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Analytical Methods

Temperature model for process impact non-uniformity in genipin recovery by high pressure processing

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ABSTRACT

A model for the process impact temperature non-uniformity during high pressure processing (HPP) of genipap fruit purees was found during genipin recovery. Purees were subjected to HPP (130–530 MPa) under quasi-isobaric non-isothermal conditions (15 min; 0, 4.6 and 9.3 mg pectinases/g fruit). Genipin and protein concentration was determined, and pH was measured. Polygalacturonase activity was quantified indirectly by protein content (mg/g fruit). First order kinetics described temperature changes (0–4 min). Polygalacturonase was activated at 130 MPa, inactivated reversibly at 330 MPa and activated again at 530 MPa. Enzyme reaction rate constant (k) was placed in the 0–4 min model and temperature from 2 to 15 min was described. Protein content and pH characterization in terms of decimal reduction time improved highly the 2–15 min model. Since temperature changes were modeled, more insight of its behavior in an HPP reactor was obtained, avoiding uniformity assumptions, making easier the industrial scale HPP implementation.

1. Introduction

Genipa americana L. is a plant native to northern South America and the Caribbean, southern Mexico (Fernandes & Rodrigues, 2012; Ueda, Iwahashi, & Iokuda, 1991). The fruit is called genipap, but it is also known as jagua, chipara, guayatil, maluco, caruto and huito (Fernandes & Rodrigues, 2012). Genipin is an iridoid cross-linking compound that constitutes *G. americana* (1–3 g/100 g) (Djerassi, Gray, & Kincl, 1960; Ramos-de-la-Peña, 2014). Genipin is able to react spontaneously with primary amine groups of amino acids, peptides or proteins to form dark blue pigments and it can be absorbed in the intestine to act as a genuine choleretic (Akao, Kobashi, & Aburada, 1994). Ramos-de-la-Peña (2014) demonstrated that the recovery of genipin using emerging technologies such as ultrasound combined with enzymatic treatment and HPP enhance the genipin recovery. During HPP, pressure acts uniformly whereas temperature heterogeneities occur due to differences in compression heating and heat transfer (Grauwet, 2010). A high concern in temperature behavior inside an HPP reactor exists, due to its effect on the impact distribution if the kinetics of change of the target attributes are sensitive to non-uniform temperature. To date, there are more than 500 known studies about modeling the enzyme and micro-organisms inactivation kinetics in fruits, vegetables, meats, seafood, dairy and egg products during HPP, but these process models should work at pilot and industrial scale. The insufficient insight of the temperature distribution in an HPP reactor (Knoerzer, Juliano, Gladman, Versteeg, & Fryer, 2007) and the assumption of uniform temperature for the proposed models as iso-thermal conditions that are used in the experiments should be avoided.

Until now, no reports on the development of a first-order model for temperature during HPP have been found. In this paper, the potential of a first-order model for temperature in the pressure transmitting medium during HPP was explored, taking into account the parameters obtained during genipin recovery from genipap fruit purees.

2. Materials and methods

2.1. Materials

Genipap fruits were obtained from Guayacan Export (Bello Horizonte, Managua, Nicaragua). Fruits were collected from wild trees (soil conditions were not controlled) in the South of Nicaragua (Buena Vista, Municipio del Castillo, Departamento de

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Río San Juan) and were stored at room temperature until use. Pectinex Ultra SP-L®, a commercial preparation from *Aspergillus aculeatus* was purchased from Novozymes (Krogshøjvej, Bagsværd, Denmark). Propylene glycol was procured from Dow Chemical Company (Horgen, Switzerland). Albumin from bovine serum ⩾98% and Bradford reagent were procured from Sigma–Aldrich (Diegem, Belgium). All other reagents were analytical grade.

2.2. Genipin recovery by high pressure processing combined with enzymatic treatment

As described by (Ramos-de-la-Peña, Renard, Montañez, Reyes-Vega, & Contreras-Esquivel, 2015), genipap fruit was cut with a stainless steel knife in two parts and the seeds were separated from the fruit, which was cut into cubes of 2 × 2 × 2 cm and 25 g was mixed with 100 mL of distilled water at 10 °C for 1 min in a kitchen mixer. The purees were packed into polyethylene plastic bags of 9 × 16 cm; 0.0, 4.6 and 9.3 mg of Pectinex Ultra SP-L/g of genipap fruit was added to the purees before the plastic bags were sealed. The bags were vacuum-packed (Multivac A300/16, Wolfertschwenden, Germany) and were pressurized to 130, 330 and 530 MPa at a rate of 500 MPa/min through indirect compression. A pilot scale vertically oriented HP equipment was used (Engineered Pressure Systems International, Temse, Belgium). Two sample bags were placed into the pressure vessel. The vessel volume is 0.5 L, the inner vessel diameter is 5 cm and the chamber length is 30 cm. A propylene–glycol mixture (60% Dowcal, Dow Chemical Co., Horgen, Switzerland) was used as a pressure transmitting medium that was injected at the reactor bottom. The pressure was kept constant for 15 min. The temperature of the vessel was controlled by a cryostat and the initial temperature inside the vessel was 10 °C. Temperature was not constant during the pressure treatment due to adiabatic heating. The vessel was decompressed after preset holding time and samples were removed after pressure release. The controls were treated at 0.1 MPa at 10 °C for 15 min in a temperature controlled water bath (Thermo Scientific, Antilles, The Netherlands). Samples were stored in ice immediately after processing. Experiments were performed in quadruplicate. Liquid phase was separated from the solid phase and it was centrifuged at 27,200*g* with JA-20 rotor (Beckman J2-HS Centrifuge, Beckman Coulter, Brea, CA, USA) at 12 °C for 25 min. Liquid phase was separated from the pellet and was stored in polypropylene tubes at −40 °C until further analysis. pH was determined after each HPP treatment at room temperature using a pH meter with a glass electrode (MeterLab PHM210, Radiometer Analytical, Lyon, France). Genipin was quantified according to Ramos-de-la-Peña et al. (2014) and protein content was determined (Bradford, 1976). Analysis of variance was carried out using Minitab, Inc. version 14 (State College, Pennsylvania, USA) software.

2.3. Kinetic modeling and parameter estimation

A kinetic study was developed in the pressure range of 130–530 MPa at initial temperature of 10 °C under quasi – isobaric non-isothermal conditions. Changes in temperature were registered for at least three runs under high pressure processing for 15 min. These temperature values were averaged, and the mean was entered into the modeling procedure. Temperature changes could be modeled by first-order reaction kinetics taking into account the initial part of the graph (0–4 min). Kinetic parameters were performed using a two-step regression approach. The time-dependent change of temperature was modeled. The reaction rate constant k at a given temperature and pressure was determined by plotting the temperature as a function of time.

$$T = T_0 \exp(-kt) \tag{1}$$

where T is the temperature at treatment time t (min) and T_0 the value of the temperature after the cut (time in which the desired pressure is reached), and k the reaction rate constant (1/min). As a measure of the ability of a model to fit all experimental data, a visual inspection of the residuals plot was performed, the corrected R^2 (Eq. (2)) and the model standard deviation (Eq. (3)) were calculated.

$$R^2_{\text{adj}} = 1 - \frac{\left(1 - \frac{\text{SSQ}_{\text{regression}}}{\text{SSQ}_{\text{total}}}\right)(x-1)}{(x-p'-1)} \tag{2}$$

$$\text{RMSE} = \sqrt{\frac{\text{MSQ}_{\text{residuals}}}{x-p'}} \tag{3}$$

pH and protein concentration were characterized in terms of the decimal reduction time D value (decimal reduction time required to reduce the initial activity or concentration by a factor of ten at constant pressure (Tucker, 2001) in min (Eq. (4)) and z (°C) value (Eq. (5)), which is the temperature change required at constant pressure to achieve a tenfold change in D value, by the use of the Thermal Death Time model (Bigelow, 1986).

$$D = \frac{\ln(10)}{k} \tag{4}$$

$$D = D_{\text{ref}} 10^{\frac{T_{\text{ref}} - T}{z}} \tag{5}$$

A first-order model was developed to describe the temperature changes during 2–15 min of pressure treatment. Protein content was found to be an indirect indicator of the enzymatic activity of the main enzyme in Pectinex Ultra SP-L, as protein content showed to be pressure–temperature dependent. The rate constants obtained for protein content (the enzymatic activation rate constants) were manually inserted in the first-order model except for 330 MPa, due to the model was based on enzymatic activation, and enzyme was inactivated at this pressure. As pH has demonstrated to affect D-values on the thermal destruction of spores of *Clostridium sporogenes* (Cameron, Leonard, & Barrett, 1980), and has shown to be highly graphic, a correlation from D-values was found to obtain a factor which was used to be subtracted from the predicted value from the first-order model. The developed model for temperature was found to be according to Eq. (6), where T represents the temperature at treatment time (min) and T_0 the temperature after the cut, respectively, and k_{enz} the enzyme activation rate constant (1/min). D_{refpH} and D_{obspH} are the decimal-reduction time at a constant pressure (min), including the reference and observed values obtained for pH.

$$T = (T_0 \exp(-k_{\text{enz}} t)) - \left(\frac{D_{\text{ref pH}}}{D_{\text{obs pH}}}\right) \tag{6}$$

3. Results and discussion

3.1. Effect of HPP on genipin recovery

Genipin concentration in extracts subjected to HPP was higher as temperature was lower. When temperature was raised, genipin concentration decreased and no further increase was observed. The yield of genipin after HPP was increased by a factor of two when compared to atmospheric pressure treatment (Table 1). The recovery of genipin by HPP could be considered as split-stream processing, which focuses on a specific end-product functionality (Houben et al., 2014), for example, the avoiding of the blue color formation of genipin during its recovery process. Immediately after HPP,

Table 1
Genipin recovery after HPP and enzymatic treatment (mg/g).

Genipin content mg/g			
Pressure (MPa)	Enzyme (mg/g)		
	0	4.6	9.3
0.1	16.2 ± 5.2	16.6 ± 2.2	11.1 ± 1.4
130	34.0 ± 1.5	23.7 ± 3.9	13.7 ± 4.0
330	13.8 ± 1.7	13.0 ± 5.8	16.3 ± 3.8
530	11.7 ± 3.4	2.7 ± 1.4	15.6 ± 6.2

non-cross-linked genipin was obtained in all samples. Absorbances at 589 nm were 0.00 ± 0.00 in all samples, but after 5 days of storage at 4.7 °C, values increased in samples with and without pectinases (Fig. 1a), suggesting the cross-linking of genipin with endogenous proteins. This is in agreement with the statement that minimal changes in fruits and vegetables based products quality occur after HPP, because the partial effects of this technology on covalent bonds of low molecular weight (Bermúdez-Aguirre & Barbosa-Cánovas, 2011), being proteins and genipin able to react after HPP, but not immediately. This was not the case in the genipin recovery by means of ultrasound, where proteins were denatured after ultrasonic treatment and they could not react with genipin (Ramos-de-la-Peña et al., 2014). This suggests that genipin was not altered after HPP, making this technology feasible for its safe recovery, but it is still necessary to implement purification technologies such as microfiltration, ultrafiltration, nanofiltration and reverse osmosis (Ramos-de-la-Peña et al., 2015) after its recovery to avoid its undesirable cross-linking. More studies need to be carried out to evaluate the organoleptic and nutritional properties of the pressurized genipap fruits purees, but it was not the purpose of this study.

3.2. Effect of HPP on protein liberation from genipap fruit

Protein content (Fig. 1b) after mashing and treatment at 0.1, 330 and 530 MPa without pectinases did not show statistical difference ($p < 0.05$). A decrease in protein content in the supernatant after centrifugation was observed when treatment was carried out at 130 MPa, this represents a reduction in proteins about 3.5 times compared to the obtained after 0.1, 330 and 530 MPa pressurizing. Protein content showed a decrease also when pectinases were added to purees subjected to atmospheric pressure. Proteins were reduced ~3 times compared to the control without enzymes, without statistical difference between enzyme amount added at 0.1 MPa. After 330 MPa processing, the protein content was increased in samples with enzymes. After 530 MPa treatment, proteins in genipap fruit extracts were lower than those found after 130 and 330 MPa. Protein content decreased after 130 MPa treatment, increased after 330 MPa and decreased again at 530 MPa when enzymes were added. Protein liberation in treatments at 0.1 MPa is attributed to the mechanical tissue disruption (Martínez-Maqueda, Hernández-Ledesma, Amigo, Miralles, & Gómez-Ruiz, 2013) that led to the highest yield of proteins in genipap fruit extracts. Proteins are contained in bodies in cell walls and the mechanical mashing contributed to their solubilization. The decrease in protein content when pectinases were added at 0.1 MPa could be addressed to the activities endogenous pectinesterase and fungal polygalacturonase, which caused the plant cell wall tissue disintegration, enhanced proteins liberation (Gruben & de Vries, 2009), and a polyelectrolyte complex formation occurred, where proteins were trapped in the complex, showing a reduction in protein content in the supernatant (Ramos-de-la-Peña et al., 2014). After 130 MPa treatments with and without pectinases, the protein content decreased due to the enhancement of the pectinesterase activity of genipap fruit and to the liberation of de-esterified water soluble pectin at 130 MPa that led to higher protein retention in the complex formed (Banjongsinsiri, 2003). After enzyme addition at 130 MPa, no protein content could be found after 9.3 mg/g treatments. This in agreement to the pectinesterase and polygalacturonase activities, that contributed to the cell wall disruption and thus a decrease in proteins in the extract, suggesting that enzymes could be active at a pressure of 130 MPa as

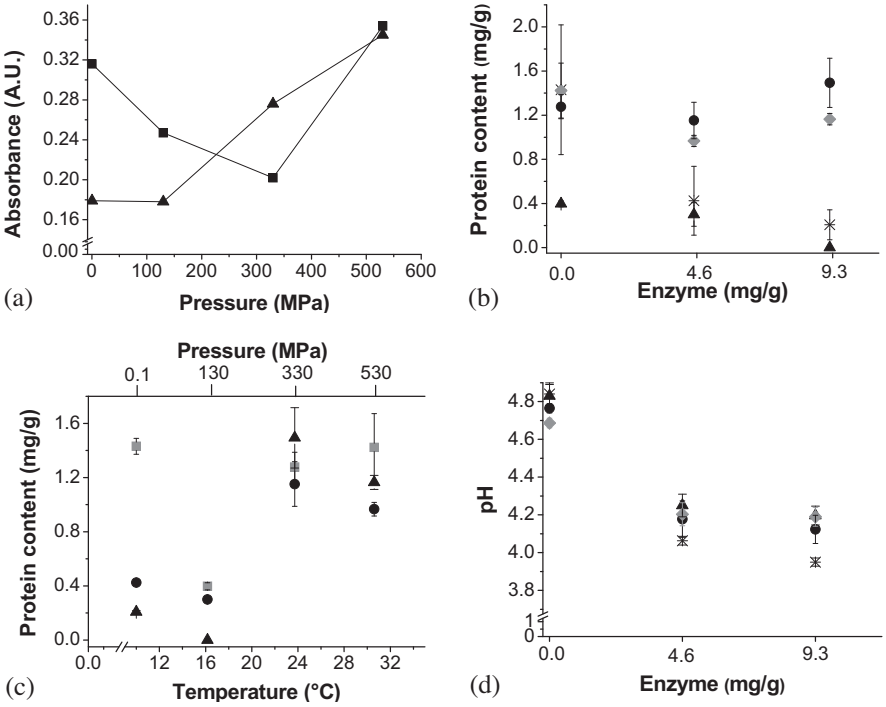

Fig. 1. (a) Effect of pressure and 0.0 (■) and 4.6 (▲) mg pectinases/g fruit on absorbance of genipap fruit extracts after 5 days of storage at 4.7 °C. (b) Protein content and pH (d) in genipap fruit purees after treatments at 0.1 MPa (✳), 130 MPa (▲), 330 MPa (●) and 530 MPa (◆). Effect of pressure, temperature and enzyme added on (c) protein content using 0 mg/g (■), 4.6 mg/g (●) and 9.3 mg/g (▲).

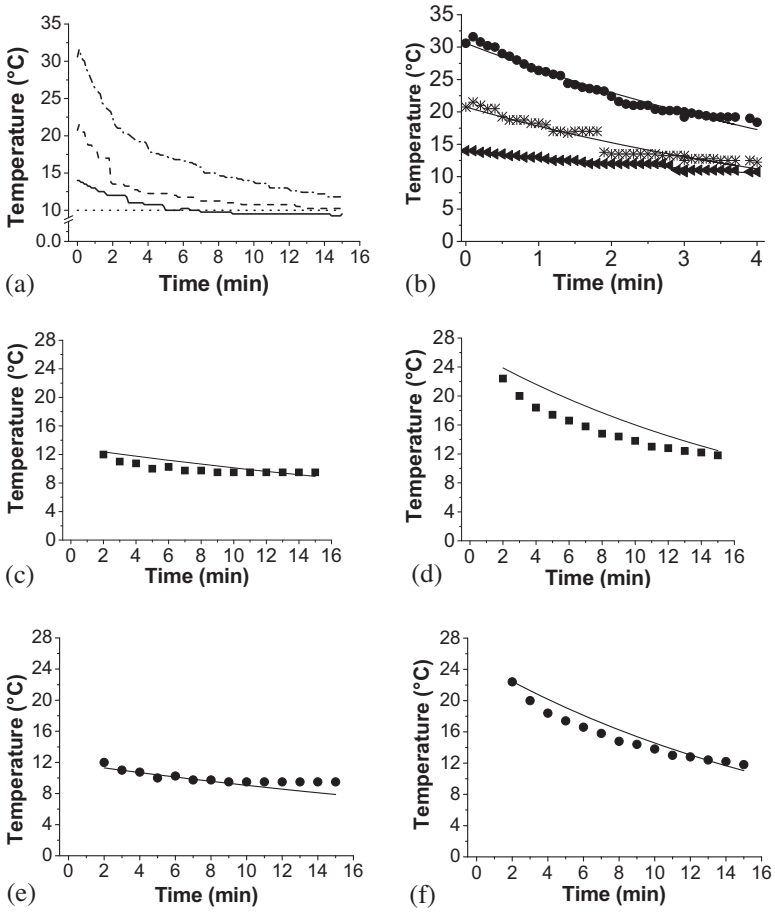

Fig. 2. Temperature profiles recorded during process of genipap fruit puree at (a) 0.1 MPa (. . .) 130 MPa (––), 330 MPa (- -) and 530 MPa (-·-). Temperature during treatments at (b) 130 MPa (◀), 330 MPa (✳) and 530 MPa (●) using the first-order model (0–4 min during holding time); at (c) 130 and (d) 530 MPa using the first order model (■); at (e) 130 and (f) 530 MPa using the improved first order model (●) (2–15 min during holding time). Solid lines represent the first-order model fit.

Table 2
Determined parameters for pH and protein content based on Thermal-Death-Time model for treatment of genipap fruit puree.

	Pressure (MPa)	k (1/min)	D_{ref} (min)	D_{obs} (min)	z (°C)
pH	130	0.011 ± 0.000	229.09 ± 15.08	214.90 ± 14.15	4.05 ± 0.00
	330	0.012 ± 0.000	222.87 ± 5.91	187.38 ± 4.97	8.47 ± 0.02
	530	0.011 ± 0.000	288.14 ± 47.91	200.48 ± 3.34	17.52 ± 0.02
Enzyme	130	0.025 ± 0.001	93.18 ± 0.00	90.75 ± 0.00	3.02 ± 0.01
	330	−0.077 ± 0.009	32.25 ± 4.76	29.76 ± 4.23	8.39 ± 0.02
	530	−0.050 ± 0.003	39.15 ± 2.43	46.04 ± 2.99	15.71 ± 0.01

has been reported for berries when using Pectinex Ultra SP-L (Hilz, Lille, Poutanen, Schols, & Voragen, 2006). After pressurizing at 530 MPa, protein content decreased slightly compared to 330 MPa treatment, but the yield was not lower than the obtained for 0.1 and 130 MPa. Because of these observations, polygalacturonase activity could be quantified indirectly by protein content (mg/g genipap fruit) in the supernatant: the lower the protein content indicates a higher polygalacturonase activity was observed and vice versa. Results suggested that the main enzyme of Pectinex Ultra SP-L (fungal polygalacturonase) could be inactivated after 330 MPa due to denaturation of the protein (enzyme) took place. This was provoked by the disruption of hydrophobic and electrostatic interactions, increasing its hydration, which was caused by the electrostriction of water molecules around charge groups (Wei, 2007). Fungal polygalacturonase showed to be activated at 0.1, 130 and 530 MPa, suggesting that the inactivation of fungal polygalacturonase at 330 MPa was reversible. Serment-Moreno, Barbosa-Cánovas, Torres, and Welti-Chanes (2014) indicated that reversible protein modifications are typically observed between 100 and 300 MPa and fungal polygalacturonase from Pectinex Ultra SP-L showed this behavior at 330 MPa.

3.3. Effect of temperature on protein liberation and pH after HPP

The effect of temperature on protein content was presented in Fig. 1c. Protein content in extracts without enzyme decreased when temperature increased after 130 MPa treatments. After temperature increased, protein content increased ~3 times in supernatants. When 4.6 mg/g of enzymes was added, no statistical difference was observed ($p < 0.05$) when temperature increased from 10.0 ± 0.0 to 14.0 ± 2.9 °C after 0 and 130 MPa processing, and protein content was lower ~3 times compared to the protein amount obtained at 10 °C without enzymes. As temperature increased during 330 and 530 MPa treatments, protein content

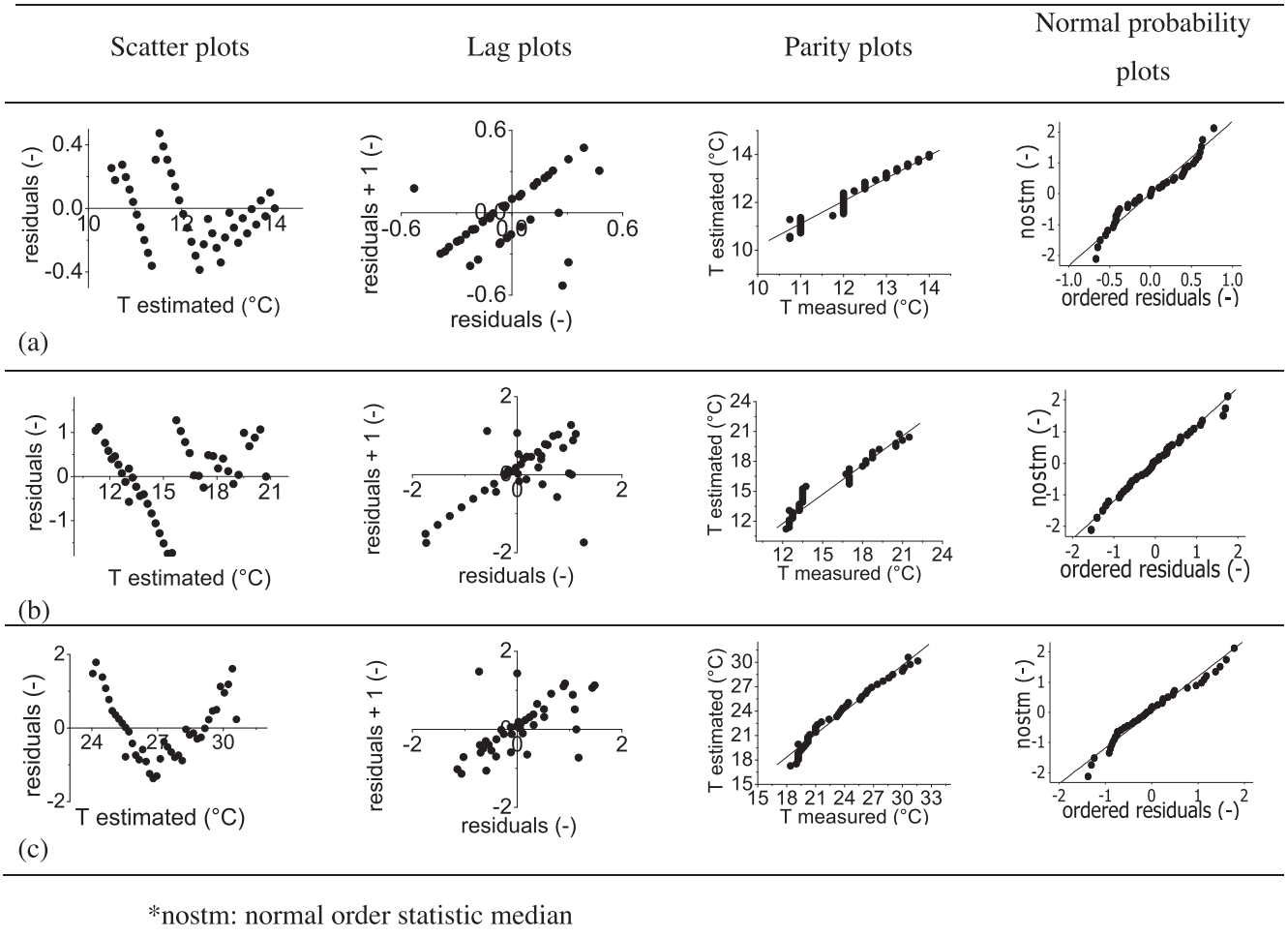

Fig. 3. Scatter plots of residuals versus the predicted values of temperature, lag plots of the residuals versus the residuals, parity plots of the estimated versus the experimental values and normal probability plots at (a) 130 MPa, (b) 330 MPa and (c) 530 MPa (0–4 min during holding time).

did, but it was not higher than the reported for extracts without enzymes. The same pattern was observed in protein amount in extracts with 9.3 mg/g of pectinases. When temperature was 14.00 ± 2.9 °C (after 130 MPa treatment and 9.3 mg/g pectinases), no protein content was registered in extracts, whereas when higher temperatures took place, protein content was increased and then slightly decreased. Protein liberation and pH showed to be pressure–temperature dependent (Fig. 1d). Data here presented fungal polygalacturonase was pressure–temperature dependent, which was similar to plant polygalacturonase from tomato (Fachin et al., 2004; Verlent, Van Loey, Smout, Duvetter, & Hendrickx, 2004), but no report was available for fungal polygalacturonase.

3.4. Modeling the time-dependent temperature

3.4.1. Pressure and temperature profiles

Once reached the pressure indicated, it showed a slight decrease when time treatment, ~10 MPa below the set point. This constant pressure behavior was in agreement with the Pascal principle, where hydrostatic pressurization allows "instant" pressure transmission in fluids and semi-solids within the pressure vessel (Barbosa-Cánovas & Juliano, 2008), contributing to the uniformity in pressure during processing and energy savings.

Temperature increased during high pressure processing as it was shown in Fig. 2a. Initial temperature was 10 °C for all pressure treatments and this value was maintained when atmospheric pressure was applied. When HPP was carried out, temperature showed an immediate increase when pressure treatments were 130, 330 and 530 MPa, respectively. During treatments, temperature showed a gradual decrease, reaching 9.5 ± 0.6, 10.2 ± 0.5 and 11.8 ± 0.8 °C after 15 min processing. Temperature increase was expected due to the adiabatic heat of compression, where the conversion of the work of compression into internal energy caused a temperature increase of compressive materials (Grauwet, 2010). Temperature decreased during high pressure processing due to heat removal by the outer vessel jacket which was maintained at 10 °C.

3.4.2. First-order model for temperature from 0 to 4 min

Fig. 2b showed temperature changes from 0 to 4 min during holding time, the range of time where temperature showed the highest non-uniformity compared to the initial temperature (10 °C). Temperature showed to be pressure dependent, as it was intensified after pressure increase when the impact was carried out. Temperature was set as the target attribute and the time-dependent temperature changes (from 0 to 4 min) were modeled by the first-order model. Parameter estimation using non-linear regression (Motulsky & Ransnas, 1987) was carried out per pressure level, k values were 0.122 ± 0.003, 0.174 ± 0.008 and 0.143 ± 0.005 /min for 130, 330 and 530 MPa, respectively. The rate constant was higher when pressure was increased from 130 to 330 MPa, but it diminished at 530 MPa. The corresponding goodness of fit is expressed by R^2_{adj} (Eq. (2)); 0.95, 0.93 and 0.97

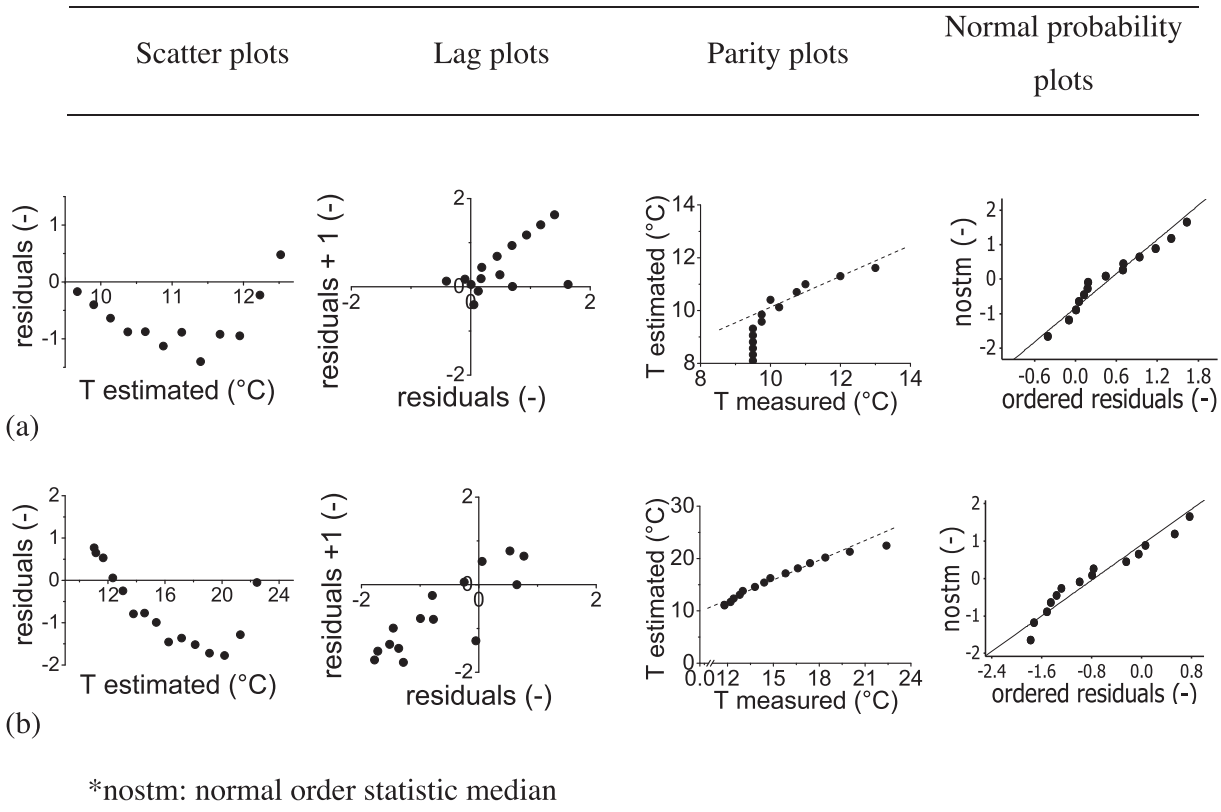

Fig. 4. Scatter plots of the residuals versus the predicted values, lag plots of the residuals versus the residuals, parity plots of the estimated versus the experimental values at (a) 130 and (b) 530 MPa (2–15 min during holding time).

for 130, 330 and 530 MPa, respectively. The RMSE values Eq. (3) varied from 0.035 to 0.120.

3.4.3. First-order model for temperature from 2 to 15 min

Although a first-order model could describe the temperature behavior of the first 4 minutes of HPP of genipap fruit puree, it was not enough to describe the temperature changes in the whole process, which took 15 min to conclude. Since pH and protein content (used to estimate indirectly fungal polygalacturonase activity) were pressure–temperature-dependent, parameter estimation was performed also for these variables per pressure level, and estimated parameters are shown in Table 2. D_{ref} and D_{obs} values in pH and protein content decreased when pressure was increased from 130 to 330 MPa, but increased again when 530 MPa were used for pressurizing. z values of both response variables were quite similar in each pressure level, indicating an evident relationship of these variables with temperature.

As protein content was considered the indirect measurement of fungal polygalacturonase activity, and it was pressure–temperature dependent, the k_{enz} value obtained for each pressure level (Table 2) was placed manually in the first model obtained previously for temperature. Fig. 2c and d showed this new model obtained for temperature at different pressure levels, except for 330 MPa, which caused enzyme inactivation instead activation. $R^2_{adjusted}$ values were 0.91 and 0.96. Despite its good fitting, this last model was not enough to describe the observed change of temperature.

3.4.4. Improved first-order model for temperature from 2 to 15 min

In order to increase the model goodness of fit, a correlation with D was found. Values obtained for D_{ref} were divided by D_{obs} in the pH context, for each pressure level (130 and 530 MPa) and the result was subtracted from the estimated values (Eq. (6)) according to the first-order model for temperature with k_{enz} parameter inserted from enzyme activity) (Fig. 2e and f).

Fig. 3a –c showed the graphical evaluation of the first model for the data obtained (0–4 min). The scatter and lag plots did not show any trend, indicating that first model fits adequately to the data. The parity plots indicated a correlation (R^2) of 0.95, 0.93 and 0.97 for each data set, corresponding to 130, 330 and 530 MPa treatments. In the normal probability plots, the deviations from the bisector observed were ($R^2_{normal\ probability} = 0.99$, 0.97 and 0.99, respectively).

Fig. 4a and b showed the graphical evaluation of the improved first modeling for temperature at 130 and 530 MPa data obtained (2–15 min). The scatter and lag plots did not reveal a specific trend. High correlation between measured and estimated values could be observed in the parity plot (R^2 = 0.95 and 0.99). In the normal probability plot, no significant deviation from the bisector could be detected ($R^2_{normal\ probability} = 0.98$ and 0.97).

Results of models evaluation indicated the models were suitable for temperature during HPP, according to recommendations for evaluation of non-linear regression models (Motulsky & Christopoulos, 2004).

Studies about the process impact on temperature have been carried out, such as the development of a computational model for temperature and sterility distributions in a pilot scale high pressure high temperature process (Knoerzer et al., 2007) and temperature sensitive indicators (pTTI) such as ovomucoid have been used for temperature mapping in a high pressure high temperature reactor (Grauwet et al., 2011); but there are no reports available in literature about a first-order model for temperature during high pressure processing. This presented model could help to the HPP implementation to industry, due to the appropriate data obtained

for temperature, which could lead to the industrial application of the developed models for inactivation of micro-organisms and enzymes.

4. Conclusion

Taking into account the parameters obtained during genipin recovery from genipap fruit purees by HPP, it was possible to establish a relationship between protein content and the main enzyme activity in Pectinex Ultra SP-L, polygalacturonase. Fungal polygalacturonase contained in Pectinex Ultra SP-L was activated at 130 and 530 MPa, but inactivated reversibly at 330 MPa. Based on polygalacturonase activation, a first-order model was obtained to describe accurately the process impact non-uniform temperature changes during treatment of genipap fruit purees at 130 and 530 MPa.

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