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Recent advances and challenges on chitosan-based nanostructures by polyelectrolyte complexation and ionic gelation for anthocyanins stabilization

Avanços e recentes desafios sobre nanoestruturas à base de quitosana preparadas por complexação polieletrolítica e gelatinização iônica para estabilização de antocianinas

Avances y desafíos recientes en nanoestructuras a base de quitosano preparadas por complejación polielectrolítica y gelatinización iónica para la estabilización de antocianinas

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Abstract
Anthocyanins are water-soluble polyphenols responsible for the color of many fruits, flowers, and vegetables. In addition to natural dyes, anthocyanins are also related to the prevention of several chronic diseases. However, anthocyanins are extremely sensitive to variations in pH, temperature, light, enzymes, and other environment variables, being necessary to employ artifices and technologies to expand their application in both the food and pharmaceutical sectors. In this context, biopolymeric nanoparticles can be used to protect and intensify the functions conferred to the anthocyanins. Among the techniques used, polyelectrolytic complexation (PC) and ionic gelation (IG) stands out due to convenience, speed, low cost, and possibility of using a versatile, biocompatible and natural polymer such as chitosan. Therefore, scoring and understanding the main factors that affect the stability of chitosan-based nanoparticles produced by PC and IG, and knowing the strategies that can be adopted to overcome these problems ~~is~~ extremely important. Thus, this review aims to provide an overview of anthocyanins and biopolymeric nanoparticles with an emphasis on PC and IG techniques. The main challenges that need to be faced when anthocyanins are incorporated into these nanoparticles will be scored, mainly when chitosan is used as a polymeric base. Also, some directions will be given to those who intend to develop new projects focusing on the stabilization of anthocyanins.
Keywords: Encapsulation; Biopolymeric nanoparticle; Nanocomplexes, Biopolymers.

Resumo
Antocianinas são polifenóis solúveis em água, responsáveis pela coloração de diversas frutas, flores e vegetais. Além de corantes naturais, antocianinas também estão relacionadas a prevenção de diversas doenças crônicas. Contudo, antocianinas são extremamente sensíveis a variações de ph, temperatura, luz, enzimas e outras variáveis do meio em que se encontram, sendo necessário empregar artifícios e tecnologias para expandir sua aplicação tanto no setor de alimentos como farmacêutico. Nesse contexto, nanopartículas biopoliméricas podem ser usadas para proteger antocianinas e até mesmo intensificar as funções conferidas a elas. Dentre as técnicas usadas, complexação

polieletrolítica (PC) e gelatinização iônica (IG) tomam posição de destaque devido a praticidade, rapidez, baixo custo e possibilidade de uso de polímeros versáteis, biocompatíveis e naturais, como a quitosana. Desse modo, pontuar e entender os principais fatores que afetam a estabilidade das nanopartículas à base de quitosana produzidas por PC e IG, e conhecer as estratégias que podem ser adotadas para contornar esses problemas são de extrema importância. Diante do exposto, essa revisão tem como objetivo fornecer uma visão geral sobre antocianinas e nanopartículas biopoliméricas com ênfase nas técnicas de PC e IG, identificando os principais desafios que precisam ser enfrentados ao incorporar antocianinas nessas nanopartículas produzidas em base de quitosana, e direcionar o desenvolvimento de novos projetos com foco na estabilização de antocianinas.
Palavras-chave: Encapsulamento; Nanopartículas biopoliméricas; Nanocomplexos; Biopolímeros.

Resumen
Antocianinas son polifenoles solubles en agua, responsables por la coloración de diversas frutas, flores y verduras. Además de los colorantes naturales, las antocianinas también están relacionadas con la prevención de varias enfermedades crónicas. Sin embargo, las antocianinas son muy sensibles a las variaciones de pH, temperatura, luz, enzimas y otras variables del medio en el que se encuentran, por lo que es necesario emplear artificios y tecnologías para ampliar su aplicación tanto en el sector alimentario como en el farmacéutico. En este contexto, las nanopartículas biopoliméricas pueden ser utilizadas para proteger las antocianinas e incluso potenciar las funciones que les confieren. Entre las técnicas utilizadas, la complejación polielectrolítica (PC) y la gelatinización iónica (IG) ocupan un lugar destacado por su practicidad, rapidez, bajo costo y la posibilidad de utilizar polímeros versátiles, biocompatibles y naturales, como el quitosano. Por lo tanto, calificar y comprender los principales factores que afectan la estabilidad de las nanopartículas a base de quitosano producidas por PC e IG, y conocer las estrategias que se pueden adoptar para superar estos problemas es extremadamente importante. Dado lo anterior, esta revisión tiene como objetivo proporcionar una visión general de las antocianinas y las nanopartículas biopoliméricas con énfasis en las técnicas de PC e IG, señalar los principales desafíos que deben afrontarse al incorporar antocianinas a estas nanopartículas cuando se utiliza quitosano como base polimérica; y dar dirección a quienes pretenden desarrollar nuevos proyectos enfocados en la estabilización de antocianinas.
Palabras clave: Encapsulación; Nanopartículas biopoliméricas; Nanocomplejos; Biopolímeros.

1. Introduction

Anthocyanins make up the largest group of water-soluble pigments in the plant kingdom and fall into the class of polyphenols, a subgroup of flavonoids, responsible for the color of various fruits, flowers, and vegetables (Akhavan & Jafari, 2017). There are more than 700 anthocyanin structures identified, which offer a range of colorings that could be explored, ranging from orange to red and purple to blue, according to the environment in which it is found (Iosub et., al, 2012).

The food and pharmaceutical industries are very interested in this natural pigment, due to its attractive color and health benefits (Lee & Choung, 2011). Additionally, there is a growing demand for the use of natural products due to the toxic effects caused by synthetic products, including dyes (Khoo et al., 2017). Besides being used as a natural dye (Chi et al., 2019), anthocyanins also play a role as an antioxidant in foods and beverages (He et al., 2017), and even as modulators of intestinal microflora. This helps prevent and even treat candidiasis and chronic diseases, such as diabetes (Suket et al., 2014; Morais et al., 2016). Other applications of anthocyanins are related to performance in inflammatory conditions in the body, including the prevention of cardiovascular diseases (Reis et al., 2016), and cancer prevention (Jeong et al., 2016).

Despite the excellent properties conferred to anthocyanins, the application is still limited due to its instability under processing and storage conditions, such as heat, high pH, and exposure to light and oxygen, which induces the degradation of the anthocyanin structure to colorless phenolic acids and aldehydes (Wang et al., 2017). Low bioavailability and rapid elimination from the body are also other limitations of anthocyanins (Peixoto et al., 2016; Kay et al., 2017; Wang et al., 2017).

In an attempt to circumvent these problems, new strategies have been adopted, such as encapsulation with biopolymer nanoparticles, especially those developed by complexation polyelectrolytic (PC) and ionic gelation (IG) techniques. Through these techniques, it is possible to produce nanoparticles simply and free of toxic solvents using biodegradable polymers (Wu et al., 2020).

Chitosan stands out in PC and IG techniques because it is a cationic, versatile, natural, and biodegradable polymer (Mohammed et al., 2017). In an aqueous medium (pH <6.5), the chitosans' amino groups are protonated and capable of forming complexes, hydrogels, or particles with natural anionic polymers and salts' polyanions GRAS (Generally Recognize as Safe), such as sodium tripolyphosphate (Zhao et al., 2011).

The formation of these different nanostructures facilitates the incorporation of anthocyanins. From there, it is possible to expand the application of anthocyanins, overcome the challenges of the pharmaceutical and food process, to produce packaging for food, beverages, and orodispersible films, among others.

Despite the great interest in PC and IG techniques, there are still points that deserve attention and need further study and discussion, such as the structure-property-function relationships of polymers with the active compound, intrinsic and extrinsic properties of the materials used in the development of nanostructures, and compatibility of these nanostructures with the matrix to which it will be added. In this review, the structural properties of anthocyanins and their limitations are addressed together with an overview of biopolymeric nanoparticles. The challenges and strategies for anthocyanin nanoencapsulation are also described using two relatively simple techniques: polyelectrolytic complexation and ionic gelatinization.

2. Methodology

A narrative and qualitative review was conducted, and searches for scientific articles were performed using the *U.S. National Library of Medicine and the National Institutes of Health* (PubMed), *Scopus*, *Web of Science* and *Google Scholar* databases. The searches were limited to articles published in the last 10 years using the keywords "biopolymeric nanoparticles", "chitosan, nanoparticles", "anthocyanin", "complexation", "nanocomplexes", "chitosan polyelectrolyte complexes", "anthocyanins microencapsulation", "anthocyanins nanoencapsulation", and "ionic gelatinization" combined with each other through the Boolean operators "AND" or "OR". The exclusion criteria adopted included particle sizes greater than 500 nm, nanocomplexation studies without the use of chitosan, nanoencapsulation studies with bioactive compounds different from those sources of anthocyanin. The reference lists of selected articles were also manually analyzed to identify other relevant studies that could be included in this review.

3. Anthocyanins

3.1 Definition, food source and consumption

Anthocyanins (from Greek *Anthos*, a "flower", and *kyanos* "dark blue color") are polyphenolic pigments of the flavonoid class, widely available in various parts of vascular plant materials. Anthocyanins are the main elements responsible for the variety of colors found in flowers and fruits (Akhavan & Jafari, 2017). These pigments are the only polyphenolic compounds capable of absorbing light both in the ultraviolet region and in the entire visible range (Brouillar et al., 2010), which makes them responsible for the red, blue, and purple colors of flowers such as red hibiscus, blue rosemary, and lavender, respectively (Khoo et al., 2017).

Fruits and vegetables, such as blueberry, juçara, açaí, grape, cranberry, red carrot, and red cabbage are known and consumed worldwide, and the colors of each one come from anthocyanins. (Yousuf et al., 2016; Ko et al., 2017). The daily consumption of anthocyanins is high (~200 mg/per capita/day), especially when you have a diet rich in colorful fruits and vegetables. (Bueno et al., 2012).

There is still no consensus on the daily amount of anthocyanins to be ingested. However, in 2013 the Chinese Nutrition Society established a daily consumption of 50 mg / per capita/day, while the FAO / WHO Committee of experts

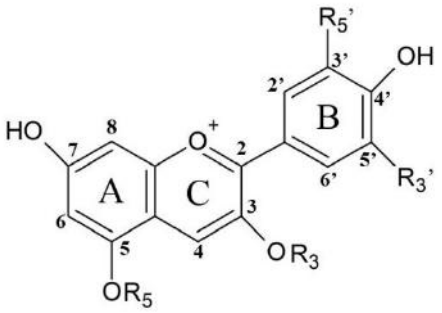
proposed consumption of 2.5 mg/kg human body weight/day for the extract of the peel grape, but not for anthocyanins in general (Wallace & Giusti, 2015).

In addition to color ability, anthocyanins also have other properties, such as antioxidant, anticancer, and anti-inflammatory (Bueno et al., 2012). This makes the aforementioned foods potentially functional, due to the fact that they are associated with reduced risk of diseases (Khoo et al., 2017), such as atherosclerosis, cardiovascular diseases, diabetes, and other chronic diseases (Kay et al., 2017). Therefore, consuming foods rich in anthocyanins can contribute to consumer health and satisfaction, since foods that are anthocyanin sources are associated with better performance of the body's biological functions (Singh et al., 2020).

3.2 Chemical structure

Anthocyanins are glycosylated forms of anthocyanidins, *flavylium* cation deriving from (2-fenilbenzopilrilium) linked only to hydroxyl and methoxyl groups. (Bueno et al., 2012). Anthocyanidins, also known as aglycone (Figure 1), are the basic structure of anthocyanins. They have a 15-carbon skeleton, containing two aromatic rings (A and B) separated by an oxygenated heterocyclic ring (C) (Castañeda-Ovando et al., 2009; Bueno et al., 2012; Tarone et al., 2020).

Figure1. Structural representation of anthocyanidin.

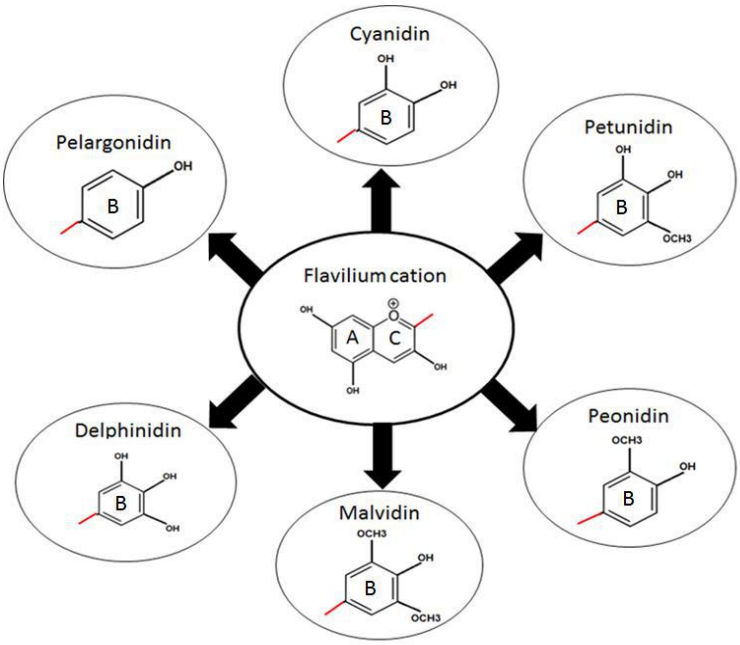


Source: Own authorship (2022).

Due to the existence of conjugated double bonds, the positive residual charge that the *flavilium* nucleus presents in an acid medium is relocated throughout the cycle, being stabilized by resonance. The electron deficiency of the *flavilium* cation makes the free aglycones highly reactive, which do not occur naturally (Srivastava & Vankar, 2010), explaining the glycosylated form with sugars that increase the stability of the molecule. There are more than 30 anthocyanidins identified in nature, differing in the number and position of the hydroxyl groups and, or methoxyl in the carbon structure. The presence of reactive hydroxyls favors the formation of covalent bonds with different sugars, such as glucose, rhamnose, galactose, xylose, and arabinose; however, glucose is the most found in anthocyanins (Santos-Buelga & González-Paramás, 2018; Tarone et al., 2020).

Aliphatic, hydroxybenzoic, or hydroxycinnamic acids may substitute the sugars. The most usual are malonic, acetic, p-coumaric, and caffeic acids (Santos-Buelga & González-Paramás, 2018). Koley et al. (2014) demonstrated that acylated anthocyanins are more stable to heating, light, and external environment than non-acylated ones. Six anthocyanidin structures are commonly found in nature (Figure 2), including cyanidin, delphinidin, pelargonidin, peonidin, malvidin, and petunidin in which the last three have methoxy groups as substitutes in their B rings. Together, these six anthocyanidins represent about 90% of all anthocyanins identified so far (Bueno et al., 2012).

Figure 2. Structures of the main anthocyanidins found in the nature.

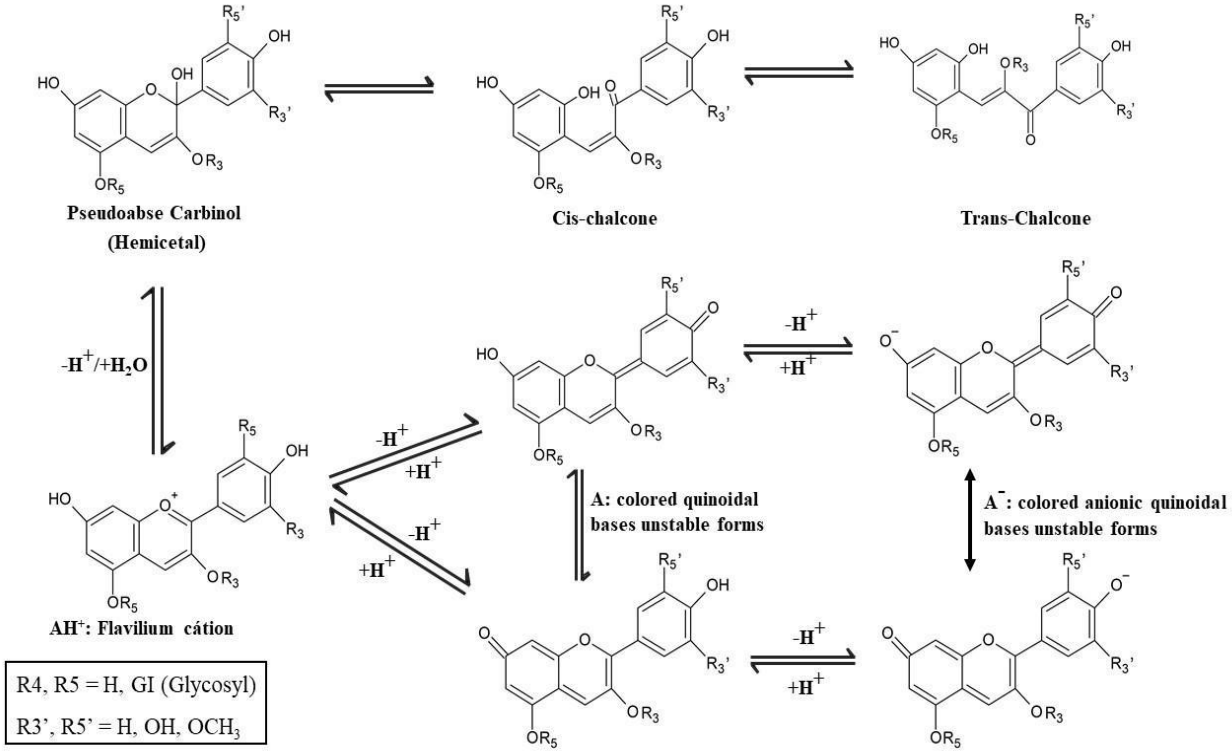


Source: Own authorship (2022).

So far, they have been described more than 700 anthocyanins from natural sources (Santos-Buelga & González-Paramás, 2018). In addition to the variations that occur in the previously mentioned anthocyanidins, anthocyanins have structures, properties, and functions affected by the physical-chemical environment in which they are inserted (Yousuf et al., 2016; Tarone et al., 2020). In aqueous solution, for example, anthocyanins have different chemical structures in equilibrium. These structures depend on the type of reaction (hydration, tautomerization, proton transfer) of the flavilium cation at certain pH values and conditions of the medium in which it is present (Figure 3) (Santos-Buelga & González-Paramás, 2018; Bueno et al., 2012).

When pH values are between 1 and 3, anthocyanins exhibits an intense red color due to the predominance of the structure in the form of the flavilium cation (AH^+). However, when the pH increases to values of 4 - 6, two reactions can occur as the same time: I) Deprotonation and formation of the purple/blue quinoid base and, II) C2 hydration of the flavilium cation as well as the appearance of the carbinol pseudo base (colorless hemiketal shape). The hemiketal shape undergoes the opening of ring C by tautomerization and gives rise to cis-chalcone. Trans-chalcone also makes up the medium when cis-chalcone is isomerized (Santos-Buelga & González-Paramás, 2018; Pina et al., 2012). In this way, with an increase in pH to values between 7 and 8, rapid deprotonation occurs, which results in the predominant formation of the blue colored quinoid base (A) (Pina et al., 2012; Bueno 2012).

Figure 3. Main structures of anthocyanin in pH-dependent equilibrium in aqueous solution.



Source: Adapted from Brouillard et al., (2010).

According to Babaloo and Jamei (2017), the anthocyanin colorimetric change as a function of pH reduces the conjugated double bonds, which causes the displacement of maximum absorption values to shorter wavelengths. A phenomenon known as hypsochromic displacement.

3.3 Stability

Elevated temperatures, oxygen, and light are some factors that promote changes in the chemical structure and influence the anthocyanins' stability, which can result in loss of the total anthocyanin content and reduction in its antioxidant capacity (Khoo et al., 2017). On oxygen presence and high temperatures exposure, the anthocyanins degradation is intensified. In this case, hydrolysis of the 3-glycosidic bond occurs, with consequent release of the aglycone and hydrolytic opening of the C ring (Sipahli et al., 2017). This process favors the formation of chalcones, which degrade to dark insoluble compounds of polyphenolic nature (Roobha et al., 2011; Bobbio, & Bobbio, 2003).

Exposure to light also accelerates anthocyanin degradation through a photooxidative mechanism, often described by first-order, low-activation degradation kinetics (Askar et al., 2015). This process is influenced by the medium pH, temperature, and exposure time (Karaoglan et al., 2019).

The pH has a direct interference in the chemical structure of anthocyanin and, consequently, in its stability, which implies changes in bioavailability, coloring power, and capabilities of antioxidant, anticancer, and anti-inflammatory (Cahyana & Gordon, 2013; Santos-Buelga & González-Paramás, 2018; Zapata et al., 2019).

Jiang et al. (2019) evaluated the effect of thermal processing at 90ºC and pH values of 3 to 7 in purple sweet potato extract. The authors found that anthocyanins degradation occurred through a first-order reaction, regardless of the pH value. The half-lives at pH values 3, 5, and 7 were 10.27, 12.42, and 4.66 h, respectively. Additionally, there were changes in the color of the extract varying with the heating time and change in the pH values, with higher values of these variables resulting

in color losses and consequent appearance of brown pigments in the extracts from 5 to 7 pH values. In their study, Atnip et al. (2017) demonstrated that anthocyanins in a medium with pH around 5 interact less with gastrointestinal tract cells, especially gastric epithelial cells (NCI-N87), than in the culture media at pH 3 and 7.4. The authors justify this fact due to the predominance of chalcones in the medium, hydrated structures and with the opening of the C ring, showing that the transport of anthocyanins in the mentioned cells is preferable for those structures with closed C rings (cation flavilium and pseudo base carbinol).

One way to stabilize and even increase the color intensity of anthocyanins is through copigmentation. Mazza and Brouillard (1987) showed that the increased concentration of anthocyanin and the proportion between co-pigments and anthocyanin promotes a greater copigmentation effect. In general, compounds that perform copigmentation alone are not colored. When mixed with anthocyanins in a solution, there is a non-covalent molecular interaction between these two components. This interaction can generate the hyperchromic effect (increased absorption intensity) and/or the bathochromic change (displacement of the maximum absorption band for longer wavelengths) in the UV-Vis spectrum as previously mentioned (Santos-Buelga & González-Paramás, 2018; Khoo et al., 2017; Bimpilas et al., 2016; Castañeda-Ovando et al., 2009; Mazza & Brouillard, 1987).

The use of copigments is very common in the food industry to increase the stability of anthocyanins in juices, jellies, concentrates, and fruit extracts, as well as to intensify the color of foods and beverages in the presence of anthocyanins. Some copigments studied include sinapic acid (Terefe et al., 2019; Ko et al., 2017); phenolic acids, such as ferulic, caffeic, gallic acid (Qian et al., 2017), tannic, benzoic (Babaloo & Jaime, 2018); and sugars, such as honey and maltose syrup (Ertan et al.,2018).

Despite the efforts made to improve the stability of anthocyanins to expand their applications, the use of copigments is not sufficient for this. Many structures of copigmented anthocyanins remain satisfactorily stable only at low pH values (Babaloo & Jaime, 2018), which makes it difficult to use these compounds as dyes in foods with pH> 4.0.

Another factor observed is that copigmentation may not affect heat-treated foods, as is the case with strawberry purees treated at 88°C / 2 minutes in the presence of sinapic acid (Terefe et al, 2019). In addition, copigmentation alters the original structure of anthocyanin (Chen & Inbaraj, 2019; Bimpilas et al., 2016), which can interfere with bioavailability and absorption into the bloodstream (Khoo et al, 2017).

In this context, based on facts and recent literatures, the use of biopolymeric nanoparticles has aroused the interest of many research groups due to the favorable properties of these nanostructures, such as good biocompatibility, simple design, easy preparation, and diversity of the structures (Meka et al., 2017). These properties make it possible to deliver bioactive compounds directly to the intended site of action. In this way, the stability and even the functionality of anthocyanins can be improved through encapsulation in biopolymeric nanoparticles.

4. Nanotechnology and Anthocyanin Stabilization

Nanotechnology is the area of science dedicated to the study, creation, and application of materials on a nano size. It has contributed to the technological development of chemistry, biology, physics, materials science, and engineering. Concerning the dimensions, the precise size that the authors considered a nanoparticle is still an issue under debate (Joye & McClements, 2014). For some scientists a nanoparticle should be ≤ 100 nm in diameter (Tong et al., 2020; Fathi et al., 2014), while for others, the sizes should be up to 500 nm (Tan et al., 2018; Wang et al.,2018; He et al., 2017).

It`s known that the use of the concept of nanotechnology is valid when materials produced at the nanoscale have surface properties superior to the same material in its original size or on the microscale. However, such properties are not always associated only with particle size (Joye & McClements, 2014). Optical, mechanical, biological, physical-chemical, and morphological properties, including size, are also extremely dependent on the type of wall material used (Bazana et al., 2019).

Therefore, excellent properties can also be found in particles with sizes between 100 and 500 nm (Ko et al., 2017; Joye & McClements, 2014). Among the nanostructures with these properties, those directed to bioactive compounds nanoencapsulation have been highlighted in the biomedical, food and material sciences areas.

In the pharmaceutical and food industries, nanoencapsulation is preferably used for compounds that need to be released in a controlled, continuous manner and at a specific location in the human organism or food (Robert & Fredes, 2015). Still, the use of nanoencapsulation in the food sector can also maintain or enhance, for a longer period, the interest properties of the active compound in a product, such as the antioxidant, antimicrobial, color capacity, and antifungal activity (Pola et al., 2019; He et al., 2017; Comin et al., 2016). Packaging and polymers area, in general, also benefits from this technology, which can be used to increase the mechanical resistance and thermal properties of different types of materials, as well as to improve their gas and water vapor barrier properties (Espitia et al., 2013).

According to Jafari (2017), nanoparticles can be classified into five groups: lipid-based nanoparticles (nanoemulsions, solid lipid nanoparticles, liposomes, nanostructured lipid carriers), polymeric nanoparticles (ionic gelatinization, coacervation, emulsification - solvent evaporation, polyelectrolytic complexation), miscellaneous nanoparticles (nanocrystals, nanostructured surfactants, inorganic nanoparticles), nanoparticles produced by special equipment (electrospraying/electrospinning, nanospray-dryer, nanofluidization), and naturally produced nanoparticles (caseins, cyclodextrins, nanostructured amyloses).

Several techniques have been used in anthocyanin nanoencapsulation to obtain smaller sizes, higher encapsulation efficiency, and greater therapeutic activity or coloring power. Table 1 presents several studies that evaluated the incorporation of anthocyanins in nanostructured systems in the last 10 years. Among the techniques employed, lipid-based nanoparticles, nanoparticles produced by special equipment, and polymeric nanoparticles are frequently used.

Despite being well studied, many lipid-based formulations are added to surfactants in relatively high amounts. This fact limits the application of these nanomaterials in some products for food and pharmaceutical purposes due to economic, legal, or sensory problems (Joye, Davidov-Pardo, & McClements, 2014). Besides, lipid-based nanomaterials are also more prone to oxidation and physical instability, as in the case of liposomes (Bryła et al., 2015; Joye et al., 2014).

Table 1. Summary of recent work involving anthocyanin nanoencapsulation.

System	Technique	Composition of wall material	Anthocyanin sources	EE Size PDI Zeta potential	Study variables	Source
Lipid-based nanoparticles	**Solid lipid nanoparticle**	Palmitic acid Span 85 Egg lecithin	Red cabbage powder extract	20.45 - 94.43% 0.414 - 59.482µm - -	**Placket-Burman**: % primary aqueous phase volume in the lipid phase / % lipid / homogenization rate / % Pluronic F127 in the secondary aqueous phase / % total surfactant / lecithin:span 85 ratio / homogenization time (minafter total lipid dispersion in the secondary aqueous phase / T[a] secondary aqueous phase / injection rate of the lipid phase into the aqueous phase / volume ratio of lipid phase to secondary water phase / co-surfactant type (ethanol, isobutanol) over EE%, size and SPAN index. **Box–Behnken**: EE%; size	Ravanfar, Tamaddon, Niakousari, & Moein, (2016)
	Pickering emulsion	Soy protein isolate Soy oil	Black rice extract	90.02 – 94.18% 186 - 675 nm - (-14) - (-18) mV	Size, zeta potential, Surface hydrophobicity, antioxidant capacity, oxidative stability, released free fatty acids, CLSM, Cryo-SEM, Creaming index	Ju et al. (2020)
	Double emulsion / Niosome	**double-emulsion**: polycaprolactone tween 20 **Niosome**: cholesterol Tween 20	Black carrot extract	**double-emulsion:** 40% 252.4 - 1003 nm - **Niosome**: 130-333.5 130-333.5 nm -	**Double-emulsion:** feeding time, T[a], mixing speed, PCL, [anthocyanin], %Tween 20 **Niosomes:** Cholesterol, Tween 20, anthocyanin amounts, feeding time, ultrasonication, ultrasonic probe and T[a]	Fidan-Yardimci et al. (2019)
	Liposome	soy lecithin	Black carrot extract	40 - 66% Day 0: 42.2 - 46.8 nm; Day 21: 41.0 - 50.8 nm; Day 0: 0.269 - 0.352; Day 21: 0.301 - 0.429; Day 0: 21.3 - 26.3 mV; Day 21: 26.3 - 28.6 mV	size, PDI, zeta potencial, EE%, phenolic content, antioxidant activity, anthocyanin content, color, lipid oxidation (hexanal content)	Guldiken, Gibis, Boyacioglu, Capanoglu, & Weiss, (2018)

	Liposome	Soy lecithin; cholesterol	Bilberry	50.6% 159 nm 0.244 -40.2 mV		Size, zeta potential, PDI, EE%; TEM; *in vitro* gastrointestinal drug release; FTIR	Zhao, Temelli, & Chen (2017)
Miscellaneous Nanoparticles	**Bicontinuous microemulsion**	Tween 80/ span 80, isopropyl palmitate, ethanol	blueberry	- 70 nm - 40.2 mV		HLB value; Km value; TEM; size; viscosity; anthocyanin content; light, T[a], NaCl, sucrose, and glucose stability	Chen, Ma, Yao, Zhang, & Zhao (2018)
Biopolymer nanoparticles	**Copigmentation + Polyelectrolite complex**	chondroitin sulfate / chitosan /cathechin	Blueberry extract	~ 60 – 70% -		Size, zeta potential, color, FTIR, CE%, EE%, Stability (ascorbic acid, T[a], auto-oxidation)	Tan, Celli, Selig, & Abbaspourrad, (2018)
	Emulsification-solvent evaporation	PLGA/PEG	-	120–165nm	60% 12mV	0.4	EE%; TEM; size, zeta potential; FTIR; In vitro drug release; cell culture, cytotoxicity; in vitro oxidative stress; ApoTox-Glo triplex assay	Amin, Shah, Badshah, Khan, & Kim (2017)
	Ionic gelation + Polyelectrolite complex	Chitosan / sodium alginate	blackrice extract	56.34 - 68.92% 35.5-635 nm - -			EE%; size, SEM, FTIR, antioxidant capacity	Bulatao, Samin, Salazar, & Monserate, (2016)
	Polyelectrolyte complex	Chitosan / Arabic gum	acai berry	- 230 - 260 nm 0.21 - 0.29 -			antioxidant capacity, size, PDI, stability (pH 4 and 7)	Shim, Lee, Nam, & Lee (2016)
	Copigmentation + Polyelectrolite complex	chitosan (CS)/ chondroitin sulfate	blueberry powder extract	~ 60 – 85% ~ 200 - 600nm - ~18 - 33 mV			copigment type; size, zeta potential, color, FTIR, EE%, ascorbic acid stability	Tan, Celli, & Abbaspourrad (2018)
	Polyelectrolyte complex	Chitosan hydrochloride/ Carboxymethyl chitosan/ β-lactoglobulin	Anthocyanins mixture (25% of purity)	41.3 - 70.8% 88.3 - 144.1 nm - -			%ATN retention, size, EE%, zeta potential, *in vitro* releasing test, *in vitro* digestion	Ge et al. (2019)
	Polyelectrolyte complex	chitosan + Sodium tripoliphosphate (TPP)	Blueberry extract	94.02% (NCC) – 32.54% (TPP) 64.8 nm (NCC) – 33887 nm (TPP) -			size; %Yield; EE%, phenolics content, antioxidant activity, anthocyanin distribuition; In vitro releasing test; FTIR; FE-SEM	Wang, Jung, Zhao (2017)

		/ cellulose nanocrystal (NCC)				
	Polyelectrolyte complex	chitosan/ chondroitin sulfate	black rice extract	~ 87% 350.1 nm 42.55 0.158 42.55 mV	size; zeta potential; PDI; loading efficiency%; TEM; AFM; FTIR, thermogravimetric analysis; cell culture; cell viability; cell apoptosis	Liang, Zhang & Jing (2019)
	Polyelectrolyte complex	carboxymethyl chitosan/chitosan hydrochloride	Blueberry extract	38.2 - 64,2% 211 - 751 nm - -	EE%; size, *in vitro* digestion; anthocyanin content; stability in a beverage system model;	He et al. (2017)
	Polyelectrolyte complex	carboxymethyl chitosan / chitosan hydrochloride	Blueberry extract	16.2 - 44.0% 178.1 - 273.6 - nm 0.315 - 0.571 15.3 - 27.8 mV	anthocyanin content; size; zeta potential; EE%; thermal properties; FTIR, TEM; storage stability (light, pH, T[a], ascorbic acid)	Ge, Yue, Chi, Liang, & Gao, (2018)
Nanoparticles produced by special equipments	**uniaxial and coaxial eletospinning**	Gelatin / Lactoalbumin	sour cherry concentrate	89.7 and 91.3% (phenolic acids); 70.3 and 77.8% (flavonoids); (-3.19) – (-16.6) - -16.6 to -3.19 mV	SEM; zeta potential; contact angle; phenolics content; flavonoids content; anthocyanins content; antioxidant capacity; EE%; *in vitro* digestion;	Isik; Altay; Capanoglu (2018)
	eletrospraying	chitosan / gelatin	black carrot extract	75.6 - 76.9% ~100 nm - ~1.5µm - -	SEM; FTIR; EE%; *in vitro* drug release in ethanol 10 % and acetic acid 3%	Isik, Altay, & Capanoglu (2018)
	electrospinning	Zein	Red Cabbage	- 444 - 510 nm - -	size; color; SEM; FTIR; contact angle	Prietto et al. (2018)
Naturally produced nanoparticles	**inclusion complex**	cycloamylose / β-cyclodextrin	Black rice extract	-	FTIR, antioxidant capacity, stability (thermal, color)	Jung, Joo, Rho, & Kim (2020)

Abbreviations: EE%, encapsulation efficiency; PDI, polydispersity index; T[a], temperature; CLSM, Confocal laser scanning microscopy; SEM, scanning electronic microscopy; PCL, polycaprolactone; PLGA/PEG, poly(lactic-co-glycolic acid)/ Polyethylene glycol; TEM, Transmission electron microscopy; FTIR, Fourier transform infrared; HLB, hydrophilic-lipophilic balance; CE%, capacity encapsulation. Source: Own authorship (2022).

Ravanfar et al., (2016) tried to nano encapsulate the anthocyanins in solid lipid nanoparticles using the microemulsion dilution method. First of all, a factor screening was performed with the Placket-Burman model, followed by an optimization of the encapsulation efficiency (EE%) and size (nm) responses by the Box-Behnken response surface methodology. Nanoparticles' average size was 479 nm with EE = 93.8%. However, 50% surfactant was required in the formulation concerning the lipid phase. The authors' conclusion regarding the surfactant content was as the higher the concentration, the smaller the particle size, and the higher the EE%.

Regarding the nanoparticles produced by special equipment, there are still difficulties in the construction, operation, and maintenance of this equipment due to the complexity they demand and the high cost involved (Tarone et al., 2020). Besides, there are techniques within this class of nanoparticles that require the use of solvents and presenting difficulties in monitoring the size of the formed particle, such as the supercritical fluid technique. Additional difficulties can also be found in sophisticated techniques, e.g. electrospinning and electrospraying can present low performance and slow process, while nanospray-dryer and freeze-dryer can produce high particle porosity (Tarone et al., 2020; Arpagaus et al., 2017; Gu, Linehan & Tseng, 2015).

In this context, biopolymeric nanoparticles have interesting properties to nanoencapsulation anthocyanins extending their use in the pharmaceutical and food areas. Aside from the similar properties that the bio polymeric nanoparticles have with those produced with synthetic polymers, they also exhibit non-toxicity, biocompatibility, biodegradability, widely available, low cost, and possibiliting their use in food processing (Luo & Wang, 2014). Furthermore, it is possible to produce biopolymeric nanoparticles with high encapsulation efficiency, absence of pores, safe (many are produced free of organic solvents and with polymers recognized as GRAS), and with bioadhesive properties (Sharif et al., 2020; Akhavan, & Jafari, 2017).

Depending on the preparation method, the biopolymer nanoparticles can be obtained as nanocapsules (vesicular system in which the bioactive compound is located inside a cavity, which consists of an internal liquid core surrounded by a polymeric membrane) or nanospheres (the bioactive compound found physically and uniformly dispersed throughout the matrix) (Esfanjani & Jafari, 2016). In general, the shape of biopolymeric nanoparticles is spherical, but they can also appear as spheroids, ellipsoids, agglomerates, disc-shaped, or needle-like when methods of macroscopic rupture, extrusion, or gel molding are used (Wen et al., 2018).

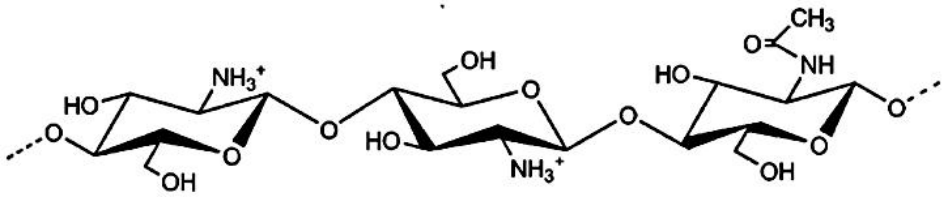
Several properties and functions conferred to biopolymeric nanoparticles come from the diversity of existing biopolymers, including polysaccharides (chitosan, gums, cellulose and their derivatives, pectin, starch) and proteins (zein, B-lactoglobulin, soy protein isolate, whey protein) (Esfanjani & Jafari, 2016).

The use of biopolymers enables the occurrence of different interactions during the formation of nanoparticles, such as electrostatic attractions, hydrogen bonds, and hydrophobic interactions (Pisoschi et al, 2018; Arroyo-Maya & McClements, 2015). Thus, biopolymeric nanoparticles can prevent the hydration of anthocyanins, maintain the flavylium cations, and improves the stability of these molecules in media considered "unsuitable" for them through associations with biopolymers (He et al., 2017).

Among the polymers used in the production of nanoparticles, chitosan is a linear polysaccharide obtained from the deacetylation of chitin and composed of randomly distributed β- (1-4) -linked D-glucosamine residues and N-acetyl-D-glucosamine units (Figure 4) (Wu et al., 2020; Ali & Ahmed, 2018). Chitosan is the second most abundant polymer in the world, behind only cellulose, and stands out because it is derived from a non-toxic natural source. Furthermore, chitosan is a versatile, biodegradable, biocompatible polymer, and has excellent functional properties, such as bio adhesiveness and anti-tumor activity (Mohammed et al., 2017; Ali & Ahmed, 2018).

Chitosan's versatility is due to the presence of active amino groups in its structure. This allows chitosan to be soluble and to have a positive charge in aqueous acidic media, which makes it capable of carrying out various interactions with other compounds of interest in the food and pharmaceutical industry (Ali & Ahmed, 2018; Zhao et al., 2011). In pH values <6.5, the amino groups located on the C2 position of the repeating-glucopyranose units of chitosan become protonated. Therefore, these groups can interact electrostatically with polyanions to form complexes and hydrogels (Hamman, 2010; Zhao et al., 2011).

Figure 4. Chitosan chemical structure.



Source: Adapted from <https://www.crq4.org.br/quimica_viva__a_quimica_das_quitosanas>

Chitosan is particularly useful for producing biopolymer nanoparticles based on electrostatic interactions. In addition, chitosan can be found with different molecular weights and acetylation degrees, which enable greater control of particle characteristics (Joye & McClements, 2014). Among the methods for preparing chitosan nanoparticles, IG and PC are the most used, since they do not require sophisticated equipment and present simple, fast and inexpensive methodologies.

Ionic gelatinization consists of the ionic interaction between the positive charges of chitosan's amino groups and the negative groups of polyanions, such as sodium tripolyphosphate (Sreekumar et al., 2018), pyrophosphate (Cai & Lapitsky, 2014), glutaraldehyde (Asiri et al., 2018), genipin (Pujana et al., 2013), among others. Among polyions studied to date, sodium tripolyphosphate (TPP) is the most used in the chitosan cross-linking, because it presents low toxicity and formation of nanoparticles with desirable sizes and shapes (Sreekumar et al., 2018; Fan et al., 2012).

Polyelectrolytic complexation involves the formation of interpolymer complexes through non-covalent interactions (electrostatic interactions, hydrophobic interactions, hydrogen bonding, and Van der Waals interactions) between polymers with opposite charges. Electrostatic interactions mainly arise from an increase in entropy due to the release of low-molecular-weight counterions (Shovsky et al., 2009). These interactions promote the formation of a three-dimensional network and particles with different sizes, depending on the polymers used, can be formed (Wu et al., 2020; Meka et al., 2017). This process is safe, ecological, simple to perform, free of chemical cross-linking agents and organic solvents, and requires little energy. (Wu et al., 2020).

4.1 Production of chitosan nanoparticles

In a stable colloidal system, the particles remain in suspension and resist aggregation or flocculation. This stability depends on the balance between repulsive and attractive forces inherent to the surface charges of the particles and the Brownian motion contained in the system (De Robertis et al., 2014). If there is little or no repulsive force, destabilizing mechanisms such as flocculation or aggregation may occur. Once destabilized, the particles in the colloidal system interact with each other and form aggregates that precipitate by gravity (Meka et al., 2017).

For the production of nanoparticles using PC and IG, the most common method is extrusion/drip with a syringe (Figure 4) (Wu et al., 2020; Bulatao et al., 2017; Meka et al., 2017; Patil et al., 2010). In this method, the crosslinking agent (IG method) or the negatively charged polymer dispersion (PC method) is added through a manual or automatic drip to another dispersion, which is kept under constant agitation (Patil et al., 2010).

Some research points to the effect of the order of addition/titration on the formation of polyelectrolytic complexes and their influence on the average size, zeta potential, and morphology of the particles formed, showing that in the same molar or mass ratio, different complexes are formed due to this effect (Wang et al., 2017; Wang et al., 2011). However, it is worth remembering that an excess of positive charges leads to the formation of nanoparticles instead of aggregates and macrogels, which does not occur when there is an excess of polyanion charges. In the latter case, more unstable aggregates and complexes are formed (Costalat et al., 2015; Wang & Roman, 2011).

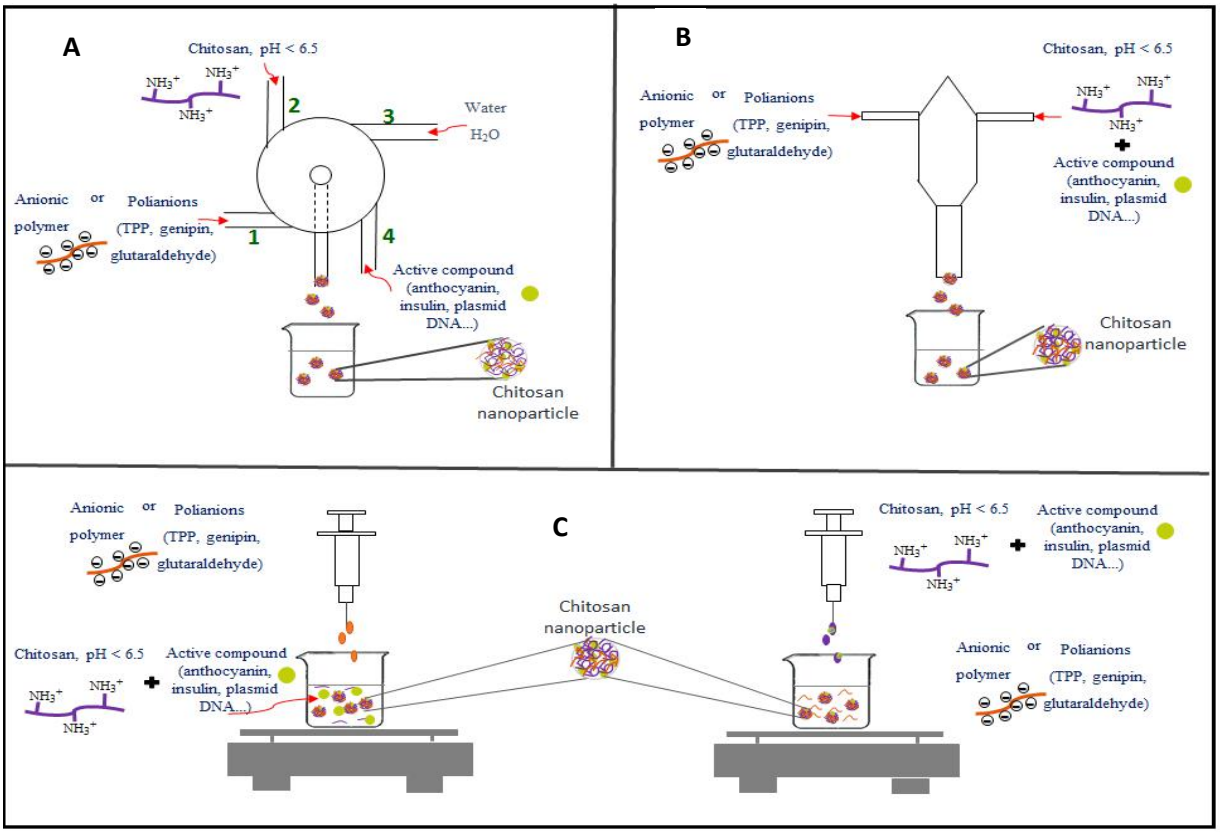
Some parameters can be controlled to achieve the desired particle size. Some of them are: the rate of addition of the dispersion to be dripped, pH and ionic strength of the dispersions, agitation speed, direction of the titration, and molar and mass ratio between the polymers or polymer-polyanion (Yuan & Huang, 2019; Sreekumar et al., 2018; Ramasamy et al., 2013; Wang et al., 2011; Wang & Roman, 2011).

In addition to the syringe extrusion/drip technique, other techniques are developed to improve the reproducibility, stability, and uniformity of nanoparticles formed by IG and PC. This allows compounds with hydrophilic characteristics to be nanoencapsulated more efficiently (He et al., 2018). An example of this is the recent use of the flash nanocomplexation (FNC) technique (Figure 5) by He et al. (2017) to produce polyelectrolytic complexes based on chitosan with small diameters and uniform size distribution.

Santos et al. (2016) used FNC for the first time through adaptations of the flash nanoprecipitation technique (FNP). The adapted technique excludes the use of organic solvents and is induced only by the formation of polyelectrolytic complexes since in the PNF technique the complexes formed are precipitated by the addition of solvents (Santos et al., 2016). For the production of uniform nanoparticles, specific equipment must be used, such as a multi-inlet vortex mixer (MIVM) or a confined impinging jet mixer (CIJ) for a continuous flow process, which allows the production of nanoparticles with sizes between 30 and 150 nm and enables the use of this technology on a large scale. (He et al., 2018; He et al., 2017; Santos et al., 2016).

Although the FNC technique is very promising, specific equipment is necessary, which still compromises research in many laboratories. Therefore, developing biopolymer nanoparticles based on chitosan through ion gelatinization or polyelectrolytic complexation, and associating them with the titration/drip technique is still the most accessible, quick, and simple option to date.

Figure 5. Methods of producing biopolymeric nanoparticles by polyelectrolyte complexation and ionic gelatinization.



*(A) multi-inlet vortex mixer (MIVM); (B) confined impinging jet (CIJ) mixer; (C) extrusion/drip method. Source: Own authorship (2022).

5. Recent Advances in Anthocyanin Nanoencapsulation by Polyelectrolytic Complexation and Ionic Gelatinization

When working with nanoparticulate systems incorporated with sensitive bioactive compounds, high efficiency of encapsulation, better performance of biological activities, and good stability in mediums with wide pH variations (mainly with pH> 6) and high temperatures are sought. In recent years, different polymers have been used in the research area, which includes proteins and polysaccharides. Cellulose nanocrystals (NCC), chondroitin sulfate (CHS), β-lactoglobulin (β-lg), and zein are some examples of biopolymers used for anthocyanins nanoencapsulation and other bioactive and nutraceutical compounds.

Cellulose nanocrystals are crystalline and compact structures, isolated from acid hydrolysis, mainly with sulfuric acid, from cellulose fibers (Abdul Khalil et al., 2016). NCCs are in the form of elongated rods or filaments and have dimensions ranging from 5 to 25 nm in diameter and from 100 to 500 nm in length (Wang & Roman, 2011).

Among the properties of NCCs, low density, low cost, abundance in nature, good mechanical properties, large surface area, flexibility, barrier properties, and low thermal expansion stand out (Abdul Khalil et al., 2016). Additionally, NCCs can increase the stiffness of nanocomposites, even at low concentrations, when incorporated into polymeric matrices. This resistance effect is due to the high length-to-width ratio and the ability of NCCs to form structures through hydrogen bonding (Yadav, Behera, Chang, Chiu, 2020; Abdul Khalil et al., 2016).

The association of NCCs with cationic polymers, such as chitosan, has attracted the attention of several researchers due to the excellent properties conferred to these materials. Furthermore, the fact that NCCs are food grade and biocompatible

makes it possible to use them in food formulations, packaging development, and development of controlled release systems for the pharmaceutical and food industries. (Mu et al., 2019; Vashist et al., 2018).

Wang, Jung, and Zhao (2017) encapsulated blueberry extract in chitosan-NCC nanoparticles for the first time and achieved EE = 98% and yield of approximately 6.9 g, with an average particle size of 64.8 nm. The authors also produced chitosan-TPP particles and used the same parameters to the NCC. As a result, microparticles with an average size of 33.88 µm, a yield of 0.3 g and EE = 32.54% were formed. Furthermore, when studying controlled release in vitro, the authors found that nanoparticles with NCC were more stable at pH 7.4 than microparticles with TPP.

Chondroitin sulfate (CHS), originated from animal tissues and some bacteria, is a heteropolysaccharide from the group of glycosaminoglycans (GAG), unbranched, and negatively charged (Liang et al., 2019). Its structure is composed of repeated units of alternating sulfated disaccharides (glucuronic acid and N-acetylgalactosamine residues), linked by glycosidic bonds β-(1 → 3). The sulfonate groups of chondroitin sulfate occur mainly in the hydroxyls in C4 and C6 of the N-acetylgalactosamine unit, and C2 of the glucuronic acid (Kumari, & Badwaik, 2019).

Besides composing a large part of the extracellular matrix of various connective tissues such as skin, bone, cartilage, ligaments, and tendons, CHS is involved in several biochemical activities such as antioxidants, anticoagulants, and anti-inflammatory. (Kumari, & Badwaik, 2019; Shariatinia, & Barzegari, 2019). This natural polysaccharide has also been the subject of much research in the field of nanotechnology, including the formation of nanostructured systems with chitosan.

Liang et al., (2019) encapsulated black rice extract anthocyanins in chitosan-CHS nanoparticles by IG method and found that there was an influence on pH, the mass proportion of polymers, and the concentration of anthocyanins in the development of nanoparticles. The encapsulation efficiency of 88.32% and an average size of 350 nm were achieved. Moreover, nanoencapsulated anthocyanins significantly reduced the viability of colon cancer cells.

Tan et al. (2018) nanoencapsulated blueberry anthocyanins copigmented with epigallocatechin gallate (EGCG) in chitosan-CHS nanoparticles. It was found that the antioxidant capacity in ascorbic acid media of nanoencapsulated anthocyanins with EGCG was greater than free anthocyanins and encapsulated anthocyanins without EGCG. Nanoencapsulated anthocyanins in the presence of EGCG inhibited 82% and 70% of DPPH and FRAP radicals, respectively.

Another advance in research to overcome the problems in the production of chitosan nanoparticles is through the modification of the chitosan surface, as in the case of chitosan hydrochloride and carboxymethyl chitosan. Good results in particle size and encapsulation efficiency have been achieved for several compounds, including doxorubicin hydrochloride, with EE = 72% and 279.3 nm (Feng et al., 2013); quercetin, with EE = 70% and 386.3 nm (Yan et al., 2018) and green tea polyphenols, with EE = 83% and 407 nm (Liang et al., 2011). For anthocyanins, the encapsulation efficiency may be further improved, and in studies of He et al. (2017) and Ge et al. (2018), the maximum values obtained for $EE_{blueberry\ extract}$ were 64.2% and 44%, respectively.

Some authors report that biopolymeric nanoparticles produced by the drip / extrusion method are not very suitable for hydrophilic compounds nanoencapsulation, because the structures formed are porous and metastable. This accelerates the oxygen permeation through the matrix and facilitates the diffusion of the bioactive compound in a shorter time. In this way, the functionality of the formed nanoparticles becomes limited, with a consequent reduction in the encapsulation efficiency. (Sharif et al., 2020; Bulatao et al., 2018; Kurozawa & Hubinger, 2017; Joye et al., 2014).

Despite the still considerable anthocyanin losses in these systems, other strategies can also be used together to minimize these effects. An example is the use of copigmentation before the formation of nanoparticles (Tan et al., 2018), the combination of IG and PC techniques (Bulatao et al., 2016), and the formation of polyelectrolytic complexes associated with proteins, such as β -lactoglobulin (Ge et al., 2019) and zein (Prietto et al., 2018) to encapsulate anthocyanins.

As for the association with proteins, zein is a promising protein in the encapsulation area. In addition to having a hydrophobic characteristic, zein is considered to be GRAS, has excellent mucoadhesive properties, and is capable of resisting the gastric environment. Thus, zein can be used as a vehicle for the controlled release of active compounds sensitive to the action of enzymes and pH in the gastrointestinal tract (Luo et al., 2012; Paliwal & Palakurthi, 2014).

Considering the instability and low bioavailability of anthocyanins in the large intestine, besides the fact chitosan-TPP nanoparticles are a metastable systems (Furtado et al., 2018; Wang et al., 2017; Tsai et al., 2011), zein could be used to coat chitosan nanoparticles loaded with anthocyanins stabilizing them. Moreover, zein can act as a polyanion in the formation of complexes with chitosan through hydrogen bonds between the hydroxyls present in its structure and the amino groups of chitosan (Ren et al., 2019). An example is the coating of chitosan-TPP nanoparticles incorporated with sodium selenite. After zein coating, EE increased from 60% to 95% and the release profile got better control compared to selenite in free form (Luo et al., 2010).

Given all the factors mentioned above and the low stability of anthocyanins, the incorporation of these molecules in chitosan nanoparticles is considered a challenge. Several factors, isolated or combined, contribute to the stability of nanoparticles formed by PC and IG. They can be classified into three groups: I) intrinsic factors of polyions, such as the nature of ionic groups, molecular weight, and charge density; II) extrinsic factors of polyions, such as polyelectrolyte concentration, pH, ionic strength of the medium, order of addition (addition of the polyanion dispersion in the polycation dispersion or vice versa); III) other factors, such as the use of ultrasound.

The structure of the nanoparticle is directly influenced by the factors described (Al-Rashed, Niknezhad, and Jana, 2019; Rampino et al., 2013), and they are not only affected by the preparation but also during storage (Rampino et al., 2013). Therefore, correct planning, with control of the variables that mainly affect EE, particle size, and the bioavailability of anthocyanins can result in highly stable systems.

5.1 pH Effect

It is known that the pH value that anthocyanin acquires in its most stable form varies between 1 and 3, which restricts the pH ranges that can be used in the preparation of polymeric dispersions. Chitosan is a promising polymer for anthocyanin nanoencapsulation because it is a weak polyelectrolyte. Chitosan charge density varies according to the solution pH, so its degree of protonation may be changed by variations in the dispersion pH (Al-Rashed et al., 2019).

The highest protonation degrees of the primary chitosan amino groups occurs in pH values <6.5. Therefore, it is possible to work with dispersions in pH values between 3 and 4 (Abdel-Hafez et al., 2014), which contributes to the greater stability of anthocyanin molecules. However, in media with pH values <4, the chitosan molecules exhibit extended conformation. This is due to the high protonation of amino groups, and even more when the degree of deacetylation of chitosan is high (> 85%). Thus, the electrostatic repulsion between the chitosan chains is intensified, which provides an increase in the exposure of the ionizable amino groups. As a result, interactions with the polyanion intensify and provide an increase in the size of the particles formed (Tsai et al., 2011).

Another point to be considered is the nature of the polyanion. Regarding ionic gelation, it is known that the most crosslinking agent used is TPP. The pH of a 0.01 M TPP aqueous solution is 9.7 (Wu et al., 2011). Depending on the volume and concentration of the TPP solution and the initial pH of the chitosan dispersion, the pH of the reaction medium may increase to a value that is capable of destabilizing the anthocyanin molecules by hydrating the *flavilium* cation. In this case, there is a nucleophilic attack on carbon 2, which leads to the formation of carbinol (colorless) pseudo bases and even chalcones. These changes in the chemical structure of anthocyanin can hinder the nanoencapsulation of these molecules and even their biological activity (Arroyo-Maya & McClements, 2015).

In polyelectrolytic complexes, the structure, morphology, and functionality of the nanoparticles formed are even more affected by pH variations, since the polyanion is also a polymeric structure and, in addition to its main functional groups, pH can also interfere in the intramolecular interactions of the anionic polymer and highlight hydrophobic interactions and hydrogen bonds (Luo & Wang, 2014). These interactions can restrict access to anionic groups since the polymeric chain will be presented in a compact form at pH values close to the pKa of its functional groups. Therefore, the nanoparticles formation and the final yield will be reduced (Laleveé et al., 2017).

5.2 Effect of deacetylation degree, molecular weight and chitosan concentration

The main disadvantage of chitosan-based nanosystems application is the lack of understanding and consensus between the different production protocols reported in scientific articles (Sreekumar et al., 2018). This hinders the ability to reproduce these nanoparticles, especially in relation to size, since the mass ratio of chitosan:polyanion is considered instead of molar ratios. Moreover, experimental factors such as deacetylation degree and molar mass of chitosan are often not prioritized. However, these factors are very important since the density of ionizable groups available for interaction is extremely significant in systems that have electrostatic interactions ~~such~~ as the main intermolecular force for interaction, besides hydrophobic interactions (Sreekumar et al., 2018; Abdel-Hafez et al., 2014).

The molecular weight refers to the average size of the individual chitosan chains. In general, high molar mass chitosan form larger particles compared to low molar mass chitosan (Wu et al. 2020; Luo & Wang, 2014). In addition, polyelectrolytes with weak ionic groups, a large difference in molecular weight and polymerization degree should be chosen during polymers and/or polyanions selection to produce stable chitosan nanoparticles. The guest-host theory confirms this claim, which the excess polymer ("host") has a higher polymerization degree than the "guest" polymer (Siyawamwaya et al., 2015). Under these conditions, the polyelectrolytes are mixed at a non-stoichiometric molar ratio, that is, with a ratio of cationic functional groups: anionic > 1.

Wu et al. (2017) were able to produce stable sodium chitosan-heparin-hyaluronate nanoparticles in a medium with physiological pH and ionic strength by selecting chitosan with an appropriate degree of deacetylation (50%) and molecular mass (10^3 kg.mol^{-1}). The nanoparticles obtained were of the core-shell type, with the shell showing hydrophilic characteristics and a diffuse interface.

As for the deacetylation degree (DD), this is related to the charge density of chitosan, which is associated with the degree of substitution of acetyl groups with free amino groups, and is very important in the development of chitosan nanoparticles. According to Laleveé et al. (2017) high degree of deacetylation (DD = 86%) of chitosan increased its charge density. Therefore, chitosan was able to interact more with the sodium hyaluronate molecules, resulting in larger and stable particles. However, by reducing the DD, the charge density of chitosan was reduced, and fewer groups of sodium hyaluronate interacted with the amino groups of chitosan. The result was smaller particles with greater polydispersity.

Another factor that should be considered to obtain high polydispersity of nanoparticulated systems based on polysaccharides is the material batch. Chitosan and other commercial polysaccharides do not have a homogeneous molar mass, that is, the chains of this polysaccharide are polydispersed. This hinders the reproducibility of the experiments, due to the great influence of the charge density and molar mass on the morphology and size of the particles formed.

Rampino et al. (2013) reported that they achieved unexpected results in terms of size or flocculation of particles upon studying the formation of nanoparticles of chitosan-TPP. The authors attributed these practical difficulties to the variation between batches of chitosan. Morris et al. (2011) also found differences in the size of polymer chains between batches and within the same batch of chitosan when producing nanoparticles cross-linked with TPP. The authors found a variation of up to

30 nm in the size of the nanoparticles, and the samples that had higher molecular mass were the same ones that originated larger nanoparticles.

Based on these reports, it is suggested to use samples from the same batch when carrying out experiments. Thus, large variations in molar mass and deacetylation degree of the collected samples can be avoided. Also, it is considered important to perform as many repetitions and replicates as possible.

5.3 Effect of ionic strength and surfactants

Particle aggregation is also an undesirable factor in colloidal systems and indicates system instability. The control of all variables mentioned so far allows stable colloidal systems to be obtained. However, some strategies can also be used to achieve these results more effectively.

The presence of monovalent ions such as NaCl in the formation of chitosan-TPP nanoparticles contributes to greater stability and smaller particle size in the system, depending on the concentration used. Jonassen et al., (2012) studied the effect of ionic strength on the formation and size of chitosan nanoparticles - TPP. It was verified that the nanoparticles prepared in medium with 50 mM and 150 mM saline concentration presented less polydispersity than those prepared without the addition of NaCl. However, nanoparticles produced in the presence of NaCl 150 mM became more aggregated and presented a larger size compared to those produced with less salt concentration.

The surfactants use in suitable proportions relative to the polymer mass also contributes to the formation of a more stable colloidal system. Yeon et al. (2019) produced chitosan-TPP nanoparticles for immobilization of the glucose oxidase enzyme and used Tween 20 (0.5% v / v) to prevent agglomeration of the particles during the ionic gelation process.

5.4 Ultrasound effect

Another device that can be used to prevent the aggregation of nanoparticles in the redispersion step is the use of an ultrasound probe. Ultrasound can help break down particles, as well as reduce size and polydispersity through cavitation effects (Floris et al., 2013). However, care must be taken to particles be not disintegrated by the action of cavitation, avoiding re-agglomeration (Gokce et al., 2014). Therefore, low powers should be used in this process, and the ideal is to carry out the preliminary study of factors that interfere in this stage.

6. Applications of Nano and Microstructured Anthocyanins

Evidence from clinical and laboratory research has shown that free anthocyanins have beneficial health properties, such as cancer (Thibado et al. 2018) and cardiovascular disease prevention (Alvarez-Suarez, et al. 2014), diabetes control (Guo et al. 2018), antioxidant (Ahmad et al. 2018), neuroprotective properties (Jeong et al. 2012), and reduction on tumor activity (Jeong et al. 2016). In addition to the benefits found in the prevention and control of various diseases, anthocyanins also have the potential to replace synthetic dyes in the food (Khoo et al. 2017) and textiles (Haddar et al. 2017) industries.

The instability of the anthocyanin structures and the degradation in the organism before its absorption in the gastrointestinal environment are factors that reduce the aforementioned properties and due to this, become major obstacles for some applications. To solve these difficulties, during the last few years, researchers have directed efforts towards the improvement of nano/microstructure development techniques to protect and minimize anthocyanins degradation by the action of extrinsic factors such as pH, temperature, light, oxygen, and strength ionic (Sharif et al., 2020).

6.1 History and lack of effective applications

Contrary to the numerous applications of free anthocyanins found in the literature, the number of studies that apply micro / nanostructures containing anthocyanins in a product is scarce.

When keywords "Anthocyanins microencapsulation" or "Anthocyanins nanoencapsulation" are searched on ScienceDirect, for example, there is a reduction of more than 900 scientific articles to a few dozen articles that performed applications and found the size of these structures.

For the development of this work and description of the application areas of the nanoparticles produced by PC and IG techniques, other criteria were also adopted: particle size up to 500 nm, studies carried out with chitosan, the encapsulated compound should be a source of anthocyanins, and PC and IG techniques for the production of nanoparticles. However, the search resulted in few studies, which justifies examples of other nanostructures in the discussion of this topic.

Therefore, we present in Table 2 the researches that focused on the development of anthocyanin stabilization techniques by nano/microstructures formation in the last 10 years and carried out application studies. Additionally, the research lines, classifications of structure type, production method, the composition of wall material, and size of the nano/microstructures were highlighted for food and packaging.

6.2 Food applications

The development of nano/microstructures with optimal characteristics for applicability in food is ~~an~~ extensive, costly, and even impractical research in a short time~~,~~ due to the numerous factors and levels of study to be analyzed. One of the ways to develop processes and technologies with greater efficiency is through the use of response surface methodology (RSM). This tool allows determining optimal conditions, among the factors and responses evaluated, to obtain an optimized treatment, which saves time, reagents, and makes it possible to evaluate synergistic or antagonistic effects between independent variables. From this, research that uses RSM for development and application of nano/anthocyanin protective microstructures is frequently observed.

Table 2. Applications in the last 10 years of anthocyanins nano/microstructures.

Research line	Application	Structure classification	Production method	Composition of wall material	Size (µm)	Source
Food	Milk	Nanoliposomes	Ultrasonication	Lecithin/Cholesterol	0.053	Chi et al., (2019)
	Beverage	Nanoparticles	Ionic gelation	Carboxymethyl chitosan /Chitosan hydrochloride	0.219	He et al., (2017)
	Soft drink	Microcapsules	Spray-drying	Maltodextrin (MC), Maltodextrin/Gum arabic (MG), and Maltodextrin/ γ-Cyclodextrin (MC)	M > 500 MG < 200 MC < 50	Burin, Rossa, Ferreira-Lima, Hillmann, & Boirdignon-Luiz (2010)
Medicine	Tumor therapy	Nanocomplex	Mixing method in water	Chondroitin sulfate/ Doxorubicin hydrochloride	0.174 to 0.269	Jeong, Bae, Park, & Na (2016)
Packaging	Active biodegradable films	Microcapsules	Freeze-drying	Maltodextrin	below 150	Stoll et al., (2015)
	Active biodegradable films	Microcapsules	Freeze-drying	Gum arabic/Maltodextrin	below 200	Stoll, Costa, Jablonski, Flôres, & de Oliveira (2015)
Controlled release	Intestinal bioavailability and accessibility in humans	Microcapsules	Emulsification, thermal gelation, and spray-drying	Whey protein (WPC), and pectin (CPC)	WPC = 200 CPC = 250 to 500	Mueller et al., (2018)
Sensors	Detection of pH and borate additives	Nanoparticles	Advanced Stöber	Mesoporous sílica matrix	0.10 to 0.50	Ha, Lien, Anh, & Lam (2017)

Source: Own authorship (2022).

Chi et al. (2019) used the RSM tool to optimize anthocyanin-loaded nanoliposomes (AN), focusing on the particle size reduction and increase of anthocyanin retention rate. The AN optimization increased the anthocyanins' stability evaluated in vitro analysis over 30 days, at 4 ºC and 25 ºC, in the presence and absence of light, compared to free anthocyanins. When applying the optimized AN in milk, improvement in the protection of anthocyanins and with slow-release rate during intestinal digestion in vitro was observed. These results indicated the use of nanoliposomes as a potential functional ingredient for milk. However, new studies should be developed in milk to assess sensory quality, combined with anthocyanin retention rate and maintenance of bioactive effects until the expiration date.

He et al. (2017) also used the RSM tool to optimize chitosan nanoparticles with hydrochloride and carboxymethyl chitosan as constituents of wall material. When comparing EE% with work developed by Chi et al.~~,~~ (2019), it was noticed that nanoliposomes had 91.13 %, while chitosan nanoparticles showed 61.80 %. However, anthocyanin retention rate was persistent over time at 4 ºC and maintained at 84.5 % after 21 days of beverage storage (He et al. 2017). On the other hand, in Chi et al.'s (2019) study practically equal retention rate in milk was possible in just five days of storage at 4 ºC. Increased protection of anthocyanins by chitosan nanoparticles is dependent on the compositional of food matrices and copigmentation effect promoted by chitosan structures, which prevents hydration of flavilium cation and reduces instability of anthocyanins (Dangles & Brouillard, 1992).

The protection promoted by chitosan structures is proven by verifying that the release rate of anthocyanins in the gastric fluid after 120 minutes was 55 % for nanoliposomes and 47.73 % for chitosan nanoparticles. However, it is recommended that further research be carried out with anthocyanins from the same source to better understand the effect of each wall material on the stabilization of anthocyanins.

Burin et al. (2010) compared degradation kinetics of microencapsulated anthocyanins with different wall materials and applied them to soft drinks. It was observed that a combination of maltodextrin/Arabic-gum produced an increase in half-life time and lowest degradation constant in all evaluated conditions. This was justified by the action of Arabic-gum on the microcapsule wall as film-forming agent, which promoted better trapping of anthocyanins and made flavilium cation less vulnerable to hydrolysis (Dangles & Brouillard, 1992).

6.3 Food Packaging applications

Anthocyanins act as primary antioxidants due to the interruption of the lipid oxidation reaction chain when donating hydrogen to peroxy radicals, forming flavonoid radicals, which in turn reacts with another free radical and ends propagation chain (Reis et al. 2016; Griendling et al. 1994). They are also secondary antioxidants. They reduce the speed of oxidation initiation through chelation with metal ions and anti-peroxidative activity forming non-radical species (Reis et al. 2016). These characteristics allow its application in another area of science, such as the development of active packaging through the incorporation of anthocyanins in a polymeric matrix for food protection, since one of the main causes of reduced shelf life is oxidation processes.

Active packages are defined as those that intentionally interact with food to improve some of its characteristics (Soares, 1998). However, the low stability of flavonoids and, in some cases, lack of compatibility with the polymeric matrix makes it necessary to form micro/nanostructures for incorporation in packaging. In this sense, Stoll et al. (2015) produced active films based on cassava starch incorporated with anthocyanin microcapsules for application in extra virgin olive oil. Oxidation stability of oil in active packaging was maintained until the 8th day, while in polypropylene packaging degradation was observed on the 4th day. Insertion of anthocyanins in polymeric packaging for foods susceptible to oxidation is promising, however, further research is needed to evaluate the efficiency of the active effect throughout the storage period and comparisons with free anthocyanins in the polymeric matrix.

Soll et al. (2015) evaluated the antioxidant activity and compatibility of anthocyanin microcapsules formed by gum arabic (GA) and maltodextrin (MD) for application in active films. GA microcapsules showed antioxidant activity 2.44 times greater than structures formed by MD. This occurrence was justified by greater solubility of GA in water, which provided greater release and performance of anthocyanins in aqueous systems. However, when incorporating microcapsules of anthocyanins and MD into cassava starch films, a greater protective effect against the peroxides formation in sunflower oil was verified, as well as better compatibility with the polymeric matrix than GA-based microcapsules. Films containing MD-based microcapsules showed greater resistance to traction, greater percentage elongation, and less permeability to water vapor than active films with GA.

Implementation of active packaging in the food industry requires research to understand the mechanisms of anthocyanin diffusion to the food matrix. Besides, it is necessary to study the stability of microcapsules, the oxygen permeability through the films, and films' biodegradability capacity. Although antimicrobial or antioxidant properties of active packaging to be evaluated throughout the storage period, the same does not occur with other physical-chemical characterizations. The mechanical, sensory, thermal and barrier properties to water vapor and oxygen are often analyzed shortly after manufacture. This is a problem for the implementation of active packaging in commerce, since these and other properties are practically not studied during storage conditions.

7. Conclusions and Future Trends

Anthocyanins are bioactive compounds extremely sensitive to changes in pH, temperature, light, enzymes, and other variables in the environment in which they are found. Therefore, it is necessary to employ artifices and technologies for their wide use in ~~both~~ the food and pharmaceutical sectors. This review addressed the main limitations of anthocyanins in terms of stability and, in particular, was directed towards a more in-depth approach on the encapsulation of this class of bioactive compounds in biopolymeric nanoparticles developed by the techniques of polyelectrolytic complexation and ionic gelatinization.

These two methodologies have been widespread in recent years due to their simplicity and because they do not require the use of toxic solvents during the preparation stage. The main limitations in the development of these nanoparticulate systems were also discussed. It can be seen that there is still a long way to go in this area of nanotechnology, especially with regard to the development of stable colloidal nanoparticles in mediums with physiological pH and high encapsulation efficiency.

Several strategies were also pointed out to improve anthocyanin nanoencapsulation, such as the use of new biopolymers for complexing with chitosan, including cellulose nanocrystal, zein, and chondroitin sulfate; modulation of ionic strength; use of non-stoichiometric proportions between polyions; adequate molar proportions; and use of surfactants in appropriate proportions.

There is a lack of studies on the behavior of nanoparticles in food or the body ~~and~~ that assesses the changes caused in these environments, leading to the development of research that aims to apply and evaluate the behavior of nanoparticles in different systems. Despite the excellent results obtained in vitro, it is inconceivable to expect the same effects in commercial products or in living organisms that present complex physicochemical conditions. It can be observed incompatibility, reduction and/or even inactivation of the effects of the active compounds in the polymeric matrix.

In addition, we suggest new research trends in order to minimize the aforementioned problems, such as: giving more attention to structure-property-function relations when planning the nanostructures; analyzing the effectiveness and possible active function of anthocyanin degradation products; characterizing the physical-chemical properties over the storage time; and optimizing micro/nanostructures in the conditions of the commercialized matrix. These proposals can lead to a greater

understanding of the effect of micro/nanostructures containing anthocyanins, in addition to providing discoveries and efficient implementation in living organisms and/or food and pharmaceutical products.

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