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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 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 trihydrate, 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 ($\bar{d}_{4,3}$) 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 ζ-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–Ciocalteu 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