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Abstract: Ribulose-1,5-biphosphate carboxylase/oxygenase (RuBisCO) is the most abundant protein on the planet, being present in plants, algae and various species of bacteria, with application in the pharmaceutical, chemical, cosmetic and food industries. However, current extraction methods of RuBisCO do not allow high yields of extraction. Therefore, the development of an efficient and selective RuBisCOs' extraction method is required. In this work, aqueous solutions of biocompatible ionic liquids (ILs), i.e., ILs derived from choline and analogues of glycine-betaine, were applied in the RuBisCO's extraction from spinach leaves. Three commercial imidazolium-based ILs were also investigated for comparison purposes. To optimize RuBisCO's extraction conditions, response surface methodology was applied. Under optimum extraction conditions, extraction yields of 10.92 and 10.57 mg of RuBisCO/g of biomass were obtained with the ILs cholinium acetate ([Ch][Ac]) and cholinium chloride ([Ch]Cl), respectively. Circular dichroism (CD) spectroscopy results show that the secondary structure of RuBisCO is better preserved in the IL solutions when compared to the commonly used extraction solvent. The obtained results indicate that cholinium-based ILs are a promising and viable alternative for the extraction of RuBisCO from vegetable biomass.

Keywords: Ribulose-1,5-biphosphate carboxylase/oxygenase (RuBisCO); spinach; solid-liquid extraction; biocompatible ionic liquids

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1. Introduction

Ribulose-1,5-biphosphate carboxylase/oxygenase, RuBisCO (EC 4.1.1.39), is the most abundant protein (enzyme) and is responsible for the atmospheric carbon fixation in photosynthetic organisms, such as plants, algae and various species of bacteria [1–5]. RuBisCO can be applied in several fields, such as food and feed, chemical, pharmaceutical and cosmetic industries [1,6–15]. In animal feed, RuBisCO is already employed as a protein source through the incorporation of a white protein concentrate, which consists of soluble leaf proteins [4,11]. Furthermore, RuBisCO gels (products from thermal denaturation) have the potential to be used in food formulation [12], while undenatured RuBisCO can be applied in foaming and emulsifying and to form gels [12]. RuBisCO peptides can be used in functional foods and nutraceuticals [2] or as additives [11], or simply as protein source [14]. Martin et al. [14] investigated RuBisCO properties, such as solubility, foam formation and stability, emulsion formation and stability, water holding capacity and gelation and fracture properties of gels, concluding that RuBisCO has the same or better results than soy and isolated whey protein. However, RuBisCO is not used in the human food industry, in which higher purity levels of RuBisCO are required, since the current extraction procedure

to obtain this protein at a high purity level is not economically viable due to the associated costs [11,16] and due to the low quality of the extract and presence of polyphenols [11]. On the other hand, when addressing applications such as cosmetics and pharmaceuticals, high-purity RuBisCO is demanded.

There are several reported methods for RuBisCO's extraction, such as percolation or maceration [17]. RuBisCO and other intracellular leaf proteins are usually extracted through the same general procedure, which involves four major steps: (i) mechanical disintegration of the plants tissue; (ii) protein solubilization; (iii) protein precipitation; and (iv) protein concentration [1,3,9]. The combination of the two first steps is designated as solid-liquid extraction (SLE), allowing soluble components to be extracted from the solid biomass using a solvent [18]. In addition, it is possible to improve the extraction of RuBisCO though the tailoring of other operating conditions, such as pH, use of given organic solvents and high temperatures. However, most of these methods lead to protein denaturation [4]. As examples, Zhang et al. [5] used a conjugation of heat and pH treatment to extract all the proteins in leaves; at optimal conditions, the authors were able to extract 95% of total protein content in 4 h at 95 °C using a solution of sodium hydroxide (NaOH) at 0.1 M and pH of 3.5. Kobbi et al. [2] studied the viability and efficiency of ammonium hydroxide (NH_4OH) to extract the protein, with further RuBisCO precipitation provoked with the adjustment of pH down to 3 through the addition of formic acid. The authors extracted RuBisCO and, at the same time, removed 80% of the polyphenols [2]. However, there are some reservations with the use of these harsh conditions, and for which the use of alternative and environmentally friendly solvents may be a viable alternative.

Ionic liquids (ILs) and their aqueous solutions have emerged as promising solvents in the solid-liquid extraction of high-value compounds from biomass [19]. When properly designed, they allow to solve some problems associated to conventional solvents due to their negligible vapor pressure, high chemical and thermal stability, ionic conductivity and non-inflammability [20,21]. ILs are ionic compounds with low melting temperatures, being constituted by a large organic cation and an organic or inorganic anion [19,21]. Although less investigated than in the extraction of low molecular weight compounds, ILs have been used in the SLE of some proteins [22–25], with interesting results that support their application to replace traditional organic solvents. For example, Martins et al. [24] extracted phycobiliproteins from the red macroalga *Gracilaria* sp. using IL aqueous solutions. The authors reported an improvement of 46.5% using a [Ch]Cl aqueous solution when compared with phosphate buffer, and obtained a similar purity to other procedures and solvents [24]. Wang et al. [25] attempted to extract proteins from Chlorella microalgae, identifying the IL 3-(dimethylamino)-1-propylamine formate able to lead to an extraction yield (protein content in the extract/total protein content in the cell) up to 12.1% [25].

Despite the ILs advantages over organic solvents, if not properly designed, they may display a non-negligible toxicity and have low biodegradability [26]. Most of the time, imidazolium-based ILs are not the best option in the field of SLE of biomolecules when considering their cost, toxicity and low biodegradability [20,24–27]. Aiming at moving towards more sustainable solvents, cholinium-based ILs and analogues of glycine-betaine-based ILs (AGB-ILs) can be seen as promising alternatives in the field of extraction of proteins from biomass [24,28]. If properly selected, cholinium-based ILs preserve the tertiary structure of proteins and have lower toxicity than imidazolium-based counterparts [28]. On the other hand, AGB-ILs also display low cytotoxicity and ecotoxicity, supporting their direct use in nutraceutical extracts envisioning human consumption [24,29]. Choline is an essential micronutrient, completely degradable under aerobic conditions and was approved as food additive, whereas glycine-betaine is a methyl derivative of glycine present in biologic fluids, plants and microorganisms [28,30–32].

Taking into account the advantages of these biocompatible ILs (cholinium-based and AGB-ILs), we studied here their application in aqueous solutions for the extraction of RuBisCO from spinach leaves. Spinach was selected since it is one of the most studied green biomass for protein extraction, along with alfalfa and tobacco [14,15]. An initial

screening of several ILs was made, in which some more traditional imidazolium-based ILs were considered for comparison purposes. A response surface methodology (RSM) for experimental conditions optimization, namely the pH, IL concentration and solid-liquid (biomass-solvent) ratio was applied, where the studied responses were the concentration of extracted RuBisCO and the extraction yield. Finally, aqueous solution of NH_4OH was applied to compare the concentration of extracted RuBisCO and the extraction yield between the extraction with conventional solvent and the extraction with ILs at the optimal conditions identified with the RSM.

2. Materials and Methods

2.1. Materials

Spinach (*Spinacia oleracea*) was purchased at a local supermarket (Continente Bomdia, Aveiro, distributed by VITACRESS, Portugal). The spinach leaves were immediately washed and frozen at −80 °C. The biomass was frozen at −80 °C in order to use the same sample along all studies and thus reduce the spinach sample variability, while keeping the proteins stability. RuBisCO standard (D-Ribulose 1,5-diphosphate carboxylase from spinach, partially purified powder, 0.01–0.1 unit/mg solid) and phosphate buffered saline (PBS) were purchased from Sigma-Aldrich (Steinheim, Germany), while ammonium hydroxide salt (NH_4OH, >99% of purity) was supplied from Fluka (Berlin, Germany). The ILs (Figure 1) applied belong to: the cholinium-based family, namely cholinium chloride ([Ch]Cl, 98% of purity, Acros Organics, Geel, Belgium), cholinium bromide ([Ch]Br, >98% of purity, TCI), cholinium acetate ([Ch][Ac], >99% of purity, Iolitec, Heilbronn, Germany), cholinium dihydrogencitrate ([Ch][DHC], 99% of purity, Sigma-Aldrich) and cholinium dihydrogen phosphate ([Ch][DHP], >98% of purity, Iolitec); the imidazolium-based family, namely 1-ethyl-3-methylimidazolium chloride ([C_2mim]Cl, 98% of purity, Iolitec), 1-butyl-3-methylimidazolium chloride ([C_4mim]Cl, 99% of purity, Iolitec) and 1-hexyl-3-methylimidazolium chloride ([C_6mim]Cl, 98% of purity, Iolitec); and the AGB-IL family, namely 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-aminobutyl-4-oxobutyl]ammonium bromide ([$Et_3NC_4NC_4$]Br, > 98% of purity) and tri(*n*-butyl)[4-aminobutyl-4-oxobutyl]ammonium bromide ([$Bu_3NC_4NC_4$]Br, >98% of purity). Ethyl 4-bromobutyrate and tri(*n*-butyl)amine (99% of purity) were acquired from Sigma-Aldrich. All ILs were commercially acquired, excepted for AGB-ILs which were synthesized by us according to the procedure reported by Diabate et al. [33,34]. Briefly, for the synthesis of [$Et_3NC_2OC_2$][Sac] and [$Pr_3NC_2OC_2$][Sac], tri(ethyl)amine or tri(*n*-propyl)amine was quaternized with ethyl bromoacetate (98% of purity, Sigma-Aldrich). The bromide salts obtained undergo anionic metathesis reaction with saccharinate (Sac) anion, allowing to obtain the corresponding two saccharinate-based AGB-ILs. In the synthesis of [$Et_3NC_4NC_4$]Br and [$Bu_3NC_4NC_4$]Br, the reaction occurred between triethylamine or tri(*n*-butyl)amine and ethyl 4-bromobutyrate (≥99% of purity, Sigma-Aldrich), respectively. The solid compounds isolated were aminolysed with n-butylamine to give the amido-functionalized glycine-betaine-based ILs. The purity of each IL was checked by ^{1}H and ^{13}C NMR.

Tris(hydroxymethyl)aminomethane (Tris, PA, Pronalab, Lisbon, Portugal), sodium dodecyl sulfate (SDS, 99% of purity, Acros Organics, Geel, Belgium), glycerol (99% of purity, Acros Organics), bromophenol blue (pure, Merck, Kenilworth, NJ, USA), dithiothreitol (DTT, 99% of purity, Acros Organics), RunBlue 20x SDS run buffer TEO–Tricine–SDS (Expedeon, Cambridge, UK), RunBlue SDS Gel 4-12%, 12 wells, GRS Protein Marker MultiColour (grisp Research Solutions, Porto, Portugal) and BlueSafe (nzytech, Lisbon, Portugal) were used to perform sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis. Sodium phosphate dibasic heptahydrate ($Na_2HPO_4.7H_2O$, 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, Barcelona, Spain) were applied in size exclusion high-performance liquid chromatography (SE-HPLC) analysis.

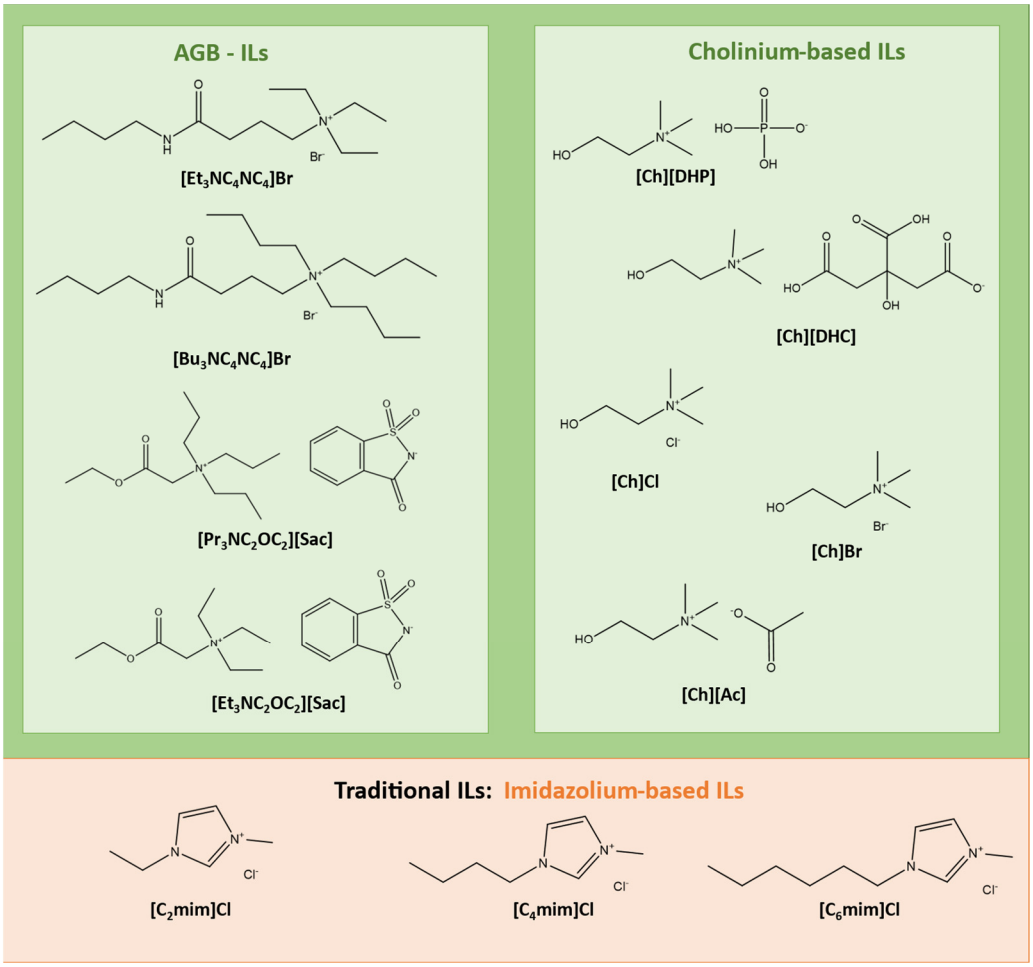

Figure 1. Structures and abbreviations of the ILs used in this work.

2.2. RuBisCOs' Extraction Using Aqueous Solutions of ILs

Before the extraction, the frozen spinach leaves were ground using liquid nitrogen in a mortar in order to obtain a homogeneous sample (biomass fragments with a similar size). Then, the RuBisCO's extraction was carried out in a Carrousel from Radleys Tech (Carousel 12 Plus Reaction Station^{TM}, Saffron Walden, Essex, UK), a commercial equipment able to both control stirring and temperature. The experimental conditions for an initial screening were adapted from Leite et al. [35], who carried out the extraction of chlorophylls from spinach leaves. The extractions corresponding to the preliminary screening were performed with a 0.10 solid–liquid ratio (biomass-solvent weight ratio), at a constant stirring of 600 rpm and temperature of (29.0 ± 0.5) °C for 30 min. The IL aqueous solutions and spinach were prepared gravimetrically within $\pm 10^{-4}$ g. To this end, aqueous solutions of $[Et_3NC_2OC_2][Sac]$, $[Et_3NC_4NC_4]Br$, $[Bu_3NC_4NC_4]Br$, $[Pr_3NC_2OC_2][Sac]$, [Ch]Cl, $[C_2mim]Cl$, $[C_4mim]Cl$ and $[C_6mim]Cl$ at 3.3 mM were first applied to identify the best IL for RuBisCO's extraction. Then, with the selected [Ch]Cl, other cholinium-based ILs aqueous solutions were tested, namely [Ch]Br, [Ch][DHC], [Ch][DHP] and [Ch][Ac], where the impact of the IL concentration (25 mM, 50 mM, 100 mM, 500 mM and 1 M) in the extraction of RuBisCO was studied. For each set of conditions, three replicas were carried out. After RuBisCO's extraction, the solutions were centrifuged at 7000 rpm for 30 min in a Neya 16R centrifuge to separate the solvent from the biomass. The supernatant enriched in protein was analyzed by SDS-PAGE and SE-HPLC (see description below). The pH of each extract was measured at (25.0 ± 0.1) °C using a Metrohm 827 pHmeter.

2.3. Response Surface Methodology (RSM)

After the initial screening using different IL aqueous solutions, [Ch]Cl and [Ch][Ac] were selected as the best solvents to be further applied in the optimization of the extraction of RuBisCO from spinach leaves. pH, solid-liquid ratio and IL concentration were the factors chosen to be applied in a 2^3 factorial planning. The 2^3 factorial planning contains a central point (level zero), factorial points (1 and −1, level one) and axial points (level α)—cf. the Supporting Information (SI), Table S1. The central and factorial points assume values that depend on the work carried out. In this case, it was assumed for the central point a pH of 7.0, a solid-liquid ratio of 0.10 and an IL concentration of 1.50 M. The factorial points were defined to analyze a broad range of operating conditions. 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 (Equation (1)) [36]:

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

Table 1. Independent variables' coded levels applied in the factorial planning for the extraction of RuBisCO from spinach.

Studied Parameters	Level				
	Axial −1.68	**Factorial −1**	**Central 0**	**Factorial 1**	**Axial 1.68**
pH	2.8	4.5	7.0	9.5	11.2
Solid-liquid ratio	0.02	0.05	0.10	0.15	0.18
IL concentration (M)	0.32	0.80	1.50	2.20	2.68

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

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

where y is the response variable, β_0, β_i, β_{ij} and β_{ij} are the adjusted coefficients for the intercept, linear, quadratic and interaction terms, respectively, and X_i and X_j are independent variables. The analysis of the surface response curves resulting from this method leads to the determination of the optimal conditions for RuBisCO's extraction.

This planning allows the analysis of various operating conditions simultaneously and identify the most significant parameters that enhance the extraction yield and the concentration of extracted RuBisCO. The remaining experimental conditions were as follows: 0.10 solid-liquid (biomass-solvent) ratio, constant stirring of 600 rpm and temperature of (29.0 ± 0.5) °C for 30 min. After RuBisCO's extraction, the solutions were centrifuged at 7000 rpm during 30 min in a Neya 16R centrifuge to separate the extracts from biomass. The supernatant enriched in protein was analyzed by SDS-PAGE and SE-HPLC. The pH of each extract was measured at (25.0 ± 0.1) °C using a Metrohm 827 pHmeter and the pH was adjusted with solutions of NaOH (0.1 M and 1 M) and HCl (0.1 M and 1 M).

The Statsoft Statistica 10.0© software was used in all statistical analyzes and to draw the response surfaces. The obtained results were statistically analyzed with a 95% confidence level, and a student t-test was applied to verify the statistical significance of the adjusted data (Supplementary Information, Tables S7–S13). 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 model's adequacy.

2.4. Sodium Dodecyl Sulphate–Polyacrylamide Gel Electrophoresis

The protein profile of the obtained extracts was analyzed by SDS-PAGE. The samples were diluted 1:1 (*v*:*v*) in a sample buffer composed of 2.5 mL of 0.5 M Tris-HCl pH 6.8, 4.0 mL of 10% (*w*/*v*) SDS, 2.0 mg of bromophenol blue, 2.0 mL of glycerol and 310 mg of DTT. After this dilution, samples were heated for 5 min at 95 °C. All samples were loaded (20 µL) on a polyacrylamide gel (stacking: 4% and resolving: 20%). The gels were impregnated with BlueSafe. GRS Protein Marker MultiColour was used as molecular weight standards, while commercial RuBisCO from spinach was used as RuBisCO standard.

2.5. Size Exclusion High-Performance Liquid Chromatography

Protein quantification was performed by size exclusion high-performance liquid chromatography (SE-HPLC). A calibration curve was determined for this purpose using commercial RuBisCO from spinach (Figure S1). For each RuBisCO solution, a characteristic RuBisCO peak with a retention time of ~13.4 min was obtained. The peaks with lower retention times than the RuBisCO peak correspond to protein aggregates. A 50 mM phosphate buffer containing NaCl (0.3 M) was used as mobile phase. Each sample was diluted 1:9 (*v*:*v*) in the phosphate buffer and then injected on a Chromaster HPLC system (VWR Hitachi). The SE-HPLC run was performed on an analytical column Shodex Protein KW-802.5 (8 mm × 300 mm). The mobile phase run isocratically with a flow rate of 0.5 mL/min with an injection volume of 25 µL. The column oven and autosampler temperatures were kept at 40 °C and 10 °C, respectively. The wavelength was set at 280 nm using a DAD detector. The obtained chromatograms were analyzed using the PeakFit version 4 software. The SE-HPLC method applied was optimized for proteins quantification. Samples of IL aqueous solutions (with no proteins) were also run in the equipment, showing that they do not interfere with protein quantification.

The purity (%$\text{Purity}_{\text{RuBisCO}}$) and yield of RuBisCO ($\text{Yield}_{\text{RuBisCO}}$) were calculated using Equations 3 and 4, respectively. The purity 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 $\text{Yield}_{\text{RuBisCO}}$ was calculated by the ratio between the mass of RuBisCO in the extract (m_{extract}) and the biomass mass (m_{biomass}). The extracted concentration of RuBisCO ($C_{[\text{RuBisCO}]\text{extract}}$, mg/mL) was also calculated, applying a calibration curve determined for this purpose using the commercially acquired enzyme:

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

$$\begin{aligned} \text{Yield}_{\text{RuBisCO}}(\text{mg of RuBisCO/g of biomass}) &= \frac{m_{\text{extract}}}{m_{\text{biomass}}} \\ &= \frac{C_{\text{RuBisCO}_{\text{extract}}} \times V_{\text{extract}}}{m_{\text{biomass}}} \end{aligned} \quad (4)$$

2.6. Circular Dichroism Spectroscopy

The RuBisCO secondary structure in [Ch][Ac] or [Ch]Cl aqueous solutions was evaluated by circular dichroism (CD) spectroscopy using a Jasco J-1500CD spectrometer. The evaluated samples were the extracts at the optimal conditions. For comparison purposes, a solution of RuBisCO standard 1 mg/mL dissolved in PBS was analyzed. CD spectra were recorded at room temperature in the far UV region, from 190 to 350 nm, using quartz CD cuvettes (0.1 cm) at a scan rate of 50 nm/min and sensitivity of 100 mdeg. The response time and the bandwidth were 4 s and 1 nm, respectively.

3. Results and Discussion

3.1. RuBisCOs' Extraction from Spinach Leaves

3.1.1. Effect of IL Aqueous Solutions on RuBisCO's Extraction

Several parameters can affect the extraction of proteins from biomass, including temperature, solid-liquid ratio, pH, solvent type, concentration and time of extraction [29,37].

Thus, to first address the effect of different ILs in the RuBisCO's extraction from spinach, the time of extraction (30 min), temperature (29 °C), IL concentration (3.3 mM) and solid-liquid ratio (0.10) were kept constant. Aqueous solutions of biocompatible ILs, i.e., AGB- and cholinium-based ILs, were evaluated in order to select the most promising ILs. More specifically, four analogues of glycine-betaine-based ILs ($[Et_3NC_2OC_2][Sac]$, $[Et_3NC_4NC_4]Br$, $[Bu_3NC_4NC_4]Br$, $[Pr_3NC_2OC_2][Sac]$) and one cholinium-based IL ([Ch]Cl) were studied. For comparison purposes, three imidazolium-based ILs were also investigated ($[C_2mim]Cl$, $[C_4mim]Cl$, and $[C_6mim]Cl$).

After the SLE using all ILs, the extracts containing RuBisCO were analyzed by SDS-PAGE and SE-HPLC. SDS-PAGE results are given in Figure 2, where a band at ~55 kDa was observed corresponding to the large subunit of RuBisCO. However, depending on the IL aqueous solution, the intensity of the band changed, revealing the different extraction performance of the different aqueous solutions of ILs applied. The aqueous solution of $[Et_3NC_2OC_2][Sac]$ (Figure 2, lane 4) presents the lowest capacity in extracting RuBisCO since a less intense band at 55 kDa is observed. On the other hand, the aqueous solutions of $[Pr_3NC_2OC_2][Sac]$, [Ch]Cl and $[C_2mim]Cl$ (Figure 2, lanes 3, 7 and 8, respectively) are the most promising IL solutions identified. Compared to the results obtained with the aqueous solutions of biocompatible and imidazolium-based ILs, only $[Et_3NC_2OC_2][Sac]$ was not able to extract RuBisCO. On the other hand, the effect of the alkyl chain length in the imidazolium-based ILs is notorious in the extraction yield of RuBisCO. The longer the cation alkyl chain length, the lower the extraction capability of the solvent. However, the results obtained with the aqueous solutions of $[Et_3NC_4NC_4][Br]$ and $[Bu_3NC_4NC_4][Br]$ indicate that there are no major differences in the extraction yield with the increase of the alkyl chain length of quaternary ammonium cation. Wang et al. [25] verified that an increase in the length of the cation alkyl chain of ammonium-based ILs leads to an increase in the protein extraction yield from *Chlorella pyrenoidosa*, meaning that the biomass nature, type and concentration of IL and type of protein play a relevant role and must be considered and optimized according to the target application.

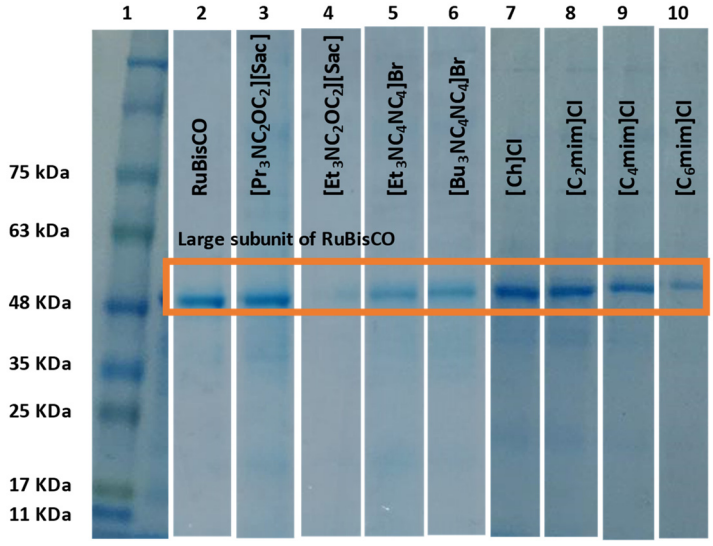

Figure 2. SDS-PAGE loaded on a polyacrylamide gel (stacking: 4% and resolving: 20%) stained with BlueSafe. Effect of IL aqueous solutions in the RuBisCO's extraction from spinach using the following experimental conditions: IL concentration of 3.3 mM, 0.10 solid-liquid ratio, solid-liquid extraction time 30 min at 29 °C. Lane 1: Standard molecular weights; Lane 2: 1 mg/mL commercial RuBisCO.

Despite the promising extraction yields of RuBisCO using IL aqueous solutions, SE-HPLC spectra (SI, Figure S2) show that there is the formation of protein aggregates in extractions carried out with AGB- and imidazolium-based ILs, being more pronounced with imidazolium-based ILs. Compared to other proteins, RuBisCO may be more susceptible to

form aggregates in the presence of ILs due to its dimensions and complexity (composed of several subunits) [13]. It has been reported that, depending on the type of protein, imidazolium-based ILs can act as protein denaturation agents [38]. This effect is mainly due to the anion coupled to the imidazolium cation, the hydrophobicity afforded by the alkyl chain length of the cation, and IL concentration [38]. On the other hand, the [Ch]Cl aqueous solution is the best solvent to avoid the formation of RuBisCO aggregates, coupled to a good extraction performance (as depicted in Figure 2). This effect seems to be related with the shorter alkyl side chain lengths present in cholinium, coupled to an anion with high hydrogen-bond basicity. The good performance of [Ch]Cl in the extraction of proteins was also observed by Martins et al. [24] in the extraction of phycobiliproteins from the red macroalga *Gracilaria* sp., in which the extraction was improved by 46.5% with a similar purity to other procedures and without compromising the secondary structure of the protein. Overall, since the aqueous solution of [Ch]Cl showed to be the most promising solvent in the RuBisCO's extraction, this IL was selected for further experiments, followed by the screening of other cholinium-based ILs, namely [Ch]Br, [Ch][DHP], [Ch][DHC] and [Ch][Ac], at different IL concentrations.

3.1.2. Effect of Aqueous Solutions of Cholinium-Based ILs and Their Concentration on RuBisCO's Extraction

Based on the previous results, different cholinium-based ILs ([Ch]Cl, [Ch]Br, [Ch][DHC], [Ch][DHP], [Ch][Ac]) at concentrations of 25 mM, 50 mM, 100 mM, 500 mM and 1 M were investigated aiming to evaluate the effect of the IL anion and IL concentration. The SDS-PAGE and SE-HPLC results, the latter allowing to determine the purity and extraction yield of RuBisCO, are given in Figures 3 and 4.

Evaluating the results obtained of the extracts by SDS-PAGE (Figure 3), it is shown that aqueous solutions of [Ch][DHP] and [Ch][DHC] did not extract any protein from spinach, as shown by the absence of bands in the gel, and further confirmed by the lack of peaks in the SE-HPLC spectra of the respective extracts (Supplementary Materials, Figures S3 and S4). On the other hand, aqueous solutions of [Ch]Cl, [Ch]Br and [Ch][Ac] were able to extract RuBisCO, with the extraction performance depending on the IL chemical structure and IL concentration (Figures 3 and 4). Relatively to [Ch]Br, through the analysis of SDS-PAGE (Figure 3, Gel B, lanes 8 to 12), it was observed that RuBisCO was extracted at all concentrations, except for 25 mM. However, the SE-HPLC spectrum (Figure S7) only presented the characteristic peak of RuBisCO at 500 mM and 1 M, although in a not accurate quantifiable concentration ($\geq$0.025 mg/mL). For aqueous solutions of [Ch]Cl at higher concentrations (500 mM and 1 M), better results were obtained at 500 mM where the yield of extraction was 1.84 mg of RuBisCO/g of biomass. With aqueous solutions of [Ch][Ac], a yield of extraction between 6.38–8.45 mg of RuBisCO/g of biomass was obtained. These data reinforce the high impact of the IL concentration on the extraction yield. The good performance of aqueous solutions of [Ch][Ac] to stabilize proteins was reported previously by Bisht et al. [39]. The authors studied if it was possible to improve the thermal stability of α-chymotrypsin with cholinium-based ILs, where [Ch][Ac], [Ch][DHP] and [Ch]Cl preserved the structural stability of the enzyme and the enzymatic activity [39].

The pH value of all IL aqueous solutions and final extracts was also determined, changing with the IL type and suffering a slight increase in the extract when compared to the initial IL aqueous solution. These results are provided in the Supplementary Materials, Table S2, ranging between 3.41 and 6.16 for the IL aqueous solutions and 3.65 and 6.45 for the same solutions after the extraction is performed. The isoelectric point of the enzyme is between 4.60 and 5.50 (depending on the species) [14,15,40], meaning that the enzyme can be slightly positively or negatively charged, as well as neutral. Moreover, it is shown that the solutions with pHs close to 6, i.e., [Ch][Ac], [Ch][Br] and [Ch]Cl aqueous solutions, are more favorable for the extraction of RuBisCO. However, it is important to highlight that RuBisCO's extractions using these ILs were not similar, since the respective chromatograms (Figures S5–S7) presented different profiles and different RuBisCO peak intensity. These

findings indicate a more relevant effect of the IL anion on the RuBisCO's extraction. Furthermore, [Ch]Cl and [Ch]Br have similar pHs before and after the extraction, reinforcing the anion effect. The ILs that do not extract any protein, [Ch][DHC] and [Ch][DHP], present the most acidic aqueous solutions. Since aqueous solutions of [Ch][Ac] and [Ch]Cl were the best solvents in terms of extraction yield, while avoiding proteins aggregation, these ILs were selected for the optimization of the extraction conditions through a response surface methodology (RSM).

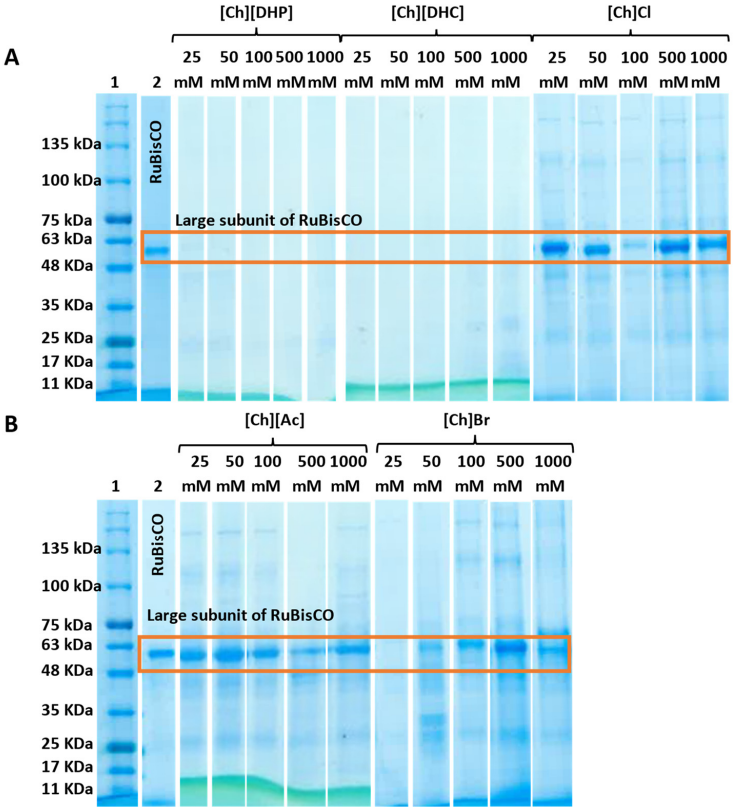

Figure 3. SDS-PAGE loaded and run on a polyacrylamide gel (stacking: 4% and resolving: 20%) stained with BlueSafe. Effect of IL concentration in the RuBisCO's extraction from spinach using the following experimental conditions: IL concentration of 25 mM–1000 mM, 600 rpm, 0.10 solid-liquid ratio, solid-liquid extraction during 30 min at 29 °C. Lanes 1, Gel (**A**,**B**): Standard molecular weights; Lanes 2, Gel A and B: 1 mg/mL of commercial RuBisCO dissolved in PBS.

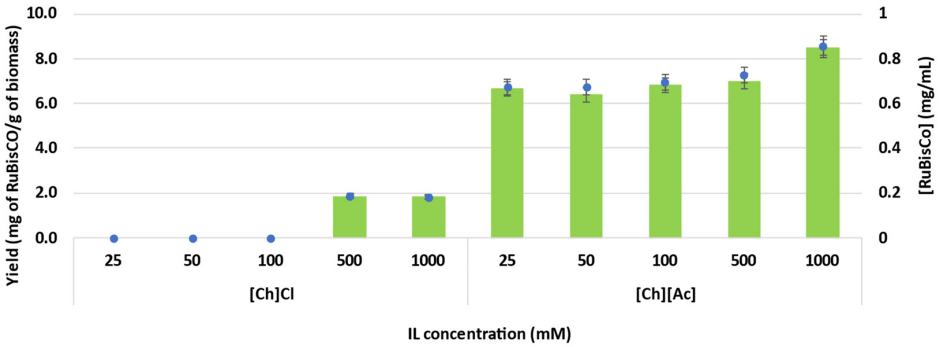

Figure 4. RuBisCO's extracted concentration (mg/mL) (blue symbols) and yield (green bars) after extraction from spinach leaves using different concentrations of IL (25 mM, 50 mM, 100 mM, 500 mM and 1 M), a solid-liquid ratio of 0.10 in a solid-liquid extraction, 600 rpm for 30 min at 29 °C.

3.2. Response Surface Methodology (RSM)

To optimize and identify the significant RuBisCO's extraction conditions, it is necessary to consider the interactions between different factors, such as pH and the solid-liquid ratio, among others. For this purpose, RSM using a 2^3 factorial planning was implemented (three factors and two levels) to improve the extraction, since it allows the extrapolation of the relationship between the dependent variables (yield of extraction and the concentration of RuBisCO extracted) and the independent variables (pH, solid-liquid ratio and IL concentration). Two independent variables were considered in the analysis, namely the extraction yield and concentration of RuBisCO. The last one allows considering the quantity of solvent employed while addressing the development of more sustainable extraction technologies.

The results obtained through RSM with the combined effects of solid-liquid ratio and IL concentration, solid-liquid ratio and pH and IL concentration and pH are depicted in Figures 5–8. The conditions, the extraction yield and concentration of RuBisCO experimentally obtained, and the respective calculated values, as well as the statistical analyzes, are provided in the Supplementary Materials, Tables S3–S18 and Figures S8–S13. Variance analysis (ANOVA) was employed to estimate the statistical significance of the variables and the interactions between them.

3.2.1. Results Obtained Using Aqueous Solutions of [Ch]Cl

The influence of the three independent variables (pH, solid-liquid ratio and IL concentration) on the concentration of RuBisCO in the extracts obtained are shown in Figure 5A–C and in the Supplementary Materials, Figure S8 and Tables S7 and S8. The obtained results were statistically analyzed with a confidence level of 95%. The data presented a R^2 of 0.9520 and an average relative deviation between the experimental and the predicted data of 0.6696%, meaning that this statistical model does a good description of the experimental results. For these data, the solid-liquid ratio and the pH are significant variables. In this case, both variables have a positive effect, i.e., the concentration of extracted RuBisCO increases with these variables. Furthermore, Figure 5B,C confirm that the solid-liquid ratio is the most significant variable, while in Figure 5B, it is shown that the pH together with the solid-liquid ratio origins the best response. A maximum concentration of RuBisCO (1.51 ± 0.07) mg/mL is observed with the maximum solid-liquid ratio applied (0.184), whereas the second highest concentration (1.37 ± 0.07) mg/mL is obtained with a solid-liquid ratio of 0.150 and a pH of 9.50. From Figure 5A,C, it is possible to conclude that the IL concentration does not affect the response in comparison with the other two variables. The relationship between the solid-liquid ratio and the pH and the global function for the concentration of RuBisCO is described by Equation (5):

$$\text{RuBisCO's concentration} = 0.3896 + 0.0097\text{pH} + 6.583\text{R} \quad (5)$$

where R is the solid-liquid ratio.

Regarding the effect of the pH, solid-liquid ratio and IL concentration on the extraction yield of RuBisCO, illustrated in Figure 5D–F and in the Supplementary Materials (Figure S9, Tables S9 and S10), it is evident that the solid-liquid ratio and IL concentration are the most significant parameters. The obtained R^2 was 0.7585, with an average relative deviation between the experimental and the predicted data of 0.8675%, meaning that this statistical model does an adequate description of the experimental results. However, and contrarily to the enzyme concentration previously discussed, the solid-liquid ratio has a negative effect in the yield, since the extraction yield does not depend on the solvent volume applied. A lower solid-liquid ratio leads to a higher yield of extraction, while the yield of extraction increases with the increase of IL concentration. A maximum yield of extraction of (14.05 ± 0.69) mg of RuBisCO/g of biomass was observed with the minimum solid-liquid ratio applied (0.016). The relationship between the solid-liquid ratio and the IL concentration and the global function for the RuBisCO's extraction yield is described by Equation (6):

$$\text{RuBisCO's extraction yield} = 8.737 - 1.267\text{C} - 75.50\text{R} \quad (6)$$

where R is the solid-liquid ratio and C is the ionic liquid concentration.

Overall, the solid-liquid ratio was the significant variable identified for both responses in the study, with a contrary effect on them. Thus, it is necessary to find a compromise between the extraction conditions to ensure a more sustainable solvent use and process. The best experimental conditions to increase both responses were a solid-liquid ratio of 0.18, pH value of 9.09 and an IL concentration of 2.68 M (Supplementary Materials, Figure S12). These predicted optimal conditions were experimentally applied, with no significant differences between the predicted (yield of 11.6 mg of RuBisCO/g of biomass and concentration of 1.72 mg/mL) and experimentally obtained results (yield of 10.6 mg of RuBisCO/g of biomass and concentration of 1.93 mg/mL).

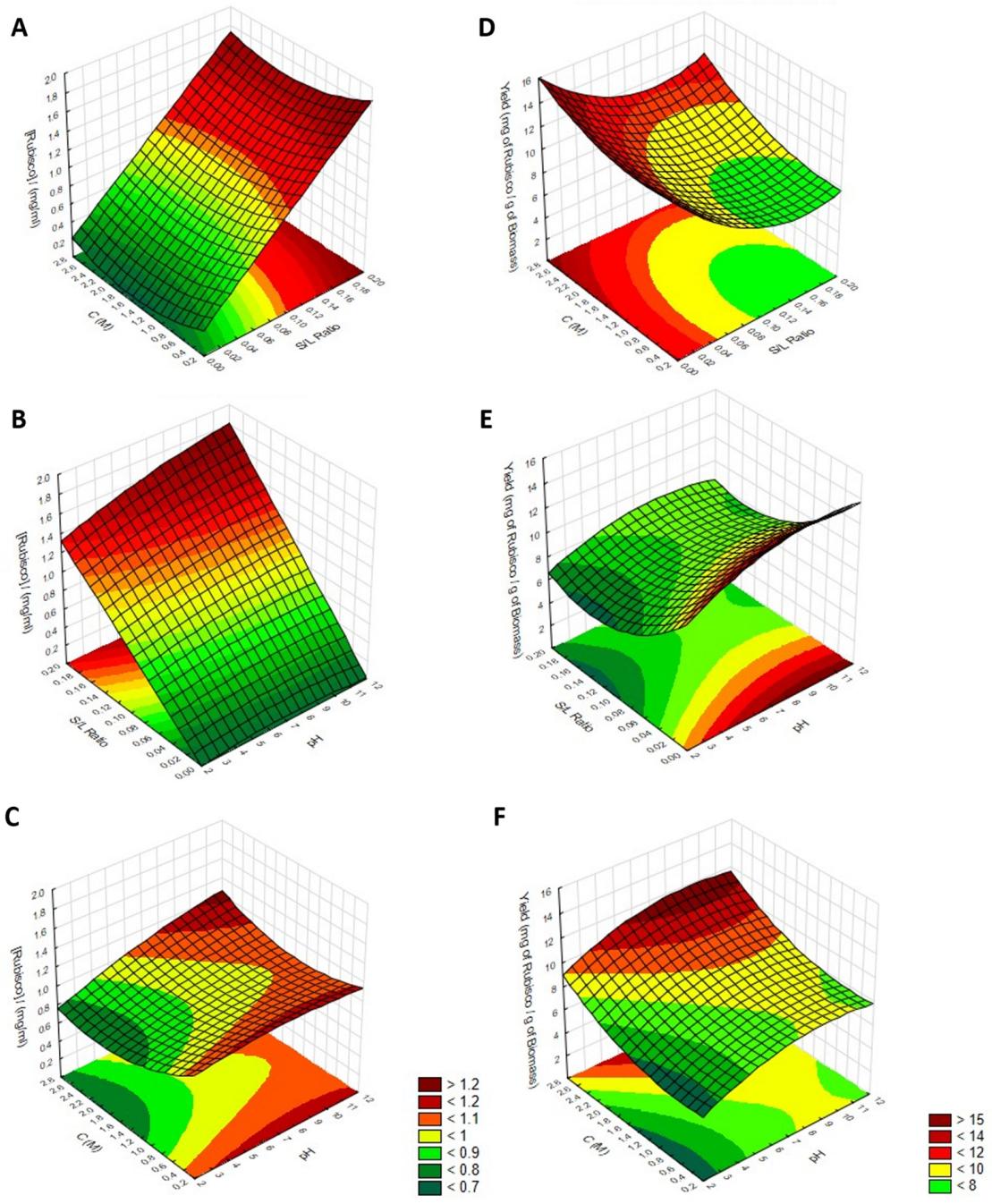

Figure 5. Response surfaces corresponding to the concentration of extracted RuBisCO (left) and yield of extraction (right) with the following combined parameters: (**A**,**D**) [Ch]Cl concentration and solid-liquid ratio; (**B**,**E**) solid-liquid ratio and pH; and (**C**,**F**) [Ch]Cl concentration and pH.

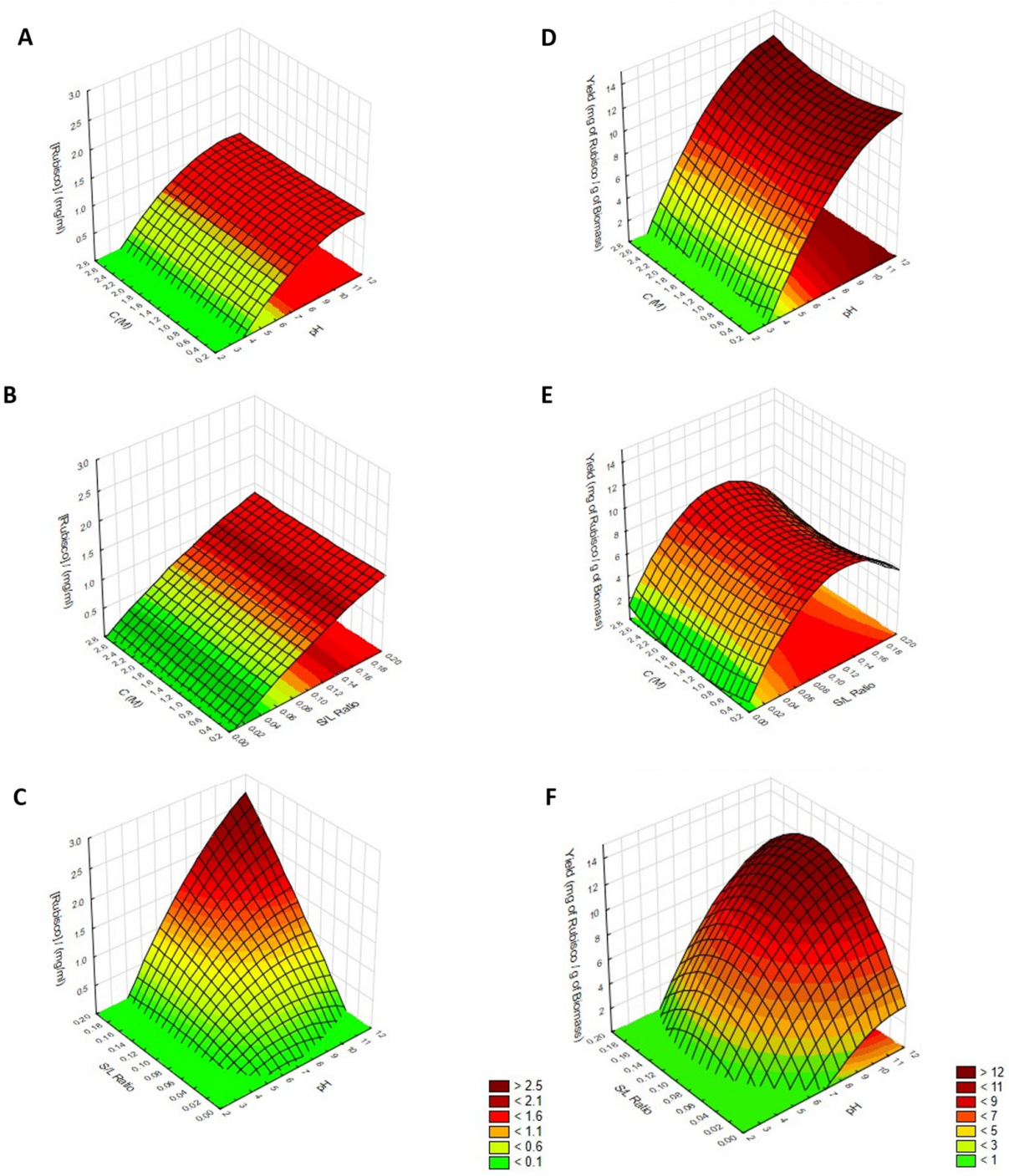

Figure 6. Response surfaces corresponding to the concentration of extracted RuBisCO (left) and yield of extraction (right) with the following combined parameters: (**A**,**D**) [Ch][Ac] concentration and pH; (**B**,**E**) [Ch][Ac] concentration and solid-liquid ratio; and (**C**,**F**) solid-liquid ratio and pH.

3.2.2. Results Obtained Using Aqueous Solutions of [Ch][Ac]

The second factorial planning was carried out using aqueous solutions of [Ch][Ac], the second solvent selected for the extraction of RuBisCO. Figure 6A–C and Figure S10 and Tables S11 and S12 in the Supplementary Materials show the relation of the concentration of extracted RuBisCO with the three independent variables investigated (pH, solid-liquid ratio and IL concentration). The obtained R^2 was 0.9087, with an average relative deviation between the experimental and the predicted data of 3.602%, meaning that this statistical

model provides a good description of the experimental results. It is evident that the solid-liquid ratio, pH and their interaction are the significant factors, and all of them have a positive effect on the response. Thus, a higher solid-liquid ratio and pH lead to an enhancement of the response variable. The maximum concentration of RuBisCO obtained was 1.59 ± 0.16 mg/mL with the maximum solid-liquid ratio of 0.184 applied, whereas the second-best concentration, 1.52 ± 0.16 mg/mL, was obtained with a solid-liquid ratio of 0.150 and a pH of 9.50. The relationship between the solid-liquid ratio and the pH and the global function for the concentration of RuBisCO is described by Equation (7):

$$\text{RuBisCO's concentration} = -0.8088 + 0.2453\text{pH} - 4.665\text{R} + 2.046\text{R} * \text{pH} \quad (7)$$

where R is the solid-liquid ratio.

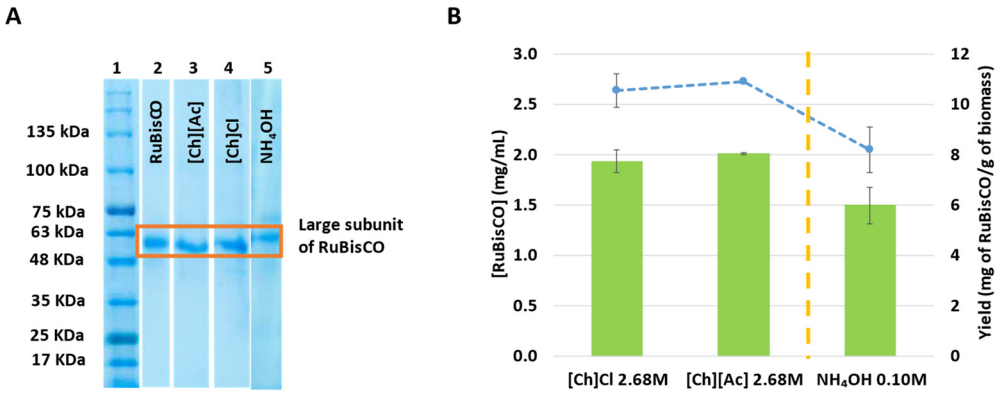

Figure 7. (**A**) SDS-PAGE loaded and run on a polyacrylamide gel (stacking: 4% and resolving: 20%) stained with BlueSafe. Lane 1: Standard molecular weights; Lane 2: 1 mg/mL commercial RuBisCO. (**B**) RuBisCO's yield (blue symbols and bar) and RuBisCO's concentration (green bars). The extractions were executed at the optimal conditions for both ILs and with a solution of NH_4OH of 0.10 M at pH 11. The agitation, time of extraction and temperature were maintained (600 rpm, 30 min, at 29 °C).

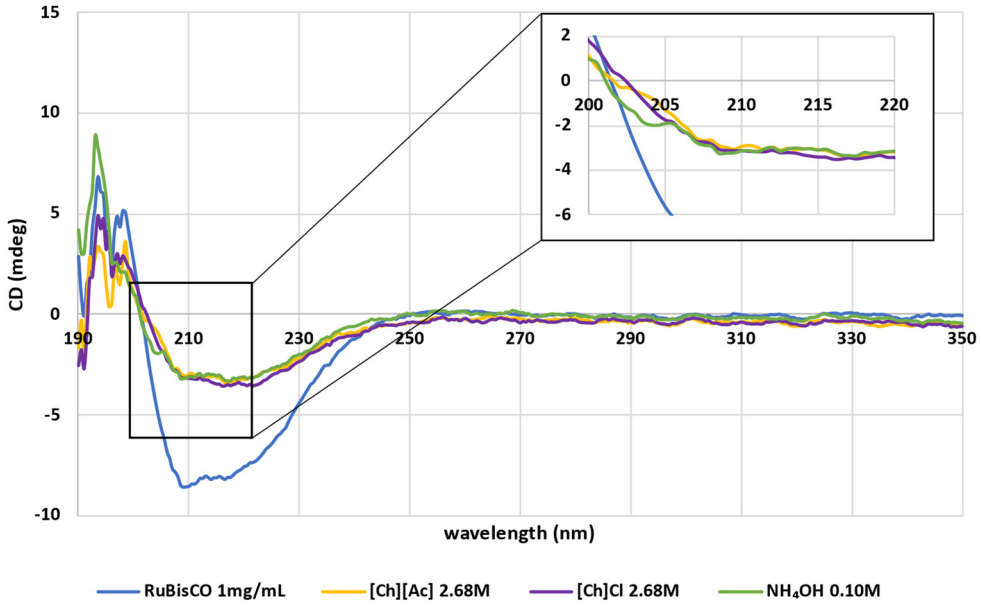

Figure 8. CD spectrums of the standard solution of RuBisCO, of the extracts with IL aqueous solutions at optimal conditions and of the extract with NH_4OH aqueous solution.

Relatively to the extraction yield of RuBisCO, from Figure 6D–F and from Figure S11 and Tables S13 and S14 given in the Supplementary Materials, it is clear that high pH values

lead to a more efficient extraction of RuBisCO from the spinach biomass. The solid-liquid ratio also has a relevant impact on the yield of RuBisCO, leading to a maximum value at a solid-liquid ratio of 0.12). In summary, the significance of the parameters with a significant effect on the extraction yield of RuBisCO, as can be seen in the Pareto chart shown in the SI, Figure S11, decreases in the following order: concentration of pH (linear, positive effect) > solid–liquid ratio (quadratic, negative effect) > solid–liquid ratio (linear, positive effect). The maximum yield of extraction obtained was (10.82 ± 0.49) mg of RuBisCO/g of biomass, when the maximum pH of 11.2 was applied. The obtained R^2 was 0.8971, with an average relative deviation between the experimental and the predicted data of 13.71%, meaning that this statistical model does a good description of the experimental results and can be described by Equation (8):

$$\text{RuBisCO's extraction yield} = -16.01 + 3.510\text{pH} + 109.5\text{R} - 489.8\text{R}^2 \quad (8)$$

where R is the solid-liquid ratio.

For both responses, pH is the most significant variable, having a positive effect with its increase. On the other hand, the solid-liquid ratio is a significant variable but acting in opposite ways in both responses. Thus, it is necessary to achieve a good compromise between the extraction conditions in order to maximize the aimed response. The best experimental conditions predicted to optimize both responses and then applied in further extractions was a solid-liquid ratio of 0.184, pH 11.2 and IL concentration of 2.68 M (Supplementary Materials, Figure S13). The determined optimal conditions were applied, and no significant differences were detected between the predicted (yield of 11.4 mg of RuBisCO/g of biomass and concentration of 2.34 mg/mL) and experimental results (yield of 10.9 mg of RuBisCO/g of biomass and concentration of 2.01 mg/mL).

3.3. Comparison between Conventional Solvents and Ionic Liquids in the Extraction of RuBisCO

To compare the performance of IL aqueous solutions in the extraction of RuBisCO from spinach with the common solvents used [2], a solution of NH_4OH 0.10M at pH 11 was applied, using the identified optimal conditions (0.184 solid-liquid ratio, 600 rpm, during 30 min, at 29 °C). The results obtained and the comparison between [Ch]Cl, [Ch][Ac] and NH_4OH 0.10 M are presented in Figure 7A,B.

The use of an aqueous solution of NH_4OH for RuBisCO's extraction, which is a commonly applied solvent, shows an inferior performance comparing with the results obtained with aqueous solutions of cholinium-based ILs: (1.49 ± 0.19) mg RuBisCO/mL aqueous solution containing NH_4OH versus (2.01 ± 0.01) mg RuBisCO/mL aqueous solution of [Ch][Ac] and (1.93 ± 0.11) mg RuBisCO/mL aqueous solution of [Ch]Cl; and extraction yield of (8.20 ± 0.89) mg of RuBisCO/g of biomass with the NH_4OH aqueous solution versus (10.92 ± 0.01) mg of RuBisCO/g of biomass with the [Ch][Ac] aqueous solution and (10.57 ± 0.67) mg of RuBisCO/g of biomass with the [Ch]Cl aqueous solution. These results reinforce that aqueous solutions of properly designed ILs allow higher extraction yields than the typically used aqueous solution of NH_4OH.

The obtained extracts were analyzed by circular dichroism (CD) spectroscopy to gain insights into possible changes in the RuBisCO's secondary structure after the extraction process. An aqueous solution prepared with commercially acquired RuBisCO at 1 mg/mL was used for comparison purposes. Both CD spectra (Figure 8) were analyzed with the K2D3 web server [41], with the percentages of α-helix (% α-helix) and β-sheet (% β-sheet) being given in Table 2.

The CD spectrum of the RuBisCO standard presents the characteristics negative peaks at 208 nm and 218 nm located in the far UV wavelength range (200–250 nm) [42]. However, it is possible to observe that for the same region (200–250 nm), associated with the integrity of the polypeptide backbone, the samples with [Ch][Ac] and NH_4OH present some alterations in their spectra [42,43]. Nevertheless, by the results obtained with K2D3, the secondary structure of RuBisCO is preserved in all IL aqueous solutions and in the NH_4OH solution, since similar values of % α-helix and % β-sheet contents are present

in the commercially acquired RuBisCO and the extracted RuBisCO with IL and NH_4OH aqueous solutions. Still, the values of % α-helix and % β-sheet contents are the most approximated for [Ch]Cl, being in accordance with the spectra. Therefore, appropriate IL aqueous solutions, such as those constituted by [Ch]Cl and [Ch][Ac] are good alternative solvents to extract higher yields of RuBisCO from biomass, without compromising the enzyme secondary structure.

Table 2. Percentage content of α-helix and β-sheet in RuBisCO.

Extracted RuBisCO	% α-Helix	% β-Sheet
Commercially acquired RuBisCO	1.91	20.11
RuBisCO extracted with aqueous solutions of [Ch][Ac] 2.68 M	1.82	19.47
RuBisCO extracted with aqueous solutions of [Ch]Cl 2.68 M	1.95	20.22
RuBisCO extracted with aqueous solutions of NH_4OH 0.10 M	2.15	20.75

4. Conclusions

IL aqueous solutions were investigated as alternative solvent aiming at developing a more sustainable approach for RuBisCO's extraction from spinach leaves. An initial screening on the effect of the IL chemical structure in the extraction of RuBisCO was carried out. For this purpose, cholinium- and imidazolium-based ILs were tested, and it was demonstrated that [Ch]Cl led to the most promising results. Using this IL, higher extraction yields are obtained. Accordingly, a new screening including other cholinium-based ILs ([Ch][DHP], [Ch][DHC], [Ch][Ac], [Ch]Cl and [Ch]Br) was performed varying the IL concentration. SE-HPLC chromatograms and SDS-PAGE analysis indicated that among the cholinium-based ILs evaluated, [Ch]Cl and [Ch][Ac] are the ones capable to extract RuBisCO in relevant amounts. Accordingly, they were then selected to apply a RSM to optimize the operating conditions. All the statistical models used have a good description of the experimental results. Based on the gathered responses, the optimal conditions found were a solid-liquid ratio of 0.18, an aqueous solution of 2.68 M of cholinium-based ILs at a pH of 9.09 and 11.2 for [Ch]Cl and [Ch][Ac], respectively. The aqueous solution of [Ch]Cl can extract 1.93 mg of RuBisCO/mL solvent with an extraction yield of 10.57 mg of RuBisCO/g of biomass. In the case of the aqueous solution of [Ch][Ac], this solvent can extract 2.01 mg of RuBisCO/mL solvent with an extraction yield of 10.92 mg of RuBisCO/g of biomass. Furthermore, these IL aqueous solutions allow higher extraction yields than the commonly applied aqueous solution constituted by NH_4OH. Furthermore, possible changes in the RuBisCO secondary structure after the extraction process were investigated by CD spectroscopy. Overall, the results showed that the secondary structure of the RuBisCO is preserved in the IL aqueous solutions after extraction, with similar values of % α-helix and % β-sheet to the commercially acquired RuBisCO.

In summary, it is here shown that aqueous solutions of cholinium-based ILs are promising alternative solvents capable of extracting high yields of the enzyme RuBisCO from biomass, while keeping its secondary structure.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/suschem3010001/s1, Figure S1: Calibration curve used to determine the concentration of RuBisCO in the samples. Equation: Area = 1.3369 × 10−4 [RuBisCO] and R2 = 0.9991, Figure S2: SE-HPLC spectra of aqueous solutions of biocompatible ILs (A) and imidazolium-based ILs (B) after RuBisCO's extraction from spinach leaves with an IL concentration of 3.3 mM. RuBisCO chromatogram presents two peaks. The peak with a retention time of ~13.4 min corresponds to the RuBisCO and at ~14.3 min corresponds to impurities. The peaks with retention times of 4.0 and 11.6 min correspond to protein aggregates, Figure S3: SE-HPLC spectra of aqueous solutions of [Ch][DHP] after RuBisCO's extraction from spinach leaves with different IL concentrations. RuBisCO chromatogram presents two peaks. The peak with a retention time of ~13.4 min corresponds to the RuBisCO and at ~14.3 min corresponds to impurities. The extracts with IL concentrations of 25 mM, 500 mM and 1 M present peaks with lower retention times (1.2, 2.0 and 8.0 min) corresponding to protein aggregates, Figure S4: SE-HPLC

spectra of aqueous solutions of [Ch][DHC] after RuBisCO's extraction from spinach leaves with different IL concentrations. RuBisCO chromatogram presents two peaks. The peak with a retention time of ~13.4 min corresponds to the RuBisCO and at ~14.3 min corresponds to impurities. The extract with IL concentrations of 25 mM and 1M present only peaks with lower retention times (1.2 and 8.0 min) corresponding to protein aggregates, Figure S5: SE-HPLC spectra of aqueous solutions of [Ch]Cl after RuBisCO's extraction from spinach leaves with different IL concentrations. RuBisCO control chromatogram presents two peaks. The peak with a retention time of ~13.4 min corresponds to the RuBisCO and at ~14.3 min corresponds to impurities. The other peaks with lower retention times (1.2, 2.4, 11.6 and 12.6 min) correspond to protein aggregates, Figure S6: SE-HPLC spectra of aqueous solutions of [Ch][Ac] after RuBisCO's extraction from spinach leaves with different IL concentrations. RuBisCO chromatogram presents two peaks. The peak with a retention time of ~13.4 min corresponds to the RuBisCO and at ~14.3 min corresponds to impurities. All the extracts present a characteristic peak for RuBisCO. The peaks with a lower retention time (1.2 and 8.8 min) correspond to protein aggregates, Figure S7: SE-HPLC spectra of aqueous solutions of [Ch]Br after RuBisCO's extraction from spinach leaves with different IL concentrations. RuBisCO chromatogram presents two peaks. The peak with a retention time of ~13.4 min corresponds to the RuBisCO and at ~14.3 min corresponds to impurities. The peaks with lower retention times (2.8 and 11.6 min) correspond to protein aggregates, Figure S8: Pareto charts for the standardized main effects in the factorial planning with [Ch]Cl for RuBisCOs' concentration. The vertical line indicates the statistical significance of the effects, Figure S9: Pareto charts for the standardized main effects in the factorial planning with [Ch]Cl for RuBisCOs' extraction yield. The vertical line indicates the statistical significance of the effects, Figure S10: Pareto charts for the standardized main effects in the factorial planning with [Ch][Ac] for RuBisCOs' concentration. The vertical line indicates the statistical significance of the effects, Figure S11: Pareto charts for the standardized main effects in the factorial planning with [Ch][Ac] for RuBisCOs' extraction yield. The vertical line indicates the statistical significance of the effects, Figure S12: Profiles for predicted values and desirability in the factorial planning for both dependent variables with [Ch]Cl, Figure S13: Profiles for predicted values and desirability in the factorial planning for both dependent variables with [Ch][Ac], Table S1: 23 factorial planning for each IL ([Ch]Cl and [Ch][Ac]), Table S2: pH values of the IL aqueous solutions and of the extracts, Table S3: Experimental data and response surface predicted values of the factorial planning for RuBisCOs' concentration, extracting with [Ch]Cl, Table S4: Experimental data and response surface predicted values of the factorial planning for RuBisCOs' extraction yield, extracting with [Ch]Cl, Table S5: Experimental data and response surface predicted values of the factorial planning for RuBisCOs' concentration, extracting with [Ch][Ac], Table S6: Experimental data and response surface predicted values of the factorial planning for RuBisCOs' concentration, extracting with [Ch][Ac], Table S7: Regression coefficients of the predicted second-order polynomial model for the RuBisCOs' concentration from RSM using [Ch]Cl, R2 = 0.95197 and radj. = 0.90875, Table S8: Effects of the variables in the second-order polynomial model for the extraction RuBisCO concentration using [Ch]Cl, Table S9: Regression coefficients of the predicted second-order polynomial model for the RuBisCOs' extraction yield from RSM using [Ch]Cl, R2 = 0.75851 and radj. = 0.54116, Table S10: Effects of the variables in the second-order polynomial model for the extraction yield of RuBisCO using [Ch]Cl, Table S11: Regression coefficients of the predicted second-order polynomial model for the RuBisCOs' concentration from RSM using [Ch][Ac], R2 = 0.90873 and radj. = 0.82659, Table S12: Effects of the variables in the second-order polynomial model for the extraction RuBisCO concentration using [Ch][Ac], Table S13: Regression coefficients of the predicted second-order polynomial model for the RuBisCOs' extraction yield from RSM using [Ch][Ac], R2 = 0.89709 and radj, = 0.80447, Table S14: Effects of the variables in the second-order polynomial model for the extraction yield of RuBisCO using [Ch][Ac], Table S15: ANOVA data for RuBisCO concentration when extracting with [Ch]Cl, Table S16: ANOVA data for the extraction yield of RuBisCO when extracting with [Ch]Cl, Table S17: ANOVA data for RuBisCO concentration when extracting with [Ch][Ac], Table S18: ANOVA data for the extraction yield of RuBisCO when extracting with [Ch][Ac].

Author Contributions: Conceptualization, M.G.F. and A.P.M.T.; methodology, A.I.V., A.M.F. and M.R.A.; software, A.I.V. and A.M.F.; validation, A.M.F., M.R.A. and A.P.M.T.; formal analysis, A.I.V., A.M.F., M.R.A. and A.P.M.T.; investigation, A.I.V.; resources, A.M., M.G.F. and A.P.M.T.; data curation, A.M.F., M.R.A., M.G.F. and A.P.M.T.; writing—original draft preparation, A.I.V.; writing—review and editing, A.M.F., M.R.A., A.M., M.G.F. and A.P.M.T.; supervision, A.P.M.T.; project administration,

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