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Development of enzymic time-temperature integrators with rapid detection for evaluation of continuous HTST pasteurization processes

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ABSTRACT

The assessment of the thermal process impact in terms of food safety and quality is of great importance for process evaluation and design. This can be accomplished from the analysis of the residence time and temperature distributions coupled with the kinetics of thermal change, or from the use of a proper time-temperature integrator (TTI) as indicator of safety and quality. The objective of this work was to develop and test enzymic TTIs with rapid detection for the evaluation of continuous HTST pasteurization processes (70–85 °C, 10–60 s) of low-viscosity liquid foods, such as milk and juices. Enzymes peroxidase, lactoperoxidase and alkaline phosphatase in phosphate buffer were tested and activity was determined with commercial reflectometric strips. Discontinuous thermal treatments at various time-temperature combinations were performed in order to adjust a first order kinetic model of a two-component system. The measured time-temperature history was considered instead of assuming isothermal conditions. Experiments with slow heating and cooling were used to validate the adjusted model. Only the alkaline phosphatase TTI showed potential to be used for the evaluation of pasteurization processes. The choice was based on the obtained *z*-values of the thermostable and thermolabile fractions, on the cost and on the validation tests.

1. Introduction

Thermal processing is one of the most widely used physical methods for food preservation. High temperature inactivates undesired microorganisms and enzymes, but also deteriorates quality and sensorial attributes. Consumer demands for minimally processed products compel food companies to optimize and redesign the existing technologies. In this context, the assessment of the process impact in terms of food safety and quality is of great importance for process evaluation and design. The *in situ* evaluation of microbial count or vitamin content is often time-consuming and expensive. Alternatively, the effect of the thermal processing can be evaluated in two ways: from the analysis of the time-temperature history and the residence time distribution coupled with the kinetics of thermal change; and from the use a time-temperature integrator (TTI) as indicator of safety and quality (Lewis & Heppell, 2000, p. 447; Van Loey, Hendrickx, De Cordt, Haentjens, & Tobback, 1996).

The first method requires the time-temperature history, which can be recorded online at the processing plant and also residence time distribution techniques. These results, combined with the knowledge of the thermal change kinetics, allow the calculation of the process impact. Alternatively, the time-temperature history can be determined from the simulation of the heat transfer and flow model of the process, as long as the model can represent the process conditions with confidence (Georgiadis & Macchietto, 2000; Grijspeerdt, Hazarika, & Vucinic, 2003; Gut & Pinto, 2009; Gut, Pinto, Gabas, & Telis-Romero, 2005; Jung & Fryer, 1999).

On the other hand, a TTI could be used for the evaluation of the process impact. The TTI must be a thermally sensitive component (intrinsic or extrinsic to the food) that allows the quantification of the thermal process impact on the safety or quality attribute. The changes that happen during the process must be irreversible and of similar dynamic of the studied attribute. The lethality calculated from the time-temperature data must agree with the lethality obtained from the TTI (Hendrickx et al., 1995; Van Loey et al., 1996).

Enzymic TTIs were for long applied to evaluate the lethality of batch thermal processes of canned or solid foods. For instance, Hendrickx, Weng, Maesmans, and Tobback (1992) developed a TTI made from the heat-stable fraction of horseradish peroxidase covalently immobilized on porous glass beads in dodecane to indicate the intensity of a delivered pasteurization process. Guiavarc'h, Deli, Van Loey, and Hendrickx (2002) and Guiavarc'h, Dintwa, Van Loey, Zuber, and Hendrickx (2002) studied the

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thermal inactivation of α-amylase from *Bacillus licheniformis* in order to develop a TTI that consisted of hollow silicon spheres containing the enzymic system to investigate the thermal impact inside particles of a liquid/solid food product in a rotary retort. Tucker, Hanby, and Brown (2009) developed an enzymic TTI that consisted of α-amylase in 10 mM acetate buffer to evaluate mild pasteurization processing of food products in sealed containers. Small samples of the TTI (20 μL) were encapsulated in silicon tubes that were later positioned inside the product container.

Some TTIs were also developed for evaluation of continuous thermal processing of liquid foods containing particles. For example, Tucker, Lambourne, Adams, and Lach (2002) sealed an enzymic TTI in small silicon particles that were incorporated randomly in batches of blackcurrant, pineapple or strawberry that were then processed in a double-pipe heat exchanger. In order to evaluate a continuous process of liquid foods without particles using an extrinsic TTI, the chosen component has to be introduced in the food product or in another liquid media (food model). Miles and Swartzel (1995a), for instance, used Blue #2 in carbonate-bicarbonate buffer to evaluate the lethality in a continuous thermal process that consisted of two double-pipe heat exchangers (heating and cooling) and a holding tube (processing temperature between 75 and 140 °C). Ellborg and Trägårdh (1994) proposed and developed a method to determine the lethality distribution in non-isothermal flow using acid hydrolysis of dextran for continuous processing in double-pipe heat exchanger.

Miles and Swartzel (1995b) determined kinetic parameters for acid hydrolysis of sucrose that well compared with literature values, using the two step Arrhenius method with tube data, and the one step equivalent point method for non-isothermal conditions. Data for this reaction were generated under continuous flow thermal processing conditions that included the UHT process temperature range. Torres and Oliveira (1999) also used the acid hydrolysis of sucrose as TTI for assessing holding temperatures in pasteurization processes. Values of temperature were estimated from the measured conversion based on kinetic data obtained in batch conditions. These results agreed with thermocouple measurements, with deviations of less than 4 °C for conversions between 0.4 and 0.7.

At the same way, Gentry and Roberts (2004) assessed the kinetic parameters for 5-hydroxymethylfurfural (HMF) formation to validate the total lethality of a continuous flow microwave pasteurization system for apple cider. The HMF concentrations were determined by gas chromatography with flame ionization detector before and after the thermal processing to determine the net increase in HMF. These values compared well with those based on the time-temperature histories.

The aim of this work was to develop and test TTIs for the evaluation of HTST pasteurization processes of liquid foods with low viscosity, such as milk and fruit juices. Instead of developing an extrinsic or intrinsic TTI for a specific food, the idea was to test general water-based TTIs that could be applied to assess different processes, as long as the viscous and thermal characteristics of the food do not differ at great extend from those of water. Therefore, these TTIs had to be able to detect under-processing and over-processing at HTST pasteurization conditions (temperatures between 70 °C and 85 °C and holding times between 10 s and 60 s). Consequently, enzymes dissolved in phosphate buffer were purposed as TTIs to evaluate continuous thermal processing of liquid foods. The buffer containing the enzyme was processed simulating the liquid food and the residual enzymic activity was assessed after the treatment. Enzymes peroxidase, lactoperoxidase and alkaline phosphatase were chosen for the tests because they are partially inactivated at pasteurization conditions and they can be rapidly assessed by reflectometric methods. Discontinuous thermal treatments at various time-temperature combinations were performed in order to adjust the kinetic parameters. The measured time-temperature history was used for the parameter adjustment instead of assuming isothermal conditions in order to improve the quality of the results. Discontinuous experiments with slow heating and cooling were used to validate the results.

2. Material and methods

2.1. Preparation of the enzymic TTIs

Three enzymes were tested in this work as TTIs, each one consisting of a commercial lyophilized powder (Sigma–Aldrich, St Louis, USA) dissolved in phosphate buffer (pH 6.6 and ionic strength 50 mM), which was prepared from mono- and dibasic sodium phosphates in distilled water. This pH value was chosen because the method used for enzymic activity assessment was originally designed for milk and the average pH or raw milk is pH = 6.6 (Wilińka, Bryjak, Illeová, & Polakovič, 2007).

The peroxidase TTI (POD) consists of horseradish peroxidase (EC 1.11.1.7, Sigma–Aldrich P6782) dissolved in the phosphate buffer. The lyophilized powder was first dissolved in distillated water (100 mg/L) and then stored in aliquots of 1.0 mL in a freezer (−30 °C) for up to three months. When for use, the stored sample was diluted with the phosphate buffer to obtain a concentration of 1.0 mg/L and this solution was stored at 5 °C for up to five days. After preparation, the enzymic activity was determined in triplicate and concentration adjustments were made, if necessary, to obtain a reading in the range 120–180 U/L (activity assessment presented further in Section 2.2).

The lactoperoxidase TTI (LPO) consists of enzyme lactoperoxidase from bovine milk (EC 232-668-6, Sigma–Aldrich L8257) dissolved in the phosphate buffer. The lyophilized powder was first dissolved in distillated water (833 mg/L) and then stored in aliquots of 1.0 mL in a freezer (−30 °C) for up to three months. When for use, the stored solution was diluted with the phosphate buffer to obtain a concentration of 20.8 mg/L and this solution was stored at 5 °C for up to five days. After preparation, the enzyme activity was determined in triplicate and concentration adjustments were made, if necessary, to obtain a reading in the range 120–180 U/L.

The alkaline phosphatase TTI (ALP) consists of enzyme alkaline phosphatase from bovine intestinal mucosa (EC 3.1.3.1, Sigma–Aldrich P7640) diluted in the phosphate buffer. The lyophilized powder was dissolved in the phosphate buffer (250 mg/L) and it was stored at 5 °C for up to five days. When for use, an aliquot of this solution was diluted in phosphate buffer to give a concentration of 0.38 mg/L. After preparation, the enzymic activity was determined in triplicate and concentration adjustments were made, if necessary, to obtain a reading in the range 7.0–9.0 U/L.

2.2. Activity assay

To simplify the practical use of the enzymic TTIs proposed in this work, a rapid method for determination of the enzymic activity was needed. In this way, a commercial reaction reflectometric kit was adapted.

The enzymic activities of the three indicators were determined using the Reflectoquant® System (Merck, Darmstadt, Germany) that consists of a portable reflectometer (RQflex plus 10) and analytical test strips. The test kit "Peroxidase in Milk" (Merck 1.16121) was used to determine the activity of the POD and LPO indicators. The test kit "Phosphatase in Milk" (Merck 1.16123) was used to determine the activity of the ALP indicator. Since the test kits were designed for milk testing (Martin et al., 2005; Sharma,

Sehgal, & Kumar, 2003), the absolute values of activity determined for the enzymic indicators in this work cannot be directly used. Instead, the residual enzyme activity defined in Eq. (1) was the variable of interest, where A is the activity reading after thermal treatment and A_0 is the activity reading before thermal treatment (average of at least three measurements). No activity was detected when testing the phosphate buffer without enzymes.

$$AR = \frac{A}{A_0} \tag{1}$$

The peroxidase test proceeded according to manufacturer's instructions (Merck, 2008), using a 1.0 mL sample, 4.0 mL of distilled water and five drops of reagent POD-1. The reaction time was 180 s at 23 °C. Phosphatase test required a 2.0 mL sample and four drops of reagent ALP-3 (originally, it would require a 5.0 mL sample with ten drops of ALP-3). The reaction time was 20 min at 37 °C (Merck, 2005). For both tests, the test strip was kept inside a semi-microcell, which was partly immersed in a water bath with temperature control. For the phosphatase test, the semi-microcell was previously filled with 15 drops of reagent ALP-1. After the reaction time, the test strip was inserted in the reflectometer, which gives the activity in U/L. Measuring ranges were 5–200 U/L for peroxidase and 1.0–10.0 U/L for phosphatase activity.

2.3. Discontinuous thermal treatment

To obtain thermal inactivation data of the enzymic indicators, several discontinuous thermal treatment tests were performed. A sample of the indicator (2.0 mL of POD, 2.0 mL of LPO or 3.0 mL of ALP) was placed in a polyethylene pouch (2.5 cm × 30 cm, thickness: 0.06 mm) with an exposed-junction type-K thermocouple placed at the center of the liquid. Polyethylene was used instead of glass because of its small thickness and, consequently, low thermal resistance. Temperature data was collected every second using a calibrated portable digital thermometer (TH-060, Instrutherm, São Paulo, Brazil). Instead of assuming isothermal conditions, the time-temperature history was obtained for each test and taken into account in the calculations, as proposed by Matsui, Gut, Oliveira, and Tadini (2008).

Thermal treatment was accomplished by immersion of the pouch in a hot water bath (controlled temperature) for a determined time, followed by immersion in an ice water bath until temperature was below 10 °C. Samples were kept in the ice bath for up to 90 min before activity measurement. Fig. 1 shows a scheme of the thermal treatment and presents some examples of obtained time-temperature histories.

Because of the volume required for the activity assay (1.0 mL for POD/LPO and 2.0 mL for ALP), it was not possible to determine the activity in duplicate or triplicate for each sample after thermal treatment. Some time-temperature conditions were repeated; however, since each run has an individual and precise time-temperature history, they were not treated as replicates.

Several combinations of hot water temperature and immersion time were tested in order to obtain values of residual activity in the range $5 \leq AR \leq 95\%$. Immersion times were between 15 s and 10 min. For indicator POD, tested temperatures were 60.0, 65.0, 70.0, 75.0, 80.0, 85.0, 90.0 and 95.0 °C. Tested temperatures for LPO were 60.0, 62.5, 65.0, 67.5, 70.0, 72.5, 75.0, 77.5 and 80.0 °C. For ALP, temperatures were 60.0, 65.0, 70.0, 75.0, 80.0 and 85.0 °C.

2.4. Kinetic model

The kinetic model used to represent the thermal inactivation of indicators POD, ALP and LPO was a first order reaction model of a two-component system (Chen & Wu, 1998; Fujikawa & Itoh, 1996; Murasaki-Aliberti, Silva, Gut, & Tadini, 2009; Tribess & Tadini, 2006). According to this model, there are two isoenzymes (with different thermal resistances) that contribute to the enzymic activity. Parameter α represents the fraction of the activity associated with the thermostable isoenzyme; accordingly, $(1 - \alpha)$ represents the contribution of the thermolabile enzyme to the activity (before thermal treatment). The thermal inactivation of the each isoenzyme follows a first order decay kinetic model, which is characterized by the parameters D-value (decimal reduction time) and z-value (temperature change necessary to obtain a tenfold decrease in the D-value). For an isothermal treatment, the residual activity at time θ can be obtained through Eq. (2), where the D-values of the thermostable and thermolabile isoenzymes are obtained from Eq. (3) and Eq. (4), respectively, where T_{ref} is the reference temperature for parameters $D_{S,ref}$ and $D_{L,ref}$.

$$AR = \alpha \cdot \text{alog}\left(-\frac{\theta}{D_S}\right) + (1-\alpha)\cdot \text{alog}\left(-\frac{\theta}{D_L}\right) \tag{2}$$

$$D_S = D_{S,ref} \cdot 10^{-(T-T_{ref})/z_S} \tag{3}$$

$$D_L = D_{L,ref} \cdot 10^{-(T-T_{ref})/z_L} \tag{4}$$

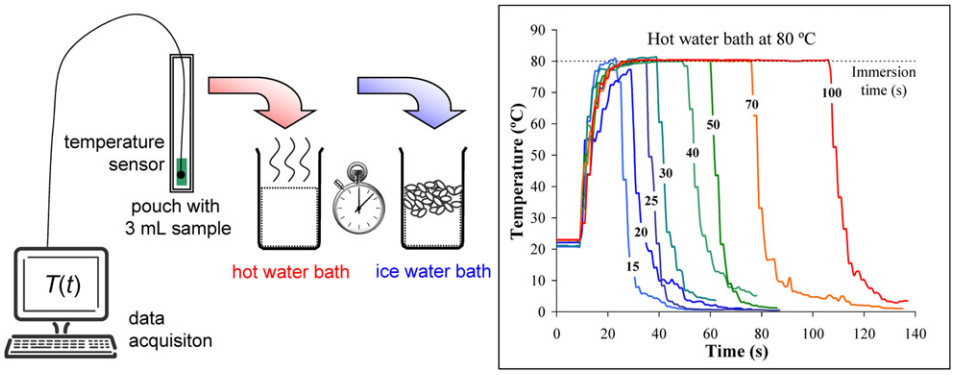

Fig. 1. Scheme of the discontinuous thermal treatment of the samples and some examples of obtained time-temperature histories for different immersion times in the hot water bath (80 °C).

Table 1
Adjusted kinetic parameters for TTIs POD (peroxidase), LPO (lactoperoxidase) and ALP (alkaline phosphatase).

TTI	n	SSE	T_{ref} (°C)	α	$D_{S,ref}$ (s)	$D_{L,ref}$ (s)	z_S (°C)	z_L (°C)
POD	56	0.57	80	0.368	808	27.0	15.1	8.22
LPO	59	1.11	70	0.568	173	149	2.40	8.91
ALP	61	0.60	75	0.585	478	3.83	7.57	6.57

For a non-isothermal treatment, where the time-temperature history $T(t)$ is known, the equivalent processing times for both isoenzymes at the reference temperature were calculated through Eq. (5a) and Eq. (5b) using the corresponding temperature dependence parameter z-value (z_S and z_L).

$$\theta_{S,ref} = \int_0^\infty \text{alog}\left(\frac{T(t) - T_{ref}}{z_S}\right) dt \quad (5a)$$

$$\theta_{L,ref} = \int_0^\infty \text{alog}\left(\frac{T(t) - T_{ref}}{z_L}\right) dt \quad (5b)$$

Combination of Eq. (2), Eq. (5a) and Eq. (5b) gives the first order reaction model of a two-component system in Eq. (6). The detailed derivation of Eq. (2) and Eq. (6) is presented by Murasaki-Aliberti et al. (2009). This kinetic model has five parameters, as follows: α (fraction of thermostable component), $D_{S,ref}$ and $D_{L,ref}$ (D-values at reference temperature of thermostable and thermolabile

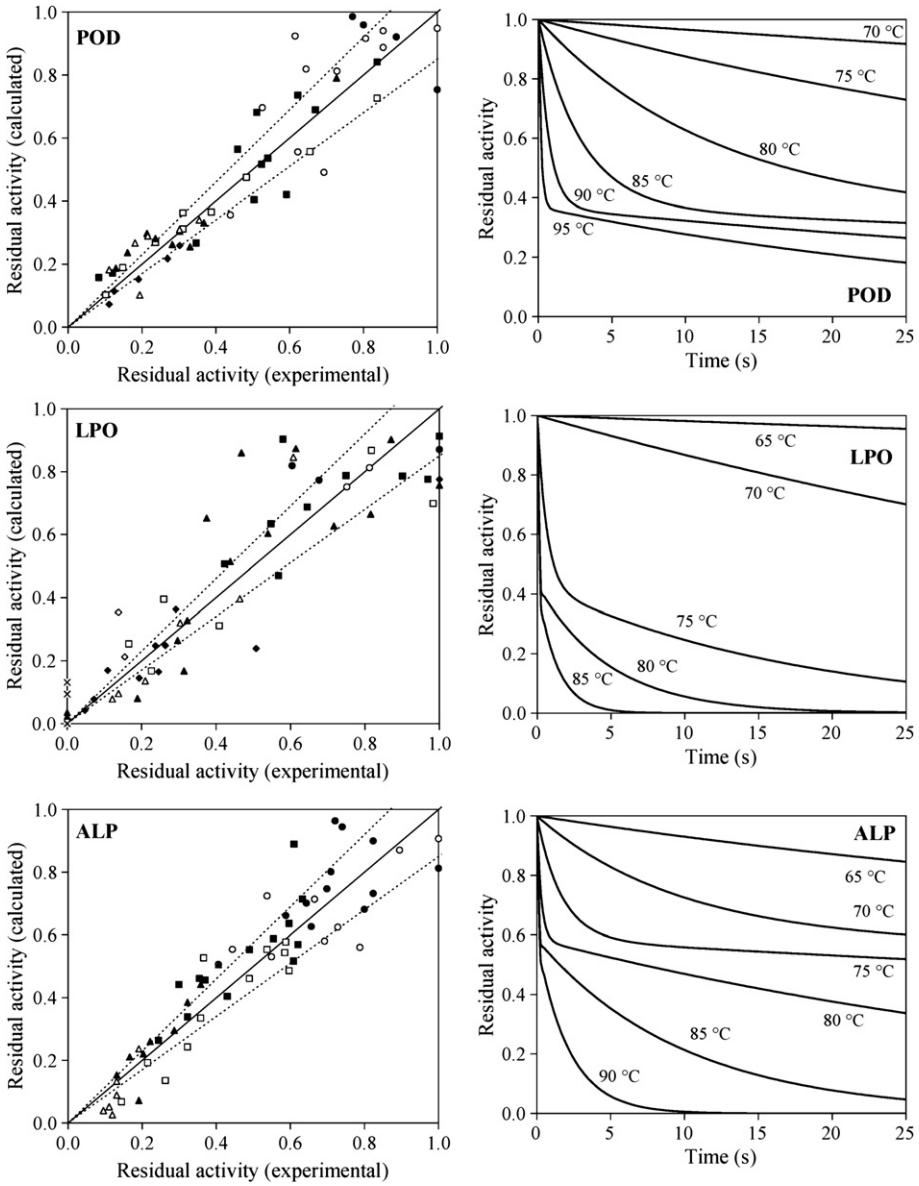

Fig. 2. Results of model adjustment for the indicators peroxidase (POD), lactoperoxidase (LPO) and alkaline phosphatase (ALP) showing the parity charts on the left (experimental vs. predicted values, — = ± 15%) and the inactivation curves of the adjusted model for significant temperatures on the right. Symbols for POD: ●: 65 °C, ○: 70 °C, ■: 75 °C, □: 80 °C, ▲: 85 °C, △: 90 °C, ◆: 95 °C. Symbols for LPO: ●: 60.0 °C, ○: 62.5 °C, ■: 65.0 °C, □: 67.5 °C, ▲: 70.0 °C, △: 72.5 °C, ◆: 75.0 °C, ◇: 77.5 °C, ×: 80.0 °C. Symbols for ALP: ●: 60 °C, ○: 65 °C, ■: 70 °C, □: 75 °C, ▲: 80 °C, △: 85 °C.

components), and z_S and z_L (z-values of thermostable and thermolabile components).

$$AR = \alpha \cdot \text{alog}\left(-\frac{\int_0^\infty \text{alog}\left(\frac{T(t)-T_{ref}}{z_S}\right)dt}{D_{S,ref}}\right) + (1-\alpha)\cdot\text{alog}\left(-\frac{\int_0^\infty \text{alog}\left(\frac{T(t)-T_{ref}}{z_L}\right)dt}{D_{L,ref}}\right) \quad (6)$$

The integrals in Eq. (6) were numerically evaluated by the trapezium method using the experimental time-temperature history data. Using an initial guess for the five model parameters, the predicted residual activity could be calculated using software Excel (Microsoft, Redmond USA). For a set of experiments, the sum of squared errors between experimental and predicted residual activities was minimized using Excel Solver to determine the optimal values of the model parameters (Matsui et al., 2008). Before using the Solver, a manual exploration of the parameters was performed to improve the initial guess and to detect large outliners. A parity chart was used to assess the error distribution and the quality of the adjustment.

2.5. Slow discontinuous thermal treatment for validation

To validate the adjusted kinetic model, indicators POD, LPO and ALP were submitted to slow discontinuous thermal treatments and the measured residual activity was compared with the predicted activity from Eq. (6) using the adjusted kinetic parameters and the acquired time-temperature histories. Samples of 3.0 mL were placed in small glass tubes (wall thickness: 1.2 mm), which were immersed in a hot water bath for 1.0 or 2.0 min before cooling in a ice water bath. Temperature of the sample was acquired through the same procedure described in Section 2.3. Tested temperatures were 65.0, 70.0, 75.0, 80.0 and 85.0 °C.

3. Results and discussion

3.1. Kinetic model

Time-temperature data of the indicators were analyzed as discussed in Section 2.4 for the adjustment of the kinetic model. Table 1 presents the adjusted parameters for indicators POD, LPO and ALP. In this table, *n* is the number of valid experiments and *SSE* is the sum of squared errors on the residual activity. The parity charts presented in Fig. 2 and the values of *SSE* in Table 1 indicate a larger experimental error for indicator LPO. The mean absolute errors in the prediction of *AR* were 21%, 27% and 20% for indicators POD, LPO and ALP, respectively. These large deviations are associated with the error on the experimental determination of *AR* and with the model error. These results indicate the need of replicate measurements when for the practical application of the proposed indicators to improve accuracy. Since each thermal treatment had a particular time-temperature history, it was not possible to run replicates in order to evaluate the variance on the measured activity. However, based on the repeated measurements of the initial enzymic activity (A_0), the average standard error for the determination of peroxidase activity was ±11 U/L (8.2% error) and the standard error for alkaline phosphatase was ±0.7 U/L (9.1% error).

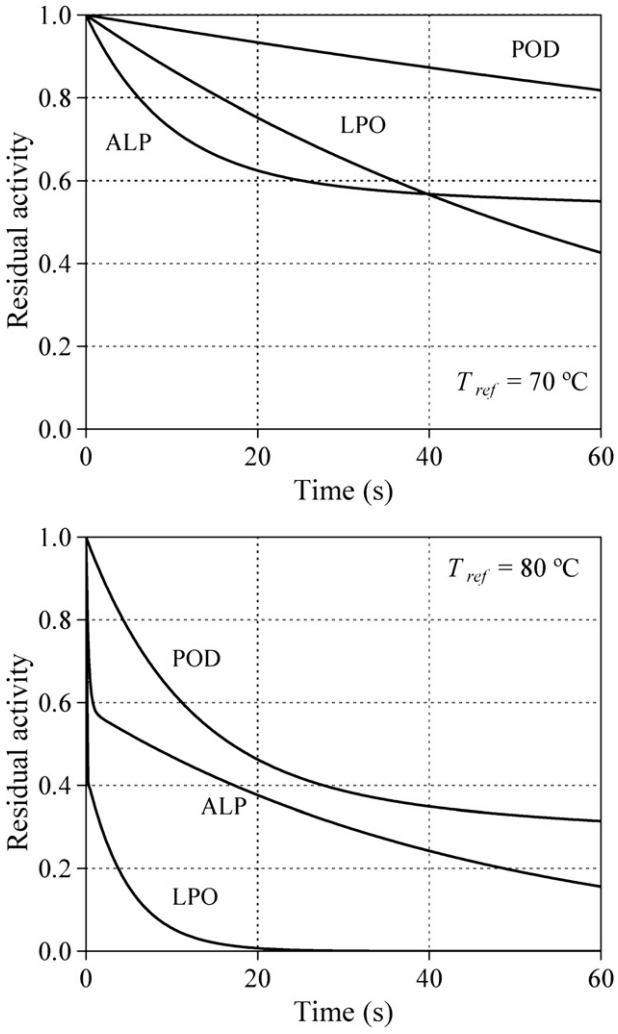

Fig. 3. Inactivation curves of indicators according to the adjusted model at temperatures 70 °C and 80 °C. POD: peroxidase, LPO: lactoperoxidase, ALP: alkaline phosphatase.

Fig. 2 also brings the inactivation curves of the indicators, as predicted by the kinetic model in Eq. (6) for isothermal conditions. It can be seen that the thermostable fraction of POD resists for up to 25 s at 95 °C. On the other hand, the thermolabile fraction of LPO rapidly inactivates at 75 °C. For temperatures above 85 °C, the POD indicator is too stable and losses sensibility to both time and temperature changes, which be disadvantageous for its use. Additionally, LPO is too unstable to be used at temperatures above 80 °C, becoming inactive in just a few seconds. Based on this curves, the thermostable fraction of ALP seems to be a good indicator for over-processing on HTST process; while its thermolabile fraction could indicate under-processing. Moreover, the values of z_1 and z_2 for the heat inactivation of indicator ALP (Table 1) are very close to those of some microorganisms in liquid foods, such as milk (Claeys, Van Loey, & Hendrickx, 2002; Sung & Collins, 1998). Fig. 3 provides a comparison between the thermal resistances of the three indicators at 70 °C and 80 °C.

The costs of the indicators must also be taken into account before they can be used in practice. Based on the initial concentrations proposed in Section 2.1 (which were determined according to the reading range of the reflectometric assay in Section 2.2), the

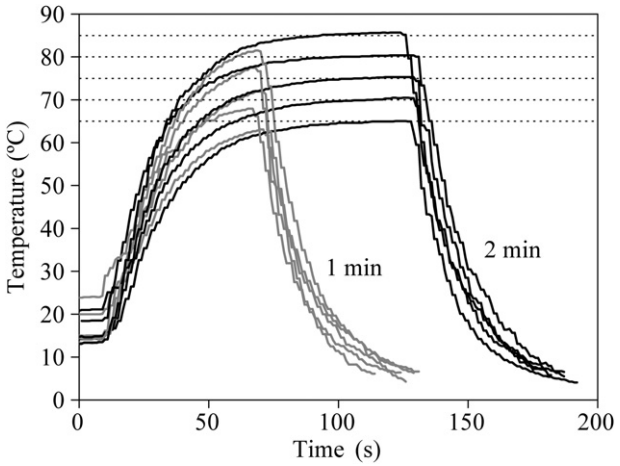

Fig. 4. Time-temperature curves obtained for the alkaline phosphatase indicator (ALP) during the validation tests with slow heating and slow cooling conditions.

specific volumetric costs of the indicators would be, approximately, 40 US$/L for POD, 2200 US$/L for LPO and 0.25 US$/L for ALP. Therefore, the application of indicator LPO would be unjustified; especially considering the large dispersion seen in the parity chart in Fig. 2. Moreover, the low value of z_1 of indicator LPO (Table 1) makes it too sensitive to temperature variations to be used as a good TTI.

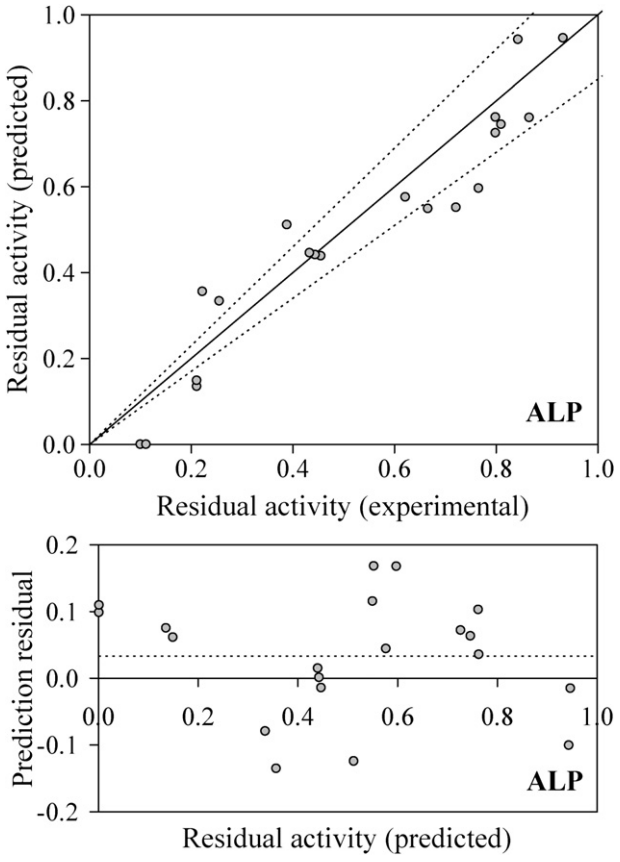

Fig. 5. Results of validation tests for the alkaline phosphatase indicator (ALP) showing the parity chart (experimental value vs. prediction from previously adjusted model) and residual distribution (experimental − predicted).

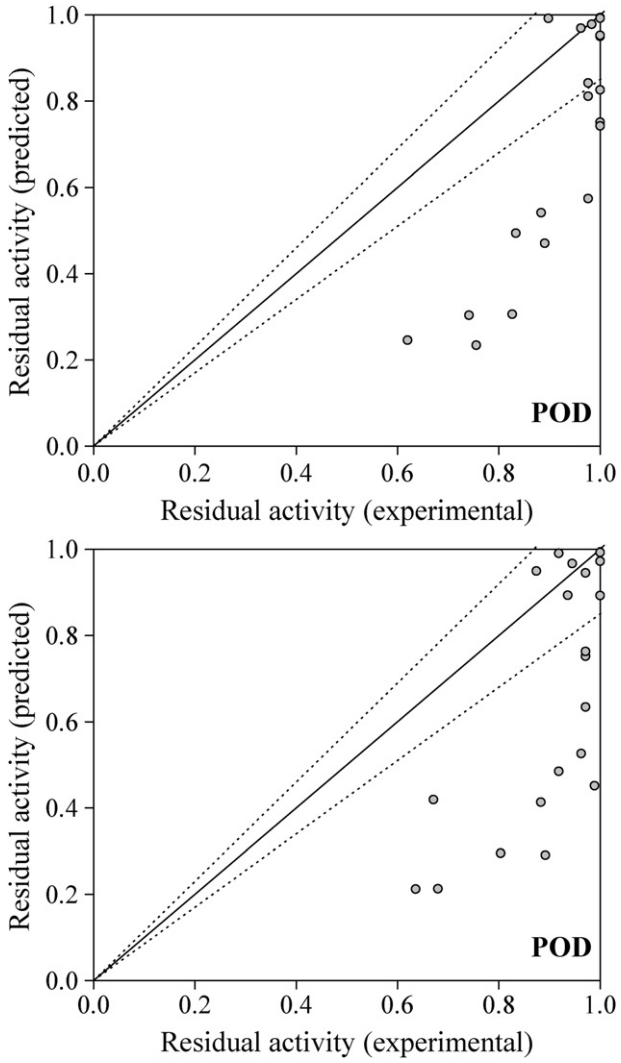

Fig. 6. Results of validation tests for the peroxidase indicator (POD) showing the parity chart (experimental value vs. prediction from previously adjusted model) for two independent sets of experiments using different batches of enzyme.

3.2. Validation tests

Indicators POD and ALP were submitted to a slow discontinuous thermal treatment for validating the adjusted kinetic model, as described in Section 2.5. Fig. 4 presents the time-temperature curves obtained for indicator ALP, which are similar to the curves obtained for indicator POD.

Fig. 5 compares the residual activity predicted by Eq. (6) with the experimental values obtained from the validation tests for indicator ALP. Since the distribution of the points in the parity chart is similar to the distribution seen in Fig. 2 and no large discrepancies can be found in the residuals plot (predicted − measured), it is possible to validate the kinetic model adjusted for ALP.

Indicator POD could not be validated, as can be seen in Fig. 6a. The experimental residual activity was much larger than the predicted activity. A new enzyme batch for prepared and all validation tests were repeated in order to confirm the discrepant behavior, and the same results were obtained (Fig. 6b). The thermal inactivation of POD showed distinct kinetics for fast heating and for slow heating conditions. This suggests that during slow heating, the POD enzyme suffers some sort of activation period. Authors have also detected activation of POD and other enzymes in thermal treatment

(Martin et al., 2005; Polakovič & Vrábel, 1996). Due to this behavior, the indicator POD is not suitable for practical use.

4. Conclusions

From the three tested TTIs, only indicator ALP showed potential to be actually used for the evaluation of continuous thermal processes under pasteurization conditions (70–80 °C). Its main advantages for practical use are: 1) the residual activity of the enzyme can be determined with an easily available commercial kit; 2) the specific cost of the TTI is low (0.25 US$/L); 3) the *z*-values of the thermostable and thermolabile fractions are very close to those of some microorganisms in liquid foods; and 4) this TTI can be considered as a multiple-response TTI because the thermostable and thermolabile fractions are inactivated in different conditions (different *D*-values). To improve the accuracy of the assessment, replicate measurements will be required. Besides continuous HTST processes, this TTI could be encapsulated in small vials to be placed in solid or semi-solid foods in order to evaluate the lethality during discontinuous thermal processing, as long as the process conditions are mild ($T < 90$ °C).

Acknowledgments

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