reduce $H_2O_2$ to water while oxidizing a variety of substrates (Robinson, 1991).

According to some researchers PPO and POD are very resistant to heat, as a consequence of this, these enzymes are considered biological indicators of thermal processing (Robinson, 1991).

The aim of this work was to obtain real temperature and time profiles during microwave heating and to obtain knowledge on the behavior of PPO and POD when submitted to electromagnetic energy and verify possible influences of the major chemical constituents in coconut water on enzyme activities.

## 2. Materials and Methods

### 2.1. Enzyme solutions

Commercial horseradish POD (Sigma-P6140) and mushroom Tyrosinase (Sigma-T3824) were used to prepare the enzyme solutions: $3.5 \times 10^{-6}$ g POD/mL distilled water and $5.4 \times 10^{-7}$g PPO/mL distilled water. Simulated solutions (PPO/Water; PPO/Water/Sugars; PPO/Water/Salts; POD/Water; POD/Water/Sugars and POD/Water/Salts) were prepared to contain sugars, salts and enzyme activity close to the average contents of coconut water. Table 1 presents salts and sugars concentrations used in this work.

**Table 1**. Concentrations of salts and sugars used to prepare 100 mL of simulated solutions.

| **Salts** | **Mass (mg) for 100 mL** | **mM** |
|---|---|---|
| $KH_2PO_4$ | 44 | 3.23 |
| $K_2SO_4$ | 336 | 192.82 |
| $Na_2SO_4$ | 31 | 21.82 |
| $CaCl_2$ | 47 | 42.30 |
| $MgCl_2$ | 20 | 20.99 |
| **Sugars** | **Mass (mg) for 100 mL** | **mM** |
| Sucrose | 280 | 81.87 |
| Glucose | 2378 | 1321.11 |
| Fructose | 2400 | 1333.33 |

### 2.2. Microwave thermal treatment

The system consisted of a microwave oven (CEM, Star System 2) at 2450 MHz. This equipment has two cavities where specific glass tubes (310 mm length and 41 mm diameter) were inserted. The maximum volume should be 20 mL to obtain homogeneity of microwave incidence. Samples of simulated solutions were, individually, submitted to a batch process in a microwave oven at different temperature/time conditions.

The microwave oven has an automatic program that controls microwave incidence. It was possible to program only exposition time and temperature, but the original probe inserted in the equipment was not in contact with the sample, therefore temperature-time profile measured was not reliable.

After microwave incidence, the glass tube was removed from the microwave oven and inserted in an ice bath to accelerate temperature decrease.

Real time-temperature profiles of samples during batch processing were determined using a fiber optic probe (Gávea Sensors, TRB, 0.1 °C precision) inserted centrally inside the glass tube. The fiber optic probe was calibrated with distilled water using a calibrated thermometer (INCOTERM, 0.1 °C precision). Temperature readings were recorded using a continuous data acquisition system. Subsequent to heating, samples of 2 mL were collected to determine the enzymatic activity and were quickly cooled in a freezer (FANEM, 349 FV, -30°C, ± 0.5 ºC). Figure 1 presents a schematic diagram of batch processing.

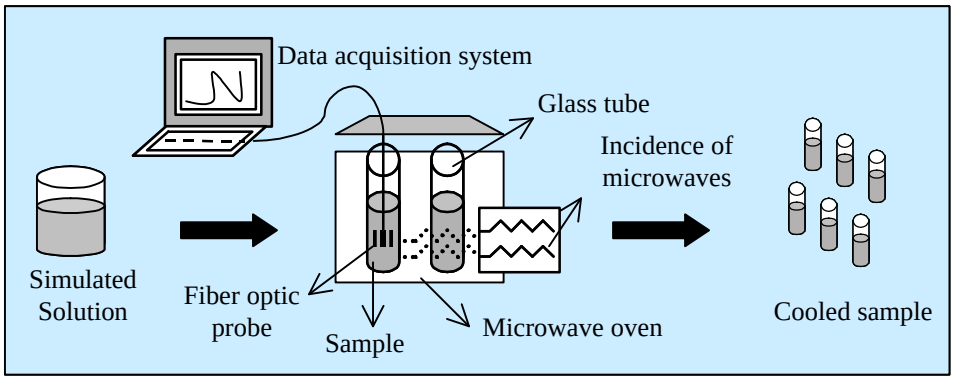



**Fig. 1**. Schematic diagram of batch processing.

### 2.3. Determination of enzymatic activity

POD activity was assessed spectrophotometrically at 470 nm in a spectrophotometer FEMTO, 700 PLUS, UV-VIS, according to the method described by Campos et al. (1996). A test tube containing 7.0 mL of 0.2 M sodium phosphate buffer pH 5.5 and 1.5 mL of 0.05 % of guayacol solution and 0.5 mL of 0.1 % hydrogen peroxide solution. The solution was immersed in a controlled temperature bath (MLW, U2C) at 35 °C for 5 min for thermal stabilization, after that an aliquot of 1.0 mL of simulated solution was added.

The reference value of POD (0.000 absorbance) was determined using a test tube containing 7.0 mL of 0.2 M sodium phosphate buffer pH 5.5 and 1.0 mL of simulated solution.

PPO activity was assessed spectrophotometrically at 425 nm in the same spectrophotometer. A test tube containing 5.5 mL of 0.2M sodium phosphate buffer pH 6.0 and 1.5 mL of 0.2 M pyrocatechol solution (15890,

FLUKA) was immersed in a controlled temperature bath (MLW, U2C) at 25 °C for 5 min for thermal stabilization and an aliquot of 1.0 mL of simulated solution was added. The reference value of PPO (0.000 absorbance) was determined using a test tube containing 5.5 mL of 0.2 M sodium phosphate buffer pH 6.0 and 1.5 mL of 0.2 M pyrocatechol solution.

The linear portion obtained in plotting absorbance as a function of time was used to compute PPO or POD activity. All enzyme activities were analysed at least in duplicate.

In both cases, one unit of enzyme activity was defined as the quantity necessary to produce an increase in absorbance of 0.001 per second per mL of sample.

The residual activity was determined by ($A/A_0$) where: A = mean residual activity (after microwave heating); $A_o$ = mean initial enzyme activity (before microwave heating).

### 2.4. Physico chemical analyses

- pH was directly measured using pH-Stat RADIOMETER, PHM-290, 0.001 pH precision;
- Soluble solids, expressed as ° Brix were determined by a refratometer CARLZEISSJENA, I, 0.1

precision and corrected by temperarute (AOAC, 1995).

## 3. Results and Discussion

Initially, some experiments were carried out with distilled water to verify the precision of the fiber optic probe when inserted in the microwave oven. Different temperature-time conditions were tested (Temperatures: 40 °C, 45 °C, 50 °C and 60 ° C; times: 30 s, 1 min., 3 min and 5 min).

Table 2 presents values of programmed temperature and time and the actual values measured by the fiber optic probe when in direct contact with water.

**Table 2**. Values of programmed temperature and time and actual values of maximum temperature and heating times measured by fiber optic probe.

| Samples (water) | Programmed time | Programmed Temperature | Real heating time | Real Maximum Temperature (°C) |
|---|---|---|---|---|
| A1 | 30 s | 40 ºC | 32 s | 71.0 ± 8.5 |
| A2 | 30 s | 45 ºC | 30 s | 91.1 ± 0.4 |
| A3 | 30 s | 50 ºC | 27 s | 95.3 ± 1.8 |
| A4 | 30 s | 60 °C | 37 s | 101.2 ± 3.9 |
| B1 | 5min | 40 ºC | 1 min 58 s | 48.1 ± 7.4 |
| B2 | 5min | 45 ºC | 1 min 36 s | 61.2 ± 1.8 |
| B3 | 5min | 50 ºC | 1 min 52 s | 70.8 ± 0.2 |
| B4 | 5min | 60 °C | 1 min 12 s | 80.5 ± 2.8 |

Thirty seconds and 1 min time conditions had a similar behavior and 3 min and 5 min conditions were not significantly different. The results presented in Table 2 showed that all real temperatures acquired by the fiber optic probe were higher than the programmed temperature.

Samples A were more difficult to control the rising temperature than B. This can be explained because temperature increased from the initial 20 ºC to programmed temperature in a short period of time (~30 s). To

reach the programmed temperature the microwave incidence was very strong, then, the microwave energy emitted was much more than necessary. This equipment was designed to work at higher temperatures.

Samples B reached the maximum temperature in the first 2 min of processing. After that, the microwaves incidence were turned off by microwave oven control and the temperature began to decrease. After reaching the maximum temperature, the glass tube was removed and inserted in an ice bath to accelerate temperature decrease.

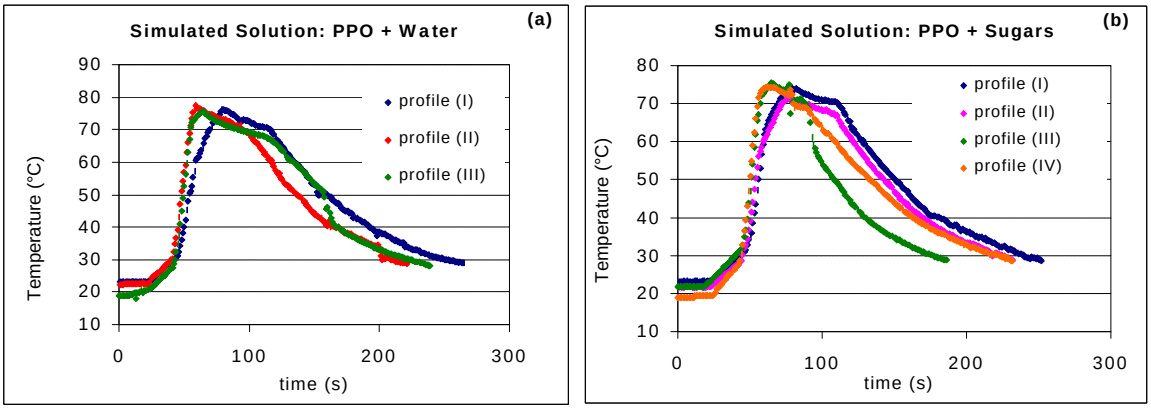
A programmed temperature of 40 °C obtained great variation in both time conditions A1 and B1. To continue this study temperatures above 45 °C and 5 min of heating time were chosen to conduct experiments with simulated solutions, as these conditions showed better control conditions.

### 4.1 Simulated Solutions

According to Tajchakavit and Ramaswamy (1997) the equivalent heating time can be estimated with some considerations. The maximum temperature obtained for each condition can be considered as the reference temperature. Applying an adequate z value for each enzyme, the heating time would be calculated using:

$$t_{equivalent} = \int_0^t L_t \cdot dt \qquad (1) \qquad \text{and} \qquad L = \int_0^t 10^{\left(T - T_{ref}/z\right)} dt \qquad (2)$$

Figure 2 presents temperature-time profiles, for a programmed temperature of 60 ºC and 5 min for PPO simulated solutions.



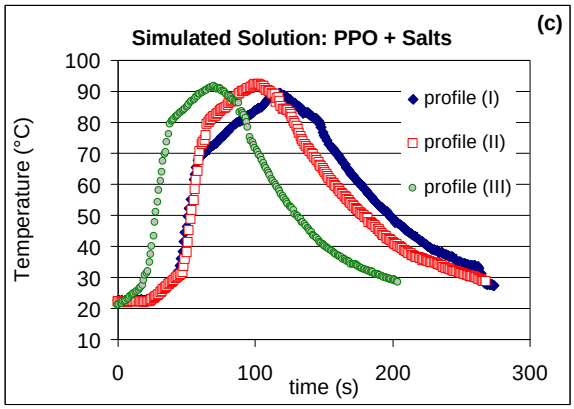



**Fig. 2.** Temperature - time profiles for: (a) PPO/distilled water, (b) PPO/Sugars, (c) PPO/Salts, at a programmed temperature of 60 °C and 5 min.

All temperature – time profiles (Fig. 2) reached higher temperatures than 60 ºC. A maximum temperature of PPO /Sugars and PPO/Water solutions was about 75 ºC. The behavior was very similar for both simulated solutions. The PPO/Salts solution reached the highest temperature, about 92 ºC. This result can be explained because ionic movement is one of the most important mechanisms that contributes to convert electromagnetic energy into heat.

Table 3 presents mean values of residual PPO activity, maximum temperature reached for each profile and equivalent heating time. A z value of 15°C was considered for the calculations.

**Table 3.** Mean values of PPO residual activity, maximum temperature of each condition and equivalent heating time for simulated solutions submitted to microwaves.

| **Programmed Temperature: 60 °C** | | | |
|---|---|---|---|
| **Simulated Solution of PPO/Water**: pH = 6.0 ± 0.01 | | | |
| Samples | Residual activity (%) | Maximum temperature (°C) | Equivalent time (s) at 77.31 °C |
| Profile (I) | 3.8 ± 0.57 | 76,32 | 30,95 |
| Profile (II) | 7.4 ± 1.29 | 77.31 | 29.76 |
| Profile (III) | 9.6 ± 0.18 | 75.62 | 29.31 |
| **Simulated Solution of PPO/Sugars**: pH = 6.3 ± 0.02 and 4.9 °Brix | | | |
| Samples | Residual activity (%) | Maximum temperature (°C) | Equivalent time (s) at 75.56 °C |
| Profile (I) | 4.9 ± 0.50 | 74.57 | 32.64 |
| Profile (II) | 5.6 ± 1.19 | 71.23 | 21.46 |
| Profile (III) | 10.6 ± 0.84 | 75.56 | 24.82 |
| Profile (IV) | 9.6 ± 1.60 | 74.43 | 27.17 |
| **Simulated Solution of PPO/Salts**: pH = 5.0 ± 0.01 | | | |
| Samples | Residual activity (%) | Maximum temperature (°C) | Equivalent time (s) at 92.32 °C |
| Profile (I) | 29.7 ± 2.23 | 89.27 | 23.68 |
| Profile (II) | 27.0 ± 3.12 | 92.32 | 34.40 |
| Profile (III) | 25.6 | 91.64 | 28.44 |

PPO/Water and PPO/Sugars solutions presented 90 % PPO activity reduction when submitted to microwaves incidence. However PPO/Salts solution presented a high residual activity (30%) when compared to other

solutions and this result was not expected. Zawistowsky et al. (1991), Campos et al. (1996), Duangmal and Apentem (1999), Mendonça and Guerra (2003) observed that in presence of salts there was a significant decrease in enzyme thermostability, this is due to salt-induced changes in enzyme conformation and possibly the dissociation of thermostable aggregated molecules.

Figure 3 shows initial PPO activity for each simulated solution, and also the increase of absorbance of catechol oxidation.

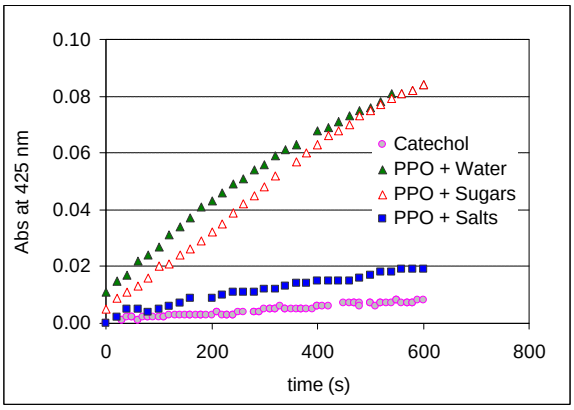



**Fig. 3.** Initial PPO activity in solutions of salts, sugars and water and catechol oxidation.

Initial PPO/Salts activity was lower than other solutions. Mean value of initial PPO/Salts activity was 4.26 x $10^{-5}$ U/ s.mL, while PPO/Water was 1.57 x $10^{-4}$ U/ s.mL and PPO/Sugars was 1.34 x $10^{-4}$ U/ s.mL.

As shown in Figure 3 the reaction rate for PPO/Salts solution and for catechol oxidation was close. This fact could superestimate the residual PPO/Salts activity, therefore the residual PPO/Salts enzyme activity solution presented in Table 3 could suggest a false interpretation.

To minimize catechol oxidation interference, more concentrated PPO solution (8.2 x $10^{-7}$g PPO/mL distilled water) are being tested. Figure 4 shows initial PPO/Salts activity and catechol oxidation.

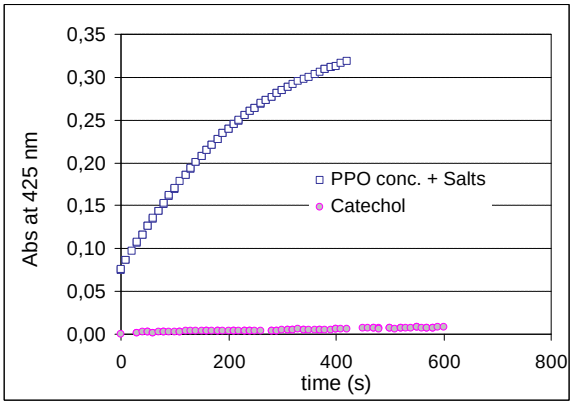



**Fig. 4.** Initial activity of PPO conc./Salts and catechol oxidation.

In Figure 4 the interference of catechol oxidation was not significant and the correct determination of PPO/Salts activity could be measured.

An increase in PPO concentration will probably reduce the effect of the salts on enzyme denaturation and the efficiency of thermal microwave processing will then be determined.

Figure 5 presents temperature-time profiles for a programmed temperature of 60 ºC and 5 min for POD simulated solutions.

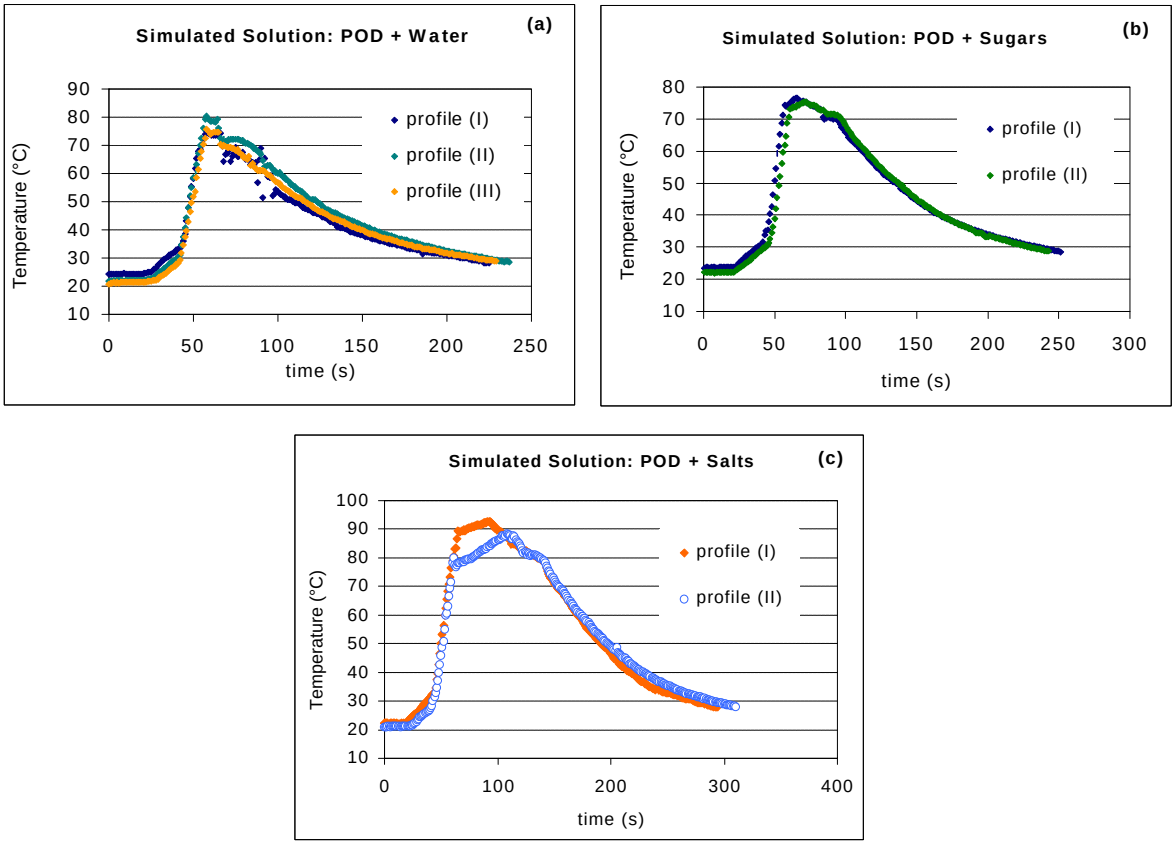



**Fig. 5.** Temperature - time profiles for: (a) POD/distilled water, (b) POD/Sugars, (c) POD/Salts, in programmed conditions at 60 °C for 5 min.

The same behavior of the PPO solutions was observed for POD solutions. Temperature-time profiles obtained for POD/Salts presented the highest temperature, 90 ºC.

Table 4 presents mean values of POD residual activity, maximum temperature for each condition and equivalent heating time. The z value of 15°C was considered for the calculations.

**Table 4**. Mean values of POD residual activity, maximum temperature of each condition and equivalent heating time for POD simulated solutions submitted to microwaves.

| **Programmed Temperature: 60 °C** | | | |
|---|---|---|---|
| **Simulated Solution of POD + Water:** pH = 6.3 ± 0.01 | | | |
| Samples | Residual activity (%) | Maximum temperature (°C) | Equivalent time (s) at 80.24 °C |
| Profile (I) | 14.52 | 75.06 | 8.35 |
| Profile (II) | 10.19 | 80.24 | 15.87 |
| Profile (III) | 14.77 | 75.70 | 8.56 |
| **Simulated Solution: POD + Sugars:** pH = 6.0 ± 0.08 and 4.9 °Brix | | | |
| Samples | Residual activity (%) | Maximum temperature (°C) | Equivalent time (s) at 76.60 °C |
| Profile (I) | 15.21 ± 1.61 | 76.60 | 29.84 |
| Profile (II) | 6.60 ± 0.97 | 75.30 | 27.35 |

Chang et al.(1988) apud Robinson (1991) showed by differential scanning calorimetry that in the presence of 10 % sucrose POD thermal stability was reduced. For a range of sugars tested, fructose was the most effective in reducing the enzyme thermostability and it was suggested that this was due to the interaction of fructose with the protein amino acids.

Mean initial POD/Salts activity value was 1.00 x $10^{-5}$ U/s.mL and the residual POD activity in POD/Salts solution was not detected, probably because of the combined effect produced by salts and microwaves. Assays with guayacol were undertaken but no change in absorbance was detected.

## 4. Conclusions

The fiber optic probe measured reliable temperature-time profiles and it was very important for this work.

POD and PPO solutions containing sugars/water or only water presented similar behavior and suffered a reduction of one logarithmic decade when submitted to microwaves.

A combined effect was observed when POD/Salts solution was submitted to microwave energy, reducing enzymatic activity to very low values, close to complete inactivation.

## Acknowledgments

The authors wish to thank FAPESP (The State of São Paulo Research Foundation) for the research grant.

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