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Effect of pH and bile salt on release pattern of nanoencapsulated of folic acid

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Abstract
Folic acid is a B9 vitamin, which requires in the human body to make new cells. So, everyone needs folic acid and particularly for the pregnant women to prevent major birth defects of her baby. The free folic acid and its bioavailability is affected by parameters like pH and bile in the gastrointestinal tract. The nanoencapsulation of such type of bioactive component can be used to protect it from adverse gastrointestinal environmental conditions as well as it improves the bioavailability, stability and controlled release at targeted site. So, in the present study, the nanoencapsulation of folic acid was carried out using electrospraying and the release profile of nanoencapsulated folic acid was studied. Release profile of nanoencapsulated folic acid was found better at 7 pH and 1.5% bile as compared to 4 pH and 0.5% bile, respectively. The highest release of nanoencapsulated folic acid i.e. 99.88 µg was observed when exposed to 7 pH up to 240 minutes. In case of bile exposure, the highest release of the nanoencapsulated folic acid i.e. 83.83 µg was observed at 1.5% bile up to 240 minutes. So, the maximum release of nanoencapsulated folic acid may help the consumers for better bioavailability.

Keywords: Folic acid, electrospraying, nanoencapsulation, release profile

Introduction
Folic acid (FA) is a water soluble vitamin and which is also known as vitamin B9. It is an essential component required for body functions and it's deficiency may lead to various disorders and diseases like atherosclerotic cardiovascular disease, shortness of breath, neurological and neuropsychiatric disorders and also congenital defects, anemia, homocysteinemia, mental confusion, irritability, depression and carcinogenesis, dementia and Alzheimer's disease (Stanger, 2002; Tucker *et al.*, 2005; Eichholzer *et al.*, 2006; Balk *et al.*, 2007; Mitchell *et al.*, 2014; Shulpekova *et al.*, 2021) [56, 59, 17, 7, 38, 54]. It takes participation in several functions like biosynthesis of amino acids, nucleotides, neurotransmitters, and certain vitamins. So, it is required at all age health group (Sijilmassi, 2019) [55].
Three chemical structures together make folic acid are (1) pteridine ring (2) p-aminobenzoic acid (PABA) and (3) glutamic acid. It is yellow in colour with molecular weight of 441.4 and is slightly soluble in water in the acid form but quite soluble in the salt form (Shane 2001; Brody and Shane 1984) [53, 9]. In nature, the folate is present in a reduced form. A carbon unit including a methylene, formyl, methyl, methenyl or formimino group is attached to the N5, N10 or in both positions of the tetrahydrofolate (THF) structure. The monoglutamate is the oxidized form of folic acid which occurs rarely in nature. This type of synthetic folic acid is contained in various fortified foods and nutritional and dietary supplements.
Absorption of dietary folates takes place in duodenum and proximal jejunum (Moll & Davis, 2017; Tappenden, 2023) [39, 57]. Before absorption, the form of folate is polyglutamate which is deconjugated to the monoglutamate form in the gut and then taken up by the mucosal cells (McNulty, 2022) [35]. Monoglutamate folate is transported across the apical membrane of enterocytes mainly by the proton coupled folate transporter (PCFT) (Zhao *et al.*, 2011; Visentin *et al.*, 2014) [65, 60]. The folate is metabolized in the enterocytes to 5- methyl-tetrahydrofolate (THF) and then exported into the portal vein. Then it is taken up by the cells during the blood circulation through the receptor mediated endocytosis of the folate receptors (FRs) or reduced folate carrier (RFC). It is reabsorbed in the intestine when the folate in hepatocyte is secreted in bile. Folylpoly-gammaglutamate synthetase (FPGS) is an enzyme responsible for the conversion of intracellular folate in to polyglutamate forms. The polyglutamylation is playing very important role as it will be used for the retention of folate within cells and for the utilization of one carbon metabolism (Shane, 2001) [53]. The bioavailability of food folates is influenced by several parameters like the intestinal

environment, chemical and physical stability/instability of folates, the composition of food and food matrix and other factors (McNulty, 2022; Crider *et al.*, 2022; McNulty and Pentieva 2004) [35, 14, 36]. The bioaccesibility/ bioavailability of folate varies from 38% to 98% from the natural sources (Brouwer *et al.*, 1999; Brouwer *et al.*, 2001; Winkels *et al.*, 2007; Moretti *et al.*, 2014; McNulty & Pentieva 2004) [11, 10, 62, 41, 36].

Yaman *et al.*, (2019) [63] conducted the study to check the bioavailability of folic acid in cereal based baby foods, infant formula samples and follow on baby milk at gastric pH of 1.5 and pH 4. They reported that the folic acid at pH 1.5 was in the range of 56% to 71% in infant formula, 59% to 78% in cereal based baby foods and 42% to 67% in follow-on baby milk. At pH 4, the folic acid was in the range of 35% to 49% in infant formula samples, 31% to 67% in cereal based baby foods and 38% to 57% in follow on baby milk. One of the reason for the fortification of folic acid in encapsulated form in food is that the free form of folic acid is not having great solubility, poor bioavailability and can gets easily oxidized (Ismail *et al.*, 2023; Ledowsky *et al.*, 2022; Naderi and House, 2018; Elliot, 2008) [23, 29, 43, 18].

Over the last few years, the nanoencapsulation of bioactive compounds is an area of improved importance which seeks to protect these products from adverse gastrointestinal environmental conditions and thus, improve their shelf-life which assures their health-promoting properties, additionally, nanoencapsulation of these components are also allows to develop new novel functional food products which has potential health benefits by incorporation into different food matrices.

Nanoencapsulation, also known as bioactive packing at the nanoscale, is a process used to encapsulate bioactive compounds in very small nanosized molecules (Lopez-Rubio *et al.*, 2006) [33]. It is a rapidly rising technology with several potential applications in areas such as the pharmaceutical and food industries. Nanoencapsulation has been used to shield bioactive compounds like polyphenol, micronutrients, enzymes, antioxidants and nutraceuticals and, finally to defend them from adverse environmental factors. It is also used for controlled release property at the targeted site (Gouin, 2004; Ezhilarasi *et al.*, 2013) [20, 19].

Nanoencapsulation includes the incorporation of different bioactive compounds in different encapsulating material to protect them from environmental changes, enzymatic modification, chemical variation, buffering against intense pH, different temperature and ionic strength variations in the form of vesicles of nanometer in diameters (Wen *et al.*, 2017) [61]. Into the body, bioactive compounds release rate in to the various sites is directly influenced by particle size (Kawashima, 2001; Hughes, 2005) [25, 22]. Reduction in the size of encapsulants to the nanoscale offers to increase or extend the retention time in the gastrointestinal conditions due to superior bio adhesiveness in the mucus covering the intestinal epithelium (Chen *et al.*, 2006) [13]. Compare to microencapsulation, nanoencapsulation has more likely to boost up bioavailability, better controlled release and help in accurate targeting of the bioactive components to a higher extent (Mozafari, 2006) [42]. Additionally, modulations or coatings of surface properties can allow the targeted release of compounds (Bouwmeester *et al.*, 2009) [8].

Moreover, improving bioavailability by nanoencapsulation technique, it also offers various benefits which include ease to handling, retention of volatile ingredients, taste masking, reduction in oxidation time, moisture, enhanced stability, pH based controlled release and successive distribution of multiple active components (Ezhilarasi *et al.*, 2013) [19].

The Electrospraying method is primarily a synthesis method which consists of the atomization of a polymer solution flowing through narrow capillary tube to produce droplets of nanometers in diameter, each carrying a miniature amount of the delivered solvent and the application of high voltage to evaporate solvent. These droplets in the flight are accelerated towards the collector plate and at the same time the solvent is evaporated through electrostatic force so there is no need for high temperatures which prevents the thermal degradation of heat-sensitive substances (Lei *et al.*, 2020) [30].

Electrospraying technique is also used for nanoencapsulation of bioactive components like ω-3 (omega-3) fatty acid (Torres-Giner *et al.*, 2010, Raval and Ramani 2018; Moomand and Lim 2015) [58, 50, 40], β-carotene (Lopez-Rubio and Lagaron, 2012) [32], folic acid (Bakhshi *et al.*, 2013), lycopene and indomethacin (Jain *et al.*, 2014) [1].

Materials and Methods

Preparation of solution and optimization study of folic acid-WPC-proline-lactate for electrospraying

The solution of folic acid (Sigma Aldrich) was prepared as per the product information sheet of Sigma Aldrich *i.e.* in 1 M NaOH (40 mg/ml) (SD Fine chemicals) in amber coloured bottle and the WPC (AS-IT-IS brand) was prepared in different concentration i.e. 10%, 20%, 30% and 40%. The different combination ratios of proline (P) (Sigma Aldrich) and lactate (LA) (Sigma Aldrich) with WPC and folic acid were used for preparation of solution for nanoencapsulation through electrospraying. Electrospraying (Model Fluidnatek LE-10, Make - Bioincia S. L., Valencia, Spain) system, equipped with a variable high voltage power supply. The prepared solutions were subjected to electrospraying at different flow rate, voltage and distance.

Standard curve preparation for the quantification of folic acid

The quantitative analysis of folic acid was carried out by using the method developed by Kshirsagar *et al.*, (2017) [28] with slight modification at λ_{max} (281 nm) in double beam spectrophotometer (Model - UV- 1900, Make - Shimadzu, Japan). The method of Olmo *et al.* (2022) [44] was used with slight modification for the preparation of the standard curve of the folic acid and the different concentrations in the range of 100 µg/ml to 500 µg/ml were prepared in 1 M NaOH solution. Measurement for the calibration was done using the 1 M NaOH solution as a blank. The stock solution was prepared by weighing 100 mg folic acid and transferred it to the 100 ml volumetric flask. The working standard solution was prepared by diluting the 0.1 ml of stock solution in 10 ml of distilled water to have concentration of 100 µg/ml.

Release profile study of nanoencapsulated folic acid

Release profile at different pH: The release profile at different pH has been studied using the method given by Črnivec *et al.*, (2020) [15] with slight modification. Črnivec *et al.*, (2020) [15] prepared the solution using an ultrasound bath whereas in this study, the magnetic stirrer was used at 600 rpm for 2 hours. Acidic and neutral pH i.e. 4 and 7 has been used to analyse the release profile of nanoencapsulated folic

acid powder. The concentration of nanoencapsulated folic acid is measured through UV-VIS spectrophotometer at an interval of 0, 30, 60, 120, 150, 180, 210 and 240 minutes.

Release profile at different bile concentration: Different concentrations of bile (HiMedia, India) i.e. 0.5% and 1.5% has been prepared. The solution of nanoencapsulated folic acid was directly mixed with the selected concentration of bile. The concentration of nanoencapsulated folic acid is measured through UV-VIS spectrophotometer at an interval of 0, 30, 60, 120, 150, 180, 210 and 240 minutes.

Statistical Analysis

The collected data were subjected to statistical analysis. Data were analysed by analysis of variance (ANOVA) and critical difference test at 5% level of significance ($p \leq 0.05$) to compare the different treatments means, with 3 replications with the help of WASP (Web Agri Stat Package) developed by the Indian Council of Agricultural Research (ICAR), New Delhi (https://ccari.icar.gov.in/wasp/index.php).

Results and Discussion

Preparation of solution and optimization study of folic acid-WPC-proline-lactate for electrospraying

The folic acid 150 µg, 10% WPC and proline to lactate ratio of 1:1 has been optimized for the nanoencapsulation process through electrospraying. The optimized flow rate, voltage and a tip-to-collector distance in the electrospraying process is mentioned in Table 1. The encapsulated powder was collected on flat collector which was wrapped with parchment paper in aluminum foil and packed in sterile sealed polythene bags and stored at 4 °C and 37 °C for analysis. The obtained powders was used for further analysis.

Table 1: Optimized process of electrospraying for nanoencapsulation of folic acid

Folic acid	WPC	P:LA ratio	Flow rate (µl/hr)	Voltage (kv)	Distance (cm)
150 µg	10%	1:1	300	15	13

Preparation of standard curve for release profile and stability study

The absorbance's of folic acid is shown in Table 2 and Figure 1. Standard curve of folic acid was prepared by using different concentration of water soluble folic acid in distilled water. The optimum wavelength for maximum absorption of water soluble folic acid (λmax) is 281 nm (Kshirsagar *et al*., 2017) [28]. The absorbance of standard folic acid solution was measured at 281 nm against distilled water as a blank. Standard curve was plotted with absorbance against concentration.

Table 2: Absorbance of folic acid at its different concentration in distilled water

No.	Concentration of folic acid (µg/ml)	Absorbance (WL- 281)
1	2	0.11
2	4	0.21
3	6	0.32
4	8	0.43
5	10	0.53

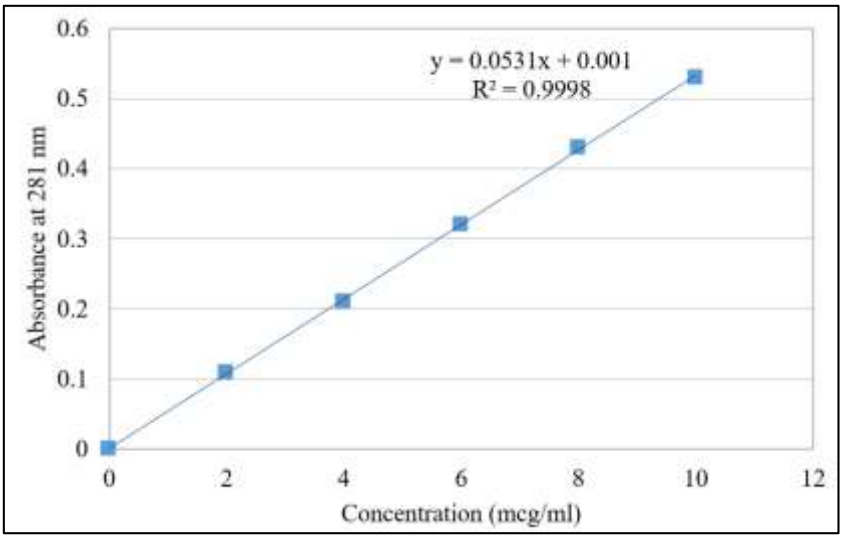

Fig 1: Standard curve of folic acid

Release profile study of nanoencapsulated folic acid

The folate derivatives are susceptible to various physical condition like light, oxygen, pH and temperature during the processing of food (Madziva *et al*., 2005) [34]. So, to check the release profile of nanoencapsulated folic acid powder in *in-vitro* conditions, it is exposed to selected pH and bile salt concentration. In this study, the nanoencapsulated folic acid was supposed to use for direct fortification in yoghurt and the average time of food transit from stomach to intestine is between 180 minutes to 240 minutes (Canon *et al*., 1904). So, all this parameters were studied after exposing the nanoencapsulated folic acid at a periodic interval of 0, 30, 60, 120, 150, 180, 210 and 240 minutes.

Release profile of nanoencapsulated folic acid at different pH

Objective of this study was to check the effect of different pH on the concentration of nanoencapsulated folic acid. After consumption of yoghurt fortified with nanoencapsulated folic acid, it reaches to the small intestine which is having pH of 6 to 7.4. The maximum absorption of folic acid takes place in the small intestine of human body (Visentin *et al.*, 2014) [60].

The release of folic acid at different time interval is given in Table 3. The nanoencapsulated folic acid was exposed at pH of 4 and 7 and at an interval of 0, 30, 60, 120, 150, 180, 210 and 240 minutes and the concentration of folic acid was quantified at each time interval. This study was carried out in triplicates. The release of folic acid was increasing and the maximum concentration of 99.98 µg folic acid release was found in 7 pH and 70.21 µg folic acid release in 4 pH after the exposure up to 240 minutes.

Table 3: Concentration of nanoencapsulated folic released acid at different pH

Concentration (µg) of folic acid						
Minutes	**4 pH**			**7 pH**		
	Rep-1	**Rep-2**	**Rep-3**	**Rep-1**	**Rep-2**	**Rep-3**
0	58.62^{h}	58.54^{h}	58.47^{h}	60.95^{h}	61.70^{h}	60.20^{h}
30	59.52^{g}	59.67^{g}	59.44^{g}	62.15^{g}	62.08^{g}	62.23^{g}
60	60.57^{f}	60.72^{f}	60.42^{f}	64.11^{f}	64.19^{f}	64.26^{f}
120	61.03^{e}	61.18^{e}	60.95^{e}	68.03^{e}	67.95^{e}	67.87^{e}
150	64.41^{d}	64.26^{d}	64.34^{d}	72.47^{d}	72.39^{d}	72.54^{d}
180	67.27^{c}	67.12^{c}	67.35^{c}	76.08^{c}	75.93^{c}	76.23^{c}
210	69.00^{b}	68.85^{b}	69.08^{b}	84.81^{b}	84.58^{b}	84.89^{b}
240	70.06^{a}	70.21^{a}	70.13^{a}	99.71^{a}	99.94^{a}	99.98^{a}

The percentage of nanoencapsulated folic acid at different pH and time interval is represented in Figure 2. The release of nanoencapsulated folic acid at pH 7 was 66.58% and at pH 4 the release was 46.76% up to 240 minutes.

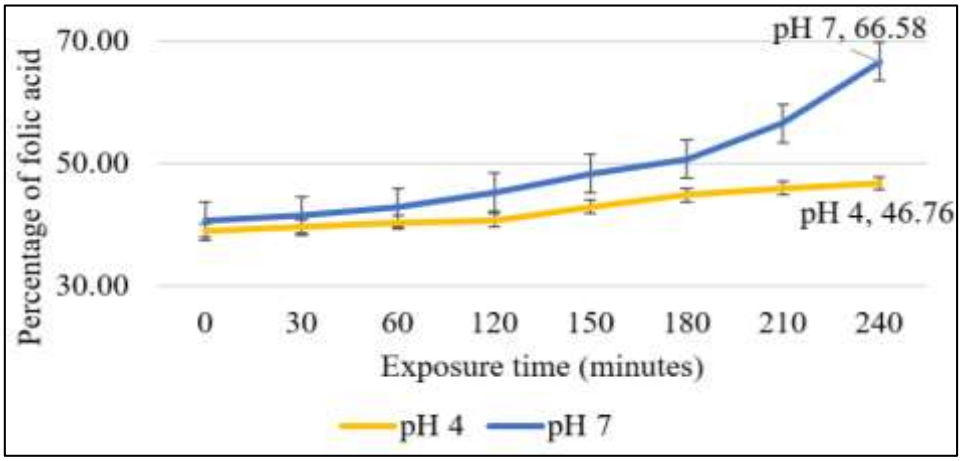

Fig. 2: Percentage of nanoencapsulated folic acid released at different pH

Prasertmanakit *et al.*, (2009) used ethyl cellulose for the folic acid encapsulation. They reported 70% release of encapsulated folic acid at 7.4 pH after 6 hours of exposure. Similarly, Alborzi *et al.*, (2014) used sodium alginate-pectin-poly(ethylene oxide) fibres for the encapsulation of folic acid through electrospinning. They reported the release of nanoencapsulated folic acid at 7.8 pH was 50% and 100% after 1 hr and 6 hr of exposure, respectively. Pandit *et al.*, (2019) prepared hydrogel for the encapsulation of folic acid using gum arabic crosslinked polyvinyl alcohol (PVA). They reported the release of nanoencapsulated folic acid at 7.4 pH and it was 21% and 78% after 1 hr and 5 hr of exposure, respectively. Shakoor *et al.*, (2022) [52] also reported the higher release of encapsulated folic acid in 6.8 pH than at 2 pH.

Assadpour *et al*., (2017) [5] studied the release of folic acid nanoparticles prepared with double emulsion (W1/O/W2) technique. Where W1 was folic acid and W2 was WPC-pectin layer. They mentioned that, in the alkaline condition, the release of folic acid was higher as compared to acidic pH of 4 and this may be due to faster dissolution of WPC in alkaline conditions as it is having low in its isoelectric point of pH 4-5. Liang *et al*., (2013) [31], prepared synthetic folic acid solutions (10 mg/L). This solution of folic acid was exposed to the pH of 1.95, 2.51, 3.51, 4.68, 5.63, 6.40, 8.05, 9.01 and 10.40. The concentration of folic acid was measured at a time interval of 2.5 hr, 5 hr, 7.5 hr, 24 hr, 48 hr and 72 hrs. They found the good stability of folic acid in alkaline condition i.e. pH 8.05 to 10.40 and reported 93.1% release of folic acid after 72 hrs. Where as in case of acidic pH, the stability of folic acid was below 55% and they also concluded that the release of folic acid is better in alkaline conditions than acidic conditions.

Hosseini *et al*., (2015) [21], measured the apparent dissociation constant (*K'*d) and apparent mole ratio of ligand for binding to B-lactoglobulin for folic acid at pH 4 and 7. They reported that the, apparent dissociation constant (*K'*d) for the folic acid at pH 7 was around 34 and at pH 4 was around 27 and the apparent molar ratio of ligand for folic acid is around 1.25 and 0.39 N at pH 7 and 4, respectively. They concluded that, at pH 7 the solubility and binding properties are better than pH 4. Kim *et al*., (2010) [27], studied the pH based release profile of folic acid loaded whey protein isolate (WPI) through cold gelation method, the particles found stable at a pH of 1.2 for more than 6 h, whereas at pH of 7.4 almost all of the folic acid was released in the dissolution media of phosphate buffer saline (PBS) within 2 h. Younis *et al*., (2009) [64], in their experiment mentioned that the folic acid showed an improved solubility with increase in pH and similar result was found in

the study of Assadpour *et al*., (2017) [5].

Perez-Esteve *et al.*, (2016) [46] purchased yoghurt of low fat and high fat from the local market, stirred and three treatments i.e. plain yoghurt (control), yoghurt with free folic acid and yoghurt with encapsulated folic acid were prepared. They reported the release of folic acid after exposure of 120 minutes in pH 2 was around 20µg and at pH 7 it was 94 µg. Madziva *et al*., (2005) [34] and Hosseini *et al*., (2015) [21] stated that the bioactive nutraceutical component should be absorbed maximally at the intestinal pH of 6 to 7.4.

Release profile of nanoencapsulated folic acid at different bile salt concentration

The objective of this study was to check the effect of bile salt on folic acid concentration. Because, after the consumption of nanoencapsulated folic acid, it will interact with the bile in the liver. However, certain studies have shown the folic acid increases the bile flow in the liver and this increase in bile flow is beneficial for the human health (Akinyemi and Adewole, 2021; Delgado-Villa *et al*., 2009; Pratt and Cooper, 1971) [2, 16, 49].

The concentrations of nanoencapsulated folic acid released is given in Table 4. Release of nanoencapsulated folic acid was studied at ambient temperature at 0.5% and 1.5% bile salt concentration. The maximum folic acid released was 83.83 (µg) at 1.5% bile concentration after the exposure of 240 minutes. In case of 0.5% bile salt, after exposure of 240 minutes the concentration of nanoencapsulated folic acid was 75.48 (µg). The concentration of folic acid was in increasing order in both the bile salt concentration.

Table 4: Concentrations of nanoencapsulated folic acid released in bile

Concentration (µg) of nano encapsulated folic acid released		
Minutes	**0.5% of bile**	**1.5% of bile**
0	56.60^{h}	56.65^{h}
30	61.40^{g}	62.00^{g}
60	64.89^{f}	66.39^{f}
120	67.42^{e}	69.51^{e}
150	69.33^{d}	71.84^{d}
180	70.96^{c}	70.94^{c}
210	72.44^{b}	80.97^{b}
240	75.48^{a}	83.83^{a}

The percentage of nanoencapsulated folic acid in 0.5% and 1.5% bile salt is represented in Figure 3. The maximum percentage of nanoencapsulated folic acid released was 55.89% in 1.5% bile salt after the exposure of 240 minutes. While the maximum percentage of nanoencapsulated folic acid was 50.32% in 0.5% bile salt after the exposure of 240 minutes.

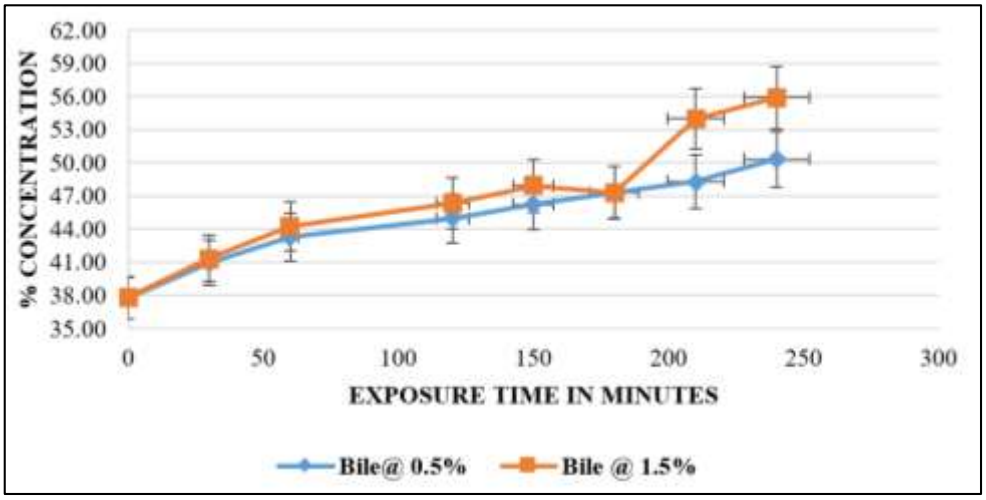

Fig 3: Percentage of nanoencapsulated folic acid released in bile

Kiaei Pour *et al*., (2020) [26] prepared the complex of alginate and pectin for the microencapsulation of folic acid. The alginate concentration used was 60%, 70%, 80% and 100% with pectin concentration of 20%, 30% and 40%. They exposed this complex in the gastric juice containing 0.6% bile salt. They reported the 50% to 70% folic acid release up to 240 minutes. Shakoor *et al.*, (2022) [52] carried out nanoencapsulation of folic acid using alginate and chickpea protein. They exposed the nanoencapsulated folic acid to the simulated GI fluid containing bile salt up to 4 hours. The reported ≈53% release of folic acid in the nanoencapsulated folic acid-chick pea complex. Agrawal *et al.*, (2014) [1] prepared the liposomes of folic acid. They reported ≈50% release of folic acid, when exposed to simulated intestinal fluid containing bile salts up to 240 minutes.

The form of folate in bile is polyglutamate (Pratt and Cooper, 1971; Arcot and Shrestha, 2005) [49, 4]. This polyglutamate in the small intestine is deconjugated to the monoglutamate form before absorption takes place and it is more stable than another forms (Melse-Boonstra *et al*., 2002) [37]. Delgado-Villa *et al*., (2009) [16] studied the effect of folic acid on bile salt. They prepared folic acid supplemented diet for the ethanol-fed rats and the study is grouped as control, alcohol, alcohol supplemented with folic acid and control supplemented with folic acid. They measured the bile acid, and folic acid supplementation significantly increased bile excretion. They also concluded that the increase in bile flow is responsible for decrease in serum and hepatic cholesterol. In our study, there is a gradual release of nanoencapsulated folic acid along with the gradual increase in the time, which may help to higher release of bile.

Conclusion

From the above studies, it may be concluded that, folic acid (150 µg), WPC (10%) and proline:lactate (1:1) ratio under specified conditions of electrospraying can be used to prepare

nanoencapsulated folic acid. Further, the maximum release of nanoencapsulated folic acid is found at pH 7 and 1.5% bile. The maximum absorption of the folic acid takes place in the small intestine, so the release of nanoencapsulated folic acid in the gastrointestinal conditions may be beneficial for the maximum bioavailability to the consumers.

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