

## Effect of Residence Time on the Fractionation of Whey Protein Isolate with ScCO<sub>2</sub> in a Continuous Reactor

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This study proposes a fractionation of proteins using solutions (5%) of Whey Protein Isolate (WPI) and supercritical carbon dioxide (scCO<sub>2</sub>) in a continuous reactor. The experiments were performed at different values of pressure, temperature, and residence times - 65.4, 34.3, and 32.3 min, respectively.  $\alpha$ -lactalbumin ( $\alpha$ -LA) and  $\beta$ -lactoglobulin ( $\beta$ -LG) levels were determined by HPLC. WPI used without treatment showed an  $\alpha/\beta$  ratio = 0.40. 16 MPa/55 °C and 16 MPa/60 °C were the conditions that suffered the most positive and negative influence of the increase and decrease in residence time, respectively. In the most severe condition, 24 MPa, 65 °C, and 4.0 mL·min<sup>-1</sup>, circular dichroism indicated that, despite not having obtained a high level of pureness, the fractionation technique employing scCO<sub>2</sub> did not denature the proteins  $\alpha$ -LA and  $\beta$ -LG.

### 1. Introduction

Whey protein isolate (WPI) has a high amount of proteins, more than 90%, based on dry basis, which is responsible for being considered a valuable dairy industry by-product (Dianin et al., 2019). The two main proteins in whey are  $\beta$ -lactoglobulin ( $\beta$ -LG) and  $\alpha$ -lactalbumin ( $\alpha$ -LA), but their sizes are similar. So, the use of membranes to separate them is not recommended. An option to overcome this problem is to use the difference between their isoelectric points ( $\beta$ -LG is 5.13 and  $\alpha$ -LA from 4.2 to 4.5). Supercritical carbon dioxide (scCO<sub>2</sub>) arises as a viable alternative for protein fractionation. scCO<sub>2</sub> mixed with protein solutions can denature, destabilize, and precipitate some proteins due to the combination of its acidifying and anti-solvent activities. Besides, the low values of criticals pressure and temperature made scCO<sub>2</sub> a green and eco-friendly solvent (Bakar et al., 2020). The precipitation of proteins is due to changes in secondary and tertiary structures induced by the decrease of pH (Bonnaillie & Tomasula, 2008). WPI fractionation using scCO<sub>2</sub> in batch reactors has been studied previously (Bonnaillie & Tomasula, 2012). However, recently, the use of continuous reactors has attracted some attention. Such way of operation allows to obtain products with better quality, lower production costs, and require equipment with small dimensions for the process implementation neither a large number of operators (Wiles & Watts, 2014).

Therefore, this study's main goal is to evaluate the residence time influence in the selectivity fractionation of the  $\alpha$ -LA and  $\beta$ -LG proteins from Whey Protein Isolate (WPI) solutions - obtained from the powder WPI dissolved in distilled water - using scCO<sub>2</sub> in a continuous flow reactor. In this way, we determined the protein contents in the precipitated and soluble fractions obtained in the fractionation process. We also got the solid and liquid fraction's secondary structure to determine the protein's irreversible denaturations.

## 2. Materials and methods

### 2.1 Protein fractionation

In the protein fractionation experiments, we used a 5% protein isolate solution prepared using powder WPI purchased from Farmácia de Manipulação Medicinal LTDA (Maringá, Paraná, Brazil). The protein solution was kept in a stirring process for 2 hours before the experiment starts. For those experiments, we also acquired carbon dioxide (95% purity) from White Martins S.A. (Osasco, São Paulo, Brazil). Lima et al. (2019) present the details about the equipment and procedures used for protein fractionation. Table 1 shows the pressures, temperatures, volumetric flow rates, and residence times used here.

Table 1: Process conditions employed in the experiment.

Binomials used (Pressure x Temperature)	$\rho$ CO <sub>2</sub> (g·mL <sup>-1</sup> ) (depended on pressure only)	CO <sub>2</sub> flow rate (mL·min <sup>-1</sup> )	WPI flow rate (mL·min <sup>-1</sup> )	Residence time (min)
8 MPa / 55 °C	0.8277	1.812	1.50	65.4
16 MPa / 55 °C	0.9118	1.645	1.50	65.4
16 MPa / 60 °C	0.9118	1.645	1.50	65.4
24 MPa / 65 °C	0.9587	1.565	1.50	65.4
8 MPa / 55 °C	0.8277	3.625	3.00	34.2
16 MPa / 55 °C	0.9118	3.290	3.00	34.2
16 MPa / 60 °C	0.9118	3.290	3.00	34.2
24 MPa / 65 °C	0.9587	3.129	3.00	34.2
8 MPa / 55 °C	0.8277	4.833	4.00	32.3
16 MPa / 55 °C	0.9118	4.387	4.00	32.3
16 MPa / 60 °C	0.9118	4.387	4.00	32.3
24 MPa / 65 °C	0.9587	4.172	4.00	32.3

Flow rates of 1.5 and 4.0 mL·min<sup>-1</sup> were chosen due to this study continues the study of Lima et al. (2019). In that work, the authors employed the flow rate of 3.0 mL·min<sup>-1</sup> and the residence time of 34.2 min. Here, the aim is to evaluate the effect of variable residence time in protein recovery using higher e lower residence time values than those used by Lima et al. (2019). We collected the samples obtained during each experiment in 15-minute intervals. Next, they were centrifugated in Falcon tubes using Quimis centrifuge - Q222TM at 2000 rpm for 40 minutes. The obtained precipitate and supernatant were nominated fraction rich in  $\alpha$ -LA and fraction rich in  $\beta$ -LG, respectively. At each condition, it was obtained 4 samples posterior to the BPR: 2 (two) precipitates and 2 (two) supernatants. The precipitated ones were resuspended in distilled water for the transference in containers for later analysis. Immediately after being properly packed, all samples were refrigerated. Next, they were frozen in liquid nitrogen, then lyophilized in proper equipment (Terroni Enterprise I). It is valid to highlight that their weighings were done before and after the lyophilization, to study the process's productivity. Next, it has proceeded with their packing in eppendorfs, and they were kept in refrigeration until the moment of posterior analysis. The pH of the reactional medium was estimated using Equation (1) (Bonnaille & Tomasula, 2012), where  $P$  and  $C$  were the pressure and the WPI solution concentration, respectively:

$$pH(P, C) = -0.248 \ln(P) + 0.216 \ln(C) + 9.365 \quad (1)$$

The pH from collected samples after depressurization was measured using a pH meter (Hanna-HI-5521). We calculated the recovery of protein  $\alpha$ -LA in the precipitate ( $reca$ ) using Equation (2). In this Equation,  $x\alpha_{precipitated}$  is the  $\alpha$ -LA concentration in the precipitate,  $x\alpha_{WPI}$  is the  $\alpha$ -LA concentration in WPI without treatment,  $m_{precipitated}$  is the precipitated protein mass, and  $m_{WPI}$  is the WPI mass collected during 15 minutes:

$$reca = \frac{(x\alpha_{precipitated} \times m_{precipitated})}{(x\alpha_{WPI} \times m_{WPI})} \quad (2)$$

We obtained the recovery of  $\beta$ -LG ( $rec\beta$ ) using an expression similar to Equation (2). In this case, we substituted the  $\alpha$ -LA concentrations to the corresponding values of  $\beta$ -LG. We considered its recovery in the supernatant fraction as all of  $\beta$ -LG that did not precipitate, given by  $1 - (rec\beta)$ .

### 2.2 Determination of $\alpha$ -LA, $\beta$ -LG, and GMP

We determined the  $\alpha$ -LA and  $\beta$ -LG contents after the fractionation using HPLC analysis (Shimadzu, Kyoto, Japan). Mobile phase A was a mixture of water and TFA (1000:1), while the mobile phase B was a mixture of acetonitrile and TFA (1000:1). The elution occurred at room temperature in a gradient regimen: 0 - 35 min (30 -

50% of B), 35 - 40 min (50 - 30% of B), using a flow rate of 1 mL·min<sup>-1</sup>, a wavelength of 215 nm and injection volume of 20 µL (Xiao-Yu et al., 2012). Samples were prepared in 5 mg·min<sup>-1</sup> concentration, heated up to 30 °C for 15 minutes and filtered in syringe filter (0.45 µm). The α-LA and β-LG contents were determined by standard curve, which was determined injecting the two proteins in the concentration ratio of β-LG/α-LA = 2, due to that being the standard ratio found in whey. The mother-solution was prepared with a mixture of proteins with α-LA and β-LG concentrations of 2.5 and 5.0 mg·mL<sup>-1</sup>, respectively. From that solution, successive dilutions were made, with α-LA concentration varying between 0.25 – 2.5 mg·mL<sup>-1</sup> and β-LG from 0.25 to 4.0 mg·mL<sup>-1</sup>. GMP content was determined via reverse phase liquid chromatography (Varian 920-LC RP-HPLC) linked to an UV/VIS detector, using C18 Gemini column (110 Å, 250 x 4.6 mm) charged with 5 µm diameter particles and a guard column (Security Guard™). Here, mobile phase A consisted of a mixture of acetonitrile, water, and TFA (100:900:1, v/v/v), while mobile phase B was a mixture of the same components in the proportion (900:100:0.8, v/v/v). Elution was made in gradient regimen: 0 - 13 min (15 - 28% of B), 13 - 22 min (28 - 32% of B), 22 - 25 min (32 - 70% of B), 25 - 30 min (70 - 70% of B), 30 - 32 min (70 - 15% of B), 32 - 40 min (15 - 15% of B). The injection volume used was 50 µL, wavelength of 220 nm, and temperature at 30 °C (Minkiewicz et al., 1996). GMP analytical curve was determined using protein concentrations from 0.125 to 2.0 mg·mL<sup>-1</sup>.

### 2.3 Circular dichroism analysis

Circular dichroism (CD) aims to estimate if a macromolecule is found doubled, exploring the fact that different protein folding states show different spectrums of dichroism (Johnson, 1990). CD analysis was made in a spectropolarimeter (JASCO J-815, Japan) with a temperature control via a Peltier system (PTC-4235/15) and optical path 0.5 mm. The sample concentration to be injected was 0.2 mg·mL<sup>-1</sup> and diluted in phosphate buffer 10 nM.

Samples submitted to that analysis were obtained upon the most drastic condition in the fractionation using scCO<sub>2</sub> - 24 MPa, 65 °C, and a flow rate of 4.0 mL·min<sup>-1</sup>. In that quantitative analysis, it was possible to evaluate the samples' thermal denaturation behavior at temperatures between 25 and 94 °C (Δt = 3 °C). For each temperature, we obtained four spectrums, in the region far-UV (190 - 260 nm).

## 3. Results and Discussion

### 3.1 Composition of obtained fractions

Table 2 shows the composition of the fractions obtained after fractionation using scCO<sub>2</sub>. That table presents pressures, temperatures, and residence times employed, as well as the amount of precipitates ( $R_{prec}$ ) obtained. At 8 MPa/55 °C, a drop in α/β ratio was observed. It was expected that with higher residence times, α-LA concentration in precipitated fraction would be higher, but that did not happen. It seems that its kinetics aggregation is fast. Thereby purer fractions are obtained in shorter residence times. The sudden drop that occurred in α/β ratio in precipitated fraction between residence times of 34.2 min and 32.3 min may be explained by the still unknown mechanism of α-LA aggregation upon this fractionation technique. There was a decrease in α-LA and an increase in β-LG in the precipitated fraction. In the supernatant fraction, we verified an increase of both α-LA and β-LG amounts. An increase in recovery of α-LA and of β-LG in precipitated fraction and a decrease in β-LG recovery in the supernatant fraction indicates that pureness was compromised with the change of residence time in the pressure of 8 MPa and 55 °C.

At 16 MPa/55 °C, an increase in the α/β ratio was observed in the precipitate, increasing and decreasing the flow rate. In the supernatant fraction, this parameter suffered a sharp drop. The pH was closer to α-LA isoelectric point, which favors its aggregation and contributes to the obtention of higher α/β ratios in the precipitated fraction. It suggests that pH influences α-LA aggregation, having contributed to obtain a fraction with higher purity, with both increase and decrease of residence time in the continuous reactor. The difference between α/β ratios in precipitated fraction using residence times of 34.2 min and 32.3 min might be related to a drop in α-LA aggregated amount during that specific length of time associated with the protein aggregation mechanism in that pH condition. There was an increase in α-LA amount and a decrease in β-LG in precipitated fraction. In the other way, there was a decrease in α-LA and an increase in β-LG in the supernatant fraction. There was an increase in α-LA recovery and an oscillation in β-LG in precipitated fraction and an oscillation in β-LG in the supernatant fraction, indicating that α-LA purity obtained was superior, both increasing and decreasing residence times at 16 MPa/55 °C.

At 16 MPa/60 °C, a drop in the α/β ratio in the precipitated was observed, increasing and decreasing flow rate. In the supernatant fraction, α/β ratio suffered an increase in both different residence times. pH was the same as the previous condition, but the temperature was higher. Solubilization of CO<sub>2</sub> in water suffers influence from temperature. Here, CO<sub>2</sub> solubilization has a limitation with temperature increase. Such fact impacts the medium acidification since chemical species responsible for pH lowering will be less produced. Besides, temperature influences the chemical reaction kinetics - tending to improve the production rate of protein fractions and

minimize residence time inside the reactor. However, the WPI solution's exposure to a residence time too long may restrain the  $\alpha$ -LA aggregation due to the difficulty in acidifying the reactional medium. The difference of  $\alpha/\beta$  ratios in precipitated fraction between residence times of 34.2 min and 32.3 min suggests that 34.2 min might be an outlier that represents an increase in aggregation of that protein or that it might be the peak of the aggregation in question. There was an increase of  $\alpha$ -LA and  $\beta$ -LG recoveries in the precipitated fraction and decreased  $\beta$ -LG recovery in the supernatant fraction. Even though there was a higher recovery in  $\alpha$ -LA in the precipitate, this fact indicates that the purity was not improved, changing the residence time at 16 MPa/60 °C.

Table 2: Composition of obtained fractions after fractionation using  $scCO_2$ .

WPI <sup>2</sup>	P/MPa T/°C	TR (min)	pH <sup>1</sup>	R <sub>prec</sub> %	Fraction rich in $\alpha$ -LA (precipitated or solid)					Fraction rich in $\beta$ -LG (solution or liquid)					
					$\alpha$ -LA %	$\beta$ -LG %	rec $\alpha$ %	rec $\beta$ %	GMP %	$\alpha/\beta$	$\alpha$ -LA %	$\beta$ -LG %	1-rec $\beta$ %	GMP %	$\alpha/\beta$
1		65.4		13.3±0.01	30.5±0.2	18.0±0.1	24.0	5.7	1.0±0.0	1.69	13.3±0.1	53.8±0.1	94.3	10.6±0.1	0.25
2	8/55	34.3	4.78	6.2±0.03	41.7±0.2	9.1±0.2	15.4	1.1	2.7±0.1	4.61	11.2±0.1	49.7±0.2	98.9	19.8±0.3	0.23
3		32.3		10.6±0.01	36.8±0.1	19.3±0.1	23.1	4.9	3.1±0.0	1.91	13.3±0.1	57.0±0.2	95.1	16.1±0.1	0.23
4		65.4		22.1±0.00	39.0±0.1	11.3±0.0	50.9	5.9	2.2±0.0	3.51	9.5±0.0	57.0±0.1	94.1	19.7±0.1	0.17
5	16/55	34.3	4.60	8.8±0.02	32.2±0.2	22.3±0.1	22.3	4.7	3.4±0.1	1.46	13.5±0.1	48.3±0.1	95.3	17.0±0.4	0.28
6		32.3		13.4±0.03	44.5±0.2	13.8±0.1	36.0	4.2	2.0±0.0	3.23	9.5±0.2	60.0±0.1	95.8	6.4±0.1	0.15
7		65.4		21.7±0.02	39.8±0.2	14.8±0.1	51.5	7.4	1.5±0.0	2.77	10.3±0.1	55.0±0.0	92.6	8.7±0.1	0.19
8	16/60	34.3	4.60	13.5±0.00	47.5±0.1	11.2±0.2	24.5	1.5	2.6±0.0	5.42	8.1±0.1	57.4±0.1	98.5	11.9±0.1	0.14
9		32.3		19.2±0.03	40.5±0.4	15.0±0.0	47.2	6.8	1.6±0.0	2.73	10.5±0.2	57.3±0.1	93.2	16.5±0.1	0.19
10		65.4		31.8±0.03	36.0±0.1	22.3±0.1	68.2	17.1	1.3±0.0	1.62	3.8±0.1	55.3±0.1	82.9	23.0±0.1	0.07
11	24/65	34.3	4.50	7.5±0.05	40.2±0.1	19.6±0.3	6.5	1.1	3.5±0.0	2.06	6.6±0.1	55.7±0.0	98.9	20.6±0.5	0.12
12		32.3		30.8±0.01	42.5±0.1	21.0±0.1	76.7	15.6	0.9±0.0	1.99	4.8±0.1	56.8±0.1	84.4	17.7±0.1	0.08

where: P: pressure in which reaction occurs; T: temperature in which reaction occurs; TR: residence time in constant flux reactor; R<sub>prec</sub>: yield in the precipitated (fraction rich in  $\alpha$ -LA); rec $\alpha$ :  $\alpha$ -LA recovery in fraction rich in  $\alpha$ -LA; rec $\beta$ :  $\beta$ -LG recovery in fraction rich in  $\alpha$ -LA;  $\alpha/\beta$ : ratio between  $\alpha$ -LA and  $\beta$ -LG in a fraction; 1-rec $\beta$ :  $\beta$ -LG recovery in fraction rich in  $\beta$ -LG.

<sup>1</sup>pH was estimated using the Equation (1);

<sup>2</sup>This line represents the composition of the 5% WPI solution with no treatment (supercritical fractionation).

At 24 MPa/65 °C, a drop in  $\alpha/\beta$  ratio in precipitated was observed, increasing and decreasing the flow rate. The  $\alpha/\beta$  ratio in the supernatant fraction suffered a reduction. The pH estimated was the lowest value, and 65 °C was the highest temperature of all experiments. Here, the medium's acidification faces even more difficulties due to the highest temperature employed, suggesting that too long residence times inside the reactor in these most extreme conditions compromise  $\alpha$ -LA protein aggregation. A decrease in residence time did not result in such a sharp drop. We observed an oscillation in  $\alpha$ -LA and an increase in  $\beta$ -LG in precipitated fraction and a reduction in  $\alpha$ -LA, and a fluctuation in  $\beta$ -LG in the supernatant fraction. There was an increase in recovery of  $\alpha$ -LA and of  $\beta$ -LG in precipitated fraction and a decrease in  $\beta$ -LG recovery in the supernatant fraction, indicating that, even though highest values of  $\alpha$ -LA recovery in precipitated were obtained, its purity was the lowest one. In contrast, it was got the highest pureness of the liquid fraction.

In every condition used, the samples' pH values after the system depressurization oscillated between 5.9 - 6.1. Before the experiments, the WPI solutions with no treatment had a pH between 6.5 - 6.6. That difference was expected since there would still be small reminiscent amounts of chemical species responsible for lowering pH – carbonic acid, bicarbonate, and hydronium ion.

Lima et al. (2019), using a WPI concentration of 1%, conditions 16 MPa/55 °C and residence time of 34.2 min, obtained  $\alpha/\beta$  ratio in precipitated fraction of 4.01 with  $\alpha$ -LA recovery of 39.6%. Here, at the same temperature, pressure, and residence time, the  $\alpha/\beta$  ratio and the  $\alpha$ -LA recovery were 3.51 and 50.9%, respectively. However, the WPI solution concentration used here was five times superior. Comparatively, and considering a much higher concentration employed here, satisfactory pureness in precipitated fraction was obtained and also higher recovery of the proteins. Comparing to that same work, under 16 MPa/60 °C conditions, it was obtained similar  $\alpha/\beta$  ratio and  $\alpha$ -LA recovery, both in precipitated fraction, considering it was employed a WPI solution concentration five times higher. Pureness values in precipitated fraction and protein desired recovery obtained here were very close to the ones obtained in that work. A higher amount of extracts obtained here is justified due to using a higher concentration of WPI solution. Bonnaille & Tomasula (2012) studied the fractionation in a batch reactor, using WPI concentration solutions of 2 and 5%. At 2%, employing pressures higher to 31 MPa,

temperatures of 60 and 65 °C and residence times of 120 min, were obtained  $\alpha/\beta$  ratios equal to 2.84 and 2.48 and  $\alpha$ -LA recoveries of 50.5% and 65.1%, respectively, both in precipitated fraction. The pressures employed by those authors were much superior to those used here, and elevated pressures directly impacted the operation costs. Here, using pressure reduced by half, residence times in which the highest of them is half of the one using in mentioned work and in a continuous regimen,  $\alpha/\beta$  ratios equal or superior were obtained and similar  $\alpha$ -LA recovery in the precipitated fraction. At 5%, using 6 MPa/ 60 °C and residence time of 270 min, it was obtained  $\alpha/\beta$  ratio equal to 2.37 and  $\alpha$ -LA recovery equal to 73.5, both in precipitated fraction. Here, employing the same concentration, intermediate pressure, residence time four times shorter, and using a continuous reactor, we obtained precipitated purer and  $\alpha$ -LA recovery in precipitated appreciable. Yver et al. (2011) conducted, also in a batch reactor, 5% concentration of WPI, pressure of 8.3 MPa, temperature of 60 °C and residence time superior to 60 min, it was obtained  $\alpha/\beta$  ratio equal to 7.0 and  $\alpha$ -LA recovery of 80%, both in precipitated fraction. Even though those results are superior to those obtained here, residence time higher than 120 min was employed.

### 3.2. Circular dichroism

This analysis aimed to obtain the thermal denaturation profile of  $\alpha$ -LA and  $\beta$ -LG proteins to estimate if the extracted fractions would be in their native forms or denatured due to the fractionation process. Since the samples had contaminating protein fractions above 5%, it was impossible to estimate their biophysical parameters. The sample from precipitated fraction and the other obtained from supernatant fraction obtained in the fractionation employing the most severe conditions – 24 MPa, 65 °C, and flow rates both of WPI and CO<sub>2</sub> of 4.0 mL·min<sup>-1</sup> – were submitted to gradual increase in temperature up to 94 °C, in 3 °C intervals. The isosbestic point indicates the moment when there is a transition between the native and denatured states. Dichroism spectrum for the obtained sample from precipitated fraction shows only one isosbestic point, near 214 nm, while in supernatant fraction rich in  $\beta$ -LG, it is not present. Figure 1 shows the variation of gross ellipticity for  $\alpha$ -LA and  $\beta$ -LG proteins as a function of wavelength during the denaturation process. The ellipticity of the samples was followed using wavelengths of 203.5 and 202 nm, respectively. The CD spectrums obtained in these cases are presented in Figure 2. We fitted a Hill equation (Equation 3) to the experimental points displayed in Figure 2.

$$y = \frac{A_1 - A_2}{1 + (x/x_0)^p} + A_2 \quad (3)$$

where  $A_1$  is the initial CD (mdeg);  $A_2$  is the final CD (mdeg);  $p$  is the transition cooperativity, and  $x_0$  is transition temperature ( $T_m$ ).

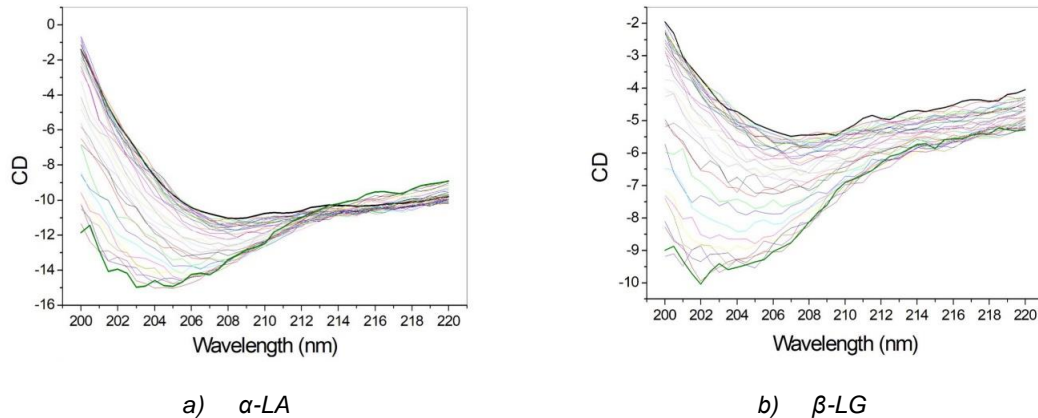


Figure 1. Variation of gross ellipticity as a function of wavelength during denaturation process – (a)  $\alpha$ -LA and (b)  $\beta$ -LG.

The model was fitted for the precipitated and supernatant fractions. According to the spectrums, both samples did not show intermediate denaturation states – called molten globule – which would be expected if the samples were pure. So, this CD analysis cannot be used to calculate thermodynamic parameters of denaturation of these proteins. However, as mentioned above, CD analysis was conducted to verify if the extracted fractions would be in their native or denatured forms. Analyzing the spectrums, we observe a transition to the unfolded state, especially between 200 and 210 nm. Denaturation of  $\alpha$ -LA and  $\beta$ -LG has its transition temperature ( $T_m$ ) around 82 °C. In this way, we obtained the fractions of the two proteins in their native forms. Even though not having provided a high pureness level in their isolation, the fractionation process did not initiate the protein denaturations.

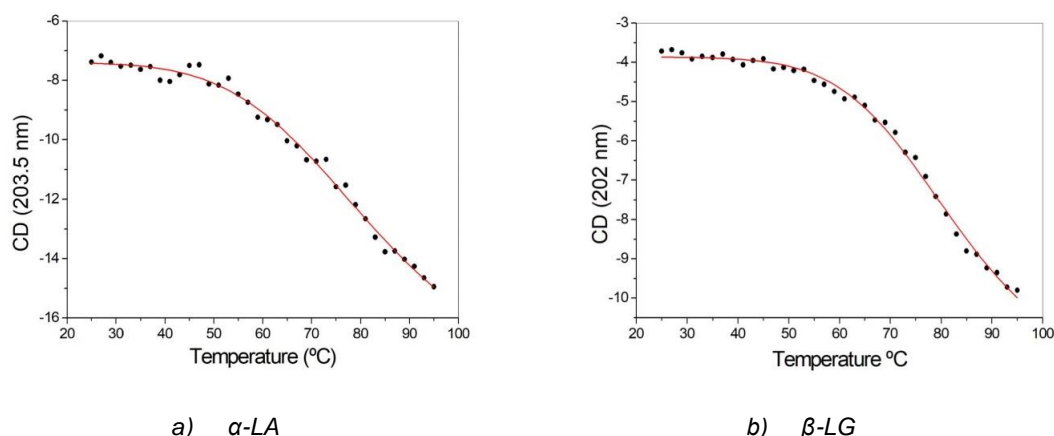


Figure 2. Variation of gross ellipticity of  $\alpha$ -LA at 203.5 nm and  $\beta$ -LG at 202 nm as a function of the increase in temperature. – (a)  $\alpha$ -LA and (b)  $\beta$ -LG.

#### 4. Conclusion

All the operation conditions employed here made it possible the fractionation of  $\alpha$ -LA and  $\beta$ -LG from concentrated WPI solution using  $scCO_2$  as an eco-friendly solvent in a continuous reactor. CD analysis in the precipitates showed no denaturation of both proteins and pureness level similar to the ones reported in open literature employing fractionation technique via  $scCO_2$ . The  $\alpha/\beta$  ratio in precipitated fraction reached values 4.05 to 8.77 times higher than the WPI with no treatment, while the ratio in supernatant reached values 1.60 to 5.71 lower. The product obtained employing this fractionation has potential for applications in new products with high food and pharmaceutical grade:  $\alpha$ -LA in its pure form can be used in order to strength infant formulas, meanwhile products enriched with  $\beta$ -LG, present an increase in the activity, emulsion stability, viscosity, and gelation properties and it is mostly applied in sport nutrition products.

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