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Emulsions Stabilized by Soy Protein Isolate Microgels: Encapsulation of β -Carotene and Incorporation into Yogurts

Diana Jimenez-Champi ¹, Matheus A. Chaves ², Juliano R. Sangalli ², Leticia S. Ferreira ¹,
Jéssica T. P. Silva ³ and Samantha C. Pinho ^{1,*}

- ¹ Laboratory of Encapsulation and Functional Foods (LEnAlis), Department of Food Engineering (ZEA), School of Animal Science and Food Engineering (FZEA), University of São Paulo (USP), Pirassununga 13635-900, SP, Brazil; djimenez@usp.br (D.J.-C.); leticia2.ferreira@usp.br (L.S.F.)
- ² Laboratory of Molecular Morphophysiology and Development (LMMD), Department of Veterinary Medicine, School of Animal Science and Food Engineering, University of São Paulo, Av. Duque de Caxias Norte, 225, Pirassununga 13635-900, SP, Brazil; matheus.chaves@usp.br (M.A.C.); julianors@usp.br (J.R.S.)
- ³ Laboratory of Microstructured Systems (LMS), Department of Food Engineering and Technology, Institute of Biosciences, Humanities and Exact Sciences (IBILCE), São Paulo State University (UNESP), Av. Cristóvão Colombo 2265, São José do Rio Preto 15054-000, SP, Brazil; jessica.tp.silva@unesp.br
- * Correspondence: samantha@usp.br

Abstract

Soy protein isolate (SPI) microgels were produced via heat-set gelation (4, 6, 8, and 10% by mass) followed by ultrasonication (400 W, 70% amplitude, 3 or 6 min) and used as stabilizers of oil–water emulsions (10% oil phase). The SPI concentration and ultrasonication time affected microgel size (236–356 nm) and polydispersity (0.253–0.550). The physical stability of the emulsions stabilized with 6 and 8% SPI microgels (6 min of ultrasonication) was evaluated for 14 d, influencing on the average size, creaming index and instability index of the emulsions, where those with 6% SPI microgels resulted in a major stability. The emulsions produced with these microgels encapsulated beta-carotene and were incorporated into whole yogurt at three concentrations: 5 (YE5), 10 (YE10), and 15% (YE15). The addition of the emulsions did not affect the physicochemical or microbiological quality of the yogurt. Rheological tests revealed that the yogurt behaved as a non-Newtonian and pseudoplastic fluid, with yogurts with more emulsions being less viscous. Sensory evaluation revealed consumer acceptance regarding color and texture; however, the perception of residual flavor was proportional to the amount of emulsion added. SPI microgels are effective stabilizers for β -carotene-loaded emulsions and a promising strategy for this compound delivery in yogurt.

Keywords: emulsion; microgel; beta-carotene; soy protein isolate



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1. Introduction

β -carotene is a lipophilic compound found as a natural pigment in several vegetables and fruits, with the highest vitamin A activity among other provitamin A carotenoids [1], and its consumption could help in the prevention of cardiovascular and cerebral illnesses and immunological development [2,3]. These properties make β -carotene a compound of great interest for the development of functional foods. However, it is poorly bioavailable from its native vegetable sources and is sensitive to various degradation factors, such as temperature, light, oxygen radicals, free radicals formed by lipid peroxidation, and transition metal cations [4]. Additionally, as β -carotene is lipophilic, it is difficult to incorporate into aqueous foods and presents low bioaccessibility after digestion. Owing to these

drawbacks, several efforts have been made to increase the incorporation, bioavailability, and shelf-life of this bioactive compound.

Microencapsulation approaches have been studied to improve the stability of beta-carotene during processing and storage, as well as to improve its bioaccessibility [5]. Lipid-based carriers are efficient for the encapsulation of highly lipophilic bioactive compounds and among the lipid-based microencapsulation systems, there are emulsions stabilized by nanoparticles or microparticles (Pickering emulsions) and emulsions stabilized by microgels. In these systems, colloidal particles adsorb at the oil/water interface, forming a thick viscoelastic coating around the droplets, protecting them from coalescence [6,7]. Food-grade emulsions are often produced with polymeric microgels as stabilizers, which are soft particles formed by a three-dimensional polymeric network that swells in aqueous solvents. Once they are adsorbed, in response to external stimuli (pH, temperature, ionic strength, and cosolvents), microgels can deform and stretch at the interface, reducing the interfacial tension and forming a strong barrier that enhances the stability of the emulsion [8–10].

Several authors have investigated the ability of plant-based protein microgels to stabilize emulsions [11–13]. Regarding microgels of soy proteins, Hou et al. [14] prepared SPI microgels (10 µm mean diameter) to be used to stabilize emulsions, and the oil encapsulation efficiency reached 99% when the oil content and SPI concentration were adjusted to 5% and 6%, respectively. In a study conducted by Yang et al. [15], SPI microgels were produced at various pH values (ranging from 3–9) with and without ultrasonication to stabilize emulsions. The authors reported that microgels at pH 9 with ultrasonication treatment presented high amphiphilicity at the oil–water interface and a superior ability to reduce interfacial tension.

More recent studies have reported that the combination of SPI with polysaccharides can further improve the stability of emulsions. Mao et al. [16] employed SPI microgels and xanthan gum to stabilize O/W emulsions; Yu et al. [17] prepared SPI microgels with sodium alginate (SA) to improve the water retention and sensory perception of low-salt pork gels; Bourouis et al. [18] incorporated SPI/chitosan (CS)-complexed microgels into soy yogurt and evaluated their structural and physicochemical properties.

Therefore, SPI microgels can effectively stabilize emulsions and allow them to act as carriers to encapsulate lipophilic bioactive compounds such as β-carotene. However, few studies have investigated the incorporation of this type of emulsion in food products. In the present study, the physicochemical properties of SPI microgels produced by ultrasonication and their potential to stabilize emulsions were evaluated. These β-carotene-loaded emulsions were then incorporated into yogurt, which was characterized physiochemically, microbiologically, and sensorially.

2. Materials and Methods

2.1. Material

SPI microgels (MSPIs) were produced via SPI (88% protein, according to the supplier) (Bremil, Arroio do Meio, RS, Brazil). Crystalline β-carotene was purchased from Sigma–Aldrich (St. Louis, MO, USA). The yogurt (Nestlé®, Araras, SP, Brazil) was purchased from a local market. The deionized water used throughout this work was obtained via a Direct-Q3 ultrapurification system (Millipore, Billerica, MA, USA). All chemicals used in this work were of analytical grade.

2.2. Production and Characterization of SPI Microgels (MSPI)

MSPI were produced at different protein concentrations (4% to 10%, *w/w*) with a pH 7.0, approximately. First, the SPI was dispersed in deionized water and magnetically stirred for 2 h at room temperature. The SPI dispersions were left at 4 °C overnight to ensure

complete hydration. Afterwards, the dispersions were heated to 90 °C for 30 min (MA 127, Marconi, Piracicaba, SP, Brazil) and immediately cooled to room temperature. The SPI gels were then subjected to ultrasonication (SFX550, Branson Ultrasonics Corporation, Danbury, CT, USA) via two processing times (3 and 6 min) at 400 W and a maximum amplitude of 70%. Table 1 shows the MSPI produced according to the processing conditions.

Table 1. Composition and preparation of each studied sample of SPI dispersion and MSPI.

Microgel	SPI Concentration (<i>w/v</i>) %	Ultrasonication Time (min)
MSPI4-3	4	3
MSPI4-6	4	6
MSPI6-3	6	3
MSPI6-6	6	6
MSPI8-3	8	3
MSPI8-6	8	6
MSPI10-3	10	3
MSPI10-6	10	6

2.2.1. Determination of the Zeta Potential, Polydispersity Index (PDI), and Average Diameter

The zeta potential, PDI, and average diameter were measured according to Silva et al. [19]. Samples were diluted 100× in deionized water and evaluated through dynamic light scattering, at 25 °C using a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK). The samples were analyzed in triplicate at 25 °C, using 1.45 as the refractive index for protein particles and 1.33 as the refractive index of the dispersant (water).

2.2.2. Protein-to-Microgel Conversion Factor

The MSPI dispersions were transferred to microtubes of 1.5 mL and centrifuged (5430 R, Eppendorf, Hamburg, Germany) (20,000× *g*, 30 min, 5 °C). The supernatant was collected, and the centrifugation process was repeated twice. The protein content in the supernatant was determined via the Dumas method with a nitrogen/protein analyzer (Leco, FP528, St. Joseph, MI, USA), with a protein conversion factor of 6.25. The results are expressed as the percentage of protein content in the supernatant in relation to the protein content in the SPI.

2.2.3. Quantification of Free Sulfhydryl (SHF) Groups

The free sulfhydryl group content was analyzed according to Jiang et al. [20] with modifications. One mL of the MSPI dispersion was diluted with 4 mL of purified water and centrifuged (5430 R, Eppendorf, Hamburg, Germany) at 7000× *g* for 15 min at 7 °C. An aliquot of 250 µL of the supernatant from each diluted sample was collected and mixed with 2.5 mL of phosphate buffer (0.1 M, pH 8). Then, 50 µL of Ellman's reagent (4 mg DTBN/mL phosphate buffer) was added to each sample, which was vortexed and left at room temperature for 15 min in the dark. Afterwards, the absorbance of each sample was measured using a spectrophotometer (Genesys 10S UV–Vis, Thermo Scientific, Waltham, MA, USA) at 412 nm. The results were evaluated against a standard curve of L-cysteine hydrochloride and expressed in µmol SH/g protein.

2.2.4. Determination of the Contact Angle

The samples of the SPI dispersions and MSPIs were carefully pipetted onto microscope slides and placed inside a desiccator for 24 h to evaporate the water, forming a thin layer on the slides. The contact angle was measured with an optical tensiometer (Attension Theta lite, KSV Instruments, Helsinki, Finland) equipped with image analysis OneAttension

software. The slides were positioned in the tensiometer, and a drop of deionized water (5 μ L) was deposited on the slide via a precision syringe. The experiment images were acquired 10 s after water was deposited on the microgel film [19].

2.2.5. Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS–PAGE)

A smooth small slab cell (Hoefer) was used with 12% acrylamide ($C = 2.6\%$ (w/w)) and slab gels (1.5 mm thickness). An amount of 500 mg of each sample was diluted in 5 mL of 1% SDS, 100 mM dithiothreitol, and 60 mM Tris HCl at pH 8.3. The diluted samples were homogenized (Polytron PT 1200, Kinematica) for 1 h and centrifuged for 20 min at 7800 rpm at 4 °C. An aliquot of 1 mL of the supernatant was collected to analyze the protein content via the Biuret method, and finally, the samples were normalized so that 3.3 micrograms of protein entered each well and diluted with buffer (125 mM Tris HCl (pH 6.8), 2.4% SDS, 50 mM DTT, 10% v/v glycerol, 0.5 mM EDTA) and bromophenol blue. Precision Plus Protein™ Dual Color standards (Bio-Rad, Cat. # 161–0374, Hercules, CA, USA) were used as ladder. A potential difference of 100 V was applied for 15 min, followed by 150 V for 1 h (max. 40 mA per gel), and then the gel was stained with Coomassie Brilliant Blue dye.

2.3. Production and Characterization of Emulsions Stabilized by SPI Microgels

The continuous phase was obtained by diluting MSPI dispersion to 1% protein content with phosphate buffer, while the disperse phase was soybean oil (10% v/v). The continuous phase and disperse phase were ultra-agitated (IKAT25, IKA, Staufen, Germany) at 9000 rpm for 3 min. The emulsions were transferred to glass tubes and stored under refrigeration for further analysis.

The production of emulsions encapsulating β -carotene included solubilization of the carotenoid in soybean oil [5]. β -Carotene was added at a concentration of 0.025% (w/w) to the soybean oil, previously heated to 50 °C. Afterwards, the mixture was vortexed (Heidolph Multi Reax, Schwabach, Germany) for 5 min and then placed in an ultrasonic bath for 5 min.

2.3.1. Determination of the Droplet Size Distribution

The emulsions were diluted with deionized water (1:1 v/v), and 40 μ L was transferred to glass slides and observed via a Leica DM500 microscope (Leica Microsystems, Heerbrugg, Switzerland) with Flexacam i5 at 40 $\times$ magnification. A set of images was acquired and processed with ImageJ 1.54 software (National Institutes of Health, Bethesda, MD, USA). Approximately 300 emulsion droplets were evaluated to obtain droplet size distribution curves.

2.3.2. Determination of Creaming Index (Ci)

Immediately after their production, the PEs were transferred to 25 mL tubes and stored at 4 °C. Cream height (H_s) and total height (H_t) were measured from 1–14 days of storage. The Ci was calculated via Equation (1):

$$Ci(\%) = \left(\frac{H_s}{H_t} \right) * 100, \quad (1)$$

2.3.3. Accelerated Stability Test

For the accelerated stability test, 0.4 mL of emulsion was placed in polycarbonate cells ($r = 130$ mm) and centrifuged at 4000 rpm for 60 min at 25 °C in a multisample analytical photocentrifuge (LUMiSizer, L.U.M. GmbH, Berlin, Germany). The instability index values were obtained with the software SepView version 4.1.

2.3.4. Morphology by Confocal Laser Scanning Microscopy (CLSM)

The morphology of the emulsions was observed via an inverted fluorescence microscope (Thunder Imager 3D Assay Leica, Leica, Wetzlar, Germany). An amount of 2.5 μL of Nile red dye solution and 5 μL of FITC were added to 1 mL of emulsion and vortexed for 30 s. Next, 20 μL of the dye-labelled emulsions were pipetted into the center of a glass-bottom dish and covered with a rounded coverslip. The slides were then observed at excitation wavelengths of 470 nm for FITC and 546 nm for Nile red.

2.4. Incorporation of β -Carotene-Loaded Emulsions in Yogurt and Characterization of Dairy Products

β -Carotene-loaded emulsions were added to the yogurt at 3 concentrations: 5, 10, and 15% (by mass, indicated as YE5, YE10, and YE15, respectively). After addition, the yogurt was stirred manually until complete homogenization. The preparation was performed within a laminar flow cabinet with previously sterilized materials to prevent microbial contamination.

2.4.1. Rheological Characterization of Yogurts

The viscosity and flow curves were determined via a rotational rheometer (AR-G2, TA Instruments, Crawley, West Sussex, England). The geometry applied was parallel plate type (60 mm, gap 800 μm). Two tests were performed for rheological analysis: a stationary test and an oscillatory test. For the stationary test, the samples were analyzed at 10 $^{\circ}\text{C}$, with a shear rate ranging from 100 to 0.01 1/s and a sample period of 10 s. The data obtained were fitted via the Herschel–Bulkley model presented in Equation (2):

$$\tau = \tau_0 + K * \gamma^n, \quad (2)$$

where τ_0 is the yield stress (Pa), k is the consistency coefficient (Pa s^n), γ is the strain rate (S^{-1}), and n is the flow behavior index (dimensionless). Oscillatory tests were also performed with a rotational rheometer to determine the storage modulus (G') and the loss modulus (G'') of the yogurt. For the frequency sweep analysis, a frequency ramp ranging from 0.1–10 Hz and a percentage strain of 0.01% was used.

2.4.2. Physicochemical Characterization of Yogurts

The yogurts were characterized in terms of pH, titratable acidity, instrumental colorimetry, and syneresis. The pH was monitored via a glass electrode pH meter. The titratable acidity was determined as described by Instituto Adolfo Lutz [21]. The degree of syneresis was determined as described by Brito-Oliveira et al. (2024) [22]. The instrumental color of the yogurt was determined via a colorimeter (Miniscan XE, Hunterlab, Reston, VA, USA) via the CIELAB color space, and the parameters L^* , a^* , b^* , chroma (C^*), and hue angle (h°) were obtained.

2.4.3. Determination of Lactic Acid Bacteria

Lactic acid bacteria (LAB) were counted via depth plating. Yogurt was diluted in peptone water (0.1% w/v) to 10%, resulting in a 10^{-1} dilution. Serial dilutions were made until a final dilution of 10^{-6} was reached. One milliliter of the 10^{-4} , 10^{-5} , and 10^{-6} dilutions was pipetted onto sterile Petri dishes, and then Man–Rogosa–Sharpe (MRS) agar was added as culture medium. After solidification, the Petri dishes were placed upside down in an incubator at 37 $^{\circ}\text{C}$ for 48 h.

2.4.4. Sensory Evaluation of Yogurts

The sensory evaluation protocol was approved by the Ethics Research Committee from the School of Animal Science and Food Engineering—University of São Paulo (code CAAE 78726624.7.0000.5422), with 120 untrained participants who first signed a term of consent. Each consumer was offered yogurt, which was placed in plastic cups and numbered randomly. Before consuming the yogurt, each consumer was asked to look at the sample and place which flavor he/she associated with according to the coloration. The yogurt was subsequently evaluated in terms of color and texture via a hedonic scale from 1–9 (1: “dislike extremely” and 9: “like extremely”). Finally, consumers were asked to say if they felt any aftertaste after tasting the yogurt and, if possible, to identify which one. All samples were microbiologically tested for coliform, mold, and yeast counts before sensory evaluation [23].

2.5. Statistical Analyses

The experiments were carried out in triplicate, and the results are reported as mean $\pm$ standard deviation. Data normality and lognormality were first assessed for all means analyzed using the Anderson–Darling and Shapiro–Wilk tests, both at a 5% significance level ($p < 0.05$), in GraphPad Prism 8.0 (v. 8.0.1.244, GraphPad Software, MA, USA). For the creaming index and instability index, comparisons between two means were performed using an unpaired t-test, with normality verified by the Shapiro–Wilk test. When three or more means were compared, variables meeting the assumptions of normality were analyzed by analysis of variance (ANOVA), followed by Tukey’s mean comparison test at a 5% significance level ($p < 0.05$). Variables that did not meet the normality criteria were analyzed using the nonparametric Kruskal–Wallis test, followed by Dunn’s multiple comparisons test at a 5% significance level. The statistical test applied in each case is indicated in the footnotes of the respective result tables. All analyses were performed using GraphPad Prism 8.0, Statistica v.12.0 (StatSoft, Hamburg, Germany), and RStudio 2025.05.1 (Posit Software, PBC, Boston, MA, USA). All graphs were constructed using Microsoft Excel® (Microsoft Corporation, Redmond, WA, USA).

3. Results and Discussion

3.1. Development of SPI Microgels (MSPIs): Characterization and Choice of Microgel for Emulsion Stabilization

3.1.1. Determination of Average Particle Size and Particle Size Distribution, Zeta Potential, and the Protein–Microgel Conversion Factor

The average particle size, polydispersity index (PDI), zeta potential, and microgel conversion factor are presented in Table 2.

The average particle size of the MSPI ranged from 236 to 374 nm. The greater the treatment time, the greater the energy input in the system, which influenced the mean particle size of the microgels, as in Table 2. Notably, the MSPIs produced with 3 min of ultrasonication presented greater mean particle sizes than did those produced with 6 min. According to Wang et al. [24], in the ultrasonication, the energy generated by cavitation could cause the microgels to experience intense agitation, resulting in a decrease in particle size. The results here obtained were consistent with those of Han et al. [25] in which SPI microgels produced in a similar way had average diameter of approximately 250 nm.

Regarding PDI values, MSPI6-6 and MSPI8-6 presented the lowest values (0.25 and 0.253, respectively), along with lower mean diameters. The ultrasonication had an effect not only on reducing the particle size but also on the size homogeneity of the MSPI, increasing the probability of success in stabilizing emulsions.

Table 2. Characterization of the MSPI dispersions: average particle size, polydispersity index, zeta potential, and conversion factor.

Microgel	Average Particle Size (nm)	PDI	Zeta Potential (mV)	Conversion Factor (%)
MSPI4-3	279 ± 2.17 ^e	0.345 ± 0.03 ^{cd}	−36.5 ± 0.29 ^a	50.63 ± 0.41 ^d
MSPI4-6	291 ± 3.37 ^d	0.393 ± 0.01 ^{bc}	−33.6 ± 0.62 ^b	47.22 ± 1.64 ^e
MSPI6-3	278 ± 3.64 ^e	0.314 ± 0.03 ^{de}	−32.5 ± 0.50 ^{bc}	56.02 ± 1.37 ^{bc}
MSPI6-6	236 ± 0.31 ^f	0.265 ± 0.01 ^e	−31.0 ± 0.44 ^d	50.94 ± 0.08 ^d
MSPI8-3	375 ± 2.43 ^a	0.550 ± 0.03 ^e	−30.3 ± 0.17 ^d	59.46 ± 0.91 ^a
MSPI8-6	236 ± 2.03 ^f	0.253 ± 0.01 ^a	−31.3 ± 1.0 ^{cd}	52.88 ± 0.35 ^{cd}
MSPI10-3	356 ± 3.50 ^b	0.413 ± 0.04 ^{bc}	−31.2 ± 0.30 ^{cd}	57.87 ± 0.34 ^{ab}
MSPI10-6	322 ± 5.93 ^c	0.416 ± 0.02 ^b	−31.1 ± 0.21 ^{cd}	55.23 ± 0.23 ^{bc}

The data are expressed as the means ± standard deviations. Different letters in the same column indicate significant difference according to one-way ANOVA and Tukey's post hoc test ($p < 0.05$).

As shown in Table 2, the zeta potential values ranged between −36.5 and −30.0 mV. For microgels, the electrostatic charges are related to the physical structure of the microgel since the compressibility of charged microgels is considered to be greater than that of uncharged microgels, so charged microgels are easier to compress and begin to interact effectively at the interface [26]. Therefore, ultrasonication forced protein unfolding, exposing more polar groups and increasing the residue charge on the surface of the SPI, increasing the probability of deformation and greater adsorption at the interface [24].

The conversion factors of SPI into MSPI were between 47.2% and 57.9%, with higher values for MSPI produced via ultrasonication for 3 min. The conversion factor varied with the concentration of proteins present in the dispersion, the yield ranged from 50.6 to 59.5% for those produced with 3 min of ultrasonication, and 47.2 to 55.2% for those with 6 min of ultrasonication. When protein concentrations are higher than a critical absorption concentration, the formation of aggregates is favored because the probability of collision between molecules (proteins-proteins and aggregates-aggregates) increases [27].

According to Iwabuchi et al. [28], heating above 70 °C causes structural changes in the β -conglycinin subunits into their constituent subunits, and the α and α' subunits form soluble aggregates. Hu et al. [29] obtained a conversion factor of 49.5% in an SPI dispersion with ultrasonication at 400 W for 15 min, concluding that ultrasound improved the conversion of insoluble precipitates in SPI into soluble protein aggregates. MSPIs produced with 6 min of ultrasonication had a lower conversion factor than those did with 3 min, possibly due to a degradation due to a longer exposure time.

3.1.2. Quantification of Free Sulfhydryl Groups

The quantification of free sulfhydryl groups (SHFs) indicates the extension of protein unfolding due to the breakage of disulfide bonds in the tertiary protein structure, which leads to increased exposure of such groups to aqueous media [30]. The SHFs of both the SPI dispersions and MSPI are shown in Table 3.

The SPI dispersions presented the lowest values of SHFs and similar to those obtained by Zheng et al. [31] and Cao et al. [32]. The major content of SHFs was found in the MSPIs, reflecting the unfolding of the tertiary structure of the SPI proteins, causing more breaking of internal disulfide bonds and exposure of SHFs by heating and ultrasonication. This is confirmed by Qin et al. [33], who reported that high-temperature heating causes disulfide bond breaking and, consequently, unfolding of the protein molecular structure and exposure of SHFs groups. Wang et al. [34] and Zou et al. [35] concluded that changes in SHFs content have significant effects on the structure and functional properties of proteins.

Table 3. Quantity of free sulfhydryl groups ($\mu\text{mol SH/g}$ protein) in the SPI dispersions and MSPIs.

Protein Concentration (%)	SPI Dispersion	MSPI-3 min	MSPI-6 min
4	3.2 ± 0.04 ^{cB}	11.36 ± 1.13 ^{aA}	12.38 ± 0.63 ^{aA}
6	3.0 ± 0.13 ^{bcA}	11.63 ± 0.25 ^{abB}	13.95 ± 0.59 ^{abC}
8	2.85 ± 0.13 ^{abB}	11.5 ± 1.00 ^{aA}	13.63 ± 1.44 ^{abA}
10	2.72 ± 0.09 ^{aB}	14.55 ± 1.74 ^{bA}	14.86 ± 0.45 ^{bA}

The data are expressed as the means $\pm$ standard deviations. Different lowercase letters in the same column and different uppercase letters in the same row indicate significant differences according to one-way ANOVA followed by Tukey's post hoc test ($p \leq 0.05$).

The number of SHFs groups in MSPI4, MSPI6, and MSPI8 were statistically similar, and only MSPI10 was different, indicating that the concentration of protein in the dispersion was not a primary factor in protein unfolding. Furthermore, there was no significant difference between the SHFs groups of the MSPIs after 3 and 6 min of ultrasonication, so it can be concluded that the unfolding of the tertiary structures of the SPI proteins and thus the exposure of the SHFs groups occurred mainly during the heating step.

3.1.3. Contact Angle

The contact angle reflects the wettability of a solid by a liquid, which is related to its adsorption at the interface. Hydrophilic particles (contact angle $\theta \sim 0^\circ$) stay in the water phase, whereas hydrophobic particles ($\theta \sim 180^\circ$) remain in the oil [19]. Thus, for emulsion stabilization, particles must present partial wettability by both water and oil phases to remain at the O/W interface.

For the SPI dispersions (Figure 1), the contact angle ranged from 33.4 to 47.4, decreasing from 0 to 10 s, and the water droplet was slightly absorbed by the surface formed on the proteins present in the SPI, indicating the affinity of the dispersion for water. The change in contact angle in the dispersions could be due to the irregular surface of the soy proteins, since SPI has different protein groups [36]. For MSPIs, the contact angle did not decrease as much as it did for the SPI dispersion; in contrast, the contact angle at 10 s remained similar to that at 0 s. Ultrasonication exposed hydrophobic groups on the surface of the microgels, preventing further absorption of the water droplet. The contact angles revealed that the microgels are more hydrophobic than the SPI dispersion is, but they still have a certain affinity for water.

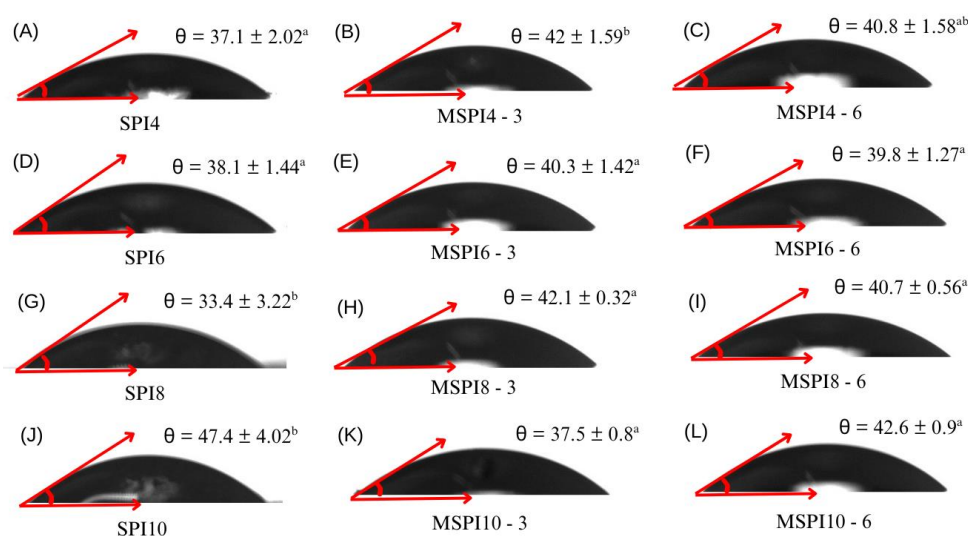


Figure 1. Contact angle in SPI dispersion and MSPI. The data are expressed as the means $\pm$ standard deviations. Different lowercase letters in the same row indicate significant differences according to one-way ANOVA followed by Tukey's post hoc test ($p \leq 0.05$).

3.1.4. Electrophoretic Patterns by Gel Electrophoresis (SDS–PAGE)

Figure 2 presents the SDS–PAGE profile of SPI and MSPI dispersions, which shows the structural changes in the SPI proteins after ultrasonication.

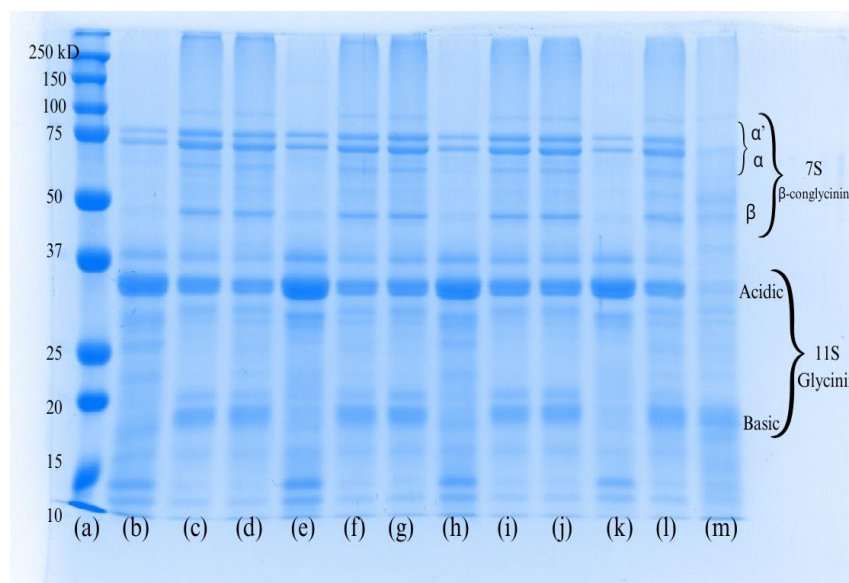


Figure 2. SDS–PAGE profile of the SPI dispersions and MSPI: (a) marker; (b) SPI4; (c) MSPI4-3; (d) MSPI4-6; (e) SPI6; (f) MSPI6-3; (g) MSPI6-6; (h) SPI8; (i) MSPI8-3; (j) MSPI8-6; (k) SPI10; (l) MSPI10-3; (m) MSPI10-6.

The SPI mainly consists of glycinin and β -conglycinin, which together represent more than 80% of the total proteins present in the SPI [36]. The glycinin (11S globulin) contains an acidic subunit (A) (32–40 kDa) and a basic subunit (B) (~20 kDa), and β -conglycinin (7S globulin) possesses three subunits: α' (~80 kDa), α (~70 kDa), and β (~50 kDa); these subunits are known for containing a series of polypeptide chains with diverse molecular weights [37].

As shown in Figure 2, the SDS–PAGE profiles of all the samples exhibited similar patterns, and protein bands of 15, 20, 35, 37, 50, and 75 kDa, characteristic of typical SPI bands, were observed in all the treatments. Among the glycinin bands, basic bands of approximately 20 kDa appeared in MSPI, and acidic bands of approximately 37 kDa remained similar to those of the SPI dispersions, indicating that more glycinin proteins were present in the MSPI dispersions than in the SPI dispersions. The β -conglycinin bands indicate the possible formation of ~50 kDa aggregates, represented by very thin lines. Finally, at the top of each MIPS band, it is possible to see a new band of higher mw, which represents some insoluble conjugated products formed by possible disulfide bonds formed during ultrasonication [37].

3.2. Characterization of MSPI-Stabilized Emulsions

As MSPI6-6 and MSPI8-6 presented lower diameter sizes and PDI values, they were selected to stabilize the emulsions, which were denoted as E6 and E8, respectively.

3.2.1. Average Particle Size and Physical Stability of MSPI-Stabilized Emulsions

Figure 3 shows optical micrographs of the emulsions during storage (14 days) and their respective droplet size distribution curves. The values of d_{10} , d_{50} and d_{90} which are the percentile values indicating the particle size below which 10%, 50%, and 90% of the total droplets in the emulsions and the Span value, calculated as $(D_{90} - D_{10})/D_{50}$, which measures the width of the distribution are shown in Appendix A.

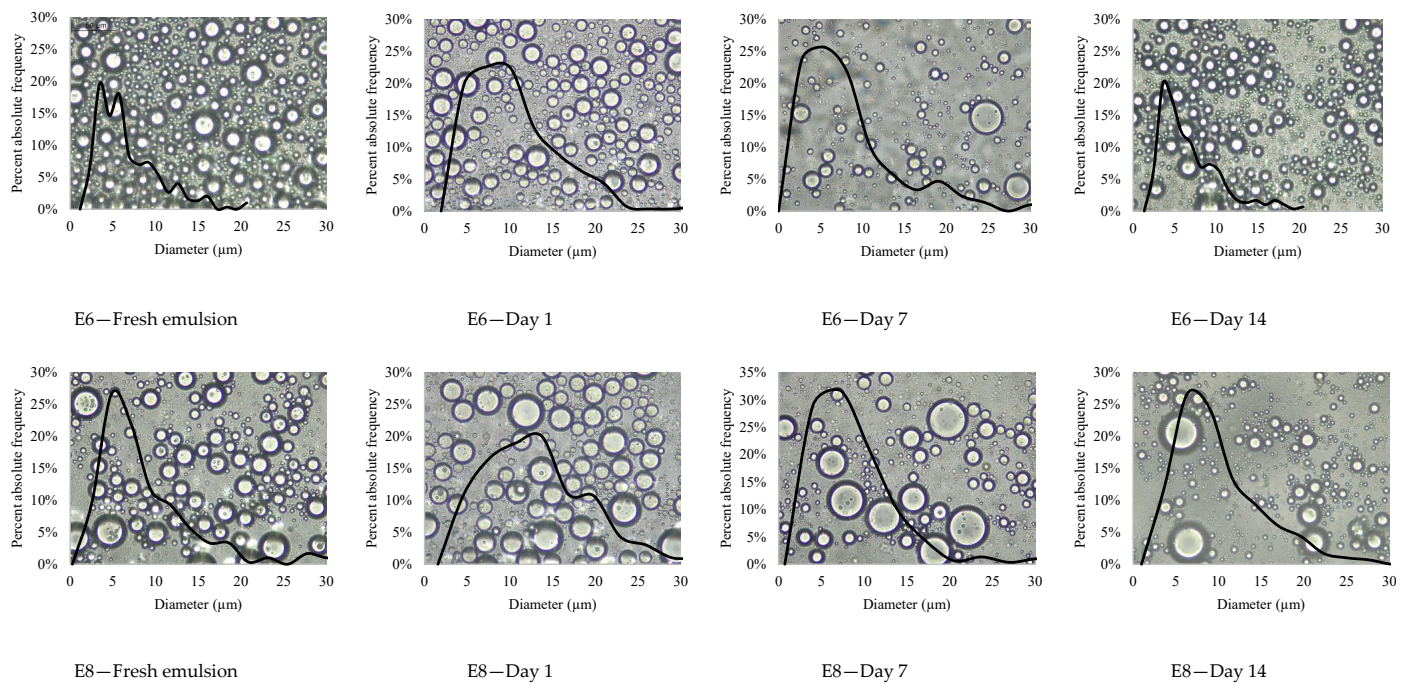


Figure 3. Micrographs of E6 and E8 with their respective droplet size distribution curves during storage.

For fresh emulsions on the 1st day of storage, the droplets in E8 were larger than those in E6, which can be corroborated with the curves in each picture, where for E6, the droplets reached a maximum diameter of 20 μm , whereas in E8, there were droplets up to 25 μm in diameter. On the 7th day, the diameter of the E8 droplets was greater than that of the E6 droplets, possibly due to coalescence. Finally, on the 14th day, few larger droplets can still be seen, indicating their destruction and the prevalence of only the smaller, microgel-covered droplets. According to Yang et al. [38], smaller droplets can result in denser packing and reduced fluidity, thereby increasing resistance to deformation. On the other hand, Dickinson [39] stated that in systems with an excess of particles present in the continuous phase, the interfacial structure can be transformed into a multilayer arrangement, with one or more layers of particles accommodated in the inner region of the droplets. Throughout storage, the droplets present in E8 remained larger than those present in E6. From day 7 onwards, the reduction in diameter occurred, with possible signs of the prevalence of smaller, more stabilized droplets that will remain in the separation of the oil/water phase.

Physical stability of the emulsions can also be evaluated by the creaming index (Ci) and instability index, which are shown in Table 4.

Table 4. Creamy indices (Ci) and instability indices of E6 and E8 during storage.

Index	Day	E6	E8
Creaming index	Fresh emulsion	6.38 ± 0.94 ^{aB}	6.69 ± 0.78 ^{aC}
	Day 1	15.15 ± 0.01 ^{aA}	16.50 ± 0.28 ^{bB}
	Day 7	14.06 ± 0.57 ^{aA}	15.15 ± 0.23 ^{bAB}
	Day 14	13.06 ± 1.62 ^{aA}	14.14 ± 0.69 ^{aA}
Instability index	Fresh emulsion	0.684 ± 0.01 ^a	0.652 ± 0.01 ^a

Different lowercase letters in the same row indicate a significant difference according to an unpaired t test ($p \leq 0.05$); different uppercase letters in the same column indicate a significant difference according to the Tukey test ($p \leq 0.05$).

As shown in Table 4, E6 and E8 had initial C_i values of 6.38 and 6.69, respectively, with no significant difference between them. The C_i increased with each day of storage, indicating the formation of creams in the emulsions; however, the C_i in E6 remained lower than that in E8, indicating better emulsion stabilization, which was related to E6 having a smaller droplet size and a more uniform distribution, resulting in less cream formation [40]. The creaming happens due to the upward movement of the relatively lower density oil globules in an aqueous phase, larger droplets floated due to gravitational separation, and eventually results in phase separation, forming the cream in the upper phase of the emulsion [41,42]. The droplet size has a significant effect on the gravitational separation of the emulsions. The larger the droplet size, the higher the creaming or sedimentation velocity [43]. Furthermore, Schröder et al. [44] reported that with high surface coverage, a dense layer of particles results in an effective barrier against droplet coalescence, resulting in physically stable emulsions.

On day 14, the C_i values decreased, possibly due to the separation of the phases (oil from water), which occurred on day 14 of storage. Ferreira et al. [45] stabilized emulsions with a thermally treated SPI dispersion (0.75 to 2.0%, w/v), obtaining C_i values between 12 and 18% after 10 days of storage, and observed that the oily phase separated from the aqueous phase. This is correlated with what was observed in the curves in Figure 3, where the droplets in E8 were larger than those in E6 and therefore had a higher C_i ; the smaller droplets had a lower buoyancy than did the larger droplets and could settle with more facilities at the bottom phase, resulting in less creaming [46].

Regarding instability index, it is inversely related to the centrifugal stability of emulsions: a lower instability index indicates better stability. This dimensionless number ranges from 0 to 1; the closer the value is to “1”, the more pronounced, or even complete, the phase separation in the emulsion [47]. The instability indices for E6 and E8 were 0.684 and 0.652, respectively, indicating no significant differences. They showed that over time, the microgels can stabilize the emulsion in a similar way, which is also corroborated by the C_i values, since from day 1, the C_i values in the emulsions were statistically similar.

3.2.2. Morphology of MSPI-Stabilized Emulsions

CLSM was used to observe the morphology of the emulsions stabilized by MSPI, as presented in Figure 4. The yellow drops represent the oil phase of the emulsions, whereas the green fluorescence observed on the droplet surface represented MSPI stain, which formed a coating on the oil–water interface surrounding the oil droplets. In E8, many droplets, with an average diameter of up to 20 μm , represented more than 30% of the total number of particles in the emulsion (Figure 3).

Wu et al. [48] reported that, in emulsions stabilized by the MSPI/chitosan complex, the oil droplets became more uniform as the particle content (MSPI) increased. Additionally, in PE8, the green fluorescence became denser, and this behavior was also observed by Zhang et al. [12] in emulsions stabilized by sea bass protein microgels, indicating that a certain amount of MSPI will surround the droplet and that the excess will form aggregates on their surface. The large amount of MSPI present at the O/W interface may be responsible for the formation of creams in the emulsions, increasing the C_i of E8 compared with that of E6.

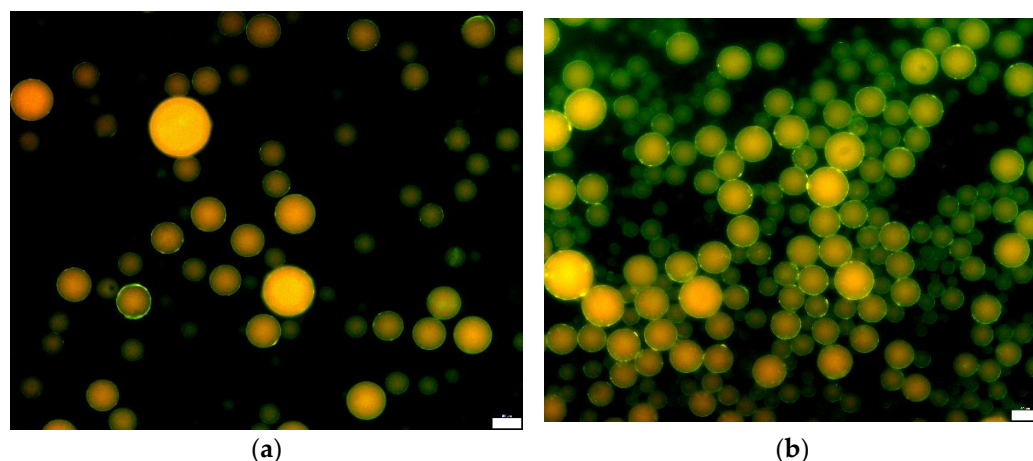


Figure 4. Micrographs obtained by confocal laser scanning microscopy for the emulsions: (a) E6; (b) E8.

3.3. Characterization of Beta-Carotene-Enriched Yogurts

Considering the previous results of the emulsion stability, E6 was selected for incorporation into the yogurt at three concentrations: 5, 10, and 15% (in mass, YE5, YE10, and YE15, respectively) and a nonincorporated sample, referred to as the “control”.

3.3.1. Physicochemical Characterization of Yogurt

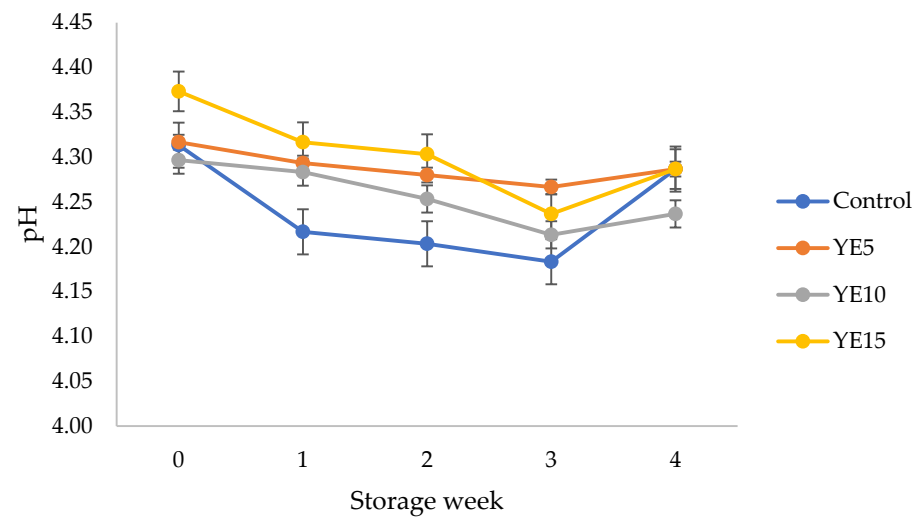
The results of the physicochemical analyses of the yogurt samples during 4 weeks of storage are shown in Figure 5.

The pH values were in the range of 4.18–4.37, in line with the Food and Drug Administration (FDA) specifications that determine a maximum pH value of 4.5 for yogurts. In each storage week, the control had the lowest pH values compared with those of the yogurt enriched with emulsions. This increase in the pH of enriched yogurts was due mainly to the inclusion of the emulsions. Nevertheless, the buffering capacity of the yogurt was sufficient to prevent noticeable changes in pH values.

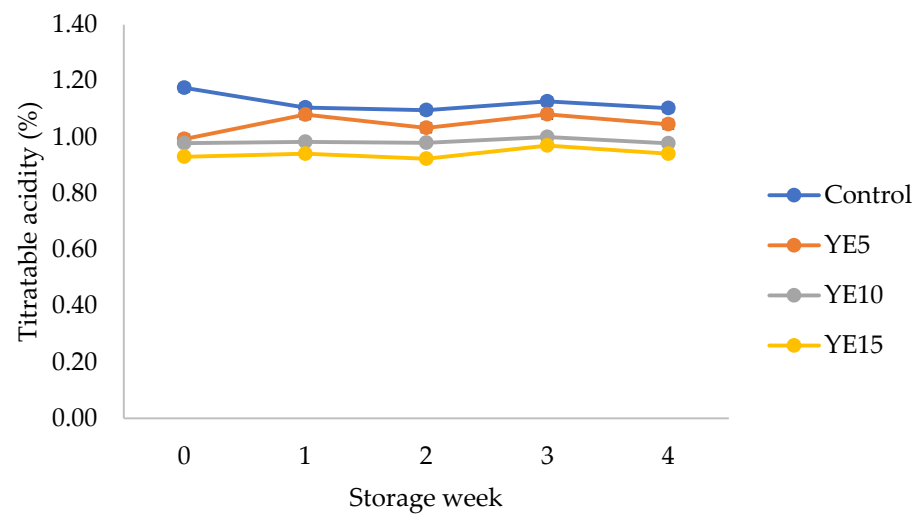
The titratable acidity tended to decrease during storage. The range of titratable acidity was between 0.92 and 1.18% (g lactic acid/100 g yogurt). The acidity values remained within the range of 0.6–1.5%, as indicated by Brazil [49].

As for syneresis, for YE5, the addition of MSPI may have contributed positively to the stability of the yogurt, preventing the loss of the aqueous phase. On the other hand, for YE10 and YE15, which had higher amounts of emulsion, the values were relatively high, indicating competition for stability between the yogurt proteins and the proteins present in MSPI, as more emulsion was added to these yogurt samples.

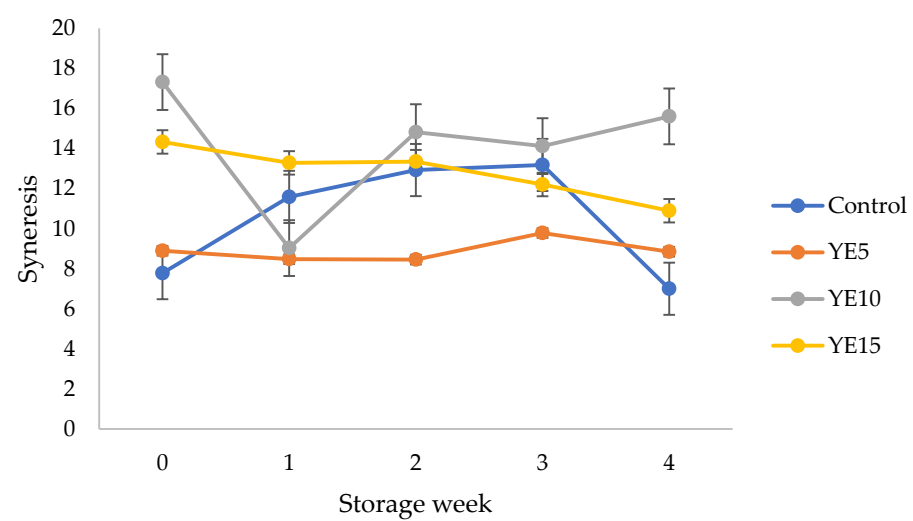
The colorimetric parameters of the yogurt samples during storage are shown in Table 5. The β -carotene gives the product an orange–red color, and it is responsible for the coloring of yogurt. There were small significant differences in the L^* values of each sample during storage, but in all the samples, the L^* was 88.20 or above, which means that the yogurt did not become darker and remained under the same light conditions until the last week. The positive b^* values indicate that the yogurt had yellow tones, which was explained by the addition of β -carotene. Moreover, as the addition of emulsions to the yogurt increased, the b^* value also increased, which was explained by the increase in β -carotene encapsulated by the emulsions.



(a)



(b)



(c)

Figure 5. Physicochemical characterization of the pH (a), titratable acidity (b), and syneresis (c) of YPEs and the control during 4 weeks of storage.

Table 5. Color parameters L*, a*, b* chroma, and hue° for the control sample and YEs during storage.

Sample	Parameter	Storage Week				
		0	1	2	3	4
Control	L*	90.89 ± 0.37 ^{ab}	90.31 ± 1.51 ^a	91.17 ± 0.84 ^{ab}	92.39 ± 1.15 ^{ab}	92.95 ± 0.16 ^b
	a*	−3.33 ± 0.04 ^b	−3.46 ± 0.08 ^{ab}	−3.49 ± 0.03 ^a	−3.65 ± 0.08 ^c	−3.49 ± 0.05 ^a
	b*	3.12 ± 0.03 ^b	3.96 ± 0.26 ^{ab}	3.60 ± 0.32 ^{ab}	4.26 ± 0.54 ^a	4.21 ± 0.26 ^a
	C*	4.56 ± 0.05 ^b	5.26 ± 0.23 ^a	5.02 ± 0.24 ^{ab}	5.61 ± 0.42 ^a	5.47 ± 0.18 ^a
	Hue°	136.83 ± 0.18 ^b	131.14 ± 1.61 ^{ab}	134.13 ± 2.46 ^{ab}	130.74 ± 3.60 ^{ab}	129.65 ± 2.01 ^a
YE5	L*	89.38 ± 1.26 ^a	89.79 ± 1.30 ^a	92.75 ± 0.67 ^a	92.180 ± 2.18 ^a	92.99 ± 0.91 ^a
	a*	−3.22 ± 0.17 ^b	−3.30 ± 0.11 ^{ab}	−3.51 ± 0.09 ^{ab}	−3.57 ± 0.16 ^a	−3.55 ± 0.07 ^{ab}
	b*	3.54 ± 0.80 ^b	4.11 ± 0.14 ^{ab}	4.43 ± 0.32 ^{ab}	4.85 ± 0.35 ^a	5.20 ± 0.17 ^a
	C*	4.79 ± 0.72 ^b	5.27 ± 0.17 ^{ab}	5.65 ± 0.30 ^{ab}	6.02 ± 0.37 ^a	6.29 ± 0.12 ^a
	Hue°	132.75 ± 4.75 ^b	128.75 ± 0.41 ^{ab}	128.43 ± 1.36 ^{ab}	126.47 ± 1.04 ^a	124.34 ± 1.24 ^a
YE10	L*	88.20 ± 1.13 ^b	90.03 ± 0.41 ^{ab}	91.28 ± 0.93 ^a	91.00 ± 0.84 ^a	91.73 ± 1.48 ^a
	a*	−3.27 ± 0.09 ^a	−3.68 ± 0.59 ^a	−3.42 ± 0.11 ^a	−3.54 ± 0.07 ^a	−3.57 ± 0.08 ^a
	b*	4.23 ± 0.09 ^b	4.84 ± 0.33 ^{bc}	5.30 ± 0.48 ^{ac}	5.67 ± 0.19 ^a	5.85 ± 0.23 ^a
	C*	5.34 ± 0.13 ^b	6.08 ± 0.55 ^{ab}	6.31 ± 0.46 ^a	6.69 ± 0.19 ^a	6.86 ± 0.23 ^a
	Hue°	127.67 ± 0.19 ^b	127.11 ± 3.54 ^b	122.93 ± 1.76 ^{ab}	121.98 ± 0.55 ^a	121.36 ± 0.52 ^a
YE15	L*	91.48 ± 1.80 ^a	88.95 ± 1.78 ^a	91.51 ± 0.52 ^a	91.91 ± 0.89 ^a	91.87 ± 1.70 ^a
	a*	−3.58 ± 0.12 ^a	−3.28 ± 0.14 ^b	−3.51 ± 0.06 ^{ab}	−3.62 ± 0.07 ^a	−3.53 ± 0.07 ^{ab}
	b*	5.79 ± 0.50 ^{ab}	5.19 ± 0.82 ^b	6.07 ± 0.23 ^{ab}	6.52 ± 0.05 ^a	6.88 ± 0.37 ^a
	C*	6.81 ± 0.48 ^{ab}	6.14 ± 0.73 ^b	7.02 ± 0.23 ^{ab}	7.46 ± 0.06 ^a	7.73 ± 0.34 ^a
	Hue°	121.80 ± 1.43 ^{ab}	122.58 ± 3.73 ^b	120.03 ± 0.58 ^{ab}	119.00 ± 0.45 ^{ab}	117.22 ± 1.16 ^a

The data are expressed as the means ± standard deviations. Different lowercase letters in the same row indicate significant differences according to one-way ANOVA followed by Tukey's post hoc test ($p \leq 0.05$).

3.3.2. Microbiological Characterization of Yogurt

Microbiological stability was measured by counting lactic acid bacteria (LAB) during each week of storage. The counts are shown in Table 6.

Table 6. Microbiological characterization of yogurt samples during storage (UFC/g).

Sample	Storage Week				
	0	1	2	3	4
Control	4×10^7	2.4×10^7	2.5×10^7	1.9×10^7	1.4×10^7
YE5	3.7×10^7	2×10^7	2.8×10^7	3.2×10^7	1.9×10^7
YE10	4.1×10^7	3.7×10^7	3.9×10^7	3.6×10^7	3.2×10^7
YE15	3.4×10^7	3.0×10^7	2.9×10^7	3.0×10^7	2.9×10^7

Table 6 shows that there was a reduction in microorganisms up to the 4th week of storage, but LAB count for the four samples would comply with the requirements of Brazilian legislation [49], which states that the count must be greater than 10^7 UFC/g. This is a strong indication that the addition of emulsions with encapsulated β -carotene did not affect the microbiological quality of the product.

3.3.3. Rheological Characterization

The rheological parameters and flow curves were studied to observe the effects of incorporating emulsions into the yogurt on the rheological characteristics, reflected in Table 7 where τ_0 is the yield stress (Pa); k is the consistency coefficient (Pa.s^{*n*}); and n is the flow behavior index. The Herschel–Bulkley model fit well with the results obtained, better representing the rheological behavior of yogurt since it considers and quantifies the initial shear stress, which is related to the internal structure of the product [22].

Table 7. Rheological parameters of the yogurt's samples.

Rheological Parameters	Control	YE5	YE10	YE15
τ_0	0.33 ± 0.07^a	0.24 ± 0.23^a	0.09 ± 0.03^a	0.35 ± 0.01^a
n	0.52 ± 0.03^a	0.41 ± 0.11^a	0.51 ± 0.03^a	0.29 ± 0.01^a
k (Pa.s ^{<i>n</i>})	3.21 ± 0.20^b	1.09 ± 0.60^a	0.76 ± 0.10^a	1.56 ± 0.10^a

The data are expressed as the means $\pm$ standard deviations. Different lowercase letters in the same row indicate significant differences according to one-way ANOVA followed by Tukey's post hoc test ($p \leq 0.05$).

The yield stress (τ_0) is defined as the value of shear stress above which the material starts to flow. Thus, it is a measure of the interactions between the interactive structures in the yogurt. Table 7 shows that τ_0 was not significantly different between the control and the yogurt with emulsions, indicating that MSPI with β -carotene did not alter the initial resistance of the yogurt to flow. The possible variations between the τ_0 values could be related with some type of interaction between the milk proteins and the added microgels in the emulsions [50].

The value of k (the consistency coefficient) is related to the viscosity of the sample. In YE5, YE10, and YE15, k was lower than that of the control but statistically similar, which was related to a lower viscosity due to the incorporated emulsions. According to Molina et al. [51], the addition of water reflects lower k values, affecting the rheology of yogurt.

Finally, the n values were less than 1, confirming that the samples were non-Newtonian fluids with pseudoplastic behavior; i.e., the apparent viscosity of the systems decreased as the shear rate increased because of the deformation and/or disintegration of the protein network and microgels as the shear rate increased. Bourouis et al. [18] explained that the reduction in viscosity is caused by the deformation of the protein network and microgels as the shear rate increases.

The flow curve in Figure 6a shows that the enriched yogurt samples exhibited weaker network structures than the control samples did, since higher shear stress values (PAs) were obtained when higher strain rates (1/s) were applied [22], which was correlated with the k values found; these values were lower for YE5, YE10, and YE15 than for the control, which reduced the shear stress (Pa) as the strain rate (1/s) increased.

At the same time, dynamic oscillatory tests were carried out on the yogurt to determine the elastic modulus (G') and viscous modulus (G'') and observe the effects on the viscoelastic properties. First, the G' and G'' moduli were more pronounced in the YE5, YE10 and YE15 yogurt samples than in the control, indicating the yogurt samples with emulsions had greater viscoelasticity. This result is characteristic of weak gels, which are typical for yogurt. In general, the G' values were greater than the G'' values, which could be attributed to the ability of MSPIs to interact with yogurt proteins and strengthen the network structure [52]. Likewise, the frequency dependence of the G' and G'' values of all the samples followed similar trends, with a slight increase in the moduli with increasing frequency. This type of dependence of both modules is consistent with a network structure maintained by physical cross-links [22].

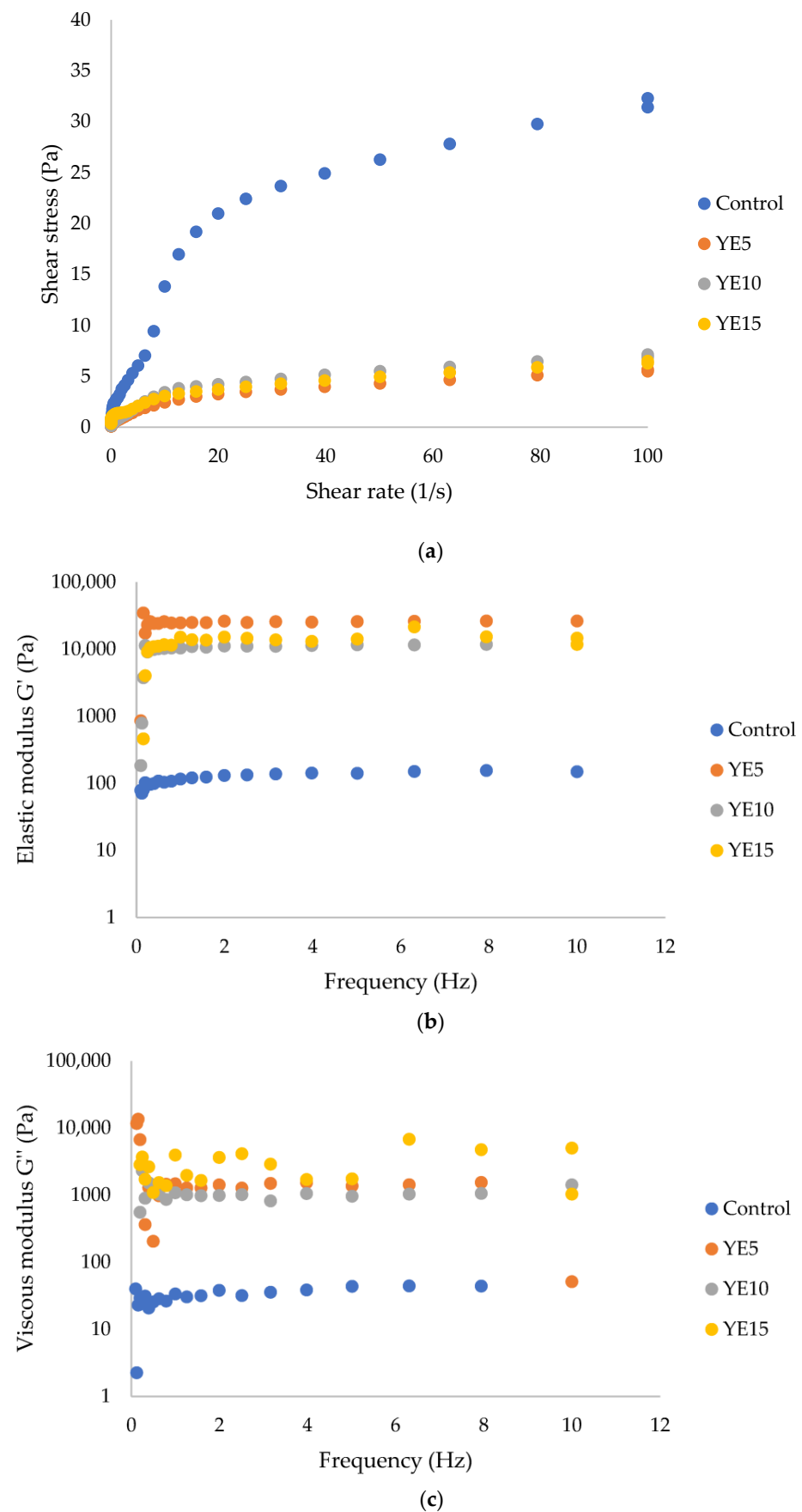


Figure 6. Flow curves obtained at 10 °C for the control and PE-enriched yogurt: (a) mechanical spectra of the elastic (G') (b) and viscous (G'') (c) moduli of yogurt.

3.3.4. Sensory Evaluation

The results for the acceptance of the yogurt in relation to the color and texture attributes and the response to the presence of aftertaste in the yogurt are shown in Table 8.

Table 8. Average scores for the color and texture attributes of the yogurt.

Yogurt	Color	Texture	Percentage of Residual Flavor Perception (%)
YE5	7.47 ± 1.58 ^a	7.69 ± 1.60 ^a	25.6
YE10	7.64 ± 1.36 ^a	7.64 ± 1.58 ^a	31.2
YE15	7.06 ± 1.47 ^b	6.98 ± 1.89 ^b	44.8

The data are expressed as the means ± standard deviations. Different lowercase letters in the same column indicate significant differences according to the Kruskal–Wallis test followed by Dunn’s multiple comparisons test ($p \leq 0.05$).

In general, the consumer gave to YE10 a higher score of 7.64, which is “I like it moderately” but not significantly higher than YE5. On the other hand, YE15 received significantly lower color scores than the other two. The averages obtained for this attribute were greater than 7, which is “I like it moderately”, and consumers accepted YE5 and YE10 in the same way with exception of YE15. The color attribute was critical for this sensory analysis since β -carotene was the main coloring agent for the yogurt. This is possibly due to the concentration of the encapsulated β -carotene, which was low; it is likely that larger quantities of β -carotene could be more easily visualized and differentiated between the samples.

On the other hand, consumers perceived differences in the texture attribute, with YE5 and YE10 scoring 7.69 and 7.64, respectively, which is “moderately liked”, whereas YE15 scored 6.98, very close to 7 “moderately liked”. However, the consumer acceptance of YE5 and YE10 was greater than that of YE15, which may be related to the perception of the usual consumption of yogurt, since YE15 was more aqueous due to the higher concentration of PE added, which made it more liquid than commercial PE, influencing consumer evaluation.

Finally, the number of panelists who perceived an aftertaste in the YE15 sample was the highest (45%). This perception was probably due to the greater amount of emulsion added, as the amount of oil was higher.

4. Conclusions

The data obtained demonstrated the viability of developing soy protein microgels with the capacity to stabilize β -carotene-loaded emulsions, which were used to incorporate this bioactivity into yogurt. Ultrasonication converted SPI proteins into microgels with efficacy, creating microstructures capable of adsorbing at the oil/water interface. Confocal laser microscopy revealed that the microgels surrounded the oil droplets, protecting them and preventing phase separation. β -Carotene was encapsulated in the emulsion and incorporated into the yogurt, and the incorporation of microgel-stabilized emulsions did not substantially affect the physicochemical and microbiological characteristics of the yogurt. The yogurt was accepted sensorially; however, the perception of oil aftertaste was proportional to the amount of emulsion added. Therefore, the data obtained in this study indicate that the use of soy proteins as microgels is an alternative for stabilizing emulsions and incorporating highly hydrophobic bioactive substances via a scalable process, facilitating the development of functional foods.

Author Contributions: D.J.-C.: Writing—review and editing, writing—original draft, methodology, investigation, formal analysis, data curation, conceptualization. M.A.C.: Methodology, investi-

gation. J.R.S.: Methodology, investigation. L.S.F.: Investigation. J.T.P.S.: Writing—review and editing, Writing—original draft, supervision, formal analysis, data curation, conceptualization. S.C.P.: Writing—review and editing, writing—original draft, supervision, project administration, funding acquisition, formal analysis, conceptualization. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

SPI	Soy protein isolate
MSPI	Microgel of soy protein isolate
E6	Emulsion stabilized with MSPI6
E8	Emulsion stabilized with MSPI8
YE5	Yogurt with 5% added emulsion
YE10	Yogurt with 10% added emulsion
YE15	Yogurt with 15% added emulsion
PDI	Polydispersity index
SHF	Free sulfhydryl
Ci	Creaming index

Appendix A

Table A1. Particle size distribution in terms of d10, d50, d90, and span of E6 and E8 during the storage.

Emulsion	Storage Day	D10 (µm)	D50 (µm)	D90 (µm)	Span
E6	Fresh emulsion	3.08	5.75	12.06	1.56
	Day 1	3.01	9.62	18.28	1.59
	Day 7	1.70	6.91	17.77	2.33
	Day 14	3.24	5.85	11.79	1.46
E8	Fresh emulsion	3.70	7.41	20.88	2.32
	Day 1	5.58	12.74	22.54	1.33
	Day 7	1.94	7.82	14.97	1.46
	Day 14	4.16	8.91	17.60	1.51

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