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HIBISCO (*HIBISCUS SABDARIFFA L.*): EXTRAÇÃO POR TERMOSONICAÇÃO SEGUIDA DE ENCAPSULAÇÃO DE ALGINATO-CA^{2+} PARA OBTENÇÃO DE CORANTE NATURAL

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Maringá

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HIBISCO (*HIBISCUS SABDARIFFA L.*): THERMOSONICATION EXTRACTION FOLLOWED BY ALGINATE-CA^{2+} ENCAPSULATION FOR NATURAL FOOD DYE OBTENTION

Dissertação apresentada ao programa de Pós Graduação em Ciência de Alimentos da Universidade Estadual de Maringá, como parte dos requisitos paraobtenção do título de mestre em Ciência de Alimentos

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BIOGRAFIA

Thainá Rodrigues Stella, nasceu em 11 de outubro de 1994 na cidade de Birigui, São Paulo.

Possui graduação em Engenharia de Alimentos pela Universidade Estadual de Maringá (UEM).

Tem experiência nas áreas de tecnologia e ciências de alimentos atuando principalmente nos seguintes temas: extração de compostos bioativos e análises antioxidantes.

Dedico

À todas as pessoas que são importantes na minha vida e que de alguma forma contribuirám para que eu conseguisse conquistar o título de mestra.

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Aos demais colegas de profissão

APRESENTAÇÃO

Esta dissertação de mestrado está apresentada na forma de um artigo científico

1 Thainá Rodrigues Stella, Carolina Moser Paraíso, Jessica dos Santos Pizzo, Jesui Vergilio Visentainer e Grasiele Scaramal Madrona,. Hibisco (*Hibiscus Sabdariffa L.*): thermosonication extraction followed by alginate-Ca^{2+} encapsulation for natural food dye obtention. Food Packaging and Shelf Life.

GENERAL ABSTRACT

INTRODUCTION.

In natural products, one of the main responsible for coloring is part of the flavonoid class, with emphasis on the anthocyanidins group. These pigments are intended for providing food coloring. Hibiscus calyxes are endowed with bioactive compounds, such as anthocyanins and phenolic compounds, which are responsible for their antioxidant properties. In this context, the replacement of synthetic dyes by natural ones has been gaining notoriety. Ultrasound is an extraction technique known as ecologically correct, cheap, fast and efficient for the extraction of phenolic compounds. Along with this fact, the encapsulation technique has also stood out in the case of natural dyes.

AIMS.

This work aims to develop a natural dye of hibiscus (*Hibiscus Sabdariffa L.*) through its aqueous extract (using ultrasound and conventional extraction) and subsequent lyophilization and encapsulation by ionic gelation. The kinetics of degradation of this dye and application in food matrix were also evaluated.

MATERIAL AND METHODS.

After being dried and crushed, the hibiscus calyxes were subjected to and phenolics were also calculated. A yogurt formulation with the addition of 1% natural hibiscus dye was subjected to analysis of rheology, color, pH, moisture, anthocyanins, phenolic compounds, flavonoids and antioxidant activity (DDPH, FRAP, ABTS). All analyses were performed in triplicate and submitted to analysis of variance and Tukey's test ($p<0.05$) for the least significant difference between means using the statistical program STATISTICA version 7.0.

RESULTS AND DISCUSSION.

Thermosonification worked effectively in extracting anthocyanins and phenolic compounds from hibiscus extracts, the antioxidant activity of the thermosified extract was higher ($p<0.05$) than that of the conventional extract, in all evaluated methods (FRAP, DPPH and ABTS), being increased by 1.16, 1.27 and 1.12 times. Regarding the color, it was observed that the samples tended towards the red color. After the lyophilization process, the same behavior is observed, the antioxidant activity was higher (1.42-fold for flavonoids, 1.07-fold for phenolic compounds, 1.48-fold for FRAP and 1.14-fold for ABTS) for the sample that has been thermosonified and lyophilized. There is even greater extraction of compounds such as quercetin and rutin, being respectively 2.67 and 1.22 times higher for thermosonified samples. In the extraction process followed by encapsulation, the powders obtained after thermosinification (TE) had a higher content of phenolic compounds (1.05 times) and gallic acid (1.23 times) when compared to the conventional process (CE). Capsules in general have a lower value probably due to the fact that the extract is dispersed within the wall material, protecting it from its direct and complete dispersion. The encapsulation efficiency was 75.94%

in the thermosonified extraction and 77.44% in the conventional one. Regarding the stability test, it is noted that light negatively affected phenolic compounds and anthocyanins during storage, with greater stability being observed in samples stored without light. The half-life was shorter for all samples submitted to light, as there was a greater degradation of phenolic compounds and anthocyanins. The lyophilized powders showed a variation of ΔE between 1.78 and 8.43. When comparing the extraction techniques, the thermosonified samples showed greater stability of ΔE, (1.78 for the powder and 2.0 for the capsule) and better half-life. In summary, the encapsulated samples showed longer half-life and lower color variations (ΔE) and, therefore, were chosen for application in food matrix. When applied to yogurt, the EC sample (freeze-dried encapsulated conventional hibiscus extract) showed an antioxidant content (ABTS) 1.09 times higher than the conventional sample. There was no significant difference for moisture, pH and rheology, which shows that the extraction techniques followed by lyophilization did not influence these characteristics evaluated. Regarding color, the encapsulated thermosonified sample (TE) was darker (lower luminosity) and with a tendency to red, while CE showed higher luminosity (L*), greater chromaticity (C), with values tending to yellow.

CONCLUSIONS.

The thermosonified extract showed better results for bioactive compounds, antioxidant activity and half-life values between 38.23 and 376.23 days. The presence of light negatively affected the samples. And when added to yogurt, the encapsulated thermosonified (TE) sample was darker (lower brightness value) and with a greater tendency to red, being potentially recommended for use as a natural food coloring. It was concluded that the extraction method using the solvent as water is ecologically correct and viable to use followed by encapsulation by ionic gelation to obtain natural hibiscus dye.

Key words: *Hibiscus sabidariffa*, natural dye, stability, thermosonification, encapsulation.

RESUMO GERAL

INTRODUÇÃO.

Em produtos naturais, um dos principais grupos responsáveis pela coloração pertence à classe dos flavonoides, com destaque para o grupo das antocianidinas. Esses pigmentos tem a finalidade de proporcionar coloração do alimento. Os cálices do Hibisco são dotados de compostos bioativos, como antocianinas e compostos fenólicos, que são responsáveis por suas propriedades antioxidantes. Diante desse contexto, a substituição de corantes sintéticos por naturais vem ganhando notoriedade. O ultrassom é uma técnica de extração conhecida como ecologicamente correta, barata, rápida e eficiente para extração de compostos fenólicos. Aliado a este fato a técnica de encapsulação também tem se destacado no caso de corantes naturais.

OBJETIVOS.

Este trabalho tem como objetivo desenvolver um corante natural de hibisco (*Hibiscus Sabdariffa L.*) por meio do seu extrato aquoso (com aplicação de ultrassom e extração convencional) e posterior liofilização e encapsulação por gelificação iônica. Avaliou-se ainda a cinética de degradação deste corante e aplicação em matriz alimentícia.

MATERIAL E METODOS.

Os cálices de hibiscos após serem secos e triturados foram submetidas à termossonificação e extração convencional, seguidos pela liofilização e encapsulação por gelificação iônica. Foram obtidos e avaliados, os extratos, os pós liofilizados e as cápsulas liofilizadas. Analisou-se nos pós obtidos umidade, pH e cor, determinação dos fenólicos totais, teor total de flavonoides e antocianinas, e atividade antioxidante (DPPH, FRAP e ABTS). O UPLC-MS / MS foi usado para identificar os compostos bioativos. Os pós obtidos foram avaliados quanto a sua estabilidade por 40 dias e armazenados à 25ºC com e sem luz. As variações de cor (ΔE), as constantes de taxa de reação de primeira ordem (k) e meias-vida (t1/2), de degradação de antocianinas e fenólicos também foram calculadas. Uma formulação de

iogurte com adição de 1% de corante natural de hibisco foi submetida à análise de reologia, cor, pH, umidade, antocianinas, compostos fenólicos, flavonoides e atividade antioxidante (DDPH, FRAP, ABTS). Todas as análises foram realizadas em triplicata e submetidas à análise de variância e teste de Tukey ($p<0,05$) para a diferença menos significativa entre as médias utilizando o programa estatístico STATISTICA versão 7.0.

RESULTADOS E DISCUSSÃO.

A termossonificação foi eficaz na extração de antocianinas e compostos fenólicos dos extratos de hibisco, onde a atividade antioxidante do extrato termossificado foi maior ($p<0,05$) que a do extrato convencional, em todos os métodos avaliados (FRAP, DPPH e ABTS), sendo aumentada em 1,16, 1,27 e 1,12 vezes. Em relação à cor, observou-se que as amostras tenderam para a cor vermelha. Após o processo de liofilização, o mesmo comportamento é observado, onde a atividade antioxidante foi maior (1,42 vezes para flavonoides, 1,07 vezes para compostos fenólicos, 1,48 vezes para o FRAP e 1,14 vezes para ABTS) para a amostra que foi termossonificada e liofilizada. Destaca-se ainda maior extração de compostos como quercetina e rutina, sendo respectivamente 2,67 e 1,22 vezes maior para amostra termossonificadas. No processo de extração seguido de encapsulação, os pós obtidos após termossinificação (TE) apresentaram maior teor de compostos fenólicos (1,05 vezes) e ácido gálico (1,23 vezes) quando comparados ao processo convencional (CE). As cápsulas no geral possuem um menor valor provavelmente pelo fato de que o extrato está disperso dentro do material de parede, protegendo-o de sua dispersão direta e completa. A eficiência de encapsulação foi de 75,94% na extração termossonificada e 77,44% na convencional. Em relação ao teste de estabilidade, nota-se que a luz afetou negativamente compostos fenólicos e antocianinas ao longo do armazenamento, sendo observada maior estabilidade nas amostras armazenadas sem luz. O tempo de meia vida foi menor para todas as amostras submetidas à luz, pois ocorreu uma degradação maior de compostos fenólicos e de antocianinas. Os pós liofilizados apresentaram variação de ΔE entre 1,78 e 8,43. Ao comparar as técnicas de extração, as

amostras termossonificadas apresentaram maior estabilidade de ΔE, (1,78 para o pó e 2,0 para a cápsula) e melhor tempo de meia vida. Em resumo, as amostras encapsuladas apresentaram maiores tempos de meia vida e menores variações de cor (ΔE) e, portanto, foram escolhidas para aplicação em matriz alimentícia. Ao aplicar em iogurte, a amostra CE (extrato de hibisco convencional encapsulado liofilizado), apresentou teor de antioxidante (ABTS) 1,09 vezes maior que a amostra convencional. Não houve diferença significativa para umidade, pH e reologia, o que mostra que as técnicas de extração seguida da liofilização não influenciaram nessas características avaliadas. Com relação à cor, a amostra encapsulada termossonificada (TE) se apresentou mais escura (menor luminosidade) e com tendência ao vermelho, enquanto CE apresentou maior luminosidade (L*), maior cromaticidade (C), com valores tendendo ao amarelo.

CONCLUSÕES.

O extrato termossonificado apresentou melhores resultados para compostos bioativos, atividade antioxidante e valores de tempo de meia vida entre 38,23 e 376,23 dias. A presença de luz afetou negativamente as amostras. E quando adicionadas em iogurte, a amostra encapsulada termosonificada (TE) mostrou-se mais escura (menor valor de brilho) e com maior tendência ao vermelho, sendo potencialmente recomendada para uso como corante alimentar natural. Concluiu-se que o método de extração utilizando o solvente como água é ecologicamente correto e viável de usar seguido do encapsulamento por gelificação iônica para obter corante natural de hibisco.

Palavras chaves: *Hibiscus sabidariffa, corante natural, estabilidade, termossonificação, encapsulação.*

Hibiscus (*Hibiscus Sabdariffa* L.): thermosonication extraction followed by alginate-Ca^{2+} encapsulation for natural food dye obtention

Abstract

The objective of this paper was to perform thermosonified and conventional extraction, followed by lyophilization and encapsulation by ionic gelation of hibiscus for use as a natural colorant in yogurt. The thermosonication process, in general, presented higher bioactive compounds and this extract when encapsulated in alginate-Ca^{2+} showed good stability over the 40-day stability evaluation. The encapsulated samples (obtained by termosonication and conventional extraction) showed longer half-lives and less color variation (ΔE), indicating that they are more stable (especially in the absence of light) and thus were chosen for application in yogurt. The yogurt with the sample obtained by the termosonified followed by alginate-Ca^{2+} encapsulation process showed to be darker (lower brightness value) and with a greater tendency towards red, being more indicated for this type of product, and potentially recommended for use as a natural food dye.

Keywords: *Hibiscus sabidariffa*, natural dye, stability, thermosonification, encapsulation.

1. Introduction

Color and appearance are sensory attributes that affect food quality, these parameters are evaluated by the consumer (Damodaran; Parkin; Fennema, 2010). Dyes are widely used, synthetic dyes are known for having high stability and low cost. On the other hand, natural dyes have lower pigmentation power and low stability (Sigurdson; Tang; Giusti, 2017).

In natural products, one of the main responsible groups for coloring belongs to the class of flavonoids, with emphasis on the group of anthocyanidins (Guimarães et al, 2012). These pigments aim to provide coloring in food, contributing to the acceptability of products by consumers

(De Barros Anastácio et al., 2016). Considering this context, the replacement of synthetic dyes by natural ones has been gaining notoriety (Menezes et al, 2015), which imposes on the industry the use of natural dyes in food products (Nachay, 2017).

Cid-Ortega and Guerrero-Beltrán (2015) mentioned food products developed from hibiscus (*Hibiscus rosa-sinensis* L.) considering it as a functional food. Hibiscus is a flower known for its relevant quantity of anthocyanins; the citrus flavor of its calyxes is a notable characteristic, it can be consumed in the form of salads, infusions, liqueurs, jellies, etc. (Silva et al., 2016). In addition, their calyxes are endowed with bioactive compounds, such as anthocyanins and phenolic compounds, which are responsible for their antioxidant properties (Cid-Ortega and Guerrero-Beltrán, 2015; Da-Costa-Rocha et al., 2014). The main anthocyanins found in the hibiscus calyx are delphinidin 3-O-sambubioside and cyanidin 3-O-sambubioside, with a content of up to 0.75 mg/mL of extract (Sinela et al., 2017).

Natural dyes usually have high costs, some of them can have lower stability under processing and storage conditions. Considering this fact, the encapsulation by different techniques of natural dyes has been widely explored by researchers as a way to increase their stability when applied to foods (Antigo et al., 2020), which is still challenging.

Thus, this is a work of innovative character, bringing the use of water as a solvent in extraction techniques combined with encapsulation, aiming to improve the stability of the natural hibiscus dye. Ultrasound is an extraction technique known as being ecologically correct, cheap, fast and efficient for the extraction of phenolic compounds, in which acoustic cavitation occurs caused by the passage of ultrasound waves. In this cavitation, the formation, growth and collapse of microbubbles on the solid surface occurs and results in the rupture of cell walls, allowing the penetration of the solvent used and improving mass transfer, resulting in a shorter extraction time and increased yield (Tao, Zhang & Sun, 2014).

Ionic gelation techniques are interesting, and the most used wall encapsulation material is alginate, a natural anionic polymer of (1,4)-linked

β-D-mannuronate (M) and α-L-guluronate (G), used in pharmaceutical and food industries, due to its biocompatibility, bioavailability and ease of gelation by adding Ca^{2+}. Thus, Romanini et al (2021) present for the first time the use of ultrasound extraction followed by encapsulation for grape residue, the authors observed that the extract encapsulated in alginate-Ca^{2+}, when stored under light, showed greater degradation of bioactive compounds, indicating that such capsules are recommended for future food applications that require light protection.

Given what was mentioned before, this work aimed to develop a natural dye of hibiscus (*Hibiscus Sabdariffa* L.) through its aqueous extract with application of ultrasound and conventional extraction and subsequent lyophilization and encapsulation by ionic gelation. The kinetics of degradation of this dye and the application in a food matrix were also evaluated.

2. Methodology

The hibiscus was purchased from a local producer in Maringá, Brazil, being from a 2021 harvest. Others components such as Ultra High Temperature milk, powdered milk, BioRich lactic culture and sugar for the yogurt production were also obtained from the Maringá, Brazil.

2.1 Hibiscus Processing

Initially, aiming to obtain hibiscus calyces, the seeds were removed, and the calyces were immersed in a 200 ppm sodium hypochlorite solution for a period of 15 minutes. After that, the calyces were sanitized (sodium hypochlorite solution 200 ppm for 15 min) and dried at 60 °C for 22 hours with a forced air convection (Cienlab). Then, the calyces were ground in an electric mill and classified according to granulometry using the Tyler sieves series. After preliminary tests, the material was chosen with an average diameter of 0.45 to 0.60 mm for conducting the experiments.

2.2 Assisted Ultrasound and Conventional Extraction

The hibiscus samples were put in an ultrasonic bath, model

Ultracleaner 1650 Unique, frequency of 40 KHz at 60 ºC for 45 minutes in a ratio of 1:10 (calyces:solvent), water was used as a solvent since it is eco-friendly. The acquired extract was filtered using Whatman No. 4 filter paper and stored in the dark at 25 °C for further analysis (Paraíso, 2020).

During the conventional extraction, the methodology described by Paraíso (2019) was followed with some adaptations, in which the samples were placed in a bath at 25ºC for 45 minutes in a calyces:water ratio of 1:10. The acquired extract was filtered using Whatman No. 4 filter paper and stored in the dark at 25 °C for further analysis.

Both samples, the thermosonified hibiscus extract and the conventional extract were obtained the way described above.

2.3 Powders obtained by lyophilization and alginate-Ca^{2+} encapsulation

The obtained extract was submitted to the lyophilization process. This process occurred for 2 days at -36ºC in order to ensure the complete drying of the product (Liobras, lyophilizer L108, Brazil). The lyophilized product was stored at -18ºC in plastic packaging for further analysis.

For the ionic gelation encapsulation process, sodium alginate at a concentration of 2 % w/v (2 g / 100 mL) was added to 100 mL of water followed by the adding of 50 mg/g of the extract under stirring and heating of 70 °C ± 4 °C for complete dispersion. The alginate capsule was made by means of the dispersion which was dripped using the Caviar Box® kit in an aqueous solution of calcium chloride at (1% w/v, 1 g/100 mL). The formed capsules were kept in the calcium chloride solution for 10 minutes, after that they were sieved and washed with deionized water for removing the calcium excess and for stopping the complexation process (Carvalho, et al., 2019). The encapsulation efficiency was measured according Selamat; Muhamad; Sarmidi (2009), were Encapsulation efficiency EE (%) is equal to ((FT-FS)/FT) × 100. Being FT the anthocyanins content inside the microcapsules and FS the compounds present on the surface.

After the encapsulation process, the capsules were frozen at -18ºC for 48 hours and then they were submitted to lyophilization (freeze L108,

Liobras) for 2 days to ensure the complete drying.

Finally, the following samples were obtained: freeze-dried thermosonified hibiscus extract (TL), freeze-dried conventional hibiscus extract (CL), freeze-dried encapsulated thermosonified hibiscus extract (TE) and (CE) freeze-dried conventional encapsulated hibiscus extract.

2.4 Characteristics of obtained powders

For controlling the process and standardizing the products, the moisture analysis was performed by drying the sample in an oven at 105ºC until constant weigh and the pH one was done by direct reading on the sample according to the methods described by the Adolf Lutz Institute (Ial, 1985).

The parameters described below were also evaluated in triplicate.

Determination of bioactive compounds

To the extraction of bioactive compounds, the disintegration of the microcapsules was carried out with distilled water, agitation and centrifugation at 4000 rpm for 10 minutes (Díaz et al., 2015).

The determination of the total phenolics was done using the methodology described by Pierpoint (2004) and Singleton and Rossi (1965). The results are shown in μg of Gallic Acid Equivalent (GAE).mg^{-1} of the product.

In order to determine the total content of flavonoids and anthocyanins, the methodology described by (Lees and Francis, 1972) was used. The total flavonoid content was expressed in mg equivalent of quercitin.100 g^{-1} of hibiscus and the total content of anthocyanins in mg equivalent of cyanidin-3-glucoside.100 g^{-1} of hibiscus.

Antioxidant Analysis (DPPH, FRAP and ABTS methods)

The stable radical DPPH (2,2-diphenyl-1-picrylhydrazyl) reduction was determined according to the methodology described by Thaipong et al. (2006). Absorbance was measured at 515 nm and antioxidant activity was calculated from the Trolox and expressed in mM TE / g of the sample. The

ABTS assay was performed at the maximum absorption of 734 nm according to Meng et al. (2017).

The Iron Reducing Antioxidant Power (IRAP) was determined according to the methodology described by Pulido, Bravo, & Saura-calixto (2000). Absorbance was measured at 595 nm and antioxidant activity was calculated from the ferrous sulfate and expressed in μM ferrous sulfate (FS) g^{-1} of sample. The spectrophotometer (Jenway 6705 UV / Vis) was used in all the analyses.

Phytochemical profile by UPLC – MS/MS

UPLC-MS/MS was used to identify the bioactive compounds in the extracts. Samples were injected into an ethane-bridged hybrid UPLC Acquity UPLC® (BEH) system coupled to the Xevo TQD™ Triple Quadrupole Mass Spectrometer (Mil Ford, MA, USA) and equipped with a Waters Electrospray Ionization Source (ESI) Zspray™ (Milford, MA, USA). The mass spectrometer was operated in both positive and negative modes using the following conditions: Collision gas pressure of 3.5 mbar, desolvation gas temperature of 500 °C, desolvation gas flow of 800 l h^{-1} and capillary voltage of 3 kV. The analysed molecules were separated on an Acquity UPLC® (BEH) hybrid ethane bridged C18 column (50 mm × 2.1 mm, 1.7 μm), maintained at 40 °C and injection volume of 4 μL. The mobile phase consisted of water, which was acidified with 0.1% of formic acid (A) and methanol (B). The gradient developed for separation was 0.22 mL min^{-1}, with 87A: 13B from 0 to 3 min, 35A:65B from 3 to 7.2 min, 0A: 100B from 7.2 to 8.3 min. After that, the mobile phase returned to the initial conditions generating a total chromatographic run of 12 minutes (Paraíso, 2021).

Color Analysis

In order to evaluate color, the portable colorimeter Minolta® CR400 with Cielab system was used, identifying the parameters of luminosity (L), red (a+), green (a-), yellow (b+) and blue (b-) and also ΔE. The instrument was calibrated on a standard white plate in each analysis at different times. The samples were placed in a white container, the calorimeter was placed

over the sample, 3 rays were fired per reading which was carried out in triplicate. The color difference (ΔE) was calculated according to equation 1, following the method detailed by da Silva et al. (2017).

$\Delta E = \sqrt{(L^* \text{ day } 40 - L^* \text{day } 1)^2 + (a^* \text{day } 40 - a^* \text{day} 1)^2 + (b^* \text{day } 40 - b^* \text{day} 1)^2}$ (1)

L*, a* and b* are color parameters; day 1 = first day of storage; day 40 = 40 days of storage

2.5 Storage stability (Kinetic degradation)

After the lyophilization, the lyophilized and encapsulated samples were stored in plastic bags in BOD's in the presence of light (two 20 W fluorescent lamps, working as an artificial light source) and in the dark at 25 °C ± 1 ºC for 40 days of storage (Santos, Rodrigues, Costa, De Cassia Bergamasco, & Madrona, 2017). During this period, the parameters of color, anthocyanins and phenolic compounds were observed as a measure of stability. The assay and readings were performed in triplicate. The results of the color analysis were expressed as a function of the ΔE value (equation 1) and the experimental data of anthocyanins and phenolics were adjusted for the first-order reaction kinetics and half-life (t1 / 2) (Stamp & Labuza, 1983) according to Eq. (2) and (3). Previous research has shown that the degradation of natural dyes follows the first-order kinetics during storage (Serris et al., 2001; Bustos-Garza et al., 2013; Cano-Higuita et al., 2015).

$$\ln(A_t / A_0) = - k \times t \quad (2)$$

$$t_{1/2=} \ln 2/k \quad (3)$$

T means the time (day), A is the level of anthocyanin or phenolics, A_0 is the initial level of anthocyanin or phenolics at time zero and k is the rate constant (day^{-1}).

2.6 Application of powders in food matrix (yogurt)

For the yogurt production was used: Ultra High Temperature milk, BioRich lactic culture, composed by *Lactobacillus acidophilus* LA-5 ($1x10^6$ CFU/g), Bifidobacterium BB-12 ($1x10^6$ CFU/g) and *Streptococcus*

thermophilus cultures, brand CRH-Hansen and sugar. Initially, the culture was prepared according to the supplier's guidelines, after that the bed was heated to 45ºC and about 3% of culture was added. Then, it was incubated for a period of 3 hours until reaching a pH between 4.5 - 4.65 and then it was cooled to 15ºC and 1% of the dyes obtained, lyophilized and encapsulated, were added.

For analyzing the antioxidant activity, it was necessary to perform the precipitation of proteins from the yogurt samples with 20% trichloroacetic acid in water, in a 1:1 proportion of yogurt and trichloroacetic acid (Zulueta et al., 2009). The samples were shaken for 30 seconds and incubated in a heating bath at 42 °C for 10 minutes. At the end, they were centrifuged (750 × g) for 15 minutes and the supernatant was used for analysis.

Just after being made, the yogurt was evaluated considering on the following aspects: antioxidants, color, pH, moisture and rheology. The rheological properties were determined considering the methodology of Fernandes et al. (2021). A viscometer model DV2T (Brookfield, Middleboro, Massachusetts, USA) was used. Steady-state flow curve tests were performed using an SC4-18 spindle at a constant temperature of 11 °C. Two rheological measurements were performed at the ascending shear rate (0 – 300 s −1) and then at the of decreasing shear (300 – 0 s−1). Data were adjusted to the power-law model using nonlinear regression analysis and Rheocalc T1.2.19 software. The rheological parameters were determined at 11 °C, since the drinks are sold and bought at refrigerated temperatures.

2.7 Statistics analysis of data

All the analyses were performed in triplicate and also submitted to the analysis of variance and Tukey's test for minimum significant difference ($p < 0.05$) between means using the Sisvar statistical program.

3. Results and Discussions

14.425 Kg of *in natura* hibiscus were obtained. The product that was submitted to the drying process and after removing the seeds, it reached a

total of 9.800 Kg and a yield of 1.045 kg (10.66%) was obtained after it was dried.

3.1 Characteristics of the extracts (conventional and thermosonified)

It was observed on Table 1 that the antioxidant activity in the thermosified extract was higher ($p<0.05$) than the one in the conventional extract, in all methods of evaluation (FRAP, DPPH and ABTS), being increased respectively by 1.16, 1.27 and 1.12 times. Regarding the color, it was observed that the samples tended towards the red color (H°), probably due to the reddish color of the hibiscus calyces, which are endowed with anthocyanins (Cid-Ortega, 2015).

Reference studies have shown that the diffusion and solubility rates, between solute and solvent, tend to increase with increasing temperature. However, excessive temperatures are associated with the degradation of anthocyanins and phenolic compounds, in which this temperature has to be limited and find the optimal extraction temperature (D'Alessandro, 2012; Pedro, 2016). In another study using a 25% ethanol solvent:water, Paraíso (2020) mentions that the sonication time variables and temperature increase had a positive effect, providing greater extraction of anthocyanins and phenolic compounds in hibiscus calyces, affirming that the best condition was 45 min, 65 °C, confirming that this method is more effective than conventional extraction. Nevertheless, in the present study, an increase in the extraction of anthocyanins and phenolic compounds was not observed, probably because it was chosen to use water as a solvent, considering having an eco-friendly process.

Table 1 – pH Analyses, moisture, color and antioxidants from thermosonified and conventionally extracted liquid extracts.

	Thermosonified	***Convencional***
Anthocyanins (mg cyanidin-3 glucoside/ g)	$0.291^{a} \pm 0.04$	$0.266^{a} \pm 0.06$
Phenolics (mgEAG/g)	$18.47^{a} \pm 0.66$	$19.40^{a} \pm 0.21$
Flavonoids (µgEG/mg)	$79.22^{a} \pm 1.88$	$74.44^{a} \pm 2.63$
FRAP (mM TE/ g)	$928.24^{a} \pm 2.31$	$802.60^{b} \pm 4.75$

DPPH (mM TE/ g)	$299.55^{a} \pm 8.27$	$234.99^{b} \pm 1.79$
ABTS (mM TE/ g)	$290.07^{a} \pm 15.26$	$259.82^{b} \pm 9.27$
Moisture (g/ 100 g)	$94.59^{a} \pm 0.02$	$95.61^{a} \pm 0.01$
pH	$2.70^{a} \pm 0.00$	$2.72^{a} \pm 0.01$
L	$19.75^{a} \pm 1.18$	$20.59^{a} \pm 0.51$
a*	$36.10^{a} \pm 1.23$	$33.07^{a} \pm 0.82$
b*	$23.62^{a} \pm 1.42$	$20.20^{a} \pm 1.09$
C*	$43.61^{a} \pm 0.56$	$43.22^{a} \pm 1.84$
H°	$31.71^{a} \pm 0.14$	$34.33^{b} \pm 0.92$

*same letters at the same line mean that there was no relevant difference between the samples in $p \leq 0,05$. EAG = equivalent gallic acid. EG equivalents of quercetin. MTE expressed in trolox.

In general, it is observed that the use of thermosonification is relevant interesting for obtaining an extract rich in antioxidants. Yet this technique did not present major influences on the other parameters evaluated such as anthocyanins, phenolics, color, pH and moisture.

On Table 2, similar to Table 1, after the lyophilization process, the same aspects are observed, in which the antioxidant activity was higher (1.42-fold for flavonoids, 1.07-fold for phenolic compounds, 1.48-fold for FRAP and 1.14-fold for ABTS) for the sample that was thermosonified and lyophilized. Romanini (2021) performed ultrasound-assisted (high-intensity) extraction of bioactive compounds from grape pomace followed by encapsulation of alginate-Ca^{2+} obtaining similar results and the levels of antioxidant capacity were higher in extracts obtained by ultrasound when compared to extraction conventional. Still according to the literature, ultrasonic energy can break the cell wall, increasing the contact between anthocyanins and solvent, which favors the reduction of sonication time (Alessandro et al. 2013; Azmir et al. 2013).

Table 2 – Physicochemical, antioxidant and UPLC analyses of samples TL (freeze-dried thermosonified hibiscus extract) and CL (lyophilized conventional hibiscus extract).

	TL	***CL***
Anthocyanins (mg cyanidin-3 glucoside/ g)	$1.57^{a} \pm 0.18$	$1.68^{a} \pm 0.13$
Phenolics (mgEAG/g)	$30.04^{a} \pm 0.56$	$28.01^{b} \pm$

		0.40
Flavonoids (µgEG/mg)	108.29[a] ± 4.08	76.14[b] ± 7.13
FRAP (mM TE/ g)	1338.76[a] ± 22.81	903.29[b] ± 16.29
DPPH (mM TE/ g)	199.80[a] ± 1.92	117.75[a] ± 1.97
ABTS (mM TE/ g)	604.75[a] ± 26.75	530.75[b] ± 34.33
Cyanidin 3-glucoside (mg/g)	0.09[a] ± 0.00	0.04[a] ± 0.02
Delphinidine (mg/g)	27.51[a] ± 2.93	34.36 [a] ± 4.44
Chlorogenic (mg/g)	2.50[a] ± 0.21	2.05[a] ± 0.10
Quercetin (mg/g)	0.16[a] ± 0.00	0.06[b] ± 0.01
Myricetin (mg/g)	0.03[a] ± 0.0	0.03[a] ± 0.0
Gallic acid (mg/g)	<LQ	<LQ
Rutin (mg/g)	0.33[a] ± 0.02	0.27[b] ± 0.01
Pcumaric (mg/g)	<LD	<LD
Ellagic (mg/g)	<LD	<LD
moisture	13.21[a] ± 0.73	15.56[b] ± 2.2
pH	2.19[a] ± 0.02	2.77[b] ± 0.03

*same letters in the same line mean that there was no relevant difference between the samples in m p≤0,05. MTE expressed em trolox. EAG = equivalente gallic acid. Quercetin EG equivalents. MTE expressed in trolox. <LD (less than detection limit), <LQ (less than quantifiable limit). * values on a dry basis.

It was also noticed that the thermosification process followed by the lyophilization (table 3), extracted more compounds such as quercetin and rutin, being respectively 2.67 and 1.22 times higher for the TL sample when compared to the conventional one (CL). Corroborating the present study, the compounds identified have already been reported in previous studies in aqueous and ethanolic hibiscus extracts (Pimentel-Moral, 2018; Sinela, 2017; Paraíso, 2019).

In the extraction process followed by the encapsulation one, the powders that were obtained after the thermosonification (TE) presented, when compared to the conventional process (CE), more phenolics compounds (1.05 times higher) and gallic acid (1.23 times higher). It is also relevant to mention that the thermosonified sample (TE) showed compounds such as chlorogenic acid, cyanidin 3-glucoside, quercetin and

rutin, that were not detected in the conventional sample (CE), probably due to the low concentration. On the other hand, the conventional sample (CE) showed a higher content of Delphinidin (on average 50%) indicating that thermosification may have degraded this compound. Previous studies have shown that, by increasing the temperature, cleavage into cyanidin and delphinidin can occur, thus forming degradation products such as protocatechuic acid and gallic acid, respectively (Kern et al., 2007; Sinela et al, 2017). Besides, the capsules have lower values due to the fact that the extract is dispersed within the wall material, protecting it from its direct and complete dispersion.

The encapsulation efficiency was similar in both types of processes, being 75.94% in thermosonified extraction and 77.44% in the conventional one. Romanini (2021) obtained similar results in the study of the same encapsulation technique for grape residue in which the use of ultrasound combined with a temperature of 55°C (thermo-sonication) was significantly better, presenting higher values (EE of 62.52 %) when compared to conventional extraction.

Table 3 – pH Analyses, moisture, antioxidants and ULPC from the TE samples (freeze-dried encapsulated thermosonified hibiscus extract) and CE (freeze-dried encapsulated conventional hibiscus extract)

	TE	***CE***
Anthocyanins (mg cyanidin-3 glucoside/ g)	0.07^{a} ± 0.01	0.06^{a} ± 0.01
Phenolics (mgEAG/g)	1.65^{a}± 0.18	$1,56^{b}$± 0.40
Flavonoids (µgEG/mg)	12.18^{a} ± 0.27	11.85^{a} ± 1.88
FRAP (mM TE/ g)	29.34^{a} ± 2.53	21.94^{a} ± 1.04
DPPH (mM TE/ g)	12.77^{a} ± 0.03	12.24^{a} ± 0.12
ABTS (mM TE/ g)	54.15^{a} ± 3.08	53.44^{a} ± 0.77
3-glycoside cyanidin (mg/g)	0.02 ± 0.00	<LQ
Delphinidin (mg/g)	0.54^{a} ± 0.14	1.27^{b} ± 0.04
Chlorogenic Acid (mg/g)	0.26 ± 0.22	<LD
Quercetin (mg/g)	0.01 ± 0.00	<LQ
Myricetin (mg/g)	<LD	<LD
Gallic Acid (mg/g)	0.64^{a} ± 0.01	0.52^{b} ± 0.01
Rutin(mg/g)	0.04 ± 0.00	<LQ
Pcumaric (mg/g)	<LD	<LD
Ellagic (mg/g)	<LD	<LD
moisture	14.10^{a} ± 0.62	9.34^{b} ± 1.89

pH	$3.42^{a} \pm 0.01$	$3.54^{b} \pm 0.03$

* same letters in the same line mean that there was no relevant difference between the samples in p≤0,05. MTE expressed em trolox. EAG = equivalente gallic acid. Quercetin EG equivalents. MTE expressed in trolox. <LD (less than detection limit), <LQ (less than quantifiable limit). * values on a dry basis.

3.2 Storage stability (Kinetic degradation)

The degradation of phenolic compounds and anthocyanins was noticed during the period of stability (Table 4), being noticeable that time and mainly incidence of light negatively affected these compounds. Regarding the half-life, it is possible to see that, in general, the incidence of light influenced the decrease, for both extracts. In a similar study, with hibiscus extract from the same region, light and temperature factors affected the stability of phenolic compounds and anthocyanins throughout storage where the levels of anthocyanins and phenolic compounds decreased significantly, for extracts with light exposure, with a loss of 41.95% and 38.02% (Paraíso, 2020). Anthocyanin loss has been reported in the literature during storage of jabuticaba bark tea, registering a loss of monomeric anthocyanins occurring after 72 hours of refrigerated storage at 5°C (Da Silva, 2017).

Table 4 – Stability to the storage (Kinetics) of the samples on ΔE, anthocyanin and phenolic compounds.

Parâmetros/amostra	***TL***	***TLLuz***	***CL***	***CLLuz***
	Anthocyanins (mg cyanidin-3 glucoside/ g)			
k	-0.01813	-0.02054	-0.02705	-0.02776
t1/2 (days)	38.23	33.74	25.62	24.97
	Phenolic Compounds (mg EAG/g)			
K	-0.00402	-0.00284	-0.00169	-0.00085
t1/2 (days)	172.51	156.20	209.66	180.45
	ΔE			
40 days	1.78	6.35	6.30	8.43
	TE	***TELuz***	***CE***	***CELuz***
	Anthocyanins (mg cyanidin-3 glucoside/ g)			
k	-0.01838	-0.01567	-0.02916	-0.04065
t1/2 days	55.45	44.23	23.77	17.05
	Phenolic Compounds (mg EAG/g)			
k	-0.00184	-0.0035	-0.00312	-0.0061

t1/2 days	376.26	198.21	222.20	113.54
	ΔE			
40 days	2.00	6.36	5.94	7.58

TL (freeze-dried thermosonified hibiscus extract), TLLight (light-freeze-dried thermosonified hibiscus extract), CL (lyophilized conventional hibiscus extract), CLLight (light-lyophilized, freeze-dried conventional hibiscus extract), TE (lyophilized encapsulated thermosonified hibiscus extract) , TELight (freeze-dried encapsulated thermosonified hibiscus extract subjected to light), CE (lyophilized encapsulated conventional hibiscus extract) and CELight (lyophilized encapsulated conventional hibiscus extract subjected to light). * values on a dry basis.

The half live time was shorter for all the samples that were exposed to light, since it suffered a greater degradation of phenolic compounds and anthocyanins. The same occurred in Dos Santos et al (2017) work, in which the process of microencapsulation with maltodextrin was efficient in protecting phenolic compounds and anthocyanins during the storage period and, regarding the loss of bioactive compounds, the influence of light was more remarkable than that of temperature. Romanini (2021) in his study shows that light degrades anthocyanins and phenolic compounds, where the extracts encapsulated in alginate-Ca^{2+} have greater efficiency when they were protected from light. In addition, Paraíso (2020) also portrays in his work that light and temperature are factors that affect the stability of phenolic compounds and anthocyanins during the storage period under the different conditions studied.

Obón et al. (2009) mentions that color changes can be measured as the modulus of the distance vector between the initial color values and the actual color coordinates in the CIELAB three-dimensional color space (ΔE). Besides, differences in perceptible color can be defined as small (ΔE < 1.5), distinct (1.5 < ΔE < 3) and highly distinct (ΔE > 3) the author also mentions that the color variation is only evident for ΔE > 5. It is observed that the lyophilized powders showed a variation of ΔE between 1.78 and 8.43. When comparing the extraction techniques, the thermosonified samples showed greater color stability (ΔE of 1.78 for the powder and 2.00 for the capsule)

and also better half-life, whereas the conventionally extracted samples showed greater color variability (ΔE) and with incidence of light.

Silva et al (2013) mentions in his study that low values of ΔE are widely wanted for the general view of color variations, they indicate that the powder pigment has the original extract color after the reconstitution. The color change can happen due to the loss of anthocyanins during the storage period, what can also be related with the co-pigmentation of anthocyanins with other phenolic compounds. A recent study evaluated the thermal processing and subsequent storage of hibiscus extract observed the clarification of the extracts with storage at 4 °C for 20 days (Sui, Bary & Zhou, 2016).

Overall, the encapsulated samples (TE and CE) presented longer half-life and smaller color variations (ΔE) showing to be more stable, mainly the absence of light. They were chosen for application in the food matrix.

3.4 Application in food matrix – yogurt

It is observed that the yogurt sample with the thermosonified capsule (Table 5) presented antioxidant content (ABTS) 1.09 times higher than the conventional sample. For the parameters of moisture, pH and texture profile, there was no relevant differences between the samples ($p<0.05$), revealing that the use of extraction techniques with encapsulation did not influence the evaluated characteristics in yogurts.

It is also observed that the yogurt sample with the thermosonified capsule (Table 5) presented antioxidant content (ABTS) 1.09 times higher than the conventional sample. For the parameters of moisture, pH and texture profile, there was no significant difference between the samples ($p<0.05$), showing that the use of extraction techniques with encapsulation, did not influence these evaluated characteristics in yogurts.

Considering color, it was noticed that thermosonified encapsulated sample (TE) was presented as darker (lower luminosity value) and tending to red, while CE showed greater luminosity (L*), greater chromaticity (C) with values that tended to yellow. A possible explanation is the type of wall material used and even though the capsule performs a controlled release,

in addition to having a part of wall material and extract. Apart from that, the homogenization of the encapsulating agents is smaller than the powder.

It is known that the yogurt consumption increases according to the need for its diversity in the market, since the most demanding consumers have been looking for dairy products with exotic and different flavors, with the incorporation of new ingredients in order to improve the flavor, color, diversifying nutritional properties and standing out in consumer preference (Barkallah et al., 2017; Mohammadi-Gouraji et al., 2019).

Table 5 - Application of samples encapsulated in yogurt

	TE	***CE***
pH	$4.68^{a} \pm 0.00$	$4.83^{a} \pm 0.03$
Moisture	$80.07^{a} \pm 0.34$	$80.36^{a} \pm 0.15$
Flavonoids (µgEG/mg)	$2.63^{a} \pm 0.10$	$2.47^{a} \pm 0.10$
Phenolics (mgEAG/g)	$0.56^{a} \pm 0.01$	$0.55^{a} \pm 0.03$
Anthocyanins (mg cyanidin-3 glucoside/ g)	<LD	<LD
DDPH (mM TE/ g)	$6.85^{a} \pm 0.20$	$7.42^{a} \pm 0.06$
ABTS (mM TE/ g)	$9.69^{a} \pm 0.11$	$8.90^{b} \pm 0.17$
FRAP (mM TE/ g)	$6.46^{a} \pm 0.13$	$6.90^{a} \pm 0.16$
L*	$88.33^{a} \pm 0.10$	$90.03^{b} \pm 0.33$
a*	$-0.04^{a} \pm 0.04$	$-1.06^{b} \pm 0.14$
b*	$10.34^{a} \pm 0.04$	$10.06^{a} \pm 0.54$
c*	$8.53^{a} \pm 0.05$	$11.12^{b} \pm 0.34$
H	$90.86^{a} \pm 0.09$	$90.77^{a} \pm 0.23$
Firmness (g)	$42.34^{a} \pm 1.71$	$44.80^{a} \pm 1.55$
Consistency (g.s)	$244.16^{a} \pm 17.18$	$248.68^{a} \pm 16.58$
Cohesiveness (g)	$-11.70^{a} \pm 0.61$	$-11.70^{a} \pm 0.80$
Viscosity Index (g.s)	$-4.86^{a} \pm 0.93$	$-4.97^{a} \pm 1.13$

* same letters in the same line mean that there was no relevant difference between the samples in p≤0,05. MTE expressed em trolox. EAG = equivalente gallic acid. Quercetin EG equivalents. MTE expressed in trolox. <LD (less than detection limit), <LQ (less than quantifiable limit). * values on a dry basis.

4. Conclusion

Overall, the thermosonified extract showed better results for bioactive compounds and antioxidant activity, in relation to stability it showed high

values of half-life (between 38.23 and 376.23 days). The presence of light negatively affected the samples (in both types of extraction evaluated), with respect to color and for the parameters of anthocyanins and phenolic compounds.

Thus, it is concluded that the ultrasonic extraction method using water as solvent is ecofriendly and feasible to use followed by the encapsulation by ionic gelation being efficient in protecting against degradation of phenolic compounds, and anthocyanins, and also color of hibiscus powders.

When applied to yogurt, the thermosonified encapsulated (TE) sample showed to be darker (lower brightness value) and with a greater tendency towards red, being more indicated for this type of product, and potentially recommended for use as a natural food dye.

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