**FELIPE ROCHA**

# COMPLEXATION OF NATURAL PIGMENTS WITH BIOPOLYMERS: AN APPROACH TO COMPLEXATION TECHNIQUES, BINDING PARAMETERS AND APPLICATION TO FOOD MATRICES

Thesis submitted to the Universidade Federal de Viçosa as part of the requirements of the Food Science and Technology Graduate Program for the degree of *Doctor Scientiae*.

Advisor: Márcia Cristina T. Ribeiro Vidigal

Co-advisors: Ana Clarissa dos Santos Pires
Valéria Paula Rodrigues Minim
Paulo César Stringheta

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Assent:

Felipe Rocha
Author

Márcia Cristina Teixeira Ribeiro Vidigal
Advisor

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## ABSTRACT

ROCHA, Felipe, D.Sc., Universidade Federal de Viçosa, July, 2022. **Complexation of Natural Pigments with Biopolymers: An Approach to Complexation Techniques, Binding Parameters and Application to Food Matrices**. Adviser: Márcia Cristina Teixeira Ribeiro Vidigal. Co-advisers: Ana Clarissa dos Santos Pires and Paulo César Stringheta.

Natural pigments are compounds that can present health-promoting bioactivities in the human body. Due to their coloring properties, these compounds have been widely used as color additives as an alternative to artificial colorants. However, since these pigments are unstable under certain conditions, such as the presence of light, oxygen, and heat, the complexation with a biopolymer is in demand. The complexation technique consists of forming a complex with the aim to make the compound less susceptible to oxidative and degrading agents. This work aims to discuss different techniques that have been used over the last years to create natural pigment-biopolymers complexes, as well as the recent advances, limitations, effects, and possible applications of these complexes in food systems. Moreover, the understanding of thermodynamic parameters between natural pigments and biopolymers to form a complex is very important. In this sense, the chapter 1 of this thesis presents a review about complexation techniques between natural pigments and biopolymers, and about thermodynamic techniques that can be used to determine binding parameters between natural pigments and biopolymers, as well as their applications, advantages, and limitations. The chapter 2 of this thesis presents the effects of natural-pigments-biopolymers complexes on food matrices, betalains (BE) nanodispersions were produced by using the solid dispersion method with polyethylene glycol (PBE) and with polyethylene glycol and low molecular chitosan (PCBE) by using the solid dispersion method followed by freeze drying. The thermal stability and stability of these pigments in acidic conditions were evaluated, as well as the effects of these nanodispersions on the color and rheology of Greek yogurt. Compared to pristine beetroot extract, PCBE nanoparticles presented increased stability for the main betalains in acidic conditions (pH 3.0 and 5.0) of 56% and 22%, respectively. Both PBE and PCBE showed enhanced relative thermal stability compared to pristine BE. Furthermore, PCBE improved commercial Greek yogurt's rheological properties and color parameters. PCBE nanodispersions can be successfully used as a color additive in the cosmetic, pharmaceutical, and food industries.



Keywords: Color. Bioactive compounds. Thermodynamic properties. Rheology.

## RESUMO

ROCHA, Felipe, D.Sc., Universidade Federal de Viçosa, julho de 2022. **Complexação de pigmentos naturais com biopolímeros: uma abordagem às técnicas de complexação, parâmetros de interação e aplicação em matriz alimentar.** Orientador: Márcia Cristina Teixeira Ribeiro Vidigal. Coorientadores: Ana Clarissa dos Santos Pires e Paulo César Stringheta.

Pigmentos naturais são compostos que podem apresentar benefícios à saúde no corpo humano. Devido às suas propriedades de cor, esses compostos têm sido amplamente utilizados como aditivos de cor como alternativo ao uso de corantes artificiais. No entanto, esses pigmentos são muito instáveis quando submetidos à presença de oxigênio, luz e calor. Nesse sentido, a complexação desses pigmentos com biopolímeros pode ser uma boa alternativa. A técnica de complexação consiste na formação de um complexo com o objetivo de fazer os pigmentos menos susceptíveis à agentes degradantes e oxidantes. Esse trabalho tem como finalidade discutir diferentes técnicas de que têm sido utilizadas nos últimos anos para criar complexos de pigmentos naturais e biopolímeros, assim como os recentes progessos, limitações, efeitos, e possíveis aplicações desses complexos em matrizes alimentares. Além disso, a elucidação sobre parâmetros termodinâmicos entre pigmentos naturais e biopolímeros para formar um complexo é muito importante. Nesse sentido, o capítulo 1 desse trabalho apresenta uma revisão de técnicas de complexação e técnicas termodinâmicas que podem ser utilizadas para determinar parâmetros de ligação entre pigmentos naturais e biopolímeros, suas aplicações, vantagens e limitações. O capítulo 2 dessa tese apresenta os efeitos de betalaínas (BE) que foram complexadas com polietileno glicol (PBE) e polietileno glicol com quitosana de baixo peso molecular (PCBE) pela técnica de dispersão sólida seguida de liofilização e a estabilidade térmica e em condições ácidas desses complexos foram avaliadas, assim como os efeitos dessas nanodispersões na cor e reologia do iogurte grego também foram avaliados. Quando comparado com o extrato na forma não complexada (BE), as nanodispersões PCBE apresentaram maior estabilidade para as principais betalaínas em condições ácidas (pH 3.0 e 5.0) de 56% e 22%, respectivamente. Ambas as nanodispersões PBE e PCBE apresentaram um acréscimo na relativa estabilidade térmica comparado com BE. Além disso, PCBE melhorou as propriedades reológicas e de cor do iogurte comercial grego. Portanto, PCBE podem ser utilizados aditivos de cor.



Palavras-chave: Cor. Compostos bioativos. Propriedades termodinâmicas. Reologia.

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**SUMMARY**

## GENERAL INTRODUCTION

Natural pigments can provide some health benefits and these compounds have great potential to be used as color additives in food matrices in contrast with artificial colorants. It is known that artificial colorants are not considered healthy due to allergenic and intolerance reactions in the human body (VARGAS-CAMPOS et al. 2018; MARTÍNEZ et al. 2019). Since natural pigments can be very unstable under certain conditions, a complexation of these pigments with biopolymers can be a suitable alternative to improve the stability of these compounds to be used in food matrices (CENOBIO-GALINDO et al. 2019; KOOP et al. 2022; LIU et al. 2022). Furthermore, the evaluation of binding parameters between natural pigments and biopolymers is very useful to determine whether a complex is formed or not, and also to determine its main driven power. The evaluation of binding parameters can also provide information about the stability of these complexes under certain conditions, such as high temperatures, pH, and the presence of salts (PACHECO et al. 2020). Therefore, the evaluation of binding parameters regarding the formation of these complexes can provide essential information for the application of these systems in food matrices.

Betalains are one of the most used red natural pigments as color additives (FERNÁNDEZ-LÓPEZ et al., 2020). Since these pigments can be used by the pharmaceutical and food industries, a complexation of these compounds with biopolymers can be a suitable alternative to improve their stability under certain conditions and their characteristics as color additives. It is known that polyethylene glycol (PEG) at low concentrations and chitosan can be safely used as food additives. In fact, PEG and chitosan are biodegradable and nontoxic biopolymers commonly used in the pharmaceutical industry (ATAY et al., 2018; YOUNES et al., 2018). In this sense, these wall materials can be potentially used to produce natural pigments-biopolymers complexes.

The aim of the Chapter 1 of my thesis was to describe the most common complexation techniques used to produce natural pigments-biopolymers complexes and also the most used techniques to evaluate binding parameters involved in the formation of these complexes. A detailed discussion about these techniques, their advantages, and their drawbacks are presented in this work. The chapter 2 of this thesis presents the effects on the stability of betalains (BE), when complexed with PEG (PBE) and PEG with chitosan (PCBE), as well as the application of these complexes in a food matrix, such as Greek yogurt.

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**CHAPTER 1**

# Complexation of natural pigments with biopolymers: an approach to encapsulation techniques and evaluation of binding parameters

This review paper was submitted to Food Research International

## 1. INTRODUCTION

Color is essential as a quality and sensory indicator in foods, and it is frequently determinant for their acceptance (DOWNHAM; COLLINS, 2000). Natural colorants are pigments found in natural sources, e.g., vegetables, minerals, and animals. Many natural pigments can be used as color additives in foods and they are usually associated with health benefits by consumers (DELGADO-VARGAS et al. 2000; PINTO et al. 2021). In this sense, studies regarding the properties of these compounds have been intensively reported, since these compounds have low toxicity, strong coloring, and biological properties, such as antioxidant activity, assisting the regulation of enzymatic activities, preventing diseases, and others (ROCHA et al. 2018; VARGAS-CAMPOS et al. 2018; MARTÍNEZ et al. 2019).

The use of natural pigments by the pharmaceutical, cosmetic, and food industries has been increasingly demanding since the health benefits of bioactive compounds have been a concern for a considerable share of consumers. Moreover, artificial colorants are linked to allergenic and intolerance reactions in humans (WROLSTAD; CULVER, 2012; SOLYMOSI et al. 2015). According to Fernández-López et al. (2020), the main natural pigments used as color additives belong to the groups of carotenoids, betalains, and anthocyanins.

Carotenoids are a class of bioactive compounds most widespread in nature. These compounds can be subdivided into carotenes, pure hydrocarbons, and xanthophylls, which often contain 2 to 4 atoms of oxygen per molecule and 40 carbon atoms (Rodriguez-Amaya 2010). The most common carotenoids present in foods are β-carotene (Fig. 1**)**, α-carotene, lutein (LUT), β-cryptoxanthin, zeaxanthin, and lycopene (LYC). These compounds are usually found in leafy and non-leafy vegetables, like carrots, watermelon, papaya, broccoli, and spinach. The carotenoids, especially the carotenes, present a very hydrophobic nature (RODRIGUEZ-AMAYA, 2010; RIVERA; CANELA-GARAYOA, 2012). It is known that carotenoids are less unstable in ranges of pH 4-8, since extreme ranges of pH can trigger

oxidation and isomerization processes (BUSTOS-GARZA et al. 2013; ORDOÑEZ-SANTOS et al. 2018).

Betalains are red water-soluble pigments that can be found in plants of the order Caryophyllales (*Centrospermae*), such as beets, cacti, and amaranths. Betacyanins, which provide the red-violet color, and betaxanthins, responsible for the yellowish color are betalains produced by the condensation of betalamic acid with cyclo-DOPA (cyclo-3,4-dihydroxyphenylalanine) and amino acids or amines, respectively. These compounds are relatively stable in pH ranging from 4 to 7, which indicates their versatile application in foods. The use of red beet betanin (Fig. 2), which is a betalain, is approved as color additive by the European Union in the Code of Federal Regulations (CFR) and by the Food and Drug Administration (FDA) (HUANG; VON ELBE, 1985; GANDÍA-HERRERO; GARCÍA-CARMONA 2013; STICH, 2016).

Anthocyanins are flavonoids that have been widely studied over the last decades due to their antioxidant activity and their abundance in nature and in many food products. These compounds are commonly found in red to purplish-blue colored leafy vegetables, grains, roots, and tubers. The most common food anthocyanins are cyanidin, malvidin (Fig. 3), delphinidin, and pelargonidin. The red color of these compounds is pH-dependent, displayed in a range of pH between 1 and 3, since their molecular structure is ionic. This pH-dependency property of the anthocyanins can contribute to their use as pH indicators (TIMBERLAKE, 1980; HE; GIUSTI, 2010; PRIETTO et al. 2018; FERNÁNDEZ-LOPEZ et al. 2020).

Solvent-based methods are very common for natural pigments extractions. For carotenoids, usually, nonpolar solvents are used due to their hydrophobic nature. In the case of anthocyanins, betalains, and xanthophylls, the use of polar solvents can be more appropriate (RORIZ et al. 2017; SAINI; KEUM 2018; SHEN et al. 2020). Non-conventional methods can also be applied and combined to assist the extraction, such as pulsed electric field, microwave, ultrasound, supercritical fluid extraction, and pressurized liquid extraction (JIANG et al. 2017; HOSSEINI et al. 2017; SANG et al. 2017; ROCHA et al. 2019; FU et al. 2020; CHUTIA; MAHANTA, 2021). In some cases, the use of non-conventional techniques can lead to a higher efficiency of extraction and preserve some natural properties of the pigments (SAINI; KEUM, 2018; CARRILLO et al. 2022).

However, natural pigments are very unstable during food processing, especially when submitted to high temperatures, light, the presence of oxygen, and extreme ranges of pH (SCHIOZER; BARATA, 2007). Therefore, the use of techniques to create natural pigments-

biopolymers complexes is in demand. Studies have shown that the use of biopolymers as carriers to natural pigments can also improve functional properties in food systems (CENOBIO-GALINDO et al. 2019; KOOP et al. 2022; LIU et al. 2022).

Several techniques can be used to improve the water dispersibility and stability of natural pigments, including spray drying (SD), electrospraying, emulsification, and freeze-drying (FD). In general, the process consists of using a wall material to make the compound of interest less susceptible to oxidative and degrading agents. Some other advantages of this process can include the controlled release of bioactive compounds and water dispersibility of hydrophobic compounds (DIAS et al. 2015; TOLVE et al. 2016). In addition, the understanding of thermodynamic binding aspects of natural pigments-biopolymers complexes can provide information about the interaction between these compounds, and the thermodynamic stability of these complexes under certain conditions (PACHECO et al. 2020).

A review about microencapsulation of natural dyes with biopolymers for application in foods was published last year by Ribeiro and Veloso (2021). However, the authors were mainly focused on the microencapsulation techniques. Our review brings innovation by providing information regarding the binding parameters between natural pigments and biopolymers, as well as by presenting an overview of the techniques used to evaluate these parameters. Moreover, a novel complexation technique, known as Pickering emulsions (PE), is presented in this work.

In this sense, this review aims to provide recent investigations, as well as the advantages, limitations, and effects of the use of complexation techniques on natural pigments, such as carotenoids, betalains, and anthocyanins. The potential applications of these complexes in food systems and binding parameters between these pigments and biopolymers are also discussed.

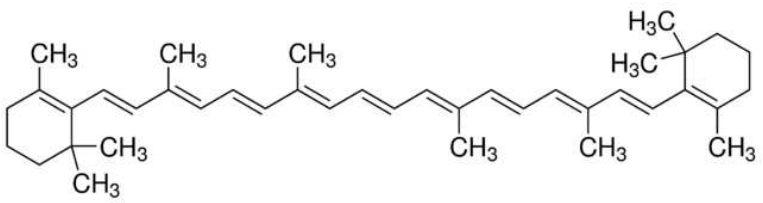


Fig. 1 – Chemical structure of beta-carotene

Source: adapted from Fernandéz-López et al. (2020)

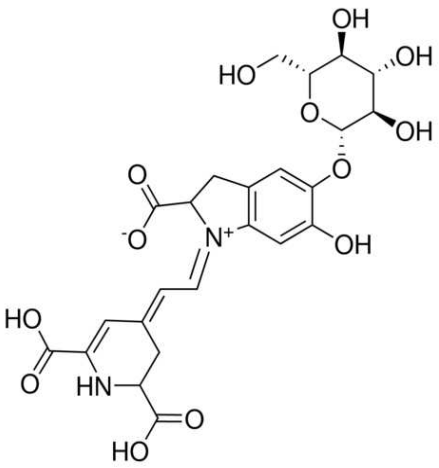


Fig. 2 – Chemical structure of betanin
Source: adapted from Fernandéz-López et al. (2020)

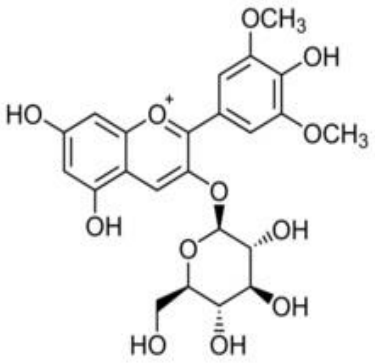


Fig. 3 – Chemical structure of anthocyanin malvidin 3-glucoside
Source: adapted from Fernandéz-López et al. (2020)

## 2. COMPLEXATION TECHNIQUES

The use of complexation techniques in natural pigments has been widely investigated over the last years to better adequate the use of these pigments in food systems (HAAS et al. 2019; KANHA et al. 2020). Some studies show that the color parameters and the thermal and storage stability of natural pigments can be improved in natural pigments-biopolymers complexes. Furthermore, an improvement in the water dispersibility of some hydrophobic natural pigments in these complexes can be also observed (BUSTOS-GARZA et al. 2013; ORDOÑEZ-SANTOS et al. 2018; XUE et al. 2019; FATHORDOOBADY et al. 2021). In

this sense, these characteristics are desirable, especially with the aim to apply these complexes to food matrices, in contrast with the use of artificial colorants (SOLYMOSI et al., 2015).

Several materials can be used as carriers to natural pigments. The most common types of materials used are polysaccharides (e.g., gums, dextrin, maltodextrin, and starch), proteins (e.g., gelatin, zein, soy proteins, and milk proteins), and lipids (e.g., nanostructured lipid carriers, liposomes, and solid lipid nanoparticles) (BOER et al. 2019; MAHALAKSHMI et al. 2020; LI et al. 2022).

Table 1 summarizes the main complexation techniques, the wall materials used for this purpose, and the results achieved for natural pigments-biopolymers complexes.

## 2.1 SPRAY DRYING

Spray dryer is the most commonly used equipment for drying or encapsulating natural pigments-biopolymers complexes. The principle of the SD technique is to create dry powder from a fluid by a gas stream. In general, the use of the equipment is very straightforward. First, the dispersion should be prepared, homogenized, and finally sprayed into a nozzle to a collector present in the equipment, where the particles are retained after drying. One of the advantages of this technique is the low cost, and the rapid and continuous process. The dry material produced by the equipment is also an advantage since the powder is a material with practical use and storage. However, the production of no uniform particles and the use of high temperatures for the process can be considered a disadvantage (GUO et al. 2020; SANTOS et al. 2021).

The particle size distribution of the product can be partially controlled by some equipment parameters, such as the inlet and outlet temperature, the nozzle diameter, and the formulation of the complex. The parameters of the SD are highly dependent on the natural pigment, the biopolymers, and the mass ratio between the natural pigment and the biopolymer. For natural pigments specifically, the use of lower temperatures can be more appropriate to reduce their thermal degradation and maintain their bioactive properties (ANTIGO et al. 2018; BOER et al. 2019).

**TABLE 1:** Overview of recent studies regarding natural pigments-biopolymers complexes

| Natural pigments | Complexation method | Materials | Results obtained | References |
|---|---|---|---|---|
| **Carotenoids** | Spray drying and Electrospraying | Zein protein in ethanol solution with β-carotene in glycerol solution | Encapsulation efficiency of 51-64% for spray dried capsules and 61-82% for electrosprayed capsules. 31-66% of encapsulated beta-carotene after in vitro digestion | (Mahalakshmi et al. 2020) |
| | Spray drying | β-carotene with β-cyclodextrin as wall material | Production of inhalable β-carotene aerosol. Encapsulation efficiency of 53-63% | (Lavanya et al. 2019) |
| | Spray drying | Lutein with β-cyclodextrin and stevioside as wall materials | Increase in lutein's *in vitro* bioaccessibility by 100% | (Xu et al. 2021) |
| | Freeze drying | Lutein, zeaxanthin, α-cryptoxanthin, α-carotene, β-carotene, and β-cyclodextrin | Increase in color stability of the carotenoids for the complexes under irradiance of 1400 lux and temperature of 25-31 ºC for 21 days | (Lobo et al. 2018) |
| | Freeze drying | Lycopene and *Chlorella pyrenoidosa* as wall material | Significant increase in storage stability for LYC-CPC loaded capsules (78.3-84.1%) compared to lycopene ex- | (Pu and Tang 2017) |

| | | | |
|---|---|---|---|
| | | tract (17.9-51%) at 4, 25, and 35 ºC for 60 days | |
| Electrospraying | β-carotene and glucuronoxylan nanostructures as wall material | Increase in β-carotene thermal stability compared with free β-carotene. Encapsulation efficiency of 73-93% | (Rostamabadi et al. 2019) |
| Emulsification | Lutein-whey protein nanoemsulsions (20% v/v oil in water) with lutein dispersed in ethanol/medium chain triglycerides oil | Approximately 95% of lutein retention after 4 weeks of storage at 4, 25, and 37 ºC | (Zhao et al. 2018) |
| Emulsification | Lactoferrin, alginate, and Ɛ-poly-$_L$-lysine as a tertiary emulsion to β-carotene | Improvement in stability under heat (≤ 70 ºC), acidic conditions (pH 2 to 5), and a concentration of 0-0.5 M of NaCl. Higher lipid digestibility (84%) and beta-carotene bioaccessibility (70%) in the tertiary after *in vitro* digestion | (Gasa-Falcon et al. 2020) |
| Emulsification | Lycopene emulsion containing whey protein isolate (1% wt) as emulsifier with corn and/or olive oil with tomato pulp as the oily phase | Increase in lycopene's bioaccessibility (61.5%) in constrast with lycopene's bioaccessibility in the tomato pulp (10%). Increase in lycopene's light and ther- | (Liang et al. 2021) |

| | | | | |
|---|---|---|---|---|
| | | | mal stability in the emulsions | |
| | Pickering emulsions | β-carotene, cellulose nanocrystals, soybean oil, and beeswax | Increase in β-carotene chemical (pH 4.0-8.0) and long-term storage (15 days at 25 ºC). Enhanced β-carotene bioaccessibility (53-68%) | (Qi et al. 2020) |
| | Pickering emulsions | β-carotene and protein/chitosan complex | The Pickering emulsions with the addition of the complex presented good storage stability after 28 days of storage. These systems presented good thermal (20-80 ºC) and oxidative stability, with no significant change in particle size | (Hu and Zhang. 2022) |
| **Betalains** | Spray drying | Betalains, maltodextrin, inulin, and whey protein isolated | Increase in betalains thermal stability (mass loss of 15-27% for the complexes compared to 39% mass loss of betalain extract) and accelerated storage stability (less color variation for the complexes after 15 weeks of stor- | (Carmo et al. 2018) |

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| | | age at 60 ºC and relative humidity of 30% | |
| Spray drying | Betalains and mucilage from *Opuntia ficus indica* | Protection against color degradation after 3 months of storage at a temperature of 4 ºC | (Delia et al. 2019) |
| Spray drying | Betalains with maltodextrin and cactus mucilage as wall materials | 90% of betalains retention after 49 days at 60 ± 2 ºC of storage for the betalains microparticles with maltodextrin. For the blends with cactus mucilage and maltrodextrin, it was reported a retention rate of 60%, in contrast with 40% for pristine betalains | (Carmona et al. 2021) |
| Spray drying and freeze drying | Betalains and pumpkin protein isolate | 92% of encapsulation efficiency for freeze dried samples and 75% for spray dried samples. Increase in antioxidant activity of betalains. The samples were easily digested in *in vitro* digestion | (Čakarević et al. 2020) |

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| Freeze drying | Betalains with maltodextrin (10% w/v) as wall material | Encapsulation efficiency of 80%. Increase in thermal stability as well as a significant increase in antioxidant activity for the encapsulated samples | (Li et al. 2022) |
| Freeze drying | Betalains with soy protein as a wall material | Encapsulation efficiency of 92% and increased antioxidant activity | (Šaponjac et al. 2020) |
| Freeze drying | Betalains from beetroot extract with polyethylene glycol and low molecular weight chitosan | An increase in betalains' stability of 56% and 22%, at pH 3.0 and 5.0, respectively. Increase in betalains' thermal stability. When applied to Greek yogurt, the betalains nanodispersions improved the rheological stability of the product after 21 days of storage at 4 ºC | (Rocha et al. 2022) |
| Emulsification | Betalains emulsion with soybean oil solution (2-8% w/w of the emulsifier CR-310) as the oil phase and phosphate buffer solution containing betanin and D-glucose | Betanin increases the overall kinetic stability of the emulsion by decreasing the dispersibility of the droplets due to electrostatic repulsion | (Pagano et al. 2018) |

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| | Emulsification followed by Spray Drying | Cactus pear extract with canola oil and polyglycerol polir-icineoleate as the emulsifier for the primary emulsion. Cactus pear extract with glycerol as the internal phase and a mixture between both emulsions as the ex-ternal phase | Encapsulation efficiency of 70% for beta-lains and maintenance of the antioxidant activity of the betalains in the emulsions | (Toledo-Madrid et al. 2018) |
| **Anthocyanins** | Spray drying and freeze drying | Anthocyanins from red-fresh apple, gum arabic, and maltodex-trin | Encapsulation efficiency of 93.8 to 96.8%. Significant increase in light stability by exposure to natural light for 12 days and heat stability for 80 ºC for 1 and 2 h for the cap-sules of antho-cyanins com-pared to non-encapsulated anthocyanin extract | (Xue et al. 2019) |
| | Spray drying | Anthocyanins extract from maqui with inu-lin or sodium alginate as wall materials | Encapsulation efficiency of 66-79%. In-crease of 10% in the bioacces-sibility for an-thocyanins in the microcap-sules compared to anthocyanins from maqui juice | (Fredes et al. 2018) |
| | Freeze drying | Anthocyanins extract | Encapsulation | (Doronio et al. |

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| | with chitosan-alginate as wall materials | efficiency of 66-79%. Increased antioxidant activity (14%) in the nanocapsules compared to pristine anthocyanins extract | 2022) |
| Freeze drying | Anthocyanins extract from berry fruits with maltodextrin, fiber from corn, modified starch from derived from waxy maize, and κ-carrageenan | After storage for 90 days at $a_w$ of 0.12-0.2 at 38 ºC, the powders presented a retention of 72-88% of the initial amount of anthocyanins. The redness color remained unchanged after storage | (Rosa et al. 2020) |
| Emulsification | Anthocyanins extract emulsions with sunflower oil as the oily phase. Black rice extract diluted in water as the inner aqueous phase, and gum arabic in water as the outer aqueous phase | Encapsulation efficiency of 99.4%. Significant stability of anthocyanins emulsion after 2h of *in vitro* digestion (3.7% of anthocyanins released). Simulated intestinal fluid showed that the total release of anthocyanins in the double emulsions was observed within 20 min | (Huang and Zhou 2019) |
| Emulsification | Black rice anthocyanins as an antioxidant to whey protein and | The addition of anthocyanins (0.02-0.06% | (Yi et al. 2020) |

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| | walnut oil emulsions | w/w) increased the physical stability of the emulsions after storage (35 ºC for 5 days) by inhibiting droplet aggregation. Moreover, the addition of anthocyanins inhibited both protein and lipid oxidation | |
| Pickering emulsions | Anthocyanin-rich black rice extract and soy protein isolate | Increase in antioxidant activity for anthocyanins PE emulsions (42-69%) compared to the control without anthocyanins (22-27%). Significant increase in oxidative stability (40 ºC for 21 days) | (Ju et al. 2020) |
| Pickering emulsions | Anthocyanin extract from bilberry with soybean oil and polyglycerol polyricinoleate as emulsifier stabilized by octenylsuccinate quinoa starch | Encapsulation efficiency higher than 95%. < 15% of anthocyanins released after 60 min of simulated stomach conditions | (Lin et al. 2020) |

The use of SD for extracts containing natural pigments can be very challenging due to the presence of some compounds, such as fructose, sucrose, glucose, and acids. These compounds can easily adhere to the walls of the drying chamber during the process, causing operational problems and potentially leading to a low yield of the final product. Therefore, the addition of wall materials with high molecular weight can contribute to the drying process by increasing the glass transition temperature of the material (JANISZEWSKA, 2014).

SD technique was used by Carmo et al. (2018) to study the effect of maltodextrin (MD), inulin (I), and whey protein isolated (WPI) as carrier agents for beetroot extract. Regarding the thermal stability, the authors observed that the mass loss at 225 ºC varied from 15 to 27% for the treatments with carrier agents, whereas the mass loss of beetroot juice was 39%, indicating a protective effect of the carrier agents. It was also reported a smaller variation of color for the complexes after storage for 7, 11, and 15 weeks of storage compared to beetroot juice. Similar results were obtained by Delia et al. (2019), who used mucilage from *Opuntia ficus indica* as a wall agent for betalains from *Escotria chiotilla* and *Stenocereus queretaroensis*. The color parameters of the samples were measured after 3 months of storage and the results showed that the encapsulated samples indicate a typical red-purple tone of betanins, whereas the non-encapsulated extract showed a yellowish tonality. In terms of encapsulation efficiency (EE), Elik et al. (2021) reported an EE of 89.8% for carotenoids enriched-flaxseed oil with low methoxylated pectin, sunflower wax, and maltodextrin as wall materials.

SD can be successfully used to produce natural pigments-biopolymers complexes, providing protection against color degradation during storage. The findings indicate that natural pigments-biopolymers complexes produced by SD can be potentially applied to food matrices, providing better stability for these products in terms of color during storage when compared to the natural pigments' extract in its pristine form.

In fact, Pal and Bhattacharjee (2018) applied lutein microcapsules obtained via SD in ready-to-serve beverages and reported a good sensory acceptance of the product according to a semi-trained panel. The yellow beverage contained 15.72 mg/100 mL of lutein. According to the authors, the beverage can be safely consumed with the aim to enhance the dietary intake of lutein. Similarly, Ursache et al. (2019) applied the carotenoids extract microencapsulated with WPI and gum acacia via SD into muffins and reported an increase in their carotenoid content and antioxidant capacity by around 100%. Moreover, the microcapsules provided more firmness and chewiness compared to the control samples. According to the authors, the muffins added with the microparticles were the most sensory preferred. Regarding the betalains, Carmona et al. (2020) applied betalains microparticles produced with maltodextrin and cactus mucilage via SD to yogurt and reported a color stability over 80% after 28 days of storage at 4 ºC.

## 2.2 FREEZE DRYING

Freeze drying is a very common method used to dry natural pigments-biopolymers complexes. The principle of this technique consists of sublimation of the water in the sample at very low temperatures under vacuum. First, the sample is prepared and frozen before drying, and the primary drying step is the direct sublimation of the sample. The secondary drying aims to remove the absorbed water from the product not eliminated in the first step. Some of the disadvantages of this technique are the cost and the time required for the process (ABDELWAHED et al. 2006; ANTIGO et al. 2018).

FD technique has been widely used to produce natural pigments-biopolymers complexes. Ahmed et al. (2018) used maltodextrin and gum arabic as wall materials to betalains. According to the authors, the encapsulation efficiency varied from 86.5 to 92.3%. It was also observed higher stability of betalains after 8 weeks of storage at 60 ºC for the complexes. In this study performed by Lobo et al. (2018), carotenoids extract-β-cyclodextrin complexes were successfully applied to isotonic drinks with promising storage stability results. After 21 days of storage, the complexes presented significantly higher color stability compared to pristine carotenoids extract. Similarly, an increase in thermal and storage degradation after 60 days at 4, 25, and 35 ºC for lycopene capsules produced with *Chlorella pyrenoidosa* as wall material by using FD were reported by Pu and Tang (2017).

Some studies comparing the use of SD and FD to dry natural pigments-biopolymers complexes are reported in the literature. Haas et al. (2019) produced microparticles of carrot concentrate powder with gum arabic and octenyl succinic anhydride (OSA)-starch by SD and FD techniques. In this study, the SD particles presented a higher size compared to the particles produced by FD. According to the authors, this difference is attributed to the agglomeration of the particles due to collision during the drying process. After storage for 91 days at 35 ºC, the complexes produced by FD presented a carotenoid loss of 87.5%, whereas the microparticles produced by SD presented a loss of 44.9%. The authors explain that this difference in the storage stability due to the porous structure of the FD particles, allowing the mobility of oxygen molecules in the material. Besides that, this difference regarding the stability can be also attributed to the higher size of SD particles and consequently lower specific surface area, which possibly played a role in delaying oxygen diffusion during storage. Differences between SD and FD regarding the morphology of the particles were also reported by Xue et al. (2019). In this study, maltodextrin and gum arabic were used as wall materials to anthocyanins. According to the authors, FD particles presented a porous structure, in contrast with smooth surfaces observed for the particles produced by SD. The porous structure as a result of the FD process can be attributed to the direct sublimation of water

present in the structure of the particles during the process. However, no difference between the treatments was found regarding the stability of anthocyanins comparing both drying methods.

Both techniques SD and FD can also play a role in the antioxidant activity of natural pigments. This difference can be explained by the fact that high temperatures are not required for the FD process, which can preserve the bioactivity of natural pigments. In some cases, the low temperature required during the freeze-drying process to dry natural pigments-biopolymers complexes can be more appropriate since most of the natural pigments are thermosensitive (ABDELWAHED et al. 2006; GOMES et al. 2018; VULIĆ et al. 2019; SHUEN et al. 2021). Regardless of the effects on the morphology, natural pigments-biopolymers complexes produced via FD can maintain their bioactive properties, meaning that besides their role as color additives, these complexes can also play a role as antioxidant compounds in food matrices. In fact, Rocha et al. (2022) applied betalains nanodispersions produced with polyethylene glycol and low molecular chitosan to Greek yogurt and reported an increase in the rheological stability of the product after 21 days of storage at 4 ºC compared to pristine betalains extract. Moreover, the betalains nanodispersions did not affect the viscoelastic properties of the yogurt. Regarding the betalains stability, it was reported a significant increase in thermal stability and in acidic conditions (pH 3.0 and 5.0).

## 2.3 ELECTROSPRAYING

The principle of electrospraying, also known as electrohydronamic atomization, consists of an application of an electric field in a dispersion to produce particles. The equipment consists of a voltage power supply, a syringe pump, a syringe with a defined diameter, and a collector for the samples. The voltage used is usually in the order of kilovolts (kV), and the electric field applied in the process can reduce the chance of aggregation of the particles by electrostatic repulsion, since the particles can be charged during the drying process (Boda et al., 2018). A schematic image is represented in Fig. 4.

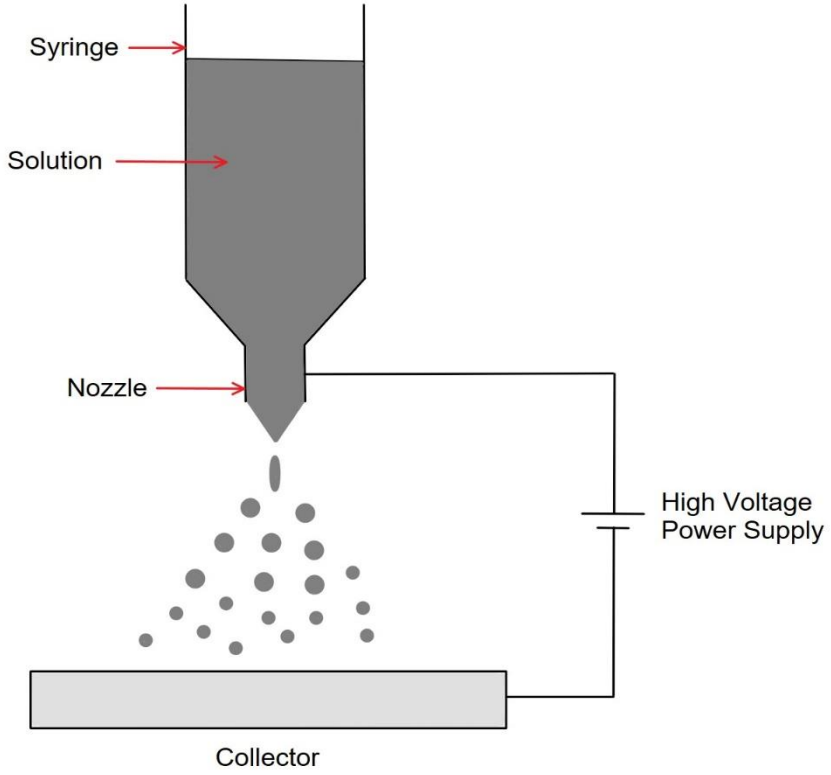


Fig. 4 – Schematic overview of an electrospraying process

The solvent dielectric constant is essential for the electrospraying process. Since the principle of the technique is the application of an electric field, the material must be able to be conducted to the cone-jet during the drying process, therefore, the use of organic solvents can be more appropriate. The vapor pressure and the volatility of the solvent is should be also taken into consideration for the drying process, since these properties can play a role in the morphology of the particles. In fact, if the solvent is very volatile, this solvent might evaporate coming off the nozzle to the collector. In the case of solvents with very high vapor pressure, the morphology of the particles can be affected due to the evaporation of these solvents before forming the dry material. The intensity of the voltage applied in the process also plays a role in determining the size of the particles. For example, a higher voltage can increase the mobility of the biopolymer within the droplet and the solution conductivity, which contributes to a reduction in the size of the particles (ALMERÍA; GOMEZ, 2014; BODA et al. 2018).

The effect of the concentration of the organic solvents on the morphology of natural pigments-biopolymers complexes was evaluated by Rodrigues et al. (2020). In this study, (WPI) was used as wall material to encapsulate beta-carotene via electrospraying, with an output voltage of 20 kV and a flow rate of 0.5 mL/h. Three solutions were produced with a

final concentration of 5, 10, and 15% of ethanol. The results obtained by Dynamic Light Scattering (DLS) suggest that higher concentrations of ethanol produced larger capsules with a higher polydispersity index, which induces potential protein aggregation. According to the authors, the solvent destabilized the protein structure, increasing the unfolding by exposing its hydrophobic reactive groups. Transmission Electron Microscopy (TEM) and Scanning Electron Microscopy (SEM) images showed that the nanocapsules maintained their spherical morphology after rehydration in water, indicating that the electrospraying process with a biopolymer enhanced the water dispersibility of the material, despite beta-carotene's hydrophobicity.

Importantly, the effect of solids content in the dispersions can also affect the electrospraying process, as reported by Atay et al. (2018). The authors evaluated the effect of different proportions of gelatin/chitosan blends (20-80% volume ratio, respectively) as wall materials, as well as the solids content (2-6.8 g/100 mL), and the concentration of acetic acid (either at 20 or 80%) to produce nanocapsules of anthocyanin-rich black carrot extract (17 wt%) via electrospraying. In this study, the electrospraying process was only possible using blends containing 2 to 8% wt solids content. For the blends with low and medium molecular weight chitosan, 6.5 to 6.8 wt% of solids content were required to produce the capsules. According to the authors, the solids' content plays an important role to promote the chain entanglement, consequently, forming the capsule structure. Regarding the anthocyanins release in ethanol (~ 13-32%) and in 3% solution acetic acid (~ 58-75%), the gelatin:chitosan ratio strongly affected the anthocyanins release, since chitosan is less soluble than gelatin in both release media. The encapsulation efficiency for the selected blends was around 76%.

The SD and electrospraying methods were compared by Mahalakshmi et al. (2020), who produced micro and nanocapsules of beta-carotene in zein protein. In general, the electrosprayed nanocapsules presented a higher encapsulation efficiency (61-82%) and a smaller average size compared to the SD microcapsules. Moreover, the authors reported a greater stability for the electrosprayed nanocapsules under *in vitro* simulated gastrointestinal digestion, and also a greater beta-carotene's bioaccessibility for the nanocapsules, compared to the SD microcapsules. This difference can be explained by the fact that the higher temperatures applied in SD processes can contribute to the thermal degradation and oxidation of natural pigments, lowering the encapsulation efficiency of these compounds.

One of the advantages of the electrospraying method is that the process can be carried out under room temperature and atmospheric pressure, meaning that the electrospraying process can be used to produce natural pigments-biopolymers complexes with

the aim to maintain their antioxidant properties and reduce the thermal degradation of natural pigments during the process (GÓMEZ-ESTACA et al. 2015; MAHALAKSHMI et al. 2020).

## 2.4 EMULSIFICATION

Thermodynamically, emulsions are unstable systems due to the elevated interfacial tension between two immiscible phases and the quantity of droplets present in the disperse phase, which significantly increases the area of interfacial tension. Oil-in-water emulsions (O/W) are classified as the ones that have oil droplets dispersed in a water phase, whereas water-in-oil emulsions (W/O) are water droplets dispersed in an oily phase (SILVA et al. 2016). Several destabilization mechanisms can occur in these systems, such as coalescence and Ostwald ripening. Coalescence is an irreversible process where one droplet is formed by the merging between two or more droplets, and the Ostwald ripening mechanism is strongly influenced by the polydispersity of the particles in the media. This phenomenon is characterized by the diffusion of smaller droplets into larger droplets due to the pressure difference between the droplets, causing the smaller droplets to be more soluble in larger monomer droplets. The emulsions are also susceptible to kinetic destabilization processes, such as flocculation, coagulation, and creaming. The creaming phenomenon is directly proportional to the radius of the droplets and the density difference between the two phases, and inversely proportional to the viscosity of the media. On the other hand, the flocculation and coagulation processes are more influenced by the electrostatic repulsion between the droplets. However, the rate and incidence of these destabilization processes can be delayed according to the properties of the systems, such as the pH, addition of salts, and the temperature disturbance (HO et al. 2017; YAMASHITA et al. 2017; MCCLEMENTS; JAFARI, 2018).

The first step to produce an emulsion is the high-energy homogenization. The formulation of the emulsions is very important, including the use of surfactants or emulsifiers, since these compounds have an amphiphilic nature and can interact with the disperse and the continuous phase simultaneously, reducing the interfacial tension between both phases (NAKAMA, 2017; YAMASHITA et al., 2017). Moreover, the electric charge on the surface of the droplets can delay droplet aggregation due to the electrostatic repulsion between the particles. In fact, the ζ-potential is a property used to measure the surface charge of the particles and involves the pH, the ionic strength and also the type of the ions of the suspension in the media where the particles are dispersed. This parameter is related to the kinetic stability

of emulsions, where a ζ-potential higher than 30 mV in absolute value is a reference to electrostatic stable emulsions. Nonetheless, the ζ-potential by itself should not be used as a kinetic stability measurement. In the case of non-charged particles, the droplet aggregation can be delayed by steric repulsion. The steric repulsion occurs due to a reduction of possible conformations between two or more molecules (CHARLES, 1992; XU 2008).

In addition, the homogenizer equipment (e.g., high pressure, ultrasound, microfluidizer) types and parameters, such as the concentration of the oil phase, play an important role in determining the morphology of the droplets and the properties of the emulsion. Preferentially, a less unstable emulsion is usually composed by particles with homogenous size distribution (MCCLEMENTS; JAFARI 2018; FU et al. 2019; SU et al. 2020).

In fact, the influence of the size of the droplets on the stability and rheological characteristics of emulsions was evaluated by Mohammed, Ishwarya, and Nisha (2021). Beet extract emulsions were prepared by rotor-stator homogenization and rotor-stator homogenization followed by ultrasound, respectively. According to the authors, regarding the thermal and pH stability of betalains, the nanoemulsions presented better results when compared to the microemulsion. Moreover, during storage, the nanoemulsion showed a lower droplet thickening rate and better color stability than the microemulsion, due to its nanoscale droplet diameter and higher viscosity. In general, based on Stokes' law, the higher the apparent viscosity and smaller the size of the emulsion droplets are important for the stability of non-Newtonian fluids. This fact can be attributed to the sluggish movement of particles in the system, as the viscosity of the continuous phase is increased (COKER, 2007).

Double emulsions consist of two types of single emulsions (O/W and W/O) and can also be used as carrier agents to natural pigments. The most common forms are water-in-oil-in-water (W/O/W) and oil-in-water-in-oil (O/W/O). In this study carried out by Paula et al. (2018), guar gum (0.75-1.75%) was used in double emulsions to increase the stability of anthocyanins, increasing the half-life time of these pigments by 2.4 times. In this system, it was also observed a high encapsulation efficiency of 90.6%.

Different homogenization parameters to produce double emulsions were evaluated by Bamba et al. (2018). The parameters evaluated by the authors were homogenization pressures (50, 100, and 200 MPa) for the final double emulsion, the stirring speed (3000, 6000, and 12,000 rpm), and time (10, 15, and 20 minutes) for the coarse double emulsion, and the stirring time only for the primary emulsion. The double emulsions containing anthocyanins and polyphenols from blueberry pomace were stabilized by whey proteins. In

general, all the parameters evaluated affected the morphology of the particles and the ζ-potential of the emulsions. Under the conditions of 50 and 100 MPa of pressure, the particles presented less polydispersity size. However, at 200 MPa, the proteins can be denatured, which modifies their structure and can affect the availability of the binding sites present in the protein, which can affect the stability of the droplets. Moreover, the denaturation of proteins can significantly modify the net charge of the system, due to the exposure of hydrophobic or hydrophilic groups of the macromolecule (Roche and Royer 2018). According to the authors, the time and the stirring speed of the homogenization also affected the morphology of the particles. In fact, it was observed that the high speed of 12000 rpm affected negatively the stability of the emulsions, due to the increase of the temperature during the process, which promotes destabilization processes, such as coalescence.

Similarly, Pagano et al. (2018) reported that the effects of the flow rate in the microchannel used during the emulsification process can also affect the stability of betalains in microcapsules in W/O/W emulsions. In general, the authors reported that the flow rate of the microchannel affected the droplet size of the particles, with the maximum polydispersity achieved with higher values of flow. Also, the lower concentration of emulsifiers seems to collaborate with emulsion breaking and coalescence occurrences. In this system, betanin acted as a stabilizer causing electrostatic repulsions between the droplets, resulting in a decrease in the polydispersity index of the particles.

In general, emulsification encapsulation can enhance the kinetic stability of natural pigments against oxidative and degrading agents (LIU et al. 2019). In this study carried out by Guo et al. (2022), the β-carotene emulsions produced with curcumin in pea protein isolate complexes presented an encapsulation efficiency for β-carotene of 92% and a bioaccessibility for this pigment of 32%. Moreover, after exposure to UV radiation and thermal treatment (85 ºC for 90 min), the authors reported a retention rate of 18 and 34%, respectively, for β-carotene. These systems can be produced at a low cost and can be applied to both hydrophobic and hydrophilic systems, meaning that they are very suitable and versatile as carriers to natural pigments (SILVA et al., 2016). In fact, betalains double emulsions were added to yogurt in this study reported by Cenobio-Galindo et al. (2019). According to the authors, betalains double emulsions were successfully added to yogurt, increasing the antioxidant activity and the bioaccessibility of antioxidant compounds in the product, as well as playing a role as a color additive. Moreover, the addition of the emulsions to the yogurt did not affect the lactic acid bacteria present in the yogurt after 36 days of storage.

### 2.4.1 PICKERING EMULSIONS

The main differences between conventional emulsions (CE) and Pickering emulsions (PE) are that CE are usually prepared by the homogenization between two immiscible components, e.g., water and oil, with the addition of an emulsifier. After the process, the disperse phase is composed by small droplets, and the continuous phase is composed by the media where the droplets are dispersed in. The PE method consists of emulsions stabilized by solid particles. The solid particles accumulated at the oil-water interface can provide a steric barrier, which helps to delay the droplets aggregation. Compared to the conventional emulsions, the PE are usually more physically stable due to the stronger adhesion of a layer of solid particles around the droplets in the disperse phase (WU; MA, 2016). Moreover, many natural biopolymers can be used as an emulsifier in PE, such as cellulose derivatives, starch, and proteins (FU et al., 2019; TAN et al., 2021). A schematic representation of conventional and Pickering emulsification processes is presented in Fig. 5.

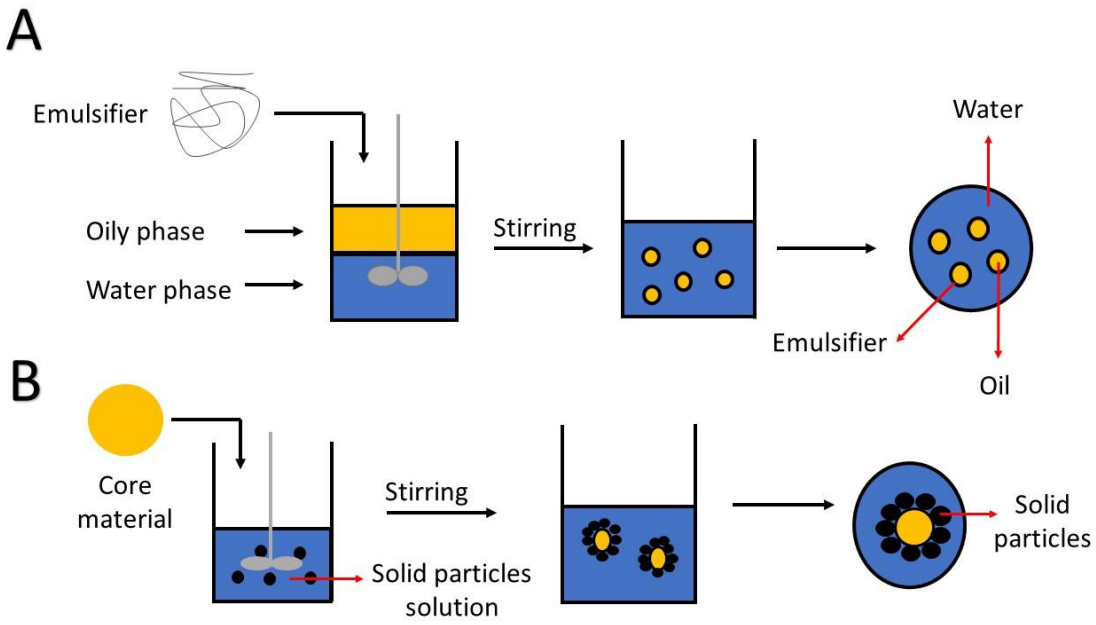


Fig. 5 – Schematic representation of conventional (A) and Pickering (B) emulsification processes

Fu et al. (2019) produced beta-carotene Pickering emulsions with wheat gluten nanoparticles (WGN) with and without the addition of xanthan gum (XG). The use of xanthan gum to produce the Pickering emulsions significantly affected the particle size (WGN-XG: 21.2 μm) when compared to the Pickering emulsions without the addition of xanthan gum

(9.4 μm). The addition of XG also affected the ζ-potential, which varied from 22.7 mV to -31 mV. Since xanthan gum is an anionic polysaccharide, this effect on ζ-potential was expected (HUBLIK, 2012). The results for ζ-potential are also an indication that the cationic WGN oil droplets are attached to the anionic groups of the biopolymer. At pH ≥ 5, the emulsions started to exhibit drop aggregation and creaming, fact attributed to the increase of negative charge in the media, which diminished the electrostatic repulsion between the oil droplets; and to the isoelectric point of the wheat gluten protein, which impacts the structure of this biopolymer and, consequently, affects the stability of the emulsion (GENNADIOS et al., 1993). However, the addition of XG in the emulsions was positive regarding the pH stability analysis, since the ζ-potential was negative for all the pH ranges evaluated (4.0-8.0), collaborating with the electrostatic repulsions between the oil droplets. The complexes presented good thermal stability, retaining a considerable amount of beta-carotene, over 98%, after exposure to 65 ºC/30 min and 90 ºC/3 minutes. Nevertheless, after storage in an incubator at temperatures of 25, 37, and 60 ºC in the darkness, the content of carotenoids dropped significantly. According to the authors, the Pickering emulsions containing xanthan gum as emulsifier presented a better stability compared to the other samples, indicating a protective effect of the biopolymer for the droplets, acting as a barrier against oxidative reactions.

Beta-carotene Pickering emulsions produced by Tan et al. (2021) presented significant stability regarding the creaming index and the water holding capacity. Medium chain triglyceride oils and gelatin nanoparticles were used as stabilizers for the Pickering emulsions. According to the authors, the difference of the density between the oils were determinant for the results of stability, since the less dense oil (corn oil, $\rho = 0.93$ kg.m$^{-3}$) tend to move more upwards when compared to medium chain triglyceride oil ($\rho = 0.95$ kg.m$^{-3}$), favoring the creaming phenomenon.

According to Ju et al. (2020), PE exhibits unique characteristics, including extraordinary emulsion stability, improved oxidative stability, and resistance to in vitro digestion, being important in the stabilization of protein-polyphenol nanoparticles. These authors produced PE using soy protein (SPI)-anthocyanin (ACN) complex nanoparticles with a soy protein solution. According to the authors, SPI and ACN were covalently bonded in the nanoparticles. The addition of anthocyanin significantly increased the ζ-potential and decreased the surface hydrophobicity of the droplets, which was attributed to the electrostatic repulsion as the net charge increases. The oxidative stability of the Pickering emulsions was also evaluated and the results showed an increase in the oxidative stability proportionally to

the concentration of anthocyanins in the complexes; similar results were found regarding the creaming index, with a reduction up to 50%. Furthermore, the addition of anthocyanin significantly increased the antioxidant activity in these systems. In this study, the authors also reported stability of the Pickering emulsions containing anthocyanins by retarding up to 6.9% release of free fatty acids after *in vitro* gastrointestinal digestion. Similarly, Qi et al. (2020) reported an increase in long-term storage, chemical stability, and bioaccessibility for beta-carotene in PE produced with cellulose nanocrystals, soybean oil, and beeswax.

In general, similarly to CE, PE can be effective carriers to natural pigments, collaborating with their increased stability under certain conditions (LIN et al. 2020). These systems can be potentially applied to food matrices.

## 3. EVALUATION OF BINDING PARAMETERS BETWEEN NATURAL PIGMENTS AND BIOPOLYMERS

The evaluation of binding parameters between natural pigments and biopolymers complexes is very useful to determine whether interactions occur between both compounds or not. If the complexation process is favored, the thermodynamic parameters can provide information about the nature of the interactions, and about their thermodynamic stability under different conditions, such as different ranges of pH, temperature, and concentration of salts. Therefore, the understanding about binding parameters is also very useful to apply a natural pigment-biopolymer complex in food systems (PAIVA et al., 2020; REZENDE et al. 2020).

The process of complexation between natural pigments and biopolymers is driven by four main interactions: hydrogen bonding, electrostatic interactions, hydrophobic interactions, and van der Waals forces. In order to evaluate the binding parameters in a natural pigment-biopolymer complex, three thermodynamic properties are taken into consideration: the standard enthalpy change of complex formation ($\Delta H^0$), which roughly indicates the gain or loss of the amount of energy during the complex formation. The standard entropy change of complex formation ($\Delta S^0$) is roughly related to the energy or the molecules' distribution in the system, and the standard Gibbs free energy change of complex formation ($\Delta G^0$) indicates whether the complex formation overcomes the interacting free molecules (MOHAMMADALINEJHAD; KUREK, 2021; LI et al., 2021; REZENDE et al., 2022).

Some techniques, such as isothermal titration calorimetry (ITC), circular dichroism spectroscopy (CD), Fourier transform infrared spectroscopy (FTIR), surface plasmon

resonance (SPR), and fluorescence spectroscopy are powerful techniques to characterize interactions between natural pigments and proteins. Recent investigations regarding their role in determining thermodynamic parameters and providing information about the nature of interactions have been intensely reported (LANG et al., 2019; PAIVA et al., 2020; MATENCIO et al., 2021). The principle of each technique and their applications in evaluating binding parameters between red natural pigments and biopolymers are discussed below.

Table 2 summarizes the most common techniques used to evaluate binding parameters between natural pigments and biopolymers, as well as their advantages and drawbacks

.

**TABLE 2:** Overview of some techniques commonly used to evaluate interactions between natural pigments and biopolymers

| Technique | Principle of the technique | Advantages | Drawbacks | References |
|---|---|---|---|---|
| **Isothermal Titration Calorimetry (ITC)** | Measurement of the amount of energy (heat) over time. This experiment is carried out under constant temperature and pressure; thus, the amount of energy is equal to the heat | Very sensitive and quick technique. Provides thermodynamic parameters $(\Delta H^0, \Delta G^0, n, K_A)$ in a single experiment | A sigmoidal curve must be obtained in the experiment in order to obtain reliable results regarding the binding parameters of complexation, which requires some pre-tests | (Dumas et al. 2016; Khalef et al. 2016) |
| **Circular dichroism** | Measurement of the | Very useful to identify | This method can be performed | (Ranjbar and Gill 2009; |

| | | | | |
|---|---|---|---|---|
| **spectroscopy** | difference in the absorption of the circularly light emitted by chromophores, comparing both directions (right-handed and left-handed) in a determined range of wavelength | structural changes, the process of folding and unfolding of proteins, and therefore, protein-natural pigments interactions. Quick and reliable measurements | only for proteins as carriers in natural pigments-biopolymers complexes. The thermodynamic parameters of interactions are not provided by the equipment | Jiang et al. 2017) |
| **Fourier Transform Infrared spectroscopy (FTIR)** | Excitation of electrons of covalent bonds by infrared radiation emitted by the equipment | Quick and sensitive method. This technique provides information about the presence of covalent bonds in the structure of the molecules | This method does not provide thermodynamic parameters of interactions | (He et al. 2016; Valand et al. 2020) |
| **Fluorescence spectroscopy** | Measurement of the fluorescence intensity emitted by fluorophores | This technique is very quick and accurate, and can provide some parameters, such | The experiment only provides information about interactions in the fluorophores | (Karoui and Blecker 2011; Ghosh et al. 2016) |

| | | | | |
|---|---|---|---|---|
| | present in the protein structure | as the binding constant ($K_A$) and the stoichiometry ($n$). Some mathematical models can be used to obtain $\Delta G^0, \Delta H^0$ and $\Delta S^0$ | present in the protein structure | |
| **Surface Plasmon Resonance (SPR)** | The concentration of analyte flowing over the surface of a sensor chip is proportional to the measured refractive index. This variation when an interaction occurs is detected by the equipment | This technique allows the evaluation of the kinetic properties of intermolecular interactions in real-time. Some mathematical models can be used to obtain $\Delta G^0, \Delta H^0$ and $\Delta S^0$ | SPR sensors require high-cost investment, component and operation. Moreover, some chemical modifications in the structure of the biopolymer might be required due to the immobilizer sensor present in the equipment | (Prabowo et al. 2018; Zhou et al. 2019; Rezende et al. 2020) |

## 3.1 ISOTHERMAL TITRATION CALORIMETRY

Isothermal titration calorimetry (ITC) is a powerful technique to evaluate binding parameters between a biopolymer and natural pigments. In the case of natural pigments-biopolymer formation process, the binding parameters are obtained from the measurement of

the amount of energy in the form of heat, per unit of time, which is released or absorbed as the bioactive compound is titrated in the biopolymer suspension. In the calorimeter, the thermodynamic processes take place under conditions of constant temperature and pressure. Under these conditions, the enthalpy change is equal to the heat resulting from the titration. This resultant heat is compared to the reference cell, which contains the non-titrated biopolymer dispersion, under the same conditions. The heat generated by the reaction and interactions between the two components is measured over time. However, it is important to consider the nonspecific effects in the total heat, such as the effect of the dilution in the solution, and the heat from mixing buffers. In order to calculate these effects, one experiment is usually performed only with the solvent (GOLAS et al., 2019; WANG et al., 2022). A schematic representation of an ITC is presented in Fig. 6.

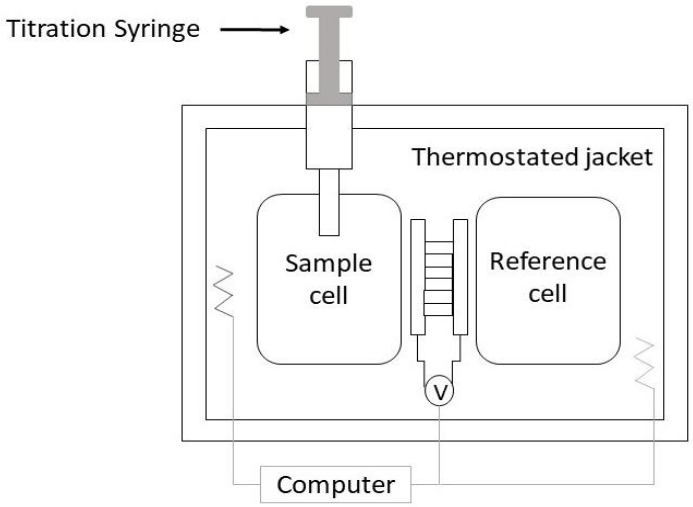


Fig. 6 – Schematic overview of an ITC

The simple equilibrium between a natural pigment and a biopolymer is given by Eq. 1, where L is the analyte and M is the biopolymer:

$$L + M \overset{K_A}{\Leftrightarrow} LM \qquad (1)$$

where the parameter $K_A$ is the binding association constant.

The experiment provides some thermodynamic parameters: the enthalpy change ($\Delta H^0$), the binding stoichiometry (n), the binding association constant ($K_A$), and the Gibbs

free energy change ($\Delta G^0$) in a single experiment. The term $T\Delta S^0$ is determined indirectly by obtaining $\Delta G^0$ and $\Delta H^0$, as shown in Eq. 3. The heat capacity change ($\partial C_p$) is strongly related to hydrophobic interactions and hydrogen bonding between the compounds, and can also be attributed to solvent-water interactions. This parameter can be determined by repeating the experiment at different temperatures (COOPER, 2005; KHALEF et al., 2016).

The thermodynamic parameters provided by the ITC are given by the following equations:

$$\Delta G^0 = -RT \ln K_A \qquad (2)$$

$$\Delta G^0 = \Delta H^0 - T\Delta S^0 \qquad (3)$$

$$\partial C_p = \frac{\partial H}{\partial T} \qquad (4)$$

## 3.2 CIRCULAR DICHROISM SPECTROSCOPY

The principle of circular dichroism spectroscopy consists of emitting a light by chromophores present in the equipment in a determined range of wavelength. The circular dichroism region is characterized by the area where there is a difference in the absorption of the circularly light comparing both directions (right-handed and left-handed). The spectrum is generated by a secondary absorbance component, which measures the final difference of absorbances of the light in both directions and provides the spectrum of this difference versus the wavelength (nm) (RANJBAR; GILL, 2009).

Circular Dichroism (CD) can identify any structural changes in the secondary structure of proteins in the ultraviolet (UV) wavelength region (~ 240 to 170 nm). Moreover, the equipment can be used to monitor the physical and chemical effects in the media in the local tertiary structure environment of aromatic amino acid residues of proteins in the UV region (~ 300 to 260 nm). Each peak present in the spectrum generated by the equipment is equivalent to a particular structure of proteins, such as α-helical and β-sheet structures. In fact, the wavelength between 270-280 nm corresponds to the vibration of C-O and N-H present in the structure of the protein (Jiang et al. 2017). The spectrum can also provide information about turns and other secondary structures of the molecules with relatively high accuracy. This method is very useful to evaluate interactional studies involving protein-ligand

interactions, as well as the fold and unfolding of macromolecules. In the case of complexation between proteins and natural pigments, the change in the structure of the carrier can provide information about the complexation process (RANJBAR; GILL, 2009).

## 3.3 FOURIER TRANSFORM INFRARED SPECTROSCOPY (FTIR)

The principle of the FTIR technique is to excite the electrons of covalent bonds by infrared radiation emitted by the equipment, which promotes vibrational transition, leading to stretching and/or bending motions of the molecular covalent bonds. The absorbance/transmittance intensity of the radiation by these groups in a specific wavelength corresponds to a specific group of covalent bonds. The Fourier transform calculation provides a spectrum of intensity versus wavenumber, where the intensity of the absorbance or transmittance is proportional to the quantity of these groups of covalent bonds present in the molecule structure (VALAND et al., 2020).

The instrument uses a signal yielded by the interference between two infrared beams as a function of the change in path length between both beams, which is the interferogram. This process is achieved by using a Michelson interferometer, and this generated signal is decomposed into the frequencies that form the signal by the Fourier transform algorithms (STUART, 2005). The Michelson interferometer is a system composed by a light source, a semi-reflecting beam splitter and two perpendicular mirrors (one moving and one stationary). In the first part of the process, the infrared radiation is equally split by a beam splitter and reflected back to the beam splitter by both perpendicular mirrors, where they are recombined and interfered. This interference is a result of the difference in path length caused by the moving mirror (STUART, 2005; ALBERT et al., 2011). A schematic overview of the FTIR instrument is presented in Fig 7.

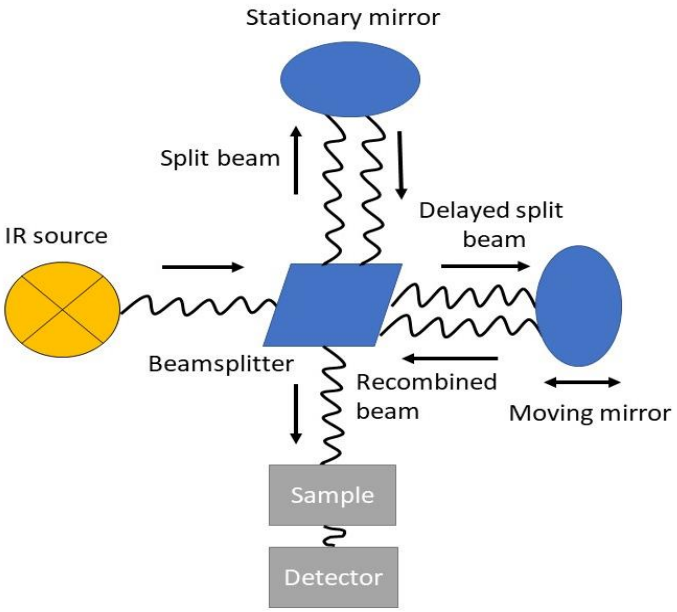


Fig. 7 – Schematic overview of a FTIR process

Even though the main interactions between natural pigments and biopolymers are driven by hydrogen bonding, electrostatic interactions, hydrophobic interactions, and van der Waals forces (FERNANDES et al. 2013; OTÁLORA et al. 2015), FTIR can be used to indicate how the complexation process between biopolymers and natural pigments can affect specific groups present in their molecular structure, as a result of the interactions between both compounds (HE et al., 2016; ROCHA et al., 2018; LI et al., 2021).

## 3.4 FLUORESCENCE SPECTROSCOPY

Fluorescence spectroscopy measures the fluorescence intensity emitted by the fluorophores present in a protein structure. When these fluorophores bind or interact with a compound, the fluorescence intensity measured by the equipment decreases, as a result of the decrease of the fluorophores sites available to interact. This restriction regarding the determination of the available specific binding sites present in the molecule structure can be a disadvantage of this method (GHOSH et al., 2016).

The principle of this technique is the following: the excitation of the molecule caused by the absorption of light, leading the molecule to transit from an electronically excited state to a lower one without any radiation. The fluorescence emission occurs after excitation, when the molecule returns to its ground stable state. This fluorescence quenching mechanism can be static or dynamic. The dynamic process involves collision and diffusion between molecules,

which can affect the complexation process, as a result of the increase of temperature and, consequently, the quenching constant, $K_{SV}$. In the static quenching, the quenching constant ($K_{SV}$) is inversely proportional to the temperature, and non-fluorescent complexes are formed by intermolecular interactions (KAROUI; BLECKER, 2011; PACHECO et al., 2020). The quenching constant ($K_{SV}$), and the quenching rate constant of the protein fluorescence ($K_Q$), can be determined by the plot of $F/F_0$ versus the concentration of the compound of interest ($c_Q$). In the case of biopolymers, the maximum scatter collision quenching constant is $2.10^{10}$ (L.$mol^{-1}.s^{-1}$); if $K_Q$ is greater than the maximum scatter collision, the quenching mechanism is considered static. The calculations for the fluorescence spectroscopy parameters for the formation of complexes are usually given by Stern-Volmer equation (Eq. 5) (GHOSH et al., 2016; MAGALHÃES et al., 2021):

$$\frac{F}{F_0} = 1 + K_{SV}c_Q = 1 + K_{SV}\tau_0 c_Q \quad (5)$$

where $F$ and $F_0$ and are the fluorescence intensities in the presence and absence of the compound, respectively. $c_Q$ is the final concentration of the compound of interest, and $K_{SV} = K_Q\tau_0$ corresponds to the biomolecular quenching constant, where $\tau_0$ is the lifetime of fluorophore in the absence of the quencher. Parameters, such as the binding constant ($K_Q$) and the stoichiometry ($n$) for the complex can be determined by this method. Furthermore, mathematical models can be applied in some cases to obtain some thermodynamic parameters, such as $\Delta G^0, \Delta H^0$ and $T\Delta S^0$ (PAIVA et al., 2020).

## 3.5 SURFACE PLASMON RESONANCE (SPR)

The SPR equipment is composed by metal-dielectric waveguides or chip, and the surface plasmon. When the biopolymer solution is injected in the equipment, part of the structure is immobilized in the biosensors. After the preparation, the solution containing the natural pigment is injected in the equipment, and the difference of the refractive index caused by the interaction between the available surface area of the biopolymer and the natural pigment is measured overtime. The electrons present in a chip or metal-dielectric surface absorb the light photons emitted by the equipment at a specific angle, generating a surface plasmon resonance. Also, it is important to note that the electric field generated by the resonance of plasmons can change the refractive angles of the light. The drop of the light

intensity is a consequence of the surface plasmon resonance, since part of the energy is absorbed by the resonance coupling. When the incident angle of the polarized light exceeds the critical value, the total reflection is achieved, and the signal of the reflected light is converted either to a resonance angle or wavelength, which is identified by the detector attached to the equipment. (PRABOWO et al., 2018; ZHOU et al., 2019). A schematic representation of the Surface Plasmon Resonance is presented in Fig. 8.

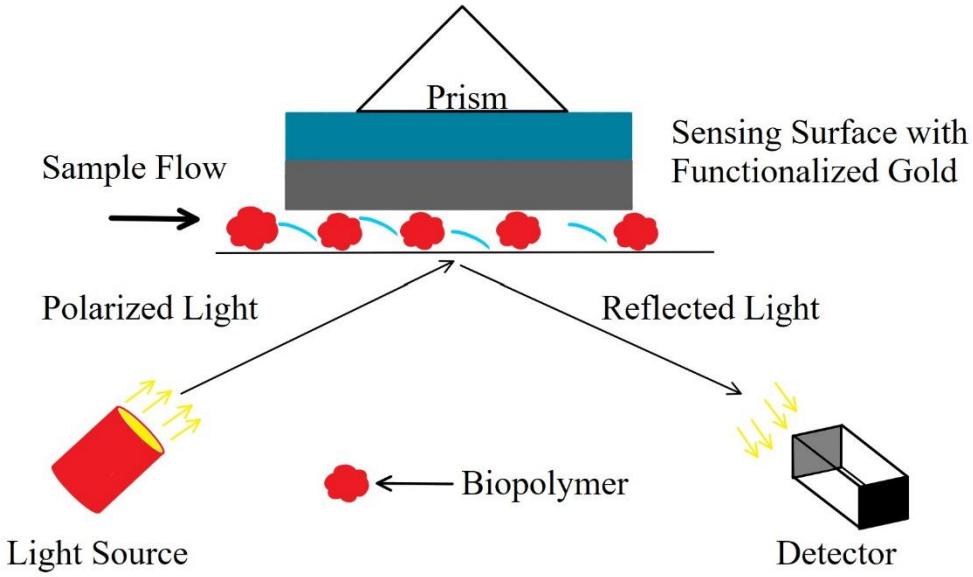


Fig. 8 – Schematic overview of a SPR process

The experiment provides a graphic of the change in response of SPR sensor, Resonance Units (RU) per time, where the processes of association and dissociation can be observed. The association phenomenon is achieved with an increase of Resonance Units (RU) within time, whereas the dissociation is characterized by the decrease of Resonance Units (RU) within time. It is possible to obtain the association ($K_a$) and dissociation constant ($K_d$) for the complex formation. In this sense, SPR can also be used to measure the kinetic stability of complexes (PRABOWO et al., 2018; REZENDE et al., 2020).

SPR is a very sensitive and non-destructive method. Other advantages of this technique can include the label-free and the possibility to evaluate the thermodynamic properties of reactions in real time (HUDSON et al., 2022).

## 3.6 BINDING PROPERTIES BETWEEN NATURAL PIGMENTS AND BIOPOLYMERS

In order to apply natural pigments-biopolymer complexes in food systems, the evaluation of binding properties between the compounds are essential to determine the main driven power of the complex formation. In this review, some studies regarding the binding properties between the most common natural pigments, such as anthocyanins, carotenoids, and betalains with biopolymers are presented under different conditions. It is important to note that the hydrophobic or hydrophilic nature of both compounds play an important role in the complexation process. Moreover, some conditions, such as the pH, temperature, denaturation of proteins can also affect these interactions between the pigments and the biopolymers (SILVA et al., 2018; LANG et al., 2019; MARCHUK et al., 2019; REZENDE et al., 2020).

Beta-carotene, LUT and zeaxanthin (ZEA) can bind with protein bovine serum albumin and glutathione S-transferase in a lipid bilayer model with egg yolk phosphatidylcholine by interacting with the protein surface and causing changes in their secondary structure. In this model, the complexes mostly interact with liposomes, as expected, due to the predominant hydrophobic nature of these pigments. Such interactions can assist the pigments transfer to the lipid membrane. Biologically, it is emphasized the potential biological application of the complex GST-zeaxanthin in transporting xanthophyll into the retina of the human eye (RESZCZYNSKA et al. 2015).

Paiva et al. (2020) investigated the thermodynamic parameters of a complex formed by bovine serum albumin (BSA) and lutein using fluorescence spectroscopy. The results obtained showed that the main interactions between BSA and lutein are hydrophobic. According to the authors, the complex formation was an endothermic process, with $\Delta G^{o} < 0$ and gradually decreasing with the increase of temperature (25-45 ºC), indicating that the complex formation is favored as the temperature increases. When submitted to high temperatures, the protein can unfold and expose its hydrophobic sites in its structure, collaborating with the formation of the complex. Silva et al. (2018) found similar results investigating the binding parameters between beta-carotene and BSA by using fluorescence spectroscopy method. In this study, binding parameters were evaluated in a range of temperature from 20 to 55 ºC, and it was reported that the complex is less unstable at high temperatures. It was possible to observe that the binding constant ($K_a$) increases, whereas $\Delta G^{0}$ and $K_{SV}$ gradually decrease with the increase of temperature, indicating that the formation of the complex is favored by the increase in the temperature. Also, it was found that beta-carotene interacts more with native BSA than with unfolded BSA, interacting mainly with the

hydrophobic cavity of the protein. According to the authors, during the process of denaturation, BSA lost its preferential binding site for beta-carotene. Regarding the effect of the complex on beta-carotene's photostability, the photodegradation constants of the beta-carotene in the protein complex decreased about 3 times.

The binding parameters between lutein and lysozyme were evaluated by Rezende et al. (2020) with a SPR. The results indicated that lutein forms a thermodynamically stable complex with the protein. For the range of temperature evaluated (12-28 ºC) at pH 7.4, it was observed that the formation of the complex is favored by the increase in temperature. At temperatures above 18.49 ºC, the authors reported that there is a predominance of hydrophilic interactions. On the other hand, hydrophobic interactions correspond to the main interactions between lutein and lysozyme at low temperatures. However, it was demonstrated that the temperature did not play a role in the thermodynamic stability of the complex. The interactions of the carotenoid β-carotene with lysozyme were evaluated by Magalhães et al. (2021) by using SPR and fluorescence spectroscopy techniques. According to the authors, the increase in the temperature (from 12 to 28 ºC) increases the constant binding affinity and decreases the Free Gibbs energy change, meaning that the formation of the complexes is favored and become more stable with the increase in the temperature. The nature of the interactions between both compounds are predominantly hydrophobic, possibly due to the high hydrophobic nature of β-carotene.

For carotenoids specifically, studies have shown that biopolymers with hydrophobic sites available for binding are more suitable to produce complexes with these pigments, due to their hydrophobic nature. In some cases, the availability of these hydrophobic sites in the biopolymer structure can be affected by some processes, e.g., denaturation of proteins (SILVA et al., 2018; REZENDE et al., 2020). Fluorescence spectroscopy carried out by Rodrigues et al. (2020) showed that the addition of ethanol contributed to the exposure of hydrophobic clusters present in WPI structure, collaborating with the interactions between the protein and beta-carotene.

The use of anionic polysaccharides to produce complexes of betalains was evaluated by Marchuk et al. (2019). The polysaccharides beet pectin, gum arabic, xanthan gum, and sodium alginate were tested at a concentration of 1% (w/w). Fluorescence spectroscopy showed that betacyanin weakly binds with these biopolymers, indicating that no complex was formed at pH of 3.2 and 5. Despite this fact, the beet pectin can act as a stabilizer for the polymer-pigment association, due to the lower variation of color at 40 ºC and pH 5 for 24 h. According to Miguel (2018), the betalains present an anionic form at pH equal or higher than

3.0, which might have contributed to weak interactions between the anionic polyssacharides and betalains at this pH, possibly due to electrostatic repulsions between betalains and the polysaccharides.

Natural and modified cyclodextrins (CD) have been widely studied due to their ability of forming complexes. The binding parameters between these polymers and betalains derivatives were evaluated by Matencio and others (2021). According to the data obtained by the fluorescence spectroscopy, only Methyl-β-CD was chosen to phenylethylamine-betaxanthin (Ph-Bx), and Hydroxypropyl-β-CD (HP-β-CD) to indoline-betacyanin (In-Bc). According to the authors, the high encapsulation constant for In-Bc and HP-β-CD at pH 7 was due to the electrostatic charge of In-Bc, becoming available to electrostatically interact with the polymer. The effect of the temperature on the formation of the complexes was negative and the thermodynamic parameters show a direct relationship with the temperature. The use of high temperatures can weaken hydrogen bonds, which significantly affects the encapsulation constant. In general, the reactions were thermodynamically favored at 25 ºC ($\Delta G^0 < 0$), and the exothermic nature of the processes is due to the interactions between both molecules. For the complex In-Bc/HP-β-CD, the entropy change was positive, possibly due to the fact that water was released from the cavity of the polymer. According to the authors, this behavior is indicative of hydrophobic interactions, and contributes to an increase in van der Waals interactions, formation of hydrogen bonds and other interactions, due to the increase in the amount of water available to interact with other molecules in the system. For the complex Ph-Bx/Methyl-β-CD, the negative enthalpy change in the process is attributed to the encapsulation process, which leads to a more ordered system, and consequently, diminishes the entropy in the system (MIRANDA et al., 2011).

Regarding the anthocyanins, Lang et al. (2019) evaluated the binding parameters between bovine serum albumin (BSA) and blueberry anthocyanins at pH 6.6 by using fluorescence and circular dichroism spectroscopies. According to the authors, the complexation was exothermic and mainly driven by electrostatic interactions. Moreover, values obtained for $\Delta G^o$ indicate that the complex formation overcomes the interacting free molecules. In addition, data obtained by the fluorescence spectroscopy indicated a decrease of the hydrophobicity and augmentation of polarity of tryptophan and tyrosine regions, suggesting that these residues were exposed, as a result of interactions between the protein and the anthocyanins. The binding with anthocyanin strongly affected the second structure of the protein, increasing by 20% the α-helix content and a decrease of 43% in the β-sheet structure of the protein.

Circular Dichroism (CD), fluorescence, and FTIR spectroscopy were used by He et al. (2016) to evaluate the complexation between bovine β-lactoglobulin and anthocyanin malvidin-3-O-glucoside. For the investigation of the binding parameters, the authors used a final concentration of β-lactoglobulin of 10 μM and 0-50 μM of anthocyanin. For the static quenching process at pH 6.3, it was reported that malvidin-3-O-glucosidase strongly binds with β-lactoglobulin in an endothermic reaction favored by the increase of the temperature. For all the temperatures evaluated (4, 14, and 24 ºC), it was reported a decrease in ΔG° decreasing with the increase of the temperature, indicating that the complex formation overcomes the interacting free molecules as the temperature increases. The results also indicated that malvidin-3-O-glucosidase binds with β-lactoglobulin mainly via hydrophobic interactions, but also via van der Waals force and hydrogen bonding. FTIR results showed changes in intensity of the hydrophobic groups of the protein molecule, validating the predominance of hydrophobic interactions. Circular dichroism analysis also showed that the turn and random coils of β-lactoglobulin significantly decreased after complexation with malvidin-3-O-glucoside.

Interactions between anthocyanin cyanidin-3-O-glucoside and β-cyclodextrin have been evaluated in some studies. Interestingly, the use of β-cyclodextrin as a wall material was not very appropriate for color additive purposes, since the effect of anti-pigmentation was observed for the complex. Furthermore, it was reported that the interaction between the compounds did not significantly alter the equilibrium and isomerization kinetic rates constant of the anthocyanins. According to the authors, the anti-pigmentation phenomena can be explained by the fact that β-cyclodextrin can act as a catalyzer to isomerization and also accelerates the hydration constant ($K_h$). Usually, the forces that mainly drive these interactions with cyclodextrin as a host molecule to natural pigments are van der Waals force, especially hydrophobic and dipole-dipole interactions, and hydrogen bonding (FERNANDES et al., 2013).

## 4. CONCLUSIONS

The use of natural of natural pigments as a color additive is increasingly demanding in the food industry. In this sense, biopolymers-natural pigments complexes can be a suitable alternative to more stable natural color additives in food matrices. In this review, the most common methods for natural pigments complexation and their binding properties with biopolymers were discussed. The findings presented in this paper show how the wall

materials and complexation methods can affect the properties of natural pigments-biopolymers complexes. Regardless the complexation method, it was shown that in most of the cases, many characteristics of the natural pigments were improved, such as color parameters, water dispersibility, thermal and storage stability, protection against color degradation, and the bioaccessibility. These findings show a great potential for these systems to be used as color additives in food matrices along with their properties as antioxidant compounds. The use of complexation methods at low temperatures showed to be more suitable for natural pigments, especially to maintain their bioactive properties and reduce their thermal degradation when complexed with biopolymers. It was also pointed out that the parameters used in the complexation method also play a role in the structure, morphology, and antioxidant activity of natural pigments-biopolymers complexes.

Given the wall materials characteristics, proteins and polysaccharides are the most used biopolymers as carriers to natural pigments, probably due to their large structure, containing more available sites to interact in their structure, and also allowing them to entangle bioactive compounds. The evaluation of binding parameters between biopolymers and natural pigments can be very helpful to choose the most appropriate wall material as a carrier to a natural pigment. Furthermore, binding parameters between natural pigments and biopolymers showed that the hydrophobic and hydrophilic nature of the biopolymer plays an important role in the interactions between both compounds. Noteworthy pointing out that the addition of solvents, salts, and thermal processes can strongly affect the availability of the sites present in the protein structure to interact with natural pigments.

Regarding the thermodynamic techniques, SPR and fluorescence spectroscopy were the most used techniques to evaluate the binding properties between biopolymers and natural pigments. These techniques are very sensitive and can provide a wide range of information about thermodynamic properties regarding biopolymers-natural pigments complexes formation.

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# CHAPTER 2

## Betalains nanodispersions: Effects on betalains stability and on rheological properties of Greek yogurt

Rocha, F., Marques, C. M., Sousa, L. S., Minim, V. P. R., Pires, A. C. dos S., Minim, L. A., Stringheta, P. C., Jones, O. G., & Vidigal, M. C. T. R. (2022). Betalains nanodispersions: Effects on betalains stability and on rheological properties of Greek yogurt. *Food Research International*. https://doi.org/10.1016/j.foodres.2022.111583

## 1. INTRODUCTION

Betalains are red natural pigments commonly found in plants, such as beets, cacti, and amaranths. The main betalains are composed of betacyanins (BC), which present a red-violet color, and betaxanthins (BX), responsible for the brownish color. Since natural pigments can present health benefits to the human body, and artificial colorants are often related to allergenic and intolerance reactions, especially in children, the use of betalains as color additives in foods can be very suitable (WROLSTAD; CULVER, 2012; SOLYMOSI et al., 2015). However, in terms of stability, these pigments can be very unstable when exposed to high temperatures, extreme ranges of pH (< 4 and > 7), and light (AZEREDO, 2009; FU et al., 2020; GANDÍA-HERRERO; GARCÍA-CARMONA, 2013).

Betalains can be extracted by using conventional (e.g., solvent extraction) and non-conventional methods. Non-conventional methods, such as ultrasound, can lead to a higher efficiency of natural pigments extraction. Moreover, ultrasound-assisted extraction presents some advantages over the conventional methods: low temperatures can be used, it is an organic solvent-free process, and less time is required for the extraction (CHEMAT et al., 2017). This technique has been widely used to extract natural pigments (SIVAKUMAR et al., 2009; LAQUI-VILCA et al., 2018; SILVA et al., 2018).

Since natural pigments are very unstable, a complexation with a biopolymer can improve betalains' stability under certain conditions, which may be interesting to obtain relatively stable betalains for use as color additives in the pharmaceutical and food industries (OTÁLORA et al., 2015). The solid dispersion method can be used for pigments' complexation, and its principle consists of using a biopolymer as a solid solvent for bioactive compounds. This technique has been widely used to improve some properties of natural pigments (SILVA et al., 2017; ROCHA et al., 2018). Polyethylene glycol (PEG) and low molecular weight chitosan have been used as encapsulants to natural pigments in many

studies with promising results (SINGH et al., 2015; BOLLA et al., 2020; GUERRERO-RUBIO et al., 2021). PEG is a non-toxic and biocompatible polymer widely used in the pharmaceutical industry (FAROOQUI, 2018; SHI et al., 2021). Chitosan is a cationic polysaccharide obtained by deacetylation of chitin (ATAY et al., 2018). PEG at low concentrations and chitosan can be safely used as food additives (ATAY et al., 2018; YOUNES et al., 2018)

The use of natural pigments as color additives has been increasingly demanding in the food industry (SOLYMOSI et al., 2015), therefore, the effects of betalains nanodispersions on the color and rheological properties of Greek yogurt are important parameters to be analyzed. Data in the literature regarding the effects of betalains nanodispersions on these properties in food matrices are scarce. In this sense, betalains nanodispersions were produced by using the polymers PEG and chitosan as dispersants. The effects of these nanodispersions regarding the main betalains' thermal and pH stability and their effects on the color and rheological properties of commercial Greek yogurt were evaluated.

## 2. MATERIALS AND METHODS

### 2.1 MATERIALS

Beetroot powder and Greek yogurt containing 15 g of carbohydrates, 3.4 g of proteins, 6.7 g of fat, and 129 mg of calcium per 100 g were purchased in the local market. Polyethylene glycol (4000 $g.mol^{-1}$, Neon, Brazil) and low molecular weight chitosan (50-190 kDa, 75-85% deacetylated, Sigma-Aldrich) were used to produce beetroot extract (BE) nanoparticles. Sodium citrate and citric acid (Dinâmica, Brazil) were used to prepare the buffer solutions (pH 3.0 and 5.0). Hydrochloric acid (HCl) and sodium hydroxide (NaOH) (Neon, Brazil) were used to adjust the pH of the solutions when appropriate. Deionized water was used to extract BE and to prepare the solutions for the determination of the ζ-potential and the average size of the particles. All the reagents used for the experiments were analytical grade.

### 2.2 EXTRACTION OF BEETROOT EXTRACT

Beetroot extract (BE) was extracted according to the methodology described by Tabio-García et al. (2021), with some modifications. Deionized water at pH ~ 6.7 was used as a solvent for the extraction, with a proportion of 1:30 beetroot powder:solvent (w/v). The extraction was carried out via ultrasound (Elma, Transsonic TI-H-10, Germany) with a frequency of 25 kHz for 60 min at 30 ºC.

## 2.3 PRODUCTION OF BEETROOT EXTRACT NANODISPERSIONS

In general, 0.1 to 1% (w/w) of betanin is sufficient to produce the desired color in food products (HENDRY; HOUGHTON, 1996). In this sense, the nanoparticles were produced according to the methodology described by Rocha et al. (2018), with some modifications. BE containing 44.4 mg of BC and 31.8 mg of BX per 100 g, with a BC:BX mass ratio of 1.4 was used to produce the nanodispersions. PEG:BE dispersions (PBE) were prepared with a mass proportion of 10:1 (w/w) of PEG:BE, and PEG:chitosan:BE dispersions (PCBE) with a mass proportion of 5:5:1 of PEG:Chitosan:BE, respectively. For PEG:BE nanodispersions (PBE), 0.18 mg of BE was mixed with 180 mg of PEG 4000. For PEG:chitosan:BE dispersions (PCBE), 0.18 mg of BE was mixed with 90 mg of PEG 4000 and 90 mg of chitosan. After that, 100 mL of deionized water was added under magnetic stirring until complete dissolution. The dispersion was placed in an ice bath and sonicated in Ultra-Turrax (IKA, 120W) for 3 min, under pulse conditions of 30 s on and 10 seconds off. After that, the samples were centrifugated at 4600 g for 10 min at 4 ºC and the supernatants were lyophilized. Finally, the samples were stored at -20 ºC in absence of light until further analysis.

## 2.4 PARTICLE SIZE AND ζ-POTENTIAL MEASUREMENTS

In order to evaluate the stability of the main betalains in the nanodispersions, the particles' hydrodynamic diameters and the ζ-potential were determined with a Zetasizer Nano-ZS (Malvern Instruments, Worcestershire, UK) in deionized water at pH ~ 6.7, and at pH 5.0 and 3.0. The size distribution of the nanodispersions was characterized by using Dynamic Light Scattering (Zetasizer Nano-ZS, Malvern Instruments). For determination of the average size of the particles, the analysis was carried out at 173 º scattering using a 632.8 nm wavelength excitation angle. Lyophilized samples were diluted before analysis with

deionized water to avoid multiple scattering effects. The experiments were carried out at room temperature and at pH ~ 6.7, 5.0, and 3.0. The pH of the solutions was adjusted by adding HCl 0.5 M.

## 2.5 FOURIER TRANSFORM INFRARED (FTIR) SPECTROSCOPY

The chemical interactions between the constituents in the nanodispersions were evaluated by Fourier Transform Infrared (FTIR, Nicolet 6700, Thermo Scientific USA) with a resolution of 2 $cm^{-1}$ from 4500 to 800 $cm^{-1}$ by coadding 64 scans.

## 2.6 THERMAL STABILITY OF THE NANODISPERSIONS

Since BE, PBE, and PCBE will be added to Greek yogurt, which is a product submitted to thermal treatment, the thermal stability of these compounds was evaluated with a TGA (DTG-60H, Japan) by heating the samples from 25 to 600 ºC under nitrogen atmosphere (50 $mL.min^{-1}$) at a rate of 10 $ºC.min^{-1}$.

## 2.7 STABILITY OF BETALAINS AT PH 3.0 AND 5.0

Since betalains are very unstable in extreme ranges of pH (< 4 and > 7) (Azeredo, 2009), and the Greek yogurt has a pH of ~ 4.7, the stability tests were carried out in acidic conditions, at pH 3.0 and 5.0. The samples were dispersed in citrate buffer (pH 3.0 and 5.0) for 21 days at 4 ºC in absence of light. The absorbances of BC and BX were measured at 538 and 480 nm, respectively, in spectrophotometer UV-VIS (Thermo Scientific, USA) every 7 days for 21 days. The main betalains were expressed as a sum of BC and BX, and the concentration of these pigments at day 0 was used as a reference (100%) for the pigments' degradation rate, expressed in percentage.

## 2.8 COLOR AND RHEOLOGY ANALYSIS OF GREEK YOGURT

Red beet betanin, which is a betacyanin, is well stablished and commonly used as color additive in food products. In fact, its use is approved by the European Union, in the

Code of Federal Regulations (CFR) and by the Food and Drug Administration (FDA) in the United States. In this sense, the yogurt added with BE was used as a control for the color and rheology measurements (Delgado-Vargas et al., 2000; Dias et al., 2020). BE, PBE, and PCBE were added to non-colored Greek yogurt to improve its sensory characteristics regarding the color, avoiding the use of artificial colorants for this purpose.

For the color and rheology measurements, 2.4% (w/w) of BE, PBE, and PCBE were manually added and mixed to the Greek yogurt 12 hours prior to the measurements at day 0, and the samples were stored at 4 ºC in absence of light until further analysis. The measurements were done in triplicate at day 0 and after 21 days of storage at 4 ºC.

The color was measured by using a colorimeter (ColorQuest XE, HunterLab). The color parameters L*, a*, and b*, and the opacity were determined. The parameter L* is related to the brightness of the samples, a* is the transition from the green color (-a*) to red (+a*), and b* corresponds to the blue color (-b*) and yellow (+b*). The change of color (ΔE) was determined by the equation (Eq. 6), as follows:

$$\Delta E = [(L_i^* - L_0^*)^2 + (a_i^* - a_0^*)^2 + (b_i^* - b_0^*)^2]^{0.5} \quad (6)$$

where $L_0^*$, $a_0^*$, and $b_0^*$ are the color parameters of the samples at zero time and $L_i^*$, $a_i^*$, and $b_i^*$ are the color parameters after 21 days of storage (Otálora et al., 2019)

The rheological tests were carried out with a rotational rheometer (Discovery Hybrid Rheometer 1, TA Instruments, USA) equipped with a stainless-steel parallel plate sensor (diameter = 25 mm, gap = 1 mm) at 25.0 ± 0.1 ºC. The flow curves were obtained by progressive applying a shear rate from 0.05 to 200.0 $s^{-1}$ in three cycles (upcycle, down cycle, and second upcycle, for 180 s each). The Hershel-Bulkley model (Eq. 7) was fitted to the experimental data from the third cycle of the flow curve. The hysteresis area obtained by the difference in area between the curves of the first and second cycles provided an estimate of the thixotropy. The apparent viscosity was evaluated at a shear rate of 50 $s^{-1}$.

$$\sigma = \sigma_0 + K\dot{\gamma}^n \quad (7)$$

where $\sigma_0$ and σ are the initial yield stress and the yield stress, respectively. $K(Pa.s^{-1})$ is the consistency coefficient, $\dot{\gamma}$ is the shear rate, and $n$ is the flow behavior index (dimensionless).

For the dynamic oscillatory tests, the linear viscoelastic region was determined by performing a strain sweep (0.0001-1%) at a constant frequency of 1 Hz. After that, a

frequency sweep was performed from 0.01 to 10 Hz, with a constant shear stress of 5 Pa, in accordance with the determined linear viscoelastic region. The results at 1 Hz were presented as storage modulus (G') and loss modulus (G"), where the tangent of the angle phase (δ) is determined by tan (δ) = G"/G'.

### 2.9 STATISTICAL ANALYSIS

All the experiments were performed in triplicate. ANOVA one-way using $p < 0.05$ was done with the software R version 4.2.0. When appropriate, the significant differences between the means of the treatments BE, PBE, and PCBE was determined by Tukey's test.

## 3 RESULTS AND DISCUSSION

### 3.1 CHARACTERIZATION OF THE NANODISPERSIONS

#### 3.1.1 SIZE AND ζ-POTENTIAL

PBE and PCBE dispersions in water presented an average size of 501 nm and 674 nm with a polydispersity index of 0.46 and 0.54, respectively (Table 3). The ζ-potential measured in the same conditions was -18.6 mV for PBE and 4.8 mV for PCBE. The difference in the ζ-potential with the addition of chitosan was expected since chitosan is a cationic polymer (REINEKE; DAVIS, 2012). In fact, ζ-potential measures the surface charge of the particles and can be used to infer the electrostatic repulsion between the particles, where higher absolute values of ζ-potential can be related to higher kinetic stability of the particles in a dispersion (Xu, 2008). Besides that, the steric repulsion also plays a role in the kinetic stability of the particles. The steric repulsion phenomenon is caused by the overlap of adsorbed polymer on the layer of the particles (KAMIYA et al., 2008). The difference in the average size of the particles between both formulations can be also explained by the difference in the ζ-potential of the nanodispersions in deionized water, which can cause the particles to electrostatically interact and agglomerate (AWAD et al., 2005).

At pH 3.0 and 5.0, PCBE presented a ζ-potential of -3.9 mV and -11.4 mV, respectively, in contrast with a ζ-potential of 4.8 mV measured at pH ~ 6.7. This change in the ζ-potential can be explained by the fact that chitosan has a pKa constant of around 6.3-7.2, depending on the molecular weight and degree of deacetylation of the polymer, meaning that at pH values below 6.3, chitosan molecules are predominantly protonated (WANG et al.,

2006). For PBE, the increase in the ζ-potential in acidic conditions was expected, since the pH can significantly affect the ζ-potential of the particles (XU, 2008).

Regarding the variation of the hydrodynamic diameter of the nanodispersions in different pH, it is possible to observe that the size of PCBE significantly decreased when dispersed at pH 5.0 compared to the nanoparticles dispersed in deionized water ($p < 0.05$), which means that the ζ-potential possibly played a role in the electrostatic repulsions between the particles. For PBE, the size of the particles did not significantly change for all the evaluated solutions.

**TABLE 3**

Average hydrodynamic diameter and ζ-potential of PBE and PCBE nanodispersions in deionized water, pH 5.0 and 3.0

| Parameter | Deionized water | | pH 5.0 | | pH 3.0 | |
|---|---|---|---|---|---|---|
| | PBE | PCBE | PBE | PCBE | PBE | PCBE |
| Hydrodynamic diameter (nm) | 501[a] | 674[b] | 513[a] | 557[a] | 522[a] | 635[a] |
| ζ-potential (mV) | -18.6[a] | 4.8[b] | -9.9[a] | -11.4[b] | -5.7[a] | -3.9[b] |

Different letters within same row mean significant differences between the samples for each solution ($p < 0.05$)

* For all parameters, the relative deviation was less than 5%

### 3.1.2 FOURIER TRANSFORM INFRARED SPECTROSCOPY

BE FTIR spectrum was similar to the one found in the literature for BE (ČAKAREVIĆ et al., 2020; TUTUNCHI et al., 2019) (Fig. 9). The band at 3400 $cm^{-1}$ corresponds to O-H groups stretching vibrations, and the bands at 2900 $cm^{-1}$ represent C-H symmetry in the stretching mode. The peaks at 1600 $cm^{-1}$ can be attributed to C=C, and the bands at 1400 $cm^{-1}$ are related to the presence of C-C. Finally, the bands at 1050 $cm^{-1}$ and 920 $cm^{-1}$ are related to C-N stretching of amine and O-H stretching of carboxylic acids, respectively. For the physical mixtures' absorption spectra, the same mass proportions used to prepare BE nanodispersions were used for BE, PEG 4000, and low molecular weight chitosan.

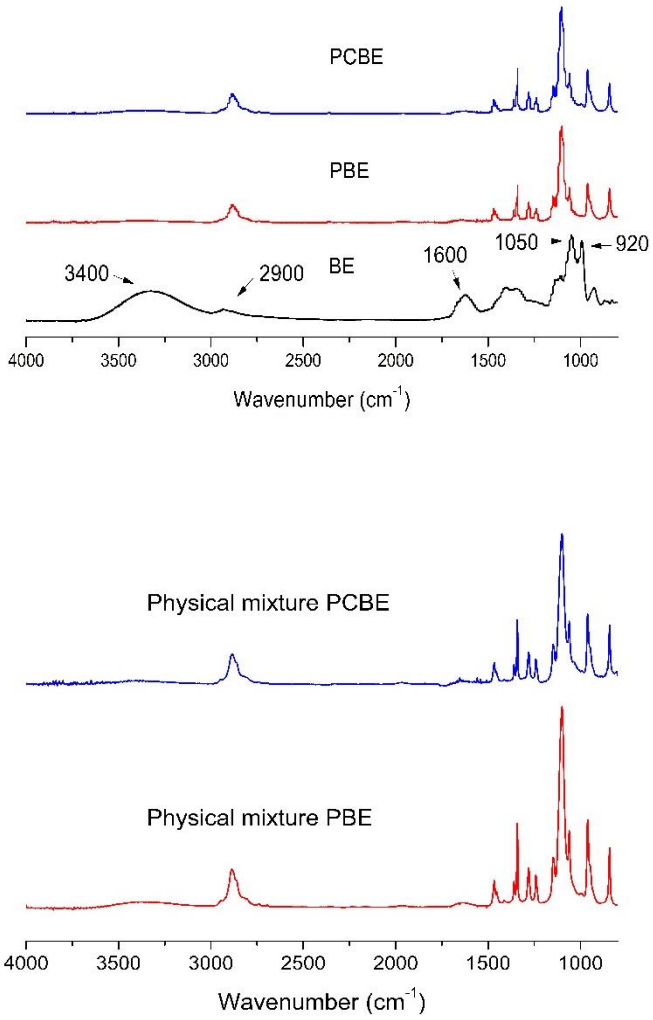



Fig. 9 – FTIR absorption spectra for BE, PBE, PCBE (top) and physical mixture PBE 10:1 and PCBE 5:5:1 (bottom)

The infrared absorption for the nanoparticles PBE and PCBE were very similar to the physical mixtures for both formulations, meaning that no interactions between BE and the polymers occurred during the transformation of BE in nanoparticles. It is possible to observe that BE peaks at 3400 $cm^{-1}$ and 1600 $cm^{-1}$ were significantly attenuated in both physical mixture and nanoparticles spectra, as expected, due to the mass proportions between BE and the polymers. Moreover, the presence of chitosan did not affect the FTIR absorption spectrum in both nanoparticles and physical mixture, since the absorption intensity spectrum for low molecular weight chitosan is neglectable compared to PEG 4000 (data not shown).

### 3.1.3 THERMAL STABILITY

The thermal stability of BE, PBE, and PCBE were evaluated by using TGA (Fig. 10). For BE, the first thermal degradation starts at 50 ºC, related to water evaporation, and can be attributed to the hygroscopic nature of the extract. The second stage above 130 ºC is related to the thermal degradation of the sample constituents, such as proteins, carbohydrates, and phenolic compounds (CARMO et al., 2018; LIANG et al., 2019).

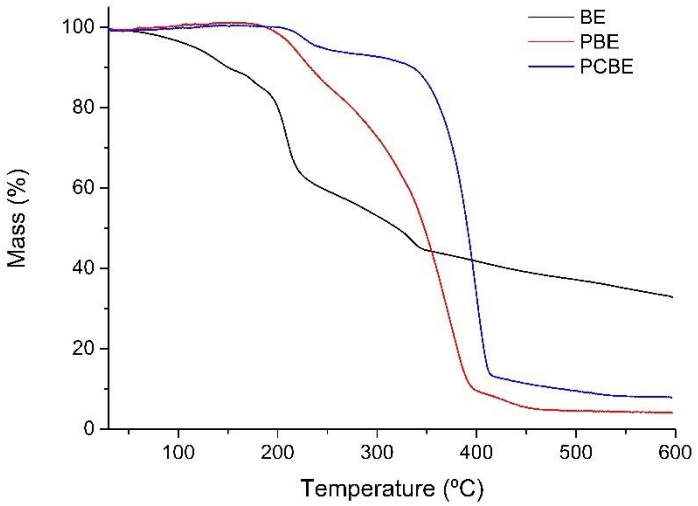



Fig. 10 – TGA curves of BE, PBE, and PCBE heated from 25 to 600 ºC at 10 ºC.min$^{-1}$ under dynamic nitrogen atmosphere

For PBE nanoparticles, the curve is related to polyethylene glycol degradation (Li et al., 2020). In the case of PCBE, the first stage of thermal degradation at 210 ºC can be attributed to non-interacted residues present in the nanodispersions. The second stage at 320 ºC is related to the thermal degradation of PCBE nanodispersions. It is known that low molecular weight chitosan starts to degrade at 220 ºC (DIAB et al., 2011; ZHENG et al., 2015). Therefore, this behavior can be explained by the interactions between both polymers with the beet extract, such as electrostatic interactions: dipole-dipole and hydrogen bonding (OTÁLORA et al., 2015).

It is possible to observe a lack of degradation for both PBE and PCBE from 100-200 ºC, despite the presence of BE in the nanodispersions. At 230 ºC, BE lost around 40% of its initial mass, in contrast with only 10% for PBE and 4% for PCBE. This behavior can be attributed to the protective effect of the polymers in the nanodispersions against thermal degradation compared to pristine BE. Similar results for TGA were reported by Carmo et al. (2018), who used oligosaccharides and whey protein as carriers to beetroot extract.

The results obtained by TGA show that PBE and PCBE present higher thermal stability compared to BE, which means that the nanodispersions can be successfully applied to food matrices that are submitted to thermal treatments at high temperatures.

## 3.2 STABILITY OF THE MAIN BETALAINS AT PH 3 AND 5

PCBE presented significantly higher stability for the main betalains (BC and BX) at pH 3.0 (Fig. 11) and 5.0 (Fig. 12), compared to PBE and BE (Table 4). Regarding the BC specifically, PCBE retained 78.3% of the initial content of this pigment after 21 days of storage, whereas BE and PBE presented stability of 47.6% and 40.2%, respectively. In pH 5.0, PCBE retained 85.6% of the initial amount of BC, whereas BE retained 60.9% and PBE 60.3%.

According to Khan (2016), betalains are very unstable in extreme ranges of pH (<4 and >7), which is in accordance with the stability results obtained in pH 3.0 and 5.0 in this study for the main betalains. At pH equal or higher than 3.0, the anionic form of betalains is predominant (MIGUEL, 2018). The stability results obtained for PCBE in acidic conditions can be explained by the interactions between BE and the polymers, possibly favored by the presence of positively charged chitosan present in the nanodispersions. That means that these interactions, such as electrostatic interactions, provided a protective effect for the main betalains. Besides that, the steric repulsion also plays a significant role in the kinetic stability of nanodispersions (CHARLES, 1992).

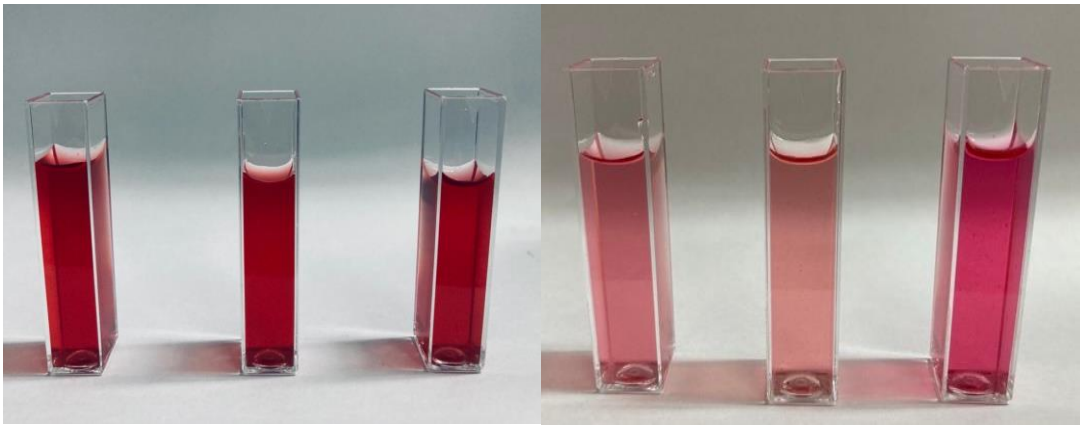


Fig. 11 – BE, PBE, and PCBE from left to right, respectively, at pH 3.0 at day 0 and after 21 days of storage

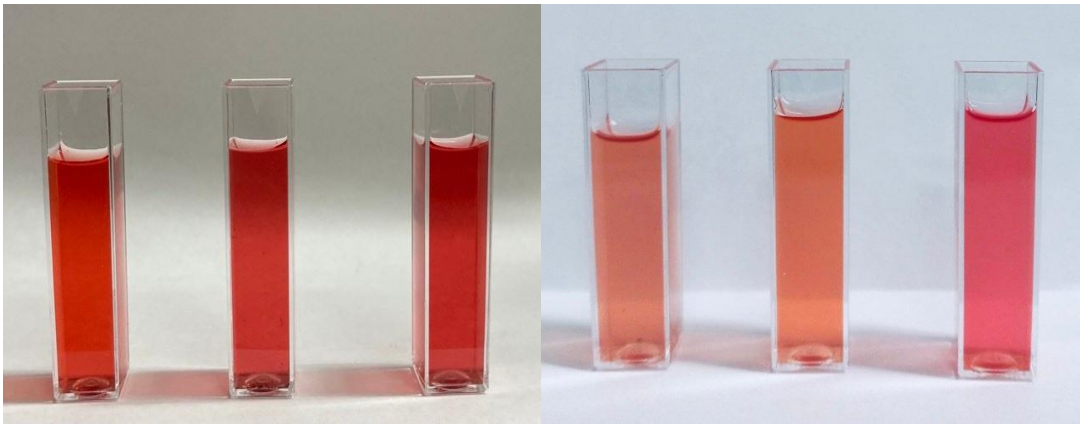


Fig. 12 – BE, PBE, and PCBE from left to right, respectively, at pH 5.0 at day 0 and after 21 days of storage

For PBE, it is possible to observe a significant increase in the ζ-potential at pH 3.0 when compared to PBE dispersed in deionized water, meaning that the charge distribution of the particles was significantly affected, as shown in Table 1. This change in the charge distribution of the particles can affect the way that the molecules interact with each other, as well as with the molecules present in the media where they are dispersed in, which strongly affects their kinetic stability (CHIRAYIL et al., 2017). On the other hand, PCBE ζ-potential did not significantly change, in absolute value, in the same conditions.

The protective effect of polymers in betalains' stability was also demonstrated by Tutunchi et al. (2019), who observed a protective effect of β-cyclodextrin against betalains degradation after 28 days of storage in pH 3.5 and 5.0 and at temperatures of 4 and 25 ºC. Otálora et al. (2019) also reported increased stability for betalains encapsulated with gelatin after storage for 30 days at 4 ºC.

**TABLE 4**

Main betalains retention (%) for BE, PBE, and PCBE in pH 3.0 and 5.0 after 7, 14, and 21 days of storage at 4 ºC

| | pH 3.0 | | | pH 5.0 | | |
|---|---|---|---|---|---|---|
| Day | BE | PBE | PCBE | BE | PBE | PCBE |
| 7 | 80.6$^{a}$ | 87.6$^{b}$ | 82.9$^{a}$ | 77.1$^{a}$ | 88.7$^{b}$ | 94.0$^{c}$ |
| 14 | 61.4$^{a}$ | 53.1$^{b}$ | 75.1$^{c}$ | 69.6$^{a}$ | 72.9 $^{a}$ | 91.8$^{b}$ |
| 21 | 43.2$^{a}$ | 39.3$^{a}$ | 67.2$^{b}$ | 65.4$^{a}$ | 65.4$^{a}$ | 80.0$^{b}$ |

Different letters within same row mean significant differences between the samples for each pH ($p < 0.05$)

* For all parameters, the relative deviation was less than 5%

## 3.3 COLOR PARAMETERS OF GREEK YOGURT

The color parameters L*, a*, and b*, as well as the opacity of the yogurt samples added with 2.4% (w/w) of BE, PBE, and PCBE were measured at day 0 and after 21 days (Fig. 13) of storage at 4 ºC (Table 5). The parameter L* is related to the brightness of the samples, where higher values for L* indicate a brighter color. In the same sense, the parameter a* indicates the transition from the color green to red, and b* is related to the transition from blue to yellow, where higher values of b* is related to a yellowish color (PÉREZ-MAGARIÑO; GONZÁLEZ-SANJOSÉ, 2003).

It is possible to observe that PBE and PCBE significantly enhanced the brightness of the samples and presented less opacity in the Greek yogurt compared to pristine BE. Moreover, PBE and PCBE increased the redness of the samples at day 0 and after 21 days of storage.

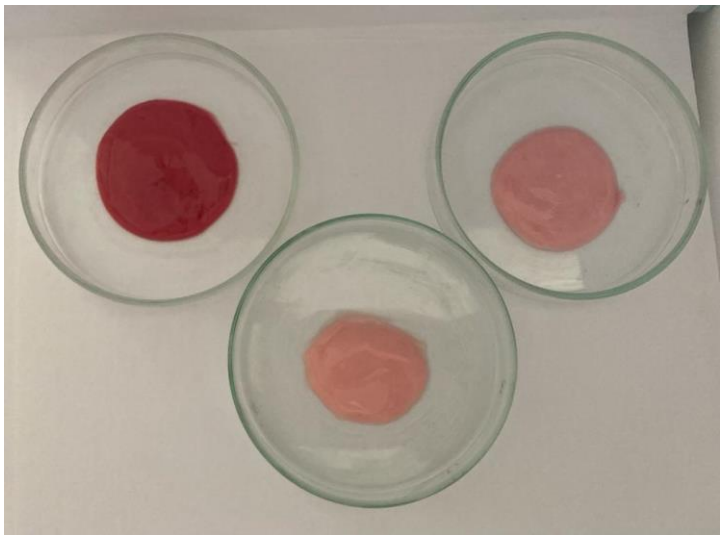


Fig. 13 – Greek yogurt added with 2.4% w/w of BE, PBE, and PCBE from left to right, respectively

The variation of color for the Greek yogurt samples added with PBE and PCBE was significantly higher compared to the yogurt added with BE (control) after 21 days of storage.

The higher variation of color for the nanodispersions can be explained by the interactions between the polymers and BE with the constituents of the yogurt, which can contribute to the release of BE from the nanodispersions to the sample (CAMPO et al., 2019). The difference in color variation between PBE and PCBE is possibly due to the stronger interactions between the polymers and BE for PCBE nanodispersions in acidic conditions, which also corroborates the stability results for the main betalains obtained for PBE and PCBE at pH 5.0, which is close to the pH of the Greek yogurt. Even though there was a significant difference in ΔE between the yogurt samples, this difference is not distinguishable by the human eye (ΔE < 2.0) (OULTON et al., 1996).

Regarding the color parameters, PBE and PCBE nanodispersions presented good results for redness, brightness and opacity at day 0 and after 21 days of storage, meaning that the nanoparticles can be successfully applied to commercial Greek yogurt as color additives.

**TABLE 5**

Color parameters and color variation of Greek yogurt added with 2.4% (w/w) of BE, PBE, and PCBE at day 0 and after 21 days of storage

| | Samples at day 0 | | | Samples at day 21 | | |
|---|---|---|---|---|---|---|
| Color parameters | BE | PBE | PCBE | BE | PBE | PCBE |
| L* | $26.2^{a}$ | $30.5^{b}$ | $30.3^{b}$ | $26.3^{a}$ | $29.9^{b}$ | $31.0^{c}$ |
| a* | $6.0^{a}$ | $7.3^{b}$ | $8.2^{c}$ | $5.6^{a}$ | $5.9^{a}$ | $7.7^{b}$ |
| b* | $-0.5^{a}$ | $-0.6^{b}$ | $-0.3^{c}$ | $-0.5^{a}$ | $-0.2^{b}$ | $0.4^{c}$ |
| Opacity | $36.4^{a}$ | $13.0^{b}$ | $13.2^{b}$ | $35.6^{a}$ | $14.0^{b}$ | $12.7^{c}$ |
| Color variation (ΔE) | - | - | - | $0.4^{a}$ | $1.5^{b}$ | $1.0^{c}$ |

Different letters within same row mean significant differences between the samples ($p < 0.05$)

* For all parameters, the relative deviation was less than 5%

## 3.4 INFLUENCE OF BE, PBE AND PCBE ON THE RHEOLOGY OF THE GREEK YOGURT

The model of Herschel-Bulkley is the most suitable (data not shown) for the yogurt samples. All the yogurt samples presented a plastic nature in rheology since their viscosity is non-constant and decreases with increasing strain rate, and their shear stress values did not

start at zero. Similar results for yogurt samples were obtained by Hassan et al. (2003) and Santillán-Urquiza et al. (2017).

The yield stress is a property that can be used to predict how structured in terms of hardness the material is, and a higher yield stress is related to higher stability against phase separation, sedimentation, or aggregation. When fitted to the Herschel-Bulkley model, the yogurts added with PBE and PCBE presented higher yield stress and flow behavior index than the yogurt added with BE at day 0 (Fig. 14). The change in flow behavior means that PBE and PCBE Greek yogurts present a behavior closer to a Newtonian fluid compared to the Greek yogurt added with BE (control). On the other hand, the consistency index was higher for the samples added with BE, compared to samples with the addition of PBE and PCBE nanodispersions. This difference in the consistency index means that a difference in the shear rate causes a greater change in the shear stress for BE Greek yogurt (CHENG, 1986; PANG et al., 2020). After 21 days of storage, it was possible to observe an increase in the yield stress with no significant difference between the samples. Regarding the viscosity, the yogurt added with PBE presented the highest apparent viscosity, which can be explained by the interactions between the constituents of the yogurt provided by the addition of PBE. This behavior also corroborates the results obtained for the hysteresis area, meaning that these weak interactions can be easily broken when submitted to shear stress (FUHRMANN et al., 2022).

The hysteresis area (area between the upward and the downward curve) is associated with the structural breakdown during shearing (HASSAN et al., 1996). After 21 days of storage, it is possible to observe that PCBE yogurt presented a significantly smaller hysteresis area, meaning that the addition of PCBE nanodispersions was able to provide stronger polymer interactions, in contrast with PBE yogurt samples, which presented a significant increase in the hysteresis area (Table 6). Moreover, the hysteresis area for the yogurt added with PCBE nanodispersions was smaller than the yogurt samples added only with BE, probably because of the addition of the polymers PEG and chitosan present in the nanodispersions, which allowed more interactions with the constituents present in the yogurt (HASSAN et al., 2003).

**TABLE 6**

Rheological parameters of Greek yogurt added with 2.4% (w/w) of BE, PBE and PCBE at day 0 and after 21 days of storage. All measurements were done in triplicate

| | Day 0 | | | Day 21 | | |
|---|---|---|---|---|---|---|
| Parameter | BE | PBE | PCBE | BE | PBE | PCBE |

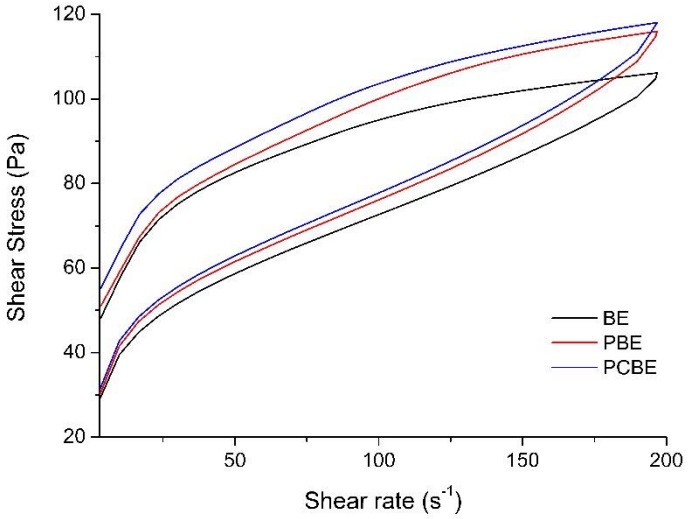
| | | | | | | |
|---|---|---|---|---|---|---|
| Yield stress, $\sigma_0$ (Pa) | 11.39[a] | 16.48[b] | 19.96[c] | 25.78[a] | 24.42[a] | 24.72[a] |
| Flow behavior index, n (-) | 0.35[a] | 0.42[b] | 0.44[b] | 0.42[a] | 0.40[a] | 0.48[a] |
| Consistency coefficient, K (Pa.s$^n$) | 12.51[a] | 9.45[b] | 8.17[b] | 11.39[a] | 21.96[b] | 8.66[a] |
| Viscosity at 50.s$^{-1}$ (Pa.s) | 1.20[a] | 1.27[a] | 1.30[a] | 1.74[a] | 2.55[b] | 1.41[a] |
| $A_{up}$ (Pa s$^{-1}$)[1] | 17556[a] | 18859[a] | 19223[a] | 25787[a] | 44142[b] | 24856[a] |
| ΔA (Pa s$^{-1}$)[2] | 3600[a] | 3900[a] | 4200[a] | 6350[a] | 13740[b] | 5700[c] |

Different letters within same row mean significant differences ($p < 0.05$) between the samples at day 0 and after 21 days of storage

[1]Area under the upward curve when plotting shear stress vs. shear rate

[2]Difference in area under the upward curve and the corresponding downward curve

* For all parameters, the relative deviation was less than 5%



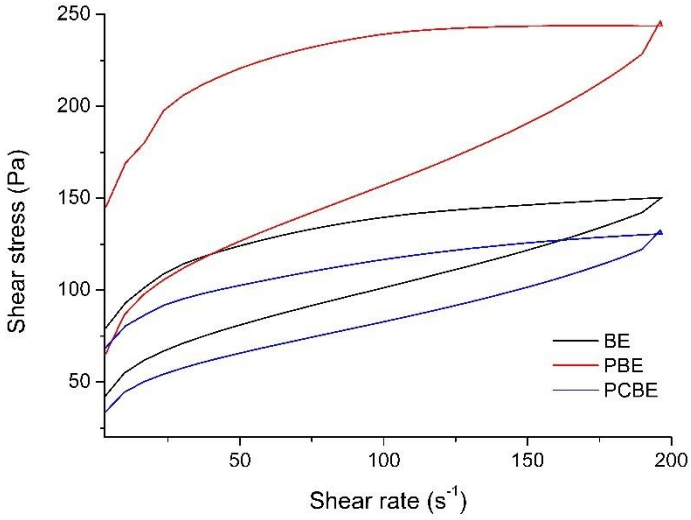



Fig. 14 – Flow curves of Greek yogurt added with 2.4% (w/w) BE, PBE, and PCBE (top) at day 0 and after 21 days of storage (bottom)

The elastic modulus (G') represents the elasticity of viscoelastic products. An increase in G' modulus is strongly related to molecular interactions or physical crosslinks that can produce harder materials, and the viscous modulus (G") is related to the viscosity of the material. When a material is submitted to a given strain level, it comprises the elastic (G') and viscous modulus (G"). This lag between the moduli response is called phase angle tangent (tg δ = G"/G') (VÉLEZ-RUIZ et al., 1997; WIDYATMOKO, 2016).

The phase angle tangent values were < 1, indicating that the Greek yogurt added with BE, PBE, and PCBE can be classified as a semi-solid food (ZHONG; DAUBERT, 2013). After 21 days of storage, there was no significant difference between the samples, meaning that despite the addition of PBE and PCBE nanodispersions, the Greek yogurt maintained its original viscoelastic properties compared to the yogurt added only with BE (Table 5).

**TABLE 7**

Viscoelastic parameters of Greek yogurt added with 2.4% (w/w) of BE, PBE, and PCBE at day 0 and after 21 days of storage. All measurements were done in triplicate

| | Day 0 | | | Day 21 | | |
|---|---|---|---|---|---|---|
| Parameter | BE | PBE | PCBE | BE | PBE | PCBE |
| Elastic modulus, G', at 1 Hz (Pa) | 338[a] | 264[b] | 421[c] | 601[a] | 1190[b] | 566[c] |
| Viscous | 95[a] | 80[b] | 116[b] | 165[a] | 313[b] | 161[c] |

| | | | | | | |
|---|---|---|---|---|---|---|
| modulus, G", at 1 Hz (Pa) | | | | | | |
| Tan (δ)[1] at 1 Hz | 0.28[a] | 0.30[a] | 0.27[a] | 0.27[a] | 0.26[a] | 0.28[a] |

Different letters within the same row mean significant differences ($p < 0.05$)
[1]Tangent of the phase angle (δ), determined by tan (δ) = (G"/G')
* For all parameters, the relative deviation was less than 5%

## 4. CONCLUSIONS

Betalains were successfully complexed with PEG and low molecular weight chitosan. PCBE presented a significant enhancement regarding the main betalains' stability in acidic (pH 3.0 and 5.0) conditions after 21 days of storage, despite the instability of these pigments in extreme ranges of pH. Furthermore, it was possible to observe enhanced thermal stability for both PBE and PCBE nanodispersions. When added to commercial Greek yogurt, it was possible to observe that the nanoparticles significantly changed the color parameters and the rheological stability of the yogurt compared to the sample added only with BE. Regarding the rheological properties, PCBE significantly reduced the area of hysteresis after 21 days of storage when compared to BE and PBE, which indicates that PCBE nanodispersions provided better rheological stability to the yogurt. Furthermore, the addition of PBE and PCBE nanodispersions did not affect the viscoelastic properties of the Greek yogurt added with BE. Therefore, PCBE nanodispersions can be successfully used as color additives to commercial Greek yogurt, as well as to improve their rheological stability.

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# GENERAL CONCLUSIONS

Natural pigments-biopolymers complexes can be a suitable alternative to artificial colorants in the food industry. Regardless the complexation method, in general, the anthocyanins, betalains, and carotenoids had their stability and properties improved. In general, the use of lower temperatures during the complexation of natural pigments can be more suitable to preserve their bioactivity and antioxidant activity.

Regarding the wall materials, proteins and polysaccharides are the most used, probably due to their large structure, which allows them to have more sites available for interactions with the compounds. Furthermore, the evaluation of thermodynamic properties showed that the nature of biopolymer plays a role in the interaction between both compounds, as well as the temperature, pH, and the concentration of salts.

The most used thermodynamic techniques used to evaluate the binding properties between natural pigments and biopolymers are SPR and fluorescence spectroscopy, probably due to their sensitivity and the wide range of information that can be provided. Moreover, since proteins and polysaccharides are the most used polymers for complexation with natural pigments, these techniques are more suitable in this context.

PBE and PCBE presented significant improvement in thermal stability when compared to BE. In acidic conditions, PCBE presented an increase in stability of 56% and 22% at pH 3.0 and 5.0, respectively. When applied to Greek yogurt, it was possible to observe that PCBE nanodispersions presented good results in terms of color and improved rheological stability of the Greek yogurt compared to BE and PBE.