Application of native fluorescence tracers for quick quantification of milk changes caused by novel processing technologies

Liu, Jinfang

Universitat Autònoma de Barcelona

Facultat de Veterinària

Department de Ciència Animal i dels Aliments

Application of native fluorescence tracers for quick quantification of milk changes caused by novel processing technologies

PhD in Food Science

Liu, Jinfang

Bellaterra (Cerdanyola del Vallès), 2017

Planta de Tecnologia dels Aliments UAB

XaRTA
Xarxa de Referència en Tecnologia dels Aliments de la Generalitat de Catalunya

www.malta-consolider.com
CONSOLIDER 2010
MALTA
Matter at High Pressure

Dr. JORDI SALDO PERIAGO, professor titular del Departament de Ciència Animal i dels Aliments de la Universitat Autònoma de Barcelona, i

Dr. MANUEL CASTILLO ZAMBUDIO, professor titular del Departament de Ciència Animal i dels Aliments de la Universitat Autònoma de Barcelona,

FAN CONSTAR que Liu, Jinfang ha realitzat, sota la seva direcció, en el àrea de Tecnologia dels Aliments de la Universitat Autonòma de Barcelona (UAB), el treball titulat "Application of native fluorescence tracers for quick quantification of milk changes caused by novel processing technologies" que presenta per optar al grau de Doctor en Ciència dels Aliments.

I perquè així consti, signa el present document a:

Bellaterra, Cerdanyola del Vallès, 26 de septiembre del 2017.

Dr. Jordi Saldo Periago

Dr. Manuel Castillo Zambudio

The research of this thesis was supported by the national research project AGL2012-33957 of the Spanish Ministerio de Economía y Competitividad by European Regional Development Fund (ERDF/FEDER) and the China Scholarship Council (CSC).

To my parents:

Ke, Yanmei and Liu, Wenji

Acknowledgements

Thanks foremost to my director Jordi Saldo, who has always been supportive to me. Thanks for your consistent encouragement, kindness and assistance in many areas. I am deeply grateful of your help in the completion of this thesis.

Thanks to my director Manuel Castillo. Your passion and detail-oriented attitude to work influenced me a lot. I am grateful for your dedication and patience for our project.

I would also like to thank Joan Miquel, Ramón and Roger, for their assistance in the pilot plant during the productions we made.

Thanks to Bibi, Sonia for their laboratory assistance, helped me gain some excellent laboratory techniques.

Thanks to Cristina, Noemí, Yanli, Ulises, Anna and all the friends who have supported and helped me during this journey, I am grateful and consider myself very lucky.

Thank you to my family who have always been there for me.

At last, many thanks to everyone who contributed to the realization of this thesis.

Abstract

This PhD dissertation is focused on using native milk fluorescence indicators to establish models for rapid evaluation and prediction of the effects of thermal (70-90 °C, 0-30 min) and ultra-high pressure homogenization (UHPH) processing (100-300 MPa, inlet temperature 22-83 °C) on bovine milk by means of front-face fluorescence spectroscopy (*FFFS*).

Rapid quantification of retinol concentration, in both thermally and UHPH processed milk, was well modeled with an R^2 of 0.92 and 0.90, and standard error of prediction of 0.025 mg/L and 0.016 mg/L, respectively. Retinol degradation originated by thermal treatments followed a first-order kinetic and the activation energy was estimated to be 52.26 kJ/mol.

In both skim milk and whole milk, the tryptophan, dityrosine and Maillard intermediate compounds fluorescence could be modeled as functions of UHPH processing parameters. In skim milk, the dityrosine and Maillard compounds fluorescence intensities were only significantly affected by inlet temperature, while all the fluorescence indicators were significantly influenced by both pressure and temperature in UHPH treated whole milk. The changes in fluorescence revealed the milk protein modifications induced by UHPH, including whey protein denaturation, casein disintegration and protein aggregation.

Particle size distribution showed that UHPH treatments could effectively reduce the particle size in whole milk. With pressure under 200 MPa, UHPH would not lead to significant protein aggregation in either skim or whole milk. On the contrary, a clear effect of UHPH treatment on milk protein structure modification could be observed above 200 MPa. These findings support and complement the conclusions from the fluorescence indicators about milk protein aggregation.

Resumen

Esta tesis doctoral se centra en el uso de indicadores nativos de fluorescencia en leche para establecer modelos de evaluación y predicción rápida de los efectos del procesamiento térmico (70-90 °C, 0-30 min) y de homogeneización a presión ultra-alta (UHPH) (100-300 MPa, temperatura de entrada 22-83 °C) sobre leche bovina entera y desnatada por medio de espectroscopía de fluorescencia *front-face* (*FFFS*).

La cuantificación rápida de la concentración de retinol, tanto en la leche procesada térmicamente como mediante UHPH, se modelizó con un R^2 de 0,92 y 0,90, y un error estándar de predicción de 0,025 mg / L y 0,016 mg / L respectivamente. La degradación de retinol originada por los tratamientos térmicos siguió una cinética de primer orden y la energía de activación se estimó en 52,26 kJ / mol.

Tanto en la leche desnatada como en la leche entera, la intensidad de la fluorescencia de los compuestos intermedios de Maillard, triptófano y ditirosina podría modelizarse como funciones de los parámetros de procesado UHPH. La fluorescencia de ditirosina y de los compuestos Maillard en la leche descremada sólo fue afectada significativamente por la temperatura de entrada, mientras que en la leche entera tratada mediante UHPH todos los indicadores de fluorescencia fueron significativamente influidos por la presión y la temperatura. Los cambios en la fluorescencia revelaron las modificaciones en las proteínas de la leche inducidas por el tratamiento UHPH, incluyendo la desnaturalización de las proteínas del suero, la desintegración de la caseína y la agregación de proteínas.

La distribución del tamaño de partícula mostró que los tratamientos de UHPH podrían reducir efectivamente el tamaño de partícula en la leche entera. Con una presión inferior a 200 MPa, el tratamiento UHPH no conduciría a una agregación significativa de proteínas ni en leche descremada ni en leche entera. Por el contrario, se pudo observar un efecto claro del tratamiento mediante UHPH sobre la modificación de la estructura de la proteína de la leche por encima de 200 MPa. Estos hallazgos apoyan y complementan las conclusiones sobre la agregación de proteínas de la leche obtenidas a partir de los indicadores de fluorescencia.

Resum

Aquesta tesi doctoral se centra en l'ús d'indicadors nadius de fluorescència en la llet per establir models d'avaluació i predicció ràpida dels efectes de processament tèrmic (70-90 °C, 0-30 min) i homogeneïtzació d'ultra alta pressió (UHPH) (100-300 MPa, amb temperatura d'entrada de 22 a 83 °C) sobre llet sencera i desnatada de boví mitjançant espectroscòpia de fluorescència *front-face* (*FFFS*).

La quantificació ràpida de la concentració de retinol, tant en la llet processada tèrmicament com en la UHPH, es va modelitzar amb una R^2 de 0,92 i 0,90, i un error estàndard de predicció de 0,025 mg / L i 0,016 mg / L respectivament. La degradació de retinol originada pels tractaments tèrmics va seguir una cinètica de primer ordre i es va estimar que l'energia d'activació és de 52,26 kJ / mol.

La fluorescència de compostos intermedis de Maillard, triptòfan i ditirosina en la llet desnatada i en la llet sencera es pot modelitzar com a funcions dels paràmetres de processament UHPH. La fluorescència de la ditirosina i de compostos Maillard en la llet desnatada només es va veure afectada significativament per la temperatura d'entrada, mentre que en la llet sencera tractada mitjançant UHPH tots els indicadors de fluorescència es van veure fortament influïts per la pressió i la temperatura. Els canvis en la fluorescència van revelar les modificacions de la proteïna de la llet induïdes pel tractament UHPH, inclosa la desnaturalització de proteïnes de sèrum de llet, la desintegració de la caseïna i l'agregació de proteïnes.

La distribució de la grandària de partícules va mostrar que els tractaments UHPH podrien reduir la mida de les partícules en llet sencera. Amb una pressió inferior a 200 MPa, el tractament UHPH no va donar lloc a cap important agregació de proteïnes en llet descremada o completa. Al contrari, es va poder observar un clar efecte del tractament UHPH sobre la modificació de l'estructura de proteïnes de la llet per sobre de 200 MPa. Aquests resultats recolzen i complementen les conclusions sobre l'agregació de proteïnes de la llet obtingudes a partir dels indicadors de fluorescència.

Table of Contents

List of Abbreviations

A	Arrhenius equation collision factor
ANOVA	Analysis of variance
Dt	Dityrosine
E_a	Activation energy
EDTA	Ethylenediaminetetraacetic acid
FFFS	Front-face fluorescence spectroscopy
F_{Dt}	Maximum fluorescence intensity of dityrosine
F_{max}	Maximum fluorescence intensity
F_{MC}	Maximum fluorescence intensity of Maillard compounds
F_{Re}	Maximum fluorescence intensity of retinol
F_{Rb370}	Maximum fluorescence intensity of riboflavin (excited at 370 nm)
F_{Rb450}	Maximum fluorescence intensity of riboflavin (excited at 450 nm)
F_{Trp}	Maximum fluorescence intensity of tryptophan
HHP	High hydrostatic pressure
HPH	High pressure homogenization
HPLC	High performance liquid chromatography
HTST	High temperature short time
k_T	Reaction rate of corresponding temperature
α-La	α-Lactalbumin
β-Lg	β-Lactoglobulin
MC	Maillard compounds
MFGM	Milk fat globule membrane
NIRS	Near infrared reflectance spectroscopy
PC	Principal component
PCA	Principal component analysis
PEF	Pulsed electric field

PLS	Partial least squares
R	Universal ideal gas constant
Rb	Riboflavin
[*Re*]	Concentration of retinol
$[Re]_0$	Initial concentration of retinol
RMSECV	Root mean square error of cross-validation
SAS	Statistical analysis system
SEP	Standard error of prediction
Trp	Tryptophan
UHPH	Ultra-high pressure homogenization
UHT	Ultra high temperature processing
UV	Ultra violet

Chapter 1

Introduction and literature review

1. Introduction and literature review

1.1 Milk overview

Milk is a kind of emulsion secreted by female mammal species. The substances in milk provide both energy and the building materials necessary for early growth. So milk meets all the nutritional requirements of newborns, including energy (lipids, lactose even proteins), protein (essential amino acids and biosynthesized nonessential acids), essential fatty acids, vitamins, inorganic elements and water (Singh, Boland, Thompson, Boland, & Singh, 2014); and is a complete food for adults. These are organized as follows: lipids in emulsified globules covered with a membrane, proteins in colloidal dispersion as micelles, and most minerals and all lactose in true solution (Jensen, 1995b). The species have been taken under research mainly including human, cow, goat, sheep, camel, horse, buffalo, yak, etc. The milk of some domestic animals and their derived dairy products have a long history in human diet, since 6000 years ago or even earlier in many areas of world (Kindstedt, 2012; Singh et al., 2014). And nowadays, with a highly-developed industry of agriculture and aquaculture, people mainly use cow, buffalo, sheep and goat milk for fresh drinking milk, yogurt, cream butter and cheese making.

1.1.1 Milk compositions

Milk from different species contain similar basic components but with different proportions. Since bovine milk is the most widely consumed and it is the research subject in present study, the milk here in after refers to bovine milk. It consists of about 87% water and 13% dry substance. The dry matter is dissolved or suspended in the aqueous phase. Depending on the type of substances there are different distribution systems of them in the water phase (Bylund, 1995).

1.1.1.1 Milk protein

All proteins are composed of linear chains of amino acids, and each protein has its own defined amino acid sequence, which is determined by the genetics of the producing

organism (Chatterjee, Sarkar, & Boland, 2014). In general, milk proteins are divided into two major categories as casein and whey (or serum) proteins. The classification is based on solubility at pH 4.6, at which caseins become insoluble, whereas whey proteins remain soluble. For physical structure, casein exists as a calcium phosphate complex in the form of colloidal suspension, while whey proteins occur in soluble form. Another type of milk protein occurs as a part of milk fat globule membrane (MFGM), covering the envelope in which milk fat is enclosed. Casein molecules in milk are organized as rough surfaced spherical particles called micelles (Chandan, Patel, Almeida, & Oliver, 2015), varying in size from 50 to 500 nm. Electron image analysis has shown that the micelles are composed of smaller particle or submicelles of 20 nm diameter or less (Chandan, 2007).

Table 1-1 Approximate concentration of protein fractions in cow's milk (Almeida et al., 2008)

Fraction	% in skim milk	% in milk protein	% total casein	% total whey protein
Total protein	3.27	100.0	—	—
Total casein	2.69	82.2	100.0	—
α-s_1-Casein	1.02	31.3	38.1	—
α-s2-Casein	0.27	8.4	10.2	—
β-Casein	0.96	29.3	35.7	—
ϰ-Casein	0.35	10.5	12.8	—
γ-Casein	0.09	2.7	3.2	—
Total whey protein	0.57	17.8	—	100.0
β-Lactoglobulin(β-Lg)	3.14	9.6	—	54.2
α-Lactalbumin (α-La)	1.23	3.8	—	16.8
Bovine serum albumin	0.45	1.4	—	7.8
Immunoglobulins, lactoferrin, Proteose peptones fraction-3	0.97	3.0	—	21.2

In addition, whey proteins are highly structured but caseins lack stable secondary structures. The inability of the caseins to form stable structures attributed to the

structure-breaking amino acid proline, being β-casein particularly rich in proline (Singh et al., 2014). Table 1-1 presents the approximate concentration of protein fractions in cow's milk.

Among milk proteins, caseins are relatively stable to heat effects. Whey proteins tend to denature progressively by severity of heat treatment, reaching 100% denaturation at 100 °C or at 80-90 °C for 10 min. In the presence of casein in milk, denatured whey proteins complex with casein and no precipitation is observed. In contrast to milk, heat treatment at 75-80 °C of whey that lacks casein results in precipitation of the whey proteins (Chandan, 2009). Serum albumin is a major protein found in blood serum; it has a principal role in the transport, metabolism, and distribution of ligands; it contributes to osmotic pressure of blood; and it imparts free radical protection. Immunoglobulin is a large, Y-shaped protein produced mainly by plasma cells that is used by the immune system to neutralize pathogens such as bacteria and viruses. Lactoferrin is an iron binding protein and has antibacterial properties. It is found in relatively low concentrations during lactation in cow milk, but is increased during mastitis and involution. Lactoferrin may also be an immunomodulator. In fact, it is the major whey protein in human milk. Proteose-peptone is a mixture of heterogeneous proteins and peptides. Its functional properties have not yet been fully understood (Almeida et al., 2008; Andrews, 1978; Farrell et al., 2004; Innocente, Corradini, Blecker, & Paquot, 1998).

1.1.1.2 Lipids

Lipids are those constituents of tissues, biological fluids or foods that are soluble in a nonpolar solvent. In general, lipids are divided into three classes: neutral lipids, polar lipids and miscellaneous lipids. Neutral lipids are esters of glycerol with one, two or three fatty acids for mono-, di- and triglycerides respectively. Neutral lipids are by far the dominant class of lipids in all foods and tissues, representing 98.5% of total milk lipids. Many polar lipids contain phosphoric acid, a nitrogen-containing compound (choline, ethanolamine or serine) or a sugar/oligosaccharide. Although present at only about 1% of total milk lipids, the polar lipids play critical roles in milk and dairy

products. Polar lipids are good natural emulsifiers and are concentrated in the MFGM, which maintains the milk lipids as discrete globules and ensures their physical and biochemical stability. Miscellaneous lipids are a heterogeneous group of compounds that are chemically unrelated to each other or to neutral or polar lipids. This group includes cholesterol, carotenoids and the fat-soluble vitamins, A, D, E and K. Milk lipids contain a very wide range of fatty acids. Up to 400 fatty acids have been reported, although most of these are present at trace levels (MacGibbon & Taylor, 2006; O'Mahony & Fox, 2014; Singh et al., 2014).

Food lipids' deterioration are in two ways: lipid oxidation, leading to oxidative rancidity, and the hydrolysis of lipids by lipases (lipolysis), leading to hydrolytic rancidity. Milk contains an indigenous lipoprotein lipase, which is normally inactive because it is separated from the triglyceride substrates by the MFGM. However, if the MFGM is damaged, lipolysis and hydrolytic rancidity is induced rapidly.

1.1.1.3 Lactose

The primary carbohydrate of the milk is lactose (4-*O*-β-D-galactopyranosyl-D-glucose), commonly called milk sugar. Moreover, milk is the sole source of lactose for all kinds of practical purposes. Bovine milks average 4.8% anhydrous lactose and about 50% of total solids of skim milk (Holsinger, 2009). In milk lactose exists in two isomeric forms, called α- and β-lactose, respectively. The molecular structures of α- and β-lactose differ in the orientation of the hydroxyl group on carbon atom number one in the glucose moiety. Both forms can spontaneously change from one to another, having an equilibrium ratio of about 40% α-lactose and 60% β-lactose at room temperature. Lactulose (4-*O*-D-fructofuranose) is an isomer of lactose, formed by molecular rearrangement, frequently under alkaline conditions, where the terminal aldose residue of lactose is converted to a ketose (Fox & Chemistry, 2009).

1.1.1.4 Minerals and vitamins

The minerals of milk constitute a small part of milk (8-9 g/L). This fraction contains calcium, magnesium, sodium and potassium for the main cations, and

inorganic phosphate, citrate and chloride for the main anions (Table 1-2). These ions play an important role in the structure and stability of casein micelles in milk.

Table 1-2 Mineral Composition of Cow milk (Gaucheron, 2005)

Mineral	Concentration (mg/kg)	Concentration (mmol/kg)
Calcium	1043-1283	26-32
Magnesium	97-146	4-6
Inorganic phosphate	1805-2185	19-23
Total phosphorus	930-992	30-32
Citrate	1323-2079	7-11
Sodium	391-644	17-28
Potassium	1212-1681	31-43
Chloride	772-1207	22-34

In milk, these macro-elements are distributed differently into diffusible and non-diffusible fractions (essentially casein micelles). Potassium, sodium and chloride ions are essentially diffusible, while calcium, inorganic phosphate and magnesium are partly bounded to the casein micelles (Gaucheron, 2005). The concentration of minerals such as chloride, phosphates, and citrates of potassium, sodium, calcium, and magnesium is important in milk processing, nutritive value, and shelf life of dairy products. Their concentration is < 1% in milk but are involved in thermal stability of milk, alcohol coagulation of milk, age-thickening of sweetened condensed milk, curdling of coffee cream, enzymatic coagulation, and clumping of fat globules on homogenization. All the minerals considered essential for human nutrition are found in milk (Chandan, 2009).

Vitamins play important roles in intermediary metabolism as co factors in numerous enzymatic reactions or in non-enzymatic physiological functions such as vision (Vitamin A or retinol), as antioxidants (carotenoids, vitamin E, C and B_2), in regulation of calcium metabolism (vitamin D), and in hematopoiesis (vitamin B_6, B_{12}

and folates). Milk and many milk products, especially fermented milk products, compared with most other foods, are rather convenient, excellent and cheap sources of those vitamins (Öste, Jägerstad, & Andersson, 2009).

Vitamin A, also known as retinol, is one of the most important micronutrients for human health. It plays an essential role in vision, bone and tooth development, and the health of skin and mucous membranes (Ball, 2006). Synthesis of retinol only occurs naturally in animals (Wilkinson, Earle, & Cleland, 1981). Generally speaking, dietary vitamin A is obtained from animal-derived foods, while plant foods provide carotenoid precursors (Ball, 2006). Good sources of retinol include eggs, dairy products (milk, cheese, yogurt, etc.) and, particularly, liver. The most common method to quantify the retinol loss during thermal treatments is high performance liquid chromatography (HPLC). The whole procedure is laborious and very time consuming. Furthermore, there is an increasing demand of the consumers and stakeholders of the food industry sector to have means of measurement allowing the simple, non-destructive, and quick characterization of raw materials (Karoui & Debaerdemaeker, 2007). In this presented research, quick quantification models for retinol in milk were established.

1.1.2 Milk processing

1.1.2.1 Thermal treatments on milk and dairy product

Heat treatment is the most conventional treatment applied on milk before it is deemed fit for drink or further processed to various dairy products. Dairy plants routinely use heat processes to make milk safe from pathogenic organisms. Depending on the different usage, different heating time and temperature would be adopted. Not only for making milk safe for consumption, but also to enhance the shelf-life of milk by destroying spoilage microorganisms and enzymes. It modifies functionality of the dairy ingredients as well. Several commonly used thermal treatments in dairy processing are listed in Table 1-3.

Table 1-3 Commonly used thermal treatments in dairy processing (Mishra & Ramchandran, 2015)

Heating process	Temperature (°C)	Time	Purpose	References
Thermization of milk	65	15 s	Destroy psychrotrophs and stabilize proteins	Vernam and Sutherland, 1994
Low temperature pasteurization	63-66	30 min	Destroy pathogens in milk and milk products	Chandan, 2009; Vernam and Sutherland, 1994
	72-75	15-20 s		
High temperature pasteurization	85	20 s-30 min	Destroy pathogens; inactivate enzymes in high viscosity products, e.g. cream	Chandan, 2009; Vernam and Sutherland, 1994
In bottle sterilization of milk	110-120	20-40 min	Destroy all pathogens and spoilage bacteria and spores	Vernam and Sutherland, 1994
Ultra-high temperature treatment	130	30 s	Destroy all pathogens and spoilage bacteria including spores	Chandan, 2009; Vernam and Sutherland, 1994
	140-145	2-6 s		
Evaporation and concentration	45-54.6 (under vacuum)	Sufficient to increase to given solid concentration	Evaporation	Farkye, 2009
Sterilization of canned concentrated milk	115-121	15-20 min	Destroy bacterial spores, enzymes	Farkye, 2009

Preheating before evaporation for production of concentrated milk	93-100 or 115-128	10-25 min or 1-6 min	Reduction of microbial load, inactivate enzymes, stabilizing proteins, reduction of viscosity for evaporated and condensed milk production	Farkye, 2009
Heating before concentration for production of powder	72-120	15 s-30 min	Reduction of microbial load, inactivate enzymes, stabilizing proteins or controlled denaturation of proteins	Augustin and Clarke, 2009; Vernam and Sutherland, 1994
Heating of milk concentrate in a spray dryer	70-95 (outlet*) and 180-230 (inlet*)	Few seconds	Drying	Vernam and Sutherland, 1994
Preheating of ice cream mix to assist in blending	45	Few minutes	To have smoother mix	Kilara and Chandan, 2009
Scalding of cheese curd	40	Sufficient to expel whey	Whey separation, final product moisture control and cheese body	Vernam and Sutherland, 1994
Heat treatment of milk for yoghurt	80	30 min	Improve viscosity and texture of yoghurt in addition to reduction in microbial load	Vernam and Sutherland, 1994

*Air temperature.

Intensive heat treatment leads to interactions of certain amino acids with lactose resulting in color changes in milk (Maillard browning) as observed in sterilized milk and evaporated milk products (Chandan, 2009). The pasteurization and sterilization temperatures and holding times employed in such treatments have profound effects on milk proteins, enzymes, MFGM, some vitamins, and physical state of minerals and other constituents. Caseins of milk are relatively stable to moderate heating regimes under conditions of normal pH and ionic balance. At 60-65 °C, β-Lg molecules begins to uncoil themselves and start interaction with κ-casein located in casein micelle forming disulfide linkages. The denaturation process is complete at 90-95 °C when milk is held for 5 min (Chandan, 2007). Under this heat treatment, α-La is relatively less vulnerable to heat, undergoing reversible denaturation. However, the immunoglobulins are fully denatured (Donato & Guyomarc'h, 2009; Jovanović, Bara, & Ma, 2005; Wijayanti, Bansal, & Deeth, 2014). In the manufacture of yogurt, this heating treatment is beneficial in increasing water- holding capacity and in reducing syneresis of the coagulum. Also, the resultant viscosity increase assists in optimizing the texture of yogurt. High heat treatment is deleterious to rennet curd formation due to rennet activity decreasing and denatured β-Lg interaction with κ-casein hindering the coagulation. It should be avoided in cheese manufacture (Chandan, 2007).

In recent years, novel thermal processing technologies have been widely investigated with a view to substitute conventional heating in dairy processing, such as ohmic heating, microwave and radio frequency heating. Advantages offered by these techniques are related to the generation of heat within dairy product (Cheng & Sun, 2015). It is possible to rapidly reduce the processing temperature and times for pasteurization and sterilization, as significant reduction of the microorganisms in milk. However, major obstacles that prevent application of these technologies in the dairy industry are lack of generalized information or validation procedures for the safety of treated dairy foods, higher capital and operating costs associated with process equipment and lack of data on the properties needed for proper designs of process and equipment. The lack of predictive models relating the electrical properties of foods to

transient time–temperature profiles (that determine product quality and food safety) has been another major obstacle in the development of these technologies (Mishra & Ramchandran, 2015; Salazar-González, San Martín-González, López-Malo, & Sosa-Morales, 2012).

1.1.2.2 Non-thermal treatment on milk and dairy product

Conventional non-thermal treatments mainly include high-hydrostatic pressure (HHP) treatment and ultra-high pressure homogenization (UHPH), among others for instance pulsed electric fields (PEF).

High-hydrostatic pressure applications on milk and dairy products between 300 and 600 MPa have shown to be an effective method to inactivate microorganisms including most infectious food-borne pathogens. Furthermore, the pressure-induced dissociation of the colloidal calcium phosphate and denaturation of serum proteins in milk may change and/or improve its technological properties (Trujillo, Capellas, Saldo, Gervilla, & Guamis, 2002). It has also been reported that it improves rennet or acid coagulation of milk without harmful effects on important quality characteristics, such as taste, flavor, and nutrients. These characteristics offer the dairy industry numerous practical applications to produce microbial safe, minimally processed products with improved performances, and to develop novel dairy products of better nutritional and sensory quality, novel texture and increased shelf-life (Trujillo et al., 2002).

The resistance of microorganisms to pressure in food is very variable depending on HHP processing conditions such as pressure, time, temperature and cycles, food constituents and the properties and the physiological state of the microorganism. The bacterial spores can survive at pressure of 1000 MPa. However, they can often be stimulated to germinate by pressures between 50-300 MPa. Germinated spores then could be killed by heat or mild pressure treatments. Gram-positive microorganisms tend to be more resistant to pressure than gram-negative microorganisms. Gram-positive microorganisms require the application of 500-600 MPa at 25 °C during 10 min to achieve inactivation, while gram-negative microorganisms are inactivated with

treatments of 300-400 MPa at 25 °C during 10 min (Smelt, 1998). Many studies on the inactivation of pathogenic and spoilage microorganisms (naturally present or inoculated) by HHP have been performed in milk during the last years and have generally demonstrated that it is possible to obtain 'raw' milk pressurized at 400-600 MPa with a microbiological quality comparable to that of pasteurized (72 °C, 15 s) milk depending on the microbiological quality of milk. However, it is not promise to sterilized milk due to HHP resistant spores (Buffa, Guamis, Royo, & Trujillo, 2001; Mussa & Ramaswamy, 1997; Trujillo et al., 2002). In addition, a number of researchers have investigated the efficacy of HHP in combination with mild temperatures (30-50 °C) and/or with bacteriocins for the inhibition of foodborne bacteria and spores, pointing that some of these combinations substantially enhance the efficiency of HHP treatment, even sometimes showing phenomenon of synergistic inactivation between HHP treatments and natural antimicrobial substances (Alpas & Bozoglu, 2000; García-Graells, Masschalck, & Michiels, 1999; Trujillo et al., 2002). Unlike thermal treatments, where covalent and non-covalent bonds are affected during the processing, HHP treatment at room and mild temperatures only disrupts relatively weak chemical bonds (hydrogen, hydrophobic, or ionic bonds). Hence, small molecules such as vitamins, amino acids, simple sugars and flavor compounds remain unaffected by the HHP treatment (Cheftel, 1992; Trujillo et al., 2002).

Regarding the two most abundant whey proteins, HHP treatment at pressures >100 MPa denatures β-Lg, while at pressures >400 MPa denatures α-La. Most denatured β-Lg in HP-treated milk associates with the casein micelles, although some denatured β-Lg remains in the serum phase or is attached to the milk fat globule membrane; HP-denatured α-La is also associated with the milk fat globules (Huppertz, Fox, & Kelly, 2004a). Casein micelles are disrupted on treatment at pressures >200 MPa; the rate and extent of micellar disruption increases with pressure and is probably due to the increased solubility of calcium phosphate with increasing pressure. On prolonged treatment at 250-300 MPa, re-association of micellar fragments occurs through hydrophobic bonding; this process does not occur at a pressure >300 MPa, leading to

considerably smaller micelles in such milk. As a result of HP-induced changes, the size, number, hydration, composition and light-scattering properties of casein micelles in HP-treated milk differ considerably from those in untreated milk (García-Risco, Olano, Ramos, & López-Fandiño, 2000; Huppertz et al., 2004a; Patel & Huppertz, 2014).

UHPH treatment is a processing technology generated by impact forces, cavitation, turbulence and shear, which combined produce destruction of microorganisms and the reduction of particle size, allowing the obtention of a physically stable product with longer shelf-life. More information is in section 1.2.

There are also novel nonthermal preservation technologies for dairy applications in recent years other than HHP and UHPH, such as pulsed electric field (PEF), ultraviolet light and ultrasounds. Research in recent years has shown that PEF processing has the potential for pasteurization of milk with improved preservation of its nutrient content and freshness. It has achieved a reduction in the microflora of milk with a shelf life similar to that of HTST pasteurized milk. It also has the potential for usage in the pasteurization of dairy beverages such as juice–milk products, etc. A combined process of thermal and PEF treatments has been suggested as an effective and energy saving strategy for the industrial pasteurization of milk. Thus, PEF processing in combination with HTST considerably extends the shelf life of refrigerated milk and may be suitable for transporting milk to distant markets (Sampedro & Rodrigo, 2015). Potential applications of UV technology in the dairy industry include: reduction of on-farm spoilage in high bacterial content milk; treatment of milk fed to calves to lower the risk of infectious diseases; reduction of bacteria not susceptible to thermal treatment; and reduction of psychrotrophic bacteria in refrigerated milk stored for extended periods. The main reasons for lack of exploitation of UV technology in milk stream processing are: low penetration of UV light into milk, and flavor impairment in milk. Further research in these areas will help develop UV technology into a cost effective and potentially viable alternative to thermal pasteurization for the dairy industry (Datta, Harimurugan, & Palombo, 2015). Applications involving the use of ultrasound for cleaning have been described in the dairy industry. Ultrasound may also

be of interest as a processing tool for the inactivation of bacteria. It may also be used effectively as a homogenization technology, yielding very fine emulsions but at very high-energy consumption (Abismail, Canselier, Wilhelm, Delmas, & Gourdon, 1999; Behrend, Ax, & Schubert, 2000; Jafari, He, & Bhandari, 2007; Wu, Hulbert, & Mount, 2000). Hurdle treatments combining ultrasound, elevated temperature, and pressure (manothermosonication) have been found to yield stronger gel structures (Vercet, Oria, Marquina, Crelier, & Lopez-Buesa, 2002). The production of hydroxyl radicals by ultrasonic treatment may induce cross-linking of food components (Almeida et al., 2008).

1.2 Ultra-high pressure homogenization treatment on milk and dairy products

1.2.1 Principles of Ultra-High Pressure Homogenization

On passing through a high-pressure homogenizer, milk encounter a number of interlinked and simultaneous physical phenomena, including high velocity, shear, collision of particles, turbulence, and rapid increases and decreases in pressure and accompanying cavitation. The strong acceleration results in elongational flow, which is probably the main mechanism of disruption of milk fat globule droplets. Velocity is further increased when the fluid accelerates into the gap between the valve and the valve seat and pressure suddenly drops and the fluid may, at pressures of 300 MPa, reach a velocity in the region of 200 m/s (Floury, Bellettre, Legrand, & Desrumaux, 2004; Floury, Legrand, & Desrumaux, 2004). These high velocities make the residence time in the homogenizing valve extremely short. During passage through the valve, high degrees of turbulence and cavitation can occur (Tobin, Heffernan, Mulvihill, Huppertz, & Kelly, 2015). Temperature increase is also observed when milk is high-pressure homogenized. Temperatures of about 65, 84 or 103 ℃ have been reported downstream of the homogenizing valve when milk at an initial inlet temperature of 40 ℃ was homogenized at 100, 200 or 300 MPa, respectively (Pereda, Ferragut, Quevedo, Guamis, & Trujillo, 2007).

1.2.2 Effects of Ultra-High Pressure Homogenization on milk fat globules and protein

When milk pass through the valve of a high-pressure homogenizer, fat globules encounter three processes simultaneously: deformation and disruption, adsorption of surface active material at the newly formed droplet interface, collision and possible re-coalescence of globules (Tobin et al., 2015).

Cow raw milk contains 3-5% fat globules with a range from 0.4 to 15 μm in diameter. These fat globules are surrounded by the MFGM (See also section 1.1.1.1 milk protein), which consists of phospholipids, proteins and neutral lipids, that protects the fat globules against flocculation and coalescence, dispersed in a continuous skimmed milk phase (Mulvihill & Ennis, 2003). Disruption of fat globules upon homogenization provokes a reduction of their size and a concomitant increase in their specific surface area (Zamora, Ferragut, Guamis, & Trujillo, 2012). The size distribution of particles in raw milk is characterized by a main peak at 3.8 μm and a second lower peak around 0.2 μm, which correspond to fat globules and casein micelle particles. Upon UHPH, these main peaks merge into a single peak between 0.1 and 0.3 μm depending on certain conditions of temperature and pressure. UHPH yielded significantly smaller fat globules than conventional homogenization (Hayes & Kelly, 2003; Zamora, Ferragut, Jaramillo, Guamis, & Trujillo, 2007).

To stabilize this surface, milk proteins, principally caseins, are absorbed, although whey proteins have also been found on the newly-formed fat surface (Dalgleish & Robson, 1985; Oortwijn & Walstra, 1979; Sharma & Dalgleish, 1993). This rearrangement of some of the milk protein has been found to affect the processing capabilities of milk (Hayes & Kelly, 2003).

Casein micelles are reported to be partially disrupted by UHPH at ~200 MPa (Hayes & Kelly, 2003; Sandra & Dalgleish, 2007). However, compared to fat globule, casein micelles are much less altered by UHPH. It is reported that casein particle size is reduced only about 10-15% in skimmed milk treated at 186 MPa by UHPH (Sandra & Dalgleish, 2005). It is likely that reductions in micelle size are the result of physical

disruption of casein micelles, because the conditions would not allow significant solubilization of micellar calcium phosphate, which governs micellar disruption at high hydrostatic pressures. UHPH of milk at pressures > 250 MPa can cause increases in casein micelle size. In the presence of calcium, considerable aggregation of casein micelles can occur during UHPH at > 200 MPa (Roach & Harte, 2008).

UHPH can also cause whey protein denaturation, the extent of which increases with increasing homogenization pressure. UHPH of skim milk at an inlet temperature of 30 ℃ , a primary stage pressure of 300 MPa and a secondary stage pressure of 30 MPa resulted in ~ 45% denaturation of β-lactoglobulin and ~ 30% denaturation of α-lactalbumin (Pereda, Ferragut, Quevedo, Guamis, & Trujillo, 2009; Serra, Trujillo, Quevedo, Guamis, & Ferragut, 2007). Whey protein denaturation during UHPH is mainly due to thermal effects encountered during the process. The extent of UHPH-induced whey protein denaturation in whole milk appears to be similar to that observed for skimmed milk (Datta, Hayes, Deeth, & Kelly, 2005; Hayes, Fox, & Kelly, 2005).

In the meantime, denaturation exposes hydrophobic amino acid residues in the protein, which had been previously protected by the protein. The exposed hydrophobic amino acids decrease the entropy of the aqueous phase surrounding them, making it favorable for surface adsorption. Hence, UHPH could cause interactions between MFGM components and whey proteins and/or caseins. The interactions between MFGM and caseins might be through adsorption of casein micelles (indirect adsorption) at the fat globule surface or through direct adsorption and/or bonding of the protein molecules (Zamora et al., 2012). β-Lg and α-La associated with MFGM proteins via disulfide bonds during the heat treatment of whole milk (Ye, Singh, Taylor, & Anema, 2004a). In UHPH-treated milk, casein micelles were discovered to be adsorbed on the MFGM in a lesser extent than in conventional homogenization-pasteurization. However, greater adsorption of directly bonded casein molecules, could be released by UHPH through the partial disruption of casein micelles, which was observed especially at high UHPH pressures. Adsorption of whey proteins on the MFGM of conventionally homogenized-pasteurized milk was mainly through intermolecular disulfide bonds with

MFGM material, whereas in UHPH-treated milk, disulfide bonding with both indirectly and directly adsorbed caseins was also involved (Zamora et al., 2012). In addition, β-Lg contains 5 cysteine residues, four of which are covalently bonded to each other, forming two disulfide bonds. When β-Lg is denatured, the fifth cysteine residue is exposed. This residue then bonds to other β-Lg proteins, including those already adsorbed to the surface (Nakanishi, Sakiyama, & Imamura, 2001).

In addition, UHPH also enhances the enzymatically induced coagulation properties of milk, and such improvement was attributed to changes at the protein-fat structures. The fat content of milk was proven to greatly influence the protein-protein interactions within rennet curds from UHPH-treated milk. UHPH triggered textural changes in fresh cheeses, such as, higher firmness, lower deformability and higher water retention, which could be explained by the reduction of fat globule size and the incorporation of caseins and whey proteins at MFGM (Zamora et al., 2012, 2007; Zamora, Ferragut, Juan, Guamis, & Trujillo, 2011). The increase in interfacial fat surface leads to the adsorption of casein and whey proteins to the newly formed fat globules. Hence, very small fat globules behave as casein micelles rather than embedded fat globules as observed in confocal micrographs. Such structures do not interfere with casein network and do not act as spacers, disrupting the network; and small fat globules covered by β-casein can act as building elements on caseinate gels. It would improve gel firmness and rate of aggregation by increasing the amount of particle associations (Trujillo, Roig-Sagués, Zamora, & Ferragut, 2016).

1.2.3 Effects of Ultra-High Pressure Homogenization on microorganisms in milk

Bacterial cells are strongly affected by the shear, cavitation, temperature and pressure occurring during UHPH, and inactivation of a wide range of bacterial species can thus be achieved by this process. UHPH-induced inactivation of bacteria increases with increasing pressure, temperature and fat content of the medium (Diels, Callewaert, Wuytack, Masschalck, & Michiels, 2004, 2005; Diels & Michiels, 2006; Pereda et al., 2007; Julieta Pereda, Ferragut, Quevedo, Guamis, & Trujillo, 2008). Roig-Sagués et al. (2009) found that milk fat content had a stronger effect on obtaining a higher death rate

of *Listeria monocytogenes,* which could be attributed to the increase of the maximum temperature reached during UHPH treatment induced by fat content, therefore enhanced the lethal effect. Moreover, no germination of bacterial spores was triggered by UHPH (Georget et al., 2014). Total bacterial counts of milk were decreased significantly by 63.3% and 88.8% respectively, for milk samples treated at 150 and 200 MPa. (Hayes & Kelly, 2003). Pereda et al. (2007) reported that the UHPH treatment performed at 300 MPa at 30 °C could be a good option to be used as a one-step homogenization and pasteurization treatment to produce commercial milk with a microbial and physicochemical shelf life equal to that of high-pasteurized milk (90 °C, 15 s) and probably with reduced heat effects due to the short holding time occurred during treatment. Gram-positive bacteria are more resistant to UHPH-induced inactivation than Gram-negative bacteria (Donsì, Ferrari, Lenza, & Maresca, 2009). Diels et al. (2004, 2005) reported that the viscosity of the medium, rather than its composition, is the crucial factor affecting the degree of bacterial inactivation by UHPH. In low viscosity media, bacterial cells are easily disrupted as a result of deformation under the forces encountered but, if viscosity is high, deformation, and hence disruption and inactivation, of bacterial cells is much more limited (Tobin et al., 2015). Because an increase in fluid viscosity will inhibit cavitation by suppressing under pressures caused by vortices. Increasing fluid viscosity will also decrease cavitation collapse pressures by dampening high localized fluid velocities. A final possible mechanism of cell disruption during high-pressure homogenization is based on the stress that cells experience in the high-stress zones close to the surface of the impingement wall, when the stress exceeds the mechanical cell wall resistance (Diels & Michiels, 2006).

1.2.4 Opportunities of Ultra-High Pressure Homogenization in the food industry

The benefits of UHPH include inactivation of microorganisms, which extents shelf-life and improvements in functionality due to increased emulsion capacity and stability, with minimal losses on nutritional value and sensory characteristics. Several European research projects have recently supported the studies of UHPH for food applications, including dairy products, vegetable milk, and fruit juices. These projects

focused on the evaluation of the effects of UHPH on various of food components, with a particular emphasis on control of enzymes and physical functionality. UHPH is a sustainable process, which has potential application in a large number of liquid foods, including milk (e.g. cow, goat) vegetal beverages (e.g. soy, rice, and almond), fruit juices (e.g. orange, apple, and grape), and to manufacture food products (yoghurt, ice-cream, and cheese, etc.) as a novel process for the production of a wide variety of safe and nutritious foods (Zamora & Guamis, 2014).

Several researchers have reported that combinations of sufficiently high homogenization pressure and inlet temperature, for example > 150 MPa and > 40 ℃ , resulted in degrees of microbial inactivation that are comparable to those achieved during HTST (High Temperature Short Time) pasteurization processes commonly applied for the production of milk for consumption. The refrigerated shelf life of such high-pressure-homogenized milk is comparable to that of traditionally pasteurized/homogenized milk (Hayes et al., 2005; Pereda et al., 2007; Picart et al., 2006; Smiddy, Martin, Huppertz, & Kelly, 2007).

1.3 Fluorescence spectroscopy

Fluorescence spectroscopy is an optical, rapid, and non-invasive technique of electromagnetic spectroscopy in which fluorescence of a sample is analyzed. It carries a beam of light in UV (ultra violet) or vis (visible) region, which is used to excite a molecules' electron from its ground state to a higher electronic state by absorbing a photon. On returning to the ground state, possibly via an intermediate state, excited electrons emit light of certain wavelengths, longer than the wavelengths of the excitation light, called fluorescence. The excitation and emission spectra are characteristics of each molecule, thereby enabling the analysis of the sample (Zettel et al., 2016).

1.3.1 Front-face fluorescence spectroscopy

The classical right angle technique is used to analysis diluted or transparent samples. When the absorbance of the sample is not higher than 0.05, the intensity of

the emitted fluorescence is proportional to the concentration of fluorophore. Thus, it is standard practice to dilute the original sample with an appropriate solvent until an absorbance lower than 0.05 has been obtained. Nevertheless, results obtained on diluted samples are not always comparable to those obtained with an original sample (Strasburg & Ludescher, 1995). To overcome this problem and examine native samples directly, the front-face fluorescence spectroscopy (*FFFS*) is much more suitable (Figure 1-1).

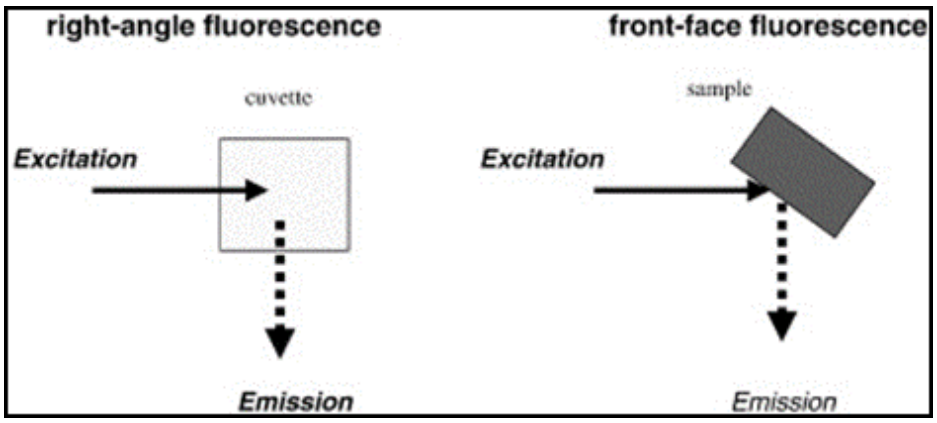


Figure 1-1 Principles of right-angle fluorescence and front-face fluorescence (Dufour, Devaux, Fortier, & Herbert, 2001)

The main difference from conventional methodology is the incidence angle. This angle is defined as the angle formed between the excitation beam and the perpendicular to the illuminated surface of the cell. It is normally around 30° in the front-face technique. The emission is measured at 90° in relation to the excitation beam. Thus, excitation of the sample and measurement of its emitted radiation are carried out in the same cell-face. Hence, it avoids the passage of radiation through the bulk solution, allowing even to study the fluorescence of solid samples. In this way, reflected light, scattered radiation and depolarization phenomena are minimized (Airado-Rodríguez, Durán-Merás, Galeano-Díaz, & Wold, 2011).

1.3.2 Potential of front-face fluorescence spectroscopy in food science

Food is a complex system composed predominantly of water, fat, proteins and carbohydrates together with various kinds of minor components. The functional properties of these components, which are determined by their molecular structure, intra- and intermolecular interactions within the food system, with different amounts

present, defines the unique characteristics of different food products. The theory and methodology of fluorescence spectroscopy have been widely exploited for studies of molecular structure and function. It is therefore applicable to food science since many foods exhibit auto-fluorescence, and it is considered highly relevant for characterization purposes and high-throughput screening. The main advantages of fluorescence spectroscopy are its sensitivity and selectivity, besides its ease of use, instrumental versatility, speed of analysis and its non-destructive character (Airado-Rodríguez et al., 2011).

The demand for high quality and safety in food production obviously demands for high standards for quality and process control, which certainly requires appropriate analytical tools to investigate food (Cheng & Sun, 2015; Nawrocka & Lamorska, 2013). So far, *FFFS* has just, recently, become quite popular as a tool in biological science related to food technology. It is usually used as a quality assessment technique in food system for online measurements of process parameters and the optimization and control of the whole processes (Zettel et al., 2016). It can measure different types of fluorophores, which are present in the food system, such as aromatic amino acids (tryptophan, tyrosine, and phenylalanine), vitamins (A and B_2, pyridoxine, and its derivatives), coenzymes (NADH, FMN, and FAD), and chlorophyll, which are present in food samples (Figure 1-2). In addition, the use of autofluorescent markers in combination with FFF configurations, allow for analysis of intact samples.

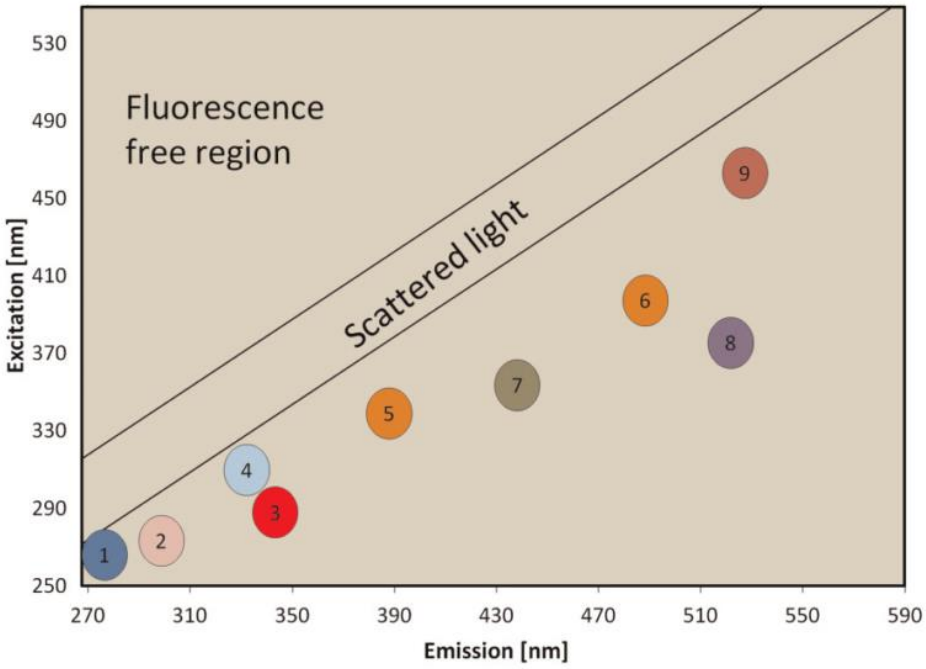


Figure 1-2 2D fluorescence spectrum showing different fluorophores: (1) phenylalanine, (2) tyrosine, (3) tryptophan, (4) Vitamin E, (5 and 6) pyridoxine and its derivatives, (7) NADH, (8 and 9) FMN, FAD and riboflavin. (Zettel et al., 2016)

As fluorescence spectra are comprised of large numbers of excitation and emission wavelength matrices, *FFFS* in combination with multivariate data analysis can be a powerful tool to extract the information to describe the structural, nutritional, and technological parameters of food systems and to calibrate and predict the model parameters for quality control, online process monitoring and optimization of processes (Airado-Rodríguez et al., 2011; Ayala, Zamora, González, Saldo, & Castillo, 2016; Kamal & Karoui, 2016; Kulmyrzaev, Levieux, & Dufour, 2005).

Chapter 2

Interest of Study, objectives and working plan

2. Interest of study, objectives and working plan

2.1 Background

UHPH is under study by several research teams all over the world. It affects not only the particle size but also other properties of the fluids processed by this technology. In the food industry, the combination of high pressure, temperature, shear force, cavitation and impingement can improve the chemical and physical stability, microbial safety and even sensory characteristics with minimal effects on the nutritional value. By treatment on raw materials, it can also enhance the quality of solid products derived from the UHPH treated fluids, such as cheese, yogurt, and soy flour (Patrignani & Lanciotti, 2016).

During the last decade, with the support of national and EU projects, the research on UHPH technology processing has been developed within the CERPTA-UAB research group. From 2004, the EU CRAFT project "UHPH 512626, Development and Optimization of a Continuous Ultra High Pressure Homogenizer for Application on Milks and Vegetable Milks" opened the research in this technology that was continued with some other EU and National funded projects aimed to study the UHPH technology for the development of safe foods (killing the pathogenic microorganisms, absence of toxic or carcinogenic substances, etc.) and to increase the working pressure. From 2008, a new EU project FUNENTECH 232603 (study of functionality, nutritional and safety aspects of liquid foods, liquid food preparations, and cosmetics processed by ultra-high-pressure homogenization) had as objective to reinforce the UHPH technology transfer processing to small and medium enterprises (SME) of liquid food and cosmetic sectors (Zamora & Guamis, 2014).

From 2012, national research project AGL2012-33957 of the Spanish Ministerio de Economía y Competitividad by European Regional Development Fund (ERDF/FEDER) supports the research of front-face fluorescence spectrum technique on thermal and high pressure treated milk. This research work is a part of the AGL2012-33957 project "Application of native fluorescence tracers for quick quantification of

milk damage during milk processing.

2.2 Interest of study

The application of UHPH treatment, which is considered as a non-thermal technique, could avoid majority of the thermal damages. However, when milk is subjected to UHPH treatments, functional, nutritional and organoleptic changes can occur, with their extent depending on treatment intensity.

In order to evaluate the damage of milk, a number of chemical indicators or tracers have been proposed. Unfortunately, the analytical methods involved are typically off-line, laborious, expensive, and/or very time consuming. Thus, optical methods such as florescence spectroscopy have shown potential as alternative, rapid method for quantification of fluorophores naturally occurring in milk that might be affected by UHPH treatments. Figure 2-1 summarizes the excitation and emission maxima of fluorophores in milk and dairy products. As it can be observed, the practical excitation ranges between 250 and 450 nm, while the emission ranges between 300 and 799 nm.

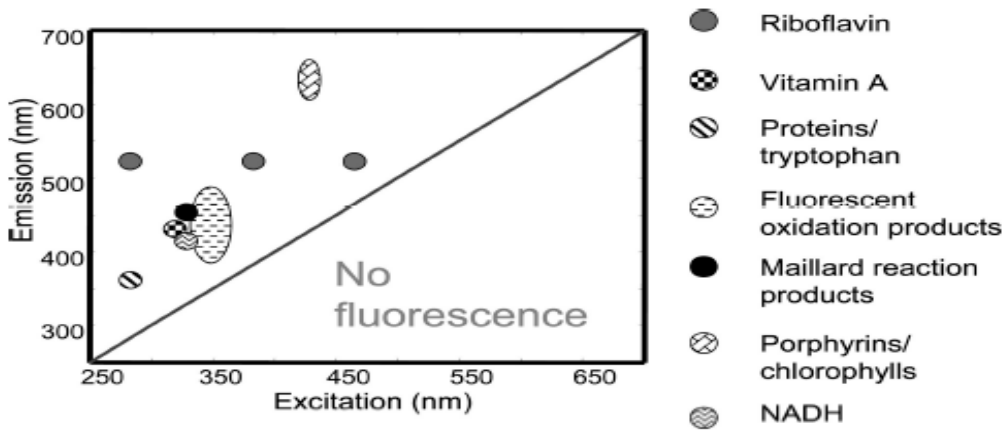


Figure 2-1 Excitation and emission maxima of fluorophores present in dairy products (Andersen & Mortensen, 2008).

Recent development of front-face fluorescence, which allows direct measurement of turbid samples, represents a unique opportunity for developing this technology as a rapid inline/at line method for quantification of damage fluorescence markers in milk. Early inline/at line detection of milk damage will not only allow classifying milk

batches to be used for their most suitable purposes but will reduce the impact of damage in milk. The impact this would have not only in organoleptic/nutritional quality and consistency of dairy foods but also on assurance of food safety and regulatory requirements, in addition to the non-existence of a simple, inexpensive, accurate, universal and rapid milk damage measurement method, motivates this research.

2.3 Objectives

The presented research work is framed within the national research project AGL2012-33957 entitled "Application of native fluorescence tracers for quick quantification of milk damage during milk processing".

The overall goal of this research was to study the application of native fluorescence tracers for quick prediction/quantification of milk damage during UHPH treatment by *FFFS*.

Specific objectives and methodological approach:

1. Setting up laboratory systems for fluorescence and physical-chemical measurement of thermal and UHPH processed milk samples.

2. Developing the fluorescence predictive equations for quantification of retinol in thermal treated milk.

3. Developing the fluorescence predictive equations for quantification of high pressure damage tracers in a skim milk model system.

4. Developing the fluorescence predictive equations for quantification of high pressure damage tracers in a whole milk model system.

5. Validating the model system predictive equations for processed milk.

2.4 Working plan

Since there were relatively few references about using *FFFS* on UHPH-treated milk, and assorted with the entire project, the overall work plan was divided into two

steps. The first step was to establish the *FFFS* model of retinol for thermal treated milk. In this step, raw whole milk samples were treated under three different temperatures in different time lengths. Then, measuring the retinol concentration by HPLC and fluorescent indicators by *FFFS* to establish the prediction model (Figure 2-2).

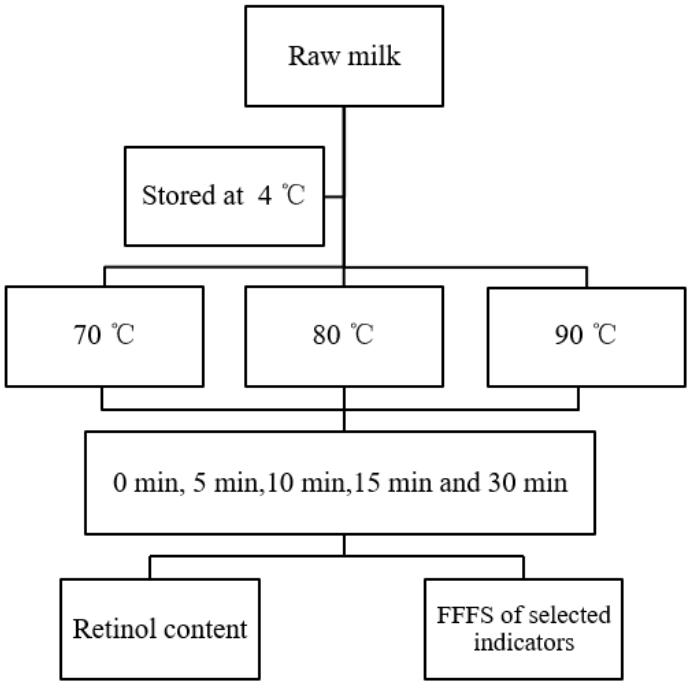


Figure 2-2 Experimental flow chart of thermal processing

The second step was to investigate the changes in fluorescence properties induced by UHPH treatment on both skim milk and whole milk. In addition, establishing the retinol prediction model in whole milk (Figure 2-3 & 2-4).

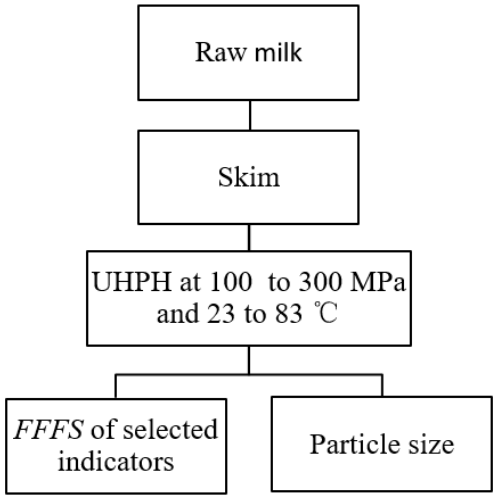


Figure 2-3 Experimental flow chart of UHPH processing on skim milk

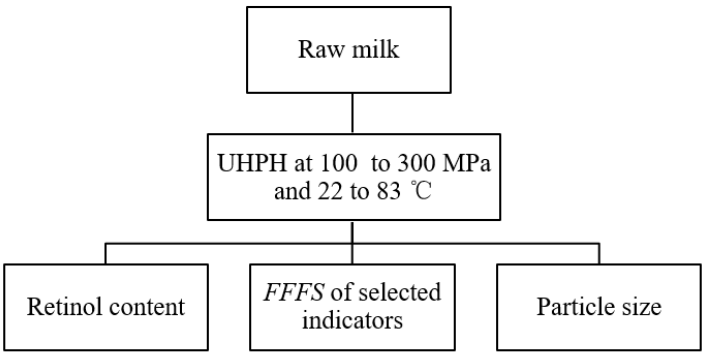


Figure 2-4 Experimental flow chart of UHPH processing on whole milk

Chapter 3

Materials and methods

3. Materials and methods

3.1 Materials

3.1.1 Milk supply

Fresh raw bovine milk was obtained from a local producer (Can Badó, la Roca del Vallès, Spain) to perform this study. It was stored at 4 °C in a refrigerator at the Servei de Planta de Tecnologia dels Aliments (SPTA) of Universitat Autònoma de Barcelona until it was used.

3.1.2 Reagents and solvents

Ethyl acetate, methanol, absolute ethanol, hexane and tetrahydrofuran (HPLC grade), ascorbic acid, standards of retinol, and δ-tocopherol, pyrogallol (purity >98 %), ethylenediaminetetraacetic acid (EDTA) and potassium hydroxide (KOH) were purchased from Sigma-Aldrich (Sigma-Aldrich Quimica,S.L., Madrid, Spain).

3.2 Methods

3.2.1 Thermal treatment

A factorial experimental design with two factors, temperature and time, and three replicates was used to heat-treat the milk samples under the following conditions: three levels of temperatures 70, 80, and 90 °C with five time levels 0, 5, 10, 15 and 30 min in a thermostatic bath (OvanTherm TC00E C, Lovango SL). One different batch of milk was used per replication and the heat treatments were applied to 45 samples in total.

A 20 mL aliquot for each sample of raw milk was filled in a 25 mL glass test tube and capped. A digital thermometer was used to monitor the internal sample temperature reached inside the test tubes to the predetermined temperature. At the different treatment time intervals indicated by the experimental design, samples were removed from the bath and placed on iced-water immediately.

3.2.2 Retinol quantification

The retinol quantification method used was based on that proposed by Salo-Väänänen et al. (2000). Milk samples were held at 40 °C for 10 min in a water bath before 1 mL was transferred to a light-protected test tube. Then, 20 µL of internal standard stock solution (δ-tocopherol, 1000 mg/L) was added to the milk sample before saponification.

After an addition of 4 mL ethanol, 0.5 g of pyrogallol and ascorbic acid, the oxygen from headspace was removed with a nitrogen flow for 10 min. Thereafter, 0.5 mL of saturated EDTA and 0.5 mL KOH (50%) were added and sample vortexed for 30 s. Further, the sample was transferred to a boiling water bath for 20 min. Last, the sample was left on ice to cool down.

The extraction was carried out by addition of 2 mL milli-Q water and 2 mL organic extraction solution (ethyl acetate: hexane, 20:80) followed by 10 min mechanical shaking. After this, the sample was centrifuged at 2,000×g for 10 min. The supernatant organic phase was transferred to another tube and held at 40 °C. The extraction step was then repeated twice without water addition. The combined supernatant was evaporated with nitrogen and the dry residue was dissolved in 500 µL of methanol:ethanol (20:80) and filtered (Polyvinyldene fluoride, 0.2 µm) prior to HPLC analysis.

The analytical column employed was a Waters Sunfire C18 (Waters, Milford, Ireland) of 4.6 mm × 150 mm and particle diameter 3.5 µm. Mobile phase A was prepared by dissolution of methanol in milli-Q water (97:3). The mobile phase B was the mixture of methanol and tetrahydrofuran (90:10). The elution was achieved with a linear gradient from 0 to 100 % of mobile phase B at flow rate of 1 mL/min and oven temperature of 25 °C. Standards for control of absorbance and calibration were daily prepared at the concentration of 30 mg/L and the spectrometry wavelengths were set at 325 and 298 nm. Retention times were around 3.70-3.90 min and 10.37-10.43 min for retinol and internal standard δ-tocopherol, respectively (recovery rate > 95 %).

3.2.3 UHPH treatment

Ultra-high pressure homogenization was performed by a benchtop ultra-high pressure homogenizer (Model: FPG12500 Stansted Fluid Power Ltd., UK). This device (flow rate of 15 L/h) is provided with two intensifiers, driven by a hydraulic pump and a high-pressure ceramic valve able to support 350 MPa. Inlet and outlet temperature of milk were controlled and monitored by a water bath located before the equipment entrance and another one located after the high-pressure valve. A central composite design was applied to both skim and whole milk as Table 3-1. The 13 conditions were run randomly. The actual operated conditions slightly differed from the coded values due to mechanical UHPH limitations. As a result, current experimental operation conditions were recorded and all the results were analyzed with the recorded actual values in chapter 5&6.

Table 3-1 The central composite design for UHPH treatment

No.	Variables		Coded Variables	
	Pressure (MPa)	Inlet Temperature (℃)	X1	X2
1	200	78	0	+1.414
2	130	70	-1	+1
3	270	70	+1	+1
4	100	50	-1.414	0
5	200	50	0	0
6	200	50	0	0
7	200	50	0	0
8	200	50	0	0
9	200	50	0	0
10	300	50	+1.414	0
11	130	30	-1	-1
12	270	30	+1	-1
13	200	22	0	-1.414

3.2.4 Fluorescence measurement

The 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 adjusted to an angle of incidence of 35°.

The dityrosine fluorescence (F_{Dt}) was scanned at excitation of 315 nm and emission between 350 and 500 nm and was read at 315/425 nm (Ex/Em). For Maillard compounds (F_{MC}) were read at 330/420 nm (Ex/Em) and excitation wavelength of 330 nm and emission range of 350-500 nm were used. For tryptophan (F_{Trp}), the wavelength used for excitation was 290 nm while for emission was 300-450 nm and was read at 290/340 nm (Ex/Em). Riboflavin was excited at 370 and 450 nm (F_{Rb370} and F_{Rb450}, respectively) the emission wavelength range was 470-570 nm and read at 370/530 nm and 450/530 nm (Ex/Em). The fluorescence spectra including retinol (F_{Re}) information was scanned at excitation between 260 and 350 nm and emission at 410 nm. All determinations were performed in triplicate.

3.2.5 Particle size distribution

The particle size distribution in milk samples was determined by Mastersizer 2000 from Matgas, UAB. The dispersant was milliQ water and the milk samples were diluted in it to obtain the appropriate laser obscuration. According to Mie theory of light scattering by spherical particles, the conditions were selected as real refractive index = 1.471; refractive index of fluid (water) = 1.332; imaginary refractive index = 0; pump speed = 21%. The size distribution was characterized by the diameter below which 50 or 90% of the volume of particles are found (D_{50} and D_{90}, respectively), the Sauter diameter (surface-weighted mean diameter, D[3,2]), and the volume-weighted mean diameter (D[4,3]) value. Additionally, the Sauter diameter of small particles (<1 μm) (surface-weighted mean diameter,) and the volume-weighted mean diameter of small particles were calculated as well, which were defined as d[3,2] and d[4,3] respectively. Each sample was measured in triplicate.

3.2.6 Statistical analysis

All data from thermal treatment were analyzed using the software “Statistical Analysis System” (SAS, version 9.4,). To study the variability of the dependent variables for the effects on retinol loss and changes in fluorescence properties, factorial

analysis of variance (ANOVA) was performed. Differences between least square means of the various treatments were considered significant when $P < 0.05$. The activation energy (*E*a) of retinol thermal degradation reaction estimation was processed by two methods. Method 1 calculated the activation energy from the slopes of linear regression between the natural logarithm of the kinetic rate constant, k, and 1/T (i.e., linearized Arrhenius equation). Method 2 used the nonlinear regression procedure (PROC NLIN) of SAS. Pearson correlation coefficients were obtained by the correlation matrices procedure (PROC CORR) and to optimize the prediction model with fluorescent markers, maximum R^2 procedure (PROC REG) of SAS was conducted.

The experiment design of UHPH on both skim and whole milk was a central composite response surface design, which is a fractional factorial design with 5 center points, augmented with a group of axial points. Results were analyzed and validated by STATISTICA (StatSoft, Inc. (2004), version 7). The principal components analysis was performed by STATISTICA as well. Evaluations were based on a significant level of $P < 0.05$.

Particle size was analyzed by Mastersizer 2000 (Malvern Application 5.61, Malvern Instruments Ltd.)

Chapter 4

Using front-face fluorescence spectroscopy for prediction of retinol loss in milk during thermal processing

(Published at *LWT-food science and technology*, 87 (2018), 151–157)

4. Using front-face fluorescence spectroscopy for prediction of retinol loss in milk during thermal processing

4.1 Results and discussion

4.1.1 Analysis of variance of retinol

In earlier studies, some researchers considered retinol as a heat-resistant compound (Andersson, Öste, & Fox, 1995; Burton, 1994). Reports on retinol stability are sometimes contradictory and depend on the analytical method involved in the separation of the isomers. Some authors reported no significant retinol losses in pasteurized and UHT milks (Le Maguer & Jackson, 1983). On the contrary, other authors reported significant losses in pasteurized, UHT and in-container sterilized milk samples using chromatographic methods, which could make the separation of all the isomers (Panfili et al., 1998). The present study also confirmed that the thermal treatment significantly influenced the retinol concentration in milk (Table 4-1). The ANOVA results suggested that time, temperature and their interaction in heat treatment have significant influence on the retinol losses. The degradation behavior of retinol shows a functional relationship with temperature and time as reported by Han et al. (2007). Moreover, the influence of replicates within the three independent experiments was significant. The reason was that initial concentration of retinol in raw milk was different due to collecting time, feeding system and even cow's individual condition and capacity. Several authors reported retinol in raw bovine milk at 0.2-2 mg/L (Bylund, 1995), 0.280-0.466 mg/L (Jensen, 1995a), 0.40 mg/L (Almeida et al., 2008) and 0.40 mg/L (Medrano, Hernandez, & Prodanov, 1994). Thus, the results obtained from the three independent experiments were within the normal variance range and the variances were all taken into consideration to ensure the prediction models could precisely and widely predict retinol irrespective of vitamin source variations.

Table 4-1 Retinol concentration in raw bovine milk after thermal treatment[1]

Code	Time (min)	Temperature (℃)	Retinol Concentration (mg/L)*
1	0	70	0.431±0.022[a]
2	5	70	0.405±0.031[a,b]
3	10	70	0.397±0.033[b]
4	15	70	0.377±0.038[b,c]
5	30	70	0.356±0.043[c,d]
6	0	80	0.431±0.022[a]
7	5	80	0.364±0.032[c,d]
8	10	80	0.343±0.026[d,e]
9	15	80	0.318±0.032[e,f]
10	30	80	0.293±0.037[f,g]
11	0	90	0.431±0.022[a]
12	5	90	0.340±0.049[d,e]
13	10	90	0.312±0.038[f]
14	15	90	0.282±0.031[g]
15	30	90	0.249±0.015[h]

[1]Values are means ± SD (n = 3 independent experiments).

a–h Different superscript letters within a column indicate significant differences ($P < 0.05$).

4.1.2 Kinetic models of retinol degradation

Typically, degradation behavior follows a specific pattern depending on the kinetics of the chemical reaction. The relationship between the reaction rate and time determines the order of the reaction (Tydeman & Kirkwood, 1984). In the present study, the retinol concentration was considered as a function of heating times to estimate the reaction at each temperature (70, 80 and 90 °C).

Retinol loss during thermal treatments (from 70 to 90 °C) showed good fitting to both first-order and second-order reaction equations, irrespectively of each temperature considered. This could be attributed to the fact that the destruction level was less than

50%. This is as expected when small percentages of degradation are evaluated. In these cases, the observed tendency might not be as clear as it might be for a larger percentage of vitamin destruction. However, it should be noted that the 50% destruction of vitamin evaluated in our study is adequate. Fox (1995) reported that overall losses of vitamin A activity during anaerobic sterilization may vary from 5 to 50%, depending on the temperature and time involved and the nature of the carotenoids. Hence, a degradation of vitamin A much higher than 50% is neither typical nor expectable during industrial thermal treatment of milk. Using a different source of retinol, Wilkinson et al. (1981) and Laohasongkram et al. (1995) reported a retinol loss in heated beef liver (103-127 °C) and in pork liver puree (80-130 °C), respectively, that could be described as first-order kinetics. So does the vitamin A degradation in enteral formula during storage (Baéz, Rojas, Sandoval-Guillén, & Ángeles Valdivia-López, 2012). Considering the available information reported in the literature and the results obtained in our study, the kinetics of the retinol degradation process was assumed to correspond to a first-order reaction. The fitting results and residuals plot are shown as Figures 4-1 and 4-2.

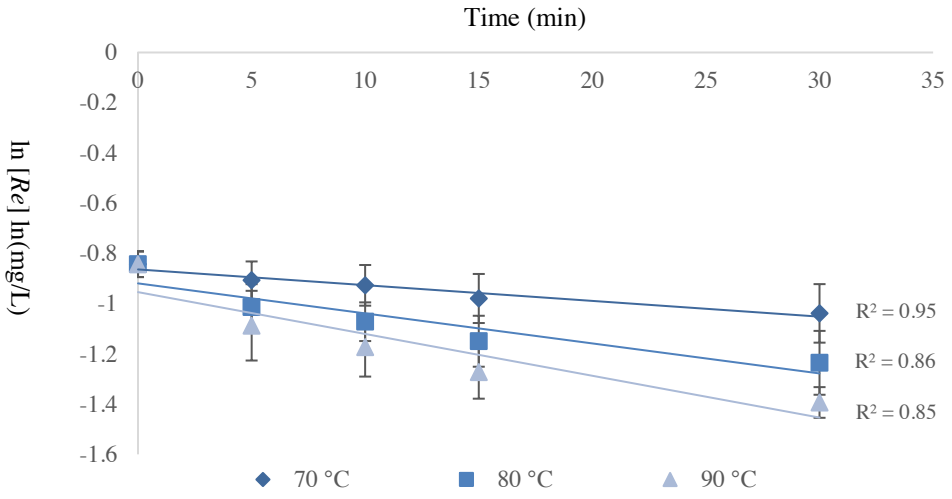


Figure 4-1 Fit of the experimental retinol concentration data to first-order kinetics

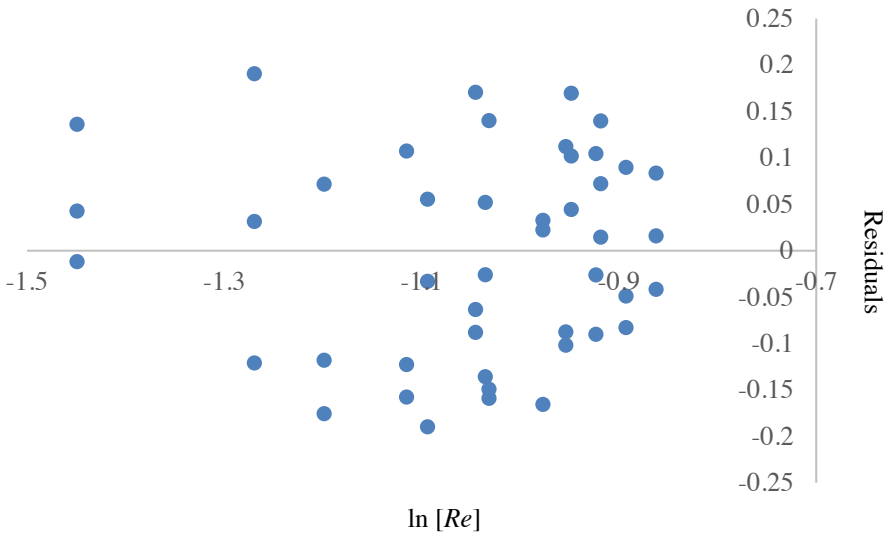


Figure 4-2 Residuals of the experimental retinol concentration data fitting to first-order kinetics (mg/L)

To estimate the activation energy, taking the natural logarithm of Arrhenius equation yields:

$$\ln\ k_T = \ln\ A - \frac{E_a}{R}\frac{1}{T} \qquad \text{Eq 4-1.}$$

where

A = Arrhenius equation collision factor for retinol (mg/min),

Ea = activation energy for the formation of retinol (kJ/mol),

R = universal ideal gas constant (kJ/mol·K),

T = reaction temperature (K),

k_T = reaction rate of corresponding temperature.

Arrhenius equation could be linearized to Eq 4-1 to allow linear regression to estimate Ea from t rate constant of reaction k at different temperatures (Method 1). Furthermore, there is another estimation of Ea (Method 2), which is solving Eq 4-1, using nonlinear estimation. The comparison of the results from these two methods is shown in Table 4-2. And both of them are lower than 112 kJ/mol reported by Wilkinson et al. (1981), 96±4 kJ/mol reported by Laohasongkram et al. (1995) and higher than 43.388 kJ/mol reported by Baéz et al. (2012).

Table 4-2 Comparison of estimation of retinol degradation activation energy using two different fitting methods[1]

Method	Equation	E_a	SEP
1	$\ln\ k_T = \ln\ A - \frac{E_a}{R}\frac{1}{T}$	52.26	0.195
2	$\ln [Re] = \ln [Re]_0 + Ae^{-\frac{E_a}{RT}} \cdot t$	50.75	0.237

[1] $[Re]$, concentration of retinol (mg/L); $[Re]_0$, initial concentration of retinol (mg/L); k_T, rate constant of reaction at temperature T; A, Arrhenius equation collision factor for retinol (mg/min); E_a, activation energy for the degradation of retinol (kJ/mol); R, universal ideal gas constant (kJ/mol·K); T, reaction temperature (K). SEP, standard error of prediction (kJ/mol); Method 1, results were calculated from slopes of the natural logarithm of k_T versus 1/T; Method 2, results were estimated by non-linear regression.

Comparing the standard error of prediction values, method 1 are slightly smaller as compared to method 2. However, the estimated activation energy values from Method 1&2 are close. This could be explained by the fact that the rate constant k used in method 1 resulted from the adjustment of the experimental data to the different kinetic equations of retinol concentration at the three evaluated temperatures. Briefly, it was an estimated value. Thus, when it was used to do further estimations, the degree of freedom diminished. Meanwhile, the method 2 estimated by the raw database thus all deviations were included. The method 1 was widely used in kinetic researches to study the correlation between the ln k and 1/T (Nikolaidis & Moschakis, 2017; Sánchez, Mateo, Fernández, & Nolasco, 2017; Trapani et al., 2017). But its disadvantage is when T values are less than three, the reliability would decrease a lot due to the deviation. In addition, method 2 could also be applied to experimental design with each sample obtained from different temperatures.

4.1.3 Correlation between the retinol concentration and the fluorescent markers

In statistics, the Pearson product-moment correlation coefficient (r) is a measure of the linear correlation between two variables. The evaluation of correlations between

retinol concentration and fluorescent markers was used as a preliminary approach to identify fluorescent markers having more potential as predictors.

Table 4-3 Pearson product-moment correlation coefficients (r)[1]

	[*Re*]	F_{MC}	F_{Trp}	F_{Dt}	F_{Rb370}
F_{MC}	-0.9419**	-	-	-	-
F_{Trp}	-0.9343**	0.8599**	-	-	-
F_{Dt}	-0.8520**	0.9432**	0.7885**	-	-
F_{Rb370}	-0.8464**	0.8055**	0.8895**	0.8568**	-
F_{Rb450}	-0.7088*	0.7099**	0.6830	0.8323**	0.9108**

[1]All the shown coefficients are significant, $p<0.05$; *$p<0.005$, **$p<0.0005$; [*Re*], concentration of retinol (mg/L); F_{Trp}, F_{MC}, F_{Dt}, F_{Rb370}, F_{Rb450}, maximum fluorescence intensity of tryptophan, Maillard compounds, dityrosine and riboflavin (excited at 370 nm and 450 nm); r is calculated by the maximum fluorescence intensity of the emission spectral scans of each fluorescent markers.

The correlation matrix (Table 4-3) shows statistically significant negative correlations between retinol concentration and the maximum fluorescence of all the studied markers. During the thermal treatment, globular milk proteins denaturalize, which involves changes in the secondary and tertiary structure and Maillard reaction between amino acids (mainly lysine) and reducing sugars takes place. Therefore, with heat treatment more tryptophan is exposed to the surface due to protein unfolding and more dityrosine and Maillard compounds are generated in the processing. Tryptophan fluorescence and Maillard compounds fluorescence increased in milk after thermal treatments (Commercial pasteurization, UHT and microwave heating) were also observed by Tu et al. (2014), Feinberg et al. (2006), Birlouez-Aragon et al. (1998) and Henle et al. (1991). But it is worth noting that the tryptophan fluorescence decreased after 10 min heat treatment at 90 °C， also found by other researchers (Ayala, Zamora, Saldo, & Castillo, 2015). Ayala et al. (2016) also reported a significant negative correlation between lactulose formation and tryptophan fluorescence during thermal

treatment in reconstructed skim milk. In addition, Scheidegger et al. (2013) verified the increase of dityrosine in milk powder production process. The most significantly correlated fluorescent markers with retinol concentration are tryptophan and Maillard compound with r = -0.93 and r = -0.94, respectively. And the least correlated with retinol loss is riboflavin fluorescence intensity with excitation at 450 nm and emission at 530 nm with r = -0.71.

4.1.4 Models for predicting the concentration of retinol by front-face fluorescence

Several retinol concentration prediction models were established from the different fluorescent markers maximum intensities (F_{max}), using retinol concentration [*Re*] as dependent variable and selected fluorescence markers and its transformations (possible ratios and products among predictors, the square, reciprocal and natural logarithm of each predictor) as independent variables. To the end, following the method Maximum R^2 of SAS, the best prediction one-, two- and three-variable prediction models were generated using the predictors indicated above (Table 4-4). The optimized model is model III with an R^2 of 0.87 and standard error of prediction of $2.47 \cdot 10^{-2}$ mg/L and the residuals are randomly distributed. The plots of fit diagnostics of model III are shown as Figures 4-3 and 4-4.

Table 4-4 Prediction models of retinol concentration in milk during thermal treatment by fluorescence markers[1]

	Model	β_0	β_1	β_2	β_3	R^2	SEP
I$^{\#}$	$[Re]= \beta_0+\beta_1 F_{Trp} \cdot F_{MC}$	$9.735 \cdot 10^{-1}$$^{\#}$	$-3.756 \cdot 10^{-5}$$^{\#}$	-	-	0.82	$2.82 \cdot 10^{-2}$
II$^{\#}$	$[Re]= \beta_0+\beta_1 F_{Trp} \cdot F_{MC} +\beta_2 F_{Rb370}^2$	1.054$^{\#}$	$-3.806 \cdot 10^{-5}$$^{\#}$	$-4.187 \cdot 10^{-5}$	-	0.83	$2.75 \cdot 10^{-2}$
III$^{\#}$	$[Re]= \beta_0+\beta_1 F_{Trp}/F_{MC}+\beta_2 F_{Trp}/F_{Rb370} +\beta_3 F_{MC}^2$	1.868$^{\#}$	-1.469$^{\#}$	0.121*	$-3.352 \cdot 10^{-5}$$^{\#}$	0.87	$2.47 \cdot 10^{-2}$

[1]N=45; *p<0.01; $^{\#}$p<0.0001; [*Re*], concentration of retinol (mg/L); F_{Trp}, F_{MC}, F_{Rb370}, maximum fluorescence intensity of tryptophan, Maillard compounds and riboflavin (excited at 370 nm); SEP, standard error of prediction (mg/L).

In addition, leave-one-out exhaustive cross-validation was conducted to check the

validity of model III. The model worked for cow milk and for the range of common thermal treatments, that is, from thermization up to in-container sterilization. The correlation coefficient obtained was 0.92 and the root mean square error of cross-validation (RMSECV) was 5.37 %.

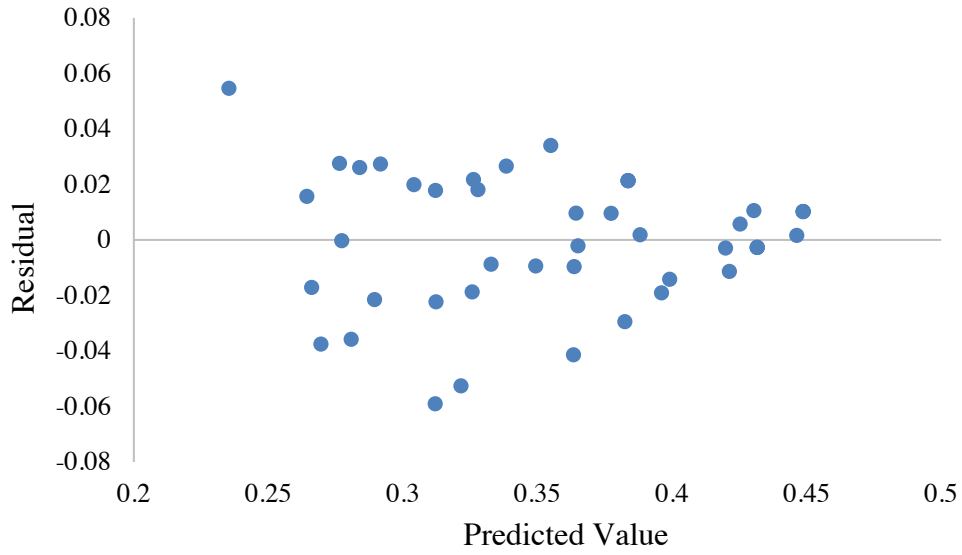


Figure 4-3 Residual plot of Model III (mg/L)

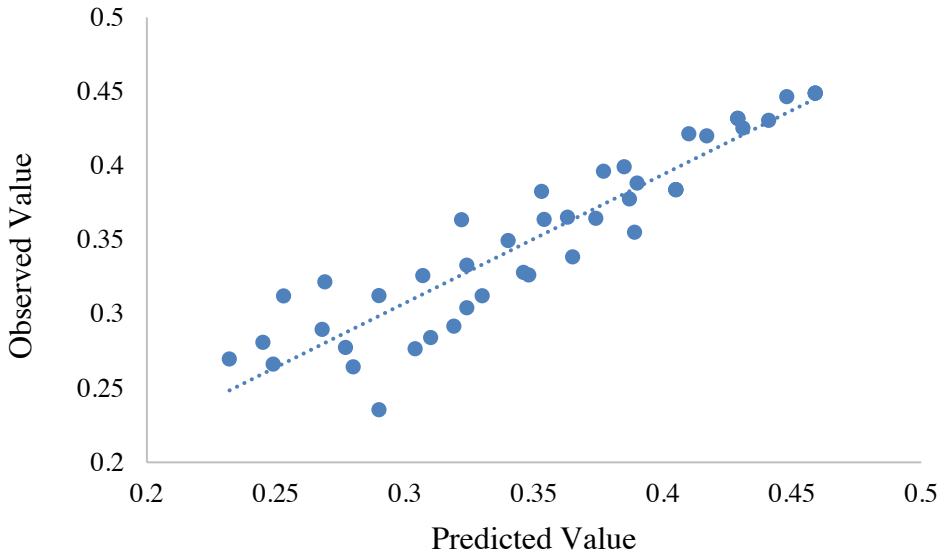


Figure 4-4 Regression between observed and predicted retinol value of model III (mg/L)

As it is shown in Table 4-3, all the five fluorophores (*Trp*, *MC*, *Dt*, Rb_{370} and Rb_{450}) are highly correlated with retinol concentration, which provides the possibility of substitution in data processing as potential indicators. This phenomena was also found

by Ayala et al. (2016) in the kinetic study of lactulose formation in heat-treated reconstituted skim milk powder using *FFFS*. So far, only using *FFFS* of Maillard compounds and tryptophan to monitor and predict nutritional impact on dairy samples has some published researches. Models were established to estimate the deterioration of the milk quality during thermal treatment. Tessier et al. (2006) found that a fast index (F_{MC}/F_{Trp}) of Malliard compounds (excitation of 330 nm and emission of 420 nm) and tryptophan (excitation of 290 nm and emission of 340 nm) could be used as a simple tool to examine the nutritional quality of microwave pasteurization milk. While, Diezet al. (2008) verified the potential of front face fluorescence of tryptophan (excitation of 290 nm and emission of 340 nm) and of Maillard products (excitation of 340 nm and emission of 420 nm) associated to partial least squares (PLS) regression to predict nutritional parameters in heat-treated infant formula. In present study, comparing model I and model II, variable F_{Rb370}^2 is added to improve the fitting but it is not significant. Besides, the variable F_{Trp}/F_{Rb370} in model III is not as significant as F_{Trp}/F_{MC} and F_{MC}^2 as well. It confirms the results in section 4.1.3 that the most significantly related fluorescent markers are tryptophan and Maillard compound, although it also suggests that the ratio between tryptophan and riboflavin fluorescence (excited at 370 nm) is also containing some complementary information.

Thermal treatment is an accelerating process to induce the inevitable retinol degradation in milk. Besides, *FFFS* was found to be the one of most sensitive methods for distinguishing the heat treatment of milk (Schamberger & Labuza, 2006). All-trans-retinol is the main form of retinol with potential as vitamin A (Weiser & Somorjai, 1992). Thermal treatment is one of the reasons that could cause retinol degradation in food. In raw milks, which are not subjected to thermal processing, there was no conversion of the predominant all-trans-isomers to cis-isomers in samples from various species (Panfili et al., 1998). Hence, the prediction model with *FFFS* for retinol is a breakthrough of quick nutritional quantification and evaluation. Similar work was also done in the lactulose formation in skim reconstructed milk. A prediction model generated with several fluorescent markers for lactulose under 90 °C treatment showed good applicability (Ayala et al., 2016). Other technique to predict retinol in milk such

as near infrared reflectance spectroscopy (NIRS) was also carried out. Between 1100 and 2498 nm, it was seen that it is possible to use NIR for routine analysis of retinol allowing to known the nutritional value of ewe's milk and identify the season of the year (winter-summer) (Revilla, Escuredo, González-Martín, & Palacios, 2017). But prediction models for dairy manufacture processing with NIRS such as thermal treatment still lacks relevant research.

4.2 Conclusion

The kinetics of retinol degradation in raw bovine milk was evaluated in this study. Reaction rate constant, k, of different heating temperatures (70, 80, and 90 °C) were obtained. Additionally, activation energy was estimated and compared using two fitting methods. It was observed that the reaction fitted best a first order kinetics. Moreover, using selected fluorescent markers in milk as predictors, four prediction models of retinol concentration were established with one to four variables. The models were established on results generated by combination of three different heat temperatures and five treating times by *FFFS*. The optimized model was model III, which used tryptophan, Maillard compound and riboflavin (excited at 370 nm) fluorescence. The proposed method indicates that front-face fluorescence, as a relatively low-cost, rapid, direct and non-destructive technique, has the potential to replace existing conventional analytical techniques practiced for retinol evaluation in heat treated milk by further improvement with inline sensor.

Chapter 5

A rapid method for monitoring the changes caused by ultra-high pressure homogenization treatment on bovine skim milk by front-face fluorescence

5. A rapid method for monitoring the changes caused by ultra-high pressure homogenization treatment on bovine skim milk by front-face fluorescence

5.1 Results

5.1.1 Temperature increase in valve during UHPH treatments

In UHPH treatment, the sample temperature reaches the desired inlet temperature by passing through the heat exchanger. Then the sample temperature increases further in the valve, which could be attributed to two reasons: one is the adiabatic heating as a result of the pressure exerted at the pressure intensifiers and the other is the high turbulence as well as the shear and cavitation forces that are produced through the valve, as a result of the release of pressure (Amador-Espejo, Suàrez-Berencia, Juan, Bárcenas, & Trujillo, 2014; Datta et al., 2005; Donsì et al., 2009; M. Hayes & Kelly, 2003). Although the valve temperature could not be controlled, it could be monitored on the screen of the equipment. The valve temperature was found corresponds to a linear regression with pressure and inlet temperature as Eq 5-1(R^2=0.98, $P < 0.0005$).

$$VT = 0.181 \cdot P + 0.440 \cdot InT + 34.720 \quad \text{Eq 5-1}$$

where P = Pressure (MPa), InT = Inlet Temperature (°C), and VT = Valve Temperature (°C).

Therefore, the valve temperature increase rate could be estimated at 18.1 °C/ 100 MPa. Several other authors using UHPH equipment with a two-stage valve design (Stansted Fluid Power Ltd.) also reported similar results. Pereda et al. (2007) obtained an increase of 19.5°C/ 100 MPa in UHPH treatments of 200 and 300 MPa at 40 °C. And Thiebaud et al. (2003) presented an 18.5 °C/ 100 MPa increase, using 100 to 300 MPa at 4, 14, and 24 °C.

5.1.2 Effect of UHPH treatment on tryptophan fluorescence

Tryptophan is one of the essential amino acids and its indole functional group absorbs strongly in the near ultraviolet part of the spectrum. Moreover, tryptophan as

an aromatic amino acid, contains a phenyl ring in its molecular structure. Due to the conjugation effect, after being excited, tryptophan molecular is prone to release energy to back to the ground state through emitting fluorescence. The measurement of tryptophan fluorescence can provide information about the molecular environment of proteins in non-diluted emulsion (Rampon et al., 2003). Therefore, tryptophan is a suitable fluorescent indicator in this research.

The results of milk tryptophan fluorescence affected by UHPH processing showed in Table 5-1&5-2 and the 3D response surface and contour plots showed as Figure 5-1.

The *P*-values of the model and lack-of-fit showed significant fitting of the tryptophan fluorescence to the response surface. However, it was noticed that the square and 2-way interaction factors were not statistically significant. It could be attributed to the highly correlation of valve temperature with pressure and inlet temperature. The multicollinearity phenomenon does not reduce the predictive power or reliability of the model as a whole, but coefficients can seem to be insignificant even when a significant relationship exists between the predictor and the response, or it may not give valid results about which predictors are redundant with respect to others. Hence, in order to reduce excessive variables (P, InT, VT, square of each of them and 2-way interactions) in model, a forward stepwise regression was applied. The regression equation was obtained Eq. 2 with R^2 of 0.99 ($P < 0.05$).

$$F_{Trp} = 2.666 \cdot InT + 8.982 \cdot VT - 0.071 \cdot InT^2 - 0.013 \cdot P \cdot VT + 386.200 \qquad \text{Eq 5-2}$$

F_{Trp} --- Maximum tryptophan fluorescence intensity (a.u.)

To validate Eq 5-2, cross-validation was performed. The coefficient of variation obtained was 1.42% and the root mean square error of cross-validation (RMSECV) was 17.23. It was noticed that, although VT and P·VT were significant in Eq 5-2, they were significant in only one replicate of the 13 replicates cross-validation in total. It

Table 5-4 The CCD design and results for UHPH processing[1]

No.	Pressure (MPa)	Inlet Temperature (°C)	Valve Temperature (°C)	F_{trp}	F_{MC}	F_{Dt}
1	200	51	93	936.9±13.0^{b,c}	73.1±0.5^{d}	74.2±1.1^{d}
2	300	51	113	931.2±19.1^{b,c}	69.5±0.8^{f}	70.7±0.8^{f}
3	200	83	105	800.1±11.0^{f}	136.7±0.9^{a}	114.9±0.7^{a}
4	130	72	91	878.7±8.7^{d}	130.1±0.8^{b}	111.6±0.3^{b}
5	200	51	96	967.0±14.3^{a}	73.9±0.6^{d}	74.9±0.9^{d}
6	100	51	75	918.3±31.7^{c}	72.7±2.0^{d}	73.4±2.2^{d,e}
7	130	34	74	931.2±6.5^{b,c}	72.5±0.378^{d}	73.5±0.2^{d,e}
8	200	23	80	931.5±8.9^{b,c}	73.5±0.2^{d}	74.8±0.2^{d}
9	270	72	116	852.4±14.5^{e}	125.1±1.5^{c}	107.6±1.6^{c}
10	200	51	92	932.8±20.9^{b,c}	70.5±1.0^{e,f}	72.0±0.9^{e,f}
11	200	51	92	941.1±6.9^{b,c}	72.2±0.7^{d,e}	73.3±0.8^{d,e}
12	270	34	97	938.7±5.5^{b,c}	70.4±0.2^{e,f}	72.0±0.2^{e,f}
13	200	51	96	951.5±11.4^{a,b}	74.0±0.3^{d}	74.8±0.7^{d}

[1]F_{Trp}, F_{MC}, F_{Dt}, maximum fluorescence intensity of tryptophan, Maillard compounds and dityrosine, respectively.

a–f Different superscript letters within a column indicate significant differences ($P < 0.05$).

Table 5-2 Variance anlysis of the CCD model for UHPH processing[1]

		F_{Trp}		F_{MC}		F_{Dt}	
Source	DF	F-Value	P-Value	F-Value	P-Value	F-Value	P-Value
Model	9	51.96	0.004	260.69	0.000	293.54	0.000
Linear	3	29.35	0.010	190.94	0.001	216.53	0.001
Pressure	1	10.69	0.047	6.14	0.089	9.28	0.056
Inlet Temperature	1	35.54	0.009	31.07	0.011	31.48	0.011
Valve Temperature	1	11.43	0.043	4.50	0.124	6.83	0.079
Square	3	0.91	0.528	23.67	0.014	29.07	0.010
Pressure*Pressure	1	0.19	0.691	2.78	0.194	3.26	0.169
Inlet Temperature*Inlet Temperature	1	0.15	0.725	10.08	0.050	12.17	0.040
Valve Temperature*Valve Temperature	1	0.36	0.592	6.21	0.088	7.19	0.075
2-way Interaction	3	0.59	0.662	42.53	0.006	52.55	0.004
Pressure*Inlet Temperature	1	0.26	0.646	6.68	0.081	7.93	0.067
Pressure*Valve Temperature	1	0.29	0.627	4.41	0.127	5.11	0.109
Inlet Temperature*Valve Temperature	1	0.37	0.587	9.35	0.055	11.04	0.045
Error	3						
Lack-of-Fit	1	0.09	0.790	11.86	0.075	6.43	0.127

[1]DF, degree of freedom; F_{trp}, F_{MC}, F_{Dt}, Maximum fluorescence intensities of tryptophan, Maillard compounds and dityrosine, respectively.

suggests the temperature influence more on tryptophan explosion and destruction than pressure.

5.1.3 Effect of UHPH treatment on dityrosine fluorescence

Tyrosine is an important amino acid in milk and tyrosyl radicals may couple to yield dityrosine due to the treatment being applied or during storage (Figure 5-2). Since ditryosine is a natural intrinsic component, it could provide structural and functional information on the cross-linked protein molecule as a fluorescence probe (Malencik & Anderson, 2003).

A forward stepwise regression was applied and the multiple regression equation for dityrosine was obtained ($R^2 = 0.92$, $P < 0.005$).

$$F_{Dt} = 0.025 \cdot InT^2 - 1.793 \cdot InT + 102.622 \quad \text{Eq 5-3}$$

F_{Dt} --- Maximum dityrosine fluorescence intensity (a.u.)

To validate Eq 5-3, ross-validation was performed and the coefficient of variation obtained was 6.24% while the RMSECV was 8.74.

Unlike the result of tryptophan, pressure and valve temperature did not significantly influence the response fitting of dityrosine fluorescence, which in agreement with Eq 5-3 where pressure and valve temperature did not appear in the regression. In the meantime, Figure 5-1 confirmed that fluorescence changes at pressure axis were very limited.

Unlike the result of tryptophan, pressure and valve temperature did not significantly influence the response fitting of dityrosine fluorescence. In the meantime, Figure 5-1 confirmed that fluorescence changes at pressure axis were very limited. This fact was also confirmed by the non-significant P-values of those two factors observed in the ANOVA analysis (Table 3).

5.1.4 Effect of UHPH treatment on Maillard compounds fluorescence

Maillard reaction is a chemical reaction between amino acids and reducing sugars. In the case of milk, lactose reacts with the free amino acid side chains of milk proteins (mainly lysine residues) to proceed to early, intermediate, and advanced stages of

Maillard reaction and forms enormous kinds of Maillard reaction products. The Maillard reaction shows various effects on milk proteins such as bioavailability, solubility, foaming property, emulsifying property, and heating stability (Shimamura & Ukeda, 2012).

As section 5.1.2 and 5.1.3, the response equation of Maillard compounds is shown as Eq 5-4 with an R^2 of 0.92 ($P < 0.005$).

$$F_{MC} = 0.038 \cdot InT^2 - 2.707 \cdot InT + 115.793 \quad \text{Eq 5-4}$$

F_{MC} --- Maximum Maillard compounds fluorescence intensity (a.u.)

To validate Eq 5-4, cross-validation was performed. The coefficient of variation obtained was 8.77% and the RMSECV was 12.85.

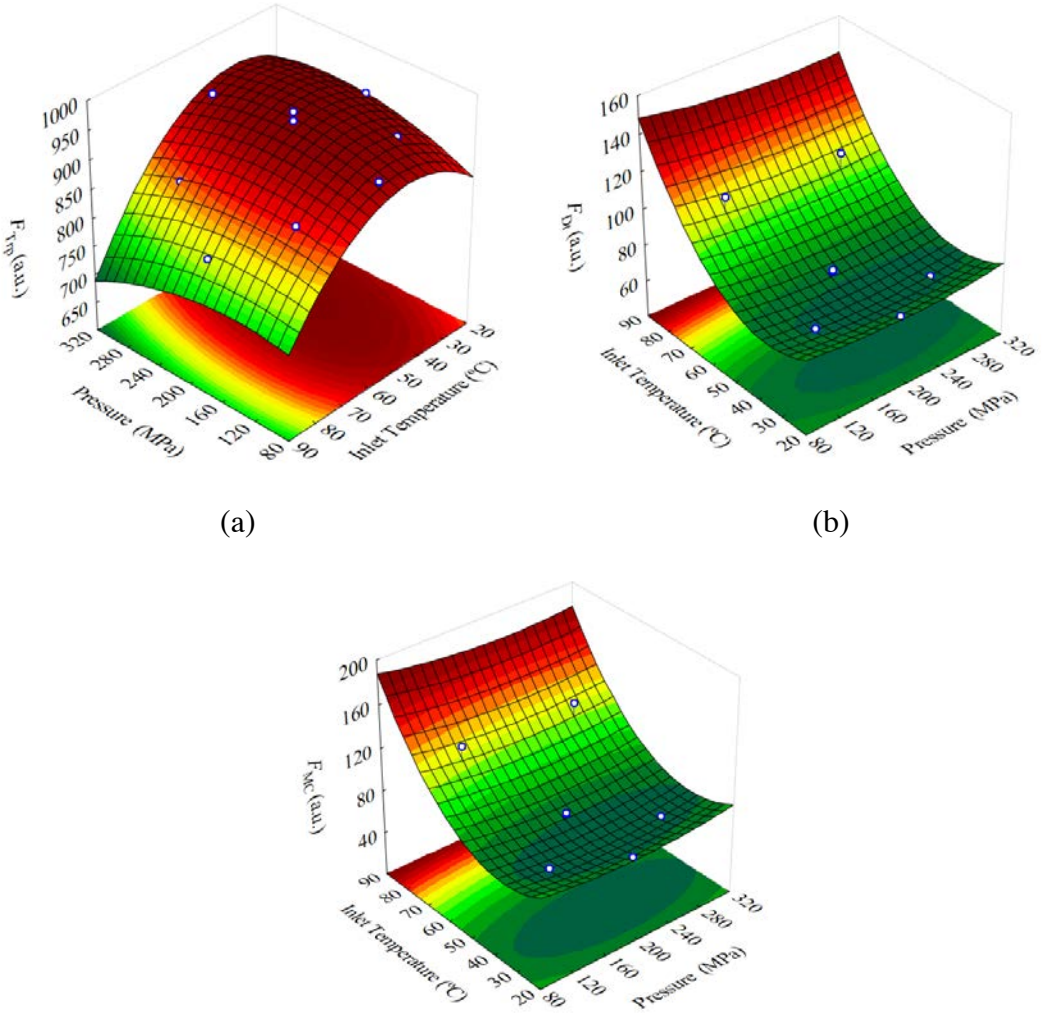
Similar to dityrosine fluorescence, inlet temperature is much more significant than other factors in the case of Maillard compounds fluorescence, which is in line with expectations as a product of thermal phenomena (Morales, 1996).

5.1.5 Prediction of UHPH treatment inlet temperature by a rapid fluorescence method

It is found that both dityrosine and Maillard compounds fluorescence could be described by a parabola of inlet temperature (Eq 5-3 and 5-4). However, it could be easily observed that there were no significant changes between about 20 to 50 °C (Figure 5-1 (b)&(c)), which means almost no accumulation of dityrosine and Maillard intermediate compounds. It could be assumed no thermal effect on milk by UHPH treatment when inlet temperature is lower than 50 °C. Thus, for rapid prediction, it is more practical to focus on the processing above 50 °C. In this case, inlet temperature corresponds to a linear regression with F_{Dt} and F_{MC} as Eq. 5-5 ($R^2 = 0.98$, SEP = 0.94 °C and $P < 0.05$).

$$InT = -3.082 \cdot F_{Dt} + 2.438 \cdot F_{MC} - 100.641 \quad \text{Eq 5-5}$$

Theoretically, pressure could be predicted by solving Eq 5-1 and Eq 5-5 into Eq 5-2. However, Table 5-1 and Figure 5-1 have shown that pressure lacks of significance upon the evaluated fluorophores. The derived equation has large SEP value. So far, the prediction of pressure by F_{Trp}, F_{Dt} and F_{MC} could not be achieved.



(a) (b)

(c)

Figure 5-1 Response surface of tryptophan(a), dityrosine(b) and Maillard compounds (c) in UHPH treatment as a function of pressure and inlet temperature

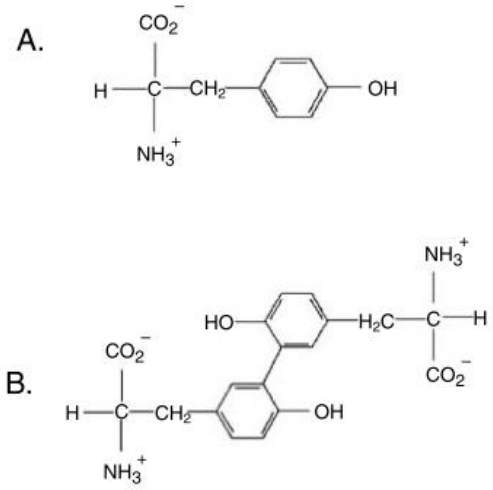


Figure 5-2 Structure of tyrosine (A) and dityrosine (B)

5.2 Discussion

According to Thiebaud et al. (2003), UHPH treatments at a pressure of 200 MPa and inlet temperature of 24 °C caused total bacterial count reductions similar to those of pasteurization processes (30 min at 63 °C or equivalent time–temperature combination). Pereda et al. (2007) discovered that the treatment performed at 300 MPa at an inlet temperature of 30 °C could obtain milk with a microbial and physicochemical shelf-life equal to that of high-pasteurized milk (90°C for 15 s). Amador-Espejo et al. (2014) reported with UHPH treatments performed at 300 MPa with InT = 75 and 85 °C produced samples without microbial growth equivalent to commercial sterilization with similar or better characteristics than UHT milk regarding to color, particle size, viscosity, buffer capacity, ethanol stability, propensity to protein hydrolysis and off-flavor. In this study, we found that there were no significant increases of dityrosine and fluorescent Maillard compounds amounts below 50 °C with pressure from 100 to 300 MPa. That means that the thermal damages could be avoided in pasteurized milk produced by UHPH. Even with the method of Amador-Espejo et al. (2014), the valve temperature was lower and duration time was shorter than conventional UHT processing. Hence, the obtained fluorescence models have great industrial application potential for industry usage for monitoring and rapid prediction in producing pasteurized-like and UHT-like UHPH milk.

During the UHPH treatment, the milk protein structure is modified by pressure and interactions of pressure and temperature. Since pressure treatment could cause direct changes in the conformation of proteins, tertiary and quaternary structure, the exposure of amino acids on the peptide chains change as well, which lead to the change of fluorescence properties.

Intrinsic fluorescence of milk proteins is caused by the three aromatic amino acids: tryptophan, tyrosine, and phenylalanine. Among these three amino acids, tryptophan dominates the major fluorescence emission mainly due to its large extinction coefficient. Thus, it is the fluorescence of this amino acid that provides most information about protein structure. The fluorescence depends on how tryptophan is exposed in the three-

dimensional configuration of the proteins (Andersen & Mortensen, 2008). The UHPH has a small effect on the diameters of casein micelles, because of the loss of some of their surface κ-casein (Sandra & Dalgleish, 2007). Since tryptophan is a non-polar amino acid, to maintain a stable protein structure, most tryptophan sites tend to be buried inside of the hydrophobic core of casein micelles and whey protein. The loss of surface κ-casein on micelle particles and whey protein denaturation would inevitably lead to more tryptophan residues exposure, thereby increasing the fluorescence intensity. Furthermore, the fluorescence intensity decrease observed at high inlet temperature (Figure 1, temperatures above ~40-50 °C) and high homogenization pressure, is assumed to be related to protein oligomerization and aggregation. Interactions between β-lactoglobulin (β-Lg) and casein micelles increase with the extent of heat treatment, and heat can cause α-lactalbumin (α-La)–β-Lg interaction after β-Lg–casein complex formation (Guyomarc'h, Law, & Dalgleish, 2003; Sandra & Dalgleish, 2005). UHPH could cause the casein micelle partial disintegration and/or reorganization and may cause modification in β-Lg binding sites. It has been reported that static high-pressure treatment of raw milk causes denaturation of α-La and β-Lg at pressures greater than 400 and 100 MPa, respectively (Huppertz, Fox, & Kelly, 2004b). Whey protein denaturation was found to increase with increasing pressure, time, and temperature, and the denatured β-Lg formed a complex with casein micelles, giving an increase in casein micelle size at pressures around 250 MPa and micelle size would also increase because of aggregation of the caseins (Garcıa-Risco, Ramos, & López-Fandiño, 2002; Huppertz, Fox, & Kelly, 2004a).

Huang and Kuo (2015) reported that vegetable proteins in soymilk could be denatured using 150 MPa UHPH treatment as evidenced by the changes in protein surface hydrophobicity and sulfhydryl contents. In bovine whole milk, Hayes et al. (2005) treating milk at 250 MPa suggested that the high turbulence, shear and cavitation forces experienced by milk during UHPH denatured β-Lactoglobulin. Moreover, Pereda et al. (2009) found a degree of denaturation of 10-17% and 32–37% for α-Lactalbumin and β-Lactoglobulin, respectively, by UHPH treatment at pressure and temperature ranging from 200-300 MPa and 30-40 °C. They also reported that

denaturation of β-Lactoglobulin was more important than that of α-Lactalbumin, and that β-Lactoglobulin was more drastically denatured by the conditions used in the thermal treatment than by pressure homogenization. This is in accordance with the results presented by Zamora et al. (2007) in cheese whey from milk treated at 300 MPa. Values of β-Lactoglobulin denaturation obtained in the study of Pereda et al. (2009) were excessively elevated to be attributed only to the thermal effect. However, in UHPH, simultaneous heating and homogenization processes exist. In agreement with this, in the present study, tryptophan fluorescence changes are apparently more related to thermal effect than to homogenization pressure (Table 5-2 & Figure 5-1).

The formation of dityrosine bonds could be indicative of certain changes of the tertiary and quaternary protein structure or oligomerization of proteins. The shear stress produced by homogenization pressure provided the chance for tyrosyl residues to expose, which is the prerequisite for cross-linking, while the required energy to form a covalent bond is offered by temperature. Both these two conditions contributed to the formation of the dityrosine. Also, the extent of dityrosine formation in milk proteins varies depending on protein structure, the matrix complexity and the presence of metal ions (Scheidegger et al., 2010; Dalsgaard, Nielsen, Brown, Stadler, & Davies, 2011; Scheidegger et al., 2013; Scheidegger, Larsen, & Kivatinitz, 2016).

The accumulation of fluorescent Maillard compounds indicates the thermal effects involving amino acids and reducing carbohydrates. The reactive carbonyl group of the sugar reacts with the nucleophilic amino group of the amino acid, and forms a complex mixture of poorly characterized molecules responsible for a range of undesired color, nutrition and flavor changes in milk. The models obtained could be used to prevent these undesirable changes in dairy products processed by UHPH.

5.3 Conclusion

UHPH treatments cause changes on milk native fluorescence. The thermal effects are more significant than pressure effects on selected fluorophores. Dityrosine and Maillard compounds fluorescence can be modeled as a function of inlet temperature. In addition, there is little formation of dityrosine and Maillard compounds when inlet

temperature is below 50 °C. Tryptophan fluorescence indicates milk protein modification such as denaturation and aggregation. The front-face fluorescence models proposed cover the commonly used UHPH treatment condition ranges proposed for improving physicochemical and sensory characteristics and microbiological safety in milk, as an alternative to industrial, conventional heat treatment. Tryptophan, dityrosine and Maillard compounds fluorescence has a promising potential to monitor and predict the structural and functional changes in milk during UHPH processing, avoid milk over-treatment and help deciding on its more appropriate usage for further production.

Chapter 6

Modeling changes in bovine milk caused by ultra-high pressure homogenization treatment using front-face fluorescence spectroscopy

6. Modeling changes in bovine milk caused by ultra-high pressure homogenization treatment using front-face fluorescence spectroscopy

6.1Results

6.1.1 Temperature increase in valve during UHPH treatments

In UHPH treatment, there is heat exchanger to ensure the sample temperature would reach to the desired inlet temperature. Then, when the sample passing through the valve, the sample temperature instantly increases, which could be attributed to two reasons. One is the adiabatic heating as a result of the pressure exerted by the pressure intensifiers and the other is high turbulence, shear and cavitation forces that are produced after the valve, through the release of pressure (Amador-Espejo, Suàrez-Berencia, Juan, Bárcenas, & Trujillo, 2014). Although the valve temperature could not be controlled, it could be monitored on the screen of the equipment (Table 6-1).

Table 6-1 The central component design of experimental parameters

No.	Pressure (MPa)	Inlet Temperature (°C)	Valve Temperature (°C)
1	200	51	95
2	300	52	116
3	200	79	111
4	130	71	90
5	200	51	97
6	100	51	78
7	130	32	75
8	200	22	84
9	270	71	114
10	200	51	98
11	200	51	98
12	270	32	102
13	200	52	99

The valve temperature was found to correspond to a linear regression with pressure and inlet temperature as Eq 6-1 ($R^2 = 0.98$, $P < 0.0001$). The valve temperature increase rate could be estimated as 18.5 °C/ 100 MPa.

$$VT = 0.185 \cdot P + 0.441 \cdot InT + 38.598 \quad \text{Eq 6-1}$$

where P = Pressure (MPa), InT = Inlet Temperature (°C), and VT = Valve Temperature (°C).

This result confirmed the obtain result from chapter 5 indirectly.

6.1.2 Effect of UHPH treatment on tryptophan fluorescence

The fluorescence of protein is a mixture of the fluorescence from individual aromatic residues. Most of the intrinsic fluorescence emissions of a folded protein are due to excitation of tryptophan residues and it is influenced by the neighboring amino acids and surrounding hydrophobicity. Hence, tryptophan fluorescence spectrum could reveal information about microenvironment of the tryptophan and proteins (Christensen, Nørgaard, Bro, & Engelsen, 2006). It can be observed that no clear red- or blue-shift occurred after the UHPH treatment in Figure 6-1. This confirmed that UHPH would not cause over-heat damage to milk within the experimental parameters range (Brodkorb, Croguennec, Bouhallab, & Kehoe, 2016).

F_{Trp} value of the UHPH treated samples showed a good fit to the quadratic response surface with P and InT ($R^2 = 0.88$, Figure 6-2). The regression equation was obtained as Eq 6-2 ($P < 0.05$).

$$F_{Trp} = 2.500 \cdot P + 5.957 \cdot InT - 0.005 \cdot P^2 - 0.022 \cdot InT^2 - 0.011 \cdot P \cdot InT + 192.906 \quad \text{Eq 6-2}$$

where F_{Trp} = Maximum tryptophan fluorescence intensity (a.u.).

To validate Eq 6-2, cross-validation by leave-one-out methodology was performed. The correlation coefficient obtained was 0.79 and the root mean square error of cross-validation (RMSECV) was 4.66%.

6.1.3 Effect of UHPH treatment on dityrosine fluorescence

It can be easily observed in Figure 6-1, that after the UHPH treatment, the dityrosine intensity of all samples increased. The peak intensities F_{Dt} respond to the pressure and inlet temperature shown in Figure 6-2. The multiple regression equation of the dityrosine with all the significant variables was obtained ($R^2 = 0.86$, $P < 0.05$).

$$F_{Dt} = 0.595 \cdot P + 2.061 \cdot InT - 0.007 \cdot P \cdot InT + 787.828 \quad \text{Eq 6-3}$$

where F_{Dt} = Maximum dityrosine fluorescence intensity (a.u.).

To validate Eq 6-3, cross-validation was performed and the correlation coefficient obtained was 0.82 and the RMSECV was 5.05%.

6.1.4 Effect of UHPH treatment on Maillard compounds fluorescence

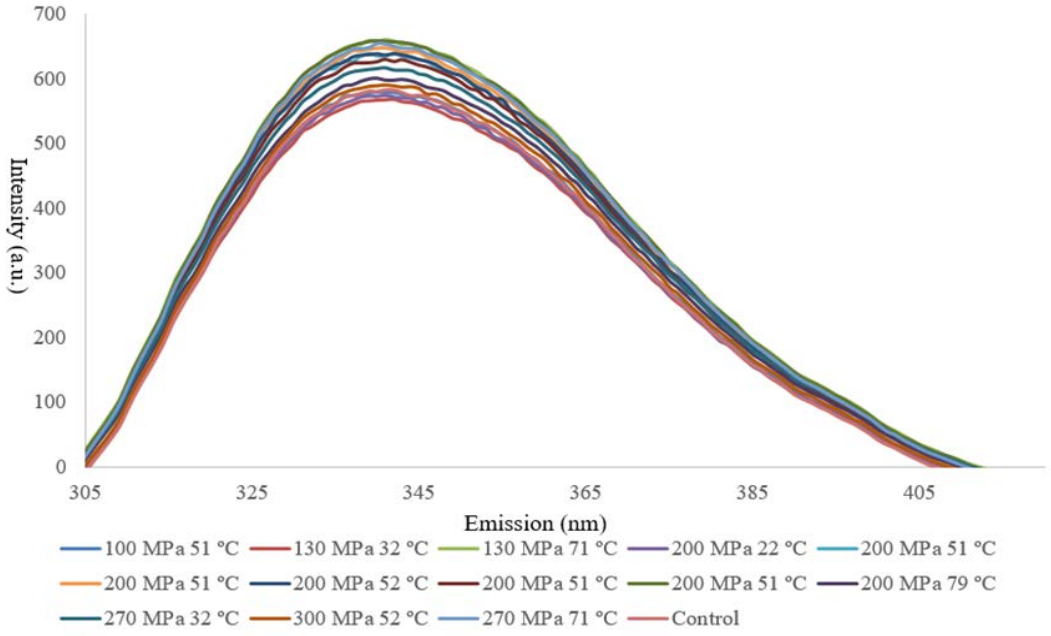
The accumulation of Maillard compounds as side products of thermal reaction with increasing inlet temperature and pressure could be easily observed in Figure 6-1.

The response surface was obtained with an R^2 of 0.85 (Figure 6-2). The regression equation was obtained as Eq 6-4 ($P < 0.05$).

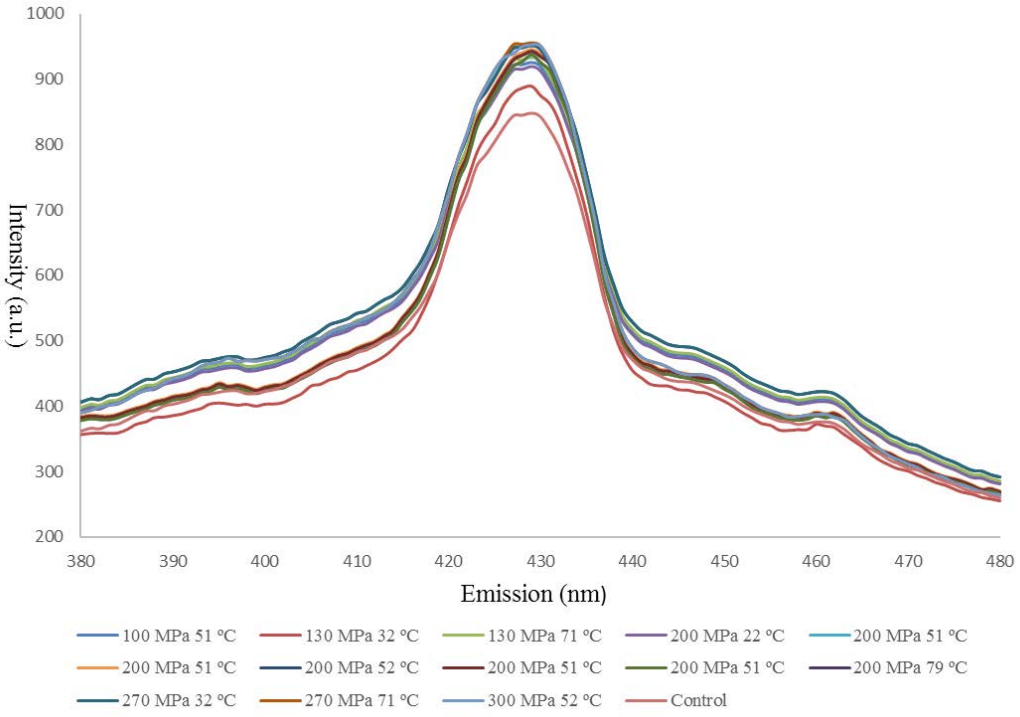
$$F_{MC} = 0.570 \cdot P + 2.132 \cdot InT - 0.008 \cdot P \cdot InT + 733.122 \quad \text{Eq 6-4}$$

where F_{MC} = Maximum Maillard compounds fluorescence intensity (a.u.).

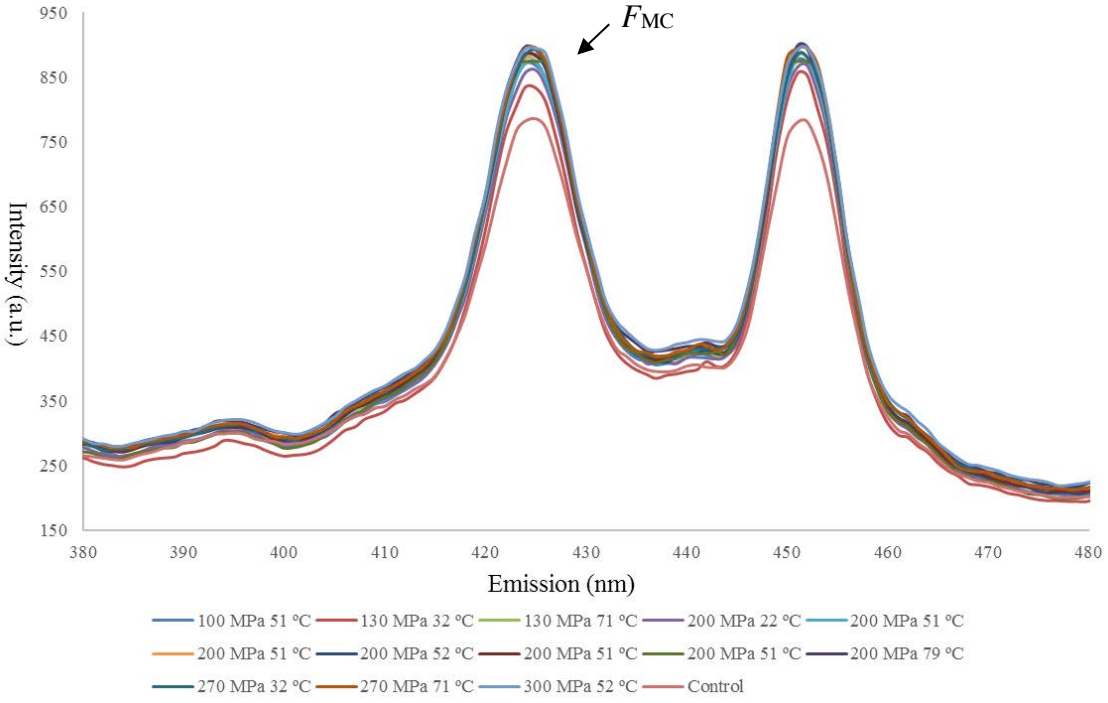
To validate Eq 6-4, cross-validation was performed and the correlation coefficient obtained was 0.87 and the RMSECV was 5.12%.



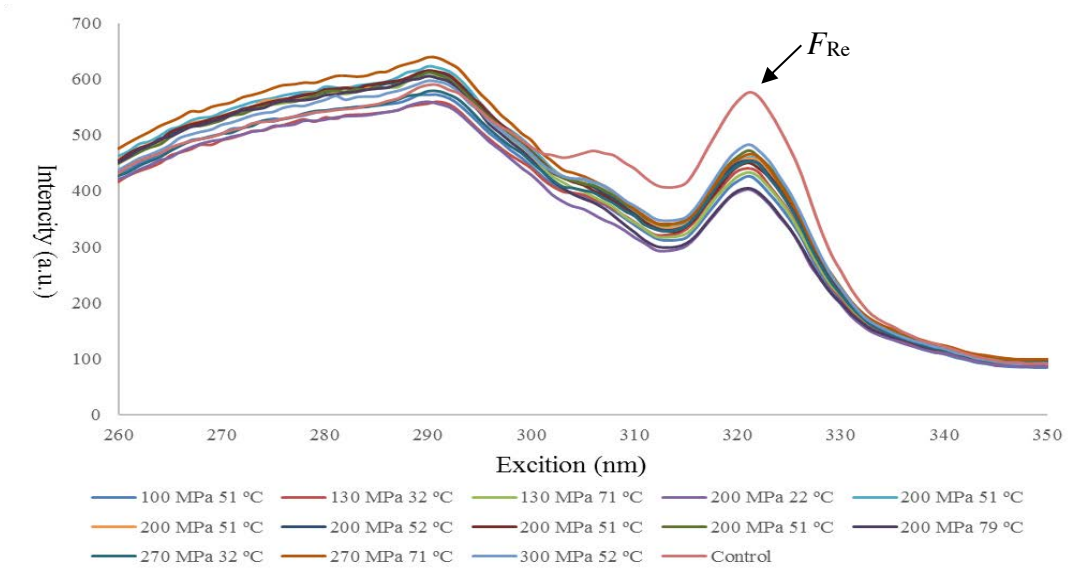
(a)


Intensity (a.u.)
Emission (nm)
100 MPa 51 °C
130 MPa 32 °C
130 MPa 71 °C
200 MPa 22 °C
200 MPa 51 °C
200 MPa 51 °C
200 MPa 52 °C
200 MPa 51 °C
200 MPa 51 °C
200 MPa 79 °C
270 MPa 32 °C
270 MPa 71 °C
300 MPa 52 °C
Control

(b)


F_{MC}
Intensity (a.u.)
Emission (nm)
100 MPa 51 °C
130 MPa 32 °C
130 MPa 71 °C
200 MPa 22 °C
200 MPa 51 °C
200 MPa 51 °C
200 MPa 52 °C
200 MPa 51 °C
200 MPa 51 °C
200 MPa 79 °C
270 MPa 32 °C
270 MPa 71 °C
300 MPa 52 °C
Control

(c)



(d)

Figure 6-1 Fluorescence spectra of tryptophan excited at 290 nm (a), dityrosine excited at 315 nm (b), Maillard compounds excited at 330 nm (c) and retinol emitted at 410 nm (d)

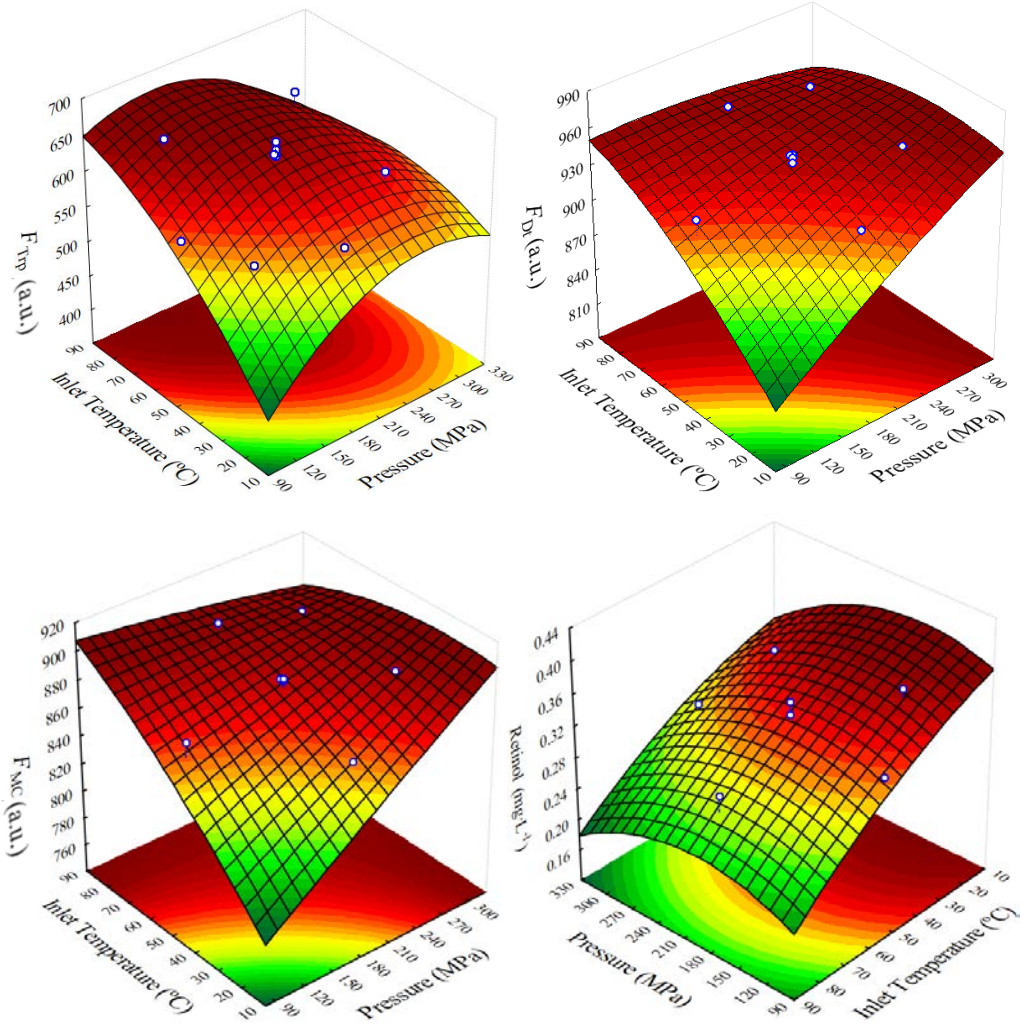


Figure 6-2 Response surface of tryptophan, dityrosine, Maillard compounds and retinol concentration in UHPH treatment as a function of pressure and inlet temperature

6.1.5 Effect of UHPH treatment on retinol fluorescence and its loss

The retinol fluorescence spectroscopy was presented as Figure 6-1. It showed significant decrease after the UHPH treatment and the retinol loss is proportional to the processing intensity. However, the attempt to modeling the actual concentration only with the peak value F_{Re} did not work out, because at this wavelength range, retinol is not the only fluorescent substance. The spectrum was also contributed by other components, for instance NADH (Andersen & Mortensen, 2008).

Table 6-2 Summary of the multiple linear regression of retinol concentration estimated by the principal components obtained from the fluorescence spectra

	Intercept	**PC1**	**PC2**	**PC3**	**PC4**	**PC5**	**P6**
Coefficient	0.32230	-0.00186	0.00351	-0.00379	-0.00438	0.01245	-0.01213
Standard error (mg/L)	0.00434	0.00057	0.00083	0.00276	0.00324	0.00292	0.00385
P-level	0.0000	0.0136	0.0039	0.2116	0.2187	0.0037	0.0161

By principal component analysis (PCA) of the spectra, it could be summarized as 6 principal components (PC). Further, the regression between retinol concentration [*Re*] obtained from HPLC (Figure 6-2) and these PCs fits linear regression with an R^2 of 0.90, SEP of 0.016 mg·L^{-1} and RMSECV of 5.22% (Table 6-2). Although the regression results suggested that PC3 and PC4 were not significant, the R^2 value would decrease a lot along with increased SEP when they were removed. Hence, the prediction equation would be established as

$$[Re]=0.32-(1.86\cdot PC1-3.51\cdot PC2+3.79\cdot PC3+4.38\cdot PC4 -12.45\cdot PC5+12.13\cdot PC6)\cdot 10^{-3} \quad \text{Eq.6-5}$$

The prediction and residuals plots have been shown as Figure 6-3 and 6-4.

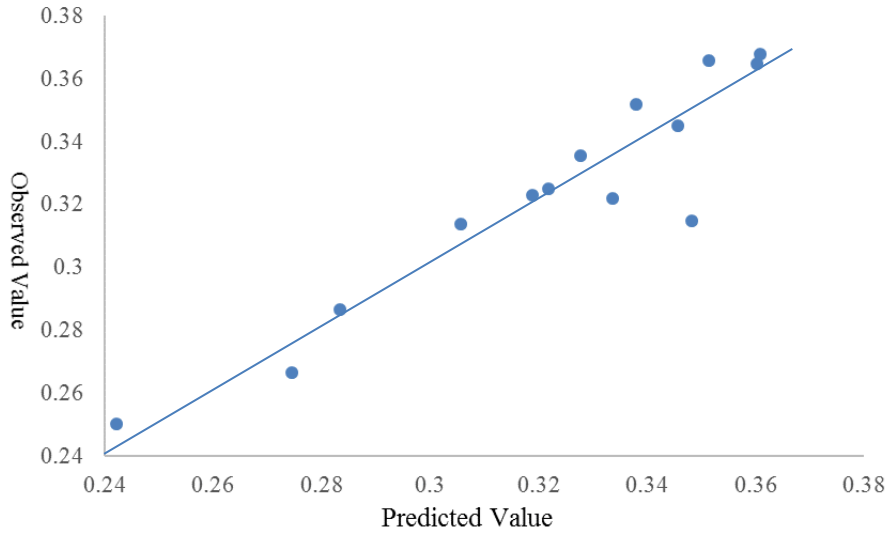


Figure 6-3 Regression between observed and predicted retinol value (mg/L)

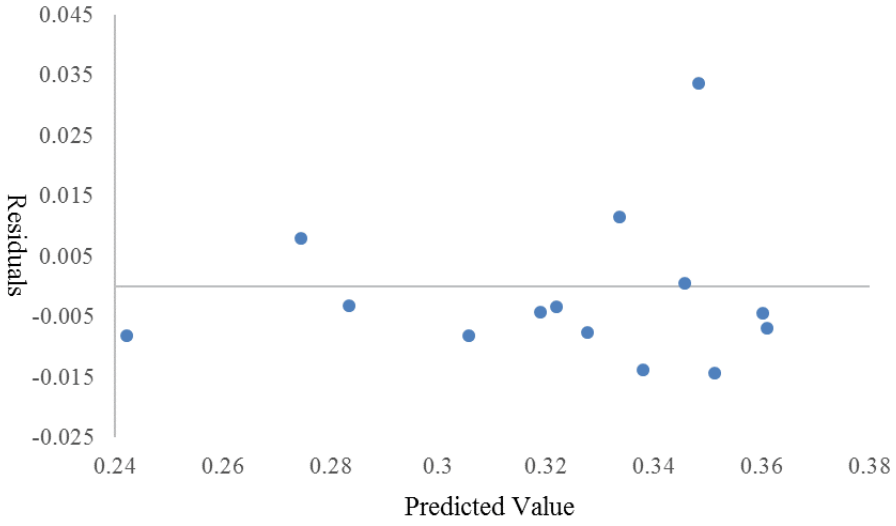


Figure 6-4 Residual plot of retinol concentration (mg/L)

6.2 Discussion

According to Eq 6-1, the valve temperature increase rate observed in this work could be estimated at 18.5 °C/ 100 MPa when inlet temperature ranged from 22 °C to 79 °C. Thiebaud et al. (2003) also presented an 18.5 °C/ 100 MPa increase, using 100 to 300 MPa at 4, 14, and 24 °C. While, Pereda et al. (2007) obtained an increase of 19.5°C/ 100 MPa in UHPH treatments of 200 and 300 MPa at 40 °C using a two-stage valve design (Stansted Fluid Power Ltd.). Comparing this increase rate to our previous work on skim milk in Chapter 5, whole milk heating rate is 0.4 °C/ 100 MPa higher.

Hayes et al. (2003) also noticed this phenomenon, observing that a higher temperature increase for UHPH treatment of liquids with higher fat contents. Also, Roig-Sagués et al. (2009) reported that fat content increases the lethality of ultra-high-pressure homogenization on *Listeria monocytogenes* in milk. They believed the maximum temperature reached during the treatments had important impact. The increment in the viscosity caused by fat content probably contributed to an increase in friction, in turn increasing the temperature reached.

To produce commercial milk, bacterial reduction is essential. Several studies showed that homogenization at pressure of 200 MPa and inlet temperatures of 24 °C caused total bacterial count reductions similar to those of pasteurization processes (30 min at 63 °C). In addition, almost 3 log units reduction after treatment performed at 300 MPa and 30 °C could obtain milk with a microbial and physicochemical shelf-life equal to that of high-pasteurized milk (90°C for 15 s) (Pereda et al., 2007; Thiebaud et al., 2003). Further, treatments performed at 300 MPa with InT = 75 and 85 °C produced samples without microbial growth after incubation (30 and 45°C) with similar or better characteristics than UHT milk in color, particle size, viscosity, buffer capacity, ethanol stability, propensity to protein hydrolysis, and lower scores in sensory evaluation for cooked flavor (Amador-Espejo et al., 2014). Thus, the experimental parameters range (P and InT) studied suits for industrial production needs.

After the homogenization, milk fat globule droplets size drastically reduced, with the surface area of MFGM increasing. In the meantime, to stabilize the newly-formed surface, milk proteins would be adsorbed to the surface of the fat globules. Thus, UHPH provokes interactions between MFGM components and whey proteins and/or caseins. Homogenization of the raw milk promoted the attachment of caseins to the MFGM (Garcıa-Risco et al., 2002). Besides, pressure treatment could cause direct changes in the tertiary and quaternary structure conformation of proteins, as well as the exposure of amino acids on the peptide chains, which lead to the change of the fluorescence spectrum. Hence, changes in the fluorescence spectra can provide information about protein conformation, subunit association, and denaturation (Elshereef, Budman, Moresoli, & Legge, 2006).

Tryptophan fluorescence depends on how tryptophan is exposed in the three-dimensional configuration of the proteins. Mainly due to its large extinction coefficient, tryptophan dominates most information about protein structure (Andersen & Mortensen, 2008; Lakowicz, 2006). In Figure 6-1(a), there is no significant red- or blue-shift of the peak, which means current UHPH processing intensity did not significantly changed tryptophan surrounding towards more hydrophobic or hydrophilic, but there is changes in intensity. Changes in casein micelle structure induced by coagulation would lead the peak to lower wavelength (Herbert, Riaublanc, Bouchet, Gallant, & Dufour, 1999), while during cheese ripening, tryptophan fluorescence shifted to longer wavelength (Karoui, Mouazen, Dufour, Schoonheydt, & De Baerpemaeker, 2006). Also, severe whey protein denaturation induced by overheating leads maximum peak to a red-shift as well (Stănciuc et al., 2012; Taterka & Castillo, 2015). In our study, the increase of the maximum intensity may be due to the changes on protein conformation. The denatured whey protein and the casein micelles that lost some surface κ-casein in the process, exposed their tryptophan sites, which was fold inside of the hydrophobic cores before (Sandra & Dalgleish, 2007). These κ-casein can be present as a disulfide-linked polymer, directly attached to the fat interface, or as κ-casein–β-Lactoglobulin complex in recombined milk (Sharma, Singh, & Taylor, 1996). Ye et al. (2004) also suggested that the casein micelles may be directly associated with the MFGM proteins through κ-casein, and indirectly through the interaction of κ-casein with β-Lg already associated with the MFGM. Further, the decrease on F_{Trp} at high inlet temperature and high homogenization pressure is assumed to be related to protein oligomerization and aggregation. Elshereef et al. (2006) reported that tryptophan fluorescence was correlated to the solubility and aggregation behavior of β-Lg. In the formation of new MFGM, interactions between β-Lg and casein micelles increase with the extent of heat treatment, and heat can also cause α-lactalbumin (α-La)–β-Lg interaction after β-Lg–casein complex formation (Guyomarc'h et al., 2003; Sandra & Dalgleish, 2005). Furthermore, UHPH could cause the casein micelle partial disintegration and/or reformation and may cause modification in β-Lg binding sites. Hayes et al. (2005), treating milk at 250 MPa, suggested that the high turbulence, shear and cavitation forces

experienced by whole milk during UHPH denatured β-Lg. Whey protein denaturation was found to increase with increasing pressure, time, and temperature, and the denatured β-Lg formed a complex with casein micelles, giving an increase in casein micelle size at pressures around 250 MPa and micelle size would also increase because of aggregation of the caseins (Garcıa-Risco et al., 2002; Huppertz et al., 2004a, 2004b). Moreover, Pereda et al. (2009) reported 10-17% and 32–37% of denaturation in the degree of α-La and β-Lg denaturation, respectively, by UHPH at 200 MPa and 300 MPa with inlet temperature at 30 °C and 40 °C. They also noticed that denaturation of β-Lg was more important than that of α-La, and that β-Lg was more drastically denatured by the conditions used in the thermal treatment than by pressure homogenization. This is in accordance with the results presented by Zamora et al. (2007) in cheese whey from milk treated at 300 MPa. Values of β-Lg denaturation obtained in the study of Pereda et al. (2009) were excessively elevated to be attributed only to the thermal effect. However, in UHPH, simultaneous heating and homogenization processes exist. While we still can observe that the curvature of the response surface and F_{Trp} intensity changed along the inlet temperature axis more than by pressure in Figure 6-2.

Dityrosine as a biomarker of protein oxidation, formation of dityrosine bonds could provide the information of tertiary and quaternary structure, oligomerization and oxidation of proteins (Malencik & Anderson, 2003; Scheidegger et al., 2013). During UHPH processing, the shear force stress and temperature rise provided the chances and energy for tyrosine residues to expose and link, which leaded to the increase in fluorescence. The flexible structure of β-casein favors radical–radical termination of tyrosyl radicals to give dityrosine, whereas the less flexible structure of globular proteins decreases the propensity for tyrosyl radicals to dimerize (Dalsgaard, Nielsen, Brown, Stadler, & Davies, 2011). The extent of dityrosine formation in milk proteins varies depending on protein structure (Dalsgaard et al., 2011), the matrix complexity and the presence of metal ions (Qi, Ren, Xiao, & Tomasula, 2015; Scheidegger et al., 2013; Scheidegger, Pecora, Radici, & Kivatinitz, 2010). In addition, oxidative changes involving tryptophan residues and formation of dityrosine have been noted by Dalsgaard & Larsen (2009) and Scheidegger et al. (2010), along with decreased

accessibility of chymosin to oxidized caseins.

Advanced Maillard reaction products such as pyrrole and imidazole derivatives are fluorescent and are formed as intermediary products during heating. It was clear that the fluorescence intensity increased with more severe heat treatment, illustrating the formation of Maillard reaction products (Andersen & Mortensen, 2008). The accumulation of Maillard compounds indicate the thermal effects with amino acids involved. Maillard reaction causes color, nutrition and flavor changes in milk, which are undesired in most dairy products. In this case, *FFFS* could be used to monitor the thermal effect on milk effectively.

As described in section 6.1.5 the peak value F_{Re} failed to model the retinol content, so it was decided to apply a PCA analysis on the whole spectrum as a dimensional reduction tool. The principal components (PCs) from retinol spectrum can be used for linear regression prediction of retinol concentration. It could be used as a routine rapid method to monitor and avoid nutritional loss in UHPH milk processing. More importantly, it was stated that the fluorescence properties of retinol provided information not only about retinol concentration, but also about the physical state of triglycerides and protein-lipid interactions (Andersen & Mortensen, 2008). Homogenization of milk breaks the protective fat globule membrane, allowing access of indigenous lipoprotein lipase and hydrolysis of free fatty acids from triglycerides. The availability of a larger number of lipid droplets upon homogenization, with greater surface area available for interaction with the peptides, led to persistence of the smaller peptide bands (Tunick et al., 2016). This phenomenon has influence on further cheese making, such as rennet coagulation time, rate of curd firming, etc. Fluorescence spectrum excited at 260-350 nm and emitted at 410 demonstrated the changes of the interactions of the fat-globule membrane with the protein network and/ or the crystallization of triglyceride during the ripening. (Karoui, Dufour, & De Baerdemaeker, 2006; Karoui, Mouazen, et al., 2006; Smoczyński, 2016). Further research of the connection is still needed.

Thus, the present work demonstrates that some milk changes can be explained by the information contained in the *FFFS* and has the potential to be used for the

development of an inline/online sensor to monitor and prevent over-treatment during UHPH processing.

6.3 Conclusion

UHPH is a promising technique to enhance milk and dairy product properties. During the process, the milk native fluorescence could be used for product development, process control, raw material determination, etc. Tryptophan, dityrosine and Maillard compounds fluorescence could be modeled as a function of inlet temperature and pressure. Tryptophan and dityrosine fluorescence indicate milk protein information such as denaturation, disintegration, reformation and aggregation and accumulation of Maillard compounds demonstrates the thermal effects occurred. What is more, by summarizing the retinol spectra to principal components, the actual concentration could be well predicted. The front-face fluorescence model covers the commonly used parameters ranges for improving physicochemical and sensory characteristics and microbiological safety. It has a promising potential to monitor and predict the structural and functional changes in milk during UHPH processing, avoid milk over-treat and decide on its more appropriate usage for further production.

Chapter 7

Particle size distribution

7. Particle size distribution

7.1 Results

7.1.2 Effects of UHPH treatments on skim milk particle size distribution

After the fat removing process, the particle size distribution curve of the control sample had one main peak at ~ 0.13 μm (from 0.04 μm to 0.55 μm) including 98.45% volume, which corresponds to casein micelle and a tiny peak at ~ 0.72 μm (from 0.55 μm to 1 μm). After the UHPH treatment, there were slight reductions in particle size of samples treated of 100 - 200 MPa. On the contrary, samples treated at pressure higher than 200 MPa show significant decreases of the main peak and a new peak between 1.94 μm to 60.08 μm appeared. Since all the particles in the control sample are smaller than 1μm, the particles bigger than 1 μm in the UHPH-treated samples indicated protein aggregation. The volume resulting from aggregation was directly proportional to the processing intensity and up to 35.92% of protein is in form of aggregates when pressure at 300 MPa and inlet temperature at 51 °C were applied. In addition, the volume-weighted mean diameter of small particles (＜1 μm) d[4,3] shifted from 0.14-0.15 μm to 0.16-0.18 μm in samples treated at a pressure higher than 200 MPa (Figure 7-1). In Table 7-1, the results of the measured diameters

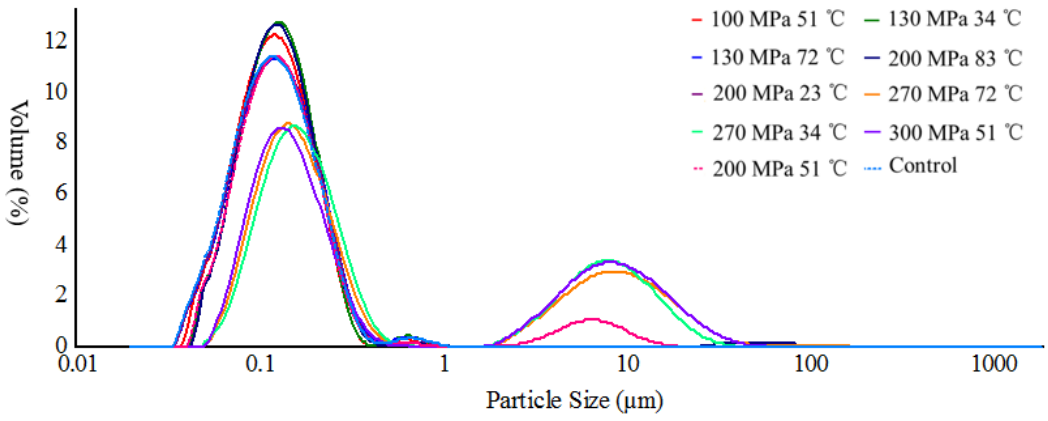


Figure 7-1 Particle size distribution in skim after UHPH treatment

Table 7-1 Particle diameters (μm) in skim milk after UHPH treatment

Pressure (MPa)	Inlet Temperature (°C)	D_{50}	D_{90}	D[3,2]	D[4,3]	d[3,2]	d[4,3]
100	51	0.123	0.222	0.108	0.137	0.108	0.137
130	34	0.128	0.227	0.115	0.144	0.115	0.144
130	72	0.125	0.240	0.108	0.145	0.108	0.145
200	83	0.129	0.234	0.117	0.713	0.116	0.142
200	23	0.124	0.237	0.107	0.143	0.107	0.143
270	72	0.209	13.189	0.209	4.151	0.141	0.170
270	34	0.221	11.076	0.219	3.254	0.149	0.181
300	51	0.203	13.114	0.205	4.013	0.133	0.159
200	51	0.133	0.295	0.121	0.648	0.105	0.141
Control	Control	0.122	0.233	0.105	0.141	0.105	0.141

Both tryptophan fluorescence and particle size distribution reveal milk protein information. In Table 7-1, the results of the measured diameters were shown. To study the relation between these two parameters, a multiple regression was obtained.

$$F_{Trp}=1645.5+10777.2\cdot D[3,2]-172.9\cdot D[4,3]-10406.8\cdot d[3,2]-200.3\cdot d[4,3] \quad \text{Eq.7-1}$$

In Eq.7-1, D[3,2], D[4,3], and d[3,2] are significant with $P < 0.05$ and R^2 value is 0.74.

7.1.2 Effects of UHPH treatments on whole milk particle size distribution

The whole raw milk particles, mainly comprised of casein micelles and fat globules, corresponded to the peaks at ~ 0.12 μm (from 0.04 μm to 0.59 μm) and at

3.87 μm (from 1.03 μm to 14.58 μm), respectively. After the UHPH processing, both peaks broadened, for samples treated at 100 - 200 MPa, the first peak appeared from 0.04 μm to 1.75 μm and the second took place from 1.75 μm to ~55 μm. The range of the first peak showed almost no shift for samples treated at pressure higher than 200 MPa, but its volume significantly decreased, with a great increase of both distribution range (extended to 100 μm) and volume of the second peak at the same time. The maximum fraction of volume of the large particles (＞1 μm) was 49.55% in sample treated at 270 MPa and inlet temperature at 71 °C. Comparing to the control sample, the volume-weighted mean diameter of small particles (＜1 μm) d[4,3] in treated samples shifted from 0.14 μm to 0.20-0.22 μm (Figure 7-2) .

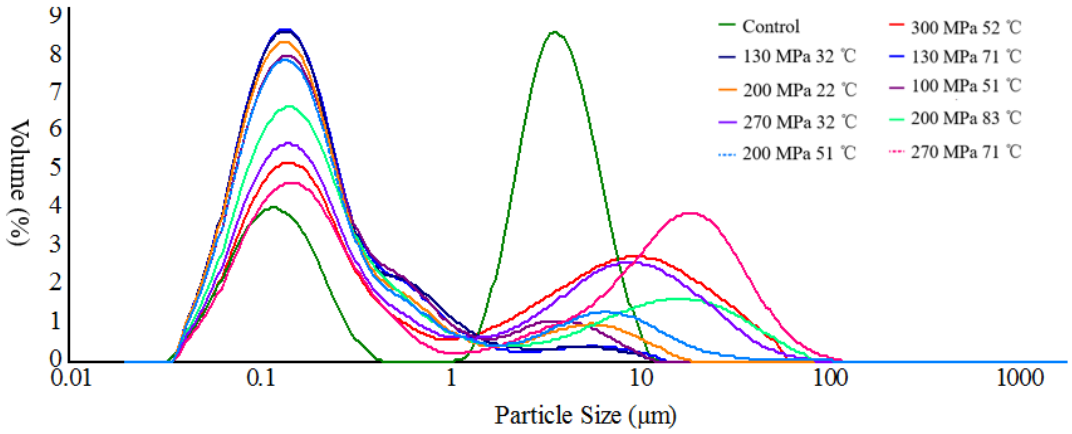


Figure 7-2 Particle size distribution in whole milk after UHPH treatment

The results of the measured diameters were shown as Table 7-2. The regression between tryptophan and particle size distribution was as following equation with an R^2 of 0.70:

$$F_{Trp} = -1147.5 - 1417.0 \cdot D[3,2] - 5.3 \cdot D[4,3] + 2730.2 \cdot d[3,2] - 16778.5 \cdot d[4,3] \quad \text{Eq.7-2}$$

In Eq.7-2, D[3,2], d[4,3], and d[3,2] were significant, with $P < 0.05$.

Table 7-2 Particle diameters (μm) in whole milk after UHPH treatment

Pressure (MPa)	Inlet Temperature (°C)	D_{50}	D_{90}	D[3,2]	D[4,3]	d[3,2]	d[4,3]
100	51	0.178	1.354	0.149	0.631	0.133	0.222
130	32	0.163	0.718	0.138	0.413	0.130	0.217
130	71	0.162	0.665	0.137	0.431	0.130	0.214
200	79	0.223	17.271	0.180	4.651	0.135	0.221
200	22	0.167	1.363	0.143	0.781	0.127	0.207
270	71	0.760	30.203	0.259	10.084	0.132	0.204
270	32	0.295	15.399	0.209	4.757	0.132	0.212
300	51	0.406	18.706	0.231	5.799	0.131	0.208
200	51	0.177	4.488	0.151	1.361	0.128	0.206
Control	Control	2.781	6.191	0.280	2.825	0.103	0.135

7.2 Discussion

According to Roach and Harte (2008) there are primarily two occurrences in particle size changes, one is the formation of β-Lg-casein complexes and the other is casein aggregation. The denaturation of whey protein increases with rising pressure (> 250 MPa) and temperature (75-80 °C) (Roach & Harte, 2008) and, therefore, increases the interaction with the surface components of the casein micelle, resulting in an increase in casein micelle size (Datta et al., 2005; Guyomarc'h et al., 2003; Sandra & Dalgleish, 2005). In another theory of high-pressure induced aggregation, the collapse or removal of κ-casein reduces the steric repulsion between the micelles and causes them to cluster together. This aggregation is due to the decreased solubility of the micelle after solubilization of κ-casein. Kappa-casein is solubilized up to 20% under

hydrostatic pressure at 200 MPa. This reduces the negative charge and electrostatic repulsions between the micelles promoting micellar aggregation, thus increasing particle diameter (Needs, Stenning, Gill, Ferragut, & Rich, 2000). While, the second theory is not as convincing as the first one is due to the mechanism action of high hydrostatic pressure and UHPH are quite different. We believe that crosslinking by disulfide or dityrosine formation or even hydrophobic interactions between caseins might create linkages among neighboring micelles.

In Figure 7-1, the slight reduction in particle size of samples treated by 100 - 200 MPa is assumed to be due to casein micelle disintegration. Sandra and Dalgleish (2005) also reported a similar view of the reduction in particle size obtained in skim milk homogenized at 114 MPa and 186 MPa. While, UHPH treatment at pressure higher than 200 MPa caused significant increase of large particles. This can be assumed to be aggregates of casein micelles, casein fragments, denatured whey proteins and whey proteins in various modified forms. Some aggregates are ~100 times larger in diameter than native caseins. Although their amount is small, these structures include a relevant fraction of volume of particles. Besides, the shift of the mean diameter d[4,3] of small particles may due to denatured whey protein attached to casein micelles. The regression between tryptophan fluorescence and particle size distribution (Eq. 7-1) also confirmed our conclusion on protein changes base on the fluorescence model. A clear effect of UHPH treatment on milk protein structure modification can be observed by particle size analysis and tryptophan fluorescence.

The most significant change of whole milk after UHPH is supposed to be a fat globules size reduction, which is the basic purpose of homogenization processing. The results we obtained confirmed this anticipation. The volume of particles smaller than 1 μm increased in all samples. On the other hand, the generation of large particles is similar to what was discovered in skim milk, but with much large aggregates, which was assumed to be related to the presence of fat globules. The absorption of casein and whey protein to the newly formed MFGM may lead to the shift between untreated and treated samples in small particles. The R^2 value of regression of particle size in whole

milk with tryptophan (Eq.7-2) was smaller than that of skim milk, which was assumed to be related to fat interference. However, it still strengthens our understanding of milk protein changes after UHPH processing. These inferences also indirectly confirm to our analysis of fluorescence spectral changes in chapter 4 and 5, especially in tryptophan.

Taterka and Castillo (2015) reported the potential for the use of optical light backscatter techniques as a measurement of changes in casein micelle particle size induced by heat treatment. Furthermore, they also formed useful correlations between the light scatter methodology and the mechanism of binding and/or aggregation providing essential information towards the development of an optical sensor for the determination of denatured whey proteins in heat-treated milk (Taterka & Castillo, 2017). Hence, measuring particle size with light scatter methodology might also be effective for UHPH-treated milk as well. Then, it provides a potential to develop a combined sensor with light scatter methodology and *FFFS* to achieve online/offline rapid prediction and evaluation of UHPH treated dairy products in the future.

7.3 Conclusion

UHPH treatments could effectively reduce the particle size in milk. Although the practical value of applying UHPH on skim milk is not as high as for whole milk, it helps to understand the protein denaturation and aggregation behavior during UHPH. Also, the changes in particle size support our findings in *FFFS*. The use of the fluorescence signal to distinguish or classify the UHPH pressure applied to the samples alone is not enough because the changes are mainly due to thermal reasons. However, the changes in particle size are mainly due to pressure. Integrating both *FFFS* and particle size information could be useful to achieve the goal of online/offline rapid prediction and evaluation of UHPH treated dairy products.

Chapter 8

Concluding summary

8. Concluding Summary

The research work in this thesis describes the application of native milk fluorescence tracers for quick quantification of milk changes caused by thermal and UHPH processing.

The objectives of the first experiment were to model the kinetics of retinol loss and assess the potential of predicting retinol loss during thermal processing using *FFFS*. The kinetics of the retinol degradation process fitted well a first-order reaction and the optimum prediction model was obtained using three fluorescent indicators, with an R^2 of 0.87, correlation coefficient of 0.92 and the RMSECV of 5.37 %.

The objectives of the second experiment were to study how UHPH influenced the fluorescence properties of skim milk through selected indicators. The response surface of all three indicators *Trp, Dt* and *MC* responded well to the conditional parameters, their square and products. Also, the indicators revealed the changes of structural, functional and nutritional diversifications of milk proteins. The particle size distribution confirmed the conclusions obtained by fluorescent compounds. Additionally, due to the significant accumulation of *MC* and formation of *Dt* when inlet temperature was over 50 °C, prediction models were also obtained. Although the influence of pressure on fluorescence was not as significant as that of temperature, pressure was the main reason for the changes in particle size distribution.

In the third experiment, with the presence of fat, the emulsion environment is more complicated than skim milk. Firstly, the instant temperature increases at the UHPH valve were higher due to the higher viscosity (Roig-Sagués et al., 2009). Secondly, the particle size reduction in fat globules and destruction and reconstruction of MFGM also influenced the fluorescence. Zamora et al. (2012) observed that newly formed particles were heterogeneous in shape and size, consisted of aggregates of very small fat globules, casein micelles and some whey proteins after UHPH at pressure of 100-300 MPa with

inlet temperature of 40 °C through electron micrographs. Individually dispersed casein micelles were slightly smaller and less electrodense, especially at 300 MPa. 100 MPa UHPH could trigger the absorption of casein micelles at the MFGM of large fat globules. However, at higher pressures (200 MPa and 300 MPa), middle size casein micelles were those adsorbing at their surface small fat globules, while some disintegrated MFGM material did not reform globular particles. This is consistent with our particle size distribution results. In addition to direct interaction with native MFGM proteins, whey proteins were adsorbed through disulfide bonding with both indirectly and directly adsorbed casein molecules. The internal changes in whole milk were much more complex than in skim milk, and as expected, the response of the CCD model was not as significant as for skim milk. Still, the obtained results showed good fit to the model and integrated the supplementary particle size results. With the achievement of light scatter methodology on heated-treated milk obtained by Taterka and Castillo (2015, 2017), there is the potential to combine *FFFS* and light scatter methodology to fulfill the goal of quick quantification and evaluation.

As to the linear regression model for retinol quick quantification, the principal components summarized from the retinol spectrum yield of an R^2 of 0.90, SEP of 0.016 mg/L and RMSECV of 5.22%. It is very practical for industrial usage compare to the conventional HPLC method, which is time-consuming and require high experimental skills.

In summary, considering the promising results obtained, the *FFFS* showed great potential on retinol quick quantification during both thermal treatments and UHPH treatments. It also reveals the protein denaturation, disintegration, aggregation and absorption during the UHPH treatments. This information is essential for the evaluation of the nutritional value and further dairy products making.

Chapter 9

Conclusions

9. Conclusions

The conclusions obtained in this thesis are lised below:

1. The degradation behavior of retinol shows a functional relationship with temperature and time. The reaction rate increases with increasing temperature and retinol loss increases with increasing reaction time.
    a. The kinetics of the retinol degradation process follows a first-order reaction.
    b. The activation energy is estimated at 52.26 $kJ \cdot mol^{-1}$.
2. The F_{Trp}/F_{MC}, F_{Trp}/F_{Rb370} and F_{MC}^2 are implemented into model that best predicted retinol concentration in thermal treated milk.
    a. There are statistically significant negative correlations between retinol concentration and the maximum fluorescence of all the studied indicators.
    b. The most significantly correlated indicator is *MC* and the least one is Rb_{450}.
3. The valve temperature corresponds to a linear regression with pressure and inlet temperature of the UHPH processing. The temperature increase rate is higher in whole milk than in skim milk.
4. Tryptophan fluorescence is significantly influenced by pressure and temperature in skim milk, and it indicates most milk protein denaturation, disintegration, reformation and aggregation information during UHPH treatments.
5. In skim milk, the dityrosine and Maillard compounds fluorescence are only significantly affected by inlet temperature during UHPH treatments and shows the thermal effects occurred.
    a. There were no significant changes of F_{MC} and F_{Dt} observed between in the range of 20 to 50 °C, which means almost no accumulation of Maillard intermediate compounds and dityrosine. It can be assumed no thermal effect on skim milk by UHPH treatment when inlet temperature is lower than 50 °C.

b. Inlet temperature is sufficiently modeled and predicted by F_{MC} and F_{Dt} using a linear equation when it is higher than 50 °C in UHPH treated skim milk.

6. The fluorescence indicators are significantly influenced by both pressure and temperature in UHPH treated whole milk. Tryptophan, dityrosine and Maillard compounds fluorescence can be modeled as a function of inlet temperature and pressure. They provide valid information on milk protein and thermal effects.

7. The retinol content in UHPH treated milk can be well predicted by principal components summarized from retinol fluorescence spectra (excitation of 260 nm and emission of 410 nm).

8. UHPH can effectively reduce fat globules size and cause protein changes in milk.

   a. Low intensity pressure (under 200 MPa) does not cause significant protein structural and functional changes.

   b. High intensity pressure (above 200 MPa) cause significant protein denaturation, disintegration, reformation and aggregation.

9. The particle size results obtained indirectly confirm our conclusions from the fluorescence and has potential for quick prediction and evaluation usage.

Chapter 10

References

10. References

Abd El-Gawad, M. A. M., Ahmed, N. S., El-Abd, M. M., & Abd El-Rafee, S. (2012). Effect of homogenization on the properties and microstructure of Mozzarella cheese from buffalo milk. In *Acta scientiarum polonorum. Technologia alimentaria* (Vol. 11, pp. 121–135).

Abismail, B., Canselier, J. P., Wilhelm, A. M., Delmas, H., & Gourdon, C. (1999). Emulsification by ultrasound: drop size distribution and stability. *Ultrasonics Sonochemistry*, *6*(1), 75–83.

Airado-Rodríguez, D., Durán-Merás, I., Galeano-Díaz, T., & Wold, J. P. (2011). Front-face fluorescence spectroscopy: A new tool for control in the wine industry. *Journal of Food Composition and Analysis*, *24*(2), 257–264.

Almeida, R. A., Aryana, K. J., Augustin, M. A., Brody, A. L., Chandan, R. C., Clarke, P. T., Fearon, A. M. (2008). *Dairy Processing and Quality Asurance*. (R. C. Chandan, Ed.). John Wiley & Sons, Inc.

Alpas, H., & Bozoglu, F. (2000). The combined effect of high hydrostatic pressure, heat and bacteriocins on inactivation of foodborne pathogens in milk and orange juice. *World Journal of Microbiology and Biotechnology*, *16*(4), 387–392.

Amador-Espejo, G. G., Suàrez-Berencia, A., Juan, B., Bárcenas, M. E., & Trujillo, A. J. (2014). Effect of moderate inlet temperatures in ultra-high-pressure homogenization treatments on physicochemical and sensory characteristics of milk. *Journal of Dairy Science*, *97*(2), 659–71.

Andersen, C. M., & Mortensen, G. (2008). Fluorescence spectroscopy : a rapid tool for analyzing dairy products. *Journal of Agricultural and Food Chemistry*, *56*(1), 720–729.

Andersson, I., Öste, R., & Fox, P. F. (1995). *Nutritional quality of heat processed liquid milk*. International Dairy Federation.

Andrews, A. T. (1978). The composition, structure and origin of proteose-peptone component 5 of bovine milk. *European Journal of Biochemistry*, *90*(1), 59–65.

Augustin, M. A., & Clarke, P. T. (2009). Dry milk products. In *Dairy Processing & Quality Assurance* (pp. 319–336). Oxford, UK: Wiley-Blackwell.

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*, 1–7.

Ayala, N., Zamora, A., Saldo, J., & Castillo, M. (2015). Effcto del tratamiento térmico sobre el espectro de emisión de fluorescencia del triptófano. In *VII Congereso CYTA/CESIA*. Badajoz.

Baéz, R., Rojas, G., Sandoval-Guillén, J., & Ángeles Valdivia-López, M. A. (2012). Effect of storage temperature on the chemical stability of enteral formula. *Advance Journal of Food Science and Technology*, *4*(5), 235–242.

Ball, G. F. M. (2006). Vitamin A: retinoids and the provitamin a Carotenoids. In *Vitamins in Food. Analysis, Bioavailability, and Stability* (pp. 39–92).

Behrend, O., Ax, K., & Schubert, H. (2000). Influence of continuous phase viscosity on emulsification by ultrasound. *Ultrasonics Sonochemistry*, *7*(2), 77–85.

Birlouez-Aragon, I., Nicolas, M., Metais, A., Marchond, N., Grenier, J., & Calvo, D. (1998). A rapid fluorimetric method to estimate the heat treatment of liquid milk. *International Dairy Journal*, *8*(9), 771–777.

Brodkorb, A., Croguennec, T., Bouhallab, S., & Kehoe, J. J. (2016). *Heat-induced denaturation, aggregation and gelation of whey proteins. Advanced Dairy Chemistry: Volume 1B: Proteins: Applied Aspects: Fourth Edition*.

Buffa, M., Guamis, B., Royo, C., & Trujillo, A. J. (2001). Microbiological changes throughout ripening of goat cheese made from raw, pasteurized and high-pressure-treated milk. *Food Microbiology*, *18*(1), 45–51.

Burton, H. (1994). *Ultra-High-Temperature Processing of Milk and Milk Products*. Boston, MA: Springer US.

Bylund, G. (1995). *Dairy processing handbook*. (A. Teknotext, Ed.)*Tetra Pak Processing Systems* (Vol. G3). Tetra Pak Processing Systems AB S-221 86 Lund, Sweden.

Chandan, R. C. (2007). Milk composition, physical and processing characteristics. In Y. H. Hui (Ed.), *Handbook of Food Products Manufacturing* (pp. 347–377).

Chandan, R. C. (2009). Dairy processing and quality assurance: An overview. In *Dairy Processing & Quality Assurance* (pp. 1–40). Oxford, UK: Wiley-Blackwell.

Chandan, R. C., Patel, D. A., Almeida, R. A., & Oliver, S. P. (2015). Mammary gland and milk biosynthesis: nature's virtual bioprocessing factory. In *Dairy Processing and Quality Assurance* (pp. 60–76). Chichester, UK: John Wiley & Sons, Ltd,.

Chatterjee, S., Sarkar, A., & Boland, M. J. (2014). Chapter 1 – The world supply of food and the role of dairy protein. In *Milk Proteins* (pp. 1–18).

Cheftel, J. C. (1992). Effects of high hydrostatic pressure on food constituents: an overview. In C. Balny, R. Hayashi, K. Heremans, & P. Masson (Eds.), *High Pressure and Biotechnology* (pp. 195–209). J. Libbey Eutotext.

Cheng, J. H., & Sun, D. W. (2015). Recent applications of spectroscopic and hyperspectral imaging techniques with chemometric analysis for rapid inspection of microbial Spoilage in Muscle foods. *Comprehensive Reviews in Food Science and Food Safety*, *14*(4), 478–490.

Christensen, J., Nørgaard, L., Bro, R., & Engelsen, S. B. (2006). Multivariate autofluorescence of intact food systems. *Chemical Reviews*, *106*(6), 1979–94.

Dalgleish, D. G., & Robson, E. W. (1985). Centrifugal fractionation of homogenized milks. *Journal of Dairy Research*, *52*(4), 539–546.

Dalsgaard, T. K., & Larsen, L. B. (2009). Effect of photo-oxidation of major milk proteins on

protein structure and hydrolysis by chymosin. *International Dairy Journal*, *19*(6–7).

Dalsgaard, T. K., Nielsen, J. H., Brown, B. E., Stadler, N., & Davies, M. J. (2011). Formation from tyrosine residues on milk proteins with globular and flexible structures as a result of riboflavin-mediated photo-oxidation. *Journal of Agricultural and Food Chemistry*, *59*, 39–47.

Datta, N., Harimurugan, P., & Palombo, E. A. (2015). Ultraviolet and pulsed light technologies in dairy processing. In *Emerging Dairy Processing Technologies: Opportunities for the Dairy Industry* (pp. 181–204).

Datta, N., Hayes, M. G., Deeth, H. C., & Kelly, A. L. (2005). Significance of frictional heating for effects of high pressure homogenisation on milk. *The Journal of Dairy Research*, *72*(4), 393–9.

Diels, A. M. J., Callewaert, L., Wuytack, E. Y., Masschalck, B., & Michiels, C. W. (2004). Moderate temperatures affect *Escherichia coli* inactivation by high-pressure homogenization only through fluid viscosity. *Biotechnology Progress*, *20*(5), 1512–1517.

Diels, A. M. J., Callewaert, L., Wuytack, E. Y., Masschalck, B., & Michiels, C. W. (2005). Inactivation of *Escherichia coli* by high-pressure homogenisation is influenced by fluid viscosity but not by water activity and product composition. *International Journal of Food Microbiology*, *101*(3), 281–291.

Diels, A. M. J., & Michiels, C. W. (2006). High-pressure homogenization as a non-thermal technique for the inactivation of microorganisms. *Critical Reviews in Microbiology*, *32*(4), 201–216.

Diez, R., Ortiz, M. C., Sarabia, L., & Birlouez-Aragon, I. (2008). Potential of front face fluorescence associated to PLS regression to predict nutritional parameters in heat treated infant formula models. *Analytica Chimica Acta*, *606*(2), 151–158.

Donato, L., & Guyomarc'h, F. (2009). Formation and properties of the whey protein/kappa-casein complexes in heated skim milk - A review. *Dairy Science & Technology*, *89*(1), 3–29.

Donsì, F., Ferrari, G., Lenza, E., & Maresca, P. (2009). Main factors regulating microbial inactivation by high-pressure homogenization: Operating parameters and scale of operation. *Chemical Engineering Science*, *64*(3), 520–532.

Dufour, E., Devaux, M. ., Fortier, P., & Herbert, S. (2001). Delineation of the structure of soft cheeses at the molecular level by fluorescence spectroscopy - Relationship with texture. *International Dairy Journal*, *11*(4–7).

Elshereef, R., Budman, H., Moresoli, C., & Legge, R. L. (2006). Fluorescence spectroscopy as a tool for monitoring solubility and aggregation behavior of β-Lactoglobulin after heat treatment. *Biotechnology and Bioengineering*, *95*(5), 863–874.

Farkye, N. Y. (2009). Evaporated and sweetened condensed milks. In *Dairy Processing & Quality Assurance* (pp. 309–318). Oxford, UK: Wiley-Blackwell.

Farrell, H. M., Jimenez-Flores, R., Bleck, G. T., Brown, E. M., Butler, J. E., Creamer, L. K.,

Swaisgood, H. E. (2004). Nomenclature of the proteins of cows' milk—sixth revision. *Journal of Dairy Science*, *87*(6), 1641–1674.

FDA. (2014). Kinetics of microbial inactivation for alternative food processing technologies - microwave. *Safe Practices for Food Processes*, (2), 1–37.

Feinberg, M., Dupont, D., Efstathiou, T., Louapre, V., & Guyonnet, J. (2006). Evaluation of tracers for the authentication of thermal treatments of milks. *Food Chemistry*, *98*(1), 188–194.

Floury, J., Bellettre, J., Legrand, J., & Desrumaux, A. (2004). Analysis of a new type of high pressure homogeniser. A study of the flow pattern. *Chemical Engineering Science*, *59*(4), 843–853.

Floury, J., Legrand, J., & Desrumaux, A. (2004). Analysis of a new type of high pressure homogeniser. Part B. study of droplet break-up and recoalescence phenomena. *Chemical Engineering Science*, *59*(6), 1285–1294.

Fox, P. F. (Ed.). (1995). *Heat-induced changes in milk* (Second Edi). International Dairy Federation.

Fox, P. F., & Chemistry, A. D. (2009). *Advanced Dairy Chemistry* (Second Edi, Vol. 1). Chapman & Hall, London.

García-Graells, C., Masschalck, B., & Michiels, C. W. (1999). Inactivation of *Escherichia coli* in milk by high-hydrostatic-pressure treatment in combination with antimicrobial peptides. *Journal of Food Protection*, *62*(11), 1248–54.

García-Risco, M. R., Olano, A., Ramos, M., & López-Fandiño, R. (2000). Micellar changes induced by high pressure influence in the proteolytic activity and organoleptic properties of milk. *Journal of Dairy Science*, *83*(10), 2184–9.

Garcıa-Risco, M. R., Ramos, M., & López-Fandiño, R. (2002). Modifications in milk proteins induced by heat treatment and homogenization and their influence on susceptibility to proteolysis. *International Dairy Journal*, *12*(8), 679–688.

Gaucheron, F. (2005). The minerals of milk. In *Reproduction nutrition development* (Vol. 45, pp. 473–483).

Georget, E., Miller, B., Aganovic, K., Callanan, M., Heinz, V., & Mathys, A. (2014). Bacterial spore inactivation by ultra-high pressure homogenization. *Innovative Food Science & Emerging Technologies*, *26*, 116–123.

Guyomarc'h, F., Law, A. J. R., & Dalgleish, D. G. (2003). Formation of soluble and micelle-bound protein aggregates in heated milk. *Journal of Agricultural and Food Chemistry*, *51*(16), 4652–4660.

Han, S.-H., Lee, J.-S., Kim, Y.-J., Kim, J.-W. J., Chang, I.-S., Chung, D. J., Kim, J.-W. J. (2007). *Quantitative characterization of degradation behaviors of antioxidants stabilized in lipid particles*. *Talanta* (Vol. 71).

Hayes, M. G., Fox, P. F., & Kelly, A. L. (2005). Potential applications of high pressure homogenisation in processing of liquid milk. *Journal of Dairy Research*, *72*(1), 25–33.

Hayes, M., & Kelly, A. (2003). High pressure homogenisation of raw whole bovine milk (a) effects on fat globule size and other properties. *Journal of Dairy Research*, *70*, 297–305.

Henle, T., Walter, H., & Klostermeyer, H. (1991). Evaluation of the extent of the early Maillard-reaction in milk products by direct measurement of the Amadori-product lactuloselysine. *Zeitschrift Fr Lebensmittel-Untersuchung Und -Forschung*, *193*(2), 119–122.

Herbert, S., Riaublanc, A., Bouchet, B., Gallant, D. J., & Dufour, E. (1999). Fluorescence spectroscopy investigation of acid-or rennet-induced coagulation of milk. *Journal of Dairy Science*, *82*(10), 2056–2062.

Holsinger, V. H. (2009). Lactose: chemistry and properties. In *Advanced Dairy Chemistry* (pp. 1–15). New York, NY: Springer New York.

Huang, Y. C., & Kuo, M. I. (2015). Rheological characteristics and gelation of tofu made from ultra-high-pressure homogenized soymilk. *Journal of Texture Studies*, *46*(5), 335–344.

Huppertz, T., Fox, P. F., & Kelly, A. L. (2004a). High pressure treatment of bovine milk: effects on casein micelles and whey proteins. *Journal of Dairy Research*, *71*(1), 97–106.

Huppertz, T., Fox, P. F., & Kelly, A. L. (2004b). Properties of casein micelles in high pressure-treated bovine milk. *Food Chemistry*, *87*(1), 103–110.

Innocente, N., Corradini, C., Blecker, C., & Paquot, M. (1998). Emulsifying properties of the total fraction and the hydrophobic fraction of bovine milk proteose-peptones. *International Dairy Journal*, *8*(12), 981–985.

Jafari, S. M., He, Y., & Bhandari, B. (2007). Production of sub-micron emulsions by ultrasound and microfluidization techniques. *Journal of Food Engineering*, *82*(4), 478–488.

Jensen, R. G. (1995a). Fat-soluble vitamins in bovine milk. In *Handbook of Milk Composition* (pp. 718–725). Elsevier.

Jensen, R. G. (1995b). General description of Milks. In *Handbook of Milk Composition* (pp. 1–3). Elsevier.

Jovanović, S., Bara, M., & Ma, O. (2005). Whey proteins-properties and possibility of application. *Mljekarstvo*, *55*(3), 215–233.

Kamal, M., & Karoui, R. (2016). Monitoring of mild heat treatment of camel milk by front-face fluorescence spectroscopy. *LWT - Food Science and Technology*, *79*, 1–8.

Karoui, R., & Debaerdemaeker, J. (2007). A review of the analytical methods coupled with chemometric tools for the determination of the quality and identity of dairy products. *Food Chemistry*, *102*(3), 621–640.

Karoui, R., Dufour, E., De Baerdemaeker, J., Dufour, É., & De Baerdemaeker, J. (2006). Common components and specific weights analysis: A tool for monitoring the molecular structure of semi-hard cheese throughout ripening. *Analytica Chimica Acta*, *572*(1), 125–

133.

Karoui, R., Mouazen, A. M., Dufour, E., Schoonheydt, R., & De Baerpemaeker, J. (2006). Utilisation of front-face fluorescence spectroscopy for the determination of some selected chemical parameters in soft cheeses. *Lait*, *86*(2).

Kilara, A., & Chandan, R. C. (2009). Ice cream and frozen desserts. In *Dairy Processing & Quality Assurance* (pp. 357–386). Oxford, UK: Wiley-Blackwell.

Kindstedt, P. (2012). Cheese and culture. Retrieved February 23, 2016, from http://www.chelseagreen.com/cheese-and-culture

Kulmyrzaev, A. A., Levieux, D., & Dufour, É. (2005). Front-face fluorescence spectroscopy allows the characterization of mild heat treatments applied to milk. relations with the denaturation of milk proteins. *Journal of Agricultural and Food Chemistry*, *53*(3), 502–507.

Lakowicz, J. R. (2006). Fluorophores. In J. R. Lakowicz (Ed.), *Principles of Fluorescence Spectroscopy* (3rd ed., pp. 63–95). Boston, MA: Springer US.

Laohasongkram, K., & Thamjariyapan, S. (1995). Kinetics of vitamin a degradation in pork liver puree during heat processing. *Thai Journal of Agricultural Science*, *28*, 229–236.

Le Maguer, I., & Jackson, H. (1983). Stability of Vitamin A in Pasteurized and Ultra-High Temperature Processed Milks. *Journal of Dairy Science*, *66*(12), 2452–2458.

MacGibbon, A. K. H., & Taylor, M. W. (2006). Composition and structure of bovine milk lipids. In *Advanced Dairy Chemistry Volume 2 Lipids* (pp. 1–42). Boston, MA: Springer US.

Malencik, D. A., & Anderson, S. R. (2003). Dityrosine as a product of oxidative stress and fluorescent probe. *Amino Acids*, *25*(3–4), 233–247.

Marra, F., Zhang, L., & Lyng, J. G. (2009). Radio frequency treatment of foods: Review of recent advances. *Journal of Food Engineering*, *91*(4), 497–508.

Medrano, A., Hernandez, A., & Prodanov, M. (1994). Riboflavin, a-tocopherol and retinol retention in milk after microwave heating. *Le Lait*, *74*(2), 153–159.

Mishra, V. K., & Ramchandran, L. (2015). Novel thermal methods in dairy processing. In *Emerging Dairy Processing Technologies: Opportunities for the Dairy Industry* (pp. 33–70).

Morales, F. (1996). Fluorescence associated with Maillard reaction in milk and milk-resembling systems. *Food Chemistry*, *57*(3), 423–428.

Mulvihill, D. M., & Ennis, M. P. (2003). Functional milk proteins: production and utilization. In *Advanced Dairy Chemistry—1 Proteins* (pp. 1175–1228). Boston, MA: Springer US.

Mussa, D. M., & Ramaswamy, H. S. (1997). Ultra high pressure pasteurization of milk: kinetics of microbial destruction and changes in physico-chemical characteristics. *LWT - Food Science and Technology*, *30*(6), 551–557.

Nakanishi, K., Sakiyama, T., & Imamura, K. (2001). On the adsorption of proteins on solid surfaces, a common but very complicated phenomenon. *Journal of Bioscience and*

Bioengineering, *91*(3), 233–244.

Nawrocka, A., & Lamorska, J. (2013). Determination of food quality by using spectroscopic methods. In S. Grundas & A. Stepniewski (Eds.), *Advances in Agrophysical Research*.

Needs, E. C., Stenning, R. A., Gill, A. L., Ferragut, V., & Rich, G. T. (2000). High-pressure treatment of milk: effects on casein micelle structure and on enzymic coagulation. *Journal of Dairy Research*, *67*(1), S0022029999004021.

Nikolaidis, A., & Moschakis, T. (2017). Studying the denaturation of bovine serum albumin by a novel approach of difference-UV analysis. *Food Chemistry*, *215*, 235–244.

O'Mahony, J. A., & Fox, P. F. (2014). Chapter 2 – Milk: An overview. In *Milk Proteins* (pp. 19–73). h

Oortwijn, H., & Walstra, P. (1979). The membranes of recombined fat globules. 2 composition. *Netherlands Milk and Dairy Journal*, *33*, 134–154.

Öste, R., Jägerstad, M., & Andersson, A. (2009). Vitamins in milk and milk products. In *Advanced Dairy Chemistry Volume 3*.

Panfili, G., Manzi, P., Pizzoferrato, L., Panfill, G., Manzi, P., & Pizzoferrato, L. (1998). Influence of thermal and other manufacturing stresses on retinol isomerization in milk and dairy products. *Journal of Dairy Research*, *65*(2), 253–260.

Patel, H. A., & Huppertz, T. (2014). Chapter 8 – Effects of High-pressure processing on structure and interactions of milk proteins. In *Milk Proteins* (pp. 243–267).

Patrignani, F., & Lanciotti, R. (2016). Applications of High and Ultra High Pressure Homogenization for Food Safety. *Frontiers in Microbiology*, *7*, 1132.

Pereda, J., Ferragut, V., Quevedo, J. M., Guamis, B., & Trujillo, A. J. (2007). Effects of ultra-high pressure homogenization on microbial and physicochemical shelf life of milk. *Journal of Dairy Science*, *90*(3), 1081–1093.

Pereda, J., Ferragut, V., Quevedo, J. M., Guamis, B., & Trujillo, A. J. (2008). Effects of ultra-high-pressure homogenization treatment on the lipolysis and lipid oxidation of milk during refrigerated storage. *Journal of Agricultural and Food Chemistry*, *56*(16), 7125–30.

Pereda, J., Ferragut, V., Quevedo, J. M., Guamis, B., & Trujillo, A. J. (2009). Heat damage evaluation in ultra-high pressure homogenized milk. *Food Hydrocolloids*, *23*(7), 1974–1979.

Picart, L., Thiebaud, M., René, M., Guiraud, J. P., Cheftel, J. C., & Dumay, E. (2006). Effects of high pressure homogenisation of raw bovine milk on alkaline phosphatase and microbial inactivation. A comparison with continuous short-time thermal treatments.

Qi, P. X., Ren, D., Xiao, Y., & Tomasula, P. M. (2015). Effect of homogenization and pasteurization on the structure and stability of whey protein in milk. *Journal of Dairy Science*, *98*(5), 2884–97.

Rampon, V., Genot, C., Riaublanc, A., Anton, M., Axelos, M. A. V, & McClements, D. J. (2003). Front-face fluorescence spectroscopy study of globular proteins in emulsions: Influence of

droplet flocculation, *51*(9), 2490–2495.

Revilla, I., Escuredo, O., González-Martín, M. I. I., & Palacios, C. (2017). Fatty acids and fat-soluble vitamins in ewe's milk predicted by near infrared reflectance spectroscopy. Determination of seasonality. *Food Chemistry*, *214*, 468–477.

Roach, A., & Harte, F. (2008). Disruption and sedimentation of casein micelles and casein micelle isolates under high-pressure homogenization. *Innovative Food Science and Emerging Technologies*, *9*(1), 1–8.

Roig-Sagués, A. X., Velázquez, R. M., Montealegre-Agramont, P., López-Pedemonte, T. J., Briñez-Zambrano, W. J., Guamis-López, B., & Hernandez-Herrero, M. M. (2009). Fat content increases the lethality of ultra-high-pressure homogenization on *Listeria monocytogenes* in milk. *Journal of Dairy Science*, *92*(11), 5396–5402.

Salazar-González, C., San Martín-González, M. F., López-Malo, A., & Sosa-Morales, M. E. (2012). Recent studies related to microwave processing of fluid foods. *Food and Bioprocess Technology*, *5*(1), 31–46.

Salo-Väänänen, P., Ollilainen, V., Mattila, P., Lehikoinen, K., Salmela-Mölsä, E., & Piironen, V. (2000). Simultaneous HPLC analysis of fat-soluble vitamins in selected animal products after small-scale extraction. *Food Chemistry*, *71*(4), 535–543.

Sampedro, F., & Rodrigo, D. (2015). Pulsed electric fields (PEF) processing of milk and dairy products. In N. Datta & P. M. Tomasula (Eds.), *Emerging Dairy Processing Technologies: Opportunities for the Dairy Industry* (First Edit). JohnWiley& Sons, Ltd.

Sánchez, R. J., Mateo, C. M., Fernández, M. B., & Nolasco, S. M. (2017). Bidimensional modeling applied to oil extraction kinetics of microwave-pretreated canola seeds. *Journal of Food Engineering*, *192*, 28–35.

Sandra, S., & Dalgleish, D. G. (2007). The effect of ultra high-pressure homogenization (UHPH) on rennet coagulation properties of unheated and heated fresh skimmed milk. *International Dairy Journal*, *17*(9), 1043–1052.

Sandra, S., & Dalgleish, D. G. G. (2005). Effects of ultra-high-pressure homogenization and heating on structural properties of casein micelles in reconstituted skim milk powder. *International Dairy Journal*, *15*(11), 1095–1104.

Schamberger, G. P., & Labuza, T. P. (2006). Evaluation of Front-face Fluorescence for Assessing Thermal Processing of Milk. *Journal of Food Science*, *71*(2), C69–C74.

Scheidegger, D., Larsen, G., & Kivatinitz, S. C. (2016). Oxidative consequences of UV irradiation on isolated milk proteins : Effects of hydrogen peroxide and bivalent metal ions. *International Dairy Journal*, *55*, 64–71.

Scheidegger, D., Pecora, R. P., Radici, P. M., & Kivatinitz, S. C. (2010). Protein oxidative changes in whole and skim milk after ultraviolet or fluorescent light exposure. *Journal of Dairy Science*, *93*(11), 5101–9.

Scheidegger, D., Radici, P. M., Vergara-Roig, V. A., Bosio, N. S., Pesce, S. F., Pecora, R. P.,

Kivatinitz, S. C. (2013). Evaluation of milk powder quality by protein oxidative modifications. *Journal of Dairy Science*, *96*(6), 3414–23.

Serra, M., Trujillo, A. J., Quevedo, J. M., Guamis, B., & Ferragut, V. (2007). Acid coagulation properties and suitability for yogurt production of cows' milk treated by high-pressure homogenisation. *International Dairy Journal*, *17*(7), 782–790.

Sharma, R., Singh, H., & Taylor, M. W. (1996). Composition and structure of fat globule surface layers in recombined milk. *Journal of Food Science*, *61*(1).

Sharma, S. K., & Dalgleish, D. G. (1993). Interactions between milk serum proteins and synthetic fat globule membrane during heating of homogenized whole milk. *Journal of Agricultural and Food Chemistry*, *41*(9), 1407–1412.

Shimamura, T., & Ukeda, H. (2012). Maillard reaction in milk – effect of heat treatment. In W. L. Hurley (Ed.), *Milk Protein*.

Singh, H., Boland, M., Thompson, A., Boland, M., & Singh, H. (2014). *Milk proteins : from expression to food*. *Science And Technology*. Amsterdam : Elsevier/Academic Press.

Smelt, J. P. P. . (1998). Recent advances in the microbiology of high pressure processing. *Trends in Food Science & Technology*, *9*(4), 152–158.

Smiddy, M. A., Martin, J.-E., Huppertz, T., & Kelly, A. L. (2007). Microbial shelf-life of high-pressure-homogenised milk. *International Dairy Journal*, *17*(1), 29–32.

Smoczyński, M. (2016). The effect of low- and high-pressure homogenization on in vitro milk fat digestion by lipase. *Polish Journal of Natural Sciences*, *31*(2).

Stănciuc, N., Aprodu, I., Râpeanu, G., Bahrim, G., Stănciuc, N., Aprodu, I., Bahrim, G. (2012). Fluorescence spectroscopy and molecular modeling investigations on the thermally induced structural changes of bovine β-lactoglobulin. *Innovative Food Science and Emerging Technologies*, *15*, 50–56.

Strasburg, G. M., & Ludescher, R. D. (1995). Theory and applications of fluorescence spectroscopy in food research. *Trends in Food Science & Technology*, *6*(3), 69–75.

Taterka, H., & Castillo, M. (2015). The effect of whey protein denaturation on light backscatter and particle size of the casein micelle as a function of pH and heat-treatment temperature. *International Dairy Journal*, *48*(July 2016), 53–59.

Taterka, H., & Castillo, M. (2017). Analysis of the preferential mechanisms of denaturation of whey protein variants as a function of temperature and pH for the development of an optical sensor. *International Journal of Dairy Technology*, *70*(2000), 1–10.

Tessier, F. J., Gadonna-Widehem, P., & Laguerre, J. C. (2006). The fluorimetric FAST method, a simple tool for the optimization of microwave pasteurization of milk, *50*(9), 793–798.

Thiebaud, M., Dumay, E., Picart, L., Guiraud, J. P., & Cheftel, J. C. (2003). High-pressure homogenisation of raw bovine milk. Effects on fat globule size distribution and microbial inactivation. *International Dairy Journal*, *13*(6), 427–439.

Tobin, J., Heffernan, S. P., Mulvihill, D. M., Huppertz, T., & Kelly, A. L. (2015). Applications of high-pressure homogenization and microfluidization for milk and dairy products. *Emerging Dairy Processing Technologies: Opportunities for the Dairy Industry*, 93–114.

Trapani, S., Guerrini, L., Masella, P., Parenti, A., Canuti, V., Picchi, M., Zanoni, B. (2017). A kinetic approach to predict the potential effect of malaxation time-temperature conditions on extra virgin olive oil extraction yield. *Journal of Food Engineering*, *195*, 182–190.

Trujillo, A. J., Capellas, M., Saldo, J., Gervilla, R., & Guamis, B. (2002). Applications of high-hydrostatic pressure on milk and dairy products: a review. *Innovative Food Science & Emerging Technologies*, *3*(4), 295–307.

Trujillo, A. J., Roig-Sagués, A. X., Zamora, A., & Ferragut, V. (2016). High-pressure homogenization for structure modification. In *Innovative Food Processing Technologies* (pp. 315–344).

Tu, Z.-C., Zhang, L., Wang, H., Huang, X.-Q., Zhang, L., & Yang, K. (2014). Structure and antioxidant activity of milk model systems after microwave heating. *Food Science and Technology Research*, *20*(2), 345–355.

Tunick, M. H., Ren, D. X., Van Hekken, D. L., Bonnaillie, L., Paul, M., Kwoczak, R., & Tomasula, P. M. (2016). Effect of heat and homogenization on in vitro digestion of milk. *Journal of Dairy Science*, *99*(6), 4124–4139.

Tydeman, M. S., & Kirkwood, T. B. L. (1984). Design and analysis of accelerated degradation tests for the stability of biological standards I. Properties of maximum likelihood estimators. *Journal of Biological Standardization*, *12*(2), 195–206.

Vercet, A., Oria, R., Marquina, P., Crelier, S., & Lopez-Buesa, P. (2002). Rheological properties of yoghurt made with milk submitted to manothermosonication. *Journal of Agricultural and Food Chemistry*, *50*(21), 6165–71.

Vernam, A.H. and Sutherland, J. P. (1994). Milk and milk Products: technology, chemistry and microbiology. Chapman & Hall, London.

Weiser, H., & Somorjai, G. (1992). Bioactivity of cis and dicis isomers of vitamin A esters. *International Journal for Vitamin and Nutrition Research*, *62*(3), 201–8.

Wijayanti, H. B., Bansal, N., & Deeth, H. C. (2014). Stability of whey proteins during thermal processing: A Review. *Comprehensive Reviews in Food Science and Food Safety*, *13*(6), 1235–1251.

Wilkinson, S. A., Earle, M. D., & Cleland, A. C. (1981). Kinetics of Vitamin A Degradation in Beef Liver Puree on Heat Processing. *Journal of Food Science*, *46*(1), 32–40.

Wu, H., Hulbert, G. J., & Mount, J. R. (2000). Effects of ultrasound on milk homogenization and fermentation with yogurt starter. *Innovative Food Science & Emerging Technologies*, *1*(3), 211–218.

Ye, A., Singh, H., Taylor, M., & Anema, S. (2004a). Interactions of whey proteins with milk fat globule membrane proteins during heat treatment of whole milk. *Lait*, *84*, 269–283.

Ye, A., Singh, H., Taylor, M. W., & Anema, S. G. (2004b). Interactions of fat globule surface proteins during concentration of whole milk in a pilot-scale multiple-effect evaporator. *Journal of Dairy Research*, *71*(4), 471–479.

Zamora, A., Ferragut, V., Guamis, B., & Trujillo, A. J. (2012). Changes in the surface protein of the fat globules during ultra-high pressure homogenisation and conventional treatments of milk. *Food Hydrocolloids*, *29*(1), 135–143.

Zamora, A., Ferragut, V., Jaramillo, P. D., Guamis, B., & Trujillo, A. J. (2007). Effects of ultra-high pressure homogenization on the cheese-making properties of milk. *Journal of Dairy Science*, *90*(1), 13–23.

Zamora, A., Ferragut, V., Juan, B., Guamis, B., & Trujillo, A. J. (2011). Effect of ultra-high pressure homogenisation of milk on the texture and water-typology of a starter-free fresh cheese. *Innovative Food Science & Emerging Technologies*, *12*(4), 484–490.

Zamora, A., & Guamis, B. (2014). Opportunities for ultra-high-pressure homogenisation (UHPH) for the food industry. *Food Engineering Reviews*, *7*(2), 130–142.

Zettel, V., Ahmad, M. H., Beltramo, T., Hermannseder, B., Hitzemann, A., Nache, M., Hitzmann, B. (2016). Supervision of food manufacturing processes using optical process analyzers - An Overview. *ChemBioEng Reviews*, *3*(5), 219–228.