

# Changes in Capsaicinoids during Development, Maturation, and Senescence of Chile Peppers and Relation with Peroxidase Activity

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The components responsible for chile hot flavor, capsaicinoids, are synthesized through the cinnamic acid pathway, and their degradation is thought to be aided by the action of peroxidases. This work describes the evolution of capsaicinoids during the development, maturation, and senescence of the fruit in three varieties of hot chile peppers widely used in Mexico [Habañero (*Capsicum chinense* Jacq.), De árbol (*C. annum* var. *Annuum*), and Piquin (*C. annum* var. *Aviculare*)] and its relation with the activity of peroxidases in these fruits. Capsaicinoids were more abundant in the fruit of Habañero, followed by De árbol and then by Piquin. Capsaicin was higher than dihydrocapsaicin in the three varieties. Capsaicinoids, capsaicin, and dihydrocapsaicin increased continuously and reached a peak after 45–50 days from fruit set (DFFS) in Habañero and De árbol and after 40 DFFS in Piquin and then declined. Peroxidase activity increased at the time when the concentration of capsaicinoids started to decrease. There was an inverse relationship between the evolution of capsaicinoids and peroxidase activity that might indicate that this enzyme is involved in capsaicinoid degradation.

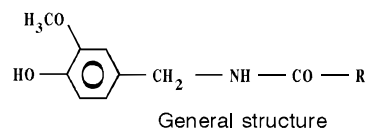
**Keywords:** *Capsicum annum*; *C. chinense*; capsaicin; dihydrocapsaicin; postharvest; Habañero; De árbol; Piquin

## INTRODUCTION

Chile peppers are popular food additives in many parts of the world, valued for their sensory attributes of color, pungency, and aroma. In Mexico, hot chile peppers represent a tradition and cultural identity. Hot chile peppers are the main elements that have characterized Mexican cuisine and culture for at least the past eight centuries. The fruit is very important economically due to the vast quantity and the diverse varieties used. The consumption of hot chile peppers is due, mainly, to their pungent flavor. The hot flavor is caused by seven closely related alkaloids or capsaicinoids (Figure 1), but capsaicin (8-methyl-*N*-vanillyl-6-nonenamide) and dihydrocapsaicin are responsible for ~90% of the pungency (Kosuge and Furata, 1970; Iwai et al., 1979b; Kawada et al., 1985; Govindarajan, 1986). Capsaicinoids are unique to the genus *Capsicum* (Govindarajan et al., 1987; Gopveindarajan and Sathyanarayana, 1991). Chile hot flavor is generally made up of at least two and perhaps all of the capsaicinoids (Collins et al., 1995).

The genus *Capsicum* (family Solanaceae) comprises >200 varieties and is the most cultivated pepper in the world. Hot pepper is widely used for its pungent flavor and also as a drug. Habañero, De árbol, and Piquin are among the most pungent varieties with heat values (Scoville units) of 200 000, 150 000, and 60 000, respectively (Bosland, 1992). The Scoville organic test, invented by W. L. Scoville in 1912, is a subjective measure of chile pungency.

De árbol fruit is 5–8 cm long, ~0.5 cm wide, and translucent when dried, and the calyx of the fruit is narrow and tapered. Piquin (or pequin), sometimes



Capsaicinoids and analogs	R
Capsaicin	
Dihydrocapsaicin	
Nordihydrocapsaicin	
Homocapsaicin I	
Homocapsaicin II	
Homodihydrocapsaicin I	
Homodihydrocapsaicin II	
N-Vanillyl octanamide	
N-Vanillyl nonanamide	
N-Vanillyl decanamide	

**Figure 1.** Chemical structures of different capsaicinoids and their analogues.

called bird pepper because birds are attracted to it, is a small, red chile of ~1 cm wide and ~2.5 cm long. Fruit of Habanero can vary from long and slender to short and obtuse and is extremely pungent and aromatic.

Fujikawi et al. (1982) determined that the biosynthetic site of capsaicinoids is in the placenta of the fruit and produced by the cinnamic acid pathway. Iwai et al. (1979a) suggested that capsaicinoid production increases with maturity until a maximum and then decreases by rapid turnover and degradation of up to

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60%. Bernal et al. (1993a,b), through in vitro studies, suggested that peroxidases are involved in the degradation of capsaicinoids. Pepper peroxidase was found to oxidize capsaicin (Bernal et al., 1993a) and dihydrocapsaicin (Bernal et al., 1993b). Oxidation of dihydrocapsaicin by *Capsicum* peroxidase was strictly dependent on the presence of H<sub>2</sub>O<sub>2</sub> (Bernal et al., 1993b). In addition, like capsaicin, peroxidase is mainly located in the placenta and the outermost epidermal cell layers (Bernal et al., 1993c, 1994b). These results were used by the authors as support for the role of peroxidase in capsaicin turnover and degradation. Several methods are available for the identification and quantification of capsaicinoids, but HPLC is considered the most reliable and rapid method (Yao et al., 1994). The objective of this work was to determine the evolution of capsaicin, dihydrocapsaicin, and total capsaicinoids during the development, maturation, and senescence of three cultivars of hot chile peppers widely used in Mexico and to relate that with changes in peroxidase activity.

## MATERIALS AND METHODS

**Plant Material.** Chile peppers var. Piquin (*C. annuum* var. Aviculare), De árbol (*C. annuum* var. Annuum), and Habañero (*C. chinense* Jacq.) were cultivated in a greenhouse in Querétaro, and field-grown Habañero fruit were obtained from Mérida, Yucatán, Mexico. Plants received standard cultural practices. Flowers were tagged at anthesis and fruit set, and fruit were harvested at different stages from immature green to senescent red. Fruit grown in the greenhouse were harvested every 10 days from fruit set (DFFS) until senescence (70 DFFS for Piquin and De árbol and 80 DFFS for Habañero). Field-grown Habañero fruit were harvested at intervals of 10–20, 20–30, 30–40, 40–50, and 50–60 DFFS.

To determine the suitable sample preparation method, and due to the unavailability of fruits from the three varieties used, capsaicinoids were determined in Jalapeño (*C. annuum*) either fresh, frozen with liquid nitrogen, or dried at 60 °C.

**Determination of Capsaicinoid Content.** At harvest, fruit were divided longitudinally; half was immediately frozen in liquid N<sub>2</sub> for peroxidase analysis, and the other half was dried in a single-wall transite oven at 60 °C until constant weight (~2 days), for capsaicinoids analysis. Capsaicinoids were quantified with HPLC according to the method of Collins et al. (1995) with some modifications as indicated below.

Dried tissue was ground, and samples of 3 g each were mixed with 30 mL of acetonitrile and kept for 4 h at 80 °C with constant shaking and without reflux, before being cooled and then filtered. A Waters HPLC equipped with a Novapak C<sub>18</sub> reversed phase column of 3.9 × 150 mm was used. The mobile phase was methanol/water at a ratio of 73:27, and the flow rate was 1 mL/min. The detector was a photodiode array, and the run was 7 min long. Capsaicin and dihydrocapsaicin were identified and quantified using standards of both compounds (Sigma, purity was 98% for capsaicin and 90% for dihydrocapsaicin). Standard curves were prepared using serial dilutions of 100, 200, 400, 600, 800, and 1000 ppm.

**Determination of Peroxidase Activity.** Samples of 5 g of lyophilized tissue were homogenized with 50 mL of acetone at –20 °C, filtered through Whatman No. 40 paper using vacuum, and then washed with acetone at –20 °C to remove pigments. The precipitate was dissolved in 10 mL of Tris-HCl (50 mM) at pH 6.0. Samples were incubated at 4 °C for 1 h and centrifuged at 3000g for 30 min at 4 °C, and the supernatant was used to determine enzyme activity. Enzyme activity was determined in a DU-65 Beckman spectrophotometer at 262 nm (Bernal et al., 1993a). The reaction mixture contained 0.1 mM H<sub>2</sub>O<sub>2</sub>, 1.0 mM capsaicin, and 0.1 M Tris-HCl at pH 6.0. The peroxidase activity was reported as millimoles of product per minute per milligram of protein.

**Table 1. Capsaicin, Dihydrocapsaicin, and Capsaicinoid Contents in Dried, Frozen, and Fresh Jalapeño (*C. annuum*) Fruit**

	capsaicin (mg/g of dry fruit)	dihydrocapsaicin (mg/g of dry fruit)	capsaicinoids (mg/g of dry fruit)
dried	17.093 (±1.032) <sup>a</sup>	2.180 (±0.124)	26.340 (±1.163)
frozen	8.790 (±0.095)	5.563 (±0.143)	13.343 (±0.807)
fresh	1.2938 (±0.673)	6.260 (±0.196)	18.825 (±0.463)

<sup>a</sup> Values in parentheses indicate standard deviation of the mean.

Peroxidase activity was determined as a function of the pH, H<sub>2</sub>O<sub>2</sub> concentration, and capsaicin concentration. The range of pH tested was 5.0, 5.5, 6.0, 6.5, and 7.0. H<sub>2</sub>O<sub>2</sub> concentrations tested were 0.025, 0.075, 0.10, 0.15, and 0.20 mM. Capsaicin concentrations tested were 0.50, 0.75, 1.0, and 1.5 mM. Protein contents were determined according to the dye binding method of Bradford (1976) using bovine serum albumin for the standard curve.

All experiments were repeated at least five times. Statgraphic 5.0 was used for statistical analysis.

## RESULTS AND DISCUSSION

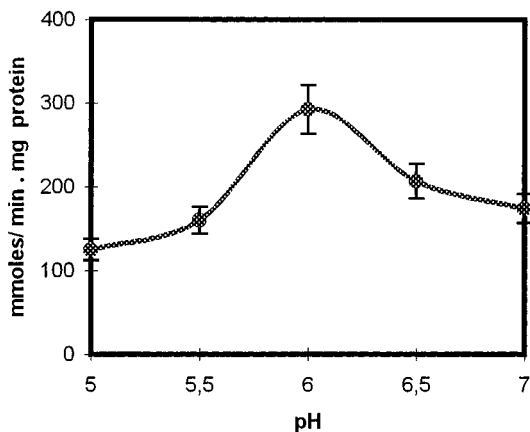
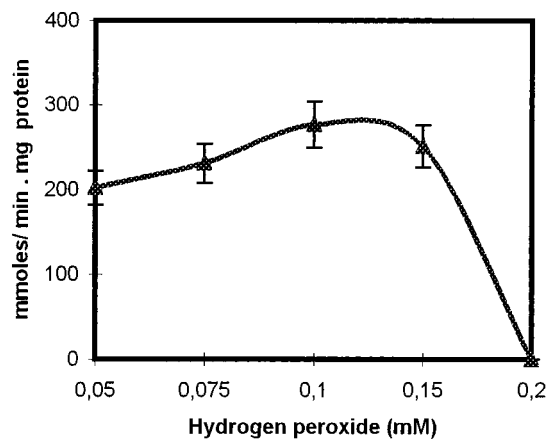
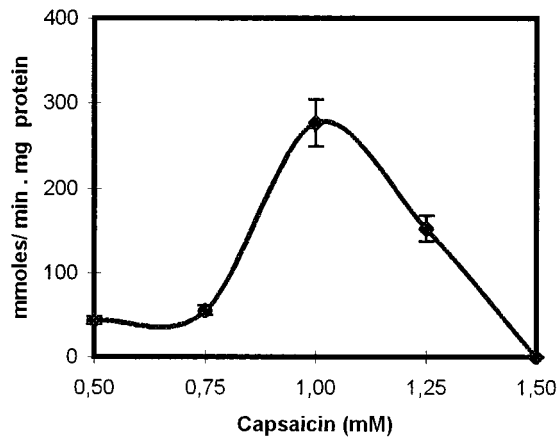
**Optimization of Capsaicinoid Extraction and Assay.** The best method for the extraction of capsaicinoids was obtained using fruit dried at 60 °C (Table 1). Dried fruits had the highest capsaicin and total capsaicinoids but the lowest dihydrocapsaicin concentration. Frozen fruit had the lowest capsaicin and capsaicinoid concentrations. Fresh fruit had the highest concentration of dihydrocapsaicin.

**Optimization of Peroxidase Assay.** Optimum pH for peroxidase activity was ~6.0, and optimum capsaicin and H<sub>2</sub>O<sub>2</sub> concentrations were 1.0 and 0.1–0.15 mM, respectively (Figure 2).

Figure 3 shows a typical HPLC chromatogram for capsaicin and dihydrocapsaicin.

**Changes in Capsaicinoid Content.** Changes in capsaicinoids and their relation with changes in peroxidase activity were similar (at least for Habañero) between greenhouse- and field-grown fruits (Figures 4 and 5). Capsaicinoids progressively accumulated during the development of the fruit, reached maxima of 200 µg/g in greenhouse fruit and 120 µg/g in field-grown fruit after 45–50 DFFS, and then started to decrease gradually. After 55 DFFS in field-grown and 80 DFFS in greenhouse-grown fruit, capsaicinoids reached minima of 60 and ~120 µg/g, respectively. Capsaicinoids also increased and reached a maximum of ~55 µg/g after 40 DFFS in Piquin and then declined and reached a minimum of ~12 µg/g after 70 DFFS (Figure 6). The maximum concentration of capsaicinoids in De árbol was ~85 µg/g and accumulated 50 DFFS, and the minimum concentration accumulated 70 DFFS was ~40 µg/g (Figure 7). Capsaicinoid concentration was significantly different in all stages of development. Accumulation of capsaicin was reported to occur over a relatively short period during fruit development (Iwai et al., 1979a; Salgado-Garciglia and Ochoa-Alejo, 1990).

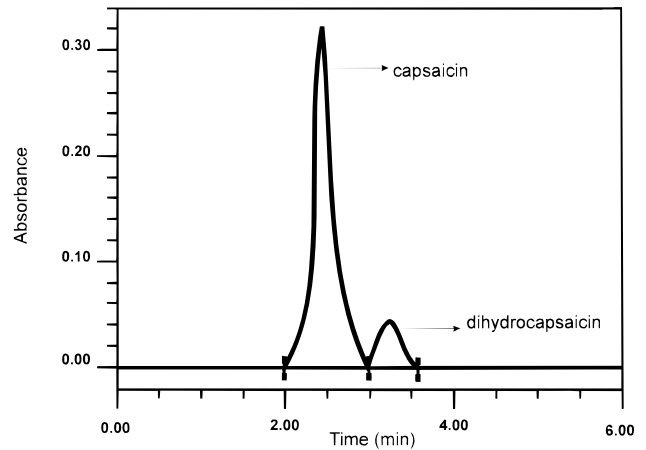
**Changes in Peroxidase Activity and Relation with Capsaicinoid Content.** Peroxidase activity in Habañero decreased in both greenhouse- and field-grown fruit until 45–50 DFFS and then gradually increased (Figures 4 and 5). This increase coincided with the initial decrease in capsaicinoids. Peroxidase activity in De árbol (Figure 7) did not change until 40 DFFS, then started to increase at the time when capsaicinoids started to decrease, reached a maximum



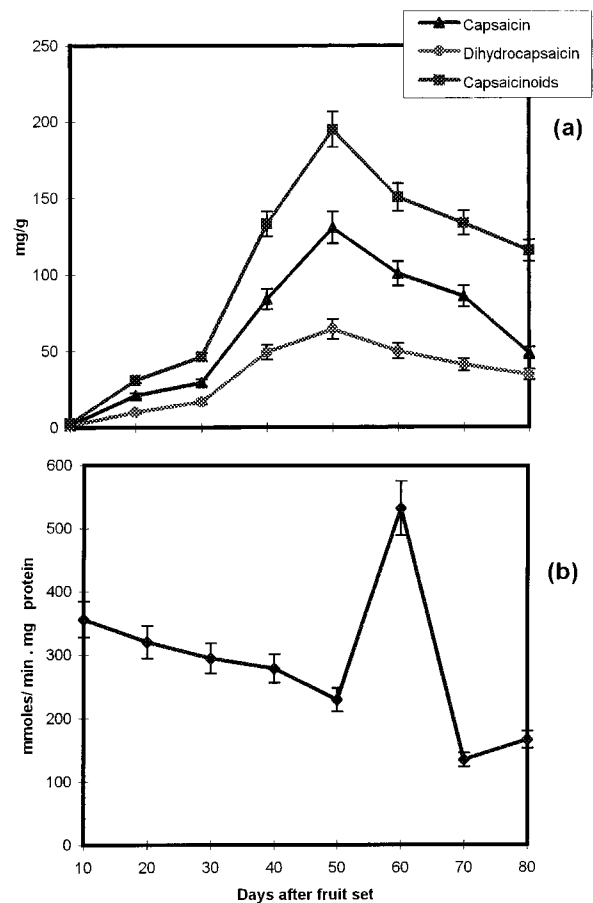
**Figure 2.** Peroxidase activity as a function of capsaicin concentration, hydrogen peroxide concentration, and pH. Vertical bars indicate standard deviation of the mean.

at 60 DFFS, and then started to decline. There were no differences in peroxidase activity in Piquin up to 30 DFFS (Figure 6), but peroxidase activity increased at 40 DFFS and reached a maximum at 60 DFFS. This increase in peroxidase activity started before the decrease in capsaicinoids.

Habañero had the highest capsaicinoids content (113.04  $\mu\text{g/g}$  in field-grown fruit and 195.38  $\mu\text{g/g}$  in greenhouse-grown fruit), followed by De árbol (80.52  $\mu\text{g/g}$ ) and then by Piquin (53.71  $\mu\text{g/g}$ ) (Figures 4–7). Capsaicin and dihydrocapsaicin followed the same trend as capsaicinoids, and capsaicin was higher than dihydrocapsaicin in the three varieties (Figures 4–7). On the other hand, De árbol had the highest peroxidase activity [2189  $\text{mmol min}^{-1} (\text{mg of protein})^{-1}$ ], followed by Piquin

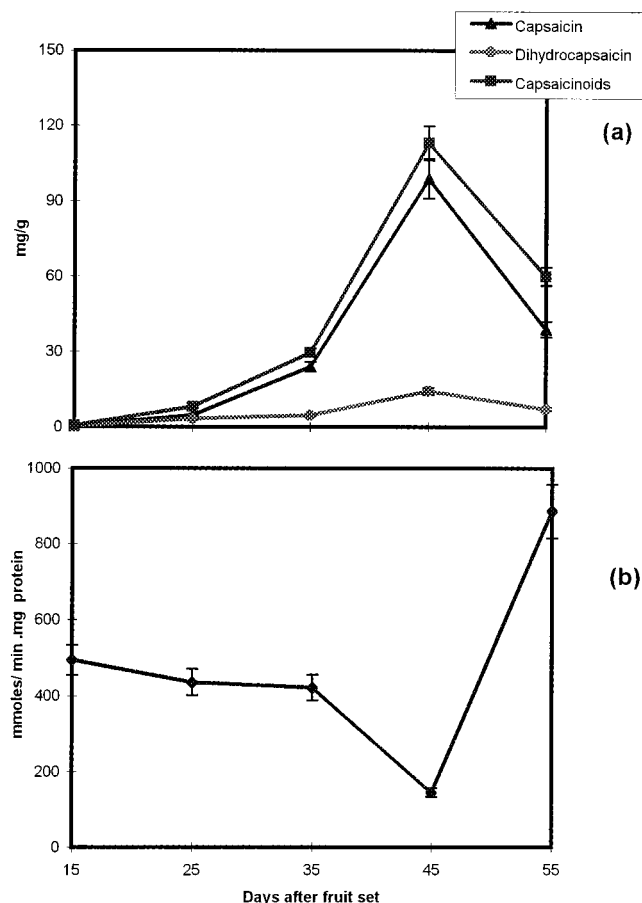


**Figure 3.** HPLC chromatogram for capsaicin and dihydrocapsaicin.



**Figure 4.** Capsaicinoid concentration (a) and peroxidase activity (b) during development, maturation, and senescence of Habañero chile peppers cultivated in the greenhouse. Vertical bars indicate standard deviation of the mean.

[1825  $\text{mmol min}^{-1} (\text{mg of protein})^{-1}$ ], and Habañero had the lowest activity [533–887  $\text{mmol min}^{-1} (\text{mg of protein})^{-1}$ ]. The maximum concentration of capsaicinoids is  $\sim 3$  times higher and the maximum peroxidase activity is  $\sim 4$  times lower in Habañero compared to that in Piquin (Table 2). Habañero grown in the greenhouse had more capsaicinoids and lower peroxidase activity than field-grown fruit (Figures 4 and 5). This is probably due to differences in soil and climatic conditions. The increase in capsaicinoid contents always coincided with a low or decreased peroxidase activity, and the decrease in their concentration always coincided



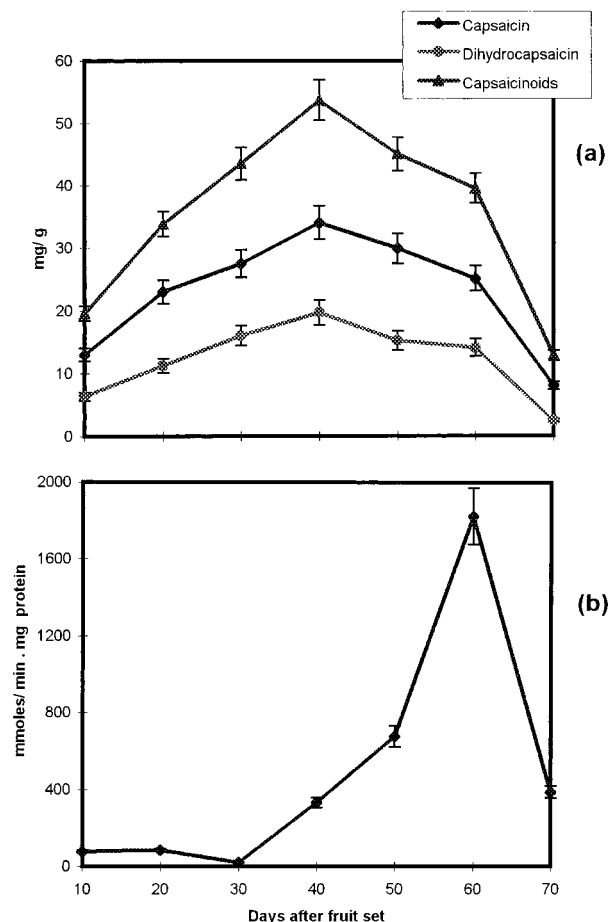
**Figure 5.** Capsaicinoid concentration (a) and peroxidase activity (b) during development, maturation, and senescence of Habañero chile peppers cultivated in the field. Vertical bars indicate standard deviation of the mean.

**Table 2. Maximum Concentration of Capsaicinoids and Maximum Peroxidase Activity during Maturation, Ripening, and Senescence of Chile Peppers**

	capsaicinoids (mg/g of dry fruit)	peroxidase activity [ $\mu\text{mol min}^{-1}$ (mg of protein) $^{-1}$ ]
Piquin	53.71 (40 DFFS) <sup>a</sup>	1825 (60 DFFS)
De árbol	80.52 (50 DFFS)	2189 (60 DFFS)
Habañero (field)	113.04 (45 DFFS)	887 (55 DFFS)
Habañero (greenhouse)	195.38 (50 DFFS)	533 (60 DFFS)

<sup>a</sup> Values in parentheses indicate days from fruit set (DFFS) when maximum concentration of capsaicinoids and peroxidase activity occurred.

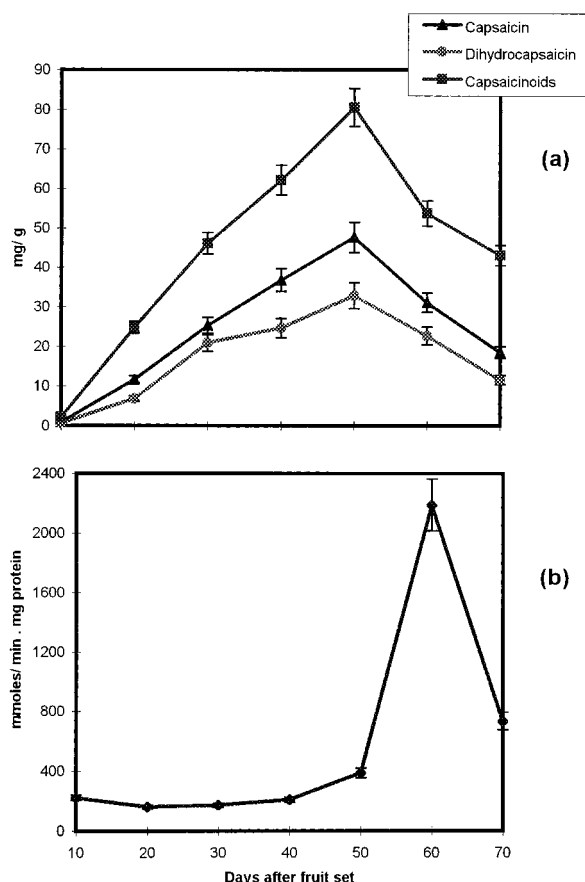
with a high or increased enzyme activity. These results clearly indicate an inverse relationship between capsaicinoid contents and peroxidase activity that might indicate an involvement of these enzymes in capsaicinoid degradation. Hot pepper peroxidases, especially peroxidase isoenzyme 6, oxidized the phenolic precursors of capsaicin biosynthesis such as caffeic acid and ferulic acid in *C. annuum* var. Annum (Bernal et al., 1995). Arguments on the involvement of peroxidase in the degradation of capsaicinoids have been based mainly on the localization of this enzyme, especially the basic peroxidase isoenzyme 6, in the placental epidermal cells (Bernal et al., 1994a) and later supported by the strong capsaicin-oxidizing activity of the basic peroxidase isoenzyme (Bernal et al., 1994b). Since basic peroxidase isoenzyme 6 is located in cell walls (Bernal et al., 1993c), Bernal et al. (1995) have suggested that it seems



**Figure 6.** Capsaicinoid concentration (a) and peroxidase activity (b) during development, maturation, and senescence of Piquin chile peppers cultivated in the greenhouse. Vertical bars indicate standard deviation of the mean.

plausible that this isoenzyme may be involved in the insolubilization of phenylpropanoid precursor and that these results are evidence for the existence of an oxidative competitive sink for phenylpropanoid intermediates of capsaicin biosynthesis, which probably competes with capsaicin itself in the cell walls of *C. annuum* var. Annum. The products of the capsaicin degradation (oxidation) were first suggested to be a lignin-like compounds (Bernal et al., 1993a) and later (Bernal and Ros Barceló, 1996) were identified as 5,5'-dicapsaicin, 4'-O-5-dicapsaicin ether, and dehydrogenation polymers with high molecular weights. Our results support previous conclusions (Bernal et al., 1993a,b) that peroxidase might be involved in capsaicinoid degradation observed in later stages of fruit development and thus the loss of pungency in chile peppers.

We conclude that the pungent compounds in hot chile peppers (capsaicinoids) decreased in the three cultivars studied 40–50 DFFS. On the basis of the evolution of the activity of peroxidases, these are probably related to the loss of capsaicinoids. The ideal harvest time from a standpoint of maximum capsaicinoid content (and thus higher pungency) is ~40 DFFS for Piquin and ~50 DFFS for De árbol and Habañero. Due to the great importance of hot chile peppers as food additives and drugs in several cultures of the world, a mechanism by which capsaicinoids are degraded and lost needs to be elucidated, and thus methods to reduce these losses need to be developed.



**Figure 7.** Capsaicinoid concentration (a) and peroxidase activity (b) during development, maturation, and senescence of De árbol chile peppers cultivated in the greenhouse. Vertical bars indicate standard deviation of the mean.

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