"Pressure effect is roughly proportional to the complexity of the molecule"

Bridgman (1915)

University of Alberta

Kinetics studies of chemical reactions and quality changes in conjugated linoleic acid (CLA) enriched milk treated with high-pressure sterilization

by

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To my wife for her love and support and to my son who gives meaning to everything

Abstract

Conjugated linoleic acid (CLA), a bioactive lipid naturally found in milk, is thermally degraded through oxidation during thermal processing. Finding alternatives to enhance the retention of CLA is challenging. In this thesis, high-pressure sterilization (HPS) was used to enhance CLA retention in milk. In addition, the effect of HPS on some quality indicators was evaluated, including lactulose formation, inactivation of alkaline phosphatase, and retention of CLA during storage. When HPS was applied (600 MPa and 120˚C), ~78% of the CLA was retained after 15 min, while only ~20% was retained after 15 min at 0.1 MPa and 120˚C. The retention kinetics was represented with Weibull model. To further improve the retention of CLA in milk, seven antioxidants, mainly phenolic acids, were evaluated. Gallic acid and catechin resulted in the highest retention of CLA after treatments at 600 MPa and 120˚C. Temperature and pressure accelerated the formation of lactulose, with a maximum value of 650 mg L^{-1} at 120˚C, 600 MPa and 15 min. The HPS conditions needed to reduce 7-log of *Bacillus amyloliquefaciens* endospores in inoculated milk were determined as well as the effect of adding nisin (4-64 mg L^{-1} milk). The inactivation kinetics of alkaline phosphatase was determined. Results showed that the addition of nisin (≥16 mg L^{-1}) significantly enhanced the inactivation of *Bacillus amyloliquefaciens* (7-log reduction) after 5 min of treatment at 600 MPa and 120˚C, while without nisin 10 min were required to achieve the same log reduction. For storage, milk treated at 600 MPa and 120˚C for 5 min was selected to evaluate the impact of HPS on the CLA retention and formation of hydroperoxides. During storage, milk with nisin added and treated with HPS delivered higher CLA retention and lower hydroperoxides concentration compared with the UHT equivalent process. The kinetic information obtained was used to build pressure-temperature diagrams for CLA retention and lactulose formation. These data can be further used to design HPS processes, achieving high CLA retention.

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List of Abbreviations

AA	Ascorbic acid
A_f	Accuracy factor
ALP	Alkaline phosphatase
AMF	Anhydrous milk fat
C_t	Concentration of a given compound at a given time
C_o	Initial concentration of a given compound
CAF	Caffeic acid
CAT	Catechin
CI95%	95% confidence interval
CLA	Conjugated linoleic acid
CUM	ρ-Coumaric acid
Cyt	Cysteine
DHA	Dehydroascorbic acid
DKA	2,3-diketogulunic acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
DSC	Differential scanning calorimetry
DO_2	Dissolved oxygen
E_a	Apparent activation energy
FAME	Fatty acid methyl esters
GA	Gallic acid
H_r	Heating rate
H-CLA	Anhydrous milk fat with high CLA content
HPP	High-pressure processing
HPLC	High performance liquid chromatography
HPS	High-pressure sterilization
HTST	High-temperature-short-time pasteurization
K	Equilibrium constant
K_a	Equilibrium constant of ionization
k	Rate constant

k_p	Rate constant at constant temperature
k_T	Rate constant at constant pressure
k_q	Rate constant of quenching
k_{pred}	Predicted values of rate constant
k_{obs}	Observable values of rate constant
k_{iso}	Isokinetic rate constant
L-CLA	Anhydrous milk fat with low CLA content
M-CLA	Anhydrous milk fat with medium CLA content
MFGM	Milk fat globule membrane
MSE	Mean square error
n	Reaction order
OSI	Oxidative stability index
PATS	Pressure-assisted thermal sterilization
PE	Phosphatidylethanolamine
pk_a	dissociation constant
PV	Peroxide value
P_{ref}	Reference pressure
r	reaction rate
R	Universal gas constant
R^2	Coefficient of determination
R^2_{adj}	Adjusted coefficient of determination
SE	Standard error
SSmin	Minimum sum of squares of residuals
TAN	Tannic acid
t	Holding time
t_c	Compression time
t_d	Decompression time
t_h	Holding time
t_l	Loading time
T_{iso}	Isokinetic temperature
T_{on}	Onset temperature of oxidation

T_{ref}	Reference temperature
T_s	Start temperature of oxidation
T_p	Maximum heat flow temperature
TVA	*trans*-Vaccenic acid
UHT	Ultra-high-temperature pasteurization
z	Pre-exponential factor
ΔV°	Reaction volume
$\Delta V^{\neq}$	Activation volume
$\Delta V_{int}^{\neq}$	Molar volume due to intrinsic parameters
$\Delta V_m^{\neq}$	Molar volume due to medium
$\Delta V_o^{\neq}$	Volume change with respect to native state
$\Delta \kappa_o^{\neq}$	Comprensibility change
$\Delta S_o^{\neq}$	Entropy change
$\Delta \varepsilon_o^{\neq}$	Expansibility change
$\Delta Cp_o^{\neq}$	Heat capacity change
ΔDO	Optical density changes

Greek letters

α	Scale parameter of Weibull model
α_{ref}	Scale parameter at a reference temperature
α_T	Scale parameter at a reference temperature
α_P	Scale parameter at a reference pressure
β	Shape constant of Weibull model
θ	Degree of conversion

Chapter 1

Introduction and objectives

Altering milk fat composition through nutritional manipulation has created new opportunities for development of functional drinks with commercial applications (Jenkins & McGuire, 2006). An attractive modification is to increase the concentration of bioactive lipids, improving the nutritional properties of milk. One group of fatty acids of particular interest is conjugated linoleic acid (CLA), which has shown health-promoting and disease-preventing properties, including cancer prevention, atherosclerosis, weight control, and bone formation (Wang et al., 2012). Remarkably, the concentration of CLA in milk has been increased up to 10-fold (Bell et al., 2006), which enhances the use of milk as a source of bioactive lipids. Unfortunately, CLA is thermally degraded through oxidation (Herzallah et al., 2005). Therefore, designing a process that guaranties food safety and retains valuable compounds is a major challenge that requires the use of emerging technologies, such as high-pressure sterilization (HPS).

The recent HPS technology that simultaneously applies high pressure (100-600 MPa) and high temperature (90-120˚C) has become a valuable alternative to process superior quality products in cases where traditional thermal treatments have failed to deliver high quality products (Heinz & Buckow, 2010; Juliano et al., 2012). Currently, there is no data available on milk rich in CLA treated with HPS conditions. In addition, research is also needed on reactions occurring in milk under HPS conditions.

The hypothesis of this thesis is that HPS can not only enhance CLA retention but also it can deliver shelf stable milk. The main objective of this thesis was to evaluate the use of HPS and antioxidants on CLA retention in CLA-enriched milk after HPS and storage conditions, aiming to understand some important chemical reactions occurring in milk at HPS conditions. The specific objectives were to:

- assess the retention kinetics of CLA in heated enriched-CLA milk at 90-120°C and model the kinetics (Chapter 3),
- evaluate the effect of CLA content on the oxidation kinetics of anhydrous milk fat (Chapter 4),
- evaluate the oxidative stability of CLA-enriched milk treated with ultra-high-temperature (UHT) processing (Chapter 5),
- evaluate the effect of HPS on CLA retention in enriched-CLA milk and enriched-CLA anhydrous milk fat (Chapter 6),
- evaluate the retention kinetics of CLA in milk treated with HPS process (Chapter 7),
- assess the use of seven different antioxidants on the retention of CLA of milk treated at HPS conditions (Chapter 8),
- evaluate lactulose formation in milk treated at HPS conditions (Chapter 9), and
- find the HPS conditions at which endospores of *B. amyloliquefaciens* are inactivated in milk with and without nisin; to evaluate the inactivation of alkaline phosphatase, to assess the effect of HPS treatment on CLA retention and hydroperoxide formation during storage, and to compare the impact of such processing conditions on the retention of CLA with the equivalent UHT treatment (Chapter 10).

The obtained results provide valuable kinetic information on CLA retention, lactose isomerization, enzyme and spore inactivation at HPS conditions, leading to new opportunities for product development of milk-based functional drinks.

1.1. References

Bell, J. A., Griinari, J. M., & Kennelly, J. J. (2006). Effect of safflower oil, flaxseed oil, monensin, and vitamin e on concentration of conjugated linoleic acid in bovine milk fat. *Journal of Dairy Science, 89*(2), 733-748.

Heinz, V., & Buckow, R. (2010). Food preservation by high pressure. *Journal of Consumer Protection and Food Safety, 5*, 73–81.

Herzallah, S. M., Humeid, M. A., & Al-Ismai, K. M. (2005). Effect of heating and processing methods of milk and dairy products on conjugated linoleic acid and trans fatty acid isomer content. *Journal of Dairy Science, 88*(4), 1301–1310.

Jenkins, T. C., & McGuire, M. A. (2006). major advances in nutrition: impact on milk composition. *Journal of Dairy Science, 89*, 1302–1310.

Juliano, P., Bilbao-Sáinz, C., Koutchma, T., Balasubramaniam, V. M., Clark, S., Stewart, C. M., Dunne, C. P. & Barbosa-Canovas, G. V. (2012). Shelf-stable egg-based products processed by high pressure thermal sterilization. *Food Engineeering Reviews, 4*(1), 55-67.

Wang, Y., Jacome-Sosa, M. M., & Spencer, D. P. (2012). The role of ruminant trans fat as a potential nutraceutical in the prevention of cardiovascular disease. *Food Research International, 46*(2), 460–468.

Chapter 2

Literature review[1]

2.1. High pressure principles

Studies on the application of high pressure processing (HPP) in different food systems have shown that pressure inactivates microorganisms in vegetative form without significantly changing the sensory and nutritional properties (Mathys et al., 2009). A recent new technology that simultaneously applies high pressure (up to 600 MPa) and elevated temperature (~90-120°C) has been successfully used for the inactivation of bacterial spores (Mathys et al., 2009). This new technology, known as pressure assisted thermal sterilization (PATS) or high pressure sterilization (HPS) was first developed to produce shelf-stable mashed potato (Sizer et al., 2002; Leadley et al., 2008). In PATS treatments where a pre-heated sample is pressurized, the rise in temperature due to adiabatic heating is used to reach the target or sterilization temperature (Ting et al., 2002). The increase in the temperature is a great advantage because changes in the temperature of a pre-heated food are rapid and uniform. It also provides a uniform distribution of temperature, reducing the thermal damage that takes place in traditional sterilization.

One important principle of high pressure treatments at either moderate or elevated temperatures is that a fluid is used to transmit the pressure throughout the food and therefore allows a true hydrostatic condition, regardless of the magnitude of the pressure, time, and space. Consequently, pressure, and therefore its effects are instantaneously and homogenously distributed within the food material, independently of food geometry and size of the equipment. This unique characteristic has enabled process development, contributing to successful commercial applications.

Another important characteristic of pressure treatment is that pressure alters interatomic distances, acting only on those weak interactions, for which

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[1] *A version of this chapter is to be submitted to Critical Reviews in Food Science and Technology for consideration for publication*

bond energy is distance dependent, such as van der Waals forces, electrostatic forces,and hydrogen bonding (**Table 2-1**). Based on the distance dependenence a pressurized sample would keep its strong bonding (mainly covalent bonds) intact. This theory has been the central hypothesis in preserving the biological activity of functional compounds, such as ascorbic acid (Oley et al., 2006), folates (Butz et al., 2004), antioxidants (Matser et al., 2004) and anthocyanins (Verbeyst et al., 2010).

Table 2-1. Possible pressure effect based on interatomic distance of different interactions (adapted from Walstra (2002)).

Type of interaction	Working distance [nm]	Distance dependence	Possible pressure effect
Coulomb	20	Proportional inversely	Highly affected
van der Waals	1-20	Optimum distance	Highly affected
Hydrogen bonding	0.2	Quadratic	Affected
Solvation	>2	Exponential	Affected
Hydrophobic	>2	Exponential	Affected
Covalent	0.2	Complicated	Unlikely affected

There are three important consequences of altering the interatomic distance due to the application of high hydrostatic pressure: 1) changes in physical properties, such as melting point, solubility, density, viscosity, etc.; 2) effects on equilibrium processes, such as dissociation of weak acids, acid-base equilibria, ionization, etc.; 3) effects on rates of processes, such as delaying or accelering the rate at which a particular reaction occurs. In pressure treated products, some intrinsic quality attributes are a result of these three phenomena occurring when affected by pressure. For instance, inactivation of microorganisms is a combination of changes in physical properties of membrane lipids, changes in the chemical equilibrium that modify the internal pH, and changes in the rate of

specific physiological functions that cause irreversible or lethal damage on bacteria cells (Molina-Guitierrez et al., 2002).

Table 2-2 provides various examples on how food quality attributes are dictated by the way of pressure affects the physical, equilibrium and rate processes. A rate process was considered when pressure increase or decrease the concentration of a particular compound.

2.2. Kinetics of chemical reactions

2.2.1 Kinetics at isobaric conditions

In general, food systems under pressure have the tendency to undergo biochemical, enzymatic or chemical changes as a function of processing and storage time (van Boekel, 2008). Chemical reactions are those interactions that result in the disappearance of existent molecules or formation of new species (atoms are arranged differently than the original reactant molecules).

A chemical reaction proceeds when the molecular positions between the reagents and products gradually rearrange into a critical position or activated state (Isaacs, 1981). This state is induced by the collision of reagent molecules. The activated state is the most energetically favorable position (Polanyi, 1937). It represents a maximum energy relative to the molecular motion and a minimum energy with respect to the reaction coordinate (Elyanov & Hamann, 1975). Although the activated state is a very short-lived complex, it has its own thermodynamic properties. The activated state concept is also known as the transition state theory and provides a useful link between kinetic data and molecular rearrangements.

During the course of a reaction at constant temperature and pressure, the rate of a reaction (r) is usually evaluated by monitoring the concentration of either reactants or products (C) over time (t), applying the law of mass action as described elsewhere (van Boekel, 2008).

Table 2-2. Examples on how food quality attributes are dictated by the effect of pressure on physical properties, equilibrium and rate processes.

Quality attribute	Effect upon pressure treatment		
	Physical properties	**Equilibrium Processes**	**Rate Processes**
Volatile formation Aroma development in onions (Butz et al., 2004)	- Disruption of cell integrity that brought phenols and enzymes together	- Changes in the pH value that affect enzyme activity	- Decrease in the concentration of dipropyldisulfide
Color and Appearance Color degradation in broccoli juice (Van Loey et al., 1998)	- Cell damage that releases different enzymes	- Shifts the equilibrium that triggers different mechanisms	- Decrease in the reaction rate
Changes in brightness in cow milk (Needs et al., 2000)	- Disruption of casein micelles - Changes in the solubility of ions and minerals	- Shifts the equilibrium between colloidal and soluble calcium - Shifts the pH value	- Upon pressure release, some changes are partially reversible
Nutritional properties Stability of thiamin and riboflavin in pork and model systems (Butz et al., 2007)	- Physically bound vitamins were released by pressure	- Shifts the pH value that might trigger different mechanisms	- Pressure increases the rate constant without affecting the activation energy
Ascorbic acid degradation (Oley et al., 2006)	- Solubility of small compounds creates a protective effect	- Degradation mechanisms of ascorbic acid are highly dependent on the pH	- Temperature and pressure act synergistically on the degradation reaction rate
Functionality Milk fat crystallization in emulsion (Buchheim & Abouelnour 1992)	- Changes in the melting curve measured by DSC - Changes in the solubility that yields different solid fat content	- Shifts the equilibrium between attractive and repulsive forces	- Pressure affects fat crystallization
Starch dispersion (Buckow et al., 2007)	- Changes in viscosity of starch suspension - Changes in rheological properties	- Shifts the chemical balance	- Retrogradation rate is affected by pressure
Microorganism inactivation Pressure induced inactivation in microorganisms (Smelt, 1998)	- Cell damage, membrane lipids change physical state	- Intracellular pH shifts - Changes in equilibrium in solute transport	- Decreased DNA synthesis
Spore inactivation Bacterial spore inactivation reviewed (Black, 2007)	- Changes in viscosity and transport properties	- Shift the osmotic equilibrium - Shifts the equilibrium of electrolytes	- Inactivation rate is affected by pressure
Sensory and texture Cheddar cheese treated by HPP to improve visual and appearance (Serrano et al., 2004)	- Surface adhesion and flexibility of protein - Disruption of the para-casein network	- Shift the equilibrium between solutes and water	- Accelerates hydrolysis of animo acids

$$r = -\frac{dC}{dt} = kC^n \qquad (2.1)$$

The term *n* is the reaction order and indicates how the reaction rate varies with the concentration of reactants and products. The reaction order is an empirical concept, which is used to mathematically describe the experimental data. Kinetics

of chemical reactions in food systems are usually expressed as a zero-, 1st-, or 2nd-order reactions (**Table 2-3**).

Table 2-3. Differential, integrated and linear forms of the kinetic models typically used in food systems. The equations represent elementary reactions in terms of reactants.

Reaction order	Differential equation	Integrated form	Linearized form
Zero	$-\frac{dc}{dt} = k$ (2.2)	$c = c_o - kt$ (2.3)	$c = c_o - kt$ (2.4)
First	$-\frac{dc}{dt} = kc$ (2.5)	$c = c_o exp^{(-kt)}$ (2.6)	$\ln c = \ln c_o - kt$ (2.7)
Second	$-\frac{dc}{dt} = kc^2$ (2.8)	$c = \frac{c_o}{1+c_o kt}$ (2.9)	$\frac{1}{c} = \frac{1}{c_o} + kt$ (2.10)

The influence of temperature on the rate of a chemical reaction is mathematically described by the Arrhenius law in the form:

$$k = z\, exp^{\left(\frac{-E_a}{RT}\right)} \quad (2.11)$$

where *z* is the pre-exponential factor, E_a is the activation energy and R is the gas constant. The activation energy is often seen as the energetic barrier that molecules need to overcome in order to be able to react. Reactive molecules collide with a certain frequency (*z*), providing the energy needed to react or overcome the energetic barrier. The frequency or pre-exponential factor (*z*) is also interpreted as the reaction rate when there is no energetic barrier (E_a = 0) (van Boekel, 2008). Although reactions in food systems are complex, the Arrhenius equation has been successfully applied to describe the influence of temperature in food systems.

2.2.2. Kinetics at isothermal conditions

Kinetics of chemical reactions as a function of pressure at isothermal conditions is governed by van't Hoff like equations:

$$\left(\frac{\partial lnK}{\partial P}\right)_T = -\frac{\Delta V^\circ}{RT} \quad (2.12)$$

$$\left(\frac{\partial ln\, k}{\partial P}\right)_T = -\frac{\Delta V^{\neq}}{RT} \quad (2.13)$$

where K and k are the equilibrium constant and the rate constant, respectively; ΔV° is the reaction volume and $\Delta V^{\neq}$ is the activation volume.

Two principles can explain the effect of pressure on kinetics of chemical reactions: i) le Chatelier principle and ii) the transition state theory. The first principle applies for a reaction system in equilibrium and it states that if a particular extensive variable changes for example pressure, the equilibrium shifts in the direction that tends to reduce the change in the corresponding intensive variable, volume (Jenner, 2004). In the case of PATS treatments that involve the simultaneous application of elevated temperature and pressure, the equilibrium not only shifts in the direction of the highest enthalpy for an endothermic reaction but also in the direction of the smallest molar volume.

The second principle states that if the molar volume of the intermediate state (activated complex) differs from that of its reacting components, the reaction velocity can increase or decrease by changing pressure, according to whether the intermediate state is less or more voluminous (Wentorf & De Vries, 2001). For a given chemical reaction, the effect of pressure favours those reactions with negative reaction volumes and those reaction pathways with negative activation volumes.

The relative short processing times and the complexity of food matrices make it difficult to reach equilibrium for a given chemical reaction. Food matrices are very complex systems with various components that simultaneously undergo chemical transformations at different rates. Consequently, the rate at which the reactions occur is often more important than the equilibrium and therefore most of

the literature considers the activation volume as an indicator whether a particular reaction is promoted or inhibited by pressure. The $\Delta V^{\neq}$ is a reflection of all volume changes that might occur during the progression of a reaction from ground to the transition state and within the transition state. The $\Delta V^{\neq}$ basically represents those changes in molar volume due to molecular reorganization, alteration of bond angles and length (known as intrinsic changes, $\Delta V_{int}^{\neq}$), and those changes due to interactions of the reactants with the solvent (known as medium or solvent changes, $\Delta V_{m}^{\neq}$).

$$\Delta V^{\neq} = \Delta V_{int}^{\neq} + \Delta V_{m}^{\neq} \tag{2.14}$$

The term $\Delta V_{m}^{\neq}$ represents the solvent effects, such as changes in polarity, electrostatic forces, dipole interactions and solvophobic interactions. If $\Delta V_{m}^{\neq}$ is zero, the reaction is isopolar and the effect of pressure is less pronounced. On the other hand, if $\Delta V_{m}^{\neq}$ is different from $\Delta V^{\neq}$, the medium plays a critical role in the reaction kinetics. The solvent effects in organic reactions at high pressures were reviewed by Jenner (2004).

The $\Delta V^{\neq}$ cannot be measured experimentally because the activated state is very unstable. Nevertheless, a common practice for estimating the $\Delta V^{\neq}$ is to evaluate the effect of pressure on the constant reaction rate at isothermal conditions in the form of equation (2.13). This equation is also known as the Eyring equation (Eyring, 1935) that considers the influence of pressure on the velocity of chemical reactions in a solution. Eventually, Evans & Polanyi (1937) exemplified this relationship for various organic reactions.

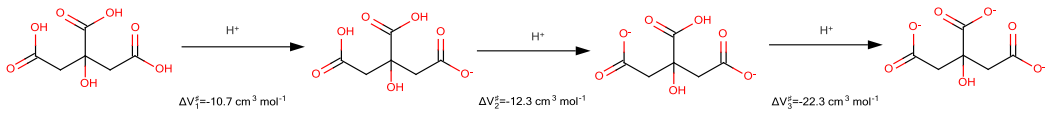
2.3. Ionization

This section includes discussion on reactions that generate a charged particle (ion) from neutral species. In these reactions, water molecules (solvent) play a critical role in the reaction pathways. When an ion is created in a liquid medium, it causes a breakdown of the structure of the surrounding liquid, creating

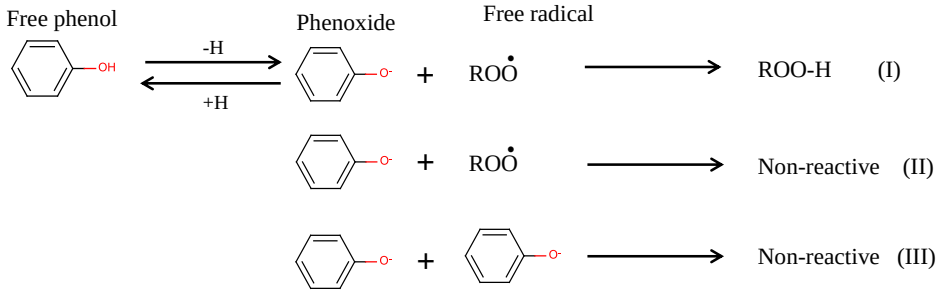
a strong electric field within the surrounding solvent molecules. The strong electric field decays rapidly with the distance from the ions. Consequently, solvent molecules in the first hydration shell are situated in a field of considerably higher strength than those molecules located outside the shell (Danielewicz-Ferchmin & Ferchmin 1998). A strengthened electric field around ions attracts more solvent molecules and therefore the volume of the solvent in the hydration shell is smaller than that in the bulk. Indeed, Monte Carlo and molecular dynamic simulations of Li^+ F^- ion pair in water showed that the first hydration shell contains between 1 and 2 more water molecules than it would have if it had the normal density of water (Hamann, 1981). This volume contraction due to a charged particle is known as electrostriction, a term coined by Drude & Nernst (1894). Electrostriction depends on the charge and size of the ion and solvent properties (Marcus, 2005). Burgess et al. (2010) correlated partial molar volumes with intrinsic volumes of different complexes of ions and cations and such correlation plots showed deviations from ideality for small complexes, reflecting the effects of electrostriction, while for large complexes the electrostrictive effects became negligible.

According to le Chatelier's principle, an increase in pressure favours those processes, which tend to reduce the volume. Pressure enhances the dissociation into ions of ionisable substances, such as salts, acids, bases, and polyelectrolytes. The formation of ions leads to a reversible and temporary change of pH that is re-established upon pressure release (Elyanov & Hamann 1975; Hamann, 1982). From a food processing perspective, the shift in the pH is an important characteristic of high pressure experiments because pH not only controls protein denaturation (Smeller, 2002) and growth of microorganisms (Molina-Guitierrez et al., 2002 Mathys et al., 2009) but also influences the kinetics of chemical reactions (Hamann, 1981; Jenner, 2004). In organic synthesis, due to its effect on the formation of ions, pressure is considered as an efficient activation method since it influences the formation of charged species in the transition state (Jenner, 2002).

Kitamura & Itoh (1987) reported that the reaction volume of ionization of citric acid becomes negatively larger in every step from -11 to -22 cm^3 mol^{-1} (Scheme 1), suggesting that citric acid became ionized by pressure. Phenols are susceptible to ionization by pressure due to charge delocalization between the oxygen and the aromatic ring, yielding activation volumes between -8 and -20 cm^3 mol^{-1} (Hamann & Linton, 1974). Phenols are well recognized for their capacity to neutralize free radicals by donating a proton. During pressure treatment, it is reasonable to suggest that phenols are ionized and therefore their ability to capture free radicals is significantly enhanced (Scheme 2). Simultaneously, the further reaction of the produced phenoxide anions with free radicals (II) and other phenoxide with the subsequent oxygen release (III) cannot be ignored. Possible reaction pathways are proposed in Scheme 2. The induced ionization of phenols and the way they react with free radicals during high pressure processing should be considered when evaluating antioxidants in a food matrix. Unfortunately, no systematic study has been reported on the effect of high pressure on phenol reaction mechanisms and therefore the reaction rates associated with it. The evaluation of these reaction mechanisms is a great challenge in high pressure treatments since the analytical methods are not suitable for *in situ* measurements.



[Scheme 1]



[Scheme 2]

Another important ionization reaction is the self-ionization of water (Scheme 3), which is greatly influenced by pressure. Water is ionized into H_3O^+ and OH^- where both ions are electrostricted, yielding a negative and large reaction volume, -22.2 ± 0.2 cm^3 mol^{-1} (Kitamura & Itoh, 1987).

$$2H_2O \leftrightarrow H_3O^+ + OH^- \qquad \text{[Scheme 3]}$$

Marshall & Franck (1981) developed an empirical equation to estimate the change in pH. The equation was based on temperature and density values. Recently, this equation was used by Mathys & Knorr (2009) to estimate the ionization equilibrium constant (K_w) of water over a wide range of pressures (0.1-1200 MPa) and temperatures (0-130˚C). Using this equation, the concentration of ions increases with pressure and temperature as reflected by important variations in the K_w values. Unfortunately, K_w does not directly represent the changes in the pH as it accounts for two contributions, $[H^+]$ and $[OH^-]$ concentration. Hayert et al. (1999) reported that the pH of water drops between 0.39 to 0.73 units per 100 MPa. Such range difference might be explained by the impurities in water (Samaranayake & Sastry, 2010). These authors proposed an overall equilibrium reaction that includes protonic ionization of a buffer (HA), dissolved CO_2, and self-ionization of water (Scheme 4).

$$HA + CO_2 + 2H_2O \leftrightarrow 4H^+ + A^- + CO_3^{2-} + OH^- \qquad \text{[Scheme 4]}$$

Table 2-4 summarizes studies in which the effect of high pressure on pH has been quantified. These studies can be classified based on those conducted in model systems or buffer solutions that allow better understanding of the pressure effect and those studies conducted in food systems where interpretation of the data is even more complex. The thermodynamic background of ionic equilibrium at high pressures has been reviewed by Stippl et al. (2005).

Buffer solutions – Changes in the pH values upon pressure treatment has been quantified inside the high pressure vessel by measuring electromagnetic frequency in a glass electrode (Disteche, 1959), optical density (Neuman et al., 1973) and fluorescent intensity (Hayert et al., 1999). Some examples of pressurized systems are provided by Neuman et al. (1973), who used buffer solutions of acetate, cocadylate, phosphate and Tris. Changes in the pH were optically measured using 2,5-dinitrophenol and *p*-nitrophenol. Shifts in the pH values were expressed in optical density changes (ΔOD) as a function of pressure. These authors calculated the molal equilibrium constant of ionization (K_a) and apparent volume of ionization (ΔV_a), according to equation (2.15).

$$\frac{\partial(pH)}{\partial P} = \frac{\partial(pK_1)}{\partial P} - \frac{\partial\left[\frac{OD_1 - OD}{OD}\right]}{\partial P} \qquad (2.15)$$

The estimated values of K_a and ΔV_a are shown in **Table 2-4**. Negative values represent acid dissociation promoted by pressure. On the other hand, the estimated values for ΔV_a and K_a for Tris buffer solution were positive, which indicates that Tris buffer is pressure insensitive. It also indicates that its acid dissociation occurs without significant volume contraction as the number of charged species is the same (scheme 5).

$$TrisH^+ + H_2O \xleftrightarrow{K_a} Tris + H_3O^+ \qquad \text{[Scheme 5]}$$

Hayert et al. (1999) measured fluorescent intensity of fluorescein to evaluate the pH at pressures up to 250 MPa. These authors correlated their experimental determinations with variations in the volume of one mole of H^+ (ΔV_{H^+}), according to equation (2.16). Therefore, this equation allows the evaluation of the pH of acidic solutions up to 250 MPa.

$$\Delta pH = \frac{\Delta V_{H+}}{\ln 10\, RT} \qquad (2.16)$$

A semi-empirical relationship (equation 2.17) that describes the influence of pressure on ionization was proposed by Elyanov & Hamann (1975). This relationship is based on Born's electrostatic theory of ionic hydration. Equation (2.17) predicts the equilibrium constant of ionization from the pressure dependency of dielectric permittivity (Stippl et al., 2005). Molina-Guitierrez et al. (2002) used the Elyanov and Hamann's equation to calculate changes in pH values of different buffer solutions during high pressure inactivation of microorganisms (**Table 2-4**).

$$\left(pK_a\right)_p = \left(pK_a\right)_o + \frac{P\Delta V^o}{\log\left(RT\left(1+bP\right)\right)} \qquad (2.17)$$

where $(pK_a)_p$ is change in the dissociation constant due to pressure; $(pK_a)_o$ is the dissociation constant at a reference pressure and temperature; P is pressure (MPa); ΔV^o is partial molar volume change of the activated dissociating acid at atmospheric pressure (m^3 mol^{-1}); R is universal gas constant (8.31 x 10^{-6}, MPa m^3 K^{-1}); T is absolute temperature (K) and b isthe constant for acids (9.2 x 10^{-4} MPa).

Although Elyanov and Hamann's equation provides a fairly good description of the effect of pressure on ionic solutions, Stippl et al. (2005) challenged the applicability of this equation. In their review, these authors highlighted that the term b in equation (2.17) is not valid for high temperatures (>40˚C). Moreover, experimental evidence showed that the dissociation reaction of ammonium hydroxide exhibited large deviations for experiments conducted at high temperatures and pressures (Stippl et al., 2005). Samaranayake & Sastry (2010) developed a pH sensor capable of measuring the hydrogen ions *in situ* up to 784 MPa. These authors coupled a Nafion membrane (permeable to water and cations but impermeable to anions) with two reverse osmosis membranes. This sensor directly relates the input voltage with the hydrogen ion concentration.

Table 2-4. Summary of studies on the effects of HPP and PATS on ionization.

Matrix	Conditions	Remarks
Raw cow milk (3.2, 1.5 and 0.1% fat) (Altuner et al., 2006)	pH = 6.4; T = 25; P = 0.1-440; t = 10 and 20	- Slight increase in pH
Cow milk (Kim et al., 2008)	pH = 6.7; T = -4; P = 200; t = 10-30	- No significant difference was observed at the pH values studied
Skim milk (Schrader et al., 1997)	pH = 6.7; T = 20; P = 400; t = 5	- pH increased 0.09 units by pressure treatment
Reconstituted skim milk (Famelart et al., 1997)	pH = 4.0-6.8; T = 20; P = 250-600; t = 30	- pH values changed between 0.3-0.42 unit per 100 MPa
Goat milk (De la Fuente et al., 1999)	pH = 6.7; T = 20; P = 400; t = 10	- pH slightly increased by pressure and caused changes in the mineral balance
Goat cheese (Buffa et al., 2000)	pH = 4.9; T = 20; P = 500; t= 15	- Pressure accelerated proteolysis
Buffer solutions: DP, NP, CC, H_2PO_4, $TrisH^+$ (Neuman et al., 1973)	pH = NR; T = 25; P = 0.1-637; t = NR	- ΔV^a, cc mol^{-1}: DP = -11.3; NP = -11.3; CC = -13.2; H_2PO_4 = -25.3 and $TrisH^+$ = +1
Buffer solutions: water, OPA, PB, MES, and acetic acid (Hayert et al., 1999)	pH = 5.8-7.2; T = NR; P = 100 and 200; t = NR	- pH changes 100 MPa: -0.31 (water), -0.92 (OPA), -0.28 (PB), +0.50 (MES), and -0.40 (acetic acid).
Buffer solutions: HCl, AA, CA, and acetate (Disteche, 1959)	pH = 7.4-7.9; T = 22; P = 147; t = 30	- Dissociation constants were in agreement with theoretical calculations
Buffer solutions: acetate, biphthalate, and sulfanilate (Samaranayake & Sastry, 2010)	pH = 4.7-6.9; T = 25; P = 0.1-784; t = 2	- pH drop ≤0.3 unit at 780 MPa - pH of water drops to 0.16
Cod (*Gadus morhua*) muscle (Angsupanich & Ledward, 1998)	pH = 7.0; T = 25; P = 100-800; t = 20	- pH of cod muscle increased with pressure
Batter of turkey meat (Chan et al., 2011)	pH = 5.5 and 5.7; T = 4; P = 50-200; t = 5	- pressure (50-100 MPa) droped the pH 0.16 units - At 150 MPa, the pH increased 0.04 units

NR – not reported; T – temperature (˚C); P – pressure (MPa); t – time (min); DP – 2,5-Dinitrophenol; NP – *p*-Nitrophenol; CC – Cocadylic acid; OPA – orthophosphoric acid; PB – potassic buffer, MES – 2-[N-morpholino]ethanesulfonic acid; AA – acetic acid, CA – carbonic acid.

Interestingly, the changes in pH values obtained with the *in situ* sensor were lower than those calculated with reaction volumes (shown in **Table 2-4**). This approach shows promising results since it allows the direct measurement of hydrogen ions at elevated pressures and it can be used for opaque samples unlike the optical methods.

All these approaches provide valuable information for the analysis of ionization reactions at high pressures and predict changes in the pH values. However, the use of these equations is limited for a few solutions, mainly buffers where the effect of co-solutes and food matrices are not considered. Additionally, a theory that satisfactorily describes the interactions between ions and solvent for high pressure processes has not yet been developed (Stippl et al., 2005). Already, Stippl et al. (2005) highlighted that the motions of ion and therefore the pH values might be affected by the structured water molecules. Indeed, Samaranayake & Sastry (2010) found two notable drops in the pH values at 392 MPa and 588 MPa after pressurizing water up to 784 MPa. These drops in the pH values were attributed to the different structured forms of liquid water. An investigation of X-ray, neutron scattering, and infrared spectroscopy of pressurized water at 300 MPa showed that pressure increases the arrangement of liquid water into denser clusters compared to the liquid water at atmospheric pressure (Khoshtariya et al., 2004). More importantly, it was found that pressure causes a weakened hydrogen-bonding structure that arranges into different dense cluster, such as cyclic, noncyclic water tetramers and water cube octomers. Consequently, liquid water upon pressurization forms a complex three-dimensional network of hydrogen bonds, coexisting simultaneously in different configurations. This novel approach challenges the applicability of Elyanov and Hamann's equation to predict pH at pressures higher than 150 MPa. As discussed above, equation (2.17) is based on electrostatic theory, which considers the movement of ions through a continuous medium and therefore the free energy of solvation of a mole of ions is proportional to the square of its charge.

Another approach used by De Vleeschouwer et al. (2010) and de Roeck et al. (2009) consisted of a combination of buffers that have positive and negative activation volumes in order to prepare a pressure independent solution. With this approach, the effect of pressure rather than the effect of pH can be determined on a particular reaction. Unfortunately, the majority of the studies reported in the

literature have focused on the effect of pressure and temperature on a particular reaction and the effect of pH is often not discussed.

Food systems –Changes in the pH value for pressurized food systems have been measured after depressurization as there is no available probe or sensor to experimentally monitor the pH values inside high pressure vessels of more than 100 MPa (Ramirez et al., 2009). This is a technological disadvantage because the induced change in the pH value is reversible upon pressure release as reported by Schrader et al. (1997), who pressurized thermally treated milk and found a completely reversed change in the pH value after 4 h at atmospheric pressure and room temperature. Therefore, in food systems, the real effect of pH on the analysis of a particular reaction cannot be measured directly. In addition, equations (2.13-2.17) that are used to predict pH changes in buffer solutions do not have application in food systems where reaction mechanisms and rates might be affected by a pH shift induced by pressure.

In high pressure processing and PATS studies, the reaction system, either buffer or food, suffers temporary and reversible modification in the pH value that might change the reaction mechanisms and therefore the associated reaction rates. One alternative to find out whether these variations on the pH values influence the reaction mechanisms and the reaction rates is to perform an additional set of experiments using one pressure-dependent buffer solution and one pressure-independent buffer solution. Using this approach, it is possible to evaluate the qualitative and quantitative effects of the pH shift on the kinetic parameters under PATS conditions.

2. 4. Volatile formation

Volatile compounds determine the development of aroma and off-flavour of food products. The aroma of food products is a result of complex mixtures of volatiles formed by different sources (proteins, fat, and carbohydrates). Thus, different reaction mechanisms occur such as Strecker degradation, dehydration,

retro-aldolisation, rearrangements, isomerization, condensation, Diels-Alder, nucleophilic and free radicals. **Table 2-5** summarizes studies on the effect of pressure on volatiles formation.

Table 2-5. Summary of studies on the effect of pressure on the formation of volatiles.

Food matrix	Conditions	Remarks
Serena cheese (Arques et al., 2007)	T = 10; P = 300 and 400; t = 10	- Pressure decelerated the formation of some volatiles
Hispano cheese (Avila et al., 2006)	T = 10; P = 400; t = 5	- Pressure treated cheeses showed lower levels of straight aldehydes
Ewe cheese (Juan et al., 2007)	T = NR; P = 300; t = 10	- Aroma intensity was not influenced by pressure
Milk (3.25% fat) with antioxidants (Vazquez-Landaverde et al., 2007)	T = 75; P = 655; t = 3-10	- Antioxidants inhibited the formation of volatile compounds
Milk (3.2% fat) (Vazquez-Landaverde & Qian, 2007)	T = 75; P = 655; t = 3-10	- The formation of volatile was inhibited with antioxidants
Strawberry (Lambert et al., 1999)	T = 20; P = 3 200-800; t = 20	- No significant change for all aromatic volatile compounds
Strawberry pulp (Butz & Tauscher, 2000)	T = 25, 40 and 60; P = 600; t = 30	- Changes in hexanal depended on the pressure level
Raspberry, strawberry, and blackcurrant purees (Dalmadi et al., 2007)	T = 25; P = 600; t = 5	- Pressure inhibited volatile formation
Strawberry puree (Navarro et al., 2002)	T = 20; P = 400; t = 20	- No change in the aromatic profile
Cherry tomato puree (Viljanen et al., 2011)	T = 20 and 60; P = 800; t = 10	- Pressure increased the concentration of hexanal, heptanal and octanal
Tomato juice (Porretta et al., 1995)	T = NR; P = 500-900; t = 3, 6 and 9	- Pressure significantly increased the concentration of hexanal
Onion (Butz et al., 1994)	T = 25 and 40; P = 150, 300 and 350; t = 5-60	- Pressure decreased the concentration of volatiles.
Oysters (Cruz-Romero et al., 2008)	T = 20; P = 256-800; t = 3-5	- Pressure increased heptanal, ketones, and dimethyl sulphide
Water solution of different aldehydes (Lewis & Wolfenden, 1973)	T = 25; P = 0.1-655; t = 2	- Pressure enhanced the equilibrium constant of aliphatic aldehydes
Cooked pork shoulder (Rivas-Cañedo et al., 2011)	T = 12; P = 400-500; t = 5, 10	- Pressure delayed the formation of aldehydes
Chicken breast and beef muscle (Schindler et al., 2010)	T = 5; P = 400 and 600; t = 15	- Pressure did not induce severe changes in the aroma profile

T – temperature (˚C); P – pressure (MPa) and t – time (min).

In a quantitative study, Vazquez-Landaverde et al. (2006) pressurized homogenized milk (3.2% fat) and evaluated the volatile profile by measuring aldehydes, ketones, and sulphur compounds. According to these authors,

moderate conditions (~500 MPa, 60˚C and up to 5 min) can be used to deliver milk with a volatile profile comparable to that obtained in high-temperature-short-time pasteurization (HTST). In addition, a shelf life of 7 weeks can be achieved at moderate conditions while the HTST milk usually has a shelf life of 3 weeks. On the other hand, at slightly more severe conditions (80˚C, 600 MPa and 5 min), the obtained volatile profile resembles that obtained at ultra-high-temperature (UHT). At conditions of 80˚C and 600 MPa for 5 min, bacterial spores are not inactivated because spores are pressure resistant and combinations of elevated temperatures (>100˚C) and high pressures (>500 MPa) are needed.

A kinetic study of milk treated with PATS shows that pressure significantly modifies the way in which ketones, sulphur compounds and aldehydes are formed (Vazquez-Landaverde et al., 2007). The development of a volatile profile encompasses not only one reaction pathway but a whole network of various reactions, occurring simultaneously at different rates. Vazquez-Landaverde et al. (2007) expressed the kinetics of formation of volatile compounds under pressure as zero- and first-order models (**Table 2-5**). Among ketones, only the formation of 2-3-butanedione was accelerated by pressure, yielding $\Delta V^{\neq}$ values from -35 to -16 cm^3 mol^{-1}. In the case of sulphur compounds, the concentration of hydrogen sulphide (H_2S) increased with pressure, reaching concentrations comparable to those obtained in UHT milk (~20 µg L^{-1}) after 5 min of holding time. Moreover, the kinetic analysis yielded negative $\Delta V^{\neq}$ values around -10 cm^3 mol^{-1}. It is believed that H_2S contributes to a cooked flavour because its sensory threshold in water is ~ 10 µg L^{-1} (Al-Attabi et al., 2009).

In the case of aldehydes, Vazquez-Landaverde et al. (2007) reported negative $\Delta V^{\neq}$ for straight aldehydes in milk treated with high pressure and moderate temperature. Straight aldehydes, such as hexanal, heptanal, and octanal result from the autoxidation of unsaturated fatty acids. A mechanistic interpretation can be derived from those $\Delta V^{\neq}$ values reported by Vazquez-Landaverde et al. (2007). One possible explanation for the formation of aldehyde

due to the application of pressure is the hydration volume of the carboxilic acid group. Lewis & Wolfende (1973) measured the hydration volume of anhydrous aldehydes at high pressure. Under pressure, the carboxylic group is solvated, yielding large negative reaction volumes. On the other hand, the substituents on the branched aldehydes exerted a great influence on the hydration volume shifting reaction volumes close to zero. According to Lewis & Wolfende (1973), branched aldehydes that possess a transition state, which resembles structurally an aldehyde hydrate would exhibit comparable effect of pressure to that observed for straight aldehydes.

In tomato juice, the application of 500 MPa for 3 min increased the concentration of hexanal (Porretta et al., 1995). Similarly, 800 MPa at 20˚C for 10 min significantly increased the concentration of straight aldehydes, such as hexanal, heptanal and octanal in cherry tomatoe puree (Viljanen et al., 2011). According to Viljanen et al. (2011), the increase in hexanal content is due to autoxidation of fatty acids. The concentration of hexanal is used as an oxidation indicator. However, hexanal is a secondary oxidation product and the conditions used (500 MPa for 3 min and 800 MPa for 10 min at room temperature) are rather moderate and might not be severe enough to induce oxidation and subsequently the formation of secondary oxidation products.

An investigation on the use of antioxidants in pressurized milk showed that the inhibition of hexanal, heptanal and octanal formation was proportional to the concentration of the antioxidant (Vazquez-Landaverde et al., 2007). These authors used BHT, epicatechin, ascorbic acid, β-carotene and L-cysteine in milk treated at 655 MPa and 75˚C up to 10 min of holding time. BHT and epicatechin inhibited aldehyde formation. Although the formation of off-flavour compounds was inhibited with the use of antioxidants, their effectiveness and the way in which free radicals are captured or react with the dissolved oxygen to retard the free radical reactions have not been studied so far under high pressure-high temperature conditions.

2.5. Maillard reaction

An important reaction in determining the final quality in heated and stored foods is the Maillard reaction. This reaction is not only responsible for the development of colour/flavour in processed foods but it is also responsible for the formation of anti-nutritive and off-flavour compounds. A review on Maillard reaction implications in food systems is provided elsewhere (Martins et al., 2001; van Boekel, 2001). The Maillard reaction involves complex sequence of reactions, including condensation, cyclization, dehydration, rearrangement, isomerization and polymerization. The velocity at which some of these reactions occur can be markedly increased or decreased by pressure according to the predominant mechanism. Thus, evaluating the effect of pressure on the overall Maillard reaction can be misleading (Schwarzenbolz et al., 2000). For simplicity, the effect of pressure on the initial, intermediate and final stages are discussed in the following section. **Table 2-6** summarizes studies where the effect of pressure on the Maillard reaction was evaluated.

2.5.1. Initial stage

Sugar-amine condensation – This reaction involves opening of the ring form of a reducing sugar, addition of a un-protonated amino group to the carbonyl group and subsequent dehydration to form an *N*-substituted glycosylamine. The open form of the sugar is considered as the reactive specie that is in equilibrium with the ring form. Sander (1943) found that pressure significantly increased the speed of mutarotation of glucose ($\Delta V^{\neq}$= -12.5 cm^3 mol^{-1}). Similarly, Andersen & Gronlund (1979) reported that pressure allowed achieving a rapid equilibrium during the interconversion of α-glucopyranose to β-glucopyranose ($\Delta V^{\neq}$= -11.7 cm^3 mol^{-1}). Upon pressurization, the equilibrium of the mutarotation of glucose moves in the direction of the open form. This is because the open form has a smaller molar volume than the closed ring, which encloses a large amount of dead space in the center of the closed ring (Andersen & Gronlund, 1979).

Table 2-6. Summary of studies where the effect of pressure was evaluated on Maillard reaction.

Food matrix	Conditions	Remarks
Carbonyl/amino solutions buffers (Tamaoka et al., 1991)	pH = 8.2; T = 50; P = 50-500; t = 15-120	- For condensation: $\Delta V^{\neq}$ = 4.3 to 8.9 $cm^3 mol^{-1}$ - For browning: $\Delta V^{\neq}$ = 12.8 to 27 $cm^3 mol^{-1}$
Equimolar solutions of N^{α}-acetyllarginine, N^{α}-acetyllysine and ribose (Schwarzenbolz et al., 2000)	T = 60; P = 200 and 400; t = 120	- Pressure promoted the formation of Amadori products while retarded their further degradation
One molal solution of glucose-lysine (Hill et al., 1999)	pH = 5.1, 6.5, 8.0, and 10.1; T = 40-60; P = 600; t = 0-22 h	- Pressure retarded the formation of Maillard browning compounds
Lysine and xylose solutions (Bristow & Isaacs, 1999)	pH = 7 and 10; T = 100; P = 100-600; t = 0-300	- Pressure retarded the decomposition of Amadori rearrangement products
Solution of tryptophan and glucose or xylose (Isaacs & Coulson, 1996)	T = 70 and 80; P = 100-800; t = 0-350	- Pressure accelerated the formation of the intermediate products (-14 cm^3/mol) but retarded their further degradation and melanoidins products (8 and 17 cm^3/mol, respectively)
Equimolar buffer solution of asparagine-glucose (De Vleeschouwer et al., 2010)	T = 100-115; P = 400-700; t = 0-60	- Pressure inhibited the formation of acrylamide compared with the control treatment (0.1 MPa)
Solution of N^{α}-acetylarginine (2 mmol) and glucose (6 mmol) (Alt & Schieberle, 2005)	pH = 7.0; T = 90-120; P = 0.1-600; t =120	- Pressure in combination with elevated temperature induced arginine modifications
Glucose-lysine model system (Moreno et al., 2003b)	pH = 5-10; T = 60; P = 0.1 and 400; t =1-3 h	- Pressure delayed the initial stages of the Maillard reaction
Dry-cured pork loins (Campus et al., 2008)	T = 20; P = 0.1-350; t =10	- Pressure affected the formation of Maillard reaction products (2-methylpropanic acid, bencenacetaldehyde and trimethylpyrazine)
Lactose solution (10%) in basic media (Moreno et al., 2003a)	T = 60; P = 0.1-400; t =3 h	- Pressure inhibited the isomerization and degradation of lactose

T – temperature (˚C); P – pressure (MPa) and t – time (min).

It can be assumed that pressure promotes the formation of the reactive form of the reducing sugar. The next step in the condensation reaction is the addition of the amino group to carbonyl. This step is governed by nucleophilic attack and involves the formation of a new bond (C=N). Tamaoka et al. (1991) condensed

glyceraldehydes with di- and tripeptides and found that the reaction was independent of pressure ($\Delta V^{\neq}$=0 cm^3 mol^{-1}) while the condensation of glyceraldehyde or glycolaldehyde with amino acids was slightly inhibited by pressure with values of $\Delta V^{\neq}$ ranging from 3.9 to 8.9 cm^3 mol^{-1}. These authors explained their results in terms of cancelation of two opposing factors, bond formation (C=N) and neutralization of the amino group. Contrarily, Isaacs & Coulson (1996) found that the condensation of 3-hydrobenzaldehyde with tryptophan was slightly accelerated by pressure ($\Delta V^{\neq}$= -7 cm^3 mol^{-1}). These authors also condensed 3-hydrobenzaldehyde with methyl ester tryptophan and found that pressure strongly accelerated the condensation ($\Delta V^{\neq}$= -21 cm^3 mol^{-1}). The difference was attributed to the internal proton transfer of the zwitterionic form of tryptophan.

Mechanistically, it is expected that pressure delays charge neutralization during bond formation because an electristrected ion needs to be released from the solvent. A charge is neutralized when the contraction volume caused by the formation of the new bond (C=N) is greater than the molar contraction volume of water by electrostriction. Bond formation by addition is expected to have negative $\Delta V^{\neq}$ of -20 cm^3 mol^{-1} (Asano & Le Noble, 1979), which would be far smaller than the molar contraction volume of water by electrostriction, -2.91 cm^3 mol^{-1} (Marcus, 2005).

Amadori rearrangement – This reaction occurs due to the isomerization of *N*-substituted glycosylamine to 1-amino-1-deoxy-2-ketoses. Amadori rearrangement involves protonation of the nitrogen atom at carbon 1, which is expected to be delayed by pressure since the proton needs to be released from the solvent. Indeed, Isaacs & Coulson (1996) estimated a positive value of $\Delta V^{\neq}$ (8 cm^3 mol^{-1}) for the rearrangement step of tryptophan and glucose condensation.

2.5.2. Intermediate stage

Sugar dehydration – Amadori rearrangement products undergo dehydration, forming furfurals, 6-carbon and reductone compounds through 1,2-enolization, 2,3-enolization, 3-deoxyosone and 1-deoxy-2,3 dicarbonyl, depending on the pH value. Furfurals are formed when the Amadori compounds dehydrate, losing 3 molecules of water. Reductones, compounds containing the group –C(OH):C(OH)-, are formed when the Amadori rearrangement products lose only 2 molecules of water.

Formation of either furfurals or reductones from Amadori rearrangement products is expected to be retarded by pressure because dissociative reactions occur that are associated with volume expansion (Asano & Le Noble, 1978). This has been exemplified by Isaacs & Coulson (1996) who heated (100˚C) a model system and found that the degradation of Amadori rearrangement products from the condensation of tryptophan and glucose was retarded by pressure with an estimated value of +17 cm^3 mol^{-1} for $\Delta V^{\neq}$. Similarly, the concentration of hydroxymethylfurfural was below the detection limit in an aspargine-glucose model system treated at high pressure sterilization conditions (110-115˚C and 400-700 MPa) (De Vleeschouwerk et al., 2010).

The impact of pressure on the formation and subsequent decay of reductones has been exemplified in a model reaction system between xylose and lysine (Bristow & Isaacs, 1999). Among the reductones identified was 5-methyl-4-hydroxy-3(2H)-furanone, a compound associated with caramel odour, which formation was found to be retarded by pressure with an estimated $\Delta V^{\neq}$ = +4.1 cm^3 mol^{-1}. More importantly, the experimental data followed a zero-order kinetic model, independent of the pressure applied (100 MPa). Consequently, the enolization step followed by rapid scavenging of the enol was proposed as the reaction mechanism.

The role of pressure in retarding the formation of 5-methyl-4-hydroxy-3(2H)-furanone was represented through the different reaction steps. Enolization involves transfer of a neutral proton without significantly changing the molecular volume. Contrarily, the elimination of the nitrogen residue was considered as the rate-limiting step because the covalent bond breaking is a dissociative reaction that results in volume expansion (Bristow & Isaacs, 1999). On the other hand, the decomposition of 5-methyl-4-hydroxy-3(2H)-furanone was slightly accelerated by pressure ($\Delta V^{\neq}$ = - 5.1 cm^3 mol^{-1}), suggesting an open ring form. Further condensation of 5-methyl-4-hydroxy-3(2H)-furanone with xylose was proposed as an alternative decomposition pathway and this reaction was found to be strongly accelerated by pressure ($\Delta V^{\neq}$ = - 35 cm^3 mol^{-1}).

Another major compound identified by Bristow & Isaacs (1999) was 5-hydroxymaltol (2-methyl-3,5-dihydroxy-(4H)-pyran-4-one), which is a precursor of furans, pyranones and carboxylic acids. The effect of pressure on the formation and further decomposition of 5-hydromaltol is similar to that observed for 5-methyl-4-hydroxy-3(2H)-furanone ($\Delta V^{\neq}$ = + 5 cm^3 mol^{-1}) in which the formation is retarded by pressure due to the elimination of nitrogen and the further decomposition is accelerated by two mechanisms: ring opening and associative reactions with other compounds. Similarly, Moreno et al. (2003b) pressurized glucose-lysine solution at 400 MPa and found low concentrations of decomposition compounds from the Amadori rearrangement products compared to the concentration obtained at atmospheric pressure. The quantified Amadori rearrangement products were α-2-furoymethyl-lysine, ϵ-2-furoylmethyl-lysine and 2-furoylmethyl-lysine. The breakdown of Amadori rearrangement products is likely to be delayed by pressure due to the elimination of nitrogen, and the covalent bond breaking. Subsequent decomposition of these intermediate compounds is likely to be accelerated by pressure due to a set of associative reactions (condensation, ring opening and bond formation).

Sugar fragmentation – The mechanism by which sugars are fragmented is retroaldolisation and oxidative fission. The produced fragments (C2, C3, C4 and C5 compounds) are reactive compounds and further react through condensation and cyclization. Frank et al. (2002) evaluated the effect of pressure on sugar fragmentation by pressurizing xylose-alanine buffer system. These authors quantified 3(2H)-furanone and 3(2H)-pyrrolinone and found that pressure retarded their formation. Similarly, the formation of 3(2H)-furanone was retarded by the use of 400 MPa in heated (100˚C) xylose-lysine system (Bristow & Isaacs, 1999).

During fragmentation of sugars, intermediate compounds such as α-dicarbonyl are formed through retroaldolisation and direct degradation of glucose. These compounds are very reactive (Gobert & Glomb, 2009). The effect of pressure on the formation of α-dicarbonyl has been reported by Alt & Schieberle (2005), who quantified 2-oxopropanal and glyoxal after pressure treatment. Remarkably, pressure enhanced the formation of both compounds by 9- and 4-fold compared with the control treatment at atmospheric pressure.

2.5.3. Final stage

In the final stage of the Maillard reaction, different reactions occur simultaneously, including Strecker degradation, aldol condensation, aldehydes-amine condensation and polymerization. The role of pressure on the cross-linking and glycation reactions of proteins is quite complex, depending on the system involved. The formation of post-translational amino acids, a cross-linked compound between amino residues and α-dicarbonyl, was promoted by pressure in N^{α}-acetylarginine and glucose (Alt & Schieberle, 2005).

The concentration of N^{7}-(1-carboxyethyl)-L-arginine, N^{5}-(5-hydro-5-methyl-4-imidazolon-2-yl)-L-ornithine, and ornithine increased with pressure, regardless of the temperature used. At 110˚C, the concentration was increased ~4-fold in those samples pressurized at 600 MPa. A hypothetical reaction mechanism was proposed by Alt & Schieberle (2005). The formation of post-translational

amino acids is explained by the reaction of 2-oxopropanal and the aldehyde function, forming a Schiff base that further rearranges through tautomerism. The effect of pressure was attributed to the increasing formation of 2-oxopropanal, which triggers the formation of post-translational amino acids. Schwarzenbolz et al. (2000, 2002) quantified pentosidine, a protein-bound marker, in a β-casein-ribose solution treated at 60˚C and 0.1-600 MPa. The concentration of pentosidine increased remarkably with pressure. But, it was below the detection limit in those samples treated at 60˚C and 0.1 MPa.

The glucosylation of bovine serum albumin and glucose was significantly reduced by pressure (Buckow et al., 2011). Similar results were reported in solutions of β-casein and saccharides where pressure inhibited the degree of cross-linking and oligomerization (Schwarzenbolz et al., 2000). In the absence of saccharides, the cross-linked products were increased with pressure. These authors concluded that pressure accelerated the degradation of sugar, delaying the cross-linking with proteins.

The development of browning was greatly inhibited in glyceraldehyde with di- and tripeptides (Tamaoka et al., 1991). Moreover, an investigation on the browning development conducted using the glucose-lysine model system showed that the rate of browning under pressure was influenced by the initial pH of the solution. In the pH range of 5.1-6.5, 7.0-7.5 and 8.0-10.5, the rate of browning was inhibited, unaffected and accelerated by pressure, respectively (Hill et al., 1996). Pressure inhibits the development of browning compared with the atmospheric treatment. However, this observation must be taken cautiously because some reactions are accelerated by pressure, resulting in a different volatile profile even at the same degree of browning. Hill et al. (1999) compared the volatile compounds of the glucose-lysine system treated at 600 and 0.1 MPa and 60˚C. The volatile profiles were obtained after the treated samples reached the same degree of browning, measured by the absorbance at 420 nm. Interestingly, these authors identified 32 compounds belonging to 8 chemical classes

(pyrrazines, ridines, pyrrolizines, pyrroles, pyrannones and furans) at 0.1 MPa and the concentration was significantly decreased at 600 MPa. Such reduction was explained by analysing the volatiles by their respective chemical class. For instance, pressure promoted further degradation of pyrazines through aldol condensation of pyrizines, containing methyl groups while the formation of pyrizines containing ethyl groups were retarded by pressure. The methyl group ionized, leading to a product condensation. On the other hand, the ethyl group induced a negative charge into the pyrazine, conferring stability. It seems that pyrazine with methyl group substituents degraded faster than its formation. Huang et al. (1996) reported that the formation of tetramethylpyrazine was slightly accelerated by pressure ($\Delta V^{\neq}$ = - 6.82 cm^3 mol^{-1}).

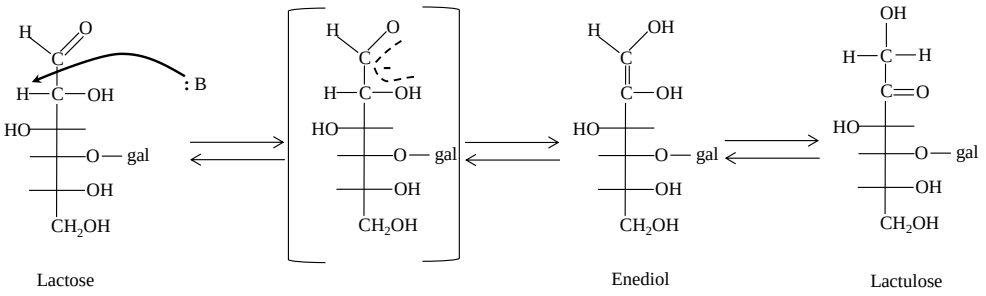
A potential carcinogen compound formed during the Maillard reaction is acrylamide, which is believed to be formed by decarboxylation of amino acids and subsequent isomerization of Schiff bases. In a model system of asparagine-glucose, De Vleeschouwer et al. (2010) found that pressure inhibited the formation of acrylamide compared to the atmospheric treatment, which can be considered as an additional benefit of the use of high pressure. Unfortunately, the exact mechanism by which the acrylamide is inhibited is still unknown. Schwarzenbolz et al. (2000) suggested that the effect of pressure should be evaluated for each food matrix. **Table 2-7** shows a simplification of the effect of pressure based on the current literature.

Table 2-7. Simplification of the effect of pressure on individual Maillard reactions.

Reaction	Expected $\Delta V^{\neq}$
Sugar mutarotation	(−)
Condensation	(−)
Amadori rearrangement	(+)
Sugar dehydration	(+)
Sugar fragmentation	(+)
Strecker degradation	(−)
Melanoidin formation	(−)

2.7. Lactose isomerization

In the case of milk, part of the reducing sugar, lactose, isomerizes to form lactulose. The effect of pressure on lactose isomerization has been reported by Moreno et al. (2003a), who pressurized (400 MPa/60˚C for 3 h) a lactose solution (10%) in basic media. Moreno et al. (2003a) found an inhibitory effect of pressure on the isomerization of lactose. The isomerization occurs through enolization, which involves removal of the proton at the α-carbon atom, followed by a shift of the double bond of the adjacent carbonyl to the α-carbon (Scheme 6). Upon pressurization, a slow proton transfer seems to be the rate-limiting step because a solvated proton from the solvent needs to be released. Further formation of enediol and lactulose, which involves molecular rearrangement and double bond shift, is believed to have a small volume change and therefore slightly affected by pressure (Isaacs, 1981).



[Scheme 6]

2.8. Lipid oxidation

The effect of pressure on lipid oxidation in meat and meat products has been reviewed by Buckow et al. (2013) and Simonin et al. (2012). In this section, the effect of pressure is discussed on the reaction mechanism rather than in the food product. Lipid oxidation involves three consecutive reactions, known as initiation, propagation and termination. In the initiation stage, free radicals are formed through homolysis, where the break of covalent bond takes place. The effect of pressure on these types of reactions has been long studied in organic synthesis, where positive $\Delta V^{\neq}$ values were reported (0.2 to 13 cm^3 mol^{-1}) (Isaacs, 1981). Bolumar et al. (2012) showed that pressure promoted the formation of free

radicals in chicken breast. These authors reported $\Delta V^{\neq}$ in the range of -17 to -26 cm^3 mol^{-1}, depending on the pressure applied. More importantly, a critical pressure (400-500 MPa, changing with temperature) was found above which the formation of free radicals was significantly enhanced. Interestingly, this critical pressure matched with the threshold pressure at which denaturation of proteins and membrane damage occur, releasing metal ions and enzyme. Whether the free radicals are formed by the released metal ions and enzymes or by a different mechanism needs to be experimentally determined.

The next oxidation stage is the propagation, which consists in the further degradation of hydroperoxides or any other primary oxidation product. There are mainly two types of degradation products from hydroperoxides. First, hydroperoxides interact with double bonds to form monomeric degradation products, such as ketones. The reaction occurs through the reduction of the hydroperoxyl group to hydroxyl derivative. Second, low molecular weight products, that results from the cleavage of the hydroperoxide chain, form aldehydes, ketones, alcohols and hydrocarbons. These low molecular weight compounds are responsible for the rancid and off-flavour produced by oxidized fats. In general, chain reactions are accelerated by pressure ($\Delta V^{\neq}$ = -5 to -15 cm^3 mol^{-1}) (Isaacs, 1981). Tauscher (1995) reported that 600 MPa had a protective effect on the autoxidation of linoleic acid, retaining 80% after 21 h. But, unfortunately, details on the investigation were not provided.

Finally, hydroperoxides and primary oxidation products undergo homolysis to form peroxyl or alkoxyl radicals that further reacts to form stable dimer-like products. The resulting compounds form viscous materials through polymerization as the oxidation proceeds. These polymers are oil insoluble and represent the termination stage of oxidation. The termination step is inhibited by pressure because the reaction is diffusion controlled.

2.9. Degradation and stability of vitamins and pigments

The stability of vitamins under pressure has been evaluated in a variety of matrices. Oley et al. (2006) reviewed the stability and bioavailability of vitamins in plant-based foods. **Table 2-8** summarizes studies on the effect of pressure and temperature on vitamins, such as ascorbic acid, anthocyanins, B1, among others. The reaction mechanisms involved in the degradation of vitamins are covalent bond breaking, Diel-Alder, ionization and condensation.

An investigation on stability of tetrahydrofolate, 5-methyltetrahydrofolate and 5-formyltetrahydrofolate in three different systems (individual standard solution, model orange juice and fresh squeezed orange juice) showed that the combination of pressure and temperature synergistically influences the folate stability (Butz et al., 2004). However, the comparison between the different systems showed that other compounds such as ascorbic acid might enhance folate stability. It was found that the degraded amount of 5-formylfolate was comparable to the amount of 5, 10-methyltetrahydrofolate that was quantified after the use of 600 MPa and 80°C. Consequently, cyclization by covalent bond formation and electrostriction were suggested as reaction mechanisms (Butz et al., 2004). In a kinetic study, Indrawati et al. (2004) evaluated the stability of 5-methyltetrahydrofolic acid towards pressure using a model system, orange juice, kiwi puree, carrot juice and asparagus.

The degradation rate constants in both model and food system increased synergistically with pressure and temperature. The addition of ascorbic acid enhanced the stability of 5-methyltetrahydrofolic acid, independently of the processing conditions. A synergistic effect was reported for the degradation of (6R,S)-5-formyltethahydrofolic acid in model systems (Nguyen et al., 2006). As the pressure and temperature increased, the degradation rate also increased, obtaining negative values of $\Delta V^{\neq}$ (Nguyen et al., 2006). Similarly, pressure and temperature synergistically affected the degradation rates of folic acid and 5-methyltetrahydrofolic acid (Nguyen et al., 2003). For both compounds, the

degradation kinetic during the simultaneous application of pressure and temperature was described by a first-order model.

Table 2-8. Summary of studies on the effect of pressure and temperature on degradation and stability of vitamins and other compounds.

Food matrix	Conditions	Remarks
Aspartame in Tris/HCl buffer (Butz et al., 1997)	pH = 7.0; T = 60; P = 600; t = 0-30	- 50% of the total aspartame degraded after 3 min at 600 MPa and 60˚C
Riboflavin and thiamin in model solutions and minced fresh pork (Butz et al., 2007)	pH = 5.5; T = 25-100; P = 600; t = 1, 3 and 18 h	- Vitamins were more stable in pork than in model solutions
Apple pectin (de Roeck et al., 2009)	pH = 6.5; T = 90-115; P = 500-700; t = 0-100	- β-elimination was retarded by pressure while demethoxylation was stimulated by pressure
Ascorbic acid in tomatoes and carrot juice (Dede et al., 2007)	T = 25 and 35; P = 150-250; t = 5-15	- The retained ascorbic acid was higher than thermal treatment
Lycopene stability in tomato juice (Gupta, et al., 2010)	T = 45 and 100; P = 600 and 700; t = 10	- All-trans lycopene was found to be stable to isomerization during processing
Folates in orange, kiwi and carrot and asparagus juice (Indrawati et al., 2004)	T = 25-65; P = 100-700; t = 5-30	- Degradation kinetics was described by first-order model - E_a values were 78-158 kJ mol^{-1}, changing with pressure
Ascorbic acid, thiamin and pyridoxal in model (Jandhyala et al., 2002)	pH = 2.2. 4.5 and 6.0; T = 21; P = 689; t = 30	- Pressure treatment yielded around 90% of retention for each vitamin
Stability of α- and β-carotene in carrot juice (Kim et al., 2001)	T = 50-70; P = 300-500; t = 10	- Pressure induced minimal changes in terms of stability of α- and β-carotene
Stability of 5-formyltetrahydrofolic acid in model system (Nguyen et al., 2006)	pH = 9.2; T = 30-70; P = 100-800; t = 0-240	$\Delta V^{\neq}$ = -3.3 to -12.4 cm^3 mol^{-1}, changing with temperature - Synergistic effect between pressure and temperature
Folic acid stability in phosphate buffer (Nguyen et al., 2003)	pH = 7.0; T = 20-65; P = 100-800; t = 0-140	$\Delta V^{\neq}$ = -5.7 to -13.9 cm^3 mol^{-1}, changing with temperature - Synergistic effect between pressure and temperature
Stability of ascorbic acid and [6S]-5-methyltetrahydrofolate (Oey et al., 2006)	T = 40 and 50; P = 100-600; t = 15	- Ascorbic acid enhanced the stability of folates upon pressure
Stability of ascorbic acid in orange juice (Polydera et al., 2005)	T = 40; P = 600; t = 4	- Retention of ascorbic acid was higher for pressurized samples compared to traditional process
Ascorbic acid in reconstituted orange juice (Polydera et al., 2003)	T = 35; P = 500; t = 5	- Ascorbic acid degradation rates were lower in high pressure treated orange juice compared the equivalent thermal process

T – temperature (˚C); P – pressure (MPa) and t – time (min).

Table 2-8. (Continued)

Food matrix	Conditions	Remarks
Stability of poliphenol and anthocyanins (Terefe et al., 2009)	T = 20-60; P = 300-600; t = 2-10	- No effect was found on the polyphenol and anthocyanin content
Stability of ascorbic acid and anthocyanin in blood orange juice (Torres et al., 2011)	T = NR; P = 400-600; t = 15	- The retention of vitamins after high pressure processing was 99 and 94% for ascorbic acid and anthocyanin, respectively
Ascorbic acid in model system (Van den Broeck et al., 1998)	T = 65-80; P = 850; t = 0-350	- Pressure and temperature act synergistically on the degradation of ascorbic acid
Degradation of chlorophyll in broccoli juice (Van Loey et al., 1998)	T = 50-120; P = 0.1-800; t = 0-70	- Degradation rate increased with temperature, following Arrhenius model
Anthocyanins in raspberry (Verbeyst et al., 2011)	T = 90-115; P = 200-700; t = 0-50	- $\Delta V^{\neq}$ = -8 to -5 $cm^3\ mol^{-1}$, changing with temperature
Anthocyanins in strawberries (Verbeyst et al., 2010)	T = 80-130; P = 200-700; t = 0-30	- $\Delta V^{\neq}$ = -2.5 to -3.66 $cm^3\ mol^{-1}$, changing with temperature
Loss of greenness in broccoli juice (Weemaes et al. 1999)	T = 70-90; P = 0.1-850; t = 0-180	- Green color was lost to chlorophyll-pheophytin conversion with no further degradation of pheophytin
Ascorbic acid in strawberries and raspberries (Verbeyst et al., 2013)	T = 60-110; P = 700; t = 0-120	- Synergistic effect of temperature and pressure
Folates in broccoli tissue (Verlinde et al., 2008)	T = 25-45; P = 0.1-600; t = 30	- Pressure yielded between 48-78% of folate losses
Anthocyanins in blueberry juice (Buckow et al., 2010)	T = 40-121; P = 100-700; t = 0-150	- Pressure and temperature increased the degradation rate - $\Delta V^{\neq}$ = -3.1 to -9.6 $cm^3\ mol^{-1}$; E_a = 51-70 kJ mol^{-1}
Stability of hydrosoluble vitamins in model system (Sancho et al., 1999)	T = NR; P = 200 and 400; t = 30	- Minor variations were found among the vitamins after pressurization

Oley et al. (2006) studied the effect of oxygen and ascorbic acid on the pressure-temperature stability of [6S]-5-methyltetrahydrofolate. These authors reported that ascorbic acid enhanced the stability of folate and, more importantly, they found the minimum concentration of ascorbic acid needed to prevent oxidation of folate (at least twice higher than the oxygen concentration). The stability of anthocyanins upon HPS has been studied in raspberries and

strawberries (Verbeyst et al., 2010 and 2011). The degradation kinetics was described by the Arrhenius-Eyring model, where the synergistic effect could be illustrated. Another synergistic effect has been suggested by Corrales et al. (2008). These authors reported that condensation reactions of anthocyanin are promoted by combinations of pressure and temperature. Their control treatment, model solutions of cyaniding-3-*O*-glucoside (Cy3gl) and pyruvate treated at 0.1 MPa and 70°C, showed around 5% of condensation, while significant degradation of Cy3gl (around 25%) was found at 600 MPa and 70°C. Prior to a high pressure treatment, De la Fuente et al. (1999) stabilized the anthocyanin content in grape juice fortified with ascorbic acid by the addition of water soluble polyphenols extracted from rosemary and thyme. In this investigation, the anthocyanin content was monitored for 21 days after 15 min of pressure treatment in the range of 400-500 MPa at room temperature. The anthocyanin content was higher in those samples that have been stabilized prior to the treatment. The authors suggested that a pressure treatment enhanced anthocyanin retention on non *o*-diphenolic such as peonidin and malvidin. More importantly, intermolecular co-pigmentation was not affected by the pressure treatment and therefore it can be used prior to pressure treatment to enhance anthocyanin retention.

The degradation of chlorophyll in broccoli juice treated at high pressure and temperature was studied by Weemaes et al. (1999). The degradation rates increased with temperature, following the Arrhenius model. These authors found that the loss of green color was due to chlorophyll-pheophytin conversion and no further degradation of pheophytin was detected. With the kinetic information reported by Weemaes et al. (1999), the reaction mechanism can be inferred. Since only chlorophyll and pheophytin were detected, the reaction Scheme 7 can be adopted:

$$Chlorophyll \overset{k_1}{\rightarrow} Pheophytin \overset{k_2}{\rightarrow} Degradation\ products \qquad \text{[Scheme 7]}$$

where k_1 and k_2 are the degradation rate constants for chlorophyll and pheophytin, respectively. Both constants were calculated as a function of pressure and temperature. The degradation products can be pyropheophytin and phytol. The calculated k values at 500 MPa and 80˚C were k_1 ($105.7 \pm 2.28 \times 10^{-3}$ min^{-1}) >> k_2 ($k_2 = 2.21 \pm 0.06 \times 10^{-3}$ min^{-1}). The conversion of chlorophyll to pheophytin was accelerated by pressure since the k_1 obtained at 0.1 MPa and 80˚C was $15.75 \pm 0.23 \times 10^{-3}$ min^{-1}. This conversion can occur through elimination of Mg in which the metal ion is electrostricted and therefore released from the chlorophyll structure. Further degradation of pheophytin might involve covalent bond breaking, which is inhibited by pressure. A multi response modelling is needed for establishing the reaction mechanisms, since other pathways can occur.

Butz et al. (2007) studied the effect of pressure and temperature on the stability of riboflavin, thiamine and thiamine monophosphate in a model system and in minced pork. These authors found that vitamins were more stable in pork than in model systems, where the reduction rate was up to 30 times faster. These results suggest that the outcomes from model systems should be used with caution when translating to food systems. Butz et al. (2007) found that 600 MPa and 100˚C for 15 min yielded a product with ~10% of vitamin loss, while the traditional thermal treatment (121˚C for 20 min) delivered a product with vitamin losses of up to 45%. The stability of lycopene was evaluated in tomato juice treated with HPS (600 MPa, 100˚C and 10 min), HPP (700 MPa, 45˚C and 10 min) and thermal treatment (0.1 MPa, 100˚C and 35 min) (Gupta et al., 2010). Samples treated with HPP and HPS showed better color retention and were more stable than the thermally treated samples.

Pressure improved the extractability of lycopene. Butz et al. (1997) compared the pressure stability of synthetic sweetener aspartame in full fat milk (pH 6.8) and in Tris/HCl buffer (pH 7). In milk added with aspartame, around 50% of active substance (diketopiperazine) is lost after 3 min at 600 MPa and 60°C while in the buffer solution, this effect was smaller. Interestingly,

simultaneous application of pressure and temperature lead to formation of two non-sweet compounds, aspartylphenylalanine and diketopiperazine. The molar sum of these two compounds corresponds to the amount of aspartame that was degraded.

Verbeyst et al. (2013) studied the aerobic and anaerobic degradation of ascorbic acid of red fruits treated with HPS. These authors used a biphasic model for the aerobic degradation, where ascorbic acid (AA) is converted to dehydroascorbic acid (DHA) and subsequently to 2,3-diketogulunic acid (DKA) (Scheme 8).

$$AA \overset{k_1}{\rightarrow} DHA \overset{k_2}{\rightarrow} DKA$$ [Scheme 8]

The k_1 calculated at HPS was higher than that obtained at atmospheric pressure (249 and 10 x 10^{-2} min^{-1}, respectively), meaning that pressure promoted the conversion of AA to DHA by the removal of two hydrogen ions through ionization. Further degradation of DHA to DKA was also accelerated by pressure (k_2 values were 101.41 and 20.79 x 10^{-2} min^{-1} for HPS and thermal treatment, respectively), opening the ring of the ascorbic acid and eliminating water by condensation (Isaacs, 1981).

HPS not only can inhibit the degradation of bioactive compounds but also can preserve the texture in fruits and vegetables. De Rock et al. (2009) studied chemical conversions of apple pectin and found that pressure inhibited the depolymerisation of the cell wall, which is the reaction responsible for tissue softening.

2.10. Kinetic studies

2. 10.1. Pressure-temperature diagram

A practical approach for expressing the simultaneous effect of pressure and temperature on the *k* values is by building pressure-temperature diagrams

through a thermodynamic model (equation (2.17)). Buckow & Heinz (2008) reviewed the fundamentals of equation (2.17). Alternatively, polynomial models (equations (2.18-2.20 in Table 2-9)) have been successfully used for modelling the *k* values as a function of pressure and temperature (Nguyen et al., 2006). Some authors used the Arrhenius-Eyring model (equation (2.19)), which provides information on the reaction mechanism (Verbesyt et al., 2010 and 2011). These diagrams are built by plotting all the possible combinations of pressure and temperature that yield the same *k* value. **Table 2-9** summarises the *k* values obtained as a function of pressure and temperature in chemical reactions for food systems.

Table 2-9 Equations and estimated model parameters to describe pressure-temperature effect on the reaction rate constant for chemical reactions in food systems.

Reaction	Equation	Parameters
Stability of folic acid (Nguyen et al., 2003)	$\ln k = \frac{\Delta\beta^{\neq}}{2RT}\cdot\left(P-P_{ref}\right)^2 - \frac{\Delta V^{\neq}}{RT}\cdot\left(P-P_{ref}\right) + \frac{\Delta S^{\neq}}{RT}\cdot\left(T-T_{ref}\right) + \frac{\Delta C_p^{\neq}}{RT}\cdot\left[T\cdot\left[ln\left(\frac{T}{T_{ref}}\right)\right] + T_{ref}\right] + \ln k_{ref}$ (2.17)	$\Delta\beta^{\neq}$ = 2.1 ± 0.3x10^{-2} $\Delta V^{\neq}$ = -8.8 ± 0.5 $\Delta S^{\neq}$ = 249.8 ± 8.3 $\Delta Cp^{\neq}$ = -1258 ± 201
Degradation of 5-formyltetrahydrofobic acid (Nguyen et al., 2006)	$\ln k = \frac{A}{T}\cdot\left(P-P_{ref}\right)^2 - \frac{B}{T}\cdot\left(P-P_{ref}\right) + \frac{C}{T}\cdot\left(T-T_{ref}\right) + \frac{D}{T}\cdot\left[T\cdot\left[ln\left(\frac{T}{T_{ref}}\right)-1\right] + T_{ref}\right] + \ln k_{ref}$ (2.18)	A = 6.8 ± 2.2x10^{-4} B = -113.3 ±1.6x10^{-2} C = 20.1 ± 1.6 D = 70.2 ± 61.3 K_{ref} = 0.18 ±0.1x10^{-3}
Degradation of anthocyanins (Verbeyst et al., 2010)	$k = k_{ref}\cdot exp^{\left(a\cdot\left(T-T_{ref}\right)\right)}\cdot exp^{\left(b\cdot\left(P-P_{ref}\right)\right)}\cdot exp^{\left(c\cdot\left(T-T_{ref}\right)\cdot\left(P-P_{ref}\right)\right)}$ (2.19)	k_{ref} = 0.018 ± 0.001 a = 0.064 ± 0.004 b = 0.001 ± 0.0001
Anthocyanins degradation (Buckow et al., 2010)	$\ln k = A + B\cdot\left(P-P_{ref}\right) + C\cdot\left(T-T_{ref}\right) + D\cdot\left(P-P_{ref}\right)^2 + H\cdot\left(T-T_{ref}\right)^3 + J\cdot\left(P-P_{ref}\right)\cdot\left(T-T_{ref}\right)^2$ (2.20)	A = -5.7 ± 0.2 B = 5.3 ± 4.7x10^{-3} C = 3.1 ± 5.3x10^{-2} D = -1.1 ± 2.1x10^{-4} H = 3.7 ± 7.1x10^{-6} J = -6.7 ± 2.3^{-7}
Degradation of anthocyanins (Verbeyst et al., 2011)	$k = k_{ref}\cdot exp^{\left(\frac{-E_a}{R}\left(\frac{1}{T}-\frac{1}{T_{ref}}\right)\right)}\cdot exp^{\left(\frac{-\Delta V^{\neq}}{RT}\cdot\left(P-P_{ref}\right)\right)}$ (2.19)	k_{ref} = 6.5 ± 0.2 E_a = 78.3 ± 1.8 $\Delta V^{\neq}$ = -5.9 ± 0.2
Free amino group for BSA-Glc conjugation (Buckow et al., 2011)	$\ln k = \ln k_{ref} - \frac{\Delta V^{\neq}}{RT}\cdot\left(P-P_{ref}\right) + \frac{\Delta S^{\neq}}{RT}\cdot\left(T-T_{ref}\right) - \frac{\Delta\beta^{\neq}}{2RT}\cdot\left(P-P_{ref}\right)^2 - \frac{2\Delta\alpha^{\neq}}{RT}\cdot\left(P-P_{ref}\right)\cdot\left(T-T_{ref}\right)$ (2.20)	k_{ref} = 2.3 ± 0.06x10^{-2} $\Delta V^{\neq}$ = 1.2 ± 0.2x10^{-5} $\Delta S^{\neq}$ = 2.3 ± 0.2x10^{2} $\Delta\beta^{\neq}$ = 1.6.1± 0.3x10^{-14} $\Delta Cp^{\neq}$ = 4.7 ± 4.4x10^{2} $\Delta\alpha^{\neq}$ = -4.7 ±1.8x10^{-8}

The parameters reported in **Table 2-9** were used to predict the *k* values for different chemical reactions (**Fig 2-1**). Each contour line represents the combinations of pressure and temperature needed to achieve the same *k* value at a given holding time. For example, 90% of the total anthocyanins of strawberries are retained at 600 MPa and 90˚C after 3 min of holding time (♦ symbol in **Fig 2-1**). For optimization purposes, this is a practical approach where an undesirable reaction can be minimized by manipulating the processing conditions. In addition, these diagrams graphically illustrate the synergistic effect of pressure and temperature.

In **Fig 2-1**, the shape of the contour lines differ from those diagrams obtained for inactivation of enzymes and microorganisms (Ludikhuyze et al., 2003; Buckow and Heinz, 2008). Enzyme inactivation diagrams are elliptical in shape while diagrams for chemical reactions are diagonal lines. In general, more severe conditions are required for observing a synergistic effect between pressure and temperature. Such difference in the shape of the diagrams allows locating the optimum processing conditions. However, the construction of P-T diagrams requires kinetic studies covering a wide range of processing conditions (Claeys et al., 2003) and heavily relies on the accuracy of the temperature-pressure dependence of rate constant.

2.10.2. Importance of kinetics studies

Chemical reactions play an important role in determining the final quality of a food product. For example, the degradation of a specific bioactive compound to produce off-flavour compounds can be the reason for a product to be rejected by consumers. Thus, understanding how a particular reaction occurs and the speed at which it occurs is important, enabling better process design and optimization and ensuring acceptable shelf-life. In addition, it has enabled off-line simulation and development of a databank (Buckow and Heinz 2008).

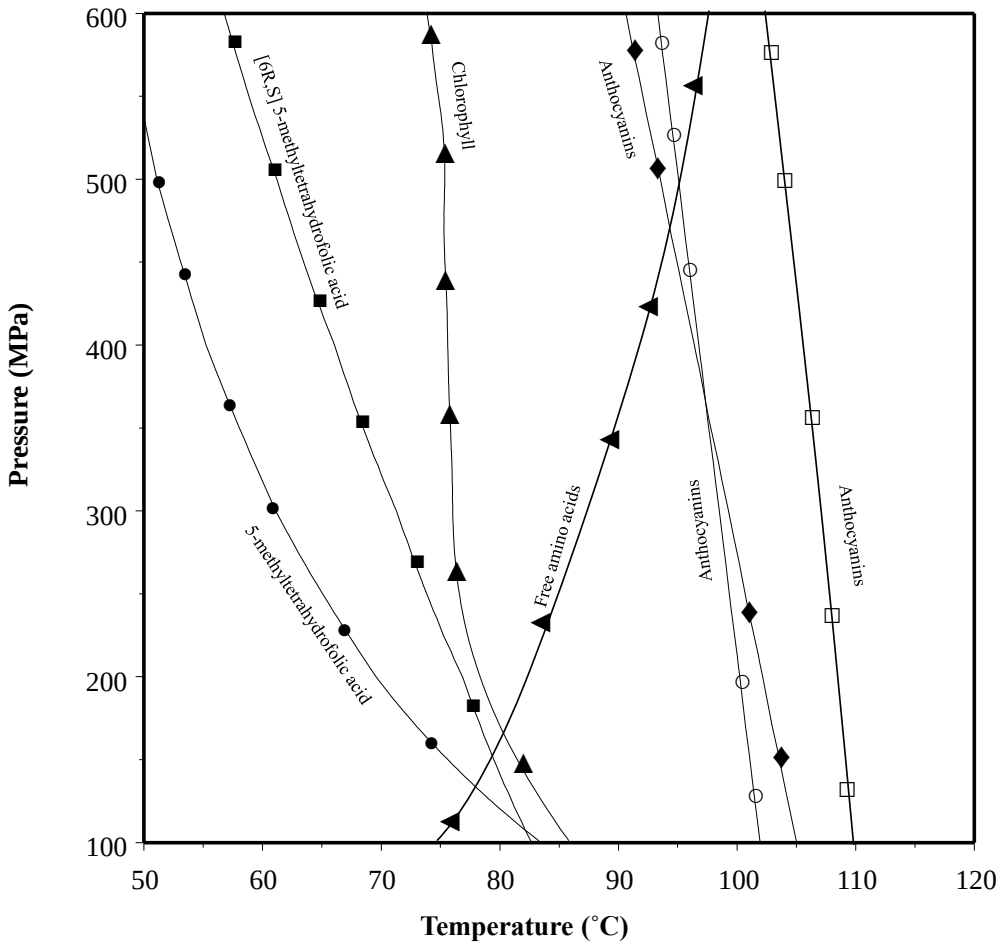


Fig 2-1. Simulated pressure-temperature diagram for different chemical reactions in model systems and real foods: (●) 40% of retention of 5-methyltetrahydrofolic acid in phosphate buffer (pH=7) after 10 min (Nguyen et al., 2003); (■) 98% of retention of (6R,S)5-formytetrahydrofolic acid in acetate buffer (pH=5) after 10 min (Nguyen et al., 2003); (▲) 90% of reduction of chlorophyll content in broccoli (Van Loey et al., 1998); (◄) 70% free amino group during the conjugation of bovine serum albumin and glucose in bicin buffer (pH=9) after 30 min (Buckow et al., 2011); (♦) 90% of retention of anthocyanins in strawberries after 3 min (Verbesyt et al., 2010); (○) 90% of anthocyanin retention in raspberry after 3 min (Verbesyt et al., 2011); (□) 50% of retention of anthocyanins in blueberry juice after 20 min (Buckow et al., 2010).

Kinetic modelling relies heavily on the adopted experimental protocols and on the accuracy of the estimated parameters. For simplicity, the use of model

systems is a common practice for kinetics studies of chemical reactions. However, the data obtained from model systems cannot be directly transferred to real food systems (van Boekel, 2008). For example, the effect of pressure and temperature on Maillard reaction has been studied at pH levels of 8 to 10 (**Table 2-6**), which are unrealistic for any real food system. An ideal model system should account for the changes in pH and ionic strength, formation of ions, changes in disassociation of electrolytes, solubility, diffusion effects, molecular exclusion, and changes in properties of transport phenomena. van Boekel (2008) provided a list of important considerations to compare model systems to real food systems, suggesting that the model system must be as close as possible to the real food system.

2.11. Conclusions and outlook

Ionization:

- Ionization is a pressure-sensitive reaction, occurring even at room temperature. Many compounds are ionisable during pressure treatment and, unfortunately, no kinetic parameters (k and $\Delta V^{\neq}$) have been reported for food systems.
- The experimental determination of the ionization rate constants of salts, phenols and vitamins as a function of pressure can be valuable in the study of chemical reactions since ionic species are more reactive.
- Although the pH has been experimentally determined inside a high pressure vessel, no commercial probe is currently available, limiting research at HPP and HPS for various food systems. More fundamental research is needed to develop a model that accounts for the changes in pH at elevated temperatures (>90˚C) and pressures.
- In model systems, the type of buffer to be used should be considered as an important part of the experimental protocol since the magnitude of the change in the pH value depends on the type of buffer.
- The use of different buffer systems, both temperature- and pressure-sensitive, seems to be an adequate approach for distinguishing the effect of pressure from that caused by the change in pH.

Volatiles formation

- Due to the complexity of the reaction network, the effect of pressure on the formation of off-flavour compounds is difficult to interpret.
- The use of antioxidants showed encouraging results in terms of inhibiting the formation of some off-flavour compounds.

Maillard reaction

- The literature data on Maillard reaction is limited and difficult to compare due to the different processing conditions used. For instance, information obtained at moderate conditions of temperature is substantially different from that obtained at severe temperature conditions.
- The effect of pressure should be evaluated for individual reactions and not as an overall effect on the Maillard reaction.

Oxidation

- Apart from meat and meat products, information on how pressure influences the oxidation of fatty acids is limited.
- Pressure can affect differently the initiation, propagation and termination stage of food systems containing fat.

Degradation and stability

- Pressure exerts a protective effect on vitamins at moderate temperatures (40-60˚C). In contrast, a synergistic effect occurs when high temperatures are used in combination with pressure, accelerating the degradation.
- The synergistic effect can be graphically illustrated through P-T diagrams.

Kinetics

- The majority of the kinetics studies consist of measuring the loss or the formation of a specific compound at a predetermined set of experimental conditions, a procedure designed to fit a kinetic model. However, for complex reactions, such as Maillard that involves oxidation and degradation, the current approach might not provide information on the reaction pathways.
- The effect of pressure on the reaction pathways is perhaps the most intriguing question that needs to be addressed. Multiresponse modelling

might help to analyse the reaction pathways in more detail by quantifying many responses at once and construct the kinetic model based on an actual reaction pathway. Recently, Verbeyst et al. (2013) developed a mechanistic model for the degradation of ascorbic acid at HPS conditions that could be used for additional kinetics studies in other systems.

- The formation of undesirable compounds, such as acrylamide and oxysterols should also be considered at HPS conditions.

2.9. References

Al-Attabi, Z., D'arcy, B. D., & Deeth, H. C. (2009). Volatile sulphur compounds in UHT milk. *Critical Reviews in Food Science and Nutrition, 49*(1), 28-47.

Alt, N., & Schieberle, P. (2005). Model studies on the influence of high hydrostatic on the formation of glycated arginine modifications at elevated temperatures. *Journal of Agricultural and Food Chemistry, 53(14),* 5789-5797.

Altuner, E. M., Alpas, H., Erdem, Y. K., & Bozoglu, F. (2006). Effect of high hydrostatic pressure on physicochemical and biochemical properties of milk. *European Food Research and Technology, 222(3-4),* 392-396.

Andersen, B., & Gronlund, F. (1979). High pressure kinetics of glucose mutarotation studied by optical rotation. *Acta chemical Scandinavica A, 33*, 275-280.

Angsupanich, K., & Ledward, D. A. (1998). High pressure treatment effects on cod (gadus morhua) muscle. *Food Chemistry, 63(1),* 39-50.

Arques, J. L., Garde, S., Fernandez-Garcia, E., Gaya, P., & Nunez, M. (2007). Volatile compounds, odor, and aroma of La Serena cheese high-pressure treated at two different stages of ripening. *Journal of Dairy Science, 90(8),* 3627-3639.

Asano, T., & Le Noble, W. J. (1979). Activation and reaction volumes in solution. *Chemical Review, 78(4),* 407-489.

Avila, M., Garde, S., Fernandez-Garcia, E., Medina, M., & Nunez, M. (2006). Effect of high pressure and a bacteriocin-producing lactic culture on the odor and aroma of Hispano cheese: Correlation of volatile compounds and sensory analysis. *Journal of Agricultural and Food Chemistry, 54(2),* 283-389.

Black, E. P. (2007). Response of spores to high-pressure processing. *Comprehensive Reviews in Food Science and Food Safety, 6(4),* 103-119.

Bristow, M., & Isaacs, N. S. (1999). The effect of high pressure on the formation of volatile products in a model Maillard reaction. *Journal of the Chemical Society, Perkin Transactions 2, 10*, 2213–2218.

Bolumar, T., Skibsted, L. H., & Orlien, V. (2012). Kinetics of the formation of radicals in meat during high-pressure processing. *Food Chemistry, 134(4),* 2114-2120.

Buchheim, W., & Abouelnour, A. M. (1992). Induction of milkfat crystallization in the emulsified state by high hydrostatic-pressure. *Fett Wissenschaft Technologie Fat Science Technology, 94(10),* 369-373.

Buckow, R., & Heinz, V. (2008). High pressure processing – a database of kinetic information. *Chemie Ingenieur Technik, 80*(8), 1081-1095.

Buckow, R., Heinz, V., & Knorr, D. (2007). High pressure phase transition kinetics of maize starch. *Journal of Food Engineering, 81(2),* 469-475.

Buckow, R., Wendorff, J., & Hemar, Y. (2011). Conjugation of bovine serum albumin and glucose under combined high pressure and heat. *Journal of Agricultural and Food Chemistry, 59*(8), 3915-3923.

Buckow, R., Kastell, A., Terefe, N. S., and Versteeg, C. (2010). Pressure and temperature effects on degradation kinetics and storage stability of total anthocyanins in blueberry juice. *Journal of Agricultural and Food Chemistry, 58(18),* 10076-10084.

Buckow, R., Sikes, A., & Tume, R. (2013). Effect of high-pressure on physicochemical properties of meat. *Critical Reviews in Food Science and Nutrition, 53(7),* 770-786.

Buffa, M., Trujillo, A. J., Royo, C., & Guamis, B. (2000). Changes in chemical and microbiological characteristics of goat cheese made from raw, pasteurized or high-pressure-treated milk. *High Pressure Research, 19(1-6),* 417-422.

Burgess, J., Guardado, P., & Hubbard, C. D. (2010). Partial molar volumes for inorganic complexes. *Journal of Coordination Chemistry, 63(14-16),* 2461-2471.

Butz, P., Bognar, A., Dieterich, S., & Tauscher, B. (2007). Effect of high-pressure processing at elevated temperatures on thiamin and riboflavin in pork and model systems. *Journal of Agricultural and Food Chemistry, 55(4),* 1289-1294.

Butz, P., Fernandez, A., Fister, H., & Tauscher, B. (1997). Influence of high hydrostatic pressure on aspartame: Instability at neutral pH. *Journal of Agricultural and Food Chemistry, 45(2),* 203-303.

Butz, P., & Tauscher, B. (2000). Recent studies on pressure-induced chemical changes in food constituents. *High Pressure Research, 19(1),* 11-18.

Butz, P., Serfert, Y., Garcia, A. F., Dieterich, S., Lindauer, R., Bognar, A., & Tauscher, B . (2004). Influence of high-pressure treatment at 25 degrees C and 80 degrees C on folates in orange juice and model media. *Journal of Food Science, 69(3),* S117-S121.

Butz, P., Koller, W.D., Tauscher, B., and Wolf, S. (1994). Ultra-high pressure processing of onions - chemical and sensory changes. *Food Science and Technology-Lebensmittel-Wissenschaft and Technologie, 27(5),* 463-467.

Campus, M., Flores, M., Martinez, A., & Toldra, F. (2008). Effect of high pressure treatment on colour, microbial and chemical characteristics of dry cured loin. Meat Science, *80(4),* 1174-1181.

Chan, J. T., Omana, D. A., & Betti, M. (2011). Application of high processing to improve the functional properties of pale, soft, and texture exudative (PSE)-like turkey meat. *Innovative Food Science & Emerging Technologies, 12(3),* 216-225.

Claeys, W. L., Van Loey, A. M., & Hendrickx, M. E. (2003). Kinetics of hydroxymethylfurfural, lactulose and furosine formation in milk with different fat content. *Journal of Dairy Research, 70(1),* 85-90.

Corrales, M., Butz, P., & Tauscher, B. (2008). Anthocyanin condensation reactions under high hydrostatic pressure. *Food Chemistry*, *110(3),* 627-635.

Cruz-Romero, M., Kerry, J. P., and Kelly, A. L. (2008). Fatty acids, volatile compounds and colour changes in high-pressure treated oysters (Crassostreagigas). *Innovative Food Science & Emerging Technologies*, *9(1),* 54-61.

Dalmadi, I., Poyak-Feher, K., and Farkas, J. (2007). Effects of pressure- and thermal-pasteurization on volatiles of some berry fruits. *High pressure Research, 27(1),* 169-172.

Danielewicz-Ferchmin, I., & Ferchmin, A. R. (1998). Hydration of ions at various temperatures: The role of electrostriction. *Journal of Chemical Physics, 109(6),* 2394-2402.

De la Fuente, M. A., Olano, A., Casal, V., & Juarez, M. (1999). Effects of high pressure and heat treatment on the mineral balance of goat's milk. *Journal of Dairy Research., 66(1),* 65-72.

Dede, S., Alpas, H., & Bayindirli, A. (2007), High hydrostatic pressure treatment and storage of carrot and tomato juices: Antioxidant activity and microbial safety. *Journal of the Science of Food and Agriculture*, *87(5),* 773-782.

de Roeck, A., Duvetter, T., Fraeye, I., Van der Plancken, I., Sila, D. N., Van Leoy, A., & Hendrickx, M. (2009). Effect of high-pressure/high-temperature processing on chemical pectin conversions in relation to fruit and vegetable texture. *Food Chemistry*, *115(1),* 207-213.

De Vleeschouwer, K., Van der Plancken, I., Van Loey, A., & Hendrickx, M. E. (2010). The effect of high pressure-high temperature processing conditions on acrylamide formation and other Maillard reaction compounds. *Journal of Agricultural and Food Chemistry, 58(22),* 11740–11748.

Disteche, A. (1959). pH measurements with a glass electrode withstanding 1500 kg/cm2 hydrostatic pressure. *Review of Scientific Instruments, 30*(6), 474-478.

Drude, P., & Nernst, W. (1894). Über elektrostriktion durch freie ionen. *Physikalische Chemiey, 15*, 79-85.

Elyanov, B. S., & Hamann, S. D. (1975). Some quantitative relationships for ionization reactions at high-pressures. *Australian Journal of Chemistry,* 28(5), 945-954.

Evans, M. G., & Polanyi, M. (1937). On the introduction of thermodynamic variables into reaction kinetics. *Transactions of the Faraday Society, 33(1),* 448-452.

Eyring, H. (1935). The activated complex and the absolute rate of chemical reactions. *Chemical Reviews, 17(1),* 65-77.

Famelart, M. H., Gaucheron, F., Mariette, F., Le Graet, Y., Raulot, K., & Boyaval, E. (1997). Acidification of pressure-treated milk. *International Dairy Journal, 7(5),* 325-330.

Frank, O., Heberle, I., Schieberle, P., & Hofmann, T. (2002). Influence of high hydrostatic pressure on the formation of intense chromophores formed from pentoses and primary amino acids. *International Congress Series, 1245*, 387-388.

Gupta, R., Balasubramaniam, V. M., Schwartz, S. J., & Francis, D. M. (2010). Storage stability of lycopene in tomato juice subjected to combined pressure-heat treatments. *Journal of Agricultural and Food Chemistry, 58(14),* 8305-8313.

Gobert, J., & Glomb, M. A. (2009). Degradation of glucose: Reinvestigation of reactive α-dicarbonyl compounds. *Journal of Agricultural and Food Chemistry, 57(18),* 8591-8591.

Hamann, S. D. (1981). Properties of electrolyte solutions at high pressure and temperatures. *Physics and chemistry of the earth, 13(4),* 89-111.

Hamann, S. D. (1982). The influence of pressure on ionization equilibria in aqueous-solutions. *Journal of Solution Chemistry, 11(1),* 63-68.

Hamann, S. D., & Linton, M. (1974). Influence of pressure on the ionization of substituted phenols. *Journal of Chemistry Society, Faraday Transactions 1: Physical Chemistry in Condensed Phases, 70,* 2239-2249.

Hayert, M., Perrier-Cornet, J. M., & Gervais, P. (1999). A simple method of measuring the pH of acid solutions under high pressure. *Journal of Physical chemistry A, 103(12),* 1785-1789.

Hill, V. M., Ledward, D. A., & Ames, J. M. (1996). Influence of high hydrostatic pressure and pH on the rate of Maillard browning in a glucose−lysine system. *Journal of Agricultural and Food Chemistry, 44(2),* 594-598.

Hill, V. M., Isaacs, N. S., Ledward, D. A., and Ames, J. M. (1999). Effects of high hydrostatic pressure on the volatile components of a glucose-lysine model system. *Journal of Agricultural and Food Chemistry, 47(9),* 3675-3681.

Huang, T. C., Fu, H. Y., & Ho, C. T. (1996). Mechanistic studies of tetramethylpyrazine formation under weak acidic conditions and high hydrostatic pressure. *Journal of Agricultural and Food Chemistry., 44(1),* 240-246.

Indrawati, Arroqui, C., Messagie, I., Nguyen, M. T., Van Loey, A., & Hendrickx, M. (2004). Comparative study on pressure and temperature stability of 5-methyltetrahydrofolic acid in model systems and in food products. *Journal of Agricultural and Food Chemistry, 52(3),* 485-492.

Isaacs, N. S. (1981). *Liquid phase high pressure chemistry.* Toronto: John Wiley.

Isaacs, N. S., & Coulson, M. (1996). Effect of pressure on processes modelling the Maillard reaction. *Journal of Physical Organic Chemistry, 13,* 639-644.

Jandhyala, M., Barbosa-Canovas, G. V., & Swanson, B. G. (2002). Note: Retention of ascorbic acid, thiamine and pyridoxal after high hydrostatic pressure or thermal treatment. *Food Science and Technology International, 8(5),* 303-308.

Jenner, G. (2002). Comparative activation modes in organic synthesis. The specific role of high pressure. *Tetrahedron, 58(26),* 5185-5202.

Jenner, G. (2004). Role of the medium in high pressure organic reactions. A review. *Mini Reviews in Organic Chemistry, 1(1),* 9-26.

Juan, B., Barron, L. J. R., Ferragut, V., Guamis, B., & Trujillo, A. J. (2007). Changes in the volatile composition of a semihard ewe milk cheese induced by high-pressure treatment of 300 MPa. *Journal of Agricultural and Food Chemistry, 55(3),* 747-754.

Khoshtariya, D. E., Zahl, A., Dolidze, T. D., Neubrand, A., & van Eldik, R. (2004). Local dense structural heterogeneities in liquid water from ambient to 300 MPa pressure: Evidence for multiple liquid-liquid transitions. *European Journal of Chemical Physics and Physical Chemistry, 5(9),* 1398-1404.

Kim, Y. S., Park, S. J., Cho, Y. H. and Park, J. (2001). Effect of combined treatment of high hydrostatic pressure and mild heat on the quality of carrot juice. *Journal of Food Science, 66(9),* 1355-1360.

Kim, H. Y., Kim, S. H., Choi, M. J., Min, S. G., & Kwak, H. S. (2008). The effect of high pressure-low temperature treatment on physicochemical properties in milk. *Journal of Dairy Science, 91(11),* 4176-4182.

Kitamura, Y., & Itoh, T. (1987). Reaction volume of protonic ionization for buffering agents. Prediction of pressure dependence of pH and pOH. *Journal of SolutionChemistry, 16(9),* 715-725.

Lambert, Y., Demazeau, G., Largeteau, A., & Bouvier, J. M. (1999). Changes in aromatic volatile composition of strawberry after high pressure treatment. *Food Chemistry, 67(1),* 7-16.

Leadley, C., Tucker, G., & Fryer, P. (2008). A comparative study of high pressure sterilization and conventional thermal sterilization: Quality effects in green beans. *Innovative Food Science & Emerging Technologies, 9(1),* 70-79.

Lewis, C. A., & Wolfende, R. (1973). Influence of pressure on equilibrium of hydration of aliphatic aldehydes. *Journal of the American Chemical Society, 95(20),* 6685-6688.

Ludikhuyze, L., Van Loey, A., Indrawati, Smout, C., & Hendrickx, M. (2003). Effects of combined pressure and temperature on rnzymes related to quality of fruits and vegetables: from kinetic information to process engineering aspects. *Critical Reviews in Food Science and Nutrition, 43(5),* 527-586.

Marcus, Y. (2005). Electrostriction, ion solvation, and solvent release on ion pairing. *Journal of Physical Chemistry B, 109(39),* 18541-18549.

Marshall, W. L., & Franck, E. U. (1981). Ion product of water substance, 1-10000 Bars new international formulation and its background. *Journal Physics Chemical Reference Data, 10(2),* 295-304.

Martins, S. F., Jongen, W. F., & van Boekel, M. J. (2001). A review of MAillard reaction in food and implication to kinetic modelling. *Trends in Foods Science & Technology, 11(9-10),* 364-373.

Mathys, A., & Knorr, D. (2009). The properties of water in the pressure-temperature lacdscape. *Food Biophysics, 4(2),* 77-82.

Mathys, A., Reineke, K., Heinz, V., & Knorr, D. (2009). High pressure thermal sterilization - development and application of temperature controlled spore inactivation studies. *High Pressure Research, 29(1),* 3-7.

Matser, A. A., Krebbers, B., van den Berg, R. W., & Bartels, P. V. (2004). Advantages of high pressure sterilisation on quality of food products. *Trends in Food Science & Technology, 15(2),* 79-85.

Molina-Guitierrez, A., Stippl, V., Delgado, A., Ganzle, M. G., & Vogel, R. (2002). In situ determination of the intercelular pH of Lactococcus lactis and Lactobacillus plantarum during pressure treatment. *Applied and Environmental Microbiology, 68(9),* 4399-4406.

Moreno, F. J., Villamiel, M., & Olano, A. (2003a). Effect of high pressure on isomerization and degradation of lactose in alkaline media. *Journal of Agricultural and Food Chemistry, 51(7),* 1894-1896.

Moreno, F. J., Molina, E., Olano, A., & Lopez-Fandino, R. (2003b). High-pressure effects on Mailllard reaction between glucose and lysine. *Journal of Agricultural and Food Chemistry, 51(2),* 394-400.

Navarro, M., Verret, P., Pardon, A., & Moueffak, El. (2002). Changes in Volatile Aromatic Compounds of Strawberry Puree Treated by High-pressure During Storage. *High Pressure Research, 22(3-4),* 693-696.

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),* 31-42.

Neuman, R. C., Kauzmann, W., & Zipp, A. (1973). Pressure-dependence of weak acid ionization in aqueous buffers. *Journal of Physical Chemistry, 77(22),* 2687-2691.

Nguyen, M. T., Indrawati, & Hendrickx, M. (2003). Model studies on the stability of folic acid and 5-methyltetrahydrofolic acid degradation during thermal treatment in combination with high hydrostatic pressure. *Journal of Agricultural and Food Chemistry, 51(11),* 3352-3357.

Nguyen, M. T., Oey, I., Hendrickx, M., & Van Loey, A. (2006). Kinetics of (6R,5) 5-formyltetrahydrofolic acid isobaric-isothermal degradation in a model system. *European Food Research Technology, 223(3),* 325-331.

Oley, I., Verlinde, P., Hendrickx, M. E., & Van Loey, A. (2006). Temperature and pressure stability of L-ascorbic acid and/or 6s 5-methyltetrahydrofolic acid: A kinetic study. *European Food Research and Technology, 223(1),* 71-77.

Polanyi, M. (1937). The transition state in chemical reactions. *Journal of chemical Society,* 629-635.

Polydera, A. C., Stofores, N. G., and Taoukis, P. S. (2005). Quality degradation kinetics of pasteurized and high pressure processed fresh Naval orange

juice: Nutritional parameters and shelf life. *Innovative Food Science and Emerging Technologies, 6(1),* 1-9.

Polydera, A. C., Stofores, N. G., and Taoukis, P. S. (2003).Comparative shelf life study and vitamin C loss kinetics in pasteurised and high pressure processed reconstituted orange juice. *Journal of Food Engineering, 6(1),* 1-9.

Porretta, S., Birzi, A., Ghizzoni, C., & Vicini, E. (1995). Effects of ultra-high hydrostatic pressure treatments on the quality of tomato juice. *Food Chemistry, 52(1),* 35-41.

Ramirez, R., Ramirez J., Perez-Lamela, C., & Torres, J.A. (2009). Reaction kinetics analysis of chemical changes in pressure-assisted thermal processing. *Food Engineering Reviews, 1(1),* 16-30.

Rivas-Cañedo, A, Juez-Ojeda, C, Nuñez M, & Fernández-García, E. (2011). Effects of high-pressure processing on the volatile compounds of sliced cooked pork shoulder during refrigerated storage. *Food Chemistry, 124(1),* 749-758.

Samaranayake, C. P., & Sastry, S. K. (2010). In situ measurement of pH under high pressure. *Journal of Physical Chemistry B, 114(42),* 13326-13332.

Sancho, F., Lambert, Y., Demazeau, G., Largeteau, A., Boovier, J. M. & Narbonne, J. F. (1999). Effect of ultra-high hydrostatic pressure on hydrosoluble vitamins. *Journal of Food Engineering, 39(3),* 247-253.

Sander, F. V. (1943). The effects of high pressure on the inversion of sucrose and the mutarotation of glucose. *Journal of Biological Chemistry, 148(2),* 311-319.

Schindler, S., Krings, U., Berger, R. G., & Orlien, V.(2010). Aroma development in high pressure treated beef and chicken meat compared to raw and heat treated. *Meat Science, 86(2)* 317-323.

Schrader, K., Buchheim, W., & Morr, C. V. (1997). High pressure effect on the colloidal phosphate and the structural integrity of micellar casein in milk. 1. High pressure dissolution of colloidal claciumphosphate in heated milk systems. *Nahrung-Food,* 41(3), 133-138.

Schwarzenbolz, U., & Henle, T. (2010). Non-ezymatic modifications of proteins under high-pressure treatment. *High Pressure Research, 30(4),* 458-465.

Schwarzenbolz, U., Klostermeyer, H., & Henle, T. (2000). Maillard-type reactions under high hydrostatic pressure: Formation of pentosidine. *European Food Research and Technology, 211(3),* 208-210.

Schwarzenbolz, U., Klostermeyer, H., & Henle, T. (2002). Maillard reaction under high hydrostatic pressure: studies on the formation of protein-bound amino acid derivatives. *International Congress Series, 1245,* 223-227.

Serrano, J., Velazquez, G., Lopetcharat, K., Ramirez, J. A., & Torres, J. A. (2004). Effect of moderate pressure treatments on microstructure, texture, and sensory properties of stirred-curd cheddar shreds. *Journal of Dairy Science, 87(10),* 3172-2182.

Simonin, H., Duranton, F., & de Lamballerie, M. (2012). New insights into high-pressure processing of meat and meat products. *Comprehensive reviews in Food Science and Food Safety, 11(3),* 285-306.

Sizer, C. E., Balasubramaniam, V. M., & Ting, E. (2002). Validating high-pressure processes for low-acid foods. *Food Technology, 56(2),* 36-38.

Smeller, L. (2002). Pressure-temperature phase diagrams of biomolecules. *Biochemica et Biophysica Acta Protein Structure and Molecular Enzymology, 1595(1-2),* 11-29.

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

Stippl, V. M., Delgado, A., & Becker, T. M. (2005). Ionization equilibria at high pressure. *European Food Research and Technology, 221(1-2),* 151-156.

Tamaoka, T., Itoh, N., & Hayashi, R. (1991). High pressure effect on Maillard reaction. *Agricultural and Biological Chemistry, 55(8),* 2071-2074.

Tauscher, B. (1995). Pasteurization of food by hydrostatic high-pressure - chemical aspects. *Zeitschrift Fur Lebensmittel-Untersuchung Und-Forschung, 200(1),* 3-13.

Terefe, N. S., Matthies, K., Simons, L., & Versteeg, C. (2009). Combined high pressure-mild temperature processing for optimal retention of physicochemical and nutritional quality of strawberries (*Fragaria x ananassa*). *Innovative Food Science and Emerging Technologies, 10(3),* 297-307.

Ting, E., Balasubramaniam, V. M., & Raghubeer, E. (2002). Determining thermal effects in high-pressure processing. *Food Technology, 56(2),* 31-35.

Torres, B., Tiwari, B. K., Patras, A., Cullen, P. J. Brunton, N., & O'Donnell, C. P. (2011). Stability of anthocyanins and ascorbic acid of high pressure processed blood orange juice during storage. *Innovative Food Science and Emerging Technologies, 12(2),* 93-97.

van Boekel, M. A. J. S. (2008). *Kinetic Modeling of Reactions In Foods.* CRC Press.

van Boekel, M. A. J. S. (2001). Kinetic aspects of the Maillard reaction: a critical review. *Nahrung/Food, 45(3),* 150-159.

Van Loey, A., Ooms, V., Weemaes, C., Van den Broeck, I., Ludikhuyze, L., Indrawati, Denis, S., & Hendrickx, M. (1998). Thermal and pressure-temperature degradation of chlorophyll in broccoli (brassica oleracea L italica) juice: A kinetic study. *Journal of Agricultural and Food Chemistry, 46(12),* 5289-5294.

Vazquez-Landaverde, P. A., Qian, M. C., & Torres, J. A. (2007). Kinetic analysis of volatile formation in milk subjected to pressure-assisted thermal treatments. *Journal of Food Science, 72(7),* E389-E398.

Vazquez-Landaverde, P. A., Torres, J. A., & Qian, M. C. (2006). Effect of high-pressure-moderate-temperature processing on the volatile profile of milk. *Journal of Agricultural and Food Chemistry, 54(24),* 9184-9192.

Verbeyst, L., Bogaerts, R., Van der Plancken, I., Hendrickx, M., & Van Loey, A. (2013). Modelling of vitamin C degradation during thermal and high-pressure treatments of red fruits. *Food and Bioprocess Technology, 6(4),* 1015-1023.

Verbeyst, L., Oey, I., Van der Plancken, I., Hendrickx, M., & Van Loey, A. (2010). Kinetic study on the thermal and pressure degradation of anthocyanins in strawberries. *Food Chemistry, 123(2),* 269-274.

Verbeyst, L., Van Crombruggen, K., Van der Plancken, I., Hendrickx, M. E., & Van Loey, A. (2011). Anthocyanin degradation kinetics during thermal and high pressure treatments of raspberries. *Journal of Food Engineering, 105(3),* 513-521.

Verlinde, P., Indrawati, O., Hendrickx, M., & Van Loey, A. (2008). High-pressure treatments induce folate polyglutamate profile changes in intact broccoli (*Brassica oleraceae L. cv. Italica*) tissue. *Food Chemistry,* 111(1), 220-229.

Viljanen, K., Lille, M., Heinio, R. L., & Buchert, J. (2011). Effect of high-pressure processing on volatile composition and odour of cherry tomate puree. *Food Chemistry, 129(4),* 1759-1765.

Walstra, P. (2002). *Physical Chemistry of Foods.* Publisher Routledge. New York, NY, United States of America.

Weemaes, C., Ooms, V., Indrawat, Ludikhuyze, L., Van den Broeck, I., Van Loey, A., & Hendrickx, M. (1999). Pressure-temperature degradation of green color in broccoli juice. *Journal of Foods Science, 64(3),* 504-508.

Wentorf, R. H., & De Vries, R. C. (2001). High-pressure synthesis (chemistry). In *Encyclopedia of Physical Science and Technology* (pp. 365-379). New York: Academic Press.

Chapter 3

Retention of bioactive lipids in heated milk: experiments and modeling[2]

3.1 Introduction

In the last two decades, the consumption per capita of fluid milk has declined (Stewart et al., 2012). One reason for the decline in milk consumption is the proliferation of sugar-sweetened beverages (Haug et al., 2007). An investigation on milk and soft drink intakes revealed that carbonated and sugar-sweetened beverages have displaced milk from the diet (Fritsche et al., 1999). Unfortunately, the overall food intake is not affected by the high consumption of sugar-sweetened beverages, creating a caloric imbalance and consequently a strong link with obesity in children and adolescents (Rae-Ellen, 2010; Mathias et al., 2013).

Dairy farmers and government are actively promoting the consumption of fluid milk through education and research (Stewart et al., 2012), highlighting the health benefits of bioactive lipids naturally found in milk fat (Merrill et al., 1997; Molkentin, 2007). Conjugated linoleic acid (CLA) and *trans*-vaccenic acid (TVA) are bioactive lipids that have been associated with health-promoting and disease-preventing properties, such as reducing the risk of cancer and artherosclerosis as well as weight control, and bone formation (Cook & Pariza; 1998; Fritsche et al., 1999; Lock & Bauman, 2004). The chemical structures of CLA and TVA are shown in **Fig 3-1**. CLA is a mixture of positional and geometrical isomers of linoleic acid, having a conjugated double bond system while TVA is a metabolic precursor of CLA (Wang et al., 2009; Jacome-Sosa et al., 2010). Unfortunately, CLA and TVA are considered minor components of milk fat (1 and 3% of the total milk fat, respectively), which limits the use of milk for delivering these bioactive compounds in the human diet.

The profile of fatty acids in milk fat can be manipulated by the feeding regime of the dairy cattle (Jenkins & McGuire, 2006). An increase of 10-fold in

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[2]*A version of this chapter is undergoing revisions for publication in the Journal of Food and Bioproducts Processing.*

the concentration of CLA and TVA was obtained by Bell et al. (2006), who used a safflower oil sumpplemented diet.

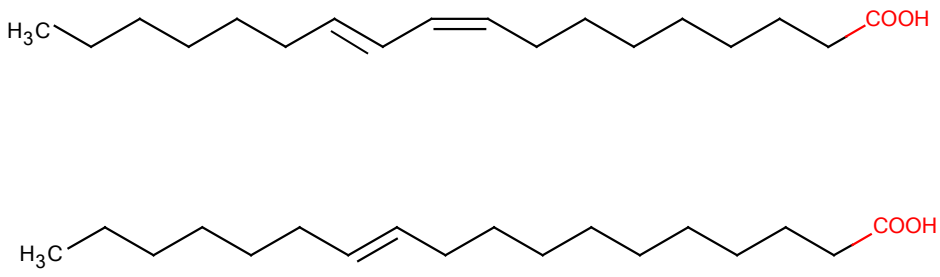

Fig 3-2. Chemical structure of conjugated linoleic acid (CLA, *cis*-9/*trans*-11) and *trans*-vaccenic acid (TVA, 18:2).

Milk is thermally processed to eliminate pathogens and make it safe for human consumption (Goff & Griffiths, 2006). The effect of thermal treatments on lactose, vitamins, proteins and fat have been extensively studied (van Boekel, 1998; Cluskey et al., 1997; Fenaille et al., 2006; Chavan et al., 2011). However, milk with a high proportion of unsaturated fatty acids would be prone to oxidation and the bioactivity of CLA and TVA might be lost or reduced by thermal treatments. Studies on the effect of thermal treatment of milk rich in CLA are scarce and those effects have been evaluated only qualitatively after UHT, microwave heating and pasteurization (Campbell et al., 2003; Herzallah et al., 2005). There are no reports on the oxidation of CLA and TVA upon thermal processing. Thus, the objective of this study was to model the retention kinetics of CLA and TVA in heated enriched milk.

3.2 Materials and methods

3.2.1 Enriched milk and anhydrous milk fat

Enriched milk was obtained from the University of Alberta Dairy Research and Technology Centre after providing the dairy cattle an appropriate diet, following the methodology proposed by Bell et al. (2006). Briefly, 12 lactating Holstein cows were first fed with a control diet for three days. Then, the

cows were fed for 16 days with grains, silage, hay and ground sunflower seed diet (6% of dry matter). The diet consisted of 60% forage and 40% concentrate. Cows were housed in tie stalls and water was available at all times. Then, milk rich in CLA and TVA (CLA/TVA-enriched) was collected and stored at -20^{o}C until needed.

3.2.2 Standardization and homogenization of milk

CLA/TVA-enriched milk was first thawed by warming the enriched milk up to 40˚C. Then, raw CLA/TVA-enriched milk was centrifuged in a Beckman Coulter apparatus (Avanti® J-E, Fullerton, CA, USA) at 15,500 x *g* and 4˚C for 40 min to obtain full-cream and skim milk. The fat content of milk was standardized by adding 2.5% (w/v) of fat into the skim milk and homogenized using a two stage APV-2000 homogenizer (Concord, ON, Canada). After homogenization, the milk was cooled immediately at 10˚C. Then, the standardized and homogenized CLA/TVA-enriched milk was thermally processed within 3 h.

3.2.3 Fatty acid determination

The fatty acid content was analyzed by gas-chromatography (GC) according to the methodology described elsewhere (Cruz-Hernandez et al., 2004). The GC (Varian 3400, Varian Inc., Walnut Creek, CA, USA) instrument is equipped with an automated cool on-column injection, a flame-ionization detector, an autosampler, and a fused-silica capillary column (SP-2560, 100 m length × 0.25 mm ID, Supelco Inc., Belfonte, PA, USA). Treated milk (2.7 mL) was mixed with 20 mL of chloroform/methanol solution (ratio 2:1 v/v). After 1 h, 8 mL of 0.9% of sodium chloride was added. The mixture was centrifuged at 839 x *g* for 5 min and the lower phase (chloroform+lipids) was transferred to a test tube. The extracted lipids were dried with nitrogen at a flow rate of 10 L min^{-1} at 30˚C. Base-catalyzed methylation was used to obtain fatty acid methyl esters (FAME). The dried lipids were mixed with 2 mL of hexane, 40 µL of methylacetate, and 80 µL of sodium methoxide (0.5 N). The mixture was allowed to react for 20 min at 60˚C. After incubation time, 2 mL of hexane, 2 mL of water

and 1 mL of internal standard were added. The internal standard was prepared by dissolving 0.05 g of methyl heptadecanoate (C17:0, Fluka #51633, purity of 99.5%, St. Louis, MO, USA) in 50 mL of hexane. The upper phase of the solution that contains the FAME was transferred to GC vials and diluted with hexane (0.4 mg of FAME in 1 mL of hexane). Samples of 1 µL of FAME solution were injected under a hydrogen flow rate of ~1 mL min^{-1}. The injector and detector temperature was 250˚C. The injected samples were heated at 45˚C for 4 min, then heated to 175˚C at 13˚C min^{-1}, held at this temperature for 27 min, then heated to 215˚C at 4˚C min^{-1}, and then held at this temperature for 35 min (Kramer et al., 2001). The chromatograms were analyzed with the Galaxi software V1.19 and the peaks were identified by comparison with a GC reference FAME standard (463-Nu Check Prep., Inc., Elysian, MN, USA). The CLA content measured by GC represents the total CLA.

3.2.4 Thermal treatment

Raw CLA/TVA-enriched milk was transferred into polypropylene tubes (Cryogenic vial, Fisher Scientific, Edmonton, AB, Canada) of ~3 mL. Then, the tubes were capped and shaken before being heated at times of up to 60 min at 90, 100, 110 or 120˚C in an oil bath (Lauda Proline RP 855, Lauda-Königshofen, Germany). The heating time was recorded at 2-3 min after the samples were immersed in the oil bath. At the end of the heating time, the tubes were removed from the oil bath and cooled down with ice water to stop further CLA and TVA oxidation.

3.2.5 Data analysis

The retention of CLA and TVA was analyzed using Weibull model, which is a convenient and flexible model (van Boekel, 2002), according to equation (3.1):

$$\frac{C_t}{C_0} = exp^{\left(-\left(\frac{t}{\alpha}\right)^{\beta}\right)} \qquad (3.1)$$

where C_t is the concentration of CLA or TVA [mmoles] at a given time; C_o is the initial concentration of CLA and TVA [mmoles]; t is the heating time (min); α is a scale parameter which is inversely proportional to the rate constant (k, min^{-1}); β is the shape constant, corresponding to first-order model when β=1. The influence of temperature on α parameter can be described by an Arrhenius-type relationship (equation (3.2)):

$$\frac{1}{\alpha} = \frac{1}{\alpha_{ref}} \cdot exp\left(\frac{-E_a}{R}\cdot\left(\frac{1}{T}-\frac{1}{T_{ref}}\right)\right) \tag{3.2}$$

where $1/\alpha_{ref}$ is the rate constant at a reference temperature (T_{ref}); E_a is the activation energy [kJ mol^{-1}]; R is the universal gas constant (8.314 J mol^{-1} K^{-1}). The influence of temperature on α was expressed in terms of a reference finite temperature, which improves the parameter estimation. The average temperature of the range tested was selected as the reference temperature (105˚C). The temperature influenced the parameter α, according to equation (3.2). In contrast, the parameter β was not affected by temperature. In order to improve the parameter estimation, equation (3.2) was incorporated into equation (3.1), yielding an overall kinetic equation (3.3). Then, a single non-linear regression analysis was applied to the whole set of experimental data using an average β value (β_{avg}).

$$\frac{C_t}{C_o} = exp\left[-\left(\frac{1}{\alpha_{ref}}\cdot exp\left(\frac{-E_a}{R}\cdot\left(\frac{1}{T}-\frac{1}{T_{ref}}\right)\right)\cdot t\right)^{\beta_{avg}}\right] \tag{3.3}$$

Once the parameters were calculated using equation (3.3), the precision and accuracy of the parameter were evaluated through joint confidence interval (Claeys et al., 2001), using equation (3.4):

$$SSR_{90\%} = SS_{min} \cdot \left(1 + \frac{n}{n-p} \cdot F_{0.9,n,n-p}\right) \quad (3.4)$$

where $SSR_{90\%}$ is the value of sum of the squares at the edge of being statistically similar to the sum of squares minimum (SS_{min}, minimum sum of squares of residuals obtained from the regression analysis at 90% confidence level); p is the number of parameters estimated simultaneously; n is the number of observations; $F_{0.9,n,n-p}$ is the statistical distribution function. All experiments were conducted in triplicate and parameters in equations 3.1 to 3.3 were obtained by non-linear least-squares regression using the Solver option in Excel Microsoft. The predictive capability of the individual and global models was assessed by the coefficient of determination (R^2), the adjusted coefficient of determination (R^2_{adj}), standard error (SE), and residual analysis. All figures were plotted using Sigmaplot software V11 for Windows (SPSS Inc., Chicago, IL, USA).

3.3 Results and discussion

3.3.1 CLA and TVA in heated milk

One way of determining the extent of oxidation in fats is by monitoring the non-reacted or remaining fraction (fatty acids) after a certain period of treatment time. **Fig 3-2** shows the extent of retention of CLA and TVA in heated milk (90-120˚C) after measuring the CLA and TVA contents by GC. In general, CLA showed lower retention compared with TVA and both bioactive lipids were oxidized to a greater extent when the temperature was increased from 90 to 120˚C.

In the case of CLA, **Fig 3-2a** shows that CLA was quite stable at 90˚C even after 60 min of heating time, obtaining a non-reacted fraction in the range of 0.68-0.71. On the other hand, the non-reacted fraction after 60 min of heating time was in the range of 0.51-0.57 and 0.20-0.24 at 100 and 110˚C, respectively. Finally, the CLA was greatly oxidized at 120˚C, yielding a non-reacted fraction in the range of 0.15-0.21 after 15 min of heating time. CLA was lost through oxidation upon thermal processing, depending on the temperature used.

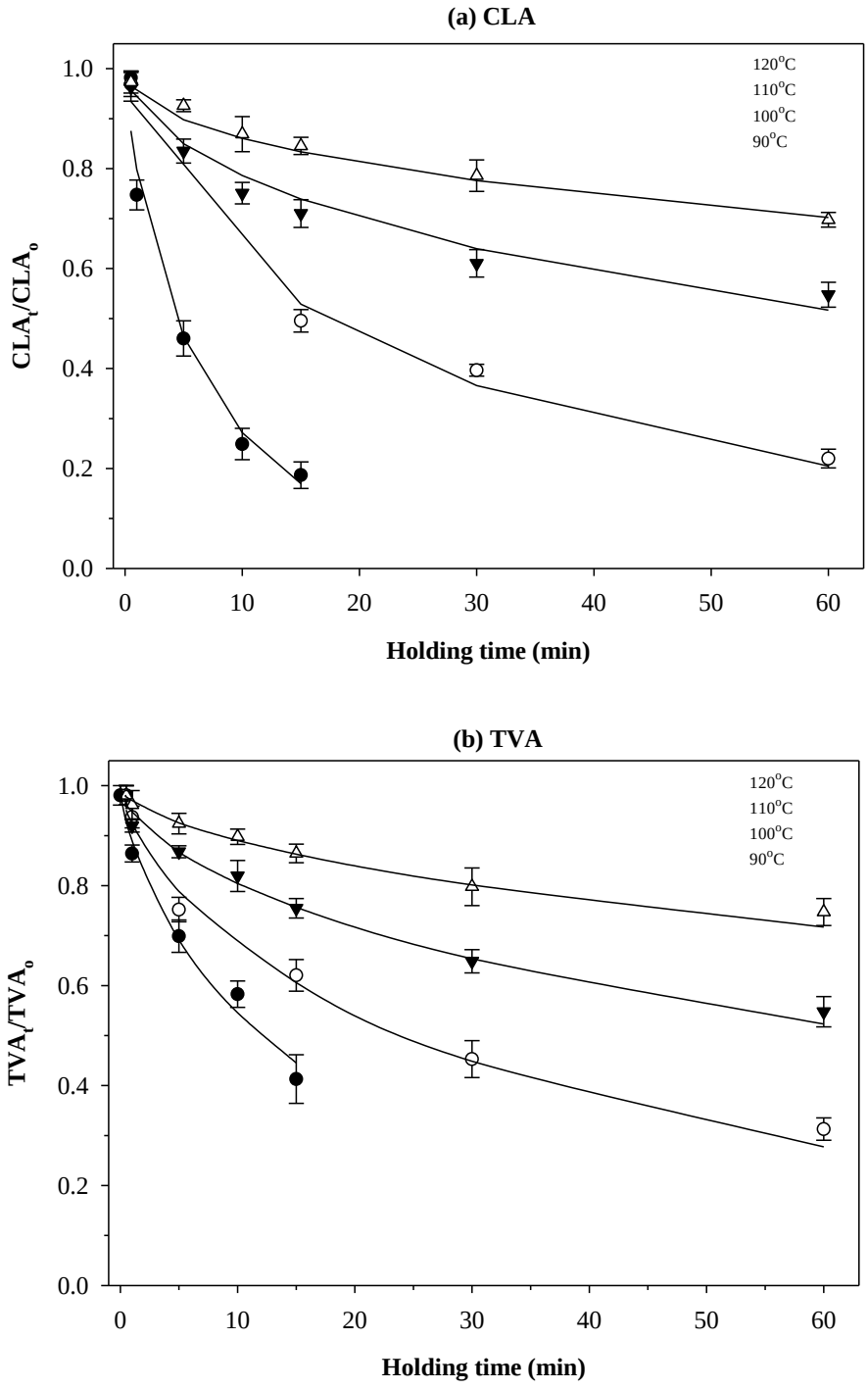

Fig 3-2. Retention of (a) conjugated linoleic acid (CLA) and (b) *trans*-vaccenic acid (TVA) at Δ 90˚C, ▼ 100˚C, ○ 110˚C and ● 120˚C. The data points are based on triplicate experiments. The curves represent the Weibull model (equation (3.3)).

Earlier reports showed that thermal treatments of 2 min at 90˚C, 5 min at 95˚C (microwave heated) and 4 sec at 140˚C (UHT) yielded a remaining fraction of 0.94, 0.79 and 0.85, respectively (Herzallah et al., 2005). Similarly, Campbell et al. (2003) retained 89% of the CLA in fortified milk after 16 s at 77˚C.

Fig 3-2b shows the retention of TVA in heated milk. Overall, TVA gradually oxidized in an exponential fashion. The non-reacted fraction obtained after 60 min of heating was in the range of 0.72-0.77, 0.51-0.57 and 0.28-0.31 at 90, 100 and 110˚C, respectively. After 15 min of heating time, the TVA was greatly oxidized at 120˚C compared with that at other temperatures. For instance, a non-reacted fraction in the range of 0.88-0.91 was obtained at 90˚C while at 120˚C the fraction was between 0.36-0.46 after 15 min. Precht et al. (1999) heated milk fat (up 300˚C for 15 min) and showed a reduction in the content of TVA. Although no general trend was observed, the TVA reduction was attributed to oxidation (Precht et al., 1999). The current study is the first investigation where the effect of heating on the content of TVA has been evaluated in milk.

3.3.2 Fitting primary models

The oxidation of CLA and TVA was assumed to follow an exponential probabilistic distribution in the form of Weibull. This is an essential assumption for applying the Weibull model. Moreover, the experimental data showed a straight line when the double logarithmic plots were applied (**Fig 3-3**). These plots are known as the hazard plots and they are used to judge the applicability of Weibull distribution when a straight line is obtained after plotting $\ln[-\ln(C_t/C_o)]$ vs $\ln(t)$, where C_t/C_o is the normalized concentration of a given fatty acid. Therefore, the oxidation of CLA and TVA in heated milk was adequately described by Weibull model in the form of equation (3.1).

The fitting parameters are displayed in **Table 3-1**. Moreover, the standardized residual against predicted values obtained with equation (3.1) showed no systematic pattern (graph not shown), allowing the estimation of

model parameters and prediction of new observations without abnormality in the methodology.

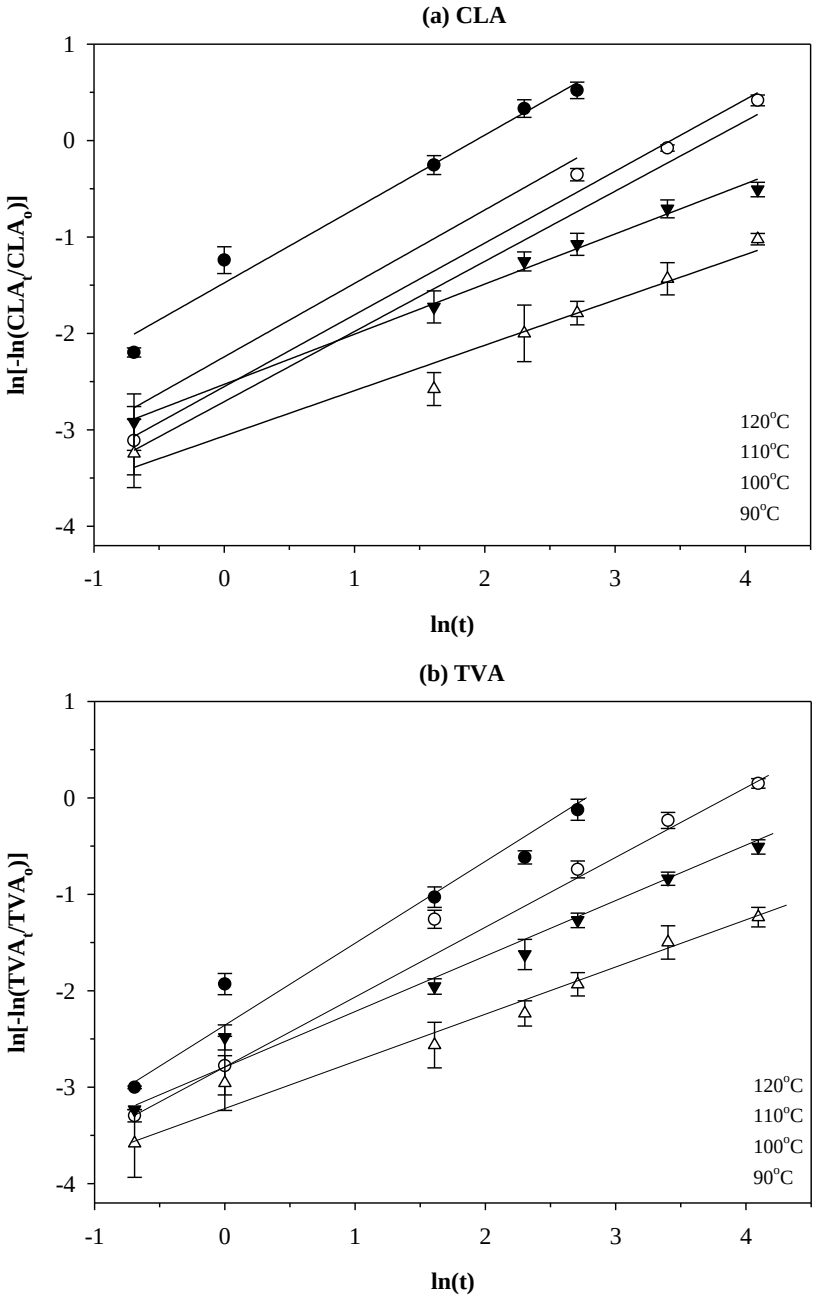

Fig 3-3. Double logarithmic plot or hazard plot for the oxidation of (a) conjugated linoleic acid (CLA) and (b) *trans*-vaccenic acid (TVA) at Δ 90˚C, ▼ 100˚C, ○ 110˚C and ● 120˚C.

The scale parameter (α) in equation (3.1) can be viewed as the time needed to achieve a fractional reduction of 1 log cycle (~63% of reduction), regardless of the β value. As expected, α decreased with increasing temperature for both CLA and TVA. For instance, 7 min was needed to oxidize 63% of the CLA at 120˚C (**Fig 3.2**), whereas ~530 min would have been enough at 90˚C. For TVA, these values would be approximate by 20 min at 120˚C and 392 min at 90˚C. On the other hand, the shape factor (β) was in the range of 0.48-0.76 for CLA and for 0.58-0.71 for TVA. These values of β suggest that the retention of CLA and TVA followed a similar behavior.

Table 3-1. Parameters of the Weibull model (equation (3.1)) for the oxidation of conjugate linoleic acid and *trans*-vaccenic acid in heated milk.

Temperature	**Conjugated linoleic acid (CLA)**					
	α	**95% CI**	β	**95% CI**	R^2	R^2_{adj}
90˚C	527.68	0.011	0.48	0.011	0.96	0.95
100˚C	125.31	0.001	0.56	0.001	0.98	0.98
110˚C	29.76	0.013	0.66	0.017	0.93	0.92
120˚C	7.07	1.11	0.76	0.015	0.91	0.90
Temperature	***Trans*-vaccenic acid (TVA)**					
	α	**95% CI**	β	**95% CI**	R^2	R^2_{adj}
90˚C	392.58	0.03	0.58	0.001	0.97	0.96
100˚C	122.26	14.18	0.61	0.061	0.95	0.94
110˚C	41.56	5.32	0.68	0.071	0.96	0.95
120˚C	20.18	4.01	0.71	0.150	0.90	0.89

The parameters were estimated using equation (3.1). 95% CI - 95% confidence interval; R^2 - coefficient of determination; R^2_{adj} – adjusted coefficient of determination.

3.3.3 Fitting secondary models

The reciprocals of α followed an Arrhenius-type relationship (equation (3.2)) for both CLA and TVA as shown in **Fig 3-4**. The results of the linear regression obtained using equation (3.2) are shown in **Table 3-2**. The use of a reference temperature reduces the correlation between $1/\alpha_{ref}$ and E_a, improving the estimation of parameters (van Boekel, 2002). The E_a for CLA was higher than that of TVA, 171.62 ± 24.62 and 119.35 ± 31.43 kJ mol^{-1}, respectively. On the

other hand, α_{ref} was higher in TVA compared with the α_{ref} obtained for CLA (77.06 ± 23.75 and 58.23 ± 13.47 min^{-1}, respectively).

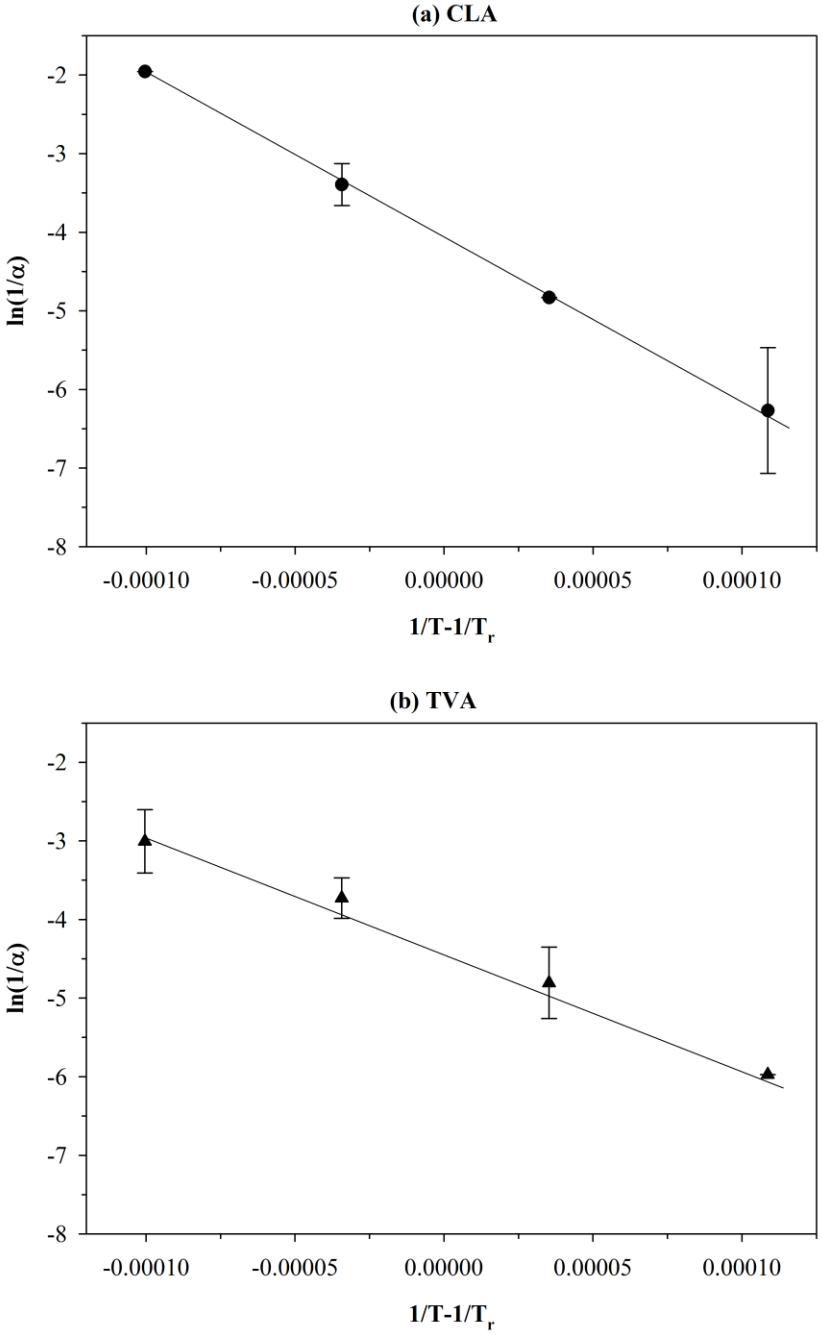

Fig 3-4. Arrhenius-type relationship between α and temperature for (a) conjugated linoleic acid (CLA) and (b) *trans*-vaccenic acid (TVA). The error bars correspond to 95% confidence interval obtained by fitting Weibull model (equation (3.1)). The reference temperature was 105˚C.

The influence of temperature on the shape factor (β) for CLA and TVA is shown in **Fig 3-5**. Although it seems to be a linear relationship between β and temperature for both fatty acids, this trend was not statistically significant ($p>0.05$), according to 95% significance level for a linear relationship. A true temperature dependence of β can be determined by adding more temperature data points (van Boekel, 2002). However, the temperature span used in this study showed a weak interrelation of α and β, meaning that the influence of temperature on α did not significantly affect β. Moreover, this has been illustrated by van Boekel (2002) who fitted experimental data from 55 publications and found that in 48 cases β was not affected by temperature.

Table 3-2. Parameters of the Arrhenius-type model (equation (3.2)) for the oxidation of conjugated linoleic acid and *trans*-vaccenic acid in heated milk.

Parameter	**Conjugated linoleic acid (CLA)**			
	Value	**95% CI**	**R^2**	**R^2_{adj}**
E_a [kJ mol^{-1}]	171.49	24.62	0.99	0.98
α_{ref} [min^{-1}]	58.23	18.47		
Parameter	***Trans*-vaccenic acid (TVA)**			
	Value	**95% CI**	**R^2**	**R^2_{adj}**
E_a [kJ mol^{-1}]	119.35	31.43	0.99	0.98
α_{ref} [min^{-1}]	77.16	23.75		

The parameters were estimated using equation (3.2). E_a – activation energy calculated with equation (3.2); α_{ref} – parameter α at a reference temperature; 95% CI - 95% of confidence interval; R^2 - coefficient of determination; R^2_{adj} – adjusted coefficient of determination.

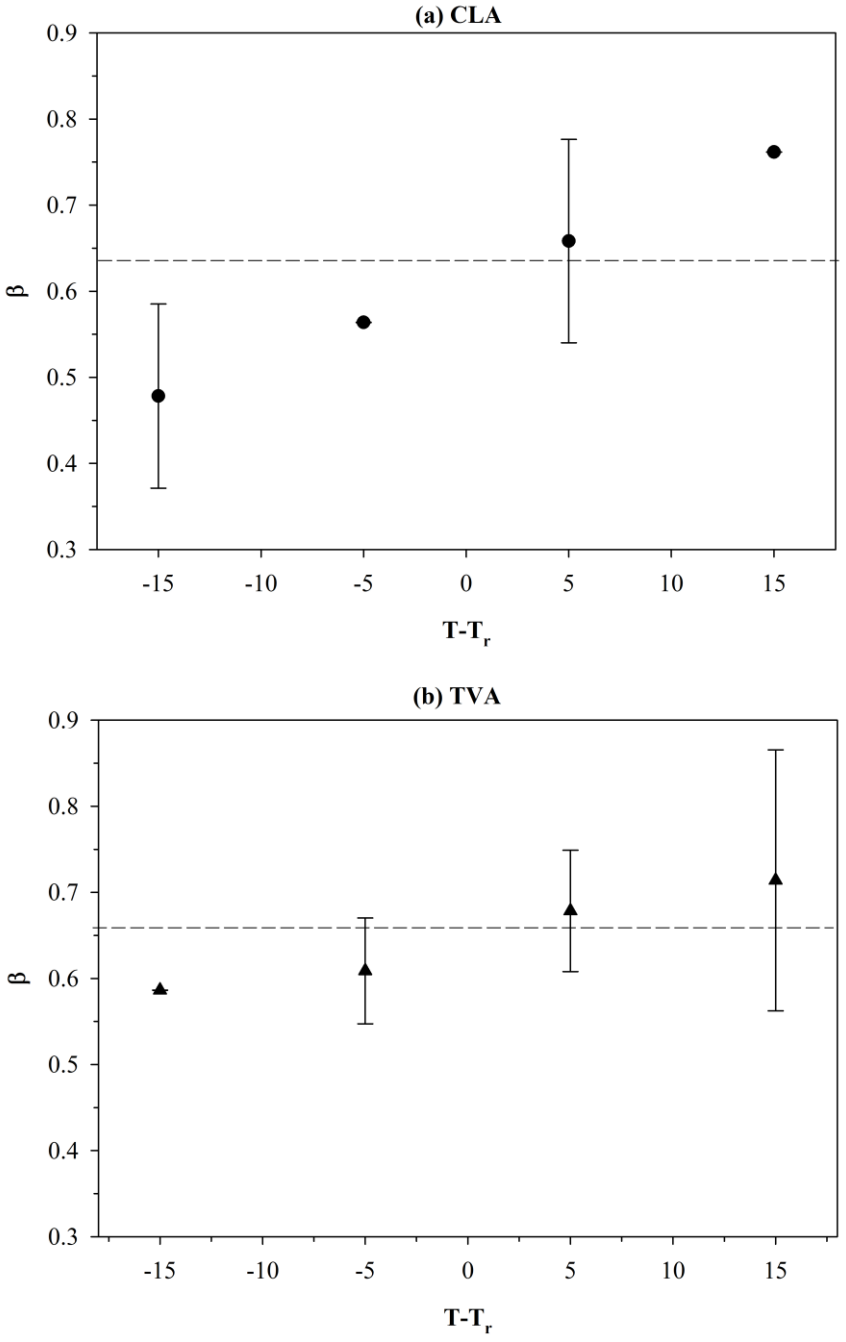

Fig 3-5. Shape parameter (β ± 95% confidence interval) as a function of heating temperature for oxidation of (a) conjugated linoleic acid (CLA) and (b) *trans*-vaccenic acid (TVA). The dashed lines represent the average value of β. The confidence intervals were obtained with Weibull model (equation (3.1)). The reference temperature was 105˚C.

3.3.4 Fitting global model

Data from **Table 3-2** were used in equation (3.3) together with the average value of 0.63 and 0.66 for β for CLA and TVA, respectively. The fitting performance of equation (3.3) is shown in **Table 3-3**. For both fatty acids, the equation (3.3) described the experimental data well using the parameters showed in **Table 3-3** ($R^2_{adj} \geq 0.93$). These parameters were estimated by a one-step procedure, including all temperatures at once into a single model (van Boekel, 2002). Once the parameters have been estimated simultaneously, the correlation between them was determined by joint confidence interval using equation (3.4). The confidence limits were built by joining the probability of two events at 95% of probability (individual confidence level). Thus, the limits of the joint region somehow coincided with the limits of the individual confidence level.

Table 3-3. Global fitting (equation (3.3)) for the oxidation of conjugated linoleic acid (CLA) and *trans*-vaccenic acid (TVA) in heated milk.

Parameter	**CLA**	**95% CI**	**TVA**	**95% CI**
E_a [kJ mol^{-1}]	151.30	13.03	99.59	12.34
α_{ref} [min^{-1}]	49.87	6.62	75.97	7.07
β	0.62	0.05	0.64	0.04
R^2	0.94		0.95	
R^2_{adj}	0.93		0.95	

The parameters were estimated using equation (3.3). E_a – activation energy calculated with equation (3.2); α_{ref} – parameter α at a reference temperature; 95% CI - 95% confidence interval; R^2 - coefficient of determination; R^2_{adj} – adjusted coefficient of determination

Fig 3-6 shows the joint confidence region for the simultaneously estimated parameters ($1/\alpha_{ref}$ and E_a) for the oxidation of CLA and TVA. The joint region was built by fixing the β parameter equal to 0.62 because β varied within a narrow range between 0.57-0.67 and 0.60-0.68 for CLA and TVA, respectively. For both fatty acids, the joint confidence region had an ellipse-like shape, indicating that the parameters were correlated. In contrast, the dashed lines correspond to the individual confidence level and form a rectangular region, which would have been the joint confidence region if the parameters were not correlated.

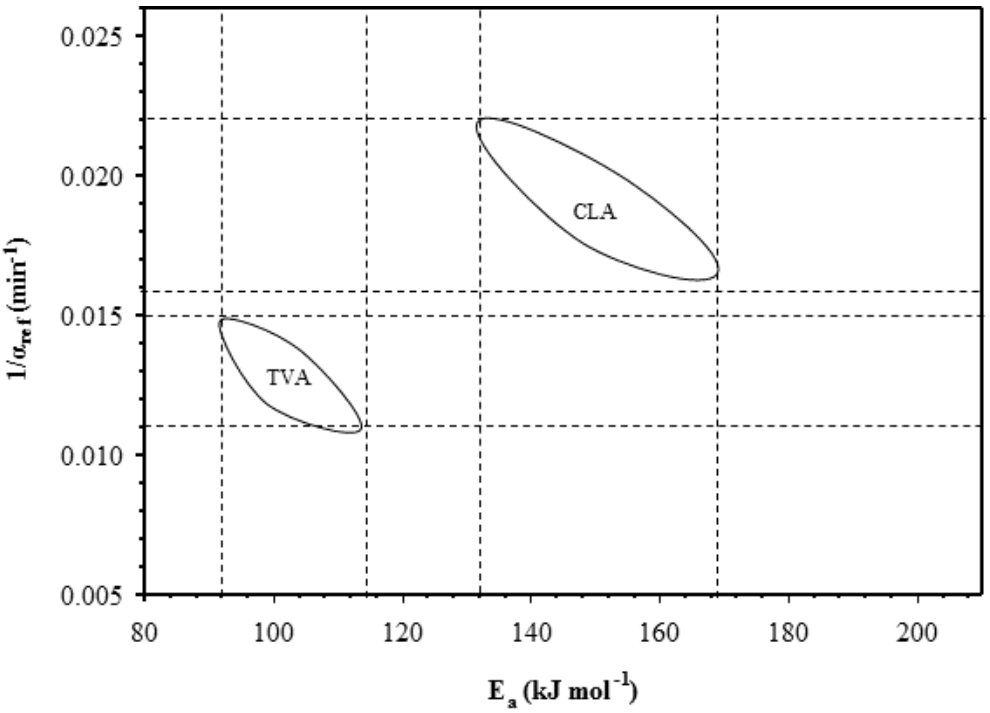

Fig 3-6. Joint confidence region (90%) for simultaneously estimated parameters ($k_{ref} = 1/\alpha_{ref}$ and E_a) for the oxidation of conjugated linoleic acid (CLA) and *trans*-vaccenic acid (TVA). Broken lines correspond to individual 95% confidence intervals. The β parameter was fixed at 0.62.

In **Fig 3-6**, the 90% confidence region for CLA and TVA did not overlap, meaning that the estimated parameters for CLA are statistically different from that of TVA. Another interpretation is that CLA oxidises differently than TVA. CLA has a conjugated double bond system while TVA has one double bond (Precht et al., 1999). Conjugated systems not only are more susceptible to oxidation than a non-conjugated one but also its oxidation mechanism occurs via addition mechanism rather than abstraction (Yang et al., 2000; Hamalainen et al., 2001).

Interestingly, the estimated values of β were lower than 1.0, which indicates that the oxidation of CLA and TVA did not follow a first-order model. One of the assumptions of a first-order model is that all the reactant molecules are in the same state unaffected by the food matrix, which is very unlikely. Indeed, CLA and TVA in milk fat are distributed throughout different triacylglycerols together with other fatty acids, resulting in different combinations of molecular

weights, chain lengths and degrees of unsaturation (Robinson & MacGibbon, 2000). Therefore, deviations from the first-order model are expected. Kinetics of chemical reactions in foods and their mechanistic interpretation are discussed by Peleg et al. (2012), Corradini & Peleg (2006), and Barsa et al. (2012).

3.3.5 Influence of temperature

A new set of data was generated using equation (3.3) and the *k* values were plotted as a function of temperature (**Fig 3-7**). As the temperature increased, the *k* values for both fatty acids increased exponentially with k, more for CLA. This is in agreement with the calculated E_a values, indicating that the oxidation of CLA is accelerated as the temperature is increased.

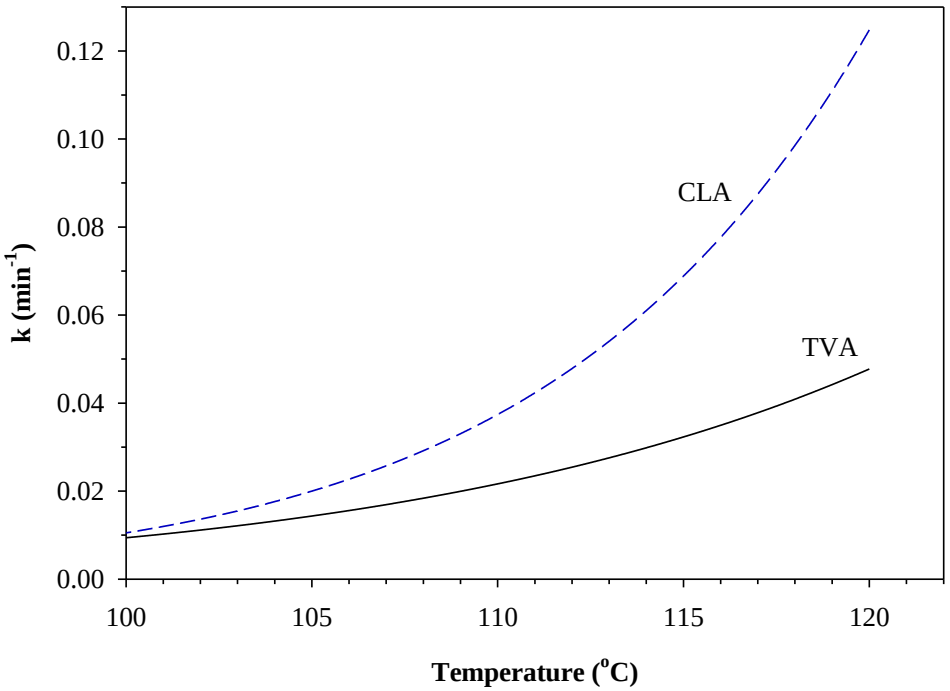

Fig 3-7. Influence of the temperature on the reaction rate constant (*k*) for the oxidation of conjugated linoleic acid (CLA) and *trans*-vaccenic acid (TVA).

Conjugated fatty acids have more than one oxidation pathway. This has been investigated by Yurawecz et al. (2003), who oxidized methyl esters of CLA

and found that 86% of the oxidation products were not formed from hydroperoxides breakdown. Similarly, other authors have quantified dimers and polymers as oxidation products of CLA linoleate (Suzuki et al., 2004). In the case of TVA, it is believed to follow an oxidation mechanism similar to that of linoleic acid; an abstraction mechanism where the double bond acts as an active site. Remarkably, the oxidation of CLA and TVA can be described by the same empirical model. The central assumption of applying the Weibull model is that the oxidation can be viewed as a failure phenomenon where a fraction of an intact lipid is reduced with time at a constant temperature, independently of the actual reaction mechanism and the whole process is governed by the probability laws. Considering the E_a and k values, it can be inferred that CLA is highly susceptible to oxidation upon heating and its reduction can be used as a marker in thermal treatment of milk rich in CLA.

3.4 Conclusions

The oxidation kinetics of CLA and TVA in milk thermally treated could be represented by the Weibull model. The influence of the temperature on α was described by an Arrhenius-type equation. The parameter β was not statistically affected by temperature. The estimated kinetic parameters indicated that CLA followed a different oxidation pathway than TVA. Deviations from a first-order model were observed. The obtained kinetic information (k and E_a) can be used to design thermal treatments for functional beverages aiming to retain bioactive lipids. The retention kinetics of CLA can be used for identifying thermal treatment indicators.

3.5 References

Barsa, C.S., Normand, M.D., & Peleg, M. (2012). On models of the temperature effect on the rate of chemical reactions and biological processes in foods. *Food Enginerring Reviews*, *4(4)*, 191–202.

Bell, J.A., Griinari, J.M., & Kennelly, J.J. (2006). Effect of safflower oil, flaxseed oil, monensin, and vitamin e on concentration of conjugated linoleic acid in bovine milk fat. *Journal of Dairy Science*, *89(2)*, 733-748.

Campbell, W., Drake, M.A., & Larick, D.K. (2003). The impact of fortification with conjugated linoleic acid (CLA) on the quality of fluid milk. *Journal of Dairy Science, 86(1),* 43-51.

Chavan, R.S., Chavan, S., Khedkar, C., & Jana, A.H. (2011). UHT milk processing and effect of plasmin activity on shelf life: a review. *Comprehensive Reviews in Food Science and Food Safety, 10(5),* 251-268.

Claeys, W.L., Ludikhuyze, L.R., & Hendrickx, M.E. (2001). Formation kinetics of hydroxymethylfurfural, lactulose and furosine in milk heated under isothermal and non-isothermal conditions. *Journal of Dairy Research, 68(2),* 287-201.

Cluskey, S.M., Connolly, J.F., Devery, R., O'brien, B., Kelly, J., Harrington, D., & Stanton, C. (1997). Lipid and cholesterol oxidation in whole milk powder during processing and storage. *Journal of Food Science, 62(2),* 331–337.

Cook, M. E., & Pariza, M. (1998). The role of conjugated linoleic acid (CLA) in health. *International Dairy Journal, 8(5-6),* 459-462.

Corradini, M.G., & Peleg, M. (2006). Prediction of vitamins loss during non-isothermal heat processes and storage with non-linear kinetic models. *Trends in Food Science & Technology, 17(1),* 24–34.

Cruz-Hernandez, C., Deng, Z., Zhou, J., Hill, A. R., Yurawecz, M. P., Delmonte, Mossoba, M.M., Dugan, M.E.R., & Kramer, J.K.G. (2004). Methods for analysis of conjugated linoleic acids and trans-18:1 isomers in dairy fats by using a combination of gas chromatography, silver-ion thin-layer chromatography/gas chromatography, and silver-ion liquid chromatography. *Journal of AOAC International, 87(2),* 545-562.

Fenaille, F., Parisod, V., Visani, P., Populaire, S., Tabet, J., & Guy, P.A. (2006). Modifications of milk constituents during processing: a preliminary benchmarking study. *International Dairy Journal, 16(7),* 728–739.

Fritsche, J., Rickert, R., Steinhart, H., Yurawecz, M.P., Mossoba, M.M., Sehat, N., Roach, J.A.G., Kramer, J.K.G., & Ku, Y. (1999). Conjugated linoleic acid (CLA) isomers: iormation, analysis, amounts in foods, and dietary intake. *Lipid / Fett, 101(8),* 272-276.

Goff, H.D., & Griffiths, M.W. (2006). Major advances in fresh milk and milk products: fluid milk products and frozen desserts. *Journal of Dairy Science, 89(4),* 1163-1173.

Hamalainen, T.I., Sundberg, S., Makkinen, M., Hase, T., & Hopia, A. (2001). Hydroperoxide formation during aautoxidation of conjugated linoleic acid methyl ester. *European Journal of Lipid Science and Technology, 103(9),* 588-593.

Haug, A., Høstmark, A.T., & Harstad, O.M. (2007). Bovine milk in human nutrition – a review. *Lipids in Health and Disease, 6(25),* 1-17.

Herzallah, S.M., Humeid, M.A., & Al-Ismai, K.M. (2005). Effect of heating and processing methods of milk and dairy products on conjugated linoleic acid and trans fatty acid isomer content. *Journal of Dairy Science, 88(4),* 1301–1310.

Jacome-Sosa, M.M., Lu, J., Wang, Y., Ruth, M.R., Wright, D.C., Reaney, M.J., Shen, J.H., Field, C.J., Vine, D.F., & Proctor, S.D. (2010). Increased hypolipidemic benefits of cis-9, trans-11 conjugated linoleic acid in combination with trans-11 vaccenic acid in a rodent model of the metabolic syndrome, the JCR:LA-cp rat. *Nutrition & Metabolism, 7(60),* 60-70.

Jenkins, T.C., & McGuire, M.A. (2006). Major advances in nutrition: impact on milk composition. *Journal of Dairy Science, 89(4),* 1302–1310.

Kramer, J.K., Cruz-Hernandez, C., & Zhou, J. (2001). Conjugated linoleic acids and octadecenoic acids: analysis by GC. *European Journal of Lipid Science and Technology, 103(9),* 600-609.

Lock, A., & Bauman, D.E. (2004). Modifying milk fat composition of dairy cows to enhance fatty acids beneficial to human health. *Lipids, 39(12),* 1197-1206.

Mathias, K.C., Slining, M.M., & Popkin, B.M. (2013). Foods and beverages associated with higher intake of sugar-sweetened beverages. *American Journal of Preventive Medicine, 44(4),* 351–357.

Merrill , A.H., Schemelz, E.M., Dillehay, D.L., Spiegel, S., Shayaman, J.A., Schroeder, J.J., Riley, R.T., Voss, K.A., & Wang, E. (1997). Sphingolipids-the enigmatic lipid class: biochemistry, physiology, and pathophysiology. *Toxicology and Applied Pharmacology*, 142(1), 208-225.

Molkentin, J. (2007). Occurrence and biochemical characteristics of natural bioactive substances in bovine milk lipids. *British Journal of Nutrition, 84(1),* 47-53.

Peleg, M., Normand, M.D., & Corradini, M.G. (2012). The Arrhenius equation revisited. *Critical Reviews in Food Science and Nutrition, 52(9),* 830-851.

Precht, D., Molkentin, J., & Vahlendieck, M. (1999). Influence of the heating temperature on the fat composition of milk fat with emphasis on cis-/trans-isomerization. *Food/Nahrung, 43(1),* 25–33.

Rae-Ellen, W.K. (2010). How Sweet it is: sugar-sweetened beverage consumption, obesity, and cardiovascular risk in childhood. *Journal of the American Dietetic Association, 110(10),* 1456-1460.

Robinson, N.P., & MacGibbon, A.K. (2000). Determination of the conjugated linoleic acid-containing triacylglycerols in New Zealand bovine milk fat. *Lipids, 35(7),* 789-796.

Stewart, H., Dong, D., & Carlson, A. (2012). Is generational change contributing to the decline in fluid milk consumption? *Journal of Agricultural and Resource Economics, 37(3),* 435–454.

Suzuki, R., Abe, M., & Miyashita, K. (2004). Comparative study of the autoxidation of tag containing conjugated and nonconjugated C18 PUFA. *Journal of the American Oil Chemists' Society, 81(6),* 563-569.

van Boekel, M.J.A. (1998). Effect of heating on maillard reactions in milk. *Food Chemistry, 62(4),* 404-413.

van Boekel, M.J.A. (2002). On the use of the weibull model to describe thermal inactivation of microbial vegetative cells. *International Journal of Food Microbiology, 74(1-2),* 139– 159.

Wang, Y., Jacome-Sosa, M.M., Ruth, M.R., Goruk, S.D., Reaney, M.J., Glimm, D.R., Wright, D.C., Vine, D.F., Field, C.J., & Proctor, S.D. (2009). Trans-11 vaccenic acid reduces hepatic lipogenesis and chylomicron secretion in JCR:LA-cp Rats. *The Journal of Nutrition, 139(11),* 2049-2054.

Yang, L., Leung, L.K., Huang, Y., & Chen, Z. Y. (2000). Oxidative stability of conjugated llinoleic acid isomers. *Journal of Agricultural and Food Chemistry, 48(8),* 3072-3076.

Yuracwecz, M. P., Delmonte, P., Vogel, T., & Kramer, J. G. (2003). Oxidation, of conjugated linoleic acid: initiators and simultaneous reactions: Theory and practice. In W. W. Christie, J. L. Sebedio & R. Adlof. *Advances in Conjugated Linoleic Acid*. American Oil Chemists Society Press.

Chapter 4

Kinetics of non-isothermal oxidation of anhydrous milk fat rich in conjugated linoleic acid using differential scanning calorimetry[3]

4.1 Introduction

The benchmark for any form of lipid-based product is the oxidative stability or resistance to oxidation (Velasco et al., 2004). Lipid oxidation is a free radical chain reaction that leads to the development of unpleasant taste and undesirable changes such as the formation of off-flavor compounds (aldehydes, ketones, etc) (Simon & Kolman, 2001). Oxidation reactions consist of initiation, propagation and termination stages. During the initiation stage, free radicals are formed through thermolysis, due to the presence of enzymes and active oxygen species. Then, these radicals react with molecular oxygen to form primary products such as hydroperoxides. These compounds are unstable and further react through free radical mechanisms to form secondary products that propagate the oxidation. The resulting compounds form viscous materials through polymerization as the oxidation proceeds. These polymers are oil insoluble and represent the termination stage of oxidation (Privett & Blank, 1962; Adhvaryu et al., 2000).

Several methods have been used to analyze and monitor lipid oxidation (Kamal-Eldin & Pokorny, 2005). These methods allow quantifying one or more of the reaction products of the different oxidation stages. Some of these methods are officially accepted by the American of Analytical Communities such as oxidative stability index (OSI) and peroxide values (PV) (Kamal-Eldin & Pokorny, 2005; Pokorny, 2005), while others are routinely conducted, such as Racimat, chemilominescence, and volumetric methods. The oxidation process cannot be measured by a single test due to its complexity. As the oxidation proceeds, several reactions occur simultaneously at different rates. These reactions release heat that can be measured using differential scanning calorimetry (DSC). Recording the heat released from a particular reaction using DSC can be conducted

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[3] A version of this chapter has been published in the Journal of Thermal Analysis and Calorimetry, 2012, 107(3), 973-981.

in either isothermal or non-isothermal mode. In general, non-isothermal methods are widely used in lipid oxidation because they can provide valuable analytical and kinetic information. In addition, DSC method is simple, convenient and fast (Agrawal, 1992; Adhvaryu et al., 2000). The non-isothermal method is based on the linear correlation between the temperature that corresponds to a specific thermal event and different heating rates (Ozawa, 1975). From this relationship that follows an Arrhenius type equation, the effective activation energy (E_a), pre-exponential factor (z) and rate constant (k) are derived. Kinetic information of lipid oxidation have been reported for soybean/anhydrous milk fat blends (Thurgood et al., 2007), unsaturated fatty acids (oleic, linoleic, and linolenic acids) (Litwinienko, 2001), saturated fatty acids (lauric, myristic, palmitic, and stearic acids) (Litwinienko et al., 1999), natural vegetable oils (canola, corn, cottonseed, and soybean oils) and genetically modified vegetable oils (Adhvaryu et al., 2000).

Anhydrous milk fat (AMF) is a versatile dairy ingredient with various applications in confectionary, bakery and dairy products. It imparts hardness to confectionary products and it is used for the manufacture of lipid shortenings to structure or plastify vegetable oils. In addition, AMF is the major dietary source of conjugated linoleic acid (CLA) in human diet. CLA are positional and geometrical isomers of linoleic acid having a conjugated double-bond system (Lock & Bauman, 2004). Epidemiological studies have positively related CLA intake with health-promoting and disease-preventing properties (Fritsche et al., 1999). Although these relationships are still under investigation, CLA has proven biological activity, including cancer prevention, atherosclerosis, weight control, and bone formation (Cook & Pariza, 1998; Park, 2009). CLA is naturally found in cow's milk (5 mg of CLA g^{-1}fat). CLA concentration in AMF can be markedly enhanced through diet manipulation and nutritional management of dairy cows, as previously reported by Kennelly & Bell (2004).

Searching alternative approaches to enhance AMF functionality by enrichment with CLA, certainly has health benefits and consequently commercial implications. However, CLA is susceptible to autoxidation upon thermal processing due to its conjugated double bond system (C=C), which serves as an active site for free radical reactions. Kinetic data derived from Arrhenius type relationship such as the effective activation energy, pre-exponential factor and constant rate are essential parameters to predict oxidative stability. The non-isothermal oxidation kinetics of AMF enriched with CLA has not been reported so far. Therefore, the objective of this study was to evaluate the effect of CLA content on the non-isothermal oxidation kinetics of AMF.

4.2 Materials and methods

4.2.1 Anhydrous milk fat with different CLA concentrations

Three different feeding regimes were provided to dairy cattle at University of Alberta Dairy Research and Technology Centre following the methodology described by Kennelly & Bell (2004), and Bell et al. (2006). Then, anhydrous milk fat was obtained from CLA enriched milk, following the procedure described elsewhere (Walstra et al., 2006) (**Fig 4-1**). Briefly, the raw milk was heated to 55˚C and centrifuged at 6000 x *g* for 6 min using an Alpha-Laval centrifuge (LAPX 202, Lundm Scania, Sweden). The cream obtained was stirred for 20 min at room temperature using a hand held Black & Decker Power Pro Mixer. The butter milk was discarded and the butter was washed with cold water to remove excess of butter milk. The butter was heated at 60˚C for 120 min until the different layers started to separate. Then, the top layer was removed and the mixture was poured through a cheese cloth to obtain AMF. This fat was stored at -18˚C until further analysis.

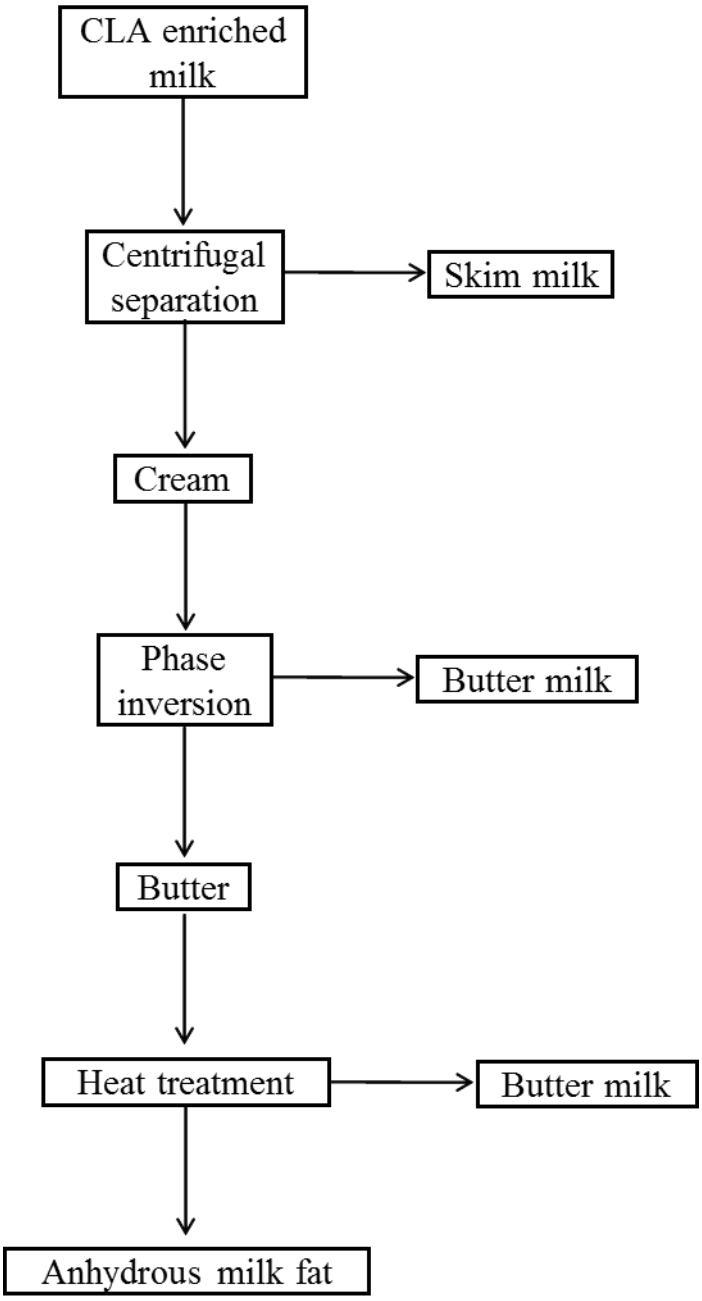

Fig 4-1. Schematic diagram of anhydrous milk fat production. Adapted from Walstra et al. (2006).

4.2.2 Fatty acids determination

The fatty acid profile of CLA-enriched AMF was determined by GC, following the methodology described in Chapter 3 (Section 3.2.5).

4.2.3 Differential scanning calorimetry determination

The oxidation kinetics of anhydrous milk fat samples was performed using a Q100 Modulated Differential Scanning Calorimeter (TA Instruments, New Castle, DE, USA). Samples of 1-2 mg were placed into aluminum pans with a pinhole lid (TA instruments, New Castle, DE, USA) and were hermetically sealed. An empty sealed aluminum pan was used as a reference and the experiments were performed under an oxygen (dry 99% pure, Praxair, Edmonton, AB, Canada) flow rate of 50 mL min^{-1} at 0.13 Pa. These conditions allowed the interaction of the sample with the oxygen and were kept constant during the entire heating protocol. The sealed pans were equilibrated at 100°C for 1 min and then heated to 350°C at linearly increased program rates (3, 6, 9, 12, and 15°C min^{-1}) to generate the oxidative profile (heat flow against temperature).

The spectra were analyzed with a TA Universal Analysis software (TA Instruments, New Castle, DE, USA) to locate the start temperature (T_s), onset temperature (T_{on}), and maximum heat flow temperature (T_p). The location of key parameters was determined using the first and second derivatives of the signal (**Fig 4-2**), a method previously proposed for lipid crystallization (Bouzidi et al., 2005). Once the DSC curves were generated, the error associated with the raw data was calculated through standard deviation. Then, the first and second derivatives were calculated. The error was obtained from the baseline, which in **Fig 4-2** corresponds to the segment of 140 to 180˚C. This is essential since the signal variability can be misinterpreted as a thermal event. In this method, a true thermal event was considered when the heat flow signal is greater than twice the standard deviation of the baseline. This criterion is known as the departure value (inlet **Fig 4-2a**). To locate the start temperature of oxidation, three criteria were considered. Firstly, the first derivative of the signal shows an inflexion point between a maximum and a minimum point of the signal (arrow (1)). Secondly, the second derivative reaches a maximum point on the heat flow signal (arrow (2)).

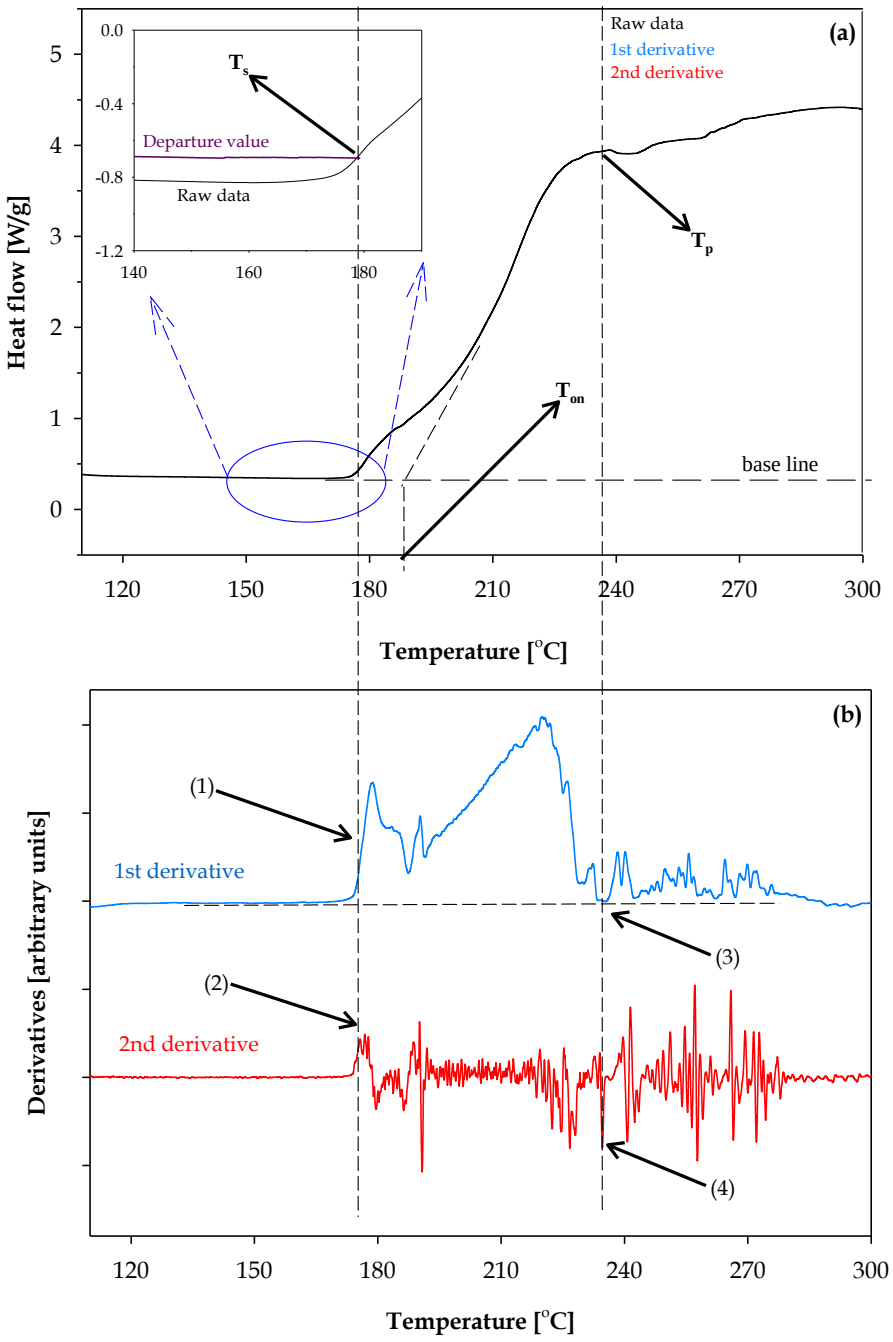

Fig 4-2. DSC oxidation curve of anhydrous milk fat with high CLA content at a rate of 15°C min^{-1}. (a) Determination of start temperature (T_s) (inlet), onset temperature (T_{on}) and maximum heat flow temperature (T_p), and (b) first and second derivatives that precisely locate the T_s, T_{on}, and T_p.

Finally, the heat flow signal should be greater than the departure value (inlet). T_p is considered when the first derivative of the signal intersects with the *x*-axis

(arrow (3)) and the second derivative has reached a maximum point on the signal (arrow (4)). T_{on} was obtained by extrapolating the tangent drawn on the steepest slope of T_p.

All these values were further used to calculate the effective activation energy (E_a) and the pre-exponential factor (z) of the Ozawa-Flynn-Wall method (Ozawa, 1975). Using this method, a set of data (T_s, T_{on}, and T_p) was obtained for each heating rate (H_r= dT/dt) from which the kinetic parameters were calculated as follows:

$$\log H_r = a\frac{1}{T} + b \tag{4.1}$$

where H_r is the heating rate (K min^{-1}) and T is the temperature T_s, T_{on}, or T_p (K). By plotting log H_r against 1/T, the effective activation energy (E_a) and the pre-exponential factor (z) can be determined directly from the slope and intercept according to:

$$a = -0.4567 \cdot \frac{E_a}{R} \tag{4.2}$$

$$b = -2.315 + \log\left(z \cdot \frac{E_a}{R}\right) \tag{4.3}$$

where a and b are the slope and intercept from equation (4.1), respectively, and R is the universal gas constant (8.314 J mol^{-1} K^{-1}). Therefore, the effective activation energy and rate constant (k) are calculated from:

$$E_a = -2.19 \cdot R \cdot \frac{d\log H_r}{dT^{-1}} \tag{4.4}$$

$$k = z \cdot exp^{\left(\frac{-E_a}{RT}\right)} \tag{4.5}$$

4.2.4 Degree of conversion

All kinetic parameters were calculated using the iso-conversional method proposed by Ozawa-Flynn-Wall in which a constant degree of conversion (θ) was

used for each point of interest in the DSC spectra (T_s, T_{on}, and T_p) based on the initial ($signal_o$) and final ($signal_f$) heat flow signals. The degree of the non-isothermal oxidation of AMF conversion was calculated as follows:

$$\theta = \frac{Signal_o - Signal}{Signal_o - Signal_f} \quad 0 \leq \theta \leq 1 \tag{4.6}$$

4.2.5 Statistical analysis

All data were collected at least in duplicates and are reported as mean values and standard deviations. The statistical analysis including Turkey test was conducted using the Sigmaplot software V11 for windows (SPSS Inc., Chicago, IL, USA).

4.3 Results and discussion

4.3.1 Fatty acid analysis

Table 4-1 shows the composition of the major fatty acids of the three different AMF samples obtained as described earlier. Overall, myristic (C14:0), palmitic (C16:0), and stearic (C18:0) acids were the saturated fatty acids present in high concentration in AMF. The concentration of these major fatty acids was within the range reported earlier by Jensen (2002), who compiled investigations on bovine milk fat from the year 1995 to 2000. Important variations were observed in the ratio of unsaturated to saturated fatty acids between feeding regimes and, consequently, the amount of CLA is also significantly different as reported and explained by Bell et al. (2006). These authors used the biohydrogenation theory to explain the increase in the CLA content and therefore the changes in the ratio of unsaturated to saturated fatty acids. Importantly, using these feeding regimes, cow milk with different concentrations of CLA were obtained (**Table 4-1**), from which AMF was separated (**Fig. 4-1**) and used in the oxidation studies.

Table 4-1. Fatty acid composition (% of total fatty acids) of low-, medium-, and high- CLA AMF.

Fatty acid	L-CLA	M-CLA	H-CLA
C4:0	0.30 ± 0.09	0.28 ± 0.01	0.20 ± 0.02
C6:0	1.37 ± 0.01	1.41 ± 0.01	0.85 ± 0.02
C8:0	0.91 ± 0.01	0.88 ± 0.08	0.50 ± 0.01
C10:0	2.04 ± 0.03	2.17 ± 0.04	1.09 ± 0.01
C12:0	2.47 ± 0.04	2.48 ± 0.16	1.47 ± 0.01
C14:0	8.87 ± 0.16	8.87 ± 0.16	6.83 ± 0.06
C15:0	1.05 ± 0.01	0.93 ± 0.08	0.79 ± 0.01
C16:0	23.94 ± 0.23	19.11 ± 0.18	17.92 ± 0.01
C16:1 *t*	0.33 ± 0.01	0.41 ± 0.02	0.73 ± 0.01
C16:1 *c*	1.81 ± 0.13	1.09 ± 0.06	1.31 ± 0.01
C18:0	9.18 ± 0.13	13.02 ± 0.22	8.69 ± 0.25
C18:1 *t11*	1.89 ± 0.06	3.97 ± 0.07	11.06 ± 0.27
C18:1 *n7*	21.99 ± 0.11	21.66 ± 0.78	21.73 ± 0.45
C18:2	1.94 ± 0.01	2.35 ± 0.02	2.27 ± 0.02
C20:0	0.142 ± 0.01	0.13 ± 0.01	0.10 ± 0.01
CLA	**0.73 ± 0.01**	**0.91 ± 0.01**	**3.23 ± 0.01**
Ratio Uns/sat	0.71	0.86	1.36

Mean ± standard deviation based on duplicates, *t* – *trans* fatty acids; *c* – *cis* fatty acids; L-CLA – anhydrous milk with low CLA content; M-CLA – anhydrous milk fat with medium CLA content; H-CLA– anhydrous milk fat with high CLA content;

4.3.2 Oxidative profile

Fig 4-3 shows the DSC thermograms of anhydrous milk fat obtained at five heating rates (3, 6, 9, 12 and 15°C min^{-1}) in the range of 100 to 350°C. As the heating rate increases, the T_s, T_{on}, and T_p temperatures increase. This behavior was similar for the three AMF samples (**Figs 4-3a-c**).

During slow heating, primary oxidation products such as hydroperoxides generated during the initial oxidation stage react with excess of oxygen to form low molecular weight compounds (intermediate oxidation products) such as aldehydes and acids that remain in solution, accelerating the degradation process. At fast heating rates, on the other hand, these intermediate products are lost through evaporation before they further react with the lipids present, shifting the threshold DSC signal to high values (Adhvaryu et al., 2000).

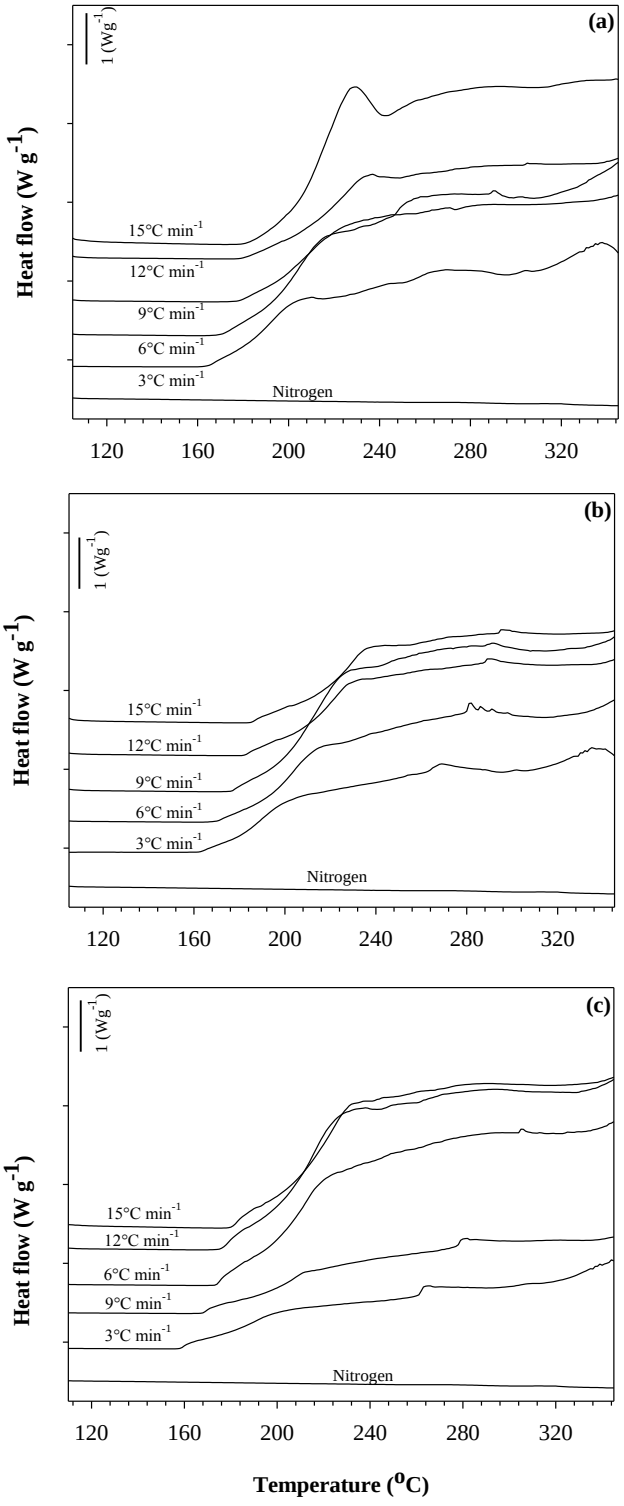

Fig 4-3. DSC oxidative profile of anhydrous milk fat with low (a) CLA content, (b) medium CLA content, and (c) high CLA content.

An additional experimental run that consisted of anhydrous milk fat heated at 3°C min^{-1} under nitrogen flow rate revealed that no significant exothermal or endothermal events were observed (bottom line in **Figs 4-3a-c**). This suggests that melting, decomposition and polymerization did not occur within the range of 100 to 350°C. Similar findings were reported by Ulkowski et al. (2005), who analyzed the weight loss of lecithin during heating under nitrogen flow. Their thermogravimetric analysis showed that in the temperature range of 100 to 250°C, only 4% of weight was lost but above 250°C, the weight lost increased considerably. Therefore, changes in the DSC signal within the range of 100 to 250°C were attributed to oxidation and those changes above 250°C corresponded to thermal degradation rather than oxidation.

Table 4-2 summarizes the T_s, T_{on}, and T_p temperatures of oxidation as a function of the heating rates. Comparing L-CLA versus H-CLA, the start, onset and maximum heat flow temperatures shifted to lower values as the CLA content increased ($p<0.05$), mainly at low heating rates (3-9 °C min^{-1}). This behavior can be a result of the different fatty acid compositions of the three AMF samples (**Table 4-1**) and the fact that unsaturated fatty acids oxidize faster at low temperatures compared to saturated fatty acids. This behavior has been exemplified by the non-isothermal oxidation of unsaturated fatty acids (oleic, erucic, linoleic and linolenic acids) (Litwinienko, 2001) and saturated fatty acids (lauric, myristic, palmitic and stearic acids, Litwinienko et al. (1999)). A decreasing tendency of the start temperature of oxidation was observed with increasing number of double bonds. For unsaturated compounds, the oxidation starts at lower temperatures due to C=C, which serves as an active site for free radical reactions such as autoxidation. Thus, in non-isothermal oxidation, the T_s values can be interpreted as the formation of peroxides (autoxidation). An investigation (Musialik & Litwinienko, 2007) of non-isothermal oxidized corn oil, linseed oil and oleic acid at different known concentrations of peroxides showed that only T_s was affected by the initial presence of peroxide, while T_p was not affected. Other investigations of oxidized linolenic acid in the presence of

different phenolic compounds showed that the oxidative stability, measured by T_s, increases with increasing concentration of an antioxidant (Simon & Kolman, 2001).

Table 4-2. Start, onset and maximum heat flow temperatures of anhydrous milk fat oxidation obtained from DSC spectra at different heating rates (H_r).

Start temperature of oxidation			
H_r °C min^{-1}	**Low CLA AMF**	**Medium CLA AMF**	**High CLA AMF**
3	164.46 ± 0.09aA	161.91 ± 1.18abA	155.74 ± 3.44bA
6	177.57 ± 1.64aBD	170.39 ± 0.94abC	164.05 ± 5.63bC
9	175.52 ± 0.11aD	176.19 ± 0.30abD	170.95 ± 3.76bBC
12	173.52 ± 0.11aC	180.54 ± 2.41bcB	174.95 ± 1.69cBC
15	179.80 ± 0.84aB	184.04 ± 1.37abB	177.93 ± 3.40bB
Onset temperature of oxidation			
H_r °C min^{-1}	**Low CLA AMF**	**Medium CLA AMF**	**High CLA AMF**
3	172.71 ± 0.20aA	173.47 ± 0.07abA	163.29 ± 1.62bA
6	184.01 ± 3.31aCE	184.71 ± 2.33aC	170.54 ± 0.85bC
9	183.16 ± 3.57aDE	192.13 ± 0.72abD	180.29 ± 3.35bD
12	187.32 ± 3.57aE	197.85 ± 3.71cB	188.50 ± 0.09bCE
15	195.07 ± 2.95aB	198.66 ± 0.39abB	191.97 ± 0.14bB
Maximum heat flow temperature of oxidation			
H_r °C min^{-1}	**Low CLA AMF**	**Medium CLA AMF**	**High CLA AMF**
3	208.70 ± 1.47aA	212.67 ± 2.28aA	202.88 ± 1.27bA
6	221.14 ± 7.42aB	223.76 ± 0.85aC	211.20 ± 0.21bC
9	229.21 ± 5.98aB	236.62 ± 1.07abD	226.40 ± 2.82bD
12	231.43 ± 3.57aB	248.10 ± 16.07aBD	237.69 ± 0.85aB
15	229.94 ± 0.38aB	247.82 ± 3.63cB	239.50 ± 2.99bB

Mean ± standard deviation (n=3) within each row with different letters (a-c) are significantly (P<0.05) different (P<0.05). Mean ± standard deviation within each column with different letter (A-E) are significantly (P<0.05) different (P<0.05). AMF – Anhydrous milk fat

4.3.3 Kinetic parameters

In DSC methods that are based on the recording of released heat in either isothermal or non-isothermal mode, the consumption of oxygen can be neglected due to the large excess of oxygen generated by a constant flow rate. Such condition allows the formation of peroxides independent of the oxygen concentration, which also means that the autoxidation is a first order reaction (Litwinienko et al.. 1999; Adhvaryu et al., 2000; Litwinienko 2001). This is an essential assumption for the calculation of kinetic parameters such as effective

activation energy (E_a), pre-exponential factor (z), and reaction rate constant (k). Using the information presented in **Table 4-2** and according to equations (4.1) to (4.4), the kinetic parameters were calculated and the values are presented in **Table 4-3**. According to Adhvaryu et al. (2000), less than 1.5 mg of sample in a hermetically sealed pan with a pinhole provides consistent results. These recommendations were followed in the present study.

Table 4-3. Kinetic and statistical parameters calculated from start, onset and maximum heat flow temperatures.

Sample	Parameters of eq. 4.1		R^2	E_a	z	k
	a	b				
Start temperature of oxidation (T_s)						
L-CLA	-8046.2	18.91	0.955	146.51	4.0×10^{14}	0.026
M-CLA	-6173.9	14.68	0.983	112.42	3.6×10^{10}	0.014
H-CLA	-4810.8	11.82	0.964	87.60	6.3×10^{7}	0.013
Onset temperature of oxidation (T_{on})						
L-CLA	-6267.1	14.57	0.974	114.11	4.8×10^{11}	0.121
M-CLA	-5714.9	13.26	0.986	104.06	2.0×10^{10}	0.065
H-CLA	-4531.2	10.92	0.947	82.42	8.7×10^{7}	0.069
Maximum heat flow temperature (T_p)						
L-CLA	-6662.1	14.31	0.981	121.31	3.5×10^{11}	0.014
M-CLA	-4800.4	10.39	0.951	87.41	3.0×10^{7}	0.006
H-CLA	-4044.1	9.02	0.943	73.64	1.2×10^{6}	0.008

L-CLA – anhydrous milk with low CLA content; H-CLA– anhydrous milk fat with high CLA content; M-CLA – anhydrous milk fat with medium CLA content; E_a – effective activation energy (kJ mol^{-1}); z – pre-exponential factor (min^{-1}); k –reaction rate constant calculated at 200°C (min^{-1}); R^2 – regression coefficient; a and b – coefficients of Eq. (4.1).

For the L-CLA samples, the values of effective activation energy, calculated from T_s, T_{on}, and T_p, were 146.51, 114.11, and 121.31 kJ mol^{-1}, respectively. For the M-CLA samples, the E_a values were 112.42 (T_s), 104.06 (T_{on}), and 87.41 (T_p) kJ mol^{-1}, respectively. For the H-CLA samples, the E_a values were 87.60 (T_s), 82.42 (T_{on}), and 73.64 (T_p) kJ mol^{-1}, respectively. The k values from T_s, T_{on}, and T_p calculated at 200°C are shown in **Table 4-3**. For L-CLA samples, the k values calculated were 0.026 (T_s), 0.121 (T_{on}), and 0.014 min^{-1} (T_p). For M-CLA samples, the values of k were 0.014 (T_s), 0.065 (T_{on}), and 0.006 min^{-1} (T_p). For H-CLA samples, the k values were 0.013 (T_s), 0.069 (T_{on}), and 0.008

min^{-1} (T_p). These variations can be attributed to differences in the ratio of unsaturated to saturated fatty acids. Thurgood et al. (2007) studied the non-isothermal oxidation of AMF and reported different activation energy values (93.56 and 57.55 kJ mol^{-1}) from onset and maximum heat flow temperatures, respectively. They also calculated the rate constant values at 200^{o}C (0.57 and 0.10 min^{-1}) from onset and maximum heat flow temperatures, respectively. In addition, these authors evaluated the non-isothermal oxidation of soybean/AMF blends. Again, the differences observed can be attributed to the ratio between unsaturated and saturated fatty acids. Thurgood et al. (2007) generated the DSC curves using excess of AMF (6.5 mg) in an open aluminum pan, a procedure that might increase the thickness of the samples and, consequently, the diffusion of oxygen might be affected, influencing the results. **Table 4-3** shows that the E_a values decrease as the CLA content increases, regardless of the selected reference temperature point (T_s, T_{on}, and T_p). This behavior might be due to the oxidation of unsaturated fatty acids that started at low temperatures. But, values of E_a should not be considered as the only parameter to compare the non-isothermal oxidation of different lipid systems (Thurgood et al., 2007). The reaction rate constant (k) should also reflect the drop in the E_a values caused by the CLA content. In **Table 4-3**, the k values calculated at 200°C from T_s decreased as the concentration of CLA increased. But, the k values calculated from T_{on} and T_p do not exhibit the same pattern as those values calculated from T_s. This suggests that the start temperature of oxidation is the most consistent reference point in this study to calculate the oxidation kinetic parameters.

4.3.4 Degree of conversion

Fig 4-4 shows the degree of conversion as a function of temperature at different heating rates (3-15°C min^{-1}). The degree of conversion was calculated from 100˚C to T_p that corresponded to the non-isothermal oxidation range of various edible fats and oils (Ulkowski et al., 2005; Musialik & Litwinienko, 2007; Arain et al., 2009).

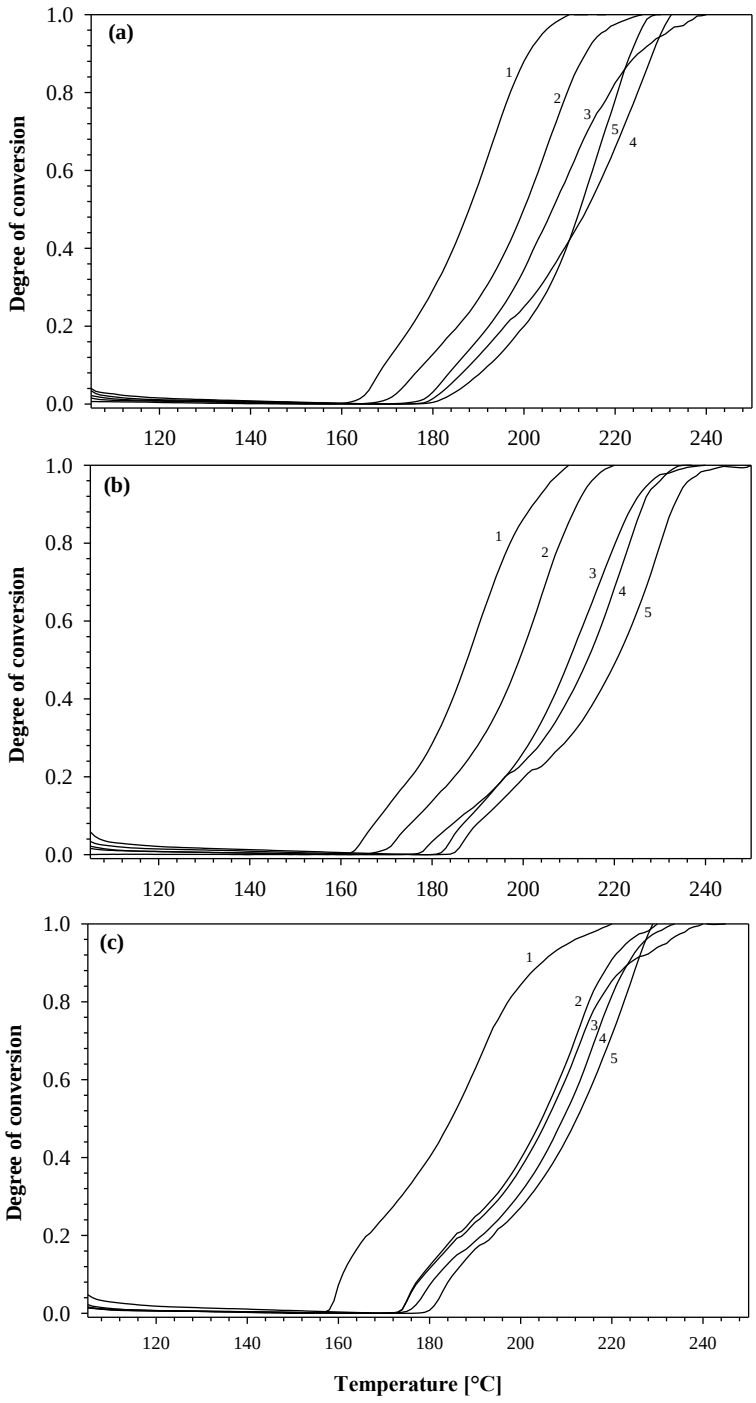

Fig 4-4. Degree of conversion as a function of temperature at different heating rates for: (a) L-CLA, (b) M-CLA, and (c) H-CLA. Line 1 (3°C min^{-1}), line 2 (6°C min^{-1}), line 3 (9°C min^{-1}), line 4 (12°C min^{-1}) and line 5 (15°C min^{-1}) represent different heating rates.

The curves for all AMF samples were sigmoid in shape regardless of the heating rate. These curves represent reaction steps, occurring during non-isothermal oxidation, which can be individually described by an Arrhenius-like model. For low heating rates, the oxidation event consists of several reactions having comparable effective activation energy values that slightly modify the general pattern of the degree of conversion curves (**Figs 4-4a-c**). Increasing the heating rate clearly modifies the general pattern of the degree of conversion curves (lines 4 and 5). Two reasons could explain such a behavior: i) reactions with high values of effective activation energy become more predominant than those reactions with low values of effective activation energy and ii) structural changes induced by the heating rate might affect the oxidation kinetics (Litwinienko et al., 1999). For the first reason, a simulation of the different reaction schemes is needed, which is beyond the scope of this study. The second reason is explored by the compensation effect.

4.3.5 Compensation effect

Table 4-3 suggests that three major reactions occur during AMF oxidation having their own kinetic parameters. The different values of effective activation energy calculated for AMF samples and therefore the oxidation rate associated with it strongly depend not only on the ratio of unsaturated to saturated fatty acids but also on the selected reference points (T_s, T_{on} and T_p). Oxidation proceeds at different reaction rates. The reactions having low values of effective activation energy (**Table 4-3**) are favored at low heating rates and low temperatures (**Table 4-2**). These reactions occur over a broad temperature range (155-224˚C). In contrast, the reactions having high values of effective activation energy are promoted at high heating rates and high temperatures (**Tables 4-2** and **4-3**), and the reaction temperature range increases (Arain et al., 2009).

During non-isothermal oxidation, the reaction systems suffer modifications of thermophysical properties such as density, viscosity, etc due to the linearly increasing temperature.

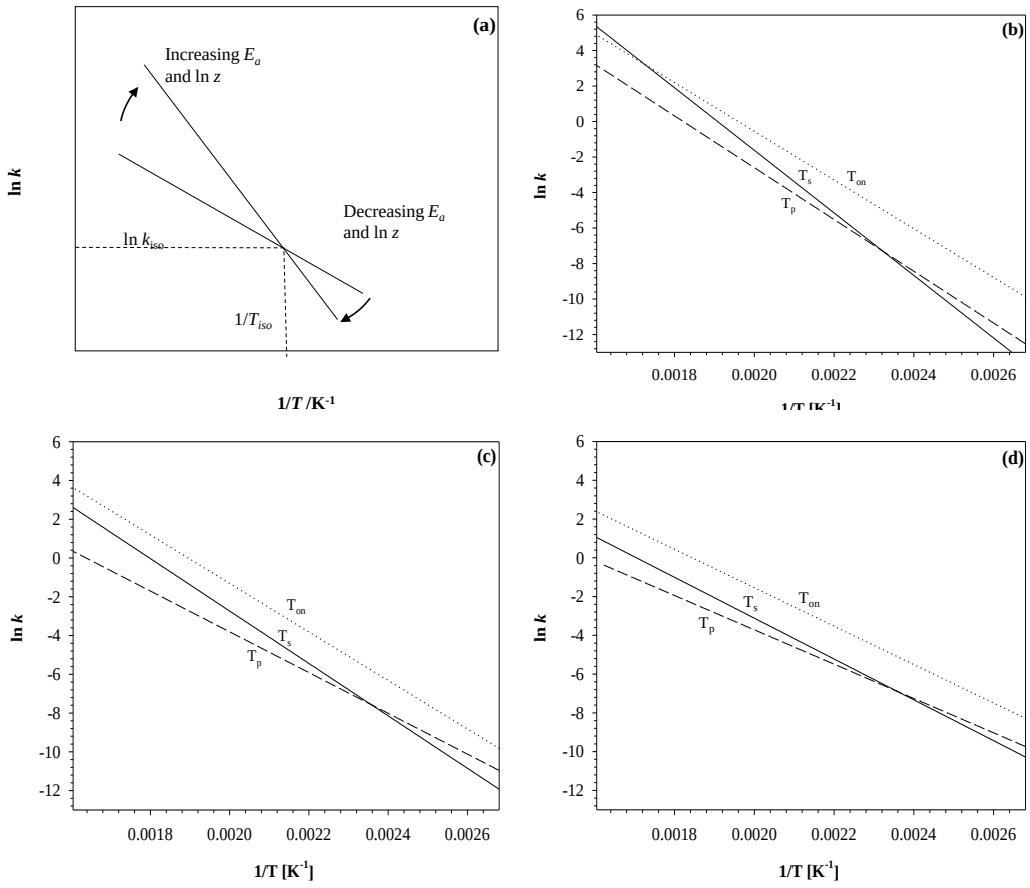

Fig 4-5. Arrhenius plots (ln k vs $1/T$) for the non-isothermal oxidation of anhydrous milk fat: (a) compensation effect (adapted from Agrawal, 1986), (b) L-CLA, (c) M-CLA, and (d) H-CLA. E_a – effective activation energy [kJ/mol^{-1}]; z – pre-exponential factor [min^{-1}]; T_{iso} and k_{iso} – the isokinetic rate constant and isokinetic temperature, respectively; T_s, T_{on}, and T_p – start, onset and maximum temperatures [K] of oxidation, respectively.

Although these structural modifications could change the kinetic parameters of effective activation energy and pre-exponential factor, these variations might not have any physical background. A proper kinetic interpretation should include the compensation effect, which is usually used to explain whether the variations on effective activation energy values have physical meaning or they are caused by either variations of process conditions or complexity of the reaction systems. Agrawal (1986, 1989) compiled and illustrated the compensation effect of several

thermally degraded materials (polymers, cellulosic materials and $CaCO_3$). The compensation effect (**Fig 4-5**) can be evaluated by plotting ln *k*, obtained by equation (4.5), against 1/T. **Fig 4-5a** shows that an increase in the effective activation energy causes an increase in ln *z* (equation 4.5). Similarly, a decrease in effective activation energy results in a lower value of ln *z*. The point of concurrence, where the different lines intersects, corresponds to ln k_{iso} and $1/T_{iso}$ (k_{iso} is the isokinetic rate constant and T_{iso} is the isokinetic temperature), which indicates the existence of compensation effect.

In the case of AMF samples, there is no concurrence at a single point (**Figs 4-5b-d**), meaning that the non-isothermal oxidation of AMF does not exhibit a compensation effect and therefore the variations in kinetic parameters (**Table 4-2**) do not have a physical background. AMF is a complex fat mainly composed of triacylglycerols that have a glycerol backbone to which three fatty acid moieties are esterified. These triacylglycerols are extremely diverse in chain length, position and degree of unsaturation of their fatty acids (Lopez et al., 2001). Moreover, Jensen (2002) reported more than 400 fatty acids in milk fat. This diversity is reflected on the oxidative profiles (**Figs 4-3 and 4-4**). Several reactions having different rate constants are simultaneously taking place and DSC only detects those reactions that have the greatest exothermal effect. This could explain why the variations in effective activation energy do not have physical meaning or no compensation effect.

4.4 Conclusions

For the first time, non-isothermal oxidation of anhydrous milk fat rich in CLA was studied. This kinetic information can be used to predict and compare oxidative stability of AMF-based products. The DSC is a fast and reliable method to evaluate the oxidation kinetics of AMF with different CLA contents. The unsaturated fatty acid content shifts the effective activation energy to lower values, while the content of saturated fatty acids increases the effective activation energy values. The shape of the DSC curves reveals the existence of different

reactions that occur simultaneously but these variations do not have any physical background, according to the compensation theory. The start temperature of oxidation (T_s) was the most consistent reference point to calculate the kinetic oxidation parameters. Therefore, changes in T_s can be used to evaluate the oxidative profile of AMF rich in CLA.

4.5 References

Adhvaryu, A., Erhan, S.Z., Liu, Z.S., & Perez, J.M. (2000). Oxidation kinetic studies of oils derived from unmodified and genetically modified vegetables using pressurized differential scanning calorimetry and nuclear magnetic resonance spectroscopy. *Thermochimoca Acta, 36(1-2),* 87-97.

Agrawal, R.K. (1986). On the compensation effect. *Journal of Thermal Analysis and Calorimetry, 31(1),* 73-86.

Agrawal, R.K. (1989). The compensation effect - a fact or a fiction. *Journal of Thermal Analysis and Calorimetry, 35(3),* 909-917.

Agrawal, R.K. (1992). Analysis of nonisothermal reaction-kinetics .1. Simple reactions. *Thermochimica Acta, 203,* 93-110.

Arain, S., Sherazi, S.H., Bhanger, M.I., Talpur, F.N., & Mahesar, S.A. (2009). Oxidative stability assessment of bauhinia purpurea seed oil in comparison to two conventional vegetable oils by differential scanning calorimetry and rancimat methods. *Thermochimica Acta, 484(1-2),* 1-3.

Bell, J.A., Griinari, J.M., & Kennelly, J.J. (2006). Effect of safflower oil, flaxseed oil, monensin, and vitamin e on concentration of conjugated linoleic acid in bovine milk fat. *Journal of Dairy Science, 89(2),* 733-748.

Bouzidi, L., Boodhoo, M., Humphrey, K.L., & Narine, S.S. (2005). Use of first and second derivatives to accurately determine key parameters of dsc thermographs in lipid crystallization studies. *Thermochima Acta, 439(1-2),* 94–102.

Cook, M.E., & Pariza, M. (1998). The role of conjugated linoleic acid (CLA) in health. *International Dairy Journal, 8(5-6),* 459-462.

Fritsche, J., Rickert, R., Steinhart, H., Yurawecz, M.P., Mossoba, M.M., Sehat, N., Roach, J.A.G., Kramer, J.K.G., & Ku, Y. (1999). Conjugated linoleic acid (cla) isomers: formation, analysis, amounts in foods, and dietary intake. *Lipid / Fett, 101(8),* 272-276.

Jensen, R.G. (2002). The Composition of bovine milk lipids: January 1995 to December 2000. *Journal of Dairy Science, 85(2),* 295-350.

Kamal-Eldin, A., & Pokorny, J. (2005). Lipid oxidation products and methods used for their analysis. In A. Kamal-Eldin, & J. Pokorny, *Analysis of Lipid Oxidation.* American Oil Chemists Society, Publishing.

Kennelly, J.J., & Bell, J.A. (2004). Increasing the concentration of conjugated linoleic acid isomers in the milk fat and/or tissue fat of ruminants, *Patent No. 20030439501 20030516.* United States.

Litwinienko, G. (2001). Autooxidation of unsaturated fatty acids and their esters. *Journal of Thermal Analysis and Calorimet*ry, 65(2), 639-646.

Litwinienko, G., Daniluk, A., & Kasprzycka-Guttman, T. (1999). A differential scanning calorimetry study on the oxidation of C12-C18 saturated fatty acids and their esters. *Journal of the American Oil Chemists' Society, 76(6),* 655-657.

Lock, A., & Bauman, D.E. (2004). Modifying milk fat composition of dairy cows to enhance fatty acids beneficial to human health. *Lipids, 39(12),* 1197-1206.

Lopez, C., Lavigne, F., Lesieur, P., Bourgaux, C., & Ollivon, M. (2001). Thermal and structural behavior of milk fat. 1. Unstable species of anhydrous milk fat. *Journal of Dairy Science*, 84(4), 756-766.

Musialik, M., & Litwinienko, G. (2007). DSC study of linolenic acid autoxidation inhibited by bht, dehydrozingerone and olivetol. *Journal of Thermal Analysis and Calorimetry, 88(3),* 781-785.

Ozawa, T. (1975). Critical investigation of methods for kinetic-analysis of thermoanalytical data. *Journal of Thermal Analysis and Calorimetry, 7(3),* 601-617.

Park, Y. (2009). Conjugated linoleic acid (CLA): Good or bad trans fat? *Journal of Food Composition and Analysis, 22(1),* S4–S12.

Pokorny, J. (2005). Volumetric analysis of oxidized lipids. In A. Kamal-Eldin, & J. Pokorny, *Analysis of Lipid Oxidation.* American Oil Chemists Society, Publishing.

Privett, O.S., & Blank , M.L. (1962). Initial stages of autoxidation. *Journal of American Oil Chemistri Society, 39(11),* 465-468.

Simon, P., & Kolman, L. (2001). DSC Study of oxidation induction periods. *Journal of Thermal Analysis and Calorimetry, 64(2),* 813-820.

Thurgood, J., Ward, R., & Martini, S. (2007). Oxidation kinetics of soybean oil/anhydrous milk fat blends: a differential scanning calorimetry study. *Food Research International, 40(8),* 1030-1037.

Ulkowski, M., Musialik, M., & Litwinienko, G. (2005). Use of differential scanning calorimetry to study lipid oxidation. 1. Oxidative stability of lecithin and linolenic acid. *Journal of Agricultural and Food Chemistry, 53(23),* 9073-9077.

Velasco, J., Andersen, M.L., & Skibsted, L.H. (2004). Evaluation of oxidative stability of vegetable oils by monitoring the tendency to radical formation. a comparison of electron spin resonance spectroscopy with the rancimat method and differential scanning calorimetry. *Food Chemistry, 85(4),* 623-632.

Walstra, P., Wouters, J.M., & Geurts, T.J. (2006). *Dairy Science and Technology.* Boca Raton: CRC Press.

Chapter 5

Oxidative stability of UHT milk rich in conjugated linoleic acid and *trans*-vaccenic acid[4]

5.1. Introduction

In many countries, bovine milk is considered to be an important part of a healthy diet, and is a source of many beneficial bioactive compounds (Molkentin, 2007). One group of fatty acids of particular interest is conjugated linoleic acid (CLA), which are a mixture of positional and geometrical isomers of linoleic acid, all having a conjugated double bond system (Lock & Bauman, 2004). The configuration of each double bond can be either *cis*-/*trans*-, *cis*-/*cis*- or *trans*-/*trans*-, but the *cis*-9/*trans*-11 isomer is the most abundant (≥80%). Studies on the health benefits of CLA were discussed in Chapters 3 and 4.

A promising approach to increase the amount of bioactive compounds such as CLA and TVA in milk fat is by feeding the dairy cattle with oilseed supplemented diets. These diets not only increase the concentration of CLA and TVA but also increment the ratio of unsaturated to saturated fatty acids compared to the control milk (Bell et al., 2006). In Chapter 4, a ratio of 1.40 ± 0.013 was reported in milk fat rich in CLA, which is higher than that obtained in normal milk (0.69 ± 0.015). This is important from a processing standpoint because unsaturated fatty acids are more susceptible to oxidation than saturated fatty acids. Furthermore, there are some reports that CLA suffers significant losses during thermal processing of milk enriched or fortified with CLA (Campbell et al., 2003; Herzallah et al., 2005).

Pasteurization processes such as High-Temperature-Short-Time (HTST, 75˚C/15 s) provide a shelf life of 15 days at 6°C. Combinations of higher temperatures and shorter holding times (130-150˚C/2-10 s) allow milk processors to achieve commercial sterilization that extends the shelf life to between 45 and180 days at room temperature (Rysstad & Kolstad, 2006). Such processing

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[4] *A version of this chapter was submitted to Journal of Dairy Science for consideration for publication*

conditions are known as Ultra-High-Temperature pasteurization (UHT). It is well known that lipid oxidation of milk leads to the development of unpleasant flavors, loss of nutrients and the formation of potentially toxic compounds, such as oxyesterols (Frankel, 1991). Studies on the effects of sterilization conditions (120-150˚C/2-15 s) on the oxidation products of CLA/TVA-milk are limited (Campbell et al., 2003; Herzallah et al., 2005). The objective of this study was to evaluate the oxidative stability of milk rich in CLA and TVA, following sterilization treatments and during storage. The oxidative stability was evaluated in terms of changes in the CLA and TVA contents, dissolved oxygen content and the formation of primary (hydroperoxides) and secondary oxidation products.

5.2. Materials and Methods

5.2.1. Milk rich in CLA and TVA

Enriched milk was obtained from the University of Alberta Dairy Research and Technology Centre (Edmonton, AB, Canada) as described in Chapter 3 (Section 3.2.1).

5.2.2. UHT treatment

Raw CLA/TVA-enriched milk was standardized and homogenized as described in Chapter 3 (Section 3.2.2). The CLA/TVA-enriched milk was processed using an indirect UHT unit (Armfield FT74P, Ringwood, Hampshire, England) equipped with a plate heat exchanger. The unit is coupled to a flow meter and either 2 or 15 s holding tube for short (<5 s) and for long (> 8 s) holding times. Different UHT conditions were used to treat the CLA/TVA-enriched milk (125˚C/2 and 15 s, 135˚C/3 and 10 s and 145˚C/4 and 20 s). Before pumping the CLA/TVA-enriched milk, the unit was run with water until the desirable flow rate, temperature and pressure (0.4 Pa) were reached. Then, the CLA/TVA-enriched milk was pumped and the first 20 mL was discarded. Afterwards, the treated milk was collected and packed in amber glass vials, leaving a headspace of 1/4 of the vial. The samples treated at 125˚C/15 s,

135˚C/10 s and 145˚C/20 s were stored at 4 and 25˚C and analyzed at 0, 7, 15, 30, 60 and 120 days. The UHT treatments were conducted in triplicate.

5.2.3. CLA and fatty acid determination

CLA and TVA contents and fatty acid composition were analyzed by gas-chromatography (GC) as described in Chapter 3 (Section 3.2.5).

5.2.4. Hydroperoxide determination

Lipid hydroperoxides were determined spectrophotometrically according to Ostdal et al. (2000). Fifty mL of 32.7 mM $BaCl_2$ was mixed with 50 mL of 36 mM $FeSO_4$ and 2 mL of 10 M HCl was added. The mixture was filtered twice to remove the precipitate. Then, 500 µL of the filtrate and 500 µL of 4 M of NH_4SCN were added to 49 mL of chloroform/methanol solution (1:1, v/v). A representative sample of treated milk (7 mL) was mixed with 10 mL of chloroform/methanol solution (2:1, v/v) for 30 min. Then, the mixture was centrifuged at 1500 x *g* for 15 min and the lower phase (chloroform+lipids) was transferred to a test tube. Two mL of the chloroform phase was mixed with 2 mL with Fe^{2+}/thiocyanate solution and the absorbance was measured at 500 nm. The lipid hydroperoxide concentration was calculated using a calibration curve of cymene hydroperoxide, obtained within the range of 5-150 µM. Samples outside this range were diluted accordingly.

5.2.5. Thiobarbituric acid reactive substance (TBARS)

Secondary oxidation products were evaluated through thiobarbituric acid reactive substances (TBARS) as reported elswere (King, 1962). Briefly, 10 mL of treated milk was mixed with 1 mL of trichloroacetic acid (1g mL^{-1}) and 2 mL of ethanol. The mixture was heated to 30˚C and kept for 10 min. The mixture was filtered using filter paper (0.45 µm pore size) (Fisher Scientific., Ottawa, ON, Canada) to obtain a clear solution (test solution). Then, 2 mL of TBA (1.4 % w/v) was added to 2 mL of the test solution and incubated at 60˚C for 60 min. After the incubation time, the mixture was cooled down and the absorbance was measured

at 531 nm. The TBARS was calculated using a calibration curve of 1,1,3,3-tetraethoxypropene (TEP) obtained within the range of 5-250 µg/mL. Samples outside this range were diluted accordingly.

5.2.6. Dissolved oxygen determination

Dissolved oxygen (DO_2) in milk was measured using an OM-4 oxygen meter (Microelectrodes, Inc., Bedford, NH, USA). The oxygen meter was calibrated using two-point calibration at 0 and 100% of oxygen saturation in water. The 0% oxygen in water solution was obtained by boiling and subsequently cooling water under nitrogen purge. The 100% oxygen in water solution was obtained by bubbling oxygen for 45 min at room temperature. For each solution, the electrode was allowed to stabilize to the sample temperature before the DO_2 measurement was recorded. All measurements of DO_2 were performed in duplicate.

5.3. Results and discussion

5.3.1. Oxidative stability of CLA/TVA-enriched milk after UHT

Retention of CLA and TVA – The high temperature used in UHT treatment induces losses of CLA and TVA through oxidation. At UHT conditions of 125-145˚C/2-20 s (**Table 5-1**), CLA and TVA losses were minor to moderate depending on the severity of the treatment. At 125˚C after 15 s of holding time, the changes in CLA were moderate (0.83 ± 0.02 retained) while minor changes were observed for TVA (0.95 ± 0.05 retained). On the other hand, the retained fraction of CLA and TVA dropped to 0.78 ± 0.02 and 0.87 ± 0.03 after 10 s of holding time at 135˚C, respectively. When the CLA/TVA-enriched milk was heated at 145˚C, 0.82 ± 0.07 and 0.92 ± 0.03 of CLA and TVA were retained after 20 s of holding time, respectively. For comparison, it was reported that 85% (0.85 fraction) of CLA was retained in conventional milk treated at 140˚C for 4 s (Herzallah et al., 2005). The same authors reported CLA retention of 94 and 79% after treatments of 5 min at 97˚C and 5 min at 97˚C (microwave heated), respectively. In another study, Campbell et al. (2003) pasteurized CLA-fortified

milk (2% of CLA in total fat) at 77˚C for 16 s. Although no change in total CLA was reported, a loss of 10% in the *cis*-9/*trans*-11 isomer and 8% in the *cis*-10/*trans*-12 isomer was reported immediately after pasteurization. To date, the effect of UHT on the distribution of isomers has not been reported.

Table 5-1. Oxidation parameters for CLA/TVA-enriched milk after UHT treatments.

UHT	CLA	TVA	Hydroperoxides	TBARS
125˚C/2 s	0.92 ± 0.03	1.02 ± 0.01	8.75 ± 2.01	128 ± 26
125˚C/15 s	0.83 ± 0.02	0.95 ± 0.04	12.23 ± 0.59	173 ± 7
135˚C/3 s	0.97 ± 0.01	1.01 ± 0.01	9.62 ± 0.51	139 ± 7
135˚C/10 s	0.78 ± 0.02	0.87 ± 0.03	13.21 ± 0.38	186 ± 5
145˚C/4 s	0.84 ± 0.07	0.97 ± 0.02	5.87 ± 1.08	90 ± 14
145˚C/20 s	0.82 ± 0.09	0.92 ± 0.03	11.31 ± 0.78	161 ± 8

CLA – conjugated linoleic acid; TVA – *trans*-vaccenic acid; hydroperoxides content was expressed in mmol mL^{-1}; TBARS was expressed in ppm of TPE

The reductions in CLA content following UHT treatment that are shown in **Table 5-1** are not surprising because CLA might be expected to partially stabilize radicals due to delocalization. These CLA-radicals can react further to form species such as furan fatty acids (Yurawecz et al. 2003, Suzuki et al. 2004). It has been reported that CLA with conjugated double bonds oxidized faster than linoleic acid (C18:2), with non-conjugated double bonds (Minemoto et al. 2003, Yang et al. 2000). In Chapter 4, the oxidation of anhydrous milk fat rich in CLA was evaluated using differential scanning calorimetry and it was found that the oxidation of the enriched sample starts at a lower temperature compared with the control.

Primary oxidation products – The hydroperoxides measured following UHT treatments are shown in **Table 5-1**. In raw milk, the hydroperoxides content was below the detection limit. An increment of hydroperoxides (5-9 mmol mL^{-1}) was observed at the beginning of the UHT treatment (2-4 s), depending on the temperature used. As the holding time increased, the hydroperoxide values slightly increased with values in the range of 11-13 mmol mL^{-1}. The measured

values are rather high compared to those reported by Smet et al. (2009) who used milk with a ratio of unsaturated to saturated fatty acids of 0.4, significantly lower than the one obtained for the CLA/TVA-enriched milk used in this study (1.38 ± 0.01). The unsaturated fatty acids that are present at a higher concentration are more prone to radical formation due to the high hydroperoxide values reported in **Table 5-1** compared to the earlier report (Smet et al. 2009).

TBARS – An indication of the formation of secondary oxidation products during UHT treatment was obtained by means of the TBARS assay. In **Table 5-1**, the effect of temperature and holding time of UHT treatments on the TBARS values is shown. Overall, the TBARS increased with the holding time at each tested temperature. At 125˚C, the TBARS values increased as the holding time increased from 2 to 15 s (128.0 ± 26.4 and 173.7 ± 7.9 ppm of TPE, respectively). At 135 and 145˚C, the TBARS values increased with an increasing holding time. At holding times of 2-4 s, the TBARS values were 139.43 ± 7.85 (135˚C) and 90.1 ± 14.28 ppm (145˚C) while at holding times of 10-20 s the TBARS values were 186.5 ± 5.1 and 161.5 ± 2.85 ppm TPE at 135 and 145˚C, respectively.

5.3.2. Oxidative stability of CLA/TVA-enriched milk during storage

Changes in CLA and TVA – Samples treated at 125˚C/15 s, 135˚C/10 s and 145˚C/20 s were stored at 4 and 25˚C to evaluate their oxidative stability up to 120 days. The CLA in samples stored at 4˚C (**Fig 5-1a**) was more stable in those samples treated at 125˚C/15 s, followed by 135˚C/10 s and 145˚C/20 s, reaching retention fractions of 0.48 ± 0.012, 0.32 ± 0.05 and 0.17 ± 0.03, respectively, after 120 days of storage. TVA was less affected by the UHT treatment and therefore more stable than CLA, obtaining retention fractions in the range of 0.45-0.48 after 120 days of storage, regardless of the UHT treatment (**Fig 5-1b**). At 25˚C, the retained fractions of CLA (**Fig 5-1c**) were lower compared with those stored at 4˚C. Unexpectedly, retention of CLA was greater at 145˚C/20 s (0.43 ± 0.018) compared with 135˚C/10 s (0.26 ± 0.031) and 125˚C/15 s (0.036 ± 0.018) during storage at 25˚C. For TVA stored at 25˚C (**Fig 5-1d**), the samples treated at

125˚C/15 s (0.57 ± 0.011) retained more TVA than those treated at 135˚C/10 s (0.32 ± 0.016) and 145˚C/20 s (0.20 ± 0.027).

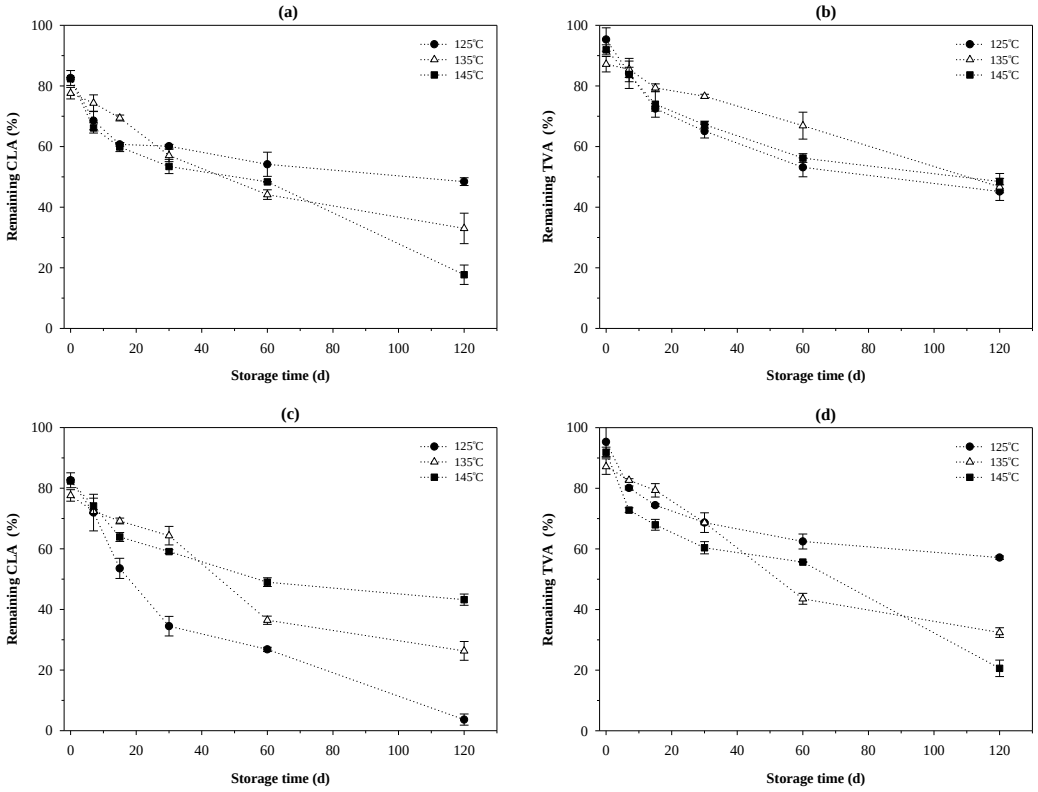

Fig 5-1. Retention of CLA and TVA in UHT enriched milk. (a) Remaining CLA in milk stored at 4 ˚C, (b) remaining TVA in milk stored at 4˚C, (c) remaining CLA in milk stored at 25˚C and (d) remaining TVA in milk stored at 25˚C. Samples were treated at 125˚C/15 s, 135˚C/10 s and 145˚C/20 s.

CLA is lost during storage through oxidation regardless of the UHT treatment as shown in **Fig 5-1**. Yurawecz et al. (2003) hypothesized that CLA is oxidized in the presence of initiators, such as enzymes, light, metal ions (Ca^{2+} and Fe^{3+}) and reactive oxygen species. These authors stored methyl esters of CLA at room temperature in the presence of light and oxygen and found that CLA deteriorate significantly while negligible changes were observed in the control treatments (methyl esters of CLA stored in dark without oxygen). In the case of UHT, the high temperature likely induced the formation of free radicals through

thermolysis. These radicals can initiate CLA oxidation during storage. Samples treated at 125˚C/15 s and stored at 4˚C yielded higher retention of CLA and TVA after 120 days of storage (**Fig 5-1**). This combination of temperature and holding time induced denaturation of protein and subsequently increased the amount of free sulfhydryl groups, which have been reported to have antioxidant activity in milk (Cluskey et al., 1997). In addition, Maillard reaction products can scavenge free radicals, enhancing the oxidative stability (Morales & Jiménez-Pérez, 2001).

Hydroperoxide formation – The level of hydroperoxides formed during storage at 4 and 25˚C are shown in **Fig 5-2**. Primary oxidation products increased after 15 days at both storage temperatures. The hydroperoxide values obtained in the samples treated at 125˚C/15 s and stored at 4˚C gradually increased reaching values of up to 101.30 ± 1.08 mmol mL^{-1} at 120 days. Similar, but less pronounced, behavior was observed in samples treated at 135˚C/10 s and 145˚C/20 s stored 4˚C reaching values of up to 75.84 ± 2.22 and 71.35 ± 4.29 mmol mL^{-1} at 120 days, respectively. On the other hand, the hydroperoxide content of samples processed at 125˚C/15 s and stored at 25˚C, reached a maximum of 115.21 ± 0.97 mmol mL^{-1} at 60 days. Then, the hydroperoxide value slightly decreased to 99.29 ± 1.25 mmol mL^{-1} at 120 days. Similarly, samples treated at 135˚C/10 and 145˚C/20 s stored at 25˚C reached values of 104.53 ± 4.48 and 110.0 ± 2.93 mmol mL^{-1}, respectively, at 120 days.

During UHT treatment, the primary oxidation products formed are typically quantified by hydroperoxides. In the case of CLA/TVA-enriched milk, these determinations can lead to an underestimation of the primary oxidation products. Conjugated fatty acids have more than one type of primary oxidation products and more than one oxidation pathway (Brimberg & Kamal-Eldin, 2003; Hamalainen et al., 2001). A kinetic analysis on autoxidation of methyl-conjugated linoleate showed that monomeric and cyclic peroxides are the major primary oxidation products rather than hydroperoxides (Hamalainen et al., 2001).

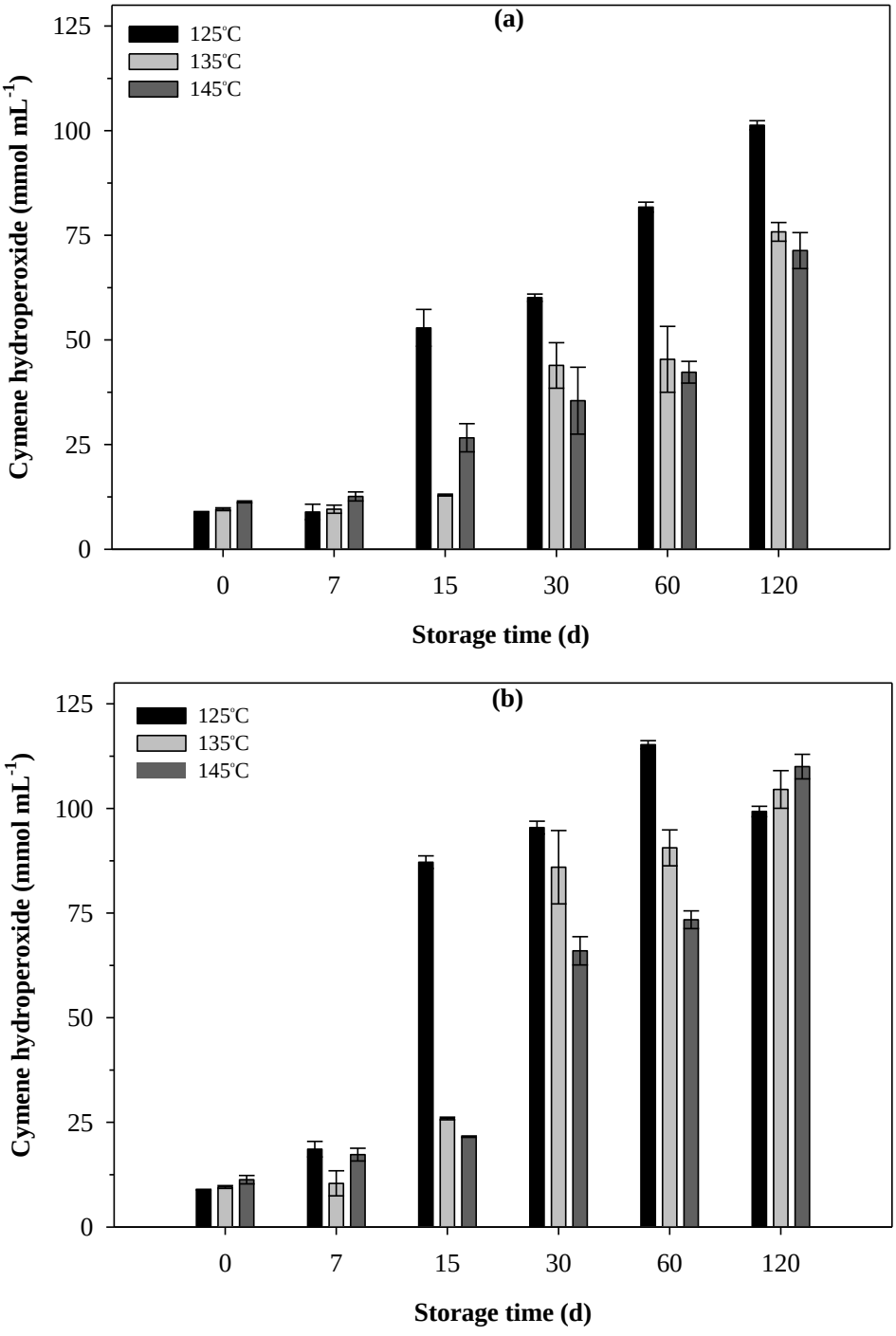

Fig 5-2. Formation of hydroperoxides in UHT treated CLA-enriched milk stored at (a) 4˚C and (b) 25˚C. Samples were treated at 125˚C/15 s, 135 ˚C/10 s and 145 ˚C/20 s. Samples were treated at 125˚C/15 s, 135˚C/10 s and 145˚C/20 s.

Moreover, an investigation of the oxidation of methyl esters of CLA exposed to oxygen showed that 86% of the oxidation products were not formed from hydroperoxides breakdown (Yurawecz et al., 2003). Similarly, Suzuki et al. (2004) quantified dimers and polymers as oxidation products of CLA linoleate. Therefore, the determination of hydroperoxides in conjugated fatty acids may not fully reflect the actual amount of primary oxidation products.

Thiobarbituric acid reactive substances (TBARS) – The TBARS values measured during storage did not show a particular trend and covered a broad range from 68-380 ppm of TPE (data not shown). TBARS values not only represent the aldehydes and ketones formed but also the degradation products from Maillard reaction. Morales et al. (2001) reported that the formation of furfural and hydroxymethylfurfural during UHT milk (140˚C for 10 s) might react with TBA, leading to an overestimation of the TBARS. Indeed, Manglano et al. (2005) reported overestimated TBARS values in commercial milk-based- infant formulas due to the formation of furfural and hydroxymethylfurfural. Luna et al. (2007) oxidized methyl ester of *cis*-9/*trans*-11 and *trans*-10/*trans*-12 and found that hydroperoxide values and TBARS did not match with the absorbed oxygen and reduction of CLA isomers.

Dissolved oxygen – The dissolved oxygen (DO_2) content was monitored during storage (**Fig 5-3**). At storage temperature of 4˚C (**Fig 5-3a**), the DO_2 content was the highest for 145˚C/20 s (~ 30%) sample followed by 135˚C/10 s (~ 25%) and 125˚C/15 s (~ 20%). But, samples stored at 25˚C (**Fig 5-3b**) showed higher DO_2 content than those stored at 4˚C after 120 days. Changes in the dissolved oxygen can be used to infer possible reaction mechanisms of CLA oxidation. For instance, a linear correlation was found between the fraction of oxygen consumed (reduction in the dissolved oxygen) and the converted fraction of CLA. The slopes obtained indicate the amount of dissolved oxygen needed to oxidize one unit of CLA. Interestingly, between 1.85 and 2.61 units of dissolved

oxygen were needed to oxidize one unit of CLA, depending on UHT and storage conditions.

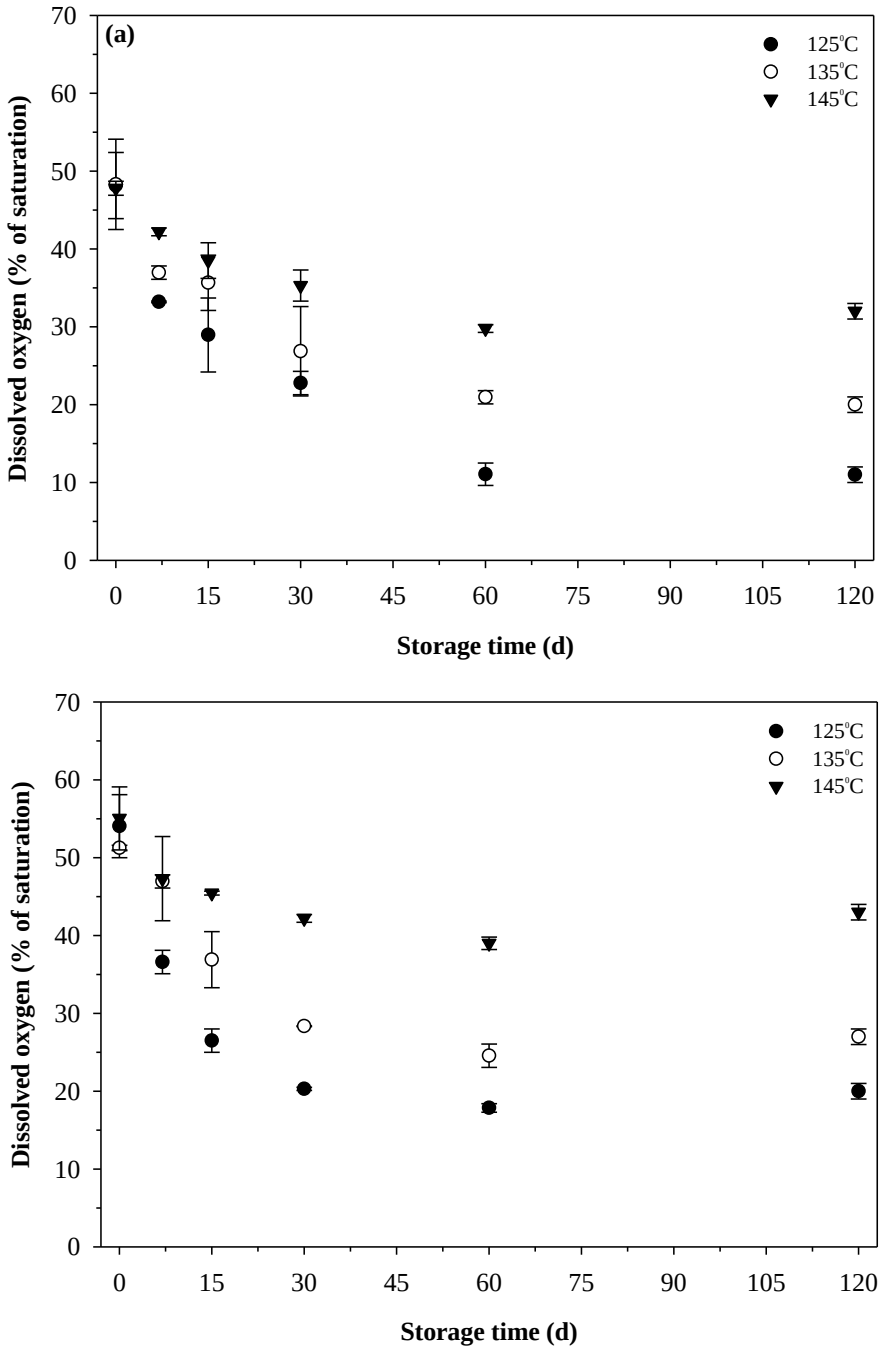

Fig 5-3. Dissolved oxygen concentration in CLA-enriched milk treated at UHT stored at (a) 4˚C and (b) 25˚C. Samples were treated at 125˚C/15 s, 135˚C/10 s and 145˚C/20 s.

Similarly, in a kinetic analysis of fatty acids with a conjugated double bond system, Brimberg & Kamal-Eldin (2003) found that one mol of oxygen oxidized one double bond of the conjugated system. More importantly, these authors proposed that the oxidation of conjugated fatty acids starts with carbon-oxygen cross-linking, forming monomeric and cyclic hydroperoxides through oligomerization and addition mechanisms.

5.4 Conclusions

The oxidative stability of CLA/TVA-enriched milk was evaluated during UHT and storage conditions. After UHT, CLA losses were minor to moderate, depending on the temperature and holding time used. Approximately 90% of CLA and TVA was retained at 125˚C/15 s while the retention of these fatty acids were 78% at 145˚C/20 s. Changes in CLA were minor to moderate (at least 79% of retention), regardless of the UHT conditions used. After 15 d of storage at 4 and 25˚C, the CLA and TVA retention values were in the range of 64-78% and 63-80%, respectively. The retention of CLA needs further correlation with other thermal damage indicators to establish a minimum retention value for CLA/TVA-enriched milk. In addition, the changes in the dissolved oxygen revealed that between 1.85 to 2.61 units of were needed to oxidize one unit of CLA.

5.5. References

Bell, J. A., Griinari, J. M., & Kennelly, J. J. (2006). Effect of safflower oil, flaxseed oil, monensin, and vitamin e on concentration of conjugated linoleic acid in bovine milk fat. *Journal of Dairy Science, 89*(2), 733-748.

Brimberg, U. L., & Kamal-Eldin, A. (2003). On the kinetics of the autoxidation of fats: substrates with conjugated double bonds. *European Journal of Lipid Science and Technology, 105*(1), 17-22.

Campbell, W., Drake, M. A., & Larick, D. K. (2003). The impact of fortification with conjugated linoleic acid (CLA) on the quality of fluid milk. *Journal of Dairy Science, 86*(1), 43-51.

Cluskey, S. M., Connolly, J. F., Devery, R., O'brien, B., Kelly, J., Harrington, D., & Stanton, C. (1997). Lipid and cholesterol oxidation in whole milk powder during processing and storage. *Journal of Food Science*, 62(2), 331–337.

Creamer, L. K., & MacGibbon, A. K. (1996). Some recent advances in the basic chemistry of milk proteins and lipids. *International Dairy Journal*, 539–568.

Frankel, E. N. (1991). Recent advances in lipid oxidation. *Journal of the Science of Food and Agriculture*, 495-511.

Hamalainen, T. I., Sundberg, S., Makkinen, M., Hase, T., & Hopia, A. (2001). Hydroperoxide formation during autoxidation of conjugated linoleic acid methyl ester. *European Journal of Lipid Science and Technology*, 588-593.

Herzallah, S. M., Humeid, M. A., & Al-Ismai, K. M. (2005). Effect of heating and processing methods of milk and dairy products on conjugated linoleic acid and trans fatty acid isomer content. *Journal of Dairy Science*, 1301–1310.

King, R. L. (1962). Oxidation of milk fat globule membrane materila. 1. Thiobarbituric acid reaction as a measure of oxidized flavor in milk and model systems. *Journal of Dairy Science*, 1165-1171.

Lock, A., & Bauman, D. E. (2004). Modifying milk fat composition of dairy cows to enhance fatty acids beneficial to human health. *Lipids*, *39*(12), 1197-1206.

Luna, P., de la Fuente, M. A., Salvador, D., & Marquez-Ruiz, G. (2007). Differences in oxidation kinetics between conjugated and non-conjugated methyl linoleate. *Lipids*, *47*, 1085–1092.

Manglano, P., Lagarda, M. J., Silvestre, M. D., Vidal, C., Clemente, G., & Farré, R. (2005). Stability of the lipid fraction of milk-based infant formulas during storage. *European Journal of Lipid Science and Technology*, 815–823.

Minemoto, Y., Adachi, S., Shimada, Y., Nagao, T., Iwata, T., Yamauchi-Sato, Y., et al. (2003). Oxidation kinetics for cis-9,trans-11 and trans-10,cis-12 isomers of CLA. *Journal of the American Oil Chemists' Society*, *80*(7), 675-678.

Molkentin, J. (2007). Occurrence and biochemical characteristics of natural bioactive substances in bovine milk lipids. *British Journal of Nutrition*, 47-53.

Morales, F. J., & Jiménez-Pérez, S. (2001). Free radical scavenging capacity of Maillard reaction products as related to colour and fluorescence. *Food Chemistry*, *72*, 119-125.

Ostdal, H., Andersen, H., & Nielsen, J. (2000). Antioxidative activity of urate in bovine milk. *Journal of Agricultural and Food Chemistry*, 5588-5592.

Rysstad, G., & Kolstad, J. (2006). Extended Shelf Life Milk - Advances in Technology. *International Journal of Dairy Technology*, *59*(2), 85-96.

Smet, K., De Block, J., De Campeneere, S., De Brabander, D., Herman, L., Raes, K., Dewettinck, K., & Coudijzer, K. (2009). Oxidative stability of UHT milk as influenced by fatty acid composition and packaging. *International Dairy Journal*, 19(6-7), 372–379.

Suzuki, R., Abe, M., & Miyashita, K. (2004). Comparative study of the autoxidation of TAG containing conjugated and nonconjugated C18 PUFA. *Journal of the American Oil Chemists' Society*, *81*, 563-569.

Wang, Y., Jacome-Sosa, M. M., & Spencer, D. P. (2012). The role of ruminant trans fat as a potential nutraceutical in the prevention of cardiovascular disease. *Food Research International*, *46*(2), 460–468.

Yang, L., Leung, L. K., Huang, Y., & Chen, Z. Y. (2000). Oxidative stability of conjugated linoleic acid isomers. *Journal of Agricultural and Food Chemistry*, *48*(8), 3072-3076 .

Yurawecz, M. P., Delmonte, P., Vogel, T., & Kramer, J. G. (2003). Oxidation of conjugated linoleic acid: initiators and simultaneous reactions: theory and practice. In W. W. Christie, J. L. Sébédio, & R. Adlof, *Advances in Conjugated Linoleic Acid Research.*

Chapter 6

Effect of high-pressure sterilization on conjugated linoleic acid (CLA) content of CLA-enriched milk[5]

6.1. Introduction

Bovine milk contains functional compounds that suffer during sterilization treatments significant losses in terms of biological activity (Claeys et al., 2002). Among these compounds, conjugated linoleic acid (CLA) has attracted consumer attention because of potential benefits. Chapters 3 and 4 reviewed the benefits of CLA. Dairy products contain the highest amounts of CLA in the human diet. Additionally, CLA concentration in milk can be markedly enhanced through diet manipulation and nutritional management of dairy cattle (Kennelly & Bell, 2004). Unfortunately, CLA is not stable during thermal processing and significant losses of biological activity occur through oxidation (Campbell et al., 2003; Herzallah et al., 2005) and isomerization (Destaillats & Angers, 2005) as also investigated in Chapters 3-5.

High-pressure sterilization (HPS) is a relatively new technology that simultaneously applies high pressure (~600-700 MPa) and high temperature (~90-120°C) to inactivate bacterial spores (Leadley et al., 2008; Mathys et al., 2009). This technology was first successfully developed to produce commercially sterile low-acid foods (Sizer et al., 2002). In HPS treatments, when the pre-heated product is pressurized, its rise in temperature due to adiabatic heating is used to reach the target or sterilization temperature (Ting et al., 2002). Although non-uniform temperature profile in high pressure vessels was reported for industrial units with a temperature variation of approximately 10^{o}C, the rapid heating in HPS processes reduces the severity of thermal treatment that occurs in traditional sterilization processes (Knoerzer et al., 2010).

Pressure alters interatomic distance acting mainly on those weak interactions, where bond energy is distance–dependent, such as van der Waals

[5] *A version of this chapter has been published in Innovative Food Science and Emerging Technologies, 2012, 16, 291-297.*

forces, electrostatic forces, hydrogen bonding and hydrophobic interactions of proteins. Based on the distance dependence, any pressurized sample would have its covalent bonds intact. This has been the central hypothesis in preserving the biological activity of functional compounds such as ascorbic acid (Oley et al., 2006), folates (Butz et al., 2004), vitamins (Matser et al., 2004), and anthocyanins (Verbeyst et al., 2010). These findings along with the inactivation kinetics of bacterial spores reported in the literature (Bartlett, 2002; Margosch et al., 2006) are promising results, suggesting that this novel technology can deliver shelf-stable milk with better quality than the traditional sterilization process; however, stability of CLA in milk at HPS conditions has not been studied. Therefore, the objective of this study was to evaluate the effect of pressure-assisted thermal sterilization on CLA retention in enriched milk and anhydrous milk fat and the possibility of increasing its retention by the addition of the antioxidant catechin.

6.2. Materials and Methods

6.2.1 Enriched milk and anhydrous milk fat (AMF)

Enriched milk was kindly provided by the University of Alberta Dairy Research and Technology Centre (Edmonton, AB, Canada). For those experiments that required AMF, the enriched milk was prepared as described in Chapter 4 (Section 4.2.1).

6.2.2. CLA content and fatty acid composition determination

CLA content and fatty acid composition were analyzed by gas-chromatography (GC) according to the methodology described in Chapter 3 (Section 3.2.5).

6.2.3. Hydroperoxide determination

The hydroperoxide content was determined as described in Chapter 5 (Sectio 5.2.4.).

6.2.4. High-pressure sterilization (HPS) treatments

HPS experiments were conducted using a high pressure multivessel system (Apparatus U111 Unipress, Warszawa, Poland) coupled with a thermostat (Lauda Proline RP 855 Low Temperature, Lauda-Königshofen, Germany). The high pressure unit consists mainly of an intensifier and four high pressure vessels (Vessel 1-Vessel 4), working in parallel, and each one with an internal volume of 8 mL **(Fig 6-1)**. The vessels were heated with a circulator thermostat using propylene glycol, which was also the pressure transmission fluid. Each vessel was equipped with a K type thermocouple located at the bottom of the vessel, which allowed recording the transmission fluid temperature (T1-T4) in each vessel. The adiabatic heating for propylene glycol is 5˚C per 100 MPa (Rasanavagam et al., 2003). Three-mL tubes (Cryogenic vial, Fisher Scientific, Canada, St. Louis, MO, USA) were filled with untreated milk and pre-heated to a determined temperature considering that the temperature of milk increases by 3°C per 100 MPa (Buzrul et al., 2008).

Once the pre-heating temperature was reached, the samples were transferred to the high pressure vessels and pressurized at a rate of ~10 MPa s^{-1}. With this experimental set up, it is assumed that the sample temperature is similar to the transmission fluid temperature as the sample volume in the polypropylene test tube is small (~3 mL) compared to the transmission fluid volume (~5 mL). At the end of the holding time, the samples were decompressed, removed immediately from the high pressure vessels and cooled down with ice to avoid CLA degradation. For the different control treatments, samples of CLA-enriched milk in closed test tubes were preheated in an oil bath at 42-100°C to simulate temperature conditions of the HPS experiments. Then, the same samples were immersed in a thermostated oil bath of the HPS equipment at a constant temperature (60, 90 or 120°C). HPS-treated and control samples were kept at -18°C until further analysis.

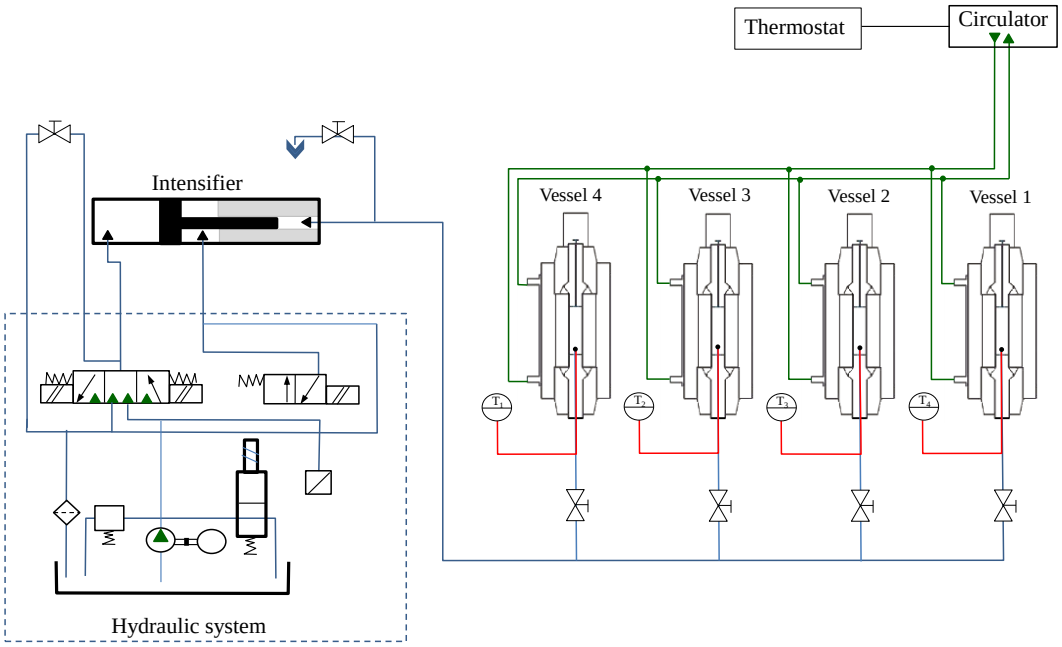

Fig 6-1. Schematic representation of the multivessel system apparatus U111 Unipress. Valve (V_1-V_4), and temperature (T_1-T_4).

Additional set of experiments adding catechin as an antioxidant to enhance CLA retention was conducted following the same experimental conditions. One gram of catechin (Sigma-Aldrich, Saint Louis, MO, USA) per kg of untreated milk was added. CLA retention was expressed as a percentage of its initial amount before treatment. All experimental data were obtained in triplicate and all figures with error bars were plotted using Sigmaplot software V11 for Windows (SPSS Inc., Chicago, IL, USA). The samples were randomly processed within the different vessels.

Additional set of experiments was performed using AMF in the presence of oxidation starters (cymene hydroperoxide, 1 mg g^{-1} fat, and Cu^{+2}, 100 mg cupric chloride per 1 g fat). The sample was heated at 120˚C and pressurized at 100 and 600 MPa for 15 min.

6.3. Results and discussion

6.3.1. Temperature-pressure profiles

Fig 6-2a shows temperature-pressure profiles obtained using the four high pressure vessels simultaneously during HPS of milk treated at 600 MPa and 120°C for 15 min of holding time. The temperature profile of each vessel was recorded for the entire HPS experiment. The loading time (t_l) was the time needed to insert the preheated sample (~102˚C), adjust the transmission fluid volume and close the high pressure vessel. At the beginning of the t_l (arrow (1) in **Fig 6-2**), a drop of ~6˚C in the medium temperature was observed when the preheated sample was inserted into the high pressure vessel.

During compression or pressure build-up time (t_c), the sample was subjected to non-isothermal and non-isobaric conditions over a relatively short period of time (~70 s). As the samples were compressed, the recorded temperature in each vessel dropped between 1 and 6˚C, depending on the location of the vessel (arrow (2) in **Fig 6-2**). For example, vessel 4 dropped its temperature by ~6˚C, while the drop in the temperature of vessel 1 was only ~1˚C. This is because the temperature of the pressurizing fluid that comes from the intensifier was lower (~75˚C) compared to the fluid temperature inside the vessel (120˚C). Vessel 4 is the nearest to the intensifier (**Fig 6-1**) and therefore was exposed to a greater temperature gradient than vessel 1. Then, the temperature of the sample and the medium increased due to adiabatic heating, reaching values up to 121˚C (arrow (3) in **Fig 6-2**). Simultaneously, all vessels reached 600 MPa. After a short period of time (~10 s), the temperature of all vessels equilibrated at 120˚C, which was considered as the start of the holding time (t_h). It was assumed that at the start of the holding time, the transmission fluid, the vessel and the sample were at the same temperature (120˚C) and therefore data obtained can be considered to be at isothermal and isobaric conditions.

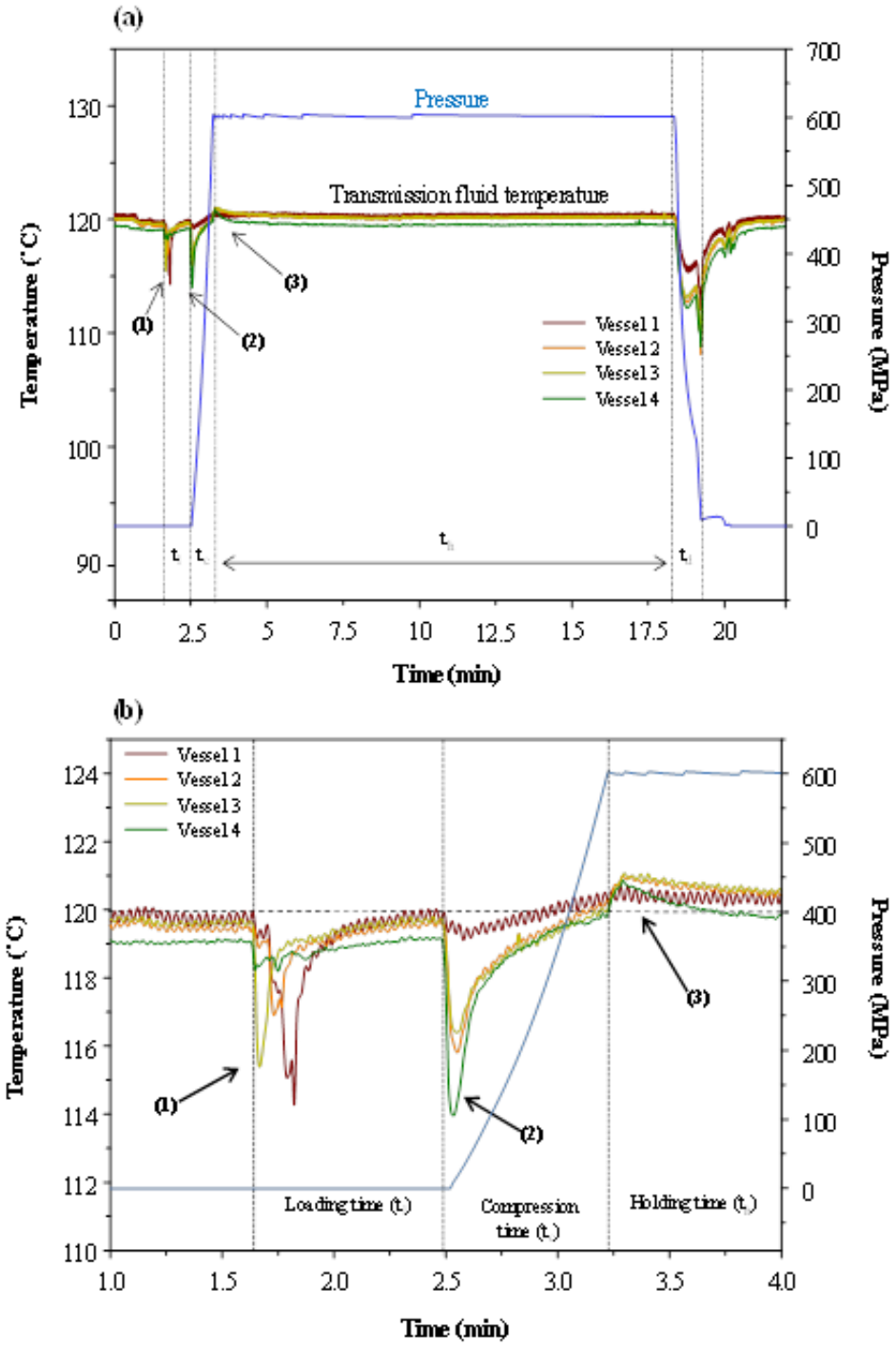

Fig 6-2. Temperature-pressure profile for high pressure sterilization treatments: (a) milk treated at 600 MPa and 120˚C with 15 min of holding time, (b) zoom into 110-124˚C and 1-4 min ranges (t_l – loading time; t_c – compression time; t_h – holding time; t_d – decompression time).

During t_c (**Fig 6-2),** the vessels experienced different temperature gradients that might affect CLA content. Consequently, the samples were randomly processed within the different vessels. At the end of the t_h, the vessels were decompressed and the samples were removed, cooled down with ice and analyzed. Similar descriptions of HPS processes have been reported elsewhere (Ting et al., 2002; Balasubramaniam et al., 2004; van den Ve et al., 2007; Mathys et al., 2009).

Knowledge of the temperature and pressure profile in HPS is needed for a correct interpretation of experimental findings (Balasubramaniam et al., 2004). **Fig 6-2** shows a detailed profile of the process variables at HPS conditions for milk, which can facilitate further comparison with the literature. However, differences in equipment size and process variables might result in different pressure and temperature profiles, affecting the final quality of the treated product.

6.3.2. CLA retention in milk treated by HPS

Fig 6-3 shows the CLA retention in CLA-enriched milk at different pressure, temperature, and treatment time conditions. CLA retention in treatments under atmospheric pressure decreased with an increase of temperature. After 14 min of treatment, CLA values were 87.0 ± 1.4, 71.3 ± 1.7, and 59.5 ± 1.6% of its original level at 60, 90, and 120°C, respectively.

During thermal processing, CLA is lost through oxidation depending on the temperature and time of treatment (see Chapter 3). In addition, Herzalla et al. (2005) determined CLA retention after treatments of 2 min at 90˚C, 5 min at 95˚C (microwave heated) and 4 s at 140˚C (UHT), obtaining values of 94, 79 and 85%, respectively. Another study showed that only 89% of CLA was retained in fortified milk treated for 16 s at 77˚C (Campbell et al., 2003). These authors as well as Yang et al. (2000) explained that the conjugated double bond system (e.g. CLA) is more susceptible to autooxidation than the nonconjugated double bond

system (e.g. linolenic acid). In Chapter 3 and 5, 20% of the CLA was retained after 15 min at 120˚C and 78% was retained when the CLA-enriched milk was treated at 135˚C/10 s.

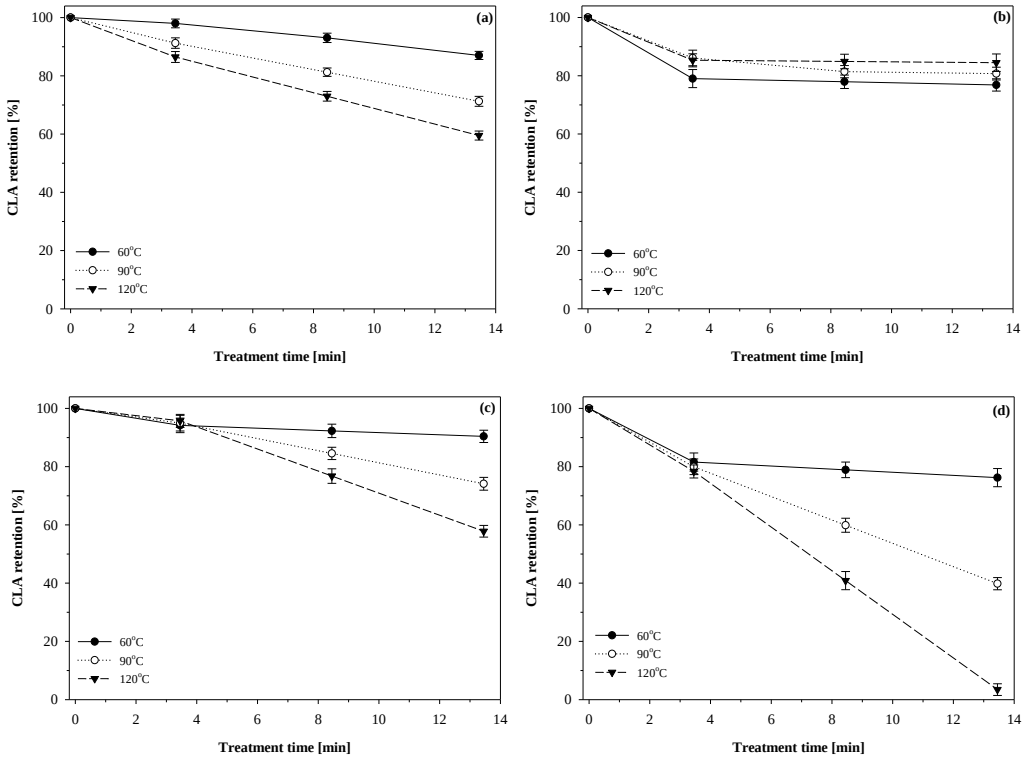

Fig 6-3. Retention of conjugated linoleic acid in milk treated at (a) 0.1 MPa, (b) 100 MPa, (c) 350 MPa, and (d) 600 MPa at different temperatures.

Two possible explanations can be used to interpret CLA losses in enriched milk (**Fig. 6-3a**). One possible reason is that AMF rich in CLA has a higher ratio of unsaturated to saturated fatty acids (1.36) than the ratio of non-enriched AMF (0.71) (Chapter 4). This ratio found in CLA-enriched AMF (same milk used to obtain AMF) is the same ratio for CLA-enriched milk. As previously reported in Chapter 4, unsaturated fatty acids also oxidize faster than saturated fatty acids at low temperatures. Under non-isothermal conditions, the start temperature of oxidation for AMF rich in CLA, as measured by differential scanning calorimetry, shifts to lower values as the ratio of unsaturated to saturated fatty acids increases.

The other possible reason is that CLA in enriched milk has 34.5 mg CLA g^{-1} fat, which is considerably higher than the content in non-enriched milk (5 mg CLA g^{-1} fat).

An initial drop in the CLA retention values was recorded between 0 and ~3 min, which corresponded to the loading and pressure built-up period (**Fig 6-3b-d**). In this period, the samples were subjected to non-isothermal and non-isobaric conditions making it difficult to interpret the initial drop. Oley et al. (2006) evaluated the initial drop in buffer solution of ascorbic acid. They concluded that the dissolved oxygen is responsible for the initial drop; however, more experimental evidence is needed for different reaction systems.

The CLA was relatively stable up to 14 min of treatment at 100 MPa, regardless of the temperature used (**Fig 6-3b**). At 60, 90, and 120°C, the CLA was retained within the range of 76 to 85% (**Fig 6-3b**). Similarly, after 14 min of treatment at 60°C in the pressure range of 350 to 600 MPa, 76 to 90% of CLA was retained (**Fig 6-3c-d**). Under these combinations of temperatures and pressures (60°C with 100-600 MPa and 100 MPa with 60-120°C), CLA losses were minor to moderate. Such combinations of pressure and temperature would allow the inactivation of vegetative microorganisms required for pasteurization (Wilson et al., 2008). Rodríguez-Alcalá et al. (2009) treated cow, goat and ewe milk using high pressure homogenization (350 MPa, and 65°C). No change in CLA content and isomers distribution was observed at any tested condition.

On the other hand, the retention values of CLA rapidly decreased when samples were heated at 90 and 120°C and pressurized at 350 MPa (**Fig 6-3c**). The same tendency was observed at 600 MPa (**Fig 6-3d**). A dramatic degradation of CLA (~3 % of retention) was observed in samples treated up to 14 min at 600 MPa and 120°C. The oxidation of CLA was accelerated by the combination of high pressure (600 MPa) and high temperature (120°C). Pressure and temperature could affect three possible reaction steps, resulting in an acceleration of CLA

oxidation. First, pressure could promote homolysis and free radical reactions, which are the most important mechanism of oxidation (O'Connor & O'Brien, 2006). This situation would occur if their activation volumes are negative or close to zero. Isaacs (1981) reported activation volumes of thermal homolysis in the range of 0.3-13 cm^3 mol^{-1} for different peroxide reaction systems. On the other hand, negative activation volumes in the range of -5 to -15 cm^3 mol^{-1} have been reported for the initiation and propagation stages of free radical reactions (Isaacs, 1981). Certainly, these investigations showed that pressure can accelerate one or more oxidation stages; however, these reported activation volumes cannot explain the dramatic decrease in the CLA retention values (**Fig 6-3d**).

An alternative effect could be that the dissolved oxygen becomes more reactive (ground oxygen) when pressure is applied. Okamoto (1992) reported that the lifetime of singlet oxygen increases significantly with pressures up to 400 MPa; however, to date, the role of oxygen under PATS conditions (90-120°C and 400-600 MPa) has not been studied. Milk fat globule membrane (MFGM) contains high concentration of phosphatidylethanolamine (PE), which is known to bind Cu^{2+}. During processing, the MFGM can be disrupted, releasing membrane phospholipids into the aqueous solution. Consequently, the proportion of PE in milk serum may increase, depending on the severity of the treatment.

6.3.3. CLA retention in anhydrous milk fat

An additional set of experiments were conducted in AMF rich in CLA (**Fig 6-4**) in which the effect of metals catalyzing the reaction would be negligible. At 60 and 90°C and 0.1 MPa, CLA retention values decreased up to 14 min of treatment (86.9 ± 1.3 and 75.5 ± 1.1%, respectively). These values were similar to those obtained at the same pressure and temperature conditions for CLA rich milk (**Fig 6-4a**); however, CLA retention in AMF dropped initially from 100 to 59.5 ± 1.3% at 120°C and 0.1 MPa and then remained relatively stable up to 14 min of treatment (53.2 ± 1.3%), reaching retention values lower than those found for milk (59.3 ± 1.6%). Fat in milk is emulsified and protected from

environmental factors such as enzymes, oxygen and metals, while the fat in AMF is exposed to environmental factors leading to a faster oxidation rate (Sharma, & Dalgleish, 1993). Moreover, oxidation in milk is a result of complex interactions between pro- and antioxidant compounds. Lindmark-Mansson & Akesson (2000) reviewed the role and occurrence of different antioxidants in bovine milk.

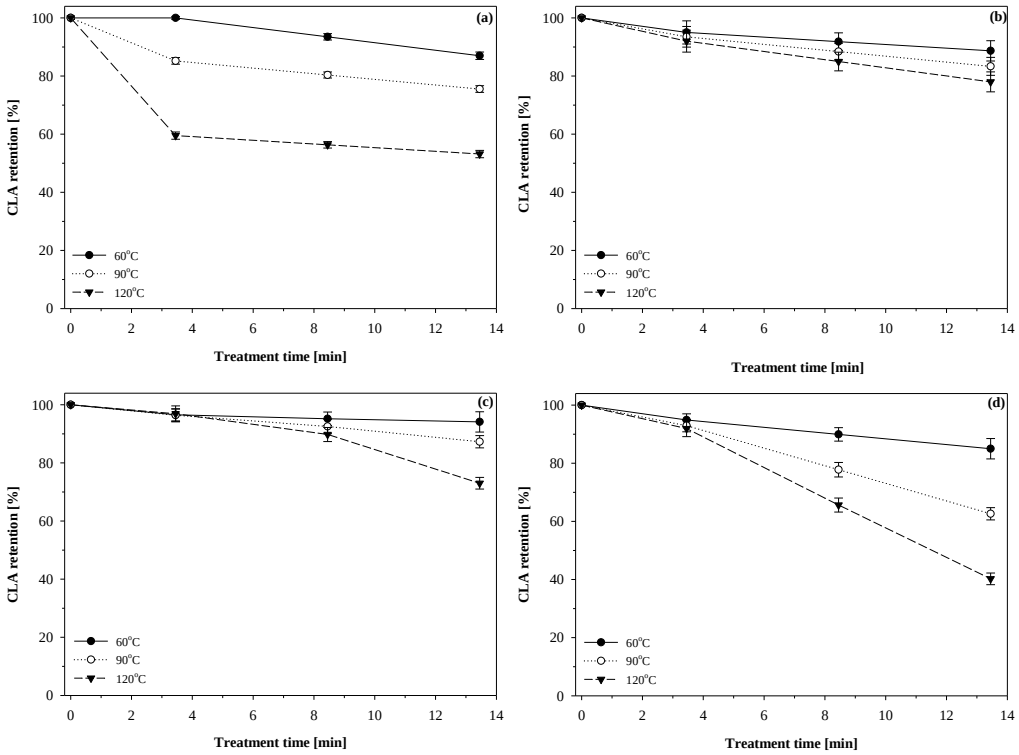

Fig 6-4. Retention of conjugated linoleic acid in anhydrous milk fat treated at (a) 0.1 MPa, (b) 100 MPa, (c) 350 MPa and (d) 600 MPa at different temperatures.

At 100 and 350 MPa, similar values of CLA were obtained for AMF as compared with those obtained for milk (**Fig 6-4b,c**); however, there is a considerable difference in the CLA retention values between those obtained in AMF and those obtained in milk at 600 MPa (**Fig 6-4d** and **6-4c**). After 14 min of treatment at 600 MPa, the CLA retention values in AMF were 91.9 ± 2.8, 65.6 ± 2.4, and 40.2 ± 2.0% at 60, 90 and 120°C, respectively, while the corresponding

CLA retention values in milk were 76.2 ± 3.1, 39.8 ± 2.1, and 3.5 ± 2.0% at the same temperature and pressure conditions. In AMF, where the presence of metals is minimal and therefore their catalytic effect can be neglected, the retention values of CLA (40.2 ± 2.0%) were considerably higher than those obtained in milk after 14 min of treatment (3.5 ± 2.0%). In milk, metals might act as catalysts, being responsible for the important losses in CLA levels.

At the beginning of the AMF treatment, there is an initial drop in the CLA values from 100 to 59.5 ± 1.3% at 0.1 MPa (**Fig 6-4a**). This might be an indication that the first stage of oxidation (radical formation by homolysis) occurred. This reaction might involve a covalent bond breaking, which would be delayed by pressure. Earlier, Tausher (1995) retarded the autoxidation of linolenic acid by using a pressure of 600 MPa. Using pressure treatments of 100-600 MPa in this study, the initial drop in the CLA values is not observed at any evaluated temperature, suggesting that pressure delays the oxidation of CLA; however, after 14 min of treatment at 600 MPa and 120°C, the retained CLA in AMF was lower than that at 0.1 MPa and 120°C (53.16 ± 1.25 and 59.25 ± 1.55%, respectively), suggesting that propagation of oxidation is accelerated by pressure. Propagation is a free radical reaction that it is expected to be accelerated by pressure. Isaacs (1981) summarized that various free radical reactions were accelerated by pressure.

6.3.4. Formation of lipid hydroperoxides

CLA is not stable upon processing and our hypothesis is that CLA is lost through oxidation. During oxidation, free radicals are formed through thermolysis and due to the presence of enzymes and active oxygen species. Then, these radicals react with molecular oxygen to form products such as hydroperoxides. These compounds are unstable and further react through free radical mechanisms to form secondary products that propagate the oxidation. Thus, the detection and quantification of hydroperoxide in HPS-treated samples can indicate an early

stage of lipid oxidation. **Fig 6-5** shows the hydroperoxide content in enriched and non-enriched milk treated at 600 MPa and 90 and 120°C.

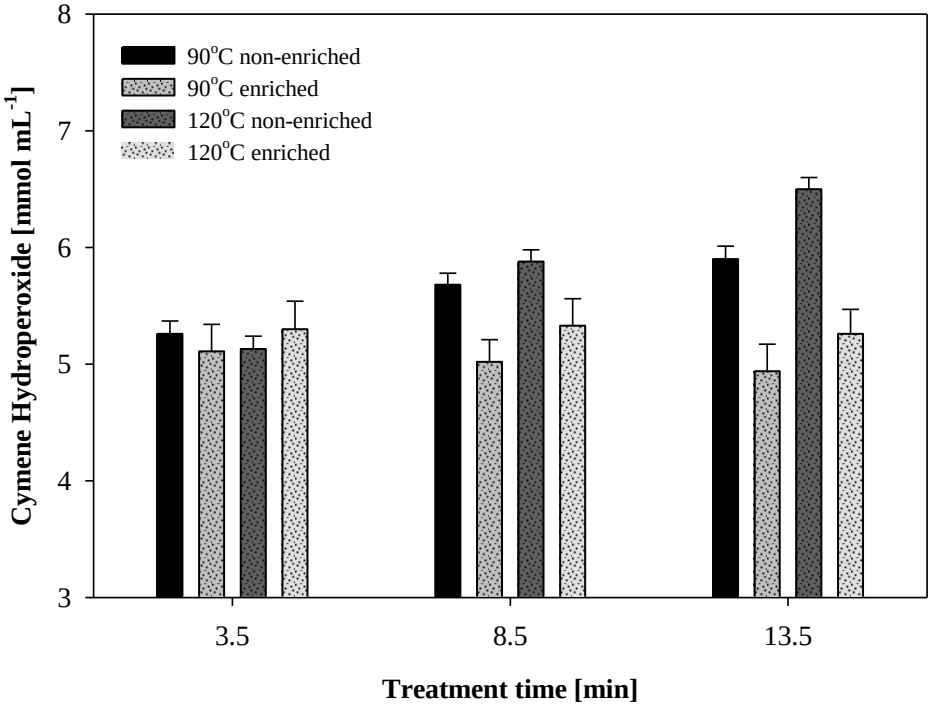

Fig 6-5. Formation of lipid hydroperoxides in enriched and non-enriched milk treated after 2, 3 and 4 min at 90 and 120°C and 600 MPa.

Interestingly, after a treatment of 14 min at 120°C and 600 MPa, the hydroperoxide content was higher in non-enriched milk than in enriched milk (6.5 ± 0.1 and 5.26 ± 0.21 mmol mL^{-1}, respectively). The relatively low content of hydroperoxides in enriched milk is not surprising since conjugated double bond systems have more than one type of primary oxidation products and more than one oxidation pathway, as reported elsewhere (Brimberg & Kamal-Eldin, 2003; Hamalainen et al., 2001). A kinetic analysis on autoxidation of methyl-conjugated linoleate showed that monomeric and cyclic peroxides are the major primary oxidation products rather than hydroperoxides (Hamalainen et al., 2001). Consequently, addition by Diels Alder-type reaction was suggested as a reaction mechanism. In general, Diels-Alder reactions are strongly accelerated by pressure

with activation volumes in the range of -30 to -40 cm^3 mol^{-1} (Isaacs, 1981). This is because cyclic compounds have smaller partial molar volumes than acyclic analogues (Walling & Waits, 1967). Another reason for the relatively low content of hydroperoxides is that CLA can act as an antioxidant capturing those free radicals responsible for lipid oxidation. This antioxidant behaviour has been demonstrated by Fagali & Catala (2008), who induced lipid oxidation in fish oil by adding tert-butyl hydroperoxide and found that CLA effectively reduced lipid peroxidation as measured by chemiluminescence. Additionally, these authors also reported antiradical or scavenging ability of CLA isomers measured by DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) technique. Antioxidant and antiradical ability depend on CLA concentration. Martinez-Monteagudo & Saldaña (2011) demonstrated that CLA preserves its antiradical ability as measured by DPPH technique within 14 min of treatment at 600 MPa and 120°C (data not shown). Thus, after a pressure treatment, CLA can still donate hydrogen to form a CLA-free radical that further reacts to inhibit hydroperoxide formation (Martinez-Monteagudo & Saldaña, 2011).

Tauscher (1995) reported that 600 MPa had a protective effect on the autoxidation of linoleic acid, retaining 80% after 21 h. Unfortunately, the temperature used in that investigation was not provided. Additional set of experiments was performed using AMF in the presence of oxidation starters (cymene hydroperoxide, 1 mg g^{-1} fat, and Cu^{+2}, 100 mg cupric chloride per 1 g fat). The sample was heated at 120˚C and pressurized at 100 and 600 MPa for 15 min. The extent of oxidation was determined by the remaining linoleic acid (**Fig 6-6**, data not published). Using AMF without starters (cymene hydroperoxide and Cu^{+2}), the retained linoleic acid was enhanced at 600 MPa, obtaining a fraction of 0.72 ± 0.04. But, the use of hydroperoxides or Cu^{+2} resulted in lower retained linoleic acid at 600 MPa. These results are in agreement with the explanation reported by Tauscher (1995), where pressure accelerates the propagation stage for the oxidation of linoleic acid without the use of any starter.

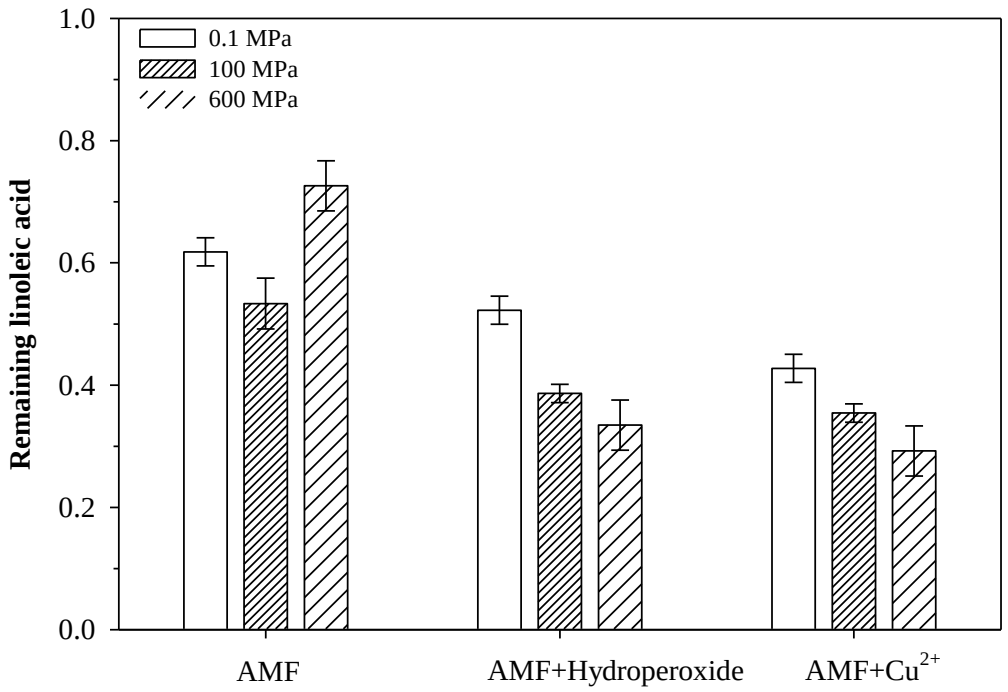

Fig 6-6. Remaining linoleic acid in anhydrous milk fat after 15 min at 120˚C and different pressures (0.1, 100 and 600 MPa). The error bars represent the standard deviation of two replicates.

6.3.5. Effect of catechin on CLA retention in milk and anhydrous milk fat

CLA is lost in a greater extent in milk compared to AMF as shown in **Figs 6-3** and **6-4**. An attempt was made to enhance the retention of CLA by the addition of catechin, which is a potent antioxidant capable of capturing free radicals and quenching metals (Yang et al., 2000). Catechin effectively retained CLA in milk and AMF regardless of the pressure and temperature used. For instance, 90.22 ± 3.5 and 90.5 ± 2.0% of CLA was retained after 14 min of treatment at 600 MPa and 120˚C (**Fig 6-7**). Further research is needed to evaluate the effectiveness of the concentration of the antioxidant and the mechanism by which this antioxidant captures free radicals or reacts with the dissolved oxygen to retard the free radical reactions under HPS conditions.

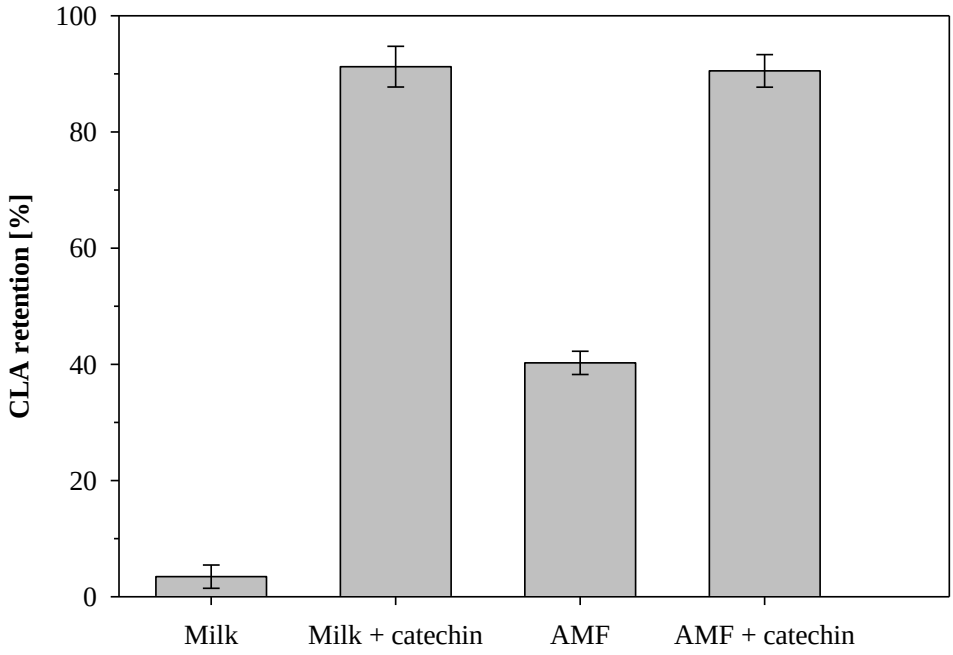

Fig 6-7. Retained CLA in CLA-enriched milk and AMF after 14 min of treatment at 600 MPa and 120˚C (catechin concentration = 1g kg^{-1} milk).

6.4. Conclusions

CLA was not stable up to 14 min of treatment at 600 MPa and 120°C with a retention value of approximately 3% in CLA-enriched milk. Under the same PATS conditions, CLA was lost to a greater extent in enriched milk than in AMF. Possibly, CLA is lost through an oxidation reaction that is catalyzed by free metal ions released when pressure is applied. This might limit the applicability of this technology in preserving the biological activity of CLA in milk; however, the addition of 1 g of catechin per kg of milk effectively enhanced CLA retention at any PATS condition tested within this study in both milk and AMF. Further research is needed to reveal the reaction mechanisms of selected antioxidants at PATS conditions (100-600 MPa; 60-120˚C and 0-14 min).

6.5. References

Balasubramaniam, V. M., Ting, E. Y., Stewart, C. M., & Robbins, J. A. (2004). Recommended laboratory practices for conducting high-pressure microbial inactivation experiments. *Innovative Food Science & Emerging Technologies, 5(3),* 299-306.

Bartlett, D. H. (2002). Pressure effects on in vivo microbial processes. *Biochimica et Biophysica Acta-Protein Structure and Molecular Enzymology*, 1595(1-2), 367-381.

Bell, J. A., Griinari, J. M., & Kennelly, J. J. (2006). Effect of safflower oil, flaxseed oil, monensin, and vitamin E on concentration of conjugated linoleic acid in bovine milk fat. *Journal of Dairy Science, 89(2),* 733-748.

Brimberg, U. I., & Kamal-Eldin, A. (2003). On the kinetics of the autoxidation of fats substrates with conjugated double bonds. *European Journal of Lipid Science and Technology, 105(1),* 17-22.

Buzrul, S., Alpas, H., Largeteau, A., Bozoglu, F., & Demazeau, G. (2008). Compression heating of selected pressure transmitting fluids and liquid foods during hydrostatic pressure treatment. *Journal of Food Engineering, 85(3),* 466-472.

Campbell, W., Drake, M. A., & Larick, D. K. (2003). The impact of fortification with conjugated linoleic acid (CLA) on the quality of fluid milk. *Journal of Dairy Science, 86(1),* 43-51.

Claeys, W. L., Van Loey, A. M., & Herndrickx, M. E. (2002). Intrinsic time temperature integrators for heat treatment of milk. *Trends in Food Science & Technology, 13(9-13),* 293-311.

Destaillats, F., & Angers, P. (2005). Thermally induced formation of conjugated isomers of linoleic acid. *European Journal of Lipid Science Technology, 107(3),* 167-172.

Fagali, N., & Catala, A. (2008). Antioxidant activity of conjugated acid isomers, linoleic acid and its methyl ester determined by photoemission and DPPH techniques. *Biophysical Chemistry*, 137(8), 56-62.

Gaucheron, F., Famelart, M. H., Mariette, F., Raulot, K., Michel, F., & Le Graet, Y. (1997). Combined effects of temperature and high-pressure treatments on physicochemical characteristics of skim milk. *Food Chemistry, 59(3),* 439-447.

Hamalainen, T. I., Sundberg, S., Makkinen, M., Hase, T., & Hopia, A. (2001). Hydroperoxide formation during autoxidation of conjugated linoleic acid methyl ester. *European Journal of Lipid Science and Techn*ology, *103(9),* 588-593.

Herzallah, S. M., Humeid, M. A., & Al-Ismail, K. M. (2005). Effect of heating and processing methods of milk and dairy products on conjugated linoleic acid and trans fatty acid isomer content. *Journal of Dairy Science, 88(4),* 1301-1310.

Isaacs, N. S. (1981). *Liquid Phase High Pressure Chemistry*. (1st ed.). Toronto: John Wiley & Sons.

Knoerzer, K., Smith, R., Juliano, P., Kelly, M., Steele, R., Sanguansri, P., & Verteeg, C. (2010). The thermo-egg: a combined novel engineering and

reverse logic approach for determining temperatures at high pressure. *Food Engineering Reviews, 2(3),* 216-225.

Knoerzer, K., Buckow, R., & Versteeg, C. (2010). Adiabatic compression heating coefficients for high-pressure processing – a study of some insulating polymer materials. *Journal of Food Engineering, 98(1),* 110-119.

Leadley, C., Tucker, G., & Fryer, P. (2008). A comparative study of high pressure sterilization and conventional thermal sterilization: Quality effects in green beans. *Innovative Food Science & Emerging Technologies, 9(1),* 70-79.

Lindmark-Mansson, H., & Akesson, B. (2000). Antioxidative factors in milk. *British Journal of Nutrition, 84(1),* S103-S110.

Margosch, D., Ehrmann, M. A., Buckow, R., Heinz, V., Vogel, R. F., & Ganzle, M. G. (2006). High-pressure-mediated survival of *Clostridium botulinum* and *Bacillus amyloliquefaciens* endospores at high temperature. *Applied and Environmental Microbiology,* 72(5), 3476-3481.

Martinez-Monteagudo, S. I., & Saldaña, M. D. A. (2011). Changes in conjugated linoleic acid (CLA) and antiradical ability of anhydrous milk fat treated with pressure assisted thermal sterilization. Abstract 43, In *24th Canadian Conference on Fats and Oils*, September 26-27th, Edmonton, AB, Canada.

Mathys, A., Reineke, K., Heinz, V., & Knorr, D. (2009). High pressure thermal sterilization - development and application of temperature controlled spore inactivation studies. *High Pressure Research, 29(1),* 3-7.

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 enzymatic coagulation. *Journal of Dairy Research, 67(1),* 31-42.

O'Connor, T.P., & O'Brien, N.M. (2006). Lipid oxidation. In Fox, PF, McSweeney P, editors. *Advanced Dairy Chemistry*, Volume 2: Lipids (3rd Edition). Springer-Verlag. pp. 557-600.

Okamoto, M. (1992). The contribution by diffusion to the cycloaddition reactions of singlet oxygen with furans in solution under high-pressure. *Journal of Physical Chemistry, 96(1),* 245-248.

Oley, I., Verlinde, P., Hendrickx, M., & Van Loey, A. (2006). Temperature and pressure stability of L-ascorbic acid and/or [6s] 5-methyltetrahydrofolic acid: A kinetic study. *European Food Research and Technology, 223(1),* 71-77.

Rasanavagam, V., Balasubramaniam, V. M., Ting, E., Sizer, C. E., Bush, C., & Anderson, C. (2003). Compression heating of selected fatty food materials during high pressure processing. *Journal of Food Science, 68(1),* 2544-259.

Rodríguez-Alcalá, L. M., Harte, F., & Fontecha, J. (2009). Fatty acid profile and CLA isomers content of cow, ewe and goat milks processed by high pressure homogenization. *Innovative Food Science & Emerging Technologies, 10(1),* 32-36.

Sizer, C. E., Balasubramaniam, V. M., & Ting, E. (2002). Validating high-pressure processes for low-acid foods. *Food Technology, 56(2),* 36-42.

Schrader, K., Buchheim, W., & Morr, C. V. (1997). High pressure effects on the colloidal calcium phosphate and the structural integrity of micellar casein

in milk . 1. High pressure dissolution of colloidal calcium phosphate in heated milk systems. *Nahrung*. *41 (3)*, 133-138.

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.

Tauscher, B. (1995). Pasteurization of food by hydrostatic high-pressure – chemical aspects. *Zeitschrift Fur Lebensmittel-Untersuchung Und-Forschung, 200(1)*, 3-13.

Ting, E., Balasubramaniam, V. M., & Raghubeer, E. (2002). Determining thermal effects in high-pressure processing. *Food Technology, 56*(2), 31-34.

Torres, J. A., & Velazquez, G. (2005). Comercial opportunities and research challenges in the high pressure processing of foods. *Journal of Food Engineering, 67(1-2)*, 95-112.

van den Ve, C., Courvoisier, C., & Matser, A. (2007). High pressure versus heat treatments for pasteurisation and sterilization of model emulsions. *Innovative Food Science & Emerging Technologies, 8(2)*, 230-236.

Walling, C., & Waits, H. P. (1967). Pressure and viscosity effects on recombination of tert-butoxy radicals from di-tert-butyl-peroxide. Journal of Physical Chemistry, *71(7)*, 2361-2362.

Wilson, D. R., Dabrowski, L., Stringer, S., Moezelaar, R., & Brocklehurst, T. F. (2008). High pressure in combination with elevated temperature as a amethod for the sterilization of food. *Trends in Food Science & Technology, 19(6)*, 289-299.

Yang, L., Leung, L. K., Huang, Y., & Chen, Z. Y. (2000). Oxidative stability of conjugated linoleic acid isomers. *Journal of Agricultural and Food Chemistry, 48*(8), 3072-3076.

Chapter 7

Modeling the retention kinetics of conjugated linoleic acid (CLA) during high-pressure sterilization of milk[6]

7.1. Introduction

Conjugated linoleic acid (CLA) is a bioactive lipid found naturally in milk fat. The term CLA refers to a mixture of octadecenoic acids with two double bonds in a conjugated form (Villegas et al., 2010). In each double bond, the configuration can be *cis*-/*trans*-, *cis*-/*cis*- or *trans*-/*trans*-. The predominant isomer is the *cis*-9/*trans*-11, which represents more than 80% of the total CLA (Luna et al., 2007). Results of human and animal intervention studies revealed that CLA from ruminant possess health-promoting properties and more importantly *trans* fatty acids from ruminants are considered as novel functional foods (Wang et al., 2012).

Dairy products can be viewed as delivery systems for bioactive lipids and efforts have been made to increase the concentration of CLA by manipulating the feeding regime of dairy cattle. A safflower oil supplemented diet was used for increasing the concentration of CLA in milk by 10-fold (Bell et al., 2006). However, changing the milkfat composition might affect the susceptibility towards oxidation during thermal processing. Indeed, Herzallah et al. (2005) reported losses of 15% of CLA in conventional milk treated at 140˚C for 4 s. Similarly, an investigation showed 10% of reduction in CLA during thermal treatments in CLA-fortified milk (2% of CLA from the total fat) (Campbell et al., 2003). The oxidation of CLA and other unsaturated fatty acids reduces the final quality of CLA-enriched product (Hillbrick & Augustin, 2002; Luna et al., 2007).

An alternative technology that reduces the thermal damage by applying hydrostatic pressure is known as high-pressure sterilization (HPS) or pressure-assisted thermal sterilization (PATS). This technology has become a viable possibility in cases where the traditional thermal treatments induce degradation of

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[6] *A version of this Chapter is to be submitted to the Journal of Agricultural and Food Chemistry for consideration for publication.*

valuable compounds. The fundamentals and applications of HPS are reviewed elsewhere (Bermudez-Aguirre & Barbosa-Canovas, 2011; Mujica-Paz et al., 2011; Juliano et al., 2012). Predicting the retention of CLA through mathematical equations derived from kinetics studies is crucial to enable technology development for process design. Kinetic models have been used for stability of vitamins at HPS (Verbeyst et al., 2010; Verbeyst et al., 2011). The Weibull model and its variations enable to fit experimental data regardless of the reaction order. This model has been used for modeling chemical reactions in various food systems (Manso et al., 2001; Corradini & Peleg, 2006). Therefore, the objective of this study is to determine the retention kinetics of CLA during high-pressure sterilization of enriched milk and to model this CLA retention with the Weibull distribution model.

7.2. Materials and methods

7.2.1. Milk rich in CLA

Milk rich in CLA was obtained as described in Chapter 3 (Section 3.2.5).

7.2.2. High-pressure sterilization (HPS) treatments

The HPS experiments were conducted as described in Chapter 6 (Section 6.2.3), with some modifications. The samples were preheated in an oil bath (2 min) at different temperatures ranging from 72 to 117˚C, considering the adiabatic heating of 3˚C per 100 MPa (Rasanayagam et al., 2003). After preheating, the tubes were transferred to a high pressure multivessel system (Apparatus U111 Unipress, Warszawa, Poland) (**Fig 6-3**). The high pressure unit was heated using a thermostat (Lauda Proline RP 855 Low Temperature, Lauda-Königshofen, Germany). Samples were compressed (~11 MPa s^{-1}) and held for 0, 5, 10, 15, 30, and 60 min. The holding time of 0 min was considered when isobaric and isothermal conditions were achieved. At the end of the holding time, samples were removed and cooled immediately with ice water. The experiments were conducted in triplicate.

7.2.3. CLA determination

CLA content was determined by gas chromatography using a 100-m SP-2560 fused-silica capillary column, adopting the methodology previously described in Chapter 3(Section 3.2.5).

7.2.4. Dissolved oxygen determination

The amount of dissolved oxygen was determined as desbribed in Chapter 5 (Section 5.2.6).

7.2.5. Modeling the CLA retention in milk treated by HPS

The retention of CLA in milk treated by HPS was analyzed using the Weibull model giving in equation (7.1). This model allows fitting kinetic data regardless of the reaction order (van Boekel, 2002).

$$\frac{CLA_o - CLA_t}{CLA_o - CLA_f} = exp^{\left(-\left(\frac{t}{\alpha}\right)^{\beta}\right)} \quad (7.1)$$

where CLA_o, CLA_t, and CLA_f are the concentrations of CLA (mg g^{-1} fat) initially, at a given time (t) and at the end, respectively; α is the scale parameter, which reciprocal represents the reaction rate constant (k); β is the shape parameter. In cases where β=1, the reaction is considered to follow a first-order model. The left side of equation (7.1) represents the CLA fraction, which indicates the retained amount of CLA at a given time divided by the minimum CLA retained at the experimental conditions.

The influences of pressure and temperature on the scale parameter were expressed by the Eyring-type and Arrhenius-type models, equations (7.2) and (7.3), respectively:

$$\frac{1}{\alpha} = \frac{1}{\alpha_P} \cdot exp^{\left(-\frac{\Delta V^{\neq}}{RT}(P - P_{ref})\right)} \quad (7.2)$$

$$\frac{1}{\alpha} = \frac{1}{\alpha_T} \cdot exp\left(\frac{-E_a}{R}\left(\frac{1}{T} - \frac{1}{T_{ref}}\right)\right) \quad (7.3)$$

where P is pressure (MPa); T is temperature (K); α_P and α_T (min) are scale parameters at a reference pressure (P_{ref}) and a reference temperature (T_{ref}), respectively; $\Delta V^{\neq}$ is the activation volume ($cm^3\ mol^{-1}$); E_a is the activation energy ($kJ\ mol^{-1}$) and R is the universal gas constant ($8.314\ J\ mol^{-1}\ K^{-1}$). The average values of the experimental pressures and temperatures were used as the P_{ref} and T_{ref}.

Once the influences of pressure and temperature were determined, equations (7.2) and (7.3) were individually incorporated into the Weibull model, yielding global kinetic equations (equations (7.4) and (7.5)). These equations describe the retention of CLA as a function of holding time and pressure at a constant temperature (equation (7.4)) and the retention of CLA as a function of holding time and temperature at a constant pressure (equation (7.5)).

$$\frac{CLA_o - CLA_t}{CLA_o - CLA_f} = exp\left[-\left(\frac{t}{\alpha_P} \cdot exp\left(\frac{-\Delta V^{\neq}}{R \cdot T}\left(P - P_{ref}\right)\right)\right)^{\beta_{avg}}\right]_T \quad (7.4)$$

$$\frac{CLA_o - CLA_t}{CLA_o - CLA_f} = exp\left[-\left(\frac{t}{\alpha_T} \cdot exp\left(\frac{-E_a}{R}\left(\frac{1}{T} - \frac{1}{T_{ref}}\right)\right)\right)^{\beta_{avg}}\right]_P \quad (7.5)$$

where β_{avg} is the average of the β values at the range of conditions studied. The relation between the kinetic parameters was evaluated using joint confidence region plots (Claeys et al., 2001).

At each experimental point, the k values calculated using equations (7.4) and (7.5) were designated as observable rate constants (k_{obs}). Then, the k_{pred} was

obtained by the Arrhenius-Eyring model (equation (7.6)) and the empirical model (equation (7.7)).

$$k = k_{ref} \cdot exp^{\left(-\frac{E_a}{R}\cdot\left(\frac{1}{T}-\frac{1}{T_r}\right)\right)} \cdot exp^{\left(\frac{-\Delta V^{\neq}}{RT}\cdot\left(P-P_{ref}\right)\right)} \quad (7.6)$$

$$\ln k = a + b \cdot \left(T - T_{ref}\right) + c \cdot \left(P - P_{ref}\right) + d \cdot \left(T - T_{ref}\right)^2 + e \cdot \left(P - P_{ref}\right)^2 \quad (7.7)$$

where k_{ref} is the reaction rate constant at reference pressure and temperature conditions and *a*, *b*, c, *d* and e are empirical parameters. All experimental data were obtained in triplicates using the same CLA-enriched milk. The non-linear regression analysis was performed using the Solver option in Excel Microsoft and the graphs were plotted using Sigmaplot software V11 for Windows (SPSS Inc., Chicago, IL, USA). The equations (7.6) and (7.7) were assessed by the coefficient of determination (R^2), the adjusted coefficient of determination (R^2_{adj}), standard error (*SE*), mean square error (MSE, equation (7.8)) and accuracy factor (A_f , equation (7.9)) where n is the number of data points.

$$MSE = \frac{\sum\left(k_{pred}-k_{obs}\right)^2}{2} \quad (7.8)$$

$$A_f = 10^{\frac{\sum \log\left(\frac{k_{pred}}{k_{obs}}\right)}{n}} \quad (7.9)$$

7.3. Results and discussion

7.3.1. Retention of CLA in milk treated by HPS

Fig 7-1 shows the normalized retained fraction of CLA as a function of holding time at various pressures and temperatures for milk treated by HPS. In general, the retained fraction of CLA followed an exponential behaviour that was well described by the Weibull equation. At a constant experimental temperature, the retention of CLA was enhanced as the pressure increased from 100 to 600 MPa.

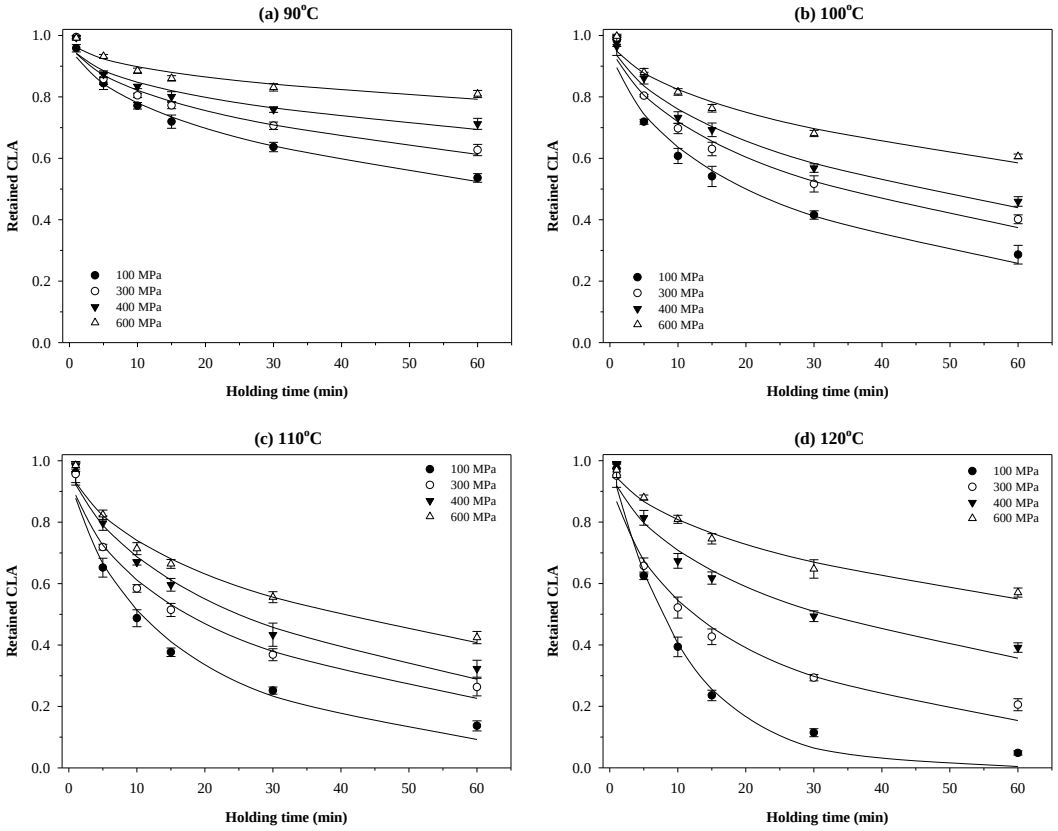

Fig 7-1. Retention of conjugated linoleic acid during high-pressure sterilization of milk at: (a) 90, (b) 100, (c), 110 and (d) 120˚C. Curves represent data obtained with the Weibull model (equation (7.4)).

A possible explanation for these results is that pressure delayed the homolysis of unsaturated fatty acids, resulting in a protective effect. Earlier reports by Neuman (1972) and Neuman & Amrich (1972) showed that thermal homolysis of azo compounds is delayed by pressure. In addition, the reported values of apparent $\Delta V^{\neq}$ of 0.3-13 cm^3 mol^{-1} for peroxides and azo compounds (Isaacs, 1981) seem to agree with the protective effect of pressure. In addition, samples treated at 100 MPa were exposed to higher temperatures during the preheating step (3˚C below the working temperature, e.g. for 120˚C, it was 117˚C) and therefore more thermal damage is expected compared with those samples pressurized at 600 MPa, where the preheating temperature was 18˚C below the working temperature, e.g. for 120˚C, it was 102˚C.

At 90˚C (**Fig 7-1a**), the normalized retained fraction of CLA after 15 min of holding time varied from 0.71 ± 0.02 to 0.86 ± 0.023, increasing with pressure from 100 to 600 MPa, respectively. **Fig 7-1a** also shows that CLA was retained (0.81 ± 0.01) at 90˚C and 600 MPa even at prolonged holding times of 60 min. At 100˚C, the retained CLA gradually decreased with holding time, obtaining retention values from 0.54-0.76 after 15 min of holding time (**Fig 7-1b)**. These values were lower than those obtained at 90˚C (**Fig 7-1a**). The decreasing tendency of the retained CLA was also observed at 110˚C (**Fig 7-1c**), where the retained fraction varied from 0.37 to 0.66 after 15 min of holding time. As the temperature increased to 120˚C (**Fig 7-1d**), the retained CLA continued to decrease in the pressure range of 100-300 MPa with retention values varying from 0.23 to 0.42 after 15 min of holding time. However, as the pressure increased to 600 MPa, the retained fraction of CLA was comparable to that obtained at 90˚C and 400 MPa after 15 min of holding time. But, the conditions of 90˚C and 400 MPa are not severe enough to achieve commercial sterilization while 120˚C and 600 MPa warrant commercial sterilization.

The high retention of CLA obtained at 600 MPa and 120˚C suggests a change in the reaction mechanism of CLA. Apart from oxidation, CLA in the presence of oxygen can be isomerised, leading to a change in the distribution of isomers without changing the total CLA content (Destaillats & Angers, 2005). The isomerization of CLA follows a free radical mechanism in which oxygen is consumed during the reaction (Destaillats et al., 2005). An attempt was made to measure the dissolved oxygen before and after HPS treatments (100-600 MPa and 120˚C), which was correlated with the retained CLA.

Fig 7-2 shows the converted fraction of dissolved oxygen against the converted fraction of CLA. The slopes obtained from the straight lines indicate the amount of oxygen needed to convert or oxidize one unit of CLA. At 100 and 300 MPa, the slopes were 0.41 and 0.64, respectively.

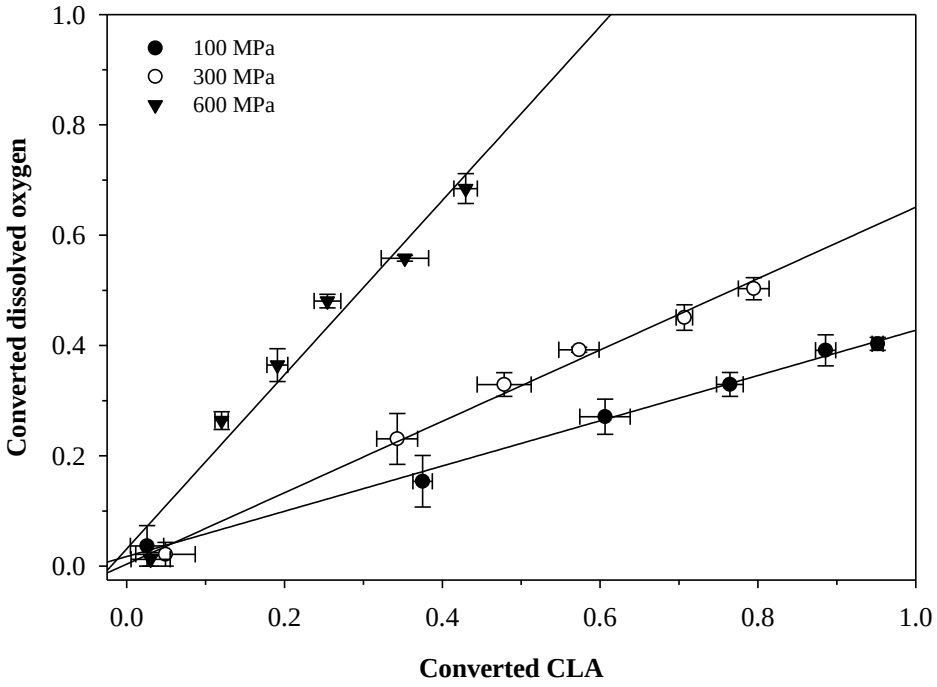

Fig 7-2. Consumed dissolved oxygen during the oxidation of conjugated linoleic acid in milk treated at 120˚C and various pressures. Full lines represent data obtained with linear regression.

An investigation on the oxidation of conjugated methyl esters in the presence of oxygen and reported that 1 molecule of oxygen was needed per conjugated double bond (ratio of 0.5 in the case of CLA) (Brimberg & Kamal-Eldin, 2003). Thus, the obtained slopes at 100 and 300 MPa and 120˚C suggest that CLA oxidized, following a similar mechanism to that observed at atmospheric pressure for conjugated methyl ester (Brimberg & Kamal-Eldin, 2003). In contrast, the slope obtained at 600 MPa was 1.57, indicating that more oxygen is needed to oxidize one unit of CLA. This experimental evidence suggests that CLA enriched milk treated at 600 MPa and 120˚C might undergo isomerisation rather than oxidation. This change in the reaction mechanism could be the reason for the high retention of CLA observed at 600 MPa and 120˚C. Quantification of the CLA isomers would confirm this hypothesis but it was beyond the scope of this study.

7.3.3. Modeling the CLA retention

The experimental data for the CLA retention of milk treated by HPS were fitted using equation (7.1) to obtain α and β parameters that are reported in **Table 7-1**.

Table 7-1. Weibull parameters for the retention of conjugated linoleic in high-pressure sterilized milk.

Pressure	**90˚C**				
(MPa)	**α**	**95% CI**	**β**	**95% CI**	**R^2**
100	135.9	28.1	0.53	0.05	0.974
300	242.8	32.4	0.51	0.0001	0.942
400	580.3	1.1	0.44	0.0002	0.906
600	1754.8	0.11	0.44	0.0001	0.892
Pressure	**100˚C**				
(MPa)	**α**	**95% CI**	**β**	**95% CI**	**R^2**
100	36.5	6.3	0.61	0.09	0.954
300	61.6	11.5	0.61	0.05	0.960
400	82.4	15.1	0.60	0.07	0.968
600	179.7	0.006	0.56	0.001	0.958
Pressure	**110˚C**				
(MPa)	**α**	**95% CI**	**β**	**95% CI**	**R^2**
100	17.7	2.3	0.71	0.11	0.967
300	31.5	0.44	0.61	0.08	0.966
400	43.4	5.6	0.66	0.08	0.968
600	71.7	0.02	0.61	0.0001	0.965
Pressure	**120˚C**				
(MPa)	**α**	**95% CI**	**β**	**95% CI**	**R^2**
100	11.1	0.98	1.1	0.15	0.982
300	22.1	3.4	0.62	0.11	0.956
400	57.2	10.7	0.61	0.09	0.957
600	146.3	0.005	0.57	0.0001	0.961

Parameters estimated using equation (7.1). 95% CI - 95% confidence interval; R^2 - coefficient of determination.

The scale parameter (α) was used to calculate the reaction rate constant by obtaining its reciprocal (1/α). The α parameter can be interpreted as the time at which ~37% of the CLA is retained (l-log cycle reduction = 63%) at a given pressure and temperature using equation (7.1) (Manso et al., 2001). For example, samples treated at 90˚C retained a high CLA content where prolonged holding times of 135-1755 min were needed to obtain ~37%, depending on the pressure

used (**Table 7-1**). On the other hand, only 11-146 min was needed to retain ~37% of the CLA at 120˚C and 100-600 MPa (**Table 7-1**). The shape parameter (β) values reported in **Table 7-1** were lower than 1, indicating that the retained CLA deviates from a first-order model. This behaviour is because CLA is distributed throughout triacylglycerols together with the other fatty acids present in milk and probably not all the CLA molecules react equally within the milk. In addition, β was not systematically affected by pressure and temperature so an average value (β_{avg}) was used for modeling. The parameters (α and β) shown in **Table 7-1** were much higher than their respective 95% confidence interval (CI95%), meaning that the calculated parameters were statistically different from zero. In addition, the R^2 values (>0.89, **Table 7-1**) indicate that the Weibull model can be used to represent the retention of CLA at tested conditions.

Pressure and temperature affected the α parameter exponentially and therefore equations (7.2) and (7.3) were used, considering the Eyring-model and the Arrhenius-model, respectively. **Fig 7-3a** shows the effect of pressure on the scale parameter, ln (1/α). Similarly, **Fig 7-3b** shows the effect of temperature on the scale parameter, ln (1/α). Both plots showed straight lines and their respective slopes are the $\Delta V^{\neq}$ and E_a. The $\Delta V^{\neq}$ values reported in **Table 7-2** are positive (9.34-16.42 $cm^3\ mol^{-1}$), indicating that pressure delayed the oxidation of CLA and consequently enhanced its retention. These positive $\Delta V^{\neq}$ values are consistent with those values reported for thermal homolysis (Isaacs, 1981) as discussed in section 7.3.1 of this chapter.

The E_a values also reported in **Table 7-2** varied from 99 to 187 kJ mol^{-1} at pressures of 100-600 MPa. The non-isothermal oxidation of anhydrous milk fat with different concentrations of CLA (7-32 mg CLA g^{-1} fat) showed E_a values within the same range (87-146 kJ mol^{-1}) (Chapter 4). Interestingly, the E_a values increased with an increase of pressure from 300 to 600 MPa (**Table 7-2**).

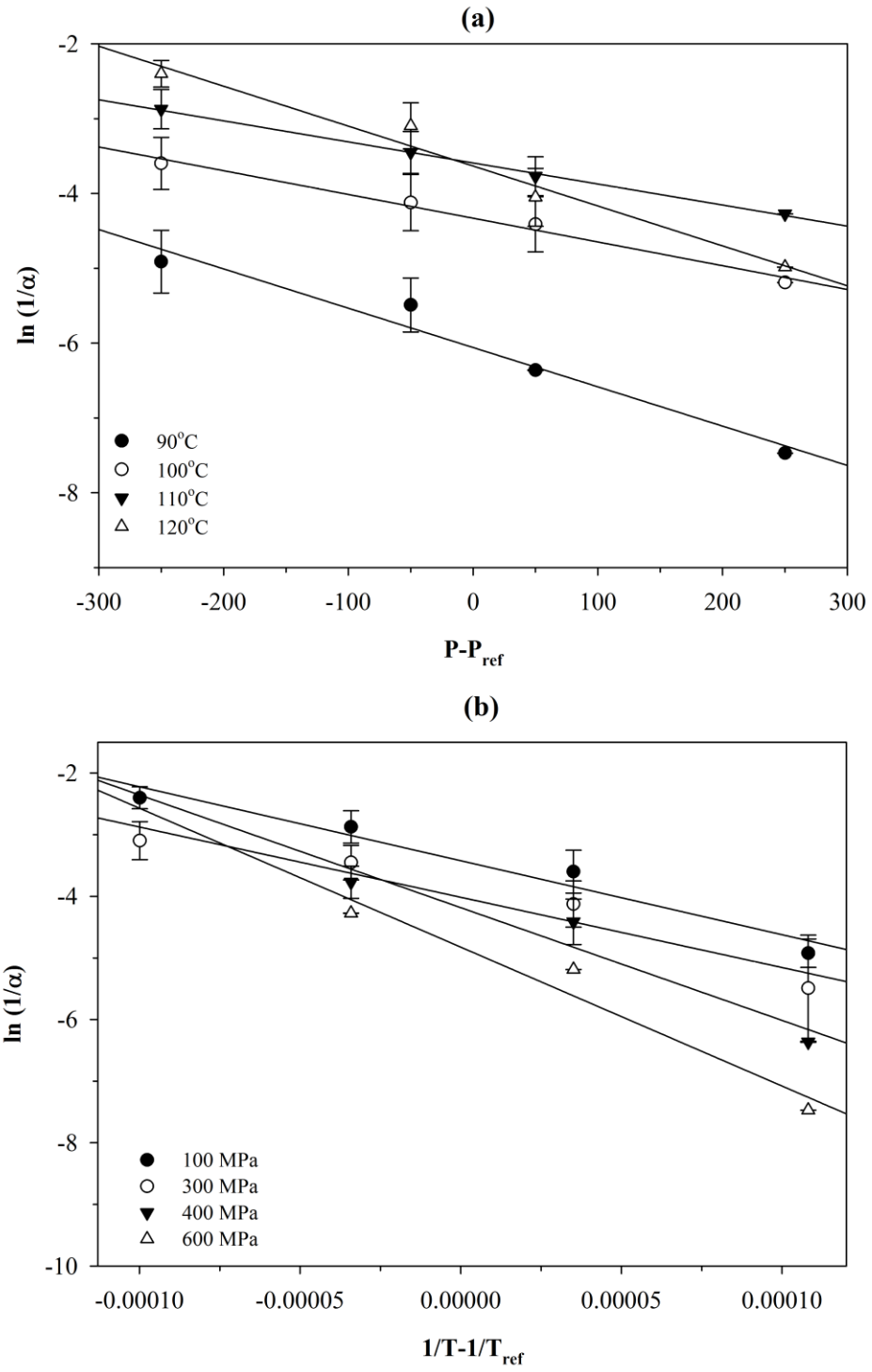

Fig 7-3. Effect of (a) pressure and (b) temperature on the scale parameter for the retention of conjugated linoleic acid in milk treated at high-pressure sterilization. The error bars correspond to 95% confidence interval. A reference pressure of 350 MPa and a reference temperature of 105˚C were used.

Statistically, k_T, k_p, E_a and $\Delta V^{\neq}$ were different from zero (CI95%) and therefore can be used for predictions. An increase in the E_a value due to pressure could be interpretated as a protective effect since more work in the form of energy is needed to overcome the energetic barrier imposed in the milk system. Likewise, an antagonist effect could be considered when the E_a decrease with pressure.

Table 7-2. Parameters of the Eyring-type and Arrhenius-type models for the retention of conjugated linoleic acid in milk treated at high-pressure sterilization conditions.

Temperature (°C)	**k_T (min^{-1})**	**CI95%**	**$\Delta V^{\neq}$ (cm^{-3} mol^{-1})**	**95% CI**	**R^2**
90	0.021	0.0005	15.17	4.51	0.964
100	0.013	0.004	9.34	3.72	0.987
110	0.028	0.009	9.61	2.95	0.972
120	0.026	0.008	16.42	4.32	0.991
Pressure (MPa)	**k_P**	**CI95%**	**E_a (kJ mol^{-1})**	**95% CI**	**R^2**
100	0.032	0.001	99.8	12.53	0.958
300	0.018	0.005	94.7	15.91	0.934
400	0.015	0.010	152.2	21.72	0.931
600	0.008	0.001	187.4	23.13	0.950

k_T is the constant reaction rate at a reference temperature; k_p is the constant reaction rate at a reference pressure; $\Delta V^{\neq}$ is the activation volume; E_a is the activation energy; 95% CI is the 95% confidence interval; R^2 is the coefficient of determination.

For the global fitting, the kinetic parameters (k_p and $\Delta V^{\neq}$) obtained with the Eyring-type model were incorporated into equation (7.1) as initial input values and these parameters were then recalculated by non-linear regression analysis. Similarly, k_T and E_a values obtained with the Arrhenius-type model were incorporated into equation (7.1) as initial input values and these parameters were then recalculated by non-linear regression analysis. At constant temperature, more than 96% (R^2>0.96) of the variability was explained by the Eyring model with the parameters shown in **Table 7-3**. On the other hand, at a constant pressure, the Arrhenius model did explain more than 87% of the variability (R^2). **Table 7-3** shows the estimated parameters (k_p, $\Delta V^{\neq}$, k_T and E_a) with the highest probability of being correct according to their 95% confidence interval. Each pair of

parameters (k_p and $\Delta V^{\neq}$ or k_T and E_a) has some degree of correlation since they were estimated simultaneously with the same equation.

Table 7-3. Global fitting for the retention of conjugated linoleic acid in milk treated at high-pressure sterilization conditions.

Parameter	**Eyring-type model (Eq. (7.4))**			
	90˚C	**100˚C**	**110˚C**	**120˚C**
k_P	0.003 ± 0.006	0.011 ± 0.002	0.003 ± 0.006	0.003 ± 0.006
$\Delta V^{\neq}$	10.88 ± 3.11	9.05 ± 2.64	10.88 ± 3.11	10.88 ± 3.11
β	0.491 ± 0.001	0.61 ± 0.02	0.49 ± 0.001	0.49 ± 0.001
R^2	0.968	0.964	0.968	0.968
R^2_{adj}	0.967	0.963	0.967	0.967
Parameter	**Arrhenius-type model (Eq. (7.5))**			
	100 MPa	**300 MPa**	**400 MPa**	**600 MPa**
k_T	0.036 ± 0.004	0.019 ± 0.003	0.012 ± 0.002	0.005 ± 0.006
E_a	75.56 ± 9.06	74.19 ± 8.08	54.64 ± 7.51	55.53 ± 7.41
β	0.71 ± 0.02	0.61 ± 0.04	0.64 ± 0.02	0.56 ± 0.001
R^2	0.953	0.956	0.913	0.878
R^2_{adj}	0.952	0.955	0.907	0.865

The error was calculated from the 95% confidence interval. E_a – activation energy calculated (kJ mol^{-1}); k_T and k_p – reaction rate constants at a reference temperature and pressure (min^{-1}), respectively; $\Delta V^{\#}$ – activation volume (cm^3 mol^{-1}); R^2 - coefficient of determination; R^2_{adj} – adjusted coefficient of determination.

A method that allows quantifying the degree of correlation between parameters is the 90% join confidence region shown in **Fig 7-4**. The joint confidence regions provide useful information on the uncertainty associated with each pair of estimates. **Fig 7-4a** shows the joint confidence region for the estimated parameters of k_p and $\Delta V^{\neq}$. The ellipses were constructed by plotting β values reported in **Table 7-3** at 0.49, 0.61, 0.65 and 0.69 for 90, 100, 110 and 120˚C, respectively. Overall, the 90% confidence region did not overlap, indicating that the parameters estimated were statistically different within the temperatures range investigated. The confidence region for those parameters estimated at 90˚C showed a squeezed-ellipse shape. In this case, the regression analysis identified the combined effect of the two parameters, but their individual effects were not precisely estimated. The confidence regions obtained for the pair k_T and E_a are shown in **Fig 7-4b**. The confidence regions were constructed by

fixing β at 0.71, 0.61, 0.61 and 0.56 for 100, 300, 400 and 600 MPa, respectively, using the non-linear regression. The confidence regions did not overlap and the parameters estimated showed a strong correlation for 400 and 600 MPa.

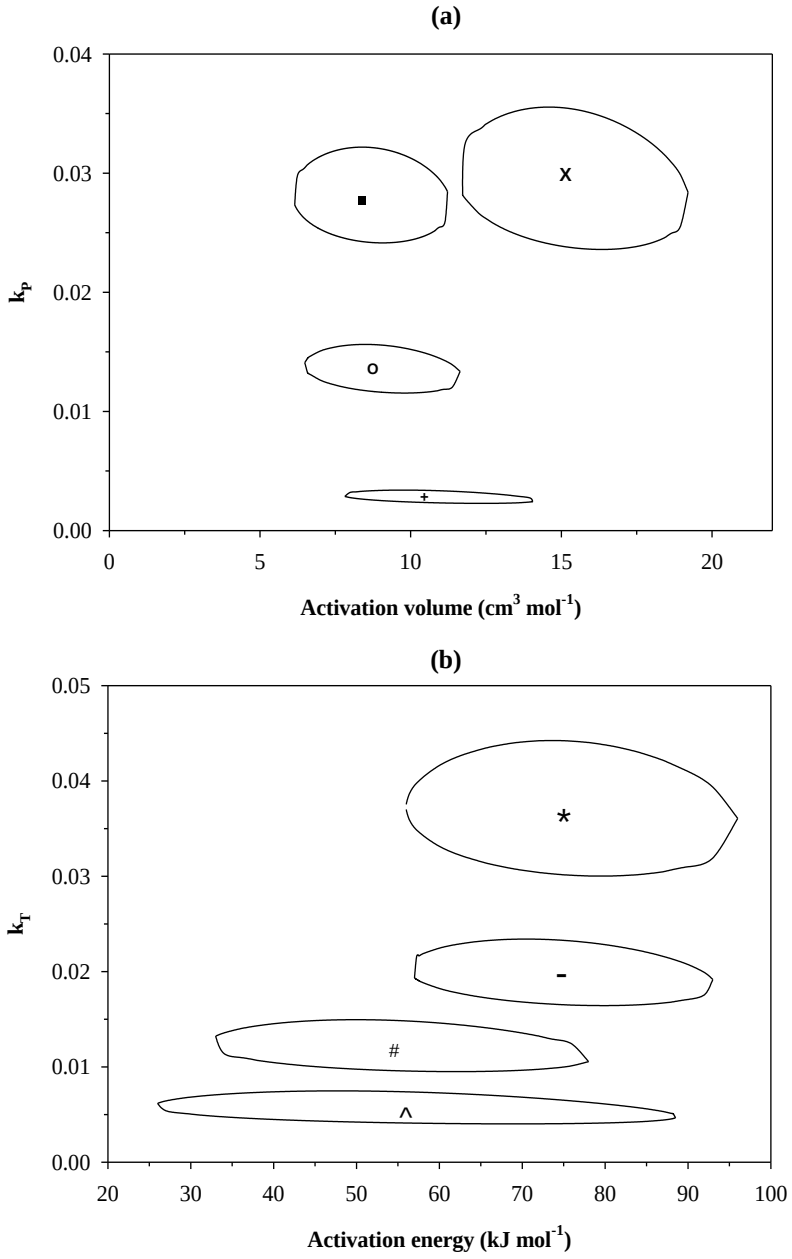

Fig 7-4. Joint confidence regions (90%) for the retention of conjugated linoleic acid in milk treated at high-pressure sterilization conditions: (a) k_P (min^{-1}) vs $\Delta V^{\neq}$ (+: 90˚C; O: 100˚C; ■: 110˚C and X: 120˚C), and (b) k_T(min^{-1}) vs E_a (*: 100 MPa, -: 300 MPa, #: 400 MPa and ^: 600 MPa).

More experimental data points could reduce the strong correlation between the parameters. However, such a correlation was at conditions where the change in the reaction mechanism was observed. For each experimental point, the k values were calculated with equation (7.4). These k values were named observed k values (k_{obs}). Two models were used to obtain the k_{pred} values and their fitting parameters and performances are shown in **Table 7-4**. The empirical model resulted in smaller mean square difference (MSD) and A_f with higher R^2 values than those of the Arrhenius-Eyring model. Moreover, the k_{pred} values were plotted against the k_{obs} values for both models (**Fig 7-5**). The k_{pred} values obtained with the empirical model (**Fig 7-5a**) showed a narrow predicted band compared with the one obtained for the Arrhenius-Eyring model (**Fig 7-5b**). Thus, the empirical equation (7.7) was further used to model the *k* values within the experimental damin.

Table 7-4. Estimated parameters for the rate constant values for the retention of conjugated linoleic acid in milk treated at high-pressure sterilization conditions.

Arrhenius-Eyring		**Empirical model**	
Parameter	**Value ± CI95%**	**Parameter**	**Value ± CI95%**
k_{ref}	0.014 ± 0.003	a	-3.84 ± 0.0001
E_a	69.64 ± 16.81	b	0.077 ± 0.0001
$\Delta V^{\neq}$	11.72 ± 2.57	c	-0.0032 ± 0.0001
R^2	0.945	d	-0.0038 ± 0.0001
MSD	0.00022	e	$1x10^{-14} \pm 5x10^{-8}$
A_f	0.57	R^2	0.987
		MSD	0.00019
		A_f	0.35

CI95% - 95% confidence interval. E_a – activation energy; and k_{ref}– reaction rate constant at a reference temperature and pressure; $\Delta V^{\#}$ – activation volume; a, b, c, d and e – regression coefficients of equation (7.7); R^2 - coefficient of determination; MSD – mean square difference; A_f – accuracy factor.

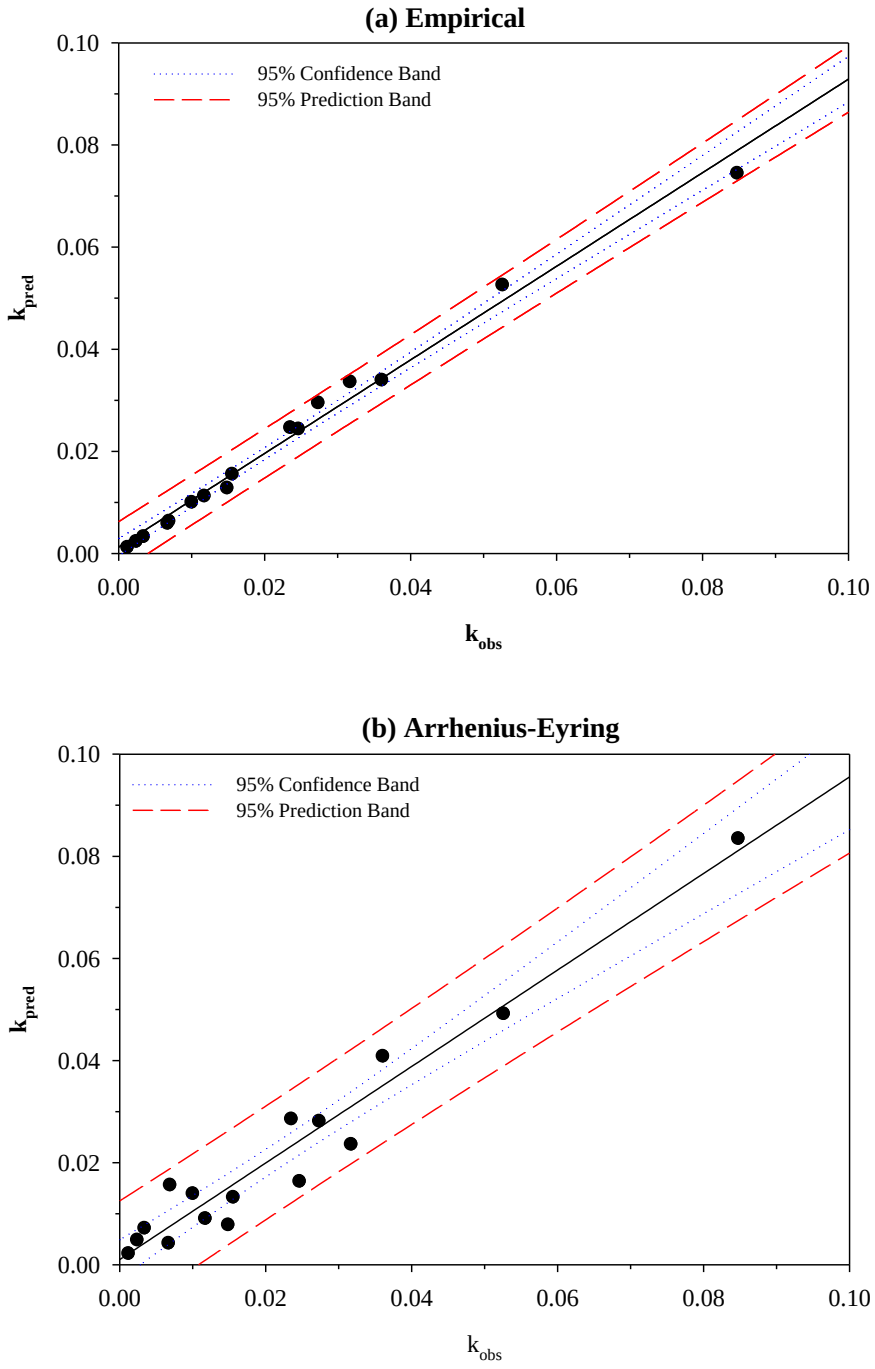

Fig 7-5. Correlation between observed *k* values (k_{obs}) and predicted *k* values (k_{pred}) for the retention of conjugated linoleic acid in milk treated at high-pressure sterilization conditions: (a) k_{pred} obtained with the polynomial model (equation (7.7)), and (b) k_{pred} obtained with the Arrhenius-Eyring model (equation (7.6)). Blue lines represent the 95% confidence band. Red lines represent the 95% prediction band.

7.3.5. Pressure-Temperature diagrams

The combined effect of pressure and temperature on the retention of CLA is illustrated through the pressure-temperature diagrams (**Fig 7-6**). The contour lines are the combinations of pressure and temperature that lead to a certain retention of CLA after holding time of 3 min (**Fig 7-6a**) and 10 min (**Fig 7-6b**). The contour lines that bent to the right can be interpreted as a change in the reaction mechanism. These diagrams can be used for engineering sterile milk beverages rich in CLA. For instance, processing conditions of 120˚C and 600 MPa for 3 min retained more than 80% of the CLA (**Fig 7-6a**). This retention is comparable to that obtained with UHT, an equivalent thermal process (Chapter 5, Section).

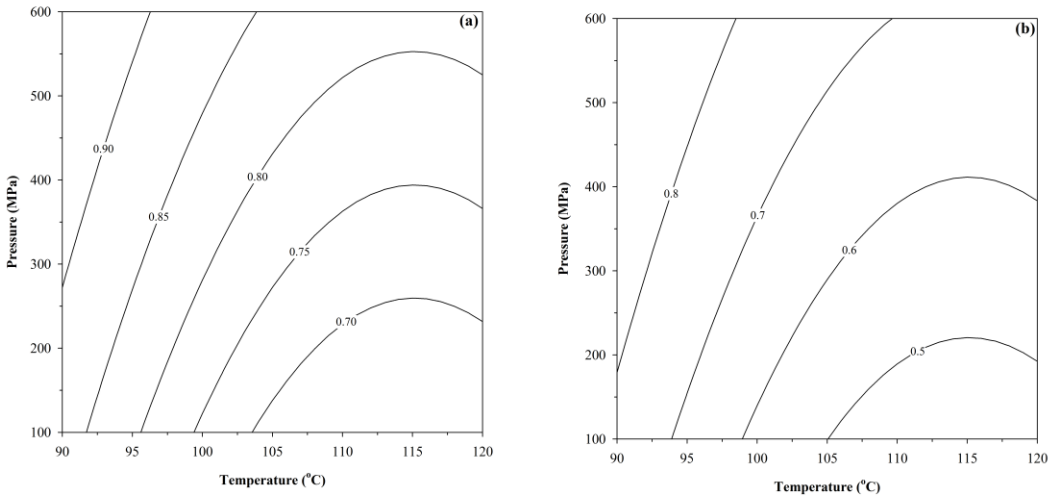

Fig 7-6. Pressure-temperature diagrams for the normalized retention of conjugated linoleic acid in milk treated by high-pressure sterilization conditions at holding times of: (a) 3 min, and (b) 10 min.

7.4. Conclusions

The retention of CLA was well described by the Weibull model in a pressure and temperature span of 100-600 MPa and 90-120˚C, respectively. At each evaluated temperature, pressure enhanced the retention of CLA, yielding positive values of activation volume (8-15 cm^3 mol^{-1}). In addition, the analysis of dissolved oxygen suggested that the isomerization of CLA was the dominant

reaction mechanism at 120˚C and 600 MPa, enhancing its retention. Pressure-temperature diagrams were also built for the retention of CLA in milk treated by HPS at different holding times, which can be used to minimize undesirable reactions by manipulating processing conditions.

7.5. References

Bell, J. A., Griinari, J. M., & Kennelly, J. J. (2006). effect of safflower oil, flaxseed oil, monensin, and vitamin E on concentration of conjugated linoleic acid in bovine milk fat. *Journal of dairy science*, 89(2), 733-748.

Bermudez-Aguirre, D., & Barbosa-Canovas, G. V. (2011). An update on high hydrostatic pressure, from the laboratory to industrial applications. *Food Engineering Reviews, 3(1),* 44-61.

Brimberg, U. L., & Kamal-Eldin, A. (2003). On the kinetics of the autoxidation of fats: substrates with conjugated double bonds. *European Journal of Lipid Science and Technology, 105(1),* 17-22.

Campbell, W., Drake, M. A., & Larick, D. K. (2003). The impact of fortification with conjugated linoleic acid (CLA) on the quality of fluid milk. *Journal of Dairy Science, 86(1),* 43-51.

Claeys, W. L., Ludikhuyze, L. R., & Hendrickx, M. E. (2001). Formation kinetics of hydroxymethylfurfural, lactulose and furosine in milk heated under isothermal and non-isothermal conditions. *Journal of Dairy Research, 68(2),* 287-201.

Corradini, M. G., and Peleg, M. (2004). A model of non-isothermal degradation of nutrients, pigments and enzymes. *Journal of the Science of Food and Agricultural,. 84(3),* 217-226.

Destaillats, F., & Angers, P. (2005). Thermally induced formation of conjugated isomers of linoleic acid. *European Journal of Lipid Science and Technology, 107(3),* 167-172.

Destaillats, F., Japiot, C., Chouinard, P. Y., & Angers , P. (2005). Rearrangement of rumenic acid in ruminant fats: a marker of thermal treatment. *Journal of Dairy Science, 88(5),* 1631–1635.

Herzallah, S. M., Humeid, M. A., & Al-Ismai, K. M. (2005). Effect of heating and processing methods of milk and dairy products on conjugated linoleic acid and trans fatty acid isomer content. *Journal of Dairy Science, 88(4),* 1301–1310.

Hillbrick, G., & Augustin, M. (2002). Milkfat characteristics and functionality: opportunities for improvement. *Australian Journal of Dairy Technology, 57(1),* 45-51.

Isaacs, N. S. (1981). *Liquid phase high pressure chemistry.* Toronto: John Wiley.

Juliano, P., Bilbao-Sáinz, C., Koutchma, T., Balasubramaniam, V. M., Clark, S., Stewart, C. M., et al. (2012). Shelf-stable egg-based products processed by high pressure thermal sterilization. *Food Engineeering Reviews, 4(1),* 55-67.

Luna, P., De la Fuente, M. A., Salvador, D & Marquez-Ruiz, G. (2007). Differences in oxidation kinetics between conjugated and non-conjugated methyl linoleate. *Lipids, 42(12),* 1085-1092.

Manso, M.C., Oliveira, F.R., Oliveira, J.C., & Frias, J.M. 2001. Modelling ascorbic acid thermal degradation and browning in orange juice under aerobic conditions. *International Journal of Food Science and Technology, 36(3),* 303-312.

Mujica-Paz, H., Valdez-Fragoso, A., Samson, C. T., Welti-Chanes, J., & Torres, J. (2011). High-pressure processing technologies for the pasteurization and sterilization of foods. *Food and Bioprocess Technology*, *4*(6), 969-985.

Neuman, R. C. (1972). pressure effects as mechanistic probes of organic radical reactions. *Accounts of Chemical Research*, *5*, 381-387.

Neuman, R. C., & Amrich, M. J. (1972). High pressure studies. X. Activation volumes for homolysis of single bonds. *Journal of the American Chemists's Society, 98(4),* 2730-2733.

Rasanayagam, V., Balasubramaniam, V. M., Ting, E., Sizer, C. E., Bush, C., & Anderson, C. (2003). compression heating of selected fatty food materials during high-pressure processing. *Journal of Food Science, 68(1),* 254–259.

van Boekel, M. J. (2002). On the use of the Weibull model to describe thermal inactivation of microbial vegetative cells. *International Journal of Food Microbiology, 74,* 139– 159.

Verbeyst, L., Oey, I., Van der Plancken, I., Hendrickx, M.E., & Van Loey, A. (2010). Kinetic study on the thermal and pressure degradation of anthocyanins in strawberries. *Food Chemistry, 123(2),* 269-274.

Verbeyst, L., Van Crombruggen, K., Van der Plancken, I., Hendrickx, M.E., & Van Loey, A. (2011). Anthocyanin degradation kinetics during thermal and high pressure treatments of raspberries. *Journal of Food Engineering, 105(3),* 513-521.

Villegas, C., Zhao, Y., & Curtis, J. M. (2010). Two methods for the separation of monounsaturated octadecenoic acid isomers. *Journal ofChromatography A,* 1217(5), 775-78.

Wang, Y., Jacome-Sosa, M. M., & Spencer, D. P. (2012). The role of ruminant trans fat as a potential nutraceutical in the prevention of cardiovascular disease. *Food Research International, 46(2),* 460–468.

Chapter 8

Role of antioxidants on the retention of conjugated linoleic acid in high-pressure sterilized milk[7]

8.1. Introduction

High-pressure sterilization (HPS) has become a valuable alternative to traditional thermal treatments (Juliano et al., 2012). This technology is also known as pressure-assisted thermal sterilization (PATS) and was first successfully developed to sterilize low acid foods (Sizer et al., 2002). Although sterilized products manufactured with HPS have not been commercialized yet, this technology has the potential to deliver a variety of novel products and its further implementation at industrial level is expected on short to medium term (De Vleeschouwer et al., 2010).

HPS offers three important technological advantages. First, the rise in the sample temperature due to adiabatic heating is used to reach the target or sterilization temperature, reducing the lack of temperature uniformity that takes place in traditional sterilization processes (Ting et al., 2010). Second, pressure reduces the interatomic distance, affecting interactions, for which bond energy is distance–dependent. Such interactions are van der Waals forces, electrostatic forces, hydrogen bonding and hydrophobic interactions of proteins. In contrast, covalent bonds are unlikely to be affected by pressure because its bonding distance can hardly be further compressed, as dicussed in Chapter 2. This has been the central hypothesis in preserving the biological activity of functional compounds, such as ascorbic acid (Oley et al., 2006), folates (Butz et al., 2004), vitamins (Matser et al., 2004), and anthocyanins (Verbeyst et al., 2010). Finally, the velocity of a chemical reaction can be increased or decreased by pressure, according to whether the molar volume of the intermediate state (activated complex) is less or more voluminous (Jenner, 2004). For a chemical reaction, the effect of pressure favours those reactions with negative reaction volume and those

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[7] *A version of this chapter has been submitted to Journal of Agricultural and Food Chemistry for consideration for publication*

reaction pathways with negative activation volume. Examples of chemical reactions related to food quality delayed by pressure are lactose isomerization (Moreno et al., 2003a), volatile formation in milk (Vazquez-Landaverde et al., 2006) and Maillard reaction (Hill et al., 1996; Moreno et al., 2003b).

Given the unique advantages of HPS, some authors have suggested that HPS can be used for producing superior quality products in cases where the traditional thermal treatments have failed to deliver high-quality products, such as egg-based and milk-based products, baby foods, desserts, gravies, soups and sauces (Heinz & Buckow, 2010; Juliano et al., 2012).

In an effort to develop high-value milk products, various investigations have highlighted the health benefits of conjugated linoleic acid (CLA), a bioactive component naturally found in milk fat (Cook & Pariza, 1998; Park, 2009). Unfortunately, CLA in milk suffers significant losses during ultra-high-temperature (UHT) as discussed in Chapter 5 and reported by Campbell et al. (2003) and Herzallah et al. (2005). In Chapter 6, the effect of PATS on the retention of CLA in milk was studied and it was found that CLA was retained up to 90% when catechin, a potent antioxidant, was added (1g per kg of milk), regardless of the processing conditions used (60-120˚C and 100-600 MPa) (see Chapter 6). These findings suggest that the use of antioxidants can enhance the retention of CLA in milk. However, their effectiveness and the way in which they capture free radicals or react with the dissolved oxygen to retard the free radical reactions have not been studied under HPS conditions. Understanding the antioxidant mechanisms under HPS conditions is important for product development and process design. Thus, the objective of this study was to evaluate the combined effect of HPS and the use of 7 different antioxidants on the retention of CLA in milk.

8.2. Materials and methods

8.2.1. Chemicals

Chloroform, methanol, hexane, sodium chloride (NaCl), barium chloride ($BaCl_2$), ferrous sulfate ($FeSO_4$), hydrochloric acid (HCl), ammonium thiocyanate (NH_4SCN), zinc acetate dehydrate, phosphotungstic acid, glacial acetic acid, cymene hydroperoxide, ascorbic acid (AA), caffeic acid (CAF), catechin (CAT), gallic acid (GA), *p*-coumaric acid (CUM), L-cysteine (Cyt), and tannic acid (TAN) were purchased from Sigma Aldrich (St. Louis, MO, USA). Methylacetate, sodium methoxide, methyl heptadecanoate (C17:0 #51633, purity of 99.5%) were purchased from Fluka Chemical Corp (Milwaukee, WI, USA). Oxygen and nitrogen (dry 99% pure) were purchased from Praxair Inc. (Edmonton, AB, Canada).

8.2.2. Obtaining CLA-enriched milk and sample preparation

Milk rich in CLA was saturated with oxygen to evaluate the effectiveness of different antioxidants at HPS conditions. The CLA-enriched milk was obtained as reported in Section 3.2.1 of Chapter 3. Prior to the HPS experiments, the CLA-enriched milk was thawed with running water at room temperature (~25˚C). Then, oxygen was bubbled for 45 min through the CLA-enriched milk at room temperature to ensure it is saturated. After that, 500 mg of either CAT, Cyst, AA, TAN, GA, CAF or CUM was added per kg of untreated milk. The antioxidants were dissolved using a bench homogenizer Diax 900 (Rose Scientific Ltd., Edmonton, AB, Canada).

8.2.3. High pressure sterilization treatments

HPS experiments were carried out as described in Chapter 6 (section 6.2.3). Briefly, the samples were preheated in an oil bath at 102˚C. After 1 min, the pre-heated samples were transferred to high pressure vessels already heated at 120˚C. Then, the samples were pressurized to 600 MPa at a rate of ~10 MPa/s. Once 600 MPa was reached, the samples were held for 1, 5, 10 and 15 min. For the control treatments, samples of raw CLA-milk in closed test tubes (Cryogenic

vial, Fisher Scientific, Edmonton, AB, Canada) were pre-heated at 102˚C and subsequently transferred to an oil bath at 120˚C to imitate the HPS experimental run. For simplicity, the control treatments are referred to samples treated at 0.1 MPa. HPS-treated and control samples were kept at -18°C until further analysis. The CLA was expressed in terms of normalized retained fraction. The experiments were conducted in triplicate.

8.2.4. Analytical determinations

CLA and fatty acid determination – CLA content and fatty acid composition were analysed by gas-chromatography (GC) according to the methodology described in Chapter 3 (Section 3.2.5).

Dissolved oxygen – The dissolved oxygen (DO_2) in milk was measured as described in Chapter 5 (Section 5.2.6).

Total phonolic content – The Folin-Ciocalteau method was used to determine the total phenolic content as reported elsewhere (Singh & Saldaña, 2011).

8.3. Results and discussion

CLA-enriched milk – **Fig 8-1a** shows the changes in CLA-content treated at sterilization conditions (0.1 MPa/120˚C or 600 MPa/120˚C up to 15 min). At 0.1 MPa, the retained fraction of CLA was 44 ± 2 and 35 ± 2 after 10 and 15 min, respectively. Similarly, our previous investigation on CLA-enriched milk treated for 14 min (4 min of pre-heating and loading time and 10 min of holding time at 120˚C and 0.1 MPa) showed that ~ 59% of CLA was retained where the initial CLA-enriched milk (28 mg g^{-1}) was different from the one used in this study (44 mg g^{-1}) (Chapter 6). In milk fortified with CLA (2% of CLA in the total fat), the retained CLA ranged from 79 to 95%, depending on the processing conditions at shorter treatment times (125-145˚C/2-20 s) (Campbell et al., 2003; Herzallah et al., 2005).

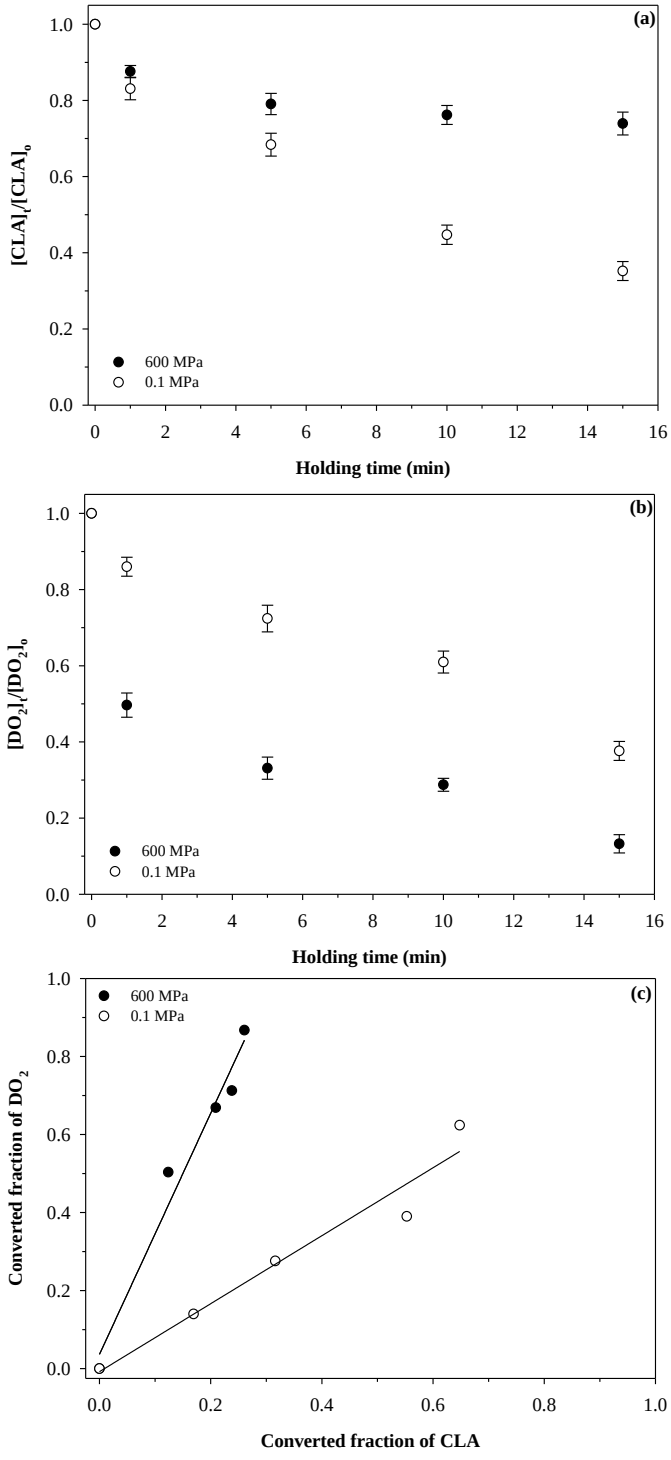

Fig 8-1. High pressure sterilization of CLA-enriched milk: changes in (a) CLA, (b) DO_2 and (c) converted fraction of DO_2 vs converted fraction of CLA. Samples were treated at 120˚C and either 600 MPa or 0.1 MPa.

CLA in milk was lost through oxidation due to the high temperature used (120˚C) that induces the formation of free radicals through thermolysis, where covalent bonds are broken. In addition, the dissolved oxygen reacts with reactive oxygen species to form oxygen radicals, such as hydroxyl (HO•), peroxyl (ROO•) and hydroperoxyl (HOO•). These radicals can easily act as initiators for the oxidation of CLA. This is in agreement with the changes in DO_2 observed in **Fig 8-1b**. At 0.1 MPa, the remaining fractions of DO_2 after 10 and 15 min were 0.61 ± 0.03 and 0.37 ± 0.03, respectively.

At 600 MPa, CLA was remarkably more stable compared to the control treatment (0.1 MPa), retaining 0.76 ± 0.02 and 0.74 ± 0.03 after 10 and 15 min, respectively. Based on these results, pressure influenced the reaction mechanism by which CLA is oxidized. Experimental data from the literature showed that thermal decomposition through homolysis of single bond and two-bond scission are delayed by the application of pressure (up to 395 MPa at 100˚C) (Neuman; 1972; Neuman & Amrich; 1972). A deceleration in the decomposition (homolysis) of unsaturated fatty acids might result in a protective effect of the CLA.

Fig 8-1b shows the DO_2 in CLA-enriched milk treated at HPS. The remaining fractions of DO_2 were 0.28 ± 0.02 and 0.13 ± 0.02 after 10 and 15 min, respectively, at 600 MPa and 120˚C (**Fig 8-1b**). The oxygen was consumed at higher rates compared with the rates at which CLA was oxidized (**Fig 8-1a**). The analysis of loss of CLA and oxygen consumption can be used to infer possible reaction mechanisms of CLA oxidation. A linear correlation was found between the oxygen consumed and the remaining or oxidized CLA (**Fig 8-1c**). The obtained slopes ($R^2 \geq 0.95$) represent the oxygen needed to oxidize one unit of CLA. For instance, 0.87 units of oxygen were needed to oxidize one unit of CLA in those samples treated at 0.1 MPa. A ratio of 0.5 (oxygen per CLA) was reported for the oxidation of conjugated methyl esters, suggesting that the oxidation of conjugated double bond systems starts with carbon-oxygen cross-

linking forming monomeric and cyclic hydroperoxides (Brimberg & Kamal-Eldin, 2003). At 0.1 MPa, it is safe to assume that CLA in naturally enriched milk oxidized through addition by Diels Alder-type reaction. On the other hand, the slope obtained at 600 MPa was 3.08, which is significantly higher than that obtained at 0.1 MPa (both samples were saturated with oxygen). During HPS, the CLA oxidized slowly and needed 3 units of oxygen. This indicates that the CLA could be isomerized upon pressure treatment. Isomerization only changes the isomer distribution of CLA but it does not change the total amount of CLA. There are two mechanisms for isomerization of CLA and the predominant mechanism depends on the oxygen content (Destaillats & Angers, 2005). The first mechanism consists of migration of double bond, known as sigmatropic molecular rearrangements, which is induced by temperature in the absence of oxygen (Destaillats & Angers, 2002). The second mechanism occurs in the presence of oxygen through free radical chain reaction, similar to the initiation step of autoxidation of linoleic acid (C18:2) (Destaillats et al., 2005). Interestingly, the initiation and propagation steps of free radical polymerization are indeed accelerated by pressure (Isaacs, 1981). Therefore, it is reasonable to hypothesize that isomerization of CLA through free radical mechanism is the predominant reaction at 600 MPa. Contrary, oxidation of CLA rather than isomerization is the predominant reaction at 0.1 MPa. To date, the effect of HPS on the isomer distribution of CLA has not been studied so far and it is beyond the scope of this study.

Ascorbic acid (AA) – Changes in CLA content in enriched milk added with AA and treated at sterilization conditions are shown in **Fig 8-2a**. The retained CLA contents after 10 and 15 min were 0.37 ± 0.02 and 0.32 ± 0.02 at 0.1 MPa, respectively. After 15 min, the retained CLA was similar to that in CLA-enriched milk without antioxidant addition (~0.35) (**Fig 8-1a**). The temperature used (120˚C) is rather severe and oxidized the ascorbic acid, losing its antioxidant activity (Smet et al., 2009).

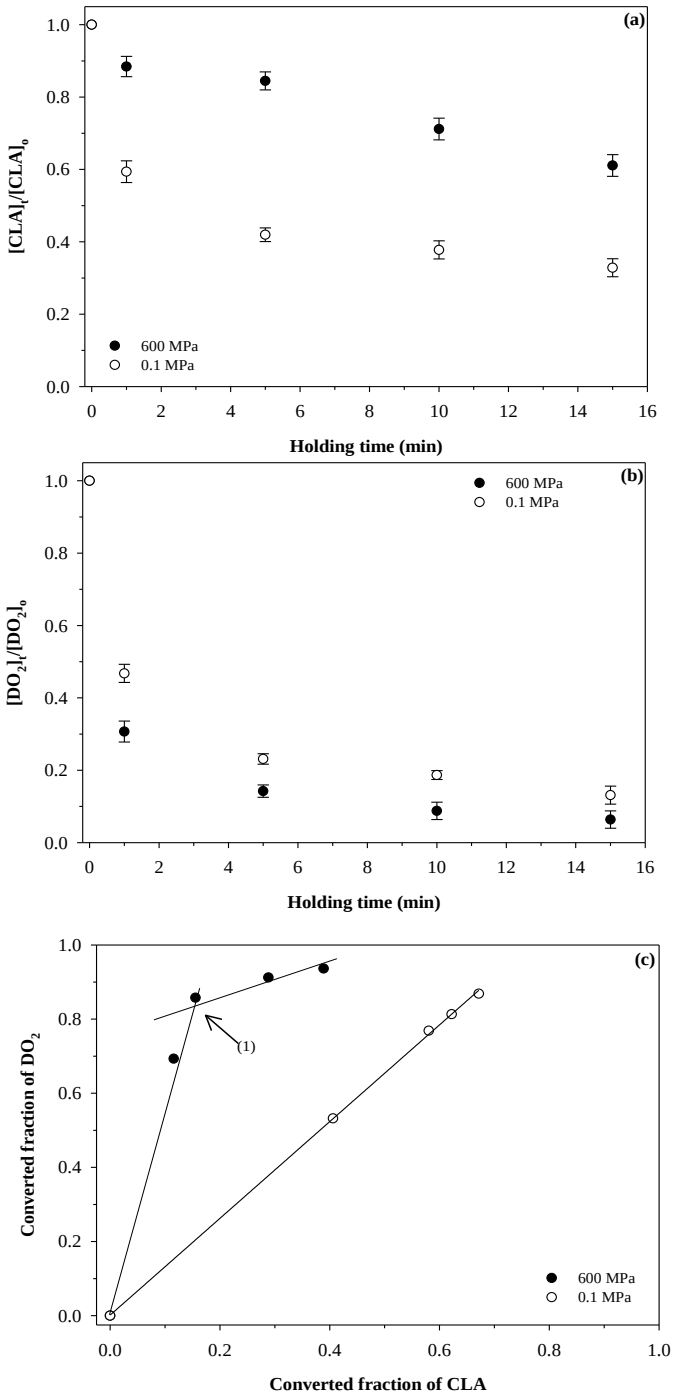

Fig 8-2. High pressure sterilization of CLA-enriched milk added with ascorbic acid: changes in (a) CLA, (b) DO_2 and (c) converted fraction of DO_2 vs converted fraction of CLA. Samples were treated at 120˚C and either 600 MPa or 0.1 MPa.

In UHT milk, ascorbic acid is lost by up to 50%, depending on the severity of the treatment time (135-150˚C) (Lindmark-Mansson & Akesson, 2000). On the other hand, the combination of AA and pressure at 600 MPa enhanced the retention of CLA compared with treatment at 0.1 MPa and milk without added antioxidant (0.71 ± 0.03 and 0.61 ± 0.01 after 10 and 15 min, respectively). One possible reason is that pressure protects the AA, which further reacts with DO_2 and free radicals. AA was used to inhibit the formation of aldehydes in milk treated at 655 MPa and 75˚C for 5 min (Vazquez-Landaverde & Qian, 2007). An investigation on the retention of AA in pressurized phosphate buffer systems showed that AA remained unchanged at moderate temperature (850 MPa and 50˚C) while at higher temperature (850 MPa and 80˚C), the degradation of AA became notorious. More importantly, AA can be used for enhancing the stability of folates in pressurized orange and tomato juices (Van den Broeck et al., 1998; Oley et al., 2006). Another investigation showed that the combination of 700 MPa and 110˚C accelerated the degradation rate of ascorbic acid up to 20 times in raspberries (Verbeyst et al., 2013).

The changes in DO_2 in milk with added AA are shown in **Fig 8-2b**. For those samples treated at 0.1 MPa, the DO_2 rapidly decreased (0.23 ± 0.01 remaining fraction) within 5 min of holding time. As the experiment proceeded, the remaining fraction of DO_2 was 0.18 ± 0.01 and 0.13 ± 0.02 after 10 and 15 min, respectively. In the case of HPS, the retained fraction of DO_2 was 0.08 ± 0.024 and 0.06 ± 0.022 after 10 and 15 min, respectively.

A close examination of the relationship between the consumed fraction of DO_2 and the oxidized fraction of CLA in milk with added AA is shown in **Fig 8-2c.** The slope obtained at 0.1 MPa (1.31, R^2 = 0.99) indicated that more oxygen was consumed in those samples with added AA compared with milk without an antioxidant treated at 0.1 MPa (**Fig 8-1c**). In the presence of oxygen, AA is converted to dehydroascorbic acid (DHA) by abstracting a proton, making it unstable (Choe & Min, 2006).

In HPS treated samples, an inflection point was observed (arrow 1), which indicates the existence of two different linear segments. Moreover, the inflection point was observed in all cases when anantioxidant was added to milk. For visibility, the segments are separated with arrows in **Figs 8-2c** to **8-8c**. In all cases, the start and end points of the linear segments were determined by first fitting a line with the five data points. Then, if the R^2 value for the line was less than 0.95, the first or last data point was not used and a new R^2 value was calculated as an approximation to detect the inflection point with at least three data points.

In **Fig 8-2c**, the first segment spans from 0 to 5 min of holding time (3 data points). Within this segment, ~ 85% of the oxygen was consumed and only ~15% of the CLA was oxidized. In the second segment (from 5 to 15 min of holding), the majority of the DO_2 has already been consumed, increasing from 85 to 93%, while the oxidized CLA increased from 15 to 39%. In the first segment, beginning of the holding time, the AA might be acting as a scavenging agent due to its relative high initial concentration (0.5 g kg^{-1}) (Yen et al., 2002). Moreover, the initial concentration of AA is 4 times higher than the initial molar concentration of oxygen ($[AA]_o$ = 2.8 mM and $[DO_2]_o$ = 0.68 mM). A molar ratio of at least 2 (AA/O_2) was reported to protect other compounds from oxidation (Oley et al., 2006). At the point where most of the DO_2 was consumed (segment two), the oxidation of CLA was evident. One possible reason is that the AA can be ionized when pressure is applied to form ascorbate, which is more reactive than AA. Both compounds oxidize to produce hydrogen peroxide that might trigger the late oxidation of CLA (segment two) (Chimi et al., 1991).

Caffeic acid (CAF), catechin (CAT,) gallic acid (GA), and *p-coumaric acid (CUM)* – The retention of CLA in milk added with different phenolic antioxidants are shown in **Figs 8-3a** to **8-6a**.

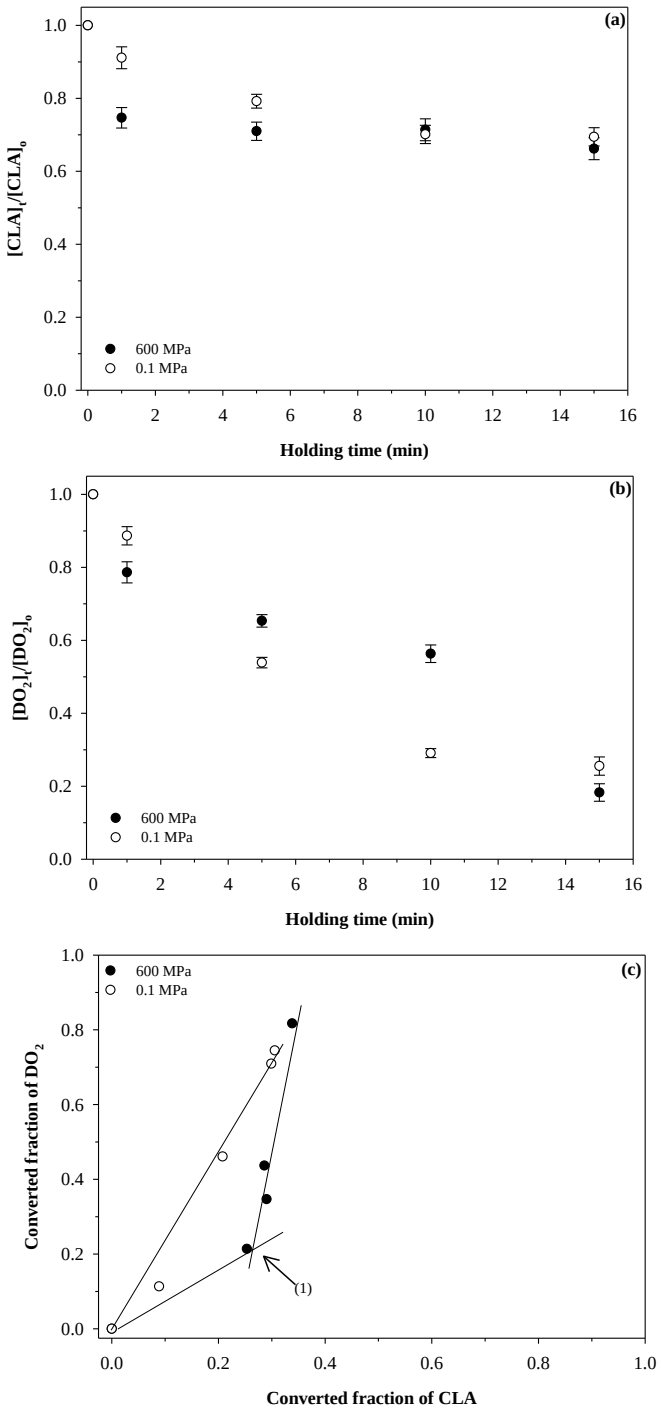

Fig 8-3. High pressure sterilization of CLA-enriched milk with added caffeic acid: changes in (a) CLA, (b) DO_2 and (c) converted fraction of DO_2 vs converted fraction of CLA. Samples were treated at 120˚C and either 600 MPa or 0.1 MPa.

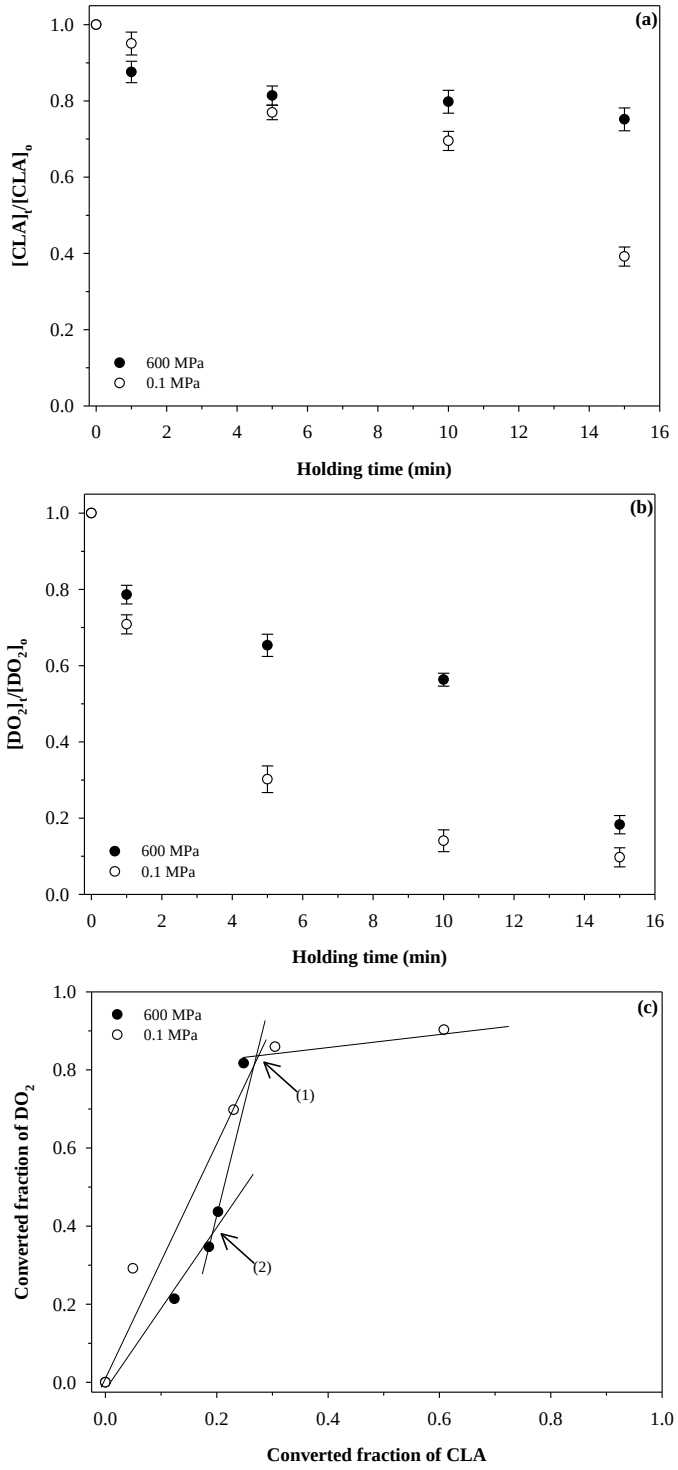

Fig 8-4. High pressure sterilization of CLA-enriched milk with added catechin: changes in (a) CLA, (b) DO_2 and (c) converted fraction of DO_2 vs converted fraction of CLA. Samples were treated at 120˚C and either 600 MPa or 0.1 MPa.

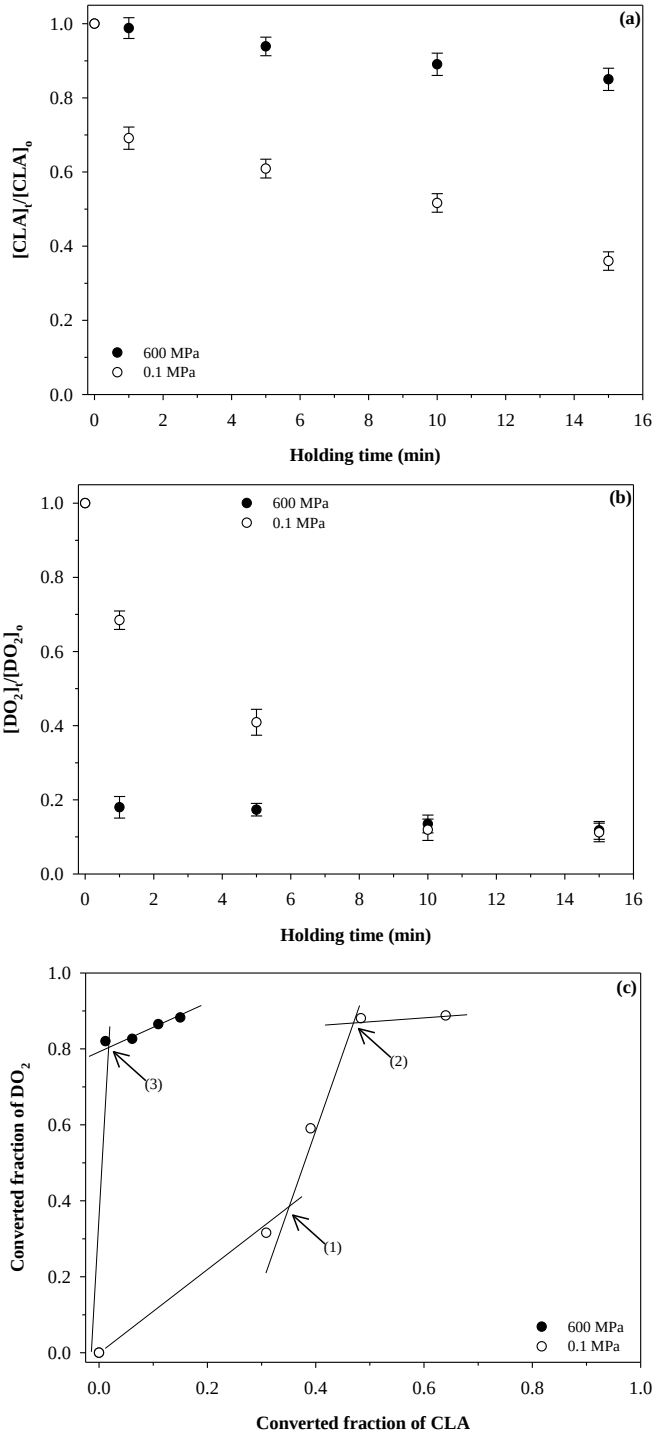

Fig 8-5. High pressure sterilization of CLA-enriched milk with added gallic acid: changes in (a) CLA, (b) DO_2 and (c) converted fraction of DO_2 vs converted fraction of CLA. Samples were treated at 120˚C and either 600 MPa or 0.1 MPa.

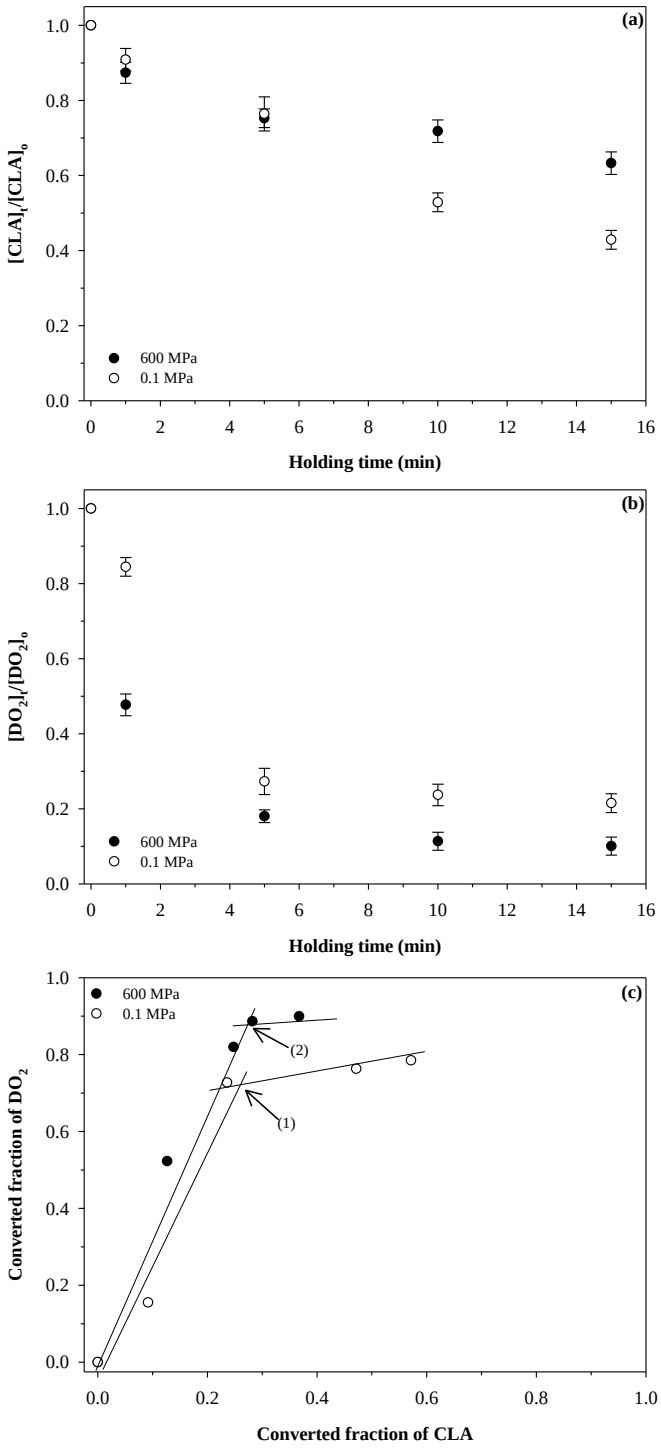

Fig 8-6. High pressure sterilization of CLA-enriched milk with added *p*-coumaric acid: changes in CLA (a), DO_2 (b) and converted fraction of DO_2 vs converted fraction of CLA. Samples were treated at 120˚C and either 600 MPa or 0.1 MPa.

The highest retention fraction of CLA in milk was obtained after adding CAF (0.69 ± 0.02), followed by CUM (0.42 ± 0.02), CAT (0.39 ± 0.03) and GA (0.35 ± 0.02) after 15 min of holding time at 0.1 MPa. On the other hand, after 15 min and 600 MPa, the highest retention fraction was obtained with GA (0.85 ± 0.03), followed by CAT (0.75 ± 0.03), CAF (0.66 ± 0.03) and CUM (0.63 ± 0.02). In general, phenolic antioxidants possess the ability to inhibit autoxidation of lipids by trapping intermediate radicals and quenching dissolved oxygen due to their low ionization potential (Hamann & Linton, 1974). **Figs 8-3b** to**8-6b** show the changes in DO_2 in milk with added phenolic antioxidants. After 15 min of holding time at 0.1 MPa, the consumption of DO_2 was larger in those samples with added CAT (0.09 ± 0.01), followed by GA (0.11 ± 0.02). After 15 min at 600 MPa, the consumption of DO_2 was larger in samples with added CUM (0.10 ± 0.02) and GA (0.11 ± 0.02).

The relationship between converted fraction of DO_2 and oxidized fraction of CLA in milk with added CAF is shown in **Fig 8-3c**. The slope obtained at 0.1 MPa was 2.51, indicating that more oxygen was needed to oxidize one unit of CLA compared with the samples without an antioxidant. This is in agreement with the remaining fraction of CLA (**Fig 8-3a**). In samples treated at HPS, two linear segments were observed (**Fig 8-3c**). The first segment spanned from 0-5 min of holding time with a slope value of 1.06, while the second segment spanned from 5-15 min with a slope value of 8.34. In the case of samples added with CAT, the relationship between converted fraction of DO_2 and oxidized fraction of CLA is shown in **Fig 8-4c**. Interestingly, two segments were observed in both treatments (0.1 MPa and 600 MPa). The inflection point at 0.1 MPa was observed at 10 min of holding time, indicating the existence of two linear segments.

The first segment spanned from 0-10 min with a slope value of 2.64. The second segment spanned from 10-15 min. This segment includes two data points and therefore the slope was not calculated. At 600 MPa, the inflexion point was observed at 5 min of holding time. The first segment only spanned two

experimental points while the second segment represented from 5-15 min of holding time. The slope value was 7.68, which indicates that a significant amount of oxygen was needed to oxidize CLA. For those samples with added GA, the relationship between converted fraction of DO_2 and oxidized fraction of CLA is presented in **Fig 8-5c**. At 0.1 MPa, two inflection points were observed, indicating the existence of three linear segments. The slope of the second segment, spanned from 1-10 min, was calculated (3.23). On the other hand, samples treated at HPS conditions showed one inflection point (arrow 3 in **Fig 8-5c**). The first segment covers the beginning of the holding time while the second segment includes 1-15 min of holding time with a slope value of 0.63. Finally, those samples added with CUM showed one inflexion point in each treatment (**Fig 8-6c**). At 0.1 MPa, the first segment was considered from 0-5 min of holding time with a slope value of 3.16 while the second segment was considered from 5-15 min with a slope of 0.16. When CUM was added into milk and treated at HPS conditions, two linear segments were observed from 0-5 and 5-15 min of holding time with slope values of 3.31 and 0.15, respectively.

Phenols are susceptible to ionization by pressure due to charge delocalization between the oxygen and aromatic ring, yielding molar activation volumes between -8 and -20 cm^3 mol^{-1} (Hamann & Linton, 1974). Phenols form hydrogen bonds with surrounding molecules. Free phenols, on the other hand, are likely to be ionized and the donated proton rapidly neutralizes the free radicals. During pressure treatment, free phenols are probably ionized and therefore their ability to quench oxygen is significantly enhanced. In HPS, the use of phenolic antioxidants not only enhances the retention of CLA but also quenches oxygen, which avoids isomerization and therefore protecting the biological activity of CLA.

Cysteine (Cyst) – The remaining CLA in samples with added Cyst is shown in **Fig 8-7a**. At 0.1 MPa, the retained fractions of CLA were 0.59 ± 0.02 and 0.56 ± 0.03 after 10 and 15 min of holding time, respectively.

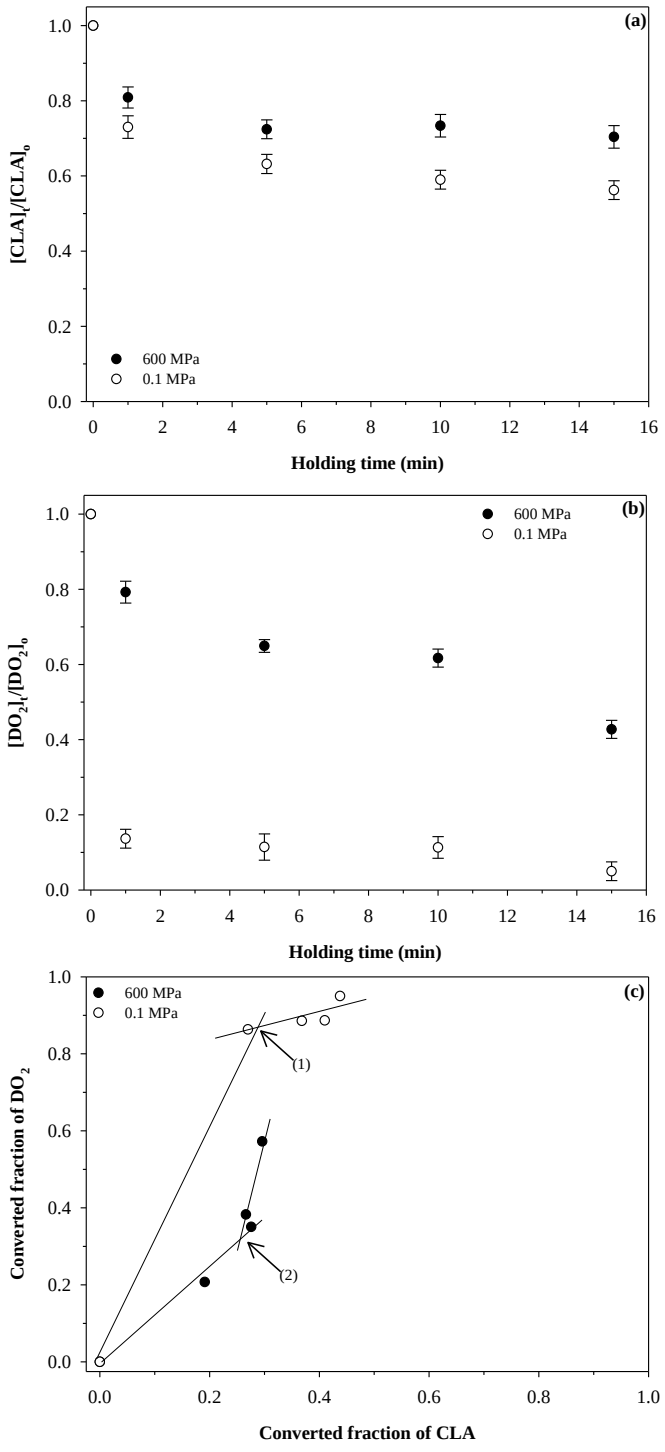

Fig 8-7. High pressure sterilization of CLA-enriched milk with added cysteine: changes in (a) CLA, (b) DO_2 and (c) converted fraction of DO_2 vs converted fraction of CLA. Samples were treated at 120˚C and either 600 MPa or 0.1 MPa.

An investigation on heated milk fat at 0.1 MPa (95˚C for several hours) showed that the addition of Cyst greatly extended the induction time of oxidation (Chen & Nawar, 1991). Another investigation reported that Cyst can be used to inhibit the formation of off-flavor compounds in UHT milk (Vazquez-Landaverde & Qian, 2007). At 600 MPa, the retained fraction of CLA was 0.73 ± 0.03 and 0.70 ± 0.03 after 10 and 15 min, respectively. The combination of Cyst and pressure yielded higher remaining fraction of CLA compared with the control treatment (0.1 MPa). Likewise, Cyst inhibited the formation of aldehydes and hydrogen sulfide in milk pressurized at 655 MPa and heated at 75˚C for 10 min (Vazquez-Landaverde & Qian, 2007).

On the other hand, the DO_2 rapidly decreased after 5 min of holding time at 0.1 MPa, resulting in values in the range of 0.11-0.05. At 600 MPa, the DO_2 fraction was considerable higher than that at 0.1 MPa with values 0.42 ± 0.02 after 15 min of holding time. In the presence of oxygen, Cyst reacts with hydroxyl radical further to form sulfonic acid (Choe & Min, 2006). This could be the reason for the substantial reduction in the DO_2 fraction observed at 0.1 MPa (**Fig 8-7b**).

In **Fig 8-7c**, the relationship between the converted fraction of DO_2 and oxidized fraction of CLA in milk with added Cyst is shown. An inflection point was observed, arrow (1), in samples treated at 0.1 MPa. At the point of inflection, ~86% of the DO_2 has been converted while approximately < 20% of the CLA has been oxidized. The slope of the first segment was not calculated since only two data points were located. The second segment spanned from 1 to 15 min of holding time yielded a slope value of 0.17, meaning that a small amount of oxygen was needed to oxidize CLA. Cyst has the ability to quench oxygen (Chen & Nawar, 1991). An investigation on the reactivity of the O_2 with amino acids showed that oxidation of amino acids was the predominant reaction rather than O_2 quenching (Michaeli & Feitelson, 1994).

In samples treated at 600 MPa, the existence of two linear segments was confirmed by the presence of an inflexion point (arrow 2). The first segment spanned from 0 to 5 min of holding time with a slope value of 1.24 while the slope of the second segment was not calculated because the data points did not fit a straight line. Cyst possesses a thiol group, which is believed to act as an antioxidant by donating hydrogen to neutralize free radicals (Khan et al., 2000). The ability of Cyst to quench free radicals depends upon the state of protonation of the functional group (Sánchez-Moreno et al., 1999). An investigation on the oxidation of milk fat with different amino acids added showed that non-protonated (NH_2) amino group, such as Cyst inhibited the oxidation (Kim et al., 2010). In HPS experiments, the reaction system suffers a temporary and reversible modification in the pH, which might change the reaction mechanism (Huppertz et al., 2006).

Tannic acid (TAN) – **Fig 8-8a** shows the remaining fraction of CLA in CLA-enriched milk with added TAN. At 0.1 MPa, the remaining fractions of CLA were 0.75 ± 0.02 and 0.64 ± 0.02 after 10 and 15 min, respectively. On the other hand, at 600 MPa, the retained fractions of CLA were 0.71 ± 0.03 and 0.62 ± 0.03 after 10 and 15 min, respectively. Interestingly, the retained fraction of CLA obtained in samples added with TAN was higher than those obtained in milk without an antioxidant and treated at 0.1 MPa. This is in agreement with the reports in the literature, indicating that TAN prevented lipid oxidation (Andrade et al., 2005; Kim et al., 2010).

Upon thermal treatment, TAN is hydrolyzed to form gallic acid and polygalloyls, which enhance the antioxidant capacity. An investigation on the oxidation of soybean oil showed that the addition of hydrolyzed TAN extended the oxidation induction period by up to 84% compared with the control treatment (Schamberger & Labuza, 2007). These authors thermally treated TAN (121˚C up to 60 min) and found that 15 min of thermal treatment was enough to hydrolyze TAN.

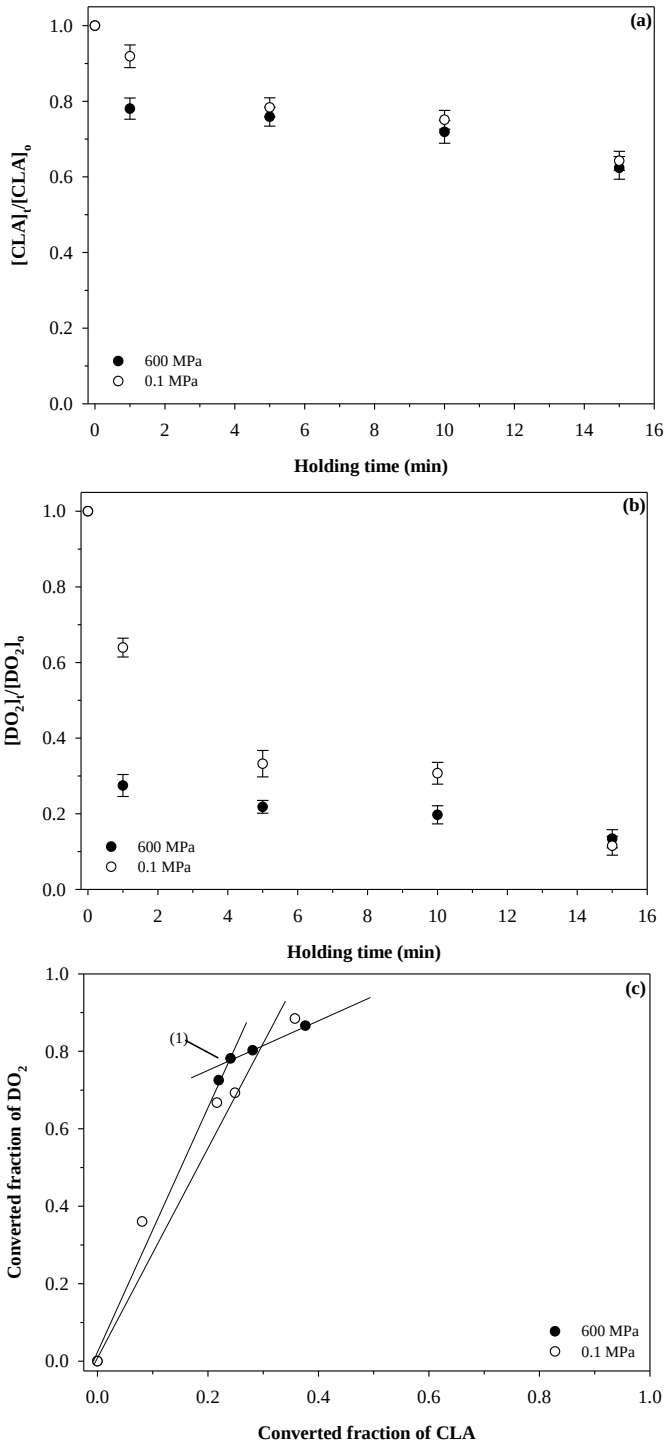

Fig 8-8. High pressure sterilization of CLA-enriched milk with added tannic acid: changes in (a) CLA, (b) DO_2 and (c) converted fraction of DO_2 vs converted fraction of CLA. Samples were treated at 120˚C and either 600 MPa or 0.1 MPa.

Fig 8-8b shows the remaining fraction of DO_2 in samples with added TAN and treated at sterilization conditions. At 0.1 MPa, the remaining DO_2 contents were 0.30 ± 0.02 and 0.11 ± 0.03 after 10 and 15 min, respectively. At 600 MPa, the remaining DO_2 contents were 0.19 ± 0.02 and 0.13 ± 0.02 after 10 and 15 min, respectively. The relationship between converted fractions of DO_2 and oxidized fractions of CLA is shown in **Fig 8-8c**. At 0.1 MPa, a straight line was obtained with a slope value of 2.3, indicating that more oxygen was needed to oxidize CLA compared with those samples without antioxidant (slope=0.87) treated at 0.1 MPa (**Fig 8-1c**). The ability of TAN to react with oxygen was demonstrated during the oxidation of ascorbate in the presence of copper where TAN not only reacted with oxygen but also chelated metal ions, delaying the oxidation process (Ohara et al., 2009). In HPS treated samples, an inflection point was observed (arrow 1), which indicates the existence of two different linear segments. The first segment spanned from 0 to 5 min with a slope value of 3.26 while the second segment spanned from 5 to 15 min of holding time with a slope value of 0.63. The existence of two linear segments suggests a change in the reaction mechanism induced by pressure in the presence of TAN. **Table 8.1** summarizes the retention of CLA and the slopes obtained in this Chapter.

Table 8-1. Summary of remaining CLA and slope values obtained after 15 min of treatment at 120˚C and either 600 or 0.1 MPa.

Antioxidant	Retained CLA*		Slope	
	600 MPa	0.1 MPa	600 MPa	0.1 MPa
Milk	0.74 ± 0.02	0.35 ± 0.02	3.08	0.87
Ascorbic acid	0.61 ± 0.02	0.32 ± 0.02	2.01	1.31
Caffeic acid	0.66 ± 0.03	0.69 ± 0.02	8.34	2.51
Catechin	0.75 ± 0.03	0.39 ± 0.03	7.68	2.64
Gallic acid	0.85 ± 0.03	0.35 ± 0.03	0.63	3.23
p-coumaric acid	0.63 ± 0.02	0.42 ± 0.02	3.31	3.16
Cysteine	0.70 ± 0.03	0.56 ± 0.03	1.24	0.17
Tannic acid	0.62 ± 0.03	0.64 ± 0.03	3.26	3.21

* The retained CLA was obtained after 15 min at 120˚C.

Different concentrations of catechin and gallic acid – An additional set of experiments was conducted using different concentrations of CAT and GA (0, 100, 200, 300 and 500 mg kg^{-1}, **Fig 8-9**). The samples were treated at 120˚C for 15 min at either 0.1 or 600 MPa. Catechin as well as gallic acid enhanced the retention of CLA at 600 MPa as opposed to the retention obtained at 0.1 MPa. In the case of milk with added CAT and treated at 0.1 MPa (**Fig 8-9a**), the retention of CLA was enhanced from 0.27 ± 0.02 to 0.44 ± 0.04 as the concentration increased up to 500 mg kg^{-1}. On the other hand, at 600 MPa, the retention of CLA was remarkably enhanced, reaching values of 0.82 ± 0.02 when 500 mg kg^{-1} of CAT was added. Similarly, the combined effect of 600 MPa and GA resulted in retention of 0.85 ± 0.03, which is considerably higher than that obtained at 0.1 MPa (0.41 ± 0.02). Catechin has been used for delaying the non-enzymatic browning in UHT milk (145˚C for 15 s) (Schamberger & Labuza, 2007).

It was demonstrated that a concentration of 0.1 mmole L^{-1} was enough to inhibit the browning of UHT milk during prolonged storage up to 36 days (Schamberger & Labuza, 2007). More importantly, consumer sensory analysis showed no difference with respect to the control samples (Schamberger & Labuza, 2007). In our study, a similar concentration of CAT was used (500 mg kg^{-1} ~ 0.17 mmole L^{-1}) and therefore changes in the sensory perceptions due to the addition of CAT are not expected; however, it needs to be confirmed requiring further research.

Possible reaction mechanisms of phenolic antioxidants – The use of phenolic antioxidants (500 mg kg^{-1}) in combination with high pressure yielded high retention of CLA (~0.85% in the case of GA) even at a prolonged holding time of 15 min. In general, the effectiveness of a given antioxidant is usually associated to the structure-function relation, in which the number of –OH groups are related to its effectiveness.

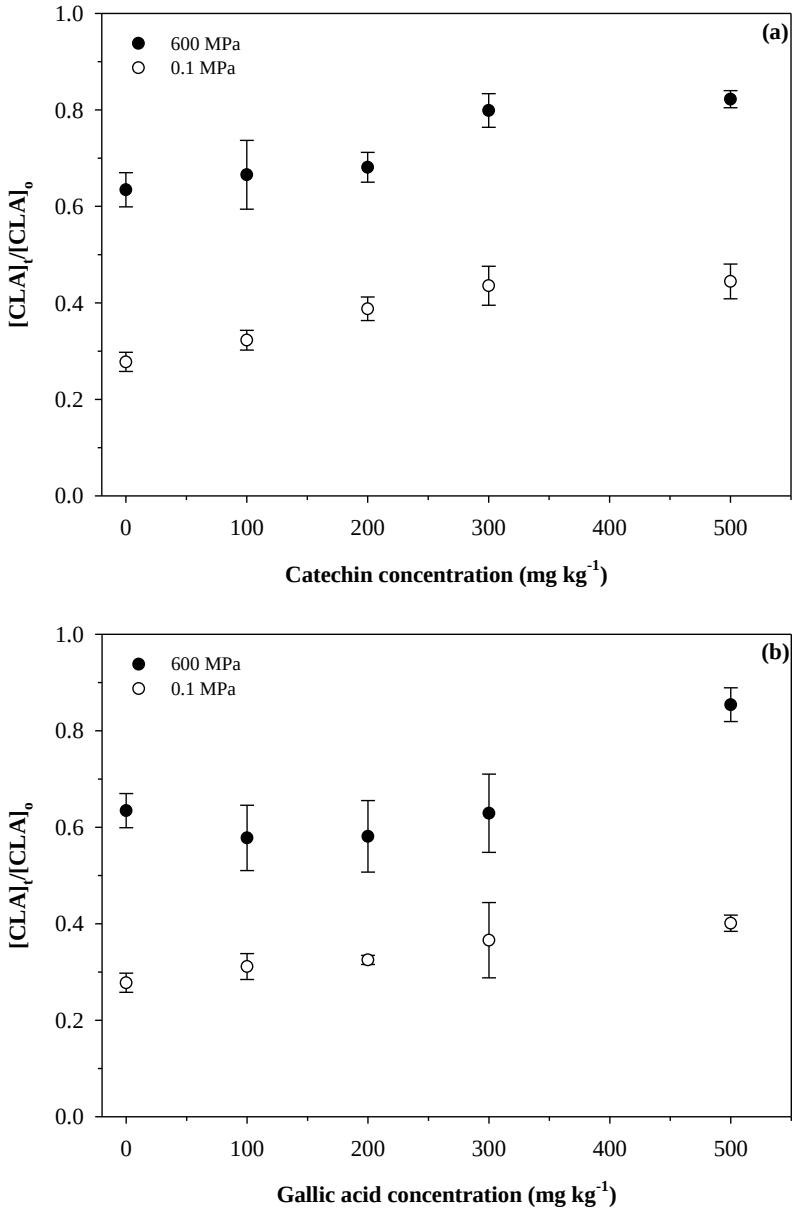

Fig 8-9. Effect of the concentration of (a) catechin and (b) gallic acid on the retention of CLA in milk treated at 120˚C for 15 min at either 0.1 or 600 MPa.

In the case of the retention of CLA upon pressure, the number of –OH groups did not reflect the antioxidant effectiveness. For instance, TAN possess 27 –OH groups in its structure but the retained normalized CLA was only 0.63 ± 0.03. In contrast, GA and CAT with 4 and 5 –OH groups, respectively, yielded higher

retention values (>0.75) than that obtained using TAN. The stability of antioxidants played an important role in enhancing the retention of CLA. For instance, GA is considered as a stable molecule proven by its thermogravimetrical analysis, which showed that decarboxilation and further degradation started at 260˚C (Garro-Galvez et al., 1996).

A closer inspection of **Figs 8-3b** to **8-5b** revealed that the oxygen was gradually consumed while the CLA remained unchanged. Phenolic antioxidants are known for their ability to quench oxygen, preventing the initiation stage of autoxidation (Ohara et al., 2009). Quenching oxygen by phenolic antioxidants has been described by bimolecular mechanism and represented as follows:

$$O_2 + Q \xrightarrow{k_q} products$$

where Q is the quenching agent or phenolic antioxidant; k_q is the apparent quenching rate constant. To evaluate the quenching ability of phenolic antioxidants, the total phenolic content was evaluated by Folin-Ciocalteau method reported elsewhere (Singh & Saldaña, 2011). **Table 8-2** shows the changes in the total phenolic content for milk samples with added GA, CAT, and CAF treated at 120˚C and 600 MPa. In general, the phenolic content followed a similar pattern to that observed for the DO_2, reaching normalized retention values in the range of 0.40-0.48 after 15 min of holding time.

Table 8-2 also shows the apparent quenching rate constant. Interestingly, GA yielded the highest k_q value followed by CAT and CAF at 120˚C and 600 MPa up to 15 min. GA also showed the greatest CLA retention followed by CAT. It seems that GA has the ability to quench oxygen and therefore delay the oxidation of CLA. Another important factor to consider is the ratio of DO_2 to the antioxidant. Ratios for GA and CAT were calculated as these antioxidants yielded the highest retention of CLA. After 10 min of holding time, ratios of 0.22 and 1.28 were obtained for GA and CAT, respectively. A value of the ratio

DO_2/antioxidant higher than 1 means that the antioxidant is consumed faster than the oxygen available, indicating that there is an excess of oxygen that can further react. On the other hand, a DO_2/antioxidant ratio of less than 1 could be viewed as the amount of oxygen being limited with respect to the amount of antioxidant (GA). These calculated ratios further demonstrated that the use of GA significantly enhanced the retention of CLA during HPS of milk. Upon pressure treatment, the role of antioxidants was strongly influenced by the dissolved oxygen content.

Table 8-2. Normalized phenolic content in CLA-enriched milk added with different phenolic antioxidants and treated at 120˚C and 600 MPa.

Holding time [min]	**Antioxidant added (500 mg kg^{-1})**		
	Gallic acid	**Catechin**	**Caffeic acid**
0	1.00	1.00	1.00
1	0.94	0.71	0.94
5	0.68	0.53	0.81
10	0.61	0.44	0.61
15	0.42	0.40	0.48
$k_{quenching}$ [min^{-1}]*	0.091 (0.003)	0.088 (0.005)	0.071 (0.005)
R^2	0.92	0.93	0.98

* Values in parenthesis are the standard errors

8.4. Conclusions

In the presence of oxygen, CLA was remarkably stable in pressurized samples compared to the control. The analysis of remaining CLA and consumed oxygen suggests that pressure induced isomerization of CLA occurs through free radical mechanism. The application of HPS in milk samples containing with phenolic antioxidants enhanced the retention of CLA. Gallic acid yielded the highest retention of CLA followed by catechin. During HPS, the rate of oxygen quenching was higher than the oxidation rate of CLA, which might avoid isomerization and therefore preserve the biological activity of CLA. Thus, the combination of HPS and the use of phenolic antioxidants can be used to produce milk-based beverages rich in CLA, addressing the growing demand for functional drinks.

8.5. References

Andrade, R. G., Dalvia, L. T., Silva, J. M., Lopes, G. K., Alonso, A., & Hermes-Lima, M. (2005). The antioxidant effect of tannic acid on the in vitro copper-mediated formation of free radicals. *Archives of Biochemistry and Biophysics, 437(1),* 1-9.

Brimberg, U. L., & Kamal-Eldin, A. (2003). On the kinetics of the autoxidation of fats: substrates with conjugated double bonds. *European Journal of Lipid Science and Technology, 105(1),* 17-22.

Butz, P., Serfert, Y., Garcia, A. F., Dieterich, S., Lindauer, R., Bognar, A., & Tauscher, B. (2004). Influence of high-pressure treatment at 25 degrees C and 80 degrees C on folates in orange juice and model media. *Journal of Food Science, 69(3),* S117-S121.

Campbell, W., Drake, M. A., & Larick, D. K. (2003). The impact of fortification with conjugated linoleic acid (CLA) on the quality of fluid milk. *Journal of Dairy Science, 86(1),* 43-51.

Chen, Z. Y., & Nawar, W. W. (1991). The role of amino acids in the autoxidation of milk fat. *Journal of the American Oil Chemists' Society, 68(1),* 47-50.

Chimi, H., Cillard, J., Cillard, P., & Rahmani, M. (1991). Peroxyl and hydroxyl radical scavenging activity of some natural phenolic antioxidants. *Journal of the American Oil Chemists' Society, 68(5),* 307-312.

Choe, E., & Min, D. B. (2006). Chemistry and reactions of reactive oxygen species in foods. *Critical Reviews in Food Science and Nutrition, 46(1),* 1-22.

Cook, M. E., & Pariza, M. (1998). The role of conjugated linoleic acid (CLA) in health. *International Dairy Journal, 8(5-6),* 459-462.

De Vleeschouwer, K., Van der Plancken, I., Van Loey, A., & Hendrickx, M. E. (2010). The effect of high pressure-high temperature processing conditions on acrylamide formation and other Maillard reaction compounds. *Journal of Agricultural and Food Chemistry, 58(22),* 11740–11748.

Destaillats, F., & Angers, P. (2002). Evidence for [1,5] Sigmatropic rearrangements of CLA in heated oils. *Lipids, 37(4),* 435-438.

Destaillats, F., & Angers, P. (2005). Thermally induced formation of conjugated isomers of linoleic acid. *European Journal of Lipid Science and Technology, 107(3),* 167-172.

Destaillats, F., Japiot, C., Chouinard, P. Y., & Angers , P. (2005). Rearrangement of rumenic acid in ruminant fats: a marker of thermal treatment. *Journal of Dairy Science, 88(5),* 1631–1635.

Garro-Galvez, J. M., Fechtal, M., & Riedl, B. (1996). Gallic acid as a model of tannins in condensation with formaldehyde. *Thermochimica Acta, 274(25),* 149-163.

Hamann, S. D., & Linton, M. (1974). Influence of pressure on the ionization of substituted phenols. *Journal of Chemistry Society, Faraday Transactions 1: Physical Chemistry in Condensed Phases, 70,* 2239-2249.

Heinz, V., & Buckow, R. (2010). Food preservation by high pressure. *Journal of Consumer Protection and Food Safety, 5*, 73–81.

Herzallah, S. M., Humeid, M. A., & Al-Ismai, K. M. (2005). Effect of heating and processing methods of milk and dairy products on conjugated linoleic acid and trans fatty acid isomer content. *Journal of Dairy Science, 88(4),* 1301–1310.

Hill, V. M., Ledward, D. A., & Ames, J. M. (1996). Influence of high hydrostatic pressure and ph on the rate of Maillard Browning in a glucose−lysine system. *Journal of Agricultural and Food Chemistry, 44(2),* 594-598.

Huppertz, T., Smiddy, M. A., Vpadhyay, V. K., & Kelly, A. L. (2006). High-pressure-induced changes in bovine milk: a review. *International Journal Dairy Technology, 59(2),* 58-66.

Isaacs, N. S. (1981). *Liquid Phase High Pressure Chemistry*. Toronto: John Wiley.

Jenner, G. (2004). Role of the medium in high pressure organic reactions. A review. *Mini Reviews in Organic Chemistry, 1(1),* 9-26.

Juliano, P., Bilbao-Sáinz, C., Koutchma, T., Balasubramaniam, V. M., Clark, S., Stewart, C. M., et al. (2012). Shelf-stable egg-based products processed by high pressure thermal sterilization. *Food Engineeering Reviews, 4(1),* 55-67.

Khan, N. S., Ahmad, A., & Hadi, S. M. (2000). Anti-oxidant, pro-oxidant properties of tannic acid and its binding to DNA. *Chemico-Biological Interactions, 125(3),* 177–189.

Kima, T. J., Silva, J. L., Kim, M. K., & Jung, Y. S. (2010). Enhanced antioxidant capacity and antimicrobial activity of tannic acid by thermal processing. *Food Chemistry, 118(3),* 740–746.

Knoerzer, K., Buckow, R., & Versteeg, C. (2010). Adiabatic compression heating coefficients for high-pressure processing — A study of some insulating polymer materials. *Journal of Food Engineering, 98(1),* 110–119.

Lindmark-Mansson, H., & Akesson, B. (2000). Antioxidative factors in milk. *British Journal of Nutrition*, S103-S110.

Matser, A. A., Krebbers, B., van den Berg, R. W., & Bartels, P. V. (2004). Advantages of high pressure sterilisation on quality of food products. *Trends in Food Science & Technology, 15(2),* 79-85.

Michaeli, A., & Feitelson, J. (1994). Reactivity of singlet oxygen toward amino acids and peptides. *Photochemistry and Photobiology, 59(3),* 284–289.

Moreno, F. J., Villamiel, M., & Olano, A. (2003a). Effect of high pressure on isomerization and degradation of lactose in alkaline media. *Journal of Agricultural and Food Chemistry, 51(7),* 1894-1896.

Moreno, F. J., Molina, E., Olano, A., & López-Fandiño, R. (2003b). High-pressure effects on Maillard reaction between glucose. *Journal Agricultural of Food Chemistry, 51(2),* 394-400.

Neuman, R. C. (1972). Pressure Effects as Mechanistic Probes of Organic Radical Reactions. *Accounts of Chemical Research, 5*, 381-387.

Neuman, R. C., & Amrich, M. J. (1972). High pressure studies. X. Activation volumes for homolysis of single bonds. *Journal of the American Chemists Society, 98(4),* 2730-2733.

Ohara, K., Ichimura, Y., & Nagaoka, S. (2009). Kinetic study of singlet-oxygen quenching by caffeic acid and related phenols. *Bulletin of the Chemical Society of Japan, 82(6),* 689–691.

Oley, I., Verlinde, P., Hendrickx, M. E., & Van Loey, A. (2006). Temperature and pressure stability of L-ascorbic acid and/or 6s 5-methyltetrahydrofolic acid: A kinetic study. *European Food Research and Technology, 223(1),* 71-77.

Park, Y. (2009). Conjugated linoleic acid (CLA): Good or bad trans fat? *Journal of Food Composition and Analysis, 22(1),* S4–S12.

Sánchez-Moreno, C., Larrauri, J. A., & Saura-Calixto, F. (1999). Free radical scavenging capacity and inhibition of lipid oxidation of wines, grape juices and related polyphenolic constituents. *Food Research International, 32(6),* 407-412.

Schamberger, G. P., & Labuza, T. P. (2007). Effect of green tea flavonoids on Maillard browning in UHT milk. *LWT-Food Science Technology, 40(8),* 1410-1417.

Singh, P. P., & Saldaña, M. D. (2011). Subcritical water extraction of phenolic compounds from potato peel. *Food Research International, 44*(8), 2452–2458.

Sizer, C. E., Balasubramaniam, V. M., & Ting, E. (2002). Validating high-pressure processes for low-acid foods. *Food Technology, 56(2),* 36-38.

Smet, K., De Block, J., De Campeneere, S., De Brabander, D., Herman, L., Raes, K., Dewettinck, K., & Coudijzer, K.. (2009). Oxidative stability of UHT milk as influenced by fatty acid composition and packaging. *International Dairy Journal, 19(6-7),* 372–379.

Ting, E., Balasubramaniam, V. M., & Raghubeer, E. (2002). Determining thermal effects in high-pressure processing. *Food Technology, 56(2),* 31-35.

Van den Broeck, I., Ludikhuyze , L., Weemaes, C., Van Loey, A., & Hendrickx, M. E. (1998). Kinetics for Isobaric−Isothermal Degradation of l-Ascorbic Acid. *Journal of Agricultural and Food Chemistry, 46(5),* 2001-2006.

Vazquez-Landaverde, P. A., & Qian, M. C. (2007). Antioxidant impacts on volatile formation in high-pressure-processed milk. *Journal of Agricultural and Food Chemistry, 55(22),* 9183–9188.

Vazquez-Landaverde, P. A., Torres, J. A., & Qian, M. C. (2006). Effect of high-pressure-moderate-temperature processing on the volatile profile of milk. *54(24),* 9184-9192.

Verbeyst, L., Bogaerts, R., Van der Plancken, I., Hendrickx, M., & Van Loey, A. (2013). Modelling of vitamin C degradation during thermal and high-pressure treatments of red fruits. *Food and Bioprocess Technology, 6(4),* 1015-1023.

Verbeyst, L., Oey, I., Van der Plancken, I., Hendrickx, M., & Van Loey, A. (2010). Kinetic study on the thermal and pressure degradation of anthocyanins in strawberries. *Food Chemistry, 123(2),* 269-274.

Yen, G. C., Duh, P. D., & Tsai, H. L. (2002). Antioxidant and pro-oxidant properties of ascorbic acid and gallic acid. *Food Chemistry, 79(3)*, 307–313.

Chapter 9

Kinetics of lactulose formation in milk treated at high-pressure sterilization conditions[8]

9.1. Introduction

Milk is naturally rich in nutrients, such as proteins, carbohydrates, fats, minerals, and vitamins. Raw milk is perishable and spoils within hours at room temperature. Therefore, milk is thermally processed to make it safe for human consumption and extend its shelf life (Claeys et al., 2002). Processes like High-Temperature-Short-Time pasteurization (HTST, 75˚C/15 s) provide a shelf life of 2 weeks at 6°C. Combinations of higher temperatures and shorter holding times (130-150˚C/2-10 s) allow to achieve commercial sterilization conditions that significantly extend the shelf life to between 45 and 180 d at room temperature (Rysstad & Kolstad, 2006). However, various reactions occur during thermal sterilization that detriment the nutritional and sensory properties of milk. These changes in milk heated at sterilization conditions have been extensively reviewed by Burton (1983) and more recently by Chavan et al. (2011). One of the main chemical changes is the isomerization of lactose to form lactulose, a compound not present in raw milk and therefore used as a heat damage indicator (Marconi et al., 2004; Claeys et al., 2004; Cattaneo et al., 2008).

A recent technology that simultaneously applied high pressure (100-600 MPa) and elevated temperature (90-120°C) has been successfully used to achieve sterilization conditions without significantly changing the sensory and nutritional properties compared to traditional thermal treatments (Matser et al., 2004). The fundamentals of high-pressure sterilization (HPS) were reviewed in Chapter 2. In the case of lactose isomerization, Moreno et al. (2003) evaluated the effect of 400 MPa and 60˚C after 3 h in aqueous lactose solution (10%) in basic media (pH= 10). The authors found an inhibitory effect of pressure on the isomerization and degradation of lactose compared to samples treated at the same temperature and

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[8] *A version of this chapter has been submitted to Innovative Food Science and Emerging Technologies for consideration for publication*

atmospheric pressure. Although the lactulose content is commonly used by the dairy industry to distinguish between sterilized and pasteurized milk, the kinetics of lactulose formation has not been studied at HPS conditions.

The kinetics of lactulose formation are relevant to evaluate the processing damage of milk during HPS treatments. Therefore, the main objective of this study was to evaluate lactulose formation at different pressures (100-600 MPa), temperatures (100-120°C) and holding times of up to 15 min. Other objectives were to analyze the kinetic data using mathematical models and to build pressure-temperature diagrams for lactulose formation at various HPS conditions.

9.2. Materials and Methods

9.2.1. Raw milk and sample preparation

Raw whole milk was collected from University of Alberta Dairy Research and Technology Centre (Edmonton, AB, Canada).

9.2.2. High-Pressure Sterilization (HPS)

HPS experiments were carried out as described in Chapter 6 (Section 6.2.3). At the end of the holding time, the vessels were decompressed and the samples were removed immediately from the high pressure vessels and cooled down with ice to minimize any further lactulose formation. The experimetns were conducted in triplicate.

9.2.3. Lactose, lactoluse and galactose quantification

Lactose, lactulose and galactose content was determined using high performance liquid chromatography (HPLC), according to the International Standard IDF 147B (1998).

9.2.4. Data analysis

The experimental data of lactose isomerization to form lactulose were analyzed using the Weibull model in the form of equation (9.1).

$$\frac{C_t - C_o}{C_f - C_o} = 1 - exp^{\left(-\left(\frac{t}{\alpha}\right)^{\beta}\right)} \quad (9.1)$$

The term on the left side of equation (9.1) is known as the fractional conversion (amount of lactulose formed at a specific time/maximum amount of lactulose formed). C_t is the concentration of lactulose (mg L^{-1}) at a specific time; C_o is the initial concentration of lactulose (mg L^{-1}); C_f is the final concentration of lactulose after 15 min of holding time; t is the heating time (min); α is a scale parameter, which is inversely proportional to the rate constant (k, min^{-1}). The influence of temperature and pressure on the α parameter can be described by the Arrhenius-type and Eyring-type relationships, equations (9.2) and (9.3):

$$\frac{1}{\alpha} = \frac{1}{\alpha_T} \cdot exp^{\left(\frac{-E_a}{R}\left(\frac{1}{T} - \frac{1}{T_{ref}}\right)\right)} \quad (9.2)$$

$$\frac{1}{\alpha} = \frac{1}{\alpha_P} \cdot exp^{\left(-\frac{\Delta V^{\neq}}{RT}(P - P_{ref})\right)} \quad (9.3)$$

Equation (9.2) was incorporated into equation (9.1), yielding a global kinetic equation (9.4), which accounts for the effects of time and temperature at a constant pressure. Likewise, equation (9.3) was incorporated into equation (9.1), accounting for the effects of time and pressure at a constant temperature (equation (9.5)).

$$\frac{C_t - C_o}{C_f - C_o} = 1 - exp^{\left[-\left(\frac{t}{\alpha_T} \cdot exp^{\left(\frac{-E_a}{R}\left(\frac{1}{T} - \frac{1}{T_{ref}}\right)\right)}\right)^{\beta_{avg}}\right]_P} \quad (9.4)$$

$$\frac{C_t - C_o}{C_f - C_o} = 1 - exp\left[-\left(\frac{t}{\alpha_P}\cdot exp\left(\frac{-\Delta V^{\neq}}{R\cdot T}\cdot\left(P - P_{ref}\right)\right)\right)^{\beta_{avg}}\right]_T \quad (9.5)$$

where β_{avg} is the average of the β values at the range of conditions studied. A new set of kinetic parameters (α_T, E_a, α_p, $\Delta V^{\neq}$ and β) were obtained using equations (9.4) and (9.5). Then, the accuracy of the estimated parameters was checked through joint confidence intervals (90%), as proposed earlier by Verbeyst et al. (2011). Once the kinetic parameters were checked for accuracy, k values were calculated for each pair of experimental temperature and pressure and were considered as the experimental rate constants (k_{exp}). Then, the k_{pred} were obtained as a function of temperature and pressure using the Arrhenius-Eyring model (equation (9.6)) and a polynomial model (equation (9.7)).

$$k = k_{ref}\cdot exp\left(-\frac{E_a}{R}\cdot\left(\frac{1}{T} - \frac{1}{T_r}\right)\right)\cdot exp\left(\frac{-\Delta V^{\neq}}{RT}\cdot\left(P - P_{ref}\right)\right) \quad (9.6)$$

$$\ln k = \ln a + b\cdot\left(T - T_{ref}\right) + b\cdot\left(P - P_{ref}\right) + d\cdot\left(T - T_{ref}\right)\cdot\left(P - P_{ref}\right) \quad (9.7)$$

where k_{ref} is the rate constant at reference conditions for equations (9.6) and (9.7), respectively, *a*, *b*, *c* and d are empirical parameters of the Weibull model obtained through non-linear regression analysis using the Solver option in Excel Microsoft. The fitting performance of the individual and global models was assessed by the coefficient of determination (R^2), the adjusted coefficient of determination (R^2_{adj}), standard error (*SE*), and residual analysis. In addition, equations (9.6) and (9.7) were assessed through mean square error (MSE) and the accuracy factor (A_f) as recently suggested by Nguyen et al. (2013).

$$MSE = \frac{\Sigma\left(k_{pred} - k_{exp}\right)^2}{2} \quad (9.8)$$

$$A_f = 10^{\Sigma \frac{\log\left(\frac{k_{pred}}{k_{exp}}\right)}{n}} \quad (9.9)$$

where k_{pred} is the k value obtained from equations (9.6) and (9.7) and n is the number of experiments.

9.3. Results and discussion

9.3.1. Formation of lactulose at HPS conditions

Fig 9-1 shows lactulose content of milk treated at HPS conditions. The data were normalized using equation (9.1) so lactulose content ranged from 0 to 1. Lactulose content equal to 1 represents the maximum formation of lactulose under the current experimental conditions (100-120˚C, 100-600 MPa and 0-15 min). At 100°C, the lactulose content was low (<0.08) throughout the holding time, regardless of the applied pressure. This lactulose content is equivalent to a concentration of ~52 mg lactulose L^{-1} milk, which is comparable to that obtained in ultra-pasteurized milk (58 mg L^{-1}) processed at 90˚C for 15 s (Pereda et al., 2009). In addition, such combinations of holding time, pressures and temperature (100-120˚C, 100-600 MPa and 0-15 min) not only yielded the same degree of thermal damage measured by lactulose concentration but also would allow inactivation of vegetative microorganisms (Wilson et al., 2008).

After 15 min of holding time at 120˚C and 100-300 MPa, a normalized lactulose content of ~0.6 was achieved. This content of lactulose is equivalent to a concentration of ~400 mg L^{-1}, which is similar to the concentration (350-600 mg L^{-1}) found in UHT milk (Pereda et al., 2009) which is within the maximum level (400 mg L^{-1} for UHT milk) allowed by the German legislation. Cattaneo et al. (2008) correlated the lactulose concentration with furosine content, a lysine damage indicator, and reported a maximum concentration of lactulose in UHT milk of 600 mg L^{-1}.

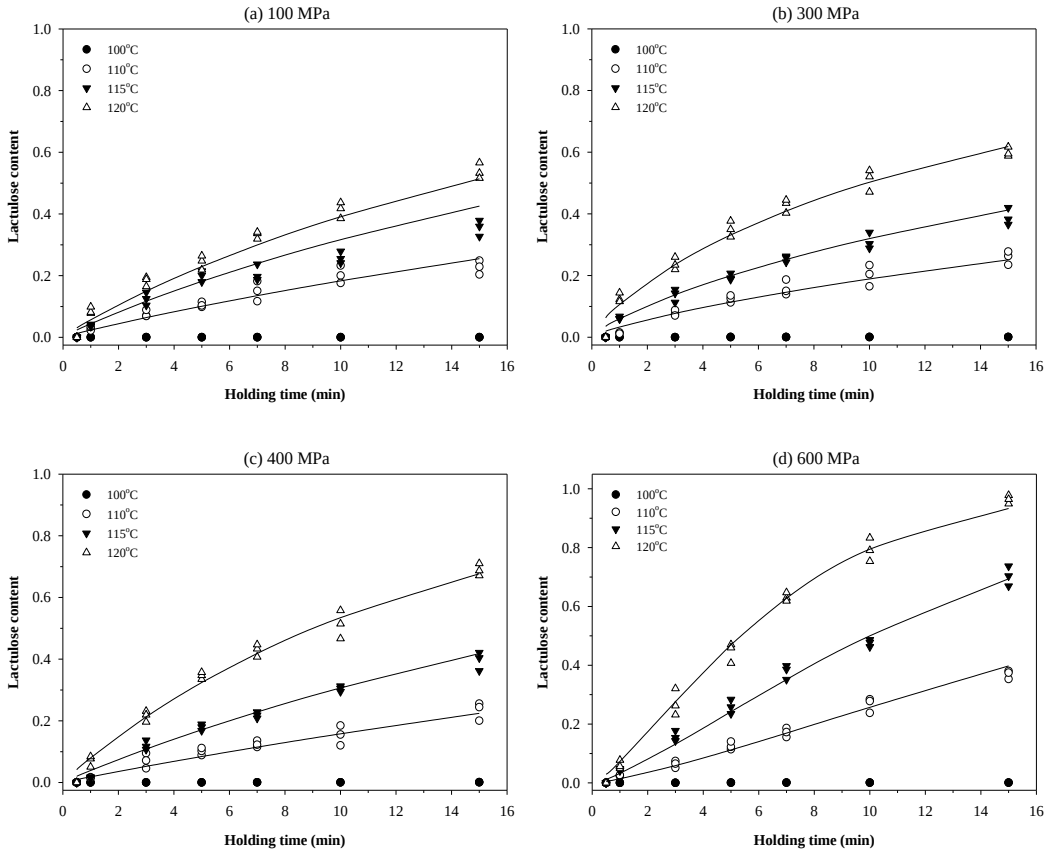

Fig 9-1. Normalized lactulose formation of milk treated at different temperatures and pressures. Curves represent the global model at a constant pressure using equation (9.4).

Milk treated at 120˚C and 600 MPa for 15 min yielded a normalized lactulose content of 0.94-0.97 (equivalent to 650 mg L^{-1}), which is lower than that obtained for UHT industrial plants (up to 850 mg L^{-1}) (Cattaneo et al., 2008) and for traditional sterilized milk cans processed at 120˚C for 30 min (800-1000 mg L^{-1}) (Andrews, 1986).

Fig 9-1 also indicates that pressure inhibited the formation of lactulose by comparing the lactulose content of high pressure sterilized milk obtained in this study (650 mg L^{-1}) with the equivalent reported contents (800-1000 mg L^{-1}) obtained with traditional thermal treatments (Cattaneo et al., 2008). This inhibitory effect of pressure on the isomerization is in agreement with the limited

data obtained by Moreno et al. (2003) who reported lactose isomerisation from lactose (10% in water) at only one high pressure condition (400 MPa/60˚C for 3 h) compared to atmospheric treatment. After 3 h, 18.8% of lactose was converted to lactulose at 0.1 MPa while only 7.8% was converted at 600 MPa (Moreno et al., 2003).

9.3.2. Modeling lactulose formation

For kinetics studies at HPS conditions where samples are preheated and compressed over a short period of time, the first kinetic data point was obtained when both isothermal and isobaric conditions have been reached at approximately 30 s after compression. Therefore, changes that occur due to preheating, loading and compression times were neglected. It was assumed that within the experimental domain, the formation of lactulose followed an exponential probabilistic distribution in the form of Weibull model. Using equation (9.1), the experimental data presented in **Fig 9-1** were fitted and the obtained Weibull parameters (α and β) are reported in **Table 9-1**. The scale parameter (α) values varied within a broad range from 5.3 x 10^5 to 7, decreasing with an increase in temperature. The α values are in agreement with the experimental data presented in **Fig 9-1** as higher temperatures (115 and 120˚C) yielded higher lactulose contents. Alternatively, the α values can be interpreted as the time needed to achieve 63% of lactulose formation (1 log cycle using equation (9.1)), regardless of the β value. According to the α values, more than 53 min were needed to achieve 63% of the maximum formation of lactulose at 120˚C and 100 MPa, while around 12 min were needed at 120˚C and 600 MPa (**Fig 9-1** and **Table 9-1**).

On the other hand, β values reported in **Table 9-1** were in the range of 1.82-0.78, 0.87-0.79, 1.50-0.89, and 1.43-0.62 for those samples pressurized at 100, 300, 400 and 600 MPa, respectively. The β values known as the shape parameter were not systematically affected by temperature and pressure (**Table 9-1**) as supported by the experimental curves presented in **Fig 9-2** that had similar exponential shapes.

Table 9-1. Parameters of the Weibull model (equation (9.1)) for lactulose formation in milk treated at high-pressure sterilization conditions.

Temp	**100 MPa**							
	α	**SE**	**95%CI**	**β**	**SE**	**95%CI**	**R^2**	**R^2_{adj}**
100˚C	2251.5	2184	1800	1.82	1.44	1.21	0.92	0.92
110˚C	76.43	14.7	0.07	0.79	0.07	0.0004	0.93	0.93
115˚C	42.81	5.03	0.011	0.78	0.05	0.0001	0.95	0.95
120˚C	20.08	0.92	1.86	0.88	0.04	0.08	0.98	0.98
Temp	**300 MPa**							
	α	**SE**	**95%CI**	**β**	**SE**	**95%CI**	**R^2**	**R^2_{adj}**
100˚C	64578	4018 1	51423	1.82	1.44	1.01	0.96	0.96
110˚C	51.18	8.45	0.015	0.85	0.065	0.0001	0.95	0.95
115˚C	35.34	2.86	0.0007	0.79	0.043	0.0005	0.97	0.97
120˚C	15.56	0.83	0.0003	079	0.046	0.0002	0.97	0.97
Temp	**400 MPa**							
	α	**SE**	**95%CI**	**β**	**SE**	**95%CI**	**R^2**	**R^2_{adj}**
100˚C	2180.2	1600	1800	1.50	0.81	1.18	0.91	0.91
110˚C	68.12	11.6	0.002	0.89	0.077	0.0002	0.94	0.93
115˚C	30.24	1.86	3.90	0.94	0.017	0.007	0.98	0.78
120˚C	12.95	0.44	0.97	0.99	0.44	0.09	0.98	0.81
Temp	**600 MPa**							
	α	**SE**	**95%CI**	**β**	**SE**	**95%CI**	**R^2**	**R^2_{adj}**
100˚C	539273	4513	63251	0.68	0.087	0.07	0.88	0.88
110˚C	28.76	1.22	1.75	1.16	0.046	0.07	0.93	0.92
115˚C	13.28	2.99	0.62	1.23	0.046	0.06	0.99	0.99
120˚C	7.07	0.12	0.27	1.43	0.055	0.11	0.99	0.99

The parameters α and β were estimated using equation (9.1). SE – standard error; CI95% - 95% confidence interval; R^2 - coefficient of determination; R^2_{adj} – adjusted coefficient of determination

Table 9-1 also summarizes the values of SE, R^2 and R^2_{adj} obtained in the temperature range of 110-120˚C, and pressure range of 100-600 MPa, suggesting that the variability was small and that the parameters were precisely estimated. But, for samples heated at 100˚C at all pressures investigated, the α and β values had large errors , meaning that they are not statistically different from zero. Therefore, the concentrations of lactulose obtained at such conditions (100˚C and 100-600 MPa) were too low to be included in the model.

The effect of temperature on the parameter α was described by the Arrhenius-type relationship (**Fig 9-2a**), where the apparent activation energy can be estimated from the slope of ln (1/α) vs ($1/T-1/T_{ref}$). The results of the linear regression obtained using equation (9.2) are reported in **Table 9-2**. A reference temperature of 111˚C was used in order to solve equation (9.2) and find $1/\alpha_T$ and E_a. The use of a reference temperature improves the estimation of these parameters as suggested earlier by van Boekel (2002).

The calculated E_a values were within a narrow range (149-193 kJ mol^{-1}) that slightly changed with pressure without any particular trend (**Table 9-2**). These values are slightly higher than those reported in the literature for lactulose formation in heated milk, 90-120˚C (105-135 kJ mol^{-1}) (Claeys et al., 2003; Andrews, 1986). Mechanistically, the inhibitory effect of pressure, for instance 100 MPa, might result in an increase of the energy needed to overcome the energetic barrier imposed in the milk system by pressure since protonation (the first step for lactulose formation) followed by a double bond shift is likely to be retarded by pressure.

Fig 9-2b shows the effect of pressure on the α parameter, which was described by the Erying-type model (equation (9.3)). The slopes obtained from the lines presented in **Fig 9-2b** were used to calculate the activation volumes ($\Delta V^{\#}$) reported in **Table 9-2**. The $\Delta V^{\#}$ values ranged from -6.19 to -7.41 cm^3 mol^{-1}. As known, the $\Delta V^{\#}$ indicates whether a particular reaction is promoted or inhibited by pressure which is calculated through the effect of pressure on the reaction rate constant at a given temperature (equation (9.3)). In the case of lactulose formation in milk, the obtained $\Delta V^{\#}$ is a reflection of all volume changes that might occur during the progression of the reaction, including reactions with proteins and molecular reorganization. Nevertheless, the small negative values of $\Delta V^{\#}$ obtained in this investigation agree with the increased formation of lactulose at high pressures (400-600 MPa).

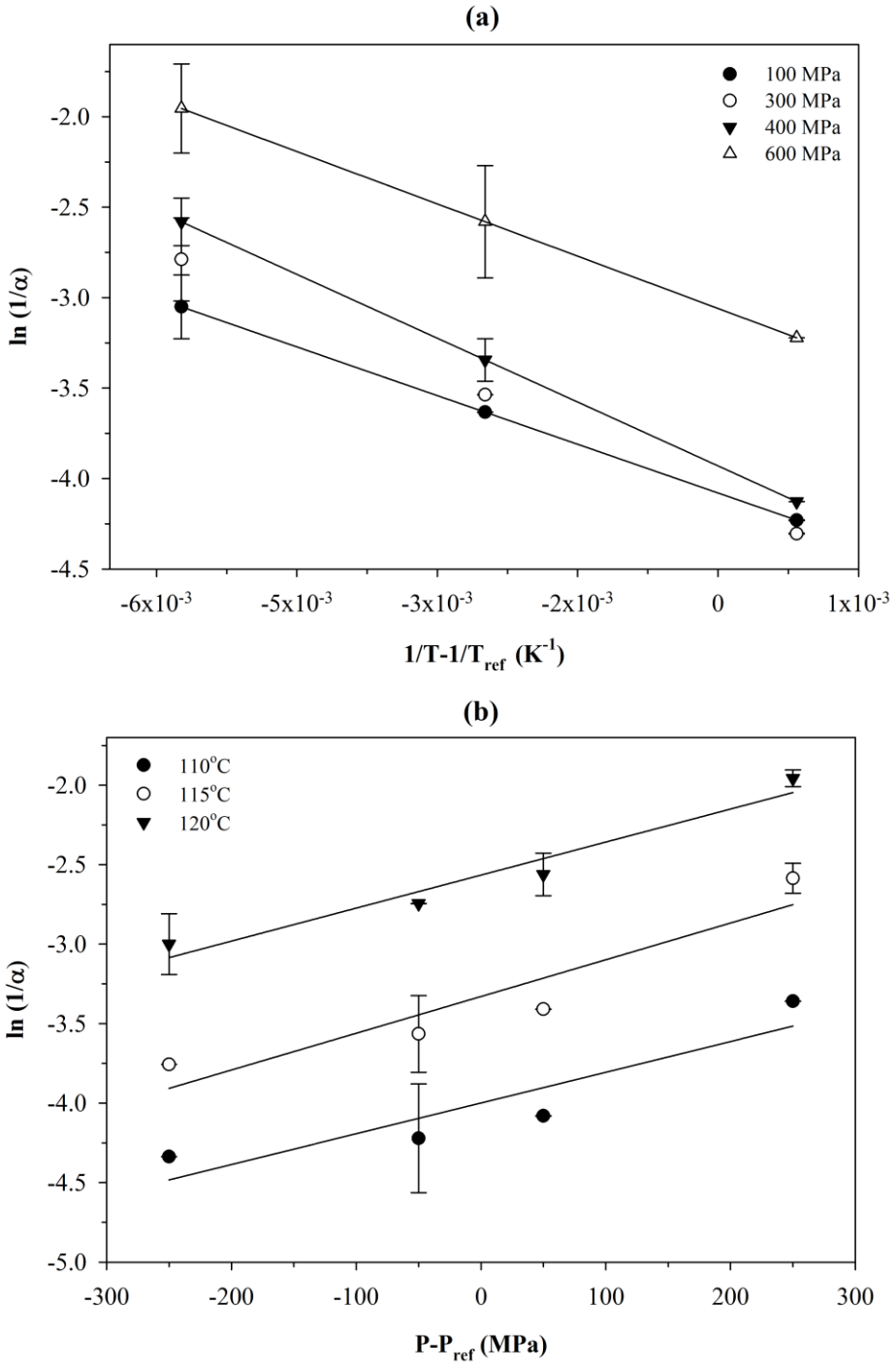

Fig 9-2. Effect of (a) temperature and (b) pressure on the scale parameter (α) for lactulose formation in milk treated by high-pressure sterilization. The error bars correspond to 95% confidence interval obtained by fitting equations (2) and (3) (T_{ref} = 111°C and P_{ref} = 350 MPa).

Pressure not only dissociates casein micelles (Needs et al., 2000; Gaucher et al., 2008) but also solubilizes the colloidal calcium phosphate (Schrader et al., 1997), shifting the equilibrium between colloidal and serum phases. This disruption of the mineral balance could increase the phosphate and citrate concentrations in the serum phase, adding a catalytic effect. Indeed, Andrews & Prasad (1987) catalyzed lactulose formation in milk by increasing the molarity of citrate and phosphate in the serum phase. Moreover, Mathys & Knorr (2009) reported that water is contracted around 15 % at 600 MPa. This reduction in volume concentrates the solutes in the serum phase, including phosphate and citrate. Therefore, the disruption in the mineral balance caused by pressure might accelerate the formation of lactulose observed at 600 MPa and 120°C.

Table 9-2. Parameters of the Arrhenius-type and Eyring-type models (equations (9.2) and (9.3)) for the formation of lactulose in milk treated at high-pressure sterilization conditions.

Parameter	**100 MPa**	**300 MPa**	**400 MPa**	**600 MPa**
E_a	148.82 ± 1.3	192.05 ± 0.98	192.88 ± 1.4	172.1 ± 24.9
k_T	0.017 ± 0.001	0.016 ± 0.004	0.021 ± 0.006	0.047 ± 0.001
R^2	0.99	0.99	0.99	0.99
R^2_{adj}	0.98	0.98	0.98	0.98
Parameter	**100˚C**	**110˚C**	**115˚C**	**120˚C**
$\Delta V^{\#}$	Not calculated	-6.19 ± 1.6	-7.41 ± 1.85	-6.63 ± 1.92
k_p	Not calculated	0.018 ± 0.003	0.036 ± 0.004	0.077 ± 0.004
R^2	Not calculated	0.87	0.87	0.97
R^2_{adj}	Not calculated	0.81	0.84	0.95

The parameters E_a, k_T, $\Delta V^{\#}$ and k_p were estimated with equations (9.2) and (9.3). The error was calculated from the 95% confidence interval. E_a – activation energy calculated (kJ mol^{-1}); k_T and k_p – reaction rate constants at a reference temperature and pressure, respectively (min^{-1}); $\Delta V^{\#}$ – activation volume (cm^{-3} mol^{-1}); R^2 - coefficient of determination; R^2_{adj} – adjusted coefficient of determination.

Data presented in **Table 9-2** were used to re-calculate the kinetic parameters (k_T, k_P, E_a, $\Delta V^{\#}$ and β) using global fitting equations (equations (9.4) and (9.5)). With this approach, the estimation of the kinetic parameters is improved because it uses all temperatures and pressures at once. The new values of the parameters (k_T, k_p, E_a and $\Delta V^{\neq}$) and the statistical analysis are summarized

in **Table 9-3**. Equation (9.) fits the experimental data really well, with an R^2_{adj} of 0.97. The E_a values calculated using equation (9.) lied within the range of 149-207 kJ mol^{-1}. On the other hand, the calculated values of $\Delta V^{\neq}$ using equation (9.5) varied from -2.57 to -8.91 cm^3 mol^{-1}, suggesting that the formation of lactulose is indeed accelerated by pressure. However, this value is rather small and the effect of pressure on the reaction rate constant is limited. The re-estimated values of β were $\sim$ 1.0, which indicates that the formation of lactulose follows a first-order kinetic reaction. In milk heated at atmospheric pressure, the lactulose formation was described by a first-order model (Claeys et al., 2004, 2003). In a complex system, such as milk, the reaction order might be viewed as empirical since no direct insight on the reaction mechanisms can be obtained from it.

Table 9-3. Global fitting (equations (9.4) and (9.5)) for the formation of lactulose in milk treated at high-pressure sterilization conditions.

Parameter	**100 MPa**	**300 MPa**	**400 MPa**	**600 MPa**
E_a	149.15 ± 32	191.6 ± 0.58	207.1 ± 0.78	172.3 ± 10.5
k_T	0.017 ± 0.001	0.01 ± 0.001	0.02 ± 0.001	0.041 ± 0.003
R^2	0.99	0.99	0.99	0.99
R^2_{adj}	0.98	0.98	0.98	0.98
Parameter	**100˚C**	**110˚C**	**115˚C**	**120˚C**
$\Delta V^{\#}$	Not calculated	-2.57 ± 1.03	-6.23 ± 1.95	-8.91 ± 1.64
k_p	Not calculated	0.02 ± 0.003	0.05 ± 0.009	0.11 ± 0.01
R^2	Not calculated	0.93	0.97	0.88
R^2_{adj}	Not calculated	0.92	0.96	0.87

The parameters E_a, k_T, $\Delta V^{\#}$ and k_p were estimated with equations (9.4) and (9.5). The error was calculated from the 95% confidence interval. E_a – activation energy calculated (kJ mol^{-1}); k_T and k_p – reaction rate constants at a reference temperature and pressure, respectively (min^{-1}); $\Delta V^{\#}$ – activation volume (cm^{-3} mol^{-1}); R^2 - coefficient of determination; R^2_{adj} – adjusted coefficient of determination.

Fig 9-3 shows lactulose formation in milk treated by HPS using equations (9.4) and (9.5) that had three parameters (k_T, k_p, E_a and $\Delta V^{\neq}$) where the correlation between them was evaluated through joint confidence regions of 90%. The joint confidence regions were built by fixing the parameter β. In **Fig 9-3a**, β was equal to 0.84, 0.79, 0.96 and 1.36 for 100, 300, 400, and 600 MPa,

respectively. In the case of **Fig 9-3b**, β was equal to 0.94, 0.95, and 1.0 for 110, 115, and 120˚C, respectively.

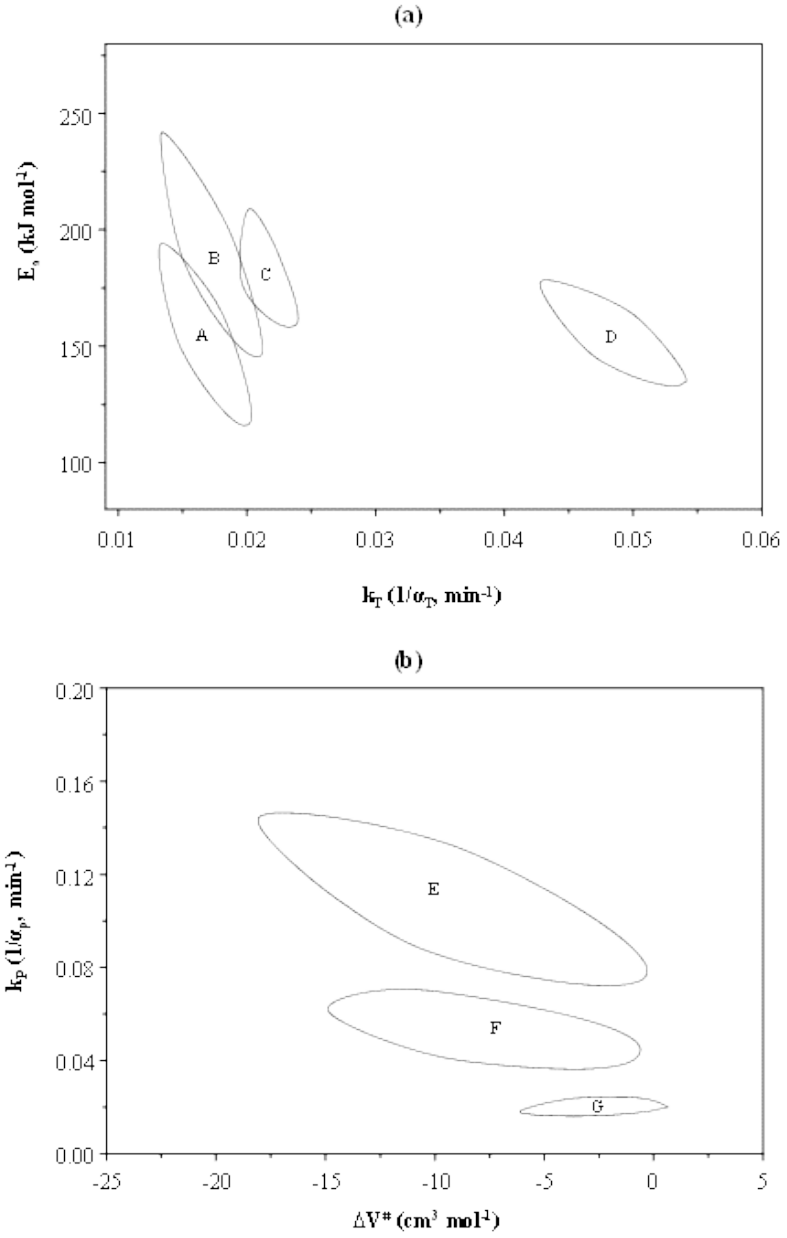

Fig 9-3. Joint confidence region (90%) for lactulose formation in milk treated at high-pressure sterilization conditions: (a) E_a vs k_T: A – 100 MPa and β = 0.84; B – 300 MPa and β = 0.79; C – 400 MPa and β = 0.96; D – 600 MPa and β = 1.32, and (b) k_P vs $\Delta V^{\neq}$: E - 110˚C and β = 0.94; F – 115˚C and β = 0.95; and G – 120˚C and β = 1.1.

At a constant pressure, **Fig 9-3a** illustrates the pairs of values of E_a and k_T that are possible solutions for the obtained model. These parameters were well correlated, judging from the shapes of the confidence regions that clearly deviate from a rectangular, which is the case if the parameters are not correlated. The confidence region obtained for 100 MPa slightly overlapped with 300 MPa, which in turn slightly overlapped with the confidence region at 400 MPa. This indicates that the calculated parameters are not statistically different within the pressure range of 100-400 MPa. In contrast, the confidence region at 600 MPa did not overlap and their parameters are statistically different from 100-400 MPa, regardless of the temperature used. Another interpretation is that the formation of lactulose at 600 MPa followed a different reaction mechanism. This interpretation supports our hypothesis that the phosphate and citrates released to the serum phase catalyzes the lactulose formation. However, further experimental validation is still needed. **Fig 9-3b** shows the confidence regions at 110, 115 and 120˚C. Similar to **Fig 9-3a**, the shape of the confidence regions indicated that the parameters were well correlated. Interestingly, the confidence regions did not overlap. However, when the confidence regions are projected over the X-axis, there is a clear overlap with respect to $\Delta V^{\neq}$. This finding indicates that $\Delta V^{\neq}$ was estimated with large error (**Table 9-3**), especially at 110˚C where the region of possible solutions of k_P and $\Delta V^{\neq}$ was quite broad. However, the confidence region at 120˚C was smaller compared with that for the other temperatures studied; suggesting that the parameters were accurately estimated.

The rate constants (k_{pred}) were calculated for each pair of temperature and pressure. These k_{pred} values were obtained using the Arrhenius-Eyring model (equation (9.6)) and the polynomial model (equation (9.7)) that describe the combined effect of temperature and pressure. Both models have been successfully used for describing chemical reactions affected by temperature and pressure (Verbeyst et al., 2011; Verbeyst et al., 2010).

Table 9-4 shows the parameters (k_T, k_p, E_a and $\Delta V^{\neq}$) for both models. The *Ea* and $\Delta V^{\#}$ revealed that high temperature and high pressure accelerated lactulose formation. In the case of the empirical model, the term *c* in equation (9.7) had an error larger than its absolute value and therefore was removed from this equation. A graphical illustration of the models' predictability is shown in **Fig 9-4**. All the predicted values by the Arrhenius-Eyring model were linearly correlated with the experimental data and laid within the 95% confidence and prediction band as observed in **Fig 9-4a**. The predicted values with the empirical model of equation (9.7) were also correlated linearly with the experimental values (**Fig 9-4b**). However, the predicted bands were slightly wider compared with those obtained by the Arrhenius-Eyring model (**Fig 9-4a**).

Table 9-4. Modeling parameters for the rate constant of lactulose formation in milk treated at high-pressure sterilization conditions.

Arrhenius-Eyring model (equation (9.6))		**Polynomial model (equation (9.7))**	
Parameter	**Value CI95%**	**Parameter**	**Value CI95%**
k_{ref}	0.021 ± 0.0004	k_{ref}	-3.82 ± 1.32
E_a	182.07 ± 8.16	*a*	0.14 ± 0.017
$\Delta V^{\#}$	-7.56 ± 0.07	*b*	0.07 ± 0.001
R^2	0.99	*c*	0.0005 ± 0.0008
R^2_{adj}	0.99	R^2	0.96
		R^2_{adj}	0.95
MSE	8.39	MSE	8.93
A_f	0.81	A_f	0.81

The parameters E_a, k_T, $\Delta V^{\#}$ and k_p were estimated with equations (9.6) and (9.7). CI95% - the 95% confidence interval. E_a – activation energy (kJ mol^{-1}); and k_{ref}– reaction rate constant at a reference temperature and pressure (min^{-1}); $\Delta V^{\#}$ – activation volume (cm^{-3} mol^{-1}); a, b, c – regression coefficients of equation (9.7); R^2 - coefficient of determination; R^2_{adj} – adjusted coefficient of determination. MSE – mean square error; Af – accuracy factor. The reference temperature and pressure used were 111˚C and 350 MPa, respectively.

In addition, the Arrhenius-Eyring model had lower MSE and A_f values (**Table 9-4**). Almost no distinction on fitting performance was detected between the Arrhenius-Eyring model and the empirical model.

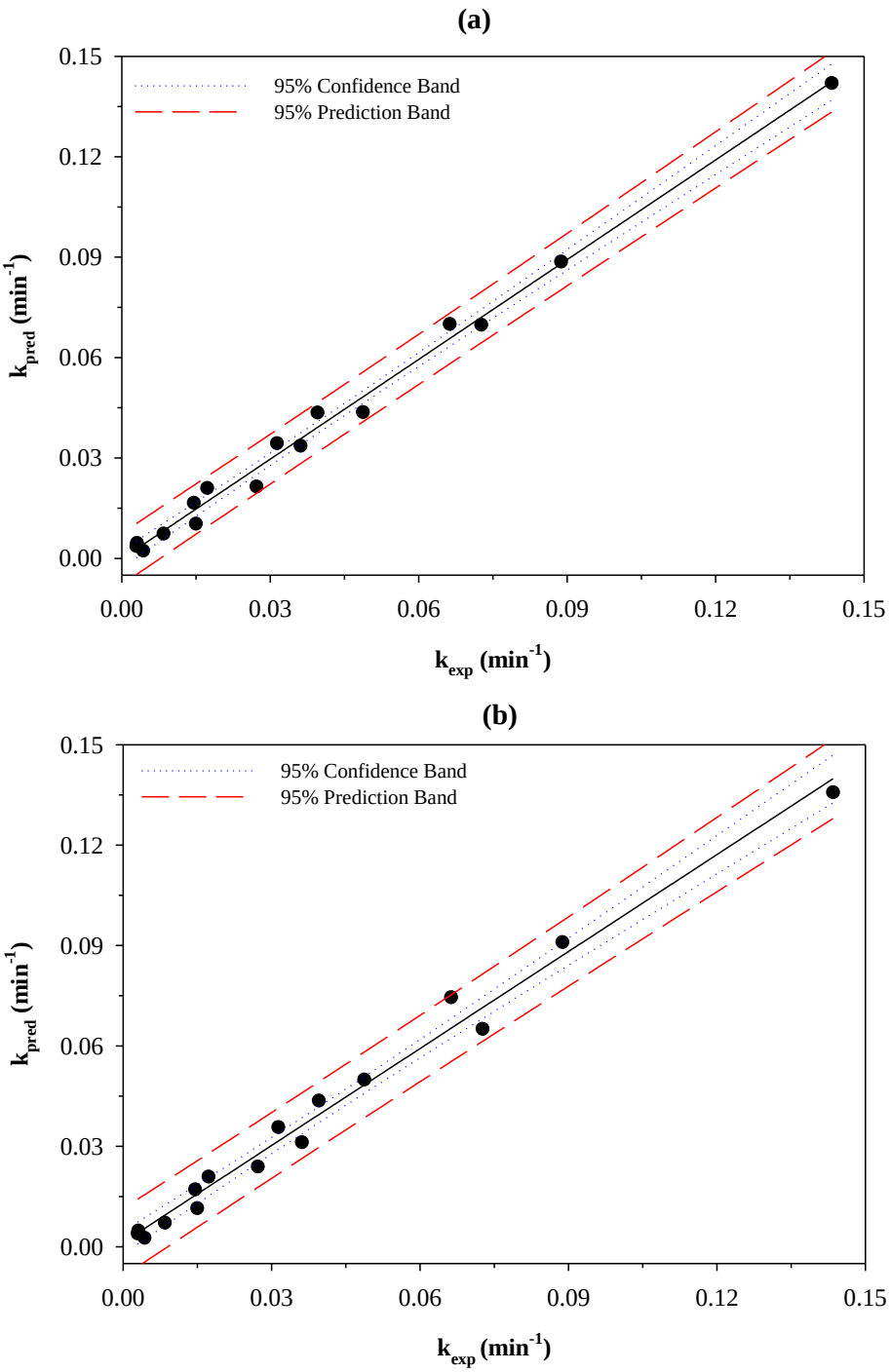

Fig 9-4. Linear relationship of *k* values obtained experimentally and predicted: (a) k_{pred} with the Arrhenius-Eyring model (equation (9.6)), and (b) k_{pred} with the polynomial model (equation (9.7)) (T_{ref} = 111°C and P_{ref} = 350 MPa). Blue lines represent the 95% confidence band and red lines represent the 95% prediction band.

Nevertheless, an ideal model would be the one for which the parameters have physical meaning and adequate statistical indeces. Unlike the polynomial model, the Arrhenius-Eyring model has theoretical background and its parameters can provide information on the reaction mechanism. The Arrhenius-Eyring model has also been reported to satisfactorily represent the combined effect of temperature and pressure on the rate constant for degradation of anthocyanins (Verbeyst et al., 2010, 2011).

9.3.3. Pressure-Temperature diagrams

The k_{pred} values predicted with the Arrhenius-Eyring model were further used to predict lactulose formation at different combinations of pressure and temperature. With this model, at any given holding time (e.g. 5 min and 10 min), the k_{pred} values were substituted into equation (9.1) and the lactulose content was obtained at different pressure and temperature combinations (**Fig 9-5**).

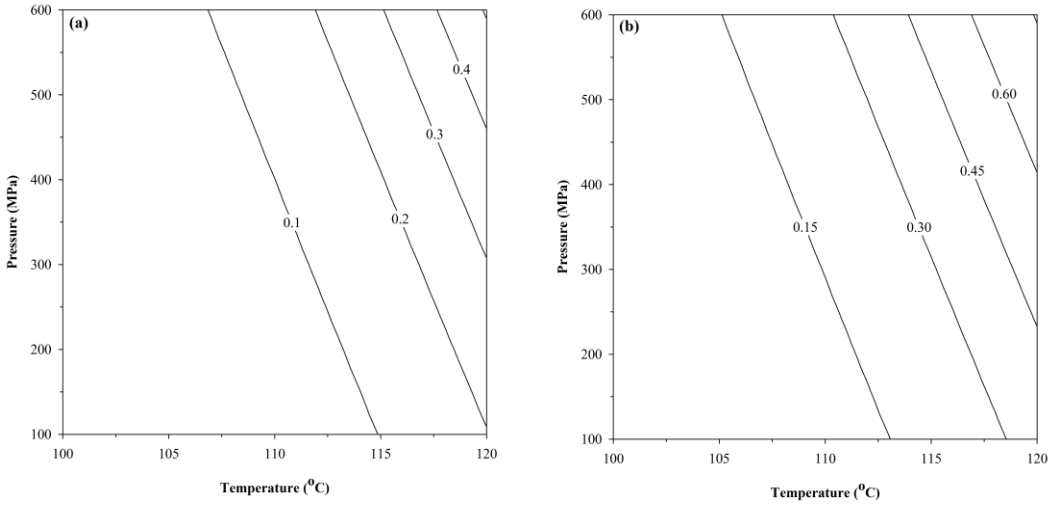

Fig 9-5. Pressure-temperature diagrams for the formation of lactulose in milk after holding times of: (a) 5 min, and (b) 10 min. The contour lines represent the required conditions to achieve a fractional conversion of lactulose (A fractional conversion of 1= 650 mg lactulose per L milk).

The lactulose content was expressed in terms of fractional conversion, considering that the maximum concentration of lactulose is reached after 15 min

at 600 MPa and 120˚C. After 5 min of holding time (**Fig 9-5a**), a lactulose content of 0.4 (260 mg L^{-1}) can be achieved at 500-600 MPa and 120˚C, which yields lower lactulose concentration compared to that found in UHT milk (400-600 mg L^{-1}). Interestingly, the lactulose concentration typically found in sterilized milk (>800 mg L^{-1}) was not achieved even after 10 min of holding time at our most drastic processing conditions studied (120˚C and 600 MPa), which would be enough to inactivate 6-log of *B. amyloliquefaciens* (Rajan et al., 2006), leading to sterilized milk with less thermal damage.

In contrast, lactulose values comparable to those typically obtained in HTST treatment, <100 mg L^{-1}, can be obtained at low temperature-low pressure and at high temperature-low pressure combinations (**Fig 9-5**). As discussed previously, lactulose concentration is used as a heat damage indicator to differentiate between pasteurized and sterilized milk. In HPS of milk, however, this interpretation must be applied cautiously because the reaction mechanism changed with the processing conditions and the resulting lactulose content might not represent the actual processing damage. Correlations with other heat damage indicators, such as furosine and hydroxymethylfurfural could provide a better description of the actual heat and pressure damage in HPS milk.

9.3.4. Reaction mechanism

Lactulose is formed via Lobry de Bruyn & Alberda van Ekenstein transformation in which glucose is isomerized to fructose via intermediate enolization (van Boekel, 1998). In a kinetic study, van Boekel (1998) reported a low value for 1,2-enediol reaction rate constant and therefore the lactulose formation can be simplified to:

$$\text{Lactose} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \text{Lactulose} \xrightarrow{k_3} \text{Galactose} + \text{C6}$$

An additional set of experiments was performed to infer the effect of HPS on the reaction mechanism. The concentrations of lactose and lactulose were determined by HPLC in samples treated at 600 MPa and 120˚C (**Fig 9-6**). The concentration of galactose was below the detection limit (data not reported). The maximum concentration of lactulose obtained was 650 mg L^{-1} that represents 13% of the total lactose. Similarly, van Boekel (1998) reported that ~11% of the lactose was isomerized to form lactulose in heated milk (0.1 MPa and 130˚C). Enolization step involves removal of the proton at the α-carbon atom, followed by a shift of the double bond of the adjacent carbonyl to the α-carbon (Izydorczyk, 2005). Upon pressure treatment, a slow proton transfer seems to be the rate-limiting step because a solvated proton from the solvent needs to be released. Further formation of enediol and lactulose, which involves molecular rearrangement and double bond shift, is believed to have a small volume change and therefore slightly affected by pressure (Isaacs, 1981).

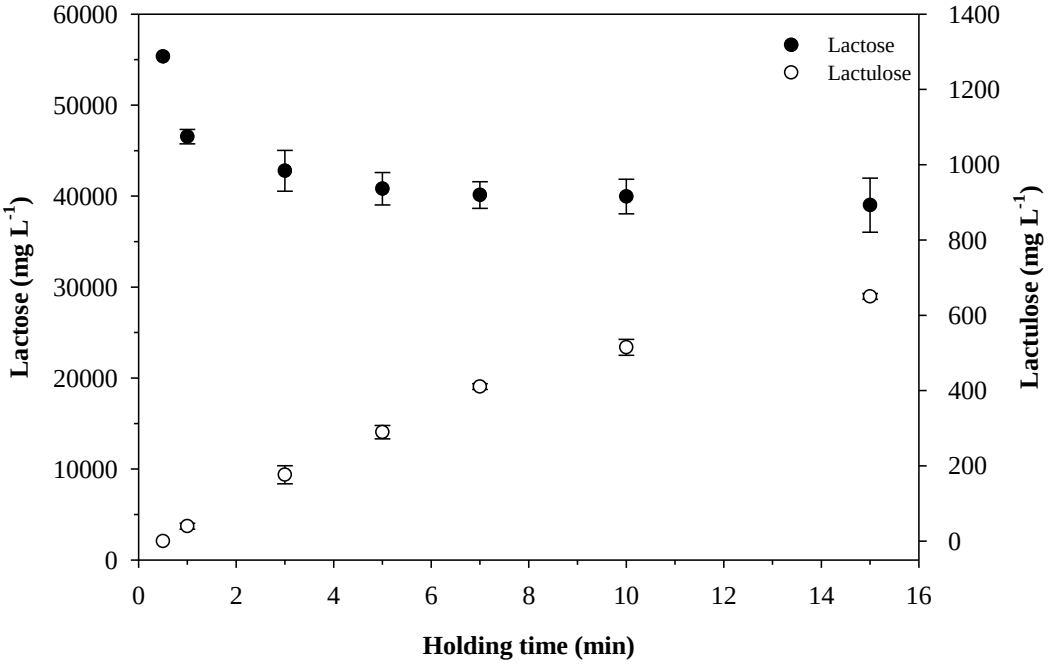

Fig 9-6. Lactose and lactulose concentrations during high-pressure sterilization of milk at 120˚C and 600 MPa.

This study showed that 22% of the total lactose is lost during HPS (600 MPa and 120˚C for 15 min). This percentage is higher than that reported by van Boekel (1998), who estimated that ~15% of the total lactose is lost in heated milk (0.1 MPa). From that 22%, the isomerization accounts for ~2% and the rest might be reacted through Maillard reaction. Indeed, the condensation reaction between a reducing sugar, glucose, and an amino group is accelerated by pressure (Isaacs & Coulson, 1996).

9.4. Conclusions

During HPS, the formation of lactulose in milk was accelerated by increasing temperature and pressure possibly because phosphates and citrates are released to the serum phase, catalyzing lactulose formation. Furthermore, the formation of lactulose was inhibited by pressure when compared with values reported in the literature for equivalent UHT processes. The experimental kinetic data on the formation of lactulose were well correlated with the Arrhenius-Eyring model and the polynomial model. With the Arrhenius-Eyring model, pressure-temperature diagrams were built and used successfully to estimate the formation of lactulose in milk at a wide range of HPS conditions. The information generated in this study can be used for minimizing the extent of processing damage in HPS milk.

9.5. References

Andrews, G. R. (1986). Formation and occurrence of lactulose in heated milk. *Journal of Dairy Research, 53(4),* 665-680.

Andrews, G. R., & Prasad, S. K. (1987). Effect of the protein, citrate and phosphate content of milk on formation of lactulose during heat treatment. *Journal of Dairy Research, 54(2),* 207-218.

Burton, H. (1983). Bacteriological, chemical, biochemical and physical changes that occur in milk at temperatures 100-150°C. *Bulletin 157* (pp: 3-16). International Dairy Federation.

Cattaneo, S., Masotti, F., & Pellegrino, L. (2008). Effects of overprocessing on heat damage of UHT milk. *European Food Research and Technology, 226(5),* 1099-1106.

Chavan, R. S., Chavan, S. R., Khedkar, C. D., & Jana, A. H. (2011). UHT milk processing and effect of plasmin activity on shelf life: A review.

Comprehensive Reviews in Food Science and Food Safety, *10(5)*, 251–268.

Claeys, W. L., Van Loey, A. M., & Hendrickx, M. E. (2002). Intrinsic time temperature integrators for heat treatment of milk. *Trends in Food Science & Technology*, *13(9-10)*, 293-311.

Claeys, W. L., Smout, C., Van Loey, A. M., & Hendrickx, M. E. (2004). From time temperature integrator kinetics to time temperature integrator tolerance levels: Heat-treated milk. *Biotechnology Progress*, *20(1)*, 1-12.

Claeys, W. L., Van Loey, A. M., & Hendrickx, M. E. (2003). Kinetics of hydroxymethylfurfural, lactulose and furosine formation in milk with different fat content. *Journal of Dairy Research*, *70(1)*, 85-90.

Gaucheron, F., Famelart, M. H., Mariette, F., Raulot, K., Michel, F., & Le Graet, Y. (1997). Combined effects of temperature and high-pressure treatments on physicochemical characteristics of skim milk. *Food Chemistry*, *59(3)*, 439-447.

International Standard IDF 147B. (1998). Heat treated milk – determination of lactulose content. Method using high-performance liquid chromatography. International Dairy Federation (IDF), Brussels.

Isaacs, N. S. (1981). *Liquid Phase High Pressure Chemistry*. (1st ed.). Toronto: John Wiley & Sons, (Chapter 4).

Isaacs, N. S., & Coulson, M. (1996). Effect of pressure on processes modelling the Maillard reaction. *Journal of Physical Organic Chemistry*, 9(9): 639-644.

Izydorczyk, M. (2005). Understanding the chemistry of food carbohydrates. In S. W. Cui (Ed.), *Food Carbohydrates Chemistry, Physical Properties, and Applications* (pp. 1-65). Boca Raton: CRC Press Taylor & Francis Group.

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 enzymatic coagulation. *Journal of Dairy Research*, 67(1), 31-42.

Nguyen, L. T., Rastogi, N. K., & Balasubramaniam, V. M. (2013). Estimation of accumulated lethality under pressure-assisted thermal processing. *Food and Bioprocess Technology*, DOI 10.1007/s11947-013-1140-6.

Marconi, E., Messia, M. C., Amine, A., Moscone, D., Vernazza, F., Stocchi, F., & Palleschi, G. (2004). Heat-treated milk differentiation by a sensitive lactulose assay. *Food Chemistry*, 84(3), 447-450.

Mathys, A., & Knorr, D. (2009). The properties of wayer in the pressure-temperature landscape. *Food Biophysics*, 4(2), 77-82.

Matser, A. A., Krebbers, B., van den Berg, R. W., & Bartels, P. V. (2004). Advantages of high pressure sterilisation on quality of food products. *Trends in Food Science & Technology, 15*(2), 79-85.

Moreno, F. J., Villamiel, M., & Olano, A. (2003). Effect of high pressure on isomerization and dgradation of lactose in alkaline media. *Journal of Agricultural and Food Chemistry*, 51(7), 1894-1896.

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

Rajan, S., Ahn, J., Balasubramaniam, V. M., & Yousef, A. E. (2006). Combined pressure-thermal inactivation kinetics of *Bacillus amyloliquefaciens* spores in egg patty mince. *Journal of Food Protection, 69*(4), 853–860.

Rysstad, G., & Kolstad, J. (2006). Extended shelf life milk – advances in technology. *International Journal of Dairy Technology*, 59(2), 85-96.

Schrader, K., Buchheim, W., & Morr, C. V. (1997). High pressure effects on the colloidal calcium phosphate and the structural integrity of micellar casein in milk. 1. High pressure dissolution of colloidal calcium phosphate in heated milk systems. *Nahrung*. 41 (3), 133-138.

van Boekel, M. A. J. S. (2002). On the use of the Weibull model to describe thermal inactivation of microbial vegetative cells. *International Journal of Food Microbiology*. 74(1-2), 139–159.

van Boekel, M. A. J. S. (1998). Effect of heating on Maillard in milk. *Food Chemistry*, 62(4), 403-414.

Verbeyst, L., Crombruggen, K. V., Van der Plancken, I., Hendrickx, M., & Loey, A. V. (2011). Anthocyanin degradation kinetics during thermal and high pressure treatments of raspberries. *Journal of Food Engineering*, 105(3), 513-521.

Verbeyst, L., Oey, I., Van der Plancken, I., Hendrickx, M., & Van Loey, A. (2010). Kinetic study on the thermal and pressure degradation of anthocyanins in strawberries. *Food Chemistry,*123(2), 269-274.

Wilson, D. R., Dabrowski, L., Stringer, S., Moezelaar, R., & Brocklehurst, T. F. (2008). High pressure in combination with elevated temperature as a amethod for the sterilization of food. *Trends in Food Science & Technology*, 19(6), 289-299.

Chapter 10

Pressure and temperature effects on the inactivation of *Bacillus amyloliquefaciens* and storage stability of conjugated linoleic acid in milk[9]

10.1. Introduction

There is growing industrial interest to change the fatty acid profile of milkfat, targeting to increase the concentration of conjugated linoleic acid (CLA), a bioactive lipid naturally found in milk. Indeed, pasteurized milk rich in CLA is already in the market in western Canada, containing 30 mg CLA g^{-1} fat (6-times higher than that in normal milk). This milk is expected to have a shelf-life of 10 d at 4˚C, which restricts its further distribution. Thus, extending the shelf-life of CLA-enriched milk is of great interest for the dairy industry.

Industrially, the alternative for producing long shelf-life milk is through UHT processing, which consists of the application of high temperature (130-150˚C) for a short holding time (2-15 s). UHT allows achieving commercial sterilization, significantly extending the shelf-life up to 180 d at room temperature (Rysstad & Kolstad, 2006; Goff & Griffiths, 2006). Unfortunately, the CLA is lost through oxidation upon heating as shown in Chapters 3 and 5. Herzallah et al. (2005) retained 85% of the initial CLA (5 mg g^{-1} fat) after UHT treatment at 140˚C for 4 s. Results presented in Chapter 5 showed that the CLA retained in CLA-enriched milk treated by UHT gradually decreased during storage. Therefore, obtaining commercially sterilized milk without losing its nutritional properties is still a great challenge for the dairy industry.

A promising approach, known as high-pressure sterilization (HPS), consists of applying high pressure (100-600 MPa) to a preheated sample (90-120˚C) (van den Ve et al., 2007). Temperature of the pressurized sample rises due to adiabatic compression, reaching the sterilization temperature. This is a great technological advantage because those changes in the temperature of the pre-heated sample are rapid and uniform, reducing the lack of temperature uniformity

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[9] *A version of this chapter was submitted to Innovative Food Science & Emerging Technologies for consideration for publication.*

that takes place in commercial sterilization treatments (Knoerzer et al., 2010). The use of HPS in CLA-enriched milk enhanced the retention of CLA compared with the thermal treatment at atmospheric pressure (Chapters 7 and 8). But, comparisons were not performed at equivalent processing conditions. Nevertheless, these results are encouraging, showing that HPS can deliver milk rich in CLA without losing its biological activity.

A further step in the development of HPS milk should consider the impact of processing conditions on the CLA content at those conditions where commercial sterilization has been achieved. This requires finding the conditions at which a pressure-resistant endospore is inactivated. Margosch et al. (2006) reported that the endospore produced by *Bacillus amyloliquefaciens* is the most pressure-resistant endospore identified so far and it is being used as a sterilization indicator (Rajan et al., 2006). Inactivation kinetics of *B. amyloliquefaciens* has been studied in model systems and food matrices (Margosch et al., 2006; Ahn et al., 2007; Ratphitagsanti et al., 2010). These reports showed that the combinations of pressure and temperature needed to inactivate the endospore varied with the food matrix. In addition, a recent investigation conducted in model systems showed that the pressure-temperature inactivation of *B. amyloliquefaciens* endospore was accelerated by the addition of nisin (Hofstetter et al., 2013). However, the combinations of pressure and temperature needed to achieve commercial sterilization in CLA-enriched milk have not been determined.

In UHT milk, the inactivation of alkaline phosphatase (ALP) is used as a process marker because it is more thermostable than *Mycobacterium tuberculosi*s, which is the most heat resistant non-sporogenic pathogen. Ludikhuyze et al. (2000) studied the inactivation of ALP under pressure (0.1-725 MPa and 23-63˚C) and found the conditions at which 90% of ALP activity was reduced. Moreover, these authors reported that ALP is not a suitable marker for pasteurization with pressure, since the complete inactivation of ALP would result in an over processed product. These results raise the question whether the residual activity of

ALP can be used as a marker in HPS, where the inactivation of endospores is aimed at high temperature and pressure within 5 min of holding time.

The objectives of this investigation were: 1) to find the HPS conditions at which endospores of *B. amyloliquefaciens* are inactivated in milk with and without nisin; 2) to evaluate the inactivation of ALP at HPS conditions, 3) to assess the effect of HPS treatment on CLA retention and hydroperoxide formation during storage, and 4) to compare the impact of such processing conditions on the retention of CLA with the equivalent UHT treatment.

10.2. Materials and methods

10.2.1. Enriched milk

The feeding regime developed by Bell et al. (2006) was used to enrich milk with CLA. Chapter 3 (Section 3.2.1) described the enrichment protocol.

10.2.2. Endospore inactivation

10.2.2.1. Endospores preparation

An endospore suspension was prepared following the methodology reported by Margosch et al. (2006). Briefly, *B. amyloliquefaciens* was grown overnight in ST1 broth (Difco, Becton, Dickinson and Company, Sparks, NV, USA) under aerobic conditions. Then, aliquots of 100 µL of the overnight culture were plated into ST1 agar and supplemented with $MnSO_4$ (10 mg L^{-1}). The cultivated plates were incubated aerobically at 37˚C for 14 d. Then, the growth of *B. amyloliquefaciens* endospores was confirmed by phase-contrast microscopy. The surface of the agar was washed with sterile saline solution (0.9%). The obtained solution was centrifuged (2,700 x *g* for 20 min), discarding the supernatant while the precipitate was re-suspended in 3 mL of saline solution. The centrifugation and re-suspension was repeated five times. Then, the obtained suspension was highly concentrated in endospores of *B. amyloliquefaciens*. Thus, the suspension was diluted with saline solution until an optical density at 600 nm (OD_{600}) with a value of 1.99 was obtained. An OD_{600} value of 1.99 corresponds to $\sim 10^9$

endospores mL^{-1} (Hofstetter, 2012). Finally, the endospore suspension was stored at 4˚C until inoculation.

10.2.2.2. Milk preparation

Raw CLA-enriched milk was centrifuged in a Beckman Coulter (Avanti® J-E, Fullerton, CA, USA) at 15,500 x *g* and 4˚C for 40 min to obtain cream and skim milk. The fat content of milk was standardized by adding 2.5% (w/v) of fat into the skim milk and homogenized using a two stage APV-2000 homogenizer (Concord, ON, Canada). Pressures of 200 and 50 kg/cm^2 were used in the first and second stages. After homogenization, the milk was cooled immediately at 10˚C and kept at that temperature until used.

10.2.2.3. Sample inoculation

A sample of 100 mL of standardized and homogenized CLA-enriched milk was inoculated by adding 1 mL of the diluted endospore suspension. The endospore suspension was vortexed before adding to the milk. Samples of 200 µL of inoculated milk were transferred into Tygon plastic tubes (Application Specific Tubing, Saint-Gobain Performance Plastics, Pittsburg, PA, USA). The tubes were sealed with heat using a hair-straightened (TONI&GUY®, Model TGST2976F, London, UK). The Tygon tubes were placed inside polypropylene containers (Cryogenic vial, Fisher Scientific, St. Louis, MO, USA) of ~3 mL volume. The polypropylene containers were filled with bleach solution (5%) to avoid contamination during the high pressure experiments. For those experiments conducted with nisin, the inoculated milk was mixed with different amounts of nisin (4, 8, 16, 34 or 64 mg of nisin per L^{-1} of milk). Then, the containers were capped and kept on ice overnight until HPS treatment.

10.2.2.4. Experimental design

Three sets of experiments were performed to select the levels of pressure, temperature and holding time to inactivate the endospore of *B. amyloliquefaciens*. Study 1 consisted of a factorial design with 2 responses (survivals after 15 and 30

min of holding time), 2 variables (pressure and temperature) at 3 levels for each variable (100, 300, and 600 MPa and 90, 100, and 120˚C, respectively). Study 2 was designed based on the results of Study 1, from which, the highest tested temperature of 120˚C was fixed and five different holding times were evaluated (0, 1, 3, 5 and 10 min) at different pressures (0.1, 100, 300 and 600 MPa). Once the conditions needed to inactivate 7-log of *B. amyloliquefaciens* endospore were established, Study 3 was performed aiming to reduce the processing holding time by using different nisin concentrations (4, 8, 16, 32 and 64 mg L^{-1}) at constant temperature and pressure conditions (120˚C and 600 MPa, respectively).

10.2.2.5. Survival enumeration

After the HPS treatment, the samples were kept at -20˚C until analysis. Surviving endospores of *B. amyloliquefaciens* were determined in RCM agar. Appropriate dilutions were plated and incubated aerobically at 34˚C for 24 h.

10.2.3. Inactivation of alkaline phosphatase (ALP)

10.2.3.1. Experimental design

An irregular factorial design was used for determining the inactivation of ALP. Temperature (60-120˚C), pressure (100-600 MPa) and holding time of up to 15 min were studied for enzyme inactivation of non-enriched milk.

10.2.3.2. Enzyme activity determination

ALP activity was determined fluorimetrically according to the standard official method ISO 1997 (standard 11816-1997). Briefly, ALP in treated milk hydrolyses a nonfluorescent substrate, Fluorophos®, to a highly fluorescent product, Fluoroyellow®. Product formation is monitored continuously during a short incubation period and enzyme activity is calculated from the rate of fluorescence increase. The detection limit of the method is 10 U mL^{-1}. In samples treated at 100 and 120˚C, the milk was centrifuged at 5000 *g* for 5 min to avoid interference with protein precipitation.

10.2.3.3. Data analysis

The inactivation of ALP under isobaric and isothermal conditions has been described by a first-order model in the form of equation (10.1) (Mussa & Ramaswamy, 1997; Ludikhuyze et al., 2000).

$$ln\left(\frac{A_t}{A_o}\right) = -k \cdot t \tag{10.1}$$

where A_t and A_o represent the enzyme activity at a given time and initial time, respectively; k is the inactivation rate constant (min^{-1}); and t is the holding time (min) at isobaric and isothermal conditions. The influence of temperature at a constant pressure on the inactivation rate constant can mathematically be expressed by the Arrhenius equation (10.2). Similarly, at a constant temperature, the effect of pressure on the k parameter can be expressed by the Eyring model (equation (10.3)).

$$k = k_T \cdot exp^{\left(\frac{-E_a}{R}\cdot\left(\frac{1}{T}-\frac{1}{T_{ref}}\right)\right)} \tag{10.2}$$

$$k = k_P \cdot exp^{\left(\frac{-\Delta V^{\neq}}{R\cdot T}\cdot\left(P-P_{ref}\right)\right)} \tag{10.3}$$

where k_T is the rate constant at a reference temperature (T_{ref}); k_P is the rate constant at a reference pressure (P_{ref}); E_a is the activation energy (kJ mol^{-1}); R is the universal gas constant (8.314 J mol^{-1} K^{-1}); and $\Delta V^{\neq}$ is the activation volume (cm^3 mol^{-1}). The simultaneous effect of temperature and pressure on the k parameter has been successfully described by a combination of thermodynamic and kinetic model, equation (10.4) (Van Loey et al., 2003).

$$\ln k = \ln k_o - \frac{\Delta V_o^{\neq}}{R\cdot T}\cdot\left(P-P_{ref}\right) + \frac{\Delta S_o^{\neq}}{R}\cdot\left(T-T_{ref}\right) - \frac{\Delta k^{\neq}}{2\cdot R\cdot T}\cdot\left(P-P_{ref}\right)^2 - \frac{\Delta \varepsilon^{\neq}}{R\cdot T}\left(P-P_{ref}\right)\cdot\left(T-T_{ref}\right) + \frac{\Delta C_p^{\neq}}{R\cdot T}\cdot\left[T\cdot\left(ln\frac{T}{T_{ref}}-1\right)+T_{ref}\right] \tag{10.4}$$

where $\Delta V_o^{\neq}$, $\Delta S_o^{\neq}$, $\Delta k_o^{\neq}$, $\Delta \varepsilon_o^{\neq}$, and $\Delta Cp_o^{\neq}$ are the volume, entropy, compressibility, expansibility and heat capacity change with respect to the native state of the enzyme. All experiments were conducted in triplicates and the parameters of equations 10.1 to 10.4 were obtained by non-linear least-squares regression using the Solver option in Excel Microsoft.

10.2.4 High-pressure sterilization

A high pressure multivessel system (Apparatus U111 Unipress, Warszawa, Poland) was used for the HPS experiments. The unit is coupled with a thermostat (Lauda Proline RP 855 Low Temperature, Lauda-Königshofen, Germany), allowing the simultaneous application of temperature and pressure up to 120˚C and 600 MPa. Details of the HPS experiments were given in Chapter 6 (Section 6.2.3).

One set of conditions resulted in equivalent microbial safety (7-log reduction of *B. amyloliquefaciens*) obtained with and without nisin. The best conditions were then selected for comparison with the equivalent UHT process in terms of CLA retention and hydroperoxide formation. The comparison was performed after 7, 15, 30 and 60 days of storage at 25˚C.

10.2.5. Analytical determinations

10.2.5.1. Fatty acid determination

The fatty acid profile of CLA-enriched milk was determined by GC, following the methodology described in Chapter 3 (Section 3.2.5).

10.2.5.2. Hydroperoxides determination

Lipid hydroperoxides were determined as described in Chapter 5 (Section 5.2.4).

10.3. Results and discussion

10.3.1. Spore inactivation

Study 1 – The effect of temperature (90-120˚C) and pressure (100-600 MPa) on the survival of *B. amyloliquefaciens* endospores in CLA-enriched milk is shown in **Fig 10-1**. The untreated milk sample had an initial endospore count of 7-log (horizontal continuous line in **Fig 10-1**). At 90˚C after 15 min of holding time, the number of survivors was high with inactivation values of less than 2-log reduction, regardless of the pressure applied (100-600 MPa). Increasing the holding time to 30 min, the survivors were still high with inactivation values of less than 3-log reduction. Similarly, 3-log reduction of the same endospore (*B. amyloliquefaciens*) for egg patty mince treated at 95˚C and 600 MPa for 10 min was reported by Rajan et al. (2006). But, Hofstetter et al. (2013) reported 1-log reduction for a suspension of *B. amyloliquefaciens* endospore treated at 90˚C and 600 MPa for 30 min of holding time.

As the temperature was increased to 100˚C, the number of surviving endospores was gradually reduced with an increase in pressure. After 15 min of holding time, the number of survivors was reduced by ~2-log and 5-log reduction at 100 and 600 MPa, respectively. After 30 min of holding time, the log reductions of endospores were ~2, 4 and 5 at 100, 300, and 600 MPa, respectively. Interestingly, for samples heated at 120˚C and held for either 15 or 30 min, the survivors were below the detection limit, regardless of the pressure applied. The 7-log reduction obtained means that sterilization conditions have been achieved and the combinations of high pressure (>100 MPa) and high temperature (>100˚C) induced structural and physiological damage to the cell. The structural damage adversely affects cellular physiology, including metabolism, membrane function, compound transport, transcription, and translation as described by Hofstetter et al. (2013).

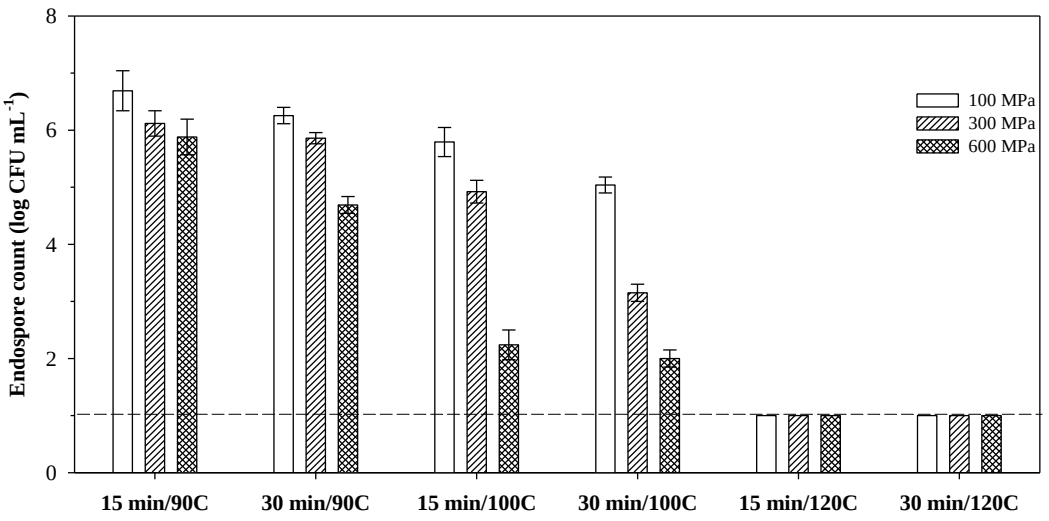

Fig 10-1. Endospore survivors of *B. amyloliquefaciens* in CLA-enriched milk treated at different high-pressure sterilization conditions. The horizontal continuous line represents the endospore count of untreated milk samples. The horizontal broken line indicates endospore counts below the detection limit.

Inactivation kinetic data of *B. amyloliquefaciens* (Rajan et al., 2006) were obtained from http://www.neptune-pt.com (continuous line in **Fig 10-2)**. The right side of the contour plot in **Fig 10-2** indicates those conditions at which 7-log reduction can be obtained after 15 min of holding time. On the left side are the conditions that were not severe enough to reduce *B. amyloliquefaciens* by 7-log. The X marks represent the conditions used for HPS treatments of CLA-enriched milk in this study (**Fig 10-2)**. Among all the pressure (100-600 MPa) and temperature (90-120˚C) conditions evaluated in this study, a sterile milk product can be obtained at 120˚C and 100-600 MPa after 15 min of holding time.

Study 2 – Considering that: i) sterilized milk was obtained at 120˚C based on the results of Study 1, and ii) a holding time of 15 min is rather long from an industrial perspective, further studies on inactivation of *B. amyloliquefaciens* endospore were conducted at a constant temperature of 120˚C while varying pressure (100, 300 and 600 MPa) and holding time (1, 3, 5 and 10 min). In this

case, the starting point (time = 0 min) of the inactivation was considered when both isothermal and isobaric conditions were achieved at ~30 s after compression. Inactivation of *B. amyloliquefaciens* at 0.1 MPa was also evaluated for comparison.

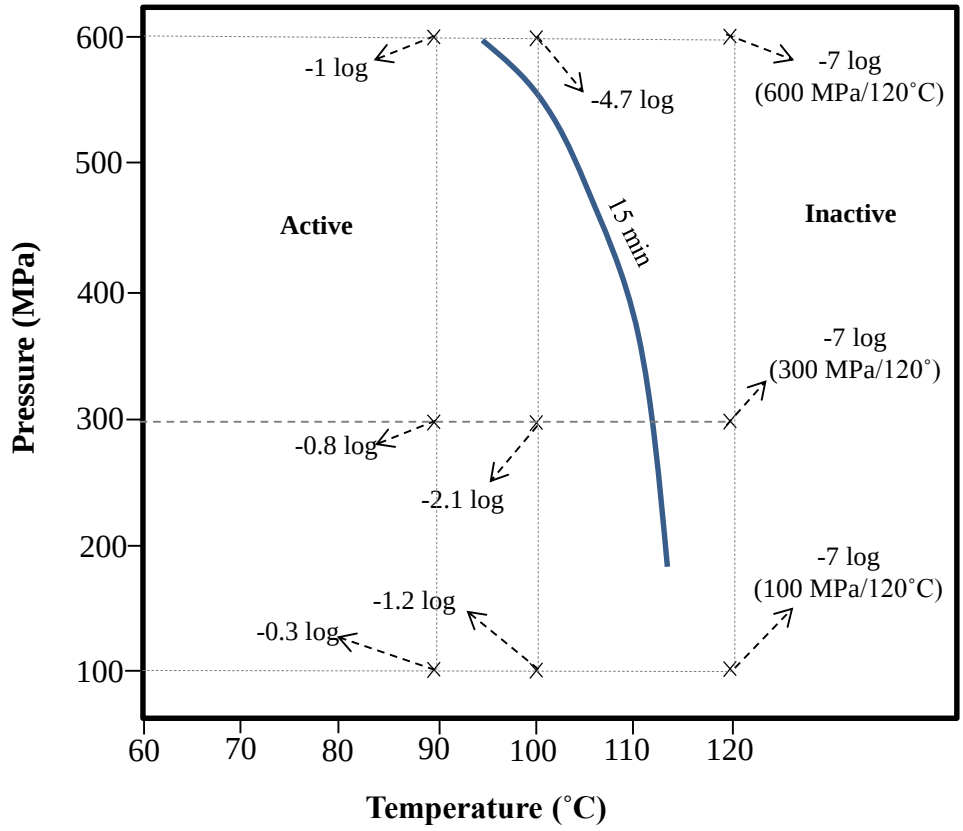

Fig 10-2. Pressure-Temperature diagram for 7-log reduction of *B. amyloliquefaciens* after 15 min of holding time (blue curve: data from Rajan et al., 2006; http://www.neptune-pt.com). The X marks represent the HPS conditions used to treatCLA-enriched milk.

Interestingly, a clear tailing was observed for the pressurized samples in **Fig 10-3**. The exact mechanism of tailing is unknown but it is thought to be caused by heterogeneous resistance of the spore population. At 100-300 MPa and 120˚C (**Fig 10-3**), the endospore inactivation showed pressure-resistant effect in comparison with that obtained at 0.1 MPa (quasi linear trend). But, milk samples pressurized at 600 MPa showed faster inactivation compared with samples treated

at other pressures, reaching ~5-log reduction. Similarly, Rajan et al. (2006) reported 7-logs reduction of *B. amyloliquefaciens* after 3 min of holding time at 121˚C and 600 MPa in egg patty mince.

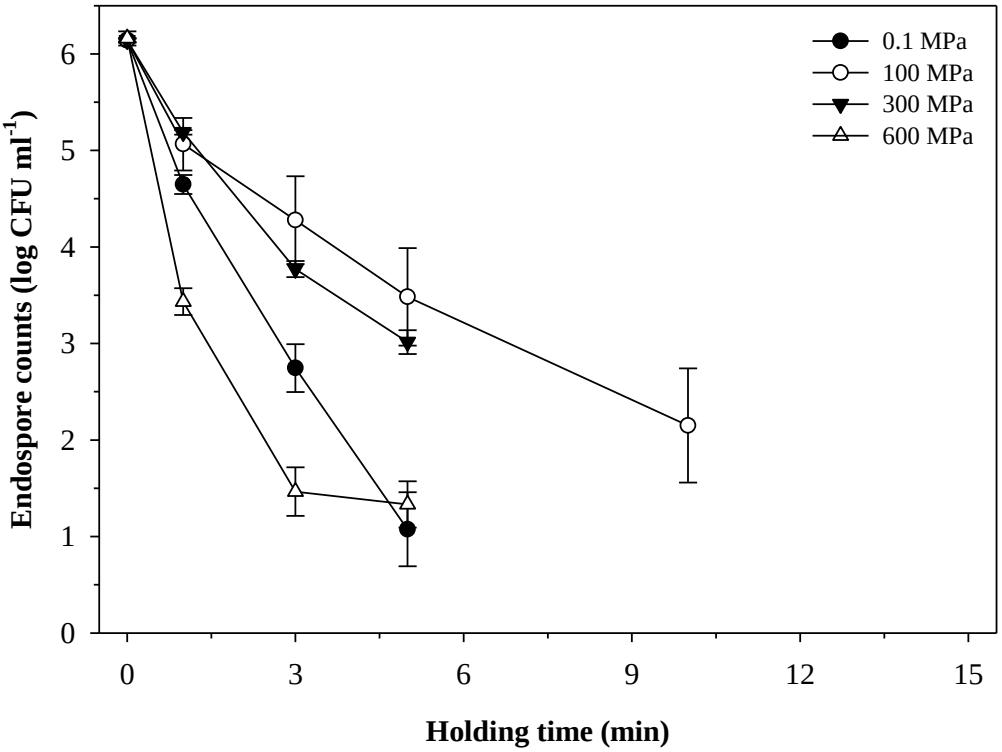

Fig 10-3. Survival of *B. amyloliquefaciens* endospore in CLA-enriched milk treated at 120˚C and different pressures. Endospore counts below the detection limit (<1-log) were not included.

Study 3 – The effect of different concentrations of nisin on the endospore counts are shown in **Fig 10-4**. Endospore counts were reduced significantly as the concentration of nisin increased until the counts of endospore were below the detection limit (<5 min and 16 mg nisin L^{-1} milk). The addition of nisin at low concentrations (4 and 8 mg L^{-1}) was not effective for endospore inactivation. The count after 5 min of holding time at 120˚C and 600 MPa was 1.1 ± 0.2 (**Fig 10-3**) while at the same conditions, the samples containing 4 mg nisin L^{-1} milk yielded endospore counts of 2 ± 0.2 (**Fig 10-4**). However, at a concentration of 16 mg nisin L^{-1} milk, the endospore counts were below the detection limit after 5 min at

120˚C and 600 MPa (**Fig 10-4**). For those samples added with 32 and 64 mg nisin L^{-1} milk, the reduction was evident, showing endospore counts below the detection limit after only 3 min of holding time. Hofstetter et al. (2013) used a concentration of 16 mg L^{-1} of nisin to enhance the inactivation of *B. amyloliquefaciens* in model systems treated at 90˚C and 600 MPa. This concentration represents an excess (16-fold) of the minimum concentration needed to inactivate the vegetive cells of *Lactobacillus sanfranciscensis* (Hofstetter et al., 2013). The mode of action of nisin has been exemplified by Hofstetter et al. (2013), who measured *in situ* membrane fluidity and found that nisin forms pores within the inner membrane, which restricts the membrane's fluidity and consequently inhibiting the cell wall synthesis.

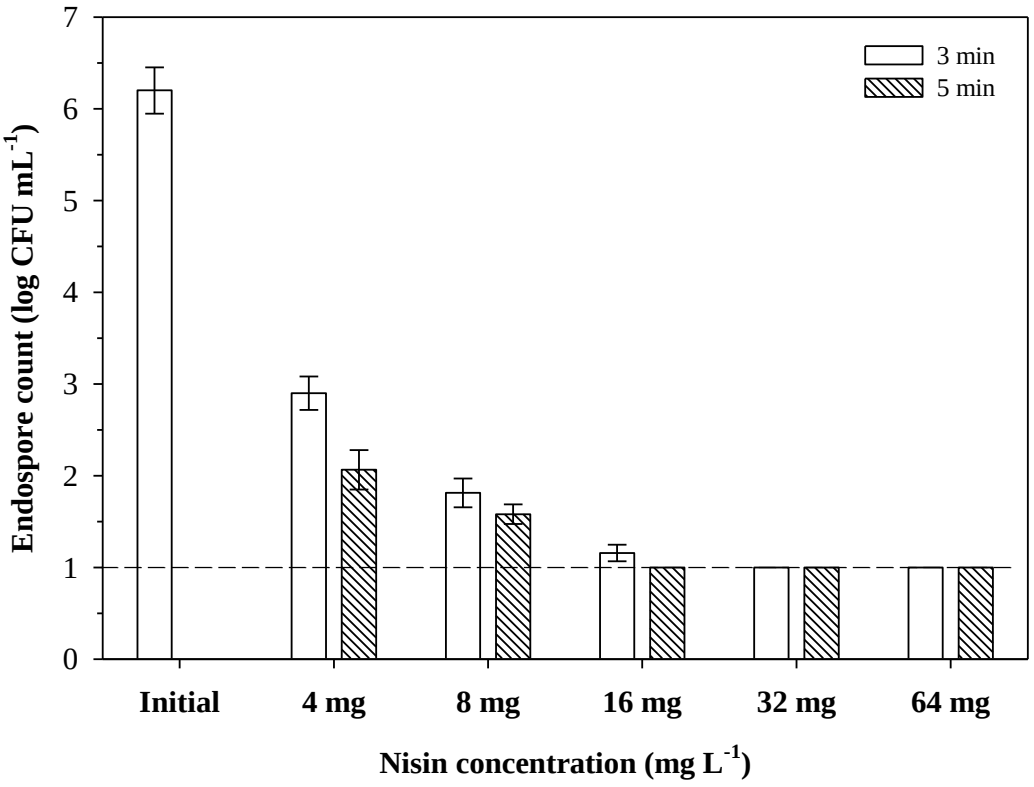

Fig 10-4. Effect of nisin concentration on the endospore counts of *B. amyloliquefaciens* in CLA-enriched milk treated at 120˚C and 600 MPa at different holding times. The horizontal broken line indicates endospore counts below the detection limit.

10.3.2. ALP inactivation

Fig 10-5 shows ALP inactivation in milk at pressures of 100-600 MPa and temperatures of 60-90˚C with holding times of up to 15 min. At pressures of 100-600 MPa, the reduction in the ALP activity was greater at 90˚C (**Fig 10-5c**) compared to that at 80˚C (**Fig 10-5b**) and 60˚C (**Fig 10-5a**). The ALP inactivation in milk followed a first-order kinetic model in the temperature range of 60-90˚C, regardless of the pressure applied (100-600 MPa). The reduction of ALP activity in milk has also been described by a first-order model in the temperature and pressure ranges of 43-63˚C and 0.1-725 MPa, respectively (Mussa & Ramaswamy, 1997; Ludikhuyze et al., 2000;). But, Rademacher & Hinrichs (2006) described the inactivation of ALP with a reaction order of 1.5 in the temperature range of 5-40˚C and the pressure range of 200-800 MPa.

The determination of the ALP activity is a routinely performed assay to evaluate the effectiveness of pasteurization (inactive to 350 U mL^{-1}). Activity values below 350 U mL^{-1} can be considered as residual activity. In the temperature range of 100-120˚C, residual activity of 185-22 U mL^{-1} was obtained for holding times of less than 7 min (not included in the model). Data presented in **Fig 10-5** was used to calculate the *k* values summarized in **Table 10-1**. The *k* values obtained at 90°C (0.26-1.95 min^{-1}) were the highest followed by values obtained at 80°C (0.19-0.49 min^{-1}) and at 60°C (0.012-0.307 min^{-1}), changing with pressure. The temperature range tested (60-90°C) in this study represents the temperature span at which the ALP is inactivated in milk at 0.1 MPa (60-86˚C) (Ludikhuyze et al., 2000). The *k* values in the pressure range of 300-400 MPa decreased for 80-90°C. Similar effects of temperature on the k values have been reported in pressure-temperature inactivation of enzymes (Ludikhuyze et al., 2003).

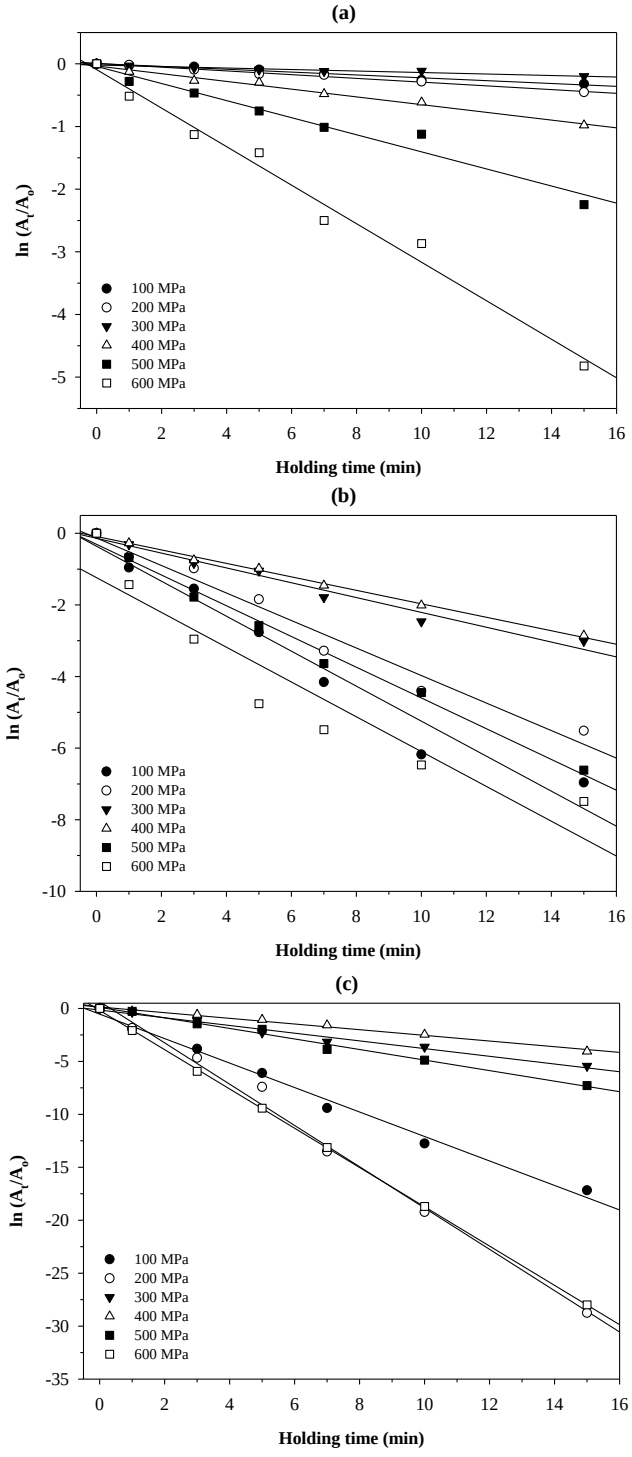

Fig 10-5. First-order pressure and temperature inactivation kinetics of ALP in milk treated at high pressures and (a) 60˚C, (b) 80˚C, and (c) 90˚C.

Table 10-1. First-order inactivation rate constant (*k*) for the pressure-temperature inactivation of ALP in milk.

Pressure	**60˚C**			**80˚C**			**90˚C**		
(MPa)	***k***	**SE**	**R^2**	***k***	**SE**	**R^2**	***k***	**SE**	**R^2**
100	0.02	0.002	0.97	0.49	0.046	0.96	1.15	0.048	0.99
200	0.03	0.001	0.99	0.38	0.029	0.97	1.95	0.071	0.97
300	0.01	0.001	0.94	0.21	0.015	0.97	0.36	0.023	0.98
400	0.06	0.003	0.98	0.19	0.006	0.99	0.26	0.012	0.97
500	0.13	0.012	0.96	0.43	0.019	0.99	0.49	0.023	0.97
600	0.31	0.017	0.98	0.48	0.075	0.89	1.18	0.010	0.98

k – first-order inactivation rate constant (min^{-1}); SE – standard error; R^2 – coefficient of determination

For each pressure used, the E_a value was calculated within the temperature span of 60-90˚C (**Table 10-2**). The E_a values obtained (41-155 kJ mol^{-1}) were calculated with standard errors of 8-21. Nevertheless, these values (41-155 kJ mol^{-1}) are comparable to those (50-415 kJ mol^{-1}) reported for inactivation of ALP in milk by Ludikhuyze et al. (2000). The $\Delta V^{\neq}$ values obtained (-14 to -30 cm^3 mol^{-1}) are consisted with those reported by Rademacher & Hinrichs (2006) at -58 to -32 cm^3 mol^{-1}, and Ludikhuyze at al. (2000) at -63 to -23 cm^3 mol^{-1}.

Inactivation of enzymes by a combination of high pressure and temperature is a complex phenomenon. The primary structure of the enzyme is minimally affected by pressure while the secondary structure suffers structural modifications only at very high pressures. The tertiary structure is greatly affected by pressure. This is because pressure disrupts the hydrophobic and electrostatic interactions, collapsing the well-organized structure (Ludikhuyze et al., 2003). Consequently, water solvates the exposed charge groups, leading to a volume reduction that inactivates the enzymes (Ludikhuyze et al., 2003).

Table 10-2. Activation energy and activation volume for the pressure-temperature inactivation of ALP in milk.

Pressure (MPa)	E_a **(kJ mol^{-1})**	**SE**	**R^2**
100	154.68	15.79	0.982
200	139.16	10.91	0.991
300	117.47	18.79	0.964
400	43.73	20.71	0.915
500	41.31	17.91	0.823
600	60.17	8.31	0.991
Temperature	$\Delta V^{\neq}$ **(**cm^3 mol^{-1}**)**	**SE**	**R^2**
60˚C	-13.92	1.94	0.954
80˚C	-25.06	1.55	0.991
90˚C	-30.06	8.35	0.934

Ea – activation energy; $\Delta V^{\neq}$ - activation volume; SE – standard error; R^2 – coefficient of determination

The experimental *k* values were modelled using equation (10.4) and the estimated parameters are summarized in **Table 10-3**. Although equation (10.4) can be used to explain the variability of the experimental data, the estimated parameters had errors larger than their absolute values, indicating that these parameters are not significantly different from zero and therefore not suitable for prediction. Alternatively, an empirical equation (10.5) was used for modelling the k values as a function of temperature and pressure.

$$\ln k = a + b \cdot \left(P - P_{ref}\right) + c \cdot \left(T - T_{ref}\right) + d \cdot \left(P - P_{ref}\right)^2 + e \cdot \left(T - T_{ref}\right) \cdot \left(P - P_{ref}\right) + f \cdot \left(T - T_{ref}\right)^2 \quad (10.5)$$

Table 10-3. Estimated parameters for the thermodynamic and empirical models to describe the pressure-temperature inactivation of ALP in milk.

Thermodynamic model (equation 10.4)			Empirical model (equation 10.5)		
Parameter	**Value**	**95% CI**	**Parameter**	**Value**	**95% CI**
k_0	2.9×10^{-5}	4.8×10^{-5}	a	-1.88	6.1×10^{-5}
$\Delta V_o^{\neq}$	-67.18	101.44	b	0.0016	3.6×10^{-7}
$\Delta S_o^{\neq}$	0.093	0.047	c	0.09	4.9×10^{-6}
$\Delta k_o^{\neq}$	-0.25	0.21	d	2.4×10^{-5}	1.6×10^{-9}
$\Delta \varepsilon_o^{\neq}$	0.911	0.41	e	-0.0002	2.8×10^{-8}
$\Delta Cp_o^{\neq}$	122.19	150.31	f	-0.0003	3.2×10^{-7}
R^2	0.957		R^2	0.955	

$\Delta V_o^{\neq}$, $\Delta S_o^{\neq}$, $\Delta k_o^{\neq}$, $\Delta \varepsilon_o^{\neq}$ $\Delta Cp_o^{\neq}$ are the volume, entropy, compressibility, expansibility and heat capacity change with respect to native state of the enzyme, respectively; a-f are regression coefficients of equation (10.5); 95% CI - 95% confidence interval; R^2 – coefficient of determination.

This equation has been used for modelling the pressure-temperature inactivation of ALP in milk (Ludikhuyze et al., 2003). The estimated parameters are provided in **Table 10-3**. The error associated with the estimation of the parameters was much lower than the absolute value. These parameters are comparable to those calculated by Ludikhuyze et al. (2003) **(Table 10-3)**.

Fig 10-6 shows the pressure-temperature diagram for the inactivation of ALP in milk using equation (10.5). After 3 min of holding time, the combinations of pressure and temperature needed to inactivate 90 and 99.9% of the ALP activity are illustrated. The regions within the dashed and continuous lines represent the residual activity of ALP. At 120˚C, there was no residual activity of ALP and therefore cannot be used as a sterilization marker. However, in cases where a significant log reduction is obtained in a temperature span of 100 to 110˚C, the residual activity can potentially be used as a sterilization marker. Rajan et al. (2006) reduced 2- and 4-log of *B. amyloliquefaciens* at 95˚C/600 MPa and 105˚C/600 MPa after 3 min, respectively. At such conditions, the contour line of

the residual activity of ALP is close to the processing conditions needed to achieve 2- and 4-log reduction in this study. If the milk product is processed until there is no residual activity, the reduction of 4-log would have been achieved. The use of residual activity as a sterilization marker shows potential in a narrow region and its implementation requires further research.

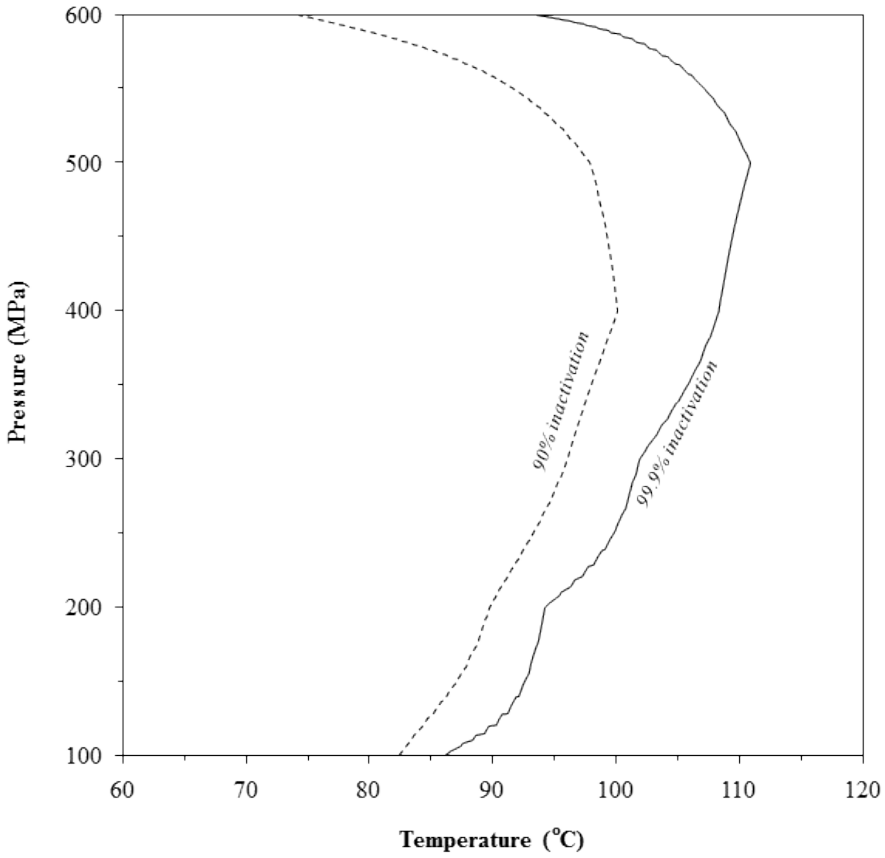

Fig 10-6. Pressure-temperature diagram for the inactivation of alkaline phosphatase (ALP) in milk. The contour lines represent the combinations of pressure and temperature after 3 min of holding time needed to inactivate 90% (dashed line) and 99.9% of the ALP activity (continuous lines).

10.3.3. Oxidative stability during storage

The impact of HPS on the oxidation of milk fat was monitored for 0, 7, 15, 30 and 60 days of storage at 25˚C. Two equivalent combinations (pressure, temperature and time) were selected for the storage study based on the

inactivation of *B. amyloliquefaciens*. The first combination was 600 MPa and 120˚C for 10 min (HPS-Milk). The second combination was 600 MPa and 120˚C for 5 min for milk with added nisin (16 mg g^{-1}, HPS-nisin). Both combinations yielded 7-log reduction of *B. amyloliquefaciens* as observed in **Figs 10-3** and **10-4**. In addition, two combinations of ultra-high-temperature (UHT) pasteurization were used for comparison (125˚C/15 s, UHT-125˚C, and 135˚C/10 s, UHT-135˚C) (data from Chapter 5).

Fig 10-7 shows the remaining CLA during storage for HPS and UHT processes. A storage time of zero represents the remaining CLA inmediately after HPS or UHT processing. At a storage time of 0 d, 88 ± 4% of the CLA was retained in those samples with added nisin. Interestingly, the combination of 600 MPa and 120˚C for 10 min, yielded CLA retention of 80 ± 1.92%, which was comparable to the retention obtained for UHT-125˚C and UHT-135˚C (82 ± 2 and 77 ± 2%, respectively). Similarly, Herzallah et al. (2005) retained 85% of the initial CLA concentration (5.6 mg g^{-1}) in UHT milk (140˚C/4 s). But, Campbell et al. (2003) found that pasteurization (77˚C/16 s) did not change the CLA content in fortified milk (2% of CLA from the total fat).

During storage, the remaining CLA decayed in an exponential fashion for all treatments. After 60 d of storage, 44.4 ± 0.5% of the CLA was retained in those samples with added nisin and treated at 600 MPa and 120˚C for 10 min while only 36 ± 1% was retained in milk samples without nisin. The remaining CLA values obtained for UHT-125˚C and UHT-135˚C were similar (26.0 ± 1.5 and 33.2 ± 2.6%, respectively) to those milk samples treated by HPS. The CLA can be oxidized due to the presence of a conjugated double bond system (Zhang & Chen, 1997), which might act as an active site for free radical reactions. An investigation on the kinetics oxidation of conjugated and non-conjugated fatty acids showed that the fatty acids in the conjugated form oxidized faster than the non-conjugated fatty acids (Tsuzuki et al., 2004). The non-isothermal oxidation of anhydrous milk fat with different CLA concentrations showed that the start

temperature decreased as the concentration of CLA increased, indicating that CLA is susceptible to oxidation (Chapter 4).

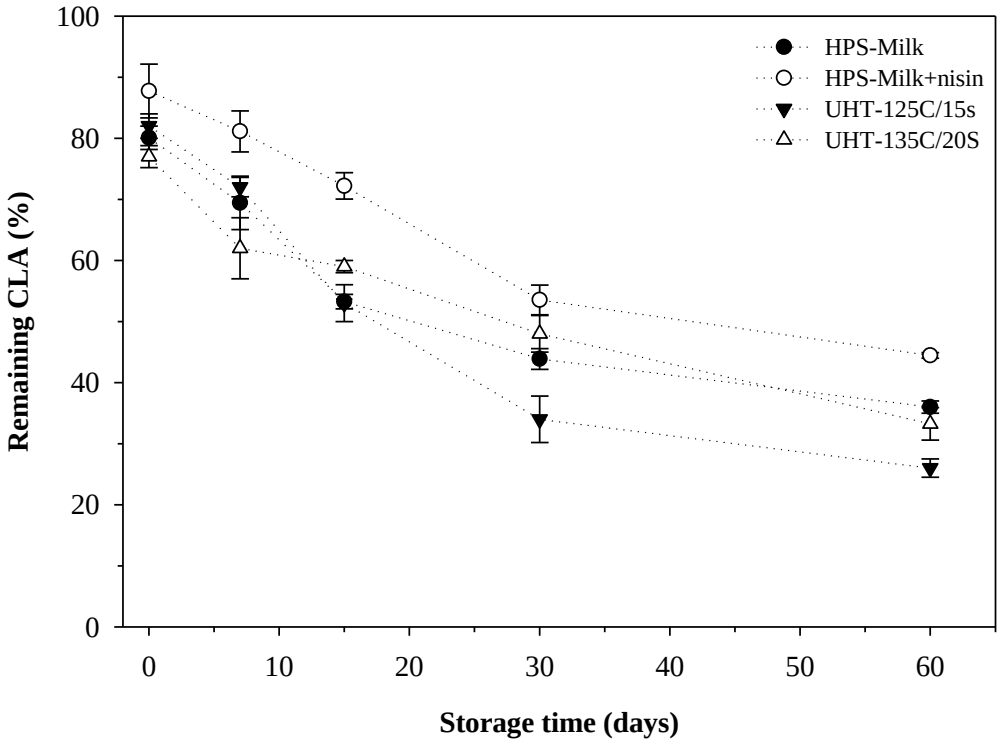

Fig 10-7. Process comparison of the remaining conjugated linoleic acid (CLA) in HPS and UHT treated milk during storage at 25˚C (HPS-Milk – milk rich in CLA treated at 600 MPa, 120˚C for 10 min; HPS-nisin – milk rich in CLA + 16 mg nisin L^{-1} milk treated at 600 MPa, 120˚C for 5 min; UHT-125˚C – milk rich in CLA treated at 125˚C for 15 s; UHT-135˚C – milk rich in CLA treated at 135˚C for 10 s). UHT data taken from Chapter 5 for comparison.

The formation of hydroperoxides, as primary oxidation products, was monitored during storage of milk treated with HPS and UHT processes (**Fig 10-8**). After processing at 0 day of storage, the hydroperoxide values were similar for both processes within a narrow range (5.7-14.2 mmol mL^{-1}). The concentration of hydroperoxides became notorious after 15 d of storage, especially for the UHT-125˚C (87.17 ± 1.52 mmol mL^{-1}) and UHT-135˚C (25.92 ± 2.91 mmol mL^{-1}) milk samples.

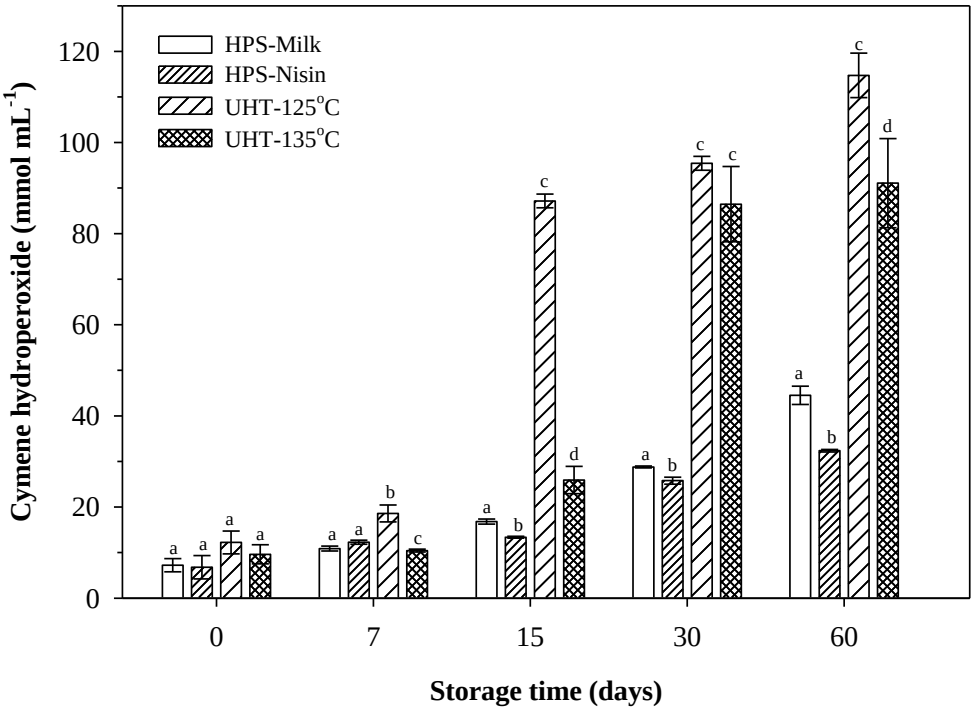

Fig 10-8. Formation of hydroperoxides during storage in milk rich in CLA treated with HPS and UHT processes. (HPS-Milk – milk treated at 600 MPa and 120˚C for 10 min; HPS-Nisin – milk + 16 mg nisin L^{-1} milk treated at 600 MPa and 120˚C for 5 min; UHT-125˚C – milk treated at 125˚C for 15 s; UHT-135˚C – milk treated at 135˚C for 10 s). UHT data from chapter 5. Bars with different letters (a-d) are significantly different ($p<0.05$).

Overall, for HPS-nisin, the concentration of hydroperoxides was lower (5-32 mmol mL^{-1}) compared with the HPS-Milk (5-32 mmol mL^{-1}), the UHT-125˚C (12-114 mmol mL^{-1}), and the UHT-135˚C (9-91 mmol mL^{-1}). Although the HPS-

Milk yielded a comparable retention of the CLA (80 ± 2%) to those obtained for the UHT-125˚C (82 ± 2%) and the UHT-135˚C (77 ± 2%), the concentration of hydroperoxides was lower for the HPS-Milk (44 ± 2 mmol mL^{-1}) compared to the UHT-125˚C (115 ± 5 mmol mL^{-1}) and the UHT-135˚C (91 ± 9 mmol mL^{-1}). The relative low concentration of hydroprexides in the HPS-Milk can be explained by the effect of pressure on the oxidation pathway. The oxidation of methyl-conjugated linoleate followed Diels Alder-type reaction rather than abstraction (Hamalainen et al., 2001). Interestingly, Diels Alder reactions are cycloaddition in which the cyclic compound possesses a smaller partial molar volume than the acyclic counterpart and therefore promoted by pressure (Fernandez-Garcia et al., 2009). Earlier, Isaacs (1981) compiled $\Delta V^{\neq}$ values of -30 to -40 cm^3 mol^{-1} for Diels Alder reaction. Our hypothesis is that pressure favours the oxidation pathway through Diels Alder reaction, forming cyclic hydroperoxides, which were not detected with the spectrophotometric technique used.

10.4. Conclusions

The used of HPS conditions (120˚C/600MPa) and nisin (≥16 mg L^{-1}) inactivated *B. amyloliquefaciens* (7-log reduction) within only 5 min of holding time, which is in the range of holding times likely to be implemented by the industry. Such holding time allows obtaining milk with high retention of CLA (for instance, 72.2 ± 2.1% of retention after 15 d of storage) and less degree of oxidation (13.3 ± 0.5 mmol mL^{-1}) compared to the equivalent UHT (for instance at 125˚C/15 s, 53.1 ± 3.1% of CLA was retained and the concentration of hydroperoxides was 87.2 ± 1.5 mmol mL^{-1}). The inactivation of ALP followed a first-order model and its residual activity showed potential for its use as a sterilization indicator within a narrow range of conditions. Other reactions, such as Maillard and formation of off-flavour compounds need to be assessed for developing high-pressure sterilized milk.

10.5. References

Ahn, J., Balasubramaniam, V. M., & Yousef, A. E. (2007). Inactivation kinetics of selected aerobic and anaerobic bacterial spores by pressure-assisted thermal processing. *International Journal of Food Microbiology, 113*(3), 321-329.

Bell, J. A., Griinari, J. M., & Kennelly, J. J. (2006). Effect of safflower oil, flaxseed oil, monensin, and vitamin e on concentration of conjugated linoleic acid in bovine milk fat. *Journal of Dairy science*, 88(2), 733-748.

Campbell, W., Drake, M. A., & Larick, D. K. (2003). The impact of fortification with conjugated linoleic acid (CLA) on the quality of fluid milk. *Journal of Dairy Science, 86*(1), 43-51.

Fernandez-Garcia, A., Butz, P., Corrales, M., Lindaur, R., Picouet, P., Rodrigo, G., et al. (2009). A simple coloured indicator for monitoring ultra high pressure processing conditions. *Journal of Food Engineering, 92*(4), 410-415.

Goff, H. D., & Griffiths, M. W. (2006). Major advances in fresh milk and milk products: Fluid milk products and frozen desserts. *Journal of Dairy Science, 89*(4), 1163-1173.

Hamalainen, T. I., Sundberg, S., Makkinen, M., Hase, T., & Hopia, A. (2001). Hydroperoxide formation during autoxidation of conjugated linoleic acid methyl ester. *European Journal of Lipid Science and Technology*, 588-593.

Herzallah, S. M., Humeid, M. A., & Al-Ismai, K. M. (2005). Effect of heating and processing methods of milk and dairy products on conjugated linoleic acid and trans fatty acid isomer content. *Journal of Dairy Science*, 1301–1310.

Hofstetter, S., Gebhardt, D., Ho, L., Gänzle, M., & McMullen, L. M. (2013). Effects of nisin and reutericyclin on resistance of endospores of Clostridium spp. to heat and high pressure. *Food Microbiology, 34*, 46-51.

Hofstetter, S., Winter, R., McMullen, L. M., & Gänzle, M. G. (2013). In situ determination of clostridium endospore membrane fluidity during pressure-assisted thermal processing in combination with nisin or reutericyclin. *Applied and Environmental Microbiology, 79*(6), 2103–2106.

Isaacs, N. S. (1981). *Liquid phase high pressure chemistry*. Toronto: John Wiley.

Knoerzer, K., Buckow, R., & Versteeg, C. (2010). Adiabatic compression heating coefficients for high-pressure processing — A study of some insulating polymer materials. *Journal of Food Engineering*, *98*(1), 110–119.

Ludikhuyze, L., Claeys, W., & Hendrickx, M. E. (2000). Combined pressure-temperature inactivation of alkaline phosphatase in bovine milk: A kinetic study. *Journal of Food Science*, *65*, 155-160.

Ludikhuyze, L., Van Loey, A., Indrawati, Smout, C., & Hendrickx, M. (2003). effects of combined pressure and temperature on enzymes related to quality of fruits and vegetables: from kinetic information to process engineering aspects. *Critical Reviews in Food Science and Nutrition, 43*(5), 527-586.

Margosch, D., Ehrmann, M. A., Buckow, R., Heinz, V., Vogel, R. F., & Ganzle, M. G. (2006). High-pressure-mediated survival of *Clostridium botulinum* and *Bacillus amyloliquefaciens* endospores at high temperature. *Applied and Environmental Microbiology, 72*(5), 3476–3481.

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

Ostdal, H., Andersen, H., & Nielsen, J. (2000). Antioxidative activity of urate in bovine milk. *Journal of Agricultural and Food Chemistry*, 5588-5592.

Rademacher, B., & Hinrichs, J. (2006). Effects of high pressure treatment on indigenous enzymes in bovine milk: Reaction kinetics, inactivation and potential application. *International Dairy Journal, 16*, 655-661.

Rajan, S., Ahn, J., Balasubramaniam, V. M., & Yousef, A. E. (2006). Combined pressure-thermal inactivation kinetics of bacillus amyloliquefaciens spores in egg patty mince. *Journal of Food Protection, 69*(4), 853–860.

Ratphitagsanti, W., De Lamo-Castellvi, S., Balasubramaniam, V. M., & Yousef, A. E. (2010). Efficacy of Pressure-assisted thermal processing, in combination with organic acids, against *Bacillus amyloliquefaciens* spores suspended in deionized water and carrot puree. *Journal of Food Science, 75*(1), M46-M52.

Rysstad, G., & Kolstad, J. (2006). Extended shelf life milk - advances in technology. *International Journal of Dairy Technology, 59*(2), 85-96.

Tsuzuki, T., Igarashi, M., Iwata, T., Ymauchi-Sato, ,. Y., Yamamoto, T., Ogita, K., et al. (2004). Oxidation rate of conjugated linoleic acid and conjugated. *Lipids*, 475-480.

van Boekel, M. A. (2008). *Kinetic Modeling of Reactions in Foods.* CRC Press, Boca Raton, florida, USA..

van den Ve, C., Courvoisier, C., & Matser, A. (2007). High pressure versus heat treatments for pasteurisation and sterilization of model emulsions. *Innovative Food Science & Emerging Technologies, 82*(2), 230-236.

Van Loey, A., Indrawati, Smout, C., & Hendrickx, M. E. (2003). Inactivation of Enzymes From Experimental Design to Kinetic Modeling. In J. R. Whitaker, A. J. Voragen, & D. S. Wong, *Handbook of Food Enzymology.* Hutgasse: Dekker.

Zhang, A., & Chen, Z. Y. (1997). Oxidative stability of conjugated linoleic acids relative to other polyunsaturated fatty acids. *Journal of the American Oil Chemists Society, 74*(12), 1611-1613.

Chapter 11
Summary and conclusions

A vast amount of literature correlates the CLA consumption with health benefits. More importantly, *trans* fatty acids found naturally in milk are associated with positive health benefits as opposed to those *trans* fatty acids formed during hydrogenation. Thus, obtaining milk with a high CLA content after processing is highly desirable for the dairy industry.

Throughout the chapters of this thesis, the CLA retention in milk after processing was extensively evaluated by means of kinetics studies, which allowed identifying those processing conditions, yielding a specific CLA retention value. Chapter 3 reported a kinetic study of the retention of CLA and its metabolic precursor, *trans*-vaccenic acid, TVA, in heated milk. In milk heated up to 120 ˚C, the CLA content decreased as a function of temperature. A practical interpretation of the kinetic study is illustrated in **Fig 11-1**, where the *ln (t)*, when α is equal to *t* (values used from **Table 3-3**), is plotted against temperature. The lines represent the time needed to reduce 63% of the CLA in milk at a specific temperature. Clearly, CLA retention is observed at low temperatures. Thus, heating milk at low temperatures for long times would result in high CLA retention.

The reduction of CLA in heated milk raises the question whether the CLA content would influence the oxidation kinetics. This question was addressed in Chapter 4, where the oxidation kinetics of anhydrous milk fat with different CLA concentrations was studied. The results obtained showed that the oxidation started at lower temperatures as the concentration of the CLA increased. Interestingly, the estimated value of E_a were higher in milk than those obtained in AMF (151.3 kJ mol^{-1} and 73-146 kJ mol^{-1}, respectively). Such differences were attributed to the adopted methodology and the food matrix. In Chapter 3, milk rich in CLA (~3 mL) was isothermally heated while in the early report anhydrous milk fat (~ 2

mg) was heated under non-isothermal conditions at a constant oxygen flow rate, a procedure that neglected the mass transfer limitations.

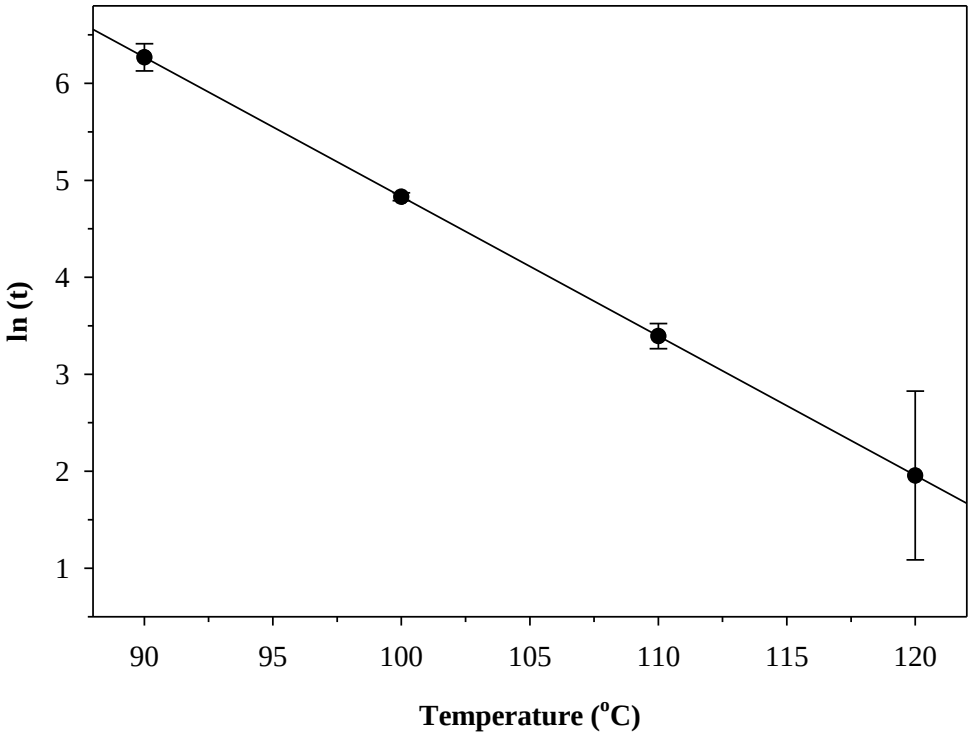

Fig 11-1. Conditions needed to reduce 63% of the CLA in heated milk. Data from Table 3-3.

The effects of UHT conditions (125-145˚C/2-20 s) and subsequent storage on the oxidative stability of CLA-enriched milk was evaluated and reported in Chapter 5. During UHT, CLA losses were minor to moderate (3 to 12% of reduction) depending on the severity of the treatment. However, significant losses occurred upon storage retaining 40% at 135˚C/15 s of the CLA after 60 days. Chapters 3-5 showed that CLA is lost through oxidation during thermal processing, leading to significant losses. The alternative proposed in this thesis consists in the simultaneous application of high pressure and elevated temperature. With this approach, the application of pressure offers three specific advantanges: 1) an instantaneous and homogenous effect, 2) protective effect, and 3) influence on the reaction mechanism.

A screening study was conducted to determine the effect of pressure, temperature and time on the retention of CLA in milk and anhydrous milk fat (Chapter 6). CLA was not stable upon HPS yielding a retention value lower than 10%. Interestingly, the addition of catechin significantly enhanced the retention of CLA (90%), regardless of the processing conditions used. The low retention of CLA challenged the applicability of HPS in CLA-enriched. This question was addressed by treating a new batch of CLA-enriched milk with HPS. It was found that pressure enhanced the retention of CLA even at elevated temperatures (120˚C). The differences in the retained CLA were attributed to sample manipulation and variations between animal to animal.

Chapter 6 provided a detailed characterization of the HPS process. In **Fig 6-2**, the temperature-pressure profile of a typical HPS run was given (120˚C and 600 MPa). The transmition fluid temperature reached 123˚C (3˚C over the working temperature) and evetuanlly the fluid temperature equilibrated the working temperature (120˚C). This is because the rise in temperature during compression not only depends on the sample composition but also on the initial temperature of the product. Such increment in the fluid temperature could have been avoided by adjusting the initial temperature, before pressurization, as function of the target temperature.

A kinetic model was developed for the retention of CLA in milk treated with HPS (Chapter 7). The kinetic model was developed using Weibull probabilistic distribution. The central assumption of the Weibull model is that the oxidation or retention can be viewed as a failure phenomenon where a fraction of an intact reactant is reduced with time at a constant temperature and pressure, independently of the actual reaction mechanism and the whole process is governed by the probability laws. Interestingly, the scale parameter (α) in the developed model was related to the Arrhenius and Eyrign equation, accounting for the temperature and pressure effects. To enhance even further the retention of

CLA, the effect of seven different antioxidants was also evaluated and the results are discussed in Chapter 8. CLA-enriched milk saturated with oxygen was used to evaluate the effectiveness of antioxidants. During HPS, catechin and gallic acid showed the ability to quench dissolved oxygen, yielding ~90% of retention of CLA even at severe processing conditions (120˚C, 600 MPa for 15 min).

The kinetics of lactose isomerization to form lactulose, a processing damage indicator, was also studied. Pressure and temperature acted synergistically on the formation of lactulose (Chapter 9). Finally, Chapter 10 reports the pressure (600 MPa), temperature (120˚C) and time (5 min) needed to inactivate 7-log of *Bacillus amyloliquefaciens*. Upon storage, the high-pressure sterilized milk showed higher retention of CLA and less oxidation compared with the equivalent ultra-high-temperature treatment (UHT, 135˚C for 15 s). The conditions of 120˚C and 600 MPa were chosen because represents a balance between cost and effectiveness.

A practical application derived from the kinetics studies of this thesis (Chapters 6-10) is illustrated in the pressure-temperature diagram of **Fig 11-2**. At a constant holding time of 3 min, equivalent combinations of pressure and temperature that satisfied certain CLA retention are represented by contour lines. In addition, milk treated by HPS can be obtained by fixing the holding time to 3 min at which 7-log reduction of *B. amyloliquefaciens* was achieved. At this holding time, CLA retention as a function of pressure and temperature are shown by contour lines and the symbol (●). Lactulose formation can also be predicted from the kinetics studies and represented by contour lines and the symbol (▲). The conditions at which 99.9% of ALP is inactivated are also shown by contour lines and the symbol (■). Summarizing the kinetics studies undertaken in this thesis, point A indicates the combination of pressure and temperature needed to reduce *B. amyloliquefaciens* in milk with added nisin (16 mg L^{-1}) by 7-log.

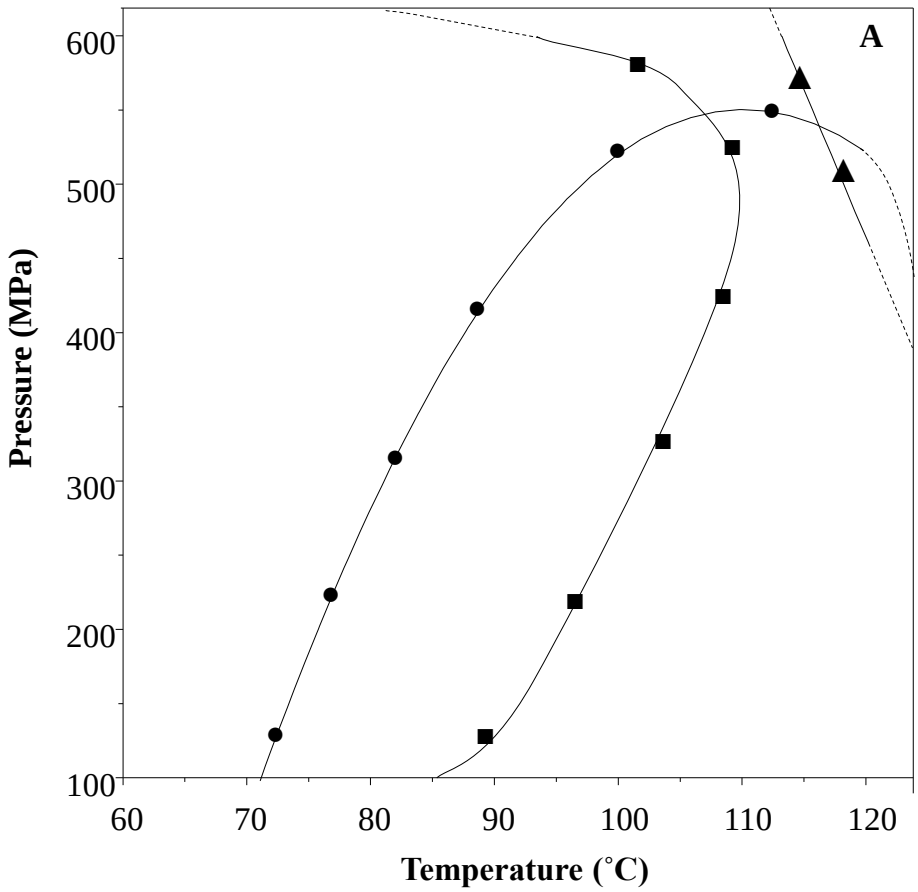

Fig 11-2. Simulated pressure-temperature diagram for milk after 3 min of holding time. (●) 80% of the retention of CLA; (▲) combinations of pressure and temperature yielding 250 mg mL^{-1} of lactulose; (■) conditions needed to inactivate 99.9% of ALP activity. Broken lines represent extrapolated data.

Such milk retains at least 80% of the CLA, with 250 mg mL^{-1} of lactulose, where the ALP was inactive. This thesis generated kinetic data for CLA retention under HPS conditions. The use of phenolic antioxidants not only enhanced the CLA retention but possibly minimized isomerization by quenching the dissolved oxygen. Other milk quality indicators were also evaluated, such as alkaline phosphatase (ALP) and endospore inactivation of *B. amyloliquefaciens*. The experimental processing conditions of 120˚C/600 MPa/5 min were enough to reduce 7-log of *B. amyloliquefaciens* spores (one of the most pressure resistant spores), inactivate ALP and retain 80% of CLA. The outcomes from this study are

of great relevance to develop functional sterile dairy drinks or other novel milk-based beverages.

11.2. Recommendations

The retention of CLA needs further correlation with other thermal damage indicators to establish a minimum retention value for the CLA-enriched milk.

The quantification of CLA isomers at selected conditions can provide additional information for process development. For example, processing conditions of 600 MPa/120˚C/5 min could have different distribution of isomers than the equivalent UHT process (125-145˚C/2-20 s).

The amount of antioxidant that can be added into milk without changing the unique sensory attributes of milk needs to be experimentally determined.

Further development of HPS milk should include other chemical reactions, such as degradation of vitamins, formation of hydroxymethyl furfural and furosine. The formation of toxic compounds, such as acrylamide and oxysterols, also need to be evaluated.

For scaling up, the developed kinetic models need to be coupled with heat and mass transfer models, which will be a significant challenge for future investigations.

More experiments on milk quality at optimized HPS conditions are needed as well as sensory analysis to generate new information for this emerging technology.