Universitat Autònoma de Barcelona

Application of native fluorescence tracers for quick evaluation of thermal damage in milk

Olga Noemí Ayala Zárate

UNIVERSITAT AUTÒNOMA DE BARCELONA

Department of Animal and Food Science

Faculty of Veterinary

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Olga Noemí Ayala Zárate

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Supervisors:

Manuel Castillo Zambudio (Academic tutor)

Jordi Saldo Periago

Anna Zamora Viladomiu

Universitat Autònoma de Barcelona

Manuel Castillo, associate professor, Jordi Saldo, associate professor, and Anna Zamora, post-doc researcher of Animal and Food Science Department, Faculty of Veterinary of the *Universitat Autònoma de Barcelona*

INFORM:

That Mrs. **OLGA NOEMÍ AYALA ZÁRATE**, MSc. in Quality of Food of Animal Origin, Degree in Human Nutrition, has conducted under our direction and advice the Doctoral Thesis titled: **"Application of native fluorescence tracers for quick evaluation of thermal damage in milk"**, and we consider that it meets the necessary conditions for its Defense by the interested part to opt for the Degree of Doctor in Food Science.

For this to happen, and for the appropriate purposes, we sign it by authorizing his presentation in *Cerdanyola del Vallès*, May 31th, 2018.

Manuel Castillo, PhD. Jordi Saldo, PhD.

Anna Zamora, PhD. The candidate, Noemí Ayala, MSc

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To my husband

To my parents

The world of fluorescence
is a world of beautiful
color. In the darkness all
the ordinary colors of our
daylight world disappear.
Only the intensely
glowing hues of
fluorescent substances
touched by the ultraviolet
beam shine out with
striking clarity.

Sterling Gleason, 1960

Publications

Ayala, N., Rinnan, Å., Zamora, A., Saldo, J., Castillo, M. (submitted May 2018). The effect of heat treatment on the front-face fluorescence spectrum of tryptophan in skim milk. *Food Research International*.

Ayala, N., Zamora, A., González, C., Saldo, J., Castillo, M. (2016). Predicting lactulose concentration in heat-treated reconstituted skim milk powder using front-face fluorescence. *Food Control*, 73, 110-116.

Publications related to the thesis

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Ayala, N., Zamora, A., Juan, B., Saldo, J., Castillo, M. (2017). *Clasificación rápida de tratamientos térmicos en leche mediante fluorescencia front-face de riboflavina*. IX Congreso CYTA-CESIA, Badajoz, 16 to 19 May, 2017.

Ayala, N., Zamora, A., Saldo, J., Castillo, M. (2017). *Discriminación rápida de tratamientos térmicos en leche desnatada*. IX Congreso CYTA-CESIA, Badajoz, 16 to 19 May, 2017.

Ayala, N., Zamora, A., Saldo, J., Castillo, M. (2016). *Classification of heat-treatment intensity in skim milk using discriminant analysis*. Food Factor I Conference, Barcelona, Spain, 2 to 4 November, 2016.

Alvarado, U., **Ayala**, **N**., Zamora, A., Saldo, J., Castillo, M. (2016). *Prediction of riboflavin concentration in heat-treated skim milk using front-face fluorescence*. Food Factor I Conference, Barcelona, Spain, 2 to 4 November, 2016.

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Zamora, A., **Ayala, N**., González, C., Rocha, A., Takerta, H., Saldo, J., Castillo, M. (2015). Application of native fluorescence tracers for quick quantification of thermal damage during milk processing. *VIII Congreso CYTA-CESIA, Badajoz,* 7 to 10 April, 2015

Zamora, A., Hebishy, E., Rocha, A., Takerta, H., González, C., **Ayala, N**., Gallardo, J., Saldo, J., Castillo, M. (2015). *Application of native fluorescence tracers for quick quantification of thermal damage during milk processing*. 12th International Congress on Engineering and Food (ICEF12), Québec, Canada*,* 14 to 18 June, 2015.

Ayala, N., Zamora, A., Liu, J., Saldo, J., Castillo, M. (2015). *Predicting lactulose concentration in heat-treated milk using front-face fluorescence*. 29th EFFoST International Conference, Athens, Greece, 10 to 12 November, 2015.

González, C., **Ayala, N**., Liu, J., Juan, B., Zamora, A., Saldo, J., Castillo, M. (2015). *Kinetic modeling of furosine formation during heat-treatment of milk and its correlation with autofluorescence markers*. 29th EFFoST International Conference, Athens, Greece, 10 to 12 November, 2015.

Zamora, A., **Ayala, N**., Gràcia-Julia, A., Rocha, A., Taterka, H., González, C., Gallardo, J., Saldo, J., Castillo, M. (2015). *Rapid quantification of milk damage during milk processing using native fluorescence tracers*. 29th EFFoST International Conference, Athens, Greece, 10 to 12 November, 2015.

Abstract

Different heat treatments are applied in milk during industrial processing in order to extend its shelf-life. However, organoleptic, functional and nutritional properties of milk can be altered by those treatments. The changes produced can be monitored analyzing the intrinsic fluorophores of milk using front-face fluorescence, which is a cheap, rapid and non-invasive technique. Since front-face fluorescence is suitable for turbid samples, this technique could be easily implemented inline. The main objective of this thesis was to study the changes produced in milk, by thermal processing, on the concentration of chemical markers and the response of intrinsic fluorescent markers for quick characterization of heat treatments.

Lactulose and furosine concentrations were chemically quantified in milk treated at different heat loads. Kinetics of both chemical markers, which followed a pseudo-zero reaction order, allowed estimating the activation energy and kinetic rate constant of the corresponding formation reactions. In parallel, fluorescent markers, such as tryptophan, Maillard compounds, riboflavin and dityrosine, were analyzed with front-face fluorescence. Changes induced by heat treatments on the fluorescent spectra of the different markers allowed to discriminate the different milk samples using Principal Component Analysis. Specifically, the response of tryptophan fluorescence was characterized. An increase in the intensity of the heat-treatment load induced both a decrease in tryptophan fluorescence intensity and a red shift. Consequently, tryptophan showed a remarkable potential as heat damage marker.

In addition, correlations between the concentration of both lactulose and furosine and the emission intensity of selected fluorescent compounds were evaluated. Prediction models for these two chemical markers were successfully obtained and validated using fluorescent markers as predictors. Commercial milk samples from different brands were purchased and analyzed in order to classify them according to their fat composition and heat treatment intensity. Particularly, pasteurized samples were allocated in a separated group on tryptophan Principal Component Analysis score-plot while UHT milk formed a different group partially overlapped with other categories.

The results obtained in this PhD dissertation contribute to establish the bases for further development of front-face florescence methodology as a quick characterization tool for the evaluation of heat damage in milk at industrial level.

Resumen

Durante el procesamiento industrial de la leche se aplican diferentes tratamientos térmicos para prolongar su vida útil. Sin embargo, las propiedades organolépticas, funcionales y nutricionales de la leche pueden verse alteradas debido a estos tratamientos térmicos. Dichas alteraciones pueden ser detectadas con el análisis de los fluoróforos intrínsecos de la leche mediante fluorescencia "*front-face*", una técnica barata, rápida y no invasiva. La fluorescencia "*front-face*" al ser adecuada para el análisis de muestras turbias, podría implementarse fácilmente para el análisis en línea. El objetivo principal de esta tesis fue estudiar los cambios inducidos, por el procesamiento térmico, en la concentración de marcadores químicos y la respuesta de marcadores fluorescentes intrínsecos de la leche para la caracterización rápida de los tratamientos térmicos.

Las concentraciones de lactulosa y furosina se cuantificaron químicamente en leche tratada térmicamente a diferentes intensidades. Las cinéticas de los dos marcadores químicos, que siguieron una cinética de reacción de orden pseudo-cero, permitieron estimar la energía de activación y la constante de velocidad de reacción. En paralelo, marcadores fluorescentes como el triptófano, los compuestos de Maillard, la riboflavina y la ditirosina se analizaron mediante fluorescencia "*front-face*". Los cambios inducidos por los tratamientos térmicos en los espectros de fluorescencia permitieron discriminar las diferentes muestras de leche mediante el Análisis de Componentes Principales. Específicamente, se caracterizó la respuesta de la fluorescencia del triptófano. Un aumento en la intensidad del tratamiento térmico indujo tanto una disminución en la intensidad de fluorescencia como un desplazamiento del pico máximo de emisión hacia una mayor longitud de onda. En consecuencia, el triptófano mostró un potencial notable como marcador de daño por calor.

Adicionalmente, se evaluaron las correlaciones de las concentraciones tanto de lactulosa como de furosina con la intensidad de emisión de los compuestos fluorescentes seleccionados. Se obtuvieron y validaron modelos de predicción para ambos marcadores químicos utilizando los marcadores fluorescentes como predictores. Se compraron y analizaron leches comerciales de diferentes marcas para clasificarlas según su composición de grasa e intensidad del tratamiento térmico. Particularmente, las muestras pasteurizadas se distribuyeron en grupos de acuerdo con el Análisis de Componentes Principales del triptófano, mientras que las muestras UHT se distribuyeron en un grupo parcialmente solapado con otras categorías.

Los resultados obtenidos en esta tesis contribuyen a establecer las bases para el desarrollo del método de fluorescencia "*front-face*" como una herramienta de caracterización rápida para la evaluación del daño térmico en leche tratada a nivel industrial.

Resum

Durant el processament industrial de la llet són aplicats diferents tractaments tèrmics per tal d'ampliar la seva vida útil. Malgrat això, les propietats organolèptiques, funcionals i nutritives de la llet poden ser alterades degut a aquests tractaments. Aquets canvis generats es poden monitoritzar a partir de l'anàlisi dels fluoròfors intrínsecs de la llet utilitzant la fluorescència "*front-face*", que és una tècnica barata, ràpida i no invasiva. La fluorescència "*front-face*" és adequada per a mostres tèrboles, pel que es podria implementar fàcilment en línia. L'objectiu principal d'aquesta tesi ha estat estudiar els canvis produïts en la llet degut al tractament tèrmic en la concentració de marcadors químics i la resposta de marcadors fluorescents intrínsecs per a la caracterització ràpida dels tractaments tèrmics.

Les concentracions de lactulosa i furosina s'han quantificat químicament en llet tractada a diferents intensitats de tractament de calor. Les cinètiques d'ambdós productes químics, les qual van seguir una reacció d'ordre pseudo-zero, van permetre estimar l'energia d'activació i la constant de velocitat de reacció. En paral·lel, es van analitzar amb fluorescència "*front-face*" marcadors fluorescents, com triptòfan, compostos de Maillard, riboflavina i ditirosina. Els canvis induïts per tractaments tèrmics sobre els espectres fluorescents dels diferents marcadors van permetre discriminar les diferents mostres de llet utilitzant l'Anàlisi de Components Principals. Concretament, es va caracteritzar la resposta de la fluorescència de triptòfan. L'augment de la intensitat del tractament tèrmic va induir una disminució de la intensitat de la fluorescència de triptòfan i així com un desplaçament de la longitud d'ona d'emissió del pic màxim cap al vermell. En conseqüència, el triptòfan va mostrar un potencial notable com a marcador de danys tèrmics.

Addicionalment, s'han avaluat correlacions entre la concentració de lactulosa i de furosina i la intensitat d'emissió dels compostos fluorescents seleccionats. Es van obtenir i validar models de predicció d'aquests dos marcadors químics utilitzant marcadors fluorescents com a predictors. Es van comprar i analitzar mostres comercials de llet de diferents marques per classificar-les segons la seva composició de greix i la intensitat del tractament tèrmic. Particularment, les mostres pasteuritzades es van distribuir en grups d'acord amb l'Anàlisi de Components Principals del triptòfan, mentre que les mostres UHT formaven un grup diferent parcialment superposat amb altres categories.

Els resultats obtinguts en aquesta tesi doctoral contribueixen a establir les bases per a un major desenvolupament del mètode de fluorescència "front-face" com a eina de caracterització ràpida per a l'avaluació del dany tèrmic en la llet tractada a nivell industrial.

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List of abbreviations

A	Arrhenius equation collision factor
ANOVA	Analysis of variance
CORR	Correlation
Dt	Dityrosine
E_a	Activation energy
Eqn	Equation
FAST	Fluorescence of Advanced Maillard products and Soluble Tryptophan
F_{Dt}	Dityrosine fluorescence intensity
FFF	Front-face fluorescence
F_{MC}	Maillard Compounds fluorescence intensity
F_{Rb267}	Fluorescence intensity of riboflavin excited at 267 nm
F_{Rb370}	Fluorescence intensity of riboflavin excited at 370 nm
F_{Rb450}	Fluorescence intensity of riboflavin excited at 450 nm
F_{Trp}	Tryptophan fluorescence intensity
$[Fu]$	Furosine concentration
GLM	General linear model
HHST	Higher heat, shorter time
HPLC	High performance liquid chromatography
HTST	High temperature, short time
k	Reaction rate constant
k_{ref}	Reaction rate constant at the reference temperature
$[L]$	Lactulose concentration
$[L]_E$	Experimental lactulose concentration
$[L]_T$	Theoretical lactulose concentration
LSM	Least square means
MC	Maillard compounds
MIXED	Linear repeated measures procedure
NLIN	Non-linear regression
PCA	Principal component analysis
Phe	Phenylalanine
$[Q]$	Quencher concentration
Q_{10}	Temperature coefficient
R	Universal ideal gas constant
Rb	Riboflavin
SAS	Statistical Analysis System
T	Reaction temperature
t	Reaction time
T x t	Temperature and time interaction

T_{ref}	Reference temperature
Trp	Tryptophan
Tyr	Tyrosine
UHT	Ultra high treatment
α-La	α-Lactalbumin
β-Lg	β-Lactoglobulin
ΔI	Total fluorescence intensity
ΔI_F	Variation in the fluorescence intensity due the peak height
ΔI_λ	Variation in the fluorescence intensity due the peak shift
$\Delta\lambda$	Shift of the fluorescence emission wavelength
κ-CN	κ-casein
λ_{max}	Maximum emission wavelength

CHAPTER 1:
Rationale and significance of the study

Milk composition is affected by several factors such as stage of lactation, breed differences, number of calvings, seasonal variations, age and health of the animal, feed and management effects including number of milking per day and herd size (Laben, 1963; Bansal et al., 2003; Walker et al., 2004; Jenkins & McGuire, 2006; FAO, 2013).

Heat-treatment is another important source of variation that affects both milk quality and composition. It is applied in dairy process as it is still the most efficient and suitable preservation method (Elliott et al., 2003). The effects of heat processing are very important for the final product properties, since it induces modifications that affect the functional and technological characteristics as well as the sensory and nutritional quality of milk and dairy products (Burton, 1984).

For the production of high quality milk, it is necessary to know the changes suffered by its components as a result of milk heat treatment (Berg, 1993). One of the most significant effects is the denaturation of whey proteins, which produces soft and high moisture enzymatic gels that are not suitable for cheese manufacturing. However, whey protein denaturation is particularly suitable for yogurt manufacture, enhancing its textural properties and minimizing wheying-off. Excessive heat treatment also induces other undesirable effects such as browning reactions, altered taste, reduction of the nutritional value, and inactivation of bacterial growth inhibitors (Mungkarndee et al., 2016).

Although several different methods to analyze heat damage in milk are available, such as chromatography, spectroscopy, colorimetry, etc., (Chen et al., 2005), most of these methods are expensive, very time consuming and/or not practical for inline application. The use of the front-face fluorescence is increasingly gaining popularity in analysis of food to monitor different aspects such as structural modifications of proteins, progress of Maillard reaction, vitamins degradation, etc. (Nielsen et al., 1985; Herbert et al., 1999; Leclère & Birlouez-Aragón, 2001; Delgado-Andrade el al., 2006; Diez et al., 2008; Ni et al., 2011).

Front-face fluorescence spectroscopy is a rapid and sensitive method to control the molecular changes. Also, the front-face fluorescence method is used as a non-destructive technique to provide information about fluorescence molecules present in milk, about milk heat treatments, or even about possible frauds such as the addition of milk powder to liquid milk (Christensen et al., 2005; Brandao et al., 2017; Kamal & Karoui, 2017). Taking into account all the above-mentioned

advantages of this method, this study focuses on setting some of the bases required for future inline determination of the heat damage in milk, using front-face fluorescence as tool. This technique would allow monitoring the quality of milk in the dairy industry and avoiding losses due to low quality of the final product.

CHAPTER 2:
Literature review

2.1. Introduction

Dairy industry moves billions of dollars in the world (IDF, 2015). Taking into account all species worldwide milk production in 2014 (802 million tonnes), ~83% (663 million tonnes) corresponded to cow milk. Near 25% of this cow milk (159.12 million tonnes) was produced in the EU-28. In the same year, the production of cow milk increased by 3.3 % (in comparison with 2013), which represented an increase of ~32.732 millions of dollars, just in the UE-28 (International Dairy Federation, 2015). Every single European Union Member State is a milk producer without exception. Furthermore, in the EU, milk is the number one single product in terms of value with ~15% of the agricultural output.

Moreover, the EU is the major player in the world dairy market as the leading exporter of many dairy products, most notably cheeses (European Commission, 2016). The majority of the dairy companies presented an increase in their turnover, which was mainly due to the increase in milk production and dairy products in the world market (Eurostat Statistical books, 2015). According to the IDF report (International Dairy Federation, 2015), milk production will increase up to 1000 million tonnes in 2025.

Milk is formed by an aqueous solution of lactose, organic and inorganic salts and many trace compounds, in which there are colloidal particles dispersed in three size ranges such as whey protein dissolved at the molecular level, caseins dispersed as large colloidal aggregates or micelles (50-500 nm) and lipids emulsified as large fat globules (1-20 μm) (Fox, 2008). As an average, cow milk major chemical compounds are distributed as follows: 3.6% fat, 4.7% lactose, 3.2% protein and 0.65% mineral substances, being the rest water (88.1%) (Pereira, 2016).

Due to its compositional characteristics, raw milk is an excellent medium for microbial growth. Consequently, it requires heat treatment in order to guarantee a safe and shelf-stable product (Claeys et al., 2002), which requires inactivation of certain enzymes and destruction of bacteria. Inactivation of enzymes and destruction of microorganisms occur depending on the temperature and time of the heat treatment. For instance, the pasteurization should destroy only some enzymes and all the pathogenic organisms, while the sterilization should inactive enzymes and destroy all microorganisms including spores. Besides, the nutritional and organoleptic properties of the products also depend on the treatments being applied. Consequently, the most common heat treatments used in the dairy industry are as follows:

a) Thermization is a heat treatment applied in order to improve the shelf-life of the milk before final milk processing. The conditions are typically 60-65 ºC for 10 to 20 s. According to Hinrichs & Kessler (1995), during this treatment the nutrients of the milk are almost unchanged, the numbers of bacteria are reduced and heat-sensitive microorganisms, such as *Pseudomonas spp*., are inactivated to a large extent. Thermized milk could be refrigerated at 7 ºC for ~3 days (Hinrichs & Kessler, 1995).

b) Pasteurization treatment conditions can vary according to the type of pasteurization been applied. For instance, conditions for batch pasteurization or low temperature long time (LTLT) pasteurization are 62-65 ºC during 30-32 min. In high temperature, short time (HTST) pasteurization, the conditions are 72-75 ºC for 15-20 s. Instead, in higher heat, shorter time (HHST) pasteurization treatment, the conditions are 105 ºC for 2 s. Pasteurization applied in raw milk increases the proteins stability. The pathogenic microorganisms are reliably destroyed under pasteurization treatments (Hinrichs & Kessler, 1995). However, pasteurized milk requires refrigeration and will have only a limited storage life since only some enzymes are inactivated and the number of spoiling microorganisms is only reduced.

c) Ultra high temperature (UHT) treatment applied in milk destroys all microorganisms that could be present, both vegetative forms and spores; this ensures a long shelf-life without refrigeration. The conditions of UHT treatment are 138 °C for 2 s (Burton, 1988; Elliott et al., 2005).

d) In-container-sterilization conditions are 120 ºC for 20 min. It is usually applied for long-life products, but it produces a number of organoleptic and chemical changes, most of which are undesirable for the consumer (Morales & Jiménez-Pérez, 1998).

Since quality is directly related to the intensity of heat treatment that is applied during milk processing, it is necessary to have indicators that can identify the treatment employed, i.e., treatment intensity, and could provide useful, inline control feedback. Among “thermal markers”, two groups can be distinguished according to the reactions and subsequent modifications undergone during heat treatment of milk. Type I markers are related to denaturation, degradation and/or inactivation of thermolabile compounds such as serum proteins, enzymes and vitamins. Type II markers include compounds which, are not present in raw milk or appear in trace amounts and are formed upon heat treatment (Pellegrino et al., 1995).

In parallel, the markers can be divided according to the severity of the heat process (Birlouez-Aragón et al., 2002). For low intensities, alkaline phosphatase is measured to distinguish

pasteurized milk from raw milk (IDF, 1999). Peroxidase is measured in HTST pasteurized milk to distinguish it from HHST pasteurized milk (peroxidase negative) (IDF, 1985; Birlouez-Aragón et al., 2002). For more intense treatments, lactose isomerization or its reaction with proteins is suitable. Changes in milk proteins induced by heat treatment depend on the severity of the treatment and the technological conditions (Baldwin & Ackland, 1991). The most important changes are a decrease in whey protein solubility, a decrease in stability of casein micelles, and modifications of organoleptic properties (Guingamp et al.,1993). It should however be highlighted that heat induces major changes in the casein micelles at temperatures above 100 ºC (Taterka, 2016).

On the other hand, the quantification of sulfhydryl and disulfide groups or of free or total sulfhydryl evolution during storage have been reported in milk heated at different intensities (Patrick & Swaisgood, 1976; Fink & Kessler, 1986a, 1986b). In addition, the near infrared spectroscopic analysis of whey proteins has been proposed as a new approach to determine protein denaturation inline (Taterka & Castillo, 2015). Heat damage markers such as lactulose, furosine and hydroxymethylfurfural have been related to the intensity in heat-treated milk. These three chemical markers are related to the Maillard compounds (*MC*), which are good indicators of heat treatments in milk, in their different stages (Nangpal & Reuter, 1990; Hewedy et al., 1994; Van Renterghem & De Block, 1996). They have been studied in different types of food from a point of view of their relation with the quality of the final product (Martins et al., 2000; Birlouez-Aragón et al., 2002; Ferrer et al., 2005; Bosch et al., 2007; Hansted et al., 2011).

Different techniques that quantify heat damage indicators are applied in milk in order to monitor changes undergone by milk components during heat exposure. They involve liquid or gas chromatography, spectrophotometry, fluorescence spectroscopy, NIR-infrared spectroscopy, colorimetry and enzymatic methods, depending on the marker to be analyzed. In recent years, the interest has been focused on non-invasive, rapid, accurate, reproducible and low cost techniques such as both fluorescence and near infrared spectroscopies. As an emerging technology in food control, front-face fluorescence spectroscopy could be a sensitive and rapid technique to monitor molecular environments and events and could provide physicochemical information according to the complexity of the sample (Christensen et al., 2005).

2.2. Theory of fluorescence

Fluorescence normally occurs from aromatic molecules although certain molecular structures containing certain carbonylic, aliphatic and alicyclic compounds as well as double bond structures that also are fluorescent. Photons of light are absorbed, exciting the molecules to a higher energy level. Then, the excited molecules, during relaxation, liberate the excess of energy returning to a ground state, re-emitting light at a longer wavelength. It occurs in nanoseconds or less and is strongly affected by the electronic structure and the chemical composition of the irradiated matter. Fluorescence can be used to detect and monitor specific molecules. Fluorophores can occur naturally (intrinsic) or can be added to generate fluorescence in the system (extrinsic) (Stavridi, 1994).

The discovery of fluorescence and further development of its application was reviewed by Berlman (1971) in his book "*Handbook of fluorescence spectra of aromatic molecules"*. It describes the fascinating history about the first experiments based on the fluorescence phenomenon. **Table 2.1** summarizes the main landmarks of the development of fluorescence spectroscopy.

Nowadays, fluorescence, an increasingly used method, is applied in different scientific disciplines and industrial sectors such as medicine, genetics, microbiology, pharmacology, oil industry, food industry, etc.

The most relevant information and processes that occur in fluorescence, which are described below, are based on the following books and other additional studies: *Principles of Fluorescence Spectroscopy* (Lakowicz, 2006), *Principles of instrumental analysis* (Skoog et al., 1998) and *Principles and Techniques of Biochemistry and Molecular Biology* (Wilson & Walker, 2010).

Fluorescence is a photoluminescence phenomenon. Photoluminescence is the emission of light by a substance as a result of photon absorption. There are two main types of photoluminescence: fluorescence and phosphorescence. The difference between them is the nature of the excited state. Phosphorescence electronic transitions involve a change of spin, presenting longer relaxation time (ms to h), while fluorescence does not involve a change of spin, lasting short periods of time (ns). The fluorescence could occur in simple or complex gaseous, liquids and solids systems (Fletcher et al., 2006).

These differences are explained by the Pauli exclusion principle, which establishes that there cannot be two electrons whose four quantum number are equal. This restriction requires no more than two electrons in the orbital. Furthermore, the spin of each electron should be opposite from the other one, as shown in **Figure 2.1**.

Table 2.1. Stages in the history of fluorescence.

Year	Scientist	Observation or achievement	Reference
1565	Nicolas Monardes	Monardes reported that water containing a certain kind of wood, then popularly known as *Lignum nephriticum*, could glow in the sunlight	Berlman, 1971
1833	David Brewster	Brewster described the red fluorescence chlorophyll in the article "*On the Colour of Natural Bodies*". Furthermore, he reported that a beam of sunlight passing through a green alcoholic extract of leaves appeared red when observed from the side	Valeur & Berberan-Santos, 2011
1845	John Herschel	Herschel reported that a solution containing sulphate of quinine emitted a strong luminescence radiation when hold in sunlight. The purpose of the report was to point out the difference between this type of radiation and ordinary scattered light	Berlman, 1971
1852	Stokes	Stokes, studying sulfate of quinine also, found that the emitted light was composed of wavelengths longer than those of the absorbed light - a phenomenon now called Stokes shift -. Stokes also discussed photochemical changes produced by sunlight. It was the major event in the history of the photoluminescence that was published in his paper entitled "*On the Refrangibility*"	Stokes, 1852; Berlman, 1971
1862	Victor Pierre	Pierre published papers where he studied solutions of single fluorescence compounds and their mixtures. He noticed that bands of fluorescence spectra were characteristic of each particular substance	Valeur & Berberan-Santos, 2011
1877	Uranin	A disodium salt of fluorescein was used for the first time as a tracer for monitoring the flow of the Danuve river	Lakowicz, 2006; Valeur & Berberan-Santos, 2011
1910	Ley and Engelhardt	Fluorescence quantum yield was measured, when determinations were made for substituting benzene derivatives	Berlman, 1971
1926	Vavilov	He was the first to find that the fluorescence yield was generally independent of the wavelength of the exciting radiation	Berlman, 1971
1948	Förster	Fluorescence resonance energy transfer was first described by Förster	Wilson & Walker, 2010

The electronic states of the molecules are described by **Figure 2.1**. First, in the lowest state, i.e., the fundamental, the spins always are paired (**a**) and is called "basic fundamental state" or "ground singlet state". In the basic state, when the molecule is exposed to a magnetic field, there are no division of energy levels. Electronic excited states are represented by **b**) and **c**). When the spins are paired during the excited state, the molecules are in a basic excited state (**b**), also called "singlet excited state". However, when the spins are not paired, the molecules are in a triplet excited state (**c**). The electrons in triplet state do not form pairs, they have parallel spins, i.e., they have the same direction.

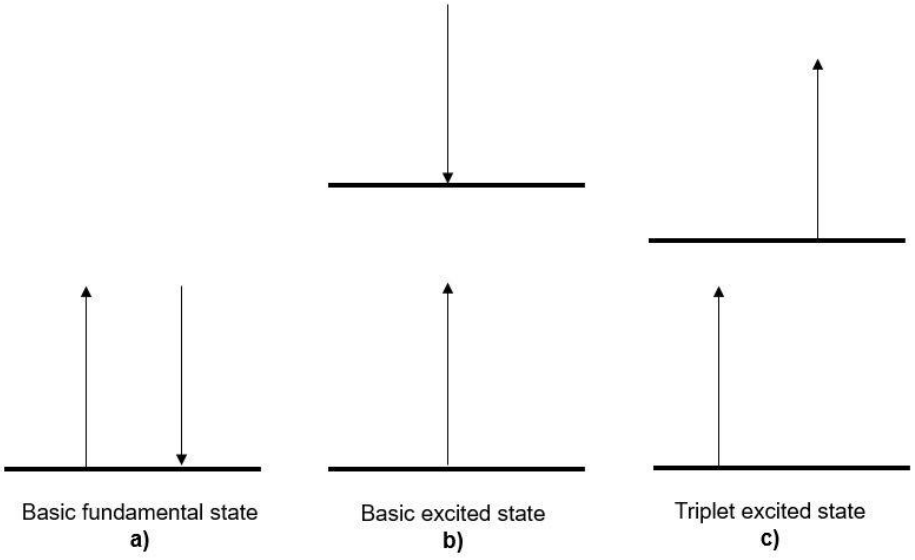


Figure 2.1. Electronic states of the molecules by Pauli principle (reproduced from Skoog et al., 2008).

The properties of triplet excited state of a molecule are very different from the basic state of the molecule. For instance, a molecule is paramagnetic in the triplet state and diamagnetic in the basic state. Most important is the fact that a ground singlet to triplet excited state transition or vice versa is a significantly less probable event than the ground singlet to excited singlet state transition or vice versa. Thus, the lifetime in the triplet excited state tends to be larger as indicated above. However, the energy level of the triplet excited state is lower than the basic excited state (**Figure 2.1**).

A scheme to explain the electronic transitions during both fluorescence and phosphorescence was presented by Jablonski (**Figure 2.2**). Horizontal lines of the Jablonski diagram show the electronic states represented by S_0, S_1 and S_2, respectively, with S_0 having the lowest energy level. There are a series of vibrational energy levels for each electronic level state, which are represented by 0, 1, 2, etc.

A fluorophore is usually excited to some higher vibrational level such as either S_1 or S_2. Regularly, the fluorescence takes place for 10^{-5} to 10^{-10} s. The transition rate from triplet to ground singlet state is lower than the transition rate from singlet excited to ground singlet state.

An excited molecule can return to its ground state by combinations of several mechanical phases. Transitions can be radiative and non-radiative. Non-radiative transitions are represented by wavy arrows. An excited state electron quickly loses its energy liberating heat by vibrational relaxation –a non-radiative transition; S_n^* to S_n–. Internal conversion is another type of non-radiative transition where electrons change from a vibrational level in one electronic state to another

vibrational level in a lower electronic state (i.e., from S_2 to S_1). Radiative transitions are indicated by straight arrows in the scheme. They only occur when the electron returns to the ground singlet state emitting light energy. **Figure 2.2**. shows two main types of radiative transitions: fluorescence and phosphorescence. Fluorescence occurs when an electron relaxes to its ground state, S_0, from a singlet excited state, S_1, emitting a photon. However, molecules in S_1 state can also undergo a spin conversion to triplet excited state (T_1). The T_1 emission is called phosphorescence and generally shifted to longer wavelengths (lower energy) relative to fluorescence. The conversion of S_1 to T_1 is called intersystem crossing.

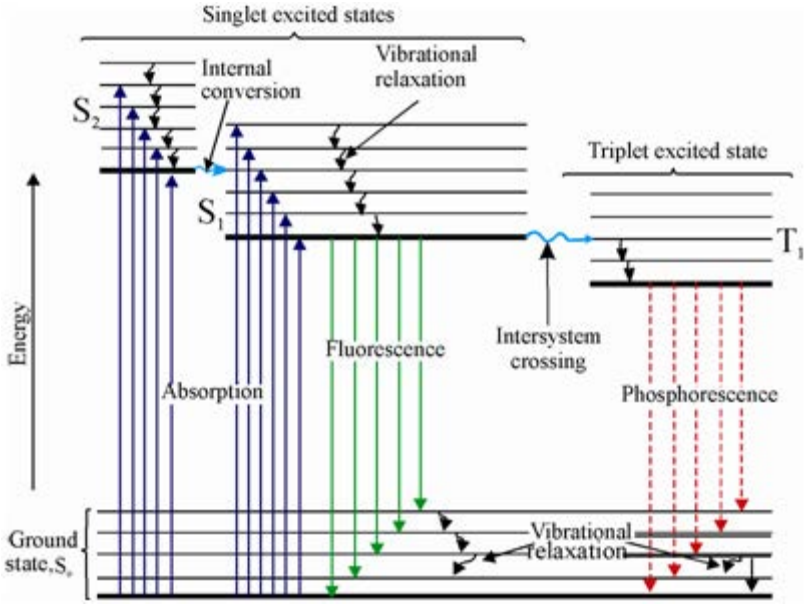


Figure 2.2. Scheme based on Jablonski diagram of the molecules energy levels (reproduced from Skoog et al., 2008).

On the other hand, the deactivation of an electronic excited state could involve the energy interaction or transfer between the fluorophore and its surroundings. This process, called external conversion, can occur between an excited molecule and the solvent or solute.

The fluorescence of a molecule is mainly determined by two factors, its molecular structure (internal factor) and the chemical environment (external factor). Molecular structure plays a major role in determining the characteristic shape and wavelength position of the fluorescence spectrum (Berlman, 1971). On the other hand, the quantum yield and the fluorescence lifetime are perhaps the most important characteristics of a fluorophore. Other variables such as temperature and solvent effects, pH, concentration, etc., can also influence in the emission intensity.

The quantum yield or quantum efficiency is the ratio between the quantity of excited molecules that presented fluorescence and the total of molecules excited, in other words, it is the number of emitted photons relative to the number of absorbed photons. The quantum yield approaches a value of one when the molecule is very fluorescent, such as fluorescein. Instead, in the case of chemical species that do not present fluorescence, its quantum yield approaches a zero value. Quantum efficiency decreases in the most of molecules that are exposed to high temperature as it increases the probability of collisions and, subsequently, the probability of deactivation by external conversion. A decrease of the solvent viscosity has a similar effect on quantum yield.

The lifetime is the characteristic time that a fluorophore remains in its excited state before returning to the ground state, emitting a photon. Thus, it is an indicator of the average time the fluorophore remains available to provide information by means of its emission profile. The transition from the excited to the ground state could be treated as first order decay process. The fluorescence lifetime indicates the global rate of deactivation of the excited state due to both radiative and non-radiative processes. As a result, lifetime can be also defined as the time elapsed from the excitation moment until the fluorescence emission intensity, I, decays to e^{-1} of its initial value (i.e., $I = I_0/e$) (Fletcher et al., 2006; Klitgaard et al., 2006; Estandarte et al., 2016).

The pH is another important factor in the fluorescence of the aromatic molecules (Christensen et al., 2006). The fluorescence of aromatic compounds with acid or basic substituents in the ring depends on pH. Probably, the excitation and the emission wavelengths are different in protonated and non-protonated forms of these compounds. As a result, the fluorescence of certain compounds as a function of pH has been used to detect the end point in certain acid-base titrations.

Often, presence of dissolved oxygen decreases the fluorescence intensity of a solution. This effect could be photo-chemically induced by oxidation of fluorescent species, although the observed reduction of intensity is more commonly the results from the paramagnetic properties of molecular oxygen, which enhances intersystem crossing and conversion of excited molecules into the triplet state.

The fluorescence intensity may decrease by a wide variety of processes, including among others, quenching and inner filter effect. The quenching process is referred as to non-radiative energy transfer from excited species to other molecules of the system. Quenching can occur by different mechanisms. Two types of quenching mechanisms can be distinguished: a) dynamic or collisional quenching, which occurs when the excited fluorophore is deactivated upon interaction with some other molecule in solution, which is called quencher. In this case, the fluorophore is returned to the ground state during a diffusive encounter with the quencher; and b) static quenching, which occurs when the non-excited fluorophore forms a non-fluorescent complex with the quencher.

That complex has a different electronic structure compared to the fluorophore alone and returns from the excited to the ground state by non-radiating mechanisms (Valeur, 2001; Klitgaard et al., 2006).

Collisional quenching is illustrated on the Jablonski diagram (**Figure 2.3**). The term Σk_i is used to represent non-radiative paths to the ground state aside from quenching.

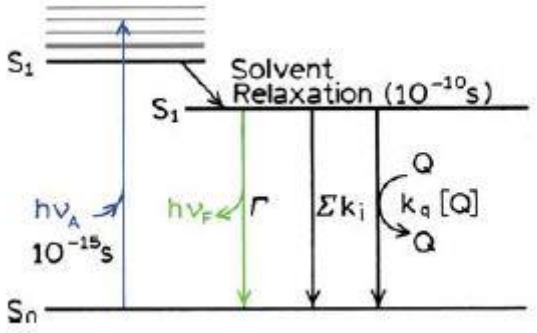


Figure 2.3. Collisional quenching (Lakowicz, 2006). Σki is used to represent non-radiative paths to the ground state aside from quenching. *r*, anisotropy.

For collisional quenching, the intensity decrease is described by Stern-Volmer equation (2.1):

$$\frac{F_0}{F} = 1 + K[Q] = 1 + k_q\tau_0[Q] \qquad [2.1]$$

where F_0 is the fluorescent intensity of the fluorophore without the quencher, F is the fluorescent intensity of fluorophore with the quencher, the Stern-Volmer quenching constant is expressed by K and indicates the sensitivity of the fluorophore to a quencher. The parameter k_q is the bimolecular quenching constant, τ_0 is the unquenched lifetime, and $[Q]$ is the quencher concentration.

Fluorescence intensity data cannot distinguish by itself between static or collisional quenching. The measurement of fluorescence lifetimes or the temperature/viscosity dependence of quenching can be used to discriminate the type of quenching process. It should be added, that both processes can also occur simultaneously in the same system.

Oxygen and iodide are highly effective quenchers of fluorescence emission. The uses of these quenchers allow the biological macromolecules surface mapping. For example, whether residues of tryptophan (*Trp*) are exposed to solvent or not could be determined using iodide.

Another important phenomenon that could generate a decrease of fluorescence intensity is the inner-filter effect. This effect occurs when the total absorbance of the solution is high. This leads

to a reduction in the intensity of the excitation radiation over the path length. The inner-filter is not usually categorized among the major quenching mechanisms. It is because this type of phenomenon is not involved in the radiative and non-radiative processes (Lindon et al., 2016).

2.2.1. Biochemical fluorophores

Fluorophores are fluorescent chemical compounds. They typically should be capable of absorbing ultraviolet or visible light; and should be rigid, and planar or cyclic with several aromatic groups and π bonds (Castro et al., 2012). Structural rigidity of the molecule diminishes the probability of vibrational energy relaxation. They can exist naturally in a matrix or could be added. Fluorophores can be used as natural indicators to monitor the structure, dynamics and metabolism of living cells. The fluorescence properties of the molecules depend on their structure and on the surrounding environment (Albani, 2007).

Fluorophores are divided in two classes: intrinsic and extrinsic. Firstly, the intrinsic fluorophores are those that occur naturally and the extrinsic fluorophores are those added to a sample, when the sample does not display the desired spectral properties.

Proteins have three intrinsic fluorophores: *Trp*, tyrosine, and phenylalanine. The latest has the lowest quantum yield (0.03) and its contribution to protein fluorescence emission is thus insignificant. The quantum yields of tyrosine and *Trp* are about one order of magnitude larger, near 0.14 and 0.13, respectively.

Tyrosine fluorescence emission is almost entirely quenched when it becomes ionized, or is located near an amino or carboxyl group, or a *Trp* residue. As a result, the indole group of *Trp* is typically the dominant fluorophore in proteins (**Figure 2.4**). The indole group absorbs near 280 nm, and emits near 340 nm. The indole emission spectrum is highly sensitive to solvent polarity. It could be blue shifted if the group is buried within a native protein (N) or could be shifted to longer wavelength (red shift) if the protein is unfolded (U) (**Figure 2.4**).

The principal application for intrinsic protein fluorescence aims at conformational monitoring. A number of empirical rules can be applied to interpret the fluorescence spectra of proteins:

- As a fluorophore moves into an environment with less polarity, its emission spectrum present hypochromic or blue shift (λ_{max} moves to shorter wavelength) and the intensity at λ_{max} (maximum wavelength) increases. Opposite behavior is observed when it moves to a more polar environment (bathochromic or red shift).
- Fluorophores in a polar environment show a decrease in quantum yield with increasing temperature. In a non-polar environment, there is a little change.
- Trp fluorescence is quenched by neighboring protonated acidic groups.

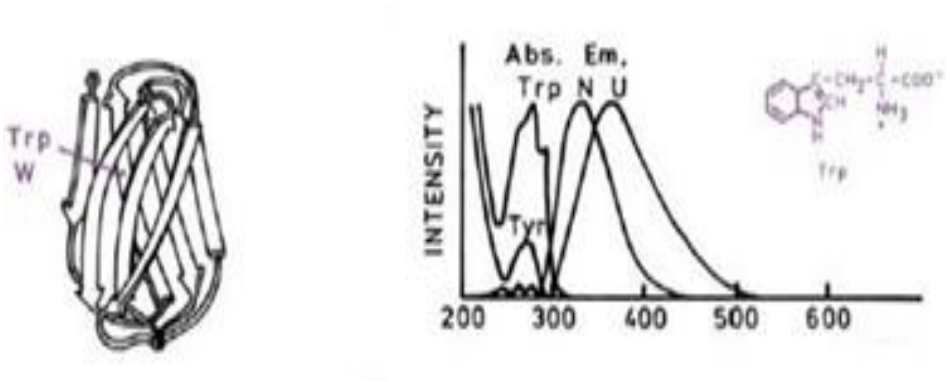


Figure 2.4. Absorption and emission of tryptophan in protein molecules (reproduced from Lakowicz, 2006).

According to the situation to be evaluated, the most specific fluorescent marker could be selected. For example, dityrosine (*Dt*), which is a product of proteins oxidative stress, may be useful to monitor oxidative conditions in biological systems. The presence of *Dt* has been tied to cross-linking in biological materials. According to literature, *Dt* fluorescence can be measured in milk samples exciting at 315 nm (Harms et al., 1997).

2.2.2. Fluorescence measurements and instrumentation

Fluorescence experiments require special care in relation with the experimental data acquisition conditions and a deep understanding of the instrumentation. There are several potential artifacts that can distort fluorescence data. Furthermore, the optical properties of samples (density or turbidity) can also affect fluorescence measurement (Lakowicz, 2006).

Fluorescence measurement can be performed using two different geometries: right angle (90º) and front-face fluorescence (between 30 to 60º) (**Figure 2.5**). The geometry used depends on the samples to be analyzed. The right-angle fluorescence has the disadvantage that can be applied only in diluted samples. The usage of the right-angle geometry is limited due to spectral phenomenon such as inner-filter effect and dispersion or reflection of light before and after excitation of the fluorophore (Wilson & Walker, 2005; Dankowska, 2016).

However, front-face fluorescence (FFF) presents a number of advantages over the right-angle fluorescence technique. Firstly, phenomena as quenching and external conversion can be minimized using this technique (Klitgaard et al., 2006; Alvarado, 2017). Secondly, front-face fluorescence does not require the dilution of samples, which means that no manipulation of foods is needed. All these advantages make front-face fluorescence a useful technique that can be applied not only to opaque liquids but also to solids and could even be suitable for inline application.

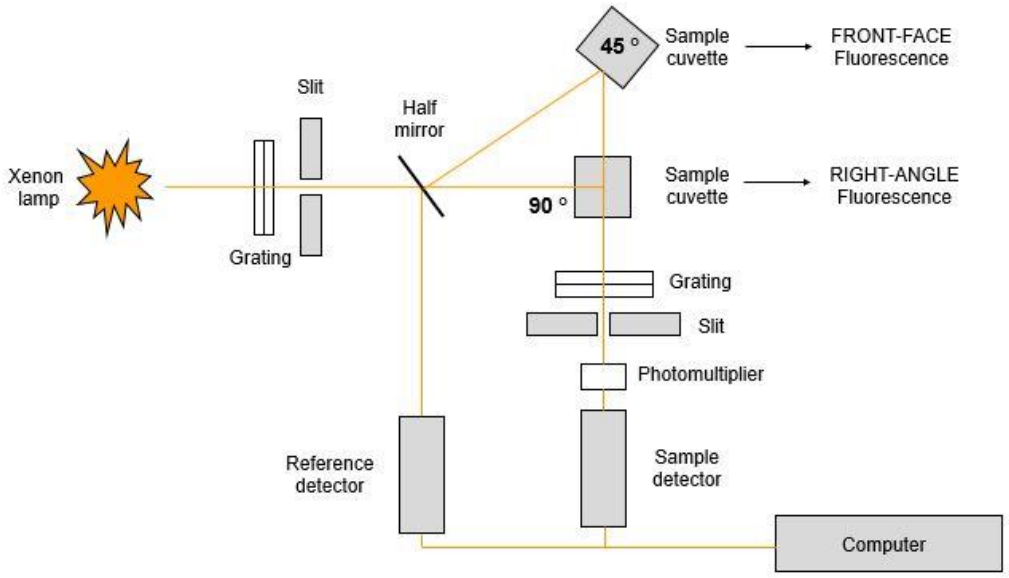


Figure 2.5. Schematic forms of front-face and right-angle spectrofluorometers (reproduced from Wilson & Walker, 2005).

The components of the instrument for measuring photoluminescence are similar to a visible ultraviolet spectrophotometer. Almost all the fluorescence instruments use optical double beam equipment, to compensate fluctuations in the radiative power. The beam from a xenon lamp, passes firstly through an excitation wavelength selector, which could be either a filter or a monochromator. It transmits the radiation at the optimum excitation wavelength but excludes or limits the radiation of the rest of the wavelengths.

The instrument is a fluorimeter if only filters are used to select the wavelengths. Furthermore, the maximum sensibility often is obtained using filters instead of monochromators, when the spectral properties of the fluorophore are well known. Instead, the spectrofluorometers are equipped with two monochromators to isolate the wavelength. Some spectrofluorometers are hybrids because they use filters to select the excitation wavelength and a monochromator to select the emission wavelength. The genuine spectrofluorometers has the ability to produce both excitation and emission fluorescence spectra (Skoog et al., 2008).

There are different methods to obtain the fluorescence data from the samples:

- One excitation wavelength / one emission wavelength. This method is the simplest and it is used to monitor just one point of the specific marker (Feinberg et al., 2006).
- One excitation wavelength / a range of emission wavelengths (**Figure 2.6**). It is used in order to explore the response of the fluorophore in a range of emission wavelengths (Liu et al., 2018; Mungkarndee et al., 2016).

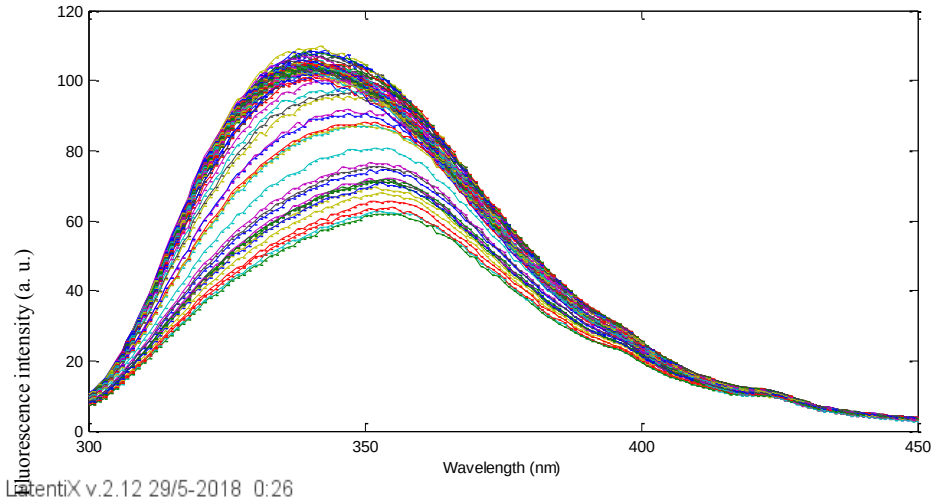


Figure 2.6. The scan obtained exciting at 290 (tryptophan) and recovering the emission from 300 to 450 nm. The data was obtained from reconstituted skim milk samples in the first experiment of this PhD study.

- Excitation - Emission Matrix (EEM), formed by multiple excitation and emission wavelengths, where data obtained generate a matrix. This technique has the benefit of scanning the maximum of possible fluorescence areas. Furthermore, it is mostly used to distinguish and quantify chemical compounds (Kulmyrzaev & Dufour, 2002; Elcoroaristizabal et al., 2016). **Figure 2.7** shows the EEM of *Trp* fluorescence. The map was made exiting *Trp* from 250 to 400 nm and recovering emission from 300 to 440 nm. The measurements were taken every 1 nm.

The EEM is also known as fingerprint method (**Figure 2.8**), and it is considered as a complete expression of the unique fluorescence characteristics of the specimen (Sugiyama & Fujita, 2014).

The fingerprint method requires data preprocessing since not all the scanned areas are used, as shown in the **Figure 2.9**, and the unnecessary excitation-emission wavelength ranges should be removed.

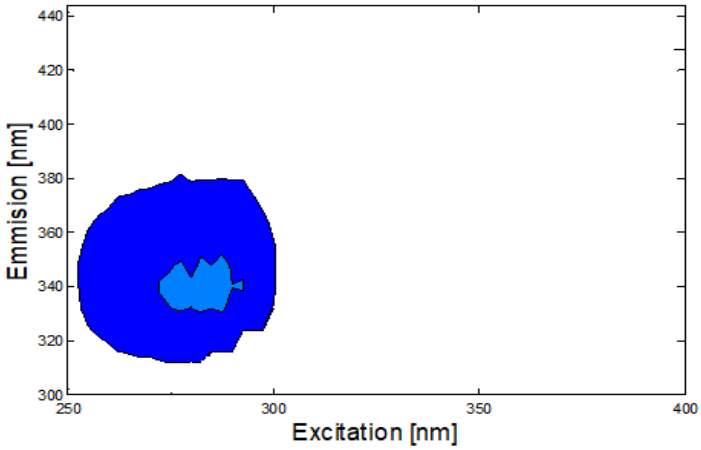


Figure 2.7. Tryptophan fluorescence excitation-emission matrix obtained from reconstituted skim milk.

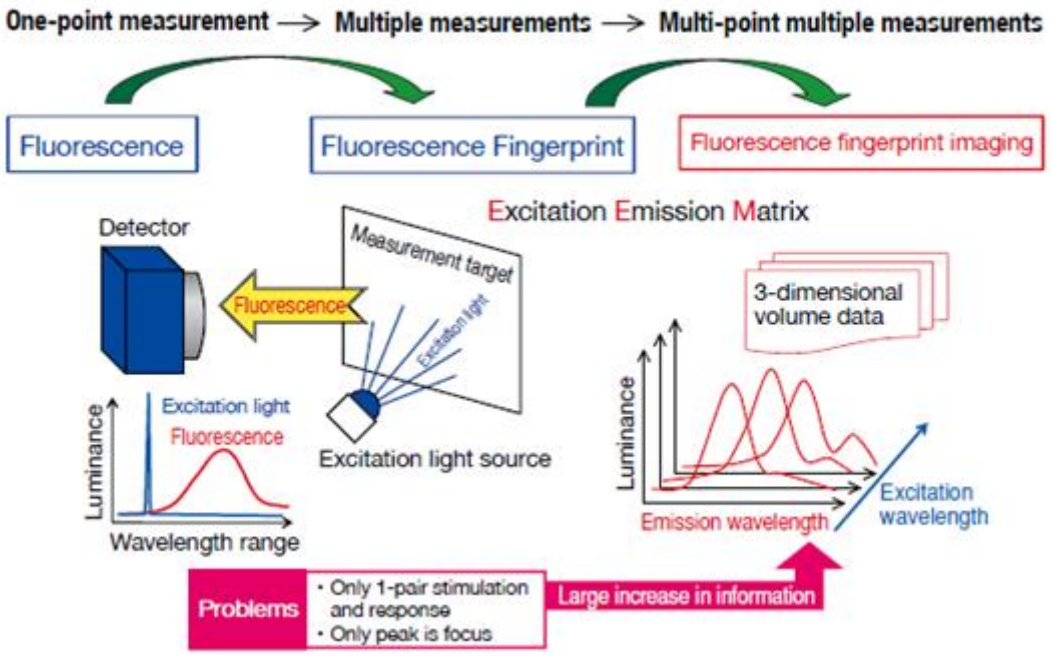


Figure 2.8. Scheme for obtaining fluorescence fingerprint from the fluorescence (reproduced from Sugiyama & Fujita, 2014).

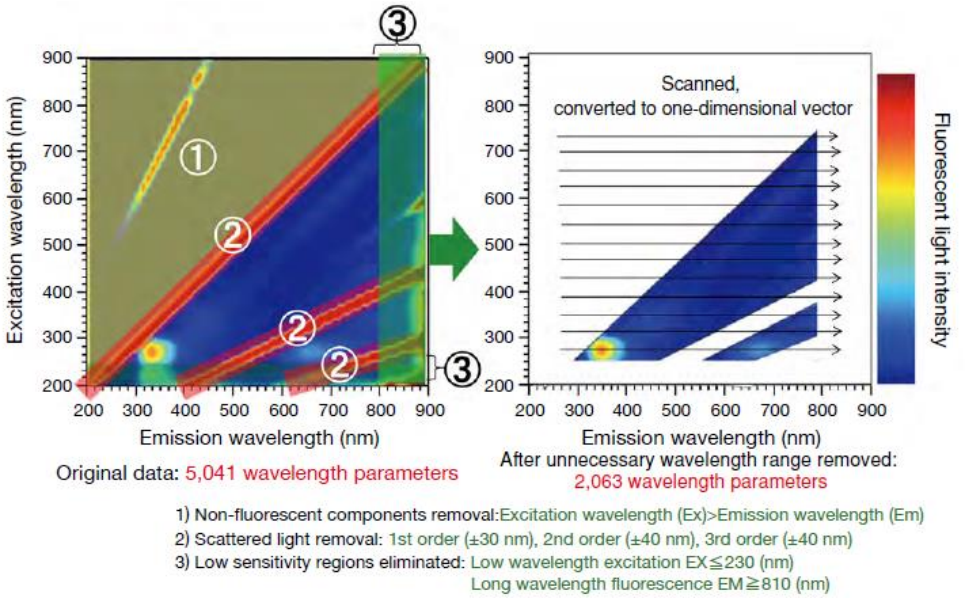


Figure 2.9. Raw and preprocessed scan of the fingerprint fluorescence data (reproduced from Sugiyama & Fujita, 2014).

2.2.3. Excitation and emission wavelengths of intrinsic food fluorophores

Table 2.2 describes the list of the most relevant fluorophores analyzed in food science either by right-angle or front-face fluorescence techniques. Sádecká & Tóthová (2007) suggested a list of food samples that could be analyzed using mainly front-face fluorescence method. The list included milk, semi-hard cheese, soft cheese, egg, fish, meat, olive oil, vegetable oil, honey, wine and beer, among others. They used the fluorescence analysis along with multivariate analysis tools in order to obtain, in most cases, classification and/or characterization of samples.

Table 2.2. The most relevant fluorophores in food analysis.

Fluorophore	Excitation (nm)	Emission (nm)
Phenylalanine	258	284
Tyrosine	276	302
Tryptophan	290	340
ATP	292	388
α-Tocopherol	298	326
Dityrosine	315	420
Pyridoxine	328	393
Maillard compounds	330	420
NADH	344	465
Retinol	346	480
Hematoporphyrin	396	614
Chlorophyll A	428	663
Riboflavin	267-370-450	530

2.2.3.1 Fluorescence in milk

Milk contains intrinsic fluorescent compounds that are used as quality markers. Furthermore, these fluorophores are analyzed in order to evaluate their changes because they emit fluorescence after chemical modifications due to heat treatments or storage. These changes suffered by the fluorescent markers can provide useful information about the intensity of heat treatment. The fluorescent molecules most typically used as markers are the aromatic amino acids (*Trp*, phenylalanine, tyrosine), vitamins A and B_2 (retinol and riboflavin (*Rb*), respectively), intermediate fluorescent Maillard reaction products and lipid oxidation products (Shaikh & O'Donnell, 2017).

Tyrosine, phenylalanine and *Trp* are responsible for the proteins absorption in UV and the associated fluorescence and have maximum absorption at wavelengths from 280 to 290 nm (Scheidegger et al., 2010). Further, F_{Trp} dominates in proteins as explained in Section 2.2.1. Although tyrosine is more fluorescent than *Trp* in solution, when in proteins, its fluorescence is weaker (Albani, 2007). The F_{Trp} has been investigated to evaluate the folding/unfolding of proteins, and it is very sensitive to local environment (Vivian & Callis, 2001; Tusell & Callis, 2012). *Trp* emits at 320 nm in an environment of low polarity. In a polar environment, the peak shifts to 355 nm. This shift is induced by the denaturation of proteins, due to the loss of the protein tertiary structure (Albani, 2007). In a recent study, protein denaturation and the formation of aggregated and micellar bound whey proteins in heat-treated milk were evaluated and predicted using F_{Trp} in combination with light backscatter response (Taterka, 2016). The fluorescence spectra of *Trp* were performed exciting at 290 nm and recovered in a range of emission between 305 and 450 nm. This study was focused on the development of an inline optical sensor for the determination of total whey protein denaturation in heat-treated milk.

Fluorescence of *MC* in milk has been studied to detect the progress of the reaction (Bastos et al., 2012). In an advanced stage of Maillard reaction, the Amadori products formed during the initial stage can form cross-links with proteins or amino acids, resulting in fluorescent aggregates (Martins et al., 2000; Bosch et al., 2007). Recently, the combination of *Trp* and *MC* fluorescence has been investigated (Alvarado, 2017). The experiment was performed in milk samples heat-treated at different intensities. The best models were selected. Best models based on the best R^2 were obtained for the prediction of the chemical concentrations of *Rb* ($R^2 = 0.75$), ascorbic acid ($R^2 = 0.90$), sulfhydryl group ($R^2 = 0.92$) and hydroxymetylfurfural ($R^2 = 0.94$).

Birlouez-Aragón (2002) patented the Fluorescence of Advanced Maillard products and Soluble Tryptophan (FAST) index. This method uses the ratio between *Trp* fluorescence (290 and 340 nm for excitation and emission wavelengths, respectively) and *MC* fluorescence (330 and 420 nm, respectively) to calculate the index. Many studies have shown that the use of the FAST method

is a very sensitive indicator of thermal damage in milk. This method was used to evaluate different heat treatments applied in milk and condensed milk samples (Birlouez-Aragon et al., 2001; Birlouez-Aragon et at., 2002). The authors suggested the FAST index as a great method to control heat processes in order to ensure a better nutritional quality of the product. Moreover, the method was applied in infant formula milk that was heated in microwave in order to evaluate the damage after treatment (Laguerre et al., 2011). Since MC fluorescence corresponds to molecular structures formed between reducing sugars and lysine residues of proteins (Birlouez-Aragon et al., 2001), this method has been proven to be useful to evaluate the nutritional and lysine damage during heat process in milk by measuring fluorescence in the pH 4.6 acetate soluble fraction of samples (Tessier et al., 2006; Bastos et al., 2012). The FAST index method was also used in combination with chemical measurements (lactulose, furosine, lactoperoxidase, β-lactoglobulin (β-LG) and α-lactalbumin (α-LA)) to evaluate the thermal treatment applied in 92 different commercial milks (Feinberg et al., 2006). The results from the discriminant analysis were encouraging, although chemicals such as lactulose and furosine were less efficient parameters to discriminate among thermal treatment intensities. The most discriminative tracers were the FAST index and α-LA concentration.

Rb is highly fluorescent like the breakdown products from photodegradation, lumiflavin and lumichrome (Fox & Thayer, 1998). This vitamin plays the role of a photosensitizer in milk (Wold et at., 2002) and can absorb light to produce the oxygen activation and radical formation leading to protein and lipid oxidation. In that way, *Rb* is considered as an important marker of photooxidation in dairy products. Furthermore, the autofluorescence attributed to this vitamin has been used to monitor light-induced changes, the oxidation stability and the quality of dairy products (Becker et al., 2003; Christensen et al., 2006; Christensen & Tomasi, 2007). Christensen et al. (2005) measured the fluorescence of *Rb* in dairy products exciting at three different wavelengths: 270, 382 and 448 nm. They evaluated in an exhaustive manner the whole spectra and then selected the wavelengths with higher fluorescence response. In another study by Liu et al. (2018), *Rb* was measured in skim milk samples at 267, 370 and 450 nm excitation wavelengths and at 530 nm of emission wavelength according to Mortensen et al. (2003). This study focused in using FFF for estimating retinol concentration in milk after thermal treatments. The best prediction model, which was selected based on the maximum R^2 ($R^2 = 0.87$), used the fluorescence of *Trp*, *MC* and *Rb* excited at 370 nm as predictors.

Dt is another fluorescent marker that can provide information about proteins. Being a product of the oxidative stress of proteins, it is useful to evaluate oxidative conditions in biological systems. The oxidation in milk involves off-flavors, making the oxidized milk unacceptable to consumers and causing a decrease in the nutritional quality (Scheidegger et al., 2010). Furthermore, *Dt* has been associated to cross-linking in biological materials (Harms et al., 1997). The mechanism of

its formation begins with the generation of a tyrosyl radical. The *Dt* is formed by the ortho-ortho cross-linkage of two tyrosyl radicals (DiMarco & Giulivi, 2007). The marker has been evaluated by FFF in heat-treated milk exciting at 315 nm and recovering the signal at an emission wavelength of 420 nm (Liu et al., 2018).

The fingerprint method has been used in several studies since it might result in accurate quantitative determinations and sample discrimination based on huge amount of data. Hougaard et al. (2013) proposed the front-face fluorescence spectroscopy to characterize 30 pasteurized milk samples treated at different levels of heating. The EEM data was recovered with excitations between 250 and 350 nm, and emission wavelengths from 260 to 500 nm. They found three interesting regions in the spectra, two of them related to proteins and the other one with vitamin A. The authors suggested vitamin A as a good fluorophore to distinguish milk samples with different intensity of treatments.

Another study showed the potential of EEM in detecting buffalo and cow milk adulteration (Durakli Velioglu et al., 2017). The measurements were recovered between 400 and 550 nm of excitation and emission wavelengths. They noted that cow and buffalo spectra were clearly different in some areas of the spectral data. The differences allowed the discrimination of buffalo and cow milk, thus the detection of adulteration.

Given its complexity, data obtained from fluorescence is mainly processed by chemometric tools. These tools are the most suitable to reduce the number of dimensions of the data. They are used to classify or discriminate samples and also to predict concentration of chemicals using the spectral data. The most used techniques are the Principal Component Analysis, Partial Least Square and PARAFAC (for three-dimensional spectral data). These methods require data preprocessing that depends on the type of spectra obtained.

In summary, several studies have reported the use of front-face fluorescence spectroscopy and have demonstrated the potential of this technique when applied to dairy products. Moreover, this technique has been proven successful in determining and providing information from milk products concerning their nature and degree of heat treatment.

CHAPTER 3:
Objectives and Working plan

3.1. Objectives

3.1.1. General objective

The general objective of this PhD work was to study the usage of intrinsic fluorescence tracers for quick estimation of furosine and lactulose, two thermal damage markers typically used by the industry. Quick estimation of heat damage will help when making process control decisions during the industrial transformation of milk, contributing to ensure high quality products and allowing rapid authentication of commercial heat-treated milk. An additional aspect of the general objective was to develop front-face fluorescence as an optical new technology to classify commercial heat-treated milks according to the treatment intensity applied.

3.1.2. Specific objectives

In order to achieve the general objective, the following specific objectives were addressed:

a) To develop laboratory techniques for both physical-chemical and fluorescence analysis. Excitation and emission wavelengths were selected in order to explore the whole set of relevant intrinsic fluorophores in milk (*Trp*, *Rb*, *Dt* and *MC*). Moreover, physical-chemical reference methods were set up for the quantification of furosine and lactulose.

b) To determine the kinetics of lactulose and furosine formation during heat treatment in reconstituted low-heat skim milk powder.

c) To compare the spectra of each of the fluorophores studied in reconstituted low-heat skim milk powder before and after applying different levels of heat treatment in order to analyze the changes induced by the heat in the fluorescence spectra.

d) To obtain prediction models for quick quantification of the two traditional heat damage markers studied (furosine and lactulose) by identifying predictors from the front-face fluorescence spectra of milk samples.

e) To validate the best prediction models using leave-one-out or cross-validation method.

f) To classify commercial samples of whole, low-fat and skimmed milks from several trademarks, subjected to different heat treatment intensities, i.e., pasteurized, UHT, spray dried and in-bottle sterilized, based on the specific spectra of the samples.

3.2. Working plan

The present PhD research was established within the frame of the National Research Project entitled "Application of native fluorescence tracers for quick quantification of milk damage during milk processing" (AGL2012-33957). In the project, several heat damage indicators have been investigated and characterized, and the evaluation of each of them was necessary to obtain complete information about the damage suffered by the milk due to the heat process. In the present dissertation, heat damage markers investigated were lactulose and furosine specifically. Two independent experiments were performed in order to address the objectives proposed in this thesis. A complete description of each experiment is presented below.

3.2.1. Experiment I

The aim of the first experiment (**Figure 3.2.1**) was to study lactulose and furosine formation and their respective kinetics in heat-treated milk samples, together with the spectra of fluorescence markers and their correlations in order to accomplish objectives a) b) c) d) and e). It was performed using reconstituted low-heat skim milk powder, that was heat-treated at four different temperatures (70, 80, 90 and 100 ºC) and six holding times (0, 10, 15, 30, 45 and 60 min). The whole experimental design was replicated three times.

For the chemical determination of lactulose three temperatures were used (80, 90 and 100 ºC) thus the number of observations was $N = a \cdot b \cdot R = 3 \cdot 6 \cdot 3 = 54$. Instead, for furosine, only milk samples with heat treatments at 70, 80 and 90 ºC were used (leaving out time zero), giving a number of observations for furosine of 45 ($N = 3 \cdot 5 \cdot 3$). Finally, for the emission spectra corresponding to *Trp*, *Dt*, *MC* and *Rb*, all four temperatures were used. But, treatments at time zero for each target temperature were considered as control and all the measurements were referred to this initial point, so the actual number of observations was $N = 4 \cdot 5 \cdot 3 = 60$ for the study about the effect of experimental factors on fluorescence spectra.

3.2.2. Experiment II

The second experiment (**Figure 3.2.2**) was performed using 59 samples from several brands of commercial milk obtained from different supermarkets. Samples were divided according to their treatment (pasteurized, spray dried, UHT, and in-bottle sterilized) and composition (whole, low-fat and skimmed milk). The aim of this experiment was to classify the samples according to their composition and heat treatment intensity using their fluorescence spectra. The objective f) was achieved through the completion of this experiment.

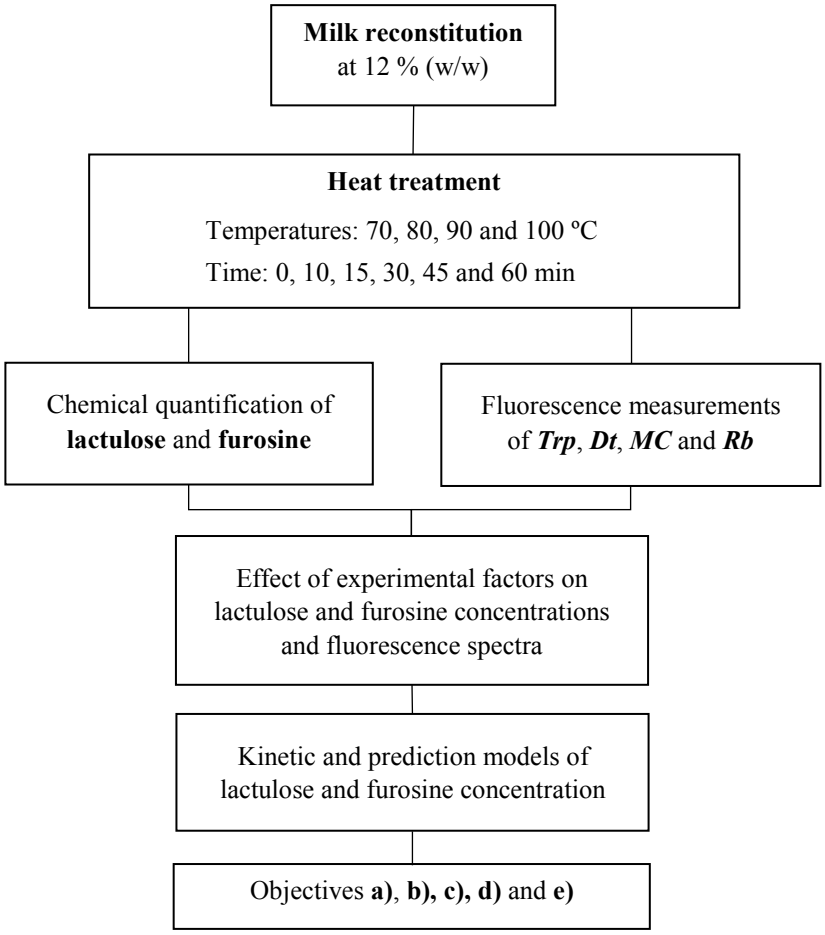


Figure 3.2.1. Working plan for lactulose and furosine formation in reconstituted skim milk powder during heat treatment and its correlation with the fluorescence markers. *Trp*, tryptophan; *MC*, Maillard compounds; *Dt*, Dityrosine; *Rb*, riboflavin.

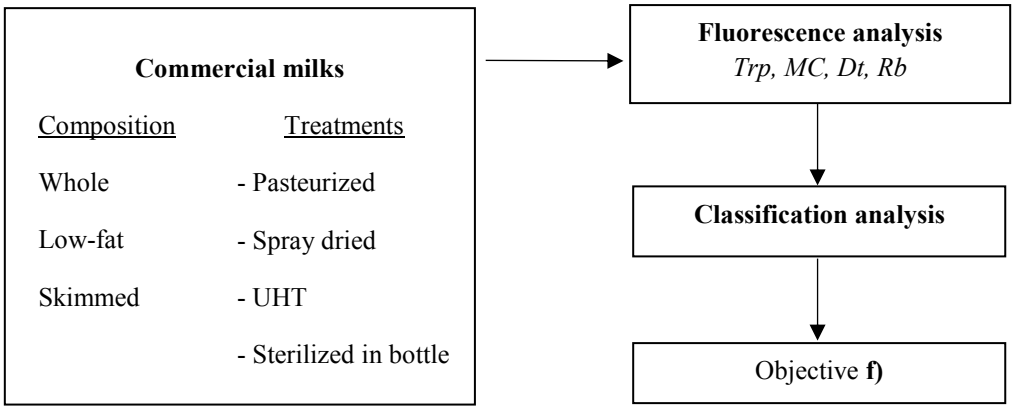


Figure 3.2.2. Working plan for the classification of the commercial milk samples. *Trp*, tryptophan; *MC*, Maillard compounds; *Dt*, Dityrosine; *Rb*, riboflavin.

CHAPTER 4:
Selected chemical markers and their formation kinetics

4.1. Introduction

Many reactions can occur in milk due to processing and storage. These reactions, which can degrade food quality, can be chemical, e.g., Maillard reactions, microbiological and biochemical. Food technology focuses on analyzing and reducing the negative consequences of thermodynamic processes in food. Thermodynamics indicates the change of direction but not the speed at which this occurs. The models that are used to analyze the food quality changes are kinetic models since they describe rate of either the degradation or the formation of compounds such as vitamins or lactulose and furosine, respectively. However, it must be pointed out that, when applying thermodynamic processes, many interactions can affect food quality (Van Boekel, 2008).

Several markers have been evaluated as chemical indicators to assess heat damage in milk (see section 2.1.). Particularly, lactulose (4-O-ß-D-galactopyranosyl-D-fructofuranose) was originally reported in heated milk by Adachi (1958) but its formation was erroneously attributed to the hydrolysis of the Amadori product, lactulosyl lysine, in addition to the lactose transformation (Adachi & Patton, 1961; IDF, 1995). It is formed in heated milks by the alkaline isomerization of lactose which is catalyzed by the free amino groups of caseins, i.e., the Lobry de Bruyn-Alberda Van Ekenstein transformation (Richards & Chandrasekhara, 1960; Adachi & Patton, 1961; Adhikari et al., 1991). The formation of lactulose depends on lactose concentration in raw milk (Aider & de Halleux, 2007) and does not occur naturally, but occurs when milk products are heat-treated (Jankowski, 2009). Furthermore, lactulose concentration in heated milk varies according to the temperature and time of the heat treatments and is also significantly affected by pH (Adachi, 1959; Martinez-Castro & Olano, 1980). Lactulose can be found in two forms: as free lactulose in solution and as N-deoxylactulosyl-L-lysine covalently bound to the amino group of the milk proteins (Henry et al., 1948; Adhikari et al., 1991). Different methods are currently used to quantify it such as high performance liquid chromatography (HPLC) (Parrish et al., 1980; Hashemi & Ashtiani, 2010), gas chromatography (Martínez-Castro, 1986) and enzymatic method (Claeys et al., 2001).

Another heat marker in milk is furosine. Since its detection 50 years ago, furosine has been used as a reliable indicator of thermal damage but also of food quality, since its concentration is related to storage conditions (Erbersdobler & Zucker, 1966; Erbersdobler & Somoza, 2007). The

compound was recognized as a quality indicator in several food products such as yogurt, pasta, milk, baking products, infant products and dehydrated fruits (Bignardi et al., 2012). Furosine is formed from the reaction between the amino acid lysine and reducing carbohydrates after acid hydrolysis of Amadori (Bignardi et al., 2012). The concentration of furosine can be quantified by HPLC. According to a report by Erbersdobler et al. (2002), furosine concentrations of 1154 and 9950 mg/kg of crude protein were detected in UHT and sterilized evaporated milks, respectively.

Kinetics of lactulose and furosine have been analyzed to monitor the changes in milk after heat treatment (Olano & Calvo, 1989; Claeys et al., 2001; Claeys et al., 2003). However, the formation of these markers was characterized by kinetic parameters such as activation energy and reaction rate constant. These parameters were obtained for the formation of lactulose and furosine in frozen raw milk samples treated at a temperature range between 90 and 140 ºC (Claeys et al., 2001). Despite the interest of lactulose as a marker of thermal damage, existing kinetic data in the literature regarding its formation in milk are certainly rare, probably due to the complexity of the chemical reactions involved and/or the limitations of the analytical methods available such as spectrophotometric (Adhikari et al., 1991) and chromatographic methods (Olano & Calvo, 1989).

Therefore, the specific objective of the present study was to determine the kinetics of lactulose and furosine formation during heat treatment in reconstituted low-heat skim milk powder.

4.2. Materials and methods

4.2.1. Experimental design

Kinetic study for the evaluation of lactulose and furosine formation during heat treatment was performed using a factorial design with two factors: *temperature* and *time*, with three *temperature* levels (80, 90 and 100 °C for lactulose; 70, 80 and 90 ºC for furosine) and five or six *time* levels (taking out time zero in the case of furosin or taking all samples as for lactulose) (see Chapter 3). The kinetic rate constant (k) and its activation energy (E_a) were estimated for both chemical markers. In addition, for the furosine formation, the temperature coefficient (Q_{10}) was estimated. The whole experiment was carried out in three independent occasions.

4.2.2. Sample preparation and heat treatment

For all trials, reconstituted standard skimmed milk was obtained from spray-dried powder with high-quality functional and microbiological grade (extra-grade, low-heat, spray-dried skim milk powder, pH= 6.5, 800 cfu g^{-1}) supplied by Chr. Hansen SL (Barcelona, Spain). Milk was reconstituted to 12% (w/w) with distilled water at ~40 ºC by stirring for 15 min at 150 rpm (Agimatic- E, JP. Selecta, Barcelona, Spain) and leaving it undisturbed at room temperature for

30 min. To study the kinetic formation of lactulose and furosine, heat treatments were applied to samples of 15 mL of milk in tubes of 30 mL capacity sealed and immersed in a thermostatic bath (mod. OvanTherm TC00E C, Lovango SL; control accuracy ±0.1 ºC) at the target temperature. At the different treatment time intervals, samples were removed from the bath and placed on iced-water to cool down to room temperature.

4.2.3. Chemical determinations

Lactulose and furosine quantifications were achieved in triplicate following the enzymatic method ISO 11285 (2004) with a Glucose/Fructose kit (Boehringer Mannheim/R-Biopharm Enzymatic BioAnalysis/Food Analysis, Germany) and the method ISO 18329 (2004), respectively.

4.2.4. Modeling the kinetic data

For the modeling of kinetic data, kinetics equations of zero, first and second order were evaluated to select the most appropriate kinetic model in each case. The effect of temperature (T) on the reaction rate was modeled with the Arrhenius equation (Peleg et al., 2012). To estimate the kinetic rate constant (k) of lactulose and furosine formations and their activation energy (E_a), once identified the order, the following equations were used (Claeys et al., 2003):

a) Zero-order rate Eqn:

$$[C] = [C]_0 + kt \qquad [1]$$

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

$$k_1 = Ae^{-\frac{Ea}{RT_1}} \qquad [2]$$

$$k_2 = Ae^{-\frac{Ea}{RT_2}}. \qquad [3]$$

Dividing equations (2) and (3) yields:

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

then, solving for k_1 yields:

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

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

$$[C] = [C]_0 + \left[k_{ref} \cdot e^{-\frac{Ea}{R}\left(\frac{1}{T} - \frac{1}{T_{ref}}\right)}\right] t, \qquad [6]$$

where:

$[C]$ = concentration of lactulose or furosine (mol L^{-1}),

$[C]_0$ = initial lactulose or furosine concentration (mol L^{-1}),

k = rate constant of reaction (mol s^{-1}),

t = reaction time (s),

A = Arrhenius equation collision factor for lactulose or furosine (mol s^{-1}),

E_a = activation energy for lactulose or furosine formation (kJ mol^{-1}),

R = universal ideal gas constant (kJ mol^{-1} K^{-1}),

T = reaction temperature (K),

k_{ref} = rate constant of the reaction at the reference temperature (mol s^{-1}), and

T_{ref} = reference temperature (K).

A bibliographic k_{ref} value was used (Earle & Earle, 2003), with a reference temperature of 120 ºC (393 K), to estimate the E_a and k values. Besides the concentration of lactulose chemically determined $[L]_E$ (experimental lactulose), concentration of lactulose at the different treatments was estimated from kinetic parameters using bibliographic information $[L]_T$ (theoretical lactulose) (Earle & Earle, 2003). To calculate the kinetic parameters of lactulose and furosine formation, Eqn (6) was fit to experimental data using nonlinear regression procedure of SAS as described below.

In addition, for furosine formation, the temperature coefficient (Q_{10}) was estimated using Eqn (7):

$$Q_{10} = \frac{k_2}{k_1} \qquad [7]$$

where k_1 and k_2 are the kinetic rate constants (mol s^{-1}) corresponding to the tested temperatures for a zero-order reaction.

4.2.5. Statistical analysis

All data were processed and analyzed using "Statistical Analysis System" (SAS, version 9.2, 2009, SAS Institute Inc., Cary, NC, USA). Nonlinear regression procedure (NLIN) was applied using the Arrhenius equation in order to calculate the kinetic parameters (activation energy and the constant of the velocity of reaction) of lactulose and furosine formation.

4.3. Results and discussion

4.3.1. Kinetics of lactulose formation

Lactulose formation was evaluated, as a function of heat treatment time at each of the tested temperatures (**Figure 4.1**), to estimate the order of reaction of lactulose formation as a chemical marker of thermal damage.

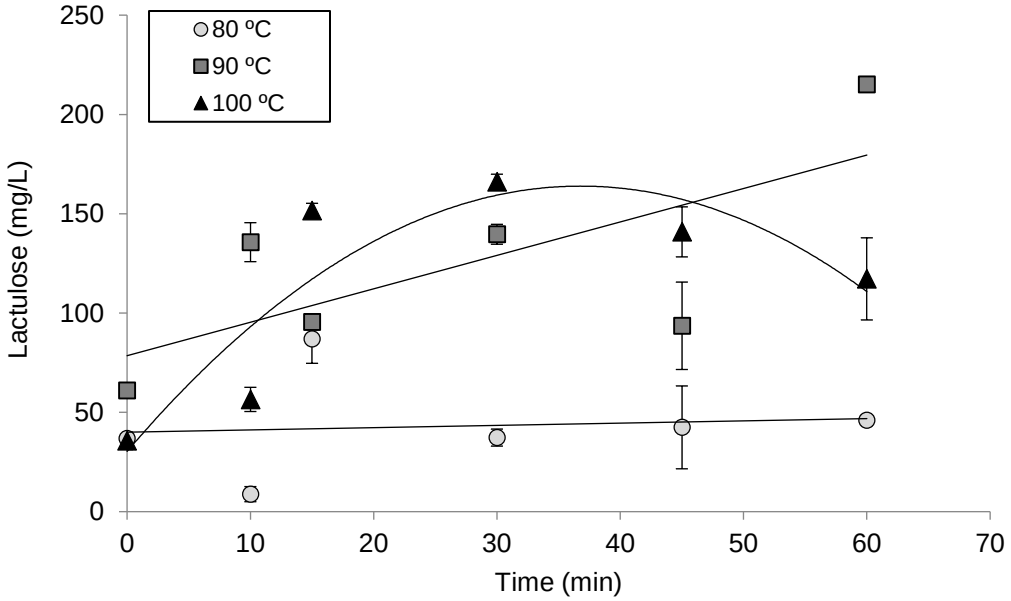


Figure 4.1. Lactulose concentration determined in skim milk powder heated at 80, 90 and 100 ºC, with the standard errors bars.

Table 4.1 shows the obtained R^2 values and *k* constants, which resulted from the adjustment of the experimental data to the different kinetic equations of lactulose formation (pseudo-zero-, first- and second-kinetic orders) at the three temperatures evaluated. Note that the order of reaction at 80 ºC could not be defined maybe as a result of the absence or little formation of lactulose (**Figure 4.1**) at this relative low temperature (Walstra et al., 2005). Indeed, type I markers are more effective for the assessment of low heat treatments than type II markers, such as lactulose (Pellegrino et al., 1995).

Table 4.1. Comparison of different kinetic equations for the formation of lactulose during heat treatment in milk.[1]

		80 °C		90 °C		100 °C	
	Equations	R^2	*k*	R^2	*k*	R^2	*k*
Order 0	$[L]_E = [L]_0 + kt$	$4 \cdot 10^{-5}$	$3 \cdot 10^{-8}$	0.86	$8 \cdot 10^{-6}$	0.18	$4 \cdot 10^{-6}$
Order 1	$\ln [L]_E = \ln [L]_0 + kt$	0.017	0.005	0.74	0.021	0.13	0.011
Order 2	$1/[L]_E = 1/[L]_0 + kt$	0.0385	-321.1	0.57	-73.4	0.044	-30.8

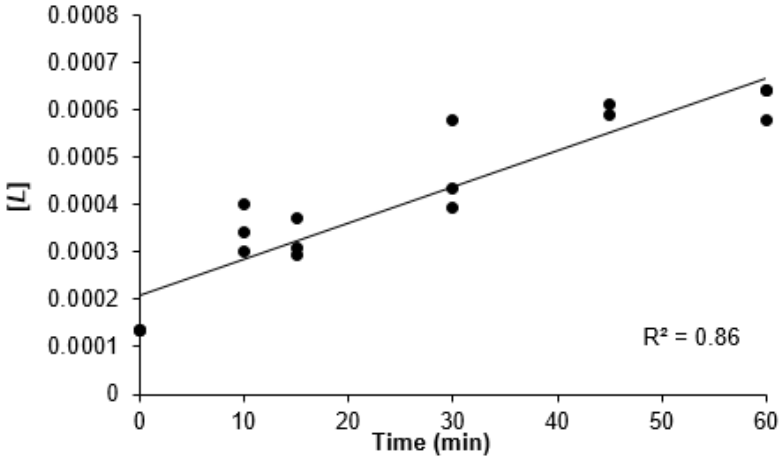
[1]N = 54; number of replicates, Nr = 3; R^2, coefficient of determination; *k*, rate constant; $[L]_E$, experimental lactulose concentration; *t*, time.

In contrast, at 90 ºC (**Figure 4.2**), R^2 values of 0.86, 0.74 and 0.57 were obtained with pseudo-zero, first- and second-orders, respectively, confirming that the kinetics of lactulose formation at 90 ºC follows an order of reaction pseudo-zero as reported by Earle & Earle (2003). Although the R^2 obtained with milk treated at 100 ºC were considerably lower than those obtained at 90 ºC, the results suggested that the reaction at 100 ºC also corresponds to a pseudo-zero order; although at 100 ºC the R^2 for pseudo-zero order was rather low, it was higher than those obtained for first and second order kinetic reactions. However, other authors mentioned that the formation of lactulose fits first-order kinetics, using milk treated between 25 and 100 ºC for 30 min or at 140-150 ºC for 10 min and determining lactulose by gas chromatography (Olano & Calvo, 1989).

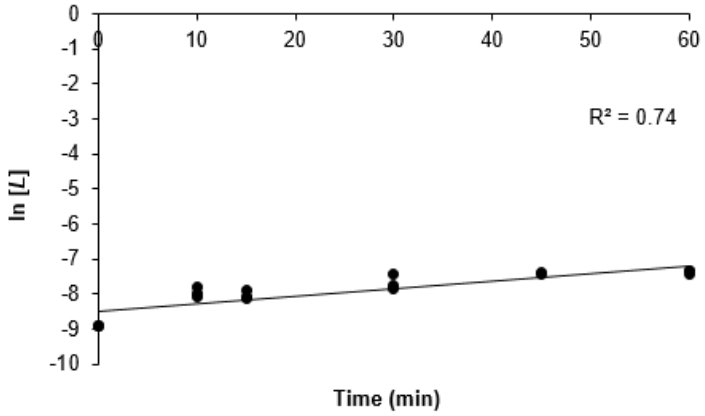
The small R^2 value obtained at 100 ºC may be due to the response of the lactulose formation, which is not linear at the temperature mentioned, and to the increase of the variability above 30 min (**Figure 4.1**). As for other studies, it was also observed that as heat load increases, the variability increases (Martinez-Castro et al., 1986; Marconi et al., 2004; Sakkas et al., 2014). For instance, in sterilized milk, Marconi et al. (2004) obtained lactulose concentrations between 493 and 1147 mg/L, which represents a considerable variability. In other studies, although much higher lactulose concentrations were observed for sterilized milks, with values ranging from 1080 up to 1400 mg/L, high variability of the measurements was also observed (Olano & Calvo, 1989; Villamiel et al., 1999; Morales et al., 2000; Elliott et al., 2003; Elliott et al., 2005; Cattaneo et al., 2008).

Eqn. (6), developed in section 4.2.4, from a zero-order rate equation (Eqn. (1)) and the Arrhenius equation (Eqn. (2)) was used to estimate E_a and k (**Table 4.2**) taking as reference data from the study carried out by Earle & Earle (2003). On one hand, Eqn. (I), using the reference value for E_a, yielded an estimated k value close to that observed in the reference study. On the other hand, Eqn. (II) used the reference value for k to estimate E_a, which resulted to be a little higher than the value stated in the reference study. When both E_a and k values were estimated from Eqn. (III), the values obtained were close to reference values. The best fit over the experimental data corresponded to the regression Eqn. III of **Table 4.2**, estimating both E_a and k, with an R^2 of 0.75.

a)



b)



c)

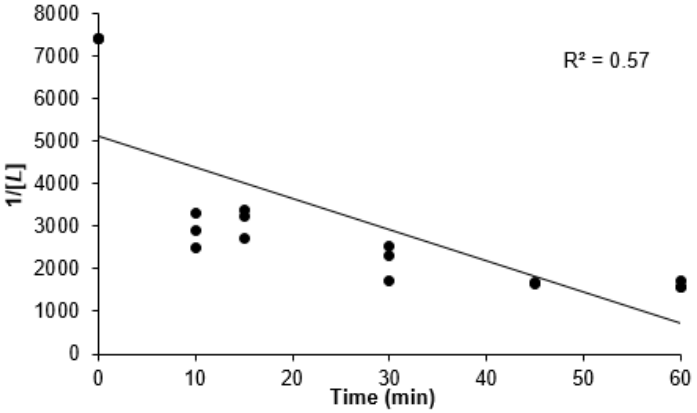


Figure 4.2. Fit of the experimental lactulose concentration data to kinetic orders a) pseudo-zero, b) first, and c) second, at a temperature of 90 ºC.

Table 4.2. Equations for determining k and E_a of lactulose formation during heat treatment of milk.

	Equations	Kinetic parameters	
		E_a (kJ mol^{-1})	k_{ref} (mol s^{-1})
I	$[L] = [L]_0 + [k_{ref} e^{-\frac{120}{R}\left(\frac{1}{T}-\frac{1}{T_{ref}}\right)}]t$	120‡	$6.85 \cdot 10^{-7}$†
II	$[L] = [L]_0 + [5.38 \times 10^{-6} e^{-\frac{Ea}{R}\left(\frac{1}{T}-\frac{1}{T_{ref}}\right)}]t$	251†	$5.38 \cdot 10^{-6}$ ‡
III	$[L] = [L]_0 + [k_{ref} e^{-\frac{Ea}{R}\left(\frac{1}{T}-\frac{1}{T_{ref}}\right)}]t$	44.8†	$1.84 \cdot 10^{-7}$†

[1]N = 54; number of replicas, Nr = 3; k, kinetic rate constant; E_a, activation energy; T, temperature; T_{ref} = 393.15 K.
† Estimated values; ‡reference data (Earle & Earle, 2003).

4.3.2. Kinetics of furosine formation

As for lactulose, the furosine formation was evaluated according to heating, in order to estimate the order of reaction.

The best fit at each temperature demonstrated that the reaction of furosine formation followed a pseudo-zero order with a kinetic constant of 1.904 mol s^{-1} at 90 ºC (**Table 4.3**), close to that reported by Claeys et al. (2001) with a value of 2.03 mol s^{-1} at 90 ºC. According to the R^2, the order of kinetic reaction of furosine formation belongs to a pseudo-zero order, which is in accordance with Montilla et al. (1996), who also found that furosine formation belonged to pseudo-zero order in whole UHT milk (135 to 150 ºC for 10 to 40 s).

Table 4.3. Comparison of equations with different kinetic order for furosine formation during heat treatment of milk at three different temperatures.[1]

		70 °C		80 °C		90 °C	
	Equations	R^2	k	R^2	k	R^2	k
Order 0	$[Fu] = [Fu]_0 + kt$	0.930	1.408	0.927	1.650	0.856	1.904
Order 1	$\ln[Fu] = \ln[Fu]_0 + kt$	0.112	0.012	0.113	0.010	0.102	0.008
Order 2	$1/[Fu] = 1/[Fu]_0 + kt$	0.367	-0.001	0.455	-0.001	0.547	-0.002

[1]N = 45; number of replicates, Nr = 3; R^2, determination coefficient; k, kinetic rate constant; [Fu], furosine concentration; t, time.

Eqn. (6) was used to estimate the activation energy (E_a) and was fitted to the 45 samples, which constituted the whole experiment, to estimate the activation energy of furosine formation (E_a), as well as its constant rate at the reference temperature (T_{ref}). Three different approaches were used:

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 x 10^{-7} mol s^{-1} (Earle & Earle, 2003) and estimating E_a;

c) estimating both parameters (E_a and k_{ref}) as are shown in **Table 4.4**.

Figure 4.3 shows the regressions summarized in **Table 4.4.** The best correlation was obtained using Model III ($R^2 = 0.92$, **Figure 4.3c**), where E_a and k_{ref} were estimated, and presented the best fit over the experimental data. The obtained values for the estimated parameters were within the same range as those found in literature (Claeys et al., 2001; Claeys et al., 2003), but underestimated.

Table 4.4. Equations for determining k and E_a of furosine formation during heat treatment of milk.

	Equations	Kinetic parameters	
		E_a (kJ mol^{-1})	k_{ref} (mol s^{-1})
I	$[Fu] = [Fu]_o + [k_{ref} e^{-\frac{81.64}{R}\left(\frac{1}{T}-\frac{1}{T_{ref}}\right)}]t$	81.637‡	4.01 x 10^{-7}†
II	$[Fu] = [Fu]_o + [4.9710^{-7} e^{-\frac{Ea}{R}\left(\frac{1}{T}-\frac{1}{T_{ref}}\right)}]t$	87.189†	4.97 x 10^{-7}‡
III	$[Fu] = [Fu]_o + [k_{ref} e^{-\frac{Ea}{R}\left(\frac{1}{T}-\frac{1}{T_{ref}}\right)}]t$	15.596†	6.73 x 10^{-8}†

[1]N = 45; number of replicas, Nr = 3; k, kinetic rate constant; E_a, activation energy; T, temperature; T_{ref} = 393.15 K.
† Estimated values; ‡reference data (Claeys et al., 2001).

For Eqn. I (**Figure 4.4a**), where fitting was restricted by assuming a bibliographic value of E_a = 81.64 kJ mol^{-1} previously estimated by Claeys et al. (2001), the estimated value of k was similar to that found in the literature with frozen raw milk samples treated at 90-140 ºC (Claeys et al., 2001). In equation II (**Figure 4.4b**), a bibliographic value of $k = 4.97 \times 10^{-7}$ mol s^{-1} was used and the obtained E_a value was also close to literature. However, the R^2 in equations I and II were lower than estimated in equation III. The E_a calculated for Montilla et al. (1996) from Arrhenius equation was 93.15 kJ mol^{-1}, of the same order of magnitude as previous results. Nangpal & Reuter (1990) estimated an E_a of 100.7 kJ mol^{-1} in milk samples heated with steam injection during direct UHT.

Finally, Eqn. (7) was used, with k_1 and k_2 corresponding to the tested temperatures for a zero-order reaction (**Table 4.4**), in order to estimate the temperature coefficient (Q_{10}) for furosine concentration in the temperature interval of the study (70 to 90 ºC). The estimated Q_{10} value between 70 and 80 ºC was 1.17 and 1.15 between 80 and 90 ºC. These estimated values were similar to those found in literature (Claeys et al., 2001).

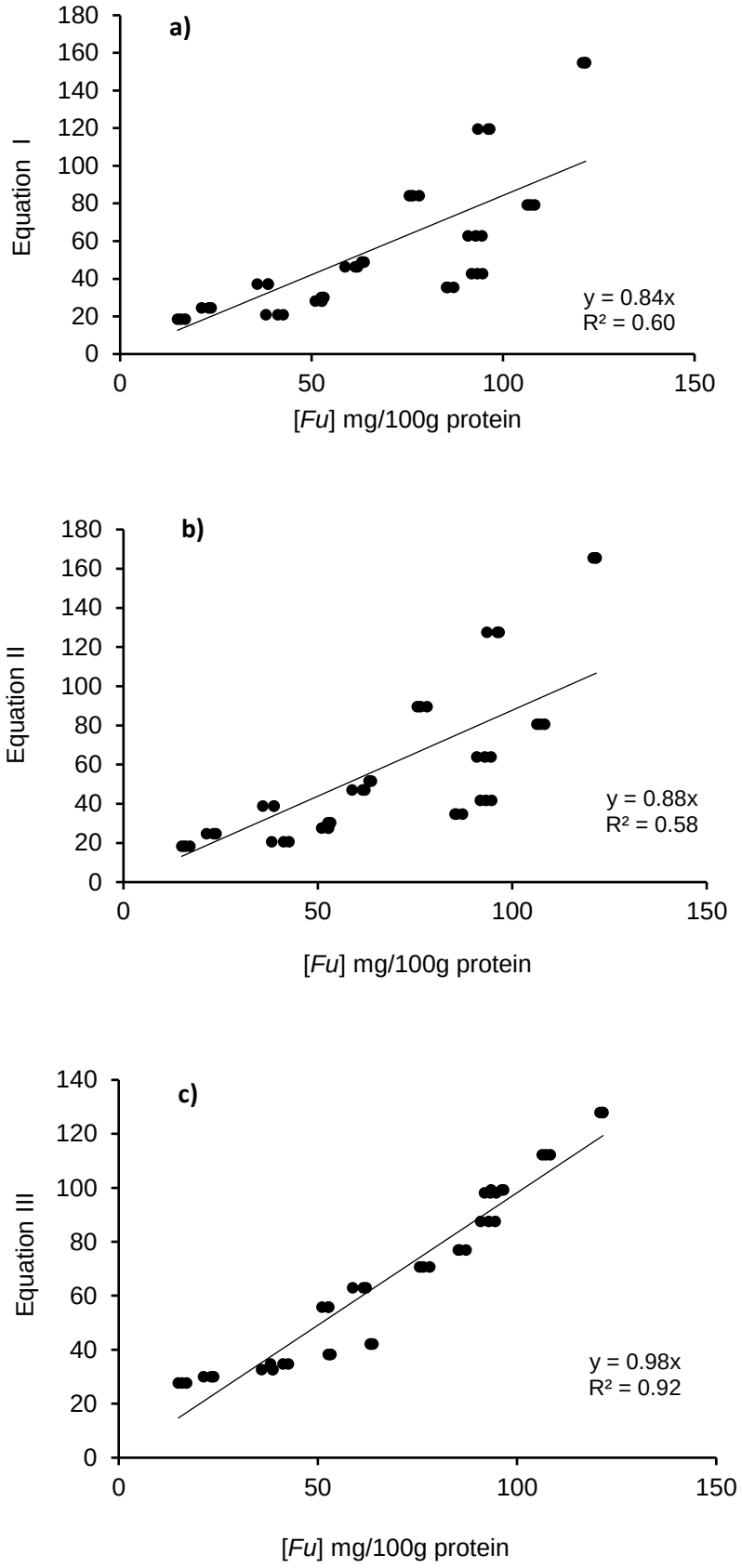


Figure 4.3. Regression between experimental and estimated values of furosine [*Fu*]. a) Equation I, b) Equation II and c) Equation III.

4.4. Conclusions

Kinetics of lactulose and furosine formation in heat-treated reconstituted skim milk powder were evaluated. Kinetic parameters as activation energy and constant of reaction were obtained from the chemical determination of both markers. In addition, the temperature coefficients were estimated in the furosine formation. Furthermore, considering the three studied temperatures, 90 ºC was found to be more adequate for determining the kinetics of lactulose formation, due to its clearer and more consistent trend between independent replicates of each heat treatment. In summary, the results obtained from lactulose and furosine formation suggest that both reactions corresponded to a pseudo-zero order with kinetic values estimated in the same magnitude as those found in literature.

CHAPTER 5:
Selected fluorescent markers

5.1. Introduction

As mentioned in Chapter 2, several techniques have been developed to monitor the quality of food, especially in the field of chromatographic and spectroscopic techniques, such as HPLC, gas chromatography, and electronic nose or fluorescence, Raman, mid-infrared, near-infrared, UV-Vis, and nuclear magnetic resonance (Ornelas-Soto et al., 2011). The use of spectroscopic methods is increasing in food research for determining the authenticity of treatments (Boyaci et al., 2015). In that way, the FFF is a useful method in the food industry to monitor several kind of food (dairy products, eggs, fish, meat, olive oils, honey, beer, sugar, etc.) (Christensen et al., 2006; Sádecká & Tóthová, 2007).

Particularly, milk is a complex biological system that can be monitored by FFF, because milk contains intrinsic fluorescent components (such as *Trp* and some vitamins). The excited state of a fluorophore is very sensitive to physicochemical properties of its environment and it is the main reason why the fluorescence technique is used frequently to study proteins structure and function. Some autofluorescent molecules might be useful as "thermal markers" since they show a change in their fluorescence due to thermal denaturation/degradation or formation. For example, *Rb* loss in milk during pasteurization and exposure to light is 10 to 20% (McDowell, 2000). But much larger losses (50 to 70%) can occur if bottled milk is left standing in bright sunlight for more than 2 h (McDowell, 2000).

Another useful fluorescent marker is *Trp* since it occurs in most proteins and biologically active peptides and its fluorescence is highly sensitive to environment, making it an ideal marker for reporting protein conformation changes and interactions with other molecules (Chen & Barkley, 1998; Pan et al., 2011). *Dt* is a marker that has been proposed to evaluate oxidative stress in proteins (Heineckes et al., 1993) and, since it forms in vivo, it may also serve as a useful intrinsic fluorescent probe of protein environments and internal reactions (Harms et al., 1997; Malencik & Anderson, 2003).

Fluorescent products have been proposed as early indicators of the Maillard reaction since, in stages prior to the formation of brown pigments, fluorescent compounds are formed (Bastos et al., 2012). In particular, the progress of the Maillard reaction in milk and milk-resembling systems during heating might be followed by monitoring free fluorescent intermediary compounds (Morales et al., 1996), hereafter referred as *MC*. In an early stage, fluorescence experiments done

by the classical right-angle fluorescence spectroscopy were carried out by diluting milk down to an absorbance of 0.1. At higher absorbances, the screening effect (or inner filter effect) provokes distortion of the excitation spectra and a decrease of fluorescence intensity (Genot et al., 1992).

Based on the foregoing, the aim of the present study was to analyze the fluorescent changes of the reconstituted skim milk samples before and after different levels of heat treatment.

5.2. Materials and methods

5.2.1. Experimental design

As described in Chapter 3, the evaluation of autofluorescent markers (*Dt*, *Trp*, *MC* and *Rb*) during heat treatment was performed using four levels of temperature (70, 80, 90 and 100 °C) and five levels of holding time (10, 15, 30, 45 and 60 min) taking time zero as reference. The whole experiment was carried out in three independent occasions.

5.2.2. Sample preparation and heat treatment

Sample preparation and heat treatments were performed as described in section 4.2.2. Briefly, low-heat skim milk powder was reconstituted to a total solids level of 12% (w/w) with distilled water at ~40 °C and stirred for 15 min at 150 rpm, then left to rehydrate for 30 min at room temperature, in the dark. Sealed tubes with 15 mL of milk were immersed in a thermostatic bath at the target temperatures for the selected holding times, after which samples were removed from the bath and placed on ice to cool down to room temperature.

5.2.3. Fluorescence analysis

Fluorescence measurements were performed using a fluorescence spectrophotometer (Cary Eclipse Fluorescence Spectrophotometer, Agilent Technologies, Madrid, Spain) equipped with 15 W lamp “press Xenon lamp” and a “front-face” geometry accessory (Solid Sample Holder Accessory and Cuvette Kit, Agilent Technologies), adjusted to an angle of incidence of 35º, which minimizes both specular phenomena from the surface of the cuvette and internal absorption, i.e., inner filtration of the sample. Measurements were made using Suprasil® quartz cuvettes (UV fluorescence cell, Agilent Technologies). Both excitation and emission wavelengths were selected based on the compound to be determined and all fluorescence determinations were performed in triplicate. Fluorescence spectra of each fluorescent compound were obtained in triplicate under the settings shown in the **Table 5.1** and readings were performed every 1 nm. The voltage of the photomultiplier detector was set to 400 V for *Trp* and *Rb* excited at 267 nm and 600 V for the rest.

Table 5.1. Fluorophores selected and their excitation/emission wavelengths.

Fluorophores		Excitation λ_{max} (nm)	Emission λ (nm)
Tryptophan	*Trp*	290	300-450
Dityrosine	*Dt*	315	350-500
Maillard compounds	*MC*	330	350-500
Riboflavin	Rb_{267}	267	470-570
	Rb_{370}	370	470-570
	Rb_{450}	450	470-570

5.2.4. Data Analysis

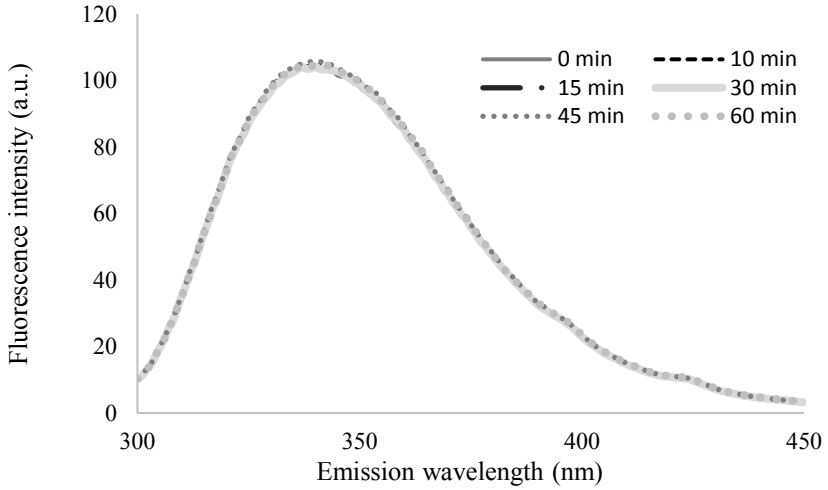
ANOVA (Analysis of variance) with GLM repeated measures (MIXED) of SAS software (version 9.4, 2012, SAS Institute Inc., Cary, NC, USA) was used to determine the effects of the two factors (temperature and time) and their interaction on the main dependent variables ($\Delta\lambda$, ΔI_λ and ΔI_F, defined in Figure 5.2). Principal Component Analysis (PCA) was performed using LatentiX © data analytical software (version 2.12, 2016, Copenhagen, Denmark).

5.3. Results and discussions

5.3.1. Changes caused by heat in the front-face fluorescence spectrum of milk induced by excitation at 290 nm

The entire FFF emission spectra, induced at an excitation wavelength of 290 nm and measured for each heat treatment intensity, were collected and analyzed. The emission from phenylalanine (Phe) in milk proteins is rarely observed because of the small quantum yield ($\Phi = 0.03$) of this amino acid. Its fluorescence is typically observed in absence of tyrosine (Tyr) and *Trp* (Alvarado, 2017) as those two aromatic amino acid residues have quantum yields one order of magnitude higher as compared to Phe (~0.14 and ~0.13, respectively). Furthermore, maximum fluorescence excitation wavelength of Phe is near 260 nm and, subsequently, is not excited at 290 nm. On the other hand, even though milk proteins contain similar number of Tyr and *Trp* residues, according to Lakowicz (2006), fluorescence emission of Tyr from most proteins is low and undetectable. It should be noted that molar extinction coefficient, ε, at 290 nm is about 19 times higher for *Trp* than that of Tyr ($\varepsilon = 4.71\ M^{-1}\ cm^{-1}$ and $0.25\ M^{-1}\ cm^{-1}$, respectively). Finally, resonance can happen from Phe to Tyr to *Trp*. This phenomenon has been observed in many proteins, which is one more reason for the minor contribution of Phe and Tyr to the emission of most proteins. As a result, for the most part, fluorescence observed from milk proteins at an excitation wavelength of 290 nm is due primarily to *Trp* emissions at the conditions used in the present study.

a)


120
100
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Fluorescence intensity (a.u.)
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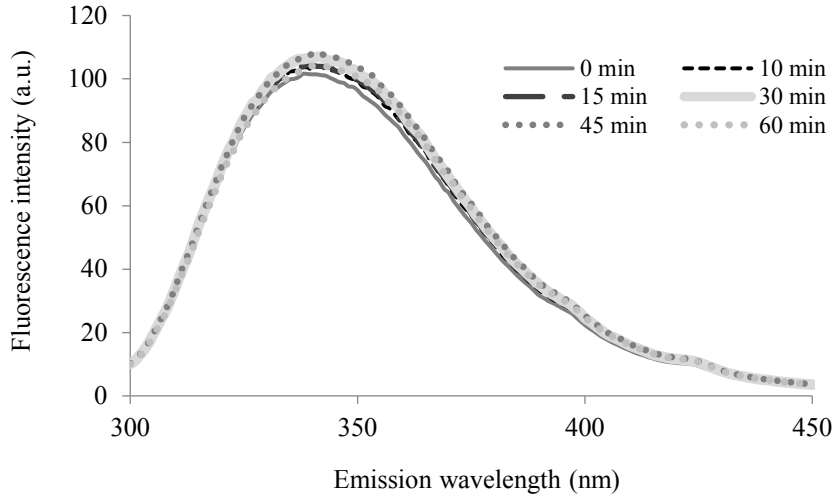
Emission wavelength (nm)

b)


120
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Fluorescence intensity (a.u.)
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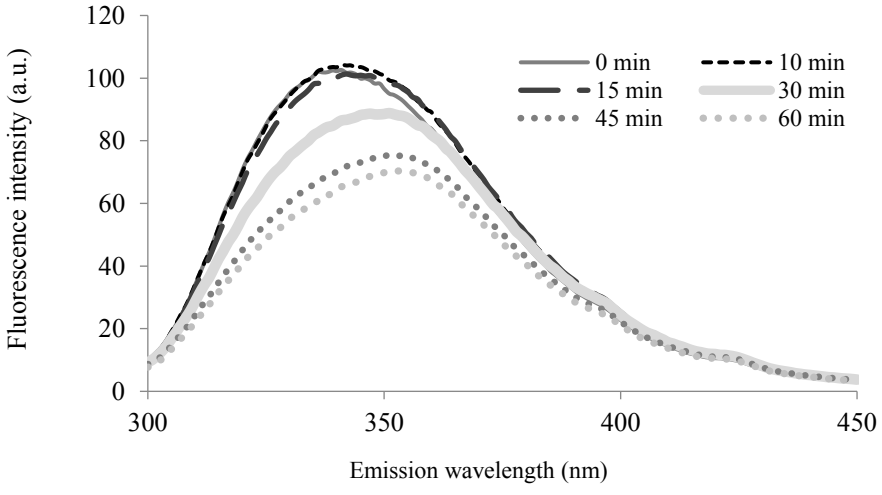
Emission wavelength (nm)

c)


120
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Fluorescence intensity (a.u.)
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60 min
300
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Emission wavelength (nm)

d)

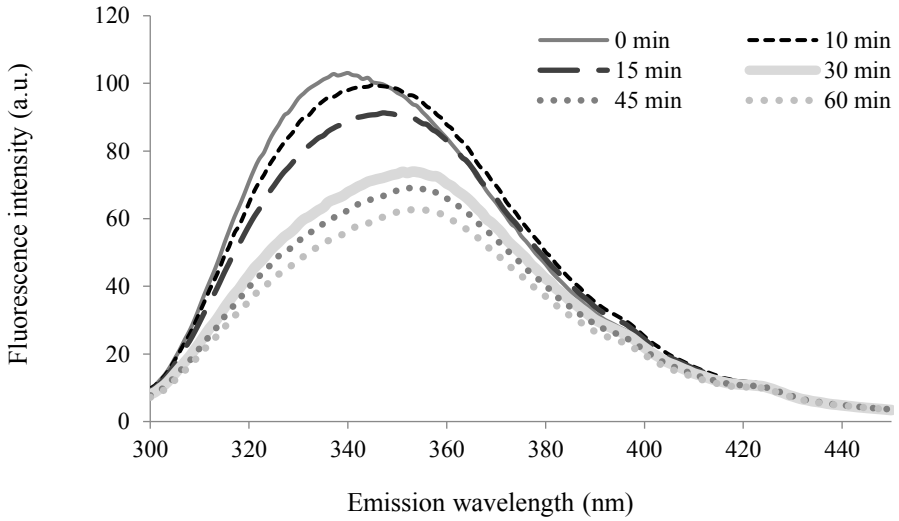


Figure 5.1. Fluorescence spectra of tryptophan, corresponding to skim milk samples treated at different holding times and at a temperature of a) 70, b) 80, c) 90 and d) 100 °C.

In general terms, it was observed that increasing heat load induced a decrease in fluorescence intensity and a red shift, especially at higher heat treatment temperatures (**Figure 5.1**). As a result, in order to further characterize this apparently systematic modification in the FFF response, the changes observed in the emission spectra, caused by heat, were described in terms of the change in intensity and the red shift occurring at the peak of maximum fluorescence emission, according to the procedure described below and represented in **Figure 5.2**. This figure shows the changes that were induced by heat treatments around the maximum emission peak, when comparing the typical FFF spectrum corresponding to non-heat-treated samples to that obtained by applying the most intense heat treatment condition tested (i.e., 100 °C, 60 min). In non-heat-treated samples, the maximum emission peak was typically observed at ~340 nm, while in samples heated at 100 °C for 60 min, it shifted to ~354 nm. These changes in the emission wavelengths were represented as $\Delta\lambda$, which corresponded to the difference between the wavelengths of the two maximum peaks in raw and in thermally treated milks, respectively (i.e., $\Delta\lambda = \lambda_0 - \lambda_T$).

The observed total decrease of fluorescence intensity, ΔI, corresponded to the subtraction of the intensity of the heat-treated and non-treated samples both measured at λ_0 (~340 nm). ΔI was further divided into other two variables, ΔI_F and ΔI_λ, which were calculated from the spectra as shown graphically in **Figure 5.2**. Note that ΔI_F represented the decrease of fluorescence intensity response associated to the change in height of the peak at a fixed λ value, while ΔI_λ represented the decrease of intensity associated to the red shift.

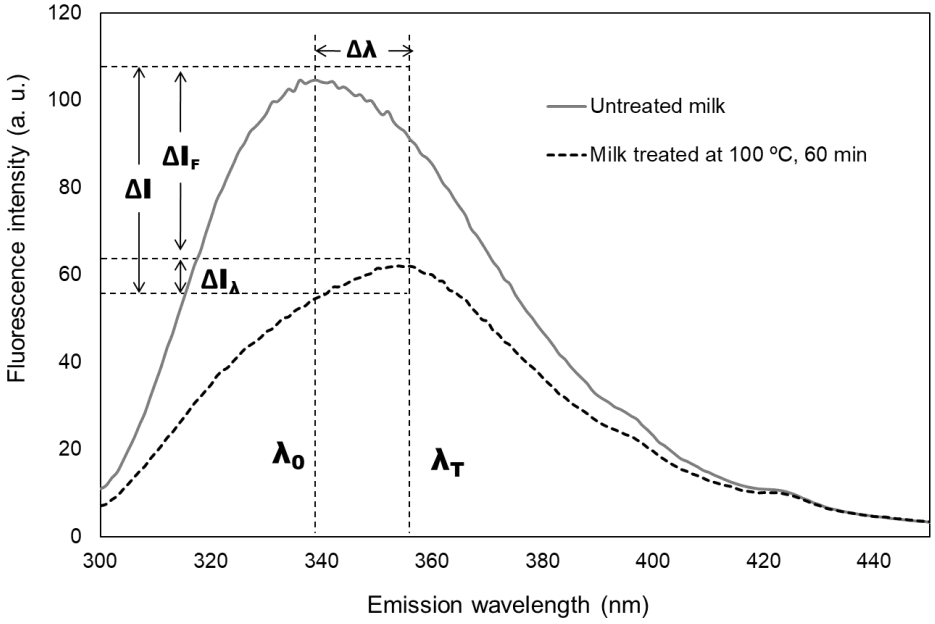


Figure 5.2. Schematic figure showing the decomposition of the decrease in total fluorescence intensity of tryptophan (ΔI) into two fractions, one associated with the change of the peak height (ΔI_F) and another with the displacement of the emission maximum (ΔI_λ). Change in the peak wavelength can be seen as a shift from λ_0 to λ_T.

The variability of the defined variables, $\Delta\lambda$, ΔI, ΔI_F and ΔI_λ, as a function of the main experimental effects: temperature (T), holding time (t), the interaction T x t, and the replication, was statistically analyzed by ANOVA using the PROC MIXED procedure of SAS. Both temperature and time, as well as their interaction, were significant at 99.9% for each one of the parameters evaluated. The replicates resulted not significant and, subsequently, this effect was removed from the model. **Table 5.2** contains the complete data matrix of the *Trp* fluorescence changes induced by the different temperature and time combinations, on the parameters characterized in **Figure 5.2**.

Major changes were not detected in samples heated at 70 ºC (**Figure 5.1a** and **Table 5.2**), as fluorescence intensity variations observed (ΔI) were small and not significant; and no red shift was clearly detected either ($\Delta\lambda$). This results are in agreement with Anema (2008), who stated that major whey proteins, β-LG and α-LA, do not denature to a significant extent below 70 ºC. Unlike in the present study, Halder et al. (2012) observed a clear red shift in samples transiently heated from 25 to 70 ºC. According to the authors, the red shift induced during heating was generated as a result of reversible ongoing protein denaturation. Right after a heating cycle, the samples were cooled down, which caused the fluorescence peak to return to the initial wavelength value. Note that the authors stated that the fluorescence emission spectra at the different evaluation temperatures were collected after 5 min of equilibration, when the target temperature was reached. However, in the present study, the fluorescence measurements of the samples were

all taken at room temperature (~22 ºC) and, in the present case, we measured permanent effects, by recording emission fluorescence spectra on already cooled samples after the thermal treatment. As a result, in the present study no temperature-induced shift should be expected. However, it is well known that a temperature increase tends to reduce the quantum yield due to increased probability of external conversion and reduced viscosity, which would affect fluorescence intensity rather than generate a wavelength shift. According to that, Halder et al. (2012) did observe a decrease of fluorescence intensity with increasing temperatures between 25 and 70 ºC, but this effect was likely not observed in the present study as the measurements were collected in treated samples at a constant room temperature, at a heating temperature that does not induce permanent denaturation of whey proteins. In a different study, Kulmyrzaev et al. (2005) found a slight decrease in the fluorescence intensity in samples treated at 72 ºC during 30 min relative to raw milk. The authors suggested that the extent of heating applied in the study was not as high as to induce considerable conformational changes that could significantly modify fluorescence properties of milk.

As for treatments at 70 ºC, samples treated at 80 ºC (**Figure 5.1b** and **Table 5.2**) at different holding times, up to 60 min, did not demonstrate significant differences in either fluorescence intensity or red shift. Thus, in samples heated at 70 and 80 ºC, the fluorescence intensity/wavelength shift changes observed were not conclusive, likely because the non-permanent nature –or reduced percentage– of the conformational changes of whey proteins originated within this temperature range.

Nevertheless, a reduce number of authors have curiously observed a slight increase in FFF intensity with application of low-intensity heat treatments, especially at temperatures up to 80 ºC. Taterka (2016) measured fluorescence of cow skim milk samples heated at 80 ºC for 0, 3, 5, 7, 12 or 25 min using an excitation wavelength of 290 nm. At an emission wavelength of 340 nm, FFF intensity increased after 25 min at milk pHs 6.3 and 7.1 by ~15.4 and 7.7%, respectively. Yazdi & Corredig (2012) and Boubellouta & Dufour (2008) also observed increasing *Trp* fluorescence in milk heat-treated at 80 ºC for 10 min and up to 50 ºC, respectively. As mentioned above, even though no significant change on FFF intensity was observed in the present study at 70 and 80 ºC, a slight but consistent increase of FFF intensity, ΔI_F, was observed at 80 ºC (**Table 5.2** and **Figure 5.3a**). Note that ΔI_λ should be a negative value (**Table 5.2**). This is logical, if the maximum peak of intensity at 80 ºC slightly red-shifts (**Table 5.2**), a small decrease of intensity measured at 340 nm, induced by the mentioned shift, is subsequently expected. As a result, the slight increase of total intensity, ΔI, observed at this temperature with increasing heating time, is less pronounced (**Table 5.2**). In agreement with the described tendency, Alvarado (2017), heating skim milk at 70, 80 and 90 ºC for 0, 5, 10, 15, 20, 25, 30, 45 and 60 min, and measuring the *Trp* emission spectrum

in the range between 300 and 450 nm, found a slight increase in fluorescence intensity at the maximum fluorescence peak followed by a significant decrease, which at 80 and 90 °C started after 20 and 5 min of heating, respectively. At both temperatures, the changes in intensity were accompanied by a clear red shift. However, no change on intensity or wavelength of emission was observed by this author at 70 ºC.

Table 5.2. Response of tryptophan fluorescence to heat treatment.[1]

Temperature (°C)	Time (min)	Δλ (nm)	ΔI_F (a.u.)	ΔI_λ (a.u.)	ΔI (a.u.)
70	10	-0.31 ± 0.54^{a,b}	0.34 ± 1.08^{a,b,c}	-0.15 ± 0.25^{a}	0.19 ± 1.23^{a,b,c}
70	15	-1.60 ± 0.58^{a,b}	0.19 ± 0.53^{b,c}	-0.47 ± 0.42^{a}	-0.28 ± 0.43^{a,b,c}
70	30	0.67 ± 1.48^{a}	-0.58 ± 0.24^{c,d}	-0.80 ± 035^{a}	-1.38 ± 0.12^{b,c}
70	45	-1.60 ± 0.46^{a,b}	1.35 ± 0.86^{a,b,c}	-0.49 ± 0.42^{a}	0.87 ± 0.99^{a,b,c}
70	60	-0.00 ± 1.73^{a,b}	-0.09 ± 0.39^{b,c}	-1.13 ± 1.12^{a,b}	-1.22 ± 1.39^{b,c}
80	10	-1.34 ± 1.50^{a,b}	1.60 ± 2.12^{a,b,c}	-0.32 ± 0.27^{a}	1.28 ± 2.38^{a,b,c}
80	15	-0.67 ± 2.01^{a,b}	2.33 ± 3.32^{a,b,c}	-0.54 ± 0.69^{a}	1.79 ± 3.42^{a,b,c}
80	30	-1.96 ± 0.92^{a,b}	4.44 ± 4.47^{a,b}	-0.54 ± 0.23^{a}	3.90 ± 4.24^{a,b}
80	45	-1.34 ± 3.17^{a,b}	5.26 ± 0.70^{a}	-1.04 ± 1.18^{a,b}	4.22 ± 1.45^{a}
80	60	-2.02 ± 1.04^{a,b}	1.42 ± 1.24^{a,b,c}	-0.69 ± 0.37^{a}	0.73 ± 1.60^{a,b,c}
90	10	-2.34 ± 2.51^{b}	0.71 ± 2.21^{a,b,c}	-0.69 ± 0.79^{a}	0.02 ± 1.91^{a,b,c}
90	15	-2.40 ± 1.52^{b}	-2.37 ± 4.14^{c,d}	-1.01 ± 0.61^{a,b}	-3.37 ± 4.06^{c,d}
90	30	-9.06 ± 2.95^{c,d}	-16.75 ± 2.03^{e}	-2.92 ± 0.86^{c}	-19.66 ± 2.66^{e}
90	45	-11.74 ± 1.15^{d,e}	-32.05 ± 6.92^{f}	-4.92 ± 1.04^{d}	-36.96 ± 7.65^{f}
90	60	-12.45 ± 2.07^{e}	-37.88 ± 1.42^{g,h}	-5.62 ± 0.83^{d,e}	-43.49 ± 2.19^{g,h}
100	10	-6.30 ± 1.51^{c}	-5.39 ± 4.59^{d}	-1.95 ± 0.81^{b,c}	-7.34 ± 5.26^{d}
100	15	-8.33 ± 1.13^{c}	-14.32 ± 5.67^{e}	-2.69 ± 1.00^{c}	-17.01 ± 6.36^{e}
100	30	-13.02 ± 1.00^{e}	-35.71 ± 1.30^{f,g}	-6.47 ± 0.52^{e,f}	-42.17 ± 1.61^{f,g}
100	45	-13.33 ± 0.58^{e}	-40.86 ± 2.42^{h}	-6.74 ± 0.16^{e,f}	-47.60 ± 2.56^{h}
100	60	-14.00 ± 2.00^{e}	-47.31 ± 1.55^{i}	-6.87 ± 0.57^{f}	-54.18 ± 1.73^{i}

[1]N = 60; Δλ, change in the wavelength of maximum fluorescence emission; ΔI_F, variation in the fluorescence intensity due to the change of the peak height; ΔI_λ, changes in fluorescence intensity due to the peak shift; ΔI, total decrease in the fluorescence intensity of tryptophan.

In the present study, changes in the fluorescence were very remarkable at 90 and 100 ºC (**Figure 5.1c** and **d**). A slight shift (-2.34 nm) was observed in treatment at 90 ºC for 10 min (**Table 5.2**), although the fluorescence intensity was similar to control milk. Schamberger & Labuza (2006) suggested that the changes observed in the fluorescence intensity of samples heated at 90 ºC for less than 10 min may be due to the denaturation of proteins and this seemed to produce a red shift. According to Tayeh et al. (2009) and Yazdi & Corredig (2012), most of the fluorescence observed in milk comes from casein micelles (80% of total proteins; α_{s1}-, α_{s2}-, β-, and κ-caseins (CN)), and whey proteins (20% of total proteins; α-LA, β-LG and bovine serum albumin). In addition, 60% of the mass proportion of whey proteins comes from β-LG, the major whey protein, while α-LA comprises a 20%. It is well known that casein micelles are quite heat stable and do not undergo

major heat induced changes below 100 °C (Goff, 2016). In general, *β*-LG is more heat sensitive than *α*-LA in that it denatures to a greater extent. Moreover, *α*-LA has a greater capability to unfold and refold back into its native state (H. Taterka & Castillo, 2018). Further, as stated by Lakowicz (2006), the emission of Tyr is relatively insensitive to solvent polarity. Thus, it was assumed that a red shift was observed during denaturation of whey proteins, and that the red shift might be dominated by conformational changes affecting *β*-LG. This whey protein contains two *Trp* residues: one is buried inside the protein structure (Trp^{19}) and a second one is located on its surface (Trp^{61}). The residue of Trp^{61} contributes to the fluorescence emission by 20%, while Trp^{19} generates the remaining 80% of the total fluorescence due to the native protein (Estévez et al., 2017). Apparently, Trp^{61} fluorescence is mostly quenched by its proximity to the Cys^{66}-Cys^{160} disulphide bond and the guanidine group of Arg^{124}, and/or self-quenched by the Trp^{61} placed in the other monomer of the *β*-LG dimeric form. During denaturation, Trp^{19} residue become exposed and moves from non-polar (folded) to a polar (unfolded) environment, i.e., bathochromic shift with increasing solvent polarity (Lakowicz, 2006). Accordingly, from data presented in **Table 5.2**, quadratic red shifts were observed with increasing holding times in milk samples treated at 90 and 100 ºC as follows:

$$\Delta\lambda_{90°C}\ (nm) = 0.0046t^2 - 0.5440t + 3.4907\ (R^2 = 0.98), \text{ and}$$

$$\Delta\lambda_{100°C}\ (nm) = 0.0049t^2 - 0.4883t - 2.0969\ (R^2 = 0.97).$$

In addition, as mentioned by Schamberger & Labuza (2006), the red shift originated by whey protein denaturation was accompanied by a significant decrease in FFF intensity, in agreement with the findings by Alvarado (2017), already mentioned above.

In fact, the relationship between ΔI_F and $\Delta\lambda$ for the different holding times at those two temperatures was quadratic ($R^2 = 0.99$) as follows:

$$\Delta I_F\ (a.u.) = -0.3071\Delta\lambda^2 - 1.0963\Delta\lambda - 1.4949.$$

Change in intensity started to be noticeable from 15 min in heat treatments applied at 90 ºC (**Figure 5.1c** and **Table 5.2**), while the samples treated at 100 ºC (**Figure 5.1d** and **Table 5.2**) showed remarkable changes in the fluorescence intensity even from the beginning of treatment. The decrease of the fluorescence intensity continued as the time of the treatment increased at both temperatures. In samples heated at 90 ºC for 60 min, the intensity decreased by ~44 a.u. (~37% of the initial peak intensity) as compared to non-heated milk samples. Similarly, in the most intensely treated samples (100 ºC, 60 min), the fluorescence intensity diminished by ~54 arbitrary units, which corresponded to a decrease of ~47% of the initial intensity peak.

The decrease of fluorescence intensity observed might be attributed to protein-protein interactions between denatured whey proteins and/or with casein micelles. It is well known that whey protein

denaturation exposes *Trp* residues that are, otherwise, buried within the native structure to the external environment. Simultaneously, the protein unfolding does also expose the free sulfhydryl groups that become reactive upon exposition. Denatured whey proteins may attach to each other forming whey protein aggregates that remain soluble in the serum or, more often, may attach by means of a disulfide bond with κ-CN on the surface of the micelle (Moro et al., 2001).

Kulmyrzaev et al. (2005) suggested that the protein-protein interactions induced by heat tend to shield the *Trp* residues exposed as a result of denaturation. Those aggregation processes have a great impact on the fluorescence intensity in heated milk samples. In summary, heat treatment induces two main chemical changes in milk whey proteins: protein unfolding and protein aggregation with other denatured whey proteins or with casein micelles. Its seems, from our results, that both unfolding and aggregation are responsible for FFF changes observed in heated milk. Protein unfolding causes a change on the hydrophobicity in the surrounding environment of *Trp* residues, inducing the shift on the maximum fluorescent emission peak, while the resulting protein-protein aggregation by disulfide bonding originated by thiol residues activation leads to a concomitant decrease in fluorescent intensity. As it is clearly observed in **Figure 5.3**, the resulting fluorescence changes are not pronounced/significant when heat treatment intensity is small, e.g., 70 and 80 °C. Likely, those slight changes are observed at the initial stages of protein denaturation, when changes are still affecting a reduced percent of the available whey protein or they are not permanent yet. If at all, a slight fluorescence intensity increase (**Figure 5.3a**, see LSM values for ΔI_F at 70-80 °C) might be observed, as it has been reported by others (González, 2014; Alvarado, 2017). However, a higher heat load, e.g., 90 °C for more than 10 min or treatments at 100 °C, would tend to induce permanent changes in proteins, generating both significant red shift and decrease in fluorescence intensity. In fact, this dual effect of temperature might be the reason why a ΔI_F change of LSMs tendency is observed at temperatures higher than 80 °C (**Figure 5.3a**) and holding times larger than 10 min (**Figure 5.3b**).

Another possible reason for the intensity decrease in the samples treated at 90 and 100 ºC may be that the *Trp* emission is absorbed by other compounds, such as Maillard intermediate compounds, which are generated when the intensity of treatments is increased. Note that such compounds are excited at optimal wavelengths of 330 nm, which are near to *Trp* emission wavelength (340 nm) (Leclère & Birlouez-Aragón, 2001). This means that Maillard intermediate compounds formation might contribute to the decrease of the *Trp* fluorescence peak, not only because *Trp* would reduce its emission due to aggregation of unfolded proteins but also because part of the emitted energy might be used by the Maillard intermediate compounds that might be absorbing and re-emitting at least part of the energy at longer wavelengths. In fact, a hump in the emission spectrum is noticeable near 420 nm (**Figures 5.1** and **5.2**), i.e., around the maximum for *MC*

fluorescent emission, although there were almost no changes in this region due to the thermal treatment intensities. If the Maillard intermediates had absorbed, at least partially, the emission energy of the *Trp* and re-emitted it at a longer wavelength, an increasing intensity at 420 nm would be expected. However, this behavior was not observed. It should be kept in mind that the red shift might had counterbalanced the mentioned phenomenon, as it would induce an ever-increasing distance between the maximum emission peak for *Trp* and the maximum excitation peak for intermediate Millard compounds. Finally, the fluorescence intensity could also decrease due the proximity between the *Trp* residues and quenching compounds (Caputo & London, 2003).

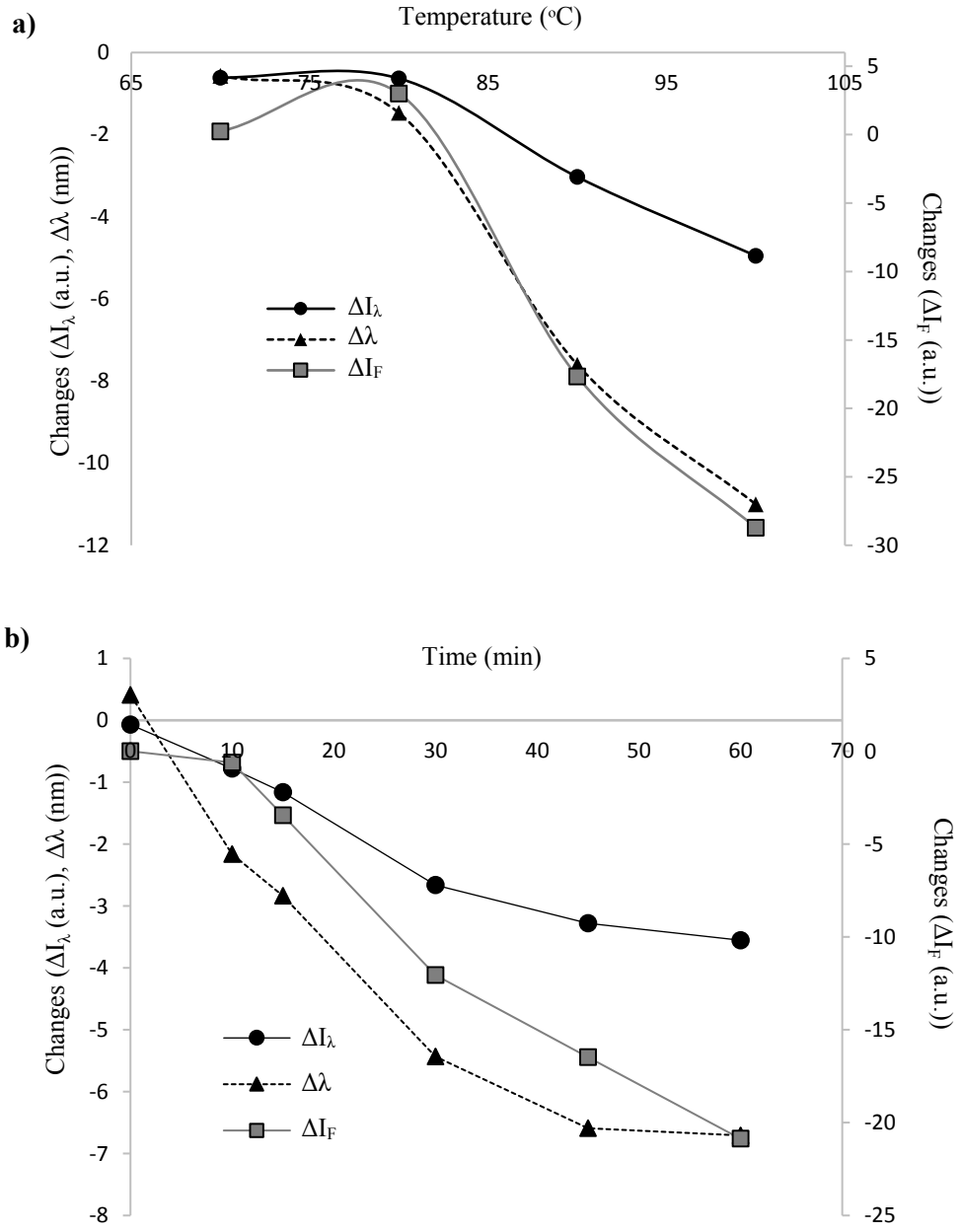


Figure 5.3. Effect of a) temperature and b) time on least square means of main FFF parameters evaluated: Δλ, fluorescence emission wavelength; ΔI_F, variation in the fluorescence intensity due the peak height; ΔI_λ, variation in the fluorescence intensity due the peak shift.

Additionally, from results shown in **Figures 5.1** and **5.2**, it is clear that measuring at a constant emission from *Trp*, e.g., 340 nm, should underestimate any initial increase of fluorescence intensity at reduced intensity loads and overestimate the subsequent decrease of intensity observed higher intensity treatments.

Finally, it should be noted that one of the advantages of FFF is its great potential for inline application in milk. This methodology has been shown to be potential for real time evaluation of whey protein denaturation and casein micelle particle size (H. Taterka, 2016) as well as for milk heat damage monitoring (Alvarado, 2017). Then, it seems convenient to collect inline parameters characterizing both changes in FFF intensity (ΔI_F, ΔI_λ) and the red shift ($\Delta\lambda$). Although this might increase the cost of a dedicated FFF inline sensor, it would be compensated as obtaining those parameters might provide useful information about different aspects of much related whey protein chemical changes such as folding/unfolding, thiol groups exposure, protein-protein interaction, i.e., micelle attachment and soluble aggregates formation, or even Maillard reaction.

Principal components analysis (PCA) was applied to the multivariate fluorescence signal, obtaining scores (**Figure 5.4**) and loading plots (**Figure 5.5**), based on *Trp* fluorescence spectra of the samples. The distribution of the samples can be observed in the score plot, according to the two principal components representing the tendencies in the fluorescence signals. The first two principal components (PC#1 and PC#2) accounted for 99.88% (**Figure 5.4**) of the total variability with a component 1 explaining 97.58%.

The organization of the heated samples was evident with a general dispersion in the two principal components throughout the heat treatment intensity. The samples treated at 70 ºC were closer together as the differences in the fluorescence spectra between several treatment times were very small. At 80 ºC, the distribution of the samples was more disperse along the axis corresponding to PC#2, when the treatment time increased. As per treatment temperatures of 90 and 100 ºC, as the treatment intensity increased, it was observed that the samples were more and more dispersed, especially not only along the axis corresponding to PC#1 but also through the PC#2 axis. From these results, it is quite clear that the degree of data dispersion in **Figure 5.4** was dependent on the combination of heating temperature and time considered.

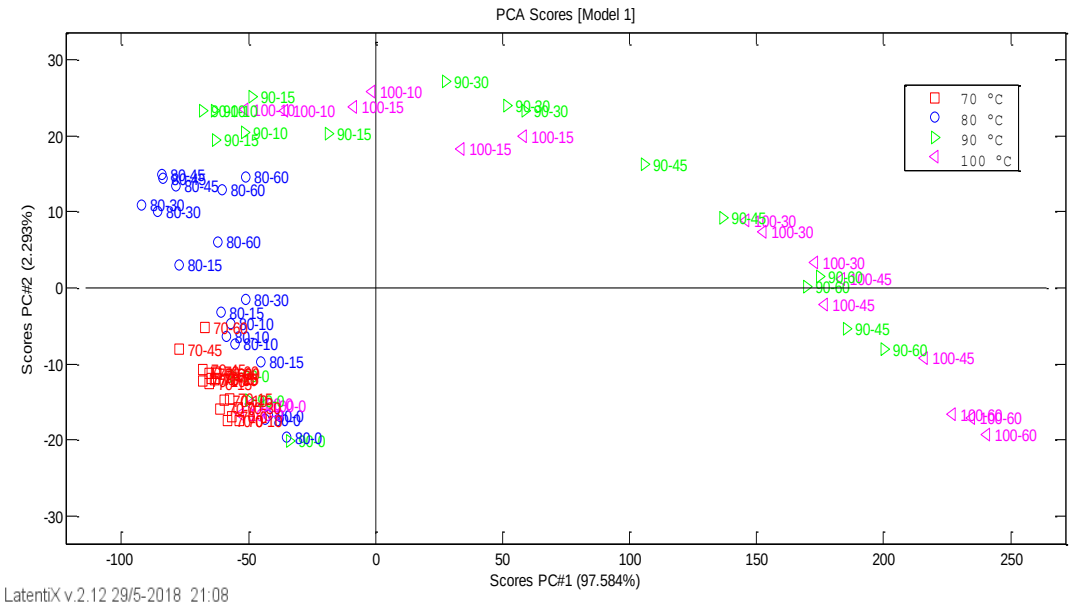
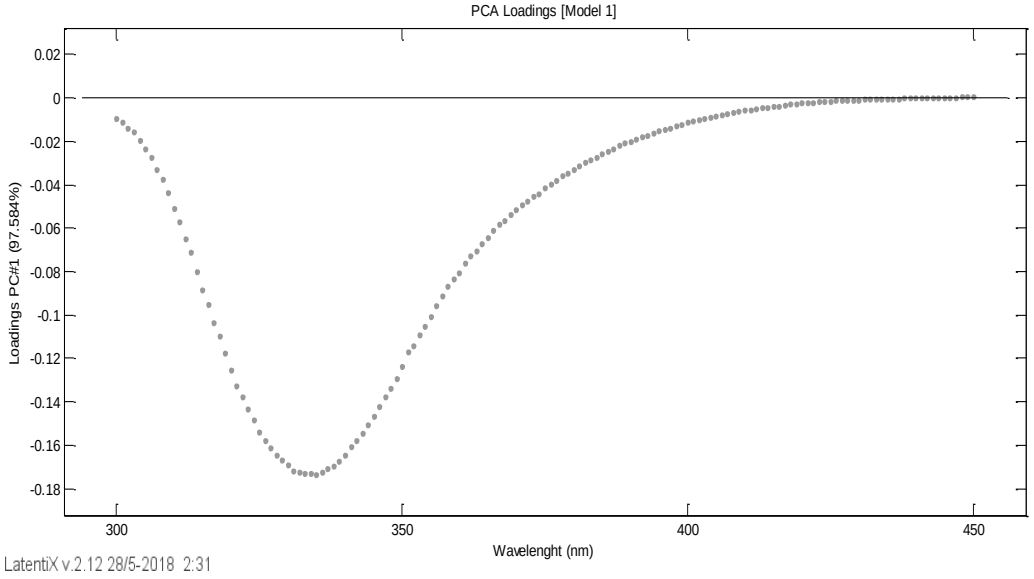


Figure 5.4. PC#1 vs PC#2 of the spectra excited at 290 nm. The observations are represented in a "Temperature-time" format of the treatment (e.g. 70 ºC-10 min).

The loading plot (**Figure 5.5**) summarizes the spectral profiles information responsible for the distribution of samples showed in the scores plot (**Figure 5.4**). The changes of the *Trp* intensity in the spectrum were largely determining for the PC#1 (**Figure 5.5a**), while the PC#2 (**Figure 5.5b**) explained the peak shift during heat treatment. Higher positive values in PC#1 scores (**Figure 5.4**) can be related with a decrease in ΔI_F near 340 nm as can be seen on the negative scores depicted in **Figure 5.5a**. Samples treated at 70 and 80 ºC showed almost the same PC#1 values in the score plot (**Figure 5.4**) for all the treatment times, in clear agreement with **Table 5.2** values. Samples treated at 90 and 100 ºC had higher PC#1 values as treatment time increased, also in agreement with the decrease in ΔI_F presented in **Table 5.2** results. PC#2 loadings illustrate the effect of peak shift (**Figure 5.5b**), with negative loadings values for samples with $\Delta\lambda$ negative or near zero and positive loading values for samples with large $\Delta\lambda$ values, with the maximum measured shift toward wavelengths near 355 nm. On the 70 and 80 ºC score plots (**Figure 5.4**), it is clearly noticeable the scatter of the samples over PC#2, reinforcing the idea of an early effect of thermal treatments on protein unfolding and thus on peak shift.

a)



b)

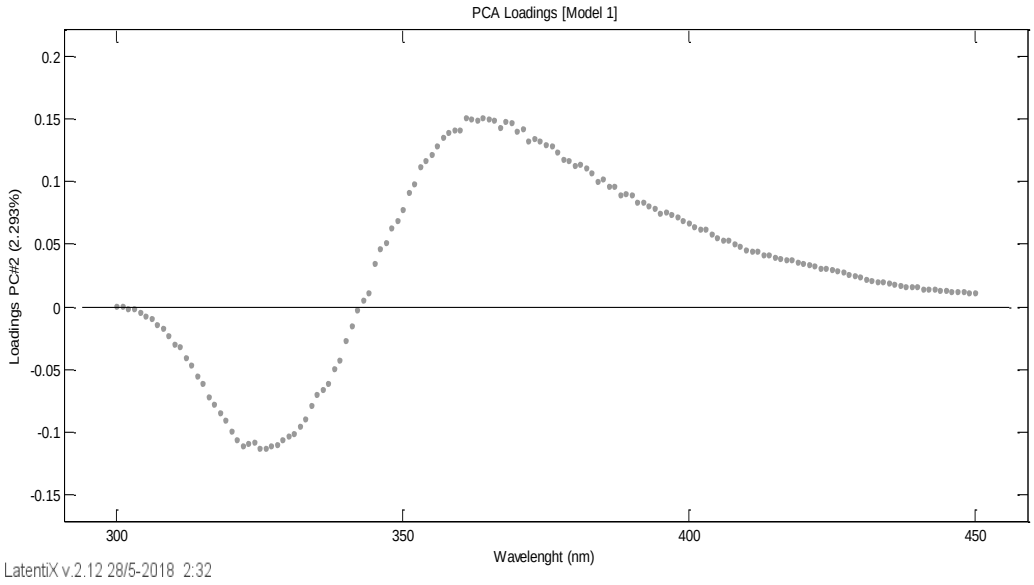


Figure 5.5. Principal component analysis loading plots for the first two components based on tryptophan fluorescence in reconstituted skim milk samples.

5.3.2. Other fluorescent compounds used in food analysis

In addition to *Trp*, others fluorescent markers present in milk were analyzed. In **Figure 5.6** the spectra associated with the *Dt* excited at 315 nm is represented. As previously mentioned in Chapter 2 and Section 5.1, the fluorescence property of *Dt* is used to monitor the oxidative stress in proteins (Polimova et al., 2011) since *Dt* is formed as a consequence of the cross-linkages between two residues of Tyr during photooxidation (Dalsgaard et al., 2007).

A peak at ~424 nm was observed and related to *Dt* (**Figure 5.6**). Furthermore, an important variation in the fluorescence intensity was noted between 350 and 380 nm. Fluorescence intensity of *Dt* (F_{Dt}) at 377 nm was statistically significant as predictor to predict the concentration of lactulose in reconstituted skim milk powder and was used in combination with *MC* and *Trp* fluorescence intensities (Chapter 6).

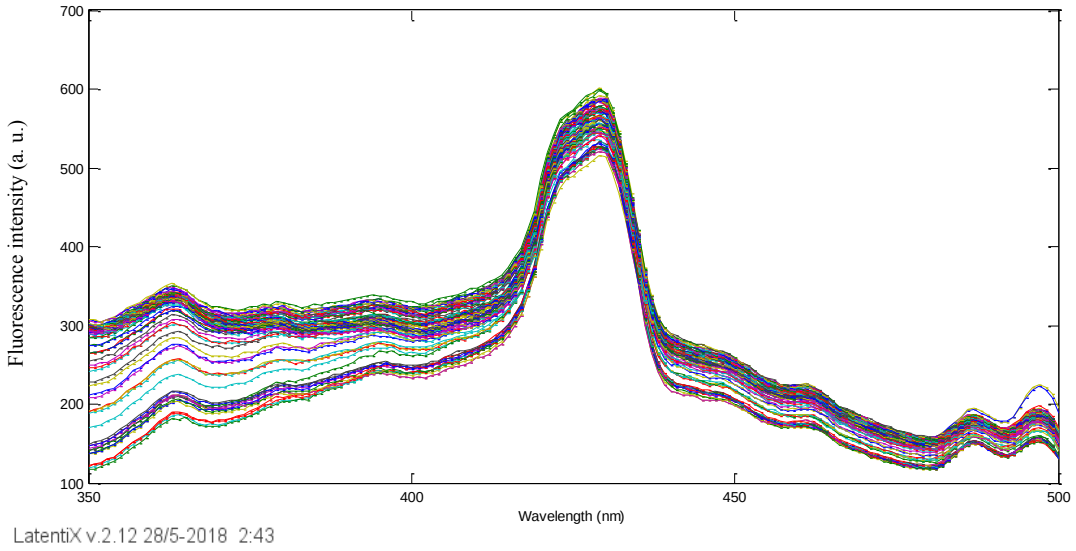


Figure 5.6. Fluorescence spectra excited at 315 nm from reconstituted skim milk powder treated at different intensities.

PCA was applied to the whole spectra to evaluate the discrimination between the different heat-treated samples. The first two components explained 99.10% of the variation of samples (**Figure 5.7**). Samples treated at 70 ºC did not show important dispersion. When treated at 80 ºC, the samples showed the same tendency than observed at 70 ºC, although the distribution of samples was more disperse at 80 ºC. The tendency of the samples treated at 90 and 100 ºC were represented by a distribution similar with a greater dispersion at long treatments. According to all the above, the discrimination of the samples was better observed when the temperature and time treatment combination was increased. The samples treated for the longer time, i.e., 60 min at 90 and 100 ºC, were distributed close to each other.

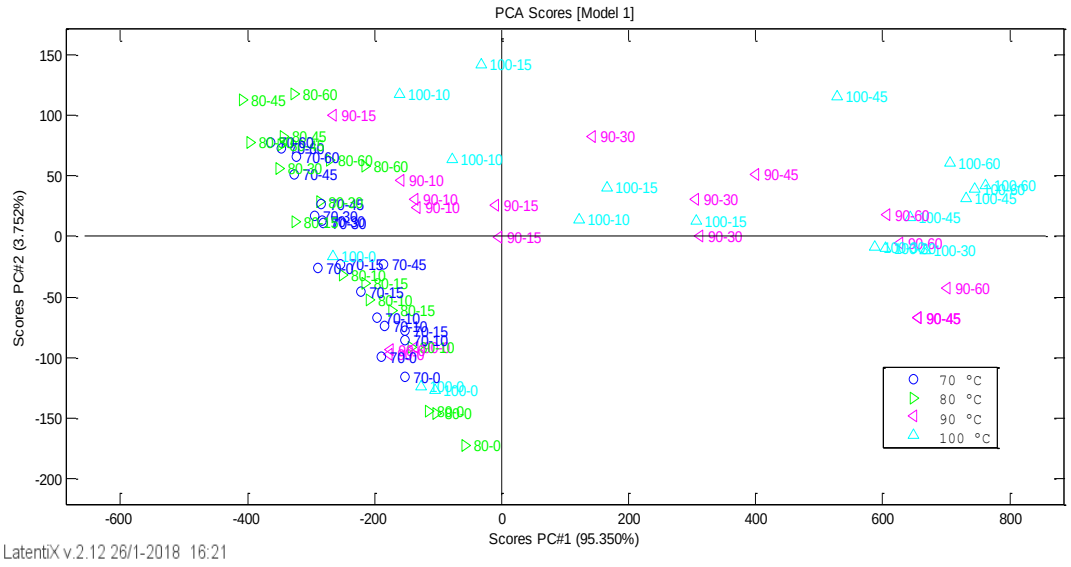


Figure 5.7. PC#1 vs PC#2 of the spectra excited at 315 nm. The observations are represented in a "Temperature-time" format of the treatment (e.g. 70 ºC-10 min).

Another heat damage marker widely used by the food industry for the quality control of the products is the *MC*. The Maillard reaction affects the final quality of the product since it can trigger changes in color, taste, aroma and nutritional values (Van Boekel, 2008). Several factors affect the Maillard reaction, such as the composition, pH and water activity of the product or the combination of temperature and time during the heating process and storage (Ames, 1998). During the initial stage of Maillard reaction, the Amadori compounds, which are not fluorescent, are formed. In advanced stages, these compounds produce cross-links with adjacent proteins or with other amino groups, giving rise to fluorescent polymeric aggregates or the so-called advanced glycation end products. In that way, the fluorescence intensity of the advanced glycation end products is what is used as marker of the Maillard reaction yield (Bosch et al., 2007). Therefore, *MC* fluorescence (F_{CM}) is used to evaluate the changes suffered by food (milk-cereal, infant foods during storage, sugar, casein) due the heat treatment process or during storage (Matiacevich et al., 2005; Delgado-Andrade et al., 2006; Bosh et al., 2007).

The spectra obtained with an excitation of 330 nm, which is related to *MC*, is shown in the **Figure 5.8**. Two important peaks were observed in the spectrum at ~425 nm and ~451 nm. Similarly, Liu and Metzger (2007) detected a peak at 423 nm with low-heat skim milk and suggested that the peak corresponded to the fluorescence of advanced Maillard reaction products. F_{MC} increased with increasing temperature and time combinations. PCA (**Figure 5.9**) showed that PC#1 and PC#2 represented the 98.12% of the distribution of the samples (89.70 and 8.43%, respectively). Similar tendencies were observed between samples treated at 70 and 80 ºC. The distributions of the samples seemed to follow a linear trend that increased when increasing the intensity of the

treatment. On the contrary, samples treated at 90 and 100 ºC followed the same trend, but the distribution of samples was more disperse when increasing heat intensity. Although F_{Dt} and F_{CM} data followed similar tendencies, the classification of the samples was clearer for F_{Dt}.

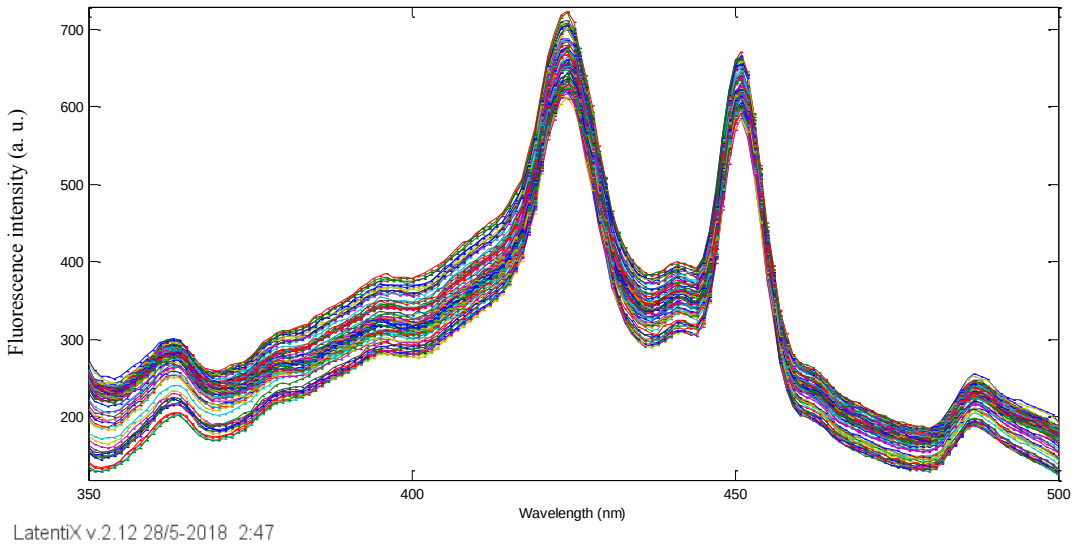


Figure 5.8. Fluorescence spectra obtained exciting at 330 nm from reconstituted skim milk powder treated at different intensities.

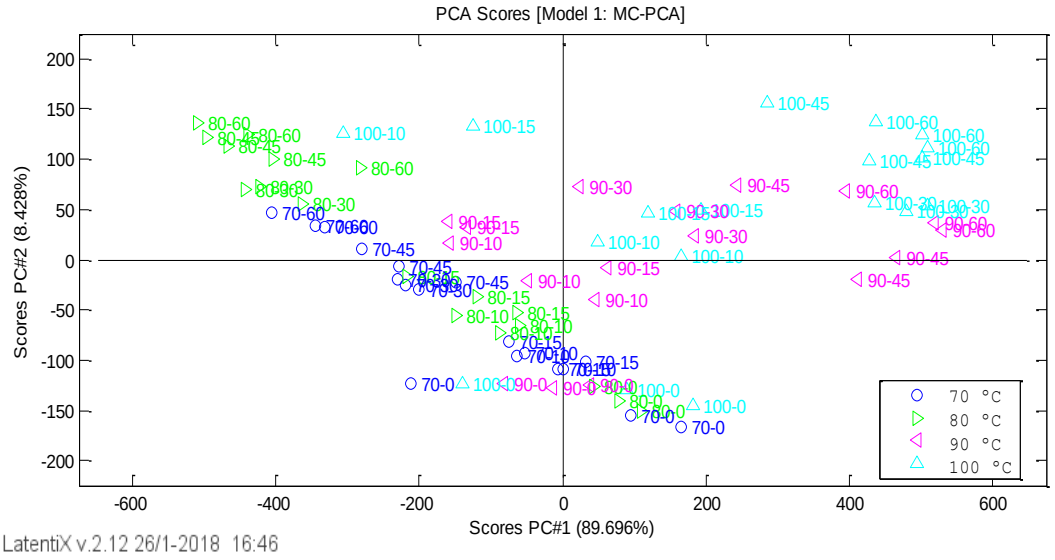


Figure 5.9. PC#1 vs PC#2 of the spectra excited at 330 nm. The observations are represented in a "Temperature-time" format of the treatment (e.g. 70 ºC-10 min).

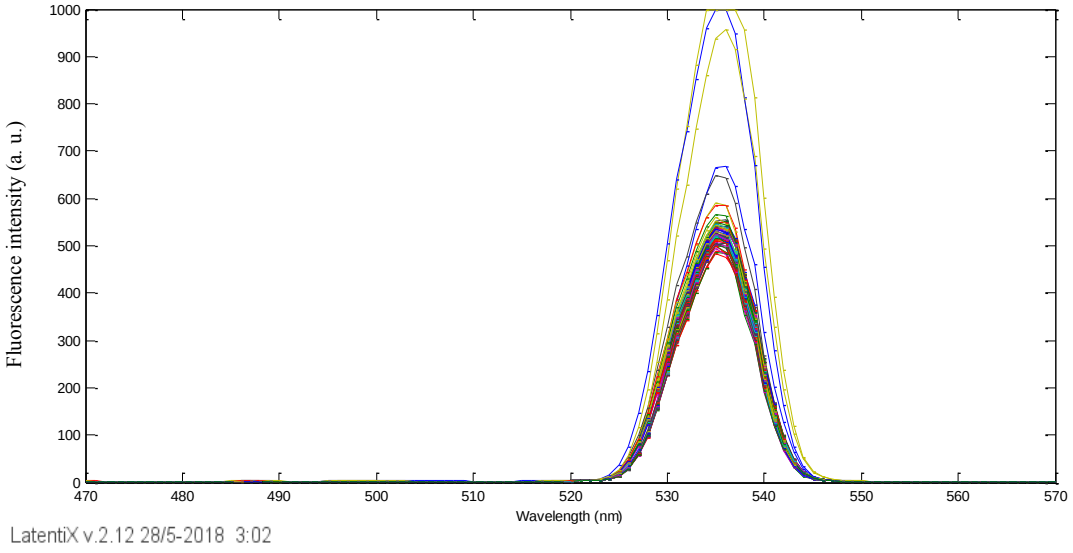
In a recent study, the F_{MC} has been evaluated in milk in order to predict retinol concentration (Liu et al., 2018). Furthermore, in combination with *Rb* fluorescence, F_{MC} was evaluated to predict the origin and the composition of milk (Ntakatsane et al., 2011). Earlier, F_{MC} emitted at 430 nm was

used in combination with F_{Trp} emitted at 340 nm in the FAST index method (the ratio between F_{Trp} and F_{MC}) to monitor heat damage in milk (Leclère et al., 2001). The value of the FAST index can be related to the modifications of molecular structures formed between sugars or oxidizing lipids and lysine residues of proteins (Feinberg et al., 2006). Furthermore, Feinberg et al., (2006) also used the FAST index with other chemical markers to discriminate between commercial milks. They suggested that at least 7 markers are needed to classify commercial milks.

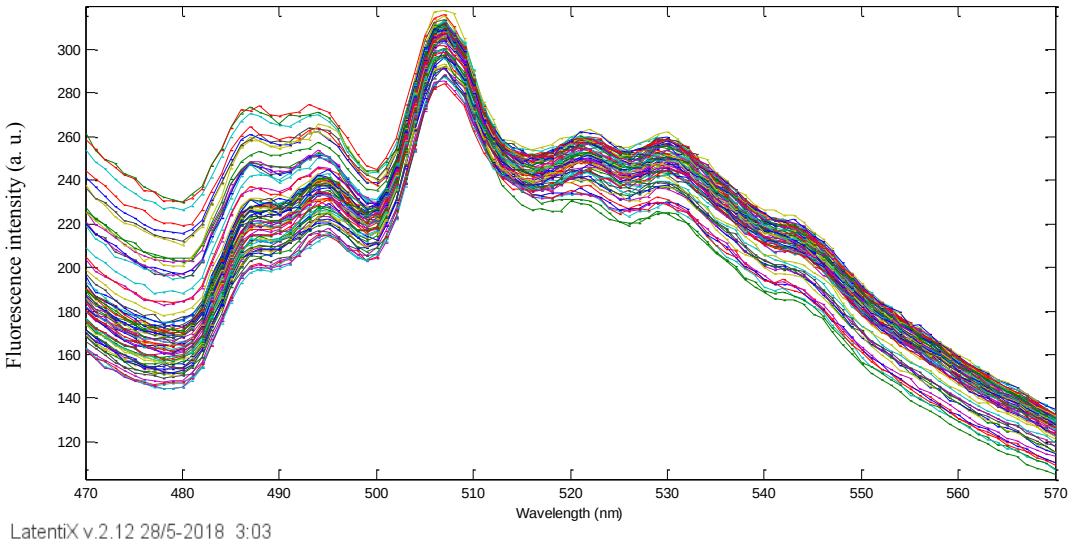
In addition to F_{Trp}, F_{Dt} and F_{MC}, fluorescence intensity of *Rb* was also evaluated. The fluorescence analysis was performed exciting the fluorophore at three different wavelengths: 267, 370 and 450 nm (**Figure 5.10)**, excitation wavelengths that have been related to *Rb* (Christensen et al., 2005). As previously described (Chapter 2 and Section 5.1), this compound has a key role related to the photosensitivity and photodegradation of dairy products; *Rb* can absorb light and reacts as a photosensitizer that involves oxygen activation and radical formation leading to protein and lipid degradation (Becker et al., 2003).

As can be observed in **Figure 5.10b**, Rb_{370} spectra were those showing greater response in the analysis. Although the highest peak was at 507 nm, a large variation in the intensity was observed between ~470 and 500 nm. Liu and Metzger (2007) reported a remarkable peak at ~510 nm when exciting skim milk at 380 nm, that was attributed to riboflavin based on literature (Wold et al., 2002; Becker et al., 2003). Finally, the Rb_{450} showed a constant variation in the fluorescence intensity with a peak at ~520 nm (**Figure 5.10c**).

a)



b)



c)

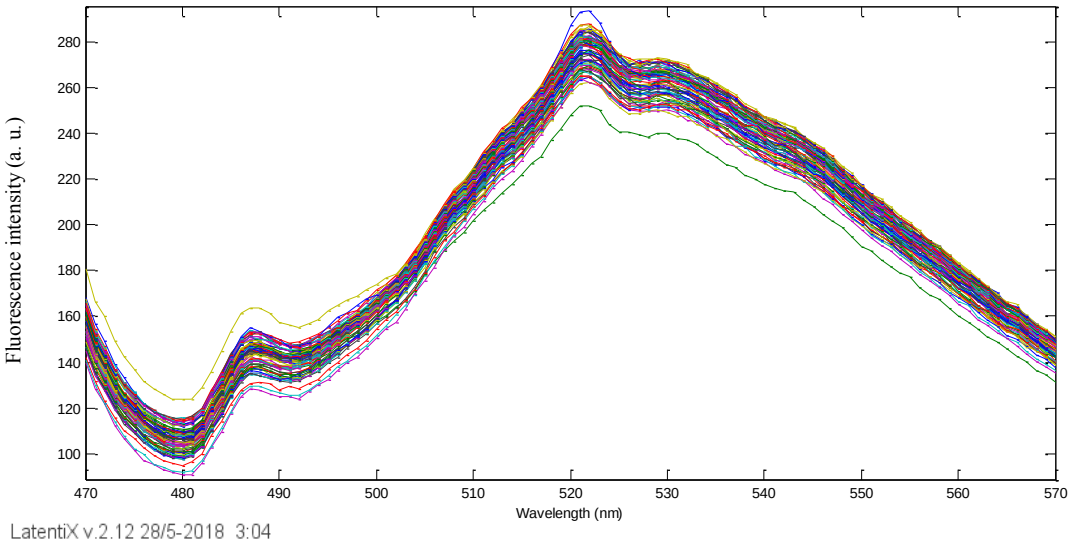


Figure 5.10. Fluorescence spectra excited at different wavelengths from reconstituted skim milk powder treated at different heat intensities. a) 267 nm; b) 370 nm and c) 450 nm

The obtained spectra were evaluated separately through PCA. **Figure 5.11** shows the score plot of Rb_{267}, which represented 99.62% of the variation between PC#1 and PC#2. Although the peak of the spectra was prominent, the discrimination between the different heat-treated samples was not clear. Moreover, the samples were separated into two groups depending on the temperature of treatment (70 and 80 ºC; 90 and 100 ºC). The separation of samples according to the time of treatment was not observed.

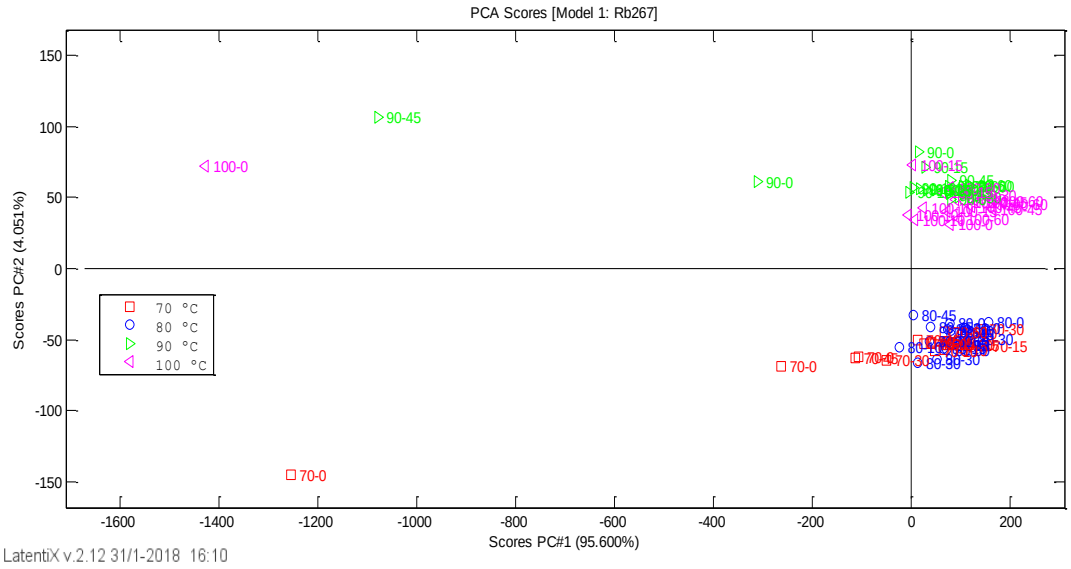


Figure 5.11. PC#1 vs PC#2 of the spectra excited at 267 nm. The observations are represented in a "Temperature-time" format of the treatment (e.g. 70 ºC-10 min).

Figure 5.12 represents the score plot of Rb_{370} by PC#1 and PC#2. The two first components explained 99.09% of the variation of samples. Two groups divided according to the temperature of the treatment were noted again (70 and 80 ºC; 90 and 100 ºC), although without a clear tendency. The samples treated at 90 and 100 ºC were more disperse with increasing time of treatment.

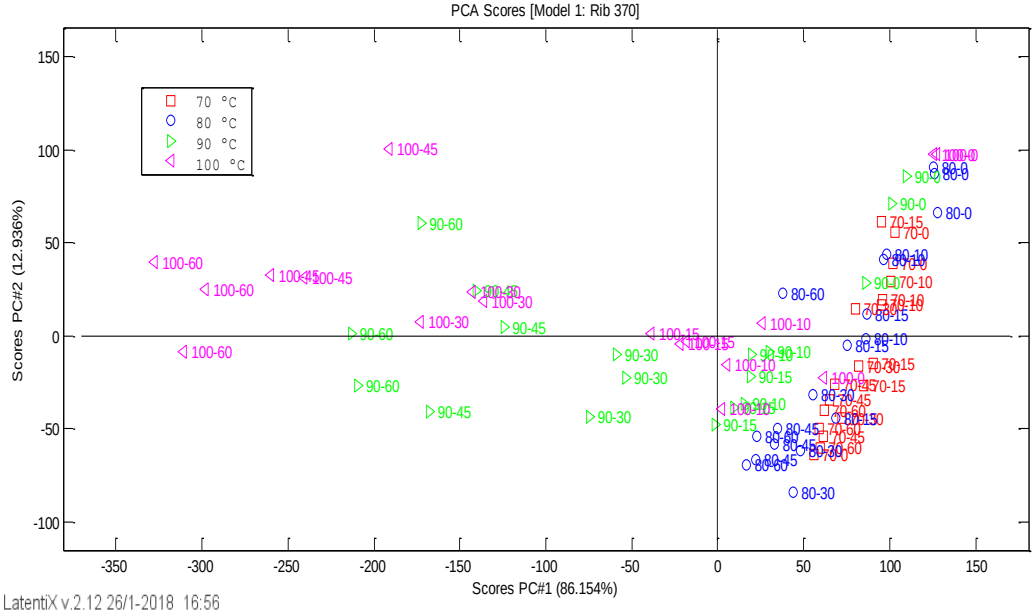


Figure 5.12. PC#1 vs PC#2 excited at 370 nm. The observations are represented in a "Temperature-time" format of the treatment (e.g. 70 ºC-10 min).

The score plot of Rb_{450} is represented in the **Figure 5.13**. The PC#1 and PC#2 explained 97.61% of the variation of the samples. Samples treated at 70 and 80 ºC form a cluster apart from the samples submitted to 90 and 100 ºC of treatments. These two groups of milk samples treated to low (70-80 ºC) or high (90-100 ºC) temperatures only overlapped partly for the shortest treatment times. There was a tendency to rank time treatments according to the scores on PC#2, a tendency much clearer for high temperature treatments.

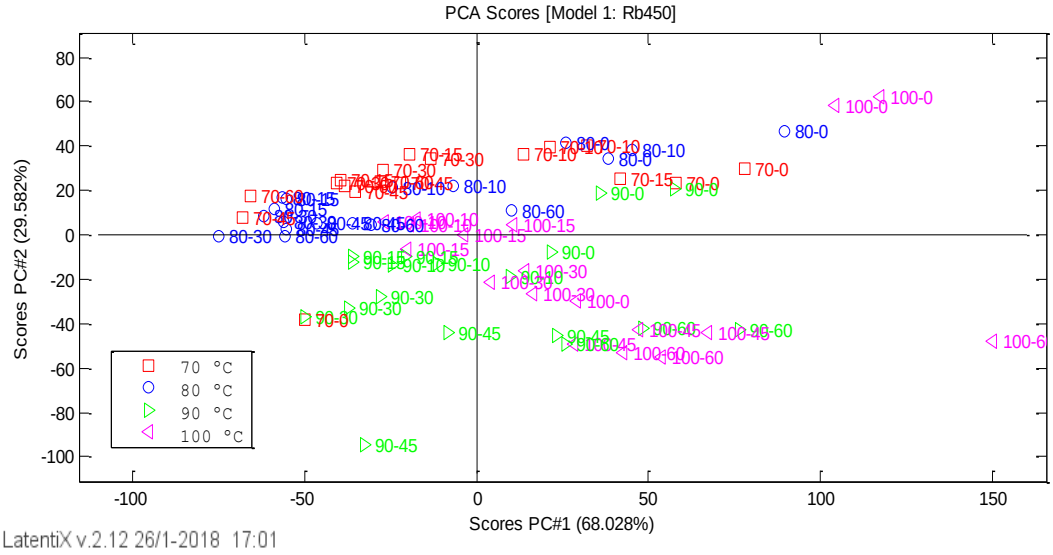


Figure 5.13. PC#1 vs PC#2 of spectra excited at 450 nm. The observations are represented in a "Temperature-time" format of the treatment (e.g. 70 ºC-10 min).

5.4. Conclusions

Tryptophan, as native fluorescence marker, was measured and characterized. In *Trp* spectra, a decrease fluorescence intensity (ΔI) and a red shift (Δλ) were detected when the intensity of heat treatments increased. The total fluorescence intensity changes were decomposed in two fractions, one associated with the change of the peak height (ΔI_F) and another with the displacement of the emission maximum (ΔI_λ). Most remarkable changes in the fluorescence intensity was noted at 90 and 100 ºC; red shift was detected in both temperatures. There are several possible reasons for the decrease of fluorescence intensity observed. Mainly, it might be attributed to protein-protein interactions between denatured whey proteins and/or with casein micelles by means of disulfide bonds formation. Another two potential reasons might be partial absorption of *Trp* emission by newly generated Maillard intermediate compounds or proximity of *Trp* residues to quenching compounds. Fluorescence spectra associated to *Trp*, *Dt*, *MC* and *Rb* excited at 267, 370 and 450 nm were studied to classify the samples of heat-treated milk. Principal component analysis showed that Rb_{267} was able to discriminate low (70-80 ºC) and high (90-100 ºC) intensity

treatments. PCA of Rb_{370} showed the samples distributed according the different treatments times. In addition, *Trp*, *Dt and MC* spectra showed differences in their intensities depending on the heat load (time and temperature) but with a partial overlapping of the samples.

In summary, between all fluorescent markers studied, F_{Trp} demonstrated potential as native marker to monitor the milk heat treatment damage with chemometric methods, suggesting that FFF spectroscopy has potential to monitor milk damage after heat processing. However, taking into account that one of the main advantages of FFF is its great potential for inline application in milk, it seems convenient to collect inline parameters characterizing the observed changes in FFF.

CHAPTER 6:
Prediction models of lactulose and furosine concentration using fluorescent markers as predictors

6.1. Introduction

Raw milk is subjected to strict sanitary control in addition to various treatment processes, all with the aim of killing pathogens that may be found in milk, prolong shelf-life and eliminate health risks. For the production of high quality milk, it is necessary to know the modifications suffered by milk components during heat treatment. Since quality in directly related to the intensity of heating that is applied during milk processing, it is necessary to have indicators that can identify the treatment employed, i.e., treatment intensity, and could provide useful, inline control feedback.

As seen in Chapters 2 and 4, lactulose and furosine are useful indicators of heat damage in milk. Lactulose is mainly formed by an isomerization reaction of lactose by the Lobry de Bruyn-Alberda Van Ekenstein transformation. Since it is a suitable marker, which provides information on the heat load to which the milk was subjected (Berg & Van Boekel, 1994), lactulose has been adopted as analytical indicator to guarantee the quality of UHT milk by differentiating between UHT and in-bottle sterilized milk (threshold of 600 mg/L of lactulose) (EC, 1992; IDF, 1993). Some studies have focused on the characterization of industrial processed milk by determining the concentration of lactulose alone or in combination with other indices (Pellegrino et al.,1995; Morales et al., 2000; Marconi et al., 2004; Feinberg et al., 2006; Sakkas et al., 2014).

Brandt and Erbersdobler (1973) demonstrated that furosine is a good indicator of lysine damage in milk products. Furosine is an artificial amino acid that arises from hydrolysis of the Amadori products (Finot et al., 1981; van Boekel, 1998) and is one of the most studied indicator of lysine damage in milk. It is generated during the fructosyl lysine hydrolysis in the early stage of Maillard reaction. The concentration of furosine can be determined by HPLC (Resmini et al., 1990), ion-exchange chromatography (Hartkopf & Erbersdobler, 1993) or also by capillary electrophoresis (Tirelli & Pellegrino, 1995).

The analysis of fluorescent markers has been evaluated in heat-treated milk in order to predict the concentrations of heat damage markers. The fluorescent markers were used as predictors and the best models were selected based on the maximum R^2; good prediction models were obtained for chemical markers such as riboflavin (R^2=0.75), ascorbic acid (R^2=0.90), sulfhydryl group

(R^2=0.92) and hydroxymetylfurfural (R^2=0.94), which are heat damage indicators widely used in milk analysis (Alvarado, 2017). The fluorescent markers used were *Trp* and *Rb* excited at 370 nm for estimated riboflavin and ascorbic acid concentration; *Trp* and *Rb* excited at 450 nm for sulfhydryl group concentration; *Trp* and *Rb* excited at 370 and 450 nm for hydroxymetylfurfural concentration. The same methodology using fluorescent compounds as predictors was applied by Liu et al. (2018) to predict the concentration of retinol (R^2=0.87) in heat-treated skim milk. The fluorescence used was from *Trp*, *MC* and *Rb* excited at 370 nm.

Finally, the use of FFF spectroscopy can provide useful information about the heat treatment that was applied to milk and consequently the damage suffered in milk (Dufour & Riaublanc, 1997; Kulmyrzaev et al., 2005; Martínez-Monteagudo & 2015). Hence, the combination of chemical and fluorescent markers could be helpful to detect the damage in heat-treated milk. Therefore, the specific objective of the present study was to obtain prediction models through kinetic modeling of lactulose and furosine formation during heat treatment of milk and the evaluation of its correlation with fluorescent markers determined by FFF.

6.2. Materials and methods

6.2.1. Experimental design

For the evaluation of correlations between chemical markers and fluorescent compounds, in order to obtain prediction models, the experimental design followed that of Chapter 4 (Selected chemical markers and their formation kinetics).

6.2.2. Chemical markers and fluorescent compounds

Data used in this chapter were those collected from the studies on the effect of experimental factors on lactulose and furosine concentrations (Chapter 4) and fluorescence spectra (Chapter 5). Specifically, for the furosine experiment, the fluorescent markers were excited at the same wavelengths as for lactulose (see **Table 5.1**) but the emission was at one single wavelength with the following excitation-emission wavelengths: for *Trp*, 290-340 nm; *Dt*, 315-420 nm; *MC*, 320-420 nm; Rb_{267}, 267-530 nm; Rb_{370}, 370-530 nm; and Rb_{450}, 450-530 nm.

6.2.3. Statistical analysis

All data were processed and analyzed using "Statistical Analysis System" (SAS, version 9.2, 2009, SAS Institute Inc., Cary, NC, USA). To study the variability caused in the dependent variables for the main effects studied, ANOVA analysis of variance was performed using the linear repeated measures procedure (MIXED) of SAS. The statistical model included as main effects the experimental design factors and replication as well as their interactions. The analysis

considered non-random effect of each of the heat treatments. Differences between least square means (LSM) of the various treatments were considered significant when $P < 0.05$. Pearson correlation coefficients between fluorescent markers and lactulose concentration were obtained by the correlation (CORR) procedure. In order to obtain the best one-, two- and three-variable lactulose/furosine concentration prediction models using predictors collected from the corresponding spectra of the different fluorescent markers, i.e., *Trp*, *Dt*, *MC*, Rb_{267}, Rb_{370} and Rb_{450}, the maximum R^2 procedure (REG, MAXR) was used. Validation of prediction models was performed with the leave-one-out method.

6.3. Results

6.3.1. Correlation between lactulose concentration and fluorescence of milk

Pearson's linear correlations were obtained between both experimental, $[L]_E$, and theoretical, $[L]_T$, concentrations of lactulose and fluorescent indicators of thermal damage as preliminary approach to identify fluorescent markers having more potential as lactulose predictors (**Table 6.1**). It can be observed that there was a negative correlation statistically significant between $[L]_E$ and F_{Trp} with r = -0.60 for maximum fluorescence emission intensity (I) and r = -0.52 for wavelength of maximum fluorescence emission intensity (λ). As expected, both *Trp* parameters were also found to correlate significantly with $[L]_T$ (r = -0.88 and r = -0.87, respectively). For the rest of markers, only Rb_{370} was found to significantly and positively correlate with $[L]_E$ (r = 0.56).

Table 6.1. Pearson correlation coefficients, r, between the concentration of lactulose and different dependent variables studied.[1]

	Trp		***Dt***	***MC***	***Rb***		
	I	λ			Rb_{267}	Rb_{370}	Rb_{450}
$[L]_E$	-0.598***	-0.518***	-0.066	-0.16	0.0455	0.557***	-0.127
$[L]_T$	-0.878***	-0.867***	-0.445	-0.337	-0.175	0.443	-0.51

[1]N = 54; * significant at 95%; ** significant at 99%; *** significant at 99.9 %; *I*, maximum fluorescence intensity of tryptophan (*Trp*); λ, wavelength of maximum fluorescence emission of tryptophan (*Trp*); *Dt*, maximum fluorescence intensity of dityrosine; *MC*, maximum fluorescence intensity of Maillard compounds; Rb_{267}, Rb_{370} and Rb_{450}, maximum fluorescence intensity of riboflavin excited at 267, 370 and 450 nm, respectively.

As this approach did not evidence potential predictors other than *Trp* and Rb_{370}, a second approach was used to generate/identify new potential predictors. With this purpose, for each fluorescent marker, and irrespectively of initial correlations observed, Pearson correlation coefficients were systematically calculated between $[L]_E$ and each individual fluorescence intensity reading comprising the corresponding fluorescence spectral scan; in other words, for each marker,

correlations between the fluorescence intensity measured at each spectrum wavelength and $[L]_E$ were studied. Then, a data matrix was generated with all selected potential predictors. The three intensity values having higher Pearson correlation coefficients were selected from the fluorescence emission spectral scans corresponding to each fluorescent marker. Finally, the matrix was expanded with variables calculated from these preselected predictors by the following transformations: possible ratios and products among predictors, the reciprocals of each predictor, as well as their squares, cubes, and natural logarithms. Finally, following the method Maximum R^2 of SAS, the best prediction one-, two- and three-variable prediction models were generated using the predictors of the data matrix.

6.3.2. Predicting the concentration of lactulose by front-face fluorescence

Results of the study on the effect of experimental factors on lactulose formation (Chapter 4) showed that the best temperature for determining the kinetics of lactulose formation was 90 °C. Thus, samples treated at this temperature were those used to obtain prediction models of lactulose concentration $[L]_E$ using the intensities of the three wavelengths of the spectrum of each fluorescent marker showing the highest correlations (**Table 6.2**).

Table 6.2. Prediction models of lactulose concentration by fluorescent markers at 90 °C.[1]

	Model	β_0	β_1	β_2	β_3	R^2	SEP
I***	$[L]_E = \beta_0 + \beta_1 \cdot F_{Trp324}/F_{Dt377}$	0.0023***	-0.0075***	-	-	0.67	0.397
II***	$[L]_E = \beta_0 + \beta_1 \cdot 1/F_{MC425} + \beta_2 \cdot F_{Trp324}/F_{Dt377}$	0.00659***	-2.21**	-0.0107***	-	0.88	0.260
III***	$[L]_E = \beta_0 + \beta_1 \cdot 1/F_{MC425} + \beta_2 \cdot F_{Trp324}/F_{Dt377} + \beta_3 \cdot F_{Trp307}$	0.0061***	-1.679**	-0.0015***	0.000345	0.90	0.178
IV***	$[L]_E = \beta_0 + \beta_1 \cdot F_{Trp324}/F_{Dt377} + \beta_2 \cdot F_{Dt377}/F_{MC425} + \beta_3 \cdot F_{Trp307}$	0.00407***	-0.0213***	0.00402***	0.000157**	0.91	0.180

[1]N = 18; R^2, determination coefficient; SEP (mg/L), standard error of prediction corrected for average; β_0, β_1, β_2, β_3, prediction coefficients; * significant at 95%; ** significant at 99%; *** significant at 99.9 %; $[L]_E$, experimental lactulose concentration; F_{Trp324}, tryptophan fluorescence emitted at 324 nm; F_{Dt377}, dityrosine fluorescence emitted at 377 nm; F_{MC425}, Maillard compounds fluorescence emitted at 425 nm; F_{Trp307}, tryptophan fluorescence emitted at 307 nm.

Even though when all generated models were significant ($P < 0.001$), the model for predicting lactulose concentration presenting the highest R^2 was model IV, which used as predictors F_{Trp} emitted at 307 nm, the ratio of F_{Trp} emitted at 324 nm and F_{Dt} at 377 nm, together with the ratio of F_{Dt} emitted at 377 nm and F_{MC} at 425 nm, to give an R^2 of 0.91 (**Figure 6.1**). Moreover, model III had the second best R^2 (0.90) and the lowest SEP value (0.178 mg/L corresponding to 0.00052 mmol/L).

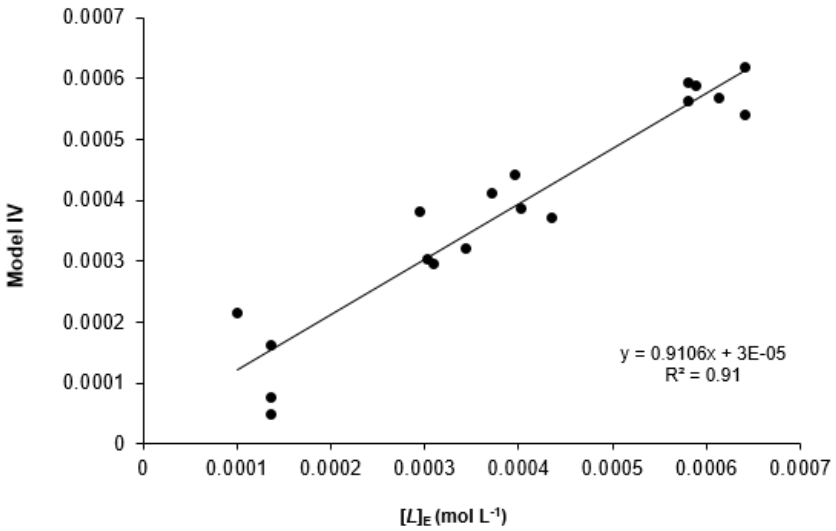


Figure 6.1. Regression between experimental and estimated values of experimental lactulose concentration by Model IV of **Table 6.2**. Points correspond to three replicates of the samples treated at 90 ºC.

6.3.3. Correlation between furosine concentration and fluorescence of milk

The ANOVA analysis showed that *temperature* (T), *time* (t) and their interaction "T x t" had significant effect on furosine concentration ([*Fu*]) (**Table 6.3**). Among the fluorescent markers, *Trp* and *MC* were the most affected by the experimental factors. Besides, replication did not present significant effect on the chemical markers or fluorescent compounds.

Table 6.3. Analysis of variance and F-statistic value for [*Fu*] and fluorescent indicators.[1]

		Heat damage indicators				
Factors	**Df**	**[*Fu*]**	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
T x t	8	24.79***	19.47***	15.00***	1.21	3.27*

[1]N = 45 ***significant at 99.9%; ** significant at 99%; * Significant at 95%; Rep, replicate; T x t, interaction temperature x time; Df, degrees of freedom; [*Fu*], 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.

Statistically significant correlations ($P < 0.05$) between [*Fu*] and F_{CM} were observed in all three temperatures (**Table 6.4**). However, correlations at 70 and 80 ºC were positive (r = 0.75 and 0.85, respectively) and that of 90 ºC was negative (r = -0.54). Additionally, at 90 ºC, [*Fu*] correlated significantly ($P < 0.05$) with F_{Trp} (r = -0.9). Considering the correlations between fluorescent compounds, only significant correlations were observed between F_{MC} and F_{Rb450} at 70 and 90 ºC

both with Pearson correlation coefficients of ~0.76. In turn, no significant correlations between [*Fu*] and fluorescence of *Rb* were observed irrespective of heat treatment.

Table 6.4. Pearson correlation coefficients, r, between the different dependent variables.[1]

70 ºC	F_{Trp}	F_{MC}	F_{Rb370}	F_{Rb450}
[*Fu*]	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 ºC	F_{Trp}	F_{MC}	F_{Rb370}	F_{Rb450}
[*Fu*]	-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 ºC	F_{Trp}	F_{MC}	F_{Rb370}	F_{Rb450}
[*Fu*]	-0.904*	-0.543*	0.361	-0.473
F_{Trp}	-	0.493	-0.423	0.35
F_{MC}		-	-0.093	0.755*
F_{Rb370}			-	0.247

[1]N = 45, * significant at 95%; [*Fu*], concentration of furosine; F_{Trp}, tryptophan fluorescence; F_{MC}, Maillard compounds fluorescence; F_{Rb370}, riboflavin fluorescence excited at 370 nm; F_{Rb450}, riboflavin fluorescence excited at 450 nm.

6.3.4. Predicting the concentration of furosine by front-face fluorescence

Several prediction models for [*Fu*] using the different fluorescent markers evaluated as predictors were obtained using all temperatures or each temperature separately. The best models were selected based on 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_{MC}, F_{Trp}, F_{Rb267}, F_{Rb450}, F_{Trp}/F_{MC}, F_{Trp}/F_{Rb450}, F_{MC}/F_{Rb450}, F_{Trp}^2, F_{MC}^2 *and* F_{Rb450}^2.

As observed in **Table 6.5**, at 70 °C all three models obtained were significant ($P < 0.05$). The model with the highest R^2 was model III with F_{MC}, F_{Rb267} and the ratio between F_{Trp} and F_{Rb450} as predictors.

Table 6.5. Models for predicting the concentration of furosine using fluorescent markers at 70 °C.[1]

	Model	β_0	β_1	β_2	β_3	R^2	SEP
I*	$[Fu]= \beta_0 + \beta_1 \cdot F_{MC}^2$	-274*	0.001	-	-	0.56	20.40
II*	$[Fu]= \beta_0 + \beta_1 \cdot F_{Trp}/F_{Rb450} + \beta_2 \cdot F_{MC}^2$	-543*	508*	0.001*	-	0.71	17.38
III*	$[Fu] = \beta_0 + \beta_1 \cdot F_{MC} + \beta_2 \cdot F_{Rb267} + \beta_3 \cdot F_{Trp}/F_{Rb450}$	-978*	1.55*	-0.068	571*	0.72	17.82

[1]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, * significant at 95%; [*Fu*], furosine concentration; F_{MC}, Maillard intermediate compounds fluorescence; F_{Trp}, tryptophan fluorescence; F_{Rb267}, riboflavin fluorescence excited at 267 *n*m; F_{Rb450}, riboflavin fluorescence excited at 450 *n*m.

As it is shown in **Table 6.6**, all the models obtained at 80 °C were significant ($P < 0.01$). The best model for predicting furosine formation was model VI, with R^2 and SEP values of 0.85 and 13.68 mg·g^{-1} protein, and employed F_{Rb267}, the ratio of F_{Trp}, and F_{Rb450}, and the ratio between F_{MC} and F_{Rb450} (**Fig 6.2**). However, Model IV, which had a R^2 of 0.73 and a SEP of 16.63 mg/100 g protein, might be more convenient due to its simplicity since it used the intercept and the ratio between F_{MC} and F_{Rb450} as predictors. Note that predictors F_{Rb267} *and* F_{Trp}/F_{Rb450} in Model VI were not significant. The model V was simplified to Model IV by eliminating the not significant predictors.

Table 6.6. Models for predicting the concentration of furosine using fluorescent markers at 80 °C.[1]

	Model	β_0	β_1	β_2	β_3	R^2	SEP
IV* **	$[Fu]= \beta_0 + \beta_1 \cdot F_{MC}/F_{Rb450}$	-528*	268***	-	-	0.73	16.63
V* **	$[Fu]= \beta_0 + \beta_1 \cdot F_{Trp}/F_{Rb450} + \beta_2 \cdot F_{MC}/F_{Rb450}$	-337*	-664*	297***	-	0.81	14.23
VI* **	$[Fu] = \beta_0 + \beta_1 \cdot F_{Rb267} + \beta_2 \cdot F_{Trp}/F_{Rb450} + \beta_3 \cdot F_{MC}/F_{Rb450}$	-412*	0.10	-571	306***	0.85	13.68

[1]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, ***significant at 99.9%; ** significant at 99%; * significant at 95%; [*Fu*], furosine concentration; F_{MC}, Maillard compounds fluorescence; F_{Rb450}, riboflavin fluorescence excited at 450 nm; F_{Trp}, tryptophan fluorescence; F_{Rb267}, riboflavin fluorescence excited at 267 nm.

Table 6.7. Models for predicting the concentration of furosine using fluorescent markers at 90 °C.[1]

	Model	β_0	β_1	β_2	β_3	R^2	SEP
VII* **	$[Fu]= \beta_0 + \beta_1 \cdot F_{Trp}^2$	139***	-0.011*	-	-	0.83	12.72
VIII* **	$[Fu]= \beta_0 + \beta_1 \cdot F_{Rb450} + \beta_2 \cdot F_{Trp}^2$	497	-1.45	-0.01***	-	0.85	12.14
IX* **	$[Fu] = \beta_0 + \beta_1 \cdot F_{Rb450} + \beta_2 \cdot F_{Trp}^2 + \beta_3 \cdot F_{Rb450}^2$	44003*	-349*	-0.01***	0.69*	0.92	9.15

[1]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, ***significant at 99.9%; ** significant at 99%; * significant at 95%; [*Fu*], furosine concentration; F_{Rb450}, riboflavin fluorescence excited at 450 *n*m; F_{Trp}^2, squared tryptophan fluorescence; F_{Rb450}^2, squared riboflavin fluorescence excited at 450 *n*m.

The best model using one predictor was the Model VII had squared F_{Trp} as predictor, with significant regression terms. The best two-variable model (Model VIII) used as predictors F_{Rb450} and the square of the F_{Trp}. Finally, in the best three-variable model (Model IX), the F_{Rb450} and its square as well as the square of F_{Trp} were used. In contrast to predictive models having three predictors at 70 and 80 °C, as shown in Table 6.7, at 90 °C, all the regression terms were significant ($P < 0.05$), with an R^2 of 0.92 (**Fig 6.3**). Note that the highest R^2 values encountered at 70 and 80 °C were 0.72 and 0.85, respectively.

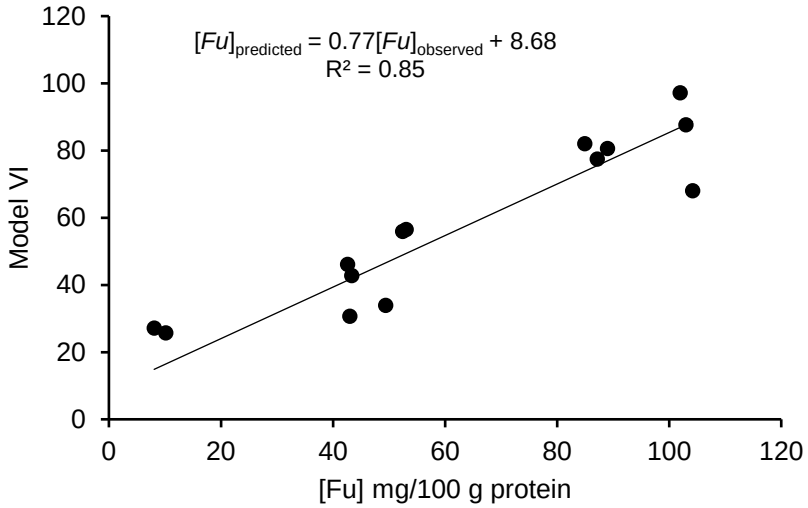


Figure 6.2. Regression between experimental and estimated values (Model VI) for [*Fu*] at 80 °C.

Finally, as it can be seen in **Table 6.7**, all the models obtained at 90 °C were significant ($P < 0.001$). Thus, predictions models were more consistent with increasing treatment temperature within the range studied, reaching a maximum R^2 value at 90 °C ($R^2 = 0.92$), which could be due to the greater changes are produced at higher temperatures.

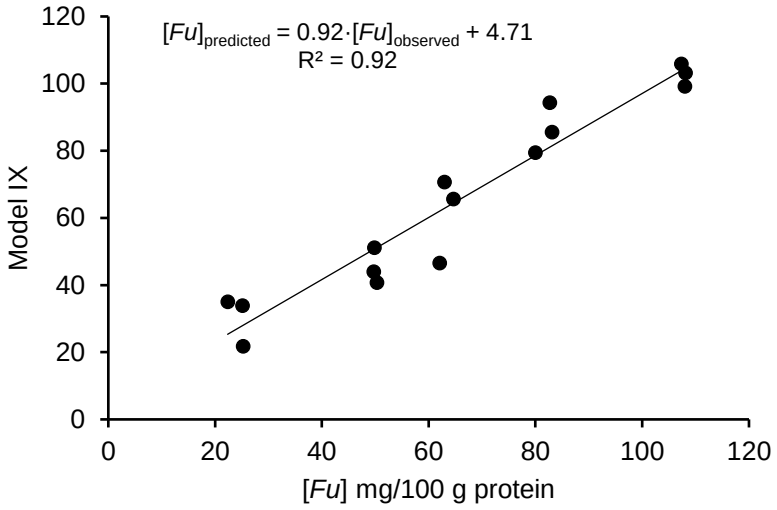


Figure 6.3. Regression between experimental and estimated values (Model IX) for [*Fu*] at 90 °C.

6.3.5. Validation of prediction models

The prediction models for lactulose and furosine concentrations were validated with the leave-one-out method. The best models obtained were at 90 ºC and they were selected for the validation purpose. The values of the validation process are described in the **Tables 6.8** and **6.9** for lactulose and furosine concentrations, respectively.

Unlike the Model I for the [*L*] prediction (**Table 6.8**), that showed a very low R^2 (0.002) and a high coefficient of variation (43.16%), the other validated models (II, III and IV), showed robust results according to the coefficients of variations and the standard error of predictions. The best model, after the validation, was the Model IV. Furthermore, it should be noted that are necessary at least four fluorescent variables to obtain a strong prediction of the [*L*].

Table 6.8. Validation of prediction models for lactulose concentration.

	Prediction models	R^2	CV	SEP
I* **	$[L]_E = \beta_0 + \beta_1 \cdot F_{Trp324}/F_{Dt377}$	0.002	43.16	$6.5 \cdot 10^{-5}$
II* **	$[L]_E = \beta_0 + \beta_1 \cdot 1/F_{MC} + \beta_2 \cdot F_{Trp324}/F_{Dt377}$	0.78	18.14	$3.4 \cdot 10^{-5}$
III* **	$[L]_E = \beta_0 + \beta_1 \cdot 1/F_{MC425} + \beta_2 \cdot F_{Trp324}/F_{Dt377} + \beta_3 \cdot F_{Trp307}$	0.74	17.83	$3.9 \cdot 10^{-5}$
IV* **	$[L]_E = \beta_0 + \beta_1 \cdot F_{Trp324}/F_{Dt377} + \beta_2 \cdot F_{Dt377}/F_{MC425} + \beta_3 \cdot F_{Trp307}$	0.92	10.60	$2.4 \cdot 10^{-5}$

[*L*] (mmol/L), lactulose concentration; R^2, determination coefficient; CV (%), coefficient of variation; SEP (mmol/L), standard error of prediction; F_{Trp324}, tryptophan fluorescence at 324 nm; F_{Dt377}, dityrosine fluorescence at 377 nm; F_{MC}, Maillard compounds fluorescence at 420 nm; F_{MC425}, Maillard compounds fluorescence at 425 nm; F_{Trp307}, tryptophan fluorescence at 307 nm

For the validated furosine models (**Table 6.9**), although the R^2 estimated in previous models were higher, the coefficients of variation and the standard errors of predictions values were good. The

best estimated and validated model was the Model IX with a good CV and a lower SEP. As in the lactulose prediction models, as much variables are used, the model was better.

Table 6.9. Validation of prediction models for furosine concentration.

Prediction models		R^2	CV	SEP
VII* **	$[Fu]= \beta_0 + \beta_1 \cdot F_{Trp}^2$	0.77	19.46	4.94
VIII* **	$[Fu]= \beta_0 + \beta_1 \cdot F_{Rb450} + \beta_2 \cdot F_{Trp}^2$	0.76	18.53	6.00
IX* **	$[Fu]= \beta_0 + \beta_1 \cdot F_{Rb450} + \beta_2 \cdot F_{Trp}^2 + \beta_3 \cdot F_{Rb450}^2$	0.87	13.97	5.59

[*Fu*] (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; F_{Rb450}, riboflavin fluorescence excited at 450 *n*m; F_{Rb450}^2 squared riboflavin fluorescence excited at 450 nm.

6.4. Discussion

At an early stage, Dufour and Riaublanc (1997) assessed the potential of FFF as a method for the characterization of raw, heated and homogenized milks. The analyses of emission fluorescence spectra of *Trp*, retinol (vitamin A) and anilino-naphthalene sulfonic acid and excitation fluorescence spectra of retinol and anilino-naphthalene sulfonic acid through principal component analysis discriminated the different types of milk as a function of heating and homogenization. More recently, Kulmyrzaev et al. (2005) demonstrated the potential of FFF for the evaluation of physicochemical changes in milk induced by low thermal treatment. Principal component analysis, applied to the normalized fluorescence spectra, successfully discriminated different milk samples according to the temperature and time of thermal treatment; and predicted the amount of the native proteins investigated, showing strong correlations between measured and predicted data for alkaline phosphatase and β-LG.

In another study, Schamberger and Labuza (2006) assessed the use of FFF for monitoring the development of Maillard browning in milk during thermal processing. They observed a strong correlation between FFF and the reference method for hydroxymethylfurfural and concluded that FFF could be useful for evaluating the effect on the first stages of the Maillard reaction caused by the heat processing of milk. Liu and Metzger (2007) detected changes in non-fat dry milk caused by manufacturing and storage conditions from the spectra of *MC*, *Trp*, and *Rb*. The study of Hougaard et al. (2013) revealed that, although FFF appeared to be a powerful tool for the discrimination of different high-heat treatments of milk, different models were needed for skimmed and non-standardized milks.

Considering other aspects of interest for the dairy sector, FFF associated to partial least squares calibration has been reported as a fast and simple method to assess the nutritional impact of heat

treatment on heat-treated infant formula models (Diez et al., 2008). Other authors demonstrated the use of FFF for monitoring light-induced changes in plain yogurt during storage (Becker et al., 2003). FFF has also been assessed with successful results for monitoring the geographic origin of milk (Karoui et al., 2005).

Special mention should be made of the study carried out by Kulmyrzaev and Dufour (2002), which demonstrated the potential of FFF in combination with chemometric methods to evaluate lactulose and furosine in heat-treated milk through the evaluation of the emission fluorescence spectra (305-450 nm) of *Trp*, and emission and excitation fluorescence spectra of *MC* and their correlation with reference methods. Under their experimental conditions, a quite linear distribution of lactulose concentration was expected in samples, since they mixed milk from two different heat treatment intensities.

In contrast, in the present study, samples were generated by combination of three different heat temperatures and six treatment times. Although the applied heat treatments diverged from industrial conditions of short heating times under turbulent flow, batch heat treatment used in the present study is substantially more practical for the development of the proposed models. It should be considered that, often, complicated kinetic models have failed to provide practical industrial solutions; while in many cases, simple and practical modeling has provided useful inline tools for food processing and control (Nieto & Castillo, 2014). Moreover, the present study, although including a larger number of fluorescent markers than the study previously mentioned (Kulmyrzaev & Dufour, 2002), the prediction models used only a few specific emission intensities.

The correlation observed in the present study between [*Fu*] and F_{MC} could be clearly attributed to the fact that furosine formation is a consequence of the Maillard reaction during heating (Erbersdobler & Somoza, 2007). In another hand, the significant correlation between F_{Trp} and [*Fu*] at 90 ºC could be related to the increasing formation of the furosine and progress of protein-protein interactions originated by denaturation of proteins. F_{Trp} shows more remarkable changes at 90 ºC may be due to the denaturation of proteins as was suggested by Schamberger & Labuza (2006).

The concentrations of furosine and lactulose have been correlated previously in milk heated for 10 to 40 s in the temperature range of 135 to 150 ºC (Montilla et al., 1996). The correlations obtained by Montilla et al. (1996), based on the R^2, had values between 0.91 and 0.93. The same correlation between [*L*] and [*Fu*] was evaluated in milk samples treated with UHT (direct and indirect) and in-bottle sterilization, and values of $R^2 = 0.99$ were obtained (Pellegrino et al., 1995). In another hand, the correlation of [*Fu*] and the concentration of β-LG was studied by Van

Renterghem & De Block (1996). The authors suggested that the correlation between the chemical markers was very poor, probably due to the considerable differences in β-LG denaturation kinetics and the furosine formation kinetics (Van Renterghem & De Block, 1996). The chemical determinations of [*Fu*] and [*L*] or β-LG can be laborious. In that way, the correlation of both [*Fu*] and [*L*] with the fluorescence markers can lower the analysis time and reduce the amount of effort. The use of fluorescence is increasing in dairy products analysis, either for the characterization or classification of samples, or to monitor the quality (Van Renterghem & De Block, 1996; Sikorska et al., 2006; Durakli Velioglu et al., 2017).

Although being thermal resistant and more sensitive to the photooxidation than to heat treatments (Fox & Thayer, 1998; Becker et al., 2003), fluorescence of *Rb* appears in the models to predict furosine concentration. Furthermore, it is important to note that F_{Trp} resulted as predictor in seven of nine prediction models of [*Fu*] as well as on the four [*L*] prediction models. This could be due to the high fluorescence response that *Trp* has shown after thermal exposure of samples (see Chapter 5) (Busti et al., 2005; Halder et al., 2012).

6.5. Conclusions

Changes in front-face fluorescence induced in milk native or generated fluorophores during heat treatment were evaluated. Furthermore, several linear correlations between chemical formation of lactulose or furosine and fluorescent markers were observed, which clearly indicates the influence of the temperature on each chemical and fluorescent markers studied.

Prediction models for lactulose and furosine concentration were obtained using the fluorescent markers as predictor. The multilinear regression prediction models were validated and the results were encouraging.

The obtained results confirm the potential of fluorescent markers naturally present in milk or generated during heat treatment in combination with front-face fluorescence measurement geometry and simple fluorescence ratios to estimate the thermal effect in milk upon processing. Although lactulose shows not to be a good heat indicator, further research about the use of front-face fluorescence spectroscopy as a predictive method to estimate heat damage indices in milk is encouraging.

CHAPTER 7:
Use of the front-face fluorescence spectroscopy to classify commercial milks

7.1. Introduction

In the industrial processing, many thermal treatments can be applied in raw milk in order to extend the shelf-life of the product. In fact, in the European Union a considerable diversity of commercial milk products can be found as result of the range of heating processes and time/temperature combinations (Feinberg et al., 2006). The most common heat conditions are thermization, pasteurizations (high-temperature, short time pasteurization, HTST; higher-heat, shorter time pasteurization, HHST), ultra-high temperature treatment (UHT) and in-container sterilization. The conditions of heating depend on the desired final product.

The treatments induce physiochemical modifications in milk molecules depending on the heat intensity. There are studies that have been carried out in order to assess the heat stability of milks (Lehmann & Buckin, 2005; Arena et al., 2017). Proteins are the most studied milk ingredient using many different analysis techniques (Kulmyrzaev et al., 2005; Stirpe et al., 2008; Taterka & Castillo, 2015). Furthermore, compounds such as furosine and hydroxymethylfurfural, along with changes in sulfhydryl groups, are evaluated as intermediate compound of the Maillard reaction (Guingamp et al., 1993; Pellegrino et al., 1995; Morales et al., 1996; van Boekel, 1998; Prakash et al., 2015;). Lactulose and epilactose, which are derived from lactose, are evaluated in order to analyze the changes that occur to lactose due to heating (Martínez-Castro, 1986; Berg, 1993; Feinberg et al., 2006).

In general, the present study proposes FFF as a tool to detect changes occurred in milk processed under industrial conditions. FFF is a rapid, non-invasive and sensitive method that can provide information on the presence of fluorescent molecules and their environment (Christensen et al., 2003). It can be even applied in milk without any dilution or previous manipulation. FFF has been evaluated to classify food samples. Specifically, fluorescence of *Trp* (290/340 nm) and *MC* (330/420 nm) were used in combination with chemical markers by Feinberg et al. (2006) to classify thermal treatments in commercial samples. Ruoff et al. (2006) used FFF for the authentication of the botanical origin and quality control and to determinate the geographical origin of honey. In another study, FFF was applied to characterize pasteurized milk samples using instant infusion at six different temperatures from 72 to 120 ºC. The fluorescence measurements were performed with excitation wavelengths from 250 to 350 nm and emission wavelengths from

260 to 500 nm (Hougaard et al., 2013). The fluorescence method also was used by Mungkarndee et al. (2016) for the identification of commercial milk samples according to their thermal treatments, analyzing a range of excitation and emission wavelengths (400-600 nm). Evaluation of yogurt was performed by front-face fluorescence spectroscopy to analyzed the products during the storage over a period of 5 weeks at 4-8 ºC (Christensen et al., 2005). In this study, fluorescence landscape was obtained with excitation wavelengths from 270 to 550 nm and recovered emissions from 310 to 590 nm. The adulteration of buffalo milk with cow milk was analyzed by fluorescence in order to discriminate the samples according to their composition. The fluorescence data was recovered from 400 to 600 nm. Durakli Velioglu et al. (2017). The changes suffered by the products due to thermal treatments or storage, as well as the profiles showed by the fluorescence markers, allows the classification, discrimination and authentication of samples.

For all the above mentioned, the aim of the present study was to evaluate differences on the fluorescence of selected markers, such as *Trp*, *Dt*, *MC* and *Rb* excited at three wavelengths: 267, 370 and 450 (Rb_{267}, Rb_{370} *and* Rb_{450}), in several commercial milks using front-face fluorescence as a tool to classify the samples according to their heat treatment and fat concentration.

7.2. Materials and Methods

7.2.1. Milk sampling

Fifty-nine milk samples coming from twenty-one different commercial milks were purchased at several supermarkets. Different batches of milk were analyzed for each commercial brand. Four classes of milk treatments were established and used in order to classify the samples according to the type of milk (fat concentration category) and heat treatment (**Table 7.1**).

7.2.2. Fluorescence determination

Front-face fluorescence measurements were performed as described in Section 5.2. Briefly, the "front-face" geometry accessory of the fluorescence spectrophotometer was adjusted to a 35 º angle of incidence and measurements were carried out using Suprasil® quartz cuvettes. Fluorescence spectra of each fluorescent compound were obtained in triplicate under the settings shown in Table 5.1 by readings performed every 1 nm at 400 V for *Trp* and *Rb* excited at 267 nm and 600 V for the rest of fluorophores.

Table 7.1. Description of samples used in the experiment.

Treatment	Fat concentration category	Code	Nº samples
UHT	Skimmed	S-UHT	15
UHT	Low-fat	LF-UHT	10
UHT	Whole	W-UHT	17
Spray drying	Skimmed	S-Powder	2
Spray drying	Whole	W-Powder	1
Pasteurization	Skimmed	S-Pasteurized	1
Pasteurization	Low-fat	LF-Pasteurized	5
Pasteurization	Whole	W-Pasteurized	4
In-bottle-sterilization	Low-fat	LF-STR	2
In-bottle-sterilization	Whole	W-STR	2

7.2.3. Data Analysis

Principal component analysis (PCA) was performed using LatentiX© data analytical software (version 2.12, 2016, Copenhagen, Denmark) taking into account two principal components; no PC with eigenvalue higher than 1 was left out of the analysis.

7.3. Results and discussion

Applying PCA, in order to overview the distribution of the samples and the spectral structure, to the results obtained from F_{Trp} spectra, PC#1 and PC#2 explained 99.03% (85.84% and 13.19%, respectively) of the variation in the distribution according to the different treatments, i.e., UHT, pasteurization, in-bottle-sterilization and spray drying (**Figure 7.1**). Replicate measurements showed good repeatability, and the points corresponding to the triplicates laid close in PC space. However, no such good results were obtained with samples of the same commercial brands but different batches. Milk samples submitted to the same categorical treatment tended to scatter, which could be due to variations in the processing conditions applied by the different factories. Pasteurized milk samples were clustered in a group separated from all the other samples. UHT milk samples were spread over a large area of the PCA scatterplot. F_{Trp} allowed discriminating thermal treatment intensities but did not discriminate by fat content.

In a study performed by Feinberg et al. (2006), F_{Trp} was used in the FAST method in order to discriminate commercial milks. They found difficult to discriminate between UHT samples

(direct or indirect) but they achieved a classification for pasteurized samples, such as in the present study, where the results of the UHT treatment were dispersed and not clear.

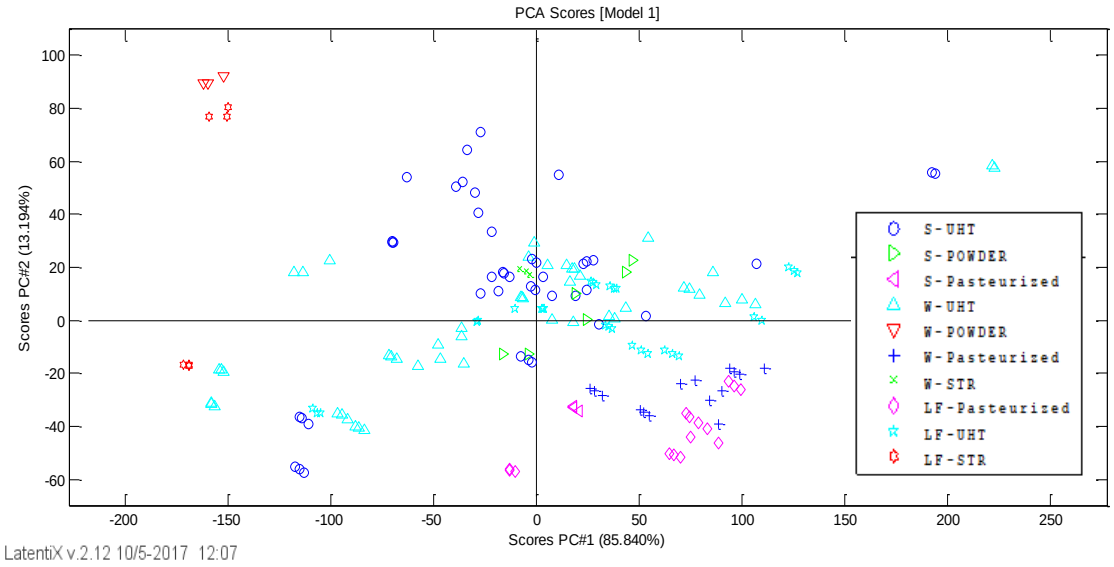


Figure 7.1. Classification of samples heat-treated at different levels using spectra obtained with an excitation of 290 nm, related to tryptophan fluorescence. S, W and LF stand for skimmed milk, whole milk and low-fat milk, respectively; STR stands for in-bottle-sterilized and Powder for reconstituted milk from spray-dried powder.

Figure 7.2 shows the classification using the excitation wavelength at 315 nm where *Dt* emits fluorescence as it is suggested by Polimova et al. (2011). The first two components explained 98.43% of the variation. Specifically, UHT whole milk samples, shown in **figure 7.2** as blue circles, were split into four different clusters, demonstrating a greater variation, followed by the UHT skim milk samples, which were divided into three different clusters.

As mentioned in the section 5.3.2, *Dt* is formed in highly oxidative environments as a consequence of the cross-linkage between two residues of tyrosine during photooxidation (Dalsgaard et al., 2007). It is often used as a marker of the oxidative stress of proteins, and it is a useful marker to evaluate oxidative conditions (Harms et al., 1997; Malencik & Anderson, 2003). The information related to the alterations in milk due to protein oxidation is limited. However, in terms of oxidation, the studies are more focused on the alteration of lipids (Capeillere-Blandin et al., 1991). For instance, antioxidants used in milk powders, e.g., secoisolariciresinol diglucoside, have been evaluated for their capacity to inhibit peroxidation and prevent off-flavor development but not on protein oxidative modifications such as protein carbonyl or *Dt* bond formation (Capeillere-Blandin *et al*., 1991; Romeu-Nadal et al., 2007; Matumoto-Pintro *et al*., 2011).

When studying the oxidation damage in Mozzarella cheese, Balestrieri *et al*. (2002) observed differences in protein carbonyl without *Dt* formation. In other words, protein carbonyl content was altered without alterations in *Dt* content (Balestrieri *et al*., 2002). Moreover, Scheidegger et

al. (2013) observed a significant difference in *Dt* content when analyzing 15 samples of commercial processed spray-dried milk powders (6 whole powders, 4 skim powders, 5 infant formula powders) before and after storage. All these mentioned changes affected *Dt* fluorescence intensity (F_{Dt}) of the samples thus could contribute in the classification of the samples.

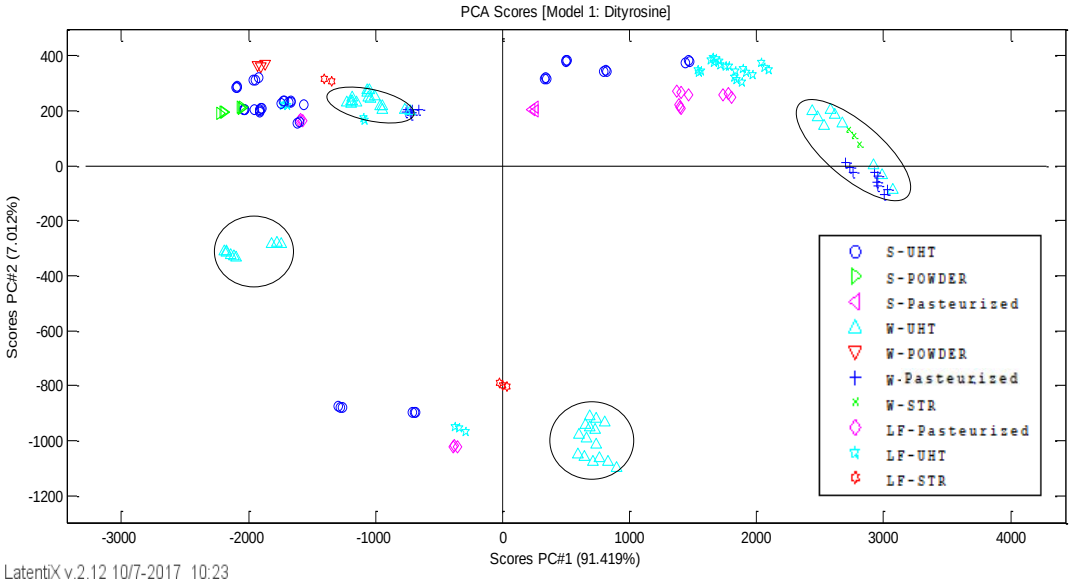
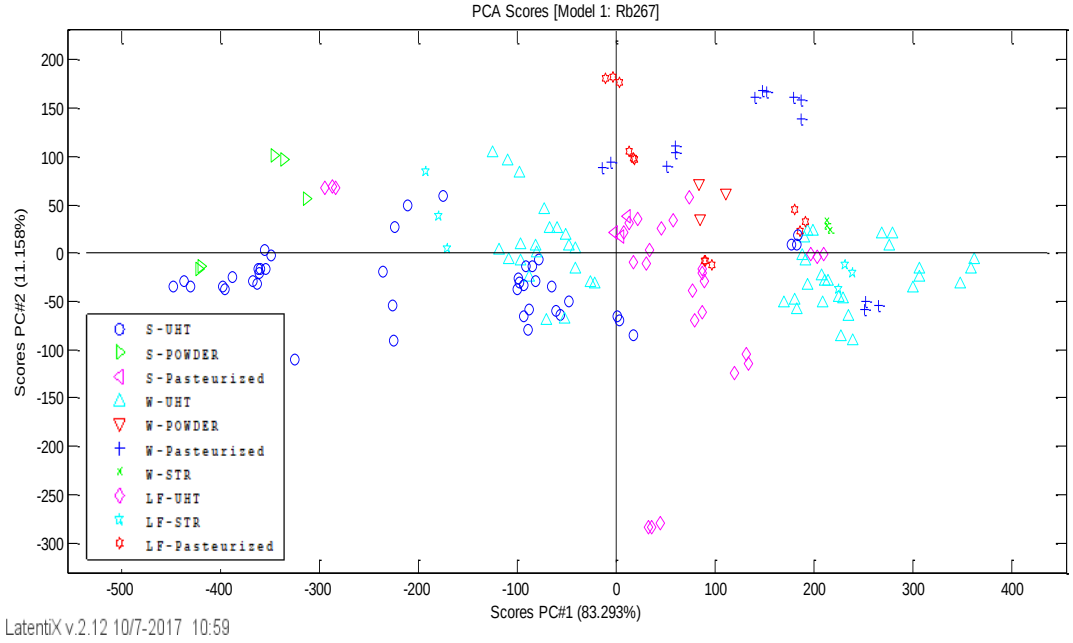


Figure 7.2. Classification of samples heat-treated at different levels using spectra excited at 315 nm, related to dityrosine fluorescence. S, W and LF stand for skimmed milk, whole milk and low-fat milk, respectively; STR stands for in-bottle-sterilized and Powder for reconstituted milk from spray-dried powder.

There are different methods to determine *Rb* content in food, which are mainly based on colorimetric, fluorometric or HPLC procedures (Elliott et al., 2003). The fluorometric method is more sensitive and free from interference and, therefore, is probably more suitable to analyze the vitamin in milk (Remington & Beringer, 2006). Although FFF technique is not related to the chemical quantification of *Rb* content, it is a sensible method to evaluate the fluorescence behavior of this vitamin. The *Rb* is a heat-stable vitamin and is known as a strong fluorescent marker (Ntakatsane et al., 2011).

The score plot of the PCA for *Rb* excited at 267 (Rb_{267}) is shown in **Figure 7.3**. The variability explained by the three first components was 97.59% (PC#1 = 83.29%, PC#2 = 11.16%, and PC#3 = 3.14%). Distribution adjusted to the different treatments and sample grouping was noted. A clearer distribution could be due to the sensitiveness of Rb_{267} showing a better discrimination of samples. The other two excitation wavelengths used for evaluating riboflavin, i.e., 370 and 450 nm, did not discriminate the different treatments, with no clear separation of the samples. According to the PCA based on the Rb_{267} (**Figure 7.3**), the grouping of the whole UHT milk samples was reduced in comparison with the PCA using other markers (*Trp* and *Dt*).

a)



b)

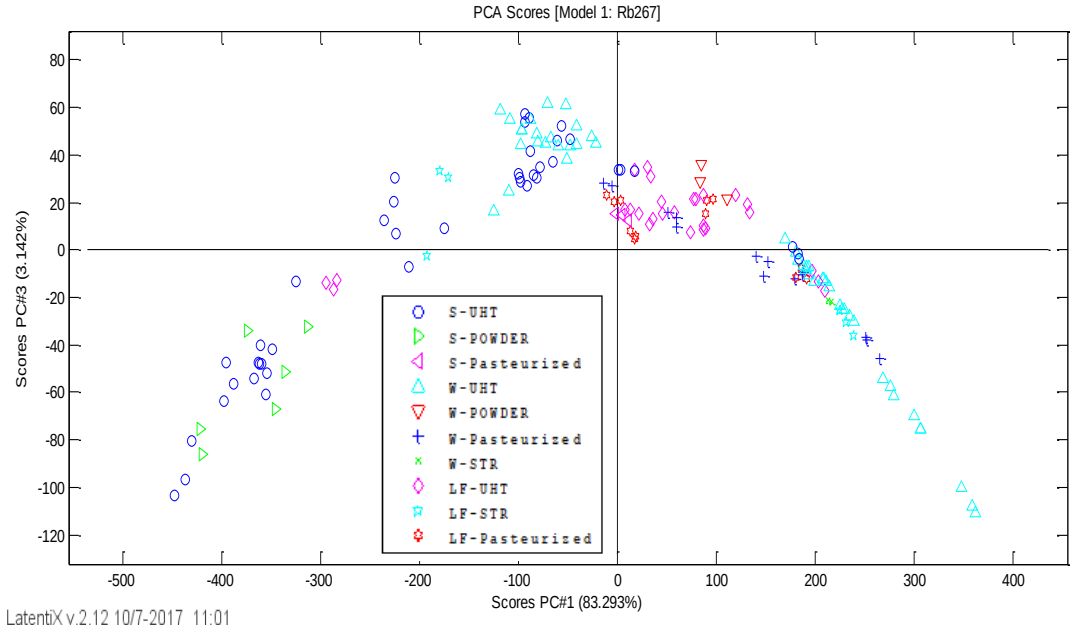


Figure 7.3. Classification of samples heat-treated at different levels using spectra excited at 267 nm, related to riboflavin fluorescence. a) PC#1 *vs.* PC#2; b) PC#1 *vs*. PC#3. S, W and LF stand for skimmed milk, whole milk and low-fat milk, respectively; STR stands for in-bottle-sterilized and Powder for reconstituted milk from spray-dried powder.

The spectrum obtained exciting at 330 nm was associated with *MC*. The PCA showed that 97.48% of the variability was explained by the first two components (**Figure 7.4**). The Maillard reaction is a darkening process extremely complex that develops in foods brown pigments from

carbohydrates and amino acids. The reaction occurs particularly during high temperature processes or storage for long periods of time (Billaud et al., 2003). The Maillard reaction is the main cause of milk damage in heat-treated milk (Leclère & Birlouez-Aragón, 2001). Advanced Maillard reaction products, such as pyrrole and imidazole derivatives, are fluorescent compounds that are formed as intermediary products during heating (Andersen & Mortensen, 2008).

Note that, increasing treatment temperature triggers higher losses of lactose than formation of Amadori products. This is due to the fact that, besides the Maillard reaction, lactose is also subjected to isomerization and degradation and its contribution is quantitatively more important (van Boekel, 1998). This suggests that when heat load is more intense, the fluorescence of *MC* (F_{MC}) could be more noticeable. Hence, theoretically, the F_{MC} could allow detecting the progress of the reaction before appearing color changes in the milk. The fluorescence generated by the Maillard reaction is attributed particularly to structures with complex molecular bonds between C and N (Bastos et al., 2012). Furthermore, the contribution of caramelized sugar to the overall fluorescence is negligible in systems containing amino acids (Matiacevich & Buena, 2006).

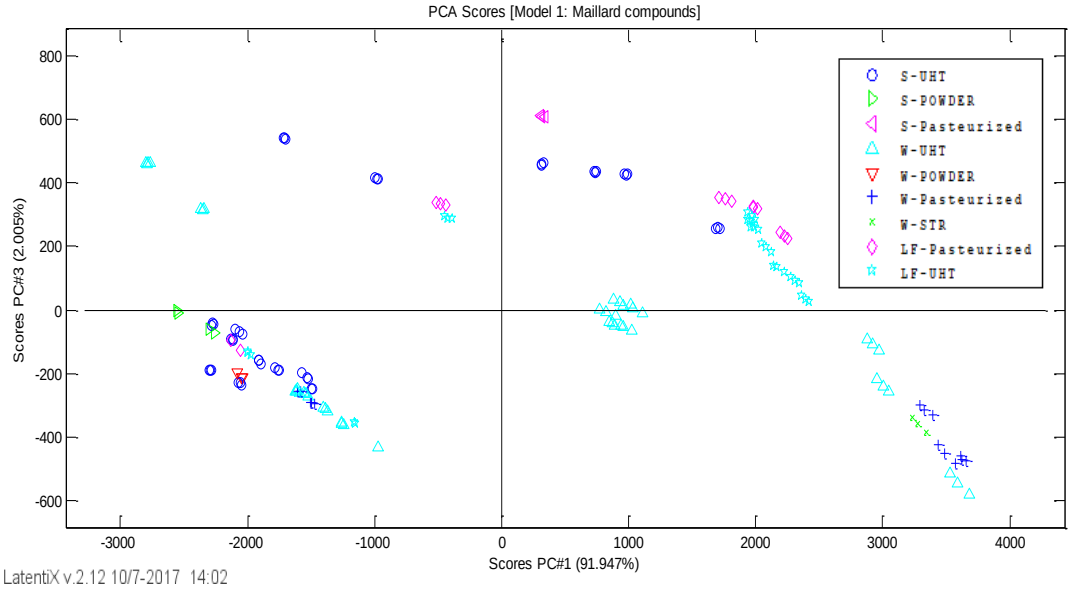


Figure 7.4. Classification of samples heat-treated at different levels using spectra excited at 330 nm, related to Maillard compounds fluorescence. S, W and LF stand for skimmed milk, whole milk and low-fat milk, respectively; STR stands for in-bottle-sterilized and Powder for reconstituted milk from spray-dried powder

Becker et al. (2003) suggested that the fluorescence variation from 370 to 470 nm (excitation) and emission around 530 nm correspond to the *Rb* and it is due to the degradation of this vitamin. However, in order to correctly interpret the classification of the samples, it should be taken into account that milk samples analyzed come from different factories. Moreover, the factories obtain raw milk from different farms. These factors could also affect the classification of the samples.

7.4. Conclusions

In this chapter whole, low-fat and skimmed milk of several commercial brands were evaluated. Fluorescence zones related to *Trp*, *MC*, Rb_{267}, Rb_{370}, Rb_{450} and *Dt* were excited in order to discriminate the commercial samples according to their treatment and fat content. *Trp* spectra showed a grouping of skimmed UHT samples represented by the two first component of PCA components according to the thermal treatment received. However, *Trp* spectra did not offer classification according to the milk composition; the samples with different fat content were dispersed within each thermal treatment group. Similarly, dityrosine PCA did not distinguish clearly the different treatments, but gave a clustering of the samples of each treatment and composition categories, split into separated groups. A discrimination of milk samples according to their fat content can be achieved by PCA of riboflavin fluorescence excited at 267 nm. The combined use of the different markers would allow a good classification of commercial samples according to their treatment with the concurrence of a chemometric approach.

CHAPTER 8:
Conclusions

Formation kinetics of heat damage indicators lactulose and furosine in heat-treated reconstituted skim milk powder followed a pseudo-zero reaction order.

- The order of reaction was successfully evaluated at the three studied temperatures for furosine, while it was only possible to stablish the reaction order for lactulose on the experiment at 90 ºC.
- Kinetic parameters such as activation energy (E_a) and reaction rate constants (k) were estimated for both chemical markers. The estimated values had the same order of magnitude as those found in literature.

An increase on the intensity of the heat-treatment load applied to milk samples induced both a decrease in tryptophan fluorescence intensity and a red shift.

- Decomposing the spectra observed for tryptophan fluorescence changes in two fractions, one associated with the change of the peak height and another with the displacement of the emission maximum, allowed the identification of the originated milk damage.
- Relatively mild milk treatments (70-80 ºC) seemed to induce a slight red shift of the tryptophan emission spectra with no reduction on the fluorescence intensity, probably due to whey protein denaturation. More intense treatments induced a clear decrease on fluorescence intensity, with a larger peak shift, likely associated with protein-protein interactions.

The spectra of the different fluorescent markers coupled to Principal Component Analysis (PCA) allowed discrimination of milk samples.

- Discrimination between temperature treatments, grouped as 70-80 ºC and 90-100 ºC, was achieved clearly on PCA analysis of riboflavin excited at 267 nm.
- Discrimination between time treatments was achieved clearly on PCA analysis of riboflavin excited at 370 nm.
- PCA of Maillard compounds, dityrosine and tryptophan contained information of time and temperature of treatments.

- The results of the PCA showed that riboflavin fluorescence excited at 450 nm was not a good marker to classify skim milk samples.

Lactulose and furosine concentrations in heat-treated milk could be predicted using models including fluorescent markers as predictors.

- Developed multilinear models were successfully validated using leave-one-out method.

Commercial milk samples can be successfully discriminated according to their treatment and fat content using the combined information of fluorescence emission spectra of tryptophan, Maillard compounds, dityrosine and riboflavin, exited at three different wavelengths, coupled with PCA.

- Pasteurized samples were placed in a separated group on tryptophan PCA score-plot irrespectively of their fat content. UHT milk formed a different group, partially overlapped with other categories.

- Different treatments tended to be split into several clusters on the PCA scatter-plot of dityrosine. UHT skim was divided into four different clusters.

- The evaluation of both Maillard compounds and riboflavin spectra showed a partial overlapping of the different treatments, yielding an incomplete discrimination for the analyzed commercial milk samples.

Front-face fluorescence is a useful, non-invasive, rapid and cheap method to evaluated heat-induced changes in milk without diluting the samples, which may have potential for in-line applications. Among the fluorescent markers studied, tryptophan has demonstrated to have a huge potential as native marker to monitor the damage in heat-treated milk when using chemometric techniques.

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