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Quantification of vitamin B12: Evaluation of the impact of nonthermal (HP, PEF and UV-C) and Thermal Technologies of processing in milk

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Preface

This thesis follows the requirements for acquiring the Ph.D. title at the Instituto de Química de São Carlos, Universidade de São Paulo, Brazil and the Department of Food Science, University of Copenhagen, Denmark, in the category of double Ph.D. degree. The project has been developed through the collaboration between both departments according to the main project entitled Novel Aging: Technologies and solutions to manufacture novel dairy products for healthy ageing, which involved the Department of Food Science - University of Copenhagen, Instituto de Química de São Carlos - Universidade de São Paulo, Embrapa Cattle and Instrumentation and Unesp - Araraquara. The project was supported by Fapesp, grant number 17/01189-0.

Firstly, I would like to express my gratitude to God for conceding me the necessary courage and commitment never to give up.

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Resumo

Até o ano de 2050, estima-se que a proporção da população mundial com mais de 60 anos quase dobre de 12% para 22%. O envelhecimento induz doenças nos idosos, tais como a deficiência de vitamina B12, considerada um problema de saúde pública. O leite é considerado uma das principais fontes desta vitamina, contendo 0,54 μg de B12 / 100 g de leite, contribuindo para o fornecimento de grande parte da dose diária recomendada (2,4 μg de B12) para uma boa saúde. No entanto, os tratamentos térmicos como pasteurização e ultra alta temperatura (UHT) amplamente empregados pelas indústrias alimentícias têm sido descritos por comprometerem os níveis de B12. Por outro lado, tecnologias não térmicas como alta pressão (HP), campo elétrico pulsado (PEF) e luz ultravioleta (UV-C) têm sido estudadas e apontadas como tratamentos suaves e de baixo impacto sobre as características químicas dos constituintes do leite. Tais efeitos foram relatados do ponto de vista da gordura, proteínas e outros componentes, mas nenhuma informação sobre o impacto dessas tecnologias sobre a B12 foi relatada para os processamentos de HP e PEF. Ademais, o impacto do calor tem sido mencionado na literatura, mas até onde sabemos, não foram feitos estudos sobre a cinética da degradação térmica da B12, considerando os efeitos de temperaturas elevadas. Assim, a presente tese objetivou investigar esses aspectos. No caso das tecnologias não térmicas, HP e PEF não impactaram os níveis de B12, sendo considerado HP a 600 MPa a condição mais promissora para tratar o leite mantendo os níveis de B12 e proporcionando um produto seguro quanto aos aspectos de inativação microbiana. Nos demais casos (HP a 400 e 500 MPa e PEF 12 μs), embora a concentração da vitamina não tenha sido afetada, obteve-se uma contagem microbiana mais pronunciada. No caso do processamento com luz UV-C, os níveis de B12 mostraram uma tendência a diminuir de acordo com a dose mais intensa aplicada nas amostras, em que 18 mJ / cm^2 levou a uma redução de 10% da concentração de B12 em relação ao controle. No entanto, não foi observada inativação microbiana nas amostras tratadas com luz UV-C. Do ponto de vista dos estudos cinéticos, a degradação térmica da B12 foi confirmada para temperaturas na faixa de 100 a 140 ^{o} C. A energia de ativação de 130 ± 5 kJ / mol foi obtida, e a entalpia e entropia de ativação foram de 126 ± 5 kJ / mol e 19 ± 14 J / mol.K, respectivamente, demonstrando que a degradação térmica desta vitamina é um processo dependente da temperatura, na qual a molécula sofre a formação de produtos não bioativos. Os estudos demonstraram a importância de se considerar tecnologias não térmicas para o tratamento do leite e a necessidade de melhor conhecimento

sobre a extensão dos tratamentos térmicos na degradação da B12. Ambos os estudos podem contribuir para que haja alternativas de processamento e/ou condições de tratamento do leite, mantendo os níveis de B12 no produto, o que é essencial para suprir as necessidades desta vitamina requeridas para uma boa condição de saúde, principalmente na população idosa.

Abstract

The proportion of the world's population over 60 years is estimated to almost double from 12% to 22% by 2050. Ageing induces diseases in the elderly, such as vitamin B12 deficiency, which is considered a public health issue. Milk is considered one of the main sources of this vitamin, containing 0.54 μg of B12 / 100 g of milk, contributing to the supply of a great part of the recommended daily dose (2.4 μg of B12) for good health. However, thermal treatments such as pasteurization and ultra high temperature (UHT) widely applied by food industries have been claimed to compromise B12 levels. On the other hand, nonthermal technologies such as high pressure (HP), pulsed electric field (PEF) and ultraviolet light (UV-C) have been studied and pointed out as mild treatments with low impact on the chemical characteristics of milk constituents. Such effects have been reported from the point of view of fat, proteins and other components, but no information has been reported on the impact of these technologies on B12 for HP and PEF processing. Furthermore, the impact of heat has been mentioned in the literature, but to the best of our knowledge, no studies have been done on the kinetics of thermal degradation of B12, considering the effects of high temperatures. So, the present thesis aimed to investigate these aspects. In the case of the nonthermal technologies, HP and PEF did not impact B12 levels, being considered HP at 600 MPa the most promising condition to treat milk keeping B12 levels and providing a safe product regarding microbial inactivation aspects. In the other cases (HP at 400 and 500 MPa and PEF 12 μs), although vitamin concentration was not affected, a more pronounced microbial count was obtained. In the case of UV-C light processing, B12 levels showed a tendency to diminish according to the most intense dose applied in the samples, in which the 18 mJ / cm^2 led to a reduction of 10% of B12 concentration compared to the control. However, no microbial inactivation was observed in the samples treated with UV-C light. From the point of view of the kinetic studies, the thermal degradation of B12 was confirmed for temperatures in the range of 100 to 140 ^{o}C. The activation energy of 130 ± 5 kJ / mol was obtained, and the activation enthalpy and entropy were 126 ± 5 kJ / mol and 19 ± 14 J / mol.K, respectively, demonstrating that the thermal degradation of this vitamin is a process dependent on the temperature, in which the molecule undergoes the formation of non-bioactive products. The studies demonstrated the importance of considering nonthermal technologies to treat milk and the need for better knowledge about the extension of thermal treatments on B12 degradation. Both studies can contribute to having alternative processing

and/or conditions to treat milk, maintaining B12 levels in the product, which is essential to supply the requirements of this vitamin for a good health condition, mainly in the elderly population.

Resumé

Andelen af verdens befolkning over 60 år anslås at være næsten fordoblet fra 12 % til 22 % i 2050. Aldring fremkalder sygdomme hos ældre, såsom vitamin B12-mangel, som betragtes som et folkesundhedsproblem. Mælk, der indeholder 0,54 µg B12/100 g, anses for at være en af hovedkilderne til dette vitamin, og bidrager til indtaget af en stor del af den anbefalede daglige dosis (2,4 µg B12) for et godt helbred. Imidlertid er termiske behandlinger som pasteurisering og ultrahøj temperatur (UHT) behandling, der er almindeligt anvendt i fødevareindustrien, blevet hævdet at kompromittere B12-niveauerne. På den anden side er ikke-termiske teknologier såsom højtryk (HP), pulserende elektrisk felt (PEF) og ultraviolet lys (UV-C) behandlinger blevet undersøgt og påpeget som milde behandlinger med lav indvirkning på mælkebestanddelenes kemiske egenskaber. Sådanne effekter er blevet rapporteret for fedt, proteiner og andre komponenter, men der er ikke rapporteret information om virkningen af disse teknologier på B12, f.eks. HP- og PEF-behandling. Ydermere er påvirkningen af varme blevet nævnt i litteraturen, men så vidt vi ved, er der ikke lavet undersøgelser af kinetikken for termisk nedbrydning af B12 ved høje temperaturer. På baggrund af dette har nærværende afhandling til formål at undersøge disse aspekter. Med hensyn til de ikke-termiske teknologier påvirkede HP og PEF ikke B12-niveauerne i mælk, og HP ved 600 MPa blev betragtet som den mest lovende behandling af mælk, der bibeholder B12-niveauerne og giver et sikkert produkt med hensyn til mikrobiel inaktivering. I de andre tilfælde (HP ved 400 og 500 MPa og PEF i 12 µs), blev vitaminkoncentrationen ikke påvirket, men der blev opnået et højere antal mikroorganismer. I tilfælde af UV-C lysbehandling viste B12-niveauer en tendens til at falde ved den mest intense dosis anvendt, idet de 18 mJ/cm2 førte til en reduktion på 10 % af B12-koncentrationen sammenlignet med kontrollen. Der blev imidlertid ikke observeret mikrobiel inaktivering i prøverne behandlet med UV-C lys. Med hensyn til de kinetiske undersøgelsers blev termisk nedbrydning af B12 bekræftet for temperaturer i området 100 til 140 °C. Aktiveringsenergien blev beregnet til 130 ± 5 kJ / mol, og aktiveringsentalpien og -entropien til henholdsvis 126 ± 5 kJ / mol og 19 ± 14 J / mol.K, hvilket viser, at den termiske nedbrydning af dette vitamin er en proces, der afhænger af temperaturen, hvor molekylet undergår dannelsen af ikke-bioaktive produkter. Undersøgelserne viste vigtigheden af at overveje ikke-termiske teknologier til behandling af mælk og behovet for bedre viden om udvidelsen af termiske behandlinger på B12-nedbrydning. Begge undersøgelser bidrager med viden til alternativ

forarbejdning og/eller betingelser til at behandle mælk, der kan opretholde B12-niveauerne i produktet, hvilket er afgørende for at opfylde behovet for dette vitamin for en god helbredstilstand, hovedsageligt hos den ældre befolkning.

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List of Abbreviations and Symbols

B12 – vitamin B12

CNCbl and CN - cyanocobalamin

OHCbl and OH – hydroxocobalamin

H_2OCbl – aquacobalamin

MeCbl, CH_3Cbl and CH_3 – methylcobalamin

AdoCbl and Ado – adenosylcobalamin

DMBz - 5,6-dimethylbenzimidazole base

CE-UV - Capillary electrophoresis-ultraviolet

PDA - photodiode array

MALDI-MS - Matrix-assisted laser desorption/ionization-mass spectrometry

HPLC-UV – High-performance liquid chromatography-ultraviolet

UHPLC-UV – Ultra-high performance liquid chromatography-ultraviolet

RP – Reversed phase

PEF – Pulsed electric field

HP – High Pressure

UV-C – Ultraviolet light (band C)

PCA – Plate count agar

MFGM – Milk fat globule membrane

β-Lg – beta lactoglobulin

α-La - alpha lactalbumin

PA – Pasteurization

CD0 – Control day zero

CD1 – Control day one

LOD – Limit of detection

LOQ – Limit of quantification

HTST – High temperature short time

UHT – Ultra high temperature

Contents

Chapter I - Introduction, Theoretical background and General objectives

1. Introduction

According to WHO (2015), between 2015 and 2050, the proportion of the world's population over 60 years will nearly double from 12% to 22%. By this year, the number of people aged 80 years or more is expected to triple, reaching 426 million. Two-thirds of this population corresponds to people who will live in low- and middle-income countries.

From a biological perspective, ageing is defined as the result of the impact of the accumulation of a range of molecular and cellular damage over time, compromising the physical and mental capacity of the person, which leads to a growing risk of diseases (WHO, 2015). Age induces physiological changes in senior citizens, such as dysphagia, loss of vision, taste and smell, and reduces nutrient absorption in the gastrointestinal tract (KANEKANIAN, 2014). These factors are known to cause a reduction in food and nutrient intake, leading to malnutrition and, consequently, to some pathologies such as sarcopenia, osteoporosis, fatigue and B12 deficiency, which is considered a public health problem (BAIK; RUSSELL, 1999; VOGIATZOGLOU et al., 2008). In 1999, Baik & Russell (1999) reported that B12 deficiency was estimated to have affected 10%–15% of the elderly. In 2004, this percentage was greater than 20% (ANDRÈS et al., 2004).

B12 participates as a cofactor in one carbon transfer through methylation and molecular rearrangements. It also acts as a coenzyme in many biochemical reactions, such as DNA synthesis, methionine synthesis and the conversion of propionyl into succinyl coenzyme A from methylmalonate. Therefore, B12 has a vital function in the regular metabolism of cells, fatty acid metabolism, cell division and synthesis of red blood cells. Due to this, the lack of this vitamin is clinically manifested in the blood and nervous system (ANDRÈS et al., 2004; RIZZO et al., 2016). Some diseases related to cobalamin deficiency are brain atrophy with consequent cognitive impairment (VOGIATZOGLOU et al., 2008), haematological problems such as pernicious anaemia (ANDRÈS et al., 2004) and hyperhomocysteinemia, which can be a risk factor for cardiovascular pathologies (ANDRÈS et al., 2004; MORELLI et al., 2002; SPLAVER; LAMAS; HENNEKENS, 2004; TINELLI et al., 2019). Low plasma levels of B12

were also suggested to be associated with a tendency to have depression (COPPEN; BOLANDER-GOUAILLE, 2005).

Deficiency of this vitamin is usually related to low intake compared to the recommended daily dose of 2.4 μg (HINE et al., 2014) or due to malabsorption conditions in the body involving the stomach, pancreas or intestine, in the process mediated by homologous proteins haptocorin (HC) and intrinsic factor (IF) that transport vitamin B12 in the human body (KOZYRAKI; CASES, 2013). Additionally, B12 deficiency has been reported to affect more vegetarians and vegans (WATANABE et al., 2013).

Cow milk is considered one of the main sources of B12, containing approximately 0.54 μg of B12 per each 100 g of milk (USDA, 2020). According to FAO (2022), more than 6 billion people consume milk, one of the most produced and valuable agricultural commodities worldwide. However, people who do not consume animal-based foods (WATANABE et al., 2013) can have a high risk of developing a lack of cobalamin (RIZZO et al., 2016) because its absorption is closely related not only to the levels ingested but also to the role played by the food matrix. Supplementation options consist of fortified foods, such as beverages, cereals and biscuits (Adams et al., 2017; Tucker et al., 2004), or supplementation by intramuscular injections and oral doses of the synthetic form of this vitamin (HINE et al., 2014). Therefore, a study comparing people who received oral daily doses of the synthetic form with others who ingested cow/ buffalo milk (MAHALLE et al., 2019) showed that individuals who received the synthetic form had a higher increase of B12 in the total plasma, which only reflects an accumulation of B12 in the inert protein-carrier haptocorrin in the blood. On the other hand, people who ingested milk presented a more efficient uptake of B12 because, in this food source, B12 is mainly bound to proteins, such as the B12-transcobalamin complex, which is rapidly absorbed by cells (FEDOSOV, 2012; GREEN et al., 2017). Juul et al. (2019) have also assessed the efficiency of vitamin B12 absorption by the body when free or bound to bovine/human transcobalamin transport proteins and showed that hydroxocobalamin passes through the simulated monolayer of human intestinal mucosal cells (RAMANUJAM et al., 1991) more efficiently (by a factor of 65-fold) when it is bound to bovine transcobalamin than compared to the human transcobalamin. Additionally, milk ingestion stimulates gastric juice production and intrinsic factor secretion (FEDOSOV, 2012; GREEN et al., 2017; IPPOLITI; MAXWELL; ISENBERG, 1976), which probably promotes greater absorptions of vitamin B12 in the body as opposed to what occurs when there is no additional substrate.

Based on this, bovine milk appears to be one of the primary vias trying to increase B12 intake as a way to eradicate its deficiency. However, studies have mentioned that thermal technologies such as pasteurization and ultra-high temperature (UHT) widely applied by the food industries to provide a safe product for consumption (QUIGLEY et al., 2013; WALSTRA; WOUTERS; GEURTS, 2005) can compromise the levels of this vitamin in food. The impact on B12 content has been described as a loss of around 7% in the pasteurization and up to 18% in the sterilization methods (BALL, 1998; OAMEN; HANSEN; SWARTZEL, 1989). Although the effect of heat has already been described, to the best of our knowledge, the effects of thermal degradation considering a kinetic study for UHT simulated temperatures have not been proposed yet.

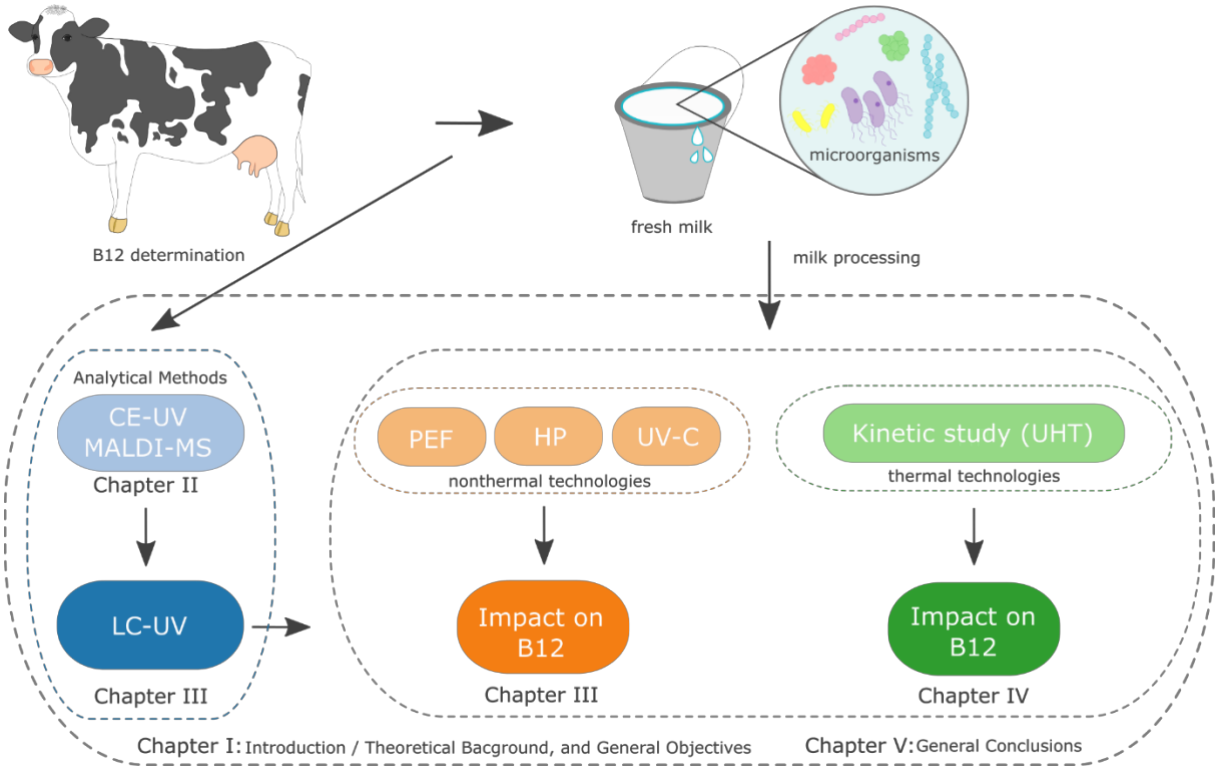
Parallelly, other techniques have been developed over the last years and presented as alternatives to traditional food processing, which are pulsed electric field (PEF), high pressure (HP) and UV-C light (UV-C) (CHOUDHARY; BANDLA, 2012; SAMPEDRO et al., 2005; YANG et al., 2020). These nonthermal technologies have been advised to reduce microorganism proliferation, and PEF and HP have been claimed to present mild effects on milk components such as fat and proteins (ALIREZALU et al., 2020; KHALIQ et al., 2021; YANG et al., 2021). To our knowledge, the effects of these treatments on B12 in milk have not been accessed so far, except for UV processing, which is known to affect vitamins (GUNESER; YUCEER, 2012), as well as B12, which is sensitive to light (FEDOSOV; NEXO; HEEGAARD, 2018; JUZENIENE; NIZAUSKAITE, 2013; SCHNEIDER, 1987).

Taking into account all these aspects, one of the objectives of the present thesis was to define an analytical method to determine and quantify vitamin B12 in milk. Another goal was to investigate the impact of nonthermal processing (PEF, HP and UV-C light) on vitamin B12 in milk. Furthermore, a kinetic study considering high treatment temperatures was proposed to evaluate the impact of the thermal degradation of B12. In both studies, vitamin B12 has been undertaken by the use of modern analytical technologies such as UHPLC-UV. The specific objectives of each study are presented in the respective chapters.

In these studies, it was possible to observe that the PEF and HP technologies do not impact B12 levels, while UV-C light can contribute to B12 depletion according to the highest doses applied, such as 18 mJ/cm^2. Regarding the kinetic study, the thermal degradation of vitamin B12 was confirmed and a mathematical model to describe the extension of the degradation was established, which provides access to the kinetic and thermodynamic parameters for this reaction. In this regard, the goals and experiments developed in this work

contributed to evaluating which technologies/conditions can be considered focusing on keeping B12 levels in the product, providing food with a high nutrient content that can reduce the B12 deficiencies or prevent the development of diseases.

These aspects are reflected in the chapters of the present thesis, as well as in this section and in the following sections in Chapter I, which includes a brief description of the basic concepts related to the study of the B12 molecule, called Theoretical background, and also the General objectives related to this work. In the sequence, Chapter II is a literature review of the methods established to analyze vitamin B12 and an explanation considering two other analytical approaches, such as capillary electrophoresis-ultraviolet and matrix-assisted laser desorption/ionization-mass spectrometry (CE-UV and MALDI-MS, respectively) as an attempt to measure B12 in this work. Chapter III is dedicated to the study of the impact of nonthermal processing (PEF, HP and UV-C) on B12 amounts measured by an improved ultra-high performance liquid chromatography–ultraviolet (UHPLC-UV) method. Chapter IV concerns the impact of heat (simulating high temperatures) on the B12 content from a kinetic perspective, and finally, Chapter V comprises the General Conclusions. Figure 1 summarizes the structure of the present thesis.



Source: Figure elaborated by the author.

Figure 1- Representative scheme of the thesis showing the structure of the chapters and the main objectives involved in this work.

2. Theoretical Background

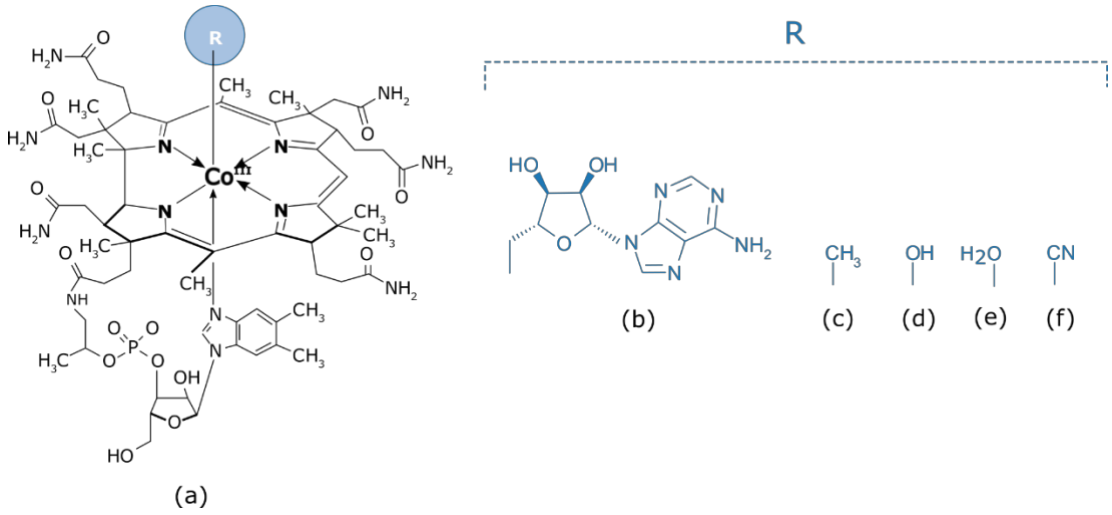
2.1. B12 molecule: forms and physicochemical properties

Vitamin B12 is a water-soluble vitamin that belongs to the B-complex group of vitamins. It is the most complex vitamin, with two Nobel Prizes associated with its studies, the first one in Medicine (1934) and the second one in 1964, due to works related to elucidating its structure (Eitenmiller & Landen, 1999).

Vitamin B12 or cobalamin is a generic name to describe a group of cobalt-coordinated compounds called corrinoids with a 5,6-dimethylbenzimidazole (DMBz) base linked to a phosphorylated ribose in the lower axial position of the molecule. Different possible ligands in

the upper axial position (R), such as 5' deoxyadenosine, CH_3, OH/H_2O and CN, attribute the names of adenosylcobalamin (AdoCbl), methylcobalamin (MeCbl), hydroxocobalamin / aquacobalamin (OHCbl / H_2OCbl) and cyanocobalamin (CNCbl) to the vitamers, respectively (WATANABE; BITO, 2018), according to Figure 2. All these forms have vitamin activity. However, only four of the B12 molecules occur naturally in food, except cyanocobalamin, which is the synthetic one (EDELMANN et al., 2016; FOX et al., 2015; GREGORY III, 2017).

In general, these molecules are tasteless, odorless and red crystalline. They have good water solubility, being soluble even in apolar solvents since they have hydroxy groups. Taking into account the spectrophotometric aspect, the maximal absorption of these molecules is at around 278, 361 and 551 nm, being the spectrum of CNCbl, OHCbl and H_2OCbl more similar, while AdoCbl and MeCbl presenting less intense absorption around 361 nm (JUZENIENE; NIZAUSKAITE, 2013) (Eitenmiller & Landen, 1999).



Source: Figure elaborated by the author.

Figure 2 - (a) Molecular structure of vitamin B12. The 6th axial coordination ligand (R) can vary according to different chemical groups such as (b), (c), (d), (e) and (f). (b) 5' deoxyadenosine group for adenosylcobalamin. (c) methyl for methylcobalamin. (d) hydroxy in the case of hydroxocobalamin. (e) aqua for aquacobalamin and (f) cyano group representing cyanocobalamin.

2.2. Main sources of vitamin B12

Vitamin B12 is synthesized exclusively by microorganisms (bacteria and archaea). Once synthesized, it is transferred along the food chain through accumulation in animal tissues, which can also occur in some species of plants and mushrooms due to microbial interaction (WATANABE; BITO, 2018). However, animal-source-based foods such as meat, liver, milk, eggs, fish and shellfish are considered the best sources of this vitamin, as a few edible plants and some mushrooms rarely offer it in a considerable amount (WATANABE et al., 2013; WATANABE; BITO, 2018).

In ruminants, vitamin B12 is synthesized by bacteria in the rumen (one of the four compartments that make up the stomach of these animals), absorbed in the intestine, transferred to the blood, and finally stored in tissues such as the liver and muscle or secreted in milk (WATANABE; BITO, 2018).

2.3. Bovine Milk and its composition

In the last three decades, milk production has increased by 59%, representing an important economic supply that can improve trade, employment, marketing and food security. In Africa and Asia, milk represents 2-4% of the dietary energy supply, 19% of the high-quality protein source in Europe and up to 14% of the dietary fat supply in Europe, Oceania and America. Cow milk represents 81% of this consumption (FAO, 2022).

According to WALSTRA; WOUTERS; GEURTS (2005), milk is denominated as the secretion of the mammary glands of mammals, whose primary natural function is the nutrition of the young. Milk can be classified as both a solution and a dispersion due to the particles in its composition, which confer its characteristic white color, presenting a pH of about 6.7 at room temperature. Milk consists of water (87%), and approximately 12 to 13% corresponds to solid elements, such as carbohydrates, proteins, lipids, minerals and vitamins.

Lactose is the main carbohydrate in milk and the distinct one composed of glucose and galactose (WALSTRA; WOUTERS; GEURTS, 2005). Proteins correspond to 3-4% of the milk solids fraction, being the caseins the predominant proteins representing about 80% (LE et al., 2011; WALSTRA; WOUTERS; GEURTS, 2005), followed by the whey proteins β-lactoglobulin (β-Lg), α-lactalbumin (α-La), bovine serum albumin (BSA) and immunoglobulins

(WANG et al., 2019). Caseins, which are composed of a mixture of four proteins (αS1-, αS2-, β- and κ-casein) together with calcium, phosphorus, and salts, are organized in the form of micelles. Milk fat is made up of small globules (triglycerides) suspended in the aqueous phase, each surrounded by a lipoprotein membrane called the milk fat globule membrane (MFGM). Lipids, together with casein micelles, are responsible for the color and consistency properties of milk (MAPA - EMBRAPA, 2019).

The main minerals in milk are calcium and phosphorus. Potassium, sodium, magnesium, zinc and iron are also present, but the last one is in low concentration. The vitamins found in milk are of both types, fat soluble and water soluble. Fatty-soluble vitamins are represented by vitamins A, D, E and K, while vitamin C and the B complex represent the water-soluble vitamins (MAPA - EMBRAPA, 2019).

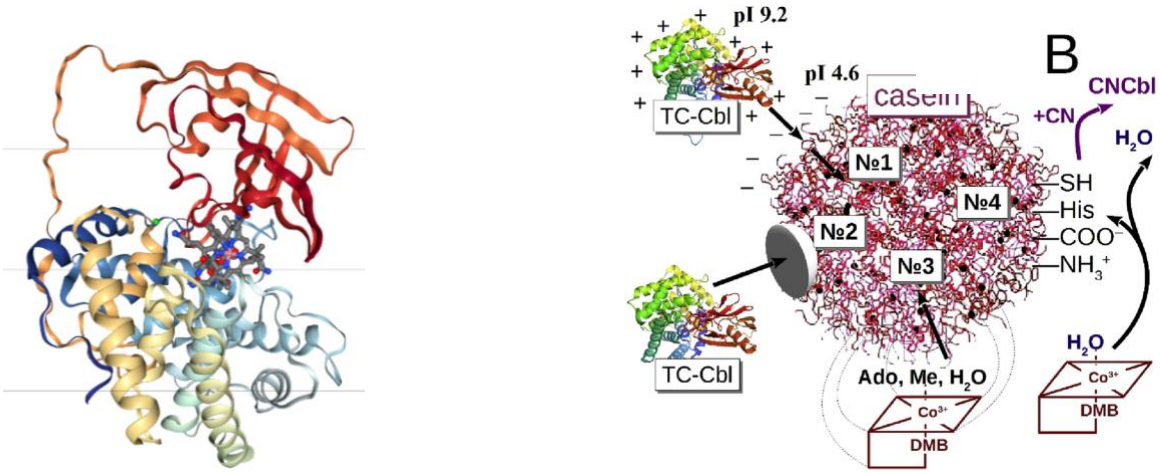
Cow milk contains approximately 0.54 µg of B12 per 100 g of milk (USDA, 2020), which can be available in free forms or complexed with proteins. In milk, B12 can occur in the possible natural forms of the molecule (AdoCbl, MeCbl, OHCbl / H_2OCbl), with hydroxocobalamin as the predominant one (FEDOSOV; NEXO; HEEGAARD, 2018). More than 95% of the hydroxocobalamin content in milk is protein-bound (FOX et al., 2015).

One of these, which appears to be the main vitamin B12 binding protein, is transcobalamin (Figure 3 (a)). It is a transport protein responsible for the preservation, uptake and targeted delivery of the vitamin, present in the whey phase, composed of around 450 amino acid residues, with 43 KDa. Based on the N-terminal sequence of this protein, it has been found to be homologous to the transcobalamin from human and rabbit plasma (FEDOSOV; NEXO; HEEGAARD, 2018; FEDOSOV; PETERSEN; NEXØ, 1996; HINE et al., 2014; NEXØ, 1998). Transcobalamin binds mainly with hydroxocobalamin and adenosylcobalamin forms (HINE et al., 2014).

β -lactoglobulins and α -lactalbumins, also whey proteins such as transcobalamin, present 18 and 14 KDa, respectively. β -lg is a globular protein with 162 amino acid residues and a β-barrel structure with multiple binding sites capable of binding small molecules in its cavity. On the other hand, α -la is a small globular protein composed of 123 amino acid residues, with a large alpha-helical domain and a small beta-sheet domain, which are separated by a gap and linked by a calcium binding loop. Due to these properties, both proteins can carry small molecules such as hydrophobic lipids, metabolites and vitamins. β -lactoglobulin and α -lactalbumin can bind to B12 in its specific adenosylcobalamin form (WANG et al., 2019).

In addition to whey proteins, caseins can carry large amounts of hydroxocobalamin / aquacobalamin based on four interaction mechanisms. The main one seems to be the interaction in which B12 directly binds casein micelles by coordination of the vitamin with the structure of the histidine residues in the casein (HOCbl via histidine-[Co3+]-Cbl). Another possibility is the nonspecific microporous adsorption of free Cbl in the micelles. Furthermore, the transcobalamin-cobalamin complex (TCCbl) can attach to the structure of the casein due to the adsorption of the positively charged complex in the negatively charged casein micelles or capture of the complex by a receptor integrated into the micelle (FEDOSOV; NEXO; HEEGAARD, 2018), such as shown in Figure 3 (b).

These properties of B12 binding proteins have been reported to enhance B12 stability and bioavailability. Wang et al. (2019) reported that β -lactoglobulin and α -lactalbumin could bind adenosylcobalamin or even cyanocobalamin (synthetic form), protecting these molecules from light and thermal decomposition, for example. Additionally, the interaction between B12 and transcobalamin can enhance the bioavailability of B12, as the vitamin is easily taken up by cells and absorbed by the organism (HINE et al., 2014).



(a) (b)

Source: (a) Reproduced from: PDB protein data bank, Bos Taurus Transcobalamin, code 2BBC, Rose et al. (2018); (b) Adapted from: Fedosov et al. (2018).

Figure 3 - (a) Transcobalamin bound-B12.; (b) Possible interaction patterns between Cbl (or the TCCbl complex) and casein: Nº1, nonspecific adsorption of TCCbl on the surface of the casein and incorporation of TCCbl into the micelle; Nº2, binding of TCCbl to a specific receptor encapsulated within the casein micelle; Nº3, nonspecific adsorption of Cbl onto the microporous surface of the casein micelle; Nº4, coordination of HOCbl (with substitution of water) to an amino acid residue of the casein.

2.4. Microbiology of milk

As milk represents a high nutritive medium with a propitious pH and water content, it also provides an ideal environment for the growth of many microorganisms. Milk is supposed to be sterile (TOLLE, 1980). However, it can be colonized by microorganisms when secreted due to many external sources, such as sanitizing conditions during milking and handling or environmental conditions (ORDÓÑES et al., 2005; QUIGLEY et al., 2013).

Milk can contain a wide variety of microorganisms, mainly bacteria and spores, as well as yeasts and molds (QUIGLEY et al., 2013). In this regard, the types of bacteria present in milk can be classified into three categories: psychrotrophic, mesophilic and thermoduric, having an affinity for growing at cold temperatures, ambient temperatures (20 – 45) °C, or around 45 - 60 °C, respectively (SAGER, 2020; WALSTRA; WOUTERS; GEURTS, 2005). *Lactobacillus* bacteria represent the dominant group of bacteria in raw bovine milk,

encompassing the genera *Lactococcus, Lactobacillus, Leuconostoc, Streptococcus* and *Enterococcus*. Furthermore, *Pseudomonas, Acinetobacter* and *Aeromonas spp*., all psychrotrophic populations, usually develop during cold storage (QUIGLEY et al., 2013; RAATS et al., 2011).

Taking into account the genera of bacteria cited, many of them originate from humans. On the other hand, molds can commonly develop in milk and dairy products, being capable of producing mycotoxins, which are toxic, carcinogenic, emetic, or mutagenic. Some examples are from the genera *Aspergillus, Penicillium* and *Fusarium*. Some protozoans can also contaminate and grow in milk, usually due to contamination by water or feces from the animal (WALSTRA; WOUTERS; GEURTS, 2005).

Due to the large number of microorganisms that can inhabit and proliferate in milk, it should be processed with the aim of reducing or inactivating the bacteria population, extending the shelf-life and providing a safe product for consumption. Regular pasteurization can kill pathogenic bacteria, spoilage microorganisms, as well as protozoa and molds. However, for some genera of bacteria capable of forming heat resistant spores, higher temperatures are required to eliminate them (WALSTRA; WOUTERS; GEURTS, 2005).

Thermal technologies have been established and extensively applied over the years, such as pasteurization and UHT, considering this objective (FIL-IDF, 2018; QUIGLEY et al., 2013; TETRA PAK, 2022; WALSTRA; WOUTERS; GEURTS, 2005). Additionally, other nonthermal technologies have emerged in the last decades as alternatives to heat treatments. They are techniques based on different principles such as pulsed electric field, high hydrostatic pressure and ultraviolet light processing (CHOUDHARY; BANDLA, 2012; SAMPEDRO et al., 2005; YANG et al., 2020) that also aim to inactivate microorganisms present in foods, preserving sensorial and nutritional characteristics and improving shelf life. The main concern of the food industry has been to produce safe food for consumers but with the minimum impact on food constituents. In this way, studies that have pointed out the strong effect that heat treatments can lead on milk components such as proteins and vitamins (BALL, 1998; MACDONALD et al., 2011; OAMEN; HANSEN; SWARTZEL, 1989) provided the basis for the development of nonthermal treatments, which are claimed as mild techniques (KNORR et al., 2011; SANCHO et al., 1999; SIERRA; VIDAL VALVERDE; LÓPEZ FANDIÑO, 2000; YANG et al., 2021), being promising technologies in maintaining milk composition, as well as, vitamin B12 content.

2. 5. Stability of vitamin B12

Vitamins are a food constituent with relative stability. Usually, it is possible to demonstrate a quantitative deterioration in content over a period due to many factors such as temperature, moisture, oxygen, light, pH, storage, oxidizing and reducing agents, presence of metallic ions (e.g., iron, copper), presence of other vitamins, other components of food (e.g., sulfur dioxide) and a combination of them. The degradation occurs based on the same principles, whether vitamins are naturally occurring or added to food from synthetic sources (OTTAWAY; OTTAWAY, 2010).

In the case of B12, the factors that contribute the most to the depletions have been cited as temperature, light, storage, pH, oxidizing and reducing agents and adverse interactions with vitamins B1, B2, B3 and C (OTTAWAY; OTTAWAY, 2010). Taking into account the temperature effect (WANG et al., 2019), the losses due to heat can achieve values around 4-18%, as described by applying milk processing as pasteurization or sterilization with high temperatures (BALL, 1998; OAMEN; HANSEN; SWARTZEL, 1989; WANG et al., 2019). According to Watanabe et al. (1998), heat leads to the losses of the lower axial ligand in the molecule and/or the propionamide side chain, leading B12 to a non-bioactive molecule. Light is expected to affect B12 stability, which induces the decomposition of the molecule in multiple steps, starting from the cleavage of the C-Co bond (in AdoCbl, MeCbl and CNCbl) to form hydroxocobalamin/aquacobalamin. Then the intermediate product (hydroxocobalamin) decomposes under light and the presence of oxygen, undergoing the cleavage of the corrin ring (Schneider, 1987; Eitenmiller & Landen, 1999; Hogenkamp, 1966; Juzeniene & Nizauskaite, 2013; Wang et al., 2019). The most stable form of the B12 molecule due to light has been reported as hydroxocobalamin (FEDOSOV; NEXO; HEEGAARD, 2018; JUZENIENE; NIZAUSKAITE, 2013). However, taking into account all degradation mechanisms (oxidative, photolytic, thermal, etc.), cyanocobalamin is the most stable of the cobalamin derivatives (SCHNELLBAECHER et al., 2019).

Studies have mentioned the degradation of B12 during storage (ARKBÅGE et al., 2003; BAJAJ; SINGHAL, 2020; REPOSSI et al., 2017). Ford et al. (1969) reported a 50% loss of B12 in milk for 90 days of storage, for example. Regarding pH, research has pointed out more drastic effects on B12 retention for low pHs, such as pH 2, than for pH 6 (BAJAJ;

SINGHAL, 2020). On the other hand, the molecule has also been described as unstable under alkali conditions (OTTAWAY; OTTAWAY, 2010).

Ascorbic acid has been described to have an antagonist effect in the B12 concentration because vitamin C causes the oxidation of cobalamin. Therefore, the B12 levels decrease with an increase in the concentration of vitamin C in the medium (BAJAJ; SINGHAL, 2020). Taking into account the other vitamins, the decomposition of vitamin B1 can also increase the rate of breakdown of cyanocobalamin, as in the cleavage of thiamin, 4-methyl-5-(P-hydroxyethyl) thiazole is formed or other related products, which can interact with B12, mainly due to extended storage time (DOERGE; RAVIN; CALDWELL, 1965; OTTAWAY; OTTAWAY, 2010). Regarding vitamin B3, Ahmad et al. (2003) mentioned that this vitamin can increase cyanocobalamin photolysis by visible light for pH lower than 6 or higher than 7, which was verified in experiments conducted in an aqueous solution. In the case of vitamin B2, it has been reported as a sensitizer, which under exposure to light, produces free radicals due to its decomposition and consequently, these subproducts lead to the photolysis of cyanocobalamin, increasing the degradation of the molecule (AHMAD et al., 2012; OTTAWAY; OTTAWAY, 2010; WATANABE et al., 2013).

The physicochemical principles involved in the other nonthermal techniques (PEF and HP) have not been described as negatively impacting B12 contents due to a lack of studies in the literature, worthy of further investigation, which justifies part of this work. On the other hand, similar to the temperature effects, UV processing is supposed to affect B12 stability. It is also a relevant objective in this study to understand better the extent of the impact of heat and light factors on B12 degradation.

3. Objectives

The general objective of this thesis is to study vitamin B12 from the point of view of the best analytical method to quantify the impact of thermal and nonthermal processing of milk on this vitamin. Specifically, it is focused on evaluating the stability of this vitamin.

The specific objectives are:

- Evaluate the viability of CE-UV and MALDI-MS to speciate B12 molecules;
- Evaluate the effect of the milk matrix on B12 analyses;

- Improve an analytical method for the B12 determination and quantification in milk using UHPLC-UV;
- Evaluate the impact on B12 in milk processed with nonthermal technologies, such as PEF, HP and UV-C light, in different conditions of the treatments;
- Investigate the microbicidal effect of nonthermal processing of milk;
- Investigate the impact of heat on the degradation of vitamin B12 in milk through kinetic studies applying different conditions of temperature;
- Determine the rate constant for the B12 degradation and thermodynamic parameters.

Chapter II - Analytical methods focused on B12 determination

1. Introduction

1.1. Main Techniques for Vitamin B12 analyses

Many methods have been developed to determine B12 vitamins over the years. Among them, we can cite polarographic, spectrophotometric, chromatographic (including paper, thin layer, open column, gas chromatographic and liquid chromatographic procedures), chemiluminescence, fluorimetric assay, capillary electrophoresis, mass spectrometry and microbiologic (Eitenmiller & Landen, 1999; Karmi et al., 2011; Selva Kumar et al., 2010; Tsiminis et al., 2015). The microbiological assay was one of the pioneering procedures and has been widely employed since 1930 (HEWITT, 2012). It was consolidated as a standard procedure employing *Lactobacillus delbruechii* (AOAC International Method), although other microorganisms have been applied, such as *Escherichia coli*, *Lactobacillus leichmanni* and *Euglena gracilis* (KARMI et al., 2011; TSIMINIS et al., 2017). The majority of data considering food analysis has been determined via this technique (Eitenmiller & Landen, 1999). This procedure can have high sensitivity (HEWITT, 2012), reaching a response of pg/ml of magnitude order (LI et al., 2019). On the other hand, since these microorganisms require corrinoids as a growth factor and inactive vitamin B12 analogs in the medium can be used by the microorganisms for this purpose, the amount of biologically active cobalamin can be overestimated, consisting of a low selective method (LI et al., 2019). Also, the process is laborious and time-consuming.

In most of the studies in the last two decades, the usual method employed has been pointed out as liquid chromatography (LC) with detection by fluorescence, spectrophotometry ultraviolet-visible (UV-vis), or mass spectrometry (MS) (TSIMINIS et al., 2017). One of the challenges associated with this method is that it requires a low detection limit, and the natural concentration of B12 in food is known to be low, which demands previous steps of sample preparation and pre-concentration (Eitenmiller & Landen, 1999). Taking into account that cyanocobalamin is the most stable of the cobalamin derivatives considering all degradation mechanisms (oxidative, photolytic, thermal, etc.) described in the literature (SCHNELLBAECHER et al., 2019), studies have been conducted preferably with this form of

the molecule, which can be achieved by usually adding potassium cyanide or sodium cyanide in the medium to convert all natural forms into the synthetic one (REPOSSI et al., 2017; ZIRONI et al., 2014). Despite the fact that cyanide is extremely toxic, it has been used in most of recent works (LI et al., 2019; SELVA KUMAR; CHOUHAN; THAKUR, 2010). Furthermore, solid phase extraction (SPE) procedures are required for the concentration and clean-up of the samples (LI et al., 2019).

Attempting to follow a different perspective for B12 analyses in which the conversion of B12 into the synthetic form was not used, an attempt of speciation (considering all B12 forms) and the possibility to see these forms was tried). The preliminary studies were developed using capillary electrophoresis with a UV detector (CE-UV). Later, another attempt was made employing matrix-assisted laser desorption/ionization-mass spectrometry (MALDI-MS) as an analytical method to determine B12. In the following sections, these topics are explored.

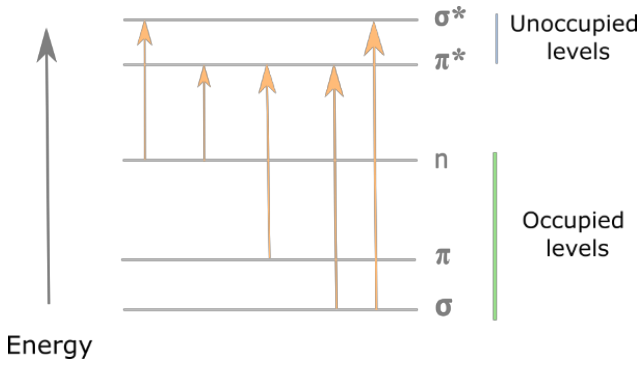
1.2. Capillary Electrophoresis

The development of high-resolution capillary electrophoresis started in 1980 due to the work developed by J. W. Jorgenson at the University of North Carolina (SMITH et al., 1993). Electrophoresis consists of a separation technique based on the movement of electrically charged particles in a liquid medium under the influence of an electric field (BAKER, 1995). The elements that make up this technique consist of the electrolyte: which is the buffer used to fill the columns and the medium in which the separation takes place; electrodes, which make the electrical connection and allow the application of the electric field in the column; capillary column, silica tube where separation takes place; sample, a mixture of neutral and ionized analytes with different charges and sizes, and the detector, region of the equipment where detection and analysis take place. There are many modes of operation in capillary electrophoresis, with Capillary Zone Electrophoresis (CZE) being the most common. One of its advantages is that it simultaneously separates cations and anions in the same run.

Different detectors can be applied, such as laser-induced fluorescence, conductivity, amperometric, radiometric, refractive index, UV absorption, fluorescence emission and MS (BAKER, 1995; SMITH et al., 1993). One of the most widely applied detectors has been UV

absorption (OLIVARES et al., 1987; SCHMITT-KOPPLIN; FROMMBERGER, 2003; SMITH et al., 1993) as the one used in this work.

UV-Vis technique is based on the absorption of UV and visible radiation by the substances analyzed. Absorption occurs when a photon of light interacts with a molecule promoting an electron from a lower to a higher energy-bound state (LOUGH; WAINER, 1996), which occurs for molecules with double bound (π electrons), or electrons not shared or bound (see Figure 4), such as olefins, aromatic compounds, among others (LANÇAS, 2009). A band represents the energy levels associated with the different transitional states that the molecules can assume, which is greater according to the number of molecules capable of absorbing light in a given wavelength (PAVIA et al., 2015).



Source: elaborated by the author.

Figure 4 – Possible transitions between electronic energy levels.

The ultraviolet-visible spectrophotometer consists of a light source, a diffraction grating and a detector. The light source is usually a deuterium lamp, which emits radiation in the UV region, whereas a tungsten lamp emits radiation in the visible region of the spectrum. The sample in the cell is directly irradiated with nonmonochromatic light. The light crosses the sample and reaches the diffraction grating, which spreads the beam of light into its component wavelengths. The light is dispersed, reaching the detector. In the detector (diode array), the intensity of the transmitted light is recorded. A diode array detector is composed of a series of photodiode detectors, each one designated to record a narrow band of the spectrum. In the end, an entire spectrum is obtained at once. The computer registers it as the absorption intensity by the function of the wavelength (LANÇAS, 2009; PAVIA et al., 2015).

One advantage of using the CE-UV technique is that it allows the analysis of the molecules in which few differences in their charge or size can be sufficient to obtain a good

separation among them (BAKER, 1995). The result represents the time the molecule requires to migrate through the capillary (expressed as an electropherogram).

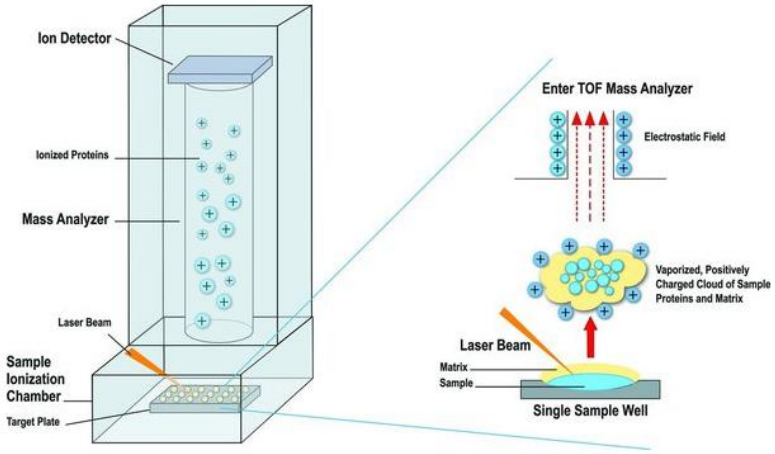
The principle of separation in CE is based on the species' electrophoretic mobility. This parameter also takes into account, among others, the charge and the hydrodynamic ratio or mass of the molecule. Considering this, a plausible expectation was that the B12 molecules could separate in different peaks during the analyses allowing the speciation of B12 forms due to their different values for these parameters. So, justifying the establishment of this method as the analytical technique to determine B12 in the present thesis.

1. 3. MALDI-MS

As an alternative to studying cobalamins by CE-UV, we tried to implement a study focused on a different technique to follow the primary goal of this work on developing an analytical method to speciate B12 forms.

Based on the work of Calvano et al. (2016), applied to the detection of cyanocobalamin in dairy products, an opportunity was tested for a new perspective for the study of B12. Additionally, other studies have shown the feasibility of employing mass spectrometry in cobalamin studies in the literature (CHEN; LING, 2002; CZERWONKA; SZTERK; WASZKIEWICZ-ROBAK, 2014; FEI; WEI; MURRAY, 1996; HE; WEI; MURRAYCOR, 1997; SCHWERTNER; VALTIER; BEBARTA, 2012; SZTERK et al., 2012).

Matrix-assisted laser desorption/ionization-mass spectrometry (MALDI-MS) consists of a technique for the ionization of molecules and separation of them based on the mass/charge ratio (m/z). In MALDI, the sample is deposited in a target plate together with a matrix, a material that absorbs UV light from a laser source (337 nm). When the laser emits a pulse, most of the radiation incident from the beam is absorbed by the matrix, leading to the ionization of the sample molecules, which are expelled from the surface of the plate. Next, the ions expelled are accelerated to a time-of-flight analyzer that separates the ions based on their speed and the criteria that the light ions are faster than the heavy ones (mass/charge ratio). Then, the ions are counted as they reach a detector, which emits a signal (electric current) proportional to the number of ions detected. In the end, this signal is registered by a computer (PAVIA et al., 2015). The data obtained in this analysis is a mass spectrum, a graphic considering the number of ions detected as a function of their mass/charge ratio. Figure 5 illustrates the parts that make up this equipment and the principle involved in this technique.



Source: Reproduced from Patel (2015).

Figure 5– Scheme showing the MALDI-MS technique, parts that make up the equipment and principles involved in the technique.

2. Objectives

The objectives of the present chapter consist of developing and testing two analytical methods for determining B12. The primary objective is to study the stability of B12 and the best conditions for analyzing this vitamin by CE-UV, with the aim of speciating its forms. The speciation of B12 is also proposed by using the MALDI-MS technique and some considerations related to the preparation of the sample for analysis of this vitamin in milk.

3. Materials and Methods

3.1. pH range and stability of cobalamins

The methods used in the study of (HAMZA et al., 2003) were employed to evaluate the contribution of chemical species in equilibrium as a function of pH, which allowed us to

evaluate the best pH range that could provide high cobalamin stability and suit CE-UV requirements.

3.2. B12 solutions for CE-UV analyses

Cyanocobalamin, hydroxocobalamin, methylcobalamin and adenosylcobalamin were purchased from Sigma Aldrich Chemie GmbH (Taufkirchen, Germany). Standard stock solutions (10mg/ml) were prepared in deionized water and stored at -4º C in an amber flask (Baker & Miller-Ihli, 2000). Subsequently, samples were diluted directly in ammonium acetate buffer, 20 mM, pH 5.5 and 8.4 for the respective analyses.

3.3. UV-vis spectrophotometry analyses

The stability of cobalamin molecules in ammonium acetate buffers pH 5.5 and 8.4 were studied by monitoring these species on a UV-Vis spectrophotometer (UV-2600 Shimadzu, Japan) every 1 hour for 24 hours. The solutions were prepared by diluting the stock solutions to 0.05 mg / ml. The analyses were performed in a wavelength range from 200 to 800 nm.

3.4. CE-UV Analyses

Cobalamin solutions were prepared by diluting the stock solutions to 2.5 mg / ml directly in ammonium acetate buffers pH 5.5 and 8.4 (20 mM), defined as the most suitable for this work. The experiments were carried out on a Beckman Coulter model P/ACETM MDQ (Fullerton, USA), with a UV-DAD detector and a data acquisition system via KaratTM 8.0 software. A fused silica capillary (internal diameter 75 µm, external diameter 375 µm, 65 cm of total length and 55 cm of distance from the detector). The experimental conditions were: a temperature of 25ºC, a potential 30 KV and monitoring channels around 278, 351 and 520 nm. The daily capillary conditioning steps consisted of washing steps with 0.1M NaOH solution (15 min), followed by washing with deionized water (15 min). The capillary conditioning between the analyses consisted of deionized water, followed by a 0.1M NaOH solution, water again and

the running electrolyte, each step of 2 min. Formamide (AccuPure Waters Millipore) was used as an electro-osmotic flow marker (prepared in the 1:1 v / v buffer solution ratio).

3.5. B12 solutions and matrix for MALDI-MS analyses

CNCbl, OHCbl, MeCbl and AdoCbl were employed in the standard stock solutions (10mg/ml) with deionized water and stored at -4º C in an amber flask. Solutions were diluted in deionized water to 20 μM for the respective analyses. 4-Chloro-α-cyanocinnamic acid (ClCCA) was used as a matrix for MALDI, as this is considered a soft matrix for the detection of cyanocobalamin, according to Calvano et al. (2016). The matrix was prepared at 20 μM in acetonitrile: water 80:20 v/v.

3.6. Evaluation of the effect of the milk matrix on B12 analyses

A calibration curve was prepared using the standard stock solution of CNCbl by dilution in deionized water, composed of 5 points: 0.05, 0.06, 0.075, 0.09 and 0.1 μM.

Raw milk was ultracentrifuged at 4ºC, 100,000 rpm for 1 h (Hitachi CS150NX, Hitachi Koki, Japan). The whey phase (in the middle) was collected and diluted (100-fold) in deionized water. The solution was used to dilute the standard cyanocobalamin stock analogously, as described above, considering the same final concentrations.

The samples considering each different concentration were analyzed and used to construct calibration curves considering the areas of the peaks.

3.7. Confirmation of the effect of the milk matrix on B12 analyses

Cyanocobalamin standard stock solution was diluted in deionized water to obtain concentrations of 20, 100, 200 and 1000 μM.

Stock solutions of β-lactoglobulin and lactose were prepared in deionized water. Simulated whey solutions were prepared by diluting the stock solutions following the same

proportion that β-lactoglobulin and lactose (2 and 4 mg/ml, respectively) occur in milk to obtain 10μL (WALSTRA; WOUTERS; GEURTS, 2005). The composition of each solution was: only lactose, only β-lactoglobulin, and both β-lactoglobulin and lactose. Cyanocobalamin (prepared from standard stock solution) was added to each solution to obtain the final concentrations of 20, 100, 200 and 1000 μM.

The solutions were analyzed to obtain two calibration curves (simulated whey and control). α-β-diphenylfumaronitrile (DPF) matrix solubilized in tetrahydrofuran (THF) 20 mg/ml was used, according to Kouchi et al. (2012).

3.8. Elimination of the effect of the milk matrix on B12 analyses

The solution of simulated whey prepared with β-lactoglobulin and lactose (containing CNCbl at 10 μM), as described in Section 3.7, was prepared to obtain a volume of 5 ml. The CNCbl was also prepared in deionized water to obtain the same volume (5 ml) as the control. C18 SPE columns were used to clean up samples (Supelclean ENVI-18, Supelco, EUA), which were conditioned with 5 ml of methanol and 10 ml of water, followed by the loaded with 5 ml of sample, 10 ml of water for washing and 1.5 ml of acetonitrile for elution. Samples were concentrated in a Speed Vac for 2 hours and subsequently analyzed using the DPF matrix and a CNCbl calibration curve in deionized water considering the concentration of 20, 80, 150 and 200 μM.

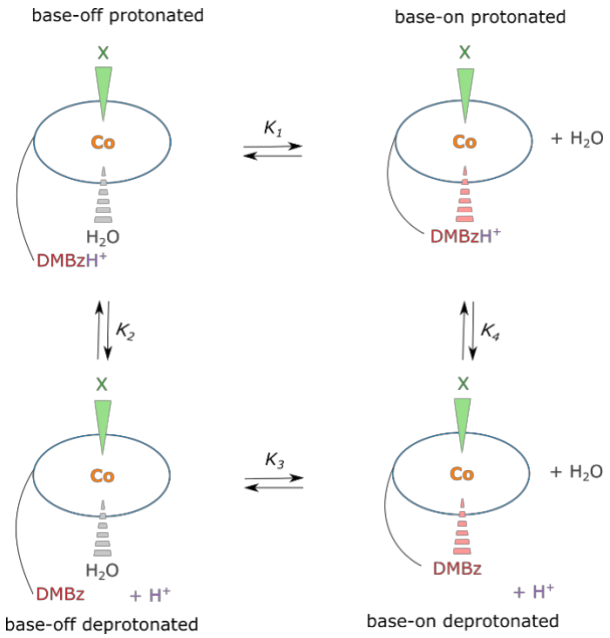
3.9. MALDI-MS analyses

A two-step sample preparation was used, in which 1 μl of the respective analyte was spotted directly on the target plate and dried. In the sequence, 1 μl of the respective matrix solution was spotted on top of the analyte and dried. The analyses were carried out at MALDI-MS (CALVANO et al., 2016). The spectra were acquired from 500-2000 m/z, using 20-30% of the laser power, positive linear mode, employing an AutoFlex Max analyzer (Bruker, EUA).

4. Results and Discussion

4.1. Preliminary study of the pH range and stability of cobalamins

Before the analyses in the CE-UV equipment, the stabilities of the cobalamins were tested by applying the study reported by Hamza et al. (2003). Cobalamin molecules in solution present the base-on/base-off equilibrium in which the axially coordinated DMBz base is displaced by water and protonated. This equilibrium is shown in Figure 6 and is related to the pH of the medium. For more acidic pH values, the protonated base-off specie is the majority form, while the deprotonated base-on form is the predominant form at neutral pH.



Source: Adapted from Hamza et al. (2003), p.268.

Figure 6– Base-on/base-off equilibria for cobalamins.

The calculation of the base-on and base-off forms involved in equilibrium can be defined. Based on the work of Hamza et al. (2003), for the system in Figure 6, the calculation of $K_{base\text{-}off}$ and fraction-off is given by Equations (1) and (2), respectively,

$$K_{base-off} = \frac{K_2\,(1+K_3)}{(1+K_1)}, \quad (1)$$

$$f_{off} = \frac{([H^+] + K_2)}{([H^+] + K_{base-off})(1+K_1)}, \quad (2)$$

where K represents the equilibrium constant for each reaction and $[H^+]$ the hydrogen concentration according to pH.

Assuming K_1 is very small, $1+K_1 \sim 1$, the equations are simplified to (3) and (4):

$$K_{base-off} = K_2(1 + K_3), \quad (3)$$

$$f_{off} = \frac{([H^+] + K_2)}{([H^+] + K_{base-off})}. \quad (4)$$

The $K_{base\text{-}off}$ values were obtained by applying in Equation (3) the pK_2 value of 5.54, which corresponds to a K_2 of $2.88.10^6$, while K_3 corresponded to 76.6, 467, $2.9.10^5$ and $4.9.10^7$ for the species AdoCbl, MeCbl, CNCbl and H_2OCbl, respectively. From these values and Equation (4), the contribution of the molecule in the base-off form was calculated for each cobalamin specie as a function of the variation in the $[H^+]$ concentration, according to Equation (5) for a pH range from 1 to 14

$$pH = -\log[H^+]. \quad (5)$$

The plots in Figure 7 show that, for all cobalamins, the best pH values were greater than or equal to 5.0 because, in this range, all molecules of each species were predominantly in the base-on form. Below this pH value, base-on and base-off forms coexist and interfere with equilibrium. Therefore, the use of ammonium acetate buffers, pH 5.5 and 8.4, were considered for subsequent analyses.

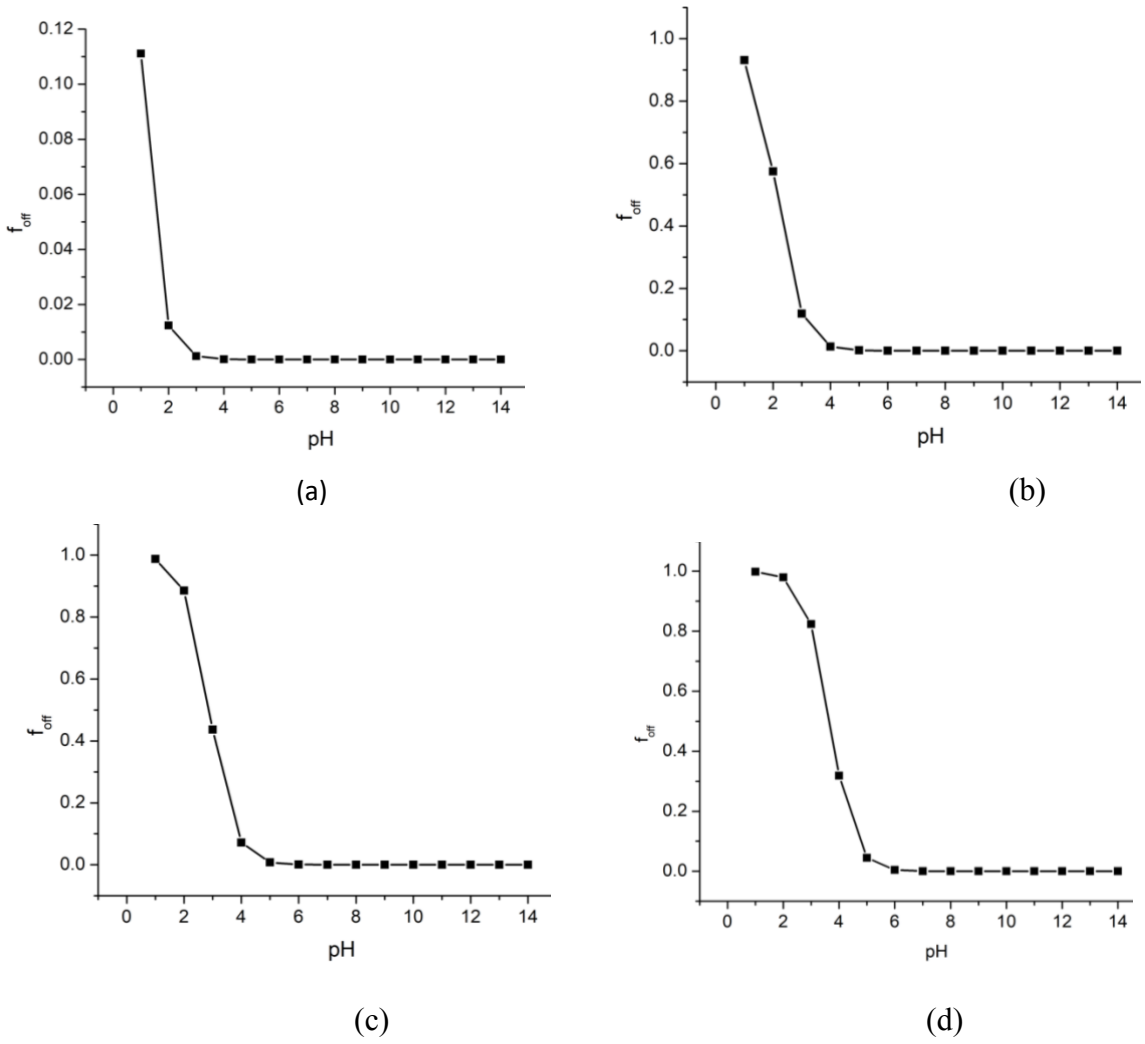
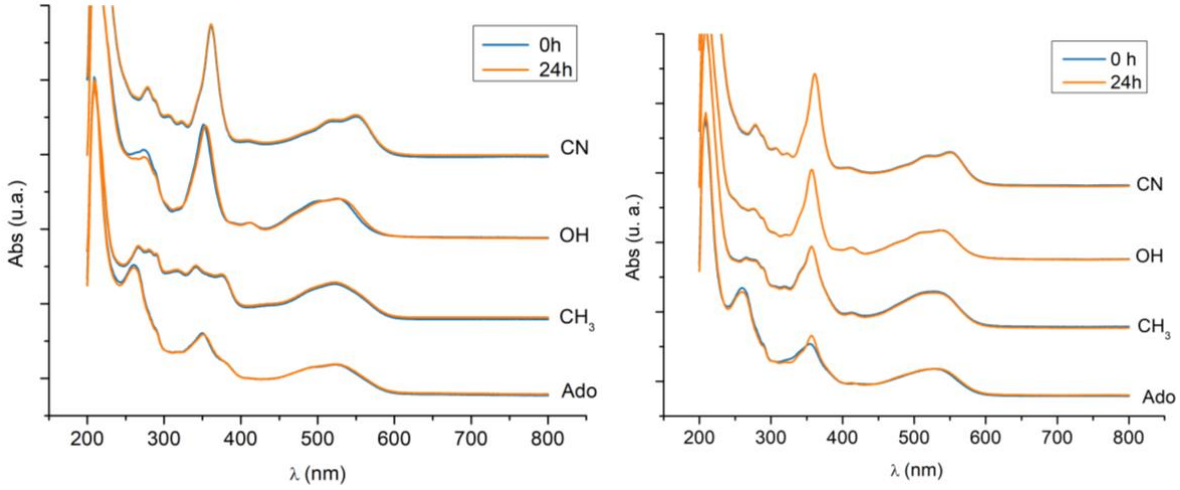


Figure 7- Graphs of the fraction of the $base_{off}$ cobalamin molecules as a function of pH. (a), (b), (c) and (d) correspond to the cyanocobalamin (CNCbl), hydroxocobalamin (OHCbl), methylcobalamin (MeCbl) and adenosylcobalamin (AdoCbl) species, respectively.

4.2. UV-vis spectrophotometry analyses

To evaluate the stabilities of B12 molecules in the buffer solutions, UV-vis analyses were carried out separately to confirm the profile of the cobalamin spectra. Figure 8 shows the spectra for each cobalamin with respect to ammonium acetate buffer at (a) pH 5.5 and (b) pH 8.4. The UV spectra of cobalamins present maximum absorption at wavelengths around 210, 278, 361 and 550 nm (JUZENIENE; NIZAUSKAITE, 2013). The wavelengths of 361 and 550 nm are the most selective.



a) b)

Figure 8 – UV-vis spectra for cyanocobalamin (CN), hydroxocobalamin (OH), methylcobalamin (CH_3) and adenosylcobalamin (Ado) in ammonium acetate buffer pH 5.5 (a) and pH 8.4 (b).

For all species, the characteristic wavelengths at which maximum absorptions occur were in agreement with the literature (JUZENIENE; NIZAUSKAITE, 2013). Absorbance at 210 nm is related to chromophore groups (N-H, O-H). This is the band with the highest intensity for all curves. Additionally, in the case of cyanocobalamin, the cyano group (-CN) also absorbs in this region. The absorbance bands around 278 and 360 nm can be attributed to chromophores such as C=O, C=N and $–CONH_2$. Regarding the band around 550 nm, it represents the presence of a polycyclic aromatic chromophore. Indeed, cobalamins absorb in the visible region because of this type of group and present a very intense red color (PAVIA et al., 2015).

It was observed that, for the cyano and methyl species, the curves remained stable, with no decrease or increase in absorption bands at pH 5.5. However, for hydroxocobalamin, a decrease in absorption was observed mainly in the 278 nm wavelength region, in addition to a slight decrease also in the 361 and 550 nm regions. This fact suggests that this minor discrepancy in the absorption bands may be related to the exchange of the X ligand at the sixth coordinate position (Figure 6), by the hydroxyl group (JUZENIENE; NIZAUSKAITE, 2013). This hypothesis was contemplated because, at this pH, the protonation/deprotonation of DMBz due to the equilibrium of base-on/base-off species was expected to be eliminated.

For pH 8.4, all cobalamins remained stable, except for adenosylcobalamin which showed a slight increase after 24 h for the band around 360 nm. The cobalamins remained

relatively stable in the buffer solutions employed, even after 24 hours, which confirmed that the chosen buffers were consistent for subsequent analyses.

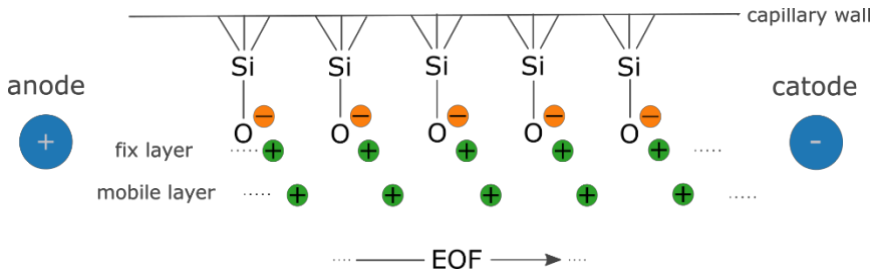
4.3. CE-UV Analyses

In capillary electrophoresis, the migration rate of a molecule (defined by Equation 6) depends on the applied electric field E and the electrophoretic mobility μ defined in Equation (7), as in the following:

$$v = \mu . E \ , \tag{6}$$

$$\mu_e = \frac{q}{6 \pi \eta r} , \tag{7}$$

where q is the effective charge of the particle (ion), η is the viscosity of the buffer and r is the total hydrodynamic radius of the particle (ion), which can also be considered as the mass of the molecule. This implies that the electrophoretic mobility of a particle and its consequent velocity are functions of the charge/size ratio, as previously cited. Positively charged molecules migrate faster than neutral molecules, and the latter migrate faster than negatively charged ones (Baker, 1995). Although neutral molecules do not have a charge, they migrate with the same velocity as the electro-osmotic flow. Electroosmosis is a fundamental phenomenon in capillary electrophoresis resulting from charges on the surface of the capillary wall. The wall, which is made of fused silica, contains silanol groups that become ionized because of the pH buffer used. These silane groups -SiO- formed provide a negatively charged capillary wall, and consequently, a double layer at the solid/liquid interface is formed naturally to preserve electroneutrality (SCHMITT-KOPPLIN; FROMMBERGER, 2003), as represented in Figure 9.



Source: Figure elaborated by the author.

Figure 9 – Representation of electro-osmotic flow in the capillary wall.

The electro-osmotic flow velocity (v) is defined by Equation (8), analogous to Equation (6),

$$v = \mu_{EOF} . E \ , \tag{8}$$

where E is the applied electric field and μEOF is the electro-osmotic mobility of the buffer (Baker, 1995). At high pH values, the silanol groups are practically all deprotonated, which leads to a more significant and constant flow within the column. At low pH, the potential at the capillary surface reduces the electro-osmotic flow due to the protonation of the silanol groups (SCHMITT-KOPPLIN; FROMMBERGER, 2003).

Another important parameter is the electrical force resulting from the applied potential that promotes the migration of particles in the capillary (Baker, 1995). The higher the applied potential, the higher the velocity of the particles, implying shorter migration times (Equations 9 and 10). However, high values of potential should not be applied indiscriminately since the greater the applied potential, the greater the electric current, according to Ohm's law (Equation 11). The transmission of electrical current via electrolytes due to buffer causes collisions between the ions and heat (Joule effect). This effect is undesirable and avoided if the linear dependence between the applied voltage and the current obtained is respected (WEINBERGER, 2000),

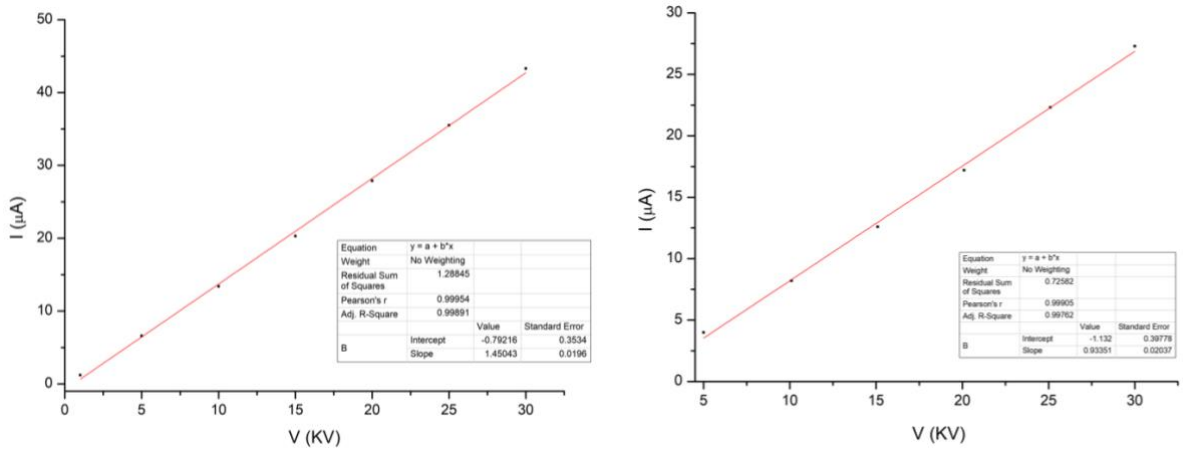
$$v = \mu E = \frac{\mu V}{L} \ , \tag{9}$$

$$t = \frac{L}{v} = \frac{L^2}{\mu V} , \tag{10}$$

$$V = R . I \ , \tag{11}$$

where v corresponds to the solute migration rate, μ is the electrophoretic mobility of the solute, E is the electric field applied to the system, V is the applied potential, L is the length of the capillary, t corresponds to the time for the solute to migrate the distance L, R is the fluid resistance and I is the electric current.

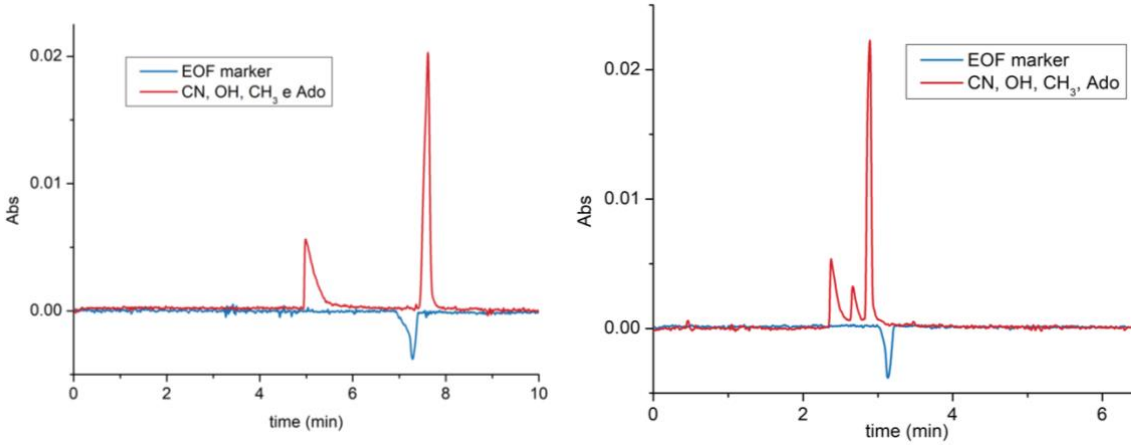
The applied potentials ranged from 5 to 30 KV, checking the corresponding current value for the buffer solutions (Figure 10). As in both cases, the maximum value of potential applied did not compromise the temperature; the nominal value of 30 KV was established for the analyses.



(a) (b)

Figure 10 – Plots for Ohm's law for ammonium acetate buffers pH 5,5 (a) and 8,4 (b), respectively.

Figure 11 shows the analyses for all cobalamins and the electro-osmotic flow marker (formamide) at pH 5.5 (a) and 8.4 (b), respectively. The electro-osmotic mobilities calculated according to Equations (10 and 9) are listed in Table 1. As expected, the analyses for more alkaline pH (Figure 9 (b)) presented faster migration times for the detected peaks (SCHMITT-KOPPLIN; FROMMBERGER, 2003).



(a) (b)

Figure 11– Electropherograms for cobalamins (red line) and electroosmotic flow marker (blue line) for ammonium acetate buffer at pH 5.5 (a) and pH 8.4 (b).

Table 1 – Electrophoretic mobilities of B12 molecules.

		$\mu .10^{-4}$ (cm^2/s.V)			
		CN	OH	CH_3	Ado
pH	5.5	4.33	5.85	4.77	3.68
	8.4	7.58	8.67	7.37	7.37

Source: Elaborated by the author.

From Figure 11 (a), observing the red line, which corresponds to the mixture of B12 molecules, we noticed that hydroxocobalamin corresponds to the peak with the shortest migration time due to its net formal charge +1 and a molecular mass of 1346.41 g/mol. The other forms analyzed, CNCbl and MeCbl, have charges of 0 and AdoCbl -3, with masses of 1355.37, 1344.41 and 1578.66 g/mol, respectively (PUBCHEM, 2004), and they correspond to the second (Figure 11 (a)) and most intense peak. The results were consistent in part with expectations since hydroxocobalamin was suggested to migrate first, followed by cyano- and methyl-. So, adenosylcobalamin. Indeed, hydroxocobalamin migrated first (according to Equation 2), while cyanocobalamin and methylcobalamin have similar net charges and masses, consequently presenting electrophoretic mobility very similar to the electroosmotic flow marker (blue line, $\mu_{EOF}=3.67.10^{-4}$ cm^2/s.V). However, despite its higher mass and charge, adenosylcobalamin also migrated together with the neutral molecules. Only the hydroxocobalamin form could be differentiated from the others for the pHs used. In the case of pH 8.4 (Figure 11 (b)), an analogous electropherogram was obtained, being the first peak with elution at around 2.5 min attributed to hydroxocobalamin and the most intense peak at around 3 min attributed to the other cobalamin molecules (with very similar electrophoretic mobility to the EOF marker, $\mu_{EOF}=6.79.10^{-4}$ cm^2/s.V, blue line). At this pH, an additional small peak appeared in the middle, probably from hydroxocobalamin, due to the equilibrium of hydroxo/aqua forms (FEDOSOV; NEXO; HEEGAARD, 2018).

As no peak was observed for each specie, it was unfeasible to continue using CE-UV as the analytical method to determine B12. This technique could not resolve the peaks that provide B12 speciation in the conditions tested. Furthermore, the possibilities of buffer solutions were restricted, as the pH range indicated to maintain the stability of the B12 molecules was also very limited. Taking into account these aspects, an approach focused on B12 analysis by MALDI-MS was tested, as described in the following.

4.4. MALDI-MS analyses

Figure 12 shows the results obtained for MALDI-MS analyses for the cobalamin standards. From top to bottom, the standards are AdoCbl, MeCbl, CNCbl and OHCbl. It is not possible to notice for all cases the characteristic peak for each B12 as an intact molecule $[M+H]^+$. Only in the case of cyanocobalamin the characteristic peak appeared $[1355+H]^+$ as m/z of 1355.62. In all cases, a peak occurred at m / z 1329.6, which corresponds to the B12 molecule without the β-ligand (Figure 2 (a), the R ligand) (CALVANO et al., 2016). The energy absorbed by the matrix and transferred to the B12 molecule during the ionization process leads to the loss of B12 ligands, which characterize the different forms of the molecule. On the basis of this, speciation considering this technique could not be achieved. However, we continued with some additional experiments using cyanocobalamin to study other aspects related to the B12 analyses directly in milk.

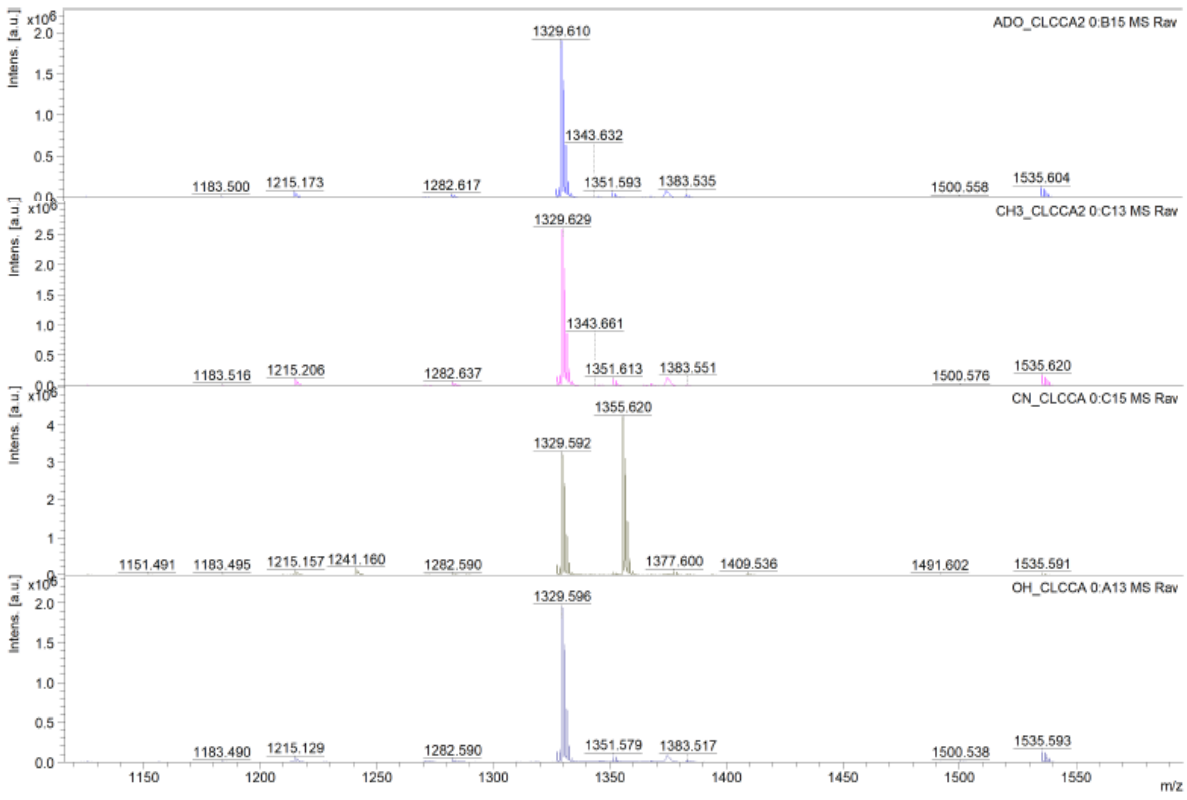
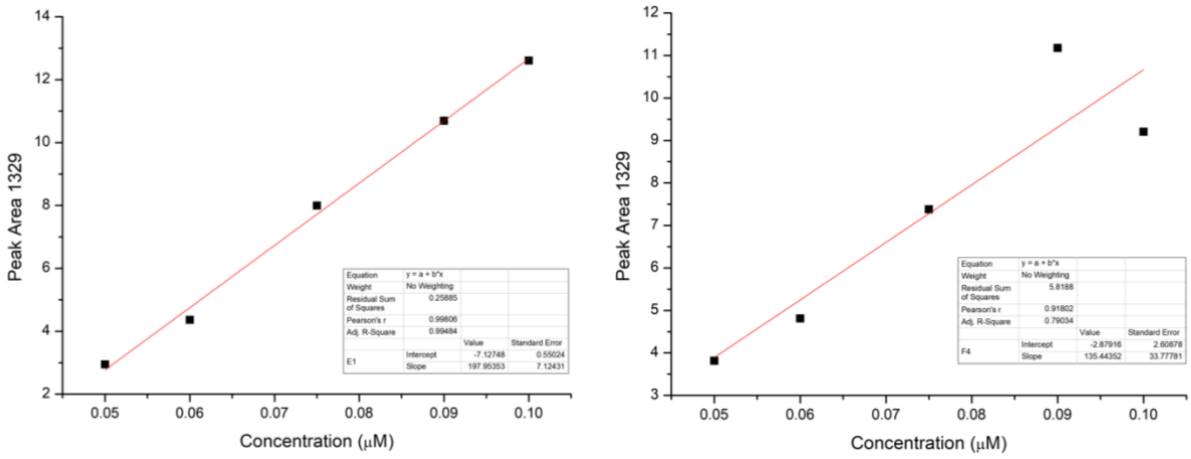


Figure 12 - Mass spectra obtained for MALDI-MS analyses of cobalamin standards. From top to bottom, the standards in order are AdoCbl (adenosylcobalamin), MeCbl (methylcobalamin), CNCbl (cyanocobalamin) and OHCbl (hydroxocobalamin).

4.5. Milk Matrix Effect on B12 Analyses

Some works in the literature have suggested the B12 analyses focused on the cyanocobalamin form – converting all forms into CNCl molecule by adding cyanide – (REPOSSI et al., 2017; ZIRONI et al., 2014), which is more stable. So, we proceed with the experiments considering this B12 molecule. In the sequence, an experiment was carried out to test how the effect of the milk matrix could interfere with the analyses, considering a simple sample preparation. As milk is a complex matrix, its constituents are expected to interfere with analyses. These effects, often caused by the alteration of ionization efficiency of target analytes, could impact on the intensity of the signals obtained, leading to a loss of response (ion suppression) or an increase in response (ion enhancement) (ZHOU; YANG; WANG, 2017).

Figure 13 (a and b) shows the calibration curve for cobalamin prepared in water and prepared directly in fresh milk (whey), respectively. When comparing both curves plotted to the peak 1329 m/z as a reference, it is possible to see that the slopes in Figure 13 varied (a) 197.9 and (b) 135.4. This variation corresponded to more than 10%, indicating a matrix effect of around 32%, with a suppression in the signal detected for B12 (AOAC INTERNATIONAL, [s.d.]). Considering the ultracentrifugation process during the sample preparation, the fatty portion and the casein pellet were supposed to be eliminated. However, the high concentration of whey proteins (mainly β-lactoglobulin) and lactose (WALSTRA; WOUTERS; GEURTS, 2005; WANG et al., 2019) probably were the elements that caused the pronounced matrix effect. Two other experiments were implemented to confirm the influence of these components on the suppression of the B12 peak and to determine if they could be efficiently eliminated, as described below.



(a) (b)

Figure 13- Calibration curves obtained for cyanocobalamin prepared in deionized water (a) and directly in whey (b), evidencing the effect of the milk matrix.

4.6. Confirmation and evaluation of the effect of the milk matrix on B12 analyses

In this case, the experiments were carried out using the DPF matrix because it is also known to cause less fragmentation of the molecules, and we could focus the analyses on the peak of m/z 1329, which appeared intensely, as shown in Figure 14.

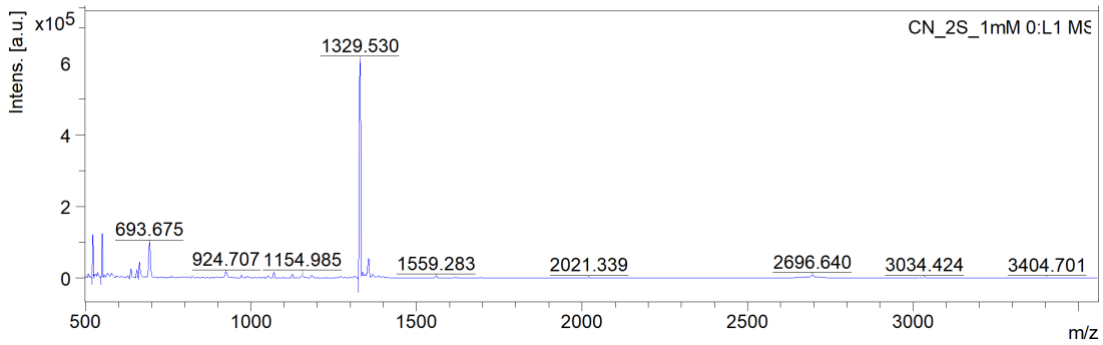


Figure 14- Cyanocobalamin mass spectrum of cyanocobalamin regarding the analyses of MALDI-MS in DPF matrix, which shows the peak of m/z 1329.5 that corresponds to the B12 molecule without the β-axial ligand.

Considering the calibration curves for CNCbl prepared directly in water (a) and in the simulated whey solutions (b, c and d) in Figure 15, the matrix effect was evidenced (AOAC INTERNATIONAL, [s.d.]), as the slopes of the curves for all simulated whey cases varied more

than 10% compared to the cyanocobalamin control solution. The values obtained for the slopes were 626, 556, 148 and 96 for CNCbl in water (a), lactose (b), β-lactoglobulin (c) and lactose + β-lactoglobulin (d), respectively. The variations suggest that lactose led to a mild matrix effect of around 11%, while the effect due to the β-lactoglobulin was more pronounced (76%). The effect considering both components resulted in a matrix effect of around 85%. Based on this, most of the cyanocobalamin suppression signal in the mass spectrometry analyses could be attributed to the presence of proteins.

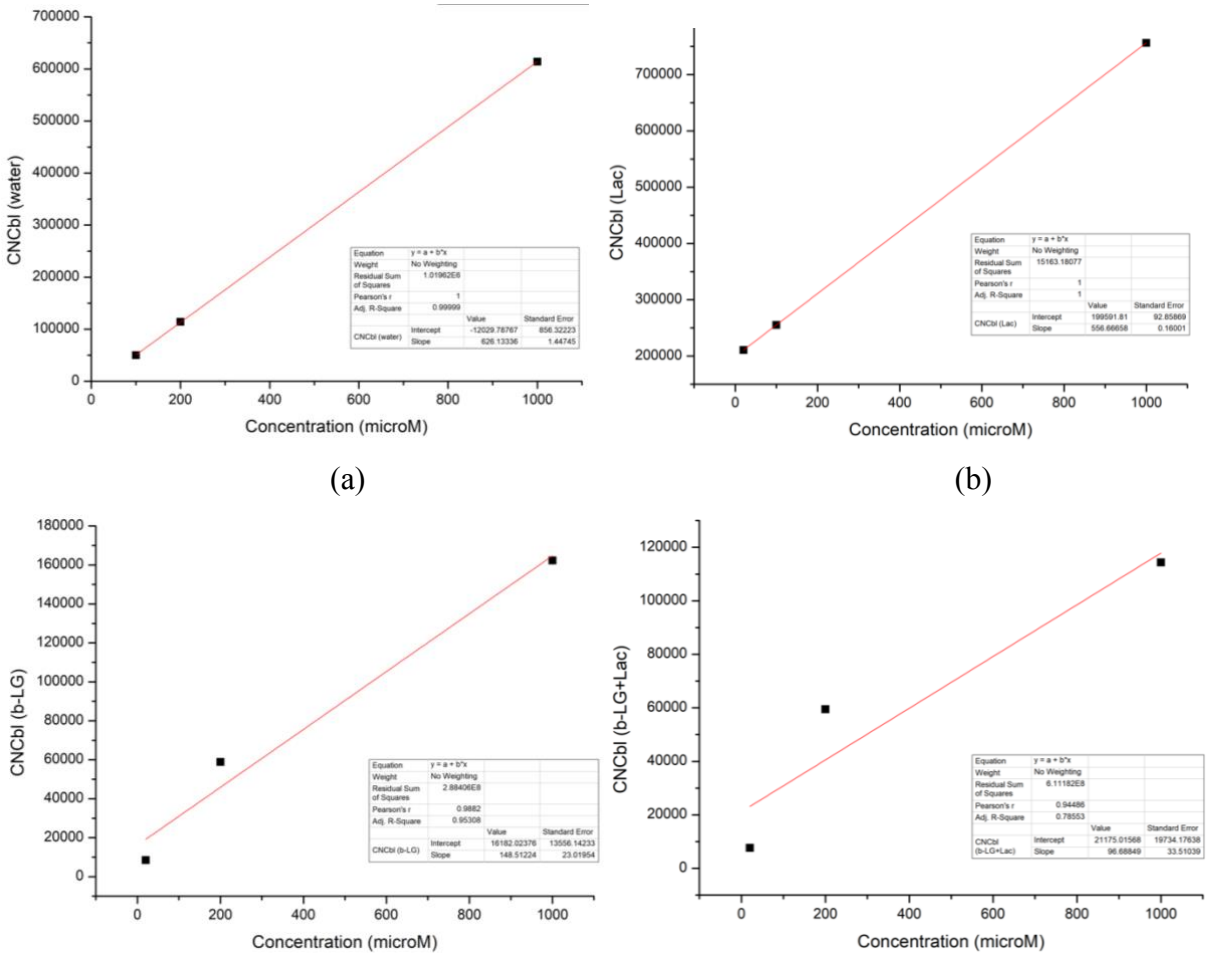


Figure 15- Calibration curves obtained for cyanocobalamin prepared in deionized water (a) and cyanocobalamin prepared directly in simulated whey such as: containing lactose (b), containing β-lactoglobulin (c) and containing lactose + β-lactoglobulin (d), evidencing the respective matrix effect caused by each of these components in milk.

4.7. Elimination of the effect of the milk matrix on B12 analyses

Regarding the pronounced milk matrix effect observed, a solid phase extraction (SPE) cartridge C18 was used to eliminate or reduce it, as recommended in some works (LI et al., 2019; REPOSSI et al., 2017; ZIRONI et al., 2013). From Figure 16, which corresponds to the cyanocobalamin calibration curve, the recovered B12 concentration was determined. As the solutions were concentrated 20 times, the CNCbl recovery was approximately 50% (102µM) for the simulated whey solution and approximately 60% for the control solution (118 µM). Although the matrix effect could be reduced, it continued to interfere with the B12 signal. Furthermore, this experiment showed that even with part of the matrix effect eliminated by the C18 column, this is not the most recommended for a study focused on B12 quantification, as at least 40% of this vitamin was lost in the SPE procedure, representing a high value. In addition, a simple sample preparation cannot be employed because this vitamin is found in low concentrations in milk and the effect of the milk matrix enhances the interference in the analyses.

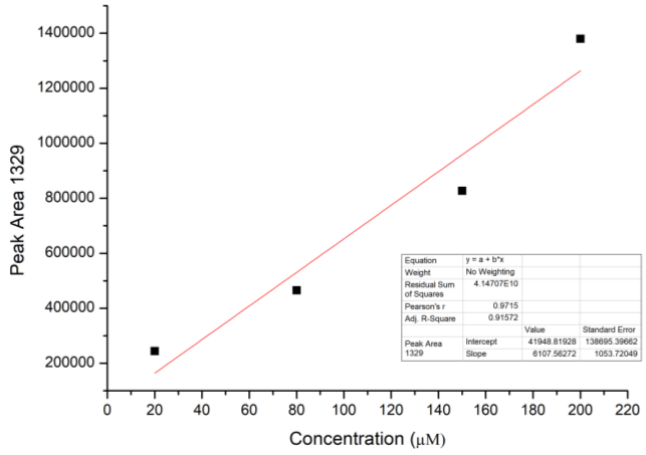


Figure 16 - Calibration curve obtained for cyanocobalamin prepared in deionized water.

Based on all exposures, we completely changed the focus of our experiments, implementing B12 analyses based only on the cyanocobalamin molecule (without speciation of B12 forms), refined cleanup steps of sample preparation instead of the simple ones and the employment of immunoaffinity columns rather than SPE C18. Although the latter can reduce the interferents of the food matrix and be suggested in the literature due to the lowest costs, the immunoaffinity columns are cited as more specific to access B12 (LI et al., 2019). Furthermore, the technique for B12 analysis was changed to UHPLC-UV, as this technique was already

described as well-established for B12 studies. In the following two chapters of the present thesis, this approach, which was well succeeded, will be presented considering all the aspects that were tried in the present exploratory chapter.

5. Conclusion

This chapter presented some preliminary studies on the stability of B12 and an overview of the main methods used to determine vitamin B12. Two techniques, which encompass CE-UV and MALDI-MS, were tested to be established as the possible analytical methods for the analyses in the present thesis. The experiments focused on the speciation of vitamin B12 carried out by CE-UV showed that although this technique could provide the detection of the molecule, it could not provide its speciation in the four different forms such as hydroxocobalamin, methylcobalamin, adenosylcobalamin and methylcobalamin. This objective was also pursued following the same type of experiments by employing MALDI-MS as an analytical technique. The results obtained showed that B12 speciation was not feasible due to the intrinsic properties of the molecules and their stability with respect to light (photochemistry of the molecule). However, the study revealed the possibility of focusing the analysis on the peak of 1329 m/z, which corresponds to the molecule with no upper axial ligand. Based on it, the experiments employing a single sample preparation to determine B12 in milk via this technique were carried out, showing the need to improve the method due to the complexity of the food matrix. The effect of the milk matrix was evaluated mainly due to the contribution of proteins and sugars (β-lactoglobulin and lactose), which can interfere with the signal response leading to a suppression of up to 87%. Although such an effect can be reduced by employing cleaning steps with a C18 column, the SPE procedure cannot completely eliminate the interferents nor provide a complete extraction of vitamin B12 in the sample. Finally, this objective is expected to be reached by using immunoaffinity columns that are highly specific for this vitamin, as mentioned in the literature. These columns were employed to access and isolate B12 properly, and an analytical approach was developed using UHPLC-UV (as described in the next chapter of the present thesis).

Chapter III - Impact on B12 due to HP, PEF and UV-C treatments

Part of this chapter's content is in a paper submitted to Innovative Food Science & Emerging Technologies journal.

1. Introduction

1.1. Analyses of B12 and HPLC/UHPLC techniques

The determination of B12 by liquid chromatography in conjunction with other techniques has been well established as a reliable procedure to achieve and quantify B12 in food sources. As our efforts to speciate B12 via other techniques were not achieved as described in the previous chapter, here and in the following, UHPLC-UV was used as the analytical method to analyze B12. Due to this, a brief description of the technique is exposed, as well as the sample preparation requirements to isolate and access B12.

As mentioned above, most of the literature considering B12 analyses has been presented based on the determination and quantification of B12 using its synthetic form, cyanocobalamin, which is the most stable of the cobalamin derivatives (SCHNELLBAECHER et al., 2019). This form of the molecule can be achieved from the natural ones, usually adding potassium cyanide or sodium cyanide in the medium to convert all natural forms into a synthetic molecule (LI et al., 2019; SELVA KUMAR; CHOUHAN; THAKUR, 2010). In this case, the purification and concentration of the sample are required, in which different solid phase extraction (SPE) cartridges have been mentioned in the literature, such as hydrophilic-lipophilic balanced (HLB), C8 and C18, the latter of which was tested in Chapter II experiments. However, they are reported to be poor selective, leading to inaccurate results (LI et al., 2019; REPOSSI et al., 2017; ZIRONI et al., 2013). Due to this, some works have reported clean-up steps based on immunoaffinity columns, such as those that were applied in the experiments of this and the following chapters, which are more expensive, but considered highly specific (CAMPOS-GIMÉNEZ et al., 2008, 2012; CHAMLAGAIN et al., 2015; HEUDI et al., 2006).

In this regard, UHPLC has been used as one of the standard methods to determine B12. Some examples are the study reported by Zironi et al. (2013), in which the determination of

B12 was made using ultraperformance liquid chromatography-tandem mass spectrometry (UPLC-MS) in raw milk and cheese samples. Zironi et al. (2014) analyzed the B12 content according to the different steps of cheese making for milk, whey, rennet, ricotta cheese, curd, mozzarella cheese and *caciota* cheese using UPLC-MS. Accordingly, Repossi et al. (2017) reported results for the analyses of milk, whey and different by-products of ricotta cheese. In addition, high-performance liquid chromatography tandem mass spectrometry with electrospray ionization (HPLC-ESI-MS) was described to measure vitamin B12 in milk powder products and multivitamin-multimineral tablets (LUO et al., 2006). Analogously, Chamlagain et al. (2015) reported the analysis of cereal matrix, bacterial cells and pig liver by UHPLC-MS. In the same way, Heudi et al. (2006) determined B12 in different kinds of samples, such as milk-based infant formula powder, infant cereals, breakfast cereals, polyvitaminated mixes, pet food, among others, by HPLC-UV. Campos-Gimenez et al. (2008) also employed HPLC-UV to determine B12 in food, some of them fortified and some non-fortified milk-based products.

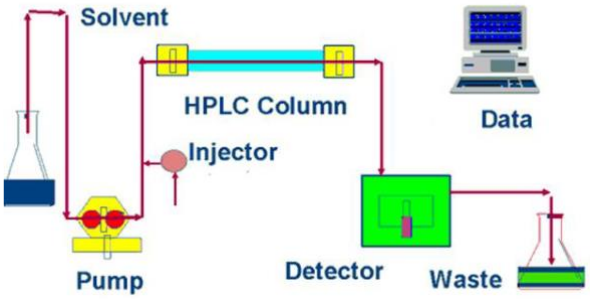
Ultra-performance liquid chromatography (UPLC), also known as Ultra-high-performance liquid chromatography (UHPLC), consists of separating the components of a compound or a mixture. The principle involved in this technique is the same as for high-performance liquid chromatography (HPLC). However, some differences in the specification of the material, configuration and properties of the system vary with respect to the typical column dimensions, particle size, typical injection run time, solvent usage per injection, pump pressure, sample usage, peak resolution and mobile phase change over (NAHAR; ONDER; SARKER, 2020). The main characteristic feature of U(H)PLC is the use of smaller particles (< 2 μm) contrarily to those employed in conventional HPLC systems (2.5 and 10 μm), requiring higher pressure to work but providing better results. Therefore, U(H)PLC represents an advance in the liquid chromatography technique (HPLC), offering faster analysis, a smaller amount of solvent employed as a mobile phase, better separation efficiency and resolution of analyte mixtures compared to HPLC (NAHAR; ONDER; SARKER, 2020).

Liquid chromatography is one of the most important techniques in analytical chemistry, providing separation, identification and quantification of compounds. The equipment consists of modules composed basically of solvents, a pump, an injector (sample), a column (composed of a porous material and where the separation occurs), a detector (where the components are identified) and a computer to process and collect the data (BHARDWAJ; DWIVEDI; AGARWAL, 2015).

In this technique, the solvents (mobile phase) are pumped at higher pressure into the column carrying the sample together. In the column (stationary phase), the analytes in the mobile phase interact in varying degrees with the solid stationary phase. The mechanisms by which these interactions occur are based on physicochemical properties such as molecular size (size exclusion chromatography), charge (ion exchange chromatography), hydrophobicity (hydrophobic interaction chromatography and reversed-phase chromatography) and specific binding interactions by adsorption or partition (affinity chromatography) (BIO-RAD, [s.d.]; CUNICO; GOODING; WEHR, 1998; LANÇAS, 2009). The separation of the analytes is due to the partitioning of each compound between the stationary and mobile phases. Each compound elutes from the column in a different order (defined as the retention time), depending on the relative strengths of the compound's interaction with the stationary or mobile phase. Each analyte is detected as a peak according to time, whose data is registered by software as a chromatogram (LANÇAS, 2009).

Reversed-phase (RP) chromatography was emloyed in the studies of this thesis. In this mode, which is opposed to normal phase chromatography, a nonpolar stationary phase is employed. The initial mobile phase is polar, such as water or buffer, with an increase in the percentage of nonpolar solvents, such as methanol, isopropanol, or acetonitrile, to effect elution. Solutes elute in increasing order of hydrophobicity or increasing order of net charge, degree of ionization and ability to participate in hydrogen bonding (CUNICO; GOODING; WEHR, 1998). For (RP) column manufacture, different packing materials can compose the stationary phase, with silica the most used as a support matrix, while the bonded phase can be a variety of hydrophobic ligands, with octadecyl (C18) being the one applied in this study (CUNICO; GOODING; WEHR, 1998).

As mentioned previously, the detector can be of different types but usually is a UV-photodiode array (PDA) or MS coupled to the system. The one used in this study was a PDA. This detector is analogous to the one described in Chapter II, Section 1.2, based on the same principles of functioning that are not described here again. Figure 17 illustrates this technique and the main parts that make up the UHPLC-UV equipment.



Source: Reproduced from Bhardwaj et al. (2015).

Figure 17- Scheme of UHPLC-UV equipment.

1.2. PEF processing

One of the nonthermal technologies applied in this work was PEF processing. This treatment consists of allocating the sample of interest, which can be liquid or solid, between two electrodes installed separately (0.1-1.0 cm), in a treatment chamber separated by an insulator, with the generation of short pulses (1-10 µs) of high voltage, 5-20 kV (SAMPEDRO; RODRIGO, 2015). This configuration generates a high intensity electric field, whose strength can be calculated by Equation 12 (Raso et al., 2016), that induces a potential difference across the food sample, particularly across the cell membrane of microorganisms.

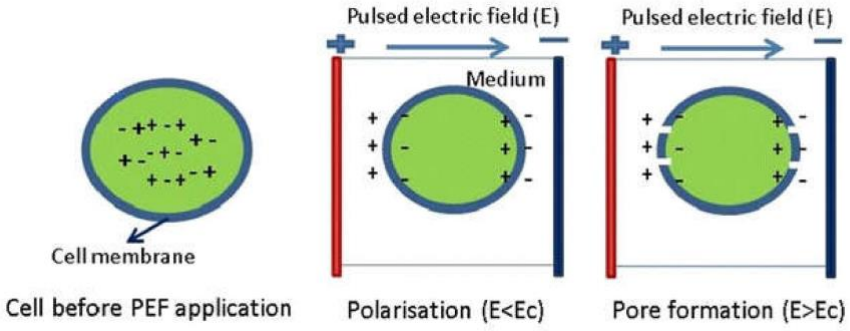
$$E = \frac{U}{d} \ , \tag{12}$$

where E corresponds to the electric field intensity, U is the applied voltage and d is the distance between the electrodes.

This potential difference (that can be estimated by Equation 13) caused by the movement of ions outside and inside the cell (BUCKOW et al., 2014) produces structural and functional changes in the cell membrane, causing its rupture and formation of pores (ZIMMERMANN, 1986), which consequently leads to the death of microorganisms (SAMPEDRO; RODRIGO, 2015).

$$\Delta\varphi = -g.E.\ R\ .\cos\theta, \tag{13}$$

where *g* is the factor of cell shape, being 1.5 for spherical cells, *E* is the intensity of the electric field, *R* is the cell radius and θ corresponds to the angle between a given membrane site and the direction of the field. Figure 18 represents the electroporation procedure.



Source: Reproduced from Szwarc & Szwarc (2020).

Figure 18- Representation of the electroporation mechanism in a biological cell.

Electric field intensities of 15-40 KV/cm are reported to provide microbial inactivation. However, the efficacy of the treatment also depends on the treatment time (which is defined according to Equation 14) for the formation of a permanent pore and irreversible damage to the membrane (MENESES et al., 2011; RASO et al., 2016):

$$t_{treat} = \frac{l}{V} . f . \tau , \tag{14}$$

where *l* is the insulator zone, *V* is the average flow velocity, *f* is the pulse frequency and τ is the pulse width.

During treatment, an increase in the temperature of the sample occurs due to the Joule effect (SAGER, 2020) and this can be measured as the energy input, described according to

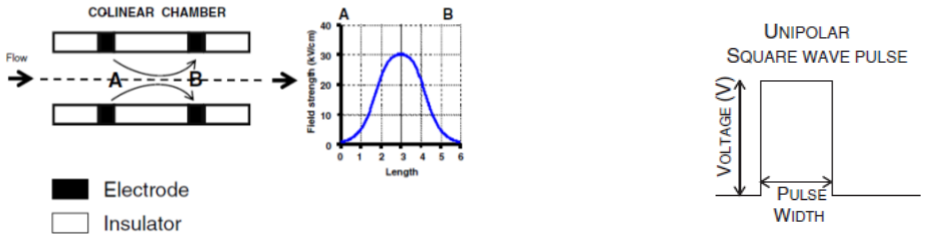
$$W = C_p . \Delta T , \tag{15}$$

where *Cp* is the specific heat capacity of the sample and ΔT corresponds to the variation in the temperature of the sample.

The basic PEF system consists of 3 parts: a voltage generator, a treatment chamber and an oscilloscope. Additional suppliers, such as pumps and others, can complement the

system. In the treatment chamber, the sample is submitted to the electric field, and the system configuration can vary according to the arrangement of the electrodes, which can be parallel or colinear (Figure 19 (a)), Raso et al. (2016). Because in the case of colinear electrodes, the electrical field strength is not homogeneously distributed as in the parallel electrodes, a conversion factor (g=1.6) is necessary to obtain the corresponding value for the colinear system (YANG et al., 2021).

The pulses applied to the sample can be of four different types, considering the polarity and shape, such as unipolar or bipolar and square wave or exponential wave, respectively. Square waves have been reported to provide a more constant voltage during the pulse, as well as bipolar pulses have been described to cause more irreversible damage to the microorganism cell membrane (RASO et al., 2016; SHARMA; OEY; EVERETT, 2014). In the present thesis, a colinear electrode configuration and unipolar square-wave pulses were employed, as represented by Figure 19 (a) and (b).



(a) (b)

Source: Adapted from Raso et al. (2016).

Figure 19- (a) Collinear electrode configuration and (b) quadratic monopolar pulse shape.

1.3. PEF effects on milk constituents and microorganisms

PEF has been described as nonthermal pasteurization that can inactivate bacteria with mild effects on milk components (ALIREZALU et al., 2020; YANG et al., 2021). According to McAuley et al. (2016), PEF used to treat milk samples (30 KV, 22 µs and 63 ºC) provided similar results compared to standard pasteurization (72 º C, 15 s) regarding the bacteria inactivation. Alirezalu et al. (2020) reported microbial reductions (3 to 6 log cycles) and improved milk and dairy products safety by using PEF (15-60 KV/cm for different treatment times, combined or not with mild heating up to 55 ºC). Regarding plasmin, the main proteolytic

enzyme of milk, application of PEF 30 a 45 kV/cm, 10–50 pulses for 2 s at 10-15º C inactivated its activity by 90% (VEGA-MERCADO et al., 2007). Sharma et al. 2014 demonstrated a reduction in the plasmin activity of 12%, and also observed a reduction in the xanthine oxidase and lipase activities of 32 and 82%, respectively, applying E = 26 kV/cm, t_{treat}= 34 µs (and heating at 55ºC in the last two cases) for raw milk.

Taking into account the milk fat globule membrane (MFGM), Barsotti et al. (2002) described that PEF (29-36 KV/cm for 0.8-1.6 µs) did not reduce the size of the MFGM in whole milk according to Garcia-Amezquita et al. (2009) for conditions of 36-42 KV/cm for 2.6 µs. However, Sharma et al. (2015, 2016) reported that PEF at 20-26 KV/cm for 34 µs (with preheating of 50º C) decreased the size of the MFGM, while (YANG et al., 2021) observed electroporation of MFGM leading to MFG protein and MFG self-aggregation for 9 KV/cm and 30 µs. Sampedro et al. (2005) and Sharma et al. (2014) pointed out that the damage caused by PEF is less than the damage caused by pasteurization, and according to Barsotti et al. (2002), these damages can be repaired by caseins.

Processing of PEF has been considered not to affect lactoferrin's physicochemical properties (SUI et al., 2011), caseins or whey proteins (LIU et al., 2015). In this way, Xu et al. (2015) demonstrated that the protein profile and bioactivity of the proteins of the MFGM fraction were not changed (37 KV/cm,1.7 ms, 50º C), but for higher temperatures (65ºC), β-lactoglobulin (β-Lg) and caseins can interact with the MFGM, leading to loss of phospholipids and bioactivity. The PEF has not been cited as affecting the primary structure of proteins, only studies that mentioned possible changes in the secondary, tertiary and quaternary structures (YANG, 2021). Consequently, Xiang et al. (2011) reported these effects for whey proteins working with an electric field range of 12-20 KV/cm, while denaturation of β-Lg, α-La and BSA (up to 40%) occurred in a study using 35.5 KV/cm and 1 ms (ODRIOZOLA-SERRANO; BENDICHO-PORTA; MARTÍN-BELLOSO, 2006).

Regarding other components of milk, such as polyphenols, isoprenoid compounds, fatty acids and most vitamins, the impact of PEF is considered low or negligible (BARBA et al., 2015; MIKHAYLIN et al., 2017). Bendicho et al. (2002) observed that PEF did not affect vitamins B1 and B2 in milk for treatment times of up to 400 µs and 18.3-27.1 KV/cm of electric field intensity. No significant changes were also observed for vitamins B2, B5, B7 and B9 by employing 15 to 40 KV/cm with treatment times of 40-700 µs (RIVAS et al., 2007). Riener et al. (2009) reported the same for liposoluble vitamins A and E, conducting experiments under 15-35 KV/cm and 12.5 to 75 µs. However, vitamin C was shown to be highly sensitive to PEF

treatment, with a loss of approximately 90% in its amount reported, but for a high input of energy (>200 kJ / l) (GRAHL; MÄRKL, 1996). Considering vitamin B12, to the best of our knowledge, there is no literature showing the impact that PEF can have on this vitamin content, which justifies one of the purposes of the present work.

1.4. HP processing

Analogously to PEF, HP is applied as a nonthermal treatment that extends shelf-life with a slight impact on food's nutritional and organoleptic characteristics (KHALIQ et al., 2021).

HP applies hydrostatic pressure in the range of 100-1000 MPa (TRUJILLO et al., 2002). The principle of this processing consists of pressure, transmitted by a fluid, applied uniformly and simultaneously from all directions through the sample, which is packaged into vacuum-sealed containers and allocated into a chamber. Therefore, the pressure of the system is increased and held during a stipulated time, usually between 2 and 30 min, after which the pressure is released (DATTA; DEETH, 1999).

The technique is based on three primary principles: Le Chatelier's, isostatic and microscopic ordering principles. Le Chatelier's principle postulates that if a sample is subjected to pressure, it will try to restore an equilibrium state by changing the molecular configuration, chemical reactions, or phase transition that reduce volume (KHALIQ et al., 2021). On the other hand, according to the isostatic principle, the pressure is transmitted uniformly and homogeneously through the sample, no matter its shape, size, or volume. The last principle states that at a constant temperature, pressure increases the degrees of the ordering of molecules, so pressure and temperature play opposite effects on molecular structures and chemical reactions (YANG, 2021; YORDANOV; ANGELOVA, 2010). The increase in pressure causes a mild rise in the temperature of food considering an adiabatic system (DATTA; DEETH, 1999).

The system consists of a high pressure vessel and its closure(s), valves, pressure generation system, temperature-control device and material handling system (YORDANOV; ANGELOVA, 2010). The composition and thermal characteristics of the pressure-transmitting fluid and the liquid-to-sample ratio must be taken into account as important factors that control the thermal behavior of the food under pressure (BALASUBRAMANIAM; MARTINEZ-

MONTEAGUDO; GUPTA, 2015). Usually, water is the most used pressure-transmitting fluid, but castor oil, glycol, aqueous glycol, sodium benzoate solution and silicon solution are also employed (KHALIQ et al., 2021). In this study, the transmission medium used was water.

1.5. HP effects on milk constituents and microorganisms

Many effects of how this process can inactivate microorganisms are described in the literature (DATTA; DEETH, 1999). All of them are based on changes that are detrimental to the microbial cell, such as in the structure of membrane macromolecules, mainly proteins, which suffer changes leading to the destruction of the cell membrane; disruption of the homogeneity of the intermediate layer between the cell wall and the cytoplasmic membrane; inactivation of the ATPase membrane impairing cellular energy supply; disruption of the nucleic acids and ribosomes involved in protein synthesis (DATTA; DEETH, 1999).

It is known that HP can affect the physicochemical properties of milk, such as reducing the turbidity of the sample and changing color and pH, being the last one not observed in the case of whole milk (YANG et al., 2020). HP has been reported to affect the noncovalent bonds in molecules, with minimum impact compressing the covalent ones. Taking this into account, the literature has noted that small molecules such as sugars, flavor compounds and vitamins such as B1 and B6 (SANCHO et al., 1999; SIERRA; VIDAL VALVERDE; LÓPEZ FANDIÑO, 2000) are not affected by HP. Although vitamin C can decrease in amount due to this treatment, the loss levels have been described as not dependent on the intensity applied in the process (SANCHO et al., 1999). For vitamin B12, to our knowledge, no study has been published reporting the effects that high pressure processing can have on the content of this vitamin.

For the other milk components, according to the suggestion by Knorr et al. (2011), the primary structure of proteins is not affected, causing the treatment only minimal compression in the covalent bonds (BALASUBRAMANIAM; MARTINEZ-MONTEAGUDO; GUPTA, 2015; YANG, 2021), even in a case in which conditions up to 1,500 MPa were used. On the other hand, Olsen & Orlien (2016) reported that the technique for pressure above 500 MPa could increase the reactivity of thiol groups, which leads to denaturation of β-Lg dissociating it and unfolding by the formation of nonactive disulfide bonds, resulting in an incorrectly folded protein. In this way, β-Lg has been described as very sensitive to pressure. However, the same

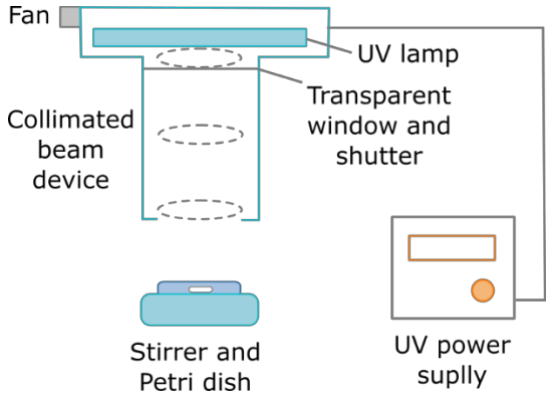
behavior was not observed for other whey proteins, as α-La has been mentioned as resistant to denaturation at pressures up to 400 MPa in milk and still stable, even after treatment at 800 MPa. Considering the casein micelles, the structure can be dissociated into smaller submicelles according to pressure conditions, treatment time and temperature. According to Orlien et al. (2006), above 600 MPa the dissociation of casein micelles dominates and they remain in this configuration without major changes.

The fat globule membrane does not appear to be affected by HP treatments at 100-400 MPa, while at pressure levels of 400-800 MPa, the MFG size can be affected due to the enlarging of the particle size as a consequence of the attempt to repair some damage caused in the membrane, leading to aggregation (HUPPERTZ; FOX; KELLY, 2003; KANNO et al., 1998; YANG, 2021). However, Yang et al. (2020) reported that HP at 600 MPa did not affect the particles of the fatty globules.

1.6. UV-C light processing

UV light processing is a nonthermal technology that can be carried out at an ambient temperature, considerably reducing the microorganisms, with low detrimental effects on the color and flavor of food (DATTA; HARIMURUGAN; PALOMBO, 2015). According to the emission wavelength, UV radiation can be classified as UV-A, UV-B and UV-C. The UV-C wavelength remains from 200 to 280 nm. For food treatment intended to inactivate pathogens and microorganisms, 254–264 nm wavelengths are commonly used (CHOUDHARY; BANDLA, 2012; DATTA; HARIMURUGAN; PALOMBO, 2015).

The technique involves exposing the sample to a light source that can be in a configuration of a laser, a UV reactor, or, as in the case of the present study, a bench-scale apparatus referred to as a collimated beam. This system is composed of a power supply, a collimated beam coupled to a beam stand, a shutter, a stirring, a radiometer and a sensor. The collimated beam is the light source emission, while the shutter is the light sensor and the exposure time controller. The radiometer and the sensor measure the irradiance to which the sample is submitted. In this system, the sample is placed on the horizontal surface below the bottom of the collimator in a container (beaker or Petri dish) under continuous stirring to ensure an equal UV dose received by the sample (BOLTON; LINDEN, 2003). Figure 20 represents the setup of the equipment.



Source: Adapted from (BOLTON; LINDEN, 2003).

Figure 20– Scheme of a bench scale device for UV experiments.

The average germicidal fluence (UV dose) received by the sample is given by Equation (16):

$$E = E'_{avg}.t\ , \tag{16}$$

where E'_{avg} is the average germicidal fluence rate and t is the exposure time.

The average germicidal fluence rate is determined according to Equation (17) (BOLTON; LINDEN, 2003; VITZILAIOU et al., 2021):

$$E'_{avg} = E_0\ .PF\ .RF\ .WF\ .DF\ .SF\ , \tag{17}$$

where E_0 corresponds to the radiometer measurement reading, PF is the Petri factor, RF is the reflection factor, WF is the water factor, DF is the divergence factor and SF is the sensor factor. All these terms are explained in more detail below:

- E_0 corresponds to the radiometer measurement reading at the center of the Petri dish;
- PF is used to correct the irradiance reading at the center of the Petri dish (I_c) to reflect the average incident fluence rate over the surface area of the liquid sample (I_{avg}), defined as the ratio of these two measurements as follows

$$PF = \frac{I_{avg}}{I_c}\ ; \tag{18}$$

- *RF* represents the real incident beam that enters the sample (Equation 19), taking into account that the refractive index (*R*) of light changes when passing between two different media,

$$RF = 1 - R \; ; \tag{19}$$

- *WF* denotes the water factor (Equation 20), which corrects the decrease in irradiance arising from absorption as the beam passes through the water, considering that the water absorbs UV at the wavelength of interest, where *a* is absorbance for a 1 cm path length and *l* is the vertical path length of the water in the Petri dish,

$$WF = \frac{1-10^{-a.l}}{a.l.ln10} \; ; \tag{20}$$

- *DF* corrects some divergences as the beam is not perfectly collimated, so for distances from the lamp more than four times the aperture diameter (Equation 21), where (*L*) is the distance from the UV lamp to the surface of the cell suspension and (*x*) is the path length of the cell suspension

$$DF = \frac{L^2}{(L+x)^2} \; ; \tag{21}$$

- *SF* takes into account the variation in the detector sensitivity over a band (200-300 nm), which is given by the sensitivity of the detector at the specific wavelength applied in the experiment (*S*) and the weighted average sensitivity of the detector over the band ($\sum_i N_{\lambda i} \, . S_{\lambda i}$), according to Equation (22)

$$SF = \frac{S}{\sum_i N_{\lambda i} \, . \, S_{\lambda i}}. \tag{22}$$

1.7. UV-C light effects on milk constituents and microorganisms

The effectiveness of UV light in the inactivation of the microorganism is mainly because UV light can induce the physical shifting of electrons and breaking bonds in deoxyribonucleic acid (DNA), as well as lead to alterations to the microbial DNA caused by

crosslinking of the pyrimidine bases with the formation of pyrimidine dimers, which block DNA transcription and replication by compromising cellular functions causing microbial cellular death. UV light can also induce damage to proteins on the cell membrane and affects metabolic processes essential to microorganisms' survival (DATTA; HARIMURUGAN; PALOMBO, 2015; GUERRERO-BELTRÁN; BARBOSA-CÁNOVAS, 2004; GUNESER; YUCEER, 2012; YIN et al., 2015). Some pieces of research have been conducted to evaluate the potential of UV light as a nonthermal alternative to thermal pasteurization for milk and dairy products (CHRISTEN et al., 2013; ENGIN; YUCEER, 2012; MATAK et al., 2005). For example, Yin et al. (2015) reported reductions of 2-3, 0.7-2.4 and 1.3-2.1 Log unities for E. coli, applying 254, 222 and 282 nm wavelengths in the treatment of milk.

Proteins can be directly photo-oxidized by UV light due to the absorption of the UV radiation by the protein or bound chromophore groups (tryptophan, tyrosine, phenylalanine, cysteine, flavins and heme), leading to the formation of excited states or radicals via photoionization. Proteins can also undergo indirect oxidation through the formation and subsequent reactions of singlet oxygen generated by the transfer of energy to the ground state (triplet) molecular oxygen by either protein bound or other chromophores (DAVIES, 2003). Consequently, unfolding, aggregating, or fragmentation of proteins can occur, compromising their secondary and tertiary structures (MANZOCCO, 2015; PATTISON; RAHMANTO; DAVIES, 2012). The literature has shown that caseins can be susceptible to photoreactions due to UV light (MANZOCCO, 2015). However, globular proteins such as lactoglobulins are more photosensitive and typically unfold under exposure to UV radiation (MANZOCCO, 2015). According to Wu et al. (2017), β-Lactoglobulin treated with UV-C at a dose of 11.8 W m^{-2} increased the β-structure, free sulfhydryl groups, dityrosine and radical scavenging activity, while the α-helicity decreased as well as, random coils and disulfide bonds (S–S).

Regarding lipids, Christen et al. (2013) and Matak et al. (2007) reported that the fatty acid profile is not significantly altered by UV irradiation. However, treatment of whole milk by UV light can lead to oxidative degradation due to the oxidation of unsaturated fatty acid residues in lipids and phospholipids, according to Koutchma (2009).

Vitamins have been described to be sensitive to light. The most sensitive was reported as vitamin C, followed by vitamins E, A and B2, showing a decrease in their content in the Guneser & Yuceer (2012) study for milk treated at 254 nm using doses of 12.6 to 88.2 J / ml. However, Choudhary et al. (2012) and Datta et al. (2015) reported an enrichment of vitamin D. B12 is known to be sensitive to light (FEDOSOV; NEXO; HEEGAARD, 2018; JUZENIENE;

NIZAUSKAITE, 2013; SCHNEIDER, 1987). Furthermore, vitamins C, B1, B2 and B3 have been described to play an antagonist effect on the depletion of B12 (AHMAD et al., 2014; SCHNELLBAECHER et al., 2019) due to light. Vitamin B2, for example, undergoes photosensitization leading to superoxide anion radicals, singlet oxygen, hydroxyl radicals and hydrogen peroxide products (CHOE; HUANG; MIN, 2005; OTTAWAY; OTTAWAY, 2010), acting as a photosensitizer that increases the photodegradation of B12 and other constituents in milk (WATANABE et al., 2013). Based on this, the behavior of vitamin B12 under light exposure deserves further investigation.

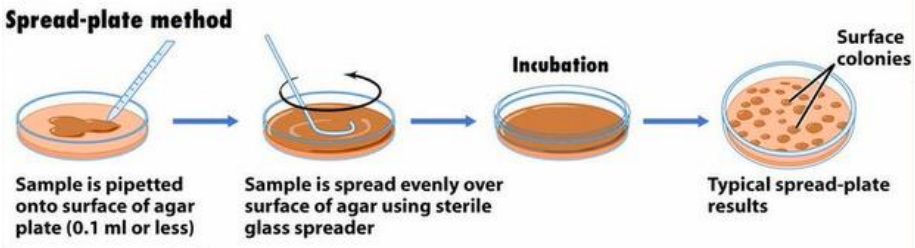
1.8. Microbiology: counting of microorganisms

Analytical tests for bacterial counts can be applied to characterize the microbial population in milk, being the standard plate counting the most traditional. In this technique, the sample is diluted (in water or saline water) according to serial dilution (usually ten-fold serial dilutions) and spread in a nutrient agar medium. This medium can be specific to the growth of a particular microorganism or non-selective media, such as plate count agar (PCA), which is propitious to the growth of bacteria, yeast and molds. The samples are incubated under aerobic conditions, and after 48 h at 32ºC, the total viable count can be determined. Plates containing 30 to 300 colonies are considered, in which each colony represents one viable cell in the original sample (WALSTRA; WOUTERS; GEURTS, 2005). This concentration (N), referred to as colony-forming units (CFU), is calculated in CFU/ml according to Equation 23:

$$N = \frac{\bar{x}}{V \,.\, d} \ , \tag{23}$$

where $\bar{x}$ is the average count per plate, V is the volume of the sample plated and d is the dilution factor (Adams et al., 2016). Figure 21 summarizes the procedure.

Although total bacteria counts are less useful for identifying the sources of bacterial contamination and assessing the risk to milk quality due to a specific bacterial population, it is still helpful to determine if milk meets specific regulations (WALSTRA; WOUTERS; GEURTS, 2005).



Source: Reproduced from (BROCK et al., 2003).

Figure 21– Illustration of the spread plate method applied in microbial determination.

2. Objectives

Chapter III of the present thesis aims to develop an analytical method to determine B12 by UHPLC-UV and investigate the impact of nonthermal technologies on this vitamin in milk, considering their relation to the microbicidal effects. Specifically, the research in this chapter is purpose to:

- Adapt and improve an analytical method for the determination and quantification of vitamin B12 in milk employing the UHPLC-UV technique;
- Processing of milk by applying nonthermal technologies such as PEF, HP and UV-C light in different conditions of treatment;
- Extract and measure the concentration of vitamin B12 in milk samples processed by the nonthermal technologies using the adapted method employing UHPLC-UV;
- Analyze the existence of significant changes in B12 levels using statistical methods of analysis;
- Investigate the impact of the nonthermal treatments on the proliferation of the microorganisms;
- Analyze microbiology results by employing statistical methods;
- Compare the efficacy of the milk treatments considering the preservation of vitamin B12 in the samples, considering the microbiological aspects.

3. Materials and Methods

3.1. Establishment and improvement of a protocol for B12 extraction and analyses by UHPLC-UV

3. 1. 1. Milk samples and chemicals

Fresh bovine raw milk from Danish Red cows was obtained from an organic farm in Denmark (Mannerup Møllegård, Osted, Denmark). The samples were stored at -20 ^{o} C and thawed overnight at 4 ^{o} C to prepare the samples of each experiment concerning the tests of the protocol.

Analytical standard of cyanocobalamin (MW: 1355.37 g/mol), sodium acetate trihydrate (puriss. pa, 99.5%), acetic acid (puriss. pa, 99.8%), porcine gastric mucosa pepsin and trifluoroacetic acid (TFA) (for HPLC, 99.0%) were purchased from Sigma Aldrich Chemie GmbH (Taufkirchen, Germany). Potassium cyanide (KCN) was obtained from J. T. Baker (Deventer, Holland). Methanol (MeOH) (for HPLC, super gradient grade) and acetonitrile (for HPLC LC-MS grade, 99.9%) were acquired from VWR BDH Chemicals (Gdańsk, Poland) and (Fontenay-sous-Bois, France), respectively.

Sodium acetate buffer (50mM, pH 4) was prepared using sodium acetate trihydrate and acetic acid. The potassium cyanide solution (1%) was prepared in deionized water. Two solutions with TFA 0.0025% were prepared, one in deionized water and the other in acetonitrile. The cyanocobalamin stock solution (1mg / ml) was prepared directly in deionized water and kept at -20^{o} C.

3. 1. 2. Extracting B12 and improvements of the protocol

Vitamin B12 was extracted from milk samples using the Easi-extract® vitamin B12 P80/P80B columns according to the protocol (R-BIOPHARM RHÔNE LTD, 2021) supplied by R-Biopharm Rhône Ltd (Glasgow, Scotland) with adaptations. First, the protocol was tested regarding the viability of some processes and the precision (repeatability) of extraction/quantitation methods in different steps of the experimental procedure. For

simplicity's sake, each part, tested on different days, is marked (I, II and III) in the main text below and described at the end of this section (in the order in which they were tested).

All procedures were performed under red light. 30 ml of milk was poured into a glass flask, followed by the addition of sodium acetate buffer (50 ml, 50 mM, pH 4) and pepsin (1 g), and left to stir for 10 minutes. Next, 1 ml of KCN (1%) was added, and the system was agitated for another five minutes. The sample was incubated in a shaking water bath at 37° C for 30 minutes and then in a shaking water bath at 100° C for 30 minutes. Subsequently, the sample was removed and placed in an ice bath to cool to room temperature. So, the extract was transferred to a volumetric flask and the volume (100 ml) was filled with sodium acetate buffer. The mixture was filtered through a Whatman 597 1/2 folded filter paper (Whatman Cytiva, Germany) (III.a).

In the sequence, the samples were concentrated and cleaned by the use of the Easi-extract® vitamin B12 immunoaffinity columns (R-Biopharm Rhône Ltd, Glasgow, Scotland). Next, a volume (I) of the filtrate was passed through the column, followed by water (10 ml), air (60 ml), and then 2.7 ml of methanol for what was performed backflushing procedure (II). A volume of 0.8 ml of methanol was added for complete extraction of the vitamin from the column without backflushing. This procedure was carried out employing one column (III.b) for each sample resulting from one bottle. During the SPE procedure, solvents and samples were allowed to pass through the column by gravity as in the following: flow rates of 1 drop/3 seconds for samples, 1 drop/1 second for water and 1 drop/3 seconds for methanol. In the end, the eluate was dried at 70° C under a nitrogen stream. The samples were kept at -45° C until chromatographic analyses (III.c).

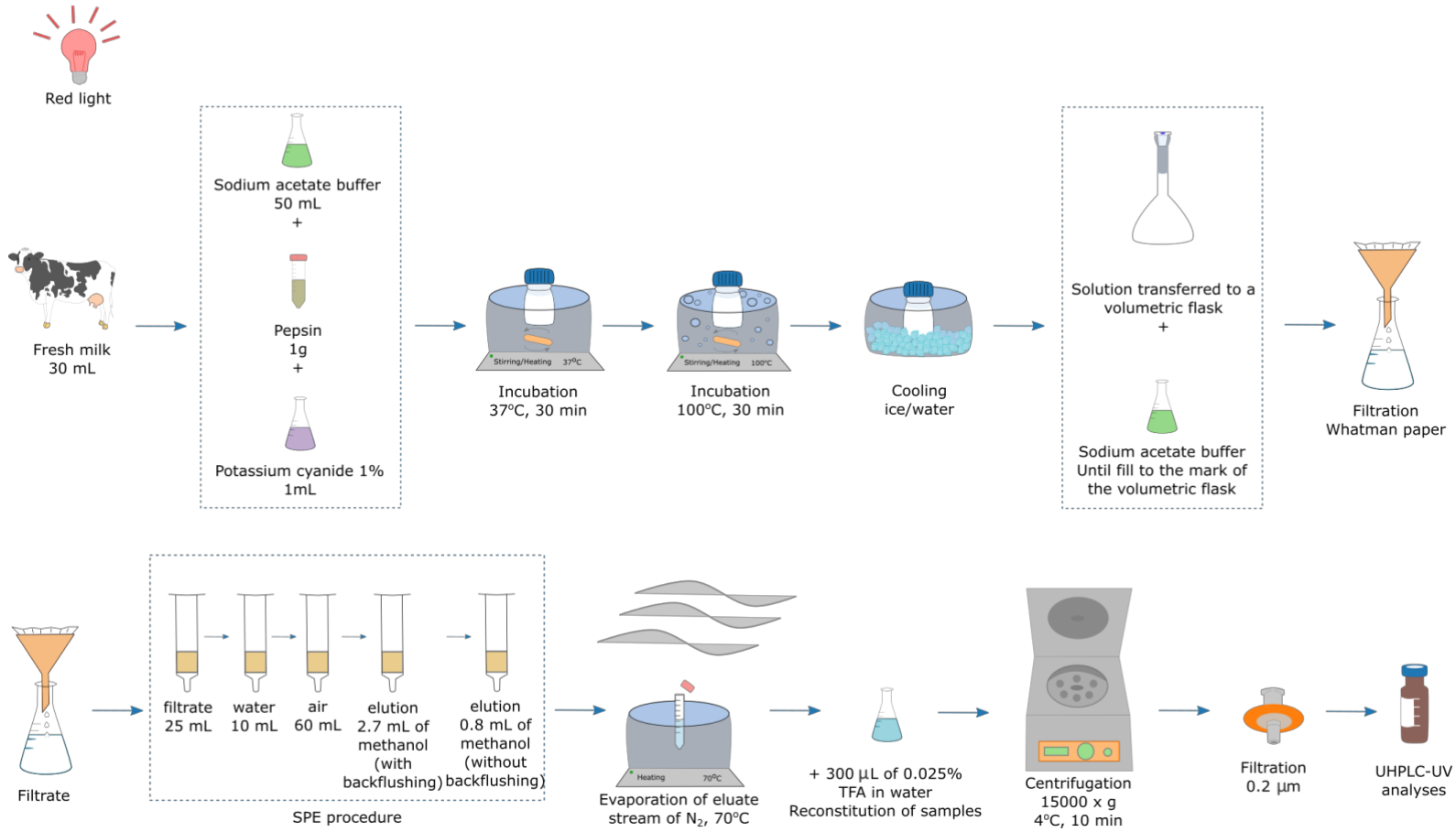
(I) Regarding the first improvement concerning the protocol, the volume of milk added to the column was tested. This procedure was performed to check if the immunoaffinity column provides a linear relationship related to the amount of milk added and the concentration of B12 recovered after the clean-up procedure. Therefore, maintaining all the conditions described in the standard protocol, the volumes of milk corresponding to 10, 20 and 50 m added to the column were tested in duplicate.

(II) The necessity of the backflushing process, which the supplier recommends, was tested. Analogously, maintaining the other steps described in the standard protocol, this part was experimented by comparing three samples prepared with backflushing and three without

backflushing, derived from the same bottle of sample preparation (each column received 10 ml of solution).

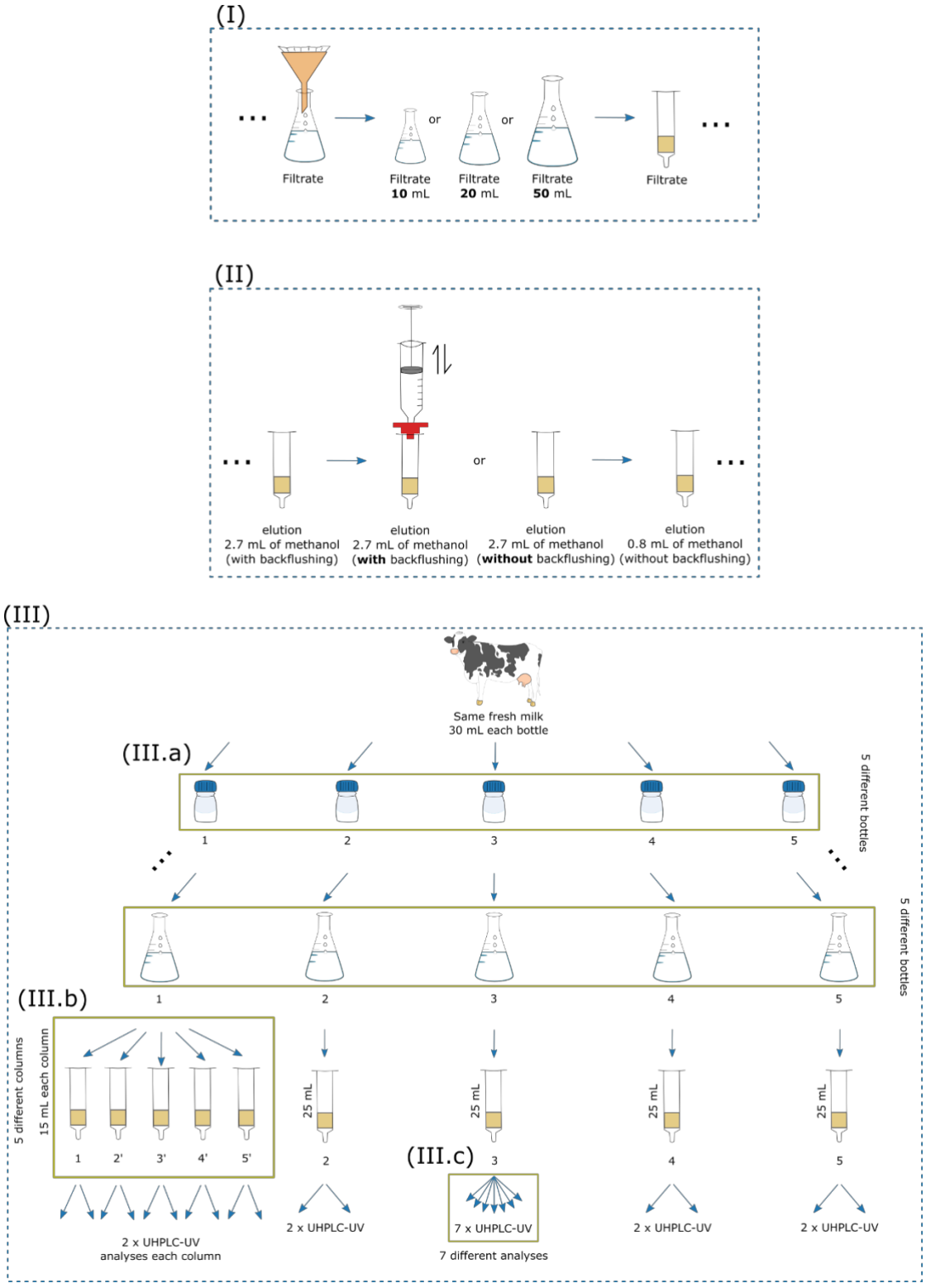
(III) (III.a, III.b, III.c) The precision of the method that encompasses the procedure of B12 extraction and quantification by UHPLC-UV was studied considering three different aspects of the protocol. The first aspect was the repeatability of the method concerning the number of samples prepared to consider the beginning of the experimental procedure. All parts described until the marker (III.a) were carried out in quintuplicate, resulting in five bottles of milk sample preparation. Therefore, it allows us to check the repeatability of the method related to different bottles. Additionally, the repeatability considering the use of different columns was tested. For one of the selected bottles (Bottle 1), the clean-up process was carried out in quintuplicate. So, for columns that received the content of Bottle 1, each column was filled with 15 ml (III.b). Finally, to verify the repeatability related to the analyses in UHPLC-UV (a step that will be depicted in the next section), chromatographic experiments were conducted seven times, specifically for the sample originating from Bottle 3 (III.c). And twice for the other samples.

The established protocol with adaptations and the steps tested considering all the different aspects of the approach described in this section are illustrated in Figures 22 and 23, respectively.



Source: Figure elaborated by the author.

Figure 22- The experimental procedure established for the preparation of milk considering the extraction of vitamin B12 using SPE columns followed by the analyses in the UHPLC-UV. Protocol of vitamin B12 extraction adapted from Easi-extract® vitamin B12 (R-BIOPHARM RHÔNE LTD, 2021).



Source: Figure elaborated by the author.

Figure 23- Adaptations and improvements made from the standard protocol regarding (I) Different amounts of sample added to the SPE column; (II) Employment or not of backflushing procedure; (III) Repeatability of the method.

3. 1. 3. Analysis of B12 by UHPLC-UV

For UHPLC-UV analyses, the dried extracts were reconstituted in 300 µl of water (0.025% TFA) and vortex mixed for 20 seconds. Then, the samples were centrifuged (15000 x g, at 4ºC, for 10 min, Ole Dich Instrumentmakers ApS, Denmark) and the supernatant was filtered employing 0.2 µm RC-membranes (Phenomenex, EUA). The samples were collected in amber vials for chromatographic analyses.

An ultra-high-performance liquid chromatography system (UHPLC) was used to conduct the experiments, with the following specifications: Thermo Dionex UltiMate 3000 (Thermo Fischer Scientific, Germany) coupled with an RP C18 Waters Acquity UPLC HSS T3 column (1.8 µm, 2.1 x 100 mm, Waters Corporation, Ireland) and a guard column Acquity UPLC HSS T3 (3/PK, 1.8 µm, 2.1 x 5 mm), both from Waters. The conditions under which the experiments were carried out were: 10 µl of injection volume, 0.32 ml / min of flow rate and 30º C regarding the temperature maintained in the column. The samples were injected and separated using a gradient of water (A) and acetonitrile (B), both containing TFA (0.025% v/v). The linear gradient employed was 5 to 40% of B in 5 min, which was then maintained for 1 min. The column was regenerated for the next analysis with a linear gradient of 40 to 5% of eluent B in 4 min and maintained at 5% B for 5 min.

The identification of the peak for vitamin B12 (with a retention time of around 6.2 min) was carried out compared to standard cyanocobalamin and confirmed by the characteristic absorption band at 361 nm detected in the UV spectrum.

3. 1. 4. Processing and quantification of B12

UHPLC-UV data were acquired and processed using the Dionex Chromeleon 7 software (Thermo Fischer Scientific, Germany). The peak areas in the samples were integrated and B12 amounts were calculated based on a linear standard curve (fitted using Origin Pro 8.5 software from OriginLab, Massachusetts, USA). This curve was prepared by measuring the peak areas in 9 cyanocobalamin standard solutions of known concentration, such as 0.015; 0.025; 0.050; 0.075; 0.100; 0.150; 0.300, 0.600 and 0.900 µg / ml (diluted from the standard stock solution in deionized water with TFA 0.025%).

3.2. Experiments regarding PEF/HP/UV treatments

3. 2. 1. Milk samples and chemicals

Fresh bovine raw milk from Danish Red cows was obtained from the organic farm in Denmark (Mannerup Møllegård, Osted, Denmark) in the morning (day 0). The samples were divided into sterile bottles and kept at 4^{o} C until processing, which was carried out the same day (in the case of pasteurization) or the next day (day 1, in the case of HP / PEF / UV treatments).

Plate count agar was obtained from Himedia (Maharashtra, India). All chemicals and solutions used in this section were prepared and used as described in the previous section (3.1) in the present chapter.

3. 2. 2. Milk composition, pH and conductivity

The composition of the whole milk before processing by pasteurization, PEF, HP, or UV-C treatments was measured in duplicate (morning of day 0) using a MilkoScan FT2 (79069, Foss Analytical S/A, Hillerød, Denmark). Furthermore, the pH and conductivity of the milk samples were measured in duplicate using a pH meter (HQ411d, HACH-LANGE, USA) and a conductivity meter (SensION+ EC71, HACH-LANGE, Germany), respectively.

Milk samples were stored at 4^{o} C until pasteurization (which was performed on the same day, denominated day 0) and overnight until day 1, when they were treated with PEF, HP and UV-C, as described in the sequence.

3. 2. 3. Pasteurization

Milk pasteurization was employed in this work only as a reference on the effect of microbial inactivation. Milk pasteurization was conducted in a pilot plant (Microthermics UHT / HTST Lab Direct & Indirect Processing System, Microthermics Inc., North Caroline, USA). The milk sample was processed by heating at 71.0^{o} C for 15 s, followed by cooling at 17.9^{o} C.

The procedure was performed in the afternoon on the same day as the sample collection (day 0). After the treatment, the sample was stored at 4° C until microbiological analyses and at -45° C until the day of B12 extraction.

3. 2. 4. Treatment with PEF

The PEF processing was carried out in a treatment chamber coupled with an electric pulse generator (M100, ScandiNova Systems AB, Uppsala, Sweden) and a digital oscilloscope (Rigol MSO 5354, Rigol Suzhou Technologies Inc., China). Milk samples (pre-cooled at 4° C on ice) were introduced into the PEF equipment using a pump (Netzsch, Pumpen & Systeme GmbH, Germany) with different flow rates for each set of experiments, such as 3.96, 5.04, 6.12 and 7.20 L/h corresponding to the treatment time of 16, 12, 10 and 8 μs, respectively. For all assays, the PEF parameters were set as in the following: generator charge voltage of 600V, monopolar pulses with a frequency of 100Hz and pulse width of 2 μs. The inlet and outlet temperatures of the milk samples were measured using a thermometer.

All procedures were carried out in triplicate. After the treatment, the samples were stored at 4° C until microbiological analyses. Later, the samples were kept at -45° C until the day of B12 extraction.

3. 2. 5. Treatment with HP

To perform HP treatment, narrow mouth bottles (VWR international, PA, USA) with a capacity of 60 ml were filled with milk samples and packed into plastic bags (PA/PE 20/70, 32 oxygen cm^3/m^2 d bar at 23°C and 75% RH, SFK, Hvidovre, Denmark) at 40% vacuum. High hydrostatic pressure processing was carried out employing a food processor (High-Pressure Press QFP-6, Quintus Technologies AB, Vesterås, Sweden) and water as the pressurization fluid, with an initial and final average temperature of 22.3 and 21.4° C, respectively. The compression rate was about 250 MPa/min, whereas the depressurization was at a rate of about 300 MPa/min (TANG; MA, 2009). Experiments were conducted in one cycle for each applied pressure condition: 300, 400, 500 and 600 MPa. The holding time was 5 min for all procedures.

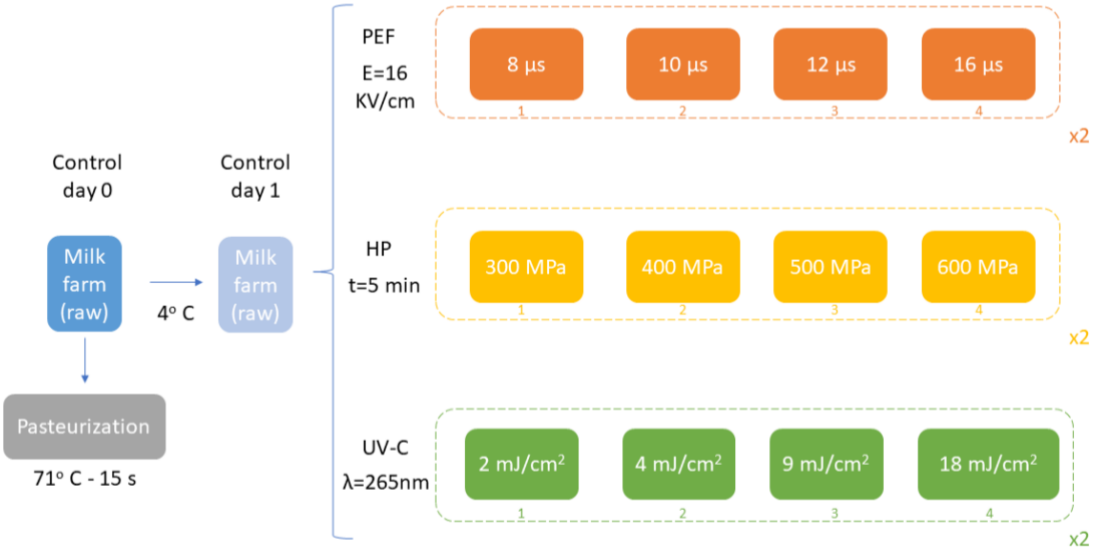
HP processing was carried out in duplicate. Subsequently, the samples were stored at 4° C until microbiological analyses and at −45 °C until further analyses.

3. 2. 6. Treatment with UV-C

Milk samples were exposed to UV-C using a UV LED collimated beam apparatus (PearlLab BeamTM, Aquisense Technologies, Erlanger, USA). The initial and final incident irradiances (which varied from 0.2182-0.2180 mW/cm^2) were measured by using a radiometer (ILT 2400) and a sensor (SED005), both from International Light Technologies (Peabody, USA).

To perform each experiment, 20 ml of milk was pipetted into a Petri dish (52mm) and a sterile magnetic stir bar (4.5 x 12 mm) was added. In the following, the Petri dish was put on a magnetic stirrer (speed 150 rpm). The PearlLab beam apparatus was placed on top of the Petri dish, so the shutter and the UV button (265nm) were turned on immediately when a timer was triggered. After achieving the desired time, the shutter and UV buttons were turned off and the sample was collected and kept at 4^o C. This procedure was repeated three times (to achieve a volume of 60 ml of milk treated for each sample). Additionally, the treatments were made in duplicate, considering each specific exposure time of the treatment. The treatment times were 2, 4, 8 and 16 min, corresponding to a dose of 2, 4, 9 and 18 mJ/cm^2, respectively. The temperatures of the samples were collected before and after the end of each procedure. After treatments, the samples were stored at 4° C until microbiological analyses and at -45^o C until the day of B12 extraction.

Additional parameters were obtained from calculations (BOLTON; LINDEN, 2003). Milk's absorbance was determined by employing a Quartz Suprasil® cuvette (QX High Precision Cell, Hellma Analytics, Jena, Germany) and a spectrophotometer at 265nm (UV 1800, Shimadzu Corporation, Kyoto, Japan). Figure 24 represents the conditions applied in each experiment with respect to the PEF, HP and UV-C treatments.



Source: Figure elaborated by the author.

Figure 24- Treatments and conditions applied in the experiments to treat milk by pulsed electric field (PEF), high hydrostatic pressure (HP) and UV light (UV-C).

3. 2. 7. Determination of microbiological inactivation

Total bacterial count in raw milk (controls from day 0 and day 1) and in milk after pasteurization and PEF / HP / UV treatments was determined with serial dilutions (in sterile 0.9% sodium chloride deionized water) of each sample, which was plated on plate count agar prepared in distilled water. In the sequence, the plates were incubated at 30° C for 48 h in a controlled temperature chamber. The experiments were performed in triplicate for each dilution. Afterward, microbial count was done.

3. 2. 8. B12 extraction, UHPLC-UV analyses and quantification

Vitamin B12 was extracted from milk samples by using the Easi-extract® vitamin B12 P80/P80B protocol (R-BIOPHARM RHÔNE LTD, 2021) and the established conditions considering the protocol improvement described in section 3.1.2. of the present chapter. The volume of sample passed through the immunoaffinity column was 25 ml, applying backflushing procedure (in a part of the extraction procedure) and for each sample: 1 bottle of sample

preparation was considered, with a clean-up step made in duplicate, followed by 4 analyses in the UHPLC-UV for each sample (2 per column).

The UHPLC-UV analyses and quantification were carried out as described in Sections 3.1.3. and 3.1.4., also in the present chapter.

3.3. Statistics

SAS 9.4 software (SAS Institute Inc., USA) was applied to conduct the statistical analyses. The student's t-test was used to evaluate the data regarding the tests for the protocol considering the use or not of backflushing. Analysis of variance (ANOVA) followed by Duncan's multiple range test was employed to compare the data due to different milk processing (PEF / HP / UV) and in the microbiological experiments analyses.

4. Results and Discussion

4.1. Sample preparation procedure

The method used in the present thesis on the determination of B12 refers to the total vitamin B12 content, which includes free molecules and the protein bound forms (LI et al., 2019).

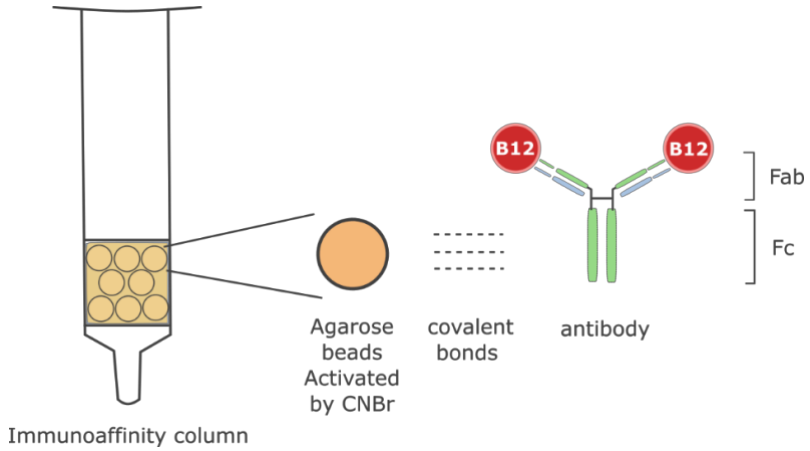
First, to access the B12 molecules protein-bound, proteins must be denatured to release the vitamin, which was achieved by using three approaches in combination: heat treatment, enzyme proteolysis and acidic precipitation (LI et al., 2019). In our procedure, the use of pepsin associated with sodium acetate buffer corresponds to enzyme proteolysis. In this step, the digestive enzyme, which is activated only in an acidic medium (pH 4), breaks the bounds in the proteins, releasing small subunits (peptides and amino acids). The use of acetate buffer also represents acidic precipitation. Finally, the use of acetate buffer (associated with potassium cyanide) under heat (37^{o} C / 100^{o} C) also promotes the denaturation of proteins, as they are

sensitive to variation in temperature, undergoing conformational changes (LI et al., 2019; LUO et al., 2006; REPOSSI et al., 2017; ZIRONI et al., 2014).

Once all B12 has been released from proteins, it is necessary to convert the natural forms of the molecule into cyanocobalamin, which is the most stable considering all possible mechanisms of B12 degradation (SCHNELLBAECHER et al., 2019). Adding potassium cyanide in excess leads to the conversion of all natural forms (AdoCbl, MeCbl and OHCbl) into cyanocobalamin, replacing natural ligands with the cyanide group (CN^-) (COOLEY et al., 1951; LI et al., 2019; SELVA KUMAR; CHOUHAN; THAKUR, 2010). According to Fedosov et al. (2018), cyanide simultaneously acts by converting B12 into the synthetic form and also releasing B12 from protein bound because it is a more potent ligand compared to the OH ligand, promoting the cyanolysis of casein-Cbl complex, for example, in a process that is enhanced by high temperatures.

As milk is a complex matrix, a step of purification and concentration of the sample was required, in which immunoaffinity extraction cleaning using Easi-extract vitamin B12® columns was employed, as described in other works in the literature (Campos-Giménez et al., 2012; Campos-Giménez et al., 2008; Chamlagain et al., 2015; Heudi et al., 2006). The immunoaffinity extraction is a liquid chromatography process representing a subcategory of affinity chromatography (MOSER; HAGE, 2010). The column in this kind of process consists of monoclonal antibodies with high affinity and specificity for B12 immobilized in a gel suspension (solid support) packed into the column (DELAUNAY-BERTONCINI; HENNION, 2004; LI et al., 2019; MARLEY; MACKAY; YOUNG, 2009). The solid support, which can be made from different types of inert materials, is agarose beads (MARLEY; MACKAY; YOUNG, 2009; MOSER; HAGE, 2010). Agarose is activated by cyanogen bromide (CNBr), which allows antibodies to link in this support (MARLEY; MACKAY; YOUNG, 2009). The mechanism by which the antibodies attach to the support material varies from covalent bonds to adsorption-based methods (MOSER; HAGE, 2010). For materials activated by cyanogen bromide, generally, the attachment occurs via an interaction between CNBr and free amine groups in the antibodies (MARLEY; MACKAY; YOUNG, 2009), which seems to be the mechanism used in the columns manufacturing applied in this study. The monoclonal antibodies (produced from an animal that has been immunized with the desired antigen) that compose the column can bind to a single epitope with identical binding affinities, being highly specific (MOSER; HAGE, 2010). The typical structure of an antibody consists of four polypeptide chains, two of which are identical heavy and the other two, which are identical light

chains linked by disulfide bonds in a Y shape. The lower region (Fc region) of this structure is highly conserved in an antibody class. In contrast, the upper region (Fab) is composed of an amino acid sequence that is identical within a single type of antibody (MOSER; HAGE, 2010). This very specific Fab region is the binding site for the antigen (Figure 25). The interaction between antibody-antigen was described as noncovalent (MOSER; HAGE, 2010). The exact mechanism by which vitamin B12 attaches to antibodies and which part of the molecule interacts is unclear. Some works suggest that the cyanide group (CN^-) is the binding site for B12 due to the high specificity the columns provide to this form of the B12 molecule (Campos-Giménez et al., 2008; Li et al., 2019; Marley et al., 2009). This theory is in agreement with the study of Campos-Giménez et al. (2008), in which the authors reported a poor recovery (<20%) when the other forms of the B12 molecule (OHCbl, MeCbl and AdoCbl) were tested in the immunoaffinity columns.



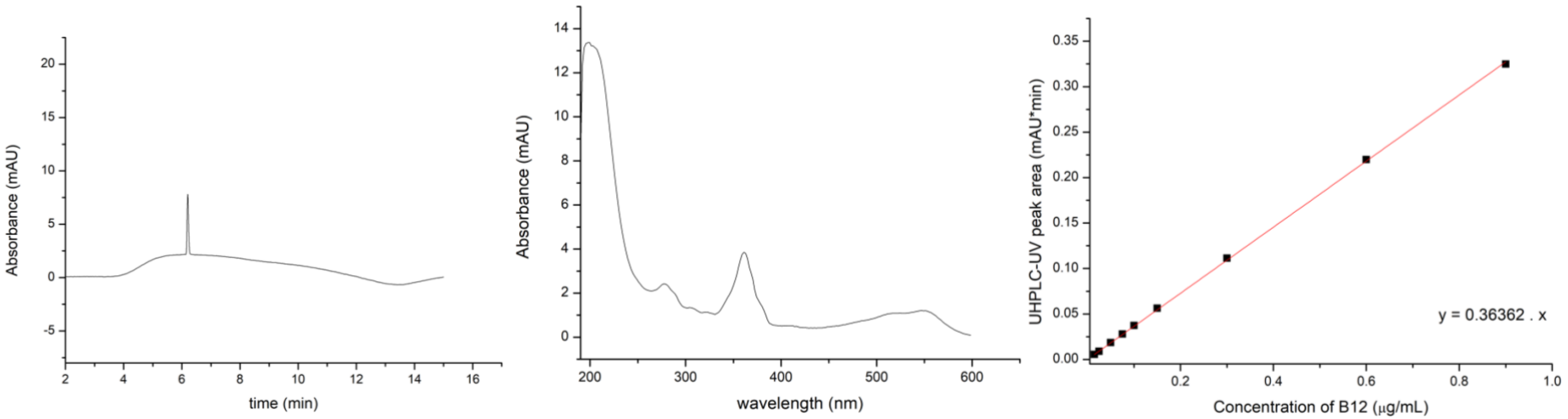
Source: Figure elaborated by the author.

Figure 25- Immunoaffinity columns and interaction between antibody and B12.

4.2. Improvements in the method for B12 extraction and quantification

As previously described, the protocol for the extraction and concentration of vitamin B12 was first tested for milk with no processing regarding the viability of some processes in sample preparation and the repeatability of methods in different steps of the experimental

procedure. Based on the calibration curves obtained for the external standard CNCbl, the concentration of B12 in milk was determined, considering the peak at 6.2 min in the chromatograms with absorption at 361 nm. Figure 26 (a), (b) and (c) illustrate the results of one of the experiments carried out.



(a) (b) (c)

Figure 26 - (a) The chromatogram obtained for one of the samples shows the peak for vitamin B_{12} with a retention time of 6.2 min. (b) The spectrum obtained for one of the samples evidences the absorption regarding vitamin B_{12} with a pronounced band at 361 nm. (c) Calibration curve obtained from standard cobalamin solutions.

Taking into account the first experiment (I) in section 3.1.2 (Figure 23) regarding different volumes of solution tested in the column (10, 20, or 50 ml), Figure 27 was obtained, which shows that the greater the amount of solution added to the column, the greater the amount of B12 captured, in a linear relationship (R^2 = 0.9990). Although the company (R-Biopharm) advises using 10 ml of the sample preparation in the column, probably because there is a limit of sites to bind B12 molecules, we concluded that the columns could support a higher sample volume. Therefore, considering the time-consuming procedure, the best volume of work was determined at 25 ml since this amount allowed us to work above the detection (LOD) (S/N=3) and quantification (LOQ) (S/N=10) limits for our method (< 0.015 and < 0.05 µg / ml), respectively.

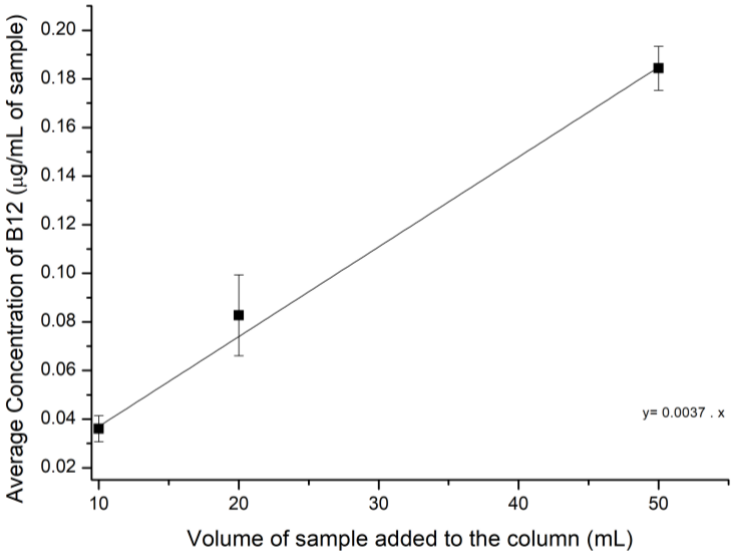


Figure 27- The relationship between the amount of volume of the sample preparation added to the immunoaffinity column (10, 20, or 50) ml and the amount of vitamin B12 detected in the sample injected into the UHPLC-UV.

For the second experiment (II) in section 3.1.2 (Figure 23), the use of backflushing was tested. The procedure was carried out for three columns in which backflushing was applied and three other columns in which the processes were carried out without backflushing from the same sample prepared. Although the statistical analysis comparing both groups did not show statistically significant differences (p> 0.05) between the two groups, we assumed that the amount of B12 recovered tended to be higher, as well as, the standard deviation lower, when

the backflushing was applied compared to the other case, (0.40±0.04) µg of B12/100g of milk, with backflushing and (0.34±0.12) µg of B12/100g of milk, without backflushing. Furthermore, the company advises the backflushing procedure, as it enhances the interaction between methanol and the B12 molecules attached to the column.

The third experiment (III) in section 3.1.2 (Figure 23) was focused on the method's repeatability considering the steps of sample preparation and analysis in the UHPLC at three different levels. The first level (III.a) was the method's repeatability in terms of the number of samples prepared from the beginning of the experimental procedure in different bottles (n = 5). Repeatability was tested considering different columns (n = 5) and different analyses in the UHPLC (n = 7) in the second (III.b) and third (III.c) levels, respectively. Table 2 summarizes the data obtained.

Table 2- Data on the repeatability of the experimental procedure of extraction and quantification of vitamin B12. Processes were studied at three hierarchical levels considering sample preparation in different bottles (n = 5), different columns (n = 5) and different analyses in the UHPLC (n = 7).

Replicate	Average B12 concentration (µg/100 g of milk)	Coefficient of Variance (CV%)
bottles	0.44 ± 0.01	3.34
columns	0.45 ± 0.04	8.07
UHPLC analyses	0.46 ± 0.01	2.06

Source: elaborated by the author.

Table 2 shows that the quantification obtained for B12 in milk were similar for all the levels studied. However, the precision of the methods tested in different steps evidenced that repeatability among different columns was the source of more variance throughout the processes (8.07%), while the UHPLC analyses presented a low variation. The results were in accordance with our expectations since the UHPLC equipment is very precise and automated. The columns could be associated with the step that inserts most variations in the method, as it depends, for example, on how the binding sites for B12 are disposed of in the column (not all

columns are perfectly uniform). Additionally, more errors can be associated with repeatability comparing different columns, as the process involves the operator being more manual, which counts for more deviations in some steps, such as the control of the flow applied in the elution, or even the backflushing handling.

Based on these results, we adopted for all sample preparation and B12 quantification for the subsequent experiments in this chapter, the use of 25 ml of sample in the immunoaffinity column (I), the use of backflushing (II) and for each sample studied, the number of analyses as in the following (III): one bottle considering sample preparation, the duplicate of columns (as this was the main source of variance) and a duplicate of analyses for each column in the UHPLC-UV equipment (besides being the most precise part of our experimental procedure, the analyses in this step were repeated to improve the quality of the statistical data).

4.3. Experiments regarding PEF/HP/UV processing

4. 3. 1. Milk composition, pH and conductivity

The composition of milk used in the experiments before processing by pasteurization and HP, PEF and UV was accessed by Milkoscan analyses, as well as analyses of pH and conductivity, as described in Table 3.

Table 3 - Composition (% w/w) of raw milk analyzed by Milkoscan, pH and conductivity (mS.cm-1). Data represent mean values ± standard deviation (n = 2).

Component/ Measurement	Raw milk (day 0)	Raw milk (day 1)
Fat	4.620 ± 0.003	4.620 ± 0.003
Protein	3.380 ± 0.002	3.380 ± 0.002
Casein	2.560 ± 0.002	2.550 ± 0.002
Lactose	4.480 ± 0.006	4.490 ± 0.006
Total solids	13.890 ± 0.002	13.900 ± 0.002
pH	6.72 ± 0.01	6.75 ± 0.01
Conductivity	4.98 ± 0.01	5.05 ± 0.01

Source: elaborated by the author.

The milk composition was as expected for healthy cows, as well as the measurements of pH and conductivity, according to Walstra et al. (2005).

4. 3. 2. Quantification of vitamin B12 in milk using the optimized method

Vitamin B12 in milk was purified, quantified by UHPLC-UV as described above (Section 3.2.9) and detected at 361 nm as a peak at ~6.2 min, analogously to the results obtained in Figure 26 (b and a, respectively). The repeatability with respect to the retention time (6.209±0.004) min for the B12 peak was 0.07% (CV) for all peaks in the standard curve. The calibration curve (Figure 28) showed high linearity (R^2 = 0.9999), with a detection limit (LOD; signal-to-noise ratio S/N=3) lower than 0.015 µg/ml (in the sample) and a limit of quantification (LOQ; S/N=10) lower than 0.05 µg/ml corresponding to 0.002 µg/ml in the milk sample.

Using the optimized procedure, the peak area for the cyanocobalamin extracted from raw milk on day 1 (CD1) was 0.04, which corresponds to 0.112±0.005 µg /ml in the sample and 0.43±0.02 µg/100g in milk. The values were determined with a precision of 4.96%. These B12 values were close to the value in the database of 0.54 µg/100g of milk (USDA, 2020), as it depends on the cow breed, diet, season and other factors (DUPLESSIS et al., 2016). Furthermore, this concentration was well above the LOQ and allowed the detection of possible decreases due to the subsequent processing of milk.

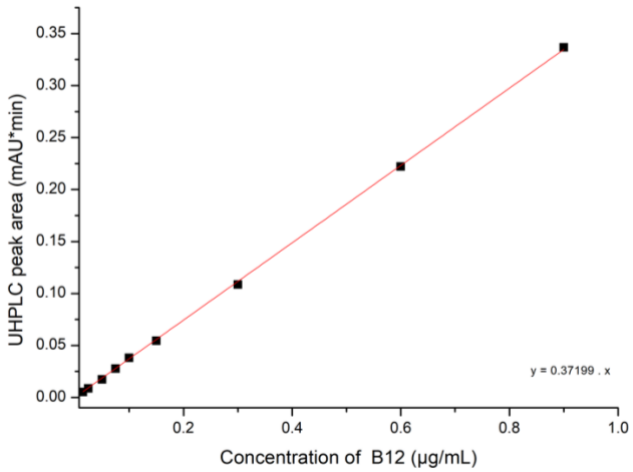


Figure 28 - Calibration curve obtained from standard cobalamin solutions.

4.3.3. Effects of nonthermal treatments on vitamin B12 levels in milk

4.3.3.1. Effects of PEF on vitamin B12 levels in milk

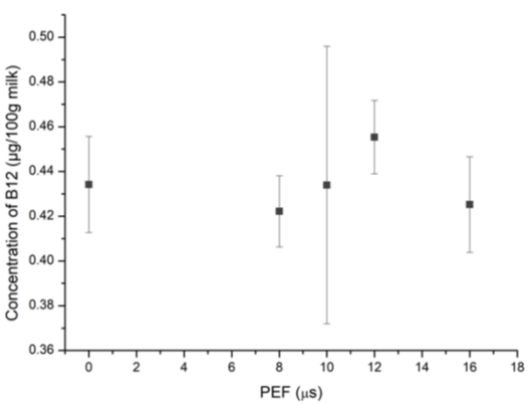
Vitamin B12 concentrations in milk subjected to PEF treatments of various durations are shown in Figure 29 (a), together with the concentration of raw milk from the same day when the processing was performed (Control Day 1 – CD1).

PEF 16μs was supposed to be the treatment to mostly affect the milk due to the most extended treatment time. However, Figure 29 (a) shows that the amount of B12 determined in the milk samples did not vary considerably, as the media values and the standard deviation of the groups overlap among them. Furthermore, the statistics (Figure 29 (d)) did not show significant differences ($p>0.05$) among the samples in these groups of treatment. This suggests that, in general, the PEF processing applied to milk in this study did not affect B12 concentration. Some works have already suggested PEF as a milk treatment, focused on the slight impact it can have on fat (SHARMA; OEY; EVERETT, 2014; YANG et al., 2021), whey proteins (LIU et al., 2015; SUI et al., 2011) and some vitamins (BARBA et al., 2015).

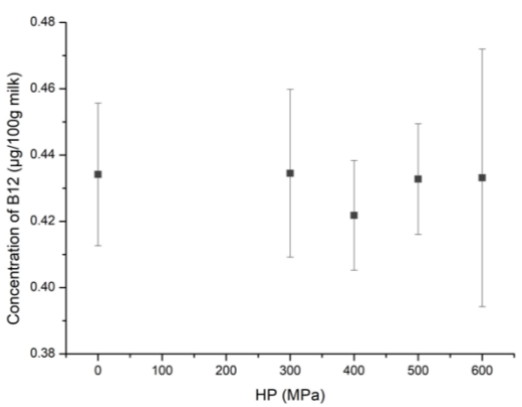
According to Knorr et al. (2011), there is little data on the effects of PEF on food constituents. To our knowledge, there is no data regarding the impact of PEF on B12, while the impact on some other water-soluble vitamins from the B-complex and C has been accessed, for example. Salvia-Trujillo et al. (2011) reported that for PEF conditions of 35KV/cm of electric field strength and a treatment time of 1800 μs (more extreme conditions than ours) applied to fruit juice-milk beverages, no losses were observed for vitamins B1, B2 and B3. However, a slight impact on vitamin C was observed, around 0.5 to 3%, after the processing. Furthermore, Bendicho et al. (2002) observed that PEF did not affect the content of vitamins B1 and B2 in milk, considering the application of treatment times of up to 400 μs and 18.3-27.1 KV/cm of electric field intensity. Accordingly, no significant changes were observed for vitamins B2, B5, B7 and B9 by employing an electric field strength range from 15 to 40 KV/cm (similar to our electric field intensity) and treatment times from 40 to 700 μs for orange juice and milk mixed beverage fortified with water-soluble vitamins (RIVAS et al., 2007). The study by Riener et al. (2009) used conditions similar to ours regarding the electric field strength and treatment times, 15-35 KV/cm and 12.5 to 75 μs, respectively, for the treatment of bovine raw milk. The results were in agreement with ours for all vitamins analyzed, such as B1 and B2, and also for the liposoluble vitamins A and E, without reducing their content.

According to the study by Yang et al. (2021), which applied the same intensity of the electric field as in our case, PEF did not have a significant effect on protein levels in milk. B12 can interact with caseins, especially hydroxocobalamin molecules, which is the predominant form in milk (FOX et al., 2015), through coordination bonds between B12 molecules and histidine residues in the protein (FEDOSOV; NEXO; HEEGAARD, 2018). Yang et al. (2021) reported that PEF changed the particle size of fat globule membranes (MFG) in milk due to their aggregation and consequent adsorption of casein micelles and also whey proteins in their structures as a mechanism to repair the induced electroporation caused in the MFG. This rearrangement can offer additional protection to proteins and, therefore, protection to vitamin B12. Regarding β-lactoglobulins (β-Lg) and α-lactalbumins (α-La), the most predominant whey proteins in milk that can interact with B12 (WANG et al., 2019), Yang et al. (2021) observed that these proteins could also adsorb on the MFG surface, which suggests additional preservation of B12.

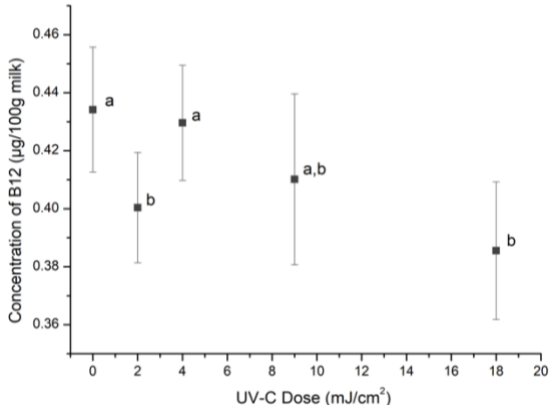
Our results and comparisons with the literature indicate that PEF can be considered a technology without affecting the B-group vitamins. This can be attributed to the fact that this technique is basically based on the intensity of the electric field. Even for conditions in which this parameter was more extreme or for the longest treatment times in the studies from the literature, no impact was observed, probably due to the protection of the proteins in the medium or the vitamin per se. Furthermore, some increases in the temperature during the processing are considered a side effect of this technique. However, the variations sometimes reach values around 50^{o} C (SALVIA-TRUJILLO et al., 2011), which is not considered a critical temperature to cause degradation, even for vitamins considered thermolabile, such as vitamin B12 (WANG et al., 2019).



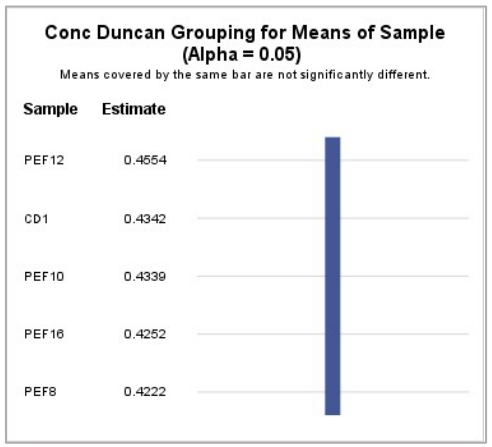
a)



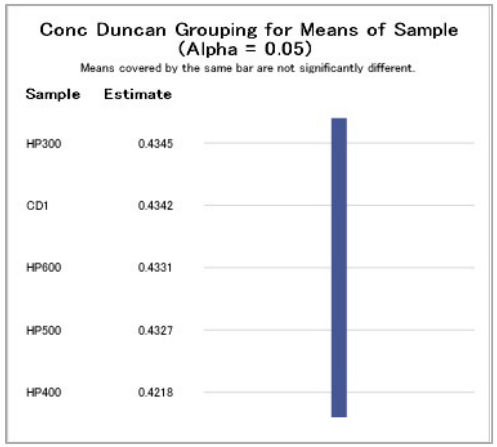
b)



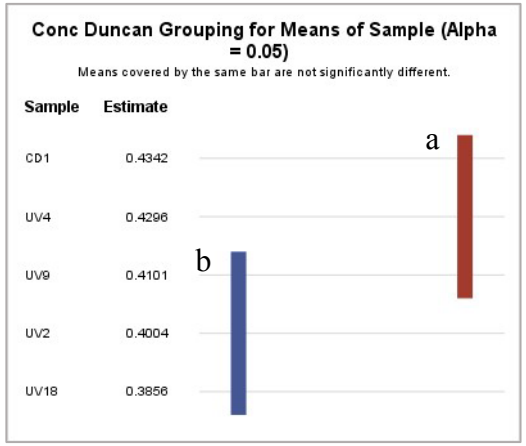
c)



d)



e)



f)

Figure 29 - (a) Average values for vitamin B12 concentration in milk determined in the Control Day 1 – CD1 sample (0) and in each condition of pulsed electric field (8, 10, 12 and 16 µs), (b) high pressure (300, 400, 500 and 600 MPa), (c) UV-C light (2, 4, 9 and 18 mJ/cm^2), (d) Statistic tests to compare the differences in vitamin B12 concentration in milk samples of (Control Day 1 - CD1) and samples treated by the pulsed electric field, (e) high pressure and (f) UV-C light. Values covered by the same bar are not significantly different.

4.3.3.2 Effects of HP on vitamin B12 levels in milk

HP processing for 5 min at 300 to 600 MPa did not cause significant differences ($p>0.05$) in B12 levels in the milk (Figures 29 (b) and (e)). From the literature, small molecules such as sugars, flavor compounds and vitamins (such as B1 and B6) (SANCHO et al., 1999; SIERRA; VIDAL VALVERDE; LÓPEZ FANDIÑO, 2000) are not impacted by HP due to the very low compressibility of covalent bonds (KNORR et al., 2011). Furthermore, the primary structure of proteins (VOIGT; KELLY; HUPPERTZ, 2015) and fat globules (GERVILLA; FERRAGUT; GUAMIS, 2001; TRUJILLO et al., 2002) remain unaffected by this kind of processing. However, vitamin C, secondary and tertiary structures of proteins can be damaged (VOIGT; KELLY; HUPPERTZ, 2015).

Analogously to the PEF treatment, the effects of HP on vitamin B12 in milk have not been accessed so far. Apparently, the only published study suggests that this kind of treatment can cause a slight impact on B12 levels, but still lower than the one caused by thermal treatments, for example. In this case, Aguayo et al. (2017) evaluated the effect of high pressure homogenization at 80 and 120 MPa in functional food supplements such as artichoke extract, royal jelly and iron supplements, observing a reduction in the B12 content of 17-24% for the artichoke extract and 4-7% for the other two products. According to the authors, the extension of the impact can be attributed to the food matrix (VILLOTA; HAWKES, 2006) and the loss of B12 can be related to the presence of lemon juice/vitamin C also present in these supplements, as acidic components can compromise the levels of vitamin B12 (BAJAJ; SINGHAL, 2020; OTTAWAY; OTTAWAY, 2010). However, the results of this study are not entirely comparable to ours since milk is a more complex matrix whose constituents can interfere / prevent more drastic HP effects in the medium.

Taking into account the interaction of B12 with caseins (FEDOSOV; NEXO; HEEGAARD, 2018), Yang et al. (2020) reported that HP treatment with 600 MPa, which is similar to our most extreme condition, can lead to a decrease in the casein particle size due to the entrance of water molecules into the caseins micelle structure, disrupting them. However, this effect is less pronounced in whole milk than in skim milk, for example, because, in the first case, the fat content can increase the protection of the micelles against the pressure effect. Since as our samples were whole milk, this could explain the protection of the casein micelles by fat and the consequent additional protection provided to the B12 molecules.

According to Yang et al. (2020), β-Lg is highly pressuring sensitive, while the α-La is more stable. Due to HP treatment, β-Lg tends to expose this free thiol group to form disulfide-linked dimers and polymers and also aggregates with other whey proteins (BOGAHAWATHTHA et al., 2018; PATEL; CARROLL; KELLY, 2016; YANG et al., 2020). However, according to our results, this does not seem to affect the interaction between this protein and B12, which binds the β-Lg by its hydrophilic sites (WANG et al., 2019). Additionally, as α-La remains unaffected by HP (YANG et al., 2020), it can suggest an improvement in cobalamin protection. According to our results, other water-soluble vitamins, such as B1 and B6, were also reported to be unaffected (100% of retention) by HP treatment of milk at 400 MPa (SIERRA; VIDAL VALVERDE; LÓPEZ FANDIÑO, 2000; TRUJILLO et al., 2002).

Based on this, as PEF treatment, HP can be considered a mild technology for milk constituents without impact on B12 content. This lack of impact can be attributed to the B12 molecule itself, as the covalent bonds in the structure of the vitamin are not affected and, consequently, do not cause degradation of the molecule. In addition, the absence of impact on the other milk constituents enhances the protection of B12. Even with some increase in temperature in the system that can occasionally occur, the increases can be considered negligible with respect to heat-sensitive components and B12 degradation.

4. 3. 3. 3. Effects of UV-C on vitamin B12 levels in milk

Unlike other processing methods, the UV-C treatment significantly affected vitamin B12 levels (Figures 29 (c) and (f)), as expected from the light sensitivity of B12 forms (hydroxocobalamin, methylcobalamin and adenosylcobalamin) in milk (EDELMANN et al., 2016). UV 4 mJ / cm^2 showed a similar B12 concentration to the control (CD1) (see column a in Figure 29 (f)), both of which are statistically significantly different ($p<0.05$) compared to the other groups, such as UV2 and UV18 mJ / cm^2 (see column b in Figure 29 (f)). UV 9 mJ / cm^2 is at the intersection between both classifications. It can be seen that there is a tendency for the longer the time of sample exposure to UV light, the lower the amount of B12 in the samples. Furthermore, for UV 18 mJ / cm^2, the longest exposure time caused a 10% of reduction in B12 levels compared to the untreated raw milk (CD1), which represented 0.38±0.02 μg / 100g of milk, the lowest amount detected in this study. Probably a UV at 18 mJ / cm^2 was a threshold

condition for some drastic effects on B12 arise, as UV-C is known to affect B12 (SELVA KUMAR; CHOUHAN; THAKUR, 2010).

Taking into account the B12 present in milk, most of it is hydroxocobalamin, and more than 95% of the molecules are protein bound (FOX et al., 2015). The other molecules are in free form and are distributed as hydroxocobalamin, methylcobalamin and adenosylcobalamin. Some processes can occur under light leading to the degradation of this vitamin, which suggests that B12-free molecules can be more susceptible to this degradation (WANG et al., 2019).

UV light is known to induce the cleavage of the C-Co β-axial bond in the methylcobalamin Co(III) and adenosylcobalamin Co(III) Cbl forms, leading to the conversion to Co(II) and methyl and adenosyl radical species (Reactions 1 and 2). In the presence of oxygen, the products tend to be oxidized: methyl became formaldehyde, and so, methyl peroxide radical or formaldehyde and hydroxyl radical; adenosine became adenosine-5'-carboxaldehyde; (HOGENKAMP, 1966; SCHNEIDER, 1987); and Co(II) photoproduct in the Cbl tends to form hydroxocobalamin-Co(III), the most stable molecule of B12 in light (FEDOSOV; NEXO; HEEGAARD, 2018) and hydrogen peroxide (Reaction 3) (DEREVEN'KOV et al., 2016). This can be described as the first step in molecule photodecomposition (FEDOSOV; NEXO; HEEGAARD, 2018; FINKE, 1998; JUZENIENE; NIZAUSKAITE, 2013; SCHNEIDER, 1987; WANG et al., 2019). Once in the hydroxocobalamin form, because of the previous photolysis of methyl- and adenosyl-cobalamins, or because of the natural form intrinsically present in milk, hydroxocobalamin also undergoes photochemical degradation by UV-C light yielding hydroxyl radical and Cbl-Co(II) (WILEY et al., 2016), (Reaction 6). The oxidative conditions generated during the Co(II)/Co(III) redox cycle and the hydrogen peroxide, as represented by the Reactions 3, 4, 5 and 6 (DEREVEN'KOV et al., 2016; SUAREZ-MOREIRA et al., 2009; WILEY et al., 2016), contribute to the degradation of the molecule with the cleavage of the vitamin corrin ring, irreversibly leading to degradation products, which are non-bioactive (AHMAD et al., 2014; WANG et al., 2019).

$$CH_3Cbl(III) \xrightarrow{light} Cbl(II) + CH_3^{\bullet} \quad (R1)$$

$$AdoCbl(III) \xrightarrow{light} Cbl(II) + Ado^{\bullet} \quad (R2)$$

$$Cbl(II) + O_2 \longrightarrow Cbl(III)\text{-}O_2^{\bullet -} \longrightarrow Cbl(III)\text{-}H_2O + H_2O_2 + O_2 \quad (R3)$$

$$Cbl(II) + O_2^{\bullet -} + 2H_3O^{+} \longrightarrow Cbl(III)\text{-}H_2O + H_2O_2 + H_2O \quad (R4)$$

$$Cbl(II) + H_2O_2 \longrightarrow Cbl(III)\text{-}OH^{-} + OH^{\bullet} \quad (R5)$$

$$OHCbl(III) \xrightarrow{light} Cbl(II) + OH^{\bullet} \quad (R6)$$

Furthermore, not only light can promote B12 degradation. Vitamins such as C, B1, B2 and B3, which are present in milk, have been reported to play an antagonist effect on depletion of B12 (AHMAD et al., 2012, 2014; AHMAD; ANSARI; ISMAIL, 2003; AHMAD; HUSSAIN; FAREEDI, 1992; SCHNELLBAECHER et al., 2019). In the case of vitamin C, a potent reducing agent that absorbs at 265 nm (the wavelength applied in our experiments), the redox cycling of Co(II)/Co(III) under aerobic conditions produces hydrogen peroxide that results in the cleavage of the B12 corrin ring, with subsequent products of degradation (AHMAD et al., 2014; DEREVEN'KOV et al., 2016). Vitamin B2 is very sensitive to light and its photosensitization leads to the formation of superoxide anion radicals, singlet oxygen, hydroxyl radicals and hydrogen peroxide (CHOE; HUANG; MIN, 2005; OTTAWAY; OTTAWAY, 2010). All these species can act on the photodegradation of other vitamins, such as A, B6 and B9 (Arrivetti et al., 2013; Guneser & Yuceer, 2012; Scurachio et al., 2011), as well as vitamin B12 in milk (AHMAD et al., 2012; WATANABE et al., 2013). However, Gaylord et al. (1986) showed that the stability of vitamin B2 increases by increasing the fat content of milk, representing a loss of only 2% of B2 in whole milk, suggesting minor or no impact from this vitamin that could interfere with B12 decomposition.

On the other hand, the photostability of B12 can be increased in the presence of β-lactoglobulin and α-lactalbumin, which can protect the vitamin from photodecomposition. Wang et al. (2019) evaluated the stability of B12 (adenosylcobalamin and cyanocobalamin forms) alone or in the presence of these proteins when subjected to UV irradiation. In all cases, the presence of proteins increased B12 protection by 20%. Although proteins can also be affected by photooxidation, in the case of β-Lg and α-La, their tryptophan residues, which are sensitive to oxidation, are in the interior part of the molecule (hydrophobic), protected and, consequently, preserving the protein structure and its interaction with B12.

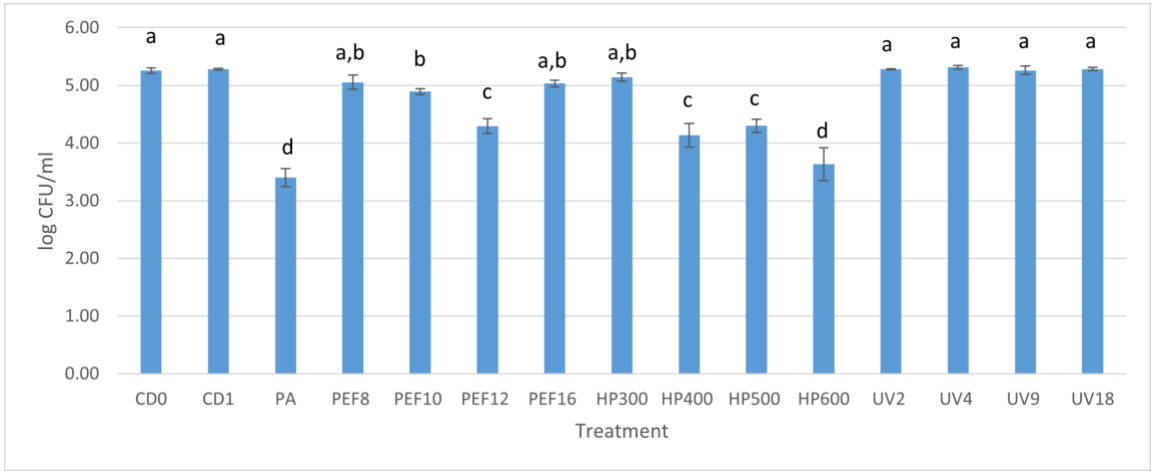
Taking this into account, we can conclude that most B12 molecules in milk are protected against photodecomposition due to milk constituents, mainly proteins, or, in some cases, due to indirect protection that other milk components (such as fat in whole milk) can offer to other vitamins that could interact with B12 increasing the degradation.

The losses observed in our study suggest that the occurrence of B12 photodecomposition may be more related to the free forms of this vitamin as a result of their interaction per se with light but also as a result of their interaction with oxygen and/or vitamin C, which is the most incompatible vitamin in terms of stability of B12 (SCHNELLBAECHER et al., 2019). For instance, the losses could be more significant in more acidic media (AHMAD;

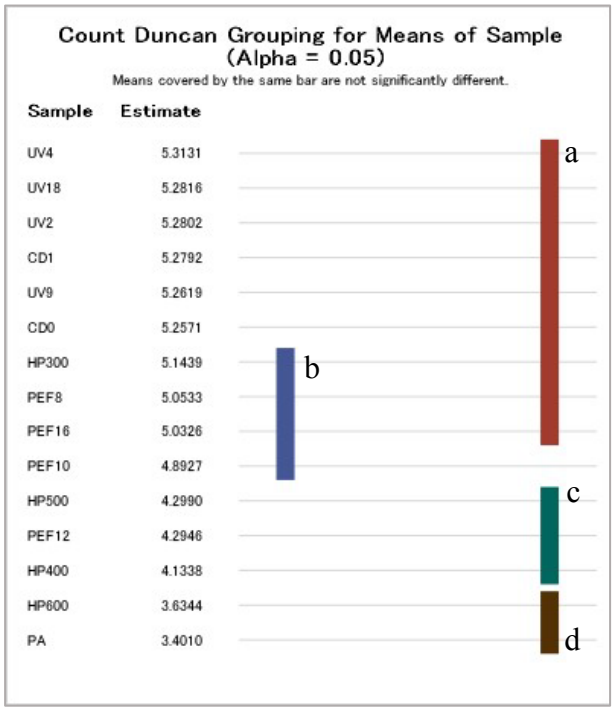
ANSARI; ISMAIL, 2003). However, the pH of milk (6.7) probably also contributed to the higher stability of milk vitamins leading to a loss of 10% for B12, even in the case of the highest applied UV dose (UV 18 mJ / cm^2).

4. 3. 4. Effects of nonthermal treatments on total bacteria counts

Microbiological analyses (Figure 30 (a)) show the average value of the log of the total bacteria counts employed for each group (n=3). The pasteurization process (as a well-established standard) was performed to provide a reference for the bacteria counting procedure, which allows us to compare the data for the other groups. The values obtained for the microbiological loads of the untreated raw milk, controls day zero (CD0) = 5.26±0.05 and day one (CD1) = 5.28±0.02 log units, followed the literature that specifies a limit of 5 log CFU / ml for this kind of milk (EUROPEAN PARLIAMENT AND COUNCIL, 2004). The groups with lower counting were pasteurized (PA), PEF12 μs, HP400, HP500 and HP600 MPa. From the statistical analysis (Figure 30 (b)), the groups also showed statistically significant differences ($p<0.05$) among them.



(a)



(b)

Figure 30 – (a) Effect of different treatments of pulsed electric field PEF8, PEF10, PEF12 and PEF16 (μs), high pressure HP300, HP400, HP500 and HP600 (MPa), and UV-C light dose (UV2, UV4, UV9 and UV18 mJ/cm^2) in the bacteria counting (average values for log CFU/ml ± SD, with n=3). CD1 corresponds to the counting of the samples of Control (Control Day 1), while PA refers to pasteurization and its respective control (Control Day 0 – CD0), (b) – Statistic tests to compare the differences among the bacteria counting in the milk samples as described for (a). The values covered by the same bar are not significantly different.

4. 3. 4. 1. Effects of PEF on total bacteria counts

Taking into account the samples treated with PEF, it is possible to observe a tendency in these treatments (except for PEF 16 µs) for a lower count of bacteria, according to the longer treatment time applied (Figure 30 (a)). The data for PEF 12 µs provided the best microorganism inactivation (4.29±0.13 log CFU / ml) of the PEF groups, presenting 0.8 log CFU / ml above the microbial stability provided by the pasteurization and 1 log CFU / ml reduction considering the controls (CD1). McAuley et al. (2016) obtained a 0.5 log CFU / ml reduction for the samples treated with PEF compared to their control. The authors also reported similar results to ours, with a log CFU / ml reduction of 1 unit considering thermal pasteurization (72º C, 15 s) compared to their control, while we obtained a 1.9 log reduction compared to our control (CD0). Furthermore, for the PEF treatment, the authors applied more extreme conditions, with electric field intensity and treatment time (30 KV/cm and 22 µs, 89.89 kJ / l – outlet temperature of 53º C). However, we proceeded with our experiments in a moderate electric field (16 KV/cm) and treatment time, which could cause less impact on milk constituents. Overall, we obtained the same count for PEF 12 µs of treatment that McAuley and colleagues observed in their PEF experiment (4.2 logs CFU / ml), which could extend the shelf life of the milk from 3-4 days compared to raw milk.

Other results in the literature that work at more similar temperature (<28º C) or electric field strength (E=25 KV/cm) that we applied for milk obtained reductions of log CFU / ml of 2.7 and 2.1 for *L. innocua* (FERNÁNDEZ-MOLINA et al., 2019) and *E. coli* (ZHAO et al., 2013), respectively. The higher reduction in microorganism counting, in this case could be attributed to the fact that in this study of the literature, the counting refers to specific bacteria, while in the present thesis it was the total microbial counting.

PEF 16 µs was expected to be the treatment that could provide the best microbial inactivation due to the longest treatment time. However, it presented similar results to the control (5 log CFU / ml). In this case, milk reached temperatures near 20º C, which is propitious to develop mesophilic bacteria that could interfere with a pronounced counting (WALSTRA; WOUTERS; GEURTS, 2005).

It is possible to infer that PEF12 µs can be potentially used to milk processing from microbiological aspects, as well as a promising treatment focused on B12 preservation, as it did not cause a reduction in B12 content (0.46±0.02 µg/100g of milk). Furthermore, this treatment presented bacteria count comparable to the results for HP 400 and 500 MPa, without statistically

significant differences among these groups (Figure 30 (b)), classified as column c, which is different from the control.

4. 3. 4. 2. Effects of HP on total bacteria counts

For samples treated with HP, we observed lower bacterial counts for higher pressure conditions applied (Figure 30 (a)), as expected, suggesting that some bacteria are more sensitive to increasing pressure than treatment time because, in all procedures, 5 min were applied (ERKMEN; DOĞAN, 2004; STRATAKOS et al., 2019). HP 300 MPa presented the same count as the control samples, while HP 400 and 500 MPa presented a reduction of approximately 1 log CFU / ml, and HP 600 MPa showed a reduction of 1.7 log CFU / ml. Although the reductions were not the same as in the literature, the tendencies to higher microbial inactivation according to the higher pressure were confirmed as presented for similar conditions of pressure (400-600 MPa) and time (5min) applied in the inactivation of *E. coli, Salmonella* and *L. monocytogenes* (STRATAKOS et al., 2019).

With 600 MPa, the best bacteria inactivation was achieved, presenting results for bacteria counting (3.63 ± 0.29 log CFU / ml) similar to pasteurization, 3.40 ± 0.20 log CFU / ml (MCAULEY et al., 2016). Yang et al. (2020), using the same conditions as our experiment at 600 MPa, reported a total microbial load below the detection limit (2.5 log CFU / ml) for whole milk and 3.2 log CFU / ml for skim milk. The discrepancies possibly have occurred in our experiment because some spores were not completely inactivated by the treatment, contributing to a higher counting, which is in accordance with the study of García-Graells et al. (1999) that showed that the inactivation of microorganisms in whole milk presents more resistance to pressure treatment. Based on this, our results suggest that this treatment could be used as a nonthermal alternative to treat milk, as in this condition (600 MPa), the total bacteria was reduced to the same levels of pasteurization and the B12 concentration was not affected (0.43±0.04 µg/100g of milk).

Treatment with lower pressures (400 and 500 MPa, respectively) could also be advised, as they presented a statistically significant difference (Figure 30 (b), $p<0.05$) considering the control, but the bacterial counts for these conditions were around 0.8 log unity above the pasteurized samples.

Finally, the reduction in bacteria caused by HP treatments was found to be statistically significantly different among them ($p<0.05$), being only HP 300 MPa comparable to the control (columns a and b of Figure 30 (b)), HP 400 and 500 MPa pertained to group c and with a slight impact on the reduction of microorganisms, while HP 600 MPa presented high values of inactivation, such as the pasteurization (column d).

4. 3. 4. 3. Effects of UV on total bacteria counts

In contrast to the other treatments, the samples treated with UV light had the highest microbial counts, even for the longest exposure time (Figure 30(a)).

For UV treatments, the counts for all groups were similar to the control on day 1 – CD1 (log CFU / ml = 5.28±0.02), with no significant differences among them ($p>0.05$), according to Figure 30 (b) (column a). In this case, a relevant aspect that could be pointed out is the wavelength used in the experiments. Many works described 255 nm as the most effective against microorganisms (VITZILAIOU et al., 2021; YIN et al., 2015) because the photons are absorbed well by their DNA, leading to more damage and mispairing in the pyrimidine bases (KOUTCHMA, 2009; OTEIZA et al., 2005), and we used 265 nm in our experiments because it was the best wavelength available.

Furthermore, possibly the time/doses applied in our experiments were not high enough to inactivate the microorganisms, even though in the literature, similar dose conditions achieved a reasonable level of inactivation for bacteria in milk. An example is a study by Yin et al. (2015), in which the doses of 5, 10 and 20 mJ / cm^2 lead to a reduction of 1.8, 2 and 3 log CFU / ml for *E. coli*. Another example is the work of Matak et al. (2005) for the inactivation of *L. monocytogenes*, which presented a reduction of 5 Log unities for a dose of 15.8 mJ / cm^2. However, in both studies, the counting of specific bacteria can present better values compared to ours, which employed the total bacteria counting.

Additionally, a relevant aspect could be the light scattering due to the colloidal particles in milk, i.e. casein micelles and fat globules (PENDYALA et al., 2021). The lower inactivation for the UV-C samples in our study was possibly likely due to the differences in milk volume/surface ratio in the experimental setup. Better microbial inactivation might be achieved in milk using other configurations that promote thin-film treatment of the milk.

Another aspect that can be pointed out is the mechanism by which microorganisms reverse UV-induced damages and regain activity (YIN et al., 2015), which can occur in photo-reactivation or dark reactivation (nucleotide excision reactivation). Photoreactivation is light dependent, requiring specific wavelengths (300-500 nm) to complete the procedure, while dark reactivation does not require light. Given that the samples were kept in the dark immediately after UV treatments, if some reactivation occurred by the microorganisms, the suggested mechanism could be dark reactivation. The process can be described as a multistep dark repair pathway, in which an abnormal or damaged base is removed by two pathways, base excision repair and nucleotide excision repair, repairing the damage caused to the DNA (RASTOGI et al., 2010; YIN et al., 2015). This mechanism could contribute to high levels of bacteria count, which can be avoided by applying higher UV doses. Again, on this, the doses applied in our experiments seemed to be one of the problems for not achieving the bacteria inactivation.

The results suggest that higher doses would be necessary to obtain a product safe for consumption in our experiments, considering the wavelength applied or keeping the doses / time applied and changing the treatment wavelength. Despite this fact, regarding the concentration of B12 in the applied UV18 mJ / cm^2 treatment, the dose was already enough to cause a depletion in the levels of this vitamin. Taking into account all these aspects, UV processing, as applied in the present work, can not be advised to treat milk from the point of view of keeping B12 in the product or sanitizing conditions.

5. Conclusion

The chapter in this thesis was dedicated to two primary goals. The first regards the determination of an analytical method to isolate and quantify B12, and the second concerns to the implementation of this method to analyze the impact of HP, PEF and UV-C light processing on this vitamin in milk. In the first case, the improvement of a protocol, taking into account the employment of immunoaffinity columns with different volumes of sample and the evaluation of the repeatability was tested and adapted for the analyses in the UHPLC-UV, being well succeeded and providing the best conditions to carry out the experiments. After establishing these conditions, the second objective was developed, which focused on employing processing with HP (300-600 MPa), PEF (16KV from 8-16 μs) and UV-C (2-18 mJ / cm^2) processing on milk. The impact of these nonthermal technologies on B12 degradation was evaluated,

considering the aspects of why these treatments are applied in food processing, such as microbial inactivation. No statistically significant differences ($p<0.05$) were observed for B12 quantification regarding HP or PEF treatments compared to the control, which was in accordance with the literature that defined these treatments with mild impacts on food components. It showed that HP (5 min) at 600 MPa can be applied to treat milk due to efficient total microbial inactivation, which provided similar results to the ones obtained for pasteurization (1.9 log reduction). The PEF that employed 12 μs of treatment time and HP 400 and 500 MPa did not affect the B12 concentration. However, under these conditions, the bacteria counting showed only 1 log unit of reduction compared to the control. For the samples treated with UV-C light, there was no reduction in microorganism totals counting until a safety condition. On the other hand, some treatments significantly impacted B12 amounts, as expected, due to the photostability of the B12 molecule. For the highest UV dose applied (18 mJ / cm^2), a tendency was observed for the lowest B12 concentration, resulting in a reduction of around 10%. However, a pronounced photodecomposition of cobalamin molecules could be expected if the experiments were not conducted for milk. In this matrix, proteins probably prevented the highest B12 decomposition, considering the interaction of the vitamin mainly with of β-Lg and α-La. Additional analyses considering the other milk components are necessary for complete knowledge of the impact of these technologies in the conditions employed in this study, but regarding the maintenance of B12 in the product and from the aseptic aspects, our outcomes suggest that PEF and mainly HP can be applied successfully.

Chapter IV - Kinetics of B12 due to thermal treatments

Part of this chapter content will be in a paper that is in preparation to submit to the Food Engineering journal.

1. Introduction

1.1. Thermal Treatments of Milk

The present introduction explains a review of the literature on the impact of heat treatments on milk. Although industrial heat processing was not used in the experiments which comprise the thesis, this is the theoretical background to understand better how heat can affect milk components and, mainly, vitamin B12. Therefore, this chapter consists of a study of the effect of heat from a kinetic perspective, considering high temperatures, whose impact can be transposed to evaluate the conditions usually applied in the industry.

Heat treatments consist of applying sufficiently high temperatures in order to reduce or eliminate pathogenic microorganisms in milk, increasing the shelf life and safety of the product for consumption (LACROIX et al., 2006). The Fédération Internationale du Lait (FIL-IDF, 2018) defines pasteurization as a process in which milk is heated at 72^{o} C for 15 s or 63-65^{o} C for 15-30 min to reduce the level of some pathogenic bacteria. In the first case, the process is also called high temperature short time (HTST) by the International Dairy Foods Association (IDFA, 2018). When the purpose is sterilization, a wide range of ultrahigh temperature (UHT) processing can be considered, being 135-150^{o} C for 1-10 s, the most used, and 110-120° C / 10-20 min or 125° C / 5 min, particularly for in-container sterilization (FIL-IDF, 2018). High temperature processing arose in France at the beginning of the XIX century and is still widely applied by industries (TETRA PAK, 2022).

In the case of UHT, the processes can still be classified as direct or indirect depending on the principles of heating involved. In direct heating, steam is injected into the milk, or milk is sprayed into the steam. Milk receives heat from the steam, which is condensed. In the sequence, the milk is cooled by evaporation under a partial vacuum to remove the additional water. In indirect heating, heat is transferred to milk through a stainless steel heating surface

that can be a tube or plate configuration (GREGORY; BURTON, 1965). The indirect method is considered to cause more chemical changes in milk, as it is a slower process compared to direct heating (DEETH; DATTA, 2011; KIERMEIER, 1972).

1.2. Impact of Heating Treatments on milk components

Reviews (LEŠKOVÁ et al., 2006; MACDONALD et al., 2011) and a meta-analysis study have presented that thermal treatments can compromise vitamin levels in food, including B12 in milk (BAJAJ; SINGHAL, 2019; EDELMANN et al., 2016; JOHNS et al., 2015; SIERRA; VIDAL-VALVERDE, 2001). Although Gille & Schmid (2015) and Arkbåge et al. (2003) have reported no losses in B12 for temperatures of 76º C for 16 s and 96º C for 5 min, which corroborates with the pasteurization of milk mentioned by (FAVIER, 1985), most of the literature has pointed out losses for B12. In this way, Callieri et al. (1959) showed that during commercial pasteurization (74º C for 16 s), part of the B12 in cow milk is degraded. Vitamin levels decreased by 12%, measured by the total vitamin activity using *E. coli*. Other examples are the experiment carried out by Burton et al. (1967), which showed B12 content after single and multiple heat treatments. A single process of high-temperature short-time (HTST) pasteurization at 71.7º C for 15 s reduced vitamin levels by 4%, while combinations of pasteurization treatments such as HTST and flash pasteurization (85º C, holding time of 2-3 s) can contribute to losses of around 10%.

Taking into account high temperatures, Favier (1985) reported 95% of retention of B12 after the UHT process. Comparisons of milk processed by direct (144º C) and indirect (141º C) UHT methods for similar sporicidal effects and under the influence of oxygen highlighted that B12 decreased by up to 13 and 4% (mean values), respectively (BURTON et al., 1970). Losses of 35% were reached for milk treated by indirect process in a tubular system operating at 135º C for 15 s. However, in the direct heating plants, when applied temperatures of 140 and 145º C during 4 s, the losses described were less than 10% (GREGORY; BURTON, 1965). Considering the UHT steam injection, after sterilization at 143º C for 3 - 4 s, a decrease of 10% was achieved by Ball (1998), while Oamen et al. (1989) reported the treatment of milk and raw deaerated milk samples at 149º C for 3.4 s and 138º C for 20.3 s, leading to an average reduction of the 17.9% of B12 content. High losses were described by Watanabe et al. (1998),

who studied the effects of microwave heating and boiling of milk, showing that applying treatments of 6 min and 30 min, respectively, can diminish the levels of B12 from 30-50%. In bottle sterilization, applying 110º C for 20 min degraded about 90% of B12 and 77% for conditions of 120º C for 13 min (BALL, 1998).

The impacts of thermal processing on B12, as well as on other vitamins, have been extensively described in the literature. For example, vitamin C has been reported to be affected by pasteurization, whose concentration has an inverse relationship with the temperature applied in the process, while for folate, the decrease in the concentration seems to be related to the duration of the process (MACDONALD et al., 2011). Decreased vitamin B2 and E levels were also mentioned (Millar & Sheppard, 1972). Other studies reported a decrease mainly in vitamins B1 and B6 when pasteurization or UHT was applied to the product, but vitamin A was essentially stable during such processes (RYLEY; KAJDA, 1994; SCHAAFSMA, 1989). Little or no losses in the amounts of vitamins B3, B5, B7 and D were reported during conventional sterilization processes (CLAEYS et al., 2014).

During thermal processing, other milk components can also be affected. Studies have mentioned that heat treatments can induce chemical and physical modifications in proteins, with many effects on their nutritional value and functional characteristics (LACROIX et al., 2006). According to Claeys et al. (2014), during classical HTST treatment, 10 to 30% of bovine milk immunoglobulin activity is lost, while for UHT processing at 138º C for 4 s, most of the specific immune activity of milk is compromised. Changes in the primary, secondary and tertiary structure of whey proteins due to thermal processing can lead to changes in their interactions with caseins (FAIRISE; CAYOT; LORIENT, 1999). These interactions can adversely affect immunoglobulin digestibility due to the unavailability of some essential amino acids (LACROIX et al., 2006).

Heat treatment mainly affects the milk fat globule membrane and some heat-sensitive components of MFGM protein, which can alter the agglomeration of fat globule particles (Claeys et al., 2014; Raynal-Ljutovac et al., 2007; Spreer, 1998). Some of these alterations can be described by the association of whey proteins and casein with MFGM via sulphydryl disulfide interchange reactions, the release of sulphydryl compounds and the removal of phospholipids at high temperatures (CLAEYS et al., 2014; VAN BOEKEL; WALSTRA, 1995). On the other hand, fatty acids have been described as not affected by thermal processing, as to occur non-oxidative decomposition of these components, temperatures above 200ºC are

necessary. So, heating is considered to have a low impact on the nutritional value of milk fat (CLAEYS et al., 2014).

2. Objectives

Chapter IV of this thesis aims to investigate the impact of heat on the degradation of vitamin B12 in milk and contribute to better understanding the effects of temperature on this molecule. Specifically, the research in this chapter aims to:

- Study the kinetics of vitamin B12 in milk subjected to different conditions of temperature;
- Determine the constant for the degradation of vitamin B12;
- Determine the activation energy and other thermodynamic parameters;

3. Materials and Methods

3.1. Chemicals

The chemicals employed in the research described in this chapter comprise the same chemicals reported in Chapter III of the present thesis, Section 3. Materials and Methods, Subsection 3.1.1.

3.2. Milk samples

Whole cow milk - 3.5% fat, 24 h from farm to shop (Arla Foods, Denmark) - was purchased from a local supermarket in Copenhagen, Denmark. CNCbl standard stock (1mg / ml) was prepared in deionized water and stored at -20^{o} C in an amber flask. 100 ml of milk was spiked with the B12 standard solution (15µg / 1 ml) and stored on ice protected from light until thermal treatment.

3.3. Determination of temperatures and dwell time

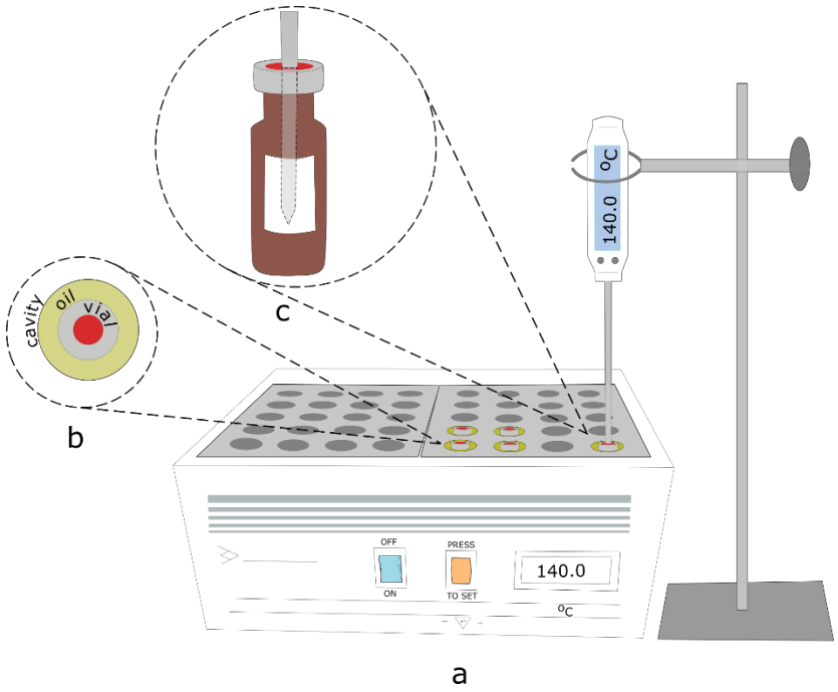
A block heater (Stuart Scientific, UK) was used to perform the thermal treatment of milk. Temperatures were defined as 100, 120, 130 and 140º C. Some tests were conducted to establish the minimum time required for milk to reach the desired temperatures. The block was first set at the desired temperature, which was checked using a digital thermometer (TFA Dostmann, Germany) placed in one of its empty cavities. When the target temperature was achieved, the block cavities were filled with silicon oil DC 200, 1070 mPas (Fluka Chemika, Switzerland). The temperature was adjusted, if necessary, with the thermometer placed in the middle of the cavity with oil until the system was ready to start the procedure. The thermometer was coupled to an amber glass vial with a PTFE aluminum crimp cap (La-Pha-Pack, GmbH) containing 1 ml of milk, which was placed in the cavity with oil precisely when a timer was triggered. The temperature was monitored for 5 minutes and the values were collected every 10 seconds. This procedure was performed for each temperature.

3.4. Heat treatment

The block heater (Stuart Scientific, UK) was used as described in the previous section, employing the same techniques to set and check the desired temperature.

Amber glass vials containing 1 ml of B12-fortified milk were placed in the cavities with oil precisely when the timer was triggered. One vial was kept in ice (control) and another one was coupled to the thermometer to provide the milk temperature during the procedure. After the specified treatment time for each vial, they were immediately cooled on ice and stored at -40º C until sample preparation.

The procedure was performed for four different temperatures and four different times at each temperature (**102.5 º C:** 120, 240, 480 and 1200 s; **122.1 º C:** 30, 180, 300 and 660 s; **131.9 º C:** 30, 120, 180 and 300 s; and **141.6 º C:** 10, 30, 60 and 120 s). The times described were considered after a dwell time of 1:40 minutes, which was taken as the minimum necessary for the temperature of milk measured in the middle of the vial to reach the specified value (previous section). Figure 31 illustrates the experimental scheme for milk treatments.



Source: Figure elaborated by the author.

Figure 31 - Representation of the experimental scheme: (a) equipment used in the heat treatment of samples composed of a block heater to place the vials and a thermometer, (b) zoom of block heater cavity filled with oil and vials with milk accommodated and (c) zoom of the vial coupled to the thermometer, showing the position of the thermometer tip immersed in milk.

3.5. Samples clean-up and B12 extraction

The sample preparation procedure developed was similar to that described in Chapter III of the present thesis, Section 3. Materials and Methods, Subsection 3.1.2., with some additional adaptations, as explained in the following.

Aliquots of 1 ml of milk (thermal treated) were inserted into a glass flask, followed by the addition of 79 ml of sodium acetate buffer (50 mM, pH 4) instead of 30 ml of milk and 50 ml of the buffer solution prescribed in column company protocol (R-Biopharm Rhone ®).

Regarding the amount of sample used in the immunoaffinity column, 1 ml of the filtrate was passed through the column, which was different from the company protocol (10 ml) and the amount established in our previous experiments (25 ml).

Finally, the steps of elution with methanol (2.7 ml with backflushing plus 0.8 ml without backflushing) were repeated four times, corresponding to a final total volume of 14 ml of methanol.

3.6. B12 analysis by UHPLC-UV and quantification

Analyses by UHPLC-UV, data processing and quantification of vitamin B12 in the samples were carried out analogously to the procedures described in Chapter III, Section 3. Materials and Methods, Subsections 3.1.3. and 3.1.4. For each temperature and time condition, four analyses in the UHPLC-UV were carried out.

Only the standard curve prepared with the cyanocobalamin standard differs from the conditions used previously. In this case, the curve was composed of 7 solutions of cyanocobalamin standard of known concentration, such as 0.1; 0.2; 0.3; 0.4; 0.5; 0.6; 0.7 µg / ml (solubilized in deionized water with TFA 0.025%).

3.7. Kinetic Calculations

The mean values obtained from the quantification of B12 were plotted against the heating time for each temperature applied (each mean value from the quadruplicate of the analyses in a respective time represents one point in the curve). The original curves were described by exponential decays, which were linearized, indicating first-order kinetics, and normalized as:

$$ln(C_t/C_o) = -k\,.\,t\,, \tag{24}$$

where *Co* indicates the concentration of B12 at time zero, C_t is the concentration of B12 at a specific time, *k* represents the reaction rate constant and *t* is the heat treatment time in seconds.

The relationship of the degradation rate constant of the vitamin to temperature was measured using the Arrhenius Equation:

$$k_T = A_0\, exp(-Ea/RT)\,, \tag{25}$$

where k is the rate constant (s^{-1}), A_0 denotes the preexponential constant (s^{-1}), E_a represents the activation energy of the reaction (kJ / mol), R indicates the universal gas constant (8.3145 J / mol.K) and T is the temperature (K).

From Equation (24), it was possible to determine the time required for the degradation of B12 to half of its initial concentration, according to Equation 26:

$$t_{1/2} = 0.693/k\,, \tag{26}$$

Additionally, to determine the thermodynamic parameters in our system, such as the enthalpy of activation (ΔH) and the entropy of activation (ΔS), the Eyring-Polanyi Equation was used:

$$\ln\left(\frac{k}{T}\right) = -\frac{\Delta H}{R}.\left(\frac{1}{T}\right) + \ln\left(\frac{kB}{h}\right) + \frac{\Delta S}{R}\,, \tag{27}$$

where k is the rate constant (s^{-1}), T is the temperature (K), ΔH is the enthalpy (kJ / mol), R indicates the universal gas constant (8.3145 J / mol.K), k_B is the Boltzmann constant ($1.38 . 10^{-23}$ J / K), h denotes the Planck constant ($6.63 .10^{-34}$ J.s) and ΔS is the entropy.

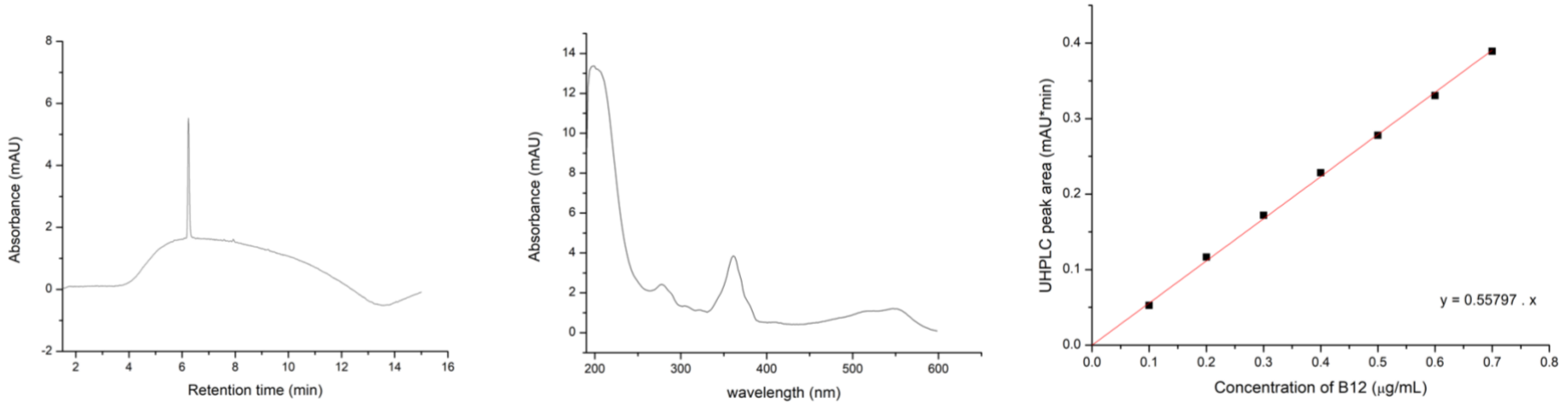
4. Results and Discussion

4.1. B12 Quantification

For all samples, a peak was observed at 6.2 min in the chromatogram, as in Figure 32 (a), with a correspondent characteristic UV spectrum recognized by a band at 361 nm (Figure 32 (b)), as the results obtained in Section 4.2. of Chapter III. From the external CNCbl standard, a linear calibration curve was obtained (R^2=0.9997; Figure 32(c)). The LOD obtained by the method was lower than 0.015 µg / ml and the LOQ was lower than 0.05 µg / ml. The mean retention time of the CNCbl peak was 6.240±0.002 min with a CV of 0.03%.

Using the modified extraction and cleanup procedure followed by the described UHPLC-UV analysis and quantification, the recovery of CNCbl from the fortified milk was (95 ± 3)%. The high value obtained for recovery can be attributed to the specificity of the column,

which contains highly selective antibodies to bind the cyanocobalamin molecule, as mentioned previously. This is suggested because of the recovery considering other natural forms of the B12 molecule (hydroxocobalamin, methylcobalamin and adenosylcobalamin) was reported as low (Campos-Giménez et al., 2008).



a) b) (c)

Figure 32- (a) Chromatogram obtained for one of the samples showing the peak of vitamin B12 with a retention time of 6.24 min. (b) The spectrum obtained for one of the samples showing the absorption of vitamin B12 with a pronounced band at 361 nm. (c) Calibration curve obtained from cobalamin standard solutions.

4.2. Kinetic for degradation of vitamin B12 in milk

The amounts of CNCbl in the samples subjected to various thermal treatments were plotted, according to Equation 24, as *ln(Ct/Co)* versus time (Figure 33) to derive the kinetic data for the thermal degradation of vitamin B12. The data for all temperatures were best described by first-order kinetics. The multiple correlation coefficient (R^2) and the correlation coefficient (Pearson r) were greater than or equal to 0.97 and -0.99, respectively, indicating a linear profile. This is in agreement with reaction orders obtained in most of the vitamin studies (KADAKAL; DUMAN; EKINCI, 2018; KWOK et al., 1998; NISHA; SINGHAL; PANDIT, 2005; SLATER et al., 1979; WILKINSON; EARLE; CLELAND, 1981). From the slopes of this graph (Figure 33), the rate constant k was obtained and the values for the half-life (Equation 26) for each temperature (Table 4).

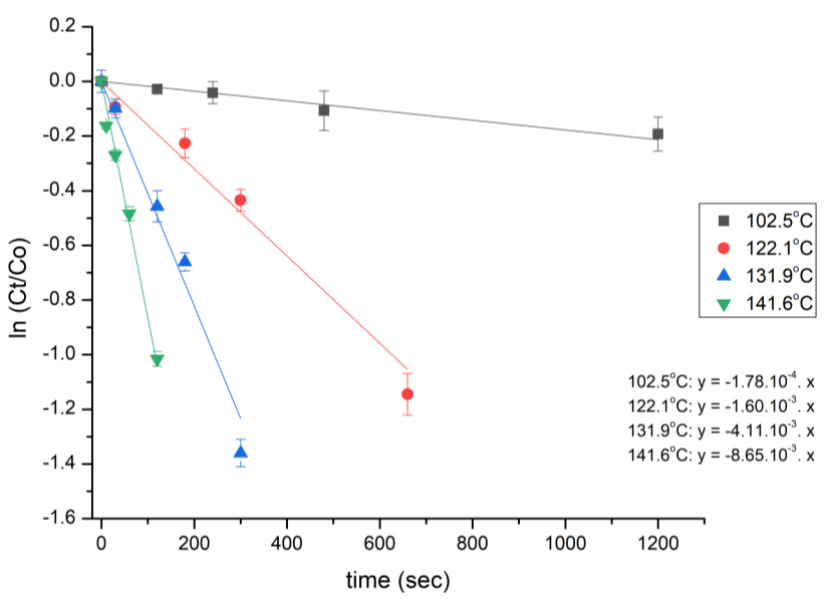


Figure 33 - Degradation rates for B12 in milk, evidencing a first-order kinetic with the logarithm of the decay of the initial concentration as a function of time for temperatures of 102.5, 122.1, 131.9 and 141.6 ºC. C_o indicates the concentration of B_{12} at time zero, C_t is the concentration of B12 at a specific time and *t* is the heat treatment time in seconds.

For all temperatures, a depletion of B12 concentration was observed. As can be seen from Figure 33 and the values in Table 4, there was a marked effect of temperature on the

degradation of CNCbl in milk. At 102.5º C, the degradation was slow, leading to a loss of only 15% after 20 min of heating. On the contrary, at 122.1, 131.9 and 141.6 º C, there was a fast degradation with half-lives of 7.2, 2.8 and 1.3 min, respectively. Therefore, the half-life obtained at 131.9 º C was approximately double that at 141.6 º C, which means that almost half of the impact on the degradation of B12 can be encountered at 131.9 º C by heating milk during the same time for both temperatures. Other insights can be extracted from Figure 33, such as the degradation (15%) of B12 at 141.6 º C for 10 s was slightly similar to that at 102.5 º C for the 1200 s. This evidenced that a longer time can compensate for a lower temperature, providing basically the same degradation effect on B12. Analogously, for 122.1 and 131.9 º C, 9% of degradation for both temperatures was obtained for a holding time of 30 s (coincident points in the graph). In contrast, a significant difference of 35% and 74% was obtained for 300 s at these temperatures, respectively. Finally, for a time of 120 s, 10 degrees of variation resulted in a substantial contrast in the degradation of 64% for 140 º C and 37% for 131.9 º C.

Contemplating the range of work for UHT processes that embrace at least our point for 140 º C in 10 s (Figure 33), we can conclude that the degradation of B12 of 15% was within the expectations. In this case, our result was comparable to the data described by Oamen et al. (1989), in which the authors reported an equivalent temperature condition (138 º C for 20.3 s) and an average reduction in the B12 content of 18% in milk treated with UHT steam injection.

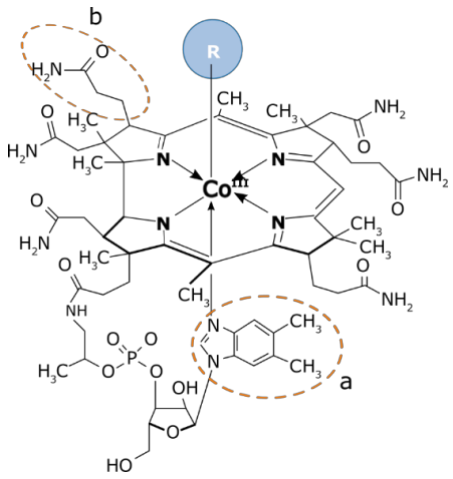
Table 4 - Data obtained for the rate constant (k) (s^{-1}) and the half-life ($t_{1/2}$, s) for the degradation of vitamin B12 in milk according to different temperatures of heating (102.5, 122.1, 131.9 and 141.6) º C.

T ± 0.1 (º C)	(k) 10^{-4} (s^{-1})	t ½ (s)
102.5	1.78 ± 0.14	3900 ± 320
122.1	16.00 ± 1.06	433 ± 29
131.9	41.10 ± 2.16	169 ± 9
141.6	86.50 ± 6.56	80 ± 6

Source: elaborated by the author.

According to Watanabe et al. (1998), the B12 degradation can be explained by the loss of the alpha-ligand (cobalt coordinated nucleotide) in the low axial position (Figure 34 (a)) or

by skeletal alterations in the propionamide side chain in the molecule (Figure 34 (b)). In the first case, it is suggested that heating promotes the elimination of the base portion, with consequent changes in the structure of the sugar moiety and methyl groups. In the latter case, the decomposition could involve the hydrolysis of the propionamide side chains and the consequent deamidation in the corrin ring. Both mechanisms of decomposition could lead to the conversion of B12 into some inactive degradation products (SCHNEIDER, 1987). This hypothesis is sustained based on a study that employed microorganisms to verify the B12 degradation due to heat. *Euglena gracillis* requires an intact B12 molecule to grow, specifically containing the α-coordinated nucleotide, while *Euglena* B12 binding proteins also require the α-ligand as well as the propionamide side chain (WATANABE et al., 1993, 1998; WATANABE; NAKANO; STUPPERICH, 1992). Without these specific parts in the molecule, the microorganisms employed in the microbiological assay are not capable of developing properly, as just vitamin B12 has 5,6-dimethyl-benzimidazole in the nucleotide moiety, being other B12 analogs - which present, for example, an adenine replacing the DMBz - not used in the biochemistry processes or even not absorbed by man and animals (SCHNEIDER, 1987).



Source: Figure elaborated by the author.

Figure 34 - Molecular structure of vitamin B12, as described in Chapter I. (a) represents 5,6-dimethylbenzimidazole (DMBz) in the cobalt-coordinated nucleotide. (b) represents the propionamide side chain. According to Schneider (1987), without both structures, B12 is non-bioactive.

Knowing the mechanism by which antibodies in the immunoaffinity column attach vitamin B12 could provide additional information to conclude what parts of the molecule are degraded during heating processing. However, no detail can be found in the literature on it. Although the immunoaffinity columns used to isolate B12 seem to have a high affinity for the cyanide group in the cobalamin structure and directly bind B12 via this part of the molecule (as described in Section in 4.1. of Chapter III), the losses of other parts of the molecule could indirectly influence the interaction between B12 and antibodies. For example, according to COOLEY et al. (1951), due to the interaction between the cyanide group with cobalt, one positive charge is transferred to the cobalt atom, which shares this charge with the nitrogen in the DMBz base. This configuration can change the electronic density around the cyanide group / cobalt atom, and thus, the interaction between it and the antibodies in the immunoaffinity column. It means that in the case of loss of the DMBz base, even with the intact cyanide ligand in the upper axial position, the electronic density of the molecule around this group could prevent its attachment to the antibody. This is a plausible assumption to explain the B12 degradation considering the method by which the molecules were isolated in the immunoaffinity columns.

From our results and comparisons with the literature, we concluded that the dependence between time and temperature is one of the most critical parameters determining the extension of B12 degradation.

4.3. Arrhenius model for the degradation of vitamin B12 in milk

The relationship between the degradation rate constant and the temperature was evidenced by plotting a graph of ln k versus 1/T (Figure 35), consistent with the Arrhenius Equation (25). From the slope of this graph (with $R^2 = 0.995$ and $r = -0.998$), the magnitude of the activation energy was inferred, that is., 130 ± 5 kJ / mol.

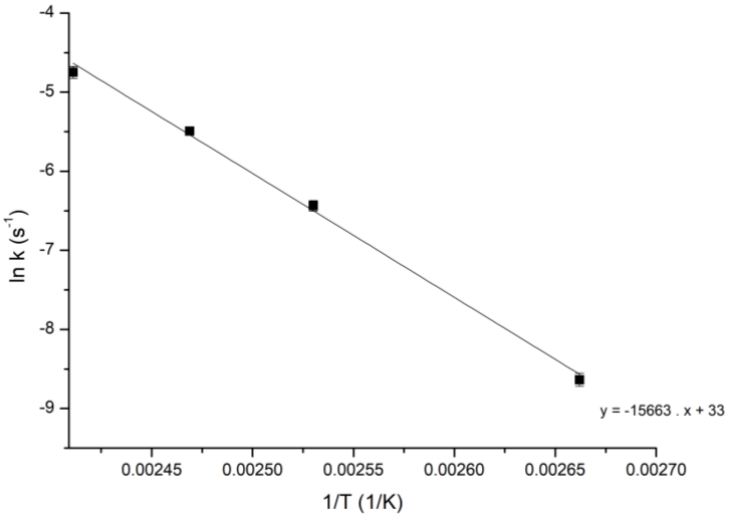


Figure 35 - Arrhenius plot for the degradation of B12 in milk indicating the natural logarithm of the rate constants *k* obtained from Figure 33 as a function of the inverse of the temperatures. T represents the temperatures of (102.5, 122.1, 131.9 and 141.6) ^{o} C converted into Kelvin unities.

The result for the activation energy was in accordance with that presented by Kessler & Fink (1986) for the same kind of matrix, cow milk. The authors obtained a value similar to ours in order of magnitude for the activation energy (100.8 kJ / mol) of vitamin B1 in a temperature range of 120-160 ^{o} C, using indirect heat exchange equipment.

Data on the kinetics of B12 are scarce in the literature. In one of the few studies reported, the B12 degradation in aqueous solutions (pH range 1-8) was explored with emphasis on the antagonistic effect of vitamin C (AHMAD et al., 2014). Complementarily, in a most recent published research (BAJAJ; SINGHAL, 2020), the pH factor and also the effect of storage time/temperature were investigated. In the study, conducted in aqueous solutions, as well as in carrot and lime juices fortified with B12, the activation energies of 10.29-41.94 kJ / mol, derived from the Arrhenius model, were much lower than the one obtained in this thesis. This disparity is possibly due to the medium since, in the study mentioned, the authors reported more drastic effects on B12 retention for low pHs, such as 2, than for pH 6. For that matter, the high activation energy obtained in our study shows that the degradation reaction of B12, considering milk, is more difficult to progress in comparison to more acid media in which vitamin C, for example, can interfere with the degradation rate, accelerating the process.

Another aspect to be considered probably is that there may also be a protective effect of the proteins in the milk.

In this regard, as described in the main introduction of the present thesis, in milk, B12 can be present in free form or complexed with caseins or whey proteins (FEDOSOV; NEXO; HEEGAARD, 2018; HINE et al., 2014; WANG et al., 2019). In the presence of β -lactoglobulin and α -lactalbumin, the two most prevalent proteins in whey, the thermostability of B12 (adenosylcobalamin and cyanocobalmin) can increase by up to 30%, as these proteins bind to the vitamin molecules protecting them from thermal decomposition (WANG et al., 2019). In the case of β -Lg, dimers and trimers are formed through disulfide bonds due to heating, protecting the thiol groups in the protein, which are hydrophobically associated products, and exposing a higher number of hydrophilic binding sites in which B12 molecules can bind. Considering the protection provided to cobalamins by cobalamin protein complexes, another whey protein that can contribute to low B12 degradation is transcobalamin, as this protein of transport can bind mainly the hydroxocobalamin and adenosylcobalamin forms in milk (HINE et al., 2014). Accordingly, Fedosov et al. (2018) reported the interaction between hydroxocobalamin/aquacobalamin (OHCbl) and caseins, which occurs through coordination bonds of the histidine residues in the protein with the vitamin molecule. Therefore, caseins could also offer protection against B12 degradation, as caseins bind to large amounts of OHCbl (loads can be estimated as up to 100 nmol of Cbl per 1 mg of caseins).

The discrepancies and similarities found for the activation energies throughout the investigations emphasize the influence of the food matrix on the kinetics of thermal degradation (VILLOTA; HAWKES, 2006). Furthermore, the extension of the degradation reaction, as previously described, can be highly affected by other parameters such as pH and the presence of other constituents, which can contribute to increasing this rate, for example, vitamins B1, B2, B3 and C (MULLEY; STUMBO; HUNTING, 1975; OTTAWAY; OTTAWAY, 2010) or contribute to reducing this rate, such as proteins in milk (WANG et al., 2019).

4.4. Thermodynamic parameters and the Eyring-Polanyi equation

Taking into account the thermodynamic parameters, the relationship between the degradation rate constant and the temperature was also evidenced by plotting a graph of *ln (k/T)*

versus *1/T* (Figure 36), consistent with the Eyring-Polanyi Equation (27). From the slope and intercept of the activation curve, we calculated the enthalpy and the entropy, which were (126 ± 5) kJ / mol and (19 ± 14) J / mol.K, respectively. Based on these values, it can be inferred that B12 thermal degradation is an endergonic process, controlled by enthalpy.

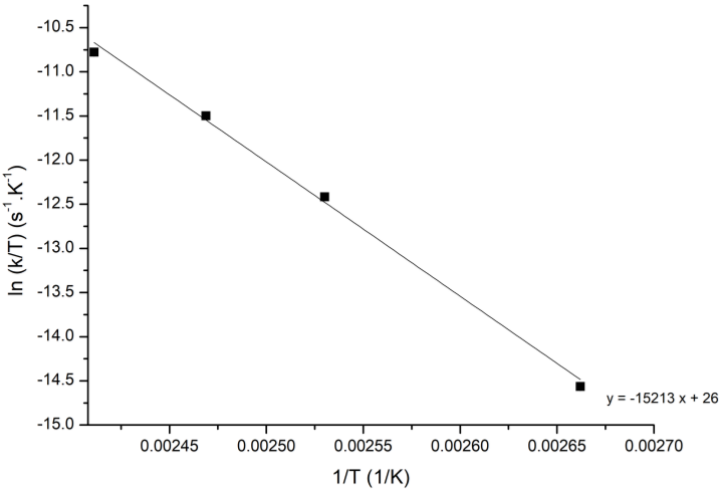


Figure 36 – Graph for the degradation of B12 in milk according to the Eyring-Polanyi equation, considering the natural logarithm of the rate constants k/T obtained in Figure 33 as a function of the inverse of the temperatures. T represents the temperatures of (102.5, 122.1, 131.9 and 141.6) ^{o}C converted into Kelvin unities.

4.5. Predictions of degradation rates for B12 and some considerations regarding industrial processing

One advantage of mathematical modeling to describe the profile of the curves related to the kinetic degradation of a vitamin in a specific medium is that it is possible to make predictions. Based on the parameters obtained from the Arrhenius graph (Figure 35), it is possible to project the rate constants and, subsequently, the degradation rate for different temperatures and times values (Equation 24), which was not necessarily measured in a practical experiment.

Although none of the UHT industrial processes, direct nor indirect, were used in this work, the temperature ranges applied were similar to those in the referred treatments. This indicates that our adapted method produced results that can be considered realistic references

for evaluations and estimations regarding industrial processing. Even for other predictions, for example, boiling milk in a conventional procedure at home (around 100 º C), it is possible to have a notion of the B12 retained and potentially ingested after a regular heating process of 2 or 4 minutes (3 - 4% of degradation).

For example, according to our theoretical data, B12 losses due to pasteurization could represent no or less than 1% for the procedures considered standards (72 º C / 15 s, 63 º C / 15 min, or 65 º C / 30 min), which is consistent with the results of Arkbåge et al. (2003). Regarding the usual UHT processes, our predicted losses varied from 0.5 to 5% for 135ºC and from 2 to 18.5% for 150 º C (holding times of 1 and 10 s), respectively, similar to data published for roughly equivalent conditions (OAMEN; HANSEN; SWARTZEL, 1989).

As previously cited in other sections, the effect of heat leads to the degradation of B12, but a variety of indirect factors can contribute to increasing this rate, such as the presence of other vitamins (such as vitamin C) (OTTAWAY; OTTAWAY, 2010), oxygen, the configuration of the treatment system (DEETH; DATTA, 2011; GREGORY; BURTON, 1965) and also the combination of all of them.

Considering the plant configuration, Burton et al. (1970) reported that the losses of vitamin B12 were higher for direct than indirect heating, being the opposite of other vitamins such as B6 and B9 (BURTON et al., 1970; FORD, 1957; KIERMEIER, 1972). In the experiments conducted by the authors, it is possible to infer that the presence of oxygen also contributes to a higher degradation of B12, as the experiment conducted with a deaerator led to lower losses of B12. In such a case, the oxygen could interact directly with B12 leading to its degradation or could interact with vitamin C resulting in its oxidation and, thus, B12 depletion (FORD, 1957; GREGORY; BURTON, 1965). This could be confirmed by the description of Ottaway (2010) on vitamin interactions, in which was mentioned that vitamin C could increase the instability of B12, but there is no information correlating vitamin C negatively with vitamin B6 (that presented higher losses when indirect heating was applied in the experiments, for example) (Burton et al., 1970). It suggests that the interaction between oxygen / vitamin C and vitamin C / B12 increases B12 depletion. Via oxygen, the B12 undergoes the hydroxocobalamin form's reduction with the corrin ring's subsequent cleavage (AHMAD et al., 2014). In the case in which vitamin C interacts with B12, this rate of degradation in the presence of oxygen is higher. In the direct heating system, the vacuum chamber removes a large proportion of oxygen in contact with milk. The reduction of oxygen amounts by ten times in the direct system

decreases vitamin C oxidation, which could avoid a pronounced B12 depletion (DEETH; DATTA, 2011).

On the other hand, the differences mentioned for vitamin losses in the literature regarding indirect and direct UHT processes could be attributed to the protection that proteins confer for B12 from the heat in milk, as also mentioned in Section 4.3. of the present Chapter. According to Deeth & Datta (2011), the slower the high temperature heating and cooling during milk processing, the greater the chemical change, which is represented by protein denaturation, protein-protein interaction (including particularly β-lactoglobulin and κ-casein), Maillard browning, formation of sulfhydryl compounds (mainly due to the degradation of β-lactoglobulin and fat globule membrane material), production of carbonyl compounds, destruction of vitamins, among others. These changes are described as more pronounced in indirect heating than the direct one due to the lower time required for heat transmission in direct heating (steam injection). As a consequence, Birlouez-Aragon et al. (1998) described a concentration of undenatured β-lactoglobulin of 79 mg / l for indirect UHT against 639 mg / l for direct UHT, which reinforces the fact that intact proteins better preserved in direct heating can enhance the B12 protection (DEETH; DATTA, 2011).

5. Conclusion

In this chapter, a study was reported focused on the kinetics of vitamin B12 in cow milk and its stability according to different heating treatments (100 – 140 °C) and times applied. The thermal degradation of B12 was quantified by UHPLC-UV and followed the Arrhenius model demonstrating a kinetic of first-order reaction, in accordance with other publications in the literature. The increase in the rate constant (k) according to the increment of temperature/time evidenced the more pronounced deleterious effects on B12 degradation, which can be attributed to the loss of the alpha ligand (cobalt coordinated nucleotide) at the low axial position of the molecule or by skeletal alterations in the propionamide side chain. The activation energy of (130 +/- 5) kJ / mol was obtained, which is comparable with the values for some other vitamins in milk. In this sense, the results suggest that the proteins present in the milk matrix, mainly β-lactoglobulin, α-lactalbumin, and also caseins, improved the protection of B12 from thermal degradation, as our activation energy was higher than those obtained for

the kinetics of this vitamin in other food sources. The activation enthalpy and entropy, 126 ± 5 kJ / mol and 19 ± 14 J / mol.K, respectively, indicated that the thermal degradation of B12 is an endergonic process. Furthermore, the mathematical model and data purposed in this study can be used to predict the thermal degradation of B12 in bovine milk during specified heating regimes. Using the equations obtained, one can indirectly calculate the concentration of B12 for samples not measured in practice, accessing the theoretical losses based on our experimental results. Although the losses described during milk processing on a commercial scale are dependent on the method applied, for example, being more pronounced in the case of the indirect heating than the direct one, this methodology is still helpful in providing reliable predictions that can be transposed more concertedly to improve knowledge about the effects of milk processing in the industry. Finally, having estimated how the thermal treatments can affect the concentration of B12 in milk, some solutions can be pursued with the intention of retaining this vitamin, which is essential for a good quality of life.

Chapter V - General Conclusions

In the present thesis, many objectives were encompassed. One of them was employing analytical methods to determine vitamin B12, using two techniques, CE-UV and MALDI-MS. The experiments focused on the speciation of vitamin B12 conducted by CE-UV showed that this technique could not provide the speciation of the molecule in its four different forms such as hydroxocobalamin, methylcobalamin, adenosylcobalamin and methylcobalamin. This objective was also pursued by employing MALDI-MS as the analytical technique, which showed that B12 speciation was not feasible due to the intrinsic properties of the molecules and their stability regarding light. Despite the fact that speciation was not possible, the study revealed the possibility of focusing the analyses on the peak of 1329 m/z, which corresponds to the B12 molecule with no upper axial ligand. Based on it, experiments focused on determining the cyanocobalamin form were carried out. Additionally, experiments to evaluate the milk matrix effect were done, showing that such an effect results mainly from the contribution of proteins and sugars (β-lactoglobulin and lactose), which can interfere with the signal response leading to a suppression of it up to 87%.

Although such an effect can be reduced by employing clean-up steps with a C18 column, the SPE procedure can not completely eliminate the interferents nor provide a complete extraction of the vitamin B12 in the sample, which can be achieved by using immunoaffinity columns. These columns (highly specific for cyanocobalamin) were employed to access and isolate B12 properly, and an analytical approach using UHPLC-UV was developed. The improvements in the primary protocol (R-BIOPHARM RHÔNE LTD, 2021) revealed a linear relationship between the amount of sample used in the immunoaffinity columns and the recovery of B12 vitamin, the importance of backflushing procedure, and the precision of the method, which was studied in three levels: regarding the beginning of sample preparation (bottles) – CV3.34%, different columns – CV8.07%, and analyses in the UHPLC-UV – CV2.06% showing that the columns were the major source of variance in the experimental procedure.

The established protocol was implemented to isolate and quantify B12 in milk samples treated by different nonthermal technologies such as PEF (16KV from 8-16 μs), HP (300-600 MPa) and UV-C light (2-18 mJ / cm^2) aiming to investigate the impact of each kind of processing and conditions on B12. No statistically significant differences ($p<0.05$) were

observed for the quantification of B12 regarding HP or PEF treatments compared to the control, which was in accordance with the literature that defined these treatments with mild impacts on food constituents. HP at 600 MPa for 5 min can be considered to treat milk due to no reductions in the B12 levels and the efficient total microbial inactivation offered by this treatment, which provided similar results to the ones obtained for the pasteurized samples (1.9 log reduction). PEF (12 μs) and HP 400 and 500 MPa did not impact B12 concentration. However, in these conditions, the bacteria counting showed only 1 log unit of reduction compared to the control. Samples treated by UV-C light showed no reduction in the microorganisms totals counting until a safety condition. However, some conditions significantly impacted B12 amounts, as suggested by the other studies considering the stability of B12 molecules due to light. At 18 mJ/cm^2, which corresponded to the highest UV dose, the impact on the B12 level was more pronounced, achieving a reduction of around 10%. Comparing the results with others in the literature, they suggest that the proteins probably prevented the highest B12 decomposition, considering the interaction of the vitamin mainly with β-Lg and α-La present in the milk matrix.

Finally, the last study focused on the kinetics of vitamin B12 in cow milk and its stability according to different temperatures (100-140^oC) and treatment times. Analogously, the B12 was determined and quantified by the protocol already mentioned with new adaptations, followed by the UHPLC-UV analyses. The degradation of B12 due to heat was described by the Arrhenius model and a kinetic of first-order reaction, in accordance with other publications in the literature. The activation energy of (130 +/- 5) kJ / mol was similar to the values for some other vitamins in milk. Again, comparing our results with those found in other works, it is suggested that β-lg, α-la, and caseins could enhance the protection of B12 from thermal degradation. The thermodynamic parameters, such as the enthalpy 126 ± 5 kJ / mol and the entropy of activation 19 ± 14 J / mol.K revealed the thermal degradation of B12 as an endergonic process. The mathematical model obtained in this study can be used to predict the thermal degradation of B12 in bovine milk for other temperatures not measured in practice. These estimations can help predict how the treatments applied in the industry can affect the B12 concentration in milk.

The studies reported in the present thesis related to the thermal degradation of B12 and the one focused on the impact of the nonthermal technologies (PEF / HP / UV) on B12 contents in milk need further investigation, but they can open avenues to find alternatives to treat milk, considering better retention of this vitamin in the final product, which is essential for a good health condition of the population. One future work could consider the use of the improvements

in the protocol (Easi-extract® vitamin B12) for B12 extraction and purification (developed in chapter III) adapted for subsequent analyses in other different analytical techniques than UHPLC-UV, for example, for analyses focused on the cyanocobalamin form of B12 and conducted on the CE-UV and MALDI-MS (as chapter II), among other techniques. Another future work could employ the conditions used in this thesis to evaluate the impact on B12 degradation with and without the influence of the other milk constituents (considering the components that can offer protection against B12 degradation, such as proteins and fatty, and the components that can lead to high B12 degradation, for example, vitamin C) in the case of nonthermal and thermal technologies. In the case of the nonthermal technologies PEF, HP and UV-C, cited in chapter III, the methodologies and parameters in this study could be used to evaluate the impact on B12 for other beverages. Considering the impact on B12 due to thermal treatments (chapter IV), a possible study could be the validation of the theoretical model obtained with the kinetic studies by using a UHT system plant. Additionally, the kinetic studies could be extended, focusing on the variation of other parameters such as pH, concentration and others. Finally, the products of the degradation of B12 due to the thermal effects could also be studied and monitored, providing additional data to better understand the mechanisms involved in the B12 degradation molecules.

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Appendix

In this appendix, we present a list of published papers, preprints and papers in preparation produced during the period in which this study was developed. Additionally, there is information regarding participation in Conferences and other contributions. First, we present the papers, events and materials that take part in this thesis as follows:

Papers:

- Kinetics of Vitamin B12 Thermal Degradation in Fortified Milk. (*In preparation*).
  Caroline Ceribeli, Jeanette Otte, Daniel Rodrigues Cardoso and Lilia Maria Ahrné
- Impact of non-thermal pasteurisation technologies on vitamin B12 content in milk. (*Submitted*).
  Caroline Ceribeli, Jeanette Otte, Markus Walkling-Ribeiro, Daniel Rodrigues Cardoso and Lilia Maria Ahrné.

Conferences (poster and oral presentation):

- Determination of vitamin B12 in milk by MALDI-MS and UPLC-UV.
  Caroline Ceribeli, Jeanette Otte, Daniel Rodrigues Cardoso and Lilia Maria Ahrné.
  The 35th EFFoST International Conference, November 2021, Switzerland.

Materials (protocol):

- Protocol for Analysis of Vitamin B12 in milk:
  Milk Sample Preparation, Extraction, Concentration and Analysis of Vitamin B12.
  Protocol prepared by Caroline Ceribeli, Jeanette Otte and Bente Danielsen based on the Easi-extract column vitamin B12 protocol (R-BIOPHARM RHÔNE LTD, 2021).

In the following, we present the list of publications also developed during the period in which this study was developed, but some works that do not take part in the present thesis or are not related to the subject of the present thesis.

Papers:

- How coupled are capillary electrophoresis and mass spectrometry?
  Caroline Ceribeli, Henrique Ferraz de Arruda and Luciano da Fontoura Costa.
  Scientometrics (2021) 126:3841–3851.

- UV-C light as a safe germicidal processing technology to bovine milk: metabolites viewpoint. (*in preparation*).
  Banny Silva Barbosa Correia, Fellipe Engel Keller, Caroline Ceribeli, Pollyana Ferreira, Joana Dias Bresolin, Teresa Cristina Alves, Luiz Alberto Colnago and Daniel Rodrigues Cardoso.

- Comparative untargeted metabolome analysis of ruminal fluid and feces of Nelore steers (*Bos indicus*).
  Jessica Moraes Malheiros, Banny Silva Barbosa Correia, Caroline Ceribeli, Daniel Rodrigues Cardoso, Luiz Alberto Colnago, Stanislau Bogusz Junior, James Mark Reecy, Gerson Barreto Mourão, Luiz Lehmann Coutinho, Julio Cesar Pascale Palhares, Alexandre Berndt and Luciana Correia de Almeida Regitano.
  Scientific Reports (2021) 11:12752.

- Effects of dietary inclusion of yerba mate (*Ilex paraguariensis*) extract on lamb muscle metabolomics and physicochemical properties in meat.
  Richard R Lobo, Banny S B Correia, Yuli A Peña-Bermúdez, Rafaela Vincenzi, Caroline M da Silva, Leticia L Panosso, Caroline Ceribeli, Luiz A Colnago, Daniel R Cardoso, Alexandre Berndt, Rafael S B Pinheiro, Ives C da S Bueno and Antonio P Faciola.
  Journal of Animal Science (2021) Sep; 99(9): skab244.

- Ruminal and feces metabolites associated with feed efficiency, water intake and methane emission in Nelore bulls. (*submitted*).

  Jessica Moraes Malheiros, Banny Silva Barbosa Correia, Caroline Ceribeli, Jennifer Jessica Bruscadin, Wellison Jarles da Silva Diniz, Priyanka Banerjee, Dielson da Silva Vieira, Tainã Figueiredo Cardoso, Bruno Gabriel Nascimento Andrade, Juliana Petrini, Daniel Rodrigues Cardoso, Luiz Alberto Colnago, Stanislau Bogusz Junior, Gerson Barreto Mourão, Luiz Lehmann Coutinho, Julio Cesar Pascale Palhares, Sergio Raposo de Medeiros, Alexandre Berndt and Luciana Correia de Almeida Regitano.

- Emerging studies of NMR-based metabolomics of fruits regarding botanic family species. (*in preparation*).

  Banny Silva Barbosa Correia, Henrique F. de Arruda, Caroline Ceribeli, Poliana Cristina Spricigo, Luísa Souza Almeida, Daniel Rodrigues Cardoso, Angelo Pedro Jacomino, Luciano da F. Costa and Luiz Alberto Colnago.

- Metabolomics of meat from cows with different genetic characteristics (Nelore and Nelore Angus) by using NMR. (*in preparation*).

  Barbara Rodrigues, Caroline Ceribeli, Banny Silva Barbosa Correia, Luiz Alberto Colnago, Daniel Rodrigues Cardoso and Leandro Samia.