University of Alberta

"Effect of Thermal Processing and Pressure Assisted Thermal Processing (PATP) on the Flavor Profile of Conjugated Linoleic Acid (CLA)-Enriched Milk"

by

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Abstract

The effect of Ultra-Pasteurization (125°C/2-15 s), Ultra High Temperature (UHT) (135°C/4-10 s and 145°C/4-20 s) and Pressure Assisted Thermal Processing (PATP) (200-600 MPa, 80-120°C for 3-30 min) on formation of flavor compounds in Conjugated Linoleic Acid (CLA)-enriched milk was studied and analyzed using headspace solid-phase microextraction gas-chromatography and mass-spectrometry. The addition of catechin (0.5 g/kg) in milk treated by PATP (400 MPa/120°C/15 min) was evaluated. Additionally, volatiles after UHT processing were investigated during storage (0-30 days at 25°C and 4°C). Overall, UHT processing had more effect on the formation of flavor compounds than with PATP. Heptanal, 2-heptanone and octanal were the main compounds found in processed CLA-enriched milk. Catechin effectively inhibited the formation of aldehydes and ketones. Finally, hexanal and heptanal decreased over storage time regardless of the storage temperature.

Key words*: catechin, Conjugated Linoleic Acid (CLA), enriched milk, gas-chromarography, milk, UHT, UP, PATP, solid-phase microextraction, flavor compounds.*

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Nomenclature

AFDP	Agri-Food Discovery Place
AMF	Anhydrous Milk Fat
AOAC	Association of Official Analytical Chemists
CANSIM	Canadian Socio-Economic Information Management System
CLA	Conjugated Linoleic Acid
CV	Coefficient of Variation
DI-SPME	Direct Imersion- Solid-Phase Microextraction
DMDS	Dimethyl Disulfide
DMTS	Dimethyl Trisulfide
DVB/CAR/PDMS	Divinlybenzene/Carboxen/Polydimethylsiloxane
FID	Flame Ionization Detector
GC	Gas Chromatography
GC-O	Gas Chromatography-Olfactometry
HHP	High Hydrostatic Pressure
HP	High Pressure
HPLC	High Pressure Liquid Chromatography
HPP	High Pressure Processing
HS-SPME	Headspace Solid-Phase Microextraction
HTST	High Temperature Short Time
IDF	International Dairy Federation
LOD	Limit of Detection
LOQ	Limit of Quantification
MS	Mass Spectrometry
MUFA	Monounsaturated Fatty Acid
NIST	National Institute of Standards and Technology
PATP	Pressure Assisted Thermal Processing
PATS	Pressure Assisted Thermal Sterilization
PEG	Polyethylene Glycol
PFTBA	Perfluorotributylamine
PTFE	Polytetrafluoroethylene
PUFA	Polyunsaturated Fatty Acid
RA	Rumenic Acid
RSD	Relative Standard Deviation
RTL	Retention Time Locking
SAFE	Solvent Assisted Flavor Evaporation
SD	Standard Deviation
SIM	Selective Ion Monitoring
SPME	Solid-Phase Microextraction
UFA	Unsaturated Fatty Acid
UHT	Ultra High Temperature
UP	Ultra Pasteurization
UV	Ultraviolet

Chapter 1. Introduction

1.1 Motivation and research

Currently, consumers are more conscious and aware about the food they eat. They have preferences for more natural, less processed, high nutritional and high sensory quality food products. Interest in CLA has increased by consumers after the discovery of its promising health promoting properties [1-6]. Dairy products are the richest source of CLA in human diet [7-8]. Additionally, CLA enrichment by diet manipulation of dairy cattle has been demonstrated [9]. However, CLA is not stable during heat processing, losing its biological activity [10-14]. Fortunately, CLA was retained during Pressure-Assisted Thermal Processing (PATP) at certain conditions [15]. In addition, CLA is more susceptible to oxidation and off-flavors may be formed during processing and storage. Moreover, flavor profile of raw and processed CLA-enriched milk has not been evaluated.

Further, fresh raw milk has a very unique bland flavor and any changes in flavor profile after processing have an impact on consumer acceptability. Flavor defects or off-flavors can be produced during milk processing [16]. Although there are negligible changes [17] on flavor profile of milk during conventional High-Temperature-Short-Time (HTST) processing, it has a limited shelf life (10-21 days) at refrigeration conditions [18-19]. While Ultra Pasteurized (UP) processing produces a strong “cooked”, ketone-like and caramelized flavor in milk [20], it increases its shelf life to 30-40 days under refrigeration conditions [18-20]. A longer shelf life (>6 months) [21] is achieved in Ultra High Temperature (UHT) processing, nonetheless strong “cooked” and “stale and oxidized” flavor notes are perceived in this milk [20, 22-26]; negatively contributing to the aroma of the UHT milk [27-28]. Pressure-Assisted Thermal Processing (PATP) is an emerging new technology investigated in recent years to improve overall quality in cases where the traditional thermal treatments have failed to deliver high quality products. Only two studies have evaluated the off-flavor formation by PATP technology at high pressure and moderate heating

(<60°C) in non-enriched CLA milk [22, 29-30]. Still, flavor formation in UP, UHT and PATP has not been studied in CLA-enriched milk. Additionally, identification and quantification of these flavor compounds is a challenge due to their high volatility, reactivity, instability and low concentrations.

1.2. Research objectives and outline of the thesis

The main objective of this thesis was to evaluate the flavor profile of CLA-enriched milk after being processed with UP, UHT and PATP. In order to accomplish this main goal, the specific objectives were:

(i) To develop an analytical technique for the identification and quantification of key volatile compounds of raw and treated CLA-enriched milk.

(ii) To evaluate the effect of UP, UHT and PATP on the generation of key volatile compounds in CLA-enriched milk.

(iii) To evaluate the addition of catechin on the formation of the identified volatile compounds at a PATP condition.

(iv) To evaluate the stability of these compounds during storage after UHT treatment.

The outline of the thesis is as follows:

Chapter 1 covers the introduction and objectives.

Chapter 2 is a review of the literature with the primary causes of off-flavor formation in milk, an overview of the effects of thermal processing treatments on the formation of off-flavor compounds in milk and an overview of PATP technology. It also includes potential problems related to CLA-enriched products and a summary of the typical analytical methods for the isolation and quantification of off-flavor compounds in fluid milk.

Chapter 3 focuses on the development of an analytical technique by Headspace Solid-Phase Microextraction Gas Chromatography/Mass Spectrometry–Selective Ion Monitoring (HS-SPM GC/MS-SIM) to identify and quantify main off-flavor compounds generated during thermal and non-thermal processing of CLA-enriched milk. Optimizations of SPME conditions as well as GC conditions were

performed. Parameters, such as linearity, reproducibility, sensitivity and precision were determined in order to validate the analytical method developed.

Chapter 4 presents the effects on the formation of key flavor compounds in CLA-enriched milk at different UHT and PATP processing conditions. Catechin addition to CLA-enriched milk at specific PATP conditions is also evaluated. A storage stability study of CLA-enriched milk treated by UHT was conducted to evaluate the behavior of these flavor compounds.

Chapter 5 summarizes the main conclusions and recommendations of this thesis.

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Chapter 2. Literature review

2.1. Milk overview

2.1.1. Fluid milk and its composition

Milk is one of the most nutritionally complete foods, which has a mildly sweet taste, with a barely perceptible odor and flavor [1]. Milk is a complex system, in terms of its physical and chemical properties. Components of milk are present in three phases. It is a diluted emulsion of fat globules combined with a colloidal suspension of casein micelles; where the aqueous phase is a solution of lactose, whey proteins, mineral, salts and other minor components such as vitamins and antioxidants [2].

As seen in Figure 2.1., milk contains a variety of components which raise its nutritional value [3]. Two main proteins are present in milk: casein and whey [3], casein being in the highest proportion (80%). Another component present in milk is lactose, which is a disaccharide containing glucose and galactose. Glucose, fructose, glucosamine and other sugars are also present in trace amounts [2]. Lactose concentration in milk varies between 3.6-6% (Figure 2.1) Along with the major components present in milk, indicated in Figure 2.1, other minor components are present, affecting nutritional and sensorial properties of milk during its processing.

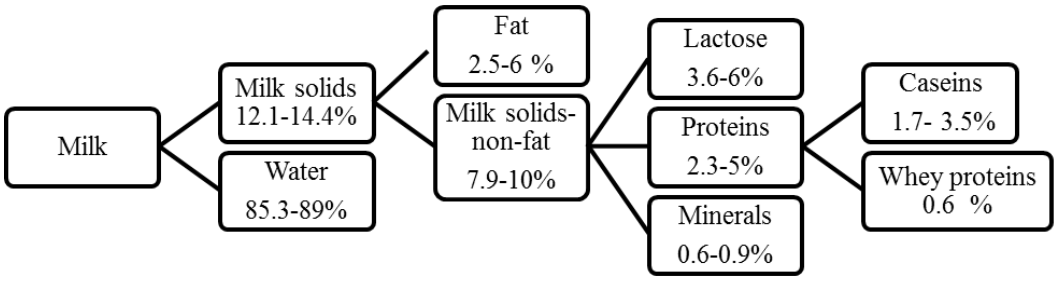

Figure 2.1. Percent composition of cow's milk. Adapted from refs. [3-6].

The fat in the milk is composed of mainly triglycerides, representing around 97% of total lipids (Table 2.1). Other lipid soluble components are present in minor concentrations, including, diglycerides, monoglycerides,

cholesterol, phospholipids, free fatty acids, sterols, carotenoids and fat soluble vitamins (A, D, E and K) [4] [7]. The fat is dispersed in small globules or droplets in the milk serum [4].

Table 2.1. Main classes of lipids in cow's milk.

Lipid class	Amount %
Triglycerides	97-98
Diglycerides	0.36
Monoglycerides	0.027
Cholesterol	0.31
Free fatty acids	0.027
Phospholipids	0.6-0.8

Information collected from refs. [2, 6-8].

More than 400 fatty acids have been identified in milk [7]. Additionally, fatty acid composition can vary in chain length from 2-20 carbon atoms and in saturation from 0 to 4 double bonds [5]. Long chain fatty acids are the most predominant occurring in milk: myristic (11%), palmitic (26%), stearic (12%), and oleic (20%); whereas short-chain fatty acids (butyric, caproic, caprylic and capric) comprise approximately 12% of total fatty acid methyl esters. Oleic acid is the most abundant unsaturated fatty acid in milk fat (Table 2.2). Various bioactive milk components have been found to have potential applications in health promotion, including CLA [9].

Table 2.2. Main fatty acids in cow's milk triglycerides

Fatty Acid (FA)	Carbon atoms: Unsaturation	% total FA content
Saturated		
Butyric	C4:0	3- 4.5
Caproic	C6:0	1.3-2.5
Caprylic	C8:0	0.8- 2.5
Capric	C10:0	1.8 -4.02
Lauric	C12:0	2- 5
Myristic	C14:0	7-11.1
Palmitic	C14:1	25- 29
Stearic	C18:0	9.6-12.2
Unsaturated		
Myristoleic	C14:1	0.8
Oleic	C18:1 cis	17.2-20.4
Oleic trans	C18:1 trans	3.9-5.3
Linoleic	C18:2	1.4- 3
CLA	C18:2 conj	1.1
α-Linoleic	C18:3	1.0

Conj: conjugated; CLA: Conjugated linoleic acid.
Data taken from refs. [4, 6, 10].

2.1.1.1. Milk consumption in Canada

Milk for human consumption should be pasteurized to be safe for the consumers. Fluid milk consumption has been gradually decreasing since 1987. The average consumption in 1990 was 95 liters per person per year, falling to 77 liters per person in 2010, representing a decrease of 19% as observed in Figure 2.2.

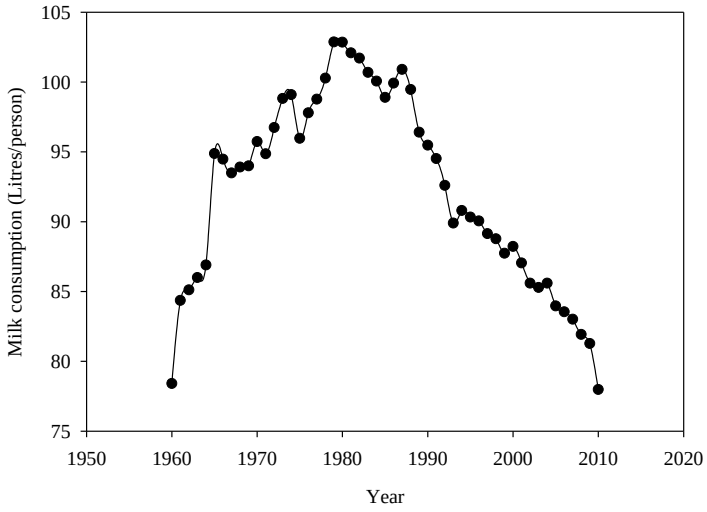

Figure 2.2. Milk consumption per capita in Canada (1960-2010). Source: Statistics Canada Food annual (2010) (liters per person, per year), CANSIM (database).

One of the reasons of this decrease is because of the consumer alertness on the nutritional components of food products and their main benefits in the body due to the high incidence in obesity and chronic diseases. Also, consumers know that besides fluid milk, there are other calcium sources like fortified juices and cereals, processed foods, vegetables and calcium supplements, drinkable yogurt, cheese, among others, which have displaced the milk market over the last few years [11]. Other factors, such as immigration, adoption of Asian diets, which are generally absent of dairy products, lactose intolerance among Middle Eastern populations [11], and promoting the idea of low fat consumption has contributed to the decline in milk consumption.

Milk consumption tendency in future years is projected to increase due to the high percentage of senior population forecast [11]. Therefore, there is an expected change in food demand and upcoming nutritional needs, especially for healthy aging.

According to Nicholson [12], emphasis on food and beverages with "healthy aging" benefits could help the U.S. Dairy business. The rate of decline might be stopped by strategically taking advantage of the current market trends, such as the introduction and promotion of flavored milk, single serving sizes,

creative and flashy packaging, sales in vending machines, school cafeterias and convenience stores [11]. Niche market opportunities are in the development of fortified and/or organic milk, and enriched milk with healthy ingredients.

In 2010, six "mega trends" and market challenges were cited at the Institute of Food Technologists (IFT) Annual Meeting & Food Expo [13]. One of them is the market niche for weight management products. Within this category, ingredients such as fiber, low-glycemic foods and CLA were included. The advantage of the dairy industry is that dairy products have nutritional benefits (proteins for muscle health, CLA, calcium, vitamin D, etc.), which gives an opportunity to this market to grow.

2.1.2. CLA-enriched milk

Dairy and meat products (beef, pork, and poultry) contain the highest amounts of CLA in human diet [14], being milk fat the richest natural source [15].

Linoleic acid (18:2) is an essential polyunsaturated fatty acid, containing 18 carbons and 2 double bonds at the 9 and 12 positions. CLA is a term referred to a collection of positional and geometric isomers of octadecanoic acids with two conjugated double bonds (double bonds adjacent to each other -C=C-C=C-, and not separated by a methylene group) at positions 9 and 11, or 10 and 12 or 11 and 13, and 12-14. Each double bond can be in either the *cis* or *trans* configuration.

The principal isomer of the CLA in milk fat is 9c, 11t-18:2 [10], also known as rumenic acid [16]. It is present in a range of 80-90% from the total CLA isomers [7, 17-18]. It is one of the most biologically active components [19] and the major form of CLA in the human diet [20].

After the discovery of its promising health promoting properties, interest in CLA has increased. CLA has been studied *in vivo* and *in vitro* experiments to confirm its positive effects, including, anticarcinogenic activity [7, 16, 21-27], prevention of cardiovascular diseases, anti-atherogenic properties [7, 16], reduction of body fat [16, 24, 28-29], improvement of the immune system [16, 24], antidiabetogenic activities [7, 22] and anti-inflammatory [24] as well as osteosynthetic effects [16, 24].

Nonetheless, more information is needed to establish a recommended CLA daily intake required by humans. Factors such as variability of CLA in foods, misconception about natural *trans* fatty acids and the *trans fats* generated during processing, and an inadequate food database challenge this assessment to be performed. Ip et al. [25] suggested an intake of 3g of CLA/day in order to obtain its beneficial effects. The average young Canadian intake of *cis* 9, *trans* 11 CLA was 0.095 ± 0.04 g/day [30]; considering that 80% of this isomer is present in total CLA isomers, CLA intake was approximately 0.118g/day.

2.1.3. Factors affecting CLA concentration in milk

There is a wide and natural variation of *cis* 9, *trans* 11 CLA content in raw milk fat. CLA commonly ranges between 3 and 6 mg/g fat [31-33] but earlier studies reported ranges of 2-30 mg/g fat [17]. Generally, milk fat content and fatty acid composition, as well as CLA values are primary influenced by breed of the cow [10, 18, 34-35], diet [9-10, 14, 18, 36], stage of lactation [34, 36], forage preservation and geographical regions [9]. However, diet is known to be the most influential factor on the CLA levels in bovine milk fat [7, 37]. The amount and type of roughage, the forage/concentrate ratio, and the carbohydrate composition of the concentrates and lipids should be considered [37, 38].

Various diets were reported in extensive studies; subsequently the fatty acid composition was determined by Martinez-Monteagudo et al. [39] in non-enriched Anhydrous Milk Fat (AMF) and CLA-enriched AMF produced by feeding the cattle with a specific feed regimen according to the method suggested by Bell et al. [40] with some modifications. Table 2.3 clearly shows the change of 6 times the concentration of CLA milk after enrichment.

Table 2.3. Fatty acids composition (% of total fatty acids) of non-enriched Anhydrous Milk Fat (AMF) and CLA- enriched AMF. Adapted from ref [39].

Fatty acid	Non-enriched AMF	CLA-AMF	Fatty acid	Non-enriched AMF	CLA-AMF
C4:0	0.31	0.21	**C18:1 n7**	21.99	23.56
C5:0	0.02	0.02	**C19:0**	0.45	0.39
C6:0	1.37	0.84	**C18:2 tt**	0.04	0.07
C7:0	0.02	0.02	**C18:2**	1.93	2.39
C8:0	0.91	0.48	**C20:0**	0.14	0.09
C9:0	0.02	0.02	**c18:3 w6**	0.02	0.01
C10:0	2.04	1.05	**C20:1 w12**	0.13	0.07
C11:0	0.26	0.25	**C20:1 w15**	0.04	0.27
C12:0	2.47	1.44	**C18:3 w3**	0.39	0.19
C14:0	8.87	6.74	**CLA**	0.53	3.21
C14:1	0.94	0.69	**C20:2**	0.03	0.02
C15:0	1.05	0.43	**C22:0**	0.03	0.03
C16:0	23.94	18.31	**C20:3 w6**	0.09	0.07
C16:1 t	0.32	0.45	**C20:4**	0.11	0.05
C16:1 c	1.81	1.12	**C20:5**	0.03	0.04
C18:0	9.18	9.53	**C22:3**	0.02	0.03
C18:1 t9	0.38	1.25	**Ratio uns/sat**	0.69	1.40
C18:1 t11	1.89	1.23			

* *t trans* fatty acids, and *c:cis* fatty acids, uns/sat ratio: unsaturated to saturated fatty acids, CLA - AMF. AMF: Anhydrous Milk Fat.

Many dietary manipulation studies suggest the possibility to manufacture CLA-enriched milk and milk products to incorporate them into human diet at levels that may be beneficial to health [40]. CLA-enriched dairy products have been produced for research purposes: butter [21], milk [40], cheese [15], and for commercialization purposes, such as CLA-enriched yogurt launched in the market by "Bles-Wold Dairy Products", and CLA-enriched milk "Vitala milk", containing 30 mg CLA/g milk fat.

2.1.4. Potential problems of CLA-enriched products

Dietary manipulations, besides enhancing rumenic acid levels, also cause a change in the saturated to unsaturated fatty acids ratio in milk fat. Greater proportions of polyunsaturated fatty acids (PUFA) are obtained along with a reduction of saturated fatty acids [41]. Additionally, MUFA and PUFA are more reactive due to their poor oxidative stability than saturated fatty acids and consequently more susceptible to oxidation during processing [34, 41], influencing flavor characteristics [42]. In addition, Yang et al. [43] and Zhang & Chen [44] proved that by increasing the amount of conjugated double bonds, the

less stable is the fatty acid to oxidation. Moreover, conjugated fatty acids are more susceptible to autoxidation than their corresponding non-conjugated ones [43]. Furthermore, increasing temperatures, fats are susceptible to oxidation, generating highly reactive/unstable compounds, such as hydroperoxides [45-46]. This autoxidative product may undergo a secondary reaction, promoting the formation of secondary oxidation products, such as aldehydes and ketones, which are undesirable in milk with some of the formed compounds possibly causing toxicity at certain levels, such as acrolein. These off-flavors, produced due to fatty acid oxidation, are a prime concern; particularly, when the proportion of unsaturated fatty acids increases in milk fat [47].

One of the greatest challenges for the food industry is to process the CLA-enriched milk without negatively altering its numerous properties. CLA content in milk is affected during heat processing at certain conditions [32, 45-46, 48-49] and during refrigeration conditions [46]. Destaillats et al. [50] demonstrated the rearrangement of rumenic acid to the trans-8, cis-10 18:2 isomer in milk fat after heat treatment, which could be attributed to fat oxidation [46] and isomerization [45]. As a result, biological activity of rumenic acid is lost [46].

2.2. Milk processing and its effects on flavor profile of milk

Fresh raw milk has a very unique bland flavor. According to Badings [51], the pleasant mouth feel and creaminess owed to the physical constitution of milk, the sweetness and saltiness taste attributed to lactose and milk salts, and finally, the delicate aroma from several odorous compounds present in low quantities [5], are the main factors responsible of its sensory properties. As well, its unique flavor is due to the combined effect of a great number of compounds from diverse origins [52]. Any change in the described characteristics would be characterized as a defect [53] and is particularly noticeable in the product [1].

Flavor results from compounds which are responsible for taste and odor; however, there are some compounds that give both sensations. Non-volatile compounds are responsible for the taste of a food product (taste perceptions: sour, sweet, salty, umami and bitter). On the other hand, volatile compounds are

responsible for the odor (aroma substances). An aroma substance or taste substance might contribute to the typical aroma or taste of a specific food, whereas in other food might case a faulty odor or taste, resulting in an off-flavor [54].

Flavor defects, malodors or “off-flavors” are sensory attributes that are not associated with the typical aroma and taste of food and beverages. Off-flavors can be produced due to several possible factors: contamination with air, water, packaging, and shipping materials, ingredients, mistakes in processing or generation (chemical or microbial) in the food itself [55]. The off-flavor compounds make the food no longer acceptable for human consumption when they are present in amounts higher than the acceptance threshold (Table 2.4).

Table 2.4. Threshold values of main volatile compounds found in raw and processed milk and aroma descriptors.

Compound	Threshold values	Odor descriptor	Tentative formation from/by:
Hexanal	4.5-10.5 ppb [1](7), 0.05 ppm [2](8), 0.15ppm [3] (8), 0.0045 ppm[1] (10)	Green (1,2,3,6,8), metallic (1), leaves (2,3)	oxidation
Heptanal	3 ppb [1], 0.12 ppm [2] (8) 0.04 ppm [3] (8)	Green (1), fatty (1), oily (2,3), putty (2,3)	fat and oxidation
Benzaldehyde	0.35 ppm [1](10)	Roasted (1), almond (1,2,3,6,9)	lactose, aminoacids
2-Methylbutanal	0.9 ppb (2) , 4 µg/L [1] (10)	Creamy (1), malty (2,3, 9,8) and cocoa (2,3)	milk as a source, milk proteins
3-Methylbutanal	0.04 ppb (2), 0.25-0.35 ppb[1] (7) , 0.2µg/L [1] (10)	Fruity(1), malty (1,2,3,9), cocoa (2,3), dark chocolate (9)	milk as a source, milk proteins
2-Methylpropanal	0.7 ppb (2), 1 ppb [1] (10)	Chemical (1), fat (1), malt (9)	milk proteins
2-Heptanone	5 ppb (2,6)	Mushroom (1,9) and earthy (1); blue cheese (3,9), soapy and fruity (9)	fat and heating
2-Nonanone	5 ppb (2), 3.5 ppm (8)	Milk (1), fatty, ketone (3), earthy, green, fruity and musty (9)	fat and heating
2-Undecanone	NA	Green (1,); ketone, floral (3)	fat and heating
Furfural	3000 ppb (2)	Almond, honey (2,3)	lactose, aminoacids, and heating
Dimethylsulfide (DMS)	2 ppb (2), 20 ppb [2](5), 14 ppb (3), 0.3-2 ppb [1] (7), 1 ppb [1] (10)	Cowy (3), cabbage and sulfurous (8)	milk as s source, light induced, aminoacids, heat
Octanal	0.7 ppb (2), 0.07 ppm [2] (8), 0.46 ppm [3] (8)	Fatty (3,9), green, soapy, ketone, musty (9)	fat and oxidation
Nonanal	1 ppb (2), 0.22 ppm [2] (8), 0.32 ppm 3 (8)	Green (9), Soapy (1), milk (1); citrus and fatty (3)	fat and oxidation
Decanal	0.1 ppb (2), 0.24 ppm [2] (8), 1.0 ppm [3] (8)	Soapy, flowery (9)	oxidation
2-Hexanone	0.4 ppm [2](8)	Fruity, ketone (9)	fat and heating
2-Octanone	0.5 ppm [2](8)	Floral, fruity, soapy, ketone, musty (9)	fat and heating

(1) Ref [56], (2) Ref [57], (3) Ref [51], (4) Ref [58], (5) Ref [59], (6) Ref [55], (7) Ref [60], (8) Ref [61], (9) Ref [62], (10) Ref [63]. Tentative formation found in refs: [51, 58, 61, 64-67]. [1] threshold values measured in water,[2] measured in milk, [3] measured in oil; NA: not available.

According to Calvo & de la Hoz [52], compounds that contribute to the flavor profile of milk can be originated from compounds from the cow's metabolism or feed, compounds produced by chemical reactions due to enzymes and microbial activity, compounds generated during heat treatment, and storage.

Marsili [68], Shipe et al. [69] and Azarra & Campbell [70] listed seven off-flavor descriptors (Table 2.5), depending on the cause of their formation: heated, lipolyzed, microbial, transmitted (from diet), light-induced, oxidized and miscellaneous. It is also reported that before processing, endogenous enzymes and microflora can produce these compounds through chemical reactions [51, 52].

The primary components of milk (fat, protein and sugars) are the most relevant precursors of milk flavor. They are also good substrates for microorganism growth and exogenous enzymes generating off-flavor metabolites. These major components are also susceptible to degradation from light and heat. In addition, the naturally bland flavor of milk is unable to mask these off-flavors [51, 68]. Therefore, milk is very susceptible to formation of off-flavors and malodors.

The majority of the flavor compounds in fresh milk, such as carbonyl compounds, aldehydes, free fatty acids, and sulfur compounds are produced in the cow's metabolism [51] but can also be transferred from the feed of the cow. Other flavor changes can also occur during milking, milk handling, manufacturing, packaging and distribution. Oxygen presence can affect lipid substances and oxidize them. Furthermore, light can be a catalyzer of many reactions, generating off-flavors; its effect on milk depends on the intensity and wavelength of the source, packaging material, and duration of the exposure [51]. Two reaction mechanisms are involved in the formation of light-induced flavors: (i) riboflavin activation by light and further breakdown of serum proteins, generating sulfur compounds, and (ii) light as an effective initiator of lipid oxidation [51].

Table 2.5. Classification of milk off flavors according to Azzara & Campbell [70] and adapted from Badings & Herman [51].

Cause	Descriptive term	Flavor compound involved
Heated	cooked (boiled eggs)	Sulfur compounds,
	caramelized	maltol, furans, pyrazines, other maillard reaction products
	UHT-ketone like	odd-numbered 2 alkanones (C5-C15),
	scorched	charred proteins
Light-induced	light, sunlight, activated	Methyl mercaptan, dimethyl disulfide, 3-methylthiopropanal
	oxidation	Aldehydes and ketones
Lipolysed	rancid, butyric, bitter, goaty	Free fatty acids (C_4-C_{18})
Microbial	acid	Lactic acid, acetic acid
	bitter	Peptides
	fruity	Ethyl butanoate, ethyl 3-methylbutanoate, ethyl hexanoate
	malty, cocoa	2-methylbutanal, 3-methylbutanal
	putrid, spoiled, rotten	Sulfides, thiols, etc
	unclean	Dimethyl Sulfide (DMS)
Oxidized	oxidation (general)	Aldehydes and ketones
	paper, cardboard, metallic	-
	oily-fatty	Vinyl alkyl ketones (C_4,C_5, C_8)
	fishy	2-alkenals (C_7-$_{10}$) and 2,4 alkadienals (C_7,C_{10})
Transmitted	feed, weed, cowy, barny, pungent	DMS, acetone
Miscellaneous	absorbed, bitter, chemical	
	flat, foreign, salty, lack of freshness	
	astringent, dry, chalky	

"Spontaneous oxidation" or lipolytic rancidity has been reported before milk processing during cold storage as a result of lipase action, causing oxidation off-flavors. Lipolytic rancidity refers to a common flavor defect generated from the hydrolysis of fatty acid ester (mostly even-numbered C_4-C_{18}) by the action of lipases [6, 51], creating a "lipolized flavor" [53]. Rancid flavors, produced from fat hydrolysis, can be originated during cold storage and during agitation of raw milk. Oxygen presence during milk manufacturing, such as blending, mixing and pumping also promotes the oxidation of milk fat. In addition, lipid oxidation can occur if milk is in contact with trace metals, or if it is exposed to sunlight and fluorescent light [71].

Secondary oxidation products, such as hexanal and propanal are also found in raw milk. Hexanal comes from cows' feed and/or from light induced lipid oxidation [52, 72]. Acetone, butanone, 2-pentanone, 2-heptanone, 2-nonanone, delta-decalactone, delta-dodecalactone, hexanal, benzaldehyde, hexanoic acid, octanoic acid, decanoic acid, ethanol, diacetyl and ethyl acetate have been identified in raw milk by Scalan et al. [58]. 2-Heptanone, 2-nonanone, 2-undecanone, and2-alkanones (odd numbered C3-C15) are formed by thermal decarboxilation of β-ketoacids naturally present in milk [51].

Many other compounds are present at a very low concentration in raw milk, such as benzaldehyde and diacetyl [52, 72], being lactose their main precursor.

2.2.1. Thermal processing and storage of milk

Raw milk must be pasteurized to be safe for human consumption by destroying microorganisms that may harm human health. Milk is an excellent medium for the growth of pathogens and physical contamination due to its composition. Thus, microbial safety is of paramount importance. A widespread existing method to minimize health risks to consumer and to prolong the shelf life of milk is thermal processing. The shelf life of processed milk is not only directly related to the severity of its processing, the cold chain carried out from producers to consumers and storage conditions but it also depends on the quality of the raw milk. Indeed, milk and milk products are thermal processed to an extent and stored. As milk has very bland flavor, extra care should be provided to prevent formation of off-flavors during processing, and while handling. According to Scalan et al. [58], temperature and time of the heat treatment strongly influence the intensity of the changes in the milk flavor profile [52]; as the heat treatment becomes more severe, cooked, heated and caramelized flavors are developed. Generally, a thermal process involves the following steps:

Figure 2.3. Typical steps for milk thermal processing.

The factors and mechanisms involved in the formation of off-flavors in milk during heat processing have been investigated [72-80]. Proteins, fat and lactose are the major milk components and may be the major precursors of off-flavor compounds. The formation of these off-flavors is mainly due to reactions between major and minor components during processing [52] and storage of milk. Sulphydril compounds are formed during denaturalization of whey proteins from milk proteins and proteinic material present in the milk fat globule membrane [52]. Generally, when milk is heat treated, aldehydes and methyl ketones are formed from milk fat.

Table 2.6 illustrates different milk processing conditions and their overall flavor profile. The step which involves the thermal treatment, pasteurization (Fig 2.4) is the one that differs in each process showed in Table 2.6. The shelf life varies depending on raw milk quality, processing conditions, microbial growth, packaging materials, temperature abuse, and exposure to light [4, 81].

Thermisation process refers to the pre-heating of the milk to a temperature below pasteurization temperature and a rapid chill to 4°C to inhibit bacterial growth in a short time (24hrs). This process is commonly used at dairy industries when it is difficult to pasteurize all the milk just after reception [4].

In Ultra Pasteurization (UP), the fundamental principle is to reduce the main causes of reinfection of the product during processing and packaging so as to extend the shelf life of the product. UP requires extremely high levels of production hygiene and distribution temperature of no more than 7°C [4].

In the case of sterilization, fat is first separated from skim milk (standarization) and then reincorporated at a desired fat content by homogenization. Later, milk is pre-heated, first at 80°C and packed in clean containers. Then, the filled containers are autoclaved at 115-120°C/20-30 min (Table 2.6). Due to the Maillard reaction and caramelization, sterilized milk becomes brownish [51] when treated at high temperatures and long period of times. Moreover, it acquires a cooked and caramel flavor. These negative effects are avoided by shortening the treatment time at high temperatures [4], such as

135°C for 4s (Table 2.6). Such sterilized and UHT milk can be stored for long periods (>6 months) without spoiling, avoiding the use of refrigeration.

The use of high temperature and long holding time processing can cause undesirable flavors or inconsistencies in fluid milk. Low pasteurized milk (HTST) treated at (72°C, 15 s) has a bland flavor. Therefore, it is one of the most accepted by consumers, although its storage stability at refrigeration conditions is only for 20 days. On the contrary, UHT milk processed at 135-150°C and 3-5 s is stable at room temperature and its shelf life is around 6 months. However, it has been reported an induction of strong "cooked" off-flavor notes [53], unacceptable to consumers. These notes are generated due to the high temperatures applied, causing several chemical changes, including a change in the flavor profile and color of milk [53].

Among all off-flavors present in milk (Table 2.6), generally consumers prefer heated flavor rather than cooked or stale flavor [53]. Some of the compounds responsible for the heated flavors are diacetyl, lactones, methyl ketones, maltol, vanillin, benzaldehyde, and acetophenone, which are Maillard reaction products [58].

A stale flavor is found in UHT milk as a result of the increase of C_2, C_{7-9} aldehydes, where lipids are the main precursors. It is probable that at high temperatures, lipids also contribute to the cooked off-flavors: hexanal, heptanal, octanal, nonanal, decanal, 3-methylbutanal, 3-methylpropanal, and 2-furaldehyde [72-73, 82-83].

Milk fat is an important contributor to milk flavor, producing some methyl ketones, lactones and aldehydes [52]. Non enzymatic autocatalytic oxidation reactions from unsaturated fatty acids form hydroperoxides, which may further react forming secondary oxidation products, such as aldehydes and ketones. Each compound has a specific aroma and usually low threshold values; depending on their concentration, these compounds could give either desirable or undesirable flavor in milk [51]. Moreover, unsaturated aldehydes and ketones have the lowest

flavor threshold values [51] and are the most abundant compounds in milk samples [84].

Table 2.6. Different milk thermal processes.

Process	Condition	Shelf life*	Flavor profile
Thermisation	63-65°C/15s (b)	24 hrs (b)	Bland (b)
LTLT pasteurization	63°C/30 min (a,c)	8-10 days at 4°C (b)	Bland (b)
HTST pasteurization	72-75°C / 15-20 s (a, b, c)	8-21 days at 5-7°C (b,f)	Bland (a)
UP	125-138°C/2-4 s (a,f)	30-40 days at <7°C (b,f)	Slight ketone-like flavor and trace of caramelized flavor (a)
UHT(sterilization)	135-140°C/4-15 s (b,c)	At ambient temperature ≥ 6 months (c)	Cooked taste, odor sulfurous, cabbage like notes (b,e)
Sterilization in container	115-120°C/20-30min (a,b)	At ambient storage (>1yr) (b)	Strong caramelized (a,d)

LTLT: Low temperature, long time; HTST: High temperature short time; UP: Ultra pasteurization; UHT: Ultra High Temperature.*Unopened package; References: (a) [51], (b) [4], (c) [85], (d) [52], (e) [69], (f) [86].

2.2.1.1. Flavors developed from autooxidation of lipids

In thermally oxidized fats, hydroperoxides are odorless and tasteless without contributing to the off-flavor of milk [65]. At high temperatures, oxidation proceeds rapidly and the rate of hydroperoxide decomposition exceeds that of hydroperoxide formation [87]. A flavor defect in cow's milk could be attributed to lipid oxidation, which often is characterized as metallic, cardboardy flavor [68].

Autoxidation of unsaturated fatty acids involves a free radical reaction, forming fat hydroperoxides and various malodorous ketones and aldehydes, resulting in rancidity flavors. Possible initiators of the chain reaction are: single oxygen, metal catalysts, heat, and light [68]. Oxygen reacts with unsaturated fatty acids (RH) yielding hydroperoxides and other oxygenated compounds. This reaction occurs by a free-radical process and can be described in three different steps: initiation, propagation, and termination. The initiation step in the autoxidation of unsaturated fatty acids is the formation of free radicals (a compound with an unpaired electron) (RO•, ROO•), which are required to start

the propagation reaction [66]. A hydrogen molecule is abstracted from the unsaturated fatty to yield a free radical. Hydrogen is removed from the carbon atom next to the double bond by the use of catalizers like trace metals, heat or light [65]. The formation of these free radicals can also take place by thermal or photodecomposition of hydroperoxides either by metal catalysis or UV radiation [64].

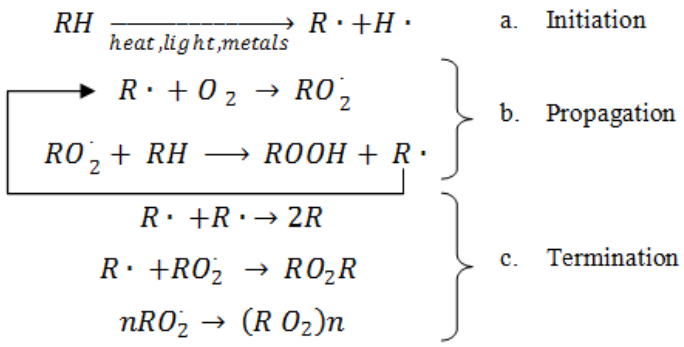

Figure 2.4. Formation of hydroperoxides from the reaction of fatty acids in the presence of oxygen. Adapted from refs. [61, 65-68].

After the free radicals (fatty acid or alkyl radical) are formed, they react with oxygen to form a peroxide-free radical (RO_2), which is highly reactive [65]. This compound is able to remove hydrogen from another unsaturated molecule with a rapid conversion, forming a hydroperoxide and another free-radical [66], starting the propagation chain reaction [65], which can be repeated several times. Hydroperoxides formed in this stage are the primary oxidation products, which are very unstable and decompose into secondary oxidation products, such as carbonyl groups like aldehydes and ketones [65]. Hydroperoxides can break down to produce more free radicals. This mechanism is responsible for the rancid off-flavors in milk [61]. Table 2.7 illustrates the breakdown products from hydroperoxides originated from oleic, linoleic, linolenic, and araquidonic acids. Free fatty acids may be considered as tertiary oxidation products. Identification and quantification of secondary oxidation products is important because they are odor-active compounds, whereas hydroperoxides are flavorless [65].

Termination occurs when the free radical chain reaction is interrupted, after free radicals (R • and ROO•) react with themselves, forming stable non-radical products.

Table 2.7. Breakdown products from hydroperoxides. Adapted from refs: [61, 64-67, 70].

Fatty acid	Hydroperoxide position	Aldehyde formed
Oleic	11	octanal
	9	2-decenal,
	8	2-undecenal, decanal
	10	nonanal
Linoleic	9	2,4 decadienal
	11	2-octenal
	13	hexanal
Linolenic	16	propanal
	14	2-pentenal
	12	2,4 heptadienal
	13	3-hexenal
	9	2,4,7 decatrienal
	11	2,5 octadienal
Arachidonic	15	hexanal
	11	2,4 decadienal

2.2.1.2. Ketones

Diketones, methyl ketones have been found in raw and heat treated milk. Acetone, 2-butanone, 2-pentanone, 2-heptanone, and 2-nonanone have been found in raw milk and heated milk in a recompilation of studies done by Vazquez-Landaverde et al. [52]. However, 2-hexanone, 2-octanone, 2-decanone, 2-undecanone and 2-tridecanone were not found in raw milk but in UHT milk and some of them in sterilized milk (Table 2.8).

Ketones are formed by β-oxidation of saturated fatty acids, followed by decarboxylation of β ketoacids, naturally present in milk [88]. Milk fat contains approximated 1% of lipids in which oxo fatty acids of various chain lengths are esterified to glycerol. These oxo-fatty acids can be liberated as β ketoacids and decarboxilated to C6-C16 methyl ketones when fat is heated in the presence of water [88]. Milk fat contains 10% C6, C8, C10, C12 fatty acids, which are precursors for odd-carbon numbered C5, 7, 9, 11 *n*-methyl ketones [73]. Fat content seems to have an influence on the concentration of methyl ketones [73].

Methyl ketones formation involves hydrolysis and decarboxilation upon heating [52, 62]. Among the most studied ketones are 2-decanone, 2-heptanone, 2-octanone, 2-undecanone, 2-hexananone and diacetyl (Table 2.8). Scalan et al. [58] found C 3, 4, 5, 7, 8, 9, 10, 11, 13 n-methyl ketones in heated milk (preheated at 82°C for 30min and heated at 146°C for 4s) and have been referred as heat-induced compounds in milk.

2-Heptanone, 2-octanone and 2-nonanone have been identified as the most intense volatile flavor compounds in UHT milk [58, 89]. According to Vazquez-Landaverde et al. [73] and Pereda et al. [77], 2-heptanone was the major contributor to off-flavor in UHT milk and considered to be a suitable marker for heat treatment. 2-Hexanone and 2-undecanone have been associated with the stale-heated flavor in UHT milk and sterilized milk [59] and their concentrations also increase in direct proportion to the severity of the heat treatment [58, 73].

The diketone diacetyl is not only present in raw milk at low concentrations but also contributes to the rich or heated note in the flavor of heated milk [58, 73]. As reported by Scalan et al. [58], diacetyl concentration increases with the severity of the heat treatment. Hodge [90] suggested that diacetyl formation comes from an intermediate produced in one of the intermediate pathways of non-enzymatic reactions (browning reactions): methyl alpha-dicarbonyl. It has been also discussed in Scalan et al. [58] that methyl glyoxal react with glycine or a derivative to produce diacetyl; and methyl glyoxal may be produced by heating lactose and trace amounts of free amino acids [58].

Table 2.8. Methyl ketones in heated milk

Methyl ketones	Raw milk	Pasteurized	UHT milk	Sterilized milk
2-Decanone	1%:0.28µg/kg (a)	0-3%-BQL (a)	1%: 0.46 µg/kg (a) 3%:1.33 µg/kg (a)	NA
2-Undecanone	1%: 1.98 µg/kg (a) 2%: 2.58 µg/kg (a)	n.r.%:8 µg/kg (h) 1%:1.46 and 0.40 µg/kg (a) 2%: 0.63 and 0.70 µg/kg (a) 3%:2.70 and 0.92 µg/kg (a)	30 µg/kg (h) 1%: 6.64 µg/kg (a) 3%: 9.70 µg/kg (a)	NA
2-Nonanone	1%:0.20 µg/kg (a) 3%:0.24 µg/kg (a)	0%:0.77-0.79 µg/kg (a) 1%-:0.43-0.44 µg/kg (a) 2%:0.33-0.59 µg/kg (a) 3%:0.53-0.61 µg/kg (a) 3%: 2.5 µg/kg (b) 0.9 µg/kg (h)	1%:35.04 µg/kg (a) 3%: 52.64 µg/kg (a) 3%:16.7 µg/kg (b) 45 µg/kg (h)	n.r.% 62.6 µg/kg (b)
2-Heptanone	1%: 1.03 µg/kg (a) 3%: 0.95 µg/kg (a) 3.2%: 1.05 µg/kg (d) 3.5%: 0.11 mg/kg (f) n.r.%: 0.14-0.17 µg/kg (g)	0%: 0.54-2.06 µg/kg (a) 0.07%: 0.90 mL/mL (e) 1% 0.45-0.87 µg/kg (a) 2% 0.55-1.79 µg/kg (a) 3% 0.72-1.12 µg/kg (a) 3%: 2.6-10.4 µg/kg (b) 3.2%: 0.76-1.11 µg/kg (d) 3.4%: 1.01 mg/mL (e) 3.5%: 0.29 mg/kg (f) 0.9 µg/kg (h)	1%: 22.32 µg/kg (a) 3%: 34.46 µg/kg (a) 3.2%: 32.4-35.6 µg/kg (d) 3%: 9.1-67.5 µg/kg (b) 83 µg/kg (h)	n.r%:64.5-253 µg/kg (f)
2-Octanone	1%: 2.11 µg/kg (a) 3% 3.82 µg/kg (a)	0%: 0.91-2.93 µg/kg (a) 1%:1.44-2.16 µg/kg (a) 2%: 1.89-2.15 µg/kg (a) 3%: 3.02-6.39 µg/kg (a)	1%: 2.65 µg/kg (a) 3%: 4.51 µg/kg (a)	NA
2-Hexanone	0.2-0.53 µg/kg (g) 0.22-0.37 µg/kg (a)	0%:0.26-0.34 µg/kg (a) 1%: 0.10-0.17 µg/kg (a) 3%: 0.06-0.16 µg/kg (a)	1%:1.46 µg/kg (a) 3%1.81 µg/kg (a)	NA
Diacetyl	5 µg/kg (g) 0.21-2.7 µg/kg (g) 1%:0.25 µg/kg (a) 3%: 0.48 µg/kg (a)	0%: 2-6.5 µg/kg (a) 1%:0.51-1.75 µg/kg (a) 2%: 9.75-1.71 µg/kg (a) 3%:0.92-2.07 µg/kg (a)	38 µg/kg (g) 1%: 3.13 µg/kg (a) 3%: 7.39 µg/kg (a)	NA

*Not detected; NA: Not applicable; n.r.:not reported BQL: Below quantification limits.
References: [a] [73], [b] [82], [c] [51], [d] [72], [e] [79], [f] [77], [g] [91], [g] [58], [h] [92].

2.2.1.3. Aldehydes

Aldehydes are derived from lipids (straight chain aldehydes), lactose [52] or amino acid degradation, generating branched-chain compounds, such as 2-methylbutanal and 3-methylbutanal [84, 89]. Presence of aldehydes varies among the type of processing, severity of temperature and treatment time. Table 2.8 shows a recompilation of the most common aldehydes in raw milk, pasteurized,

UHT and sterilized milk. More importantly, aroma impact is not only dependent on concentration, but also on sensory threshold.

Saturated aldehydes, such as hexanal, heptanal, octanal, nonanal and decanal result from the autoxidation of unsaturated fatty acids (C18:1 and C18:2) and also from the spontaneous decomposition of hydroperoxides promoted by heat [88]. According to Vazquez-Landaverde et al. [73], fat percentage did not seem to influence the concentration of aldehydes. Octanal has been found in raw and heated milk in Table 2.7. Hexanal is produced from hydroperoxides of linoleic acid, which has been detected detected in heated, raw and pasteurized milk [52, 58]. Heptanal was found in heated milk at higher concentrations in UHT milk [73]. Nonanal has been found in raw, pasteurized, and UHT milk despite its low concentration, it could contribute to the aroma of heated milk [73]. Decanal is a contributor to the aroma of raw, pasteurized, and UHT milk with higher concentrations found in UHT milk [73].

As stated by Labuza & Dugan [61], and Belitz & Grosch [63], 3-methylbutanal may be formed by non-enzymatic browning reactions from leucine, andincreasing with the severity of the heat treatment [82], contributing to the aroma of the UHT milk [73]. Microbial growth of *Streptococcus lactis* var. *maltigenes* or *Lactobacillus malaromicus* can also produce this compound [68]. Whereas, Calvo & de la Hoz [52] demonstrated the formation of 2-methylbutanal via Strecker degradation, from the reaction of methylglyoxal with isoleucine [61] in aqueous solutions. The aminoacid catabolite of 2-methylpropanal is suggested to be valine [63]. 2-Methylpropanal can be formed via Strecker degradation from methyl glyoxal and valine [52, 61, 63, 73]. 2-Methylpropanal, 3-methylbutanal and 2-methylbutanal have been identified in pasteurized UHT and sterilized milk [52].

Scalan et al. [58] reported that benzaldehydes formed in higher concentrations in heated milk than in raw milk. Scalan suggested its formation from lactose degradation when milk is subjected to several thermal conditions. It might be formed from phenylalanyl residue and also from sugar molecules in the non-enzymatic browning reaction [52]. In the other hand, formation of furfural is

induced by heating, forming melanoidins compounds during Maillard reactions between lactose and the amino group, such as lysine [3]. Thus, furfural content can be a considered as a good indicator of the severity of the heat treatment [93]. Furfural has been found in UHT milk [58].

Table 2.9. Aldehydes in heated milk.

Aldehydes	Raw milk	Pasteurized	UHT	Sterilized
Octanal	1%: 0.43 µg/kg (a); 3%: 0.52 µg/kg (a);3.2%: 1.44 µg/kg (b); 3.2%: 0.24 µg/kg); n.r.%: 0.02 µg/kg (d)	0%: 0.08-0.14 µg/kg (a); 1%: 0.07-0.1 µg/kg (a); 2%: 0.09-0.21 µg/kg (a); 3%: 0.12-0.15 µg/kg (a); 3.2%: 0.13-0.14 g/kg (b); n.r. %: 0.04 (d); 0.7 µg/kg (i);	1%: 0.48 µg/kg (a); 3%: 0.95 µg/kg (a);3.2%: 0.91-1.02 µg/kg (b); n.r.%: 2 µg/kg (i)	NF
Benzaldehyde	NF	1.2 µg/kg (h)	3%: 0.2 µg/kg (e); 5 µg/kg (h)	n.r.%: 4.9 µg/kg (e)
Heptanal	1%: 0.22 µg/kg (a); 3%: 0.2 µg/kg (a); 3.2%: 1.05 µg/kg (b); 3.2%: 0.26 µg/kg (c) n.r.%: 0.08 µg/kg (d); n.r.%: 0.38-1.17 µg/kg (g)	0%: 0.03-0.37 µg/kg (a); 0%: 0.09 mg/mL (f); 1%: 0.04-0.11 µg/kg (a); 2%: 0.08-0.12 µg/kg (a); 3%: 0.07-0.14 µg/kg (a); 3.2%: 1.68-1.73 µg/kg (b); 3.2%: 0.98 mg/mL (f); 3%: 2.4 µg/kg (e); n.r. %: 0.09-0.12 µg/kg (d); 0.7 µg/kg (i)	1%: 0.49 µg/kg (a); 3%: 1.68 µg/kg (a) 3%: 2.1 µg/kg (e); 3.2%: 1.68-1.73 µg/kg (b); n.r.%: 2.1 µg/kg (i)	NF
Hexanal	n.r.%: 3.21-38.48 µg/kg (g) 1%: 4.77 µg/kg (a); 3%: 2.68 µg/kg (a); 3.2%: 13.43 µg/kg (b) 3.2%: 1.51 µg/kg (c); n.r.%: 0.26 µg/kg (d)	0%: 2.8-5.21 µg/kg (a); 1%: 0.75-1.62 µg/kg (a); 2%: 0.82-1.65 µg/kg (a); 3%: 0.81-0.74 µg/kg (a); 3%: 3.8 µg/kg (e); 0%: 0.64 mg/mL (f); 3.2%: 0.59 mg/mL (f); n.r.%: 0.26-0.29 (d); 2.3 µg/kg (i);	1%: 1.58 µg/kg (a); 3%: 12.97 µg/kg (a); 3.2%: 12.76-13.52 µg/kg (b); 3%: 2.5 µg/kg (e); 3.3*%: 4.5 ng/mL (h); 4 µg/kg (i);	n.r.%: 3.6 µg/kg (e)
3-Methylbutanal	n.r.%: 8.42-74.93 µg/kg (g)	0%: 0.02-0.03 µg/kg (a); 1%:0.02-0.03 µg/kg (a); 2%: 0.17 µg/kg (a); 3%: 0.06-0.08 µg/kg (a)	1%: 0.85 µg/kg (a); 3%: 0.85-1.14 µg/kg (a)	NF
2-Methylbutanal	n.r.%: 1.1-6.79 µg/kg (g)	0%: 0.14-0.18 µg/kg (a); 1%: 0.09-1.13 µg/kg (a);2%: 0.11-0.14µg/kg (a); 3%: 0.09-0.13 µg/kg (a)	1%: 0.57 µg/kg (a); 3%: 0.91 µg/kg (a)	NF
Nonanal	n.r.%: 0.2-0.46 µg/kg (g)	2.5 µg/kg (i)	5 µg/kg (i);	NF
Decanal	1%: 2.42 µg/kg (a) 3%: 2.72 µg/kg (a)	0.8 µg/kg (i)	1.6 µg/kg (i)	NF
Furfural	3%: 0.20 µg/kg (a)	0.2 µg/kg (i); BQL (a); 0%: 0.14 µg/kg (a); 2% : 0.13 µg/kg (a)	2 µg/kg (i); 1%: 0.52 µg/kg (a); 3%: 0.38 µg/kg (a)	NF

References: [(a)] [73], [(b)] [72], [(c)] [76], [(d)] [77], [(e)] [82], [(f)] [79], [(g)] [91], [(h)] [94]; * UFA-enriched milk (1.86% CLA), (i) [92]; NF: not found

2.2.1.4. Sulfur compounds

Dimethyl sulfide (DMS) and other sulfur compounds (i.e. hydrogen sulfide (H_2S), methethiol (MeSH), carbon disulfide (CS_2), dimethyl disulfide (DMDS), dimethyl trisulfide (DMTS), dimethyl sulphoxide, and dimethyl sulphone) have related to the cooked flavor defect specially in UHT and sterilized milk [53, 69, 95]. Typical concentration ranges of DMS in various types of milk is included in Table 2.10. Milk proteins containing aminoacids with sulfur groups, cystine, cysteine and methionine often release volatile sulfur compounds, which are affiliated to strong cooked flavors [70]. Sulfur compounds can be formed by heat [92]. When whey proteins, mainly β-lactoglobulin and proteins present in the fat globule membrane, are denaturated during heat treatment, sulphydryl and volatile sulphides are liberated. The presence of these compounds is correlated to the intensity of the cooked, cabbage and sulfur flavor in heated milks [52]. Pasteurization of milk can impart a slight cooked and sulfurous note; however, it is still acceptable at low levels for most consumers.

DMS has been observed in raw milk from the feed of cows [52] and heated milk [58]. Its concentration increases upon thermal treatment [96], as confirmed by Jaddou & Manning [59], who found higher concentrations of DMS in UHT milk than in raw milk (Table 2.10).

Table 2.10. Concentration of DMS in various types of milk.

Type of milk	Processing conditions	DMS concentration	Reference
Raw	NA	32 µg/L	[97]
	NA	0.6-0.9 µg/L	[59]
	NA	8.16 µg/kg	[68]
	NA	7.40-8.16 µg/kg	[73]
Pasteurized	Not given	8.49 µg/kg	[68]
	Not given	6.61-16.08 µg/kg	[73]
UHT	140°C, 3s	2 µg/L	[59]
	Not given	21.4-22.4 µg/kg	[73]
Sterilized	120°C, 30 min	3.3 µg/kg	[82]

NA: Not applicable

2.2.1.5. Other compounds

The presence of other compounds in thermal treated milk, such as lactones, organic acids and alcohols has been documented [58]. Organic acids, such as hexanoic, and octanoic, decanoic acids are also found in heated milk (UHT) [58]. Alcohols, such as 3-methyl-1-butanol and ethanol have been found in raw milk [84]. Moio et al. [89] stated that these compounds are generated from microbial degradation of its respective aldehydes; however, due to its low concentrations, it is unlikely to have a negative impact on fresh milk.

2.2.2. High-Pressure (HP) Technologies

Non-thermal processing is an alternative technology to conventional thermal food processing. But, thermal processing is currently the most commonly used technology in the food industry. The use of high temperatures during conventional processing may have an impact on the degradation of vitamins, antioxidants, bioactive food components and sensorial characteristics, reducing the quality of the product itself. High Pressure Processing (HPP) is one of the most promising non-thermal technologies. One of the main advantages of HPP versus thermal processing is that high temperatures are not employed; thus, no thermal degradation of food components occurs [98]. Moreover, hydrostatic pressure can destroy microorganisms without heat [99]. Hence, overall quality, flavor and nutritional properties are retained and/or preserved. Due to consumers' preference for less processed foods or minimally processed foods, commercial products have already been launched into the food market demonstrating the industrial scale feasibility of this technology.

A fruit jam was first introduced in Japan in 1990 [100]. Then, different HPP food products have been commercialized in the USA (fresh salsas, fresh oysters, organic fruit juices, chicken fajitas), Spain (sliced meats, spinach in cream, cooked sausages), United Kingdom (lobsters), France (orange juice), Portugal (fruit puree) and Mexico (avocado paste).

HPP has been used for food products alone or in combination with conventional processes (pressure-temperature combinations) [101]. Interest in HPP applications in milk and dairy products has increased as it offers a new technology for food preservation to the industry.

Pressure assisted thermal processing (PATP) is a relatively new technology that simultaneously apply high hydrostatic pressure (600-700MPa) and temperature (high temperatures (70-120°C) to preheated food (50-90 °C)) to achieve spore inactivation and enhance shelf life It is suitable for low acid foods. According to Tovar-Hernandez et al. [102], shelf life of milk can be extended (>45 days) at moderate temperature and high pressure (586 MPa) and 5 min.

Unfortunately, very few reports have been published on PATP effects on milk changes and flavor profile [76]. Vazquez et al. [76] and Parada-Rabell [103] reported an increase of aldehydes formation, promoted by high pressure.

2.3. Instrumental analysis of flavor compounds in milk

2.3.1. Typical extraction methods

In the case of food products and in particular for fluid milk, aroma perception is one of the primary criteria of a consumer for its preference. Sensory analysis (descriptive) and instrumental analysis play a key role to define quality in milk and consequently consumer acceptability.

In terms of analytical instrumentation, in order to obtain the flavor profile of a food product, it is required to isolate the target compounds from their matrix sample. As a result, the extraction removes interferences and concentrates the analytes of interest. Many extraction techniques have been widely used for the study of flavor composition in dairy products, such as static headspace [32], purge and trap [82, 94, 104-107], solvent-assisted thermal evaporation [108] and others

[58, 89, 91]. However, most of these methods can cause artifact formation from heat sensitive compounds, such as DMS, acetone, acetaldehyde, hexanal, heptanal, among others [82, 109].

Similarly, volatile compounds in milk have been studied to understand the effects of animal feeding [56, 75, 110-112], heat treatment [73-74, 82-83, 94], non-heat treatments (Ultraviolet light and ultrasound, HPP, and PATP) [72, 76, 113], storage studies [56, 74, 83, 107-108, 114-115], by different extraction techniques. However, sample preparation is complicated due to numerous factors [116]: (i) volatile compounds are generally at low concentrations of ppm, ppb or ppt, being a challenge to isolate and concentrate them; (ii) milk is also a very complex matrix, containing non-volatile components such as proteins, lipids and carbohydrates, which may interfere in the isolation process; (iii) aromatic composition of milk is also very complex, many flavor compounds or components have been identified (Tables 2.7 and 2.8), all of them differing in polarities, solubilities, pHs and boiling temperatures and; (iv) many flavor compounds are very unstable and might be heat labile, unstable to light and oxygen, consequently easily oxidized, and rapidly decomposed to other degradation products.

2.3.2. Solid-Phase Micro Extraction (SPME)

SPME is a relatively new technique developed in 1990 by Arthur & Pawliszyn [117]. This technique is simple, rapid, inexpensive and solventless, widely used for the analysis of volatiles in foods [118-124], CLA-rich oil [125-128].

SPME is a versatile technique that combines sampling, pre-concentration, and transferring of the analytes into a GC [117]. Moreover, it has the ability to extract very low levels of trace organic components and when sampling the headspace, it reduces interference from high-molecular weight components, such as proteins, fats, etc [75].

SPME allows the extraction and concentration of volatiles or semi-volatiles. Analytes establish equilibrium between the sample matrix, the headspace above the sample [129] and the fused silica fiber acting (stationary

phase). A typical automated SPME apparatus (Figure 2.7) consists of a SPME adapter where the fiber holder is placed and a syringe-like device, also known as fiber support rod where the SPME fiber is held for its protection; the small inert fiber is coated with an adsorbent.

First, sample vials are transported into the heated incubator for preconditioning. Subsequently, the fiber support rod penetrates the vial allowing the fiber exposure. Penetration depth (fiber position) allows extracting compounds by immersion in a liquid sample or in the headspace (HS) area above the liquid or solid samples. At this point, the samples can be agitated and heated simultaneously, therefore reducing the extraction time for semi-volatile compounds. At this time, complete adsorption of analytes takes place until equilibrium between sample matrix, headspace and fiber coating is reached. The adsorbed compounds are now thermally desorbed from the fiber to the capillary GC column and the released compounds are identified. Lastly, the fiber can be cleaned at high temperatures (230-320°C) in the special fiber conditioning station to avoid carry over from sample to sample.

Commercially available options of coated SPME fibers diverge and its selection depends on the application and specific properties of analytes.

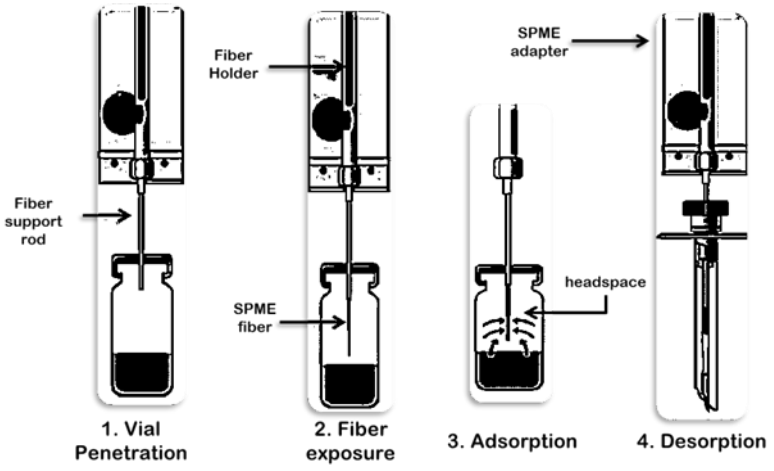

Figure 2.5. Mechanics of automated HS-SPME extraction process. Adapted from ref. [130].

2.3.2.1. Isolation of aroma volatiles from milk and other dairy products

SPME has been used to detect volatile compound in a number of applications, including environmental, pharmaceutical, clinical and forensic applications. Huaquing et al. [131] studied chemicals in tobacco smoke. Also, Liao et al. [132], Zhang et al. [133], Zhipei et al. [134], and Sugaya et al. [135] have conducted studies of pollutants in water. In the food research area, numerous volatile compounds have been studied by SPME, such as palm sugar [118], watermelon [119], malt whisky [120], freeze-dried chicken myofibrils [121], orange juice [122], black and white truffles [123], butter [124], CLA-rich oil [125], fruit-flavored malt beverages [126], wines [127], oxidation compounds in oils [125], coffee [128], among others.

This technique has been widely used to extract many volatile compounds from dairy products, such as yogurt [136], liquid infant foods based on milk and cereals [137, 114], ewe's dairy products [111], cream and anhydrous milk fat (AMF) [78], cheese [109, 138-141], and butter [105, 124]. Thus, many authors selected SPME technique to isolate volatile flavor compounds in milk [72-80].

Few studies on volatile compounds formation in CLA and/or unsaturated fatty acid (UFA)-enriched products were reported [32, 108, 94, 115], using mainly SPME technique. Hexanal was monitored as an indicator of the oxidative stability on UFA-enriched UHT milk by Smet et al. [94]. Similarly, Campbell et al. [32] identified and quantified hexanal, pentanal, 2,4 decadienal and nonanal in CLA fortified milk. Also, Mallia et al. [115] identified 68 odour-active compounds in UFA/CLA enriched butter and eighteen important odorants [108], but isolated with solvent-assisted thermal evaporation technique and SPME. Lastly, Martinez et al. [125] isolated and quantified nine major volatile oxidation compounds in CLA rich oil.

2.3.3. Gas-Chromatography and Mass-Spectrometry (MS) Ionization-SIM/SCAN

After the compounds are isolated from the matrix sample, the adsorbed compounds are desorbed in the injection port in the GC column, where the

compounds are separated according their differences in partitioning behavior between the mobile phase and affinity to the stationary phase, following the detection of analytes. As elution of components takes place, aroma compounds can be identified. FID and MS detectors are the most commonly used due to its high sensitivity, essential for analysis in the flavor research.

MS measures atoms and molecules, providing structural information and molecular weight of the mixture. A charge is applied to the analyte of interest (ionization), and the trajectories of the resulting ions respond to vacuum to different combinations of magnetic and electric fields. Separation is according to mass/charge ratio. A mass spectrometer is typically operated in two different modes: full scan or selected (or single) ion monitoring (SIM). Typical MS can operate simultaneously in SIM and SCAN modes; but functions can be also operated individually. A full spectrum analysis collects compounds within a target range of mass fragments. This mode is useful when determining unknown compounds in a sample and to obtain structural information. In contrast, SIM mode is used for quantitative target analysis. Specific ion fragments are selected for the developed method and the mass spectrometer only detect those fragments set to scan over a very small mass range. As the instrument only searches for specific mass fragments during each scan, only compounds with the selected mass are detected and plotted. The mass spectrometer can dwell for a longer time over a small mass range. As a result, there are less matrix interferences. The detection limit is lower, giving more sensitivity (as the mass range is narrower, it is more specific the assay) and better selectivity when operating in SIM mode.

Both FID [32, 72-73, 75-76, 104, 109, 114] and MS [32, 73, 75, 105, 109, 111, 113-115, 136, 139] have been used for the quantification and identification of volatile compounds in dairy products. However, only few studies are reported with SIM as operating mode [77, 108, 114, 124, 142] for quantitation purposes.

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Chapter 3. A headspace solid-phase microextraction gas-chromatographic mass-spectrometric method (HS-SPME-GC/MS) to quantify volatile compounds in CLA-enriched milk

3.1. Introduction

Flavor is one of the most important characteristics that determine flavor quality of milk [1]. Many extraction techniques have been widely used to study flavor composition in dairy products, such as static headspace [2], purge and trap [3-8], solvent-assisted thermal evaporation [9] and others [10-12]. However, most of these methods can cause loss of heat sensitive compounds, such as dimethyl sulfide (DMS), acetone, acetaldehyde, hexanal, heptanal, among others [4, 13]. Solvents can cause artifact formation during analysis [14] and also require preparative steps which are labor intensive and time consuming [15].

Solid-phase microextraction (SPME) is a relatively new technique developed in 1990 by Arthur & Pawliszyn [16]. This is a simple, rapid, inexpensive and solventless technique, widely used for the analysis of volatiles in foods such as palm sugar [17], watermelon [18], malt whisky [19], orange juice [20], black and white truffles [21], butter [22], and commercial CLA-rich oil [23]. SPME allows fast extraction and concentration of volatiles or semi-volatiles in the headspace. The principle of headspace SPME is an equilibrium partitioning between analytes among the sample matrix, fiber coating and the headspace above the sample [24].

Volatile compounds in non-enriched CLA milk have been studied to understand the flavor profile of raw milk and the effects of heat treatment [4, 8, 25-27]. Volatile compounds chosen for evaluation in this study are important thermally derived off-flavor compounds in processed non-enriched CLA milk. Methyl ketones, such as 2-pentanone, 2-heptanone and 2-octanone, 2-nonanone have been found in heated milk and considered as predominant odorants [11, 12, 25, 28-29]. Also, 2-hexanone, 2-decanone and 2-undecanone have been associated with the stale-heated flavor in UHT milk and sterilized milk [30]. Saturated aldehydes, such as hexanal, heptanal, octanal, nonanal and decanal have

been detected in raw, pasteurized milk and UHT milk [11, 25, 29]. Benzaldehyde has been reported in high concentrations in heated milk [11] and furfural in UHT milk [11,31]. 2-Methylpropanal, 3-methylbutanal, 2-methylbutanal and have been identified in pasteurized UHT and sterilized milk [29]. Dimethyl sulfide (DMS) among other several sulfur compounds have been identified in heated milk and correlated to the cooked flavor of UHT and sterilized milk [32-34]. These compounds have been also found in milk treated under High Pressure Processing (HPP) and Pressure Assisted Thermal Processing [35-37].

An analogous matrix was used to study off-flavor compounds (hexanal, 2-4 decadienal, nonanal and pentanal) in CLA-fortified milk by Campbell et al. [2] by static headspace gas-chromatographymass-spectrometry (S-GC/MS). Similarly, Mallia et al. [9] reported the oxidative stability of butter enriched with unsaturated fatty acids (UFA) and conjugated linoleic acid (CLA) by isolating eighteen odorant compounds by solvent-assisted flavor evaporation (SAFE) and SPME techniques followed by GC/MS.

Moreover, no research has been previously done in the volatile profile of raw CLA-enriched milk. CLA-enriched products have been recently marketed, and it is of interest to evaluate its flavor composition after processing and during storage. Thus, the aim of this study was (1) to develop an analytical method to identify and quantify flavor compounds in raw CLA-enriched milk, and (2) to validate the method. In this thesis, the combination of SPME and GC/MS in SIM mode was used. Various SPME conditions were investigated (extraction temperature, extraction time and sample size). A reliable and efficient method for the analysis of volatile compounds at parts per billion in CLA-enriched milk using SPME-GC-MS/SIM technique was developed, validated and applied to raw CLA-enriched milk. The methodology developed was used to study volatile compounds in processed CLA-enriched milk (Chapter 4).

3.2. Materials and methods

3.2.1. Chemicals and reagents

Chemicals, such as 3-heptanone, 3-octanonene,4-methyl-2-pentanone, trans-2-hexenal, 2-pentanone, octanal, benzaldehyde, acetaldehyde, decanal, nonanal, propanal, 3-methylbutanal, 2-methylbutanal, 2-methylpropanal, 2-nonanone, 2-heptanone, 2-undecanone, 2,3 butanedione, heptanal, furfural, 2-octanone, hexanal, 2-pentanone, 2-hexanone, 2-methylbutanal, 2-decanone, and dimethyl sulfide were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada); 4-decanone was bought from TCI America (Portland, OR, USA). Methanol (HPLC grade) and sodium azide were bought from Fisher Scientific (Ottawa, ON, Canada); Milli Q deionized water was acquired from a Milli-Q water purification system from Millipore (Bedford, MA, USA) located at the Department of Agricultural, Life and Environmental Sciences at the University of Alberta (UofA). Liquid nitrogen was obtained from Praxair (Edmonton, AB, Canada).

3.2.2. Milk samples

CLA-enriched raw milk was obtained at University of Alberta Dairy Unit. It was produced by feeding the cattle with a specific feed regimen according to the protocol suggested by Bell et al. [38] with slight modifications. After milk was collected, sodium azide (0.02%) was added and then stored at -20°C until further use.

3.2.3. Headspace solid-phase microextraction (HS-SPME) procedure

A 2-cm 50/30 µm divinlybenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) coated StableFlex SPME fiber (Supelco analytical; Bellemonte, ON, Canada) was used for the extraction of the following target volatile compounds: hexanal, heptanal, octanal, nonanal, decanal, furfural, benzaldehyde, 2-methylpropanal, 2-methylbutanal, 3-methylbutanal, 2-pentanone, 2-hexanone, 2-heptanone, 2-octanone, 2-nonanone, 2-decanone, 2-undecanone and DMS. The fiber was conditioned in the fiber cleaning station at 270°C for 1h

before the first run of the day to remove adsorbed compounds, and cleaned after each run to eliminate carry-over of non-desorbed compounds.

All samples were prepared using the scheme presented in Figure 3.1. First, by weighting approximately 5g of milk was placed in a 20 mL amber headspace glass vial, fitted with a 18 mm magnetic screw cap and a silicon/ polytetrafluoroethylene (PTFE) septa from Canadian Life Science (Peterborough, ON, Canada). Samples were equilibrated at 35°C for 5 min in the equilibration station with an agitation speed of 250 rpm. After equilibration, the SPME fiber was exposed to the headspace for volatile extraction at 35°C for 30 min) and then desorbed in the GC-injection port at 250°C (5 min) using a CombiPAL system injector autosampler (CTC Analytics, Zwingen, BL, Switzerland) and analyzed in the GC/MS.

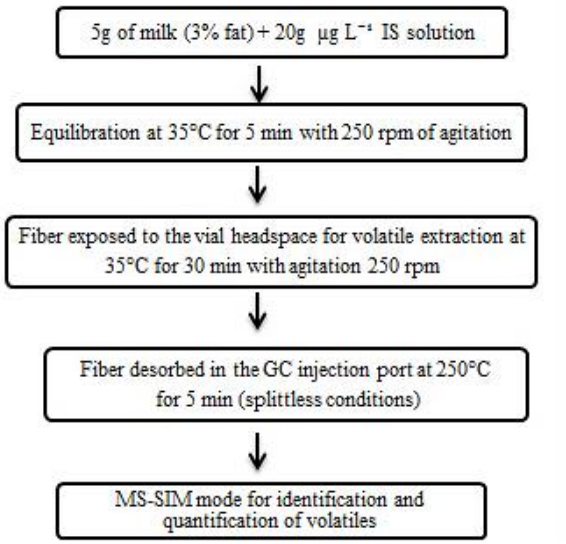

Figure 3.1. Analytical method for the extraction of milk volatiles using HS-SPME technique. Internal standard solution: 20 µg L-1 each of 4-methyl-2-pentanone, trans-2-hexenal, 3-heptanone, 3-octanone, and 4-decanone.

3.2.4. Evaluation of SPME parameters

The effect of extraction time (5-90 min), extraction temperature (35-65°C) and sample size (5-10 g) on the amount of volatile compounds in CLA-enriched milk adsorbed by the SPME fiber was studied. The response recorded was the total peak area of the compounds of interest. First, the samples were extracted at

different temperatures (35, 45 and 65°C) and a fixed extraction time of 5 min and a sample size of 5g. After extraction temperature was selected, the extraction time was studied from 5-120 min; sample size was fixed to 5g. Once extraction time was selected, two samples sizes were evaluated (5g and 10g). These analyses were done in duplicate in HS-SPME GC-MS/SCAN mode.

3.2.5. Gas-chromatography mass-spectrometry (GC/MS) apparatus and conditions

Analyses were carried out with an Agilent 7890A series GC system coupled with a 7975C series mass analyzer detector (Agilent Technologies; Mississauga, ON), a CTC CombiPAL autosampler system (CTC Analytics, Zwingen, Switzerland) and a Rxi-5ms capillary column (29 m x 250 µm x 0.25 µm; Restek, Bellefonte, PA, USA). An injector liner of 0.75-mm i.d. (Supelco; Bellenfonte, PA, USA) was used to reduce peak width, especially for early-eluting compounds.

Liquid nitrogen was used as cryogenic coolant for the capillary column. The temperature of the oven was initially at -5 °C for 1 min (equivalent to the first 1.12 m of the column to cryofocus the volatiles desorbed from the fiber), and was increased to 10 °C at a rate of 10°C min^{-1}, then to 95 °C at a rate of 20°C min^{-1} to 150 °C for 1 min at a rate of 10°C min^{-1} and finally held from 150°C to 230°C for 10 min at a rate of 30°C min^{-1}, yielding a total run time of 26 min. Helium was used as a the carrier gas at a flow rate of 1 mL min^{-1}. The purge flow rate was 20 mL min^{-1} starting, at 5 min. Pressure in the column was 4.09 psi. The injector, detector transfer line, and ion source temperatures were 250, 240, and 230°C, respectively. The electron impact (EI) ionization mode was used at a voltage of 70 eV and tune to perfluorotributylamine (PFTBA). The mass spectra was obtained at a mass-to-charge ratio scan range from 35 to 200 atomic mass units (amu) collected at 7.76 scans s^{-1}, which was used for the identification of the compounds of interest. The instrument control and data analysis were performed using MSD ChemStation (Agilent Technologies; Mississauga, ON, Canada). Identification of target compounds and internal standards (Table 3.1) was carried out by comparing

GC retention times with those of authentic pure standard compounds. NIST 8.0 MS library was used to identify the target compounds.

3.2.6. Mass spectra of volatile compounds

To achieve the best sensitivity, in this study, HS-SPME-GC/MS was run at selective ion mode (SIM) for data acquisition, strategically selecting quantitation ions and identification ions (monitor ions) for each off-flavor compound. Each target compound was assigned to a group number according to its retention time and chemical similarity. Seventeen groups were created for the SIM method, where one retention time locking (RTL) compound. (4-methyl-2-pentanone) was selected. Retention time of each off-flavor compound, the monitored ions and group numbers are shown in Table 3.1. Minimum dwell time per ion was 3 milliseconds (ms) and maximum dwell time per ion was 25 ms.

Table 3.1. Retention time and SIM monitor ions of volatile compounds in CLA-enriched milk.

Compound	Retention Time (min)	Monitor Ion	Group No.
Propanal	2.39	43†, 58	1
DMS	2.59	62†, 47, 45	2
2-Methylpropanal	3.16	43.1†, 72, 41.1	3
Diacetyl	3.63	43†, 86	4
2-Methylbutanal	4.62	57.1†, 41.1, 86.1	5
3-Methylbutanal	4.49	41.1†, 58.1, 71.1	5
2-Pentanone	4.92	43.1†, 86.1	6
4-Methyl-2-pentanone*	5.56	43.1†,85.1, 100.1	7
2-Hexanone	6.11	43.1†, 58.1, 100.1	8
Hexanal	6.22	56.1†,44, 72.1	8
Furfural	6.58	96†, 39	9
Trans-2-hexenal*	6.77	41.1†, 55.1, 98.1	10
3-Heptanone*	7.10	57.1†, 85.1, 114.1	11
2-Heptanone	7.14	43.1†, 58.1, 71.1	11
Heptanal	7.25	70.1†, 96.1, 44	11
Benzaldehyde	7.91	106†, 77, 51	12
3-Octanone*	8.15	57.1†, 72.1, 99.1	13
2-Octanone	8.19	58†, 71.1	13
Octanal	8.33	41.1†, 57, 84	13
2-Nonanone	9.35	58†, 43	14
Nonanal	9.50	57.1†, 41.1, 84	14
4-decanone*	10.34	43.1†, 86.1	15
2-decanone	10.58	58†, 43	16
Decanal	10.75	57.1†, 82.1, 112.1	16
2-undecanone	11.86	58†, 71.1	17

**Internal Standard; † Quantitation ions*

3.2.7. Qualitative and quantitative analysis of volatile compounds

Full scan mode was first used to identify the compounds present in the sample, to determine their retention times and also to obtain the mass fragment fingerprint, which are needed to develop the SIM method.

Identification of target compounds was done using GC/MS in scan mode (GC/MS-SCAN). Selective ion monitoring mode (GC/MS-SIM) was used for the

quantitative analysis to achieve high sensitivity. Quantitation ions of the target compounds are shown in Table 3.1.

Part of our protocol for quantitative off-flavor analysis of milk was adapted from Vazquez-Landaverde et al. [25]. A standard stock solution, containing 10g L^{-1} each of 2-methylpropanal, dimethylsulfide, 2-pentanone, 2-methylbutanal, 2-hexanone, furfural, benzaldehyde, heptanal, hexanal, 2-undecanone, 2-octanone, 2-decanone, 2-nonanone, octanal, decanal, nonanal and 2-heptanone, was prepared in methanol. It was then diluted with Mili-Q water to obtain the following concentrations: 0.02, 0.1, 0.2, 1, 2, 6 and 10 mg L^{-1}. Aliquots (25µL) of each diluted standard stock solution were used to spike 5 g of raw milk, obtaining final concentrations of 0.1, 0.5, 1, 5, 10, 30 and 50 µg L^{-1}. An internal standard stock solution, containing 5 g L^{-1} each of 4-methyl-2-pentanone, trans-2-hexenal, 3-heptanone, 3-octanone, and 4-decanone, was prepared in methanol. It was then diluted with Milli-Q-water to obtain a solution of 2 mg L^{-1}. An aliquot (20 µL) of the diluted standard solution was added to milk to yield a final concentration of 20 µg L^{-1}.

Milk samples were equilibrated at 35°C for 5 min and extracted for 30 min at the same temperature. Calibration curves for compounds of interest were carried out based on the multiple internal standard addition technique and applying linear and nonlinear regression analysis on concentration ratio (concentration of compound of interest/ concentration of internal standard) and peak area ratio (peak area of compound of interest/peak area of internal standard). Analyses were carried out in triplicate at each concentration level.

For the quantification of processed samples, 20 µL of the diluted internal standard solution (2 mg L^{-1}) was added to the 5g milk sample and further analyzed following the protocol described previously.

Concentration of each analyte is calculated according to the peak area of the analyte and to the peak area of internal standard using the calibration curve generated for each compound.

3.2.8. Statistical analysis

Significant differences between each condition for each SPME parameter studied were established using *t*-test and regression equations were obtained using SigmaPlot software V11 (SPSS Inc., Chicago, IL, USA). Significance was established at $P < 0.05$.

3.3. Results and discussion

3.3.1. Selection of target compounds and Internal Standards (IS)

Eighteen thermally derived off-flavor compounds, including aldehydes and ketones, were selected for this study. This selection was based on previous studies discussed in Section 2.2. Molecular masses for the selected compounds range from 58-170 units, whereas boiling points ranged from 45-209°C. An early, middle and late retention times were considered for the selection of these compounds. Other characteristics were also considered, such as stability of the compound, no overlapping with other compounds, similar analytical behavior to the target compounds and not expected to be found in the samples to be analyzed. Initially, trial analyses were attempted with different internal standards (3-octanone, 3-heptanone, trans-2-hexenal, trans-2-nonenal, 4 decanone, 4-methyl-2-pentanone and propylacetatate) previously reported in flavor analysis of dairy products [2, 8, 25, 39-40].

Internal standards, such as 3-octanone, 3-heptanone, trans-2-hexenal, trans-2-nonenal and 4 decanone were used for the quantification of volatile compounds in milk [25]. However, trans-2-nonenal has been identified in UFA/CLA enriched butter [41], heated butter oil [42], commercial CLA-rich oil [23] and cheese [43] which is suggested to be formed from oxidation of *cis* 9, *trans* 11 CLA isomer [23]. The same compound was also identified in experimental trials with processed CLA-enriched milk (data not shown). Therefore the use of this compound as an internal standard was discarded.

4-Methyl-2-pentanone was used for quantification of hexanal and pentanal in liquid infant foods and powdered infant formulas [39] and for quantification of hexanal in milk [8, 40]. 3-Heptanone was also used for quantification of volatiles

in milk [2]. Both compounds demonstrated to be suitable for our analytical method along with 3-octanone, trans-2-hexenal and 4-decanone. Propylacetatate was also used in trials; however, it interfered with one of our target compounds. Five internal standards were chosen (4-Methyl-2-pentanone, trans-2-hexenal, 3-heptanone, 3-octanone, and 4-decanone), as the use of multiple internal standard technique has been suggested to be more precise for quantification purposes [44].

3.3.2. Evaluation of HS-SPME parameters

3.3.2.1. Selection of SPME sampling mode

SPME can be commonly used in two sampling modes depending on the properties of the analytes of interest. In direct immersion sampling (DI-SPME), the fiber is immersed directly into the sample (usually an aqueous matrix) thus low detection limits are obtained. However, as the fiber is in contact with the sample, it can get contaminated with compounds with high molecular weights. Thus, this mode is generally used for semi-volatile compounds and for clean matrices. On the other hand, in headspace sampling (HS-SPME), the fiber is exposed to the vapor phase of the sample (liquid/solid) for adsorption of the analytes. Therefore, headspace sampling is generally used for medium to high volatile compounds and for complex matrixes.

Hence, HS-SPME technique was used in our study because of its fast equilibration time [16] and better selectivity [45] in comparison to DI-SPME. Other advantage is that, there is no interference with proteins and fats or undesirable semi-volatile compounds during adsorption. HS-SPME has been used for identification of volatiles in milk [4, 25, 46, 47-48], butter [22], powdered infant formulas [39] and cheese [13, 49].

3.3.2.2. Selection of fiber coating material

Several fiber coatings are commercially available (Supelco Analytical; Bellemonte, ON, Canada) and they are basically selected based on selectivity and polarity. For analysis of target compounds from milk samples, a DVB/CAR/PDMS fiber was mainly selected due to its sensitivity to a wider range of volatiles and semi-volatiles than other fiber coatings [50]. The specifications of

the SPME manufacturer [51] and literature reports on the determination of similar volatiles in milk were considered for the selection of the fiber [4, 25, 46, 52].

3.3.2.3. Selection of extraction temperature, time and sample size

Extraction efficiency is affected by experimental parameters such as extraction temperature, time and sample size [16]. Fig. 3.2 showed chromatographic peak areas of total target compounds (*y axis*: total abundance) for temperatures below 35 °C , volatile compounds are less likely to be released the from the matrix [25] and temperatures above 45°C can cause artifact formation [25] which can interfere with the compounds studied. At 65°C, the total peak area is higher than at lower temperatures, suggesting that increasing temperature probably increased *ex novo* formation of aldehydes (i.e. hexanal [22]) and ketones (Figure 3.2).

As expected, a decrease in DMS is seen as temperature increased most probably because of its high volatility (Figure 3.3). In contrast, an increase of straight aldehydes and methyl ketones is observed as of temperature increased (45 to 65°C). No difference on total abundance was found between 35°C or 45°C on high molecular weight compounds (C_7-C_{11}) (Appendix, Figure A1). But, at 65°C, the adsorption of these compounds was twice greater than at lower temperatures. One explanation to this could be that at lower extraction temperatures (35°C and 45°C), an exclusion of high molecular weight compounds and concentration of lower molecular weight compounds. While, at 65°C a higher concentration of high molecular weight compounds is observed, probably because of an enhancement on the transfer of high molecular weight compounds from the sample matrix to the headspace. Extraction temperature of 35°C was selected to prevent matrix alterations (i.e. oxidation) and production of aldehydes during sampling [22].

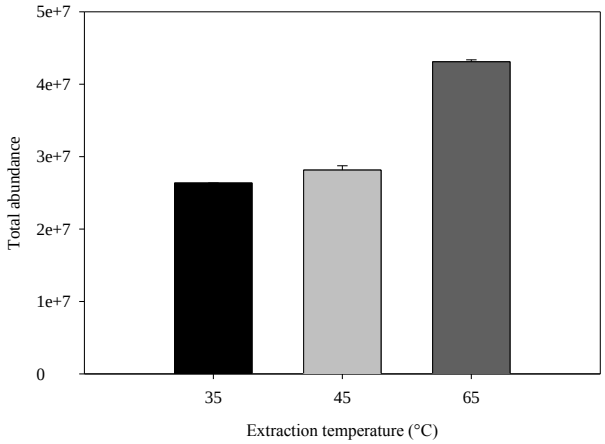

Figure 3.2. Effect of temperature on the equilibrium of volatiles between the SPME coating and the headspace of CLA-enriched milk. Sample size fixed at 5g and 5 min of extraction time. Total abundance = Sum of the target compounds' peak areas. Target compounds: propanal, hexanal, heptanal, octanal, nonanal, decanal, 3-methylpropanal, 2-methylbutanal, 3-methylbutanal, furfural, benzaldehyde, diacetyl, 2-pentanone, 2-hexanone, 2-heptanone, 2-octanone, 2-nonanone, 2-decanone,2-undecanone. Analyses were carried out in duplicate.

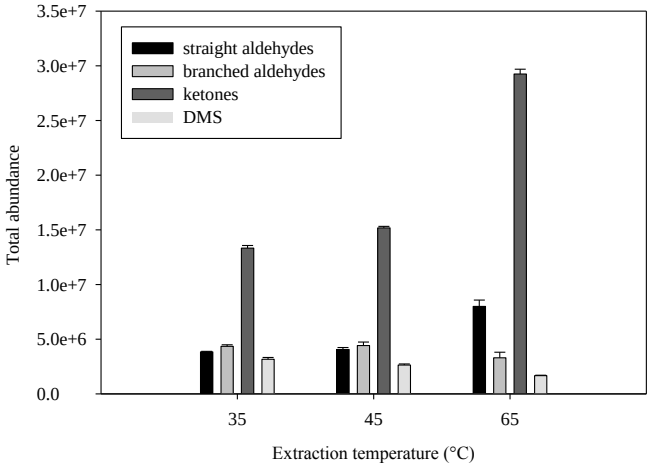

Figure 3.3. Effect of temperature on the equilibrium of volatile compounds (by groups) between the SPME coating and the headspace of CLA-enriched milk. Sample size fixed at 5g and 5 min of extraction time. Total abundance = Sum of the target compounds' peak areas. Analyses were carried out in duplicate. Group categories are designed as follows: Straight aldehydes (propanal, hexanal, heptanal, octanal, nonanal and decanal), branched aldehydes (3-methylpropanal, 2-methylbutanal, 3-methylbutanal, furfural and benzaldehyde), ketones (diacetyl, 2-pentanone, 2-hexanone, 2-heptanone, 2-octanone, 2-nonanone, 2-decanone and 2-undecanone) and DMS.

After selecting the temperature, time was studied. Both analyte and matrix properties have an effect on the time required to reach equilibrium during SPME sampling [53]. The SPME fiber was exposed to the headspace of previously equilibrated samples (5 min at 35°C with stirring at 250 rpm at different times (5,

15, 30, 45 90 and 120 min). An increase of the total peak area was observed by increasing the extraction time (Fig 3.4). The maximum response was acquired at 60 or 90 min, as high standard deviation is observed at 60 min. After this period, a plateau is reached, showing equilibrium between the phases (milk, headspace and fiber coating), which is in agreement with Perkins [46], who found the highest peak areas at 90 min for milk volatiles using similar conditions (extraction temperature, 40°C; sample size, 7 mL). Also, at 90 min better reproducibility of the values was obtained (RSD=1.36%) than at 60 min (RSD=9.01%). For our study because of productivity limitations, 30 min was selected (RSD=2.68%) for analysis of the treated milk samples. Moreover, according to Ai [53] for quantitative purposes, it is not necessary to reach adsorption equilibrium if time and agitation conditions are kept constant throughout all experiments. However, in cases where a compound is present at very low concentrations, extraction times should be increased. The time selected for adsorption (30 min) allows the entire analysis to be completed in 1 hr (5 min of equilibration time+30 min of adsorption time+25 min of GC/MS analysis).

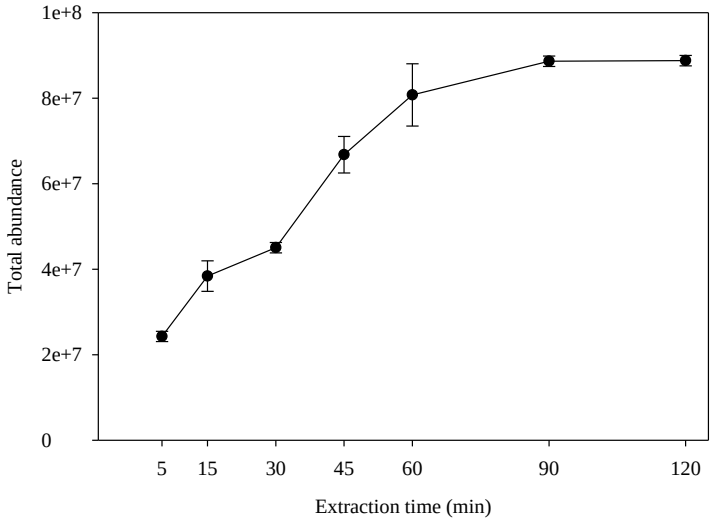

Figure 3.4. Effect on time on the equilibrium of volatile compounds between the SPME coating and the headspace of CLA-enriched milk. Sample size fixed at 5g and 35°C of extraction temperature. Total abundance = Sum of the target compounds' peak areas. Target compounds: propanal, hexanal, heptanal, octanal, nonanal, decanal, 3-methylpropanal, 2-methylbutanal, 3-methylbutanal, furfural benzaldehyde, diacetyl, 2-pentanone, 2-hexanone, 2-heptanone, 2-octanone, 2-nonanone, 2-decanone,2-undecanone. Analyses were carried out in duplicate.

Earlier, Garcia-Llatas et al. [39] selected 37°C as extraction temperature for determination of hexanal and pentanal and a similar equilibration adsorption time of 30 min with a CAR/PDMS fiber, which was more suitable for small-molecules. Perkins et al. [46], selected 90 min and 40°C using the same fiber as in this study, for the determination of seventeen aldehydes and ketones in milk. Vazquez-Landaverde et al. [25] found that sensitivity was improved by increasing extraction time to three hours (35°C); however, due to their productivity limitations the time selected was 60 min. Contarini & Polovo [4] used similar conditions of 40 °C and 30 min for analysis of milk volatiles. In our study some compounds were not observed with 15 min of extraction time (data not shown).

Lastly, after temperature (35°C) and time (30 min) were set, sample size was studied. Two milk sample sizes (5 and 10 g) were evaluated. Significant differences (P>0.05) were found among the two sample sizes studied (Figure 3.5). As the amount of milk increased from 5 to 10 g in total abundance decreased from 4.91 to 3.00 (1×10^7). This decline might be attributed to the increase of water vapor pressure when sample was increased to 20 g. As suggested by Lee et al. [49], water vapor competes with volatile compounds for the active sites of SPME coating phases reducing total peak areas, particularly affecting food with high content of water [54], thus reducing the absorption of certain volatile compounds. Therefore, 5g sample size was selected.

Vazquez-Landaverde et al. [25] reported that sample size had no significant effect on the extraction of milk volatiles at the time and temperature evaluated (extraction temperature: 5-35°C; extraction time: 10-180 min and sample size: 5-30 g) (data of this experiment was not shown nor described in detail). They indiscriminately selected 20 mL of milk sample and placed on a 40 mL vial. Gandy et al. [52] previously used 10 mL of milk in a 40 mL vial for the identification of volatiles and in other study, Perkins et al. [46] used 7 mL of milk in a 10 mL vial. However, they did not evaluate the effect of using different sample sizes.

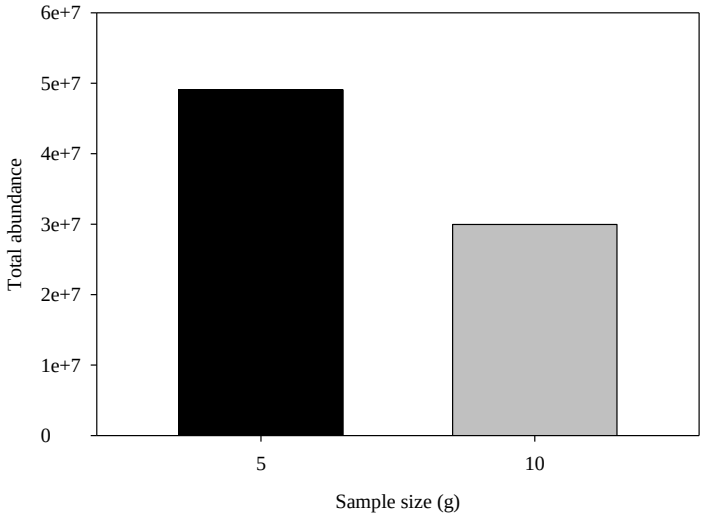

Figure 3.5. Effect of sample size on the equilibrium of flavor compounds between SPME coating and the headspace of CLA-enriched milk. Extraction temperature fixed to 35°C and 30 min of extraction time. Total abundance= Sum of the target compounds' peak areas. Analyses were carried out in duplicate. Target compounds: propanal, hexanal, heptanal, octanal, nonanal, decanal, 3-methylpropanal, 2-methylbutanal, 3-methylbutanal, furfural benzaldehyde, diacetyl, 2-pentanone, 2-hexanone, 2-heptanone, 2-octanone, 2-nonanone, 2-decanone, 2-undecanone. Analyses were carried out in duplicate.

3.3.3. GC/MS conditions

It was seen that with cooling the first meter of the column, a narrow starting band profile was achieved. Good separation was obtained by combination of SPME extraction and a cool starting temperature (-5°C) in the ramp temperatures for the chromatographic column. Volatiles in milk were also identified in other study [52] using the same fiber and the same column. A cross-linked polyethylene glycol column was used (HP Innowax) (60mx 0.32mmx 0.5 μm film thickness) by Contarini & Povolo [4] to identify and quantify volatiles in milk.

3.3.4. Standard calibration curves

The concentration of the target compound was calculated by determining the value of 'y', which is known, as it is defined as area of the target compound divided by the area of the internal standard. The 'x' value represents the concentration ratio (concentration target compound/concentration IS), where the concentration of the IS added is known. Thus, by solving the regression equations for the unknown variable "x", concentration of the target compound present in the

sample is obtained by multiplying "x" by the concentration of the internal standard added to the sample.

Standard curves for 18 compounds were constructed and coefficients of determination were obtained ($R^2 > 0.73$) (Table 3.2.). Only linear sections were considered for quantification of volatiles in processed milk (Chapter 4). As linearity was low for 4 out of the 18 compounds (i.e. furfural, 2-decanone, 2-undecanone, decanal), the samples needed to be re-run after constructing new calibration curves. This allows verifying if there was an error because of the high volatility, reactivity and instability of these compounds during sampling or if the method does not fit very accurately to these compounds. Moreover, it is complicated to obtain high linearity where many compounds are targeted in a single method. For these reasons, the results provided here, are not reported as with concentration levels but peak areas, which provides the tendency of the compounds adsorbed on the fiber and are included in Appendix B.

3.3.5. Method validation

Under the most favorable experimental SPME conditions for this study (i.e. temperature, 35°C; time, 30 min and sample size, 5g) and other conditions selected based on trials (i.e. pre-incubation time, 5 min; injection time, 5 min and desorption time, 5 min) along with the GC-MS/SIM method, analytical parameters, such as linearity, precision, detection limit and recovery were determined.

3.3.5.1. Calibration and linearity

Standard calibration curves were also generated to ensure that the experiments were operated within the linear response range of each flavor compound. Calibration curves for each target compound are included in Appendix A.

Table 3.2. Regression equation for milk volatile compounds spiked in raw CLA-enriched milk (n=3).

Compound	Internal standard	Regression equation[1]	R^2 adjusted
DMS	4-methyl-2-pentanone	y = 0.01+ 0.24 *x	0.93
2-Methylpropanal		y = 0.002+ 0.075*x	0.91
2-Methylbutanal		y= 0.53*(1-exp(-0.66*x))	0.97
3-Methylbutanal		y=0.56*(1-exp(-0.47*x))	0.94
2-Pentanone		y=1.11*(1-exp(-0.59*x))	0.96
2-Hexanone	trans-2-hexenal	y= 44.67*(1-exp(-0.17*x))	0.91
Hexanal		y=6.5069*(1-exp(-1.686*x))	0.92
Furfural		y= -0.002 + 0.44*x	0.72
2-Heptanone	3-heptanone	y=1.595*(1-exp(-0.781*x))	0.96
Heptanal		y=0.6053*(1-exp(-0.745*x))	0.98
Benzaldehyde	3-octanone	y= 1.6243*(1-exp(-0.3726*x))	0.93
2-Octanone		y=3.942*(1-exp(-0.5823*x))	0.98
Octanal		y=1.203*(1-exp(-0.437*x))	0.90
2-Nonanone	4-decanone	y=22.07*(1-exp(-0.165*x))	0.95
Nonanal		y= 0.03+0.81*x	0.96
2-Decanone		y=9.02*(1-exp(-0.44*x))	0.82
Decanal		y= -0.001+0.5*x	0.85
2-Undecanone		y=4.27*(1-exp(-1.69*x))	0.80

[1]y=peak area compound/peak area IS; x=compound concentration/IS concentration; R^2, coefficient of determination; R^2 adj, adjusted coefficient of determination. Only linear section was considered for quantification of compounds in Chapter 4.

3.3.5.2. Limit of detection (LOD) and limit of quantification (LOQ)

LOD and LOQ of all off-flavor compounds were estimated according to the following relationships proposed by American Chemical Society [55].

$$LOD = 3 * \frac{SD_o}{slope} \quad \text{.....(Eq. 1)}$$

$$LOQ = 10 * \frac{SD_o}{slope} \quad \text{.....(Eq. 2)}$$

The detection and quantification limits of volatiles in CLA-enriched milk are shown in Table 3.3. LOD's were lower than values previously reported [46] in milk using HS-SPME GC/FID, including 2-hexanone (1.4 µg/L), hexanal (1.4 µg/L), 2-heptanone (1.5 µg/L), heptanal (3.7 µg/L), octanal (1.1 µg/L), 2-nonanone (4.5 µg/L), nonanal (16.6 µg/L), 2-decanone (3.7 µg/L), decanal (2.9

µg/L) and 2-undecanone (4.0 µg/L). Only LOD value for 2-octanone (0.8 µg/L) was lower than in our study. Quantitation limits were higher than reported by Vazquez-Landaverde et al. [25]. No values corresponding to LOD and/or LOQ for these volatiles have been previously reported in CLA-enriched milk.

Table 3.3. Regression equations for milk volatile compounds spiked in raw CLA-enriched milk (n=3).

Compound	Residual standard deviation (SDo)	LOD (µg L^{-1}) (n=3)	LOQ (µg L^{-1}) (n=3)
DMS	0.05	0.62	2.05
2-Methylpropanal	0.05	2.25	7.48
2-Methylbutanal	0.02	0.14	0.45
3-Methylbutanal	0.08	0.43	1.44
2-Pentanone	0.05	0.16	0.53
2-Hexanone	0.60	0.04	0.14
Hexanal	0.39	0.18	0.61
Furfural	0.23	1.60	5.32
2-Heptanone	0.11	0.22	0.73
Heptanal	0.02	0.12	0.39
Benzaldehyde	0.09	0.17	0.56
2-Octanone	0.15	0.12	0.40
Octanal	0.08	0.21	0.70
2-Nonanone	0.63	0.09	0.29
Nonanal	0.12	0.48	1.58
2-Decanone	0.86	0.29	0.95
Decanal	0.19	1.15	3.83
2-Undecanone	0.85	0.60	2.00

3.3.5.3. Precision

The precision of the method was expressed as the relative standard deviation (RSD) of replicate relative peak areas obtained from the determination of three analyses of CLA-enriched milk. The RSD ranges from 1.05 to 53.35%, which is also observed in other study by Contarini & Povolo [4] (Table 3.5). Similar values were reported for quantification of hexanal in butter (4.94%) [22] and in liquid infant foods (2.87%) [39]. Although these compounds were obtained from a standardized method, the high volatility of these compounds have a strong effect on the variability of the results obtained.

Table 3.4. Repeatability of relative peak areas of the target compounds of raw CLA-enriched milk.

Flavor compound	Replicate [a]			Average	SD	RSD%
	1	2	3			
DMS	0.004	0.005	0.006	0.005	0.001	18.63
2-Methylpropanal	0.004	0.009	0.004	0.006	0.002	40.00
2-Methylbutanal	0.001	0.0007	0.001	0.001	0.0001	15.25
3-Methylbutanal	0.001	0.0007	0.001	0.001	0.0004	28.57
2-Hexanone	0.02	0.03	0.02	0.02	0.001	6.411
Hexanal	0.19	0.17	0.17	0.18	0.011	5.99
Furfural	0.01	0.01	0.01	0.01	0.002	13.73
2-Heptanone	0.01	0.01	0.01	0.01	0.0001	1.05
Heptanal	0.01	0.009	0.009	0.009	0.0004	3.80
Benzaldehyde	0.008	0.006	0.008	0.007	0.0008	10.10
2-Octanone	0.003	0.004	0.003	0.003	0.0004	11.84
Octanal	0.03	0.03	0.03	0.03	0.002	5.83
2-Nonanone	0.005	0.006	0.005	0.005	0.0006	10.26
Nonanal	0.01	0.01	0.01	0.01	0.002	11.68
2-Decanone	0.0005	0.001	0.0005	0.0008	0.0005	53.35
Decanal	0.002	0.002	0.002	0.002	0.0002	10.38
2-Undecanone	0.0005	0.0005	0.0006	0.0005	0.00	8.84

[a] peak area of target compound divided by peak area of internal standard. SD= Standard deviation, RSD= Relative standard deviation.

Table 3.5. Repeatability expressed as the RSD (%) of volatile compounds in different milks by SPME (μg kg $^{-1}$ ± SD) [4].

	DMS	2-hexanone	2-heptanone	2-nonanone	benzaldehyde	2-undecanone
Pasteurized	58.33	0	0.79	16	0	0
UHT	0	0	75.11	77.24	300	75
Sterilized*	90.9	68.75	22.76	26.51	28.57	21.47

*in bottle-sterilized

This HS-SPME-GC/MS method was used to analyze off-flavor compounds of processed CLA-enriched milk described in the following chapter. Reproducibility of the method was also determined in processed CLA-enriched milk (Chapter 4).

3.4. Conclusions and recommendations

The technique developed in this study allowed the identification and an acceptable quantification of a broad range of methyl ketones and saturated aldehydes from the headspace of CLA-enriched milk. The method is simple, fast and precise for some of the compounds and can be used to analyze large number of samples. The recommended optimized SMPE method for extraction of volatiles involves use of 2cm fiber coating of 50/30 um DVB/CAR/PDMS and extraction conditions of 30 min and 35°C with constant stirring and using 5 mL of CLA-enriched milk. This method can be applied to the quantitative analysis of off-flavor compounds in CLA-enriched milk after processing and during storage. This method can be adapted and applied for different dairy beverages. No data have been previously reported on the identification and/or quantification of these volatiles in CLA-enriched milk.

3.5. References

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Chapter 4. Volatiles formation of processed CLA-enriched milk by UP, UHT and PATP technologies

4.1. Introduction

Fresh raw milk has a delicate aroma from several odorous compounds present in low quantities [1]. Cow's milk contains Conjugated Linoleic Acid (CLA) which has many promising health promoting properties [2-7]. As a result there is consumer interest in CLA-enriched products. Milk fat is the richest natural source of CLA [8]. Earlier, dietary modifications have been explored, pursuing an increase on CLA levels in milk [9-12]. However, a change in the saturated fatty acids to unsaturated fatty acids ratio in milk fat as a consequence of dietary manipulations is a prime concern in the formation of off-flavors due to the higher susceptibility of fatty acids to oxidation [13-15], such as aldehydes and ketones. These secondary oxidation products can also be generated during processing and storage hence negatively affecting the flavor of dairy products and reducing the shelf life [16].

The auto-oxidation of lipids in dairy products and the resulting off-flavors have been studied [17-18]. However, only one study reported the volatile oxidation of CLA fortified milk [19] and commercial CLA rich oil [20]. Moreover, the oxidation patterns of CLA are still unknown and few studies indicated the secondary products of CLA auto-oxidation [16, 21-22].

Milk is highly perishable because of its composition and must be pasteurized for human safe consumption. Thermal treatment is the most common way to pasteurize and extend milk shelf life; which inactivates spoilage microorganisms and enzymes [23]. The shelf life varies depending on raw milk quality, processing conditions, microbial growth, packaging materials, storage temperature, and exposure to light [24-25]. Conventional High Temperature Short Time (HTST) pasteurized milk is heated at 72-75°C for a minimum of 15 s [24, 26, 27] where "cooked" flavor is negligible [28]. This treated milk is one of the most accepted by consumers, with a limited shelf life of 10-21 days at refrigeration conditions [24, 29].

Ultra Pasteurized (UP) milk has a shelf life of 30-40 days under refrigeration conditions [24, 26, 29] and a strong "cooked", ketone-like and caramelized flavor is perceived [26]. Ultra High Temperature (UHT) milk (135-140°C for 4-15 s) [24, 27] and UP milk (125-138°C for 2-4 s) [26] have similar processing conditions. However, UHT milk is aseptically packaged, extending its shelf life (>6 months) [30], but it has strong "cooked" flavor notes [31]. Further, other chemical changes occur during storage of UHT milk. A "stale and oxidized" flavor is perceived due to the increase of methyl ketones and saturated aldehydes [32]. More than a six-month shelf life is achieved through sterilization processing (115-120°C for 20-30 min) [24, 26] [24]. However, milk acquires a "cooked" and caramel flavor; and a brownish color is developed due to Maillard reaction, forming furfural and other furosine derivatives and caramelization [23, 26]. Other chemical reactions, such as Strecker degradation generates branched aldehydes, such as 3- methylbutanal, 2-methylbutanal and 2-methylpropanal from leucine, isoleucine, and methyl glyoxal/valine, respectively [33-34]. Branched aldehydes increase with the severity of the heat treatment [35] and negatively contribute to the aroma of the UHT milk [36-37].

A stale flavor is found in UHT milk due to the increase of C_2, C_{7-9} aldehydes, where lipids are the main precursors. At high temperatures, these compounds together with 3-methylbutanal, 3-methylpropanal, and furfural also contribute to the cooked off-flavor [35, 38]. On the other hand, odd carbon methyl ketones are naturally present in raw milk but can also be generated during thermal processing [35], producing heated notes [26, 39]. In addition, CLA is not stable during thermal processing, losing its biological activity by oxidation [19, 40-43].

Furthermore, Pressure Assisted Thermal Processing (PATP) (also referred to as Pressure Assisted Thermal Sterilization (PATS)) is an alternate new technology to heat treatment. It involves exposing a product to high pressure and high temperature for a given time. Although, there are no studies on the formation of off-flavor compounds in CLA enriched milk during PATP, two studies with non-enriched CLA milk treated by High Pressure Processing (HPP) and PATP have been reported recently, showing that specific reactions are inhibited

increasing the shelf life [38, 44]. Additionally, CLA was retained during PATP at certain conditions [45]. Other studies have demonstrated a significant loss during heat treatment [19, 40-43]. But, currently, there are no commercial milk and/or CLA-enriched milk treated by PATP in the market.

Headspace Solid Phase Microextraction (HS-SPME) and GC/MS have been used for the analysis and quantification of off-volatiles as described in Chapter 3. Also, more studies are needed on the stability of CLA during processing and storage, especially on the implications on the flavor profile of CLA-enriched milk as little research has been done in this area. Campbell et al. [19] have monitored compounds, such as hexanal, 2-4 decadienal, nonanal and pentanal which are indicators of lipid oxidation in CLA-fortified milk (2% fat, 1 and 2% CLA).

The objectives of this study were: (1) to identify and quantify main flavor compounds responsible of processed CLA-enriched milk, (2) to evaluate flavor profile in CLA-enriched milk treated by UP processing (125°C/2 s and 125°C/15 s), UHT (135°C/4 s and 10 s, and 145°C/4 s and 20 s) and PATP (0-600 MPa, 80-100°C for 3-15 min), (3) to evaluate the stability of these flavor compounds during shelf-life after UHT processing, and (4) to investigate the effect of the addition of catechin, as an antioxidant on inhibition of flavor compounds in milk under PATP conditions. To the best of our knowledge, this is the first study using CLA-enriched milk to evaluate its flavor profile after processing. In addition, this is the first study on CLA-enriched milk treated by PATP to study flavor compounds by analytical techniques.

4.2. Materials and methods

4.2.1. Chemicals and solvents

Chemicals, such as 3-heptanone, 3-octanone, 4-methyl-2-pentanone and trans-2-hexenal, were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). Catechin and 4-decanone were bought from TCI America (Portland, OR, USA) and Sigma-Aldrich (Saint Louis, MO, USA), respectively. Sodium azide was bought from Fisher Scientific (Ottawa, ON, Canada); Milli Q deionized

water was acquired from a Milli-Q water purification system from Millipore (Bedford, MA, USA) located at the Department of Agricultural, Life and Environmental Sciences at the University of Alberta (UofA). Liquid nitrogen was obtained from Praxair (Edmonton, AB, Canada).

4.2.2. CLA-enriched milk samples

CLA-enriched raw milk (~3%) was obtained from the University of Alberta Dairy Unit. It was produced by feeding the cattle with a specific feed regimen according to the method suggested by Bell et al. [46]. After the CLA-enriched milk was collected, sodium azide (0.02%) was added to inhibit microbial growth and subsequently stored at -20°C until further processing and flavor analysis. The same batch of milk was used for all treatments.

4.2.3. Commercial milk samples

UHT milk (1% fat) purchased in Mexico (Leche Lala-Ultra Lala, Durango, Mexico) was stored at 4°C and used for flavor analysis before its expiration date (June 2012). Pasteurized milk (3.25% fat) obtained from a local store was stored under refrigeration conditions and used before its expiration date. No information about the specific processing conditions was given for these samples.

4.2.4. Conventional thermal processing: UP and UHT treatments

4.2.4.1. Standarization and homogenization protocol

CLA-enriched raw whole milk (~3 % fat) was obtained from the University of Alberta Dairy Unit. Milk cream was separated from the fluid milk using a centrifuge Model Avanti J-20 Beckman Coutler (Mississauga ON, Canada) at 4°C and at a speed of 8,000 rpm for 40 min. Sequentially, the skim and cream were combined to obtain a final 2.6% fat content using a two-stage homogenizer (first stage at 1.4 MPa, and second stage at 14.3 MPa) Model APV-2000, SPX Corporation) (Concord, ON, Canada) to assure an uniform distribution of the milk fat to process with a HTST/UHT processing unit. The protocols for UP and UHT treatments are described in the following section (Figure 4.1).

4.2.4.2 Thermal treatments

After CLA-enriched milk was homogenized, milk was processed at different conditions (Table 4.1.) in an indirect HTST/UHT processing unit Model FT74P miniature heat exchanger, Armfield Company (Ringwood, Hampsire, UK) at Agri-Food Discovery Place (AFDP), University of Alberta.

Three processing temperatures were used (125, 135 and 145°C) with two different holding times (2 and 15 s, 4 and 10 s and 4 and 20 s, respectively), resulting in a total of six different thermal treatments (Table 4.1). After milk samples were treated, they were stored in amber glass vials at -18°C to minimize any changes in the flavor profile of the milk until analysis of volatiles were carried out.

Table 4.1. Process conditions for heat treatment of CLA-enriched milk (2.7% fat).

Process	Flow rate (mL/min)	Heat treatment	
		°C	*holding time (s)
Raw milk	NA	NA	NA
UP	210	125	2
UP	169	125	15
UHT	104	135	4
UHT	250	135	10
UHT	110	145	4
UHT	128	145	20

NA: Not applicable; * estimated using the flow rate and the length of the holding tube used.

For UP and UHT treatments, the same lot of milk was used. For each processing condition, a single batch of milk was used. For instance, for the processing condition at 125°C/2 s just one batch was processed and 2 samples (5g/each) were taken to be analyzed. Volatile analysis was done in duplicates.

Eight off-flavor compounds were identified and monitored, but not quantified in this study due to the high variability of results (See Appendix B.). Furfural, 2-undecanone and 2-nonanone concentration was quantified but included in Appendix B due to the variability of results in some of the milk samples treatments.

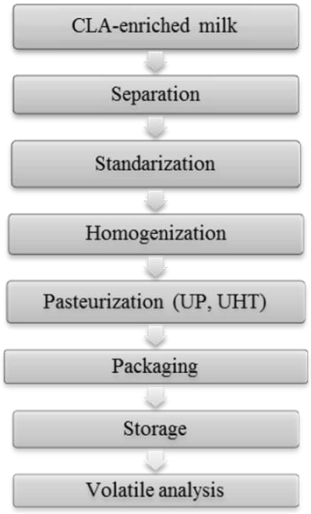

Figure 4.1. General overview of the protocol used for conventional thermal treatments of milk (UP and UHT).

4.2.4.3. Storage stability protocol

The treated milk was stored in amber glass vials with screw caps and silicon/polytetrafluoroethylene (PTFE) septum caps from Canadian Life Science (Peterborough, ON, Canada). The containers were stored in the refrigerator at 4°C and in an incubator at 25°C.

Selected processing conditions for this study are summarized in Table 4.2. CLA-enriched milk was treated at three different temperatures (125°C, 135°C, 145°C) and holding times (15 s, 10 s and 20 s). For each treatment condition, 2 samples (10g each) were stored at 4°C and 25°C and volatile analysis was carried out at 0, 7, 15 and 30 days (Table 4.2). A headspace of ¼ was left for all the samples. All the containers were opened once, just before volatile analysis.

Table 4.2. Processing conditions for the storage stability study of CLA-enriched milk (2.7%).

Process	Heat treatment	Storage Conditions	
	Temperature/time	Temperature	Time (days)
UP	125°C/15 s	4°C and 25°C	0, 7, 15, and 30
UHT	135°C/10 s	4°C and 25°C	0, 7, 15, and 30
UHT	145°C/20 s	4°C and 25°C	0, 7, 15, and 30

4.2.5. PATP treatments

PATP treatments were carried out in a high-pressure (HP) multivessel Apparatus U111 (Warwanza, Poland), which is located at Agri-Food Discovery Place (AFDP), University of Alberta in South Campus. The system is coupled

with a thermostat (Lauda Proline RP 855 Low Temperature Thermostat) connected to a Data Acquisition System (OMB-DAQ-54) to record processing data from the U111 unit. The HP unit has four vessels (8mL of internal volume), working in parallel. At the bottom of each vessel, there is a type K-thermocouple which allows the recording of the temperature profile of the pressure transmitter fluid. Propylene glycol was used as the pressure transmitter fluid where adiabatic heating is 5°C/100 MPa [47]. This unit operates at high pressures (up to 600 MPa) and high temperatures (up to 120°C). Cryogenic vials from Fisher Scientific (Ottawa, ON, Canada) were filled with non-homogenized raw milk (3%) (2.7 mL) and pre-heated to a determined temperature, considering an increase of 3°C in milk for every 100 MPa applied [48]. Once the samples were pre-heated in a propylene glycol thermostat, the samples were transferred to the high pressure vessels and pressurized at a rate of 6.37 MPa/s. After the samples were pressurized for a specific holding time, the pressure was released and the samples were then removed from the high pressure vessels and placed in an ice-bath to cool down and stop further degradation reactions.

The following conditions and combinations were selected: pressure (0.1-600 MPa), temperature (80-120°C) and holding time (3-15 min). A condition (90°C/200 MPa/30 min) equivalent to sterilization was also run with CLA-enriched milk and compared with the thermal treatments and commercial milk. The four pressure vessels were used at each studied condition. Treatments at 0.1 MPa were considered as control samples. All control treatments were run at atmospheric pressure to simulate the temperature conditions during PATP treatments. Cryogenic vials were filled with CLA-enriched milk and pre-heated (62-114°C) in a propylene glycol bath. After that, samples were transferred to the HP unit and kept at a constant temperature (80-120°C). Once the holding time was reached, samples were transferred to an ice-bath to stop further degradation reactions. After each treatment, control and PATP samples were kept in the freezer at -18°C until their analytical analysis. Volatile analysis was done in duplicates.

4.2.5.1. PATP treatments and catechin addition

Additional set of PATP experiments were carried out following the same protocol described in the previous Section 4.2.5. Catechin, previously investigated in our laboratory [45] was selected as an antioxidant to study the effects of its addition on the formation and/or inhibition of off-flavor compounds in CLA-enriched milk treated by PATP at 400 MPa, 120°C and 15 min.

CLA-enriched non-homogenized raw milk was placed in a 200 mL Erlenmeyer flask and then heated to 20°C. Afterwards, 0.5g of catechin was added to 1 kg of untreated milk with constant stirring. The mixture was covered from the light with aluminum foil. To determine the effectiveness of catechin, the concentration of each off-flavor compound was expressed as a percentage of its initial concentration before treatment. All experimental data were obtained in duplicate and all figures with error bars were made using SigmaPlot software V11 SPSS Inc. for windows (Chicago, IL, USA).

4.2.6. Analysis of volatile compounds

Analysis of the twenty off-flavor compounds were determined by HS-SPME-GC/MS in Single Ion Monitoring (SIM) described in detail in Chapter 3, Section 3.2.

4.2.6.1. Screening study: Selection of target compounds

As discussed in Chapter 2 Section 2.2, many off-flavor compounds can be generated after the milk is obtained (e.g. during handling, processing and storage) until the milk is consumed. The selection of the off-flavor compounds for this study was based on: (1) previous literature on main off-flavor compounds found in milk, (2) off-flavor compounds with more aroma impact in milk, and (3) similarity of their chemical/physical properties.

The presence of these compounds was also confirmed and monitored in untreated CLA-enriched milk and CLA-enriched milk after processing at different conditions using HS-SPME-GC/MS in Scan mode to identify the milk volatile profile. Scan mode was used for the identification of volatiles. Protocol is described in detail in Section 3.2.

4.2.7. Statistical and data analysis

Significant differences between the treated samples were established by using t-test using SigmaPlot software V11. Significance was established at $P < 0.05$. For the storage stability study, significant differences among 0, 7, 15 and 30 days of storage were determined for each off-flavor compound with *t*-test ($P < 0.05$) using SigmaPlot software V11.

Data reported was expressed as mean and relative standard deviation (RSD) or Standard Deviation (SD). Microsoft Excel 2010 (Microsoft Corporation, Redmond, WA, USA), and Design expert v.7.0.0 software were used. All the graphs were constructed using SigmaPlot software V11.

4.3. Results and discussion

4.3.1. Compositional analysis

Milk fat, total solids, protein and lactose contents (Table 4.3) were determined according to the standard procedure (IDF 141C, 2000) using a Foss System MilkoScan FT 6000 (Foss Electric, 2005, York, UK) at Central Milk Testing Laboratory (Edmonton, AB, Canada). Ash content was determined by the gravimetric method following the AOAC official method (923.03, 930.30, 930.22) [49].

Table 4.3. Average compositional analysis of CLA-enriched milk.

Component (%)	CLA-enriched milk Homogenized†	CLA-enriched milk Non-homogenized*
Fat	2.7	3.0
Protein	3.3	3.4
Lactose	4.3	4.5
Ash	0.7	0.7
Total solids	11.3	11.9

†Homogenized milk was used for UP and UHT treatments;
* non-homogenized milk was used for PATP treatments.

4.3.2. Screening study: Selection of target compounds

Off-flavor compounds found in CLA-enriched milk samples are shown in Figure 4.2, Figure 4.3 and Figure 4.4. A total of 38 different compounds were identified in different CLA-enriched milk samples. In general, aldehydes and ketones were the major off-flavor compounds. However, in Figure 4.3, thermal treatment had an effect on the oxidation of long chain saturated fatty acids,

resulting in the occurrence of some other fatty acids. Also, in Figure 4.3, peaks correspond to *n*-hexadecanoic acid (peak **33**), *cis* 13 octadenoic acid (peak **29**), *trans* 13 octadenoic (peak **30**) acid and 17-octadecynoic (peak **32**) acid after 15 min of retention time. These compounds were not observed in untreated milk and PATP milk (Figure 4.2 and Figure 4.4). Campbell et al. [19] identified and quantified hexanal, nonanal, 2-4 decadienal and pentanal in CLA-fortified milk. Of these four compounds, only two (hexanal and nonanal) were identified in our study. However, Campbell et al. [19] did not report other volatiles in their study.

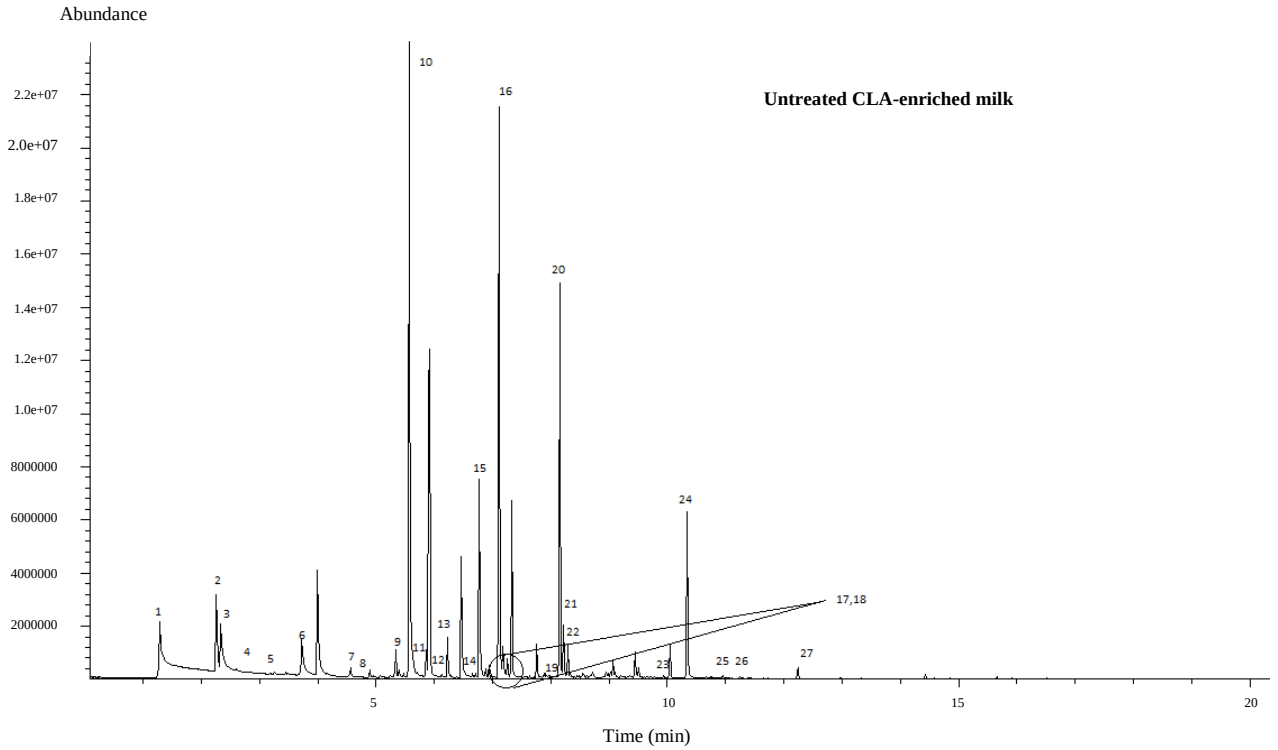

Figure 4.2. GC/MS chromatogram (SCAN mode) for raw CLA-enriched milk obtained by SPME technique. Peaks numbered correspond to **1**, carbon dioxide; **2**, hydrogen azide; **3**, acetone (coeluting with propanal); **4**, DMS; **5**, 2-methyl propanal; **6**, 2-butanone; **7**, 3-methyl butanal; **8**, 2-methyl butanal; **9**, 2-pentanone; **10**, 4-methyl 2 pentanone (IS); **11**, toluene; **12**, 2-hexanone; **13**, hexanal; **14**, furfural; **15**, trans-2-hexenal (IS); **16**, 3-heptanone (IS); **17**, 2-heptanone; **18**, heptanal; **19**, benzaldehyde; **20**, 3-octanone (IS); **21**, 2-octanone; **22**, octanal; **23**, nonanal; **24**, 4-decanone (IS); **25**, decanal; **26**, 2-decanone; **27**, 2-undecanone; **28**, lactic acid amida; **29**, *cis* 13 octadenoic acid; **30**, *trans* 13 octadenoic acid; **31**, pentadecanoic acid; **32**, 17-octadecynoic acid; **33**, *n*-hexadecanoic acid; **34**; 2-furanmethanol; **35**, 4-methyl 2 heptanone; **36**, 3-methyl 2 pentanone; **37**, 2,4 dimethyl furan; **38**, 2-nonanone. Not labeled peaks are possible contamination from the column or fiber used. DMS: Dimethyl sulfide; IS: internal standard.

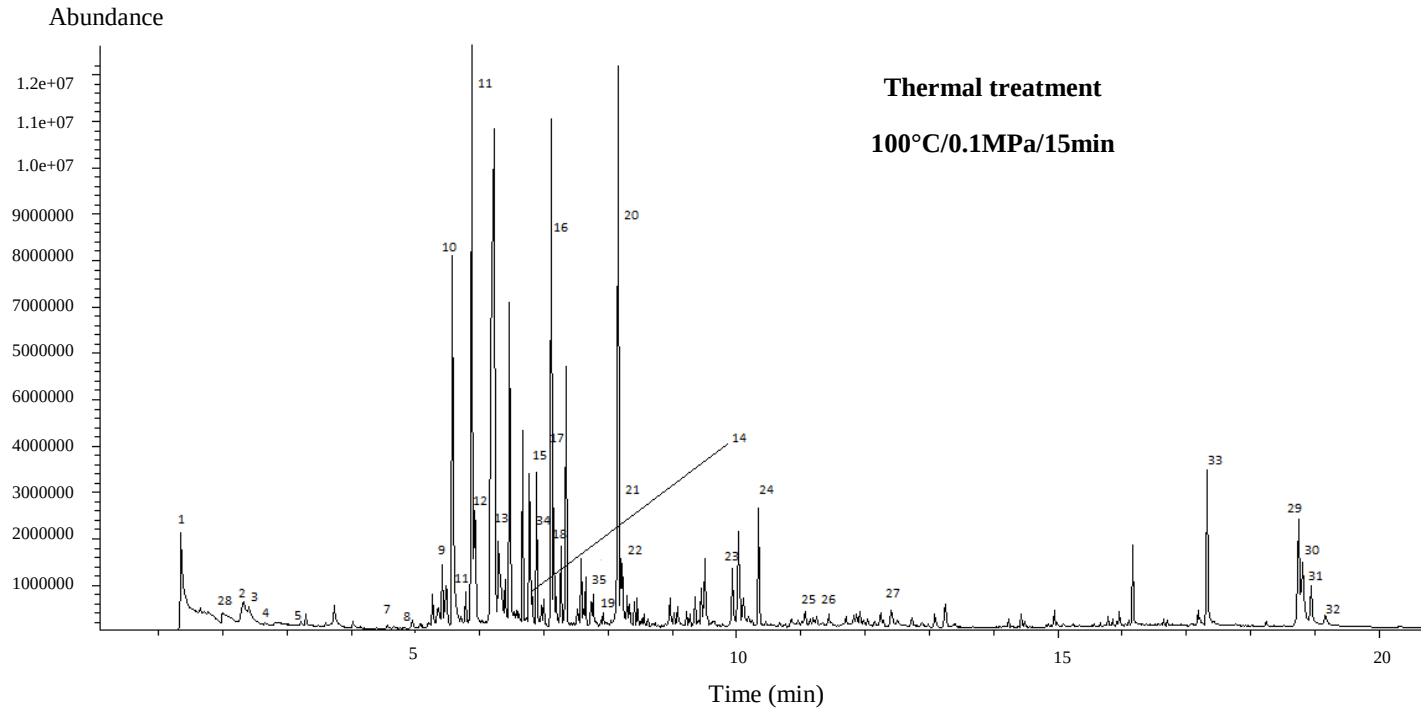

Figure 4.3. GC/MS chromatogram (SCAN mode) for thermal treated CLA-enriched milk obtained by SPME technique. Peaks numbered correspond to **1**, carbon dioxide; **2**, hydrogen azide; **3**, acetone (coeluting with propanal); **4**, DMS; **5**, 2-methyl propanal; **6**, 2-butanone; **7**, 3-methyl butanal; **8**, 2-methyl butanal; **9**, 2-pentanone; **10**, 4-methyl 2 pentanone (IS); **11**, toluene; **12**, 2-hexanone; **13**, hexanal; **14**, furfural; **15**, trans-2-hexenal (IS); **16**, 3-heptanone (IS); **17**, 2-heptanone; **18**, heptanal; **19**, benzaldehyde; **20**, 3-octanone (IS); **21**, 2-octanone; **22**, octanal; **23**, nonanal; **24**, 4-decanone (IS); **25**, decanal; **26**, 2-decanone; **27**, 2-undecanone; **28**, lactic acid amida; **29**, *cis* 13 octadenoic acid; **30**, *trans* 13 octadenoic acid; **31**, pentadecanoic acid; **32**, 17-octadecynoic acid; **33**, *n*-hexadecanoic acid; **34**; 2-furanmethanol; **35**, 4-methyl 2 heptanone; **36**, 3-methyl 2 pentanone; **37**, 2,4 dimethyl furan; **38**, 2-nonanone. Not labeled peaks are possible contamination from the column or fiber used. DMS: Dimethyl sulfide; IS: internal standard.

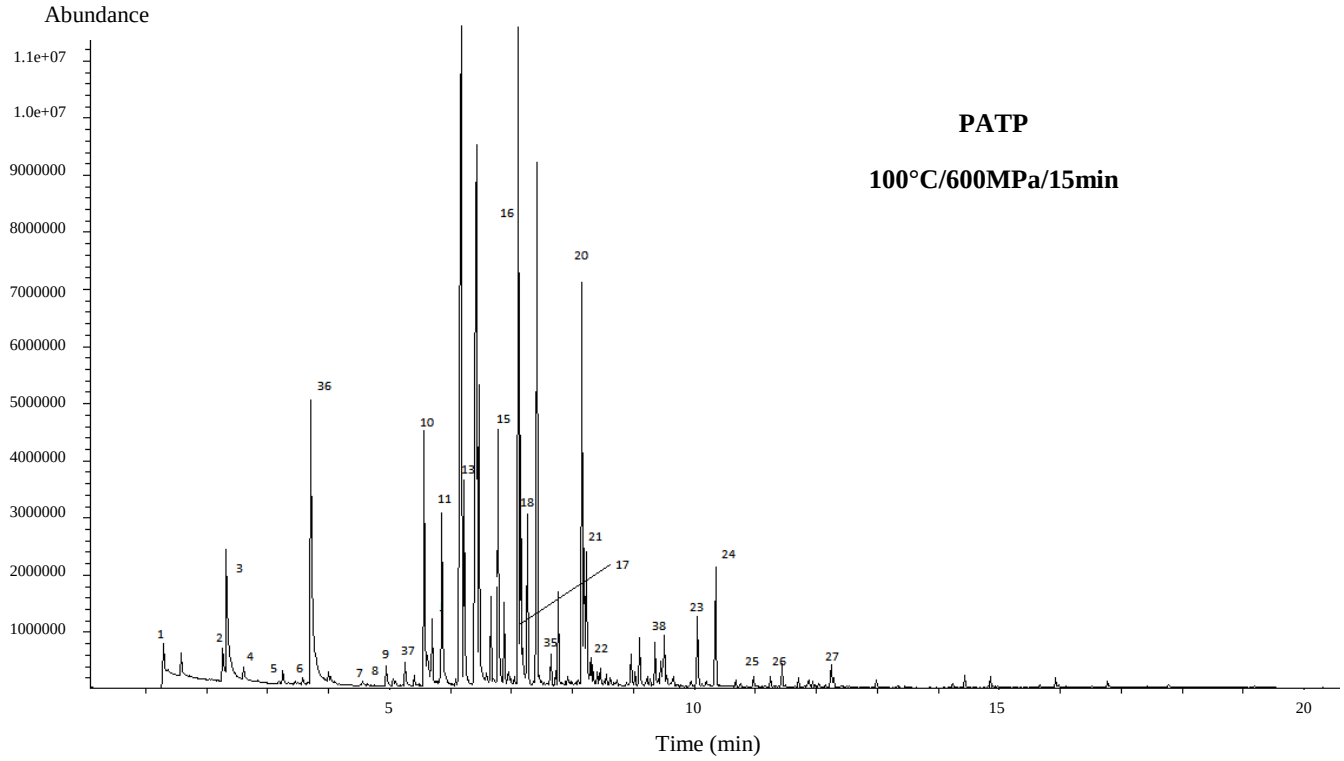

Figure 4.4. GC/MS chromatogram (SCAN mode) for PATP treated CLA-enriched milk obtained by SPME technique. Peaks numbered correspond to **1**, carbon dioxide; **2**, hydrogen azide; **3**, acetone (coeluting with propanal); **4**, DMS; **5**, 2-methyl propanal; **6**, 2-butanone; **7**, 3-methyl butanal; **8**, 2-methyl butanal; **9**, 2-pentanone; **10**, 4-methyl 2 pentanone (IS); **11**, toluene; **12**, 2-hexanone; **13**, hexanal; **14**, furfural; **15**, trans-2-hexenal (IS); **16**, 3-heptanone (IS); **17**, 2-heptanone; **18**, heptanal; **19**, benzaldehyde; **20**, 3-octanone (IS); **21**, 2-octanone; **22**, octanal; **23**, nonanal; **24**, 4-decanone (IS); **25**, decanal; **26**, 2-decanone; **27**, 2-undecanone; **28**, lactic acid amida; **29**, *cis* 13 octadenoic acid; **30**, *trans* 13 octadenoic acid; **31**, pentadecanoic acid; **32**, 17-octadecynoic acid; **33**, *n*-hexadecanoic acid; **34**; 2-furanmethanol; **35**, 4-methyl 2 heptanone; **36**, 3-methyl 2 pentanone; **37**, 2,4 dimethyl furan; **38**, 2-nonanone. Not labeled peaks are possible contamination from the column or fiber used. DMS: Dimethyl sulfide; IS: internal standard.

Acrolein was identified in milk treated samples (data not shown). This toxic compound was not found in GC runs of blanks. However, more experimental evidence is necessary to demonstrate the formation mechanism and its concentration as it was not the purpose of this study.

4.3.3. Fatty acid composition

Diet can markedly affect the composition of the fatty acid profile of milk. As previously reported by Martínez-Monteagudo et al. [50], the use of safflower oil diet (high in unsaturated fatty acids) incremented the content of UFA/CLA in AMF compared with the non-enriched milk. In addition, a higher ratio of unsaturated to saturated fatty acids was reported in the enriched samples than for the non-enriched samples (0.71 and 1.36, respectively). The high amount of UFA contained in the CLA-enriched milk (UFA: 56.38 % of total fatty acids; CLA: 6.77% of total fatty acids) (Table 4.4) together with the low concentration of minerals present in milk [18] can promote lipid oxidation. Linoleic acid (C18:2) is highly susceptible to photo-oxidation and make milk more susceptible to oxidation than normal milk [51].

Raw CLA-enriched milk was analyzed to determine CLA and fatty acid content by Martinez-Monteagudo et al. [52]. This determination was performed following the protocol described in [45, 50]. Table 4.4 entails fatty acid composition of the CLA-enriched milk used for the whole study.

Unsaturated lipids are prone to oxidation [13-14], and may negatively affect the flavor of dairy products [53]. Off-flavors due to fatty acid oxidation are of prime concern mainly when the proportion of unsaturated fatty acids in the milk fat was increased.

Table 4.4. Fatty acid composition (% of total fatty acids) of CLA-enriched milk adapted from Martinez-Monteagudo et al. [52].

Fatty acid	CLA-enrriched milk
C4:0	0.12±0.02
C5:0	0.37±0.03
C6:0	0.80±0.02
C8:0	0.45±0.03
C10:0	1.24±0.06
C11:0	0.19±0.05
C12:0	1.78±0.09
C13:0	0.06±0.01
C14:0	7.93±0.29
C14:1	0.78±0.27
C15:0	0.79±0.23
C15:1	0.18±0.02
C16:0	17.84±0.64
C16:1 t	0.89±0.10
C16:1 c	1.05±0.28
C17:0	2.30±0.27
C17:1	0.09±0.02
C18:0	9.04±0.31
C18: t9	2.01±0.65
C18:1 t11	13.66±0.44
C18:1 n9	28.04±0.78
C18:1 n7	0.66±0.19
C18:2	2.25±0.06
CLA	6.77±0.05
Ratio of unsat/sat	1.38

For the UP and UHT treatments, homogenized milk was used (2.7% milk fat) and for the PATP treatments, non-homogenized milk was used (3% milk fat), containing 43 mg CLA/g fat.

4.3.4. Effect of thermal processing and UHT storage on the formation of flavor compounds

4.3.4.1. Temperature and pressure history during PATS treatments

Very little research is available on the use of high pressure and its effects on flavor generation. Up to 18 volatile compounds were identified and selected after PATP processing. Only 10 volatile compounds (2-hexanone, hexanal, heptanal, 2-heptanone, octanal, 2-octanone, 2-nonanone, 2-undecanone, benzaldehyde and furfural) were quantified while the rest of the compounds were monitored only (Appendix B).

4.3.4.2. Total aldehydes and methyl ketones in milk samples

Volatile analysis showed that concentration of total aldehydes and total methyl ketones are significantly higher in samples treated at 125°C for 15 s compared to the treatments at 135°C and 145 °C at any holding time (Figure 4.5). At 125°C, as time increases, there is a higher concentration of the total methyl ketones and total aldehydes; however, as temperature increases to 135°C, at longer processing time, concentration of these two groups of compounds decrease. These data suggest that as time increases, some of these ketones and aldehydes could be decomposed into other compounds. At 145°C and both processing times (4 and 20 s), concentration of either total aldehyde or total methyl ketones remained constant. These findings might suggest that the velocity of the reactions involved in the formation of these compounds is faster at this temperature, which is in agreement with observations of Nawar & Wassef [54]. In addition, the SPME fiber might be saturated with non-target compounds with samples treated at 145°C.

At these conditions used, oxidation of unsaturated fatty acids occur forming peroxide-free radical (RO_2). This free-radical is able to remove hydrogen from an unsaturated fatty acid to rapidly convert it into hydroperoxides, which are the primary oxidation products [55-56]. As hydroperoxides are very unstable, they decompose easily into secondary oxidation products, such as aldehydes and ketones [33, 56]. In addition, secondary oxidation products might also decompose into other compounds.

Although some authors [36, 57] convey the idea that methyl ketones are the main compounds that show a surge in their concentration during heat processing, Figure 4.5 shows that total aldehyde concentration increased significantly in contrast to total methyl ketones (See also Appendix, Table C1 and Table C2). In addition, Contarini & Povolo [35] found that methyl ketones had a higher correlation to the severity of the heat treatment in fluid milk than aldehydes, which is not in agreement with our findings. One reason could be that the proportion of main precursors of these methyl ketones, and the saturated fatty

acids (C6:0, C8:0, C:10 and C12:0) are significantly lower (4.3%) than those reported in the other study that used non-enriched CLA milk (10%) [36]. These saturated fatty acids are the main precursors for the formation of 2-pentanone, 2-heptanone, 2-nonanone and 2-undecanone [36, 57]. In particular, at 125°C/15 s and 125°C/4 s (Figure 4.5 A.), total aldehydes had almost a 4 fold and 2 fold increase, respectively, when compared to total methyl ketones at the same conditions (Figure 4.5 B). Total methyl ketones (2-pentanone, 2-heptanone, 2-nonanone, 2-decanone, 2-undecanone, diacetyl and propanone) were evaluated in non-enriched CLA milk [44] after pasteurization (78°C/18 s) and UHT treatment (139°C/2 s), obtaining 61 µg kg $^{-1}$ and 115 µg kg $^{-1}$, respectively; being the major contributors 2-pentanone, 2- heptanone and diacetyl. On difference, in our study, lower concentrations were found (6.19 µg L^{-1}) of total methyl ketones at a similar treatment (135°C/4 s). 2-Heptanone and heptanal were the major contributors in heat treated milk (Figure 4.6 D and Figure 4.9 B).

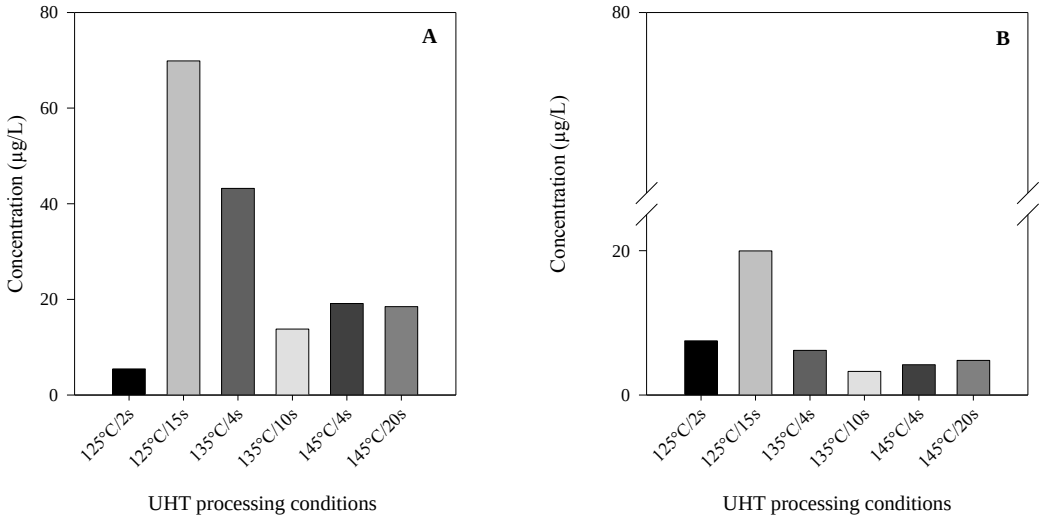

Figure 4.5 Concentration of (A) total aldehydes, and (B) total methyl ketones generated in heat treated CLA-enriched milk at different processing conditions. Total aldehydes include hexanal, heptanal, octanal, benzaldehyde and furfural. Total methyl ketones include 2-hexanone, 2-heptanone, 2-octanone, 2-nonanone and 2-undecanone.

Aldehydes

The formation of off-flavor compounds in milk from oxidation during processing or storage, involves a free radical reaction, forming hydroperoxides,

which later forms various malodorous ketones and aldehydes [51]. Straight aldehydes, such as hexanal, heptanal and octanal, are generated from autoxidation of unsaturated fatty acids (C18:1 and C18:2), where single oxygen, metal catalysts, heat, and light could be the possible initiators of the chain reaction [51]. Major precursors of the straight aldehydes (C6-C8) are oleic, linoleic, linolenic, and arachidonic acids [33, 39, 55-60]. Also, CLA can be considered as a source of odorants, like hexanal and heptanal; however, their formation is unlikely to be from free-radical mechanisms [22].

In our study, hexanal, heptanal and octanal were formed at thermal (Figure 4.6) and PATP processing conditions (Table 4.5). Concentration of these compounds was significantly different ($P<0.05$) between samples at 125°C/2 s and 125°C/15 s. On the contrary, no significant difference was found on samples treated at 145°C/2 s and 145°C /20 s ($P>0.05$). It has been reported that hexanal concentration (pasteurized: 0.75-5.21 ppb; UHT: 1.58-12.57 ppb) increases with the severity of the heat treatment (UHT commercial treatments) [36]. Although the concentration of hexanal is generally lower at higher temperatures than at 125°C, it should not be inferred that hexanal formation is inhibited at high temperatures. Hexanal may decompose to other oxidative products, such as free fatty acids. This might explain the presence of hexanal at low concentrations at high temperatures.

Formation of hydroperoxides from CLA was reported to be negligible at 30°C; however, in our study, temperatures were much higher (>125°C). Another proposed mechanism for the formation of volatile compounds from CLA is due to the cycloaddition of oxygen [22]. Moreover, CLA content in milk is affected by heat processing [19, 40-43] and during refrigeration conditions [40]. However, more studies are needed on the stability of CLA during processing and storage, especially on the implications on the flavor profile of CLA-enriched milk.

Hydroperoxide formation and CLA stability in heat treated milk rich in CLA was studied by Martínez-Monteagudo et al. [52]. The same technological conditions were used to evaluate volatile generation in this study. They observed

an increment on the formation of hydroperoxides at the shortest treatment times, regardless of the temperature treatment. However, at the highest treatment temperature (145°C) and shorter time (4 s), a lower increment is observed when compared to the other two treatments (125°C/2 s and 135°C/3 s). This finding suggests that formation of free radicals and consequently the formation of hydroperoxides is much faster at the highest temperature (145°C). As hydroperoxides are very unstable compounds, they can decompose into other secondary products, such as aldehydes and ketones. Hydroperoxides can also break down to produce more free radicals [56]. Therefore, one reason that can explain the lowest concentration of hydroperoxides at 145°C/4 s is because hydroperoxides are decomposing into secondary products, such as hexanal, heptanal and octanal.

Hexanal and heptanal have been suggested to be formed from the oxidation of arachidonic acid [61], linoleic acid [56] and CLA [22]. Yurawetz et al. [22] found that when *cis*9, *trans*11-CLA was kept in glass vials exposed to oxygen and ambient light for 8 days, heptanal and other compounds were formed. On the contrary, *trans*10, *cis*12-CLA formed mainly hexanal, suggesting that different CLA isomers generate different volatiles as secondary oxidation products, which is in agreement with the findings of Mallia et al. [21] and Garcia-Martinez et al. [20]. Although the amount of individual isomers of CLA present in the CLA-enriched milk used was not quantified (only total CLA) by Martínez-Monteagudo et al. [45], it might be possible that, *trans*10,*cis* 12-CLA isomer is present at lower percentages than *cis*9, *trans*11-CLA isomer as significantly high concentrations of heptanal (4 to 66 µg/L) were found in heat treated CLA-enriched milk (Figure 4.6 D). Hexanal was formed in a range of 0.06-1.26 µg/L (Figure 4.6 A).

Holding time had a significant effect on the concentration of heptanal at 125°C (2 s-15 s). A 13 fold increase in concentration was found among these two treatments, which is higher compared with other studies [35-36, 38, 62]. Variability of results might exist among the studies due to different amounts of fat in milk and the analytical method used. Commercial and non-commercial UHT

milk (1-3.2%) were analyzed and reported a range of heptanal concentration from 0.49 to 2.1 µg/L [35-36, 38, 62], which is considerably lower than our findings. However, no data is reported about fatty acid composition.

Hexanal has been considered as an oxidation marker in dairy products [17-18]; however, our results clearly show that heptanal can be used as a marker of the oxidation progress in CLA-enriched products which is in agreement with Garcia-Martinez et al. [20]. Nevertheless, heptanal is not a good marker for PATP treated samples (Table 4.5).

Additionally, hexanal, heptanal, octanal, and benzaldehyde were identified in pressure-treated milk (Table 4.5). For PATP samples, hexanal concentration ranged from 0.83-6.23 µg/kg at the conditions studied, obtaining the highest concentration for samples treated at 100°C/600 MPa/3 min. Remarkably, much higher concentrations of hexanal (79-91µg/kg) were reported by Parada-Rabell [44] at similar treatment conditions (105°C/650 MPa at 0,1 and 5 min) in milk samples being this compound one of the major off-flavors contributors in his study, which is not in agreement with our findings. Moreover, in another study [63], milk samples were treated at a lower temperature (75°C) and 655 MPa for 10 min and hexanal concentration was found even higher than in the previous (144.5 µg/kg).

The concentration of hexanal from 0.58 to 2.96 µg/L increased with the severity of the heat treatment, regardless of the holding time in control treatments (PATP study). Heptanal, on the other hand, had a different behavior than hexanal when milk was treated at the same conditions. Heptanal increased its concentration when temperature changed from 80 to 100°C as temperature continued to rise from 100 to 120°C; a reduction in its concentration is observed regardless of the holding time. Higher concentrations of heptanal (2.78 and 2.24 µg/L at 100 and 120°C, respectively) were found at a holding time of 9 min and at 100°C/15 min (2.46 µg/L). Martínez-Monteagudo et al. [45] studied CLA retention in CLA enriched milk and reported a retention of CLA in control samples (0.1 MPa) of 87, 71 and 59% in samples treated at 60, 90 and 120°C,

respectively, after 14 min of treatment, which might explain the formation of these compounds, as CLA is lost as processing temperature increases.

Heptanal was present at 3.55, 3.74 and 4.09 µg/L when milk was treated at 100°/600 MPa for 3, 9 and 15 min, respectively (Table 4.5), suggesting that pressure slightly affects the formation of heptanal. Whereas Vazquez-Landaverde et al. [63] measured 36.9 µg/kg of heptanal in samples treated at 75°C/655 MPa/10 min and a range of 1.60-5.63 µg/kg was found in samples treated at 580-620 MPa, 60°C and 1-5 min [38]. Although treatment conditions are quite similar dissimilarities in the CLA content of the milk used in our study and ratio of unsaturated to saturated fatty acids play a significant role in the obtained results. In addition, CLA-enriched milk might be protected from oxidation by its high content of antioxidants, such as CLA and probably because of α-tocopherol presence [16].

Octanal is suggested to be formed by oxidation of oleic acid [33, 55-56, 59-60], and linoleic acid [56]. In our study, octanal concentrations were found for UHT milk 0.32-3.08 µg/L) (Figure 4.6 B). Octanal has been found in raw milk (0.02 -1.44 µg/kg) [36, 38, 63-64], pasteurized milk (0.04-0.21 µg/kg) and UHT milk (0.48-2 µg/kg) [36, 38, 62, 64]. Higher concentrations were found in UHT milk with high percentage of fat [36].

Octanal concentrations were below 2 µg/L in almost all pressure treatments; except when milk was treated at 120°C in combination with 400 MPa/15 min, 600 MPa/9 min and 600 MPa/15 min (Table 4.5). Combination of moderate (400 MPa) and high pressures (600 MPa) and long holding times (15 min) at the highest temperature (120°C) triggered the formation of this compound at very similar extent (74.62 and 75.55 µg/L, respectively). Likewise, lower concentrations (20.90µg/L) were formed at shorter holding times (9 min) when milk was treated at 600 MPa. However, at 600 MPa/3 min, octanal concentration was only 0.45µg/L. At 80°C/600 MPa/9 min, octanal was found at 1 µg/L, whereas Vazquez-Landaverde et al. [63] reported 18.3 µg/kg of milk treated at a similar condition (75°C/655 MPa/10 min). Overall, octanal concentration

increased as temperature, pressure, and holding times increased. An overall trend was difficult to observe in octanal formation on control treatments. However, a decline in concentration was observed when temperature shift from 100 to 120°C at 3, 9 and 15 min of holding time.

In our study, the concentration of all the straight aldehydes had not the same behavior among them with the increase of the severity of the heat treatment. But, Vazquez-Landaverde et al. [38] found that straight aldehydes increased with the severity of heat treatment under atmospheric conditions.

Milk (3.2%) was pre-treated at 60°C/3.2 min following a more severe treatment of 79°C/3 and 5 min [38]. Hexanal, heptanal and octanal concentrations were found at a range of 11.48-11.87 µg/kg, 1.53-1.78 µg/kg and 2.35-2.73 µg/kg, respectively. When a pre-treatment at 60°C/3.2 min and subsequently to 100°C/3 and 100°C/5 min was used, hexanal, heptanal and octanal were found at a range of 15.24-16.98 µg/kg, 2.14-2.61 µg/kg, and 3.73-5.13 µg/kg, respectively. Hexanal concentrations were at least 10 times higher in their samples than in our findings at either 80°C or 100°C (0.58-1.77 µg/L and 100°C 1.33-1.68 µg/L respectively). Octanal, in the other hand was 3 to 5 times higher in their treatments, and heptanal concentration range was very similar in both studies.

An aromatic aldehyde was also included in our study. Benzaldehyde seemed to be less affected during thermal treatment. Even at the highest treatment temperatures, concentration remained very alike (0.08-1.11 µg/L). Benzaldehyde has been found in UHT heated milk at 0.2 µg/kg [35] and at 5 µg/kg [65]. Scalan et al. [66] suggested that benzaldehyde occurred in large concentrations in heated milk than in raw milk. It may be formed from lactose degradation when milk is subjected to thermal conditions. It might be formed from phenylalanyl residue and also from sugar molecules in the non-enzymatic browning reaction [58]. Samples treated at 125°C/2 s and 125°C/15 s were found to have a significant difference ($P<0.05$) (Figure 4.6 C). In general, a slight increment is seen in the concentration of benzaldehyde as temperature treatment increases, but no significant difference was found at the highest temperatures (Figure 4.6 C).

Benzaldehyde is the least dominant of all the compounds studied in the PATP treatments (below 2 µg/L) (Table 4.5). Formation of this compound is suggested to be from lactose degradation when milk is subjected to thermal conditions [66], however little information is found about lactose behavior during PATP. Overall, the highest concentrations are observed in samples treated at 100°C regardless of the pressure or holding time used. Similar concentrations were also found in UHT CLA-enriched milk in our study. On the other hand, higher concentrations (4.9 µg/kg) were found in "in-bottle" sterilized milk where conditions are equivalent to the following PATP conditions: 100°C/ 400 MPa/15 min.

2-Nonanone, nonanal and hexanal were the most prevalent compounds detected by GC-Olfactometry (GC-O) in fresh UFA/CLA enriched butter, also other aldehydes were detected, such as heptanal, octanal, and decanal. [21]. In our study, nonanal, which is suggested to be formed by oxidation of oleic acid [56] was found to be below quantification limits (BQL) at concentrations lower than 0.1µg/L in UHT samples. Also, it has been found in pasteurized milk at 2.5 µg/kg and in UHT processed milk at 5 µg/kg [62].

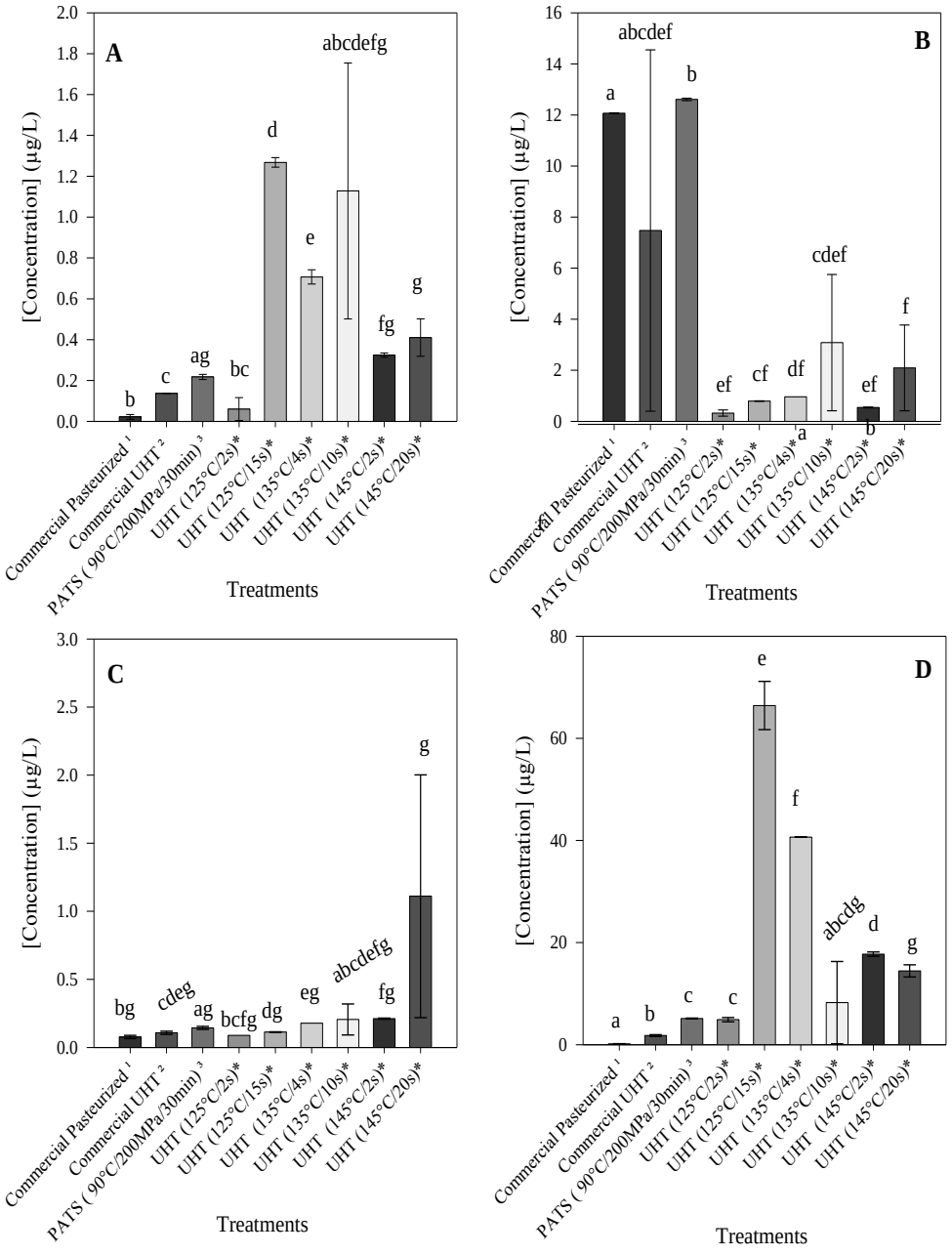

Figure 4.6. Concentration of (A) hexanal, (B) octanal, (C) benzaldehyde and (D) heptanal after using different processing technologies. Superscripts: ¹ 3.25% fat, ² 1% fat, ³ 3% fat, * 2.6% fat. Commercial samples are not enriched with CLA. Letters with different superscript are significantly different (n=2). No specifications about processing conditions were given for commercial samples.

In PATP treatments, high increments (3.07-87.32 µg/L) of furfural were observed in milk samples treated However, low precision was observed (SD:

10.16-82.06) in these results (Appendix, Figure D.6.). Thus, it is difficult to make assumptions based on the results. Nevertheless, milk acquired a brownish color as it was subjected to a more severe treatment (120°C/600/15 min), perhaps due to the formation of furfural or other furan derivatives. In other study [38], furfural concentration increased (0.98-3.77 µg/kg) with temperature and time under atmospheric pressure. In high pressure treatments, furfural was found in milk at a range of 1.60-2.40 µg/kg when milk was treated at 60°C/580 MPa and 1-5 min, decreasing its concentration as holding time increased; however, when pressure was increased to 620 MPa under the same conditions, slight change is seen with no trend observed (1.99-2.78 µg/kg). Furthermore, generally lower concentrations were observed in pressure-treated samples than in the corresponding control treatments in their study [38].

Table 4.5. Mean concentrations (µg/L ± SD) of aldehydes in CLA-enriched milk during PATP (n=2).

Treatment No.	Temperature (°C)	Pressure (MPa)	time (min)	hexanal	heptanal	octanal	benzaldehyde
1	80	0.1	3	0.58±0.21	1.75 ±1.00	0.98 ±0.01	0.28 ±0.04
2	80	0.1	9	1.77 ±0.33	0.96 ±0.04	0.37 ±0.01	0.35 ±0.08
3	80	0.1	15	0.96 ±0.11	0.50±0.50*	0.35 ±0.01	0.36 ±0.11
4	80	200	3	1.10 ±0.25	0.47±0.47*	0.35 ±0.21	0.22 ±0.05
5	80	200	9	0.83 ± 0.08	2.11±0.78	0.86 ±0.33	0.90 ±0.01
6	80	200	15	0.94 ±0.19	0.64±0.64*	0.37 ±0.21	0.37 ±0.07
7	80	400	3	2.77 ±0	0.80 ±0.80*	0.58±0.05	0.61 ±0.31
8	80	400	9	2.54 ±0.09	1.66 ±0.06	0.44 ±0.08	0.42 ±0.16
9	80	400	15	1.37 ±0.17	1.56±1.56*	1.19 ±0.05	0.62 ±0.34
10	80	600	3	1.67 ±0.23	1.48 ±1.48*	0.62 ±0.05	0.60 ±0.52
11	80	600	9	1.20 ±0.04	4.11 ±0.02	1.00 ±0.06	1.34 ±0.01
12	80	600	15	1.65 ±0.05	2.80 ±0.46	0.61 ±0.05	NA
13	100	0.1	3	1.68 ±0.37	1.52 ±0.48	0.42 ±0.26	0.48 ±0.18
14	100	0.1	9	1.33 ±0.21	2.78 ±0.48	1.20 ±0.26	1.40 ±0.40
15	100	0.1	15	1.52 ±0.06	2.46 ±0.48	1.11 ±0.26	1.21±0.48
16	100	200	3	1.47 ±0.12	2.72 ±0.01	1.05 ±0.06	1.01±0.44
17	100	200	9	1.71 ±0.50	3.04 ±0.44	0.81 ±0.06	1.0±0.05
18	100	200	15	1.77 ±0.18	3.65 ±0	1.18 ±0.06	1.18 ±0.53
19	100	400	3	2.31 ±0.18	3.24 ±0.30	1.02 ±0.11	1.20 ±0.18

*data not precise, high SD (>); NA: data not available.

Table 4.5 Continue

Treatment No.	Temperature (°C)	Pressure (MPa)	time (min)	hexanal	heptanal	octanal	benzaldehyde
20	100	400	9	2.56 ±0.63	3.14 ±0.30	0.83 ±0.11	1.16±0.15
21	100	400	15	3.22 ±0.34	4.22 ±0.30	0.89 ±0.11	1.12±0.25
22	100	600	3	6.23 ±2.42	3.55 ±0.02	0.76 ±0.07	0.91 ±0.44
23	100	600	9	2.94 ±1.29	3.74 ±0.53	0.65 ±0.04	0.99 ±0.16
24	100	600	15	1.61 ±0.28	4.09 ±0.01	0.79 ±0.07	1.01±0.06
25	120	0.1	3	2.92 ±0.40	1.19 ±0.49	0.39 ±0.11	0.61±0
26	120	0.1	9	2.01 ±0	2.24 ±0.11	0.50 ±0.05	0.83±0.32
27	120	0.1	15	2.96 ±0.39	1.71 ±0.40	0.43 ±0.08	0.92±0.02
28	120	200	3	1.48±0.85	2.40 ±0.78	0.99 ±0.38	1.52±0.19
29	120	200	9	2.36 ±0.09	3.33 ±0.09	0.74±0.06	1.02±0
30	120	200	15	1.73±0.19	1.88 ±0.25	0.45 ±0.04	0.93±0.04
31	120	400	3	2.03 ±0.44	2.23 ±0.95	0.59 ±0.24	0.99±0.03
32	120	400	9	3.8± 0.59	3.73 ±0.09	0.90 ±0.05	1.27±0.13
33	120	400	15	1.30 ± 0.71	5.93 ±0.04	74.62 ±3.84	0.85±0.19
34	120	600	3	2.74 ±0.19	1.72 ±0.24	0.45 ±0.02	0.75±0.09
35	120	600	9	2.42 ±0.16	3.54 ±0.25	20.90 ±0.01	0.84±0.21
36	120	600	15	2.39 ±0.12	5.88 ±0.25	75.55 ±0	0.98±0.33

*data not precise, high SD (>); NA: data not available.

For the storage stability study, CLA-enriched milk was processed at 125℃/15 s, 135℃/10 s and 145℃/20 s and stored at 4℃ and 25℃ under dark conditions (Figure 4.7). During cold storage (4℃), hexanal concentration of milk treated at UP: 125℃/15 s decreased from 0-7 days, whereas no change was observed from day 7 to 15. For the last 15 days, hexanal increased significantly (Figure 4.7 A). On difference, hexanal concentration (10 µg/kg) of UFA-CLA enriched butter did not change during 42 days of cold storage [21].

Mallia et al. [67] monitored hexanal of UFA/CLA enriched butter by GC/MS/O at 0 (fresh), 28, 42 and 56 days of storage. An increase in signal intensity of hexanal was observed from 0-24 days of storage from weak (fresh), medium (28 days) and strong (42 days). From day 42 to 56, the intensity was constant. But, hexanal was still not significantly higher in UFA/CLA butter when compared to conventional butter. Although this study can provide an idea of hexanal behavior in UFA/CLA milk, milk and butter are not produced in the same way as different processing conditions are used. Moreover, butter generally has more fat than milk, and milk has more than 85% of water. These factors, among others might make a difference in the stability study of both dairy products.

At the same conditions (UP:125℃/15 s) but at 25℃ storage (Figure 4.7 B), hexanal started to decrease after it was processed until day 15, but it is uncertain if concentration increased or decreased at day 30 because of the high variability in this condition. At the most severe treatment (145℃/20 s), and storage time at 4℃ (Figure 4.7 A) or at 25℃ (Figure 4.7 B), similar hexanal concentrations are observed up to day 15.

A similar scenario was observed in the behavior of heptanal during storage at 4°C with the same processing conditions (UP:125℃, 15 s) (Figure 4.7 C), but was not the same case at 25°C (Figure 4.7 D). Generally, at 135°C/10 s either at 4 or 25°C storage similar concentrations are observed. However, an unexpected increase is seen at 145°C/20 s at 25°C storage on day 30.

Heptanal in conventional butter were twice high compared to the UFA/CLA enriched butter exposed to the same conditions. On the contrary, at 0

days of storage heptanal concentration was twice high in UFA/CLA enriched butter than conventional butter [21]. These authors suggested that the higher levels of retinol and α-tocopherol in UFA/CLA enriched butter along with the protective effect of CLA contributed significantly to the inhibition/retardation on the formation of hexanal and heptanal. However, only a statistical significant difference was found in the levels of α-tocopherol between the conventional and UFA/CLA enriched butter samples (25 and 36 mg/kg, respectively). Although α-tocopherol in the CLA enriched milk was not determined in this study, diets high in safflower oil and rich in α-tocopherol can be transferred from the diet to the milk. Heptanal concentration was significantly higher in UFA/CLA enriched samples than in conventional butter after 8 weeks of storage (20 mg CLA/g fat and 12 mg CLA/g fat, respectively) [21]. Additionally, their study indicates that *cis* 9, *trans*11-CLA was the major CLA isomer (19 mg/g fat) present in the UFA/CLA enriched butter, suggesting that this isomer might play a significant role in the formation of heptanal. UFA/CLA-enriched butter during cold storage (6°C) for six weeks seems to affect levels of heptanal [21]. Odorants including hexanal, heptanal and nonanal significantly increased in UFA/CLA-enriched butter after 56 days [16].

Although initial concentrations (day 0) of octanal were below 5 µg/L (Figures 4.7 D and 4.7 E) regardless of processing conditions, an increase in concentration is observed in samples stored at 4°C, especially at day 30 during 4°C of storage. At both storage temperatures, high concentrations of this compound (~12-14 µg/L) were present. The high percentage (28% from total fatty acids) of oleic acid present in the CLA-enriched milk might be inducing the generation of this compound during storage.

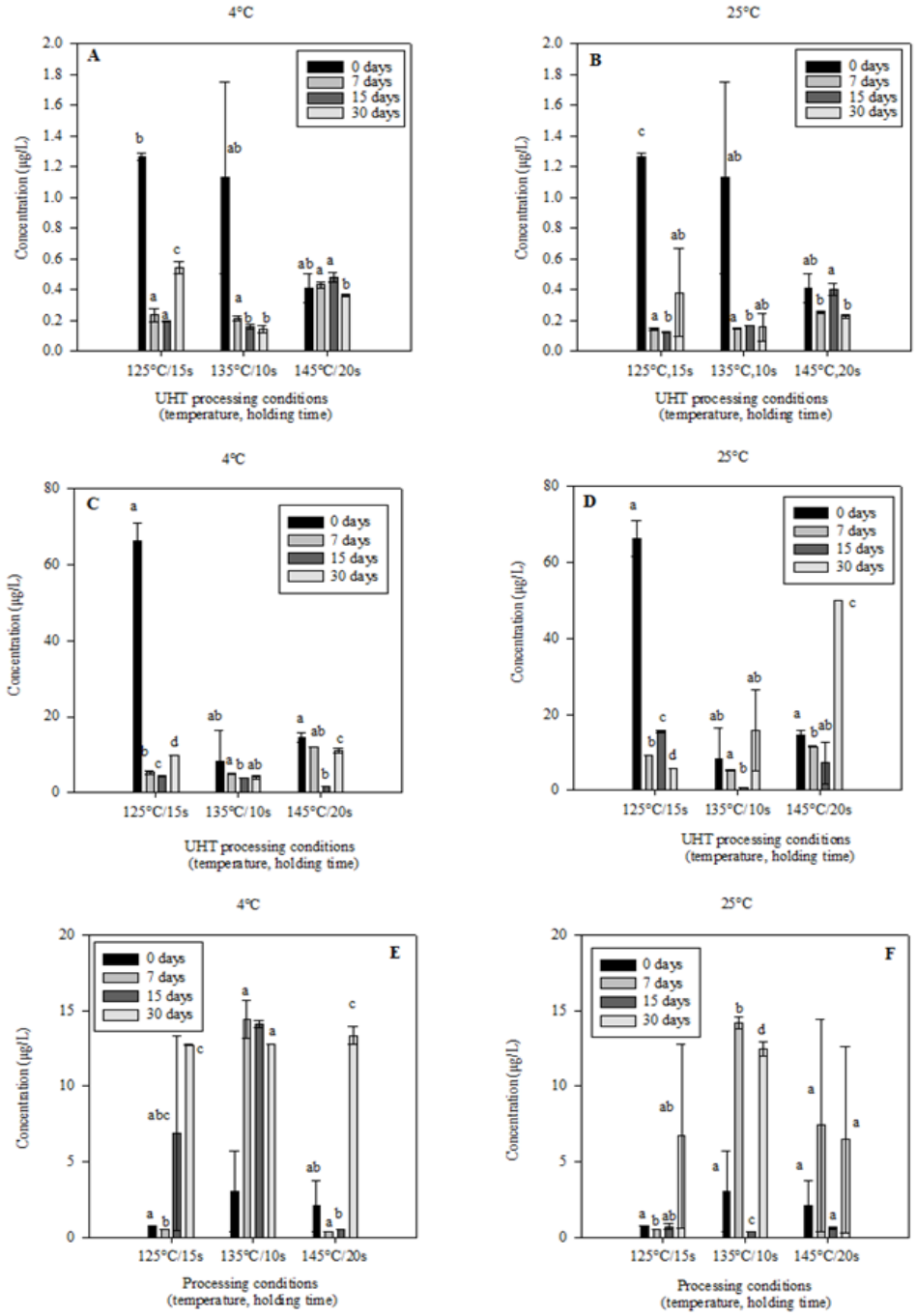

Figure 4.7. Hexanal (A,B), heptanal (C,D) and octanal (D, E) formation at 0, 7, 15 and 30 days of storage at two storage temperatures (4°C and 25°C). Means with the same processing condition within a plot with different letters are significantly different ($P < 0.05$). Presence of same letters or absence of letters means they are not significantly different between the samples ($P > 0.05$).

Benzaldehyde was more stable during cold storage (Figure 4.8) in agreement with Potineni & Peterson [68] that determined benzaldehyde during

storage at 5°C after milk was treated under more severe heat treatment conditions (i.e. UP and UHT). At 145°C, concentration was almost equal during the storage period at both storage temperature conditions. On the other hand, at lower processing conditions (Figure 4.8 A and B) and a storage of 25°C, benzaldehyde increased over time except at 135°C/10s from day 15-30. Mallia et al. [16] found that benzaldehyde started to have a weak odor intensity after day 28, that remained the same during the following days of the study (until 56 days).

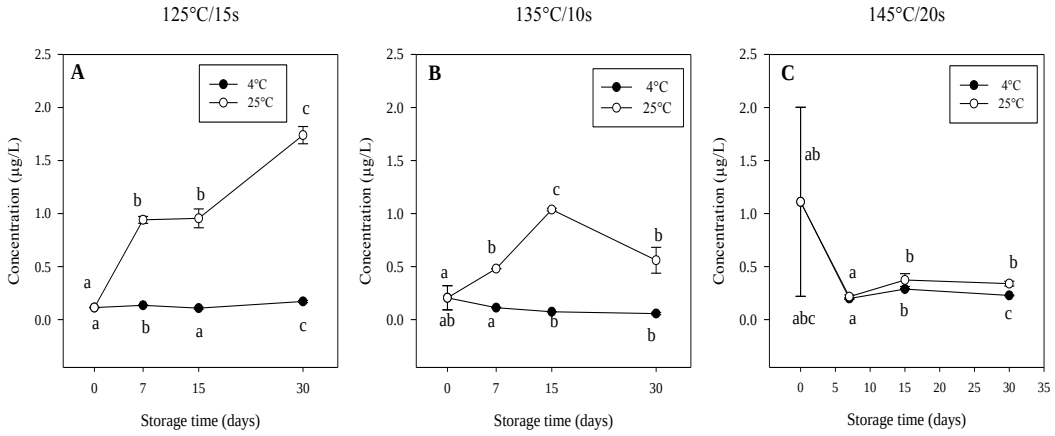

Figure 4.8. Benzaldehyde formation during storage at 4°C and 25°C for 0, 7, 15 and 30 days after thermal treatment at: (A) 125°C/15 s, UP; (B) 135°C/10 s, UHT, and (C) 145°C/20 s, UHT. Means within the same graph with different letters are significantly different ($P < 0.05$). Absence of letters means they are not significantly different between the samples ($P > 0.05$).

Based on empirical observations, intensity of honey color increases with the severity of the heat treatment, suggesting an increase of furfural in the heat treated milk samples, or perhaps the formation of other furan derivatives such as furosine and HMF [69] that were not analyzed in this study. Browning of milk and formation of furfural are induced by heating during Maillard reactions between lactose and an amino acid such as lysine [70]. Thus, it is considered a good indicator of the severity of the heat treatment [66, 69]. Although, its odor threshold is high (3000 ppb) [71] to be considered as an important aroma contributor, furfural is a precursor for the formation of melanoidins in Maillard reaction negatively affecting color of milk. Although concentration increase is not directly proportional to the increase of processing temperature (Appendix, Figure E.2.), might be inferred that furfural is decomposing to other furan derivatives.

The highest concentration is observed at 135℃/10 s and 125℃/ 15 s. At the lowest holding times of each treatment temperature (125℃/2 s, 135℃/4 s and 145℃/4 s), the lowest formation of furfural were observed. No significant (P>0.05) change was found in the treatments at 145℃ (4 s and 20 s), generating 0.32 and 0.41µg/L of furfural, respectively. Furfural was also determined in commercial milk samples and the lowest concentration was found in commercial pasteurized samples. Furfural has been found in milk treated by UHT processing by Scalan et al. [66]. Also, Vazquez-Landaverde et al. [36] quantified furfural in raw milk (3%), pasteurized (0% and 2%), and UHT milk samples (1% and 3%) at the following concentrations: 0.20 µg/kg, 0.14 µg/kg, 0.13 µg/kg, 0.52 µg/kg and 0.38 µg/kg, respectively.

Overall, furfural concentration was not affected by storage temperature at any treatment condition (Appendix, Figure F.1.). Either at 125°C or 135°C, a decrease in furfural is observed from day 0 to 7. However, at 145°C (Appendix F.1.), a slight increase was observed within the days of storage (0-15 days).

Methyl ketones

Methyl ketones are naturally present in raw milk [66]. High concentrations of 2-hexanone (0.2-0.53 µg/kg) were found in raw milk by Toso et al. [72]. 2-Hexanone concentrations below 0.2 µg/L were found in our study for commercial pasteurized and UHT non-enriched CLA milk, and CLA-enriched milk after UP, UHT and PATS treatments (Figure 4.9 A). Higher concentrations of 2-hexanone were obtained in UHT commercial milk (1.46-1.81 µg/L) and pasteurized milk (0.06-0.34 µg/kg) with different fat contents (0, 1 and 3%) [36]. One possible reason is the lower percentage of saturated fatty acids present in CLA-enriched milk.

2-Hexanone was also affected by pressure (Appendix, Figure D.1.). Little change was observed when temperature treatment increased from 80 to 120℃ at atmospheric conditions. However, when milk was treated at a high temperature in combination with a high pressure (600 MPa and 120℃), 2-hexanone concentration increased significantly (1.5-10.7 µg/L). Vazquez-Landaverde et al.

[38] reported a concentration of 1.19-1.64 µg/kg in treated milk at 580 and 620 MPa, 60°C and 1-5 min of holding time and concentration was not significantly affected by increasing the pressure and holding time. 2-Hexanone was formed at 5-7 times higher in our study, perhaps because of high temperature (120°C) used.

2-Heptanone is also a major contributor of off-volatiles in UHT milk [26, 35, 64]. The highest concentration of 2-heptanone at 125°C/15 s (19.59 µg/L) was significantly different (P<0.05) among the rest of the samples (Figure 4.9 B). A similar concentration was found in UHT commercial milk (22.32 µg/kg) with 1% fat [36]. However, no information was reported about the specific UHT conditions used. Contarini et al. [73] reported that 2-heptanone concentrations increased with the severity of the heat treatment (pasteurization-UHT-sterilization).

Combinations of high temperature and high pressure severely affected the formation of this compound, positioning as the major contributor among the methyl ketones studied (Appendix, Figure D.2.), which is in agreement with Parada-Rabell [44] who studied non-enriched CLA milk under PATP. When milk was subjected to control treatment (0.1 MPa), 2-heptanone concentration gradually increased from 0.31 to 7.74 µg/L as temperature increased (Appendix, Figure D.2). A negligible increase from 0.3±0.01 to 1.30±0.29 µg/L was observed as pressure increased at 80°C (Appendix, Figure D.2.). On the other hand, at 100°C, 2-heptanone concentration slightly increased as pressure increased (1.4±0.10 to 6.2±2.42 µg/L). At 120°C, similar concentrations were found in samples treated at 0.1 and 200 MPa, while at 400 and 600 MPa high increments were observed as holding time increased (5.0±2.8 - 55±0.40 µg/L). Parada-Rabell et al. [44] assessed the effects of HPP and PATP in the formation of methyl ketones, such as 2-pentanone, 2-heptanone, 2-nonanone, 2-decanone, 2-undecanone, diacetyl and propanone, finding that HPP treatment at 650 MPa/72 °C for 1 and 5 min resulted in the formation of these methyl ketones at 54 and 58µg/kg, respectively. However, when milk was treated under PATP conditions (650 MPa/105°C for 0-5 min), total methyl ketones ranged from 71-161 µg/kg, suggesting an increase of these compounds. 2-Heptanone was found to be one of

the most discriminating compounds within the chemical class of methyl ketones, and the possibility of using this volatile as a marker of thermal and PATP treatments in CLA-enriched milk.

In addition, in two different studies of pressured-treated milk, major contributors of methyl ketones were reported as 2-heptanone [44] and 2-hexanone [38]. Vazquez-Landaverde et al. [38] observed a dramatic increase in methyl ketones by thermal treatments (80°C) at atmospheric conditions, being 2-decanone and 2-heptanone the most affected, whereas concentrations of 2-octanone was not affected.

2-Octanone concentration (1.35±1.01 µg/L) was the highest at 135°C/10 s (Figure 4. 9 C). This is not in agreement with previous studies [36, 74], that state that 2-octanone is a predominant odorant in UHT milk. Vazquez-Landaverde et al. [36] reported concentrations of 2.65 and 4.51 µg/kg in UHT milk with 1 and 3% fat, respectively.

At 0.1 and 200 MPa, a similar pattern is observed for 2-octanone (Figure 4.9 C) where concentration increases as temperature increases from 80-100°C and longer holding times. However, concentration is negligible at any pressure studied at 120°C. One explanation might be that at 120°C, this compound decomposes into other compounds. Pressures of 400-600MPa seemed to accelerate the formation of this compound (Appendix, Figure D.3.). Nevertheless, concentrations did not exceed 2 µg/L. But, higher concentrations (3.70-4.79 µg/kg) were reported in milk treated at 580 and 620 MPa and 60°C for 1-5 min [38].

In the case of 2-nonanone, concentration slightly increased with the severity of the heat treatment (Appendix, FigureD.4.). This observation is consistent with previous literature reports [35-36, 73]. However, it is not the most intense compound found in UHT milk samples as Moio et al. [74] stated earlier. Significant difference (P <0.05) was only found at 125°C treatments. Vazquez-Landaverde et al. [36] quantified 2-nonanone in UHT milk with 1 and 3% milk fat and reported concentrations of 22.32 and 34.46 µg/kg, respectively, whereas in

our study the highest concentration was found at 1.41 µg/L (Appendix, Figure E.1.), which was the most severe condition (145°C/20 s).

2-Nonanone had a similar trend in its formation at 80°C and 100 °C regardless of the pressure (Appendix, Figure D.4.); but, a different behavior was observed at 120°C. At any studied pressure, a sudden increase in 2-nonanone concentration is observed on samples treated at 9 min followed by a reduction in concentration at 15 min. 2-Nonanone concentration ranged from 0.06 ±0.01 to 11.25±4.89 µg/L among all treatments. 2-Nonanone was also quantified by Vazquez-Landaverde et al. [38] at 60°C/580 MPa and 1, 3 and 5 min, and a slightly decrease in concentration was observed as holding time increased (2.63, 2.37 and 2.05 µg/kg, respectively). But, when samples were subjected to 620 MPa under the same conditions, concentration slightly increased (1.36, 1.40, 2.03 µg/kg respectively). At similar conditions in our study (80°C/600 MPa/3 min and 80°C/600 MPa/9 min) concentrations were found to be 0.15 µg/L and 0.94 µg/L, respectively, lower than the ones found in their study [36].

2-Undecanone, associated with the stale-heated flavor in UHT milk, has a ketone-floral odor [26]. Very low concentrations were found in our study (<0.15 µg/L) (Appendix, Figure E.1.). Vazquez-Landaverde et al. [36] observed that concentration increases with the severity of the heat treatment but concentration levels were much higher (6.64 and 9.7 µg/kg in 1 and 3% UHT milk, respectively) than those in our study.

In pressurized milk samples, the highest concentration of 2-undecanone was found at 100°C/400 MPa/3 min, although a high standard deviation was obtained (14.49±7.62 µg/L) (Appendix, Figure D.5.). 2-Undecanone was found at less than 0.02 µg/L in milk treated at 80°C/600 MPa and 3-9 min (Appendix, Figure D.5.). Whereas, in another study [38], 2-undecanone was found at ranges from 0.47 to 0.61 µg/kg at similar conditions (580 and 620 MPa, 60°C and 1-5 min).

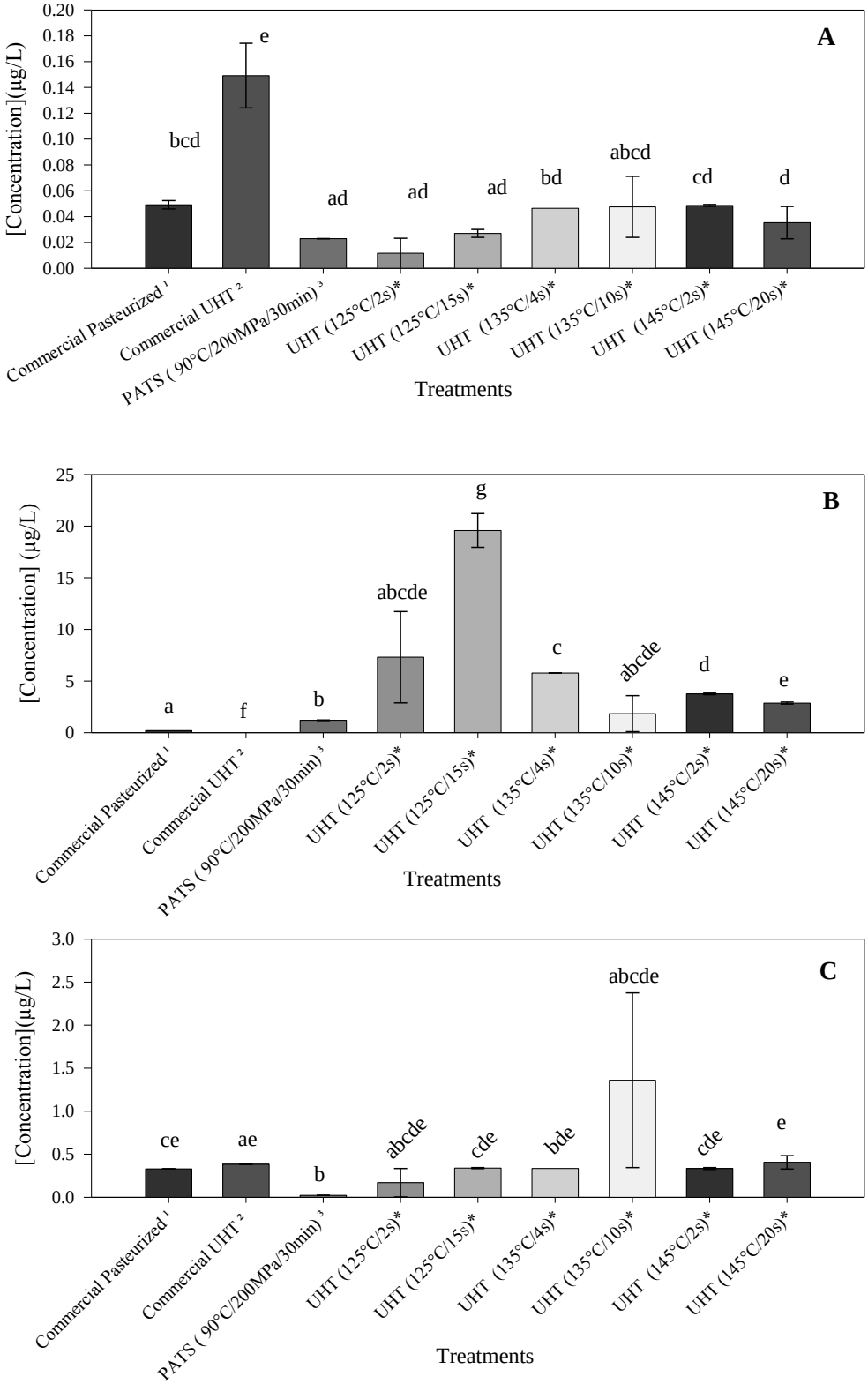

Figure 4.9. Concentration (µg/L) of (A) 2-hexanone, (B) 2-heptanone, and (C) 2-octanone in milk treated bydifferent processing technologies. Superscripts: ¹ 3.25% fat,² 1% fat, ³ 3% fat,*2.6% fat. Commercial samples are not enriched with CLA. No specifications about processing conditions were given for commercial samples. Means within the same graph with different letters are significantly different ($P < 0.05$). Absence of letters means they are not significantly different between the samples ($P > 0.05$).

After processing milk at 125°C/15 s (Figure 4.10 A and B), a high concentration of 2-heptanone is observed (~20 µg/L) but it decreased during storage time at 4°C (Figure 4.10). This compound was constant when treated at 145°C and cold storage (4°C). In difference, a sudden increase was observed from day 15 to day 30 when stored at room temperature (25°C). During cold storage of the treated samples at 125°C, a little increase of 2-hexanone concentration is observed from day 15 to day 30 (Figure 4.10 E); the same behavior is observed at 145°C during 25°C of storage (Figure 4.10 F).

During the storage study, samples treated at 145°C/20 s and day 0, concentration of 2-undecanone started to decrease over time (Appendix, Figure F.3.). Moreover at the same treatment conditions, the highest concentration for 2-nonanone is observed just after milk processing and slightly decreases within the storage study at 4°C and 25°C. When milk was treated at 125°C, a slight increase is observed on day 30 during storage at 25°C (Appendix, Figure F.3.).

In addition to the depletion of antioxidants and quenchers during storage of milk, oxidation products are formed. For lipid oxidation, secondary oxidation products were monitored. Although antioxidant content was not measured in this study, the concentration of flavor compounds during storage might be affected due to the presence of antioxidants in CLA-enriched milk, such as α-tocopherol. Moreover, CLA might be protecting the CLA-enriched milk from lipid oxidation [75].

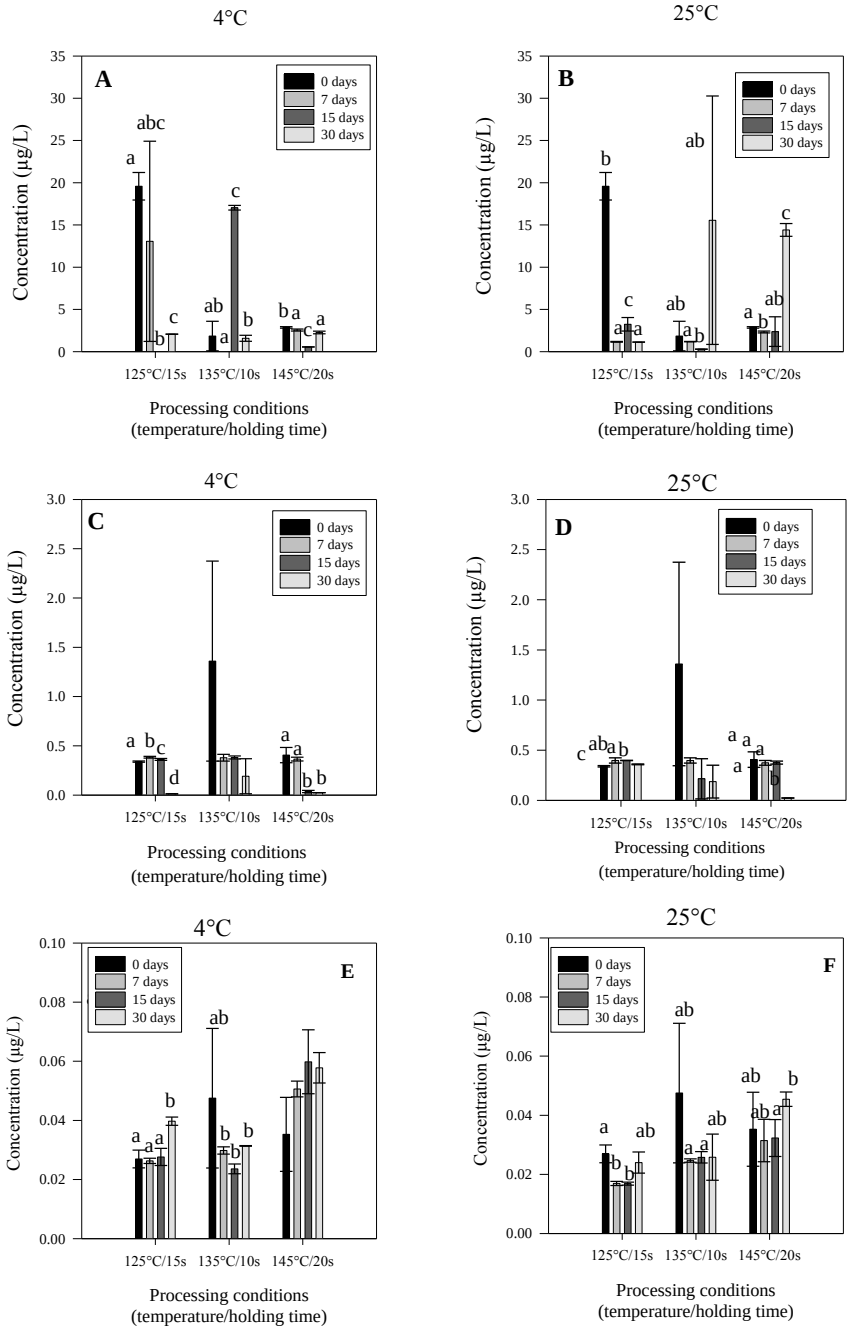

Figure 4.10. 2-Heptanone (A,B), 2-octanone (C,D), and 2-hexanone (E, F) formation after UP and UHT during 0,7, 15 and 30 days of storage at 4°C and 25°C. Means with the same processing condition within a plot with different letters are significantly different ($P < 0.05$). Use of same letters or absence of letters means they are not significantly different between the samples ($P > 0.05$).

4.3.5. Precision of the method using UP, UHT and PATP treated CLA-enriched milk

Precision of the method was evaluated by analyzing different samples of UP, UHT and PATP in duplicate. Although the standard deviation obtained was not as the expected for the normally obtained with chromatographic analysis, the high variability of the results is attributed to the high volatility of the extracted compounds and the change in composition of the matrix after milk processing and after the storage. Also, partition coefficients might be altered among each treated sample. Coefficient of variations (CV%) changed significantly from processing treatment conditions and the target compound (Appendix, Table G.1. and Table G.2.).

Similar variability was found in a study of volatile compounds in heated milk using purge and trap technique, GC and FID by Contarini et al [73]. They identified different compounds, belonging to the classes of ketones, aldehydes, terpenes and sulfur compounds, obtaining a (CV%) of 3.6-31.8%. Six of the compounds identified were similar to our compounds with the following CV: 3-methylbutanal (13.8%), 2-pentanone (5.3%), DMS (4%), hexanal (4.9%), 2-heptanone (10%) and heptanal (31.8%).

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4.3.6. Odor Activity Values (OAVs) in processed CLA-enriched milk

To estimate the sensory contribution of the odorants to the overall odor of the CLA-enriched milk, odor activity values (OAVs) were calculated by dividing the concentration (µg/L) by the nasal/retronasal odor threshold determined in different matrixes (oil, water or milk). OAVs were higher than 1 in heat treated milk indicated that they are important aroma contributors, such as heptanal, octanal and 2-heptanone in most of the treatments (Table 4.6). Although hexanal had a low threshold, OAVs were lower than 1, suggesting that hexanal is not an important aroma contributor in heated CLA-enriched milk. Octanal was a potential odor contributor in PATP treated milk. The rest of the compounds have high sensory thresholds, suggesting that these compounds are of less importance for the development of off-flavors in heated milk.

Odor threshold values were obtained from different studies in different matrixes like water [34, 71] and milk [33]. In each study, different compounds were studied. Odor threshold values were compiled from different literature sources, as there was no a single study source that included all of the target compounds used in our study.

Table 4.6. Odor Activity Values (OAV)[1] of some volatiles quantified treated CLA-enriched milk.

		Odor Activity Values (OAV)							
Compound	Odor threshold (µg/L)	UP milk 125°C/2s	UP milk 125°C/15s	UHT milk 135°C/4s	UHT milk 135°C/10s	UHT milk 145°C/4s	UHT milk 145°C/20s	PATP (90°C/200 MPa/30 min)	PATP (120°C/600 MPa/ 15min)
Hexanal	4.5[a]	0.01	0.28	0.15	0.24	0.06	0.09	0.05	0.79
Heptanal	3[a]	1.63	22.13	13.55	2.74	5.91	4.81	1.71	1.96
Octanal	0.7[a]	0.44	1.14	4.4	1.37	0.77	2.98	18.01	107.93
2-Hexanone	400[b]	0.00002	0.00005	0.0001	0.0001	0.0001	0.00007	0.0001	0.01
2-Heptanone	5[a]	1.46	3.91	1.15	0.36	0.74	0.57	0.24	10.64
2-Octanone	500[b]	0.0003	0.0006	0.0006	0.0027	0.0006	0.0008	0.00004	0.00004
Benzaldehyde	350[c]	0.0002	0.0003	0.0004	0.0005	0.0006	0.0031	0.0004	0.002

[1]OAV were calculated by dividing the concentration (µg/L) by the odor threshold (µg/L). Odor thresholds reported in: [a] [71] measured in water, [b] [33] measured in milk and [c] [34] measured in water.

4.3.7. Effect of catechin on the inhibition of flavor compounds

Enhancing rumenic acid levels along with a higher ratio of unsaturated fatty acids to saturated fatty acids in milk fat makes milk more susceptible to oxidation [76], allowing the formation of off-flavor compounds [53]. Thus, inhibition of volatile off-flavor compounds would be desirable. Catechins are effective in retarding oxidation of polyunsaturated fatty acids [77]. The effectiveness of catechin at 1 g/kg on the retention of CLA in milk and AMF under different high pressure and temperature conditions was reported by Martínez-Monteagudo et al. [45].

CLA-enriched milk samples containing 0.5 g catechin per kg were treated at 400 MPa, 120°C and 15 min. These processing conditions were selected as most of the off-flavor volatiles concentrations were high at these conditions. Catechin successfully reduced more than 80% of the off-flavor compounds studied, except for heptanal (Table 4.7). Milk was also treated under high pressure and mild temperatures (75°C/655 MPa for 3, 5 and 10 min) and epicatechin was effective in the inhibition of aldehydes at 0.1 and 1 g/kg [78]. More importantly, 2-heptanone was inhibited by 97% (Table 4.7).

It is not clear if catechin addition was effective on the reduction of certain volatiles, such as hexanal, 2-octanone and 2-hexanone, considering that these compounds have a high standard deviation in milk treated without antioxidant with no statistical significant difference. CLA-enriched milk can be treated with PATP and added with catechin to develop a commercial product without significant flavor changes.

Table 4.7. Effect of catechin addition on CLA-enriched milk on the formation of milk volatiles after PATP (400 MPa/120°C/15 min).

Off-flavor compounds	Treated milk <u>with</u> antioxidant (µg/L±SD)	Treated milk <u>without</u> antioxidant (µg/L±SD)	Reduction† %
Hexanal	0.19 ± 0.02	1.30 ± 0.71*	85
Heptanal	4.45 ± 0.35 [a]	5.93 ± 0.04 [b]	24
Octanal	13.24 ± 0.22 [a]	74.62 ± 3.84 [b†]	82
2-Heptanone	1.37 ± 0.009 [a]	54.99 ± 0.40 [b]	97
Benzaldehyde	0.09 ± 0.001 [a]	0.85 ± 0.19 [b]	88
2-Octanone	0.36 ± 0.006	4.11 ± 3.76*	91
2-Hexanone	0.09 ± 0.02	2.59 ± 1.72*	96

Means ±SD within the same row with different letters are statistically different ($P<0.05$). †Untreated milk concentrations are considered as 100% for the calculation of the percentage of the concentration reduced.* High SD; † Concentration above quantification limits (QL<50 µg/L)

4.4 Conclusions and recommendations

Eighteen flavor compounds in processed CLA-enriched milk were identified and/or quantified. Formation of methyl ketones and aldehydes were observed in CLA-enriched milk after thermal processing and PATP to a different extent. No trend was observed as pressure, temperature or holding time increased within groups of compounds (i.e. aldehydes and/or ketones). Moderate pressure and low temperatures causes minimum change of volatile compounds in milk. Combinations of pressure, temperature and holding time had a different effect on each flavor compound. 2-Heptanone, 2-hexanone and octanal were the most predominant compounds. 2-Heptanone is a suitable marker for PATP treatments (400-600 MPa and 100-120°C). High pressure and high temperature not only favored the formation of aldehydes in milk but also methyl ketones, which is not in agreement with previous studies.

Heat treatment promoted the formation of aldehydes and methyl ketones. Overall, the highest concentration of total aldehydes and methyl ketones was observed at 125°C/15 s. Aldehydes were more dominant compounds than methyl ketones. Generally, UHT processing had a more severe effect on both groups (except for octanal) over PATP, when comparing at a 90°C/200 MPa/30 min of treatment. Similar concentrations of 2-octanone were obtained in PATP and heat treated milk. 2-Heptanone and heptanal seemed to be suitable markers for heat treatment of CLA-enriched milk. A particular trend was not observed in the concentration of the off-flavor compounds within the days of storage. Hexanal and heptanal concentrations decreased over storage time regardless of the storage temperature; except for an increase in concentration of heptanal at day 30 at 25°C. Octanal tend to increase over storage time (4°C). Benzaldehyde concentration was not affected during storage at 4°C; however, an increase is observed at 125°C/15 s and 135°C/10s at day 30 and 15, respectively. 2-Octanone and 2-heptanone tend to decrease over storage time regardless of temperature storage. In contrast, 2-hexanone slightly increased over time at 4°C.

Major aroma contributors for the CLA-enriched treated milk were heptanal, octanal and 2-heptanone in UHT treated milk and PATP (200 MPa/90°C/30 min). However, 24, 82 and 97% of heptanal, octanal and 2-heptanone inhibition, respectively, was achieved when milk was treated at 400 MPa/120°C/15 min with the addition of catechin (0.5 g/kg). Evaluation of catechin addition under other PATP conditions is needed. Moreover, more research is needed to evaluate the effectiveness of catechin at different concentration levels, and the mechanisms involved when used under PATP conditions. The addition of catechin to UHT milk samples might reduce the formation of 2-heptanone and heptanal which were found at high concentrations, allowing to develop a commercial product that is more shelf-stable while possibly reducing the "cooked and stale" flavor. Therefore, future research is needed.

Even though positive outcomes were obtained regarding flavor formation during PATP, before recommending this technology as an alternative to heat treatment, studies on the effect of PATP on certain microorganisms in milk are needed. Moreover, more information is needed to understand the formation of these compounds under high pressure. Commercialization of the product might be feasible. However, a sensory study with different levels of CLA should be evaluated for consumer acceptance.

4.5. References

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Chapter 5. Conclusions and recommendations

For the first time, aldehydes and methyl ketones in raw CLA-enriched milk and after processing (UP, UHT and PATP) were quantified. The HS-SPME GC/MS technique developed in this study for the analysis of flavor compounds in CLA-enriched milk was accurate and sensitive for specific aldehydes and methyl ketones (C6-C8) and was used in the analysis of flavor compounds in processed CLA-enriched milk. From 18 volatiles identified, hexanal, heptanal, octanal, 2-hexanone, 2-heptanone and 2-octanone were quantified in processed CLA-enriched milk.

The method was less sensitive to compounds with lower volatility (i.e. 2-pentanone, 2-methylbutanal, 2-methylpropanal, 3-methylbutanal, etc). For some compounds, reproducibility was not acceptable; particularly for processed milk where formation of other compounds not originally present (i.e. acids) in the CLA-enriched milk might interfere with retention times of the target compounds. In addition, high variability was observed on chromatographic peak areas of internal standards of processed milk.

Reproducibility can be improved using an isotope standard, such as D_{12}-hexanal. To improve sensitivity over these compounds, it would be recommended to use a more polar capillary column than the DB-5 capillary column, such as a polyethylene glycol (PEG) column with a thicker film (i.e.1µm). Based on the results of this thesis and OAVs for each compound, the number of compounds to be monitored could be reduced to heptanal, 2-heptanone and octanal. The development and standardization of the analytical procedure based on only one or two compounds might result in a simple method for application in the analysis of CLA-enriched milk in dairy products.

Formation of methyl ketones and aldehydes were observed in CLA-enriched milk after thermal processing and PATP to a different extent. Aldehydes were more dominant compounds than methyl ketones in heat treated milk. Overall, the highest concentration of total aldehydes and total methyl ketones was observed at 125°C/15 s. Generally, UHT processing had a more severe effect on

both groups (except for octanal) over PATP, when comparing at a 90°C/200 MPa/30 min of treatment. 2-Heptanone and heptanal were found to be the most discriminating compounds, with the possibility to be used as markers for heat treatments. Only 2-heptanone was found to be the most discriminating compound in PATP treatments. These findings suggest that mechanisms of formation of these flavor compounds could follow different reaction pathways to those treated at atmospheric pressure. However, it is unknown if pressure and/or CLA were the main inhibitors on the formation of some of these flavor compounds. These speculations on flavor formation are possible to verify in further studies with non-enriched CLA milk after thermal and PATP processing.

An overall trend was not observed in the concentration of the flavor compounds within storage. Hexanal and heptanal concentrations decreased over storage time regardless of the storage temperature; except for an increase in concentration of heptanal at day 30 at 25°C. Octanal tended to increase over storage time (4°C). Benzaldehyde concentration was not affected during storage at 4°C; however, an increase is observed at 125°C/15 s and 135°C/10s at day 30 and 15, respectively. 2-Octanone and 2-heptanone tend to decrease over storage time regardless of temperature storage. In contrast, 2-hexanone slightly increased over time at 4°C.

Finally, major aroma contributors for the CLA-enriched treated milk were heptanal, octanal and 2-heptanone in UHT treated milk and PATP (200 MPa/90°C/30 min). However, 24, 82 and 97 % of heptanal, octanal and 2-heptanone inhibition, respectively, was achieved with the addition of catechin at 0.5 g/kg in milk treated at 400 MPa/120°C/15 min. However, more research is needed to evaluate the effectiveness of catechin at different concentration levels, and the mechanisms involved under PATP conditions. The addition of catechin to UHT milk samples might reduce the formation of 2-heptanone and heptanal which were found at high concentrations, allowing to develop a commercial product that is more shelf-stable while possibly reducing the “cooked and stale” flavor. Therefore, future research is needed.

Even though positive outcomes were obtained regarding flavor formation during PATP, before recommending this technology as an alternative to heat treatment, studies on the effect of PATP on certain microorganisms in milk are needed. In addition, a sensory study with different levels of CLA should be evaluated for consumer acceptance after milk processing.

Appendix A: SPME optimization and calibration curves

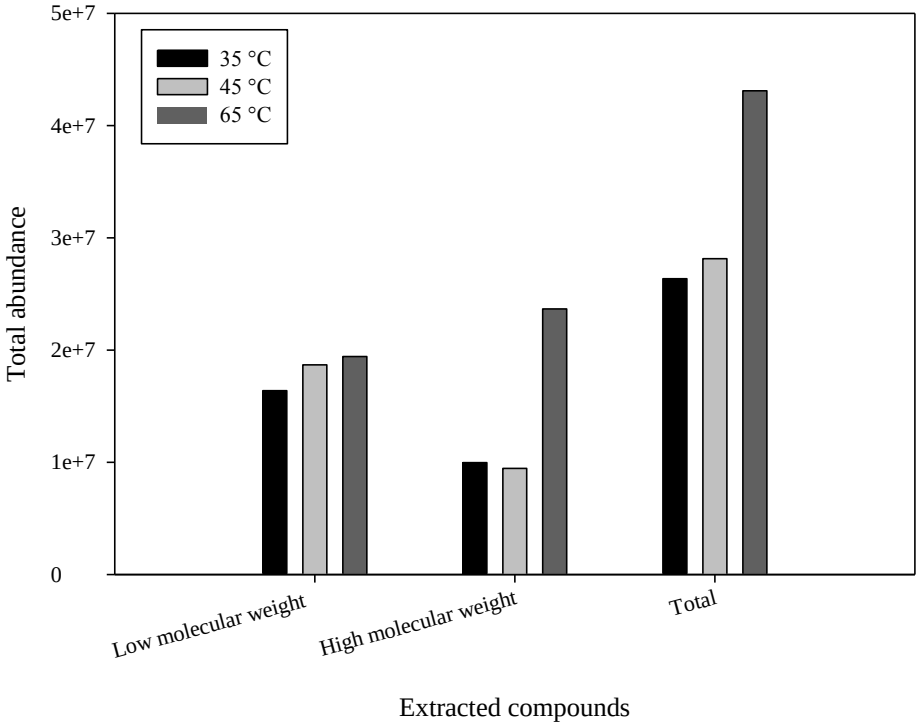

Figure.A.1. Effect of extraction temperature on the solid-phase microextraction sensitivity to milk volatiles in equilibrated raw milk (35°C, 5 min, at 250 r.p.m). Sample size fixed at 5g and 30 min of extraction time. Sum of the low molecular weight compounds' peak areas ($C_3 – C_6$) and high molecular weight compounds (C_7-C_{11}). Total= sum of low and high molecular weight compounds' peak areas. Analyses were carried out in duplicate.

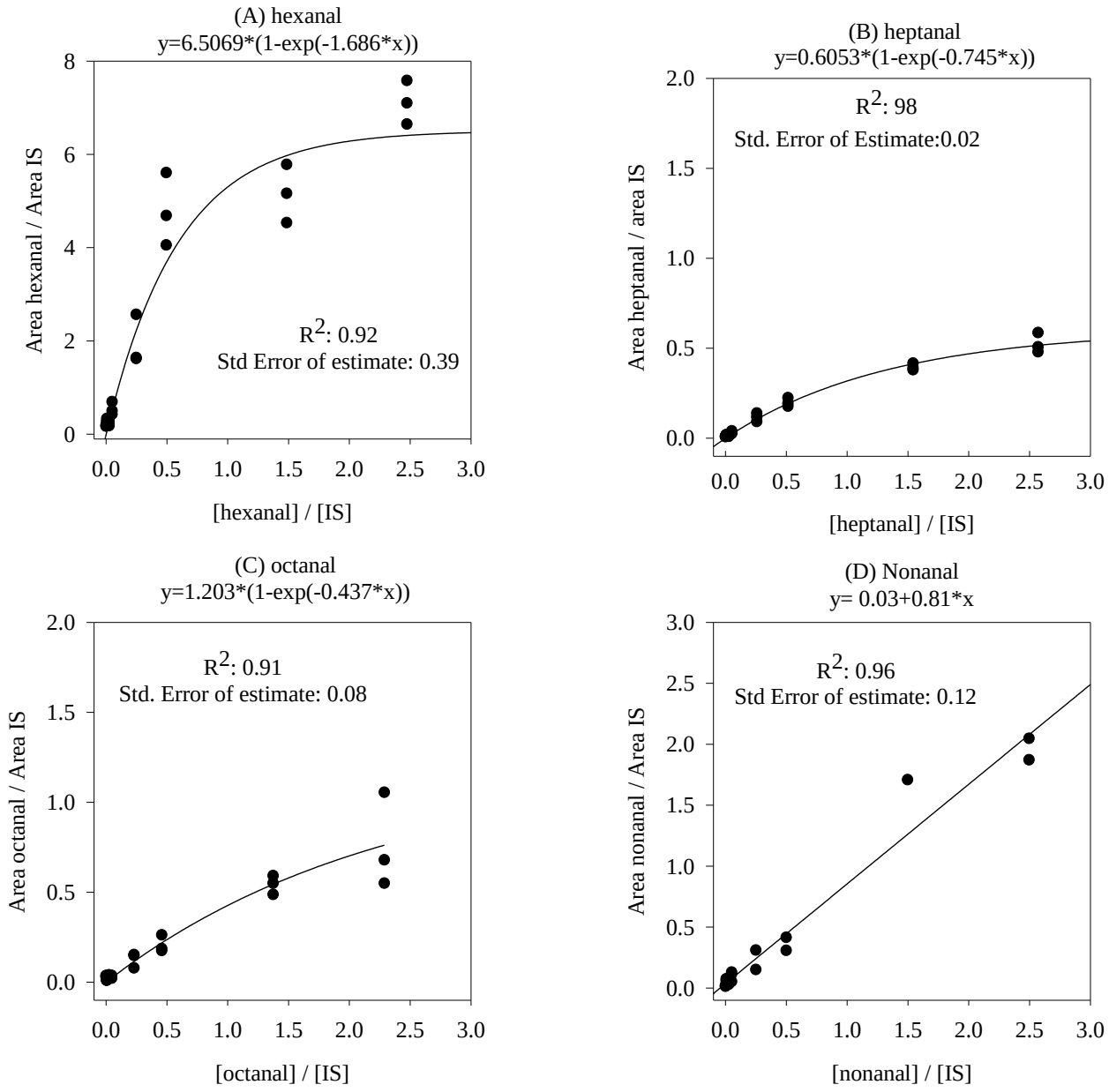

Figure A.2. Calibration curves constructed for (A) hexanal, (B) heptanal, (C) octanal and (D) nonanal in raw CLA-enriched milk.

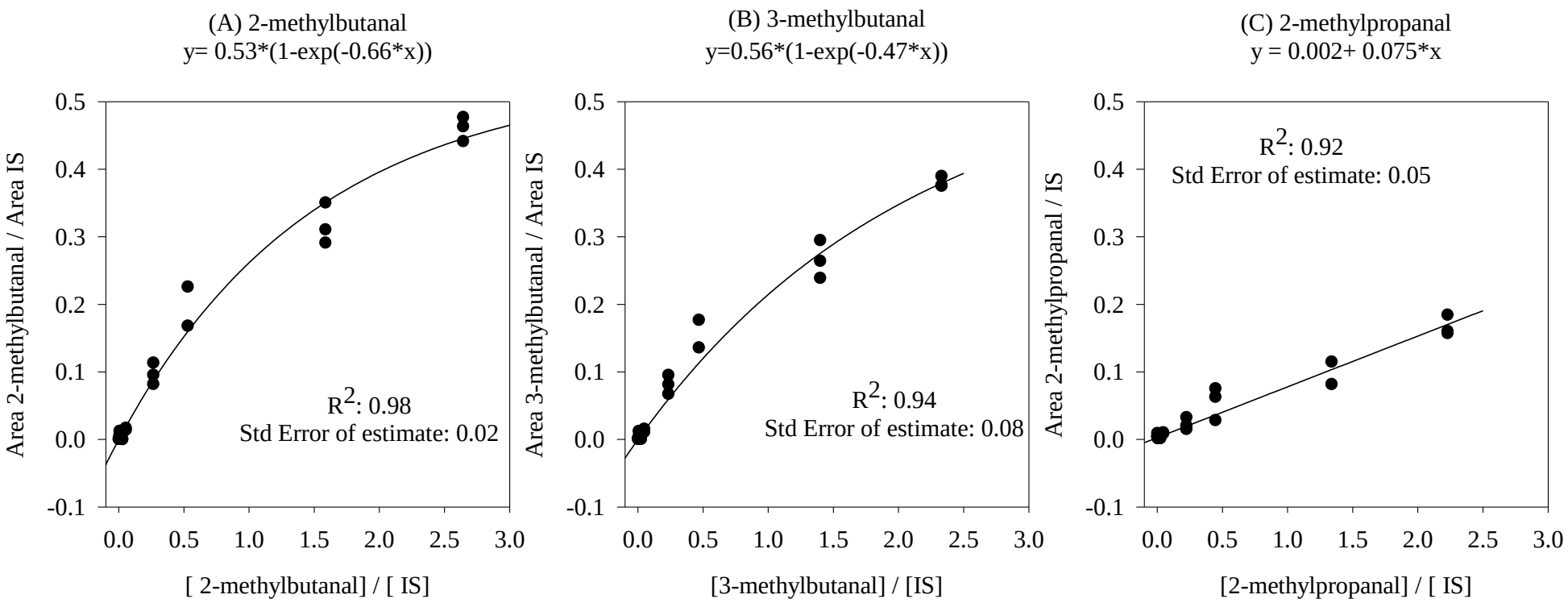

Figure A.3. Calibration curves constructed for branched aldehydes: (A) 2-methylbutanal, (B) 3-methylbutanal, and (C) 2-methylpropanal in raw CLA-enriched milk.

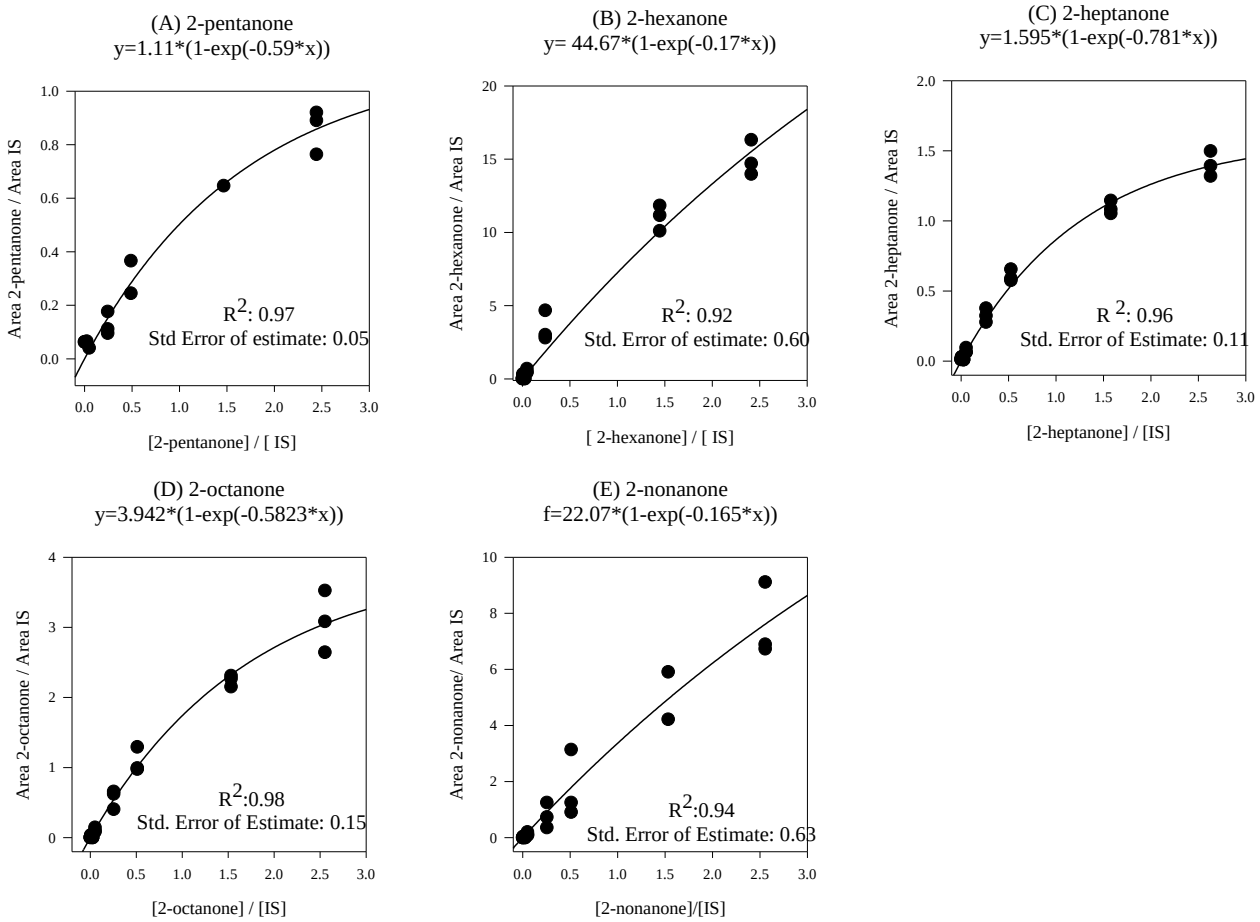

Figure A.4. Calibration curves constructed for methyl ketones (A: 2-pentanone, B: 2-hexanone, C: 2-heptanone, D: 2-octanone and E: 2-nonanone) in raw-CLA enriched milk.

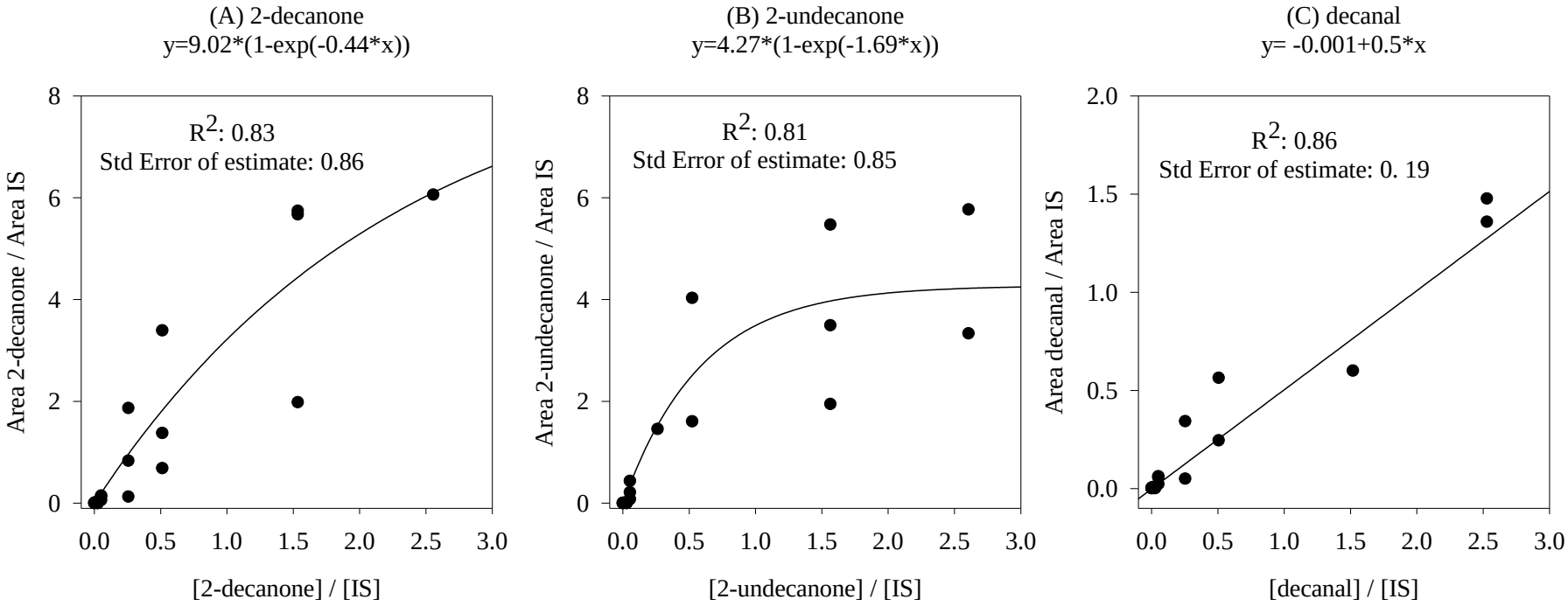

Figure A.5. Calibration curves constructed for (A) 2-decanone, (B) 2-undecanone and (C) decanal in raw CLA enriched milk.

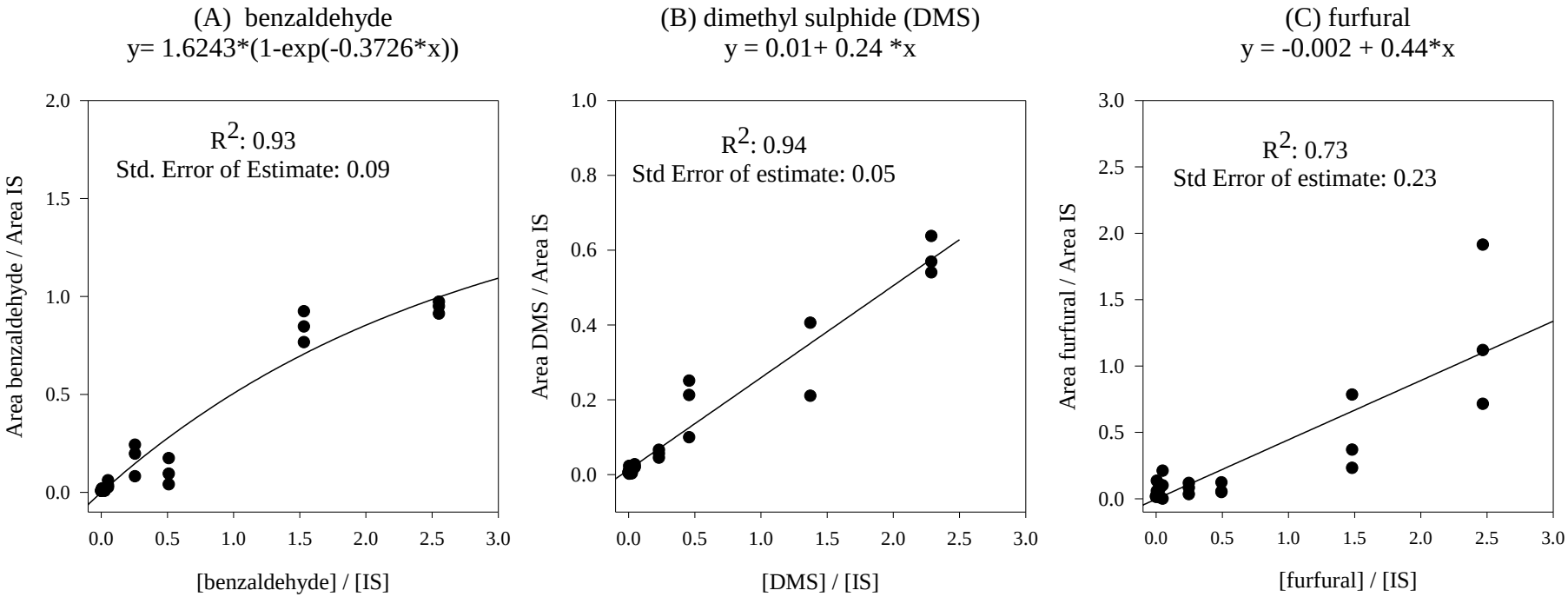

Figure A.6. Calibration curves constructed for (A) benzaldehyde, (B) DMS and (C) furfural in raw CLA enriched milk.

Appendix B: Identification of other aldehydes and methyl ketones in PATP treated CLA-enriched milk.

Table B.1. Peak areas of aldehydes and ketones in CLA-enriched milk after PATP. * Compounds were identified and monitored but not quantified

T (°C)	P (MPa)	t (min)	2-methylpropanal	2-decanone	3-methylbutanal	2-methylbutanal	DMS	2-pentanone	nonanal	decanal
80	**0**	**3**	27,025.00	3149	7939	18534	83890	16699	46693	925
	0	**3**	16,679.00	117	8853	7960999	64115	0	22921	1251
	0	**9**	19,861.00	199	311	7318998	64024	0	33452	2234
	0	**9**	14,109.00	1767	138	7116983	40926	0	76018	4682
	0	**15**	52,863.00	8359	11755	17019	83716	30951	47362	1912
	0	**15**	39,670.00	2685	9383	7485032	75464	0	90112	3647
	200	**3**	63,249.00	632	41342	14795	45446	33208	51426	888
	200	**3**	9,427.00	650	3422	7444049	24121	794947	71593	2057
	200	**6**	25,825.00	1003	4615	3871193	12548	0	104953	4511
	200	**6**	32,884.00	437	3660	4256957	10419	0	76170	2540
	200	**9**	14,544.00	5900	5807	5832654	20737	0	498407	20414
	200	**9**	144,761.00	4695	9284	9391845	13526	0	386767	16016
	200	**12**	16,141.00	4830	597	4647128	12554	0	310207	16377
	200	**12**	3,698.00	819	128	4394115	6116	406395	83634	3416
	200	**15**	6,542.00	484	69177	18813	23849	29091	30307	822
	200	**15**	32,895.00	10655	6678	7620701	29587	0	68379	3587
	400	**3**	12,614.00	1659	12494	23761	60927	33837	72220	1330
	400	**3**	14,667.00	21013	275	7428751	31326	0	899706	17149
	400	**6**	ND	ND	ND	ND	ND	ND	ND	ND
	400	**6**	ND	ND	ND	ND	ND	ND	ND	ND
	400	**9**	51,077.00	4170	8211	6671667	22951	509830	129988	5038
	400	**9**	9,408.00	6600	3947	6666246	32241	0	150042	5057
	400	**12**	28,407.00	1073	4829	4022864	19168	336733	78756	3107
	400	**12**	5,670.00	846	4333	3616593	18512	0	64306	2510
	400	**15**	10,516.00	1560	16330	19721	41096	27738	60456	1193
	400	**15**	13,657.00	2874	7799	6316092	20491	0	445981	13945
	600	**3**	115,715.00	4915	40383	16343	35856	29365	27848	727
	600	**3**	898,421.00	6467	7650	4350546	9853	0	158261	10218
	600	**6**	30,636.00	1071	4390	4066423	19947	338779	102476	3199
	600	**6**	52,696.00	2780	2504	4530678	17977	0	169325	7575
	600	**9**	41,983.00	27215	16577	2002608	37658	60567	774840	6823
	600	**9**	26,001.00	11067	10039	5408966	7676	0	229534	9407
	600	**12**	12,801.00	3015	3360	4009636	14185	0	135721	6155
	600	**12**	13,001.00	3238	8705	4354261	13140	0	108721	5068
	600	**15**	3,776.00	5762	31479	4956144	876	10132	16268	1182
	600	**15**	11,375.00	3074	825	4956144	12664	264371	113427	6709

Table B.2. Peak areas of aldehydes and ketones in CLA-enriched milk after PATP. * Compounds were identified and monitored but not quantified

T (°C)	P (MPa)	t (min)	2-methylpropanal	2-decanone	3-methylbutanal	2-methylbutanal	DMS	2-pentanone	nonanal	decanal
100	**0**	**3**	16,757.00	7880	17175	46713	97291	35735	62612	1670
	0	**3**	10,438.00	1112	10612	1986302	31062	0	91540	3998
	0	**9**	13,133.00	91971	6862	1691523	46337	109783	581404	16407
	0	**9**	32,561.00	9260	4462	1679181	21756	97611	270322	14006
	0	**15**	15,152.00	7931	22631	37208	77010	108180	76983	1770
	0	**15**	31,237.00	14879	181	4415022	22888	225218	262213	10991
	200	**3**	17,726.00	5301	18975	19027	79858	64104	41393	877
	200	**3**	18,316.00	4754	369	3301654	48395	0	205959	8474
	200	**6**	16,157.00	1529	3469	3972510	67155	127514	74182	2562
	200	**6**	10,668.00	1555	1959	4303909	20335	246623	112852	4092
	200	**9**	27,170.00	13699	7348	5552538	71318	174103	198308	12572
	200	**9**	10,159.00	6522	10609	2008902	64367	172493	276610	13080
	200	**12**	9,837.00	1694	149	3742624	43560	111493	97279	2781
	200	**12**	12,366.00	1382	3190	1650159	50833	146824	97407	2579
	200	**15**	15,239.00	4273	133435	31314	53152	119716	47856	735
	200	**15**	27,575.00	5773	5377	3984266	59399	180033	221664	8524
	400	**3**	81,207.00	2350	23886	30175	68580	142601	70462	1583
	400	**3**	11,957.00	2384	4937	3717230	29649	115326	157741	4617
	400	**6**	ND	ND	ND	ND	ND	ND	ND	ND
	400	**6**	ND	ND	ND	ND	ND	ND	ND	ND
	400	**9**	16,559.00	8246	5211	5827199	0	162790	183663	7728
	400	**9**	28,432.00	13458	4298	4147659	55193	131859	167755	6912
	400	**12**	ND	ND	ND	ND	ND	ND	ND	ND
	400	**12**	ND	ND	ND	ND	ND	ND	ND	ND
	400	**15**	40,092.00	5334	29697	43268	114649	96655	62990	808
	400	**15**	8,854.00	3304	4753	1263463	27665	155977	138820	4428
	600	**3**	105,510.00	5253	22545	22807	56976	88841	39784	1187
	600	**3**	47,029.00	4147	4398	5555678	217659	649769	74521	4974
	600	**6**	12,288.00	2351	133	4465822	57497	130231	114058	4722
	600	**6**	12,272.00	1341	3380	4465822	68205	159766	86027	2342
	600	**9**	57,884.00	3308	3665	6371105	166599	690884	50644	2943
	600	**9**	35,475.00	2956	2772	6428582	106504	244344	70521	5576
	600	**12**	4,493.00	4107	3451	2354089	71175	174878	70629	5332
	600	**12**	8,064.00	2061	182	4081959	29921	151439	131117	4882
	600	**15**	3,802.00	3176	23395	24925	35671	181678	95697	1865
	600	**15**	28,011.00	6010	5225	208547	141068	349814	83980	7226

Table B.3. Peak areas of aldehydes and ketones in CLA-enriched milk after PATP. * Compounds were identified and monitored but not quantified

T (°C)	P (MPa)	t (min)	2-methylpropanal	2-decanone	3-methylbutanal	2-methylbutanal	DMS	2-pentanone	nonanal	decanal
120	**0**	**3**	74,342.00	1464	38335	39918	136431	109106	130003	3491
	0	**3**	27,404.00	1701	4531	9022453	93824	914608	66130	2684
	0	**9**	54,448.00	4229	7126	8524954	32268	597987	65964	4346
	0	**9**	17,227.00	6256	8770	9267078	32268	1673475	73914	6987
	0	**15**	65,919.00	13596	42599	76710	79025	636872	65392	4714
	0	**15**	179,950.00	4468	32900	8412556	45954	706684	148062	6164
	200	**3**	116,460.00	10926	63312	80209	241074	516861	83010	8905
	200	**3**	100,270.00	8565	11282	6619551	277310	716299	25873	5578
	200	**9**	124,080.00	10035	12725	3176194	115055	1015192	433155	18035
	200	**9**	100,037.00	3711	14742	8305236	56321	2312961	117015	7300
	200	**15**	71,476.00	14959	55868	83630	92200	759060	99041	4850
	200	**15**	121,366.00	14093	14929	6499222	128790	2263710	319530	12738
	400	**3**	15,856.00	10630	170399	65528	100907	1143591	470873	2316
	400	**3**	82,039.00	4015	13009	9022344	73839	870414	407867	14189
	400	**9**	194,896.00	18287	19098	7613040	99609	1238361	461027	27235
	400	**9**	102,692.00	15034	18389	7493773	85284	1044750	591649	22948
	400	**15**	28,296.00	5276	11675	5148313	1020	699321	28494	3190
	400	**15**	30,562.00	2230569	38441	5148313	162698	572682	186326	19820
	600	**3**	21,772.00	29696	54952	63807	218965	620011	76830	5766
	600	**3**	103,289.00	8565	18074	7023583	150118	1721608	301040	14855
	600	**9**	ND	ND	ND	ND	ND	ND	ND	ND
	600	**9**	164,316.00	9713	24162	8990781	214058	974757	101095	8203
	600	**15**	59,983.00	54076	47539	83416	224787	790520	67801	5327
	600	**15**	48,749.00	8086766	32651	5684663	77355	614333	162125	2397487

Appendix C: Concentration of aldehydes and methyl ketones in heat treated milk.

Table C.1. Concentration (µg L^{-1}) of individual and total aldehydes in treated milk.

	Processing Conditions					
Average Concentration (µg/L)	**125°C/2s**	**125°C/15s**	**135°C/4s**	**135°C/10s**	**145°C/4s**	**145°C/20s**
Hexanal	0.06	1.27	0.71	1.13	0.32	0.41
Heptanal	4.91	66.42	40.66	8.24	17.73	14.45
Octanal	0.32	0.79	0.96	3.08	0.55	2.09
Benzaldehyde	0.09	0.11	0.18	0.21	0.21	1.11
Furfural	0.06	1.27	0.71	1.13	0.32	0.41
Total aldehydes	5.44	69.86	43.21	13.79	19.14	18.48

Table C.2. Concentration (µg L^{-1}) of individual and total ketone in treated milk.

	Processing Conditions					
Average Concentration (µg/L)	**125°C/2s**	**125°C/15s**	**135°C/4s**	**135°C/10s**	**145°C/4s**	**145°C/20s**
2-Hexanone	0.01	0.03	0.05	0.05	0.05	0.04
2-Heptanone	7.31	19.58	5.78	1.84	3.75	2.87
2-Octanone	0.17	0.34	0.33	1.36	0.33	0.41
2-Nonanone	0.01	0.02	0.03	0.02	0.06	1.42
2-Undecanone	0	0	0	0	0	0.07
Total ketones	7.50	19.97	6.19	3.27	4.19	4.79

Appendix D: Aldehydes and methyl ketones in PATP treated milk.

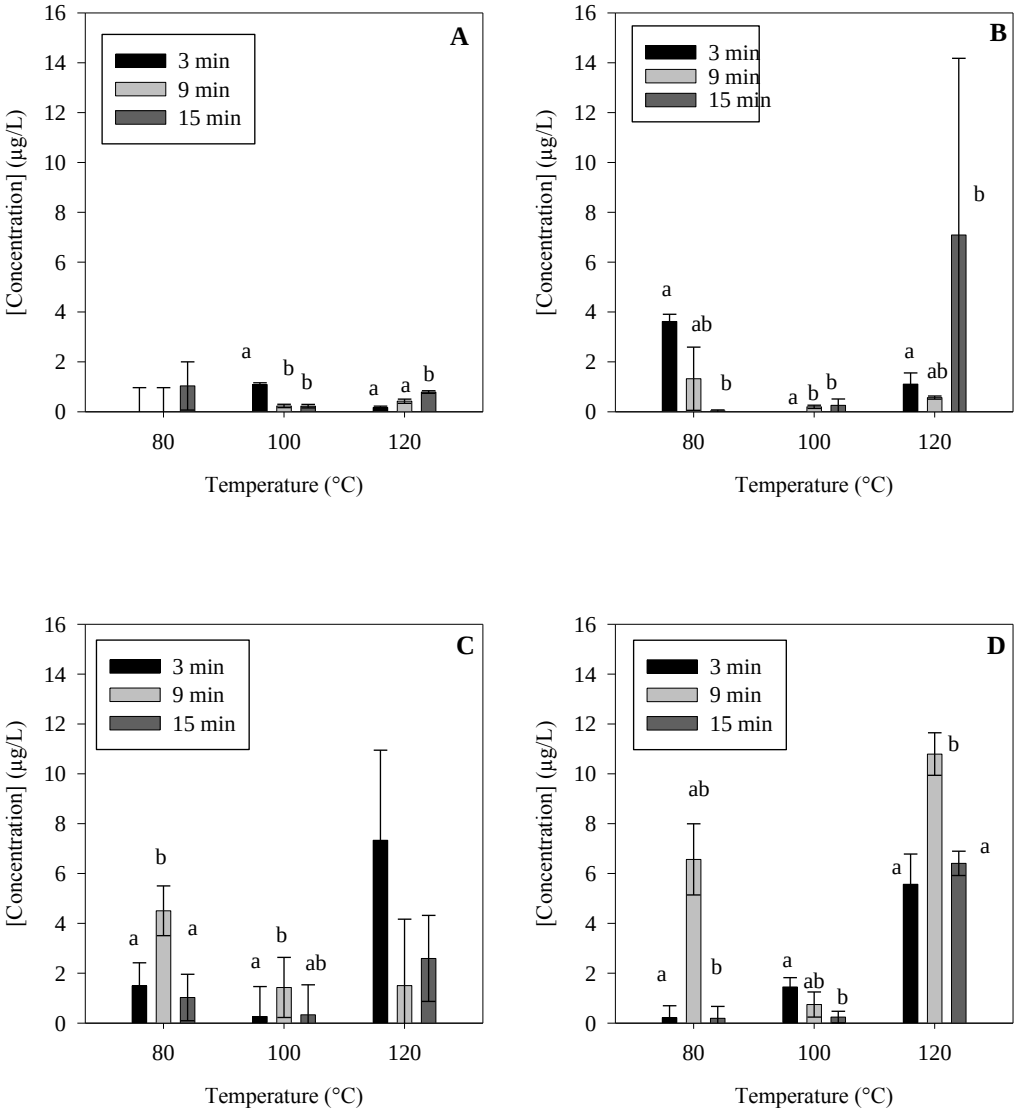

Figure D.1. 2-Hexanone formation in CLA-enriched milk treated at 0.1 (A), 200 (B), 400 (C) and 600 MPa (D) and temperatures of 80, 100 and 120 °C. Means within each graph with the same temperature with different letters are significantly different ($P < 0.05$). Presence of same letter or absence of letters means they are not significantly different between the samples ($P > 0.05$).

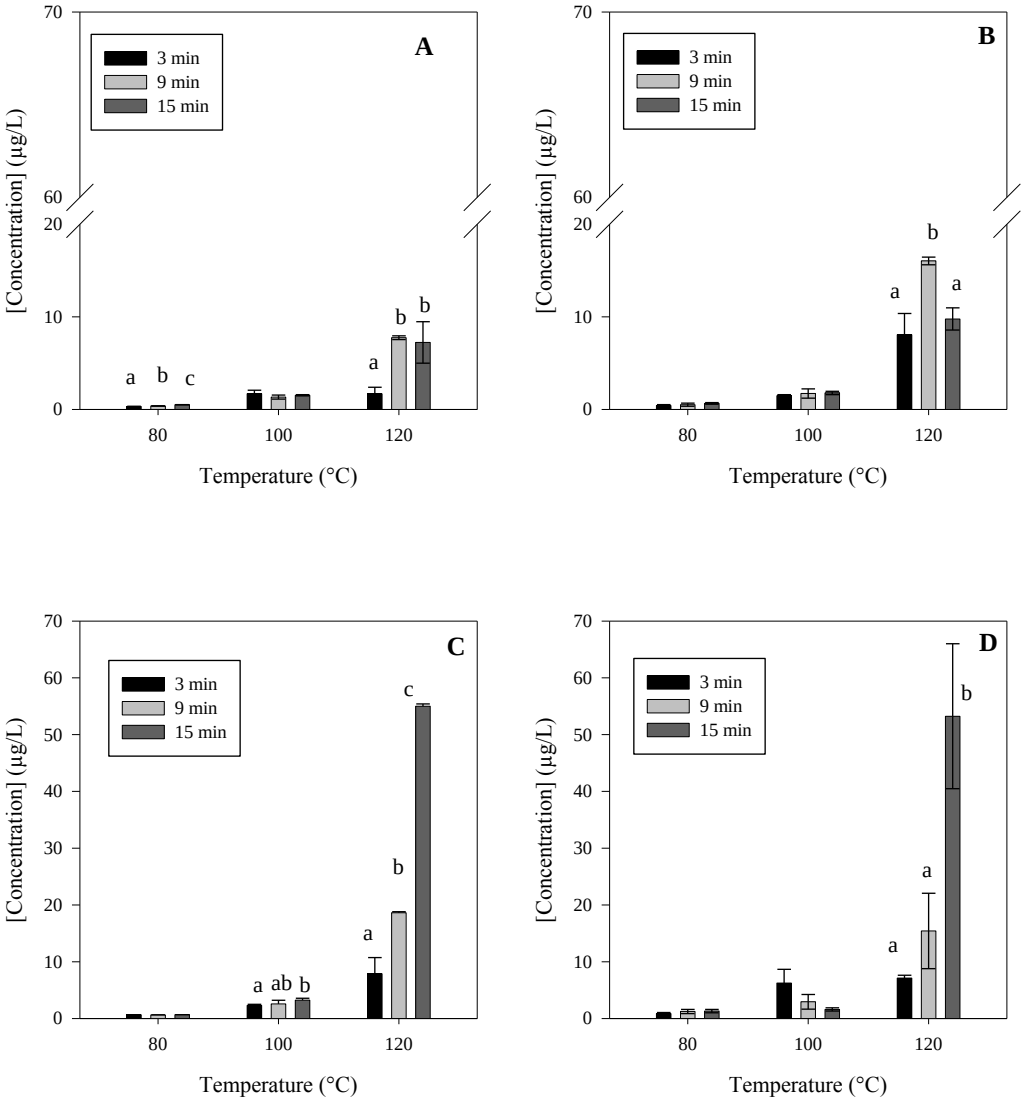

Figure D.2. 2-Heptanone formation in CLA-enriched milk treated at 0.1 (A), 200 (B), 400 (C) and 600 MPa (D) and temperatures of 80, 100 and 120 °C. Means within each graph with the same temperature with different letters are significantly different ($P < 0.05$). Presence of same letter or absence of letters means they are not significantly different between the samples ($P > 0.05$).

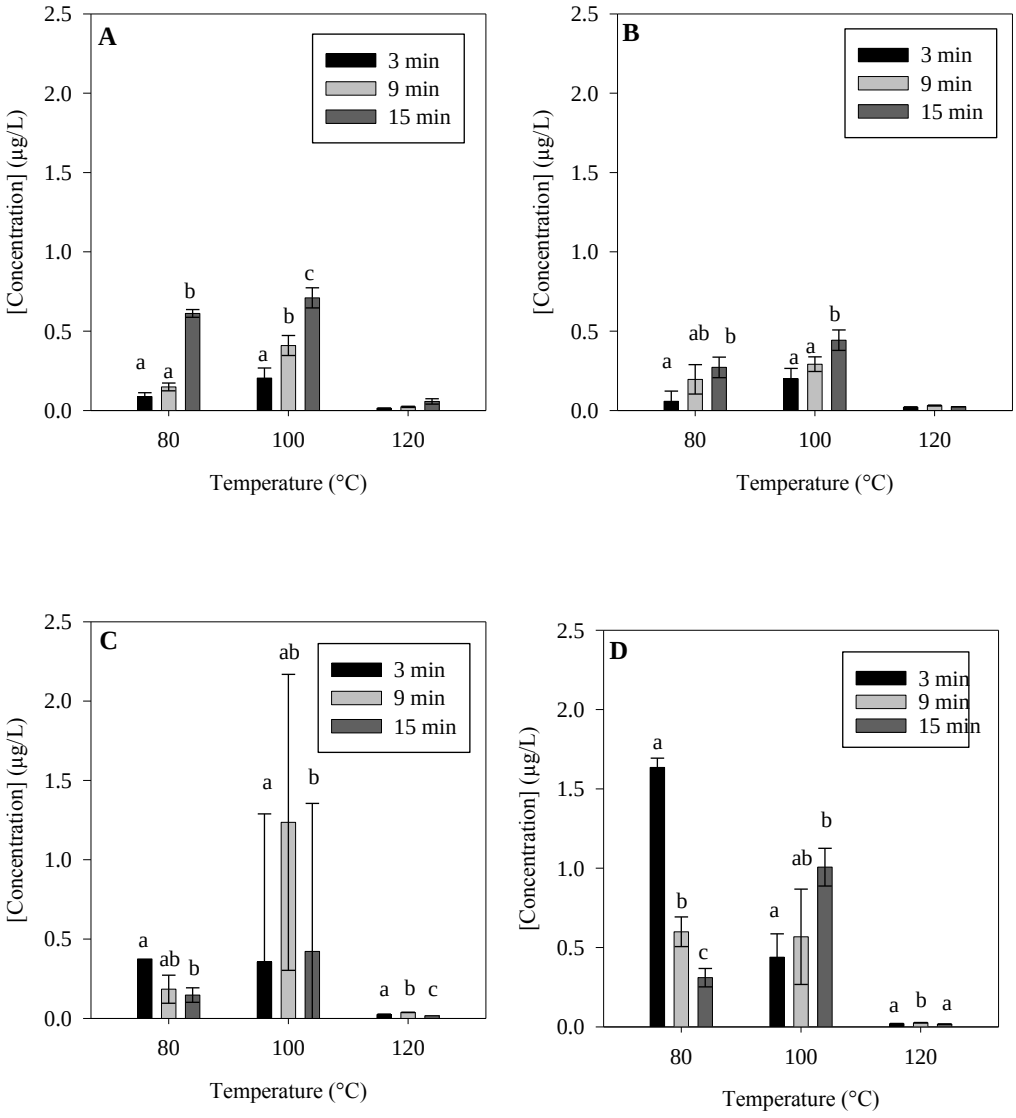

Figure D.3. 2-Octanone formation in CLA-enriched milk treated at 0.1 (A), 200 (B), 400 (C) and 600 MPa (D) and temperatures of 80, 100 and 120 °C. Means within each graph with the same temperature with different letters are significantly different ($P < 0.05$). Presence of same letter or absence of letters means they are not significantly different between the samples ($P > 0.05$).

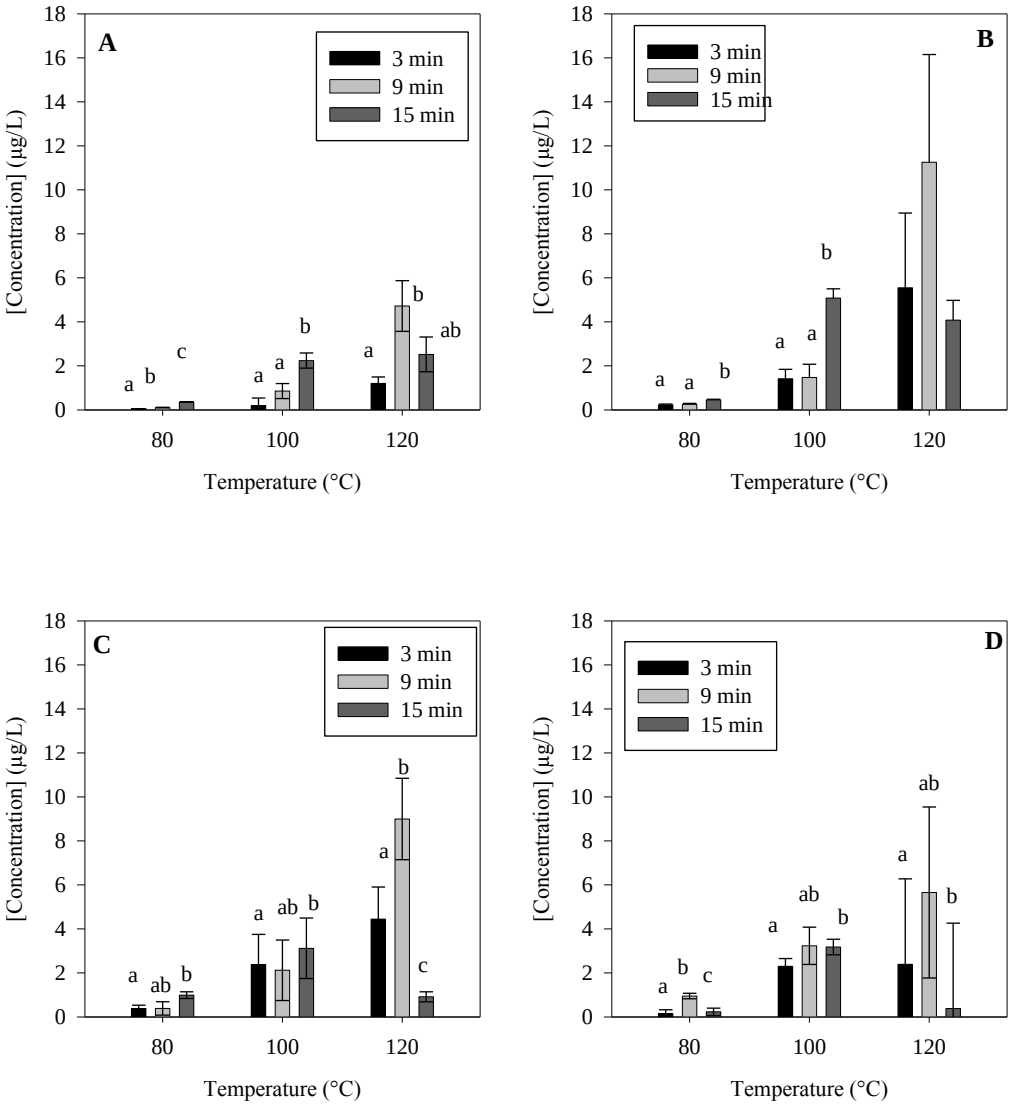

Figure D.4. 2-Nonanone formation in CLA-enriched milk treated at 0.1 (A), 200 (B), 400 (C) and 600 MPa (D) and temperatures of 80, 100 and 120 °C. Means within each graph with the same temperature with different letters are significantly different ($P < 0.05$). Presence of same letter or absence of letters means they are not significantly different between the samples ($P > 0.05$).

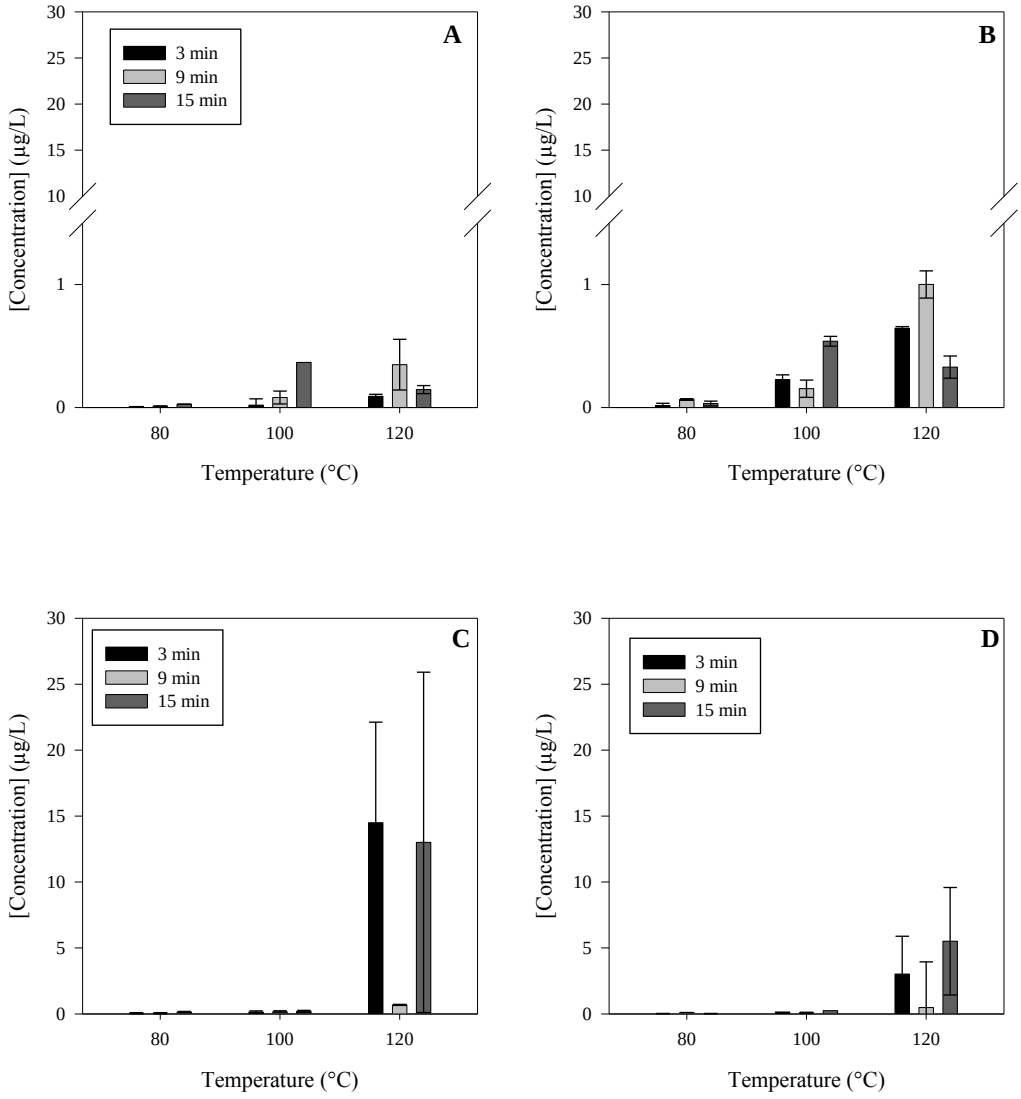

Figure D.5. 2-Undecanone formation in CLA-enriched milk treated at 0.1 (A), 200 (B), 400 (C) and 600 MPa (D) and temperatures of 80, 100 and 120 °C in CLA-enriched milk during PATP. Means within each graph with the same temperature with different letters are significantly different ($P < 0.05$). Presence of same letter or absence of letters means they are not significantly different between the samples ($P > 0.05$).

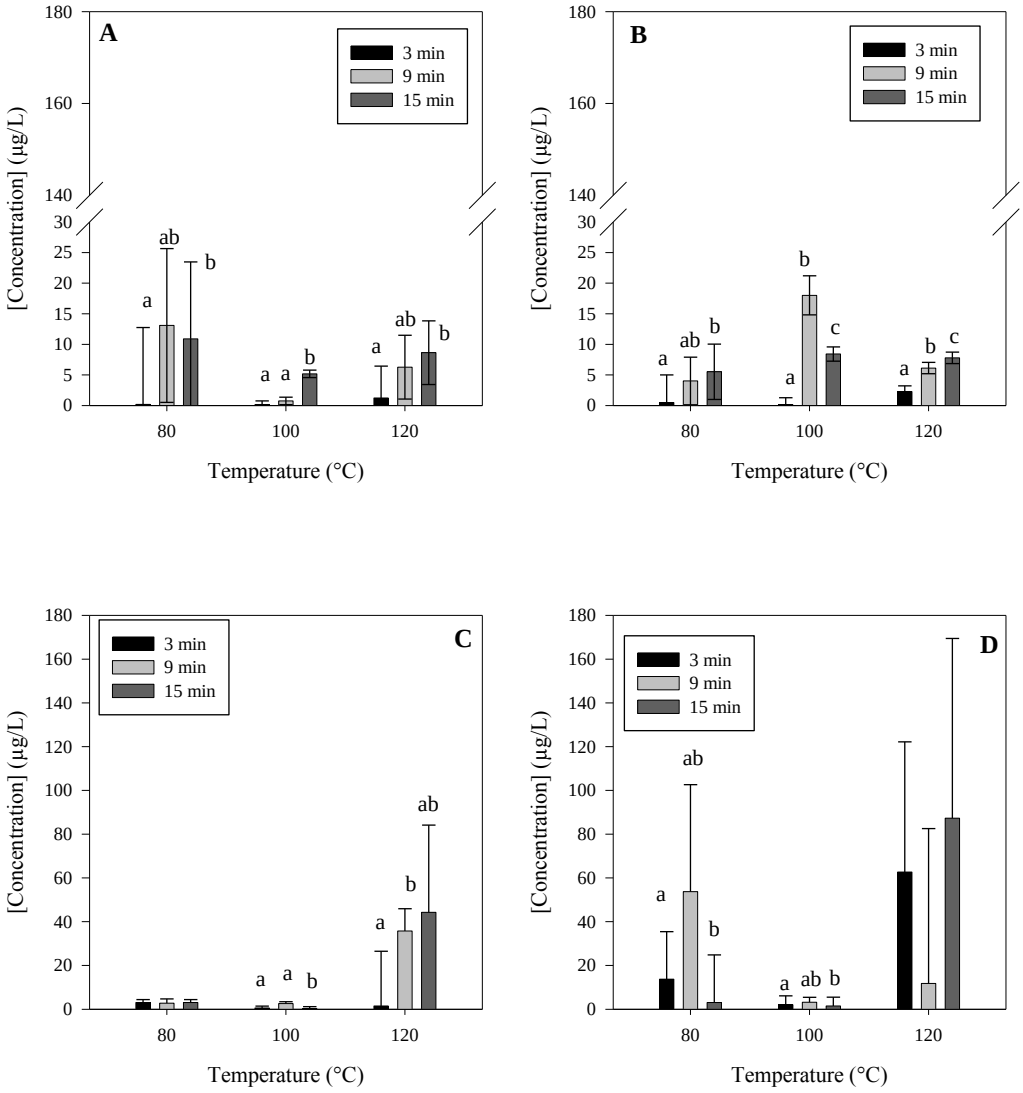

Figure D.6. Furfural formation in CLA-enriched milk treated at 0.1 (A), 200 (B), 400 (C) and 600 MPa (D) and temperatures of 80, 100 and 120 °C.. Means within each graph with the same temperature with different letters are significantly different ($P < 0.05$). Presence of same letter or absence of letters means they are not significantly different between the samples ($P > 0.05$).

Appendix E: Volatiles in CLA-enriched milk and non-enriched milk after different processing technologies.

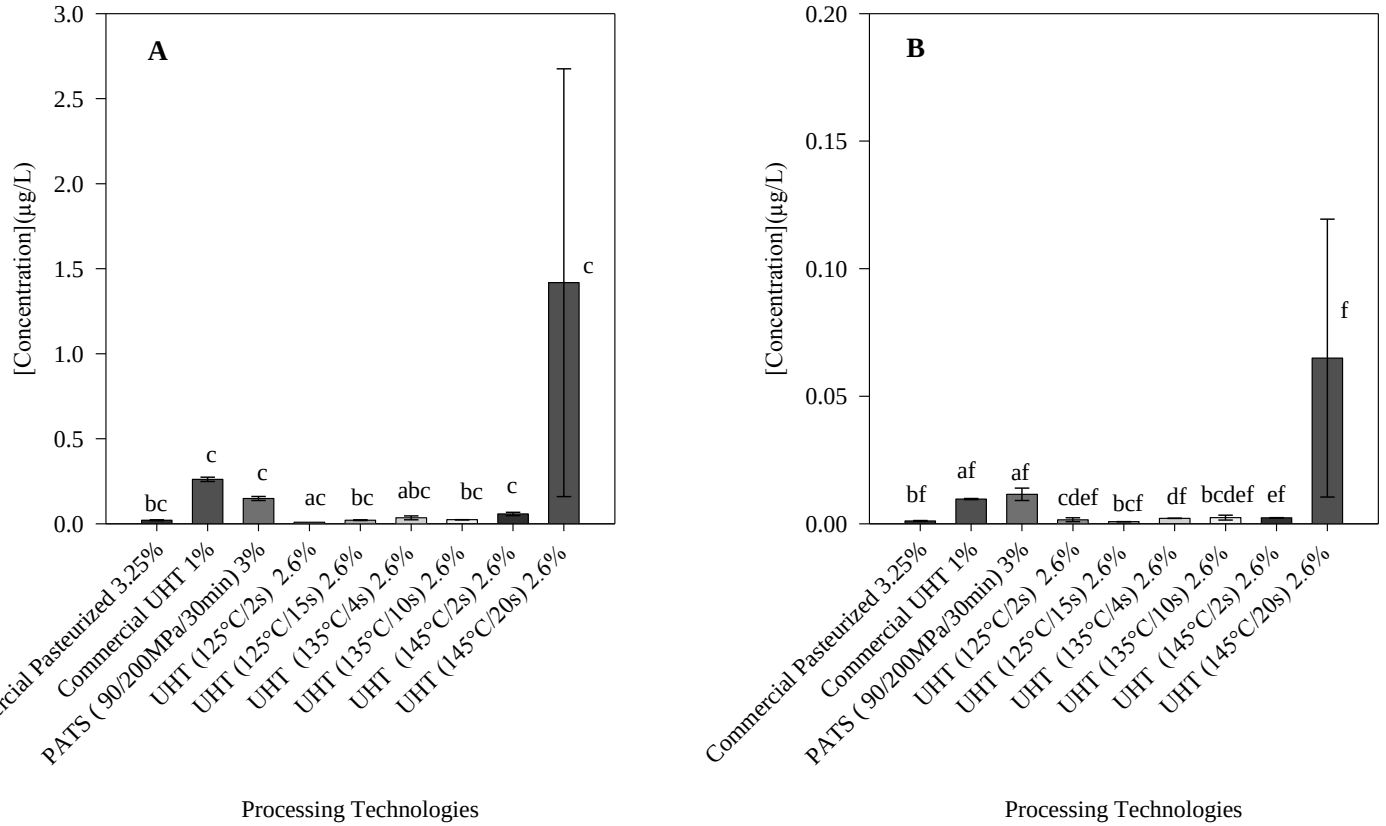

Figure E.1. Concentration of (A) 2-nonanone and (B) 2-undecanone using different processing technologies. Non-commercial samples are enriched with CLA. Letters with different superscript are significantly different (n=2).

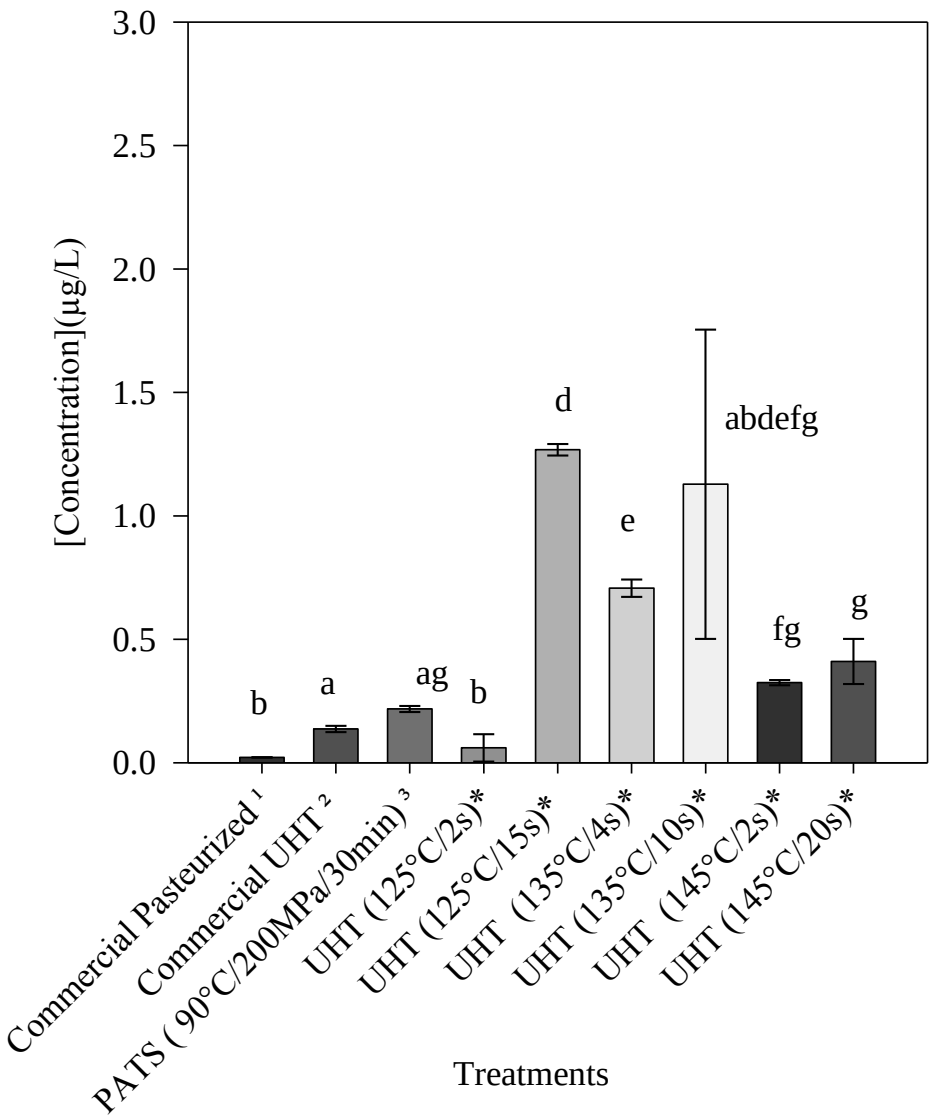

Figure E.2. Concentration of furfural after using different processing technologies. Superscripts: [1] 3.25% fat, [2] 1% fat, [3] 3% fat, * 2.6% fat. Commercial samples are not enriched with CLA. Letters with different superscript are significantly different (n=2). No specifications about processing conditions were given for commercial samples.

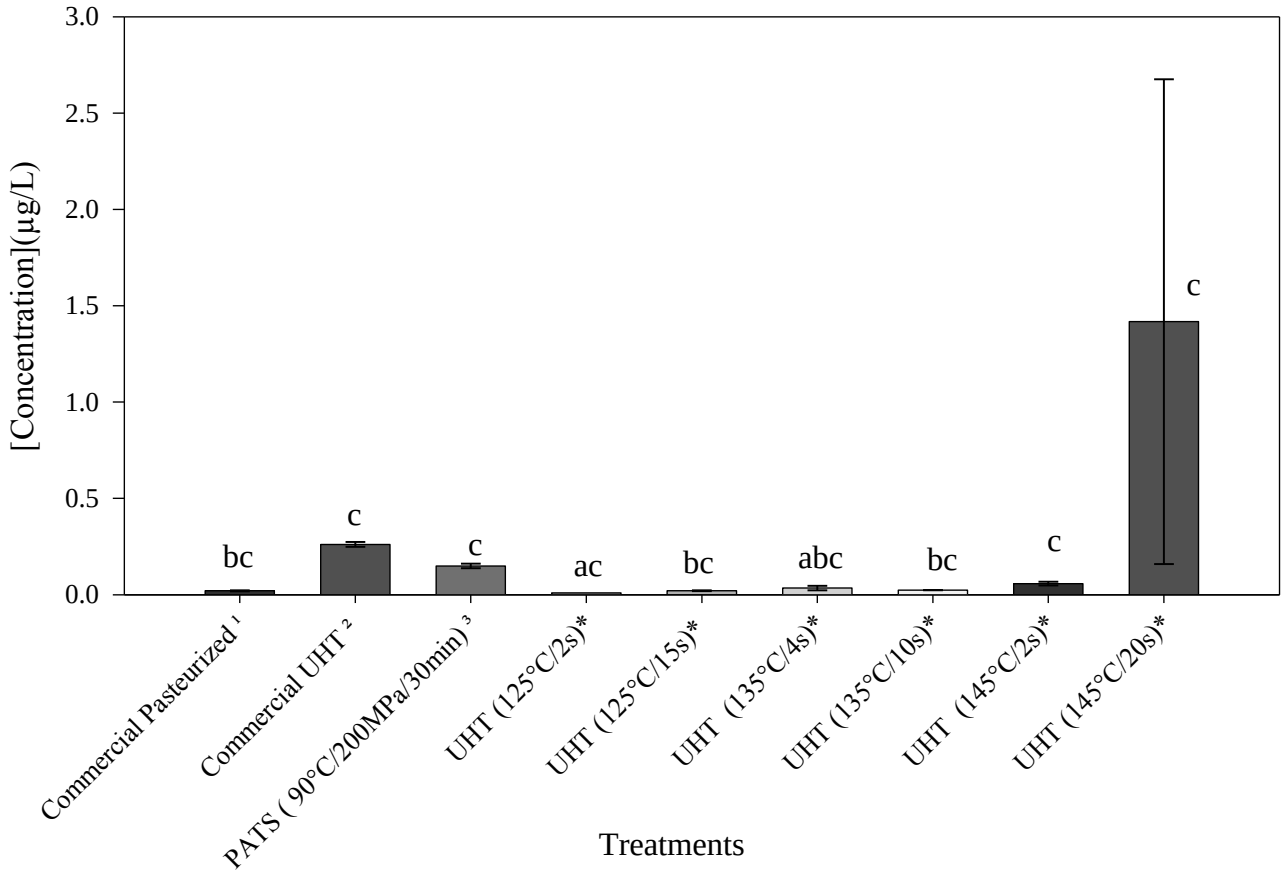

Figure E.3. Concentration of 2-nonanone (µg/L) in milk using different processing technologies. Superscripts: ¹ 3.25% fat,² 1% fat, ³ 3% fat, * 2.6% fat. Commercial samples are not enriched with CLA. No specifications about processing conditions were given for commercial samples. Means within the same graph with different letters are significantly different ($P < 0.05$). Absence of letters means they are not significantly different between the samples ($P > 0.05$).

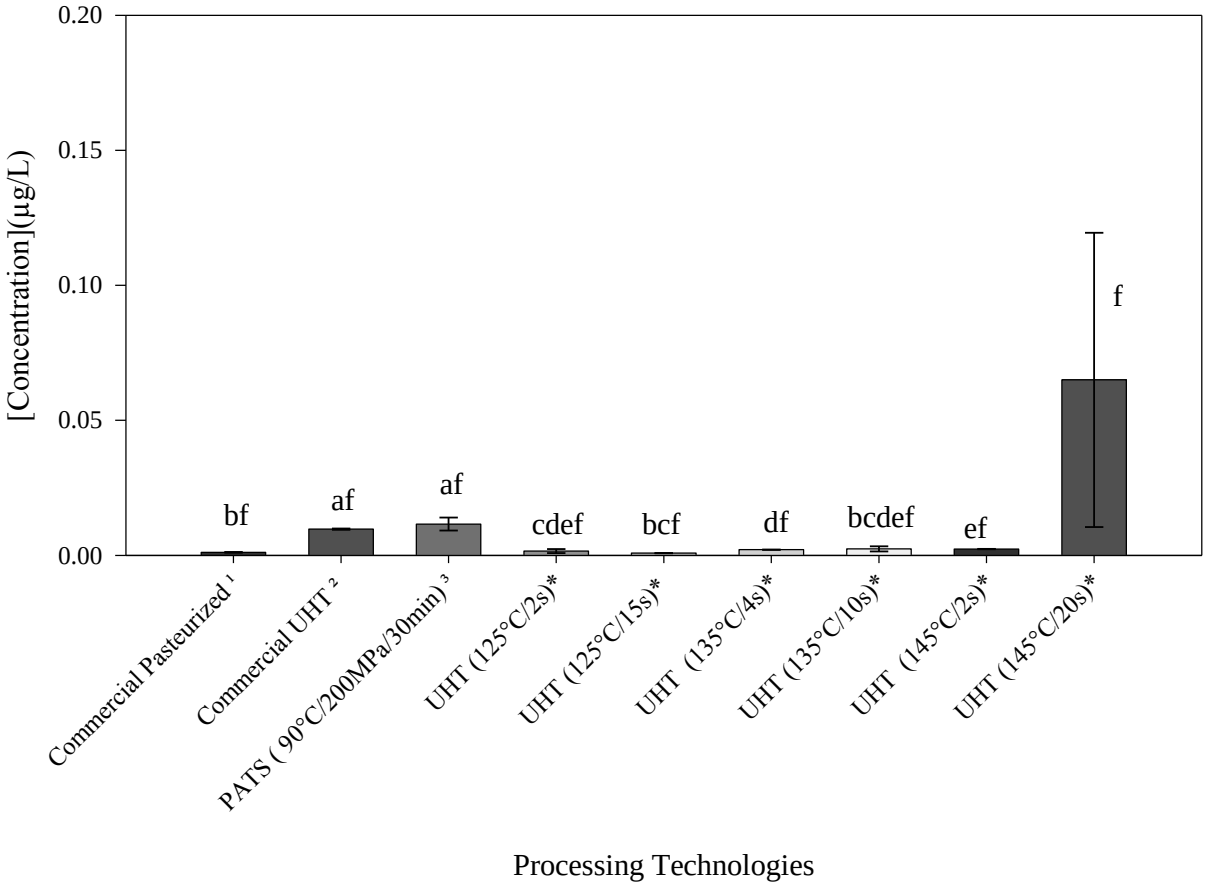

Figure E.4. Concentration of 2-undecanone (µg/L) in milk using different processing technologies. Superscripts: ¹ 3.25% fat,² 1% fat, ³ 3% fat, * 2.6% fat. Commercial samples are not enriched with CLA. No specifications about processing conditions were given for commercial samples. Means within the same graph with different letters are significantly different ($P < 0.05$). Absence of letters means they are not significantly different between the samples ($P > 0.05$).

Appendix F: Volatile compounds in UHT treated CLA-enriched milk during storage.

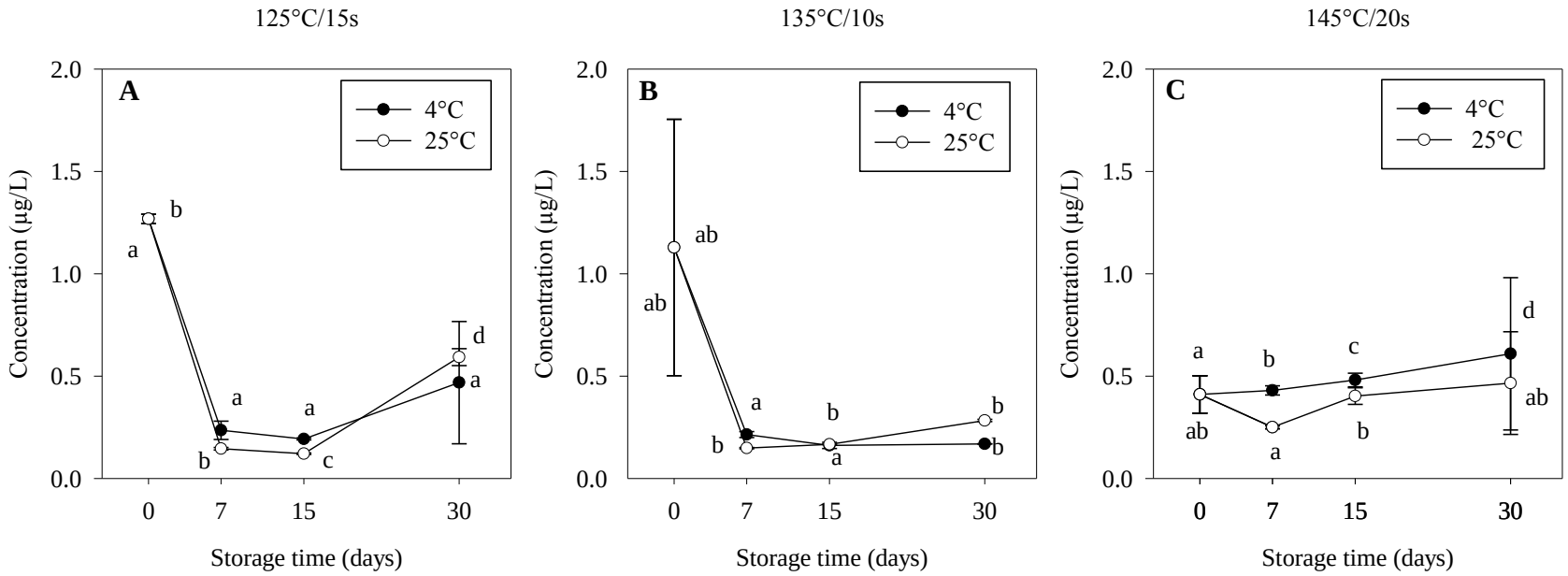

Figure F.1. Furfural concentration during storage at 4°C and 25°C for 0, 7, 15 and 30 days after thermal treatment at: (A) 125°C/15 s, UP; (B) 135°C/10 s, UHT and (C) 145°C/20 s, UHT. Means within the same graph with different letters are significantly different ($P < 0.05$). Absence of letters means they are not significantly different between the samples ($P > 0.05$).

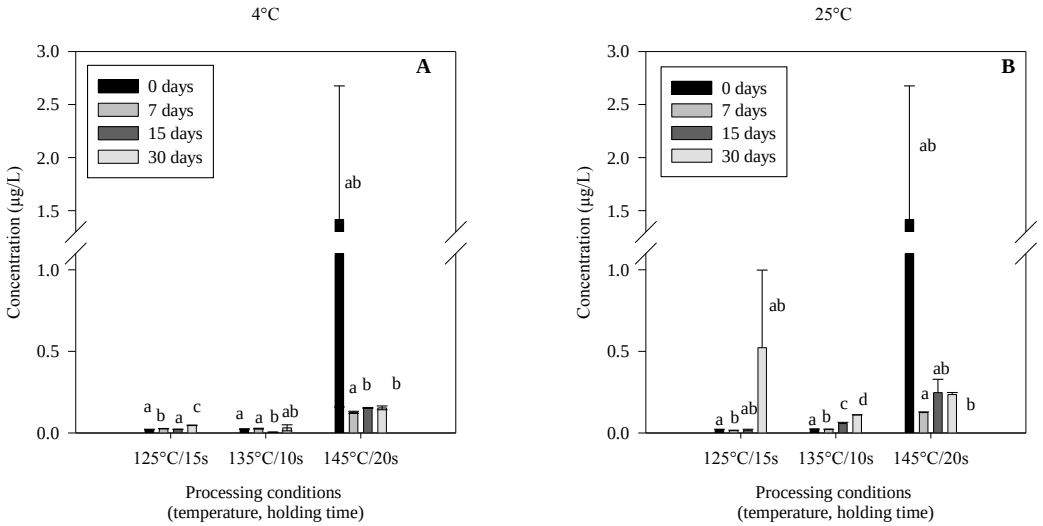

Figure F.2. 2-Nonanone formation after: UP: 125°C/15s; and UHT: 135°C/10s and (C)145°C/20s during 0,7, 15 and 30 days of storage at 4°C (A) and 25°C (B). Means with the same processing condition within a plot with different letters are significantly different ($P < 0.05$). Presence of same letters or absence of letters means they are not significantly different between the samples ($P > 0.05$).

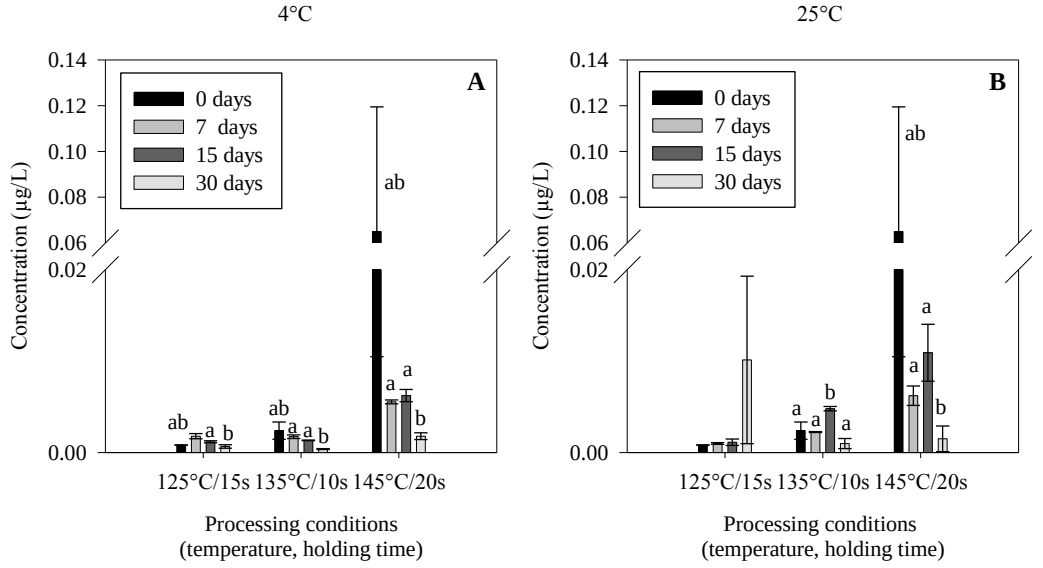

Figure F.3. 2-Undecanone formation after: UP: 125°C/15s; and UHT : 135°C/10s and 145°C/20s during 0,7, 15 and 30 days of storage at 4°C (A) and 25°C (B). Means with the same processing condition within a plot with different letters are significantly different ($P < 0.05$). Presence of same letters or absence of letters means they are not significantly different between the samples ($P > 0.05$).

Appendix G: Precision of the method using CLA-enriched milk after processing by UP, UHT and PATP.

Table G.1. Precision of the analytical method obtained from off-flavor compounds (µg/L)* in UP and UHT milk samples (n=2).

Treatment conditions	**UP (125°C, 2s)**		**UP (125°C, 15s)**		**UHT (135°C, 4s)**		**UHT (135°C, 10s)**		**UHT (145°C, 4s)**		**UHT (145°C, 20s)**	
Compound	*mean ± SD	CV (%)	*mean ± SD	CV (%)	*mean ± SD	CV (%)	*mean ± SD	CV (%)	*mean ± SD	CV (%)	*mean ± SD	CV (%)
Hexanal	0.06±0.05	92.8	1.26±0.02	0.9	0.70±0.01	2.42	1.12 ±0.62	55.52	0.31±0.01	3.23	0.41±0.09	14.35
Heptanal	4.90±0.41	8.4	66.41±4.72	7.1	40.65±0.04	0.10	8.24±8.06	97.82	17.73±0.42	2.38	14.45±1.2	8.25
Octanal	0.31±0.12	37.9	0.80±0.004	0.6	3.08±2.66	86.57	0.96±0	0	0.54±0.01	2.89	2.09±1.67	80.11
Benzaldehyde	0.08±0	0	0.11±0.002	1.87	0.17±0	0	0.20±0.11	55.29	0.21±0.004	2.02	1.11±0.89	80.25
Furfural	0.06±0.05	92.3	1.26±	1.82	0.70±0.03	4.91	1.12±0.62	55.52	0.32±0.01	3.23	0.31±0.04	14.36
2-hexanone	0.01±0.002	16.6	0.02±0.003	11.2	0.04±0	0	0.04±0.02	49.71	0.04±0	1.52	0.03±0.01	35.46
2-heptanone	7.30±4.41	60.4	19.57±1.63	8.33	5.77±0.01	0.18	1.84±1.75	95.12	3.74±0.07	2.06	2.86±0.09	3.18
2-octanone	0.17±0.16	97.3	0.33±0.003	0.95	0.33±0	0	1.35±1.01	74.62	0.33±0.01	3.23	0.40±0.07	19.01
2-nonanone	0.009±0	0	0.02±0.001	9.25	0.03±0.01	33.33	0.02±0	0.19	0.05±0.004	8.43	1.41±1.25	88.76
2-undecanone	0.001±0.0003	31.3	0.0008±0	1.21	0.002±0	0	0.002±0	39.41	0.002±0	3.23	0.06±0.05	83.88

*mean concentration ± standard deviation (SD), CV (%)= coefficient of variation.

Table G.2. Precision of the analytical method obtained from aldehydes (µg/L)* in CLA-enriched milk after PATP (n=2).

Temperature	Pressure	time	hexanal		heptanal		octanal		Benzaldehyde	
(°C)	(MPa)	(min)	mean±SD	CV%	mean±SD	CV%	mean±SD	CV%	mean±SD	CV%
80	0.1	3	0.58±0.21	36.21	1.75 ±1.00	57.14	0.98 ±0.01	1.02	0.28 ±0.04	14.29
80	0.1	9	1.77 ±0.33	18.64	0.96 ±0.04	4.17	0.37 ±0.01	2.70	0.35 ±0.08	22.86
80	0.1	15	0.96 ±0.11	11.46	0.50±0.50*	100.0	0.35 ±0.01	2.86	0.36 ±0.11	30.56
80	200	3	1.10 ±0.25	22.73	0.47±0.47*	100.0	0.35 ±0.21	60.00	0.22 ±0.05	22.73
80	200	9	0.83 ± 0.08	9.64	2.11±0.78	36.97	0.86 ±0.33	38.37	0.90 ±0.01	1.11
80	200	15	0.94 ±0.19	20.21	0.64±0.64*	100.0	0.37 ±0.21	56.76	0.37 ±0.07	18.92
80	400	3	2.77 ±0	0.11	0.80 ±0.80*	100.0	0.58±0.05	8.62	0.61 ±0.31	50.82
80	400	9	2.54 ±0.09	3.54	1.66 ±0.06	3.61	0.44 ±0.08	18.18	0.42 ±0.16	38.10
80	400	15	1.37 ±0.17	12.41	1.56±1.56*	100.0	1.19 ±0.05	4.20	0.62 ±0.34	54.84
80	600	3	1.67 ±0.23	13.77	1.48 ±1.48*	100.0	0.62 ±0.05	8.06	0.60 ±0.52	86.67
80	600	9	1.20 ±0.04	3.33	4.11 ±0.02	0.49	1.00 ±0.06	6.00	1.34 ±0.01	0.75
80	600	15	1.65 ±0.05	3.03	2.80 ±0.46	16.43	0.61 ±0.05	8.20	NA	NA
100	0.1	3	1.68 ±0.37	22.02	1.52 ±0.48	31.58	0.42 ±0.26	61.90	0.48 ±0.18	37.50
100	0.1	9	1.33 ±0.21	15.79	2.78 ±0.48	17.27	1.20 ±0.26	21.67	1.40 ±0.40	28.57

Table G.2. Continue.

Temperature (°C)	Pressure (MPa)	time (min)	hexanal		heptanal		octanal		benzaldehyde	
			mean±SD	CV%	mean±SD	CV%	mean±SD	CV%	mean±SD	CV%
100	0.1	15	1.52 ±0.06	3.95	2.46 ±0.48	19.51	1.11 ±0.26	23.42	1.21±0.48	39.67
100	200	3	1.47 ±0.12	8.16	2.72 ±0.01	0.37	1.05 ±0.06	5.71	1.01±0.44	43.56
100	200	9	1.71 ±0.50	29.24	3.04 ±0.44	14.47	0.81 ±0.06	7.41	1.00±0.05	4.96
100	200	15	1.77 ±0.18	10.17	3.65 ±0	0.08	1.18 ±0.06	5.08	1.18 ±0.53	14.29
100	400	3	2.31 ±0.18	7.79	3.24 ±0.30	9.26	1.02 ±0.11	10.78	1.20 ±0.18	22.86
100	400	9	2.56 ±0.63	24.61	3.14 ±0.30	9.55	0.83 ±0.11	13.25	1.16±0.15	12.93
100	400	15	3.22 ±0.34	10.56	4.22 ±0.30	7.11	0.89 ±0.11	12.36	1.12±0.25	22.32
100	600	3	6.23 ±2.42	38.84	3.55 ±0.02	0.56	0.76 ±0.07	9.21	0.91 ±0.44	48.35
100	600	9	2.94 ±1.29	43.88	3.74 ±0.53	14.17	0.65 ±0.04	6.15	0.99 ±0.16	16.16
100	600	15	1.61 ±0.28	17.39	4.09 ±0.01	0.24	0.79 ±0.07	8.86	1.01±0.06	5.94
120	0.1	3	2.92 ±0.40	13.70	1.19 ±0.49	41.18	0.39 ±0.11	28.21	0.61±0	0
120	0.1	9	2.01 ±0	0.10	2.24 ±0.11	4.91	0.50 ±0.05	10	0.83±0.32	38.55
120	0.1	15	2.96 ±0.39	13.18	1.71 ±0.40	23.39	0.43 ±0.08	18.60	0.92±0.02	2.17
120	200	3	1.48 ±0.85	57.43	2.40 ±0.78	32.50	0.99 ±0.38	38.38	1.52±0.19	12.50
120	200	9	2.36 ±0.09	3.81	3.33 ±0.09	2.70	0.74±0.06	8.11	1.02±0	0

Table G.2. Continue.

Temperature (°C)	Pressure (MPa)	time (min)	hexanal		heptanal		octanal		benzaldehyde	
			mean±SD	CV%	mean±SD	CV%	mean±SD	CV%	mean±SD	CV%
120	200	15	1.73 ±0.19	10.98	1.88 ±0.25	13.30	0.45 ±0.04	8.89	0.93±0.04	4.30
120	400	3	2.03 ±0.44	21.67	2.23 ±0.95	42.60	0.59 ±0.24	40.68	0.99±0.03	3.03
120	400	9	3.81± 0.59	15.49	3.73 ±0.09	2.41	0.90 ±0.05	5.56	1.27±0.13	10.24
120	400	15	1.30 ± 0.71	54.62	5.93 ±0.04 5	0.76	74.62 ±3.84	5.15	0.85±0.19	22.35
120	600	3	2.74 ±0.19	6.93	1.72 ±0.24	13.95	0.45 ±0.02	4.44	0.75±0.09	12
120	600	9	2.42 ±0.16	6.61	3.54 ±0.25	7.06	20.9 ±0.01	0.05	0.84±0.21	25
120	600	15	2.39 ±0.12	5.02	5.88 ±0.25	4.25	75.55±0	0	0.98±0.33	33.67

*data not precise, high SD (>); NA: Not available.