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Comparing different methods for estimating kinetic parameters of whey protein heat-induced denaturation in infant milk formulas

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ABSTRACT

Modeling the heat-induced denaturation of milk proteins is a relevant issue because of heating processes in the manufacturing of several dairy products. In this study, four different parameter estimation methods were evaluated to estimate the kinetic parameters of the heat-induced protein denaturation of β-lactoglobulin (β-LG) and lactoferrin (LF) in infant milk formulas (IMF). The methods were: a two-step method, nonlinear least-squares (NLS), one-step linearized, and weighted least-squares (WLS). The WLS was the best alternative, avoiding biases observed when applying other methods besides producing consistently low residuals. Using the second proposed weight function, the WLS produced sum of squared errors and mean absolute percentage errors with average values of respectively 0.18 and 12.3% versus 0.27 and 13.3% considering all methods. However, the WLS requires good initial guesses for parameters, and previous knowledge of sampling and residuals variance. Those can be provided by previously performing the two-step and NLS methods, respectively.

1. Introduction

Modeling, predicting, and controlling the extent of whey protein denaturation is a critical issue to optimize the quality of the dairy products and the performance of the processing unit. Most dairy products are heat processed. Heating takes place immediately after the raw milk is delivered to the dairy companies and/or is a step during the manufacture of dairy products. Aside improving the microbial safety and extending the shelf life of dairy products, heat treatments induce the denaturation of the whey proteins that have positive or negative impacts on the quality of the final products and impair the performance of the processing unit. Heat-induced whey protein denaturation is used to improve yoghurt firmness (Sodini et al., 2004). However, in the manufacture of high protein yoghurts, the heating of the concentrated milk and consequently the extent of whey protein denaturation has to be reduced to achieve the optimal texture of the final gels. Otherwise, the gel is too strong, too pasty and sticky compared to standard yoghurts (Purwanti et al., 2010). Heating cheese milk usually improves cheese yield as more denatured whey proteins and more water are retained in the curd (Kelly et al., 2008). Whey protein retention in the curd also improves the nutrient value and it modifies the functional properties of the cheeses (Hinrichs, 2001). However, if the heating and the extent of whey protein denaturation is too high, the gel time of rennet-induced coagulation is delayed (Vasbinder et al., 2003), and the gel elasticity decreases. In the context of infant milk formula, the extent of whey proteins denaturation affect the physical stability of the liquid formulation during manufacture (Buggy et al., 2017) and modify the nutritional quality of the proteins (Peram et al., 2013; O' Loughlin et al., 2012). Moreover, the denaturation of the whey proteins is mainly responsible for the fouling of the dairy equipment's (Burton, 1968; Fickak et al., 2011 Fickak et al., 2011; Truong et al., 2017).

β-LG is often described as the protein responsible for the above-mentioned effects in dairy products. The heat denaturation of β-LG proceeds along various stages and it is considered irreversible above around 75 °C (Wit, 2009; Brodkorb et al., 2016). Lactoferrin (LF) is one of the most heat-labile whey proteins with a denaturation temperature around 65 °C (Brisson et al., 2007) and it exhibits a wide spectrum of biological properties (Baker and Baker, 2009; Tomita et al., 2009). Thus, this study was focused in the denaturation of β-LG and LF.

The relationship between protein denaturation, heating time and temperature is described by a nonlinear mathematical expression. In such case, linear regression techniques must be extended, which introduces considerable complexity (Bates and Watts, 1988). Fogler (1999) suggests rearranging non-linear equations to obtain linear

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relationships between measured variables and perform a linear regression, then using parameters estimated by the linear regression as initial guesses to perform a nonlinear regression. Other possible approaches are performing a weighted least-squares regression (Shalizi, 2015; DuMouchel and Duncan, 1983) or a general least-squares regression (Mardia et al., 1979).

In some situations, there is no generic formula of one parameter estimation method that overcomes all others. Kohberger (1980) has examined three different methods for evaluating kinetic parameter estimation of an enzymatic reaction described by the Michaelis-Menten equation: a direct linear plot, nonlinear least-squares and weighted least squares. His conclusions reinforce the importance of previous knowledge of the error structure to perform precise estimations, and that the direct linear plot cannot be used as a substitute for the least-squares method when precision is required.

Loveday (2016) has compared estimation of kinetic parameters of β-lactoglobulin (β-LG) denaturation performed in one-step method (estimating the energy of activation, kinetic constant and reaction order simultaneously) to estimation performed in two-step method (estimating kinetic constant for different temperatures in the first step, then estimating the activation energy by the Arrhenius equation). One important issue when estimating kinetic parameters of protein heat-denaturation, is that the kinetic rate constant k_n is dependent on the temperature. Thus, to be estimate k_n the heating temperature must be stationary. Loveday (2016) points the uncertainty as to when stationary temperature is reached, because it takes a finite time that is often unknown, and some protein denaturation may occur during temperature equilibration. Although the system quickly reaches stationary temperature, the progress of protein denaturation while temperature increases can't be ignored, so Van Loey et al. (2002) suggest that to heat a sample then stop heating as soon as stationary temperature is reached and use this time as the time course 'zero point' whose concentration of native proteins is designated C_0. Another approach is to apply mathematical methods, to convert experimental heating times, in the equivalent heating times that, at the stationary temperature T of each series, would lead to the same denaturation level (Halabi et al., 2020a). When performing estimations redefining the "zero point", as suggested by Van Loey et al. (2002), there was no evidence of significant difference in the results. Considering the rapid heating-up time of the protein solutions, some authors consider all experimental points in the fit of the kinetic parameters (Kehoe et al., 2007; Croguennec et al., 2004).

The objective of the current study was to evaluate performance of different parameter estimation methods in estimating kinetic parameters of the heat-induced denaturation of individual whey proteins (β-LG and LF) in different infant milk formula. The methods under study were a two-step method, nonlinear least-squares (NLS), one-step linearized, and weighted least-squares (WLS). In case of the latter, applying two different weight functions.

2. Materials and methods

2.1. Preparation of the IMFs

Three different IMFs were used in this study. The IMFs were formulated in agreement with the European regulation (EU, 2016/127) for the bovine-milk-based IMFs regarding the contents of protein (1.3% w/w), lactose (5.7% w/w) and the principal minerals (Calcium: 42, sodium: 22 potassium: 62, iron: 0.42, chloride: 68, inorganic phosphate: 21 mg/100g of IMF). Skimmed milk (SMP), whey protein isolate (WPI, Prolacta®95, Lactalis Ingredients, Bourgbarré, France), LF (Prodiet Lactoferrin®, Ingredia Dairy Experts, Arras, France) and α-lactalbumin (α-LA, Agropur Inc, Appleton, USA) powders, lactose powder (Armor Proteines, Saint-Brice-en-Coglès, France) and $Na_3C_6H_5O_7{\cdot}2H_2O$ (Carlo Erba Reagents, Val-de-Reuil, France), $Na_3PO_4{\cdot}12H_2O$ (Merck, Darmstadt, Germany), KCl (Panreac, Barcelona, Spain), $CaCl_2{\cdot}2H_2O$ (AnalaR, Leuven, Belgium) and $FeSO_4{\cdot}7H_2O$ (Sigma-Aldrich, St-Louis, USA) salts were used for IMF formulation. Lactose was first dissolved in Milli-Q water and then protein powders were added. After protein dispersion, the solutions were supplemented by minerals, and stirred during 10 min at room temperature. The IMFs were adjusted at pH 6.8 with 1 M KOH and stored overnight at 4 °C to ensure the complete rehydration and powder equilibration. The only difference between the IMFs is that standard IMF contained β-LG (0.51 ± 0.01%) and no added LF. LF1 and LF2 are IMFs that contained the same amount of added LF (0.16 ± 0.01%) but different amount of β-LG (0.33 ± 0.01% and 0.06%, respectively). α-LA powder was used to vary β-LG content in LF1 and LF2.

2.2. Thermal treatment and denaturation kinetics

The IMFs (800 μL) in glass capillary tubes (8 mm inner diameter and 40 mm length, Waters, USA), were heated in a thermally controlled water bath (Fisherbrand™ Isotemp™ water bath, Thermo Fisher Scientific, Newington, USA) set at different temperatures ranging from 67.3 °C to 79.6 °C by intervals of about 2.5 °C. Model IMFs were not heated above 80 °C because the mechanism of β-LG denaturation change leading to a change of b- β-LG activation energy (Hillier et al., 1979; Petit et al., 2011). For each temperature, the heating ramp (i.e. the time to reach the target temperature) was controlled with a YC-747UD Data Logger Thermometer inserted in a reference tube. The heating ramp was converted into a heating time at target temperature (Halabi et al., 2020a). At various time intervals, tubes were removed and instantaneously chilled on ice bath to stop whey protein denaturation/aggregation reactions. The unheated IMFs were used as reference. Three independent experiments were performed for the heat treatment at 75 °C, considering the standard deviation equivalents at all heating temperatures investigated.

2.3. Determination of the contents of β-LG and LF

Native whey proteins were separated from the caseins and denatured/aggregated whey proteins, which precipitate after IMF pH adjustment at 4.6, by centrifugation at 14,000g for 20 min at 25 °C (Eppendorf Microcentrifuge MicroStar 17R, VWR, Leuven, Belgium). The supernatants of the IMFs were recovered for native β-LG and native LF separation by reverse phase-HPLC (Dionex UltiMate 3000 HPLC System, Thermo Fisher, Dreieich, Germany) using a PLRP-S 300 Å, 8 μm, 150 × 3 mm column (Agilent Technologies, UK). Protein were eluted from the column at a flow rate of 0.2 mL/min using a gradient of acetonitrile obtained by mixing the mobile phase A (0.106% (v/v) of trifluoroacetic acid in Milli-Q water) and the mobile phase B (0.1% (v/v) of trifluoroacetic acid in acetonitrile). Native whey proteins in unheated (C_0) and heated samples (C_t) were quantified using a calibration curve established by direct injection of β-LG and LF standards.

2.4. Kinetics of denaturation

The kinetics of milk protein denaturation is usually described by equation (1), which integrated for n $/=$ 1 and rearranged results in equation (2). (Oldfield et al., 1998; Croguennec et al., 2014; Halabi et al., 2020a).

$$-\frac{dC}{dt} = k_n{\cdot}C_t^n \tag{1}$$

$$\frac{C_t}{C_0} = \sqrt[(1-n)]{1 + (n-1){\cdot}k_n{\cdot}C_0^{(n-1)}{\cdot}t} \tag{2}$$

In which C_t is the native protein concentration in time t, C_0 is the native protein concentration before heating, k_n is the reaction rate constant at a defined temperature T_n, and n is the reaction order according to the protein concentration. Units might be arbitrary, but, in the current study, concentration is given in (g.L^{-1}), time in min, k_n in

($g^{(1-n)}.L^{(n-1)}.min^{-1}$), n is dimensionless.

The correlation between the reaction rate constant k_n and given temperature T is given by the Arrhenius relationship given by equation (3), as described by Oldfield et al. (1998).

$$k_n = k_{n0} \cdot e^{\frac{-Ea}{R} \cdot \frac{1}{T}} \tag{3}$$

In which, Ea corresponds to the activation energy, k_{n0} to a pre-exponential constant, R to the ideal gas constant and T to the temperature. Once again, defining units is arbitrary but in the current study Ea is in ($J.mol^{-1}$), k_{n0} is in ($g^{(1-n)}.L^{(n-1)}.min^{-1}$), R is in ($J.mol^{-1}.K^{-1}$), and T is in K.

Substituting equation (3) in equation (2), results in equation (4) that might be rearranged to equation (5) by substituting k_{n0} by k_{n1} of an arbitrary temperature T_1 from equation (3):

$$\frac{C_t}{C_0} = \sqrt[(1-n)]{1 + (n-1) \cdot k_{n0} \cdot e^{\frac{-Ea}{R} \cdot \frac{1}{T}} \cdot C_0^{(n-1)} \cdot t} \tag{4}$$

$$\frac{C_t}{C_0} = \sqrt[(1-n)]{1 + (n-1) \cdot k_{n1} \cdot e^{\frac{Ea}{R}\left(\frac{1}{T_1} - \frac{1}{T}\right)} \cdot C_0^{(n-1)} \cdot t} \tag{5}$$

2.5. Methods for parameter estimation

2.5.1. Two-step method

The two-step method consists of the estimation of the kinetic parameters in two steps: one for estimating kinetic constants for each temperature and another for estimating the activation energy and global kinetic constant (Dannenberg and Kessler, 1988; Petit et al., 2011; Halabi et al., 2020a).

Sometimes, the reaction order is fixed arbitrary to 1, 1.5 or 2 in reference to previous studies (Dannenberg and Kessler, 1988; Galani and Owusu Apenten, 1999) to determine the rate constant. In the two-step method used in the current study, the reaction order n for the denaturation of LF and β-LG at each sample was defined plotting $(C_t/C_0)^{(1-n)}$ versus time for each temperature and retaining the n value in the range 1–2 with the best R^2.

Using the mean of retained values of n, it was determined the kinetic constant k_n for each temperature, by the linear regression slope of $(C_t/C_0)^{(1-n)}$ versus time, according to equation (6).

$$\left(\frac{C_t}{C_0}\right)^{(1-n)} = 1 + (n-1) \cdot k_n \cdot C_0^{(n-1)} \cdot t \tag{6}$$

In the following step, the activation energy Ea and logarithm of the kinetic pre-exponential constant $\ln(k_{n0})$ were determined respectively by the slope and intercept of the linear regression for the logarithm of the kinetic constant k_n (estimated at each temperature) versus the inverse of the heating temperature, described by equation (7)

$$ln(k_n) = ln(k_{n0}) - \frac{Ea}{R} \cdot \frac{1}{T_n} \tag{7}$$

It is remarkable that a linearization is performed in both steps of this method to correlate to dependent variable to the independent variable. Linearization is a technique described by Bates and Watts (1988) that allows to find algebraic solutions to estimate parameters of nonlinear models. It is particularly useful because, since there is an algebraic solution, the method does not demand an initial guess. However, it has the disadvantage of modifying the profile of residuals distribution and so might lead to bad estimations.

2.5.2. Nonlinear least-squares

The nonlinear least-squares (NLS) method was employed to estimate in one step the kinetic parameters activation energy Ea, kinetic constant k_{n1} (defined at an arbitrary $T_1 = 345$ K), and reaction order n, for each protein in each IMF, fitting experimental data do equation (5).

Comparing to the two-step method we must highlight some differences. The NLS method estimates simultaneously (in a single step) the parameters Ea, n and k_{n1}, as performed by Oldfield et al. (1998). Moreover, in the NLS method the dependent variable is nonlinearly related to the independent variables, while each step in the two-step method is based on a linear relationship.

According to Bates and Watts (1988), a nonlinear regression model can be written as by equation (8).

$$y_i = f(\boldsymbol{X}i, \boldsymbol{\theta}) + z \tag{8}$$

In which y_i corresponds to the experimental observation of index i, f to the expectation function of the model, $\boldsymbol{X}_i$ is a vector associated to the independent variables of index i, $\boldsymbol{\theta}$ is a vector of parameters, and z corresponds to residuals of mean 0 and homogeneous variance σ^2.

The NLS regression was performed in Python 3.7 using the *curve_fit* function with least-squares method from *scipy* library (Jones et al., 2001).

As described by Bates and Watts (1988), Fogler (1999), and by Edgar et al. (2001), a nonlinear least-squares regression consists in, starting from an initial guess, applying optimization algorithms to estimate parameters that minimize the sum of squared residuals from a given model, represented in equation (9).

$$SSe = \sum_{i=1}^{no} (y_i - \hat{y}_i)^2 \tag{9}$$

In which, SSe is the sum of squared errors (residuals), i is the index for each observation or estimation, no is the number of experimental points, y_i is the experimental observation of index i, and $\hat{y}_i$ is the y value calculated by the model for the observation of index i, using estimated parameters.

Another possible approach based on the same assumptions would be minimizing the sum of absolute errors (least absolute deviation), but it is a method of difficult convergence as described by Li and Arce (2004).

The parameters errors were calculated according to Bates and Watts (1988) from the standard deviation of each parameter, with assumptions of linearity of the model in the neighborhood of the optimal solution, and residuals of mean 0 and constant variance. The standard deviations were obtained as described by Silveira et al. (2017) and by Bates and Watts (1988).

The errors (residuals) variance σ^2 was calculated by equation (10):

$$\sigma^2 = \frac{SSe}{no - p} \tag{10}$$

In which, p is the number of estimated parameters and no is the number of observation points (experimental points).

The covariance matrix of the parameters $\mathbf{V}(\boldsymbol{\theta})$ (as returned by the *curve_fit* function), can be calculated by equation (11).

$$\mathbf{V}(\boldsymbol{\theta}) = \sigma^2 \cdot \left(\boldsymbol{J}^T \cdot \boldsymbol{J}\right)^{-1} \tag{11}$$

In which, $\boldsymbol{J}$ is a matrix of shape (no, p) with the partial derivatives of each $\hat{y}$ of index i, to each parameter $\boldsymbol{\theta}$ of index j, according to equation (12).

$$\boldsymbol{J}_{ij} = \frac{\partial \hat{y}_i}{\partial \boldsymbol{\theta}_j} \tag{12}$$

In which, i is in range from 1 to no, and j in range from 1 to p.

The standard deviation for each parameter $s(\boldsymbol{\theta}_j)$ is given by equation (13).

$$s(\boldsymbol{\theta}_j) = \sqrt[2]{\mathbf{V}(\boldsymbol{\theta})_{jj}} \tag{13}$$

At last, the error associated to each parameter was obtained by the product of $s(\boldsymbol{\theta}_j)$ and *t-value* considering a valid t-distribution.

For a matter of comparison to the parameters estimated by the two-step method, it was calculated the natural logarithm of the pre-exponential constant k_{n0}, which was calculated substituting k_n by k_1

and T by T_1 at equation (7).

To calculate errors for $\ln(k_{n0})$, the same methods applied to Ea, k_{n1}, and n, were performed. This time, in a model considering Ea, $\ln(k_{n0})$, and n, according to equation (14).

$$\frac{Ct}{C_0} = \sqrt[(1-n)]{1 + (n-1)\cdot e^{ln(k_{n0})}\cdot e^{\frac{-Ea}{R\cdot T}}\cdot C_0{}^{(n-1)}\cdot t} \tag{14}$$

As expected, the estimates for Ea and n and their respective confidence intervals remain the same, and the only additional information after the new estimation in the covariance matrix $\mathbf{V}(\boldsymbol{\theta})$ is the value associated to $\ln(k_{n0})$, used to calculate its confidence interval.

2.5.3. One-step linearized

To distinguish effects of the data linearization from effects of performing estimation in one or two steps, it was developed a one-step method applying nonlinear optimization algorithms to estimate kinetic parameters Ea, k_{n1}, and n that would best fit the linearized kinetic equation (15), in which the dependent variable is linear in respect to time, but not to temperature. In this study this method is referred to as "one-step linearized method" or just "linearized method".

Both the two-step method and the one-step linearized method consider the dependent variable as being linear versus time, while they differ in estimating simultaneously the kinetic parameters Ea, k_{n0}, and n or estimating in two-steps. Hence, comparing results of the two methods it is possible to assess the difference in performing estimations in one or two steps. Moreover, comparing the one-step linearized method to the NLS method it is possible to assess effects of the linearization with respect to time (both estimations being performed in one step).

$$\left(\frac{C_t}{C_0}\right)^{(1-n)} = 1 + (n-1)\cdot k_{n1}\cdot e^{\frac{Ea}{R}\cdot\left(\frac{1}{T_1}-\frac{1}{T}\right)}\cdot C_0^{(n-1)}\cdot t \tag{15}$$

Although being linear according to time, the linearized method presented in this study was applied to a dataset considering different temperatures, and since the dependent variable has a nonlinear relationship with temperature, there isn't algebraic solution and so it isn't possible to apply standard linear regression methods.

Once again, the solution was obtained using Python 3.7, but this time applying the *minimize* function using nonlinear default optimization algorithms from *scipy.optimize* module (Jones et al., 2001). In this method, the objective function was maximizing the R^2 (minimizing $-R^2$), described by equation (16), in which y is described by equation (17).

$$R^2 = 1 - \frac{\sum_{i=1}^{no}(y_i - \widehat{y}_i)^2}{\sum_{i=1}^{no}(y_i - \overline{y}_i)^2} \tag{16}$$

$$y = \left(\frac{C_t}{C_0}\right)^{(1-n)} \tag{17}$$

It is important to remark that since it is a nonlinear problem, R^2 can't be interpreted as it is in a multivariable linear regression method, where it means variance described by the model over total variance Montgomery and Runger (2003).

The parameters errors were calculated the same way as using the nonlinear least-squares method, and so keeping assumptions of residuals with mean = 0 and constant variance σ^2.

2.5.4. Weighted least-squares

The weighted least-squares (WLS) is an alternative to unweighted nonlinear least-squares method for reducing a possible bias caused by the sampling scheme (DuMouchel and Duncan, 1983). Shalizi (2015) suggests weighting residuals according to the inverse of the local variance in order to minimize the parameters variance. Besides, by attributing higher weights for residuals where there is less variance, the model is more likely to match observations where there is less noise and so where observations are more reliable.

The WLS method takes as objective function minimizing the weighted sum of squared errors, described by equation (18).

$$SSe_w = \sum_{i=1}^{no} w_i\cdot(y_i - \widehat{y}_i)^2 \tag{18}$$

Kohberger (1980) uses the inverse of the predicted values squared as weights. Campos et al. (2018) use the inverse of the observation values squared as weights. In this study, weights were proposed by equations (19) and (20).

$$w_i = \frac{1}{y_i\cdot\widehat{y}_i} \tag{19}$$

$$w_i = \frac{(t_i + 0.25\cdot\overline{t(T)})}{s(t(T))}\cdot\frac{no}{no(T)\cdot len(Temps)} \tag{20}$$

In which w_i is the weight for observation of index i, t_i is the time of observation of index i, $\overline{t(T)}$ is the average time of heating at temperature T, $s(t(T))$ is the standard deviation of the times of observations at temperature T, no is the total number of observations, $no(T)$ is the number of observations at the given temperature, and $len(Temps)$ is the number of unique temperatures evaluated in the experimental dataset.

The weights of equation (19) were chosen to avoid remarkably high relative residuals at low concentrations. The rate of denaturation is lower at lower concentrations, because of the first derivative (equation (1)). So, it is expected that the concentration is more stable at lower values and there's lower local variance. It is important to remark that the WLS regression with this weights function is of easier convergence than using the inverse of squared model values, described by Kohberger (1980).

Weights of equation (20) were chosen after analyzing the samples distribution to avoid sampling bias of having more observations at initial times than at ending times and of having more observations at some temperatures.

To perform the WLS, experimental data was fitted to equation (5), applying *least_squares* function from *scipy.optimize* module (Jones et al., 2001), to minimize the output of a weighted squared residuals function (equation (18)).

The parameters variance matrix was calculated by modifying equation (21) for weighted linear regression (DuMouchel and Duncan, 1983).

$$\mathbf{V}(\boldsymbol{\beta}_w) = (\boldsymbol{X}^t\cdot\boldsymbol{W}\cdot\boldsymbol{X})^{-1}\cdot(\boldsymbol{X}^t\cdot\boldsymbol{W}^2\cdot\boldsymbol{X})\cdot(\boldsymbol{X}^t\cdot\boldsymbol{W}\cdot\boldsymbol{X})^{-1} \tag{21}$$

Substituting $\boldsymbol{X}$ by $\boldsymbol{J}$ in analogy to Bates and Watts (1988) to transform from linear to nonlinear regression, it results in equation (22).

$$\mathbf{V}(\boldsymbol{\theta}w) = (\boldsymbol{J}^t\cdot\boldsymbol{W}\cdot\boldsymbol{J})^{-1}\cdot(\boldsymbol{J}^t\cdot\boldsymbol{W}^2\cdot\boldsymbol{J})\cdot(\boldsymbol{J}^t\cdot\boldsymbol{W}\cdot\boldsymbol{J})^{-1} \tag{22}$$

In which, instead of using $\boldsymbol{\beta}_w$, it was used $\boldsymbol{\theta}_w$ as the vector of the estimated parameters. $\boldsymbol{W}$ corresponds to the weight matrix, and $\boldsymbol{J}$ to the matrix of partial derivatives of the model to each parameter.

As to the NLS method, parameters errors were calculated as a product of *t-value* and the standard deviation calculated for each parameter from the diagonal of the variance matrix.

2.6. Methods of evaluation

2.6.1. Statistical

The methods for parameter estimation Ea, n and $\ln(k_{n0})$ were evaluated for each protein at each sample by the sum of squared errors from equation (9), and by adjusted mean absolute percentage errors (MAPE), given by equation (23), described by Armstrong (1985).

$$\overline{MAPE} = \frac{1}{no}\sum_{i=1}^{no}\frac{2\cdot|y_i - \widehat{y}_i|}{(y_i + \widehat{y}_i)} \tag{23}$$

2.6.2. Graphical

Each method was evaluated by graph analysis, plotting C/C_0 versus

Table 1
Kinetic parameters and statistical indicators for estimations performed by different methods.

Protein	IMF	Method	Ea (x10^3)	n	k1 (x10^2)	ln(kn0)	SSe (x10^2)	MAPE
β-LG	Std	NLS	326±7	1.81±0.06	0.71±0.05	108±5	4	5.5%
		linearized	346±11	1.61±0.1	0.76±0.09	116±7	11	6.1%
		WLS1	339±15	1.67±0.15	0.77±0.11	113±13	5.9	5.1%
		WLS2	331±8	1.73±0.08	0.75±0.07	110±6	4.4	4.9%
		two-step	337	1.6	0.79	112	8.9	5.8%
	LF1	NLS	390±18	2.03±0.14	2.56±0.29	132±12	11.7	14.6%
		linearized	412±28	1.5±0.21	2.63±0.45	140±19	36.4	14.1%
		WLS1	408±57	1.56±0.43	2.82±0.55	138±49	24.6	12.8%
		WLS2	396±24	1.8±0.19	2.82±0.36	134±16	13.5	12.2%
		two-step	416	1.6	2.79	141	22.5	12.9%
LF	LF1	NLS	179±19	2.2±0.17	11.78±0.98	60±13	32.6	27.0%
		linearized	189±25	1.53±0.24	7.29±0.78	63±17	91.7	18.3%
		WLS1	180±99	1.6±0.66	8.76±4.63	60±83	59.5	16.3%
		WLS2	177±23	1.9±0.21	10.99±0.99	59±16	36.4	18.4%
		two-step	195	1.65	8.7	65	57.5	16.8%
	LF2	NLS	220±11	1.6±0.12	6.9±0.38	74±8	17	14.3%
		linearized	197±12	1.6±0.13	7.48±0.46	66±8	20.2	13.6%
		WLS1	200±55	1.48±0.7	6.73±3.88	67±68	19.9	14.9%
		WLS2	215±14	1.57±0.17	6.68±0.47	72±9	17.6	13.8%
		two-step	212	1.77	9.2	71	36	18.2%

time for each heating temperature for each protein at each sample.

By equation (5), it is possible to infer that the shape of the model curves is related to the estimation of *n*, while the general trend of the curve is related to the kinetic constant, and the accuracy of fitting different temperatures is related to *Ea*. Underestimations of *n* lead to model curvatures less accentuated than experimental points while overestimations of *n* lead to model curvatures more accentuated than experimental points, perhaps intercepting them. Bad estimations of the

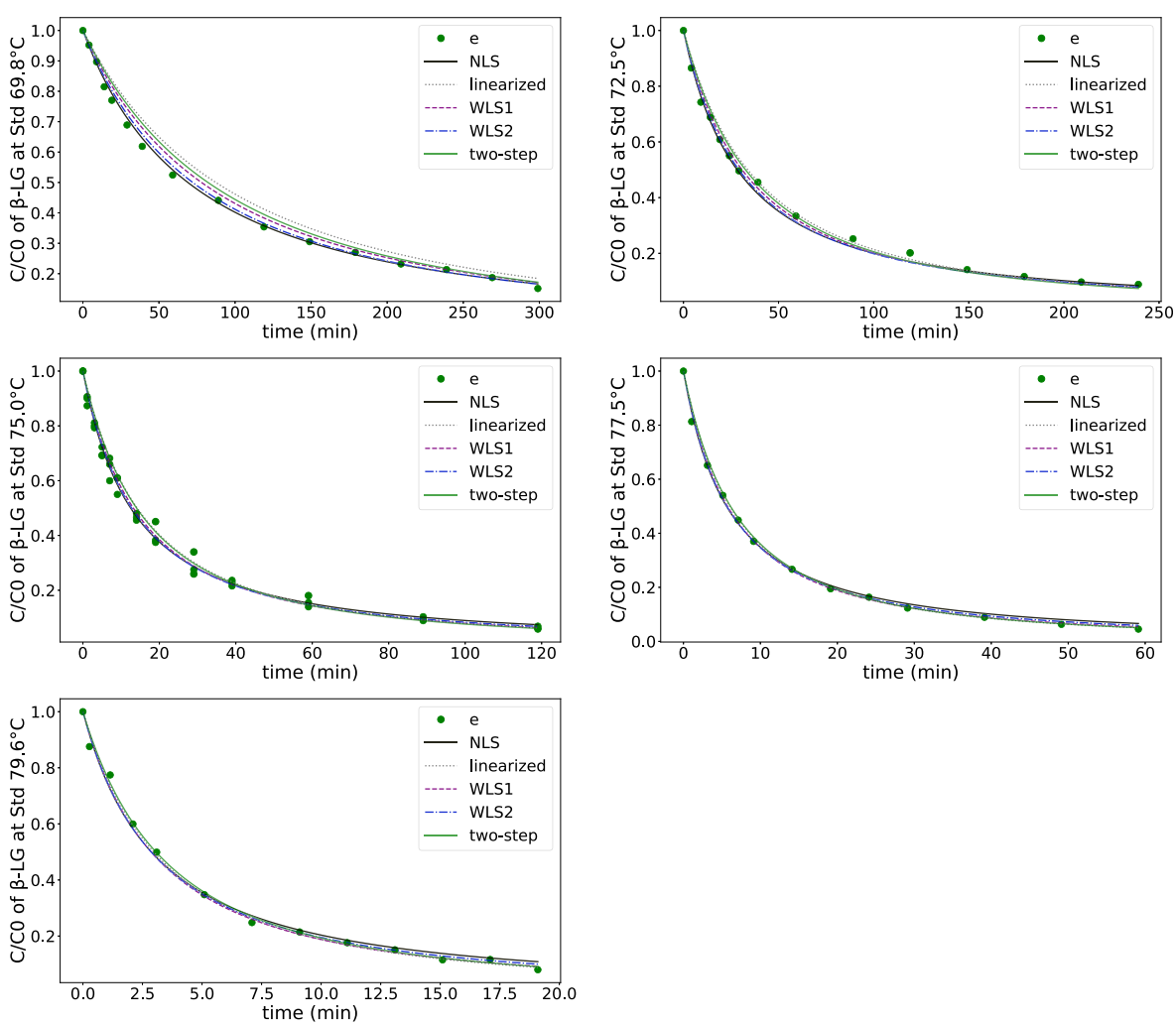

Fig. 1. Experimental points and kinetic model curves for concentration ratio versus time using estimations obtained by different methods for β-LG at the standard sample.

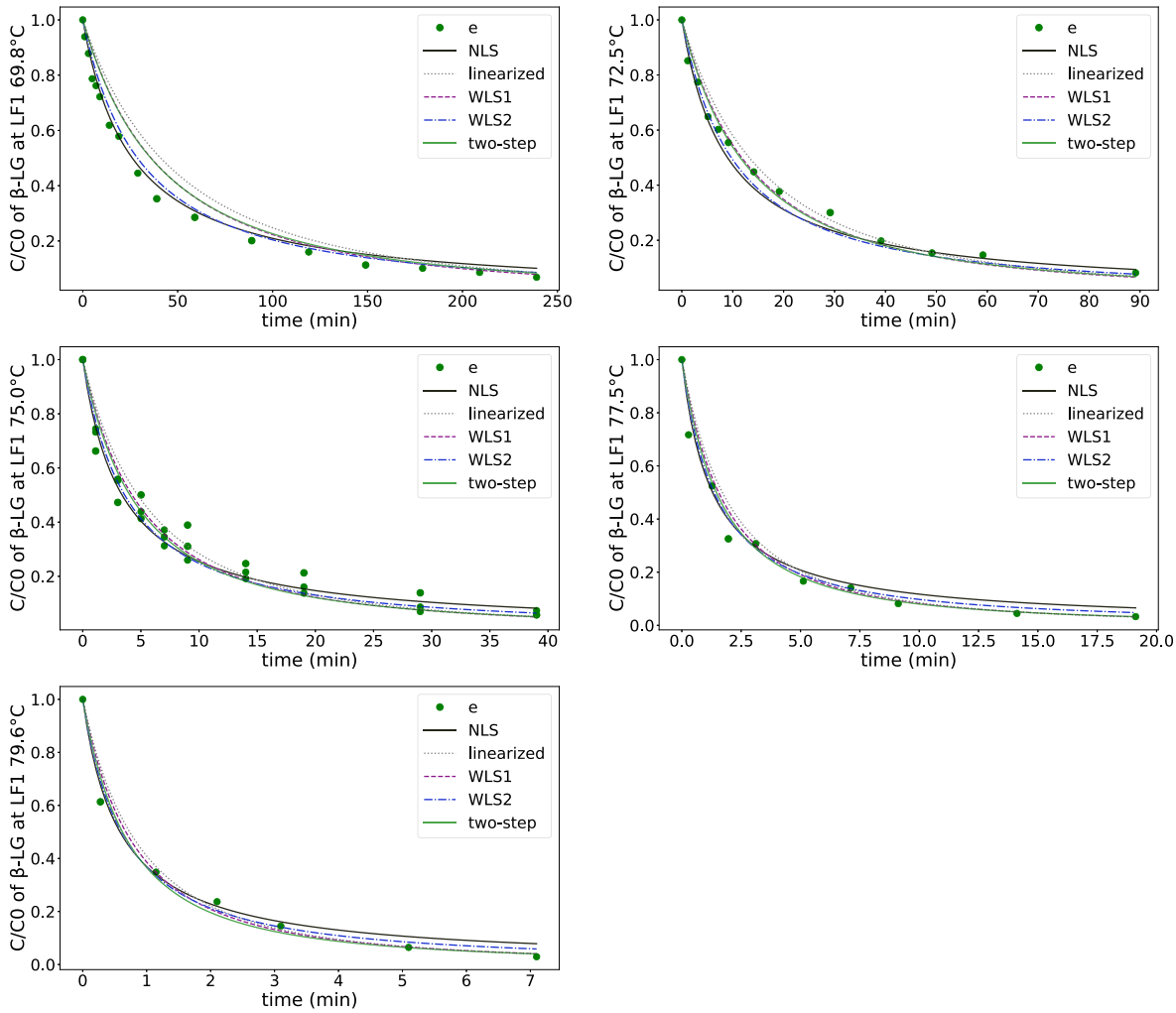

Fig. 2. Experimental points and kinetic model curves for concentration ratio versus heating time using estimations obtained by different methods for β-LG at LF1 sample.

kinetic constant will probably affect the general trend of the curve. At last, bad estimations of *Ea* lead the curve not to fit well all temperatures, but also if the Arrhenius equation can't be extended to some temperature interval, it is expected to have a bad fit for estimations at temperatures beyond the limits.

According to Bates and Watts (1988), before characterizing the precision of estimates using confidence intervals, one must check the residuals for signs of model inadequacy. Edgar et al. (2001) suggest plotting residuals versus the dependent variable and versus independent variables to check detection of outliers, trends in the residuals, abrupt shifts, and changes in error variance. According to Montgomery and Runger (2003), plotting residuals versus the dependent and independent variables is also a way to check the accuracy of the model, verifying if modeling assumptions are valid, as mean = 0 and constant variance. Shalizi (2015) suggests plotting residuals and squared residuals in order to determine an equation to describe weights as the inverse of the local variance.

A graphical analysis was performed in this study by plotting residuals multiplied by the square root of the weight function versus time and versus C/C_0, for each protein at each sample. Multiplying the residuals by the square root of the weight function represents how the local residuals impact the objective function of each method, and so to what interval each model tends to better adjust.

3. Results

Applying the methods described above it was possible to estimate the kinetic parameters of the denaturation of β-LG and LF in different IMFs (Table 1). The parameters *Ea* (J.mol^{-1}), k_1 (g$^{(1-n)}$.L$^{(n-1)}$.min^{-1}), and *n*, correspond to kinetic parameters from equation (5). The arbitrary temperature of k_1 was defined as 345K. The parameter k_{n0} (g$^{(1-n)}$.L$^{(n-1)}$.min^{-1}), corresponds to the kinetic pre-exponential term from equation (4).

The procedures for parameter estimation were performed in the temperature range from 67.3 °C to 79.6 °C for LF, while for β-LG the data obtained at 67.3 °C at both samples was not considered by the model because of evidence of poor adjustments at this temperature. Poor adjustments for β-LG at 67.3 °C are consequence of the very slow denaturation of the protein and the possible interference of concomitant reactions such as protein lactosylation through Maillard reactions that prevented the precise quantification of the native protein at longer heating times. To distinguish the two different weight functions used in the WLS method they are referred as WLS1 and WLS2.

For all proteins at all samples, the NLS method produced the lowest values in terms of *SSe*, as it would be expected once it is the objective function of the method, with mean 0.16. It was followed by the WLS method using the second weight function, that resulted *SSe* with mean 0.18. This result was significantly better than using the first weight function, that produced *SSe* with mean 0.27. The linearized method

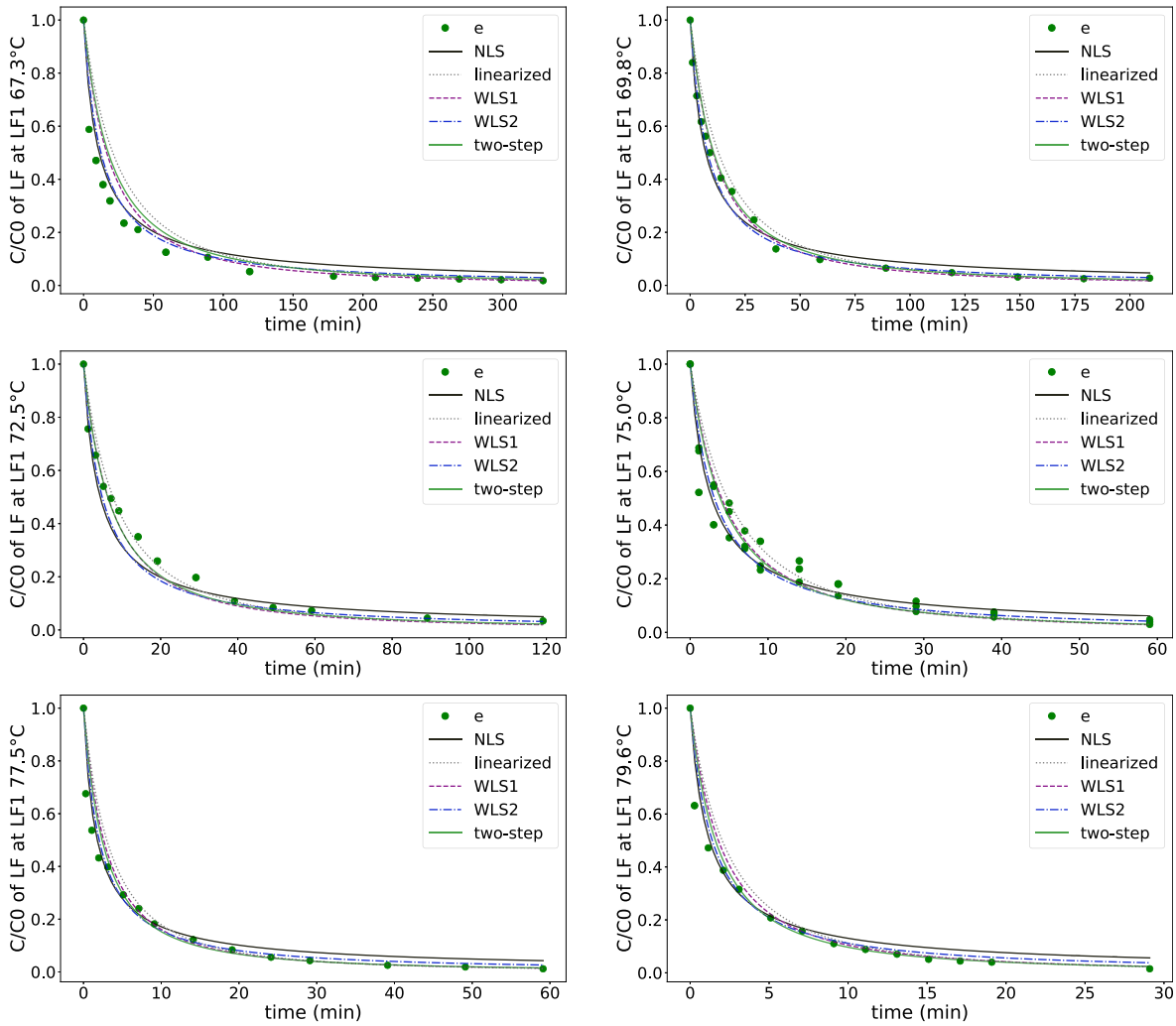

Fig. 3. Experimental points and kinetic model curves for concentration ratio versus heating time using estimations obtained by different methods for LF at LF1 sample.

performed in one step usually led to the worst estimations in terms of *SSe* with mean 0.40, except for LF at LF2, in which the two-step method resulted in higher values. The mean *SSe* considering all methods was 0.27. In terms of adjusted MAPE, results obtained by all methods were quite similar for each protein at each IMF sample. Exceptions were observed for LF at the LF1 sample, in which the NLS method led to significantly higher values, and for LF at the LF2 sample, in which the two-step method led to significantly higher values. Thus, the mean value of adjusted MAPE for the NLS was the highest (15.4%), followed by the two-step method (13.4%). The best results in terms of adjusted MAPE were obtained by WLS1 and WLS2 with mean values of 12.3% for both. The mean adjusted MAPE considering all methods was 13.3%.

Whatever the method, the residuals were the lowest for β-LG at the standard sample, while quite similar for others. It reinforces the reliability of experimental data for β-LG at the standard sample.

Besides analyzing the residuals numerically, it was performed a graphical evaluation. Figs. 1–4 show the results of different methods in modeling the denaturation of β-LG at the standard sample, β-LG at the LF1 sample, LF at the LF1 sample and LF at the LF2 sample, respectively.

Fig. 1 reinforces the reliability of results obtained in Table 1, once the model curves were precise in fitting experimental points. However, linearized, two-step and WLS1 had slightly worse results at 67.3 °C, in which it is possible to observe a bias of the model curve being above experimental points.

At Figs. 2 and 3, it is possible to notice more significant differences between estimation methods, perhaps because of the magnitude of experimental errors. Differences in terms of *n* observed in Table 1 are reinforced, as the model curves differ in shape, specially obtained using the NLS method, in which there is a remarkable bias of assuming values strictly higher than experimental points for longer holding times (or lower concentrations).

When analyzing the shape of the curves using parameters estimated by the linearized method from Figs. 2 and 3 it is possible to notice that the magnitude of residuals is significantly lower for lower concentrations. This can lead to poor fittings at initial holding times, what was specially noticed for lower temperatures. Compared to the two-step method, there was no evidence of improvement, despite what is suggested by Loveday (2016).

At Fig. 4 it is possible to notice a significantly worse performance of the two-step method compared to others including the linearized one-step method, reinforcing conclusions from analyzing residuals at Table 1. However, this was not a behavior observed at other series.

Comparing the model curves obtained by the two different weight functions WLS1 was more effective in describing the denaturation of β-LG at the standard (Fig. 1) and LF1 (Fig. 2) samples at higher temperatures while WLS2 was more effective in describing it at lower ones. Moreover, the WLS1 was more effective in describing the denaturation of LF at the LF1 sample (Fig. 3) while WLS2 was more effective in describing of LF at LF2 sample (Fig. 4).

Weighted residuals for LF at LF1 sample were of significant relevance

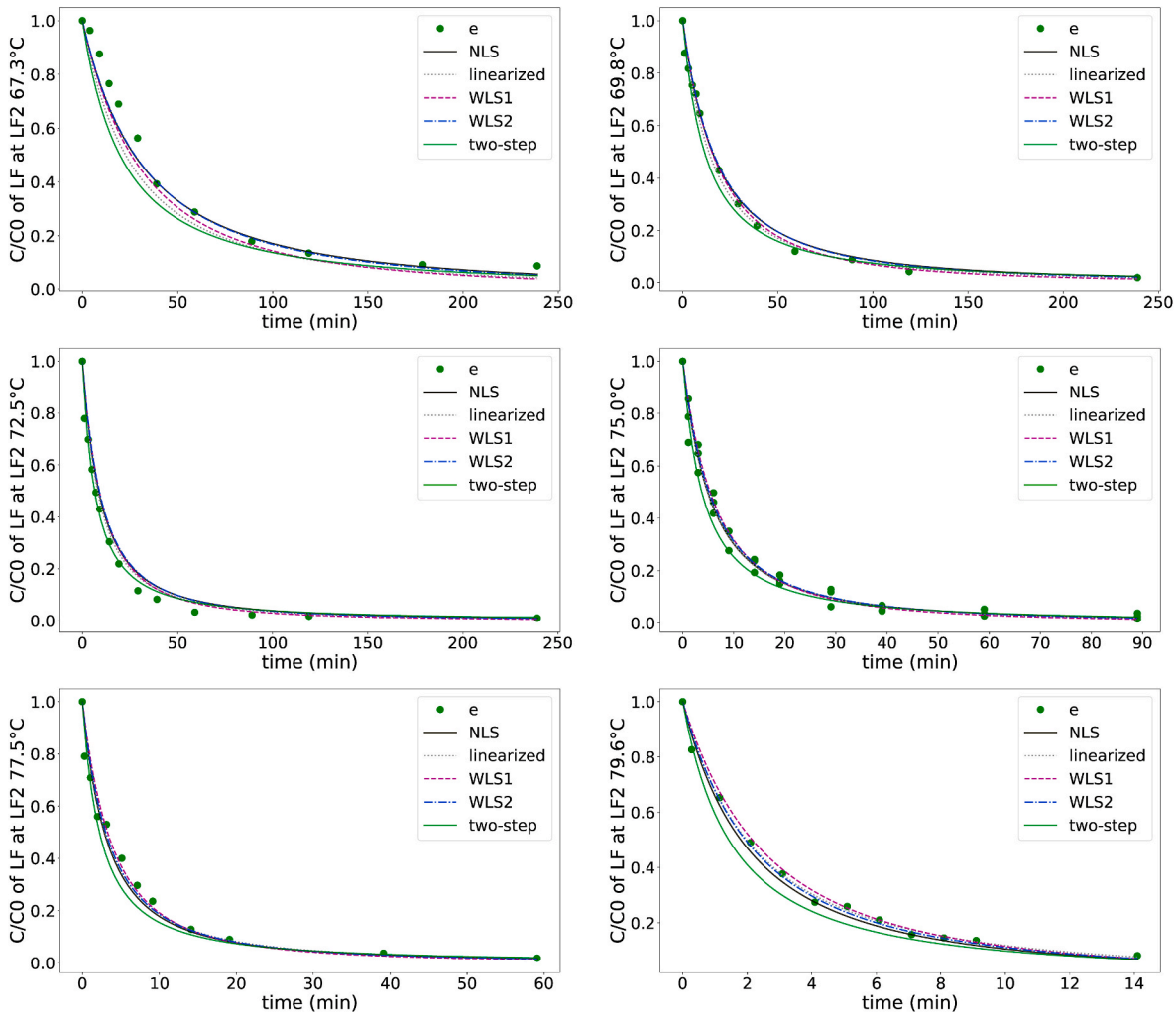

Fig. 4. Experimental points and kinetic model curves for concentration ratio versus heating time using estimations obtained by different methods for LF at LF2 sample.

to compare methods performances and validate model's assumptions. They can be analyzed by Fig. 5.

It is evident that the residuals variance is not constant neither in respect of time nor concentration for any method. It is a remarkable point specially about the NLS method because residuals constant variance is one of the method assumptions. The assumption of residuals with mean 0 also is not valid, once NLS led to residuals not equally distributed between positive and negative values, with bias of negative values at low concentrations (or long holding times) for the NLS method. This bias is reduced by both weight functions, specially the first.

4. Discussion

4.1. Methods performances

By analyzing *SSe*, adjusted MAPE (Table 1) and by graphical analysis, it is observed that the two-step method resulted in accurate estimations for kinetic parameters. While other methods need accurate initial estimations in order to converge, both steps from the two-step method have analytical solution, so it can be a powerful strategy to perform initial estimations.

Estimations using the two-step method were especially accurate for LF at LF1 sample, while there was lack of accuracy for LF at LF2 sample, as its possible to notice by analyzing Fig. 3 and adjusted MAPE (Table 1), For both linearized and two-step methods the most significant distancing from experimental points was noticed at lower temperatures, especially for LF.

Compared to the two-step method, there was no evidence of consistent improvement performing estimations by the one-step linearized method, despite what is suggested by Loveday (2016). However, there was a consistent improvement in terms of residuals when performing the NLS method. This might evidence that the data linearization versus time can have a more negative impact on the accuracy of estimations than performing in two steps instead of one.

Despite the positive effect of minimizing *SSe* using the NLS method, there was evidence of poor estimations in terms of n and of biased residuals distribution proving the assumptions of residuals' constant variance and constant mean = 0 not to be valid. This might be consequence of sampling and/or of variance bias. However, the WLS method proved to be a good alternative to improve estimations reducing model curves biases observed when using the NLS method. By analyzing residuals (Fig. 5), we can conclude that both weight functions were effective in producing more homeostatic weighted residuals compared to unweighted, what can be a source of reducing bias in parameter estimations.

Comparing the two weight functions in terms of *SSe* and adjusted MAPE, the second produced more consistent results. Besides, as observed in Table 1, the confidence intervals for parameters estimated using WLS1 were much broader than when estimated using WLS, what evidences that the first weight function is of harder convergence to

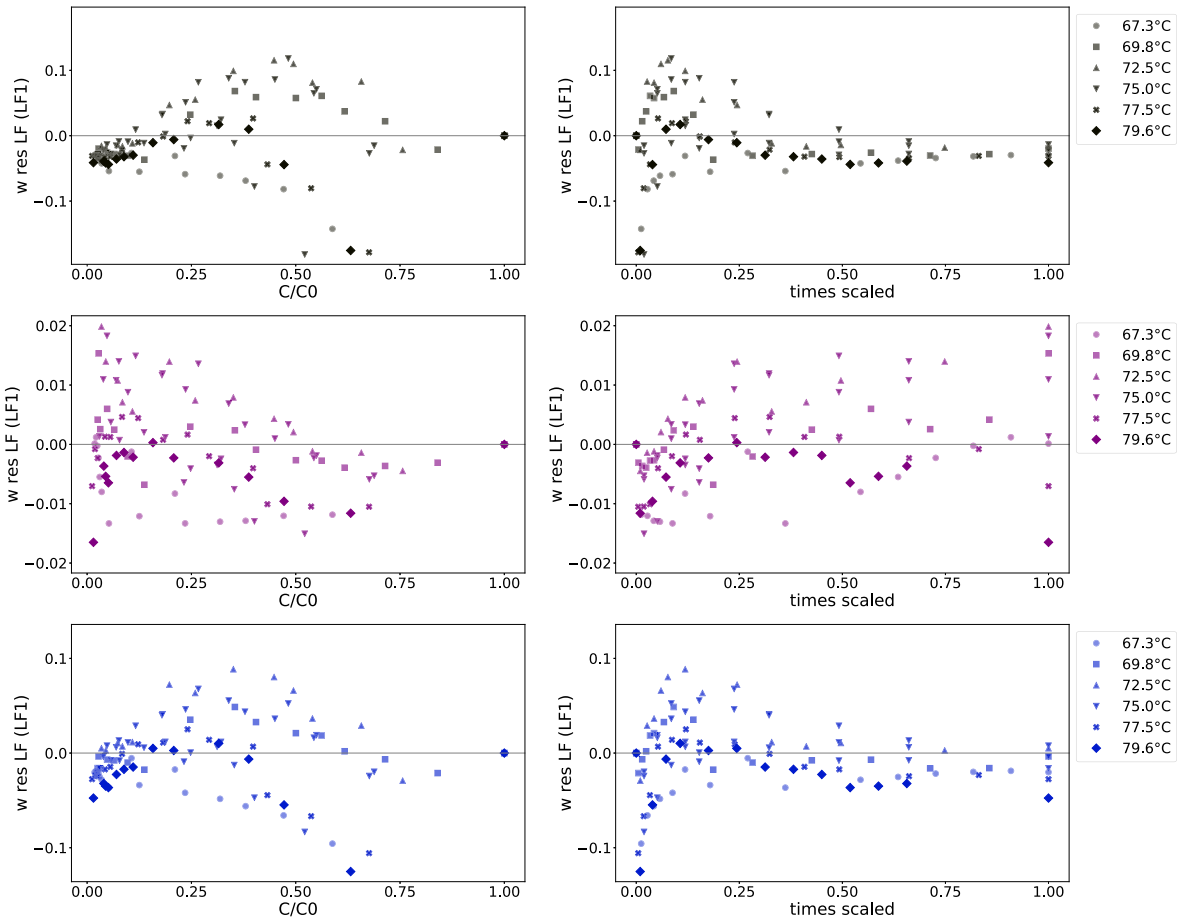

Fig. 5. Experimental weighted residuals versus C/C_0 and versus time for kinetic model using estimations obtained by NLS (black), WLS1 (magenta), and WLS2 (blue) for LF at LF1 sample. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

optimal solution than the second one.

4.2. Denaturation of β-LG and LF

Whatever the method and the proteins under study, the reaction order is fractional meaning that the kinetics of denaturation of β-LG and LF in infant milk formulae are complex kinetics. Except for LF in LF1 infant milk formula using NLS method (reaction order of 2.2 ± 0.17), the reaction order ranged between 1.5 and 2. The mechanism of denaturation of the whey proteins is described in two steps, an unfolding step consisting on protein conformational changes leading to reactive molecular species and an aggregation step in which the reactive species participate to several aggregation reactions (Roefs and de Kruifs, 1994). The former reaction is considered of first-order while the latter reactions are considered of second-order. Due to the intricacy of these two steps, unfolding and aggregation, during whey protein denaturation, the reaction order is expected to be in the range 1–2 in agreement with reported values. The reaction orders obtained in this study for β-LG denaturation are slightly higher than the reaction order determined during skimmed milk heating as summarized in Table 1 of the publication of Loveday (2016). This difference could be explained by the higher whey protein/casein ratio in infant milk formulae compared to skimmed milk. In the absence of caseins, the reported reaction orders were mainly >1.5 (Loveday (2016)).

Although Liu et al. (2020) assumed reaction order of $n = 1$ to predict the kinetic parameters for the denaturation of lactoferrin in bovine milk. The most reliable predictions in this study are close to $n = 1.6$ for all samples. To our knowledge, no other study investigates the reaction order of the denaturation of LF in infant formulae.

For β-LG, it was observed that the highest denaturation rate was at the LF1 sample, which might evidence that the presence of LF increases the denaturation rate of β-LG. Moreover, in the LF1 sample, the denaturation of β-LG was more sensitive to a variation in temperature as evidenced by the higher *Ea*. In the temperature range investigated, *Ea* of β-LG denaturation in skimmed milk is between about 260 and 300 kJ mol^{-1} (Dannenberg and Kessler, 1988; Anema, 2000). *Ea* slightly increases when the total solid of skimmed milk was increased (Anema, 2000). In the standard IMF, the *Ea* of β-LG denaturation was around 330–340 kJ mol^{-1} whereas the β-LG concentration was in the same range in infant milk formula and in skimmed milk. This higher *Ea* indicates that the denaturation of β-LG is retarded in infant milk formula compared to in skimmed milk. This difference could come from different whey proteins/casein ratio or the difference in the amounts of lactose and minerals between infant milk formulae and skimmed milk. For instance, calcium content was shown to increase the *Ea* of β-LG denaturation (Petit et al., 2011). $Ea > 300$ kJ mol^{-1} were determined in some studies dealing with the heating of whey protein solutions or pure β-LG solutions (Hoffmann et al., 1996; Le bon et al., 1999; Tolkach and Kulozik, 2007; Petit et al., 2011). The *Ea* of β-LG denaturation is even higher in the presence of LF (around 400 kJ mol^{-1}) whereas β-LG denaturation rate is faster than in the absence of LF. At first sight, it appears contradictory but the presence of LF in infant milk formulae could have different impacts on the IMF components. First, it was observed that in the presence of LF, the casein micelle partially disintegrated (Anema and de Kruifs, 2012; Halabi et al., 2020b) and the presence of free αS- and β-caseins is known to interact with partially unfolded β-LG on heating (Morgan et al., 2005). Secondly, LF exhibits a lower temperature of denaturation than β-LG (Bengoechea et al., 2011;

Brodkorb et al., 2016) and a lower *Ea* than β-LG. Consequently, LF is readily unfolded on heating and it exposed many sites for aggregation reactions with β-LG.

For LF, it was observed that the denaturation rate was slightly lower in the LF2 sample than in the LF1 sample. However, the difference was not as significant as for β-LG and it makes sense if we consider that LF denatured earlier than β-LG.

5. Conclusions

The assumption of residuals with constant variance in which the nonlinear least-squares method is based proved not to be valid to this dataset, because the variance of residuals was observed to be constant neither versus time nor versus concentration and mean of the residuals was biased not equally distributed between positive and negative values. The linearized method was less effective than NLS and WLS in making good predictions because of uncontrolled distortion of residuals due to the linearization, and, although being performed in one step, it did not result in more accurate estimations when compared to the two-step method. The WLS with the proposed weight functions has been the best method for estimating kinetic parameters, avoiding bias observed in the NLS estimations. Analyzing *SSe* and confidence intervals of parameters we can infer that the second weight function produced even more accurate results than the first. However, defining the weight function requires previous knowledge of sampling biases and residuals variance, that are particular to each dataset. This can make it harder to implement the WLS without previously performing another regression method. Moreover, to perform the WLS method (in nonlinear problems) it is necessary to provide initial guesses for the parameters, as for any nonlinear regression.

Probably a good approach when performing estimations of kinetic parameters in future studies could be to start by the two-step method, use obtained estimations as initial guesses in the NLS method, analyze residuals distribution, and then define weights and apply WLS.

CRediT authorship contribution statement

Bruno Leite: Methodology, Software, Formal analysis, Writing - original draft, Writing - review & editing, Visualization. **Thomas Croguennec:** Validation, Writing - original draft, Resources, Project administration. **Amira Halabi:** Validation, Investigation, Data curation, Resources. **Esly Ferreira da Costa Junior:** Methodology, Formal analysis, Supervision, Project administration.

Declaration of competing interest

The authors whose names are listed immediately below certify that they have NO affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers' bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements), or non-financial interest (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript. **Author names:** Bruno Leite, Thomas Croguennec, Amira Halabi, Esly Ferreira da Costa Junior.

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