AN ABSTRACT OF THE DISSERTATION OF

Nattaporn Chotyakul for the degree of Doctor of Philosophy in Food Science and Technology presented on November 25, 2014.
Title: Extraction and Quantification of Vitamins from Raw, HTST, UHT, and PATP Milk and of Diatom-produced Chitin and Glucosamine

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Analytical chemistry is an essential qualitative and quantitative tool to describe foods and biomaterials and their changes during production, processing and distribution. In this work, rapid analytical procedures for the extraction and quantification of components in fluid milk and diatom fermentation broth were developed. Analytical methods to measure the concentration of vitamin A, E & C in fluid milk were developed and applied to quantify these vitamins in milk distributed commercially in Spain and their retention in milk subjected to pressure-assisted thermal processing (PATP). Analytical procedures were also developed for the quantification, recovery and purification of chitin in diatom photobioreactor broths and for its conversion into glucosamine by HCl acid hydrolysis. This information was required for a techno-economical and life-cycle analysis of diatom-produced chitin for food and biomedical applications, and of its conversion into glucosamine to be marketed as a nutraceutical. This analytical development work lead to the following five studies.

In the first study, total ascorbic acid (AA) in whole/semi-skim/skim raw/pasteurized/UHT milk packaged in opaque bags, transparent plastic, cardboard, and Tetra Brik™ and collected from processing plants and retail outlets was extracted and quantified by HPLC using a C-18 column. AA content ranged 0.21-10 and 3.4-16 mg/l in milk from retail outlets and processing plants, respectively, and was higher in organic milk. For same processor/lot samples, pasteurized milk showed higher AA content than UHT milk but this was not true for retail outlets samples. Among UHT samples, the AA content trend was whole<semi-skim<skim and lower for UHT milk in opaque plastic and Tetra Brik™ containers. In milk stored 14 d at 4ºC in the dark, AA losses ranged 35-83% depending on milk type. AA retention was higher in unopened milk containers.

In the second study, an extraction and HPLC quantification method was improved to allow the simultaneous quantitation of retinol, α-tocopherol, α-tocotrienol and β-carotene in commercial whole/semi-skim/skim samples of raw/pasteurized/UHT milk commercialized in transparent plastic/glass bottles and Tetra Brik™ containers. The fat-soluble vitamin content in raw, pasteurized conventional/organic, and UHT milk ranged 0.055-5.540 (retinol), 0.135-1.410 (α-tocopherol), and 0.040-0.850 mg/l (β-carotene). No significant differences ($p>0.05$) were observed on the losses of retinol, α-tocopherol, and β-carotene content in UHT whole milk after 5 d at 4ºC in the dark. After 14 d at 4ºC in the dark the contents of retinol, α-tocopherol, and β-carotene remained higher in milk with higher fat content and were higher in unopened milk containers. In UHT whole milk samples containing 0.02% NaN_3, retinol (33%) and α-

tocopherol (11%) but not β-carotene (2%) decreased significantly ($p<0.05$).

The third study focused on the effect of elevated pressure (200-705 MPa) at moderate temperatures (30-75º) on the AA, vitamin A (retinol, β-carotene), and vitamin E (α-tocopherol) retention in raw-whole and pasteurized-skim milk. This study showed minimum losses of retinol, α-tocopherol and β-carotene after high pressure treatments at moderate temperatures. However, even the least severe treatment (400 MPa/31°C/5 min) resulted in a statistically significant AA loss ($p<0.05$) in raw whole (20%) and pasteurized skim milk (13%) when compared with untreated controls. AA losses reached a maximum loss of 55 and 68% in raw whole and pasteurized skim milk, respectively, when treated for 5 min at 705 MPa and 72ºC.

The focus of the fourth study was the determination of the chitin amount present in fermentation broths produced in a diatom photobioreactor. This was achieved by acid hydrolysis conversion of chitin into glucosamine quantified by HPLC. This information was essential for a techno-economical and life-cycle analysis of the chitin production by diatom cells and its subsequent conversion into glucosamine. Diatoms are single-cell algae with intricately structured cell walls made of nanopatterned silica (SiO_2) previously studied for applications in nanotechnology. This study focused on the long chitin fibers extruded by *Cyclotella* spp. diatoms to be used as is for biomedical and food applications or it can be hydrolyzed into glucosamine to be marketed as a dietary supplement. Kinetics of the glucosamine (GlcN) monomer production by acid hydrolysis of commercial diatom chitin was determined in 4M, 6M, 8M, 10M and 12M HCl at 90°C for up to 3 h. The GlcN

produced was quantified by HPLC (Dionex MA-1 column, 30°C, 0.4 ml/min 0.75M NaOH isocratic mobile phase, ED-40 PAD detector). Acid hydrolysis in 8M HCl at 90°C for 2 h of commercial chitin suspended in distilled water showed a high GlcN conversion (98.0% ± 0.04, n=2). Tests using reagent-quality GlcN showed that the chitin monomer was stable under these hydrolysis conditions (>88% retention). However, the 8M HCl hydrolysis of commercial diatom chitin suspended in two different formulations of sterile diatom fermentation broths for 1, 2 and 3 h showed that the chitin conversion to glucosamine was fermentation media dependent. Further tests of chitin produced by diatoms in the photobioreactor confirmed that the acid hydrolysis of chitin is media dependent. To overcome this media-dependence limitation, diatom photobioreactor broth samples corresponding to initial, mid and final fermentation time points were spiked to obtain an average media correction factor. This factor was determined for each photobioreactor run to quantify chitin production using glucosamine yield after 8M HCl hydrolysis at 90ºC for 3h. The correction factor allowed reliable and low-variability determinations of the chitin production kinetics in the diatom photobioreactor.

The fifth study focused on the development of a chitin separation and purification method from the diatom photobioreactor broth. A mild centrifugation step to remove diatom cells in the pellet was optimized (1500g, 1 min) and yielded a high retention of chitin in the supernatant (96.6±0.18 and 84.6±0.02) when evaluated using two different fermentation broth formulations. Intense centrifugation (11000g, 30 and 60 min) was then used to recover chitin fibers in a second pellet. Analysis of the chitin

content in the supernatant of the second centrifugation showed losses of less than 10%. Therefore, the two centrifugation steps allowed a recovery exceeding 80%. The chitin pellet was then purified using 1M HCl at 70^{o}C to solubilize calcium and other salts, 0.5% w/w SDS to remove insoluble proteins, and 95% ethanol to remove chlorophylls and other organic materials yielding chitin with a 70.9±16.6% purity.

Extraction and Quantification of Vitamins from Raw, HTST, UHT, and PATP Milk and of Diatom-produced Chitin and Glucosamine

by
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I understand that my dissertation will become a part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

Nattaporn Chotyakul, Author

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CONTRIBUTION OF AUTHORS

Dr. J. Antonio Torres as major professor provided the laboratory facilities needed for this research and contributed to the research design, analysis of findings and the preparation of all manuscripts here included.

In the work presented in Chapters 3, 4, and 5, Dr. Concepción Pérez-Lamela provided laboratory facilities in Spain and contributed to the experimental design plan. Dr. Miriam Pateiro-Moure assisted with experimental work and contributed to the data analysis and preparation of the manuscripts included in these three chapters. Dr. Jorge A. Saraiva assisted in manuscript preparation and participated in numerous discussions on pressure processing effects and data reporting. Dr. Elena Martínez-Carballo provided guidance on HPLC analysis and in the preparation of the manuscript presented in Chapter 3.

In Chapters 6 and 7, Bob Durst contributed to the experimental design and provided technical support on HPLC analysis. Xulei Wu contributed to experimental design, data generation and manuscript preparation. In chapter 6, Dr. Greg Rorrer and Omar Chiriboga generated the diatom photobioreactor samples while Kai Deng assisted in experimental work when the number of samples to be processed at the same time was too large. Finally in chapter 7, Dr. Bettye Maddux in the Chemistry Department assisted in experimental planning and Vinicio Serment-Moreno contributed to experimental design and laboratory work.

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CHAPTER 1

Introduction

Analytical chemistry is one of the most powerful tools available to describe food qualitatively and quantitatively (DeVries and Silvera 2000). It is applied by industry and all sciences to understand the chemistry of a substance (Skoog and others 2013). The use of analytical chemistry applies not only directly to the analysis of the food itself but also to the monitoring of the effects of the food after it is prepared. Analytical chemistry is used to determine the bioavailability of essential constituents and to assess the toxicity of food components (DeVries and Silvera 2000). The use of analytical methods and systems in research, product development, and industrial production is essential for technological progress (Nijenhuis 1991).

Consumers are more conscious and proactive about their well-being including the practice of positive (selecting foods with desirable nutrients) and negative eating (avoiding undesirable ingredients and rejecting excesively processed foods). While technologies such as thermal processing eliminate microbial risks and extend product shelf-life, they cause extensive chemical changes. On the other hand, minimal processing strategies cannot eliminate many microorganisms creating safety risks or limiting the product shelf life. In the case of extended refrigerated shelf-life (ESL) milk and dairy beverages, flavor and nutrient losses increase when the severity of thermal treatments is increased over those used in conventional pasteurization. Furthermore, UHT sterilization can yield shelf-stable milk and dairy beverages but it generates flavors rejected by consumers and nutrient losses increase also. Thermal

processing destroys nutrients, particularly vitamins naturally present in milk. The extent of these losses depends on the nutrient and the processing method used. Nutrients lost during processing can be replaced through milk fortification. Temperature, oxygen, light and transition metals frequently play an active role in accelerating or promoting nutrient loss (Ryley and Kaida 1994).

Pressure processing technologies are being investigated as alternatives for food pasteurization and sterilization. High pressure processing (HPP) and pressure-assisted thermal processing (PATP) inactivate enzymes and microorganisms with less impact on food composition (Norton and Sun 2008; Mújica-Paz and others 2011; Verbeyst and others 2012; Verbeyst and others 2013). HPP of foods was first reported in 1899 when treating milk at 670 MPa for 10 min achieved a 5-6 log-cycle total counts reduction (Hite 1899). Equipment advances, product commercialization successes, and a demand for less processed, high quality and safer foods have led to considerable interest in this technology (Torres & Velazquez 2005, 2008; Torres *et al*. 2009a,b). New units for combined pressure and thermal treatments is allowing the development of PATP, i.e., the simultaneous application of elevated pressure (~600–700 MPa) and temperature (~90–120 °C) to preheated foods (~60–90 °C) (Pérez Lamela & Torres 2008a,b). Consumer acceptance has also been superior to that of other emerging processing technologies (Cruz and others 2011). Moderate temperature PATP ($T<100\ ^{o}C$, LT-PATP) can be used when an HPP pasteurization process is not commercially feasible due to long processing times, e.g., 15-30 min for pressure-pasteurized milk (Mussa and Ramaswamy 1997; Mussa and others 1998). PATP relies

on compression-heating/decompression-cooling for a rapid and uniform change in food temperature reducing the severity of thermal treatments (Meyer and others 2000).

Milk, dairy beverages and other dairy products are important sources of nutrients for consumers particularly for children and teenagers. Vitamin A was analyzed in two vitamer forms (retinol and β-carotene) because nothing had been published on its stability under PATP treatments. Vitamin C was chosen as a vitamer form of ascorbic acid (AA) for this study because several publications have shown it to be lost at high rate under PATP conditions (Taoukis and others 1998; Polydera and others 2003; Oey and others 2006; Sanchez-Moreno and others 2006; Castro and others 2008; Moltó-Puigmartí and others 2011; Verbeyst and others 2013). However, all those publications cover fruits and vegetables only. Formation of off-flavor volatiles including aldehydes formation was the only group of off-flavors that was accelerated by PATP treatments in milk (Vazquez and others 2007). The effectiveness of tocotrienols, a vitamer of vitamin E, in reducing the formation of these compounds needed to be confirmed experimentally. Therefore, the degradation of vitamin E (α-tocopherol and α-tocotrienol) was also important to study.

In the USA, the National Center for Food Safety and Technology (NCFST), Illinois Institute of Technology (IIT), and Avure Technologies, Inc., announced in 2009 that the U.S. Food and Drug Administration (FDA) accepted the institute's filing of a PTAP as a new food sterilization process (Anonymous 2009b; Anonymous 2009a). It was the first petition to the FDA for the commercial use of this alternative technology in the production of low acid foods. However, acceptance of this

sterilization technology in the EU requires compliance with novel food laws. Novel foods were originally defined as all foods and food ingredients not used for human consumption to a significant degree within the EU prior to May 1997. In Europe and countries following similar regulations (e.g., Canada), information on the loss of nutrients and the formation of new, possibly toxic compounds, is now required (Hepburn and others 2008).

Algae and microalgae are sources of bioactive compounds for use as functional food ingredients (Plaza and others 2008; Plaza and others 2009). Microalgae, including diatoms, are single celled photosynthetic organisms that grow, reproduce themselves, and biosynthesize energy-dense molecules through metabolic processes that utilize only sunlight, atmospheric CO_2, water, and nutrients principally nitrate and phosphate salts as inputs. In this study, the development of a method to recover and purify chitin from diatom cell broths was investigated. Chitin or polymerized *N*-acetylglucosamine (p-GlcNAc) is used as the raw material for chitin derived products such as chitosan, oligosaccharides, and glucosamine (Einbu and Vårum 2008). Commercial sources of chitin production are various crustaceans, particularly shrimps and crabs. The production of natural polysaccharides from marine source is already large and expanding rapidly (Laurienzo 2010). Microalgae have become particularly interesting because of the possibility to easily control the growth conditions in a bioreactor together with the demonstrated biochemical diversity of these organisms (Laurienzo 2010). Marine microorganisms as algae are now considered efficient producers of biologically active and chemically novel compounds

(Mayakrishnan and others 2013). Research on the design of photobioreactors to cultivate photosynthetic cells of microalgae is being extensively investigated (e.g., Harun and others 2010). A target for the development of diatom biorefineries is chitin for biomedical applications such as drug/gene delivery and wound healing (Jayakumar and others 2011; Tamura and others 2011). However, it can be also hydrolyzed into glucosamine to be marketed as a dietary supplement (Jeon and others 2000; Vaclavikova and Kvasnincka 2013), or used as is in foods as an antimicrobial agent, edible film component, emulsifier, thickening, or stabilizing agent (Shahidi and others 1999; Agulló and others 2003). Chitin can be quantified by acid hydrolysis cleaving glycosidic bonds between monomers and removing the acetyl group in GlcNAc residues yielding GlcN (Zamani and others 2008).

Under current U.S. regulations, glucosamine is a dietary supplement subject to the rules of production and marketing outlined in the 1994 Dietary Supplement Health and Education Act, DSHEA (Barnhill and others 2009). Glucosamine used in the U.S.A. since the 1960s to treat osteoarthritis (OA), a disease affecting joints in elderly populations, is now widely used all over the world for this purpose (Nakamura 2011). The extensively investigated conversion of chitin into glucosamine indicate that acid hydrolysis is the preferred method to yield glucosamine (Zhu and others 2005).

Dissertation objectives

The objectives of five studies were identified as:

1. The objectives of the first study were to develop and apply an improved extraction

and HPLC quantification method to determine the total AA in commercial cow milk samples; to evaluate the influence of heat treatment, packaging material, production system, and milk type on total AA content; and, to quantify losses of total AA in milk samples stored at 4ºC for up to 14 d under dark conditions.

2. The objective of the second study were to improve the extraction of vitamins A and E using organic solvent as *n*-hexane and to develop an HPLC method to allow the simultaneous quantification of retinol, retinyl acetate, β-carotene (all with vitamin A activity), α-tocotrienol, and α-tocopherol (all with vitamin E activity) in samples of raw, pasteurized, and UHT milk with different fat content (whole, semi-skim, and skim), also organic milk contained in different container types. Finally, the losses of vitamin A and E during consumer milk handling were determined including determinations for milk stored refrigerated in close and opened containers with and without the addition of antimicrobial as sodium azide.
3. The objectives of the third study were to determine the effect of elevated pressure (100-700 MPa), moderate temperature (30-75°C) and time (0-10 min) on the retention of ascorbic acid (AA), vitamin A (retinol, β-carotene), and vitamin E (α-tocopherol) in raw whole and pasteurized skim milk to observe the effects on chemical changes at high temperature and pressure levels treatments of milk.
4. The overall objective of the fourth study was the quantification of the long chitin fibers extruded by *Cyclotella* sp. diatoms. Acid hydrolysis into glucosamine was a simple and convenient method for quick and reliable quantifications of the chitin content in diatom cell broths. Glucosamine was quantified by HPLC using an

electrochemical detector.

5. The overall objective of the fifth study focused on a quick and reliable recovery and purification of chitin from the diatom *Cyclotella* spp. cell broth independent of fermentation conditions and time while minimizing its degradation.

CHAPTER 2

Literature Review

Analytical chemistry procedures involve the following main steps: (1) selecting a representative sample; (2) extracting the analyte from its matrix; (3) converting the analyte into a measurable form; (4) developing a measurement method; and, (6) calculating and interpreting the values measured (Krupadanam and others 2001; Skoog and others 2013). Among measurement methods, high-performance liquid chromatography (HPLC) has made major contributions towards nutritional studies of food processing. HPLC has been very successful in the last decade in separating and quantifying individual vitamers (Ryley and Kaida 1994).

2.1. Milk

Milk, one of the most nutritious foods, is rich in high quality proteins providing all ten essential amino acids and contributes to total daily intake of essential fatty acids (Early 1998; Miller and others 1999). Cow's milk is the predominant type in several countries, although goat, buffalo, sheep, and camel milks are also widely consumed (Vaclavik and Christian 2007). Milk is also consumed in fermented forms such as cheese, yogurt, kefir, and buttermilk, and as butter. Commercially available milk can be classified into two major groups: liquid milk and dried or powdered milk. Milk is available with different fat content including whole (3.25%), reduced fat (2%), low-fat or light (0.5, 1%), nonfat/fat-free/skim (<0.5%) (Vaclavik and Christian 2007).

2.1.1. Raw milk

Raw milk is neither heated nor treated by the producer but its composition including fat content can be modified. It should be sold only by labeling it “raw milk to be boiled before consumption” due to the sanitary risks involved (Spreer 1998). Raw milk must be processed within 36 and 48 h from acceptance by the dairy processing plant if milk has been kept at no more than 6ºC and 4ºC or lower, respectively. Preservation alternatives to extend milk shelf life are thermal processes such as pasteurization, ultra-pasteurization and ultra-high-temperature (UHT).

2.1.2. Pasteurized milk

Milk preservation treatments are an unavoidable step to inactivate pathogenic microorganisms and reduce the spoilage microflora. The most commonly used pasteurization methods are low-temperature/long-time (LTLT) pasteurization, also known as holder pasteurization, in which the milk is heated at 62.5 ºC in a water bath for 30 min (Updegrove, 2005), and high-temperature/short-time (HTST) heating at 71.7 ºC for 15 s (Ball 2006). The shelf-life of pasteurized milk kept under refrigeration (4 ºC) varies from 7-14 d depending upon the initial microbial load (Early 1998).

2.1.3. UHT milk

Ultra-high-temperature (UHT) milk refers to milk heated to over 130 ºC for 1 s or more which is then aseptically packaged (Ball 2006). Commercial UHT treatment of milk vary from country to country, including temperatures in the 130-150ºC range

and holding times of 1-8 s commonly used (Robinson 2005). Manufacturers usually claim a 6-month shelf life for UHT milk, although in practice the quality of the milk may remain of satisfactory quality for several months longer (Early 1998).

2.2. Vitamins

Vitamins are sensitive to different extent to heat, light, water activity, and the presence of oxidizing and reducing agents. Recent advances in technology have enabled the production of commercial forms of vitamins with improved stability and compatibility with other micronutrients. They can be added to products to compensate the amounts destroyed during thermal processing and product storage. For example, milk contains both liposoluble (A, D, E, and K) and hydrosoluble vitamins (C) and it is often enriched with vitamins A, C, and E (MacGibbon and Taylor 2003).

2.2.1. Vitamin A

Vitamin A refers to a variety of chemical substances showing similar biological activity including retinol, retinal, retinoic acid, retinyl esters, and provitamin A carotenoids such as β-carotene (Chotyakul and others 2014b). Retinol (vitamin A) is important in the protein metabolism of cells. Lack of vitamin A affects negatively epithelial tissue and also causes night blindness (Belitz and others 2009). The rate of vitamin A loss in milk is a function of enzyme activity, water activity, light, storage condition and temperature. During thermal treatments (72ºC for 15 s, 92ºC for 2 sec, 85ºC for 16 s) vitamin A concentration changes were not observed (Mahindru 2009).

2.2.2. Vitamin E

Eight compounds are known to have vitamin E activity and they include α-, β-, γ- and δ-tocopherols containing saturated phytol side chains and α-, β-, γ- and δ-tocotrienols with three double bonds in the side chain. The vitamin E bioactivity of α-tocotrienol is ~30% compared to that of α-tocopherol but tocotrienols have been shown to be potent antioxidants (Suarna and others 1993; Suzuki and others 1993; Kamat and Devasagayam 1995) and better than α-tocopherol which is typically used as an antioxidant in the 0.02 to 0.1% level (Suzuki and others 1993). In clinical trials, tocotrienols in doses as small as 42 mg/day have been found to reduce blood cholesterol levels by 5-35% (Qureshi and others 1991; Tan and others 1991; Qureshi and others 1995; Theriault and others 1999). Tocotrienols are found in cereal grains (rye, barley, oat) and certain vegetable oils (palm oil, rice bran oil). No recommended dietary allowance (RDA) for tocotrienols has been established but commercial products supply 10-50 mg of mixed tocotrienols per day. Human studies have shown no adverse effects with consumption of 240 mg/day for 18-24 months (Tomeo and others 1995; Kooyenga and others 1997).

Tocotrienols and tocopherols are present in milk at a concentration of around 0.09 mg/100 g. Extraction procedures from milk are performed by organic solvents (Tuan and others 1989) or supercritical fluid extraction (Mathiasson and others 2002). In general, the analytical procedures involve saponification in milk and subsequent quantification by HPLC (Indyk 1988; Rodas-Mendoza and others 2003).

2.2.3. Vitamin C

Vitamin C, a water soluble vitamin synthesized by many organisms but not by humans, should be consumed every day. It is sensitive to heat and light and it is destroyed over time when exposed to atmospheric oxygen. Vitamin C activity is due to both ascorbic acid (AA) and its oxidized form, dehydroascorbic acid (DHAA). The main AA degradation pathway via oxidation to DHAA yields diketo-L-gulonic acid (DKGA) (Murchie and others 2005). This reaction is the basis for the use of AA as an antioxidant (Chotyakul and others 2014a). Oxidation of AA to DHAA can be reversed by reducing agents which are used in analytical procedures to quantify the total amount of AA. Losses of vitamin C in pasteurized milk have been reported to be as little as 0-10% but more severe pasteurization (e.g., 73ºC for 10 min) results in about 26% losses (Mahindru 2009). Losses reaching 80% during UHT treatment but lower levels are observed when the oxygen level is lower. Presence of oxygen in milk is more detrimental to AA than storage temperature, therefore should have low oxygen permeability.

2.3. Shelf life

Shelf-life is the period of time before a food product reaches an unsatisfactory or unacceptable state under specific processing, packaging and storage conditions. In other words, it is the period of time during which it will retain an acceptable level of eating quality from a safety, nutritional and sensory point of view. The four critical factors affecting this evaluation are composition, processing, packaging and storage

conditions (Singh and Cadwallader 2004). Shelf-life considerations should include also the currently increasing consumer demand for fresh, convenient, safe and superior quality foods. Food processor should approach the shelf-life determination methods with the necessary care to ensure that consumers will receive a high product quality with the added convenience of extended shelf-life (Singh and Cadwallader 2004). Also, food quality is a consumer-based perceptual construct which is relative to person, place and time.

The estimation of shelf-life is today an important food development requirement to meet safety regulations and to deliver consistently products with the quality required to meet consumer expectations. Ensuring that products do not exceed its shelf-life before consumption is an important responsibility for everyone in the food chain including ingredients suppliers, food processors, warehouse managers, supermarket operators, and even the final consumer. A consumer should know the length of time that a product can be kept at home before it can no longer be used. A retailer should know the length of time that a product can stay on store shelves while a manufacturer should know when a product is no longer marketable. Every food product should be described as having a specific maximum microbiological, chemical and sensory shelf-life because every food will deteriorate at a different rate (Man 2002). Although, shelf-life defined by the processor refers only to the unopened package, once a package is opened and its contents is not consumed in a single event, the rest should be stored under the conditions, particularly time and temperature, recommended by the manufacturer. In 1997, FDA determined that the labeling of

potentially hazardous foods that need refrigeration should be more specific about the types of hazards present and the necessary storage conditions after the food is opened by the consumer and issued labeling guidance to food manufacturers (Anonymous 1997a; Marth 1998).

2.4. Thermal processing

Thermal processing is defined as a temperature and time combination required for the inactivation of undesirable microorganisms and enzymes while inducing an acceptable level of chemical changes in foods (Ryley and Kaida 1994; Toledo 2007). The term commercial sterility refers to treatments inactivating microorganisms that cause illness, and are capable of growing under non-refrigerated storage and distribution conditions (Toledo 2007). Thermal processing has focused on the inactivation in low-acid foods of spores of *Clostridium botulinum*, a heat resistant organism of high public health risk to consumers (Clark 2002). However, the effect of thermal processing must be taken into consideration when assessing vitamin stability in foods because they can be subjected to a number of adverse factors during processing.

2.5. High Pressure Processing

High pressure processing (HPP) was first reported by Hite (1899) who treated milk at 670 MPa for 10 min achieving a 5-6 log-cycle reduction in total counts. Research at Oregon State University began in the 1990's with constant and pulsed

pressure processing of fruits and juices (Aleman and others 1994; Aleman and others 1998). Equipment advances, successful commercialization of several products and a consumer demand for minimally processed, high quality and safe foods have led to considerable interest in HPP research (Torres and Velázquez 2005; Torres and Rios 2006; Torres and Velazquez 2008; Ramirez and others 2009; Torres and others 2009a; Torres and others 2009b; Mújica-Paz and others 2011; Valdez-Fragoso and others 2011; Serment-Moreno and others 2014). HPP at refrigeration or ambient temperature inactivates pathogenic and spoilage microorganisms with minor changes in food texture, color and flavor as compared to conventional technologies. Five decimal reductions in pathogens including *Salmonella typhimurium*, *S. enteritidis*, *L. monocytogenes*, *Staphylococcus aureus* and *Vibrio parahaemolyticus* can be achieved (Metrick and others 1989; Styles and others 1991; Mackey and others 1994; Patterson and others 1995; Stewart and others 1997; Ahn and others 2007). Unlike thermal processing and other preservation technologies, HPP effects are uniform and nearly instantaneous throughout the food and thus independent of food geometry and equipment size. This has facilitated the scale-up of laboratory findings to full-scale production for a rapid commercialization of this processing alternative, particularly in the USA, Europe and Asia. However, since Appert's invention driven by the need to supply safe food to Napoleon's armies, thermal processing has been the prevailing processing method to achieve microbial stability and safety. Although thermal processing is effective, economical and readily available, in many cases it has undesirable effects on food quality. Higher quality and elimination of many microbial

safety risks have been the drivers for the successful commercial introduction of many HPP products. Also important has been the high consumer acceptance of pressure treated products (Cruz and others 2011).

2.6. Nanotechnology and biotechnology

The simplest definition of nanotechnology is "technology at the nanoscale" (Ramden 2011). Nanotechnology is concerned with materials and systems whose structures and components exhibit novel and significantly improved physical, chemical and biological properties, phenomena and processes due to their nanoscale size (Ramden 2011). Nanoscience and nanotechnology have already been applied in various fields, such as computer electronics, communication, energy production, medicine and also in the food industry (Sozer and Kokini 2009). Since primary energy supply provided by renewable energy technologies is required urgently, technologies such as hydrogen fuel, solar cell, biotechnology based on nanotechnology are being developed (Guo 2011).

Bioprocessing and bioproducts have gained commercial interest because of the perceived green advantage of using biomass rather than fossil energy for the chemical production and industrial products (Yang 2007). The rapid increase in energy demand has generated a growing global interest in marine algae biorefineries to tap them as a source of biofuels, biomass and biomolecules (Sandun and others 2006; Bozarth and others 2009). The latter products are often included to make algal biofuel production

financially feasible.

2.7. Diatoms

Diatoms are unicellular marine organisms with self-assembled nanoporous silica outer structure or frustule. They use photosynthesis as an energy source and convert dissolved carbon dioxide into sugars (Yang and others 2011). Diatom algae require both CO_2 and dissolved silicon as substrates in addition to inorganic salts and light for cell growth and division. As a photosynthetic organism, diatoms grow in fresh, sea, or brackish water (Lopez and others 2005; Bozarth and others 2009).

2.8. Chitin

Chitin is a linear 1,4-β-linked *N*-acetyl glucosamine (GlcNAc) (Figure 2.1) and the most abundant carbohydrate after cellulose (Chen and others 2010). Diatoms, producers of chitin, have significant ecological importance (Durkin and others 2009). They convert uridine diphosphate *N*-acetylglucosamine (UDP-GlcNac) into β-crystalline chitin (Ogawa and others 2011) via chitin synthesis pathway catalyzed by a plasma membrane-bound chitin synthase (Tesson and others 2008; Durkin and others 2009). Chitin is biocompatible, biodegradable, non-toxic, and exhibits anti-bacterial and anti-inflammatory properties for applications in wound dressing (Vournakis and others 2008; Jayakumar and others 2011). It is sold commercially under various trade names including Syvek-Patch (Marine Polymer Technologies, Burlington, MA) using chitin derived from diatoms. Chitin can be used also as an antimicrobial agent, edible

film component, emulsifier, thickener, or as a stabilizer for food applications (Shahidi and others 1999; Agulló and others 2003). When hydrolyzed into glucosamine it can be marketed as a dietary supplement (Jeon and others 2000; Vaclavikova and Kvasnincka 2013). Chitin is used also used as raw material for the industrial production of chitosan, chitin and chitosan derivatives, and oligosaccharides (Einbu and others 2007).

2.9. Glucosamine

Glucosamine (2-amino-2-deoxy-α-D-glucose) is the de-acetylated monomer of chitin obtained by acid or enzymatic hydrolysis (Santhosh and Mathew 2007). Glucosamine is a precursor in the biosynthesis of fundamental connective tissue molecules such as hyaluronic acid, proteoglycans, glycosaminoglycans, glycolipids, and glycoproteins (Miller and Clegg 2011). Glucosamine has reportedly relieved the symptoms of osteoarthritis (Anderson and others 2005; Nakamura 2011). Glucosamine is sold in different salt forms depending on the acid used during hydrolysis (Figure 2.2 A-C). The major source of glucosamine is chitin from shrimp shells, which is not generally recognized as safe (GRAS) by the FDA, and it is not a suitable source for vegetarians and consumers with shellfish allergies (Barrow 2010). The only commercially available vegetarian, kosher, and FDA-approved GRAS glucosamine source is Cargill's Regenasure, produced from the fungus *Aspergillus niger* (Barrow 2010). Attempts to produce glucosamine by genetically engineered *E. coli* or fungi has faced many technical challenges (Barrow 2010; Sitanggang and others 2010; Chen and

others 2012). However, the production and characterization of glucosamine from the chitin of diatoms has not been investigated.

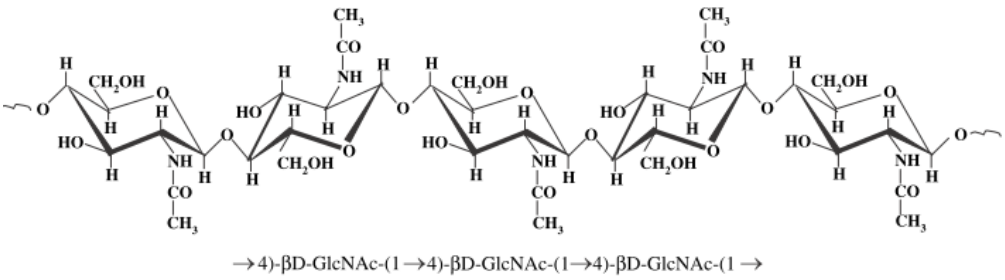

Figure 2.1. Primary structure of chitin (Harish Prashanth and Tharanathan 2007)

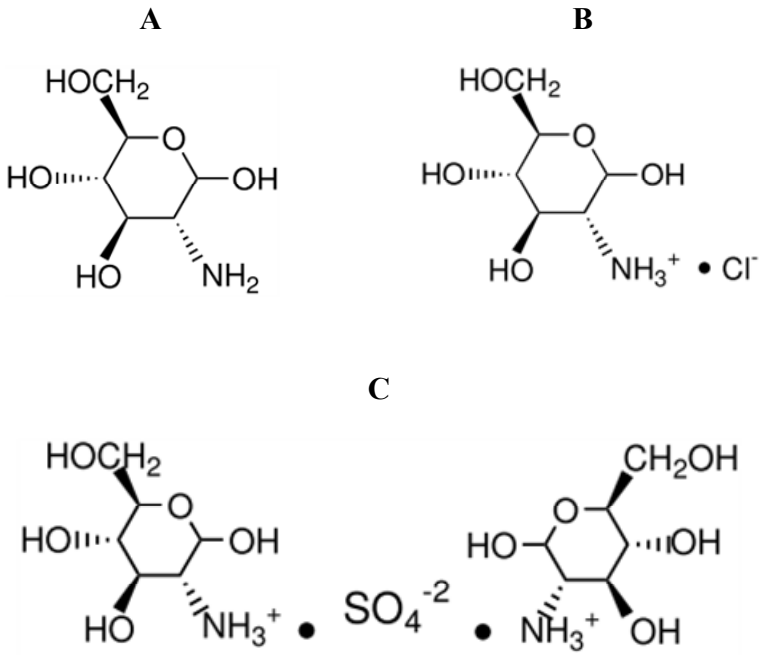

Figure 2.2. A-C. Structure of glucosamine (A); glucosamine hydrochloride (B); glucosamine sulfate (C) (Barnhill and others 2009)

CHAPTER 3

Development of an improved extraction and HPLC method for the measurement of ascorbic acid in cows' milk from processing plants and retail outlets

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ABSTRACT

An improved extraction (2.5% HPO_3, 5 mM dithiothreitol) and HPLC quantification using a C-18 column at 35ºC and 0.1M acetic acid (98%) and acetonitrile (2%) mobile phase was developed to quantify total ascorbic acid (AA) in commercial whole/semi-skim/skim raw/pasteurized/UHT milk packaged in opaque bags, transparent plastic, cardboard, and Tetra Brik™. AA content ranged 0.21-10 and 3.4-16 mg/l in milk from retail outlets and processing plants, respectively, and was higher in organic milk. For same processor/lot samples, pasteurized milk showed higher AA content than UHT milk. This was not true for retail outlets samples. AA content was similar for whole/semi-skim and semi-skim/skim milk but not for whole/skim comparisons. Among UHT samples, the AA content trend was whole<semi-skim<skim and lower for UHT milk in opaque plastic and Tetra Brik™ container. After 14d at 4ºC in the dark, AA losses ranged 35-83% depending on milk type and preservation method with a higher AA retention in unopened containers.

Keywords: Cow milk; vitamin analysis; ascorbic acid; HPLC-DAD

Running Title: Vitamin C in retail/processing plant cow milk

3.1. Introduction

Vitamins are essential in physiological processes including vitamin C which cannot be synthesized by humans and thus must be present in the diet. Some foods are supplemented with vitamins such as specialized dairy products for infants, children, and teenagers. Significant vitamin losses occur during food processing and storage reflecting food processing temperature and time, pH, redox potential, packaging material, and storage conditions such as light, temperature, oxygen, and others (Oamen and others 1989; Ochiai and Nakagawa 1992; Albala-Hurtado and others 2000). Vitamin C, a water-soluble vitamin present naturally in foods mostly as ascorbic acid (AA), is an excellent antioxidant with multiple metabolic functions in both plant and animal cells (Lindmark-Mansson and Akesson 2000). AA is used as a technological marker of the severity of processing and storage effects on food quality (Pizzoferrato 1992; Romeu Nadal and others 2008a; Romeu Nadal and others 2008b). AA is also important when assessing novel alternatives to stabilize foods including pressure processing. AA losses during pressure-assisted thermal processing have been reported (Ramirez and others 2009).

The main AA degradation pathway is oxidation to dehydroascorbic acid (DHAA) yielding diketo-L-gulonic acid (DKGA). This reaction is the basis for the use of AA as an antioxidant. Oxidation of AA to DHAA can be reversed by reducing agents which are used in analytical procedures to quantify the total amount of AA. The first reducing agent investigated for this purpose was dithiothreitol (DTT) used at room temperature at a concentration of 10 mM (Scott and Bishop 1986). DTT has

been effectively used in determinations of total AA in several foods including milk (Weiss 2001) but its efficiency is pH-dependent (Ryley and Kaida 1994). Takayanagi *et al*. (2009) showed that 5 mM DTT was sufficient to protect AA against degradation during sample handling. Although other reductants such as mercaptoethanol, homocysteine, and cysteine can prevent AA oxidation during analytical determinations, DTT is the most common alternative (Ryley and Kaida 1994; Mesías-García and others 2010).

The AA content in cow milk is low varying with the season and the feed used. Walstra *et al.* (2001) reported that the total AA content in raw cow milk ranged between 1.7 and 2.8 mg/100 g, while Bilic (1991) found values for AA and DHAA of 0.59 mg/100 g and 0.15 mg/100 g, respectively. In pasteurized cow milk, Marconi and Panfili (1998) reported a value of 0.70 mg/100 g while Scott *et al.* (1984) found AA values of 1.45 mg/100 ml. Pizzoferrato (1992) reported amounts between 0.25-0.51 mg/100 ml in UHT cow´s milk. The main problem associated with AA determinations in milk is its low concentration. Avoiding potential interferences from other food components is also a concern. High-performance liquid chromatography (HPLC) is frequently used to quantify AA in foods including milk (Okamura 1980; Gliguem and Birlouez Aragon 2005; Eitenmiller and others 2008).

The aims of this study were to develop and apply an improved sample extraction and HPLC quantification method to determine the total AA in commercial cow milk samples; to evaluate the influence of various conditions (heat treatment, packaging material, production system, and milk brand) on their total AA content; and,

to quantify losses of total AA in milk samples stored at 4ºC for up to 14 d in the dark.

3.2. Materials and Methods

3.2.1. Chemicals and reagents

Reagents were all of analytical grade including the AA standard (Grupo Productos Aditivos, Madrid, Spain), DL-dithiothreitol (DTT) and phosphotungstic acid hydrate (Fluka Analytical, Madrid, Spain), zinc acetate (Merck, Darmstadt, Germany), glacial acetic acid and *meta*-phosphoric acid (Panreac, Barcelona, Spain), and HPLC grade water (Sigma-Aldrich, Madrid, Spain). The AA standard stock solution, and dilutions in 2.5% *meta*-phosphoric acid (HPO_3) to yield a pH value similar to the solution used to extract AA from milk samples, were prepared quickly and stored immediately in amber bottles. DTT (5mM) was added to minimize AA oxidation. Seven AA standard solution concentrations in the 0.25-10 mg/l range were used to build an HPLC calibration curve. AA standards were prepared daily and kept at 4 °C until used. Standard solutions were injected only once to minimize possible effects of AA degradation.

3.2.2. Chromatographic quantification method

The HPLC system (Thermo Fisher Scientific Inc., Waltham, MA) used consisted of a Spectra system SCM 1000 vacuum degasser, a Spectra system P4000 pump, a Spectra system AS3000 autosampler, and a Surveyor PDA^+ detector

connected to a computer running ChromQuest™ 5.0. Different columns and mobile phases with a 0.5 ml/min flow rate were tested during the quantification method optimization (Table 3.1). Milk sample AA peak identification for the optimized method was carried out by comparison of retention time, wavelength and spectrum with those obtained for AA standards. AA quantification in milk was performed in triplicate samples.

3.2.3. Milk samples

Organic/conventional, whole/semi-skim/skim, raw/pasteurized/UHT milk in various packaging types and from seven commercial trademarks was investigated (Figure 3.1). Raw whole milk was obtained from two processing plants (brands A and D), while pasteurized and UHT milk were obtained from the same two processing plants, and also from local retail outlets (brands B, C, E, F, and G). Raw milk samples were collected in 100 ml plastic containers filled with no headspace and kept covered with aluminum foil until analyzed. Pasteurized whole, semi-skim and skim milk were obtained in three packaging types (opaque plastic bags, transparent plastic bottles, cardboard boxes). UHT whole, semi-skim, and skim milk were obtained in opaque plastic bottles and Tetra Brik™ containers. Transparent packages were covered with aluminum foil before analysis completed usually within a few hours after sample collection. When this was not possible, samples were kept at -20 ºC for a few days and thawed at 4 ºC before analysis. Composition of milk samples is shown in Table 3.2.

3.2.4. Sample extraction

3.2.4.1 Method I. This method was based on the work reported by Zafra-Gómez *et al.*(2006) modified to improve AA extraction. Milk samples (4 ml) were mixed with 1 ml of an extracting solution prepared by dissolving 9.1 g zinc acetate, 5.5 g phosphotungstic polyhydrated acid in 5.8 ml glacial acetic acid, and adding then HPLC grade water to reach a 100 ml final volume. The milk and extraction solution mix was vortexed for 10 s and centrifuged at 4000 rpm and 10 °C for 10 min. The clear layer was filtered through 0.45 μm Chromafil® Xtra into an amber vial and injected directly into the HPLC system. The analysis was completed in less than 1 h from extraction to chromatographic separation.

3.2.4.2. Method II. Sample preparation was carried out as in Method 1 but using 2.5% *meta*-phosphoric acid (HPO_3) as extraction solution to improve AA extraction from milk as suggested by Romeu-Nadal et al. (2006).

3.2.5. AA Degradation during storage at 4 °C

The degradation of AA during storage was analyzed assuming first order kinetics (İpek and others 2005; van Loey and others 2005):

$$\ln \frac{A_t}{A_o} = -kt \qquad (1)$$

where A_o and A_t are the AA concentration (*mg/l*) at time 0 and t (*day*), respectively, and k is the reaction rate constant (day^{-1}). The time period considered in this study was based on the assumption that milk consumers open a container, store it under

refrigeration, and then open it once a day for one week. The study was extended to 14 d which was the maximum shelf-life expected.

3.2.5.1 Study 1: *Consumer milk handling effect*. AA degradation was studied in raw whole, pasteurized semi-skim and skim and UHT whole milk for brand A, and in raw and pasteurized whole milk for brand D. Samples from UHT containers opened at time 0 were taken after 0, 0.25, 0.5, 1, 2, 5, 7, and 14 d at 4ºC. Thus, milk was subjected to microbial and atmospheric oxygen conditions representing consumer use. Before analysis, samples were protected from light and kept at 4ºC.

3.2.5.2. Study 2: *Effect of sodium azide addition on the consumer milk handling effect*. AA degradation was studied in UHT whole milk (brand A) by sampling from the same open container, without and with 0.02% sodium azide added to control microbial growth, and following the above periodicity.

3.2.5.3. Study 3: *Milk storage effect.* AA degradation was studied in UHT whole milk (brand A) by opening new containers at each sampling time following the above periodicity to determine storage effects in closed containers stored at 4ºC.

3.2.6. Statistical analysis

Statistical analysis was performed with SPSS (Windows Version 18.0). Data was tested for normality by the Kolmogorov-Smirnov (K-S) test. One and multi-way analysis of variance (ANOVA) were used to test for differences between mean AA content ($\alpha = 0.05$) in raw, pasteurized and UHT milk, using fat content, packaging, and brand as factors. A paired two-sample test was used to compare conventional and

organic milk, pasteurized and UHT milk, and type of container for UHT milk from the same brand.

3.3. Results and Discussion

3.3.1. Extraction procedure

The extracting solution used in Method I was immiscible with acetonitrile and DTT causing several problems including column clogging. The Method II extracting solution (based on *meta*-phosphoric acid) was miscible with acetonitrile and DTT, and was used in this work as the preparation procedure was quicker and easier. *Meta*-phosphoric acid as extracting solution for AA analysis has been reported in previous works, with indications that it precipitates proteins and stabilizes AA (Kim and others 1987; Eitenmiller and others 2008).

3.3.2. Column and mobile phase selection

Several mobile phases and three columns were tested to establish optimum analysis conditions yielding the retention times and absorption wavelengths reported in Table 3.1. Using an amino column (150 x 2.1 mm, 3 μ particle size, Hypersil® APS1, Thermo Electron Corporation, UK) with mobile phases containing high acetonitrile content (mobile phases 1-3) caused precipitation of the extracted milk sample in the column and could not be used. Using the same column with mobile phases 4 and 5 resulted in retention times with high standard deviation values, i.e., 2.9±0.6 and 4.9±0.3, respectively, and variable maximum absorption wavelengths (Table 3.1).

Tests with the C_{18} Kinetex column (100 x 4.6 mm, 2.6 µ particle size, Phenomenex®100A, Phenomenex, Torrance, CA) with mobile phases 6 and 7 showed an unacceptably low AA retention time (about 0.8 min). Tests with the C_{18} Luna column (150 x 4.6 mm, 5µ particle size, Phenomenex® 100A) using mobile phase 6 showed the same co-elution problems as the C_{18} Kinetex column while a 5 min AA retention time and good separation were observed when using mobile phase 5. However, 2.0% acetonitrile was necessary to retain column efficiency, resulting in mobile phase 8, which showed good peak separation and slightly shorter retention times. In conclusion, mobile phase 8, column Luna C_{18}, and a guard column containing the same packing material were selected. A 35 ºC column temperature and 0.50 ml/min isocratic elution rate resulted in a 20 min quantification time. The injection volume was 20 µL and the detection was at 245 nm. Figure 3.2 shows an analysis for AA in 2.5% *meta*-phosphoric acid and 5 mM DTT using these conditions.

3.3.3. Ascorbic acid quantification

After optimization of the extraction, column, mobile phase, and elution conditions, the method was evaluated in terms of linearity, limit of detection (LOD), limit of quantification (LOQ), reproducibility, and precision. HPLC run time was set at 20 min ensuring that the column was ready for the next sample. The calibration curve showed r^2 values of 0.999. LOD and LOQ values were defined as the concentration in the milk sample extract (n = 3) with signal to noise ratio of 3 and 10, respectively

(MacDougall and Crummett 1980; Vial and Jardy 1999). The LOD (0.050 mg/l) was lower than values reported for fortified foods (around 1 mg/kg, Fontannaz and others 2006), baby foods (30 mg/kg, Su and others 1995) and milk (1 mg/kg, Mannino and Pagliarini 1988) but higher than values (0.010 mg/l) reported by Rodríguez-Bernaldo de Quirós *et al.* (2009) for fruit juices and soft drinks. In addition, the LOQ (0.10 mg/l) was lower than all the reported values in the works cited for LOD comparison. Method reproducibility was determined by analyzing six replicates (n=6) of three standards (0.5, 1, and 5 mg/l) showing about 5.0% relative standard deviation (RSD). Method precision (2.0%) was estimated as the RSD value for multiple extractions of the same milk sample (UHT whole milk, n=6). Finally, the results showed high recovery levels (tested by spiking the samples with 10 mg/l of AA, Table 3.3) ranging 77-83%, 62-82%, and 53-88% for whole, semi-skim, and skim milk, respectively with RSD values lower than 5.0%. When DTT was used in the analysis, the corresponding values increased up to 90%, 95% and 96% with RSD values lower than 3.0%. These results indicate that the method was suitable for AA analysis in milk.

3.3.4. Commercial milk samples

Milk fat content ranged 3.5-3.9%, 1.5-1.6%, and 0.23-0.30% for whole, semi-skim milk and skim milk (Table 3.2). AA concentration ranged 0.21-10 mg/l in brand B, C, E, F, and G samples collected from retailers, and 3.4-16 mg/l in brand A and D samples collected from the processing plant (Table 3.4). Brand D (organic milk) showed the highest values, while brands B, C (except for Tetra Brik™ container milk),

and F showed the lowest, with the remaining brands (A, E, and G) presenting intermediate values.

3.3.4.1. Milk collected from processing plants. Three sample lots (I, II, and III) of brand A and one of brand D were analyzed (Table 3.4). AA content in brand A raw milk ranged from 4.9 ± 0.14 (Lot II) to 9.8 ± 0.41 mg/l (Lot I), and was 16 ± 0.17 mg/l for brand D organic milk. UHT processing of brand A milk reduced AA content by about 20 to 40%. Pasteurization of brand A and D milk caused lower losses ranging 10-20%. Multi-way variance analyses showed statistically significant differences between lots ($p<0.05$). Multi-range ANOVA tests showed AA content similarities between lots I and III for raw and pasteurized milk but not for UHT milk from all three lots (Table 3.4). Different feeding, sampling date, and thermal treatments are possible causes for this observation. Processing temperature and time are the most important factors affecting AA degradation (Steskova and others 2006). UHT milk can be processed by direct (~2 s at 150 ºC) and indirect heating (15 s at 142 ºC) (Walstra and others 2001) while pasteurization requires at least 15 s at 72 ºC, although actual time and temperature used by the processor may be higher than these values (Salgado and others 2011). Pasteurization conditions of brand A and D were 15-20 s at 66-68 ºC and 14 s at 75 ºC, respectively, while the thermal treatment for the brand A UHT milk was 2 s at 148 ºC. When comparing samples from the same brand A lot, pasteurized milk showed higher AA content than UHT milk for all lots, an observation consistent with previous reports (MacDougall and Crummett 1980; Ryley and Kaida 1994) and reflecting thermal process severity differences (Okamura 1980; Haddad and

Loewenstein 1983). For the same brand A milk processing lot, significant differences ($p<0.05$) were observed between pasteurized and UHT whole and semi-skim milk (Lots III and I, respectively), but not for skim milk (Lot II). Therefore, thermal treatment severity was not the only factor influencing the AA content.

3.3.4.2. Milk collected from retail outlets. Thermal pasteurization conditions for brand B milk were 12 s at 78 ºC but this information was unavailable for brand C. Both brands showed similar low AA content (under 0.36 mg/l and 0.42 mg/l, respectively) with statistical differences only between whole and skim milk from the same brand ($p<0.05$). The UHT thermal treatment for brands E and G was similar, i.e., 3 and 2.4 s at 148 ºC, respectively, while the brand C conditions were more severe than those used typically (9 s at 150 ºC). Processing conditions for brand F were 2.8 s at 142 ºC**.** One and multi-way variance analyses ($p<0.05$) were conducted to assess milk fat content effects. With regards to pasteurized milk, statistical differences in AA content were found for the fat content for brands B and C, using multi-way analysis. Multi-range tests showed similarities between AA content for whole/semi-skim and semi-skim/skim milk samples but not for whole/skim comparisons (Table 3.4). For the 5 UHT milk samples, statistical differences in AA content were found also for fat content showing a whole < semi-skim < skim trend.

The packaging effect was studied for UHT milk comparing opaque plastic bottles and Tetra Brik™ containers. Statistical differences ($p<0.05$) were found for UHT milk as affected by packaging type with lower AA content observed in opaque plastic as compared to Tetra Brik™ containers, except for brand B whole milk which

showed very low AA content. AA levels ranged from 0.21-10 mg/l and 0.22-3.4 mg/l in Tetra Brik™ and opaque plastic containers, respectively (Table 3.4). These results are similar to previous reports on the effect of packaging on the AA stability in milk. For example, Gliguem and Birlouez Aragon (2005), reported that after 1 month at room temperature AA degradation in UHT milk reached 99% and 51% in 3-layered and in 6-layered packaging, respectively. AA degradation differences may reflect oxygen permeation differences and a higher milk exposure to light in opaque plastic as compared to Tetra Brik™ containers (Gliguem & Birlouez Aragon, 2005). Although **s**tatistical differences in AA content were found, the retail outlet samples storage time and conditions were factors that could not be controlled, and the AA content for the raw milk corresponding to the pasteurized and UHT milk samples collected was unavailable. However, considering that AA indicates the process and storage severity, these large differences in AA content do suggest quality differences in the commercial retail outlet milk.

3.3.4.3. Effect of milk type (conventional/organic). Organic milk brand D showed the highest AA content (Table 3.4). Lund (1991) reported also that organic milk showed higher AA concentration than conventional milk and reported AA values of about 15 mg/l. Since organic cows graze on fresh grass and clover, the milk they produced has been reported to have a higher vitamin content (Kalac 2011).

3.3.4.4. Consumer storage effects. AA concentration in milk stored at 4ºC decreased gradually as reported by Oamen et al. (1989) following first order kinetics for up to 6-7 d of storage (Figure 3.3). Çakmakçi & Turgut (2005) reported that AA in

pasteurized milk decreased significantly for the first 24 h of 4ºC storage, then gradually between1-3 d, changing much less afterwards (3-5 d). After 14 d, conventional (brand A, Lots I, II, and III) and organic (brand D) whole milk showed a 35-66% and 83% AA content decrease, respectively, with respect to raw milk. The AA degradation rate constant (k, day^{-1}) was significantly higher ($p<0.05$) in brand D (0.347) than in A with non-significant differences among lots (0.153, 0.166, and 0.154 for lots I, II and III, respectively, Figure 3.3A). At the same temperature (4ºC), the k value for AA loss in orange juice was lower than in milk (Kennedy et al., 1992).

The thermal process effect (brand A, Lot III) on AA losses (Figure 3.3B) showed a significantly higher reaction rate constant ($p<0.05$) for pasteurized milk (0.298 day^{-1}) than for raw (0.154 day^{-1}) and UHT (0.057 day^{-1}) milk. In the case of the fat content level effect on AA losses analyzed for pasteurized milk (Figure 3.3C), the reaction rate constant changed from 0.397, 0.345 and 0.298 day^{-1} for brand A semi-skim, skim and whole milk, respectively, and were statistically different ($p<0.05$). The AA degradation rate constant (k, day^{-1}) for pasteurized whole milk was significantly higher for organic brand D milk (0.0353 day^{-1}) than in brand A, Lot I (0.298). However, since after 6-7 d of faster AA degradation following first order kinetics, the AA loss rate decreased or approached zero, AA losses after 14 d was about 81%, 62%, and 52% for UHT, pasteurized, and raw milk, respectively. Gliguem & Birlouez Aragon (2005) reported a 51% AA decrease in fortified UHT milk kept one month at room temperature, and up to 75% decrease after four months. Other authors have reported AA losses during milk storage also (Kennedy and others 1992; Rodríguez-

Comesaña and others 2002; Castro and others 2004; Burdurlu and others 2006). A rapid decrease of about 90% in AA levels was reported during the first 48 h for pasteurized milk in plastic or glass containers (Öste and others 1997), whereas in this work a 48% reduction was observed within the first 48 h in pasteurized whole milk (brand A, Lot III) in transparent glass containers covered with aluminum foil. Steskova *et al.*, (2006) reported no significant of AA losses during 7 days after pasteurization when milk packages were opened, but another work reported that AA was converted to other forms (Schroeder and others 1985).

3.3.4.5. *Storage studies*. AA degradation rate constants were 0.006, 0.022 and 0.057 day^{-1}, respectively with statistical differences ($p<0.05$), for new container, same container, and same container with sodium azide added after opening it (Figure 3.4). After 14 d, AA loss was highest (84%) in milk with 0.02% sodium azide, whereas the lowest reduction (18%) was observed when a new container was opened at each sampling time. When sampling always from the same container, an AA loss of 50% of was observed. These results indicate that adding sodium azide increased AA losses, whereas milk stored in unopened bottles retained more AA. Further research is required to fully interpret the sodium azide effect but it may reflect an interaction with ascorbic acid.

3.4. Conclusions

An extraction and HPLC methodology was developed and optimized to analyze AA content in commercial milk samples obtained from processing plants and

retail outlets. AA concentration in samples of raw, pasteurized, and UHT conventional and organic milk with different fat content of several brands, and commercialized in various container types ranged widely, i.e., from 0.21 to 16 mg/l. AA content variation could be attributed to processing conditions, feed composition, and container type. AA levels varied for pasteurized and UHT milk with different fat levels, with a whole<semi-skim<skim AA concentration observed as a general trend. Organic milk showed the highest AA level. In spite of the unknown factors for milk collected from retail outlets, it was concluded that the Tetra Brik^{TM} container is a packaging choice superior to opaque plastic bottles. After 14 d of storage at 4ºC in the dark, the decrease of AA ranged from 83% to 35% depending on milk type and preservation method used. A higher retention of AA was observed when the milk container was kept unopened.

Acknowledgements

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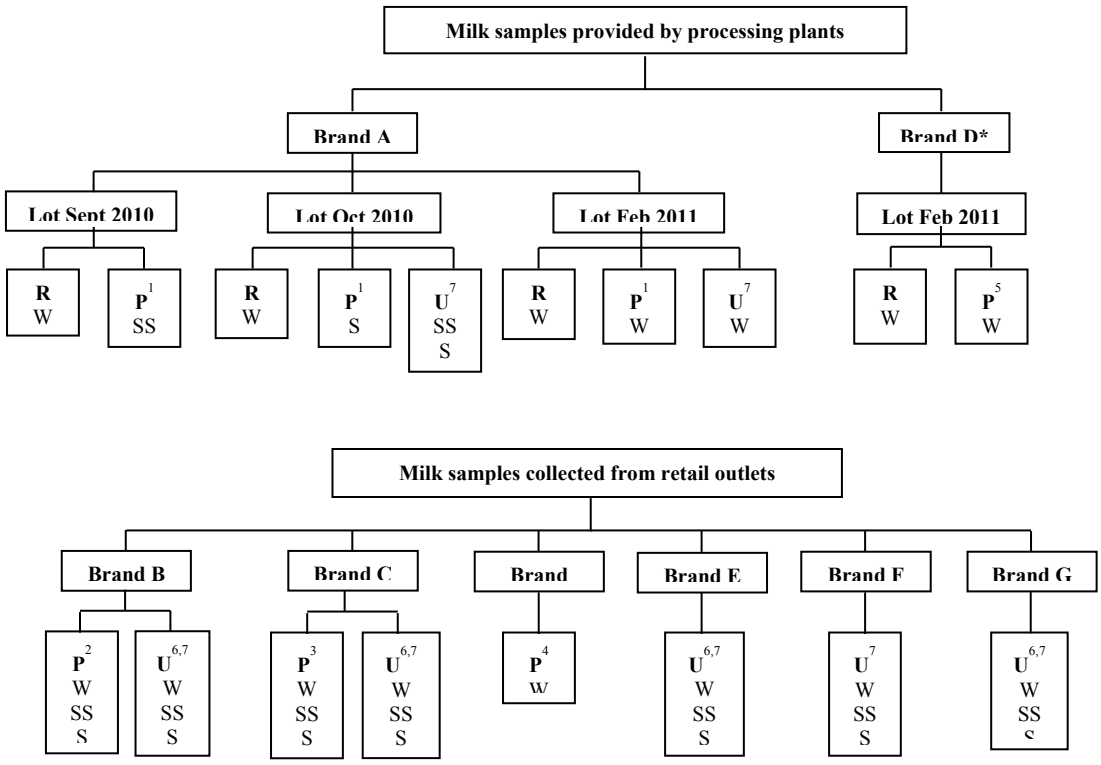

Figure 3.1. Milk samples collected from processing plants and retail outlets indicating brand (A to G), fat content (W: whole, SS: semi-skim, S: skim), container type (1: transparent plastic bottle covered with aluminum foil before analysis, 2: transparent plastic bottle, 3: cardboard boxes, 4: opaque plastic bag, 5: transparent glass bottle covered with aluminum foil before analysis, 6: opaque plastic bottle, 7: Tetra Brik™ container), type of milk (*: organic), and thermal treatment (R: raw, P: pasteurized, U: UHT). Roman numerals in milk samples provided by processing plants indicate samples from the same milk lot

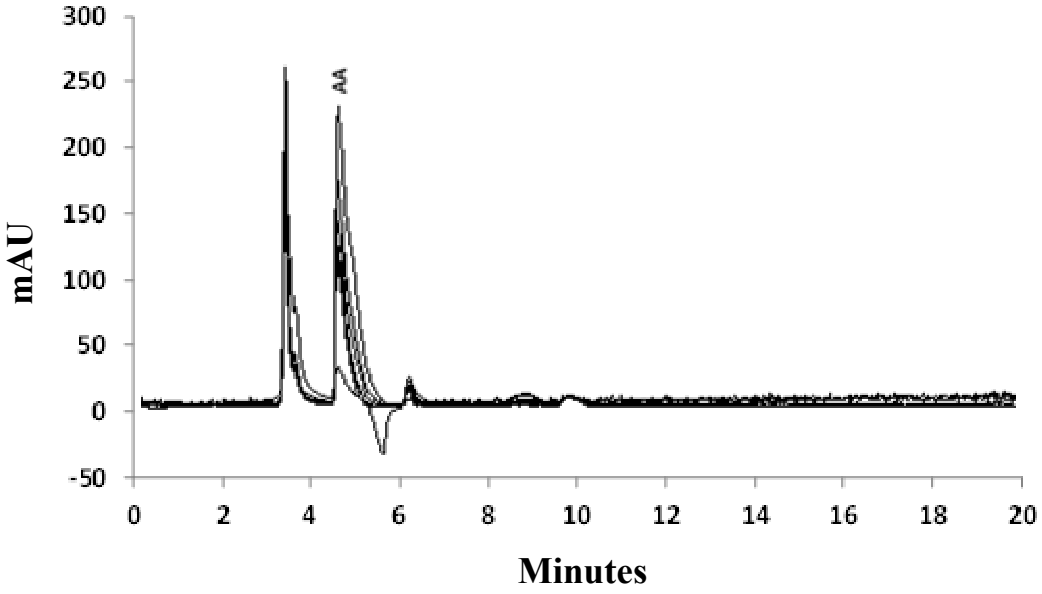

Figure 3.2. Chromatograms for 0.25 - 10 mg/l ascorbic acid (AA) standards in 2.5% *meta*-phosphoric acid (HPO_3) and 5 mM dithiothreitol (DTT). HPLC quantification using a C_{18} Luna column (150 x 4.6 mm, 5µ particle size, Phenomenex® 100A) at 35ºC and a 0.1M acetic acid (98%) and acetonitrile (2%) mobile phase.

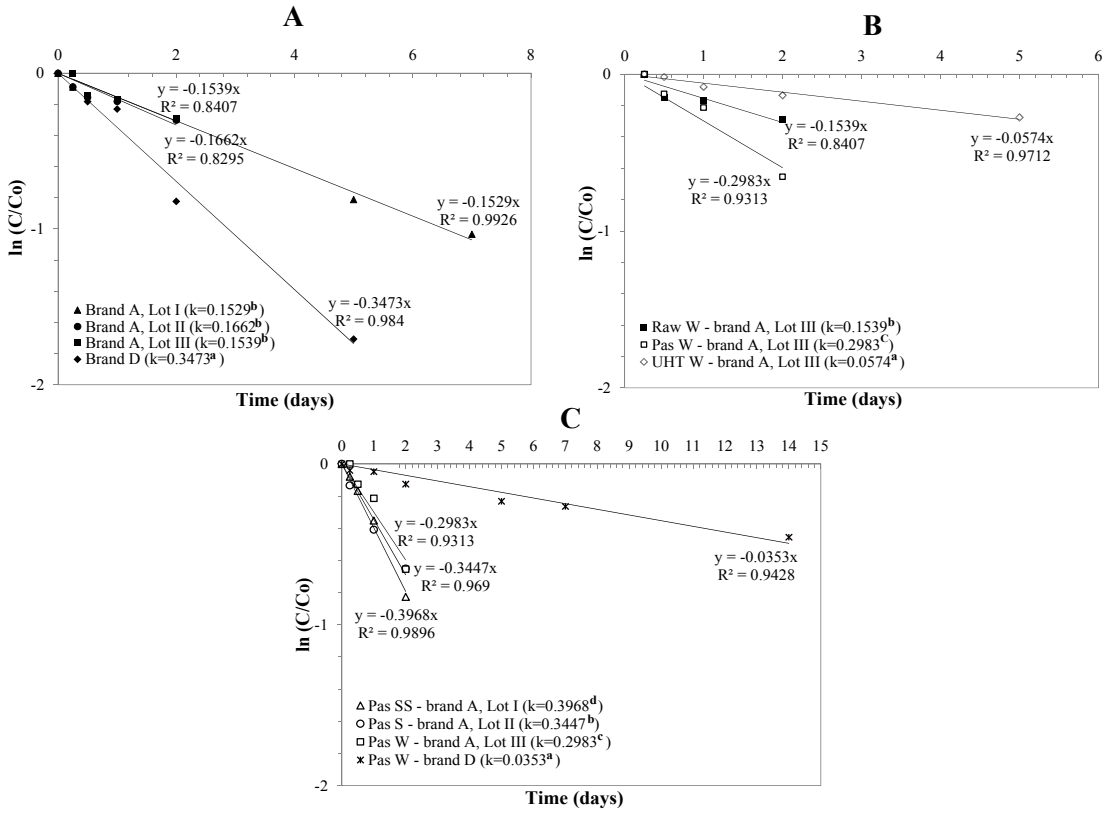

Figure 3.3. A-C. Ascorbic acid degradation kinetics in milk packaged in transparent glass bottles covered with aluminum foil before analysis: raw whole milk (A); effect of thermal process on milk brand A, Lot III (B); effect of fat content level on pasteurized whole, semi-skim and skim milk (C). Different low case subscript letters on the first order kinetic constant values (*k*) shown in each graph for the milk studied indicate statistically significant differences ($p<0.05$).

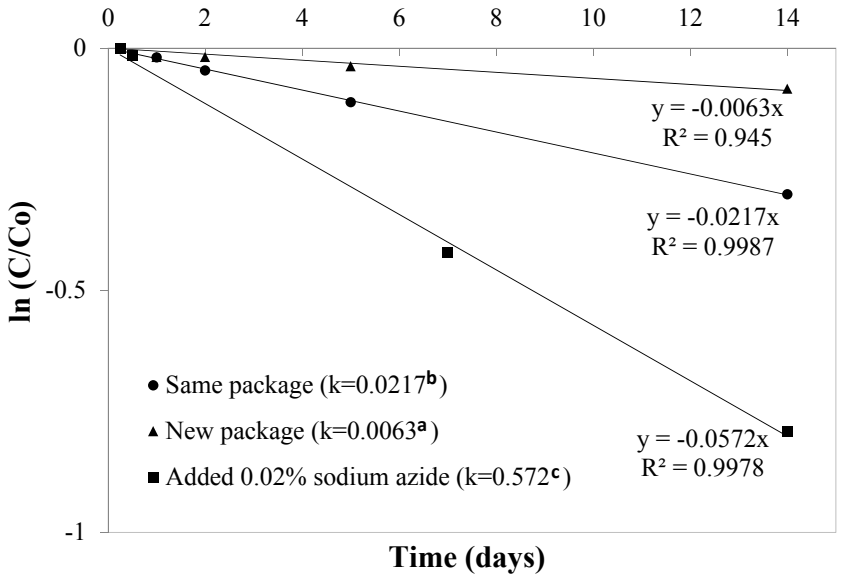

Figure 3.4. Ascorbic acid degradation in UHT whole milk in Tetra Brik^{TM} container: (●) sample from the same container, (▲) sample from new containers, (■) sample from same container after adding 0.02% sodium azide (NaN_3). Different low case subscript letters on the first order kinetic constant values (k) indicate statistically significant differences ($p<0.05$).

Table 3.1. Milk ascorbic acid analysis conditions

Mobile phase and column		Retention time (min) mean± SD, n = 2	Absorption (nm)
No.	***Amino column (150 x 2.1 mm)***		
1	75% ACN + 25% 0.05M KH_2PO_4	6.6 ± 1.9	253, 267
2	50% ACN + 50% 0.05M KH_2PO_4	2.7 ± 0.5	240, 244
3	19% ACN + 81% 0.005M H_2SO_4	3.2 ± 1.2	243
4	95% 0.08M CH_3COONH_4 in ACN + 5% ACN	2.9 ± 0.6	250, 256
5	0.1M Acetic acid	4.9 ± 0.3	236, 245
	Kinetex C18 column (100 x 4.6 mm)		
6	95% 0.01M CH_3COONH_4 in ACN + 5% ACN	0.8 ± 0.00	258, 259
7	2% Acetic acid	n.d.[(1)]	n.d.
	Luna C18 column (150 x 4.6 mm)		
6	95% 0.01M CH_3COONH_4 in ACN + 5% ACN	1.8 ± 0.01	265
5	0.1M Acetic acid	5.1 ±0.01	245
8	98% 0.1M Acetic acid + 2% ACN	4.9 ± 0.00	245

[(1)] n.d. = not determined

Table 3.2. Composition of commercial milk samples

Treatment	Brand	Type	Energy value (kcal)	Protein (g/100ml)	Carbohydrate (g/100ml)	Fat content (g/100ml)
Raw[8]	A	Whole I	n.a	3.2	4.7	3.8
	A	Whole II	n.a.	3.2	4.7	3.9
	A	Whole III	n.a.	3.2	4.7	3.6
	D*	Whole	n.a.	n.a.	n.a.	n.a.
Pasteurized	A[1]	Whole	n.a.	3.2	4.7	3.5
	A[1]	Semi-skim	n.a.	3.3	4.8	1.5
	A[1]	Skim	n.a.	3.3	4.8	0.24
	B[2]	Whole	51	3.1	4.6	3.5
	B[2]	Semi-skim	45	3.1	4.6	1.5
	B[2]	Skim	34	3.1	4.6	0.30
	C[3]	Whole	62	3.0	4.5	3.6
	C[3]	Semi-skim	43	3.0	4.6	1.5
	C[3]	Skim	33	3.1	4.7	0.30
	D*[, 5]	Whole	n.a.	n.a.	n.a.	n.a.
UHT	A[7]	Whole	n.a.	3.2	4.7	3.5
	A[7]	Semi-skim	n.a.	3.3	4.7	1.5
	A[7]	Skim	n.a.	3.3	4.9	0.23
	B[6]	Whole	64	3.1	4.7	3.6
	B[6]	Semi-skim	46	3.2	4.7	1.6
	B[6]	Skim	34	3.2	4.7	0.30
	C[7]	Whole	62	3.0	4.5	3.6
	C[7]	Semi-skim	43	3.0	4.6	1.5
	C[7]	Skim	33	3.1	4.7	0.30
	E[7]	Whole	63	3.1	4.6	3.6
	E[7]	Semi-skim	45	3.2	4.7	1.6
	E[7]	Skim	34	3.2	4.7	0.25
	F[7]	Whole	61	3.0	4.6	3.6
	F[7]	Semi-skim	45	3.1	4.6	1.6
	F[7]	Skim	32	3.2	4.6	0.30
	G[7]	Whole	64	3.0	4.8	3.6

Table 3.2. (Continued) Composition of commercial milk samples

Treatment	Brand	Type	Energy value (kcal)	Protein (g/100ml)	Carbohydrate (g/100ml)	Fat content (g/100ml)
UHT	G[7]	Whole	64	3.0	4.8	3.6
	G[7]	Semi-skim	46	3.0	4.8	1.6
	G[7]	Skim	35	3.0	4.8	0.30

Raw milk samples were collected in 100 ml plastic containers filled with no headspace and covered with aluminum foil before analysis while the container for other milk samples were as follows: (1) Transparent plastic bottle covered with aluminum foil before analysis; (2) Transparent plastic bottle; (3) Cardboard box; (4) Opaque plastic bag; (5) Transparent glass bottle covered with aluminum foil before analysis; (6) Opaque plastic bottle; (7) Tetra Brik™ container.
* = organic milk and n.a. = not available

Table 3.3. HPLC ascorbic acid (AA) determination and recovery from commercial milk samples with and without dithiothreitol (DTT) in the extracting solution (n = 3)

Sample	Concentration (mg/l) ± RSD(*)		Recovery (%)	
	w/o DTT	w/ DTT	w/o DTT	w/ DTT
Transparent plastic bottle, Brand B				
Pasteurized whole milk	0.26±0.014	0.37±0.020	83±0.80	90±1.70
+ 10 mg/l AA	8.6±0.08	9.3±0.08		
Pasteurized semi-skim milk	0.36±0.024	0.44±0.002	62±1.7	67±0.8
+ 10 mg/l AA	6.6±0.17	7.2±0.08		
Pasteurized skim milk	0.36±0.031	0.40±0.071	88±1.4	87±1.7
+ 10 mg/l AA	9.1±0.12	9.1±0.15		
Cardboard box , Brand C				
Pasteurized whole milk	0.35±0.080	0.47±0.010	77±1.5	90±2.3
+ 10 mg/l AA	8.1±0.15	9.5±0.23		
Pasteurized semi-skim milk	0.33±0.040	0.46±0.030	75±0.30	95±0.60
+ 10 mg/l AA	7.8±0.03	10±0.06		
Pasteurized skim milk	0.42±0.050	0.47±0.043	84±0.9	96±0.9
+ 10 mg/l AA	8.8±0.092	10±0.093		
Tetra Brik^{TM} container, Brand E				
UHT whole milk	6.7±0.020	7.5±0.23	81±2.1	78±0.9
+ 10 mg/l AA	15±0.21	15±0.090		
UHT semi-skim milk	5.4±0.35	6.4±0.18	82±3.2	75±0.5
+ 10 mg/l AA	13±4.85	14±4.60		
UHT skim milk	8.4±0.32	8.5±0.17	55±2.5	70±0.9
+ 10 mg/l AA	14±4.85	16±4.85		

(*) RSD = relative standard deviation

Table 3.4. Ascorbic acid content (n = 3, average ± standard deviation, mg/l) in raw, pasteurized and UHT milk samples†

Treatment	Container†††	Fat content	Processing plant samples		Retail outlet samples				
			Brand A††††	Brand D*	Brand B	Brand C	Brand E	Brand F	Brand G
Raw††		Whole	9.8 ±0.41[a] (I)	16 ±0.17[a]		n.a.	n.a.	n.a.	n.a.
			4.9 ±0.14[b] (II)	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
			9.6 ±0.23[a] (III)	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Pasteurized		Whole	9.1 ±0.15[1,a] (III)	12 ±0.23[4, b] 14 ±0.33[5, b]	n.a.				
					0.26±0.010[2,A]	0.35±0.08[3,A]	n.a.	n.a.	n.a.
		Semi	10 ±0.23[1,a] (I)	n.a.	[2,AB]	0.33±0.04[3,AB]	n.a.	n.a.	n.a.
		Skim	3.4 ±0.070[1,c] (II)	n.a.	0.36±0.031 0.30±0.030[2,B]	0.42±0.05[3,B]	n.a.	n.a.	n.a.
	1-5	Whole	7.9 ±0.10[d] (III)	n.a.	0.21±0.010[A]	3.3 ±0.050[D]	6.7 ±0.020[A]	0.96±0.11[A]	5.1 ±0.14[A]
	7	Semi	6.2 ±0.38[e] (I)	n.a.	0.35±0.010[B]	8.9 ±0.34[E]	5.4 ±0.35[A]	0.87±0.10[A]	7.1 ±0.17[B]
		Skim	4.0 ±0.22[c] (II)	n.a.	0.35±0.0010[B]	7.4 ±0.19[E]	8.4 ±0.32[B]	0.41±0.11[B]	10 ±0.17[C]
UHT		Whole	n.a.	n.a.	0.22±0.030[A]	0.41±0.00[B]	2.9 ±0.0040[C]	n.a.	1.0 ±0.010[D]
	6	Semi	n.a.	n.a.	0.28±0.020[A]	[C]	3.4 ±0.030[C]		1.2 ±0.060[D]
		Skim	n.a.	n.a.	0.29±0.010[A]	0.81±0.13[C]	1.3 ±0.030[D]	n.a.	1.3 ±0.070[D]

n.a. 0.70±0.0010 n.a.

† Different low case subscript letters indicate statistically significant differences among samples collected from the same plant, brand, processing lot and container type. Different upper case subscript letters indicate statistical differences ($p<0.05$) among samples collected from retail outlets. For samples of brands B, C, E, F and G, the multi-way factor ANOVA analysis considered fat content and packaging type.

†† Raw milk samples collected in 100 ml plastic containers with no headspace and covered with aluminum foil before analysis

††† Commercial containers were: (1) Transparent plastic bottle covered with aluminum foil before analysis; (2) Transparent plastic bottle; (3) Cardboard box; (4) Opaque plastic bag; (5) Transparent glass bottle covered with aluminum foil before analysis; (6) Opaque plastic bottle; (7) Tetra Brik™ container.

†††† Milk samples from the same plant A lots identified with Roman numerals I to III

* = organic milk and n.a. = not available.

CHAPTER 4

Simultaneous HPLC-DAD quantification of vitamins A and E content in raw, pasteurized, and UHT cow's milk and their changes during storage

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ABSTRACT

An improved extraction and HPLC method for the simultaneous extraction and quantitation of retinol, α-tocopherol, α-tocotrienol and β-carotene was developed to analyze commercial whole/semi-skim/skim samples of raw/pasteurized/UHT milk in transparent plastic/glass bottles and Tetra Brik™ containers. The sample preparation method required prior saponification at 40ºC for 15 min followed by n-hexane extraction. An isocratic acetonitrile:methanol (65:35 v/v) mobile phase and UV detection were chosen for HPLC quantification. The liposoluble vitamin content in raw, pasteurized conventional/organic, and UHT milk ranged 0.055-5.540 (retinol), 0.135-1.410 (α-tocopherol), and 0.040-0.850 mg/l (β-carotene). No significant differences ($p>0.05$) were observed on losses of retinol, α-tocopherol, and β-carotene content in UHT whole milk after 5 d at 4ºC in the dark. After 14 d at 4ºC in the dark the contents of retinol, α-tocopherol, and β-carotene remained higher in milk with higher fat content and were higher in unopened containers. In UHT whole milk samples containing 0.02% NaN_3, retinol (33%) and α-tocopherol (11%) but not β-carotene (2%) decreased significantly ($p<0.05$).

Keywords: Cow's milk; Food analysis; Fat soluble vitamins; HPLC; vitamin A; vitamin E

4.1. Introduction

Milk contains the liposoluble vitamins A, D, E and K and it is often enriched with vitamins A, C, and E (MacGibbon and Taylor 2003). Assuming the recommended daily intake (RDI) for dairy products in Spain, consuming three milk servings/day provides about 16% and 3% of the RDI for vitamins A (1 mg/d) and E (15 mg/d), respectively (Herrero and others 2002). Vitamin A is a generic term referring to a variety of chemical substances showing vitamin A activity including retinol, retinal, retinoic acid, retinyl esters, and provitamin A carotenoids such as β-carotene. In dairy and animal food products it is most often present as the retinyl palmitate ester which is hydrolyzed in the small intestine cells to the alcohol retinol (Sommer and West 1996). Retinol and its derivatives are found in animal tissues and dairy products, while β-carotene is present in foods of plant and animal origin. In the case of milk, the conversion of β-carotene in the feed to retinol may occur in the mammary gland of lactating dairy cows (Baldi and Pinotti 2008).

Vitamin E inhibits the oxidation of polyunsaturated fatty acids (Miquel and others 2004) and is found in the form of eight isomers differing in biological activity, namely four tocopherols (α-, β-, γ- and δ-) and the four corresponding tocotrienols (Kamal-Eldin and others 2000). In milk, the vitamin E content is highly variable and present mostly as α-tocopherol (McDowell 2000). Compared to the other isomers, α-tocopherol is less stable during processing and storage, but it has the highest biological activity with beneficial antioxidant effects on the prevention of chronic diseases (Brigelius-Flohe and Traber 1999; Chavez-Servin and others 2006). Environmental factors such as oxygen, light, temperature, and

also the intrinsic food factors water activity, lipid content and pH influence their stability (Miquel and others 2004). Liposoluble vitamins content vary with processing conditions, season, cow species, and cow feed composition. Krukovsky et al. (1950) determined the carotenoid and α-tocopherol content in milk from Holstein, Guernsey, Brown Swiss and Jersey cows during both pasture and barn feeding. Vitamin E is bound to milk lipids and thus it is expected to decrease when milk fat content is reduced (Kaushik and others 2001). The composition and milk fat content depend also on feed, season, quality/quantity of feed, and lactation stage. The presence of α-tocopherol in milk may be attributed to the cow feed α-tocotrienol content, particularly that coming from grasses (Kaushik and others 2001). Guernsey and Holstein milk showed the highest and lowest α-tocopherol level, i.e., 3 and 2 mg per 100g milk fat, respectively. The season effect was studied by Agabriel et al. (2007) who analyzed bulk milk in French farm tanks. The difference in α-tocopherol content was attributed to the proportion of grazed grass or grass silage in the feed. Kalac (2011) reported also that the α-tocopherol content in milk fat depends on cow feeding showing higher values for cows fed grass (0.38 mg per 100 g milk fat) than maize silage (0.21 mg per 100 g milk fat). Higher content of α-tocopherol in summer than in winter milk has been reported and associated to cow feed (Ball 1998; Lindmark-Månsson and others 2003).

Extremely sensitive detection methods are required to quantify tocopherols and tocotrienols in dairy products (Podda and others 1996). Liposoluble vitamins are analyzed by direct solvent extraction or after saponification (Chavez-Servin and others 2006). Efficiency of these extraction methods varies and thus must be

optimized for milk. HPLC is most often used to quantify vitamins including liposoluble vitamins in milk (Bernal and others 2011), typically using UV-visible and diode-array detection (DAD) methods (Liyanage and others 2008; Plozza and others 2012; Szlagatys-Sidorkiewicz and others 2012; Turner and Burri 2012).

In this study, an improved method was developed by studying the simultaneous extraction of vitamins A and E using *n*-hexane directly, or by using *n*-hexane or dichloromethane after saponification following three methods. Also developed was a rapid and sensitive HPLC method for the simultaneous quantification of retinol, retinyl acetate, β-carotene (all with vitamin A activity), α-tocotrienol, and α-tocopherol (all with vitamin E activity). The extraction and HPLC method developed was used to quantify these compounds in samples of raw, pasteurized, and UHT milk with different fat content (whole, semi-skim, and skim) obtained from retail stores and processing plants. Since vitamin content differences have been reported for organic and conventional raw, whole, semi-skim and skim milk distributed in different types of packaging (Moyssiadi and others 2004; Zygoura and others 2004; Ellis and others 2007), organic milk and different container types were included in this study. Finally, the losses of vitamin A and E during consumer milk handling were also studied. This included the refrigerated storage of containers kept close and opened (to simulate contact with the environment) with and without the addition of sodium azide.

4.2. Materials and Methods

4.2.1. Milk samples

Organic/conventional, whole/semi-skim/skim, raw/pasteurized/UHT milk in various packaging types was collected in Spain from commercial sources. Raw whole milk was obtained from two processing plants (brands A and B), while pasteurized and UHT milk was obtained from the same two processing plants and also from retail outlets (brands C, D and E). Samples of raw and pasteurized whole, semi-skim, skim milk were transported in plastic and glass bottles for brands A and B. UHT whole, semi-skim and skim milk were obtained in Tetra Pak^{TM} containers. All transparent containers were covered with aluminum foil to minimize light exposure before analysis which was conducted usually within a few hours after samples were collected. When this was not possible, samples were kept at -20 ºC for a few days and thawed at 4 ºC before analysis. Composition of the milk samples reported by the milk suppliers is shown in Table 4.1.

4.2.2. Chemicals and reagents

The calibration standards retinol, β-carotene and α-tocopherol and the internal standards retinyl acetate and α-tocotrienol were obtained from Sigma-Aldrich (Madrid, Spain) (Table 4.2). Tert-butylhydroquinone (TBHQ, 97%), n-hexane (Chromasolv®) and acetonitrile (Chromasolv®) were obtained from the same supplier. Dichloromethane, phenolphthalein, absolute HPLC-grade ethanol, anhydrous sodium sulfate, and potassium hydroxide (85%) were supplied by

Panreac (Vigo, Spain). Stock standard solutions of 100 mg/l α-tocotrienol and 100 mg/l of retinol, retinyl acetate, β-carotene, α-tocopherol, and β-carotene were prepared in acetonitrile, except for β-carotene which was prepared in *n*-hexane. To minimize vitamins loss by oxidation, all solutions were bubbled with nitrogen for 1 min and stored in amber vials under refrigeration (4ºC).

4.2.3. Chromatography and detection procedures

The chromatographic system used was a Liquid Chromatography HPLC system from Thermo Fisher Scientific (Waltham, MA, USA) consisting of a Spectra System SCM1000 vacuum degasser, a Spectra System P4000 pump, a Spectra System AS3000 autosampler, and a Surveyor PDA plus detector connected to a PC computer running ChromQuest™ 5.0. The most common mobile phases used in the determination of vitamins A and E are methanol (MeOH), acetonitrile (ACN), and mixtures of these solvents with water (Escrivá and others 2002). Methanol:acetonitrile and methanol:water mixtures were tested as mobile phase (Table 4.3) on a 150 mm×4.6 mm 5 μm particle Luna C_{18} analytical column (Phenomenex, Madrid, Spain) with a 4 mm×2 mm guard column with the same packing material. Isocratic conditions of the mobile phase at a flow rate of 1 ml/min for 30 min and DAD detection were used to optimize all quantification methods tested. HPLC column temperature was held constant at 35 ºC. Sample injection volume initially set at 20 μL was increased to 100 μL to detect low vitamin concentrations. The wavelengths used were 296 nm for α-tocopherol and α-tocotrienol, 326 nm for retinol and retinyl acetate, and 450 nm for β-carotene (Table 4.2).

4.2.4. Milk saponification and solvent extraction procedures

Four extraction methods were tested for the extraction of vitamins from milk with antioxidants added to prevent the oxidation of fat soluble vitamins during extraction (Table 4.4). Butylated hydroxytoluene (BHT) and ascorbic acid (AA) are antioxidants commonly used for this purpose. Lee et al. (2003) found that BHT was more suitable than AA and caused fewer background interferences in the chromatographic separation of tocopherols by yielding a single 292 nm peak when using UV detection. However, BHA and BHT are only fairly heat stable, whereas TBHQ is more heat-stable (Reische et al. 2002). Therefore, TBHQ was used instead of BHT to minimize vitamin losses during the saponification heating steps. In all cases, saponification was conducted in the dark under a nitrogen atmosphere.

4.2.4.1. Method I. Saponification was not used in this extraction method based on the work by Rodas-Mendoza et al. (2003). As recommended by Eitenmiller et al. (2008), 1 ml milk samples without saponification were extracted with n-hexane with and without 1 g TBHQ added as antioxidant (Method I). Milk (1 ml) was mixed with 4 ml absolute ethanol, stirred for 2 min, and then for an additional 4 min after adding 400 µL n-hexane. After centrifugation at 2,500 rpm for 5 min at room temperature, the clear organic top layer was concentrated to dryness under nitrogen gas at 40 °C using a TurboVap LV concentration workstation (Caliper Life Sciences, Barcelona, Spain), reconstituted in 2 ml acetonitrile-methanol (65:35), and passed through 0.45 µm Chromafil®Xtrafilter before HPLC injection.

4.2.4.2. Method II. This extraction procedure is based on the work by

Bognar (1986) and Albalá-Hurtado et al. (1997). AA (0.5g) was added to a 25 ml milk sample and then mixed with 50 ml absolute ethanol and 10 ml 60% potassium hydroxide solution. The mixture was then kept overnight at room temperature with mild stirring. After saponification, the mixture was extracted 5 times with n-hexane (3x50 ml plus 2x25 ml). The pooled extracts were then washed with 50 ml water until the aqueous layer appeared colorless when adding 2-3 phenolphthalein drops. Then, 1 g TBHQ and 2 g anhydrous sodium sulfate (Na_2SO_4) were added to the organic fraction as antioxidant and to remove water traces, respectively. The mixture was filtered through a 0.45 µm MS®Nylon membrane filter and concentrated to dryness under nitrogen gas at 40 °C using a TurboVap LV concentration workstation. The extract was reconstituted in 10 ml acetonitrile-methanol (65:35) and passed through 0.45 µm Chromafil®Xtrafilter before HPLC injection.

4.2.4.3. Method III. In this method published by FAO (Anonymous 1997b), 0.25 g AA, 50 ml methanol, and 5 ml 50% KOH were added to 5 ml milk samples, held under bubbling nitrogen for 1 min, and then stirred in a water bath set at 80°C for 45 and 15 min, and at 40°C for 15 min. Saponified samples were then extracted with 4x50 ml of n-hexane. As in method II, the mixture was washed with 50 ml of water and 2-3 phenolphthalein drops were added. After washing the organic phase with water, the remaining procedures were conducted as in method II.

4.2.4.4. Method IV. In Method IV suggested by Rodas-Mendoza et al. (2003), 150 ml dichloromethane, 75 ml methanol and 2 g TBHQ were added to 25

ml milk samples and shaken at room temperature for 30 min. The saponified sample was extracted with 2x10 ml dichloromethane and 2x50 ml water and then the remaining procedures including adding 2-3 drops phenolphthalein were followed as in methods II and III.

4.2.5. Vitamins A and E determination by HPLC-DAD

Vitamin content determinations in milk were conducted in duplicates. Individual vitamins and their mixture were spiked into milk samples at concentrations of 1 mg/l for the retinol and β-carotene standards and the internal standard retinyl acetate, while 10 mg/l was used for the α-tocopherol standard and the α-tocotrienol internal standard. Milk sample peaks were identified by comparisons of retention time and wavelength at maximum absorbance for the standards and the internal standards. Six concentrations in duplicates were used to prepare calibration curves in the 0.025-1 mg/l range for vitamin A (retinol, retinyl acetate and β-carotene), and 0.25-10 mg/l for vitamin E (α-tocopherol and α-tocotrienol) yielding the linearity parameters shown in Table 4.3.

Based on 6 replications of blank sample injections, limits of detection (LOD) and quantification (LOQ) were estimated as 3 and 10 times, respectively, the value of the standard deviation of the peak height at the retention time for retinol, α-tocopherol, α-tocotrienol and β-carotene (Vial and Jardy 1999). Reproducibility was determined by analyzing six replications (n=6) of each of the four standards (retinol, α-tocotrienol, α-tocopherol, β-carotene). The precision was

estimated as the RSD (%) value of the injection of multiple extractions of the same milk sample (UHT whole milk, n=6). Finally, recovery studies were performed by spiking milk samples and solvents used to dilute standards with known amounts of each vitamin. The recovery response was calculated as the vitamin content difference between spiked and non-spiked samples (Table 4.4).

4.2.6. Degradation of retinol, α-tocopherol, and β-carotene during consumer storage

The effect of consumer milk storage at 4ºC on the retention of retinol, α-tocopherol, and β-carotene was studied for brand A (raw conventional whole milk) and brand B (raw whole, pasteurized whole, and pasteurized semi-skim organic milk). The test conditions considered in this study were based on the assumption that consumers store open milk bottles under refrigeration for up to 2 weeks. In the first study, a bottle of each milk type was opened and samples taken from the same container after 0, 2, 5, 8, and 14 d were kept protected from light until analyzed. In the second study, the losses of retinol, α-tocopherol, and β-carotene in UHT whole milk (brand A) was studied i) by sampling at 0, 3, 10, 20, 30 d always from the same bottle, as above, but with 0.02% sodium azide (NaN_3) when opening it to control microbial growth; and, ii) by sampling from a new milk bottle at and of 0, 4, 11, 21, 31 d to simulate refrigerated storage of milk in closed containers.

4.2.7. Statistical analysis

Statistical analysis was performed with Minitab Statistical Software (Minitab Inc., State College PA). A one-way ANOVA was performed using Tukey post hoc honestly significant difference (HSD) for differences between mean liposoluble vitamin contents (α = 0.05) considering the same thermal treatment (raw, pasteurized and UHT milk) using fat content, packaging, and brand as factors.

4.3. Results and discussion

4.3.1. Development of the HPLC analysis protocol

The optimization of the chromatographic conditions for the determination of vitamins A (retinol, β-carotene) and E (α-tocopherol) was performed using the analytical column Luna C_{18}. The first mobile phase tested was methanol:water (94:6, v/v) using 20 and 100µL volume loops with the latter improving the resolution of the β-carotene peak. The chromatographic separation was generally good but resolution of the α-tocotrienol peak was particularly incomplete and the α-tocopherol retention time was excessively short. Using the larger loop size, methanol was tested to improve the chromatographic separation to quantify β-carotene. Sensitivity improved but the retention times were excessively long suggesting that methanol may be too polar for the elution of β-carotene (Nelis and De Leenheer 1983). Although a methanol mobile phase has been used by other authors to quantify retinol and α-tocopherol (Arnaud and others 1991), it did not improve the separation of the vitamers in this work. Similar results were observed

for a 90:10 (v/v) MeOH:ACN mobile phase. A ternary ACN:MeOH:H_2O (91:8:1, v/v/v) mobile phase as reported by Gruszka and Kruk (2007) produced variable retention times and incomplete peaks resolution for retinol, retinyl acetate and β-carotene. It was modified to a binary ACN:MeOH mobile phase and tested at small methanol increments but this did not improve the peak shape and chromatographic separation. Peak separation for all the vitamers in this study was achieved (Figure 4.1) when using a higher methanol concentration (i.e., ACN:MeOH at 65:35, v/v) and a higher column temperature (35 ºC) as in the work by Lee et al. (2003) but using in this study only one C_{18} column instead of two, and by increasing the injection volume to 100 μL to compensate for the lower sensitivity of UV detectors such as the one used in this study. The retention times obtained at a flow rate of 1 ml/min were 3.5, 9.6 and 26 min for retinol, α-tocopherol and β-carotene, respectively, while the internal standards retinyl acetate and α-tocotrienol eluted at 4.3 min and 5.4 min, respectively. In milk samples, retinol and β-carotene appeared to co-elute with retinyl acetate even though this was not observed when injecting solutions of these compounds as suggested by the high value for β-carotene in method II (Table 4.4). Consequently, spiking of milk with retinyl acetate was not used when analyzing commercial milk samples even though other authors have used it as an internal standard (Escrivá and others 2002; Chauveau-Duriot and others 2010; Gentili and others 2013).

4.3.2. Development of the extraction protocol

Samples without (method I), or with saponification to facilitate vitamin

separation from milk fat (methods II-IV), were extracted with *n*-hexane, a most commonly used solvent (Bognar 1986; Albalá-Hurtado and others 1997), or dichloromethane (Table 4.4). The latter, used in a novel, fast and simple milk analysis procedure developed by Stefanov et al. (2010) was evaluated in this study because its stronger polarity may dissolve milk compounds better than the frequently-used diethyl ether reported to yield higher recoveries (89.9±1.4%) than n-hexane (14.1±0.7%) when quantifying *α*-tocopherol (Stancher and Zonta 1983; Escrivá and others 2002). However, *n*–hexane yielded higher recoveries (83-103%, method III) than dichloromethane (44-61%, method IV). Three saponification methods were tested in this study. In method II, the saponification time was excessive and caused large vitamin losses whereas in method IV precipitation interfered with the extraction of the saponified solution. However, method III adapted from FAO (1997) and tested with samples stirred for 15 min in a 40°C water bath, yielded high recoveries. During the saponification and evaporation steps, AA and TBHQ were used as antioxidants. According to Lee and others (2003), good antioxidants allow separation of individual compounds as single peaks without interferences. TBHQ used in method III (Table 4.4) allowed separating α-tocopherol and α-tocotrienol in standards and milk extracts (Figure 4.2). In summary, method III was chosen to determine vitamins A and E in milk in this work because it resulted in high recoveries with low standard deviations while methods I, II and IV showed lower efficiency and highly variable standard deviations (Table 4.4).

4.3.3. Performance of the extraction and quantification method selected

The reproducibility of the protocol method III yielding high recoveries of 86, 103, 83, and 99% for retinol, β-carotene, α-tocotrienol, and α-tocopherol, respectively (Table 4.4), was determined by analyzing six replicates (n=6) of three standard solutions (0.05, 0.25, 1 mg/l for retinol and β-carotene and 0.5, 2.5, and 10 mg/l for α-tocotrienol and α-tocopherol). The relative percentage standard deviation (% RSD) showed variability under 2% for retinol, α-tocotrienol and α-tocopherol, and about 2% for β-carotene. The precision was estimated as the RSD value of the injection of six extractions of the same milk sample (n=6) yielding values of 3, 2, and 5% for retinol, α-tocopherol and β-carotene, respectively, in UHT whole milk. However, α-tocotrienol could not be quantified because the LOQ in this study was higher (0.1 mg/l) than the reported concentration in raw whole milk (0.0176 mg/L, Kaushik and others 2001).

The quantification linearity (R^2) for the HPLC method here reported was about 0.999, with a lowest value of 0.975 for the simultaneous quantification of vitamins A and E. LOD values were 6, 53, 27 and 4 μg/l for retinol, α-tocotrienol, α-tocopherol and β-carotene, respectively, while the corresponding LOQ values were 10, 100, 100 and 10 μg/l. These values are better than those in previous reports. For example, Karpinska et al. (2006) reported for retinol and α-tocopherol LOD values of 3.83 and 1.81 mg/l, respectively, and corresponding LOQ values of 12.7 and 6.37 mg/l. Albalá-Hurtado et al. (1997) reported for infant milk formulae LOD/LOQ limits also higher than those found in this study, i.e., 0.01/0.02 mg/l for vitamin A (retinol) and 0.30/0.40 mg/l for vitamin E (α-tocopherol). Higher values

were also found by Lee et al. (2003) for retinol, α-tocopherol and β-carotene.

4.3.4. Analysis of commercial samples

Fat content of the samples included in this study ranged 3.5-3.92%, 1.5-1.6% and 0.24-0.30% for the whole, semi-skim and skim commercial milk samples (Table 4.1), respectively. Alfa-tocotrienol was not detected in all milk samples because of the lower LOD values of this study as compared to previous reports (Karpińska and others 2006). Samples of brand A (lots I, II, and III), B, C, D, and E were compared (α=0.05) as the difference between mean values for each vitamer in raw, pasteurized and UHT milk using fat content as factor from the same processed milk in different brands.

The vitamin A and E content in raw and pasteurized conventional (brand A) and organic milk (brand B), and UHT samples of conventional milk (brands A, C, D, and E) samples measured in this study (Table 4.5) were compared with published values (Table 4.6). These comparisons required conversion of published values from w/w to w/v units using reported density values for milk (Chandan 2007).

Retinol: The retinol levels in conventional raw whole milk brand A lots I to III, ranging from 0.730 to 0.895 mg/l, were higher than the minimum of the corresponding range 0.269-0.362 mg/l reported by Paul and Southgate (1985) for whole milk (3.8% fat) and also higher than the reported average vitamin A content in milk of 0.413 mg/l (range 0.103-1.033 mg/l) reported by Renner et al. (1989).

For raw milk with similar fat content (3.92%), the retinol level in raw whole milk brand A lot II (0.73 mg/l) was higher than the value reported by Ollilainen and other (1989). Whereas organic raw whole milk brand B lots I and II showed retinol content of 1.020±0.27 and 1.075±0 mg/l, respectively were lower than the corresponding concentrations reported by Ellis et al. (2007) in raw organic milk (14.575±2.60 mg/l) and even in raw conventional milk (16.785±3.74 mg/l). No significant differences ($p>0.05$) were observed for raw whole milk brands A and B from different lots. The retinol content of raw organic versus conventional whole milk was reported by Bergamo et al. (2003). Retinol was 6% lower in conventional milk, whereas in this study it was almost 30% lower. Pasteurized milk brand A showed 1.065±0.01, 5.540±0.27 and 4.140±0.23 mg/l for whole, semi-skim, and skim milk, respectively, i.e., higher than the corresponding values of 0.537 mg/l, 0.258 mg/l, and traces, reported by Early (1998). Pasteurized semi-skim and skim milk of brand A yielded significantly higher ($p<0.05$) retinol content than whole milk reflecting the vitamin A and D fortification declared on the container label. Organic pasteurized whole milk brand B lots I and II showed retinol contents of 0.930±0.11 and 0.880±0 mg/l, with non-significant statistic differences ($p>0.05$) among lots, but with statistic significant difference ($p<0.05$) when milk fat was removed for thermal milk treatment for semi-skim brand B lot II which contained only 0.450 mg/l retinol. Results for UHT commercial milk samples brands A (lot III), C, D and E showed that milk fat content affect the retinol content with statistically significant differences ($p<0.05$) for each brand (Table 4.5). The retinol content range in whole, semi-skim, skim UHT milk brands C, D, and E was 0.745-

0.820, 0.230-0.290, and 0.055-0.085 mg/l. Ollilainen et al. (1989) reported an average content of all-trans retinols of 0.168, 0.337, 0.539 mg/l in 1.9%, 3.9% fat cow's milk and human milk, respectively (Table 4.6).

α-tocopherol: The α-tocopherol level in conventional raw whole milk brand A lots I-III varied from 0.590 to 0.730 mg/l, while organic raw whole milk brand B yielded values of 1.105±0.01 (lot I) and 1.125±0 mg/l (lot II) lower than the corresponding concentrations reported by Ellis et al. (2007) in raw conventional milk (44.665±9.85 mg/l) and raw organic milk (42.372±9.85 mg/l). No significant differences ($p<0.05$) in α-tocopherol were detected for raw whole milk samples of the same brand except for brand A lot I. An about 47% higher α-tocopherol content was also found in organic as compared to conventional milk whereas Ellis et al. (2007) reported that α-tocopherol was 5% higher in conventional than in organic milk. Pasteurized milk brand A yielded values of 0.700±0.03, 0.815±0.01 and 0.400±0.01 mg/l for whole, semi-skim, and skim milk, respectively and the statistic showed significant effect ($p<0.05$) of fat content. Organic pasteurized semi-skimmed and whole milk brand B showed that *α*-tocopherol content varied from 0.445 to 0.930 mg/l, with statistically significant differences ($p<0.05$) among lots and milk with different fat level. The α-tocopherol content range in whole, semi-skim, skim UHT milk brands C, D, and E was 0.135-0.620 mg/l. Kaushik and others (2001) reported a 0.453±2.2 mg/l α-tocopherol concentration in raw whole milk (3% fat) that was higher than in reduced (2% fat, 0.273±3.9 mg/l), low (1% fat, 0.147±1.7 mg/l) and non-fat milk (0.5% fat, 0.047±0.5 mg/l). The level of α-tocopherol in UHT commercial milk samples

brand A (lot III), C, D and E showed a significant effect ($p<0.05$) of fat content for all brands except for brand D skim and semi-skim UHT milk ($p>0.05$) (Table 4.5).

β-carotene: Significant differences ($p<0.05$) were found among lots of raw whole milk samples with values ranging from 0.160 to 0.180 mg/l for brand A lots I-III which were higher than the minimum of the corresponding range 0.134-0.238 mg/l reported by Paul and Southgate (1985) for whole milk (3.8% fat) and also higher than the reported average β-carotene 0.207 mg/l (range 0.031-0.516 mg/l) by Renner et al. (1989). Organic raw whole milk brand B lots I and II yielded levels of 0.330±0.002 and 0.290±0.001 mg/l, respectively lower than the corresponding concentrations reported by Ellis et al. (2007) in raw organic milk (5.526±1.35 mg/l) and raw conventional milk (5.154±2.10 mg/l). A significant effect ($p<0.05$) of the milk fat content was observed for brands A and B pasteurized milk samples. Organic samples contained about 45% more β-carotene than conventional samples which was higher than reported by Ellis et al. (2007) (about 7%). The β-carotene of pasteurized milk brand A samples was 0.165±0.002, 0.100±0.200 and 0.040±0.0003 mg/l for whole, semi-skim, and skim milk, respectively. The β-carotene content for organic pasteurized milk brand B ranged from 0.125 to 0.270 mg/l lower than the concentration (5.681 mg/l) reported by Hulshof et al. (2006). UHT milk samples brand A (lot III), C, D and E showed a significant effect ($p<0.05$) of the milk fat content (Table 4.5). The liposoluble vitamin content range in whole, semi-skim, skim UHT milk brands C, D, and E was 0.125-0.140, 0.070-0.85, and 0.04-0.06 mg/l for β-carotene. Ollilainen et al. (1989) reported an average content of β-carotene for 0.099, 0.173, 0.031 mg/l in

1.9%, 3.9% fat cow's milk and in human milk, respectively (Table 4.6).

4.3.5. Degradation of retinol, α-tocopherol, β-carotene

The analysis of retinol, α-tocopherol, and β-carotene in milk samples differing fat content showed losses generally increasing during the 14 d storage time (Table 4.7). In most cases, short storage periods (up to 5 d) had no statistically significant effect ($p>0.05$) on the retinol, α-tocopherol, and β-carotene content. In most cases, storage for 5-14 d led to significant losses ($p<0.05$) for retinol and β-carotene, and similarly also for α-tocopherol after 8-14 d ($p<0.05$). Retinol, α-tocopherol, and β-carotene concentration at the end of storage time (14 d) remained higher in milk with higher fat content. After 14 d, loss of retinol was 18% (raw, brand A and B), 15% (pasteurized whole milk brand B) and 20% (pasteurized semi-skimmed milk brand B) whereas α-tocopherol loss was 27% (raw milk brand A), 23% (raw milk brand B), 15% (pasteurized whole milk brand B), and 9% (pasteurized semi-skimmed milk brand B). Finally, β-carotene showed losses of 11% (raw milk brand A), 19% (raw milk brand B), 20% (pasteurized whole milk brand B), and 10% (pasteurized semi-skimmed milk brand B).

The effect of storage on the loss of retinol, α-tocopherol, and β-carotene concentration in UHT whole milk is shown in Table 4.8. Retinol concentration decreased significantly ($p<0.05$) to 33% after 30 d in milk samples containing 0.02% NaN_3 (condition i), whereas when opening new containers (condition ii) at every sampling time, smaller (0-6%) but significant ($p<0.05$) losses were found

after 11 d and only 8% reduction after 31 d. In general, the α-tocopherol content determined for each storage time under conditions (i) and (ii) were statistically different ($p<0.05$) after 10 and 11 d, respectively, and became constant for longer storage period (20-31 d). Losses were about 11% and 13% for condition (i) and (ii), respectively. No changes in β-carotene were found under conditions (i) and (ii) during storage time of this study except for the latter showing a loss of only 9% after 31 d.

4.4. Conclusions

Four extraction methods to simultaneously determine by HPLC the concentrations of retinol, *α*-tocotrienol, *α*-tocopherol, and *β*-carotene in cow's milk were compared. Method III chosen in this work using small sample and solvent volumes yielded minimum losses of all target vitamins (86±2%, 103±0%, 83±1%, and 99±4% recovery values for retinol, α-tocotrienol, α-tocopherol, and β-carotene, respectively). When combined with HPLC/DAD it allowed the simultaneous determination of vitamers in several brands of raw, pasteurized conventional and organic milk and commercial UHT milk with different fat content ranging 0.055-5.540 (retinol), 0.135-1.410 (α-tocopherol), 0.040-0.850 (β-carotene) mg/l. Short storage periods at 4ºC in the dark (up to 5 d) had no statistically significant effect ($p>0.05$) on the retinol, α-tocopherol, and β-carotene content. After 14 d, the concentration of retinol, α-tocopherol, and β-carotene range was higher in milk with high fat content. The effect of storage on the loss of retinol, α-tocopherol, and β-carotene concentration in UHT whole milk, retinol concentration decreased

significantly ($p<0.05$) in milk samples containing 0.02% NaN_3 whereas unopened containers (condition ii) every sampling time, significant ($p<0.05$) losses were found after 11 d. The content of α-tocopherol determined for each time of storage for both condition i) and ii) became constant for longer storage period (20-31 d). No significant changes ($p>0.05$) in β-carotene were found in both cases for condition i) and ii) over the time period excepted for condition ii) at 31 d.

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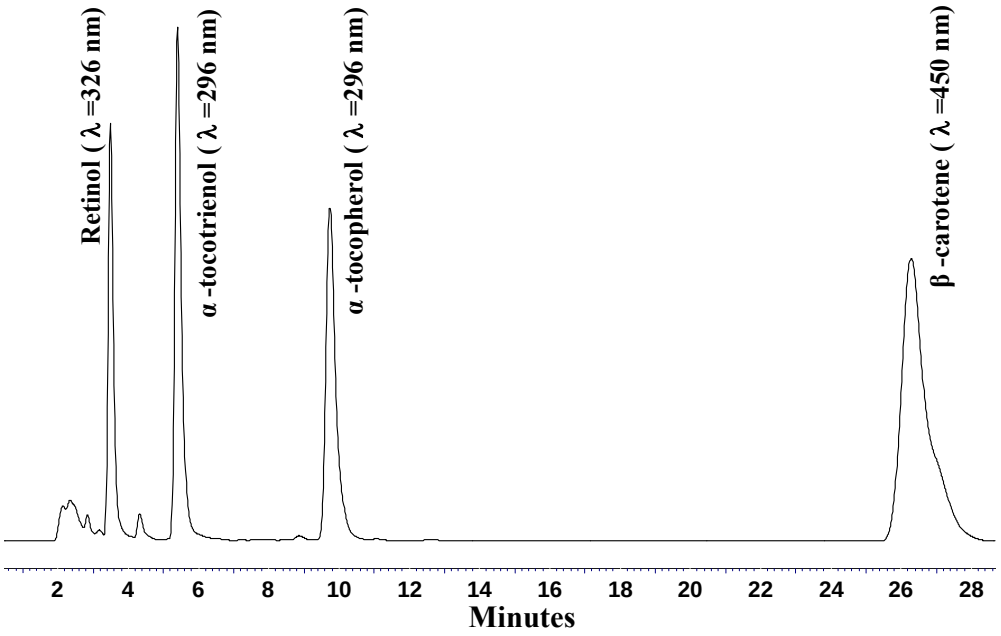


Figure 4.1. Chromatogram for a 1 mg L^{-1} and 10 mg L^{-1} standard solutions of vitamin A (retinol and β-carotene) and vitamin E (α-tocotrienol and α-tocopherol), respectively

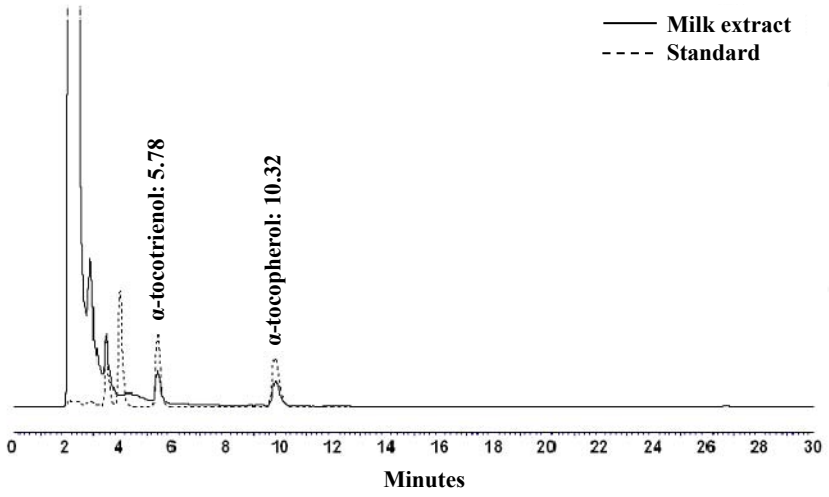


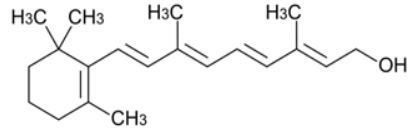
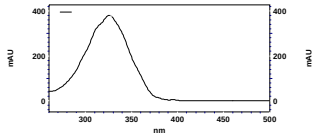
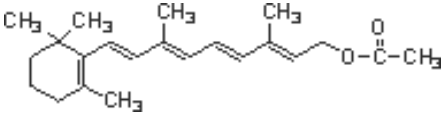
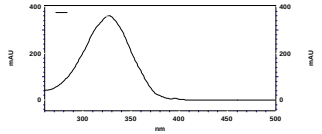
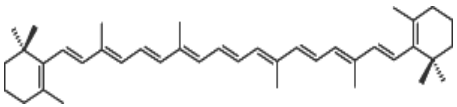
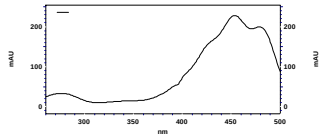
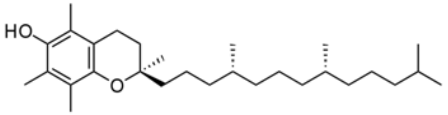
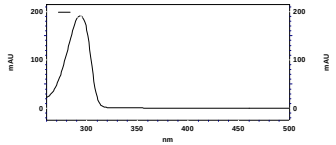
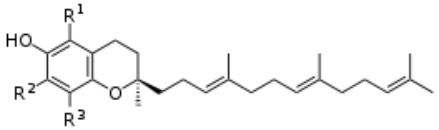
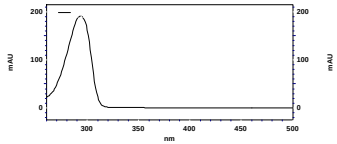
Figure 4.2. Chromatograms of 10 mg/ L standard solutions of vitamin E (α-tocopherol and α-tocotrienol) and milk samples with antioxidant TBHQ (2 mg/l) and spiked with 10 mg/l vitamin E

Table 4.1. Characteristics of commercial milk samples

Treatment	Brand	Type	Fat (g/100 ml)	Protein (g/100 ml)	Carbohydrate (g/100 ml)	pH
Raw	A	Raw (I)	3.77	3.20	4.65	6.74
	A	Raw (II)	3.92	3.22	4.65	6.73
	A	Raw (III)	3.62	3.24	4.72	n.a.
	B*	Raw (I)	3.2-3.6	n.a.	n.a.	n.a.
	B*	Raw (II)	3.2-3.6	n.a.	n.a.	n.a.
Pasteurized	A	Whole	3.51	3.22	4.74	n.a.
	A	Semi-skim	1.50	3.25	4.81	6.73
	A	Skim	0.24	3.31	4.82	6.74
	B*	Whole	3.2-3.6	n.a.	n.a.	n.a.
	B*	Semi-skim	1.2	n.a.	n.a.	n.a.
UHT	A	Whole	3.50	3.21	4.74	n.a.
	C	Whole	3.60	4.50	3.00	n.a.
	C	Semi-skim	1.50	3.00	4.60	n.a.
	C	Skim	0.30	3.10	4.70	n.a.
	D	Whole	3.60	3.00	4.60	n.a.
	D	Semi-skim	1.55	3.10	4.60	n.a.
	D	Skim	0.30	3.15	4.60	n.a.
	E	Whole	3.60	3.00	4.80	n.a.
	E	Semi-skim	1.60	3.00	4.80	n.a.
	E	Skim	0.30	3.00	4.80	n.a.

* denotes organic milk sample; n.a. = information not available

Table 4.2. Physical and chemical characteristics of the studied vitamins.

	Compound	Chemical structure	Formula	Absorbance [d] λ_{max} (nm)	Spectrum
Vitamin A	**Retinol**		$C_{20}H_{30}O$	326	
	Retinyl acetate		$C_{22}H_{32}O_2$	326	
	β-carotene		$C_{40}H_{56}$	450	
Vitamin E	**α-tocopherol**		$C_{29}H_{50}O_2$	296	
	α-tocotrienol		$C_{29}H_{44}O_2$	296	

[a]Molecular weight
[b]Melting point (ºC)
[c]Density at 20ºC (g ml^{-1})
[d]Maximum absorbance in ACN: MeOH (65:35)

Table 4.3. Effect of the mobile phase on the calibration curve based on the HPLC peak area for liposoluble vitamins A and E

Mobile phase	Compounds	Concentration (mg/l)	Calibration line	
			Slope[a]	r^2
MeOH:Water (94:6 v/v)	Retinol	0.025-2.5	9.3×10^5	0.998
	Retinyl acetate	0.10-5.0	6.4×10^5	0.998
	β-carotene	0.50-10.0	1.4×10	0.975
	α-tocopherol	1.0-20.0	8.3×10^3	0.999
MeOH (100%)	β-carotene	0.050-5.0	3.2×10^6	1.000
ACN:MeOH:Water (91:8:1 v/v)	α-tocopherol	0.25-15.0	4.0×10^4	0.999
	α-tocotrienol	0.25-15.0	2.3×10^4	0.999
ACN:MeOH (65:35 v/v)	Retinol	0.025-1.0	4.0×10^6	0.999
	Retinyl acetate	0.025-1.0	4.0×10^6	0.999
	β-carotene	0.025-1.0	1.0×10^7	0.999
	α-tocopherol	0.25-10.0	4.3×10^5	0.999
	α-tocotrienol	0.25-10.0	2.3×10^5	0.999

Table 4.4. Effect of the extraction method on the % recovery for vitamins A and E

Vitamin	Compound	Solvent extraction[a]		Saponification and solvent extraction[a]		
		Method I[b]	Method I[c]	Method II[d]	Method III	Method IV
A	Retinol	68 (23)	93 (12)	47 (13)	86 (2)	61 (35)
	β-carotene	12 (2)	10 (46)	168 (6)	103 (0)	25 (9)
E	α-tocotrienol	64 (3)	61 (4)	74 (2)	83 (1)	39 (11)
	α-tocopherol	62 (4)	70 (4)	80 (3)	99 (4)	44 (10)

[a]Values in brackets are CV (%)
[b]extracted without antioxidant
[c]extracted with antioxidant (TBHQ)
[d]spiked with retinyl acetate intended to be used as internal standard

Table 4.5. Liposoluble vitamin content (average ± standard deviation, mg/l) in commercial raw, pasteurized and UHT milk samples of the same brand and container type but differing in fat content

Vitamin	Brand	Raw whole[1,2]	Pasteurized[1,2]			UHT[3]		
			Whole	Semi-skim	Skim	Whole	Semi-skim	Skim
Retinol	A (I)	0.895 ± 0.07[1,a]	n.a.	5.540 ± 0.27[1,d]	n.a.	n.a.	n.a.	n.a.
	A (II)	0.730 ± 0[1,a]		n.a.	4.140 ± 0.23[1,c]	n.a.		n.a.
	A (III)	0.790 ± 0[1,a]	1.065 ± 0.01[1,a]		n.a.	1.260 ± 0[d]		n.a.
	*(I)	1.020± 0.27[2,a]	0.930 ± 0.11[2,a]	n.a.	n.a.	n.a.	n.a.	n.a.
	B*(II)	1.075 ± 0[2,a]	0.880 ± 0[2,a]	n.a.	0.450 ± 0.03[2,b]	n.a.	n.a.	n.a.
B	C	n.a.	n.a.	n.a.	n.a.	0.775 ± 0.12[c]	0.230 ± 0.06[b]	0.085 ± 0.01[a]
	D	n.a.	n.a.	n.a.	n.a.	0.820 ± 0.05[c]	0.260 ± 0.01[b]	0.055 ± 0[a]
	E	n.a.		n.a.	n.a.	0.745 ± 0.01[c]	0.290 ± 0.02[b]	0.055 ± 0.01[a]
α-tocopherol	A (I)	0.730 ± 0.03[1,b]		0.815 ± 0.01[1,c]	n.a.	n.a.	n.a.	n.a.
	A (II)	0.635 ± 0.01[1,a]		n.a.	0.400 ± 0.01[1,a]	n.a.	n.a.	n.a.
	A (III)	0.590 ± 0.02[1,a]	0.700 ± 0.03[1,b]		n.a.	0.685 ± 0.02[e]		n.a.
	B*(I)	1.105 ± 0.01[2,c]	0.930 ± 0.03[2,d]	n.a.	n.a.	n.a.	n.a.	n.a.
	B*(II)	1.125± 0[2,c]	0.825 ± 0.01[2,c]	n.a.	0.445 ± 0.01[2,a]	n.a.		n.a.
	C	n.a.	n.a.	n.a.	n.a.	1.410 ± 0.03[g]	0.305 ± 0[b]	0.620 ± 0[d]
D		n.a.	n.a.	n.a.	n.a.	0.600 ± 0[d]	0.295 ± 0.01[b]	0.300 ± 0.01[b]

Table 4.5. (Continued) Liposoluble vitamin content (average ± standard deviation, mg/l) in commercial raw, pasteurized and UHT milk samples of the same brand and container type but differing in fat content

Vitamin	Brand	Raw whole[1,2]	Pasteurized[1,2]			UHT[3]		
			Whole	Semi-skim	Skim	Whole	Semi-skim	Skim
α-tocopherol	E	n.a.	n.a.	n.a.	n.a.	0.825 ± 0.03^{f}	0.425 ± 0^{c}	0.135 ± 0.01^{a}
β-carotene	A (I)	$0.160 \pm 0^{1,a}$		$0.100 \pm 0.20^{1,b}$		n.a.	n.a.	n.a.
	A (II)	$0.170 \pm 0^{1,ab}$		n.a.	$0.040 \pm 0^{1,a}$	n.a.		n.a.
	A (III)	$0.180 \pm 0.01^{1,b}$	$0.165 \pm 0^{1,d}$		n.a.	0.160 ± 0^{e}		n.a.
	B^{*} (I)	$0.330 \pm 0^{2,d}$	$0.270 \pm 0^{2,f}$		n.a.	n.a.		n.a.
	B^{*} (II)	$0.290 \pm 0^{2,c}$	$0.225 \pm 0^{2,e}$	$0.125 \pm 0^{2,c}$		n.a.		n.a.
	C	n.a.	n.a.	n.a.	n.a.	0.125 ± 0^{d}	0.070 ± 0^{b}	0.040 ± 0^{a}
	D	n.a.	n.a.	n.a.	n.a.	0.140 ± 0.01^{d}	0.075 ± 0^{bc}	0.050 ± 0^{ab}
	E	n.a.	n.a.	n.a.		0.135 ± 0.01^{d}	0.850 ± 0^{c}	0.060 ± 0.01^{b}

[1] Transparent plastic bottle covered with aluminum foil
[2] Transparent glass bottle covered with aluminum foil
[3] Tetra Pak™ container
Different subscript letters indicate statistical differences among samples collected from the same processing plant, same brand. Finally, *denotes organic milk and n.a. = not available; n.d. = not detectable. I, II, and III indicate different lots of the same milk brand and type.

Table 4.6. Vitamins A and E concentrations in cow's milk reported in several works in the literature

Milk sample	Retinol	α-tocopherol	β-carotene	References
Raw conventional milk (brand A), mg/l	0.73-0.89	0.59-0.73	0.16-0.18	*
Raw organic milk (brand B), mg/l	1.02-1.08	1.11-1.12	0.29-1.02	*
Conventional milk, mg/l	16.785±3.74	44.665±9.85	5.154±2.10	Ellis et al. (2007)
Organic milk, mg/l	14.575±2.60	42.372±9.85	5.526±1.35	Ellis et al. (2007)
Bovine milk, mg/l	0.45	1.5	0.12	Plozza et al. (2012)
3.8% milk fat, mg/l	0.269-0.362	n.a	0.134-0.238	Paul and Southgate (1985)
3.9% milk fat, mg/l	0.337	n.a	0.173	Ollilainen et al. (1989)
1.9% milk fat, mg/l	0.168	n.a	0.099	Ollilainen et al. (1989)
Whole milk (3% fat), mg/l	n.a	0.453±2.2	n.a	Kaushik et al. (2001)
Reduced fat (2% fat), mg/l	n.a	0.273±3.9	n.a	Kaushik et al. (2001)
Low-fat (1% fat), mg/l	n.a	0.147±1.7	n.a	Kaushik et al. (2001)
Non-fat (0.5% fat), mg/l	n.a	0.047±0.5	n.a	Kaushik et al. (2001)
Pasteurized conventional whole milk (brand A), mg/l	1.07	0.07	0.16	*
Pasteurized conventional semi-skim milk (brand A), mg/l	5.54	0.82	0.1	*
Pasteurized conventional skim milk (brand A), mg/l	4.14	0.4	0.04	*
Pasteurized organic whole milk (brand B), mg/l	0.88-0.93	0.82-0.93	0.22-0.27	*
Pasteurized organic semi-skim milk (brand B), mg/l	0.45	0.44	0.12	*
Pasteurized whole milk, mg/l	0.537	n.a	n.a	Early (1998)
Pasteurized semi-skim milk, mg/l	0.258	n.a	n.a	Early (1998)
Pasteurized skim milk, mg/l	Trace	n.a	n.a	Early (1998)
UHT whole milk (brand C – E), mg/l	0.74-0.82	0.6-1.41	0.06-0.08	*
UHT semi-skim milk (brand C – E), mg/l	0.23-0.29	0.3-0.43	0.03-0.62	*
UHT skim milk (brand C – E), mg/l	0.06-0.08	0.07-0.09	0.04-0.06	*

* Values reported in this work. α-tocotrienol was not detected and was not reported in the references here listed

Table 4.7. Effect of storage at 4°C in the dark on the retinol, α-tocopherol, β-carotene content (n = 3, average ± standard deviation, mg/l) in milk with different fat content

		Time, d					
		0	**2**	**5**	**8**	**14**	**Final % Loss**
Retinol	*Brand A*[1]						
	Raw whole milk	0.835 ± 0.005^{c}	0.825 ± 0.005^{c}	0.809 ± 0.005^{c}	0.767 ± 0.004^{b}	0.685 ± 0.003^{a}	18%
	Brand B[*,2]						
	Raw whole milk	1.075 ± 0.001^{b}	1.071 ±0.022^{b}	0.929 ± 0.005^{a}	0.903 ± 0.009^{a}	0.886 ±0.006^{a}	
	Pasteurized whole milk	0.878 ± 0.001^{e}	0.851 ± 0.003^{d}	0.833 ± 0.001^{c}	0.814 ± 0.002^{b}	0.744 ± 0^{a}	
	Pasteurized semi-skim milk	0.452 ± 0.025^{b}	0.426 ± 0^{b}	0.362 ± 0.001^{a}	0.366 ± 0.018^{a}	0.363 ± 0.008^{a}	
α-tocopherol	*Brand A*[1]						18%
	Raw whole milk	0.715 ± 0.003^{c}	0.671 ± 0.006^{b}	0.653 ± 0.003^{b}	0.650 ± 0.008^{b}	0.521 ± 0.014^{a}	15%
	Brand B[*,2]						20%
	Raw whole milk	1.122 ± 0.005^{b}	1.124 ± 0.034^{b}	0.944 ± 0.006^{a}	0.888 ± 0.084 ^{a}	0.866 ± 0.002^{a}	
	Pasteurized whole milk	0.824 ± 0.010^{b}	0.786 ± 0.049^{b}	0.787 ± 0.016^{b}	0.787 ± 0.016^{b}	0.697 ± 0.004^{a}	27%
	Pasteurized semi-skim milk	0.445 ± 0.010^{b}	0.448 ± 0.007^{b}	0.448 ± 0.015^{b}	0.443 ± 0.006^{b}	0.404 ± 0.004^{a}	
β-carotene	*Brand A*[1]						23%
	Raw whole milk	0.179 ± 0.001^{b}	0.174± 0.001^{b}	0.174 ± 0.001 ^{b}	0.173 ± 0.003^{b}	0.159 ± 0.001^{a}	15%
	Brand B[*,2]						9%
	Raw whole milk	0.293 ± 0.001^{d}	0.279 ± 0.001^{c}	0.247 ± 0.003^{ab}	0.252 ± 0.005^{b}	0.239 ± 0.005^{a}	
	Pasteurized whole milk	0.225 ± 0.003^{b}	0.225 ± 0.020^{b}	0.203 ± 0.001^{ab}	0.198 ± 0.002^{ab}	0.179 ± 0.003^{a}	
	Pasteurized semi-skim milk	0.125 ± 0.002^{b}	0.124 ± 0.004^{b}	0.121 ± 0.001^{ab}	0.119 ± 0.003^{ab}	0.113 ± 0^{a}	
							19%
							20%
							10%

† Container type: [1] transparent plastic bottle covered with aluminum foil before analysis, [2] transparent glass bottle covered with aluminum foil before analysis. Average values with same superscript in a row are not significantly different (p> 0.05). Finally, * denotes organic milk

Table 4.8. Effect of storage at 4°C in the dark on the retinol, α-tocopherol, β-carotene content (n = 3, average ± standard deviation, mg/l) in UHT whole milk in Tetra Pak™ containers†

	Time, d					
	0	**3**	**10**	**20**	**30**	**Final % Loss**
Same container with 0.02% sodium azide [1]						
Retinol	0.80 ± 0.001^{e}	0.766 ± 0.008^{d}	0.671 ± 0.009^{c}	0.586 ± 0.002^{b}	0.534 ± 0.004^{a}	33%
α-tocopherol	0.481 ± 0.001^{c}	0.459 ± 0.008^{b}	0.451 ± 0.002^{b}	0.434 ± 0.004^{a}	0.428 ± 0.006^{a}	11%
β-carotene	0.124 ± 0.005^{a}	0.129 ± 0^{a}	0.128 ± 0.001^{a}	0.127 ± 0.001^{a}	0.122 ± 0.05^{a}	2%
	0	**4**	**11**	**21**	**31**	**Final % Loss**
- New bottle [2]						
Retinol	0.80 ± 0.001^{d}	0.767 ± 0.014^{c}	0.755 ± 0.002^{bc}	0.747 ± 0.001^{ab}	0.737 ± 0.001^{a}	8%
α-tocopherol	0.482 ± 0.001^{b}	0.463 ± 0.001^{b}	0.46 ± 0.011^{b}	0.423 ± 0.011^{a}	0.417 ± 0.010^{a}	13%
β-carotene	0.124 ± 0.005^{b}	0.130 ± 0^{b}	0.127 ± 0.002^{b}	0.130 ± 0.003^{b}	0.113 ± 0.001^{a}	9%

† Testing conditions included 1: 0.02% sodium azide (NaN3) added once with milk analyzed from the same container, 2: milk analyzed from a new container for every sampling time. Average values with the same superscript in a row are not significantly different ($p > 0.05$)

CHAPTER 5

Vitamins A, C and E retention in milk subjected to high pressure treatments at moderate temperatures

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ABSTRACT

Heat treatments can lead to undesirable biochemical and nutritional changes in milk. On the other hand, high pressure processing (HPP) could improve the retention of nutrients and non-nutrient phytochemicals. Additives could improve the microbial inactivation effect of HPP but are generally not allowed in milk. This study on pressure treatments (200-705 MPa) at moderate temperatures (30-75º) focuses on the effect of elevated pressure at moderate temperatures on the retention of ascorbic acid (AA), vitamin A (retinol, β-carotene), and vitamin E (α-tocopherol) in raw whole and pasteurized skim milk. This study showed that retinol, α-tocopherol and β-carotene were not greatly influenced by these treatments. However, even the least severe treatment (400 MPa/31°C/5 min) resulted in a statistically significant AA loss ($p<0.05$) in raw whole (20%) and pasteurized skim milk (13%) when compared with untreated controls. AA losses reached a maximum of 55 and 68% in raw whole and pasteurized skim milk, respectively, when treated for 5 min at 705 MPa and 72ºC. The results suggest the need for future studies to identify conditions optimizing AA retention.

KEYWORDS: ascorbic acid, retinol, β-carotene, α-tocopherol, high pressure/temperature treatments, HPLC, milk

5.1. Introduction

Conventional thermal pasteurization treatments can lead to undesirable biochemical and nutritional changes in processed products (e.g., color changes, losses of amour, aroma, and vitamins) affecting product quality, consumer appeal and even food safety (Choi and Nielsen 2005; Eisenbrand and others 2007; Suárez-Jacobo and others 2011). Pressure processing technologies are being investigated as an alternative preservation technique to achieve pasteurization and sterilization at lower process temperatures and/or shorter treatment times than thermal processing (Norton and Sun 2008; Mújica-Paz and others 2011; Verbeyst and others 2012; Verbeyst and others 2013). High pressure processing (HPP) is an innovative emerging technology with the potential to improve the retention of nutrients, non-nutrient phytochemicals, sensory attributes, perceived freshness and the safety of "fresh" or "just prepared" foods (Houska and others 2006; McInerney and others 2007; Cruz and others 2011; Picon and others 2013). HPP at ambient and refrigerated temperatures allows an improved retention of nutrients and delivers the sensory attributes of (Cano and De Ancos 2005). During HPP treatments, small molecules such as volatile compounds, pigments, amino acids, and vitamins are mostly retained as pressure alone is unable to break covalent bonds (Serment-Moreno and others 2014). Pasteurization and sterilization by combined high pressure and elevated temperature treatments is required to inactivate baroresistant enzymes and bacterial spores (Farr 1990). Pressure treatment in combination with heat is commonly referred to as pressure-assisted thermal processing (PATP) while pressure-assisted thermal sterilization (PATS) or high pressure

temperature sterilization (HPHT) should be used only if the commercial sterilization of the food is achieved (Nguyen and others 2010; Valdez-Fragoso and others 2011).

Milk is heat-processed to ensure microbial safety and quality preservation. However, heating can adversely affect nutritional quality, a critical factor when the milk is consumed by infants. In this market sector, manufacturers must ensure that milk deterioration is reduced to a minimum, since infant nutrition may rely wholly or largely on this single food. When treating raw whole milk at 600 MPa, Dogan and Erkmen (2004) reported for *Listeria monocytogenes* a 2.4 min *D* value with a 578 MPa pressure resistance value (z_p). These authors compared this value with smaller values observed for the same pathogen in juices, and concluded that fat and protein content in whole milk appear to increase its baroresistance. *L. monocytogenes* inactivation and injury levels after combined high pressure and temperature treatments were determined by quantifying survivors on nonselective and selective media, respectively (Mishra and others 2013). At 400 MPa, the injured decimal reduction values observed after 15 min at 27 and 43°C were 2.10 and 3.46, respectively, whereas at 60ºC, 4.58 decimal reductions were reached in 1 min. These authors concluded that 6 decimal reductions of this pathogen should be possible in less than 2 min at 400 MPa and 60ºC. Sequential mild-heating and moderate pressure treatments, i.e., 37 °C for 240 min or 50 °C for 10 min followed by 5 min at 550 MPa and 25°C has been proposed to inactivate *L. monocytogenes* in milk (Koseki and others 2008). Guan et al. (2005) reported that pressures of 500, 550, and 600 MPa at ambient temperature achieved ~4.5 to 5.1 decimal reductions of *Salmonella*

typhimurium DT 104 within 10 min. Combining HP and nisin can reach greater inactivation levels of *Listeria innocua* in milk than either one applied individually. For example, 8 decimal reductions were achieved in 5 min at 500 MPa and 20ºC when 500 IU/ml nisin were added (Black and others 2005). However, identity standards in the U.S. limit the use of additives in milk while European regulations limit also their use in infant formula (Anonymous 2002; Anonymous 2006). *Bacillus cereus*, an ubiquitous spore-forming bacterial foodborne pathogen with *D* values at 100ºC of 2.2–5.4 min, can survive milk pasteurization representing a safety risk since vegetative cells strains can grow down to 4ºC (van Opstal and others 2004). Furthermore, mild heat treatment might activate bacterial spores. Van Opstal et al. (2004) showed that 6 decimal reductions in spore counts were possible by a single step treatment at 500 MPa and 60ºC for 30 min, or by a two-step treatment consisting of 30 min at 200 MPa and 45ºC inducing germination followed by 10 min at 60ºC to inactivate germinated spores. Commercial implementation of these promising combined pressure and temperature treatments requires information on their effect on the flavor and nutrient content of milk (Vazquez and others 2007). However, only a few studies have focused on the effects on chemical changes at high temperature and pressure levels treatments of milk (Ramirez and others 2009; Martínez-Monteagudo and others 2012). Therefore, the objective of this study was to determine the effect of elevated pressure (100-700 MPa), moderate temperature (30-75°C) and time (0-10 min) on the retention of ascorbic acid (AA), vitamin A (retinol, β-carotene), and vitamin E (α-tocopherol) in raw whole and pasteurized skim milk.

5.2. Materials and methods

5.2.1. Chemicals and reagents

Reagents were all of analytical grade including the AA standard (Grupo Productos Aditivos, Madrid, Spain); retinol, α-tocopherol and β-carotene (Sigma-Aldrich, Madrid, Spain); DL-dithiothreitol (DTT) (Fluka Analytical, Madrid, Spain); tert-butylhydroquinone (TBHQ, 97%), n-hexane and acetonitrile (Chromasolv®), glacial acetic acid and *meta*-phosphoric acid (Panreac, Barcelona, Spain); HPLC grade water (Sigma-Aldrich, Madrid, Spain); and, absolute HPLC-grade ethanol, potassium hydroxide (85%), and anhydrous sodium sulfate (Panreac, Vigo, Spain).

5.2.2. Milk samples

Raw whole and pasteurized skim milk from three lots were provided by the same commercial processing plant in 2L plastic containers filled with no headspace. The milk was then poured into 50 ml plastic containers and covered with aluminum foil before pressure treatments no later than 24 h after sample collection.

5.2.3. Pressure treatments

Pressure treatments were carried out with an experimental system (Model S-FL-850-9-W HP food processor, Stansted Fluid Power Ltd., Harlow, UK) including hydraulic pressure intensifier, pressure vessel, and control panel. The pressure vessel (3.7 cm inner diameter, 30 cm height, approximately 120 ml volume) is designed to

withstand pressures up to 900 MPa in the temperature range from -20 to 80ºC.

Milk samples (35 ml) collected in 50 ml plastic containers were vacuum-sealed into small polyethylene bags (9 x 6 cm) and kept protected from light at 4°C. Six sealed bag samples of raw whole and pasteurized skim milk (3 each) were placed as pairs at three vessel positions (top, middle, bottom) which was then filled with pressurizing medium (water:monoethylene glycol, 70:30). The treatment time reported in this study does not include the time for pressure increase and release. The pressurizing fluid temperature for all treatment runs was recorded every 15 s. After pressure treatment, the sealed bag samples were removed from the vessel and immediately cooled at 4°C before vitamin analysis performed within 2 h for AA and 24 h for retinol, α-tocopherol and β-carotene.

5.2.4. Experimental design

The degradation of AA, retinol, α-tocopherol and β-carotene was studied at 5 pressure (95-705 MPa), 3 temperature (31-73°C), and 5 pressure-holding times (0.43-9.58 min) yielding a total of 15 experimental runs. Experimental runs (R1-R15) were arranged from lowest to highest temperature. Due to the time required to adjust and stabilize the equipment temperature, the implementation of the experimental design required 3 d and thus three untreated control samples were collected (U_{1-3}, Table 5.1). Conditions R1-R3, R4-R5, and R6-R15 were tested on day 1, 2 and 3, respectively, and corresponded to untreated milk U_{1-3}. Finally, only raw whole milk was chosen to study pressure treatment effects on retinol, α-tocopherol and β-carotene retention to

have detectable initial levels of these fat-soluble vitamins without the need to work with vitamin-enriched samples.

5.2.5. Vitamin Analysis

The vitamin quantification procedures by high-performance liquid chromatography (HPLC) with photo diode array (PDA) detection were described in recently published reports (Chotyakul and others 2014a; Chotyakul and others 2014b). Briefly, the HPLC unit (Thermo Fisher Scientific, Waltham, MA, USA) consisted of a Spectra System SCM1000 vacuum degasser, a Spectra System P4000 pump, a Spectra System AS3000 autosampler, and a Surveyor PDA^{plus} detector connected to a PC computer running ChromQuest™ 5.0. An isocratic mobile phase (98% 0.1M Acetic acid + 2% CAN, 0.5 ml/min for 20 min), a 150 mm × 4.6 mm 5 µm particle Luna C_{18} analytical column (Phenomenex, Madrid, Spain) with a 4 mm × 2 mm guard column with the same packing material were used for AA quantification. The same column was used for retinol, α-tocopherol and β-carotene analysis also with an isocratic mobile phase (ACN:MeOH, 65:35 v/v, 1 ml/min for 30 min). In both analyses, the HPLC column temperature was 35 ºC. Sample injection volume was 20 µl for AA and 100 µl for the vitamins A and E. The PDA detector wavelengths used were 245, 296, 326, and 450 nm for AA, α-tocopherol, retinol, and β-carotene, respectively. Procedures for the preparation of vitamin standards and extraction of these vitamins from milk were conducted also as previously reported (Chotyakul and others 2014a; Chotyakul and others 2014b). Determinations were conducted in triplicate for AA and

in duplicate for retinol, α-tocopherol and β-carotene, since the latter group showed a significantly lower variability among replicated measurements (data not shown).

5.2.6. Statistical analysis

Statistical analysis was performed using Minitab Statistical Software (Minitab Inc., State College PA). A one-way ANOVA was performed and followed by Tukey post hoc honestly significant difference (HSD) for differences between mean vitamin content ($\alpha = 0.05$).

5.3. Results and discussion

The pressurizing medium temperature values during the pressure processing of milk samples are reported in Table 5.1. For example, the experimental pressurizing medium temperature for runs R3 (200MPa/46°C/8 min) and R10 (400MPa/75°C/5 min) treatments were 42 and 70°C instead of the experimental design values of 46 and 75ºC, respectively (Figure 5.1). Sample position in the pressure vessel (top, middle, bottom) showed non-significant effects on vitamin losses ($p>0.05$) reflecting isostatic pressure conditions (Rivalain and others 2010) and vessel location temperature differences insufficient to have a detectable (data not shown). Therefore, vitamin loss values could be summarized as averages for samples at the three vessel locations. The vitamin content of pressure-treated milk samples was compared to the corresponding untreated milk control ($U_{1\text{-}3}$, Table 5.2) used during sequential experimental days at increasingly higher temperatures and were reported as vitamin loss values (Figures

5.2-5.3).

5.3.1. Analysis of the variability of the pressure treatment effect on vitamin retention

Some variability in determinations of vitamin losses between experimental runs at the same pressure, temperature and holding time is to be expected. However, if the variability is excessively high it would be difficult to select an optimum processing condition for commercial implementation. In this study, six replicated runs of the same pressure treatment condition (400MPa/75°C/5min, R8-R13) were used to determine the variability of AA losses in raw whole and pasteurized skim milk (Figure 5.2A), and of the fat soluble vitamers retinol, α-tocopherol and β-carotene losses in raw whole milk (Figure 5.2B). No significant differences (p>0.05) were observed between these six runs in the losses of AA in raw whole milk and pasteurized skim milk (Figure 5.2A) and for the four fat soluble vitamers in raw whole milk (Figure 5.2B).

5.3.2. Effect of pressure treatments on ascorbic acid (AA) retention

AA losses for treatments at different pressure but at the same temperature showed non-significant differences (p>0.05). Figure 5.3A shows that the heat-sensitive AA at even the lowest temperature tested, sample R1 (400MPa/31°C/5 min), showed a statistically significant decrease (p<0.05) in raw whole (20%) and pasteurized skim milk (13%) when compared with untreated control U_1. This is consistent with work by Oey et al. (2006), who reported that AA stability during pressure processing is

sensitive to temperature and also to oxygen and light. In this study, the effect of the latter was minimized by protecting samples using aluminum foil. Oey et al. (2006) reported that AA oxidation was accelerated during pressure build-up even at relatively low-pressure levels. The treatments at intermediate temperatures combined with high pressure as in runs R2-5 showed AA losses reached values up to 39 and 22% in raw whole and pasteurized skim milk, respectively (Figure 5.3A). For example, raw whole milk treated at 200MPa/46°C for 2 (R2) and 8 min (R3) reached 35 and 39% AA loss, respectively. Pressure treatments at higher temperatures (R6-R15) showed even higher losses reaching for experimental run R13 losses of 55 and 68% in raw whole and pasteurized skim milk, respectively. AA losses of 10-25% and above 25% were reported by Miller et al. (1999) when subjecting milk to commercial thermal pasteurization (72°C/15 s) and sterilization (138 °C/at least 2 s), respectively. Some noticeable decreases of initial AA content in apple puree were found around 86% and 43% losses for 600 MPa and 400 MPa at 20 °C for 5 min, respectively (Landl and others 2010). In tomato puree, AA loss was 46% after thermal treatment (70°C, 2min) but only 6.3% after pressure treatment (600 MPa/20 °C/15 min) (Patras and others 2009b). A similar AA loss (6%) was observed in blackberry puree after the same pressure treatment (600 MPa/20 °C/15 min) (Patras and others 2009a). In this study (Table 5.1), temperature had more effect on AA losses than pressurizing time and pressure. Although activation values could not be obtained in this study, Baéz et al. (2012) reported a 52.0 kJ/mol activation energy value for AA losses during storage of enteral formula, a value lower than the 56-106 kJ/mol determined by Burdurlu et al.

(2006) in a juice concentrates storage study, and the 68.5 and 53.1 kJ/mol obtained by Polydera et al. (2005) for pressure- and thermally-treated fresh Navel orange juice, respectively.

5.3.3. Pressure treatment effects on retinol, α-tocopherol and β-carotene retention in raw milk

Retinol, α-tocopherol and β-carotene losses (Figure 5.3B) were much lower than those observed for AA (Figure 5.3A), particularly at high temperature. AA in fruits and vegetables has been shown to be stable when pressure-treated at low temperatures, but when subjected to high pressures at high temperatures (>65 °C), oxidation degradation reactions are enhanced (Indrawati and Hendrickx 2002; Oey and others 2008). Most studies on the effect of high pressure on fat soluble vitamins have been limited to carotene, vitamin A (retinol) and vitamin E (α-tocopherol) in juices and purees showing that HPP has minimal effect on these vitamins (Indrawati and Hendrickx 2002; Oey and others 2008). In this study, no significant differences (p>0.05) were observed in retinol, α-tocopherol and β-carotene concentration for raw milk subjected to the low-temperature R1 treatment (400MPa/31°C/5 min) when compared with the untreated control (U_1). No retinol and α-tocopherol loss was detected in raw milk treated at 200 MPa and 46 °C for 2 or 8 min (samples R2-R3, Figure 5.3B). However, β-carotene in samples from the same two treatments showed significant losses (p<0.05). The same pattern was observed in the higher pressure treatments R4 (600MPa/46°C/2 min) and R5 (600MPa/46°C/8 min) (Figure 5.3B).

Application of more severe treatments (R6-R15) showed losses under 10% for both retinol and α-tocopherol and 16% for β-carotene (Figure 5.3B). Comparable losses of β-carotene in raw apple juice treated at 4°C and 100-200 MPa (18%) or 300 MPa (33%) were reported by Suárez-Jacobo et al. (2011). Cilla et al. (2012) reported for whole milk and skim milk treated at 400 MPa/40°C/5 min a significant decrease (p<0.05) in the bioaccessibility of carotenoid and AA but not of tocopherol. Bioaccessibility was determined using an in vitro simulated gastrointestinal digestion procedure mimicking the upper digestive tract (stomach and small intestine) developed by Cilla et al. (2008). HPLC determinations showed that after pressure processing, losses of AA, α-tocopherol and β-carotene in whole milk were 2%, 27%, and -6%, respectively (i.e., the observed concentration for β-carotene increased), and 11%, 13% and 8%, respectively, in skim milk (Cilla and others 2012). In milk, casein micelles protect β-carotene from degradation during heat and pressure processing. During heating at 80°C for 8 h, β-carotene concentration in casein micelles decreased only by 30.9% (Sáiz-Abajo and others 2013). In studies on the preservation of human breast milk by treatments at 65°C or 80°C at 300, 600, and 900 MPa), a significant decrease (p<0.05) in α-tocopherol was observed (Delgado and others 2013). Although this study was not designed to determine storage effects, Baéz *et al.,* (2012) reported the loss of vitamin A (retinol) in enteral formula was 19.7, 34.0, and 51.5% when stored 105 d at 22, 37 and 45ºC, respectively. These values are higher than the 30% loss after 18 months at 40ºC reported by Chávez-Servín *et al.* (2008) for children's milk formula.

5.4. Conclusion

High pressure processing has the potential to retain nutrients with health-promoting properties such as the vitamins included in this study. Regarding milk pasteurization by pressure treatments at moderate temperatures, an optimized pressure/temperature/time combination could minimize vitamin losses. Fat-soluble vitamins A & E were found to be far more stable than water-soluble vitamin C. Milk concentration of retinol, α-tocopherol and β-carotene was not greatly influenced by pressure treatments and their degradation does not seem to be accelerated by pressure. However, treatments at the more severe pressure and temperature combinations could result in extensive degradation of AA. Therefore, a careful selection of pressure around 200-600 MPa and low temperature (<46°C) appears essential to retain AA in milk. Previously published reports suggest the need to determine the role of dissolved oxygen in milk on AA degradation.

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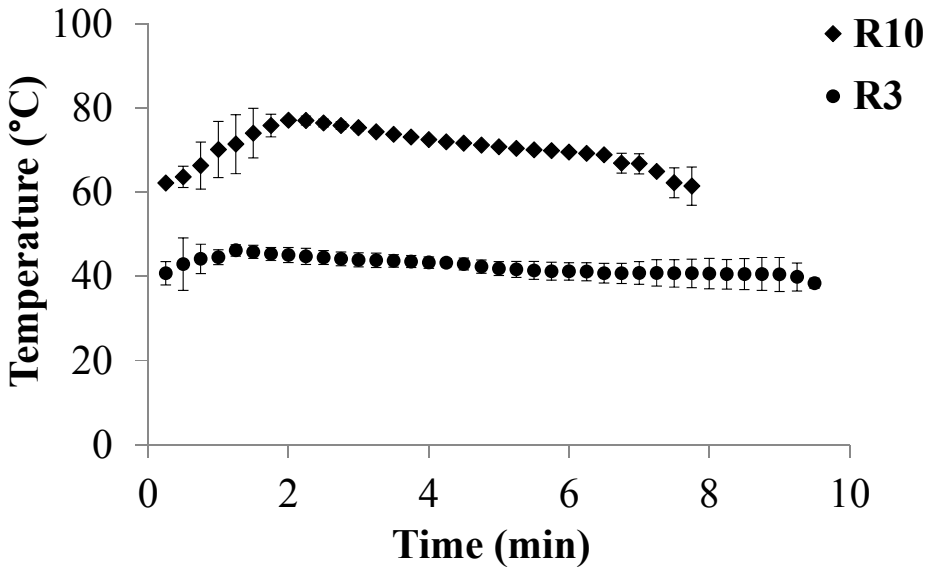

Figure 5.1. Temperature profiles during processing of samples R10 (400MPa/75°C/5 min) and R3 (200MPa/46°C/8 min)

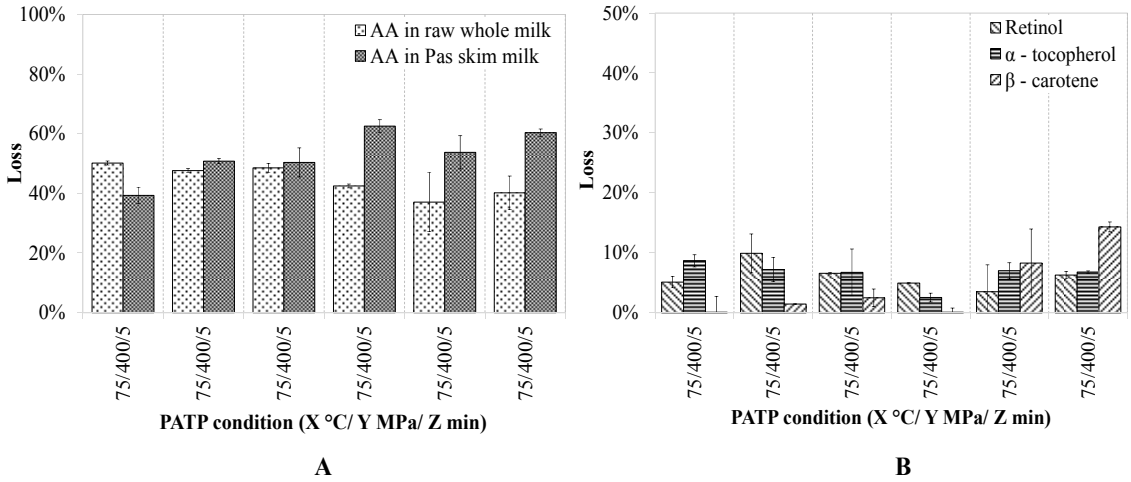

Figure 5.2. Effect of pressure treatment variability showing no significant differences ($p > 0.05$) between the six treatment replicates for losses of (A) AA in raw whole & pasteurized skim milk, and (B) fat-soluble vitamins in raw whole milk

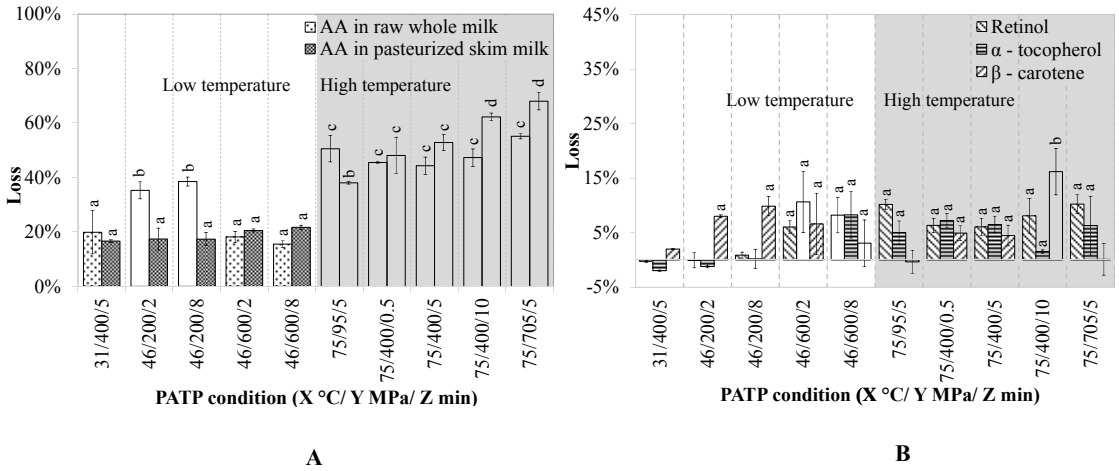

Figure 5.3. Effect of pressure treatments on (A) AA losses in raw whole and pasteurized skim milk and of (B) fat-soluble vitamins in raw whole milk. Mean values w/ same superscript indicate no significant differences ($p > 0.05$) between treatments. Lack of superscript indicates no significant difference ($p > 0.05$) from the corresponding untreated control.

Table 5.1. Pressure treatment conditions

Run	Treatment Conditions T ºC	P MPa	t min
R1	30 (31)*	400	5
R2	44 (46)	200	2
R3	44 (46)	200	8
R4	46 (46)	600	2
R5	41 (46)	600	8
R6	61 (75)	95	5
R7	67 (75)	400	0.43
R8	73 (75)	400	5
R9	73 (75)	400	5
R10	72 (75)	400	5
R11	72 (75)	400	5
R12	70 (75)	400	5
R13	72 (75)	400	5
R14	66 (75)	400	9.58
R15	62 (75)	705	5

* Values in parenthesis indicate the equipment setting temperature which was higher than the values measured for the pressurizing fluid in the vessel surrounding the milk samples.

Table 5.2. Ascorbic acid and liposoluble vitamin content (average ± standard deviation, mg/L) in pasteurized and raw whole milk samples

Untreated control	Pasteurized milk	Raw whole milk			
	AA	AA	Retinol	α-tocopherol	β-carotene
	mg/L	mg/L	mg/L	mg/L	mg/L
U_{1*}	4.10±0.21	7.27±0.19	0.80±0.001	0.46±0.005	0.15±0.002
U_2	3.16±0.06	5.76±0.42	0.80±0.003	0.522±0.006	0.13±0.004
U_3	3.31±0.13	4.91 ± 0.09	0.79±0.011	0.519±0.017	0.13±0.003

*U = Untreated, * = a numeric sub index is used to indicate that each of group of runs were compared to a different untreated sample due to the need to use three experimental days to run the unit at increasingly higher temperatures (U_1; R1-R3, U_2; R4-R5, U_3; R6-R15)

CHAPTER 6

Chitin content in diatom fermentation broths determined by acid hydrolysis to glucosamine quantified by HPLC

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ABSTRACT

An accurate, simple and rapid chitin quantification method was required for a techno-economical and life-cycle analysis of chitin production in a diatom photobioreactor. Chitin fibers extruded by *Cyclotella* spp. are used as is for biomedical applications or hydrolyzed into glucosamine for the dietary supplement market. Among reported chitin quantification methods, direct acid hydrolysis of the fermentation broth stood out for its simplicity, accuracy and sensitivity. Kinetics of the acid hydrolysis of purified chitin into glucosamine (GlcN) monomer was determined in 4M, 6M, 8M, 10M and 12M HCl at 90°C for up to 3 h. GlcN was quantified by HPLC (Dionex MA-1 column, 30°C, 0.4 ml/min 0.75M NaOH isocratic mobile phase, and ED-40 PAD detector). Tests using reagent-quality GlcN confirmed the stability of the monomer under these hydrolysis conditions. Acid hydrolysis of diatom fermentation broths chitin was conducted in 8M HCl for 3 h at 90ºC even though glucosamine yields were influenced by the fermentation broth composition. The latter was solved by determining the glucosamine concentration of three spiked and unspiked broth samples to determine an average correction factor. This allowed a low-variability monitoring of the chitin production in the diatom photobioreactor (7.8% and 18.3% for 2 broths differing in N content).

KEYWORDS: chitin, diatom, photobioreactor, glucosamine, acid hydrolysis

6.1. Introduction

The rapid increase in energy demand has generated a growing global interest in marine algae biorefineries to produce biofuels, biomass and biomolecules (Sandun and others 2006; Bozarth and others 2009). The latter products are often included to make algal biofuel production financially feasible. Diatoms are single-cell organisms with intricately structured cell walls made of nanopatterned silica (SiO_2) previously studied for applications in nanotechnology (Jeffryes and others 2008; Qin and others 2008; Wang and others 2009; Jeffryes and others 2011). Diatoms are also producers of chitin and have significant ecological importance (Durkin and others 2009). They convert uridine diphosphate *N*-acetylglucosamine (UDP-GlcNAc) into β-crystalline chitin (Ogawa and others 2011) via chitin synthesis pathway and catalyzed by plasma membrane-bound chitin synthase (Tesson and others 2008; Durkin and others 2009).

Major product targets for the development of diatom biorefineries are the fine and inert siliceous frustule structure (Round and others 1990), chitin as is for biomedical applications such as drug/gene delivery and wound healing (Jayakumar and others 2011; Tamura and others 2011) or hydrolyzed into glucosamine to be marketed as a dietary supplement (Jeon and others 2000; Vaclavikova and Kvasnincka 2013). Chitin can be used as an antimicrobial agent, edible film, emulsifier, thickener, or stabilizer for food applications (Shahidi and others 1999; Agulló and others 2003). Chitin is used as a raw material for the industrial production of chitin-derived products such as chitosan, derivatives of chitin/chitosan, oligosaccharides and glucosamine (Einbu and others 2007). Obtained from renewable sources, chitin is biodegradable

and biocompatible and has been the subject of numerous past and current research efforts to identify commercial applications (Mireles and others 1992; Torres and others 1999; Savant and Torres 2000; Anitha and others 2014). However, the market competitiveness of chitin and of other potential products from diatoms depends on cost and environmental impact considerations (Lebeau and Robert 2003).

6.1.1. Chitin production by diatoms

As a photosynthetic organism, diatoms require to grow fresh, sea, or brackish water in addition to carbon dioxide, inorganic salts, and light (Lopez and others 2005; Bozarth and others 2009). Chitin is composed of β-(1→4) linked 2-acetamido-2-deoxy-β-D-glucose (N-acetylglucosamine) units and constitutes the second most abundant biopolymer after cellulose (Durkin and others 2009). In the entire marine environment, this biopolymer has been estimated to represent 1560 million tons (Agulló and others 2003). Since crustacean exoskeletons are the largest biomass source for its commercial production, most chitin studies have been carried out on materials obtained from crab and shrimp (Durkin and others 2009). Transmission and scanning electron microscopy has been used to investigate the β-chitin fibrils formation by the centric diatom algae *Cyclotella cryptica*, *Cyclotella nana*, *Cyclotella meneghiniana*, and *Thalassiosira fluviatilis* (Herth and Barthlott 1979). McLachlan et al. (1965) reported the glucosamine and chitin synthesis by *Thalassiosira fluviatilis* diatom species. The synthesis of highly crystalline extracellular β-chitin fibers by

Thalassiosira spp. was reported by Brunner et al. (2009).

6.1.2. Glucosamine health benefits

Glucosamine (2-amino-2-deoxy-α-D-glucose) is the deacetylated monomer produced by acid hydrolysis of chitin (Santhosh and Mathew 2007). It is a precursor in the biosynthesis of fundamental components of connective tissue molecules such as hyaluronic acid, proteoglycans, glycosaminoglycans, glycolipids, and glycoproteins (Miller and Clegg 2011). Glucosamine has been reported to relieve the symptoms of osteoarthritis (Anderson and others 2005; Barnhill and others 2009; Nakamura 2011; Chen and others 2012). Wang and others (2007) observed in rabbits that glucosamine treatments reduced during the early stages of experimentally-induced osteoarthritis the extent of changes occurring in the turnover, structure and mineralization of the subchondral bone which provide support for the articular surface cartilage. This suggests a possible mechanism by which glucosamine could protect cartilages from degeneration and implies a disease-modifying effect of glucosamine on the subchondral bone in patients suffering from osteoarthritis. In a study of a 3-year glucosamine sulfate treatment to modify the progression of joint structure and changes in symptoms in knee osteoarthritis by Pavelká and others (2002), approximately two hundred patients with this disease as defined by the American College of Rheumatology criteria were randomized to receive oral glucosamine sulfate (1500 mg, once a day) or placebo. The treatment retarded the progression of knee osteoarthritis. However, Kwoh and others (2014) who studied 201 patients with knee osteoarthritis

subjected to short-term oral glucosamine treatments found no evidence of structural benefits from glucosamine supplementation among individuals with chronic knee pain. Glucosamine is often combined with chondroitin sulfate and the combination has been reported to be probably effective in the treatment of knee osteoarthritis patients with moderate-to-severe knee pain (354 patients), while it was not effective in reducing pain in the overall group (1582 patients) (Clegg and others 2006).

6.1.3. Glucosamine market

The glucosamine market appears steady with healthy worldwide growth expected to be driven by an aging population, and companies promoting the benefits of glucosamine as a vital dietary supplement for joint health (Anonymous 2011). According to Mintel's Global New Products Database (GNPD), 48% of joint mobility products contain glucosamine. The global glucosamine supplement market was about $2 billion in 2008, reflecting a massive 62% increase from 2003 and equivalent to a 10% annual growth rate. In 2008, the largest markets were the U.S. and Japan accounting for sales of $872 and $300 million, respectively. Compared to the annual worldwide rate of 10%, the U.S. glucosamine supplement market grew from 2003 to 2009 at a much slower annual rate of 2.7%. However, this reflected mostly a lowering in glucosamine prices rather than a reduction in demand (Heller 2009).

World-wide production of glucosamine is expected to reach 46,600 metric tons by 2017 (Anonymous 2011). Williams and Laurens (2010) estimated $7,500 per metric ton to be the annualized total capital and operating costs for algal biofuel

produced by photobioreactors. Using a conservative price of $20/kg for glucosamine and $3/gal for biofuel, the annual revenue for this facility would be $10 million/year for glucosamine and only $540,000/year for biofuel. This indicates clearly that the economic viability of photosynthetic biofuels biorefineries could be significantly improved by adding glucosamine as a co-product stream.

Glucosamine is sold in different salt forms depending on the acid used during hydrolysis. The major source of glucosamine is chitin from shrimp shells, which is not generally recognized as safe (GRAS) by the FDA, and it is not a suitable source for vegetarians and consumers with shellfish allergies (Barrow 2010). Vegetarian glucosamine is mainly targeted at individuals allergic to shellfish, and to a specific section of population who strictly follow Jewish food habits (Anonymous 2011). The only commercially available vegetarian, kosher, and FDA-approved GRAS glucosamine source is Cargill's Regenasure produced from the fungus *Aspergillus niger* (Barrow 2010). Attempts to produce glucosamine by genetically engineered *E. coli* or fungi have faced many technical challenges (Barrow 2010; Sitanggang and others 2010; Chen and others 2012).

6.1.4. Quantification of chitin production by diatoms

The overall objective of this study is the quantification of the long chitin fibers extruded by *Cyclotella* spp. diatoms. The method to be developed must allow quick and reliable quantification of the chitin content of diatom cell broth without sample fractionation to reduce chitin losses. Acid hydrolysis is a simple and convenient means

to obtain a wide range of chitin fragments of different molecular weights by changing the reaction time, temperature and/or acid concentration (Chang and others 2000; Zhu and others 2005). In this study, methods based on the formation of anhydro mannose, reaction with the Ehrlich's reagent, and enzymatic and HCl hydrolysis followed by HPLC quantification of the monomers obtained were compared.

6.2. Materials and methods

6.2.1. Chitin and chitosan sources

Shrimp chitin powder was obtained from Spectrum Chemical (Gardena, CA). Shrimp chitosan, chitin flake, and glucosamine hydrochloride (part no. G4875) used as a reference standard for HPLC analysis were obtained from Sigma-Aldrich (Milwaukee, WI). Commercial glucosamine dietary supplements purchased locally (Corvallis, OR) are described in Table 6.1. Commercial diatom chitin suspensions and dry matrices of poly-*N*-acetyl glucosamine fibers isolated from microalgae (Talymed®) were provided by Marine Polymer Technologies, Inc. (Burlington, MA).

Several samples of fermentation broths, sterile and after diatom cultivation in a 5 liter bubble column photobioreactor using a modified Harrison's artificial seawater medium (Harrison and others 1980) were received from the Chemical Engineering Department at Oregon State University (Corvallis, OR). *Cyclotella* spp. were cultivated in single-stage batch growth mode under silicon and sodium nitrate co-limitations. The initial constant dissolved silicon concentration of 0.8 mM was targeted to provide three to four doublings while the sodium nitrate was varied from

0.5-5 mM to reach its depletion before, during, and after silicon depletion. The experiments were run until diatom cells reached the stationary phase, and when the concentrations of silicon and sodium nitrate had been depleted. Fermentation broths were stored under refrigeration (4°C) before analysis within 24 h. To observe the influence of fermentation media composition on chitin hydrolysis, sterile fermentation broth type 1 and 2 were used to dissolve commercial diatom chitin corresponding to the lowest (0.5 mM) and highest (5 mM) sodium nitrate concentration, respectively.

6.2.2. Chemicals and reagents

Reagent quality hydrochloric acid (HCl) was obtained from EMD Millipore (Billerica, MA). Reagent quality sodium hydroxide (NaOH) pellet and monobasic potassium phosphate (KH_2PO_4) were obtained from Macron Fine Chemicals™ (Center Valley, PA). Ultrapure HPLC grade water was obtained from Alfa Aesar (Ward Hill, MA). Thiobarbituric acid (TBA) and DAB-p-dimethylaminobenzaldehyde were obtained from Sigma-Aldrich and Avantor Performance Materials, Inc. (Center Valley, PA), respectively. *Streptomyces griseus* chitinase was obtained from Sigma-Aldrich.

6.2.3. Chitin quantification

The following four methods were compared as alternatives for chitin quantification:

6.2.3.1. Method I: The anhydro-mannose spectrophotometric method for chitin

quantification described by Badawy (2012) was assessed as follows. First, 0.5 mg chitin was deacetylated into chitosan using 10 ml of 45% NaOH. After overnight steeping of chitin in 45% NaOH it was transferred to a 25 ml glass tube which was then placed for 6 h in a 110°C dry heating block (Model 113004, Boekel Scientific, Feasterville, PA). Next, chitosan collected on a vacuum filter flask membrane (Whatman® Filter Paper No.42) and washed with distilled water till reaching neutrality was dried overnight at room temperature (23^{o}C). A 1.9 ml suspension of chitosan in distilled water (200 μg/ml) was depolymerized (80^{o}C, 30 min) into 2,5-anhydro-D-mannose using 0.1 ml of 0.5 M sodium nitrite. Following depolymerization, 0.2 ml of 3 M NaOH was added to reach pH 8, and after careful mixing, 0.5 ml of a 0.04 M thiobarbituric acid solution was added. After 10 min incubation in an 80^{o}C water bath, the anhydro-mannose reaction with TBA yielded a pink color (λ = 555 nm).

6.2.3.2. Method II: This colorimetric quantification method reported by Pagnussatt et al. (2014) was implemented as follows. First, 5 ml of 6 M HCl were mixed with 0.2 g commercial chitin/chitosan/GlcN/GlcNAc and then kept at 100°C for 40 min. The hydrolysate solution was filtered, neutralized with 3 M NaOH, and then 4-5 drops of 1% (w/v) $KHSO_4$ were added. After placing a 1 ml solution aliquot in boiling water for 20 min and cooling it in a room temperature water bath, 6 ml ethanol, 1 ml Ehrlich reagent (2.67 g DAB-p-dimethylaminobenzaldehyde dissolving in 15 ml of ethanol) and 15 ml HCl were added. Glucosamine was quantified colorimetrically

by absorbance measurements at 530 nm.

6.2.3.3. Method III: A third method, chitin hydrolysis by a chitinase from *Streptomyces griseus* (Ohno and others 1996) was investigated as follows. An enzyme stock solution was prepared in 0.05M KH_2PO_4 buffer and stored at -20°C. A suspension (2 ml) containing 5 mg of chitin was mixed with 0.075U of enzyme and incubated at 37°C for 0.5, 1, 2 and 20 h. The mixture was centrifuged at 12,000 g for 10 min and then the glucosamine content in the supernatants was quantified by HPLC.

6.2.3.4. Method IV: Hydrochloric acid has been frequently reported for the hydrolysis of chitin but only for chitin from sources other than diatom fermentation broths (Chen and Johnson 1983; Chang and others 2000; Novikov 2004; Zhu and others 2005). Acid hydrolysis in 4M, 6M, 8M, 10M, and 12M HCl at 90°C was compared to optimize the diatom chitin depolymerization into glucosamine. For hydrolysis in 4, 6, 8, or 10M HCl, 5 mg of commercial diatom chitin was accurately weighed into a 25 ml glass tube, then gently suspended in 2 ml of distilled water or in sterile fermentation broth type 1 or 2, and finally adding 1, 2, 4, or 10 ml of 12M HCl, respectively. All samples were vortex-mixed at low speed before glass tubes were loosely capped and incubated in a dry heating block set at 90°C. After 1, 2 and 3 h, aliquots of 0.3 ml for 4M, 8M, 10M HCl samples and 0.4 ml for 6M HCl samples were collected in Eppendorf tubes. Acid hydrolysis in 12 M HCl was tested by adding 0.5, 1, and 5 mg commercial diatom chitin into 4 ml of 12M HCl. After incubation in a dry heating block set at 90°C, 0.3 ml aliquots were collected after 0.5, 1, 2, and 3 h.

All aliquots were cooled in a room temperature water bath and the hydrolysis reaction stopped by adding gradually 0.2, 0.4, 0.4, 0.5, and 0.4 ml of 6M NaOH to reach pH 11-12 for the 4, 6, 8, 10 and 12M HCl reactions, respectively. The hydrolysates collected in Eppendorf tubes were centrifuged at 12,000 g for 10 min before transferring the supernatant into HPLC injection vials for subsequent glucosamine quantification. All test conditions were run in duplicates.

6.2.4. Hydrolysis of deacetylated chitin

The small number of amino groups in chitin explain why it does not dissolve nor swell in common solvents (Kurita 2001). Following an industrial suggestion, deacetylation of chitin into chitosan prior to acid hydrolysis was evaluated as follows. Three commercial chitin samples (diatom chitin, shrimp chitin flake and shrimp chitin powder) were deacetylated by overnight soaking in 45% NaOH at room temperature (23ºC), followed by 6 h incubation at 110 °C (Kurita 2001) or autoclaved at 121 °C for 15 min (No and others 2000). After cooling in a room temperature water bath, the reaction mixture was filtered, washed on the filter paper (Whatman® Filter Paper No.42) with distilled water until reaching neutrality, and after overnight drying in a 60°C oven, the deacetylated chitin was recovered and hydrolyzed (8M HCl, 90°C, 3h) into glucosamine which was then quantified by HPLC. Hydrolysis reaction, sample cooling and neutralization with NaOH were done as described before. Tests with all three commercial samples were conducted in duplicate.

6.2.5. Monitoring chitin concentration during batch fermentation processes

The precision of a quantification method can be improved by spiking experimental samples with known amounts of the analyte, in this case, diatom chitin. Three samples from the fermentation broth Type 1 and 2 were spiked with 0.25 mg commercial diatom chitin and then hydrolyzed in 8M HCl at 90°C for 3 h. The samples selected for spiking corresponded to the first, middle and end point of broth Type 1 (14 samples) and 2 (18 samples). Spiked and unspiked hydrolyzed samples were analyzed in duplicate with glucosamine determined by HPLC. Again, hydrolysis reaction, sample cooling and neutralization with NaOH were done as described before. All determinations were done in duplicate.

6.2.6. Effect of hydrolysis condition on glucosamine hydrochloride

Samples of the glucosamine hydrochloride were subjected for 3 h to 8M HCl at 90°C to determine the stability of the monomer obtained by acid hydrolysis of chitin. Samples of the standard (5 mg) in 25 ml glass tube were suspended in 2 ml of distilled water or sterile fermentation broth 1 and then vortex-mixed. After adding 4 ml of 12 M HCl, all samples were vortex-mixed at low speed before glass tubes were loosely capped and incubated in a dry heating block set at 90ºC for up to 3 h. Hydrolysis reaction, sample cooling and neutralization with NaOH procedures were as described before. Experiment was run in duplicate.

6.2.7. Quantification of glucosamine content in commercial dietary supplements

Four commercial dietary supplements (Table 6.1) were dissolved in a 25 ml glass tube with 10 ml distilled water and vortexed at high speed until fully dissolved. This stock solution was diluted 1,000 times with distilled water to yield solutions in the 7.5-15 mg/ 100 ml range. Glucosamine solution collected in Eppendorf tubes were centrifuged at 12,000 g for 10 min before transferring the supernatant into HPLC injection vials for subsequent glucosamine quantification.

6.2.8. HPLC quantification of glucosamine

HPLC detection of glucosamine was done on a Dionex modular HPLC unit consisting of a GP50 pump, ED40 detector, LC30 column oven and AS3500 autosampler (Thermo Fisher Scientific Inc. Sunnyvale, CA). Conditions were: Dionex MA-1 column (4x250mm) with MA-1 guard column (4x50mm) @ 30°C, isocratic 750mM NaOH mobile phase at 0.4 ml/min, and 10µl injection. Detection was via pulsed amperometric mode using the quad potential waveform with a disposable Au on PTFE electrode.

6.3. Results and discussion

6.3.1. Chitin quantification

6.3.1.1. Method I: The anhydro-mannose spectrophotometric chitin quantification method described by Badawy (2012) requiring as a first step the deacetylation of chitin into chitosan was rejected for the following reasons. First,

measured (e.g., 168.4 μg/ml) and actual chitin concentration measurements (200 μg/ml) suggested that the deacetylation reaction was incomplete (Table 6.2). Moreover, ensuring consistent deacetylation in spite of diatom media formulation differences and changes during fermentation time would be challenging. In addition, glucosamine solutions (200 μg/ml) yielded an apparent concentration of 71.1 μg/ml, which indicated that any glucosamine in the fermentation broth would interfere with chitin content determinations. As expected, chitin and *N*-acetylated glucosamine did not react (Table 6.2). Finally, the method was complex and long as it required chitin deacetylation and depolymerization into 2,5-anhydro-D-mannose, which was then detected by reacting it with a thiobarbituric acid solution to generate the colored pigment.

6.3.1.2. Method II: The Ehrlich's reagent chitin quantification method was tested using 200 μg/ml suspensions/solutions of chitosan, chitin, glucosamine and *N*-acetylated glucosamine. This method was rejected because only chitosan reacted with the Ehrlich's reagent (Data not shown). The alternative of deacetylating chitin prior to reaction with the Ehrlich's reagent was rejected for the reasons previously stated.

6.3.1.3. Method III: Enzymatic hydrolysis of chitin, explored as an alternative to its acid hydrolysis, was rejected because chitinase showed extremely low glucosamine recovery values (< 10%) even after 20 h incubation (data not shown). All microbial chitinases can hydrolyze GlcNAc-GlcNAc and GlcNAc-GlcN linkages but only the *Streptomyces griseus* chitinase used in this study can hydrolyze GlcNAc-GlcNAc or GlcN-GlcNAc linkages and chitosanase was required to hydrolyze GlcN-

GlcN linkage (Ohno and others 1996). All these linkage forms may be present in chitin because most but not all monomers are acetylated. Therefore, enzymatic hydrolysis results in a mixture of mostly N-acetylated glucosamine with similar retention time but much lower detector response than glucosamine. This may explain the low recovery values observed in this study since glucosamine was the HPLC calibration standard. The alternative of chitin deacetylation prior to enzymatic hydrolysis was rejected for the reasons previously stated.

6.3.1.4. Method IV: HCl hydrolysis deacetylated chitin and cleaved the glycoside bonds between monomers yielding *N*-acetyl-D-glucosamine monomers (Navikov 1999) which were quantified by HPLC to estimate chitin concentration (Harrison and others 1980). However, in addition to acid concentration and hydrolysis temperature and time, broth media composition affected the hydrolysis extent of the chitin present in the sample. Chitin hydrolysis was found to depend on the broth composition which is varied on whether the media is designed for maximum cell mass, lipid, or chitin production.

The acid hydrolysis method followed by glucosamine quantification by HPLC was found to be sensitive (see Section 6.3.4) and up to 30 samples could be run in duplicates at the same time. When compared with the anhydro-mannose and Ehrlich's reagent spectrophotometric methods, acid hydrolysis did not require the long (>6h) chitin deacetylation first step. The alternative hydrolysis method using an enzyme was discarded for the reasons previously described. As summarized in Table 6.3, acid hydrolysis followed by HPLC glucosamine quantification allowed the monitoring of

chitin concentration during diatom batch fermentation process requiring a time-efficient method (~200 min/sample + 14 min/sample HPLC run time).

6.3.2. Optimization of glucosamine yield by chitin acid hydrolysis

Table 6.4 compares the glucosamine production after hydrolysis at 90°C in 4M, 6M, 8M and 10M HCl of commercial diatom chitin (5 mg) suspended in distilled water or sterile fermentation broth Type 1 and 2. Hydrolysis in 4M and 10M HCl were discarded because they yielded the lowest chitin conversion into glucosamine. For chitin suspended in distilled water, the highest glucosamine yield (98.0±0.04, n =2) was achieved after 2h hydrolysis in 8M HCl at 90°C. However, in sterile fermentation broths, the glucosamine yield reached the highest value (73.7±0.02 and 97.2±0.02 in broth type 1 and 2, respectively, n =2) after 3 h hydrolysis in 6M HCl at 90°C. The optimum condition reported by Zhu et al (2005) for the acid hydrolysis of chitin present in biomaterials and raw foods, and including crab and shrimp, included a more severe hydrolysis time and temperature (6h in 8M HCl at 110ºC).

The same HCl molar concentration and reaction time at 90ºC yielded different glucosamine levels depending on whether chitin was suspended in water, fermentation broth type 1, or fermentation broth type 2. When comparing 6M and 8M HCl hydrolysis conditions, the higher acid concentration favored higher but different glucosamine yield for chitin suspended in the sterile fermentation broth 1 and 2 while 6 M HCl yielded better conversion for chitin suspended in pure water (Table 6.4). This

implies that the fermentation broth would interfere with the hydrolysis of chitin into glucosamine and thus with the determination of chitin produced in the diatom photobioreactor.

To test the viability of using even higher HCl concentrations, different amounts of commercial diatom chitin were hydrolyzed in concentrated HCl (12 M). The highest glucosamine yields were achieved after 30 min incubation at 90°C (Table 6.5). However, higher chitin concentrations resulted in higher glucosamine yields, e.g., a 71.3% conversion to glucosamine was observed when 5 mg diatom chitin were reacted with 4 ml of 12M HCl after 30 min at 90°C, while 66.6% and 68.5% values were observed when 0.5 mg and 1 mg chitin reacted with the same 12M HCl amount. This suggested that glucosamine may degrade in 12 M HCl and was therefore discarded.

An additional attempt to stabilize the yield of glucosamine obtained by HCl hydrolysis of chitin was to deacetylate chitin before hydrolysis. However, hydrolysis of commercial chitosan and of deacetylated commercial chitin under the same conditions yielded lower glucosamine values (4-28.8% range) than hydrolyzing chitin directly (19.9-48.8% range). This may be due in part to the loss of material during the deacetylation step. Consequently, deacetylation of chitin prior to HCl hydrolysis was discarded.

6.3.3. Effect of acid hydrolysis conditions on the glucosamine stability

Extreme acid hydrolysis conditions reduce analysis time but may degrade the

glucosamine produced from chitin. However, even after 3 h in 8M HCl at 90°C the glucosamine hydrochloride retention was 88±0.02 (n = 2). This indicated that the degradation of the monomer under the hydrolysis conditions chosen would have a minor effect when quantifying the chitin produced in the diatom photobioreactor.

6.3.4. HPLC quantification of glucosamine

The HPLC glucosamine determination yielded a good chromatographic separation with a retention time of about 8.5 min and a total 14 min run time ensured that the column was ready for the next sample (Figure 6.1). The calibration curve for glucosamine standard range 0.05 to 20 mg/100 ml showed r^2 values between 0.972-0.997. The limit of detection (LOD) and limit of quantification (LOQ) defined as a concentration in glucosamine standard (n=4) with signal to noise ratio of 3 and 10, respectively, were 10 and 30 µg/100 ml, respectively.

6.3.5. Commercial glucosamine dietary supplement quantification

To verify the accuracy of the glucosamine quantification by HPLC, the labeled glucosamine amount, mg/tablet, of commercial supplements were compared with experimental determinations (Table 6.1). Product 1, 2, 3 and 4 yielded glucosamine levels of 1071, 688, 1429 and 923 mg/tablet, respectively, which were similar to label values. The difference of analytical result and the amount reported on label was less than 10% for all products. The highest consistency was obtained in product 1. The

reason was probably because the supplier use different grade of material. However, the HPLC method showed high enough efficiency for quantitative method.

6.3.6. Monitoring chitin concentration during batch fermentation processes

In this study, the key requirement for an acceptable chitin quantification method was to avoid its extraction from the fermentation broth (Chotyakul and others 201X-b) prior to acid hydrolysis and HPLC glucosamine quantification. Therefore, acid hydrolysis of diatom fermentation broths chitin was conducted in 8M HCl for 3 h at 90^{o}C even though the yields for fermentation broth 1 and 2 shown in Table 6.4 were different (47.5±0.24 and 86.8±0.08, respectively). The limitation of the influence of the fermentation broth type was solved by sample spiking, i.e., adding a known amount of commercial diatom chitin into the fermentation broth and using the glucosamine concentration for the spiked and unspiked samples to determine an average concentration correction factor (Figure 6.2). Though the correction factor rate varied from 30% to 90% in different diatom fermentation broth samples, for the same fermentation run, the values obtained were stable (Data not shown). This allowed reliable chitin content determinations as required to monitor and optimize chitin production in the diatom bioreactor. Figure 6.3 shows chitin diatom production for tow photobioreactor runs with the same initial silica content (0.8 mM) but diatom broth type 2 with a higher nitrogen concentration (4 mM) yielded higher chitin amounts than the broth type 1 with a lower nitrogen concentration (3 mM). The average chitin determination error was 7.8% and 18.3% for broth type 1 and 2 samples, respectively.

These are low error levels considering the heterogeneity of the fermentation broth samples.

6.4. Conclusions

Ongoing work in the Department of Chemical Engineering at Oregon State University on the Life Cycle Analysis and Techno-Economic Analysis of the diatom photobioreactor production of biofuels and chitin (data not shown) have highlighted the importance of high values for yield, recovery and conversion of chitin into glucosamine. These determinations required a reliable quantitative determination of the chitin produced in the diatom photobioreactor. Direct acid hydrolysis for 3 h in 8M HCl at 90^{o}C of chitin suspended in the diatom fermentation broth allowed reliable and low-variability determinations of total chitin production.

Acknowledgements

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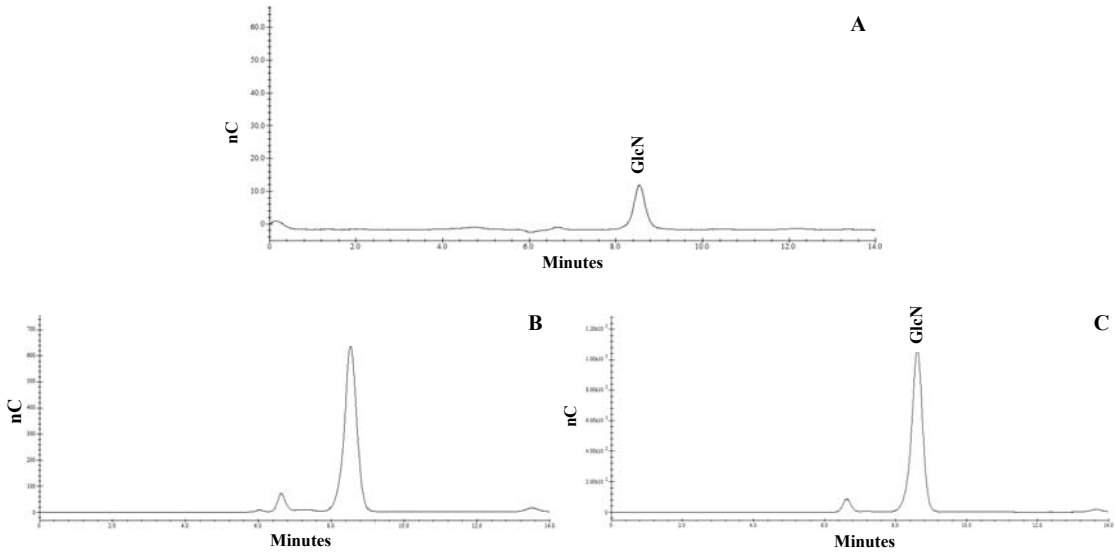

Figure 6.1 Chromatograms of 0.25 mg/100 ml glucosamine standard (A); glucosamine in 20 mg/100 ml hydrolyzed commercial diatom chitin (B); glucosamine in 20 mg/100 ml hydrolyzed glucosamine hydrochloride (C)

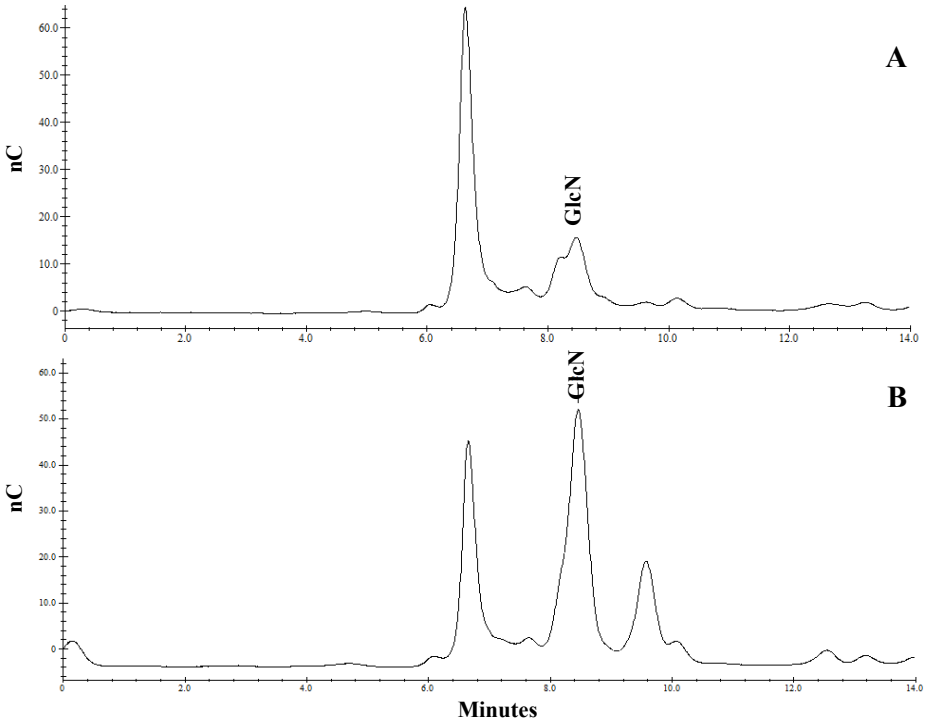

Figure 6.2 Chromatograms of glucosamine in fermentation diatom broth (A); glucosamine in fermentation diatom broth spiked with 0.25 mg commercial diatom chitin (B)

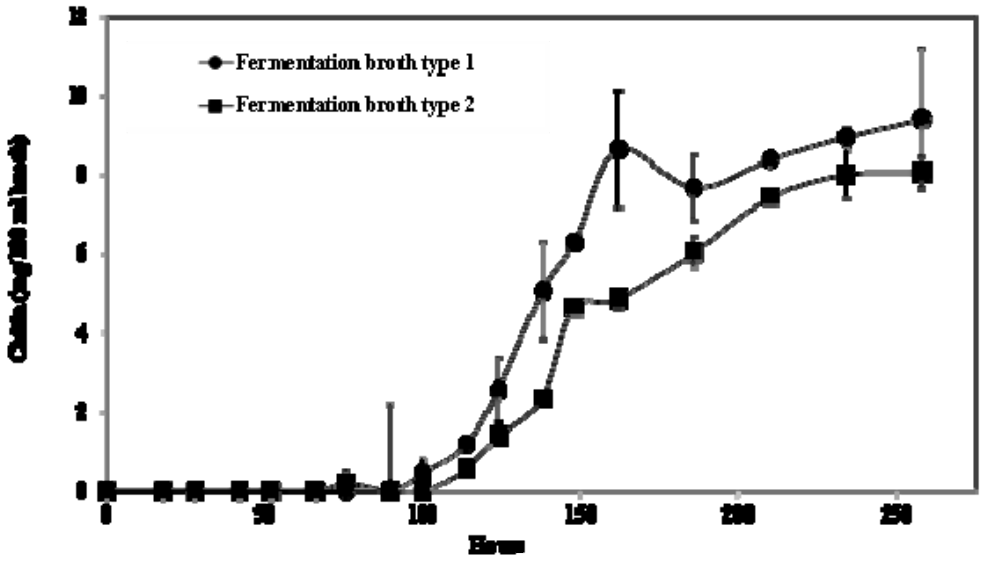

Figure 6.3 Chitin content (mg/100 ml) after 8M HCl hydrolysis at 90°C for 3 h in 2 types of fermentation diatom broth formulations cultivated in photobioreactor

Table 6.1. Commercial dietary glucosamine supplements

#	Lot #	Tablet Dose (mg GlcN)	Source	Supplier	GlcN content by HPLC quantification (mg/tablet)
1	171012A	1,000	Shrimp, crab, lobster, and crayfish	Schiff Nutrition Intl., Salt Lake City, UT	1,071
2	missing	750		Safeway	688
3	769374-01 S4525	1,500		Safeway	1,429
4	1285BO2612	1,000	Non-shellfish	General Nutrition Corp., Pittsburgh, PA	923

Table 6.2. Quantification of suspensions/solutions of chitin and associated compounds (200 µg/ml) using the anhydro-mannose spectrophotometric method

Sample	Absorbance @ 555nm	Concentration (µg/ml)
Chitosan	0.085	200.0
Deacetylated chitin	0.074	168.4
Glucosamine-HCl	0.044	71.1
Chitin	nr[1]	-
N-acetylated glucosamine	nr	-

[1] nr = no reaction

Table 6.3. Comparison of chitin quantification methods

Method	Advantage	Disadvantage
Anhydro-mannose Spectrophotometric	Fast detection, ~1h Low-cost equipment	Long/uncertain deacetylation degree, >6h Less specific color reaction
Ehrlich's reagent Spectrophotometric	Fast detection, ~1.5h Low-cost equipment	Long/uncertain deacetylation degree, >6h Less specific color reaction
HCl hydrolysis HPLC	Least overall time, ~6 h High precision	Hydrolysis correction factor for each sample
Chitinase hydrolysis HPLC	Mild condition Chitinase is inducible	Expensive chitinase Hydrolysis correction factor Yields a GlcN + GlcNAc mixture

Table 6.4. Glucosamine production from diatom chitin (5 mg) hydrolyzed by HCl in distilled water and two sterile formulations

Hydrolysis conditions			Production %		
HCl, M	Temperature, ˚C	Time, h	Water	Sterile broth 1	Sterile broth 2
4	90	1	4.1±0.01	1.6±0.01	6.8±0.02
		2	6.9±0.01	2.4±0.00	11.8±0.02
		3	9.7±0.01	5.6±0.00	17.6±0.02
6	90	1	44.2±0.02	20.9±0.02	36.5±0.06
		2	80.3±0.05	53.8±0.01	68.8±0.06
		3	97.7±0.03	73.7±0.02	97.2±0.02
8	90	1	97.8±0.05	56.3±0.26	93.1±0.46
		2	98.0±0.04	56.5±0.19	85.2±0.02
		3	81.4±0.12	47.5±0.24	86.8±0.08
10	90	1	64.1±0.15	57.7±0.08	57.0±0.03
		2	57.9±0.09	53.1±0.01	47.4±0.00
		3	57.4±0.01	61.1±0.06	59.0±0.03

Table 6.5. Glucosamine production from varied amount of diatom chitin hydrolyzed by concentrated HCl (12M)

Temperature, °C	Diatom chitin, mg	Time, h	Production, %
90	0.5	0.5	66.6±0.36
		1	32.0±0.19
		2	17.0±0
		3	21.4±0.10
	1.0	0.5	68.5±0.13
		1	38.1±0.16
		2	23.9±0.04
		3	30.5±0.12
	5.0	0.5	71.3±0.11
		1	41.6±0.22
		2	40.4±0.20
		3	43.8±0.22

CHAPTER 7

Chitin recovery from diatom photosynthetic biorefinery broths and its purification and conversion into glucosamine

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ABSTRACT

A quick and reliable quantification method for chitin separation and purification from diatom cell broth as a function of fermentation condition and time was developed. To ensure homogeneity of broth samples, and to help dislodge chitin from diatom cells, samples were first treated for 10 s in a laboratory blender. The liquid supernatant containing the chitin fibers after mild centrifugation (1500*g*, 1 min) was subjected to an intense centrifugation step (11000g, 30 and 60 min) to recover cell-free chitin as a pellet. Acid hydrolysis in 8M HCl at 90°C for 3 h of the supernatants obtained after mild and intense centrifugation yielding glucosamine quantified by HPLC was used to determine the unpurified chitin recovered exceeding 80% of the total amount produced by the diatom photobioreactor. Chitin purification was achieved using 1M HCl at 70ºC to solubilize calcium and other salts, 0.5% w/w SDS to remove insoluble proteins, and 95% ethanol to remove chlorophylls and other organic material. This treatment yielded diatom chitin with 70.9±16.6% purity.

KEYWORDS: chitin, glucosamine, diatom, photobioreactor, recovery, purification

7.1. Introduction

The marine environment contains a vast array of organisms with unique biological properties but remains a most underutilized biological resource. However, marine microorganisms such as algae are now considered efficient producers of biologically active and/or chemically novel compounds (Mayakrishnan and others 2013). For example, biologically active compounds extracted from diatom cells have been proposed for a range of biotechnological applications (Lopez and others 2005). Algae and microalgae are potential sources of functional food ingredients (Plaza and others 2008; Plaza and others 2009). The organic components in brown algal biomass include alginates, fucans, and other biopolymers and carbohydrates of commercial interest (Mayakrishnan and others 2013). Biopolymers of marine origin have been tested for a wide variety of applications (e.g., Torres and others 1999).

Chitin, the second most abundant biopolymer found in nature, can be obtained from many marine sources including diatoms and krill of ecological importance, and as a byproduct from crustaceans harvested for food consumption. This biopolymer is also present in the exoskeletons of insects and the cell walls of fungi and some algae (Zhang and others 2005). Chitin is used as raw material for the production of chitosan, oligosaccharides, and glucosamine (Einbu and Vårum 2008).

In nature, chitin can be found in three polymorphic forms (α-, β-, γ-chitins) differing in the orientation of the chitin microfibrils (Zhang and others 2005). The α-chitin form with chains packed in antiparallel is found in shrimps, crabs and fungi

while β-chitin with parallel chains occurs mainly in algae and squid pens. The γ-chitin form refers to a mixture of α- and β-chitins (Jang and others 2004). Chitin degradation studies by thermogravimetric analysis (TGA) in the 20 to 700°C have shown that the thermal decomposition activation energies of α-chitin, β-chitin, and γ-chitin (60.6, 58.2, and 59.3 kJ/mol, respectively) reflect differences in intersheet and intrasheet hydrogen-bonds which are stronger for α-chitin than β-chitin and γ-chitin (Jang and others 2004).

Diatoms are unicellular algae with a solid silica shell composed of two parts or frustules fitting into each other like a petri dish termed epitheca (larger) and hypotheca (smaller). The silica shell is used to classify them into centric and pennate diatoms. The symmetry of pennate diatoms is radial while that of the centric diatoms, more frequently found in marine environments, is bilateral (Alexopoulos and Bold 1967; Round and others 1990). Although various crustaceans, principally shrimps and crabs, are important current sources of raw material for commercial chitin production, the field of natural polysaccharides of marine origin is expanding rapidly. Microalgae have become particularly interesting because of their biochemical diversity and the possibility to control its growth using bioreactors (Laurienzo 2010). *Cyclotella* and *Thalassiosira* are examples of photosynthetic marine diatoms biosynthesizing lipids and chitin as well as nanostructured biosilica (McLachlan and others 1965; Herth and Barthlott 1979; Hoagland and others 1993).

In most biomass sources, chitin is closely associated with lipids, proteins,

minerals and pigments requiring harsh conditions for their extraction (Percot and others 2003) while diatoms produce extracellular chitin (McLachlan and others 1965; Herth 1979; Herth and Barthlott 1979). A particularly pure form of β-chitin can be found in the monocrystalline spines of the marine diatom *Thalassiosira fluviatilis* Hustedt (McLachlan and others 1965) as confirmed by chitinase susceptibility tests (Walsby and Xypolyta 1977). Some species of diatom such as *Cyclotella* spp. produce an extracellular spine made of essentially pure β-chitin fibrils (Herth and Barthlott 1979). Transmission and scanning electron microscopy has been used to investigate the formation of β-chitin fibrils by centric diatom algae including *Cyclotella cryptica*, *Cyclotella nana*, *Cyclotella meneghiniana*, and *Thalassiosira fluviatilis* (Herth and Barthlott 1979). These extracellular sources of chitin have the potential of being useful when manufacturing chitin/chitosan-based structures and ingredients for biomedical and food applications (Knorr 1986; Agulló and others 2003; Noishiki and others 2003; Laurienzo 2010).

Chitin can be extracted and isolated by chemical, physical, enzymatic and microbiological methods (Ghorbel-Bellaaj and others 2012). Although many methods can be found in the literature for the removal of proteins and minerals to obtain pure chitin or chitosan, detrimental effects on their molecular weight and degree of acetylation may be difficult to avoid (Percot and others 2003). Therefore, the objectives of this study were to optimize the recovery of chitin produced by the diatom *Cyclotella* spp. and while minimizing its degradation to lower impurity levels for its potential use in food and biomedical applications.

7.2. Materials and methods

Previous studies have reported the recovery of chitin microfibers from diatom cells by selective dissolution of biosilica with hydrofluoric acid (Brunner and others 2009; Tsuji and others 2009). This alternative was discarded for hydrofluoric acid toxicity reasons (Bertolini 1992). In this study, a multiple step approach was used to recover chitin from diatom fermentation broths and then depolymerize it to obtain *N*-acetyl glucosamine. In step 1, the long chitin fibers (>60 μm) were detached or dislodged from the *Cyclotella* cells by shear forces during mixing in a blender (Tsuji and others 2009; Nishiyama and others 2011). In step 2, the diatom cells were separated from the chitin suspension by low speed centrifugation. In step 3, high speed centrifugation was used to harvest a chitin pellet for further purification. Finally in step 4, HCl hydrolysis was required to obtain glucosamine.

7.2.1. Chitin sources

Samples of a commercial suspensions and dry matrices (Talymed®) of diatom chitin fibers obtained from microalgae were provided by Marine Polymer Technologies, Inc. (Burlington, MA). Fermentation diatom broth samples received from the Chemical Engineering Department at Oregon State University (Corvallis, OR) were prepared in a 5 liter bubble column photobioreactor using a modified Harrison's artificial seawater medium (Harrison and others 1980). The photobioreactor runs were designed to cultivate *Cyclotella* spp. in a single-stage batch growth mode under silicon and sodium nitrate co-limitations. The initial dissolved silicon concentration was

targeted to provide three to four doublings, and the sodium nitrate was varied to obtain depletion before, during, and after silicon depletion occurred. The experiments were run until the cells reached the stationary phase, and when the concentrations of silicon and sodium nitrate were depleted from the medium. Fermentation broths were stored under refrigeration (4°C) before analysis within 24 h.

7.2.2. Chemicals and reagents

Reagent grade glucosamine hydrochloride (Part # G4875) used as a reference standard for HPLC analysis was obtained from Sigma-Aldrich (Milwaukee, WI). Reagent quality hydrochloric acid (12.2M) and sodium hydroxide pellets were obtained from EMD Millipore (Billerica, MA) and Macron Fine Chemicals™ (Center Valley, PA), respectively. Ultrapure HPLC grade water and sodium n-dodecyl sulfate (SDS, 99%) were obtained from Alfa Aesar (Ward Hill, MA). Finally, ethanol (95%) was purchased from the Oregon State University Chemistry Stores (Corvallis, OR).

7.2.3. Recovery of unpurified chitin

Chitin fibers were dislodged from the diatom cells in the fermentation broth in a laboratory blender (10 s) (Waring® Blender 700A, Waring Laboratory Science, New York City) alone or followed by sonication (Bransonic B2200R-1 Ultra Sonic Cleaner, Fischer Scientific, Waltham, MA) for 10 min. Samples of the resulting suspension (20 ml) were centrifuged at 750*g* for 10 min, 1500*g* for 1, 3, 5, 10, and 15 min and 3000*g*

for 10 min (Figure 7.1). “Supernatant 1” was expected to retain chitin fibers and “Pellet 1” to remove diatom cells with only trace amounts of chitin still attached to them. “Supernatant 1” samples obtained after 750, 1500 or 3000*g* for various amount of time were then centrifuged at 11000*g* for 30 or 60 min yielding “Pellet 2” and “Supernatant 2” containing unpurified chitin and the soluble broth components with only trace amounts of chitin, respectively. Samples of the broth suspension (2 ml) after blending or blending/sonication, and of supernatants 1 and 2 were analyzed for chitin content following the procedures previously reported (Chotyakul and others 201X-a). Chitin recovery after the first (CR_1) and losses after the second centrifugation (CL_2) was determined as follows:

$$CR_1 = \left(\frac{Chitin_{Supernatant\,1}}{Chitin_{Broth\ Suspension}} \right) * 100 \quad (1)$$

$$CL_2 = \left(\frac{Chitin_{Supernatant\,2}}{Chitin_{Broth\ Suspension}} \right) * 100 \quad (2)$$

7.2.4 Chitin purification

The chitin purification method evaluated in this study was adapted from the work by Vournakis et al. (1997) and Tsuji et al. (2009). First, calcium and other salts contained in the unpurified chitin “Pellet 2” (about 3.5 ml obtained from 20 ml of fermentation broth) were solubilized by adding 30 ml of 1M HCl at 70°C followed by mild stirring for 30 min. The suspension was then decanted into a Buchner filtration

unit with a polytetrafluoroethylene (PTFE) membrane filter with 1 μm pore size (Pall® Life Science, Pall Corp., Port Washington, NY). Fibers collected on the membrane filter were washed with four times the volume of the original suspension of distilled water. Chitin fibers were then detached from the membrane filter and further purified by resuspension in 50 ml of 0.5% sodium dodecyl sulfate (SDS) and mild overnight stirring at room temperature (23ºC) to remove insoluble proteins. The suspension was then transferred to a Buchner filtration unit where the fibers collected on the PTFE membrane filter were washed with distilled water until no foam formed in the Buchner flask. Finally, organic residues in the chitin fibers were removed by resuspending the chitin fibers in 50 ml of 95% ethanol followed by mild stirring at room temperature for 30 min. Thereafter, chitin fibers were allowed to sediment without disturbing for 1 h. The top clear ethanol layer (40 ml) was removed and the chitin fibers in the remaining 10 ml ethanol were dried for 4 h in a 45°C oven.

7.2.5. Glucosamine production by chitin acid hydrolysis

Based on previous work (Chotyakul and others 201X-a) acid hydrolysis was used in this study to obtain glucosamine from the purified chitin. Purified recovered chitin (2.5 mg) was resuspended in 2 ml distilled water in a 25 ml glass tube to which 4 ml 12M HCl were added to yield 8M HCl. Samples were then vortexed at low speed before glass tubes were loosely capped and incubated for 3 h at 90°C in a dry heating block (Model 113004, Boekel Scientific, Feasterville, PA). After cooling in a room

temperature water bath, the reaction was stopped by adding gradually 8 ml of 6M NaOH to neutralize the solution (pH 11-12). Aliquots (1 ml) of the reaction mixture were transferred to Eppendorf tubes and centrifuged at 12000*g* for 10 min before transferring into vials for HPLC quantification of glucosamine.

7.2.6. Glucosamine quantification by HPLC

HPLC detection of glucosamine was done as described by Chotyakul and others (201X-a). Briefly, the Dionex modular HPLC unit consisted of a GP50 pump, ED40 detector, LC30 column oven and an AS3500 autosampler (Thermo Fisher Scientific Inc. Sunnyvale, CA). Conditions for analysis were Dionex MA-1 column (4x250mm) with MA-1 guard column (4x50mm) operated at 30°C. The isocratic 0.4 ml/min mobile phase was 750mM NaOH. Glucosamine detection after 10µl sample injection was via pulsed amperometric mode using the quad potential waveform with a disposable Au on PTFE electrode.

7.3. Results and discussion

7.3.1. Chitin separation and purification

Step 1 (10 s in a laboratory blender) in the recovery of chitin from the diatom photobioreactor broth (Figure 7.1) ensured homogeneity of the broth and helped dislodge chitin from the diatom cells. A mild centrifugation (750-3000*g*, Step 2) was then optimized to recover diatom cells in pellet 1 while leaving chitin fibers in supernatant 1. The ratio of the chitin content in 2 ml aliquots of supernatant 1 and the

broth suspension (CR_1, Eq. 1) showed that centrifugation at 1500g for 1 min removed diatom cells from the supernatant as confirmed by microscope observations and yielded the highest chitin recovery in the supernatant (Table 7.1). Considering that it could help separating chitin from diatom cells, a 10 min sonication treatment prior to the mild centrifugation was tested but microscopic examination showed fractured diatom cells. This may explain why mild centrifugation could not separate the diatom cell fractions from the chitin and why only 11.6 and 35.5% of the total chitin was recovered in the supernatant Table 7.1). Broth samples subjected to mild centrifugation without blending showed lower chitin recovery than those pretreated in the blender (Table 7.1). The 1500*g* mild centrifugation optimum was then confirmed by treating samples of fermentation broth type 1 and 2 for 1, 3 and 5 min. Again, the highest chitin recovery values were observed after 1 min (96.6±0.18 and 84.6±0.02% for broth 1 and 2, respectively Table 7.2). These values confirmed that chitin loss after the mild centrifugation (1500*g*, 1 min) was less than 10%.

An intense centrifugation (11000*g*, Step 3) was then applied to recover chitin fibers for further purification and subsequent hydrolysis to obtain glucosamine. No significant differences were observed when supernatant 1 was treated for 30 or 60 min at 11000*g* showing less than 10% chitin loss in supernatant 2 for both centrifugation times (CL_2, Table 7.1). Therefore, the optimized centrifugation for both mild and intensive conditions showed exceeding 80% unpurified chitin recovery. The optimum conditions identified in this study, 1500g/1 min followed by 11000g/30 min, were less severe than the one reported by Nishiyama et al. (2011) who proposed 2000*g* and

19000*g* centrifugation steps to collect a cell-free chitin spine pellet.

7.3.2. Chitin purification and optimization of chitin hydrolysis into glucosamine

Acid treatment was tested to remove calcium and other salts contained in the diatom chitin pellet 2 obtained after mild (1500*g*, 1 min) and intensive centrifugation (11000*g*, 60 min). Although hydrofluoric acid has been used for this purpose (Vournakis and others 1997; Tsuji and others 2009), 1M HCl was used in this study followed by 0.5% SDS and 95% ethanol to remove proteins and organic residues, respectively. These three purification steps yielded chitin with average 70.7±16.6 purity (Table 7.3). Unfortunately, the purity of the glucosamine ingredients used by the dietary supplements industry, and of the chitin used to produce it, is not available in the published literature.

7.4. Conclusion

In summary, unpurified chitin recovery exceeding 80% was achieved by two centrifugation steps, 1 min at 1500*g* and 30 min at 11000*g*. The purification method using 1M HCl, 0.5% SDS and 95% ethanol yielded 70.9±16.6% purity for chitin recovered from diatom fermentation broths.

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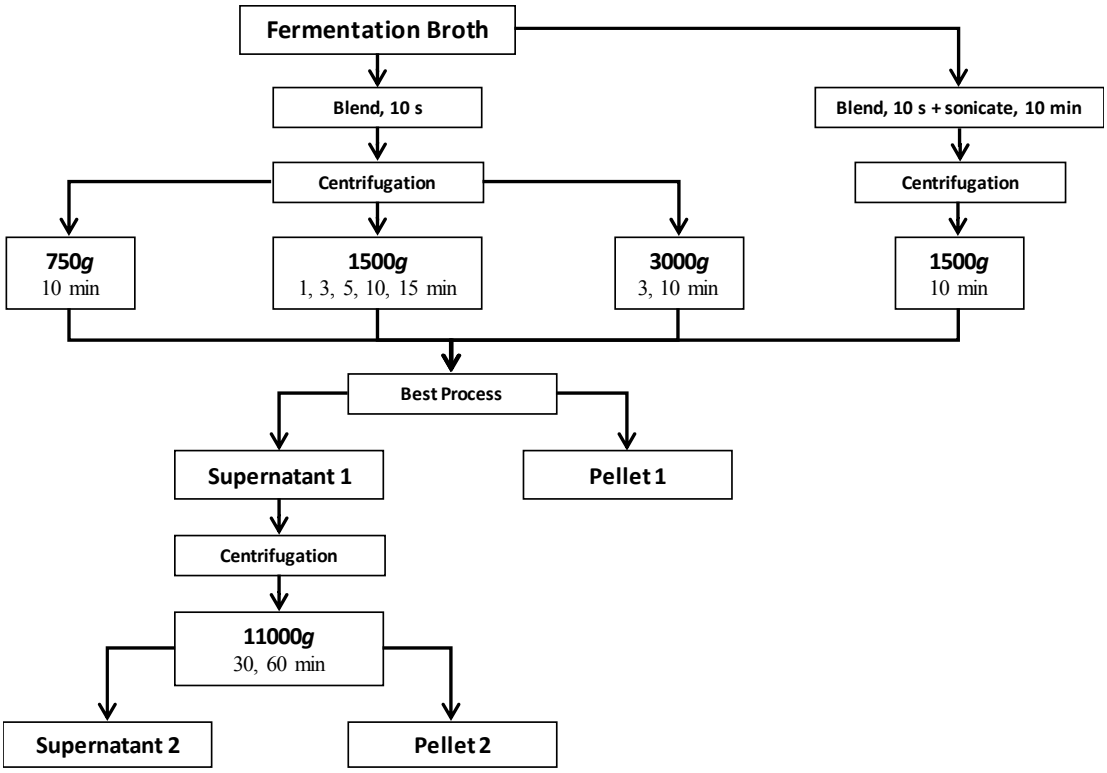

Figure 7.1. Diatom chitin isolation and preparation by centrifugation

Table 7.1. Chitin recovery (CR_1) after mild centrifugation to remove diatom cells (average ± standard deviation, %, n = 2)

Pre-treatment	Mild centrifugation			Intensive centrifugation		
	Speed, g	Time, min	CR_1, Chitin recovery %	Speed, g	CL_2, Chitin loss % Time, min	
					30	60
10 s blender	750	10	61.8±0.01	11000	6.5±0.02	7.0±0.01
	1500	1	**96.6±0.18**		5.7±0.02	5.3±0.02
		3	52.1±0.04		5.7±0.02	5.7±0.02
		5	50.7±0.06		8.2±0.00	8.7±0.00
		10	42.5±0.07		7.5±0.05	6.5±0.00
		15	45.2±0.02		8.1±0.01	6.5±0.02
	3000	10	26.9±0.02		7.5±0.02	6.5±0.02
10 s blender + 10 min sonication	1500	3	11.6±0.01		-	-
		10	35.5±0.06		7.5±0.00	6.5±0.00
no blender	1500	1	81.6±0.09		4.6±0.02	5.0±0.03

Table 7.2. Chitin recovery (CR_1) after 1500*g* centrifugation for 1-5 min to remove diatom cells and loss (CL_2) after 1500g for 1 min and 11000*g* centrifugation for 30 and 60 min (average ± standard deviation, %, n = 2)

Centrifugation		Chitin recovery, CR_1 %	
Speed, g	Time, min	Broth 1	Broth 2
1500	1	96.6±0.18	84.6±0.02
	3	52.1±0.04	82.1±0.03
	5	50.7±0.06	69.1±0.03

Table 7.3. Chitin recovery from purification after intensive centrifuge (11000*g*) for 60 min and hydrolyzed in 8M HCl, 90°C for 3 h

Run	Chitin weight (mg)	GlcN from HPLC (mg/100 ml)	Chitin purity (%)
1	0.90	5.75	89.40
2	0.90	3.69	57.35
3	0.80	3.73	65.26
Average			70.7±16.6

CHAPTER 8

General Conclusion

Analytical chemistry was shown to be critically important in the diverse studies included in this dissertation seeking effective methods to describe the effects of processes, packaging, distribution and storage on the retention of vitamins in milk. The same was true when determining the production rate, recovery and purification of chitin produced in a diatom photobioreactor.

In a first study, an extraction and HPLC method was developed and optimized to quantify the hydrosoluble and labile ascorbic acid (AA) vitamin in commercial milk samples obtained from processing plants and retail outlets. Raw, pasteurized and UHT milk of different brand and fat content commercialized in various containers were analyzed for AA content ranging values from 0.21 to 16 mg/l. AA levels showed the highest values for organic milk and varied for pasteurized and UHT milk with different fat levels, indicating a whole<semi-skim<skim general trend in AA content. Analysis of milk collected from retail outlets showed that the Tetra Brik^{TM} container was a packaging choice superior to opaque plastic bottles. A shelf life study indicated that after 14 d of storage at 4ºC in the dark, the decrease in AA content ranged 83% to 35% depending on milk type and preservation method used. A higher retention of AA was observed when the milk container was kept unopened. Liposoluble vitamins (A & E) in milk were the focus of a second study. Four methods to simultaneously determine by HPLC the concentrations of retinol, α-tocotrienol, α-tocopherol, and β-carotene in cow's milk were compared. The simultaneous determination of these

vitamers in several brands of raw, pasteurized conventional and organic milk and commercial UHT milk with different fat content showed values ranging 0.055-5.540 (retinol), 0.135-1.410 (α-tocopherol), 0.040-0.850 (β-carotene) mg/l. Most processed foods today are heat treated to kill bacteria, which often diminishes product quality. In a third study, high pressure processing treatments at moderate temperatures showed that the liposoluble vitamins A & E were found to be more stable than the hydrosoluble vitamin C. Milk concentration of retinol, α-tocopherol and β-carotene was not greatly influenced by pressure treatments and their degradation did not seem to be accelerated by pressure. However, even the least severe pressure and temperature combinations resulted in extensive AA degradation.

The Life Cycle Analysis and the Techno-Economic Analysis of a diatom photobioreactor has been reported to highlight the importance of high yield and recovery of chitin from the fermentation broth, and also for high rates of its conversion into glucosamine. Therefore, the fourth study focused on the quantitative assay of total chitin in the diatom fermentation broth. Acid hydrolysis of the whole diatom cell suspension and the fermentation broth was used to convert chitin into glucosamine quantified by HPLC analysis. In the fifth study, two centrifugation steps were optimized to recover chitin from the diatom fermentation broth achieving recovery of unpurified chitin exceeding 80%. A three-steps purification procedure yielding chitin with 70.9±16.6% purity.

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APPENDICES

A. Additional Information

Several samples of fermentation broths, sterile and after diatom cultivation in a 5 liter bubble column photobioreactor using a modified Harrison's artificial seawater medium (Harrison and others 1980) in chapter 6 and 7 were received from the Chemical Engineering Department at Oregon State University (Corvallis, OR).

Table A-1. Biofermentor experimental parameters

Experiment parameters	Values	Units
Silicon	0.7	mM
Nitrate	0.5, 1, 2, 5	mM
Initial cell density	4.5	L
Inoculum age	14	days
Initial pH	8.1	-
Air flow	1400	ml/min
Temperature	20	°C
Light intensity	150	mE/m^2s
Photoperiod (Light/Dark)	14/10	h

B. Modified Harrison's artificial seawater medium

Artificial seawater enriched with nutrients, trace metals and vitamins used to cultivate diatom cells in bioreactor.

Harrison's artificial seawater medium preparation:

Zinc Sulfate Heptahydrate Superstock

1. Measure out the following masses of components and add to 1 L volumetric flask
   - 0.1897 g Zinc Sulfate Heptahydrate ($ZnSO_4$ $7H_2O$) FW 287.56
2. Fill with deionized H_2O to the 1 L mark
3. Mix with a stir bar until all salts have dissolved
4. Pour solution into a 1 L kimax bottle and label "Zinc Sulfate Heptahydrate, 0.1897 g/L"

Sodium Molybdate Dihydrate Superstock

1. Measure out the following masses of components and add to 1 L volumetric flask Record the lot number, expiration date, and actual mass added
   - 0.0347 g Sodium Molybdate Dihydrate (Na_2MoO_4 $2H_2O$) FW 241.95
2. Fill with deionized H_2O to the 1 L mark
3. Mix with a stir bar until all salts have dissolved
4. Pour solution into a 1 L kimax bottle and label "Sodium Molybdate Dihydrate, 0.0347 g/L"

Copper Sulfate Pentahydrate Superstock

1. Measure out the following masses of components and add to 1 L volumetric flask
    - 0.0452 g Copper Sulfate Pentahydrate ($CuSO_4$ 5 H_2O) FW 249.69
2. Fill with deionized H_2O to the 1 L mark
3. Mix with a stir bar until all salts have dissolved
4. Pour solution into a 1 L kimax bottle and label "Copper Sulfate Pentahydrate, 0.0452 g/L"

Sodium Selenite Superstock

1. Measure out the following masses of components and add to 1 L volumetric flask
    - 0.0329 g Sodium Selenite (Na_2SeO_3) FW 172.94
2. Fill with deionized H_2O to the 1 L mark
3. Mix with a stir bar until all salts have dissolved
4. Pour solution into a 1 L kimax bottle and label "Sodium Selenite, 0.0329 g/L"

Cobalt (II) Sulfate Heptahydrate Superstock

1. Measure out the following masses of components and add to 1 L volumetric flask
    - 0.2776 g Cobalt (II) Sulfate Heptahydrate ($CoSo_4$ $7H_2O$)
2. Fill with deionized H_2O to the 1 L mark
3. Mix with a stir bar until all salts have dissolved
4. Pour solution into a 1 L kimax bottle and label "Cobalt (II) Sulfate Heptahydrate, 0.2776 g/L"

Nickel Chloride Hexahydrate Superstock

1. Measure out the following masses of components and add to 1 L volumetric flask
    - 0.0284 g Nickel Chloride Hexahydrate ($NiCl_2$ $6H_2O$) FW 237.69
2. Fill with deionized H_2O to the 1 L mark
3. Mix with a stir bar until all salts have dissolved
4. Pour solution into a 1 L kimax bottle and label "Nickel Chloride Hexahydrate, 0.0284 g/L"

Stock Solution Preparation, 2L, 10x:

Measure out the following components in a 2 L volumetric flask.

- 0.212 g Iron(III) Chloride Hexahydrate ($FeCl_3$ $6H_2O$)
- 0.308 g Ethylenedinitrol tetraacetic Acid Disodium Salt ($C_{10}H_{14}O_8N_2Na_2$ $2H_2O$)
- 0.0204 g Manganese (II) Sulphate Tetrahydrate ($MnSO_4$ $4H_2O$)
- 5 mL of "Zinc Sulfate Heptahydrate Superstock"
- 5 mL of "Cobalt (II) Sulfate Heptahydrate Superstock"
- 5 mL of "Sodium Molybdate Dihydrate Superstock"
- 5 mL of "Copper Sulfate Pentahydrate Superstock"
- 0.1 mL of "Sodium Selenite Superstock"
- 1 mL of "Nickel Chloride Hexahydrate Superstock"

1. Fill with deionized H_2O to the 2 L mark on a volumetric flask
2. Mix with a stir bar until all salts have dissolved

4. Pour solution into a 2 L kimax bottle and label “Stock Solution 5, 10x”.