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Nanoencapsulation improved water solubility and color stability of carotenoids extracted from Cantaloupe melon (*Cucumis melo* L.)

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ABSTRACT

Cantaloupe melon carotenoids were encapsulated in porcine gelatin, whey protein isolate and concentrate by emulsification O/W to evaluate which agent could promote an increase in water solubility, and color stability in yogurt. The average particle size obtained was 59.3 (2.60) nm–161.0 (27.30) nm. Encapsulated crude extract in porcine gelatin presented the smallest size and polydispersity index [0.4 (0.04)], and showed sphericity, smooth surface and low agglomeration in SEM. These results associated to the good chemical interaction between the raw materials shown by FTIR, justify the increase in water solubility [0.072 (0.007) $mg.mL^{-1}$] compared to the crude extract [0.026 (0.003) $mg.mL^{-1}$]. The yogurt added with this nanoencapsulate remained stable for 60 days, unlike the crude extract. The results show that the nanoencapsulation using gelatin increased water solubility and the potential of application of melon carotenoids in food as natural dyes.

1. Introduction

Color is associated with many aspects of life and influences decisions, including those involving food selection and, therefore, eating habits. Sensory characteristics, especially the appearance of food at the time of purchase, allows rapid identification and influences acceptance. Thus, the acceptability of food is directly affected by color and, this generates an increase in the use of food and beverage colorants, either in the natural or artificial form (Oliveira, Jacques, Nery, & Abrantes, 2010).

Dyes are included in the group of food additives and are defined as any substance that confers, enhances or restores color. However, it is necessary to be aware of the possible toxicological risks that may be caused by frequent ingestions, especially those above the daily acceptable intake index (ADI) (Polônio & Peres, 2012).

Consumers preoccupation with the adverse health effects associated with the intake of synthetic dyes has led to an increase in the demand for natural dyes, considered to be healthy substances (Borges, Tejera, Díaz, Esparza, & Ibáñez, 2012).

Among the most commonly used natural dyes, are carotenoids that attribute color to food and have important biological activities, promoting health benefits (Martins, Roriz, Morales, Barros, & Ferreira, 2016). They are natural pigments responsible for the orange, red and yellow coloring found in plants, algae, and microorganisms. Commonly located inside the membranes associated to proteins and to a variety of cellular structures, such as fibers and polysaccharides, they can function as antioxidants and perform specific functions of protection of tissues against damage caused by light, and the presence of oxygen (Gonnet, Lethuaut, & Boury, 2010).

Melon (*Cucumis melo* L.) is a specie of plant belonging to the Cucurbitaceae family, subdivided into botanical groups, among them Reticulatus, which is considered noble, because of its better quality fruits, with characteristic aroma and flavor (Khoo, Prasad, Kong, Jiang, & Ismail, 2011).

Cantaloupe melon is a fruit rich in bioactive compounds that contribute to improve health. In addition to containing phenolic

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compounds, it is considered a source of β-carotene, which contributes to the orange coloration of the pulp (Özkan & Bilek, 2014). This pigment can undergo asymmetric cleavage to generate β-apocarotenoids, which presents biological functions essential for the human organism, such as potent activity as provitamin A and acting as a transcriptional regulator (Fleshman et al., 2011).

One of the major limiting factors for the use of carotenoids as a food coloring is their unstable nature, very susceptible to chemical degradation, by isomerization and oxidation. Moreover, they present low solubility in water, which makes it difficult to apply in food matrices (Gutiérrez et al., 2013).

According to studies, nanoencapsulation is a promising technique that is capable of promoting increased water solubility of highly hydrophobic bioactive compounds, such as carotenoids. Because of nano scale (< 100 nm) particle size, it increases the dissolution rate. This occurs due to the larger contact surface for interaction with the water molecules (Chu, Ichikawa, Kanafusa, & Nakajima, 2007a). Thus, among emulsification encapsulation techniques, it is an effective method to increase the uptake of bioactive compounds in order to generate new products, aiming to increase the application potential of carotenoids in industrialized foods (Lamba, Sathish, & Sabikhi, 2015).

Based on this, the present study aimed to produce and characterize a carotenoid-rich extract from Cantaloupe (*Cucumis melo* L.) melon pulp, encapslutaling this extract in gelatin or whey proteins aiming to promote its solubility in water. Subsequently, the best encapsulating combination was added to yogurt, for the evaluation of the color stability as a natural dye.

2. Materials and methods

2.1. Materials

The fruits of the species *Cucumis melo* L. (var reticulatus Naud.) were purchased in local markets of Natal city, Rio Grande do Norte state (RN), northeast Brazil. Porcine gelatin (Type A), Tween 20 surfactant and β-carotene standard were obtained from Sigma-Aldrich. Whey proteins concentrate (76% protein) and isolate (90% protein) were obtained from Alibra®. Soya bean oil (Lizza®) and purchased in local markets of Natal (RN).

2.2. Processing of the melon pulp (Cucumis melo L. reticulatus Naud.) and extraction of the extract containing the carotenoids

The extraction of carotenoids from Cantalupe melon pulp followed the protocols already established in the routine of the laboratory. The melons were washed in water. The husks and seeds were removed and the pulp was separated (6.5 kg) and cut into pieces of approximately 2 cm of thickness, to be dehydrated in a ventilated oven (TE 394/2 TECNAL®) (55 °C/24 h). Subsequently, the material was grounded in an industrial blender and subjected to maceration using 95% ethanol (1:4 w/v). Under light protection, the solvent was changed every other day until exhaustion of color and the extract was refrigerated at 4 °C.

The ethanolic extract obtained was partitioned using hexane (1:1 v/v) and 10% NaCl solution (1:10 v/v) until the hexane phase was colorless. The solvent was removed on a rotary evaporator (Buchi-R-215) under low pressure at 28 °C. The resulting dry solvent-free extract was weighed for yield determination, using the following formula: (weight of melon extract/dry melon pulp in ventilated oven) × 100. Then, the extract was characterized and encapsulated in different wall materials. The physicochemical characterization, incorporation efficiency and water solubility were subsequently evaluated.

2.3. Cantaloupe melon extract characterization

2.3.1. Absorption spectrophotometry in UV–Vis

The Cantaloupe melon carotenoid extract was analyzed by absorption spectrophotometry in the UV-Vís (Bel photonics 1105) by scanning (250–700 nm), for determination of the maximum absorption wavelength.

To calculate the mean carotenoid concentration, the following equation was used based on Biehler, Mayer, Hoffmann, Krause, and Bohn (2010) at the maximum absorption wavelength obtained by scanning spectrophotometry: C (mol/L) = A450 × FD/2592 (d = 1 cm), where A450 is the average absorbance obtained at the wavelength of maximum absorption, FD is the dilution factor adjusted for the absorbance determinations of the hexane solubilized dry extract, and 2592 is the coefficient of absorption (ε) of β-carotene. The mean molar mass of β-carotene was used, according to Biehler et al. (2010) and the result was expressed in microgram/g of melon pulp (μg/g).

Afterward, a calibration curve was constructed, using the Cantaloupe melon carotenoid crude extract (CE), to obtain the concentration versus absorbance ratio at the determined wavelength, which was used for the quantification of the efficiency of incorporation of the extract in porcine gelatin (PG), whey protein concentrate (WPC) and whey protein isolate (WPI), and for solubility assay.

2.3.2. Ultra high-efficiency liquid chromatography (UHPLC)

The Cantaloupe melon carotenoid extract was analyzed by UHPLC diode array detector (UHPLC-DAD), to the determination of β-carotene concentration and absorption spectra at 450 nm of according to Rodriguez-Amaya (2001) with modifications.

The UHPLC (Shimadzu) presented a binary analytical pump (LC-20A3 XR), automatic injector (SIl-20AD XR), degasser (DGU-20A3), column kiln (CTO-20AC) diode array detector (SPD-M20A), with a system controlled by LC Solution® software. The column used was typed XR-ODS, Shim-Pak®, C18, 30 × 20 mm; 2.2 μm. Prior to the injection, the extract was solubilized in ethyl acetate at a concentration of 0.4 $mg.mL^{-1}$ and 5 μL were injected. The mobile phase used was an elution gradient with acetonitrile and water (900: 99 v/v – phase A) and ethyl acetate (phase B) in the following composition: 0 min, 100% A, 0% B; 5 min, 75% A, 25% B; 10 min, 30% A, 70% B; 13 min, 0% A, 100% B; 14 min, 100% A, 0% B; 20 min, 100% A, 0% B. The flow used in the column was 0.5 mL/min. A calibration curve for the β-carotene standard (Sigma Aldrich) (450 nm) was previously constructed.

2.4. Obtainment of encapsulates rich in Cantaloupe melon carotenoids extract

The particles were obtained by emulsification in O/W technique with subsequent dispersion of the solution containing encapsulating agent and tensoative in the emulsion obtained, based on Qian, Decker, Xiao and McClements (2012) protocol with modifications. The drying was done by lyophilization. The encapsulates were formulated using three different encapsulating agents: 1) porcine gelatin (EPG), 2) whey protein concentrate (EWPC) and, 3) whey protein isolate (EWPI).

Two aqueous phases were formulated: AF 1 (90 mL) containing 1.5% Tween 20 (w/v) solubilized in distilled water, and AF 2 (100 mL) containing 4% encapsulating agent (w/v), and 1.5% Tween 20 (w/v) solubilized in distilled water. For the oily phase (10 mL), 0.5% of Cantaloupe melon carotenoid extract (w/v) solubilized in soybean oil was used.

To promote the solubilization of the carotenoid-rich extract in soybean oil, magnetic stirring was used for 30 min at room temperature under light protection. The same procedure was performed for the preparation of AF 1. For AF 2, the solubilization occurred for 1 h under magnetic stirring, at 40 °C and ambient temperature, respectively, for porcine gelatin and whey proteins (isolate and concentrate).

To promote the formation of the emulsion, the aqueous phase 1 was homogenized with the oil phase under ultra-dispersion (17,000 rpm/10 min) (Ultra-Turrax, IKA®T18 basic). Then, AF 2 was dispersed in emulsion obtained using the same conditions mentioned above. At the end of the process, this final emulsion was dried by lyophilization

(LioTop L101) at −57 °C and pressure of 43 μHg. Subsequently, for the gelatin-based encapsulation, the lyophilized extract was triturated in a blender. The experiments were performed in triplicate.

2.5. Particles characterization

2.5.1. Laser diffraction

For the determination of particle size, protocols already established in the routine of the laboratory to promote the desagglomeration of the particules and facilited the mensurement were used: 10 mg of EPG and 30 mg of EWPI and EWPC were dispersed in 4 mL of acetone under magnetic stirring at room temperature for 2 min. Subsequently, 2 mL formaldehyde PA were added to dispersions and stirred for 10 min (EWPI and EWPC), and 30 min (EPG) to promote particle crosslinking.

After this time, the dispersions were filtered and the particles retained on the qualitative filter paper were collected for Laser Diffraction. The particles were redispersed in 5 mL of acetone, placed in glass cuvettes and read at 5 runs.min^{-1} for the measurement of the mean diameter and the polydispersity index in the NanoBrook ZetaPlus Zeta Potential Analyzer, Brookhaven Instruments – ZetaPALS Particle Sizing software. Experiments and measurements were performed in triplicate.

2.5.2. Zeta potential

For measurement of the Zeta Potential, 10 mg of EPG was dispersed in 12 mL of water, and 20 mg of EWPI and EWPC in 4 mL of water and then placed in acrylic cuvettes with side electrodes. Ten runs were performed for 1 min each, on the NanoBrook ZetaPlus Potential Analyzer, coupled with the softwareBrookhaven Instruments - PALS Zeta Potential Analyzer. Measurements were performed in triplicate.

2.5.3. Scanning electron microscopy (SEM)

The powdered encapsulates were dispersed in acetone PA and dripped onto a silicon chip support, fixed in stub using carbon tape. After evaporation of acetone, the particles were analyzed at various magnifications using a high vacuum, 2–3 kV voltage, without metallization, under SEM-FEG ZEISS (AURIGA) type microscopy.

2.5.4. X-ray diffraction

The encapsulating agents and powdered encapsulates were analyzed on a high-resolution X-ray diffractometer (SHIMADZU, model XRD 7000), with Seifert ID3000 generator, to evaluate whether the dominant phase in the materials was crystalline or amorphous. For this analysis, the materials were placed in the cylindrical sample port and analyzed at 2θ diffraction angle between 0 and 100°.

2.5.5. Fourier transform infrared spectrophotometry (FTIR)

The encapsulating agents, Tween 20, crude extract rich in carotenoids from Cantalupe melon, and powdered encapsulates were mixed with potassium bromide (KBr), macerated and pressed for the formation of the pellets, and subjected to FTIR analysis to obtain the spectra in a 400–4000 cm^{-1} range, using the Shimadzu, model FTIR-8400S, series IRAFFINITY-1, software IRSOLUTION, version 1.60, with a scanning number of 32 and resolution 4 cm^{-1}.

2.6. Determination of the incorporation efficiency of carotenoids

The amount of encapsulated material inside the particles was obtained from the formula: EI (%) = (carotenoids in particles/total carotenoid extract used) × 100 (Hu et al., 2015).

For the accomplishment of this methodology, 150 mg of the encapsulates dispersed in 1 mL of hexane was used. This was sonicated (Ultra Cleaner 1650 Unique®) for 3 min and centrifuged (Micro Centrifuge 6000 RPM HT) at 947.52 × *g* for 20 min to separate the supernatant containing the carotenoids. The procedure was performed in triplicate and repeated until exhaustion of the color.

2.7. Solubility assay

The solubility analysis of the powdered encapsulates and the crude extract containing the carotenoids was based on protocols already established in the laboratory. An excess of crude and encapsulated extract (10 mg and 105 mg) was added to 4 mL of distilled water and placed in glass tubes. The tubes were placed on an orbital shaker table (ACB LABOR®) at 120 rpm for 45 h at 27 ± 2 °C.

Subsequently, separation of the fraction of the non-solubilized carotenoid extract was performed by a separating funnel partition using hexane and a 10% NaOH solution (1:10 v/v). After partition, the groups were analyzed by UV–visible absorption spectrophotometry (450 nm), using the equation of the line obtained previously, to determine the concentration of carotenoid extract that did not solubilize in water and, thus, discounting the total amount of extract in the particles (Table 1 – Supplementary material). Experiments and measurements were performed in triplicate.

2.8. Color stability evaluation of the encapsulated carotenoids extract in yogurt

The evaluation of the color stability of the encapsulated extract containing the carotenoids added to the yogurt followed the protocols already established in the routine of the laboratory. In the preparation of the yogurt, 1 L of sterilized milk, 10% of milk powder, and 170 g of natural yogurt were used. The lactic fermentation was done at 45 °C until pH 4.1, from the inoculation of the natural yogurt containing *Streptococcus thermophilus* and *Lactobacillus bulgaricus*. After fermentation and coagulation, the gel was shaken to give creaminess to the yogurt. The product was then cooled to 5 °C.

The porcine gelatin encapsulate, which presented the best characterization and solubility results, was evaluated for its color stability in yogurt. This was evaluated comparing three groups: 1) Yogurt without the addition of the natural dye (YWD); 2) Yogurt with the addition of the carotenoid crude extract from Cantaloupe melon (YCE); 3) Yogurt with the addition of the Cantaloupe melon carotenoids encapsulated with porcine gelatin (YEPG).

In the preparation of YCE, 50 mg of crude extract/g of yogurt was added and in the YEPG group, 2 g of the encapsulated material based on carotenoids and gelatin/g of yogurt was used. These concentrations were established based on the quantities of dyes (crude and encapsulated) required to achieve the color parameters of commercial pineapple yogurt (L^* = 83.81; a^* = −3.83; b^* = 24.06).

All groups of yogurt were refrigerated (0–4 °C) for 60 days, representing the shelf life of commercial yogurt. The instrumental color analysis was performed at 0, 15, 30, 45 and 60 days, by reflectance spectrophotometry (INSTRUTHERM, ACR-1023) in the RGB System and converted to CIElab using the OpenRGB program. The measured color parameters were L^* = luminosity (0 = black and 100 = white); a^* (−80 to zero = green, from zero to +100 = red) and, b^* (−100 to zero = blue, from zero to +70 = yellow). Experiments and measurements were performed in triplicate.

2.9. Statistical analysis

The efficiency of incorporation obtained and the concentrations of solubilized extracts in water (solubility assay) were expressed as a mean and standard deviation, and evaluated by ANOVA with Tukey post-test ($p < 0.05$).

For the evaluation of color stability in yogurt, the color indexes determined in each time point were compared using ANOVA. A p value < 0.05 was adopted to determine significant differences between storage days for each evaluated group. If there was a significant difference, the means were compared to time zero using the Dunnet post-test. Statistical analysis was performed using Graph Pad Prism 5.0 software.

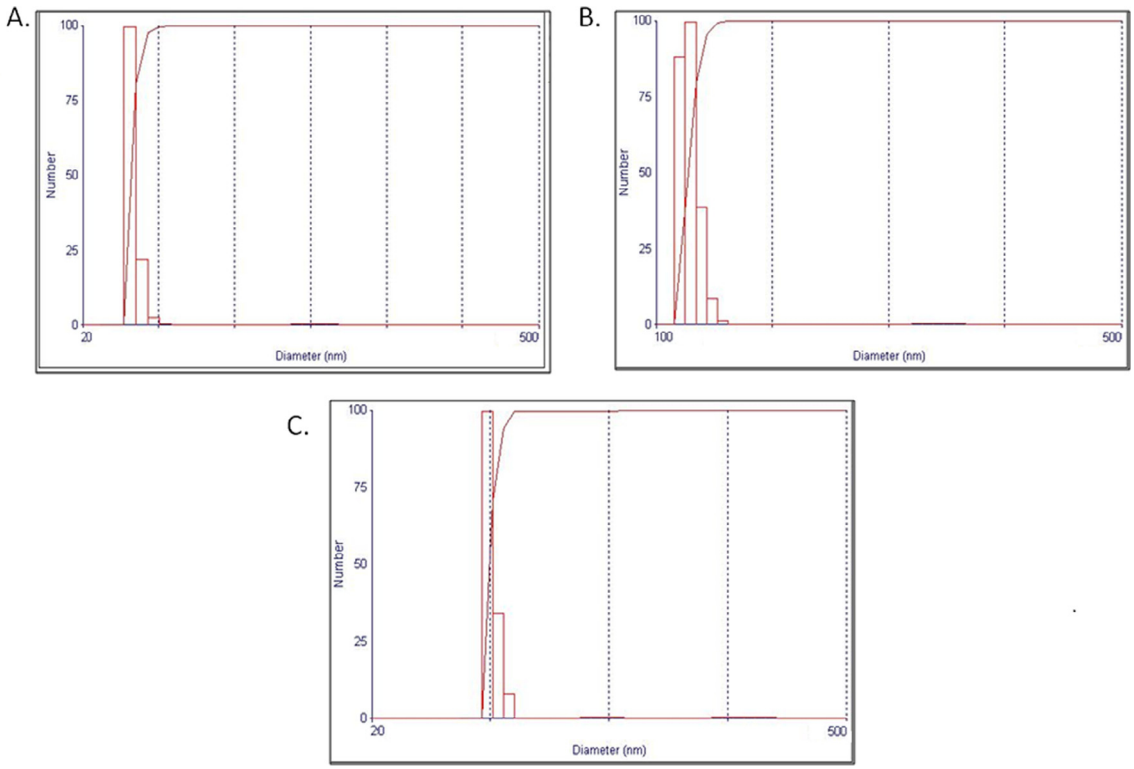

Fig. 1. Particle size distribution by laser diffraction of powder particles, which were crosslinked and dispersed in acetone for the measurement, based on a crude extract of carotenoids of the Cantaloupe melon pulp (*Cucumis melo* L. reticulatus Naud.) and encapsulating agents obtained by emulsification O/W technique. (A) EPG: encapsulated in porcine gelatin, (B) EWPI: encapsulated in whey protein isolate, (C) EWPC: encapsulated in whey protein concentrate.

3. Results and discussion

3.1. Characterization of Cantaloupe melon pulp extract

In the present study, Cantaloupe melon was used as a raw material for the extraction of carotenoids, presenting a high content of these pigments. After the process of obtaining the solvent-free carotenoid-rich extract, the yield of the obtained dry extract was 2.6% (0.007). This extract was used in all subsequent experiments.

The total carotenoid content found in the extract was of 46.2 (4.9) $\mu g.g^{-1}$ of Cantaloupe melon pulp. According to Fleshman et al. (2011), the foods containing more than 20 $\mu g\, g^{-1}$ of total carotenoids are very important for health. Thus, the carotenoid extract of Cantaloupe melon (var. reticulatus Naud.) presented a significant total carotenoid content.

The maximum wavelength absorption was 450 nm, so this was used in the construction of the calibration curve. The equation of the line obtained was y = 5,1574x + 0,018 (R^2 = 0,997), which was used to determine the total crude extract carotenoids present in encapsulates, and to evaluate the solubility of carotenoids.

The presence of β-carotene was confirmed by the peaks observed at 425, 450 and 478 nm by UHLPC. The line equation obtained from the calibration curve with the β-carotene standard was $y = 10^7x - 77{,}163$ (R^2 = 0.998), this was used to determine the pigment content present in the pulp. UHPLC indicated the presence of β-carotene (*all-trans* isomer), in a total of 28.2 (2.93) $\mu g.g^{-1}$ Cantaloupe melon pulp (Fig. 1 in the Supplementary material), due to the similar retention time (≅8 min) and spectrophotometric profile with the standard. This carotenoid was the most abundant pigment in the Cantaloupe melon pulp extract, and possibly responsible for its orange coloration.

This result was similar to that obtained by Fleshman et al. (2011), who observed that β-carotene was the only carotenoid detected in the Cantaloupe melon pulp at 450 nm by HPLC, with more than 98% was the *all-trans* isomer.

Esferas et al. (2018) concluded that orange pulp melons compared to other groups (yellow, green or white pulp) present β-carotene as the main carotenoid, followed by β-cryptoxanthin and lutein.

Among the carotenoids, the highest activity as pro-vitamin A is attributed to β-carotene, due to the presence of two β-ionone rings (Ambrósio, Campos, & Faro, 2006), which reduces the risk of degenerative diseases (cardiovascular diseases, cataracts, and cancer) and promotes protection by the immune system (Gonnet et al., 2010).

3.2. Encapsulates characterization

Carotenoids are susceptible to chemical degradation, especially during food processing and storage (Chen et al., 2014). This characteristic seems to be related to some of its properties, especially its lipophilic nature and the sensitivity to heat, light and the presence of oxygen (Gutiérrez et al., 2013).

Thus, the application of carotenois as dyes in food matrices depends particularly on their solubility, commonly limited because of the low water dispersibility, chemical instability and bioavailability. Therefore, an alternative to minimize these limitations is the use of techniques such as encapsulation, since they guarantee greater stability to the bioactive compounds (Dias, Ferreira, & Barreiro, 2015).

In this study, the carotenoids extracted were encapsulated using whey proteins (concentrate and isolate) and porcine gelatin as wall materials. The whey proteins were chosen based on the numerous beneficial properties, such as flexible molecular chain, solubility, viscosity and emulsifying power (Madene, Jacquot, Scher, & Desobry, 2006).

And gelatin was chosen as a low cost, non-toxic encapsulating agent and widely used in the food industry as an emulsion stabilizer and thickener.

Afterwards, the powdered encapsulates were evaluated for their physical and chemical characteristics, in order to select the encapsulated particles with a greater efficiency of incorporation,

solubility and, stability in an alimentary matrix.

Several techniques are used to characterize and evaluate the success of the encapsulated production, among them particle size, polydispersion index, Zeta Potential, SEM, FTIR and XRD.

3.2.1. Laser diffraction

Particle size and polydispersion index (PDI) obtained for the encapsulates (Fig. 1) were, respectively, 59.3 (2.60) nm and 0.4 (0.04) for EPG; 161.0 (27.30) nm and 0.6 (0.07) for EWPI and; 123.3 (37.45) nm and 0.5 (0.02) for EWPC.

Particle size is a fundamental parameter for deciding the application of the encapsulates obtained (Onwulata, 2012). Thus, particles of nanometric size (< 100 nm) with homogeneous distribution are adequate to increase water solubility of carotenoids. The Polydispersion Index (PDI) is used as an indicator of stability and particle uniformity. Therefore, the PDI value reflects the particle size distribution, that is, samples with a wider range of sizes have higher PDI values, whereas samples with uniform-sized particles have lower values (Masarudin, Cutts, Evison, Phillips, & Pigram, 2015).

In the present study, the group with the lowest mean diameter was EPG, compared to the other groups, which may indicate a better interaction between the carotenoids and the encapsulating agent, provided by the use of Tween 20 surfactant. This promoted the reduction of surface tension and surface viscosity, promoting uniform distribution of particle size. Therefore, adequate concentrations of surfactant results in lower diameters and polydispersion indices (Anarjan & Tan, 2013).

Chu, Ichikawa, Kanafusa and Nakajima (2007b) also obtained particles based on β-carotene and WPC with a wide range of medium-sized distribution, attributed to the lower percentage of protein present in WPC compared to WPI. With this, there is a smaller amount of proteins, insufficient to stabilize the pigment, promoting the formation of aggregate particles. The same authors also encapsulated β-carotene in WPI and observed that it presents heterogeneous molecular weight chain, which generates less structural flexibility and forms larger particles (> 100 nm) containing the pigment.

3.2.2. Zeta potential

The results obtained for Zeta Potential were 21.2 (0.10) mV, −10.8 (0.55) mV and −14.2 (0.25) mV, respectively, for the particles based on carotenoids and gelatin (EPG), whey protein isolate (EWPI) and whey protein concentrate (EWPC).

This measurement indicates the stability of the particles. So, according to parameters described by Bhattacharjee (2016), the particles are highly unstable around ± 0–10 mV, moderately stable in the range of ± 20–30 mV and, highly stable above ± 30 mV. From this, it can be inferred that EWPI and EWPC presented results that classify them in the instability range, which may generate aggregation or flocculation between the particles in solution. Moreover, the determined Zeta Potential was negative, since the whey protein isolate had an isoelectric point in the pH range of 4.8–5.34 (Lee & Hong, 2009), and presented negative charges (pH 6.0–7.0).

The Zeta Potential result obtained for EPG, shows the presence of positive charges on the surface of the particles, indicating the influence of the pH of the relatively high isoelectric point (pI ≥ 7.0) for porcine gelatin, which makes it possible to formulate emulsions and dispersions with a positive charge over a wider range of pH values. Accordingly, this encapsulating agent may be suitable for obtaining food emulsions with high oxidative stability since it could, for example, repel metal ions, such as iron, from the surfaces of oil droplets over a wide pH range found in various foods (Dickinson & Lopez, 2001).

3.2.3. Scanning electron microscopy (SEM)

Fig. 2 shows the micrographs obtained for EPG, EWPI, and EWPC. EPG presented spherical particles, with a smooth surface, without cracks or depressions, and homogeneous sizes at the nanoscale (30–60 nm). EWPI presented particles with a smooth surface and some with a spherical shape, but extremely agglomerated. For the EWPC, we observed smooth and non-cracked particles, however heterogeneous sizes (40–150 nm).

Micrographs obtained in the present study showed that EPG presented spherical particles, with a smooth surface, and sizes at the nanometer scale (30–60 nm), which confirms the result obtained in the Laser diffraction. The other two encapsulations obtained showed particles with similar characteristics in relation to the surfaces. However, EWPI was extremely agglomerated, compared to EPG, with a lower number of particles at the nanoscale. Despite EWPC having less agglomerated nanoparticles, the observed physical sizes showed a very heterogeneous material, with a larger particle size compared to EPG.

Thus, from the observations of the micrographs, the encapsulated carotenoids obtained by the emulsification technique were well protected by all the encapsulating agents used, but presented satisfactory size and distribution characteristics only with the use of porcine gelatin.

3.2.4. Fourier transform infrared spectrophotometry (FTIR)

The FTIR spectra (Fig. 3) shows the results for the encapsulating agents, Tween 20, crude extract rich in carotenoids from Cantaloupe melon and, for the powdered encapsulates obtained (EPG, EWPI, EWPC).

In the spectrum of the carotenoid-rich crude extract of the Cantaloupe melon, the presence of hydrocarbon groups, such as the bands of 2927 cm^{-1} and 2847 cm^{-1} (C–H bond), 1562 cm^{-1} (C═C stretching vibration), and in the region between 1450 and 1082 cm^{-1} (C–C bond) were observed.

The presence of bands around 3500 cm^{-1} (–OH), 1700 cm^{-1} (C═O bond), and 843 cm^{-1} (double bond of the methacrylate molecule) were observed in relation to Tween 20.

The spectra obtained for porcine gelatin showed, mainly, an elongation around 1670 cm^{-1}, which characterizes the stretching of the C═O (amide I) bond, an absorption band of 1549 cm^{-1}, indicating the presence of the amide (N–H), and another band near the 3580 cm^{-1} region relative to stretches of N–H and O–H bonds, which may form hydrogen bonds with the carbonyl group of the peptide bond.

For the whey protein isolate the presence of characteristic bands in the region between 1392 and 1239 cm^{-1} (elongation of the C–O bond), 1450–1392 cm^{-1} (folding of hydroxyl groups), 1668 cm^{-1} (amide I) and, 1530 cm^{-1} (folding of N–H bonds and C–N elongation of amide II) were observed. And for the whey protein concentrate the absorption bands for amides I and II, respectively, were observed at 1659 cm^{-1} and 1542 cm^{-1}.

In the EPG spectra (Fig. 3A), were observed absorption bands which were present in the raw materials used or the displacement of these bands. The absorption bands of Cantaloupe melon crude extract at 2927 cm^{-1} and 2847 cm^{-1} were attenuated, and the bands at 1450 cm^{-1} and 1082 cm^{-1} were not observed, indicating that the crude extract was protected by gelatine. As well as the appearance of new bands (3014 cm^{-1}, 1659 cm^{-1}, and 1554 cm^{-1}), which may indicate that the non-polar amino acids of gelatine made hydrophobic interactions with the carbonic chain of the crude extract rich in carotenoids.

For EWPI (Fig. 3B), no new bands were observed that demonstrate new interactions between the materials used. Besides that, no displaced absorption bands similar to those presented in the crude carotenoid extract were observed, only for Tween 20 (1754 cm^{-1} and 1655 cm^{-1}), and whey protein isolate (3292 cm^{-1}, 3011 cm^{-1}, 1546 cm^{-1}). Bands identical to those present in the crude extract were observed in the encapsulate (2927 cm^{-1} and 2847 cm^{-1} not attenuate and at 1450 cm^{-1} attenuate), showing that the extract rich in carotenoids had low chemical interaction with the hydrophobic region of the protein chain isolated from whey protein. This in fact shows that the whey protein isolate did not adequately encapsulate Cantaloupe melon extract.

In EWPC (Fig. 3C) it was possible to observe an absorption band

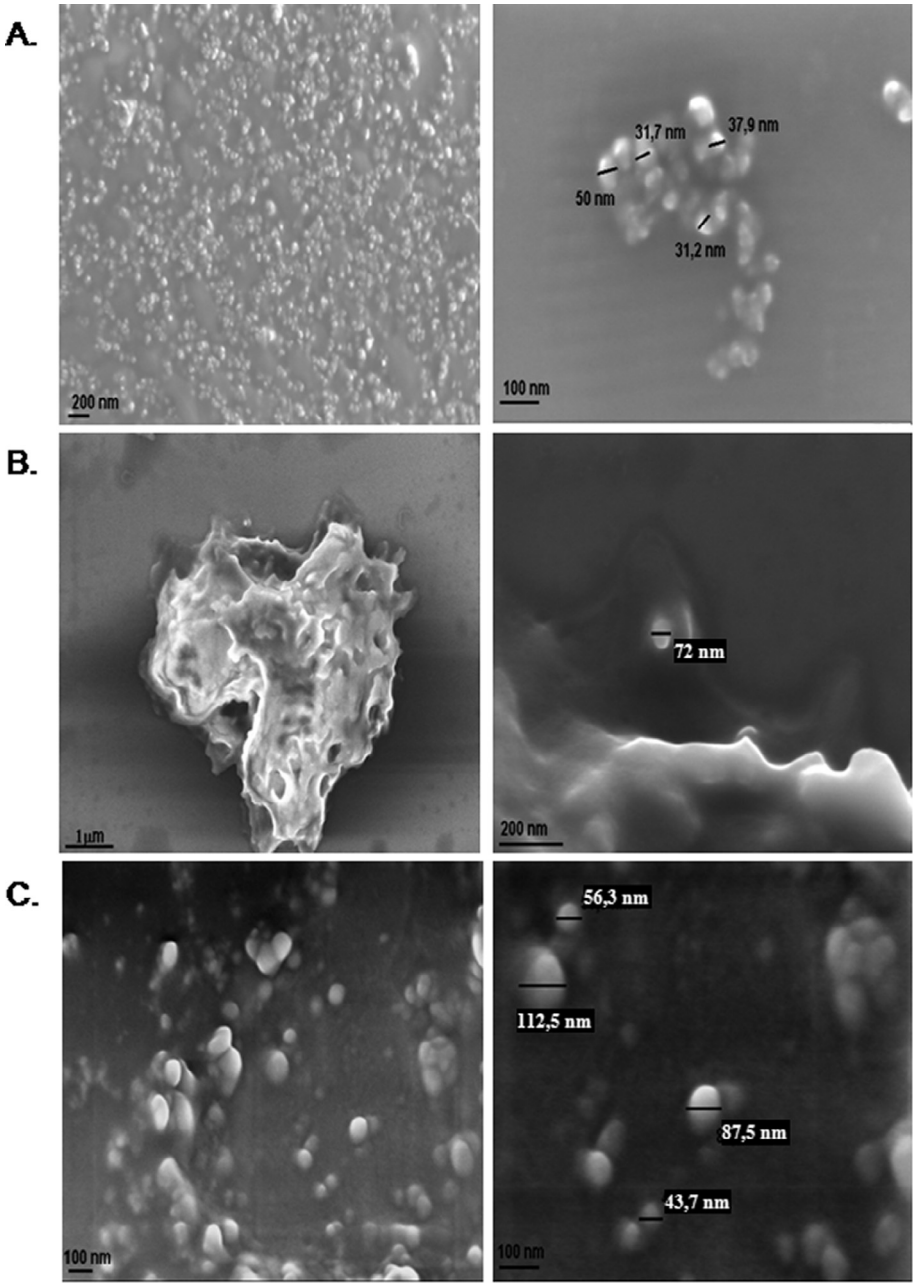

Fig. 2. Scanning electron microscopy micrographs of the powder particles, dispersed in acetone, based on a crude extract of carotenoids of the Cantaloupe melon pulp (*Cucumis melo* L. reticulatus Naud.) and encapsulating agents obtained by emulsification O/W technique. (A) EPG: encapsulated in porcine gelatin, (B) EWPI: encapsulated in whey protein isolate and, (C) EWPC: encapsulated in whey protein concentrate.

shift similar to that observed in Tween 20 and whey protein concentrate. And bands of Cantaloupe melon crude extract attenuated (2927 cm^{-1}) and displaced (2856 cm^{-1}, 1542 cm^{-1} and 1462 cm^{-1}). This reflects that there was a greater interaction of melon carotenoid extract with WPC compared to WPI, probably due to the greater number of hydrophobic interactions between the chains.

According to the manufacturer (Alibra), WPI presents higher percentages of polar amino acids (threonine, cysteine, tyrosine, aspartic acid, glutamic acid, arginine and lysine) compared to WPC, which consequently hinders interaction with the apolar extract chain of carotenoids of the melon rich in β-carotene. McSweeney and O'Mahony (2016) state that WPI is rich in sulfur amino acids, responsible for polar interactions, such as sulfur bonds, which turns it more difficult to interact with carotenoids.

The appearance of new bands in the EPG and EWPC encapsulates reflects the interaction between the materials in the formed particles, showing that the emulsification O/W technique was efficient to encapsulate the carotenoids of the Cantaloupe melon pulp in porcine gelatin, and whey protein concentrate. This was not observed for the encapsulation with the whey protein isolate.

3.2.5. X-ray diffraction (XRD)

The diffractograms for the raw and encapsulated materials are shown in Fig. 4. The encapsulating agents used had a semi-crystalline structure, as a function of some defined and intense peaks observed in the diffractograms.

The XRD showed diffraction signals in 2θ equal to ~20° and between 60 and 80° for the whey proteins used, being close to that observed by Bastos, Gonçalves, Andrade, Araújo, and Leão (2012) and, for gelatine, 2θ equal to ~20° and between 37 and 80°, which according to

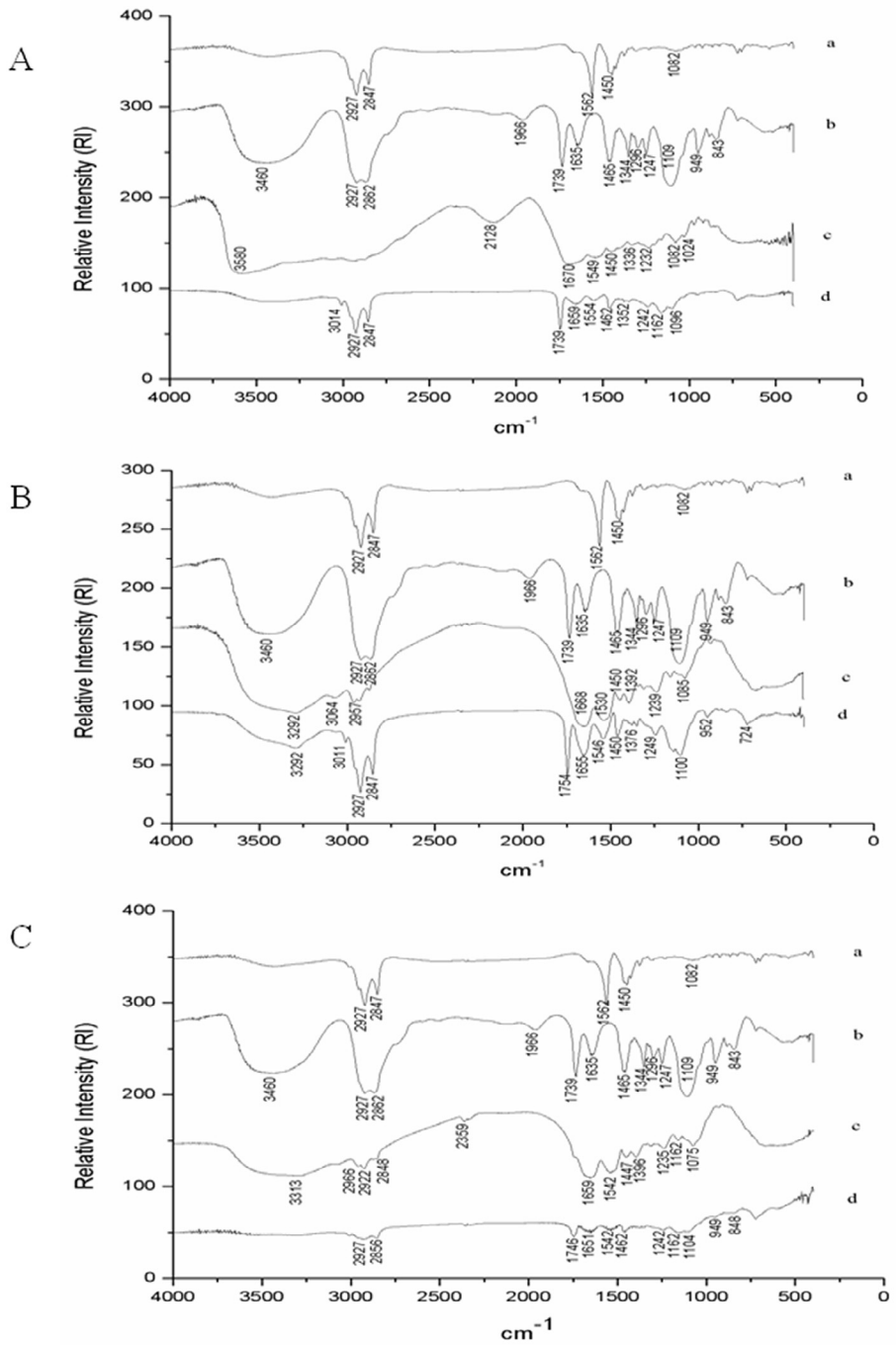

Fig. 3. FTIR spectra of the powder particles based on a crude extract of carotenoids of the Cantaloupe melon pulp (*Cucumis melo* L. reticulatus Naud.) and encapsulating agents obtained by emulsification O/W technique. (A) EPG: encapsulated in porcine gelatin. a: crude extract of carotenoids; b: Tween 20; c: porcine gelatin, and d: EPG; (B) EWPI: encapsulated in whey protein isolate. a: crude extract of carotenoids; b: Tween 20; c: whey protein isolate, and d: EWPI; (C) EWPC: encapsulated in whey protein concentrate. a: crude extract of carotenoids; b: Tween 20; c: whey protein concentrate; and d: EWPC.

Pena, La Caba, Eceiza, and Mondragon (2010) that can be due to the crystal structure of the triple helix collagen.

The encapsulates obtained presented low crystallinity compared to the encapsulating agents. These data show a predominance of disorganized molecular chain behavior. Two reflections were present in the whey proteins, and these were not observed in the base encapsulates of these materials (EWPC and EWPI). Therefore, it can be inferred that the interactions between the chains of encapsulating agents were replaced by hydrophobic interactions between these and the crude extract rich in carotenoids from Cantaloupe melon, and also by the bonds that the Tween 20 can establish with the polar fraction of the whey proteins (Ghadetaj, Almasi, & Mehryar, 2018) and porcine gelatin (Uricanu, Duits, Filip, Nelissen, & Agterof, 2006).

3.3. Evaluation of the efficiency of incorporation (EI)

In addition to the physical and chemical characterization demonstrated for the carotenoids encapsulated in this study, the incorporation efficiency was also performed. This is one of the most important parameters in the characterization of the encapsulation process. Its efficiency depends on the ratio of core and encapsulating agent, on the conditions under which the encapsulation is performed, and on the

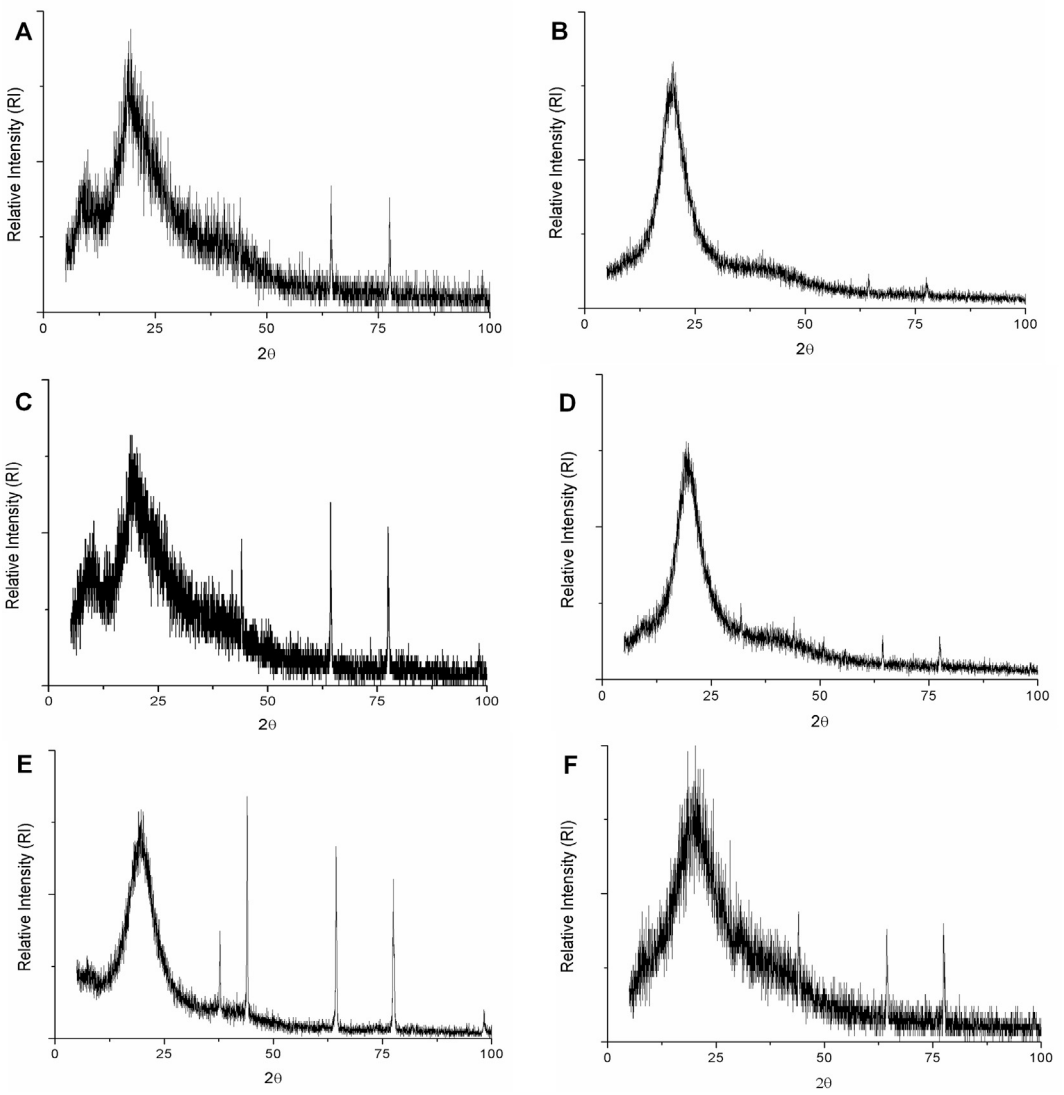

Fig. 4. X-ray diffraction of the powder particles based on a crude extract of carotenoids of the Cantaloupe melon pulp (*Cucumis melo* L. reticulatus Naud.) and encapsulating agents obtained by emulsification O/W technique. (A) WPC: whey protein concentrate; (B) EWPC: encapsulated in whey protein concentrate; (C) WPI: whey protein isolate; (D) EWPI: encapsulated in whey protein isolate; (E) PG: porcine gelatin; and (F) EPG: encapsulated in porcine gelatin.

technique or method of production employed (Tavares, Croguennec, Carvalho, & Bouhallab, 2014).

The efficiency of incorporation for the encapsulated extracts was 58% (0.71), 77% (8.65) and 90% (7.39) for the EWPI, EWPC and EPG materials, respectively. Only EPG and EWPC did not differ significantly ($p > 0.05$).

In the present study, the result for EI (%) was smaller and significant in relation to the others, when whey protein isolate was used as the encapsulating agent, which confirms the results obtained for the infrared spectroscopy of this material, showing low chemical interaction between this wall material and the crude extract rich in carotenoids.

However, when the wall material was porcine gelatin, the result found was superior to that shown in other studies that also encapsulated carotenoids, however by different techniques and without the use of Tween 20 as a surfactant. In the study of Lemos, Marfil, and Nicoletti (2017), encapsulating buriti rich carotenoid oil by coacervation, using alginate and gelatin as wall materials, incorporation efficiency was of 80%. In another study, an EI of 65% for the encapsulation of carotenoids from the yeast *Phaffia rhodozyma*, by lyophilization, using the soy protein as encapsulating agent was found (Nogueira, Prestes, & Burkert, 2017)

Possibly the high efficiency of incorporation obtained in this study was, among other factors, due to the use of the surfactant in low concentrations in the two aqueous phases. The emulsification technique requires the use of surfactants with high hydrophilic-lipophilic balance (HLB) to stabilize the interface between oil and water (Lamba et al., 2015). In the present study, Tween 20, a non-ionic surfactant, was used, with an HLB around 16.7. This surfactant is from the polysorbates group and considered to be the most hydrophilic emulsifier among the non-ionic group (Yuan, Gao, Zhao, & Mao, 2008).

In the study by Anarjan and Tan (2013), among the four emulsifiers used (Tween 20, 40, 60 and 80) in the production of nanodispersions of astaxanthin by emulsification, Tween 20 was the surfactant that produced the smallest particles [75.0 (3.20) nm].

3.4. Solubility assay

The incorporation of carotenoids into the food matrix is difficult because they are insoluble in water and partially soluble in oils at room temperature. Therefore, the elaboration of nanoparticles containing carotenoids is considered a very promising strategy, as a result of increasing the solubility in water and, in addition, increasing the bioavailability of these substances that promote several benefits to human health (Bitencourt et al, 2018).

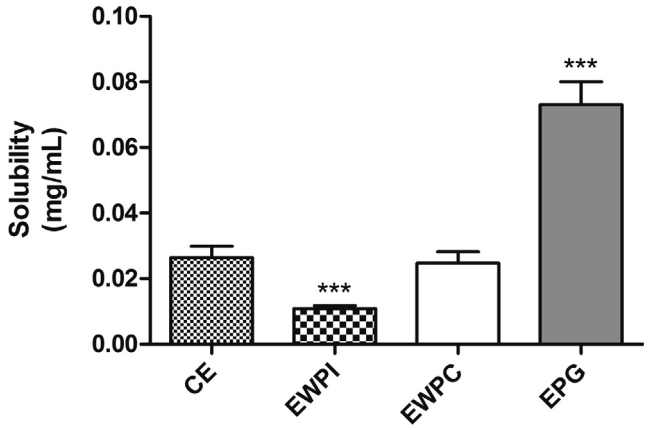

Fig. 5. Solubility indexes for the crude extract of carotenoids from Cantaloupe melon solubilized in water, and encapsulated crude extract in different agents by the emulsification O/W technique. The values are presented as mean ± standard deviation and compared to each other by means of Analysis of Variance with Tukey post-test ($p < 0.05$). CE: crude extract of carotenoids from Cantaloupe melon solubilized in water; EWPI: crude extract encapsulated in whey protein isolate solubilized in water; EWPC: crude extract encapsulated in whey protein concentrate solubilized in water; EPG: crude extract encapsulated in porcine gelatin solubilized in water. *** Comparison of solubility of all groups ($p < 0.001$).

The results obtained in the solubility assay are shown in Table 1 (supplementary materia1) and Fig. 5, where the concentrations of solubilized carotenoids present in the crude extract rich in carotenoids from Cantaloupe melon (CE), EWPC, EWPI, and EPG are shown. The Fig. 2 of Supplementary material shows the crude extract from Cantaloupe melon and encapsulates solubilized in water after the incubation time of 45 h.

EWPI presented the lowest solubility [0.010 (0.001) $mg.mL^{-1}$] when compared to CE [0.026 (0.003) $mg.mL^{-1}$] and the other encapsulating agents ($p < 0.05$). EPG presented the highest solubility [0.072 (0.007) $mg.mL^{-1}$] amongst the encapsulating agents ($p < 0.05$), about 267% than CE. Therefore, EPG was chosen for the color stability study in yogurt.

In addition, the solubility test revealed that the EPG encapsulation compared to the crude carotenoid extract was three times more soluble in water, which justified its use in the stability study in yogurt. However, the results obtained for EWPC [0.025 (0.003) $mg.mL^{-1}$] and EWPI were lower, and EWPI showed a solubility even lower than that of the crude extract itself. This is justified by the low chemical interaction between the materials involved in the encapsulation and to the worst values found for particle size and Zeta Potential, which reflects the rapid phase separation (oil and water) presented by these materials when submitted to the solubility assay.

Porcine gelatine is considered one of the most important macromolecules with diverse applications in the food, pharmaceutical, cosmetic and photographic industries and presents properties for new applications in functional foods (Silva, Lourenço, & Pena, 2017).

On the other hand, carotenoids have high hydrophobicity, due to the long chains containing exclusively carbons and hydrogens, making them difficult molecules to incorporate into a food matrix, thus reducing their application potential (Wackerbarth, Stoll, Gebken, Pelters, & Bindrich, 2009).

Therefore, the use of porcine gelatin as an encapsulating agent possibly improved the solubility of the core. In addition to its important physicochemical properties, the use of porcine gelatin as a wall material in the present study led to the formation of nanoparticles with lower sizes (59.3 nm) and also more homogeneous when compared to those obtained with the other encapsulating agents. This provided an increase in the contact surface of the encapsulated with the water, favoring the solubilization of the carotenoids, thus adding a new functionality to this nucleus. For the other encapsulating agents, this was not observed due to the physical state of the particles obtained and the larger particle size, with a wide observed size range (40–150 nm). Thus, this did not favor the increase of carotenoid interaction with water.

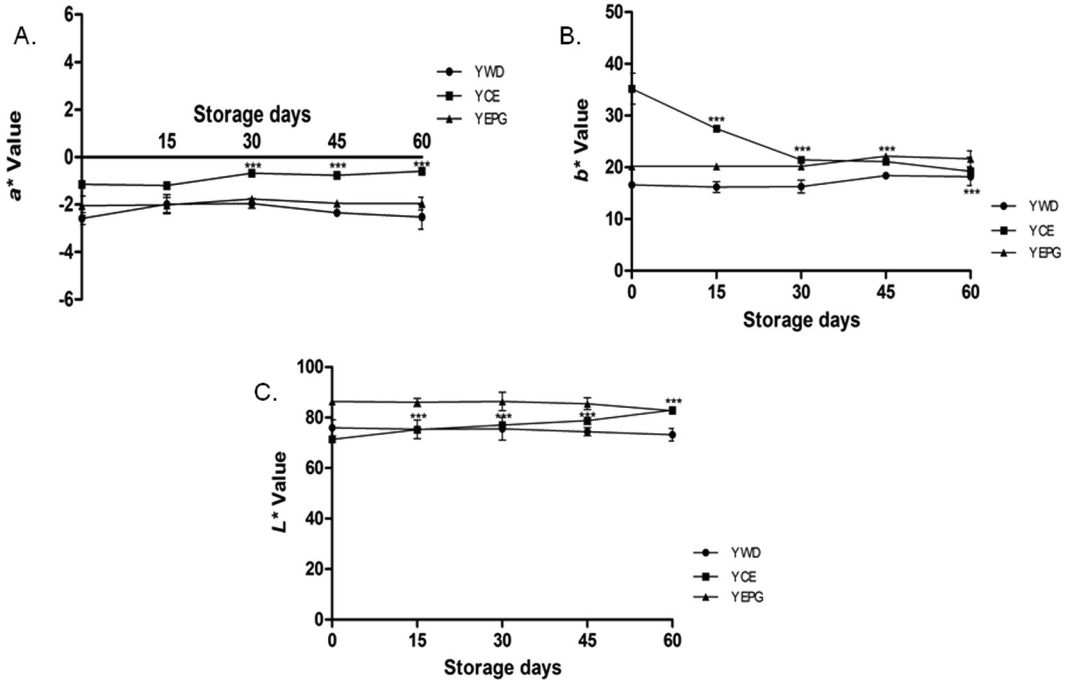

Fig. 6. Colorimetry (CIElab) of yogurts without natural dye (YWD), added with crude extract (YCE), encapsulated based on gelatine and extract rich in carotenoids of Cantaloupe melon (YPGE) evaluated for 60 days of storage (mean ± standard deviation). A. L^* index = luminosity (0 = black and 100 = white); B. a^* index = −80 to zero = green, from zero to +100 = red; C. b^* *index* = −100 to zero = blue, from zero to +70 = yellow. *** Comparison of time zero with the other evaluated times ($p < 0.001$).

3.5. Color stability of crude and encapsulated extracts containing carotenoids added to yogurt

Yogurt was chosen because it is a food with excellent nutritional value, being widely consumed by individuals of all ages, commonly with the addition of artificial dyes. When added to yogurt, the extract rich in carotenoids from Cantaloupe melon pulp gave yellow color, simulating the presence of yellow fruits like pineapple, passion fruit, peach, and mango (Fig. 3 – Supplementary material).

The yogurt added with carotenoids encapsulated in porcine gelatine (YEPG), presented stability in all index colors up to 60 days, when compared to time 0 (Fig. 6). This was not observed when only the crude extract containing carotenoids (YCE) was added, where the green color (a^* index) stability was lost at 30 days (Fig. 6A), and the yellow color (b^* index) stability lost from 15 days on (Fig. 6B). These results explain the maintainence of the L^* index in the yogurt added with carotenoids encapsulated in porcine gelatine (YEPG), which was not observed in the other tested conditions (Fig. 6C).

According to the labels of industrialized yellow fruit yogurts commercialized in Brazil, they receive the addition of artificial dyes, such as Sunset Yellow and Tartrazine, which are cheaper and more stable compared to carotenoids. However, several studies have related excessive consumption of these substances to short- and long-term health problems, such as allergies, asthma, childhood hyperactivity and even tumor development (Hamerski, Rezende, & Silva, 2013). Thus, it is extremely important to respect the acceptable daily intake (ADI) of these additives, in milligram per kilogram ($mg.kg^{-1}$) of the individual's weight. Of note, children may consume a large number of artificially colored industrialized foods and easily extrapolate the ADI, because of their lower weight compared to adults (Polônio & Peres, 2012). Besides, the negative health effects associated with the presence of increasingly critical consumers helped to increase the preference for foods containing a natural dyes (Hamerski et al., 2013).

In this study, adding carotenoids in its crude form to yogurt has proved that these molecules do not present color stability to conventional shelf-life of industrialized products. Important changes in the colorimetric parameters were observed, suggesting susceptibility of the Cantaloupe melon carotenoid-rich extract to the food matrix and storage conditions, especially at acidic pH (4.0), due to the observed changes for indexes a^* and b^* with marked loss of green and yellow staining, respectively. A reduction of the a^* index of 48% was observed for YCE between 0 and 60 days. Using EPG reduced this to only 4%. Regarding the b^* index, the YCE presented a high reduction (45%), whereas EPG totally prevented loss in this color parameter.

Therefore, for YEPG, staining was preserved over the 60 days of storage, showing that gelatine promoted carotenoids protection against adverse factors. In addition, EPG presented excellent solubility in yogurt, with homogeneous yellow coloration. This was not observed in the YCE, which presented loss of staining and unsolubilized orange dots throughout the sixty days of storage (Fig. 3 – Supplementary material).

4. Conclusion

The encapsulation using the emulsification technique, with porcine gelatin, was a promising strategy to promote the production of nanoparticles in order to minimize the problems related to the formulation, turning the carotenoids more dispersible in water, and easier to incorporate in a food matrix. Thus, the encapsulated extract rich in carotenoids of Cantaloupe melon pulp can be applied in yogurt to simulate the coloration of yellow fruit flavored yogurts, promoting homogeneity and high color stability compared to the addition of the crude extract. This can increase their functionality to human health and further studies should address this issue.

6. Declarations of interest

None.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.foodchem.2018.07.099.

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