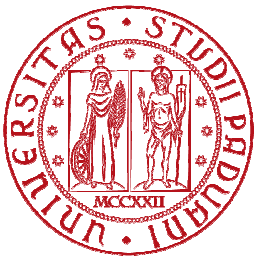

UNIVERSITÀ DEGLI STUDI DI PADOVA

DIPARTIMENTO DI INGEGNERIA INDUSTRIALE

Corso di Laurea Magistrale in
Ingegneria chimica e dei processi industriali

PROTEIN SEPARATION AND CHARACTERIZATION FROM *Neochloris Oleoabundans*

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Dedicata a mia mamma Anna

“Il segreto della esistenza umana non sta soltanto nel vivere,
ma anche nel sapere per cosa si vive”

Fëdor Michajlovič Dostoevskij

Contents

Abstract

The production of proteins from microalgae was investigated to find sub products with an exploitable market value.

Water soluble proteins from three different strains (*Neochloris Oleabundans*, *Chlorella vulgaris* and *Desmodesmus*) were examined. pH range of highest precipitation (80%, 76%, 89%) was found around 3.5. Anionic Exchange Chromatography was used as the separation system for microalgae's proteins. A mixture of amines solutions was used as eluent between pH 3 and 10.5. Unlikely the titration curves several proteins eluted at very high pH (> 10.0). For *Neochloris Oleabundans* most part of proteins, according to chromatograms, eluted in the first 150 minutes (10.5<pH<9.5). Eluted fractions were submitted to electrophoresis (native and SDS) and the most recurrent molecular weights were in the range 50-70kDa. Large proteins were detected (500-800kDa) and the last eluting proteins (5.2<pH<3.5) had similar molecular weights (~110kDa with subunits of 30kDa and 95kDa) at steady and exponential growth phase of *Neochloris Oleabundans.* Although the chromatographic patterns were different for both growth phases, the electrophoretic ones seem to have similar protein composition.

Based on the experimental results and on literature data, two processes have been designed for the production and separation of RuBisCO from microalgal biomass.

Sommario

Introduzione

L'obiettivo di questo lavoro è stato quello di studiare la separabilità delle proteine contenute nelle microalghe e successivamente di caratterizzarle. Lo scopo era il confronto delle proteine solubili in acqua contenute nella *Neochloris Oleoabundans* durante le fasi di crescita esponenziale e stazionaria. L'attenzione è stata poi focalizzata sul RuBisCO, la proteina più abbondante negli organismi fotosintetici, poiché accreditata di idoneità agli usi alimentari.

Le microalghe costituiscono la fonte dei biocombustibili di terza generazione.
Le prime volte di cui si è parlato di biocombustibili risale a circa venti anni fa, ma solo negli ultimi anni, a causa dell'aumento notevole della richiesta dei carburanti ottenuti da fonti fossili, ha preso piede l'idea che investimenti in quel campo possano cambiare l'economia dei trasporti. Con il termine biocombustibili di prima generazione si fa riferimento alla produzione di bioetanolo da fermentazione dell'amido di mais, o dal processo dei semi oleosi. I biocombustibili di seconda generazione sono invece quelli derivati da trattamenti di conversione della biomassa lignocellulosica: i principali processi di sintesi sono la pirolisi, da cui si ottengono prevalentemente oli vegetali, la gassificazione in presenza di aria e acqua da cui si ottengono syngas, eteri, o Diesel attraverso il processo di Fischer Tropsch e la fermentazione volta a produrre bioetanolo. Le microalghe rappresentano appunto la terza generazione che si sta sviluppando e su cui vengono investiti capitali consistenti. Le microalghe sono microorganismi che possono vivere sia in acqua marina che dolce purché in presenza di sufficienti nutrienti. Grobbelaar, (2004) ha dato una delle prime stime della richiesta di nutrienti e l'ha espressa con una formula:

$$CO_{0.48}H_{1.83}N_{0.11}P_{0.01}$$

Successivamente è stato stilato un elenco più preciso delle sostanze necessarie alla crescita della *Neochloris Oleoabundans*, fra cui magnesio, manganese, potassio, sodio, molibdeno, facendo in modo che l'unico fattore limitante per la crescita fosse la luce (Pruvost, Van Vooren, Cogne, & Legrand, 2009). Per la lista dettagliata si rimanda alla Tabella 1, pagina 5990 dell'articolo citato.

Le classi dei prodotti principali ottenibili dalle microalghe sono tre: oli, proteine e carboidrati. Gli oli sono prevalentemente costituiti da acidi grassi polinsaturi, con un grado di insaturazione medio, maggiore rispetto ad altri oli vegetali, e ciò li rende di più difficile trattamento e stoccaggio in quanto più suscettibili all'ossidazione. A livello europeo lo standard di insaturazione richiesto non deve superare 1% molare di composti con grado di insaturazione maggiore o uguale a 4 (Chisti, 2007). La trans esterificazione è il processo chimico necessario per trasformare gli oli vegetali grezzi in biodiesel. Nella Tabella 1.2 tratta dall'articolo di Yusuf Chisti,

(2007), si può notare come, assumendo realisticamente un contenuto di olio del 30% sul peso secco, se il 2.5% della superficie agricola USA fosse dedicato alla coltivazione delle microalghe, esso sarebbe sufficiente per soddisfare la metà del fabbisogno di carburante per trazione degli USA stessi.

Un altro prodotto delle microalghe sono le proteine: lo studio di (Wolkers, Barbosa, Kleinegris, Bosma, Wijffels, & Harmsen, 2011) propone una stima del contenuto di materiale commercialmente utile all'interno delle microalghe, e le proteine ricoprono circa la metà del prodotto valorizzabile. La principale destinazione pensata per il contenuto proteico è l'arricchimento di prodotti alimentari e mangimi. Gli esperimenti sono stati condotti esclusivamente sulla frazione proteica solubile in acqua, in quanto il recupero delle proteine precipitate o intrappolate nella fase solida aumenterebbe notevolmente i costi di lavorazione. Il terzo prodotto principale sono i carboidrati, contenuti all'interno della parete cellulare.

Materiali e metodi

In Fig.2.1 è raffigurato il processo schematico che consente l'analisi del contenuto proteico partendo dalla microalga stessa. Dopo aver raccolto le alghe, il primo step è la rottura della parete cellulare per permettere il rilascio del contenuto. Il funzionamento dell'apparecchiatura adottata allo scopo è raffigurato in Fig.20. La rottura delle cellule è ottenuta per attrito in un piccolo volume di forte agitazione mediante pellets di zirconia-ittrio; il macinato viene osservato periodicamente al microscopio per valutare se la rottura delle cellule sia sufficiente. Ultimata la frattura cellulare, il liquido cellulare è centrifugato per separare il surnatante, contenente le proteine solubili in acqua, dal precipitato, formato prevalentemente dalla membrana cellulare e dalle proteine intrappolate nella precipitazione. Il liquido surnatante viene poi microfiltrato e sottoposto a una misura della concentrazione delle proteine mediante il metodo di Lowry (Lowry, 1951). Il surnatante può essere così dializzato nel buffer di equilibrazione della cromatografia liquida per ridurre la conduttività dei campioni eliminando gran parte dei sali residui dell'ambiente di crescita delle alghe. A questo punto il campione è pronto per essere studiato mediante cromatografia anionica liquida per separare le proteine della miscela grezza. Per effettuare questa separazione si è adottato un gradiente di pH nel range [10.6-3]. Le proteine eluiscono in prossimità del loro punto isoelettrico, pH al quale la carica netta della proteina è quasi nulla e le interazioni con la resina ionica della colonna cromatografica molto deboli. Il campionamento viene eseguito manualmente in corrispondenza di aumenti del segnale (picchi) rilevati dalla cella UV che lavorava sulla lunghezza d'onda di 280nm, frequenza alla quale gli amminoacidi Istidina e Triptofano mostrano risonanza ed emettono quindi un segnale. I campioni sono poi dissalati per rimuovere le ammine presenti nei buffer utilizzati nel HPLC (High Performance Liquid Chromatography) e liofilizzati per aumentare notevolmente la concentrazione delle proteine e renderle quindi rilevabili negli esperimenti successivi. Dopo questi trattamenti i campioni sono pronti per lo studio mediante elettroforesi e analisi immunologiche. L'elettroforesi è stata condotta su gel di poliacrilamide nativi, volti a rilevare il

peso molecolare totale della proteina non denaturata, e riducenti, per rilevare il peso molecolare delle sub unità o il peso molecolare della proteina stessa nel caso in cui non presenti struttura quaternaria. Dal confronto fra i pesi molecolari rilevati nelle due fasi di crescita della *Neochloris Oleoabundans* è stato possibile rilevare le similarità ma soprattutto le proteine differenti fra fase esponenziale e stazionaria. Per la rilevazione delle proteine è stata utilizzata la colorazione argentica, in quanto la colorazione a blu Coomassie non si è dimostrata sufficientemente sensibile. L'esperimento finale condotto è stato il Western blot, un metodo immunologico volto alla rilevazione di una proteina obiettivo mediante l'impiego degli anticorpi. Essi hanno infatti funzione di ligandi che successivamente permettono il legame con una sostanza otticamente attiva rilevatrice per visualizzare la presenza della proteina.

Risultati e discussione

Per fare il design sperimentale della cromatografia liquida è stata necessaria una titolazione con acido della miscela proteica grezza. In particolare questo studio serve a identificare il range di pH in cui si ha la massima precipitazione, e dove quindi il pH deve variare più lentamente durante la cromatografia. I risultati della titolazione sono mostrati nelle Figure 3.2, 3.3 e 3.4 ,rispettivamente per le specie microalgali di *Chlorella Vulgaris*, *Neochloris Oleoabundans* e *Desmodesmus,* ed evidenziano come il valore minimo di solubilità delle proteine, detto punto isoelettrico di miscela, sia compreso fra pH 3.5 e 4.5. Questo intervallo è ragionevole in quanto il RuBisCO, la proteina più abbondante, ha un punto isoelettrico di 4.4-4.7 (Barbeau & Kinsella, 1988) e durante la sua precipitazione può avvenire del trascinamento di altre proteine. Un'ipotesi interessante formulata riguarda la ridissoluzione delle proteine per pH inferiori al pI di miscela. In Fig. 3.1 (Antonov & Soshinsky, 2000) viene mostrato il comportamento del RuBisCO al variare del pH. In particolare si può notare come a pH inferiore del punto isoelettrico di miscela il RuBisCO si ridissolva. Considerato che il RuBisCO è, come già detto, la proteina più abbondante nella miscela considerata, le specie *Chlorella vulgaris* e *Desmodesmus* sulla base di quanto detto, hanno una presenza maggiore di questa proteina, in quanto le proteine ridissolvono rispettivamente del 77% e 67% a fronte del 42% della *Neochloris Oleoabundans*.

Le soluzioni buffer per la cromatografia liquida sono state preparate seguendo quanto già fatto da (Ahamed, et al., 2007) e la composizione finale delle due soluzioni buffer è riportata in Tabella 3.4. Il buffer per l'equilibrazione serve a fare partire gli esperimenti sempre dalle stesse condizioni e uniformare il sistema a pH=10.6, mentre il buffer per l'eluizione genera il gradiente di pH programmato, riportato in figura 3.7. Abbassando il pH, cala selettivamente la solubilità delle proteine, ovvero avvicinandosi al punto isoelettrico, la proteina non ha più energia sufficiente per rimanere legata alla resina anionica ed esce.
Nonostante la conduttività sia stata uniformata per ridurre interferenze nella cromatografia per presenza disomogenea di ioni, i cromatogrammi presentano similarità solo nei primi 150 minuti e negli ultimi 50 minuti. Nella parte centrale, molte proteine sono rilevate solo nella *Neochloris Oleoabundans* in fase esponenziale, mentre nella stazionaria risultano assenti. Lo studio dei pesi

molecolari si è basato sul confronto dei risultati dei gel nativi e di quelli denaturanti, per avere simultaneamente più informazioni riguardo la proteina, ovvero peso molecolare, peso molecolare delle sub-unità e punto isoelettrico. La quantificazione è stata sviluppata svolgendo l'integrazione dei picchi del cromatogramma, ma essendo la frequenza di rilevamento del segnale propria dei soli due amminoacidi nominati in precedenza,questo metodo risulta non accurato. Si noti infatti come in corrispondenza di segnali molto elevati nel cromatogramma non sempre corrispondano bande rilevanti nei gel dell'elettroforesi. Questo effetto è dovuto alla possibile sottostima/sovrastima del segnale in funzione di quanta Istidina e Triptofano siano presenti; un secondo fattore è la non linearità della colorazione argentica che non può quindi essere usata per la determinazione del quantitativo di proteine contenute nel campione.

E' stato poi quantificato quanto RuBisCO fosse contenuto nelle diverse microalghe. L'esperimento è stato condotto su un gel riducente di poliacrilamide e il calcolo è stato fatto mediante integrazione ottica della banda rilevante tale proteina attraverso bande di calibrazione fatte con una quantità nota di RuBisCO (Fig.3.25). Il risultato ha confermato l'ipotesi proposta basandosi sulla titolazione con acido cloridrico, ovvero hanno confermato il contenuto sensibilmente più elevato di RuBisCO delle specie *Chlorella Vulgaris* e *Desmodesmus* rispetto alla *Neochloris Oleoabundans*. In particolare il contenuto del RuBisCO nelle prime due specie è di circa il 30% mentre nella *Neochloris Olabundans* varia dal 9% al 20% rispettivamente per fase di crescita stazionaria ed esponenziale.

Sviluppo del processo

Sulla base di risultati di laboratorio dai dati di letteratura vengono proposte due soluzioni di processo e di impianto per la purificazione del RuBisCO. Il primo processo riflette fedelmente la procedura adottata in laboratorio per la purificazione delle proteine ed è quindi orientato al recupero della frazione proteica solubile in acqua. È stato calcolato che mediante questo processo possono essere recuperati 15 g RuBisCO/kg biomassa. Un processo alternativo prevede la dissoluzione di tutte le proteine all'interno della fase liquida e a tal fine è prevista l'addizione di solvente la cui composizione è uguale al buffer di equilibrazione utilizzato nella separazione cromatografica. In questa maniera si possono recuperare tutte le proteine, consentendo anche un recupero maggiore in RuBisCO. Per entrambi i processi il surnatante contenente le proteine è separato dai frammenti di membrana cellulare mediante centrifugazione e il precipitato viene inviato a fermentazione per produzione di biogas. Il biogas prodotto nei due casi può sviluppare una potenza di 0.4 KW nel primo caso e 0.16 KW nel secondo adottando una base di calcolo di 2kg di biomassa/ora.

Conclusioni

Lo scopo di questo progetto di tesi sperimentale è stato ampliare la conoscenza sulle proteine solubili in acqua contenute nelle microalghe. Mediante titolazioni con HCl si è provato come l'intervallo di massima precipitazione delle proteine sia fra pH 3 e pH 4. Successivamente separazioni cromatografiche hanno evidenziato un profilo di eluizione diverso fra la fase esponenziale e stazionaria della *Neochloris Oleoabundans*. Studi elettroforetici in gel nativi e riducenti hanno mostrato punti in comune nei pesi molecolari fra le due fasi di crescita della microalga in esame. Dopo aver quantificato il RuBisCO presente nelle diverse specie è stato possibile spiegare la diversa ridissoluzione delle proteine una volta raggiunto un pH minore del punto isoelettrico di miscela. Il RuBisCO misurato nella fase esponenziale della *Neochloris Oleoabundans* è circa il 20% delle proteine totali, mentre nella fase stazionaria è circa il 9%.
Per lo sviluppo del processo ci si è basati quindi sulla raccolta dell'alga nella fase esponenziale, ed è stato prevista una produzione di 15g RuBisCO/kg microalghe.

1.INTRODUCTION

Since the last decades, many efforts have been spent in the direction of green fuels and reduction of CO_2 emissions. A strong investment in researches in this field has been needed due to the limited amount of available petroleum in the oil fields and to the increasing temperature of the Earth. Anyway, research efforts may be not enough if the human and politic sensibility do not help the spread of results and methodology to improve production systems.

Since a couple of decades the term "biofuels" has started its wide-spread among the world. The aim of the companies that produce these "biofuels" is to make economically affordable fuels from renewable resources. The US Council in 2000 defined the term biomass as "organic matter that is available on a renewable or recurring basis (excluding old growth timber), including dedicated energy crops and trees, agricultural food and feed crop residues, aquatic plants, wood and wood residues, animal wastes, and other waste materials". A significant improvement has taken place in biofuels field and that is why a clarification about their stages of progress is needed. The first difference is about the utilization: primary biofuels consist on biomass (wood, pellets, vegetable wastes) that is burnt to produce heat. Instead of a direct utilization, secondary biofuels are obtained from chemical processes and they have a further classification in three different classes (Naik, Goud, Rout, & Dalai, 2010). The first generation of bio-fuels has been intended to produce fuels from corn starch for producing bio-ethanol but the economic value of the product is just slightly higher than all the production costs (Bounds, 2007). The first way to produce oil was by a thermochemical process called pyrolysis. In this process the biomass is heated at 500-800°C in absence of oxygen: in this way many products could be observed like acidic oils, that need further treatments, charcoal and sub-products such as CO_2 and H_2O. Pyrolysis has not been used for long time because the efficiency was around 50% and it was not profitable. Gasification is another thermochemical process, but it is not meant to produce oil from biomass. It has been largely used in syngas (mixture of CO and H_2 and traces of CO_2) production. The process consists in a high temperature treatment of biomass with air and water steam. The products are mainly CO and H_2 that could be used directly in turbines or for the synthesis of chemicals such as methanol, dimethyl ether and Fischer Tropsch Diesel. Another example is the transesterification of vegetable oil to produce bio-diesel that consists on the mixture of esters produced by this chemical process. The best known process is FAME (Fatty Acid Methanol Esterification, Fig. 1.1). For this purpose rapeseeds and soy were used and chemically transterified to produce biodiesel. Many efforts were also spent to produce biodiesel by transesterification of cooking oil and waste of animal fats.

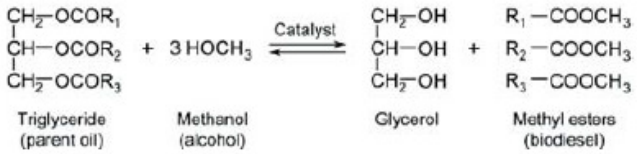

Fig.1.1. The figure represents the FAME process (Chisti, 2007)

In the second generation of biofuels, researches were focused on the conversion of the cellulose into glucose by enzymes and the further fermentation of the obtained sugars to ethanol. Many raw materials that were used for the production were lignocellulosic materials such as straw, wood and grass. Another route was gasification of agricultural residues to obtain syngas (mainly CO and H_2) then used for Fischer Tropsch applications or methanol production, and pyrolysis of agricultural residues. In the third generation efforts have been focused on microalgae and especially on how to produce bioethanol, biodiesel and hydrogen from microalgae (Dragone & Fernandes, 2010). The third generation is represented by biofuels produced by microalgae. Microalgae are cell factories that convert CO_2 to primitive compounds that can be processed to produce biofuels, foods, feed and other high value bioactives. Microalgae produce several biofuels such as methane obtained by anaerobic digestion of biomass and biodiesel from microalgal oil. Biodiesel is commercially produced from plant and animal oils, but the option of manufacturing it from microalgae is now taken into account more seriously due to the enhancing petroleum price (Chisti, 2007).

1.1 Microalgae

Microalgae are small plant-like organisms having a size of 1-50 micrometres in diameter. They make part of the aquatic biomass together with macroalgae and large aquatic plants. There are hundreds of thousands of existing microalgal species but just a few tens of thousands have been described in literature. They live both in freshwater and seawater and a single cell cannot be seen with naked eye, but usually when the concentration increases they turn the water colour to green, brown blue or orange (Fig. 1.2). Most species contain chlorophyll, so they can use sunlight to convert carbon dioxide into oxygen and biomass. Many products make part of the biomass, such as fatty acid, proteins, colorants, anti-oxidants and starch that can all be used in many everyday utilities (Pulz & Gross, 2004).

Fig.1.2 On the left an example of microalgae cultivation. On the right some fed-batch reactors of different varieties of microalgae.

Microalgae need a specific living setting to perform the highest productive condition. They should be exposed to light and the medium should provide the right compounds in the right

quantity. Nutrients amount can be estimated from the approximate molecular formula for the microalgal biomass:

$$CO_{0.48}H_{1.83}N_{0.11}P_{0.01}$$

presented by (Grobbelaar, 2004) even if other nutrients such as iron should be added. A dedicated analysis was made on *Neochloris Oleoabundans* by (Pruvost, Van Vooren, Cogne, & Legrand, 2009). They discovered that optimal growing conditions for this microalgal strain requires magnesium, sodium, copper, molybdenum, and calcium; all the salts required are reported in Table 1, page 5990 of the quoted scientific paper.

Phosphorus should be added in excess because phosphates add complex with metal ions. Microalgae growth depends on many factors such as the size of the inoculum, the specific growth rate of the strain, and the capacity of medium and culturing conditions to support the growth. Main phases of growth are (Fig.1.3): lag, exponential, stationary, declining. Lag phase happens especially after inoculums in different growth conditions from the previous. Lag phase is usually proportional to the time that the inoculums spent in declining-death phase. Exponential phase is the measure of the growth of cells in the time and it depends on the culture parameters and the relation between medium, growth rate and size of the inoculum. Declining phase usually occurs when the biomass content becomes very high and the medium is running out of nutrient salts. Cells enter the stationary phase when the net growth is equal to zero and depending on the limiting nutrient, cells can undergo biochemical variations. Fig.1.3 shows the common behaviour of microalgae growth.

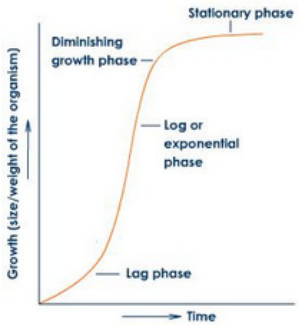

Fig.1.3 Typical example of growth rate and growth phases of microorganisms.

Microalgae oil content is various, and high quality fatty acids like omega-3 and omega-6 are even contained. Microalgae may become the source for omega-3, presently obtained from fishes (Wolkers, Barbosa, Kleinegris, Bosma, Wijffels, & Harmsen, 2011). From further analysis it was developed that the lipid mass in *Dunaliella* specie is comprised between 45% and 55% of the total organic mass and the most abundant fatty acids are palmitic (16 Carbon atoms), linoleic (18 C) and palmitoleic (16 C) fatty acids (Sheelan, Dunahay, Benemann, & Roessler, 1998). The second important class of compounds found in microalgae are proteins. Most part of protein content estimations are based on so called crude proteins, mainly used in food and feed. The

protein has to be declared safe for human consumption before being used in food applications. Tests have to be performed to gain the quality and safety certification and especially for microalgae, which are encountered in unconventional protein sources, several requirements have to be satisfied. Many tests have already been taken and serious anomalies were not found neither in short or long term experiments nor in analysis on chronic toxicity. None of the tests taken have revealed any restriction for using properly processed microalgae for human scopes. That is why from these results microalgae seem to be promising for the quality of proteins even higher than conventional plants proteins (Becker, 2007). The third most important compound-class found in microalgae are carbohydrates. Unlike the most part of land plants, microalgae usually do not contain simple carbohydrates or easily hydrolysable polysaccharides. Linear carbohydrates can be found but they are often derivatized with acids or complexing groups like sulphate group. That means that industrial fermentation is not such an easy task because there are not known industrial yeast strains able in fermenting the most part of microalgae carbohydrates. The above mentioned are the most important compound-classes that can be observed in microalgae. So many products can be achieved from processing these microorganism that the efforts and researches performed in that field seem to be fully justified.

1.2 Biofuels

Microalgae represents the third generation of biofuels. At the beginning of the researches reduction of the cost was not the top priority, because the market of these products was small and the market price of the products very high. But in larger market of raw materials, such as fatty acids for producing biodiesel, the reduction of costs is of vital importance to make affordable the comparison between microalgal raw products and other sustainable raw materials. Many efforts have been spent to increase the productivity of this system because the production of biofuels does not pay-back fixed and production investments. One alternative to such an economic situation is the profiting of more sub-products from microalgae instead of focusing the attention just on biofuels (Wolkers, Barbosa, Kleinegris, Bosma, Wijffels, & Harmsen, 2011) . It is estimated that a reduction of the growing costs down to 0.5€/kg biomass would make feasible the use of microalgae for biofuel production (Wolkers, Barbosa, Kleinegris, Bosma, Wijffels, & Harmsen, 2011) since the typical composition of the algal biomass is 40% lipids, 50% of proteins and 10% of sugars. With this assumption an approximate economic value of the biomass is possible and Fig.1.4 shows the distribution of the economic potential for every product that can be achieved. With the composition assumed the total economic value of the biomass would be 1.65 €/kg biomass. Biorefinery costs are not included in both estimations, but the range of income is wide and allows to affirm that with improvements in the productive chain, biofuels will be economically feasible.

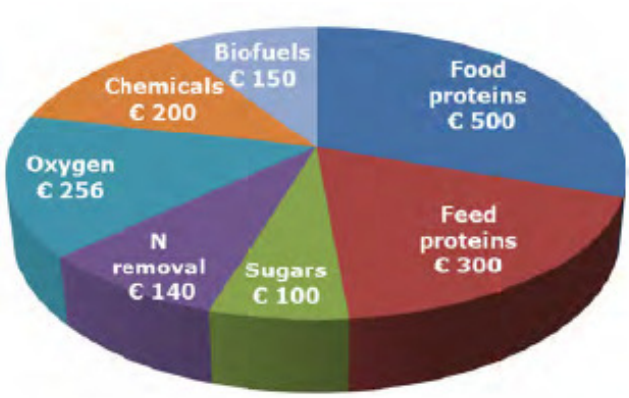

Fig. 1.4. The picture represents the distribution of various products from microalgae on a base scale of 100 kg of microalgal biomass (Wolkers, Barbosa, Kleinegris, Bosma, Wijffels, & Harmsen, 2011)

The production of methane from microalgae has been proposed since the 50's using wastewater as inoculums, however not much work has been done in this field. Recently the U.S. Department of Energy listed three main biobased fuels as the main ones from microalgae, namely:

i. Production of methane gas via biological or thermal gasification
ii. Production of ethanol via fermentation
iii. Production of biodiesel

A potential fourth option would be burning directly the biomass to produce steam and electricity. However, it would miss the transportation fuel purpose and its emphasis as an environmentally sustainable fuel, which is one of the most important issues for developed economies (i.e. USA). (Sheelan, Dunahay, Benemann, & Roessler, 1998)

Microalgae store organic matter through the photosynthesis. Microalgae find CO_2 in atmosphere as well as anthropogenic gases like flue gases from fossil power plants. SunChem process was designed to produce methane from gasification of microalgae. It is mainly composed by 5 different steps as shown in Fig. 1.5. The first step is represented by the production of the biomass in the PBR (photobioreactor). In this stage the fixation of CO_2 via the photosynthesis leads to the synthesis of biomass. In the second step excess water is removed mechanically and recycled to the PBR. The algae concentration reached is approximately 20 wt% dry mass. The biomass is then liquefied by heating it up to 450°C at a pressure of 30 MPa . In the fourth step the organic stream is catalytically gasified under hydrothermal conditions to Bio-syngas, with methane as the main product. In the last step methane is separated from CO_2, that is recycled to the PBR and methane is partially used to provide the heating needed in the process. (Haiduc, Brandenberger, S., F., Bernier-Latmani, & Ludwig, 2009).

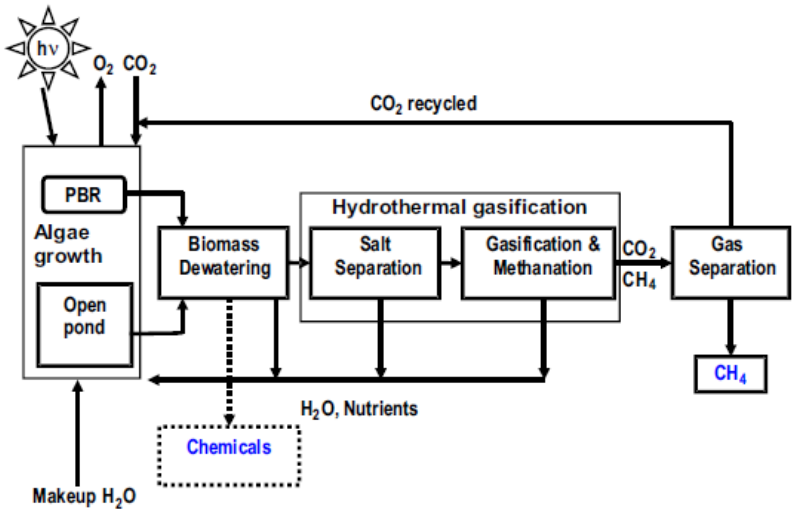

Fig.1.5. The figure shows the Block flow diagram of the SunChem process. (Wolkers, Barbosa, Kleinegris, Bosma, Wijffels, & Harmsen, 2011)

Some species of algae, such as *Chlorella vulgaris* and *Chlamydomonas perigranulata* can produce ethanol and other alcohols through heterotrophic fermentation of starch. The microalgaes synthesizes starch by CO_2 fixation (photosynthesis) with further anaerobic fermentation under dark condition. The process usually consists on closed photobioreactors, with metabolically enhanced microorganism producing alcohols while resisting to high temperature, high salinity and even high ethanol levels. One of the key parameters is the use of CO_2 coming from power plant to feed microalgae and accelerate their growth. This technology is expected to produce up to 10,000 gallons per acre per year in the next years (Krassen, 2007). Also other alcohols, such as methanol and butanol can be produced in microalgae and the heavier alcohols have an energy density almost equal to gasolines but they are not produced for commercial purposes (Sheelan, Dunahay, Benemann, & Roessler, 1998)

Microalgae can produce oils and every specie has its own capacity, as shown in Table 1.1 (Becker, 2007). Microalgae can accumulate significant quantities of triacylglycerol's (TAG) that are important fuel precursors indeed biodiesel can be achieved by a transesterification process, and FAME (Fatty Acid Methanol Transesterification), is the most used and is shown in Fig.1.1. Three moles of alcohol are required to esterify the triglyceride, but the reaction is reversible so 6 alcohol moles per each mole of triglyceride are used on industrial scale to make the highest percentage possible of triglycerides react (Chisti, 2007). Chemical and enzymatic catalysts were studied to improve the process. (Fukuda, Kondo, & Noda, 2001) shows that alkali-catalysts can react with a 6:1 butanol:soybean ratio, despite of acid-catalysts that requires a 30:1 ratio. Furthermore (Chisti, 2007) proposed some scenarios about the 50% conversion of the USA transports from fossil fuels to bio-based fuels (Table 1.2).

Table 1.2 shows that microalgae can provide the highest concentration of biofuels per land unit. Even considering the lowest concentration of oil in microalgae, investing 5% of the whole US cropping area would be enough to cover the 100% of US fuel demand with biofuel of microalgaes. These data show clearly that biofuels from microalgae require less land for growing

and they does not exploit feed source, that would be a strong response to the ethical question about the investment of ground for producing fuels instead of food. It is acknowledged that fossil fuels cannot last forever and that is why researches about microalgae are required to improve the yield of fuel production.

Table 1.1. General composition of different algae (% of dry matter) (Becker, 2007)

Alga	***Protein [% on dry matter]***	***Carbohydrates [% on dry matter]***	***Lipids [%on dry matter]***
Anabaena cylindrica	43-56	25-30	4-7
Aphanizomenon flos-aquae	62	23	3
Chlamydomonas rheinhardii	48	17	21
Chlorella pyrenoidosa	57	26	2
Chlorella vulgaris	51-58	12-17	14-22
Dunaliella salina	57	32	6
Euglena gracilis	39-61	14-18	14-20
Porphyridium cruentum	28-39	40-57	9-14
Scenedesmus obliquus	50-56	10-17	12-14
Spirogyra sp.	6-20	33-64	11-21
Arthrospira maxima	60-71	13-16	6-7
Spirulina platensis	46-63	8-14	4-9
Synechococcus sp.	63	15	11

Table 1.2. Comparison of some sources of biodiesel (Chisti, 2007)

Crop	***Oil yield (L/ha)***	***Land area needed (Mha)***	***Percent of existing US cropping area[a]***
Corn	172	1540	846
Soybean	446	594	326
Canola	1190	223	122
Jatropha	1892	140	77
Coconut	2689	99	54
Oil palm	5950	45	24
Microalgae[b]	136900	2	1.1
Microalgae[c]	58700	4.5	2.5

a For meeting 50% of all transport fuel needs of the US **b** 70% oil (by wt) in biomass **c** 30% oil (by wt) in biomass.

1.3 Proteins

Proteins are organic macromolecules made of amino acids which are molecules containing an amine group, a carboxylic group and a side-chain. The side-chain determines the nature of the amino acid. Key elements of amino acids are carbon, hydrogen, oxygen and nitrogen. Amino acids are bound with a peptide bond and that is why proteins are also called polypeptide. Peptide bond is generated with the formation of a molecule of water. Proteins are fundamental for organism life and they take part in several processes. Their structure is characterized at four different levels. The primary structure refers to amino acid sequence. Amino acids are bound together by peptide bonds. The ends of the polypeptide chain have a carboxyl group and an amine group. The secondary structure is referred to local sub-structures. Main types of secondary structures are alpha helix and beta sheets and they present a regular structure. The tertiary structure is referred to the 3-dimensional structure of the proteins. It is referred to the arrangement among secondary structures. The structure is stable only when specific tertiary interactions take place such as salt bridges and disulfide bonds. The quaternary structure is the gathering of multiple protein chains (polypeptides) and its stabilized by the same interactions of the tertiary structure. Tertiary and quaternary structures makes a protein functional, while the secondary structures itself do not.

Protein are thermo-sensitive molecules, and that is why protein samples should be kept in a fridge or in a freezer. According to (Kampinga, 1995) proteins start to denaturate. This change in protein structure can be due to thermal vibrations and collision between molecules. Protein solubility is pH-depending. (Schwenzfeier, Wierenga, & Gruppen, 2011) showed that water soluble proteins from *Tetraselmis* present the lowest solubility value between the pH values of 3 and 4. Fig.1.7 presents their results changing the pH and the salinity of the solution with different kinds of microalgal protein solution. This is a consequence of the Isoelectric Point, that changes from protein to protein and it is a pH value at which the protein gains a neutral electric charge. Fig.1.6 shows the behaviour of Zeta potential that is depending by the electric charge while changing the pH. The solubility is strictly connected to the charge, because if the protein does not have any electric charge on it, intermolecular interaction are not possible.

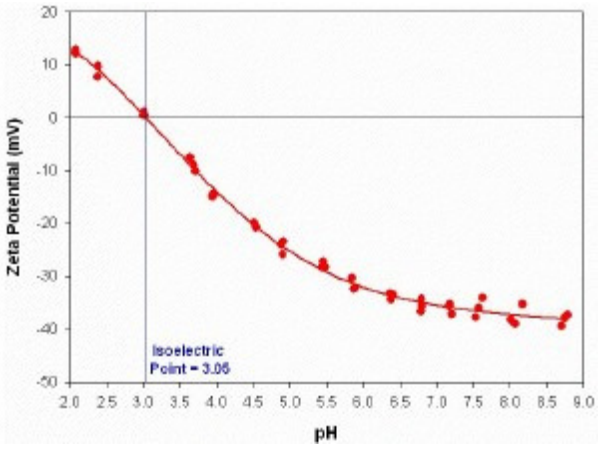

Fig.1.6 The picture represents the behaviour of Zeta potential at pH-variations

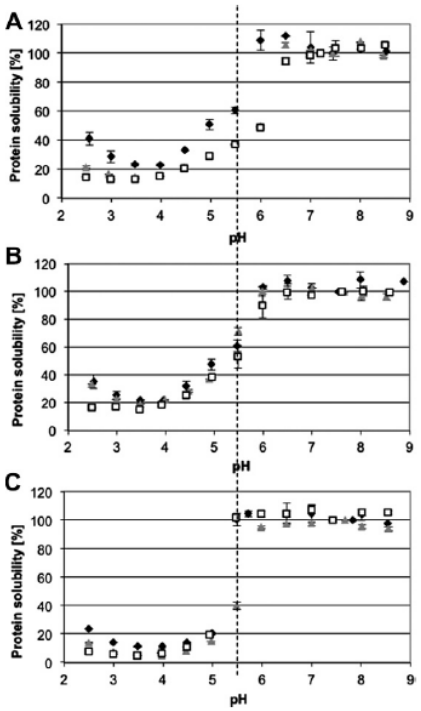

Fig.1.7 Protein solubility as a function of pH for algae juice (A), crude algae protein isolate (B) and final algae protein isolate (C) at different ionic strengths (I=0.03M (▪), 0.2M (▲) I=0.5M (□). 100% =soluble protein concentration at pH 7.6.
Dashed lines indicates pH 5.5. Error bars According to standard deviation (Schwenzfeier, Wierenga, & Gruppen, 2011)

1.3.1 Protein quantification method

Concentration of total proteins was determined by Lowry method that was published by Oliver H. Lowry in 1951. The method is based on the reactions of copper ions with the peptide bonds under alkaline conditions. The Lowry method is based on the reaction of Cu^{+} ion produced by the oxidation of peptide bonds, with Folin-Ciocalteu reagent (a mixture of phosphotungstic acid and phosphomolybdic acid in the Folin-Ciocalteu reaction). This method is recommended for this type of analysis because it is 10 to 20 times more sensible than ultraviolet (280nm) absorbance reading, it is less disturbed by turbidity, and it is 100 times more sensible than Biuret reaction. The list of all interfering substances with the Folin-Ciculteau's reagent is elsewhere reported (Lowry, 1951). The main disadvantages of this method are that the amount of color can change with different proteins and that the optical density is not strictly correlated to the protein concentration. Despite of this disadvantages the Lowry assay is still the most recommended way to measure low protein concentration (Lowry, 1951). For this assay the recommended protein concentration range, according to (Lowry, 1951) is [0.1-1 g/L] to avoid proteins concentration values on the hysteresis range of the calibration line. The experiment is conducted under alkaline condition by adding NaOH to the samples that enables peptide bonds to become negative charged. It is even noticed that in presence of copper, working in alkaline conditions increase several times the color of the samples. Samples are then incubated at 95°C to make the proteins more linear and to increase the rate of the copper reaction. After the addition of Folin's reagents the samples has to be placed into the darkness and then the absorbance can be read at 750nm. In Fig.1.8 (Lowry, 1951) is shown that the highest value of the absorbance is reached after 30 minutes of adding the Folin reagents.

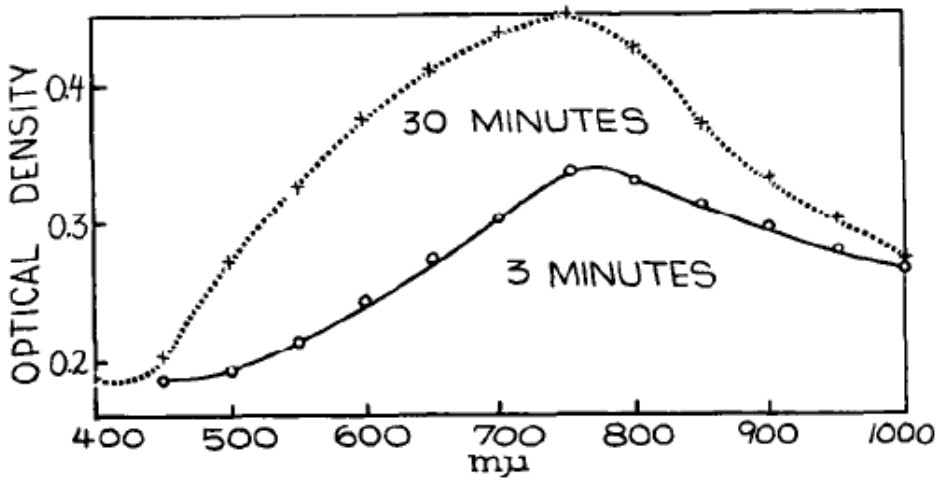

Fig.1.8. Dependence of the Optical density of the samples treated with the Folin-Ciculteau's phenol by the time passedin the darkness (Lowry, 1951).

1.3.2 Protein separation

To purify soluble proteins from microalgae some steps are required and they are shown in Fig.1.9.

After cell disruption, the rich protein aqueous phase is separated from the cell debris by centrifugation. The supernatant must be submitted to further protein fractionation/purification. Many different ways of separating proteins are feasible: liquid chromatography, electrophoresis on gel, immunologic methods, precipitation, extraction and filtration. Many types of liquid chromatography are known: the basic liquid chromatography is based on adsorption of proteins on an insoluble granular matrix. Smaller proteins are characterized by a larger retention time than larger proteins. This fact is because small proteins can enter the pores of the beads so the way through the gel is longer than for larger molecules that cannot get retained within particles. Ionic exchange chromatography is another kind of liquid chromatography and is itself divided in two kinds: isoelectrofocusing (salt gradient) and chromatofocusing (pH gradient) and they both take place in a charged chromatographic resin. Protein's charge depends on the pH: the higher the pH, the lower the charge. In this way the different proteins charge allow different retention times due to the different binding strength to the resin, which is modulated by the salt gradient (isoelectrofocusing) or by the different protein isoelectric points in the pH gradient (chromatofocusing). The affinity chromatography is specific for the detection and isolation of one protein. The target protein is retained into the column by specific ligands. The other proteins are eluted by washing with an eluent solution. The target protein is then released by washing the system with a solution at high concentration of the retaining ligands. The stationary phase must be stable, made of specific substances retaining the target protein, just weak interactions with the target compound to not compromise its elution pattern. The chromatography can be improved with HPLC (High performance Liquid Chromatography, Fig. 9) or UHPLC (Ultra-high performance Liquid Chromatography, Fig.10). For the calibration, the

injection of a sample pure protein with a known concentration is required. In this way the calculation of the concentration of the target proteins in the sample will be possible (van Dongen & van Berkerl, 2010).

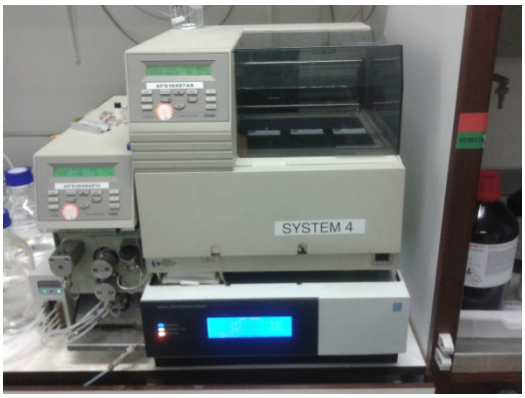

Fig.1.9. HPLC instrumentation (Picture of BPE HPLC)

Fig.1.10. UHPLC instrumentation

Another way of separating proteins is electrophoresis. A molecule with electric charge can move in an electric field. On a gel that act as a molecular sieve, proteins are separated by the electric charge differences. In SDS-PAGE (Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis), the SDS breaks all the non-covalent bonds and the complex protein is negatively charged. The migration is then possible due to the protein charge and the migration of the proteins into the gel is related with the molecular weight of the protein, larger proteins migrate slower than the small ones. Isoelectric focusing (IEF) is another gel-based technique in which separation is based on the differences between isoelectric points of proteins. The gel has a pH gradient in itself and the electric field is used to allow the migration of the proteins through the gel. Once the proteins reach their isoelectric point their net charge is zero and they stop. 2-Dimensional PAGE (Fig.1.12) is the union of IEF followed by SDS-PAGE. The first dimension is carried out with Isoelectric focusing on a gel that allows separation of proteins by their pH in an electric field. The second dimension consists on a SDS-PAGE that allows proteins with isoelectric points close to each other to be separated by molecular dimensions to guarantee a higher resolution.

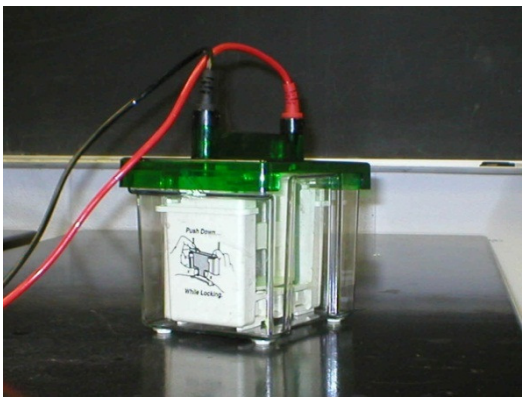

Fig.1.11. SDS-PAGE instrumentation

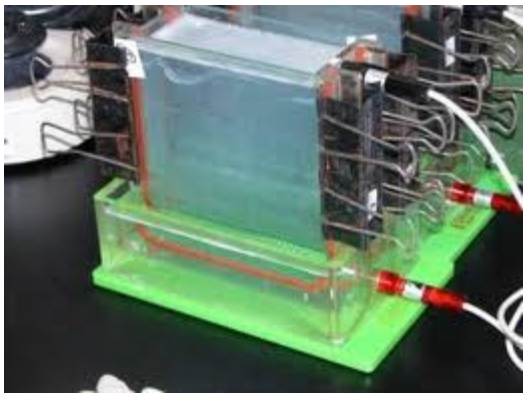

Fig.1.12. A 2-Dimensional PAGE instrumentation

Precipitation and extraction is a further method to separate proteins. Due to surface properties of proteins, they can interact with salts, other proteins and solvents that allow them to be transferred to a solid phase (precipitation) or liquid (extraction). Precipitation takes place when protein solubility reaches a critical value due to increasing (salting out) or decreasing (salting in) in salt concentration, or adding chemical compounds like PEG (polyethylene glycol). Commonly used salts are ammonium sulphate: EDTA is used to remove impurities often contained into ammonium sulphate.

A summary of the main ways of proteins precipitation and the dominating mechanisms is shown in Table 1.3.

Table 1.3. Ways of precipitation and mechanism ruling the process

Agent Class	***Example***	***Mechanism***
Polar solvent	Water	Salting-in (decrease in ionic strength)
Salt	Ammonium sulfate Potassium chloride	Salting-out (increase in ionic strength)
Polymer	Polyethylene glycol Polyethyleneimine	Salting-out
Temperature	Heat	Denaturation and aggregation
pH	Acid	Reduce ionic charge of proteins
Non-polar solvent	Ethanol	Reduce activity of water

Most precipitates are a mixture of proteins with close elution point. A first precipitation can be done to remove less soluble proteins than target protein, and the second to precipitate the product.

Liquid-liquid extraction happens when two different liquid phases are created. Starting solution is often an aqueous solution with PEG and when a salt is added, two different phases are formed. The top solution is usually enriched in polymer, and the lower, more dense, is enriched in salt. Partition coefficient K is defined as:

$$K = \frac{c_t}{c_b} \tag{1}$$

where c_t is the concentration of the substances in the top phase and c_b is the concentration of the substances in the bottom phase. Distribution of proteins depends on the protein behaviour and the nature of the two phases. Several parameters can be changed to reach the optimal settings for the separation such as the chemical nature and the molecular weight of primary and secondary polymers, the type of salt, the concentration of salt and polymer, pH and temperature.

Filtration is a separation by size exclusion of compounds. It consists of a selective barrier that does not allow transition of all the proteins. Filtration is usually divided into microfiltration

when the range is between 0.05µm and 2µm. Ultrafiltration requires smaller pores in the membrane and their size are usually between 0.2µm and 1nm.

Microfiltration is mainly used to remove suspensions from liquid phases. Ultrafiltration can be used to retain dissolved molecules. The pores are small enough to avoid passing of proteins but passing of salts is still allowed. Fig.1.13 shows diameter molecular ranges for different filtrations.

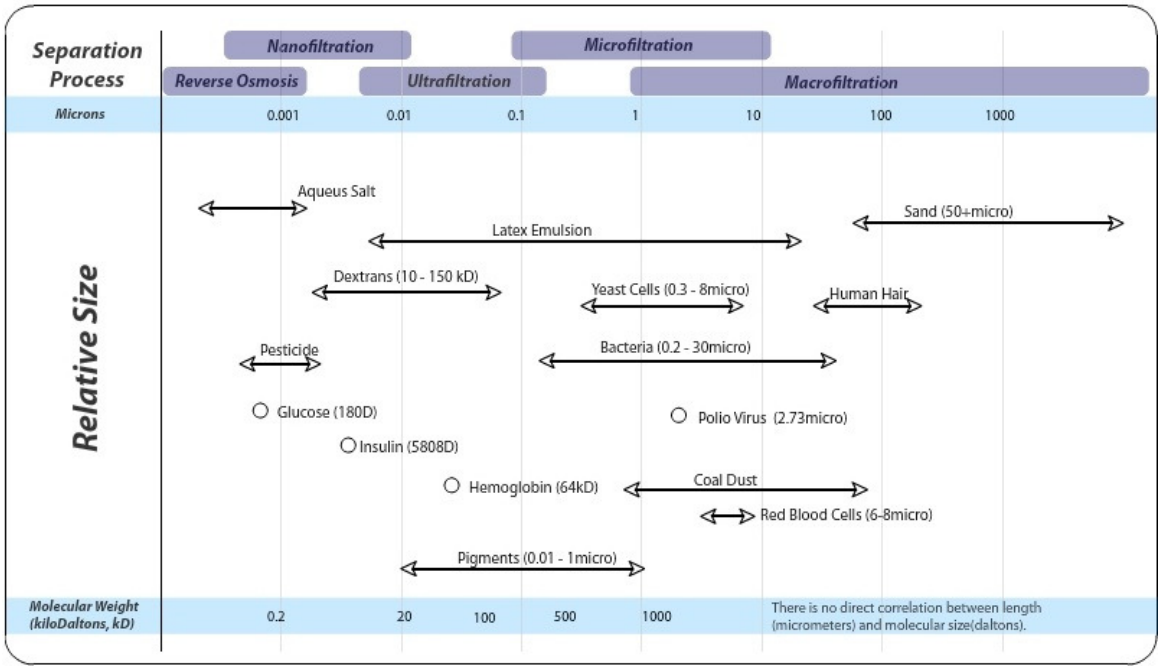

Fig.1.13. Range of molecular diameter of filtration

According to (Ahamed, Ottens, Nfor, van Dedem, & van der Wielen, 2006) chromatography is very important for biomolecules separation because of its high-resolution. Many different types of chromatography are possible and the most important aspect that has to be considered is the partitioning behaviour of target and contaminant molecules.

Ionic exchange chromatography allows separation of molecules due to the charge that they carry. IEC is made by a stationary phase and a mobile phase. The stationary phase is a matrix gel that acts as the ion carrier. The mobile phase consists of solutions adopted for the separations plus the sample that has to be fractionated. Separation of molecules is possible because of differences in charge, charge density and distribution of charge on molecules surface. Control of these differences in interactions between molecules and the resin can be handled by changing the ionic strength or the pH of the mobile phase. There are two different IEC, namely, Anionic Exchange Chromatography (AEC or AEX) and Cation Exchange Chromatography (CEC or CEX). In AEC the charges on the resin are positive and they exchange anions, in the CEC the resin is negatively charged and cations are exchangedd. The separation in both cases can be conducted through a pH gradient or a salt gradient. The pH gradient is based on the change of the electric charge that proteins get at different pH. Proteins get detached from the column when the pH

reaches a value close to its own isoelectric point, for the weakening of the bond between the resin and the protein. The salt gradient is based on the different strength of the bonds that proteins can make with the resin which is weakened by the increase of salt concentration in the eluent. pH-gradient was designed as the target separation because it allows the detection of isoelectric points of the eluting proteins giving further information on proteins behaviour. Ionic exchange chromatography conducted by pH gradient is based on reaching the isoelectric points of proteins, that gives further informations on protein's behaviours. At the isoelectric points proteins reach the lowest solubility, and the elution into the column happens very close to this pH value. The chosen IEC was the Anionic Exchange Chromatography because at hight pH the proteins are completely dissolved into the solution as will be seen in further chapters (§3.1 HCl titration of protein solutions). The pH-gradient starts at 100% in equilibration (or loading) buffer and finish at 100% in elution buffer. For a chromatography by pH gradient, some steps are required. At first the column needs to be equilibrate with at least 5 CV (Column Volume) with the loading buffer to run the experiments from the same initial conditions. After equilibration the sample can be injected and proteins are adsorbed in the gel due to their negative charges. Substances not bound to the column are washed from the equilibration buffer. Desorption of molecules bound to the gel occurs switching from the application conditions to elution conditions by decreasing the pH.
Effectiveness of this technique has already been proved. (Ahamed, et al., 2007) used this method with several proteins to compare elution-pH with the isoelectric point. Results showed different protein behaviour between protein with acidic, basic and neutral pI. Elution point for acidic (pI<6) and basic (pI>8) test proteins was found to be usually within 0.5 pH units from the isoelectric point. Neutral test proteins (6<pI<8) tend to elute at a higher pH than the isoelectric point, usually at about a pH value of 9.

1.3.3 Protein characterization

Once proteins are separated by AEC informations about them are required. Many different methods are suitable for gaining different informations about proteins. Electrophoresis allows proteins separation due to the differences in molecular weights. Electrophoresis can be conducted in native or reducing gels. Native gels are meant to separate proteins by their whole molecular weight. SDS (sodium dodecyl sulphate) gels denaturate proteins by detaching them into their subunits that are separated by molecular weight.

A more specific manner to identify target protein is Immunoblotting or Western blotting. Western blotting is a technique that allows detection of the presence of specific proteins by inserting the specific antibody into the system. At first separation of proteins is required by 3-D structure for native proteins or polypeptide length for denatured proteins. Proteins are then transferred to a membrane typically made of nitrocellulose, where proteins are probed with the specific antibody.

FT-IR (Fourier transformate InfraRed) is used to detect secondary structures of proteins. With this method, the sample is irradiated by all the wavelengths of the infrared spectrum. In the range of the "functional groups", between 3800 and 1300 cm^{-1}, functional groups such as N-H, O-H, C-H, C=C, C=O, N=O are stretched or deformed. (Ruegg, Metzger, & Susi, 2004) explained what was the effect of some wavelengths on proteins. $1585cm^{-1}$ and $1400cm^{-1}$ causes an asymmetric and symmetric stretching of side-chain COO-, respectively.

MALDI-TOF made of a TOF (Time Of Flight) analyzer and a MALDI (Matrix Assisted Laser Desorption Ionization) source gives accurate information about structure and molecular weight of biomolecules such as peptides, carbohydrates and proteins. The sample is mixed with the matrix and then placed on a steel target to crystallize before entering the vacuum chamber. Ions are extracted from the target with a laser and with the time of flight through an electric field the molecular weight can be determined.

1.3.3.1 SDS-PAGE

SDS- PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) is a technique widely used to separate proteins. It is used to estimate molecular mass, relative concentration and distribution of proteins among the fractions. The aim of this method is separation of proteins by molecular mass through an electric field (electrophoresis). The SDS is a very common separating method that uses a discontinuous polyacrylamide gel as a support medium and sodium dodecyl sulfate for denaturation of secondary and non-disulfide-linked tertiary structures. The aim of SDS PAGE is to separate denatured proteins, that means a separation based just on the primary structure. SDS is an anionic detergent, so when macromolecules are dissolved in it, they are negatively charged, that enables protein migration through the gel. These charges swamp the inherent charge of the proteins and give to every protein the same charge-to-mass ratio. The velocity of movement of macromolecules is directly proportional to the electric field strength and the charge of molecules and inversely proportional to the size of molecules and the viscosity of the gel. The sieving properties of the gel allow the proteins moving with different velocities, depending on their molecular size. Fig.1.14 shows the procedure for a electrophoresis with a SDS (or Native) gel.

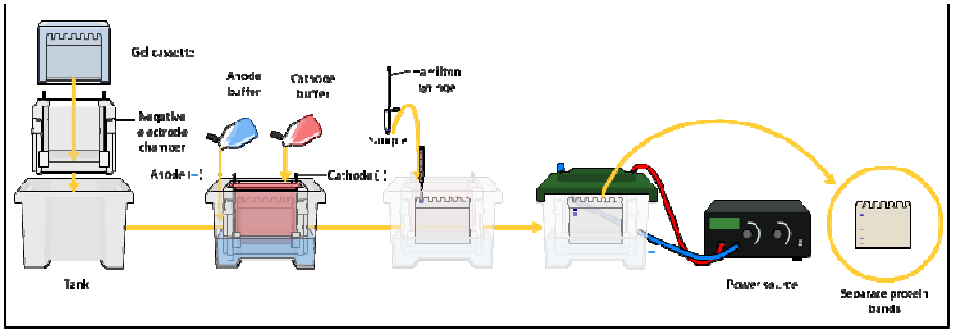

Fig.1.14. General procedure for making a PAGE experiment running.

Inserting a known protein sample is required: this sample is called “ladder” and it allows to recognize molecular weight of the unknown samples in the remaining channels. In Fig. 15 the ladder protein is ran simultaneously with the samples to have the protein molecular weight standard, and molecular weights of samples are then detected. When buying a ladder protein, the place of the bands is related with the molecular mass of proteins contained with an exponential function. Development of the gel can be done by Coomassie blue staining or silver staining.

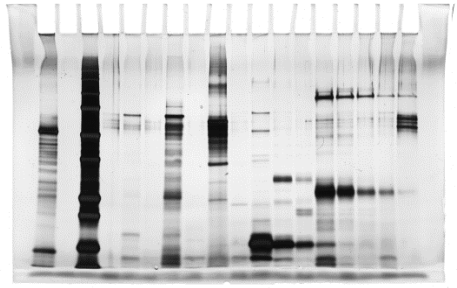

Fig.1.15. A SDS-PAGE gel example. In the 3rd well the ladder is clearly visible and unknown samples in other wells are detected

1.3.3.2 Native – PAGE

Native Page is an electrophoretic way for separating proteins and is run in non-denaturing conditions. Due to their native conditions, proteins are separated not only by their molecular weight and intrinsic charge but also by the cross-sectional area: this separation process then, depends also on the molecular shape. Native PAGE results can be important for studying changes in charge and conformation due to degradation and it is used also for revealing protein molecular weights even if this measurement is not completely reliable because of the dependence of migration on the shape of the protein.

1.3.4 RuBisCO

RuBisCO (Ribulose-1,5-bisphosphate carboxylase oxygenase), is the most common and large protein in green microalgae. It is considered as the most abundant protein on earth because of its presence in every photosynthesizing cell. RuBisCO is predominant in microalgae and may contribute up to 50% of all the protein cell content (Feller, Anders, & Mae, 2008). RuBisCO’s molecular weight is around 550kDa, but it can change from plant to plant. The protein structure is made of 16 subunits, 8 bigger (LS) and 8 smaller (SS), respectively about 55 kDa and 13 kDa. Small differences have been found in large subunits of RuBisCO from different plants, while rather wide variations were seen in the smaller subunits. RuBisCO from tobacco has a polypeptide chain of 477 aminoacids and shows 90% and 92% homology grade with RuBisCO’s Large Subunit from maize and spinach. Small subunits’ chains are made of 123 aminoacids but

the homology grade is respectively 70% and 75%. (Barbeau & Kinsella, 1988). The subunit model was confirmed with X-ray diffraction, electron microscopy and optical diffraction measurements. Several studies have been conducted on tobacco leaves and in Fig.1.17 the arrangement of the subunits in two layers about a cylindrical hole of around 20 Å is shown.

RuBisCO's main function in plant cells is to catalyze CO_2 fixation for the photosynthesis. According to (Barbeau & Kinsella, 1988) it loses rapidly its enzymatic function once isolated. Since the enzymatic activity of RuBisCO is difficult to preserve, this protein could have bulk applications in the food industry rather than as isolated catalyst. As seen in Fig.1.4 almost half of the value of the sub-products from microalgae is gained from the food and feed applications of proteins. RuBisCO's food potential is very high because it has an excellent set of aminoacids. Based on FAO/WHO reference patterns RuBisCO can reach a chemical score of 98 over 100, as can be seen in Table 1.4 (Barbeau & Kinsella, 1988). RuBisCO has been found to have also good emulsifying properties, greater than ovalbumin but lower than BSA. It was also found that RuBisCO has a good solubility that is usually the problem by application of protein as emulsifying agents.

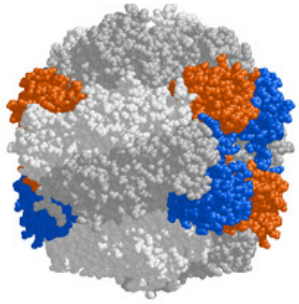

Fig.1.16. RuBisCO's structure:
the large subunits (grey),
the small subunits. (orange and blue, Wikipedia)

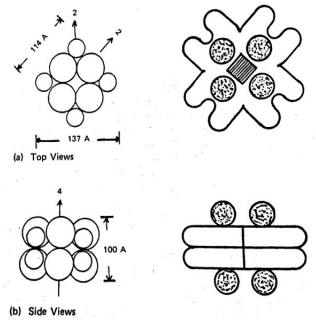

Fig.1.17. RuBisCO's top and side views.
(Barbeau & Kinsella, 1988)

Table 1.4. Comparison of the essential amino acid composition of RuBisCO with the FAO/WHO Reference Pattern (Barbeau & Kinsella, 1988)

Amino acid	*FAO/WHO [-]*	*Whole egg [-]*	*Casein [-]*	*Soybean meal [-]*	*RuBisCO [-]*	*Chemical score [-]*
Lysine	5.5	6.4	8.0	6.9	6.5	>100
Tryptophan	1.0	1.2	1.3	1.3	2.7	>100
Threonine	4.0	5.0	4.3	4.3	5.3	>100
Cystine and methionine	3.5	5.5	3.5	2.4	3.4	98
Isoleucine	4.0	6.6	6.6	5.1	4.9	>100
Valine	5.0	7.4	7.4	5.4	6.7	>100
Leucine	7.0	8.8	10.0	7.7	9.4	>100
Tyrosine and phenylalanine	6.0	10.1	11.2	8.9	12.8	>100

Proteins for food application in most cases cannot be used as crude powders and they should have a similar behaviour to proteins already present in foods and the final result is that organoleptic properties such as taste and colour have become more important than the nutritional value. Proteins give the food some physicochemical properties called functional properties like solubility, gelation, emulsifying and foaming. In Table 1.5 is shown a comparison between RuBisCO from spinach, soybean and tobacco. Tobacco RuBisCO is taken as a reference of comparison for Soy proteins isolates and RuBisCO from spinach (Barbeau & Kinsella, 1988).

Table 1.5. The table shows a comparison between Tobacco (reference), spinach RuBisCO and soy proteins isolates (Barbeau & Kinsella, 1988)

Properties		***Tobacco RuBisCO***	***Soy proteins***	***Spinach RuBisCO***
Solubility		Reference	Worse	Better
Gelation		Reference	Worse	Comparable
Emulsifying	Low pH	Reference	Worse	---
	High pH	Reference	Better	---
Foaming		Reference	Worse	Comparable

1.4 Aim of the work

This thesis is aimed to study protein separation and characterization from microalgae and to propose a possible industrial process to recover target proteins such as RuBisCO. The developed procedure is general and it was applied to Neochloris Oleoabundans since this micro algal strain has a very high lipids productivity.

2.MATERIALS AND METHODS

One aim of this work was the separation of the water soluble fraction of proteins by Anionic Exchange Chromatography with a later characterization by electrophoresis. Attention is focused on the differences between exponential and steady phase of *Neochloris Oleabundans*, using *Chlorella vulgaris* to improve HPLC parameters and electrophoresis procedure. In Fig.2.2 is shown the growth profile of *Neochloris Oleabundans* used for comparison between the growth phases. Algae in Exponential and Steady phase were taken at 6^{th} and 13^{th} day cultivation, respectively. The process started from the whole microalgal solution but for this study just the water soluble fraction has been examined because extracting trapped proteins into the precipitate would cost more money in terms of chemicals and process steps. So thinking about a practical application, at first the economical feasibility would have been studied just on this protein fraction.Milling of the cells was at first required to release water soluble proteins. After some purification steps (centrifugation, microfiltration, dialysis) samples were ready for AEX. Samples from chromatography were desalted, freeze dried and later used for electrophoresis in native and reducing gels. In fig. 2.1 the summary of the process is shown.

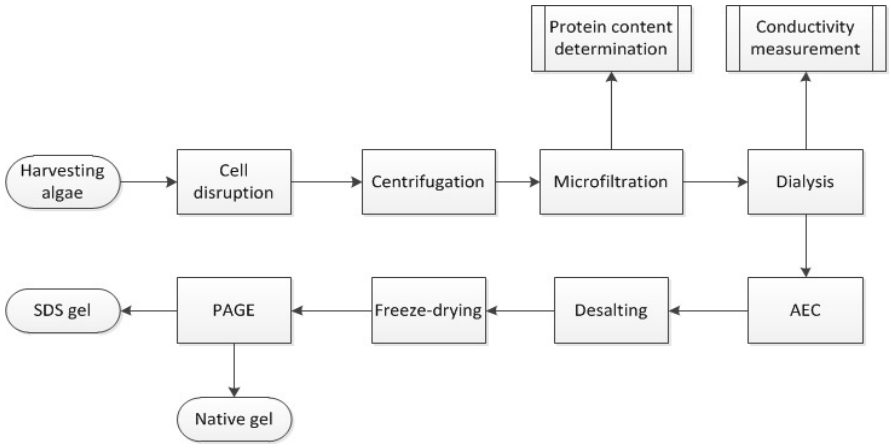

Fig.2.1. Scheme of the process used to analyze soluble proteins content of microalgae

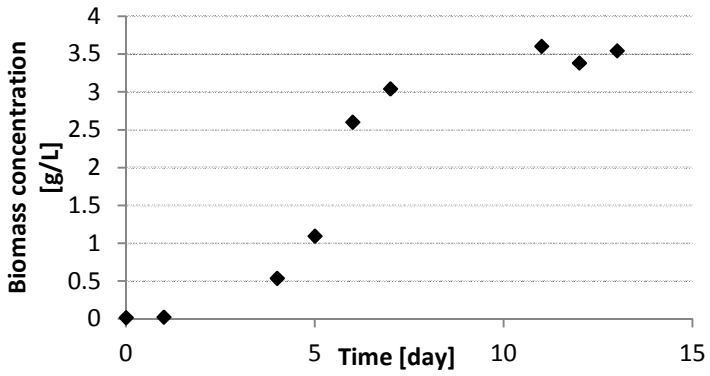

Fig. 2.2. Growth of *Neochloris Oleabundans*.

2.1 Cell disruption

Proteins are not directly analyzable, because they are enclosed in the cell, so the breakdown of the cell wall was necessary. Bead milling was the way used to break the cell wall. Thermal treatments were discarded because integrity of proteins would have been compromised.
"Dyno Mill Multi Lab" was the instrument used and in Fig. 2.3 a scheme of it is reported.

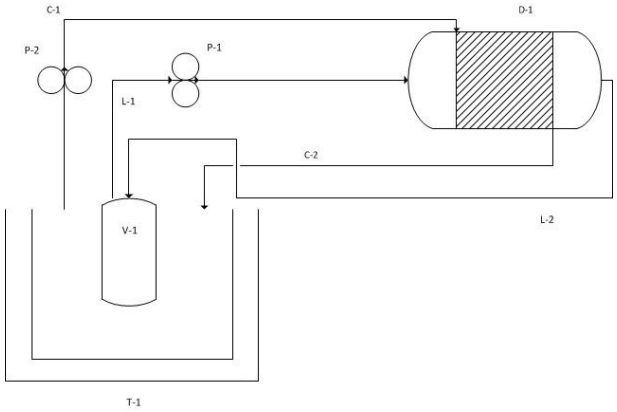

Fig. 2.3. The scheme of the "Dyno Mill Multi Lab" is represented. Microalgae are contained in the jar V-1, L-1 and L-2 are respectively the inlet and the outlet pipelines for microalgal cell solution in the instrument (D-1), T-1 is a jacketed-open vessel for cooling of V-1 (vessel containing the microalgal mixture) and C-1 (milling chamber) and C-2. P-1 and P-2 are peristaltic pumps.

Disruption of cells was done by strong mixing between the liquid microalgal solution and zirconia-yttrium beads. The mixing chamber was cooled to avoid a temperature increase. The algae solution is pumped by peristaltic pumps P-1 and P-2. Pressure (2 bar) and temperature (50°C) were controlled to avoid degradation of the products. V-1 was dipped in the jacketed vessel T-1 that was also necessary to chill the cooling water for the mixing chamber of D-1. Depending on the micoralgal specie the process lasted between 30 and 50 minutes. The duration was estimated by the experience of previous milling processes with the same instrument. Samples during cell-breakage were visualized in an optical microscope. Three microalgal species were used: *Neochloris Oleabundans*, *Desmodesmus sp.* and *Chlorella vulgaris* and the durations of the process were 30 minutes for Neochloris Oleabundans and 50 minutes for *Desmodesmus sp.* and *Chlorella vulgaris*, respectively.

2.2 Protein content

The procedure for the determination of protein content is the Lowry assay, based on (Lowry, 1951). Several compounds are used to perform this experiment and they are listed in Table 2.1.

BSA (Bovine Serum Albumin) is used to do the calibration line (0.4 g/L). Details of the calibration line are given in Table 2.2.

Table 2.1. Composition of compounds used in the Lowry's method

Name	***Composition (g.L^{-1})***
Folin I	20 Na_2CO_3
Folin II	10 $CuSO_4 \cdot 5H_2O$
Folin III	20 g/l sodium potassium tartrate
Folin IV	9 g/l NaCl
Folin V	80 g/l NaOH
Folin complex	Mix of Folin I : Folin II : Folin III in a ratio 100:1:1
Folin-Ciculteau reagent	50% v/v Folin Ciculteau's in water

Table 2.2. Samples composition for the standard line.

Calibration line samples	***0***	***1***	***2***	***3***	***4***	***5***
BSA (0,4 g/l)[µL]	150	120	90	60	30	0
Folin IV [µL]	0	30	60	90	120	150
BSA [g/L]	0.0	0.08	0.16	0.24	0.32	0.4

In each tube a protein solution of 150 µL should be prepared, making sure that concentrations of unknown sample proteins are within the concentrations range of the standard line [0.0-0.4] g/L. Then 75 µL of Folin V were added to each sample. After mixing, samples were placed at 95°C for 30 minutes. Afterwards, samples were cooled down and 750 µL of Folin complex and 150 µL of Folin Ciculteau's reagent were added. The samples were mixed, centrifuged and placed in darkness for 30 minutes. After this time the absorbance was read by a Beckman DU640 spectrophotometer at 750nm. Lowry procedure is summarized in Table 2.3.

Table 2.3. Procedure for the Lowry experiment.

Protein sample size [µL]	***Adding Folin V [µL]***	***Action***	***Action***	***Adding Folin complex [µL]***	***Adding Folin reagent [µL]***	***Action***	***Action***	***Action***
150	75	Vortex	95 ^{o}C – (30 min)	750	150	Vortex	Darkness (30 min)	Absorbance (750 nm)

2.3 Centrifugation

After bead milling the cells a first centrifugation was conducted at 18,879g for 15 minutes in a Beckman J2-MC centrifuge. A second centrifugation was carried out at 31,360g for 12 minutes and it is made twice. Both of the relative G-forces are calculated at the average radius. Between the first and the second centrifugation of both stages the precipitate should be removed from the bottom of the bottle. After centrifugations the final supernatant has a light green color and

is ready for microfiltration, to remove all the remaining solid (pore size 0.2 µm). Used centrifuge is shown in Fig.2.4.

2.4 Dialysis

Dialysis is the final purification stage. Dialysis cassettes (Thermo Scientific "Slide-A-Lyzer® Dialysis Cassette G2", 3.500 MWCO, 15 mL) were filled with the microfiltrated protein solution and submerged into 4 liters of the loading buffer solution used in the HPLC. Dialysis aim is reducing conductivity of the samples and remove low molecular weight compounds. The process lasts 2 days, and takes place on a magnetic stirrer at 5 °C in darkness. Buffer solution were changed twice.

Fig.2.4.. Beckman J2-MC Centrifuge with the biggest rotor on it.

2.5 Anionic Exchange Chromatography

HPLC with Spectrasystem P4000 pump model equipped with a Alltech Elite™ Degassing System, a Spectrasystem AS3000 auto-sampler model and a DIONEX Ultimate 3000 UV detector was used. Chromeleon 7.1 software was used to record data. The column was a Mono Q™ 4.6/100 PE from GE Healthcare. It is a strong anion exchanger made of quaternary aminoethyl, on a matrix made of rigid and porous beads of polystyrene, the flow rate range is [0.5-3] ml/min and the maximum tolerated pressure 40 bar. The used buffer solutions and their molar compositions are shown in Table 2.4.

Fig.2.5 shows the inlet column pH used for separation of proteins from *Chlorella vulgaris and Neochloris Oleabundans*. Comparison between pH profiles at the inlet and outlet of the column for separation of proteins from *Neochloris Oleabundans* is shown in Fig.2.6. The duration of the

separation process was set to 625 minutes, the maximum time allowed from the instrumentation. All experiments were conducted with a flow rate of 0.5 ml/min. Samples were taken when a peak appeared on the screen.

Table 2.4. Composition of adopted buffer solution for AEC. Composition expressed in millimolarity.

Compound	***Equilibration buffer (pH=10.5) (mM)***	***Elution buffer(pH=3.00) (mM)***
Piperazine	20	20
Piperidine	20	20
Triethanolamine	20	20
N-methyl piperazine	20	20
Bis tris propane	20	20
Glycine	20	20
HCl	-	to pH=3

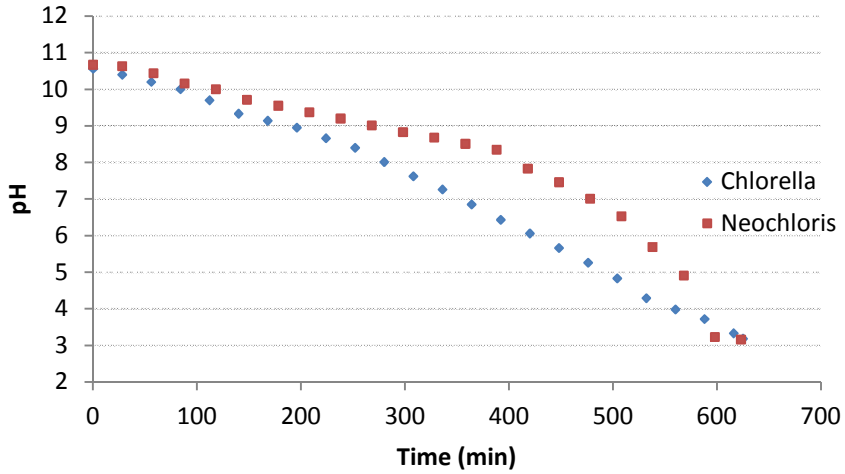

Fig.2.5. pH profiles for separation of *Chlorella vulgaris* and *Neochloris Oleabundans*

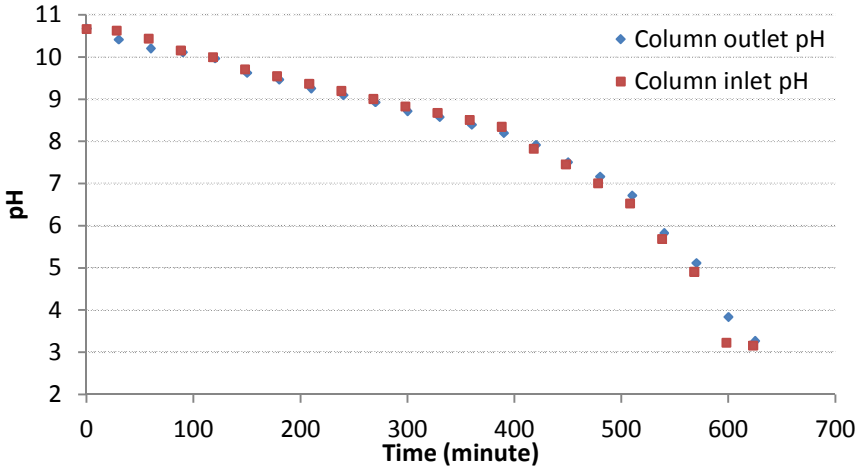

Fig.2.6. pH profile for separation of proteins from *Neochloris Oleabundans*

2.6 Desalting

Samples taken from the HPLC were desalted to avoid interference of the amines with the further silver staining step of electrophoresis analysis. GE Healthcare PD-10 Desalting columns were used for this purpose. The separation technique is gel filtration and the exclusion limit for molecular weights is 5.000 Da. 2.5ml samples were eluted through the column and retained protein were eluted by 3.5ml milli Q water . A typical elution profile is shown in Fig.2.7 taken from GE Healthcare PD-10 Desalting columns protocol.

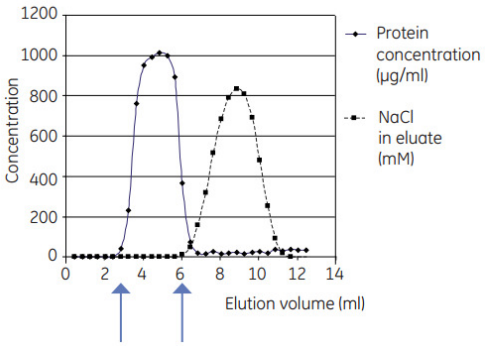

Fig.2.7. Elution profile for GE Healthcare PD-10 Desalting columns

2.7 Freeze-drying

After desalting every sample was freeze-dried. Frozen samples (-80 ^{o}C) were placed in freeze-drier Zirbus Sublimator 2x3x3 during 16 hours according to Table 2.5.

Table 2.5. Set points for the freeze drying process

Step	*Temperature [°C]*	*Pressure [atm]*	*Duration [min]*
1	-50	1	60
2	-50	0.04	600
3	Room temperature	0.04	300

2.8 SDS-PAGE

2.8.1 SDS-PAGE materials

PAGE: BIO-RAD Criterion Cell

Polyacrilamide gel: BIO-RAD “Criterion™ XT Precast Gel, 12% Bis-Tris, 18 Well Comb, 30µl 1.0mm”

Running Buffer: BIO-RAD MOPS 20x concentrated

XT Sample Buffer: BIO-RAD 4x concentrated

XT Reducing Agent: BIO-RAD 20x concentrated

Protein Marker: BIO-RAD Precision Plus Protein™ All Blue Standards.

The general procedure for making SDS-PAGE is shown in Fig.1.14.

2.8.2 SDS-PAGE protocol

Running buffer MOPS was prepared according to the instructions of the manufacturer (BIO-RAD) and kept away of the light. Then, samples for well-injections were made as follows:
1,5 µl of Reducing agent
5,5 µl of loading buffer
8 µl of protein sample.
Samples were boiled for 10 minutes at 95° C. After filling the instrument with the running buffer the wells were filled with 14µL of samples. Electrophoresis chamber was run for 50 minutes (300V, 400mA).

2.9 NATIVE-PAGE

2.9.1 NATIVE-PAGE materials

PAGE: BIO-RAD Criterion Cell
Polyacrilamide gel: BIO-RAD "Criterion™ TGX™ Precast Gels 4-20% , 18 Well Comb, 30 µl 1.0mm"
Running Buffer: BIO-RAD 10x Tris/Glycine Buffer
Loading Buffer: BIO-RAD Native Sample Buffer
Protein Marker: Native Mark™ Protein Std

2.9.2 Native-PAGE protocol

Samples were prepared as follow:
8 µl of Loading Buffer
8 µl of protein sample
16µL samples were placed into the well and the instrument ran for 20 minutes (300V, 400mA)

2.10 Staining

Proteins were revealed in some cases by Coomassie Brilliant Blue R-250. It consists in an anionic dye that binds non-specifically to the proteins. Coomassie Brilliant Blue R-250 staining can detect down to 8 ng of protein (BIO-RAD Bio-safe Coomassie Stain). If a higher sensibility was required, due to a too low concentration of proteins, then Silver Staining was the used method. It is more expensive than the CBB R-250, but the higher sensitivity allows to detect proteins up to 0.25 ng (Thermoscientific®)

2.10.1 Coomassie Brilliant blue protocol

The gel was washed for 5 minutes in ultrapure water and stained by Coomassie Brilliant Blue (100ml) for at least 1 hour and then washed 2 or 3 times with ultrapure water until the background was clear enough.

2.10.2 Silver staining protocol

Used staining solution was Thermo Scientific Pierce Silver Stain Kit. The gels were washed twice with ultrapure water for 5 minutes. To fix the gel, it was washed with a solution of 10% acetic acid, 30% ethanol and further rinsed with ultrapure water for 30 minutes. Further the gels were incubated in 10% ethanol 90% ultrapure water 5 minutes two times. The Sensitizer working Solution was made of 80 µl of Silver Stain Sensitizer in 40 ml of ultrapure water and the gel had to be submerged in it for exactly one minute. Then the gel was washed twice with ultrapure water for one minute. Finally the staining was done with 0.8ml of Enhancer in 25ml of Silver Stain and this Staining step lasts 30 minutes. Developer Working solution (0.8ml of Enhancer in 25ml of Developer) and the Stop Solution (5% acetic acid in ultrapure water) were prepared. Gel was further washed in ultrapure water and then submerged in the Developer Working Solution. Bands begins to appear within 30 seconds and the optimal time was between 2 and 3 minutes depending on the protein concentration. To stop the reaction gels were incubated for 10 minutes in the Stop solution. Finally, gels were washed with ultrapure water to remove traces of previous solutions. After staining, proteins should be visible and a scan of the gel should be taken to process the visual data.

2.11 Western Blot

Immunoblot was the final proof to understand which peaks from the chromatograms were correlated to Rubisco. For this experiments specific antibodies for the target proteins are required and in this case the used antibody detects the smaller subunit of Rubisco.
At first sample proteins must be carried into a SDS-gel, with the protocol explained in §2.8. Then gels has to be detached from the plastic box and washed for a few seconds in the Nu-Page sample buffer that is a solution made of 5% methanol in ultra-pure water. Filter paper has to be cut of the exact size of the gel, washed for a few seconds in the Nu-page buffer and placed on the top of plastic support in the Western Blot electrical device. The gel has to be placed carefully above the filter paper, trying to avoid stacked bubbles between the filter paper and the gel. The membrane has to be cut of the same size of the gel, washed for a minute in pure methanol and then for one more minute in the Nu-Page sample buffer and placed on the gel. Finally, another piece of filter paper has to be placed on the filter membrane. Now the plastic support should be

closed.

The tank for the experiment is filled with the Running Buffer that is made of 0.1% of Nu-Page antioxidant (Invitrogen) in ultra-pure water. The box has to be closed and the blotting is performed at 30V for 2 hours and the temperature of the Running buffer has to be kept at 4°C inserting cold buffer or ice-blocks.

The membrane needs to be washed in ultra-pure water for 3 minutes on a shaker at room temperature. The membrane must be placed in a plastic box to prevent drying until the incubation phase is carried out.

Now the membrane has to be blocked for 1 hour in a 2% low-fat milk powder in TBS-T that is a precasted solution made of: 20mM Tris-Base, 137mM NaCl, 0.1 TWEEN 20x at pH 7.6. Blocking solution is discarded and the membrane is incubated in 10ml of the primarily antibody solution made of 0.01% antibody solution in blocking solution. This incubating stage lasts 2 hours. Primarily antibody solution has to be discarded and the membrane has to be washed 5 times with 20ml of TBS-T for 5 seconds, 15 minutes and the last three times for 5 minutes. Now the membrane can be incubated for 2 hours in the secondary antibody solution made of 0.005% secondary antibody in 10ml of blocking solution. The antibody solution is discarded and the membrane is washed as described before, pending the signal detection. Developer solution is now prepared with 100mM Tris/HCl, 100mM NaCl, 5mM $MgCl_2$ at pH equal to 9.5 by adding 33µl of NBT (Nitro-Blue Tetrazolium, made up 50mg/ml in ultrapure water) and 33µl of BCIP-T (5-bromo-4chloro-3indolyl phosphate, p-toluidine salt, made up 50mg/ml in ultrapure water). Developing phase lasts up to one hour even if first bands become visible within 5 minutes.

3. RESULTS AND DISCUSSION

This work points to set an instrumental method (AEX technique) capable to separate the proteins present in microalgae. In addition, differences between proteins profiles of *Neochloris Oleabundans* at the exponential growth phase and steady state phase are analyzed.

Preliminary works has been conducted to understand behavior of the proteins at pH variations and later to find proper buffer solutions to generate the pH gradient in the HPLC.
Chlorella vulgaris was not studied as *Neochloris Oleabundans* since the HPLC encountered many problems and time was not enough to go deeper. The most time was spent working with the chromatography by an AEC to find the best pH profile. Later, processed samples taken from the HPLC were analyzed by electrophoresis on Native and SDS gels. The aim of this step was correlating peaks of the chromatograms with molecular weights of entire proteins and their subunits. Since the pH profile in the HPLC was not modified between Exponential and Steady phase of *Neochloris Oleabundans*, it is also possible to check different relative quantities of proteins between phases matching the elution times and Native PAGEs between phases. Most attention was paid on *Neochloris Oleabundans* and the comparison between the different behaviors between the Exponential and the Stationary phases. *Chlorella vulgaris* was used to standardize methods and quantities both for the HPLC and PAGE so results from *Chlorella Vulgaris* will be also presented even if they have not been investigated in detail as results from *Neochloris Oleabundans*.

3.1 HCl titration

The aim of these experiments is understanding at which pH proteins of every strain have its own lowest solubility point. Separation throughout a pH-gradient was the desirable chromatography way, then knowledge about proteins precipitation behaviour pH variations was required. When the pH changes, solubility of proteins is expected to change due to the isoelectric point of each protein in the mixture. Titration with HCl was made for three different species of microalgae: *Neochloris Oleabundans*, *Chlorella Vulgaris* and *Desmodesmus spp*. Titration experiments were conducted on the micro filtrated protein solution obtained from the milled and then centrifuged cells (§2.3 and §2.4). Starting pH for titrations is the pH at which cells are grown (6.05 , 6.18, 7.91). Titrations were conducted with 6 Molar HCl in ultrapure water in a small beaker, continuously stirred. Samples were taken every 0.5 pH units, and afterwards centrifuged to remove suspended precipitate. Further the concentration of dissolved proteins was quantified by (Lowry, 1951). Figures 3.2, 3.3, 3.4 show proteins behaviour of respectively: *Chlorella vulgaris, Neochloris Oleabundans, Desmodesmus.* In Fig.3.4 (*Desmodesmus*), data between pH 6 and 8.5

have to be carefully considered as the protein concentration cannot exceed the starting value but they can be interpreted as a negligible protein precipitation in this pH range.

Starting protein concentrations for these microalgal strains are shown in Table 3.1.

Table 3.1. Starting concentration of strains from Fig.3.2, Fig.3.3, Fig.3.4.

Strain	***Protein concentration [gL^{-1}]***
Chlorella vulgaris	4.38
Neochloris oleabundans	6.38
Desmodesmus	4.18

The behaviour of these three strains is very similar: *Neochloris Oleabundans*, *Chlorella vulgaris* and *Desmodesmus* show the lowest concentration of proteins at pH [3.5-4.5]. A similar precipitation profile has already been seen for *Tetraselmis* and is reported in Fig.1.7 (Schwenzfeier, Wierenga, & Gruppen, 2011). A common characteristic among *Neochloris Oleoabundans*, *Chlorella vulgaris*, *Desmodesmus*, and *Tetraselmis* is that they are all green algae (*Chlorophyceae*). Therefore, these common behaviour can be explained with the high presence of RuBisCO among all of them. According to (Barbeau & Kinsella, 1988) RuBisCO's isoelectric point is at pH 4.4-4.7, so a massive precipitation is expected next to this pH value as RuBisCO is the most common protein and while precipitating, co-precipitation of other proteins could happen. *Desmodesmus* shows the highest amount of precipitation at pH=4 with a recovery up to 89% (Fig. 3.4). *Chlorella* and *Neochloris* reach 76% and 80%, respectively (Fig. 3.2 and 3.3). A very different behaviour can be observed when the pH goes lower than the "solution-pI". For example, *Chlorella vulgaris* and *Desmodesmus* at pH lower than the lowest solubility point show an increase of protein. Redissolved proteins in solution reach up to 77% and 67% of the starting protein concentration. *Neochloris oleabundans*, however, does not go beyond 42%. This effect can be due to different percentages of RuBisCO's content. In §3.5.2 results will show that *Neochloris* has the lowest RuBisCO's concentration among these three strains. Fig. 3.1 (Antonov & Soshinsky, 2000) shows correlation of particle size in a solution water-RuBisCO in a wide pH range. Increase of particle size causes a higher precipitation and it is clear from the figure. At lower pH a wide decrease in particle size can be observed so RuBisCO is redissolving into the solution. With these considerations, a higher content of RuBisCO in *Chlorella vulgaris* and *Desmodesmus* can explain the behaviour shown in Fig.3.2-3.4.

Once titration curves from Fig.3.2-3.4 were made, AEX was chosen as the chromatography technique since starting from high pH was required to avoid earlier protein precipitation. From figure 26-28 can be concluded that the most part of the proteins elutes in the between pH 3 and pH 4.

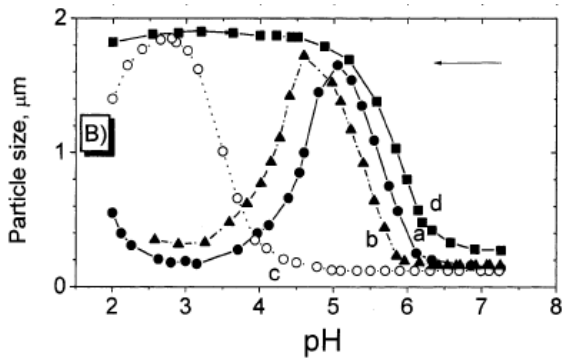

Fig.3.1. Effect of pH on the middle size of particles for binary and ternary system. (•) Water-RuBisCO (0.008%). (Antonov & Soshinsky, 2000).

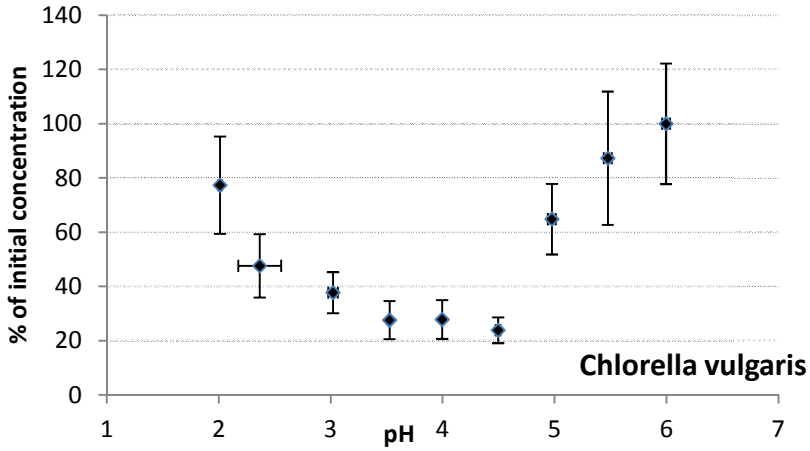

Fig.3.2 pH precipitation profile of *Chlorella vulgaris* by HCl 6 M. Unprecipitated proteins (%) vs pH.

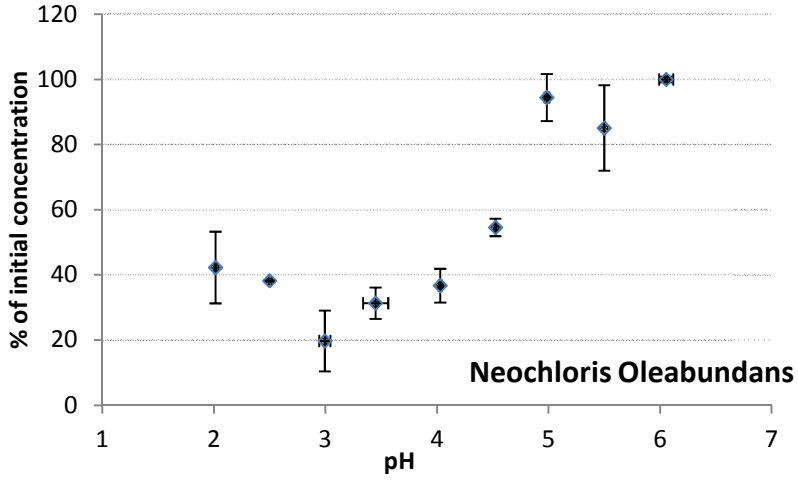

Fig.3.3 pH precipitation profile of *Neochloris Oleabundans* by HCl 6 M. Unprecipitated proteins (%) vs pH.

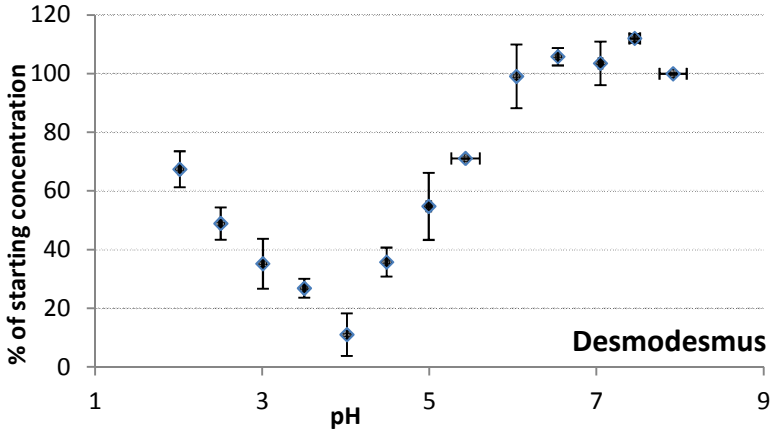

Fig.3.4 pH precipitation profile of *Desmodesmus* by HCl 6 M. Unprecipitated proteins (%) vs pH.

3.2 Choosing buffer solutions

Choosing the buffer solutions to create the pH gradient in the HPLC column was the first step for setting chromatography as the separation method. As seen in §3.1 at the microalgal natural pH the solubility reaches the maximum value and the lowest solubility happens in the pH range of [3.5-4.5]. Starting pH should be as far as possible from the protein isoelectric point to ensure solubility of them (Ahamed T., 2007). To separate a complex mixture of proteins a wide pH range is required [10.5-3.7]. Buffer solutions published elsewhere has been tested (Ahamed T., 2007). Piperidine was omitted because the starting pH was lower than 10.5 and its pKa is 11.12 so the buffer capacity is expected to be in the range 10.5-12.0 (Sigma Aldrich). The elution buffer resulted to be at pH = 1.5 as it was made of 50 mM HCl in ultrapure water. As seen in Fig. 3.2, Fig. 3.3, Fig. 3.4, solubility of proteins is still lower than the maximum at pH lower than 3.7, then some proteins are still eluting at pH lower than 3.7 and that is why a lower pH for the elution buffer was required.

The first trial, that will be called “Amines 1”, was made of: 20mM piperazine, 20mM, N-methyl piperazine, 20mM bis tris propane, 20mM triethanolamine according to (Ahamed T., 2007) but avoiding the use of HCl.

Fig.3.5 shows the pH gradient generated by mixing the amine solution and the 50mM HCl. Y-axis values are defined as the ratio between amines volume divided by the volume of 50mM HCl solution. The profile was considered not satisfactory because of the pH step, that would make more difficult the pH control in the range of proteins expected maximum elution. Further experiments were made, trying to achieve a higher linearity of the gradient. Modifications of composition and compounds of the solutions were based on the pK_a of the amines that are shown in Table 3.2.

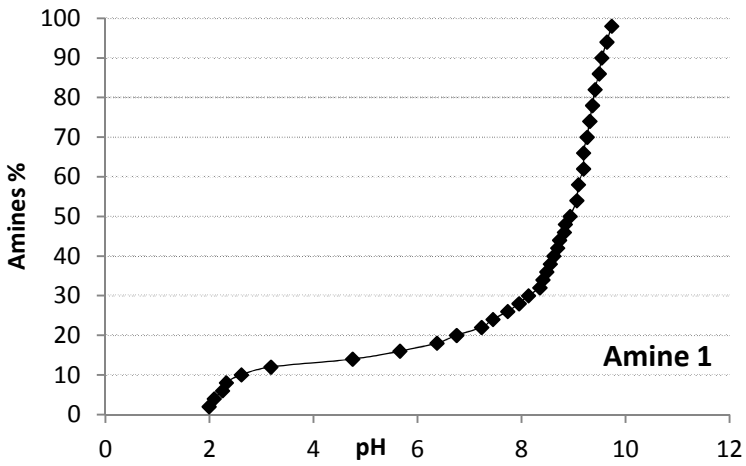

Fig.3.5 pH profile of the "Amine 1" solution: 20mM piperazine, 20mM, N-methyl piperazine, 20mM bis tris propane, 20mM triethanolamine.

Several trials were made to achieve a higher linearity in the pH profile by adding new components and changing concentrations. Trials composition and profiles are shown in Table 3.3 and Fig.3.6. The best solution found is "Amine 4" as it shows the most linear pH due to the addition of glycine, that has a pK_a of 2.35 and a buffer capacity between of 2.2-3.6. Y-axis values are defined as the ratio between amines volume divided by the volume of 50mM HCl solution.

Table 3.2. pKas and effective pH range of different amines
1: Values taken from (Khalili, 2009)
2: Values taken from Sigma-Aldrich

Amine	*pK_a(1)*	*pH(1)*	*pK_a(2)*	*pH(2)*
Piperazine[2]	5.33	5.0-6.0	9.73	9.5-9.8
Piperidine[2]	11.12	10.5-12.0		
Triethanolamine[2]	7.76	7.0-8.3		
N-methyl piperazine[1]	9.14		4.63	
Bis tris propane[2]	6.8	-	9.0	-
1,4 dimetil piperazine[1]	8.38	-	3.81	-
Glycine[2]	2.35	2.2-3.6	9.78	8.8-10.6

Table 3.3. The table shows the composition of the trials for amine buffer solutions. Compositions are expressed in milliMolarity of the substance

Compound	*Amine 2*	*Amine 3*	*Amine 4*	*Amine 5*
Piperazine	20	20	20	5
Piperidine	20	-	-	-
Triethanolamine	20	20	20	20
N-methyl piperazine	20	20	20	20
Bis tris propane	20	20	20	20
1,4 dimetil piperazine	-	20	-	-
Glycine	-	-	20	40

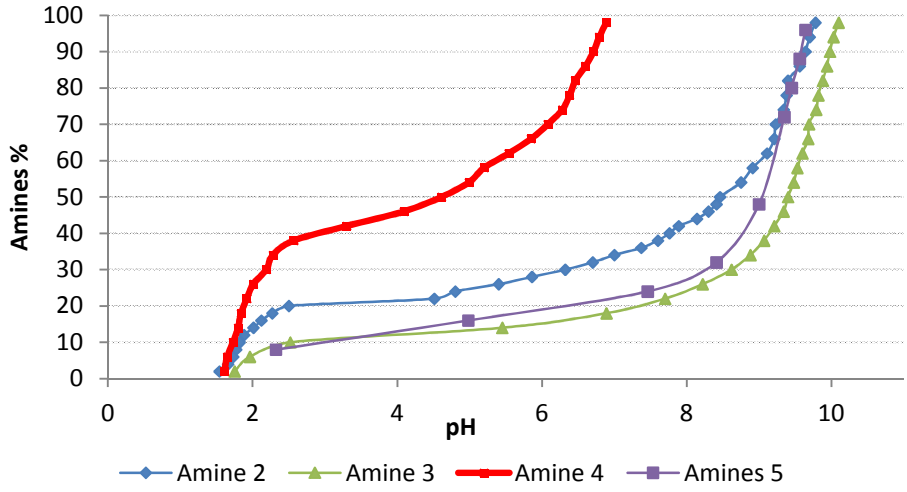

Fig. 3.6. Column inlet pH profile of the different "amine" trials from Table 3.3.

A further improvement was made. Piperidine was added at the "Amine 4" to create the equilibration buffer at pH higher than 10.5 so the elution buffer was made of "Amine 4" plus Piperidine with the addition of pure HCl until reaching pH=3. With these buffers the pH range is between 10.6 and 3, the composition of the definitive solutions is shown in Table 3.4 and the achieved pH profile mixing the loading and elution buffers is shown in Fig. 3.7. The Y-axis is expressed as the ratio between the volume of loading buffer and the volume of the elution buffer. The data correspond to the pH profile at the inlet of the column.

Table 3.4. Composition of the buffer solutions to generate the pH gradient.

Compound	***High pH buffer solution (mM)***	***Low pH buffer solution (mM)***
Piperazine	20	20
Piperidine	20	20
Triethanolamine	20	20
N-methyl piperazine	20	20
Bis tris propane	20	20
Glycine	20	20
HCl	-	to pH=3

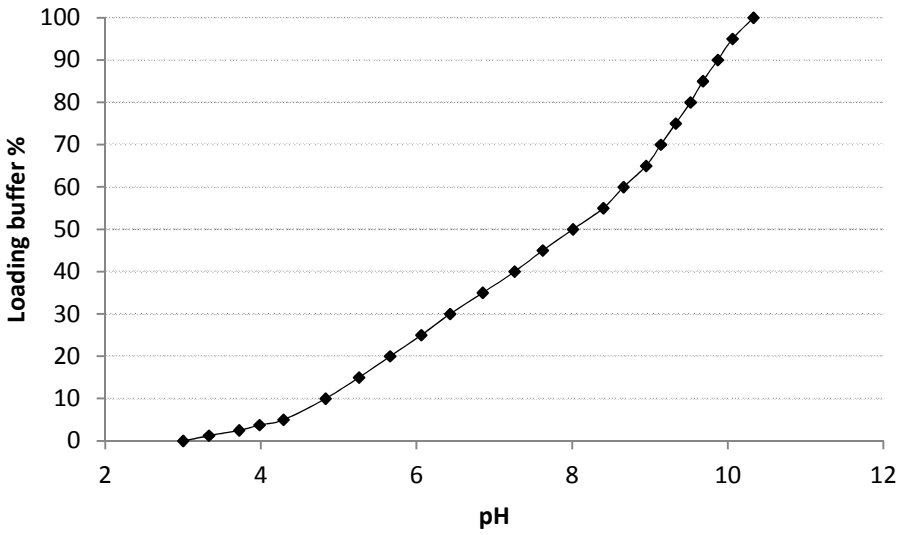

Fig.3.7. The figure shows the pH profile achieved with the definitive buffer solutions. Composition is showed in Table 3.4.

3.3 Chromatograms

Chlorella vulgaris was used as the first trial to improve settings of Anionic Exchange Chromatography. Buffer solutions, flow rate, duration and pH profile were the ruling variables of the process and with several experiments on *Chlorella vulgaris* process sensitivity at variations of these variables was clearer. The HPLC profile of proteins from *Chlorella vulgaris* is shown in Fig.3.8 and the pH profile adopted at the column inlet is shown in Fig.3.7. Dialysis was used to reduce conductivity of samples for AEC. In Table 3.5 conductivity, protein concentration and amount of samples for AEC are reported. Samples from *Neochloris Oleabundans* were concentrated 5 times otherwise protein content could have not been enough for detection with electrophoresis.

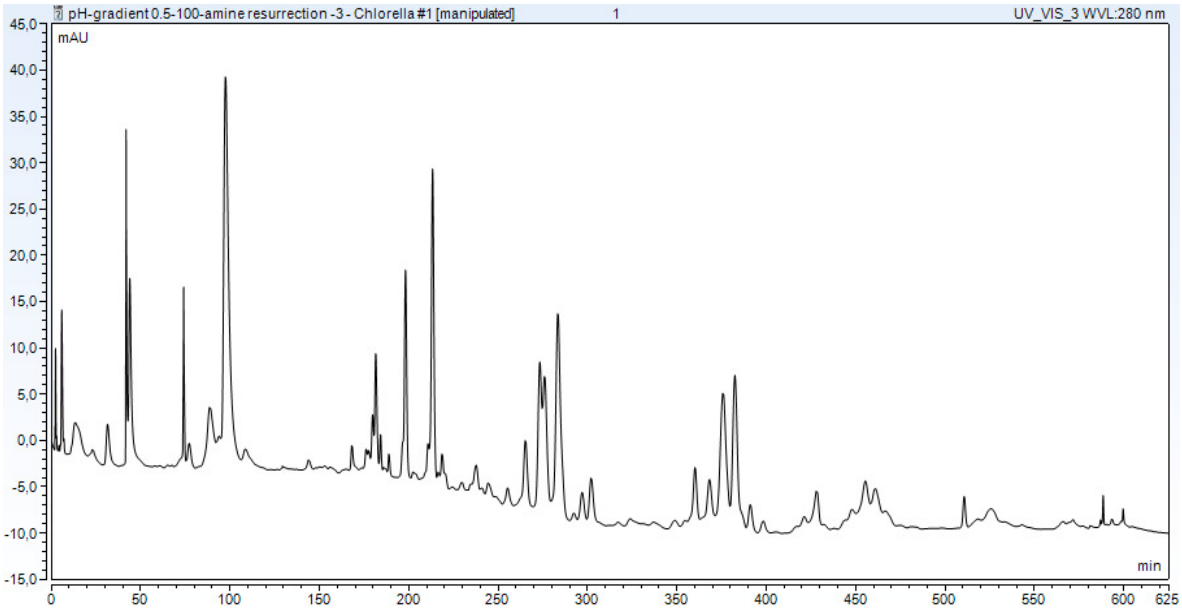

Fig.3.8. *Chlorella vulgaris* chromatogram.

Table 3.5. Conductivity, protein content and sample injection for AEC

Sample	***Conductivity [mS]***	***Protein concentration [g/l]***	***Sample volume [µL]***
Chlorella vulgaris	1.761	4.38	200
Neochloris Oleabundans (Exp phase)	4.400	16.57	100
Neochloris Oleabundans (Steady phase)	4.530	11.37	200
RuBisCO	3.563	5.68	100

Gained resolution for chromatograms in Fig. 3.8-3.10 was considered to be satisfactory, since the starting proteins solution was the crude mixture of soluble proteins from microalgal strains.

Once the standardization of AEX was completed with samples from *Chlorella vulgaris*, focus was moved on *Neochloris Oleabundans* during Exponential and Stationary Phase. pH profile for proteins elution was the same for both the growth phases. Fig.3.9 shows the chromatogram from *Neochloris Oleabundans* during the Exponential phase and Fig.3.10 shows the chromatogram from *Neochloris Oleabundans* during the Steady phase.

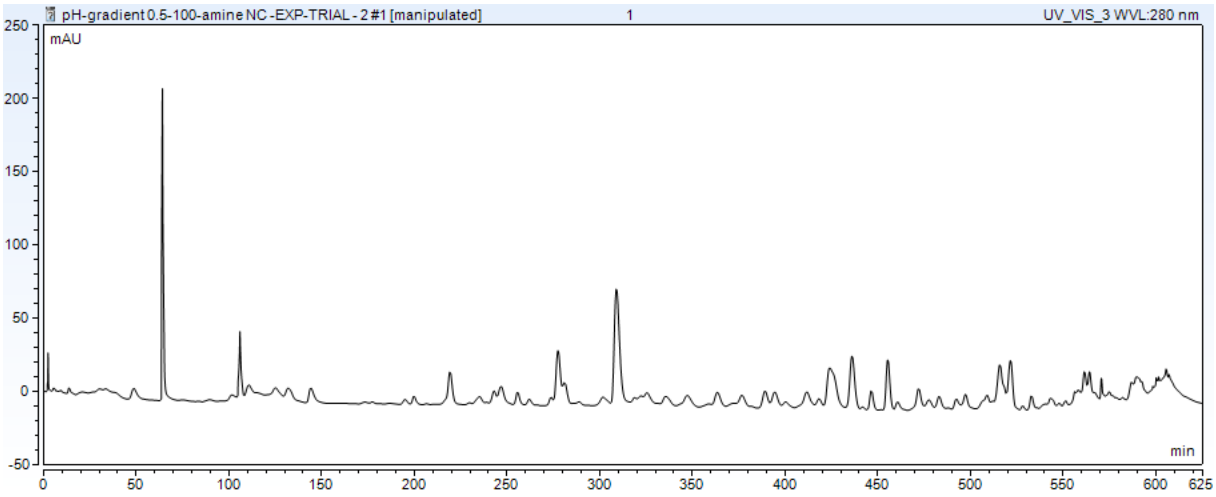

Fig.3.9. Chromatogram from *Neochloris Oleabundans* during the Exponential phase.

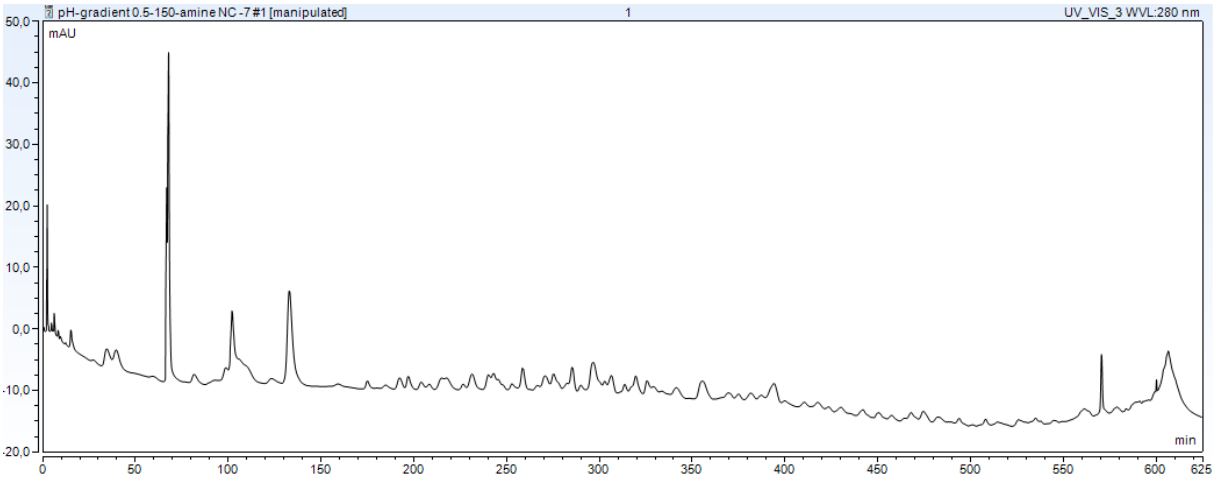

Fig.3.10. Chromatogram from *Neochloris Oleabundans* during the Steady phase.

The pH profile used for *Neochloris Oleabundans* separation was different from *Chlorella vulgaris* profile and is shown in Fig.2.5 because *Chlorella*'s pH profile was found to have not enough peaks resolution in the pH range of [10.6-9.5] for *Neochloris Oleabundans* samples.

pH profile used for separation of crude protein mixture from *Neochloris Oleabundans* during Exponential and Steady phase was the same. Proteins eluting at the same time in chromatograms from Fig.3.9 and Fig.3.10 have close Isoelectric points and are expected to be very similar to each other. Early elution of proteins, above pH 9, is observed. According to (Ahamed, et al., 2007) neutral protein are likely to elute at higher pH than their own pI and most of the times they elute at about pH=9. High pH elution can be also a consequence of protein denaturation due to the high pH so shorter aminoacids chains are eluting at a different pH than the native protein.

Similar elution behavior can be observed in the first 15 minutes in chromatograms from *Neochloris Oleabundans* and *Chlorella vulgaris*. First proteins eluting between 0 and 20 minutes, at high pH can be featured from a high pI, but there is also the possibility of not-binding with the column because their retention time was proved to be dependant only on the flowrate. This behavior was demonstrated by changing the flow rate, noticing that the elution time changed by the exact ratio of flow rate modification. Similar peaks for elution time and height, between the two growth phases of *Neochloris Oleabundans*, are visible between 60 and 70 minutes and 100 and 110 minutes. A similar behavior is clearly noticed also in the last part of the *Neochloris Oleabundans* chromatograms where the elution profile shows a very wide peak (575-615 minutes). In Fig.3.11 comparison between chromatograms is shown by overlapping Fig. 3.9, Fig.3.10 and partially Fig.3.8.

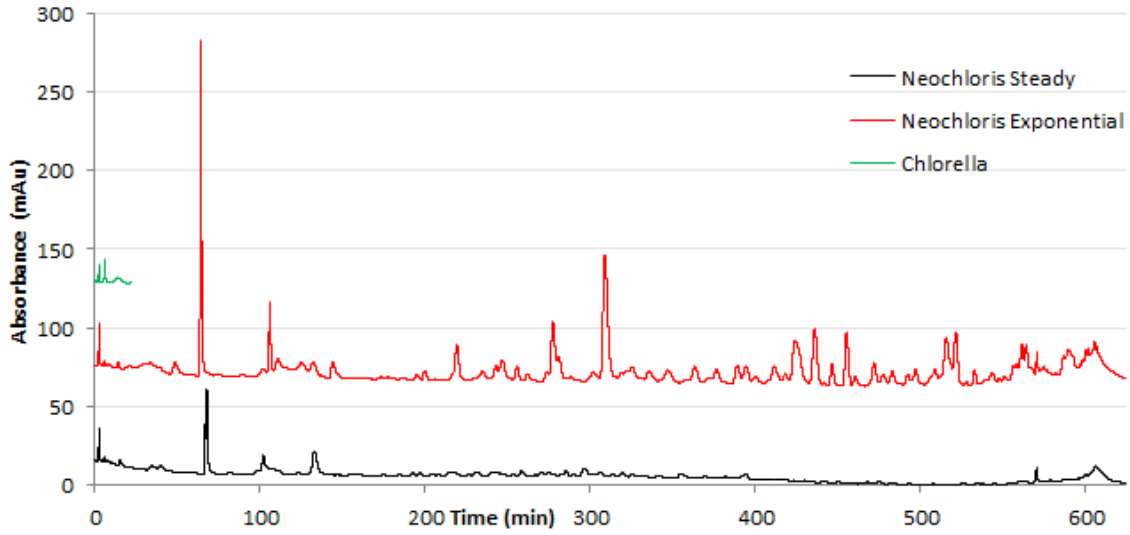

Fig.3.11. Comparison of chromatograms from Fig.3.8,3.9,3.10.

3.4 Characterization of proteins

In this section samples taken from AEX are submitted to electrophoresis experiments with Native and SDS gels. The aim is detection of molecular weights, quaternary structure of proteins and similarity between *Neochloris Oleabundans* during the Exponential and the Steady phase. Each picture will show red numbers that are referred to the molecular weight in kDalton. Black numbers are meant just to make easier the well count.

3.4.1. Comparison of Native gels between exponential and steady phases

In this section, molecular weights of proteins from *Neochloris Oleabundans* during exponential and steady phase are compared. These experiments were conducted in Native gels (see Materials And Methods). Samples are chronologically ordered as they were taken from the HPLC.

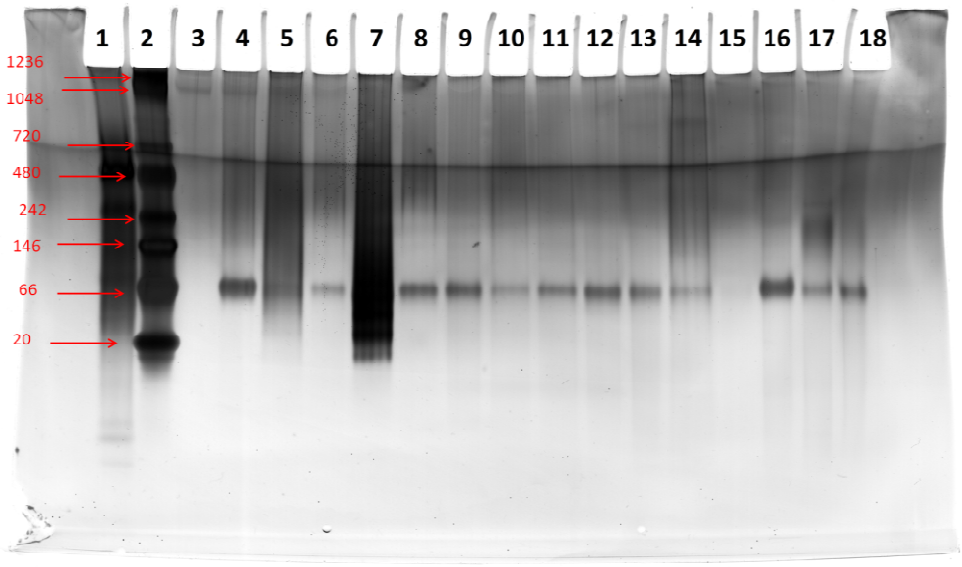

Fig.3.11a. Native gel from *Neochloris Oleabundans* in Exponential phase (sampling time 2'-221').Correlation well-retention time. 1 [RuBisCO], 2 [Ladder], 3 [Amines], 4 [2'05"-3'10"], 5 [5'10"-7'00"], 6 [12'50"-15'20"], 7 [30'30"-33'40"], 8 [47'38"-50'57"], 9 [63'37"-66'50"], 10 [100'35"-103'41"], 11 [109'10"-112'30"], 12 [125'37"-128'40"], 13 [131'11"-134'30"], 14 [143'00"-148'00"], 15 [193'49"-197'00"], 16 [198'30"-201'45"], 17 [215'00"-218'00"], 18 [221'00-223'00"].

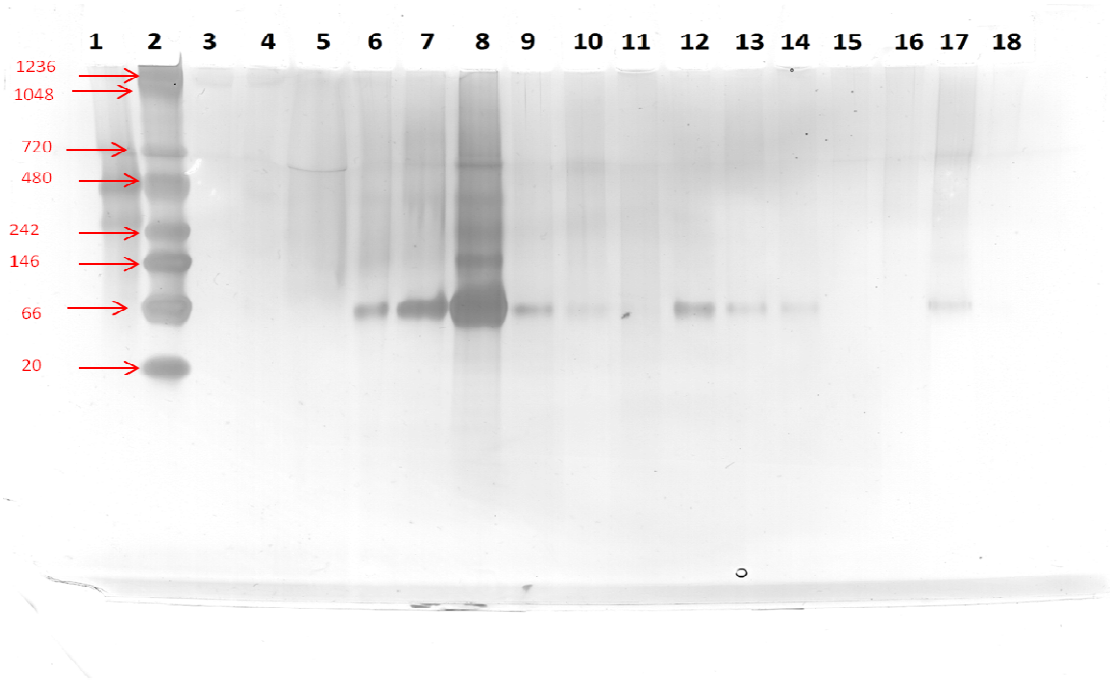

Fig.3.11b. Native gel from *Neochloris Oleabundans* during Exponential phase (sampling time 222'-327'). Correlation well-retention time: 1 [RuBisCO], 2 [Marker], 3 [218'000"-221'00"], 4 [232'00"-235'00], 5 [235'30"-237'30"], 6 [242'10"-245'13"], 7 [245'13"-250'00"], 8 [254'30"-257'42"], 9 [257'42"-259'00"], 10 [260'40"-264'00"], 11 [273'44''-275'20''], 12 [275'20''-280'00''], 13 [280'00''-284'30''], 14 [300'00''-305'00''], 15 [306'50''314'00''], 16 [317'00''-320'00''], 17 [320'06''-323'42''], 18 [324'30''-327'30''].

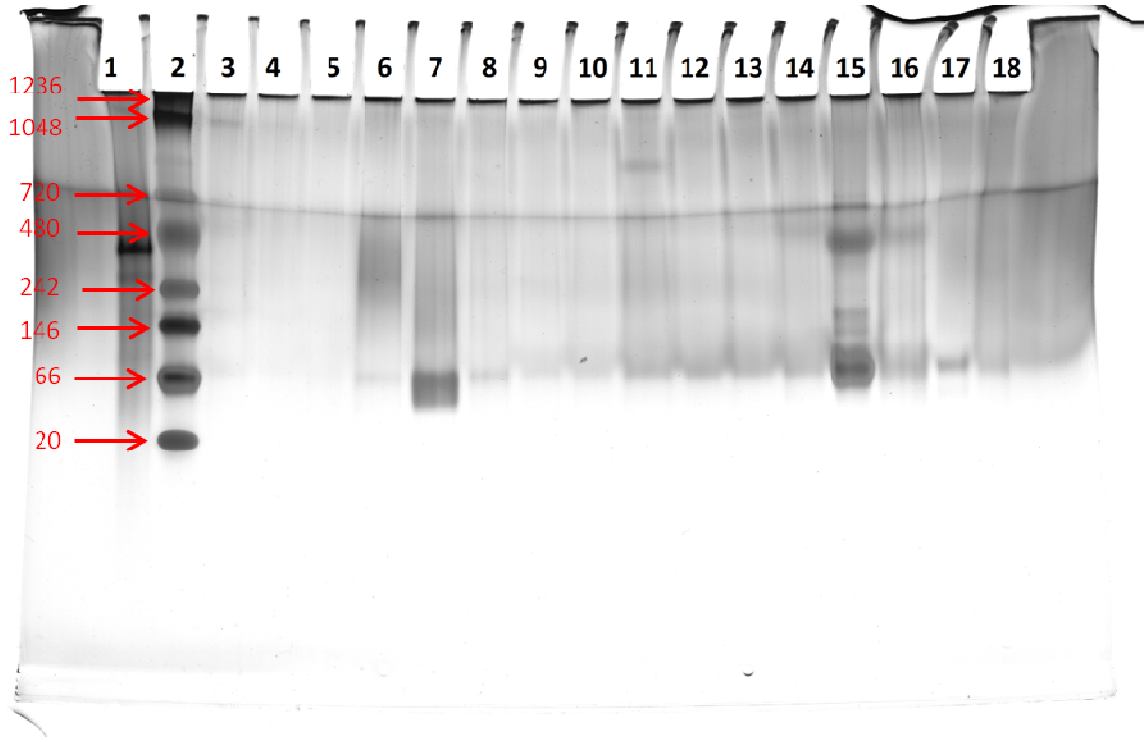

Fig.3.12. Native gel from *Neochloris Oleabundans* during Steady phase (sampling time 2'-271'). Correlation well-retention time: 1 [RuBisCO], 2 [Marker], 3 [2'00-4'15''], 4 [6'00''-7'15''], 5 [15'30''-17'00''], 6 [33'00''-41'00''], 7 [66'16''-69'25''], 8 [97'00''-100'00''], 9 [100'00''-103'00''], 10 [103'00''-106'00''], 11 [131'30''-136'00''], 12 [196'50''-199'00''], 13 [202'50''-206'00''], 14 [213'20''-218'20''], 15 [228'35''-233'00''], 16 [238'40''-244'40''], 17 [257'30''-260'30''], 18 [268'40''-271'40''].

These gels (Fig. 3.11a, 3.11b) contain samples from the first part of chromatograms of *Neochloris Oleabundans* Exponential phase from 2 to 327 minutes and Steady phase (Fig.3.12) from 2 to 271 min. The 3rd well of the gel from Fig.3.11a contains the buffer solution used for the AEC to recognize bands caused from amines smear. In the 4th, 5th and 6th wells of Fig.3.11a and in the 3rd well of Fig.3.12, a band is detected at the same height of the band generated by the amines (1100 kDa). Therefore, either amine contamination due to an incomplete desalting or proteins could be responsible of this band. In further sections will be shown that this band (1100 kDa) corresponds to proteins with an electrophoretic pattern similar to the amine solution (see §3.4.2). Presence of proteins will be point out in the SDS gel that will show the detached protein. It is clearly visible that in the exponential phase (Fig.3.11a and Fig.3.11b) bands in the range of 54kDa-65kDa are very recurrent. In the gel from the Steady phase (Fig.3.12) many bands in this range of molecular weights are detected too, but bands in Fig.3.12 should be recognized carefully due to the background generated by the silver staining. Despite of the background, clear bands of 65kDa are observed in 6 , 7, 8, 11, 12, 15, and 17 wells in the Native gel from the Steady phase (Fig.3.12). A protein of 834kDa is detected in the 11th well of the Steady phase (Fig. 3.12, 131'30''-136'00'') and a band at a very similar Molecular weight is visible in the 14th well of the Exponential phase(Fig.3.11a, 143'00''-148'00''). Proximity of the elution points and similar molecular weights suggest that probably these bands correspond to the same protein or at least proteins with similar molecular weight and similar behavior. Confirmation will be

achieved in a later section (§3.4.2) since both bands will show the same native and SDS pattern, indicating that indeed it is the same protein. One more similarity in pattern is shown in 15th and 16th (Steady phase; retention time=228-233 minutes at pH=9.14; 239-244 minutes at pH=9.08) wells from Fig.3.12 and in the 8th (Exponential phase; retention time=254-257 minutes at pH=9.00) well from Fig.3.11b. In the first case (Steady phase), detected molecular weights are 490kDa (16 well), 448kDa (15 well), 175kDa, and 130kDa and the retention time was 228'-244'. In the second case (Exp phase), molecular weights of 605kDa, 372kDa, 232kDa, 151kDa, 68kDa are visible and the retention time for this sample is 254'-257'. Electrophoretic patterns are similar, and even if retention times differ in 20 minutes, it is worth to mention that the pH difference is lower than 0.2. Native gel of the same sample may show differences in electrophoretic patterns as will be demonstrated in later section of this work (§3.5) so same proteins can even be identified in different native gel by close but not equal molecular weights.

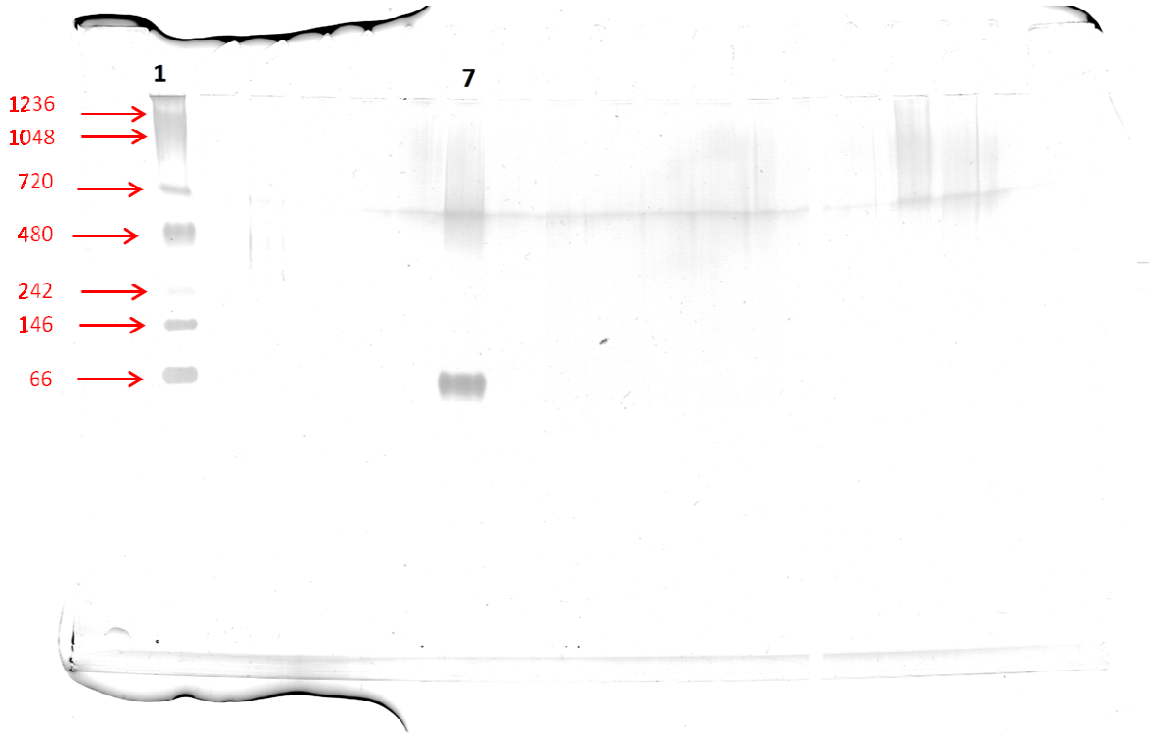

Fig.3.13a Native gel from *Neochloris Oleabundans* in Exponential phase (sampling time 327'-485') Correlation between well and retention time: 1 [Marker], 7 [391'45''-397'09''].

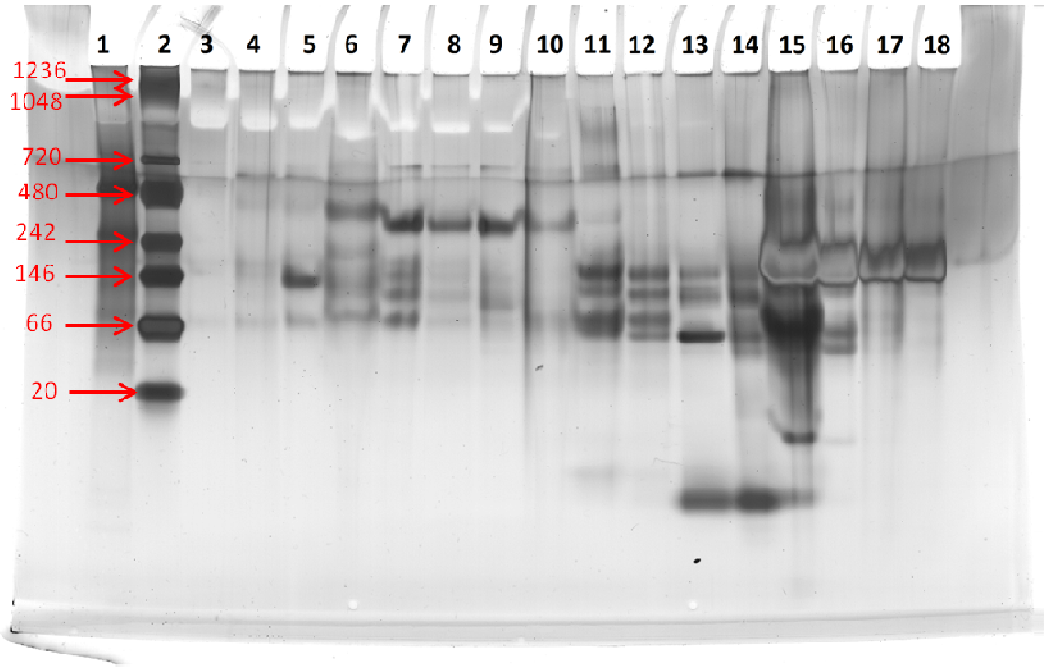

Fig.3.13b Native gel from *Neochloris Oleabundans* during Exponential phase (sampling time 485'-609'). Correlation between well and retention time: 1 [RuBisCO], 2 [Marker], 3 [490'50''-494'30''], 4 [494'41''-500'00''], 5 [506'00''-509'30''], 6 [514'00''-519'30''], 7 [519'30''-522'30''], 8 [522'30''-525'00''], 9 [532'00''-535'30''], 10 [539'00''-545'00''], 11 [556'20''-560'40''], 12 [560'40''-563'20''], 13 [563'20''-566'45''], 14 [570'06''-573'20''], 15 [585'05''-591'05''], 16 [591'05''-594'05''], 17 [598'10''-604'10''], 18 [604'10''-609'10''].

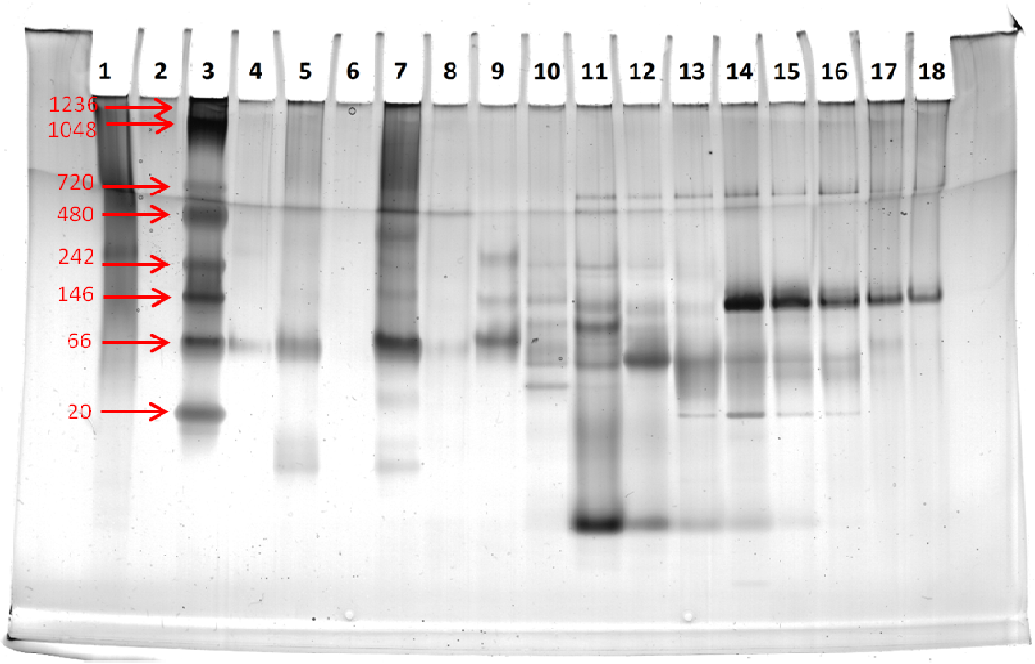

Fig.3.14 Native gel from *Neochloris Oleabundans* during Steady phase (sampling time 271'-612'). Correlation well-retention time: 1 [RuBisCO], 2 [275'40''-278'00''], 3 [Marker]. 4 [282'25''-287'25''], 5 [295'10''-300'10''], 6 [353'20''-359'00''], 7 [392'30''-395'30''], 8 [466'20''-478'00''], 9 [527'37''-537'00''], 10 [556'00''-565'00''], 11 [569'00''-574'00''], 12 [576'00''-581'00''], 13 [582'00''-587'00''], 14 [587'00''-592'00''], 15 [592'00''-597'00''], 16 [597'00''-602'00''], 17 [602'00''-607'00''], 18 [607'00''-612'00''].

In Fig.3.13a only two bands are detected in the 7th well . Sample shown in the 7th well has a retention time of 392'-397' and corresponding molecular weights of 574kDa and 64kDa. With the same retention time (392'-395') the 7th well of Fig.3.14 detects bands at 400kDa, 247kDa, 140kDa, 67kDa, 26kDa and 8kDa and bands at about 64kDa are also shown in 4th and 5th well. Sampling time of gel from Fig.3.13a (Exp phase) was 327-490 minutes. Samples from the Exponential phase were taken for presence of small peaks in the chromatogram. Clearly the proteins amount was not enough to be detected by electrophoresis and almost total absence of proteins in 327-490 minutes is the result. This evidence is also confirmed from Fig. 3.14 (Steady phase) where, in the same time interval, just the 7th well shows bands. Molecular weights between 550 and 650kDa are widespread in Fig. 3.14 and Fig.3.13b. In the gel from the Exponential phase (Fig.3.13b) this range of molecular weights is visible throughout almost all the gel from the 4th to the 14th wells covering 79 minutes of chromatogram from 494' to 573' minutes. In Fig. 3.14 this range of molecular weights is present too and it is visible from 11th to 16th wells with retention time of 569'-602'. In the 12th and 13th wells from Fig.3.13b a clear band is visible at 55kDa and the related retention time is 561'-567'. A similar behaviour can be observed in 11th and 12th wells of Fig.3.13b where similar bands in molecular weight (47kDa) and shape are detected. Behaviour of proteins eluting in the last hour presents clear similarities in molecular weights and it can be observed in Fig.3.14 (steady phase) and Fig.3.13b (exp phase). The first analogy is visible in Fig.3.14 (14th to 18th well) and Fig.3.13b (15th to 18th well) with molecular weights in the range of 130kDa-155kDa. Retention time is 587'-612' for samples from Fig.3.14 and 585'-609' for samples from Fig.3.13b. A second similarity is seen from 11th to 15th wells of Fig.3.14 and from 13th to 15th wells of Fig.3.13b. Detected molecular weights are around 3kDa and respectively retention times are 569'-597' and 563'-591'but molecular weights that are not in the range provided by the protein marker (1236kDa-20kDa) are not completely trustful. Furthermore, molecular weights lower than 5kDa are hardly the accurate evaluation of protein size since the desalting process has a 5kDa cut-off. That evidence highlights that Native gels are not completely reliable for molecular weight evaluation of non-denatured proteins.

3.4.2 Matching molecular weights from Native and SDS PAGE

In this section, results from Native and SDS PAGE are compared matching molecular weights of subunits forming whole proteins detected in the Native gels in order to understand if proteins got detached during the sample processing. It is clearly visible that the most part of detected proteins present a molecular weight between 40 and 65 kDa even if higher molecular weights were expected. In the first three gels from *Neochloris* Exponential phase this range of molecular weights is recurrent. This evidence can be due to the high pH (up to 10.6) of the equilibration buffer during AEC and the duration of treatment (milling, centrifugation, microfiltration, dialysis, AEC, desalting, freeze-drying)of samples before electrophoresis. This aspects may increase the chances of protein denaturation, so they would not be detected integer any more.

As stated in §2.9, native gels are not fully reliable for measuring the molecular weight of proteins, but is a very easy and quick way for doing it and that is why this method was carried out. References to results from native gels will be given precisely but the uncertainty given from native gels has to be taken into account. Appendix A.1 gives a more accurate overview on matching peaks from chromatograms and molecular weights.

3.4.2.1 *Neochloris Oleoabundans* during the exponential phase

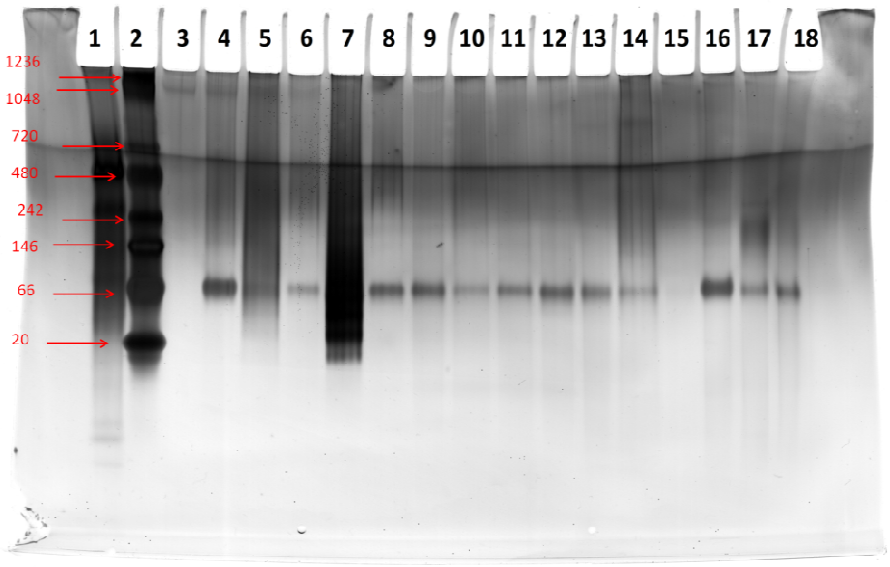

Fig.3.15a. Native gel from *Neochloris* Exponential. Sampling Time 2'-223'. Description of wells is given in Table 3.6a.

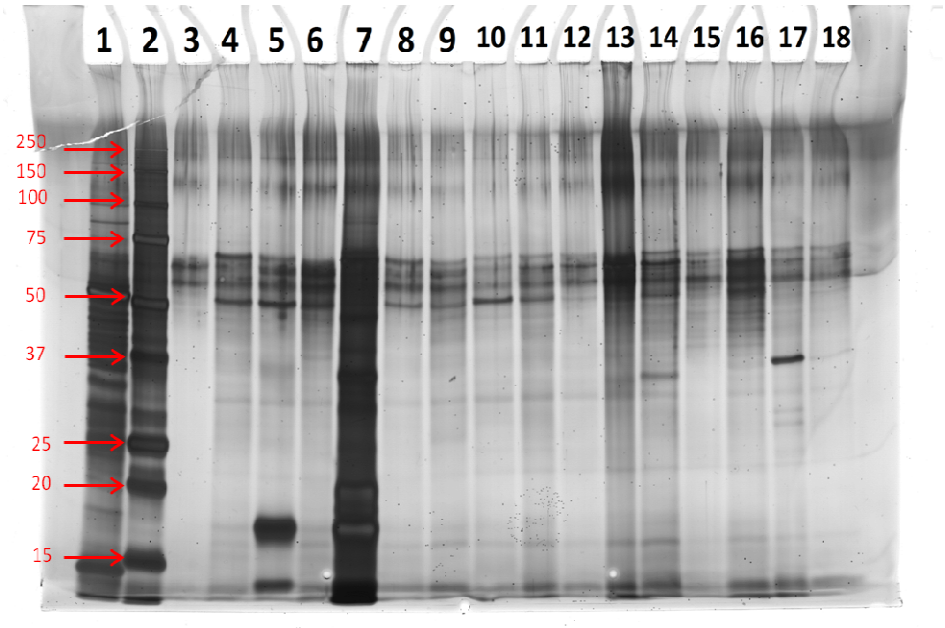

Fig.3.15b. SDS gel from *Neochloris* Exponential. Sampling Time 2'-223'. Description of wells is given in Table 3.6b.

These are gels made from the first 16 samples taken from the HPLC. The pH range of samples is 10.68-9.18 and they were taken into the time interval of the chromatogram is 2-221 minutes. Temporal order of sampling goes from the left to the right.

In the first gel (Fig.39a) well, pure RuBisCO from spinach is inserted. Its native molecular weight is expected to be around 550 kDa, but the measured one is 452.25 kDa (Table 16a). This variance is due to the non-denaturizing properties of native gels. In the 3rd well of Fig.3.15a equilibration buffer is inserted as sample and a band is detected at 1103.24 kDa and at 62.96 kDa and 56.65 kDa in SDS gel. So light bands at these height can be associated to amine traces into samples.

Molecular weights of RuBisCO whole proteins detected from the native gels are mostly between 55 kDa and 60kDa. This result is confirmed from the SDS gel that detects more proteins/subunits in the same range. In the SDS gels more molecular weights are detected due to its better resolution and accuracy but values are still close to the ones found in the Native. About 30 kDa and 15 kDa subunits are also detected, so some proteins can split into subunits into the SDS gel and the molecular weight of the whole protein is about 55-60 kDa. Both of the 7th wells result to be very dark, this can be caused both from a high quantity of proteins and lots of different molecular weights. Tables 3.6a and 3.6b show molecular weights associated with gel wells, retention time and pH of sampling.

Table 3.6.a Correlation of gel wells from Fig. 3.15a with molecular weights. In brackets in the first ladder, pH of sampling.

***WELL** (pH)*	***Retention time (min)***	***Molecular Weight from Native PAGE (kDa)***
1(RuBisCO)	---	521.26
2 (Marker)	---	1236 1048 720 480 242 146 66 20
3 (Amines)	---	1103.24
4 (10.65)	2'05"-3'10"	1098.33 59.47
5 (10.62)	5'10"-7'00"	1099.76
6 (10.55)	12'50"-15'20"	56.72
7 (10.41)	30'30"-33'40"	46.92 34.65 23.95
8 (10.31)	47'38"-50'57"	57.8
9 (10.22)	63'37"-66'50"	56.6
10 (10.01)	100'35"-103'41"	56.7
11 (9.95)	109'10"-112'30"	57.8
12 (9.86)	125'37"-128'40"	56.1
13 (9.82)	131'11"-134'30"	54.61
14 (9.74)	143'00"-148'00"	800.00 54.61
15 (9.39)	193'49"-197'00"	Nd
16 (9.36)	198'30"-201'45"	60.6
17 (9.24)	215'00"-218'00"	59.47
18 (9.20)	221'00-223'00"	55.65

Table 3.6b Correlation of gel wells from Fig. 3.15b and molecular weights. In brackets pH at which sample was taken. Every well corresponds to the same sample of Table 3.6a.

***WELL** (pH)*	***Molecular Weight from SDS PAGE (kDa)***										
1(RuBisCO)	52.39	14.75									
2 (Marker)	250	150	100	75	50	37	25	20	15		
3 (Amines)	62.96	56.65									
4 (10.65)	67.13	56.35	50.44	29.91	13.47						
5 (10.62)	60.44	55.7	49.75	29.91	16.99	13.65					
6 (10.55)	60.44	55.05	49.25	29.91							
7 (10.41)	64.44	55.21	49.13	33.79	47	40.6	29.15	19.73	18.76	16.85	13.63
8 (10.31)	65.01	59.56	55.37	49.25							
9 (10.22)	64.07	59.56	54.73	46.76	15.94	13.56					
10 (10.01)	66.74	62.05	56.35	51.03							
11 (9.95)	66.74	62.41	56.35	51.03	30.26	15.94					
12 (9.86)	66.55	63.14	57.01	51.03	30.26	13.61					
13 (9.82)	67.33	63.14	57.51	47.79	30.26	16.1	13.61				
14 (9.74)	68.52	64.44	57.51	52.8	33.66	30.26	16.1	13.65			
15 (9.39)	68.92	64.44	58.19	16.1							
16 (9.36)	70.13	64.63	58.36	54.41	47.08	16.34					
17 (9.24)	70.13	64.63	58.7	36.21	28.92	27.26	13.74				
18 (9.20)	70.13	64.63	58.7	13.74							

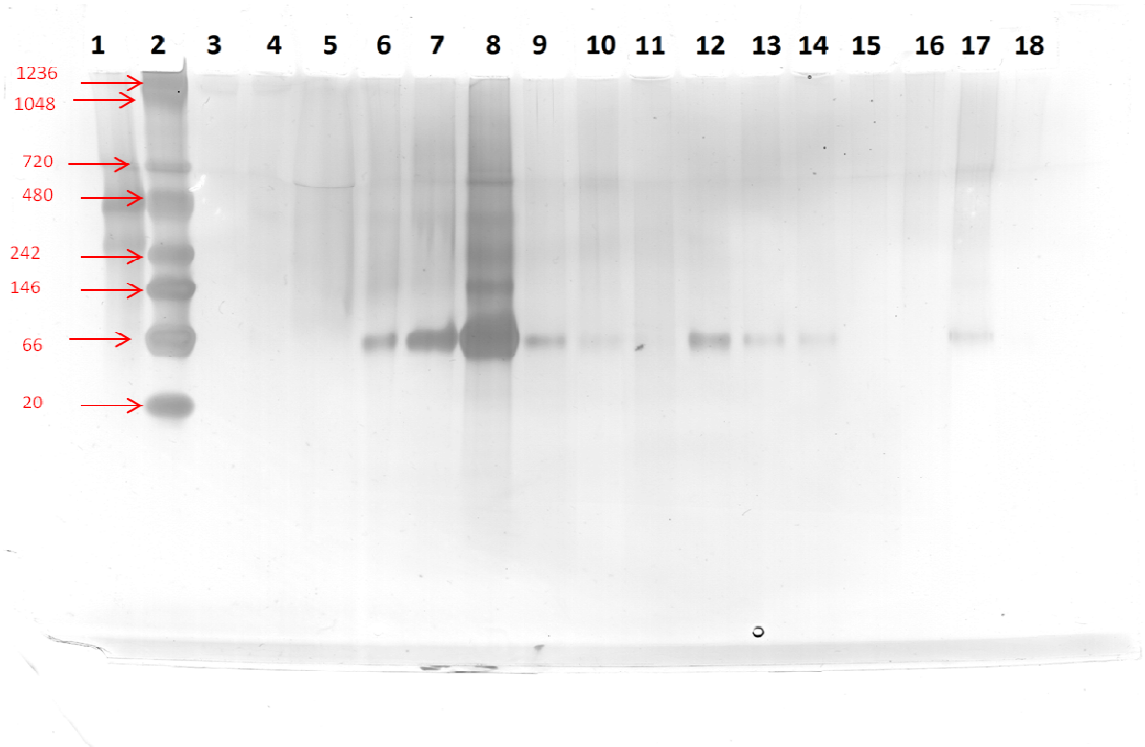

Fig. 3.16a. Native gel from *Neochloris* Exponential. Sampling Time 218'-327'. Description of wells is given in Table 3.7a.

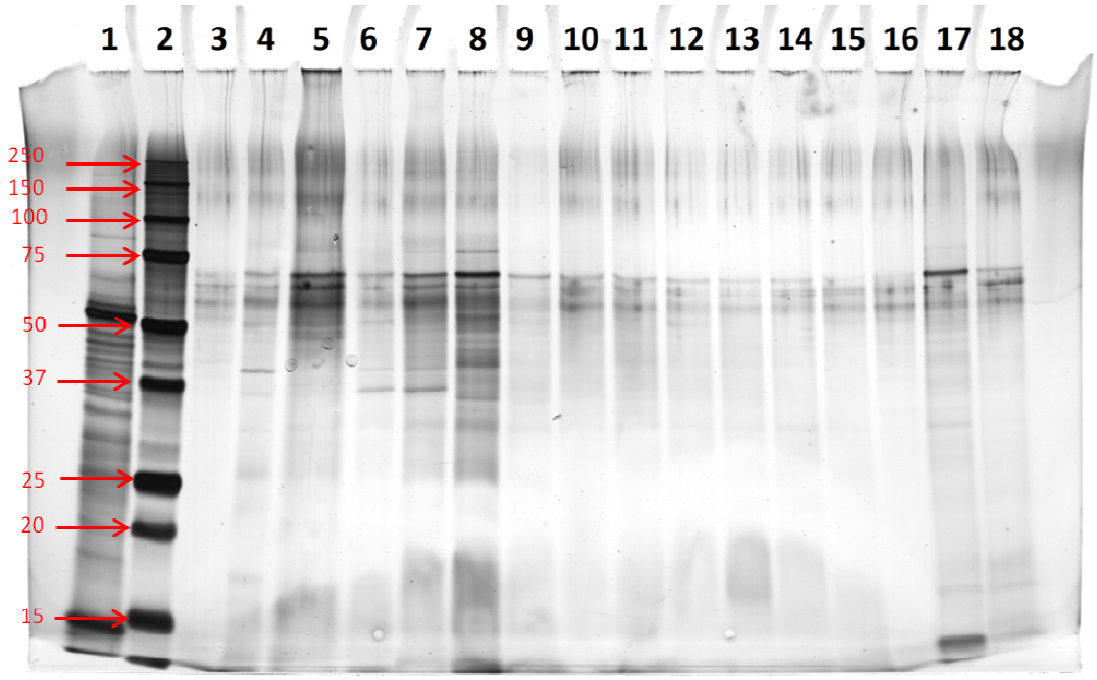

Fig. 3.16b. SDS-gel from *Neochloris* Exponential. Sampling Time 218'-327'. Description of wells is given in Table 3.7b.

Tables 3.7a and 3.7b show correlation between gel-wells, molecular weights, retention time and pH of gels from Fig.3.16a and Fig.3.16b.

Table 3.7a Correlation between gel wells from Fig. 3.16a and molecular weights. In brackets in the first ladder, pH of sampling.

***WELL** (pH)*	***Retention time (min)***	***Molecular Weight from Native PAGE (kDa)***							
1(RuBisCO)	---	452.25							
2 (Marker)	---	1236	1048	720	480	242	146	66	20
3 (9.22)	218'000"-221'00"	Nd							
4 (9.13)	232'00"-235'00	Nd							
5 (9.11)	235'30"-237'30"	Nd							
6 (9.06)	242'10"-245'13"	63.55							
7 (9.03)	245'13"-250'00"	66.00							
8 (8.98)	254'30"-257'42"	605.62	371.56	231.78	151.5	67.46			
9 (8.97)	257'42"-259'00"	62.92							
10 (8.94)	260'40"-264'00"	62.05							
11 (8.87)	273'44''-275'20''	Nd							
12 (8.85)	275'20''-280'00''	56.74							
13 (8.82)	280'00''-284'30''	63.33							
14 (8.70)	300'00''-305'00''	63.02							
15 (8.66)	306'50''314'00''	59.99							
16 (8.62)	317'00''-320'00''	Nd							
17 (8.60)	320'06''-323'42''	Nd							
18 (8.58)	324'30''-327'30''	67.95							

Table 3.7b. Correlation between gel wells from Fig. 3.16b and molecular weights . In brackets in the first ladder, pH of sampling. Every well corresponds to the same sample of Table 3.7a.

***WELL** (pH)*	***Molecular Weight from SDS PAGE (kDa)***								
1(RuBisCO)	52.45	15.21							
2 (Marker)	250	150	100	75	50	37	25	20	15
3 (9.22)	63.88	57.4							
4 (9.13)	67.39	63.16	57.4	39.84	36	25.45			
5 (9.11)	67.77	63.34	57.56	52.16	48.86	46.55			
6 (9.06)	67.77	63.34	57.56	52.16	48.86	46.55	39.84	36.49	
7 (9.03)	67.77	63.7	58.05	54.41	40.00	36.57	32.88		
8 (8.98)	67.96	63.52	58.38	53.2	41.61	36	32.88		
9 (8.97)	67.01	62.46	55.8						
10 (8.94)	67.01	62.81	56.59						
11 (8.87)	66.9	62.81	55.02						
12 (8.85)	66.8	61.93	61.15	55.96					
13 (8.82)	66.8	61.93	61.15	55.96					
14 (8.70)	66.8	62.11	61.24	55.96					
15 (8.66)	62.11	61.24	55.96						
16 (8.62)	61.24	56.91							
17 (8.60)	68.35	63.16	56.91	31.00	15.00				
18 (8.58)	69.31	63.16	56.91						

These gels are made with samples taken from the 223rd minute to the 327th of the chromatogram and the pH range of samples is 9.18-8.58 from left to right of gels.

From the native gel the most part of molecular weights are in the range of 55-68 kDa with the exception of the 8th well that presents some bigger proteins. Results of the native are confirmed in the SDS gel with the most part of molecular weights within the range 55-68 kDa. This similarity of molecular weights between native gel and SDS gel can mean that proteins are not constituted by attached polypeptides or their subunits are already detached due to sample processing before HPLC injection. In the 8th well of the native gels large proteins are detected and a good response is found in SDS gel with many different subunits molecular weights, that include the 67.46 kDa protein detected in the native that can even be a subunit of the large proteins. Table 3.7b highlights a recurrence of proteins with a molecular weight about 65kDa, confirming molecular weights detected from Native gel (Fig. 3.16a).

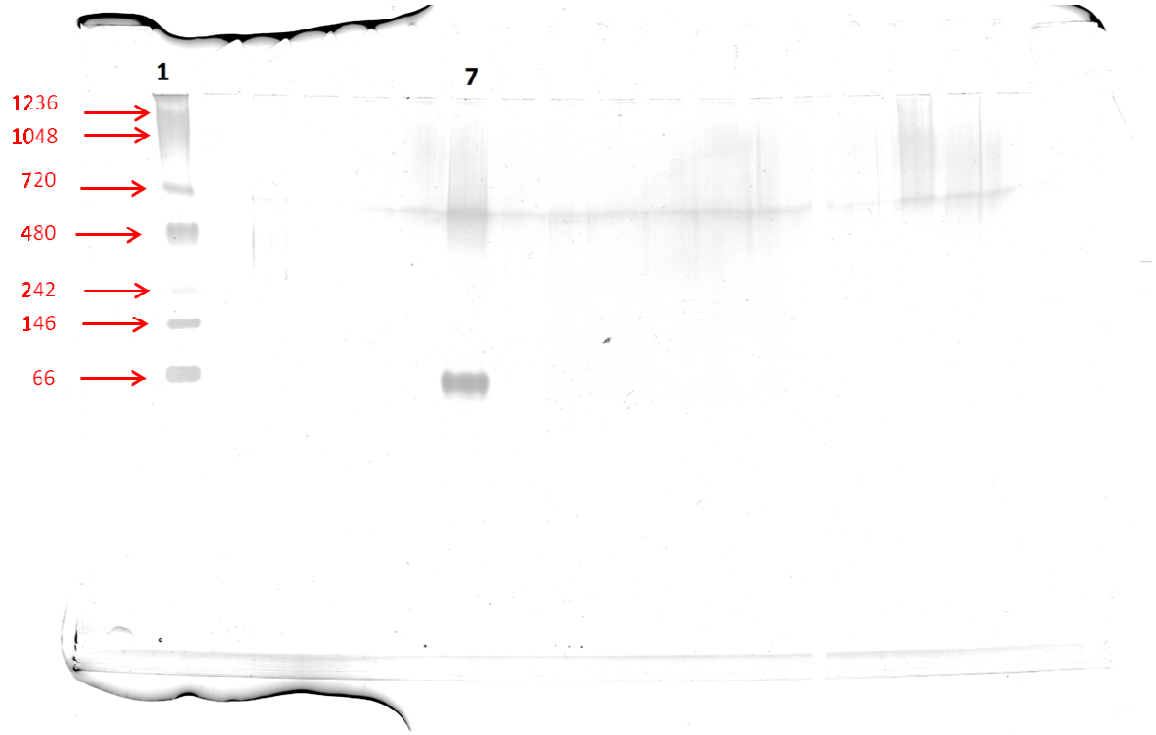

Fig.3.17a. Native gel from *Neochloris* Exponential. Sampling Time 333'-485'. Description of wells is given in Table 3.8a.

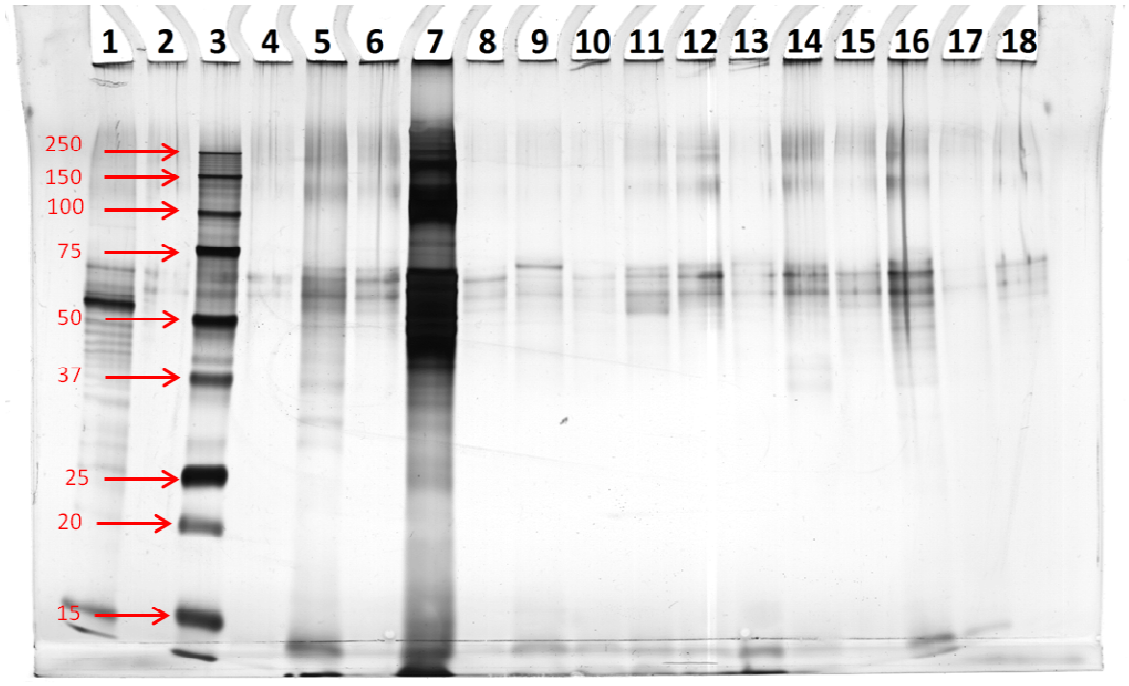

Fig.3.17b. SDS-gel from *Neochloris* Exponential. Sampling Time 333'-485'. Description of wells is given in Table 3.8b.

Tables 3.8a and 3.8b show correlation between gel-wells, molecular weights, retention time and pH of gels from Fig.3.17a and Fig.3.17b.

Table 3.8a Correlation between gel wells from Fig.3.17a molecular weights and retention time. In brackets in the first ladder, pH of sampling.

***WELL** (pH)*	***Retention time (min)***	***Molecular Weight from Native PAGE (kDa)***
1 (Marker)	---	1236 1048 720 480 242 146 66 20
2 (8.57)	333'28''-339'00''	Nd
3 (Empty)		
4 (8.50)	346'40''-350'00''	Nd
5 (8.40)	361'36''-365'00''	Nd
6 (8.20)	387'06''-391'45	Nd
7 (8.16)	391'45''-397'09''	574.18 64.03
8 (8.11)	398'16''-402'30''	Nd
9 (8.00)	409'50-415'23''	Nd
10 (7.94)	416'55''-420'00''	Nd
11 (7.85)	421'40''-430'00''	Nd
12 (7.73)	434'40''-440'00''	Nd
13 (7.60)	445'20''-448'50''	Nd
14 (7.48)	453'30''-458'30''	Nd
15 (7.43)	459'00''-462'00''	Nd
16 (7.25)	470'30''-474'00''	Nd
17 (7.17)	475'30''-479'00''	Nd
18 (7.07)	481'20''-484'50''	Nd

Table 3.8b. Correlation between gel wells from Fig.3.17b molecular weights . In brackets, pH of sampling. Every well corresponds to the same sample of Table 3.8a.

***WELL** (pH)*	***Molecular Weight from SDS PAGE (kDa)***
1 (Marker)	55.46 15.56
2 (8.57)	63.14 57.35
3 (Empty)	250 150 100 75 50 37 25 20 15
4 (8.50)	63.14 57.35
5 (8.40)	64.82 57.51 36.27 31.02
6 (8.20)	Nd
7 (8.16)	201.16 196.83 119.16 115.94 100.00 79.44 65.77 59.91 53.16 48.49 46.43 44.92 39.78 12.00
8 (8.11)	64.07 57.85 53.16
9 (8.00)	68.92 64.26 57.85
10 (7.94)	64.26
11 (7.85)	68.52 64.63 58.53 52.85
12 (7.73)	68.52 64.82 58.7
13 (7.60)	65.39 58.87
14 (7.48)	69.32 65.39 59.04
15 (7.43)	65.97 59.39 55.7
16 (7.25)	69.32 65.97 59.39 55.7 48.49
17 (7.17)	68.92 62.05
18 (7.07)	68.92 62.05 57.85

Samples of gels from Fig. 41a and Fig.41b correspond to peak of the chromatogram between the 333th and 485th minutes. The pH range for the chromatogram for these samples is 8.58-7.17. In the native gel bands appeared just in two wells, the ladder and one unknown sample. From a first impression it could be said that all the other gel wells are empty but this idea is not supported from SDS result. A first hypothesis is that Native and SDS gels have different sensitivities and concentrations were too low to be detected from the native gel. The second hypothesis is that something went wrong in sample preparation of the native gel. The 7th native well detects only two bands, and proteins from the heaviest one are split in several subunits as shown in the 7th SDS well.

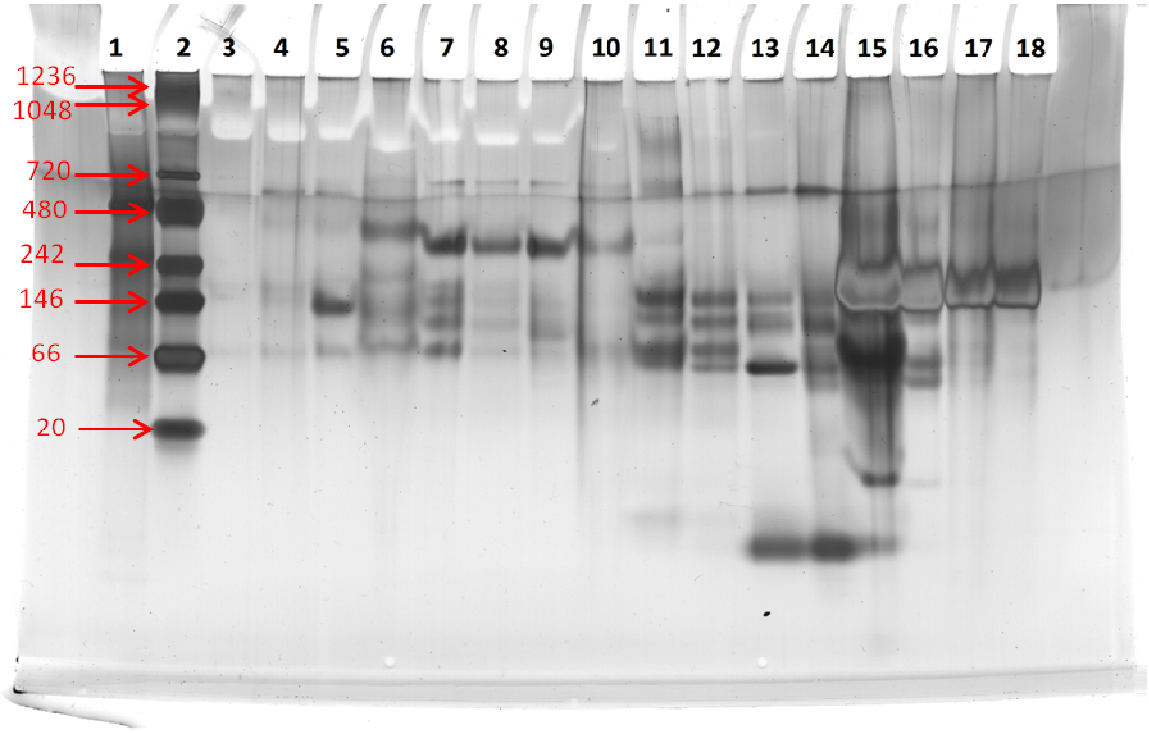

Fig. 3.18a. Native gel from *Neochloris* Exponential. Sampling Time 491'-609'. Description of wells is given in Table 3.9a.

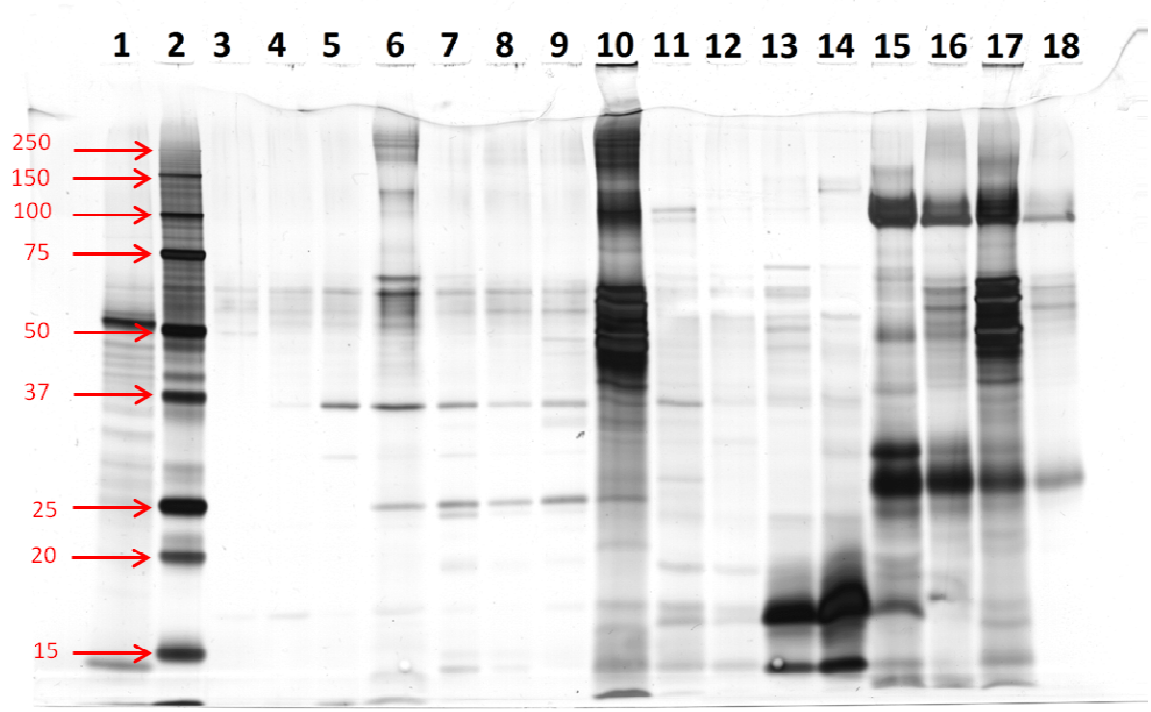

Fig. 3.18b. SDS-gel from *Neochloris* Exponential. Sampling Time 491'-609'. Description of wells is given in Table 3.9b.

Table 3.9a and Table 3.9b show correlation between gel-wells, molecular weights, retention time and pH of gels of Fig.3.18a and Fig.3.18b.

Table 3.9a. Correlation between gel wells from Fig. 3.18a molecular weights and retention time. In brackets the pH of sampling.

***WELL** (pH)*	***Retention time (min)***	***Molecular Weight from Native PAGE (kDa)***							
1 (RuBisCO)	---	496.27							
2 (Marker)	---	1236	1048	720	480	242	146	66	20
3 (6.90)	490'50''-494'30''	172	71.21						
4 (6.83)	494'41''-500'00''	589.51	400	149.56	101.26	71.21			
5 (6.61)	506'00''-509'30''	560.77	385	142.02	72.7				
6 (6.43)	514'00''-519'30''	367.65	213.28	128.94	74.73				
7 (6.34)	519'30''-522'30''	651.5	302.83	172.79	151.37	11.54	73.71		
8 (6.27)	522'30''-525'00''	658.78	302.83	178.06	149.56	101.26	72.7		
9 (6.04)	532'00''-535'30''	651.5	290.25	178.06	149.56	88.81	71.21		
10 (5.84)	539'00''-545'00''	655.13	290.25	88.21	71.21				
11 (5.42)	556'20''-560'40''	640	154.12	110.01	72.7	4.37	3.07		
12 (5.30)	560'40''-563'20''	579.77	151.37	109.25	72.7	55.16			
13 (5.22)	563'20''-566'45''	599.42	151.37	109.25	54.3	2.76			
14 (5.01)	570'06''-573'20''	599.42	154.12	107.75	71.7	58.25	41	2.76	
15 (4.50)	585'05''-591'05''	154.12	116.25	68.79	8.75	2.96			
16 (4.33)	591'05''-594'05''	154.12	59.17	42.63	7.96				
17 (4.05)	598'10''-604'10''	154.12	72.7	59.17					
18 (3.83)	604'10''-609'10''	154.12	72.7	54.3					

Table 3.9b. Correlation between gel wells from Fig. 3.18b and molecular weights. In brackets the pH of sampling. Every well corresponds to the same sample of Table. 3.9a.

***WELL**(pH)*	***Molecular Weight from SDS PAGE (kDa)***										
1 (RuBisCO)	52.4	14.57									
2 (Marker)	250	150	100	75	50	37	25	20	15		
3 (6.90)	61	55.22	50								
4 (6.83)	60.98	55.22	52.26								
5 (6.61)	60.98	55.22	52.26	35.66	29.64	16.59					
6 (6.43)	309.3	272.22	227.17	125.12	65.88	60.48	55.33	52.26	35.52	24.77	16.98
7 (6.34)	65.88	60.98	55.53	35.79	25.14	24.5	19.5	14.37			
8 (6.27)	60.98	55.22	35.92	25.37	19.5						
9 (6.04)	60.98	58.51	55.22	47.67	44.47	35.99	33.67	25.61	24.94	20	
10 (5.84)	309.3 43.42	272.22 38.44	227.17 35.92	185.58 33.67	125.12 25.7	61.49 20.9	57.71 17.37	55.22 16.09	50 14.57	47.33	44.47
11 (5.42)	105.48 14.5	96.19	65.34	61.49	52.26	35.85	27.42	23.92	19.31	17.11	16.52
12 (5.30)	53.42	36	31.6	19.31	17.11	16.52	14.5				
13 (5.22)	69.23	62	59.98	53.42	50.42	46.5	36.39	23.59	16.88	14.41	
14 (5.01)	124	69.23	53.42	36.39	30.19	23.59	17.27	14.57			
15 (4.50)	109.49 14.55	97.32	69.23	48.7	37.89	30.19	27.07	24.65	19.58	18.81	17.11
16 (4.33)	108.91	97.89	64.62	60.98	56.14	50.89	48.47	45.33	30.36	27.27	25.09
17 (4.05)	111.26 16.44	97.89 14.75	64.62	61.49	59	56.3	50.42	45.33	39.85	30.36	27.27
18 (3.83)	97.69	64.62	61.49	59	56.3	27.27					

Samples from gels of Fig.3.18a and Fig.3.18b, correspond to peaks between 485th and 625th minutes of the chromatogram and the pH range is 7.17-3.27. This was the pH range of expected maximum proteins elution (§3.1), many more molecular weights were detected in the native gel and consequently a higher number of molecular weight was expected in the SDS gel. A higher number of heavy proteins are detected in gel from Fig. 3.18a than detected proteins from gel of Fig.3.15a, Fig.3.16a, Fig.3.17a. Many molecular weights are detected into the Native gel (Fig.3.18a) especially from 11th to 18th wells. Most recurrent native molecular weights are 150kDa, 115kDa, 72kDa, 8kDa. The reducing gel (Fig.3.18b) shows that some proteins detected in the Native gel do not have a quaternary structure since many bands are detected at molecular weight between 98Da and 125kDa; smaller subunits are also detected at molecular weights between 15kDa and 65kDa that can represent either a sharper detection of the entire protein or subunits from the quaternary structure. This gel shows samples from the last part of the chromatogram and suggests that acidic proteins are more stable than basic as they seem to maintain their original molecular weight without detachments. Molecular weights out from the protein marker range have to be considered carefully, because the exponential correlation of the molecular weights of the marker could be not valid any more. Molecular weights lower than 5kDa are hardly acceptable because of the excluding size of dialysis (3.5kDa) and desalting

(5kDa). These values can be seen in 11th, 13th, 14th,15th, and 16th wells from the native gel (Table 3.9a-Fig.3.18a) and 6th and 10th wells of the SDS gel (Table 3.9b-Fig.3.18b).

3.4.2.2 *Neochloris Oleoabundans* during the stationary phase

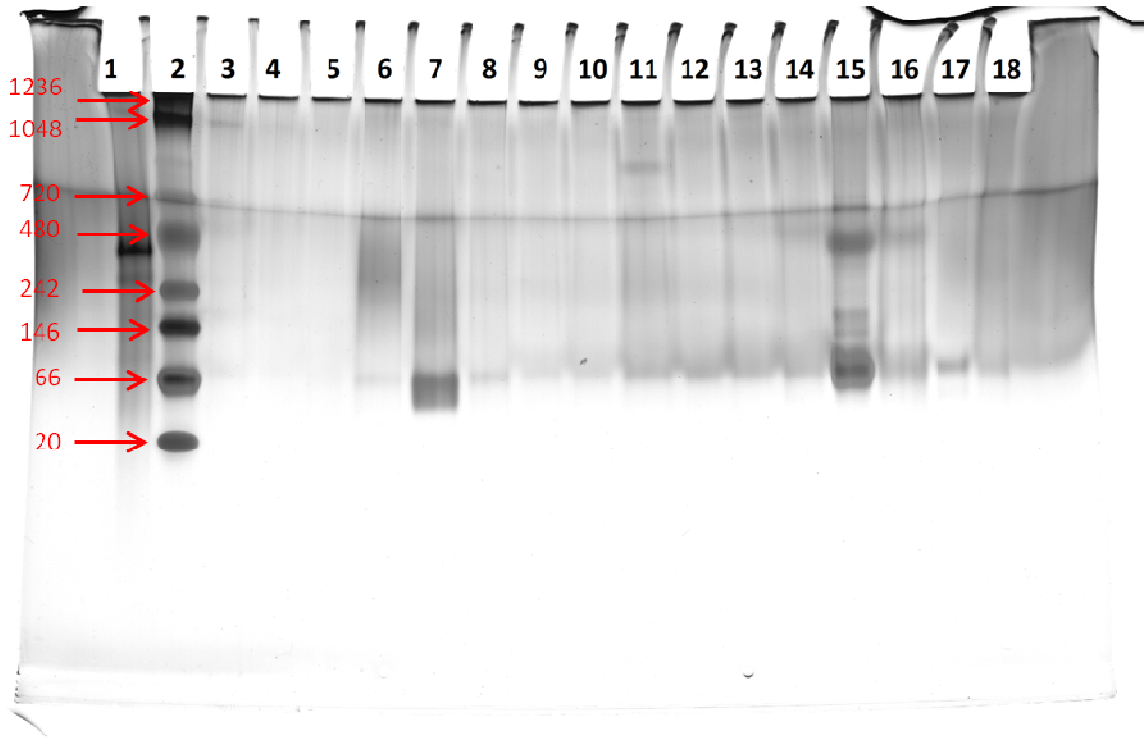

Fig.3.19a. Native gel from *Neochloris* Steady state. Sampling Time 2'-271'. Description of wells is given in Table 3.10a.

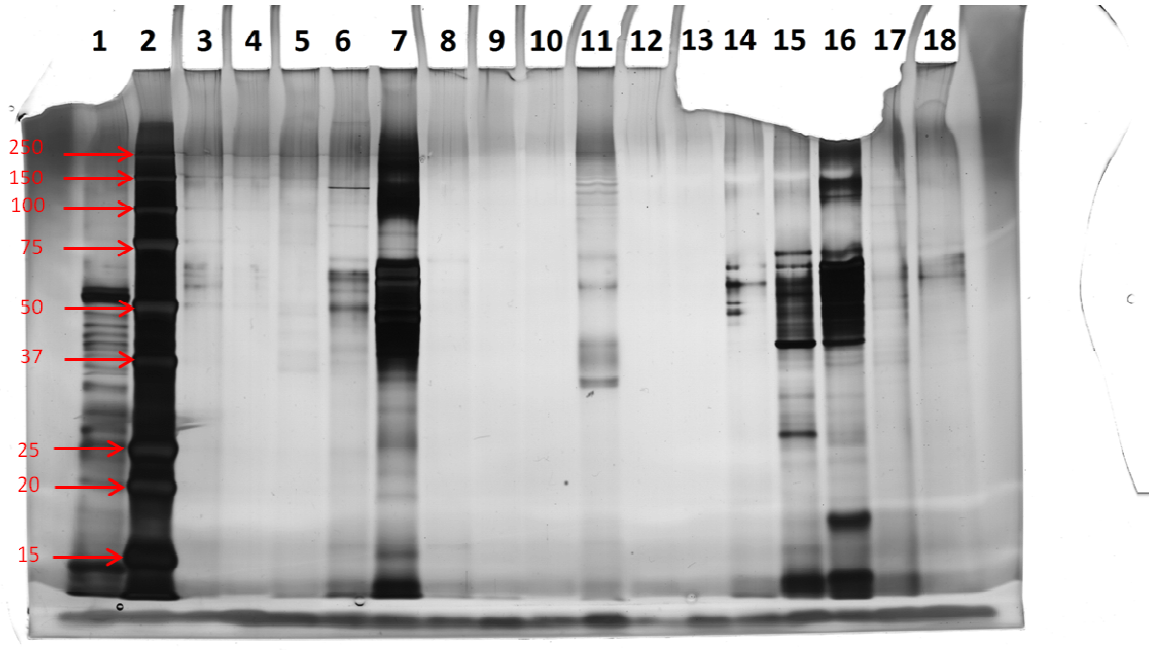

Fig.3.19b. SDS-gel from *Neochloris* Steady State. Sampling Time 2'-271'. Description of wells is given in Table 3.10b.

Table 3.10a and Table 3.10b show correlation between gel-wells, molecular weights, retention time and pH of gels from Fig.3.19a and Fig.3.19b.

Table 3.10a. Correlation between gel wells from Fig.3.19a molecular weights and retention time. In brackets the pH of sampling.

WELL	***Retention time (min)***	***Molecular Weight from Native PAGE (kDa)***
1 (RuBisCO)	---	399.26
2 (Marker)	---	1236 1048 720 480 242 146 66 20
3 (10.61)	2'00-4'15''	1025.88
4 (10.59)	6'00''-7'15''	Nd
5 (10.56)	15'30''-17'00''	Nd
6 (10.45)	33'00''-41'00''	650.00 287.61 65.43
7 (10.25)	66'16''-69'25''	650.00 56.42
8 (10.03)	97'00''-100'00''	Nd
9 (10.01)	100'00''-103'00''	Nd
10 (9.98)	103'00''-106'00''	Nd
11 (9.77)	131'30''-136'00''	834.16 77.00
12 (9.33)	196'50''-199'00''	77.00
13 (9.29)	202'50''-206'00''	Nd
14 (9.23)	213'20''-218'20''	Nd
15 (9.14)	228'35''-233'00''	447.97 175.76 130.35 77.14
16 (9.08)	238'40''-244'40''	489.96 80.49
17 (8.99)	257'30''-260'30''	78.24
18 (8.93)	268'40''-271'40''	Nd

Table 3.10b. Correlation between gel wells from Fig.3.19b molecular weights. In brackets the pH of sampling. Every well corresponds to the same sample of Table. 3.10a.

WELL *(pH)*	***Molecular Weight from SDS PAGE (kDa)***
1 (RuBisCO)	52.39 14.6
2 (Marker)	250 150 100 75 50 37 25 20 15
3 (10.61)	63.29 60.65 55.71 13.35
4 (10.59)	63.29 60.65 55.71
5 (10.56)	54.39 47.63 40 38.75 36.16 13.41
6 (10.45)	120.34 81 64 60.78 54.39 48.08 38.56 13.55
7 (10.25)	220.6 115.39 104.89 79.81 63.86 58.63 50.45 47.63 43.64 38.75 36.85 34.47 25.21 20.00 15.36 13.65
8 (10.03)	60.78
9 (10.01)	Nd
10 (9.98)	Nd
11 (9.77)	133.14 119.59 68.95 55.88 40.28 36.92 33.69 13.60
12 (9.33)	Nd
13 (9.29)	Nd
14 (9.23)	62.62 58.38 50 47.51
15 (9.14)	68.96 63.39 57.11 47.36 40.4 31.58 26.56 13.07
16 (9.08)	133.14 112.67 70.84 63.39 54.06 48.33 45.15 41.17 37.81 36.69 17.57 14.00
17 (8.99)	70.64 59.34 31.58 26.56 13.67
18 (8.93)	65.91 59.34

Samples in gels of Fig. 3.19a and 3.19b represent peaks between 2nd and 271st minutes of the chromatogram from *Neochloris Oleabundans* during the steady phase. The pH range in which they were taken is 10.68-8.93. Most recurrent molecular weights are in the 65kDa and 78kDa but many heavier proteins are present.

In the 3rd, 6th, 7th, 11th, 15th, 16th heavy proteins are detected into the native gel, and correspondence is found in the SDS gel with many different molecular weights that can identify protein subunits and a sharper detection of the molecular weights of non-detaching proteins. SDS wells (4th, 5th, 8th, 14th, 18th) that detect bands do not find a match with the same native wells. This evidence can strengthen the hypothesis of a higher sensitivity of the SDS gel.

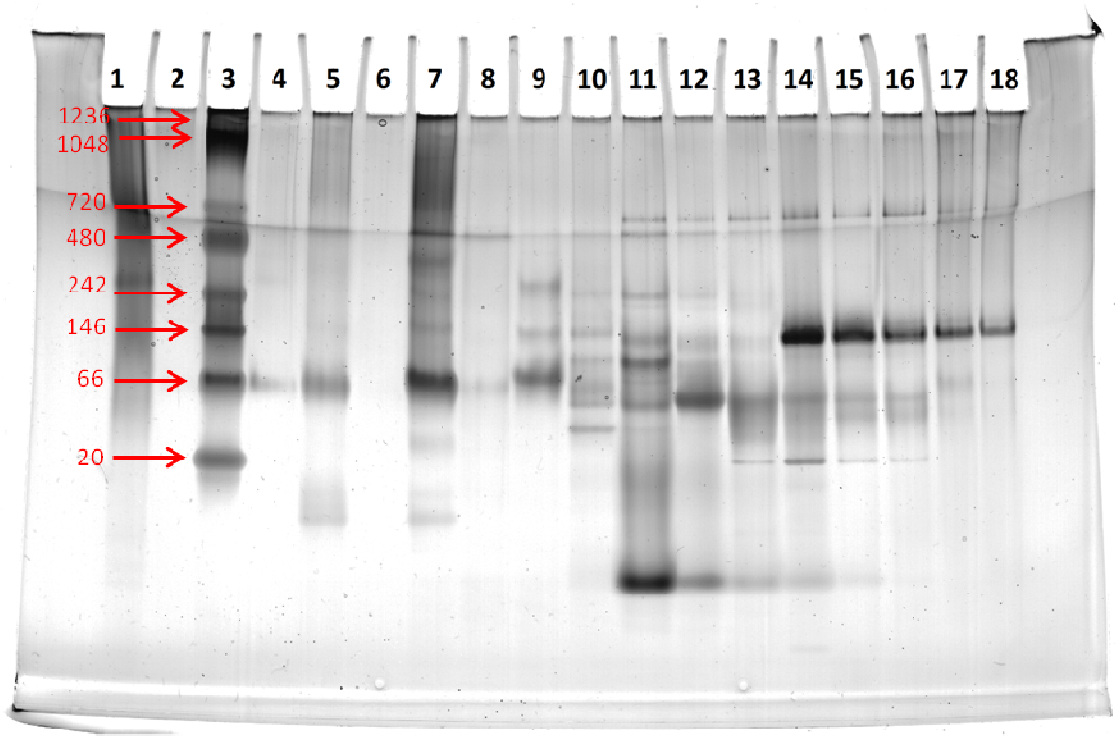

Fig.3.20a. Native gel from *Neochloris* Steady state. Sampling Time 275'-612'. Description of wells is given in Table 3.11a.

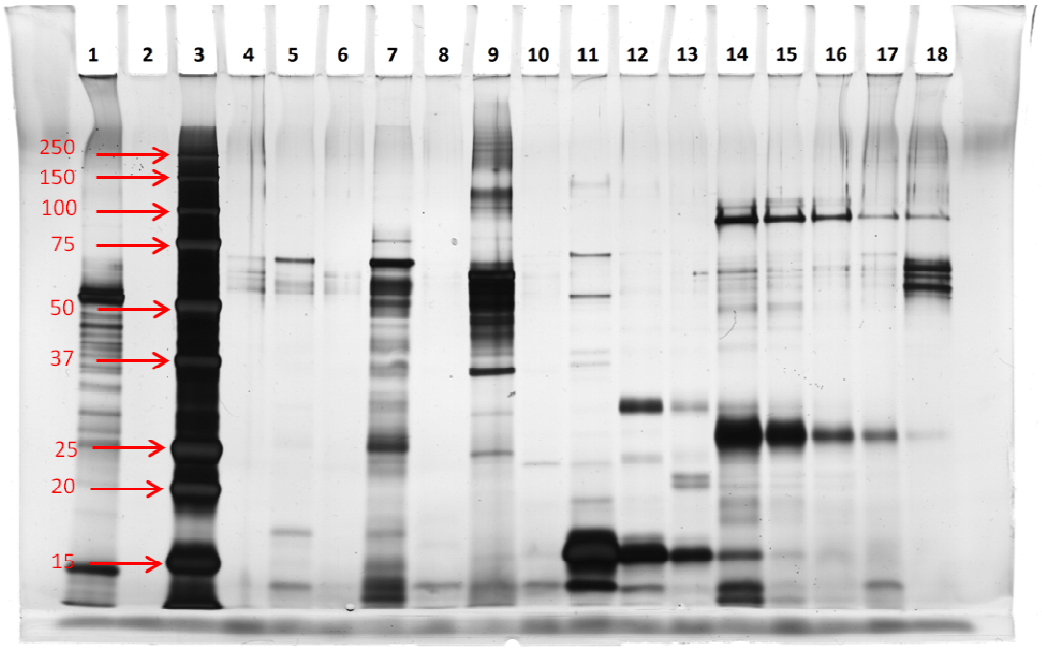

Fig.3.20b. SDS-gel from *Neochloris* Steady State. Sampling Time 275'-612'. Description of wells is given in Table 3.11b.

Table 3.11a. Correlation between gel wells from Fig.3.20a molecular weights and retention time. In brackets the pH of sampling.

***WELL** (pH)*	***Retention time (min)***	***Molecular Weight from Native PAGE (kDa)***							
1 (RuBisCO)	---	520.00							
2 (8.89)	275'40''-278'00''	61.57							
3 (Marker)	---	1236	1048	720	480	242	146	66	20
4 (8.85)	282'25''-287'25''	61.57							
5 (8.78)	295'10''-300'10''	62.43	8.23						
6 (8.44)	353'20''-359'00''	Nd							
7 (8.16)	392'30''-395'30''	400	140	67.47	26.22	8.11			
8 (7.25)	466'20''-478'00''	64							
9 (6.09)	527'37''-537'00''	267.09	139.7	66.98					
10 (5.35)	556'00''-565'00''	133.67	86	46.97	31.84				
11 (5.01)	569'00''-574'00''	649.89	242	133.67	86.00	45.68	3.13		
12 (4.79)	576'00''-581'00''	653.03	47.96	3.18					
13 (4.60)	582'00''-587'00''	660.34	47.3	18.92	3.16				
14 (4.43)	587'00''-592'00''	665.46	129.8	49.65	19.45	3.13			
15 (4.26)	592'00''-597'00''	672.12	131.72	50.35	19.72	3.2			
16 (4.08)	597'00''-602'00''	676.48	134.66	50.35	38.94	19.72			
17 (3.94)	602'00''-607'00''	137.00	63.31	38.94					
18 (3.72)	607'00''-612'00''	143.87							

Table 3.11b. Correlation between gel wells from Fig.3.20b molecular weights. In brackets the pH of sampling. Every well corresponds to the same sample of Table 3.11a.

***WELL** (pH)*	***Molecular Weight from SDS PAGE (kDa)***										
1 (RuBisCO)	52.42	14.68									
2 (8.89)	Nd										
3 (Marker)	250	150	100	75	50	37	25	20	15		
4 (8.85)	66.04	60.43	54.32								
5 (8.78)	65.45	55.29	54.32								
6 (8.44)	60.43	55.29									
7 (8.16)	74.56 13.66	64.68 13.13	55.29	49.88	48.02	44.52	36.02	30.7	25.26	16.74	14.95
8 (7.25)	Nd										
9 (6.09)	117.25 24.49	101.03 13.69	63.92	60.07	58.15	54.97	49.88	45.8	42.76	38.89	35.28
10 (5.35)	23.03	13.61									
11 (5.01)	68.02	52.27	38.98	36.47	23.03	18.85	15.58	13.76			
12 (4.79)	30.19	23.63	15.49	13.64							
13 (4.60)	30.07	21.66	20.41	15.47							
14 (4.43)	88.83	61.33	59.36	48.6	26.55	19.09	15.49				
15 (4.26)	90.34	61.33	48.94	26.61	15.47						
16 (4.08)	91.5	62.61	26.61	15.47							
17 (3.94)	93.06	68.22	26.55	13.78							
18 (3.72)	92.67	65.26	62.61	59.36	55.62	52.74	26.5				

Samples of gels from Fig.3.20a and Fig.3.20b represent peaks of the chromatogram between 271st and 625th minute.

The pH range in which these samples were taken is 8.93-3.16. These gels contains samples from the most acidic region in which higher elution of proteins is expected (§3.1). Clear bands are detected both in native and in the SDS gels at the same molecular weights especially from the 11th to the 18th. This repetition can point to the abundance of proteins with these molecular weights that elute very slowly and at different pH. Bands from the Native gel (Fig.3.20a) at around 3.1kDa are present through 5 different wells (11th to 15th) which cover 28 minutes of the chromatogram. As previously stated molecular weights lower than 5kDa are not acceptable, so a sharper detection is the aim of the SDS-gels that identify that proteins at 15.5kDa. A similar behavior can be observed again in the Native gel through 5 wells (14th to 18th) covering 25 minutes of elution. Bands from the native at about 132kDa from 14th to 18th well could be detected in the SDS gel as a whole protein (around 90-92kDa). Other bands are detected in the same SDS wells and they are likely to be the detached proteins into subunits (around 26.5 kDa) as both of the configuration are detected in the same correspondent wells. One more hypothesis is that all proteins got detached and subunits sizes are about 90kDa and 26.5KDa, which sum is not too far from the supposed native molecular weight of 132kDa. Differences in migration pattern are not allowed in a reducing gel, that is why more than one protein with same molecular weights is expected to be detected into the native gel.

As previously said, molecular weights values overcoming limits of the markers and especially lower than 5kDa are not completely reliable.

3.4.3 Western Blot characterization

Western Blot was made to detect with no uncertainties which peaks from the chromatograms were related to RuBisCO. Samples. Many problems with buffers were encountered and several shift and rinse of SDS-gels were required that one gel broke and some proteins may have gone. For the experiments, in the SDS gels samples from pure RuBisCO and from the whole microalgal protein mixture were run, to have a reference for molecular weight and to ensure correct functioning of the antibodies.

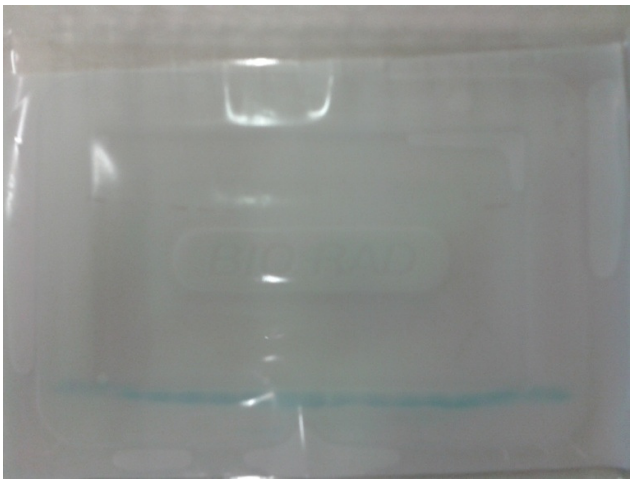

Fig. 3.21. Western blot membrane. On the left at middle height, RuBisCO bands from samples are detected.

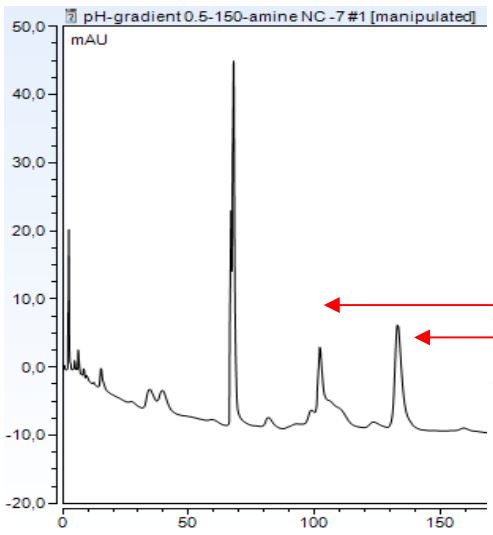

Fig. 3.22a. Part of the chromatogram from steady *Neochloris Oleoabundans.*

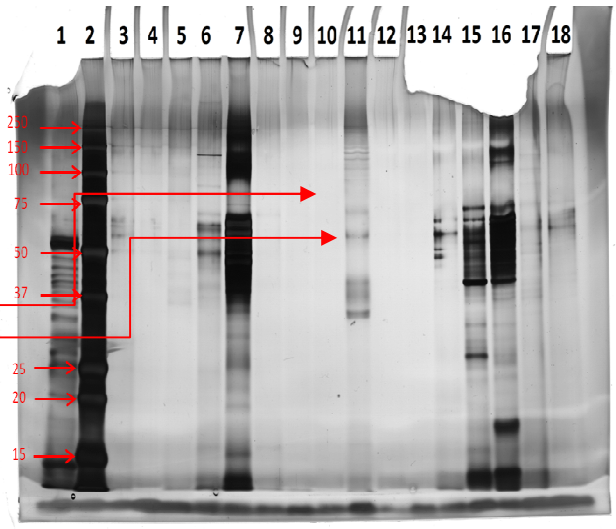

Fig.3.22b. SDS-gel with samples from chromatography from *Neochloris Oleoabundans* with sampling corresponding to time range of Fig.3.22a.

Totally, 6 bands from samples are visible, plus the RuBisCO's reference band. RuBisCO was detected by Immunoblot in the peaks pointed out by the red arrows in samples taken from chromatography of *Neochloris Oleoabundans* during the steady phase. Peak eluting around

minute 100th do not display bands into the related well into the SDS gel. This is caused by the different sensitivity of the two experiments. Immunoblot detects down to femtograms, while silver staining can detect nanograms. The other 4 bands refer to the full microalgal mixture from *Neochloris Oleoabundans* during the exponential and steady phase, *Desmodesmus* and *Chlorella vulgaris*. *Neochloris Oleoabundans* during the exponential phase did not show any band even if the amount of water soluble proteins of this microalgal strain is more than double than during the steady phase. This may be cause to the several washings required to change running buffers for the electrical device and that the gel containing these proteins broke.

3.5 RuBisCO detection

Standard RuBisCO was analyzed in the AEC system . At first an experiment by AEC was made using pure sample of RuBisCO from spinach to understand its retention time. Eluted fractions were taken for further examination by electrophoresis. This procedure was made to understand if the protein was likely to elute in the natural form or detached in subunits. If RuBisCO was eluting in the detached form, seeing which subunit was eluting first would have been possible. Experiments were also made to understand by visual resolution, RuBisCO's concentration in three different strains: *Chlorella vulgaris*, *Neochloris Oleabundans* during Exponential phase, *Neochloris Oleabundans* during Steady phase and *Desmodesmus spp*.

3.5.1 RuBisCO's chromatogram

A chromatogram with pure RuBisCO was made to understand the retention time of this protein, using the same pH profile as *Neochloris Oleabundans* showed in Fig. 2.4. From literature (Barbeau & Kinsella, 1988), RuBisCO is expected to have its Isoelectric point at pH=4.4-4.7 but elution of the most part of it happens at the beginning. Conductivity was excluded as the cause of the earlier elution because it is very close to the values from *Neochloris Oleabundans* samples, as shown in Table 3.5.

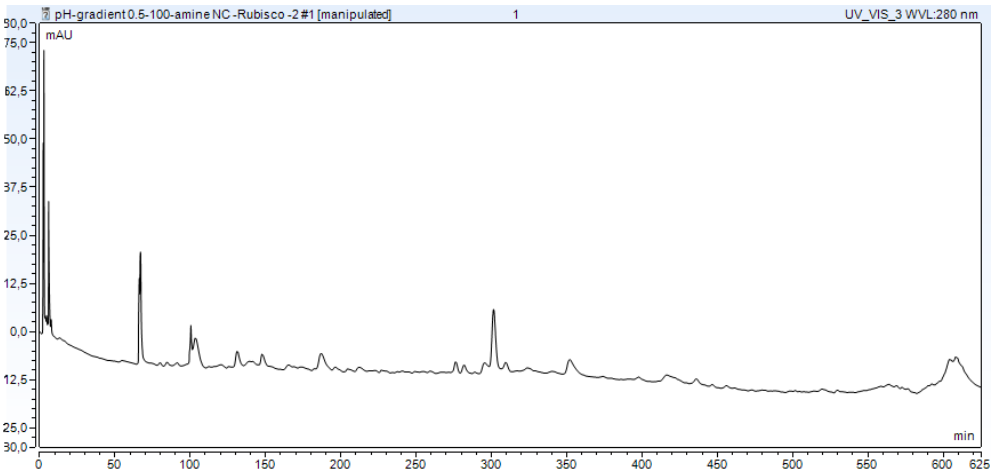

Fig.3.23. Chromatogram of pure RuBisCO from spinach.

3.5.2. Native and SDS PAGE from HPLC run

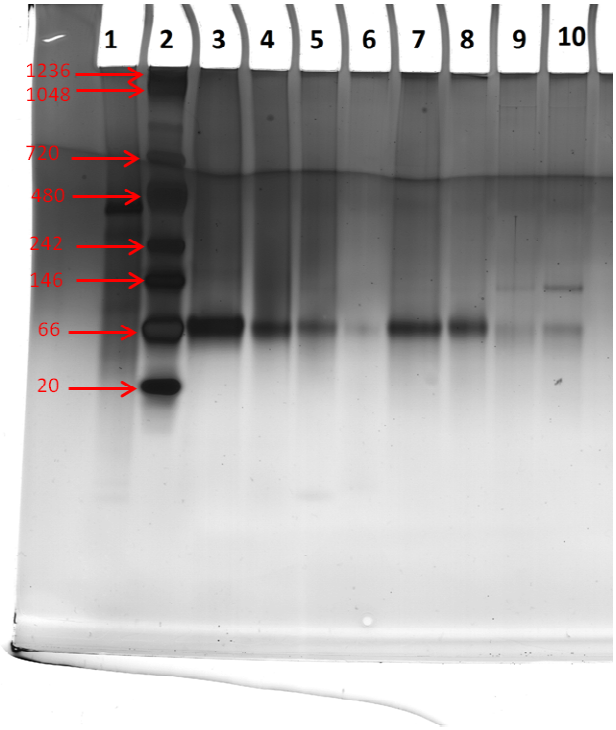

Fig.3.24a. Native gel with samples from pure spinach RuBisCO experiment with AEC. Description of wells is given in Table 3.12a.

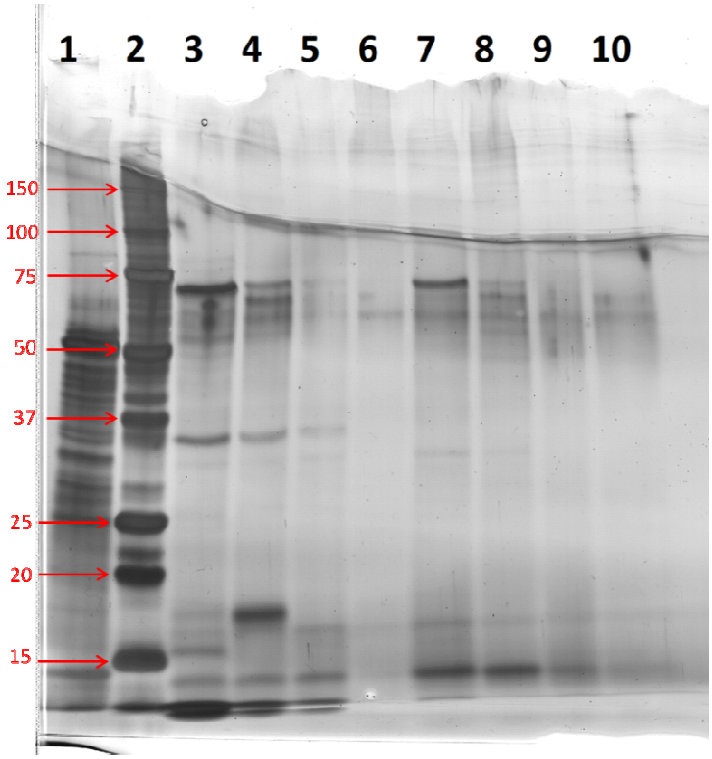

Fig.3.24b. SDS gel with samples from pure RuBisCO experiment with AEC. Description of wells is given in Table 3.12b.

Table 3.12a. Molecular weights from Native gel of Fig.3.24a. In brackets pH of sampling.

***Well** (pH)*	***Retention time (min)***	***MW from Native gel (kDa)***							
1 (RuBisCO)		397.99							
2 (Marker)		1236	1048	720	480	242	146	66	20
3 (10.61)	1’35’’-2’42’’	69.36							
4 (10.61)	2’42’’-3’25’’	70.35							
5 (10.59)	5’49’’-8’00’’	69.85	2.04						
6 (10.26)	65’50’’-68’05’’	410.01	68.87	2.2					
7 (9.40)	185’30’’-191’00’’	410.01	67.90						
8 (8.76)	301’10’’-304’20’’	410.01	69.36						
9 (3.97)	600’20’’-606’30’’	400.56	127.60	62.38					
10 (3.75)	606’20’’-612’30’’	400.56	128.51	66.94					

Table 3.12b. Molecular weights from SDS gel of Fig.3.24b. In brackets pH of sampling.

***Well** (pH)*	***MW from SDS gel (kDa)***									
1 (RuBisCO)	52.54	14.41								
2 (Marker)	150	100	75	50	37	25	20	15		
3 (10.61)	69.32	61.33	57.68	53.63	34.12	31.15	17.5	16.38	15.47	13.97
4 (10.61)	70.75	65.39	59.56	54.57	34.34	17.4	14.07			
5 (10.59)	71.79	67.13	61.33	35.57	31.93	16.56	14.2			
6 (10.26)	60.79	16.56	14.2							
7 (9.40)	71.58	59.58	32.6	17.15	14.49					
8 (8.76)	71.58	66.35	59.39	55.54	48.53	32.53	17.08	14.54		
9 (3.97)	59.39	17.27	14.51							
10 (3.75)	65.77	59.39	14.54							

In the first well of figures 3.24a and 3.24b the injected solution was made of standard RuBisCO from spinach.

Samples in the gels are chronologically ordered as they were taken from the HPLC. A clearer view about matching peaks and molecular weights will be given in §3.4.3.

At a first sight it seems that RuBisCO in the natural form does not almost appear, except for well from 5^{th} to 10^{th} of Fig.3.24a in which a very light band is detected at the molecular weight of 400kDa. In literature (Mangino, 2007) is found that denaturation of proteins can be observed at high pH (>>pI) or lower pH (<<pI) and the Isoelectric point for RuBisCO is 4.4-4.7 (Barbeau & Kinsella, 1988).

The small subunit can be seen in the 5^{th} and 6^{th} wells of Fig.3.24a. The detected molecular weight is about 3kDa, but the value is not reliable since the extrapolation of data from fitting is not recommended. From the SDS-gel (Fig.3.24b) a different result is then achieved because in all the gel wells the smallest subunit is visible. Why the full molecule is not detected from the native gel from the 3^{rd} to 5^{th} wells is still not clear. One possible explanation is that RuBisCO got detached after the elution for denaturation at high pH.

In a reducing gel (Fig.3.24b) RuBisCO was expected to detach into subunits about 55kDa and 13kDa. Into 3^{rd},4^{th} and 7^{th} wells of Fig.3.24b the darkest bands are detected at about 70kDa. Three explanations are possible: the first is a non-complete detachment of subunits , the second

is a wrong migration of proteins. Both of them are hardly acceptable as a non complete detachment in a reducing gel is not considerable since the standard RuBisCO (Well-1, Fig. 3.24b) appears with a higher amount than the other wells and is completely detached. Wrong migration is also not really acceptable since it would seem to affect just the bands at about 70kDa (the supposed big RuBisCO subunit) and the small subunits reached the level of the standard used. The third hypothesis is proteins subunits cross-linking. (Provansal, Cuq, & Cheftel, 1975) explains that if proteins are kept in alkaline conditions, formations of unusual aminoacids and crosslink can happen. Arginine is the most susceptible amino acid with loss up to 100%. Same results were found from (Whitaker, Robert E. Feeney, & Sternberg, 2009) and what they found is that main factors inducing these chemical transformations were high temperature and long exposure to alkaline conditions.

3.5.3 Visual concentration and subunits size detection

Next gels will show the comparison between pure RuBisCO from spinach and crude protein samples from *Chlorella vulgaris*, *Neochloris Oleabundans* during the Steady phase, *Neochloris Oleabundans* during the Exponential phase and *Desmodesmus*. The adopted solution of RuBisCO has a concentration of 1g/l and for both the gels the 3rd, 5th, 7th, 9th wells shows samples of pure RuBisCO with different amounts injected. All the samples from microalgal proteins solution were diluted to 1g/l. In Table 3.14 measurements of whole RuBisCO and its subunits are reported.

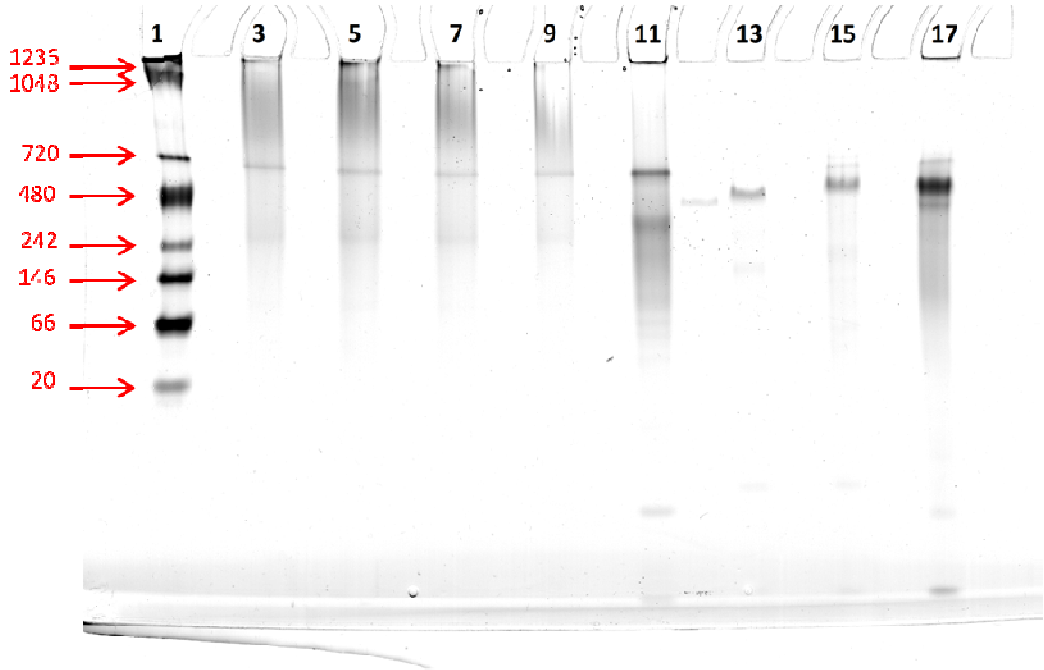

Fig.3.25. Native gel with samples from standard spinach RuBisCO, *Chlorella vulgaris, Neochloris Oleabundans* Steady phase, *Neochloris Oleabundans* Exponential phase and *Desmodesmus*. Samples order, injection amount and concentration of starting solutions are reported in Table 3.13.

Table 3.13. Correlation between well of gel from Fig.3.25 and sample injected. All microalgal strain was diluted at $1gL^{-1}$ and the injected amount was the same per every microalgal sample. From well 3 to 9 Standard solution from RuBisCO spinach ($1gL^{-1}$) was used.

WELL	*Sample*	*Amount in Fig.47[μL]*	*Amount in Fig. 48-49[μL]*
1	Marker	7	6
3	RuBisCO	12	10
5	RuBisCO	10	8
7	RuBisCO	8	6
9	RuBisCO	6	4
11	*Chlorella vulgaris*	*9*	*7*
13	*Neochloris Steady phase*	*9*	*7*
15	*Neochloris Exponential phase*	*9*	*7*
17	*Desmodesmus*	*9*	*7*

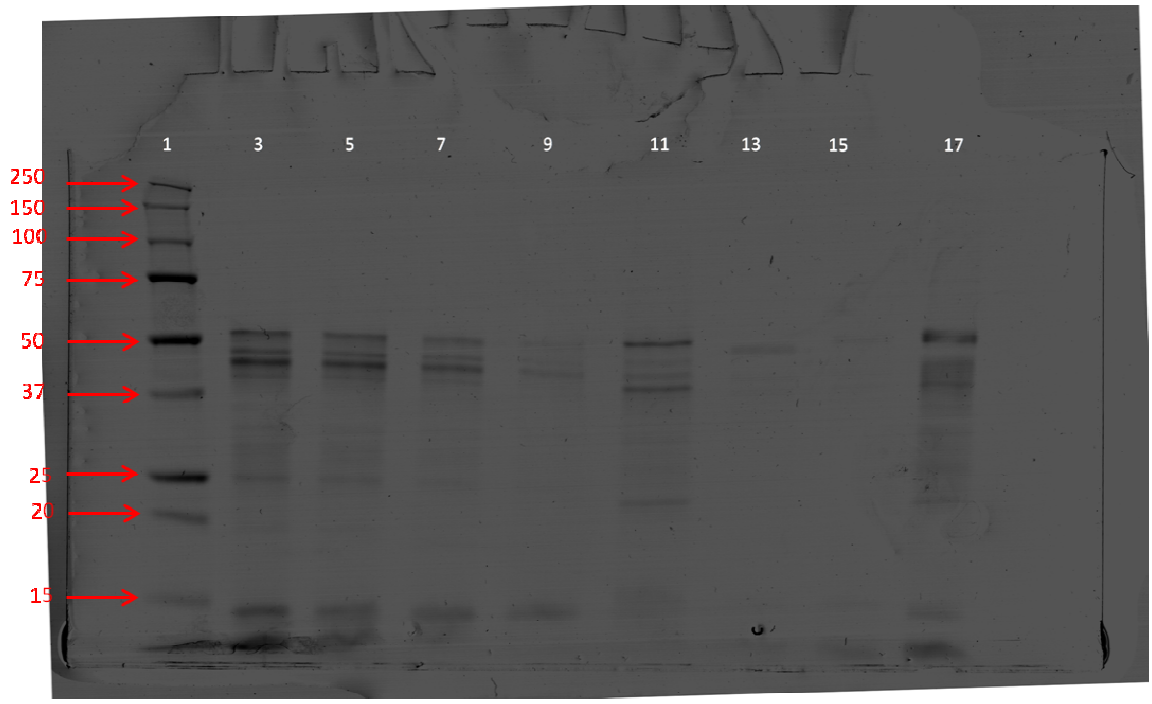

Fig.3.26. SDS gel with samples from standard spinach RuBisCO, *Chlorella vulgaris, Neochloris Oleabundans* Steady phase, *Neochloris Oleabundans* Exponential phase and *Desmodesmus*. Samples order, injection amount and concentration of starting solutions are reported in Table 3.13.

Before doing the calculation from the visual results from Fig.3.25, a protein determination of a solution containing pure RuBisCO ($0.2gL^{-1}$) was measured with Lowry protocol (§2.2) and the result was $0.15gL^{-1}$. That indicates that 0.2 g of standard RuBisCO is quantified s 0.15 g using the Lowry method. It is worthwhile to remind that Lowry method was relative to BSA.

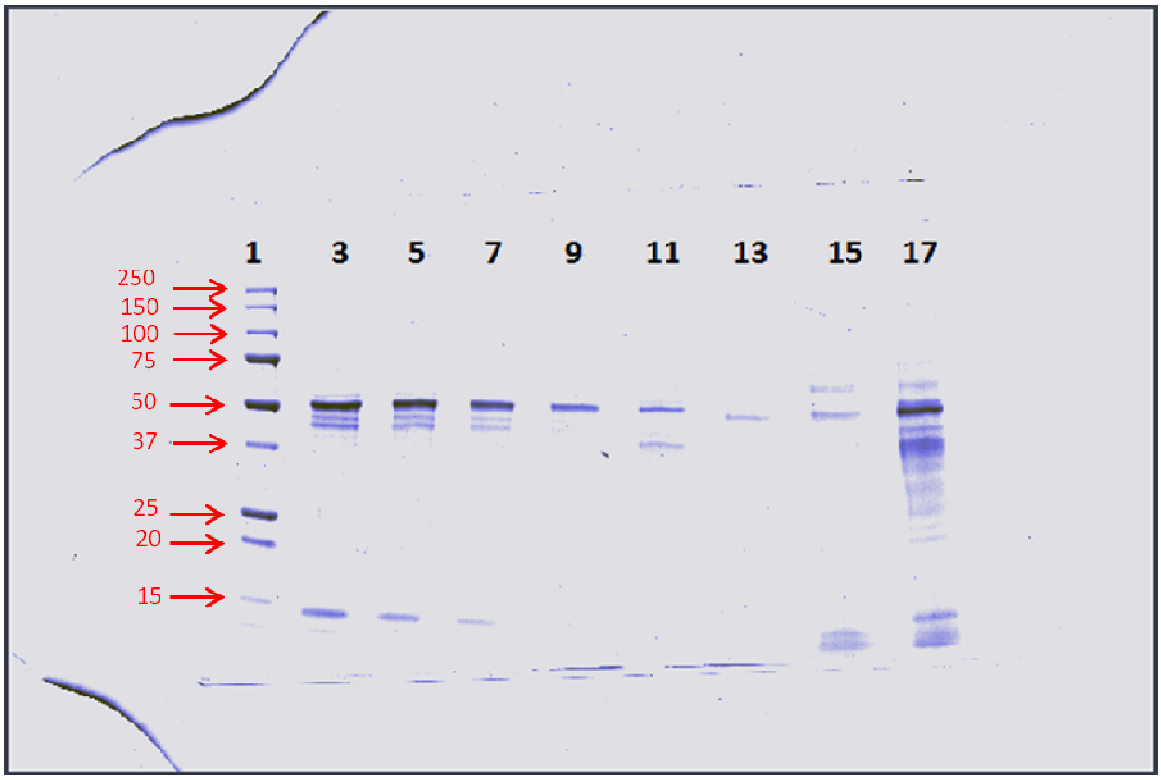

Fig.3.27. The figure shows the SDS gel from which from which RuBisCO was quantified. Samples are from standard spinach RuBisCO, *Chlorella vulgaris, Neochloris Oleabundans* Steady phase, *Neochloris Oleabundans* Exp phase and *Desmodesmus*. Samples order, injection amount and concentration of starting solutions are reported in Table 3.13.

Table 3.14. Molecular weights of whole RuBisCO from different organisms and its subunits. The last column shows RuBisCO % on measured proteins content.

Sample	***WELL***	***Native MW (kDa)***	***SDS MW (kDa)***		***RuBisCO %***
Spinach RuBisCO	3,5,7,9	491.21	53.94	12.66	/
Chlorella vulgaris	11	489.65	52.32	12.82	29.13
Neochloris Oleabundans exponential phase	15	468.20	53.39	13.15	19.96
Neochloris Oleabundans steady phase	13	455.91	50.68	12.93	8.86
Desmodesmus	17	468.86	53.93	12.46	30.7

From a visual elaboration of the SDS gel from Fig.3.27 with the software ImageJ calculation of RuBisCO's quantity inside each microalgal sample was possible and in Table 3.14 results are showed. RuBisCO's content is expressed as percentage of the all protein content. The starting concentration was the same (1 g/l) for all the microalgal strains and was detected according to Lowry procedure §2.2. Calculation of RuBisCO percentage in *Desmodesmus* was made as ratio of the 55kDa band and all the other bands. This value can be overestimated since some protein may not be stained for the Coomassie brilliant Blue sensitivity.

Gel from Fig.3.27 was used for RuBisCO quantification, but SDS-gel from Fig.3.26 was required to make the molecular weight detection. Results for quantification seem to be antithetical but the

origin are technical problem encountered during inserting samples in gels of Fig.43.25 and Fig.3.26.

Table 3.14 shows achieved results from Fig.3.25, Fig.3.26 and Fig.3.27. Similarity between molecular weights of RuBisCO's subunits from different microalgal strains can be observed. Expected RuBisCO's molecular weight was about 550kDa (Barbeau & Kinsella, 1988) and results were detected according to the Native gel-reliability for molecular weight of non-denatured proteins detection. This property is clear checking the 12th and 13th well from Fig.3.25, where the same sample was injected, but revealed molecular weights are different.

Measurements were made to calculate *Neochloris* protein content per unit of biomass and 5.2% for the Steady phase and 8.3% for the Exponential phase were found. (Gatenby, Orcutt, Kregger, Parker, Jones, & Neves, 2003) already studied proteins content profile for *Neochloris Oleabundans* and their results are shown in Fig.3.28. Values are then lower than expected. Two explanation are possible: the first is the incomplete cell disruption that did not allow the release of all the soluble proteins. The second explanation is that soluble proteins are a small fraction of the total proteins and the most part of the proteins is trapped into the solid phase. As previously stated, the aim of this work was studying the water soluble protein fraction since protein extraction from the precipitate would cost more in terms of equipments since a solvent would be required.

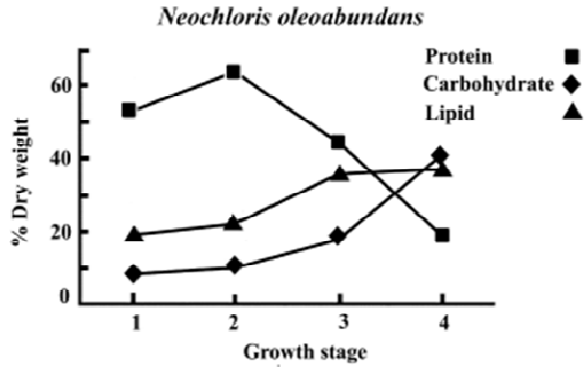

Fig.3.28 Carbohydrate, protein and lipid contents of *Neochloris Oleabundans* at different growth stages (1=early log/log; 2=late log; 3=stationary; 4=late stationary)

A resume of the amount of proteins injected in the AEX column, RuBisCO percentage on the total amount of proteins and size of its subunits are reported in Table 25.

Table 3.15. Main results and parameters: HPLC injections, RuBisCO% among all the proteins, size of RuBisCO's subunits.

	HPLC injections volume[μL]/conc [gL^{-1}]	*RuBisCO %*	*RuBisCO subunits [kDa]*	
			BIG	SMALL
Chlorella vulgaris	200 / 4.37	29.13	52.32	12.82
Neochloris Exp phase	100 /16.57	19.96	53.39	13.15
Neochloris Steady phase	200 / 11.37	8.86	50.68	12.93
Desmodesmus	---	30.7	53.93	12.46

4. PROCESS DEVELOPMENT

4.1 Process fundamentals

An industrial scale recovery of the water soluble protein fraction from microalgae has not yet been developed. The aim of this chapter is matching the literature state-of-the-art and the results of the experiments that were made on separation and characterization of the water soluble fraction of the proteins in order to develop a possible industrial process for producing proteins from micro algal biomass.

For a large scale plant, lowering waste quantities and energy consumption must be considered carefully to aim to a higher economic profict. For protein recovery the whole cell will not be necessary and the cell wall would be discarded. Anyway, the cell wall is mainly composed by polysaccharides that according to (Mussgnug, Klassen, Schlüte, & Kruse, 2010) can be exploited as energy, for instance by fermentation, to produce biogas composed by methane and carbon dioxide.

In Figure 4.1 a block flow diagram of the process is represented, where for each stage many options are available to achieve the target but some are better than others. The selection among them will be clarified since some techniques are more easily scalable than others. *Neochloris Oleoabundans* has not been widely studied yet and that is why almost all the articles cited in this section refer to other microalgal strains. However, explanations given do not lose significance since all the microalgae that are considered make part of the same division as *Neochloris Oleoabundans*, the Chlorophyta.

Figure. 4.1. A Block Flow Diagram of the process to produce microalgae aimed to protein recovery.

4.1.1 Photobioreactor

The photobioreactor is a system in which microalgae can grow in optimal conditions. Several kind of PBR have been developed until now but only two of them are said to be suitable for large scale application. The first one is the raceway pond, that is an open reactor and is widely used for microalgae strains that are less affected from contamination like *Arthrospira Platensis.* From the productive point of view, a tubular photobioreactor is the best available for industrial applications since it is sealed and is optimized for light irradiance. Tubular photobioreactor costs more than raceway ponds, both for the initial investments and for the operational costs, but

they provide a more efficient use of the sunlight and a higher specific productivity and and better control of operative conditions (Ugwy, Aoyagi, & Uchiyama, 2008).

The photobioreactor represents definitively an important part of the total costs and its choice must be carried out carefully.

4.1.2 Harvesting

Microalgae harvesting has been widely studied for species such as *Arthrospira Platensis* that has found many investors thanks to its healthy properties and its size, that make harvesting easier to be accomplished. Efficiency in biomass recovery and economic impact must be verified since every technique has pros and cons, but according to the literature, some harvesting paths may have a lower economic impact on the whole process than others

4.1.2.1 Filtration

Filtration is the technique chosen to harvest the culture since it requires a low energy consumption that is mainly represented by the pumping energy to face the pressure drop into the filter.
(Petrusevski, Bolier, Van Breemen, & Alaerts, 1994) found that Tangential Flow Filtration is an affordable and reliable way to harvest microalgae. According to the article a membrane with pore size equal to 0.45μm is enough to concentrate microalgae. Their results are summarizes in figure 4.2.
During the filtration, the inlet stream is split into a concentrate stream whose concentration values are shown in Fig. 4.2b, so that its water content decreases by the same factor. The quantification of chlorophyll-α is necessary to understand the filtration pattern and for the mass balance of the filtration system. For the quantification of chlorophyll-α content, we refer to the data from (Pruvost, Van Vooren, Cogne, & Legrand, 2009) where lipids from *Neochloris Oleoabundans* were quantified and characterized. From the values reported in the quoted article, the filtration pattern shown in fig. 4.2b has to be taken into account. Biomass recovery is expressed with a "Concentration Factor C" that is defined as the ration between the inlet and the outlet flow rates. With a C factor equal to 40, the biomass lost through the filter is 30% (Petrusevski, Bolier, Van Breemen, & Alaerts, 1994).

Advantages of this technique are low energy requirements and the easy repair or manutention of the membrane.

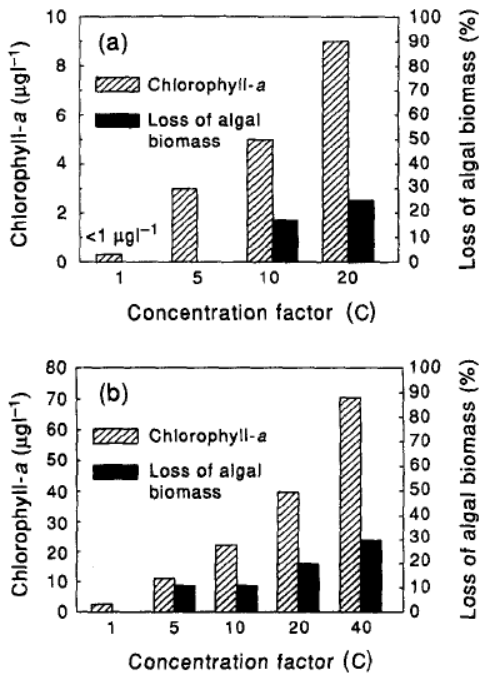

Fig.4.2 Recovery of algal biomass, expressed as chlorophyll-α, and percentage of biomass lost at different concentration factors: (a) reservoir water with a very low algal concentration (chlorophyll-α < 1µg/l); (b) reservoir water with a moderate algal concentration (chlorophyll-α ≥ 2.5 µg/l). (Petrusevski, Bolier, Van Breemen, & Alaerts, 1994)

4.1.2.2 Centrifugation

Centrifugation is a reliable technique that allows the recovery of the biomass. Centrifugation forces used in laboratory for biomass settling were 19.000 and 31.000g. According to (Molina Grima, Belarbi, Acien Fernandez, Robles Medina, & Chisti, 2003) 95% biomass recovery is achieved with g-forces higher than 13.000g while the recovery lowers to 60% and 40% with g-forces equal to 6.000g and 1.300g respectively. Centrifugation implies a high energy consumption but its reliability is an advantage. Mechanical troubles may occur and a back up centrifuge may be necessary to ensure continuity of this process.

4.1.2.3 Settling

Another method used for biomass separation from the culture medium is natural settling. Anyway natural settling is affected from operating variables such as contamination, retention time and salt concentration.

In addition chemical flocculation of microalgae is the most common way of flocculation. Chemical compounds such as zinc, aluminum, iron salts and polyelectrolytes have been studied since 1988 from (Bilanovic & Shelef, 1988). Chemical flocculation is advisable only if non toxic

compounds are used because, even if biomass is usually washed from the medium, traces of flocculants may remain into the biomass.
(Collet, Hélias, Lardon, Ras, A., & Steyer, 2011) made an evaluation of the life cycle of *Chlorella vulgaris* aimed to the production of biogas, and natural settling provided a concentration increase from 0.5 g/l to 10 g/l. The substantial difference is that *Chlorella vulgaris* was not destined to food application. Further experiments on natural settling are required to understand its feasibility and technological improvements to prevent biomass degradation.

4.1.3 Cells breakage

4.1.3.1 Milling

In the laboratory scale process cells were broken by friction with beads into a bead miller. The high friction forces created in the mixing chamber caused cells breakage. Beads used in laboratory at this purpose are made of zirconia-yttrium. From laboratory experiments, it was seen that efficiency was up to 95% and this stage lasted about 30 minutes. Proteins did not denaturize thank to the cooling chamber in which the mixture is circulated. Cell miller used in the experiment is described in §2.1.

4.1.3.2 Enzymatic treatment

Another way to break microalgal cell wall is with enzymes. Until now, there are just a few scientific articles about this technique. Experiments were carried out from (Sander & Murthy, 2009), but enzymatic disruption was satisfactory only with preliminary treatments by addition of chemicals. This way would increase the cost impact of cell disruption and insert potentially dangerous chemicals into the process.

4.1.4 Protein separation

Many different ways are used for protein separations and the topic has been described in §1.3

4.1.5 Biomass utilization

4.1.5.1 Thermal methods

Combustion is the easiest thermal way for producing energy from biomass. Two ways of biomass burning are possible: the first is with an auxiliary fuel, the second is with the biomass only (DeMartini, Aho, Hupa, & Murzin D., 2012). The choice among this two possibilities depends on the fire holding capacity of the biomass and its humidity grade. An example of combustion with auxiliary fuel is co-firing with coal. The main aim of this utilization is reduction of NO_x and SO_x

emissions and this technique has already been verified in more than 100 power plants around the world (Baxter, 2005).

Another thermochemical method is biomass pyrolysis, which is used for oil production from biomass. Pyrolysis oil, also known as biooil is interesting since it may become a petroleum substitute. This process is conducted at 500° C and the products of pyrolysis is the formation of solid, liquid and gaseous residues. The solid fraction also called "tar", is made of coal impregnated with the liquid residue that is mainly composed by oil with traces of organic compounds. The liquid residue is a dark-brown oil made from depolymerization of the three main components of biomass: cellulose, hemicelluloses, lignin (Oasmaa & Czernik, 1999). The gaseous residue is syngas, made of carbon monoxide, methane and carbon dioxide with traces of sulfides.

Biomass gasification is the third most known way to transform biomass by thermal treatments. It requires a preliminary burner chamber at 900°C where the biomass is dried and heated for the later gasification stage where the temperature rises to 1400°C for a fixed bed, or remains constant at 900°C for a fluidized bed (Ruggeri, Mancuso, & Collodi, 2010). In both cases the biomass is transformed into carbon monoxide and methane. At lower temperature methane and Tar production are higher and an advanced method for Tar purification is required.

4.1.5.2 Fermentation

Since oil extraction can cost up to 50% of the cost of the entire process (Moheimani, 2005) and thermal treatments requires large investments for the high temperature units of the plant. A new process to recover energy from the biomass would be welcome (Collet, Hélias, Lardon, Ras, A., & Steyer, 2011).

Fermentation is the easiest way to valorize biomass since it does not require high temperatures. The aim of this process is the biogas production from the three most common components of the cellular membrane. An economical evaluation of anaerobic fermentation was made from (Collet, Hélias, Lardon, Ras, A., & Steyer, 2011) for *Chlorella vulgaris* strain, that makes part of the Chlorophyta plant division, like *Neochloris Oleoabundans*. The biogas yield depends on the strength of the cell membrane. Mussgnug, Klassen, Schlüte and Kruse (2010) has conducted a broad study on several microalgal strains. They report that the yield is comprised between 287 and 652 ml/g biomass, and the methane content in the biogas is between 56% and 67% and the remaining was CO_2 .

Fermentation can be made by adding sludge from a tertiary waste water depuration pond (Ras, Lardon, Bruno, Bernet, & Steyer, 2011).

4.2 Process flow diagrams

The process design was made referring all the calculations to a volumetric flow rate of 1000 l/h with a biomass concentration coming out from the photobioreactor of 2g/l.
Two different process designs are shown: the first one is meant to the recovery of the only RuBisCO from the water soluble fraction of the proteins while the second is meant to recovery also the proteins stuck into the cell wall debris.

For the first process, harvesting of the biomass is the first required step to increase concentrations and reduce the volume of downstream units. The technology chosen for the harvesting is Tangential Flow Filtration (TFF) with values measured from (Petrusevski, Bolier, Van Breemen, & Alaerts, 1994) for 6 different microalgal strains. *Neochloris Oleoabundans* was not among the six examined strains, so reference values, reported in Fig.4.2, were assumed for this calculation. With a chlorophyll-α content higher than 2.5µg/l. Chlorophyll-α values were taken from (Pruvost, Van Vooren, Cogne, & Legrand, 2009).
The Tangential Flow Filtration operates with a Concentration Factor "C" equal to 40 and the related biomass loss factor is between 11% and 30% (for our calculation 30% has been assumed). Membrane pores are 0.45µm since smaller pores would cause a more frequent clogging. Two TFF units are required to recover microalgae and recycle water with a lower concentration of biomass . From the first unit the concentrate stream has a total volumetric flow rate of 25 l/h, a mass flow rate of 1.40kg/h and a biomass concentration of 56 g/l. The rest of the inlet flow passes through the membrane and has a flow rate of 975 l/h, mass flow rate of 0.6 kg/h and a concentration of 0.60 g/l. This stream is fed to a second Filtration unit, where the concentrate has a volumetric flow rate of 24.40 l/h, a mass flow rate of 0.42 kg/h and a biomass concentration of 17.2 g/l The stream passed through the membrane has a flow rate of 950.6 l/h, a mass flow rate of 0.18 kg/h and a concentration of 0.19 g/l and is recycled back to the photobioreactor.
The two concentrated streams are mixed together forming a stream with 49.40 l/h, 1.82 kg/h, 36.85 g/l which is then centrifuged at low speed to increase further the biomass concentration and to reduce volume and solvent flow rates of the chromatography separation section. This centrifugation step will be done with a G-force between 10.000 and 13.000g, reaching 22% of solid concentration in the precipitate(according to Christenson & Sims,(2011). The precipitate is now concentrated up to 220 g/l, with a total volumetric flow rate of 10.12 l/h, composed by 1.82 kg/h of biomass and 8.27 l/h of water. Supernatant from the centrifuge is recirculated to the photobioreactor since it still contains nutrients.
Precipitate from the centrifuge is sent to the cell miller in order to break the cell membrane. One more centrifugation step is required to precipitate cell wall debris and clear the supernatant that contains the water soluble fractions of the proteins. This centrifugation phase should be carried at least at 25.000g, to reach 60% solids (FSA Environmental, 2002). Cell wall debris flow rate is 1.67 kg biomass/h is sent to the fermenter. According to (Collet, Hélias, Lardon, Ras, A., &

Steyer, 2011) the biomass can produce 438 l/h of biogas with a heating value of 0.4KW. Values for conversion of the biomass to biogas are valid for *Chlorella vulgaris* but they are considered to be consistent reference values for *Neochloris Oleoabundans* since they make part of the same microalgal division (Chlorophyta).
Supernatant from the centrifuge is sent to a microfiltration unit with a membrane pore size of 0.2µm to remove completely traces of the cell wall and avoid clogging of the chromatographer. Liquid from the microfiltration is then sent to a dialysis unit where the liquid flows into a membrane system where on the opposite side the equilibration buffer for the HPLC is run. This operation is required to reduce conductivity of the protein solution, to operate the liquid chromatography in steady starting conditions and to increase the efficiency of the chromatofocusing. The buffer used for salinity decrease is regenerated in a close system with ionic exchange resins. According to (Krokhin & Ying, 2006) dialysis time should be about 6 hours but this value strongly depends on the ions concentration of the cultivating medium. Anionic exchange chromatography has been chosen as the way for protein fractionation. A flow rate of 7.16 l/h of 21.1 g/l proteins has to be fractionated. The target protein for the separation is RuBisCO, that represents 19.96% of the water soluble proteins. The solvent to sample ratio used in lab was 1562 to 1, that is absolutely unbearable on a larger scale. Elution time can be 10 times reduced since isolation of only RuBisCO is required and the solvent required for protein purification decrease to 156.2 times the sample volume meaning a consumption of 1117 l of solvents/h. RuBisCO's fraction has to be separated from the rest of the proteins eluted. It has to be desalted and then freeze dried to be concentrated to powder. The expected quantity of purified RuBisCO is 30.15 g/h.
The scheme explained above represents the process made in laboratory with some adaptations due to the larger scale. It is shown in Figure 4.3, where also the main material balances are reported.

An alternative process is also presented (Fig. 4.4) to recover the entire protein fraction in *Neochloris Oleoabundans*, that is indeed high (63% of dry weight biomass). This is achieved by extracting all the proteins with a solution that has the same composition of the "equilibration buffer and in figure 54 this process is presented. The difference is the solvent extractor after the cell miller. The aim of this new unit is dissolving the proteins stacked into the cell wall debris that did not solubilize in the supernatant. The solvent chosen is the equilibration buffer used for the HPLC and its composition is reported in Table 9. Since during laboratory experiments the proteins have never showed an incomplete solubility, the assumption of infinite proteins solubility is made and only one liter of buffer is added to the mixture.

The supernatant from the second centrifugation increases by one liter and the solvents for HPLC fractionations become 1275 l/h. Despite of the increase of buffer solutions required, with the solvent extraction all the proteins are dissolved in the liquid phase and they are 1183 g/h, instead of 151 g/h of the process for the only water soluble fraction. According to experimental

evidences and to the scientific article of (Antonov & Soshinsky, 2000), also RuBisCO recovery would be enhanced.
This process guarantees a higher proteins recovery and that is why a lower quantity of biomass is sent to fermentation. Biogas production 167 l/h with an enthalpy flow rate of 557 KJ/h. Quantification of RuBisCO recovered in this way is not achievable but it will be reasonably higher than the value obtained from the first process.

The second process can represent a good pathway for the complete recovery of proteins from microalgae and would increase the global yield of the process since proteins recovery is almost 10 times higher than the first process. On the other hand, the first process produces more than twice the biogas than the second one because more biomass is sent to the fermenter. Wise economic evaluation should be made once a market value for RuBisCO has been estabilished.

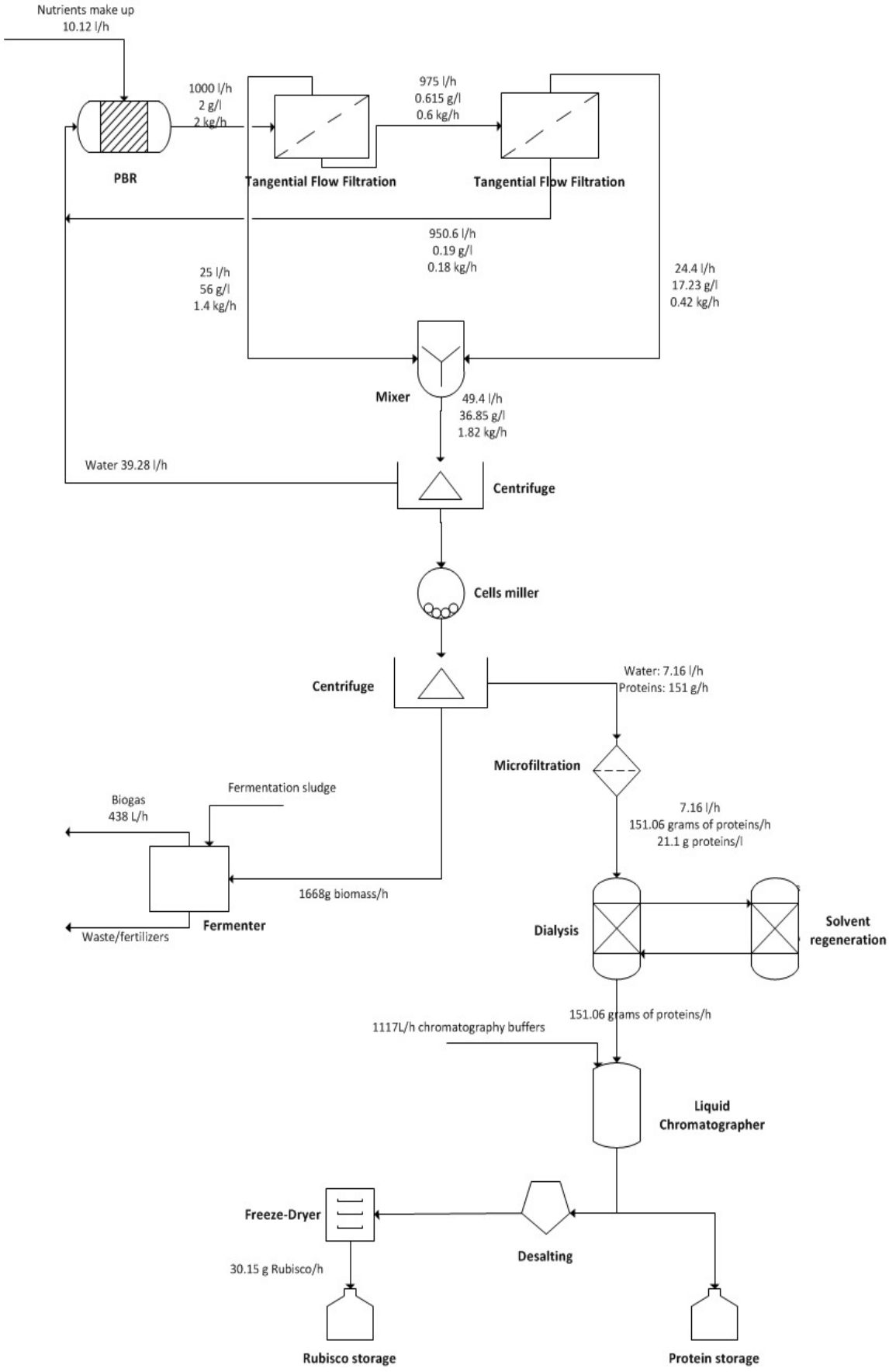

Fig.4.3. The figure represents the process for the purification of the only water soluble proteins fraction.

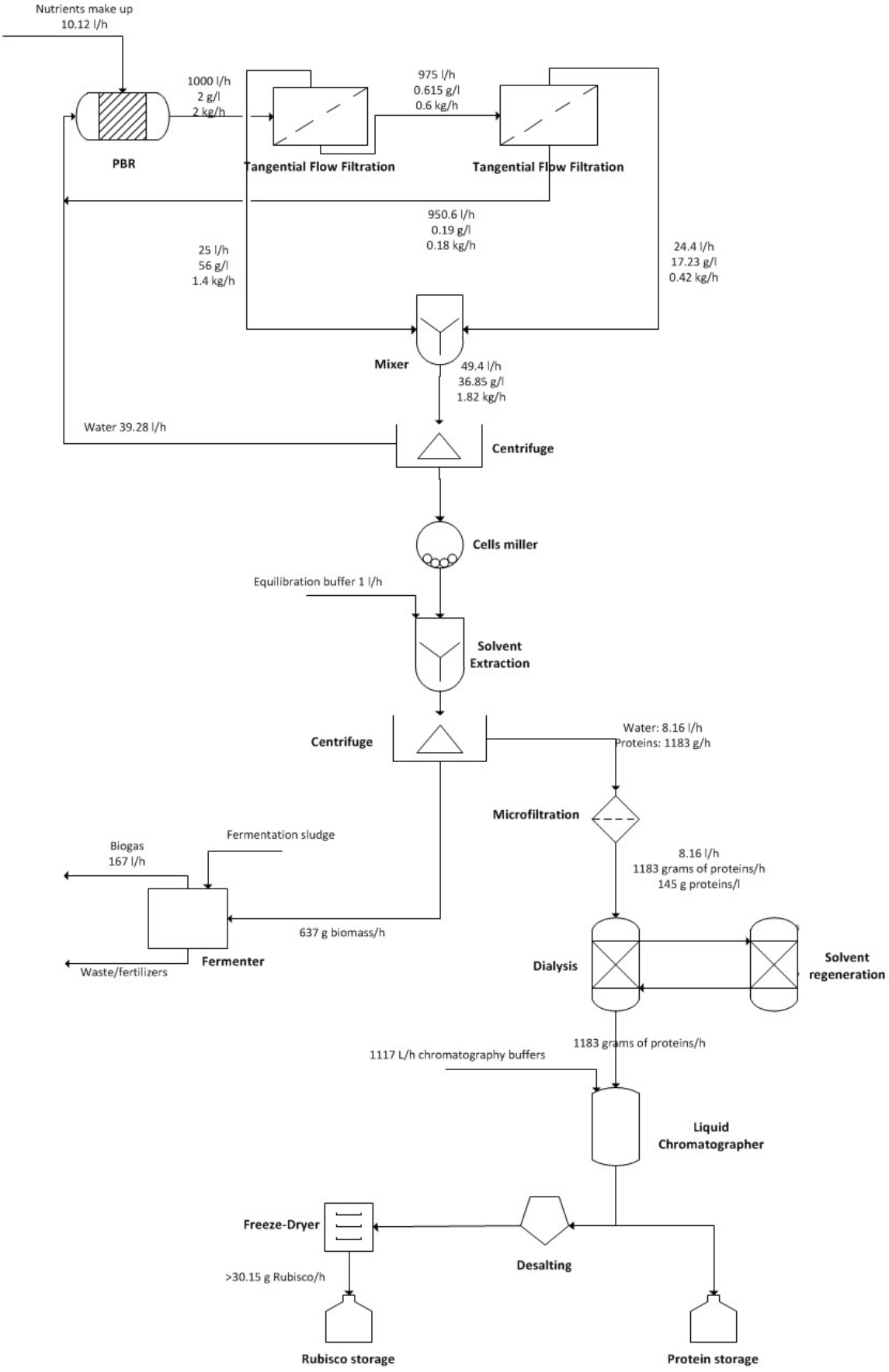

Fig.4.4. The process represents RuBisCO recovery and protein separation with solvent extraction.

5.CONCLUSIONS AND RECOMMENDATIONS

This thesis project is focused on the possibility to recover proteins, in particular RuBisCO, from microalgae, and the attention was focused on *Neochloris Oleoabundans*.
A first aim was understanding the pH of maximum precipitation. This experiment was made by HCl titration and the behavior of water soluble proteins from three different strains was then acknowledged. A similar behavior was seen for *Chlorella vulgaris*, *Neochloris Oleoabundans* and *Desmodesmus*. They all have the maximum proteins precipitation in the range [3-4]. A further decrease of pH shows that *Chlorella vulgaris* and *Desmodesmus* return to a higher percentage (77.4% and 67.4%) of proteins dissolved than *Neochloris Oleoabundans* (42.3%).
The water soluble protein content of *Neochloris Oleabundans* was found to be 8.3% and 5.2% (on dry-weight basis) for the exponential and the steady phase of growth respectively. Accordingly RuBisCO's content in the water soluble protein fraction is about 15g/kg biomass.

Chromatography pattern of *Neochloris* obtained in the exponential and steady growth phases are quite different. Even if analogies can be seen in the first 20 minutes, between 50 and 110 minutes with big peaks eluting and a wide peak from 580 to 605 minutes, the exponential phase present many peaks between 200' and 550' that do not appear in the chromatogram of the steady phase. The last peak from the two growth phases shows recurrences in molecular weights (140-150kDa from Native gels and 95kDa and 26kDa from reducing gels).
An experiment with pure RuBisCO demonstrated that most part of RuBisCO eluted in the first 5 minutes (pH=10.6).

With native gels it was found that in the first 200 minutes of the chromatogram (10.6<pH<9.3) the most recurrent molecular weight of the proteins from native gels is between 55 and 70 kDa, that was later confirmed by reducing gels. These results suggest that protein denaturation is due to the long residence time at the initial pH (10.5<pH<9).
From electrophoresis experiments on samples taken from chromatography made with a pure RuBisCO sample, the most intense bands were detected at 67-69 kDa instead of 55 kDa (RuBisCO's bigger subunit Mw) and a possible explanation is cross-linking of subunits. From RuBisCO quantification, the different behaviors of protein precipitation percentage was explained since *Chlorella vulgaris* and *Desmodesmus* has a higher amount of RuBisCO than *Neochloris Oleoabundans*.

Further analysis for proteins characterization would be required to gather more informations.
Western blotting would be necessary to identify RuBisCO and MALDI-TOF to understand if cross-linking really occurs. Chromatography can be improved reducing the starting pH value to reduce the risk of protein denaturation or crosslinking.

Large scale application of protein production from microalgae was studied by developing two possible processes: the first one reflects the steps made in the laboratory, extracting RuBisCO

from the only water soluble protein fraction. The second process provides the full recovery of the proteins with an extraction with the same solvent used for the chromatography. Recovery of RuBisCO in the first process is 15g RuBisCO/kg biomass while in the second is surely higher, since all the proteins have been dissolved. Biogas production in the first process is more than twice higher than in the second one because here all the proteins are recovered and a lower biomass quantity is fed to the fermenter.

With the large scale process development, more than 550 l/kg biomass of solvents are required, which value should be reduced by process optimization to minimize the environmental impact and to improve its economic impact on the process.

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Appendix

In this section, chromatograms from the different microalgal strains analyzed are presented with the related table reporting molecular weight of the proteins corresponding to the related peak.

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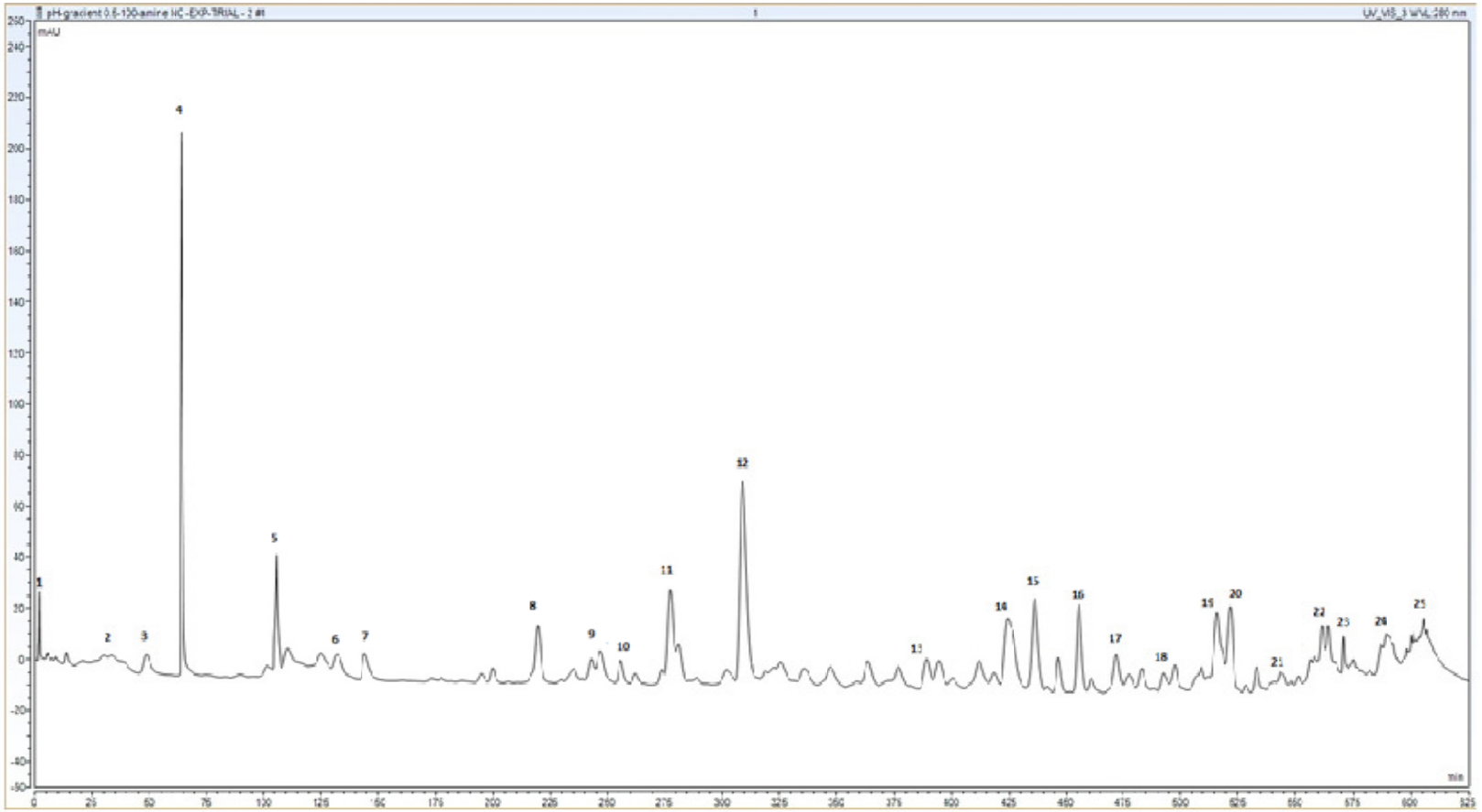

Fig.A.1 Chromatogram from *Neochloris Oleabundans* during exponential phase

Table A.1 Chromatogram peaks with retention time and molecular weights from PAGE experiments with samples taken from the chromatogram of *Neochloris Oleabundans* during exp. phase

Peak	*Ret time*	*NATIVE PAGE MW (kDa)*							*SDS PAGE MW (kDa)*									*Rel %*
1	2'05"-3'10"	1098.33	59.47						67.13	56.35	50.44	29.91	13.47					0.37
2	30'30"-33'40"	46.92	34.65	23.95					64.44	55.21	49.13	33.79	47.00	40.60	29.15	19.73	18.76	2.73
									16.85	13.63								
3	47'38"-50'57"	57.80							65.01	59.56	55.37	49.25						0.9
4	63'37"-66'50"	56.60							64.07	59.56	54.73	46.76	15.94					7.5
5	109'10"-112'30"	57.80							66.74	62.41	56.35	51.03	30.26	15.94				9.29
6	131'11"-134'30"	54.61							67.33	63.14	57.51	47.79	30.26	16.1	13.61	13.65		9.29
7	143'00"-148'00"	54.61							68.52	64.44	57.51	52.80	33.66	30.26	16.10	13.65		1.21
8	215'00"-223'00"	55.65	59.47						70.13	64.63	58.7	63.88	57.4	36.21	28.92	27.26	13.74	2.27
9	242'10"-245'13"	63.55							67.77	63.34	57.56	52.16	48.86	46.55	39.84	36.49		2.22
10	254'30"-259'00"	605.62	371.56	231.78	151.5	67.46	62.92		67.96	67.01	63.52	62.46	58.38	55.8	53.2	41.61	40.00	0.68
									36.57	36.00	32.88							
11	275'20"-284'30"	63.33	56.74						66.8	61.93	61.15	55.96						5.47
12	306'50"314'00"	59.99							62.11	61.24	55.96							9.26
13	387'06"-391'45	574.18	16.45						Nd									2.36
14	421'40"-430'00"	Nd							68.52	64.63	58.53	52.85						4.90
15	434'40"-440'00"	Nd							68.52	64.82	58.70							3.85
16	453'30"-458'30"	Nd							69.32	65.39	59.04							2.84
17	470'30"-474'00"	Nd							69.32	65.97	59.39	55.7	48.49					1.25
18	490'50"-494'30"	172.00	71.21						61.00	55.22	50.00							0.87
19	514'00"-519'30"	367.65	213.28	128.94	74.73				309.3	272.22	227.17	125.12	65.88	60.48	55.33	52.26	35.52	5.23
									24.77	16.98								
20	519'30"-525'00"	658.78	651.5	302.83	178.06	172.79	151.37		65.88	60.98	55.53	55.22	35.92	35.79	25.37	25.14	24.5	4.17
		149.56	101.26	88.81	72.7	71.21			19.5	14.37								
21	539'00"-545'00"	655.13	290.25	88.21	71.21				309.3	272.22	227.17	185.58	125.12	61.49	57.71	55.22	50.00	0.90
									47.33	44.47	43.42	38.44	35.92	33.67	25.7	20.9	17.37	
									16.09	14.57								
22	560'40"-563'20"	579.77	151.37	109.25	72.7	55.16			53.42	36	31.6	19.31	17.11	16.52	14.5			4.62
23	570'06"-573'20"	599.42	154.12	107.75	71.7	58.25	41	2.76	124	69.23	53.42	36.39	30.19	23.59	17.27	14.57		1.27
24	585'05"-594'05"	154.12	116.25	68.79	59.17	42.63	8.75	7.96	108.91	97.89	64.62	60.98	56.14	50.89	48.47	45.33	30.36	3.09
		2.96							27.27	25.09								
25	598'10"-609'10"	154.12	72.7	59.17	54.30				111.26	97.89	97.69	64.62	61.49	59.00	56.30	50.42	45.33	6.34
									39.85	30.36	27.27	16.44	14.75					

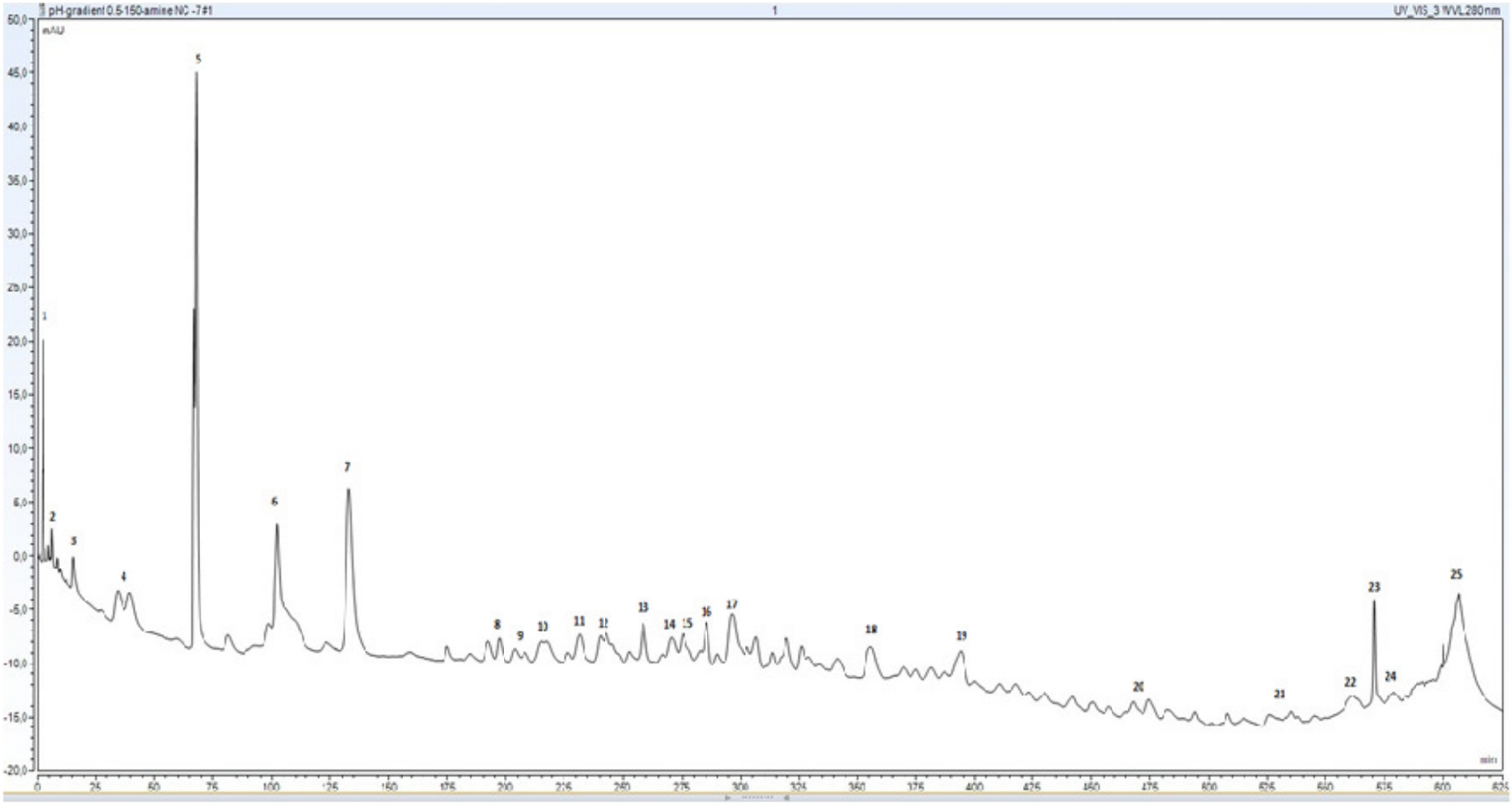

Fig.A.2 Chromatogram from *Neochloris Oleabundans* during steady phase

Table A.2 Chromatogram peaks with retention time and molecular weights from PAGE experiments with samples taken from the chromatogram of *Neochloris Oleabundans* during steady phase

Peak	*Ret Time (min)*	*NATIVE PAGE MW (kDa)*					*SDS-PAGE MW (kDa)*									*Relative %*
1	2'00-4'15''	1025.88					63.29	60.65	55.71							1.56
2	6'00''-7'15''	Nd					63.29	60.65	55.71							0.88
3	15'30''-17'00''	Nd					54.39	47.63	40.00	38.75	36.16					0.69
4	33'00''-41'00''	287.61	65.43				120.34 220.6	81.00 115.39	64.00 104.89	60.78 79.81	54.39 63.86	48.08 58.63	38.56 50.45	33.55 47.63	43.64	3.88
5	66'16''-69'25''	68.38					38.75	36.85	34.47	25.21	20	15.36	13.65			14.24
6	97'00'' 106'00''	Nd					60.78									11.93
7	131'30''-136'00''	834.16	77.00				133.14	119.59	68.95	55.88	40.28	36.92	33.69	13.60		8.89
8	196'50''-199'00''	77.00					Nd									0.78
9	202'50'' 206'00''	Nd					Nd									0.88
10	213'20''-218'20''	Nd					62.62	58.38	50	47.51						2.03
11	228'35''-233'00''	447.97	175.76	130.35	77.14		68.96	63.39	57.11	47.36	40.4	31.58	26.56	13.07		1.82
12	238'40'' 244'40''	480.96	80.49				133.14 36.69	112.67 17.57	70.84 14.00	63.39	54.06	48.33	45.15	41.17	37.81	3.01
13	257'30''-260'30''	78.24					70.64	59.34	31.58	26.56	13.67					1.79
14	268'40''-271'40''	Nd					65.91	59.34								2.29
15	275'40'' 278'00''	61.57					Nd									
16	282'25''-287'25''	61.57					66.04	60.43	54.32							1.60
17	295'10''-300'10''	62.43	8.23				65.45	55.29	54.32							2.57
18	353'20'' 359'00''	Nd					60.43	55.29								2.27
19	392'30''-395'30''	400	140	67.47	26.22	8.11	74.56 16.74	64.68 14.95	55.29 13.66	49.88 13.13	48.02	44.52	36.02	30.7	25.26	2.00
20	466'20''-478'00''	64.00														2.17
21	527'37'' 537'00''	267.09	130.7	66.08			117.25 38.89	101.03 35.28	63.92 24.49	60.07 13.09	58.15	54.97	49.88	45.8	42.76	1.44
22	556'00''-565'00''	133.67	86	46.97	31.84		23.03	13.61								1.23
23	569'00''-574'00''	242	133.67	86	45.68	3.13	68.02	52.27	38.98	36.47	23.03	18.85	15.58	13.76		1.92
24	576'00''-581'00''	47.96	3.18				30.19	23.63	15.49	13.64						
25	582'00''-612'00''	143.87 63.31 49.65	137 50.35 19.72	134.66 49.65 19.45	131.72 47.3 3.16	129.8 38.94 3.13	93.06 59.36 20.41	92.67 55.62 19.09	91.5 52.74 15.49	90.34 48.94 15.47	88.83 48.6 13.64	68.22 30.07	65.26 26.61	62.61 26.55	61.33 21.66	18.88

VI.

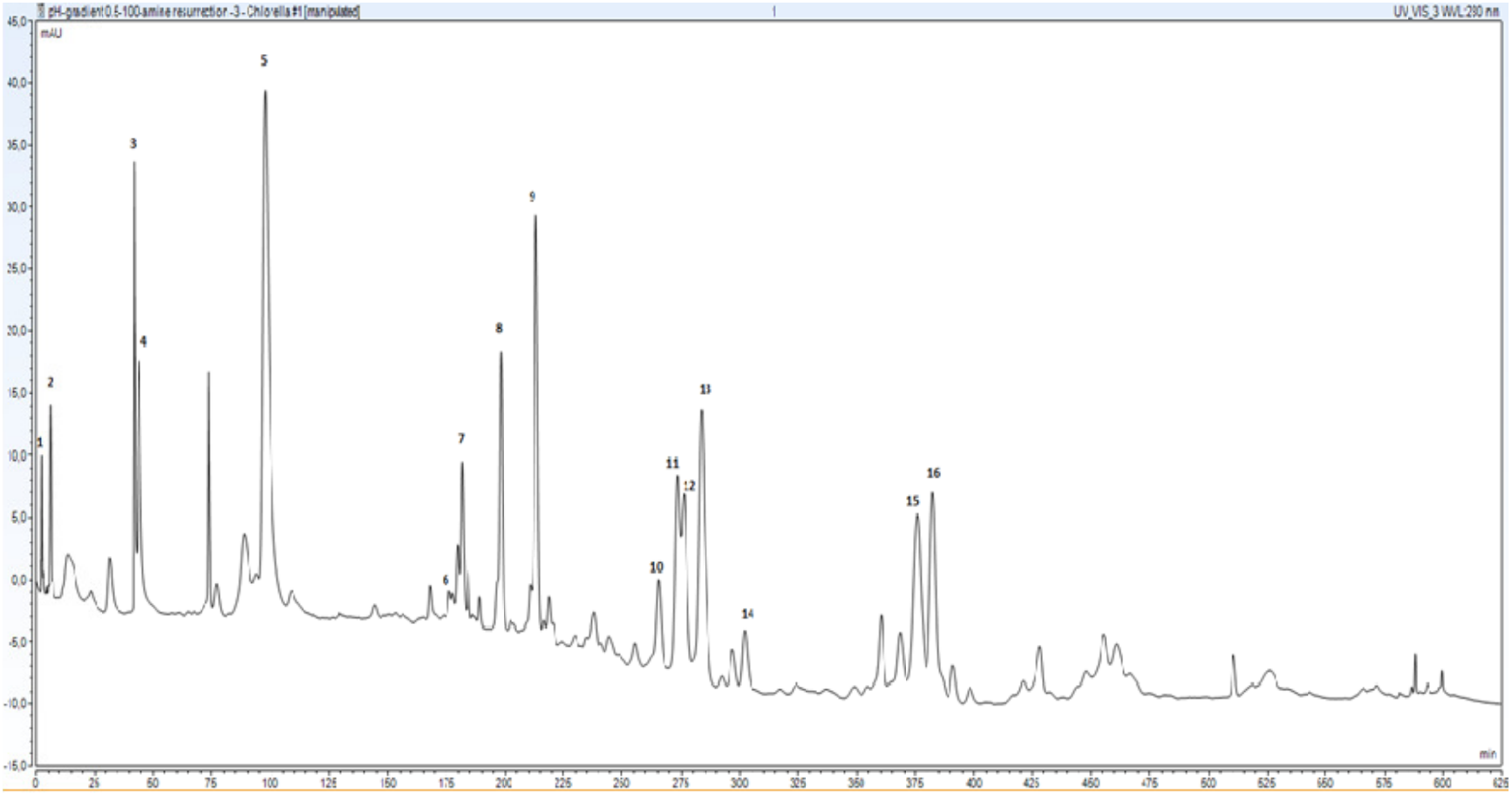

Fig.A.3. Chromatogram from *Chlorella vulgaris*

Table A.3. Chromatogram peaks with retention time and molecular weights from Native PAGE experiment with samples taken from the chromatogram of *Chlorella vulgaris*

Peak	*Retention time (min)*	*NATIVE PAGE MW (kDa)*										*Relative %*
1	1 23''-4'00''	229.58	135.83									0.48
2	5 00''-8'04''	247.45	141.02	69.17								1.22
3	41 41''-45 00 '	450.80	146.40	71.82	40.93							5.70
4	45 01''-47 13 '	338.02	141.02	71.82	40.17							
5	96'00''-101'50''	146.40										19.32
6	175 00''-177'30''											4.31
7	182 43''-185'15''	152.00										
8	196 46''-199'47''	151.99										3.68
9	212 00''-221'40''	643.54	315.73	29.21	22.46	17.94	12.80					6.81
10	263 32''-265'20''	434.21	135.83	71.82	42.49							1.90
11	273 35''-277'00''	504.45										7.03
12	277 00''-279'00''	658.23	193.94	110.52	42.49	32.68	20.45	18.28	16.96	14.87	12.80	7.03
13	281 00''-287'00''	141.02										6.57
14	299 57''-305'02''	146.40										2.38
15	372 00''-379'00''	733.89	149.17	83.43								15.98
16	380 00''-385'00''	1047.85	747.78	146.40	77.41							

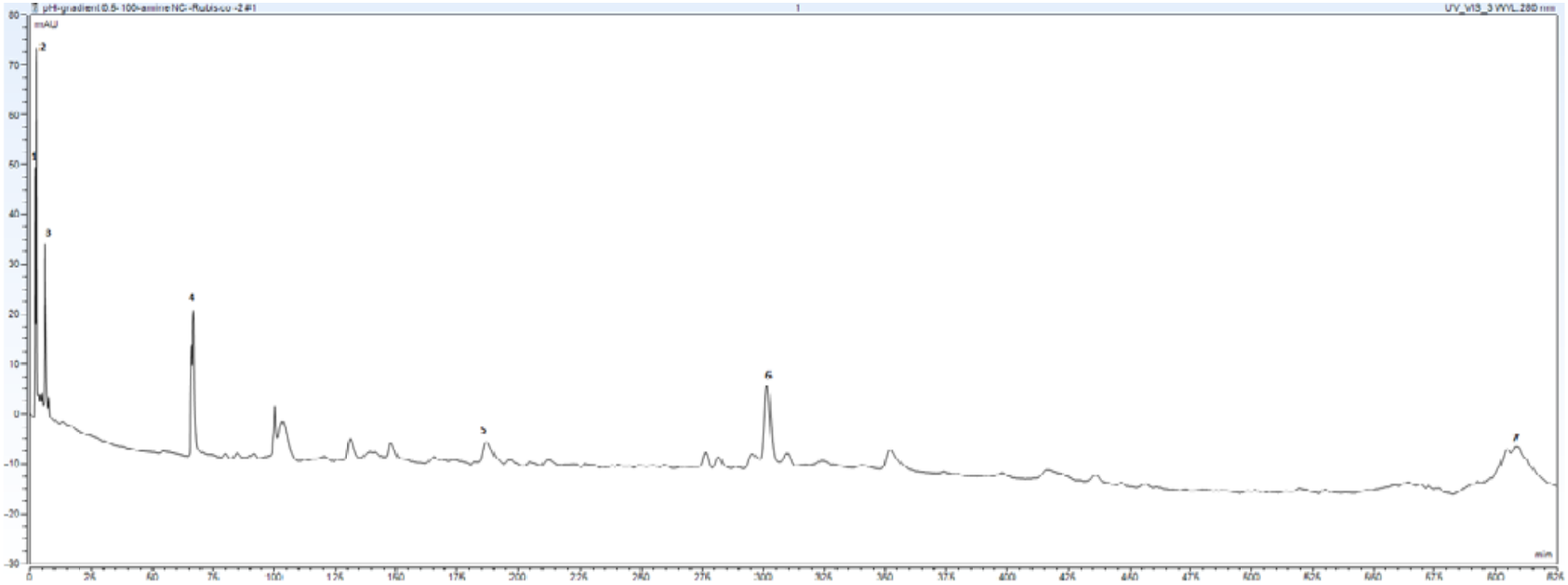

Fig.A.4. Chromatogram from a pure Rubisco sample, made with the same pH profile used for *Neochloris Oleabundans*

Table A.1. Chromatogram peaks with retention time and molecular weights from PAGE experiments with samples taken from the chromatogram of Rubisco

Peak	Retention time	*NATIVE PAGE MW (kDa)*				*SDS-PAGE MW (kDa)*										*Rel %*
1	1'35''-2'42''	69.36				69.32	61.33	57.68	53.63	34.12	31.15	17.5	16.38	15.47	13.97	2.84
2	2'42'' 3'25''	70.35				70.75	65.39	59.56	54.57	34.34	17.4	14.07				8.14
3	5'40''-8'00''	60.85	2.04			71.79	67.13	61.33	35.57	31.03	16.56	14.2				4.49
4	65'50''-68'05''	68.87	2.2			60.79	16.56	14.2								13.37
5	185'30''-191'00''	67.9				71.58	59.58	32.6	17.15	14.49						4.61
6	301'10''-304'20''	69.36				71.58	66.35	59.39	55.54	48.53	32.53	17.08	14.54			17.22
7	600'20''-612'30''	128.51	127.6	66.94	62.38	65.77	59.39	17.27	14.54	14.51						22.86