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Ultra high pressure treatment of orange juice: a kinetic study on inactivation of pectin methyl esterase

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Ultra high pressure treatments ranging from 0 to 720 min were applied to freshly squeezed, non-pasteurized orange juice and reconstituted frozen orange juice at pressures in the range 100 to 400 MPa to investigate the pressure inactivation kinetics of pectin methyl esterase (PME) activity. Freshly squeezed orange juice was adjusted to pH 3.7 or 3.2 (12.6° Brix) while the reconstituted frozen concentrate orange juice was adjusted to 10, 20, 30 and 40° Brix at pH 3.7. The frozen concentrated orange juice was initially heat treated to inactivate the natural enzymes and then fortified with commercial PME prior to inactivation studies. Analyses of kinetic data revealed the presence of a dual-effect of pressure inactivation: the first one, designated as an instantaneous pressure kill (IPK), which depended only on the pressure level; and a second one, which depended on the holding time at each pressure level, described by first-order reaction kinetics (characterized by a rate constant, k, or by the more familiar decimal reduction time, D). The associated IPK were higher and D values were lower at the lower pH and lower soluble solid contents. The pressure sensitivity of D values were adequately described by a z_p value which represented the range of applied pressure between which the D values change by a factor of 10. © 1997 Published by Elsevier Science Ltd on behalf of the Canadian Institute of Food Science and Technology

Keywords: ultra high pressure, pectin methyl esterase, enzyme inactivation, pasteurization, orange juice.

INTRODUCTION

A common problem associated with citrus juices (fresh squeezed, concentrated and preserved) is the loss of cloudiness and concentrate gelation which has been directly related to the activity of pectin methyl esterase (PME) (Wenzel *et al.*, 1951; Versteeg *et al.*, 1980). PME, commonly present in all citrus fruits is a cell-wall bound enzyme, is present as a complex with pectin through electrostatic interaction. During the juice extraction process, the enzyme is released into the juice and hydrolyses the pectin (methyl esters of polygalacturonic acid) transforming it gradually into low methoxy pectin or pectic acids. The resulting low methoxy pectin complexes with calcium to form insoluble calcium flocculation resulting in cloud loss (Joslyn and Pilnik, 1961). Inactivation of PME is generally used as an indicator of the adequacy of pasteurization because it is known to be more heat resistant than the common micro-organisms.

Pasteurization (90°C for 1 min) is the conventional method used to inactivate PME (Eagerman and Rouse, 1976). However, this pasteurization treatment may also result in thermal damage (non-enzymatic browning) and reduce the freshness of the juice flavors (Reynolds, 1963). Other alternative methods or novel combinations of existing methods are continually being investigated by the industry in the pursuit of producing better quality foods more economically. One of the most innovative technology for processing of thermosensitive products is pressure treatment which utilizes ultra high pressures (UHP) to bring about microbial destruction

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and significantly retard the rate of enzyme reaction (Morlid, 1981; Knorr *et al.*, 1992). Hence, high pressure processing technology offers an alternative method for the preservation of fruit juices without undesirable sensory changes often associated with thermal treatment.

The effect of high pressure treatment of various enzymes have been investigated and found to behave differently. Thus, it may cause reversible or irreversible inhibition, partial or complete inactivation/activation depending on the chemical nature of the enzymes being studied, the nature of the substrate, temperature, the level and duration of applied pressure and the influence of environmental parameters (Cheftel, 1992; Kunugi, 1992; De Cordt *et al.*, 1995). Ohmori *et al.* (1991) observed that lipase in fish muscle, amino and carboxypeptidases in beef muscle were markedly inactivated, while the corresponding acid protease appears to be pressure resistant. Glucose and ascorbate oxidases were significantly retarded (Aoyama *et al.*, 1993). In contrast, peroxidase, lipoxygenase and polyphenoloxydase remained partly inactivated after pressurization treatment (Knorr *et al.*, 1992). In some studies, initial activation, subsequently followed by inactivation, of polyphenoloxydase in pear and apple and peroxidase in carrot have been reported (Asaka and Hayashi, 1991; Anese *et al.*, 1995).

A number of studies have been conducted on the application of high pressure as a substitute to thermal treatment for inactivation of PME and preservation of citrus fruit juices (Ogawa *et al.*, 1990; Takahashi *et al.*, 1993) and have already showed some promise. The effect of juice concentration and different levels of applied pressure on the inactivation of PME and destruction of micro-organisms have been investigated to some extent (Ogawa *et al.*, 1990). However, no detailed study on the pressure-induced PME inactivation kinetics have been reported although similar studies have been published on thermal inactivation kinetics (Nath and Rangana, 1977*a*,*b*; Marshall *et al.*, 1985; Wicker and Timelli, 1988) and the analyses have been mostly qualitative. The main objective of the present investigation was therefore to investigate the high pressure inactivation kinetics of PME as influenced by pH and soluble solid concentrations with a secondary objective of gathering the kinetic data needed for establishing the UHP pasteurization process.

MATERIALS AND METHODS

Preparation of orange juice

Preparation of pH-adjusted orange juice

Unpasteurized freshly squeezed orange juice (Old South Brand) was obtained from a local market to investigate the pressure inactivation of the endogenous PME (initial activity, 3–4 PEu/ml). The pH of juice was adjusted to 3.7 or 3.2 by using citric acid. Test samples at different pH were maintained at the naturally present soluble solids content of 12.6° Brix.

Preparation of orange juice of different soluble solid contents

Frozen concentrated orange juice (Metro brand, 41° Brix) was pre-heated to boiling to inactivate the naturally occurring enzymes and was blended with different volumes of distilled water to adjust the soluble solids content to 10, 20, 30, and 40° Brix, measured by a hand refractometer. The prepared samples were all adjusted to pH 3.7. A stock solution of commercial citrus PME (Sigma Chemical Co., St Louis, MO) was prepared in 1.70 M NaCl and its activity was determined (procedure detailed later). A calculated amount of this enzyme stock solution was added to 160 ml of prepared test samples of four different soluble solids concentrations to obtain an initial activity of about 3–4 PEu/ml. The enzyme enriched test samples were stored at −10 to −15°C prior to use.

Measurement of PME activity

PME activity was evaluated by titrating the free carboxyl groups at pH 7.5 and at a temperature of 30°C employing the method of Rouse and Atkins (1955) with the modification of alkali strength, pH and temperature. 2 ml of juice sample was added to 50 ml 1% pectin solution containing 0.3 M NaCl which was previously adjusted to pH 7.5 with 0.2 N NaOH at 30°C. After readjustment of the reaction mixture to pH 7.5 which was kept constant by titrating the mixture with 0.02 N NaOH, consumption of 0.02 N NaOH was recorded during the 30 min reaction period. The PME unit was expressed in microequivalents of ester hydrolysed per minute per milliliter of juice sample at pH 7.5 and 30°C. The units were multiplied by 1000 for easy interpretation and were calculated according to eqn 1:

$$\text{PME(unit)} = \frac{(\text{ml of NaOH})(\text{N of NaOH})10^3}{(\text{ml of juice})(\text{time in min})} \quad (1)$$

Pressure treatment

The juice samples were used to fill plastic pouches (Dual Peel sterilization pouch, 10×20 cm; Baxter, Mississauga, Ont.). After expelling as much air as possible, the pouches were sealed. Pressure treatment was given in an ultra high pressure isostatic press (Model #CIP 42260, ABB Autoclave Systems—Autoclave Engineers, Erie, PA). The pouch was submerged in water containing 2% soluble oil (Autoclave Engineers, part no. 5019) which acted as the hydrostatic fluid medium during pressurization. Samples were subjected to pressure treatment at pressures ranging from 100 to 400 MPa.

The pressure levels and holding times for the different sets of experiments are shown in Table 1. Pressurization varied from 1–3 min and depressurization varied from 10–30 s. Since the time intervals used in this study were relatively long, pressure come-up and come-down corrections were expected to be small and hence not applied. All experiments were carried out in duplicate and the PME assay was carried out in triplicate, and the results were averaged.

Data analysis

The kinetic data on the inactivation of PME were initially analyzed using a conventional first-order model, i.e.

$$\log[A/A_o] = (k/2.303)t \quad (2)$$

where A is the mean residual enzyme activity (PME units) at time t (min), A_o the mean initial enzyme activity and k the reaction rate constant (min^{-1}) at a given pressure. The values of k were obtained from the regression of $\log[A/A_o]$ versus time as −slope/2.303.

The decimal reduction time (D value) defined as the pressure treatment time needed for 90% inactivation of initial activity at a given pressure level was obtained:

$$D = 2.303/k \quad (3)$$

The pressure sensitivity of D values were obtained using procedures analogous to that employed in thermal death time studies by plotting the logarithm of D values versus pressure. The pressure sensitivity parameter, z_p, was the pressure range between which the D values change tenfold. Mathematically,

$$\log[D_1/D_2] = (P_2 - P_1)/z_p \quad (4)$$

where P_2 and P_1 were pressures corresponding to decimal reduction times D_1 and D_2, respectively. The value of z_p was obtained as the negative reciprocal slope of the regression line representing log D versus P relationship.

Table 1. Test conditions used for the current study

Condition	Pressure (MPa)	Holding time (min)	Pressurization time (min)
pH 3.7	100	120–720	0.33
	200	90–450	1.07
	300	60–300	1.46
	400	0–180	2.8
pH 3.2	270	30–120	1.32
	300	30–120	1.46
	330	10–60	1.57
	400	0–20	2.8
10,20,30 or 40° Brix (pH 3.7)	200	30–90	1.07
10,20,30 or 40° Brix (pH 3.7)	300	30–90	1.46

A careful look at the plot of logarithm of residual activity versus time indicated that generally the conventional semi-logarithmic linearity resulted only after an initial quick drop in the activity. While calculating the first-order rate constants, therefore, the initial activity (i.e., activity at time zero prior to pressure treatment) was not included in the regression. From the intercept value of the regression equation extrapolated to time zero, the drop in the enzyme activity which would be a result of the instantaneous application of pressure (no holding time) was calculated. This drop in the activity was designated as the instantaneous pressure kill (IPK) value. It represents the percentage reduction in the enzyme activity due to a single application of instantaneous pressure and it does not depend on the holding time.

RESULTS AND DISCUSSION

Pressure inactivation kinetics of PME as influenced by pH

Figures 1 and 2 show semi-logarithmic plots of percentage residual activity versus holding time at various pressures for PME in orange juice at two pH levels, 3.7 and 3.2, respectively. The results generally indicated a linear relationship confirming a first-order rate of pressure inactivation of PME, although, generally, it happened only after an initial drop in the activity. The first-order kinetics were applied only to the latter linear region, and the intercept from the regression line extended to the zero holding time was used to calculate the instantaneous pressure kill value.

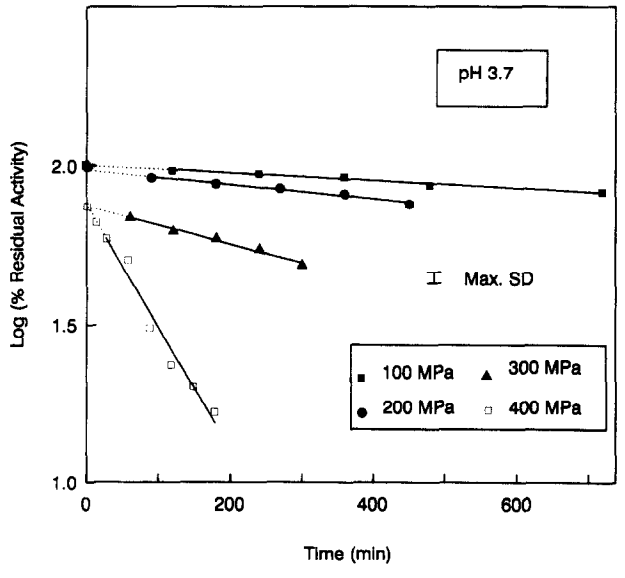

Fig. 1. Pressure inactivation kinetics of PME in orange juice at pH 3.7 at various pressures (the extrapolated intercept to time zero is used in calculating the instantaneous pressure kill value).

From the results, the pressure inactivation of pectin methyl esterase, is hypothesized to be bi-phasic. However, the bi-phasic nature observed is somewhat different from most conventional bi-phasic reactions. For example, Versteeg *et al.* (1980) reported the thermal inactivation of PME to be bi-phasic and hypothesized that the two phases are due to two typical pseudo-first-order inactivation reactions comprising of reacting mixtures of two fractions of differing thermal sensitivity. Such a concept would result in two straight line segments in the inactivation plots (Figs 1 and 2) with one having a steeper slope than the other. This is not the case in the present study. There is no evidence supporting the presence of a steeper first line at the beginning of the plot. Most experimental data points belong homogeneously to one segment. It may be argued that if shorter time intervals were employed for pressure treatment, this first segment will become more obvious. Initially, the treatment time-intervals were kept between 15 and 30 min depending on the pressure level. When the dual-behavior described above was observed, the times were reduced for selected conditions to 10, 5 and finally 0 min holding time (see Figs 1 and 2; 400 MPa). Once again, all these points formed a smooth continuum of previous data points. Hence, it becomes obvious that there are no kinetics involved in the first phase inactivation and the bi-phasic nature is not due to two first-order inactivations. On the other hand, it is due to the dual effect of the pressure treatment; the first one being an instantaneous pressure kill (IPK) which depends only on the pressure level, and the second one, which depends on both pressure level and holding time, for which the first-order rate kinetics apply. The instantaneous effect results in a step change reduction in the activity after which the conventional first-order inactivation behavior prevails.

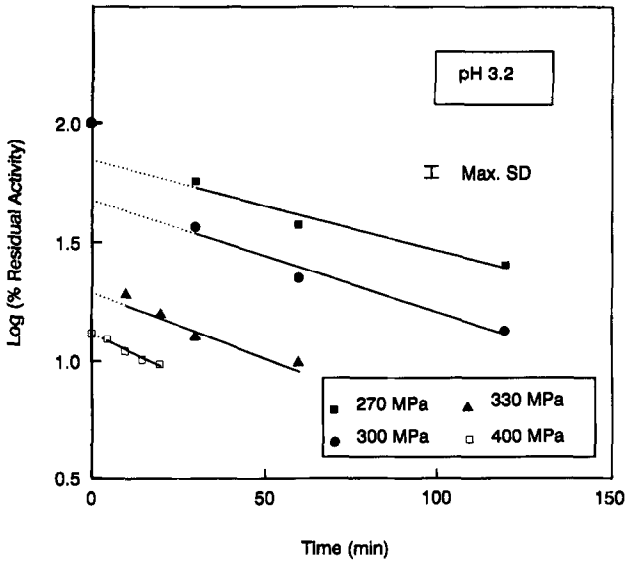

Fig. 2. Pressure inactivation kinetics of PME in orange juice at pH 3.2 at various pressures (the extrapolated intercept to time zero is used in calculating the instantaneous pressure kill value).

The first-order rate parameters, k and D, computed from regression of $\log(A/A_o \times 100)$ vs. time plots (Figs 1 and 2) are tabulated in Table 2 generally indicating a good fit ($R^2 > 0.90$; $P \leq 0.05$). The rate constants clearly depended on the pressure level as well as pH with higher pressures resulting in lower D values (higher k) which decreased (k increased) further at the lower pH. These results appear to be consistent with those reported earlier (Ogawa *et al.*, 1990; Balny and Masson, 1993) that application of higher pressures cause higher inactivation of enzymes due to more rapid denaturation of protein. Comparison of data at the two pH levels indicated a modest to strong dependence of pressure inactivation kinetics on pH. At the higher pH, an increasing level of pressure produced much steeper slopes in contrast to that at lower pH. At pH 3.2, the initial drop in the activity was high and the associated D value was generally lower than at pH 3.7, however, the relative change in slopes at different pressures were smaller. In general, the associated D values at pH 3.7 were two- to eight-fold higher than at pH 3.2 (direct comparison available at 300 and 400 MPa). The associated k values at pH 3.2 were higher than at pH 3.7 by similar margin. Similar observations have been reported for thermal inactivation of PME (Nath and Rangana, 1977*a*,*b*). Owusu-Yaw *et al.* (1988) reported that inactivation of PME can be accomplished by lowering pH without heat treatment. The k and D values reported in Table 2 were not corrected for the pressure come-up and come-down periods. Since the associated D values were considerably large (>90 min) relative to the come-up period (<3 min), and only values beyond the come-up periods were included in their computation, any come-up correction would change all the treatment times by the same margin and hence, would not affect the calculated D or k values.

The pressure sensitivity of kinetic parameters can be obtained by the parameter z_p which indicates the pressure difference that will result in a ten-fold change in the D values. The z_p values are generally obtained as the negative reciprocal slopes of regression plots of logarithms of D values versus pressure (Fig. 3). The z_p

Table 2. Kinetic parameters for the pressure inactivation of PME in orange juice at different pressures and pH (12.6° Brix)

pH	Pressure (MPa)	D value (min)	k value (min^{-1})	R^2 ($P \leq 0.05$)
3.7	100	8250	0.000279	0.97
	200	4490	0.000513	0.98
	300	1690	0.00136	0.99
	400	260	0.0089	0.97
3.2	270	264	0.00873	0.96
	300	211	0.0109	0.97
	330	180	0.0128	0.78
	400	145	0.0159	0.96

values were also pH dependent and the value (525 MPa) at pH 3.2 was about 2.5 times that at 3.7. Although the *D* value at any given pressure at pH 3.2 was lower than the corresponding *D* value at pH 3.7 within the pressure range studied, the difference in slopes of the two curves indicate that beyond a pressure of about 600 MPa, a reversal of the effects might be a possibility.

The instantaneous pressure kill (IPK) values under the different test conditions are tabulated in Table 3. The instantaneous pressure kill was clearly dependent on the treatment pressure level as well as pH of the test sample, generally increasing with pressure and with dramatically higher values at the lower pH. At the natural pH of 3.7, IPK values were relatively small (up to 25%) and would have probably been ignored as experimental error. However, the effect was clearly noticeable at pH 3.2 with values increasing from about 25% at 100 MPa to as high as 90% at 400 MPa. Thus, application of pressure alone, without any holding time, could have a significant role in the inactivation of PME if assisted by environmental parameters such as pH. The elevated pressure kill at the lower pH is obviously a result of higher protonization, an observation which has been noted previously for other enzymes like peroxidase and polyphenoloxidase. The activity of these deterioratory enzymes have been shown to be greatly affected by pH of the enzymatic crude juice (Anese *et al.*, 1995). Aoyama *et al.* (1993) also showed that very short time treatment of pressurization and depressurization could retard the activity of the enzyme.

Pressure inactivation kinetics of PME as influenced by total soluble solids

These studies were carried out only at pH 3.7 and at two pressure levels. The effect of pressure hold time on PME inactivation is shown in Fig. 4 which shows semi-logarithmic plots of residual activity versus treatment time at the two pressure levels (200 and 300 MPa). As mentioned previously, at the higher pressure, these effects were more prominent. The kinetic parameters calculated from the data are summarized in Table 4. At both pressure levels employed, a decrease in PME inactivation rate (higher *D* value) was observed with an increase in the soluble solid content. These results

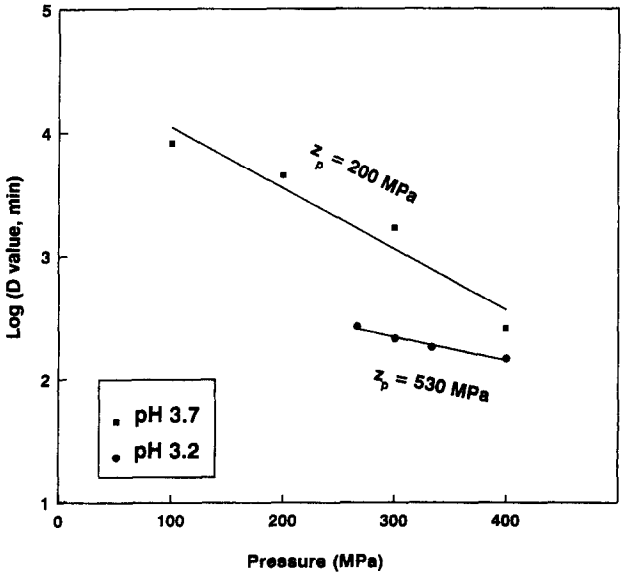

Fig. 3. Pressure sensitivity of PME inactivation rates in orange juice at two pH levels.

Table 3. Calculated instantaneous pressure kill (IPK) value for PME in orange juice under different conditions

pH	TSS (° Brix)	Pressure (MPa)	IPK (%)
3.7	12.6	100	0
		200	2.3
		300	26
		400	26
3.2	12.6	270	26
		300	53
		330	81
		400	90
3.7	10	200	8.8
		300	6.7
	20	200	4.5
		300	0
	30	200	0
		300	0
	40	200	0
		300	0

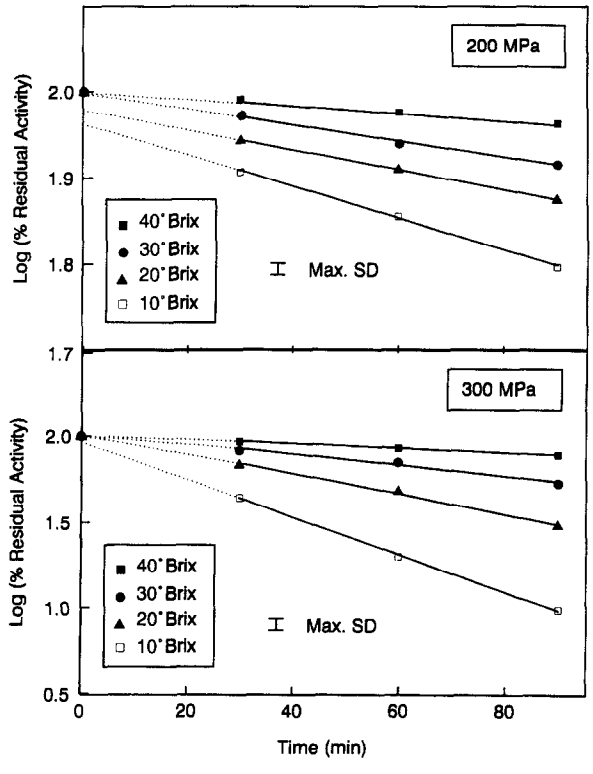

Fig. 4. Inactivation kinetics of PME in orange juice at pH 3.7 as influenced by soluble solids concentration at two pressure levels (200 and 300 MPa).

Table 4. Kinetics parameters for the pressure inactivation of PME in orange juice at various soluble solids content and two pressure levels (pH 3.7)

Pressure (MPa)	Total solids (° Brix)	D value (min)	k value (min^{-1})	R^2 ($P \leq 0.05$)
200	10	455	0.00506	0.99
	20	731	0.00314	0.99
	30	1080	0.00212	0.99
	40	2440	0.00949	0.99
300	10	91	0.0252	0.99
	20	170	0.0136	0.99
	30	311	0.00742	0.97
	40	750	0.00307	0.99

confirm some of those reported previously (Marshall *et al.*, 1985; Cheftel, 1992). Higher concentration levels thus may offer some protection against pressure inactivation. Higher concentrations of sugar has also been frequently shown in literature to offer protection against thermal destruction of microorganisms. Marshall *et al.* (1985) found increased levels of soluble solid contents (40° Brix) in juice to decrease the PME inactivation rate suggesting a protective effect by the solid content. Arakawa and Timasheff (1982) demonstrated that the primary reason for the sugar stabilization of proteins is the preferential hydration of proteins in the presence of sugars which result in surface free energy perturbations. They also reported that exclusion of volume of the sugars in the reacting mixture and the chemical nature of the protein surface to be other contributing factors in sugar stabilization. However, there are also reports in the literature which contradict the protective action of sugars on enzymes. Ogawa *et al.* (1990), Bissett *et al.* (1957), Carroll *et al.* (1957) and Versteeg *et al.* (1980) reported that the inactivation rate increases with the soluble solids content.

Effects of total soluble solids on instantaneous pressure kill or IPK values of PME are also included in Table 3 using the approach described earlier. It is apparent that the IPK values are more influenced by the pH than the soluble solids content with inactivation values of < 10%. Especially, the higher soluble solids (40° and 30° Brix) had almost no effect on IPK value.

CONCLUSIONS

The inactivation of PME in orange juice was dependent on the pressure level, pressure–hold time, pH and total soluble solids. The instantaneous pressure kill was directly proportional to the pressure level and was higher at the lower pH. Pressure–hold inactivation of pectin methyl esterase in orange juice followed first-order rate kinetics. Pressure sensitivity of kinetic parameters was well described by the z-value approach traditionally followed in handling of kinetic data in thermal process calculations. IPK values ranged from under 25% at pH 3.7 to 90% at pH 3.2 and 400 MPa pressure. The D values varied from 260 to 8250 min in the pressure range of 100 and 400 MPa at pH 3.7. The D value range at pH 3.2 was 140 to 260 min in the pressure range of 270 to 400 MPa. indicating a large reduction in the D value as the pH was lowered. Total soluble solid content in orange juice (10–40° Brix) also influenced the inactivation rate of PME with a decrease in the inactivation rate at higher Brix suggesting some protective action at high solid content.

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