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Prediction of riboflavin and ascorbic acid concentrations in skimmed heat-treated milk using front-face fluorescence spectroscopy

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ABSTRACT

The objective of this work was to obtain degradation kinetic models of riboflavin and ascorbic acid, and to evaluate the potential of front-face fluorescence (FFF) spectroscopy to predict their concentration in milk after thermal processing. A factorial design with three temperatures (70, 80 and 90 °C) and nine times (from 0 to 60 min) of heat treatment was used. Degradation kinetics of both vitamins were of first order. Predictive kinetic models using the Arrhenius equation had a variability coefficient of 2.26 and 3.64% for riboflavin and ascorbic acid, respectively. With fluorescent predictors such as tryptophan, Maillard compounds and riboflavin, prediction models with coefficients of variation smaller than 3.57% for riboflavin and ranging from 4.24 to 14.25% for ascorbic acid were obtained. In conclusion, FFF is a viable alternative to predict riboflavin and ascorbic acid content of milk which could allow in-line monitoring and control of thermal damage.

1. Introduction

Bovine milk is an excellent source of vitamin B_2, which can occur as free riboflavin or in the form of one of the two coenzymes, flavin mononucleotide and flavin adenine dinucleotide (Koop et al., 2014). Much of riboflavin in milk is present as free riboflavin in up to 1 mg/L and so milk is one of the main contributors to the dietary intake of riboflavin (Saedisomeolia and Ashoori, 2018), fulfilling recommended allowance of 1.1 and 1.3 mg for adult females and males, respectively (Nohr et al., 2011). This vitamin is characterized by being the most heat resistant of the water-soluble family, but it is very labile to the action of visible and ultraviolet light (Lešková et al., 2006). Conventional sterilization causes losses of up to 18%, in skimmed milk (Nohr et al., 2011), whilst in whole milk, the destruction percentage does not exceed 10% (Veisseyre, 1988). This difference could probably be due to the fact that riboflavin would be more protected in whole milk than in skimmed milk, because fat globules diminish heat transfer (Fox et al., 2015).

Like riboflavin, vitamin C or ascorbic acid is an essential vitamin, which fulfils vital processes in the human body. This compound has an antioxidant function that delays or inhibits oxidation, reduces the concentration of free radicals and provides protection against diseases (arthritis, atherosclerosis, cancer, diabetes, ischemia, etc.) that involves oxidative stress (Rajendran et al., 2014). It is ingested mainly through a diet rich in fresh fruits and vegetables; however, milk is not an important source of the recommended daily intake (Morrissey and Hill, 2011). Being the most unstable and labile vitamin, ascorbic acid is very easily altered not only by temperature, but also by the action of light, changes in pH, and metal ions (Morrissey and Hill, 2011). Thus, in the food industry, ascorbic acid is considered as an index of nutrient retention and an indicator of thermal damage (Sun et al., 2012).

There are several techniques to quantify these two vitamins in milk, being the chromatographic methods the most widely used and in particular high-performance liquid chromatography (HPLC) technique (Ardö et al., 2011; Romeu-Nadal et al., 2006; Bueno-Solano et al., 2009; Poulsen et al., 2015). Most of these methods are accurate, but require sophisticated laboratory implementation, trained personnel, large sample amount, complex sample preparation, appreciable amounts of reagents and long analysis times (Huang et al., 2021). Currently, the food industry needs to develop fast and inexpensive alternative methods. In this sense, electrochemical sensors are being developed to quantify vitamins in foods (Huang et al., 2021). However, owing to the fact that other alternative methods providing real-time information could guarantee process control to ensure food safety and security, optical methods, such as NIR and fluorescence spectroscopies, are gaining

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more interest (Shaikh & O'Donnell, 2017; Pu et al., 2020). In particular, front-face fluorescence spectroscopy (FFF), which measures the fluorescence on the surface of the analyzed sample, can be directly applied to cloudy samples such as milk, showing great application potential (Karoui and Blecker, 2011). So far, several markers of thermal damage, such as furosine, lactulose and retinol have been estimated using FFF detecting fluorophores such as tryptophan, dityrosine, Maillard's compounds and riboflavin found in milk (Kulmyrzaev and Dufour, 2002; Ayala et al., 2017; Liu et al., 2018).

As was previously mentioned, both riboflavin and ascorbic acid could play a major role in evaluating the thermal damage of milk. However, to the best of our knowledge, no kinetic studies about riboflavin and ascorbic acid degradation due to heat treatment of milk have been published. However, two studies have shown that it is feasible to quantify both vitamins using FFF. Diez et al. (2008) built mathematical models that allowed estimating ascorbic acid with a relative error of 12% during heat treatment at seven temperatures (72–115 °C) and six different times (2–9.5 min) in infant milk formula serum-based. Recently, Alvarado et al. (2019) also developed and validated a mathematical model for the prediction of riboflavin concentration in milk. The validation of the model yielded a standard error of prediction of 0.13 mg/L and a coefficient of variation of 4.14%. Finally, the riboflavin concentration prediction model based on FFF data developed by Alvarado et al. (2019) was compared with the concentration measured using two other HPLC conventional techniques. Results obtained with commercial milk showed no significant differences between the vitamin concentration estimated using FFF and HPLC. Thus, both investigations suggest the great potential of FFF as it is a simple and fast method compared to conventional measurements.

Due to the lack of studies of ascorbic acid and riboflavin degradation kinetics in heated milk and the need to lay the foundations for the development of an optical sensor that allows monitoring and evaluating thermal damage in milk, the present study proposes to develop kinetic prediction models of the loss of riboflavin and ascorbic acid in milk during heat treatment and its evaluation with fluorescent markers applying FFF.

2. Materials and methods

2.1. Sample preparation

The study was carried out with standard skimmed milk powder dried by atomization, of high functional and microbiological quality (extra-grade, "low heat"), supplied by Chr. Hansen S.L. (Barcelona, Spain). The milk was reconstituted at 12% (w/w) with distilled water tempered at 40 °C, magnetically stirring for 15 min at 150 rpm (Agimatic-E, JP. Selecta, Barcelona, Spain). It was then allowed to stand for 30 min at room temperature in the dark to facilitate proper hydration of the milk constituents.

In order to study the kinetic evolution of riboflavin and AA, heat treatments were applied to 5 mL samples of milk, using Pyrex test tubes of 30 mL capacity, tightly sealed and immersed in a thermostatic bath (OvanTherm model TC00E C, Suministros Grupo Esper SL, Badalona, Spain). The following conditions were evaluated: three levels for temperature (70, 80 and 90 °C) with nine levels for time (0, 5, 10, 15, 20, 25, 30, 45 and 60 min). At the different treatment time intervals, the samples were removed from the bath and placed in cold water until reaching room temperature to measure fluorescence, while for the kinetic study of the vitamins they were frozen at −30 °C until their subsequent chemical analysis.

2.2. Fluorescence determination

Spectroscopic fluorescence measurements were performed in triplicates in Suprasil quartz cuvettes (Agilent Technologies, Madrid, Spain) with a spectrofluorometer model Cary Eclipse (Agilent Technologies). This device was equipped with a 15 W xenon pulsed lamp and an accessory with "front-face" geometry adjusted to an angle of incidence of 35°, which minimizes both the phenomena of specular reflection by the surface of the cuvette and of internal absorption by the sample.

Excitation and emission wavelengths were selected based on the fluorophore to be determined, using a 5 nm slit in all cases. Tryptophan fluorescence spectrum (F_{TRP}) was scanned at 290 nm excitation and the emission range of 300–450 nm. For Maillard compounds (F_{MC}) the excitation wavelength was 330 nm, and between 350 and 500 nm for the emission. Dityrosine (F_{DT}) was read at the excitation wavelength of 315 nm with an emission range from 350 to 500 nm. Finally, riboflavin was excited at 267, 370 and 450 nm (F_{Rb267}, F_{Rb370} and F_{Rb450} respectively) with a wavelength emission range of 470–570 nm.

The maximum fluorescence intensity value of each fluorophore was used to construct the mathematical prediction models using fluorescent markers.

2.3. Chemical quantification of vitamins

2.3.1. Riboflavin

Riboflavin concentration was analyzed by the method of Albala--Hurtado et al. (1997). Thawed milk samples (5 g) were accurately weighed in a centrifuge tube to which 0.5 g of trichloroacetic acid was added. The mixture was stirred for 10 s and subsequently centrifuged for 15 min at 2,500 rpm (Sigma laboratory centrifuge, 4K-15, SN. 93250, Osterode am Harz, Germany). Supernatants were stored and 0.5 mL of 4% trichloroacetic acid was added to the residual pellets for a second extraction by mixing for 1 min before being centrifuged at 2,500 rpm for 10 min. The two acid extracts were combined in a 10 mL tube and were kept protected from light. Just before injection into HPLC, extracts were filtered through 0.22-μm filters.

A stock solution of 100 mg L^{-1} of riboflavin in the form of flavin mononucleotide (R9504, Sigma Aldrich, Saint Louis, Missouri) prepared in 2.4% (v/v) of acetic acid was used to obtain calibration solutions (0.5, 1.0, 2.0, 4.0 mg L^{-1}), diluting the stock solution in 2.4% aqueous acetic acid. All standard solutions were filtered through a 0.22-μm filter just before injection into HPLC.

Chromatography measurements were performed in triplicate using a Dionex P680 HPLC UV–Vis detector (Dionex, Germering, Germany). The analytical column used was Waters Spherisorb ODS 2 C18, with a particle filling of 5 μm in diameter, 4.6 × 150 mm (Tecknokroma, Barcelona, Spain). Chromatographic separation was carried out under isocratic conditions in a mobile phase of 5 mM octane sulfonic acid, 0.5% triethylamine, 24% glacial acetic acid and 15% methanol. The eluent flow rate was 1.0 mL min^{-1} and the column temperature was 25 °C. Time analysis of each sample was 20 min.

2.3.2. Ascorbic acid

The same equipment with an analytical column Tracer Extraxil ODS 2 C18; 0.46 mm × 250 nm and particles of diameter 5 × 5 mm I.D. (Tecknokroma, Barcelona, Spain) was used for the quantification of ascorbic acid following the method of Romeu-Nadal et al. (2006) with some modifications. Thawed milk samples (1.50 mL) were centrifuged at 12,000 rpm for 15 min (Hettich Universal, Mikro 12–24, Germany). 0.4 mL of the supernatants were carefully extracted, mixed with 1.00 mL of 0.55% (w/v) metaphosphoric acid solution and manually stirred for 30 s. Supernatants obtained by a second centrifugation at 12,000 rpm for 10 min were filtered through a 0.22-μm filter just before injection into HPLC.

Chromatographic separation was performed in triplicate by isocratic elution using a mobile phase of Milli-Q water with acetic acid (0.1% v/v) and methanol in a ratio of 95:5 (v/v). The eluent flow rate was 0.7 mL min^{-1}, the column temperature was 25 °C and the analysis time of each sample was 20 min. Ascorbic acid was identified by comparing the retention time of the sample peak at 254 nm. Quantification was carried out using a calibration curve of ascorbic acid standard solutions (1, 5,

10, 20 and 30 ppm) prepared by dissolving ascorbic acid (Sigma-Aldrich, Spain) in 0.55% (w/v) metaphosphoric acid dissolved with Milli-Q water.

2.4. Kinetic data modeling

Kinetic equations of zero, first and second order were used to fit the data of riboflavin and ascorbic acid degradation during milk heat treatment and obtain corresponding kinetic data through regression analysis using MS Excel software 2013 (Microsoft, USA). Determination coefficients (R^2) were used to select the order of the kinetic model that better explains the degradation kinetics, according to the following equations:

Zero order as

$$[C] = [C]_0 + k_T t$$

First order as

$$ln[C] = ln[C]_0 + k_T t$$

Second order as

$$1 / [C] = 1/[C]_0 + k_T t,$$

where, $[C]_0$ and $[C]$ = initial and final concentration of vitamin respectively (expressed in mg·L^{-1}); k_T = reaction rate as a function of temperature (expressed in different units depending on the reaction order); t = time (expressed in min).

The effect of temperature on the reaction rate was modeled using the Arrhenius equation (Van Boekel, 2008). Furthermore, the following thermal parameters described by Ordóñez et al. (2013) were determined:

Decimal reduction time (D_T) as

$$D_T = \frac{Ln(10)}{k_T}$$

with k_T being the reaction rate.

Half-life time ($t_{0.5}$) as

$$t_{0.5} = \frac{Ln(0.5)}{k_T}.$$

Thermal coefficient (Q_{10}) as

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

with k_1 *and* k_2 corresponding to the rate constants at T_1 = 70 °C and T_2 = 90 °C, respectively.

Thermal resistance constant or *z* value

$$z = \frac{10Ln(10)}{LnQ_{10}}.$$

Arrhenius law

$$k_T = A_0 e^{-E_a/RT}$$

where A_0 is the constant or pre-exponential factor, E_a the activation energy of the process (expressed in J·mol^{-1}), R is the general gas constant (8.3141 J $mol^{-1}K^{-1}$) and, finally, T is the absolute temperature expressed in K (°C + 273).

2.5. Prediction models using fluorescent compounds

Data were processed and analyzed using "Statistical Analysis System" (SAS, version 9.2, 2009, SAS Institute Inc, Cary, NC, USA). Pearson's correlation coefficients (r) were determined by the correlation procedure (CORR). The maximum R^2 procedure (REG, MAXR) was utilized to obtain the best one, two- and three-parameter models for predicting riboflavin concentration with F_{Trp}, F_{Dt}, F_{MC}, F_{Rb267}, F_{Rb370} and F_{Rb450}. The procedure to obtain the prediction models of the two vitamins was correlated with the maximum fluorescence intensities obtained from the emission spectra for each fluorescent marker.

3. Results and discussion

3.1. Effect of heat treatment on riboflavin and ascorbic acid degradation

Fig. 1a shows the effects of heat treatment on riboflavin content of reconstituted skimmed milk. An initial concentration of 1.72 mg L^{-1} was found in non-heat-treated milk, equal to that found by Poulsen et al. (2015). Variations of riboflavin in fresh cow milk are mainly due to breed and/or biotypes, diet, and climate effects (Fox et al., 2015). At the highest temperature evaluated (90 °C), riboflavin loss was 2.99 and 22.55% after 5 min and 60 min, respectively, showing that this vitamin is not very sensitive to heat. Meha (1994) found decreases of 2 and 4% of riboflavin during heat treatments for 30 min at 80 and 90 °C, respectively, in whole milk; values that are lower than the results of the present study. This difference could be due to the fact that riboflavin shows higher degradation in high-fat milk than in skim milk (Fox et al., 2015). Saad (1980), cited by Meha (1994), found 12% loss of riboflavin as a result of low pasteurization (63 °C, 30 min). Such loss was only observed at 90 °C for 30 min in the present work. Asadullah et al. (2010) found a decrease of 16.5 and 27% in riboflavin concentration of whole cow milk after heating at 100 °C for 10 and 15 min, respectively.

In many cases, ascorbic acid is considered as an indicator molecule in food processing because it is highly reactive and very sensitive to the physicochemical conditions in the environment (Al Fata et al., 2017). Therefore, although milk is not the main source of this vitamin, it could be used as a potential marker of thermal damage to milk during processing. Fig. 1b shows the effects of time and temperature of heat treatment on the concentration of ascorbic acid in reconstituted skimmed milk. The amount found in non-heat-treated milk was 19.50 mg L^{-1}, which is in the range of concentrations reported by other authors (Esteire et al., 2014; Fox et al., 2015; Nalame et al., 2009). Its concentration depends on the biological conditions (breed, food, etc.) and, possibly, the season of the year, being apparently higher in winter (Tamime, 2009). The loss of ascorbic acid significantly increased ($p < 0.05$) with increasing heat treatment temperature and time.

As mentioned, ascorbic acid is quite thermolabile and is quickly destroyed when the milk is subjected to heat treatment. The results obtained in the present study confirm its thermolability. Meha (1994) observed degradation values of 26 and 36% for treatments of 30 min at 80 and 90 °C, respectively. Damjanovic and Birlouez-Aragon (2011) found losses of up to ~23% due to treatments at 80 °C for 9.5 min in infant food, being therefore slightly greater than our results. In other investigations, the reduction was even greater. For example, Bendicho et al. (2002) found losses of up to ~50% after 30 min treatment at 63 °C in skim milk, while Haddad and Loewenstein (1983) found ~21% loss after treating milk at 80 °C for 16 s. It has been shown that temperature and matrix are important factors that affect the stability of ascorbic acid (Stešková et al., 2006).

3.1.1. Kinetic models for riboflavin degradation

Different kinetic equations for the degradation of riboflavin in milk during heat treatment were obtained (Table 1). The first-order kinetic is the model that had the best fit in all treatments, showing in general the highest R^2. It was not possible to find information about riboflavin thermal degradation in milk, but for other products, disappearance of the riboflavin responds also to a first-order kinetics, like in soy milk (Kwok et al., 1998), whole green soybeans (Nisha et al., 2005a) or spinach (Nisha et al., 2005b).

Table 2 shows the estimated kinetic parameters assuming a first-order for degradation of riboflavin in milk. As expected, the values of

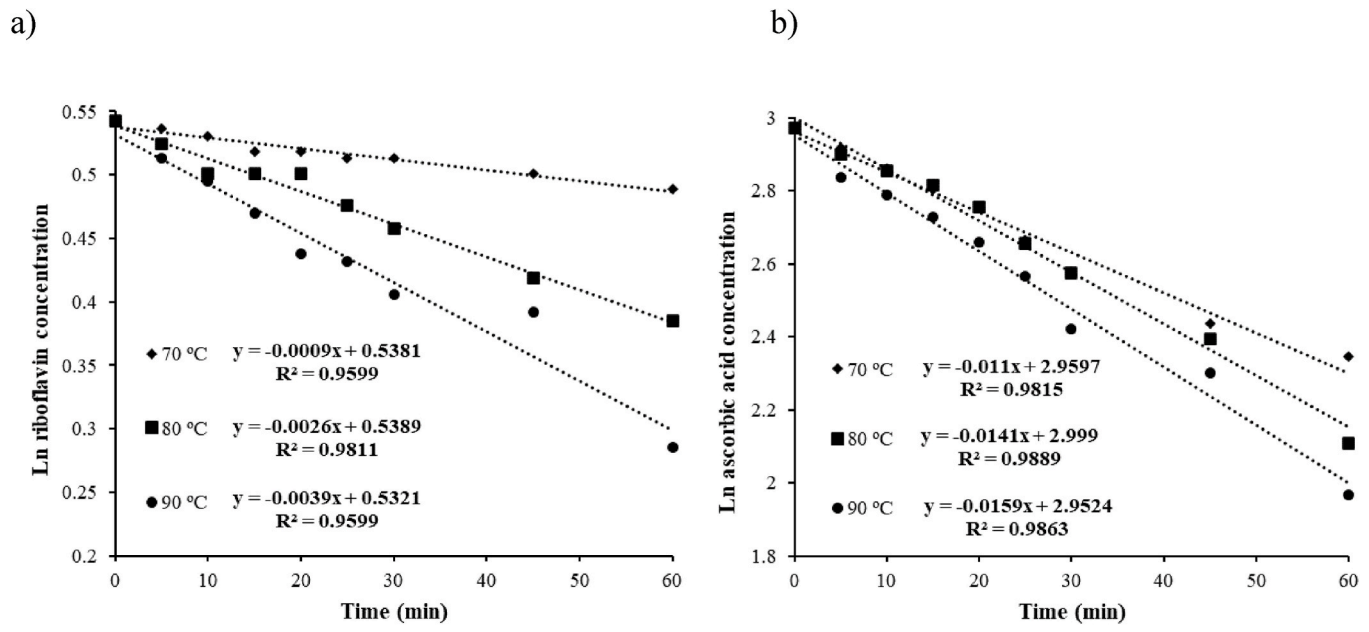

Fig. 1. Thermal degradation of a) riboflavin and b) ascorbic acid in skimmed milk (first order kinetics).

Table 1
Comparison of different kinetic equations of riboflavin and ascorbic acid degradation during heat treatment in skim milk.

Equations		70 °C		80 °C		90 °C		k_T units
		R^2	k_{70}	R^2	k_{80}	R^2	k_{90}	
Zero-order	Rb	0.969	−0.0015	0.971	−0.0042	0.973	−0.0062	$mg{\cdot}L^{-1}{\cdot}min^{-1}$
	AA	0.989	−0.187	0.992	−0.187	0.964	−0.194	
First-order	Rb	0.965	−0.0009	0.983	−0.0027	0.983	−0.0041	min^{-1}
	AA	0.990	−0.011	0.986	−0.014	0.980	−0.016	
Second-order	Rb	0.965	0.0005	0.959	0.0017	0.956	0.0027	$mg^{-1}{\cdot}L{\cdot}min^{-1}$
	AA	0.962	0.001	0.951	0.001	0.947	0.001	

N = 27; number of replicates, Nr = 3; Rb, riboflavin; AA, ascorbic acid; k_T, reaction rate; t, time (min); R^2, coefficient of determination.

Table 2
First order kinetic parameters of riboflavin and ascorbic acid degradation in milk.

Vitamin	T °C	k_T (min^{-1})	D_T (min)	$t_{0.5}$ (min)	Q_{10}	z (°C)	E_a ($kJ{\cdot}mol^{-1}$)
Rb					2.13 ± 0.33	30.41 ± 4.08	75.84 ± 10.59
	70	−0.0009 ± 0.00007	2560.79 ± 203.2	770.87 ± 61.2			
	80	−0.0026 ± 0.00023	864.84 ± 85.2	260.34 ± 25.6			
	90	−0.0039 ± 0.00062	563.11 ± 89.3	169.51 ± 26.9			
AA					1.28 ± 0.004	96.96 ± 7.96	19.07 ± 2,85
	70	−0.0110 ± 0.0002	221.78 ± 8.64	63.08 ± 1.10			
	80	−0.0141 ± 0.0002	160.59 ± 3.28	49.11 ± 0.76			
	90	−0.0159 ± 0.0010	137.93 ± 6.41	43.66 ± 2,90			

N = 27; number of replicates, Nr = 3; Rb, riboflavin; AA, ascorbic acid; k_T, kinetic constant; D_T, decimal reduction time; $t_{0.5}$, half-life; Q_{10}, thermal coefficient; E_a, activation energy; z, z-value.

the reaction constant (k_T) increased with temperature. Fig. 2a shows the relation between Ln k_T and the inverse of absolute temperature. In green soybeans, decimal reduction time (D_T) and half-life ($t_{0.5}$) of riboflavin at 90 °C were 3268 min and 122 min, respectively (Nisha et al., 2005a). The z value found in the present study was slightly lower than that found for thermal degradation of riboflavin in soy milk (36 °C) (Kwok et al., 1998). Using the Arrhenius equation based on a first-order kinetic model with R^2 ~0.95, the obtained activation energy (E_a) was 75.8 kJ mol^{-1}. This value was higher than that found by Singh et al. (1975) for light-induced riboflavin loss in whole milk (~27.5 kJ mol^{-1}), and close to 83.3 kJ mol^{-1} reported for thermal degradation of riboflavin in soy milk (Kwok et al., 1998). Likewise, Galdi et al. (1989) found a wide range of E_a values from 34 to 44 kJ mol^{-1} in infant milk, stored at 20–45 °C up to 12 months.

Few authors have approached the kinetic study of the degradation of riboflavin in milk during heat treatment. However, a considerable variation in the kinetic parameters of this compound has been documented in other foods. For example, E_a was reported to be 21.7 kJ mol^{-1} in spinach (Nisha et al., 2005b), 29.8 kJ mol^{-1} in green soybean (Nisha et al., 2005a) and 37.2 kJ mol^{-1} in rosehip nectar (Kadakal et al., 2018). Thermal degradation of riboflavin at 50–70 °C produced β-keto acid and a dioxo compound as the isoalloxazine ring cleavage products at pH 9–13 (Sheraz et al., 2014). Little information is available regarding riboflavin thermal degradation in aqueous solution. Riboflavin destruction rate increased when oxygen was present during storage and with increasing water activity. Other components in food may also affect

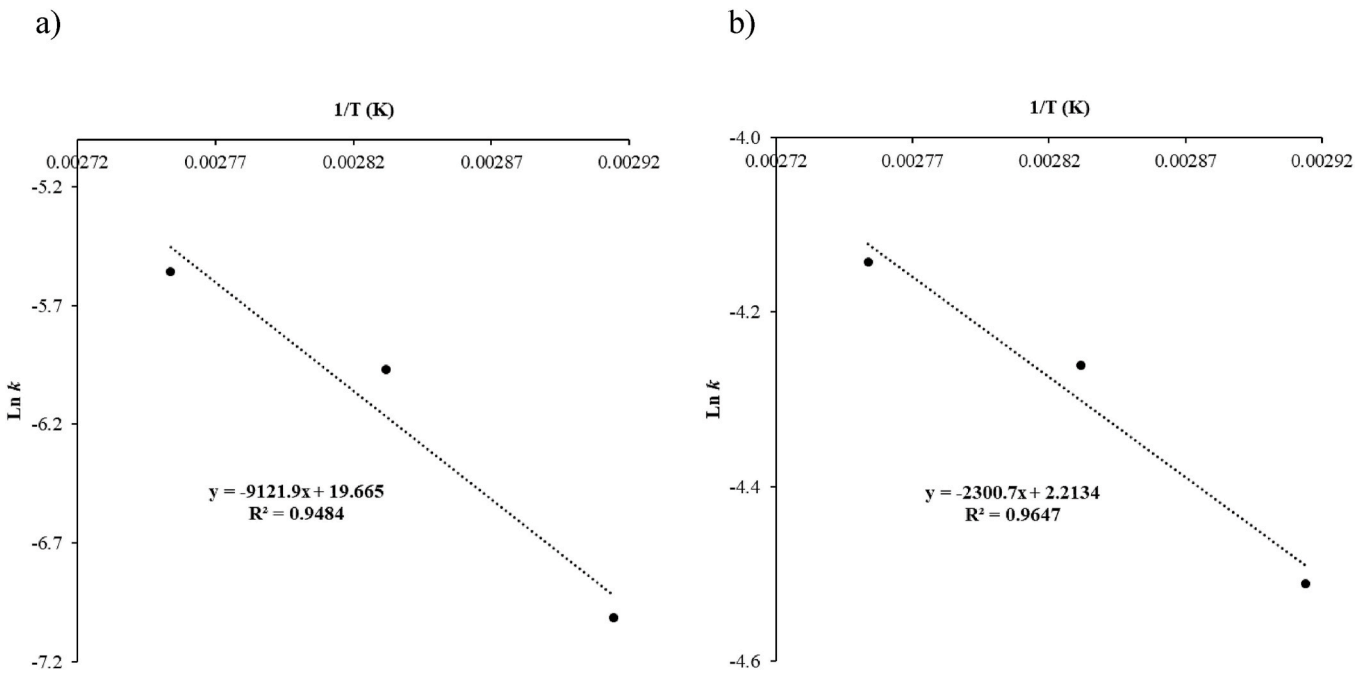

Fig. 2. Effect of temperature (70, 80 and 90 °C) on the degradation rate of a) riboflavin and b) ascorbic acid in skim milk based on Arrhenius model (*k* values used corresponded to first order kinetic models).

the riboflavin degradation rates (Choe et al., 2005). The differences found in the results with respect to those of other studies may be a consequence of temperature ranges evaluated, the chemical composition of food, pH and the presence of light during the production process (Saidi and Warthesen, 1995).

The kinetic equation to predict the concentration of riboflavin as a function of time and temperature is given by the following mathematical model:

$$[Rb]_t = [Rb]_0 \times e^{\left(3.47\times10^8 \times t \times e^{\frac{-9121}{T}}\right)}$$

where $[Rb]_t$ is the concentration of riboflavin at time t ($mg{\cdot}L^{-1}$); $[Rb]_0$, the initial concentration ($mg{\cdot}L^{-1}$); t, time (min); T, temperature (K); the value 3.47×10^8 min^{-1}, is the pre-exponential factor (A_0), determined using Arrhenius equation. With this equation it was possible to predict riboflavin thermal degradation with a standard error of prediction (SEP) and a variation coefficient (CV) of 0.03 mg L^{-1} and 2.26%, respectively

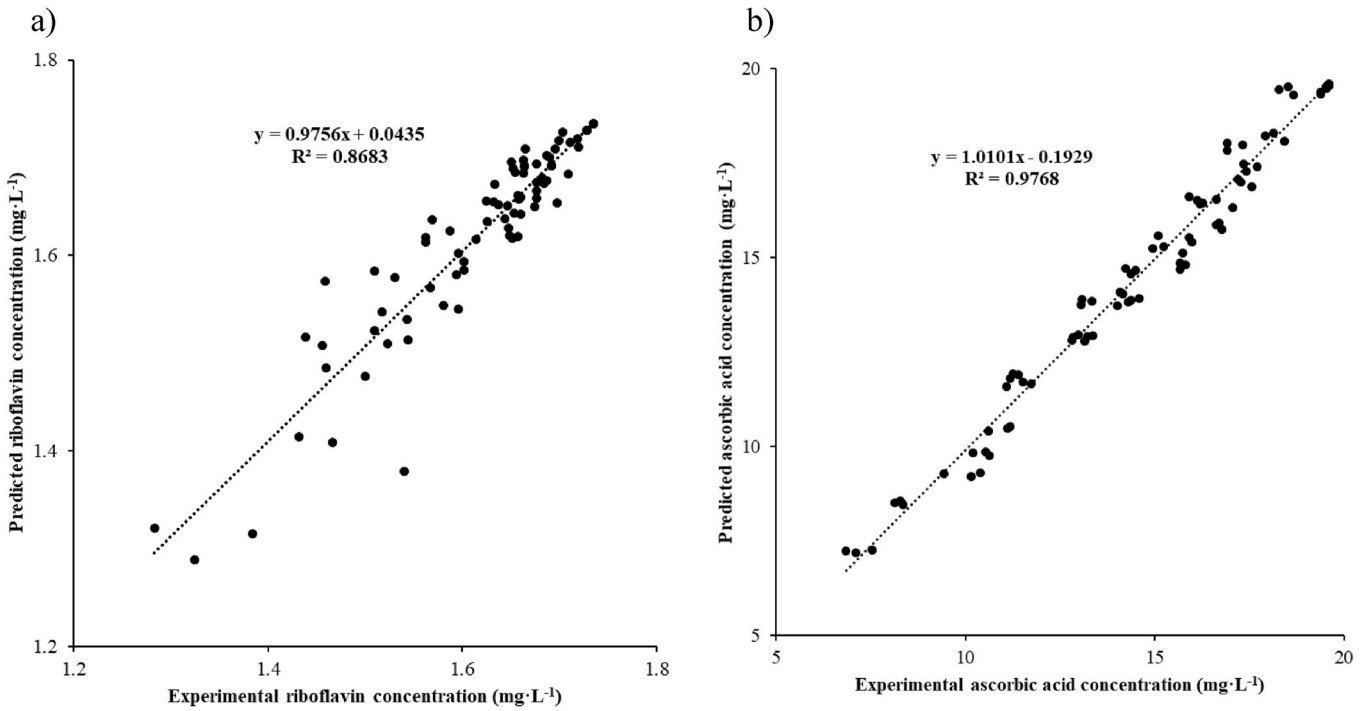

Fig. 3. Concentration of experimental values with respect to those predicted for a) riboflavin and b) ascorbic acid are estimated according to the kinetic model of order one in skim milk.

(Fig. 3a).

The results confirm that riboflavin is quite thermostable since it only degraded in small percentages (e.g., ~22% at 90 °C for 60 min). In the dairy industry, riboflavin has been studied normally during products storage to evaluate its photodegradation. However, results indicate that it can also be a potential marker to monitor thermal damage during high heat treatments.

3.1.2. Kinetic models for ascorbic acid degradation

Comparison of different kinetic equations for ascorbic acid degradation at different temperatures, according to zero, one and two-order models are shown in Table 1. The data confirm that ascorbic acid disappearance follows first-order kinetics (Fig. 2b), in accordance with that obtained, in the same biological matrix, by Bendicho et al. (2002) and in other foods (Karhan et al., 2004; Stešková et al., 2006). Table 2 shows the first-order kinetic parameters of ascorbic acid degradation in milk. The obtained $k_{90\ °C}$ was 0.0159 min^{-1}, much lower than 0.08 min^{-1} reported by Bendicho et al. (2002) but similar to 0.014 min^{-1} observed by (Kadakal et al., 2018) in rosehip nectar. The obtained $t_{0.5}$ at 70 °C was higher than the 49 min found by Ordóñez-Santos and Yoshioka-Tamayo (2012) in mango pulp treated at the same temperature. For D_T, the obtained values were lower than those found by Dhuique-Mayer et al. (2007) at the same temperatures in citrus juice; but, higher than those found in guava fruits, at 75 and 95 °C (2.88 and 1.5 min) (Ordóñez et al., 2013). The z value was higher than those observed in guava (~77 °C) and in mango pulps (66 °C) (Ordóñez-Santos and Yoshioka-Tamayo, 2012; Ordóñez et al., 2013). Furthermore, the Q_{10} value found was similar to that of some fruits e.g. 1.35 in guava (Ordóñez et al., 2013), 1.4 in mango pulp (Ordóñez-Santos and Yoshioka-Tamayo, 2012) and 1.2 in rosehip pulp (Karhan et al., 2004). The obtained E_a was lower than that found by Bendicho et al. (2002) and Damjanovic and Birlouez-Aragon (2011), which reported values of 63.66 kJ mol^{-1} for skim milk and 67.94 kJ mol^{-1} for infant formula respectively. A wide range of E_a values (7.54–125.6 kJ mol^{-1}) were found in liquid foods (Lee and Labuza, 1975). Ascorbic acid can be reversibly oxidized to dehydroascorbic acid (DHAA), without losing vitamin C functionality. Further degradation of DHAA (e.g. to 2,3-diketogulonic acid) results in the loss of biological activity. These conversion reactions are influenced by pH, moisture content, temperature, presence of oxygen and metal ions (Belitz et al., 2004; Mendoza-Corvis et al., 2015). Oxygen is probably the most determining factor in vitamin C degradation, temperature is frequently the accelerating factor, whereas anaerobic degradation occurs mainly when oxygen is depleted (Oey et al., 2008; Hiatt et al., 2010). In thermal experiments, each of the two degradation reactions was found to follow first-order kinetics (Lima et al., 2010; Verbeyst et al., 2013).

Substituting the Arrhenius equation into the first-order kinetic equation, we obtained the following mathematical model to predict the concentration of ascorbic acid at a given time and at a given heat treatment temperature.

$$[AA]_t = [AA]_0 \times e^{\left(9.15 \times t \times e^{\frac{-2300.7}{T}}\right)}$$

Where $[AA]_t$, final concentration of ascorbic acid ($mg{\cdot}L^{-1}$); $[AA]_0$, its initial concentration ($mg{\cdot}L^{-1}$); t, time (min); T, temperature (K); the value 9.15, is the pre-exponential factor (A_0), which is given from the intercept of linear regression. Prediction of ascorbic acid concentration using this equation had SEP = 0.03 mg L^{-1} and CV = 2.26% (Fig. 3b).

From the results obtained, it is confirmed that ascorbic acid is very sensitive to processing by heat treatment. Therefore, it can be used as an indicator to assess thermal nutritional damage in the dairy industry (Sun et al., 2012).

3.2. Prediction of riboflavin and ascorbic acid using fluorescent compounds

3.2.1. Riboflavin prediction

Riboflavin is a fluorescent compound with 3 excitation maxima, reason why it might be complicate to use its fluorescence to directly assess its concentration due to the interference of other self-fluorescent compounds naturally present in milk. Among those, we can highlight some of the most relevant aromatic amino acids as tryptophan, tyrosine and phenylalanine, having this last one a low quantum yield and contributing little to protein fluorescence. Di-tyrosine is a product of proteins oxidative stress, which is also fluorescent and can provide information on changes undergone by proteins. Some other self-fluorescent compounds, as NADH or even chlorophylls, are present in low amounts and have a limited stability.

One-, two- and three-parameter models were obtained from fluorescent markers for prediction of riboflavin concentration at 70, 80 and 90 °C using the maximum R^2 procedure of SAS (Table 3). At 70 °C, F_{MC} and F_{Dt} were the main predictors, both being inversely proportional to the concentration of riboflavin and with Pearson's correlation coefficients of −0.89 ($p < 0.0001$). However, at 80 °C, the main predictors were F_{Trp} and λ_{FTrp} with r values of 0.73 and −0.79 ($p < 0.0001$), respectively. Similarly, at 90 °C, both F_{Trp} and λ_{FTrp} showed the highest r values with respect to riboflavin concentration (0.91 and −0.81 with $p < 0.0001$, respectively). Except in model IV, R^2 values for all prediction models, even for those with only one-parameter, were between 0.80 and 0.86 with SEP below 0.06 mg L^{-1}. These results show that some FFF parameters are good predictors of riboflavin concentration in milk heat-treated at different temperatures.

Table 3 also shows the general mathematical models for the estimation of riboflavin concentration obtained with data from the three temperatures studied. The three-parameter model (XII) included data at all temperatures and is the one with a better fit. This model explained 81% of the variability of riboflavin concentration with a SEP of 0.043 mg L^{-1} (Fig. 4a). Both F_{Trp} and λ_{FTrp} had a high relationship with riboflavin concentration, with $r \geq 0.80$, while the relationship with the third parameter, F_{MC}, was low ($r = 0.26$). Predictor λ_{FTrp} is attributed to the redshift of F_{Trp} due to the denaturation of whey protein as a consequence of exposure of tryptophan residues from a non-polar environment to the aqueous phase of milk (Alvarado, 2017; Ayala et al., 2020). At first, Becker et al. (2003) studied the potential use of FFF with the statistical method partial least squares (PLS) for the estimation of riboflavin in yogurt during storage (5 weeks at 4 °C), constructed a two-component multiple regression model that was attributed to F_{Rb} (450 nm excitation and 530 nm emission) and F_{Trp} (290 nm excitation and 350 nm emission). Multivariate regression showed a correlation of 0.99 and a prediction error of 0.092 μg of riboflavin per g of yoghurt. Christensen et al. (2005) also took advantage of the FFF with the application of parallel factor analysis (PARAFAC) to predict the same vitamin with the same biological matrix and under the same environmental conditions as the previous study. The PARAFAC analysis with the multiple linear regression method showed an R^2 of 0.97, and a prediction error of 0.094 ppm, with F_{Rb} and lumichrom (photochemical degradation of riboflavin) being the main predictors. Recently, Alvarado et al. (2019) established a prediction model based on F_{Rb} with point mediated at 370 nm of excitation and 530 nm of emission to estimate the concentration of riboflavin in commercial milk which was compared with HPLC methods. The validation of the model was $R^2 = 0.99$ and SEP = 0.13 mg L^{-1}. It should be noted that in these investigations there was no prior manipulation of the sample for analysis.

Therefore, all these results suggest the convenience of using FFF spectroscopy as a simple and non-destructive method for online and offline determinations of riboflavin in dairy products.

3.2.2. Ascorbic acid prediction

Using maximum R^2 procedure of SAS, highly significant models with

Table 3
Prediction models of riboflavin concentration in heated milk by means of front-face fluorescence.

Mathematical models	β_0	β_1	β_2	β_3	R^2	SEP	CV	T
I [Rb]*** $= \beta_0 + \beta_1 F_{MC}$	2.40***	−0.001***	–	–	0.80	0.014	0.84	
II [Rb]*** $= \beta_0 + \beta_1 F_{MC} + \beta_2 F_{Dt}$	2.55***	-5.5×10^{-4}	-8.4×10^{-4}	–	0.83	0.013	0.80	70[a]
III [Rb]*** $= \beta_0 + \beta_1 F_{MC} + \beta_2 F_{Dt} + \beta_3 F_{Rb450}$	2.43***	-5.8×10^{-4}	-9.8×10^{-4}*	-8.4×10^{-4}	0.84	0.013	0.78	
IV [Rb]*** $= \beta_0 + \beta_1 \lambda_{FTrp}$	11.1***	−0.027***	–	–	0.62	0.051	3.14	
V [Rb]*** $= \beta_0 + \beta_1 F_{Trp} + \beta_2 F_{MC}$	1.39***	0.013***	−0.002***	–	0.83	0.054	2.81	80[a]
VI [Rb]*** $= \beta_0 + \beta_1 F_{Trp} + \beta_2 F_{MC} + \beta_3 F_{Rb267}$	1.31***	0.015***	−0.002***	2.1×10^{-4}*	0.86	0.032	1.99	
VII [Rb]*** $= \beta_0 + \beta_1 F_{Trp}$	0.98*	0.006*	–	–	0.83	0.049	3.15	
VIII [Rb]*** $= \beta_0 + \beta_1 F_{Trp} + \beta_2 F_{MC}$	1.51*	0.007*	−0.001	–	0.85	0.047	3.00	90[a]
IX [Rb]*** $= \beta_0 + \beta_1 F_{Trp} + \beta_2 F_{MC} + \beta_3 F_{Rb450}$	1.15*	0.007*	−0.001*	0.002	0.86	0.046	2.96	
X [Rb]*** $= \beta_0 + \beta_1 \lambda_{FTrp}$	6.81***	−0.015***	–	–	0.64	0.06	3.57	
XI [Rb]*** $= \beta_0 + \beta_1 F_{Trp} + \beta_2 F_{MC}$	1.87***	0.008***	−0.001***	–	0.72	0.05	3.06	All[b]
XII [Rb]*** $= \beta_0 + \beta_1 F_{Trp} + \beta_2 F_{MC} + \beta_3 \lambda_{FTrp}$	4.07***	0.006***	−0.001***	−0.006*	0.81	0.04	2.65	

*p < 0.05, **p < 0.001, ***p < 0.0001.
[a] N = 27.
[b] N = 81; R, determination coefficient; SEP (mg·L^{-1}), standard error of prediction; CV (%), coefficient of variation; T (°C), temperature; β_0, β_1, β_2, β_3, regression coefficients; [Rb], riboflavin concentration; λ_{FTrp}, maximum intensity wavelength of tryptophan fluorescence; F_{Trp}, F_{MC}, F_{Dt}, F_{Rb267}, F_{Rb450}, maximum fluorescence intensity of tryptophan, Maillard compounds, dityrosine and riboflavin (excited at 267 and 450 nm).

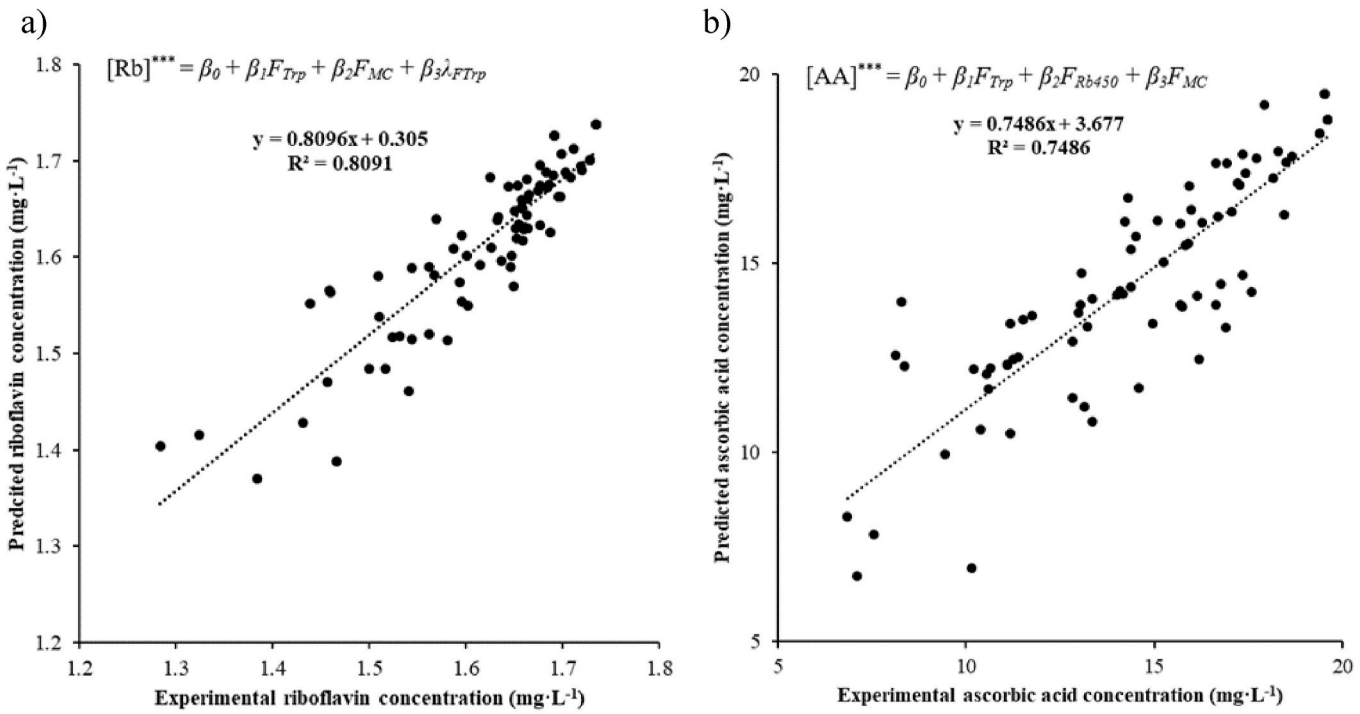

Fig. 4. Relationship between the experimental values of a) riboflavin and b) ascorbic acid versus the predicted values of model XII from Tables 3 and 4, respectively.

high R^2 and small SEP values were obtained for prediction of ascorbic acid concentration at 70 and 90 °C but using different fluorescent markers for each temperature and having as main predictors F_{MC} and F_{Trp}, respectively (Table 4). At 80 °C, the fit of the models was lower because the main predictor (λ_{FTrp}) had a moderate negative correlation coefficient ($r = -0.78$, $p > 0.0001$). This predictor is related to the fact that there is a shift in the maximum wavelength of fluorescence emission throughout the heat treatment, both at 80 and 90 °C, while at 70 °C no change in the wavelength of the maximum fluorescence emission peak is observed. Similar results were found by Ayala et al. (2020) in the same biological matrix. Model I was the best one-parameter model found to predict the concentration of ascorbic acid at 70 °C. It included an intercept (β_0) and the predictor F_{MC}, which had an inverse correlation with ascorbic acid ($r = -0.97$, $p > 0.0001$). The intersection and the predictor were significant, and this simpler model explained the variability of ascorbic acid concentration in 95%, with a SEP of 0.69 mg L^{-1}. Little improvement was achieved by the two or three-parameter models. However, at 90 °C the best model was VIII, which included two predictors to estimate the concentration of ascorbic acid; F_{Trp} and F_{MC} had a positive correlation with ascorbic acid ($r = 0.95$, $p > 0.0001$ and 0.59, $p > 0.001$, respectively). Although it is true that at high temperatures and prolonged times Maillard reactions take place, causing changes in the color, taste and odor of milk, in the present study both at 80 and 90 °C, the F_{MC} shows an increase up to the minute 30 and 5, respectively, and subsequently a decrease which could be related to the disappearance of intermediate compounds and production of advanced stages of the Maillard reaction or, in a lesser extent, to the internal filter effect caused when the fluorophores are in very high concentrations (Geddes and Lakowicz, 2006).

The general models to estimate the concentration of ascorbic acid for the three temperatures studied are shown, also in Table 4. All the mathematical models were highly significant ($p < 0.0001$). It was found that F_{Rb450} and F_{Rb370} were inversely proportional to ascorbic acid concentration with r values of −0.13 and −0.72, respectively. As with

Table 4
Prediction models of ascorbic acid degradation in heated milk by means of front-face fluorescence.

Mathematical models	β_0	β_1	β_2	β_3	R^2	SEP	CV	T
I [AA]*** $= \beta_0 + \beta_1 F_{MC}$	91.22***	−0.11***	–	–	0.95	0.69	4.53	
II [AA]*** $= \beta_0 + \beta_1 F_{MC} + \beta_2 F_{Rb450}$	82.92***	−0.12***	0.05	–	0.95	0.65	4.29	70[a]
III [AA]*** $= \beta_0 + \beta_1 F_{MC} + \beta_2 F_{Rb450} + \beta_3 F_{Trp}$	76.97***	−0.12***	0.05	0.06	0.95	0.65	4.24	
IV [AA]*** $= \beta_0 + \beta_1 \lambda_{FTrp}$	423.3***	−1.19***	–	–	0.62	2.12	14.25	
V [AA]*** $= \beta_0 + \beta_1 F_{Trp} + \beta_2 F_{MC}$	4.56	0.57***	−0.07***	–	0.70	1.39	9.33	80[a]
VI [AA]*** $= \beta_0 + \beta_1 F_{Trp} + \beta_2 F_{MC} + \beta_3 F_{Rb370}$	19.96	0.53***	−0.05*	−0.08	0.85	1.33	8,97	
VII [AA]*** $= \beta_0 + \beta_1 F_{Trp}$	−5.315**	0.22***	–	–	0.91	1.07	7.78	
VIII [AA]*** $= \beta_0 + \beta_1 F_{Trp} + \beta_2 F_{Dt}$	16.75*	0.28***	−0.05*	–	0.94	0.84	6.08	90[a]
IX [AA]*** $= \beta_0 + \beta_1 F_{Trp} + \beta_2 F_{Dt} + \beta_3 \lambda_{Trp}$	36.51	0.27***	−0.05*	−0.05	0.95	0.84	6.14	
X [AA]*** $= \beta_0 + \beta_1 F_{Rb370}$	105.7***	−0.30***	–	–	0.51	1.39	10.13	
XI [AA]*** $= \beta_0 + \beta_1 F_{Rb370} + \beta_2 F_{Rb450}$	76.28***	−0.39***	0.21***	–	0.64	1.06	7.74	All[b]
XII [AA]*** $= \beta_0 + \beta_1 F_{Trp} + \beta_2 F_{Rb450} + \beta_3 F_{MC}$	38.84***	−0.29***	0.04	−0.09***	0.75	0.97	7.05	

*p < 0.05, **p < 0.001, ***p < 0.0001.
[a] N = 27.
[b] N = 81; R, determination coefficient; SEP (mg L^{-1}), standard error of prediction; CV (%), coefficient of variation; T, temperature (°C); β_0, β_1, β_2, β_3, regression coefficients; [AA], ascorbic acid concentration; λ_{FTrp}, maximum intensity wavelength of tryptophan fluorescence; F_{Trp}, F_{MC}, F_{Dt}, F_{Rb370}, F_{Rb450}, maximum fluorescence intensity of tryptophan, Maillard compounds, dityrosine and riboflavin (excited at 370 and 450 nm), respectively.

riboflavin, it was necessary to include three parameters in model XII, which contained data at the three temperatures, to obtain an acceptable goodness of fit ($R^2 = 0.75$, SEP = 0.97 mg L^{-1}) as shown in Fig. 4b. Diez et al. (2008) demonstrated that FFF spectroscopy associated with PLS calibration is a quick and simple method to evaluate the nutritional impact of heat treatment at seven temperatures (72, 80, 87, 95, 100, 110 and 115 °C) for six different times (from 2 min to 9.5 min) in serum-based infant food samples. The model allowed to obtain an estimate of ascorbic acid concentration with a relative error of 12% using F_{Trp} and F_{MC}.

4. Conclusions

Using FFF spectroscopy markers, it was possible to obtain prediction models for predicting of riboflavin and ascorbic acid degradation during milk heat treatment, being tryptophan fluorescence the most important predictor.

The proposed technique to predict and/or quantify riboflavin and ascorbic acid is a viable alternative to control thermal damage but it could also be used to evaluate milk powder. FFF spectroscopy has the advantage of revealing information quickly, directly and non-destructively. In addition, it is friendly to the environment as does not require reagents. Therefore, the implementation is encouraging enough to promote future applications for in-line process monitoring and control.

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