**Universidade de Aveiro** Departamento de Química
**Ano 2020**

**Ana Isabel Bastos Valente**

# Extração e purificação de RuBisCO: da biomassa residual à proteína de valor acrescentado

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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biotecnologia Industrial e Ambiental, realizada sob as orientações científicas da Doutora Ana Paula Mora Tavares, Investigadora Auxiliar do Departamento de Química da Universidade de Aveiro e da Doutora Ana Mafalda Rodrigues Almeida Rocha, Investigadora do Departamento de Química da Universidade de Aveiro

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**“Matar o sonho é matarmo-nos. É mutilar a nossa alma. O sonho é o que temos de realmente nosso, de impenetravelmente e inexpugnavelmente nosso.”**

-Fernando Pessoa-

**o júri**

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**palavras-chave**

Ribulose-1,5-biphosphate carboxylase/oxygenase, extração sólido-líquido, sistemas aquosos bifásicos, líquidos iónicos, extração, purificação

**Resumo**

Com o crescimento da população mundial são necessárias novas fontes de proteínas. A biomassa vegetal é uma fonte promissora, pois permite a utilização de resíduos de culturas agrícolas com a capacidade de ser uma fonte contínua de biomassa sem prejudicar os ecossistemas já existentes, e a extração de proteínas de biomassa ainda fresca. Atualmente, com os métodos utilizados, é obtido um produto final que não pode ser aplicado na indústria alimentar além de que, os métodos até hoje desenvolvidos não são seletivos nem permitem atingir simultaneamente purezas e eficiências de extração próximas de 100 %.

A RuBisCO (Ribulose-1,5-bifosfato carboxilase/oxigenase) é a proteína (enzima) mais abundante no planeta e pode ser aplicada nas mais diversas áreas, desde a farmacêutica à alimentar e de rações. Deste modo, nesta tese de mestrado foi desenvolvido um novo método de extração da RuBisCO e posterior separação e purificação. Para tal, foram usadas soluções aquosas de líquidos iónicos (LIs) biocompatíveis (análogos da glicina-betaína e derivados de colínio) na extração sólido-líquido da RuBisCO da folha do espinafre. Através da metodologia de superfície de resposta, foram otimizadas as condições da extração (pH, razão sólido-líquido e a concentração de LI). Nas condições ótimas, foi obtida uma extração máxima de 2.00 mg /mL de RuBisCO e um rendimento de 10.93 mg de RuBisCO /g de biomassa para o acetato de colínio ([Ch][Acetato]) e 1.86 mg /mL e um rendimento de 10.12 mg de RuBisCO /g de biomassa para o cloreto de colínio ([Ch]Cl). Os pontos ótimos determinados foram: razão sólido-líquido de 0.184, concentração de LI de 2.68 M e um pH de 9.09 para o [Ch]Cl e 11.2 para o [Ch][Acetato].

Após a extração, a separação e a purificação da RuBisCO foi realizada através da aplicação de sistema aquoso bifásicos (SABs). Os extratos provenientes da extração sólido-líquido com [Ch]Cl e [Ch][Acetato] que contêm LI e RuBisCO foram misturados individualmente com polipropileno glicol 400 $g.mol^{-1}$ (PPG 400) ou polietileno glicol 1000 $g.mol^{-1}$ (PEG 1000) e fosfato dipotássico ($K_2HPO_4$) formando-se dois tipos de SABs compostos por LI e polímero ou sal. Após este processo foi possível obter uma eficiência de extração da RuBisCO de 100% para a fase de LI no sistema composto por PPG400+ [Ch][Acetato] e por $K_2HPO_4$ + LI ([Ch][Acetato] e [Ch]Cl), no entanto, não foi possível separar a RuBisCO das restantes proteínas, tendo demonstrado estes SABs (LI+polímero e LI+sal) não serem seletivos para a purificação da RuBisCO.

**Keywords**

Ribulose-1,5-biphosphate carboxylase/oxygenase, solid-liquid extraction, aqueous biphasic systems, ionic liquids, extraction, purification

**Abstract**

With the growth of the world population, new sources of proteins are needed. Vegetable biomass is a promising source, as it allows the use of agricultural crop residues with the ability to be a continuous source of biomass without harming existing ecosystems, and the extraction of biomass proteins while still fresh. Currently, with the methods used, a final product is obtained that cannot be applied in the food industry. Furthermore, the methods developed until today are not selective and do not allow simultaneously reaching purities and extraction efficiencies close to 100%.

RuBisCO (Ribulose-1,5-bisphosphate carboxylase/oxygenase) is the most abundant protein (enzyme) on the planet and can be applied in the most diverse areas, from pharmaceuticals to food and feed. Thus, in this master's thesis, a new method of extracting RuBisCO and subsequent separation and purification was developed. For this purpose, aqueous solutions of biocompatible ionic liquids (ILs) (glycine-betaine analogues and cholinium derivatives) were used in the solid-liquid extraction of RuBisCO from the spinach leaf. Through the response surface methodology, the extraction conditions (pH, solid-liquid ratio, and IL concentration) were optimized. Under optimum conditions, a maximum extraction of 2.00 mg/ mL of RuBisCO and a yield of 10.93 mg of RuBisCO/ g of biomass for cholinium acetate ([Ch][Acetate]) and 1.86 mg/ mL and a yield of 10.12 mg RuBisCO / g biomass for cholinium chloride ([Ch]Cl). The optimum points determined were: solid-liquid ratio of 0.184, IL concentration of 2,68 M and a pH of 9.09 for [Ch]Cl and 11.2 for [Ch][Acetate].

After extraction, the separation and purification of RuBisCO were performed through the application of aqueous biphasic systems (ABSs). The extracts from the solid-liquid extraction with [Ch]Cl and [Ch][Acetate] containing IL and RuBisCO were individually mixed with polypropylene glycol 400 $g.mol^{-1}$ (PPG 400) or polyethylene glycol 1000 $g.mol^{-1}$ (PEG 1000) and dipotassium phosphate ($K_2HPO_4$) forming two types of ABSs composed of IL and polymer or salt. After this process, it was possible to obtain a RuBisCO extraction efficiency of 100% for the IL phase in the system composed of PPG400 + [Ch][Acetate] and $K_2HPO_4$ + IL ([Ch][Acetate] and [Ch]Cl), however, it was not possible to separate RuBisCO from the remaining proteins, having demonstrated that these ABSs (IL + polymer and IL + salt) are not selective for the purification of RuBisCO.

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# List of abbreviations

**[$Bu_3NC_4NC_4$]Br -** Tri(n-butyl)[4-butylamino-4-oxobutyl]ammonium bromide

**[$C_2$mim]Cl -** 1-ethyl-3-methylimidazolium chloride

**[$C_4$mim]Cl** - 1-butyl-3-methylimidazolium chloride

**[$C_6$mim]Cl** - 1-hexyl-3-methylimidazolium chloride

**[Ch][Acetate] -** Choline acetate

**[Ch][DHC] -** Choline dihydrogencitrate

**[Ch][DHP] -** Choline dihydrogen phosphate

**[Ch]Br -** Choline bromide

**[Ch]Cl** - Choline chloride

**[Cho][Pro] –** Choline proline

**[$Et_3NC_2OC_2$][Sac] -** Tri(ethyl)[2-ethoxy-2-oxoethyl]ammonium saccharinate

**[$Et_3NC_4NC_4$]Br -** Tri(ethyl)[4-butylamino-4-oxobutyl]ammonium bromide

**[Mim$NC_4NC_4$]Br -** 1-(4-(butylamino)-4-oxobutyl)-1-methylimidazol-1-ium bromide

**[Mpyr$NC_4NC_4$]Br -** 1-(4-butylamino-4-oxobutyl)-1-methylpyrrolidin-1-ium bromide

**[$Pr_3NC_2OC_2$][Sac] -** Tri(n-propyl)[2-ethoxy-2-oxoethyl]ammonium saccharinate

**[Pro][$NO_3$] –** Proline nitrate

**AAGB-IL –** Analogue of glycine-betaine ionic liquid

**ABS –** Aqueous biphasic system

**AGB-IL –** Glycine-betaine ionic liquid

**DTT –** Dithiothreitol

**HPLC –** High performance liquid chromatography

**IL –** Ionic Liquid

**LLE –** Liquid-liquid extraction

**PBS –** Phosphate buffer saline solution

**PEG 1000 –** Polyethylene glycol with a molecular weight of 1000 g/mol

**PEG 400 –** Polyethylene glycol with a molecular weight of 400 g/mol

**PEG 600 –** Polyethylene glycol with a molecular weight of 600 g/mol

**PPG 400 -** Polypropylene glycol with a molecular weight of 400 g/mol

**RSM –** Response surface methodology

**RuBisCO –** Ribulose-1,5-biphosphate carboxylase/oxygenase

**SDS –** Sodium dodecyl sulfate

**SDS-PAGE –** Sodium dodecyl sulfate polyacrylamide gel electrophoresis

**SE-HPLC –** Size-exclusion high performance liquid chromatography

**SLE –** Solid-liquid extraction

**TL –** Tie lines

**TLL –** Tie line length

**Tris –** tris(hydroxymethyl)aminomethane

# List of symbols

***K* –** Partition coefficient (dimensionless)

**$R^2$ –** Correlation coefficient (dimensionless)

**wt% -** Weight percentage (%)

**[RuBisCO] –** Concentration of RuBisCO (mg/mL)

***EE%* -** Extraction efficiency (%)

# 1. Introduction

## 1.1. Scopes and objectives

The global population is increasing and, therefore, the search for new sources of protein is a demand (1,2). Proteins from green biomass are seen as an alternative in countries having the need to import them for feed and food production (1). In fact, it is possible to use green biomass, discarded by industries or biorefineries and from the waste of crops where the consumables are the fruits or roots, as a new protein source for both humans and animals (3,4). The crops' waste is the best source of green biomass, as these residues can be transported directly from the farm to the biorefinery. Furthermore, it can be a continuous source of green biomass since various crops are harvested at different times of the year. However, the efficiency of the methods used in protein recovery and their nutritional value maintenance are very important factors for the industries (5). Nowadays, it is already possible to extract proteins like RuBisCO with relatively high efficiency extraction and purity, using classical methodologies such as alkaline extraction, aqueous ammonia extraction, among others (5). Some authors have tried to develop an extraction method for RuBisCO, but have never achieved simultaneously a high extraction efficiency, a high purity or even a high specificity for RuBisCO (6,7). Nonetheless, its application in the food industry is not allowed due to the low value and concentration of the extraction product, the inefficient processing and the loss of insoluble proteins (3,7). RuBisCO is found at high concentrations in leaves (50 % - 60 % of total protein), which represents a great percentage of the green biomass (6,8–11). RuBisCO can be applied in several areas such as pharmaceutical, food and feed industries. However, its extraction and purification procedures have to be improved (1,3–7,9,12–14), in order to allow its widespread use.

Solid-liquid extraction with ionic liquids (ILs) combined with aqueous biphasic systems (ABS) seems a possible strategy to extract and then purify RuBisCO. The extraction of proteins with ILs maintaining their biological activity is already reported using ILs with high biocompatible and biodegradability (11,14,15).

Taking this into account, this work aims to use aqueous solutions of biocompatible ILs (derived from cholinium and glycine-betaine analogues) to extract RuBisCO from spinach (one of the most used plant for the development of extraction protocols and rich in RuBisCO) (1,2). Later on, the formation of ABS constituted by the same ILs, for RuBisCO purification is addressed. This investigation aspires to the development of a sustainable and, low-cost procedure for the extraction and purification of RuBisCO from spinach, allowing the

maintenance of protein structure and activity. Lastly, the major goal will be the replacement of spinach for any green biomass.

## 1.2. RuBisCO

### 1.2.1. Structure, characterization, and mechanisms of action

Ribulose-1,5-biphosphate carboxylase/oxygenase, RuBisCO (EC 4.1.1.39), is the most abundant protein (enzyme) in nature and it is present in a wide number of photosynthetic organisms such as plants, algae and various species of bacteria (3,8,9,11,12,16).

RuBisCO is present in nature in three different forms: I, II and III, as depicted in Figure 1 (8,17). The most common RuBisCO is the form I and it has 2 subclasses: green-type, present in proteobacteria, cyanobacteria, green algae and plants, and red-type, present in photosynthetic bacteria and nongreen algae. The form II occurs in dinoflagellates and bacteria and form III is exclusive of archaea (8,17).

Structurally, form I of RuBisCO consists of a hexadecameric complex, with about 550 kDa, and constituted by eight large subunits (RbcL, circa 50-55 kDa) and eight small subunits (RbcS, circa 12-18 kDa) (1,3,5,8,13,17). The complex's core is formed by a tetramer of two antiparallel dimers of RbcL, which have all the active sites of the enzyme, and RbcS are located in complex's borders (four RbcS in each top) (1,8,13,17). On the other hand, form II is constituted by one or more dimers of RbcL and form III has a ring shape formed by three to five dimers of RbcL and none of them have RbcS (8,17). Furthermore, form III has other functions besides being involved in photosynthesis, as D-ribulose-1,5-biphosphate (RuBP) regeneration produced in nucleotides metabolism (8).

RuBisCO has an isoelectric point between 4.6 and 5.5, varying with the species, a solubility above 80% at pH lower than 4 and higher than 6, and a denaturation temperature of 66.5 ºC and 67.5 ºC in alfalfa case and 64.9 ºC for spinach RuBisCO (1,2,18).

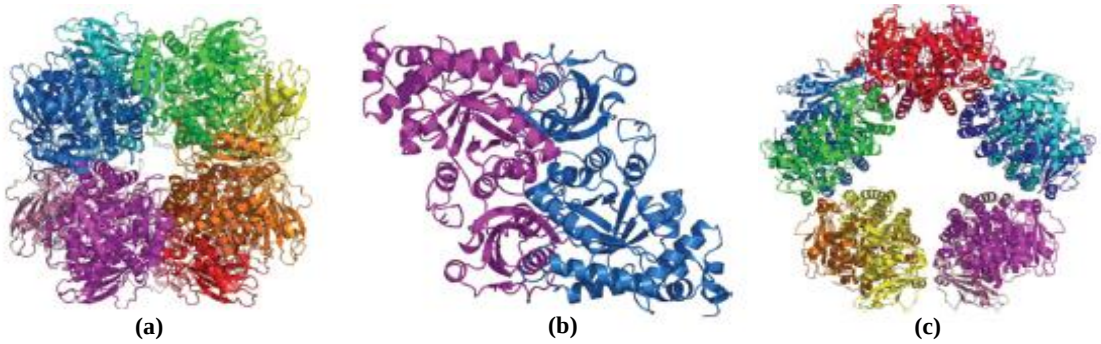


**Figure 1.** Structures of RuBisCO's forms I (a), II (b) and III (c). Adapted from Tabita et al. (17).

RuBisCO is the principal way to fixate atmospheric carbon in photosynthetic organisms (3,8,10,16,19). In this conversion, RuBisCO catalyses a chain of five reactions (Figure 2): the enolization of C3 from RuBP (RuBisCO's substrate); the carboxylation of C2 ($CO_2$ fixation); protonation of the bond established between the $CO_2$ and C2 which lead to C3 hydration and the cleavage of the C2-C3 bond of the unstable intermediate originating 2 molecules of 3-phosphoglyceric acid (3PGA) (8,16,19). 3PGA will enter in Calvin-Benson-Bassham cycle where it will be converted in glyceraldehyde-3-phosphate which is the predecessor of several biomolecules (sugars, fatty acids, and amino acids)(8).

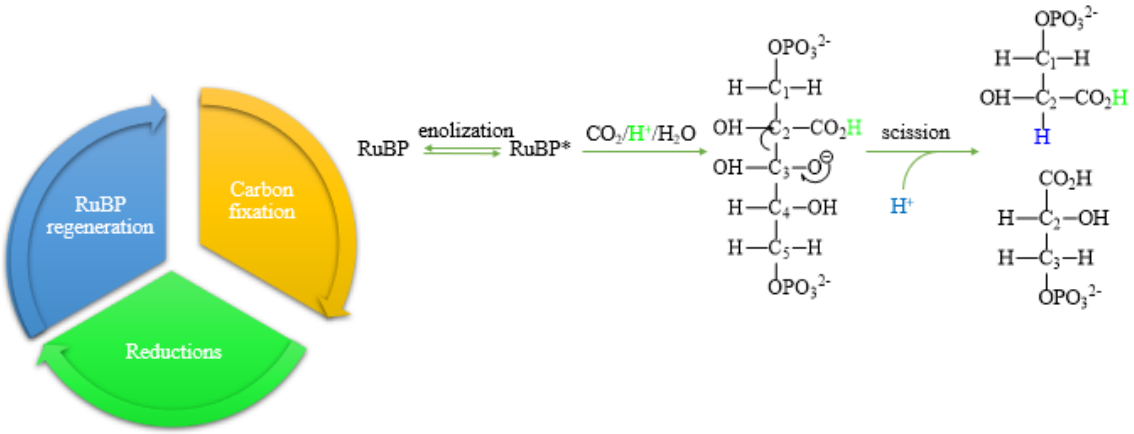


**Figure 2.** Scheme of the reactions chain catalyzed by RuBisCO in the Calvin-Benson-Bassham cycle. Adapted from Cummins et al. (19).

As previously mentioned, the complex composed by RbcLs have all the active sites of the enzyme, and presents a high structural stability and its sequence has circa of 60% of identity, meaning a relation between the complex structure and RuBisCO's performance (8). To RuBisCO be competent, Lys201 (on plants) must be carbamylated and then a $Mg^{2+}$ will attach to this group, in order to RuBP bound in the active site (8,17). When RuBP linked to the active site, the flexible part of RuBisCO structure closes the active site, creating an interior space, allowing the electrophilic attack of RuBP from $CO_2$ or $O_2$ (8,17). Although RbcSs are in the extremities of Form I complex, they are needed for the enzyme to be functional and their sequence is more diversified and has more than 30% of identity. It was observed that both the subunits are affected by sulphur deficiency and cadmium stress (high concentration), although RbcL is more susceptible to cadmium presence and RbcS to sulphur deficiency since, if the plant has sufficient quantity of sulphur, the amount of RbcS increased in cadmium presence (20).

Organisms like plants and green algae have different families of RuBisCO's isoforms that have different expression rates affecting their catalytic activity and, it was though that RbcS emerge as an evolutionary adaptation to the increase of $O_2$ in the atmosphere (8). However, RuBisCO has three major questions affecting its role as $CO_2$ acceptor: efficiency related to the capability to accept $O_2$, its reactions are error-prone and inhibitions (8–10,19). Regarding to the efficiency problem, RuBisCO only fixates two-five molecules of $CO_2$ per second in plants (it has a substrate-saturated $K_{cat}$ in the range 1-12$s^{-1}$ and a $K_{cat}/ K_C$ ratio in the range 2-40 x $10^4$ $M^{-1}s^{-1}$ which is substantially lower than the ratios registered to other enzymes ($10^8$-$10^9$ $M^{-1}s^{-1}$)) (8,9). This disadvantage obligates the plant to produce large amounts of RuBisCO, representing in some cases, 50% of the total soluble protein in leaf tissues (6,8–11). However, these amounts of enzyme or the catalytic activity can be lower if the plant is in an environment with nitrogen deficiency or presence of cadmium (20,21). Furthermore, RuBisCO can accept $O_2$, but the intermediate scission will produce only one molecule of 3PGA instead of two, and other of 2-phosphoglycolate (2PG) (8,9,19). This last one is toxic for chloroplasts and it can be converted in 3PGA by photorespiration (8). To avoid an unnecessary energy loss, $CO_2$ emission and disturbances in carbon and nitrogen cycles, some organisms developed strategies to concentrate $CO_2$ around RuBisCO like the existence of compartments with machinery for $CO_2$ concentration (for example carboxysomes in cyanobacteria and pyrenoids in green algae) (8,9). Nevertheless, $CO_2$ concentration affects

the RuBisCO's affinity by RuBP: in spinach, when the RuBP concentration is approximate to zero $K_M$ is 1.5±0.5 μM and when concentration tends to infinite $K_M$ is 0.8±0.2 μM (22). The second problem is related to the fact that reactions catalysed by RuBisCO are error-prone, as mention above. When errors occur, the intermediate is D-xylulose-1,5-biphosphate (XuBP) and the reactions products are D-glycero-2,3-pentodiulose-1,5-biphosphate (PDBP) and 2-carboxytetritol-1,4-biphosphate (CTBP). The formation of these sugar phosphates leads to enzyme inhibition since they had a strong affinity with RuBisCO's active site (8,10). In addition to the wrong protonation of the intermediate and the formation of sugar phosphates, RuBisCO can be inhibited for several reasons: with the bound of a RuBP uncarbamylated to the active site; the denominated autoinhibition which is the binding of RuBP to an uncarbamylated RuBisCO when the enzyme losses the ion $Mg^{2+}$ from its active site in conjunction with the carbamylated group; and the production of 2'-carboxy-D-arabinitol-1-phosphate (CA1P) in plants in dark environment (8). In all these cases, the inhibition can be reverted by RuBisCO activase (Rca), which mediates RuBisCO's activity, with the exception in inhibition by sugar phosphates, were specific phosphatases must intervene for inhibitors degradation, which will later be converted in RuBP on Calvin-Benson-Bassham cycle (8,10,23).

### 1.2.2. RuBisCO's applications

RuBisCO can be applied in several fields, like food and feed industries, chemical industry, pharmaceutical, cosmetics, among others (1–7,9,12–14).

In animal feed, RuBisCO is already employed as a protein source through a white protein concentrate. The hydrolysis of this concentrate results in peptides with high solubility and nutritional value that can be incorporated in feed (12). However, in the food industry RuBisCO is not used yet, due to the extraction procedure applied, which leads to a lower quality than required because of polyphenols presence (12), and economic disadvantages (6). Nevertheless, many studies have been carried out for future applications, such as RuBisCO gels, products of thermal denaturation, which can be used in food formulation (13); undenatured RuBisCO to be applied in foaming, emulsifying and to form gels (13); peptides resulted from RuBisCO hydrolysis, which can be used in functional foods and nutraceuticals (3) and as additives (12), or simply as protein source (1). In fact, Martin et al. (1) investigated properties such as solubility, foam formation and stability, emulsion

formation and stability, water holding capacity, gelation and fracture properties of gels, and conclude that RuBisCO has the same or better results than soy and whey protein isolates.

For pharmaceutical and cosmetic industries, peptides resulted from the RuBisCO hydrolysis are the most desired due to their multiple properties, such as immunostimulant, antioxidant, opioid glucose uptake stimulant (5,12). Peptides like RuBisCO hydrolysate (with a degree of hydrolysis of 18.8%) can inhibit linoleic acid oxidation, ferric ion reduction, stabilization of 2.2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid free radicals ($ABTS^{*+}$) (3).

Recently, artificial photosynthesis was developed and is about 12% more efficient and simpler compared to natural photosynthesis. Based on this information, Zhu et al. (24) immobilized RuBisCO in polydopamine-modified microfluidic reactors to produce glucose precursor. Their results are very promising because the immobilization improved the thermal and storage stabilities and at the end of 5 cycles. Moreover, RuBisCO had 90.4% of its initial activity and after 10 cycles, 78.5% of the initial activity was preserved. Furthermore, 3PGA production was continuous, if the injection of $CO_2$ and RuBP were constant and, in this way, the feedback inhibition was avoided, and the efficiency of the enzyme maximized (24).

## 1.3. RuBisCO extraction and purification methods

### 1.3.1. Traditional methods

The leaves used to extract and purify RuBisCO can be provided by biorefineries that discard higher amounts of green biomass or from crop residues (3,4). There are several methods of extraction such as percolation, maceration or Soxhlet extraction but commonly, RuBisCO and other intracellular leaf proteins are extracted through the same general procedure which involves four major steps (Figure 3): i) mechanical disintegration of the tissue, ii) protein solubilization, iii) protein precipitation and iv) protein concentration (3,4,10,25). Usually, the mechanical disintegration occur at pH 10-11 to promote, at the same time, the protein solubilization, while protein precipitation uses for pH treatment an acidic pH value (*e.g.* pH 3) at 4ºC (3,10).

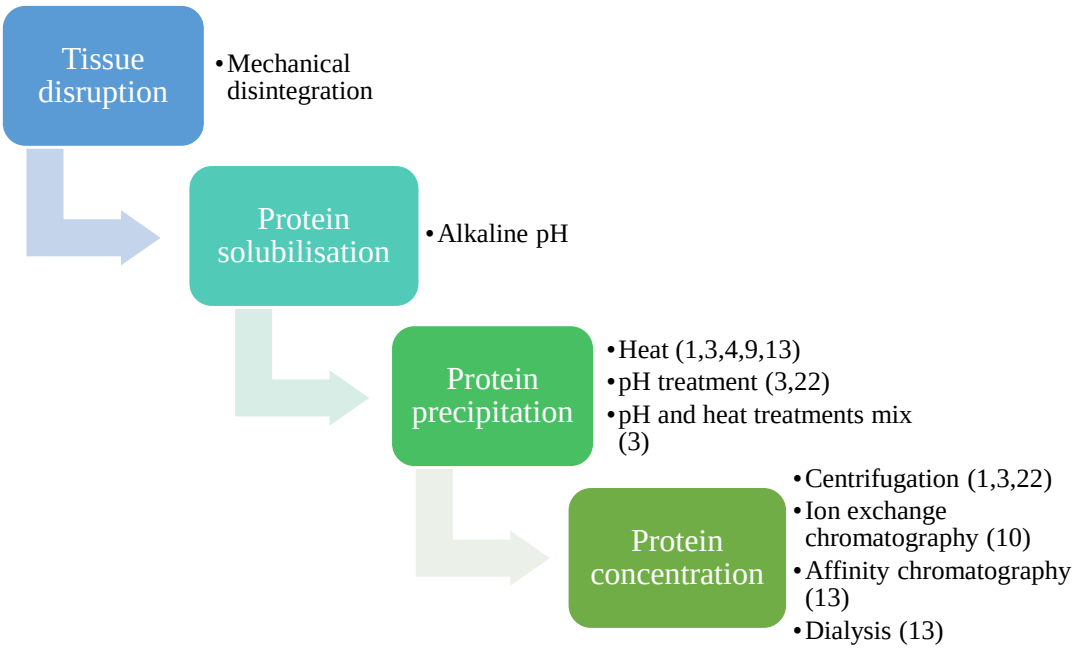


**Figure 3.** General scheme of the currently processes used in RuBisCO extraction.

From mechanical disintegration is obtained a green juice with soluble proteins, fibers, sugars, polyphenols and chlorophylls (6,12). Then, chlorophylls and fibers are removed by thermal precipitation, however fibers can retain water and, consequently, retain proteins with it (1,6,26). In order to recover the retained proteins in the precipitate, Tenorio et al. (6) tried to re-dissolve the proteins trapped in the precipitate with surfactants but failed in the improvement of the extraction efficiency of their procedure (mechanical disintegration followed by heating to 50ºC and finishing with centrifugation). They obtained a solution with 41.1 wt% in protein content, and after a purification step, recovered 6% of RuBisCO with 90% purity. During thermal precipitation, depending on temperature, can also occur protein denaturation. Due to ionic bonds cleavage, the release of divalent anions and the capture of divalent cations, protein gelation resulted from the aggregation of denatured proteins when the concentration of proteins is higher than the percolation limit (13). Since polyphenols at alkaline pH are highly reactive with hydroxyl and aromatic groups from proteins (creating cross-linked between proteins and oxidized polyphenols (4)) and sugars, causing a brown coloration to the solution resulted from their oxidation, they have to be removed after mechanical disintegration, to avoid a decrease on the final product quality (12). For example, France – Luzerne (12) produced a white protein concentrate allowed to be used in feed but not in food industry because of phenolic compounds presence. To remove

them, without changing the nutritional value and the functional properties of the final product, ultrafiltration was introduced in the protocol but with no significant decrease in the polyphenol's concentration and the color intensity. To improve these results, ultrafiltration was conjugated with sorption techniques and the best result was with a polystyrene resin where the polyphenols decreased 94% (12).

This general procedure with 4 major steps is the most relevant due to its high extraction efficiencies, but there are other protocols, like alkaline extraction (5). As an alkaline extraction example, Zhang et al. (7) used a conjugation of heat and pH treatment to extract all the proteins in leaves and they were able to extract 95% of total protein content in 4 h at 95ºC with a v/w of 40 mL/g NaOH 0.1M (pH 3.5), where 85% of the protein has 52% purity. Nevertheless, this procedure does not have the efficiency needed to be executed on a large scale.

Kobbi et al. (3) also studied the feasibility and efficiency of ammonium hydroxide and protein precipitation at pH 3 without prejudice the biological activity. They were able to remove 80% of polyphenols and extract RuBisCO with around 90% of purity. Furthermore, comparing their results with commercial products, there are no significant differences between them.

The application of methods with extreme conditions such as alkaline pH, the use of organic solvents and high temperatures improve the extractions of these biomolecules, although it leads to protein activity loss (6). Therefore, other techniques have been applied for the RuBisCO extraction and purification like crystallization, aqueous biphasic systems (ABS), pulsed electric fields, extraction with ionic liquids, ultra-sonification, microwaves, among others (4), to improve the extraction yields.

### 1.3.2. Solid-liquid extraction

#### *1.3.2.1. General concepts*

Solid-liquid extractions (SLE), one of the oldest unit operations in the chemical industry, is another possible approach to extract RuBisCO from biomass. This type of extraction allows soluble components to be removed from solids using a solvent, where operational conditions such as temperature, extraction time, and solid−liquid ratio are optimized, with subsequent separation of the phases by decantation, filtration or centrifugation (Figure 4).

The selection of the solvent is carried out taking into consideration its selectivity, capability for dissolving the solute, density, viscosity, surface tension, toxicity, boiling temperature, chemical and thermal stabilities and cost (27). Obviously, due to the toxicity of some organic solvents, there are some restrictions to their use in the food, cosmetic and pharmaceutical industries (27). Furthermore, the organic compounds commonly employed display major drawbacks in what concerns safety and environmental issues.

In summary, SLEs involves the extraction and dissolution of a given compound from a solid matrix in a given solvent and without the identification of selective solvents, extractions from biomass usually result in a complex extract (28). An example is the extraction used to extract the leaf proteins (including RuBisCO), described in Section 1.3, were the SLE is the combination of the two first steps: the mechanical disintegration and protein solubilization. Therefore, after the extraction step, induced precipitation, distillation, and chromatography, among others, are used as separation/purification techniques. Most of these techniques generally involve the use of volatile and often toxic organic solvents, additionally leading to environmental and human concerns (28). Furthermore, the costs associated with the final product are strongly dependent on the downstream processing, namely the type of techniques used and the number of steps required (28). Thus, it is of crucial relevance to explore alternative solvents environmentally friendly and develop cost-effective and sustainable extraction and purification techniques, which could be ideally incorporated in an integrated process. A possible alternative includes the separation and purification of the target compounds from complex extracts using liquid−liquid extractions (LLE).

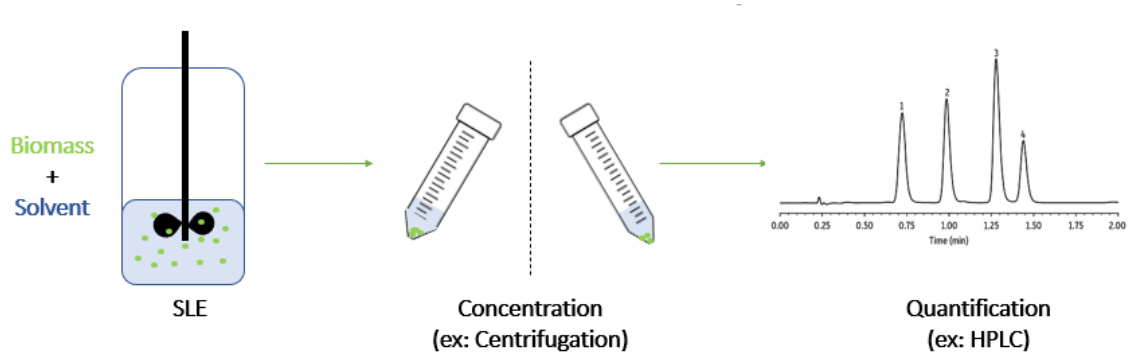


**Figure 4.** General scheme of solid-liquid extraction applied to solid biomass samples.

### *1.3.3.2. Solid-liquid extraction using Ionic Liquids*

ILs are ionic compounds with fusion temperatures lower than 100ºC, because of the lack of an ordered crystalline structure (11,14,29). Usually, they are constituted by a large organic cation and an organic or inorganic anion (Figure 6). Due to these characteristics, they have a negligible vapor pressure, a high chemical and thermostability, ionic conductivity non-inflammable and are liquid in a wide range of temperatures (11,29). These characteristics make ILs a good alternative to organic solvents. The first IL was developed in 1914 and one of the firsts applications was its use as a solvent to store and preserve cellulose and then its application in aluminium electrodeposition (29,30). In 1992, ILs stable at air and water presence were developed with other cations, as imidazolium (29), to prevent denaturation. Divya et al. (31) proved that imidazolium-based ILs prevent protein aggregation and unfolding with an increase in thermal stability. However, imidazolium present some toxicity and is why other authors like, Sahoo et al. (32) studied cholinium-based ILs for the same purpose of Divya et al. (31) and concluded that solutions with a low concentration of choline proline ([Ch][Pro]) and proline nitrate ([Pro][$NO_3$]) preserve the cytochrome-c tertiary structure.

However, the major benefit in use ILs comes from the possibility to change one or the two ions of the IL, tuning its hydrophobicity, polarity, and viscosity (11,29).

ILs have been used in the SLE of several biomolecules with interesting results that support its application instead of organic solvents. As examples, Martins et al. (15) extracted phycobiliproteins with ionic liquid aqueous solutions. They improved extraction by 46,5 % with a purity similar to other procedures (like solid-liquid extraction, enzymatic process, etc) without compromising the secondary structure of the protein; Cláudio *et al.* (33) extracted caffein with a yield up to 9 wt% per guaraná dry weight at 70 ºC for 30 min with the confirmation that the IL solutions are recyclable and reusable and, Martins *et al.* (34) developed a cost-effective and efficient process for chlorophylls that allowed the maintenance of the stability of the final product for more than a month.

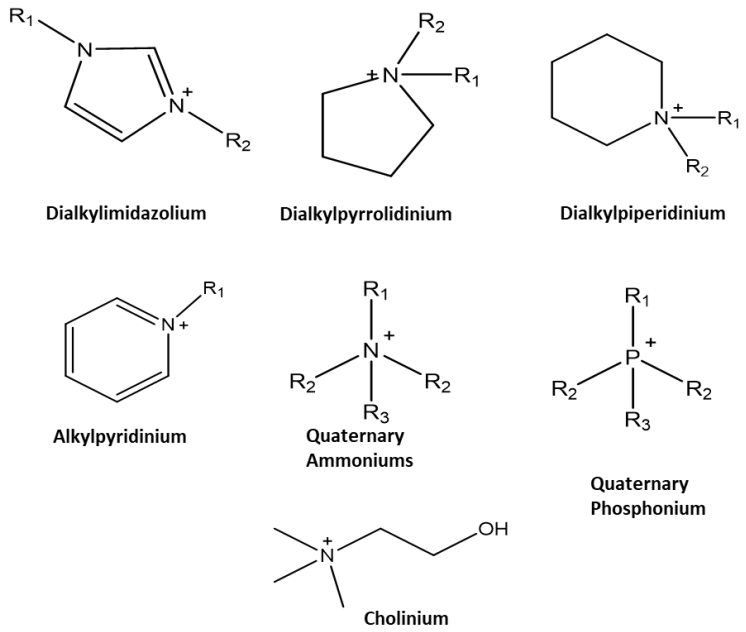


**Figure 5.** Examples of cation structures of the most studied IL families. Adapted from Ferreira et al. (29)

### 1.3.3. Aqueous Biphasic Systems (ABS)

#### *1.3.3.1. General concepts*

LLE is usually performed using organic solvents immiscible with water (35). Compared to chromatography, liquid-liquid systems offer technological simplicity and low cost, as well as the capability to provide high yields, improved purification factors, enhanced selectivity and the possibility of combining the recovery and purification steps (35). Aiming at avoiding the use of organic solvents in LLE, in 1958, Albertsson introduced the aqueous biphasic systems (ABS) for the separation of (bio)molecules by their partitioning between two liquid aqueous phases (36). ABS are good alternatives for the purification of several (bio)molecules because of their simplicity, low cost, the possibility to use components non-inflammable, non-volatile and with low toxicity. Moreover, they can be improved to separate multiple products and are easy to scale-up (14,29,37–39). This type of liquid-liquid extraction method can combine the separation and purification in just one step (29,39).

ABS are composed by two incompatible solutes, above certain conditions (temperature, concentration, etc), like two polymers or salts, or one polymer and one salt. ABS's phases have a high content in water, creating biocompatible systems (11,14,29,37–39).

The prediction of an ABS formation (Figure 5) is based on their binodal curve, which separates the monophasic (below the curve) and the biphasic regions (above the curve). In the biphasic region, the mixture of the two compounds forms an ABS, and the larger the area of the biphasic region the greater the capability to form an ABS (29). Empirically, binodal curves can be determined by several methods (40), nevertheless, cloud point titration and turbidimetric method are simpler since the systems' compositions are determined by weight quantification (41,42). For the cloud point titration method, the salting-out agent solution is added dropwise to the solution of the second constituent until obtaining a cloudy solution and, at this point, the salting-out agent solution is replaced by water until the cloudy solution becomes clear and this procedure is repeated until the solution losses the capability to become cloudy or clear (41,43). In the turbidimetric method case, several mixture points are made in the biphasic region, and water is added dropwise until the solution becomes clear (42).

Additionally, to characterize the ABS, the tie-lines (TLs) can be determined, which provide information about the composition of each phase. To this end, a mixture point in the biphasic region is selected (with a precise quantity of each compound) and, after the formation of the biphasic system, both phases are separated and weighted and with their weight percentage, and using an appropriate software, a line is drawn, intersecting the mixture point and the binodal curve in two points (TL) (44). The two intersections points between the binodal curve and the TL gives the composition of each phase and the phases of every mixture points that coincide with that TL have the same composition but different phase volumes, as represented in Figure 5 (29,44). The length of the TL (TLL) is used as an indicator of the differences between the compositions of the phases and enables to predict the product partition. It is possible to have a TLL of zero and in this case, the mixture point is known as critic point and the bottom and top phases have the same compositions (29).

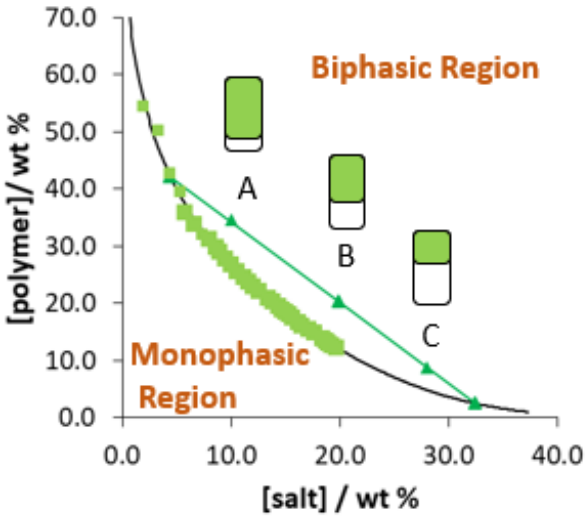


**Figure 6.** Example of a binodal curve (black line) that fits experimental data (green squares) and a TL with 3 mixtures points (A, B and C).

The extraction of solutes in ABS can be evaluated by the partition coefficient (*K*) and extraction efficiency (EE%) of the solute, which quantifies the solute distribution between both phases of the ABS (39) and between each phase and the total mixture (14,38,45), respectively. *K* is the ratio between the solute concentration in both phases and, in a case where *K* is determined in function to top phase, if *K* is lower than 1, the product of interest is major present in the bottom phase, and if *K* is higher than 1, most of the product of interest is in the top phase. A *K* value close to 1, means that the product of interest has partitioned similarly between the two phases (39). The partition of the product of interest is highly dependent on the affinity between the product and the phase component and physicochemical parameters such as pH, temperature, and TLL (11).

Nowadays, it's possible to recover products using conventional ABS (polymer-based ABS), with yields between 65 and 100 %, from different sources, like biologic suspensions, fermentation broths, etc (29,39,46). However, the conventional ABS presents some disadvantages, like viscosity, opacity and a limited range of polarity (37). So as alternatives to polymers and salts, ionic liquids (ILs) have emerged, and combinations between ILs and polymers/amino acids/ carbon hydrates have been used today (29,39).

#### *1.3.3.2. Ionic liquid-based ABS (IL-based ABS)*

Nowadays, ILs are used as solvents in chemical reactions, synthesis, chromatography and liquid-liquid extractions of biomolecules from water solutions through the ABS formation and the application of hydrophobic or hydrophilic ILs (29). As referred previously, ABS can be formed by the conjugation of ILs with other compounds (polymers, salts, etc.) (11,29). These ABS had advantages compared to the conventional ones, since ILs can be designed according to the final purpose of the work. Moreover, the lower viscosity of IL-based ABS leads to a quicker phase separation and higher extractions efficiencies (29).

Several biomolecules, including amino acids (47–49), proteins (50,51), and antibiotics like tetracycline and ciprofloxacin (52) have been extracted with IL-based ABS with extraction efficiencies close to 100%.

RuBisCO extraction with IL-based ABS was already studied. Ruiz et al. (14) compared a polymer-salt ABS (PEG 400 - Potassium citrate), a polymer-IL (PEG 400 - Cholinium dihydrogen phosphate) and an IL-salt ABS (Iolilyte 221PG - Potassium citrate). PEG was chosen because of its capacity to stabilize proteins, Iolilyte 221PG because of its higher capability to form ABS and cholinium-based ILs because they had buffer properties, low toxicity, are biodegradable, low cost and maintain the protein structure and biological activity (11,14). Ruiz et al. (14) concluded that ABS with Iolilyte 221PG had a better extraction efficiency, 98.8% by a single extraction, in comparison to the 96.6 % for PEG 400 – Potassium citrate and the 79.6 % for PEG 400 - Cholinium dihydrogen phosphate. However, the ABS with PEG 400 preserve the protein integrity so, with no surprise, they observed that RuBisCO migrate to PEG-rich phase. Contrarily, in the ABS with Iolilyte 221PG occur formation of aggregates and they noticed that the increase of IL concentration affects protein stability.

Desai et al. (11) also report the RuBisCO migration to Iolilyte 221PG-rich phase and Iolilyte 221PG-sodium potassium buffer with a $K$ 3-4 times higher than the $K$ registered to PEG-potassium citrate. However, with the increase of IL concentration, RuBisCO began to aggregate and fragment.

Due to these results and their natural origin glycine-betaine ILs (AGB-ILs) are an alternative for the extraction and purification of RuBisCO. Betaine is a methyl derivative of glycine and is present in biologic fluids, plants, microorganisms, among others when the cells are exposed to a stress environment (53). Pereira et al. extracted 5 amino acids with

ABSs composed by salt and analogues of glycine-betaine ILs (AAGB-IL) and extracted 65% to 100% in just one step. AAGB-ILs with hydrophobic properties were already used in pesticides and metallic ions extraction (53,54). Furthermore, Capela et al. (55) were able to extract monoclonal antibodies with ABSs composed by AAGB-ILs with recovery yields up to 100% and a purification factor up to 1.6 preserving, at the same time, the biological activity of the biomolecule.

## 1.4. Main goal of the thesis

Because of the high costs, time or complexity of various processes, integrated processes began to be developed and consists of the combination of several unit processes at the same equipment (56). One of the purposes of this thesis is the development of an integrated process where in the first step occur the RuBisCO's SLE and in the second and last step, through the addition of a second compound, is formed an ABS for the separation and purification of the enzyme. The application of aqueous solutions of ILs in the SLE allows a more selective extraction of RuBisCO thanks to some of the characteristics of ILs (such as the capability to change one of the ions) and allying to the high extraction efficiencies of IL-based ABSs it is possible the development of a more selective extraction procedure for RuBisCO. For this thesis the chosen ILs are cholinium- and AGB-based, increasing the interest of this alternative because beyond the selectivity, this procedure will be biodegradable with low toxicity and cost.

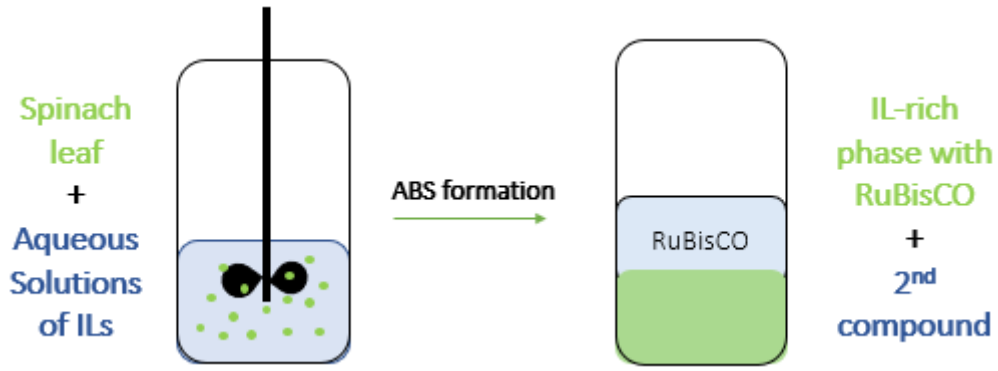


**Figure 7.** Scheme of the extraction procedure proposed in this thesis.

# 2. Experimental Section

## 2.1. Materials

RuBisCO was extracted from spinach (ready to cook) acquired in a local supermarket, at Aveiro. RuBisCO standard (D-Ribulose 1,5-diphosphate carboxylase from spinach) and phosphate buffered saline (PBS) were purchased from Sigma-Aldrich. The solvent applied in the conventional extraction method of RuBisCO used was ammonium hydroxide ($NH_4OH$, 25% of purity, Fluka). In the SLE were used the following ILs (Figure 8): choline chloride ([Ch]Cl, 98 % of purity, Acros Organics), choline bromide ([Ch]Br, > 98 % of purity, TCI), choline acetate ([Ch][Acetate], > 99 % of purity, Iolitec), choline dihydrogencitrate ([Ch][DHC], 99 % of purity, Sigma-Aldrich) and choline dihydrogen phosphate ([Ch][DHP], > 98 % of purity, Iolitec); 1-ethyl-3-methylimidazolium chloride ($[C_2mim]Cl$, 98 % of purity, Iolitec), 1-butyl-3-methylimidazolium chloride ($[C_4mim]Cl$, 99 % of purity, Iolitec), 1-hexyl-3-methylimidazolium chloride ($[C_6mim]Cl$, 98 % of purity, Iolitec); and the AGB-ILs, tri(n-propyl)[2-ethoxy-2-oxoethyl]ammonium saccharinate ($[Pr_3NC_2OC_2][Sac]$, > 98 % of purity), tri(ethyl)[2-ethoxy-2-oxoethyl]ammonium saccharinate ($[Et_3NC_2OC_2][Sac]$, > 98 % of purity), tri(ethyl)[4-butylamino-4-oxobutyl]ammonium bromide ($[Et_3NC_4NC_4]Br$, > 98 % of purity), 1-(4-butylamino-4-oxobutyl)-1-methylpyrrolidin-1-ium bromide ($[MpyrNC_4NC_4]Br$, > 98 % of purity), 1-(4-(butylamino)-4-oxobutyl)-1-methylimidazol-1-ium bromide ($[MimNC_4NC_4]Br$, > 98 % of purity), tri(n-butyl)[4-butylamino-4-oxobutyl]ammonium bromide ($[Bu_3NC_4NC_4]Br$, > 98 % of purity). AGB-ILs were synthetized and kindly supplied by Professor Aminou Mohamadou from Institut de Chimie Moléculaire, Univesité de Reims Champagne-Ardenne.

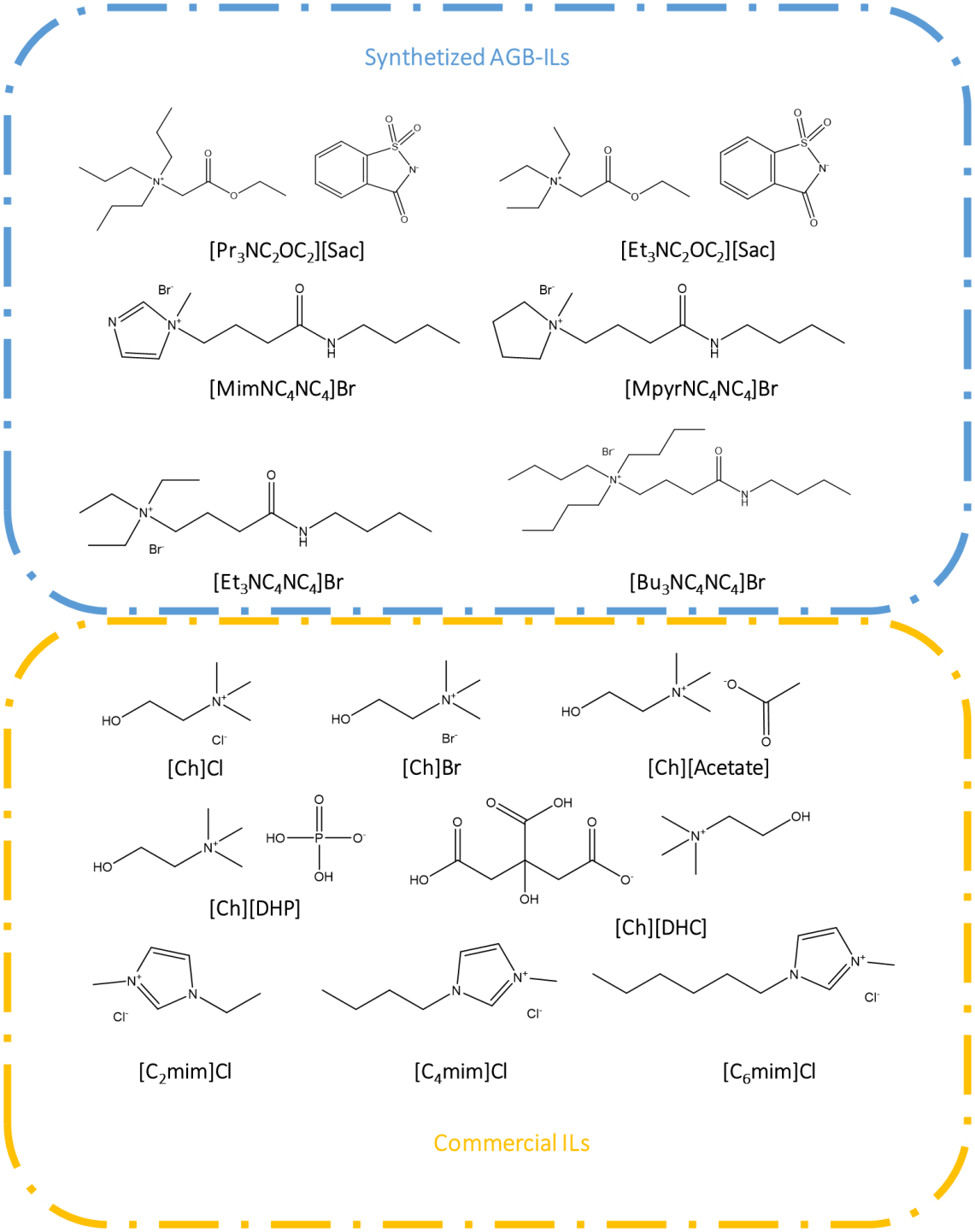


**Figure 8.** Structures and abbreviations of the ILs applied in this work.

To perform sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) were used: tris(hydroxymethyl)aminomethane (Tris, PA, Pronalab), sodium dodecyl sulfate (SDS, 99 % of purity, Acros Organics), glycerol (99 % of purity, Acros Organics), bromophenol blue (pure, Merck), dithiothreitol (DTT, 99 % of purity, Acros Organics),

RunBlue 20x SDS run buffer TEO – Tricine – SDS (Expedeon), RunBlue SDS Gel 4-12%, 12 wells, GRS Protein Marker MultiColour (grisp Research Solutions), and BlueSafe (nzytech).

For size-exclusion high-performance liquid chromatography (SE-HPLC) quantification it was used sodium phosphate dibasic heptahydrate ($Na_2HPO_4 \bullet 7H_2O$, 98.0-102.0 % of purity, Sigma-Aldrich), sodium dihydrogenphosphate ($NaH_2PO_4$, 99 % of purity, Sigma-Aldrich) and sodium chloride (NaCl, 99.5 % of purity, Panreac).

To form the ABSs it was used polypropylene glycol 400 $g.mol^{-1}$ (PPG 400), polyethylene glycol 1000 $g.mol^{-1}$ (PEG 1000), both from Sigma Aldrich, and di-potassium hydrogen phosphate trihydrate ($K_2HPO_4 \bullet 3H_2O$, 98% of purity, Scharlau).

## 2.2. RuBisCOs' solid-liquid extraction

Fresh spinach leaves were stored at -80 ºC and exactly before the extraction were ground with liquid nitrogen in a mortar. The RuBisCOs' extractions were carried out in a Carrousel from *Radleys Tech* able to both stir and maintain the temperature within ± 0.5 ºC. The experimental conditions were adapted from Leite *et al.* (57). All the experiments were performed at 600 rpm, (29.0 ± 0.5) ºC for 30 min with a 0.1 solid-liquid ratio. The aqueous solutions containing known amounts of IL and biomass were prepared gravimetrically within $\pm 10^{-4}$ g. The impact of different concentrations of IL and pH in the extraction of RuBisCO were studied. For each set of conditions were prepared three replicas and the extractions evaluated by SDS-PAGE and HPLC quantification. After the extraction, the solutions were centrifuged (at 7000 rpm for 30 min in a *Neya 16R* centrifuge) to separate the extracts from biomass. The pH of the extracts was measured at (25.0 ± 0.1) ºC with a *Metrohm 827 pH lab* equipment.

## 2.3. Response surface methodology (RSM)

In order to analyse several operational conditions and identify the most significant parameters in RuBisCOs' extraction, a response surface methodology was applied. In a $2^k$ RSM, k are the factors that provide a different response and through the adjustment of the data to a second order polynomial equation (Equation 1):

$$y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum_{i<j} \beta_{ij} X_i X_j \quad (1)$$

where y is the response variable, $\beta_0$, $\beta_i$, $\beta_{ij}$ and $\beta_{ij}$ are the adjusted coefficients for the intercept, linear, quadratic and interaction terms, respectively, $X_i$ and $X_j$ are independent variables. The analysis of the surface response curves resulted from this method, leads to the determination of the optimal conditions.

After the initial screening using different ILs, [Ch]Cl and [Ch][Acetate] showed to be the best option as solvents to optimize extraction of RuBisCO from spinach leaves. Operational conditions, namely, pH, solid-liquid ratio and IL concentration were optimized by a $2^3$ factorial planning to simultaneously analyse various operational conditions and to identify the most significant parameters that enhance the extraction yield and the extracted concentration of RuBisCO. The reaming extraction conditions used were the same as the SLE extraction protocol described in section 2.2.

The $2^3$ factorial planning is defined by the central point (level zero), the factorial points (1 and -1, level one) and the axial points (level α), see Table B 1 in Appendix B. The independent variables coded levels used in the factorial planning are presented in Table 1. The axial points are encoded at a distance α from the central point (58):

$$\alpha = (2^k)^{1/4} \quad (2)$$

**Table 1.** Independent variables' coded levels applied in the factorial planning.

| Studied parameters | Level | | | | |
|---|---|---|---|---|---|
| | **Axial** | **Factorial** | **Central** | **Factorial** | **Axial** |
| | **-1.68** | **-1** | **0** | **1** | **1.68** |
| **pH** | 2.8 | 4.5 | 7.0 | 9.5 | 11.20 |
| **Solid-liquid ratio** | 0.02 | 0.050 | 0.100 | 0.150 | 0.184 |
| **Concentration (M)** | 0.323 | 0.800 | 1.500 | 2.200 | 2.676 |

The obtained result was statistically analysed with a 95 % confidence level and a student t-test was applied to verify the statistical significance of the adjusted data (Appendix B, Table B 4.1 – Table B 5.2). The regression coefficient ($R^2$), the lack of fit and the F-value obtained from the analysis of variance (ANOVA) were evaluated to determine the adequacy

of the model. The Statsoft Statistica 10.0© software was used in all statistical analyses and to drawing the response surfaces. Contour plots of the yield of extraction and of the extracted concentration of RuBisCO were generated from adjusted models, and through their analysis, the optimal conditions can be determined.

## 2.4. SDS-PAGE analysis

The proteins profile of the obtained extracts was determined by SDS-PAGE. The samples were diluted at a 1:1 (v/v) ratio in a sample buffer composed by 2.5 mL of 0.5 M Tris-HCl pH 6.8, 4.0 mL of 10 % (w/v) SDS solution, 2.0 mg of bromophenol blue, 2.0 mL of glycerol and 310 mg of DTT. After this dilution, the samples were heated for 5 min at 95 ºC, to break up the quaternary structure and deconstruct part of the tertiary structure by reducing the disulfide bonds and denaturing the proteins. All samples were loaded and run on a polyacrylamide gel (stacking: 4 % and resolving: 20 %). To stain the proteins, the gels were impregnated with BlueSafe and stirred in an Heidolph™ rotamax 120 orbital shaker at 50 rpm for 40 minutes at room temperature. GRS Protein Marker MultiColour (grisp Research Solutions) was used as molecular weight standards while commercial RuBisCO from spinach (Sigma-Aldrich) was used as RuBisCO standard.

## 2.5. SE-HPLC analysis

Protein quantification was performed by size-exclusion high-performance liquid chromatography (SE-HPLC). A calibration curve was determined for this purpose (Appendix A, Figure A 2) using commercial RuBisCO from spinach (Sigma-Aldrich).

A phosphate buffer solution (1000 mL), used as mobile phase, was prepared using 47 mL of a Solution A (27.8 g of $NaH_2PO_4$), 203 mL of a Solution B (53.65 g $Na_2HPO_4 \bullet 7H_2O$) and 17.5 g of NaCl. Each sample was diluted at a 1:9 (v/v) ratio in the phosphate buffer and then injected on a *Chromaster HPLC system* (*VWR Hitachi*). The SE-HPLC was performed on an analytical column *Shodex Protein KW-802.5* (8 mm x 300 mm). The mobile phase, a 50 mM phosphate buffer + NaCl 0.3 M, ran isocratically with a flow rate of 0.5 mL/min and the injection volume was 25 µL. The column oven and autosampler temperatures were kept at 40 ºC and at 10 ºC, respectively. The wavelength was set at 280 nm using a DAD detector. The obtained chromatograms were treated and analysed using the PeakFit version 4 software.

The purity and yield of RuBisCO were calculated based on Equations 3 and 4, respectively. The purity (%Purity, Equation 3) was determined by the ratio between the peak area of RuBisCO ($A_{RuBisCO}$) and the area of all peaks of the chromatogram, corresponding to other proteins present in the samples ($A_{Total}$). The yield (Equation 4) was calculated by the ratio between the concentration of RuBisCO in the extract ($C_{extract}$) and the biomass mass ($m_{biomass}$).

$$\%Purity = \frac{A_{RuBisCO}}{A_{Total}} \times 100 \quad (3)$$

$$Yield = \frac{C_{extract}}{m_{biomass}} \quad (4)$$

## 2.6. RuBisCOs' purification using ABS

The extract containing RuBisCO, other biomolecules and IL was used to form the ABS. Additionally, from the phase diagrams determined by Pereira et al. (59), ternary mixtures composed of IL + PPG 400 (15 wt% of [Ch][Acetate] + 19 wt% of water + 67 wt% of polymer and 12 wt% of [Ch]Cl + 21 wt% of water + 67 wt% of polymer) and IL + PEG 1000 (15 wt% of [Ch][Acetate] + 19 wt% of water + 67 wt% of polymer and 12 wt% of [Ch]Cl + 21 wt% of water + 67 wt% of polymer), which lead to the formation of two-phase systems, were chosen to carry out the separation and purification of RuBisCO. Other ABSs composed by IL+salt were studied. The ternary mixtures were selected from the phase diagrams of [Ch][Acetate] + $K_2HPO_4$ (27 wt% of IL + 44 wt% of water + 29 wt% of salt) and [Ch]Cl + $K_2HPO_4$ (24 wt% of IL + 48 wt% of water + 29 wt% of salt) determined by Belchior *et al.* (60) and Osloob *et al.* (61) respectively. Each mixture was stirred, centrifuged for 15 min at 4000 rpm to reach the biomolecules equilibrium and partition between the coexisting phases. After a careful separation of the phases, RuBisCO present in each phase was quantified by SE-HPLC and the protein profile was analysed by SDS-PAGE. At least three independent ABS were prepared, and three samples of each phase were quantified. Control or "blank" solutions at the same mixture point used for the extraction studies (with no biomolecules) were used in all systems

The partition coefficient (*K*), the yield of extraction and the extraction efficiency (*EE%*) of each system to RuBisCO were defined by Equations 5 to 7. The equations are written for the ABS with PPG 400, where most of the enzyme has partitioned for the bottom phase. For the ABS with $K_2HPO_4$, Equation 5 is inverted and in Equations 6 and 7, the numerator is related to the top phase instead of the bottom.

$$K = [RuBisCO]_{bottom}/[RuBisCO]_{top} \quad (5)$$

$$Yield\ (\%) = ([RuBisCO]_{bottom}/[RuBisCO]_{ABS}) \times 100 \quad (6)$$

$$EE\% = (m_{RuBisCO\,bottom}/(m_{RuBisCO\,top} + m_{RuBisCO\,bottom})) \times 100 \quad (7)$$

# 3. Results and Discussion

## 3.1. RuBisCOs' solid-liquid extraction

In general, the extraction methods applied in the protein extraction of green biomass involves the use of organic solvents with extreme temperatures or pH and sometimes the combination of both. These conditions can able the extraction but with the cost of the protein activity loss (6). ILs have been used in the SLE of several biomolecules, such as proteins (15), caffein (33) and chlorophylls (34) with interesting results that support its application instead of organic solvents.

### 3.1.1. Effect of aqueous solutions of ILs on the RuBisCO's extraction

In this work, for RuBisCO extraction from spinach, the effect of aqueous solutions of biocompatible glycine-betaine- ILs, cholinium- and imidazolium-based ILs was firstly addressed in order to select the most promising ILs. Specifically, the ILs used were: $[Et_3NC_4NC_4]Br$, $[MpyrNC_4NC_4]Br$, $[MimNC_4NC_4]Br$, $[Bu_3NC_4NC_4]Br$, $[Pr_3NC_2OC_2][Sac]$, $[Et_3NC_2OC_2][Sac]$, [Ch]Cl, $[C_2mim]Cl$, $[C_4mim]Cl$ and $[C_6mim]Cl$ (Figure 8). The operational conditions were adapted from Leite et al. (57) and kept constant in all the experiments, namely the IL concentration of 3.3 mM, the solid-liquid ratio of 0.1 and the extraction time of 30 min and temperature at 29 ºC. After the extraction, all extracts were analysed by SDS-PAGE (Figure 9) and SE-HPLC (Figure 10).

The electrophoresis (Figure 9) demonstrates, by the presence of the large subunit (~55 kDa) and small subunits (12-18 kDa) of RuBisCO, that all ILs are capable to extract RuBisCO, from spinach. However, depending on the IL the intensity of the large subunit is distinct revealing the different performance of all ILs. $[Et_3NC_2OC_2][Sac]$ (Figure 9, lane 4) is the IL with the lower capacity to extract RuBisCO, since presents a less intense bands around 55 kDa. On the other hand, $[Pr_3NC_2OC_2][Sac]$, [Ch]Cl and $[C_2mim]Cl$ (Figure 9, lanes 3, 9 and 10, respectively) are the most promising ILs. Comparing the results obtained with the imidazolium-based ILs, it's it possible to conclude that all of them extracted RuBisCO but the increase of the carbon chain length decreases the extraction yield due to the intensity of the band decrease (Figure 9, lanes 10 to 12).

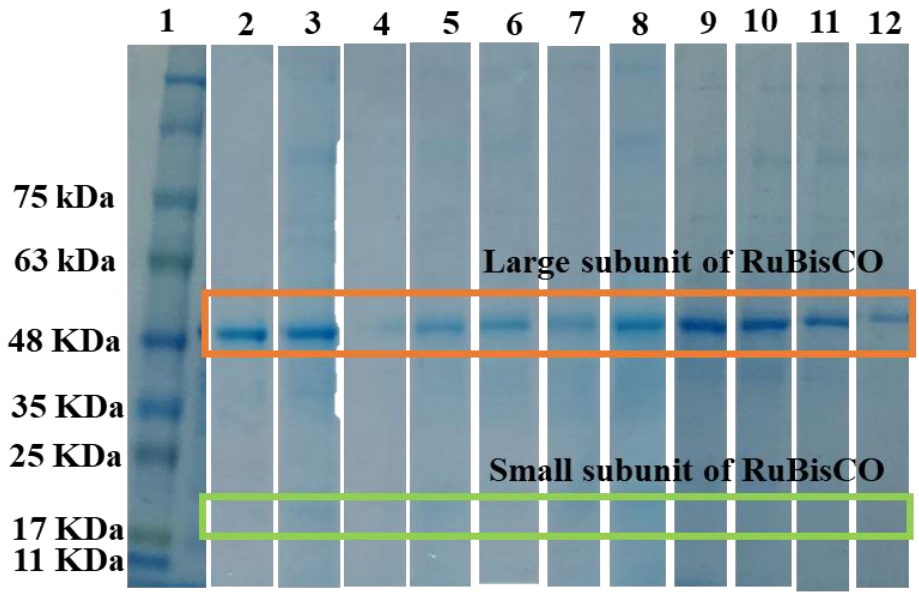


**Figure 9.** SDS-PAGE stained with BlueSafe. Effect of different aqueous solutions of ILs in the RuBisCO extraction from spinach using the following experimental conditions: IL concentration of 3.3 mM, 0.1 solid-liquid ratio, solid-liquid extraction during 30 min at 29 ºC. Lane 1: Standard molecular weights; Lane 2: 1 mg/mL commercial RuBisCO, dissolved in PBS; Lane 3: extract with $[Pr_3NC_2OC_2][Sac]$; Lane 4: extract with $[Et_3NC_2OC_2][Sac]$; Lane 5: extract with $[Et_3NC_4NC_4][Br]$; Lane 6: extract with $[Bu_3NC_4NC_4][Br]$; Lane 7: extract with $[MpyrNC_4NC_4][Br]$; Lane 8: extract with $[MimNC_4NC_4][Br]$; Lane 9: extract with [Ch]Cl; Lane 10: extract with $[C_2mim]Cl$; Lane 11: extract with $[C_4mim]Cl$; Lane 12: extract with $[C_6mim]Cl$.

SE-HPLC spectra (Figure 10) demonstrate that [Ch]Cl is the only IL capable to extract RuBisCO without the formation of aggregates and, for the others ILs, most of the protein is in the form of aggregates where the peaks from $[MpyrNC_4NC_4]Br$ is the one with a greater area and $[Et_3NC_4NC_4][Br]$ is the worst IL for RuBisCO extraction. Crossing information with SDS-PAGE, $[MpyrNC_4NC_4]Br$, $[MimNC_4NC_4]Br$, $[Pr_3NC_2OC_2][Sac]$ and [Ch]Cl was tested in other concentrations (from 25 mM to 1M) to check the influence of the concentration in the extraction yield.

Comparing to other proteins, RuBisCO is more susceptible to form aggregates in the presence of ILs due to its dimensions and complexity (composed for several subunits) and due to this fact (14), it is not suspicious that only [Ch]Cl were able to extract RuBisCO without some degree of denaturation at low concentration (3.3 mM). As a matter of fact, this response to [Ch]Cl in the extraction of proteins was observed by Martins et al. (15) when they chose [Ch]Cl because it was able to extract phycobiliproteins at a low concentration and at the same time avoid the extraction of chlorophylls.

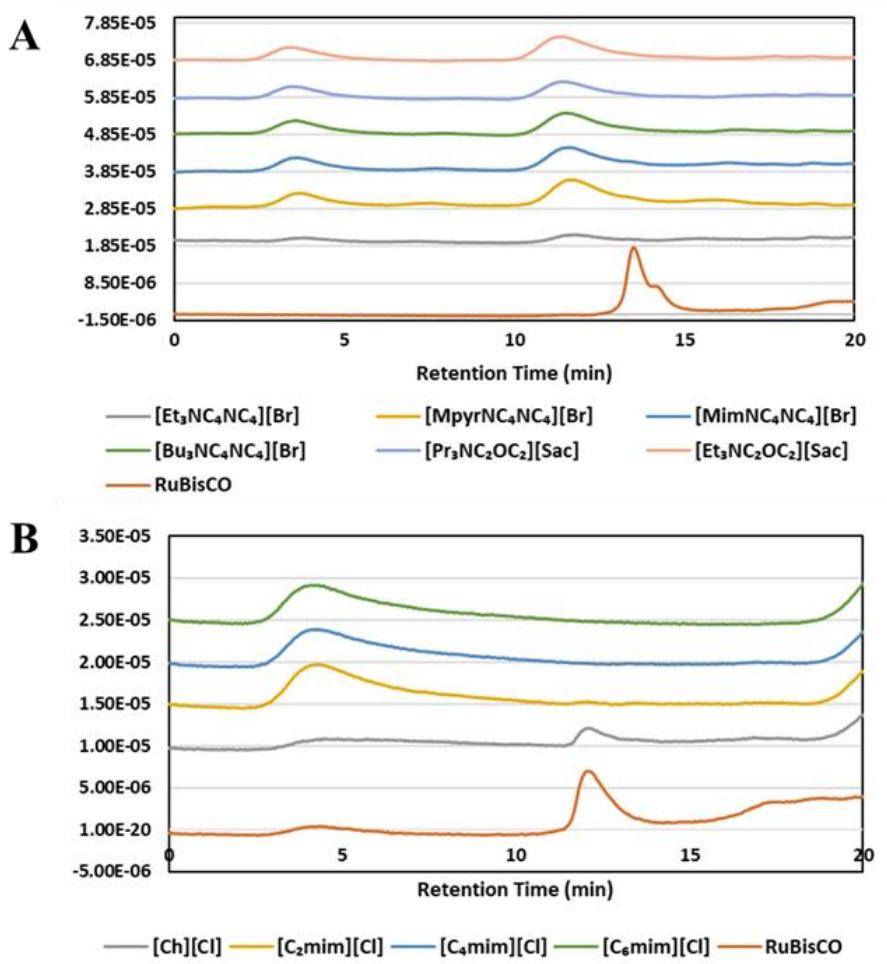


**Figure 10.** SE-HPLC spectra of aqueous solutions of synthesized ILs (A) and commercial ILs (B) after RuBisCO extraction from spinach leaves with an IL concentration of 3.3 mM.

### 3.1.2. Effect of ILs concentration on the RuBisCO's extraction

Based on the data previously shown, the most promising ILs in the extraction of RuBisCO were applied in different concentration, 25 mM, 50 mM, 100 mM, 500 mM and 1M, with the exception of [MpyrNC$_4$NC$_4$]Br and [Pr$_3$NC$_2$OC$_2$][Sac] due to their disposable amount in the moment and solubility, respectively, to investigate the effect of ILs concentration in the extraction of RuBisCO. Additionally, [Ch]Br was introduced in this studied to evaluate the effect of bromide anion in the extraction. As in the previous assays, all the experimental conditions were kept constant (600 rpm, a solid-liquid ration of 0.1 and an extraction during 30 min at 29 ºC). After the extraction, all extracts were analysed by SDS-PAGE (Figure 12) and SE-HPLC to determine the purity and yield of RuBisCO (Figure 11) according to Equations 3 and 4, respectively.

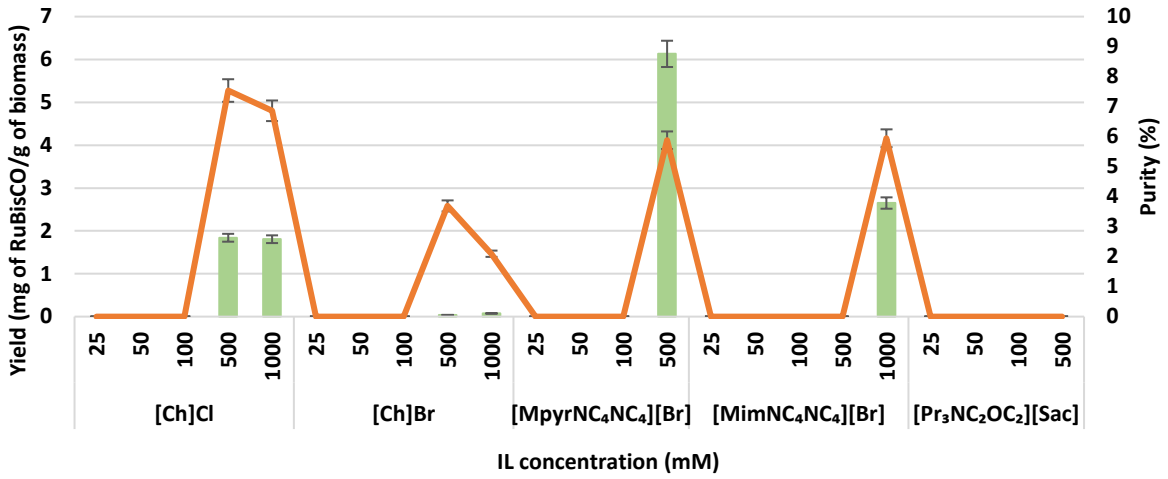


**Figure 11.** RuBisCO's purity (%), orange line, and yield, green bars, after extraction from spinach leaves using different concentrations of IL (25 mM, 50 mM, 100 mM, 500 mM and 1 M), a solid-liquid ratio of 0.1 in a solid-liquid extraction during 30 min at 29 ºC.

Comparing the results of Figures 11 and 12 it is possible to conclude that $[Pr_3NC_2OC_2][Sac]$ didn't extract any protein from spinach, due to the absence of bands in the SDS-PAGE (Figure 12, Gel A, lanes 3-6) and peaks in SE-HPLC chromatograms leading to a RuBisCO yield and purity of zero (Figure 11). Regarding the ILs $[MpyrNC_4NC_4]Br$ and $[MimNC_4NC_4]Br$ (Figure 12, Gel A, lanes 7-15), the presence of the large subunit of RuBisCO is more intense when a higher concentration of IL was applied. These results are in agreement with the analysis by SE-HPLC (Figure 11), where only samples from extraction with aqueous solutions of ILs with higher concentrations (500 mM and 1M) have a yield and purity higher than zero. Contrarily to glycine-betaine-based ILs, [Ch]Cl is the only IL able to extract RuBisCO without denaturation at all concentrations as confirmed by SDS-PAGE (Figure 12, Gel B, lanes 3-7) but with an extraction performance depending on the IL concentration. This is confirmed by yield and purity of RuBisCO determined by SE-HPLC (Figure 11), with samples from extraction with aqueous solutions of ILs with higher concentrations (500 mM and 1M) having a yield and purity higher than zero. Relatively to these two concentrations, at the concentration of IL of 500 mM that a higher yield (6.13 mg of RuBisCO /g of biomass) is achieved with a purity of 5.88 %. This low value of purity, similar to purity values when $[MpyrNC_4NC_4]Br$ 500 mM and $[MimNC_4NC_4]Br$ 1 M were applied is due to other proteins present in the extract and visible in the SDS-PAGE gels (Figure 12). [Ch]Br were used in this investigation to evaluate the effect of bromide anion

in the extraction, however based in Figures 11 and 12 worst results were obtained when compared to [Ch]Cl.

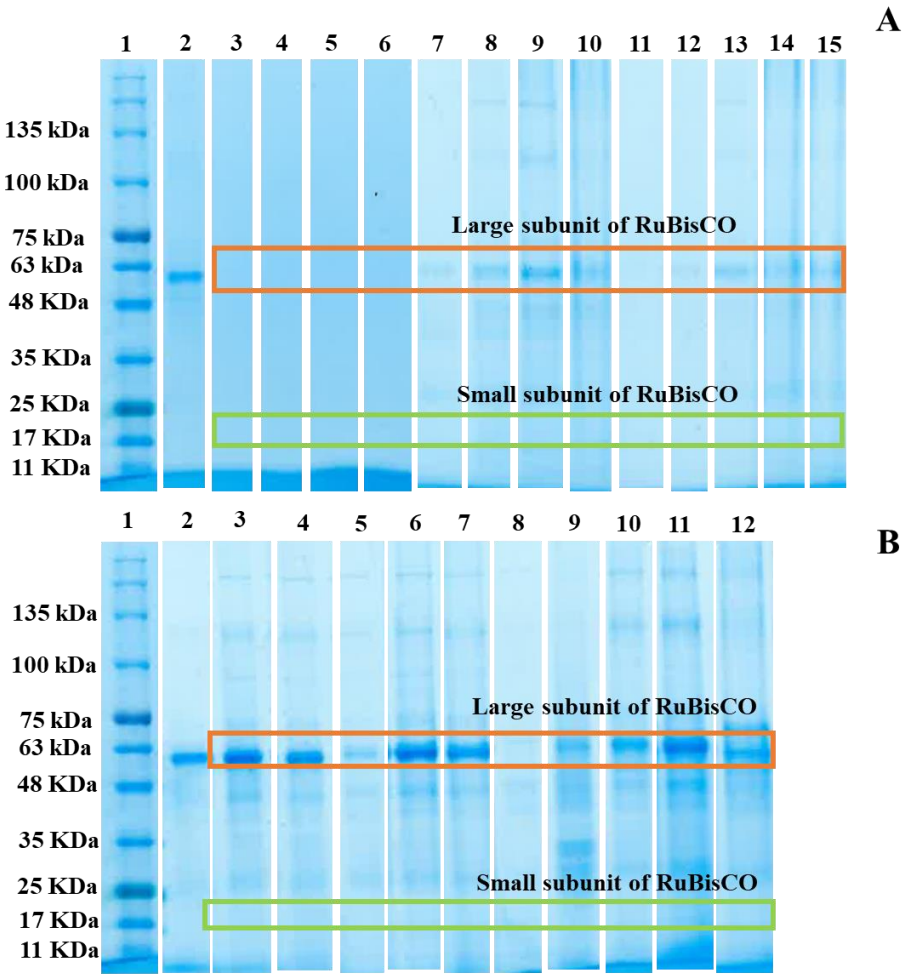


**Figure 12.** SDS-PAGE stained with BlueSafe. Effect of IL concentration in the RuBisCO extraction from spinach using the following experimental conditions: IL concentration of 25 mM – 1M, 0.1 solid-liquid ratio, solid-liquid extraction during 30 min at 29 ºC. **Gel A**: Lane 1: Standard molecular weights; Lane 2: 1 mg/mL commercial RuBisCO, dissolved in PBS; Lanes 3 to 6: extracts with [$Pr_3NC_2OC_2$][Sac] with the following order of concentrations: 25 mM; 50 mM; 100 mM and 500 mM; Lanes 7 to 10: extracts with [$MpyrNC_4NC_4$]Br with the following order of concentrations: 25 mM; 50 mM; 100 mM and 500 mM; Lanes 11 to 15: extracts with [$MimNC_4NC_4$]Br with the following order of concentrations: 25 mM; 50 mM; 100 mM; 500 mM and 1M. **Gel B:** Lane 1: Standard molecular weights; Lane 2: 1 mg/mL commercial RuBisCO, dissolved in PBS; Lanes 3 to 7: extracts with [Ch]Cl with the following order of concentrations: 25 mM; 50 mM; 100 mM; 500 mM and 1 M; Lanes 8 to 12: extracts with [Ch]Br with the following order of concentrations: 25 mM; 50 mM; 100 mM; 500 mM and 1 M.

Taking all these results into account, [Ch]Cl was chosen for RuBisCO extraction and other commercial ILs were tested, for comparison, at the previous concentrations. The reason why [MpyrNC$_4$NC$_4$]Br and [MimNC$_4$NC$_4$]Br were discarded since they only extracted RuBisCO without aggregation at the highest concentrations (500 mM and 1M), although [MpyrNC$_4$NC$_4$]Br was able to extract an unquantifiable concentration of RuBisCO at 100 mM.

Beyond these analyses, the pH value in all the extracts was evaluated. The results show a variation in the pH value between 4.42 and 7.89, with the best results of yield and purity being achieved where, after the SLE, pH value range between 6 and 8. These results are in agreement with the know data about RuBisCO, since between 4.42 and 6 the pH is close to the isoelectric point of the enzyme which is located in the range between 4.6 and 5.5 (depending on the specie), and the best results are located in one of the pH range where the solubility of the enzyme is above 80 % (1,2,18).

### 3.1.3. Effect of cholinium-based ILs and their concentration on the RuBisCO's extraction

Since [Ch]Cl reveal promising results in the previous assays, other cholinium-based ILs were evaluated in this study, namely [Ch][Acetate], [Ch][DHP] and [Ch][DHC]. Moreover, the IL concentration used in the RuBisCO's extractions were also investigated: 25 mM, 50 mM, 100 mM, 500 mM, and 1 M. The remaining experimental conditions were kept constant (600 rpm, a solid-liquid ratio of 0.1, an extraction during 30 min at 29 ºC).

The RuBisCO's purity and yield of extraction were calculated according to Equations 3 and 4, respectively and the results are displayed in Figure 13. SE-HPLC chromatograms from extracts, where [Ch][DHP] and [Ch][DHC] were applied, didn't show any peak, confirming that these ILs are not able to extract RuBisCO. These results were also confirmed by the SDS-PAGE gel (Figure 14) since no bands of protein were detected. Therefore, the results regarding these ILs were not included in Figure 13. Concerning the results obtained with [Ch]Cl and [Ch][Acetate], the last one reveals better RuBisCO's purity ((15.76-57.05)%) and yield of extraction ((2.84-8.45) mg of RuBisCO /g of biomass). These data are in agreement with the SDS-PAGE gel (Figure 14) with a lower number and less intense bands corresponding to other proteins than RuBisCO. The best performance of [Ch][Acetate] can be related to higher protein stability in the presence of this IL. In fact,

[Ch][Acetate] was able to increase the conformational and thermal stability of chymotrypsin with a mild increase in its activity (62).

Relatively to the pH value of extracts, it was observed that the samples from extractions with [Ch][DHP] and [Ch][DHC] have a pH between 3.65 and 5.37, explaining the poor performance of both ILs. As previously reported, the best results are verified when the pH value after extraction is between 6 and 8 since the solubility of the enzyme is above 80 % (1,2).

Selecting [Ch][Acetate] and [Ch]Cl as the best ILs for the extraction of RuBisCO, the next step was the optimization of two parameters: yield of extraction and RuBisCOs' concentration. The purity was not considered since the development of the ABS is to separate and purify the protein in a single step.

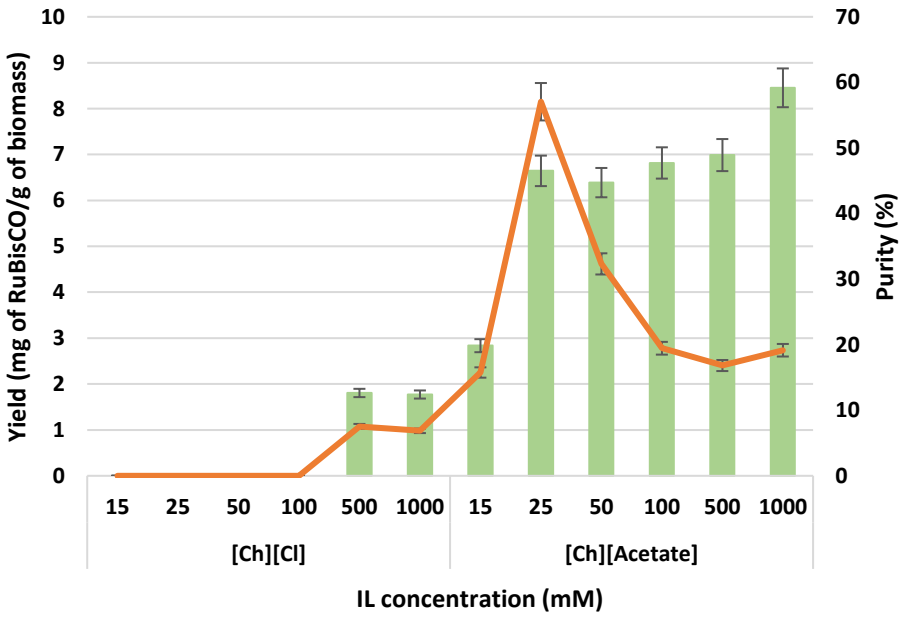


**Figure 13.** RuBisCO's purity (%), orange line, and yield, green bars, after extraction from spinach leaves using different concentrations of IL (25 mM, 50 mM, 100 mM, 500 mM, and 1 M rpm), a solid-liquid ratio of 0.1 in a solid-liquid extraction during 30 min at 29 ºC.

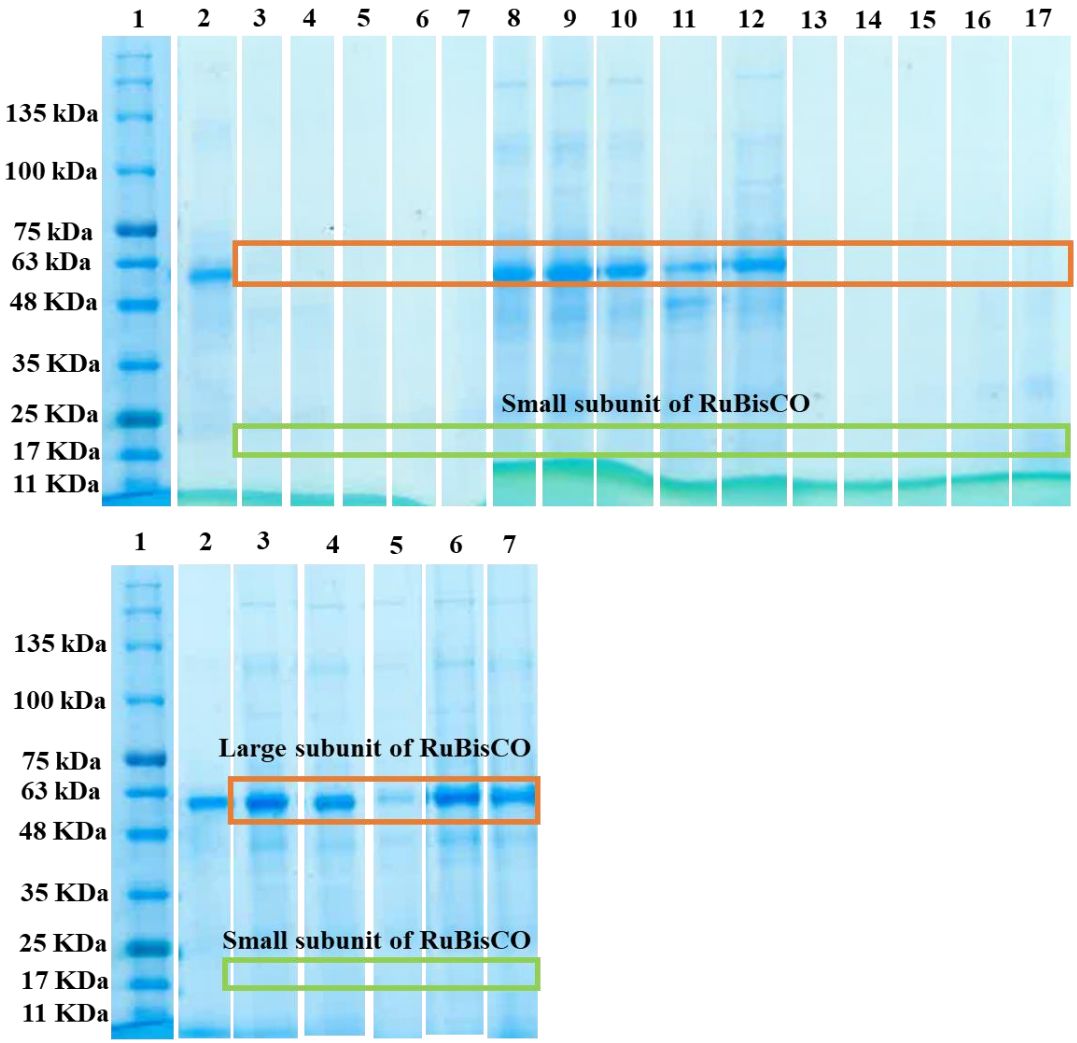


**Figure 14.** SDS-PAGE stained with BlueSafe. Effect of IL concentration in the RuBisCO extraction from spinach using the following experimental conditions: IL concentration of 25 mM – 1M, 0.1 solid-liquid ratio, solid-liquid extraction during 30 min at 29 ºC. **Gel A**: Lane 1: Standard molecular weights; Lane 2: 1 mg/mL commercial RuBisCO, dissolved in PBS; Lanes 3 to 7: extracts with [Ch][DHP] with the following order of concentrations: 25 mM; 50 mM; 100 Mm; 500 mM and 1 M; Lanes 8 to 12 extracts with [Ch][Acetate] with the following order of concentrations: 25 mM; 50 mM; 100 mM; 500 mM and 1 M; Lanes 13 to 17 extracts with [Ch][DHC] with the following order of concentrations: 25 mM; 50 mM; 100 mM; 500 mM and 1 M. **Gel B:** Lane 1: Standard molecular weights; Lane 2: 1 mg/mL commercial RuBisCO, dissolved in PBS; Lanes 3 to 7: extracts with [Ch]Cl with the following order of concentrations: 25 mM; 50 mM; 100 mM; 500 mM and 1 M.

## 3.2. Response surface methodology (RSM)

The previous extractions allowed the selection of the best ILs but is necessary to consider the interaction between different factors such as pH, solid-liquid ratio, among others. With the objective to optimize the extraction of RuBisCO and identify the significant conditions, an RSM applying a $2^3$ factorial planning was implemented (3 factors and 2 levels). RSM permit the extrapolation of the relationship between the dependent variables (yield of extraction and the concentration of RuBisCO extracted) and the independent variables that can improve the extraction. The factorial planning was performed using [Ch]Cl and [Ch][Acetate] with an extraction time of 30 min, at 29 ºC and 600 rpm. The independent variables studied were pH, solid-liquid ratio, and IL concentration.

The results obtained through RSM with the combined effects of solid-liquid ratio and IL concentration, solid-liquid ratio and pH, IL concentration and pH are illustrated in Figures 14-17. The model equation, the experimental conditions, the extraction yield of RuBisCO and the concentration of RuBisCO obtained experimentally and the respective calculated values, as well as the statistical analyses, are provided in Appendix B (Sections B 2 to B 11). Variance analysis (ANOVA) was employed to estimate the statistical significance of the variables and the interactions between them.

### 3.2.1. Analysis of results for [Ch][Acetate]

For the extractions using [Ch][Acetate], results from the statistical analysis, represented in the pareto chart (Appendix B, Figure B 6.1), show that the significant variables for the yield of extraction are pH and solid-liquid ratio (linear and quadratic). The results of Figure 15 and Appendix B, Table B 4.1 – Figure B 4.1.1 show that pH is the variable with the most significant effect (positive effect in the response) followed by solid-liquid ratio where the linear variable had a positive effect and the quadratic a negative one. The maximum yield of extraction obtained was (10.824 ± 0.485) mg of RuBisCO /g of biomass, for a pH of 11.2, an 0.1 solid-ratio and an IL concentration of 1.5 M. However, above a 0.12 solid-liquid ratio there is a slight decrease in the yield of extraction (Figure 15, (D) and (E)) caused by the effect of the quadratic variable.

Relatively to the concentration of extracted RuBisCO, pareto chart (Appendix B, Figure B 6.2) evidenced that solid-liquid ratio, pH, and their interaction are the significant variables. Taking into account the data represented in Figure 15 and Appendix B, Table B 4.2 – Figure

B 4.2.1, pH is the parameter that has a higher effect on the concentration of extracted RuBisCO and with solid-liquid ration and their interaction are responsible for the increase of the response. The maximum concentration of extracted RuBisCO obtained was (1.594 ± 0.068) mg/mL, for a pH of 7.0, an 0.184 solid-ratio, and an IL concentration of 1.5 M.

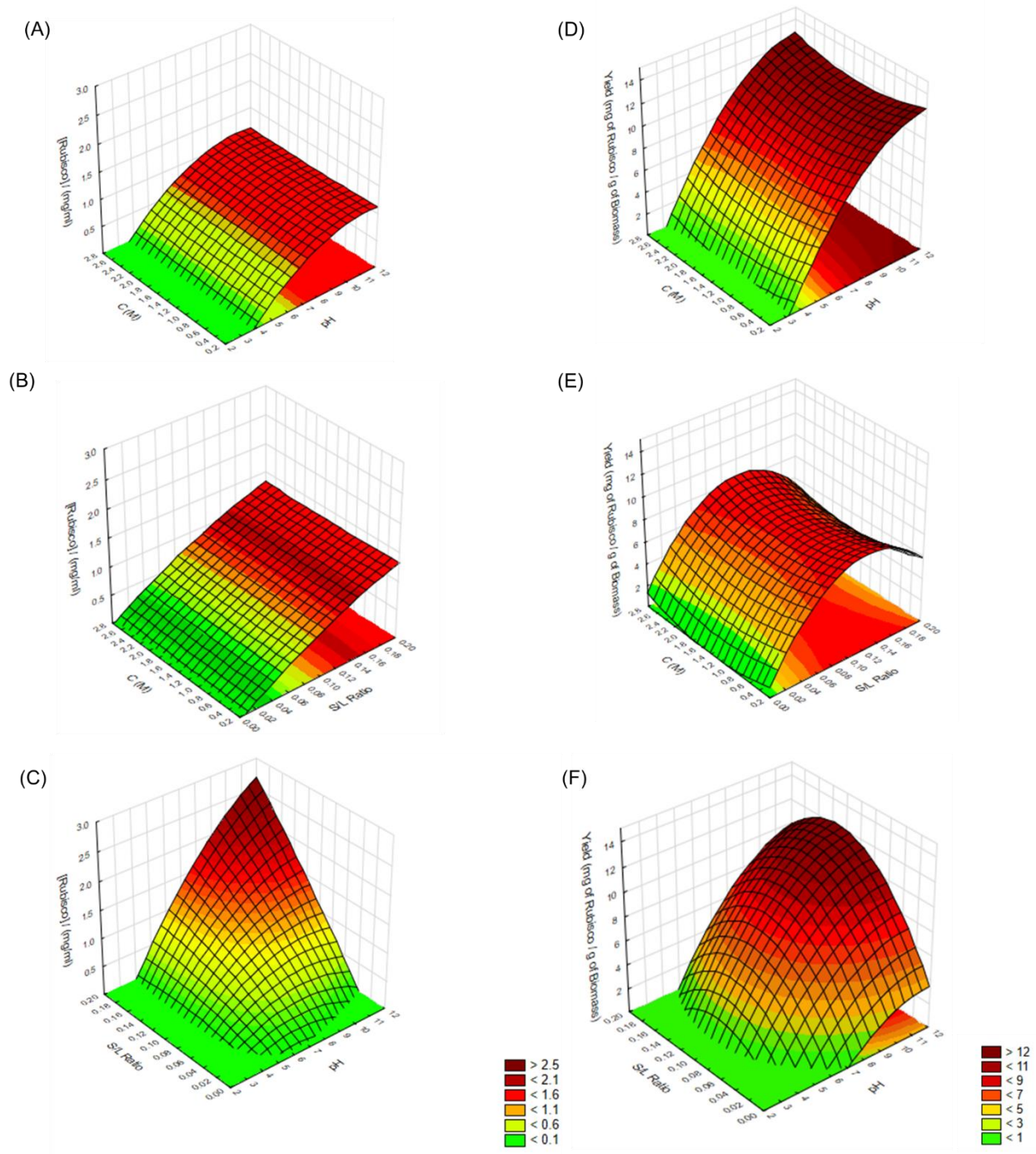

**Figure 15**. Response surfaces corresponding to the concentration of extracted RuBisCO (left) and yield of extraction (right) with the following combined parameters: (A) and (D) [Ch][Acetate] concentration and pH; (B) and (E) [Ch][Acetate] concentration and solid-liquid ratio; and (C) and (F) solid-liquid ratio and pH.

For both responses, pH is the most significant variable however, other variables affect the responses differently so, it was calculated the best experimental conditions to optimize both responses and were applied in further extractions: a 0.184 solid-liquid ratio, a pH value of 11.2 and an IL concentration of 2.68 M (Appendix B, Figure B 10). The application of a high concentration of IL is this extraction an advantage because in the next step (ABS formation) IL is going to be the salting-out agent and a high concentration facilitates the process. The optimal conditions were tested, and no significant differences were detected between the predicted and observed results, since the concentration of extracted RuBisCO presented only a slight discrepancy between the predicted and the observed value, as shown in Table 2.

**Table 2.** Results of the extraction with [Ch][Acetate] at the optimal conditions.

| | **Predicted Results** | **Observed Results** | **Relative error (%)** |
|---|---|---|---|
| **[RuBisCO] (mg/mL)** | 2.34 | 2.00 | -16.7 |
| **Yield (mg of RuBisCO /g of biomass)** | 11.4 | 10.9 | -3.97 |

### 3.2.2. Analysis of results for [Ch]Cl

The statistical results represented in the pareto chart (Appendix B, Figure B 7.1), showed that in the extractions using [Ch]Cl as a solvent, the solid-liquid ratio and IL concentration are the significant parameters on the yield of extraction. The results of Figure 16 and in Appendix B, Table B 5.1 – Figure B 5.1.1, showed that the solid-liquid ratio has a negative effect and pH and [Ch]Cl concentration have a positive effect on the response. The maximum yield of extraction obtained was (14.05 ± 0.69) mg of RuBisCO /g of biomass, for a pH of 7.0, an 0.016 solid-ratio, and an IL concentration of 1.5 M.

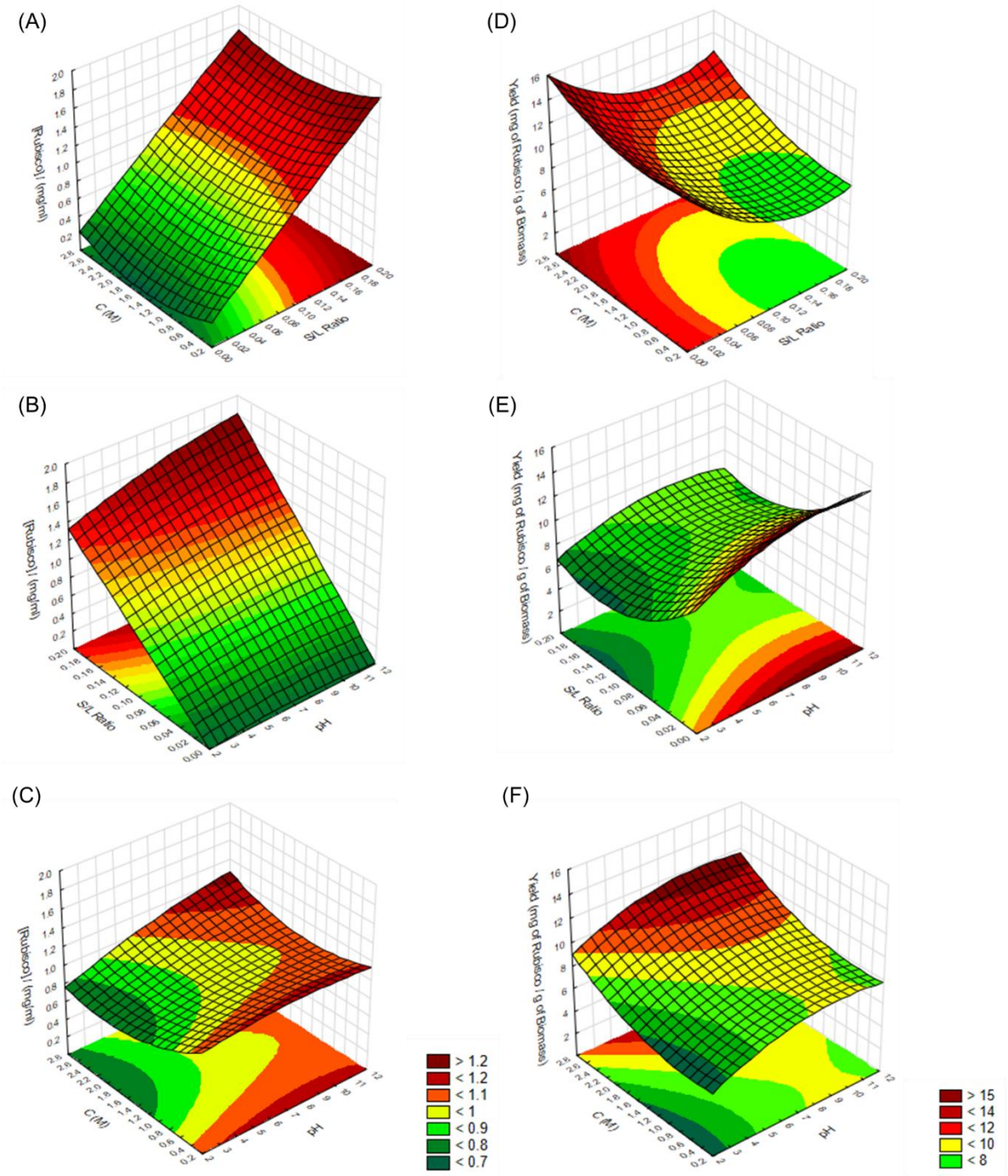


**Figure 16.** Response surfaces corresponding to the concentration of extracted RuBisCO (left) and yield of extraction (right) with the following combined parameters: (A) and (D) [Ch]Cl concentration and solid-liquid ratio; (B) and (E) solid-liquid ratio and pH; and (C) and (F) [Ch]Cl concentration and pH.

For the concentration of extracted RuBisCO, the pareto chart (Appendix B, Figure B 7.2) evidenced that the solid-liquid ratio and pH are the significant variables. Taking into account the data represented in Figure 16 and Appendix B, Table B 5.2 – Figure B 5.2.1, the solid-liquid ratio is the parameter that has a higher effect on the concentration of extracted RuBisCO with a positive effect on the response, and pH and the concentration of [Ch]Cl (quadratic) did not affect in a significant way the increase of the response. The maximum concentration of extracted RuBisCO obtained was (1.37 ± 0.07) mg/mL, for a pH of 9.5, an 0.15 solid-ratio and an IL concentration of 0.80 M.

Since each response is affected by different variables, the best experimental conditions, to increase both responses, were calculated and applied in further extractions: a 0.184 solid-liquid ratio, a pH value of 9.09 and an IL concentration of 2.68 M (Appendix B, Figure B 11). As said before, also here the application of a high concentration of IL is this extraction an advantage because in the next step (ABS formation) IL is going to be the salting-out agent and a high concentration facilitates the process. The optimal conditions were tested, and no significant differences were detected between the predicted and observed results, as shown in Table 3.

**Table 3.** Results of the extraction with [Ch]Cl at the optimal conditions.

| | **Predicted Results** | **Observed Results** | **Relative error (%)** |
|---|---|---|---|
| **[RuBisCO] (mg/mL)** | 1.72 | 1.86 | 7.51 |
| **Yield (mg of RuBisCO /g of biomass)** | 11.6 | 10.1 | -14.1 |

In order to compare the performance of ILs in the extraction of RuBisCO from spinach with the solvent commonly used, the $NH_4OH$ (3) was also evaluated. An aqueous solution of $NH_4OH$ 0.1M at pH 11 was applied in the RuBisCO's extraction from spinach, and the remaining extraction conditions were maintained (600 rpm, a solid-liquid ratio of 0.1, during

30 min, at 29 ºC). The results obtained and the comparison with [Ch]Cl and [Ch][Acetate] are demonstrated in Figures 17 and 18.

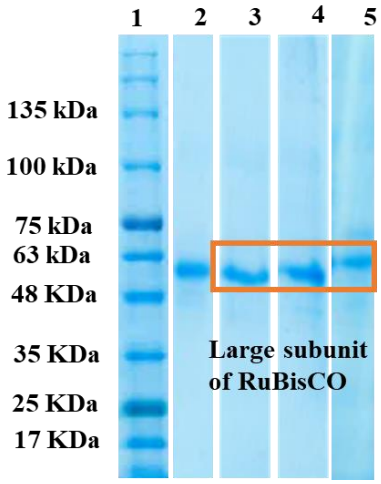


**Figure 17.** SDS-PAGE stained with BlueSafe. Lane 1: Standard molecular weights; Lane 2: 1 mg/mL commercial RuBisCO, dissolved in PBS; Lane 3: spinach extracts with [Ch][Acetate]; Lane 4: spinach extracts with [Ch]Cl; Lane 5: spinach extract with $NH_4OH$.

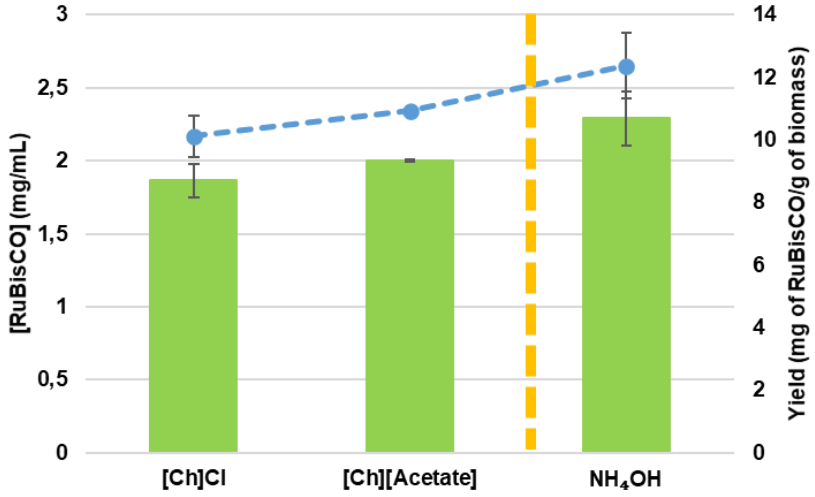


**Figure 18.** RuBisCO's yield, blue stroke, and RuBisCO's concentration, green bars, after extraction from spinach leaves at the optimal conditions and with $NH_4OH$, a solid-liquid ratio of 0.1 in a solid-liquid extraction during 30 min at 29 ºC.

The application of $NH_4OH$ for RuBisCO extraction reveals results slightly better than the ones obtained with aqueous solutions of ILs, since higher concentration of RuBisCO

(2.29 mg/mL *versus* 2.00 mg/mL and 1.86 mg/mL) and extraction yield (12.38 mg of RuBisCO /g of biomass *versus* 10.12 mg of RuBisCO /g of biomass and 10.93 mg of RuBisCO /g of biomass) were obtained with the conventional solvent, using the same conditions of extraction. However, it should keep in mind that other variables must be evaluated, such as protein stability, if it is possible to integrate with another process/technique. Furthermore, cholinium-based ILs were developed as possible substitutes to others with toxicity problems that put in cause the biocompatibility and biodegradability of the systems (14,32).

### 3.3. RuBisCOs' purification with ABS

Considering that the extracts previously obtained in the SLE, specifically with the most promising ILs ([Ch]Cl and [Ch][Acetate]), are complex and contain several biomolecules, a purification step is required in order to obtain a higher pure sample of RuBisCO. In this sense, ABS was applied to separate RuBisCO from one of the aqueous phases while the remaining proteins are expected to migrate to the opposite phase. However, cholinium-based ILs tend to be highly hydrophilic and only form ABS with strong salting-out salts (*e.g.*, $K_3PO_4$ and $K_2CO_3$) or with polymers (*e.g.*, PPG 400 and 1000, PEG 400, 600 and 1000), in which the IL may act as the salting-out species (63). Therefore, in this work PPG 400, PEG 1000 were selected as the second compound of the ABS due to strong evidence that proteins are stable in the presence of certain polymers (14). The mixture compositions of the ABS were selected based on the report of Pereira et al. (59). Using PPG 400, the mixture point 15 wt% of [Ch][Acetate] + 19 wt% of water + 67 wt% of polymer and 12 wt% of [Ch]Cl + 21 wt% of water + 67 wt% of polymer were chosen while for PEG 1000 the biphasic mixture 15 wt% of [Ch][Acetate] + 19 wt% of water + 67 wt% of polymer and 12 wt% of [Ch]Cl + 21 wt% of water + 67 wt% of polymer were selected. For all the systems, the weight percentage of IL and water derived from the extracts. Briefly, after extraction with each IL, it was added to an aliquot of 1 g of extract 2 g of each polymer. The obtained ABSs are showed in Figure 19 and as depicted in Figure 19, the top phase (polymer-rich phase) of the ABS composed of PEG 1000 has a very small phase. Consequently, only PPG 400 was selected for the RuBisCOs' purification. Additionally, in this ABS the formation of a precipitate in the interphase that extends to the top phase was observed (Figure 19). This precipitated may be RuBisCO or other biomolecules present in the spinach extract. To clarify

and verify if the RuBisCO purification was succeeded both phases and interphase precipitated were analysed by SDS-PAGE and SE-HPLC.

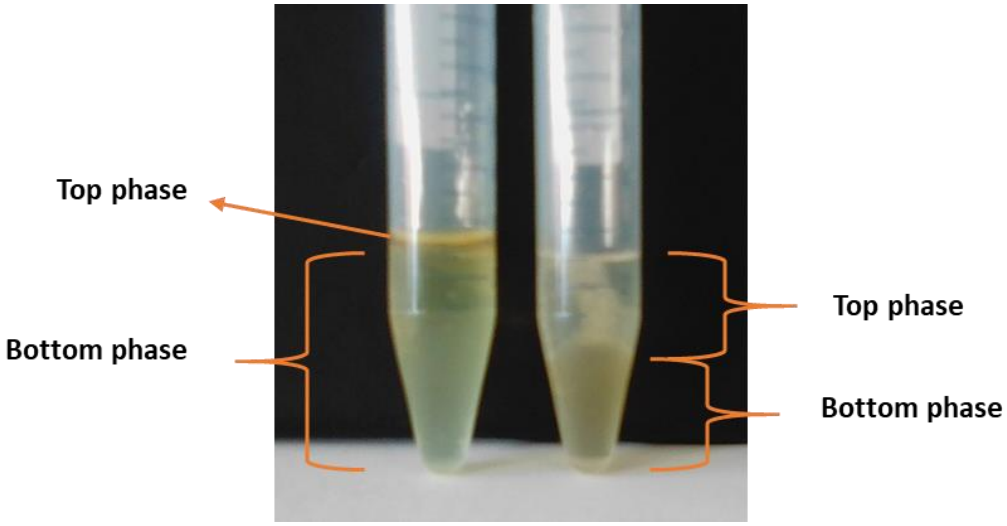


**Figure 19.** ABS composed by PEG 1000+ spinach extract with [Ch][Acetate] (left) and PPG 400 + spinach extract with [Ch][Acetate] (right).

Only with the ABS composed of PPG 400 + [Ch][Acetate] the presence of RuBisCO in the bottom-phase (IL-rich phase) is noticed in the protein profile analysed through SDS-PAGE. This result agrees with the SE-HPLC analysis (Figure 20) since only in the chromatograms of the bottom phase of this ABS the presence of different peaks, including the peak corresponding to RuBisCO, appear. With ABS composed of PPG 400 + [Ch][Acetate] an extraction efficiency (*EE%*) of RuBisCO of 100% was obtained and a recovery of (1.34 ± 0.36) mg of RuBisCO of the initials (1.36 ± 0.006) mg of RuBisCO, meaning a yield of 100%. Although a good yield was obtained, a lower value of purity was attained ((22.67 ± 2.06) %). Despite the absence of other bands in SDS-PAGE, the SE-HPLC spectra show a great amount of protein eluted between the 15 and 20 min, decreasing the purity of the protein. Taken the purity into account it is evidenced that this ABS is not selective for RuBisCO and consequently is not adequate for its purification. There are some studies about RuBisCO partitioning in ABS with polymer and ILs. Preferably, the polymer used is PEG due its capability of stabilize the proteins and, in fact in the examples with RuBisCO when PEG was present in the ABS, RuBisCO partitioned in majority for the PEG-rich phase. In this work the selected compounds were PPG 400 and cholinium-based ILs in which the used ILs are well-known for their biocompatibility, biodegradability, low toxicity,

and the capability to stabilize the target protein. So, it was expected that on PPG 400 + cholinium-based ILs studied in this work, RuBisCO partitioned in its majority for the IL-rich phase (bottom phase) (11,14,32,64).

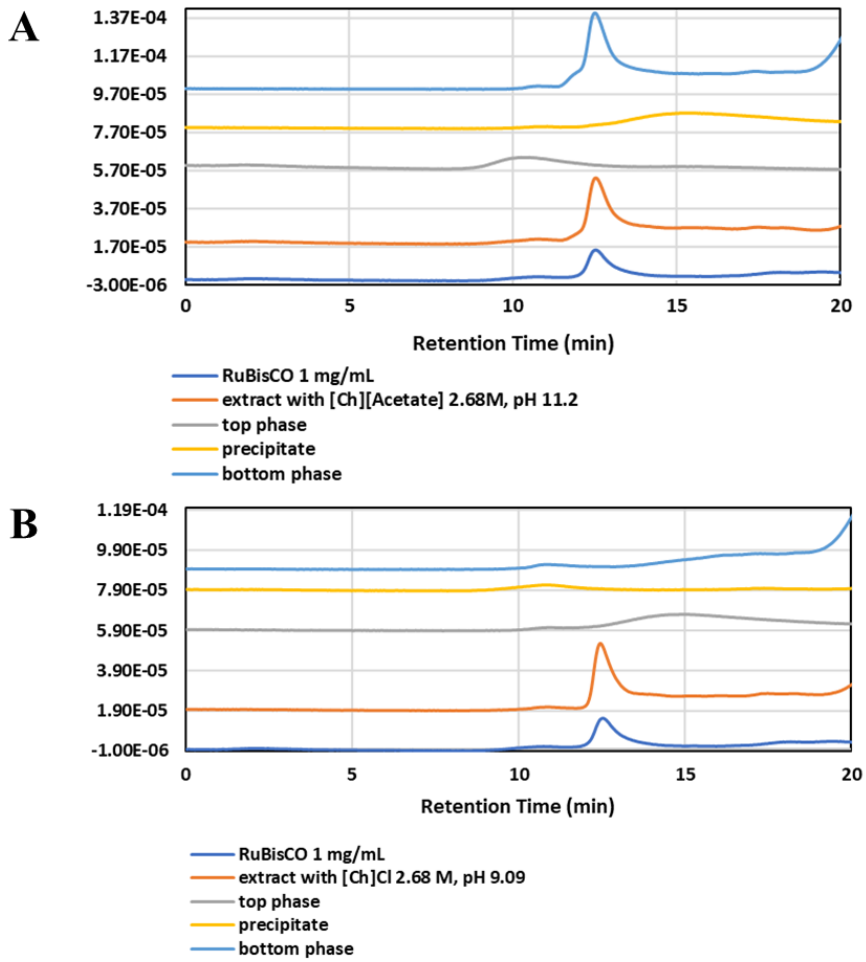


**Figure 20.** SE-HPLC spectra of the different extracts, phases, and precipitates of the ABSs composed by IL and PPG 400, where (A) is for [Ch][Acetate] and (B) for [Ch]Cl.

Since the ABS with PPG 400 is not selective for RuBisCO, a ternary mixture composed by the extract (contributor for the water and IL weight percentages) and $K_2HPO_4$. The selected ternary mixtures 27 wt% of [Ch][Acetate] + 44 wt% of water + 29 wt% of $K_2HPO_4$ and 24 wt% of [Ch]Cl + 48 wt% of water + 29 wt% of $K_2HPO_4$ determined by Belchior *et al.* (60) and Osloob *et al.* (61) respectively. As in the previous ABSs, after the extractions with each IL, it was added to an aliquot of 1 g of extract 0.6 g of $K_2HPO_4$. After the phases' separation the ABS showed in Figure 21 were obtained and each phase was analysed by SDS-PAGE and SE-HPLC.

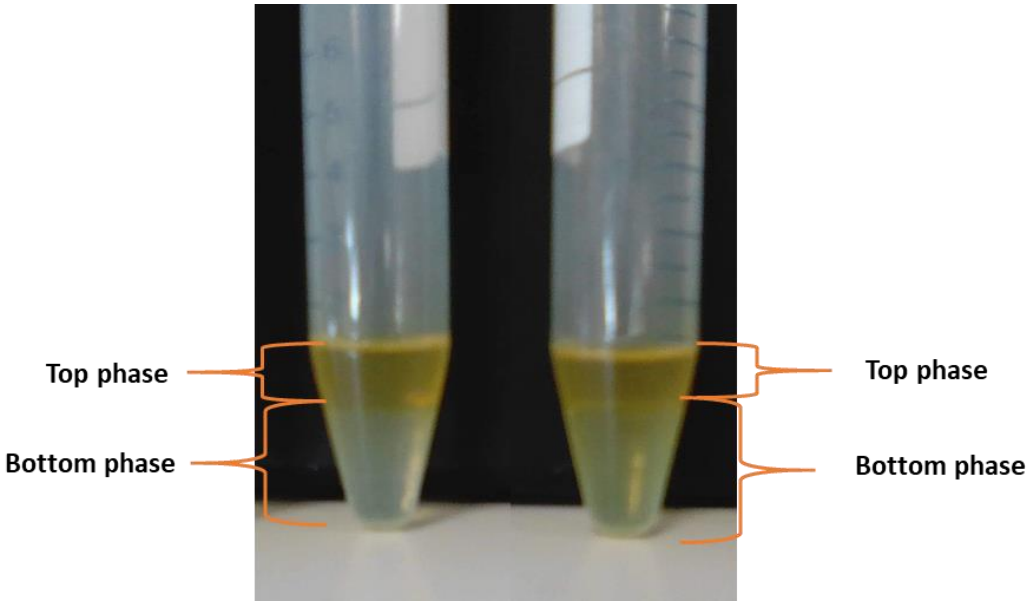


**Figure 21.** ABS composed by $K_2HPO_4$ + spinach extract with [Ch][Acetate] (left) and $K_2HPO_4$ + spinach extract with [Ch]Cl (right).

In both ABSs, by the analysis of the protein profile in SDS-PAGE, RuBisCO partitioned to the top phase (IL-rich phase) and for the precipitate. This result agrees with the SE-HPLC analysis, Figure 22, since only in the chromatograms of the top phase and precipitate the presence of different peaks, including the peak corresponding to RuBisCO appears although in unquantifiable amounts in the precipitate. With these ABSs, extraction efficiencies (*EE%*) for RuBisCO of 100% were obtained and the *K* confirm the complete extraction of RuBisCO. Relatively to the extraction yield, for the system with [Ch][Acetate] it was possible to recover (0.72 ± 0.09) mg of RuBisCO of the initials (1.43 ± 0.097) mg of RuBisCO, meaning a yield of (50.4 ± 9.96) % and, for the system with [Ch]Cl it was possible to recover (0.77 ± 0.30) mg of RuBisCO of the initials (1.55 ± 0.389) mg of RuBisCO, meaning a yield of (54.5 ± 0.639) %. These results show that the ABSs composed of PPG 400 had the best yield of extraction, but in terms of purity ((35.1 ± 1.40) % for [Ch][Acetate] and (12.9 ± 0.668) % for [Ch]Cl) both ABSs with salt and polymer are not selective for RuBisCO. The results are in agreement with the conclusions of several publications like the work from Belchior *et al.* (60) that were able to extract several amino acids with the ABS composed by [Ch][Acetate] + $K_2HPO_4$. They obtained extraction efficiencies above 95 % although they only achieve a complete extraction for L-tryptophan. The ABS composed of

[Ch]Cl and $K_2HPO_4$ was studied by Osloob *et al.* (61) for the partition of penicillin G and 96 % of the biomolecule partition to the IL-rich phase. Relatively to RuBisCO, Desai *et al.* (11) report the RuBisCO migration to IL-rich phase in an ABS composed by Iolilyte 221PG and sodium potassium buffer and Ruiz *et al.* (14) observe the same behavior for the ABS with Iolilyte 221PG and potassium buffer. For biological matrices, an ABS with $K_2HPO_4$ and 1-butyl-3-methylimidazolium chloride was studied to extract the proteins of human urine by Du *et al.* (65), where it was observed that all the proteins partitioned to the top phase (IL-rich) and the metal species migrated to the salt-rich phase.

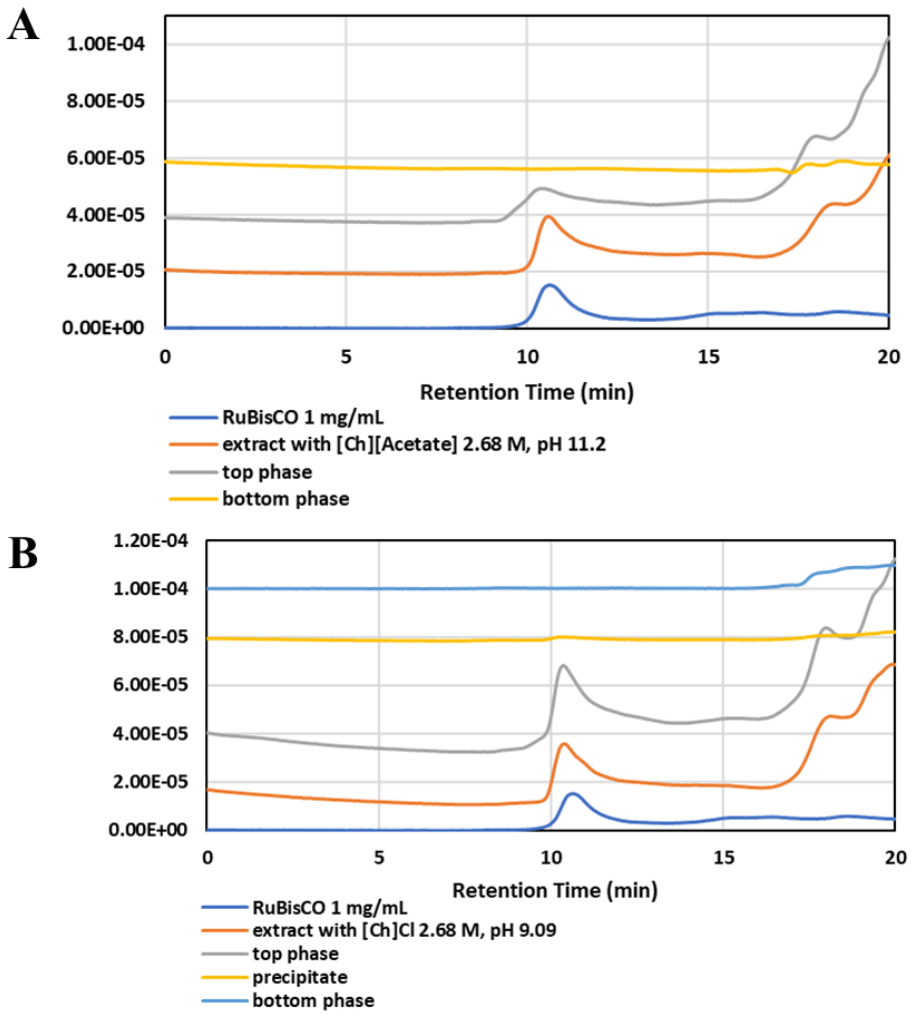


**Figure 22.** SE-HPLC spectra of the different extracts, phases, and precipitates of the ABSs composed by IL and $K_2HPO_4$, where (A) is for [Ch][Acetate] and (B) for [Ch]Cl.

# 4. Final Remarks

## Conclusions

In this work it was developed a new SLE method for RuBisCO from spinach with aqueous solutions of ILs with the aiming of integrating the extract in a separation/purification step through the formation of an ABS. An initial screening, where the effect of ILs in the extraction of RuBisCO from spinach was evaluated showed that [$Et_3NC_4NC_4$][Br] and [$Et_3NC_2OC_2$][Sac] are the worst ILs to extract RuBisCO and, that [$MpyrNC_4NC_4$]Br, [$MimNC_4NC_4$]Br, [$Pr_3NC_2OC_2$][Sac], [Ch]Cl and [$C_2$mim]Cl were the most promising ILs. However, the extractions using [$C_2$mim]Cl, [$C_4$mim]Cl and [$C_6$mim]Cl were affected by the length of the carbon chain, where the increase of the carbon chain length decreased the extraction yield. In fact, [Ch]Cl was the only IL able to extract RuBisCO without the formation of aggregates. A new screening with variation of IL concentration was made and [Ch]Cl was the only one able to extract RuBisCO without denaturation at all concentrations. The study of other cholinium-based ILs bring to this work [Ch][Acetate].

The extractions were optimized through a RSM and the optimal conditions were: a 0.184 solid-liquid ratio, an IL concentration of 2.68 M and a pH of 9.09 for [Ch]Cl and a pH of 11.2 for [Ch][Acetate]. These results were verified and validated for both ILs and comparing with an extraction where the solvent of the conventional method was applied, the results are similar although the results with the conventional solvent are slightly better. However, it is necessary to evaluate the further steps for separation and purification in the choice of the best solvent.

To separate and purify RuBisCO from the other proteins and contaminants, it was studied two ABSs composed by IL + water + PPG 400 and IL + water + $K_2HPO_4$. For the ABS with PPG 400, the one with [Ch][Acetate] was the only one to achieve the goal without the degradation of the target biomolecule with an EE% of 100% since RuBisCO partitioned in totality for the bottom phase; the same happened in the ABS with $K_2HPO_4$ with the exception that both ABS (with [Ch][Acetate] and [Ch]Cl) achieve the goal and the protein partitioned to the top phase. Nevertheless, theses ABSs are not selective for RuBisCO for the reason that in ABSs with PPG 400 all the phases have protein (in the bottom phase there are others in addition to RuBisCO) and in $K_2HPO_4$ case, all the protein migrates to the top-phase and due to this they cannot be applied in the purification of RuBisCO.

## Future work

In this work, it was developed a new SLE method for RuBisCO from spinach leaves with aqueous solutions of ILs with the intention of integrating with a separation/purification step through the formation of an ABS. Numerous ILs were studied and the best were selected. The extraction was optimized, and it was created an ABS with the addition of a polymer or a salt to the extract. Even though RuBisCO partition in totality for one of the phases it is necessary further investigations to find a better ABS since these are not selective.

Based on the results, the next step should be focused on the improvement of the ABS selectivity. After that, it can be interesting to investigate if it is possible to do consecutive extractions until the IL solution is saturated with RuBisCO and applied the ABS in the end. Another possibility related to the sustainability of the process that can be investigated is if it is possible to recycle the phases of the ABS. Of course, that all of these improvements in the method have to consider the stability and activity of the target protein.

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# Appendix A

## A 1. Calibration curve for the quantification of the total amount of proteins

Figure A 1 depicts the calibration curve obtained through SE-HPLC and made with BSA.

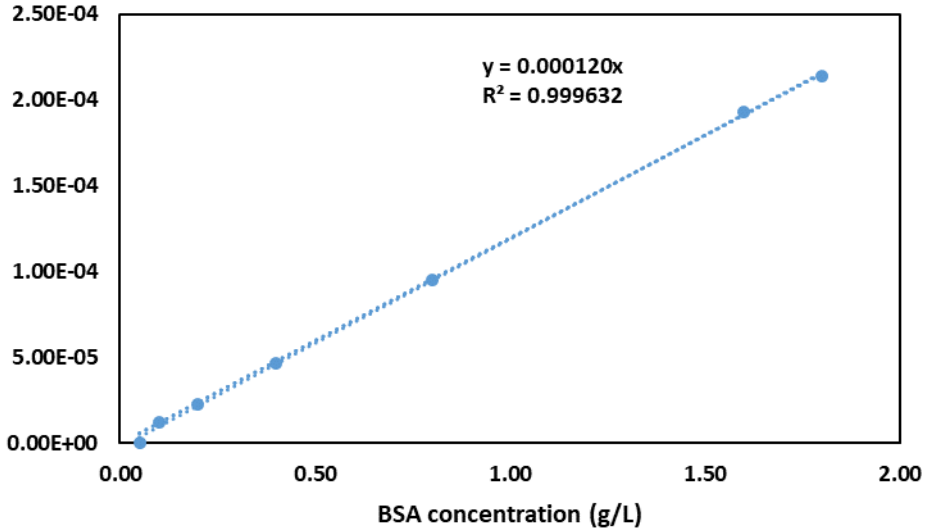


**Figure A 1.** Calibration curve for total amount of protein.

## A 2. Calibration curve for the quantification of the amount of RuBisCO

Figure A 2 depicts the calibration curve obtained through SE-HPLC and made with commercial RuBisCO.

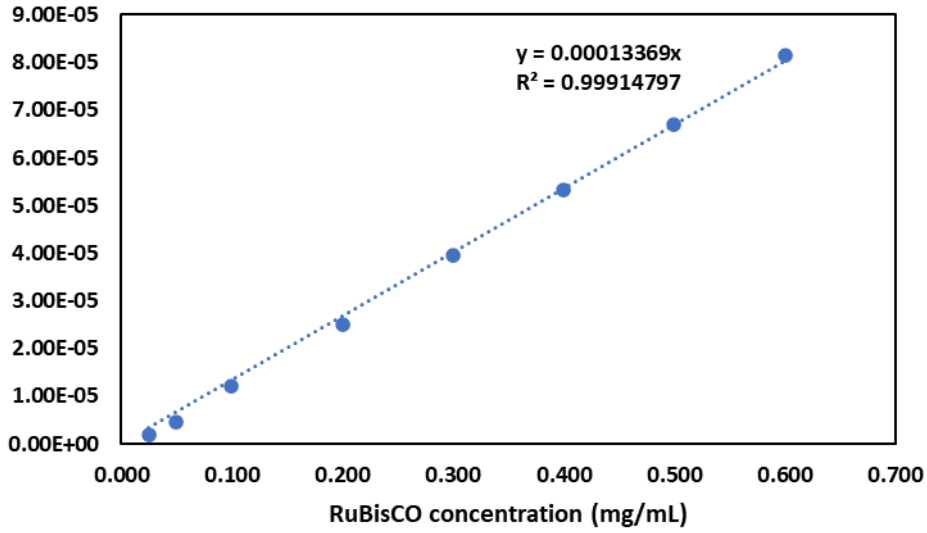


**Figure A 2.** Calibration curve for RuBisCO quantification.

# Appendix B

## B 1. Factorial planning for both ILs

Table B 1. exhibits the $2^3$ factorial planning for each IL.

**Table B 1.** $2^3$ factorial planning for each IL ([Ch]Cl and [Ch][Acetate]).

| Experiment | $X_1$ | $X_2$ | $X_3$ |
|---|---|---|---|
| **1** | -1 | -1 | -1 |
| **2** | 1 | -1 | -1 |
| **3** | -1 | 1 | -1 |
| **4** | 1 | 1 | -1 |
| **5** | -1 | -1 | 1 |
| **6** | 1 | -1 | 1 |
| **7** | -1 | 1 | 1 |
| **8** | 1 | 1 | 1 |
| **9** | -1.68 | 0 | 0 |
| **10** | 1.68 | 0 | 0 |
| **11** | 0 | -1.68 | 0 |
| **12** | 0 | 1.68 | 0 |
| **13** | 0 | 0 | -1.68 |
| **14** | 0 | 0 | 1.68 |
| **15** | 0 | 0 | 0 |
| **16** | 0 | 0 | 0 |
| **17** | 0 | 0 | 0 |
| **18** | 0 | 0 | 0 |
| **19** | 0 | 0 | 0 |
| **20** | 0 | 0 | 0 |

## B 2. Experimental data and response surface predicted values of the factorial planning for [Ch][Acetate]

Table B 2.1 exhibits the experimental data and response surface predicted values of the factorial planning for RuBisCOs' extraction yield.

**Table B 2.1.** Experimental data and response surface predicted values of the factorial planning for RuBisCOs' extraction yield.

| Nº | pH | S/L RATIO | C (M) | Predicted Results | Observed Results | Relative deviation (%) |
|---|---|---|---|---|---|---|
| 1 | 4.50 | 0.05 | 0.80 | 0.280 | 0.00 | |
| 2 | 9.50 | 0.05 | 0.80 | 7.95 | 8.79 | 9.56 |
| 3 | 4.50 | 0.15 | 0.80 | 2.18 | 0.00 | |
| 4 | 9.50 | 0.15 | 0.80 | 10.8 | 9.94 | -8.73 |
| 5 | 4.50 | 0.05 | 2.2 | 0.400 | 0.00 | |
| 6 | 9.50 | 0.05 | 2.2 | 8.09 | 9.00 | 10.2 |
| 7 | 4.50 | 0.15 | 2.2 | 2.11 | 0.00 | |
| 8 | 9.50 | 0.15 | 2.2 | 10.8 | 9.76 | -10.1 |
| 9 | 2.80 | 0.10 | 1.5 | -2.34 | 0.00 | |
| 10 | 11.2 | 0.10 | 1.5 | 11.4 | 10.8 | -5.10 |
| 11 | 7.00 | 0.02 | 1.5 | 1.75 | 0.500 | -250 |
| 12 | 7.00 | 0.18 | 1.5 | 5.60 | 8.65 | 35.3 |
| 13 | 7.00 | 0.10 | 0.32 | 8.06 | 8.93 | 9.73 |
| 14 | 7.00 | 0.10 | 2.7 | 8.11 | 9.04 | 10.3 |
| 15 | 7.00 | 0.10 | 1.5 | 7.14 | 7.43 | 3.96 |
| 16 | 7.00 | 0.10 | 1.5 | 7.14 | 6.98 | -2.28 |
| 17 | 7.00 | 0.10 | 1.5 | 7.14 | 7.69 | 7.18 |
| 18 | 7.00 | 0.10 | 1.5 | 7.14 | 6.29 | -13.4 |
| 19 | 7.00 | 0.10 | 1.5 | 7.14 | 7.22 | 1.11 |
| 20 | 7.00 | 0.10 | 1.5 | 7.14 | 6.90 | -3.43 |

Table B 2.2 exhibits the experimental data and response surface predicted values of the factorial planning for RuBisCOs' concentration.

**Table B 2.2.** Experimental data and response surface predicted values of the factorial planning for RuBisCOs' concentration.

| Nº | pH | S/L RATIO | C (M) | Predicted Results | Observed Results | Relative deviation (%) |
|---|---|---|---|---|---|---|
| 1 | 4.50 | 0.05 | 0.80 | 0.280 | 0.00 | |
| 2 | 9.50 | 0.05 | 0.80 | 7.95 | 8.79 | 9.56 |
| 3 | 4.50 | 0.15 | 0.80 | 2.18 | 0.00 | |
| 4 | 9.50 | 0.15 | 0.80 | 10.8 | 9.94 | -8.73 |
| 5 | 4.50 | 0.05 | 2.2 | 0.400 | 0.00 | |
| 6 | 9.50 | 0.05 | 2.2 | 8.09 | 9.00 | 10.2 |
| 7 | 4.50 | 0.15 | 2.2 | 2.11 | 0.00 | |
| 8 | 9.50 | 0.15 | 2.2 | 10.8 | 9.76 | -10.1 |
| 9 | 2.80 | 0.10 | 1.5 | -2.34 | 0.00 | |
| 10 | 11.2 | 0.10 | 1.5 | 11.4 | 10.8 | -5.10 |
| 11 | 7.00 | 0.02 | 1.5 | 1.75 | 0.500 | -250 |
| 12 | 7.00 | 0.18 | 1.5 | 5.60 | 8.65 | 35.3 |
| 13 | 7.00 | 0.10 | 0.32 | 8.06 | 8.93 | 9.73 |
| 14 | 7.00 | 0.10 | 2.7 | 8.11 | 9.04 | 10.3 |
| 15 | 7.00 | 0.10 | 1.5 | 7.14 | 7.43 | 3.96 |
| 16 | 7.00 | 0.10 | 1.5 | 7.14 | 6.98 | -2.28 |
| 17 | 7.00 | 0.10 | 1.5 | 7.14 | 7.69 | 7.18 |
| 18 | 7.00 | 0.10 | 1.5 | 7.14 | 6.29 | -13.4 |
| 19 | 7.00 | 0.10 | 1.5 | 7.14 | 7.22 | 1.11 |
| 20 | 7.00 | 0.10 | 1.5 | 7.14 | 6.90 | -3.43 |

## B 3. Experimental data and response surface predicted values of the factorial planning for [Ch]Cl

Table B 3.1 exhibits the experimental data and response surface predicted values of the factorial planning for RuBisCOs' extraction yield.

**Table B 3.1.** Experimental data and response surface predicted values of the factorial planning for RuBisCOs' extraction yield.

| Nº | pH | S/L RATIO | C (M) | Predicted Results | Observed Results | Relative deviation (%) |
|---|---|---|---|---|---|---|
| 1 | 4.50 | 0.05 | 0.80 | 8.73 | 7.36 | -18.7 |
| 2 | 9.50 | 0.05 | 0.80 | 10.2 | 8.80 | -15.5 |
| 3 | 4.50 | 0.15 | 0.80 | 6.36 | 6.00 | -5.96 |
| 4 | 9.50 | 0.15 | 0.80 | 7.41 | 6.72 | -10.3 |
| 5 | 4.50 | 0.05 | 2.2 | 10.4 | 10.0 | -4.29 |
| 6 | 9.50 | 0.05 | 2.2 | 11.9 | 11.2 | -6.82 |
| 7 | 4.50 | 0.15 | 2.2 | 8.56 | 8.80 | 2.73 |
| 8 | 9.50 | 0.15 | 2.2 | 9.66 | 9.92 | 2.56 |
| 9 | 2.80 | 0.10 | 1.5 | 6.47 | 7.07 | 8.52 |
| 10 | 11.2 | 0.10 | 1.5 | 8.61 | 9.59 | 10.3 |
| 11 | 7.00 | 0.02 | 1.5 | 12.3 | 14.1 | 12.8 |
| 12 | 7.00 | 0.18 | 1.5 | 8.35 | 8.14 | -2.59 |
| 13 | 7.00 | 0.10 | 0.32 | 7.84 | 9.55 | 17.9 |
| 14 | 7.00 | 0.10 | 2.7 | 11.2 | 11.1 | -1.13 |
| 15 | 7.00 | 0.10 | 1.5 | 8.45 | 9.02 | 6.30 |
| 16 | 7.00 | 0.10 | 1.5 | 8.45 | 8.72 | 3.09 |
| 17 | 7.00 | 0.10 | 1.5 | 8.45 | 7.39 | -14.4 |
| 18 | 7.00 | 0.10 | 1.5 | 8.45 | 7.67 | -10.2 |
| 19 | 7.00 | 0.10 | 1.5 | 8.45 | 8.77 | 3.64 |
| 20 | 7.00 | 0.10 | 1.5 | 8.45 | 8.87 | 4.70 |

Table B 3.2 exhibits the experimental data and response surface predicted values of the factorial planning for RuBisCOs' concentration.

**Table B 3.2.** Experimental data and response surface predicted values of the factorial planning for RuBisCOs' concentration.

| Nº | pH | S/L RATIO | C (M) | Predicted Results | Observed Results | Relative deviation (%) |
|---|---|---|---|---|---|---|
| 1 | 4.50 | 0.05 | 0.80 | 0.516 | 0.519 | 0.530 |
| 2 | 9.50 | 0.05 | 0.80 | 0.526 | 0.581 | 9.49 |
| 3 | 4.50 | 0.15 | 0.80 | 1.25 | 1.34 | 6.74 |
| 4 | 9.50 | 0.15 | 0.80 | 1.37 | 1.37 | -0.036 |
| 5 | 4.50 | 0.05 | 2.2 | 0.410 | 0.370 | -10.9 |
| 6 | 9.50 | 0.05 | 2.2 | 0.541 | 0.410 | -31.9 |
| 7 | 4.50 | 0.15 | 2.2 | 1.12 | 1.03 | -9.36 |
| 8 | 9.50 | 0.15 | 2.2 | 1.37 | 1.33 | -3.29 |
| 9 | 2.80 | 0.10 | 1.5 | 0.703 | 0.709 | 0.892 |
| 10 | 11.2 | 0.10 | 1.5 | 0.921 | 0.973 | 5.31 |
| 11 | 7.00 | 0.02 | 1.5 | 0.189 | 0.237 | 20.2 |
| 12 | 7.00 | 0.18 | 1.5 | 1.50 | 1.51 | 0.673 |
| 13 | 7.00 | 0.10 | 0.32 | 1.05 | 0.940 | -11.4 |
| 14 | 7.00 | 0.10 | 2.7 | 0.956 | 1.12 | 14.8 |
| 15 | 7.00 | 0.10 | 1.5 | 0.854 | 0.898 | 4.90 |
| 16 | 7.00 | 0.10 | 1.5 | 0.854 | 0.901 | 5.21 |
| 17 | 7.00 | 0.10 | 1.5 | 0.854 | 0.744 | -14.9 |
| 18 | 7.00 | 0.10 | 1.5 | 0.854 | 0.777 | -9.98 |
| 19 | 7.00 | 0.10 | 1.5 | 0.854 | 0.905 | 5.59 |
| 20 | 7.00 | 0.10 | 1.5 | 0.854 | 0.890 | 4.04 |

## B 4. Regression coefficients of the predicted second-order polynomial model from RSM using [Ch][Acetate]

Table B 4.1 exhibits the regression coefficients of the predicted second-order polynomial model for the RuBisCOs' extraction yield from RSM.

**Table B 4.1.** Regression coefficients of the predicted second-order polynomial model for the RuBisCOs' extraction yield from RSM, $R^2 = 0.89709$ and $r_{adj,} = 0.80447$.

| | Regression Coefficients | Standard deviation | t-student (10) | p-value |
|---|---|---|---|---|
| **Interception** | -16.01 | 7.663 | -2.097 | 0.0624 |
| **pH** | 3.510 | 1.309 | 2.682 | 0.0230 |
| **pH $^2$** | -0.1482 | 0.0757 | -1.958 | 0.0787 |
| **Solid-liquid Ratio** | 109.5 | 59.39 | 1.844 | 0.0950 |
| **Solid-liquid Ratio $^2$** | -489.8 | 189.2 | -2.589 | 0.0270 |
| **IL Concentration** | -1.905 | 4.311 | -0.4418 | 0.6680 |
| **IL Concentration$^2$** | 0.6838 | 0.9653 | 0.7083 | 0.4949 |
| **pH x Solid-liquid Ratio** | 1.918 | 5.075 | 0.3779 | 0.7134 |
| **pH x IL Concentration** | 0.0025 | 0.3625 | 0.0069 | 0.9946 |
| **Solid-liquid Ratio x IL Concentration** | -1.407 | 18.13 | -0.0776 | 0.9396 |

**Table B 4.1.1.** Effects of the variables in the second-order polynomial model for the extraction yield of RuBisCO.

| | Regression Coefficients | Standard deviation | t-student (10) | p-value |
|---|---|---|---|---|
| **Interception** | 7.137 | 0.7318 | 9.752 | 0.0000 |
| **pH** | 8.158 | 0.9713 | 8.399 | 0.0000 |
| **pH $^2$** | -1.852 | 0.9460 | -1.958 | 0.0787 |
| **Solid-liquid Ratio** | 2.287 | 0.9713 | 2.354 | 0.0404 |
| **Solid-liquid Ratio $^2$** | -2.449 | 0.9460 | -2.589 | 0.0270 |
| **IL Concentration** | 0.0326 | 0.9713 | 0.0336 | 0.9739 |
| **IL Concentration$^2$** | 0.6701 | 0.9460 | 0.7083 | 0.4949 |
| **pH x Solid-liquid Ratio** | 0.4795 | 1.269 | 0.3779 | 0.7134 |
| **pH x IL Concentration** | 0.0088 | 1.269 | 0.0069 | 0.9946 |
| **Solid-liquid Ratio x IL Concentration** | -0.0985 | 1.269 | -0.0776 | 0.9396 |

Table B 4.2 exhibits the regression coefficients of the predicted second-order polynomial model for the RuBisCOs' concentration from RSM.

**Table B 4.2.** Regression coefficients of the predicted second-order polynomial model for the RuBisCOs' concentration from RSM, $R^2 = 0.90873$ and $r_{adj.} = 0.82659$.

| | Regression Coefficients | Standard deviation | t-student (10) | p-value |
|---|---|---|---|---|
| **Interception** | -0.8088 | 0.9426 | -0.8580 | 0.4110 |
| **pH** | 0.2453 | 0.1610 | 1.524 | 0.1586 |
| **pH $^2$** | -0.0200 | 0.0093 | -2.144 | 0.0577 |
| **Solid-liquid Ratio** | -4.665 | 7.305 | -0.6386 | 0.5374 |
| **Solid-liquid Ratio $^2$** | -13.08 | 23.27 | -0.5620 | 0.5865 |
| **IL Concentration** | -0.0179 | 0.5303 | -0.0338 | 0.9737 |
| **IL Concentration$^2$** | 0.0128 | 0.1187 | 0.1079 | 0.9162 |
| **pH x Solid-liquid Ratio** | 2.046 | 0.6243 | 3.277 | 0.0083 |
| **pH x IL Concentration** | -0.0006 | 0.0446 | -0.0145 | 0.9887 |
| **Solid-liquid Ratio x IL Concentration** | -0.0793 | 2.230 | -0.0356 | 0.9723 |

**Table B 4.2.1.** Effects of the variables in the second-order polynomial model for the extraction RuBisCO concentration.

| | Regression Coefficients | Standard deviation | t-student (10) | p-value |
|---|---|---|---|---|
| **Interception** | 0.7488 | 0.0900 | 8.318 | 0.0000 |
| **pH** | 0.8479 | 0.1195 | 7.097 | 0.0000 |
| **pH $^2$** | -0.2494 | 0.1164 | -2.144 | 0.0577 |
| **Solid-liquid Ratio** | 0.6922 | 0.1195 | 5.794 | 0.0002 |
| **Solid-liquid Ratio $^2$** | -0.0654 | 0.1164 | -0.5620 | 0.5865 |
| **IL Concentration** | 0.0113 | 0.1195 | 0.0948 | 0.9264 |
| **IL Concentration$^2$** | 0.0126 | 0.1164 | 0.1079 | 0.9162 |
| **pH x Solid-liquid Ratio** | 0.5115 | 0.1561 | 3.277 | 0.0083 |
| **pH x IL Concentration** | -0.0023 | 0.1561 | -0.0145 | 0.9887 |
| **Solid-liquid Ratio x IL Concentration** | -0.0056 | 0.1561 | -0.0356 | 0.9723 |

## B 5. Regression coefficients of the predicted second-order polynomial model from RSM using [Ch]Cl

Table B 5.1 exhibits the regression coefficients of the predicted second-order polynomial model for the RuBisCOs' extraction yield from RSM.

**Table B 5.1.** Regression coefficients of the predicted second-order polynomial model for the RuBisCOs' extraction yield from RSM, $R^2 = 0.75851$ and $r_{adj.} = 0.54116$.

| | Regression Coefficients | Standard deviation | t-student (10) | p-value |
|---|---|---|---|---|
| **Interception** | 8.737 | 5.258 | 1.662 | 0.1276 |
| **pH** | 1.043 | 0.8981 | 1.161 | 0.2726 |
| **pH $^2$** | -0.0518 | 0.0519 | -0.9967 | 0.3424 |
| **Solid-liquid Ratio** | -75.50 | 40.75 | -1.853 | 0.0936 |
| **Solid-liquid Ratio $^2$** | 262.2 | 129.8 | 2.020 | 0.0710 |
| **IL Concentration** | -1.267 | 2.958 | -0.4283 | 0.6775 |
| **IL Concentration$^2$** | 0.7607 | 0.6624 | 1.149 | 0.2775 |
| **pH x Solid-liquid Ratio** | -0.7645 | 3.483 | -0.2195 | 0.8306 |
| **pH x IL Concentration** | 0.0080 | 0.2487 | 0.0320 | 0.9751 |
| **Solid-liquid Ratio x IL Concentration** | 3.471 | 12.44 | 0.2791 | 0.7859 |

**Table B 5.1.1.** Effects of the variables in the second-order polynomial model for the extraction yield of RuBisCO.

| | Regression Coefficients | Standard deviation | t-student (10) | p-value |
|---|---|---|---|---|
| **Interception** | 8.453 | 0.5021 | 16.83 | 0.0000 |
| **pH** | 1.269 | 0.6665 | 1.903 | 0.0862 |
| **pH $^2$** | -0.6470 | 0.6491 | -0.9967 | 0.3424 |
| **Solid-liquid Ratio** | -2.320 | 0.6665 | -3.481 | 0.0059 |
| **Solid-liquid Ratio $^2$** | 1.311 | 0.6491 | 2.020 | 0.0710 |
| **IL Concentration** | 1.985 | 0.6665 | 2.978 | 0.0139 |
| **IL Concentration$^2$** | 0.7455 | 0.6491 | 1.148 | 0.2775 |
| **pH x Solid-liquid Ratio** | -0.1911 | 0.8706 | -0.2195 | 0.8306 |
| **pH x IL Concentration** | 0.0279 | 0.8706 | 0.0320 | 0.9751 |
| **Solid-liquid Ratio x IL Concentration** | 0.2430 | 0.8706 | 0.2791 | 0.7859 |

Table B 5.2 exhibits the regression coefficients of the predicted second-order polynomial model for the RuBisCOs' concentration from RSM.

**Table B 5.2.** Regression coefficients of the predicted second-order polynomial model for the RuBisCOs' concentration from RSM, $R^2 = 0.95197$ and $r_{adj.} = 0.90875$.

| | Regression Coefficients | Standard deviation | t-student (10) | p-value |
|---|---|---|---|---|
| **Interception** | 0.3896 | 0.4504 | 0.8649 | 0.4074 |
| **pH** | 0.0097 | 0.0769 | 0.1261 | 0.9021 |
| **pH $^2$** | -0.0024 | 0.0044 | -0.5342 | 0.6049 |
| **Solid-liquid Ratio** | 6.583 | 3.491 | 1.886 | 0.0886 |
| **Solid-liquid Ratio $^2$** | -1.257 | 11.12 | -0.1130 | 0.9122 |
| **IL Concentration** | -0.4671 | 0.2534 | -1.843 | 0.0951 |
| **IL Concentration$^2$** | 0.1067 | 0.0567 | 1.880 | 0.0895 |
| **pH x Solid-liquid Ratio** | 0.2366 | 0.2983 | 0.7932 | 0.4461 |
| **pH x IL Concentration** | 0.0172 | 0.0213 | 0.8084 | 0.4377 |
| **Solid-liquid Ratio x IL Concentration** | -0.1247 | 1.065 | -0.1170 | 0.9092 |

**Table B 5.2.1.** Effects of the variables in the second-order polynomial model for the extraction RuBisCO concentration.

| | Regression Coefficients | Standard deviation | t-student (10) | p-value |
|---|---|---|---|---|
| **Interception** | 0.8540 | 0.0430 | 19.85 | 0.0000 |
| **pH** | 0.1297 | 0.0571 | 2.271 | 0.0465 |
| **pH $^2$** | -0.0297 | 0.0556 | -0.5342 | 0.6049 |
| **Solid-liquid Ratio** | 0.7801 | 0.0571 | 13.66 | 0.0000 |
| **Solid-liquid Ratio $^2$** | -0.0063 | 0.0556 | -0.1130 | 0.9122 |
| **IL Concentration** | -0.0545 | 0.0571 | -0.9546 | 0.3623 |
| **IL Concentration$^2$** | 0.1045 | 0.0556 | 1.880 | 0.0895 |
| **pH x Solid-liquid Ratio** | 0.0592 | 0.0746 | 0.7932 | 0.4461 |
| **pH x IL Concentration** | 0.0603 | 0.0746 | 0.8084 | 0.4377 |
| **Solid-liquid Ratio x IL Concentration** | -0.0087 | 0.0746 | -0.1170 | 0.9092 |

## B 6. Pareto charts for the standardized main effects in the factorial planning with [Ch][Acetate]

Figure B 6.1 exhibits the pareto charts for the standardized main effects in the factorial planning for RuBisCOs' extraction yield from RSM.

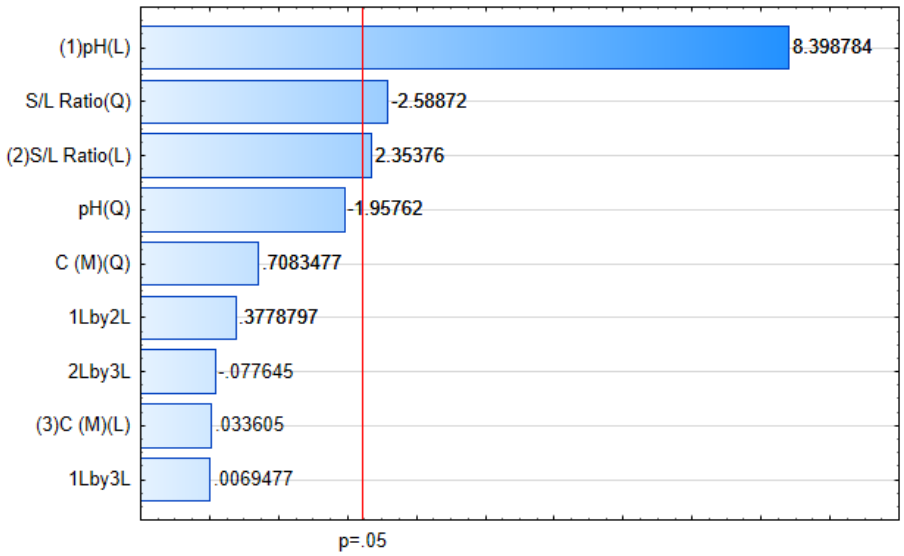


**Figure B 6.1.** Pareto charts for the standardized main effects in the factorial planning for RuBisCOs' extraction yield. The vertical line indicates the statistical significance of the effects.

Figure B 6.2 exhibits the pareto charts for the standardized main effects in the factorial planning for RuBisCOs' concentration from RSM.

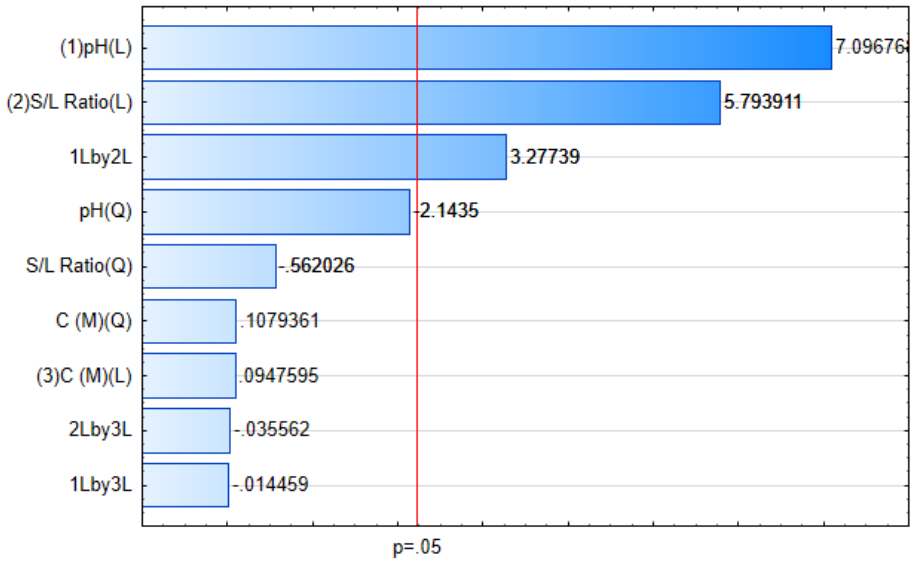


**Figure B 6.2.** Pareto charts for the standardized main effects in the factorial planning for RuBisCOs' concentration. The vertical line indicates the statistical significance of the effects.

## B 7. Pareto charts for the standardized main effects in the factorial planning with [Ch]Cl

Figure B 7.1 exhibits the pareto charts for the standardized main effects in the factorial planning for RuBisCOs' extraction yield from RSM.

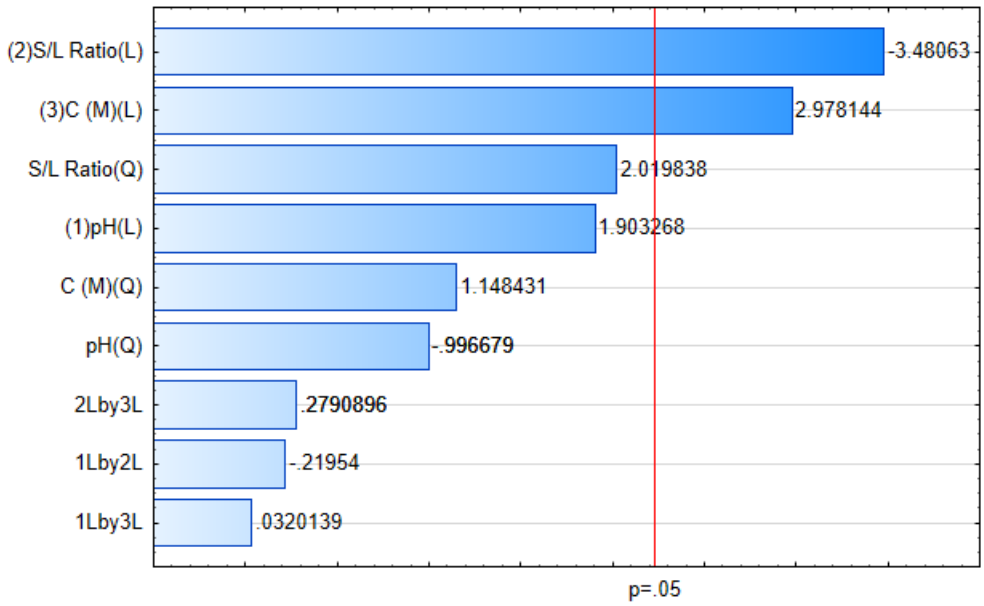


**Figure B 7.1.** Pareto charts for the standardized main effects in the factorial planning for RuBisCOs' extraction yield. The vertical line indicates the statistical significance of the effects.

Figure B 7.2 exhibits the pareto charts for the standardized main effects in the factorial planning for RuBisCOs' concentration from RSM.

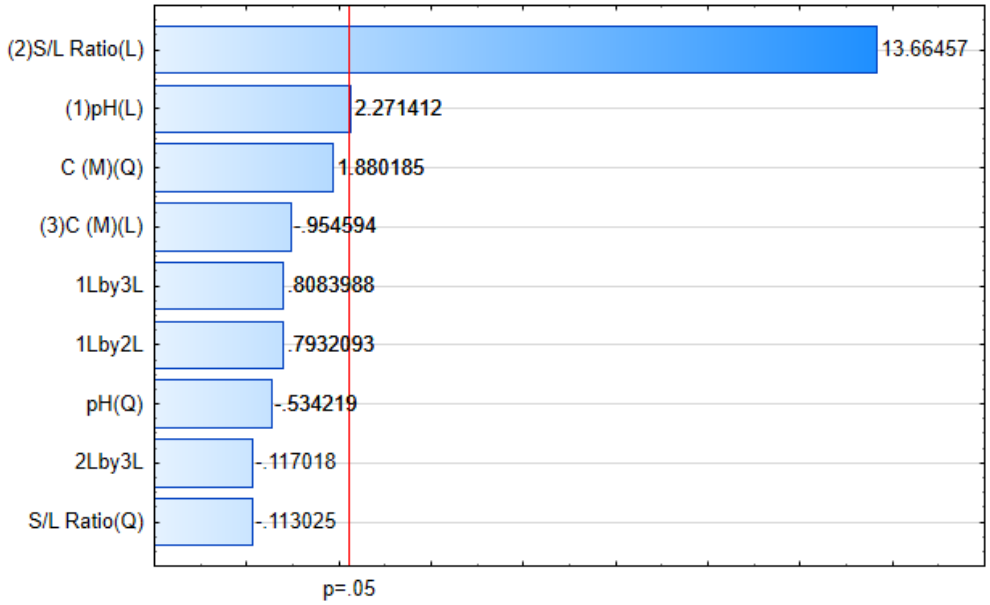


**Figure B 7.2.** Pareto charts for the standardized main effects in the factorial planning for RuBisCOs' concentration. The vertical line indicates the statistical significance of the effects.

## B 8. ANOVA data for the extraction with [Ch][Acetate]

Figure B 8.1 exhibits the ANOVA data for the extraction yield of RuBisCO.

**Table B 8.1.** ANOVA data for the extraction yield of RuBisCO.

| | Sums of squares | Degrees of freedom | Mean square | F-value | p-value |
|---|---|---|---|---|---|
| **Regression** | 280.7 | 9.00 | 31.19 | 9.686 | 0.0007 |
| **Residuals** | 32.20 | 10.0 | 3.220 | | |
| **Total** | 312.9 | | | | |

Figure B 8.2 exhibits the ANOVA data for RuBisCO concentration.

**Table B 8.2.** ANOVA data for RuBisCO concentration.

| | Sums of squares | Degrees of freedom | Mean square | F-value | p-value |
|---|---|---|---|---|---|
| **Regression** | 4.850 | 9.00 | 0.5400 | 11.06 | 0.00 |
| **Residuals** | 0.490 | 10.0 | 0.0500 | | |
| **Total** | 5.340 | | | | |

## B 9. ANOVA data for the extraction with [Ch]Cl

Figure B 9.1 exhibits the ANOVA data for the extraction yield of RuBisCO.

**Table B 9.1.** ANOVA data for the extraction yield of RuBisCO.

| | Sums of squares | Degrees of freedom | Mean square | F-value | p-value |
|---|---|---|---|---|---|
| **Regression** | 47.61 | 9.00 | 5.291 | 3.490 | 0.0322 |
| **Residuals** | 15.16 | 10.0 | 1.516 | | |
| **Total** | 62.77 | | | | |

Figure B 9.2 exhibits the ANOVA data for RuBisCO concentration.

**Table B 9.2.** ANOVA data for RuBisCO concentration.

| | Sums of squares | Degrees of freedom | Mean square | F-value | p-value |
|---|---|---|---|---|---|
| **Regression** | 2.20 | 9.00 | 0.2450 | 22.02 | 0.000019 |
| **Residuals** | 0.11 | 10.0 | 0.0110 | | |
| **Total** | 2.32 | | | | |

## B 10. Profiles for predicted values and desirability in the factorial planning with [Ch][Acetate]

Figure B 10 exhibits the profiles for predicted values and desirability in the factorial planning from RSM.

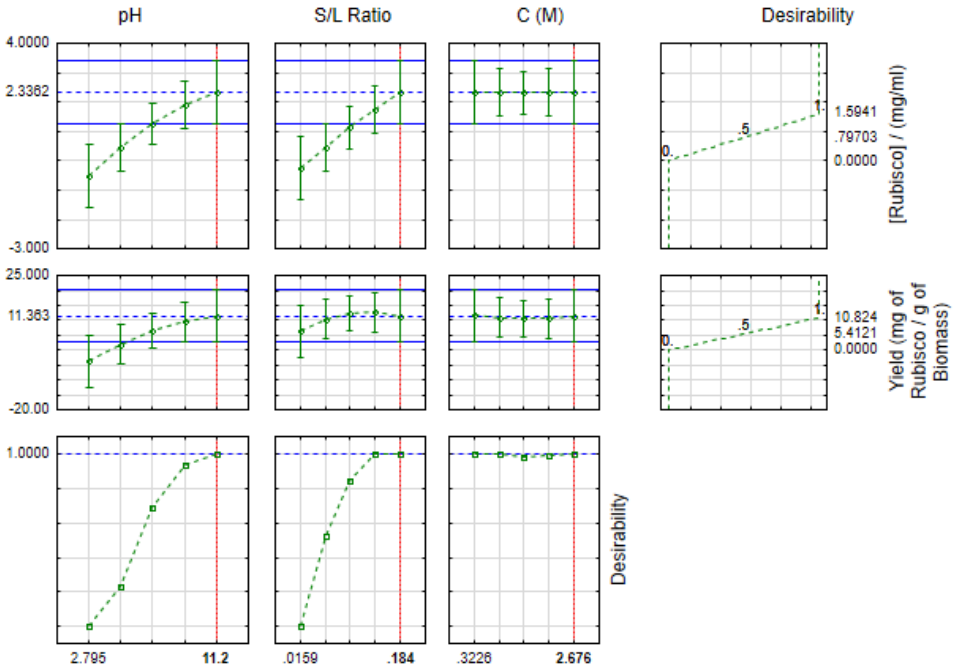


**Figure B 10.** Profiles for predicted values and desirability in the factorial planning for both dependent variables.

## B 11. Profiles for predicted values and desirability in the factorial planning with [Ch]Cl

Figure B 11 exhibits the profiles for predicted values and desirability in the factorial planning from RSM.

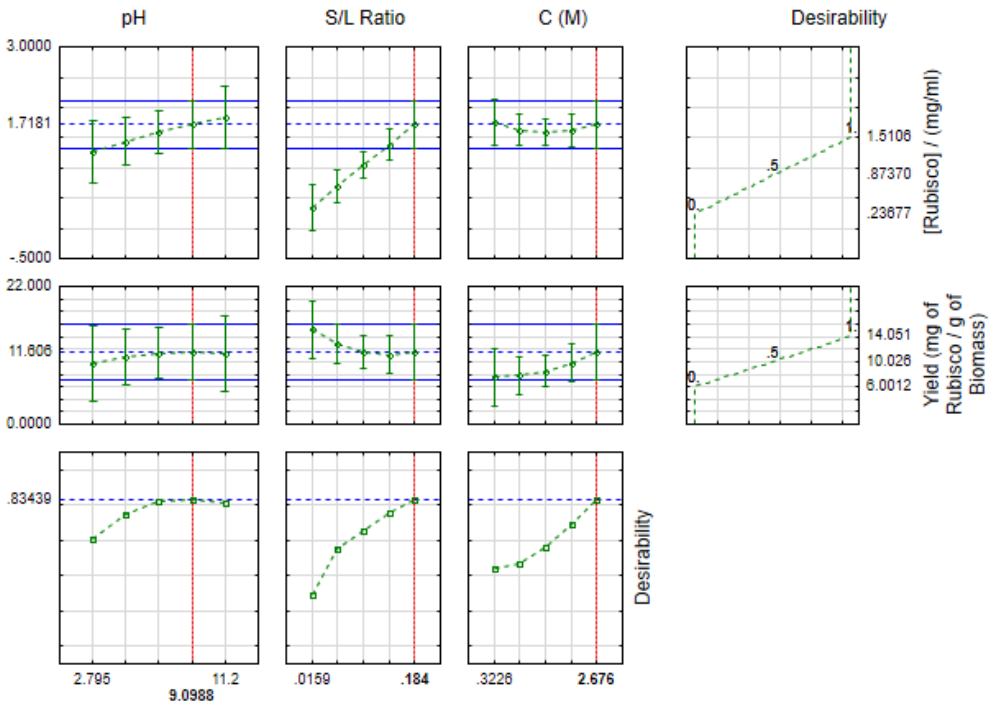


**Figure B 11.** Profiles for predicted values and desirability in the factorial planning for both dependent variables.

# Appendix C

## C 1. Experimental binodal data for systems composed of PPG 400 + [Ch][Acetate] + $H_2O$ and PPG 400 + [Ch]Cl + $H_2O$

The phase diagrams of the ABS used in this work and reported in literature are shown in Figure C 1.

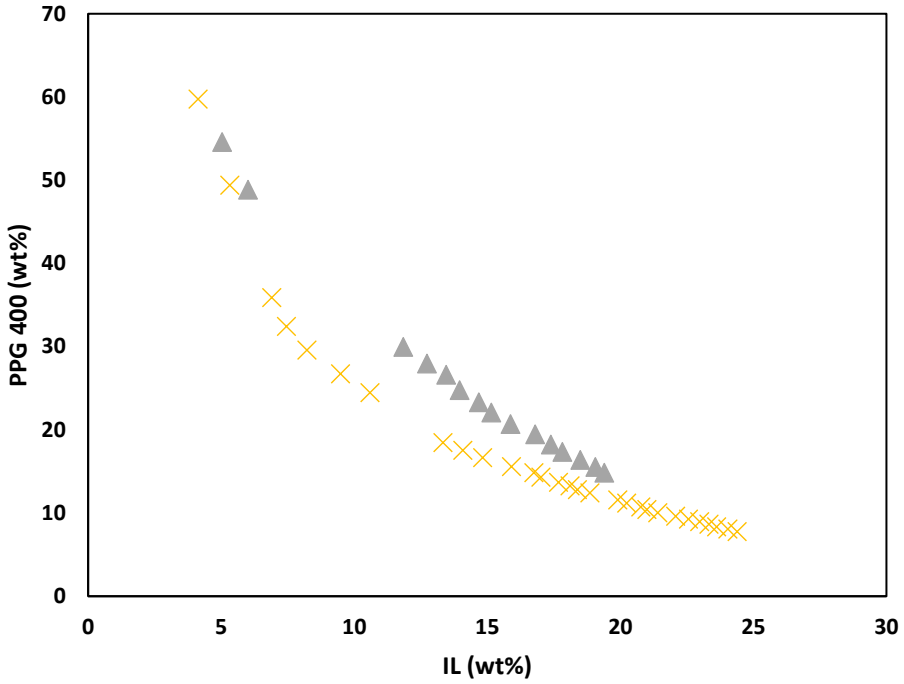


**Figure C 1.** Phase diagrams for systems composed of PPG 400 + [Ch][Acetate] + $H_2O$ (grey triangles) and PPG 400 + [Ch]Cl + $H_2O$ (yellow crosses) at 25 ºC.

The experimental weight fraction data for the phase diagrams of the systems PPG 400 + [Ch][Acetate] + $H_2O$ and PPG 400 + [Ch]Cl + $H_2O$ are presented in Table C 1.

**Table C 1.** Experimental weight fraction data for the phase diagrams of the systems PPG 400 (1) + [Ch][Acetate] (2) + $H_2O$ (3) and PPG 400 (1) + [Ch]Cl (2) + $H_2O$ (3).

| **[Ch][Acetate]** **Mw = 163.2 g/mol** | | **[Ch]Cl** **Mw = 139.6 g/mol** | | | |
|---|---|---|---|---|---|
| **100 $w_1$** | **100 $w_2$** | **100 $w_1$** | **100 $w_2$** | **100 $w_1$** | **100 $w_2$** |
| 54.603 | 5.039 | 59.753 | 4.139 | 12.819 | 18.398 |
| 48.866 | 6.010 | 49.418 | 5.325 | 12.429 | 18.866 |
| 29.966 | 11.852 | 35.897 | 6.910 | 11.610 | 19.910 |
| 27.991 | 12.734 | 32.455 | 7.452 | 11.194 | 20.242 |
| 26.648 | 13.465 | 29.602 | 8.232 | 10.746 | 20.780 |
| 24.773 | 13.970 | 26.751 | 9.491 | 10.434 | 21.003 |
| 23.336 | 14.687 | 24.479 | 10.598 | 10.068 | 21.411 |
| 22.087 | 15.158 | 18.497 | 13.336 | 9.617 | 22.091 |
| 20.697 | 15.883 | 17.556 | 14.086 | 9.301 | 22.570 |
| 19.477 | 16.800 | 16.682 | 14.837 | 8.985 | 22.989 |
| 18.261 | 17.389 | 15.583 | 15.916 | 8.657 | 23.342 |
| 17.346 | 17.830 | 14.864 | 16.754 | 8.352 | 23.627 |
| 16.393 | 18.499 | 14.319 | 17.023 | 8.084 | 24.051 |
| 15.560 | 19.067 | 13.697 | 17.682 | 7.773 | 24.390 |
| 14.877 | 19.400 | 13.283 | 18.122 | | |

## C 2. Experimental binodal data for systems composed of $K_2HPO_4$ + [Ch][Acetate] + $H_2O$ and $K_2HPO_4$ + [Ch]Cl + $H_2O$

The phase diagrams of the ABS used in this work and reported in literature are shown in Figure C 2.

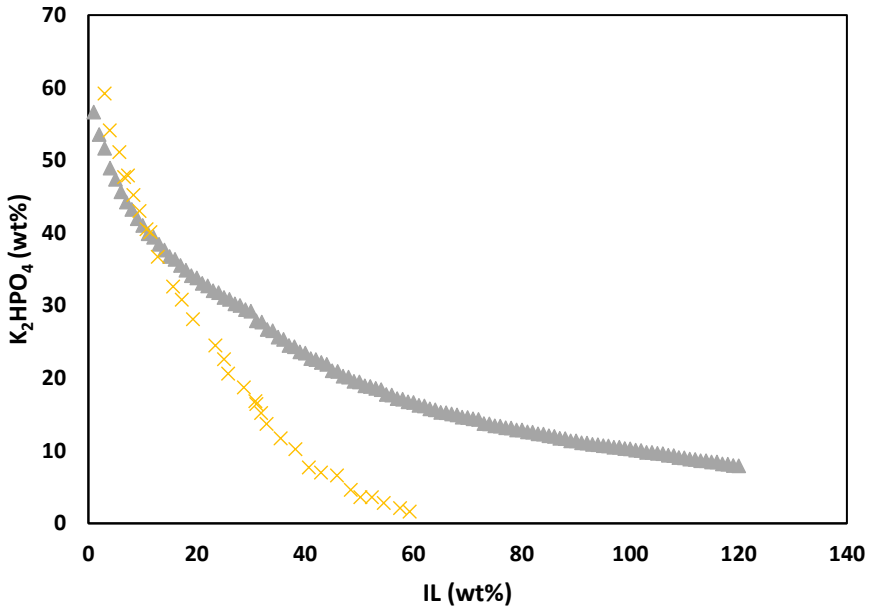


**Figure C 2.** Phase diagrams for systems composed of $K_2HPO_4$ + [Ch][Acetate] + H2O (grey triangles) and $K_2HPO_4$ + [Ch]Cl + H2O (yellow crosses) at 25 ºC.

The experimental weight fraction data for the phase diagrams of the systems $K_2HPO_4$ + [Ch][Acetate] + $H_2O$ and $K_2HPO_4$ + [Ch]Cl + $H_2O$ are presented in Table C 2.

**Table C 2.** Experimental weight fraction data for the phase diagrams of the systems $K_2HPO_4$ (1) + [Ch][Acetate] (2) + $H_2O$ (3) and $K_2HPO_4$ (1) + [Ch]Cl (2) + $H_2O$ (3).

| **[Ch]Cl** | | **[Ch][Acetate]** | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **Mw = 139.6 g/mol** | | **Mw = 163.2 g/mol** | | | | | | | |
| **100 $w_1$** | **100 $w_2$** | **100 $w_1$** | **100 $w_2$** | **100 $w_1$** | **100 $w_2$** | **100 $w_1$** | **100 $w_2$** | **100 $w_1$** | **100 $w_2$** |
| 59.2 | 3 | 56.62 | 7.79 | 26.71 | 23.34 | 15.21 | 33.89 | 10.3 | 38.98 |
| 54.1 | 3.9 | 53.55 | 7.37 | 26.53 | 23.18 | 15.05 | 34.17 | 10.27 | 38.88 |
| 51.1 | 5.7 | 51.66 | 9.23 | 25.64 | 24.42 | 14.91 | 33.87 | 10.06 | 39.3 |
| 47.9 | 7.3 | 48.9 | 8.74 | 25.31 | 24.1 | 14.61 | 34.4 | 10.01 | 39.1 |
| 47.7 | 6.6 | 47.35 | 10.36 | 24.48 | 25.27 | 14.55 | 34.27 | 9.78 | 39.58 |
| 45.2 | 8.3 | 45.67 | 9.99 | 24.32 | 25.11 | 14.4 | 34.53 | 9.75 | 39.48 |
| 43 | 9.4 | 44.23 | 11.57 | 23.61 | 26.13 | 14.29 | 34.26 | 9.62 | 39.77 |
| 40.5 | 10.7 | 43.21 | 11.31 | 23.45 | 25.96 | 13.72 | 35.28 | 9.59 | 39.67 |
| 40.1 | 11.4 | 41.95 | 12.73 | 22.68 | 27.09 | 13.67 | 35.15 | 9.36 | 40.16 |
| 36.7 | 12.8 | 41.02 | 12.45 | 22.53 | 26.91 | 13.43 | 35.6 | 9.31 | 39.94 |
| 32.6 | 15.6 | 39.86 | 13.8 | 22.15 | 27.48 | 13.37 | 35.46 | 9.06 | 40.47 |
| 30.8 | 17.2 | 39.41 | 13.64 | 21.91 | 27.18 | 13.17 | 35.83 | 9.01 | 40.26 |
| 28.1 | 19.3 | 38.4 | 14.83 | 21.02 | 28.5 | 13.12 | 35.71 | 8.83 | 40.66 |
| 24.5 | 23.4 | 37.64 | 14.54 | 20.9 | 28.33 | 12.87 | 36.16 | 8.81 | 40.56 |
| 22.6 | 25 | 36.74 | 15.63 | 20.25 | 29.31 | 12.83 | 36.03 | 8.64 | 40.94 |
| 20.6 | 25.8 | 36.36 | 15.47 | 20.14 | 29.15 | 12.58 | 36.5 | 8.62 | 40.85 |
| 18.7 | 28.7 | 35.52 | 16.51 | 19.57 | 30.02 | 12.53 | 36.36 | 8.46 | 41.19 |
| 16.8 | 30.8 | 34.85 | 16.2 | 19.47 | 29.86 | 12.31 | 36.78 | 8.42 | 41 |
| 15.2 | 31.9 | 34.1 | 17.14 | 18.94 | 30.67 | 12.26 | 36.64 | 8.17 | 41.56 |
| 16.4 | 31 | 33.78 | 16.98 | 18.85 | 30.53 | 12.05 | 37.06 | 8.13 | 41.36 |
| 13.7 | 32.9 | 33.04 | 17.91 | 18.59 | 30.93 | 11.97 | 36.83 | 7.95 | 41.79 |
| 11.7 | 35.5 | 32.71 | 17.74 | 18.41 | 30.62 | 11.69 | 37.37 | 7.91 | 41.58 |
| 10.2 | 38.2 | 32.02 | 18.63 | 17.75 | 31.66 | 11.65 | 37.24 | | |
| 7.7 | 40.7 | 31.77 | 18.48 | 17.65 | 31.48 | 11.37 | 37.79 | | |
| 7 | 42.9 | 31.1 | 19.36 | 17.18 | 32.24 | 11.33 | 37.66 | | |
| 6.6 | 45.9 | 30.85 | 19.2 | 17.1 | 32.08 | 11.09 | 38.14 | | |
| 4.6 | 48.4 | 30.24 | 20 | 16.71 | 32.71 | 11.05 | 38.01 | | |
| 3.6 | 50.2 | 29.98 | 19.83 | 16.64 | 32.57 | 10.88 | 38.36 | | |
| 3.6 | 52.3 | 29.43 | 20.57 | 16.24 | 33.22 | 10.84 | 38.24 | | |
| 2.8 | 54.5 | 29.2 | 20.41 | 16.16 | 33.06 | 10.68 | 38.57 | | |
| 2.1 | 57.5 | 27.89 | 22.18 | 15.75 | 33.75 | 10.64 | 38.45 | | |
| 1.6 | 59.3 | 27.69 | 22.01 | 15.62 | 33.47 | 10.49 | 38.77 | | |
| 59.2 | 3 | 56.62 | 7.79 | 15.28 | 34.04 | 10.46 | 38.66 | | |