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Bioaccessibility of folic acid in egg white nanocarriers and protein digestion profile in solution and in emulsion

Carolina Arzeni[a,b,**], Ana M.R. Pilosof[a,b,*]

[a] Universidad de Buenos Aires, Facultad de Ciencias Exactas y Naturales, Departamento de Industrias, Buenos Aires, Argentina
[b] CONICET - Universidad de Buenos Aires, Instituto de Tecnología de Alimentos y Procesos Químicos (ITAPROQ), Buenos Aires, Argentina

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ABSTRACT

The objective of this work is to assess bioaccessibility of folic acid in egg white (EW) nanocarriers after digestion of solutions and emulsions containing the vitamin-protein complexes. Furthermore, the influence of folic acid binding on the peptides profile after digestion is also evaluated. To this end an *in vitro* gastrointestinal digestion was performed and the pattern of digestion products was determined by high performance liquid chromatography. When folic acid was bound to nanocarriers, the vitamin peak area was approximately 80% lower than the one for folic acid alone. In emulsion, even less amount was detected. After digestion, no significant differences on folic acid peak areas were observed. Folic acid was released from nanocarriers after digestion, thus indicating that the vitamin would be bioaccessible in the site of absorption. Moreover, it was demonstrated by tricine-SDS-PAGE that folic acid binding induced a slight aggregation of proteins, although it did not alter the profile of peptides obtained after digestion. Binding of folic acid to nanocarriers proved to cause no negative effect on EW proteins digestibility. On the contrary, it seemed to slightly enhance their digestion. These nanocarriers would constitute an adequate system for the preservation, transport and target delivery of this bioactive.

1. Introduction

Folic acid, 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). However, folic acid is unstable to heating and light exposure, especially at acid pH (Akhtar, Khan, & Ahmad, 1999; Gazzali et al., 2016; Liang, Zhang, Zhou, & Subirade, 2013; Liang, Zhao, & Hao, 2013; Shrestha, Arcot, & Yuliani, 2012).

The design of food grade nanocarriers for the delivery of bioactives to specific sites within the consumer's body is an active field of research. Food proteins can be used to prepare a wide range of biocompatible 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 foods. Furthermore, the ability to control the particle size of proteinaceous materials is of primary importance not only for determining food product properties such as taste, aroma, texture, 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 (Escobal et al., 2001).

High intensity ultrasound (HIUS) technology has been proved to be effective in controlling particle size of different biopolymers (Chen, Chang, & Shyur, 1997; Gordon & Pilosof, 2010; Müller, Radtke, & Wissing, 2002a; Radomska-Soukharev & Muller, 2006; Wibowo et al., 2013). By controlling the conditions used for the sonication treatment, i.e. temperature and processing time, as well as pH conditions, it is possible to control particle size of proteins.

The formation of complexes between folic acid and protein nanoparticles might enhance its stability during food manufacturing processes where the vitamin is incorporated or during its passage through the gastrointestinal tract (Liang, Zhang et al., 2013; Müller, Radtke, &

* Corresponding author. Departamento de Industrias, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Ciudad Universitaria (1428), Buenos Aires, Argentina.
** Corresponding author. Departamento de Industrias, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Ciudad Universitaria (1428), Buenos Aires, Argentina.
E-mail addresses: carzeni@di.fcen.uba.ar (C. Arzeni), apilosof@di.fcen.uba.ar (A.M.R. Pilosof).

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Wissing, 2002b; Uner, 2006; Weiss et al., 2008).

In previous studies, the generation of egg white (EW) nanoparticles was investigated (Arzeni, Pérez, & Pilosof, 2015). Two kinds of nanoparticles were obtained by sonicating EW solutions either with or without simultaneous heating (TS and S nanoparticles, respectively). The ability to bind folic acid was also studied in order to assess the possibility of designing EW nanocarriers for this vitamin, namely TS_F and S_F, respectively (Arzeni, Pérez, LeBlanc, & Pilosof, 2015). EW nanocarriers for folic acid showed a high folic acid binding capacity (about 80%), thus being a potential carrier for folic acid (Arzeni, Pérez, LeBlanc et al., 2015). However, folic acid bioaccessibility needs to be assessed in order to establish the potential use of EW nanoparticles as folic acid carriers. On the other hand, protein digestion profile must also be monitored so as to elucidate if folic acid binding enhances, hinders or makes no interference on its digestibility. Additionally, it is well known that changes in protein structure can also affect its digestibility. Emulsification of protein is one of the processes that changes protein structure. Proteins with relatively disordered, open structures (such as casein) undergo relatively rapid conformational changes upon adsorption in the oil droplet surface, whereas more rigid globular proteins (such as serum albumin) exhibit slow conformational changes. Such conformational changes can be seen as a form of interfacial denaturation of the protein. The adsorbed and unadsorbed proteins are likely to exist in different conformational states, so their susceptibility to pepsin could be different (Singh & Ye, 2013). For example, it has been shown that under a given set of simulated gastric conditions, adsorbed β-lactoglobulin was more susceptible to pepsin hydrolysis (85% decrease in the intact protein) than the unadsorbed protein (about 50% decrease in the intact protein). In contrast, a native β-lactoglobulin solution (not in an emulsion) was largely resistant to pepsin digestion (about 20% decrease in the intact protein) under the same conditions (Singh et al., 2013). Understanding the processes involved in protein digestion is also of great importance in food allergy since many proteins used in the food industry are food allergens (Mackie & Macierzanka, 2010). In this regard, Jiménez-Saiz, Ruiz-Henestrosa, López-Fandiño, and Molina (2012) studied the *in vitro* digestibility and human IgE binding capacity of egg proteins in solution and in emulsion. In their research work, they found that the digestion of egg proteins was slightly favoured when they formed part of the emulsions. The most abundant egg white protein, ovalbumin, was digested faster in emulsion than in solution during the gastric phase, as shown by SDS-PAGE gels, and this result was corroborated by RP-HPLC analyses. Similarly, lysozyme, also recognized by its high stability to gastric digestion, was more resistant to hydrolysis by pepsin in solution than in emulsion. The fact that egg protein digestibility was slightly increased in the emulsion was also reflected in the IgE-binding, leading to a slightly lower IgE-binding ability in the emulsion digests as compared to the solution digests. For all the reasons above, the pattern of protein digestion in aqueous or emulsified samples was also evaluated in the present contribution.

Consequently, the objective of this work is to assess folic acid bioaccessibility after digestion of solutions and emulsions containing the vitamin-protein complexes. Furthermore, the influence of folic acid binding on the peptides profile after digestion is also evaluated. To this end an *in vitro* digestion model was used.

2. Materials and methods

2.1. Preparation of EW and folic acid solutions

EW powder gently provided by Ovoprot International S.A. (Buenos Aires, Argentina) was used as starting material. EW solutions (100 ml) at 5% w/w were prepared with deionized water and 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 1N HCl.

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

2.2. Design of EW nanoparticles

EW solutions were sonicated for 20 min using an ultrasonic processor Vibra Cell Sonics, model VCX 750 (Newtown, Connecticut, USA) 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 the sonication process, or at 85 °C, in order to generate S or TS nanoparticles, respectively. The acoustic power dissipated in the liquid was 4.27 ± 0.71 W, determined by a calorimetric method according to a previous work (Arzeni et al., 2012).

2.3. Generation of EW nanocarriers for folic acid

An aliquot of 100 μl of folic acid solution was added to 4 ml of EW nanoparticles with continuous stirring for 5 min. The ratio of folic acid:EW nanoparticles was 1:200 (folic acid 0.025% w/w: EW nanoparticles 5% w/w). The mixtures were covered with aluminium foil and prepared daily for each experiment.

2.4. Emulsion preparation

Chia oil (O) (Sturla, Argentina) and the thermosonicated mixtures prepared as described in Section 2.3 were emulsified in a 10:90 ratio, respectively, for 10 min using the ultrasonic processor described in Section 2.2 with the cooling bath temperature set at 0.5 °C.

2.5. In vitro gastrointestinal digestion

An *in vitro* gastrointestinal digestion was performed according to (Moreno, Mellon, Wickham, Bottrill, & Mills, 2005) with some modifications. An aliquot of 5 ml of samples (sonicated and thermosonicated nanoparticles in solution or in emulsion with and without folic acid before and after digestion) was dissolved in 4 ml of a simulated gastric fluid (SGF, 0.15 mol/L NaCl, pH 2.5). The pH was adjusted to 2.5 with 1 mol/L 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 mol/L $NaHCO_3$ to inactivate pepsin (maximum volume = 1 ml). If less volume was required, deionized water was added until 1 ml was reached. Then, 3 ml of a simulated intestinal fluid (SIF, 39 mmol/L K_2HPO_4, 150 mmol/L NaCl y 30 mmol/L $CaCl_2$, pH 7.0) was 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 of SIF were incorporated. For emulsion digestion, 26.76 mg lipase (L3126, 243 U/mg enzyme) was also incorporated at this stage. Samples were incubated at 37 °C for 2 h. Finally, enzymes were inactivated in a water bath at 80 °C during 5 min. The digested solutions were then freeze-dried. All the biochemical agents were purchased from Sigma-Aldrich.

2.6. Reversed phase – high performance liquid chromatography (RP-HPLC)

Folic acid, EW, nanocarriers and their corresponding hydrolysates powders were rehydrated in deionized water, filtered by a 0.45 μm Nylon syringe filter (Sartorius AG, Germany) and analysed in a Waters 1525 HPLC system (Waters, Milford, MA, USA) using a BioSuite™ pC18 500, 7 μm column (150 × 4.6 mm, Waters, Milford, MA, USA), with a flow rate of 1 ml/min, an injection volume of 20 μl and the following mobile phase: solvent A, 0.37 ml/L TFA in deionized water; solvent B, 0.27 ml/L TFA in HPLC grade ACN. All solvents were filtered by a

0.45 μm membrane prior to use. A linear gradient of solvent B in A, from 0 to 65% in 55 min was used. Absorbance was recorded at 220 and 280 nm with a 2998 Photodiode Array Detector (Waters, Milford, MA, USA). The software Breeze Waters was used.

2.7. Tricine–sodium dodecyl sulphate-polyacrylamide gel electrophoresis (tricine-SDS-PAGE)

Tricine–SDS–PAGE was conducted according to the method by (Schägger, 2006) with some modifications. Samples were diluted with a reducing sample buffer (12% w/v SDS, 6% v/v 2-mercaptoethanol, 30% w/v glycerol, 0.1% w/v bromophenol blue, 150 mmol/L Tris/HCl pH 7.0) in a ratio 3:1, incubated at 37 °C for 15 min and centrifuged at 12,857 × *g* for 5 min. An aliquot of 20 μl of each supernatant was then loaded into the wells under the cathode buffer. The gel consisted of a 16%/4 mol/L urea separating gel, overlaid with a 10% spacer gel and a 4% stacking gel. The run was performed in a vertical slab gel electrophoresis unit, model Hoefer SE 660 (Amersham Biosciences Ltd, Buckinghamshire, England), connected to a constant voltage power supply, model 1000/500 (Bio-Rad, New York, USA), for 7 h 30 min with the following running conditions: an initial voltage of 90 V was applied until the samples completely entered the stacking gel. Then, it was raised up to 150 V and finally, near the end of the run, a 200 V voltage was applied. The gels were incubated into a fixing solution, stained with Coomasie Blue and destained in 10% acetic acid and 40% ethanol.

2.8. 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. Release of folic acid after in vitro gastrointestinal digestion of EW nanocarriers in solution

Detection of peptides and proteins in RP-HPLC generally involves detection between 210 and 220 nm, which is specific for the peptide bond, or at 280 nm, which corresponds to the aromatic amino acids tryptophan and tyrosine (Aguilar, 2004). Regarding folic acid, it is well known that this vitamin shows a peak of absorption at 280 nm (Aceituno-Medina, Mendoza, Lagaron, & López-Rubio, 2015; Chaudhary, Rindhe, & Bhusari, 2018; Off et al., 2005; Qu et al., 2010; Rathinaraj, Lee, Park, & Kang, 2015). In the present work, two wavelengths of detection were used: 220 nm, in order to register protein and peptides peaks and, 280 nm, for better appreciation of folic acid peak, as it exhibits a stronger absorbance than proteins or peptides at this specific wavelength. Fig. 1 shows the chromatograms of S, S_F and folic acid (F) samples before (Fig. 1A) and after (Fig. 1B) the digestion process at 280 nm. Folic acid eluted at a retention time of 10.5 min. Before digestion, the intensity of this peak was lower in S_F sample, in comparison to folic acid alone (Fig. 1A). The same behaviour was observed for TS_F sample (Fig. 2A). All samples were diluted in order to obtain the same protein/folic acid concentration, thus comparisons of the peak areas could be made. Table 1 shows folic acid peak areas calculated for the different samples. As it can be clearly seen, the areas for S_F and TS_F samples are significantly lower than the one calculated for folic acid sample, approximately 80% lower. This could indicate an interaction between folic acid and EW nanoparticles in such a way that cannot be separated in the chromatographic conditions used in the present work. Other authors have reported the formation of complexes between folic acid and different proteins. For example, Liang and Subirade (2002) found, by fluorescence spectroscopy, that folic acid could be bound to β-lactoglobulin and that the complexation reduced folic acid photodegradation. In another work, Jha and Kishore (2011) found, by isothermal titration calorimetry in combination with fluorescence and circular dichroism spectroscopies, that bovine serum albumin could effectively bind folic acid and that the binding was dominated by electrostatic interactions with a contribution of hydrogen bonding and hydrophobic interactions. After *in vitro* gastrointestinal digestion, on the other hand, no significant differences on the areas of folic acid peak (Table 1) were observed for $S_{F,d}$ and F_d samples (Fig. 1B) and the same was evidenced for $TS_{F,d}$ sample (Fig. 2B). This could indicate that the vitamin was released from the nanocarriers at the end of the gastrointestinal digestion, leaving folic acid bioaccessible in the site of absorption. In a previous contribution, the influence of pH on folic acid binding capacity of EW nanoparticles was investigated (Arzeni, Pérez, & Pilosof, 2015). The percentage of bound folic acid was high below the protein isoelectric point but lowered when the pH was raised above it, pointing out the electrostatic nature of the binding of folic acid to EW proteins. Thus under gastrointestinal conditions, folic acid would be bound to protein under gastric digestion (very low pH) and released at the final pH of duodenum that is close to neutrality.

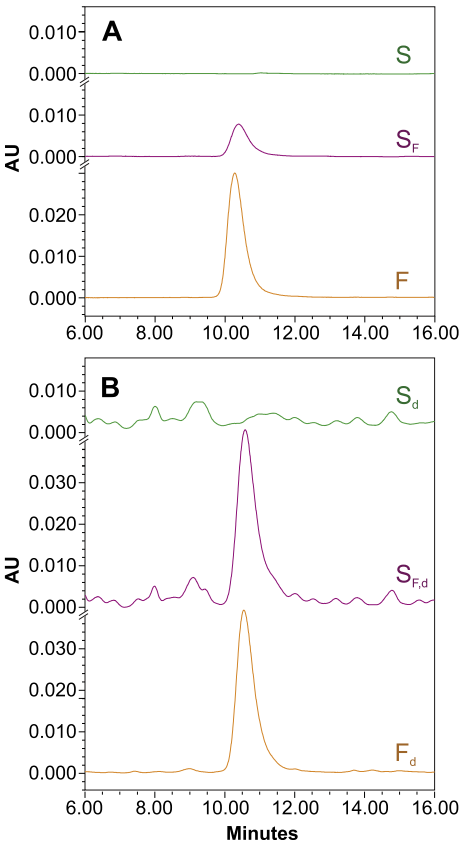

Fig. 1. RP-HPLC chromatograms at 280 nm of folic acid and sonicated nanoparticles with and without folic acid before (A) and after (B) digestion. F = folic acid; S = sonicated nanoparticles; S_F = sonicated nanoparticles with folic acid; F_d = digested folic acid; S_d = digested sonicated nanoparticles; $S_{F,d}$ = digested sonicated nanoparticles with folic acid.

3.2. Release of folic acid after in vitro gastrointestinal digestion of EW nanocarriers in emulsion

With the purpose of evaluating the effect of emulsification on folic acid release, a 10:90 O/W emulsion of chia oil and TS_F nanocarriers was prepared. The initial mean diameter of the droplets, D[3,2], was 0.239 ± 0.006 μm, and did not change significantly within a day but

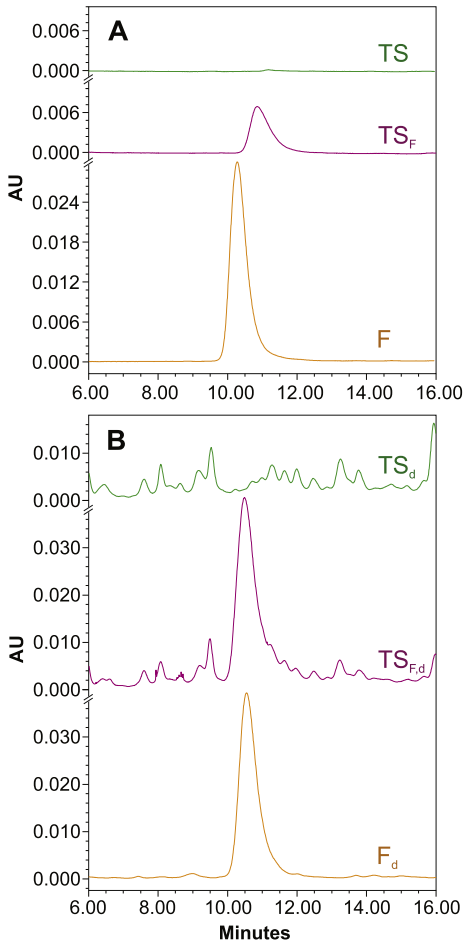

Fig. 2. RP-HPLC chromatograms at 280 nm of folic acid and thermosonicated nanoparticles with and without folic acid before (A) and after (B) digestion. F = folic acid; TS = thermosonicated nanoparticles; TS_F = thermosonicated nanoparticles with folic acid; F_d = digested folic acid; TS_d = digested thermosonicated nanoparticles; $TS_{F,d}$ = digested thermosonicated nanoparticles with folic acid.

Table 1
Folic acid peak area obtained from the chromatograms registered at 280 nm.

Sample	Area (μV.seg x 10^6)
F	12.7 ± 3.2 ^{b}
F_d	15.4 ± 0.6 ^{c}
S_F	2.7 ± 0.2 ^{a}
$S_{F,d}$	16.5 ± 0.1 ^{c}
TS_F	2.9 ± 0.3 ^{a}
$TS_{F,d}$	16.3 ± 2.1 ^{c}
O:TS_F	1.5 ± 0.1 ^{a}
O:$TS_{F,d}$	14.7 ± 1.4 ^{bc}

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

presence of floccules was detected over longer periods of storage. Thus, in order to perform the experiments in this work a freshly prepared emulsion was used in each case.

Fig. 3.A shows the chromatograms for TS_F nanocarriers in solution in comparison with the emulsified sample and folic acid alone. As it can be appreciated, a low amount of folic acid was detected in the emulsified samples (O:TS_F), the calculated peak area was the lowest of all measured samples (Table 1). This emulsion was white before filtration prior to HPLC measurement, but after filtering through a 0.45 μm membrane a clear solution was obtained, indicating that a great amount

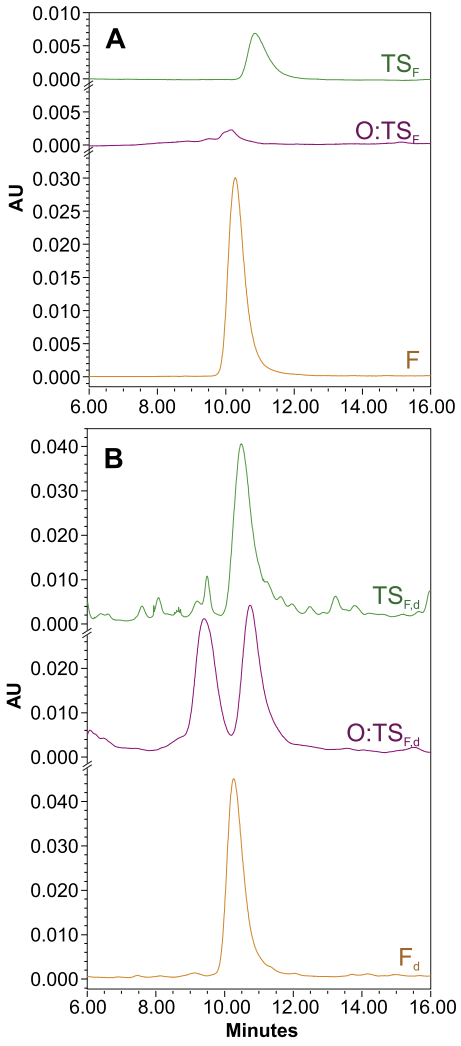

Fig. 3. RP-HPLC chromatograms at 280 nm of folic acid and thermosonicated nanoparticles with folic acid in solution and in emulsion before (A) and after (B) digestion. F = folic acid; TS_F = thermosonicated nanoparticles with folic acid; O:TS_F = emulsified thermosonicated nanoparticles with folic acid; F_d = digested folic acid; $TS_{F,d}$ = digested thermosonicated nanoparticles with folic acid; O:$TS_{F,d}$ = digested emulsified thermosonicated nanoparticles with folic acid.

of the components were retained in the filter. It is well known that folic acid contains aromatic rings in its structure that allows it to interact with hydrophobic compounds (Jha et al., 2011; Ma et al., 2015; Wibowo et al., 2013). In this particular case, in addition to the proteins, the triglycerides contained in the oil could interact with folic acid. The protein-folic acid complex might be participating in the stabilization of oil droplets, which cannot pass through the filter. After digestion, in O:$TS_{F,d}$ samples folic acid peak was a slightly lower than F_d and $TS_{F,d}$ samples, although no statistical differences were found (Table 1). This shows that when the nanocarriers are emulsified, folic acid interactions seem to be stronger, thus leading to a lower release of the vitamin. However, regardless of this observation no significant differences were found on the release of folic acid.

3.3. Influence of folic acid binding on the digestion profile of EW proteins

As detailed above, the chromatograms were also registered at 220 nm, in order to detect protein and peptides. The three main peaks that were present in S sample (Fig. 4A), identified as OM, LZ and OVA, correspond to ovomucoid, lysozyme and ovalbumin, in accordance with

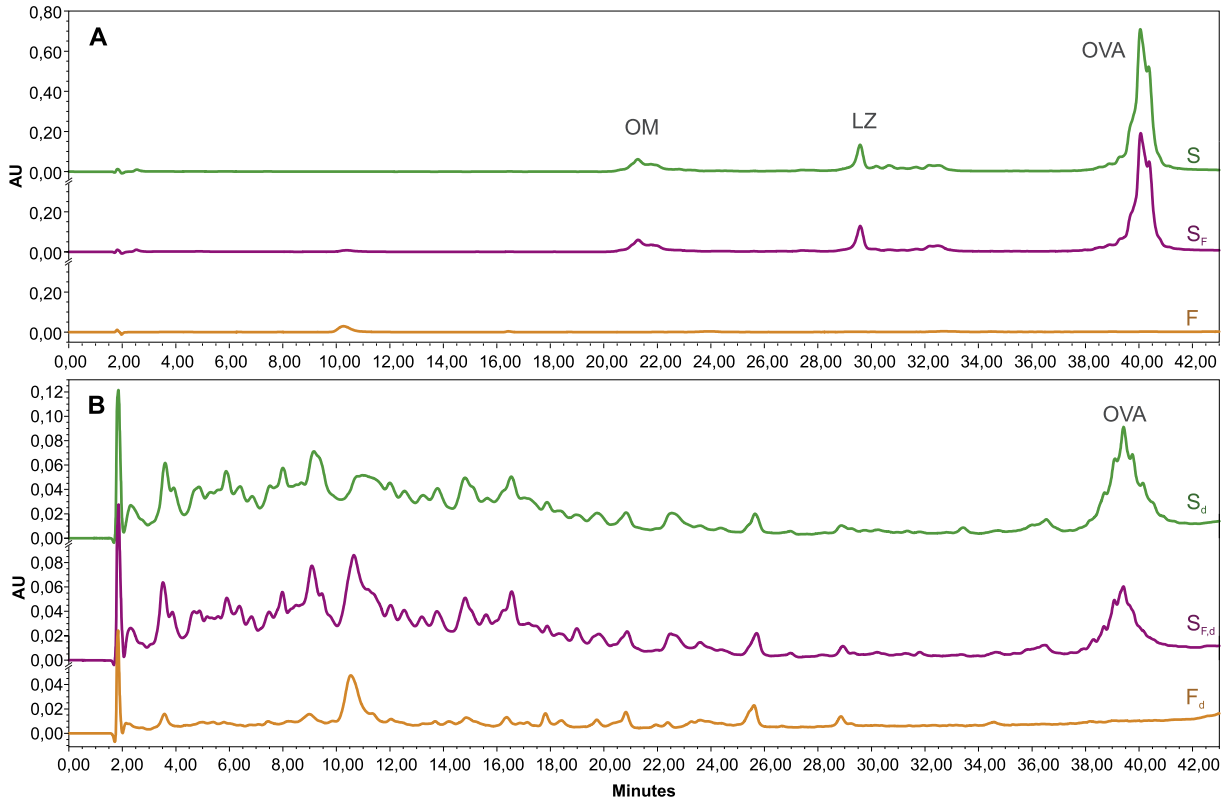

Fig. 4. RP-HPLC chromatograms at 220 nm of folic acid and sonicated nanoparticles with and without folic acid before (A) and after (B) digestion. F = folic acid; S = sonicated nanoparticles; S_F = sonicated nanoparticles with folic acid; F_d = digested folic acid; S_d = digested sonicated nanoparticles; $S_{F,d}$ = digested sonicated nanoparticles with folic acid.

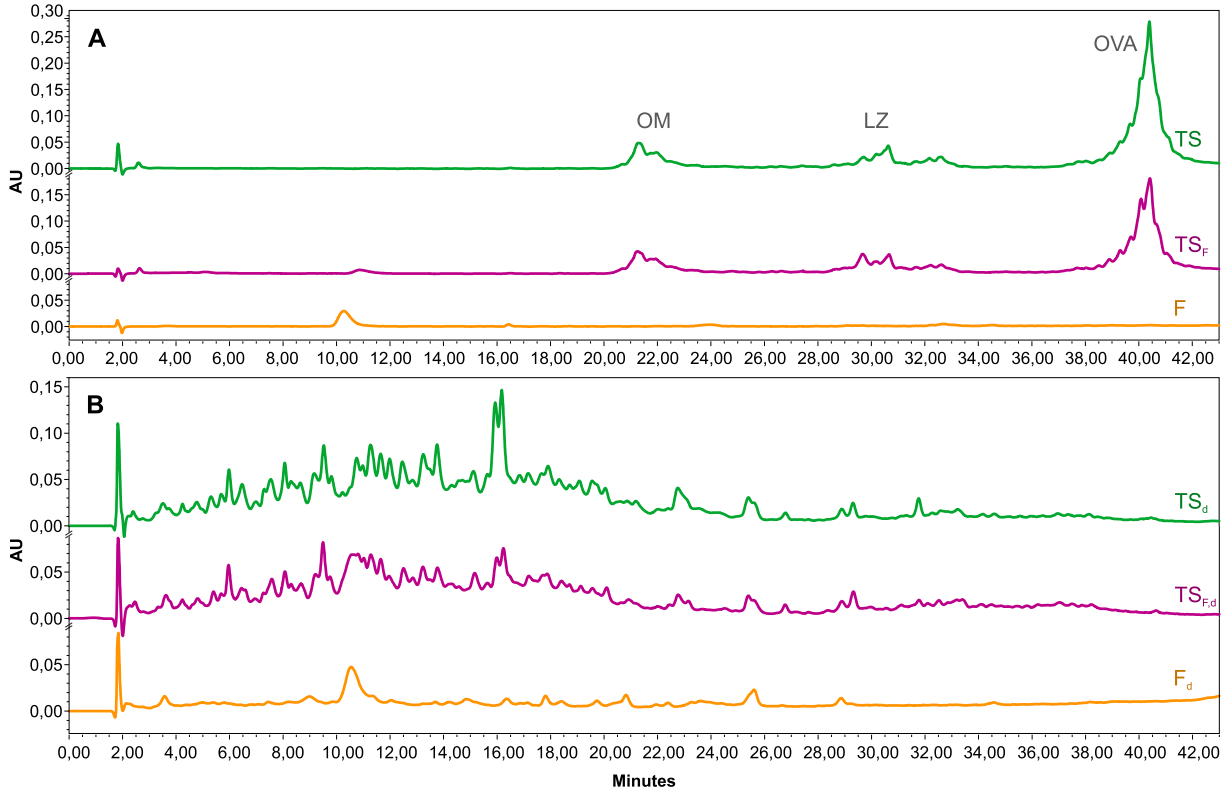

Fig. 5. RP-HPLC chromatograms at 220 nm of folic acid and thermosonicated nanoparticles with and without folic acid before (A) and after (B) digestion. F = folic acid; TS = thermosonicated nanoparticles; TS_F = thermosonicated nanoparticles with folic acid; F_d = digested folic acid; TS_d = digested thermosonicated nanoparticles; $TS_{F,d}$ = digested thermosonicated nanoparticles with folic acid.

Table 2
Ovomucoid, lysozyme and ovalbumin peak areas obtained from the chromatograms registered at 220 nm.

Sample	OM	LZ	OVA
	Area (μV.seg x 10^6)	Area (μV.seg x 10^6)	Area (μV.seg x 10^6)
S	30.6 ± 2.3 ^{c}	28.8 ± 2.0 ^{c}	305.5 ± 13.6 ^{f}
S_F	31.2 ± 2.6 ^{c}	29.4 ± 1.3 ^{c}	256.9 ± 18.2 ^{e}
S_d	N/D	N/D	53.7 ± 8.9 ^{b}
$S_{F,d}$	N/D	N/D	32.6 ± 6.4 ^{a}
TS	27.3 ± 1.8 ^{c}	15.8 ± 1.4 ^{b}	153.9 ± 6.9 ^{d}
TS_F	25.3 ± 2.1 ^{b}	10.0 ± 1.0 ^{a}	100.8 ± 4.4 ^{c}
TS_d	N/D	N/D	N/D
$TS_{F,d}$	N/D	N/D	N/D
O:TS_F	2.5 ± 0.2 ^{a}	N/D	N/D
O:$TS_{F,d}$	N/D	N/D	N/D

Different letters within a column indicate significant difference ($p < 0.05$). N/D = not detected.

the peaks identified for EW in a previous research (Martos, Lopez-Fandino, & Molina, 2013). It should be clarified that no protein or folic acid standards were used in the HPLC method because the aim of the present work was not to calculate the actual concentration of the species but to compare the differences arose between the samples containing folic acid or not. Fig. 5A shows the chromatogram for TS sample. As for S sample, the same peaks corresponding to EW proteins were observed, though in a much lower intensity. This difference might be related to the presence of aggregates in TS sample generated during the thermosonication process (Arzeni, Pérez, & Pilosof, 2015). These aggregates could be retained in the 0.45 μm membrane used in the preparation step prior to HPLC separation, thus reducing the intensity of the peaks corresponding to the native proteins. Table 2 shows the peak areas calculated for the different EW proteins detected. The areas of these proteins in TS samples were significantly lower than in S sample, except for OM, where no statistical difference was found. On the other hand, when folic acid was added to the nanoparticle solutions, the area of OVA peak decreased (Table 2), both for S_F and TS_F samples in comparison to the samples that did not contain the vitamin, probably due to the interaction of this protein with folic acid, as previously showed (Fig. 4A and 5.A, respectively). No significant differences on the peak area of OM and LZ were found were comparing S sample with S_F. However, on TS_F all protein peaks were significantly lower than for TS sample. In the emulsified sample, OVA and LZ were not detected (Fig. 6A). This might occur because the protein stabilized oil droplets may be retained by the filter membrane.

Fig. 4B, 5B and 6B show the chromatograms of digested samples recorded at 220 nm. Intact OVA was found in S_d samples, indicating that this protein was only partially digested *in vitro* (Fig. 4B). Previous works available in the literature report the resistance of OVA to digestive enzymes (Pardeike, Hommoss, & Muller, 2009; Teeranachaideekul, Muller, & Junyaprasert, 2007; Varshosaz, Eskandari, & Tabakhian, 2010). On the other hand, the area of OVA peak in $S_{F,d}$ sample was lower than the corresponding to S_d sample, as shown in Table 2, which could indicate that folic acid enhances OVA degradation by the digestive enzymes. This change in OVA digestibility might be related to a different conformation that the protein would adopt when interacting with folic acid. Other studies reported that the stability of proteins to digestion may be altered in the presence of various components of the food matrix, such as soluble polysaccharides (Lepoint & Lepoint-Mullie, 1998), lipids (Henglein, 1993) or protease inhibitors (Riesz, 1991). The peaks corresponding to OM and LZ were not observed in the digested samples, suggesting that their proteolysis was complete and no differences were found between S_d and $S_{F,d}$ samples. Regarding thermosonicated samples, no intact proteins were found in TS_d and $TS_{F,d}$ samples (Fig. 5B). However, the profile of peptides of TS_d sample showed little differences in comparison with $TS_{F,d}$ sample, indicating that the presence of folic acid would influence the digestion pattern. Fig. 6B shows the profile of O:$TS_{F,d}$ sample. As it can be appreciated, emulsification of TS_F nanocarriers enhanced protein digestion. In this regard, Jiménez-Saiz et al. (2012) found that OVA and

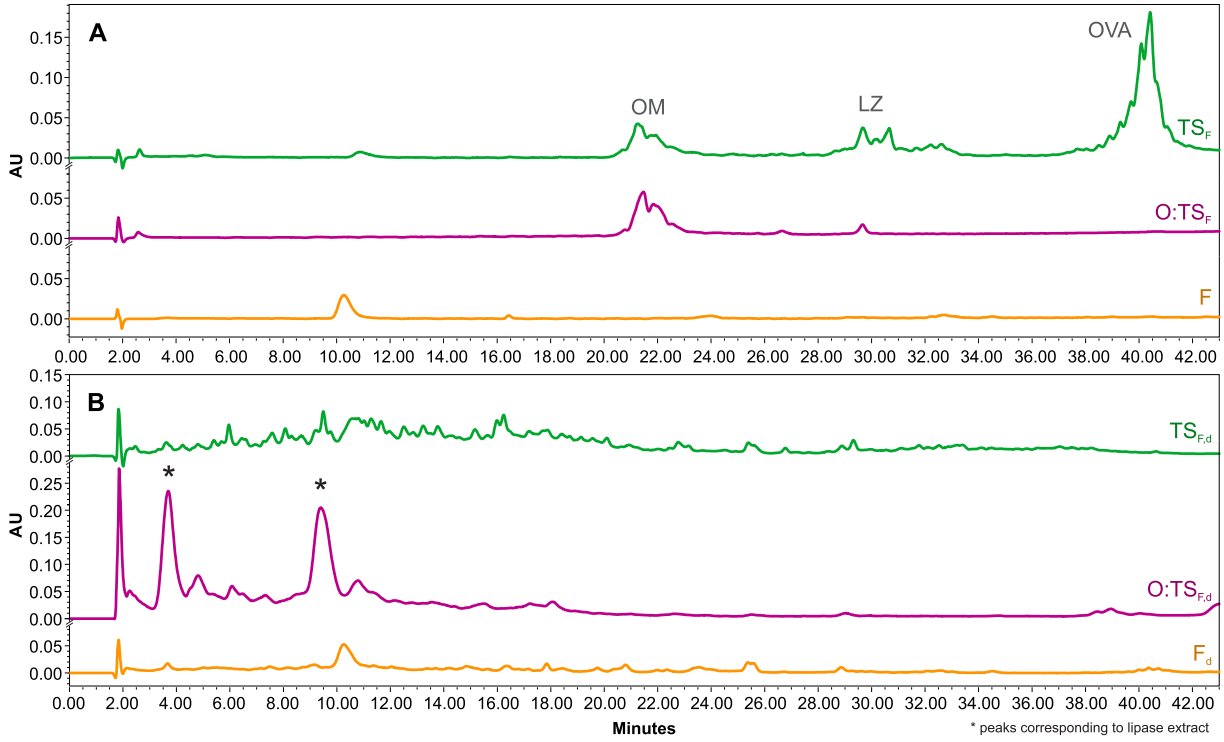

Fig. 6. RP-HPLC chromatograms at 220 nm of folic acid and thermosonicated nanoparticles with folic acid in solution and in emulsion before (A) and after (B) digestion. F = folic acid; TS_F = thermosonicated nanoparticles with folic acid; O:TS_F = emulsified thermosonicated nanoparticles with folic acid; F_d = digested folic acid; $TS_{F,d}$ = digested thermosonicated nanoparticles with folic acid; O:$TS_{F,d}$ = digested emulsified thermosonicated nanoparticles with folic acid.

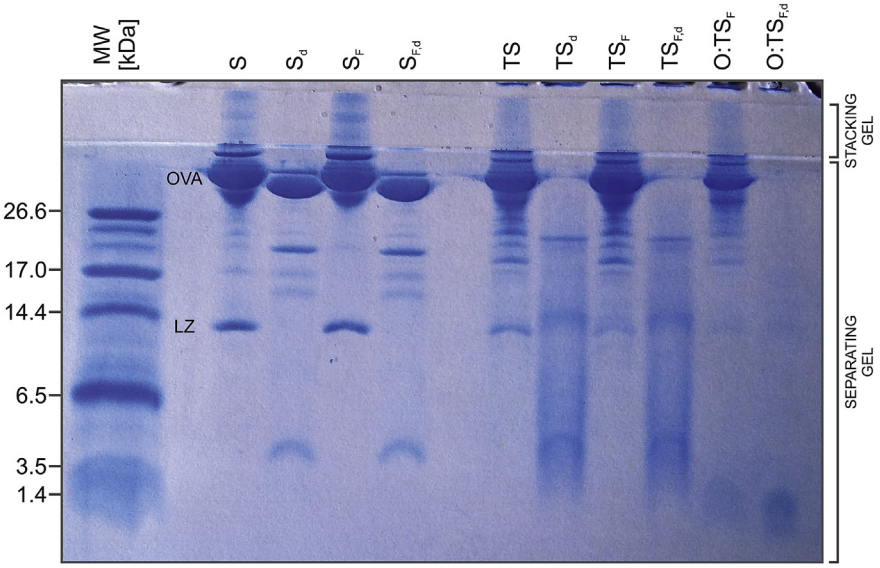

Fig. 7. Comparison of tricine-SDS-PAGE patterns for sonicated and thermosonicated nanoparticles in solution or in emulsion with and without folic acid before and after digestion. First lane shows the bands of the peptide molecular weight marker.

LZ digestibility was slightly improved when these proteins were in emulsion rather than in solution.

Lastly, a tricine-SDS-PAGE assay was performed, as an additional technique to assess if folic acid binding modified the proteins and peptides profiles. Fig. 7 shows the image of the bands in the gel. Regarding the undigested samples without folic acid, S sample contained more proteins/aggregates than TS sample with MW higher than 50 kDa that could not enter the separating gel, but TS sample presented aggregates of even higher MW that were not able to enter the stacking gel. TS sample also presented more protein fragments between 17.0 and 26.6 kDa but less LZ (14 kDa) than S sample. In accordance with HPLC results, OVA was not completely digested in S_d sample but LZ band disappeared after digestion. All these bands did not appear in TS_d sample. On the other hand, TS_d sample presented more peptides between 1.4 and 6.5 kDa than S_d while O:TS_d sample seemed to be more extensively digested, containing peptides below 1.4 kDa, which verifies the results found by HPLC. Regarding samples with folic acid, S_F did not present the protein fragments between 17.0 and 26.6 kDa that were present in S sample, but contained more proteins/aggregates above 45 kDa. After digestion, no differences were found between these two samples. TS_F samples seemed to contain less LZ than TS sample. After digestion, no differences were found between these samples. In summary, it was demonstrated that folic acid binding induced a slight aggregation of proteins. However, this did not alter the profile of peptides obtained after digestion of the samples. On the other hand, the electrophoretic technique performed seemed to have poor sensitivity for peptide differentiation, as compared with the results obtained by RP-HPLC.

4. Conclusions

The release of folic acid from EW nanocarriers after *in vitro* gastrointestinal digestion of the samples is an indication that folic acid would be bioaccessible in the site of absorption. Therefore, this work corroborates the findings of a microbiological assay (Arzeni, Pérez, LeBlanc et al., 2015) that provided a preliminary indication that folic acid was bioaccessible after *in vitro* gastrointestinal digestion. Moreover, binding of folic acid to EW nanoparticles proved to cause no negative effect on EW proteins digestibility. On the contrary, it seemed to slightly enhance their digestion, which would provide an extra benefit for their use as nanocarriers. To sum up, EW nanocarriers would constitute an adequate system for the preservation, transport and target delivery of folic acid.

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