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Synthesis, characterization, stability evaluation and release kinetics of fiber-encapsulated carotene nano-capsules

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SUMMARY: In the present work, carotenoids were isolated (1.2%) from crude palm oil and encapsulated with isabgol fiber (*Psyllium husk*). The efficiency of encapsulation was 82.23±1.42%. The morphology of the capsules showed rough surface texture with minimal pores. The amorphous natures of the nano-capsules was obvious from X-ray diffraction patterns. DSC studies showed high thermal stability of the nano-capsules between 20–120 °C. *In vitro* release studies revealed that controlled release from the nano-capsules could be achieved using isabgol fiber as encapsulant. However it was observed that the nano-capsules followed a non-Fickian diffusion pattern. Good DPPH-radical scavenging and metal-chelation activities were observed for encapsulated carotenoids. Shelf-life studies showed that the nano-capsules gradually degraded at 97% relative humidity, as the moisture-induced rancidity was evidently not extensive.

***Keywords*:** *Carotenoids; Isabgol fiber; Nano-capsules; Release; Release kinetics; Stability*

RESUMEN*: Síntesis, caracterización, evaluación de la estabilidad y cinética de liberación de caroteno encapsulado en nanocápsulas de fibra*. En el presente trabajo los carotenos fueron aislados (1,2%) a partir de aceite de palma crudo y encapsulados con fibra de isabgol (cáscara de psyllium). La eficiencia de encapsulación fue 82,23±1,42 %. La morfología de las cápsulas mostró una textura áspera de la superficie con mínimos poros. La naturaleza amorfa de las nanocápsulas fue evidenciada a partir de patrones de difracción de rayos X. Los estudios de DSC mostraron una alta estabilidad térmica de las nanocápsulas entre 20–120 °C. En los estudios de liberación *in vitro* se vió que la liberación controlada de las nanocápsulas se podría lograr mediante fibra isabgol como encapsulante. Sin embargo se observó que las nanocápsulas siguieron un patrón de difusión no-Fickian. Se observaron buenas actividades DPPH captadoras de radicales y actividades de quelación de metal para carotenoides encapsulados. Los estudios sobre la vida útil mostraron que las nanocápsulas se degradan gradualmente con una humedad relativa del 97%, y aunque la humedad indujo el enranciamiento, este hecho no se puede generalizar.

PALABRAS CLAVE: *Carotenoides; Cinética de liberación; Estabilidad; Fibra isabgol; Liberación; Nanocápsulas*

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1. INTRODUCTION

Due to their antioxidant activities, carotenoids possess important health-promoting functions which are helpful in the prevention and protection against a number of serious health disorders such as cancer, cardiovascular disease, colorectal adenomas and so on (Albanes, 1999). Hence there is immense interest in using carotenoids as a functional ingredient in food products. However, carotenoids are insoluble in water and only marginally soluble in lipids at room temperature. This makes it difficult for them to be incorporated into different food formulations. Additionally, carotenoids are susceptible to environmental stress conditions like heat, light and oxidation, which poses a challenge to the food and nutraceutical industries and limits their application due to their rapid deterioration (Rodriguez-Huezo *et al*., 2004). Palm oil is well-known for being a rich source of carotenoids such as α-, β-, γ-carotene and lycopene. It was shown that the β-carotene concentration of whole palm oil extract was significantly reduced when it was stored at room temperature for three months (Fauzi and Sarmidi, 2011). Hence to improve the solubility and bioavailability of carotenoids, attempts have been made to incorporate them into fine particles in the form of nano-dispersions (Tan and Nakajima, 2005). The use of natural fibers finds profuse application in medical, pharmaceutical and nutraceutical fields. The immense potential of fibres lies in their molecular structure, which offers excellent possibilities as a matrix for the design of bioactive, biocompatible, and structured materials, which is why fiber has been chosen as an encapsulant in the present work.

An effective technique for the preservation and proper utilization of functional food ingredients is encapsulation. Furthermore nano-encapsulation would insinuate controlled release and help to avoid undesired side-effects generated by the consumption of antioxidants in unregulated doses. In the present study, carotenoids were encapsulated using a natural fiber as the encapsulant. The dried nano-capsules were subsequently characterized with respect to the efficiency of encapsulation, morphology, crystallinity, release, release kinetics, antioxidant activity of released matter, thermal stability and shelf–life. The effect of water content on the structure of the nano-capsules was also assessed, which in consequence would affect the quality of the encapsulated bioactive component. The study would help to evaluate the efficacy of the nano-encapsulation of a bioactive product with a unique fiber encapsulant.

The final product is symbolic of the application of nano-capsules in the controlled release and precision targeting of nutraceuticals which enhance the increased bioavailability of these components. Apart from the beneficial antioxidant activity imparted by the encapsulated carotenoids upon being released in the human body, the added advantage of using fiber as encapsulant lies in the fact that they lower total and LDL cholesterol, regulate blood sugar, increase food volume without increasing caloric content, and speed-up the passage of foods through the digestive system, which facilitates regular defecation (Eastwood and Morris, 1992; Spiller *et al*., 2001).

2. MATERIALS AND METHODS

2.1. Materials

Crude palm oil imported from Indonesia was collected from a local Vanaspati manufacturer. Isabgol (*Psyllium husk*), a natural fiber, and rice bran oil were purchased from the local market. Lecithin was obtained from Ruchi Soya Industries Ltd., Haldia, India. Polysorbate 20 (tween 20) was purchased from Sisco Research Laboratories Pvt. Ltd., Mumbai, India. All other reagents were of analytical grade and purchased from Merck India Pvt. Ltd., Mumbai.

2.2. Isolation of carotenoids from crude palm oil

Carotenes in crude palm oil consists of 91% α- and β-carotene, with the proportion of other carotenoids being minimal (Ping, 2007). The oil was chemically transesterified with methanol and sodium hydroxide until complete transesterification was achieved (synthesis of ester was monitored by thin layer chromatography). Oil and alcohol were taken in 1:10 molar ratios and stirred at 200 rpm while maintaining a high temperature of 80 °C for 3 hours with a 0.2% NaOH catalyst (Nitsche *et al*., 1999). A two-phase mixture comprising of a glycerol phase and an ester phase consisting of fatty esters and carotene was obtained. The ester phase was distilled at 300 °C at 100 Pascal pressure (SIBATA, Japan, Serial no. N40394). The residue was diluted 5 times and saponified with ethanolic potassium hydroxide at 80 °C. The carotenoids were extracted from the saponified residue with a mixture of hexane and water where the extract phase was rich in carotenoids. A pure product was obtained after solvent removal which was crystallized and re-crystallized with acetone to get 1.2% carotenoids. The nano-capsules synthesized by the application of the developed methodologies can be used directly in the foods, cosmetics and pharmaceutical industries.

2.3. Isolation of isabgol fiber

The soluble fiber was extracted according to the modified procedure of Cartano and Juliano (1970) and the extraction time and temperature were determined according to Graham *et al*. (1988) who suggested that the extraction of soluble fibers with pH

5.0 acetate buffers for 4 hours at 60 °C gave significantly higher values than other conditions. The starch free residue (50g) was blended in a colloid mill with 1L of reagent and extracted by shaking for 4 hour at 60 °C. The reagent used was 4% HCl (pH 0.5). The extract was centrifuged and neutralized with 5(N) NaOH. The neutralized supernate was treated with trichloroacetic acid to a final concentration of 7%. After centrifugation the extract was stirred while being poured into four volumes of 95% of ethanol and stored overnight. The precipitate was collected by centrifugation, re-dissolved in water, and freeze-dried to get 0.2% of a brown powder-like mass.

2.4. Encapsulation of carotenoids and size-reduction

An oil-in-water emulsion was prepared. The carotenoids in crystalline form were mixed with rice bran oil (1:1 on weight basis) and stirred well. Rice bran oil contains a significant amount of natural antioxidants like triterpenyl esters of ferulic acids, tocopherols and tocotrienols. Hence it has a relatively high smoke point of 232 °C and a mild flavor making it considerably stable with its own set of health benefits. Thus it was blended with the carotenoids and used as the core material thereafter, to impart dual efficacy in terms of health promoting effects.

An emulsifier proportion of lecithin:tween20 (70:30) was used, where the core lipid:emulsifier ratio was maintained at 15:1. The proportions of each emulsifier added and the core:emulsifier ratio were standardized after repeated experiments to obtain the most stable emulsion (data not shown). Lecithin was mixed with the core lipid and the entire blend was well-mixed using a homogenizer at 2000 rpm. A buffer of pH 7 was prepared with distilled water and mixed with isabgol fiber and stirred well until completely soluble. The core material (carotene and oil) to coating material (fiber) ratio was maintained at 1:2 by weight as optimized after repeated experiments. Then a measured amount of tween 20 was added to the mixture. The fat phase was blended with the aqueous solution using a homogenizer at 8000 rpm with 6 re-circulations using the homogenizer of Universal Motors (Type: RQ-127A, Remi Motors Ltd., Mumbai-53, India) at 1.1 Amp and 220 V to give pre-emulsions for 1 hour. The resulting emulsions were further homogenized with a high speed stirrer (OMNI International GLH; Model: GLH-220, Ser. No.-76014) at 30,000 rpm for 15 minutes. Followed by homogenization, shear forces were generated in the system by ultrasonication (Ultrasonicator, Vibronics, 3172 Meco-G) for 1 hour (carried out intermittently) at 200 Volts while maintaining a temperature of 10 °C throughout by placing on an ice bath. Thereafter the size-reduction of the emulsion was achieved by the high-pressure homogenization technique (NanoDebee [8799], B.E.E. International Inc., Easton, MA 02375, USA) with a hydraulic pressure of 3000 psi and a homogenization pressure of 40,000 psi and 5 cycles at 5 °C.

2.5. Particle-size analysis followed by powder production

The particle size distribution and mean droplet diameter of the emulsion was measured using the dynamic light scattering technique (Nano-ZS, Malvern Instruments, Worcestershire, UK). Mean particle diameters were reported as Z-average diameter or the scattering intensity-weighted mean diameter. The sample was diluted prior to making the particle size measurements to avoid multiple scattering effects, using a dilution factor of 1:10 sample-to-deionized water. The data reported is a mean of 3 consecutive readings.

Thereafter the emulsion was initially frozen with liquid nitrogen (−196 °C) and then stored overnight at −70 °C. The next day the frozen emulsion was freeze-dried to remove the crystallized water (Cerimedo *et al*., 2008). An Eyela Freeze Dryer (Type - FD-5N, Ser. No.-10160657, AC100V, and 50/60Hz 500W, Tokyo Rikakikai Co. Ltd., Japan) were used for freeze-drying which was operated at −20 °C and a chamber pressure of 13.3 Pa. The dried product was grain-like in nature which was further crushed using a mortar and pestle to make it powdery.

2.6. Observation under microscopes

A drop of the prepared emulsion was placed on the slide, air-dried and observed under an optical microscope (Leitz Laborlux, 12 POL S, Germany) with polarized light. The overall surface morphologies of the freeze-dried capsules were observed using scanning electron microscopy [Carl Zeiss EVO18 (Special Edition) Germany]. Prior to observation, the samples were mounted on metal grids, using double-sided adhesive tapes and coated in gold under vacuum.

2.7. Efficiency of encapsulation

The antioxidant content was estimated by initially analyzing its free fraction and then hydrolyzing the capsules and extracting the antioxidant with suitable solvent to determine the actual loading of the bioactive product. Approximately 10 mg nanocapsules were first washed with petroleum ether to extract the surface carotenoids. The capsules were then hydrolyzed with 5 mL of 0.1N HCl. After complete hydrolysis, the oil-soluble components were extracted with petroleum ether. Solvents from both the processes were removed and the amounts of oil

and antioxidant collected were noted quantitatively and confirmed spectrophotometrically. The mean of three readings is reported.

$$\text{Encapsulation efficiency} = \frac{\text{actual loading of lipid+ bioactive product-free lipid+ free bioactive product}}{\text{actual loading of lipid+ bioactive product}} \times 100$$

2.8. Crystallinity

Crystallinity was analyzed by X-ray diffraction (XRD). A diffractometer (XPERT-PRO from Panalytical Diffractometer) using Cuα (λ=1.5406) was used as X-ray source. $K\alpha_1\alpha_2\beta$ radiation from copper was used at 40 kV and 30 mA. The $K\alpha_2/K\alpha_1$ ratio was 0.50000. A scanning velocity of 1°·minute^{-1} from 2° to 80° was maintained. The experiments were performed at ambient temperature (25 °C).

2.9. Thermal stability

Pressurized DSC experiments were conducted for assessing the thermal stability of the nanocapsules using a TGA/DSC1 thermal analyzer from Mettler, Toledo, Switzerland, using the software STAR^e System. Typically, 1 mg of sample was placed in an aluminum pan hermetically sealed with a pinhole lid and oxidized in the presence of dry air (Gateway Airgas, St. Louis, Mo, USA), which was pressurized in the module at a constant pressure of 200 psi. A 5 °C·minute^{-1} heating rate from 20 to 120 °C was used during each experiment. The oxidation onset was calculated from a plot of heat flow (W/g) versus temperature for each experiment. The sample was run in triplicate, and average values rounded to the nearest whole degree are reported.

2.10. Release of bioactive components

About 10 g nanocapsules were placed in a glass bottle containing 30 mL dissolution media consisting of a phosphate buffered saline (PBS) with pH 7.4. The selected pH corresponded to the pH found in the small intestine. It was incubated in a shaking bath at 37 °C and 100 rpm. At time intervals of 0, 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 hours, 10 mL samples were withdrawn and filtered. At the same time 10 mL of fresh PBS was added to the mixture. The residues filtered from the withdrawn reaction mixture were also added back to the dissolution media. The filtrate was then extracted with petroleum ether and the absorbance of the petroleum ether layer was measured at 450 nm. The concentrations in each case were calculated according to equation (1).

$$C = A/\varepsilon L \quad (1)$$

Where C = concentration of the active molecule
A = absorbance of the specified molecule at maximum wavelength (λ_{max})
ε = molar extinction coefficient of the specified molecule [for α-carotene =2800 dL·g^{-1}·cm^{-1}; β-carotene =2560 dL·g^{-1}·cm^{-1} at 450 nm (Biehler *et al*., 2010)].

2.11. Release kinetics

The *in vitro* carotene release patterns were fitted to various release kinetic models like zero order (Hadjiioannou *et al*., 1993), first order (Bourne, 2002), Higuchi model (Higuchi, 1963), Hixson-Crowell cube root law (Hixson and Crowell, 1931) and Korsmeyer-Peppas model (Korsmeyer *et al*., 1983).

2.12. Antioxidant assay

2.12.1. DPPH-radical scavenging activity

The antioxidant activity of the encapsulated antioxidants was studied after its release. The nanocapsules were hydrolyzed and the antioxidant was extracted with petroleum ether, evaporated to dryness and the final product dissolved in ethanol for the assay. Antioxidant activity was determined by the scavenging activity of the stable DPPH free radical. The method was described by Katerere and Eloff (2005). Absorbance was measured at 517 nm. Radical scavenging activity was expressed as the inhibition percentage and was calculated using the following formula:

$$\% \text{ Radical scavenging activity} = [(A_0 - A_1)/A_0] \times 100$$

where A_0 is the absorbance of the control and A_1 is the absorbance of the sample

2.12.2. Metal chelation activity

The Fe^{2+} chelating ability of antioxidants was estimated by the method of Dinis *et al*. (1994). Absorbance was thereby measured spectrophotometrically at 562 nm. The percentage of inhibition of ferrozine-Fe^{2+} complex formation was calculated as follows:

$$\% \text{ Inhibition} = [(A_0 - A_1)/A_0] \times 100$$

where A_0 is the absorbance of the control and A_1 is the absorbance of the sample.

The antioxidant activity of the free carotenoids was also determined by the two methods.

2.13. Shelf-life of the nano-capsules

Evacuated, sealed desiccators filled with calcium chloride at 30 °C and a beaker with a saturated solution of potassium sulphate (a_w=0.97) was kept

overnight. 1g of the dried nano-capsules was kept in a beaker inside the desiccator for 30 days. After 0, 5, 10, 15, 20, 25 and 30 days, samples were removed and analyzed for moisture content and amount of bioactive component retention.

2.13.1. Moisture content

Aweighed amount of capsules on a watch glass was placed in oven at 110 °C for 6 hours. The gravimetric difference in weight before and after drying gave the moisture content. Moisture contents were measured at 0, 5, 10, 15, 20, 25 and 30 days.

2.13.2. Bioactive component retention

A weighed amount of capsules was hydrolyzed with 0.1(N) HCl (20 mL) for 15 hours at 300 rpm. The retention of the active components was estimated on the basis of the amount of β-carotene retained. After 15 hours the samples were filtered. The filtrate was extracted with petroleum ether and absorbance was determined at 450 nm. The concentrations were evaluated according to equation (1). The retention of the bioactive component was measured at 0, 5, 10, 15, 20, 25 and 30 days.

2.14. Statistical Analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA). When ANOVA detected significant differences between mean values, means were compared using Tukey's test. For statistical studies OriginLab software (OriginLab Corporation, Northampton, UK) was used. Statistical significance was designated as $p<0.05$. Values are expressed as Mean±SEM.

3. RESULTS AND DISCUSSION

3.1. Particle-size analysis of the emulsion

The particle-size analysis of the emulsion revealed an average droplet-size of 255.9±1.63 nm where sizes of all the lipid droplets were below 1 μm. By development of a nano-emulsion, an alteration of the solubility of functional lipids was performed, which overwrought the poor water solubility of lipids making them problematic in food formulations. Tan and Nakajima (2005) successfully prepared β-carotene nano-dispersions and investigated the influence of phase ratio and homogenization conditions on droplet size and β-carotene content. Here the focus was on the production of nano-capsules in dried forms after the development of stable nano-emulsions by homogenization followed by ultrasonication based on the emulsification–evaporation technique. The emulsion droplets correspond to single nano-capsules. Hence a particle-size estimation of the emulsion droplets by the DLS technique, allows us to evaluate the size (in terms of diameter) of the nano-capsules. This cannot be done by a simpe morphology study (SEM) as this gives us a broad idea about the features of the dried powders which are basically an assemblage of nanocapsules. Size reduction was performed by application of force in the form of ultrasonic vibrations and the degree of control in size reduction influenced the properties of the produced material (Sanguansri and Augustin, 2006). In general, the average diameters of the emulsion droplets were considerably small (255.9 nm) after the homogenization and sonication processes. The oil/water mixture was subjected to intense shear forces and cavitations by ultrasonic vibrations resulting in atomization of the dispersed phase. Due to the high interfacial tension between the organic and aqueous phases, a mixture of lecithin and Tween 20 emulsifiers were used to lower the interfacial tension and hence the stress needed to reduce droplet sizes, which is crucial for the formation of stable droplets (Tadros *et al*., 2004). Marie and co-workers (Marie *et al*., 2002) have shown that an emulsifier concentration higher than 1% in the aqueous phase did not induce a decrease in particle size and hence in total a 1% emulsifier concentration was maintained. Tween 20, a non-ionic emulsifier that absorbs very quickly at the oil–water interface, has shown results in the development of small particles for various applications. Moreover emulsifiers have an effect on the long-term stability of nano-capsules (absence of aggregates) but do not affect the shape, structure or functionality of the produced nano-capsules (Cheong *et al*., 2008).

3.2. Observation under microscopes

Typical surface morphologies of dried emulsion are shown in Figure 1 (A, B, C and D). Granular and conical shaped structural morphology of the powders containing several nano-capsules in dried forms were observed (Figure 1A and 1C). The structure of the powders was very irregular and had a rather rough surface with small gaps (Figure 1B and 1D). However, the integrity of the isabgol-emulsifier membrane was retained after the drying process, as observed that the dried capsules maintained a more or less uniform and homogeneous structure, without the observation of pores and ruptures on the surface. This is essential for the protection of the easily oxidizable carotenoids. Due to the presence of a fibrous encapsulant, the mechanical strength of the nanocapsules were relatively high. A dense but poorly-defined structure of the group of nanocapsules (Figures 1C and 1D) was probably due to the preparation process where the gelling isabgol

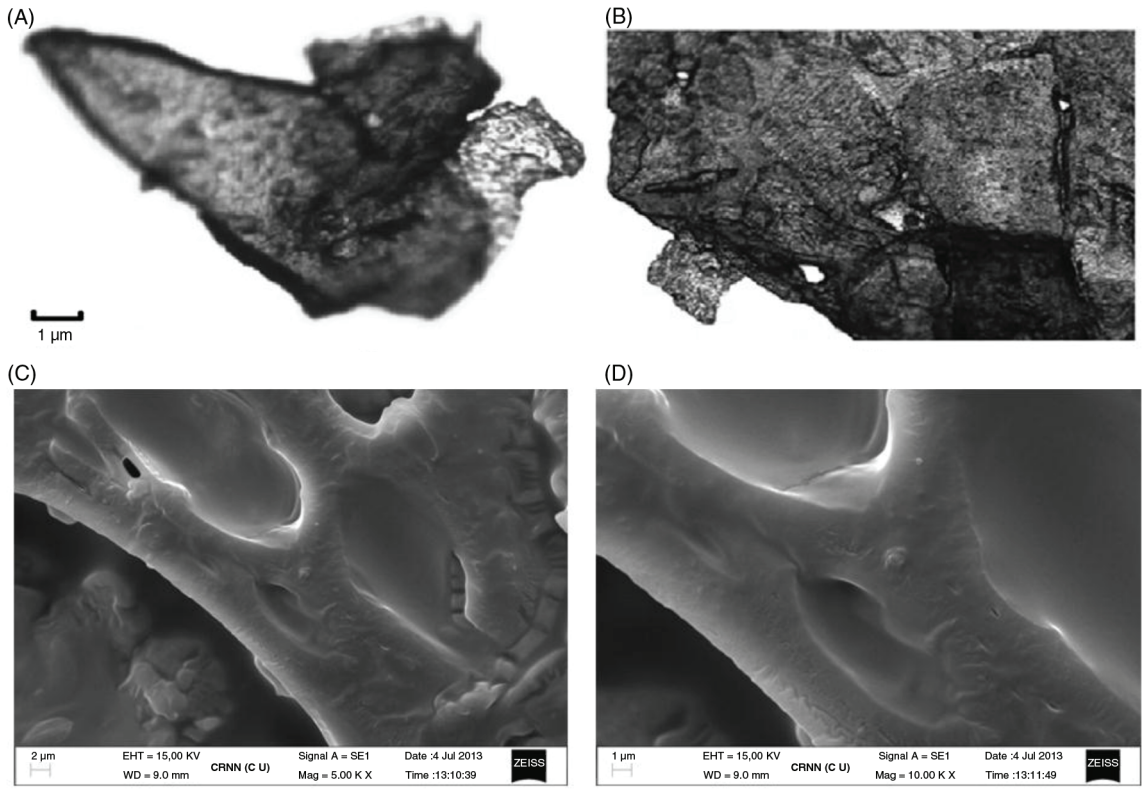

FIGURE 1. Observation of carotene nano-capsules under polarized and scanning electron microscopes (SEM) respectively: (A) Crushed and powdered products under polarized microscope. Each powder granule contains several dried emulsion droplets corresponding to nano-capsules; (B) Surface morphology of the dried powders under polarized microscope; (C) SEM graph of surface of the dried powders containing several nano-capsules, at 5000X magnification; (D) SEM graph of surface morphology of the dried powders at 10000X magnification.

in an aqueous solution was rapidly cooled by freeze-drying which led to a rigid structure.

3.3. Efficiency of encapsulation

The efficiency of encapsulation was found to be 82.23±1.42%. The amount of free lipid, tantamounting to the non-encapsulated lipid, was sufficient, indicating that higher homogenization time is essential for complete emulsification of the entire lipid core. pH also had an important effect on the encapsulation efficiency generated due to fiber encapsulants. At acidic pH (0.1N HCl at which the nano-capsules were hydrolyzed) fibers become smooth and swell up resulting in an increase in the compatibility of the aqueous solution of fiber with the core material. The efficiency of any coating material depends on its strength, flexibility, impermeability and non-reactivity with the core material. The combination of the two emulsifiers, lecithin and tween 20 also produced a system with high internal lipid-carotenoid content due to the production of a highly stable oil-in-water nano-emulsion. So a system was formed which was considerably stable, thus increasing its efficiency of encapsulation.

3.4. Crystallinity

Overlaid XRD patterns of carotene nanocapsules, coated with isabgol fiber are shown in Figure 2. The XRD pattern of the capsules exhibit two sharp peaks at 2θ scattered angles of 20.027° and 72.645°, indicating a slight crystalline nature. A single peak with decreased peak intensity was observed at the 2θ scattered angle of 43.481° showing that the degree of crystallinity of the fiber was actually considerably reduced. The XRD study suggested that the X-ray diffractogram of dried nano-capsules and fibers indicated the presence of a slight crystalline state with two sharp principle peaks and a diffused peak, though in general the nanocapsules were mostly amorphous in nature. This could be due to the re-structuring of the fiber molecules during the rapid cooling procedure of lyophilization. A similar trend was observed in DSC studies.

3.5. Thermal stability

Figure 3 shows the DSC data of lyophilized isabgol nano-capsules. The heat flow curves did not show the melting peak for either isabgol or the inner core

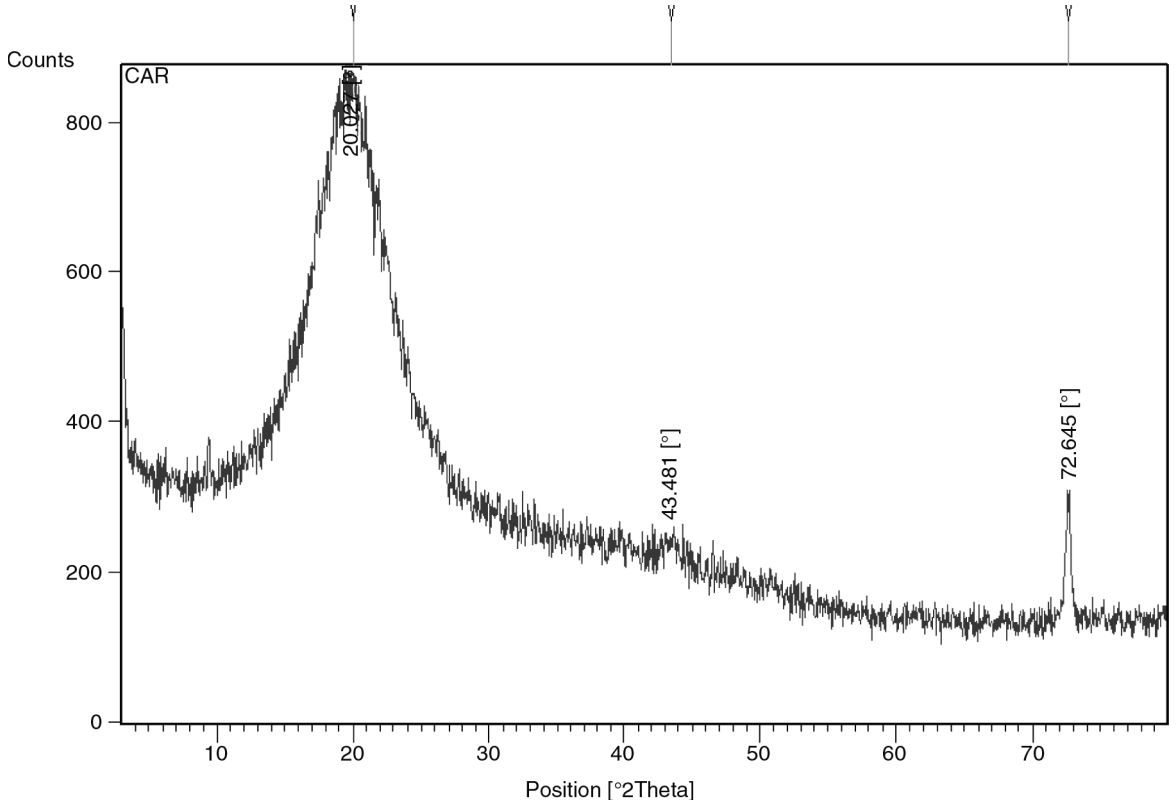

FIGURE 2. Pattern of XRD peaks of isabgol fiber nano-capsules.

materials in the tested range of 20 to 120 °C. The curves reveal that no rupture of the outer shell took place in this range and hence core was not released into the amorphous medium as also corroborated by XRD studies. The highly amorphous nature of the fiber capsules could be attributed to the rapid quenching of the nano-emulsion during the powder formation procedure, which does not allow the core or the encapsulant to properly crystallize. Lipids and the aqueous solution of isabgol were mixed well and subsequently, the solvent water was evaporated. This allowed for a homogeneous dispersion of lipid components finally leading to the development of proper nano-capsules. Furthermore, the method of preparation (homogenization followed by ultrasonication) and the presence of emulsifiers did not allow the core and coating to crystallize. The type of emulsifier used affects the crystallinity of nanoparticles and, consequently, the degradation velocity (Olbrich and Kayser, 2002). The emulsifier combination chosen stabilized the system and produced an amorphous structure. The results were verified by evaluating the sample weight and heat flow at different Tr and Ts values, where Tr and Ts are the reference and sample temperatures. Initially at Tr 30 °C and Ts 36.9 °C the sample mass was found to be 10.5669 mg. At Tr 100.6 °C and Ts 108.6 °C the sample mass was found to slightly reduce to about 10.3025 mg indicating only the loss of adsorbed water. At Tr 119.8 °C and Ts 128.8 °C sample mass was finally reduced to 10.2366 mg. In a similar manner at the above specified Tr and Ts values, the heat flow was found to increase from an initial negative value of −0.717 mW to −0.315 mW to +0.479 mW. In general, the isabgol fibers displayed immense thermal stability in the range tested in terms of thermodynamic stability of the encapsulant.

3.6. Release of bioactive components

The behaviour of carotene through the small intestine was simulated *in vitro* with a phosphate buffered saline at pH 7.4 in order to determine if the bioactive component was being released prematurely or gradually with time. Figure 4 depicts the release profile of carotene from nano-capsules under simulated conditions of the small intestine. The release was once again studied with respect to the concentration of β-carotene released. Initially, at time zero, the amount of carotene released as reported in terms of $mol \cdot L^{-1}$ was 0. Then gradually with increasing time it was observed that after 1 hour 0.12×10^{-5} $mol \cdot L^{-1}$ carotene was released. Thereafter more or less a steadily increasing concentration of the carotene was detected in the released medium for almost 6 hours. After that a slow decline in the carotene concentration

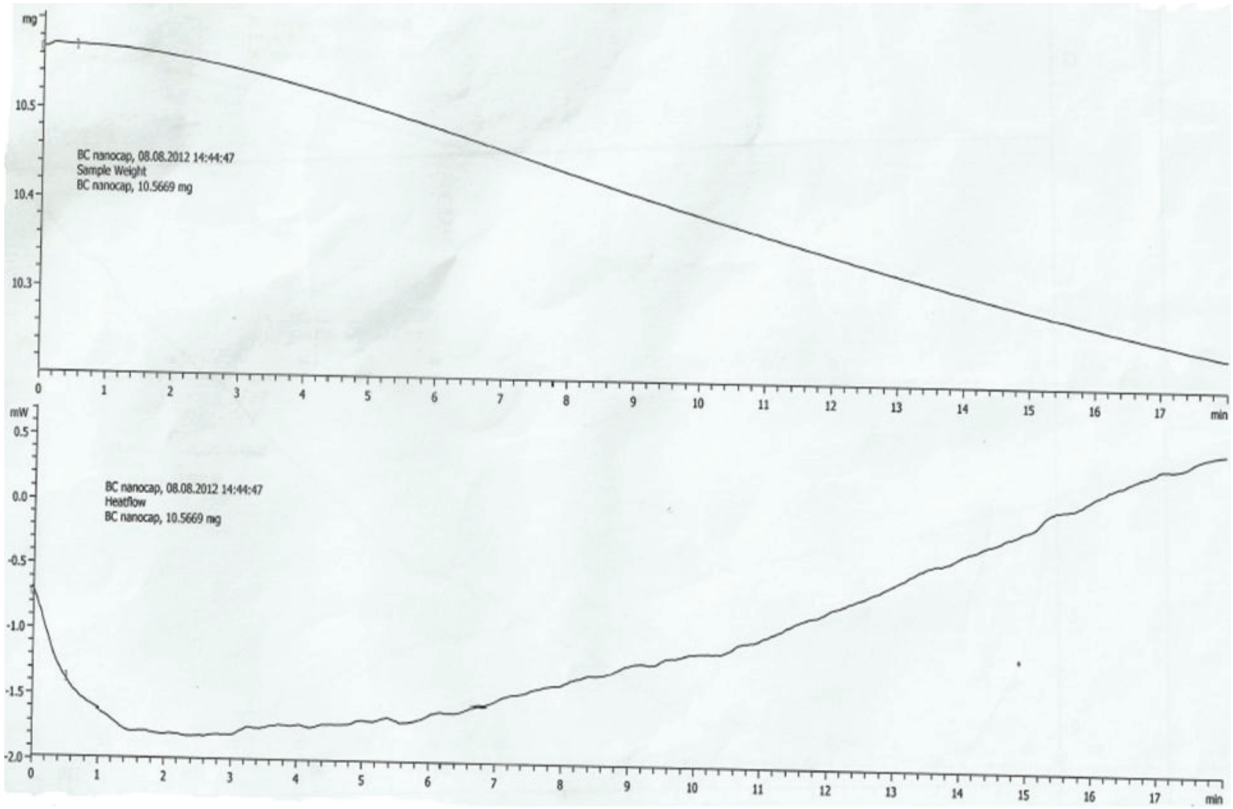

FIGURE 3. Heat flow curves of isabgol fiber nano-capsules.

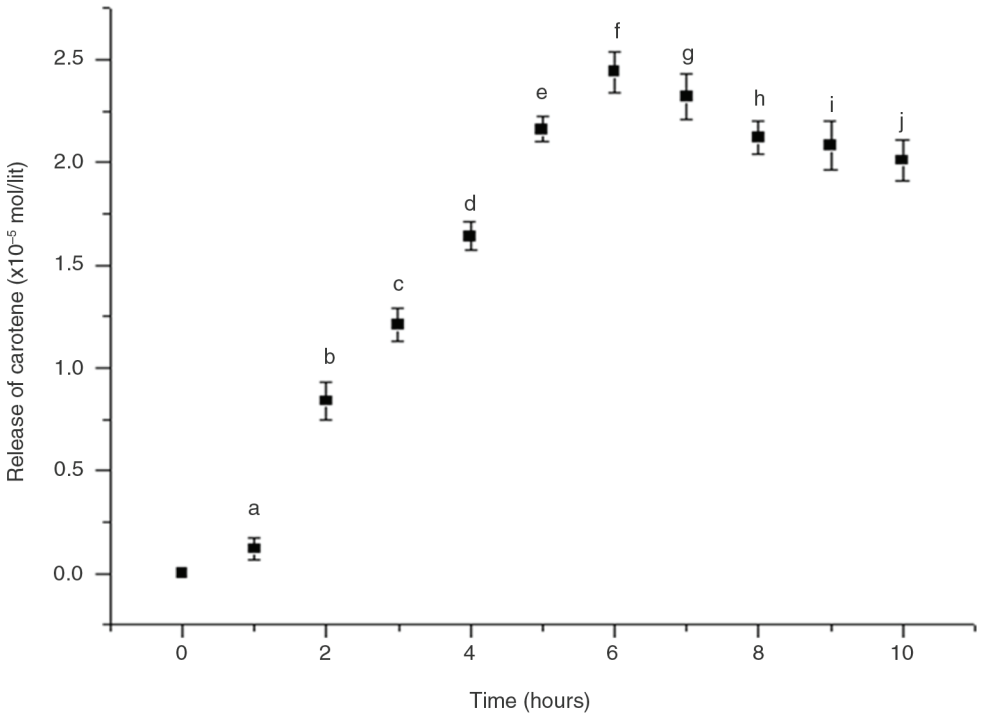

FIGURE 4. Release pattern of carotene from fiber nano-capsules. Values were expressed as mean±S.E.M, n=3. Means were compared using Tukey's test. Values not sharing a common superscript in the release curve are statistically significant at ($p<0.05$).

was noted, although the fall was only minimal. Even after 10 hours 2.01×10^{-5} mol·L^{-1} of carotene was released into the medium. The evidently slow release was likely due to the fiber forming a compact gel structure at the slightly alkaline pH of 7.4, reducing permeability and potentially stabilizing the bioactive component. Hence, a gradual release was observed for up to 6 hours. Beyond 6 hours, the proportion of the encapsulated carotene had been considerably reduced, leading to the detection of lower concentrations of the bioactive product. This can be explained by the fact that the amount of carotene released was less than that withdrawn, indicating that the amount of encapsulated carotene was gradually depleted beyond 6 hours. The pH-dependent bioactive product release and also fiber porosity had a significant influence on the carotene release profile. Product release from such fibrous encapsulant matrices may be modulated by a dissolution–erosion process. At low pH, fibers became rigid, resulting in a compact and impermeable matrix retaining the release of carotene in an acidic environment such as that of the gastrointestinal tract (results not shown). But at potentially neutral/higher pH values, this problem was overcome; however the release occurred in a time-dependant manner over 10 hours. In fact here swelling of the fibrous matrix was further enhanced by the presence of the phosphate ion PO_4^- from the phosphate buffer used and by the counter-ions such as Na^+ (Ramdas *et al*., 1999). Carotene is slowly released from the nano-capsules once in neutral conditions. It was found that the fibrous coating acted efficiently in regulating the carotene release, which is indicative of a slow core material release. The values of release of carotene varied significantly from each other with time when the means were compared among themselves ($p<0.05$).

3.7. Release Kinetics

Various kinetic models were used to analyze the controlled release kinetics from the fiber nano-capsules. The zero order rates (Hadjiioannou *et al*., 1993) describes a system where the release rate from capsules is independent of its concentration. For the first order model (Bourne, 2002), the release occurred in a concentration dependent manner. According to the Fickian diffusion (Higuchi, 1963) release occurs as a function of the square root of time. Release from systems where there is a change in surface area and diameter of capsules can be explained by the Hixson-Crowell cube root law (Hixson and Crowell, 1931). Finally the lipid release form a polymeric system can be described by a simple relationship according to the Korsmeyer–Peppas model (Korsmeyer *et al*., 1983).

Different plots were made based on the release of the core materials from the nano-capsules: the cumulative release of carotene in percentages vs. time (zero order kinetic model) (Figure 5A); log cumulative of percentage carotene remaining vs. time (first order kinetic model) (Figure 5B); cumulative carotene release in percentage vs. square root of time (Higuchi model) (Figure 5C); log cumulative carotene release in percentage vs. log time (Korsmeyer model) (Figure 5D) and cube root of carotene expressed in percentage remaining in matrix vs. time (Hixson-crowell cube root law) (Figure 5E).

Dissolution data of the nano-capsules were fitted into the five kinetic models. The data from the different kinetic models for the release of carotene from the nano-capsules are given in table 1. From table 1, it can be seen that the 'n' value of the Korsmeyer-Peppas model, which is a diffusion component for the kinetic models, indicates the carotene release mechanism. When n=0.5, it indicates that the lipid is released from the nano-capsules with the Quasi-Fickian diffusion mechanism, if (n>0.5), then non-Fickian or zero order release exists (Korsmeyer *et al*., 1983). In this study n=0.977. Hence from the above theory it can be said that the release of carotene from fiber nano-capsules followed a non-Fickian or zero order release rate.

Figure 4 displays the release of carotene from the nano-capsules according to the various kinetic models which follow a linear relationship in all the cases. In the zero order plot (Figure 5A), the R^2 value obtained was 0.73. Again, the first order (Figure 5B) plot had an R^2 value of 0.74. For the Higuchi model (Figure 5C), R^2 was 0.84. From the Hixson-Crowell cube root law, R^2 was 0.53 (Figure 5E).

The different kinetic models described the release rate of carotene. The best linearity was found in the Higuchi model (R^2=0.84). Hence, the plots of the Higuchi model indicated that the release of carotene from nano-capsules as a function of the square root of time was based on a non-Fickian diffusion and not a zero order release rate. By incorporating the first 65.6% (4 hours) and 97.6% (6 hours) of release of carotene from nano-capsules, the mechanism of release was predicted by the Korsmeyer-Peppas model where n is the release exponent, indicating the mechanism of release. The Fickian diffusional release and a case-II relaxation release are the limits of this phenomenon (Korsmeyer *et al*., 1983). For the Korsmeyer-Peppas model the R^2 value was 0.78, close to that for the Higuchi model. The value of the release exponent (n) in this sustained release mechanism was found to be 0.977 which is greater than the defined limit of 0.5. Furthermore the Hixon-Crowell model showed an extremely low R^2 value of 0.53, indicating that its disparity with the release rate of carotene was the highest.

The value of the kinetics constant (k=3.418) was in accordance with the value of n (Table 1). Thus the release of carotene from the fiber-based nano-capsules showed a considerably delayed diffusion rate when release occurred in a medium of pH 7.4. Hence a sustained release mechanism can be formulated using fibers like isabgol as encapsulant.

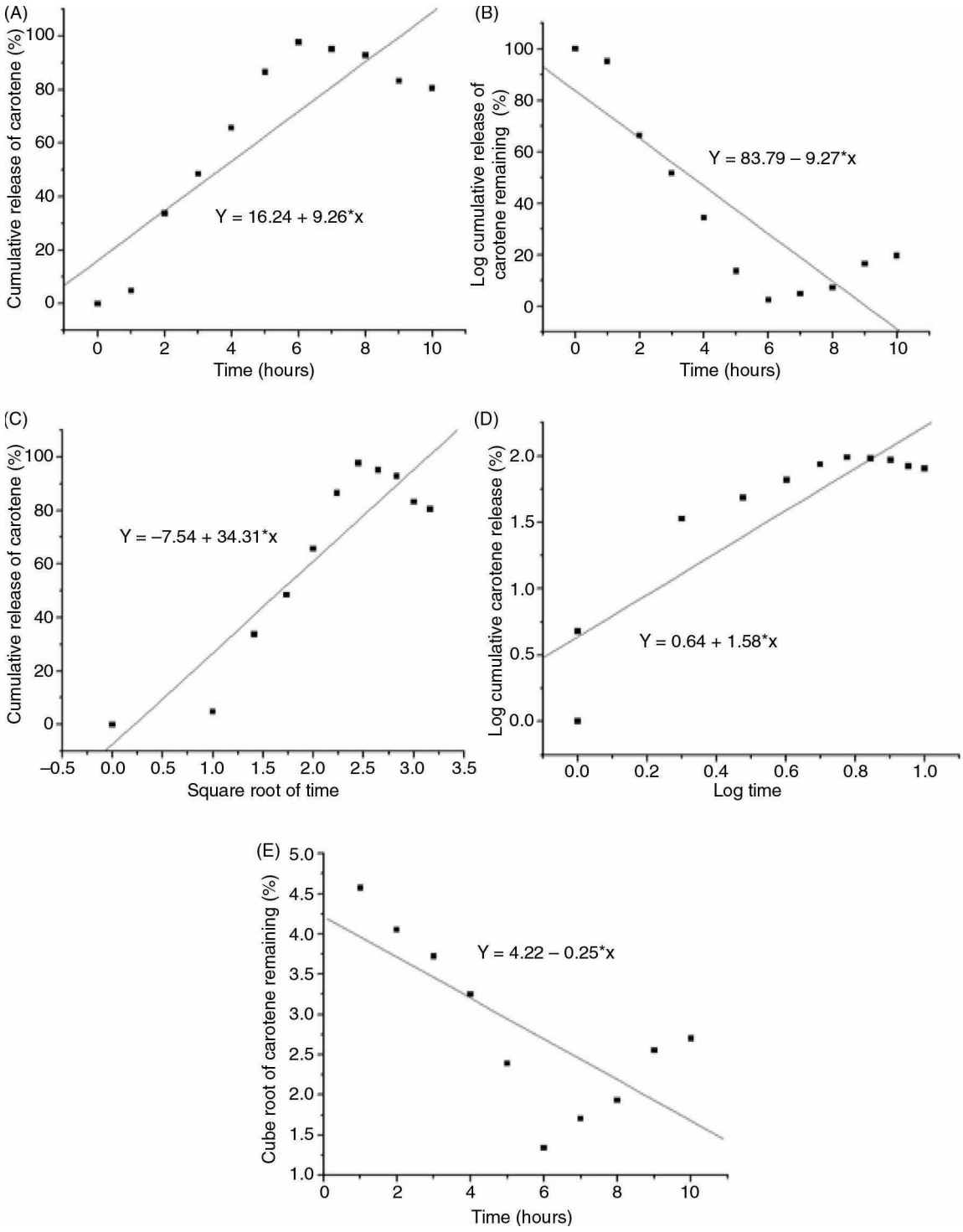

FIGURE 5. Kinetics study for the release of carotene from fiber-based nano-capsules (A) Zero order kinetic model; (B) First order kinetic model; (C) Higuchi model; (D) Korsmeyer model; (E) Hixson-crowell cube root law.

3.8. Antioxidative assay

The DPPH radical scavenging and metal-chelation activities of the encapsulated carotene were evaluated (EC) and then compared with the activities of the carotene that was not encapsulated (FC). The comparative study is essential to understand the efficacy of the encapsulation process for retaining the basic properties of an oxidation-prone bioactive natural product. The encapsulation procedure entails

TABLE 1. Linear fits of carotene released from fiber coated nano-capsules

Zero Order	First order	Higuchi	Hixson-Crowell	Korsmeyer-Peppas
R^2 k_o ($mg{\cdot}h^{-1}$)	R^2 k_1 (h^{-1})	R^2 k_H ($h^{-1/2}$)	R^2 k_{Hc} ($h^{-1/3}$)	R^2 n value K_{KP} (h^{-n})
0.73 2.2025	0.74 0.267	0.84 32.8	0.53 0.347	0.78 0.977 3.418

gyrating temperature, pressure and mixing conditions. This is suitable enough to deteriorate the quality of any sensitive nutraceutical. Hence an analysis to evaluate the amount of antioxidant potential retained by the compound after encapsulation is necessary. In the present study both groups of carotenoids show good antioxidant activity and the encapsulated carotene had retained its bioactivity. The metal-chelation activity of the carotenoids was much lower than the DPPH radical scavenging activity (Figure 6).

In case of DPPH radical scavenging activity, the activity was directly proportional to the concentration of the anti-oxidants. The DPPH radical scavenging method was based on the reduction in the methanol DPPH solution in the presence of a hydrogen donating antioxidant, due to the formation of the non-radical form DPPH-H (Li and Zhou, 2007). It was observed that the carotenoids were able to reduce and decolor 1,1-diphenyl-2-picrylhydrazyl, via their hydrogen donating ability both in encapsulated form and before encapsulation. This is an electron transfer based assay which measures the capacity of anti-oxidants in the reduction of an oxidant. Hence the degree of color change was correlated with the sample's anti-oxidant activities and the results of the encapsulated carotene were promising enough and indicated that fiber was an appropriate encapsulant.

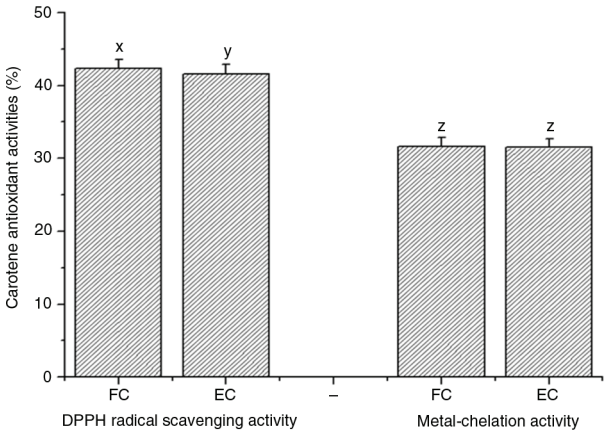

FIGURE 6. DPPH radical scavenging and metal-chelation activities of released carotene where EC: encapsulated carotene and FC: free carotene. Values were expressed as mean±S.E.M, n=3. Means were compared using Tukey's test. Bars not sharing a common superscript are statistically significant at ($p<0.05$).

Metal-chelating activity is an example of a complexation reaction. Ferrozine quantitatively formed a complex with Fe^{2+}. In the presence of chelating agents the complex formation was hampered resulting in a decrease in the color of the complex. The measurement of color reduction therefore allowed for estimating the metal chelating activity of the coexisting chelator (Yamaguchi *et al.*, 2000). It was reported that chelating agents that form *s*-bonds with metal are effective as secondary antioxidants because they reduce the redox potential, thereby stabilizing the oxidized form of the metal ion (Keowmaneechai and McClements, 2006). Here both encapsulated carotenoids and those before encapsulation, interfered with the formation of ferrous complex and ferrozine. This suggested that the antioxidant has chelating activity and captured the ferrous ion before ferrozine. The chelating agents forming *s*-bonds with a metal were effective as a secondary anti-oxidant because they reduced the redox potential and stabilized the oxidized form of the metal ion (Sen Gupta and Ghosh, 2012). Although the DPPH-radical scavenging activity of encapsulated and non-encapsulated carotene were significantly different from each other ($p<0.05$), the metal-chelation activity showed no such variations (Figure 6).

3.9. Resistance to relative humidity

A storage stability test to evaluate the effect of humidity was performed on the fiber encapsulated powders. On the basis of the fact that the nano-encapsulated carotene was a result of a freeze-drying process, humidity is a critical factor for preserving the quality of the product during storage. The samples were taken in vials and placed in evacuated desiccators exposed to a relative humidity of 97% for a period of 30 days at 30 °C (Table 2). From table 2 the obtained data revealed that the degradation of the bioactive product occurred with time due to the presence of sufficient moisture in the system due to a high relative humidity of 97%. This fact can be explained by the oxidation of carotenoids in the presence of moisture. Initially the high moisture content led to the swelling of the outer fibrous cover, promoting its rupture and releasing the antioxidant into the medium. Previous works showed that such reactions followed a pseudo-first-order kinetics (Ersus and Yurdagel, 2007). Here the degradation kinetics of the nano-encapsulated carotene showed a gradually approaching steady value, as the moisture content of

TABLE 2. Moisture content and bioactive product retention for a water activity of 0.97 for 30 days

Water activity of 0.97		
Time (days)	**Moisture content (%)[a]**	**Bioactive product retention (%)[b]**
0	20.2+0.53	98.75+2.92
5	18.1+1.89	86.25+2.63
10	13.4+1.43	71.25+1.98
15	12.7+0.16	68.75+2.33
20	6.20+0.34	65.0+2.49
25	5.14+1.68	56.25+2.18
30	2.80+0.18	46.25+2.55

[a,b]Values are expressed as mean±S.E.M, n=3.

the powder products decreased in the evacuated environment. Total carotene content suffered no apparent change over the time of study. In fact, the stored powder was stored away from light and oxygen in hermetically sealed desiccators. Therefore according to our results, the oxidation of carotene would only be possible by the auto-oxidation of the outer fiber encapsulant which would consecutively lead to further oxidation of the carotenoids and take a minimum of 3 weeks to produce any noticeable degradation of it. Moreover, the collapse of the matrix might have caused a release of oil from the matrix, but the associated caking phenomenon reduced the powder's available surface for extraction and reduction of the amount of extractable oil.

4. CONCLUSIONS

In summary, it can be concluded that nanocapsules obtained by the emulsion dispersion of isabgol fiber, have potential for an oral dosage system for carotene and other relevant nutraceuticals. Data obtained from this study demonstrates that the fiber nanospheres were successfully prepared and this fact was corroborated by an incorporation efficiency of almost 82% and unimodal size distribution. The *in vitro* assay to measure the released amount of carotene was done in a new way by evaluating the concentrations of the antioxidant released *in situ*. Furthermore, the release kinetics study led to the affirmation that release was feasible for the selected system. Homogenization followed by the ultra-sonication method proved suitable to produce the capsules in the nano-meter range. The nanocapsules also displayed considerably good shelf-life.

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