Agronomy Research **12**(3), 825–834, 2014

Liposomal beta-carotene as a functional additive in dairy products

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Abstract. Encapsulation is of growing use in food technologies for various bioactive compounds. The study addresses the aspect of possible improved physiological activity that encapsulated form can enable. Liposomes obtained by dehydration/rehydration method were used as carriers of beta carotene. The liposomal additive was used for fortification of low-fat milk drink. Formation, structure and stability of the liposomes were controlled in the product matrix by transmission electron microscopy. Fraction-dispersed composition and aggregate stability of liposomal particles were investigated by means of dynamic light scattering. Liposomal beta-carotene was found to maintain stability during storage at 4 ± 2ºC within 15 days. Hexobarbital sleeping time test performed on mice fed with the product showed enhanced hepatostimulation effect achieved by the liposomal additive compared to free beta carotene. Liposomal beta-carotene is suggested to be used for development of functional health-promoting dairy products.

Key words: liposomes, beta-carotene, bioactive compounds, encapsulation, hepatostimulation activity.

INTRODUCTION

Dietary supplements are bioactive substances of natural origin for nutritional balance normalization and health promotion. Dietary supplements are the most efficient way of micronutrients deficiency elimination if their dosage corresponds to physiological requirements (Gichev & Gichev, 2009). Many dietary supplements contain substances of adaptogenic and restorative action, which stimulate body defense, enhance general resistance and vitality, physical and mental performance and reduce negative environmental effects and stress. Dietary supplements are the ingredients that provide health benefits beyond nutritional value, enhance physiological performance and prevent diseases (Paliyath et al., 2011).

Beta-carotene is a hydrocarbon with molecular formula $C_{40}H_{56}$ containing 11 conjugated double bonds. It is a biologically active substance playing important role in vital functions (pro-vitamin A). Beta-carotene is also an antioxidant involved in free radical defense. Fortification of food products is the most rational way of ensuring a

daily intake of β-carotene. However, beta-carotene is sensitive to environmental factors and rapidly loses activity being exposed to high temperature, light or oxygen (Krichkovskaya et al., 2001).

Combination of instability and hydrophobicity of crystal beta-carotene hampers its direct application in food processes. Development of acceptable β-carotene forms is required for introduction to food compositions. Encapsulation enables carotenoids stability optimization, dispersion in aqueous media and bioavailability improvement. The methods of microencapsulation of bioactives are used in medicamental compositions. Both natural substances (lipids, proteins, polysaccharides, etc.) and synthetic polymers (nylon, polyethylenimine, polylysine) are utilized as a wall material (Zuidam & Nedovic, 2010; Abbas, 2012).

Microencapsulation technology combines various physical and chemical methods including coacervation, precipitation, melt injection, fluidized bed coating, extrusion, vapor condensation, cross-linked polymers, polycondensation and polymerization. The method choice is mainly determined by properties of encapsulated ingredient (Sotnikov et al., 1999). For instance, the most complete encapsulation of flavorings can be achieved by spray drying. In this method encapsulation composition includes water-dispersed wall material, active ingredient, antioxidants and emulsifier (Merritt, 1981; Rich & Reineccius, 1988).

Preparation of water-dispersed forms of hydrophobic beta-carotene using microencapsulation includes several stages. Beta-carotene is dissolved in lipid fraction with emulsifiers and solvent at elevated temperature and pressure. Aqueous solutions of protectant colloid (gelatin or polymer) and softener (sugar) are prepared separately. The components are mixed and cooled down by pressure relieve. This results in formation of microparticles, namely, oil drops containing dissolved beta-carotene coated in protective shell. Fine powder is obtained after drying; it can be dispersed in water (Antoshkiw et al., 1982; Agapova et al., 1994).

Solubilization of beta-carotene can be achieved by using Tween 80 (polyoxyethylene sorbitan monooleate). Authors propose to use the solubilizate both for fatty and aqueous food systems (Sotnikov, 1995). Microemulsion 'Vetoron' produced in Russia includes alpha-tocopherol acetate, ascorbic acid and other bioactives in different proportions. There are successful results of using 'Vetoron' for medical applications (Sorokina & Lokshina, 2010). The possibility of using 'Vetoron' for fondant manufacture is currently investigated (Davidovich, 2009).

It should be noted that microencapsulates contain, as a rule, 1.5–10% of beta-carotene, up to 10% of stabilizer and antioxidants and 80–88.5% of non-ionogenic surfactants. In other words, they are mainly surfactants with beta-carotene addition and their combined biological effect requires special investigation (Shih, 2004). A way to obtain aqueous dispersion of beta-carotene at concentration of 80% requires application of extensive amounts of chlororganic solvents (dichlorethane, ethyl chloride, chlorophorm) or toluene (Bocharov, 2000). This makes application of this technique in food industry impossible (Zuidam & Nedovic, 2010).

Beta-carotene oil-in-water emulsions are prepared in few stages. At first stage β-carotene solution in non-polar solvent is added to vegetable oil at high temperature. This is followed by emulsification in aqueous phase containing stabilizer (Zuidam & Nedovic, 2010). Oil forms of beta-carotene increase its absorbtion *in vitro* and *in vivo*.

However, their use is limited and not applicable for products with reduced or zero fat (Berketova et al., 1995).

Liposomal encapsulation is a process that has been widely employed in medicine, pharmacology, cosmetology and genetic engineering (Lipowsky & Sackmann, 1995; Jesorka & Orwar, 2008; Mozafari et al., 2008). Liposomes are one of the most well studied supramolecular structures. There are researches on the diversity of lipid vesicles in terms of structure (size, number of layers), trapping capacity and localization in supramolecular structure (Gregoriadis, 2006). Liposomes can be objects of many applications because of independent biological activity of intact liposomes and enhanced efficacy of encapsulated ingredients (Bolshunova et al., 2010; Umnova, 2010). At present, liposomes are studied as an independent object in colloidal chemistry. They are considered as a target carrier of chemicals to cells, tissues and organs and as a model for biological membranes studies. However, their structure and properties still require investigation.

One of the promising liposomes applications is encapsulation of bioactive compounds and design of functional additives for food products. In the review by Gresler et al. (2008) liposomal technologies are considered as one of prospective lines in food industry. Benefits provided by embedding of active ingredients into liposomes include following: improved bioavailability of encapsulated ingredients; protection from environmental factors, e.g. oxygen, acids, processing temperature; ability to turn hydrophobic compounds into a water-dispersed form for better delivery; controlled release of an active ingredient (Mozafari et al., 2008).

The liposomes production processes known from literature could be adapted for the production of carotenoid-loaded liposomes as described by Ax (2003). In order to obtain the desired liposome size distribution, the dispersion is subjected to high-pressure homogenization, for example, by a microfluidizer.

The aim of this study was to show the possibility of designing a functional additive with beta-carotene in liposomal form and its usage for producing a dairy product. The additive should enhance biological activity and, therefore, improve health benefits of a product for daily consumption.

MATERIALS AND METHODS

Soya lecithin powder LeciPRO 90C (Orison Chemicals Ltd., China) was used as a liposomal wall material. Active ingredient for liposome encapsulation was crystalline beta-carotene (DSM Nutritional Products AG, Switzerland). Oil solution of α-tocopherol acetate 300 mg ml^{-1} (GalenoPharm, Russia) was used for antioxidant protection. Bioactive additive 'Vetoron' (Akvion, Russia) containing 20 mg ml^{-1} of beta-carotene and 8 mg ml^{-1} of vitamin E in water solution was used for preparation of non-liposomal carotene.

Carotene-loaded liposomes were prepared by dehydration/rehydration technique followed by mechanical homogenization (Weissig, 2010; Zabodalova et al., 2011). Soya lecithin was dissolved in approximately ten-fold n-hexane (Komponent-Reaktiv, Russia). Beta-carotene was dissolved in the minimal amount of chloroform (approximately 1 ml per 50 mg beta-carotene) and added to lipid solution to ensure the level of active ingredient being 0.5% (w/w) of lecithin. Antioxidant protection was achieved by addition of α-tocopherol in form of oil solution at the amount of 0.2%

(w/w) of lipid to the mixture. The blend was evaporated by the rotary evaporator EV311-V Plus (LabTech Inc., Italy) until lipid film was obtained at the flask bottom. Ethanol 50% solution was introduced to the dry film for better emulsification in the amount equal to soya lecithin weight, thoroughly stirred and the resulting mixture was left overnight. Next day the mixture was diluted with distilled water to ensure the lipid level of 10% (v/v). This mixture was an object of study as a water suspension of liposomes. The mixture was homogenized using mechanical mixer Biomix LE-402 (LaborMIM, Hungary) at the stirring rate of 15,000 rpm.

Empty liposomes were prepared in the same manner excluding the step of beta-carotene solution addition.

Reconstituted skim milk was used as a dairy product for fortification with liposomal beta-carotene. The skim milk powder contained 1% of fat (w/w), 36% of protein (w/w), 52% of lactose (w/w), 6% of ash (w/w) and 5% of water (w/w); it was obtained from the Bellakt Company (the Republic of Belarus). The skim milk powder was dissolved in distilled water (9 g skim milk powder per 100 ml distilled water) at 40–45°C. The mixture was left for 40 min for protein hydration and then pasteurized for 20−25 s at 76 ± 2°C. Liposomal additive was introduced into refrigerated milk with temperature of 4 ± 2°C in form of water suspension with 10% of lipid (v/v) and thoroughly stirred. Liposomal beta-carotene was added in the amount of 40% of daily adult requirement (5 mg) in beta-carotene established by the national health authorities (Rospotrebnadzor, 2009; Tutelyan, 2009) if consumed with 1 portion of the product (200 ml). Thus, the final levels of soya lecithin and beta-carotene in the product were 0.2 g and 1 mg per 100 ml of product, respectively.

Formation and internal structure of the liposomes were studied using electronic microscope JEM-100C (JEOL, USA). Two methods were used: negative contrast and ultra-thin slices technique. The first method was used as a preliminary to determine whether the desired structures were formed. Negatively stained samples were examined on support films with grids. The sample was mixed with a contrast solution. After 1 min of incubation, it was applied to the grid, the excess solution was drained, and the preparation was examined immediately (Lichtenberg et al., 1981; Podgorny & Ovchinnikov, 2002). Ultra-thin slices technique is more informative as it allows not only to determine the size, but also to obtain information about the internal structure. Ultra-thin slices were embedded in epoxy resin Araldite (Fluka, Switzerland) (Sora, 2007).

Fraction-dispersed composition and aggregate stability of liposomal particles were investigated by means of dynamic light scattering using spectrometer LKS-03 (Intoks, Russia). The method is based on scattered light intensity shift resulting from molecular movement in liquid. The technique provides information on particles size distribution. The percentage of diffused light corresponding to each fraction is related to surface area of diffusing particles which gives an indirect estimate of fraction share (Chernjavskij et al., 1985; Torchilin & Weissig, 2003). Since the dairy product matrix is too complex, fraction-dispersed composition of carotene-loaded liposomes was studied in the water suspension.

Titratable acidity and pH were controlled in the dairy product during storage to detect the effect of liposomal additive on milk stability. Titratable acidity was controlled according as follows: 10 ml of the product were mixed with 20 ml of distilled water and 3 drops of 1% phenolphthalein in a flask. The mixture was titrated

with 0.1 M NaOH until the defined pink color appeared. Titratable acidity was expressed as the volume of NaOH multiplied by ten (°T).

Beta-carotene concentrations in the dairy product and in the water suspension of liposomes during storage were determined using spectrophotometric method on UV-2,600 spectrophotometer (Shimadzu, Japan). The samples were diluted with distilled water to have 1% of fat (v/v). Beta-carotene was extracted from 1 ml aliquots of the dairy product or the water suspension using 2 ml of chloroform. Then 0.7 ml of the extract was collected and 3 ml of chloroform were added. The absorbance of carotenoid was measured in 1 cm cuvettes at a wavelength of 451 nm. Concentration was determined using standard curve (Dawson et al., 1989).

Biological activity of the dairy products was investigated using hexobarbital sleeping time test on white outbred mice (Dearing et al., 2006). Hexobarbital is a drug used for general anesthesia. It is degraded by microsomal enzymes in the liver that results in awakening. The test was performed on five groups with 10 animals in each (males, weight of 18–20 g). The dairy product samples were injected intragastrically in the dose of 0.2 ml per animal daily during 5 days. The last injection was made 30 min before hexobarbital injection. Hexobarbital was injected to all groups intraperitoneally in the dose of 60 mg kg^{-1}. The sleeping time was determined in seconds.

All measurements were performed at least in triplicate. Statistical analysis was performed using Microsoft Office Excel tools at theoretical frequency of 0.95.

RESULTS AND DISCUSSION

The liposomes characteristics

Fraction-dispersion composition and aggregative stability of liposomal dispersion in water and dairy product matrix were studied using transmission electron microscopy. Electron microscopy of the dairy product with carotene-loaded liposomes on the first day of storage proved the presence of continuous hollow spherical structures (Fig. 1). Unilamellar hollow vesicles and several multi-layered hollow structures can be distinguished clearly. The dark circles are probably slices of lipid drops with phospholipid and protein coatings (Gorbatova et al., 2011).

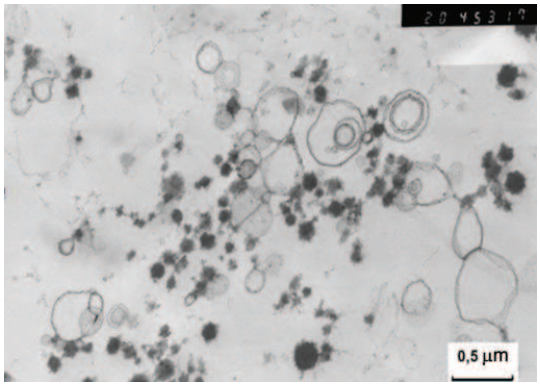

Figure 1. Electron microscopy image of ultra-thin slice of beta-carotene loaded liposomes in the dairy product matrix on the first day of storage.

The stability of lammelar structures of liposomes was studied in the dairy product during 10 days of refrigerated storage at 4 ± 2°C (Fig. 2). No structure disruption or aggregation of lipid vesicles were observed, however monolamellar liposomes dominate in the field of vision. Lipid drops tend to aggregate. This indicates the stability of liposomal additive in the dairy product during refrigerated storage for the time of observation.

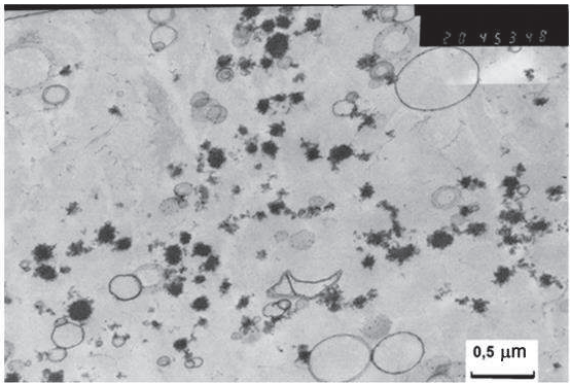

Figure 2. Electron microscopy image of ultra-thin slice of beta-carotene loaded liposomes in the dairy product matrix after 10 days of storage at 4 ± 2°C.

It should be noted that homogenization transfer of multi-layered liposomes to mono-layered ones and reduction of their mean diameter also occurs after mechanical stirring or sonication (Weissig, 2010).

Liposomes of different size and characteristics can be obtained depending on a preparation technique. The size is important in terms of liposomes application. Although the ultra-thin slices images can give a brief estimate of size distribution, it cannot be considered reliable as during sample preparation certain deformations of shape and structure occur.

More objective data on fraction-dispersed composition was obtained by means of dynamic light scattering (Table 1). It should be noted that the actual mean diameter is lower than the mean hydrodynamic diameter as measured by this method.

Table 1. Mean hydrodynamic diameter (d, nm) and percent of scattered light (%) of carotene-loaded liposomes in water suspension during storage at 4 ± 2ºC

Method	Storage duration, days					
	0		7		27	
	d, nm	%	d, nm	%	d, nm	%
without homogenization	1,100	12	–	–	–	–
	430	60	530	27	350	85
	126	28	220	63	115	11
with homogenization	17,500	30	21,000	40	22,000	12
	730	33	525	40	500	45
	260	33	170	19	190	42
	80	3	–	–	–	–

It is established that most of the particles in the solution have a size less than 1 μm. Different stereometry of lipids from the raw material may cause erratic distribution of formed lamellar particles size. Changes in particles size in the unhomogenized sample were not observed during the period of study (27 days at 4 ± 2ºC). It can be said that in this sample liposomes have stable sizes of about 0.5 μm or less. First fraction with the size of 1.1 μm could be formed by united particles which are subsequently separated.

The fraction with sizes of 17–22 μm was established in the water suspension liposomes samples subjected to the homogenization. Since there were no particles of this size in the original solution before homogenization, it can be assumed that this fraction is represented by a group of smaller particles joined together. This supposition is supported by this fraction part reduction from 30% to 12% in 27 days, which may be associated with the detachment of liposomes from aggregates formed during homogenization. Large fraction formation indicates the necessity of selecting another conditions or equipment for homogenization.

The products stability during storage

Stability of beta-carotene in liposome form during storage at 4 ± 2ºC was determined in two samples: the fortified dairy product and the water suspension of liposomes. Oxidized beta-carotene does not absorb at a wavelength of 451 nm. The oxidation rate of beta-carotene and the residual amount of non-oxidized beta-carotene in liposomes can be studied by the concentration in an extract from liposomes. The concentrations of beta-carotene in the chloroform extracts from the product and the suspension did not change during the observation period of 0–15 days and were 4.7 ± 0.5 μmol l^{-1} and 3.3 ± 0.7 μmol l^{-1}, respectively. This means that the liposomal beta-carotene is not exposed to oxidation.

The stability of the dairy product was evaluated by controlling titratable acidity and pH in reconstituted skim milk, in the product fortified with liposomal beta-carotene and in water suspension of liposomes. Both reconstituted skim milk with and without liposomal beta-carotene had pH of 6.75 ± 0.13 and titratable acidity of 18.9 ± 1.1°T for 0–13 days of storage at 4 ± 2ºC. Water suspension of liposomal additive had pH of 7.04 ± 0.09 for the same period and conditions of storage. Thus the liposomal additive did not affect pH and titratable acidity and did not lead to earlier spoilage of the product compared to the control sample.

Bioactivity of liposomal beta-carotene

Liposomal additive has a wide range of bioactivity due to its composition. The effect of the fortified dairy product consumption on the liver function of mice was chosen to study its bioactivity. The hexobarbital sleeping time test was performed using three samples: reconstituted skim milk, reconstituted skim milk fortified with liposomal beta-carotene and reconstituted skim milk fortified with water-soluble beta-carotene (‘Vetoron’). The two latter samples contained equal amounts of beta-carotene ensuring 40% of daily requirement of an adult – 1 mg%. The first control group received no treatment; the other control group received distilled water. The results are given in Table 2.

Table 2. The effect of the different products on hexobarbital sleeping time of mice

Group	Hexobarbital sleeping time	
	min	% of control
Control (intact animals)	40 ± 5	100
Distilled water	41 ± 4	100
Skim milk	39 ± 3	98
Skim milk + 'Vetoron'	30 ± 5	75
Skim milk + liposomal beta-carotene	24 ± 4	60

The data indicates decrease in hexobarbital sleeping time of animals treated with the samples fortified with beta-carotene. The sample with liposomal beta-carotene is more efficient than the one with water-soluble beta-carotene.

It should be noted that the lipids in liposomal wall bear not only structural function. Phospholipids also possess bioactivity and employed in the drugs stimulation of a liver function. An activation of liver detoxication function due to the functional additive intake was shown in the hexobarbital sleeping time test. This may indicate the ability of liposomes to penetrate hepatocytes and the effect of the functional additive on the liver detoxification rate (Shleikin et al., 2006). Further study is required to determine which components of the liposomal additive, jointly or separately, and in what form affect the liver function.

Liposomal beta-carotene has two active components: beta-carotene itself and lecithin as the wall material. Both contribute to hepatostimulation activity of the additive. However, it is not the only bioactive property. The additive can give a wide range of health benefits including anti-inflammatory, immunostimulatory activities, etc.

The results are in accordance with studies of other authors. Liposomal form doubles bactericidal and bacteriostatic activity of the phytochemical compositions as shown in the bioactivity comparison of their native and liposomal forms (Umnova, 2010). It was shown in the same research, that liposomal form of bioactives increases their membrane-stimulating properties by 40% and antioxidant activity by 25%. Liposomal form of *Bergenia crassifolia* leaves extract had a three times high immunomodulatory effect under cold stress as native extract (Bolshunova et al., 2010). Stimulating effect of the empty liposomes was also noted. Researches confirm liposomal form to be promising for phytocompositions and encapsulation of bioactives.

CONCLUSIONS

The novel functional additive for dairy products is proposed. The method used for liposome preparation is low-cost, simple and enables the production of unilamellar vesicles with the mean diameter below 1 μm. The stability of liposomes in dairy product matrix during storage is confirmed by transmission electron microscopy. The liposomal beta-carotene is not exposed to oxidation during storage for 15 days at 4 ± 2ºC.

The fortified low-fat milk drink was prepared with addition of liposomal beta-carotene to pasteurized reconstituted skim milk. The intake of 200 ml of the fortified product can supply 40% of daily requirement in beta-carotene. The liposomal additive

did not lead to earlier spoilage of the product as the product had pH of 6.75 ± 0.13 and titratable acidity of 18.9 ± 1.1°T for 0–13 days of storage at 4 ± 2ºC.

The activation of liver detoxication function due to the fortified dairy product intake was shown in the hexobarbital sleeping time test on mice. The contribution of each component of the liposomes in this bioactive property requires further study as well as other possible health benefits of the functional additive.

ACKNOWLEDGEMENTS. This work was partially financially supported by the government of the Russian Federation, Grant 074-U01.

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