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ENCAPSULATION OF ZINC-CHLOROPHYLL DERIVATIVES IN WHEY PROTEIN MATRIX BY EMULSION/COLD-SET GELATION

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ABSTRACT

Encapsulation of spinach zinc-chlorophyll (Zn-chlorophyll) derivatives in whey protein matrix by emulsion/cold-set gelation seems to be a promising alternative method for handling stabilized green natural colorant. The main important parameter was the pH of the emulsion system that caused precipitation of the whey protein. The shape of the beads containing 1 and 5% Zn-chlorophyll derivatives were spherical, while beads containing 10% Zn-chlorophyll derivatives production resulted in precipitation of whey protein due to isoelectric point (pI= 4.9). Encapsulation efficiency was determined for different loads of active material, whereas, the highest value was obtained for 1% Zn-chlorophyll derivatives containing beads. Beads with 5% Zn-chlorophyll derivatives showed the best stability for color values (L*=31.25 ± 0.06, a*= -2.91 ± 0.11, and b*= 25.85 ± 0.08), and a 74% protection of the total chlorophyll content was maintained at the end of 3 months of storage at 4 °C.

Keywords: zinc chlorophyll derivatives, whey protein, emulsion/cold-set gelation, and bead

ÇİNKO-KLOROFİL TÜREVLERİNİN PEYNİR ALTI SUYU PROTEİNİ İLE EMÜLSİYON/SOĞUK JELLEŞME YÖNTEMİ İLE ENKAPSÜLASYONU

ÖZ

Ispanakta bulunan çinko-klorofil (Zn-klorofil) türevlerinin peynir altı suyu protein kullanılarak emülsiyon/soğuk-tip jelleşme yöntemi ile enkapsülasyonu, kararlı yapıda doğal yeşil renklendirici eldesinde umut verici alternatif bir yöntem olarak görülmektedir. Emülsiyon sisteminde, peynir altı suyu proteininin çökelmesi nedeniyle en önemli parametre pH olarak belirlenmiştir. %1 ve 5 oranında Zn-klorofil türevleri içeren tanecikler küresel bir yapıya sahipken, %10 oranında Zn-klorofil türevleri içeren taneciklerin üretimi peynir altı suyu proteinlerinin izoelektrik noktaya (pI= 4.9) ulaşması nedeniyle çökelme ile sonuçlanmıştır. Enkapsülasyon verimliliği aktif maddenin farklı yükleri için hesaplanmış, en yüksek değer %1 oranında Zn-klorofil türevleri içeren tanecikler ile elde edilmiştir. 4 °C'de 3 aylık depolama süresi sonunda en iyi renk stabilitesi (L*=31.25 ± 0.06, a*= -2.91 ± 0.11 ve b*= 25.85 ± 0.08) ve %74 oranında korunan toplam klorofil içeriği değerlerine %5 oranında Zn-klorofil türevleri içeren tanecikler ile ulaşılmıştır.

Anahtar kelimeler: çinko klorofil türevleri, peynir altı suyu proteini, emülsiyon/soğuk-tip jelleşme ve tanecik

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INTRODUCTION

Development of food colorants from natural sources has an increasing interest to synthetic dyes due to legislative action and consumer concern (Giusti, 1996). Even though, the Food and Drug Administration have approved artificial colors using by food companies, consumer advocacy groups such as the Center for Science in the Public Interest have argued that they could be harmful to a person's health, leading to hyperactivity, allergic reactions, and other illnesses. Additionally, a marketing research firm Nielsen showed that according to global respondents, the absence of artificial colors (42%) and flavors (41%) is important to their food purchase decisions (Nielsen, 2016).

Chlorophylls as lipophilic compounds are synthesized and accumulated in specialized organelles including chloroplasts and chromoplasts and they are responsible for green color of vegetables and mostly unripe fruits (Britton and Hornero-Méndez, 1997). Biological importance of chlorophyll pigment stem from its antioxidant and antimutagenic activities, modulation of xenobiotic metabolising enzyme activity, and induction of apoptotic events in cancer cell lines, prevention of cancer and other degenerative diseases (Dashwood, 1997; Ferruzzi and Blakeslee, 2007). Chlorophyll is a stable pigment in nature, however when it is extracted from plant tissues, it could degrade due to heat, light, oxygen, acid, and enzymes (Koca et al., 2007). After its degradation, bright green colored natural chlorophyll transform into chlorophyll derivatives such as pheophytin, pyropheophytin, pheophorbide, pyropheophorbide and chlorophyllide (Rodriguez-Amaya, 2016). To preserve the green chlorophyll, replacing the Mg^{++} ion in the porphyrin ring of chlorophyll pigment with Cu^{++} or Zn^{++} salts produces a regreening effect. These metallo-chlorophyll complexes are more stable and more resistant to acid and heat (Leunda et al., 2000). In addition, these alternative metallo-chlorophyll complexes have greater antioxidant properties than their natural form (Mg^{++}-chlorophyll) (Tonucci and Von Elbe, 1992). Özkan and Bilek (2015) reported that the formation of Zn-chlorophyll derivatives from spinach leaf was performed by the reaction of fresh leaves with 300 ppm $ZnCl_2$ at pH 7, followed by a heating process at 110 °C for 15 min. Zinc-pheophytin was also identified in the stabilized chlorophyll. For increasing the efficiency of the extraction, an enzyme-assisted release of zinc-chlorophyll derivatives from the spinach pulp was applied, and Pectinex Ultra SP-L (Novozymes, Denmark) was used as the enzyme to increase the extraction efficiency. Pretreatment of the pulp with enzyme and extraction in ethanol resulted in a 39% increase in the Zn–chlorophyll derivative yield (Özkan and Bilek, 2015). There are a limited number of researches about chlorophyll as a natural food colorant. One of the technology for production of colorant as solid form is microencapsulation. This technology is used for the protection, stabilization, and the slow release of the active materials in the microbead system (Özkan and Bilek, 2014). However, in aqueous environments, water-soluble microparticles generally disintegrate and lose their stabilizing effect for the encapsulated compounds. Thus, water-insoluble encapsulation systems appear promising because of their structure and core material-stabilizing function after immersion in water (Betz and Kulozik, 2011).

In recent years, microencapsulating properties of whey proteins have been investigated and reported (Rosenberg, 1997). Whey protein concentrates and isolates exhibit high nutritional value, good emulsification, gelation, and film-forming properties (Lee and Rosenberg, 2000; Lefevre and Subirade, 2000; Leung et al., 2005). Besides, whey proteins have the ability to form heat-induced gel matrixes, capable of holding large amounts of water and entrapping active agents for delivery (Oztop et al., 2012). Whey protein gels are usually formed by either heat set or cold set mechanisms (Nicolai et al, 2011; Banerjee and Bhattacharya, 2012). Heat set gelation is a one-step process that involves heating a solution of globular proteins above their thermal denaturation temperature (Nicolai et al., 2011). Cold-set gelation method is a two step process that involves the denaturatşon of whey protein isolate (WPI) solution at a concentration

below the critical concentration for gelation, followed by the gelation of the denatured protein solution at ambient temperature by either changing the pH of the solution (Britten and Giroux, 2001), or the saline conditions (Barbut and Foegeding, 1993). Microencapsulation by means of the emulsion/cold-set gelation method comprises the generation of the whey protein-zinc chlorophyll derivatives containing extract/sun flower oil emulsion and the cold-set gelation of this emulsion to acquire the dispersed beads.

The objective of the present study was to evaluate the ability of the newly developed whey protein based microencapsulation system to preserve the stability of the encapsulated zinc-chlorophyll extract with desirable characteristics. The usage of water insoluble protein based encapsulation systems for Zn-chlorophyll compounds and process related factors during bead production were investigated. In order to optimize the encapsulation process, different loads of active material was used. The encapsulation systems were examined and compared to choose the best process conditions with respect to entrapment efficiency and color and chlorophyll stability over storage period.

MATERIALS AND METHODS

Materials

Spinach leaves were purchased from the local markets in İzmir, Turkey between October and November, 2013. The Pectinex Ultra SP-L enzyme (containing polygalacturonase, hemicellulase, cellulase, protease, and amylase) was obtained from Novozymes (Novozymes, Denmark). It is a brown liquid manufactured from *Aspergillus aculeatus*, and manufactured to an activity specification of 3800 PGNU/mL. The whey protein isolate was obtained from Davisco Foods International Inc. (North America). All reagents used for the analyses were of analytical grade.

Methods

Formation of Zn-chlorophyll derivates

The formation of metallochlorophyll complexes in spinach leaf extract was performed according to Ozkan and Bilek (2015) as follows: The fresh spinach leaves were washed, chopped into small pieces, and homogenized with a Waring blender (Waring Commercial, Torrington, CT) for 1 minute at the highest speed. The ratio of spinach leaves to water was 1:4. For the formation of the Zn–chlorophyll derivatives, the spinach pulp containing 300 ppm $ZnCl_2$ at a pH 7 was thermally processed at 110 °C for 15 min in a autoclave. Finally, enzyme-assisted extraction by Pectinex Ultra SP-L was carried out to thermally processed spinach pulp to obtain Zn-chlorophyll derivatives. Optimum enzyme-assisted extraction condition was determined as 8% (v/v) Pectinex Ultra SP-L, 30 min reaction time and 45 ºC incubation temperature. Additionally, the pH of the Zn-chlorophyll containing spinach pulp was adjusted to 5 by addition a 1 N citric acid solution for the maximum enzyme activity. Extraction was performed under constant stirring conditions at 120 rpm in a Gerhard thermoshake shaker (C. Gerhardt GmbH & Co. KG Konigswinter, Germany) continuously. After the enzymatic pretreatments, solvent extraction of total chlorophyll from spinach pulp was performed. Based on preliminary studies, the solvent extractions were carried out using an ethanol/pulp ratio (2.5:1, v/v), at 60 ºC extraction temperature for 45 min extraction duration, and stirring at 120 rpm in a Gerhard Thermoshake shaker. The obtained Zn-chlorophyll derivatives in liquid form were concentrated to 30º Brix by using rotary evaporator (Heidolph, Germany) to use for the emulsion/cold-set gelation microencapsulation process.

Emulsion preparation

A WPI solution (8% w/v of protein content) was prepared and stirred with a magnetic agitator for 1 h at room temperature (25 ± 1 °C), then the solution was left overnight at 4°C to ensure full hydration of the proteins. The final pH of the solution was 6.8 without any adjustments. The following day, this WPI solution was denaturated at 80 °C for 30 min in a temperature-controlled water-bath (Buchi 461 CH-9230, Switzerland) and simultaneously mixed at 900 rpm with a IKA stirring device (RW 20 D, IKA-Werke GmbH & CO. KG, Staufen, Germany). After heat

treatment, the denatured WPI solution was cooled rapidly in an ice bath to 25 °C and was kept at this temperature before further use (Egan et al., 2014).

Oil-in-water emulsion (O/W) consisting of the denatured 8% (w/v) WPI solution as an aqueous phase; 1, 5, and 10% (v/v) of Zn-chlorophyll extract, and 20% (v/v) sunflower oil as an oil phase were prepared according to the emulsification procedure adapted from Rosenberg and Lee, (1993). Briefly, the emulsion was prepared using an Ultra-Turrax homogenizer (Ultra-Turrax T25 basic IKA-WERKE) operated at 15500 rpm for 1 min. The O/W emulsions prepared as described above were used for the production of beads by cold-set gelation method.

Preparation of beads

The preparation of beads was adapted from Wichchukit et al., (2013). The O/W emulsion containing the Zn-chlorophyll was dropped into 200 ml of mildly agitated 3% (w/v) calcium chloride ($CaCl_2$) solution using a 5 ml syringe with a 21 G (0.8 x 38 mm) needle. Tween 80 was added to the $CaCl_2$ at a final concentration of 1% (w/v) to obtain spherical beads and decrease the interfacial tension. The beads remained in the $CaCl_2$ solution for 1 hour to allow for sufficient gel matrix formation. Then, the beads were washed with 0.02% (w/v) sodium azide containing deionized water to remove the excess $CaCl_2$. Before storage, they were dried for 12 h in a vacuum oven at 35 °C.

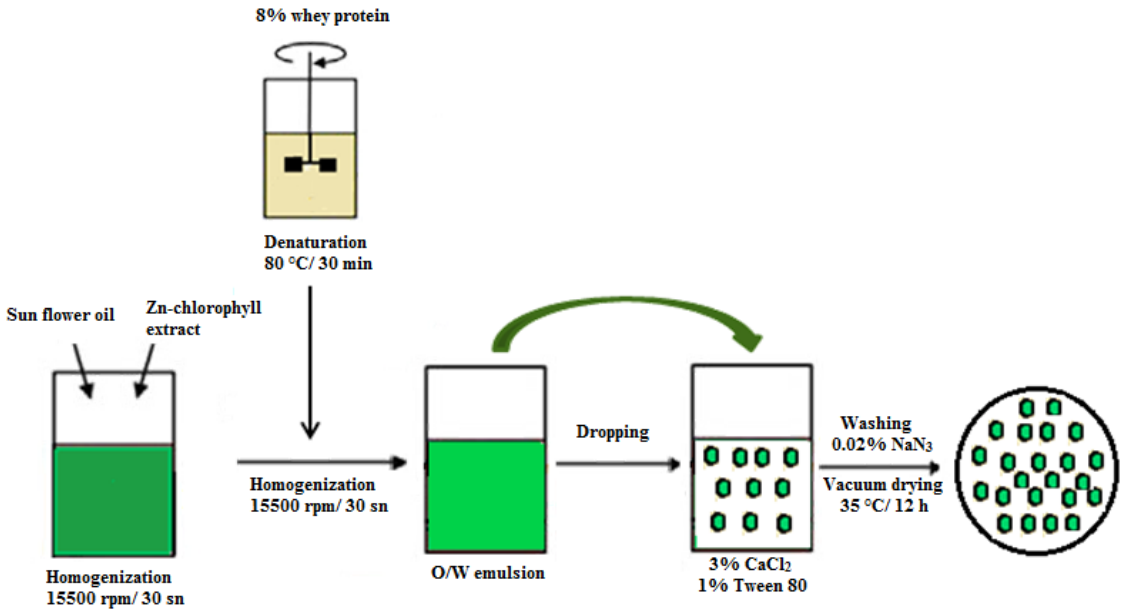


Figure 1. Bead production from stabilized spinach chlorophyll in whey protein by emulsion/external cold-set gelation

Determination of chlorophyll content by spectrophotometric method

To measure the chlorophyll content, 20 ml of 80% acetone was added to 1 g of each of the chlorophyll loaded beads and mixed with a homogenizer (Ultra-Turrax T25 basic IKA-WERKE) for 1 min at 15,500 rpm, then centrifuged at 8000 rpm for 5 min at 4 °C, and then filtered through Whatman No.1 filter papers. The volume was adjusted to 25 ml and the absorbance values were measured at 663 and 645 nm by spectrophotometer (UV-Visible VARIAN Cary 50) (Vernon, 1960).

Color measurement

A Hunterlab Colorflex CFLX 45-2, VA colorimeter was used to determine the CIE L*, a*, and b* values in which L* is the lightness of the

color (100= white and 0= black), a* value (+a*= red and -a*= green), and the b* value (+b*= yellow and -b*= blue).

Total dry matter content
The dry matter content (%) of the beads was determined according to Young et al., (1993) using a vacuum oven (Nüve EV 018, Turkey) to dry the samples to a temperature of 65 °C at 517.17 mmHg of pressure.

Encapsulation efficiency
The encapsulation efficiency (EE) was calculated as the ratio between the initial mass of Zn-chlorophyll to be encapsulated and its mass in the final product (Shu et al., 2006).

Scanning electron microscopy (SEM)
The outer structural features of the dry beads were monitored by SEM. The beads were washed four times in absolute ethanol and then dried for 12 h in a vacuum oven at 55 °C. The procedure was carried out using the scanning electron microscope (Tescan) operated at 20 kV (Rosenberg and Young, 1993).

Zinc content
Zinc content of the beads was determined by AOAC (2005) 999.10 using inductively coupled plasma–optical emission spectrometry (ICP-OES) method (Porrarud and Pranee, 2010).

Protein content
The total protein content of the samples was determined by means of the Dumas method using the nitrogen gas analyser system FP-528 (LECO, Moenchengladbach, Germany). The whey protein concentration was calculated from total nitrogen content using a factor of 6.38 (Betz and Kulozik, 2011).

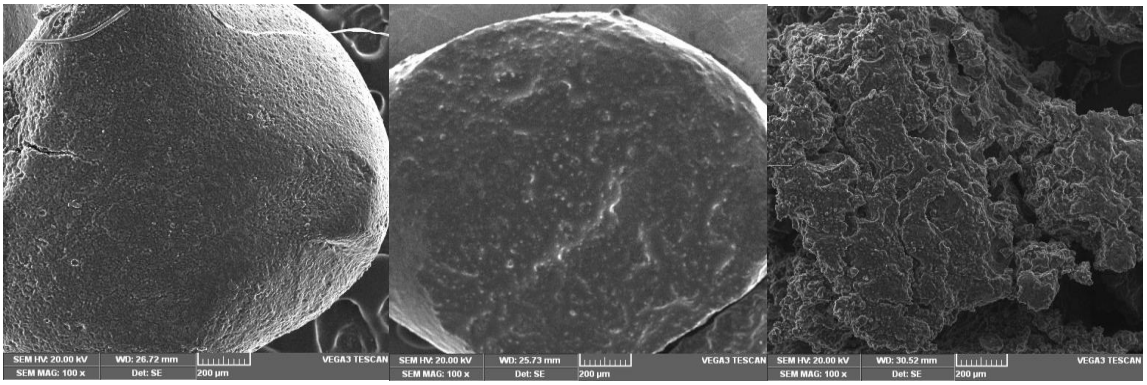
Storage stability of beads
The storage stability of the WPI based zinc-chlorophyll loaded beads was performed at +4 °C in a glass jar for 3 months. The beads were assessed in terms of color (-a*, greenness) and spectrophotometric total chlorophyll contents at 30 day intervals. The aim of the storage was to investigate the effect of time on the physical and chemical properties of the zinc-chlorophyll under constant storage conditions.

RESULTS AND DISCUSSION

Generation of zinc-chlorophyll extract-loaded cold-set gelled beads
The zinc-chlorophyll loaded WPI based spherical beads, 1-2 mm in diameter, were generated by changing the zinc-chlorophyll concentration as 1, 5, and 10% (v/v) in order to investigate the influence of the amount of the active material on the EE. It was found that the EE was influenced by the concentration of the zinc-chlorophyll. When the zinc-chlorophyll concentration increased from 1% to 5% and then 10%, the EE was found as 65.89, 47.05, and 39.82%, respectively. Addition of 1% zinc-chlorophyll extract was found to be the optimal choice in terms of EE for the emulsion/cold-set gelation method. This situation was effected from several factors including polymer hydrophilicity, porosity, crosslinking as well as, interaction between polymer and extract components (Trifković et al., 2014). Moreover, a SEM analysis was conducted to obtain information about the surface morphology and shape of whey protein beads as well as to confirm the influence of the content of active material. Visual observations showed that stable beads were obtained with 1% and 5% active material concentration (Fig 2). The pH value of the whey protein-Zn-chlorophyll/sunflower oil emulsion decreased with the addition of increasing amount of Zn-chlorophyll extract at pH 5. Hence, the zeta-potential of the WPI-zinc chlorophyll derivatives with a 10% chlorophyll content was approaching around zero. Due to reaching of the whey proteins isoelectric point (pI near 4.9) by addition of 10% chlorophyll extract, precipitation of the whey protein occurred where the stable emulsion matrix could not maintained.

These results are in agreement with earlier studies of the pH stability of oil-in-water emulsions which were prepared with WPI (Chanamai and McClements 2002; Charoen et al., 2010). According to Charoen et al. (2010), extensive aggregation at pH 5 and a large increase in mean diameter around the pI ($4 < pH < 6$) occurred in

the WPI-stabilized emulsion. It was also observed that creaming stability was fluctuating at pH values around their pI (Charoen et al., 2010). The unstability of the WPI-stabilized emulsions around their pI may be due to poor electrostatic repulsion between the oil droplets that results with the flocculation of oil droplets (McClements 2005). At relatively high H^+ concentrations (pH << pI), the amino groups are positively charged ($-NH_3^+$) and the carboxyl groups are neutral (-COOH), so the net protein charge is positive. At relatively low H^+ concentrations (pH >> pI), the carboxyl groups are negatively charged ($-COO^-$) and the amino groups are neutral ($-NH_2$), so the net protein charge is negative. At the pI, net charge of the protein approach to zero due to balance in positively and negatively charged groups. Therefore, around the pI, aggregation of the droplets occures because of decreasing stability to aggregation and increasing van der Waals forces (Charoen et al., 2010).



(a) (b) (c)

Figure 2. The morphology of the WPI based beads as observed with SEM with different chlorophyll contents 1% (a), 5% (b), 10% (c)

Qian et al. (2012) investigated the pH effect on the physical and chemical stability of β-carotene enriched nanoemulsions. According to the results, β-lactoglobulin-coated lipid droplets were unstable to aggregation at pH values close to the pI of the protein (pH 4 and 5). A large increase in mean particle size and phase separation due to droplet creaming was observed from the nanoemulsions stored at pH 4 and 5 (Qian et al., 2012).

Effect of storage on bead stability

Color values of beads

According to the results, the -a* value representing green color was found to be 0.62 ± 0.06, -2.91 ± 0.11, and -2.07 ± 0.02 for 1, 5, and 10% zinc chlorophyll derivatives containing beads, respectively (Table 1). The results also showed that the $-a^*$ value of the 1% zinc chlorophyll derivatives containing beads decreased significantly ($P \leq 0.05$) during the 3 months of storage. This result may be attributed to the loss of zinc-chlorophyll as a consequence of diffusion through the large specific area of the whey protein particles. On the other hand, there was no significant difference ($P>0.05$) on the color change of the 5% zinc-chlorophyll containing beads during the storage period. Moreover, there was a slight decrease in the 10% zinc–chlorophyll containing sample.

Total chlorophyll content of the beads

Table 2 shows the change of total chlorophyll contents of beads. According to the results, the total chlorophyll contents of the 1, 5, and 10% Zn-chlorophyll containing beads decreased significantly ($P \leq 0.05$) during the 3 months of storage at 4 °C. Higher losses (55% reduction) for 1% Zn-chlorophyll derivatives containing beads may be due to the high diffusion rate of Zn-chlorophyll throughout the large specific area of the whey protein particles.

Table 1. Change in the a* values of the beads during the 3 months at 4°C

Zn-chlorophyll concentration (%)	0. month	1. month	2. month	3. month
1	0.62 ± 0.06^{a}	0.89 ± 0.04^{b}	1.37±0.03^{c}	1.70±0.06^{d}
5	-2.91 ± 0.11^{a}	-2.98 ± 0.11^{a}	-3.05± 0.11^{a}	-2.75±0.03^{a}
10	-2.07±0.02^{a}	-1.57±0.02$^{a.b}$	-1.59±0.15^{b}	-1.82±0.07^{b}

Means in the same row identified by the same letter are not significantly different.

Table 2. Change in the total chlorophyll content of the beads during the 3 months of storage at 4°C

Zn-chlorophyll concentration (%)	0. month	1. month	2. month	3. month
1	1.83±0.07^{a}	1.34±0.19$^{a.b}$	1.31±0.24$^{a.b}$	0.83±0.70^{b}
5	6.54±0.27^{a}	5.33±0.45^{a,b}	5.28±0.42^{a,b}	4.84±0.13^{b}
10	11.07±0.08^{a}	10.91±0.29^{a}	8.57±0.18^{b}	8.47±0.42^{b}

Means in the same row identified by the same letter are not significantly different.

Storage stability of Zn-chlorophyll derivative containing WPI based beads was shown in Fig 3. Regression equation of *ln* (pigment retention %) against storage time expressed the linear relation with negative slope when plotted on a natural logarithmic scale. Decrease of the Zn-chlorophyll derivative content can be concluded as first-order kinetics with rate constant (*k*) of 23×10^{-3}, 0.9×10^{-3} and 1.045×10^{-3}/day for the 1, 5 and 10% Zn-chlorophyll derivatives containing beads, respectively. From this experiment results, it was clear that 5% Zn-chlorophyll was the appropriate concentration in which the most of the Zn-chlorophyll derivatives was retained with the lowest rate constant.

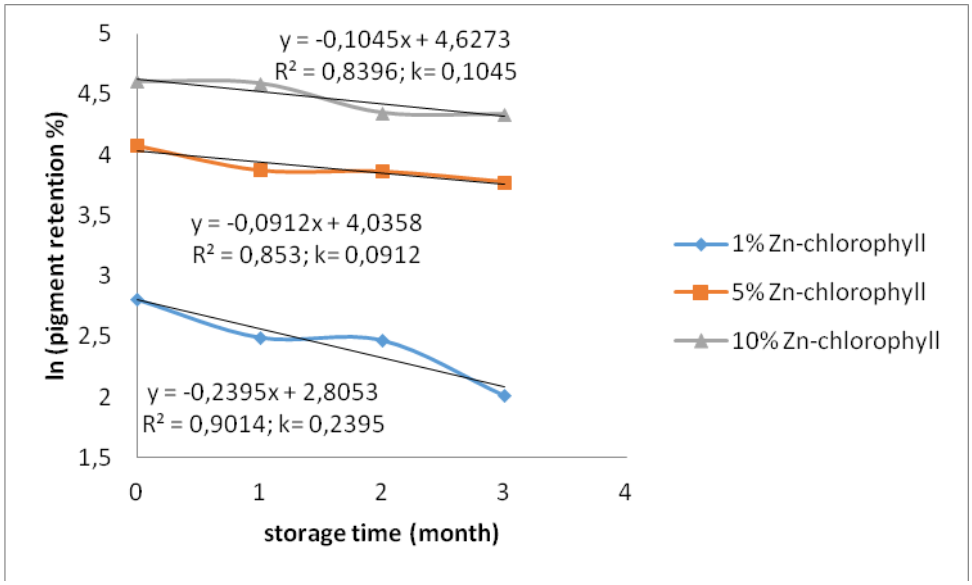


Figure 3. Degradation of Zn-chlorophyll derivatives in WBI beads with (a) 1% Zn-chlorophyll, (b) 5% Zn-chlorophyll, (c) 10% Zn-chlorophyll content

Similar rate constants for determining decay of Zn-chlorophyll derivatives was supported with another study. Porrarud and Pranee (2010) investigated the influence of different wall materials including gum arabic, maltodextrin and osa-modified starch (contains both hydrophobic and hydrophilic groups) on stabilities of encapsulated Zn-chlorophyll containing powder. The osa-modified starch as a wall material provided a longer shelf life ($k= 1.5\times10^{-3}$ /day) compared to the gum arabic ($k= 2.1\times10^{-3}$ /day) and the maltodextrin ($k= 1.8\times10^{-3}$ /day) powders (Porrarud and Pranee, 2010).

Zinc content of the beads

The zinc content of the samples did not change during the storage period. The average zinc contents of the beads during storage were found to be 55, 232, and 383 mg/kg for 1, 5, and 10% Zn-chlorophyll derivatives loads, respectively. The FDA limit for the concentration of Zn^{2+} in the food product cannot exceed 75 ppm (LaBorde and Von Elbe, 1994). Hence, the concentrations of the Zn^{2+} of the bead samples are acceptable for the given limits when they are added to the food products for different concentrations. Similarly, Porrarud and Pranee (2010) reported that the zinc content of the zinc-chlorophyll powders produced by different wall material types such as gum arabic, maltodextrin, and osa-modified starch were found to be 14.45, 13.79, and 13.12 mg/kg, respectively which were also found in the range of acceptable values (Porrarud and Pranee, 2010).

Protein content of the beads

The initial protein concentration of WPI based beads (8% w/v) increased after drying due to removal of the moisture from bead structure. The protein content of the samples did not change during the storage period. The average % protein contents of the beads during storage were found to be 30.06, 31.94 and 30.81% for 1, 5, and 10% Zn-chlorophyll derivatives loads, respectively. Emulsion/cold-set gelation methods seems to be a promising encapsulation method on account of high protein recovery and protein protection. Egan et al., (2012) investigated the immobilization of a lipid phase within whey protein microgels prepared by emulsification/cold-set whey protein gelation method. There was very little loss of protein phases from microgels after removal from the $CaCl_2$ solution. Hence, this method can be approved as an efficient immobilization of active material within the whey protein matrix with very high level of protein recovery in the range of 95.95-100% (Egan et al., 2012).

CONCLUSION

The whey protein based beads were used for entrapment of zinc-chlorophyll extract using emulsion/cold-set gelation aiming to improve the functionality and stability of the bioactive materials. It was found that the concentration of zinc-chlorophyll affected the bead formation in both shape occurrence and stability. Stable beads were obtained with 1% and 5% active material concentration, whereas 10% zinc-chlorophyll containing beads were prone to precipitation due to reaching the pH values of whey proteins to the pI. The encapsulation efficiency was found to be the highest as 65.83 for 1% zinc-chlorophyll loaded beads. On the other hand, beads obtained with 5% zinc- chlorophyll loading showed the best results in terms of color and total chlorophyll content during the 3 months of storage. The results of this study showed that zinc-chlorophyll derivatives could be successfully entrapped in whey protein matrix. These beads have a potential for usage as food colorants and food additives incorporated into dietary supplements, functional food, and pharmaceuticals.

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