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Egg albumin–folic acid nanocomplexes: Performance as a functional ingredient and biological activity

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ABSTRACT

The ability of egg white (EW) nanoparticles to bind folic acid (FA) and protect it through the gastrointestinal tract and the resulting properties of the mixtures as functional ingredient was investigated. Two kinds of EW nanoparticles (USN and TSN) were mixed with FA to generate nanocarriers (USF and TSF). The particle size distribution of USN remained unaltered after the binding of FA, while a little increase in particle size was observed for TSN. Zeta (ζ) potential and fluorescence intensity did not show any significant change after FA addition for both nanoparticles. The percentage of bound folic acid (% BFA) was 78.0 ± 9.1 and 79.7 ± 9.0, for USF and TSF, respectively. A slight formation of aggregates in the samples was observed after freeze-drying and redispersion of the nanocarriers, which was also confirmed by confocal laser scanning microscopy. Nanocarriers particle size did not change after adjusting the pH from 3 to 4, but strongly increased after adjusting it to 5, 6 or 7. The % BFA at pH 4 was similar to that at pH 3, but greatly decreased at pH 7. The bioavailability of FA for *Lactobacillus rhamnosus* was enhanced when the vitamin was incorporated in the form of digested nanocomplexes USF or TSF. The interaction of EW nanoparticles with FA has proven to be beneficial for the transport and release of FA after *in vitro* digestion.

1. Introduction

In recent years, the food industry has increasingly incorporated bioactive compounds to their manufactured products. These compounds are usually highly susceptible to environmental, processing and/or gastrointestinal conditions and therefore, encapsulation has been proposed as an approach for their effective protection (Nedovic, Kalusevic, Manojlovic, Levic, & Bugarski, 2011). Food proteins can be used to prepare a wide range of matrices and multicomponent matrices in the form of hydrogel, micro- or nanoparticles, all of which can be tailored for specific applications in the development of innovative functional food products. The ability to control the particle size of proteinaceous materials is of primary importance not only for determining food properties such as taste, aroma, texture,

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and appearance, but also for determining the release rates of the carried bioactive compounds and ultimately how much is absorbed into the body and hence the overall efficacy of the compounds (Chen, Remondetto, & Subirade, 2006).

Folate, an important B-group vitamin, participates in many metabolic pathways such as DNA and RNA biosynthesis and amino acid interconversions. It is involved in essential functions of cell metabolism such as DNA replication, repair and methylation and synthesis of nucleotides, amino acids, and some vitamins. Mammalian cells cannot synthesize folate; therefore, an exogenous supply of this vitamin is necessary to prevent nutritional deficiency (Iyer & Tomar, 2009). Thus, the World Health Organization established the recommended daily amounts (RDA) of 400 dietary equivalents (DFE) for adults, 600 DFE for pregnant women, and 500 DFE for nursing mothers (World Health Organization, 2006). For this reason and due to the appearance of deficiencies in the general population, the FDA has recommended fortifying commercial flours with folic acid (FA) since 1998 (Food and Drug Administration, 1996). The folate form commercially used in supplements and fortified foods is the fully oxidized monoglutamate form, FA. In aqueous solution, FA is stable at 100 °C for 10 h in a pH range of 5.0–12.0 when protected from light, but becomes increasingly unstable as the pH decreases below 5.0 (Akhtar, Khan, & Ahmad, 1999; Paine-Wilson & Chen, 1979). Alkaline hydrolysis under aerobic conditions promotes oxidative cleavage of the FA molecule to yield *p*-aminobenzoylglutamic acid and pterine-6-carboxylic acid, whereas acid hydrolysis under aerobic conditions yields 6-methylpterin (Tannenbaum, Archer, & Young, 1985). FA is also sensitive to light exposure in aqueous solutions and is degraded to pterine-6-carboxylic acid and *p*-amino-benzoyl-L-glutamic acid; the rate of photolysis is gradually decreased on moving from the acid to the alkaline region (Akhtar et al., 1999; Akhtar, Khan, & Ahmad, 2003).

Many researchers have focused on the study of the interaction between FA and proteins in order to generate complexes for different applications (Bourassa, Hasni, & Tajmir-Riahi, 2011; Jha & Kishore, 2011; Pérez, David-Birman, Kesselman, Levi-Tal, & Lesmes, 2014), for target delivery of nanoencapsulated drugs (Teng, Luo, Wang, Zhang, & Wang, 2013; Zhao et al., 2010) and to provide FA protection against photodegradation (Liang, Zhang, Zhou, & Subirade, 2013), among other purposes. However, the interaction between FA with egg white (EW) proteins has never been investigated yet.

High intensity ultrasound (HIUS) technology has been effective in controlling particle size of different biopolymers. In a previous research, EW nanoparticles have been developed by application of HIUS or thermosonication (Arzeni, Pérez, & Pilosof, 2015). By applying HIUS without heating for 20 min to an EW solution at pH 3, a monomodal population of 220 nm of diameter was obtained as evaluated by dynamic light scattering. On the other hand, when applying thermosonication at 85 °C for 20 min to an EW solution at pH 3 it was possible to obtain a bimodal distribution, with particles of 295 and 70 nm of diameter, the last one being the main population of the distribution. These two kinds of nanoparticles would have the potential to act as food grade carriers for FA. Therefore, the objective of this work was to assess the ability of EW nanoparticles to bind FA and protect it through the gastrointestinal tract and to evaluate the resulting properties of the mixtures as functional ingredients.

2. Materials and methods

2.1. *Preparation of EW and FA solutions*

EW powder was provided by Ovoprot International S.A. (Buenos Aires, Argentina) and was used as starting material. The moisture content was 5.66% (wet basis) and the pH was 7.34, as specified by the manufacturer. The protein content (total basis) of the powder was 88.93 ± 1.18% (N × 6.25) (AOAC, 1980). A thorough characterization of reconstituted EW (10% (w/w) solutions) was carried out in a previous research (Arzeni et al., 2012). Solutions at 5% (w/w) were prepared with double distilled water. Sodium azide (0.02% w/w) was added in order to prevent microbial growth, except for the determinations described in Sections 2.5 and 2.6. Solutions were centrifuged for 1 h at 12,857 × *g* and 20 °C (Centrifuge 5804 R, Eppendorf, Hamburg, Germany). The supernatant was used for the determinations and the pH was adjusted to 3 with 1M HCl.

FA powder (DSM Nutritional Products Argentina S.A., Buenos Aires, purity: 99.5%, dry basis) was kindly provided by Laboratorios Bagó S.A. (La Plata, Argentina). FA solutions at 1% (w/w) were prepared daily for all the experiments. The pH was adjusted to 7 with NaOH 1N (for mixtures) or to 3 with HCl 1N (control). The solutions were covered with aluminum foil to prevent exposure to light.

2.2. *Design of EW nanocarriers*

EW nanoparticles were designed according to a previous work (Arzeni et al., 2015). Briefly, EW solutions (5% (w/w), pH 3 and 7) were sonicated for 5, 10, 15 and 20 min using an ultrasonic processor Vibra Cell Sonics, model VCX 750 (Newtown, CT, USA) with a maximum net power output of 750 W at a frequency of 20 kHz and an amplitude of 20% (maximum amplitude 40%, 228 μm). Temperature was kept constant at 0.5 °C to dissipate the heat produced during sonication in order to evaluate the effect of HIUS alone or, at 80 and 85 °C, for thermosonication treatment. In order to generate EW nanocarriers for FA, an aliquot of 100 μl of FA solution (1% (w/w), pH 7) was added to 4 ml of EW nanoparticles, with continuous stirring for 5 min. The ratio of FA:EW nanoparticles was 1:200 (v/v). The mixtures were covered with aluminium foil and prepared daily for each experiment.

2.3. *Particle size and zeta potential determinations*

Particle size was measured by dynamic light scattering (DLS) with a Zetasizer Nano-Zs analyser from Malvern Instruments (Worcestershire, UK) as described elsewhere (Martínez, Carrera Sánchez, Rodríguez Patino, & Pilosof, 2009). The samples were diluted at 0.1% (w/w) in double distilled water and measured without any further filtering. In the present work, the mean particle size of each peak is reported in intensity and the plot of the distribution is reported in volume, in order to visualize the relative contribution of each population.

Measurements of zeta potential (ζ) were made with the same analyzer at a fixed angle of 17°. The solutions were diluted at 0.01% (w/w) with double distilled water and placed into special folded capillary cells (DTS1060C, Malvern Instruments,

Worcestershire, UK). Average values of two replicates per sample are reported.

2.4. Fluorescence measurements

Fluorimetric measurements were carried out in order to prove the interaction between EW nanoparticles and FA. The fluorescence emission spectrum was recorded by means of a Kontron S25 spectrofluorometer at λ_{exc} = 280 nm and λ_{em} from 320 to 550 nm. For all samples, 200-fold dilutions were used. All measurements were performed in standard 10 mm quartz cuvettes. Mean values of two replicates per sample are reported.

2.5. Evaluation of FA binding

The percentage of FA bound to EW nanoparticles was evaluated by difference between the amount of added and unassociated (free) FA. The amount of free FA was determined by ultrafiltration using centrifugal filter devices with a molecular weight cut-off of 10,000 Da (Amicon® Ultra-15, Millipore, Ireland). First, 5 ml of the mixtures or the blank (FA 0.025%, pH 7) were centrifuged in 15 ml plastic tubes at 12,857 × *g* for 15 min at 20 °C. Then, the two parts of the filter device (filter and centrifugal tube) were separately weighted. After that, 3 ml of supernatant were added to the filter device and reweighted. The device was assembled properly and centrifuged at 5000 × *g* for 15 min at 20 °C. The centrifuge tube containing the filtrate was weighed. The concentration of FA in the filtrate was determined according to Zhang et al. (2011) with some modifications. Samples were diluted at 50% in a mixture of dimethyl sulphoxide (DMSO)/H_2O 50/50 (v/v) and the absorbance was read spectrophotometrically at 365 nm. A calibration curve of FA was made for this purpose (Zhang et al., 2011). The amount of bound FA (% BFA) was calculated as follows, considering the density of diluted solutions nearly equal to that of the water:

$$\%\ \mathrm{BFA} = \left[1 - \frac{(W_1C_1 + F_{ad})}{W_0C_0}\right] \times 100 \quad (1)$$

where

$F_{ad} = W_{0,F}C_{0,F} - W_{1,F}C_{1,F}$ is the amount of FA lost by adsorption on the filter membrane

$W_{(X)}$ is the weight of (X): (0) the initial solution; (1) the filtrate; (0,F) the initial blank solution; (1,F) the blank filtrate

$C_{(X)}$ is the concentration of (X): (0) the initial solution; (1) the filtrate; (0,F) the initial blank solution; (1,F) the blank filtrate. Mean values of two replicates per sample are reported.

2.6. Confocal microscopy

Images of samples were recorded with a confocal laser scanning microscope (Model FV300, Olympus, Tokyo, Japan), provided with an He–Ne laser (543 nm). An objective PLAN APO 60× and a digital zoom of 2.5× were used. Non-covalent labelling of protein was performed with a few drops of 0.02% w/w rhodamine B solution (excitation wavelength 560 nm; emission maximum 625 nm). Digital image files were acquired in multiple.tif format in 1024 × 1024 pixel resolution.

2.7. Nanocarriers drying and rehydration

The mixtures of EW nanoparticles and FA were lyophilized for 24 h in an ALPHA 1–4 LD freeze-dryer (Christ, Osterode am Harz, Germany). After that, the dried powder was resuspended in double distilled water at the same original concentration. The particle size distribution and zeta potential were measured as described in Section 2.3. Images of the samples before and after freeze-drying were taken as detailed in this section.

2.8. Evaluation of biological activity of FA in nanocarriers of EW

2.8.1. In vitro *gastrointestinal digestion protocol*

This was performed according to Moreno, Mellon, Wickham, Bottrill, and Mills (2005) with some modifications. An aliquot of 5 ml of samples was dissolved in 4 ml of a simulated gastric fluid (SGF, 0.15 M NaCl, pH 2.5). The pH was adjusted to 2.5 with 1 M HCl, if necessary. A solution of pepsin (P7000, 182 U/mg of protein) dissolved in 1 ml of SGF was added. The mixture was incubated at 37 °C for 2 h with continuous stirring. At the end of this stage, pH was adjusted to 7.0 with 1 M $NaHCO_3$ to inactivate pepsin (maximum volume = 1 ml). If less volume was required, double distilled water was added until 1 ml was reached. Then, 3 ml of a simulated intestinal fluid (SIF, 39 mM K_2HPO_4, 150 mM NaCl and 30 mM $CaCl_2$, pH 7.0) were added. Next, 80 mg of bile salts (B3883) dissolved in 1 ml of SIF, trypsin (T8003, 34.5 U/mg of protein) and α-chymotrypsin (C4129, 0.44 U/mg of protein), each one dissolved in 0.5 ml double distilled water were incorporated. Samples were incubated at 37 °C for 2 h. At the end of this stage, trypsin and α-chymotrypsin were inactivated in a water bath at 80 °C during 5 min. The digested solutions were then freeze-dried.

2.8.2. *Microbiological model*

Lactobacillus casei subsp. rhamnosus (NCIMB10463) was activated in MRS medium (Britania, Buenos Aires, Argentina) and incubated at 37 °C for 24 h. The culture was then centrifuged (8000 × *g* for 5 min, at 21 °C), washed and resuspended to its original volume with sterile physiological solution (0.85%, m/v). The cell suspension was inoculated at 2% (v/v) in vitamin-free FACM medium (Folic Acid Casei Medium, Difco, Argentina) and incubated at 37 °C for 24 h. After that, the culture was washed and inoculated in 2× FACM, and different concentrations of commercial FA (Sigma, Buenos Aires, Argentina) or the samples were added in the same volume as 2× medium. The inoculated media with FA or samples were incubated at 37 °C for 48 h and the optical density (OD) was read at 580 nm. A calibration curve of microbial growth as a function of FA concentration was also measured. Blanks of the digested nanoparticles without FA were also assessed.

2.9. Statistical analysis

All the determinations were made at least in duplicate. Significant differences between samples were determined by analysis of variance (one way ANOVA) using the general linear model procedure (Statgraphics Centurion XV). An alpha level of 0.05 ($p < 0.05$) was used to determine significance. The values statistically different are indicated by different superscripts.

3. Results and discussion

3.1. *Binding of FA to EW nanoparticles*

Based on the results of previous experiments, two kinds of EW nanoparticles were used to assess their ability to bind FA and act as potential carriers for the vitamin. The first type of EW nanoparticles (USN) was obtained by HIUS treatment for 20 min at pH 3 and presented a monomodal distribution of 220 nm of diameter. The other type of EW nanoparticles (TSN), obtained by TS at 85 °C, for 20 min and pH 3, was chosen because it was constituted mostly by a population of small particle size (~70 nm), though the distribution also included particles of 295 nm (Fig. 1).

In order to generate FA loaded nanoparticles, three strategies were tested. First, the nanoparticle solutions at pH 3 were mixed with a solution of FA at the same pH. At this pH, FA has a low solubility (Wu, Li, Hou, & Qian, 2010) and exhibits acicular crystals, as can be seen by optical microscopy (not shown). When the mixture was made at this pH, FA crystals did not dissolve, and therefore, did not interact with the protein nanoparticles, as was observed by optical microscopy.

In a second attempt, the mixture was performed at pH 7, since FA has a high solubility in water at this pH, and then pH was adjusted to 3. In this trial, EW nanoparticles were not stable at pH 7 and aggregated, being this state irreversible when re-adjusting the pH to 3. Finally, nanoparticle solutions at pH 3 were mixed with the solution of FA at pH 7, with slight and continuous agitation. The mixtures were observed by optical microscopy and FA crystals were not detected. The pH of the mixtures was measured and remained constant at a value of 3, after addition of FA. The mixtures of USN and TSN with FA are hereafter referred as USF and TSF, respectively.

Once the mixtures were achieved, their particle size distribution was measured and compared with the distributions obtained for the nanoparticles alone. As can be seen in Fig. 1A the particle size distribution of USN remained unaltered after the binding of FA. However, a little increase in particle size was observed for TSN when these were bound to FA (Fig. 1B).

Surface charge, expressed as ζ potential, did not show any significant change after FA addition for both nanoparticles as can be appreciated in Table 1. On the one hand, major EW proteins have their pI at a pH between 4 and 5. On the other hand, FA has a pKa value of 2.3. Below this pH, most cationic species

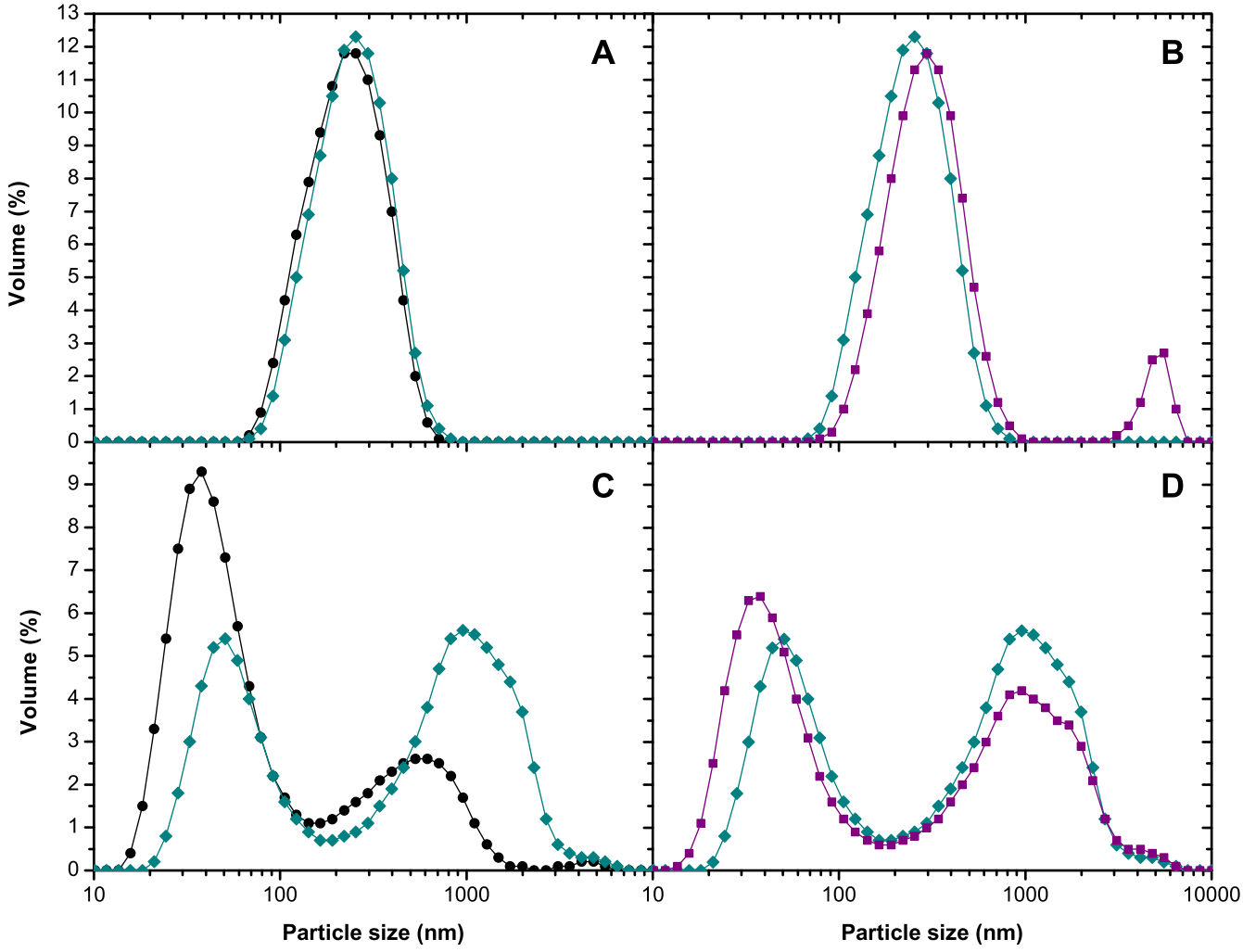

Fig. 1 – Volume particle size distributions of US (A) and TS (B) nanoparticles with (♦) and without (●) FA and; USF (C) and TSF (D) nanocarriers before (♦) and after (■) freeze-drying.

Table 1 – Fluorescence intensity and ζ potential of EW nanoparticles (US and TS) and FA nanocarriers (USF and TSF).

Sample	FI (a.u.)	ζ Potential (mV)
US	2.24 ± 0.09[a]	+20.7 ± 2.3[a]
USF	2.02 ± 0.05[a]	+20.9 ± 1.2[a]
TS	2.31 ± 0.16[a]	+31.5 ± 4.0[a]
TSF	1.90 ± 0.24[a]	+28.4 ± 2.2[a]

Different letters within columns indicate significant difference ($p < 0.05$).

are present. Above this pH, neutral species appear and as the pH further increases anionic species become predominant (Akhtar et al., 1999; Ye, Eitenmiller, & Landen, 2007). Thus, at pH 3, where the complexes are formed, EW proteins are positively charged while neutral species of FA predominate, and this might be the reason why zeta potential of nanoparticles does not vary after addition of FA. Other authors have reported no differences in the zeta potential of poly(DL-lactide-co-glycolide) (PLGA) nanospheres after the addition of folic acid, independently from the ratio of PLGA and FA used (Stevanovic, Radulovic, Jordovic, & Uskokovic, 2008).

The fluorescence intensity was recorded for the protein nanoparticles alone and with FA (Table 1). It is known that in proteins, the dominant fluorophore is the indole group of tryptophan. Indole absorbs near 280 nm and emits near 340 nm. The emission spectrum of indole is highly sensitive to solvent polarity. The emission of indole may be blue shifted if the group is buried within a native protein, and its emission may shift to longer wavelengths (red shift) when the protein is unfolded (Lakowicz, 2006). As previously reported in the literature, the main egg white proteins, ovalbumin, conalbumin and lysozyme, contain 3, 12 and 6 tryptophan residues, respectively (Levine & Federici, 1982; Spies, 1967). The results for both nanoparticles alone showed a maximum of fluorescence intensity at 334 nm, and this maximum emission wavelength stayed constant with the addition of FA, which indicates that tryptophan residues may be buried within the protein and that the conformation remains stable when mixed with the vitamin. A slight reduction of the fluorescence intensity at the maximum emission length (334 nm) was observed for the mixtures, which could be attributed to the quenching effect of FA due to its interaction with protein nanoparticles. Other authors have already reported a quenching of the fluorescence of different proteins by FA and correlated it with their interaction (Bourassa et al., 2011; Jha & Kishore, 2011; Liang & Subirade, 2002; Liang et al., 2013). However, in the present study, a statistical analysis of the results showed that the reduction of fluorescence intensity observed was not significant, thus the results of this experiment were not conclusive.

Therefore, an evaluation of bound FA to EW nanoparticles was performed by the spectrophotometric determination of free FA permeated after ultrafiltration of the samples. The percentage of bound folic acid (% BFA) was calculated as the percentage ratio between bound FA and the amount added to the mixtures. To determine the amount of bound FA, the difference between the amount of added FA and the amount present in the filtrate was calculated. The % BFA was 78.0 ± 9.1 and 79.7 ± 9.0, for USF and TSF, respectively, which confirmed that FA was bound to EW nanoparticles, constituting EW–FA nanocomplexes. The amounts of BFA found here are comparable to those reported by Verwei, Arkbage, Mocking, Havenaar, and Groten (2004) for the binding of FA to folate-binding protein, which would indicate that EW could have a similar affinity for the vitamin. The % BFA expressed in milligrams per gram of protein represent 3.90 ± 0.46 and 3.99 ± 0.45 mg BFA/g protein, for USF and TSF mixtures, respectively. These values prove to be above the recommended levels of fortification (Food and Drug Administration, 1996), thus allowing the use of these nanocarriers of FA in small amounts as food additives. As a consequence, the nanocarriers should be dehydrated in order to obtain a powder product, which would be easier to handle, transport and store. For this reason, the stability of the complexes to a drying process was assessed. Fig. 1 shows the particle size distribution of USF and TSF samples before and after freeze-drying. For USF sample, the main population did not suffer any significant variation in size after freeze-drying and redispersion, but a very low proportion of a new population appeared near the upper limit of detection, probably due to aggregation of some protein particles during the drying process. On the other hand, the freeze-drying process did not cause any apparent modification on the particle size distribution of TSF sample, according to the standard deviation of the duplicates (not shown here for the sake of clarity). Fig. 2 shows images of confocal laser scanning microscopy for EW nanoparticles and FA nanocarriers. These images confirmed what was found by DLS, and also revealed a certain degree of aggregation in TSF sample after freeze-drying, which was not appreciated in the particle size distribution obtained by DLS.

3.2. *Evaluation of biological activity of FA in nanocarriers of EW*

3.2.1. *Stability to pH*

As during gastrointestinal digestion food is subjected to pH variations, the stability of the binding of FA to nanocarriers was first evaluated in the pH conditions involved in that process. Fig. 3 shows the change in appearance of the nanocarriers after adjusting the pH from 3 to 7. When the pH was adjusted from 3 to 4, the macroscopic aspect of the solutions was similar to the one of the nanocarriers solutions at pH 3. However, when the pH was adjusted to 5 or higher values, an increase in turbidity and a loss of the yellow colour of the solutions was observed. The pH value from which most noticeable changes were observed coincides with the isoelectric point of most EW proteins (~5). This fact could be related to the change in the net charge of particles, from a positive value (pH < pI) to a negative one (pH > pI), which could cause structural modifications on the proteins and on the type of interactions between the protein molecules and/or between the proteins and FA.

Particle size distribution and % BFA was assessed on USF nanocarriers at pH 4 and 7. The particle size distribution remained unaltered after adjusting pH from 3 to 4. Table 2 shows the average diameters (Z-av) derived from the size distributions. Contrarily, when pH was adjusted to 5, 6 or 7 the size distribution could not be measured by DLS because these samples presented large sedimenting particles, whose sizes would be higher than the upper limit of detection of the

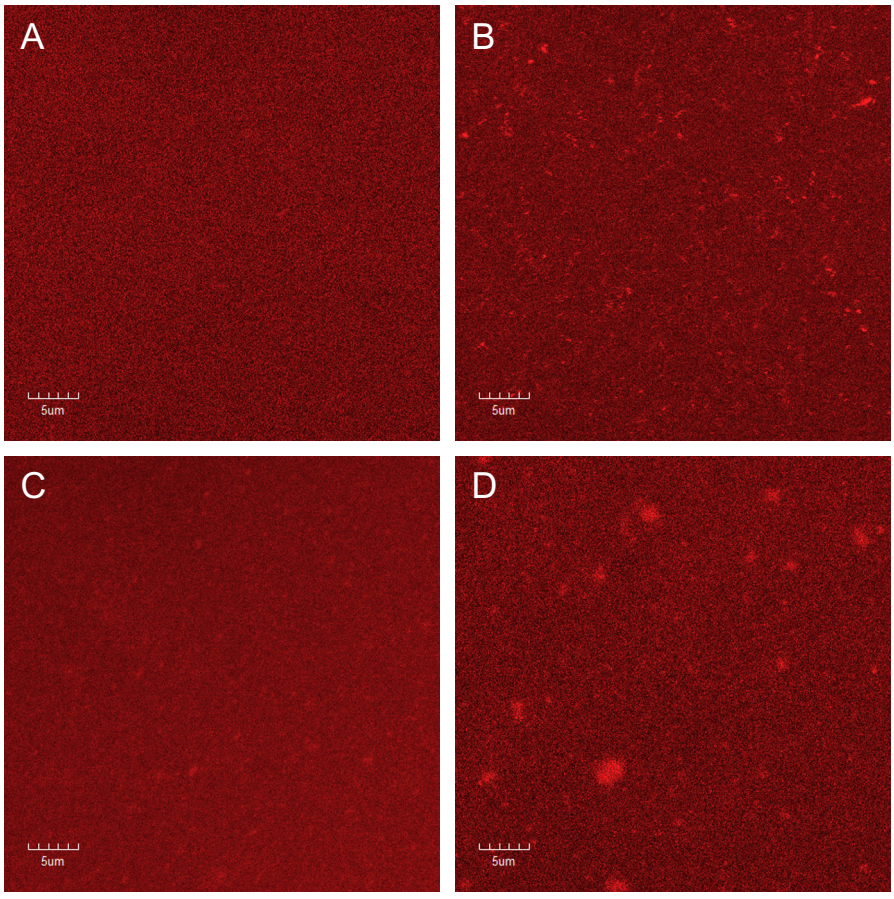

Fig. 2 – Images of confocal laser scanning microscopy of USF (A), freeze-dried USF (B), TSF (C) and freeze-dried TSF nanocarriers (D).

equipment (6 μm). Then, the % BFA was determined in the samples adjusted to pH 4 and 7 by the ultrafiltration assay previously mentioned. Table 2 shows that the % BFA was not reduced significantly after adjusting the pH from 3 to 4, which would indicate that the complex might be stable in this pH range. However, when adjusting the pH to 7, the % BFA decreased to less than the half of that at pH 3, i.e. that the nanocarrier would be unstable in those conditions and there would be a partial dissociation at pH 7. This could indicate that the binding would, to some extent, be mediated by electrostatic interactions. Many authors have reported electrostatic as well as hydrophobic interactions involved in FA binding with proteins (Bourassa et al., 2011; Jha & Kishore, 2011; Liang & Subirade, 2010; Liang et al., 2013; Teng et al., 2013). In the present work, hydrophobic interactions may be involved at pH 3, where FA shows a predominant neutral charge and the hydrophobic moiety of the molecule could interact with hydrophobic residues present in EW proteins. However, as pH increases (e.g. during gastrointestinal digestion), both EW proteins and FA acquire a negative charge that causes them to repel from each other with the subsequent release of the vitamin. Therefore, the binding of FA to the nanoparticles at pH 3 and its subsequent liberation at pH 7 would be a premise for the probable protection of the vitamin during its passage through gastric digestion and its subsequent liberation in the site of absorption that would be produced during intestinal digestion at pH 7.

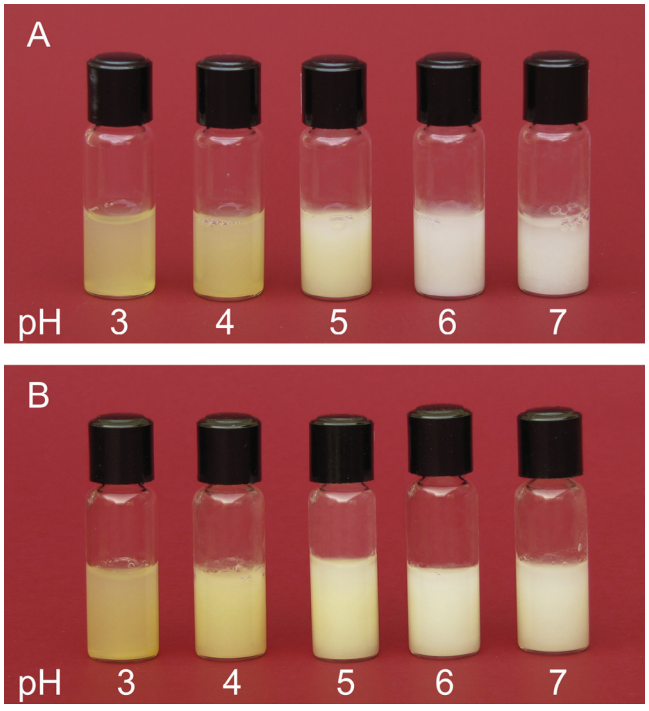

Fig. 3 – Macroscopic appearance of USF (A) and TSF (B) nanocarriers at different pH values.

Table 2 – Percentage of bound FA and Z-average of USF nanoparticles at different pH values.

pH	% BFA	Z-av (nm)
3	78.0 ± 9.1[a]	187.3 ± 3.6[a]
4	75.9 ± 0.8[a]	185.1 ± 4.5[a]
7	27.0 ± 0.5[b]	ND

Different letters within columns indicate significant difference ($p < 0.05$).
ND = not determined.

3.2.2. *Estimation of FA biological activity*

A microbiological assay was carried out to determine the biological activity of FA in the digested samples. All the samples contained the same FA concentration; therefore, if there were any differences in microbial growth, these could be originated by a different biological activity of FA in each sample that would lead to apparently different concentrations of FA. For this reason, the results of the microbiological assay are expressed as apparent FA concentrations ($[AF]_{ap}$) and these values represent the differences in microbial growth due to a higher or lower biological activity of FA in each sample. When digested US and TS nanoparticles were incorporated into the culture media, *L. rhamnosus* did not grow. Then, when digested FA was added to the growth medium it was equivalent to having a $[AF]_{ap}$ of 64 ± 10 μg/ml. However, when the vitamin was added in the form of the digested nanocomplexes USF or TSF, the growth was equivalent to an $[AF]_{ap}$ of 179 ± 3 and 211 ± 7 μg/ml, respectively. This would indicate a synergistic effect between the protein and FA, visualized as an increment in the vitamin bioavailability for *L. rhamnosus*. Other authors have already reported synergistic effects in the FA bioavailability bound to proteins. Swiatlo, O'Connor, Andrews, and Picciano (1990) found that the incorporation of human or bovine milk into rat diets enhanced total folate bioavailability and, on the opposite, goat milk reduced it. On the other hand, de Jong et al. (2005) reported that milk is an adequate matrix for the fortification to increase folate status in humans. There are many researches that focus on the interaction of FA and folate binding protein (FBP). Several *in vivo* and *in vitro* studies indicated that bovine FBP contribute to the absorption and/or retention of folates from milk, especially during the neonatal period (Colman, Hettiarachchy, & Herbert, 1981; Ford, 1974; Ford, Knaggs, Salter, & Scott, 1972; Mason & Selhub, 1988; Said, Horne, & Wagner, 1986; Salter & Mowlem, 1983; Swiatlo et al., 1990; Tani & Iwai, 1984). However, some results are contradictory, because enhancement of folate absorption by FBP was observed in studies performed with isolated rat intestinal mucosal cells (Colman et al., 1981) and intestinal brush border membrane vesicles (Salter & Mowlem, 1983), whereas Said et al. (1986) and Tani and Iwai (1984) found lower jejunal and equal ileal transport in rats of FBP-bound folate compared with unbound folate. Mason and Selhub (1988) showed that FBP-bound folate is absorbed in the small intestine by a different mechanism than the absorption of unbound folate. The absorption of FBP-bound folate occurs more gradually and slowly. It has been suggested that a slower absorption rate prevents the occurrence of high plasma folate levels, which could promote rapid excretion by the kidneys (Tani & Iwai, 1984). In the present study, the microbiological assay provides only an approach to understanding the bioavailability of FA in EW nanocarriers, but cannot be directly extrapolated to what would occur in the human digestive system.

4. Conclusion

The results presented here demonstrated the development of EW proteins based nanocarriers for FA, a bioactive compound with enormous implications in public health. The binding of FA to EW nanoparticles was confirmed by the spectrophotometric determination of free FA. The particle size distributions and zeta potential remained almost unaffected by this interaction. EW nanoparticles could constitute an important carrier for FA; their interaction has proven to be beneficial for the transport and release of FA after *in vitro* digestion as an enhancement of its bioavailability for *L. rhamnosus* was found. However, other assays should be performed in order to assess the bioavailability of FA in EW nanocarriers for the human metabolism.

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REFERENCES

Akhtar, M. J., Khan, M. A., & Ahmad, I. (1999). Photodegradation of folic acid in aqueous solution. *Journal of Pharmaceutical and Biomedical Analysis*, 19(3–4), 269–275. doi:10.1016/S0731-7085(98)00038-7.

Akhtar, M. J., Khan, M. A., & Ahmad, I. (2003). Identification of photoproducts of folic acid and its degradation pathways in aqueous solution. *Journal of Pharmaceutical and Biomedical Analysis*, 31(3), 579–588. doi:10.1016/S0731-7085(02)00724-0.

AOAC. (1980). Official methods of analysis of the Association of Official Analytical Chemists (13th ed.). Washington DC, USA.

Arzeni, C., Martínez, K., Zema, P., Arias, A., Pérez, O. E., & Pilosof, A. M. R. (2012). Comparative study of high intensity ultrasound effects on food proteins functionality. *Journal of Food Engineering*, 108(3), 463–472.

Arzeni, C., Pérez, O. E., & Pilosof, A. M. R. (2015). Power ultrasound assisted design of egg albumin nanoparticles. *Food Biophysics*, doi:10.1007/s11483-015-9407-2 (in press).

Bourassa, P., Hasni, I., & Tajmir-Riahi, H. A. (2011). Folic acid complexes with human and bovine serum albumins. *Food Chemistry*, 129(3), 1148–1155. doi:10.1016/j.foodchem.2011.05.094.

Chen, L., Remondetto, G. E., & Subirade, M. (2006). Food protein-based materials as nutraceutical delivery systems. *Trends in Food Science & Technology*, 17(5), 272–283. doi:10.1016/j.tifs.2005.12.011.

Colman, N., Hettiarachchy, N., & Herbert, V. (1981). Detection of a milk factor that facilitates folate uptake by intestinal cells. *Science*, 211(4489), 1427–1429.

de Jong, R. J., Verwei, M., West, C. E., van Vliet, T., Siebelink, E., van den Berg, H., & Castenmiller, J. J. M. (2005). Bioavailability of folic acid from fortified pasteurised and UHT-treated milk in humans. *European Journal of Clinical Nutrition*, *59*(8), 906–913.

Food and Drug Administration (1996). Food standards: Amendment of standards of identity for enriched grain products to require addition of folic acid; final rule (vol. 21 CFR parts 136, 137, and 139). *Federal Register*, 8781–8797.

Ford, J. E. (1974). Some observations on the possible nutritional significance of vitamin B12- and folate-binding proteins in milk. *British Journal of Nutrition*, *31*(2), 243–257. doi:10.1079/BJN19740030.

Ford, J. E., Knaggs, G. S., Salter, D. N., & Scott, K. J. (1972). Folate nutrition in the kid. *The British Journal of Nutrition*, *27*(3), 571–583.

Iyer, R., & Tomar, S. K. (2009). Folate: A functional food constituent. *Journal of Food Science*, *74*(9), R114–R122. doi:10.1111/j.1750-3841.2009.01359.x.

Jha, N. S., & Kishore, N. (2011). Thermodynamic studies on the interaction of folic acid with bovine serum albumin. *The Journal of Chemical Thermodynamics*, *43*(5), 814–821. doi:10.1016/j.jct.2010.12.024.

Lakowicz, J. R. (2006). Introduction to fluorescence. In J. R. Lakowicz (Ed.), *Principles of fluorescence spectroscopy*, 15. New York, USA: Springer US.

Levine, R. L., & Federici, M. M. (1982). Quantitation of aromatic residues in proteins: Model compounds for second-derivative spectroscopy. *Biochemistry*, *21*(11), 2600–2606.

Liang, L., & Subirade, M. (2002). Study of the acid and thermal stability of β-lactoglobulin-ligand complexes using fluorescence quenching. *Food Chemistry*, *132*(4), 2023–2029. doi:10.1016/j.foodchem.2011.12.043.

Liang, L., & Subirade, M. (2010). β-lactoglobulin/folic acid complexes: Formation, characterization, and biological implication. *The Journal of Physical Chemistry B*, *114*(19), 6707–6712. doi:10.1021/jp101096r.

Liang, L., Zhang, J., Zhou, P., & Subirade, M. (2013). Protective effect of ligand-binding proteins against folic acid loss due to photodecomposition. *Food Chemistry*, *141*(2), 754–761. doi:10.1016/j.foodchem.2013.03.044.

Martínez, K. D., Carrera Sánchez, C., Rodríguez Patino, J. M., & Pilosof, A. M. R. (2009). Interfacial and foaming properties of soy protein and their hydrolysates. *Food Hydrocolloids*, *23*(8), 2149–2157.

Mason, J. B., & Selhub, J. (1988). Folate-binding protein and the absorption of folic acid in the small intestine of the suckling rat. *The American Journal of Clinical Nutrition*, *48*(3), 620–625.

Moreno, F. J., Mellon, F. A., Wickham, M. S. J., Bottrill, A. R., & Mills, E. N. C. (2005). Stability of the major allergen Brazil nut 2S albumin (Ber e 1) to physiologically relevant *in vitro* gastrointestinal digestion. *FEBS Journal*, *272*(2), 341–352. doi:10.1111/j.1742-4658.2004.04472.x.

Nedovic, V., Kalusevic, A., Manojlovic, V., Levic, S., & Bugarski, B. (2011). An overview of encapsulation technologies for food applications. *Procedia Food Science*, *1*, 1806–1815. doi:10.1016/j.profoo.2011.09.265.

Paine-Wilson, B., & Chen, T. S. (1979). Thermal destruction of folacin: Effect of pH and buffer ions. *Journal of Food Science*, *44*(3), 717–722. doi:10.1111/j.1365-2621.1979.tb08484.x.

Pérez, O. E., David-Birman, T., Kesselman, E., Levi-Tal, S., & Lesmes, U. (2014). Milk protein–vitamin interactions: Formation of beta-lactoglobulin/folic acid nano-complexes and their impact on in vitro gastro-duodenal proteolysis. *Food Hydrocolloids*, *38*, 40–47. doi:10.1016/j.foodhyd.2013.11.010.

Said, H. M., Horne, D. W., & Wagner, C. (1986). Effect of human milk folate binding protein on folate intestinal transport. *Archives of Biochemistry and Biophysics*, *251*(1), 114–120. doi:10.1016/0003-9861(86)90057-3.

Salter, D. N., & Mowlem, A. (1983). Neonatal role of milk folate-binding protein: Studies on the course of digestion of goat's milk folate binder in the 6-d-old kid. *The British Journal of Nutrition*, *50*(3), 589–596.

Spies, J. R. (1967). Determination of tryptophan in proteins. *Analytical Chemistry*, *39*(12), 1412–1416. doi:10.1021/ac60256a004.

Stevanovic, M., Radulovic, A., Jordovic, B., & Uskokovic, D. (2008). Poly(DL-lactide-co-glycolide) nanospheres for the sustained release of folic acid. *Journal of Biomedical Nanotechnology*, *4*(3), 349–358. doi:10.1166/jbn.2008.321.

Swiatlo, N., O'Connor, D. L., Andrews, J., & Picciano, M. F. (1990). Relative folate bioavailability from diets containing human, bovine and goat milk. *The Journal of Nutrition*, *120*(2), 172–177.

Tani, M., & Iwai, K. (1984). Some nutritional effects of folate-binding protein in bovine milk on the bioavailability of folate to rats. *The Journal of Nutrition*, *114*(4), 778–785.

Tannenbaum, S. R., Archer, V. R., & Young, M. C. (1985). Vitamins and minerals. In O. R. Fennema (Ed.), *Food chemistry* (2nd ed.). New York: Marcel Dekker.

Teng, Z., Luo, Y., Wang, T., Zhang, B., & Wang, Q. (2013). Development and application of nanoparticles synthesized with folic acid conjugated soy protein. *Journal of Agricultural and Food Chemistry*, *61*, 2556–2564.

Verwei, M., Arkbage, K., Mocking, H., Havenaar, R., & Groten, J. (2004). The binding of folic acid and 5-methyltetrahydrofolate to folate-binding proteins during gastric passage differs in a dynamic in vitro gastrointestinal model. *Journal of Nutrition*, *134*(1), 31–37.

World Health Organization (2006). Defining and setting programme goal. In L. Allen, B. de Benoist, O. Dary, & R. Hurrell (Eds.), *Guidelines on food fortifications with micronutrients*, 145. Switzerland: WHO Press.

Wu, Z., Li, X., Hou, C., & Qian, Y. (2010). Solubility of folic acid in water at pH values between 0 and 7 at temperatures (298.15, 303.15, and 313.15) K. *Journal of Chemical & Engineering Data*, *55*(9), 3958–3961. doi:10.1021/je1000268.

Ye, L., Eitenmiller, R. R., & Landen, W. O. (2007). Folate and folic acid. In R. R. Eitenmiller, L. Ye, & W. O. Landen, Jr (Eds.), *Vitamin analysis for the health and food sciences* (2nd ed., pp. 443–505). Boca Raton, FL, USA: CRC Press.

Zhang, Y., Li, J., Lang, M., Tang, X., Li, L., & Shen, X. (2011). Folate-functionalized nanoparticles for controlled 5-Fluorouracil delivery. *Journal of Colloid and Interface Science*, *354*(1), 202–209.

Zhao, D., Zhao, X., Zu, Y., Li, J., Zhang, Y., Jiang, R., & Zhang, Z. (2010). Preparation, characterization, and *in vitro* targeted delivery of folate-decorated paclitaxel-loaded bovine serum albumin nanoparticles. *International Journal of Nanomedicine*, *5*, 669–677.