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Protection of folic acid through encapsulation in mesoporous silica particles included in fruit juices

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ABSTRACT

Folic acid (FA) is a synthetic vitamin commonly used for food fortification. However, its vulnerability to processing and storage implies loss of efficiency, which would induce over-fortification by processors to obtain a minimum dose upon consumption. Recent studies have indicated potential adverse effects of FA overdoses, and FA protection during processing and storage could lead to more accurate fortification. In addition, sustained vitamin release after consumption would help improve its metabolism. The objective of this work was to study controlled FA delivery and stability in fruit juices to reduce potential over-fortification risks by using gated mesoporous silica particles (MSPs). The obtained results indicated that FA encapsulation in MSPs significantly improved its stability and contributed to controlled release after consumption by modifying vitamin bioaccessibility. These results confirmed the suitability of MSPs as support for controlled release and protection of bioactive molecules in food matrices in different food production and storage stages.

Keywords: bioaccessibility modification; folic acid; fruit juice; mesoporous silica particles; stability**.**

1. Introduction

The generic term ‘‘folates’’ refers to a group of naturally-occurring B vitamins essential for the human body. An adequate folate status provides health benefits by preventing birth defects during pregnancy and cardiovascular diseases (Hoag, Ramachandruni, & Shangraw, 1997), Alzheimer’s disease (Clarke, Smith, Jobst, Refsum, Sutton, & Ueland, 1998) or colorectal cancer (Stover, 2004). The European Food Safety Authority (EFSA) recommends a daily intake of 200-400 µg folate/day for adults, and an additional intake of 400 µg for women of childbearing age (ESCO, 2009).

Folates are present in diverse food products, including liver, egg yolk, green vegetables, certain beans, citrus fruits, and cereal products (Ball, 2005). However, folate intake is strongly influenced by the degradation of its vitamers during food processing. Hence folic acid (FA), a synthetic form of the vitamin, is commonly used for folate supplementation and food fortification thanks to its reported enhanced bioaccessibility and stability. Food fortification should guarantee the FA concentration indicated on the label until the expiry date, which usually entails having to add large amounts of the vitamin (up to 50%) to compensate for the losses that occur during processing and/or storage (Frommherz, Martiniak, Heuer, Roth, Kulling, & Hoffmann, 2014). The folates degradation process depends on several factors, such as high temperature, light, low pH, oxygen and overall food composition (Nguyen, Oey, Verlinde, van Loey, & Hendrickx, 2003; Fukuwatari, Fujita, & Shibata, 2009; Jastrebova, Axelsson, Strandler, & Jägerstad, 2013). Some studies have reported a marked deviation of FA content from the labeled values of fortified foods (Lebiedzińska, Dbrowska, Szefer, & Marszałł, 2008; Frommherz *et al.*, 2014), which can result in health risks. In particular, FA, which requires metabolic activation before it can function, appears unmetabolized in the bloodstream when the tolerable upper intake level of 1 mg/day (EFSA, 2006) is

exceeded. Unmetabolized FA has been associated with some neoplasia, cognitive damage among seniors due to the masking of vitamin B12 deficiency, and also to the reduced efficacy of anti-folate drugs used to treat rheumatoid arthritis or psoriasis (ESCO, 2009; Crider, Bailey, & Berry, 2011).

Considering the importance of FA for human health, its vulnerability to external agents and the potential risks associated with excessive intake, FA encapsulation could be an opportunity to diminish its degradation and improve its bioavailability. Diverse micro- and nanoencapsulation systems have been recently reported for folates fortification based on organic encapsulation supports (Aceituno-Medina, Mendoza, Lagaron, & López-Rubio, 2015; Bakhshi, Nangrejo, Stride, & Edirisinghe, 2013; Madziva, Kailasapathy, & Phillips, 2006; Shrestha, Arcot, & Yuliani, 2012; Tomiuk, Liu, Green, King, Finglas, & Kitts, 2012). Some proposed systems have improved the stability of this vitamin in different food matrices and food processing. However, these systems present some instability during processing or ingestion processes, a poor ability to control the FA rate release or provide targeted delivery (Pérez-Esteve, Ruiz-Rico, Martínez-Máñez, & Barat., 2015b). As an alternative, inorganic encapsulation systems, such as mesoporous silica particles (MSPs), can be useful in food fortification thanks to large loading capacity, biocompatibility, stability during digestion conditions and controlled release capability (Aznar, Oroval, Pascual, Murguía, Martínez-Máñez, & Sancenón, 2016, Li, Barnes, Bosoy, Stoddart, & Zink, 2012; Pérez-Esteve et al., 2016; Slowing, Vivero-Escoto, Wu, & Lin, 2008; Song & Yang, 2015). Despite some toxicological limitations need to be overcome before starting to use MSP as smart delivery systems in food industry, several studies have assessed the impact of MSPs. Diverse and contradictory results have been observed in some cells or animals treated with MSPs, but the use of functionalized mesoporous silica microparticles seems a good

strategy to minimize the risks associated with using MSPs as supports to develop smart delivery systems (Pérez-Esteve *et al.*, 2015b).

In this scenario, we previously reported the design and synthesis of a smart FA delivery system capable of controlling and modifying FA release in different digestion steps (Pérez-Esteve *et al.*, 2015a). As a step forward, the present work aimed to evaluate the protective effect of MSPs in relation to FA stability under real food industry conditions. To accomplish this goal, the bioaccessibility and stability of FA encapsulated into a MCM-41 silica support functionalized with amines that acted as molecular gates was investigated after its incorporation into fruit juices. Apple and orange juices were selected as model food systems for their low pH, which should hinder the delivery of the vitamin, and because different amounts of protective active ingredients were present, such as ascorbic acid. In order to establish the influence of MSPs encapsulation, the stability of free and entrapped FA against processing and storage agents, such as high temperature, light and juice composition, was investigated. As far as we know, this is the first study dealing with the protective effect of MSPs on the stability of biomolecules in real food systems.

2. Materials and methods

2.1.Chemicals

Tetraethylorthosilicate (TEOS), *N*-cetyltrimethylammonium bromide (CTABr), sodium hydroxide (NaOH), triethanolamine ($TEAH_3$), *N*-(3-trimethoxysilylpropyl)diethylenetriamine (N3) and phosphoric acid were provided by Sigma-Aldrich (Madrid, Spain). FA was purchased from Schircks Laboratories (Jona, Switzerland). Acetonitrile HPLC grade was provided by Scharlab (Barcelona, Spain).

Two fruit juice types (apple and orange) were purchased from local supermarkets. The composition of these juices is shown in Table 1. HPLC analysis revealed a concentration of folic acid below the limit of quantification in both juices. Juices were stored at 4 ºC until analyzed.

Table 1. Main nutrients and pH values from apple and orange juices.

	Apple juice	**Orange juice**
Carbohydrates (g/100 mL)	11.3	9.9
Proteins (g/100 mL)	0.1	0.7
Fats (g/100 mL)	0.1	0.1
Vitamin C (mg/100 mL)	-	40
pH	3.53	3.64

2.2.*Synthesis of encapsulated folic acid (**E-FA**)*

Synthesis of microparticulated MCM-41 was carried out using CTABr as the structure-directing agent and TEOS as the silica source, and a molar ratio fixed at 7 $TEAH_3$: 2 TEOS:0.52 CTABr:0.5 NaOH:180 H_2O. CTABr was added to a $TEAH_3$ and NaOH solution that contained TEOS at 118 ºC. Then water was slowly added, with vigorous stirring at 70 ºC. A white suspension was formed after a few minutes of stirring. This mixture was aged in an autoclave at 100 °C for 24 h. The resulting powder was collected by filtration. Then it was washed with distilled water and ethanol and dried at 70 °C. The as-synthesized solid was calcined at 550 °C for 5 h to remove the template phase (Bernardos *et al.*, 2008).

FA was loaded in the calcined MCM-41 microparticles by the impregnation method described by Pérez-Esteve *et al.* (2015a). FA (10 mg/mL) dissolved in phosphate-buffered saline (PBS) was added to 300 mg of MCM-41 in three addition cycles (1.5 mL per cycle). After each addition cycle, the solid was dried at 37 ºC to remove water content. After loading and drying, the solid was functionalized with 1.29 mL of N3 in acetate buffer at pH 2. The final mixture was stirred for 5.5 h at room temperature, isolated by vacuum filtration, washed with 300 mL of acetate buffer at pH 2, and dried at room temperature for 24 h.

2.3.Characterization of supports

Powder X-ray diffraction (PXRD), transmission electron microscopy (TEM), N_2 adsorption-desorption isotherms and zeta potentials were used to characterize the synthesized materials. PXRD was performed in a BrukerD8 Advance diffractometer using CuKα radiation (Bruker, Coventry, UK). For the TEM analysis, particles were dispersed in dichloromethane and deposited onto copper grids coated with a carbon film (Aname SL, Madrid, Spain). Imaging of the MSPs samples was performed with a JEOL JEM-1010 (JEOL Europe SAS, Croissy-sur-Seine, France) at an acceleration voltage of 80 kV. Single-particle size was estimated by averaging the measured size values of 50 particles. The N_2 adsorption-desorption isotherms were recorded with a Micrometrics ASAP2010 automated sorption analyzer (Micromeritics Instrument Corporation, Norcross, USA). Samples were degassed at 90 ºC in vacuum overnight. Specific surface areas were calculated from the adsorption data within the low pressure range by the BET model. Pore size was determined following the BJH method. Zeta potential measurements were taken by a Zetasizer Nano ZS (Malvern Instruments, U.K.). Samples were dispersed in water at a concentration of 1 mg/mL. The zeta potential was

calculated from the particle mobility values by applying the Smoluchowski model. Measurements were taken at 25 ºC in triplicate.

2.4.Release and bioaccessibility studies

The release kinetics of **E-FA** in fruit juices was performed to assess the capability of polyamines to hinder vitamin release at the juice's natural pH (pH ca. 3.5), and to evaluate if juice composition had any influence on vitamin delivery when the gate was open (pH 7.5). Moreover, the release studies allowed us to calculate the maximum FA release from 1 mg of support in both juices. In a typical experiment, 10 mg of the solid **E-FA** were placed in 25 mL of the corresponding juice. The same procedure was carried out with the juices neutralized with NaOH 5 M to simulate the pH after reaching the small intestine, where folic acid should be delivered by its absorption and body assimilation. At certain time points (0, 2, 5, 15, 30, 60, 120, 180 and 240 min), aliquots were separated, centrifuged, filtered, and the solution was analyzed by HPLC to establish the amount of released FA.

FA bioaccessibility from fruit juices was determined by simulating human digestion according to Versantvoort, Oomen, Van de Kamp, Rompelberg, & Sips (2005). For these assays, 4 mg of **E-FA,** or the equivalent amount of free FA (**F-FA**, 0.3 mg), were suspended in 10 mL of apple or orange juice. The *in vitro* digestion procedure started by adding 6 mL of simulated saliva and the incubation of the mixture for 5 min at 37 °C. Then 12 mL of gastric juice were added and the sample was maintained with stirring at 37 ºC for 2 h. Lastly, 12 mL of duodenal juice, 6 mL of bile and 2 mL of bicarbonate solution (1M) were added simultaneously, and the mixture was incubated for 2 h. At certain time points (0, 2, 5, 10, 60, 115, 120, 135, 150, 180 and 240 min), aliquots were taken and centrifuged. Supernatants were filtered and analyzed by HPLC to determine

FA concentrations. All the chemicals for the digestive fluids were provided by Sigma-Aldrich (Madrid, Spain).

2.5.Stability assays

Stability experiments were run to establish the influence of diverse parameters, such as temperature, light and food composition on free and entrapped FA stability during the shelf life of juices.

In a typical experiment, 4 mg of the entrapped vitamin (**E-FA**), or the equivalent amount of free FA (**F-FA**, 0.3 mg), were dissolved in 10 mL of fruit juice. For all the stability assays (thermostability, photostability and shelf life experiments) the free folic acid was previously incubated (37 °C, 96 h) in order to simulate the loading and functionalization process of the entrapped vitamin.

In order to determine the thermostability of free and entrapped vitamin, **E-FA** and **F-FA** were added to the fruit juices' insight opaque containers (Ø 24 mm, h 45 mm) and subjected to thermal treatment at 121°C and 1 bar at different time points (5, 10 and 15 min). After treatment, samples were cooled in an ice bath and FA was released from the MCM-41 voids before quantification. For the photostability experiments two light sources, visible (intensity ca. 8 mW/cm^2) and ultraviolet (intensity ca.4 mW/cm^2) lamps, were used. **E-FA** and **F-FA** were incorporated into the fruit juices and kept inside closed transparent borosilicate glass vessels (Ø 24 mm, h 45 mm). Samples were placed under visible and ultraviolet (UV) lamps for different times (16, 18, 20, 22 and 24 h) to simulate an indirect light-induced exposure of the food products. Shelf life experiments were carried out to study the influence of juice composition on vitamin stability during the storage period. Free (**F-A**) and encapsulated (**E-FA**) FA were added to the corresponding juice inside the opaque containers and samples were maintained

refrigerated at 4 ºC for 28 days. Samples were analyzed by HPLC on certain days (0, 7, 14, 21 and 28 days). Before the analysis in all cases, samples were adjusted to neutral pH and stirred at 37 ºC for 2 h to solubilize (**F-FA-**containing samples) or release (**E-FA-**containing samples) the FA vitamin before the HPLC analysis. All the stability experiments were performed in triplicate. The control solutions, with the same amount of entrapped and free FA, at pH 7.5 with no treatment, were also studied for comparison purposes. Vitamin recoveries after treatments were calculated as the ratio between FA content in treated and control juices (non-exposed to light, heat or acid media).

2.6.Folic acid quantification

FA was determined by reversed-phase HPLC according to the method described by Johansson, Jastrebova, Grahn, & Jägerstad (2005) with some modifications. The HPLC instrument consisted of a Hitachi LaChrom Elite liquid chromatograph (Hitachi Ltd., Tokyo, Japan) equipped with an auto-sampler (module L-2200) and UV detector (model L-2400). A Kromaphase 100 C18 (250 mm x 4.6 mm i.d., 5-µm particle size analytical column) (Scharlab, Barcelona, Spain) was used for separations. The wavelength of the UV detector was set at 280 nm. The mobile phase consisted of (A) 30 mM potassium phosphate buffer at pH 2.3 and (B) acetonitrile. The gradient program was isocratic for 10 min with 90% A and 10% B. FA was quantified according to the external standard method (since no matrix effect was observed) using a calibration curve of the peak area against the compound concentration. For all samples, the applicability of this method was evaluated by performing a recovery study. For this purpose, juice samples were fortified with FA at three different concentration levels. In all cases, recovery values, which were estimated from measured versus added amounts of FA, were close to 100%.

Thus, the results obtained demonstrated the applicability of the proposed methodology for the accurate determination of FA in juice samples.

2.7.Data analysis

Data were statistically processing using Statgraphics Centurion XVI (Statpoint Technologies, Inc., Warrenton, VA, USA). The influence of different factors on the release and stability of the vitamin was analyzed by a one-way analysis of variance (One-way ANOVA). The LSD (least significant difference) procedure was used to test the differences between means at the 5% significance level.

3. Results and discussion

3.1.Synthesis and material characterization

FA was entrapped in MCM-41 microparticles functionalized with diethylenetriamine moieties (solid **E-FA**), which acts as capping systems to control payload delivery at a neutral pH, but was able to hinder the delivery of the cargo at an acidic pH. This pH-responsive capping system has already been reported by some of us (see Bernardos *et al.*, 2008; Casacús *et al.*, 2008; Pérez-Esteve *et al.*, 2015a).

The final **E-FA** support was characterized by standard techniques. The X-ray patterns of MCM-41 solids as synthesized (a), calcined (b) and loaded with FA and functionalized with polyamines (c) are shown in Supplementary Figure S1. The PXRD of MCM-41 as synthesized (curve a) shows the expected four peaks of a hexagonal-ordered array indexed as (100), (110), (200) and (210) Bragg reflections. After calcination (curve b), a significant shift of the (100) reflection was clearly observed, which relates to cell contraction by the condensation of silanols in the calcination step. The loading and

functionalization (curve c) produced the loss of reflections (110), (200) and (210) However, the fact that a (100) peak appeared in the PXRD patterns in all the solids clearly indicates that the pore loading and functionalization process did not modify mesoporous MCM-41 scaffolding to a large extent. In addition, FESEM and TEM analysis were used to characterize the shape and size of the solids and the particle mesostructure (Supplementary Figure S2). The MCM-41-based particles were irregular in shape, while the size of particles was 853±67 and 862±59 nm for bare calcined MCM-41 and **E-FA**, respectively.

The N_2 adsorption-desorption isotherms of the MCM-41 calcined material and **E-FA** are shown in Supplementary Figure S3. The calcined MCM-41 material showed a type IV isotherm, which is typical of mesoporous supports. In contrast, **E-FA** displayed the characteristic curves of supports with filled mesopores. The calculated pore size (2.98 nm), pore volume (0.467 cm^3/g) and specific surface area (1075.36 m^2/g) of the starting MCM-41 material clearly reduced in **E-FA** (pore volume 0.058 cm^3/g and specific surface area 124.21 m^2/g) due to cargo loading and external functionalization.

The functionalization process was also verified by zeta potential determinations of the bare MCM-41 and MCM-41 loaded with FA and amine-functionalized (**E-FA**). The starting particles showed a zeta potential of -23 mV, which changed to +54 mV for **E-FA.** This is in agreement with the presence of amines in the final solid.

3.2.Juice fortification with entrapped FA

New strategies for food fortification are based on FA encapsulation in order to improve both the stability and bioavailability of the added vitamin in food products. Some FA encapsulation strategies based on food-grade nanoencapsulation systems based on chitosan nanoparticles (de Britto, de Moura, Aouada, Mattoso, & Assis, 2012), alginate

nanoparticles (Bakhshi *et al.*, 2013) or amaranth protein-based electrospun fibers (Aceituno-Medina *et al.*, 2015) have been described in recent years. Moreover, Pérez-Esteve *et al.* (2015a) developed a new FA delivery system based on MSPs capped with amines to assess the modulation of vitamin release and bioaccessibility in *in vitro* assays. Herein the latter pH-responsive delivery support was incorporated into apple and orange juice to study its release behavior in real food systems. Fruit juices were chosen because they are liquid foods commonly used as vehicles for FA fortification (Sherstha *et al.*, 2012). The reported amounts of endogenous folates are 0.3 μg/100 g and 25 μg/100 g for apple and orange juice, respectively (Vahteristo *et al.*, 2002). More importantly, orange juice is rich in ascorbic acid, while apple juice only contains a small amount of this vitamin (Gardner, White, McPhail, & Duthie, 2000). Besides its nutritional importance, ascorbic acid is considered significant because its content guarantees the presence of other nutrients, and it is usually added to process orange juice in recommended values of around 40 mg/100 mL (Esteve, Frígola, Rodrigo, & Rodrigo, 2005).

3.2.1. FA release profile in juices

Release studies were carried out to confirm the ability of the amine-gated **E-FA** support to control vitamin release according to the pH of food. The pH-dependent releases of the encapsulated FA in the apple and orange juices are shown in Figure 1. Delivery profiles exhibited the typical progression, which has already been found in the work of Pérez-Esteve *et al.* (2015a) in PBS at pH 2 and pH 7.5. As this figure depicts, delivery from **E-FA** was largely inhibited at the typical pH of fruit juices (pH 3.5) and only 10% FA release values were detected with time. This allowed us to confirm that vitamin

delivery from **E-FA** was hindered by the combination of low FA solubility at a low pH and the gating effect of the polyamines anchored to the surface of MSPs due to electrostatic repulsions, and also to the interaction between the polyammonium moieties and anionic species present in juices. Polyamines are transformed into polyammonium groups at an acidic pH by adopting a rigid-like conformation due to Coulombic repulsions, and are able to interact with anions via electrostatic forces, which result in the capping of pores and cargo release inhibition (Bernardos *et al.*, 2008). Conversely, sustained FA release with time was observed in neutralized fruit juices (pH 7.5.). At a neutral pH, delivery took place because FA solubility increased and polyamines were less protonated, which reduced Coulombic repulsion and affinity for anions. The delivery profile of the entrapped vitamin from **E-FA** in fruit juices at a neutral pH was similar to that obtained in a buffer solution (Pérez-Esteve *et al.*, 2015a). So it can be concluded that the matrix composition of fruit juices had no significant influence on the delivery functionality of the gated support. Maximum vitamin release at a neutral pH was achieved in less than 2 h with an average value of 83.9±6.2 mg FA/g (a similar delivered amount was found for both juices). This was much higher than for other previously described FA encapsulation systems, such as that obtained by Madziva *et al.* (2006), which allowed maximum FA encapsulation of 3.6 μg/g in alginate-pectin capsules. The maximum release amount of FA was used to calculate the equivalent amount of entrapped and free FA needed in the stability assays (*vide infra*).

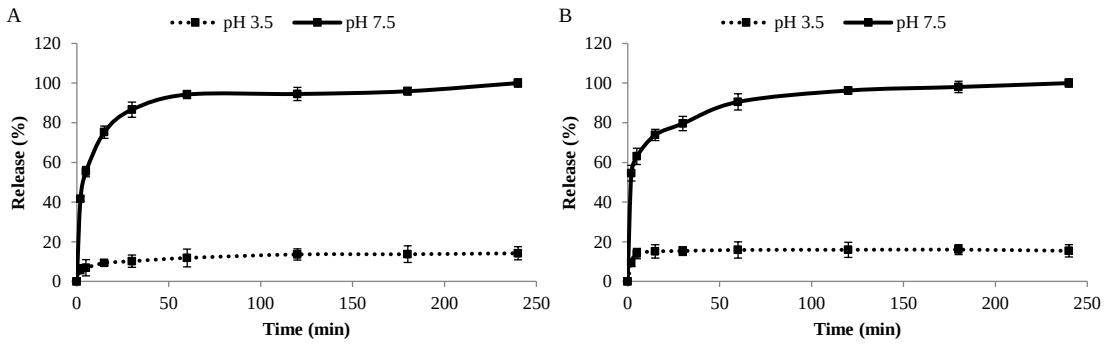

Figure 1. Release profiles of the vitamin from the pores of **E-FA** in apple (A) and orange juice (B) at pH 3.5 (dotted lines) and pH 7.5 (solid lines).

3.2.2. FA bioaccessibility during the in vitro digestion of juices

The pH-responsive delivery of FA from the amine-gated MSPs (solid **E-FA**) suggested that it is suitable for vitamin release in the gastrointestinal tract (closed gates in the stomach and opened gates in the intestine). Therefore, bioaccessibility of the vitamin during the simulated digestion of fruit juices was investigated. Figure 2 shows the behavior of entrapped (**E-FA**) and free (**F-FA**) FA in different *in vitro* digestion stages. As we can see, a small amount of FA was detected in the juices that contained free or entrapped FA in the buccal and stomach stages (2 h). The oral phase was too short to solubilize and/or release the vitamin, and the low pH of the gastric phase inhibited FA release and solubilization. In contrast, **E-FA** and **F-FA** displayed different behaviors in the intestinal stage. The bioaccessibility of free FA rapidly increased after adding intestinal juices. With apple juice, free FA recovery did not reach 100% of the vitamin added because of degradation after exposure to acidic pH according to the shelf life stability results (*vide infra*). Conversely, sustained vitamin delivery from **E-FA** was observed in the digestion phase for both juices. In apple juice, progressive FA release after adding intestinal juices was detected and the maximum release took place at ca.

240 min. With orange juice, FA release was faster and a 100% release was observed after ca. 180 min of digestion. This suggests that apple juice better controls vitamin release than orange juice, and the differences in delivery rates were most likely due to differences in the food composition (Table 1). Overall these studies demonstrated that **E-FA** can protect FA in early digestion stages, but progressive FA release in the intestine can occur (where it is absorbed). This could allow the metabolic activation of the entire vitamin and could prevent the potential risks associated with presence of unmetabolized FA in the bloodstream.

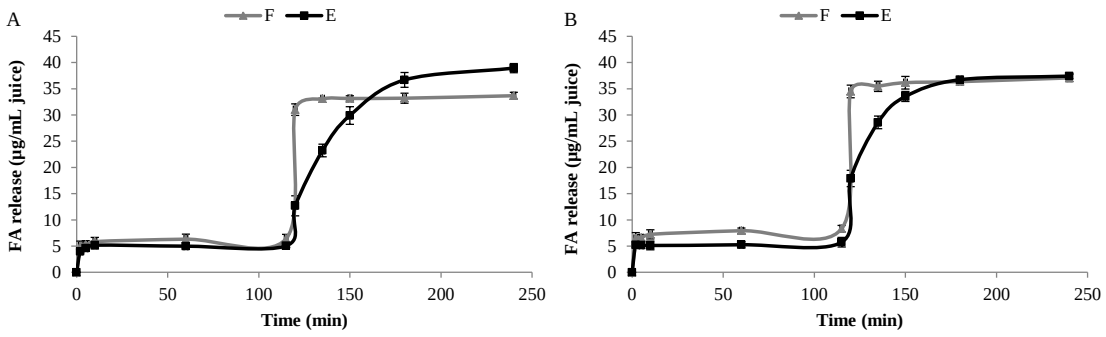

Figure 2. Bioaccessibility of **F-FA** (grey lines) and **E-FA** (black lines) during an *in vitro* digestion procedure in apple (A) and orange (B) juice.

3.3.Stability assays

Besides folates encapsulation and the controlled release from the **E-FA** support, some studies have reported the ability of encapsulation systems to enhance the stability of entrapped vitamers after food production and storage. Based on this idea, the stability of entrapped (**E-FA**) and free (**F-FA**) FA incorporated into fruit juices after simulating food production and storage processes was further studied.

3.3.1. Temperature stability

Heat treatment is a key step in food processing to ensure microbiological food safety in different food production and storage stages. The impact of high temperature on the stability of encapsulated (**E-FA**) and non-encapsulated (**F-FA**) FA was studied by simulating the sterilization conditions (121 °C, 1 bar) at different times. Figure 3 shows the recoveries of FA (FA present in the solution) from **E-FA** and **F-FA** in the apple (A) and orange (B) juices after heat treatment. With apple juice, entrapped FA showed significantly better stability than the free vitamin at the different exposure times ($p < 0.05$). Heat treatment led to major free FA loss of between 34% and 42% due to the high temperature and acidic pH combination. According to the literature, FA is considered stable at 100 ºC for several hours within the 5.0–12.0 pH range, but becomes increasingly unstable as pH goes below 5.0 (Ball, 2005). In contrast, when **E-FA** and **F-FA** were added to orange juice, excellent FA recovery was observed at different times. Typical FA recovery values of 81-91% and 81-85% for the encapsulated (**E-FA**) and non-encapsulated (**F-FA**) vitamin were respectively observed (Fig. 3B). This improved FA stability in orange juice compared with apple juice is most likely related to presence of ascorbic acid in citrus juice, which has been reported to strongly protect folates against pressure and heat treatments (Arcot, Shrestha, & Gusanov, 2002; Butz *et al.*, 2004; Liu, Green, Wong, & Kitts, 2012).

These results demonstrated that encapsulation in MSPs enhanced FA stability. A simulated sterilization process has revealed the protective effect of MSPs on the vitamin's thermostability, mainly for the apple juice samples in which ascorbic acid content is negligible (Fig. 3A). Other encapsulation systems have obtained similar results in previous studies after different food processing treatments. The processing of

bread flour fortified with folates has resulted in a 20-30% FA loss (Johansson, Witthöft, Bruce, & Jägerstad, 2002; Gujska & Majewska, 2005). However, the microencapsulation of folates significantly improved their stability during the course of bread making, while toasting obtained similar recoveries to those obtained when sodium ascorbate was added. Furthermore, co-encapsulating the reducing agent with folates provided better vitamin recovery after baking and greater stability during storage than the free compound (Liu *et al.*, 2012; Tomiuk *et al.*, 2012). Shrestha *et al.* (2012) encapsulated 5-methyltetrahydrofolic acid by spray-drying and exposed microcapsules to extrusion (100-150 ºC) to obtain enhanced folate stability (84–94.5% retention) compared to the free form (65.3–83.2%) in all the extruded products.

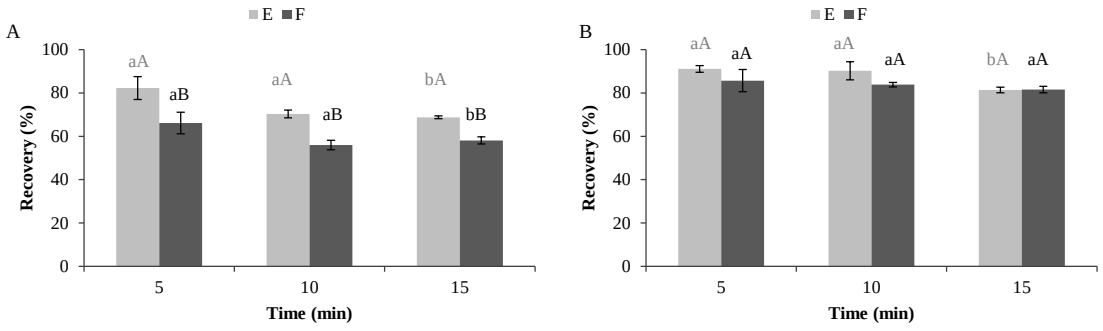

Figure 3. Influence of temperature exposure on the stability of encapsulated (**E-FA**) and free (**F-FA**) vitamin incorporated to apple (A) and orange (B) juices. Different letters in the bars indicate statistically significant differences ($p<0.05$) from levels of time exposure (small letters) and differences between encapsulated or free FA (capital letters). Values are Means ± SD, n = 3.

3.3.2. Light stability

Folic acid is photosensitive to visible or ultraviolet light, which results in the cleavage of the bond between C9-N10, and then the excision of the p-teridin moiety from p-

aminobenzoylglutamate (Akhtar, Khan, & Ahmad, 2003). In this section, free (**F-FA**) and encapsulated (**E-FA**) FA were added to apple and orange juices, and were exposed to visible and UV light. Recoveries were compared with those of the non-irradiated samples. The preliminary experiments revealed no or very little degradation after 14 h of treatment (data not shown), so assays were ran from 16 h to 24 h. Figure 4 displays the different degradation profiles of the vitamin after exposure to visible light in apple juice (A) and orange juice (B). FA, when added as **F-FA,** progressively declined over time and with a maximum degradation of ca. 76% after 24 h for the apple juice samples. The FA from **E-FA** underwent a similar degradation rate to **F-FA**, but recoveries were significantly greater in all the samples, which confirmed the protective effect of the encapsulation system. In contrast, neither the free nor encapsulated FA included in the orange juice showed degradation during visible light exposure. The high antioxidant (ascorbic acid) content in this food system was most likely responsible for the high FA stability against oxidative photodegradation (Ball, 2005).

The stability behavior of the FA from **E-FA** and **F-FA** was similar after ultraviolet light exposure. With apple juice (Fig. 4C), the FA concentration decreased over time and non-significant differences were found between encapsulated and non-encapsulated FA, which obtained only 44% and 36% of the FA originally contained in the samples, respectively. In orange juice, the vitamin displayed better stability behavior (Fig. 4D). Despite the fact that the FA included in orange juice displayed slight degradation during the exposure time, after 24 h of irradiation more than 73% and 83% of the vitamin had been recovered for **E-FA** and **F-FA**, respectively. Nevertheless, the protective action of the mesoporous silica support against photodegradation was evidenced when ascorbic acid was absent. The mechanism of action was not clear, but enhanced stability could be

a result of confining the vitamin in the mesopores of the silica support, which hindered the oxidation of the C9-N10 bond (Song & Yang, 2015).

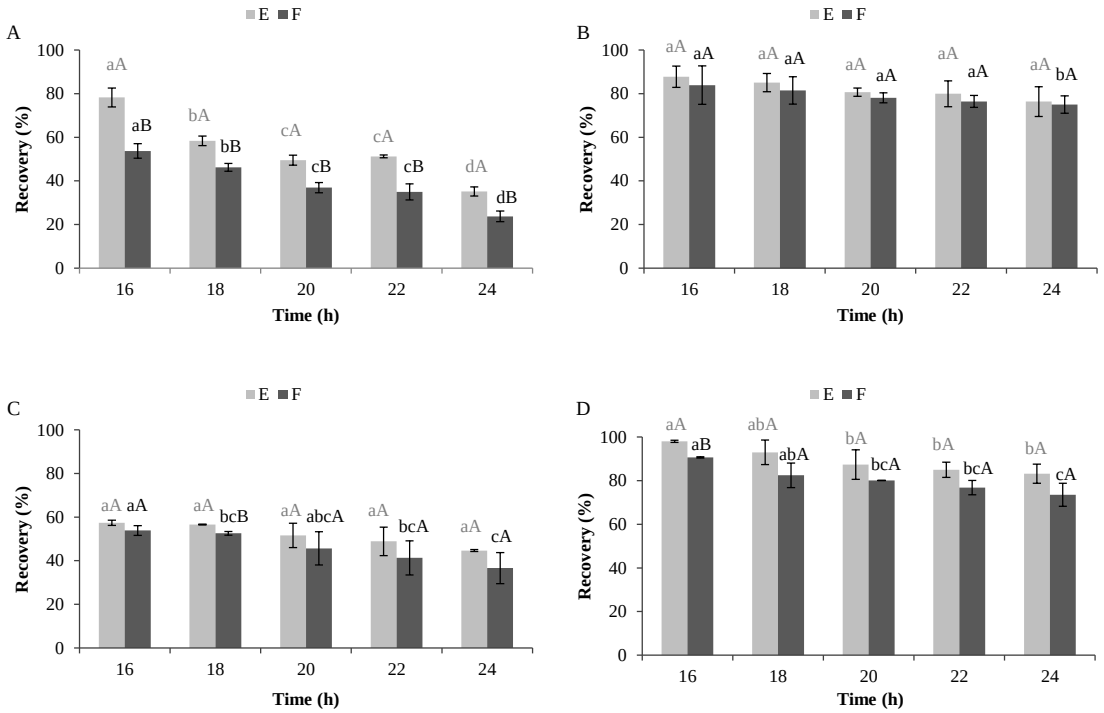

Figure 4. Influence of visible (A, B) and ultraviolet (C, D) light exposure on the stability of encapsulated (**E-FA**) and free (**F-FA**) vitamin incorporated to apple (left) and orange (right) juices. Different letters in the bars indicate statistically significant differences ($p<0.05$) from levels of time exposure (small letters) and differences between encapsulated or free FA (capital letters). Values are Means ± SD, n = 3.

3.3.3. Composition influence throughout shelf life

Another factor that could affect FA stability was the composition of foodstuffs during the storage period. The shelf life of fruit juices has been established to be around 1 month when considering them to be refrigerated pasteurized juices (Öhrvik *et al.*, 2008). Figure 5 presents the stability of FA when encapsulated (**E-FA**) and non-encapsulated

(**F-FA**) in apple (A) and orange juice (B) throughout storage time (0, 7, 14, 21 and 28 days). FA added in the **F-FA** form brought about a significant decrease in the recoveries of both juices, but the degradation profile differed according to juice type. With apple juice, the free vitamin was quickly degraded with an obtained recovery of 90% at day 0 according to previous studies due to oxidative degradation (De Brouwer *et al.*, 2007). Moreover, FA recovery diminished to values of 77% after 28 days of refrigerated storage. However, **F-FA** added to orange juice was almost fully detectable with a 98% recovery on day 0. After the storage period (28 days), a slight decrease was observed with final FA recovery values of ca. 80%. These results are in accordance with previous studies, which have demonstrated a significant decrease in FA concentration (46%) during a 12-month period in fortified fruit juices, which indicates FA instability in an aqueous acidic matrix (Yakubu & Muazu, 2010; Frommherz *et al.*, 2014). A higher degradation rate was obtained in month one due to a reaction with oxygen, which was eventually exhausted (Frommherz *et al.*, 2014). Some other studies have considered orange juice a proper matrix to contain FA due to high ascorbic acid content (De Brouwer, Zhang, Storozhenko, Van Der Straeten, & Lambert, 2007; Öhrvik *et al.*, 2008), which can also be potentially lost during storage (Esteve *et al.*, 2005). Kabasakalis, Siopidou, & Moshatou (2000) reported ascorbic acid degradation in commercial fruit juices and showed complete antioxidant loss when containers were opened for consumption and stored refrigerated for 31 days. Therefore, alternatives to the presence of ascorbic acid are needed to maintain FA content in fruit juices. As depicted in Figure 5, FA recoveries reached values of ca. 100% in all cases when **E-FA** was used, which indicates the good stability of the encapsulated vitamin in the acidic environment of these fruit juices. These results confirmed the suitability of MSPs as a

support for FA encapsulation and protection in fortified juices in the same way as for other reported encapsulation systems (Madziva *et al.*, 2006).

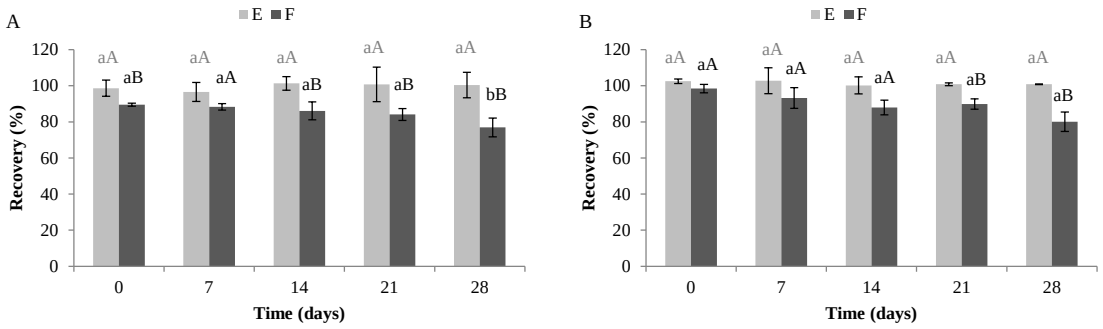

Figure 5. Stability of encapsulated (**E-FA**) and free (**F-FA**) vitamin incorporated to apple (A) and orange (B) juices during the shelf life period. Different letters in the bars indicate statistically significant differences ($p<0.05$) from levels of time exposure (small letters) and differences between the encapsulated or free FA (capital letters). Values are Means ± SD, n = 3.

4. Conclusions

A smart delivery system of FA based on amine-gated MSPs has been successfully applied to improve both FA bioaccessibility and stability after being incorporated into fruit juices. The pH-responsive delivery effect of the functionalized support conferred the vitamin protection during food production simulation, as well as its controlled release under digestion conditions. A simulated *in vitro* digestion of fortified juices showed that entrapped FA was protected in the buccal and gastric stages, and that vitamin bioaccessibility was modified by the amine-gated MSPs in the intestinal stage, which would prevent unmetabolized synthetic FA being present in the bloodstream. Moreover, fortified juices were exposed to different environmental agents, which led to improved FA stability when the pH-responsive **E-FA** mesoporous support was used,

particularly when ascorbic acid was absent. This preservation strategy could allow a smaller amount of FA needed for food fortification and could reduce the potential risks associated with high exposure to the vitamin. The impact of different processing and storage factors on FA stability has been individually evaluated herein. Bearing in mind these findings, we suggest considering MSPs to be smart delivery systems for the encapsulation, protection and controlled release of different food-related bioactive molecules in diverse food systems.

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