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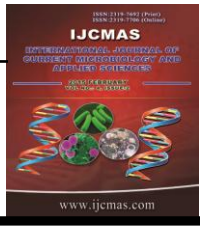

Original Research Article

Encapsulation of Nano Carotenoids; Evaluation of Stability and Safety

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Keywords

Carotenoid; Micro encapsulation, Nanopaticles, Antioxidant, Thermal Stability

A B S T R A C T

The goal of this study was to produce food capsulated nanoparticles from natural sources that help a lot in solving problems concerning with food supplementation, producing more effective and efficient nutraceuticals able to be delivered through safer active target system. A Supercritical Co_2 Fluid system was used to extract carotenoids from carrot waste samples obtained from Egyptian factories led to nanoparticle compounds after mechanical treatment. The carotenoid nanoparticles were measured by using Transmission Electron Microscopy. The microencapsulation of nanocarotenoids caused oxidation prevention and increased the thermal stability.

Introduction

The current global population is nearly 9 billion with 50 % living in Asia, and 90 million living in Egypt. A large proportion of those living in developing countries face daily food shortage as a result of environmental impacts or political instability.

In the developed countries, the food industry is driven by consumer demand which is currently for fresher and healthier food stuff. Nanotechnology creates hope and excitement about possible breakthroughs for solving some of society's pressing problems.

Nanotechnology is considered to be new tools for delivering health giving substances to reach the right part of the body. Now consumers may choose to eat food products containing microencapsulated ingredients, which set up to work on providing specific health benefits to the target area. The impact of nanotechnology in the food industry has become more apparent over the last few years with the potential to revolutionize the agricultural and food industry with new tools for the molecular treatment of diseases, rapid disease detection, enhancing the ability of plants to absorb nutrients… etc. (Joseph and Morrison 2006).

Due to consumer concerns for food safety and strict government regulations, the consumption of synthetic colorants is decreasing and the demand for natural colorants is growing. Carotenoids are one of the most important natural food colorants. Besides their application as natural colorant, carotenoids also play an important role as food ingredients due to their provitamin A

activity and antioxidant function. (Block *et al.*, 1992; Steinmetz and Potter 1993). The most important sources of carotenoids are fruits and vegetables. Among these, carrots are excellent sources of carotenoids and are commonly consumed. According to (Chen *et al.*, 1995), ß-carotene constitutes a large portion (60-80%) of the carotenoids in carrots followed by α-carotene (10-40%) and lutein (1-5%).

The two essential biological functions of carotenoids in photosynthetic membranes are to collect light energy, and to exert protection photo damage (Astorg, 1997). Many other bioactives have been claimed in addition to the antioxidant properties of carotenoids. Because of them, a lot of industrial interest has emerged to isolate carotenoid pigments from biological substrates for the use as nutraceuticals and related applications. Supercritical fluid extraction has established itself as an alternative to traditional, low-selectivity, and questionable isolation processes using organic solvents, because of relatively low critical temperature, inertness, and non-toxic of carbon dioxide, the most commonly used supercritical solvent, Supercritical CO_2 (SC-CO_2) extraction processes are typically carried out near-environment temperatures and in the absence of air, which reduces heat requirements, and avoids thermal and oxidative damage of labile compounds (Brunner, 1994, Durante, *et al.*, 2014).

Pigments extracts can be produced by conventional (Soxhlet extraction, using organic solvents) or supercritical fluid extraction (usually by the use of carbon-dioxide as supercritical solvent). However, there are some disadvantages of using solvents : long term of extraction, large solvent waste (which implies some economical and environmental problems), relatively high working temperature, as the samples are extracted at the boiling point of the solvent, so thermal decomposition of some compounds can occur. Moreover, after the removal of the solvent (in Vacuum), some residues remain in the extract. On the other hand, supercritical requires shorter time, smaller amount of solvent, CO_2 is inflammable, chemically inert and less toxic and there is no need for solvent removal, and the extract is solvent-free. (de Castro and Garcia-Ayuso, 1998).

Microencapsulation / Nanoencapsulation is defined as a technology of packing solids, liquids or gaseous materials in miniature, sealed capsules that can release their contents at controlled rates under specific conditions (Desai and Park, 2005). These capsules protect the encapsulated product from the light and oxygen and consequently prevent its degradation. Antioxidants can be added to systems where they might degrade, become hazardous by oxidation reactions and change the original physical properties. Nanoencapsulation is one technology that can be used to overcome these problems and also permit the dilution of small amounts of the active compound in a uniform dispersion. The efficiency of the nanoencapsulation process was tested by different techniques and the results obtained show that the objectives were achieved: quantification of the encapsulated antioxidant relatively to the shell material; protection of the antioxidant from the environment; stability of the encapsulated antioxidant for long period of time; release of the antioxidant from the shell under specific conditions. This process is particularly suitable for encapsulation of natural antioxidants that will be used in food, pharmaceutical and cosmetic industries (Kandaswami and Middleton 1994).

Building on the concept of "on-demand" food, the idea of interactive foods is to allow consumers to modify food depending on their own nutritional needs or tastes. The concept is that thousands of nanocapsules containing flavour or colour enhances, or added nutritional elements (such as vitamins), would remain dormant in the food and only be released when trigged by the consumers (Dunn, 2004). In general four broad areas of food science and technology where nanotechnology is very likely to have substantial impact were identified; health and nutrition, development of noval materials, food processing and food safety.

This study aims to produce food nanoparticles from natural sources with controlled released property capable to be introduced in our diet and affecting the targeted organs that unsurely will help a lot in solving problems concerning with food supplementation, producing more effective and efficient nutraceuticals able to be delivered through safer active target system.

Materials and Methods

Carrot Samples Preparation

Carrots wastes obtained from food Egyptian factories (in Cairo) were washed, shopped into 5 mm cubes at room temperature with a food chopper. Desirable particle size (nano-size) was achieved by grinding the cubes, then sieved and homogenized prior to extraction using Sonicator (Ultrasonic processor) XL No. 2015 in a dark place. The samples were placed in petri dish, packed with aluminium foil and stored in a refrigerator at –80°C until freeze. Then, carrot samples were freeze – dried (LABCONCO, Kansas City, USA) at –50 & 0.014 mbar for 48 hrs to reach moisture content 1 %. Carrot samples powder were ground and stored in a refrigerator at –80°C in brown glass bottle to prevent oxidative damage until extraction.

Supercritical Fluid CO2 Extraction of Carotenoids

Carotenoids were extracted from carrots powder by Supercritical (SCF) at 70 °C and 276 bar pressure (Sun and Temelli, 2006)) until no significant amount of extracted carotenoids could be collected.

Physical Properties

Transmission Electrom Microscopy (TEM)

The morphology of the extracted carotenoids was examined by the transmission electron microscopy (TEM) (JED 1230, JEOL Ltd., Tokyo, Japan) using freeze-fraction replica method (Nobuo, 2008).

Fourier Transform Infrared Spectroscopy (FT-IR)

The spectra or finger print of the extracted carotenoids sample were obtained using FT-IR spectroscopy.Crotenoids sample of FT-IR (FT-IR-6100, Jasco,Japan) were prepared by using potassium bromide disks as described by Adt *et al.,* (2006).

Thermal Properties of carotenoids Nanoparticles

The thermal properties of carotenoids extracted from by supercritical Co_2 under various conditions i.e. pH, temperature and day light were determined according to Van den Berg *et al.,* (2000). Carotenoids were protected from light and the stability was calculated from the reduction in the content of carotenoids.

Thermal Stability of Extracted Carotenoids (Differential Scanning Coliremetry DSC)

Thermal stability of carotenoids extracted sample was determined according to the methods described by Pérez-Alonso *et al.*, (2008). All extracted samples between 4 and 5 mg were placed in the furnace of the TA Instruments DSC model 2010 (New Castle, DE.USA), and were subjected to heating rates (β) of 4,6,8 and 10 °C min^{-1} from 30 to 230 °C or 400 °C, when required, using an oxygen flow rate of 25 cm^3 min^{-1}. A blank was run using N_2 in order to determine if the exothermic peaks of the samples were due to oxidation. Measurements were done in duplicate.

Microencapsulation of extracted carotenoids

Microencapsulation of extracted carotenoids were done with sodium alginate (6 % (w/v), *d*p = 3 mm) by using standard ionotropic gelation through a syringe as described by Kubic *et al.*, (2004).

Transmission Electron Microscope of all encapsulated products

The morphology of all microencapsulated carotenoids was examined by transmission electron microscopy (TEM) using freeze-fraction replica method as described above.

Thermal Stability of all Microencapsulated products (Differential Scanning Colorimetry DSC

The thermal stability of all microencapsulated carotenoids was determined as described before.

Safety of Microcapsulated Nano Carotenoids

Toxicity Assay

The preliminary Safety evaluation of the microcapsulated carotenoids was determined by using supercritical Co_2 was determined using shrimp lethality test according to Simionatto *et al.*, (2005) with some modification. This brine shrimp (Artemia salina) lethality assay is considered to be a useful tool for preliminary assessment of cytoxicity (Solis *et al.*, 1993). Brine shrimp assays have also been used for the analysis of pesticides residues (Grosch, 1967), to monitor the toxicity of organic waste to marine organisms and active plant constituents (Meyer *et al.*, 1982). The brine shrimp eggs were provided from (Hobby, Eine Marke der Dohse Aquaristik, Germany), and were hatched in artificaial sea water (38 g salt per litre of water). After 24 hours, the hatched nauplii suspension was left to stand for 1 hour without aeration, and then the nauplii were collected by pipetting from middle layer of solution, in which most of nauplii were swimmed. The crude nano extracts was dissolved in distilled water to various concentrations and the shrimp larvae were placed into them (duplicate). Sea water without extract was used as a negative control, while potassium dichromate had a LC_{50} = 20µg/ml as a positive control. Fifteen naulpii were withdrawn through a glass capillary and placed in each vial containing 4.5 ml of brine solutions. Final concentration of extracts in each experiment were sed 5.000, 2.5000, 1.250, 0.625, 0.312, 0.156 and 0.078 mg/ml. 0.5ml of the nano extracts was added to 4.5 ml of brine solution and maintained at room temperature for 24 hours under the light and surviving larvae were counted.

Result and Discussion

Supercritical Fluid CO2 Extraction of Bioactive compound from natural sources

In this study, one type of additives has been evaluated and already being utilised in food and drink products. This additive was obtained from food processing wastes and showed a greater added value.

In addition, the high degree of purity and safety of the product obtained by means of supercritical extraction is one of the factors that should be studied over the medium to long term, since it constitutes a factor of considerable importance, in the context of the possible implementation of this technology on industrial scale. In this respect, the degree of selectivity presented by carbon dioxide as a supercritical solvent contributes to reduce the number of substance co-extracted, thus increasing the weight of principle extract compounds (Herrero *et al.,* 2009). Results showed that the weight of the extracted carotenoids was 5102 µg/mg respectively which is much higher than that extracted by conventional methods being 500 µg/gm. This result would encourage the use of this recent technology for the production of natural pigments from food plant wastes in an industrial scale with higher quality, purity and safety products.

Physical Properties

Transmission Electron Microscope

The morphology of carotenoids after freeze-drying was obtained by using transmission electron microscopy (JED 1230, JEOL Ltd., Tokyo, Japan).

Figure (1) Shows pictures of carotenoids nanoparticles with diameter range from 7nm to 20 nm.

FT-IR analysis

FT-IR spectral peaks of supercritical carotenoids sample were shown was shown in Fig. 2. The spectra consist of different groups of absorption bands at wave ranging from 4000-700 cm^{-1}. Fig.2 showed stretching oH at 3441.35 cm^{-1}. This band is identified only in the high pressure extracts. Although this is quite surprising, the spectra of extracts at high pressure not affected and still provide very meaningful information. The c-H stretching bands were identified at 2925 and 2857 cm^{-1}. The region of 1800-700 cm^{-1} of the individual bands of the functional groups.

The sharp bands at 1739 cm^{-1} are assigned to c=o stretching vibration and may be characterised by the presence of high amounts of carboxylic acid in the extract. Under the SCF extracted conditions 70 °C and 276 bar, sharp peak at 1455 cm^{-1} assigned to V symmetrical of cH_2 of lycopene, a small peak at 1378.85 cm^{-1} assigned to bending cH_2 and a small sharp peak identified at 723.12 cm^{-1} assigned out of plane =c-H. The peak at 1163.83 cm^{-1} may be assigned to polysaccharides compounds.

Thermal properties of extracted carotenoids

Effect of PH on Carotenoids Stability

The stability of the extracted carotenoids from carrot and orange peels waste by using Supercritical extraction method was tested at different PH values (i.e. 1-13) as presented in Table (1).

Results in (Table 1 and Fig.3) showed that the stability of the extracted carotenoids was greatly affected by change in pH values. The extracted carotenoids was increased by

increasing pH values from 1 to 8 then decreased. The maximum stability of the extracted carotenoids was noticed at pH 8 (i.e. 91.7%) and reduced below and above this pH. Results also revealed that around pH values from (12-13) the extracted lycopene complete degradation. These results are in accordance with those obtained by Rizk *et al.*, (2002), Yi *et al.* (2014) in that the degradation percentage of carotenoids increased, as the pH value was decreased. These indicate that the alkaline media was very efficient and effective in maintaining carotenoids.

Treatment of the extracted carotenoids at different pH values and incubated for 60 or 120 min. exhibited the same trend as those after 30 min with a lower stability values. The carotenoids were completely decreased at 12 and 13 pH values while the optimum pH was at 7-8.5.

Effect of temperature

The stability of carotenoids (percent retention) after treatment at different temperature values (i.e. 20, 40, 80 and 120°C) for (30, 60 and 90 min) was evaluated as presented in Table 2 & Fig. 4).

Results in (Table 2 & Fig. 4) showed that the stability of crude carotenoids were varied according to heat treatment (Temperature and Time). Increasing temperature values from 20 to 60 °C and after 30 min. caused a reduction in the percent retention of the studied carotenoids. The maximum retention was achieved at 20°C then decreased till complete destruction at 120°C. The same trend was recorded after 60 and 90 min, as those after 30 min. and more reduction as the time of treatment increased. Results also indicated that the highest retention was achieved at 20 followed by that at 40 °C.

Effect of day light

The stability of carotenoids extracted by Supercritical CO_2 from carrot & orange peels as affected by day light was tested as presented in the following results (Table 3 & Fig.5).

Results in (Table 3 & Fig.5) showed that the percent retention of the extracted carotenoids was varied according to the periods of daylight. Subjecting the studied carotenoids to day light reduced the percent retention which also decreased by increasing the time of exposure. However storage for 4 and 8h in dark had almost no effect on the percent retention for extracted carotenoids. The reduction in percent retention was increased by subjecting the extracted carotenoids to daylight for 4 to 8 h. The lower retention in carotenoids was noticed for carotenoids after 8h in sun light being 31.0 %. From (Table 3 & Fig. 5) it could be concluded that storage in dark completely retained all carotenoids extract. However the exposure to day light caused a great degradation in the extracted carotenoids. Therefore these carotenoids should be stored in dark conditions to prevent its degradation

Thermal Stability DSC of extracted carotenoids (Differential Scanning Colirmetry)

DSC of extracted carotenoids were measured using Differential Scanning Colirmetry. Results in Fig. (6) Show that the melting point of extracted nano carotenoids is 322.62°C.

Microencapsulation of carotenoids nanoparticles

Figure (7) indicated that sodium alginate beads exhibited better wall material for encapsulation of carotenoids. This results

indicates that microencapsulation of carotenoids in carrier matrices can provide protection against the degradative reaction, prevent loss and enhance stability

Transmission Electron Microscope

The morphology of the encapsulated nanocarotenoids was obtained by using ransmission electron microscopy. Figure (8) shows the TEM of carotenoids nanoparticles with diameter range from 89 to 176 nm.

Thermal Stability DSC of encapsulated carotenoids (Differential Scanning Coliremetry)

The thermal stability of the encapsulated carotenoids was determined by using Differential Scanning Coliremetry Figure (9). Higher exothermal peaks occurred at much higher temperature 368.62 °C than that for the pure extracted carotenoids. This result indicated that microencapsulation caused oxidation prevention and increased the thermal stability of extracted carotenoids.

Toxicity Assay

Brine shrimp lethality assay is a convenient method for general screening for toxicity of the extracts or compounds towards brine shrimp, and it can give an indication regarding possible cytotoxicity of the test samples. No toxic symptoms mortality was observed in the shrimp larvae for 24 hours of the study. The results support the traditional food supplements and medicinal uses.

Carotenoid compounds were extracted from natural sources (carrots) by Supercritical Co_2 fluid extraction after mechanical treatment.

The compounds were elucidated as nanoparticles and microencapsulated.

The microencapsulation prevent oxidation and increase the stability of carotenoids nanoparticles.

The rising need to extract functional compounds and nutraceuticals from natural sources continues searching for economically and ecologically feasible extraction technologies. The large amount of solvent used in traditional extraction techniques not only increases operating costs but also causes additional environmental problems, moreover, in the isolation of antioxidant compounds a non-oxidizing extraction media and mild extraction conditions are required. Therefore compressed fluids have become an interesting alternative to obtain antioxidants from different vegetal sources.

Carrots waste samples obtained from food processing industries (in Cairo) were homogenized using Sonicator and Carotenoids nanoparticles were extracted from freeze-dried carrot samples after using supercritical CO_2. Not only the hydrocarbon compounds such as α-and ß-carotene but also the oxygenated carotenoids such as lutein were recovered with supercritical CO_2. The microencapsulation caused oxidation prevention and increased the thermal stability of extracted carotenoids.

In summary, SFE is an efficient extraction technique for recovery of natural food colorants with high antioxidants nanoparticles

Table.1 Effect of PH on stability of natural carotenoids extracted by Supercritical

pH	Carotenes %		
	After 30 min	After 60 min	After 120 min
1	Zero	Zero	Zero
2	1.5	Zero	Zero
3	10.3	4.3	Zero
4	26.7	21.4	12.3
5	31.6	26.9	19.6
6	44.8	39.0	31.7
7	85.6	81.5	80.1
8	91.7	89.7	86.3
9	50.8	43.8	38.5
10	35.4	29.7	19.9
11	23.9	16.2	8.9
12	9.6	Zero	Zero
13	Zero	Zero	Zero

Table.2 Heat stability of extracted carotenoids by supercritical from carrot and orange peels

Temperature	Carotenoids retention (%)		
˚C	30 min.	60 min.	90 min.
20	99.8	99.7	99.7
40	99.6	97.8	95.3
60	91.5	81.4	21.4
80	80.1	62.6	3.9
100	31.3	1.4	Zero
120	Zero	Zero	Zero

Table.3 Effect of day light on stability of the extracted carotenoids by Supercritical from carrot & orange peels

Sample	Carotenoids retention %					
	Dark			Sun light		
	0	4h	8h	0	4h	8h
Carotenoids	100	98.4	96.9	100	59.2	31.0

Fig.1 TEM of Carotenoids nanoparticle extracted by Supercritical CO2

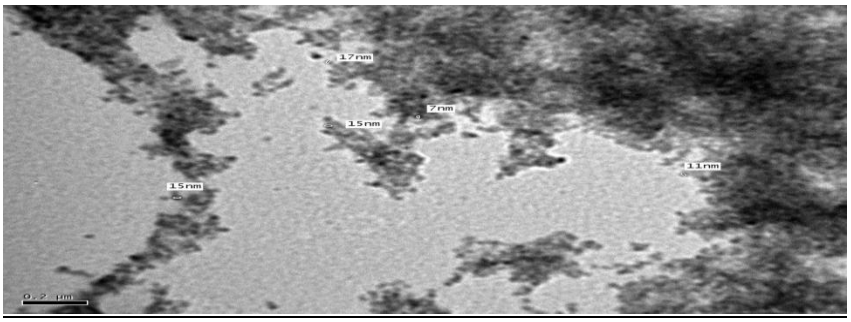


Fig.2 FT-IR spectral peaks of supercritical carotenoids sample

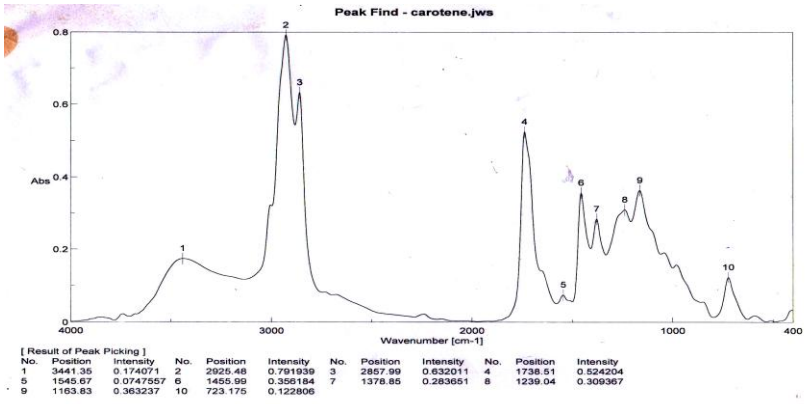


Fig.3 Effect of PH on stability of natural carotenoids extracted by Supercritical CO2

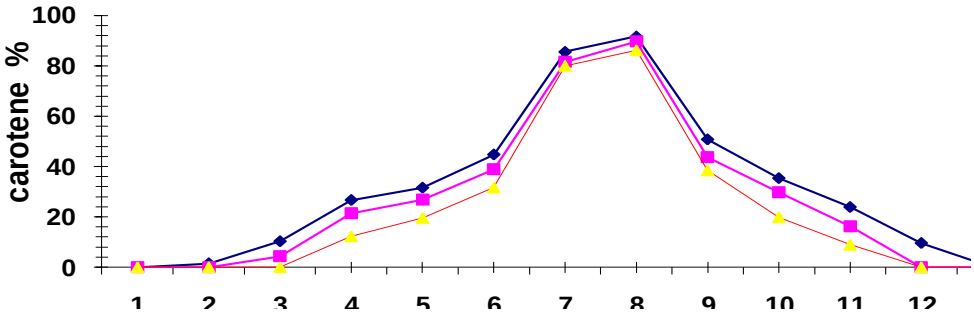


Fig.4 Heat stability of the extracted carotenoids by using supercritical from carrot and orange peels

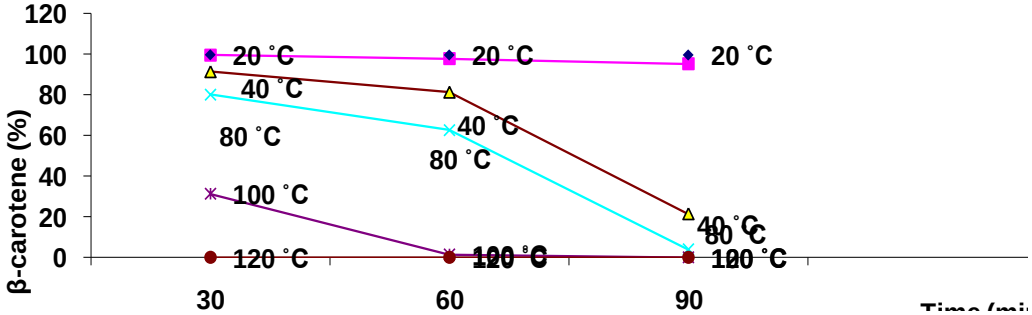


Fig.5 Effect of day light on stability of the extracted carotenoids by Supercritical from carrot and orange peels

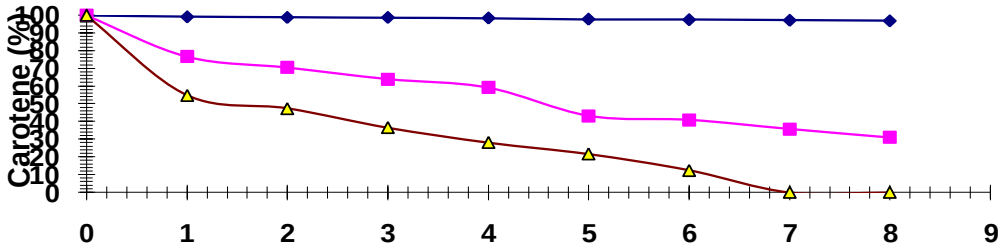


Fig.6 DSC of extracted carotenoids

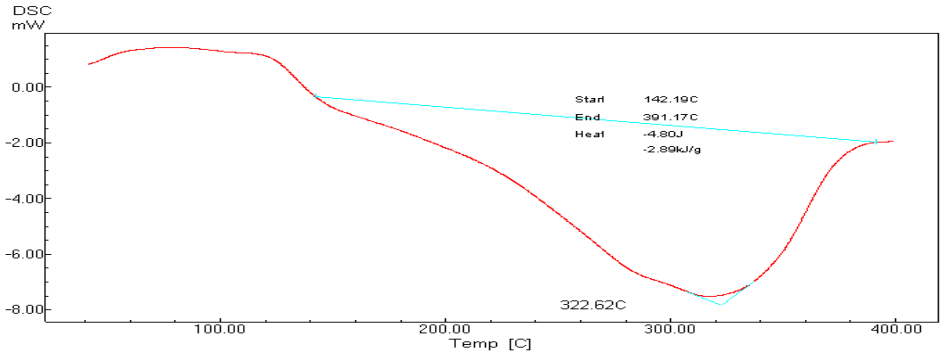


Fig.7 Microencapsulated Carotenoids

Fig.8 TEM of the encapsulated nano carotenoids

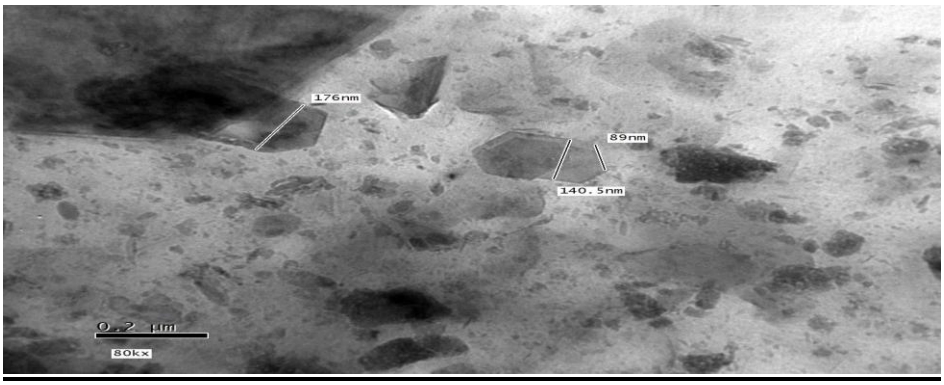
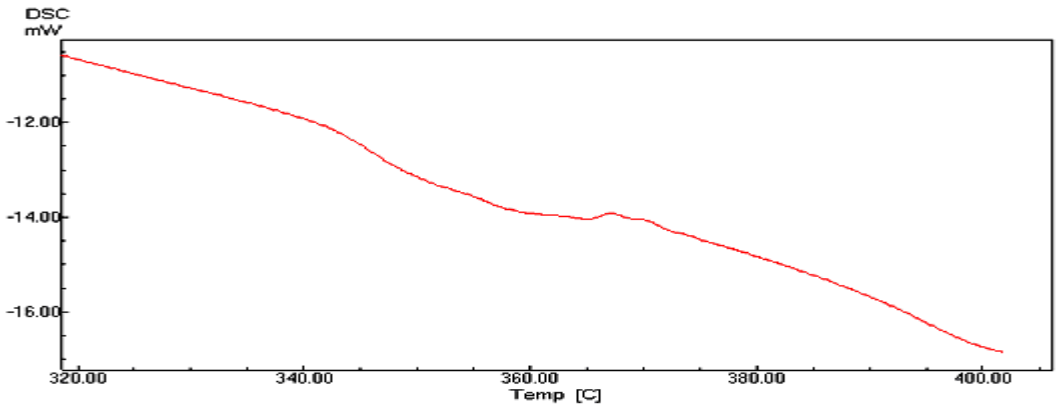


Fig.9 DSC of encapsulated carotenoids



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