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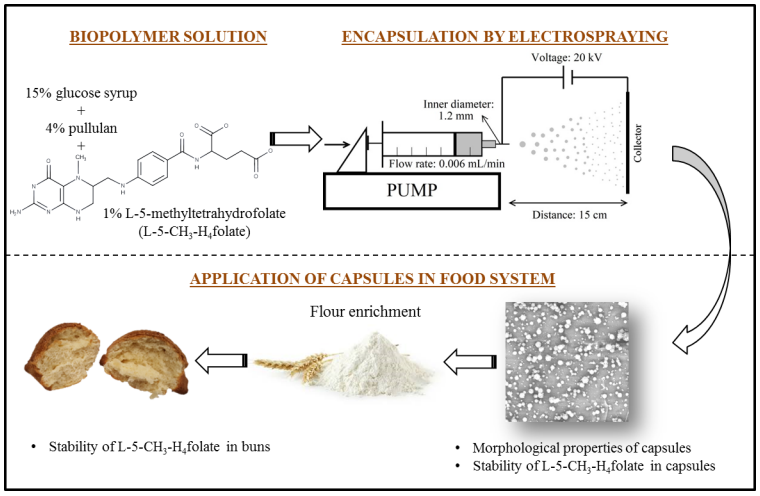

Title:

Encapsulation of L-5-methyltetrahydrofolate by electrospraying for food applications

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Abstract:

Biologically active natural folate form L-5-methyltetrahydrofolate is less stable than synthetic folate form folic acid commonly used for food fortification. The production of electrosprayed capsules using a combination of carbohydrates such as glucose syrup and pullulan was investigated to provide higher stability of L-5-methyltetrahydrofolate in food products. Additionally, the protective effect of ascorbic acid, if added to the biopolymer solution for capsules production was investigated. During the production of electrosprayed capsules containing L-5-

methyltetrahydrofolate, both with and without ascorbic acid, the recovery of folate was >97%. During storage at 22°C for 21 days, the electrosprayed capsules (with or without ascorbic acid) showed significantly higher folate recovery compared to free L-5-methyltetrahydrofolate (91% vs. 61%, $p \leq 0.05$). In buns baked with all-purpose flour fortified with the free form or the electrosprayed capsules, which were stored at 22 ºC for 9 days, no significant difference was shown for the retention of the L-5-methyltetrahydrofolate.

Keywords:

L-5-Methyltetrahydrofolic acid; Fortification; LC-MS/MS; Food; Stability; Retention

Abbreviations:

NTDs, Neural tube defects; 5-CH_3-H_4folate, 5-methyltetrahydrofolate, natural form; L-5-CH_3-H_4folate, L-5-methyltetrahydrofolate, synthetic form; Asc, Ascorbic acid; *Capsules – Asc*, Capsules without ascorbic acid; *Capsules + Asc*, Capsules containing ascorbic acid

1. Introduction

5-methyltetrahydrofolate (5-CH_3-H_4folate) is the predominant naturally occurring form of folate or vitamin B_9 group. This group of biologically active compounds plays an important role in human health as it is vital for one-carbon transfer and normal cellular functions such as methylation and regeneration of DNA, where 5-CH_3-H_4folate is involved in biologic processes (Scaglione and Panzavolta, 2014). Adequate folate intake prevents the development of neural tube defects (NTDs), anemia, cardiovascular diseases, cancer, and degenerative cognitive diseases (Bailey et al., 2015). Even though naturally occurring folate is widely distributed in food, its intake is often insufficient (Obeid et al., 2016). More than 260,000 pregnancies worldwide are estimated to be affected by NTDs (Garrett and Bailey, 2018), whereas 44% of the pregnancies are unintended, which also

contributes to the birth defects risk as folate plays a significant role in the first months after conception (Bearak et al., 2018). In order to reduce the prevalence of mentioned diseases and to improve the nutritional quality of the food supply, FDA established mandatory fortification of cereal-grain products among the others; flour, bread, macaroni products in the USA and Canada by folic acid, a synthetic and the most stable folate form (Food and Drug Administration, 1996). In the following years, 81 countries mandated fortification of wheat flour, maize flour, or rice by folic acid, which resulted in a significant reduction of NTDs. This led to the conclusion that food fortification by folic acid should be part of most national public health strategies (Garrett and Bailey, 2018). However, folic acid as a synthetic folate form has to pass additional metabolic processes in order to convert to biologically active folate form 5-CH_3-H_4folate. Upon the chronic intake of synthetic form (>200 µg/day), un-metabolized folic acid is circulating in the body, which results in the occurrence of adverse health effects (Stolzenberg-Solomon et al., 2006) and masking of vitamin B_{12} deficiency (Green et al., 2013). The use of supplements and voluntary fortification of food products with folic acid are recommended in the European Union, but not mandatory (European Union, 2002, 2006). There is a concern about the occurrence of adverse health effects, which presumably resulted in a decrease of voluntary fortification by folic acid in some countries such as Spain (Samaniego-Vaesken et al., 2017). A fortification of 150 µg folic acid per 100 g of food showed to be effective in the reduction of the prevalence of NTDs and the increase in blood folate concentrations in the USA and Canada (Berry et al., 2010).

L-5-CH_3-H_4folate, Metafolin, a synthetic form of natural 5-CH_3-H_4folate is a biologically active folate form that does not have to undergo additional metabolic conversion, it does not mask B_{12} deficiency as folic acid does and it is more suitable for food fortification (Obeid et al., 2013; Pietrzik et al., 2010). However, L-5-CH_3-H_4folate is very unstable under various environmental conditions such as changes in pH, temperature, oxygen, or light exposure, which makes its

incorporation to food systems challenging (Liu et al., 2012). Green et al. (2013) studied the bioavailability of encapsulated L-5-CH_3-H_4folate and folic acid from fortified wheat rolls and found no differences in bioavailability, indicating that replacing folic acid by encapsulated L-5-CH_3-H_4folate would reduce concerns regarding the masking of B_{12} deficiency and adverse health effects of chronic consumption of folic acid.

Due to its low stability, the protection of L-5-CH_3-H_4folate in the food system and gastrointestinal tract is desirable. Encapsulation has been proposed as a new technology for packing materials in the form of micro- or nanostructures by entrapping bioactive compounds and protecting them by another substance in order to increase the stability of bioactive compounds during processing and storage (Dias et al., 2015). Spray-drying is the most commonly used encapsulation technique. However, despite the short processing time, spray-drying can cause deterioration of labile ingredients such as folate due to the contact of the droplet/particle with air at high temperature (100-220 °C) in the drying chamber (Jacobsen et al., 2018). Electrospray has shown to be a good alternative to spray-drying since no high temperatures are used during the process (García-Moreno et al., 2018a). Its relevance for the encapsulation of thermally sensitive bioactive compounds is supported by recent research based on the increase of the productivity of the process (Busolo et al., 2019). This technique is based on the use of a high voltage electric field to generate dry polymeric particles. In electrospray, the jet ejected from the Taylor cone breaks down into fine charged droplets from where the solvent evaporates during the flight to a grounder collector, and the result is the formation of nanoparticles (Ghorani and Tucker, 2015). Previous studies examined the encapsulation of L-5-CH_3-H_4folate using coating materials such as pectin, skimmed milk powder, and modified starch during spray-drying(Kitts and Liu, 2015). The recovery, defined as a ratio of concentrations of L-5-CH_3-H_4folate in the final product after the encapsulation and the one initially added to the dry mixture, ranged from 70 to 91%.

92 The properties of biopolymer solutions (e.g., viscosity, surface tension, conductivity), as well as of
93 the solvent (e.g., vapor pressure), highly affect electrospraying process such as stability of Taylor
94 cone and morphology of the capsules (Jacobsen et al., 2018; Tapia-Hernández et al., 2017). Pectin
95 and alginate are often used for encapsulation of folic acid due to the low cost and biocompatibility,
96 even though coating materials such as stearate, skimmed milk powder, and modified starch have
97 also been used (Alborzi et al., 2013; Kitts and Liu, 2015). In this study, glucose syrup was used as
98 the main carbohydrate due to its ability to form capsules of appropriate morphology by
99 electrospraying, whereas pullulan was used to stabilize the electrospraying process (García-Moreno
100 et al., 2018b; Hermund et al., 2019). Glucose syrup is a common and low-cost coating material used
101 for the encapsulation of bioactive compounds for application in food (García-Moreno et al., 2018b).
102 Furthermore, glucose syrup-electrosprayed capsules have lower oxygen permeability when
103 compared to higher molecular weight carbohydrates such as dextran (Boerekamp et al., 2019). The
104 latter is of special importance for the protection of oxidative unstable bioactives such as L-5-CH_3-
105 H_4folate. Besides, the protective effect of ascorbic acid (Asc) against oxidation was also examined,
106 as L-5-CH_3-H_4folate is instable under various environmental conditions (Kitts and Liu, 2015).

107 To the best of our knowledge, the encapsulation of L-5-CH_3-H_4folate by electrospray, that could
108 provide even higher protection of the bioactive compound than spray-drying, remains to be
109 investigated. Electrospraying is performed at room temperature, which is convenient for
110 encapsulation of thermally sensitive compounds such as L-5-CH_3-H_4folate. Furthermore, the
111 reduced capsule size compared to spray-drying enables easier incorporation in a food matrix (Kitts
112 and Liu, 2015). As the main ingredient in bakery products being already used for fortification by
113 folic acid, all-purpose flour seems to be a good candidate to be fortified with 5-CH_3-H_4folate. Due
114 to the costliness of the pure 5-CH_3-H_4folate, the synthetic Metafolin form of L-5-CH_3-H_4folate was
115 used in this study as it was proposed by previous studies (Liu et al., 2015, 2013).

116 The study aimed to investigate the encapsulation of L-5-CH_3-H_4folate by electrospraying using a
117 combination of carbohydrates, such as glucose syrup and pullulan, as coating materials. The study
118 included optimization of the electrospraying process and testing the oxidative stability of the
119 capsules loaded with L-5-CH_3-H_4folate during 21 days of storage. In addition, the protecting effect
120 of encapsulation during processing was tested by studying the fortification of buns produced with
121 all-purpose flour containing electrosprayed capsules loaded with L-5-CH_3-H_4folate.

122 ## 2. Materials and methods

123 ### 2.1. Materials

124 Glucose syrup (DE38, C*Dry 1934) was kindly provided by Cargill Germany GmbH (Krefeld,
125 Germany). Pullulan (molecular weight = 200,000 Da) and Tween-20 (polyethylene glycol sorbitan
126 monolaurate) were provided by Hayashibara Co., Ltd. (Okayama, Japan) and Sigma-Aldrich
127 (Steinheim, Germany), respectively. L-5-Methyltetrahydrofolic acid (L-5-CH_3-H4folate, (6S)-5-
128 methyltetrahydrofolic acid, calcium salt, Metafolin) was provided from Merck & Cie
129 (Schaffhausen, Switzerland). All-purpose flour and granulated sugar (Vores bagværk), salt, instant
130 dry yeast (Malteserkors), eggs (Barkholt), and sunflower oil were bought from the local
131 supermarket (Copenhagen, Denmark).

132 ### 2.2. Optimization of biopolymer solutions for electrospraying process

133 The main compounds of the electrospraying solution, such as glucose syrup (15%) and pullulan
134 (4%), were selected based on previous work (García-Moreno et al., 2018b). The experiments
135 involved in the optimization of the electrospraying process included the addition of 1% Asc, 1%
136 Tween-20 surfactant, and the use of 3-5 Microfluidizer passes (M110L Microfluidics, Newton, MA,
137 USA). The addition of Asc to the electrospraying solution was carried out to test its effect on the
138 stability of L-5-CH_3-H_4folate in a further study during the electrospraying process, storage, and

processing of the capsules. Tween-20 addition, which reduces the surface tension of the biopolymer solution, and passing the biopolymer solution through a high-pressure homogenizer (e.g., Microfluidizer), which breaks biopolymer chains reducing polymer entanglements, were also carried out in order to evaluate their influence on electrosprayability.

Briefly, pullulan and glucose syrup were dissolved in distilled water under constant stirring (700 rpm) for 30 min at room temperature. Asc was added, dissolved, and further homogenized in a Microfluidizer (M110L Microfluidics, Newton, MA, USA) equipped with a ceramic interaction chamber (CIXC, F20Y, internal dimension 75 µm). The solutions for electrospraying were homogenized at a pressure of 9,000 psi, with 3 passes, whereas 5 passes were also tested. In order to investigate the effect of the surfactant in the process of electrospraying, 1% Tween-20 was added to the solution before and after homogenization. L-5-CH_3-H_4folate was included in the optimization process after the homogenization of the solutions produced in optimal conditions. Solution with L-5-CH_3-H_4folate was stored in a brown glass vial, atmospheric oxygen was substituted by nitrogen, and the vial was slowly turned up and down by hand for 3 min until L-5-CH_3-H_4folate was completely dissolved. The resulting solutions for electrospraying contained 1% wt L-5-CH_3-H_4folate. Two different solutions were tested: one without Asc (*Capsules – Asc*) and one with 1% Asc (*Capsules + Asc*). The biopolymer solutions were prepared up to 3 days before electrospraying and stored at room temperature in brown glass vials. L-5-CH_3-H_4folate was added to the solutions right before the electrospraying process in order to avoid oxidation and possible light degradation. All techniques and analysis including L-5-CH_3-H_4folate were performed under subdued light using brown glass in order to protect photo-sensitive folates.

2.3. Electrospraying process

Electrospraying process was performed at room temperature and environmental conditions of relative humidity (26-50 %). Biopolymer solutions were added to a syringe, which was placed in a syringe pump (New Era Pump Systems, Inc., USA). A 16G needle (Proto Advantage, Canada) was used. A high-voltage electrostatic field was supplied by a high voltage power supply (Gamma High Voltage Research, USA), and applied between the spinneret and a stainless steel 15x15 cm collector plate. The system was set horizontally, where the distance between needle tip and collector was 15 cm. In order to stabilize the Taylor cone and avoid dripping of the solution on the collector, different flow rates and voltages in the ranges of 0.001-0.007 mL/min and 16-20 kV were tested, respectively.

The productivity of the electrospraying process was calculated as the weight (mg) of powder collected in 1 h, and it was used during the optimization of the process.

2.4. Characterization of electrosprayed capsules

2.4.1. Morphology

A Scanning Electron Microscopy (SEM) (Phenom-World B.V., Eindhoven, The Netherlands) was used in order to examine the morphology of the capsules produced by electrospraying. The capsules from different experiments were collected on the foil and kept in a desiccator in order to examine the effect of Asc and surfactant addition, as well as the use of different Microfluidizer passes. Approximately 0.5cm^2 of aluminum sheet covered with a thin layer of electrosprayed capsules was placed on carbon tape and sputter-coated with gold, 8s, 40 mA utilizing a Q150T Quorum Coater (Quorum Technologies Ltd, East Sussex, UK). The capsule diameter distribution was determined from the micrographs by using an image processing program ImageJ (National Institutes of Health). Size distributions were obtained from a minimum of 100 measurements.

2.4.2. Oxidative stability of L-5-CH_3-H_4folate

Immediately after the electrospraying, a part of the capsules was stored in a desiccator in darkness at room temperature, and the other part at -80 °C. The content of L-5-CH_3-H_4folate was quantified the day the capsules were produced and after a 1-month storage at -80 °C. Furthermore, 1% L-5-CH_3-H_4folate in water solution was prepared and used as a control representing non-encapsulated folate. It was stored at room temperature (22 °C) in darkness for 21 days together with fresh *Capsules + Asc* and *Capsules – Asc*, to simulate storage conditions of cereal-grain products that are commonly stored at room temperature. Vials (4 mL) contained approximately 1 mL of control solution and 150 mg of capsules in order to maintain a similar headspace. At days 0, 3, 7, 14, and 21, three subsamples of each sample were analyzed for the content of L-5-CH_3-H_4folate.

2.5. Stability study in fortified buns

2.5.1. Flour fortification

L-5-CH_3-H_4folate was added, in a free form, or the encapsulated forms (C*apsules – Asc*, and *Capsules + Asc*) to a small amount of flour. This small amount of the mixture was dispersed using mortar and pestle before being mixed with the rest of the flour by a commercial-grade mixer. The final concentration of L-5-CH_3-H_4folate was aimed to be ~150 µg/100 g of flour. Six subsamples of each of the fortified flour were sampled for quantification of L-5-CH_3-H_4folate content, in order to ensure even distribution. Unfortified flour was also analyzed for the folate content (n=3) and was used for the preparation of buns, which was necessary for the calculation of folate retention.

2.5.2. Buns production

12 g of active dry yeast was mixed with 20 g of sugar and 100 mL of water. Yeast was mixed with one egg, 40 g of oil and 400 g of flour was added with 1 tsp salt. The elastic dough was kneaded and separated into 15 balls, from which 12 were placed on a baking tray as presented in Supplementary online material (SOM, Fig. S1), and 3 were analyzed as the dough. Buns were

baked at 200°C for 7.5 min until they were risen and golden brown. Three baking batches were carried out, representing three replicates of processing conditions, where four buns were baked in each batch. They were separated after baking to represent different storage conditions, as it is shown on Fig. 1. The buns were weighed before and after the processing, and folate content was analyzed before and after the thermal procedure.

Figure 1 The design of the storage experiment in buns. It included retention study during baking (1) and stability study during storage for 0, 3, 6, and 9 days (2). Three replicates were sampled at each sampling point. *indicate dough samples sampled for the calculation of the retention (n=3).

2.6. Quantification of 5-CH_3-H_4folate

The quantification of 5-CH_3-H_4folate was performed with a slightly modified version of the LC-MS/MS method described in detail elsewhere (Ložnjak et al., 2019). Instead of 1 g of samples, 0.01 g of capsules were dissolved in 25 mL of folate extraction buffer (50 mM phosphate buffer, 1% Asc, 0.1% β-mercaptoethanol, pH 6.0), and diluted to the concentration of approximately 500 ng/mL. When fortified flour or buns were analyzed, 0.5 g of the sample was mixed with 10 mL of folate extraction buffer. $^{13}C_5$-5-CH_3-H_4folate internal standard was added for quantification. Samples were shaken vigorously after 15 min and cleaned by strong anion exchange solid-phase extraction prior to detection and quantification by LC-MS/MS (Ložnjak et al., 2019).

2.7. Recovery and retention of 5-CH_3-H_4folate

Calculations of recovery and retention of L-5-CH_3-H_4folate was exemplified by 5-CH_3-H_4folate in Eq. 1 and 2. The recovery of 5-CH_3-H_4folate in capsules was calculated according to Eq. 1:

$$Recovery\ (\%) = \frac{concentration\ of\ 5\text{-}CH_3\text{-}H_4\text{folate}_{\ quantified}}{concentration\ of\ 5\text{-}CH_3\text{-}H_4folate_{added}} * 100 \quad [1]$$

The retention of 5-CH_3-H_4folate in buns was calculated according to Eq. 2:

$$Retention\ (\%) = \left(\frac{\mu g\ 5\text{-}CH_3\text{-}H_4\text{folate}\ \ per\ 100\ g\ of\ buns * amount\ of\ buns}{\mu g\ 5\text{-}CH_3\text{-}H_4\text{folate}\ \ per\ 100\ g\ of\ dough * amount\ of\ dough}\right) * 100 \quad [2]$$

2.8. Statistical analysis

All results from the stability study of L-5-CH_3-H_4folate in capsules and buns were presented as means of three independent experiments, i.e., buns were prepared in three batches. One-way ANOVA was used to test the agreement in the recovery of L-5-CH_3-H_4folate between various samples within the day, and between different treatments. Tukey-Kramer test was used to examine the differences between the recovery of L-5-CH_3-H_4folate in the samples with free and two different types of encapsulated folate in capsules and buns. A p-value ≤ 0.05 was classified as a significant difference. Statistical analyses were performed using JMP® Statistical Discovery software version 13.0 (SAS Institute Inc. Cary, NC, USA). The results were given as a mean ± standard deviation (SD).

3. Results and discussion

3.1. Optimization of biopolymer solutions and electrospraying process

Processing conditions leading to stable Taylor cone, while maintaining high productivity, were needed to be investigated for the new biopolymer solutions with folate and Asc. Table 1 presents the overview of the experiments performed during the optimization of the electrospraying process showing the productivity from each experiment. The processing conditions and biopolymer solution used in former studies, containing 15% glucose syrup and 4% pullulan, were optimized for encapsulation of fish oil (20% load) (García-Moreno et al., 2018b). The recommendation for dietary intake of folate is 300 µg/day (NNR, 2014). Therefore, a low amount of folate vitamer L-5-CH_3-H_4folate should be added in a biopolymer solution, which in contrast to the addition of

macronutrients should not affect the electrospraying process. Pullulan, which has excellent spinnability properties (García-Moreno et al., 2018b), was added to enhance the stability of the Taylor cone during electrospraying at increased flow rates. This increases the productivity of the electrospraying process, which is required when producing encapsulates for food applications.

Table 1 Chronological overview of the experiments used in optimization of electrospraying process and the results for the productivity

During the optimization, the voltage and flow rate parameters were changed from the ones used by García-Moreno et al. (2018b), because of the instability of the process and formation of droplets during electrospraying. Even though previously optimized conditions (17 kV and 0.007 mL/min) provided the highest productivity (Exp. 3 and 5), reduced flow rate (0.006 mL/min) and increased voltage (20 kV) enabled a stable electrospraying process without dripping and droplets in the collector. It should be noted that, despite the considerable difference in bioactive load (20% fish oil vs. 1% folate), the optimum processing conditions slightly varied. This indicates that the electrosprayability of the solutions tested is mainly controlled by the concentration and molecular weight of the biopolymers used.

Table 1 also shows that the addition of Tween-20 enhanced productivity, as observed when comparing Exp. 2 and 3, or 4 and 5. Tween-20 reduced the surface tension of the biopolymer solution, which implies the use of low voltages to eject the jet from the Taylor cone, leading to a more stable electrospraying process (e.g., less dripping) (García-Moreno et al., 2017). As expected, the addition of Asc also led to higher productivity due to an increase in the solid content (Exp. 8 and 9 in Table 1). Finally, increasing the number of Microfluidizer passes from 3 to 5, which could imply more severe breaking of polymer chains, and thus less polymer chain entanglements did have a clear effect on productivity. For instance, productivity was increased for solutions without Asc

274 when increasing the number of passes (exp. 7 and 9), whereas the opposite was observed for
275 solutions with Asc (Exp. 8 and 10) (Table 1). New optimized conditions used for the production of
276 capsules are shown in Fig. 2.

277 **Figure 2** Scheme of the optimized electrospraying setup and conditions used in this study

278 ### 3.2. Morphology and distribution of the capsules

279 Fig. 3 shows the morphology and particle size distribution of produced capsules. The overall
280 capsule sizes ranged from 0.72 ± 0.41 µm for capsules without Asc to 0.55 ± 0.34 µm for capsules
281 with Asc, which is in accordance to the previous study using the same biopolymers where 60-70%
282 of the capsules were below 1 µm (García-Moreno et al., 2018b). Particles evaluated after production
283 had a small particle size and smooth and round shape (Fig. 3), enhanced with the addition of the
284 surfactant Tween-20 (SOM, Fig. S2). Minor fibrils also occurred during electrospraying. Thus, an
285 increased number of Microfluidizer passes (5x) was tested in order to break polymer chain
286 entanglements and reduce fibrils formation. However, a minor occurrence of fibrils was still visible
287 (SOM, Fig. S3), which suggests that no further disruption of biopolymer chains (e.g., pullulan) was
288 carried out after 3 passes. The capsules produced by electrospraying had smaller sizes compared to
289 spray-drying (10-50 µm), which enables better incorporation and more homogeneous distribution in
290 a food product (Kitts and Liu, 2015).

291 **Figure 3** SEM images and particle size distribution of electrosprayed capsules containing 1%
292 Tween-20 surfactant after 3 Microfluidizer passes: (a) Capsules – Asc, (b) Capsules + Asc
293 produced at optimum processing conditions (flow rate 0.006 mL/min, voltage 20 kV)

294 ### 3.3. Stability of L-5-CH_3-H_4folate-loaded electrosprayed capsules

295 Recovery of L-5-CH_3-H_4folate from electrosprayed capsules was 98 ± 6% (n=5) and 97 ± 5% (n=5)
296 in the *Capsules – Asc* and + *Asc*, respectively. Recovery was calculated as the mean from 5
297 measurements from two separated batches for each biopolymer solution. Batch 1 was used for the
298 stability study of the capsules, whereas batch 2 was used for the fortification of flour. Stability of
299 produced capsules was tested during storage in the freezer (-80 °C), which indicated that keeping
300 the capsules in the freezer does not affect the stability of capsules (e.g., morphological changes and
301 recovery of L-5-CH_3-H_4folate) (data not shown). Fig. 4 shows the oxidative stability of L-5-CH_3-
302 H_4folate in capsules during storage at room temperature in darkness. The use of Asc in the capsules
303 did not significantly improve the recovery of L-5-CH_3-H_4folate. However, *Capsules + Asc* showed
304 higher recovery between the days, as *Capsules – Asc* differed significantly between the days. From
305 day 7, recovery of free L-5-CH_3-H_4folate was significantly lower ($p \leq 0.05$) than the recovery of
306 encapsulated L-5-CH_3-H_4folate, and an almost linear decrease was observed within the 21 days.

307 **Figure 4** Recovery (%) of 5-CH_3-H_4folate, free and in electrosprayed capsules during 21 days
308 storage in darkness at room temperature (22°C). Different letters *abc* indicate significant
309 differences between different samples within a day, whereas *uxyz* indicate significant
310 differences within the samples analyzed on different days ($p \leq 0.05$).

311 ### 3.4. Stability of buns enriched with L-5-CH_3-H_4folate

312 The distribution of L-5-CH_3-H_4folate, free form, and capsules with or without Asc in flour is shown
313 in Table 2. Blending the free L-5-CH_3-H_4folate into the flour resulted in a more uniform
314 distribution, whereas the distribution in *Capsules – Asc* varied as indicated by high SD.
315 Furthermore, the concentration of measured 5-CH_3-H_4folate in *Capsules + Asc* was significantly
316 lower ($p \leq 0.05$) than in the other two fortified flours. These results indicate that some oxidation

317 during blending may have occurred and that it is possible that using mortar and pestle during the
318 integration of capsules into flour destroyed some capsules.

319 **Table 2** Distribution of free and encapsulated L-5-CH_3-H_4folate in flour prior to dough preparation

320 **Figure 5** Retention (%) of 5-CH_3-H_4folate from buns enriched with L-5-CH_3-H_4folate, free or in
321 capsules, during 9 days storage at room temperature (22°C) in darkness. Non-enriched
322 buns represent 5-CH_3-H_4folate naturally occurring from the other ingredients in buns,
323 whereas the other samples have added L-5-CH_3-H_4folate. Different letters *abc* indicate
324 significant differences between different samples within the day, whereas *xy* indicate
325 significant difference within the same sample between different days ($p \leq 0.05$).

326 Fig. 5 presents the retention of L-5-CH_3-H_4folate in enriched buns during 9 days of storage at room
327 temperature (22 ºC). No significant difference was found in the control and the buns containing
328 *Capsules + Asc* over 9 days of storage. The recovery of L-5-CH_3-H_4folate in *Capsules – Asc* was
329 significantly different from 100%, and it was equal to 93 ± 0.3% after production, which indicates
330 that part of L-5-CH_3-H_4folate oxidizes during the production. Electrospraying is a suitable
331 substitute for the spray-drying method in terms of working with thermally sensitive compounds like
332 folate. In contrast to our results, in spray-drying sodium ascorbate have shown a protective effect on
333 the stability of L-5-CH_3-H_4folate in capsules using modified starch as a coating material by
334 providing >95% recovery during the production of the capsules (Kitts and Liu, 2015; Liu et al.,
335 2013).

336 In general, the use of electrosprayed capsules did not show any protective effect on the L-5-CH_3-
337 H_4folate when incorporated into buns. Many different research works have evaluated the potential
338 of microencapsulation for the protection of food bioactives. However, data about their incorporation
339 into food systems are scarce, even though they are important for the evaluation of capsules'

behavior under processing conditions. Encapsulation by electrospraying has already been proven to stabilize bioactive ingredients, but just a few studies tested the effect of capsules incorporation into a food matrix and how food processing influences the stability of the fortified product (Gómez-Mascaraque et al., 2017; Hermund et al., 2019; Miguel et al., 2019). Liu et al. (2013) encapsulated L-5-CH_3-H_4folate by spray-drying and studied storage stability for 7 days. >50% loss of free L-5-CH_3-H_4folate was reported after baking, and degradation pattern in the recovery of L-5-CH_3-H_4folate was observed in pilot baking bread study between the days 3 and 7. Our results showed high retention (100%) of free L-5-CH_3-H_4folate, which was significantly higher than the retention of the encapsulated form. The same observation was made by Kitts & Liu (2015), who used long-chain fatty acid stearate derived from soybean oil for coating material in encapsulation by spray-drying. Both encapsulated L-5-CH_3-H_4folate and folic acid showed lower recovery than corresponding free forms, which was explained by exceeded melting points of stearate and destruction of the capsules that caused oxidation and degradation. Furthermore, Liu et al. (2012) reported a protective effect of skim milk powder and soy milk powder if added to a solution of L-5-CH_3-H_4folate, which indicates that some antioxidative properties of the food matrix may occur. In non-enriched buns, the retention of 5-CH_3-H_4folate was 50% following baking at 200°C for 7.5 minutes, evidencing the instability of the naturally occurring 5-CH_3-H_4folate. Baked buns were stored for 9 days, and no significant difference in 5-CH_3-H_4folate content was observed during this period, which was considered as a limit after which they would not be consumed. The retention of free L-5-CH_3-H_4folate added to buns significantly decreased from 100% to 88% ($p \leq 0.05$), whereas no significant difference was observed in the retention of encapsulated L-5-CH_3-H_4folate after 9 days of storage. However, a significant difference between retention of L-5-CH_3-H_4folate in two types of capsules was observed between days 0 and 3, which could be connected to reduced uniformity of the capsule dispersion in the flour in *Capsules – Asc* as shown in Table 2. As the

stability of free L-5-CH_3-H_4folate started to decrease, there was no significant difference ($p \leq 0.05$) between the stability of buns containing free L-5-CH_3-H_4folate and capsules on day 9. The decrease in retention of free L-5-CH_3-H_4folate in buns followed the pattern similar to the one observed in the recovery of free L-5-CH_3-H_4folate that was equal to 88% after 7 days of storage (Fig. 4).

Even though electrospraying enabled higher recovery than spray-drying (98% vs. 91%), which supports the hypothesis that thermally labile compounds such as folate may be preserved better than if using spray-drying, no protective effect from encapsulation was observed during buns fortification. Further investigation in alternative food products with a longer shelf life is recommended in order to examine if these electrosprayed capsules would improve the stability of folate vitamers during processing. However, our results were in accordance with Gómez-Mascaraque et al. (2017), who found no significant effect of electrosprayed encapsulates loaded with green tea extract when incorporated into biscuits. These findings emphasize the importance of the further step of incorporation of developed capsules to a real food system as their performance varies depending on numerous parameters, such as physical properties of fortified products, as well as production and processing conditions.

4. Conclusions

High oxidative stability of electrosprayed capsules loaded with L-5-CH_3-H_4folate was obtained within 21 days, as no significant difference was observed on L-5-CH_3-H_4folate recovery during storage, whereas a significant decrease of 40% was reported for the free L-5-CH_3-H_4folate. The use of 1% ascorbic acid as an antioxidant did not show any effect on the stability of the produced capsules. Buns were baked with the capsules and stored for 9 days, which showed no significant difference in retention between enrichment with free and encapsulated L-5-CH_3-H_4folate. However, non-enriched buns containing naturally occurring folate from buns' ingredients showed

significantly lower retention of folates (>50%) during processing. Results showed that food processing is a critical step in the preservation of bioactive compounds even when a protection technique such as encapsulation by electrospraying is applied. Therefore, the study may serve as a basis for further research.

Declarations of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be considered as a potential conflict of interest.

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508

List of captions:

Figure 1 The design of the storage experiment in buns. It included retention study during baking (1) and stability study during storage for 0, 3, 6, and 9 days (2). Three replicates were sampled at each sampling point. *indicate dough samples sampled for the calculation of the retention (n=3).

Figure 2 Scheme of the optimized electrospraying setup and conditions used in this study

Figure 3 SEM images and particle size distribution of electrosprayed capsules containing 1% Tween-20 surfactant after 3 Microfluidizer passes: (a) Capsules – Asc, (b) Capsules + Asc produced at optimum processing conditions (flow rate 0.006 mL/min, voltage 20 kV)

Figure 4 Recovery (%) of L-5-CH_3-H_4folate, free and in electrosprayed capsules during 21 days storage in darkness at room temperature (22°C). Different letters *abc* indicate significant differences between different samples within a day, whereas *uxyz* indicate significant differences within the samples analyzed on different days ($p \leq 0.05$).

Figure 5 Retention (%) of L-5-CH_3-H_4folate from buns enriched with L-5-CH_3-H_4folate, free or in capsules, during 9 days storage at room temperature (22°C) in darkness. Non-enriched buns represent 5-CH_3-H_4folate naturally occurring from the other ingredients in buns, whereas the other samples have added L-5-CH_3-H_4folate. Different letters *abc* indicate significant differences between different samples within the day, whereas *xy* indicate significant difference within the same sample between different days ($p \leq 0.05$).

Table 1 Chronological overview of the experiments used in optimization of electrospraying process and the results for the productivity

Table 2 Distribution of free and encapsulated L-5-CH_3-H_4folate in flour prior to dough preparation

Table 1 Chronological overview of the experiments used in optimization of electrospraying process and the results for the productivity

Exp.	Solutions	Asc (1%)*	Tween-20 (1%)**	L-5-CH_3-H_4folate (1%)*	Voltage [kV]	Flow rate [mL/min]	Productivity [mg/h]
1	15% glucose syrup + 4% pullulan	+	-	-	17	0.007	44.5
2	15% glucose syrup + 4% pullulan	+	-	-	16	0.007	0.9
3	15% glucose syrup + 4% pullulan	+	+	-	17	0.007	74.9
4	15% glucose syrup + 4% pullulan	+	-	-	16.5	0.007	29.7
5	15% glucose syrup + 4% pullulan	+	+	-	17	0.007	85.3
6	15% glucose syrup + 4% pullulan	+	+	-	20	0.006	63.1
7	15% glucose syrup + 4% pullulan	-	+	+	20	0.006	45.4
8	15% glucose syrup + 4% pullulan	+	+	+	20	0.006	73.9
9#	15% glucose syrup + 4% pullulan	-	+	+	20	0.006	51.4
10#	15% glucose syrup + 4% pullulan	+	+	+	20	0.006	55.9

Exp., experiment; Asc, ascorbic acid; L-5-CH_3-H_4folate, L-5-methyltetrahydrofolate.

*percentage of the compound in capsule

**percentage of the compound in biopolymer solution

#5 Microfluidizer passes

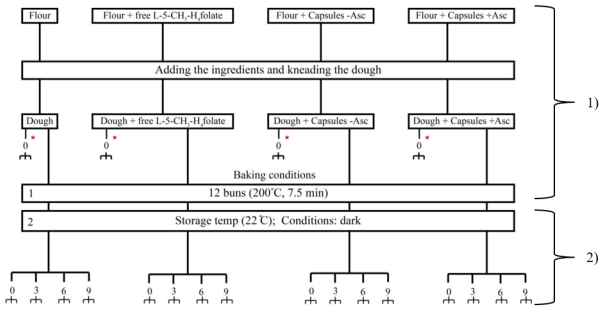
Table 2 Distribution of free and encapsulated L-5-CH_3-H_4folate in flour prior to dough preparation

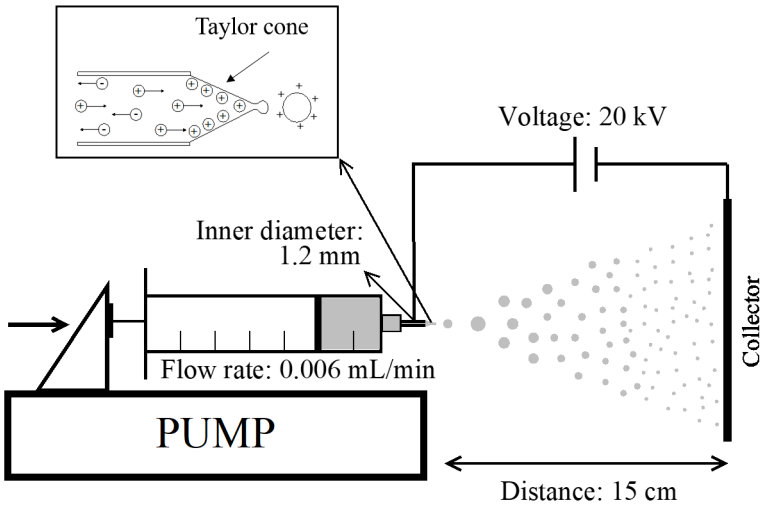
Fortificant	Targeted L-5-CH_3-H_4folate concentration (µg/100 g)[a]	Required weight for enrichment (mg/kg)	Measured mean 5-CH_3-H_4folate (µg/100 g)[b]	Standard deviation (µg/100 g)[b]
Free L-5-CH_3-H_4folate	150	1.50	163[x]	3.5
Capsules - Asc	150	150	155[x]	19.2
Capsules + Asc	150	150	127[y]	9.0

[a]150 µg of L-5-CH_3-H_4folate/100 g of flour represent 1.5 mg/kg of flour fortification recommendation by FDA

[b]Six determinations of each dry mix

Letters *xy* indicate significant difference tested by Tukey-Kramer ($p \leq 0.05$).





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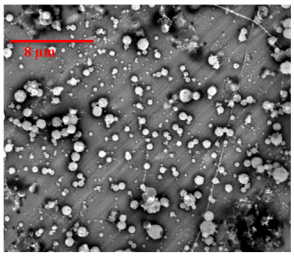

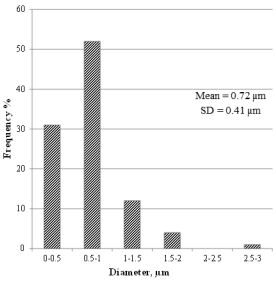

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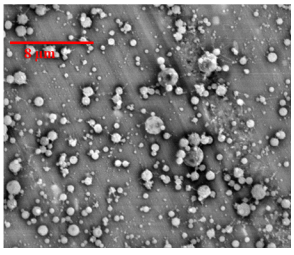

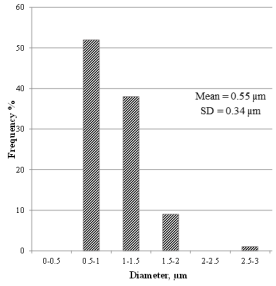

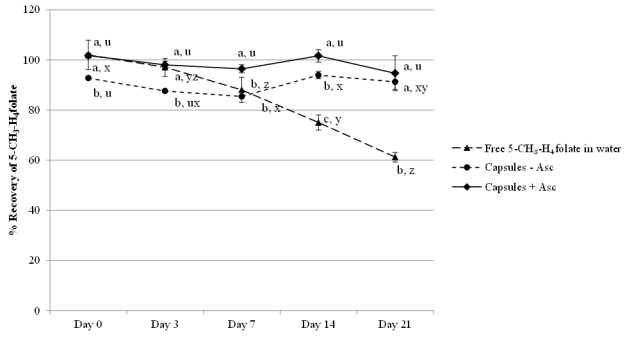

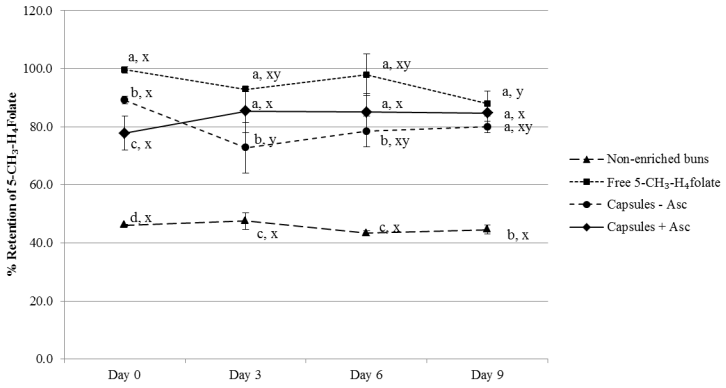

Highlights:

- Glucose-pullulan capsules containing L-5-CH_3-H_4folate were made by electrospraying
- Capsules are stable against oxidation during 21 days of storage in darkness at 22 ºC
- Ascorbic acid added to capsules does not improve the stability of L-5-CH_3-H_4folate
- Encapsulation does not improve the retention of L-5-CH_3-H_4folate in buns

Author Contribution Statement:

The authors responsibilities were as follows: Petra Ložnjak Švarc, Pedro J. García-Moreno, Ana C. Mendes, Elnaz Z. Fallahasghari and Jette Jakobsen contributed to the design of the study; Elnaz Z. Fallahasghari performed electrospraying optimization, and examined physical properties of the capsules; Petra Ložnjak Švarc conducted stability studies including folate analysis, data analysis and wrote the manuscript. All authors helped in interpretation of data. Petra Ložnjak Švarc, Pedro J. García-Moreno, Ana C. Mendes, and Jette Jakobsen read, reviewed and approved the final manuscript.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: