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Polyethyleneimine as a tool for compounds fractionation **by flocculation** in a microalgae biorefinery context

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Abstract

In the context of emerging biorefinery for microalgae, polyethyleneimine (PEI), has been tested in order to achieve separation of fat-soluble and water-soluble compounds from *Haematococcus pluvialis*. Several parameters were taken into account (ratio between sample and PEI, pH, and ionic strength) and **2 conditions (0.075% PEI pH 7.4, and 0.100% PEI pH8.5)** were studied for up-scalability, with a recovery of flocculated compounds (lipids and pigments), and a complete characterization of both phases. Using 0.075% PEI, pH7.4, 100% sugars and 89.8% proteins were retained in the supernatant, but some trace of beta-carotene were also detected. For 0.100% PEI, pH 8.5, a loss in proteins content was highlighted (61.2% proteins retained), but no residual lipids or pigments were detected. PEI could therefore be considered as an efficient method to fractionate fat-soluble and water-soluble compounds from microalgae.

Keywords

Microalgae, biorefinery, polyethyleneimine, flocculation

Highlights

- Polyethyleneimine can be used for fractionation of *H. pluvialis* compounds
- Water-soluble and fat-soluble fractions can easily and efficiently be separated
- 95% of fat-soluble compounds can be recovered in the pellet
- 100% of sugars and 90% of proteins remained in the supernatant
- Technofunctional properties of the water-soluble fraction were not altered

1. Introduction

Microalgae are eukaryotic photosynthetic microorganisms with significant ecological importance, for example for CO₂ capture and oxygen production through photosynthesis. Industrially, they can also be source of bioactive compounds such as lipids, proteins, carbohydrates and pigments. In this context, they have been mostly studied as potential raw materials in biofuel production (Gerde et al., 2013). However, the economic production cost of this sustainable energy source is still too high. In order to reduce the biofuel production cost, a more efficient approach for the sustainable valorization of microalgae biomass is the integrated production of energy and biobased value added products (Batista et al., 2013; Schwenzfeier et al., 2011). However, attention has to be paid to reducing the number of unit operations, sources of additional costs.

The green microalgae *Haematococcus pluvialis* belongs to the group of chlorophyceae. This freshwater organism has an atypical life cycle in that it is able to go from a vegetative cell state rich in chlorophylls and proteins to an encysted state under stress. At this encysted state, *H. pluvialis* is surrounded by a thick cell wall and can produce high amounts of secondary metabolites including carotenoids (Mendes-Pinto et al., 2001). Especially, astaxanthin, which has attracted considerable attention in recent years, is a fat-soluble carotenoid, accumulated in oil droplets following nitrogen depletion, light stress, or when submitted to a high salinity condition (Damiani et al., 2010 ; Guedes et al., 2011). Astaxanthin from *Haematococcus pluvialis* presents three forms (free, monoester, and diester) and is commercialized as an oleoresin obtained by extraction of the microalga with supercritical carbon dioxide. Beside the synthetic form of astaxanthin is the most used nowadays, the natural astaxanthin from *Haematococcus pluvialis* is allowed for salmon's feed in Canada and Japan, as well as for food as natural dye. The market price for this high value molecule is between 2500 and 7000 \$/kg whereas the production cost is estimated to be around 1000 \$/kg (Li et al., 2011).

However, other compounds from *H. pluvialis* can present commercial interest, such as lipids for biofuels, polysaccharides for food or cosmetic, and proteins for food and feed. As an example, study of Ba et al., (2016) have highlighted the strong potential of its proteins as emulsifier for food applications. Concerning the red state, rich in lipids, carotenoids and sugars, some applications in biofuels (biodiesel and bioethanol), in cosmetic, nutraceutical or pharmaceutical areas are possible (Boussiba, 2000 ; Lopez et al., 2006). It seems then necessary to develop mild and efficient methods for fractionation of microalgae compounds for a sustainable biorefinery approach.

Due to the high mechanical resistance of its cell wall, *Haematococcus* requires strong methods such as high pressure homogenization in order to release intracellular compounds. As described by Safi et al., (2014), high pressure homogenization is the most efficient cellular lysis method, even on this extremely resistant microalga. Main drawback of this method is that the recovered extract is then a stable emulsion of lipids in aqueous phase, and that additional step is required to fractionate fat-soluble (lipids and pigments) and water-soluble (proteins and sugars) compounds.

During the last decades, some papers described the use of ionic liquids (ILs) for microalgae biorefinery. The first intention was to use them for lipids or hydrophobic pigments extraction (Teixeira et al., 2012; Orr et al., 2016), and later for proteins extraction. However, studies were often focused on a specific protein such as RuBisCo (Lee et al., 2017; Desai et al., 2014; Suarez Ruiz et al., 2018) and application to crude microalgae extracts is rather scarce. Moreover, if ILs could be considered as “green solvents”, their main drawback is their high cost, and the operating conditions to recover hydrophobic compounds or proteins are often not compatible. It seems then mandatory to find a new method, allowing recovery of both fractions without alteration.

In this paper, we propose to use a polymer, polyethyleneimine (PEI), in order to achieve separation of fat-soluble and water-soluble compounds from *Haematococcus pluvialis*. This polymer can be linear or branched, with a high degree of polymerization ranging from 700 to 2000. This organic molecule is rich in amine groups and has a pKa between 10 and 11, which gives it a cationic behavior at neutral pH (Burgess, 1991). With a relatively low molecular weight (2 kDa), PEI is a stabilizer of proteins in solution (Mazzaferro et al., 2010; Andersson et al., 2000), while the high molecular weight PEI can be used for proteins purification (Burgess, 2009). The principle of **flocculation** of the PEI is assumed to be based on a difference in charge between the polymer and its target molecule. Indeed, the positive charges of the PEI may bind to macromolecules negatively loaded such as nucleic acids or other anionic molecules. A molecular network could form from these connections with acidic compounds, ending up becoming a precipitate recoverable by centrifugation (Burgess, 1991). At pH above 3, astaxanthin from *H. pluvialis* wear a negative charge, as demonstrated by Dermiti et al., (2009) by zeta-potential measurements. The PEI flocculation method could then be an efficient tool for microalgae compounds fractionation. However, PEI has never been tested in this context. Several parameters influence the **flocculation** efficiency of the PEI such as the ionic strength, pH as well as the concentration in PEI (Milburn et al., 1990; Salt et al., 1995; Salt et al., 1996). In our study, **these** parameters were taken into account (ratio between sample and PEI, pH, and ionic strength), with an evaluation of efficiency following turbidity, proteins and sugars amounts in the supernatant after flocculation. Finally, 2 conditions were studied for up-scalability, with a recovery of flocculated compounds (lipids and pigments), and a complete characterization of both phases.

2. Materials and Methods

2.1. Cellular lysis

Suspensions of 2% dry weight **in water** were prepared **as previously described (Ba et al., 2016)** from frozen paste of *Haematococcus pluvialis* (Microphyt, Baillargues, France).

Cellular lysis was carried out by high-pressure cell disruption using a TS HAIVA (Constant systems LTD) device. Suspension was submitted to a pressure of 2.7 kbar, followed by centrifugation at 10,000 g for 30 min at 20 °C. The pellet was discarded and the supernatant that contained the solubilised compounds, was used for the rest of the study.

2.2. Flocculation with PEI

The PEI used was in the branched form, with high molar mass (Mw average: 750 kDa). It is available from the society Sigma-Aldrich in the form of a solution at 50 per cent in mass of PEI. Its use as an agent of purification of water-soluble protein has been tested on the supernatant of *H. pluvialis*, retrieved after cellular lysis and centrifugation. The pH of the solutions is previously adjusted up to the desired value (pH = 6.3, 7.4, or 8.5).

Subsequently, the flocculation is carried out by adding a volume of sample and a volume of PEI. The final concentrations of PEI after mixing with sample was between 0 and 0.125%. PEI solutions were prepared with pure water or with a solution of NaCl, the concentration of which can reach 1 M. The sample/PEI mixtures were homogenized by two successive reversals of the tube. They are then centrifugated at 2500 g for 10 minutes and 20°C. The supernatant and pellet of flocculation were kept for analyzes.

2.3.Extraction and quantification of lipids

Extraction was performed using a modified Bligh and Dyer (1959) method. A mixture of chloroform: methanol was prepared (1: 2, v/v) and 3.75 mL were added to 1 mL of sample (prepared in triplicates). Then 1.25 mL of chloroform were added and the tube was vigorously mixed, before addition of the same volume of distilled water and mixed again. The final mixture is centrifuged at 2000 g for 10 minutes and the lower organic phase was recovered and transferred in tubes previously dried and weighed. The organic phase is evaporated in a centrifugal evaporator Speed-Vac (Savant™ SPD111 SpeedVac™, ThermoScientific) at 40°C under a vacuum of 950 mbar, up to stabilization of the mass of the tubes. The fat content is then quantified gravimetrically but corresponds to the sum of lipids and fat-soluble pigments (chlorophylls and carotenoids) that are concomitantly extracted. Depending on further analysis, this pellet is resuspended in 100 µL chloroform (for fatty acids analysis) or 500 µL of acetone (for pigments quantification).

2.4.Spectrophotometric assays

Flocculation efficiency on pigments was evaluated by measuring absorbance of samples at 750 nm (further called “turbidity”). This wavelength has been chosen to reflect the presence of both lipids and pigments, as these fat-soluble compounds will be present as small droplets in the aqueous phase. Proteins are quantified following the colorimetric assay developed by Lowry et al., (1951), using bovine serum albumin as a standard. Protein content was reported to the dry mass of sample (%DW), and further expressed as “% of retained proteins” as compared to the appropriate reference. This dry weight of samples is obtained by freeze-drying using Heto L1150 Powerdry freeze-dryer (Fisher scientific), with vacuum set at 500mTorr, and initial temperature of samples at -80°C. Total carbohydrates were quantified using resorcinol-sulphuric method described by Monsigny et al., (1988) using glucose as a

standard, and expressed on a dry weight basis (%DW). The content in chlorophyll and carotenoids pigments was evaluated according to the equations (1) of Lichtenthaler and Buschmann (2001) for samples in acetone:

$$\begin{aligned}Ca(\mu\text{g/mL}) &= 11,24 * A_{661,6} - 2,04 * A_{644,8} \\Cb(\mu\text{g/mL}) &= 20,13 * A_{644,8} - 4,19 * A_{661,6} \\Cx+c(\mu\text{g/mL}) &= (1000 * A_{470} - 1,90 * Ca - 63,14 * Cb) / 214\end{aligned}\tag{1}$$

With Ca, the chlorophyll a concentration, Cb, the chlorophyll b concentration, and Cx+c, the total carotenoids concentration.

2.5. Chromatographic methods

Molecular weight distribution of proteins was evaluated in non-denaturing conditions by size exclusion chromatography (SEC). SEC was carried out on an AKTA FLPC chromatograph (Amersham Biosciences, UK) equipped with an UV-VIS detector. The Superdex 200 10/300GL column (Amersham Biosciences, UK) was calibrated using a standard proteins solution ranging between 670 and 1.35 kDa (bovine thyroglobulin: 670kDa; bovine γ -globulin: 158 kDa; chicken ovalbumin: 44 kDa; horse myoglobin: 17 kDa; vitamin B₁₂: 1.35 kDa). This standards proteins mix allowed determining void volume, column volume, and to establish a calibration curve between elution volume and molecular mass. Samples containing 1 g.L⁻¹ of proteins were filtered at 0.2 μm , and 0.1 mL of those was injected. The elution was carried out at 0.5 mL.min⁻¹ with sodium phosphate buffer (50 mM) at pH=7. The proteins eluted through the column were detected at 280 nm. Two other wavelengths, 661 and 470 nm for chlorophylls and carotenoids respectively (Lichtenthaler and Buschmann, 2001), made possible to follow presence of pigments.

The fatty acids were quantified by gas phase chromatography after transformation in methyl esters following method of Morrison and Smith (1964). Eight hundred μL of a methanol-BF₃

solution at 14% p/p (FLUKA) were mixed with 100 μ L of chloroform containing the lipids in sealed tubes. The mixture was boiled in a water-bath for 15 minutes and cooled to room temperature. 1.5 mL of distilled water and 750 μ L of hexan (containing 1% (v/v) of heptadecan as internal standard) were added. The mixture was vortexed for 2 minutes and then decanted. The upper phase contained the methylated fatty acids. The gas chromatography was an Agilent 6850 Series II, equipped with a autosampler G4513A and a flame ionization detector (FID). One μ L of sample was injected. The column used was a polar column HP 88 (112-88A7E) of 100 m x 0.25 mm X 0.2 μ m. The oven was set at an initial temperature of 140°C for 5 minutes followed by a ramp of 4°C/min up to 240°C. Finally, the temperature was maintained at 240°C for 5 minutes. Nitrogen was used as a carrier gas, with the injector and the FID temperatures fixed at 260°C and the split ratio at 1: 20. Acquisition and data processing were done using Chemstation software. The fatty acids methyl esters were identified and quantified using standards, methylated according to the same method (25 injected standards). Mass of fatty acids was then reported to the dry mass of the original sample.

The chlorophyll and carotenoids pigments were identified by high-pressure liquid chromatography (HPLC), using an Agilent 1100 Series, equipped with a module of pumps, an automatic injector and a Diodes Array Detector (DAD). The acquisition of the data and their processing was done using Chemstation software (version A.08.03). The pigments were extracted according to the method previously described and resuspended in dichloromethan : methanol (25:75, v:v) according to Yuan et al. (1997). The free astaxanthin (Glentham), Lutein (Glentham), chlorophyll a (Sigma-Aldrich), chlorophyll b (Sigma-Aldrich) and β -carotene (Glentham) were used as standards. Other pigments were identified according to their retention times (Yuan et al., 1997). The samples, filtered through 0.45 μ m, were separated using a C18 column (150 mm x 4.6 mm x 5 μ m, Phenomenex) at 40°C. The mobile

phase consisted in methanol, dichloromethan, acetonitrile and water (69:17:11.5:2.5, v:v:v:v). The flow rate was fixed at 0.7 mL/min and 20 μ L of samples were injected. The acquisition was performed at 445 nm.

2.6.Evaluation of techno-functional properties

2.6.1. Emulsification capacity (EC)

Emulsification capacity (EC) was evaluated using the procedure given by Thakur et al. (2008). Rapeseed oil was continuously added to the protein solution homogenized by an Ultra-Turrax device (T25, Ika-Werke GmbH, Germany). At the same time, the conductivity of the emulsion was monitored using a CDM210 conductimeter (Radiometer Analytical, France) until destabilization of the emulsion. EC corresponds to the maximal oil volume emulsified per gram of proteins.

2.6.2. Emulsification activity index (EAI)

Emulsification activity index (EAI) was quantified using the procedure given by Pearce and Kinsella (1978). Twenty milliliters of a 0.1% (w/v) protein solution at pH=7 was homogenized with 6.6 mL of rapeseed oil for one minute under agitation by Ultra-Turrax device. Immediately after homogenization, 50 μ L of the emulsion were diluted in a 5 mL SDS solution at 0.1%. The absorbance (A_0) of the solution was determined at 500 nm and EAI was deduced by the following equation:

$$\text{EAI (m}^2 \cdot \text{g}^{-1}\text{)} = \frac{2.303 \cdot A_0}{L} * \frac{2 \cdot d}{10^6 \cdot C \cdot \varphi} \quad (2)$$

where A_0 is the optical density measured after emulsion homogenization, L is the absorbance path length ($L=10^{-2}$ m), C is the protein concentration ($C=2$ g.L⁻¹) before dilution in SDS, φ is the oil volume fraction in the emulsion ($\varphi=0.25$), and d is the dilution factor ($d=100$).

2.6.3. Emulsification stability (ES)

Emulsification stability (ES) was measured using the method given by Yasumatsu et al. (1972). A protein solution with an equivalent volume of rapeseed oil was homogenized by an Ultra-Turrax device. An aliquot of the emulsion was placed in a tube and heated at 80°C for 30 minutes. The sample was then cooled and kept at room temperature for 24 hours. ES was expressed as a ratio of the height of the remaining emulsified layer to initial height. All analyses were done in triplicate to estimate the standard deviation.

2.7. Experimental design

2.7.1. Dilution of samples

In order to evaluate effect of PEI on the **flocculation** of compounds, a first step was conducted regarding influence of the sample's dilution prior mixing with PEI solution. A factorial design was used : the supernatant obtained after cellular lysis was diluted (1/1, 5/6, 2/3, 1/2, 1/3, 1/6) and then mixed (v/v) with water (references) or with a 0.075% PEI solution.

All experiments were conducted at least in triplicate.

2.7.2. Influence of pH and PEI concentration

The effect of pH and PEI concentration on fractionation of compounds was studied by using a factorial design: six concentrations of PEI were tested (PEI solutions at 0.025, 0.050, 0.075, 0.100 and 0.125%), and three pH (6.3 which is the native pH of the samples, 7.4, and 8.5). The aim of the pH variation was to modify molecules charge, and then their interaction with PEI. Previous studies have been conducted at neutral pH (Milburn et al., 1990; Salt et al., 1995; Mazzaferro et al., 2010). **All experiments were conducted at least in triplicate.**

2.7.3. Salt effect

A factorial design **has been used** to study the interaction between NaCl concentration (0 M, 0.125 M, 0.250 M, 0.500 M, 0.750 M and 1 M) and PEI concentration (0.025, 0.075 and 0.100%) for an extract diluted by 2, a pH set at 7.4. **All experiments were conducted at least in triplicate.**

2.8. Statistical treatment.

All experiments were conducted at least in triplicates. In each case, average value and standard deviations were calculated.

All statistical analyses were performed using R 3.6.1 software (R Core Team, 2019) and the statistical packages agricolae (de Mendiburu, 2019), HH (Heiberger and Holland, 2015) and multcomp (Hothorn et al., 2008). All the data obtained were statistically analyzed by two-ways ANOVA to test the level of statistical significance of factors. *Post-hoc* procedures were used when the F-test was significant ($p < 0.05$). Multiple comparisons among means were examined by a Tukey's HSD test. This test was chosen because it finds means that are significantly different from each other. The level of statistical significance was set at $p < 0.05$.

3. Results and Discussion

3.1. Tuning of flocculation conditions

3.1.1. Dilution of samples

Results of turbidity and % of retained proteins were reported on **Figure 1**. Logically, retained proteins were about 100% while turbidity decreased with dilutions for references. When adding 0.075% PEI, modulation of turbidity and retained proteins was observed. For the non-diluted sample, turbidity was about 7-fold lower (0.14 uA) than the corresponding reference

(1.005 uA), while retained proteins were about 50%. When further diluted (5/6, 2/3), turbidity was found even lower (respectively 0.04, and 0.03 uA), while retained proteins remained constant (~50%) in the supernatant. For dilution 1/2, the turbidity was found at the same level than for dilution 1 (0.13 uA), but 89% of proteins remained in the supernatant. For the last 2 dilutions (1/3 and 1/6), protein content in the supernatant was found higher than the corresponding references, leading to deviant estimation of retained proteins (respectively 150 and 230%). These abnormal results could probably be attributed to interference of PEI with the Lowry assay. Indeed, [Gupta et al., \(2000\)](#) have described an overestimation of protein content by colorimetric assays for samples containing PEI in solution. Our hypothesis is that for these dilutions, the PEI amount could be too high regarding the compounds to flocculate, resulting in an excess of PEI, remaining in the supernatant. Concerning sugar assays, almost 100% were retained in the supernatant whatever condition tested.

A two-factors (dilution factor and presence/absence PEI) ANOVA with interaction between the two factors was performed on turbidity. The two factors and their interaction have a highly significant effect on turbidity. The decreasing slopes (i.e. the dilutions for which adding PEI allow decreasing the turbidity) were obtained for dilutions in the order 2/3, 5/6 1, 1/2 and 1/3, whereas no significant effect was noticed for 1/6 (**Figure 1 B**). Similarly, a two-factors ANOVA was performed on proteins remaining in the supernatant. Only, factor dilution had a highly significant effect. Positive slopes observed for dilutions 1/3 and 1/6 (**Figure 1 C**) correspond to the overestimation of proteins content has previously discussed, whereas a negative slope relate to a loss of proteins. According to these considerations, the dilution 1/2 seems to be the more suitable to prevent a too high loss of proteins. This is confirmed by the HSD Tukey test (lowercase letters on **Figure 1 A**), for which we found that dilutions 1, 5/6 and 2/3 led to significant loss of proteins, whereas for the dilution 1/2, the amount of proteins kept in the supernatant was found not significantly different from the

reference. A half volume dilution before mixing with PEI solution has then been retained as the best one.

3.1.2. Influence of pH and PEI concentration

The pH may have an effect on the flocculation efficiency. As pKa of PEI is 10-11 (Burgess, 2009), the polymer is cationic at neutral pH. However, charge density or level of repulsion of compounds in the supernatant can be modified, modifying interactions with PEI and their capacity to flocculate. Three pH were tested, which are the native pH of the supernatant (6.3 **Figure 2A**), a neutral pH (7.4, **Figure 2B**), and a more basic pH (8.5, **Figure 2C**).

The two first tested pH (6.3 and 7.4) have substantially the same protein profile. The proteins were precipitated with the other compounds at low levels of PEI, and are kept in full in the supernatant when the percentage of PEI increased. The same trend was observed for turbidity, very low for 0.025 % of PEI and rising beyond. It may be explained by a re-solubilization of non-proteic compounds (lipids and pigments), due to an excess of PEI as previously observed by Salt et al. (1995). At pH 8.5, the pattern was found different when compared to other pH conditions. In that case, it was needed to achieve 0.05% of PEI to observe a drop of turbidity with a minimum of 0.007 uA for 0.100% of PEI. Similar trend was observed for proteins. They were kept in the supernatant for 0.025 % of PEI, decreased around 50% of retained proteins for 0.050 and 0.075% of PEI and finally rised back to 62% for 0.100 %, and 89% at the maximum level of PEI. As previously, and whatever condition tested (pH or PEI concentration), almost 100% of sugars were retained in the supernatant.

Analysis of variance on turbidity results shows that the two factors (pH and PEI concentration) and their interaction are highly significant. Concerning Tukey's HSD test (**indicated by letters on Figure 2**), the most significant differences were observed for pH 8.5 (0.075% and 0.1%), and at pH 7.4 (0.025%). The condition at pH 7.5 and 0.075% PEI was

found significantly different from the references but also from the best ones. Concerning proteins, only PEI concentration has a highly significant effect and all results were found significantly different one from the other. Tukey's HSD test indicates that intermediate concentrations (0,025 % and 0,05 %) correspond to low concentration of proteins remaining in the supernatant. **These results thus confirm that the PEI flocculation efficiency is strongly dependent on pH. However, as the composition of the supernatant is not precisely resolved regarding the charge and pKa of molecules composing it, it seems difficult to fully understand the mechanism leading to the observed results.**

3.1.3. Salt effect

As PEI is a cationic polymer, the ionic strength may also have an effect on the flocculation efficiency modifying interactions between compounds. Especially, salts can act as counter-ions for charged molecules. The work of Salt et al. (1995) have shown that by varying the ionic strength (adding sodium chloride, NaCl), the amount of lost proteins was minimized. For their *E. coli* extract, adding 1M NaCl allowed them to retain 86% of proteins in the supernatant, instead of 60% without salt. Burgess (2009) also described strategies for fractionation by PEI who are in function of the nature of the proteins (neutral, acidic or basic) and the ionic strength.

Results of turbidity and % of retained proteins in the supernatant **as function of NaCl concentration, for 3 PEI concentrations (0.025%, **Figure 3A**, 0.075%, **Figure 3B** and 0.100%, **Figure 3C**), with pH set at 7.4.**

Results of ANOVA show that the two studied factors (% PEI and NaCl concentration) were both highly significant as well as their interaction in the case of turbidity and proteins in the supernatant. Interaction plots of turbidity (**Figure 4A**) or of proteins (**Figure 4B**) don't show clear effect of ionic strength for 0.025% PEI. These observations were confirmed by the HSD

Tukey test (indicated by letters on **Figure 3**), for which no significant difference was noticed. For 0.075% PEI, the means of turbidity were found lower while increasing NaCl concentration (500 mM or more), but in the same time, the % of retained proteins was also lower, highlighting the fact that both pigments and proteins were more flocculated. For 0.100% of PEI, adding NaCl had an unfavorable effect, as % of retained proteins was high (but not higher than for the reference at the same PEI concentration without NaCl), and turbidity was found greater than the reference. At pH 7.4, the addition of NaCl therefore failed improving the efficiency of the fractionation.

Salt et al., (1995) had shown that the addition of NaCl allows to decrease from 40 to 14% the percentage of flocculated proteins with contaminants of a yeast extract (*S. cerevisiae*). However, an excess of NaCl or PEI leads to the opposite effect with a resolubilisation of flocculated compounds. In our case, the pre-clarification with sodium tetraborate was tested but no difference was noticed (data not shown). Once more, no influence was noticed regarding sugars concentration in the supernatant which remained at 100%.

3.2. Scale up

The cationic polymer used in this study had never been tested previously on a microalgae extract. It allowed the flocculation of some compounds and required determining some physico-chemical parameters allowing an improved fractionation. The aim of this first part of the study was to evaluate effect of different parameters (dilution of sample, pH, ionic strength and % of PEI), on the **flocculation** of compounds from a cellular extract from *H. pluvialis*. Unlike the work of Salt et al. (1995), the modification of the ionic strength did not help improving the fractionation, but the results have shown that, depending on the conditions tested, this method allowed to flocculate lipids and pigments while maintaining all of the sugars and an important part of the proteins in the supernatant.

The addition of successive parameters helped to consider two scenarios. The first was to select the conditions that allow the flocculation of a maximum of lipids and pigments (i.e. the lower turbidity), but with a substantial loss of proteins. This result was obtained by diluting the cellular extract to half and by mixing it with a 0.100% PEI solution at pH 8.5. These conditions allowed to obtain a supernatant with a turbidity of 0.007 uA and 61% of the proteins present initially. The second case was to select a condition that does not eliminate all the lipids and pigments but allowing to retain more proteins. This was the case when diluting to half and mixing with 0.075% or 0.100% PEI solutions at pH 7.4. These conditions allowed to obtain 89 to 100% of retained proteins, with a turbidity of approximately 0.14 uA.

Nevertheless, these tests have been conducted on volumes of 25 mL. It appeared therefore essential to validate the conditions of flocculation on larger volumes in order to assess the possibility to scale-up the method and to characterize the different fractions. Indeed, if the chemical methods of fractionation are known to be quite effective in the purification of the compounds of interest, they are sometimes difficult to extrapolate to the pilot scale or even industrial.

The 3 chosen conditions had then been tested for volumes of 500 mL, which corresponded to a scale-up x20. In terms of analysis, as previously, sugars and proteins have been measured in the supernatant and these latter were analyzed by steric exclusion chromatography.

Concerning lipids and pigments, they were extracted with solvents (chloroform/methanol) both in the supernatants and pellets of flocculation in order to quantify and analyze them (gas chromatography for lipids, HPLC and spectrophotometry for pigments).

3.2.1. Characterization of water-soluble fraction

Concerning the water-soluble fraction, analyzed only in the supernatant of flocculation, results obtained for the 3 conditions and those obtained for smaller volumes are presented in **Table 2**.

Regarding the proteins content, **no significant difference were noticed for results obtained with 0.075% of PEI at pH 7.4 and 0.100% at pH 8.5 between 25 mL and 500 mL volumes** ($89.8\% \pm 3.3\%$ of proteins are retained in the supernatant at pH 7.4 against $89.1 \pm 4.9\%$ previously, and 61.2% at pH 8.5 against $61.3 \pm 7.5\%$ for volumes of 25 mL). In contrast, the condition with a PEI solution at 0.100% and pH 7.4, which was the more favorable to the maintenance of the proteins in the supernatant ($104.9 \pm 13.3\%$) was found disappointing for 500 mL volumes as only $89.6\% \pm 4.1\%$ of the proteins were retained in the supernatant, value **no more significantly different** to what was obtained for 0.075% of PEI. In the light of these results, this condition was discarded, and the rest of the analysis was performed only on the samples obtained with 0.075 % of PEI at pH 7.4 and 0.100% at pH 8.5. **Concerning turbidity measurements, the scale change had no impact on results as not significant difference was noticed with volumes. The molecular weights profiles of the proteins were analyzed by steric exclusion chromatography for the extract after cell lysis, of the supernatant of flocculation obtained with 0.075 % of PEI at pH 7.4, and the supernatant of flocculation obtained with 0.100% of PEI at pH 8.5 (Supplementary material).** The results showed that for the supernatant obtained at pH 7.4, the absorbance at 660 nm is zero on the whole elution time, whereas a peak of significant intensity was observed for the cellular extract. This showed that the chlorophylls, which strongly absorb at 660 nm, were absent from the supernatant after flocculation. Regarding the carotenoids, which can be followed by the absorbance at 470 nm, the main peak had disappeared but a low intensity peak was still observed between 10 and 15 mL of elution. This result was in agreement with the turbidity measurements obtained on this

sample, which was 0.14 uA, and which reflected the residual presence of pigments. For proteins, the high molecular weight proteins have predominantly disappeared, even if a part of them were preserved. For the supernatant obtained at pH 8.5, the turbidity was very low (0.007 uA). SEC analysis is in accordance with this low turbidity value, as no peak was detected neither at 660 nor 470 nm. Concerning the proteins profile, once again, mainly the high molecular weight proteins have disappeared, but also a fraction of approximately 30 kDa. Ursu et al., (2014) has observed aggregates between chlorophylls and high molecular weight proteins from *Chlorella vulgaris* after high pressure homogenization. Same kind of aggregates may have occurred in our study, explaining the loss of these proteins, driven with the chlorophylls in the flocculated pellet.

Ionic liquids (ILs) have been studied for microalgae biorefinery. ILs are salts which generally consist of a combination of an organic cation with an anion and have a melting point below 100 C. They can vary considerably in their physiochemical properties such as polarity, hydrophobicity, toxicity, and thermostability (Petkovic et al., 2011) and have often been described as green solvents. ILs have been shown to facilitate the extraction of lipids from microalgae, primarily by disrupting microalgal cell structure (Texeira et al., 2012; Orr et al., 2016), allowing auto partitioning of the lipids. However, high temperatures (100–140°C) were often necessary to achieve the complete dissolution and cell lysis (Texeira et al., 2012) which are not compatible with the further extraction of proteins. Milder temperature conditions were also described but required the use of co-solvent such as methanol or ethyl acetate (Desai et al., 2016) which also induce denaturation of proteins. Partitioning of proteins in IL-based Aqueous two-phase system (ATPS) is a complex phenomenon **which** depends on several factors including type and concentration of phase-forming components, pH, temperature, ionic strength (Ventura et al., 2017). In the case of proteins, hydrophobicity, isoelectric point, molecular weight and conformation play a critical role (Dreyer et al., 2009).

Some studies focused on extraction of *C-phycoerythrin* from *Spirulina* strains (Luo et al., 2016), *B-phycoerythrin* from *Porphyridium cruentum* (Benavides et al., 2006), and proteins from *Chlorella pyrenoidosa* (Waghmare et al., 2016), and *Chlorella sorokiniana* (Phong et al., 2017). However, it is to notice that these proteins concentrates could be still significantly contaminated by pigments (Waghmare et al., 2016). Additionally, Suarez Garcia et al., (2018) reported proteins extraction efficiency of about 80% for *Neochloris oleoabundans* and *Tetraselmis suecica* but didn't verified the functional properties of the protein extract. In our case, the amount of recovered proteins was then found greater (nearly 90%), with very low pigment remaining.

3.2.2. Characterization of the fat-soluble fraction

The fat-soluble fraction was analyzed both in the supernatant and pellet of flocculation, in order to quantify and characterize the lipids and the pigments present. The first step has thus been to extract lipids and pigments by the modified method of Bligh and Dyer as described in section 2.3. This gravimetric method allowed to quantify the whole fat-soluble fraction. In the cellular extract, lipids and pigments represent 45 % DW. For the pellets of flocculation obtained with 0.075 % of PEI at pH 7.4, and 0.100% of PEI at pH 8.5, the fat-soluble fractions represented respectively 42% and 43% of the initial dry weight of the cellular extract, which then corresponded to 94 and 96% of this initial fraction. This result showed that both PEI treatments allowed an efficient flocculation of lipids and pigments, but also that the interaction was reversible since nearly 100% of the fraction was recovered. For the supernatants obtained after flocculation, the direct application of the Bligh and Dyer method did not allow the quantification of the fat-soluble fraction because the gravimetric method is not suitable for diluted samples. An aliquot was therefore freeze-dried, and then the fat-soluble fraction extracted according to the same method. Nevertheless, it was still not possible to quantify the fat-soluble fraction, the amounts of residual lipids and pigments in the

supernatants being too low. However, the supernatant of flocculation obtained for 0.075% of PEI at pH 7.4 presented a visible color and a measured turbidity of 0.14 uA. An acetone extraction was conducted on the freeze-dried supernatants, in order to achieve a spectrophotometric quantification according to the formula of Lichtenthaler and Buschmann (2001). This quantification has been also carried out for the cellular extract and results are presented in **Table 3**.

The pigments analysis of the cellular extract showed a distribution of 33 % of chlorophylls and 67 % of total carotenoids. For pigments extracted from flocculated pellets, the repartitions were **not significantly different**. Concerning supernatant obtained after flocculation with the 0.100% PEI solution at pH 8.5, the quantities of pigments were too low to be detected. For the supernatant of flocculation obtained with the 0.075% PEI solution at pH 7.4, no chlorophyll was detected, but a low amount of carotenoids has been highlighted (0.34 ± 0.08 mg/g initial DW). These results seemed in accordance with the previously measured turbidities which were greater for this second condition of flocculation, but also with the small peak detected at 470 nm during SEC analysis. Even if this carotenoids content appeared to be low, it seemed important to go further and to identify which kind of carotenoid was present. The HPLC spectrum (**Supplementary material**) of the cellular extract revealed a very important number of peaks, corresponding to a complex pigments mixture. Spectra obtained from pellets of flocculation presented profiles not fully superimposable but very similar. For the supernatant of flocculation obtained for 0.075% of PEI at pH 7.4, a peak of esterified astaxanthin of very low intensity was detected, but the main peak corresponded to the β -carotene. This result is of interest in *Haematococcus pluvialis* biorefinery context. Among pigments from this strain, the higher value-added one is clearly astaxanthin, with a growing commercial market and a price between 2500 to 7000\$/kg (Li et al., 2011).

Initial cellular extract and pellets obtained after flocculation were analyzed by gas chromatography for their fatty acids content and repartition. A comparison of spectra obtained for the cellular extract and the flocculation pellet obtained with a 0.075 % PEI solution at pH 7.4 is proposed in **Supplementary material**. The quantitative results of the analysis are presented in **Table 4**. The results showed a strong similarity between the different samples and no significant difference was noticed. In the conditions of flocculation tested, the PEI therefore didn't show specificity of flocculation and all the lipids were recovered. Indeed, in all cases, the main fatty acids (in bold in the **Table 4**) are the palmitic acid (18.1 to 20.6%), oleic acid (13.1 to 14.3%), linolelaïdic acid (28.6 to 29.5%) and alpha-linolenic acid (13.2 to 13.9%).

Concerning flocculation mechanism, it was assumed that the cationic polymer PEI may interact with negatively charged molecules, leading to aggregates formation and then flocculation. Concerning *H. pluvialis*, astaxanthin is negatively charged at the working pH range. However, most of the lipids are neutral, but were found to flocculate. Our hypothesis is that the network formed by the interaction of PEI with anionic molecules may have driven other fat-soluble compounds due to their hydrophobicity. Nevertheless, other experiments will be needed to confirm this hypothesis. As *Haematococcus pluvialis* contains only neutral sugars (starch and hemicellulose) this flocculation mechanism may explain that addition of PEI had no effect on these molecules.

3.3. Technofunctional properties of protein extracts

In a biorefinery context, the fractionation of compounds must be done in mild conditions, without alteration of recovered fractions. Concerning proteins, their possible use as technofunctional compounds implies not to alter their native conformation. The emulsification index, emulsification capacity, emulsion stability of proteins recovered after PEI flocculation

have thus been evaluated and compared to the crude extract obtained just after cellular lysis.

These results are presented in **Table 5**.

The emulsification capacities of crude extract and of the supernatant obtained after 0.075%PEI/pH7.4 treatment were of the same order of magnitude (respectively 2074 ± 27 and 1921 ± 12 mL of oil/g protein). These values were similar to those previously described by Ba et al. (2016) on a crude extract from the green vegetative form of *H. pluvialis* used at the same concentration (2 g/L). This result confirms that the PEI flocculation had no impact on the native conformation of proteins. For this supernatant, nearly 90% of the proteins initially present in the crude extract were retained, explaining that the emulsifying capacity was only slightly decreased. On the other hand, the emulsification capacity measured for the 0.100%PEI/pH8.5 condition was significantly lower (1343 ± 21 mL of oil / g protein). Concerning emulsification stability (ES) values and emulsification activity index (EAI), similar trend was observed, with a decrease observed for the 0.100%PEI/pH8.5 condition, while no significant difference was noticed for the other flocculation's supernatant as compared to the crude extract. These differences in EC, ES and EAI for the sample obtained after 0.100%PEI/pH8.5 treatment can be explained by the loss of high molecular weight proteins observed by size exclusion chromatography. Indeed, it had been shown that the molecules of high molar masses limit the phenomenon of coalescence (Dickinson and Stainsby, 1982) and act against the degradation of the emulsion by the presence of steric forces (Damodaran, 2005; Choplin et al., 2006). In contrast, the low molecular weight molecules have very little steric forces, which reduces their ability to maintain a stable emulsion.

3.4.Economical aspects

All these results therefore confirmed the interest of the flocculation using PEI, who had never been tested on microalgae, for the fractionation of water-soluble and fat-soluble fractions of

Haematococcus pluvialis. The condition of treatment with a 0.075 % PEI solution at pH 7.4 appears to be the best choice since it allows a good fractionation, with nearly 100% of lipids that can be recovered from the pellet, and with limiting losses in proteins. In addition, as the required PEI amount is lower, the cost of treatment will be lower too. This economic aspect is obviously an important point to take into consideration in the establishment of a scheme of biorefinery. In the last years, ionic liquids have gained interest for the fractionation of microalgae compounds, as phase forming components in aqueous two-phase systems due to their chemical versatility and physicochemical properties (Suarez Garcia et al., 2018). However, even if ionic liquids have been found efficient for proteins fractionation on different microalga species, main drawback of using ionic liquid remains their expensive cost. In the case of flocculation using PEI, the cost of treatment seems reasonable. In the case of this study, the PEI has been purchased from the Sigma-Aldrich company with a cost of 70euros/kg, but it is likely that the cost would be lower with increased purchased amount. Nevertheless, on this cost basis, and knowing that the treatment requires 75g of PEI for 1 kg DW of biomass, the cost would therefore be 5.5 euros per kg of dry biomass. Moreover, the PEI is known to be non-toxic at low concentration (Pandey and Sawant, 2016), which gives it a significant advantage in the approach of biorefinery.

Several tracks exist to further optimize this process. First of all, the possibility of reusing the PEI must be considered after recovery of the fat-soluble fraction, which would further decrease the process cost. In order to facilitate this reuse, it would be interesting to test the fractionation efficiency of PEI fixed on a support. Indeed, the PEI exists grafted on silica beads for chromatographic applications. If the flocculation performances are not altered by this grafting, the use of this type of support could allow a separation of the flocculated fraction by direct settling (instead of a centrifugation), and the reuse of beads for a number of

cycles which remains to be determined. Indeed, this kind of support could perhaps avoid the half dilution step of the cellular extract.

4. Conclusion

The flocculation using PEI thus enables an effective fractionation of water-soluble and fat-soluble fractions, with 89.8% of proteins and 100% of sugars retained in the supernatant (0.075 % PEI at pH 7.4), while nearly 100% of lipids and pigments were easily recovered from the pellet **after flocculation**. The analysis of molecular weight distribution showed that the proteins lost corresponded mainly to the high molecular weight proteins, perhaps "driven" by the flocculation of lipids and pigments. However, the emulsification properties were found unchanged as compared with the crude extract, confirming that this method is non-destructive.

Supplementary material for this work can be found in e-version of this paper online

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Figures caption

Figure 1 Influence of sample dilution on turbidity (**lines**) and % of retained proteins (**bar charts**) (A). In blue: samples mixed with water (references), In red: samples mixed with 0.075% PEI solution. Results correspond to the **mean** value of at least 3 experiments, and bars are **standard deviations**. Letters correspond to Tukey's HSD test results for turbidity (a-f) and % of retained proteins (x-z). Different letters correspond to significant different results. Variance analysis interaction plot for turbidity (B) and % of retained proteins (C).

Figure 2 Influence of concentration of PEI solution on turbidity (**line**) and % of retained proteins in supernatant (**bar charts**) at pH 6.3 (A), 7.4 (B) and 8.5 (C). Results correspond to the **mean** value of at least 3 experiments, and bars are **standard deviations**. Letters correspond to Tukey's HSD test results for turbidity (a-f) and % of retained proteins (s-z). Different letters correspond to significant different results.

Figure 3 Influence of NaCl concentration (0 to 1000 mM) on turbidity (**line**) and % of retained proteins in supernatant (**bar charts**), for 0.025% of PEI (A), 0.075% of PEI (B) and 0.100% of PEI (C). **All experiments were performed with a pH set at 7.4**. Results correspond to the **mean** value of at least 3 experiments, and bars are **standard deviations**. Letters correspond to Tukey's HSD test results for turbidity (a-g) and % of retained proteins (v-z). Different letters correspond to significant different results.

Figure 4 Variance analysis interaction plot for turbidity (A) and % of retained proteins (B) for the influence of NaCl concentration (0 to 1000 mM) at pH 7.4.

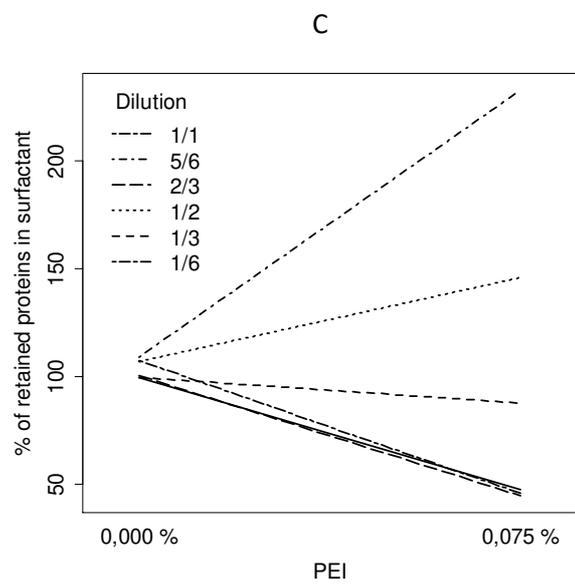
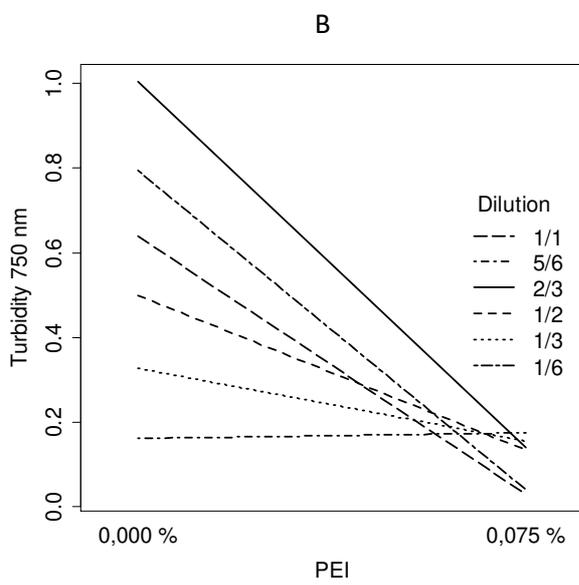
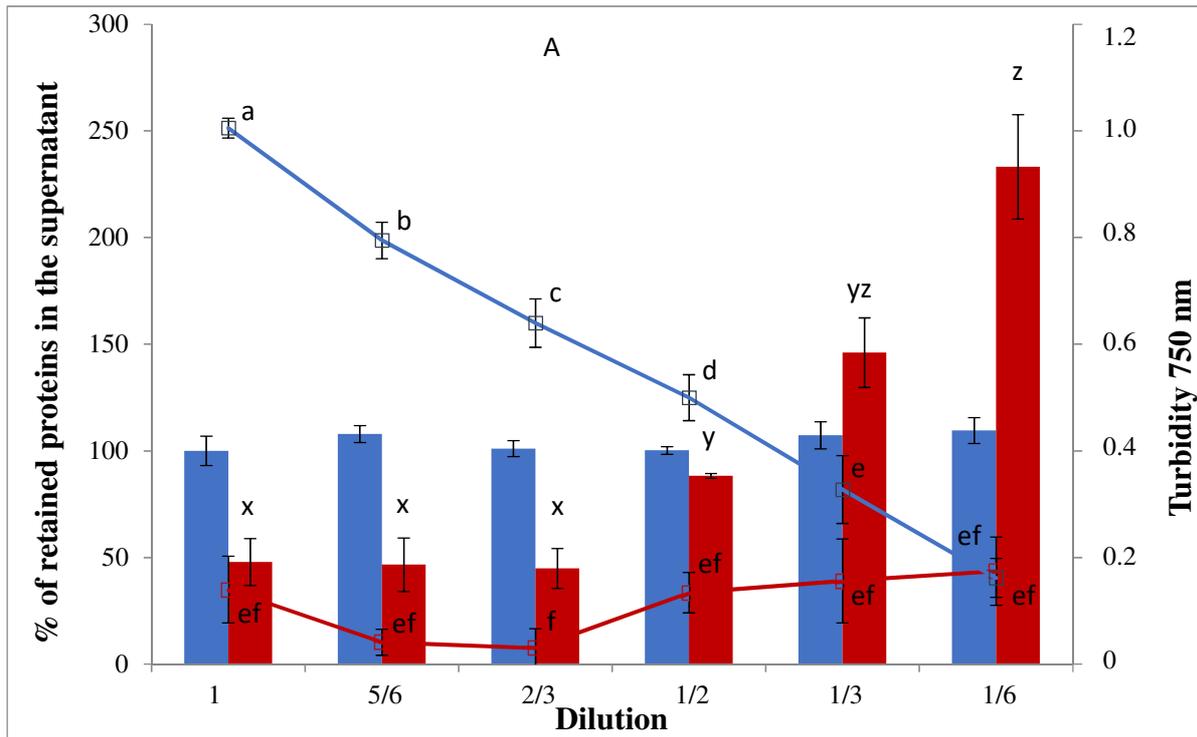


Figure 1

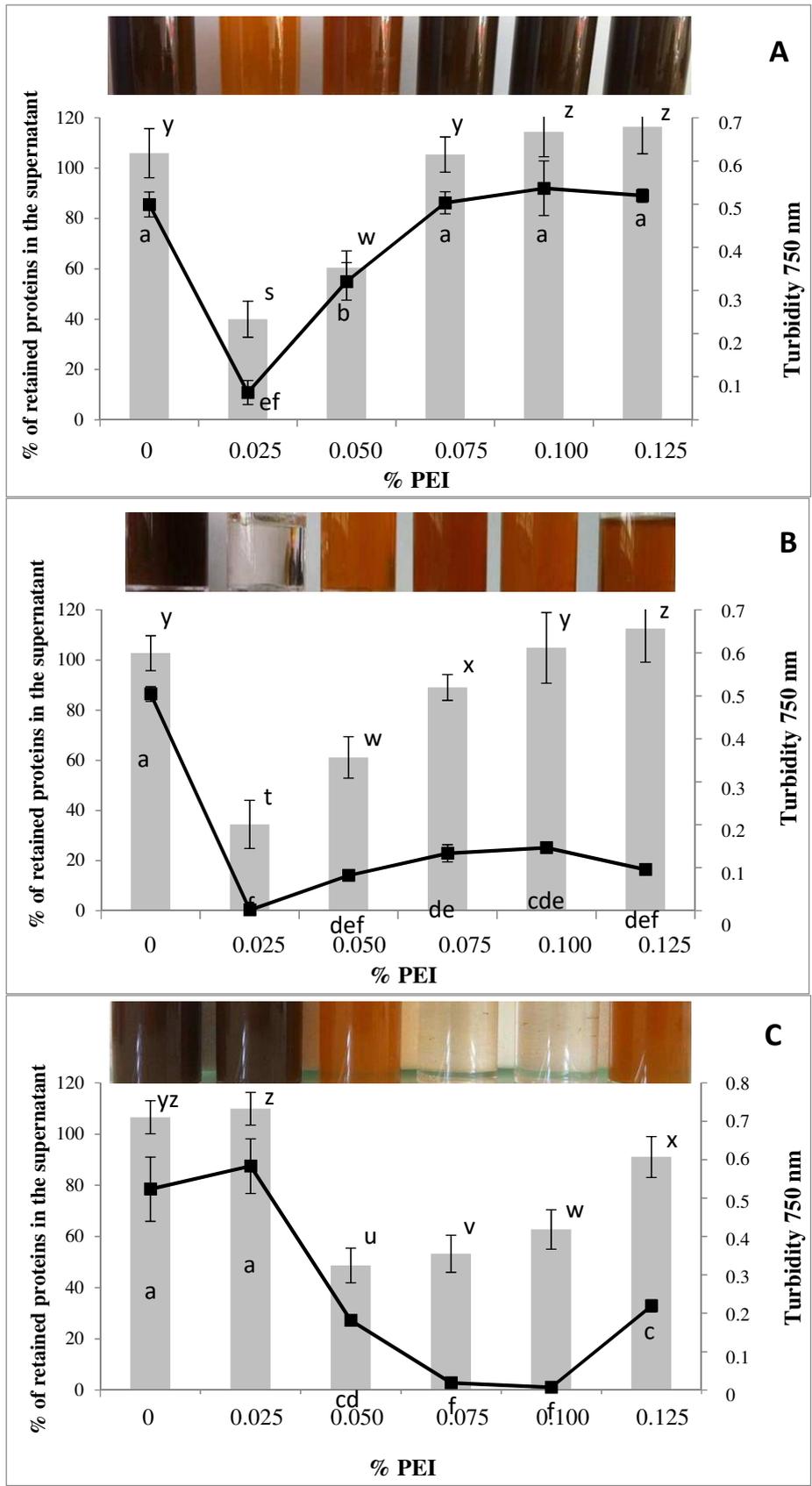


Figure 2

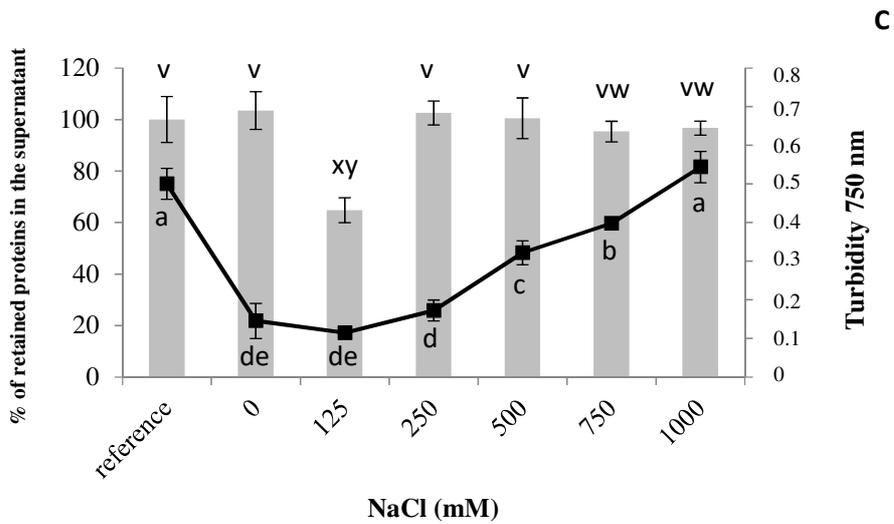
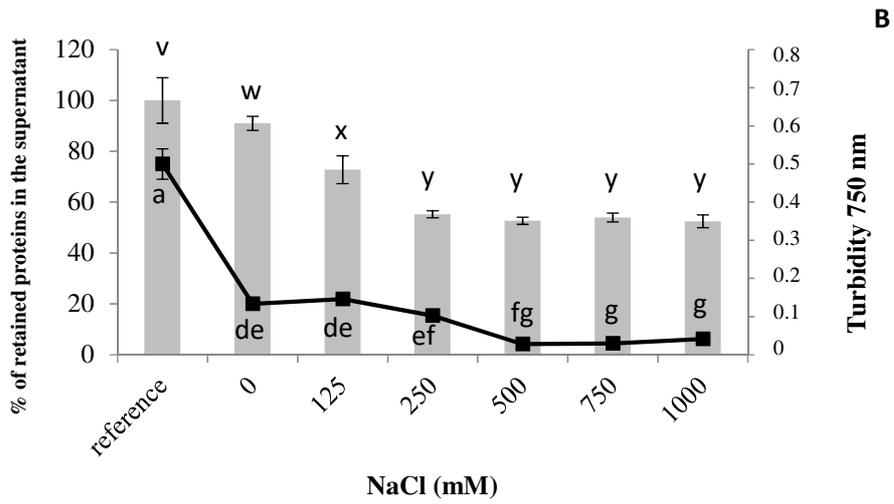
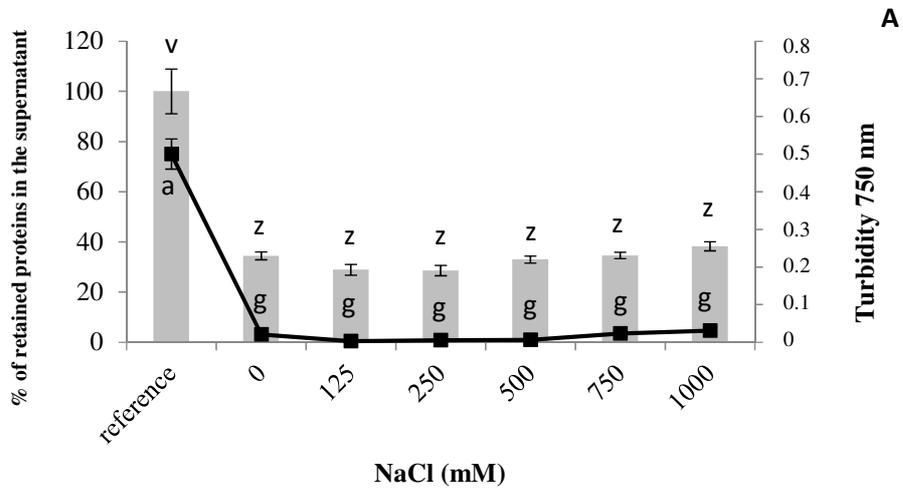


Figure 3

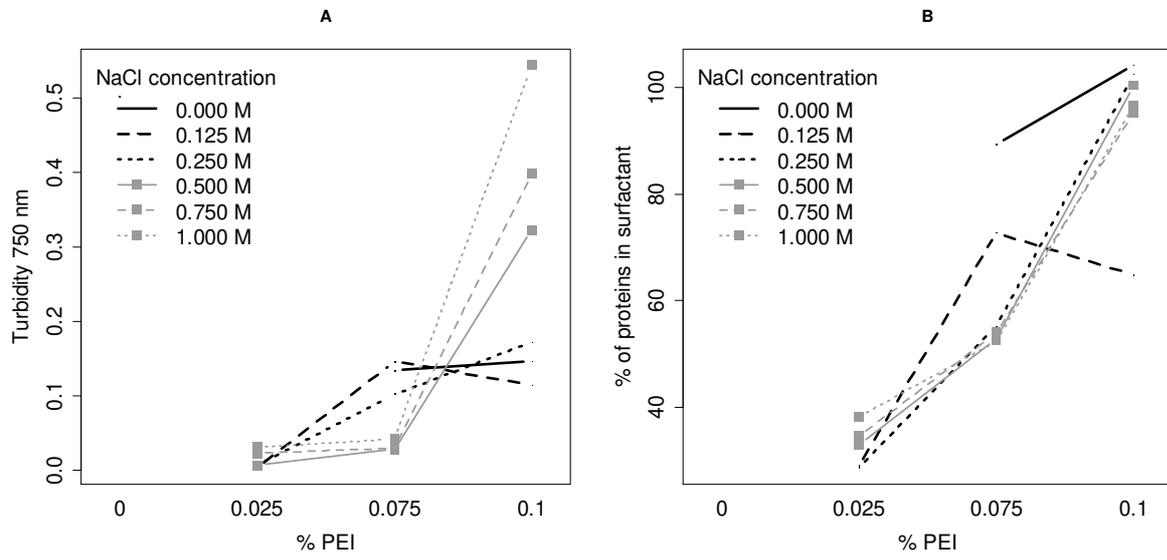


Figure 4

Table 1 ANOVA

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
PEI	1	1.8934	1.8934	802.18	< 2e-16	***
Dilution	5	0.5998	0.1200	50.82	5.46e-12	***
PEI:Dilution	5	0.8831	0.1766	74.83	7.66e-14	***
Residuals	24	0.0566	0.0024			

Table 2 Comparison of turbidity measurements at 750 nm, proteins and sugars assays obtained for 25 mL and 500 mL volumes for the 3 conditions. Experiments were conducted **at least** in triplicates and standard deviations are mentioned. **Different letters indicate different significance of results.**

	0.075% PEI, pH 7.4		0.100% PEI, pH 7.4		0.100% PEI, pH 8.5	
	25 mL	500mL	25 mL	500mL	25 mL	500mL
Proteins (% retained in the supernatant)	89.1 ± 4.9% a	89.8%± 3.3% a	104.9 ± 13.3% b	89.60% ± 4.1% a	61.3% ± 7.5% c	61.2%± 3.7% c
Sugars (% retained in the supernatant)	101.0 ± 7.5% d	99.0%± 1.1% d	103.0 ± 8.3% d	94% ± 3.5% d	89.5% ± 10.8% d	101%± 2.4% d
Turbidity 750 nm (uA)	0.135 ± 0.04 e	0.141 ± 0.01 e	0.14 ± 0.07 e	0.17 ± 0.01 e	0.007 ± 0.05 f	0.006 ± 0.01 f

Table 3 Spectrophotometric quantification of chlorophylls a, b and carotenoids according to Lichtenthaler et Buschmann (2001) method. Results correspond to the average value of at least 3 experiments, % indicate repartition of chlorophylls and carotenoids among analyzed pigments, \pm are standard error deviations, nd non-detectable.

	mg/g initial DW			
	Chlorophyll <i>a</i> (Ca)	Chlorophyll <i>b</i> (Cb)	Total Chlorophylls (Ca+b)	Total Carotenoids (Cx+c)
Cellular extract	4.60 ± 0.06	3.55 ± 0.06	8.15 ± 0.13 33 %	16.82 ± 0.32 67 %
Flocculation pellet (0.075 %. pH 7.4)	5.18 ± 0.11	4.24 ± 0.09	9.42 ± 0.20 37 %	15.91 ± 0.18 63 %
Flocculation pellet (0.100 %. pH 8.5)	5.08 ± 0.17	4.06 ± 0.12	9.14 ± 0.30 34 %	17.79 ± 0.21 66 %
Flocculation supernatant (0.075 %. pH 7.4)	nd	nd	- 0 %	0.34 ± 0.08 100 %
Flocculation supernatant (0.100 %. pH 8.5)	nd	nd	- 0 %	- 0 %

Table 4 Fatty acids repartition in lipids extracted from cellular extract and flocculation pellets obtained with a 0.075% PEI solution at pH 7.4 and 0.100 % PEI solution at pH 8.5.
Main fatty acids are indicated in bold.

AG		Cellular extract	Flocculation pellet 0.075% PEI pH 7.4	Flocculation pellet 0.100% PEI pH8.5
Myristic acid	14:00	0.7%	0.6%	0.7%
Palmitic acid	16:00	20.6%	19.1%	18.1%
Stearic acid	18:00	2.0%	1.9%	2.5%
Oleic acid	18:1n-9c	13.1%	14.3%	14.1%
Elaidic acid	18:1n-9t	5.2%	4.9%	4.9%
Linoleic acid	18:2n-6c	8.0%	9.3%	8.1%
Linolelaidic acid	18:2n-6t	29.1%	28.6%	29.5%
Gamma-linolenic acid	18:3n-6	2.7%	2.5%	2.3%
Alpha-linolenic acid	18:3n-3	13.3%	13.2%	13.9%
Arachidic acid	20:00	2.6%	2.6%	2.7%
Arachidonic acid	20:4n-6	1.3%	1.4%	1.6%
Docosadienoic acid	22:2n-6	1.3%	1.4%	1.5%

Table 5 Emulsification properties of crude extract and supernatants obtained after flocculation with 0.075 % PEI / pH 7.4 or 0.100 % PEI / pH 8.5. EC : Emulsification Capacity (mL oil / g proteins), ES : Emulsification Stability (%), EAI : Emulsification Activity Index (m²/g).

	EC (mL oil / g proteins)	ES (%)	EAI (m ² /g)
Crude extract	2074 ± 27	71 ± 2	114 ± 9
0.075 % PEI pH 7.4	1921 ± 12	75 ± 1	121 ± 5
0.100 % PEI pH 8.5	1343 ± 21	42 ± 3	43 ± 8