Fortification of dark chocolate with spray dried black mulberry (Morus nigra) waste extract encapsulated in chitosan-coated liposomes and bioaccessability studies

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ABSTRACT

Fine-disperse anionic liposomes containing black mulberry (Morus nigra) extract (BME) were prepared by high pressure homogenization at 25,000 psi. Primary liposomes were coated with cationic chitosan (0.4, w/v%) using the layer-by-layer depositing method and mixed with maltodextrin (MD) (20, w/v%) prior to spray drying. After that, spray dried liposomal powders containing BME were added to chocolates with alkalization degrees (pH 4.5, 6, 7.5) at conching temperatures of 40 °C, 60 °C, and 80 °C. The results showed that, compared to spray dried extract, chitosan coated liposomal powders provided better protection of anthocyanin content in both increased temperature and pH. In addition, encapsulation in liposomes enhanced in vitro bioaccessability of anthocyanins. Chocolate was fortified with encapsulated anthocyanins maximum 76.8% depending on conching temperature and pH.

1. Introduction

Black mulberries (Morus nigra) are very rich sources of flavonoids, particularly anthocyanins. However, their capacity and health benefit potential are limited since they are unstable during food processing, distribution or storage, or in the gastrointestinal tract (Munin & Edwards-Lévy, 2011). Temperature, pH, oxygen, and water activity, enzymes, the presence of other nutrients like proteins are factors which might influence their stability. Moreover, degradation and polymerization formed during heating usually lead to their discoloration (Munin & Edwards-Lévy, 2011; Tsai, Delva, Yu, Huang, & Dufosse, 2005). On the other hand, only a small proportion of the polyphenols including anthocyanins are absorbed due to insufficient gastric residence time, low permeability and/or low solubility (Fang & Bhandari, 2010). Therefore in various studies, it had been shown that activity of polyphenolic compounds may be protected by encapsulation which might be performed by different techniques such as spray-drying, freeze drying, extrusion coating, fluidized bed coating, cocrystallization, coacervation, inclusion complexation, emulsions, suspensions and liposome entrapment (Fang & Bhandari, 2010; Lu, Li, & Jiang, 2011).

Liposomes, microscopic bilayer vesicles from dispersion of membrane-like lipids in aqueous solvents, are biocompatible, biodegradable, nontoxic and their use in the biomedical, food, and agricultural industries is gaining increasing popularity in recent years for their ability to act as targeted release-on-demand carrier systems for both water- and oil-soluble bioactive compounds (Fang & Bhandari, 2010; Reza Mozafari, Johnson, Hatziantoniou, & Demetzos, 2008). However liposomes are generally unstable when suspended in aqueous systems for prolonged periods, including vesicle fusion, aggregation and leakage of encapsulated material. Recently, the layer-by-layer electrostatic deposition method has been shown to be an effective way to enhance the stability of liposomes (Chun, Choi, Min, & Weiss, 2013; Laye, McClements, & Weiss, 2008). Since much physical and chemical deterioration processes take place in an aqueous environment, one possible approach to increase the stability and to make the use of such delivery systems industrially applicable might be converting them into dry forms. This could be achieved by several methods, such as freeze-drying, power bed grinding, fluidized bed drying, or spray drying. Due to being less expensive, time- and energy consuming process and its use is extremely well known in food processes, the use of spray drying might be preferable in the production of dry liposomal powders (Moraes et al., 2013).

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Chocolate represents functional properties due to its high level of flavonoid content, namely catechins and procyanidins, and beneficial impacts of chocolate consumption on human health (Wollgast & Anklam, 2000). However, consumers are becoming more demanding in food market and they would like to have more options to choose from than ever before. Therefore, manufacturers desire to broaden their product ranges such as having organic chocolate, high-cocoa polyphenol-rich chocolate, probiotic chocolate, and prebiotic chocolate rather than ordinary chocolate. We recently showed that dark chocolate ensured a high probiotic survival rate (Erdem et al., 2014).

A substantial amount of mulberry fruit is processed into juice and juice concentrate, which is subsequently used in beverages, syrups, and other food products. Nevertheless, juice processing generates a waste by-product called press cake. Since mulberry presscake has high amounts of anthocyanins and polyphenols, it is a potential source for antioxidants.

In this current study, we explored the possibility of using dry liposomal delivery systems containing black mulberry (M. nigra) waste extract to retain anthocyanin content against increased pH and temperature during the industrial production of dark chocolate. Therefore, first, primary liposomes by high pressure homogenization method were produced and coated with cationic chitosan by the layer-by-layer deposition method. To characterize primary and coated liposomes, particle size distribution, and zeta potential were measured. Then, liposomes which retained their structure upon addition of maltodextrin were spray dryed. After getting liposomal dry powders, they were added into chocolate formulation at different alkalization degrees (pH 4.5, 6, 7.5) and conching temperature during the industrial production of dark chocolate. Therefore, first, primary liposomes by high pressure homogenization method were produced and coated with cationic chitosan by the layer-by-layer deposition method. To characterize primary and coated liposomes, particle size distribution, and zeta potential were measured. Then, liposomes which retained their structure upon addition of maltodextrin were spray dryed. After getting liposomal dry powders, they were added into chocolate formulation at different alkalization degrees (pH 4.5, 6, 7.5) and conching temperatures (40, 60, 80 °C) to observe the change in anthocyanin content and the level of protection provided by liposome encapsulation compared to spray dried extract. Within our knowledge, no previous study to date evaluated the potential of liposome encapsulated phenolics in chocolate.

2. Materials and methods

2.1. Materials

Chitosan with 80% DDA (degree of deacylation) was donated from Primex (Siglufjordur, Iceland). Lecithin (Soybean phospholipids, 97%-Ultralec® P) was kindly provided by Rotel, Turkey and maltodextrin was a gift from Tunckaya Kimyevi Maddeler Ticaret ve Sanayi Inc., Turkey. Sephadex G50 was purchased from GE Healthcare Life Sciences (Uppsala, Sweden). Folin-Ciocalteu's phenol reagent was purchased from Merck KGaA (Darmstadt, Germany). Triton X100 was purchased from Carl Roth GmbH (Karlsruhe, Germany). Sodium acetate hydrate, acetic acid, sodium hydroxide, hydrochloric acid, potassium chloride, gallic acid were purchased from Sigma–Aldrich Co. (St. Louis, USA). Industrial black mulberry waste was provided from a local fruit juice factory in the form of frozen puree waste and milled after freezing by liquid nitrogen. Milled samples were kept at -80 °C. Natural chocolate liquor (pH 4.5) was provided from Nestle Turkey Gida Inc.

2.2. Preparation of black mulberry extract

Aqueous two-phase extraction method described by Wu et al. (2011) was employed. 20 g of ammonium sulfate was dissolved in 40 ml water and mixed with milled frozen mulberry (10 g) and ethanol (30 g) for 15 min. pH was adjusted to 4.5 by 0.01 N HCl and placed in a water bath at 35 °C, 100 rpm for another 30 min. Two phases (top-ethanolic phase containing anthocyanins, bottom-aqueous phase containing sugars) were formed after centrifugation of the mixture at 4000 rpm for 5 min. The mulberry residues accumulated at the interface of two phases were discarded. The mixture was kept at 4 °C overnight, upper phase was mixed with two more volumes of ethanol, then centrifuged and ethanol was removed in a rotary evaporator at 40 °C. The remained extract was freeze dried.

2.3. Preparation of uncoated and chitosan coated liposomes

2% (w/v) lecithin powder in acetate buffer (pH = 3.5 ± 0.1: 0.1 M) was stirred overnight at room temperature and BME (0.05–1%, w/v) were dissolved in lecithin solution. Blank liposomes and liposomes containing BME were prepared by homogenizing solutions with a high shear disperser (DI-25 Yellowline, IKA) for 10 min at 9,500 rpm, it passed five times through a high pressure homogenizer (Microfluidizer Processor M-110L, Microfluidics, Newton, USA) at homogenization pressure of 25,000 psi. The homogenization chamber was cooled with ice to prevent the overheating of samples. Negatively charged liposomes were coated by electrostatic deposition of positively charged chitosan layer. To this purpose, liposome suspensions (2% w/v) were added to chitosan solutions (0.001–0.5%, w/v prepared in acetate buffer and stirred overnight at room temperature.

2.4. Measurements of particle size distribution and zeta (ζ) potential

The particle size distribution of uncoated and coated liposomal dispersions was measured using a static light scattering instrument (Mastersizer 2000, Malvern Instruments). A refractive index for lecithin of 1.44 and 1.33 for the aqueous phase was used to calculate particle size distributions. The volume mean diameter (d4,3) was used to report average particle diameters. All particle size measurements were made on at least two freshly prepared samples with three readings made per sample. Liposomal dispersions were diluted to a particle concentration of approximately 0.005% (w/v) with acetate buffer and zeta-potential was measured by a Zetasizer (Zetasizer 2000, Malvern Instruments). Results are reported as the average and standard deviation of measurements made from two freshly prepared samples. The powder samples were reconstituted by dissolving 0.5 g powder in 4.5 ml of acetate buffer (pH = 3.5). The particle diameter and zeta potential were detected after reconstitution using the methods mentioned above.

2.5. Removal of unencapsulated extract by gel filtration

Sephadex gel filtration was used to remove both chitosan which had not bound to liposomal surfaces, and extract which had not been encapsulated in liposomes. Sephadex G50 solution (5 wt% in deionized water) was filled in syringes (6 ml) until a layer of about 3 cm of gel had been formed. 1.5 ml of acetate buffer was added on top of the gels. Syringes were placed into falcon tubes and the Sephadex G50 column then centrifuged at 3000 rpm for 10 min. Then, 1.5 ml of sample was added on top of the gels and the centrifugation repeated. Gel filtered samples collected in the falcon tubes were then further used (Gibis, Vogta, & Weiss, 2012).

2.6. Determination of total phenolic content

Total phenolic content (TPC) of liposomal dispersions was measured according to Folin–Ciocalteau reagent test by Gibis et al. (2012). For the determination of TPC in samples, 1 ml of the diluted sample was mixed with 5 ml of the diluted (1:10) Folin–Ciocalteau reagent, stirred with a vortexer and left to stand for 3 min. Then, 4 ml of the sodium carbonate solution (7.5%, w/v) was added and again stirred with a vortexer. The sample was left to stand for 60 min in dark, and its extinction
A light microscopy (Clemex Nikon Eclipse L150, Japan) was used to detect potential aggregation of liposomes. A drop of sample on a glass slide was observed at a magnification of 100x.

### 2.9. Spray drying

Chitosan coated liposomal dispersions with and without BME, and BME (0.05% w/v) were mixed with maltodextrin solutions prior to spray drying to obtain mixtures containing 20% (w/v) MD, 0.5% (w/v) lecithin and 0.2% (w/v) chitosan. The dispersions were dried at a feed rate of 2.5 cm³/min. at an inlet temperature of 140–150 °C resulting in an outlet temperature of 90 °C, and 0.67 m³/min. air flow using a laboratory scale spray drier equipped with a 1.5-mm nozzle atomizer operated at an atomizing air flow of 5 cm³/min. (Mini spray dryer B-290, BUCHI, Switzerland). Dried powders were stored in airtight containers and placed in a desiccator at room temperature.

### 2.10. Scanning electron microscopy (SEM)

Spray dried powders were mounted onto adhesive-coated aluminum pin stubs. Excess powder was removed by blowing dry air across. The stubs were sputter coated with a thin layer of gold in a Leica vacuum coating unit at 40 mA for 100 s 3 times, at a working distance of 50 mm by using an argon gas purge (Quorum SC7620 Sputter Coater). The samples were examined using a Quanta FEG 250 SEM. The SEM was operated at high vacuum with an accelerating voltage of 5 kV. Images were taken at 4000 and 12,000 magnifications.

### 2.11. In vitro digestion

In vitro bioaccessibility was determined to evaluate the effect of liposome encapsulation on anthocyanins. The method of McDougall, Dobson, Smith, Blake, and Stewart (2005) was applied with some modifications. An initial pepsin/HCl digestion for 2 h at 37 °C was followed by a digestion with bile salts/pancreatin for 2 h at 37 °C to simulate gastric and small intestine conditions, respectively. The simulated stomach solution was prepared with pepsin, NaCl and pH was adjusted to 2.0. 20 ml stomach solution was mixed with 5 g of liposomal dispersions with and without BME, and BME (0.05% w/v) samples and incubated in shaking water bath for 2 h at 37 °C and 100 rpm. At the end of time, the mixture was cooled down immediately and 2 ml aliquots of the post-gastric (PG) digestion was collected. After addition of 4.5 ml of 4 mg/ml pancreatin and 25 mg/ml bile salt mixtures, segment of cellulose dialysis tubing containing sufficient NaHCO₃ to neutralize the titratable acidity was placed into the beaker. After incubation in shaking water bath for 2 h at 37 °C and 100 rpm, the solution in the dialysis tubing was collected as IN (material entered in serum) while the solution outside the dialysis tubing was collected as OUT (material remained in the GI tract). PG, IN and OUT samples were stored at –80 °C until further analysis. Before analysis, samples were thawed and centrifuged at 18,000 rpm for 10 min.

### 2.12. Addition of liposomal powders to chocolate formulation

Spray dried liposomal powder with BME, and spray dried BME were added to natural (pH 4.5) and alkalized cocoa liquors (pH 6 and 7.5) during the last hour of conching at temperatures of 40, 60, and 80 °C, respectively. The application was performed triplicate. After removing lipids with hexane from chocolate samples, total anthocyanin contents were determined according to the pH differential method mentioned above.

### 2.13. Statistical analysis

IBM SPSS Statistics 21 (Chicago, IL, USA) software was employed for statistical analysis. All measurements were repeated at least three times using triplicate samples. Differences were analyzed by Tukey’s test comparisons and p value of <0.05 was chosen to determine significant differences.

### 3. Results and discussion

#### 3.1. Characterization of uncoated liposomes with and without BME

Mean particle diameter and zeta potential of samples were measured to characterize primary liposomes with and without BME (Fig. 1A). Mean particle diameter of primary liposomes without BME was approximately 150 nm at 25,000 psi homogenization pressure. The size of primary liposomes containing BME had higher values than liposomes that did not contain any extract at the same homogenization pressure and the particle size was affected by the presence of extract until approximately 0.2% (w/v). All liposomes had negative surface charges whether they contained extract or not, ranging between −27.6 and −36.0 mV.

#### 3.2. Influence of chitosan addition on the properties of liposomes

The coating of primary liposomes with chitosan was assessed by measuring the change in surface charge and particle size by the addition of polymer in the range of 0–0.5% (w/v). The uncoated primary liposomes was highly anionic (−36 mV) and addition of chitosan changed the surface charge of liposomes from negative...
to positive (∼ + 50 mV). At concentration of 0.05 (w/v%), the net surface charge on the particles became zero due to charge neutralization. At approximately 0.1 (w/v%) and higher chitosan concentration, the \( \zeta \)-potential became constant at ∼ + 49 mV, which indicates that chitosan fully covered the surfaces of vesicles (Fig. 1B).

The size of liposomes was dependent on the chitosan concentration, and it was increased from ∼150 nm in the absence of chitosan to around 80 μm if the chitosan concentration was lower than 0.2 (w/v%) which might be attributed to bridging flocculation (Karadag et al., 2013, Chun et al., 2013). The mean diameter of aggregates was decreased with increasing chitosan concentration and became minimum around 300 nm at 0.4 chitosan concentration (w/v%). We used this level of chitosan to cover the surfaces of liposomes containing BME.

3.3. Content and location of phenolics in uncoated and chitosan coated liposomes

Content and location of phenolics in uncoated and coated liposomes were determined according to the method of (Gibis et al., 2012). Total phenolic content was determined in the aqueous phase containing liposomes and in the aqueous phase after removing unencapsulated phenolics by gel filtration. Then, liposomes were destabilized by the addition of Triton X-100 to make the phenolics in the interior of liposomes accessible to reagent. TPC consists of phenolics on the surface or in the membrane plus the amount located in the interior of liposomes. The amount of phenolics inside the liposomes was calculated by subtraction of the measured amount on intact liposomes and the measured amount on destabilized liposomes.

To determine the optimum phenolic concentration in primary liposomes, BME concentrations of 0.05–1% (w/v) were studied. Between 51.0% and 84.7% of the added extract was incorporated on the surface and to the interior of the liposomes, calculated as gallic acid. Our findings are comparable with the results reported by Gibis et al. (2012) who found that 83.5% of the added grape seed extract (0.1%) was incorporated on the surface and to the interior of the liposomes. Although the highest efficiency % was calculated for 0.05% concentration of black mulberry extract, 0.2% (w/v) concentration of extract with 76.8% efficiency was selected for further experiments to make the spectrophotometric measurements easier due to multiple dilution in the following steps.

According to the results, at 0.2% (w/v) extract concentration, subtraction of the concentration of polyphenols detected on the surface of liposomes after gel filtration (80.5 ± 7.19 mg L\(^{-1}\)) from the concentration detected after Triton treatment (108.0 ± 7.00 mg L\(^{-1}\)) yielded an amount of 27.5 mg L\(^{-1}\). After addition of chitosan layer, 60.23 mg L\(^{-1}\) ± 3.21 of phenolics was detected in intact liposomes while concentration of 85.00 ± 1.61 mg L\(^{-1}\) was found after
Fig. 2. Microscopic images (at 100× magnification) of dispersions of (A) liposome (2 w/v%), (B) liposome (2 w/v%) with BME (C) liposomes (1 w/v%) after addition of chitosan A (0.4 w/v%), (D) liposomes (1 w/v%) with BME after addition of chitosan A (0.4 w/v%), (E) liposomes (0.05 w/v%) after addition of MD (20 w/v%), (F) liposomes (0.05 w/v%) with BME after addition of MD (20 w/v%), (G) chitosan (0.4 w/v%) coated liposomes (0.05 w/v%) after addition of MD (20 w/v%), (H) chitosan (0.4 w/v%) coated liposomes (0.05 w/v%) with BME after addition of MD (20 w/v%).
breakage with Triton X-100. This decrease in the amount of detectable phenolics in the intact liposomes showed that phenolics on or in the surface of primary liposomes became less accessible after chitosan coating similar to the finding in the study of Gibis et al. (2012).

3.4. Spray drying of liposomes

In our previous study (Karadag et al., 2013), we found that chitosan absorbed on liposome surfaces provided a protective layer against subsequent depletion flocculation induced by addition of maltodextrin by increasing the thickness of the interfacial layer and altering its charge. In this study, similar flocculation and phase separation had been observed in both blank and BME containing liposomes after maltodextrin addition to facilitate spray drying (Fig. 2E and F). Therefore, liposomes coated with 0.4 (w/v%) chitosan were used to prepare spray dried powders (Fig. 2G and H). In chitosan coated liposomes with and without BME, spray drying resulted in a decrease in mean particle diameter (Table 1) due to the osmotic driving force (Karadag et al., 2013). Addition of maltodextrin to liposomes caused a slight but not significant decrease in $\zeta$-potential values.

The moisture content of spray dried chitosan coated liposome with BME, chitosan coated liposome without BME, and BME was

<table>
<thead>
<tr>
<th>Liposome</th>
<th>Liposome with BME</th>
<th>Chitosan coated liposome without BME</th>
<th>Chitosan coated liposome with BME</th>
<th>BME mixed with MD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial diameter (nm)</td>
<td>149 ± 7.8</td>
<td>173 ± 1.2</td>
<td>312 ± 8.0$^a$</td>
<td>473 ± 12.7$^a$</td>
</tr>
<tr>
<td>Mean diameter of pre-dried dispersions (nm)</td>
<td>Phase separation</td>
<td>Phase separation</td>
<td>300 ± 1.2$^a$</td>
<td>470 ± 50.9$^a$</td>
</tr>
<tr>
<td>Mean diameter of reconstituted dispersions (nm)</td>
<td>–</td>
<td>–</td>
<td>279 ± 10.7$^b$</td>
<td>362 ± 45.7$^b$</td>
</tr>
<tr>
<td>Initial $\zeta$-potential</td>
<td>–33.47 ± 0.12$^a$</td>
<td>–32.47 ± 0.06$^a$</td>
<td>46.10 ± 0.50$^a$</td>
<td>41.87 ± 1.37$^a$</td>
</tr>
<tr>
<td>$\zeta$-Potential of pre-dried dispersions</td>
<td>–32.43 ± 0.15$^a$</td>
<td>–30.23 ± 0.32$^a$</td>
<td>45.17 ± 0.35$^a$</td>
<td>40.62 ± 0.78$^a$</td>
</tr>
<tr>
<td>$\zeta$-Potential of reconstituted dispersions</td>
<td>–30.17 ± 0.12$^a$</td>
<td>–29.43 ± 0.06$^a$</td>
<td>44.30 ± 0.36$^a$</td>
<td>39.94 ± 0.80$^a$</td>
</tr>
</tbody>
</table>

Pre-dried dispersion means liposome dispersion mixed with MD prior to spray drying. Values with different superscript letters within the column are significantly different ($p < 0.05$).

![Fig. 3. SEM images of chitosan coated liposomes with BME (A), chitosan coated liposomes (B). Pictures were taken at 4,000× and 12,000× magnifications, respectively.](image-url)
found to be 5.10 ± 0.05, 5.10 ± 0.45, and 4.60 ± 0.52, respectively. Water activity value of all spray dried samples was below 0.10. There was no significant difference among samples (p > 0.05) in terms of moisture content 4.60 to and water activity. Meanwhile, yield of the powders was changed between 66.0% and 75.13% (p < 0.05).

3.5. Powder morphology

The SEM images of liposomal powders are given in Fig. 3. They showed mostly spherical structures with some small indentations and wrinkles on their surfaces. Most of the particles had average diameter of less than 5 μm. When compared to Figures 3A2 and 3B2, particle size of secondary liposomes prepared with MD is generally smaller than secondary liposomes. This finding is collaborated with particle size values given in Table 1. Dents and wrinkles seen in SEM images were also reported before by Peres et al. (2011) using maltodextrin during spray drying. High evaporation rates caused skin formation around the droplet due to wall solidification (Shen & Quek, 2014). When this shell forms earlier in the evaporation process on a larger droplet, wrinkles formation on particles surface are observed. Thermal expansion of air or water vapors inside the drying particles can smooth out dents, which depends on the drying rate and viscoelastic properties of the wall matrix (Peres et al., 2011). Finally, such expansion may lead to collapse of particles (Fig. 3). As shown in Table 1, mean particle diameter of reconstituted powders were slightly smaller than those of pre-dried dispersions (p > 0.05). Meanwhile, a slight decrease in ε-potential of particles (p > 0.05) was observed, as well (Table 1).

3.6. Effect of spray drying on stability of BME

Chitosan coated liposomes with and without BME and MBE were spray dried using MD (20% w/v) as a carrier. TPC and anthocyanin (ACN) retentions were calculated according to the following formulas based on dry matter measurements (Fang & Bhandari, 2011).

\[
\text{TPC retention} = \frac{\text{TPC in spray dried powder}}{\text{TPC in feed solution}} \times 100
\]

\[
\text{ACN retention} = \frac{\text{ACN in spray dried powder}}{\text{ACN in feed solution}} \times 100
\]

TPC and ACN retention of spray dried liposomal powder was 69.23 ± 5.1%, and 56.19 ± 4.8%, respectively. Meanwhile, 42.85 ± 4.3 of TPC and 39.94 ± 4.1 of ACN was remained in spray dried powder. The results showed that chitosan layer protected anthocyanins compared to the BME dried in the presence of only maltodextrin (p < 0.05). However, Fang and Bhandari (2011) found higher retention of TPC and ACN (96% and 94%) values when they dried bayberry polyphenols probably due to the milder operating conditions applied. In another study, Ersus and Yurdagel (2007) also found greater black carrot anthocyanin losses when they applied higher inlet (>160–180 °C) and outlet temperatures (107–131 °C).

3.7. In vitro gastrointestinal digestion

Encapsulating phenolics in liposomes as a delivery system preserves their biological activities due to stabilizing and protecting them from degradation. Encapsulation also enhances bioavailability of phenolics since encapsulation in delivery systems of nanometric size contributes more significantly to the improvement of its cell uptake (Sessa, Tsao, Liu, Ferrari, & Doni, 2011).% Recovery of anthocyanins from the bioavailability procedure is given in Table 2. Due to acidic pH of gastric conditions, anthocyanins were stable in PG samples with % recovery of 103–130%. However, % recovery of control anthocyanins was the lowest (1.8%) among the IN samples due to poor bioavailability. Our results are in agreement with McDougall et al. (2005) who reported % recovery of raspberry anthocyanins to be in the range of 1.7% and 12.6%. Entrapment of anthocyanins in liposomes increased their recovery in the IN samples (2.1%). However, liposomes undergo digestion in the GI tract by bile juice containing bile salts which work as surfactants. Bile salts interact with liposomes and burst them by extracting lipids from the liposomal membrane.

% Recovery of the chitosan coated liposome with extract in the IN samples (3.7%) was the highest. Surface coating of liposomes with chitosan could avoid the direct interaction of bile salts with lipid membrane (Iwanaga et al., 1997).

3.8. Addition of liposomal powders to chocolate formulation

Since dark chocolate mainly consists of cocoa butter and cocoa mass, it is not possible to fortify it with aqueous lipid dispersions with BME. Therefore, chitosan coated liposome dispersions with BME was spray dried to convert liquid dispersions into dry powders. BME containing pre-determined amounts of anthocyanins was added to cocoa liquor samples with alkalization degrees of pH 6.0 and 7.5. pH of natural cocoa liquor was 4.5. To mimic chocolate production, powders were mixed in the last hour of conching at temperatures of 40 °C, 60 °C and 80 °C since lecithin is recommended to be added as late as possible to get max viscosity reduction which means that keeping lecithin on the surface of the particles is necessary (Minifie, 1989). At the end of time, samples were collected to determine total anthocyanin contents. Results of ACN amounts and % loss of anthocyanins in liposomes upon change in alkalization degrees (pH) and conching temperatures are given in Table 3. According to the results, % loss of anthocyanins increased from 36.8% to 60.3% in spray dried BME by increasing temperature from 40 to 80 °C at 4.5 pH. However, % loss of anthocyanins in chitosan coated liposomes was in the range of 32.4% and 49.8% with increasing temperature from 40 and 80 °C at 4.5 pH. Increasing pH from 4.5 to 6.0 and 7.5 resulted in higher anthocyanin loss in all samples. % Loss of anthocyanins in spray dried BME increased from 50.0% to 79.8% with increasing temperature from 40 to 80 °C at 6.0 pH. % Loss of anthocyanin was lower (between 47.5% and 57.2%) in chitosan coated liposomes at this pH. At pH 7.5, % loss of anthocyanins was between 76.3% and 100% in spray dried BME with increasing temperature from 40 to 80 °C. However, % loss of anthocyanins in chitosan coated liposomes was in the range of 65.8 and 73.1. Based on statistical analysis, formulation, pH, and temperature, interactions of formulation * pH, formulation * temperature, pH * temperature, formulation * temperature * pH have significant effects on ACN loss.

Table 2

<table>
<thead>
<tr>
<th>% Recovery of anthocyanins in liposomes.</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
</tr>
<tr>
<td>PG</td>
</tr>
<tr>
<td>IN</td>
</tr>
<tr>
<td>OUT</td>
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</table>

IN sample: anthocyanins survived gastric digestion, represents the serum available material.
OUT sample: anthocyanins remains in gastrointestinal tract, and pass through to the colon. PG, post-gastric.
Values with different superscript letters within the same row are significantly different (p < 0.05).
ACN concentration (mg cyanidin-3-glucoside/L) and % ACN loss upon change in alkalization degrees (pH) and conching temperatures.

<table>
<thead>
<tr>
<th>pH</th>
<th>Control</th>
<th>Spray dried liposomal powder</th>
<th>Spray dried BME</th>
<th>Spray dried liposomal powder</th>
<th>Spray dried BME</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.5</td>
<td>21.0 ± 0.73</td>
<td>31.1 ± 1.27</td>
<td>24.8 ± 1.61</td>
<td>9.6 ± 0.36</td>
<td>12.3 ± 1.36</td>
</tr>
<tr>
<td></td>
<td>16.8 ± 2.00</td>
<td>25.8 ± 1.30</td>
<td>18.3 ± 0.30</td>
<td>11.3 ± 0.53</td>
<td>16.4 ± 0.77</td>
</tr>
<tr>
<td>6.0</td>
<td>20.3 ± 0.57</td>
<td>38.2 ± 2.4a</td>
<td>42.4 ± 2b</td>
<td>17.7 ± 1.07</td>
<td>60.5 ± 3c</td>
</tr>
<tr>
<td>7.5</td>
<td>15.5 ± 1.61</td>
<td>49.8 ± 2a</td>
<td>60.3 ± 3c</td>
<td>15.7 ± 1.43</td>
<td>57.2 ± 2b</td>
</tr>
<tr>
<td></td>
<td>11.7 ± 0.69</td>
<td>79.8 ± 3d</td>
<td></td>
<td></td>
<td>79.8 ± 3d</td>
</tr>
</tbody>
</table>

*(Values with different superscript capital letters within the same column are significantly different (p < 0.05).)

(p < 0.05). Encapsulation of extract in chitosan coated liposomes protected ACN content.

4. Conclusion

Chocolate was fortified with encapsulated anthocyanins maximum 76.8% depending on conching temperature and pH. In further studies, we are planning to focus on fortification/enrichment of food products with other liposome encapsulated bioactive compounds. However, more research is needed to investigate chemical stability of liposomes during storage. In conclusion, the results are quite interesting for food and pharmaceutical industry.

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References


