



Research paper

Using front face fluorescence for rapid estimation of furosine concentration in heat treated milk: a kinetic study

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ABSTRACT

This study focused on characterizing the kinetics of furosine formation during milk heat treatment and establishing correlations between furosine concentration and native autofluorescent markers to develop predictive models with inline applicability. Furosine generation and fluorescence changes were monitored at 70, 80, and 90 °C. At 90 °C, furosine increase and tryptophan fluorescence loss followed apparent zero-order kinetics. The activation energy, E_a , and thermal coefficient Q_{10} of furosine formation were estimated at 15.72 kJ mol⁻¹ and 1.15, respectively. Heat treatment significantly affected autofluorescent marker intensity. Using these data, fluorescence-based models were developed to predict furosine concentration, with the best performance at 90 °C. A model incorporating riboflavin and tryptophan fluorescence intensities was validated through leave-one-out cross-validation, yielding an R^2 of 0.87 and a standard error of 5.59 mg/100 g protein. Overall, front-face fluorescence shows strong potential as a process analytical technology to estimate furosine inline and heat-induced changes in milk.

1. Introduction

Heat treatment of milk is required by the dairy industry for food safety, quality and functionality reasons. However, heat treatment can also cause undesirable effects in milk quality such as Maillard reactions, nutrient disappearance and the generation of distasteful odors and flavors. For this reason, improving control in milk thermal processing has potential implications for consumer health (Erbersdobler & Somoza, 2007).

An extensive range of chemical and biological markers has been investigated to evaluate the thermal damage in milk. These markers are classified into two groups (Richardson & Philip, 2010): a) type I markers, represented by thermolabile compounds, such as alkaline phosphatase or serum proteins which are degraded, denatured or inactivated during heat treatment and b) type II markers, which correspond to compounds that are generated as a result of the heat treatment, such as furosine, lactulose, or hydroxymethylfurfural.

One of the most typical chemical markers is furosine (ε-N-2-fuorolymethyl-L-lysine) that is generated during hydrolysis of fructosyl lysine in the early stages of the Maillard reaction (Bignardi et al., 2012; Skibsted et al., 2010). Furosine determination requires a

time-consuming sample preparation procedure, sophisticated and expensive instrumental equipment, and the use of costly reagents (Singla et al., 2018). Another drawback of traditional furosine determination is that its concentration does not increase linearly with the intensity of heat treatment and, in addition, a long heat treatment may cause degradation of furosine (Erbersdobler & Somoza, 2007; Van Boekel & Berg, 2005). This causes it to behave as a Type II marker, conditioned by Type I conversion (i.e., a mixed, type I-II marker).

Despite the interest of furosine as a marker of thermal damage, furosine formation kinetic data existing in milk is rare in the literature, probably as a result of the complexity of the chemical reactions involved or the limitations of the analytical methods available such as chromatographic (Resmini et al., 1990) or electrophoretic methods (Tirelli, 1998). Because of these limitations, front-face fluorescence (FFF) represents a cheaper, faster and nondestructive alternative technique to characterize the thermal damage with clear potential for inline application (Ayala et al., 2017; Liu et al., 2017; Hougaard et al., 2013). FFF is one of the best alternatives because it is a very fast and sensitive methodology that allows the investigation of fluorescence in powder, turbid and concentrated samples, with no sample preparation (Birlouez-Aragón, 2002; Birlouez-Aragón et al., 2001; Kulmyrzayev &

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Dufour, 2002). In addition, *FFF* is a rapid and sensitive method to monitor the molecular changes affecting the fluorophores environment. As a result, *FFF* has been used as a non-destructive technique to provide information about fluorescent molecules present in milk, milk heat treatment, or even about possible frauds such as the addition of milk powder to liquid milk (Brandao et al., 2016; Christensen et al., 2005; Kamal & Karoui, 2017).

In milk, there are several native fluorophores compounds having fluorescent behavior that can work as Type I or Type II markers like tryptophan, dityrosine, intermediate compounds of the Maillard reaction, retinol and riboflavin, among others (Alvarado et al., 2022; Heinicke et al., 1993; Kulmyrzaev & Dufour, 2002; Liu et al., 2018a, Liu et al., 2018a; McDowell, 2000; Schamberger & Labuza, 2006; Taterka & Castillo, 2018). Additionally, according to Markowicz et al. (2012), the autofluorescent markers can be used as early indicators of the Maillard reaction, as they are formed in stages prior to the formation of brown pigments.

Currently, there is no simple, cost-effective, accurate, universally applicable, and rapid method for assessing heat-induced damage in milk. As mentioned above, monitoring of native fluorescent milk compounds by using *FFF* spectroscopy has the potential to be used as inline process analytical technology (PAT) tool for real time detection of thermal damage in milk. Such technology would allow evaluation of functional quality of milk, and the adoption of preventive actions during processing, avoiding milk over processing and reducing losses due to low quality/functionality of the final product.

The objective of this study was to model the kinetics of furosine formation during milk heat treatment and correlate it with milk autofluorescent markers in order to develop a furosine concentration prediction equation based on *FFF* predictors. To that end, the effect of heat treatment on both furosine concentration and fluorescent markers was evaluated in parallel, the kinetics of furosine formation was modeled and, finally, the correlation between furosine concentration and the fluorescence signal was evaluated to obtain fluorescence models to predict the concentration of furosine.

2. Materials and methods

2.1. Experimental design

For the study, low-heat skimmed spray-dried milk powder (pH = 6.5, 800 cfu g⁻¹) supplied by Chr. Hansen SL (Barcelona, Spain) was used. One single milk powder batch was used in the study to reduce the experimental variability. Before running the experiment, linearity of the correlation between *FFF* fluorescence determinations and furosine concentration in milk, determined by HPLC, was evaluated using a small preliminary assay as follows: low-heat milk powder was reconstituted as described below. An aliquot of the reconstituted milk was sterilized in an autoclave (in-bottle sterilization at 120 °C for 20 min) while the remaining reconstituted milk was not heat treated. Four mixtures of sterilized and unheated milk were prepared in proportions of 0, 25, 75 and 100 % treated milk. A total of 24 furosine assays (i.e., 4 dilutions x 3 replicates x 2 determinations by HPLC) and 36 fluorescence assays (i.e., 4 dilutions x 3 replicates x 3 fluorimetric determinations) were performed.

Following this preliminary assay, kinetic data of furosine formation and selected fluorescence indicators (Maillard Compounds, *MC*; Tryptophan, *Trp*; Riboflavin, *Rb*) were evaluated in parallel during heat treatment of low-heat reconstituted skim milk heated at different temperature-time combinations (at three different temperatures and five times, detailed below). Three independent replicates were performed in the study. Chemical furosine determinations were performed in duplicate while fluorescence determinations were obtained in triplicate. The results were expressed using the mean of each determination.

A volume of 25 mL of reconstituted milk was heat-treated in Pyrex test tubes of 30 mL of capacity, sealed and immersed in a thermostatic

bath (OvanTherm TC00E C, Lovango SL, Spain) at 70, 80 and 90 °C for 10, 15, 30, 45 and 60 min. Fifteen different treatment combinations were evaluated under this experimental design, which was replicated three times. A total of 45 tubes (i.e., 3 temperatures x 5 times x 3 replicates) containing 25 mL of reconstituted milk were treated. After heat treatment, the samples were immediately cooled by immersion in crushed ice. At the different treatments considered, furosine chemical determinations and fluorescence measurements for the different fluorescent markers studied were performed in parallel to evaluate the correlations between the concentration of furosine and the corresponding fluorescence intensity values, for fluorescent indicators evaluated at the different treatments considered.

The formation of furosine was evaluated as a function of time of heat treatment to establish the kinetic order of the reaction and estimate the corresponding kinetic rate constants at the different reaction temperatures. Kinetic order and rate constants of changes associated with each of the fluorescent markers as a function of heat treatment time were also studied. Additionally, for furosine formation reaction, temperature coefficient Q_{10} and its activation energy were estimated, as described below.

A total amount of 120 g of standard, low-heat skimmed milk powder was reconstituted with 880 g of distilled water (12 % w/w) at ~40 °C in a volumetric flask covered with aluminum foil, using a hot plate with temperature control (Agimatic-E, JP. Selecta, Barcelona, Spain) and stirring for 15 min at 150 rpm. Then, the milk sample was allowed to stand for 30 min at room temperature, in the dark. Using low-heat skim milk allowed reducing the variability of the dependent variables evaluated decreasing the experimental error.

2.2. Determination of furosine concentration

ISO 18329 (2004) reference method was used for determination of furosine concentration. For sample preparation, a 2 mL sample of milk was hydrolyzed with 6 mL of 10.6 M HCl in pyrex test tubes. Then, they were bubbled with nitrogen for 2 min and capped. Bubbled samples were heated at 110 °C for 23 h in a convection oven (mod. Beschickung 100-800, Memmert, Germany) and filtered through Whatman N° 42 filter paper to obtain filtered sample.

For solid phase extraction, a 5 mL sample of methanol was placed in Octadecyl cartridges C18/18 Mini Spe-ed (Applied Separations, Allentown, PA, USA) and then, a 10 mL aliquot of distilled water was added. Subsequently, a 0.5 mL sample of the filtered hydrolysate was injected and a 3 mL aliquot of 3 M HCl was added to collect the purified hydrolysate.

For chromatographic determination, first the furosine column (C8-dedicated Furosine Alltech column, Alltech, Italy) was cleaned for 12 h with methanol at a flow of 1 mL s⁻¹. Subsequently, the solvents A and B were prepared with milliQ water. Solvent A was glacial acetic acid at a concentration of 0.4 % (v/v) while solvent B was a solution of 3 g L⁻¹ KCl in solvent A. The system was equilibrated for 35 min with a mixture 50:50 of solvents A and B.

The determination was performed at a temperature of 34 °C, with a flow of 1.2 mL min⁻¹ at a wavelength of 280 nm, with an injection of 50 mL of purified hydrolysate.

An external commercial furosine standard (Polypeptide, Strasbourg, France) was used for quantification. Formation of furosine is highly dependent on protein concentration; therefore, data were expressed as milligrams of furosine per 100 g of protein. When necessary, concentration units were converted into molarity.

2.3. Fluorescence determination

The fluorescence measurements were performed using a bench top spectrofluorimeter (Cary Eclipse Fluorescence Spectrophotometer, Agilent Technologies, Madrid, Spain) equipped with a 15 W xenon pulsed xenon lamp and a front-face geometry accessory (Solid Sample Holder

Accessory and Cuvette Kit, Agilent Technologies) adjusted to an angle of 35°, which minimized the phenomena of specular reflection on the surface of the cuvette and internal absorption (i.e., inner filtration of the sample). Measurements were made directly on ~3 mL undiluted milk samples using Suprasil® quartz cuvettes (UV fluorescence cell, Agilent Technologies) as described by Ayala et al. (2017). Both excitation and emission wavelengths were selected depending on the compound to be determined, as is reported in Table 1, using a slit of 5 nm. Readings were performed every 1 nm. The voltage of the photomultiplier detector was set to 400 V for *Rb* excited at 267 nm and *Trp* and 600 V for the rest.

2.4. Kinetic data modelling

For kinetic data modeling, zero-, first-, and second-order kinetic equations were evaluated to select the most appropriate kinetic model in each case. The effect of temperature on the reaction rate was modeled by the Arrhenius equation. To estimate the kinetic rate constant of furosine formation and its activation energy, once identified the reaction order (zero-order), the following equations (Claeys et al., 2003) were used:

a) zero-order kinetic equation:

$$[F] = [F]_0 + kt, \quad [1]$$

b) Arrhenius equation applied to two conditions of temperature T_1 and T_2 :

$$k_1 = Ae^{-\frac{E_a}{RT_1}} \quad [2]$$

$$k_2 = Ae^{-\frac{E_a}{RT_2}} \quad [3]$$

Dividing eqns. (2) and (3) yields

$$\frac{k_1}{k_2} = e^{\left(-\frac{E_a}{RT_1} + \frac{E_a}{RT_2}\right)} \quad [4]$$

Then rearranging Eqn. (4) and solving for k_1 yields

$$k_1 = k_2 e^{\frac{E_a}{R} \left(\frac{1}{T_1} - \frac{1}{T_2}\right)} \quad [5]$$

where assuming T_1 and T_2 as working and reference temperatures, respectively, and substituting Eqn. (5) into Eqn. (1) gives:

$$[F] = [F]_0 + \left[k_{ref} e^{\frac{E_a}{R} \left(\frac{1}{T} - \frac{1}{T_{ref}}\right)} \right] t \quad [6]$$

where:

$[F]$ is the furosine concentration (mol L⁻¹)

$[F]_0$ is the initial furosine concentration (mol L⁻¹)

k is the reaction rate constant (M s⁻¹)

t is the reaction time (s)

Table 1

Excitation and emission wavelengths used for the fluorimetric determination of the analyzed fluorophores.^a

Fluorophore	Excitation (nm)	Emission (nm)	Parameter
MC	330	420	F_{MC}
Trp	290	340	F_{Trp}
Rb	267–450	530	$F_{Rb267} - F_{Rb450}$

^a **MC**: Maillard Compounds; **Trp**: Tryptophan; **Rb**: Riboflavin.

Source: (Becker et al., 2003; Birlouez-Aragón et al., 2002; Bosch et al., 2007; Dufour & Riaublanc, 1997; Schamberger & Labuza, 2006; Wold et al., 2002).

A is the Arrhenius equation collision factor for furosine formation (M s⁻¹)

E_a is the activation energy for furosine formation (kJ mol⁻¹)

R is the universal ideal gas constant (kJ mol⁻¹ K⁻¹)

T is the reaction temperature (K)

k_{ref} is the reaction rate constant at the reference temperature (M s⁻¹)

T_{ref} is the reference temperature (K).

A bibliographic k_{ref} value was used (Earle & Earle, 2003), with a reference temperature of 393 K (120 °C), to estimate the E_a and k values. Based on bibliographic kinetic data for furosine formation during heat treatment of milk published by Earle and Earle (2003), Eqn (6) was used to estimate the activation energy of furosine formation, E_a , and/or its rate constant, k_{ref} , at a reference temperature, $T_{ref} = 393$ K (120 °C), using three different approaches: a) assuming an E_a of 81.64 kJ mol⁻¹ (Earle & Earle, 2003) and estimating k_{ref} , b) assuming k_{ref} of 4.97×10^{-7} M s⁻¹ (Earle & Earle, 2003) and estimating E_a , and c) estimating both E_a and k_{ref} .

2.5. Statistical analysis

All data were processed and analyzed using "Statistical Analysis System" (SAS, version 9.2, 2009, SAS Institute Inc., Cary, NC, USA). An analysis of variance (ANOVA) was performed using the mixed linear (MIXED) procedure of repeated measures. This procedure is the most appropriate one when in an experimental design there are combined random and fixed effects, as in our case. The Pearson correlation coefficients, r , were determined by the correlation (CORR) procedure.

The maximum R² (REG, MAXR) procedure of SAS was used to obtain the best one-, two-, and three-parameter prediction models for predicting the concentration of furosine $[F]$ from fluorescence emission intensities due to Maillard intermediate compounds, tryptophan, riboflavin excited at 370 nm and riboflavin excited at 450 nm (F_{MC} , F_{Trp} , F_{Rb267} and F_{Rb450} , respectively). Non-linear regression (NLIN) procedure was applied to both kinetic models and Arrhenius equation to estimate the activation energies and the kinetic reaction rate constants. Validation of prediction models was performed with the leave-one-out method, using SAS.

3. Results and discussion

In the preliminary assay for linearity (results not shown), the ANOVA showed that percentage of treated milk significantly affected ($P < 0.01$) the concentration of furosine and the fluorescence of tryptophan, intermediate Maillard compounds, and riboflavin at an excitation of 450 nm. As expected, increasing the percentage of treated milk resulted in higher $[F]$ and changes in the fluorescent markers. A statistically significant negative correlation ($P < 0.05$) was identified between $[F]$ and F_{Trp} ($r = -0.89$). Additionally, significant correlations ($P < 0.01$) were observed between the percentage of treated milk and $[F]$ ($r = 0.97$), F_{Trp} ($r = -0.96$), and F_{MC} ($r = 0.80$).

3.1. Kinetics of thermal damage markers

The formation/disappearance of chemical and fluorescent markers was evaluated as a function of the heat treatment time to estimate the order of each of the reactions. The ANOVA analysis (repeated measures) evaluating the effect of the main experimental factors, heating temperature (T) and time (t), replication (Rep) and temperature $\times$ time interaction (Txt) is presented in Table 2.

It was observed that the temperature, time and interaction Txt had a statistically significant effect on $[F]$ and tryptophan fluorescence, whereas in the case of fluorescence of Maillard intermediate compounds only time and the interaction Txt had a statistically significant effect. Additionally, Txt interaction was also found to be significant for fluorescence of riboflavin excited at 450 nm. As expected, the replica did not

Table 2

Analysis of variance and F-statistic values for furosine concentration and several autofluorescent indicators.^a

Factors	Df	Heat damage indicators				
		[F]	F_{Trp}	F_{MC}	F_{Rb267}	F_{Rb450}
Rep	2	0.87	1.23	0.24	2.37	1.04
Temperature (T)	2	905.98***	23.20*	0.62	2.85	0.06
Time (t)	4	5150.23***	19.71***	4.22**	0.34	0.11
Txt	8	24.79***	19.47***	15.00***	1.21	3.27*

^a N = 45 ***significant at 99.9 %; ** significant at 99 %; * Significant at 95 %; Rep, replicate; Txt, interaction temperature x time; Df, degrees of freedom; [F], concentration of furosine; F_{Trp} , tryptophan fluorescence; F_{MC} , Maillard intermediates compounds fluorescence; F_{Rb267} , riboflavin fluorescence at excitation of 267 nm; F_{Rb450} , riboflavin fluorescence at excitation of 450 nm.

present any significant effect on the thermal damage indicators studied.

3.2. Furosine

The fit of the experimental data points corresponding to the change in furosine concentration, as a function of heat treatment time, to zero, first, and second order kinetic equations was evaluated. Table 3 shows that the best fit at each temperature was a zero order kinetics, with a kinetic constant of $2.97 \times 10^{-7} \text{ Ms}^{-1}$ at 90 °C, close to that of $2.03 \times 10^{-7} \text{ Ms}^{-1}$ at 90 °C reported on previous studies (Claeys et al., 2001). According to the R^2 values, the order of reaction of furosine formation corresponds to zero order, which is in agreement with Montilla et al. (1996) and Rafael et al. (1997), who also observed that type of kinetics in milk heated at 135–150 °C for 10–40 s and 100–120 °C for 20–60 s.

In Fig. 1, the fit of experimental data to a zero kinetic reaction order is shown for each of the temperatures studied with linear k values as a function of temperature as expected ($2,956 \times 10^{-7}$, $2,965 \times 10^{-7}$, $3,085 \times 10^{-7}$; Table 3). Equation (6), which combined a zero-order kinetic equation with the Arrhenius equation (respectively, Eqn. 1 and 2-3 in section 2.3), was used for estimation of the activation energy. Eqn (6) was fitted to the 45 tests, which constituted the whole experiment, to estimate the activation energy of furosine formation, E_a , as well as its rate constant at the reference temperature (at $T_{\text{ref}} = 393 \text{ K}$) using the three different approaches described in section 2.3: a) assuming an E_a of $81.64 \text{ kJ mol}^{-1}$ (reference value from literature; Earle & Earle, 2003) and estimating k_{ref} , b) assuming k_{ref} of 4.97×10^{-7} (reference value from literature) and estimating E_a , and c) estimating both E_a and k_{ref} . The E_a and k_{ref} values estimated are shown in Table 4. Fig. 2 shows graphically the fit of the regressions summarized in Table 4. The best correlation was obtained using model III ($R^2 = 0.92$) (Fig. 2c). In this model, both the activation energy (E_a) and the kinetic rate constant (k_{ref}) were estimated, and the model presented the best fit over the experimental data. The estimated E_a values were within the same range as those reported in the literature (Claeys et al., 2001), although slightly higher. However, for the case of equation I (Fig. 2a), where fitting was restricted by assuming a bibliographic value of $E_a = 81.64 \text{ kJ mol}^{-1}$, previously estimated by Claeys et al. (2001), the estimated value of k ($4.01 \times 10^{-7} \text{ M s}^{-1}$) was very similar to that found in the literature with frozen raw milk samples treated at 90–140 °C (Claeys et al., 2001). In equation II (Fig. 2b), a bibliographic value of $k = 4.97 \times 10^{-7} \text{ M s}^{-1}$, previously calculated by

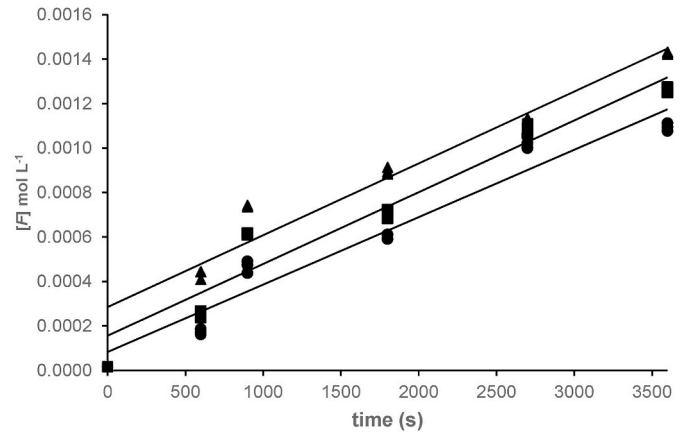


Fig. 1. Fit of experimental data for furosine formation to a zero order kinetic equation during heat treatment at three temperatures 70 °C (●) 80 °C (■) and 90 °C (▲) of cow, skim milk powder reconstituted in distilled water.

Table 4

Equations to determining k and/or E_a of furosine formation during heat treatment of milk.^a

Equations	Kinetic parameters	
	E_a (kJ mol ⁻¹)	k_{ref} (M s ⁻¹)
I $[F] = [F]_0 + \left[k_{\text{ref}} e^{\frac{81.64}{R} \left(\frac{1}{T} - \frac{1}{T_{\text{ref}}} \right)} \right] t$	81.637 ^c	4.01×10^{-7b}
II $[F] = [F]_0 + \left[4.9710^{-7} e^{\frac{E_a}{R} \left(\frac{1}{T} - \frac{1}{T_{\text{ref}}} \right)} \right] t$	87.189 ^b	4.97×10^{-7c}
III $[F] = [F]_0 + \left[k_{\text{ref}} e^{\frac{E_a}{R} \left(\frac{1}{T} - \frac{1}{T_{\text{ref}}} \right)} \right] t$	15.715 ^b	6.73×10^{-8b}

^a N = 45.

^b Estimated values.

^c bibliographic values reported by Earle and Earle (2003); k , kinetic rate constant; E_a , activation energy. $T_{\text{ref}} = 393 \text{ K}$.

Claeys et al. (2001) was used, and the estimated value of E_a ($87.189 \text{ kJ mol}^{-1}$) was also very close to literature. However, in the last two cases, the R^2 values in equations I and II were 0.80 and 7.58, respectively lower than that obtained using equation III. The E_a values estimated by Montilla et al. (1996), Nangpal et al. (1990), and Schlimme et al. (1996) ranged from 86.2 to $100.2 \text{ kJ mol}^{-1}$, of the same order of magnitude as those obtained in the present study.

Finally, to estimate the temperature coefficient, Q_{10} , for furosine formation in the temperature interval of the study (70–90 °C), Eqn (7) was used:

$$Q_{10} = \left(\frac{k_2}{k_1} \right)^{\frac{10}{T_2 - T_1}} \quad [7]$$

where k_1 and k_2 are the kinetic rate constants (M s^{-1}) corresponding to

Table 3

Comparison of different order kinetic equations for furosine formation during heat treatment of milk at three different temperatures.

	Equations	70 °C ^a		80 °C ^a		90 °C ^a	
		R^2	k (M s ⁻¹)	R^2	k (M s ⁻¹)	R^2	k (M s ⁻¹)
Order 0	$[F] = [F]_0 + kt$	0.930	2.956×10^{-7}	0.927	2.965×10^{-7}	0.856	3.085×10^{-7}
Order 1	$\ln[F] = \ln [F]_0 + kt$	0.112	5.113×10^{-4}	0.113	4.370×10^{-4}	0.102	3.326×10^{-4}
Order 2	$1/[F] = 1/[F]_0 + kt$	0.367	-1.120	0.455	-0.744	0.547	-0.413

^a N = 15; number of replicas, Nr = 3; R^2 , determination coefficient; k , kinetic rate constant; [F], furosine concentration (mol L^{-1}); t , time (s).

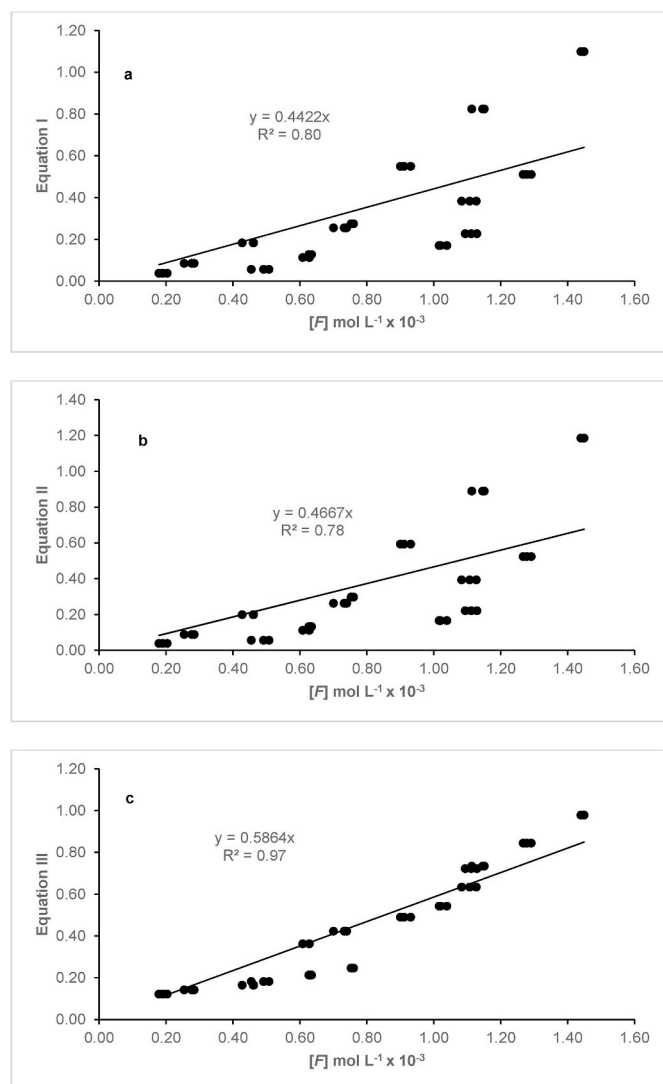


Fig. 2. Regression between experimental and estimated values of furosine concentration $[F]$. a) Equation I, b) Equation II and c) Equation III. See Table 4 for equations details.

the tested temperatures for a zero order reaction (Table 3; mol L^{-1}). Estimated Q_{10} between 70 and 80 °C was 1.17 while between 80 and 90 °C was 1.15. The observed values were similar to those (1.10) found in the literature (Claeys et al., 2001).

3.3. Tryptophan, Maillard reaction intermediate compounds and riboflavin fluorescence

As shown in Table 2, the changes in tryptophan fluorescence intensity (F_{Trp}) were significantly influenced by the heat treatment temperature ($P < 0.05$) as well as by holding time and the interaction of T_{ht} ($P < 0.001$), as argued by Ayala et al. (2020), who observed that fluorescence at an excitation wavelength of 290 nm in milk was mostly due to Trp fluorescence. At this wavelength, Phenylalanine (Phe) is not significantly excited (maximum excitation wavelength ~ 260 nm) and its small quantum yield is one order of magnitude lower than those of Tyrosine (Tyr) and Trp. Further, molar extinction coefficient at 290 nm is ~ 19 times higher for Trp as compared to Tyr, which, in combination with potential resonance phenomena from Phe to Tyr to Trp, decreases substantially the contribution of Phe and Tyr to the observed fluorescence signal at the mentioned excitation wavelength. About 80 % of total milk proteins is represented by casein (α_{s1} -, α_{s2} -, β -, and κ -CN),

which do not experience relevant changes due to heat at temperatures below 100 °C. The remaining ~ 20 % of total milk proteins are comprised of whey proteins. Major whey protein components, β -lactoglobulin (β -LG) and α -lactalbumin (α -LG), make up ~ 80 % of whey proteins, coming up to 50–60 % of the whey protein mass proportion from β -LG.

According to Geddes (2006) and Taheri-Kafrani et al. (2010) the fluorescence of bovine β -lactoglobulin (β -LG) depends on the microenvironment of the two Trp residues, Trp¹⁹ and Trp⁶¹ contained on that protein. In particular, about 80 % of fluorescence due to native protein is attributed to Trp¹⁹ of β -LG, which is buried inside the globular protein structure and are exposed during denaturation (Moro et al., 2001). Exposing Trp¹⁹ of β -LG to a hydrophilic environment as a result of the thermal denaturation modifies fluorescence behavior of serum proteins and induces protein-protein interactions, all of which contribute in a complex manner to observed fluorescence changes.

As shown in Fig. 3, the temperature had no clear effect on tryptophan fluorescence, at 70 °C (Fig. 3a). However, a slight tendency to increase in fluorescence can be observed in F_{Trp} up to $t \sim 30$ min, that might be mainly dominated by incipient, reversible whey protein denaturation. At 80 °C (Fig. 3b), the slight increase in tryptophan fluorescence up to ~ 30 min, followed by the subsequent decrease became more evident. The curved shape of F_{Trp} profile as a function of heat exposure time was attributed to the effect of a greater presence of unfolded whey protein chains, as a consequence of denaturation, and the subsequent initiation of preliminary protein-protein interactions. Apparently, those two interrelated phenomena might be inducing opposed trends in the fluorescent behaviors of whey proteins, as it will be argued below.

Finally, at 90 °C (Fig. 3c), a marked, sound decrease in F_{Trp} with holding time took place. Note that complete denaturation of whey proteins at 90 °C requires at least a treatment for 10 min (Fox et al., 2015). In Fig. 3c, a consistent decrease of F_{Trp} is observed from the very first holding time considered, $t = 10$ min.

The apparent erratic behavior of F_{Trp} at 70 °C, which is transformed into a clearer peak-shaped tendency at 80 °C, finally turning into a consistent and, for the most part, significant ($P < 0.05$) decrease at 90 °C was as expected considering that emission was measured at 340 nm and taking into account the changes on photo-physical properties of tryptophan that are induced by heat.

First, in a study performed in milk mixtures obtained by mixing increasing proportions of in-bottle sterilized milk with unheated milk by Alvarado et al. (2024), F_{Trp} tryptophan fluorescence significantly and steadily decreased with the increase of heat-treated milk proportion. This behavior was also observed in the small preliminary linearity assay conducted in the present study. F_{Trp} gradually and significantly ($P < 0.0014$) decreased with the increasing percent of autoclaved milk (0, 25, 75, 100 %) in the milk mixture. However, the observed decrease was not linear but slightly asymptotic, contrarily to what it was expected (Veisseyre, 1980, pp. 11–32). When milk is in-bottle sterilized at 115 °C for 15 min (note that in the present study, sterilizing conditions applied were 120 °C for 20 min), all whey proteins have been completely denatured and for the most part covalently attached by disulfide bonds to either κ -casein (κ -CN) on the micellar surface or each other as whey protein aggregates, depending on pH. Further, Lakowicz (2006) attributed spectral shifts of protein emission to three main phenomena: binding of ligands, protein-protein association, and protein unfolding. Additionally, this author stated that tryptophan fluorescence is subjected to quenching by nearby disulfide groups. Since high intensity heating, such as in-bottle sterilization, induces both complete whey protein unfolding and very extensive permanent disulfide bond formation, in-bottle sterilized milk is expected to present maximum red shift and F_{Trp} diminution. Subsequently, increased proportions of autoclaved milk in the mixtures should necessarily translate into F_{Trp} emission attenuation, when measured a 340 nm.

Second, according to Walstra et al. (2005), denaturation of whey protein is a reversible process as the original conformation of a globular protein can be recovered if the inducing denaturation factor reverts to its

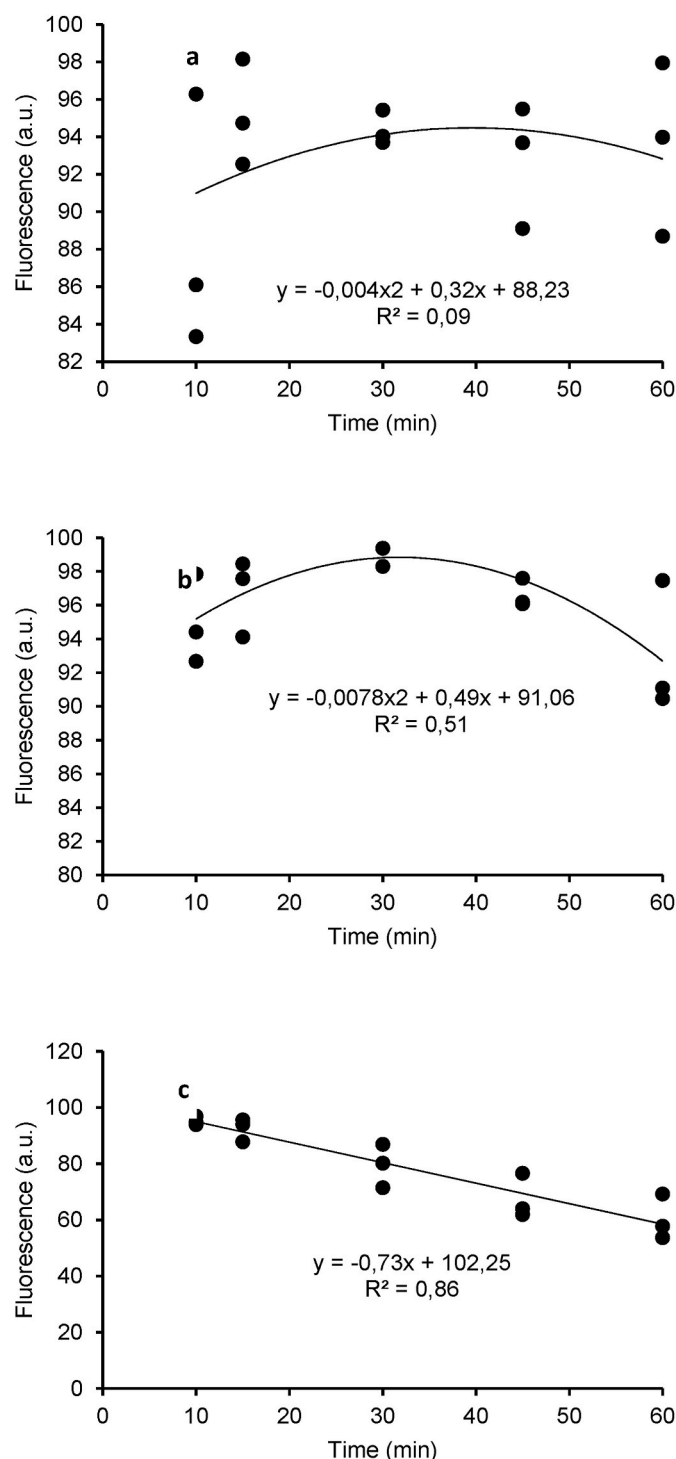


Fig. 3. Fluorescence intensity of tryptophan in milk measured using excitation and emission wavelengths of 290 and 340 nm, respectively, at: a) 70 °C, b) 80 °C y c) 90 °C; a.u., arbitrary units.

initial value, e.g., by cooling. But in practical conditions, such regeneration does not take place when the more reactive unfolded state (i.e., exposure and subsequent activation of -SH groups) induces formation of covalent bonds. In that sense, [Steventon et al. \(1991\)](#) divided denaturation of globular proteins in milk, such as whey proteins, in two reversible stages: denaturation/unfolding and initiation of aggregation (i.e., dimers formation) and one irreversible stage, propagation of aggregation (i.e., further polymerization). Then, if fluorescence is measured at a constant emission wavelength, for instance 340 nm, both

a red shift and an increase of F_{Trp} up to a maximum value and then a subsequent decrease should be expected due to reversible denaturation of whey proteins and successive irreversible protein-protein interactions, induced by heat.

Using reconstituted skim milk powder, [Ayala et al. \(2020\)](#) characterized systematically the front-face emission spectrum of tryptophan excited at 290 nm as a function of the heat-treatment intensity, varying temperature from 70 to 100 °C and holding time from 0 to 60 min. It was demonstrated that the fluorescence intensity change observed at a single wavelength was twofold: direct change in emission intensity and indirect change in intensity due to the red shift of the maximum emission wavelength. These authors also observed a slight tendency for F_{Trp} to increase between 70 and 80 °C and a significant decrease at 90 and 100 °C. Results obtained in the present study clearly confirm the results obtained by [Ayala et al. \(2020\)](#).

From the results of the present study, it seems that heat treatment is inducing red shift and either increase or decrease of fluorescence intensity depending on reversibility/irreversibility of changes induced by heat on whey proteins. This behavior would have been even more evident if measurements were not taken at a single wavelength, as at a single wavelength the red shift tends to minimize the initial and slight fluorescence intensity increases due to reversible denaturation and maximize successive intensity decrease originated by permanent disulfide bonding.

Our results, and the limited number of studies available, suggest the interest of further studying the kinetics of tryptophan fluorescence during heat treatment of milk to better understand how whey protein denaturation and subsequent protein-protein interaction modify the fluorescence characteristics of tryptophan.

Considering the complexity of the fluorescence response to milk heating, the fit of fluorescence data to kinetic models might help to better understand the changes in fluorescence as a function of heating temperature and holding time. As shown in [Table 5](#), at 70 and 80 °C, F_{Trp} did not fit any kinetic model in particular. This is logical; in this temperature range, reversible denaturation, likely inducing an increase of F_{Trp} , takes place simultaneously with early but permanent disulfide bond formation due to exposed -SH groups, probably inducing a F_{Trp} decrease. However, at 90 °C, F_{Trp} data better met a zero order kinetics ($R^2 = 0.97$) although a good fitting was also observed for first and second order kinetics (R^2 of 0.85 and 0.82, respectively). The observed dependence of F_{Trp} with time of heat treatment could be, at least in part, related to the complex kinetic behavior of whey proteins denaturation and subsequent protein-protein interaction steps in milk. According to [Anema \(2020\)](#), although early studies about kinetic of whey denaturation “did not follow a simple exponential law” -i.e., it was not first order, the complex nature of whey protein denaturation was categorically demonstrated by [Lyster \(1970\)](#). It is precisely the complex nature of the denaturation phenomena that may be responsible for difficulties in establishing the kinetic order of reaction. Several whey proteins suffer denaturation phenomena, each one with structural specificities that are responsible for differences in thermal sensibility, and degree of both reversibility and protein-protein interactions. At a first stage, protein varieties which often appear under dimeric/oligomeric forms such β -LG, are required to reversibly dissociate into monomers before they can unfold. Although the unfolding step is reversible, it exposes an intern thiol group, which activates allowing irreversible aggregation reactions with other unfolded whey proteins or with casein micelles, depending on pH. [Lyster \(1970\)](#) concluded that α -LA and β -LG appeared to follow first and second order kinetics, respectively. However, after thermodynamic evidences about complex temperature dependence of denaturation kinetics of those two whey proteins, demonstrated by [Dannenberg and Kessler \(1988\)](#), it is now generally accepted that denaturation of β -LG has an order of ~ 1.5 , while denaturation of α -LA is considered to be pseudo first order. Recently, [Anema \(2017\)](#) studied denaturation traying both whey proteins in milk, α -LA and β -LG, as a “single total whey protein entity”, observing a second order kinetic behavior.

Table 5

Comparison of different kinetic equations for the decrease of tryptophan fluorescence intensity during heat treatment of milk.

	Equations	70 °C ^a		80 °C ^a		90 °C ^a	
		R ²	k	R ²	k	R ²	k
Order 0	$F_{Trp} = F_{Trp0} + kt$	0.034	0.041	0.073	-0.042	0.972	-0.661
Order 1	$\ln F_{Trp} = \ln F_{Trp0} + kt$	0.036	0.001	0.452	-0.0004	0.848	-0.010
Order 2	$1/F_{Trp} = 1/F_{Trp0} + kt$	0.039	-5×10^{-6}	0.075	5×10^{-6}	0.823	0.0001

^a N = 15; number of replications, Nr = 3; R², determination coefficient; k, kinetic rate constant; F_{Trp}, tryptophan fluorescence; t, time.

As a result, the unspecific kinetic behavior observed in our study for whey protein denaturation and subsequent protein-protein interactions was attributed to a few facts: a) several whey protein varieties, with specific denaturation behaviors (heat sensitivity, distribution of -SH groups, reversibility tendencies, etc.), are combined in milk at somewhat varying proportions; b) denaturation phenomenon is comprised of several successive stages; c) temperature dependence and potential for acting as rate-determining step of each denaturation stage seems playing a “confounding” role to some extent.

Table 6 shows that fluorescence of the intermediate compounds of the Maillard reaction did not fit well with any particular kinetics, regardless of the processing temperature, as suggested by the low and similar magnitude of the R² values obtained for zero, first, and second-order kinetic models. The lack of fit can be attributed to the simultaneous formation and degradation of Maillard intermediates, each with distinct reaction rates and temperature dependencies. Higher temperatures accelerate both processes, creating a balance that complicates kinetic modeling (Ayala et al., 2017; Claeys et al., 2001). This dual behavior suggests that no single kinetic model can capture these dynamics effectively. In other studies, a noticeable hump in the tryptophan emission spectra around 425 nm has been observed (Alvarado et al., 2022; Ayala et al., 2020; Henihan et al., 2018). It is important to note that Maillard reaction intermediate compounds are excited at 330 nm and emit at 420 nm. Thus, the observed hump may potentially be linked to the emission wavelength of Maillard reaction intermediate compounds. This phenomenon can be explained by the process of Fluorescence Resonance Energy Transfer (FRET), which is a non-radiative energy transfer that occurs when the donor emission wavelength and the acceptor excitation wavelength are in close proximity (typically 2–10 nm), and there is significant overlap between the donor's emission spectrum and the acceptor's excitation spectrum. This results in a decrease in the donor's fluorescence intensity and a corresponding increase in the fluorescence emission of the acceptor (Yang et al., 2024).

In the study by Henihan et al. (2018) a tryptophan emission spectrum shows a distinctively higher hump around a wavelength of 400 nm in infant milk formula that has been stored at 37 °C, as compared with samples stored at 15 °C, which is compatible with the described hypothesis.

In the case of riboflavin fluorescence intensity at both excitation

Table 6

Comparison of different kinetic equations for the changes of Maillard compounds fluorescence intensity during heat treatment of milk.

	Equations	70 °C ^a		80 °C ^a		90 °C ^a	
		R ²	k	R ²	k	R ²	k
Order 0	$F_{MC} = F_{MC0} + kt$	0.554	0.726	0.684	0.923	0.409	-0.972
Order 1	$\ln F_{MC} = \ln F_{MC0} + kt$	0.552	0.002	0.682	-3×10^{-6}	0.416	-0.002
Order 2	$1/F_{MC} = 1/F_{MC0} + kt$	0.550	-3×10^{-6}	0.683	0.002	0.425	3×10^{-6}

^a N = 15; number of replications, Nr = 3; R², determination coefficient; k, kinetic rate constant; F_{MC}, Maillard reaction intermediate compounds fluorescence; t, time.

wavelengths tested (267 and 450 nm), very limited fit to any evaluated kinetic reaction order was observed (results not shown). This was attributed to an unexpectedly high variability in the fluorescence data obtained, possibly due to inconsistencies in the experimental setup or sample composition. The fluorescence response in riboflavin, as highlighted in similar studies using front-face fluorescence spectroscopy (FFF), tends to exhibit sensitivity to environmental factors such as sample composition, processing, and thermal treatments, which may affect its stability and detection reliability (Alvarado et al., 2020).

However, surprisingly, in several studies (Alvarado et al., 2022, 2024; Liu et al., 2018b), riboflavin fluorescence consistently emerges as a reliable predictor of thermal damage markers in milk. This suggests that apparent variability of riboflavin fluorescence might be underscoring its potential role in monitoring heat treatment in milk. Then, despite the variability observed, riboflavin remains a key compound for assessing thermal effects, as is shown below in section 3.1.4 and further research is necessary to refine its use as a marker.

3.4. Correlation between furosine concentration and individual fluorescent markers

Pearson's linear correlation between furosine concentration, [F], and each of the different fluorescent markers evaluated, F_{MC}, F_{Trp}, F_{Rb370}, and F_{Rb450}, as well as among the different fluorescent markers were studied at each milk heat treatment temperature. In Table 7, a statistically significant and positive correlation (P < 0.05) between [F] and F_{MC} (r = 0.75) was observed at 70 °C and 80 °C, it also shows that there were no other statistically significant correlation except for that observed between F_{MC} and F_{Rb450} (P < 0.05, r = 0.76). Similarly, at 80 °C, a statistically significant and positive correlation (P < 0.05) between [F] and F_{CM} was encountered. However, the observed r value was higher at 80 °C (r = 0.85) as compared to 70 °C. At 80 °C, no other statistically

Table 7

Pearson correlation coefficients, r, between the different dependent variables.

70 °C ^a				
	F _{Trp}	F _{MC}	F _{Rb370}	F _{Rb450}
[F]	0.265	0.751*	0.144	0.353
F _{Trp}	–	–0.094	0.264	–0.389
F _{MC}		–	0.116	0.763*
F _{Rb370}			–	–0.254
80 °C ^a				
	F _{Trp}	F _{MC}	F _{Rb370}	F _{Rb450}
[F]	–0.201	0.849*	0.028	–0.252
F _{Trp}	–	0.039	–0.289	0.072
F _{MC}		–	–0.225	–0.028
F _{Rb370}			–	0.096
90 °C ^a				
	F _{Trp}	F _{MC}	F _{Rb370}	F _{Rb450}
[F]	–0.904*	–0.543*	0.361	–0.473
F _{Trp}	–	0.493	–0.423	0.350
F _{MC}		–	–0.093	0.755*
F _{Rb370}			–	0.247

***significant at 99.9 %; ** significant at 99 %; * significant at 95 %; [F], concentration of furosine; F_{Trp}, tryptophan fluorescence; F_{MC}, Maillard intermediate compounds fluorescence; F_{Rb370}, riboflavin fluorescence excited at 370 nm; F_{Rb450}, riboflavin fluorescence excited at 450 nm.

^a N = 15.

significant correlation was observed. Finally, at 90 °C, a statistically significant and negative correlation ($P < 0.05$) was observed between $[F]$ and F_{Trp} ($r = -0.9$), which was not observed at the previous temperatures, 70 and 80 °C. Additionally, a significant negative correlation between $[F]$ and F_{MC} ($r = -0.54$) similar to the correlations found at 70 °C and 80 °C, but considerably lower and of the opposite sign, was encountered. It is expected that the increase in the concentration of furosine is directly related to the increase in the fluorescent signal of Maillard intermediates compounds due to the heat treatment applied to milk.

The negative correlation between furosine and tryptophan fluorescence at 90 °C could be explained because the complete denaturation of the whey proteins at this temperature, as discussed before (section 3.1.2).

3.5. Predication models for furosine concentration using front-face fluorescence predictors

Several prediction models for $[F]$ combining the different fluorescent markers evaluated as potential predictors were obtained, at each temperature, using the Maximum R^2 procedure of SAS. One, two and three-parameter best prediction models were generated using as potential predictors the following fluorescence variables: F_{Trp} , F_{MC} , F_{Rb267} , F_{Rb450} , $F_{\text{Trp}}/F_{\text{Rb450}}$, $F_{\text{MC}}/F_{\text{Rb450}}$, F_{Trp}^2 , F_{MC}^2 and F_{Rb450}^2 . As observed in Table 8, at 70 °C all the models obtained were significant ($P < 0.05$). The best model for predicting furosine formation was model III, which utilized F_{MC} , F_{Rb267} , $F_{\text{Trp}}/F_{\text{Rb450}}$. The four regression coefficients were significant ($P < 0.05$), and the model had an R^2 of 0.72 and SEP values of 17.82 mg/100 g. Fit of experimental data to model III is shown in Fig. 4.

As it is shown in Table 8, all the models obtained at 80 °C were also significant but with higher significance level ($P < 0.001$). The best model for predicting furosine concentration was model VI, which employed F_{Rb267} , $F_{\text{Trp}}/F_{\text{Rb450}}$ and F_{Rb450} and $F_{\text{MC}}/F_{\text{Rb267}}$ as predictors. Model VI had R^2 and SEP values of 0.85 and 13.68 mg/100 g, respectively. Fit between observed and predicted $[F]$ values using Model VI is shown in Fig. 4. Thus, Model VI was simplified to Model V by eliminating the not significant predictors. Model V, which only used an intercept, $F_{\text{Trp}}/F_{\text{Rb450}}$ and $F_{\text{MC}}/F_{\text{Rb450}}$ as predictor, was more convenient due to its simplicity and had a R^2 of 0.81 and a SEP of 14.23 mg/100 g.

Finally, as it can be seen in Table 8, all the models obtained at 90 °C were significant ($P < 0.001$). Note that significance of models increased with increasing temperature.

The best model using one predictor (Model VII) included F_{Trp}^2 corresponding to squared tryptophan fluorescence, with significant

regression terms. The best two-variable model (Model VIII) used as predictors riboflavin fluorescence excited at 450 nm (F_{Rb450}) and F_{Trp}^2 . Finally, the best three-variable model (Model IX) used F_{Rb450} , F_{Trp}^2 and F_{Rb450}^2 , where F_{Rb450}^2 was the square of riboflavin fluorescence excited at 450 nm. In model IX (Table 8), at 90 °C, all the regression terms were significant ($P < 0.05$), with an R^2 of 0.92 and the smallest SEP value (9.15 mg/100 g). Note that the highest R^2 values encountered at 70 and 80 °C were 0.72 and 0.85, respectively. Fig. 4 shows the fit of the prediction of $[F]$ using Model IX.

Thus, predictions models were more consistent with increasing treatment temperature, reaching a maximum R^2 value at 90 °C, within the range of temperatures studied, which might be caused by the higher correlations existing between $[F]$ and fluorescent markers at 90 °C, as mentioned above. It needs to be highlighted that, even when trends of riboflavin fluorescence as a function of heat treatment time observed in this study were not completely understood, it seems clear from statistical results, that an important piece of information, regarding furosine formation, is carried out by riboflavin fluorescence changes, which in addition to tryptophan fluorescence and intermediate Maillard compounds seems to provide useful information to predict $[F]$. These results are consistent with those found in lactulose by Ayala et al. (2017).

3.6. Validation of prediction models

The prediction models for furosine concentrations were validated with the leave-one-out method. The best models obtained were at 90 °C and they were selected for the validation purpose. The statistical parameters corresponding to the validation of the prediction model are described in Table 9.

For the validated furosine models, although the R^2 estimated in previous models were slightly higher, the standard errors of prediction and the coefficients of variation values were good. Validation of Model IX had a higher determination coefficient and lower SEP and CV values. These simple and practical models based on a few FFF predictors show potential as inline tools for food heat treatment processing and control in industrial environments.

4. Conclusions

Few studies have investigated the kinetics of furosine formation during heat treatment of milk. Experimental furosine concentration data points obtained at the three temperatures studied were successfully fitted to a zero order kinetic model in combination with the Arrhenius equation. The activation energy, E_a and the kinetic constant, k , for

Table 8
Models for predicting the concentration of furosine using fluorescent markers.

70 °C. ¹								
	Model	β_0	β_1	β_2	β_3	R^2	SEP	
I*	$[F] = \beta_0 + \beta_1 F_{\text{MC}}^2$	-274* (± 77.84)	0.001 (± 0.0003)	–	–	0.564	20.40	
II*	$[F] = \beta_0 + F_{\text{Trp}}/F_{\text{Rb450}} + \beta_2 F_{\text{MC}}^2$	-543* (± 128.86)	508* (± 208.69)	0.001* (± 208.69)	–	0.708	17.38	
III*	$[F] = \beta_0 + \beta_1 F_{\text{MC}} + \beta_2 F_{\text{Rb267}} + \beta_3 F_{\text{Trp}}/F_{\text{Rb450}}$	-978* (± 207.07)	1.55* (± 0.3)	-0.068* (± 0.1)	571* (± 234)	0.719	17.82	
80 °C.								
IV***	$[F] = \beta_0 + \beta_1 F_{\text{MC}}/F_{\text{Rb450}}$	-528* (± 97.49)	268*** (± 44.87)	–	–	0.733	16.63	
V***	$[F] = \beta_0 + \beta_1 F_{\text{Trp}}/F_{\text{Rb450}} + \beta_2 F_{\text{MC}}/F_{\text{Rb450}}$	-337* (± 115.21)	-664* (± 276.41)	297*** (± 40.28)	–	0.810	14.23	
VI***	$[F] = \beta_0 + \beta_1 F_{\text{Rb267}} + \beta_2 F_{\text{Trp}}/F_{\text{Rb450}} + \beta_3 F_{\text{MC}}/F_{\text{Rb450}}$	-412* (± 123.09)	0.10 (± 0.07)	-571 (± 273.88)	306*** (± 39.21)	0.847	13.68	
90 °C.								
VII***	$[F] = \beta_0 + \beta_1 F_{\text{Trp}}^2$	139*** (± 9.96)	-0.011* (± 0.001)	–	–	0.825	12.72	
VIII***	$[F] = \beta_0 + \beta_1 F_{\text{Rb450}} + \beta_2 F_{\text{Trp}}^2$	497 (± 237)	-1.45 (± 0.96)	-0.01*** (± 0.001)	–	0.853	12.14	
IX***	$[F] = \beta_0 + \beta_1 F_{\text{Rb450}} + \beta_2 F_{\text{Trp}}^2 + \beta_3 F_{\text{Rb450}}^2$	44003* (± 13668)	349* (± 109.1)	-0.01*** (± 0.001)	0.69* (± 0.22)	0.924	9.15	

^a N = 15, R^2 , determination coefficient; SEP (mg/100 g protein), standard error of prediction corrected for average; β_0 , β_1 , β_2 , β_3 , prediction coefficients ($\pm$ Standard error), ***significant at 99.9 %; ** significant at 99 %; * significant at 95 %; $[F]$ (mg/100 g protein), furosine concentration; F_{Rb267} , riboflavin fluorescence intensity excited at 267 nm; F_{Rb450} , riboflavin fluorescence intensity excited at 450 nm; F_{MC} , Maillard intermediate compounds fluorescence; $F_{\text{Trp}}/F_{\text{Rb450}}$, tryptophan fluorescence intensity/riboflavin fluorescence intensity excited at 450 nm; $F_{\text{MC}}/F_{\text{Rb450}}$, Maillard intermediate compounds fluorescence intensity/riboflavin fluorescence intensity excited at 450 nm; F_{Trp}^2 , squared tryptophan fluorescence intensity; F_{MC}^2 , squared Maillard intermediate compounds fluorescence intensity and F_{Rb450}^2 , squared riboflavin fluorescence intensity excited at 450 nm.

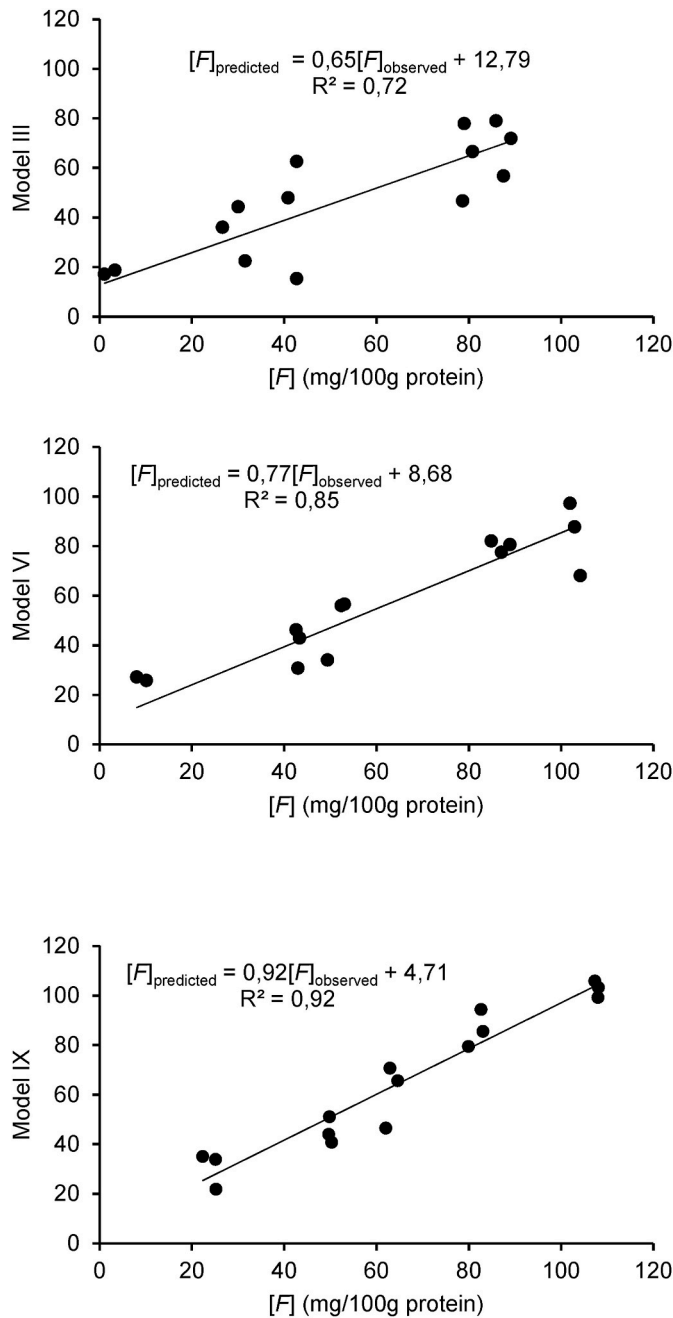


Fig. 4. Regression between experimental and estimated values for furosine concentration $[F]$ at 70 °C (Model III), 80 °C (Model VI) and 90 °C (Model IX).

Table 9

Validation of prediction models for furosine concentration.

Prediction models	R^2	CV	SEP
VII*** $[F] = \beta_0 + \beta_1 F_{\text{Trp}}^2$	0.77	19.46	12.73
VIII*** $[F] = \beta_0 + \beta_1 F_{\text{Rb450}} + \beta_2 F_{\text{Trp}}^2$	0.76	18.53	12.12
IX*** $[F] = \beta_0 + \beta_1 F_{\text{Rb450}} + \beta_2 F_{\text{Trp}}^2 + \beta_3 F_{\text{Rb450}}^2$	0.87	13.97	9.14

$[F]$ (mg/100 g protein), furosine concentration; R^2 , determination coefficient; CV (%), coefficient of variation; SEP (mg/100 g protein), standard error of prediction; F_{Trp}^2 , squared tryptophan fluorescence intensity; F_{Rb450} , riboflavin fluorescence intensity excited at 450 nm; F_{Rb450}^2 squared riboflavin fluorescence intensity excited at 450 nm.

furosine formation reaction obtained were within the range of magnitude described in the literature. The temperature coefficient, Q_{10} , values obtained between 70 and 80 °C and between 80 and 90 °C were similar to those found in the literature.

Kinetic changes induced by heat treatment in milk fluorophores were for the most part inconclusive, except for tryptophan fluorescence changes measured at 90 °C, which seemed to follow a zero kinetic order. These changes could be attributed to whey protein denaturation and subsequent protein-protein interaction.

Several linear correlations were observed between $[F]$ and various fluorescent markers at the different temperatures studied. Prediction models for $[F]$ using front-face fluorescence measurements alone were obtained at each temperature level, which clearly indicates the influence of the temperature on each of the markers studied and strongly suggest the potential for inline front-face determination of both furosine formation and thermal damage of milk during heat treatment. The mentioned potential of the front-face measurements and the inconclusive trends observed during milk heat treatment for some of the studied markers such as Maillard intermediate compounds and riboflavin warranty further studies.

CRedit authorship contribution statement

C. González-Gallardo: Writing – review & editing, Writing – original draft, Methodology, Formal analysis. **N. Ayala:** Resources, Project administration, Investigation. **F. Ali:** Methodology, Investigation, Formal analysis. **A. Zamora:** Validation, Methodology, Data curation, Conceptualization. **J. Saldo:** Methodology, Data curation. **M. Castillo:** Visualization, Validation, Supervision, Software, Resources, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

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