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Optimization of folic acid nano-emulsification and encapsulation by maltodextrin-whey protein double emulsions

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ABSTRACT

Due to susceptibility of folic acid like many other vitamins to environmental and processing conditions, it is necessary to protect it by highly efficient methods such as micro/nano-encapsulation. Our aim was to prepare and optimize real water in oil nano-emulsions containing folic acid by a low energy (spontaneous) emulsification technique so that the final product could be encapsulated within maltodextrin-whey protein double emulsions. A non ionic surfactant (Span 80) was used for making nano-emulsions at three dispersed phase/surfactant ratios of 0.2, 0.6, and 1.0. Folic acid content was 1.0, 2.0, and 3.0 mg/mL of dispersed phase by a volume fraction of 5.0, 8.5, and 12%. The final optimum nano-emulsion formulation with 12% dispersed phase, a water to surfactant ratio of 0.9 and folic acid content of 3 mg/mL in dispersed phase was encapsulated within maltodextrin-whey protein double emulsions. It was found that the emulsification time for preparing nano-emulsions was between 4 to 16 h based on formulation variables. Droplet size decreased at higher surfactant contents and final nano-emulsions had a droplet size < 100 nm. Shear viscosity was higher for those formulations containing more surfactant. Our results revealed that spontaneous method could be used successfully for preparing stable W/O nano-emulsions containing folic acid.

1. Introduction

Folates are generally thought of as water-soluble vitamins belonging to the B group complex. As folates are synthesized only by microorganisms and plants, humans depend on a variety of dietary sources for the vitamin [1,2]. Folic Acid (FA), also known as pteroylglutamic acid, is the simplest form of folate, which is essential to human health facilitating the single carbon transfer reaction for the synthesis of basic constituents of DNA and RNA which provide the genetic basis of life [3]. FA is composed of three moieties (Fig. 1): a bicyclic 6-methylpterin ring, p-amino benzoic acid and a single molecule of L-glutamic acid, each of which has no vitamin activity when separated [4].

Research over recent decades has shown that low or inadequate folate concentrations may contribute to some malfunctions and disorders [2]. In many countries, particularly the developing populations, people suffer from folate deficiency. Cereal flours have been a primary candidate for fortification as they are consumed by most of the people [3,5,6]. Fortification with FA has some possible limitations since added vitamin may be lost during processing and handling due to its high sensitivity to heat, oxidation, pH, and other environmental conditions. Due to its properties as a vitamin and medicinal compound, FA must be protected against environmental conditions and should have a controlled release. There are several ways to encapsulate and protect hydrophilic bioactive components such as FA including liposomes, multiple emulsions, solid fat particles, biopolymer complexes, niosomes, and biologically derived systems [7,8].

Nano-emulsions are gaining increasing attention in the food and pharmaceutical industry as a novel delivery system for bioactive ingredients [9,10]. Real nano-emulsions are those containing dispersed phased droplet sizes of less than 100 nm, which are called micro-emulsions [11]. The potential benefits of micro-emulsions include optical clarity, high and favorable stability to gravitational separation, flocculation and coalescence, and improved absorption and bioavailability of functional and bioactive components [12,13]. A micro-emulsions is a thermodynamically stable system which forms spontaneously with a totally clear appearance [14].

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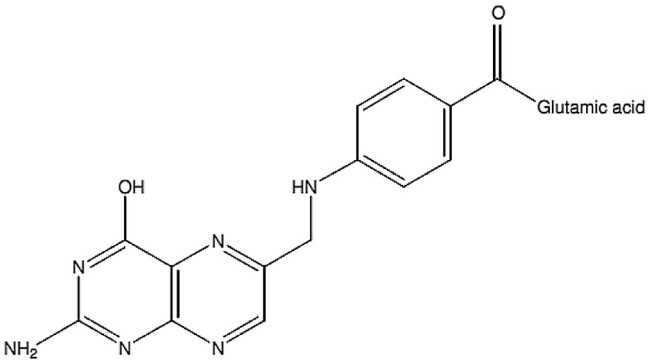

Fig. 1. Chemical structure of folic acid.

The pharmaceutical industry is the dominant field where most applications of nano-emulsions are proposed. Extensive research has been conducted on a variety of drug delivery systems with enhanced solubilization of poorly soluble drugs and improved bioavailability following incorporation into nano-emulsions [15,16]. In the food industry, many food-derived bioactive compounds demonstrate significant health benefits when consumed in relatively high concentrations. Unfortunately, most of these compounds exhibit poor solubility and bioavailability in aqueous-based foods. Recently the development of nano-emulsions loaded with lipophilic and hydrophilic food components has demonstrated the potential of nano-emulsions as a carrier to deliver these bioactive ingredients in food applications [17–19].

Nano-emulsions can be produced by high energy or low energy techniques [20]. High energy methods include homogenizing and sonication devices which are traditionally used in industrial operations because of the flexible control of emulsion droplet size distribution and ability to produce fine emulsions from a wide variety of materials [21,22]. Nano-emulsions produced using low energy methods are called micro-emulsions, which are produced using techniques such as phase inversion composition (PIC), phase inversion temperature (PIT), and spontaneous emulsification [23].

High levels of surfactant and co-surfactant, and long emulsification time limits application of low energy methods for food and pharmaceutical industries. The main goal of this research was optimization of producing micro-emulsions containing folic acid dispersed in canola oil through response surface methodology and its encapsulation within double emulsions of maltodextrin-whey protein in order to protect it from deteriorating environmental and process conditions. Our specific target was to reduce the usage of surfactant and reach the minimum time of emulsification by a low energy nano-emulsion formation technique.

2. Materials and methods

Folic acid (purity > 97%, molecular weight 441.4) and Span 80 (sorbitan mono oleate) was purchased from Sigma-Aldrich Co. (St. Louis, MO), and Merck Chemicals Co. (Germany), respectively. Canola oil was purchased from a local market. Double distilled water was used for preparing W/O micro-emulsions. Whey protein concentrate (80% protein) and maltodextrin (DE = 16–20) were obtained from Arla (Denmark) and Qinhuangdao Starch Co., respectively.

2.1. Micro-emulsion production

W/O micro-emulsions were prepared by spontaneous emulsification according to previously mentioned procedures for making O/W emulsions [24,25] with some modifications. Aqueous phase was prepared by mixing FA solution and Span 80 using a magnetic stirrer (IKA, Germany) at 1000 rpm and then added drop wise to oil phase while magnetically stirring.

2.2. Biopolymer solution preparation

Firstly, a 50% w/w maltodextrin solution was prepared by dissolving maltodextrin into deionized powder. Then, aqueous solution of whey protein concentrate was prepared by dispersing 8 g of WPC powder into deionized water to obtain 100 g solutions containing 0.02% sodium azide as an antimicrobial agent. Solutions were gently stirred for at least 30 min on a magnetic stirrer. Maltodextrin and WPC solutions (in a ratio of 1–1) were mixed together and stored overnight at room temperature for complete hydration of biopolymers and their pH was adjusted to 6.0 using HCL (0.1 M).

2.3. Preparation of W/O/W double emulsions

W/O/W double emulsions were prepared by gradually adding W/O nano-emulsions into the outer aqueous phase of mixed biopolymer solutions (WPC/maltodextrin) while blending by a homogenizer (Heidolph Silentcrusher, Germany) at 12000 rpm for 5 min at 10 °C, and then these coarse emulsions were further emulsified using mentioned homogenizer at 15000 rpm for 8 min at 10 °C [8].

2.4. Droplet size measurement

Droplet size of micro-emulsions was measured firstly through microscopic pictures taken from a Zeisss optical microscope (Germany) and analyzed by ImageJ software [26]. Some samples were analyzed simultaneously using a dynamic light scattering method (Zetasizer Nano Zs, Malvern Instrument, Malvern, UK). To avoid multiple scattering, all samples diluted using 0.1% SDS.

2.5. Shear viscosity

Effect of composition and preparation conditions on viscosity was measured using a Brookfield viscometer (LVDV Pro II, Brookfield Engineering Laboratories, USA) by a spindle S34 [27].

2.6. Color measurement

For evaluating the effect of composition and formulation conditions on color of micro-emulsions, color values ($L^*a^*b^*$) were measured by image analysis using Image J software [26]. We have reported just L value as it corresponds well with the transparent appearance of micro-emulsions.

2.7. Spray drying of double emulsions

The infeed double emulsions were transformed into encapsulated powders in a lab spray drier (Model SP1500, Fanyuan Instrument Co., Shanghai, China) equipped with a pressure air atomizing nozzle at 2.5 bar air pressure, inlet air temperature of 180 ± 5 °C, and outlet air temperature of 90 ± 5 °C with a feed flow rate of 450 mL/h. The dried powder was collected and stored in dark bottle, air tight containers at 4 °C until further analysis.

2.8. Encapsulation efficiency of folic acid

It was necessary to analyze encapsulated folic acid powders in terms of total content and surface content of folic acid in final powders. For surface content, 0.5 g of each sample was dispersed in 20 mL hexane, vortexed for 2 min and filtered using Whatman filter paper no. 41. After adding 10 mL ethanol, the folic acid content was determined spectrophotometrically at 282.5 nm

(Matias, Ribeiro et al., 2014). For total content, 0.1 g of powders was dissolved in 7 mL hexane and 3 mL phosphate buffer (pH 9.5). These solutions were ultrasonicated for 5 min and centrifuged at 3500 rpm for 20 min. The supernatant was withdrawn carefully without disturbing the cake left at bottom of Eppendorf, diluted sufficiently and analyzed for estimation of total folic acid available in nano-encapsulated powders. The encapsulation efficiency of folic acid nano-encapsulated within powders was calculated using formula [22]:

$$\%\,\text{Encapsulation efficiency} = \frac{\text{total amount of FA in formulations - amount of free FA present in supernatant}}{\text{total amount of FA within formulations}} \times 100.$$

2.9. Statistical analysis

Experiments (Table 1) were designed and analyzed by Central Composite Design pattern of Response Surface Methodology through Design Expert Software (version 7). There were three independent variables of FA content, dispersed phase to surfactant ratio, and dispersed phase volume fraction.

3. Results and discussion

3.1. Influence of formulation variables on droplet size of nano-emulsions

Our results (Table 2 and Fig. 2) revealed that in general, droplet size of produced nano-emulsions was smaller than 100 nm which shows it is possible to produce successfully real nano-emulsions containing folic acid through spontaneous emulsification. The smallest droplet size (32.5 nm) was obtained for treatment No. 4 (Table 2), i.e., the micro-emulsion prepared with 12% dispersed phase containing 1 mg/mL folic acid and a dispersed phase/surfactant ratio of 0.2. As it can be seen in Fig. 2A, by the increase of dispersed phase volume fraction and with the lower contents of Span as the surfactant, droplet size is increasing. This is quite predictable since in these conditions, number of dispersed water droplets containing folic acid and their surface area is increasing dramatically and due to limited surfactant concentration, these droplets cannot be stabilized by surfactant molecules and they tend to flocculate and merge together, resulting in coalescence and higher size of droplets [28–30]. Folic acid content didn't have any obvious influence on droplet size (Fig. 2B), but our experiments with higher concentrations of folic acid showed surprisingly some sedimentation (crystallization) of encapsulant. This phenomenon has been observed previously and is a results of temporary collisions between emulsion dispersed droplets [31–33]. Crystallization in micro-emulsions proceeds initially through the material exchange which can occur when two droplets collide and form a transient dimer. If one colliding droplet contains a nucleus, then its surrounding solute concentration (folic acid in our case) will be depleted, and the resulting concentration gradient in the transient dimer produces a solute flow into this depleted region, allowing the nucleus to grow [32].

Fig. 3 shows DLS results of emulsion droplet size and their distribution. As it can be seen, for all prepared nano-emulsion samples, the average peak of droplet size for dispersed phase (water containing folic acid) was below 100 nm. Interestingly, dynamic light scattering (DLS) results for size analysis of the emulsions were well compatible with microscopic image analysis results for the size of droplets. When comparing two similar nano-emulsion formulations with different surfactant concentrations (treatment 13 vs. treatment 16 with a dispersed phase to surfactant ratio of 0.2 and 1.0, respectively), it is clear that the sample with higher surfactant content results in a nano-emulsion with smaller droplet size (Fig. 3A). As mentioned before, higher surfactant content can stabilize emulsion droplets well against coalescence and by reducing surface tension at a higher rate, facilitates disruption of big droplets and formation of newly tiny droplets which are highly stable in micro-emulsion [20]. Fig. 3C is related to a formulation with medium surfactant content and surprisingly, its emulsion size distribution is something between highest surfactant content (Fig. 3A) and lowest one (Fig. 3B).

3.2. Viscosity of folic acid nano-emulsions

One of the most important parameters in preparing emulsions using low energy methods is minimizing viscosity to facilitate droplet break up and nano-emulsion formation [27,34]. Our results showed that by decreasing water/surfactant ratios, viscosity of nano-emulsions increased (Fig. 4A and C). In other words, at higher concentrations of Span surfactant, we observed higher viscosity levels which could be related to the higher viscosity of Span itself and also, formation of free micelles of surfactant which could result in higher viscosity. On the other hand, when dispersed phase volume fraction increases, again there is an increasing trend for viscosity which could be indirectly a result of higher consumption of surfactant. As it can be seen in Fig. 4, folic acid content does not have any influence on viscosity since its concentrations (1, 2, and 3 mg/mL of dispersed phase) are not such high to influence the viscosity of final nano-emulsions. Generally, we found that the most important factor affecting the viscosity of final nano-emulsions containing folic acid was surfactant concentration.

3.3. Influence of formulation variables on color of nano-emulsions

For evaluating the influence of formulation variables on color values of produced nano-emulsions, we analyzed "*L* value" or lightness since it reflects well the transparency of the samples which is very important for characterizing the formation of real micro-emulsions [35]. In fact, when droplet size of emulsions falls well below the visible wavelength of light (400–700 nm), their appearance would be observed like a transparent solution as there is no scattering of light by present particles [11,36]. So, the conversion of emulsion appearance from a milky state (nano-emulsions with big droplets > 700 nm) into a transparent display distinguishes the formation of real nano-emulsions with very small droplet sizes (<100 nm).

By looking at Fig. 5A, it is obvious that when the dispersed phase/surfactant ratio increases, lightness of nano-emulsions goes higher which could be explained by more contribution of water content in final color of emulsions and lower share of surfactant color. In fact, Span is brownish yellow liquid which lowers the L value, so when lower concentrations of Span are used, we expect higher L value. This trend could also be seen in Fig. 5C for the influence of dispersed phase/surfactant ratio on color.

3.4. Emulsification time of nano-emulsion preparations

Required time of emulsification in the preparation of nano-emulsions by low energy methods such as spontaneous emulsification is one of the limiting factors. Contrary to high energy emulsification techniques like ultra-sonication and high pressure homogenization which result in the formation of nano-emulsions in a very short time (less than a few minutes), the emulsification time in low energy techniques is very high (a few hours) due to

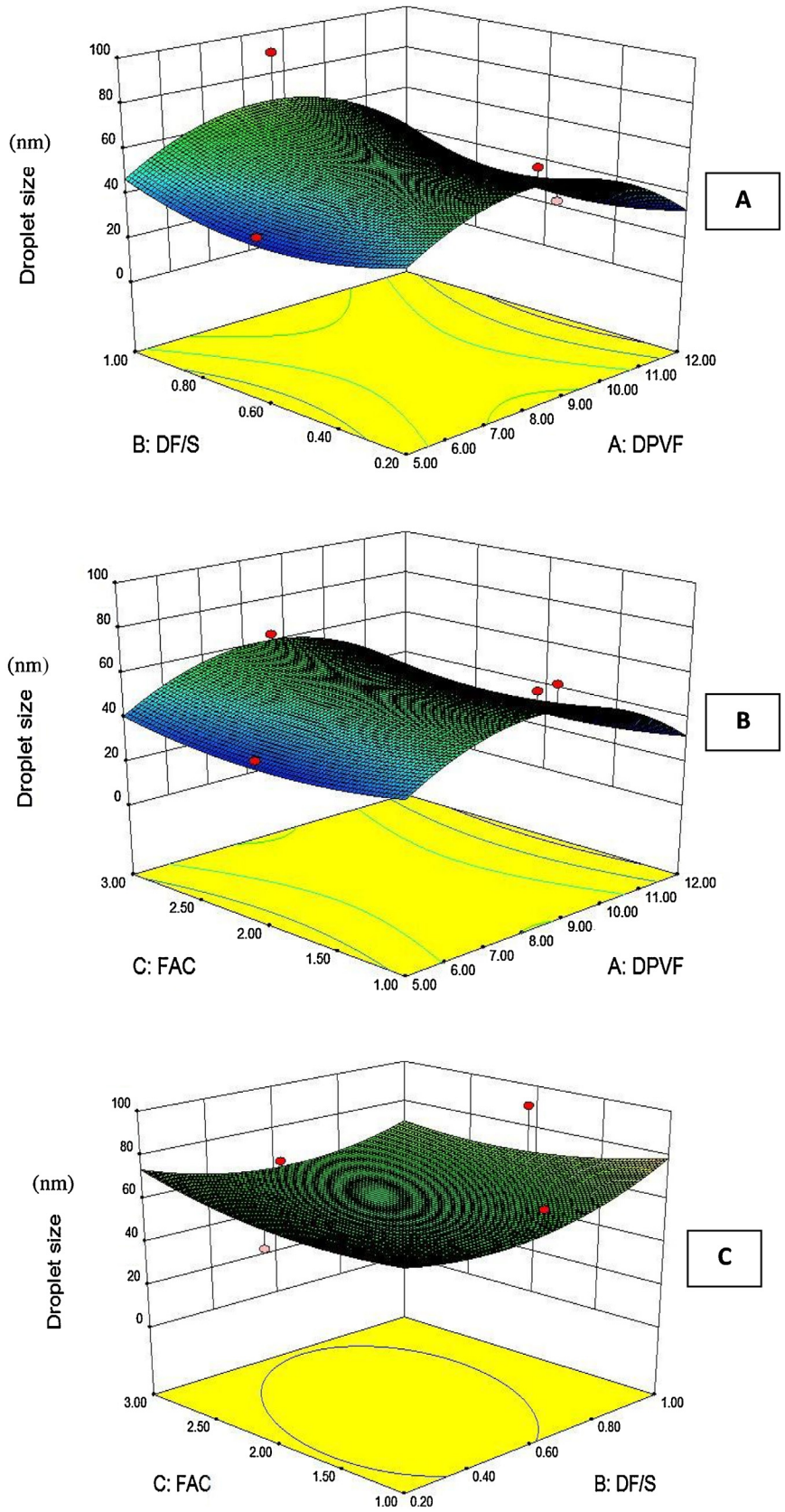

Fig. 2. RSM plots showing the influence of formulation variables on droplet size of nano-emulsions containing folic acid: FAC (folic acid content, mg/ml); DPVF (dispersed phase volume fraction,%); DF/S (dispersed phase to surfactant ratio).

Table 1
Experimental variables and their levels.

No	Variable (independent)	Level
1	Dispersed phase volume fraction[a] (%)	5.0, 8.5, 12.0
2	Dispersed phase/surfactant ratio	0.2, 0.6, 1.0
3	Folic acid content (mg/ml)	1, 2, 3

[a] By definition, it is the volume of dispersed phase divided by the volume of total emulsion multiplied by 100.

Table 2
Obtained results for different treatments designed by RSM methodology: DPVF (dispersed phase volume fraction); DF/S (dispersed phase to surfactant ratio); FAC (folic acid content).

Run no.	DPVF (%)	DF/S ratio	FAC (mg/ml)	Droplet size (nm)	Viscosity (mPa.s)	Color (*L* value)	Emulsification time (h)
1	8.5	0.6	3	63.5	76.3	66	4
2	5.0	0.6	2	38.5	67.5	66	7
3	8.5	0.6	2	44	76.3	64	10.5
4	12.0	0.2	1	32.5	266.4	55	14
5	5.0	0.2	1	49.5	94.6	59	6
6	12.0	1.0	1	47	73.3	66	15
7	12.0	0.6	2	36.5	87.8	62	14
8	5.0	1.0	3	46.5	65.4	65	9.25
9	5.0	1.0	1	51	63.7	70	7
10	5.0	0.2	3	58	96.0	61	6
11	8.5	0.6	2	45	76.3	66	10.5
12	8.5	1.0	2	90	68.6	68	12
13	12.0	0.2	3	49	267.9	58	16
14	8.5	0.6	2	47	77.3	66	10
15	8.5	0.6	1	71	75.8	66	8.5
16	12.0	1.0	3	38.5	75.2	66	13.5
17	8.5	0.2	2	54	152.7	57	15

DPVF (dispersed phase volume fraction,%); DF/S (dispersed phase to surfactant ratio); FAC (folic acid content, mg/ml).

lower energy density (amount of energy in a specified volume of emulsion) and enough time required to break dispersed droplets through stirring and adsorption of surfactant molecules onto interface between water and oil phases [21,37].

For preparation of nano-emulsions containing folic acid by spontaneous emulsification, we recorded a time between 4 to 16 h based on the formulation parameters. The lowest emulsification time was related to treatment no. 1 (Table 2) with 8.5% dispersed phase volume fraction, a water/surfactant ratio of 0.6, containing 3 mg/mL folic acid within the dispersed phase. On the other hand, the longest emulsification time was recorded for treatment no. 13 with 12.0% dispersed phase content, water/surfactant ratio of 0.2 and 3 mg/mL folic acid (Table 2). In fact, when we applied the highest dispersed phase content, and the maximum folic acid concentration, it resulted in the longest emulsification time. This is quite predictable since by increasing dispersed phase volume fraction, number of droplets and their surface area for preparation of nano-emulsions increases exponentially [20]. At the same time, there is limited concentration of surfactant molecules to stabilize these tiny droplets with the highest content of folic acid. Therefore, the required time for emulsification and production of final nano-emulsions increases dramatically, almost 4 times higher than the required time for treatment no. 1. Our results in Fig. 6 confirm these trends.

3.5. Optimization results for nano-emulsification of folic acid

Finally, in order to obtain the best formulation based on spontaneous emulsification in terms of lowest usage of surfactant (or higher dispersed phase to surfactant ratio), lowest emulsification time, and highest concentration of folic acid, we applied the optimization tool of RSM technique (Table 3). It was found that a nano-emulsion formulation with 12% dispersed phase volume fraction, a water to surfactant ratio of 0.9 and folic acid content of 3 mg/mL of dispersed phase could be suggested to have the optimum conditions. For validation, we reproduced the same emulsion

Table 3
Optimization procedure and results for optimum formulation to produce nano-emulsions containing folic acid.

Name	Goal	Lower Limit	Upper Limit	Lower Weight	Upper Weight	Importance
A:DPVF	Maximize	5	12	1	1	4
B:DF/S	Maximize	0.2	1	1	1	4
C:FAC	Maximize	1	3	1	1	5
Droplet size	Maximize	32.5	90	1	1	3
Viscosity	Maximize	63.7	267.9	1	1	3
Color L	Maximize	55	70	1	1	2
Time	Maximize	4	16	1	1	2

Solutions	DPVF	DF/S	FAC	Drop size	Viscosity	Color L	Time	Desirability	
1	12	0.89	3	39.6811	77.5928	65.7592	11.677	0.843	Selected
2	12	0.89	3	39.8816	77.1304	65.7612	11.732	0.843	
3	12	0.89	3	39.4454	78.1656	65.7575	11.612	0.843	
4	12	0.89	3	40.1256	76.5939	65.7597	11.8	0.842	
5	12	0.89	3	40.3886	76.051	65.7582	11.872	0.842	

DPVF (dispersed phase volume fraction,%); DF/S (dispersed phase to surfactant ratio); FAC (folic acid content, mg/ml).

by suggested formulation conditions and resulted responses for droplet size, emulsion viscosity and color, and emulsification time were not significantly different compared with those values predicted by the software.

3.6. Folic acid encapsulation efficiency within double emulsions

The encapsulation efficiency for spray dried powders produced from double emulsions of maltodextrin-WPC containing folic acid within the internal aqueous phase (Fig. 7) was equal to 86.60%. In other words, more than 86% of total folic acid was encapsulated within powder particles and less than 14% was remained un-encapsulated at the surface of particles. This finding reveals that double emulsions prepared by 25.0% maltodextrin and 4.0% WPC had a highest stability and low release of folic acid from

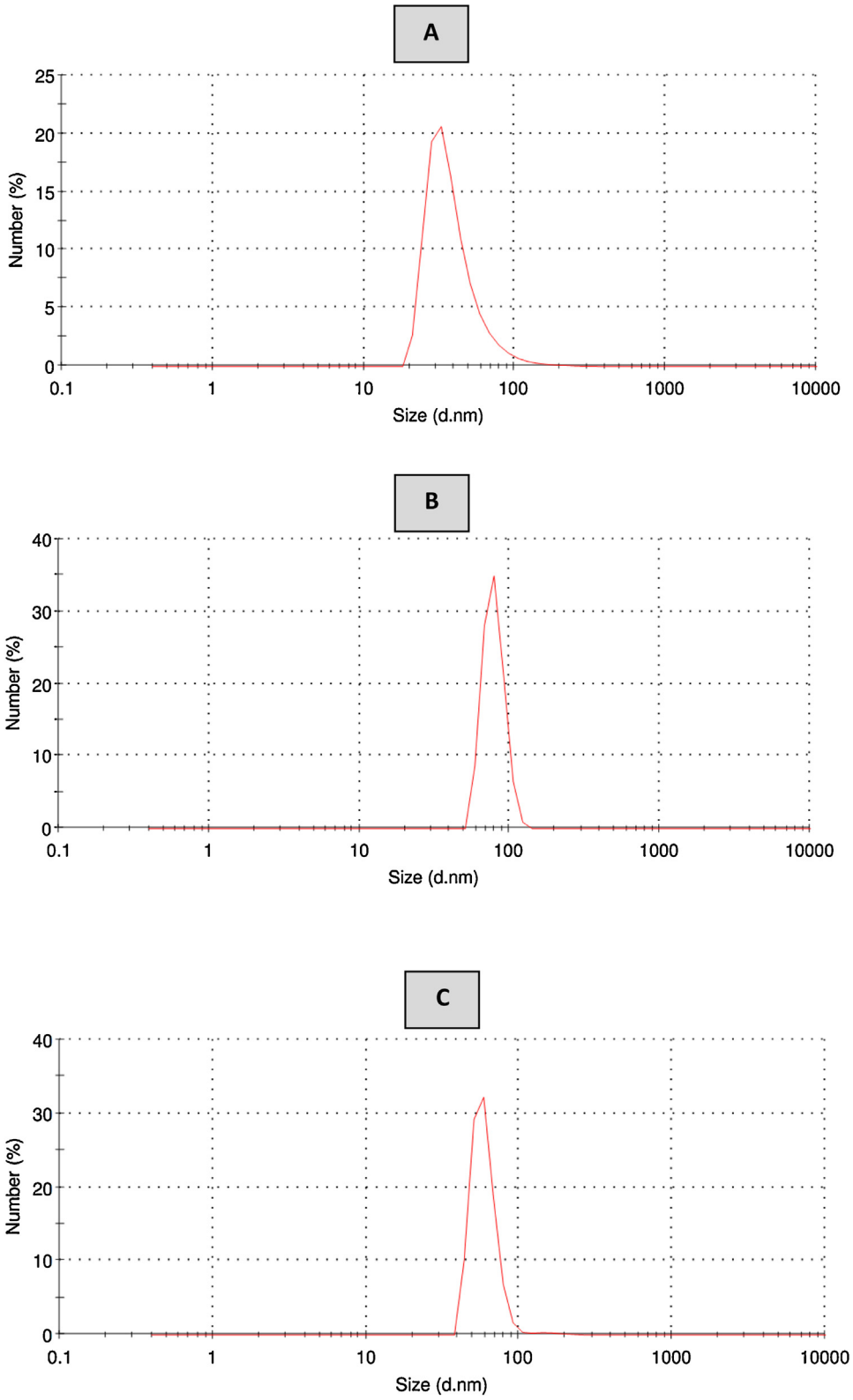

Fig. 3. Droplet size analysis results of nano-emulsions containing folic acid by Dynamic Light Scattering (DLS) technique. (A) 12% dispersed phase volume fraction containing 3 mg/mL folic acid with a dispersed phase to surfactant ratio of 0.2 (treatment 13); (B) 12% dispersed phase volume fraction containing 3 mg/mL folic acid with a dispersed phase to surfactant ratio of 1.0 (treatment 16); (C) 12% dispersed phase volume fraction containing 2 mg/mL folic acid with a dispersed phase to surfactant ratio of 0.6 (treatment 7).

internal nano-emulsions into outer aqueous phase of double emulsions, which results in high encapsulation efficiency. In this study, dispersed phase itself was a W/O nano-emulsion composed of surfactant (Span), oil, and water containing folic acid. Increase in dispersed phase content results in an increase in the dispersed phase volume of double emulsions which could result in bigger

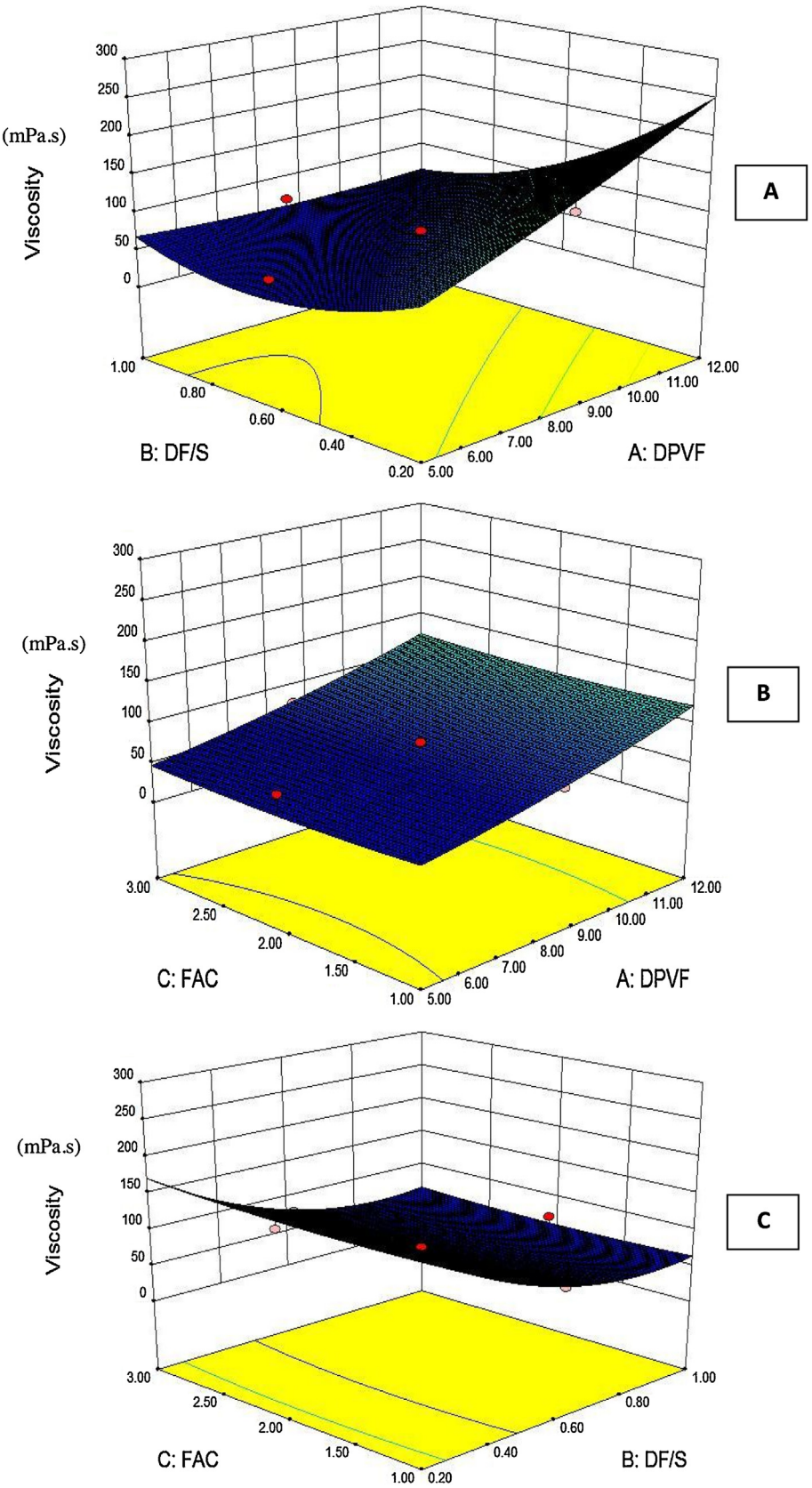

Fig. 4. RSM plots showing the influence of formulation variables on viscosity of nano-emulsions containing folic acid: FAC (folic acid content, mg/ml); DPVF (dispersed phase volume fraction,%); DF/S (dispersed phase to surfactant ratio).

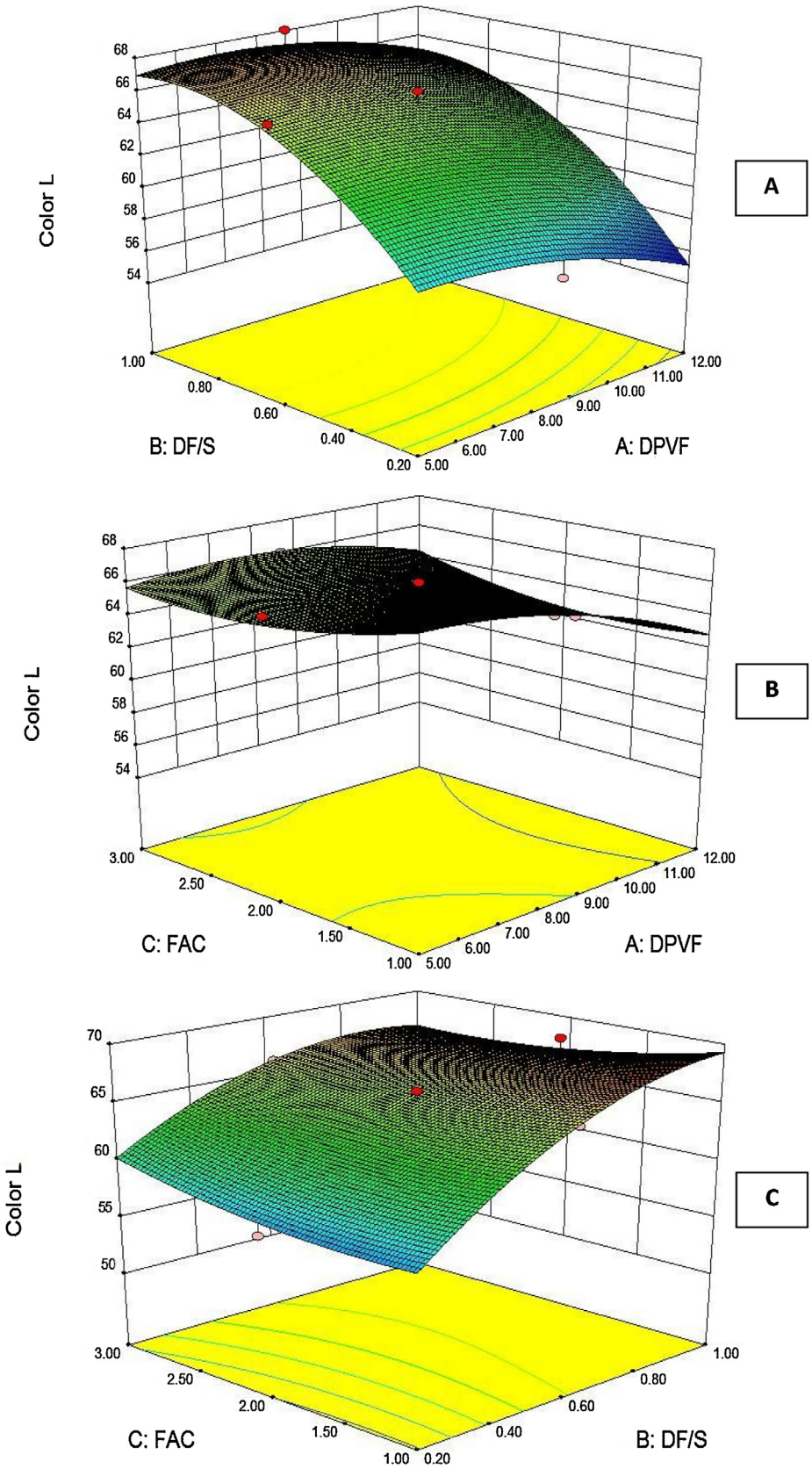

Fig. 5. RSM plots showing the influence of formulation variables on color (L value) of nano-emulsions containing folic acid: FAC (folic acid content, mg/ml); DPVF (dispersed phase volume fraction,%); DF/S (dispersed phase to surfactant ratio).

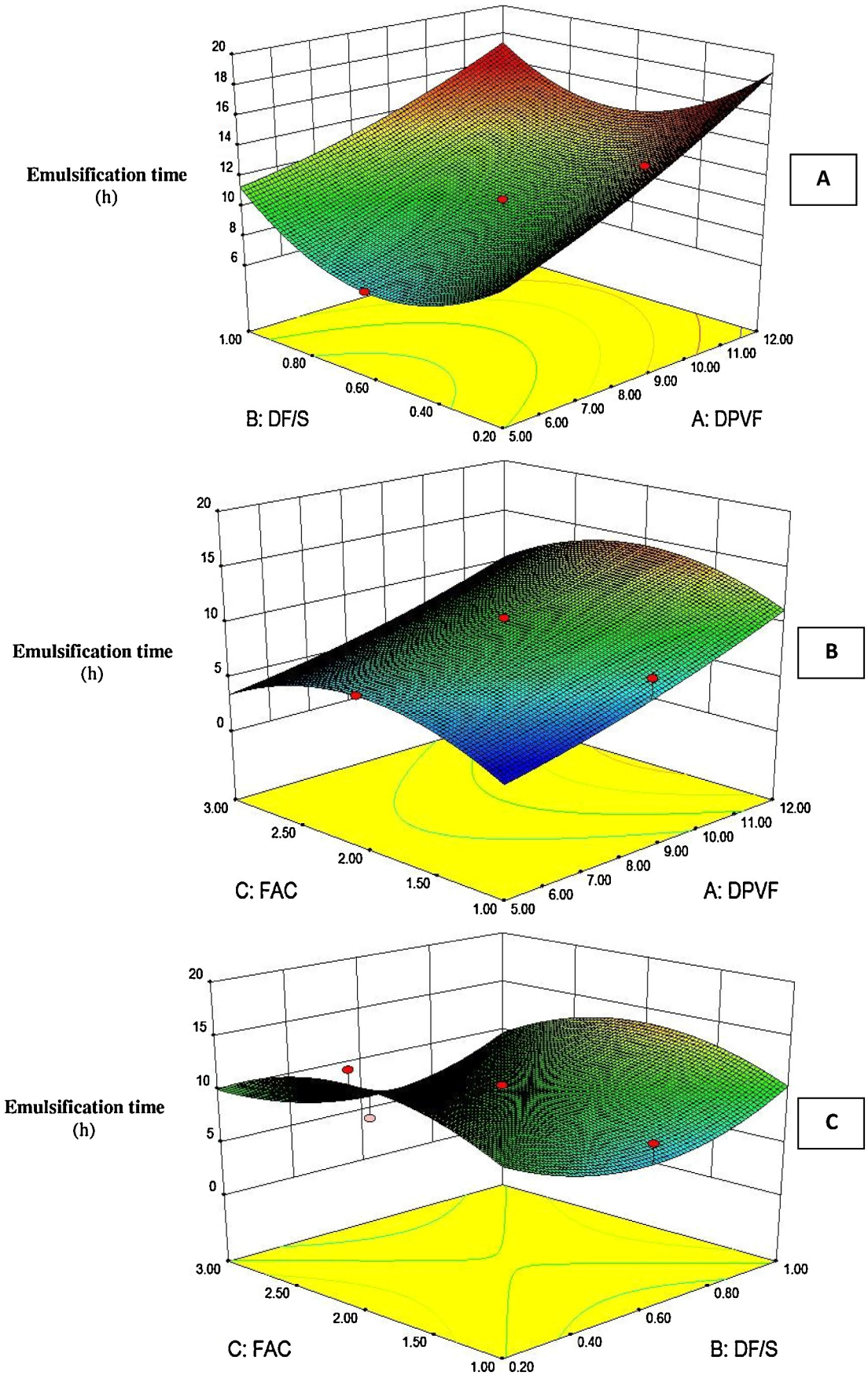

Fig. 6. RSM plots showing the influence of formulation variables on preparation time of nano-emulsions containing folic acid: FAC (folic acid content, mg/ml); DPVF (dispersed phase volume fraction,%); DF/S (dispersed phase to surfactant ratio).

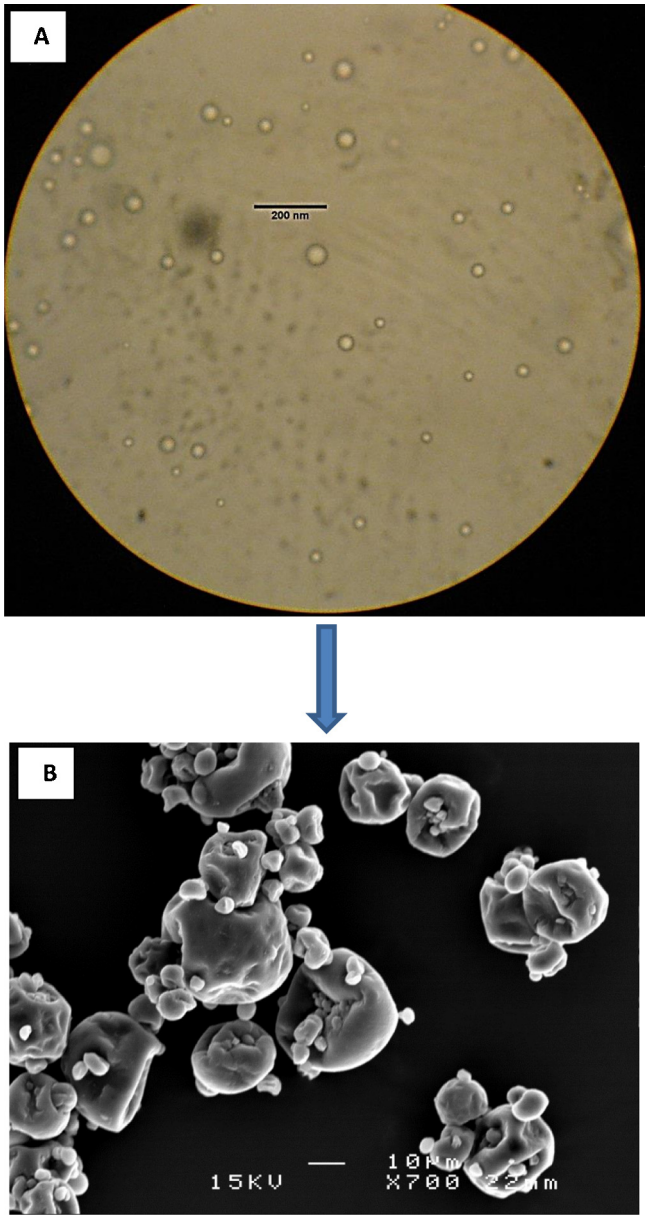

Fig. 7. (A) Microscopic image showing initial prepared nano-emulsions (scale bar = 200 nm) containing folic acid within dispersed droplets; (B) Final spray dried encapsulated powders produced from maltodextrin-whey protein double emulsions containing initial nano-emulsions.

dispersed droplets with lower emulsion stability [20]. Thus, folic acid molecules within the internal phase (dispersed droplets) could easily release and exit into external aqueous phase of final double emulsions without any protection from biopolymer layer of maltodextrin-WPC and led to lower encapsulation efficiency.

pH strongly affects both the net charge of biopolymers and the interactions between them. Moreover, the net surface charge (Zeta- potential) of the emulsion droplets affects the emulsion stability. In our preliminary tests, it was found that at pH 5, which is the isoelectric point of the solution of WPC biopolymers, they are almost neutralized, forming relatively large precipitating aggregates (flocs). Because the absorption of the large flocculated aggregates on the oil/water interface is poor and incomplete, the repulsion between the droplets is also insufficient. At pH 6, there are several positively charged groups on the WPC backbone, adding some beneficial repulsive forces between the droplets and improved emulsion stability.

4. Conclusion

Since folic acid is a vital bioactive component for the body, in this study we evaluated preparation of nano-emulsions containing folic acid by a low energy emulsification technique and its encapsulation within maltodextrin-whey protein double emulsions in order to protect it from detrimental conditions of the environments and possible processes during fortification of foods. The main goal was to introduce a simple nano-emulsion preparation method with the lowest emulsification time and surfactant usage. By analyzing some important nano-emulsion properties including their droplet size and color, it was found that the most important parameter affecting final emulsion responses was surfactant concentration. Another interesting result was crystallization of folic acid molecules at higher concentrations of this vitamin and lower surfactant content which was attributed to droplet collision within micro-emulsions and then, precipitation. Analysis of our results through RSM technique revealed that a nano-emulsion formulation with 12% dispersed phase volume fraction, a water to surfactant ratio of 0.9 and folic acid content of 3 mg/mL of dispersed phase was the optimum formulation. These nano-emulsions can be applied in fortification of liquid foods such as beverages and fruit juices. But for many food products such as flour, bakery products, etc., it is necessary to convert them into a powder form which was achieved by spray drying encapsulation of optimum folic acid nano-emulsions within double emulsions of maltodextrin-whey proteins with high encapsulation efficiency.

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