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Scale-up production of vitamin loaded heteroprotein coacervates and their protective property

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ABSTRACT

The production of biocarriers for the protection and controlled delivery of bioactives is of great interest to develop functional foods. The ability of two whey proteins (WP), Beta-Lactoglobulin (BLG) and Lactoferrin (LF), to efficiently entrap vitamin B9 by complex coacervation has been reported at laboratory scale. In the present work, we report on the scaling-up production of B9 loaded BLG-LF coacervates (B9-WP coacervates). Complex coacervation was performed at bench scale, using commercial protein solutions. Under optimized conditions, B9-WP coacervates were produced by static mixing at a flow rate of 300 mL/min. Bench scale efficiency similar to that found for laboratory scale was reached with a coacervation yield of 65% and the B9 entrapment of 98%, demonstrating an efficient scaling-up. B9-WP coacervates showed good protection property for B9 during storage treatments, confirming the efficiency of this type of biocarrier for the development of natural functional foods.

1. Introduction

A current trend in the development of new food products is to add bioactives molecules in their formulation to provide specific nutritional and health benefits (vitamins, minerals, essential fatty acids) and/or added value (new flavors). The protection and controlled delivery of such molecules is a key factor to guarantee the final quality and properties of the resulting functional food products. As a result, the food industry have been investigating more and more controlled release technologies, with a special emphasis on microencapsulation technologies (Gouin, 2004). As one of the microencapsulation technologies, the complex coacervation of two biopolymers to form a biocarrier for third molecules is of great interest.

Complex coacervation is a liquid-liquid phase separation of a colloidal system occurring between two oppositely charged biopolymers through electrostatic interactions. It results in the formation of two phases: one is the polymer-poor continuous phase and the other is the polymer-rich dense phase, named coacervate. The concept behind the formation of biocarriers by complex coacervation is to first dispersed the bioactive in one of the initial biopolymer solution and then use the phase separation to newly form the coacervate phase containing the bioactive ingredient (Sánchez et al., 2016). The formation of biocarriers by coacervation presents numerous advantages for the protection of high-value and labile functional ingredients. It only requires mild preparation conditions with no use of neither organic solvent, drastic temperature nor high pressure which is of interest to reduce the industrial cost of biocarriers formation (Yan and Zhang, 2014). Coacervation also offers high shell integrity, high encapsulation efficiency and good controlled-release properties (Gouin, 2004). Moreover, this method has the advantage that a specialized equipment is not required to complete the coacervation process. Hence, it appears as potentially scalable to the industrial production of biocarriers (Shewan and Stokes, 2013). Numerous biopolymers systems have been studied to use complex coacervation as a technology to form biocarriers. Most of them studied the interactions between proteins and polysaccharides (Doublier et al., 2000; Schmitt and Turgeon, 2011) to form biocarriers for specific core bioactives such as essential oils or lipophilic vitamins (Matalanis et al., 2011), flavors (Koupantsis et al., 2014; Xiao et al., 2014) or probiotics (de Vos et al., 2010) for example. Nevertheless, few studies deal with the complex coacervation of a two proteins systems.

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Proteins are major nutrients for a balanced diet and contribute to the final structure, texture and stability of foods (Doublier et al., 2000). They are generally recognized as safe (GRAS) by the food industry and present excellent functional properties (Chen et al., 2006). Thus, they stand as interesting biomaterials for biocarriers. Among food proteins, whey proteins display interesting properties as biomaterials (Tavares et al., 2014). They are also a natural by-product from the dairy industry. Thus, it is relevant to seek enhanced functionalities for them, creating benefit to the sector. Diverse strategies have been investigated to exploit whey proteins as biocarriers for bioactive molecules, such as the formation of simple complexes with ligands, the formation of gel networks, or their complex coacervation through electrostatic interactions (Bouhallab and Croguennec, 2014; Diarrassouba et al., 2015; Schmitt et al., 2009).

The potentiality of using the complex coacervation of two whey proteins (WP), the lactoferrin (LF) and the beta-lactoglobulin (BLG), to form a biocarrier for a bioactive, the vitamin B9 (B9), was previously investigated (Chapeau et al., 2016). This work had established the proof of concept that LF and BLG can spontaneously co-assemble in presence of B9 by complex coacervation. The complex coacervation of WP with B9 occurred preferentially for mixing solutions of medium proteins concentrations (10–15 g/L) and lead to the formation of spherical supramolecular co-assemblies from 8 to 15 μm, appearing as small droplets suspended in solution. These small droplets tend to coalesce into a dense phase called *the B9-WP coacervate*. This dense phase can be obtained by the centrifugation of the coacervated solution of B9-WP. The B9-WP cocervates recovered after centrifugation is highly concentrated in whey proteins (≈320 g/L) and B9 (4–16 mg B9/g proteins) (Chapeau et al., 2016). Other encapsulation technologies such as the nano-spraydring or the electrospraying of whey proteins showed similar biocarrier efficiency for B9 (Pérez-Masiá et al., 2015). Thus, it was concluded that B9-WP coacervate demonstrated good potential as biocarrier for B9. Nevertheless, this work was carried out at a laboratory scale (μL). For a possible industrial application of B9-WP coacervate, the scale-up production to bench scale (L) has to be considered. However, coacervation is known to be a process highly dependent of the biomaterials system involved and each coacervation system operates under a unique set of conditions (Yan and Zhang, 2014). Moreover, the scale-up of the complex coacervation process is often achieved empirically, leading to possible trial and errors setup and may involve expense of cost and resources (Lemetter et al., 2009; Paul et al., 2004). That is why the specific conditions of complex coacervation of such proteins systems have to be specifically investigated, especially when industrial applications are considered to maximize process yield and minimize loss and batch-to-batch variations (Thies, 2007).

The aim of this study was to investigate the scale-up production of B9-WP coacervates from laboratory scale (μL) to bench scale (L) using batch and continuous mixing systems. Both systems allowed obtaining encapsulation yields for B9 up to 98%, similarly to yields obtained at the μL scale. Coacervation yields of whey proteins varied from 55% for the batch system (similar at μL scale) and up to 65% for the continuous system, which is higher than the yield obtained at μL scale. Finally, the protective effect of B9-WP coacervates against B9 degradation was shown for two types of food storage treatments, freezing and freeze-drying.

2. Materials and methods

2.1. Materials

Vitamin B9 (B9) was purchased from Sigma Aldrich (Folic acid, purity > 97%, Sigma Aldrich, St. Louis, MO, USA). Lactoferrin (LF) from bovine milk, (purity 90% and iron saturation level of 10%–20%) was purchased from the Fonterra Cooperative Group, New Zealand. LF powder was used without further modification. Beta-Lactoglobulin (BLG) powder was obtained from a confidential industrial source. Its composition (w/w) was: protein 93.5%, moisture 4% and ash < 1.8%. Protein purity was determined by reversed-phase HPLC and no proteins other than BLG were detected.

2.2. Stock solutions

B9 stock solution was prepared by solubilizing the vitamin powder in milliQ water and then adjusted to pH 5.5 using 1 M HCl solution. B9 solution was centrifuged at 28,000 g for 30 min at room temperature (centrifuge Hereaus Biofuge primo, KENDRO Laboratory products, Courtaboeuf, France) to remove the insoluble fraction of B9. The exact vitamin B9 concentration was determined by absorbance at 283 nm (spectrometer UVmc^2, Safas, Monaco) using 25.1 L g^{-1} cm^{-1} as extinction coefficient.

Before use, BLG was purified. BLG powder was dispersed in deionized water (45 g/L), adjusted to pH 4.6 with 1 M HCl and kept at 30 °C for 5 min in order to precipitate non-native forms of BLG. The dispersion was centrifuged at 20,000 g for 10 min at room temperature (Heraeus Biofuge Primo, Thermo Scientific, Waltham, MA, USA). BLG suspension was then freeze-dried and stored at -20 °C until use. Then, LF and BLG stock solutions were prepared by solubilizing the protein powders in milliQ water adjusted to pH 5.5 using 1 M HCl. The protein solutions were filtered through a 0.45 mm and a 0.2 mm membrane (cat. no. 4612, Pall Corporation, Ann Arbor, MI, USA). The exact proteins concentrations were determined by absorbance at 280 nm (spectrometer UVmc^2, Safas, Monaco) using 1.47 L g^{-1} cm^{-1} and 0.96 L g^{-1} cm^{-1} as extinction coefficients for LF and BLG respectively. B9, LF, and BLG stock solutions were stored at 4 °C and protected from UV light radiation.

2.3. Production of B9-whey protein coacervates

The production diagram of B9-whey proteins (B9-WP) coacervates is depicted in Fig. 1A. First, a solution of B9-LF complex was prepared by blending B9, LF and milliQ water, to have final concentrations of 4.15 g/L for LF and 0.11 g/L for B9. The B9-LF complex solution was let to equilibrate by itself for 10 min at 20 °C. Then, BLG solution was added to the B9-LF complex solution to reach a final BLG concentration of 9.15 g/L, in order to induce complex coacervation. Addition of BLG was performed under either batch or continuous mixing conditions. The coacervated phase recovered by centrifugation at 38,000 g for 45 min at 20 °C (Centrifuge Avanti J-26S XP, Beckman Coulter Inc., Brea, CA 92821 USA), was weighed and kept in the dark at 4 °C.

Batch mixing by mechanic stirring was performed by stirring the solution in a vessel containing a propeller pale of 2.5 cm diameter, with 3 blades (Fig. 1B). The propeller was set in rotational motion by the mean of an electric motor set at the desired stirring speed, during 2 min. Unstirred solution was also equilibrated during 2 min prior centrifugation.

Continuous mixing by static stirring was made using a static mixer (Fig. 1C) constituted by a series of 10 static mixer units of 0.4 × 0.6 cm diameter, set in-line, for a total length of 6 cm. The input pipes for B9-LF and BLG solutions were of 0.4 cm diameter and 30 cm length. The output pipes for B9-WP coacervates solution was of 0.2 cm diameter. According to preliminary tests to set the system, permanent regime of the static mixer was achieved after 20 s. Aliquots were withdrawn over time for further analyses.

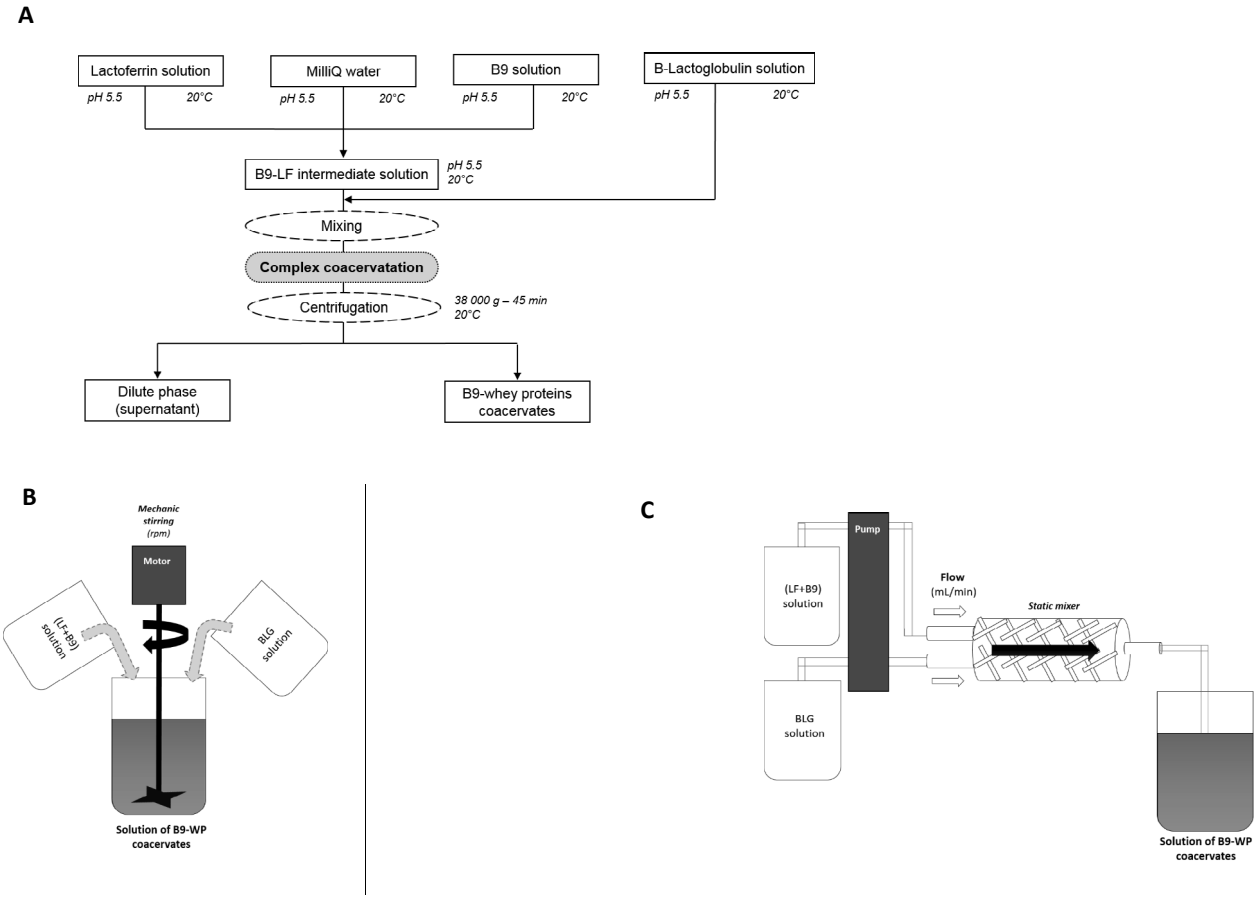

Fig. 1. Schematic diagram for the of production of B9-WP coacervates (A) and the device used for batch mixing by mechanic stirring (B) or continuous mixing by static stirring (C).

2.4. Storage treatments

Two storage treatments were applied to B9-WP coacervates: freezing at −20 °C and freeze-drying. For the freezing, a sample of 0.5 g was placed at −20 °C, in the dark, for 8 h. Freeze-drying of samples were performed using a freeze-dryer (RP2V-22304, S.G.D Group, 95100 Argenteuil, France). A 0.5 g aliquot of B9-WP coacervates was placed in plastic tubes and was frozen at −25 °C in 50 min. The frozen samples were dried under vacuum conditions for a night.

2.5. Turbidity analysis

Turbidity measurements at 600 nm (A_{600nm}) (spectrometer UVmc^2, Safas, Monaco) was used to monitor complex coacervation and the formation of B9-LF-BLG coacervates in the coacervated solutions. Absorbance measurements were converted to turbidity (t, cm^{-1}) using the following relationship: $t = (2.303A_{600nm})/l$, where l is the light path length ($l = 1$ cm) (Melik and Fogler, 1983). The stock solutions of vitamin B9, LF and BLG (purified and filtered) had no detectable turbidity before mixing.

2.6. Phase contrast microscopy

The formation of B9-WP coacervates in the coacervated solution was observed by a phase contrast optical microscope (Olympus BX51TF, Olympus, Hamburg, Germany) set at the magnification ×40. A 10 mL sample of the B9-LF-BLG coacervated solutions was taken, deposed on standard microscope slide, recovered by a coverslip (22 mm × 22 mm) and immediately observed. The samples and observations were performed three times on each solution to ensure the representability of the results.

2.7. Particle size distribution by laser light scattering

The size distribution of B9-whey proteins coacervates in the coacervated solution prior to centrifugation was determined by laser light scattering using a Mastersizer 2000 (Malvern instruments, Malvern, UK), with two different wavelengths (He/Ne laser: 633 nm; electroluminescent diode: 466 nm). The samples (about 0.2–0.5 mL of B9-WP coacervates solution) were diluted in 100 mL of milliQ water at pH 5.5directly in the measurement cell of the apparatus in order to reach 10% obscuration. The refractive indexes used in this study were 1.52 for whey proteins coacervates and 1.33 for water.

2.8. Quantification of B9 and proteins in the coacervates

B9 and total whey protein concentrations recovered in the B9-whey protein coacervates were quantified using a PLRPS column (S/N 1006329-5, Varian Inc., Shropshire, UK) connected to a Waters 26 95 HPLC. MilliQ water containing 1.06‰ (v/v) of trifluoroacetic acid and an 80/20 acetonitrile/milliQ water (v/v) mixture containing 1.0‰ (v/v) of trifluoroacetic acid were used for elution. The absorbance was monitored at 280 nm using a Waters 2487 detector. B9-WP coacervates were resuspended and diluted in solution of MilliQ water containing 1.06‰ (v/v) of trifluoroacetic acid. According to the dilution factor and the measured density of B9-WP

coacervates, i.e. 1.356 g/mL, the B9 and WP concentrations were expressed in g/kg of B9-WP coacervates.

2.8.1. Biocarrier efficiency: entrapment and coacervation yields

The efficiency of the B9-WP coacervates as biocarrier was characterized by two yields: the entrapment yield of B9 in the coacervates and the coacervation yield of the WP. These two yields were calculated according to the following equations:

Entrapment yield for B9 (%),

$$Entrapment\ yield = \frac{Q_{B9\ coacervate}}{Q_{B9\ initial\ solution}} \quad (1)$$

Coacervation yield of the whey proteins (%),

$$Coacervation\ yield = \frac{Q_{WP\ coacervate}}{Q_{WP\ initial\ solution}} \quad (2)$$

where Q is the quantity of B9 (g) and whey proteins (g) recovered in the B9-WP coacervates or in the initial solution. Q were calculated depending on the B9 whey proteins concentrations recovered in the B9-WP coacervates and the total quantity of B9-WP coacervates (g) recovered for each experiment.

3. Results and discussion

The optimal conditions for coacervation between BLG and LF and entrapment of vitamin B9 were previously established at laboratory scale (Chapeau et al., 2016). These conditions constitute the starting point for the present work.

3.1. Scaling-up the complex coacervation of whey proteins in the presence of B9

3.1.1. Effect of increasing the volume

The first step of scaling-up was the gradual increase of the mixing volume of coacervated solutions to increase the quantity of B9-WP coacervates recovered after centrifugation, without hindering the coacervation process. In fact, complex coacervation process is known to be highly sensitive to volume and biopolymer concentrations (Schmitt and Turgeon, 2011; Yan and Zhang, 2014). Firstly, coacervation phenomena were compared in three solutions with total mixing volume of 0.1, 5 and 100 mL, using purified and filtered whey proteins solutions as in (Chapeau et al., 2016). Fig. 2A represents the final turbidity of the three coacervated solutions prior to phase separation and centrifugation. For the three mixing volumes using purified and filtered whey proteins, the turbidity values were high with similar magnitude around 3.3. This high turbidity is characteristic of complex coacervation between whey proteins in solution (Chapeau et al., 2016; Yan et al., 2013). The formation of coacervates in the three solutions was attested by phase contrast microscopy (Fig. 2C a,b,c). The formed coacervates appeared spherical, with a mean diameter between 5 and 10 μm, similarly as previous results (Chapeau et al., 2016). After centrifugation, 0.23 mg of B9-WP coacervates were recovered for the 0.1 mL solution and 1 g for the 100 mL solution, attesting that increasing the volume increased the recovered dense phase of B9-WP coacervates. Fig. 3A shows the concentrations of B9 ([B9]) and whey proteins ([WP]) in the B9-WP coacervates recovered after centrifugation of the three coacervated solutions. For the three mixing volumes, [B9] was between 5.0 and 5.5 g/kg and [WP] was between 330 and 360 g/kg. So, [B9] and [WP] yields recovered in the B9-WP coacervates can be considered constant, without significant impact of the mixing volume from 0.1 mL to 100 mL. As a result, a 1000 fold increase of the mixing volume of B9, LF and BLG solutions do not affect the complex coacervation between the three molecules. The B9-WP coacervates recovered after centrifugation presented similar compositions for small and high studied mixing volume.

3.1.2. Effect of whey protein's purity

The production of a high amount of coacervates requires the preparation of important volume of WP solutions. Subsequent purification and filtration steps usually performed at laboratory scale to remove residual small protein aggregates may constitute a limit for a scaling-up purpose. Thus, it would be more efficient to perform heteroprotein coacervation with commercial WP samples, without any purification and filtration of the WP solutions. Nevertheless, it has been demonstrated that proteins aggregation may impair and compete with coacervation in WP systems (Yan et al., 2013). To explore this observation here, two types of LF and BLG solutions were tested:

- BLG was purified prior to reconstitution and then the LF and BLG solutions were filtered to remove any aggregated proteins (as described in 2.2),
- LF and BLG solutions were directly reconstituted from commercial LF and BLG powders, without any purification or filtration steps.

The presence of small aggregates in commercial BLG is clearly visible to the eye (Fig. 2B). The turbidity value of non-purified and non-filtered BLG commercial solution (Fig. 2B a) was higher than that measured after purification and filtration (Fig. 2B b). However, the turbidity of the coacervated solutions obtained with commercial proteins solutions was equivalent to the ones obtained with purified and filtered proteins solutions at both working volumes of 5 and 100 mL (Fig. 2A). Similar formation of B9-WP coacervates was observed for mixing solutions using commercial WP, at 5 mL and 100 mL (Fig. 2C d, e) compared to B9-WP coacervates obtained using purified and filtered WP solutions. Regardless the purity of the used BLG solution, similar concentrations of [B9] and [WP] was recovered in the coacervates (Fig. 3A). Therefore, the presence of small amounts of aggregates in WP solutions did not hinder the coacervation to occur under the present conditions. As a result, complex coacervation of B9, LF and BLG can be performed at larger volume using commercial protein samples without further purification steps.

3.1.3. B9 entrapment efficiency

As expected, the increasing mixing volumes allowed the increase of the recovered weight of B9-WP coacervates (Fig. 3B). For 0.1 and 5 mL, the entrapment yield of B9 was between 96 and 99%. The same trend can be observed for the coacervation yield of WP, with a high coacervation yield of WP at 50% for 0.1 and 5 mL. These entrapment and coacervation yields are in accordance with the previous results obtained by Chapeau et al. (2016). Nevertheless, for the higher volume tested, the B9 entrapment and coacervation yields decreased to 66% and 35%, respectively. Such yield decrease could be linked to the processing parameters. It has been reported that shear, pressure or residence time can impact greatly the complex coacervation process and the coacervation yield (Lemetter et al., 2009; Yan and Zhang, 2014).

3.2. Effect of the solutions mixing mode

Mixing is defined as the reduction of inhomogeneity in order to achieve a desired process result. Secondary effects such as chemical reaction and changes in product properties are usually the critical objectives (Paul et al., 2004). In the present study, the step of mixing was defined at the addition of BLG solution and the

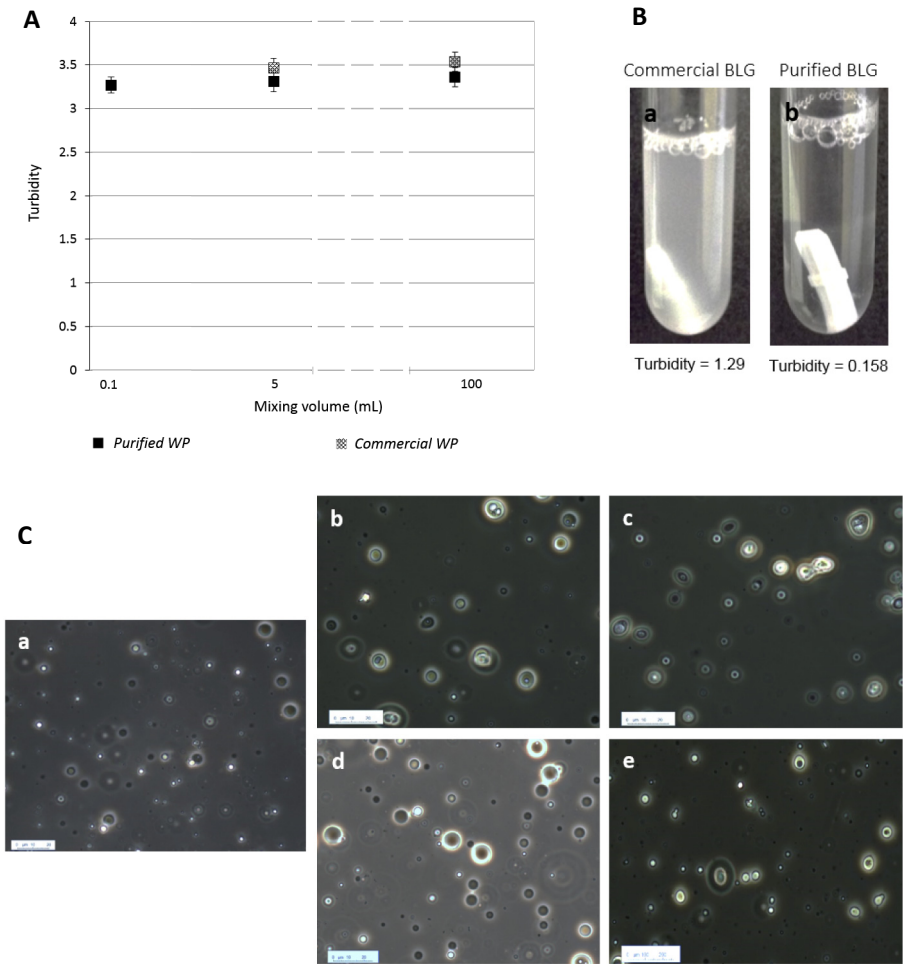

Fig. 2. **A**: Turbidity of B9-WP coacervate solutions at different mixing volumes for purified (■) and commercial proteins samples (▒). **B**: Visual difference between commercial (a) and purified BLG (b). **C:** Phase contrast microscopy (×40) of B9-WP coacervates obtained at different mixing volume and WP samples: purified WP solution (a) 0.1 mL, (b) 5 mL, (c) 100 mL; and commercial WP solutions, (d) 5 mL, (e) 100 mL.

chemical reaction required was complex coacervation (Fig. 1). At laboratory scale, the complex coacervation between B9-LF intermediate solution and BLG occurred instantaneously after the addition of the BLG solution. This was possible because the step of mixing (homogeneous equilibration) of the solutions was also instantaneous due to the very small volumes used. Increasing the mixing volume at fixed speed may impair the mixing efficiency and consequently the coacervation yield. Thus, the mixing step appeared to be crucial for the feasibility of a scale-up production of B9-WP coacervates. For this study, the mixing system was defined as a liquid-liquid mixing system where the two liquids are miscible together (two aqueous solutions). According to the literature, two types of mixing can be considered for this system: a batch mixing by mechanic stirring and a continuous mixing by static stirring (Paul et al., 2004).

3.2.1. Batch mixing by mechanic stirring

The set-up used to produce B9-WP coacervates by batch mixing using mechanic stirring is described in Fig. 1B. An increasing speed of stirring was applied at the mixing step between B9-LF and BLG solutions in a total volume of 100 mL: no stirring (0 rpm), low stirring (50 rpm) or high stirring (150 rpm). Above this last stirring value, the formation of foam is observed on the top of the solution. This constitutes a limit for coacervation scale up as already reported by (Lemetter et al., 2009). Due to a vortex created by the propeller speed, air was incorporated into the system, inducing the foaming of the protein solutions and prevented the occurrence of complex coacervation.

Fig. 4A presents the B9 and WP concentrations recovered in the coacervates obtained at the three stirring rates. The concentration of the two entities and the overall composition of the B9-WP coacervates were not affected by the applied stirring rate. Interestingly, while the composition remained constant, increasing the stirring speed induced an increase of the B9 entrapment and the coacervation yields (Fig. 4B). Thus, B9 entrapment as well as the coacervation yield doubled when the solution was stirred at 150 rpm. It has to be noted that stirring at 150 rpm allowed reaching the maximum yields found at laboratory scale (Chapeau et al., 2016).

Fig. 4C presents the size of the B9-WP coacervate droplets recovered in the coacervated solutions after the stirring step as measured by laser light scattering. For unstirred solution, the coacervate droplets presented a heterogeneous size distribution that evolved with post-stirring time. The droplets of the coacervated solution are highly polydisperse but evolved to a rather homogeneous population of particles with a mean diameter of 10 μm after 15 min (Fig. 4C, a). At low stirring rate, the polydispersity of the particles was lesser, with two main populations of droplets around 2–3 μm and 25 μm at T0 (Fig. 4C, b). These two populations remained stable over time (Fig. 4C, b). At high stirring

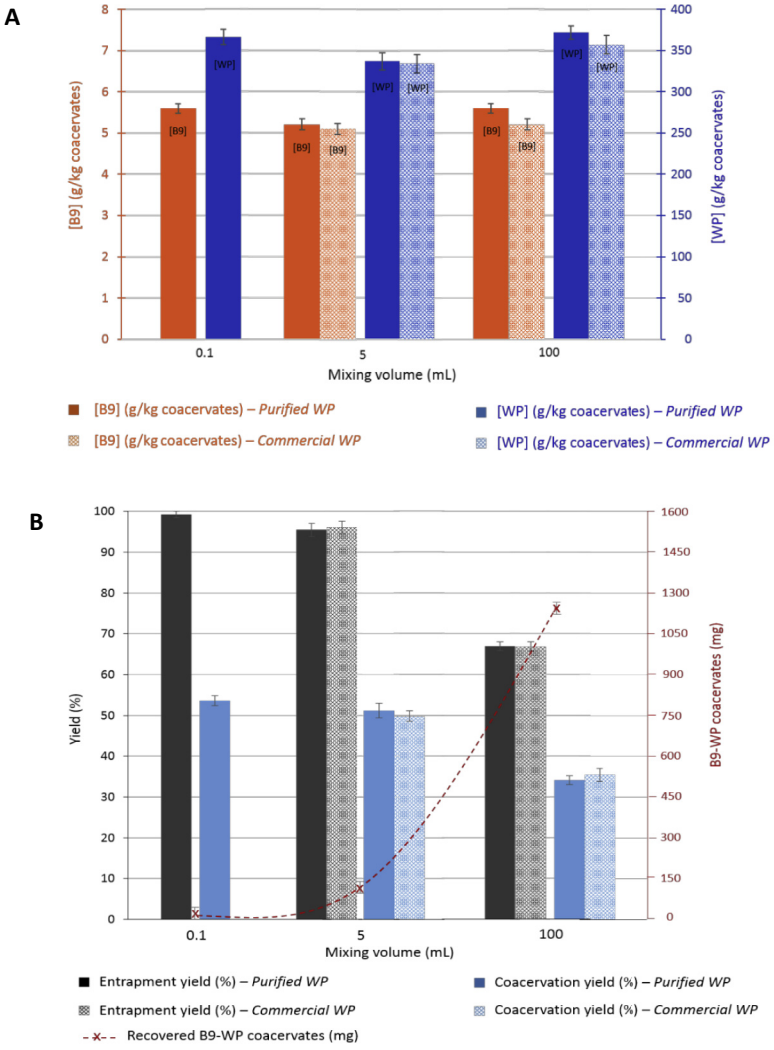

Fig. 3. A: B9 and WP concentrations recovered in B9-WP coacervates at different mixing volumes and the type of whey proteins used, purified (▉) versus commercial protein samples (▒). **B:** Entrapment, coacervation yields and total weight of B9-WP coacervates obtained at different mixing volumes with two types of whey proteins purified (▉) versus commercial samples (▒).

rate, the coacervate droplets were rather monodisperse, with a mean diameter of particles of 2–3 μm and stayed stable overtime (Fig. 4C, c). These results can be explained by stirring-induced acceleration of the liquid-liquid phase separation phenomenon throughout coalescence of the coacervate droplets. With no stirring, the formation of droplets of B9-WP coacervates was spontaneous as soon as BLG was added. The formed droplets were rather polydisperse and unstable in solution, as illustrated by the schematic representation of Fig. 4C, a. They tended to coalesce leading to liquid-liquid phase separation overtime, characterizing the completion of the complex coacervation. These observations were in agreement with other works performed with no stirring but at small volume scale (Flanagan et al., 2015; Tavares et al., 2015; Yan et al., 2013). Increasing stirring induced an increase of the coalescence between the droplets of B9-WP coacervates. The size of resulting droplets of coacervates became large enough to decant forming the concentrated phase of B9-WP coacervates, as illustrated by the schematic representation of Fig. 4C, b. At high stirring, it can be supposed that the formation of the concentrated phase of B9-WP coacervates was complete right after the two minutes of stirring. Only the smaller droplets of B9-WP coacervates that were stable and in equilibrium with the dense phase remained in solution, as illustrated by the schematic representation of Fig. 4C, c.

As a result, increasing the stirring rate favored the complete phase separation of the dense phase of B9-WP coacervates. Then, the B9-WP coacervates were fully recovered by centrifugation. Thus, the B9-WP coacervation yield increased with the increase of the mechanic stirring speed. The B9-WP coacervates obtained with high level of stirring showed the highest biocarrier efficiency. This biocarrier efficiency was similar to the biocarrier efficiency obtained at laboratory scale.

3.2.2. *Continuous mixing by static stirring*

The set-up used to produce B9-WP coacervates by continuous mixing using static stirring is described in Fig. 1C. Four flow rates were set for mixing the B9-LF and BLG solutions: 50, 100, 200, and 300 mL/min. As stated above for mechanic stirring, the maximum flow rate used was 300 mL/min to avoid the formation of foam in the solution.

Fig. 5A presents the turbidity of the B9-WP solutions obtained

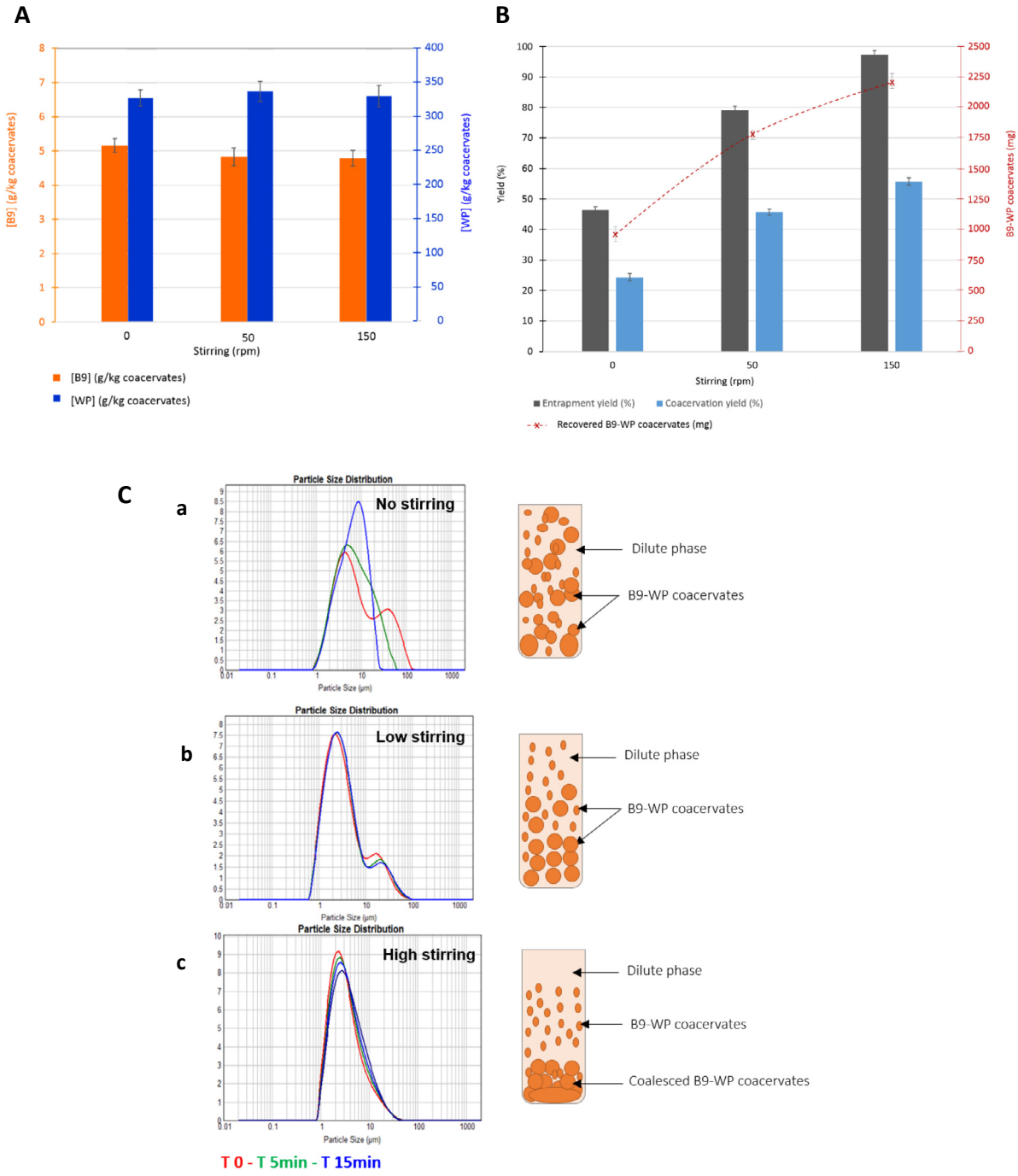

Fig. 4. Batch coacervation at different mechanic stirring. **A**: B9 and WP concentrations in B9-WP coacervates. **B**: Entrapment, coacervation yields and total weight of B9-WP coacervates, **C**: Particle size distribution obtained by laser light scattering and associated schematic representation of B9-WP coacervated solutions after (a) no stirring (0 rpm), (b) low stirring (50 rpm) or (c) high stirring (150 rpm). Measurements were performed at 0, 5 and 15 min after the end of the mixing step.

by continuous mixing by static stirring depending on the flow of the initial solutions (mL/min), and using commercial WP. At 50 mL/min, turbidity of B9-WP solution was low, around 1.05. Then, the turbidity increased with increasing the flow rate to reach an optimum value at 200 mL/min. Microscopy analysis indicates the presence of aggregates at low flow rate (Fig. 5B, image a). At flow rate >200 mL/min only coacervates are observed (Fig. 5B, image c). At 100 mL/min, the formation of B9-WP coacervates also occurred (Fig. 5B, image b) but to a lesser amount than at higher flow rate. The formation of aggregates rather than coacervates following a change in the processing conditions was also reported for gelatin and gum Arabic mixture (Lemetter et al., 2009). It was attributed to an insufficient level of stirring that induced an incomplete mixing of the solutions preventing the occurrence of complex coacervation. As a result, increasing flow rate favored complex coacervation between B9 and WP, with a critical flow rate for coacervation around 100 mL/min.

Fig. 5C presents the B9 and WP concentrations recovered in the B9-WP coacervates obtained by continuous mixing by static stirring at flow rates where coacervation was favored, and using

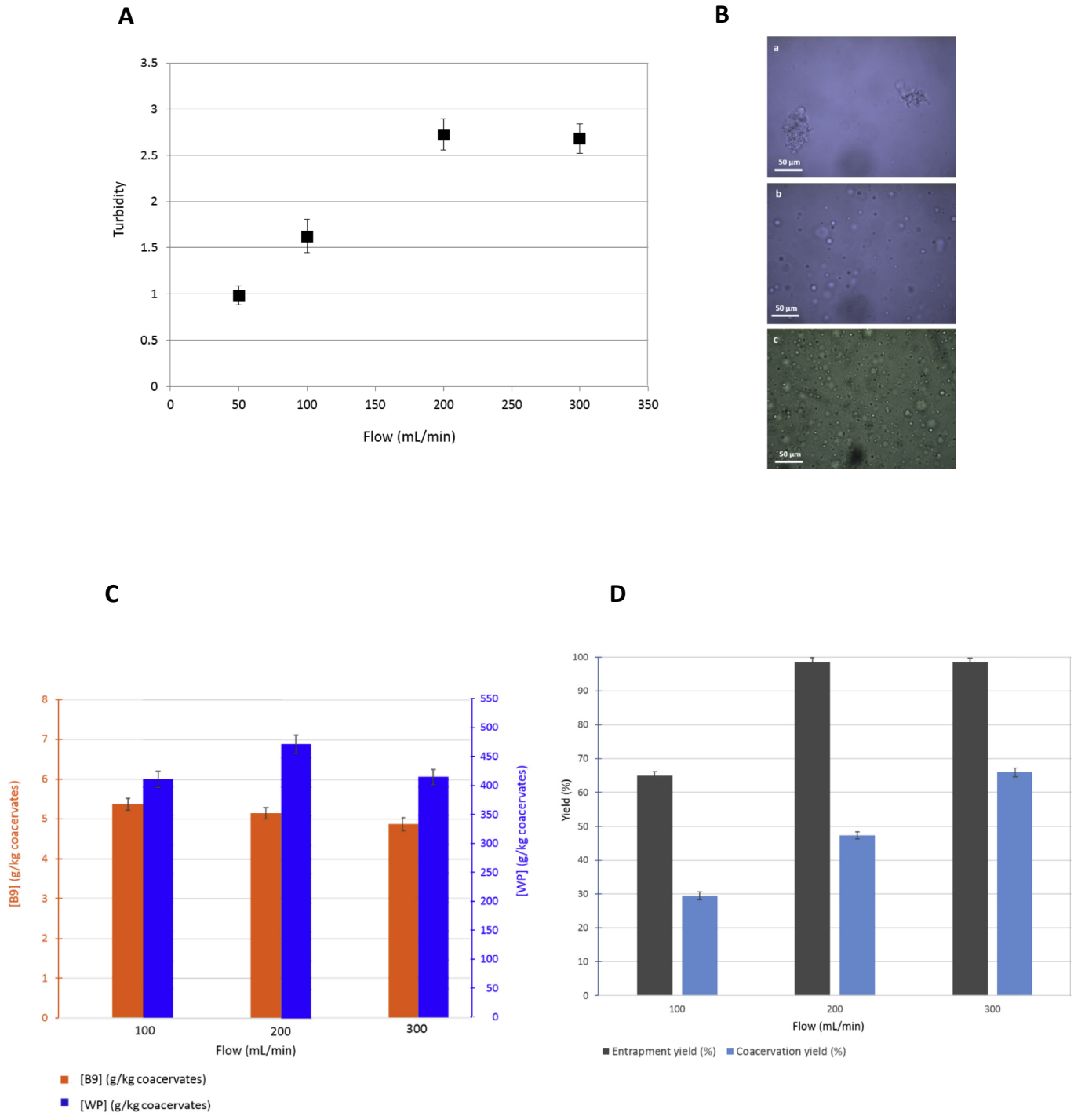

Fig. 5. Continuous coacervation of B9-WP depending on initial flows. **A:** Turbidity of B9-WP solutions measured at different initial flows (mL/min) through the static mixer, **B:** Corresponding particle morphology prior centrifugation: (a) flow = 50 mL/min, (b) flow = 100 mL/min, (c) flow > 200 mL/min, **C**: B9 and WP concentrations in B9-WP coacervates, **D**: Entrapment of B9 and coacervation yield after centrifugation.

commercial WP. B9 concentrations were relatively constant varying from 5.3 to 4.9 g/kg of coacervates. WP concentrations in the coacervates reached 400 g/kg under flow rates of 100 and 300 mL/min. A higher WP concentration value of 465 g/kg was recovered under a flow rate of 200 mL/min. Fig. 5D presents the entrapment and coacervation yields of the B9-WP coacervates. For 100 mL/min, the entrapment yield was around 65% and the coacervation yield was low, around 30%. For 200 mL/min and 300 mL/min, the entrapment yield was around 97%, which is in agreement with previous results obtained at laboratory scale (Chapeau et al., 2016). The coacervation yield increased from 48 to 65% when the flow rate increased from 200 to 300 mL/min. This coacervation yield was superior to the coacervation yields obtained up to now for B9-WP coacervation. Hence, increasing the flow rate favored the coacervation yield offering the possibility to entrap more B9 molecules throughout an increase of initial B9 concentration.

3.2.3. Batch versus continuous mixing for scale-up production of B9-WP coacervates

We showed that without mixing, the entrapment yield for B9 in the coacervates was 46% and the coacervation yield reached only 24%. These yields corresponded to a low biocarrier efficiency, demonstrating that a mixing device is required for the scale-up production of B9-WP coacervates. For batch mixing by mechanic stirring, the optimum conditions were found for a stirring at 150 rpm, during 2 min. It lead to the formation of B9-WP coacervates with a coacervation yield of 55% and an entrapment yield of 97%. These yields demonstrated a satisfying biocarrier efficiency, equivalent to the biocarrier efficiency obtained at laboratory scale. For the continuous mixing by static stirring, B9 entrapment yields up to 98% were obtained at flow rates of 200 and 300 mL/min with a highest coacervation yield of 65% reached at 300 mL/min. Hence, the continuous mixing by static stirring seems to be the best mode for BLG-LF coacervation and entrapment of a hydrophilic bioactive molecule such as the vitamin B9. Moreover, the continuous mixing by static stirring presents other advantages for coacervation at large scale production. For the coacervation using polysaccharides or polyelectrolytes systems, a device by continuous flow such as microfluidic can be successfully use to reach a good yield of coacervates production and enable to control more precisely the amount of initial biomaterials required (Simone, 2016; van Swaay et al., 2015). In that regard, the static stirring that enable the B9-WP coacervates production by continuous flow can be viewed as more efficient than the batch mixing by mechanic stirring. Largely, literature on industrial mixing presents static stirring as highly engineered for continuous process or reactions. Static stirring devices are presented as a good choice when a high degree of homogeneity is required in a very short term (Etchells and Meyer, 2003). This characteristic applies to the coacervation process here as the formation of B9-WP coacervates is known to be instant at the addition of BLG. As a result, the continuous mixing by static stirring at a flow rate of 300 mL/min of initial solutions can be viewed as an efficient device for the large scale production of B9-WP coacervates, offering the possibility of high scale production for potential industrial application.

3.3. Protection of B9 for storage treatments by B9-WP coacervates

An interesting property of using coacervates as biocarrier is to potentially stabilize and protect the bioactive carried against physical or chemical degradations. For instance, vitamin B9 is known to be sensitive to degradation in frozen food products (Czarnowska and Gujska, 2012), resulting in a loss of the vitamin's bioactivity (Lucock, 2000). To evaluate the protective effect of B9-WP coacervates, two storage conditions routinely applied to food products or ingredients were tested: a standard freezing treatment at − 20 °C representative of a frozen food product, and a freeze-drying treatment representative of the freeze-drying of an ingredient. Fig. 6 presents the evolution of B9 concentrations, alone or entrapped in the WP coacervates after the two storage treatments. Initially, B9 concentrations in B9 solution and in B9-WP coacervates were similar, at 6.5 g/kg (±0.21) for the solution and 6.7 g/kg (±0.15). For the two treatments, WP coacervates reduced the degradation of B9. After the freezing treatment, the concentration of B9 in control solution decreased to 4.3 g/kg, corresponding to a B9 loss of 33%. In the B9-WP coacervates, the B9 concentration after freezing were at 5.4 g/kg, corresponding to a B9 loss of 19%. Alternatively, after the freeze-drying processing, the 5.7 g/kg of B9 were recovered in the control powder corresponding to a B9 loss of 12%, against 6.4 g/kg in the powder of B9-WP coacervates, corresponding to a loss of only 3%. As a result, WP coacervates offer a protection for B9 during storage treatments. Similar protective effect was also found for WP capsules formed by electrospraying for another bioactive compound, the beta-carotene (López-Rubio and Lagaron, 2012). Studies are in progress to confirm the observed protective effect after longer storage period and using higher B9 concentrations.

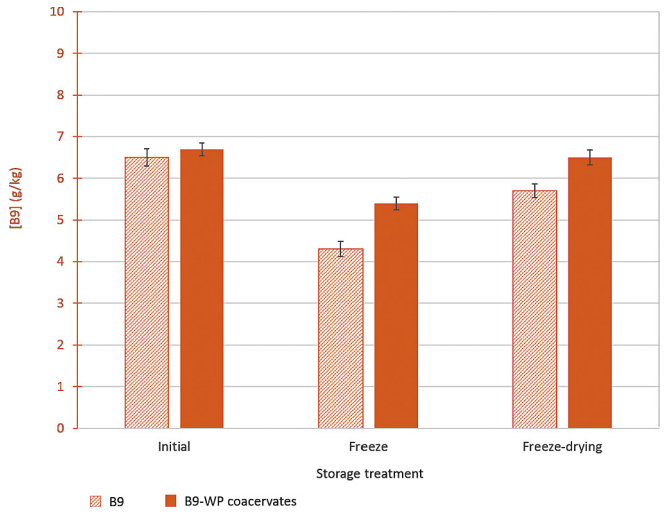

Fig. 6. B9 concentrations before and after storage treatments of B9 free in solution or entrapped in WP coacervates.

4. Conclusion

This work has demonstrated that WP coacervates with entrapped significant amount of B9 was produced at a large volume scale, showing the possibility of using the coacervation of whey proteins to develop biocarrier for bioactives. The preparation of initial protein solutions for coacervation was optimized for industrial implementation, using commercially available protein samples. The feasibility of scaling-up the production of B9-WP coacervates from µL scale to bench scale (1–2 L) has been achieved. The next step is to enlarge the volumetric range from bench to pilot scale. Static mixing appeared to be an efficient device to produce B9-WP coacervates at a large scale and this equipment is easily available in food plants. This device enabled to reach a coacervation yield of 65% and a B9 entrapment of 98%, which was equivalent to the biocarrier efficiency obtained at laboratory scale, demonstrating an efficient scaling-up. Moreover, B9-WP coacervates has shown an interesting protection property for B9 against the degradations induced by food storage treatments such as freezing or freeze-drying. Thus, B9-WP coacervates constitute an interesting ingredient to develop novel natural and functional foods. Now, the stability into a food matrix and the controlled release conditions of B9 from the B9-WP coacervates during its digestion have to be further investigated.

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