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1 Biochemical and physical-chemical characterisation of leaf proteins extracted
2 from *Cichorium endivia* leaves

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8 **Abstract**

9 This study provides a detailed characterisation of a leaf protein concentrate (LPC) extracted from *Cichorium*
10 *endivia* leaves using a pilot scale process. This concentrate contains 74.1% protein and is mainly composed of
11 Ribulose-1,5-BISphosphate Carboxylase/Oxygenase (RuBisCO). We show that the experimentally determined
12 extinction coefficient (around $5.0 \text{ cm}^{-1} \text{ g}^{-1} \text{ L}$ depending on the pH) and refractive index increment (between 0.27
13 and 0.39 mL g^{-1}) are higher than the predicted ones (about $1.6 \text{ cm}^{-1} \text{ g}^{-1} \text{ L}$ and 0.19 mL g^{-1} , respectively). In addition,
14 the UV-visible absorption spectra show a maximum at 258 nm. These data suggest the presence of non-protein
15 UV-absorbing species. Chromatographic separation of the concentrate components in denaturing conditions
16 suggests that RuBisCO SC may be covalently bounded to few phenolic compounds. Besides, the solubility of LPC
17 proteins is higher than 90% above pH 6. Such high solubility could make LPC a good candidate as a functional
18 food ingredient.

19 **Key-words:**

20 Leaf protein concentrate, RuBisCO, UV-visible spectroscopy, Protein solubility

21 **1. Introduction**

22 Leaf proteins were first described by Rouelle in 1773. Researches on leaf proteins were stimulated in the early
23 1940s when Norman Pirie discussed the potential of leaf protein concentrates (LPC) as a source of proteins for
24 human consumption to overcome wartime food shortages (Pirie, 1942). Proteins constitute between 15 to 20%
25 of the leaf dry mass depending on the species. Leaf proteins are often classified according to their affinity with
26 water. Water-insoluble proteins, also called "green proteins", are mainly embedded in the membrane of plant
27 organelles and often associated with lipophilic pigments such as chlorophyll (Thornber, 1975). Water-soluble
28 proteins, also called "white proteins", are mainly composed of chloroplastic and cytoplasmic enzymes. The first
29 commercial LPC, produced from alfalfa leaves, was launched in 1967 and consisted in a sole green concentrate
30 rich in proteins and xanthophylls intended for monogastric and poultry feeding (Knuckles, Spencer, Lazar, Bickoff,
31 & Kohler, 1970). Once improved, the pilot-scale process allowed the fractionation of water-soluble and water-
32 insoluble proteins, resulting in a green concentrate with a lower protein content and a white protein concentrate,
33 suitable for human consumption (Edwards et al., 1975). This white LPC is mainly composed of a protein named
34 Ribulose-1,5-BISphosphate Carboxylase/Oxygenase (RuBisCO).

35 RuBisCO is a key enzyme in photosynthetic carbon assimilation, catalysing the first step of CO₂ fixation in the
36 Calvin cycle. RuBisCO is found in most autotrophic organisms from prokaryotes to eukaryotes. Despite its
37 deficient specificity and its low carboxylase activity, RuBisCO fulfils its carbon fixation functions in plants thanks
38 to its high level of expression in the growing leaf. The enzyme represents up to 30-50% of the soluble proteins in
39 leaves. RuBisCO is therefore claimed to be the most abundant protein on Earth (Ellis, 1979). RuBisCO is an
40 hexadecameric protein, consisting of 8 large chains (LC) and 8 small chains (SC) arranged around a four-fold axis.
41 The quaternary structure of RuBisCO mainly relies on electrostatic interactions although it was shown that LC
42 tends to cross-link by disulphide bridges in oxidative stress conditions (Mehta, Fawcett, Porath, & Mattoo, 1992).
43 In addition to its abundance, it was mentioned from the early 1940s that RuBisCO was interesting for human
44 nutrition because of its balanced amino acid profile (Pirie, 1942). Some authors even compare the nutritive value
45 of RuBisCO to that of casein (Hood, Cheng, Koch, & Brunner, 1981).

46 Several LPC extraction processes have been described (Edwards et al., 1975; Knuckles et al., 1970). LPC have up
47 to now not been used as an ingredient in human food due to the difficulty of combining the economic viability
48 of the extraction process with the quality of the protein concentrate (reviewed in Chiesa & Gnansounou, 2011).

49 Recent advances in extraction process methods such as membranes technologies are encouraging research on
50 the use of LPC as a functional ingredient in human food (Ducrocq, Boire, Anton, Micard, & Morel, 2020; Kiskini,
51 2017; Martin, Castellani, de Jong, Bovetto, & Schmitt, 2019). These studies agree that LPC show very interesting
52 functional properties, especially gelling properties, for industrial applications. In addition, the production of LPC
53 could contribute to the valorization of green agro-industrial waste. Leaves from crop residues, such as sugar beet
54 leaves, or from plant processing discards, such as ready-to-eat salad leaves, may be use as raw materials for LPC
55 production. This would contribute to an optimized biomass valorization while providing an alternative protein
56 source. In this paper, we were interested in proteins extracted from Escarole (*Cichorium endivia*) leaves, a
57 vegetable consumed as fresh or packaged Ready-To-Eat salad. Its chemical and nutritional composition depends
58 greatly on the variety and on the cultivation conditions (Otalora et al., 2018). In average, the protein content is
59 about 1-1.5% of the fresh leaves (Otalora et al., 2018). Therefore, significant amount of protein may be extracted
60 from the by-product of ready-to-eat salad processing.

61 Numerous characterisation studies have been carried out on highly purified RuBisCO to understand its structure
62 and its enzymatic activity to improve its carbon fixation rate. However, in the field of Food Science and
63 Technology, the characterisation of the LPC is fragmented and, to our knowledge, no exhaustive study
64 characterises in details the biochemical and physical-chemical properties of the LPC as a food ingredient. The
65 present paper describes in detail the biochemical and physical-chemical characterisation of a leaf protein
66 concentrate extracted from *Cichorium endivia* leaves using a pilot scale process. A combination of techniques
67 based on biochemical assay, chromatography, and spectroscopy was used to investigate the composition of the
68 LPC and the physical-chemical properties of RuBisCO, its major protein. The experimentally measured
69 parameters are compared with values from the literature as well as with theoretical values computed from
70 known RuBisCO sequences.

71 **2. Material and methods**

72 *2.1 Leaf protein concentrate extraction and storage*

73 Leaf protein concentrate (LPC) was provided by Florette (Lessay, France). LPC was extracted from *Cichorium*
74 *endivia* leaves using the extraction conditions of the WO 2014/104880 patent but excluding the hydrophobic
75 column adsorption step. This process is based on the classical process of protein extraction from leaves which
76 has been studied for many years and widely described in the literature (Edwards et al., 1975; Knuckles et al.,

77 1970). Indeed, the process we use includes the classical steps of the leaf protein extraction process: i) separation
78 of the fibres by pressing, ii) heat precipitation of the coloured proteins iii) concentration of the proteins of
79 interest and removal of residual contaminants. Briefly, the juice was first extracted from raw material by pressing
80 using a twin-screw press with addition of a buffering solution (1 M MES buffer pH 6.5) and a reducing agent (e.g.
81 sodium metabisulphite) to avoid phenolic oxidation and cross-linking. Membrane proteins and pigments were
82 then coagulated upon heating at 55°C for 20 minutes. After cooling and decantation of the coloured pellet, a
83 cross-flow microfiltration step on a 0.2 µm membrane allows the sterilisation of the supernatant. The protein-
84 rich supernatant was then concentrated by ultrafiltration using a 100 kDa molecular weight cut-off and
85 subsequently diafiltrated to remove salts, phenolics, and other impurities. The liquid protein concentrate was
86 then freeze-dried. At least 14 days before every experiment, the protein powder was placed in a humidity-
87 controlled chamber with a K₂CO₃ saturated salt solution at 20 °C to maintain 43.2% relative humidity to ensure
88 a constant moisture content. All experiments were performed with Milli- 100 Q water (Millipore Systems,
89 Guyancourt, Molsheim, France) and all chemicals used were of analytical grade and were purchased from Sigma
90 (Bornem, Belgium), VWR 99 international (Leuven, Belgium) or Carlo Erba (Peypin, France).

91 *2.2 Amino acids composition and nitrogen to protein conversion factor determination*

92 Amino acid analysis was carried out with an amino acid analyser (L-8900, Hitachi, Tokyo, Japan) as described in
93 Margier et al. (2018). Briefly, four types of hydrolysis were performed before the analysis. Acid hydrolysis with 6
94 N HCl was performed for 24 hours at 110 °C to determine the amount of most amino acids. Oxidation with
95 performic acid was performed before the hydrolysis to assay sulphur-amino acids. Leucine, Isoleucine and valine
96 were quantified after an acid hydrolysis with 6 N HCl for 48 hours at 110 °C. The amount of tryptophan was
97 determined after basic hydrolysis with 4 N Ba(OH)₂ for 16 hours at 110 °C. For each hydrolysis, norleucine was
98 used as internal standard. The relative standard deviation for the concentration of each amino acid standard is
99 comprised between 0.6 and 2.5 % with this method and equipment. From the amino acid composition, the
100 chemical score of essential amino acids (EAA) was calculated as the ratio between the EAA content in the
101 concentrate over its content in the reference amino acid profile established by the French Food Safety Agency
102 (AFSSA, 2007). As white LPC is generally mainly composed of RuBisCO, the LPC amino acid composition was
103 compared with the amino acid composition of RuBisCO from *lactuca sativa* was computed from the sequences
104 of its subunits (accession numbers of LC and SC in UniProt database: P48706 and Q40250). The whole RuBisCO

105 sequence was obtained by adding the sequences of 8 large chains and 8 small chains, excluding the signal
106 peptides.

107 The Nitrogen to Protein conversion factor (N:P factor) was computed from the ratio of total anhydrous mass of
108 amino acids to the total mass of nitrogen, as described in Sosulski & Imafidon (1990). The anhydrous molecular
109 weight of an amino acid corresponds to its molecular weight minus the molecular weight of a molecule of water
110 ($18 \text{ g}\cdot\text{mol}^{-1}$). The Asparagine (Asn) and Glutamine (Gln) were assayed in their acidic form. Therefore, they could
111 not be distinguished from Asparagic acid (Asp) and Glutamic acid (Glu) content in the amino acid profile analysis.
112 We estimated the content in Asn and Gln in LPC by taking the proportion of Asn/Asp and Gln/Glu from lettuce
113 RuBisCO amino acid sequence.

114 *2.3 Composition of the leaf protein concentrate*

115 Water and ash contents of LPC powder were determined by thermogravimetric analyses (TGA 2050, TA
116 instruments, New Castle, England) under nitrogen atmosphere. About 10 mg of powder was heated at $3 \text{ }^\circ\text{C}/\text{min}$
117 until $130 \text{ }^\circ\text{C}$ for 30 min. The measured mass loss was attributed to water content. The powder was further heated
118 at $600 \text{ }^\circ\text{C}$ for 180 min to determine ash content. Measurements were done in triplicates. The total nitrogen
119 content of LPC powder was determined using the Kjeldahl procedure (NF V 03–050, 1970). Protein content was
120 obtained using a N:P factor determined from LPC amino acid composition.

121 Uronic acid was determined by an automated m-hydroxybiphenyl method (Thibault, 1979). Neutral sugars were
122 analysed as to their alditol acetate derivatives by gas-liquid chromatography after hydrolysis (Englyst &
123 Cummings, 1988). The total free phenolic compounds were determined using the Folin-Ciocalteu reagent.
124 Polyphenols were extracted in acetone (80%) with a liquid:solid ratio of 40. The extraction was performed at 4°C
125 in an ultrasounds bath for 45 min. The mixture was centrifuged ($9000 \times g$, 15 min) and the extraction was
126 repeated once. Both supernatants were combined and evaporated under nitrogen stream. Dry extracts were
127 then diluted in ultrapure water so as to obtain an absorbance within the linearity range of the test. In a
128 microplate, $25\mu\text{L}$ of sample was mixed with $125\mu\text{L}$ of Folin Ciocalteu (10%) and $100\mu\text{L}$ Na_2CO_3 ($75 \text{ g}\cdot\text{L}^{-1}$). The
129 microplate was then incubated at $40 \text{ }^\circ\text{C}$ for 5 min before absorbance reading at 735 nm. Gallic acid was used as
130 standard and results were expressed as gallic acid equivalent. Measurements were done in triplicates.

131 *2.4 SDS-PAGE and Western Blot*

132 SDS-PAGE was performed in non-reducing and reducing conditions. LPC was solubilised at 2 g.L⁻¹ in sodium
133 phosphate buffer (0.1 M, pH 8, 1 mM EDTA) and diluted twice in Laemmli buffer. β-Mercaptoethanol at 50 µg.mL⁻¹
134 ¹ was added to the Laemmli buffer for electrophoresis under reducing conditions. Both unreduced and reduced
135 samples were then heated at 100 °C for five min. Gels (4-12 % Bolt Bis-tris-Plus, 8 x 8 cm², Novex) were run in
136 MES running buffer at a constant voltage of 200 V for 40 min in the Mini Gel Tank (ref A25977, ThermoFisher
137 scientific). For the non-specific labelling of proteins, electrophoresis gel was incubated one hour in Coomassie
138 blue stain (InstantBlue, Expedeon, San Diego, CA, USA), rinsed several times in distilled water and scanned.

139 A Western-Blot was performed using rabbit anti-RuBisCO large chain (anti-Rbcl, Agrisera) as described in
140 O'Donnelly et al. (2014) with some modifications. Briefly, the gel was equilibrated in transfer buffer (25mM Tris,
141 192mM Glycine, SDS 0.1% (w/v), Ethanol 20% (v/v)) for 15 min after SDS-PAGE running. Polypeptides
142 fractionated on SDS-PAGE gels were transferred to a nitrocellulose membrane (162-0112, Bio Rad) at 250 mA for
143 90 min (mini Trans-Blot cell, BIO RAD). The membrane was then washed with the saturating solution (5% (w/v)
144 milk powder in PBS - 0.1% (v/v) Tween20) for 30 min at room temperature, and rinsed three times 10 min in PBS-
145 tween buffer. The membrane incubation with primary antibody solution (rabbit anti-Rbcl dissolved in PBS, 2%
146 (w/v) milk powder at a ratio 1/10000) was performed for 1 hour. The membrane was then rinsed 3 times 10 min
147 in PBS-Tween. The secondary Antibody Solution (AntiRabbit HRP, A-8025, sigma) was dissolved in PBS 1X, 2%
148 milk powder at a ratio 1/25000. The membrane was incubated with the secondary Antibody Solution for 1 hour
149 before being rinsed twice in PBS-tween and once in PBS. Western Bright™ Quantum (Advansta) reagent was
150 mixed with the membrane for 2 min; the membrane was then imaged with the Fujifilm LAS 3000 camera.

151 *2.5 UV-visible spectra*

152 *Determination of the specific extinction coefficient*

153 The specific extinction coefficients of LPC dispersion were determined at pH 5, pH 7 and pH 8. To obtain the same
154 ionic strength for all buffers, specific ratio of acid and base were used to achieve the desired pH. The molecular
155 species used were as follows: acetate buffer (CH₃COOH/CH₃COO⁻) 0.025 M pH 5, phosphate buffer (H₂PO₄⁻/
156 HPO₄²⁻) 0.01 M pH 7, phosphate buffer (H₂PO₄⁻/ HPO₄²⁻) 0.01 M pH 8. When needed, minimal amount of NaCl
157 was added to reach an ionic strength of 25 mM. LPC was dispersed in buffer overnight at room temperature at
158 3 mg.mL⁻¹; dispersions were centrifuged the day after for 30 min at 39191 x g. The total nitrogen content of

159 supernatant was determined using the Kjeldahl procedure (NF V 03–050, 1970). Protein content was obtained
160 using the N:P factor determined from the LPC amino acid composition.

161 The UV-visible spectra were recorded on the supernatant and after six dilutions (30, 12, 6, 4, 3 and 1.5 times)
162 using a UV-compatible microplate and a plate reader (Spark®, Tecan Trading, Ltd., Switzerland). The liquid
163 pathlength in each well was determined as described in Lampinen, Raitio, Perälä, Oranen, & Harinen (2012).

164 *Calculation of the theoretical UV-visible spectrum*

165 The theoretical UV-visible spectrum of the LPC proteins was calculated from LPC amino acid profile. First, the
166 concentration of tyrosine, tryptophan and phenylalanine was calculated for 1 g.L⁻¹ LPC dispersion. Then, the
167 theoretical UV-visible spectrum was calculated as a linear combination of individual spectrum of each AA
168 extracted from the photochemcad database (<http://photochemcad.com/>). Tyrosine and tryptophan spectra
169 were measured in phosphate buffer (0.1 M, pH 7) and phenylalanine in water.

170 *2.6 Refractive index increment*

171 The dn/dc was determined at 589.3 nm using a multi-wavelength Abbe refractometer (Anton Paar GmbH,
172 Graz/AUSTRIA) at 20 °C for LPC dispersions, similarly to the specific extinction coefficients, at pH 5 (25 mM
173 acetate buffer), pH 7 (10 mM phosphate buffer) and pH 8 (10 mM phosphate buffer). Theoretical dn/dc was
174 calculated from the amino acid composition of LPC according to Zhao, Brown, & Schuck (2011).

175 *2.7 Protein solubility*

176 LPC was solubilised at 5 g.L⁻¹ in water for 30 min at room temperature. The pH was adjusted to the desired value
177 with 0.1 or 0.5 M NaOH or HCl. Dispersions were mixed on a rotary shaker for 2 hours; the pH was checked and
178 adjusted if needed at the end. Dispersions were then centrifuged (10 000 x g, 20 °C, 15 min) to recover soluble
179 proteins. The nitrogen content of total dispersion and of the supernatant was analysed according to the Dumas
180 method using a rapid MAX N exceed (Elementar, Langenselbold, Germany). About 1 mL of protein dispersion
181 was placed in a steel crucible and injected into an oven under a stream of oxygen and helium. The combustion
182 was operated at 900°C with an oxygen flow rate of 100 mL/min for 120 s. The gas mixture was then reduced in
183 a reduction tube and separated on selective trap columns. Glycine was used as a standard for nitrogen content.
184 Measurements were done in triplicates.

185 *2.8 Electrophoretic mobility*

186 To determine LPC isoelectric point, electrophoretic mobility was measured on LPC dispersions at pH ranging from
187 3 to 11. LPC was solubilised at 5 g.L⁻¹ in water for two hours before being centrifuged at 12 000 xg for 20 min.
188 One milliliter of supernatant was adjusted to the desired pH using 0.1 M HCl or 0.1 M NaOH. Electrophoretic
189 mobility was measured in triplicate at each pH using a zetasizer Nano Series (Nano-ZS, Malvern instrument,
190 Germany). The conductivity was about 0.7 +/- 0.3 mS.cm⁻¹. The whole experiment was performed twice. The
191 theoretical net charge of lettuce RuBisCO as a function of pH was computed using the webserver pdb2pqr
192 (<http://server.poissonboltzmann.org/pdb2pqr>), for unfolded and folded spinach RuBisCO (ProteinDataBank
193 entry for spinach RuBisCO: 1aus). The computation was performed on spinach RuBisCO because the 3D-structure
194 of lettuce RuBisCO was not available. Note that the theoretical net charges of unfolded spinach RuBisCO and
195 unfolded lettuce RuBisCO were similar.

196 *2.9 Dynamic light scattering*

197 Dynamic light scattering (DLS) measurements were performed using a zetasizer Nano Series (Nano-ZS, Malvern
198 instrument, Germany). The measurements were performed at 20°C in manual mode: 10 runs of 30 sec were
199 collected and repeated twice for each sample. From the correlation function, the size distribution was calculated
200 using the “general purpose” analysis, provided by the commercial software. It consists in a distribution analysis
201 with a sum of ideal exponential decays. All LPC dispersions were performed overnight, at room temperature
202 before being centrifuged at 12 000 x g for 20 min. The protein concentration was determined after centrifugation
203 using UV absorbance. To study the effect of ionic strength, LPC was dispersed in 0.01 M phosphate buffer pH 7
204 to which was added NaCl to achieve given ionic strengths: 25 mM, 50 mM, 100 mM or 150 mM. To determine
205 the effect of pH, LPC was dispersed in MES buffer 0.03 M pH 6, phosphate buffer 0.01 M pH 7 or phosphate
206 buffer 0.01 M pH 8. When needed, minimal amount of NaCl was added to reach a ionic strength of 25 mM. The
207 effect of protein concentration was measured on dispersions at 1.5 g.L⁻¹, 3 g.L⁻¹ and 9 g.L⁻¹ in phosphate buffer
208 0.01 M pH 7. For each physical-chemical condition, three samples were prepared and measured.

209 *2.10 Size-exclusion chromatography analysis*

210 LPC proteins were extracted as described in Ducrocq et al. (2020) with some modifications. Proteins were first
211 extracted in 0.1 M sodium phosphate buffer pH 6.8 with 1% SDS (v/v) at a solid to liquid ratio of 8 g.L⁻¹. Extraction
212 was performed on a rotary shaker set at 60 rpm at 60 °C for 80 min. The supernatant containing SDS-soluble
213 proteins was recovered after centrifugation (39 191 x g, 30 min, 20 °C). The SE-HPLC apparatus (Waters model

214 LC Module1 plus) was equipped with an analytical column, TSK G4000-SW (7.5 x 300 mm) and a guard column,
215 TSK G3000-SW (7.5 x 75 mm) (both from Merck, Darmstadt, Germany). The columns were eluted at 25°C with
216 0.1 M sodium phosphate buffer pH 6.8 containing 0.1% SDS (v/v). The flow rate was 0.7 mL.min⁻¹ and absorbance
217 was recorded from 210 nm to 700 nm.

218 **3. Results and discussion**

219 *3.1 Composition of the leaf protein concentrate*

220 The composition of the LPC was investigated through the analysis of the amino acid composition and of the
221 nitrogen, ash and sugar contents. Results are expressed on a dry matter basis. Amino acid composition of LPC is
222 detailed in Table 1 and is compared to the theoretical amino acid composition of lettuce RuBisCO. Experimental
223 data obtained on the LPC are very similar to the calculated amino acid composition of lettuce RuBisCO, except
224 for the cysteine content. A 2.5 fold higher amount of cysteine than the amount calculated for lettuce RuBisCO is
225 found in LPC. According to the literature, this difference in cysteine amount is not related to species (Gerloff,
226 Lima, & Stahmann, 1965). The LPC amino acid composition was used to compute the chemical score
227 (Supplementary Table S1). Chemical scores of all essential amino acids are above 100%, meaning that LPC
228 includes all essential amino acids needed for human diet. The composition in amino acids allows the calculation
229 of the N:P factor, which is essential for calculating the protein content from a nitrogen assay. From the amino
230 acid composition described in Table 1, we obtained a nitrogen-to-protein conversion factor of 5.79. This value is
231 within the range of nitrogen-to-protein conversion factor estimated for the leaf proteins of 90 plant species,
232 which varies from 5.15 to 5.93 (Yeoh & Wee, 1994). The nitrogen content of LPC is of 12.8% according to Kjeldahl
233 method, which leads to a protein content of 74.1%. LPC contains about 8.2% neutral sugars and 1.2% uronic acid
234 (Supplementary Table S2). The significant content of sugars, detected in the LPC, probably results from leaf
235 polysaccharides. Besides neutral sugars, the presence of uronic acid suggests the presence of pectin (Kiskini,
236 2017). The LPC contains less than 5 mg of free phenolics (in gallic acid equivalents) in 100 g of powder. Ashes
237 represent 7.8% of the LPC mass and 8.7% of LPC dry mass is of unknown origin.

238 *3.2 Evaluation of protein purity and subunit molecular weights*

239 LPC dispersions were analysed by SDS-PAGE under reduced and non-reduced conditions to evaluate the protein
240 purity and determine the molecular weight of the major proteins in denaturing conditions. Resulting gels are
241 shown in Figure 1A. Three major bands are observed on the SDS-PAGE gels in both non-reducing and reducing

242 conditions at 50, 40 and 14 kg.mol⁻¹. Bands at 50 and 14 kg.mol⁻¹ may respectively correspond to the large-chain
243 (LC) and the small-chain (SC) of RuBisCO. Both bands at 50 and 40 kg.mol⁻¹ are revealed by anti-Rbcl (RuBisCO
244 LC) antibody (Figure 1B) during the Western Blot analysis. This confirms the presence of the RuBisCO LC at 50
245 kg.mol⁻¹ and shows that the band at 40 kg.mol⁻¹ also contains RuBisCO LC. It may correspond to a hydrolysed
246 form of RuBisCO LC, as previously suggested by Hood et al. (1981). Several small bands of medium intensity are
247 observed in non-reducing conditions around 100 kg.mol⁻¹. Their intensity decreases upon reduction and they
248 react with the antibody against RuBisCO LC. These bands may correspond to disulphide bonded dimers of LC.
249 Disulphide linked dimers of RuBisCO LC have already been identified *in vivo* and *in vitro* (Mehta et al., 1992;
250 Rintamaki, 1989) and related to an oxidative stress *in planta* and/or during the extraction process. A low intensity
251 band is observed at ~35 kg.mol⁻¹. Anti-Rbcl antibodies reveal this band and the intensity of the band increases
252 after reduction. This band may also correspond to a hydrolysed form of LC, which would be part of the disulphide
253 bonded aggregates in non-reducing conditions. Apart from identified RuBisCO bands, less intense bands were
254 observed between 10 and 55 kg.mol⁻¹. It is not possible to precisely quantify the RuBisCO purity of the LPC based
255 on SDS-PAGE patterns due to the protein-type dependency of Coomassie staining (Fountoulakis, Juranville, &
256 Manneberg, 1992). However, the SDS-PAGE analysis highlights that RuBisCO is the major protein of LPC.

257 *3.3 Determination of the UV-visible spectroscopic and refractometric parameters*

258 In the wavelength range of 235-300 nm, the UV-visible spectrum of a protein is mostly driven by its content in
259 UV-absorbing amino acids namely tyrosine, tryptophan, and phenylalanine. The UV-visible spectrum of a protein
260 also varies with the environment of the aromatic amino acids and their solvent exposure but this variation is
261 expected to be small (<5%) (Pace, Vajdos, Fee, Grimsley, & Gray, 1995). Therefore, from the proportions of the
262 three UV-absorbing amino acids in LPC and their respective individual spectra, a theoretical UV-visible spectrum
263 of the protein was calculated for a 1 g.L⁻¹ LPC dispersion in aqueous condition (Figure 2A). LPC theoretical
264 spectrum shows a maximum of absorbance at 275 nm due to a major contribution in tryptophan and tyrosine.
265 Experimental UV-visible spectra of LPC dispersion in water at several protein concentrations are represented in
266 Figure 2B. Above 245 nm, the absorbance reaches a maximum at 258 nm and displays residual absorption
267 between 300 and 420 nm. Other spectra obtained at pH 5, pH 7 and pH 8 present the same profile and maximum
268 absorbance around 258 nm (Supplementary Figure S1). This suggests the presence of non-protein compounds
269 that absorb around 260 nm and 300-450 nm. Phenolic compounds are common non-protein contaminants in

270 LPC (Pedone, Selvaggini, & Fantozzi, 1995). Among them, hydroxycinnamates and flavonols are well-known
271 phenolic compounds previously identified in *Cichorium endivia* leaves exhibiting maximum absorbance in the
272 300-330 nm and in the 340-370 nm ranges, respectively (Mascherpa, Carazzone, Marrubini, Gazzani, & Papetti,
273 2012). Nucleic acids absorb in the range 230-300 nm with a maximum of absorbance around 260 nm (Groves,
274 W.E., Davis, F.C., & Sells, B.H., 1968). They could contribute to the UV-visible spectra of LPC dispersions below
275 300 nm. Absorbance at higher wavelength (400-420 nm) may also correspond to brown-yellow phenolic
276 oxidation products that would have been generated during the preparation of the LPC. Some of them, such as
277 rutin, have high absorption around 260 nm and 400 nm and could correspond to the non-protein UV-absorbing
278 species (Mirgorod, Borodina, & Borsch, 2013).

279 These results point to a significant contribution of non-protein species to the LPC absorbance at 280 nm.
280 Therefore, the use of extinction coefficients values found in literature or calculated from the amino acid
281 sequence is not adapted to LPC dispersions. The specific extinction coefficient at 280 nm was determined for LPC
282 in buffers at pH 5, pH 7 and pH 8. The linearity of Beer Lambert's law was checked in triplicate for each physical-
283 chemical condition (inset in Figure 2B). The specific extinction coefficients are of $4.96 \text{ cm}^{-1} \text{ g}^{-1} \text{ L}$ at pH 5, 5.82 cm^{-1}
284 $\text{g}^{-1} \text{ L}$ at pH 7 and $4.58 \text{ cm}^{-1} \text{ g}^{-1} \text{ L}$ at pH 8. Several studies carried out on purified RuBisCO report specific extinction
285 coefficients in the range of 1.41 to $1.82 \text{ cm}^{-1} \text{ g}^{-1} \text{ L}$ depending on the species and the method of extraction of the
286 proteins (reviewed in Douillard & de Mathan, 1994). Moreover, the specific extinction coefficient calculated from
287 the LPC amino acid profile is $0.94 \text{ cm}^{-1} \text{ g}^{-1} \text{ L}$. This latter value is quite lower than the previously reported values,
288 which may be due to the difficulty of assaying tryptophan in protein concentrates (Oelshlegel, Schroeder, &
289 Stahmann, 1970). These values are much lower than those measured in our study, supporting the presence of
290 non-protein UV-absorbing species in the LPC.

291 We computed the dn/dc from the amino acid composition of LPC and obtained 0.189 mL.g^{-1} at 589.3 nm and
292 25°C . This value is close to the consensus value of 0.185 mL.g^{-1} generally accepted for proteins (Zhao et al., 2011).
293 The dn/dc values measured on LPC dispersions are of 0.27 at pH 8, 0.31 at pH 7 and 0.39 at pH 5. These values
294 are high as compared to calculated values, suggesting the presence of non protein species. Polysaccharides and
295 nucleic acids have dn/dc values of 0.15 and 0.17-0.19 (Theisen, Johann, Deacon, & Harding, 2000). Tannins are
296 expected to have dn/dc around 0.25-0.26 mL/g at 620 nm (Vernhet A., Dubascoux S., Cabane B., Fulcrand H.,
297 Dubreucq E., Poncet-Legrand C., 2011). The presence of polysaccharides, nucleic acids or tannins seems

298 therefore not to explain such high dn/dc values. To our knowledge, no experimental dn/dc are available for
299 flavonol and hydroxycinnamates that are compatible with the UV-visible spectra (e.g. caffeic acid).

300 *3.4 Insight into the interactions between UV-absorbing species and RuBisCO*

301 To identify possible covalent bound between RuBisCO and the UV-absorbing species, LPC proteins were
302 extracted in a denaturing buffer containing SDS and analysed using size-exclusion HPLC. Signal was recorded
303 from 200 to 700 nm. The elution profile obtained at 214 nm is represented on Figure 3A. Three major peaks are
304 visible. The main fraction was eluted with an apparent molecular weight of about 15 kg.mol⁻¹ and was attributed
305 to RuBisCO SC. The peak eluted at 14.3 min (~45 kg.mol⁻¹) was attributed to the RuBisCO LC and the peak eluted
306 at 13.15 min (~100 kg.mol⁻¹) corresponds to the LC dimer. A shoulder is visible between the LC and the SC peaks,
307 corresponding to a specie of about 28 kg.mol⁻¹. Smaller peaks are detected below 10 kg.mol⁻¹, they correspond
308 to small peptides or non-protein species.

309 UV-visible spectra recorded for these four main peaks are represented on Figure 3B. Spectra associated with
310 RuBisCO LC and LC dimer have the same profile with a maximum absorbance at 276 nm. This wavelength is close
311 to the wavelength of the maximum absorbance of the theoretical spectrum of LPC (Figure 2A). This result
312 suggests that RuBisCO LC and LC-dimer are not covalently linked with any UV-absorbing species. In contrast, the
313 absorption spectrum associated with the RuBisCO-SC displays a maximum at 265 nm. Despite the absence of
314 molecular weight change, few phenolic compounds may be covalently-bound to RuBisCO-SC. In addition,
315 molecules eluted before and after the SC have high absorbance at 260 nm resulting in high A_{260 nm}/A_{214 nm} ratio
316 (0.37 and 0.79 as compared to 0.16 for SC peak). The distortion of the RuBisCO SC spectrum may also be due to
317 the absorbance of these two adjacent species. The absorption spectra associated with the peak detected around
318 28 kg.mol⁻¹ displays a maximum at 259 nm. A similar 28 kg.mol⁻¹ molecule is also detected on a commercial
319 RuBisCO (Sigma-Aldrich, St. Louis, MO). Its maximum absorbance is measured at 277 nm, supporting a proteic
320 nature (Supplementary Figure S2).

321 All these data suggest that RuBisCO LC is not covalently bonded with a UV-absorbing species. In contrast, RuBisCO
322 SC and a protein specie of about 28 kg.mol⁻¹ may be covalently bounded to few phenolic compounds.

323 *3.5 Effect of pH on protein solubility and surface charge*

324 The solubility of LPC proteins was determined as a function of pH. For pH > 6, the solubility was higher than 90%
325 as shown in Figure 4A. A minimum of solubility was obtained for pH around 4. The overall features of the solubility
326 curve are consistent with literature data on LPC proteins (Kiskini, 2017). The minimum of solubility of the LPC
327 proteins is consistent with the electrophoretic mobility measurements as presented in Figure 4B (empty
328 symbols). The isoelectric point (IEP) of LPC dispersion is around pH 3.8 and it has a negative charge for pH > 4.0.
329 The IEP of the LPC is in the range of sugar beet leaves protein concentrate as determined by zeta potential
330 measurement (Kiskini, 2017). The IEP of the LPC is however lower than other experimental values obtained using
331 isoelectric focusing: between 5 and 5.5 for spinach RuBisCO (Iwanij, Chua, & Siekevitz, 1974; Matsumoto,
332 Sugiyama, & Akazawa, 1969), around 7 for lettuce RuBisCO (Rabinowitz, Reisfeld, Sagher, & Edelman, 1975), and
333 6 for alfalfa RuBisCO (reviewed in Douillard & de Mathan, 1994). The isoelectric point of maize RuBisCO was
334 reported around 4.6 as obtained by isoelectric focusing (Reger, Ku, Pottert, & Evans, 1983). For comparison, the
335 pH-dependence of the calculated total net charge of spinach RuBisCO is reported (full symbols). For unfolded
336 spinach RuBisCO, an equal number of positive and negative charges, the isoionic point (IIP), is expected at pH
337 6.6. The IIP of spinach RuBisCO computed with PROPKA software (version 3.0) is 6.0 instead of 6.6, a shift related
338 to RuBisCO conformation. The experimental IEP of LPC is much lower than the theoretical IIP of RuBisCO. Such
339 discrepancy may be ascribed to the presence of the absorbed species previously highlighted.

340 In a previous work, it was suggested that the discrepancy between experimental IEP and theoretical IIP on LPC
341 dispersions was related to the presence of pectin (Kiskini, 2017). Pectin that have been highlighted in our LPC
342 may indeed reduce the IIP of RuBisCO. Moreover, several studies report an acidification of proteins after their
343 derivatisation by phenolic compounds. Higher degrees of derivatisation induced lower IEP of soybean glycinins
344 (Rawel, Czajka, Rohn, & Kroll, 2002) and of lysozyme (Rawel, Kroll, & Rohn, 2001).

345 *3.6 Impact of pH on the size distribution of proteins*

346 DLS measurements were performed to highlight the effect of pH on LPC dispersion hydrodynamic properties.
347 Representative correlation functions are shown in Figure 5A. Two major peaks are found with a mean
348 hydrodynamic radius (R_h) of 7.5 and 45 nm, respectively (Figure 5B). The smallest size population could
349 correspond to RuBisCO hexadecamer since the R_h is close the Stokes radius of 6.4 nm estimated for RuBisCO
350 from citrus leaves (Penarrubia & Moreno, 1988) and of 7.4 nm from oat (Steer, M.W., Gunning, B.E., Graham,
351 T.A., & Carr, D.J., 1968). This is also in the range of the one computed for lettuce RuBisCO using the Hullrad server

352 (6.4-6.5 nm). An increase in pH from 6 to 8 favors the proportion of smaller particles. Electrostatic repulsions at
353 basic pH, as suggested by the electrophoretic mobility results, may prevent protein aggregation. At the other pH,
354 part of the RuBisCO may no longer be in the form of hexadecamer. The protein concentration, up to 9.5 g.L⁻¹,
355 and the ionic strength, up to 150 mM, do not affect the size distribution of the LPC dispersion (Supplementary
356 Figure S4).

357 **4. Conclusion and perspectives**

358 We showed that the LPC has a protein content higher than 74% and mainly consists of RuBisCO. The presence of
359 non-protein compounds was evidenced, mostly pectin and UV-absorbing species. These compounds affect the
360 extinction coefficient, the refractive index increment and the surface charge of RuBisCO. Despite these changes,
361 a solubility higher than 90% was observed for pH > 6.0. Such a high solubility may provide interesting techno-
362 functionalities for the use of LPC as a food ingredient. Beyond the characterisation of a leaf protein concentrate,
363 this study raises the effect of minor compounds on the spectroscopic and physical-chemical properties of protein
364 ingredients. This is especially true for plant-based protein concentrate that are increasingly investigated to
365 replace proteins of animal origins. Since such minor compounds may affect the techno-functional properties of
366 protein ingredients, a thorough characterisation of such ingredients would help in better understanding and
367 controlling their use in food applications.

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372 performing Western-Blot and Nouredine Hafnaoui (UNH, INRAE) and Didier Remond (UNH, INRAE) for
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377 **Declaration of competing interest**

378 The authors declare that they have no known competing financial interests or personal relationships that could
379 have appeared to influence the work reported in this paper.

380 **CRediT authorship contribution statement**

381 Maude Ducrocq: Conceptualization, Methodology, Investigation, Writing - original draft, Writing - review &
382 editing. Marie-Hélène Morel: Conceptualization, Methodology, Writing - review & editing. Marc Anton: Project
383 administration, Funding acquisition. Valérie Micard: Writing - review & editing, Project administration, Funding
384 acquisition. Sylvain Guyot: Conceptualization, Writing - review & editing. Valérie Beaumal: Methodology,
385 Investigation. Véronique Solé-Jamault: Investigation, Resources. Adeline Boire: Conceptualization, Methodology,
386 Investigation, Writing - original draft, Writing - review & editing, Supervision.

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495 **Table captions**

496 **Table 1** Amino acid composition determined on LPC in $\text{mg}\cdot\text{g}^{-1}$ of protein as compared to calculated amino acid
497 composition of lettuce RuBisCO (based on lettuce RuBisCO sequence found in UniProt database: *lactuca sativa*;
498 accession numbers of LC and SC: P48706 and Q40250).

499 **Figure captions**

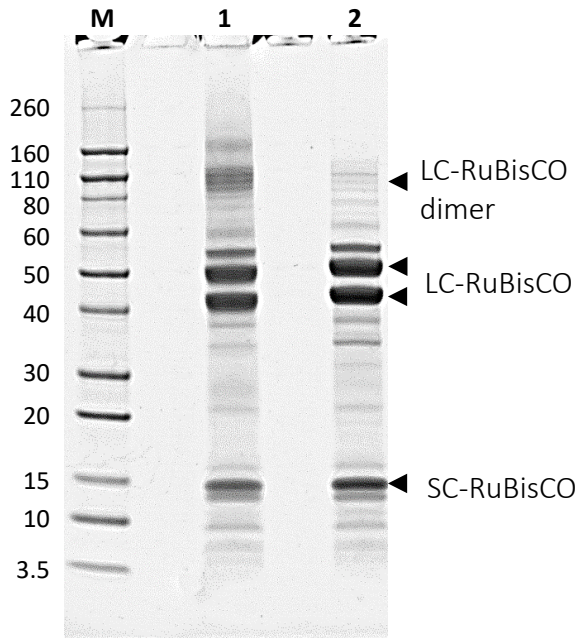
500 **Figure 1** SDS-PAGE (**A**) and Western blot (**B**) patterns of LPC in non-reducing (lane 1) and reducing conditions
501 (lane 2). Arrows indicate RuBisCO sub-units. LC-RuBisCO stands for RuBisCO large chain and SC-RuBisCO stands
502 for RuBisCO small chain. Lane M corresponds to molecular weight standards in $\text{kg}\cdot\text{mol}^{-1}$.

503 **Figure 2 A.** UV-visible spectra calculated for phenylalanine (dotted line), tyrosine (dashed line) and tryptophan
504 (dashdotted line) for a $1\text{ g}\cdot\text{L}^{-1}$ LPC dispersion; and total calculated UV-visible spectra (solid line) for a $1\text{ g}\cdot\text{L}^{-1}$ LPC
505 dispersion. Calculations are based on the amino acid composition of LPC. **B.** UV-visible spectra of LPC dispersion
506 in water at several protein concentrations: 0.43 (full line), 0.21 (dashdotted line) and 0.11 (dashed line) $\text{mg}\cdot\text{mL}^{-1}$.
507 ¹. The inset represents the Beer-Lambert law established for RuBisCO in water.

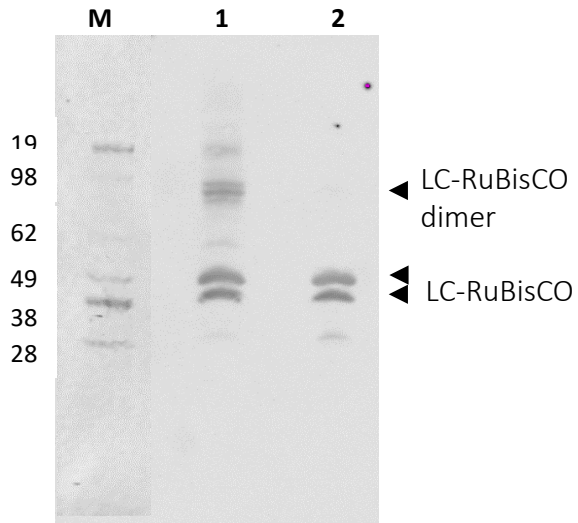
508 **Figure 3 A.** Elution profile, recorded at 214 nm, of SDS soluble proteins extracted from LPC. Symbols represent
509 peaks of interest: RuBisCO small chain (SC; ∇), possible UV-absorbing species (o), RuBisCO large chain (LC; \diamond)
510 and RuBisCO large chain dimer (LC dimer; \blacklozenge). **B.** UV-visible spectra acquired at the elution times corresponding
511 to the peaks at 214 nm of SC, contaminant, LC and LC dimer.

512 **Figure 4 A.** Evolution of the LPC protein solubility in water from pH 3 to pH 10. **B.** Experimental electrophoretic
513 mobility (empty symbols) of LPC dispersion (at $2.9\text{ g}\cdot\text{L}^{-1}$ for diamonds and $2.8\text{ g}\cdot\text{L}^{-1}$ for crosses) according to pH
514 and estimated total net charge (full symbols) of unfolded (circles, pointed line) and folded (triangles, dashed line)
515 spinach RuBisCO. Spinach RuBisCO total net charge was estimated using PROPKA (3.0) with spinach RuBisCO
516 structure from PDB (entry: 1AUS).

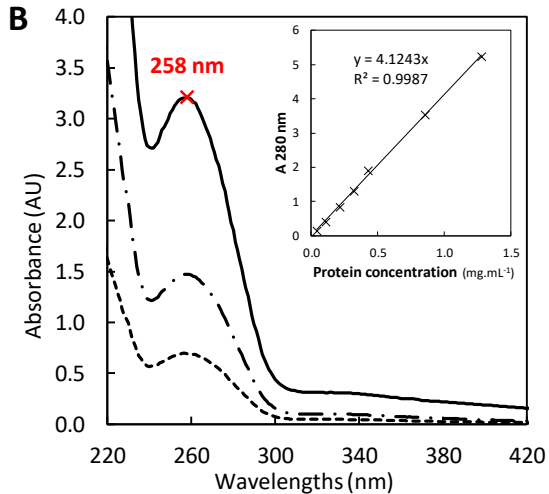
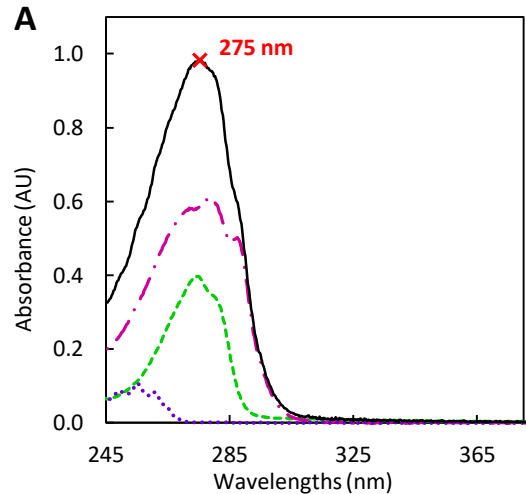
517 **Figure 5** Correlation functions (**A**) and size distribution (**B**) obtained by dynamic light scattering according to pH.
518 Correlation functions are representative of at least six observations. Size distribution curves are the mean of six
519 observations. For readability reasons, the standard deviations are not shown; they suggest a significant
520 difference between the mean curves.

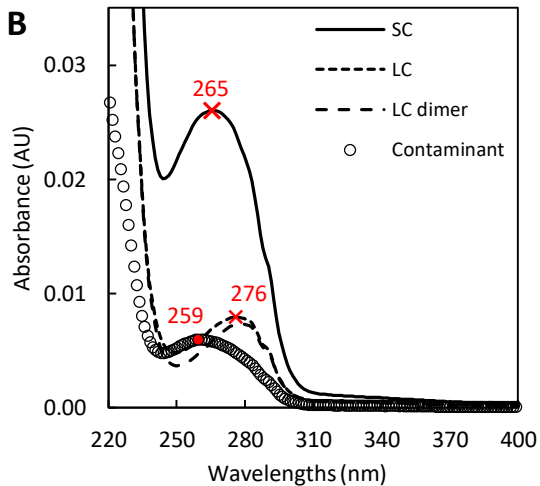
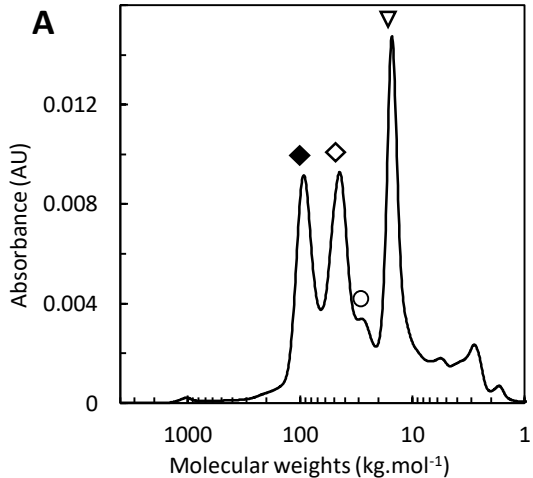


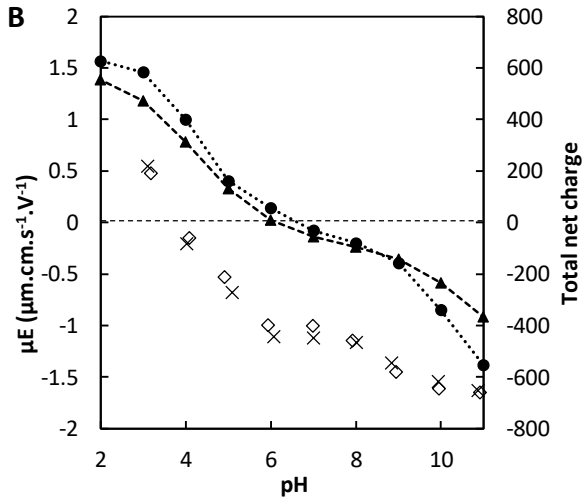
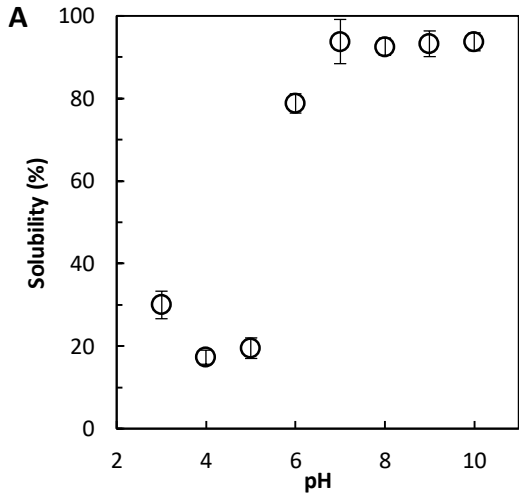
A



B







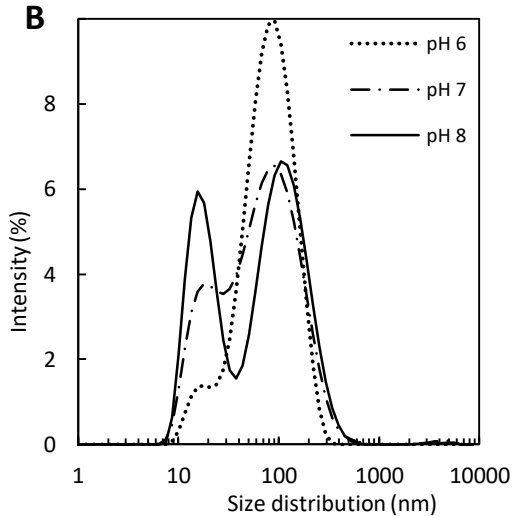
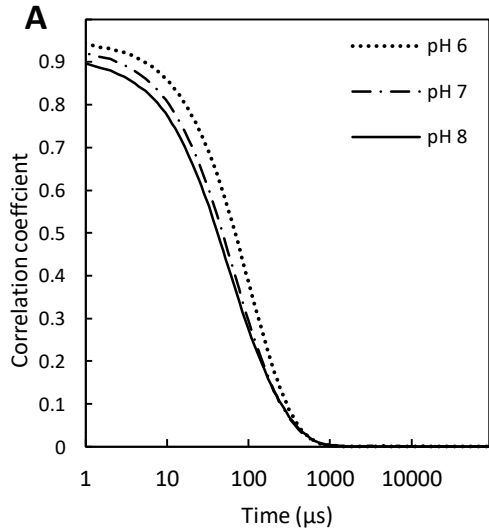


Table 1 Amino acid composition determined on LPC in mg.g⁻¹ of protein as compared to calculated amino acid composition of lettuce RuBisCO (based on lettuce RuBisCO sequence found in UniProt database: *lactuca sativa*; accession numbers of LC and SC: P48706 and Q40250).

Amino acids	Amino acid residue content (mg.g ⁻¹ total amino acid residues)	
	Leaf protein concentrate	Lettuce RuBisCO
Ala	55.6	49.7
Arg	56.7	76.7
Asp	82.3	83.6
Cys	45.0	18.4
Glu	116.6	116.9
Gly	49.9	46.7
His	33.7	36.7
Ile	45.4	53.9
Leu	91.1	84.2
Lys	62.9	64.8
Met	29.3	25.4
Phe	56.7	61.3
Pro	63.9	46.2
Ser	24.4	29.8
Thr	55.5	54.2
Trp	21.4	33.2
Tyr	48.4	60.7
Val	61.4	57.5