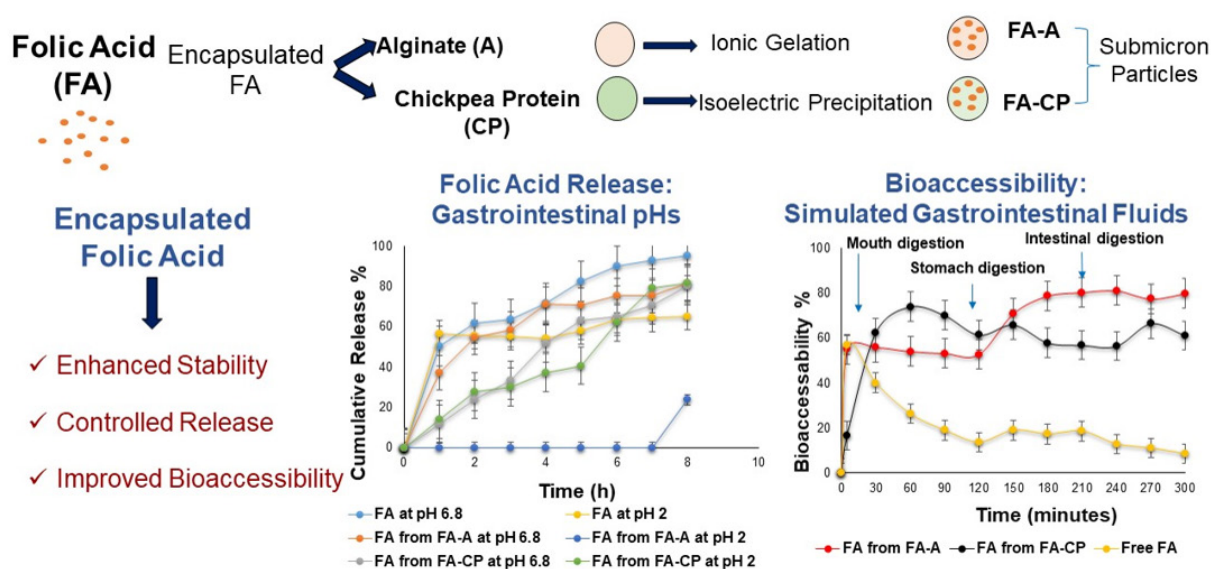


Effect of matrix composition on stability, release and bioaccessibility of encapsulated folic acid

I.F. Shakoor, G.K. Pamunuwa* and D.N. Karunaratne



Highlights

- Folic acid encapsulated alginate and chickpea protein submicron particles were prepared.
- Alginate supersedes protein in improving photo-/thermal-stability of folic acid.
- Release of folic acid follows matrix and pH-dependent kinetics.
- Enhanced bioaccessibility of folic acid is observed with the alginate submicron particles.
- Alginate promises better properties over chickpea protein as the delivery matrix.

RESEARCH ARTICLE

Effect of matrix composition on stability, release and bioaccessibility of encapsulated folic acid

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Abstract: Folic acid is a fast degradable vitamin, hence showing reducing functionality. Therefore, engineering of an efficacious vehicle for oral delivery of folic acid via encapsulation using an appropriate matrix is important. As eligible matrices, alginate and chickpea protein were compared in terms of stability, release kinetics, transport mechanism and bioaccessibility. Both matrices yielded particles of submicron-range with high encapsulation efficiency. Stability, *in vitro* release and bioaccessibility studies displayed reduced degradation, protection against gastric pH and increased bioaccessibility (80%) by alginate particles. The protein matrix also displayed slower release and higher bioaccessibility of folic acid compared to the free compound. Release of folic acid from alginate particles showed Fickian diffusion, while release from chickpea protein particles showed quasi Fickian diffusion and super case II transport mechanism at gastric and intestinal pHs, respectively. In conclusion, alginate particles were superior in affording thermal and photo stability, pH dependent release and higher bioaccessibility.

Keywords: Bioaccessibility; Chickpea protein; Folic acid; Release kinetics; Transport mechanism.

INTRODUCTION

Folic acid (FA) or pteroyl-L-glutamic acid, the synthetic form of folate otherwise known as vitamin B₉, is involved in several significant physiological biochemical processes such as nucleic acid biosynthesis and protein metabolism in the human body (Gazzali *et al.*, 2016). Presently, it is consumed either naturally or mostly as a supplement by children, adults and, especially, pregnant women to overcome the impediments of FA deficiency (Czeizel *et al.*, 2013). However, degradation of FA when exposed to dynamics such as light (UV), temperature, pH and reducing or oxidative environments which poses a challenge to FA supplementation has been examined (Gazzali *et al.*, 2016). Also, due to the unstable nature of FA in acidic media than in alkaline media, its premature degradation in gastric pH before arriving at its site of absorption is foreseeable. In fact, the tendency of FA to degrade rapidly in low pH conditions along with poor water solubility (0.01 mg mL⁻¹ at 25 °C) and stability (Gazzali *et al.*, 2016) adversely affect its bioavailability after oral administration (Jafari and

McClements, 2017). Therefore, protected, controlled and targeted delivery of FA would be beneficial for its overall functionality at its specific site of absorption (Pamunuwa *et al.*, 2020).

Nano-encapsulation is an effectual approach to upsurge the stability, efficacious delivery and bioavailability of bioactive components including vitamins and minerals that are highly sensitive, poorly soluble and primarily employed in food fortification. Therefore, researchers currently focus on engineered edible biopolymeric nanoparticles with encapsulated bioactive agents (Jafari and McClements, 2017) which facilitate site specific and slow and/or controlled release (Boostani and Jafari, 2020) compared to the free form of bioactive agents. It follows that the type of delivery vehicle utilized for encapsulation is essential to be well-matched with the bioactive agent while exhibiting a pH dependency to attain controlled release and a protective role in harsh physiological environments along the gastrointestinal tract (Kharat and McClements, 2019). In order to satisfy the aforementioned requirements, selecting the most appropriate polymeric matrix is significant (McClements, 2018) in engineering polymeric nanoparticles for the encapsulation of bioactive agents. Also, the selection of appropriate materials and strategies for the engineering of such encapsulated nano-particulate systems is critical to minimize the risks of instigating antagonistic or toxic effects. Therefore, particularly, the chosen delivery matrices should possess a limited life span and be biodegradable to avoid accumulation in the liver and spleen (Gustafson *et al.*, 2015) in addition to possessing mainly protective, and slow and/or controlled release properties.

Considering the aforesaid facts, two types of major biopolymers (a polysaccharide and a protein) which are obviously structurally, characteristically and functionally dissimilar (Joye and McClements, 2014) were utilized in order to ascertain the most suitable type of delivery vehicle for the encapsulation of FA. Owing to the characteristics such as biodegradability, biocompatibility, non-toxicity, mucoadhesive properties and cost effectiveness, the most frequently utilized anionic biopolymer, alginate

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(George and Abraham, 2006), has been chosen as the polysaccharide matrix for the preparation of bulk, macro, micro or nano hydrogel formations (Pacho *et al.*, 2019). As alginate particles are progressively engineered to achieve controlled release of encapsulated bioactive agents, alginate displays applications in many important areas such as pharmaceutical, nutraceutical as well as food fortification leading to functional foods (Črnivec and Ulrih, 2019). Interestingly, alginate has offered improved stability of the incorporated bioactive agents with more precise controlled release (Estevinho *et al.*, 2020). In fact, encapsulation of FA in alginate or in alginate in combination with other polymers has emphasized properties such as improved stability, slow and controlled release much desirable in numerous areas including food (Madziva *et al.*, 2005; Camacho *et al.*, 2019).

So far, protein matrices including soy, whey, casein and zein proteins (Ding and Yao, 2013; Peñalva *et al.*, 2015; Pérez-Masiá *et al.*, 2015) have been utilized in encapsulating FA. However, chickpea protein (CP), although known for its high protein content (Chavan *et al.*, 1987) has been utilized scarcely in delivery vehicle formulations. Inspired by the previous work of FA encapsulation in CP (Ariyaratna and Karunaratne, 2015), which has proved to be effective in achieving a controlled release while maintaining the quality of the encapsulated FA during processing, handling and storage, we compared the performance of CP with that of commonly employed alginate as a matrix for FA encapsulation. Although the aforementioned literature related to FA encapsulation in either alginate/alginate combinations or chickpea protein has reported many interesting findings, *in vitro* digestion studies which could mimic the physiological and physical conditions of *in vivo* environment have not been reported. Therefore, bioaccessibility of ingested FA which undergoes mouth, stomach and intestinal digestion processes and the influence of digestive enzymes on encapsulated FA were evaluated in this work.

In this study, a comparison is carried out between FA encapsulated alginate and CP particles on physical, morphological and chemical structural properties. Also, thermal and photo stability, release in gastrointestinal pHs, and bioaccessibility of the encapsulated FA, are compared. Much emphasis is given to *in vitro* release studies where kinetic modelling and release mechanisms are described in relation to the release media of gastrointestinal pHs, and to *in vitro* digestion studies where the overall performance of the delivery matrices is evaluated. The said studies of the submicron particles provide a superior platform to understand the suitability of the biopolymers, scarcely used CP and commonly used alginate, as matrices of FA delivery vehicles.

MATERIALS AND METHODS

Materials

FA (meets USP testing specifications), alginic acid sodium salt, dialysis tubing cellulose membrane (avg. flat width 43 mm/12,000 MWCO), alpha-amylase

(≥ 5 units mg^{-1}), pepsin (from porcine stomach mucosa, pepsin; ≥ 250 units mg^{-1}), pancreatin (from porcine pancreas; 8×USP specifications) and bile salt extract, sorbitan monooleate (Span 80) and glyoxal were purchased from Sigma Aldrich Company (St. Louis, MO, USA). Chickpea was purchased from a local supermarket and all other chemicals used were of analytical grade.

Preparation of folic acid-alginate encapsulate (FA-A)

FA-A particles were prepared using the ionic gelation method (Pamunuwa *et al.*, 2020) with slight modifications under obscure light conditions as follows. Alginate solution (0.3% w/v, pH adjusted to 5), was stirred well after adding a few drops of Span 80 followed by the addition of FA 0.01% (w/v). Next, CaCl_2 0.1% (w/v) was added dropwise under continuous stirring. Finally, the solution was refrigerated, centrifuged (Sigma 3-18KS, Germany) and freeze dried (Alpha 1-2 LDplus) to obtain FA-A particles. Plain alginate particles were prepared as the control.

Preparation of folic acid-chickpea protein encapsulate (FA-CP)

CP free flowing powder and FA-CP particles were prepared by using the alkaline extraction method and isoelectric precipitation method (Ariyaratna and Karunaratne, 2015) under obscure light conditions, respectively, with slight modifications as follows. Chickpea powder was subjected to defatting, alkaline extraction and acidification to isoelectric point (pH 4.5). Then, the mixture was centrifuged and freeze dried to obtain a free flowing powder.

Extracted CP (1% w/v) was dissolved in 1 M NaOH solution followed by the addition of 0.010 g of FA and was stirred well to obtain a homogeneous solution. Next, the solution was acidified to pH 4.5 by adding 1 M HCl, and glyoxal was added as a crosslinker. The solution was refrigerated, centrifuged and freeze dried to obtain FA-CP particles. Plain CP particles were prepared as the control.

Determination of particle size, poly-dispersity index (PDI) and zeta-potential

The average particle sizes with PDIs and zeta-potentials were determined using a Particle Size Analyser (Cilas Nano DS, France) and Zeta-Potential Analyser (Zetasizer Nano ZS, Malvern Instruments, UK), respectively. To obtain the readings, a small aliquot of FA-A particles was dispersed in distilled water, while FA-CP particles were dispersed in pH 4.5 buffer solution. Replicate measurements were taken.

Determination of encapsulation efficiency (% EE) and loading capacity (% LC)

% EE and % LC of FA-A and FA-CP particles were analysed using UV-Vis Spectrophotometer (Evolution 220/Thermo fisher Scientific, USA). The amount of FA in the supernatant was quantified after centrifugation of the particle suspensions at the λ_{max} (286 nm) of FA. The following equations 1 and 2 (Pamunuwa *et al.*, 2016) were used in the calculations.

$$\% EE = \frac{FA_{tot} - FA_{sup}}{FA_{tot}} \times 100 \quad (1)$$

where, FA_{tot} and FA_{sup} are total mass of FA in the system and mass of FA in supernatant, respectively.

$$\% LC = (\text{Mass of FA entrapped}) / (\text{Mass of particles}) \times 100 \quad (2)$$

Fourier transform infrared (FTIR) spectroscopy

The spectral data of FA, alginate, CP, and FA encapsulated particles were recorded using FTIR-ATR (Attenuated Total Reflectance) (Alpha, Bruker, Germany) technique within a scanning range from 500 to 4000 cm^{-1} of absorbance.

Scanning electron microscopy (SEM)

The characterization of FA-A and FA-CP particles via surface morphology was performed by scanning with SEM (ZEISS EVO/LS15) using an acceleration voltage of 5.0 kV after sputter coating the particles pasted on a double adhesive tape with a thin layer of gold. The images were obtained with a magnification of 50.00 K X.

Photo-stability and thermal-stability

The photo-stability study was conducted by keeping two sets of FA encapsulated particles separately in the dark and exposed (to daylight) conditions. Similarly, the thermal-stability study was carried out by keeping two covered sets of FA encapsulated particles separately at room temperature (RT) and in the refrigerator at 4 °C. Free FA was used as the control. The experiment was continued for 3 months and a small amount from each encapsulate was tested for FA with one month intervals for three-month duration. Methanol and NaOH were used to breakdown the FA-A and FA-CP particles, respectively. The supernatants of centrifuged sample aliquots were quantified using UV-Vis spectrophotometer.

In vitro release study

In vitro release studies of FA, FA-A and FA-CP particles were carried out in simulated gastro-intestinal solutions (Ariyaratna and Karunaratne, 2016) with slight modifications under obscure light conditions as follows. Buffer solutions of simulated gastric juice of pH 2 and simulated intestinal fluid of pH 6.8 were prepared without enzymes as mentioned in previous studies (Stippler *et al.*, 2004; Corcoran *et al.*, 2007).

A specified amount of FA powder/FA-A/FA-CP particles were suspended in 10 mL of pH buffer solution and the solution was inserted in a dialysis membrane, which was then dipped in 100 mL of the same buffer solution. Then, while stirring, an aliquot was pipetted from the release medium at one hour intervals (up to 8 h) and the amount of released FA was quantified by using a UV spectrophotometer at the respective maximum wavelengths (λ_{max}) of FA obtained at pH 2 and pH 6.8. The aliquots taken out at each trial was replaced with a fresh buffer solution. Always, the temperature of the solution was maintained at 37 °C \pm 2 °C with a stirring speed of 400 rpm. The experiment was conducted in triplicate.

Cumulative release percentage (% cumulative release) was calculated by using the following equation 3 (Ariyaratna and Karunaratne, 2015);

$$\% \text{ Release} = \frac{\text{Amount of FA in release medium}}{\text{Initial amount of FA in the encapsulated system}} \times 100 \quad (3)$$

Finally, the release data were fitted in to seven drug release kinetic models (Dash *et al.*, 2010) as shown in the following equations (4-10). The model with the highest adjusted R-square value was selected as the model of best fit.

$$\text{Zero Order: } Q_t = Q_0 + K_0 t \quad (4)$$

Where, Q_t - Amount of FA dissolved in time t

Q_0 - Initial amount of FA in the solution

K_0 - Zero order release constant

$$\text{First Order: } \log C = \log C_0 - Kt/2.303 \quad (5)$$

Where, C_0 - Initial concentration of FA

C - Concentration of FA at time t

K - First order rate constant

$$\text{Higuchi: } Q_t = K_H \times t^{1/2} \quad (6)$$

Where, Q_t - Amount of FA released in time t

K_H - Higuchi dissolution constant

$$\text{Hixson-Crowell: } M_0^{1/3} - M_t^{1/3} = \kappa_{HC} t \quad (7)$$

Where, M_0 - Initial amount of FA in the matrix

M_t - Remaining amount of FA in the matrix at time t

κ_{HC} - Hixson-Crowell constant

$$\text{Baker-Lonsdale: } f = \frac{3}{2} \left[1 - \left(\frac{M_t}{M_\alpha} \right)^{2/3} \right] - \frac{M_t}{M_\alpha} = kt \quad (8)$$

Where, M_t/M_α - Fraction of FA released at time t

k - Release constant

$$\text{Weibull: } M = M_0 \left[1 - e^{\left\{ -\frac{(t-T)^b}{a} \right\}} \right] \quad (9)$$

Where, M - Amount of FA dissolved as a function of time t

M_0 - Total amount of FA being released

T - Lag time

b - Shape parameter

a - Scale parameter

$$\text{Gompertz: } X(t) = X_{max} X e^{\{-\alpha e^{\beta \log t}\}} \quad (10)$$

Where, $X(t)$ - Percentage of FA dissolved at time t

X_{max} - Maximum dissolution

α - Undissolved proportion at time t (location/scale parameter)

β - Dissolution rate per unit of time (shape parameter)

In order to evaluate the release transport mechanism of FA from the FA-A and FA-CP particles, Korsmeyer-Peppas equation 11 (power law) (Korsmeyer *et al.*, 1983) was used. The first 60% of FA release data were fitted into the model and $M_t/M_\infty \leq 0.6$ versus $\log t$ linear plots were obtained. Here, the diffusional exponent (n value) which is indicative of the transport mechanism is given by the slope of the graph obtained. Hence, n value was used to characterize different drug release mechanisms (Table 1) for particles of spherical shape (Peppas and Sahlin, 1989).

$$\frac{M_t}{M_\infty} = K t^n \quad (11)$$

where,

M_t/M_∞ - FA release at time t as a fraction

t - Release time

K- Kinetic constant characteristic of the FA/polymer system

n- Diffusional exponent

Linearized power law (equation 12)

$$\log \frac{M_t}{M_\infty} = \log K + n \log t \quad (12)$$

***In vitro* digestion study**

In vitro digestion study was conducted in simulating saliva, gastric juice, and intestinal fluids to evaluate the bioaccessibility of encapsulated FA according to previous studies (Elless *et al.*, 2000; Tan *et al.*, 2000). *In vitro* digestion (Gawlik-Dziki *et al.*, 2009) of FA-A and FA-CP particles were carried out with slight modifications as follows. Meanwhile, *in vitro* digestion of FA powder was conducted as a control experiment. All the steps for each particle system were carried out in physiological temperature (37 ± 2 °C) with a shaking speed of 400 rpm.

Mouth digestion (pH 6.75): Encapsulated particles were shaken for 5 minutes in 30 mL of simulated salivary fluid. Stomach digestion (pH 1.2): The pH of the solution was brought up to pH 1.2 by the addition of 5 M HCl. Next, 30 mL of simulated gastric juice was added, and the mixture was shaken for 2 h. Intestinal digestion (pH 6-8): 30 mL of the bile extract and pancreatin mixture (in 0.1 M of NaHCO_3 ; pH 6) was added to the previous solution and the pH was adjusted to 7 by the addition of 1 M NaOH

followed by the addition of 0.12 M NaCl and 0.005 M KCl (5 mL each) (Gawlik-Dziki *et al.*, 2009). The solution mixture was shaken for 3 h. Small aliquots pipetted out at 5 minutes first and then at 30 minute intervals were centrifuged and FA in supernatants was quantified using UV absorbance measurements. The aliquots taken out each time were put back in the solution. The experiment was conducted in triplicate.

Statistical analysis

All data are presented as mean \pm standard deviation (SD) of parallel experiments. Microsoft Office Excel 2010 was used to produce FTIR graphs, release graphs and stability study graphs. Origin Pro 9.1 software was used to model fit the release data. Release kinetic model fitting and stability studies were completed by using Minitab 16 software (ANOVA) with a significance value of $\alpha = 0.05$.

RESULTS AND DISCUSSION

Particle size, PDI and zeta-potential of submicron particles

The median diameters of FA-A and FA-CP particles were 405.0 nm and 214.4 nm, respectively. The diameter of FA-A particles was much larger and almost twice that of FA-CP particles and these sizes represent the hydrodynamic diameters. A recent study by Ariyaratna and Karunaratne (2015), reported the particle size of folate encapsulated in CP particles to be in the range of 0.5–3.5 μm . The decreased particle size observed in the present study may be attributed to the addition of glyoxal which leads to crosslinking via mainly lysine side chains of proteins (Marquié, 2001). In fact, crosslinking has shown to decrease swelling of proteins leading to reduced particles sizes (Asor *et al.*, 2019). FA-CP particles were prepared under basic conditions. In basic conditions, glyoxal is transformed into glycolate (CH_2OHCOOH) and therefore, the reaction between amino groups of protein and glyoxal is favourable under alkaline conditions. This crosslinking reaction leads to enhanced mechanical performance and water resistance with high crosslinking density and cohesive strength of the particles (Wu *et al.*, 2021). Therefore, decreased hydration in aqueous (PBS) solution might have led to decreased size of FA-CP particles.

Table 1: Diffusional exponent (n) values and release transport mechanisms based on spherical shape vehicles.

Diffusional Exponent (n) for Spherical Shape Particles	Release Mechanism
$n < 0.43$	Less Fickian
$n = 0.43$	Fickian Diffusion
$0.43 < n < 0.85$	Anomalous Transport
$n = 0.85$	Case II Transport
$n > 0.85$	Super Case II Transport

In the case of alginate particles, it has been reported that the increase of CaCl_2 concentration has led to a decrease in alginate polymer hydration and thereby, polymer swelling. In fact, increased Ca^{2+} ions would enhance the binding sites and 3D network formation with COO^- of alginate leading to a higher degree of crosslinking and rigidity of the structure. However, in this study, 0.1 (w/v %) of CaCl_2 was used for crosslinking of alginate. Therefore, lower concentrations of CaCl_2 would have led to insufficient binding sites of Ca^{2+} ions and less molecular chain entanglement and thereby, led to increased hydration and higher alginate polymer swelling (Pillay *et al.*, 1998; Li *et al.*, 2016) during the particle size analysis in the aqueous medium. As explained above, the low calcium ion density of the alginate particles must be the reason for the larger size of alginate particles than chickpea protein particles.

However, PDIs of FA encapsulated particles were different and quite large, with FA-A particles showing a value of 106.6% while FA-CP particles showing a lower value of 90.9%. These results indicate that despite the difference in the particle sizes, both types of particles showed a broad size distribution in the suspension. Zeta-potentials of the FA-A and FA-CP particles were -11.10 ± 7.79 mV and -12.90 ± 7.65 mV, respectively, which are almost similar. These results indicate that both the particle systems show only transient stability in aqueous systems (Kumar and Dixit, 2017).

Percentage EE and LC

Both FA-A and FA-CP particles showed high EEs and the values were $79.67 \pm 5.24\%$ and $85.37 \pm 4.15\%$, respectively. However, both types of particles exhibited low, yet acceptable, LCs. In fact, LCs of FA-A and FA-CP particles were $2.65 \pm 0.18\%$ and $0.43 \pm 0.03\%$, respectively. In the previous study by Ariyaratna and Karunaratne (2015), the EE of folate was much lower ($62.19 \pm 2.05\%$) than that of the present study but their LC was higher ($10.18 \pm 0.89\%$). The cause of having lower FA loading capacities by both the polymer particles may be due to the utilization of a lower drug/polymer ratio (i.e. 1%) in this study (Ji *et al.*, 2012).

FT-IR spectroscopy

Structural analysis of FA and FA encapsulated particles were carried out via FTIR-ATR for the confirmation of the success of FA encapsulation process. Figure 1 displays the FTIR spectra of free FA, free alginate, free CP as well as FA encapsulated particles.

The spectrum of alginate (A) showed important absorption bands related to hydroxyl, ether and carboxylic functional groups. The peak related to O-H stretching vibrations that appeared at 3259.5 cm^{-1} in the spectrum of alginate indicated a shift to 3325.4 cm^{-1} in the spectrum of FA-A particles. Further, strong peaks between $1630\text{--}1690\text{ cm}^{-1}$ which correspond to C=N and C=O amide stretching of the spectrum of FA were absent in the spectrum of FA-A particles. Similarly, the peak at 1601.9 cm^{-1} corresponding to N-H bending vibration of

CONH group of FA (Mohammed, 2014; Rana *et al.*, 2016) has been masked.

Furthermore, peaks related to C-O stretching vibrations at 1085.1 cm^{-1} and 1027.4 cm^{-1} have shifted to higher wavenumbers of 1087.2 cm^{-1} and 1029.5 cm^{-1} , respectively; while the peaks at 1124.2 cm^{-1} and 941.0 cm^{-1} corresponding to CO stretching (Fenoradosa *et al.*, 2010) have shifted to 1122.2 cm^{-1} and 943.0 cm^{-1} , respectively, in the spectrum of FA-A particles, indicating the encapsulation of FA.

In the spectrum of CP, a broad peak related to hydroxyl group and N-H stretching vibrations (Palaniappan and Vijayasundaram, 2009) was observed in the range $3300\text{--}3600\text{ cm}^{-1}$ and around 3265 cm^{-1} , respectively. However, due to encapsulation, a change in the peak shape from a broad curved peak to a broad pointed peak was observed.

Also, a weak sharp peak corresponding to C=O stretching of amide I groups was observed at 1639.0 cm^{-1} (Barth, 2007).

In the spectrum of FA-CP particles, two bands related to C=O stretching were observed. The amide band I (C=O) has been shifted to 1630.8 cm^{-1} while amide band II (N-H and C-N) at 1523.7 cm^{-1} (Liang and Wang, 2018) was quite visible. Strong peaks at $1638\text{--}1690\text{ cm}^{-1}$ corresponding to C=O bond stretching of $-\text{CONH}_2$ (Mohammed, 2014) of FA were observed more prominently in FA-CP particles. These spectral data confirm the presence of FA in FA-CP particles.

SEM

SEM images of FA-A and FA-CP particles are shown in Figure 2. The diameters of freeze-dried samples of both particle systems – FA-A (Figure 2a) and FA-CP (Figure 2b) were less than 200 nm although the hydrodynamic diameters were much larger. Furthermore, the spherical nature of both FA encapsulated particles is visible.

Photo-stability and thermal-stability of FA on storage

The high sensitivity of FA towards external conditions necessitates the evaluation of the potency of different matrices to improve the stability of FA upon encapsulation. This study evaluated the thermal and photo protective ability of alginate and chickpea protein polymers, as matrices of submicron particles, on FA. In fact, lyophilized FA-A and FA-CP particles were subjected to different photo and thermal conditions as shown in Figure 3. Here, free FA was used as a control.

Generally, all particles in dark conditions (i.e. covered particles) showed a significantly lower extent of degradation (16-18%) than particles exposed to light (i.e. exposed particles) (Figure 3a) as expected. Both covered and exposed particles degraded gradually with time exhibiting higher degradation at the end of the three-month storage period. Interestingly, photo-degradation of the three types of covered samples was similar. However, FA in alginate particles remained more stable showing

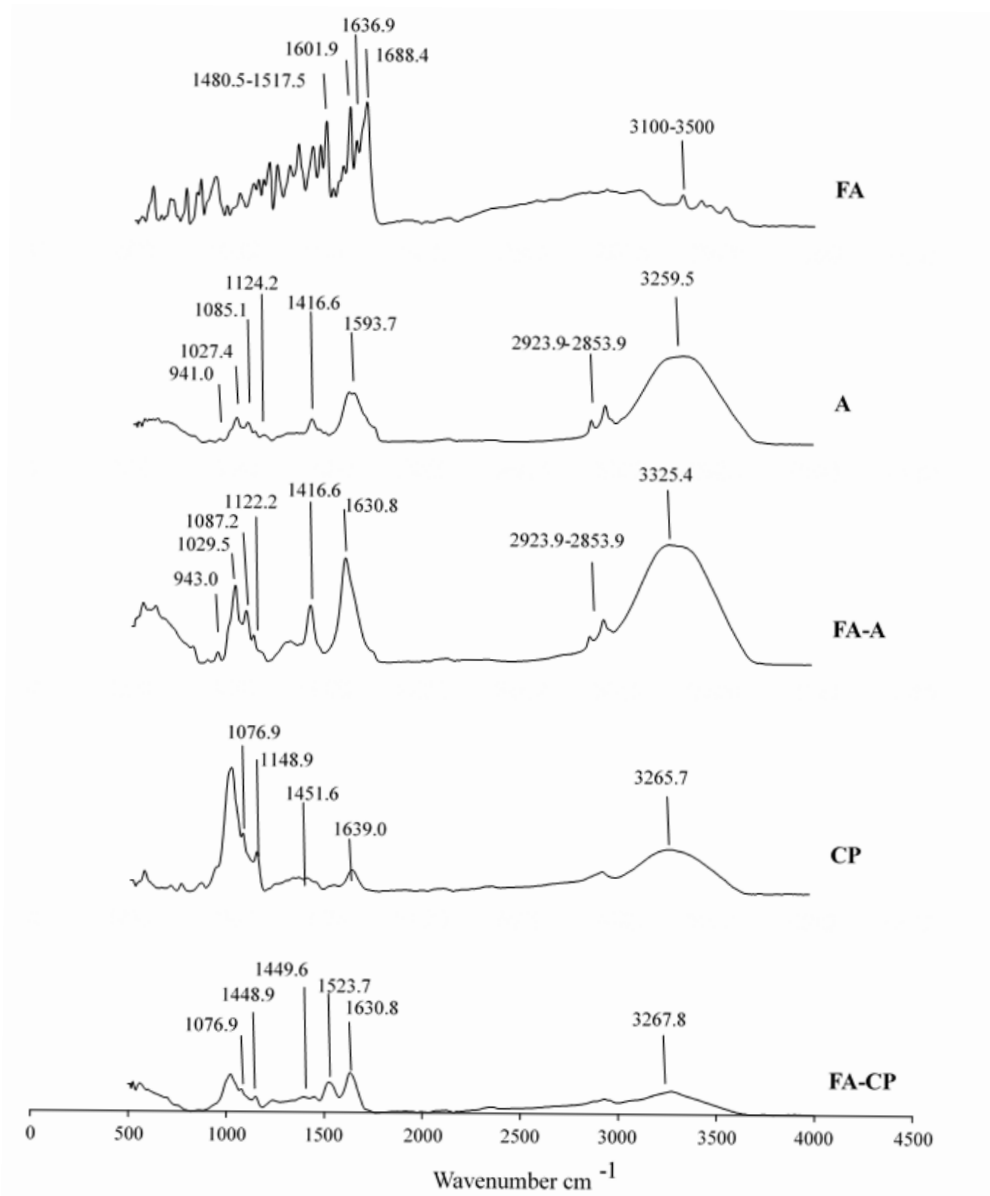


Figure 1: FTIR spectra of FA (folic acid), A (alginate), FA-A (folic acid encapsulated alginate particles), CP (chickpea protein), and FA-CP (folic acid encapsulated chickpea protein particles).

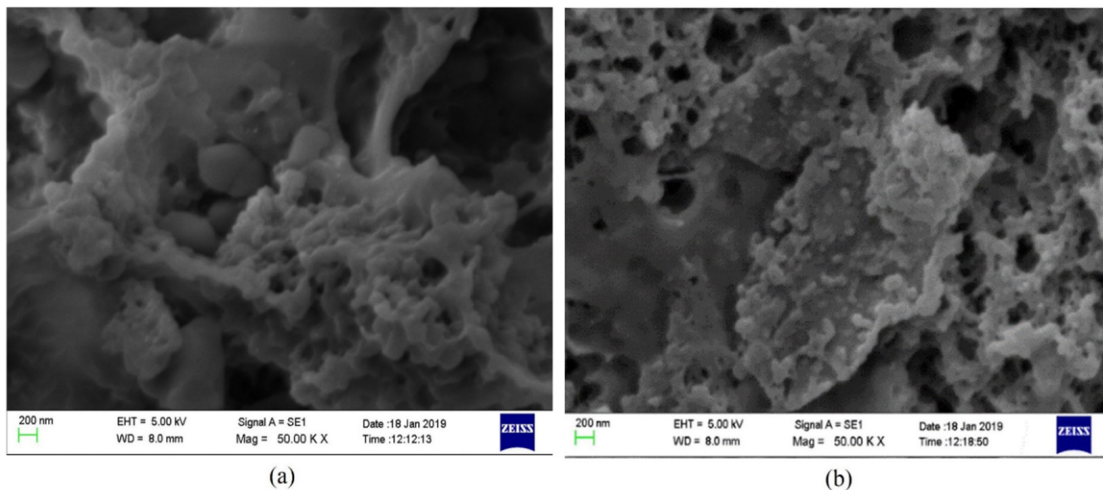


Figure 2: SEM images of (a) FA-A (folic acid encapsulated alginate) and (b) FA-CP (folic acid encapsulated chickpea protein) particles.

significantly a lower extent of degradation (30.5%) than both free FA (44.6%) and FA in protein particles (40.7%) that showed a similar degree of degradation, upon exposure to day light. Thus, alginate matrix may be used effectively for protecting FA against photolysis, especially, when exposed to day light.

According to the study of thermal effect on encapsulated and free FA samples, alginate afforded the best protection. The degradation of free FA and FA in FA-CP particles was similar and greater than that of FA-A particles (Figure 3b). Samples stored at refrigerated temperature (4 °C) exhibited a considerably lower FA degradation than those stored at room temperature displaying the temperature effect on degradation of FA.

Accordingly, alginate appears to be far better than CP as a protective matrix for FA encapsulation against thermal degradation. Overall, covered and refrigerated conditions are suggested for enhancing the stability of FA.

In vitro release

Figure 4 exhibits the cumulative release percentages of free FA and FA from the FA-A and FA-CP particles with time. Free FA displayed fast release at both pH 2 and pH 6.8 reaching 65% and 95%, respectively at 8 h. Comparatively, encapsulated FA exhibited slower and controlled release at the beginning. Notably, release of FA from FA-A particles was very low at pH 2 compared to release at pH 6.8, while the pattern was opposite with FA-CP particles up to several hours.

At pH 2, FA-A particles exhibited no release up to 7 h. However, by the eighth hour, a cumulative release of nearly 24% was observed. The seven hour lag phase may be due to the decreased hydrophilicity of alginate polymer in acidic media upon alginic acid formation followed by polymer chain shrinkage that may lead to decreased polymer swellability (Goswami *et al.*, 2014). Alginate molecules tend to swell in alkaline media due to

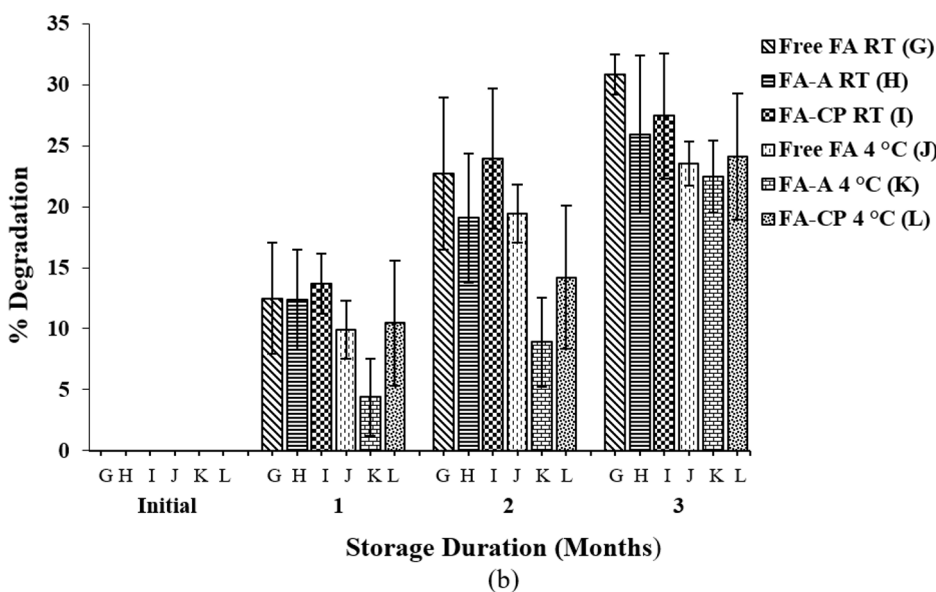
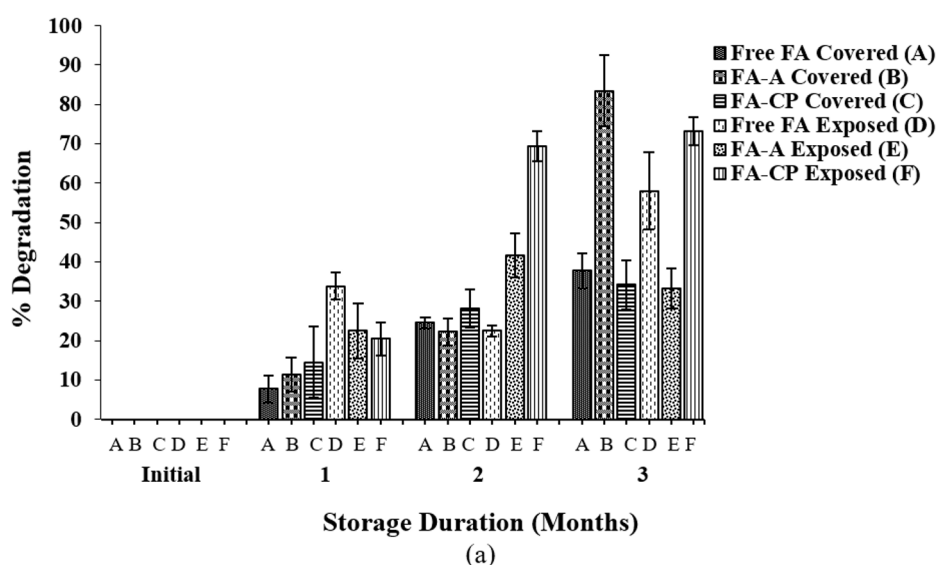


Figure 3: Stability (a) Photo degradation (b) Thermal degradation, of free FA (folic acid), FA in FA-A (folic acid encapsulated alginate) and FA-CP (folic acid encapsulated chickpea protein) particles on storage, stored for 3 months, (RT – room temperature).

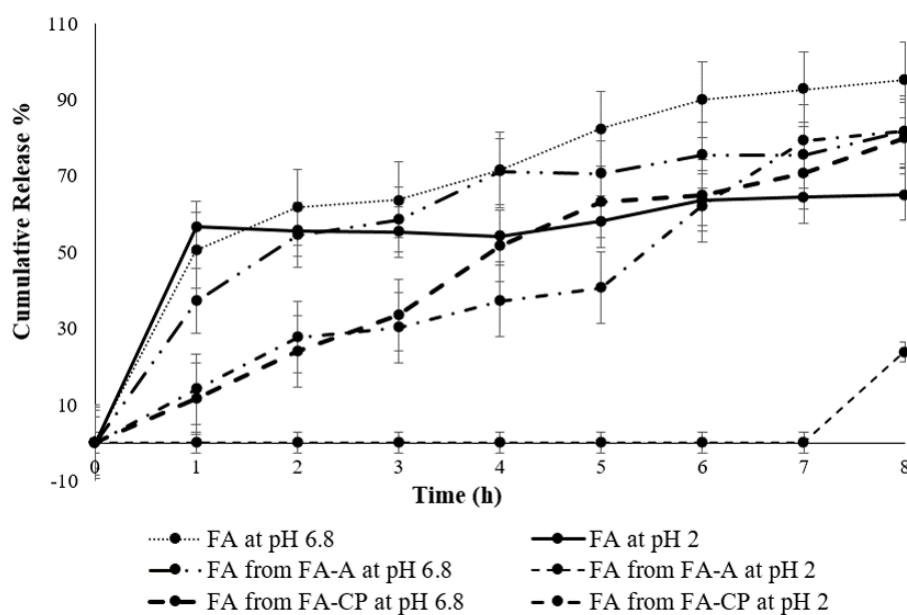


Figure 4: *In vitro* release of free FA (folic acid), FA from FA-A (folic acid encapsulated alginate) and FA-CP (folic acid encapsulated chickpea protein) particles at pH 2 and 6.8.

de-protonation of carboxylic acid groups causing strong electrostatic repulsion between the negatively charged alginate polymer chains (Bajpai and Sharma, 2004). Accordingly, the release of FA from the FA-A particles at pH 6.8 showed a much higher release of 82% than at pH 2, by the end of the release period. Thus, swelling and rearrangement of alginate polymer at alkaline pH assist in the release of FA from the matrix (Pacho *et al.*, 2019). Basically, the protonation/deprotonation behavior of alginate has led to the controlled release behavior shown by FA-A particles.

The release behavior of FA from FA-CP particles was dissimilar to that from FA-A particles due to the unfolding nature of protein structure in both high acidic and basic media. A high FA release from FA-CP particles (around 80%) was observed at both the physiological pHs at 8 h. At pH values above or below the isoelectric point (pH 4.5), the protein tends to denature or unfold and destabilize the structure (Ariyaratna and Karunaratne, 2016). This destabilization leads to a high release of FA into the media at both high and low pH values. Pertaining to the fact that FA degrades easily in acidic media (Akhtar *et al.*, 2003) in the stomach showing low stability, it is obvious that alginate particles are more capable of providing a protective and efficacious FA delivery than chickpea protein particles across the gastric phase to the main FA absorption site which is the intestine (duodenum and jejunum) (Bernstein *et al.*, 1970).

Model fitting and release mechanism

Table 2 displays the adjusted R-squared values of the fitted mathematical model at gastrointestinal pHs. As indicated in Table 2, the release profiles of FA-A particles at pH 6.8 fitted best with Weibull model showing a sigmoidal pattern. However, no lag time was observed may be due to fast relaxation of alginate polymer chains in alkaline media leading to fast release (Pacho *et al.*, 2019). Model

fitting was omitted for the release profile at pH 2 due to the absence of release up to 7 h of release time.

At pH 6.8, the release profile of FA-CP particles fitted best with Gompertz model, which is a simple exponential model, showing a sigmoidal pattern. According to the model, release of FA does not proceed at a constant rate, but the rate changes with time. Release of FA shows a steep increase at the beginning and converges slowly to its asymptotic maximal dissolution (Easton, 2002). At pH 2, the release profile of FA from FA-CP particles fitted best with Higuchi model. The model suggests a diffusion release mechanism of encapsulated FA from the CP matrix (Costa and Lobo, 2001).

In evaluating the transport mechanisms (Table 1) of FA encapsulated particles, FA-A at pH 6.8 exhibited a Fickian diffusion ($n=0.43$) where the solvent penetration rate into the hydrogel is slower than the polymer chain relaxation rate (Bruschi, 2015). In fact, alginate matrix swells in intestinal pH leading to diffusion of FA (Tønnesen and Karlsen, 2002). However, for FA-CP particles, the n value at pH 2 was 0.40 displaying a less Fickian mechanism where the rate of water penetration is much lower than the rate of polymer chain relaxation (Gierszewska-Drużyńska and Ostrowska-Czubenko, 2012).

This observation was confirmed as the release profile of FA from FA-CP particles at pH 2 fitted best into the Higuchi model which is purely based on Fickian diffusional release (Hans *et al.*, 2019). At pH 6.8, the n value was obtained as 1.05 displaying a super case II transport mechanism in which the release occurs mainly due to matrix breaking and solvent crazing until finally the vitreous nucleus breaks (Bruschi, 2015).

Basically, the two different types of FA encapsulated particles, FA-A and FA-CP, exhibited different release patterns and mechanisms in gastric pH and intestinal pH,

displaying matrix type and pH dependent release of FA. This information will be much valuable in food engineering as improving the bioaccessibility at the site of absorption of FA is the prime target of FA fortified food engineering.

In vitro digestion

To evaluate the bioaccessibility of FA along the gastrointestinal tract, until it is actively absorbed by the specific transporters (Reduced Folate Carrier and the Proton-Coupled Folate Transporter) in the jejunum (Zhao *et al.*, 2009) of the small intestine, an *in vitro* digestion model which mimics the mouth, stomach and intestinal digestion under simulated conditions was used. Figure 5

displays the bioaccessibility of free FA and FA encapsulated in alginate and CP particles during *in vitro* mouth, stomach and intestinal digestion.

During the five minutes of mouth digestion, nearly 57% of free FA was released to simulated saliva. However, during the 2 h of stomach digestion, high degradation of FA occurred decreasing bioaccessibility of FA to nearly 14%. Degradation of FA continued in the intestine further decreasing the bioaccessibility of free FA to less than 9% at 300 minutes.

Release of FA from FA-A particles (55%) was quite similar to that from free FA in salivary pH (i.e. pH 6.75). However, bioaccessibility of FA decreased only negligibly

Table 2: Correlation coefficient (adjusted R-squared) values of mathematical models obtained for the folic acid encapsulated alginate and chickpea protein particle systems at pH 2 and pH 6.8.

Models	Folic acid encapsulated particles				
	Adjusted R-squared values (Mean \pm SD)				
		FA-A		FA-CP	
		pH 2	pH 6.8	pH 2	pH 6.8
Zero order	No fit	0.7363 \pm 0.035 ^b	0.8896 \pm 0.046 ^a	0.8896 \pm 0.046 ^a	0.9463 \pm 0.028 ^a
First order	No fit	0.3673 \pm 0.019 ^d	0.8884 \pm 0.061 ^a	0.8884 \pm 0.061 ^a	0.7722 \pm 0.153 ^a
Higuchi	No fit	0.9456 \pm 0.017 ^a	0.9085 \pm 0.059 ^a	0.9085 \pm 0.059 ^a	0.9148 \pm 0.023 ^a
Hixson-Crowell	No fit	0.2245 \pm 0.015 ^c	0.2518 \pm 0.090 ^b	0.2518 \pm 0.090 ^b	0.1382 \pm 0.023 ^b
Baker-Lonsdale	No fit	0.6248 \pm 0.035 ^c	0.4060 \pm 0.200 ^b	0.4060 \pm 0.200 ^b	0.2000 \pm 0.375 ^b
Weibull	No fit	0.9862 \pm 0.007 ^a	0.8840 \pm 0.082 ^a	0.8840 \pm 0.082 ^a	0.9635 \pm 0.004 ^a
Gompertz	No fit	0.9483 \pm 0.007 ^a	0.8626 \pm 0.070 ^a	0.8626 \pm 0.070 ^a	0.9701 \pm 0.003 ^a

^{a-c}Different superscripts of each column represent significantly different means. n=3.

(SD – standard deviation, FA-A – folic acid encapsulated alginate particles, FA-CP – folic acid encapsulated chickpea protein particles).

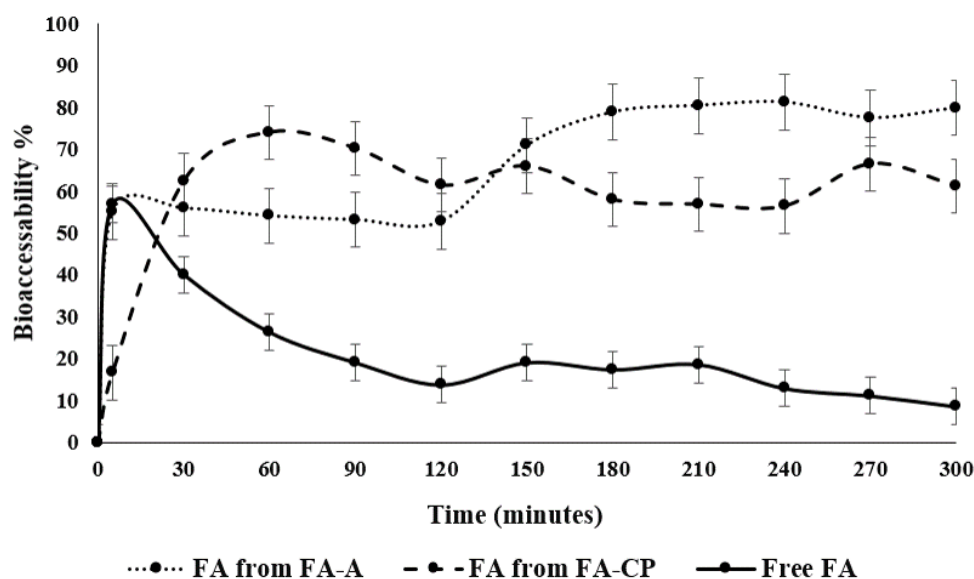


Figure 5: Bioaccessibility of free FA (folic acid), FA from FA-A (folic acid encapsulated alginate) and FA-CP (folic acid encapsulated chickpea protein) particles during *in vitro* mouth, stomach and intestinal digestions.

with FA-A particles, unlike with free FA, up to 2 h in stomach pH. The said observation may be totally due to the formation of alginic acid in the acidic media prohibiting the release of FA as shown in the release profile of FA-A particles in gastric pH. In intestinal digestion, the bioaccessibility of FA increased to nearly 80% due to swelling of alginate polymers in alkaline pHs (pH 6-8) (Bajpai and Sharma, 2014) facilitating release of FA. Moreover, the stability of FA in alkaline pH (Gazzali *et al.*, 2016) makes it more bioaccessible during the absorption process.

Release of FA from the FA-CP particles in mouth digestion was low (17%), while a higher release corresponding to higher bioaccessibility (62-74%) was observed during stomach digestion. Denaturation of CP structure in the presence of acidic pH and influence of pepsin on breaking of the peptide bonds (Cornish-Bowden and Knowles, 1969) in the protein may facilitate release of FA from protein particles.

However, released FA has undergone degradation to a small extent due to the acidic pH (Gazzali *et al.*, 2016) of the stomach. At the end of intestinal digestion, the remaining FA for absorption was 61% which was quite similar to the FA bioaccessibility at the beginning of intestinal digestion. Although FA is an essential vitamin, a high intake of FA (>1 mg) might show toxic symptoms in the gastrointestinal tract and nervous system, including nausea, abdominal distension and discomfort, irritability, sleep disturbance and many more (Butterworth and Tamura, 1989). Further, the required dosage of FA depends upon the physiological factors and FA absorption might depend on certain clinical conditions of a person (Herbert, 1968). Therefore, slow controlled delivery of FA via encapsulation is highly advantageous. According to the *in vitro* bioaccessibility study, submicron particles made of either alginate or CP may be used to enhance the bioaccessibility of FA. Interestingly, alginate particles show higher capability than CP particles as a protective controlled delivery vehicle carrying FA to the absorption site in the intestine.

CONCLUSION

In this study, FA encapsulated alginate and chickpea protein submicron particles were prepared successfully, characterized and evaluated for enhancing stability, release and bioaccessibility of FA. Overall, the alginate matrix was found to be more effective than chickpea protein matrix for FA delivery due to its excellent structural behaviour (pH dependency) in gastrointestinal pH conditions, which is slowing down of FA release in gastric pH while facilitating release in intestinal pH. Both matrices were capable of increasing the bioaccessibility to a considerable level than the free compound. Model fitting and evaluation of transport mechanisms suggested that FA-A particles at pH 6.8 display diffusional transport with Fickian behavior, while FA-CP particles at pH 6.8 and at pH 2 display super case II transport mechanism and diffusion-based release of FA, respectively. The alginate matrix imparted higher stability to FA under photo and thermal conditions than the chickpea protein matrix. Further, covered-refrigerated

conditions are preferred for greater stability of encapsulated FA. In summary, alginate supersedes chickpea protein as the better matrix for encapsulating FA for achieving higher stability, controlled release and bioaccessibility of FA.

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STATEMENT OF CONFLICTS OF INTEREST

The authors report no conflict of interest.

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