NOVA SCHOOL OF SCIENCE & TECHNOLOGY

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Licenciado em Ciências de Engenharia Química e Bioquímica

MICROALGAE PROCESSING TOWARDS BIOREFINERY ROUTES

PROCESING OF SPIRULINA FOR PHYCOCYANIN EXTRACTION AND PURIFICATION

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MESTRADO EM ENGENHARIA QUIMICA E BIOQUIMICA

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Microalgae Processing Towards Biorefinery Routes: Processing of spirulina for phycocyanin extraction and purification

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To my parents and brother.

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"He who knows all the answers has not been asked all the questions."
(Confucius)

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ABSTRACT

Natural pigments from photosynthetic micro-organisms have stood out as one of the main alternatives to synthetic dyes and pigments. *Arthropira (Spirulina) platensis* is considered to be one of the main sources of phycocyanin, a protein pigment with great applicability in the industry due to its functional properties as both an antioxidant and an anti-inflammatory.

The demand for natural pigments has been growing rapidly in recent years, with its market expected to grow at a Compound Annual Growth Rate (CAGR) of 5% by the year 2026, the food pigments market itself is expected to grow at a CAGR of 12,4 % between 2019 and 2027. As such, extraction processes for phycocyanin from dried Arthrospira were developed and optimized. Meanwhile, the extracting process for phycocyanin from fresh Arthrospira has not yet been extensively investigated to this day.

In the present study, it was developed and optimized, the laboratory and pilot-scale processes associated with the thermal treatment of fresh Arthrospira for phycocyanin stabilization and the milling of fresh Arthrospira biomass. Additionally, a protocol for the quantification of phycocyanin was also developed. In this study, the potassium phosphate buffer was shown to be effective in extracting and stabilizing phycocyanin (CPC). The heat treatment performed at "T" ^{o}C enhanced both the extract's stability and shelf life successfully. Performing an extraction without milling was found to be the most effective extracting method, with salting out proving to be an efficient purification method. The salting out method resulted in a pure and concentrated CPC extract with a recovery yield of 96% and a concentration gain of 139%.

Keywords: Microalgae, Spirulina, Biomass Stabilization, Heat Treatment, Biorefinery

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RESUMO

Actualmente, uma das principais alternativas aos pigmentos sintéticos utilizados na indústria são os pigmentos naturais provenientes de microrganismos fotossintéticos. A *Arthrospira (Spirulina) platensis* é considerada uma das principais fontes de ficocianina, um pigmento proteico, com ampla aplicabilidade na indústria devido às suas propriedades funcionais como antioxidante e anti-inflamatório.

A procura por pigmentos de origem natural tem vindo a aumentar rapidamente nos últimos anos. Prevê-se que este mercado cresça com uma Taxa de Crescimento Anual Composta (CAGR) superior a 5% até 2026, sendo que o mercado dos pigmentos alimentares estima-se que cresça 12,4 % entre 2019 e 2027. Neste sentido têm-se desenvolvido e optimizado processos de extracção de ficocianina proveniente de Arthrospira seca. Não existindo, no entanto, um estudo extenso relativo à extracção de ficocianina proveniente de Arthrospira fresca.

No presente estudo foram optimizados os processos à escala laboratorial e piloto referentes ao tratamento térmico de Arthrospira fresca para estabilização de ficocianina e moagem da biomassa de Arthrospira fresca, foi ainda desenvolvido um protocolo de quantificação de ficocianina. Os resultados deste trabalho revelaram a eficácia do tampão fosfato de potássio na extracção e estabilização de ficocianina (CPC), foi possível observar que um tratamento térmico realizado a "T" ºC promoveu a estabilidade e estendeu a vida útil do extracto de CPC. Verificou-se que a extracção sem moagem é o processo mais vantajoso e que o salting out mostra-se como um método de purificação eficiente. Denotando este uma recuperação de 96% e um ganho de concentração por parte da ficocianina superior a 139%.

Palavras chave: Microalgas; Spirulina; Estabilização de Biomassa; Tratamento Térmico; Bio refinaria

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LIST OF UNITS

Finance	USD	United States Dollar
Temperature	^{o}C	Celsius Degree
Time	s	Second
	min	Minute
	h	Hours
Distance	nm	Nanometer
	cm	Centimeter
	m	Meter
Volume	mL	Milliliter
	L	Liter
	m^3	Cubic meter
Weight	mg	Milligram
	g	Gramma
	kg	Kilogram
Concentration	%	Percent
	mg/g	Milligram per gram
	g/L	Gram per liter
	mM	Millimolar
	M	Molar
Quantity	mol	Mol
Speed	rpm	Revolutions per minute
	m/s	Meters per second
Acceleration	xg	Times Gravity (gravity force)
Frequency	kHz	Kilohertz

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LIST OF VARIABLES

A=illuminated surface area (m^2),
μ=Specific growth rate (h^{-1}),
X=Biomass concentration ($g.l^{-1}$),
V=Culture volume (m^3),
Y=Growth yield ($g.J^{-1}$).
DW (%): Dry weight expressed in percentage (%).
P_f: Total mass, the plate plus dried sample (*g*).
P_r: Plate mass (*g*).
P_i: initial sample mass (hydrated) (*g*).
P_C: represents crucible mass (*g*).
P_S: represents sample mass [salt-free] (*g*).
AFDW (%): weight value of dried and burned biomass sample expressed in percentage.
Ash (%): weight value of ashes expressed in percentage.
SFDW (%): weight value of Salt-Free Dry Weight biomass sample expressed in percentage.
PC: CPC Concentration (*mg*/*mL*).
A_{620}: Maximum absorbance for CPC.
A_{652}: Maximum absorbance for APC.
CPC_{AFDW} (%): percentage (mg/mg) of CPC per milligram of AFDW of a given sample (*mg*/*mg*);
$m_{CPC1st\ extraction}$: mass (mg) of CPC extracted in the first step (*mg*);
$m_{CPC2st\ extraction}$: mass (mg) of CPC extracted in the second/resuspension step (*mg*);
$m_{Arthrospira}$: the Ash-free dry weight mass of the sample (*mg*);
$CS_{CPC\ 1st\ extraction}$: Concentration in the supernatant from the first extraction (*mg*/*ml*);
$V_{buffer\ 1st\ extraction}$: Volume of buffer used in the first extraction (*mL*);
$CS_{CPC\ 2nd\ extraction}$: Concentration in the supernatant from the second extraction (*mg*/*mL*);
V_{beads} : Volume of supernatant non removed from the first extraction (interstitial volume) (*mL*);
$V_{buffer\ 2nd\ extraction}$: Volume of buffer used in the second extraction (*mL*).
TP: Total Proteins
A_{280}: Maximum absorbance for proteins.
Chl: chlorophyll;
A_{430}: Maximum Absorbance for chlorophyll.
R_E: Recovery Efficiency (%)

$\bar{\mathbf{m}}_{\mathbf{CPCsediment}}$: Average CPC weight recovered in the sediment (precipitate) (*mg*)

$\bar{\mathbf{m}}_{\mathbf{CPCextract}}$: Average CPC weight in the initial extract (*mg*)

CG: Concentration Gain (%),

CI CPC: CPC concentration on the initial extract (*mg*/*mL*),

CP CPC: CPC concentration on the precipitate (after salting out) (*mg*/*mL*)

LIST OF ABREVIATIONS

ATP	Adenosine Triphosphate
A4F	Algaefuel, SA
APC	Allophycocyanin
AS	Ammonium Sulfate
AFDW	Ash Free Dry Weight
AOAC	Association of Official Analytical Chemists
Bi	Billion
CO_2	Carbon Dioxide
CRW	Cascade raceway
Chl	Chlorophyll
CAGR	Compound Annual Growth Rate
CG	Concentration Gain
DSP	Downstream Processing
DW	Dry Weight
HTST	High Temperature Short Time
LED	Light Emitting Diode
LDPE	Low Density Polyethylene
LTLT	Low Temperature Long Time
MO	Microscopical Observation
MHTPBR	Multilayer horizontal tubular photobioreactor
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
OD	Optical Density
O_2	Oxygen
PBR	Photobioreactors
CPC	Phycocyanin
PQP	Phycocyanin Quantification Protocol
PE	Phycoerythrin
R-CPC	Phycoerythrocyanin
pH	Potential of Hydrogen
RE	Recovery Yield
RT	Room Temperature
SDS	Sodium Dodecyl Sulphate
TT	Thermal Treatment
UV-VIS	Ultraviolet - Visible Radiation
UHTPBR	Unilayer horizontal tubular photobioreactor
USP	Upstream Processing
Δ	Variation

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1. INTRODUCTION

1.1. Framework

Nowadays, one of the major alternatives to synthetic dyes used in the food industry is natural dyes obtained from microorganisms with the capacity to produce photosynthetic pigments and accessories. The cyanobacterium Arthrospira (Spirulina) platensis, due to its high contents in protein and bio-pigments, has been widely studied. It is considered one of the major sources of the protein-pigment phycocyanin, which has large applicability in the food industry due to its functional properties as antioxidant and anti-inflammatory.

In order to obtain products with applicability for the food industry, the processes capable of extending product shelf life were already studied by the industry, such as thermal treatment. At the same time, this thermal process may affect the biochemical composition of the biomass. For this purpose, optimization and stabilization of these conditions is an essential first step in, order to extract the products of interest. Additionally, for the food industry, it is necessary to extract and purify phycocyanin extracts with a certain degree of purity. For this reason, optimization of phycocyanin extraction and purification strategies is required to obtain a product with relevant commercial value. However, there is a lack of knowledge regarding the processing and stabilization of fresh bio-mass, namely Arthorspira through thermal procedures.

Taking into account the aforementioned, the main objective of this study was to develop, at a pilot scale, all the steps required to reach a food-grade quality phycocyanin extract from fresh Spirulina biomass. In order to fulfill this global objective, some partial objectives were considered as follows: 1) the development and optimization of a thermal treatment protocol for fresh Spirulina biomass; 2) the development and optimization of a cell rupture protocol for the thermally treated biomass; 3) the development and optimization of extraction and purification protocols, with focus on obtaining a purified phycocyanin extract. Biomass and fractions obtained will be biochemically characterized for the products of interest.

1.2. State Of The Art

1.2.1. Microalgae

Microalgae are categorized into eight groups as follows: Cyanophyta, Chlorophyta, Ochrophyta, Dinophyta, Rhodophyta, Euglenophyta, Haptophyta, and Prymnesiophyta. [1]–[3] These non-obligatory photosynthetic microorganisms occur in both freshwater, saline environments, and as has recently been studied, wastewater may also be adequate for these species to strive.[4], [5] These kinds of microorganisms are widely spread across the globe, and it is estimated at, at least, 200 000 different species.[6]

Some microalgae are classified as being mixotrophic, meaning they can either execute autotrophic or heterotrophic processes in order to produce cellular energy. For autotrophic microalgae, photosynthesis is essential as they convert solar energy (radiation) and CO_2 into reducing power (NADPH), for the production of chemical energy (adenosine triphosphate - ATP), O_2, and 3-phosphoglycerate, which are then used to support growth and overall cell functioning.[7] As stated by Pirt, Stephenson, and Mondal, photosynthesis-wise, microalgae can be more efficient than land plants.[8]–[10] Microalgae alone produce as much as 40 to 60% of the available O_2 in the atmosphere, due to the photosynthetic process. [11]–[13] The following Figure 1 illustrates an ultrastructure belonging to a microalga (Cyanobacteria).

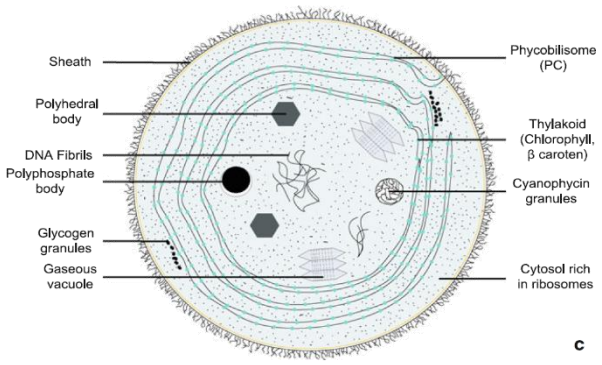

Figure 1 - Ultrastructure of microalgae: Cyanobacteria [16]

Throughout the years microalgae have had several different uses, chronologically, two periods stand out. Initially, microalgae were used (raw) as feeds for aquaculture and fertilizer for the agricultural industry. [14], [15] Later, microalgae found in the Food Industry a new market, as food supplements. The pharmaceutical and cosmetics industries also took an interest in Microalgae as a raw material for the extraction of specific molecules of high value.

In the past decade, the concerns over the three major environmental issues (global climate change, greenhouse emissions growth, and the depletion of traditional fuel reserves) have been rising. This led to a surge in interest in microalgae cultivation for biodiesel production, wastewater treatment, and CO_2 mitigation by biological means. Photosynthetic CO_2 fixation is thought to be a feasible technology as it is energy-efficient, sustainable, and environmentally benign. [10].

These developments are only economically viable if all the microalgae fractions are valorized in a biorefinery strategy. [16]

1.2.2. Microalgal cultivation

Most principles from microbial cultivation can also be applied to microalgae production, however, the latter has a unique capability of photosynthesis, setting them apart from most microorganisms. Whether microalgae are cultivated autotrophically or mixotrophically, light inputs must be constant and adjusted for the growth stage.

Regarding the cultivation methods, one may divide them into batch and semi-continuous. In a batch system, both the inoculum and the complete medium (containing all the necessary nutrients) are placed in a container, under conditions that promote culture growth. Agitation may come from three means, namely, physically shaking the container, agitation employing an impeller, or bubbling the aeration. In the aeration, usually, CO_2 enriched air is supplied to the reactor, it may be provided continuously or interspersed.[17] The source of light may be natural, LEDs, or even provided as "sunlight through optic fiber".[18]–[23] In general, batch processes are commercially successful due to their ease of operation and can be broken down into three stages.

An initial "lag" phase, where growth is suboptimal, usually represents a phase of adjustment/adaptation of the culture, to a change of concentration (dilution), change of luminosity.

The lag phase ends with the beginning of the "exponential growth" phase, in this second stage, the cells are well adjusted to the environment. In this stage as long as the nutrient saturation is maintained the cellular growth will be accurately represented by an exponential equation (growth per period). Culture growth-wise the amount of light absorbed is determined by the culture's concentration, instead of the photon flux density, until this stage. Therefore, the exponential phase remains until the cellular concentration is such that it absorbs all the irradiated light. [17]

Upon ending the exponential phase, the "linear" stage begins, in this phase the relation between cellular growth and absorbed light is linear, so it can express by the following equation[17], [24]:

Equation 1 - Linear Light vs Growth relation

$$I * A = \mu X * \frac{V}{Y}$$

Where,

I=Photon flux density in the photosynthetically available range $(Jm^{-2}h)^{-1}$,

A=illuminated surface area (m^2),

μ=Specific growth rate (h^{-1}),

X=Biomass concentration ($g.l^{-1}$),

V=Culture volume (m^3),

Y=Growth yield ($g.J^{-1}$).

In a semi-continuous regimen, there is a continuous or intermittent supply of fresh medium to the reactor. [17] In order to avoid what may be compared to the end of an exponential stage in the batch process, biomass is removed from time to time, and its volume is replaced

by a fresh medium. By doing so, the culture is kept in a "pseudo-exponential" state for a longer period, since it never exceeds the concentration that would turn the absorbed luminosity into a limiting factor. Additionally, the biomass removed from the reactor may be refined or used as inoculum for a new reactor. Literature suggests one can fix a growth rate by adjusting a concentration and dilution.[17] "Chemostat" is an example of a continuous regimen, where the total volume remains constant. This regimen, also known as "Constant Chemical Environment" is largely used in research, as it allows to maintain the growth rates pre-determined as well as study the cellular adaptations provoked by variations on the amount of light absorbed, culture concentration.[24]–[27]

As a living organism, adaptation and response to stimuli are inherent characteristics of microalgae. This stimulus can be classified in two ways, either as a limiting factor or as a stress factor if it provokes a response from the culture. Generally, a limiting factor will cause a change in the growth rate without an adaptation or response to it. On the other hand, a stress factor presupposes an adaptive response by the organism, this response is caused by a biochemical and metabolic imbalance.[17] A new steady state is reached after an adaptation stage, provoked by the latter factor.

According to Richmond (2004), one can divide stress factors into circadian factors, if they change during the day (temperature, available light), or seasonal factors, if the change occurs in a longer if these factors change over a longer time horizon (climate, seasons). In highly concentrated cultures, a third cycle, the light-dark cycle, can be described, as the culture is so concentrated that it absorbs all light radiation. This cycle is marked by fluctuations of fractions of seconds as opposed to the hours or months of the other two cycles referred to above. [17]

Among the main growth stressors experienced by microalgae, excessive solar energy absorbed by photosynthesis, nitrogen depletion, salinity, and high temperatures result in lipid storage.[9], [28]–[35] Although microalgal growth varies with sun exposure, microalgae production is not seasonal, therefore the production and harvesting process may occur daily. [36].

Microalgae are cultivated in photobioreactors (PBRs), and they can be categorized into two groups:

- Open-systems such as circular ponds, conventional and cascade raceway-CRW;
- Closed systems as multilayer horizontal tubular photobioreactor-MHTPBR, unilayer horizontal tubular photobioreactor-UHTPBR, flat-panel photobioreactor-FPPBR, and glass panel photobioreactor.

Cascade raceway modules' typically occupy about 3000 to 4000 square meters in an industrial setting. Among the main factors that influence the construction of raceways are the shape, depth, and mixing method.[5] These modules usually consist of two sloped ramps with converging inclinations (Figure 2). Cultures flow in a circuit across these ramps getting collected at the end of the second ramp and pumped back to the first sloped ramp. The first set of sloping culture units was designed and operated by Setlik et al. in Czechoslovakia. [5], [15], [37]

Figure 2 - General representation of a Cascade Raceway (CRW) [37]

As an alternative and among the most popular technologies for microalgae production, tubular photobioreactors (Figure 3) offer flexibility for both a monolayer and multilayer structure. In this configuration, tube rows are arranged horizontally on the ground and parallel to each other, increasing the volume to area ratio. [37]

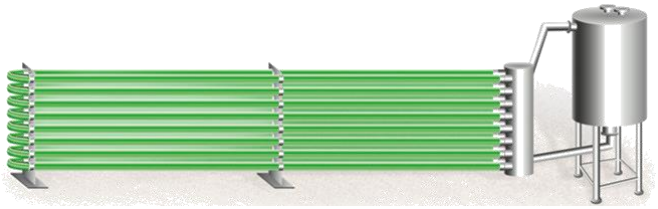

Figure 3 - General representation of a Tubular Photobioreactor (UHTPBR) [37]

Flat-panel photobioreactors can serve as a step in between laboratory scale and industrial reactors. The FPPBR (Figure 4) consists of a disposable low-density polyethylene (LDPE) film enclosed in a metal frame. This reactor solution prevents contamination and achieves higher volumetric productivities. Within closed systems, this is considered to be the most versatile and economical option available.[37]

As a general consensus closed systems are characterized as having better yields than open ones, are easily controlled in terms of growth parameters, have a better surface to volume ratio, in addition, are less prone to contamination. Notwithstanding, there are some limitations to this system that jeopardize its industrial scaling, such as temperature control, which may be a major barrier adding to the already problematic scalability.

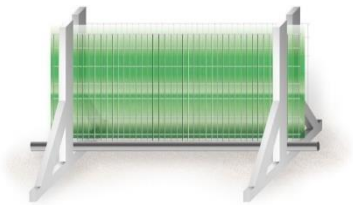

Figure 4 – General representation of a Flat Panel Photobioreactor (FPPBR) [37]

The choice of setup is not clear, since both systems have their limitations, the option should be based on the intended use of each system.[38]

Within the company's facilities, there are both types of reactors available, each used for specific scales and stages of the scaling process.

1.2.3. Biorefinery Systems

Biorefining is the procedure for processing biomass efficiently and economically to produce Biobased products. [39]–[41] This innovative approach uses, similarly to traditional refineries, different fractions of the raw material for the elaboration of different products. Biomass products range from energy and biofuels to food, pharmaceuticals, nutraceuticals, chemicals, and materials. Biorefining makes it possible to use fractions that have been discarded up till now, extracting gains from them. Therefore, biorefining presents itself as a key element in the implementation of a circular economy.[39]

Although there has recently been a new approach to biorefineries and biorefining, the process itself already exists and is already applied in the food, and "pulp and paper" sectors. Among the most recent biorefineries, biofuel and bioenergy stand out as the main products. Although product-based biorefinery manufactures primarily products of high commercial value they can also produce bioenergy in smaller quantities as a means of saving energy.[39], [42] Usually, a biorefinery would produce multiple products with inherently different values and rates. One may produce a high-value product in a low volume, such as chemical or nutraceutical, and a lower-value product but at a faster rate. In addition, by doing so, it would take advantage of the whole biomass and its components, thereby maximizing the value derived from the biomass feedstock.[43]

When analyzing the evolution of biorefineries, four periods stand out, also called generations. The first generation aimed to produce biofuel derived from sugarcane, corn, and rapeseed. Although this was a good start it competed directly with the food industry and ended up failing. The generation that followed used derived food residues (lignocellulosic) to produce energy, chemicals, and biomaterials.[44] The third generation had its primary focus on biodiesel production from microalgae transesterification. Lastly, the fourth generation produces hydrocarbons from microalgal hydroprocessing. [39], [41]

The following Figure 5 exemplifies the idea behind the biorefining process.

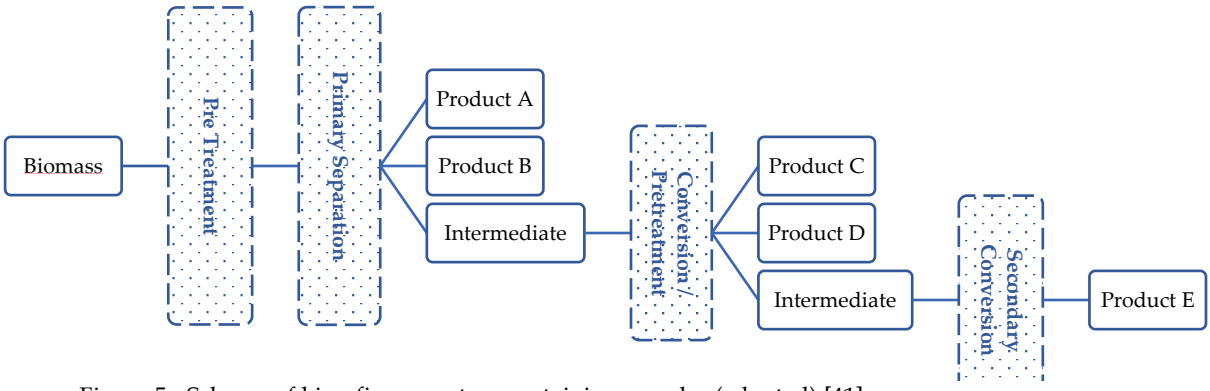

Figure 5 - Scheme of biorefinery system containing recycles (adapted) [41]

1.2.3.1. Microalgae Biorefinery

The initial focus of the use of microalgae in biorefining was the production of biofuels, as mentioned above. However, this focus changed due to the associated costs that could jeopardize the viability of the project. Kit Chew argues that the key for a sustainable biorefinery is to have a balance between a mix of diverse high commercial valued products and biofuel.[45] Taking a similar approach to that of traditional refineries, an attempt was made to profit from the different fractions by extracting high commercial value products from co-products. Another advantage of using microalgae for the extraction of high-value products is that they can be cultivated by utilizing only water and atmospheric CO_2, which may be available at minimum cost. It does not create competition for land and food crops as microalgae can grow on degraded land. In this scenario, while the high-value products increase profitability, the CO_2 consumption may help either, to reduce the factory's overall carbon footprint or be outsourced as an alternative way to treat carbon emissions by other plants in the surroundings. [45]

As examples of these added-value products pigments, proteins, lipids, carbohydrates, and vitamins stand out. These products are desired by the food, nutrition, and pharma industries. Regarding the use of the above products, lipids stand out in the production of biodiesel, in addition to long chains of fatty acids from microalgae are used in the food and health supplement sector. Proteins and Pigments have their main use in the pharmaceutical sector, which is used to treat certain diseases.[46] Carbohydrates are mainly used as alternative carbon sources for industrial fermentations.[45]

Microalgae biorefineries may be divided into two stages: upstream processing (UPS) and downstream processing (DSP). The effectiveness and efficiency of USP have four critical factors, being microalgal strain, CO_2 supply, nutrients source, and light source.[47]

Regarding DSP, this stage presents conventional methods, of which stand out the bead milling, homogenizers, high-pressure heating, and chemical methods. The lack of standardization of an economic process added to the high costs of these methods mentioned leads to the need for a multi-step process in order to guarantee extraction yields as high as economically feasible, as reported by Jacob-Lopes. [45], [48] It should also be noted that as it doesn't use solvents or high-pressure techniques, this processing prevents damage to the fractions underlying the fraction in extraction. [47]

1.2.4. Spirulina (Arthrospira) platensis

Cyanophyta (as referred earlier), Cyanobacterium, or Cyanobacteria are a phylum of Gram-negative bacteria, with individualized organelles/structures. [49] In Cyanobacteria the photosynthetic process is assured by Thylakoids, individualized structures that mimic the chloroplasts. Arthrospira platensis is a photosynthetic organism that was considered to be algae (as a eukaryotic organism), until 1962 when it was reclassified as prokaryote and fit in the Cyanobacteria phylum.[50]

The two currently accepted designations for the genus Arthorspira (Arthrospira and Spirulina) may occasionally create confusion. Stinzenberger (1854) and Gomont (1892-1893) identified two different types of microalgae, a first with helical shape and presence of visible

septa which was called Arthrospira Stinzenberger 1852 and the second with a clear absence of septa, called Spirulina Turpin 1829. [13] This was the main differentiating criterion within the order of Oscillatoreales.[51]–[53] These two genera were merged in 1925 (reinforced in 1932) by Geitler in his revision, under the argument of the presence of helical trichomes over the entire length of the cell, regardless of the presence of "more or less visible cross-walls under the microscope" as he stated.[54], [55] It wasn't up until more recent times that the division of these species was reconsidered by their morphological characteristics.[56], [57] This division was officially recognized in Bergey's Manual of Systematic Bacteriology by Castenholz in 1989. [13], [58] According to several authors, this division makes more sense in the light of current knowledge. Among the singular differences found by the authors are helicity, trichomes size, motility, and fragmentation of trichomes, as well as GC content and oligonucleotides. [13], [59], [60]

As a result of this inaccuracy, which lasted for a few years today, there are some cyanobacteria with the general epithet "Spirulina" erroneously. Therefore, according to Vonshak and Tomaselli, they should be renamed Arthrospira in the place of Spirulina.[61]

Morphologically, Arthrospira (Figure 6) usually has a left-handed spiral shape as a distinctive feature. When in the helical form its mobility is due to a gliding rotation along its axis. Some cultures of Arthrospira present themselves with either linear or helix-like shape forms depending on the culture medium in use.[62] Although Arthrospira is denser than water, there are gas vesicles as an evolutionary adjustment so that floats. [63], [64] These vesicles often mask as cross walls. Arthrospira has a Gram-Negative-like cell wall with peptidoglycan as an inner layer. This carbohydrate offers osmotic protection and overall integral strength to the microorganism cell wall.

Figure 6 - Microscopical view at 10x2 of Arthrospira Platensis

1.2.5. Phycocyanin

Phycobilisomes, Figure 7, allow cyanobacteria to absorb light, over a wide range of wavelengths in the visible part of the spectrum and transfer the excitation energy by radiationless processes to the reaction centers in the photosynthetic membranes for conversion to

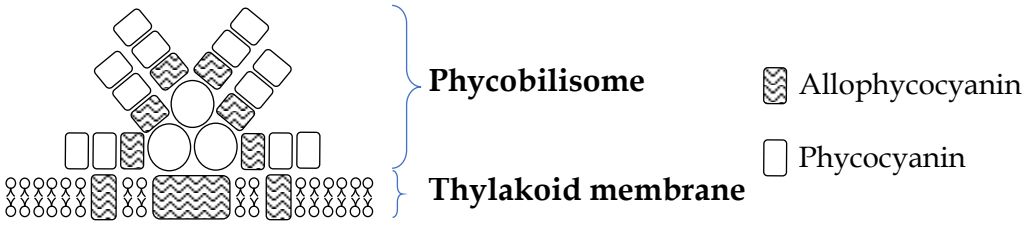

Figure 7 - Photosynthetic complex - Structure & location of phycobilisomes (Adapted) [78]

chemical energy.

An interesting application for the phycobiliproteins is their use as natural dyes in foods and cosmetics replacing the synthetic dyes since the latter are, generally, toxic, carcinogenic, or

otherwise unsafe. In some countries where algal cultivation is an established industry, natural pigments from phycobiliproteins have already been patented. In Japan, Dainippon Ink and Chemicals Inc., patented both in 1979, and 1987 their products. [65]

One can divide these pigments according to their absorption, phycoerythrins (PE, λmax 540–570 nm), Phycocyanin (CPC, λ_{max} 610–620 nm), and Allophycocyanin (APCs, λ_{max} 650–655 nm). Some cyanobacteria, such as Arthrospira platensis, have a fourth type of biliprotein in place of PE, the phycoerythrocyanin.[66], [67] Visually, while phycoerythrins appear red, CPCs range from purple (phycoerythrocyanin, R-CPC) to deep blue (CPC), and the APCs are blue with a hint of green.[68]–[70] The use of this biliprotein has been somewhat limited due to the difficult and long purification procedures. Pure phycobiliproteins from crude algal extracts are usually obtained by a combination of different and, until now, non-scalable methods. [65], [71]–[74] Additionally there are some questions regarding its instability both due to pH conditions and to thermal processing, as CPC may lose its color activity when in contact with low pH values and high temperatures.[75], [76] This factor is conducive to denaturation, constraining its application in the industry, as color is the most important techno-functional property. [77] Phycocyanin powders' intensity degradation is an indirect descriptor of underlying chemical reactions. As CPC intensity decreases it is noticeable an increase in absorbance at 360 nm. As this degradation occurs the secondary, tertiary, or quaternary structure of the apoprotein rearranges itself from a linear chromophore into a cyclic form. [78], [79]

Although this thermic sensitivity issue is widely known, the kinetics of color degradation has not been extensively studied to this day. Researchers analyzed other solutions for this problem including techniques of encapsulation and stabilization with sodium dodecyl sulfate (SDS), the addition of sugars may be an alternative to SDS as similar results were obtained.[29], [81], [34]

1.2.6. Processual Strategies

1.2.6.1. Thermal Treatment

Usually, Arthrospira is sold as dried biomass, in order to improve stability, extended lifespan, and ease of transportation, thus methods such as spray drying, air drying, and freeze-drying were developed and optimized. [82], [83] Despite that, dried biomass usually loses some properties as it decreases in protein, lipid, and pigments contents. [62], [83]–[85] A possible alternative to the drying process for preserving fresh biomass is a pasteurization-like treatment, preserving fresh biomass while still disabling enzymes (such as Lysozyme, which degrades peptidoglycan, a structural polysaccharide[86]). According to the International Dairy Federation pasteurization may be defined as: ''(…) a process applied to a product to minimize possible health hazards arising from pathogenic microorganisms associated with the product which is consistent with minimal chemical, physical, and organoleptic changes in the product.''[87]. Pasteurization may be performed either as a batch process where all its steps with extended holding times or as a continuous process. [88] Any pasteurized product goes through a variable time/temperature combinations treatment to kill/inactivate bacteria

or pathogens. [88] These combinations range from a low-temperature long time (LTLT) to a high-temperature short time (HTST). Both Arthrospira and CPC are thermally sensitive since Arthrospira samples put through temperatures over "T+20" ºC have a burnt appearance (as it's already company's knowledge) and CPC values decrease both with high temperatures and/or extended periods exposed to mildly high temperatures ("T+10" ºC or higher).[89]

Hence, in this thesis, it is proposed a thermal process for enzymatic inactivation as an alternative to pasteurization. With reduced periods of exposure and lighter temperature ranges, thus protecting both the desired thermal sensitive pigment (CPC) and the Arthrospira integrity (by disabling the enzymes). This process mimics the pasteurization process with three steps: temperature increase, hold period, and finally a cooling step, as is illustrated in Figure 8.

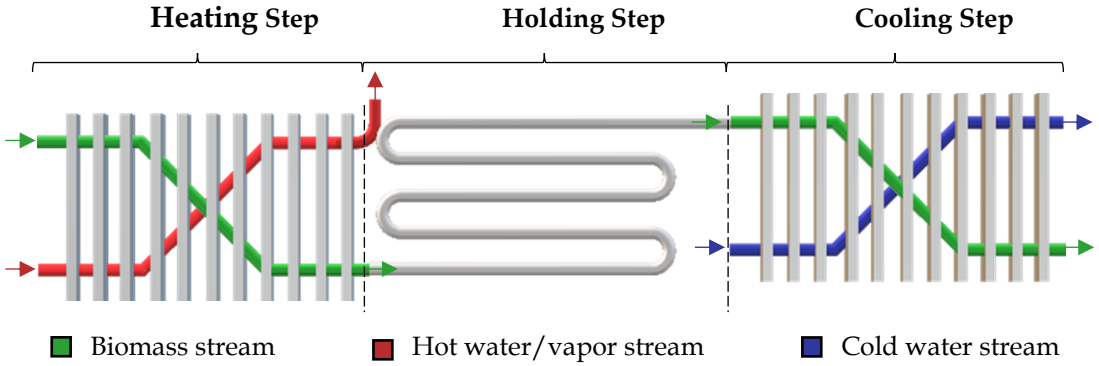

Figure 8 - Pasteurization/Thermal Treatment General Representation

1.2.6.2. Cellular Rupture

After the harvesting process, it is necessary to rupture the Arthrospira cell wall for later extraction of its intracellular components. [90]–[92] There is a variety of methods for cell wall disruption ranging from ultra-Sonification, bead milling to frost/defrost cycles, or even enzymatic digestion. As for the degree of rupture, one can identify two types of cell rupture: mild disruption, which largely means that there was some extent of rupture in the cell wall, or a more severe rupture leading to total cellular disintegration. The latter, in most cases, leads to a non-selective release of all intracellular components hence must be avoided. Many rupture methods are harsh and provoke severe rupture due to either high pressures, high shear levels, or high temperatures.[93]

Historically bead mills' main use in the chemical industry was restricted to paints/lacquers and grinding minerals.[94] Although, more recently disintegration of fungi, cyanobacteria, and microalgae was studied.

The bead mill has been successfully adapted for cell disruption both in the laboratory and on an industrial scale.[95], [96] It provides a simple and effective means for disrupting different types of microorganisms. Although different designs are offered, the basic scheme is a jacketed grinding chamber, either vertical or horizontal, with a rotating shaft running through its center. This shaft has an agitator(s) of varied design that induces kinetic energy to the chamber filling; usually, this filling consists of small beads made out of either glass or ceramic, forcing them to collide with each other.[97]–[100] The following Figure 9 illustrates the general concept present in a bead mill.

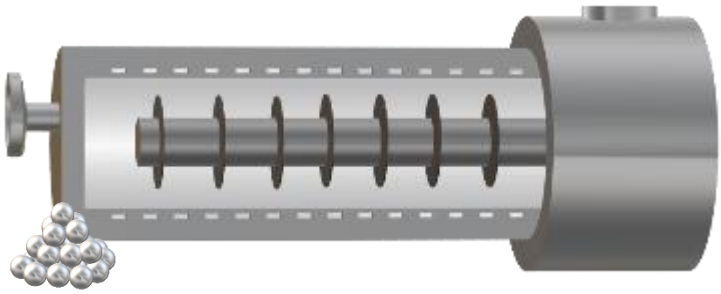

Figure 9 - General bead mill representation

The most important parameters for bead milling, and overall cell disruption efficiency, are listed below:[37], [98]

- Chamber and agitator geometry;
- Agitator speed (tip speed);
- Biomass concentration;
- Suspension flow rate;
- Bead filling ratio;
- Bead type and diameter.

As the disruption effectiveness level increases with bead loading since it also increases the bead-to-bead interaction, both heating and power consumption increase, as a consequence of this interaction. As bead loads over 90% present an increase in both heating and power consumption that usually outweigh the disruption efficiency, the usual bead loading is at the 80% to 85% range.[94] Smaller beads diameter usually led to faster cell disruption. The approximate "optimal" diameter range for beads diameter is at 0,10 to 0,15 mm to bacteria and 0,25 to 0,75 mm to yeasts.[101]

Industrial mills must use beads in a range greater than 0,4 to 0,6 mm in diameter due to the mechanism for separating the beads from the suspension. Even though increasing the impeller tip speed will increase the disruption effect, it also increases both the power usage and heat generation. [94]

The probability of cellular destruction increases with the extension of exposure, the longer a cell remains in the chamber higher is the probability of rupture. Therefore, increasing either the milling time, if in a batch operation, or the mean residence time, if in a continuous operation, will normally increase the level of disruption.

1.2.7. Uses and Market for pigments

Economically, several Added Value Products can be extracted from Arthrospira. Those products may be divided by their final market use: as a dietary supplement - it can be extracted both proteins and lipids (α- Linoleic Acid and γ-Linoleic Acid); as a natural dye - pigments such as CPC, APC, PE, a-chlorophyll (Chl), and carotenoids can also be extracted.[102]

In 2020 dietary supplements market size was valued at USD 140.3 Bi and is expected to expand at a compound annual growth rate (CAGR) of 8.6% from 2021 to 2028. Furthermore, protein supplements alone are expected to reach USD 36.05 Bi by 2028. [103]

CPC, specifically, is mostly used as a food colorant and cosmetic such as lipstick and eyeliners. [104] It was also shown to have therapeutic value (immunomodulating activity and anticancer activity).[105] Owing to its fluorescence properties it has gained importance in the development of phycofluor probes for immunodiagnostics.[106]

As for pigments, the demand for naturally derived materials is rising at a rapid pace for the past few years. This market is expected to grow with a CAGR of over 5% by 2026[107], the global natural food colors market was valued at USD 1.3 Bi, the food colors market size was valued at USD 2,1 billion in 2019, and is estimated to reach USD 3.5 Bi by 2027, registering a CAGR of 12.4% in that period.[108]

2.
MATERIALS AND METHODS

2.1. Materials

2.1.1. Spirulina (Arthrospira) platensis strain

Arthrospira is most often used as a raw material in the form of dried biomass as discussed earlier due to ease of transportation.

In the vast majority of cases, it is beneficial for a refinery or any given processing industry that its location is close to raw materials, in order to avoid time-consuming transportation. Since the company complex comprises both production and processing facilities, the issue of lengthened transportation, where dried biomass would have an advantage over its counterparts loses importance. Furthermore, it is already known within the company that dried biomass has a lower nutrient content (e.g., CPC) than biomass from a culture or paste (concentrated culture). Skipping this step prevents a drastic increase in energy consumption during the drying stage. As a logistical comparison, the paste is more efficient than culture since it avoids the unnecessary transport of large volumes of water associated with culture transport while, also reducing the cost of internal transport, storage, and processing.

Arthrospira biomass used in the trials had two sources:

- Initially, as it was intended to have fewer variables and unknown factors more controlled biomass was used from the company's laboratory;
- During the second phase, following the establishment of both quantification and thermal treatment processes, biomass from green walls / flat panels reactors, from the production facilities, was used for testing, since it will be used in the refinery.

The Arthrospira used in this work was characterized by the company with 56,8% Proteins, 30,5% Carbohydrates, 5,9% Lipids, and 7,4% Ashes.

2.2. Methods

2.2.1. Optical Density

As a preliminary test for the culture dry weight, an optical density reading is performed on a Spectrophotometer (Genesys 10S UV-VIS). In this method, the tested culture is diluted, and the returned value from the preprogrammed setting for Cyanobacteria read, the returned values inserted into a calibration line which returned the concentration in grams per Liter (g/L).

2.2.2. Dry weight

To have a more accurate reading on the actual dry weight biomass samples were put through a moisture content tester (MS-70, A&D Weighing) at 105°C, according to the AOAC method. [109]

The empty plate was weighed until reached three equal measures then, approximately 1 mL of the concentrate of fresh Arthrospira was poured into the plate and weighed finally after 105 ^{o}C drying process the plate was again weighed until reach three equal measures. The following Equation was used to return the dry weight value:

Equation 2 - Determination of Dry Weight (g/g)

$$\%\ DW = \frac{P_f - P_r}{P_i} * 100$$

Where,

% DW: Dry weight expressed in percentage (%).

P_f: Total mass, the plate plus dried sample (g).

P_r: Plate mass (g)

P_i: initial sample mass (hydrated) (g)

2.2.3. Ash-Free Dry weight

The Ash-Free Dry Weight (AFDW) content was measured using the official AOAC methods by weighing the burned sample in a muffle furnace (LE 6/11, Nabertherm) at 575 °C and by following the protocol already implemented at the company. [109] The ash weight was always measured with duplicates to have a more accurate reading. When utilizing this method firstly the ash content is obtained and then the AFDW is calculated by difference with the SFDW value from the. With the assistance of Equation 3, the ash content of Arthrospira biomass was determined.

Equation 3 - Determination of Ash contents (%)

$$\%Ash = \frac{P_f - P_c}{P_s}$$

Wherein,

% Ash: weight of ashes expressed in percentage.

P_C: represents crucible mass (g).

P_S: represents sample mass [salt-free] (g).

The value returned in the previous equation should be subtracted to the SFDW in order to obtain the Ash-Free Dry weight. [109]

Equation 4 - Determination of Ash-Free Dry Weight

$$\%\,AFDW = \%\,SFDW - \%\,Ash$$

Where:

% AFDW: weight value of dried and burned biomass sample expressed in percentage.

% Ash: weight value of ashes expressed in percentage, obtained in Equation 3

% SFDW: weight value of Salt-Free Dry Weight biomass sample expressed in percentage.

2.2.4. Phycocyanin Quantification

The quantification of CPC aims to characterize both the cultures of Arthrospira samples from different reactors and processed samples, to better assess its impact on the sample. As the standard procedure applied by the company was developed for dried biomass, it did not present satisfactory results for fresh biomass. During the course of this thesis, a new quantification procedure was developed and applied.

The previously used method when applied for fresh biomass struggled with the influence of chlorophyll as it also absorbs at the same wavelength of CPC.

2.2.5. Thermal Treatment

Protocols were developed in order to stabilize fresh biomass to improve its shelf life. As the existing procedures could not be applied to Arthrospira due to its thermal sensitivity this method was developed during this work, and it can be consulted in the results chapter where it is described in detail.

2.2.5.1. Laboratory scale Thermal Treatment

This method was created to simulate pilot-scale thermal treatment in laboratory conditions, particularly to study the effectiveness of pilot-scale thermal treatment of Spirulina for enzyme inactivation. In this method three heat ramps are tested T1which emulates the company's pilot pasteurizer (default treatment 45min), the remaining T2 (default heating + rapid cooling) and T3 (shock treatment) emulate an industrial scale pasteurizer with shortened holding periods and a quicker cooling step.

2.2.5.2. Pilot Thermal Treatment

This method was created, after optimization of the laboratory protocol for TT, to emulate the T2 ramp, simulating industrial-scale thermal treatment, particularly to study the effectiveness of industrial-scale thermal treatment of Spirulina for enzyme inactivation. In this method.

2.2.6. Cellular Rupture

The following procedure was adapted from the current milling procedure by the company. This method tests three different flow rates being F L/h, 2F L/h, and 3F L/h.

For preparation, all connection hoses and connections should be checked, jerry cans containing biomass should be homogenized, and poured into a bucket. Connect the feed hose to it on one end, and the other end to a pump for feeding the biomass to the mill. Parameters such as tip-speed, bead volume/filling ratio were chosen accordingly with the company's milling protocol in place.

For the milling, firstly turn on the mill's refrigeration, then the pump at F L/h, and finally, turn on the mill. During milling outlet temperature and foam, height should be registered and monitored. The first fraction should be discarded (approx. F L) and the following F L should be acquired and stored refrigerated for later analysis. Change the flowrate from F L/h to 2F L/h, change the first fraction should, once again, be neglected and discarded (approx. F L); The following f L should be acquired and stored refrigerated for later analysis; Change the flowrate from 2F L/h to 3F L/h. Once again, the first fraction should be neglected and discarded (approx. F L), and the following F L should be acquired and stored refrigerated for later analysis. All three milled fractions should be processed according to the CPC Quantification Protocol describe in this work, skipping the milling stage in test tubes as they are already ruptured.

2.2.7. Salting out - CPC Purification Protocol

Phycocyanin extract was added to a saturated solution of ammonium sulfate ($(NH_4)_2SO_4$) (being referred forward as "AS") to be purified as far as protein contents. In this method, soluble and non-soluble proteins get separated as two phases appear. Phycocyanin along with other non-soluble proteins was retrieved from the sediment as the soluble proteins remained in the supernatant.[37], [93]

The following plot is a result of the studies already conducted by the company. Only validating tests were performed with three different concentrations (Figure 10).

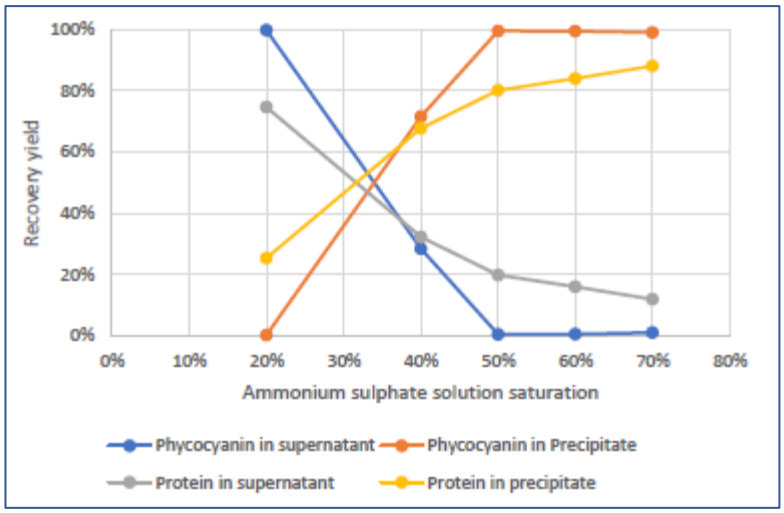

Figure 10 - Proteins and CPC Curves vs AS solution saturation [93]

After comparing with previous results, the most efficient saturation percentage of salt solution was selected, by comparing the results of 40, 50, and 60% of saturation.

In this method, concentrated solutions were prepared to reach the target concentration after the addition of the phycocyanin extract, being prepared one "stock" solution for each concentration used in all trials. The AS was pipetted into the test tubes (previously weighed) as the volumetric mass could not be approximated to the water, as for the CPC extract it was weighed into the already filled with AS test tubes. As far as the volumetric mass of each stock solutions I, II, and III were prepared corresponding to 40, 50, and 60% (Figure 11).

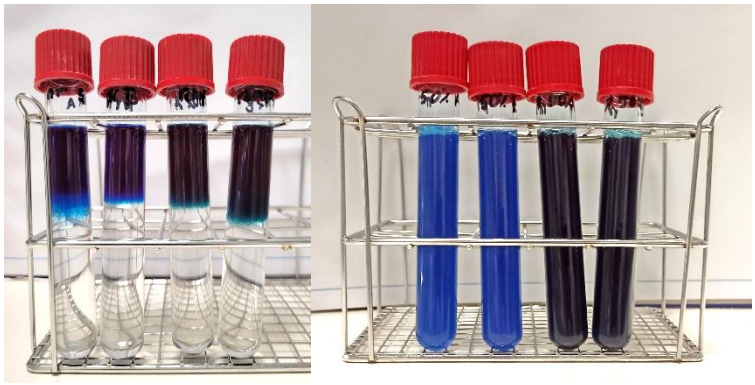

Figure 11 - General Representation of Salting Out tubes before and after homogenization

After this addition and in order to ensure homogeneity, all samples were vortexed for 30 seconds before being stored overnight at 4 ºC, covered from the light with aluminum foil. After the overnight period, all samples were taken from storage and centrifuged (Ortoalresa 21) for 20min at 2400 xg. Supernatants and sediments were separated for weighing and analysis. Both phases were weighed, as far as the supernatant, it was pipetted and weighed, the sediment was weighed in the test tube and calculated its weigh by subtraction to the previous empty weighed tube.

Although the supernatants can be read in the spectrophotometer without dilution, the sediments undergo two dilutions to be read. In an initial dilution, 1 ml of sediment is added to 10 ml of 0,1 M potassium phosphate buffer, pH 6.9; in the second dilution, from the diluted solution, 1 ml is added to 2 ml of potassium phosphate buffer. After these dilutions, both e samples can effectively be read on the spectrophotometer.

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3. Results and Discussion

3.1. Characterization of *Arthrospira* biochemical Composition - CPC quantification

The previously applied method for quantification of phycocyanin was designed for dried biomass and had been used uninterruptedly since its validation. This method is based on the high solubility of phycocyanin in aqueous phases and the low solubility of chlorophyll in them.[79], [114]–[117] The existing protocol was applied to fresh biomass paste samples, as the equivalent of 15mg DW was weighed and added to 10mL of potassium phosphate buffer and milled in the vortex for 12 min. In the next step, the milled biomass was centrifuged according to the protocol for 25 min. Despite centrifugation under the conditions described in the protocol, the results, as present in the following Figure 12, showed a green supernatant, indicating the presence of chlorophyll, which would lead to an inaccurate reading in the following step (spectrophotometry).

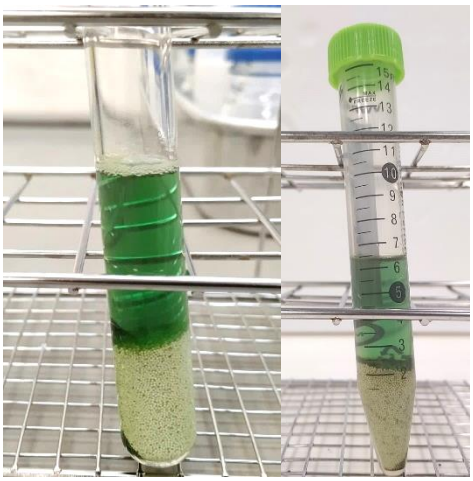

Figure 12 - CPC Extract Previous Protocol with Chl contamination

As the results using this protocol were not satisfactory a new protocol, suited for fresh Arthrospira was developed and applied.

As phycocyanin is the main focus of this thesis and even for the company regarding Arthrospira refining it is of utmost importance to have an accurate reading on its value and purity. The company suggested that this 'new' method should be an improvement over the existing method, rather than a new method. It should also be as timely as possible to provide real-time results. After implementation, this method would also be used to monitor the phycocyanin levels in the production cultures.

As part of the method development process, the main parameters to be studied were determined in collaboration with the company. The following Table 1summarizes the different aspects/parameters to be studied as well as the correspondent trial and letter. This list was developed with the input of the company as some of these parameters had already shown significance in other microalgae analysis performed in the company's laboratory.

Table 1 - Different trials and variables studied

Trial	Variables								
	Initial State	Centrifugation Cycles	Temperature (°C)	pH	Extraction Time	Biomass Concentration	Bead to Biomass volume	No. Resuspensions	Milling Time
A		✓							
B	✓								
C						✓			
D								✓	
E					✓				
F			✓						
G									✓
H				✓					

Considering the low solubility of Chl in aqueous solutions already mentioned above, in ruptured biomass chlorophyll stays in the cellular debris. As such, the influence of centrifugation time on chlorophyll and phycocyanin values was analyzed in the first test. [79], [114]–[117] As the tube centrifuge used in the previous protocol was already being operated at maximum power and revealed to be ineffective, an Eppendorfs centrifuge with a higher power was used in its place.

The second trial studied the influence of the biomass' initial state in the quantification process, as the previous protocol was developed for dried biomass. In this assay, fresh biomass and freeze-dried biomass were compared.

The following trials studied the influence of what was called process parameters such as the feed concentration, the number of needed resuspensions for a complete extraction, pH (plus the influence of potassium phosphate buffer), Temperature, the needed extraction time for a complete extraction, the required milling. In each trial each parameter was studied individually in order to prevent doubt on the origin of the interference, by the company's indication conjugated interferences should be neglected as only individual interferences were ought to be studied. The values presented are average values from repeated tests regarding each trial, in these tests duplicates were used. Values from CPC contents and, consequently, the calculated ratios varied from test to test as they directly depend on the specific conditions of the tested biomass at testing time. As mentioned in the microalgae cultivation chapter CPC

and other nutrients may vary with sun exposure, aeration, and biomass concentration, with that in mind each value should only be directly compared with values from the same trial. Parallelisms between different trials should be made cautiously, as the total nutrient value may vary between each test and consequently, in each trial.

3.1.1. Trial A - Improving CPC/Chl ratio through centrifugation

This first trial aimed to uncover the correlation between the influences of chlorophyll in the phycocyanin spectra versus the centrifugation time. As per the company's advice, the centrifuge MiniSpin Plus from Eppendorf was used after the Megafuge as the following step, as the latter did not present the needed output. Given that the Chl remains in the cellular debris improving the separation of liquid/debris would decrease the chlorophyll value. In this trial, it was analyzed the whole spectra from samples centrifuged at 10min, 30min, and 50min at 14100 xg to observe the Chl and CPC peaks variation. Both samples were harvested, centrifuged in the Megafuge for 20 min, separated supernatant and pellet and resuspended in Potassium Buffer as it was described in the previous protocol, milled in test tubes for 15 min with the described volume of beads in a vortex. The following plots illustrate both the absorbance variations as well as the purity (CPC/Chl) ratio for the different periods of centrifugation (Figure 13).

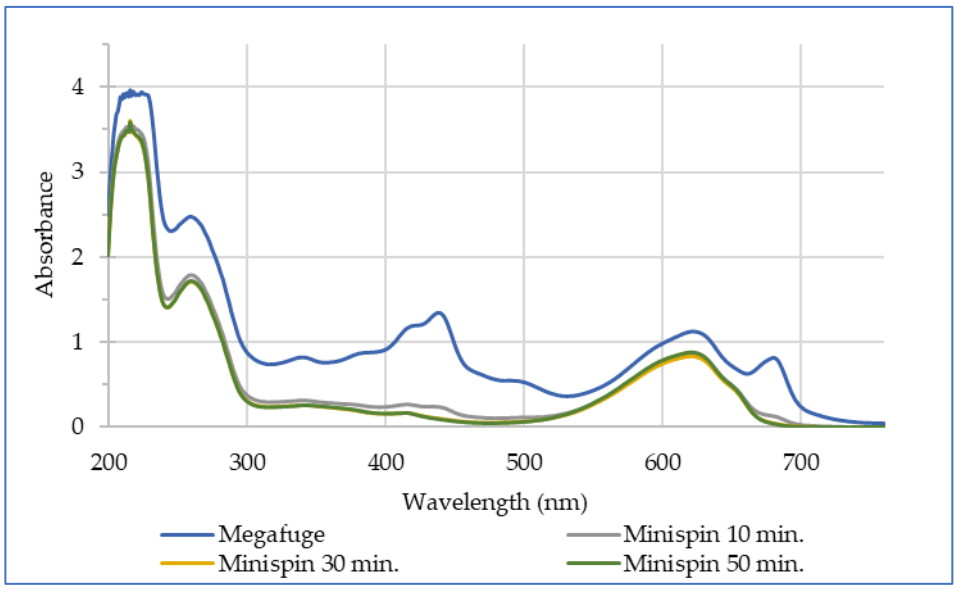

Figure 13 - Phycocyanin Extract Spectra (200-760 nm)

The initial sample from the megafuge centrifuge (150 mL) was divided, the supernatant retrieved, and the sediment discarded. The retrieved supernatant was homogenized and poured into 1mL Eppendeorfs. Each after each type B centrifuge cycle before reading in the spectrophotometer the eppendorfs were changed (supernatant retrieved and poured into a new Eppendorf) to prevent resuspension that would lead to chlorophyll contamination

The power improvement in centrifugation (from 4100 to 14100 xg) appears to lead to a major improvement in the overall Chl removal as shown in the above plot. The sample after centrifugation in the megafuge shows a clear peak at 439 nm and 684 nm (known peaks for chlorophyll). The samples centrifuged at 14100 xg appear to have successfully eliminated both

thee peaks, the peak near 684 nm is the main responsible for interference in the CPC's value and seems to be completely removed in both 30min and 50 min centrifuged samples.
The following plot Figure 14 illustrates both chlorophyll absorbance and the ratio between the phycocyanin and chlorophyll absorbances.

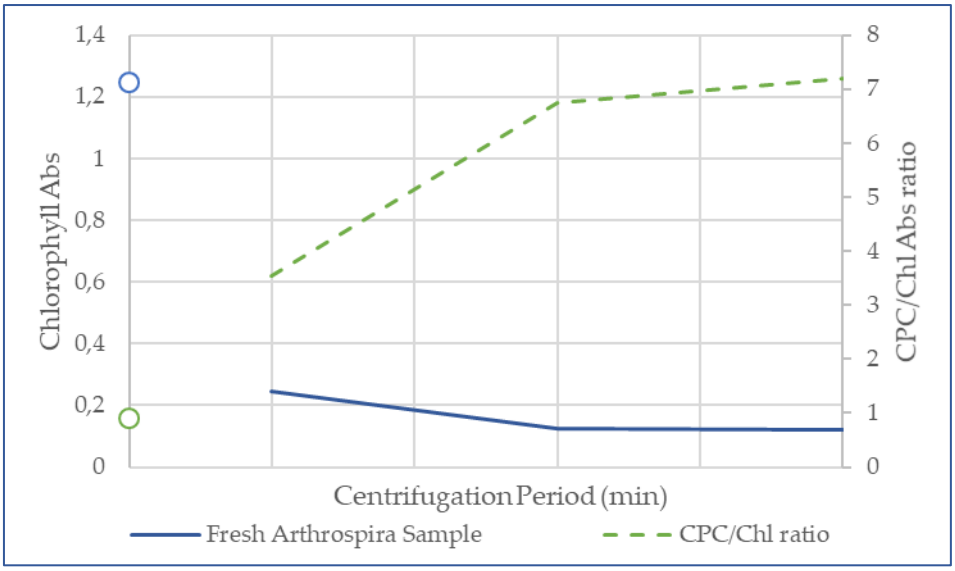

Figure 14 - Chlorophyll and CPC/Chl values across centrifugation

After the first centrifugation, as shown in the plot above, Figure 14, the Chl absorbance is at 1,25 and a ratio (CPC/Chl) 0,906. After 10 min of centrifugation at 14100 xg the chlorophyll dropped 90,0% to 0,245 and the CPC/Chl ratio improved 191,8% to 3,55. This improvement in both indicators may imply a path for optimization at this point. At midpoint the chlorophyll absorbance-dropped 0,124 (49,4% decrease in Chl concentration), the ratio maintained a similar slope to a value of 6,75 (90,1% improvement in chlorophyll contamination). Finally, at the last measurement, the absorbance for Chl was at 0,123 and the ratio (CPC/Chl) at 7,2. As shown in the Figure 14 the centrifugation appears to be very effective until midpoint for Chl absorbance reaching a plateau at that point with limited decline. This may indicate that for this specific equipment the ceiling for effectiveness is reached at the midpoint of centrifugation at 14100 xg. For the ratio CPC/Chl the pseudo-plateau where still hints at a linear increase relation between centrifugation time and overall purity, although, with a poorer slope, appears approximately at $\frac{3}{4}$ of the centrifugation period. Figure 14 hints that with more time of centrifugation the CPC/Chl would improve until reaching a plateau, as happened with de absorbance with minimal improvement. This method was designed to be as precise and as quick as possible. As the ratio, CPC/Chl is used as a relative measure for the purity of the final extract and the assessment of the overall reliability of the CPC results compromises were made to establish the last measurement mark as the maximum centrifugation time. In the following results, all samples were centrifuged for a longer period tested to ensure the best ratio, and as a result most purity from the CPC at a reasonable timeframe.

3.1.2. Trial B - Fresh vs freeze-dried biomass in the CPC quantification

As the previous protocol was developed for dried biomass it was of interest to study the potential differences in the effectiveness of the new method between fresh biomass and dried biomass. For these tests, fresh biomass from the company laboratory (2L Schotts) was used and compared to freeze-dried biomass. This trial tried to assert if the lack of effectiveness from the previous protocol was eradicated and the CPC values from both dried and fresh biomass matched. If that were the case, then the following steps would be to perfect the method rather than correct it.

In this assay fresh biomass was harvested according to the current protocol in place, then both samples were resuspended in potassium phosphate 0,1 M, milled for 15 minutes. As decided after the previous trial both subjects were centrifuged for 15 min, 4 ºC, at 4100 xg plus the previously tested period at 14100 xg. Between each cycle, the sediment and supernatant were separated. After the last centrifugation cycle, each sample was poured from the individual Eppendorfs and stored in test tubes in dark until reading to prevent light deterioration.

The following plot Figure 15 summarizes the results from this trial as the whole spectra from Arthorspira were read and CPC contents were analyzed and plotted with CPC/Proteins and CPC/Chl ratios.

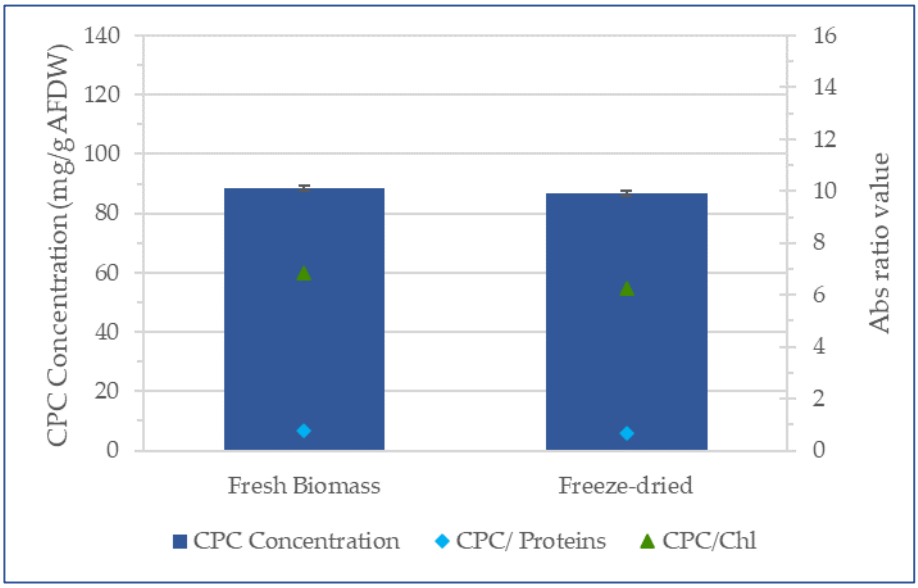

Figure 15 - CPC Quantification - Biomass Origin: Fresh Biomass vs Freeze-dried

As the chart above illustrates, the contents of phycocyanin in both samples are similar, with values in the error margin. As for the ratios, both are comparable as the CPC/Proteins, the main indicator of purity for commercial use. Both samples show a 0,1-difference favoring the fresh biomass, which may be linked to the slightly higher (1,8 mg/g AFDW) CPC content. As is the case of CPC/Chl, the indicator used in the company for the reliability of results, both present a similar value being 6,8 for fresh biomass and five-tenths lower at 6,3 for freeze-dried Arthorspira. These results appear to corroborate the idea that the main issue with the previous

protocol was solved in trial A as the interference from Chl appeared to have disappeared, solving the first stage of this method developing.

3.1.3. Trial C - Influence of the biomass concentration

In order to improve and optimize the quantification, this trial C studied if the current biomass concentration was ideal or if it was otherwise saturated for CPC extraction. In an extraction procedure, it is of the utmost importance to ensure that the solvent is not saturated, to guarantee a complete extraction. Consequently, trial C was performed in order to better assess this issue. In each test Arthorspira from the company's laboratory was harvested and resuspended into three different concentrations: 2C g/L the current concentration from the previous protocol, 1,3C g/L similar concentration to the biomass culture from the laboratory at the time of testing, and finally C g/L being half the current concentration used. The tree samples plus duplicates after resuspension were milled with beads for 20 min, then centrifuged by type A followed by type B centrifugation. As the available Eppendorf centrifuge has 12 slots, waiting samples were stored in dark to prevent light deterioration (Figure 16).

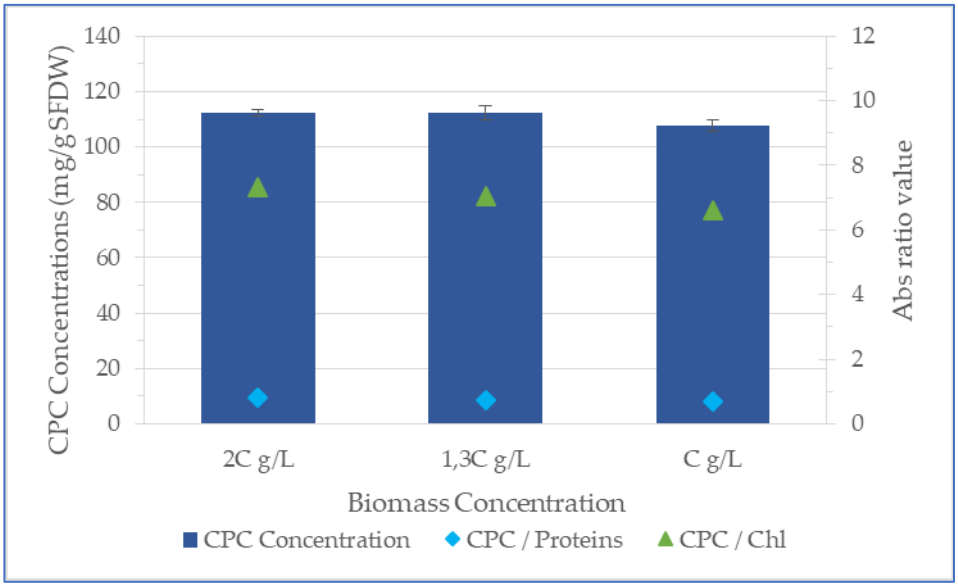

Figure 16 - CPC Quantification - Different concentrations: 2C g/L vs 1,3C g/L vs C g/L

It seems that the concentration of the biomass does not influence the purity ratios as proteins appear to be extracted similarly in either sample. Although 2C g/L shows a slightly better ratio of phycocyanin vs proteins (0,1 higher than its counterparts) it falls into the reading error. The ratio (CPC/Protein) obtained in this trial is considered as fit to enter the Food Grade [111] category as far as the phycocyanin. For CPC/Chl ratio, although it appears that the lower concentration (C g/L) extracts more Chl than the other two samples being the lower purity ratio from the three samples, this lower ratio may be a consequence of the lower value in CPC contents. Finally, the CPC value is comparable in both 2C g/L and 1,3C g/L, at 112,47 mg/g AFDW and 112,52 mg/g AFDW respectively, and 4,2% lower in the C g/L, at 107,76 mg/g AFDW. As these differences fall into the error range it is not possible to find a connection between the biomass concentration and an improvement in the extraction overall efficiency. As there was no clear relation between concentration and efficiency, and as procedure-

wise, the dilution of biomass leads to an increase in the total volume to be processed, plus the intention of getting results in a reasonable timeframe, in the following trials, along with the company, the concentration of 2C g/L was chosen as default.

3.1.4. Trial D - Number of Resuspensions

When centrifuging microalgae, as it is already the company's knowledge, the main component by weight in the sediment/pellet is the medium/solvent as humidity, being 80% at the most concentrated samples. In this case after milling the intracellular components are extracted into the potassium phosphate buffer solution (acting as a solvent for the extraction). After centrifugation, some of these intracellular components will be trapped in the pellet in its humidity. To prevent this entrapment, this trial studied the possibility of resuspension of the centrifuged pellets. In this trial the pellet from the type-A centrifuge cycle was resuspended three times and read, each time homogenized in a vortex for the "milling period", sediment from the type-B centrifuge cycle was also resuspended. Procedure-wise after a type A centrifuge it wasn't possible to remove all the liquid/supernatant from the test tube without removing the pellet, in order to prevent this artifact in later data sorting the exact volume added and removed each time was registered and its interference eliminated in the calculations (e.g., if 5,1 mL were added and only 4,1 mL were removed in later calculations 1mL at the concentration of the initial sample was ought to be removed (Figure 17).

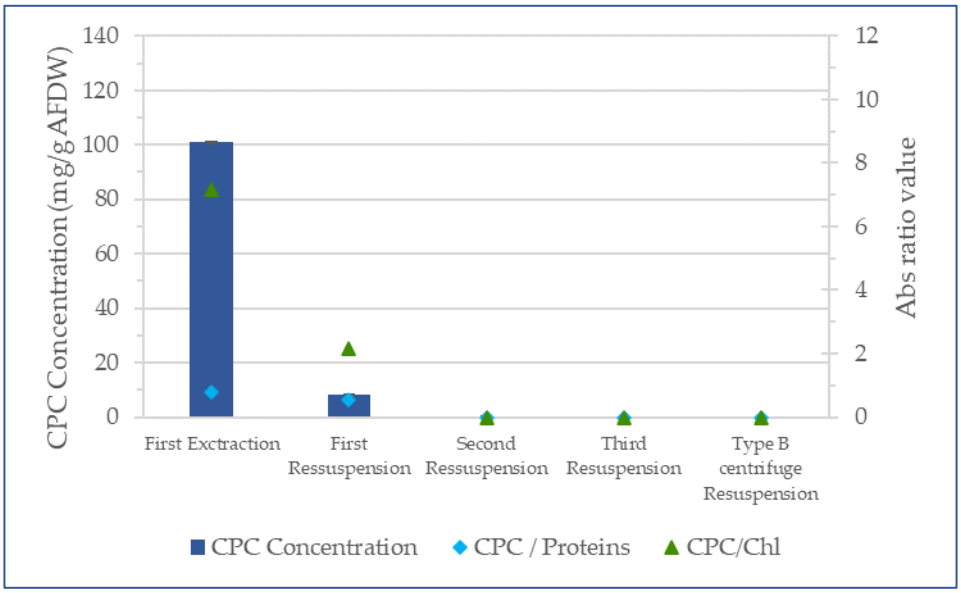

Figure 17 -CPC Quantification - No. Resuspensions

The first extraction showed comparable values to the other trials as far as the ratios go, the protein ratio averaged 0,8 (0,1 above the minimum limit for the Food Grade), regarding the Chl contamination as it appears the ratio is similar to previous trials, being acceptable and denoting reliable results. [111] The first extraction was able to extract 92,6% of the total phycocyanin. The remaining 7,4% were extracted in the first resuspension, this value carries a degree of uncertainty linked to the lower CPC/Chl ratio. This decrease in purity from the

extract was observed in all tests from this trial. The fact that this sample (resuspended) presents a lower Chl ratio may be related to either a lower CPC content and a similar Chl or the fact that the biomass sample experiences double the time in the vortex, possibly disintegrating photosynthetic complexes, and thus releasing more chlorophyll. The following extractions (2nd, 3rd, and the resuspension from the MiniSpin pellet) averaged either zero or approximately zero milligrams of CPC per gram of ash-free dry weight of fresh biomass. Consequently, none of the ratios were calculated for either sample. This trial suggests that for a complete extraction it is important to resuspend once each pellet after a type-A centrifuge, although this procedure may lead to uncertainty in the total value. From this trial forward as an indication from the company, all tests were performed with one resuspension after the first extraction.

3.1.5. Trial E - Extraction Period

Trial E studied the influence of the period of extraction on the total CPC content extracted. This trial was initially performed with 15min increments and later was repeated for longer periods. In order to ensure a complete extraction, the period of contact between the solvent and the microalgae may play a crucial role in the overall process efficiency. As in the previous trials, Arthorspira from the company's lab was harvested, resuspended at 2C g/L, and milled as described in the previous protocol.

The following Figure 18 illustrates the variation in the CPC contents, proteins, and Chl ratios.

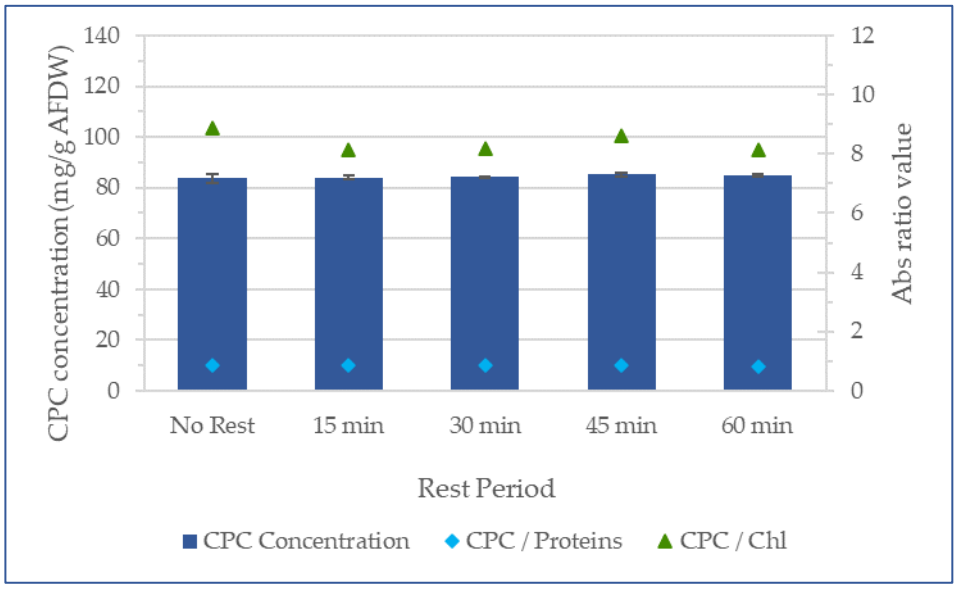

Figure 18 - CPC Quantification - Extraction Time 0-60 min

The proteins ratio does not vary significantly across the studied timeframe; thus, it is possible to assert that for this time frame there does not seem to exist an influence from the extraction period on the extraction effectiveness for proteins. The minimum value for CPC/Proteins ratio in this trial averaged 0,84 (~0,8) from the 60 min sample being fit to be labeled as Food Grade. [111] There was a decimal variation on the Chl ratio (Δ=0,8) across the maximum and minimum values in this trial. This ratio does not appear to have a particular pattern as the highest value is reached by the non-rested sample, a second maximum at 45min,

and the lowest value at both 15 min and 60 min periods. The best value of CPC/Chl ratio may be responsible for the lower phycocyanin value in this trial, since having a higher phycocyanin ratio means having less chlorophyll and therefore less influence on phycocyanin quantification.

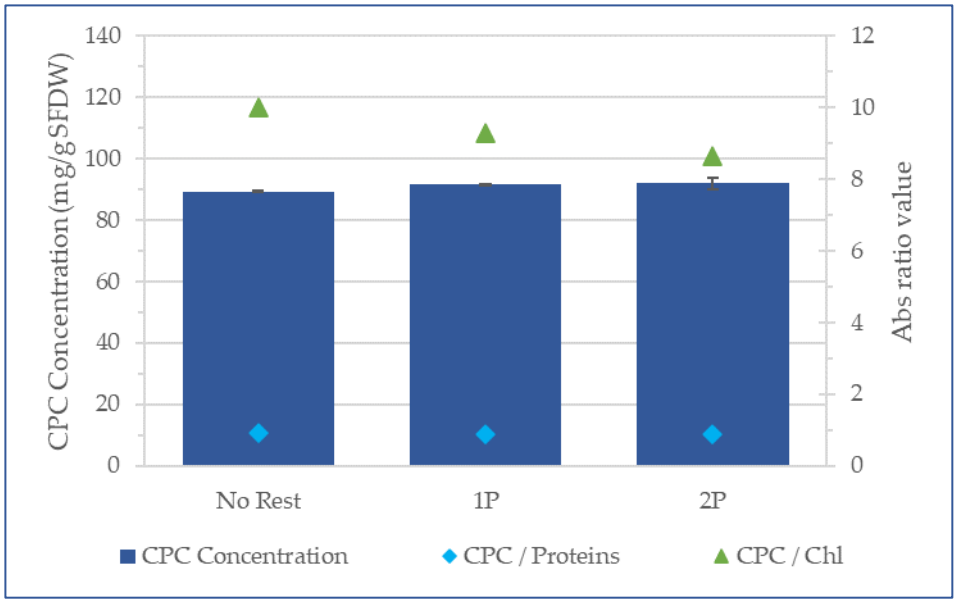

Figure 19 - CPC Quantification - Extraction Time 0-120 min

As there were no major differences, in the studied timeframe, either for any of the ratios or CPC values the timeframe was extended with 1-hour increments. Figure 19 illustrates the variation across the three periods (No rest; 1P rest; and 2P rest).

This expansion in the timeframe does not seem to impact the extraction of the overall protein content, as the ratio of CPC to proteins is approximately constant throughout this trial at 0,9. The highest CPC/Chl value was achieved in the "No- rest" at 10,0. It appears to exist a descending trend in the chlorophyll ratio with the extending of the period of extraction after milling. As far as phycocyanin contents it seems that the value is approximately constant in the three tested periods, not existing a direct connection to the extraction. As there is no visible advantage of extending the extraction period after milling over the current period, along with the company it was determined to dispense the resting period. Although, in a later trial where the temperature influence is studied, it was necessary to put the samples through a warm bath, in that specific case with was necessary to have a resting period to ensure that the whole sample was at the desired temperature, thus as a resting period of 1 P was chosen as it did not show major setbacks.

3.1.6. Trial F - Temperature's influence on CPC quantification

As it was the company's knowledge the increase in temperature might improve the extraction, this trial aimed to study the influence of the increase in temperature on the CPC extraction. The chosen temperature was "T- 10" ºC as the company already knew that Arthorspira would appear burnt at "T+20" ºC, thus a "T- 20" ºC safe range was chosen by indication from the company. In this trial, Arthorspira from the company's laboratory was harvested, resuspended at 2C g/L in potassium phosphate buffer, and milled. The milled sample was divided into four tubes, two rested for 1 hour in a dark environment at room temperature (RT) the remaining tubes were placed in a warm bath at "T-10" ºC covered from the light in both cases this procedure was performed to avoid any light deterioration (Figure 20).

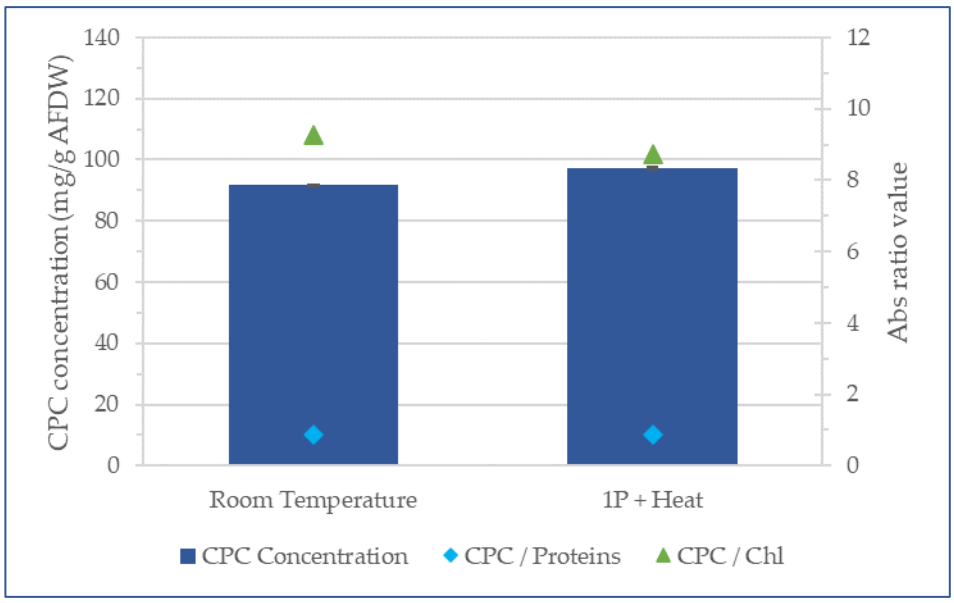

Figure 20 - CPC Quantification - Temperature: RT vs "T-10" ºC

In this trial, the temperature does not seem to influence the CPC/Proteins ratio as both averages a similar value. These values are comparable with the obtained values in previous trials as averaging 0,9 in the proteins' ratio. Chlorophyll ratio was on average slightly worse in the heated sample, being 9,3 de ratio from de RT sample and 8,7 the heated. The difference in these ratios can be caused by a greater propensity to extract chlorophyll with heat. This ratio alone is not enough to assertively assess that temperature will extract more chlorophyll as the difference between the RT and the T40. Additionally, the fact that the heated sample has a worse ratio and displays a higher phycocyanin content may suggest that chlorophyll is either overestimating partially the upset on the total content of phycocyanin or even it is responsible for the entire difference of 5,67 mg/g AFDW. This trend appeared in various tests for the quantification and will appear again later in the Thermal Treatment chapter.

3.1.7. Trial G - The influence of the milling time

After the harvesting stage, optical density reading, concentration step, and resuspension it is necessary to rupture the biomass for later extraction. [90]–[92] The importance of the milling period lies in the fact that a sample that is not properly milled will not have a maximum extraction efficiency, on the other hand, if the cell is disintegrated and the photosynthetic complexes destroyed there will be an increased release of chlorophyll that will influence the subsequent readings of CPC. In addition, due to the procedure used (milling in test tubes), the increase in milling time without rest combined with the absence of a cooling system may lead to an increase in the internal temperature of the test tube and, consequently, of the biomass. As for indication from the company, the optimal period for milling would range from 20 min to 40 min. As Arthorspira milling at laboratory scale had already been the subject of study a period under 20 min was already rejected. Trial G investigated the effect of milling time with incremental steps of 7,5 minutes after the initial milling time of 20 minutes (Figure 21).

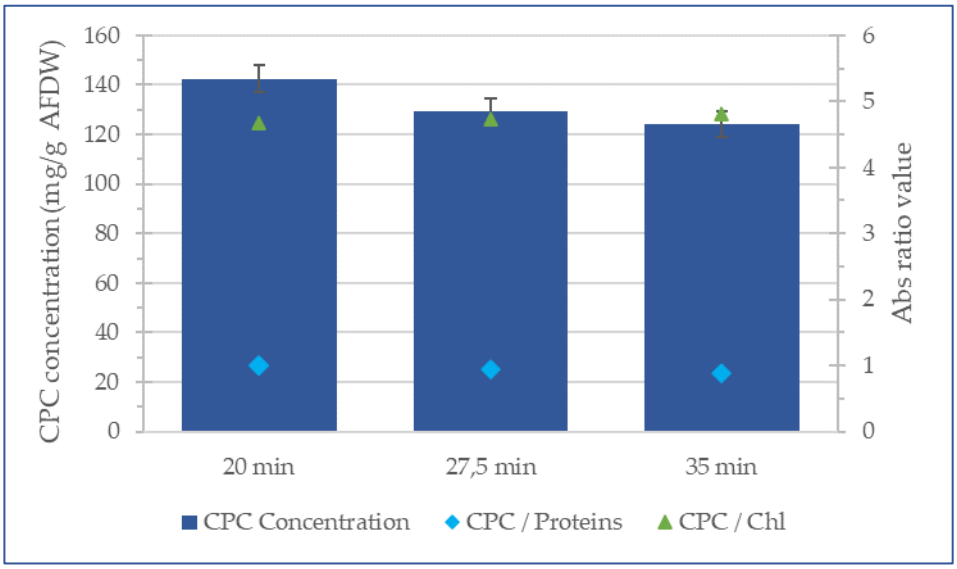

Figure 21 - CPC Quantification - Milling Cycle 20 min 27,5 min vs 35 min

In this trial the proteins' ratio was mostly constant with the variation falling in the reading error, it averaged 0,99 (~1,0) for 20 min, 0,94 (~0,9) for 27,5 min, and finally 0,88 (~0,9) for the longer milling period, 35min. As it seems the extended time of milling does not influence the extraction of either CPC or other proteins in ratio. Although this indicator alone cannot be used for a global assessment, it is possible to infer that any variation in the extraction would happen proportionally between the two. The results as far as this ratio go allow any of these extracts to be classified as Food Grade. [111] The chlorophyll ratio maintained a constant value across the studied timeframe averaging 4,67 (~4,7) for the default period (20 min), 4,74 (~4,7) for the 27,5 min period, and finally 4,81 (~4,8) for the 35min milling. The values of these ratios seem to imply that the milling is complete in the default time (20 min), since the ratios remain approximately constant it is possible to infer that there is no increase in the degree of chlorophyll release with the increase of the milling time. As far as the CPC values it appears that the increase in milling time is linked to the degradation of this pigment. Phycocyanin value decrease with the extending of milling time. Although in this trial CPC values might have been

overvalued by the presence of chlorophyll as the separation was not efficient. Since CPC/Chl ratios are equated in the various samples, even though CPC values are overvalued, they are relatively comparable to each other in this trial.

The default milling period averaged 142,4 mg/g AFDW being the highest value from the three studied periods, the second period decreased 9,3% to 129,2 mg/g AFDW, finally, the 35 min milling period averaged 124,2 mg/g AFDW, dropping 12,8% when compared to the default time. There seems to be a clear descending trend with the increase in milling time. Given these results, the company opted to use 20min milling as the optimal procedure.

3.1.8. Trial H - Influence of pH

As it has already been studied phycocyanin stabilization is deeply linked to pH stability, being even possible to stabilize CPC extracts in higher temperatures by decreasing the pH value to a more acidic environment. [75], [76], [118] In order to study this influence of pH and the effect of a buffer solution it was compared the extraction and reading effectiveness of potassium phosphate buffer (Potassium Phosphate Buffer with Deionized Water; pH=6,9), Deionized Water, Acidified Medium (Culture Medium + Nitric Acid; pH=6,9), and a "Scalable Buffer" (Potassium Phosphate Buffer salts diluted in Culture Medium; pH=6,9). As per the company's indication, if needed to decrease the culture medium pH value, nitric acid would be the choice as it is food-grade resulting in a marketable product. The extraction with buffer is the most commonly used as described by Doke. [119]

In case of the acidic medium shows positive results, it might be an indicator for the industrial process as there would not be a need for "solvent" replacement after the centrifugation step, this topic will be further elaborated ahead in the Thermal Treatment chapter. In this trial, all samples were harvested by type-A centrifugation, and its dry weight was measured by a humidity scale for dilution to de desired concentration in buffer (Potassium Phosphate buffer; pH=6,9), Deionized Water, Acidified Medium (Culture Medium + Nitric Acid; pH=6,9), and a "Scalable Buffer" (Potassium Phosphate Buffer salts diluted in Culture Medium; pH=6,9).

The following Figure 22 demonstrates the results from the four tested diluters.

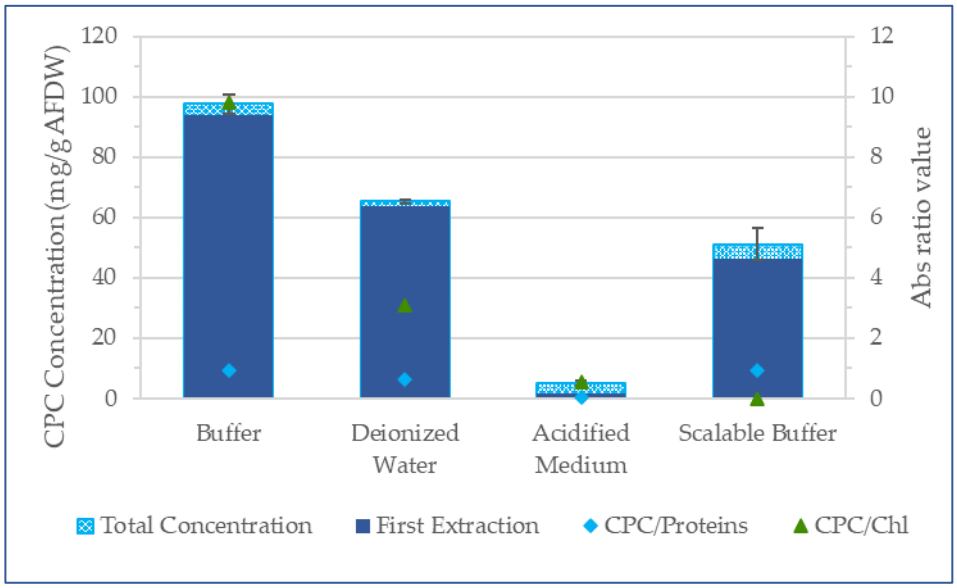

Figure 22 - CPC Quantification - pH/Buffer Influence - Potassium Phosphate Buffer vs Deionized Water vs Acidified Medium Scalable Buffer

In this trial it is visible a variation in both ratios, denoting possible significant differences in the four tested solvents. The proteins' ratio ranged from 0,02 (~0,0) in the Acidified Medium sample and 0,934 in the Scalable Buffer. As per the various trials, only the Buffer and the Scalable Buffer showed Food Grade values from the proteins' ratio. This seems to suggest a positive interference by the Buffer in the CPC/protein separation as both samples with the presence of those salts averaged the better results.

As far as CPC/Chl it averaged 9,8 on the Buffer sample, 3,1 on the Deionized Water, 0,6 in the Acidified Medium, and lastly in the Scalable Buffer there was no reading at the 430 nm wavelength in any of the tests, thus this ratio will be disregarded for this sample. Among the remaining samples, only the Buffer sample displayed high purity from CPC/Chl, indicating reliable and reproducible results. The second-best option when it comes to purity was deionized water, although its value averaged just one-third of the buffer sample. Acidified Medium did not present satisfactory results as it averaged one-tenth of the best result.

As far as phycocyanin concentration had its maximum in the buffer sample with 97,5 mg per gram of biomass (AFDW), and most of its content in CPC was obtained in the first extraction (~96,5%). Deionized water and scalable buffer averaged comparable values at 65,3 mg/g AFDW and 51,1 mg/g AFDW respectively, corresponding to drops of 33,1%, and 47.6%when compared to the buffer sample. Finally, the acidified medium did not perform either comparable or satisfactory results averaging 5,19 mg/g AFDW (94,8% drop when compared with the buffer sample). It seems to exist a positive influence of both the buffer salts and de deionized water as when comparing both buffers the scalable buffer averaged the worst result in almost all categories (CPC value, CPC/Chl ratio), having a similar value in the CPC/Proteins ratio (both 0,9 in absorbance ratio). When comparing the two samples using deionized water, were the two better options in the majority of the categories. Among the two, the sample labeled as "buffer" had the better result across all categories.

As this trial studied the buffer prepared with deionized water presented the best results as there does not appear to exist a viable alternative among the studied diluters. After discussion with the company, it was decided to use potassium phosphate buffer (0,1 M, pH=6,9) as default in the Quantification Protocol.

3.1.9. Parameters Summary

These eight trials aimed to, firstly, correct the available protocol to obtain more accurate, reliable, and reproducible results. As per company indication, this renewed method was meant to extract the most phycocyanin with the most reliable values, however, it should not be so extensive that it would take too long to achieve results after beginning the process. If needed to be, some compromises would be made in order to better balance the accuracy/time spent. The first stage (correction) was achieved after trials A and B (with B being the confirmation). As it appears the previous protocol was deficient in centrifugation for biomass, possibly due to different behavior in centrifugation of dried and fresh biomass. Although, Figure 15 shows a poorer improvement after 1P min, as CPC/Chl is the main indicator of reliability it is of the utmost importance to maximize this indicator, so the longer period of centrifugation was chosen.

Trial C initiated the improvement/optimization step as it was meant to extract most of the available CPC, as it was shown in Figure 16 at the current concentration, there was no saturation as there was no improvement in the extraction was observed in either of the diluted biomass. Trial D aimed to study the number of resuspensions needed for complete extraction. In the previous protocol, there were no references to performing a second extraction, as Figure 17 illustrates there is gain in resuspending the tested sample. Through various tests across different trials, the increment in concentration extracted ranged from 5 to 10% in the second resuspension. The following resuspensions were revealed to be ineffective as there were no gains in CPC extraction. This second extraction lead to a gain in concentration up to 10% of the total value, increased the operating time in of 1h and 20 min (between milling and centrifuging) plus transfers and reading.

Trial E did not present significant variations on the CPC contents, as the mean value was 84,52 mg/g AFDW and the furthest value at 83,75 mg/g AFDW a difference of 0,9%. With no significant improvement in any of the tried periods the default period "no rest" was maintained as the optimal, being the only one that would not increase the operating time, thus leading to faster results. Trial F aimed to study the influence of heat in the extraction process. Having comparable CPC/Chl values, both phycocyanin concentrations would have similar reliability. In this trial, the heated sample showed the best results as present Figure 20. The heated sample showed a 5,8% improvement when compared with the non-heated one. In discussion with the company, it was decided to neglect this improvement as it would be overshadowed by the improvement already made with the resuspension. If this procedure would have been incorporated in the protocol it would delay the results by two hours as both the first and second extraction would be heated for 1 hour after milling.

Trial G investigated the influence of the milling time. In this trial, the main objective was to analyze the degree of rupture, plus the possible overheating at longer milling periods.

As previously there were already tested shorter milling periods that option was not tested. In the tested periods it was beneficial to use the shorter period as that sample demonstrated the best values with both alternatives losing in average 13,2 and 18,2 mg/g AFDW, respectively. With lower CPC and comparable purity ratios, there was no improvement in increasing the milling time further than 20 min.

The last trial (Trial H) tested four different "solvents" in this case the word solvent is loosely used to describe the liquids used in the dilution of the biomass to the desired concentration. The option that would be economically cheaper did not present satisfactory results in any indicator. As it was later tested if an extraction with this solvent and then a second extraction with buffer, however, it seems that something in the culture medium degrades phycocyanin, since this second extraction, with buffer, could not extract more CPC than the initial attempt with culture medium. Both deionized water and "scalable medium" presented comparable results with 14 mg/g AFDW of difference between them. The best result was achieved with the buffer (prepared with deionized water) with better results than the latter two across all indicators.

The following Table 2 summarizes the chosen parameters/optimizations chosen in the development of the Phycocyanin Quantification Protocol.

Table 2 - Optimized Parameters for PQP

Chosen Parameters for Phycocyanin Quantification Protocol (PQP)						
Centrifugation period	Concentration	No. Resuspension	Extraction Period	Temperature	Milling Period	pH
50 min	2C g/L	1	0 min Rest	No heating	20 min	6,9 w/ buffer

3.1.10. Phycocyanin Quantification Protocol Description

For this new method starting with culture, firstly the biomass concentration should be calculated by measuring its optical density. Centrifuge the needed volume for an equivalent of 7,5 mg of AFDW (as the volume depends on the culture concentration) for 5 min at 14500 rpm. After separation, the culture medium should be discarded while resuspending the pellets into 5 mL phosphate buffer, 0.1 M, pH 6.9. To ensure that all the biomass is recovered, “rinsing" the Eppendorfs with the buffer itself may be needed. As CPC is protected by membranes inside the Arthrospira cell a rupture step is needed for the extraction. As such the equivalent of 2.5 mL of beads to the glass tube and vortex (Vortex Genie2) is for a period of 20 minutes, full speed, to promote cellular disruption. Transfer the entire contents of the glass tube to a 15 mL Falcon, including beads, liquid, and foam (formed during cell disruption). These Falcon tubes should be centrifuged for 10 min at 4200 rpm at 4 ºC to better separate all the cellular debris from the supernatant. From here forward these conditions in this centrifuge will be simply referred to as type A centrifuge. From the centrifuged supernatant transfer 3 mL to Eppendorfs (1 mL per Eppendorf). Only the liquid on top of the beads should be collected and the volume quantified in a test piece, like the rest of the supernatant (present in the interstices of the beads may contain cellular debris. This step is extremely important as the

debris/pellet will be resuspended later for the second extraction. Centrifuge the eppendorfs in the MiniSpin Plus from Eppendorf for 50 min at 14500 rpm, from here forward these conditions in this centrifuge will be simply referred to as type B centrifuge. Then analyze the pigments in the spectrophotometer (Genesys 10S UV-VIS). The CPC concentration (mg/ml) of the samples submitted to the spectrophotometer is determined from the sample absorbance at 620 and 652 nm according to Equation 5 as studied by Bennett e Bogorad (1973) [110]:

Equation 5 - CPC concentration (mg/mL)

$$PC = \frac{A_{620} - 0{,}474 * (A_{652})}{5{,}342}$$

Where,
PC: CPC Concentration (mg/mL).
A_{620}: Maximum absorbance for CPC.
A_{652}: Maximum absorbance for APC.

To ensure that all CPC is quantified a second extraction is performed, resuspending the cellular pellet and the volume of liquid present in the beads in 5 mL buffer phosphate, 0.1 M, pH 6.9. Although the first extraction usually presents a higher CPC concentration the amount of liquid present in the bead's interstices will not interfere with the value of the second extraction as its CPC value is discarded in the data treatment. Shake Falcon in the vortex for 1 minute to both promote resuspension and homogenization from the sample and then transfer the entire contents of the 15 mL, Falcon, into a glass tube, including the beads. Vortex the glass tubes for 20 min., at maximum speed, for a full cell rupture. Once again centrifuge for 10 min., 4200 rpm, 4°C, in the refrigerated centrifuge, to sediment most cellular debris. The following steps are similar to the first extraction.

The CPC concentration (mg/ml) of the samples submitted to the spectrophotometer is determined from the absorbances reading at the wavelength of 620 nm and 652 nm according to Equations 6Equation 6, 7, and 8. The concentration of CPC in the initial biomass is obtained by combining the amount of CPC recovered after the first extraction step and after pellet resuspension (2nd extraction).

Equation 6 - CPC Concentration % (w/w)

$$CPC_{AFDW}(\%) = \frac{mCPC_{(1st\ extraction)} + mCPC_{(2nd\ extraction)}}{m_{Spirulina(AFDW)}} * 100$$

Equation 7 - Auxiliary equation for Equation 5

$$mCPC_{(1st\ extraction)} = CS_{CPC(1st\ extraction)} * V_{buffer(1st\ extraction)}$$

Equation 8 - CPC Extracted from resuspension

$$mCPC_{(2nd\ extraction)} = CS_{CPC(2nd\ extraction)} * \left(V_{beads} + V_{Buffer(2nd\ extraction)}\right) - CS_{CPC(1st\ extraction)} * V_{beads}$$

Where,
CPC_{AFDW} (%): percentage (mg/mg) of CPC per milligram of AFDW of a given sample (mg/mg);
$mCPC_{1st\ extraction}$: mass (mg) of CPC extracted in the first step (mg);
$mCPC_{2st\ extraction}$: mass (mg) of CPC extracted in the second/resuspension step (mg);
$m_{Arthrospira}$: the Ash-free dry weight mass of the sample (mg);
$CS_{CPC\ 1st\ extraction}$: Concentration in the supernatant from the first extraction (mg/ml);
$V_{buffer\ 1st\ extraction}$: Volume of buffer used in the first extraction (mL);
$CS_{CPC\ 2nd\ extraction}$: Concentration in the supernatant from the second extraction (mg/mL);
V_{beads} : Volume of supernatant non removed from the first extraction (interstitial volume) (mL);
$V_{buffer\ 2nd\ extraction}$: Volume of buffer used in the second extraction (mL).

For the development of this method, the following variables were tested:

- Centrifugation cycles;
- The initial state (fresh vs dried);
- Variation of temperature;
- A variation on the pH;
- A variation on the extraction time;
- Variation of biomass concentration;
- A variation on the bead to total volume ratio;
- Number of resuspensions;
- Milling time.

In addition to the CPC quantification, supplementary tests were also performed, including CPC purity (vs other protein) and contamination by chlorophyll.

3.1.10.1. Phycocyanin / Total Proteins ratio

As the ratio CPC per total protein is of interest in order to establish a grade of purity, whenever the quantification is performed it is also calculated.[111] Commonly this ratio is calculated as shown in Equation 9:[112]

Equation 9 - CPC / Protein ratio

$$\frac{CPC}{TP} = \frac{A_{620}}{A_{280}}$$

Where,
TP: Total Proteins
A_{280}: Maximum absorbance for proteins.

However, this method involves some imprecision as it does not distinguish between both CPC and APC, in addition, the wavelength at maximum absorption is influenced by the aggregation state, and hence by pH, as stated by MacColl, 1998. [78]

3.1.10.2. Phycocyanin-chlorophyll ratio

Although a-chlorophyll's maximum used for calculations is at 430 nm it also absorbs at 620 nm, which is the maximum for CPC. [113] To calculate this ratio, it was chosen chlorophylls' farther peak from the phycocyanin's peak, giving a more accurate reading on the "pure" chlorophyll reading. As at this time it is not possible to determine the exact influence of the chlorophyll on the CPC reading, this ratio intends to assess the degree of influence on the readings and accuracy on the value obtained for the CPC quantification. This ratio is calculated according to Equation 10, which illustrates the pigment's spectra for auxiliary information regarding each peak.

Equation 10 - CPC / Chlorophyll ratio

$$\frac{PC}{Chl} = \frac{A_{620}}{A_{430}}$$

Where,
Chl: chlorophyll;
A_{430}: Maximum Absorbance for chlorophyll.

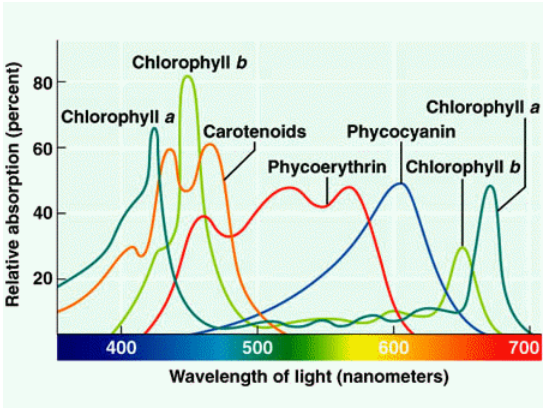

Figure 23 - Pigments Spectra [113]

3.2. Method development for a thermal treatment for Arthrospira platensis

The heat treatment in the case of Arthrospira has been proposed to be used as a stabilizing method to avoid biomass degradation during the industrial process in case of a stoppage. In this sense, it would be necessary to stabilize the biomass for a period ranging between 24 to 72 h. In terms of processing capability in the company, two scales stand out, pilot-scale and industrial scale. The heat treatment proposed in this chapter was developed so that it could be applied to the pilot-scale with the equipment present in the industrial complex. The company's indications were that pilot equipment would have more restrictions than industrial equipment, so a model developed for the pilot-scale could be scaled for industrial equipment.

Although pasteurization to Arthrospira was not performed, the HTST and LTLT Pasteurization models were equated as a way to provide this treatment. The method used was based on the method described by Dumalisile for long pasteurization at low temperatures, since as already known by the company Arthrospira does not tolerate high temperatures appearing burned at 70ºC. [37], [120] Given the restrictions in the pilot equipment, the following premises were deemed as mandatory for the acceptance of the proposed treatment method. In addition to the need to stabilize biomass for the defined period, it would also be necessary to comply with the following criteria for the treatment ramp, heating should be restricted to 1,5 ºC/min and cooling between 2 ºC/min and 3 ºC/min.

3.2.1. Thermal Treatment - Laboratory trials

3.2.1.1. Trial 0 - Hydraulic testing

In these tests both heating and cooling speed were tested to emulate the pilot pasteurizer existing on the industrial site. As for simplification, a Greek letter was given to each ramp, resulting in the following Table 3, with the results plotted in Figure 24.

Table 3 - Tests description and corresponding letters

Letter	Description
α	Air Cooled Sample
β	Sample Cooled in a chilled water bath (4 ºC)
γ	Sample chilled in three progressively colder baths
δ	Samples chilled in the bath, with surrounding water being replaced.

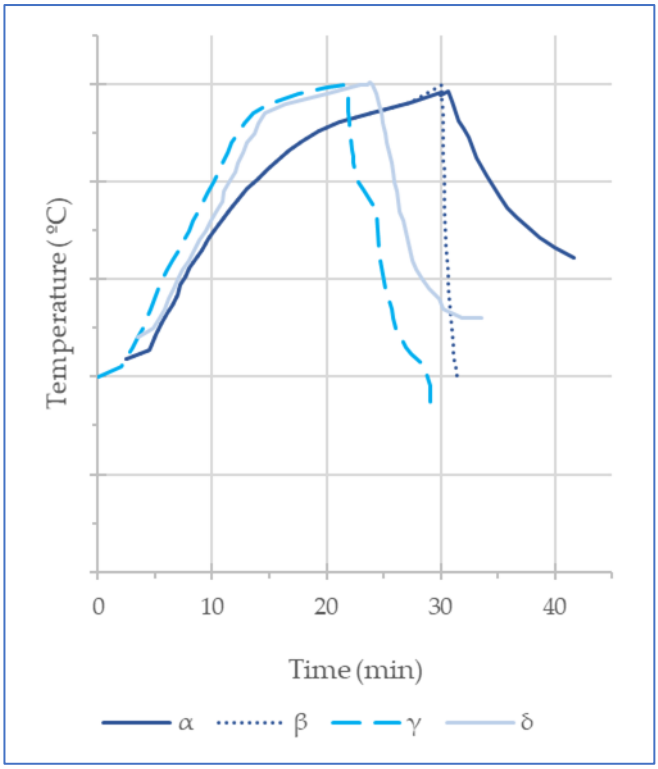

Figure 24 - Thermal Treatment - Hydraulic Testing

As the bath used is regulated only according to the chosen temperature there is no possibility to control the temperature rise speed. The thermostat present in the bath controls the power supply to avoid a possible overshoot. In order to draw the temperatures-plot above, a thermocouple was used in order not manually register the temperatures at 30-second intervals. The alpha ramp was the first to be tested with heating with a "T" ºC set point (intended temperature) and air cooling. In this case, after reaching the desired temperature, the tube holder was removed from the bath and placed on a tray.

In this trial inefficient heating was observed, not complying with the premises for correct emulation. Two stages of heating are denoted, in the first, from the initial temperature to the "T- 10" ºC there is faster heating, which loses intensity as the temperature approaches the setpoint. As the graphic above shows, in this case, the cooling was quite slow, not fulfilling the premises for the emulation of the pilot pasteurizer. In addition, this cooling process is conditioned by the temperature of the room. This implies not only an increasing slowness when approaching the value of room temperature but a cooling ceiling (the temperature of the room).

As the previous test showed an insufficient cooling speed the following test studied the cooling speed in an iced bath. This test is based on the assumption that if the tubes were put in a sufficiently larger cooling mass, then the thermal equilibrium would be reached quicker and at a similar temperature to the cooling body. This test, similar to the previous one, demonstrated warming with two distinct phases. The first phase, from the initial temperature to "T-10" ºC showed faster heating, after "T-10" ºC the bath control systems regulated the power supply to avoid an overshoot, resulting in a decrease in the heating speed.

In order to correct the ineffective heating found in the two previous tests, a battery of tests was conducted resulting in the correction factor "a" that was used in the following tests (e.g., if the desired temperature is "T" ºC then the bath should be set at Ta ºC and cut the heating at T-a ºC). This correction proved effective during the remaining tests.

In the gamma test, an intermediate between alpha and beta testing was attempted. In this test, the heating was performed with the temperature correction (setpoint at Ta ºC), and the cooling was done in three stages. In the first stage, cooling water at 20 ºC was used to avoid too fast cooling that would not be reproducible in the pilot pasteurizer. After remaining in this primary cooling bath, the tube holder would move to a second bath at 10 ºC. After staying in this bath, the support would be transferred to a bath at 4 ºC where would finish the ramp with stabilization of the temperature.

The heating in this trial was effective and showed a reproducible heating speed with the existing pilot equipment. The cooling stage has achieved an average speed that met the premises, being accepted. It is possible to observe the formation of plateaus during this stage, indicative of the possibility of further optimization. A possibility for the reduction of these plateaus would be the exchange of baths at higher temperatures, in order to avoid this flattening of the curves present in the graph.

The delta test was an attempt to optimize the plateaus present in the previous test. In this test, instead of passing the tube holder between several containers with refrigerant at different temperatures, it was decided to pass water at two temperatures through the hot bath equipment. Thus, considering a minimum transfer at 10 ºC difference, from "T" ºC to "T-20" ºC, tap water would be delivered for cooling the bath. From "T- 20" ºC to the final temperature, water at 4 ºC would be delivered from a previously filled Jerry can. Using the heating bath as the coolant tank eliminates a possible logistical problem since there is no physical transfer of the tube holder. There is only transfer of the cooling fluid, on one side, water delivery via a hose, and on the other hand water removal by a pump. This setup is illustrated by the following Figure 25.

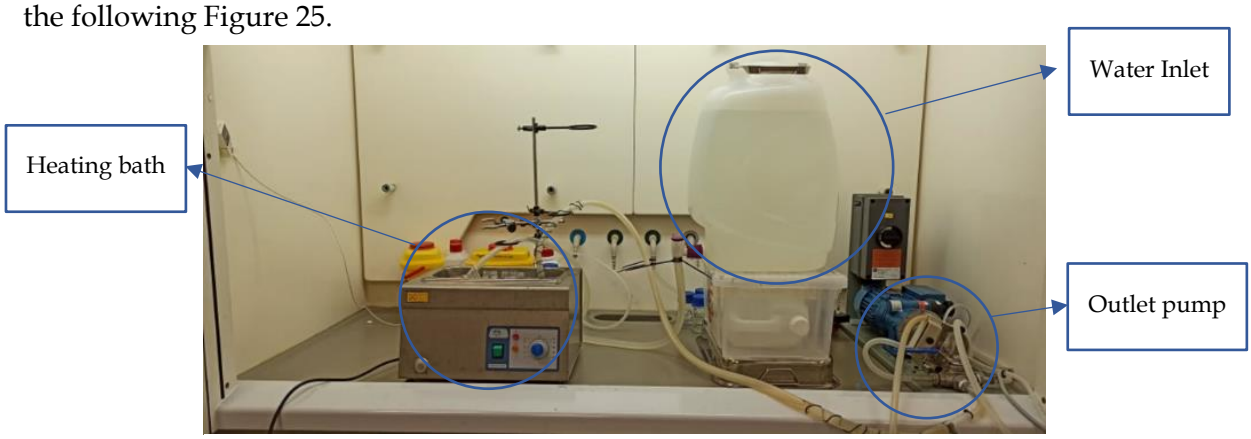

Figure 25 - Thermal Treatment Setup

This test showed acceptable values for both heating and cooling. When compared to the previous trial, the plateaus were successfully removed, the logistical problems were also removed in the vast majority. Two new logistical problems appeared with this test since the water at 4ºC (approximately 25 L of water for a 5 L bath and 12 sample tubes) would have to be stored in a refrigeration chamber during the previous night at least, occupying plenty of

space. Despite the thermal inertia associated with large volumes, the waiting during the heating stage sometimes resulted in heating of the refrigerant and consequently a decrease in cooling power.

After discussion with the company and presentation of the possible proposals, the delta test was accepted and assumed as the procedure to be followed thereafter.

3.2.1.2. Laboratory Testing - List of trials

Heat treatment was carried out before the milling stage, and after one concentration step, treating intact and concentrated biomass. In these laboratory tests, the influence of temperature, treatment efficiency, biomass conservation at different temperatures and storage times, fluid for biomass dilution, and cellular integrity was studied and compared (Table 4).

Table 4 - Thermal Treatment - Trial Overview

Trial	Variables				
	Temperature (ºC)	Effectiveness (%)	Conservation at extended periods	Solvent	Cell Integrity
1	✓				
2				✓	
3		✓			
4	✓	✓	✓		
5	✓	✓			✓
6	✓	✓			✓

3.2.1.3. Trial 1 - Temperature influence on the thermal treatment - "T" ºC vs "T+10" ºC

It was already known by the company that the biomass of Arthrospira had a burnt appearance when subjected to temperatures of "T+20" ºC, additionally, the literature refers to the fragility of the CPC when exposed to temperatures above "T+10" ºC. [37], [75], [76], [121] Thus, assuming a safety margin of 10 ºC, tests at "T" ºC and "T+10" ºC were proposed using/adapting the delta ramp for each test. In these tests, the CPC variation was studied both after treatment and after 48 hours of storage. The variation at day 0 (day of treatment) would show the impact of temperature on the CPC concentration, with an indication of the limit temperature for CPC destruction, on the other hand reading these samples after 48 hours would reveal the effectiveness of this treatment as a way to preserve CPC.

Performing the treatment at "T" ºC represents the safest alternative to biomass, being the lowest temperature, if there is thermal destruction of CPC at "T" ºC then it will also be destruction at any higher temperature. In this test (Figure 26), the delta temperature ramp was followed, with the addition of a cooling step after the end of the heat treatment. This addition does not alter the ramp, since after the use of the cooling water the tubes holder is placed in the refrigerator at 4 ºC.

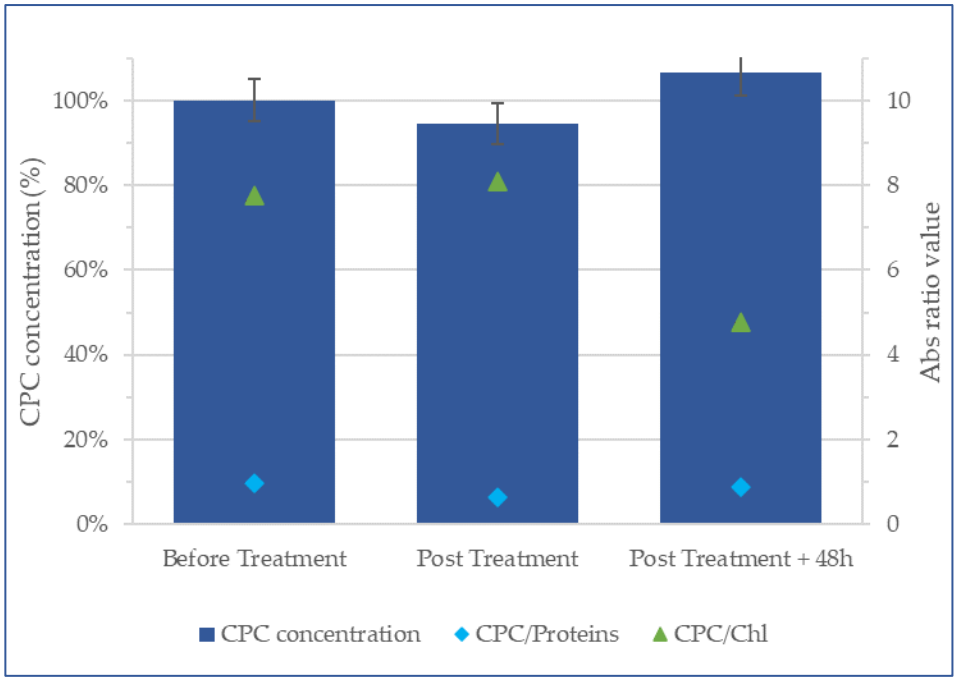

Figure 26 - Thermal Treatment - T="T" ºC

The test at "T" ºC appears to be influenced differently by both purity ratios. The protein ratio averaged 0,98 for the initial sample and 0,65 for the thermally treated sample, although this variation is slightly over the margin of error it should be taken just as an indication for the relative purity. Chlorophyll ratio averaged 6,65 and 6,73 respectively, showing no significant variation. There seems to have been a loss of 6% in the sample after heat treatment, however, this variation is contained in the margin of error, so it seems in general that the heat treatment performed at "T" ºC, on average, does not negatively impact the phycocyanin value. Comparing with the results obtained in the development of the Phycocyanin Quantification Protocol, where the sample subjected to heating ("T-10" ºC) had about 10% more than the initial sample, there may have been a loss of CPC due to the increase of 10ºC ("T-10" ºC) concerning these tests. Due to the enzymatic activity being most active at temperatures ranging from "T- 20" ºC to "T- 10" ºC, further lowering of the temperature was not possible.

After 48h of storage seems like the protein's ratio maintained similar values and 0,90 for the treated sample. These values may be slightly overvalued as CPC/Chl ratio decreased in the stored sample (from 8,1 to 4,8). Phycocyanin seems to either maintain its value or slightly decrease, CPC value may be overvalued as some chlorophyll may be read in CPC's place.

The test at "T+10" ºC (Figure 27) may provide better results from the point of view of preservation over time, however, the fact that this test is performed at a higher temperature than the previous test (plus 10 ºC) there may be a more pronounced decrease in phycocyanin concentration. The experimental conditions were similar in everything to the conditions used in the test at "T" ºC, the delta ramp was used with the addition of a cooling step at 4 ºC after the end of the treatment.

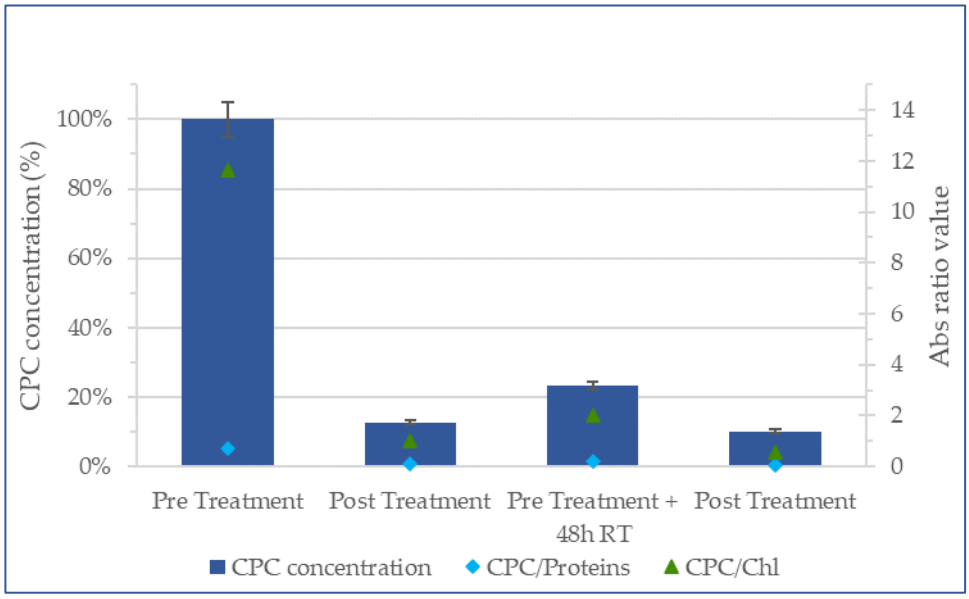

Figure 27 - Thermal Treatment - T="T+10" ºC

The temperature of "T+10" ºC seems to have a drastic impact on the phycocyanin concentration, bringing down the purity indicators. Both ratios seemed to be more influenced by the temperature than the storage time. The initial sample, before treatment, presents mean values of 0,69 regarding the protein ratio, after treatment, this value decreases 82,8% to a value of 0,12. Regarding the Chlorophyll ratio, the initial sample showed a value of 11,7 which dropped 79,5% to 2,4 after treatment. Phycocyanin-wise, there was a clear drop in contents as the sample after treatment dropped 87%.

After storage, the non-treated sample dropped 77% in CPC contents whereas the treated sample only dropped 3% (starting from a lower initial value). These values suggest that for storage of 48h the treatment at "T+10" ºC is more harmful than the storage without any treatment.

Complementarily, in order to better understand the influence of temperature increase in the CPC concentration a fresh extract, following the CPC Quantification Protocol for extraction and CPC reading was heated with 10 ºC increments from room temperature (approximately 20 ºC) to "T+10" ºC (Figure 28). After each reading, the sample was poured back into the same tube and put into a warm bath. After each reading, the temperature was increased and held for 30s to ensure thermal homogeneity.

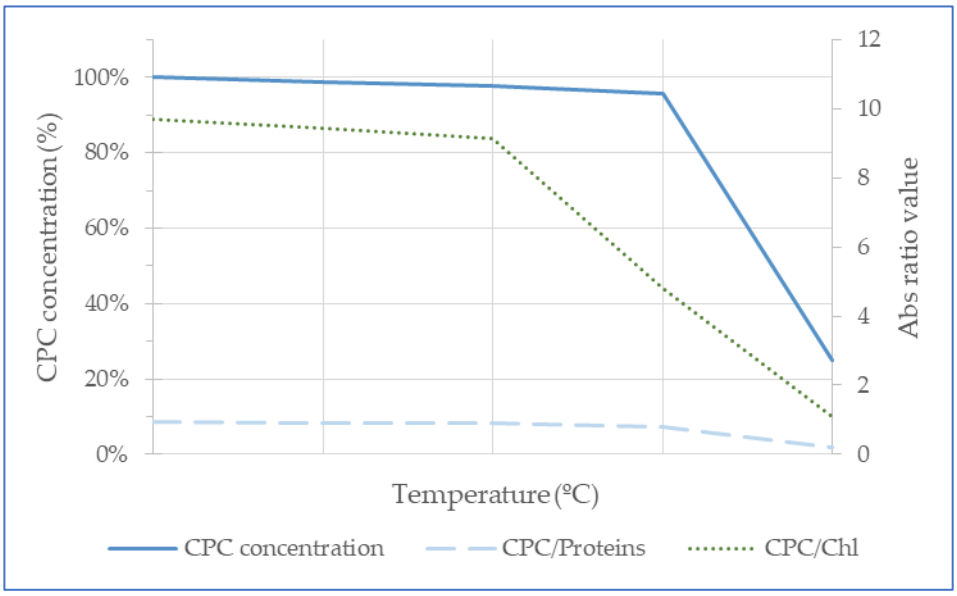

Figure 28 - Thermal Treatment - CPC Thermal Degradation

The results from this test are in line with the two temperature tests carried out for temperatures of "T" ºC and "T+10" ºC. There seems to be a slight decrease in all indicators due to the temperature until "T- 20" ºC, after which the decrease emphasizes itself.

In relation to the chlorophyll ratio, between temperatures of "T- 20" ºC and "T" ºC, two periods stand out. In the first period ("T- 20" ºC to "T- 10" ºC) a decrease of 6% is observed in CPC/Chl ratio. In the second period, the decrease reaches 50% of the initial value. On the other hand, CPC and protein ratio values appear to maintain the initial variation trend up to a temperature of "T" ºC. Considering the data from the three indicators, it appears that up to "T" ºC the variation in the chlorophyll ratio is due to an increase in the wavelength of 430 nm, rather than a significant decrease in the amount of phycocyanin. Since there is a variation in CPC concentration similar to the initial trend.

After "T" ºC, all indicators decrease rapidly, from "T" ºC to "T+10" ºC there is a 79% decrease in CPC/Chl purity, corresponding to an 89% loss compared to the initial value. The protein ratio value decreases 80% when compared to the initial value, and CPC concentration decreases to 75% of the initial value. The values in this test appear to indicate that phycocyanin can tolerate temperatures up to around "T" ºC and deteriorate at higher temperatures. Possibly using shorter temperature ranges, one could have a more accurate notion of the critical temperature. The literature defines 57,5 ºC as the temperature at which 50% of the proteins present in phycocyanin are denatured at a pH of 7. [76], [79]

3.2.1.4. Trial 2 - Solvent trials at extended conservation periods

Similarly, to trial H from the Phycocyanin Quantification Protocol, this trial was tested and the efficacy and efficiency of different solvents, in this case, the resuspension was performed before the heat treatment. From an industrial point of view, logistically and economically it would be advantageous to use as close as possible to the growing medium, as substantial volumes will be processed. In the first phase of this test use of a two-source buffer was tested, the dissolution of salts was made in a sample by culture medium and in the next by deionized water. The samples were tested before TT, after TT, and after a week of storage (Table 5 and Figure 29).

Table 5 - Trial 2 Samples

Sample Code	Description
I-1	Pretreatment Acidified Medium
I-2	Pretreatment Buffer Solution
II-1	Post Treatment Acidified Medium
II-2	Post Treatment Buffer Solution
I-1 + 1 week	Pretreatment Acidified Medium + 1 week
I-2 + 1 week	Pretreatment Buffer Solution + 1 week
II-1 + 1 week	Post Treatment Acidified Medium + 1 Week
II-2 + 1 week	Post Treatment Buffer Solution + 1 Week

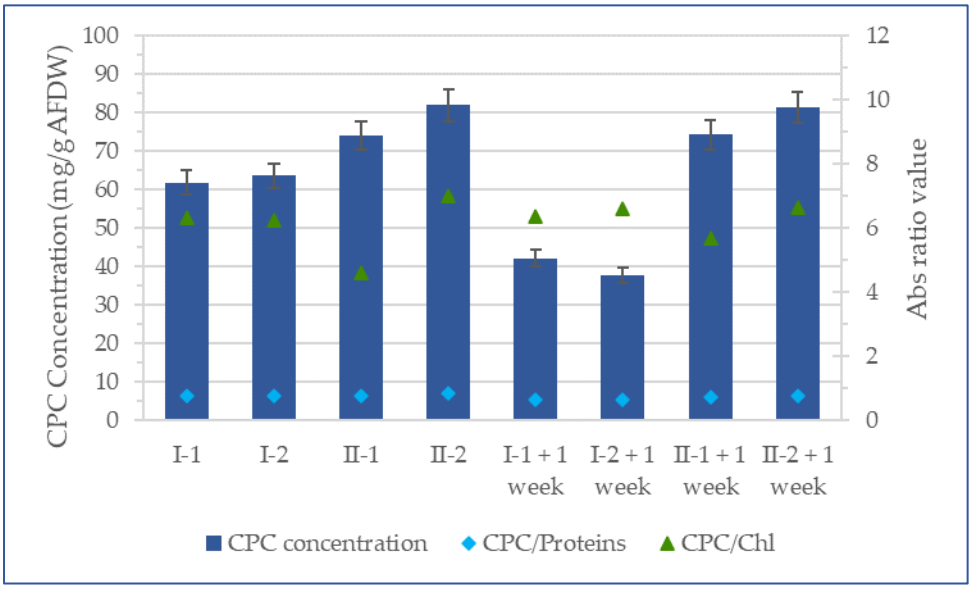

Figure 29 - Thermal Treatment - pH Influence + 1 Week

The results in the above graph appear to indicate that for pre-treatment samples between Culture medium-plus Salts ("Scalable buffer" from the previous chapter) and buffer (prepared with deionized water) there are no significant differences in the protein ratio, that is, the solubility of phycocyanin or other proteins present has not been changed by the fluid

variation. Regarding the chlorophyll ratio, there was no significant change before treatment. The variations of the CPC values in both cases are within the error margin.

After-treatment samplers have similar protein ratio values, with a slight improvement in buffered samples. There is a clear difference in the chlorophyll ratio, with the sample prepared with buffer at significantly higher values (a 55,6% improvement). Post-treatment CPC values favor the sample prepared with the buffer, being not only superior but with more accurate values, having a much better purity/interference ratio.

After a week of storage at 4 ºC, the untreated samples maintained similar chlorophyll ratios, although they showed a significant decrease in CPC content. With 7-day storage, the scalable buffer sample appears to have a slightly higher CPC value.

The samples treated after one week show similar values in all indicators, except for an improvement in the chlorophyll ratio for the scalable buffer sample, which can be explained by degradation of chlorophyll, or variations in the analytical process.

The second phase tested the possibility of using deionized water, comparing its results with both buffer samples. In these tests, the three samples were tested before and after treatment on day 0 (Figure 30) and day 3 (Figure 31).

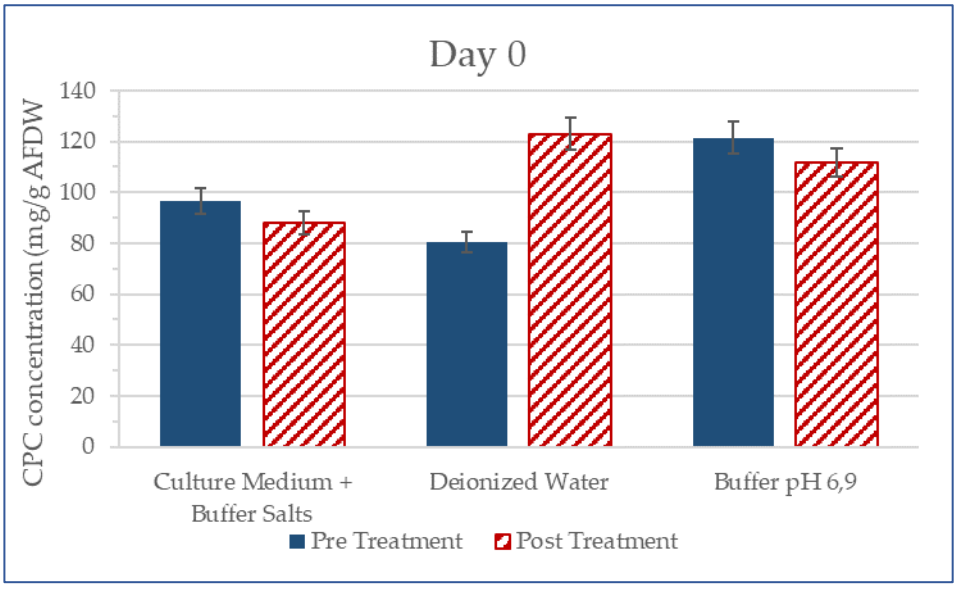

Figure 30 - Thermal Treatment - pH Influence - Day 0

On day 0 apart from the buffer prepared with culture medium the remaining samples showed better results after treatment. The variation on the scalable buffer is within the margin of error being neglectable, the deionized sample before treatment seems as it did not extract the whole amount of CPC available as the post-treatment extraction is appreciably larger, possibly needing more extraction time or multiple extractions. The buffer sample has the highest pretreatment value and a post-treatment value comparable to the higher values observed in this test (buffer pretreatment and the post-treatment deionized water sample), being within the margin of error.

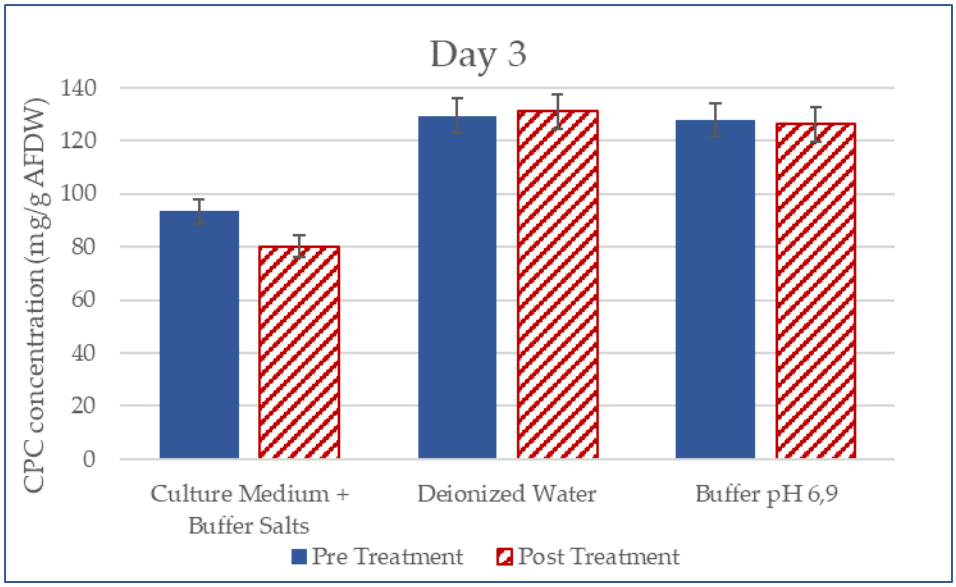

Figure 31 - Thermal Treatment - pH Influence - Day 3

After the storage, the scalable buffer appears to have the same CPC values with slight variations to the initial values being contained by the margin of error. Deionized water sample appears as it extracted the remaining CPC after storage, as both pre and post-treatment show similar values being also higher than the post-treatment at day 0. The buffer sample after storage also extracted more CPC over the three days, having similar contents to the deionized water sample.

After analyzing the results collected in this trial, the decision was made to proceed with the use of phosphate buffer prepared with deionized water, since the results from buffer prepared in culture medium had consistently lower values, with great variability among them. These results are aligned with the literature as far as the importance and deep link connecting the pH value and stability to the CPC stability. [74], [79]

The results shown are aligned with the results present in the development chapter of the Phycocyanin Quantification Protocol (PQP), the buffer made with deionized water and deionized water itself obtain the best results. Implying that in the industrial process it may be necessary to change the fluid where the biomass is contained (represented in this work by the loosed application of the term "solvent").

3.2.1.5. Trial 3 - Thermal Treatment Effectiveness

In order to understand the efficiency of the heat treatment in the stabilization of biomass and particularly of phycocyanin in Trial 3, the concentration of phycocyanin was analyzed before and after treatment in the day 0 and after a week stored at 4ºC (since it would be the temperature closest to the temperature used in the industrial process) (Figure 32).

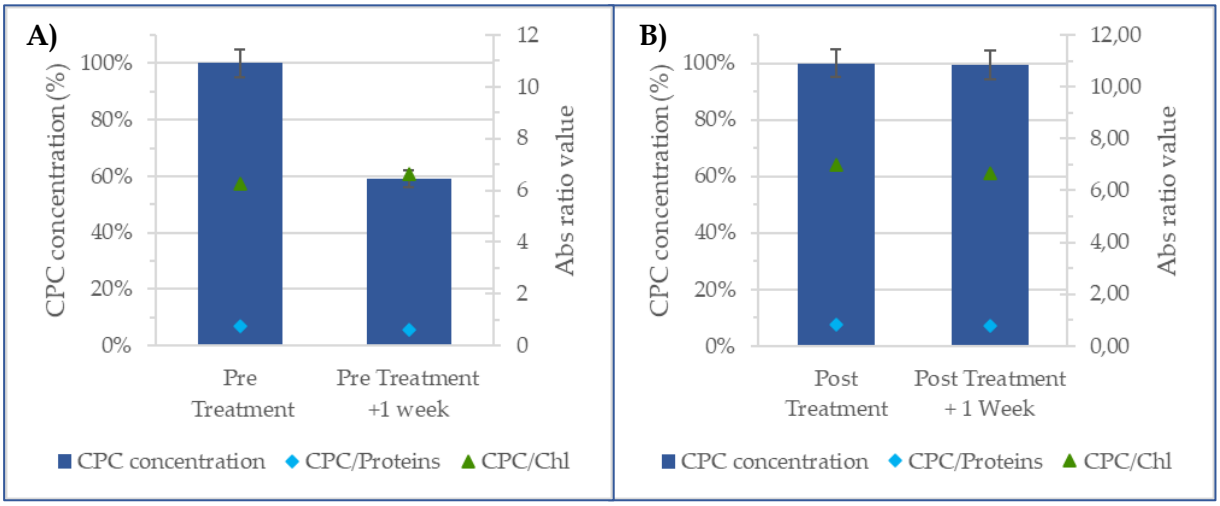

Figure 32 - 1 Week Variation on A) Non-Treated Biomass; B) Treated Biomass

In this test, there was no significant variation in the purity indicating ratios. In the untreated samples, there was a slight increase in purity relative to chlorophyll within the margin of error, the protein ratio there was a slight decrease, after storage, also contained in the margin of error. Regarding phycocyanin content, this decreased 41% in the untreated sample after one week of storage.

The sample treated at "T" ºC with the delta ramp and the additional cooling showed a slight improvement in the chlorophyll ratio when compared to the untreated sample, the protein ratio in turn is similar to the value before treatment. After storage, there were, on average, minimum decreases in the two purity ratios studied in the treated samples. The protein ratio decreased seven hundredths (0,84 to 0,77); the chlorophyll ratio decreased 35 hundredths (7,01 to 6,66). Finally, there was, on average, a 1% decrease in phycocyanin concentration (mg/g AFDW), being within the margin of error of this test.

The values obtained in this test indicate the success of the heat treatment to stabilize biomass and particularly phycocyanin. When comparing the loss from treated and untreated samples, assuming, on average, a total loss of CPC of 5% (1% from this trial plus 4% from the trial A), compared to the 41% loss observed in this trial, it shows a clear advantage in the use of heat treatment performed under these conditions ("T" ºC, buffer as solvent and storage at 4 ºC for one week).

3.2.1.6. Trial 4 - Improving ramps

After the previous tests indicated the benefit from heat treatment, this test assessed two possible ways of improvement and scalability to the industrial scale. The premises by which the previous tests were conducted were dictated by the limitations of the pilot-scale equipment in this test, starting from the same heating tested on the delta ramp (being limited by the equipment/bath used), different cooling options, and their impact on phycocyanin concentration was tested over 72h with an intermediate measurement at 48h into the test. In this trial were compared untreated samples versus 3 thermal ramps, T1 represents the delta ramp already studied; T2 experiences equal heating but when at "T" ºC the tubes are placed on a support in a refrigerated chamber at 4ºC, having no contact with the chamber walls (the cooling is accomplished only by the heat transfer of the walls of the tubes with the air inside the chamber) and T3 a shock treatment resembling HTST, where the sample to be treated was placed on the already heated ("T" ºC) bath held for 30 s and removed (Figure 33).

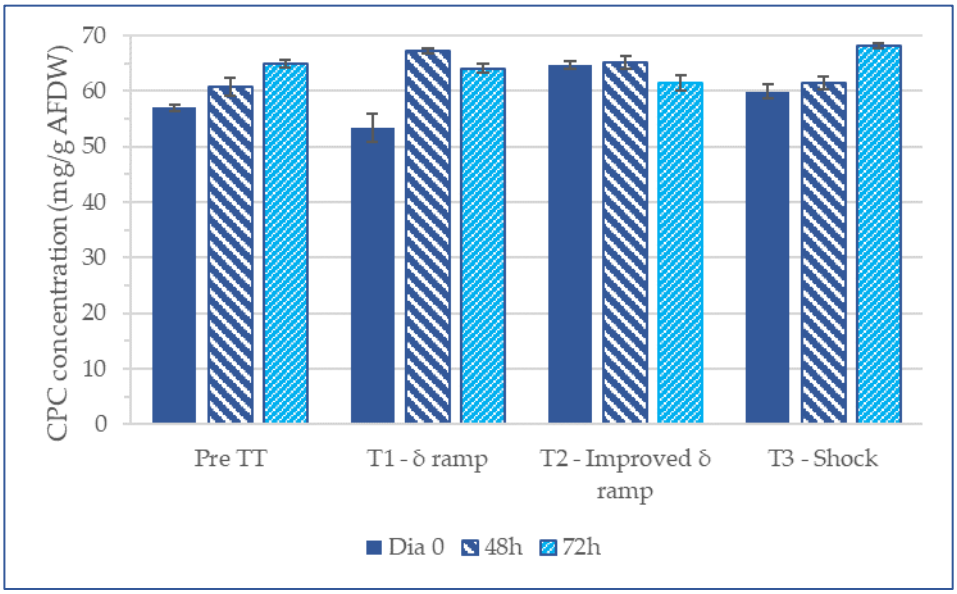

Figure 33 - Thermal Treatment - Heat-ramps Comparison

There appears to be no appreciable variation in protein ratio throughout this trial. After treatment, the T1 sample shows a decrease as already verified in trials 1 and 2 as a consequence of the TT. Both T2 and T3 samples on average did not show this decrease associated with TT, on the contrary, show the increase also observed in the Trial F performed in the development of the Phycocyanin Quantification Protocol, in which, the sample was subjected to a temperature increase before rupture had a higher phycocyanin value.

At the second moment of analysis (48h of storage), the untreated sample appears to have increased its phycocyanin value due to a decrease in purity relative to chlorophyll, similarly, the T1 sample appears to have increased the CPC value as a result of a Chl increase, there is a decrease in the purity relative to chlorophyll. The T2 and T3 samples do not present a variation outside the margin of error, so it is assumed that during the first 48h of storage the CPC concentration remained constant in the two ramps.

After 72h of storage, the decreasing trend of CPC/Chl purity remains present in the untreated sample, the interference of this pigment seems to be responsible for the apparent increase of CPC. The T1 sample presents a similar CPC/Chl ratio and a slight decrease in CPC concentration, these results are similar to the previous trial where stabilization had been tested with one week of storage. Sample T2 showed a slight decrease (8.6%) in the CPC/Chl purity ratio and a 5.6% decrease in the CPC content. The T3 sample appears to have increased both the CPC value and the Chl ratio, which may be related to the decomposition of chlorophyll into pheophytins, not only decreasing the total Chl but causing the same interference in the CPC reading that Chl would do. After discussion with the company and presentation of the results, the T2 ramp was chosen as the preferred one, considering the current logistical conditions. Although at the moment, it was not possible to scale the T3 ramp, this test could be repeated when the industrial pasteurizer starts. The following Figure 34 illustrates in detail the T2 ramp.

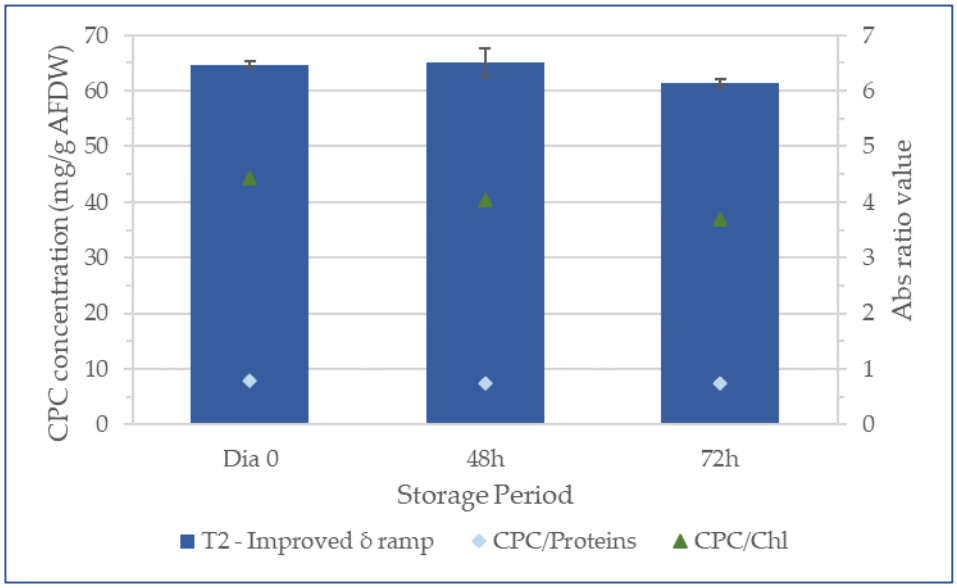

Figure 34 - Thermal Treatment - Improved δ ramp Overview

Ramp T2 seems to be an improvement on the previous ramp delta, in the case of this T2 it seems to have eliminated the decrease related to the TT that had been observed in the previous trial, additionally, it seems to be able to take advantage of the temperature as had been observed in the aforementioned trial of the development of the Phycocyanin Quantification Protocol. It should also be noted that the T2 ramp presents more constant values with a higher CPC final value than the T1 value and a lower percentage decrease after one week of storage.

3.2.1.7. Trial 5 - Cellular fragilization by thermal treatment

As through the past trials, it was observed a release of CPC from intact stored samples, this trial tested the need for cellular rupture for CPC extraction after thermal treatment. In this trial, extractions from four different samples (non-treated, T1, T2, and T3) were compared on day 0 and after an overnight period. The samples were processed as described in the following Figure 35.

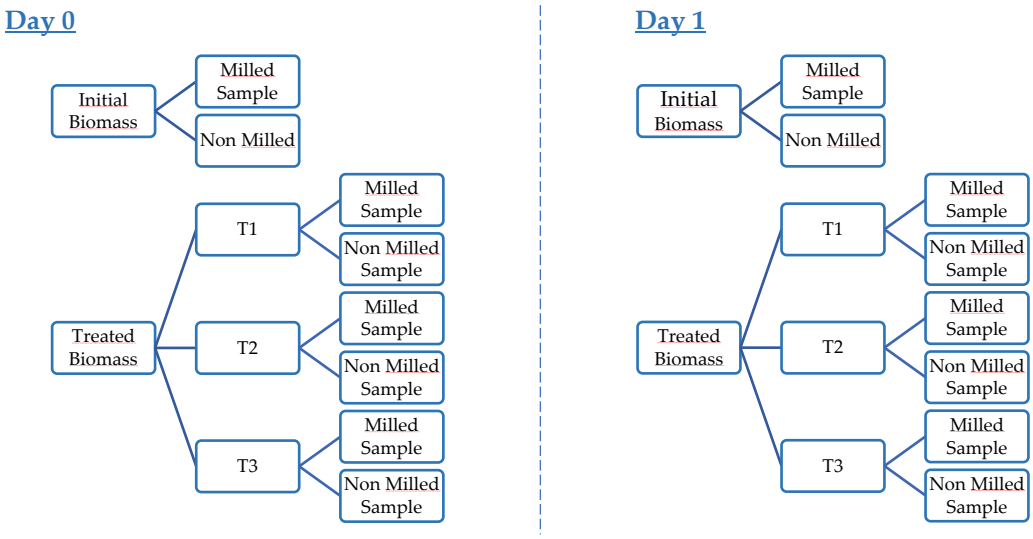

Figure 35 - Trial 5 Sample Schedule Diagram

The values for the tests performed on day zero are present in the following graph. Biomass samples before and after treatment were analyzed in relation to their CPC content as well as purity ratios. Additionally, processed samples were analyzed both in the presence and absence of Beads (Figure 36 and Figure 37).

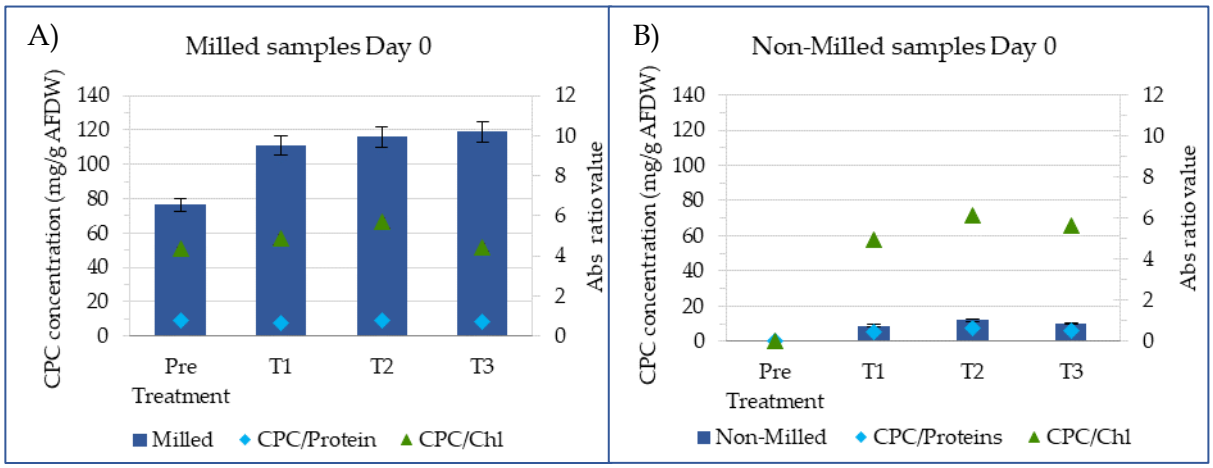

Figure 36 - Thermal Treatment - Cellular Fragilization - A) Milled Sample Day 0; B) Non-Milled Sample Day 0

Day 0 results vary greatly depending on whether or not the samples have been milled. On average, the treated and milled samples presented, on day 0, very similar results among themselves, varying within the margin of error of the test, the pretreatment sample presented a lower CPC value than the treated samples. Without storage time, the non-milled samples show much lower values than the milled samples. Although they had similar purity ratios, the CPC was very low, with its maximum value occurring in the sample relative to T2 (corresponding to 10,3% of the T2 sample when milled).On day 1 the samples were removed from the cooling chamber, waited for thermal balance, each tube was homogenized in the vortex and were processed with and without beads.

The results of the overnight extraction with and without Beads are found in the following bar chart Figure 37.

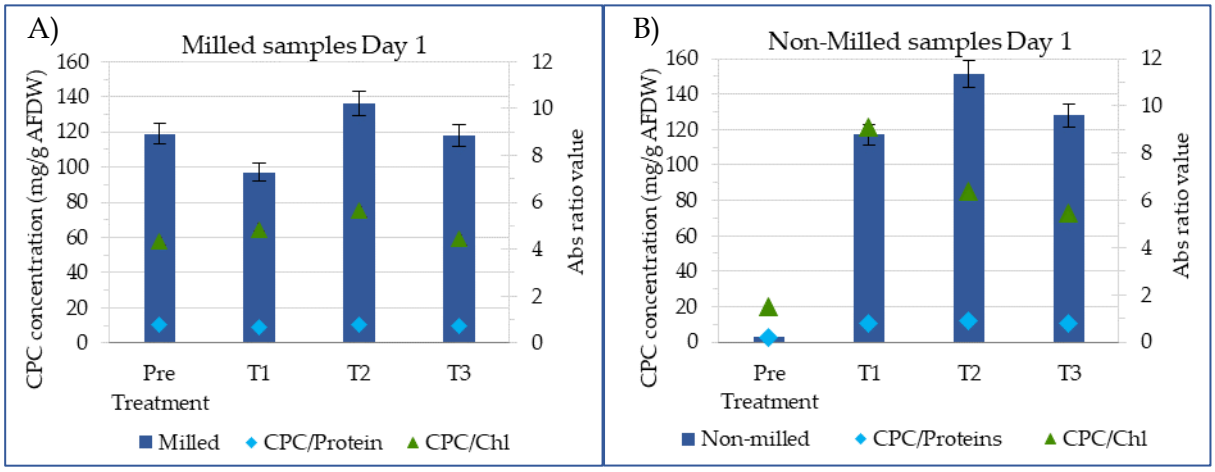

Figure 37 - Thermal Treatment - Cellular Fragilization - A) Milled Sample Day 1; B) Non-Milled Sample Day 1

As expected, milled T2 presented the best results on day 1 with higher CPC values and better purity ratios. T3 was the second best with a low to none decrease in CPC value after treatment, T1 presented the worst result in this trial.

The non-milled samples on day 1 presented on average better results, higher CPC values with better purity ratios than milled biomass. Pretreatment samples after the overnight store did not extract CPC hinting at a relation between the thermal treatment and fragilization of the Arthrospira's cell wall. All treated samples performed better CPC results in the absence of rupture, in addition, the CPC/Chl purity ratio improved in all samples representing a more accurate result.

It seems clear that samples extracted on the same day/ without rest period require milling, and the studied alternative was not shown to be viable. On the other hand, if the sample can be exposed to an extended rest period (up to 8h) the processing in the absence of Beads appears as an alternative not only viable but presents clear advantages when compared to milling.

This test seems to indicate the possibility of using a longer (overnight) extraction instead of milling. According to the values present in the graphs above, all samples obtained, on average, higher CPC values with better purity when undergoing an overnight extraction rather than milling on the initial day. It seems clear that heat treatment has an influence on the fragilization of the Arthrospira's cell walls, and only the treated samples were able to perform significant CPC extractions with very positive CPC/Chl ratios. In examining the non-ruptured samples compared to those that went through the default process, it was also evident that the extract was much more intense, as illustrated in Figure 38 on the right.

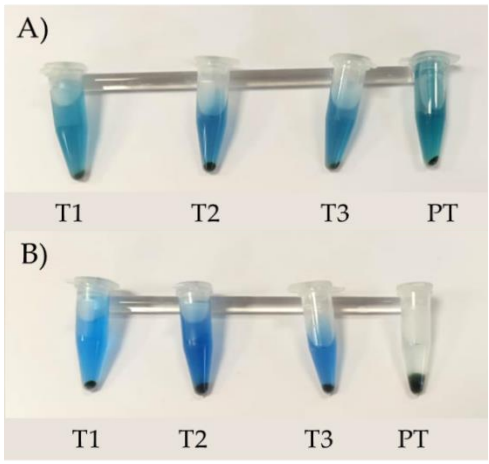

Figure 38 - Thermal Treatment - Milled vs Non-Milled samples

3.2.1.8. Trial 6 - Final lab test

Trial 6 had two main objectives, to test the reproducibility of the T2 ramp, as well as its results (i.e., not to decrease the CPC content, and the ability to take advantage of the temperature in order to optimize the extraction of CPC with the increase of temperature); the second objective being to test whether the heat treatment would be sufficient to weaken the biomass so that the milling step could be skipped if so, it could be an indicator for the pilot testing. Throughout the various trials, non-milled samples stored in the refrigerated chamber systematically showed a blue supernatant.

In this trial to study CPC contents from treated and untreated samples were compared on day 0, on day 1 only the pasteurized samples were read. Day 1 samples were processed differently, as the "with Beads" sample was processed as described in the Phycocyanin Quantification Protocol present in this work, the sample without Beads would undergo the same procedure, but in the absence of Beads (Figure 39).

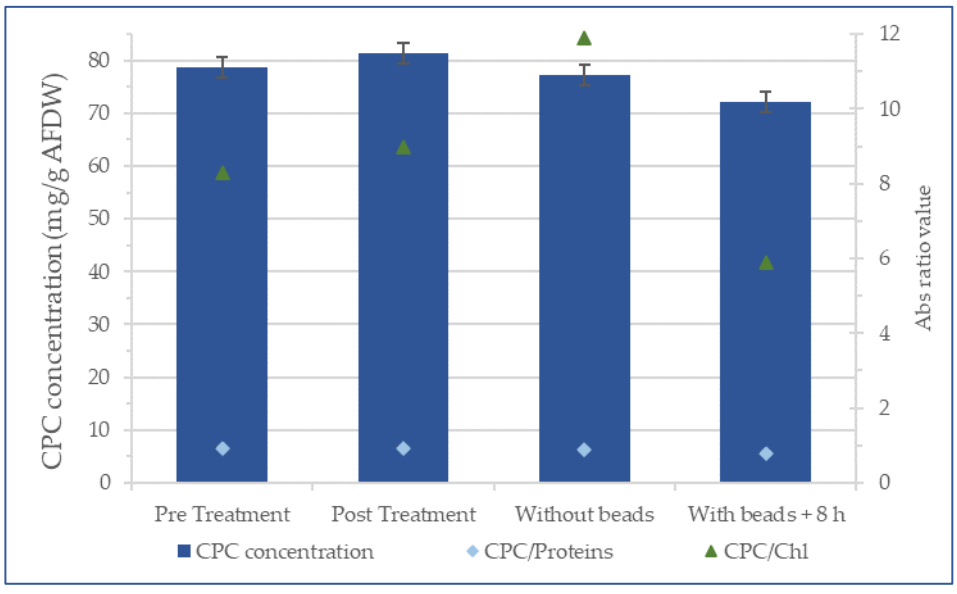

Figure 39 - Thermal Treatment - Final Test

In this assay, the protein ratio varied within the margin of error with the minimum observed at 0,80 from the sample milled after the overnight period, therefore all samples are within the category Food Grade. [111]

The overnight sample processed without beads presents a higher purity value concerning chlorophyll, with a similar total phycocyanin value which seems to indicate a higher actual phycocyanin value (being less overvalued). In relation to Day 1 samples, it is possible to observe that the samples stored overnight have different ratios of CPC/Chl being the post-treatment without Beads being the highest of the test. As regards the CPC value, the two samples show similar values and a variation in the margin of error.

The overnight extraction may be a viable alternative to milling according to the values of this test, as the variation between the treated sample on day 0 and the sample that underwent overnight extraction (day 1) without beads is contained in the margin of error, although the sample without beads has a higher purity.

3.2.2. Thermal Treatment - Pilot trials:

The pilot tests were carried out in two phases, in the first phase the ramp possible in the pilot pasteurizer was optimized to better emulate the T2 curve performed in the laboratory. After being able to optimize the treatment ramp in the pilot pasteurizer, thermal treatment tests were carried out where the CPC reading, and its purity ratios were used to analyze the feasibility of the process.

3.2.2.1. Pilot trials - Hydraulic testing

The hydraulic tests (Figure 40) aimed to optimize the ramp in the equipment present in the industrial site, to be able to accommodate the T2 curve, coming from the laboratory tests. In the cooling step, to increase the temperature/time gradient between "T" ºC and 20 ºC (critical range for enzyme activity), crushed ice was added to the pasteurizer. With this addition, it was possible to achieve the intended cooling speed in the critical range, similar to the laboratory curve. From a logistical point of view, the addition of ice was the best alternative to ensure rapid cooling of biomass without either compromising the ease of process execution or exposing the biomass to the elements. Due to the equipment specifications, the ice had to be crushed before it was placed, which added a step to the preparation of each test. In this way, the ice was previously crushed and placed in a freezer container until needed. The fact that the ice is crushed rather than drop as plates/blocks, prevented the biomass from being burnt by direct contact with ice.

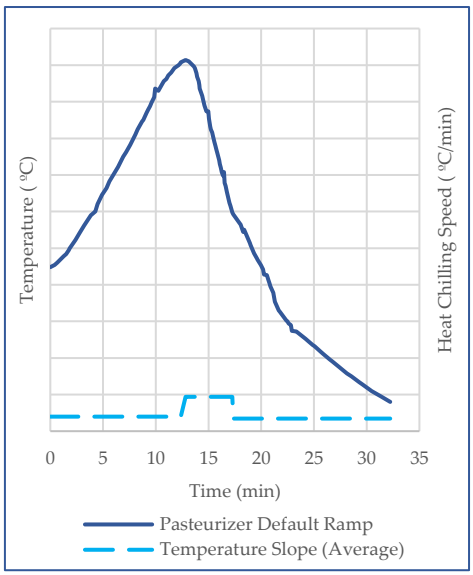

Figure 40 - Thermal Treatment - Pilot Hydraulic Test

3.2.2.2. Pilot trials - Scaling up

For logistical reasons, due to the high volumes processed, the pilot pasteurization tests were carried out in conjunction with the pilot milling tests, with the pre and post-pilot-treatment samples being the starting point for the milling tests. The results obtained during pilot tests related to the thermal treatment were summarized in the following Figure 41.

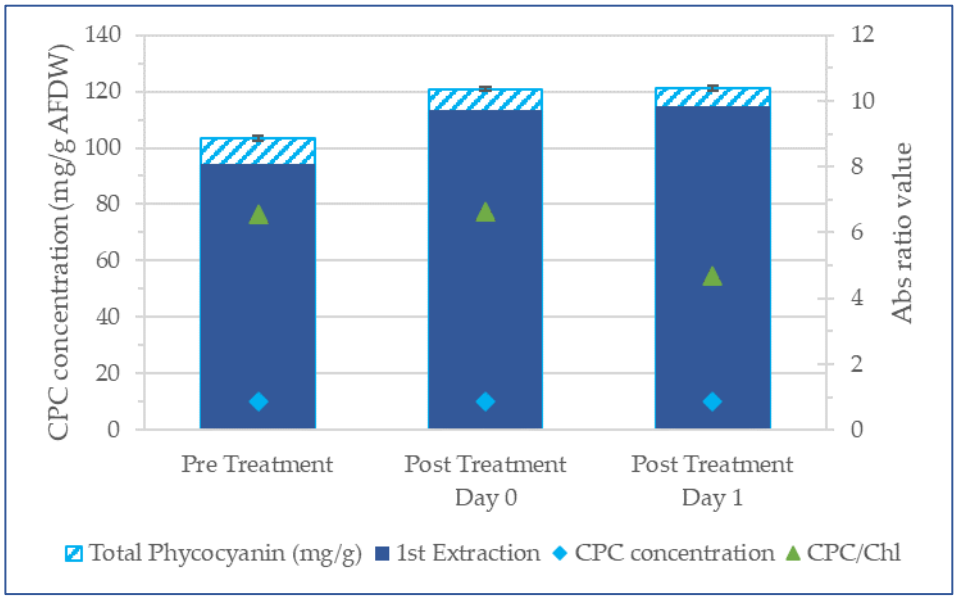

Figure 41 - Thermal Treatment - Pilot Testing with Biomass

It is possible to observe the increase of CPC content characteristic of the T2 ramp, while the treated samples maintain the CPC/Chl ratio. The ratio relative to proteins, improved when compared to the untreated sample. On day 1, the post-treatment sample does not have observable variations in the CPC values, there is only a decrease in the phycocyanin vs chlorophyll ratio, which may indicate the CPC value in the stored biomass may be overvalued. Overall, this trial was successful as it managed to accurately emulate de T2 laboratory ramp.

The diverse battery of tests in this work resulted in the improvement and optimization of the process and phycocyanin stabilization from fresh biomass. From the developed curves in the laboratory, the improved delta curve and shock are clearly the most effective with the choice of the first rather than the second having been in charge of the company due to logistical conditions. The improved delta curve Scale-up was successfully performed, with a heat treatment resulting in similar values to those obtained in the laboratory, considering all the limitations related to scalability.

3.2.3. Thermal Treatment Protocol Description

3.2.3.1. Laboratory Scale Treatment Description

Briefly, the culture Optic Density (OD) was measured to estimate the dry weight composition, and consequently the needed volume of culture. After that, the culture should be centrifuged (type A centrifuge) for concentration. Collect the supernatant for pH and conductivity analysis. Perform a fast dry weight reading (humidity scale) and resuspend the resulting pellet biomass in potassium phosphate buffer (1M, pH=6,9) for the desired concentration. Prepare 12 glass tubes of the new suspension and number each tube. If the three ramps are being tested place T1 and T2 tubes in the bath and the place of T3 place blank tubes (tubes filled with culture medium. If due to lack of biomass volume there is any empty tube, it should be filled with culture medium. Position the glass tubes in a way to prevent thermal gradient in the warm bath and ensure homogeneity in the thermal spread. To control the actual temperature inside the tubes instead of the bath temperature this procedure is performed with all tubes being opened and a thermocouple shall be placed inside a representative tube, so the temperature can be effectively measured.

If all T1 ramps are being tested the preparation for the cooling system follows, if T1 is not of interest skip the next paragraph about cooling system preparation.

For the cooling system preparation place a hose (hose A) for delivering fresh water (either tap water or water from a jerry can previously filled) in the warm bath and a second hose (hose B) for suction and disposal of warm water from the bath. The suction hose should be connected to a pump which should match the water inlet from hose A to maintain the bath volume constant. Connections between the bath and the water source should be double-checked for leakage. A testing first trial should be done with the bath's heat turned off to ensure the correct functioning of the cooling system. To ensure better efficiency from the cooling system, the suction hose should be as apart as possible from the delivering hose.

After preparation is done and without pre-heating the warm bath, place the tubes holder inside the bath and start the heating process (temperature setpoint = Ta ^{o}C, where "a" represents an empirical factor, to prevent a decrease in the heating speed around the setpoint temperature), measuring temperature variations (time vs temperature with 1 ^{o}C slope). One degree from the desired temperature is reached (T-1 ^{o}C) set a new set point at "T-20" ^{o}C (as a precaution).

If all 3 Temperatures are tested the procedure continues as it follows in the next paragraph, if otherwise only T2 ramp is performed skip the next paragraph, instead the tubes should be closed with the correspondent lids and the tube's holder should be taken to a refrigerated chamber (at the cooling temperature) until the tubes reached final temperature. At that point, the TT stage is finished, and CPC reading should be done according to the CPC Quantification Protocol as it is described in this work. If only T3 is being studied skip the next paragraph, instead the blank tubes in T3 place should be replaced by the actual biomass tubes and wait until thermal equilibrium is reached. After that, these tubes should be closed with the correspondent lids and the tube's holder should be taken to a refrigerated chamber. At that point, the TT stage is finished, and CPC reading should be done according to the CPC Quantification Protocol as it is described in this work.

Heat ramp 2 tube's should be closed with the correspondent lids and the tube's holder should be taken to a refrigerated chamber until reaches the final temperature, blank tubes in the T3 place should be replaced by the actual biomass tubes and wait until thermal equilibrium. After that, these tubes should be closed with the correspondent lids and the tube's holder should be taken to a refrigerated chamber (-25 ºC). Initiate the cooling system and measure temperature variations, when at "T- 10" ºC (10 ºC before the setpoint), the heat should be turned off and temperature variations measured. To maintain the cooling velocity, when at 25 ºC the inlet water should be switched from the tap water to the pre-chilled jerry-can (the final temperature), register all variations until the temperature stabilizes (temperature vs time at 1 ºC slope). At that point, the TT stage is finished, and CPC reading should be done according to the CPC Quantification Protocol (PQP) as it is described in this work.

3.2.3.2. Pilot Scale Treatment Description

Succinctly, for the preparation crush the whole amount of ice needed and store it in the refrigerated container (1 kg per 5L of biomass); Prepare a potassium phosphate buffer solution for dissolution as it follows: weigh the needed amounts of salts to prepare the phosphate buffer (0,1M, pH= 6,9) Store the needed volume of distilled water the buffer solution. As melting ice will dilute the solution its volume should be considered when preparing the buffer.

After preparation, Arthrospira should be harvested, and its final concentration measured by a quick dry weight reading; the water previously stored should be poured into the pasteurizer and the previously weighed salts for the potassium phosphate buffer added. In a non-heating setting mix the water with the salts. After dissolution, pour the concentrated biomass onto the pasteurizer repeat the non-heating mixing program to ensure homogeneity. Start pasteurization in a pre-set program that heats and cools down at 2 ºC, programmed to heat to "T" ºC, hold 1min and cool down to the final temperature. Register time vs temperature at 1 ºC slope in heating until "T-20" ºC, after which two measures should be taken each 1 ºC increment. In the cooling step, two measures should be taken per 1 ºC until "T-20" ºC, then only a 1 ºC slope is required until the final temperature (end of treatment). When at Ta ºC pour the, previously crushed, ice, maximizing the cooling speed. Pasteurization ends at the final temperature, after which, the pasteurizer should be emptied of its contents into jerry cans for either being milled and perform CPC reading at that time, following the milling procedure as present in this work, or stored at the final temperature for later milling (next day) and CPC reading according to the CPC Quantification Protocol as it is described in this work.

3.3. Milling

Milling was tested as a form of phycocyanin extraction, so the process was optimized within the following restrictions. Despite the different optimizable parameters, this trial optimized flow rates and residence times. The parameters for the chamber are restricted to the existing equipment, and parameters such as filling ratio and agitator speed, and beads (material and geometry) had already been optimized by the company previously. Two main indicators were used to analyze the chosen flow rates F L/h, 2F L/h, and 3F L/h, as the milled

samples were examined under a microscope and the ruptured cells were counted, Phycocyanin was measured in addition, and the usual ratios were calculated.

3.3.1. Mill testing:

In this chapter, the tests carried out studied the three flowrates mentioned, as well as the possibility of skipping the milling step performing an extended extraction. To analyze the results obtained, the following tests were performed:

- The microscopic analysis aims to evaluate the degree of rupture in the different flows studied and thus to study the efficiency of biomass rupture.
- The extraction efficiency is given by reading the sample in CPC content, as well as by protein and chlorophyll ratios.

Regarding the flow rates and their residence times, a prolonged residence time ensures a more complete rupture, however, they can disintegrate photosynthetic complexes and cause a greater release of Chl, influencing the CPC readings and their accuracy.

3.3.1.1. Microscopical analysis

All the samples milled in the company's pilot mill came from the same initial homogeneous sample treated in the pilot pasteurizer. The three flowrates tested were analyzed under a microscope to study the degree of milling efficiency, the degree of milling efficiency is determined by the degree of breakage observed under the microscope. The microscopic analysis (MO) was performed by the company's laboratory technicians.

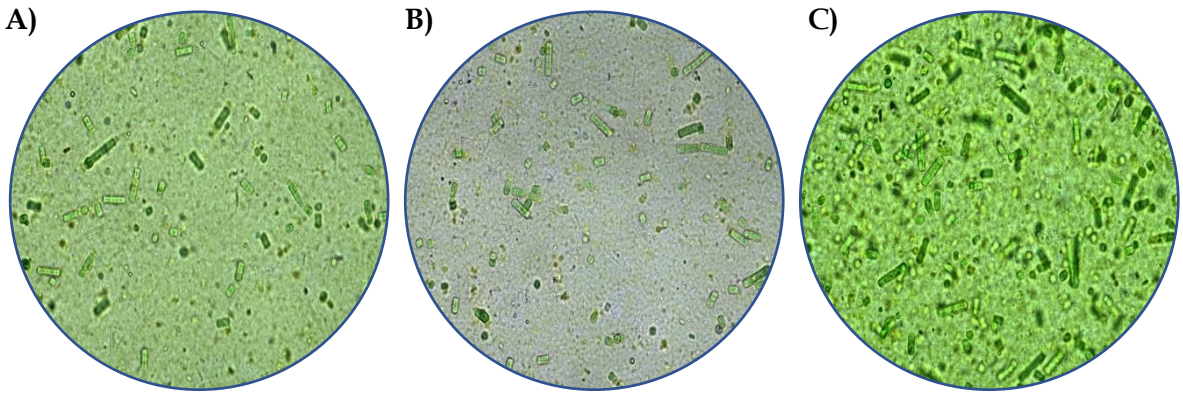

Figure 42 - Ruptured Arthrospira - Microscopical Imaging. A) F L/h; B) 2F L/h; C) 3F L/h

According to the notes of the microscopic analysis, all three flows presented a degree of rupture greater than 90%. The flow rates of F L/h and 2F L/h showed a very similar rupture efficiency with few long filaments. It was also observed the absence of vacuoles in the existing filaments. It was possible to witness the release of cellular content to the external medium, indicating extraction. The flow rate of 15L/h (with shorter residence time) showed a slight decrease in the rupture efficiency. The number of large filaments was slightly superior when compared to the other flow rates. However, the release of intracellular contents into the external environment and the absence of vacuoles were observable, so it seemed to indicate a full extraction as well.

3.3.1.2. Phycocyanin values - Milling

In addition to the microscopic analysis, the samples from the different tests that compose this trial were analyzed in their CPC contents. Allowing to study both the influence of the mill residence time in the extraction of phycocyanin as well as to study which is the most effective flow rate for higher extraction efficiency. Due to the high operating times of the pilot tests (linked to the operation of large equipments and processing of large volumes from the harvesting phase of thermal treatment), the milling by the pilot mill was always carried out the day after treatment. In order to reduce any possible deterioration, the biomass is stored overnight at 4ºC. Since the samples were going to be milled with a rest period of 8h (the same period used in the laboratory testing for "overnight" samples) these samples were added for testing without alteration of the test protocols.

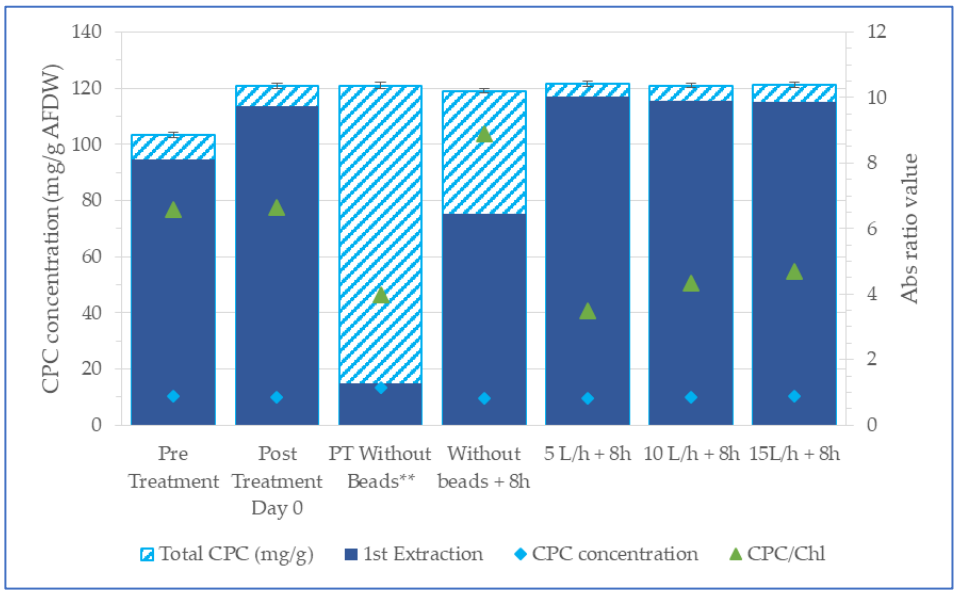

Figure 43 - Flowrate testing

NOTE:
** Sample processed in the absence of beads for the first extraction and milled for resuspension.

The samples treated and read on day 0, corresponding to the day of treatment, have very similar total values, and their variation is restricted to the margin of error. The treated samples present slightly higher CPC values than the untreated sample, as observed in the previous chapters regarding the development of Phycocyanin Quantification and Heat Treatment Protocol. The sample treated and processed in the absence of beads presents a poor first extraction requiring a second extraction step. In this case, the first extraction can only extract 12,2% being the second extraction mandatory to achieve the total CPC. In this case, the second extraction resulted in a decrease in the purity ratio associated with chlorophyll contamination, adding uncertainty to the CPC value presented. Generally, this decrease in CPC/Chl is observed in all of the second extractions (resuspensions), possibly due to the vortex time. Given this contamination is of the utmost importance to extract the maximum possible in the first extraction step.

The sample treated and stored in the refrigeration chamber has been subjected to different extraction processes, namely, beads-free extraction, pilot mill extraction at F, 2F , 3F L/h. Regardless of the extraction process used in each sample, all samples showed similar total CPC values, varying within the error margins. The sample processed on day 1 in the absence of Beads showed an improvement in the efficiency of the first extraction after the overnight period was possible to extract about 63,3%, with a total value aligned with the other extraction processes. In relation to the three flow rates studied, there are two indicators to be considered. As the flow rate decreases, the residence time increases, as a result, the degree of rupture increases with the flow rate (as evidenced in the microscopic analysis). As shown in the graphic above, the increasing flowrate slightly reduces the extraction efficiency across the three samples, but, at the same time, improves the Chl ratio (as a result of less destruction of photosynthetic complexes). In Figure 44 on the right are positioned the extracts after a centrifugation type A, from left to right are the extracts free of Beads; milled in the laboratory, and the third extract milled in the pilot mill. In terms of separation and purification of cellular debris, ocularly, it is clear the greater efficiency of the non-milled sample.

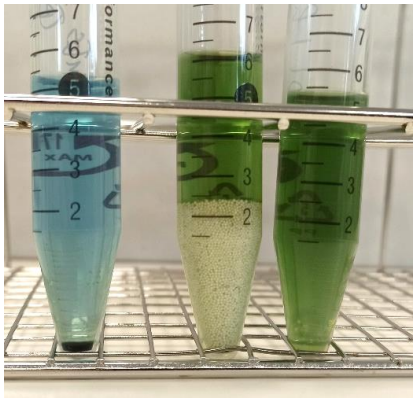

Figure 44 - CPC Extract from Non-Milled, Lab Milled; Pilot Mill

Two main conclusions can be drawn from the analysis of these results, there is a major improvement in the Chl ratio when in the absence of beads, although a second extraction may be required to complete the extraction, on the other hand, there is not a clear advantage in any flowrate over the next from the efficiency standpoint, probably the faster flowrate would be beneficial as it, on average, shows better purity ratios.

After extraction of the samples corresponding to the 2F L/h flowrate and beads-free extraction, both were purified by centrifugation in order to better separate CPC extract from cellular debris. Each of the two samples was centrifuged using three consequent centrifugations type-A with pellet discard followed by two type-B cycles. The resulting sample of each of these steps is illustrated in Figure 45 below.

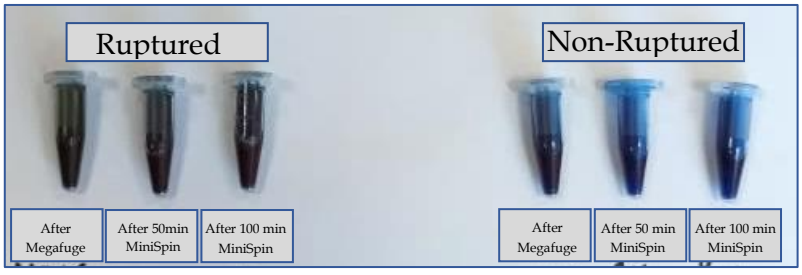

Figure 45 - Phycocyanin Extracts from Pilot Mill and Non-Milled samples at each Centrifugation Stage.

The color differences can clearly be observed in both milled and non-milled extracts in Figure 45. For the milled samples, centrifugations do not seem to have a significant impact on

the separation efficiency. Despite the pellet discarded in each cycle, the final supernatant was dark green after a total of 45mins at 4100 xg and 100 min at 14100 xg.

Along the three stages of centrifugation, the non-milled biomass seemed always brighter, with blue color opposing the milled extract. After the third type-A centrifugation cycle, the non-milled extract appeared to have a clearer appearance with a strong blue color, although there were noticeable some dark green traces. The first type-B cycle seemed to be effective at eliminating this green interference. The second cycle did not present a noticeable difference in the blue color, although it resulted in a small pellet in the eppendorf's bottom.

The results presented in this trial show a similar efficiency in the three studied flowrates, performing equally. In this trial was possible to prove the clear benefit in skipping the milling stage as far as the CPC purity and total values. Given the better purity ratios, the "same" total CPC value is an indication of a higher extraction in this processing route.

3.4. Phycocyanin Purification - Salting Out

A study concerning the salting out method was proposed by the company to investigate its efficiency for CPC purification. According to Bodzon-Kulakowska, Ammonium Sulfate (AS) is recognized as the most widespread precipitant for the effect.[122] This method is based on the addition of high amounts of AS salt to a protein extract, resulting in increased protein interactions causing protein aggregation and in turn precipitation. This methodology allows selective separation as different proteins precipitate at different AS concentrations. This selective separation may be verified by the CPC/Proteins ratio presented in the following trial results plot. This methodology had already been extensively investigated by the company in previous studies with dry biomass. This work firstly confirmed whether the results with fresh biomass presented similar values (Figure 46). Preliminary studies confirmed the effectiveness of this method, as it was observed in the previous tests there is a linear growth between AS concentrations of 40% and 50% of the saturation concentration, the slope reduces considerably between 50% and 60% reaching a plateau after "T+10" ºC. After the 50% concentration as can be observed from Figure 10, there is a decrease in the CPC/proteins separation efficiency as more proteins start to precipitate. After this confirmation, in the preliminary tests, the efficiency of salting out in both milled and non-milled samples was investigated.

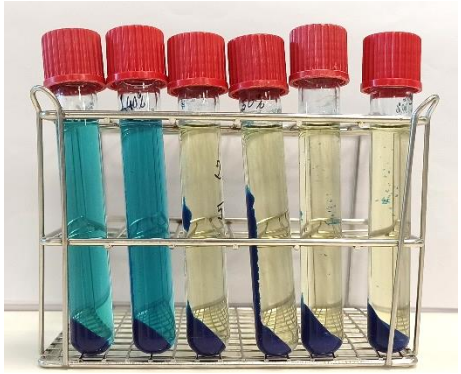

Figure 46 - Salting Out Precipitates: 40%; 50%; 60%

In the preliminary tests, it was observable that the sample 40% did not precipitate the entire content of phycocyanin, and the supernatant still has a bluish color, as illustrated by Figure 46.

The following samples, from the third to the sixth tubes, present few visual differences mirrored in phycocyanin results, with little to no difference in CPC contents. From the samples tested, the concentration of 50% concentration was chosen for the following tests as it presented a better CPC/Proteins separation Efficiency.

The tests that compose this trial analyzed two different indicators, the degree of CPC recovery and the concentration gain. Starting from the samples processed in the pilot process, treatment, and milling, the salting out procedure was carried out as described in the previous works carried out in the company.

The average values from the initial extracts used in the salting out tests are detailed in Table 6.

Table 6 - Salting Out - Initial Extracts

Non-Milled Extract			Milled Extract		
CPC concentration (mg/mL)	CPC/Proteins Ratio	CPC/Chl Ratio	CPC concentration (mg/mL)	CPC/Proteins Ratio	CPC/Chl Ratio
4,84	0,86	10,33	5,47	0,74	2,27

The initial samples in this test were called "extract" presented with similar CPC values. Although the milled extract has a higher CPC content, its purity ratio is much lower and may indicate an overvaluation of this indicator.

3.4.1. Phycocyanin Recovery Efficiency (R_E)

In this test, milled and non-milled samples are compared side by side. Following the procedure described in the Methods chapter, both samples were processed and CPC, CPC/Proteins, and CPC/Chl ratios plots were drawn. Phycocyanin recovery efficiency measures, as a percentage, the amount of CPC recovered in the precipitate from the total CPC existing in the initial extract. The percentage recovery was calculated as shown in the following Equation 11.

Equation 11 - Recovery Efficiency

$$R_E = \frac{\overline{m}_{CPCsediment}}{\overline{m}_{CPCextract}}$$

Where,

R_E: Recovery Efficiency (%)

$\overline{m}_{CPCsediment}$: Average CPC weight recovered in the sediment (precipitate) (mg)

$\overline{m}_{CPCextract}$: Average CPC weight in the initial extract (mg)

The percentage of CPC recovered from the initial sample is represented in Figure 47 below.

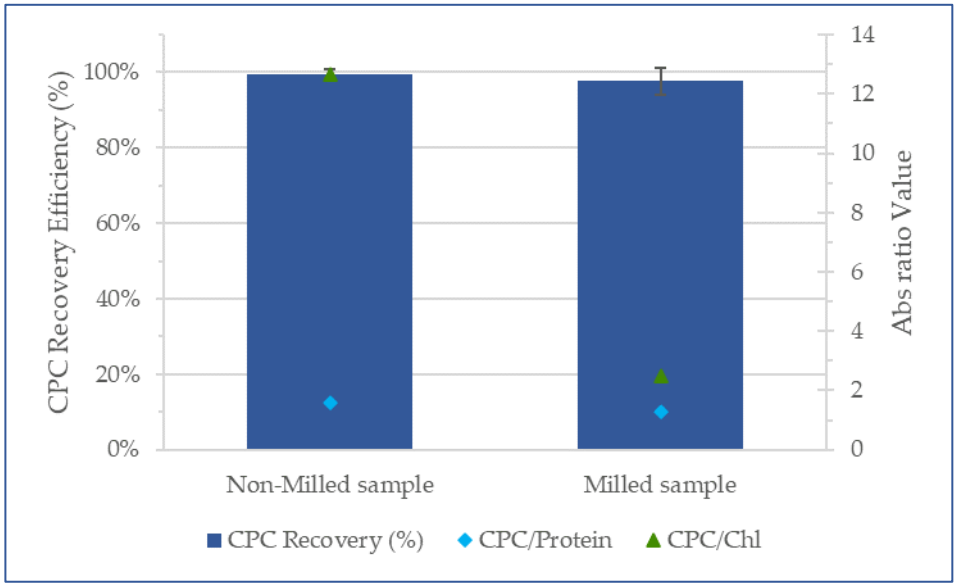

Figure 47 - Salting Out - Recovery Efficiency (%)

Selective separation benefited both samples with an improvement of the CPC/Proteins ratio of 87,2% and 70,9% for the non-milled and milled samples, respectively.

After the Salting Out process, both samples improved their purity, being within the Food Grade category, with ratios above the 0,7 minimum limit.[111] Chl ratios also changed after salting out. The milled sample shows an improvement of 22,7%, while the sample that suffered milling shows an improvement of 10,1% in the indicator of purity relative to chlorophyll. These values seem to indicate that salting out has a positive impact on the separation of phycocyanin from chlorophyll.

AS for phycocyanin recovery both samples present results above 97% recovery. The sample processed in the absence of beads presented the highest recovery value in this trial with a R_E of 99,6%, whereas the sample from milled extract had a recovery efficiency was at 97,6%.

3.4.2. Concentration Gain (G_C)

In addition to the recovery of phycocyanin, the other main objective of salting out is to concentrate said CPC. This concentration brings advantages especially in the logistic aspect (being able to transport smaller volumes), but also in the commercial aspect, where a more concentrated product will bring advantages. Regarding this indicator, the gain in concentration was calculated, the ratio of CPC/Proteins and CPC/Chl will also be represented even if they are mean values, as such, equal to the values presented in the recovery efficiency graph.

The concentration gain values, present in Figure 48, were calculated with the following Equation 12.

Equation 12 - Concentration Gain Expression

$$C_G = \frac{|C_{ICPC} - C_{PCPC}|}{C_{ICPC}}$$

Where,
C_G: Concentration Gain (%),
C_{ICPC}: CPC concentration on the initial extract (mg/mL),
C_{ICPC}: CPC concentration on the precipitate (after salting out) (mg/mL)

Figure 48 below summarizes the results of the concentration gain from this trial.

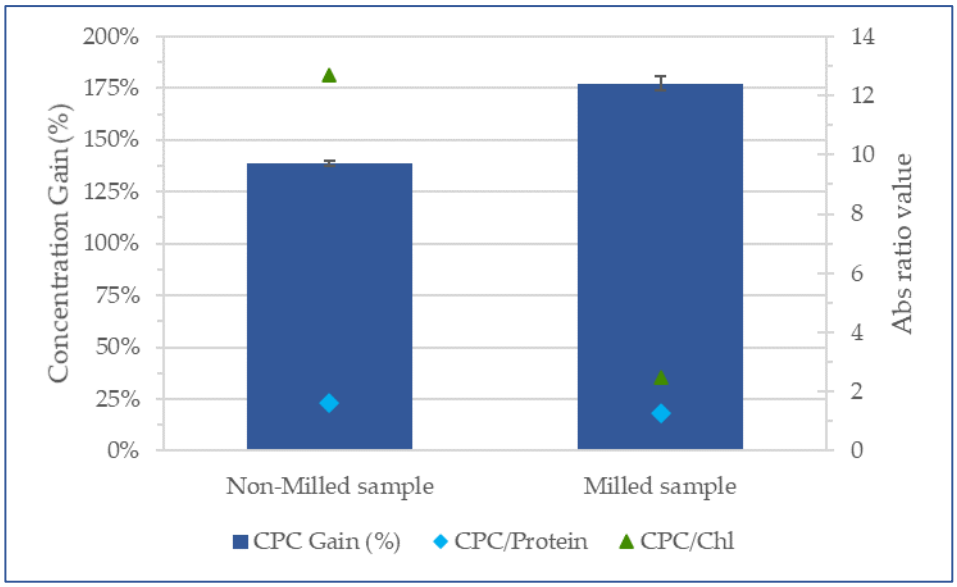

Figure 48 - Salting Out - Concentration Gain (%)

The purity values, either in relation to proteins or chlorophyll, are the same as those shown in the CPC recovery indicator. As such, it is possible a priori to notice that the results obtained in the uncut sample have a higher degree of accuracy than the sample from the pilot mill, due to the CPC/Chl value being substantially higher. CPC values in this trial showed a 138,6% increase in CPC from the initial extract to the precipitate on the bead-free sample, the milled sample showed a superior growth in CPC concentration with an increase of 177,3%, These values imply the success of this method within the proposed objectives. Although the milled samples show much higher concentration gain values when compared to the milled sample, the significant differential in the initial purity in these samples does not allow them to be compared directly.

Through the analysis of the results of this test, it seems clear that CPC extracts benefit from salting out and there is improvement in all indicators studied. There is an increase in significant concentration in phycocyanin, a high recovery rate, and an improvement in the two purity indicators, relative to total proteins and chlorophyll.

3.5. *Arthrospira (Spirulina) platensis* biorefining route

As the salting out trial closed the laboratory and pilot trials it was possible to draw a proposal for a biorefining route for Arthorspira to extract phycocyanin with the highest purity achievable. The following Figure 49 illustrates the proposed biorefining route developed for phycocyanin extraction based on the results achieved in this work. This proposal summarizes the options which resented the best results throughout this work being the optimal route for a pure and concentrated phycocyanin extract.

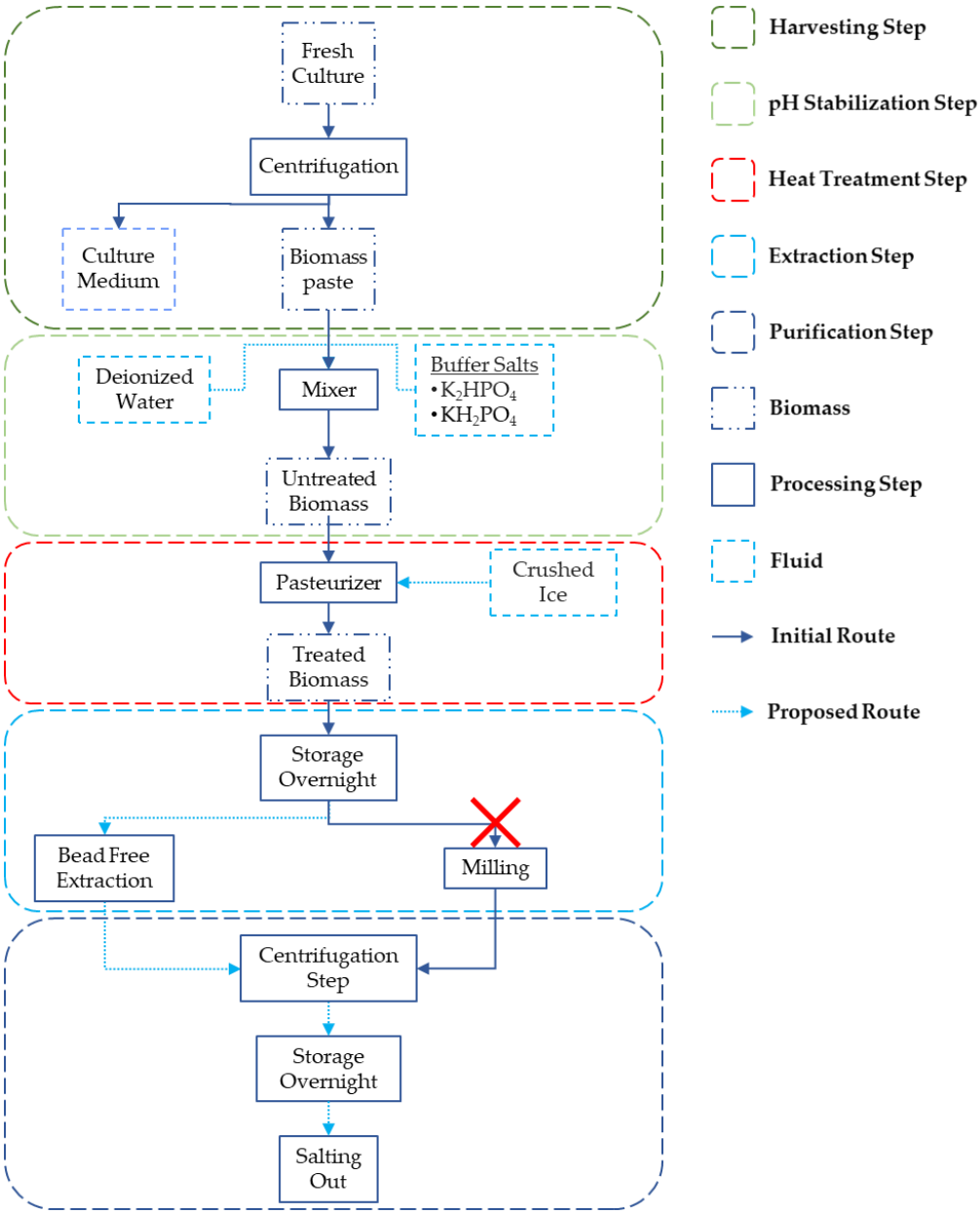

Figure 49 - Biorefining route proposal

4.
Conclusions and Future Perspectives

4.1. Conclusion

During the development of PQP, it was possible to conclude that among the different centrifugation times analyzed the best results were obtained in the interval ranging from 30 min to 50 min of centrifugation with the best result obtained at 50 min, with no remarkable influence from the freezing cycles. As far as the ideal concentration for extraction, the most concentrated sample (2C g/L) presented the best results, proving not to be saturated. The dilution solvent chosen was potassium phosphate buffer confirming the close link between pH stability and phycocyanin stability. As regards the extraction time, as there was no benefit in extending the extraction beyond the milling time when at room temperature, it was decided not to carry out any rest period, in contrast, a minimum resting period at the temperature of "T-10" ^{o}C has a positive impact on phycocyanin extraction, ensuring, on average, a 10% increase in phycocyanin extracted.

The heat treatment procedure was successfully developed and, at the longer period, was able to stabilize phycocyanin for a week at 4^{o}C. Among the ramps tested in the hydraulic tests, only the delta ramp was able to emulate the natural cycle of the pilot pasteurizer on the premises, being only overhauled by the improved delta ramp, which implies cooling as the most relevant aspect in the ramp optimization. The tests with different fluids corroborated the tests performed in the development of the phycocyanin quantification protocol, being the potassium phosphate buffer solution that showed better results.

After the optimization of the temperature curve in hydraulic tests, the treatment ramp was tested with biomass and successfully fulfilled the objectives, achieving results similar to those obtained in the laboratory, being able to stabilize phycocyanin.

The milling and extraction chapter presented new and unexpected results as a new and improved extraction route was discovered. Although the milling tested, presented the three flowrates performing similarly with no major differences distinguishing them from one another, and the microscopic analysis revealed that the samples milled at F and 2F L/h had very similar degrees of rupture, the sample from milling at 3F L/h appeared to rupture degree slightly lower than the rest. The best result was achieved with the new procedure a bead-free extraction designed, developed, and applied in this work. The overnight extraction in the absence of beads proved to be the best option, assuming the second extraction in a mandatory

way. This new alternative showed very promising results, obtaining the highest and purest phycocyanin extracts from all trials.

The preliminary salting out experiments tested the precipitation yield and successfully compared the values obtained with the company's internal previous values, the concentration of 50% of saturation proved to be the optimal point between a higher CPC recovery and lower precipitation of the remaining proteins. Salting out improved purity indicators in all assays performed, with a lower concentration of proteins and chlorophyll when compared to phycocyanin.

The results obtained during this work achieved the objectives initially proposed for the heat treatment and milling of fresh Arthrospira Platensis in addition to the quantification, extraction, and purification of phycocyanin, pigment of interest in this work.

The most important conclusions are the possibility of removing the influence of chlorophyll for the first time by centrifuging for 50 minutes and achieving a complete extraction through resuspension. Regarding the heat treatment, it was possible to design a root process and proved effective for the desired time window.

4.2. Future Perspectives

While achieving the objectives from this work new topics for further study were discovered.

Although this work presented a feasible proposal to correctly quantify the phycocyanin, the influence of chlorophyll on its value is not to this day quantifiable, therefore establishing a correlation between the two pigments at different concentrations may be of use. Regarding the thermal treatment, it could be of interest to study the effectiveness of the three studied ramps in the industrial equipment in order to scale up the process from the current pilot procedure. In relation to the extraction process, one might optimize the rest period for extraction, from the current "overnight" to the minimum effective time, by testing different resting periods. Additionally, the possibility of constant agitation may improve both the extraction period (decreasing it) and the first extraction efficiency. Although the methods developed in this work guarantee a food-grade extract, it would be interesting to study different pathways to increase its purity, which will bring more commercial value.

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