Enzyme Inactivation Analysis for Industrial Blanching Applications: Comparison of Microwave, Conventional, and Combination Heat Treatments on Mushroom Polyphenoloxidase Activity

Carlos Devece,[‡] José Neptuno Rodríguez-López,[†] Lorena G. Fenoll,[†] José Tudela,[†] José Manuel Catalá,[‡] Elias de los Reyes,[‡] and Francisco García-Cánovas*[,†]

Grupo de Investigación de Enzimología (GENZ), Departamento de Bioquímica y Biología Molecular-A, Facultad de Biología, Universidad de Murcia, E-30100 Espinardo, Murcia, Spain, and Grupo de Calentamiento por Microondas, Departamento de Comunicaciones, Universidad Politécnica de Valencia, Camino de Vera s/n, Valencia, Spain

Browning reactions in fruits and vegetables are a serious problem for the food industry. In mushrooms, the principal enzyme responsible for the browning reaction is polyphenoloxidase (PPO). Microwaves have recently been introduced as an alternative for the industrial blanching of mushrooms. However, the direct application of microwave energy to entire mushrooms is limited by the important temperature gradients generated within the samples during heating, which can produce internal water vaporization and associated damage to the mushrooms texture. A microwave applicator has been developed, whereby irradiation conditions can be regulated and the heating process monitored. Whole edible mushrooms (*Agaricus bisporus*) were blanched by conventional, microwave, and combined heating methods to optimize the rate of PPO inactivation. A combined microwave and hot-water bath treatment has achieved complete PPO inactivation in a short time. Both the loss of antioxidant content and the increase of browning were minor in the samples treated with this combined method when compared to the control. This reduction in processing time also decreased mushroom weight loss and shrinkage.

Keywords: *Mushroom polyphenoloxidase; thermal inactivation; blanching; microwaves*

INTRODUCTION

The shelf life of minimally processed mushrooms, such as the commercial button mushroom *Agaricus bisporus*, is limited to a few days, because of enzymatic browning during storage. These browning reactions have been linked to mechanical damage during handling and processing, abrasions, washing, senescence, and bacterial infestations. In mushrooms, polyphenoloxidase (PPO, monophenol, dihydroxy-L-phenylalanine: oxygen oxidoreductase, EC 1.14.18.1) is considered the primary enzyme responsible for browning (Mayer and Harel, 1979). PPO is a widely distributed copper-containing protein that catalyzes two different reactions, both of which use molecular oxygen: the hydroxylation of monophenols to *o*-diphenols (monophenolase activity) and the oxidation of *o*-diphenols to *o*-quinones (diphenolase activity) (Mason, 1955; Makino and Mason, 1973). The quinones thus formed lead by polymerization to the formation of brown pigments, which generally decrease the quality of processed food (Prota, 1988).

Inactivation of PPO by heat or the application of antioxidants or enzyme inhibitors is essential to prevent enzymatic browning. Current conventional techniques to avoid browning include autoclave and blanching methods, whereby the mushrooms are immersed in a liquid at 80−90 °C for 8−9 min or passed through a forced steam flow. These conventional processes are inherently linked to important weight and nutritional quality losses in the product (Konanayakam and Sastry, 1988), pointing to the need for alternative industrial blanching techniques. One of the alternatives which has been proposed is microwave energy. Few works have been published regarding microwave blanching since the first promising study on this subject appeared (Proctor and Goldblith, 1948). In most of these works it was concluded that, although the combination of microwave heating techniques with a conventional process enhances the final quality of the product, neither weight loss nor texture degradation is considerably reduced (Ponne *et al.*, 1994). This undoubtedly is the main reason microwave blanching has not enjoyed great success in industrial blanching except for some isolated applications (Decareau, 1985).

In a previous paper we studied the thermal inactivation of mushroom PPO using 2450 MHz microwave radiation (Rodriguez-Lopez *et al.*, 1999). Nevertheless, direct application of microwave energy to entire mushrooms is limited by the important temperature gradients generated within the samples during heating, which can produce internal water vaporization and associated damage to the mushrooms texture. Therefore, in this study we analyze three different thermal methods for blanching whole mushrooms. A microwave applicator for treating mushrooms has been developed

* Author to whom correspondence should be addressed (fax 00-34-968-363963 or 00-34-968-364147; e-mail canovasf@fcu.um.es).
[†] Universidad de Murcia.
[‡] Universidad Politécnica de Valencia.

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whereby radiation conditions can be regulated and the heating process monitored. Promising results are obtained in terms of weight loss, shrinkage, browning, and antioxidant activity of treated mushrooms.

MATERIALS AND METHODS

Reagents. Edible mushrooms (*Agaricus bisporus*) were kindly supplied by COVEMUR, S.A. (Archena, Murcia, Spain). 3,4-Dihydroxyphenylporpionic acid (DHPPA), 2,2′-azinobis(3-ethyilbenzothiazolinesulfonic acid) (ABTS), and 3-methyl-2-benzothiazoline hydrazone (MBTH) were purchased from Sigma Chemical Co. (Madrid, Spain). Reagent grade H_2O_2 (30% v/v) was obtained from BDH/Merck (Poole, U.K.), and its concentration was determined by iodide titration with HRPC (Cotton and Dunford, 1973). Reducing substrates were prepared in 0.15 mM phosphoric acid to prevent autoxidation. All other chemicals were of analytical grade and supplied by Merck (Darmstadt, Germany).

Apparatus. To carry out microwave heating treatment a laboratory microwave oven was implemented. This applicator is a multimode cavity with an additional mode stirrer to achieve better electric field uniformity across the sample. Microwave power can be regulated electronically between 0 and 700 W, measuring simultaneously both incident and reflected power at the input port besides. The internal temperature profiles have been recorded during the course of the experiments using a Luxtron 790 fluoroptic thermometer. This optical thermometer can measure a temperature range between −195 and 200 °C with an accuracy of ±0.5 °C. To prevent the core mushroom temperature from surpassing a prefixed value, a proportional plus integral plus derivative control was developed to regulate the microwave power supplied by the generator (de Swardt and Siebrits, 1997).

Thermographic images using the Agema 400 thermovision camera (Agema Infrared Systems, Danderyd, Sweden) were also taken during the process to obtain information of gradient temperature distributions within the mushroom core by means of longitudinal slices cut after treatment.

Heat Treatments. Microwave exposure was carried out in the multimode oven described above, using 200 g of mushrooms for each test. The mushrooms were placed on a nonstick sieve and exposed to 2.45 GHz microwave radiation at different times and power regimes. Conventional thermal treatment was carried out using a Tectron 3473100 circulating bath, with 200 g of whole mushrooms introduced in a bath at 92 °C for different times. Mixed mode combined microwave/conventional heating was carried out by exposing the mushroom to 2.45 GHz microwave radiation for different times and final temperatures and then introducing the samples in a water bath at 92 °C for 20 s.

Extraction of PPO. Once treated, the outermost layers (∼1−2 mm) of the cap covering (cap skin) were removed. Whole and partial stem tissue was also removed from the cap. Cap, cap skin, and stem were frozen in liquid nitrogen and stored at −80 °C. Frozen tissues (20 g) were blended in 50 mL of 30 mM sodium phosphate buffer (pH 7.0) for 1 min and then centrifuged at 100000*g* for 15 min. The supernatant was filtered, collected, and used as a source of PPO. The purification steps were kept to a minimum so that PPO maintained its natural substrates and to facilitate the study of browning after different thermal treatments.

Enzymatic Assays. PPO activity was assayed spectrophotometrically using the MBTH method (Rodríguez-López *et al.*, 1994) and DHPPA as a diphenolic substrate (Espín *et al.*, 1997). Product formation was measured at 466 nm [$\epsilon_{466\ nm} = 20\,000\ M^{-1}\ cm^{-1}$ (Espín *et al.*, 1997)]. The standard reaction mixture included 2 mM DHPPA, 4 mM MBTH, and 2% *N*,*N*′-dimethylformamide (DMF) in 0.1 M sodium phosphate buffer (pH 6.8). Kinetic assays were carried out with a Perkin-Elmer Lambda-2 UV-vis spectrophotometer interfaced online with a compatible PC for further data analysis. Activity was determined at 25 °C, and the temperature was controlled with a Haake D1G circulating water bath equipped with a heater/cooler. Apparent rate constants for the inactivation of PPO after different heating treatments were determined by fitting the relative activities (A_R) versus treatment times curves to exponential function by nonlinear regression. Initial estimations were obtained by linear regression of the plot of $\ln(A_R/100)$ versus time. A_R was calculated as a percentage of a control not subject to heating.

Determination of Mushroom Shrinkage. Each mushroom was weighed and labeled, and its diameter cap was measured individually before treatment. The samples were immersed into a 20 °C water bath immediately following the heat process for 2 min to cool them, then weighed, and measured again. The mushroom diameters ranged from 15 to 40 mm.

Total Antioxidant Activity Determinations. TAA was spectrophotometrically determined using the method proposed by Cano et al. (1998). The reaction mixture contained 0.1 mM ABTS, 0.01 mM H_2O_2, and 0.25 μM horseradish peroxidase isoenzyme C (HRPC) in 50 mM glycine-HCl buffer (pH 4.5) in a total volume of 1 mL. The reaction was monitored at 414 nm (corresponding to the ABTS radical) until absorbance was stable. Different amounts of mushroom extracts were then added, and the decrease in absorbance was determined. The relative TAA remaining in the mushroom extracts after use of the different blanching methods was determined as a percentage of the TAA calculated in an untreated sample.

Measurement of Browning. Whole mushrooms were treated by different thermal methods at different treatment times. Once treated, the extracts were obtained as described in the extraction of PPO section. One milliliter of extract was placed in a 1 mL cuvette, and soluble pigments were evaluated by the measurement of absorbance at 400 nm (Weurman and Swain, 1955).

RESULTS AND DISCUSSION

Temperature Distribution in Mushrooms. Figure 1 is a thermographic image representing temperature distribution in mushroom slices. Figure 1A shows the temperature distribution after a conventional 1 min 92 °C hot-water treatment. The center of the mushroom core remains colder than the surface in this conventional treatment. The main effect of this temperature gradient is the overtreatment of the hotter areas, while in the colder areas the enzymes may not be completely inactivated. Conventional and microwave blanching treatments generate opposite heating profiles. This can be confirmed by observing Figure 1B, where the temperature distribution in mushroom slices is depicted for a microwave heating treatment of 1 min at 85 °C. In this case the surface of the mushroom was cooler than the center. A more uniform temperature distribution can

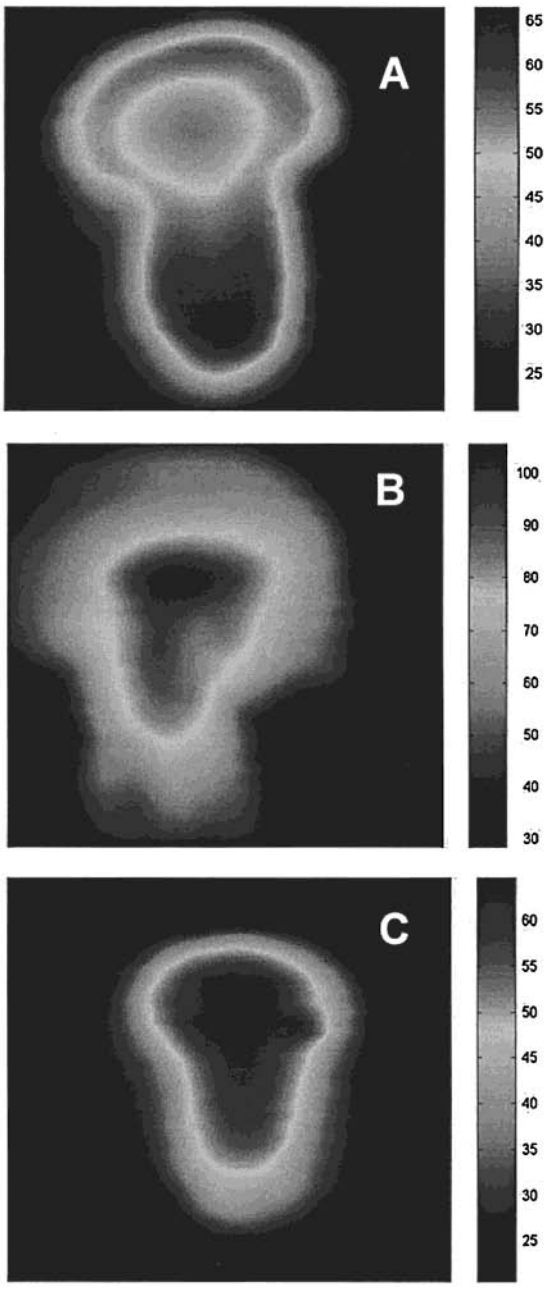

Figure 1. Temperature distribution in mushroom slices after (A) conventional 1 min 92 °C hot-water treatment, (B) 1 min microwave treatment at 85 °C, and (C) combined 1 min microwave at 85 °C plus 20 s 92 °C hot-water treatment. Temperature scale is indicated in Celsius.

be viewed in Figure 1C, where a combined 1 min microwave at 85 °C plus 20 s 92 °C hot-water treatment has been performed. Heat diffusion from the hot-water through the mushroom is the main factor in a conventional heating process. However, the temperature distribution in microwave heating depends on the electric field distribution within the sample. For cylinders and spheres with a relative dielectric constant 52 and loss factor 14, and diameters between 15 and 40 mm, the power density at 2.45 GHz concentrates in the center (Ohlsson and Risman, 1978). This effect can be viewed in Figure 1B. Another effect that cannot be neglected during microwave heating is the refrigeration, which occurs at the mushroom surface, mainly due to water vaporization (Devece *et al.*, 1997). This refrigeration prevents total enzyme inactivation in the skin when microwave treatment alone is applied.

Thermal Inactivation of Mushroom PPO. From the results obtained in the previous section, it seems reasonable to expect that enzymes might be completely inactivated with a comparatively rapid combined hot-water (surface heating) and microwave (internal heating) treatment. To verify such predictions mushrooms were separated into cap, cap skin, and stem after treatment, and a crude extract was obtained as indicated in the Material and Methods section. The distribution of PPO activity in mushroom tissues was determined in a collection of 50 individual mushrooms. In agreement with previous results (Boiret *et al.*, 1985) the relative PPO activity (determined as $\Delta A_{466\ nm}\ s^{-1}\ g^{-1}$) was higher in the stem (ca. 45%) than in the cap skin (40%), where it was higher than in the mushroom cap itself (15%). However, the total amount of PPO activity in the various mushroom tissues (43% in cap, 41% in stem, and 16% in cap skin) reflected the weight of the respective parts (69, 21, and 10%, respectively).

Figure 2 shows the time required for total PPO inactivation in the different mushroom tissues using microwave, conventional, and combined heat treatments. Conventional treatment was represented by a 92 °C water bath, while in the microwave treatment the power generator was controlled so that the mushroom core temperature did not exceed 85 °C, thus avoiding damage to the mushroom tissue. In the combined treatment the samples were heated by microwaves at 85 °C for different times and then immediately immersed in a 92 °C water bath for 20 s. Residual activity was determined by measuring the oxidation of DHPPA in the presence of MBTH (Espin *et al.*, 1997) and then calculating this as a percentage of a control not subjected to heating. In mushroom cap PPO inactivation was complete after 2 min of microwave irradiation, whereas conventional methods needed more than 6 min for thermal inactivation to be complete (Figure 2A). The fastest PPO inactivation (in less than 20 s) was obtained by employing a combination of microwave and conventional heating (Figure 2A). These results are confirmed in Table 1, which shows the apparent first-order constants for PPO inactivation after different thermal treatments.

A different PPO inactivation profile was obtained for the cap skin (Figure 2B). In this case, inactivation was slower with microwave irradiation, and the PPO inactivation constant was two times lower than for the mushroom cap (Table 1). Cap skin PPO was inactivated faster than cap PPO when a conventional heating method was used. However, as in the case of the cap, the mixed heating method was the most efficient for PPO inactivation with an inactivation constant close to 100 min^{-1} (Table 1). These results show a direct relationship between the temperature gradient observed by using thermographic images and PPO inactivation.

The inactivation of PPO in the mushroom stem was similar to that obtained in the cap (Figure 2C). The microwave treatment was faster than conventional heating, and the mixed mode the fastest, only the combination of both treatments leading to the total inactivation of PPO. A final residual activity (ca. 6% for conventional and 3% for microwave treatments) was achieved. The lower sensitivity of mushroom PPO to inactivation in the stem could be related with the

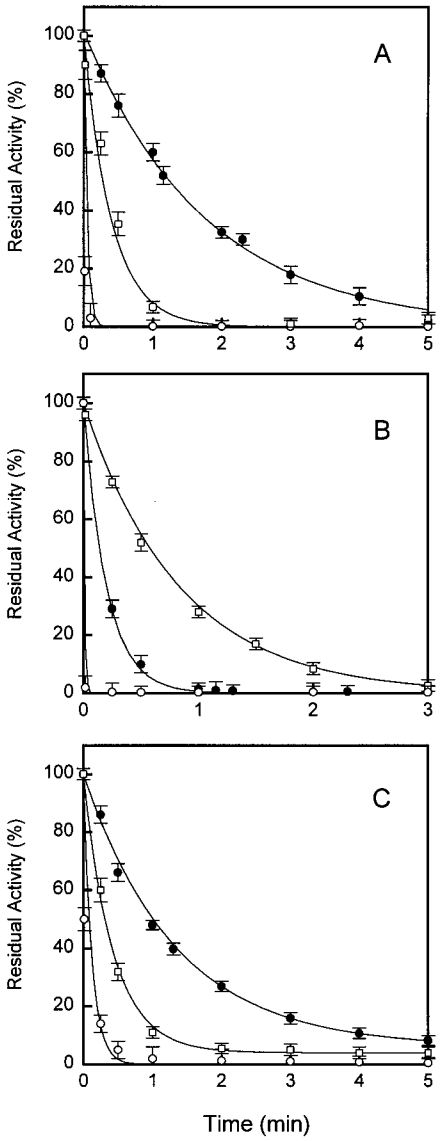

Figure 2. Time-course of PPO inactivation in (A) cap, (B) cap skin, and (C) stem using different thermal treatments: (●) conventional hot-water bath, (□) microwave, and (○) mixed mode. Each point is the average of three different inactivation experiments.

Table 1. Apparent Rate Constants (*k*) for the Inactivation of PPO in Mushroom Tissues after Different Thermal Treatments

	k^a (min^{-1})		
heating method	cap	cap skin	stem
conventional water bath	0.57	5.03	0.75
microwave	2.50	1.20	2.35
mixed method	20.5	93.1	8.10

[a] Rate constants were obtained by fitting the data depicted in Figure 2.

presence of different isoenzymes (Ingerbrigtsen *et al.*, 1989) with different thermal stability.

The basic principle of microwave heating is the interaction of polar molecules with the electric component of the electromagnetic field, which generates heat due to the friction produced as the molecules attempt to orient themselves within the oscillating field. Water and salts are the major determinants of microwave absorptivity in most food systems (Kermasha *et al.*, 1993). The effect of microwave energy on enzyme inactivation has generally put down to thermal effects, although there is some evidence for nonthermal effects (Porcelli *et al.*, 1997). The latter effects are regarded as controversial since the energy associated with microwaves is many orders of magnitude below that required to break covalent bonds. However, microwave energy could break down lower energetic interactions such as the hydrogen bonding between the protein and water molecules associated with protein structure. This would explain the different inactivation profiles observed in mushroom PPO when conventional or microwave treatments were used (Rodriguez-Lopez *et al.*, 1999). However, more studies are needed to determine whether the faster inactivation rates using microwave energy is due to thermal or nonthermal effects.

Table 2. Dependence of the Weight Loss and Shrinkage on Blanching Treatment

heating method	weight loss (%)	shrinkage (%)
conventional water bath[a]	(18.0 ± 2.0)	(24.5 ± 2.4)
microwave[b]	(13.6 ± 2.0)	(8.3 ± 0.9)
mixed method[c]	(0.9 ± 0.1)	(13.9 ± 2.6)

[a] Conventional water bath treatment was 6 min at 92 °C. [b] Microwave treatment was 3 min at 85 °C. [c] Mixed mode treatment was 1 min microwave at 85 °C plus 20 s 92 °C water bath.

Weight Loss and Shrinkage in Treated Mushrooms. To show the advantages of combined techniques versus individual conventional hot-water and microwave treatments for industrial blanching applications we measured different parameters in mushrooms after the various treatments. These included two physical-related parameters, weight loss and shrinkage, and two biochemical parameters, TAA and browning. Weight loss or shrinkage due to water evaporation is a major problem in commercial mushroom canning operations. The loss of canned product weight which occurs during blanching and thermal processing usually ranges between 30 and 40%, and most of this shrinkage (average 25%) occurs during the blanching process (McArdle and Curwen, 1962). It is therefore important to study these parameters under different heating conditions. Weight loss and subsequent shrinkage in mushroom blanching depends mainly on the sample size, heating temperature, and heating time (Biekman *et al.*, 1996). Therefore, a reduced treatment time ought to lead to an improvement in mushroom shrinkage. Table 2 shows the percentages of weight loss and shrinkage (determined by the diameter of the mushroom cap) in whole mushrooms after the time needed to inactivate 99% of PPO by the different thermal treatments. Similar weight loss and shrinkage of previously published data (McArdle and Curwen, 1962) were obtained after treatment in a hot-water bath at 92 °C for 6 min, with a higher percentage of shrinkage than weight loss. In the microwave treatment, both shrinkage and weight loss were considerably improved due to the reduction in processing time (3 min of treatment) and the lower maximum temperature reached in the samples (85 °C). The lower weight loss with respect to shrinkage can be explained by the microwave water pumping effect. With a combined method (1 min microwave heating at 85 °C plus 20 s in a 92 °C water bath), the weight loss is optimized, and any shrinkage is due to the conventional treatment.

Effects of Thermal Treatments on the TAA. Processing and storage can profoundly alter the antioxidant composition of fruits and vegetables (Halliwell, 1995). Figure 3 represents the loss of TAA in mushroom

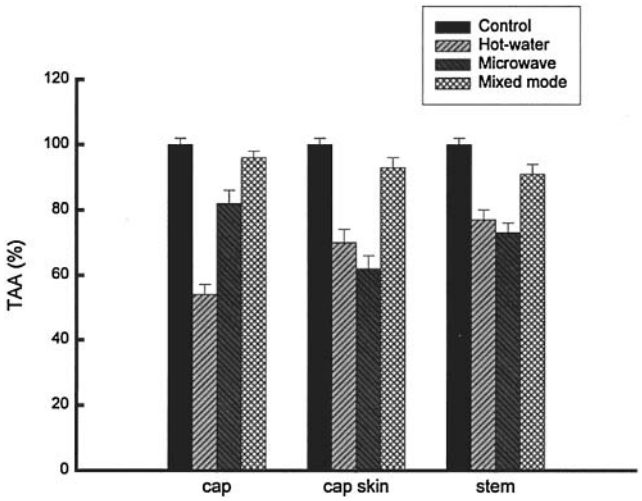

Figure 3. Relative total antioxidant activity (TAA) in mushroom tissues after using different blanching methods. The control is the TAA calculated in an untreated sample. Description of the methods may be found in the text.

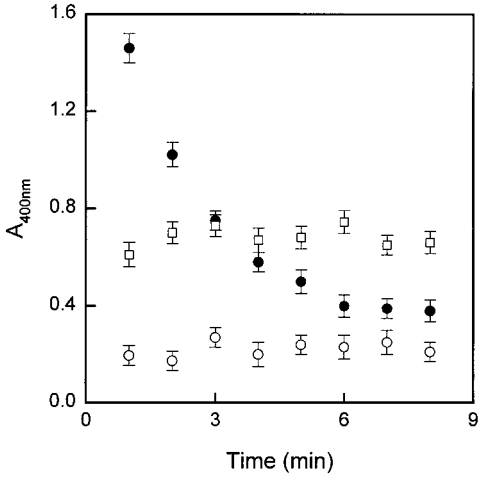

Figure 4. Browning of mushroom tissues as a function of time and heating treatment: (●) conventional hot-water bath, (□) microwave, and (○) mixed mode treatment.

tissues after the application of different blanching treatments. TAA loss was calculated as a percentage of the TAA in nontreated mushrooms. Conventional treatment in a hot-water bath at 92 °C for 6 min produced an important loss in TAA, which was greater in the cap (ca. 46% of the total) than in the other mushroom tissues. These data agree with the lower rate of PPO inactivation in the mushroom core using a hot-water bath. Using microwave blanching for 3 min at 85 °C the greatest loss of TAA was observed in the cap skin, due to the effect described in the thermal distribution section of this paper. The residual TAA was very high in all the tissues (ranging from 91 to 96%) of the mushrooms treated with a mixed method (1 min of microwave heating plus 20 s of immersion in a water bath at 92 °C). Oxidase enzymes, including PPO, are believed to be responsible for the decrease in antioxidant capacity, either directly through the oxidation of reducing substrates or indirectly though the oxidation of ascorbic acid by the quinones and oxidized products generated in the enzymatic oxidation of their substrates (Ros *et al.*, 1993). The results obtained in this section confirm the direct relationship between PPO activity and loss of TAA and point to the importance of rapid PPO inactivation for maintaining the nutritional value of mushrooms.

Effects of Thermal Treatments on the Browning of Mushroom Extracts. The PPO-catalyzed enzymatic browning of phenolic compounds is of vital importance in mushroom processing due to the formation of undesirable colors and flavors and the loss of nutrients. The dependence of the browning of mushroom extracts on the treatment time can be observed in Figure 4. In mushrooms treated by a conventional heating method the degree of browning decreased with the treatment time, reaching a minimum at 6 min and being proportional to PPO inactivation. Using a mixed method (microwaves plus hot-water bath), browning was very low and constant with the time of treatment. Rapid inactivation of PPO using this method prevents the oxidation of phenolic compounds. Although the browning obtained by mixed or longer conventional methods was similar, the evolution of this parameter with time was different. Thus, the mixed mode treated mushroom extracts showed the same level of browning after 24 h at room temperature (data not shown). However, the extracts treated during 7 min in a hot-water bath evolved to brown pigments in the next 24 h (data not shown). In the extracts treated by conventional thermal methods, PPO remained active for more than 6 min before being completely inactivated. This time saw the generation of numerous oxidation products, such as *o*-quinones, which may react with phenolic compounds. After the inactivation of PPO, the oxidation products would remain in the sample, generating melanin products. In contrast, the extracts treated with mixed techniques quickly inactivated PPO, and the enzyme could not therefore generate oxidation products.

An intermediate degree of browning was observed using microwave blanching alone. Browning remained constant with irradiation time, but it was not possible to obtain such a low level of browning as that obtained using mixed or longer conventional methods. The higher level of browning observed using microwave treatment could be explained in two different ways. On one hand, in a short time the treatment did not inactivate PPO completely so that brown pigments are produced (Figure 2). On the other hand, at longer treatment times PPO is inactivated, but microwave heating may increase internal cell pressure, leading to rupture and a loss of cell contents, oxidation, and related degradative reactions (Cano *et al.*, 1990). The results described here indicate the importance of the rapid inactivation of PPO, using a combined heating method to avoid browning reactions in mushrooms.

CONCLUSIONS

Microwave heating techniques for the industrial blanching of mushrooms have shown promising results for improving product quality and, particularly, for shortening the processing times currently used. The better results in terms of temperature distribution, PPO inactivation, weigh loss, shrinkage, TAA content, and browning of the samples were obtained by using a combination of heating treatments involving microwaves plus conventional heating. Temperature distribution in whole mushrooms using only microwaves or hot-water blanching produces an overtreatment of the hotter areas, while in the colder areas the PPO is not completely inactivated. This effect leads to longer time treatments with a consequent loss of weight and nutritional values. The objectives of our study were to

determine the optimal process conditions for pilot-scale microwave inactivation of PPO in mushrooms. The results of this work indicate the importance of the use of special microwave cavities for mushrooms treatments, where the irradiation conditions can be regulated. Further research involving microwave heating aided by pressure and/or water vapor is envisaged.

ABBREVIATIONS USED

PPO, tyrosinase or polyphenoloxidase (EC 1.14.18.1); TAA, total antioxidant activity; DMF, *N*,*N*′-dimethylformamide; ABTS, 2,2′-azinobis(3-ethyilbenzothiazoline-sulfonic acid); MBTH, 3-methyl-2-benzothiazoline hydrazone; DHPPA, 3,4-dihydroxyphenylpropionic acid.

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