UNIVERSIDADE ESTADUAL DE CAMPINAS
Faculdade de Engenharia de Alimentos

MARIA ISABEL LANDIM NEVES

Corante natural azul à base de leite e *Genipa americana* L. para a indústria de alimentos: Obtenção, mecanismo de reação e design de partículas utilizando carreadores prebióticos

An all-natural blue colorant based on milk and *Genipa americana* L. for the food industry: Obtaining, reaction mechanism, and particle design using prebiotic carriers

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To my parents, Maria do Carmo and João Vandir

Soli Deo gloria

"Let your light shine before others, that they may see your good deeds and glorify your Father in heaven"

Jesus Christ, Matthew 5:16

"I was taught that the way of progress was neither swift nor easy"

Marie Curie

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Quanto custa um doutorado?

Custa conhecimento e conhecimento nos faz enxergar nossa pequenez, o quanto ainda temos que aprender. Também custa sacrifícios, lapidação, humildade, aceitar erros e acertos também. Custa frustrações, surpresas, oportunidades, vontade de aprender, mas também desânimo de ter que fazer algo novo, ou de novo. Custa estar longe de pessoas que amamos, mas também amar pessoas que a caminhada nos aproxima. Nos ensina a perceber que nunca fazemos nada sozinhos e que todos que passam pelo nosso caminho nos deixa pegadas, ensinamentos e exemplos. Por perceber tão claramente que essa tese não seria feita se não fossem as pessoas e suas pegadas, tentarei verbalizar minha gratidão a cada um, ou cada grupo, que fez parte desse caminho.

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RESUMO

Corante natural azul à base de leite e *Genipa americana* L. para a indústria de alimentos: Obtenção, mecanismo de reação e design de partículas utilizando carreadores prebióticos

O interesse por alimentos saudáveis tem crescido devido ao aumento da disponibilidade de informações acerca dos problemas de saúde ocasionados por uma alimentação inadequada. O novo perfil de consumidores busca por ingredientes naturais e funcionais que trarão benefícios à saúde. Deste modo, para atender a essa nova demanda, a indústria de alimentos tem atualizado a formulação de seus produtos, visando substituir compostos sintéticos pela utilização apenas de ingredientes naturais. Dentre os ingredientes mais requisitados por essa atualização estão os corantes, uma vez que os sintéticos podem apresentar efeitos alergênicos e, um consumo a longo prazo, pode provocar sérios problemas a saúde. Atualmente, o maior desafio da indústria de corantes está em ampliar sua paleta de cores naturais, dentre elas, a cor azul é o maior desafio tecnológico. Junto aos precursores naturais de corante azul, a genipina, obtida a partir do fruto típico da América Latina, o jenipapo (*Genipa americana* L.), é uma excelente candidata. Agenipina é um agente reticulante que interage com aminas primárias produzindo um composto azul.Vários estudos visam a obtenção do corante utilizando genipina e aminoácidos. Porém, a nível industrial, a produção de um ingrediente a partir de compostos puros é economicamente inviável. Deste modo, o objetivo desta tese foi desenvolver um corante em pó azul natural utilizando precursores de reação viáveis e acessíveis para a indústria de ingredientes naturais. Inicialmente, o sistema coloidal do leite em diferentes composições foi avaliado como solvente de extração, meio de reação e carreador para os compostos azuis obtidos a partir da reação entre a genipina e as proteínas do leite. O efeito dos principais tratamentos comerciais do leite (tratamentos térmicos e secagem por atomização) sobre a formação da cor azul também foi avaliado. Em seguida, objetivou-se compreender, desde o ponto de vista molecular até a nível complexo extrato de genipina/proteínas do leite, os mecanismos de reação para a formação do corante. Por fim, para viabilizar e facilitar a micronização do corante líquido, foram produzidas partículas utilizando fibras dietéticas prebióticas como agentes carreadores. Para isso, foram estudadas diversas fibras que já são usadas como carreadores, e, estudou-se um novo prebiótico como possível carreador, os xilooligossacarídeos (XOS). A escolha do uso de carreadores prebióticos visou agregar valor ao ingrediente aumentando sua funcionalidade, a fim de promover efeitos benéficos à saúde dos consumidores. Após sua produção, as partículas foram caracterizadas

em relação às suas propriedades físicas, químicas, morfológicas e tecnológicas, como o seu poder corante e estabilidade a variações de temperatura e pH. Por fim, os corantes em pó foram aplicados em diferentes bases alimentícias. Os resultados demonstraram o potencial da genipina extraída utilizando o leite como solvente, meio de reação e fornecedor de aminas primárias para fornecer um novo corante azul, o qual foi quimicamente estável durante 96 h sob refrigeração para posterior atomização. A composição do leite afetou a intensidade da cor azul, uma vez que apresenta diferentes teores de proteína, e, consequentemente, de aminas primárias para reagir com a genipina.Porém, o uso de leite integral, apesar de apresentar proporcionalmente menos proteína, favoreceu o espalhamento da luz, resultando em um corante azul mais intenso, mas com maior luminosidade. Os diferentes tratamentos térmicos e a secagem aplicados ao leite também influenciaram naintensidade do azul: tratamentos menos severos, como a pasteurização lenta e pasteurização rápida LTLT e HTST, respectivamente, resultaram em um corante azul com coloração mais intensa. Verificou-se que o leite é um meio ideal para a reação com a genipina, pois apresenta muitos aminoácidos básicos, nos quais estão em a uma baixa relação NH_3^+/NH_2 promovendo maiorvelocidade e intensidade de reação. Adicionalmente, o pH 6.7, que é o pH natural do leite, está dentro da faixa ideal para a reação. Também se descobriu que o complexo corante apresenta tamanho molecular de aproximadamente 200 kDa. Em relação ao corante em pó, as partículas carreadas com prebióticos apresentaram menores tamanhos de partícula, melhores propriedades de reidratação, maior retenção da cor azul, alto poder corante e estabilidade a altas temperaturas e a variações de pH, comparado às partículas sem carreador. Adicionalmente, o novo prebiótico XOS pôde ser usado como carreador, apresentando alta estabilidade térmica, maior proteção e manutenção da cor azul, além de características similares àquelas carreadas com maltodextrina (MD) e (frutoologissacarídeo) FOS. Por último, as partículas puderam ser devidamente aplicadas em diferentes bases alimentícias: *Chantilly*, muffin e beijinho, e foram estáveis ao processo de produção sem perder o seu poder corante.

Palavras-chave: *Genipa ameriana* L.; xylooligossacarídeos; alimentos funcionais; spray-dryer; HPLC.

ABSTRACT

An all-natural blue colorant based on milk and *Genipa americana* L. for the food industry: Obtaining, reaction mechanism, and particle design using prebiotic carriers

Interest in healthy foods has grown due to the increased availability of information about health problems caused by inadequate nutrition. The new consumer profile is looking for natural and functional ingredients that will bring health benefits. Thus, to meet this new demand, the food industry has updated the formulation of its products, aiming to use only natural ingredients. Among the most needed ingredients for this substitution are the colorants, since synthetics can have allergenic effects and, long-term consumption, can cause health problems. Currently, the colorant industry is looking for expanding its palette of natural colors, among them, the color blue is the biggest technological challenge. Among the natural blue precursors, genipap (*Genipa americana* L.), a Brazilian native fruit, is an excellent candidate. This fruit is source of genipin, a natural crosslinker in which interacts with primary amines forming a blue colorant. Several studies aim to obtain the blue colorant using genipin and amino acids. However, at the industrial level, the production of an ingredient from pure compounds is economically unfeasible. Therefore, the aim of this thesis was to develop a natural blue powder colorant using viable and affordable reaction precursors for the natural ingredients industry. Milk is used as a genipin extraction solvent, reactionmedium and amine supplier. Initially, a study was developed using the colloidal system of milk, in different compositions, as extraction solvent, reaction medium and carrier for the blue compounds obtained from the reaction between genipin and milk proteins. Subsequently, the effect of the main commercial milk treatments (thermal treatments and spray drying) on the formation of the blue color was evaluated. Then, we aimed to understand, from the molecular point of view to the complex level of genipin extract/milk proteins, the reaction mechanisms for the colorant formation.Finally, to enable and facilitate the micronization of the liquid colorant, particles were produced using prebiotic dietary fibers as carrier agents. For this, several fibers that are already used as carriers were studied, and a new prebiotic was studied as a possible carrier, the xylooligosaccharides (XOS). The choice of using prebiotic carriers aimed to add value to the ingredient by increasing its functionality, in order to promote beneficial effects to the health of consumers. After their production, the particles were characterized regarding their physical, chemical, morphological and technological properties, such as their coloring power and stability to temperature and pH variations. Finally, the powdered

colorants were applied in different food bases. Our results demonstrated the potential of extracted genipin using milk as a solvent, reaction medium, and primary amine supplier to provide a new blue colorant. Which was chemically stable for 96 h refrigerated for further atomization. The milk composition affected the intensity of the blue color, since it has different proportions of protein and, consequently, of primary amines to react with genipin. However, the use of whole milk, despite having proportionally less protein, favored light scattering, providing a more intense blue color with greater luminosity. Additionally, less severe treatments such as slow pasteurization and fast pasteurization LTLT and HTST, respectively, resulted in an intensely colored blue. We could observe that milk is an ideal medium for this reaction, presenting a sufficient amount of basic amino acids, which are at a low NH_3^+/NH_2 ratio, promoting high reaction velocity. Additionally, pH 6.7, which is the natural pH of milk, is within the ideal range for the reaction. The blue colorant complex presents approximately 200 kDa molecular size. The particles formed by this complex loaded with prebiotics showed smaller particle sizes, better rehydration properties, greater retention of the blue color, high coloring powerand stability at high temperatures and pH variations, compared to particles without a carrier. Additionally, the new prebiotic XOS could be used as a carrier, presenting high thermal stability, protection and maintenance of the blue color, in addition to presenting particle characteristics similar to those carried with maltodextrin (MD) and fructologsaccharide (FOS). Finally, the particles could be properly applied in different food bases: *Chantilly*, muffin and *Beijinho*, and were stable in the production process without losing their coloring power.

Keywords: *Genipa ameriana* L.; xylooligosaccharides; functional foods; spray-dryer; HPLC.

RESUMEN

Colorante natural azul a base de leche y *Genipa americana* L. para la industria alimentaria: Obtención, mecanismo de reacción y design de partículas utilizando portadores prebióticos

El interés por los alimentos saludables ha crecido debido a la mayor disponibilidad de información sobre los problemas de salud causados por una nutrición inadecuada. El nuevo perfil de consumidor busca ingredientes naturales y funcionales que, además de promover una sustitución de los sintéticos, aporten beneficios para la salud. Así, para atender esta nueva demanda, la industria alimentaria ha actualizado la formulación de sus productos, buscando utilizar únicamente ingredientes naturales. Entre los ingredientes más requeridos para esta actualización se encuentran los colorantes, ya que los sintéticos pueden tener efectos alergénicos y, su consumo a largo plazo, puede causar problemas de salud. Actualmente la industria busca por ampliar su paleta de colores naturales, entre ellos, el color azul es su mayor desafío tecnológico. Junto con los precursores naturales del colorante azul, la genipina, obtenida de una fruta latinoamericana, el genipap (*Genipaamericana* L.), es una excelente candidata. Genipin es un agente de reticulación que interactúa con aminas primarias produciendo un compuesto azul. Varios estudios obtuvieron el colorante utilizando genipina y aminoácidos. Sin embargo, a nivel industrial, la producción de un ingrediente a partir de compuestos puros es económicamente inviable. Por lo tanto, el objetivo de esta tesis fue desarrollar un colorante en polvo azul natural utilizando precursores de reacción viables y asequibles para la industria de ingredientes naturales. Inicialmente se evaluó el sistema coloidal de la leche en diferentes composiciones como solvente de extracción, medio de reacción y portadorde los compuestos azules obtenidos de la reacción entre la genipina y las proteínas de la leche. Posteriormente se evaluó el efecto de los principales tratamientos lácteos comerciales (tratamientos térmicos y atomización) sobre la formación del color azul. Luego, se buscó comprender, desde el punto de vista molecular hasta el nivel complejo de extracto de genipina/proteínas de la leche, los mecanismos de reacción para la formación del colorante. Finalmente, para permitir y facilitar la micronización del colorante líquido, se produjeron partículas utilizando fibras dietéticas prebióticascomo agentes portadores. Para ello, se estudiaron varias fibras que ya se utilizan como transportadores, y se estudió un nuevo prebiótico como posible transportador, los xilooligosacáridos (XOS). El propósito de utilizar portadores prebióticos fue de agregar valor al ingrediente aumentando su funcionalidad, para promover efectos beneficiosos para la salud de los consumidores. Luego de su producción, las partículas fueron

caracterizadas, aplicadas en bases alimenticias y verificadas por su poder colorante y estabilidad a las variaciones de temperatura y pH. Los resultados demostraron el potencial de la genipina extraída usando leche como disolvente, medio de reacción y proveedor de amina primaria para producir el colorante, que fue químicamente estable durante 96 h refrigerado para la etapa siguiente: la atomización. La composición de la lecheafectó la intensidad del color azul, ya que tiene diferentes proporciones de proteína y, en consecuencia, de aminas primarias para reaccionar con la genipina. Sin embargo, el uso de leche entera, a pesar de tener proporcionalmente menos proteína, favorecía la dispersión de la luz, proporcionando un color más azulado, pero con mayor luminosidad. Los diferentes tipos de leche tratada térmicamente también influyeron en la intensidad del azul: los tratamientos menos severos, como la pasteurización lenta y la rápida LTLT y HTST, respectivamente, resultaron un azul más intenso. De los resultados obtenidos, también se observó que la leche es un medio ideal para llevar al cabo esta reacción, ya que contiene muchos aminoácidos básicos, en los cuales se encuentran en una baja relación NH_3^+/NH_2, favoreciendo una mayor velocidad e intensidad de reacción. Además, el pH 6,7, que es el pH natural de la leche, está dentro del rango ideal para la reacción. También se encontró que el complejo colorante tenía un tamaño molecular de aproximadamente 200 kDa. En relación al colorante en polvo, las partículas portadas con prebióticos mostraron tamaños departícula más pequeños, mejores propiedades de rehidratación, mayor retención del color azul, alto poder colorante y estabilidad a altas temperaturas y variaciones de pH, en comparación con las partículas sin portador. Además, el nuevo prebiótico XOS presentó alta estabilidad térmica, protección y mantenimiento del color azul, además de presentar características de partículas similares a las que se realizan con maltodextrina (MD) y fructologsaccharide (FOS). Luego las partículas pudieron ser aplicadas adecuadamente en diferentes bases alimenticias: *Chantilly*, muffin y *Beijinho*, y fueron estables en el proceso de producción sin perder su poder colorante.

Palabras clave: *Genipa ameriana* L.; xilooligosacáridos; alimentos funcionales; atomización; HPLC.

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CHAPTER 1

General introduction, justification, objectives, and thesis structure

1.1 Introduction

Color is an important sensorial attribute because brings quality perception in a food product by the consumers. In this way, colorants enhance the product's acceptability, making it more attractive. For many years, the food industry used synthetic colorants, featuring a diverse color palette for making foods very attractive from a sensory viewpoint. However, as consumers are increasingly informed about the importance of healthy foods for preventing disease, products made with synthetic colorants are being rejected. Given this new consumer profile, the use of natural colorants has been one of the biggest trends in the food production sector (Faustino, Veiga, Sousa, Costa, Silva, & Pintado, 2019; Neves, Silva, & Meireles, 2019). Accordingly, the global natural colorants market is expected to earn about $37.49 billion in 2025 (Silveira, Barcelos, Vespermann, Pelissari, & Molina, 2019). Some natural colors as the yellow, red, and green can already be found in the market. However, since blue color matters, the scenario is scarce, and natural existents resources have low stability, like the anthocyanins for example. For this reason, the blue is still used in a synthetic form. Consequently, especially when dealing with the color blue, consumers label the product as “an artificial food”, claiming that it does not bring health benefits (Spence, 2018).

Regarding the possible blue sources, the colorant produced from the reaction between genipin obtained from unripe fruits of *Genipa americana* L., known as genipap, and primary amines has great potential for industrial application (Echeverry, Zapata, & Torres, 2011; Náthia- Neves & Meireles, 2018; Silva, Saint-Jalmes, de Carvalho, & Gaucheron, 2014). Genipin reacts spontaneously with primary amines of amino acids, peptides or proteins, producing a high molecular weight polymer with an intense blue coloration (Buchweitz, 2016; Neri-Numa, Angolini, Bicas, Ruiz, & Pastore, 2018).

Studies and patents focused on the production of blue colorant from genipin, used pure compounds, or genipin extracted reacted with the amines of genipap's own proteins (Echeverry, Zapata, & Torres, 2011; Cano et al., 2014; Nathia-Neves et al., 2018). However, both cases are not viable, the first, because it uses pure compounds, the colorant becomes economically unfeasible to be used in industry. The second one, the primary amines content available for reaction may vary according to the fruit, not being possible to ensure that the extract will present the intense blue color necessary for its subsequent application. In this sense, it is necessary to use an easy-available and economic both genipin and primary amines sources. To solve this problem, the genipin can be

extracted from *Genipa americana* L. using milk as a solvent and primary amines supplier. The primary amines could be provided by both caseins and whey proteins. Likewise, the blue colorant can be carried by the milk as it is a colloidal system. However, since milk is a complex matrix with proteins, fats, sugars and minerals, its composition may affect the blue color formation. The component with the greatest variation in milk composition is fat, including natural variations and changes due to processing (Walstra, Walstra, Wouters, & Geurts, 2005). Changes in lipid content led to changes in composition concerning all constituents of milk, including proteins. Thus, the composition of milk can affect the kinetics of blue color formation from genipap extract. Additionally, differences in protein conformation and amino acid proportion due to the heat milk commercial process may affect the formation of the blue color, as they can reduce the exposed sites of free primary amines available for the reaction with genipin.

There are no studies that clearly explain the interaction of milk proteins and genipin, nor is the literature fully elucidated on how the reaction proceed using pure compounds. There are still many lacks about the molecular structure, formation mechanism, reaction kinetics, preferred amino acids and optimal conditions for the reaction. For the reason that this colorant is a polymeric compound and is hence difficult to analyze separately, also, there are many steps and intermediate compounds until produce the blue colorant. Thus, much effort has been focused on understanding the genipin and primary amine interaction (Di Tommaso, David, Gomar, Leroy, & Adamo, 2014; Dimida, Demitri, De Benedictis, Scalera, Gervaso, & Sannino, 2015; Tsutsumiuchi et al., 2021). Because of that, some authors dedicated on working with the simplest compounds to produce the colorant: genipin and a pure primary amine group, seeking to unsterstand the reaction (Fujikawa, Fukui, Koga, Iwashita, Komura, & Nomoto, 1987; Touyama, Takeda, Inoue, Kawamura, Yatsuzuka, Ikumoto, Shingu, Yokoi, & Inouye, 1994; Di Tommaso et al., 2014; Dimida et al., 2015). Hence, although blue colorant-loaded milk has enormous potential as a colorant, it is important to deeply understand how this interaction takes place, firstly from a molecular standpoint, about the mechanism, kinetics, and preferential conditions of the reaction. Later, in a particle-scale, the influence of complex matrices on the production and characteristics of the colorant, and how interactions happen from the macro approach.

After the production step, the colorants pass to a micronization or drying process tofacilitate their use in various foodstuffs. This manufacturing step is essential to maintain the chromophilic properties, facilitate transport and promote greater dissolution of these in various food bases

(Santos & Meireles, 2013). In this regard, carrier matrices are used to assist the drying process and protect the natural ingredient. Carriers such as proteins, lipids or carbohydrates are largely used (Celli, Ghanem, & Brooks, 2015). The chemical and physical properties of carrier materials define the technological characteristics of the final product. They influence the size,shape and structure of microparticles besides determine their stability during production, storage and consumption regarding the external environment (Botrel, de Barros Fernandes, Borges, & Yoshida, 2014).

Among the possibilities of carriers based on prebiotic dietary fibers, fructans have the potential to improve the functional and sensory characteristics of foods (Vervoort & Kinget, 1996). During digestion, they undergo little hydrolysis, not increasing the glycemic index. Therefore, they can be used in sugar-free foods and beverages (Bakowska-Barczak & Kolodziejczyk, 2011). Fructans, such as fructooligossacharides (FOS) and inulin (IN), are prebiotic carbohydrates because when consumed they become carbon sources for bifidobacterias and lactobacillus present in the colon, increasing the volatile fatty acids production, and anti-gastric ulcer activity Femat- Castañeda, Chávez-Rodríguez, Chávez-Rodríguez, Flores-Martínez, Farías-Cervantes, & Andrade-González, 2019. As polymers, they have excellent functional properties depending on their molecular chain size to act as carrier agents (Femat-Castañeda et al., 2019). Some studies have demonstrated the feasibility of using inulin-type fructas as a wall material for rosemary essential oil (Fernandes, Borges, Botrel, & Oliveira, 2014), fish oil (Botrel et al., 2014), annatto seed oil (Silva & Meireles, 2015), chlorophyll (Femat-Castañeda et al., 2019), oleuropein (Pacheco,González, Robert & Parada, 2018), *Lactobacillus acidophilus* (Raddatz et al., 2020), anthocyanins (Lacerda et al., 2016), and betalains (Kuhn, Azevedo & Noreña et al., 2020).

Additionally, xylooligosaccharides (XOS) are prebiotic oligosaccharides made up of xylose units found in bamboo shoots, fruits, vegetables, milk and honey (Vazquez, Alonso, Domınguez,& Parajo, 2000). The use of XOS as a prebiotic ingredient has some advantages over FOS and IN, as they are more stable over a wide range of pH values (2.5 - 8.0) and at temperatures up to 100 °C. Also, XOS require a lower dosage to give the same prebiotic effect (> 1.4 g) compared to fructans (Ferrão et al., 2018; Yang, Liang, Balakrishnan, Belobrajdic, Feng & Zhang, 2020). The consumption of XOS has several advantages, such as improving the immune system, modulating the intestinal microbiota and reducing the risk of cancer (Aachary & Prapulla, 2011; Morshedi, Agh, Noori, Jafari, Ghasemi & Mozanzadeh, 2019). The use of XOS as a carrier matrix of spray-dried

products are still scarce. However, as well as inulin and FOS, which have already studies showing potential for this purpose, XOS has promising properties to be used as a carrier matrix with a potential prebiotic effect.

1.2 Justification

This work proposed to provide a functional blue colorant to meet consumer demand for natural products that promote healthiness while maintaining a high sensory standard.

Forming a blue colorant from pure compounds, such as amino acids and genipin is important to understand the reaction. Nonetheless, may not be economically attractive and possibleto produce in scale-up. Therefore, the justification for this work is obtaining an available and not expensive new natural ingredient using emerging technologies, such as high-intensity ultrasound and spray-drying. Also, the addition of prebiotic carriers aims to develop a functional particle, increasing its potential as a healthy food.

Finally, the promotion of a new natural and functional colorant with the most needed color by the industry is an excellent achievement for the food sector that seeks innovation, sensory appeal and, at the same time, health and well-being for consumers.

1.3 General objective

Developing novel blue colorants for the food industry using unripe *Genipa ameriana* L. pulp, milk, and prebiotic fibers to produce innovative all-natural ingredients.

1.3.1 Specific objectives:

- Study the milk potential as a genipin extract solvent and reaction medium for the blue compound.

- Evaluate the effects of milk composition on blue color formation.

- Evaluate the effects of thermal treatments on milk proteins and, consequently, on the availability of $-NH_2$ for the reaction.

- Study the reaction mechanisms from the molecular approach until the formation of the genipin-milk protein complex.

- Comprehend why milk is an ideal reaction medium for the blue color formation.

- Evaluate the powder characteristics of the new blue colorant particle.

- Evaluate the properties of inulin as a wall material and verify the effect of the fructans' polymerization degree on the particle characteristics.

- Study the potential of xylooligosaccharides (XOS) as a new carrier matrix with prebiotic effects.

- Study the potential of the blue colorant when applied to different foodstuffs, its coloring power and stability to different pH and heat.

1.4 Thesis structure

This thesis is assembled in 9 chapters. This chapter (***Chapter 1 - General introduction, justification, objectives, and thesis structure***) briefly presents the theme, state of the art, hypotheses, objectives, and steps involved for its realization. The flow diagram highlights and linked the experimental activities carried out in this thesis (Figure 1.1).

The **Literature Review** is presented in ***Chapter 2 – What is the state of art and future trends regarding natural blue colorants? –*** we approached the current scenario of the use of blue colorants in the industry, the synthetic ones used and the dangers related to their consumption. We discussed about the biggest difficulties encountered in the replacement by natural colorants, possible sources and respective limitations. Finally, the future perspectives for the use of natural blue colorants, from a sensory and technological perspective.

In ***Chapter 3*** **- Experimental article I** - ***Could milk be a primary amines supplier to produce the blue colorant?*** – content the experimental results involving the obtaining of a new natural blue colorant from the reaction between an extract of genipin and primary amines from milk. Genipin is extracted from the unripe genipap using milk as a solvent, primary amine supplier and carrier of the blue compound formed. Specifically, chapter 3 aimed to investigate the effect of milk composition on the kinetics of blue color formation.

Chapter 4 **- Experimental Article II** - ***Any heat treated-milk could be used as a blue colorant amine supplier, reaction medium, and carrier?*** - addresses the experimental results of the influence of milk heat processes in the availability of primary amines to react with genipin and form the blue colorant.

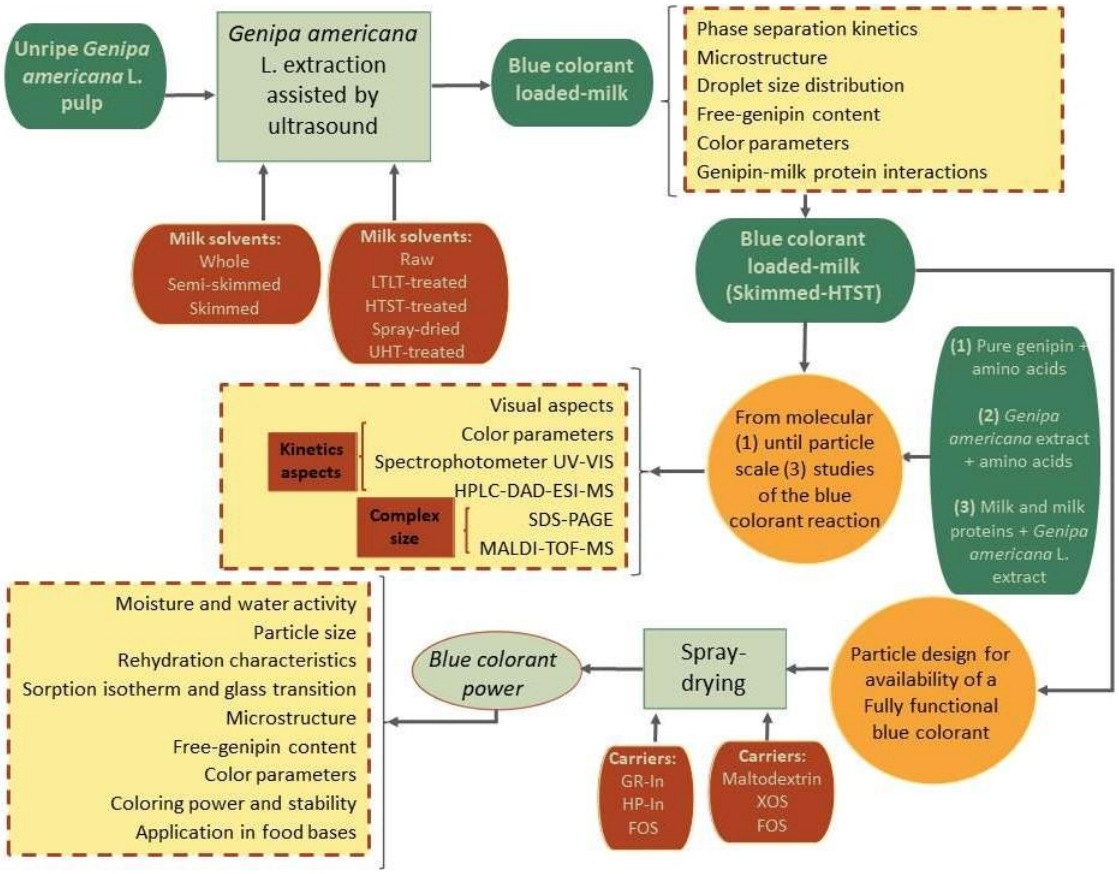

Figure 1.1. Flow diagram of activities carried out in the thesis.
LTLT: Low-temperature and long-time; HTST: High-temperature and short-time; UHT: ultra-high temperature; HPLC-DAD-ESI-MS: High-performance liquid chromatography with diode array detection coupled to mass spectrometry using the electrospray ionization interface; SDS-PAGE: Sodium Dodecyl Sulfate - Polyacrylamide Gel Electrophoresis; MALDI-TOF-MS: Matrix-Assisted LaserDesorption/Ionization - Time of Flight - Mass Spectrometry; GR-Inulin: inulin with degrees of polymerization ≥10 ; HP-Inulin: inulin with degrees of polymerization ≥ 23; FOS: fructooligosaccharides; XOS: Xylooligosaccharides.

Below are highlighted the main items comprise each chapter of the thesis (Figure 1.2).

Figure 1.2. Scheme of representation of the subjects covered in the chapters of this thesis.

In ***Chapter 5*** - **Experimental Article III** - ***Why Genipa americana L. extract and milk are an ideal medium to produce the blue colorant?*** - we studied the reaction mechanism for the colorant formation, seeking to answer which compounds are formed, the reaction kinetics, preferential reaction conditions and size of the genipin-protein complex.

Both ***Chapters 6*** and ***7,*** contain papers in which we developed the blue colorant in powder carried by prebiotic matrices. The **Experimental article IV** - ***How are the characteristics of the***

colorant carried by prebiotic matrices regarding powder properties, colorant power, and stability, and **Experimental Article V** - ***Could blue colorant improve its functionality being carried by xylooligosaccharides and be applied in some foodstuffs?***

The **General Discussion** of this thesis is in ***Chapter 8*** – ***What did this thesis bring?*** - we explain the main observed results, the link between each article, and the core to which this thesis contributed to scientific development.

Finally, in ***Chapter 9*** – **General Conclusions** - we presented briefly the main conclusions for each thesis chapters, linking all the knowledge generated in response to the general objective of the thesis. At the end of this chapter, we presented the **Memorial of doctorate period** for records of all activities involved: academic papers published in the period (first and co-authorship), co-supervision, participation/organization of conferences, disciplines, and teaching internships carried out.

Furthermore, in the **APPENDIX** section there are some published articles, supplementary material, and other unpublished files of a relevant order to be included in this thesis.

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CHAPTER 2

Literature review

What is the state of art and future trends regarding natural blue colorants?

Natural blue food colorants: Consumer acceptance, current alternatives, trends, challenges and future strategies

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Natural blue food colorants: Consumer acceptance, current alternatives, trends, challenges, and future strategies

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ABSTRACT

Background: The food industry seeks to expand the color palette with natural colorants. Nevertheless, in nature, the blue color is rare compared to other colors. Despite synthetic colorants' effects on human health, synthetic blue colorants are largely used by the food industry. In this way, consumers usually associate the blue color with artificial ingredients. However, whether blue colorants obtained from natural sources were used in food production, would consumers better accept these products?

Scope and approach: The current demand for manufacturing food products with natural ingredients has increased the market interest for natural blue colorants. Therefore, this review summarizes the current scenario of synthetic blue colorants' use and the possible sources of natural colorants that the industry can use to replace them. Also, the challenges, alternatives, and future strategies are discussed.

Key findings and conclusions: Although a considerable effort has been dedicated to searching for new blue colorants, they present some limitations: physical and chemical instability, the unfamiliarity of the blue color compound, and high-production cost. Among the blue colorant options, the compound formed by the reaction between genipin and primary amines seems to have the highest potential for industrial application. However, its composition, toxicity, physical and chemical stability in adverse processing and storage conditions are still unknown.

1. Introduction

Color is an essential attribute for the sensory quality of a food product. In this way, colorants can improve the product's acceptability, making it more attractive to consumers (Carocho, Morales, & Ferreira, 2015). The food industry leans towards choosing synthetic colorants instead of natural colorants because, in general, they present higher chemical and physical stability, coloring power, and, mainly, lower cost than natural colorants (Amchova, Kotolova, & Ruda-Kucerova, 2015). However, non-synthetic additives are the current trend in the food sector due to food safety demand, reinforced by natural nutrients' health benefits (Faustino et al., 2019; Neves, Silva, & Meireles, 2019). Furthermore, natural colorants can contribute to ensuring functional properties in foods (Bagchi, 2006). This market sector exhibited an annual growth rate of 8.5% between 2018 and 2019 (Granato et al., 2020; Jędrusek-Golińska et al., 2020).

Nature offers a wide variety of colors that can be used as natural ingredients (Newsome, Culver, & Van Breemen, 2014). Most colorants are extracted from plant sources. They are usually recovered from leaves, roots, flowers, rhizomes, barks, or fruits. Bixin, anthocyanins, betalains, chlorophylls, caramel, curcumin, carminic acid, lycopene, and carotenoids are examples of natural colorants (Martins, Roriz, Morales, Barros, & Ferreira, 2016; Náthia-Neves, Tarone, Tosi, Júnior, & Meireles, 2017; Neves, Silva, & Meireles, 2019). Likewise, algae, insects, and microorganisms are other natural sources (Martins et al., 2016).

Despite the several natural sources, the manufacturing of food products containing only natural colorants is still a big challenge. The variety of commercially available natural colorants is still limited (Neves, Silva, & Meireles, 2019; Rodriguez-Amaya, 2016; Sigurdson, Tang, & Giusti, 2017). Several natural colorants are degraded during food processing and storage due to their chemical instability (Neves, Silva, & Meireles, 2019). High temperatures can promote color degradation (Torres-Valenzuela, Ballesteros-Gómez, & Rubio, 2020). The colorant extracts are subjected to drying processes, which may decrease their color intensity (Neves, Silva, & Meireles, 2019; Rodriguez-Amaya, 2016). It is necessary to ensure color maintenance during product storage since reactions between the colorant, and the food can occur (Chaiklahan, Chirasuwan, & Bunnag, 2012; Ravanfar, Comunian, &

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Abbaspourrad, 2018).

Among the natural colors, blue is probably the biggest challenge since blue's natural sources are limited. Therefore, especially when dealing with the color blue, consumers label the product as "an artificial food", claiming that it does not bring health benefits (Spence, 2018). There is no natural blue colorant with the same physical and chemical stability, coloring power, and easy scale-up production in the market yet, comparing to the synthetics. Thus, the industry trends to use synthetic ones.

There are natural blue sources such as *Gardenia jasminoides*, *Spirulina platensis, Indigofera, Haslea ostrearia, Genipa americana* L., and *Allium*. However, they have at least a single disadvantage limiting their industrial application (Sigurdson et al., 2017). Anthocyanins present blue color with low intensity in a pH range from 6 to 7. Another precursor of the blue colorant is the phycocyanin. Recently, the US Food and Drug Administration (FDA) approved phycocyanin as a spirulina extract. Nevertheless, its use is quite limited due to the lack of stability to high temperatures, light, and pH variations, in addition to its high-cost production (Pojer, Mattivi, Johnson, & Stockley, 2013; Somavat, Kumar, & Singh, 2018; Tarone, Cazarin, & Junior, 2020). Also, iridoids can be blue color precursors. They are colorants derived from *Genipa americana* and *Gardenia jasminoides* cannot yet be commercialized due to limited approval. Such permission is pending due to the compound's lack of knowledge responsible for the coloration (Brauch, Zapata-Porras, Buchweitz, Aschoff, & Carle, 2016; Buchweitz, 2016).

Another issue for the colorant application is the lack of chemical structure knowledge, which hinders advances in their use validation. Despite these limitations, natural blue colorants are the future for the food industry that seeks to develop novelties that draw consumers' attention in the face of so much competition in the market. Furthermore, it will undoubtedly gain even more interest when the limitations are solved. Therefore, this review highlights the challenges and trends for applying natural blue colorants in the food industry, consumers' perception of the industrial possibilities for applying the blue color from natural sources, and blue sources that stand out as future strategies in the search for a blue colorant.

2. Why is blue food perceived as less natural?

Fruits and vegetables have been recognized as essential components of a healthy and balanced diet (Leong, Show, Lim, Ooi, & Ling, 2018). That is why colorful food brings a feeling of health and pleasure to the consumer. Therefore, differently to other colors, blue fruits and vegetables are rare in nature. Perhaps, as a result, the blue color is not a part of nature's appetite-inducing palette and tends to be associated with artificial food color (Spence, 2018). Hence, some consumers consider blue an 'unnatural' color for food applications (Coultate & Blackburn, 2018).

Food color promotes psychological effects. The uncommon blue color brings the sensation of artificial food and is not satiable. Studies by Suzuki et al. (2017) proved this effect. The authors observed the tasters' behavior when eating identical soups, with different colors: yellow, white, and blue. They aimed to verify whether the color could affect the satiety feeling. After ingestion, blue soups showed significantly lower satiety rates than white and yellow soups. The authors concluded that food color might be associated with the expected flavor and produce different effects depending on their experiences. Since blue foods are rare, the visual signs of blue color may have caused cognitive unfamiliarity due to the difficulty of associating color with some type of sensory experience.

Paakki, Sandell, and Hopia (2016) investigated the consumer's preference comparing the "traditional" yellow potatoes and blue potatoes for verifying the feeling that blue foods bring. Only 28% of consumers selected blue potatoes due to their desire to try new and unusual things. The tasters who preferred blue potatoes tended to be more neophilic and younger than those who chose yellow potatoes. The yellow potato choosers preferred eating familiar foods. In this study, the impact of an atypical color on food perception and consequent food choices was verified. From this study, we can observe two groups of consumers: traditional and neophiliacs. The latter responds positively to the consumption and application of atypical colors in foods since they are consumers who seek varieties and trends (Carter, 2011; Paakki et al., 2016).

Therefore, despite being considered by traditional consumers as unpleasant and unacceptable, blue foods have been used for creating novelty and differentiation, especially in categories aimed at children and young people (Coultate & Blackburn, 2018). Therefore, blue has become more popular because of its benefits for quickly capturing consumer attention (Carter, 2011).

Some blue foods are already considered "accepted", such as sweets, candies, and cakes, and draw much attention from consumers attracted by the color differential (Buchweitz, 2016). However, the consumer still associates blue foods as artificially colored (Buchweitz, 2016; Spence, 2018).

Although rare, there are many natural blue products (Fig. 1). These include blue potatoes such as the blue Congo variety (Paakki et al., 2016), blueberries, blue corn, blue lobsters, red cabbage, cornflowers, borage, blue butterfly pea, blue cheese, blue-foot, and blewit mushrooms (Spence, 2018). These food matrices can be used in the formulation of blue products instead of synthetic additives. Thus, studies on the sensory acceptability of blue foods aim to expand this color by applying naturally blue foods in the formulation.

Vázquez-Carrillo, Aparicio-Eusebio, Salinas-Moreno, Buendía--Gonzalez, and Santiago-Ramos (2018) blindly tested the acceptability of blue polvorones, using blue corn flour. First, they found an effect of the polvorones' furnishing process on reducing anthocyanins, responsible for the blue color in corn, directly affecting the product's final color. Second, they found that blue cornmeal flour powder had better overall acceptance than non-colored and commercial wheat samples. They even reported that this better acceptability was due to the novelty that the product brought. However, they observed an unstable color at pH values above 6.0. They also verified a possible color deterioration during storage. Bord, Guerinon, and Lebecque (2017) compared the acceptance of blue cheeses heated or consumed fresh. The authors concluded that for both heated and unheated cheese, the amount of "Blue-veined" is a sensory attribute directly proportional to the acceptability of blue cheeses. The tasters correlate "Blue-veined" with a more intense flavor in the cheese. Gaytos and Lumagbas (2019) performed an ice cream acceptability test by adding Blue Pea flower to their formulation. The tasters appreciated blue ice cream. They also pointed out that the blue pea flower added favored its acceptability. Moskowitz (2002) studied the most attractive color combinations for candies by children. They found that all combinations with blue played an essential role in attracting them. Galetović et al. (2020) added phycobiliproteins to milk drinks, presenting satisfactory sensorial acceptance. Additionally, when the testers were informed that the blue milk drink offered antioxidant properties, the acceptability was improved.

Natural blue-colored products may attract consumers due to their atypical differential color, bringing the "novelty" feeling (Cardello, 1996, pp. 1–82; Granato et al., 2020). Also, an expectation is to reduce the association of "artificial" or "less satiable" regarding blue food (Buchweitz, 2016; Paakki et al., 2016).

3. What are the synthetic blue colorants currently used by the food industry and their dangers?

The synthetic blue colorant available to the food industry are E 131 ceruleum protectum, Patent Blue; E 132 indigocarminum, Indigo Carmine (FD&C blue No. 2); E 133 ceruleum nitens, Brilliant Blue (FCF blue No. 1) (Amchova et al., 2015; Buchweitz, 2016).

The "No-Observed-Adverse-Effect" (NOAEL) is the highest concentration of a substance that causes no detectable adverse alteration of

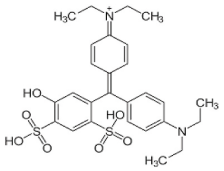
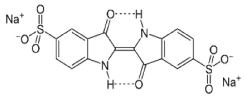
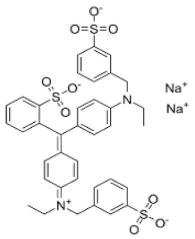
Fig. 1. Some examples of naturally blue foods: lobster, blue-foot mushroom, blueberries, blue cheese, borage, blue corn, and blue potatoes.

functional capacity, growth, or development of the target organism under defined conditions exposure. For clinical recommendations, this value is divided by a safety factor that is usually 100. Which is called acceptable daily intake (ADI), expressed as mg per kg of body weight (Amchova et al., 2015). Usually, artificial colors have low ADI, confirming the problem in their application in food products. Table 1 shows the approved artificial blue colorants, their chemical formula, ADI, and the cause of the harmful effects on the human organism. All blue synthetic colorants present a risk to the consumer's health if consumed above the established dosages (ADI). The values determined by regulatory authorities cannot eliminate the risk of possible adverse reactions to particular substances, especially in vulnerable populations or hypersensitive individuals (Amchova et al., 2015; Jindal et al., 2014; Kus & Eroglu, 2015).

The colorant Patent blue V did not show any mutagenic activity or reproductive toxicity. However, the ability to reduce hemoglobin was detected in patients who consumed high doses (Amchova et al., 2015). Maranhão, da Nóbrega, Anunciação, Maia, and Mariano (2016) presented a case of an allergic reaction to Patent blue V, which is commonly used by anesthetists to locate lymph nodes. Twenty minutes after the intravenous colorant application, the patient presented a rapid heart rate. Patent blue V presents an ADI of 5 mg/kg body weight/day and must be at least 90% pure. Even at high doses such as 10 mg/kg body weight/day of its ingestion, it did not present dangers of genotoxicity, intolerance, or carcinogenicity in animal tests (EFSA, 2013). Patent blue V is usually applied in scotch eggs and jelly (Martins et al., 2016).

The Brillant blue colorant is also known as Blue 1. It is a water-soluble colorant applied in dairy powders, beverages, candies, condiments, icings, syrups, and jelly (Lucová, Hojerová, Pažoureková, & Klimová, 2013; Martins et al., 2016). The human gastrointestinal tract can absorb approximately 8% of this compound, presenting an ADI of 6 mg/kg body weight/day, higher than the other blue synthetic colorants (Amchova et al., 2015). This value approximately corresponds to the European population's real consumption (Lucová et al., 2013). However, EFSA (2010) warned that even low doses could cause hypersensitive reactions in susceptible individuals. Kus and Eroglu (2015) reported that Blue 1 also presented potential cytotoxic and genotoxic effects on cultured human lymphocytes. Brilliant blue, used on the retina for coloring the membrane and facilitating macular hole diagnosis, caused a decrease in the foveal and hyperpigmentation in three patients'

Table 1
The synthetic blue colorants, their chemical formula, acceptable daily intake (ADI), and possible effects in human health.

Colorant	Chemical formula	ADI	Effect	Reference
E131, Patent blue		5 mg/kg of body weight per day	-Reduces value for hemoglobin; -Promotes severe allergic reactions; - Even in reduced doses may cause hypersensitive reactions in susceptible individuals.	(EFSA, 2013; Maranhão et al., 2016)
E132, Indigo carmine		2.5 mg/kg of body weight per day	- Atrioventricular blocking capacity; - Promotes severe allergic reactions; - One case of severe acute hypotension; - Can cause cerebral ischemia.	(EFSA, 2014; Lee et al., 2015; Takeyama et al., 2014)
E133, Brillant blue		6 mg/kg of body weight per day	- Cytotoxic and genotoxic potential on human blood; - Even in reduced doses may cause hypersensitive reactions in susceptible individuals.	(EFSA, 2010; Jindal et al., 2014; Kus & Eroglu, 2015)

foveal peripheral region (Jindal et al., 2014).

The studies by Lucová et al. (2013) with Patent blue V and Brilliant blue indicated that both were able to penetrate the bloodstream through the tongue's back and in slightly damaged skin. Therefore, the use of these colorants in lollipops and topical products should be avoided.

The colorant Indigo Carmine did not demonstrate genotoxicity, cytotoxicity, or changes in the blood in chronic toxicity studies when consumed at 2.5 mg/kg body weight/day (EFSA, 2014). Dixit and Goyal (2013) reported an adverse effect on testicles at a dose of 17 mg/kg body weight/day. Case studies report an atrioventricular blocking capacity of indigo carmine (Takeyama, Sakamoto, Yoshikawa, & Suzuki, 2014). Lee, Baek, and Jeon (2015) related severe acute hypotension. The patient decreased his blood pressure 30 min after indigo carmine intravenous application to medicinal diagnostics. Another case study suggests that indigo carmine's intravenous use induced an allergic reaction causing cerebral ischemia due to vasospasm (Kawaguchi, Hashimoto, Kitayama, & Hirota, 2007). Indigo Carmine is widely used in ice-creams, sweets, confectionery products, and biscuits (Lee et al., 2015; Martins et al., 2016).

Despite the controlled use of these colorants in doses evaluated to ensure consumer safety, their purity specifications allow concentrations of aromatic non-sulfonated amines. These amines can reach 100 mg/kg of colorant. Assuming the maximum permitted average concentration of colorants in food (500 mg/kg of food), these amines' concentration can reach 50 μg/kg of food. Estimates of intake of colorants at levels up to ADI of 15 mg/kg body weight/day can reach the intake of these aromatic amines at levels of up to 1.5 μg/kg body weight/day. In other words, synthetic blue colorants may damage human health since aromatic amine's indirect consumption presents genotoxicity and carcinogenicity effects (EFSA, 2013). There are some compounds allowed in these colorants' composition, which are not added intentionally but are residual, migrated substances or are present in these additives due to their production, namely, arsenic, mercury, cadmium, and lead, which have toxic effects (EFSA, 2010, 2013, 2014). These components may present long term effects as a result of their accumulation in the organism. Arsenic can cause skin illnesses (hyperpigmentation and hyperkeratosis), gastrointestinal, vascular, diabetes mellitus, and peripheral neuropathies. The consequences of consuming mercury include damage to the brain, kidneys, and lungs. Prolonged exposure to lead and cadmium can damage numerous organs, such as the testicles, kidneys, liver, pancreas, thyroid, adrenal gland, bone, central nervous system, and lungs (Bertoli, Carvalho, Cannata, Bastos, & dos Santos Augusto, 2012; Zenebon et al., 2004; Patel et al., 2021).

Unfortunately, most of these artificially colored foods are consumed daily by children, such as candies, sweets, ice cream, snacks, breakfast cereals, and cakes (Feketea & Tsabouri, 2017). Amounts within the range of 0.3–33 mg in sweets, 9.4–41.3 mg in breakfast cereals, 1.9–6 mg in ice cream, and 2.8–14.4 mg in snacks can be ingested daily by children (Stevens, Burgess, Stochelski, & Kuczek, 2015). It is even possible for a child to consume, from different sources, accumulated amounts of colorants far above the recommended ADI levels. Each food can have colorant concentrations at the maximum allowed. There are no reports of side effects in children caused by the aforementioned blue colorants. However, a major study called "Southampton study" indicated behavioral disorders, irritability, restlessness, and sleep disorders after high levels of ingestion of other artificial colorants (McCann et al., 2007). Possible allergic reactions can occur, including reactions triggered by immune (immediate and late hypersensitivity) and non-immune (intolerance) mechanisms, which arise more quickly in children. Furthermore, prolonged intake of these colorants can have long-term effects due to the accumulation of these components in the organism (Feketea & Tsabouri, 2017; Martins et al., 2016).

The health's dangerous concerning the consumption of these artificial colorants claims to replace them with the natural ones. However, we face a significant obstacle since artificial colors are more stable, cheap, and available than natural colors (Delgado-Vargas, Jiménez, & Paredes-López, 2000; Neves, Silva, & Meireles, 2019; Rodriguez-Amaya, 2016).

4. What is the current scenario regarding the use of natural colorants?

The current industrial demand is manufacturing food products using natural coloring compounds extracted from fruits, vegetables, algaes, and microorganisms. The concept of "coloring foodstuffs" is also growing, based on the food ingredients use, such as fruits, vegetables and herbs; or even processed foods such as wine and coffee, whose side effect of their application is providing color (Oplatowska-Stachowiak, Elliott, 2017).

The most common natural colorants used as food ingredients are anthocyanins, betalains, carotenoids, curcumin, bixin, chlorophyll, caramel, phycocyanin, canthaxanthin, carbon black, and carminic acid (Table 2). Despite the numerous stability limitations and restrictive application, there is already a substitution of synthetic for natural colorants in some foods (Hendry & Houghton, 1996; Martins et al., 2016). Dairy products, cereals, candies, confectionery, and beverages sectors present high adherence to natural colorants (Bartoe, 2018; Myers, 2015). For the colorants already approved by the European Commission Regulation, there are two different statuses: "approved at quantum sadis" or "approved with combined maximum limit", the first means that a maximum numerical level allowed for use is not specified, provided that the additive is added according to acceptable manufacturing practices; the second, the additive presents limit of use according to each food in which it is applied.

Carotenoids are the most studied and widely used colorants by industry (Rodriguez-Amaya, 2016). They are relatively thermostable compounds compared to other natural dyes. The primary instability restrictions are due to their degradation by oxygen and acidic pH (Rehman et al., 2020). However, encapsulation techniques can enable their use in hydrophilic foods and minimize losses due to exposure to oxygen and acidic media (Abreu, Turmo-Ibarz, Piccoli, Martín-Belloso, & Salvia-Trujillo, 2021; Comunian, Silva, Moraes, & Favaro-Trindade, 2020; Etzbach et al., 2020; Hari, Francis, Rajendran Nair, & Nair, 2018). Carotenoids provide yellow, orange, and red colors. They are usually extracted from plants such as corn, carrot, papaya, tomato, and watermelon. Nevertheless, they can also be obtained from algae and insects (Khalid, Bilal, Iqbal, & Huang, 2019; Rehman et al., 2020). Most orange and yellow fruits are sources of carotenoids, especially α-carotene and β-carotene. Orange, papaya, and mandarin are sources of lutein and crocin. Tomato has high levels of lycopene. Annatto seed is the primary source of bixin and norbixin. Finally, several plant leaves are sources of lutein (Khalid et al., 2019). Regarding legislation, carotenoids are approved by the Food and Drug Administration (FDA) and European Commission Regulation (FDA, 2001). These are β-carotene, norbixin, bixin, paprika, crocin lutein, and lycopene (Hendry & Houghton, 1996; Rehman et al., 2020). Carotenoids present therapeutic effects, including anticancer, immunomodulatory, anti-inflammatory, antibacterial, antidiabetic, and neuroprotective (Khalid et al., 2019; Nabi et al., 2020). On the other hand, the lack of carotenoids in the diet leads to corneal deficiencies, including night blindness, xerophthalmia, corneal ulcers, and even irreversible blindness (Sommer, 2008). The main carotenoid applications are sauces, spices, margarine, beverages, and dairy products (Baines & Seal, 2012).

Anthocyanins are water-soluble colorants with a greater range of colors. Additionally, there are some natural anthocyanin resources. They can be extracted from flowers, fruits, leaves, and other plants (Rodriguez-Amaya, 2016; Tarone et al., 2020). Many factors affect their stability, such as pH, temperature, light, oxygen, humidity, and salinity (Tarone et al., 2020). Anthocyanin as a food colorant presents low physical stability and the possibility of interaction with the food matrix, causing color changes (Calderaro et al., 2020, pp. 177–195; Martins et al., 2016; Neves, Silva, & Meireles, 2019; Tarone et al., 2020). The

Table 2
Some natural colorants and their color, approbation status, E number, primary sources, solubility, and physical and chemical stability.

Colorant	Color obtained	Approbation status	E number	Primary sources	Solubility	Physical and chemical stability	References
Curcumin	Yellow	Approved with combined maximum limit	E 100	*Curcuma longa* (rizome)	Oil soluble	Curcumin gives a lemon-yellow color in acidic media with a distinct green shade. In alkaline conditions (above pH 9.0) the color becomes orange; thermolabile (>100°C); light and sufur doixide sensible	(EC, 2011; Hendry & Houghton, 1996; Khanji et al., 2018)
Riboflavin	Yellow	Approved at quantum satis	E 101	Some plants, animals and microorganisms	Oil soluble	Thermolabile (> 50°C); light and sufur doixide sensible	(EC, 2011; Hendry & Houghton, 1996)
Chloropyll	Green	Approved at quantum satis	E 140	Can be extracted from a range of green leaves, but usually grass, nettles or alfalfa is used	Oil soluble	Heat stable; light and acid pH sensible	(EC, 2011; Hendry & Houghton, 1996; Janiszewska-Turak, Pisarska, & Królczyk, 2016)
B-carontene	Yellow	Approved with combined maximum limit	E 160a	Extracted from a number of sources including algae, carrots and palm oil	Oil soluble	Natural oxidation effect; stable to heat; light and sufur doixide sensible	(Carocho, Morales, & Ferreira, 2015; EC, 2011; MacDougall, 2002)
Norbixin	Orange	Approved with combined maximum limit	E 160b	*Bixa orellana* (Seeds) (formed in alkaline hydrolysis)	Water soluble	It should be precipitates from an acid solution; and with divalent cations (particularly calcium) it will form salts. Thermolabile (>100°C and <0°C); stable to light; suphur dioxide sensible	(EC, 2011; Hendry & Houghton, 1996; Leong, Show, Lim, Ooi, & Ling, 2018)
Bixin	Orange	Approved with combined maximum limit	E 160b	*Bixa orellana* (Seeds)	Oil soluble	Thermolabile (>100°C and <0°C); Easy oxidation; stable to light; suphur dioxide sensible	(EC, 2011; Hendry & Houghton, 1996; Leong et al., 2018)
Paprika	Reddish orange	Approved at quantum satis	E 160c	*Capsicum annum*	Oil soluble	Thermolabile (>100°C and <0°C); Easy oxidation; stable to light; suphur dioxide sensible	(EC, 2011; Hendry & Houghton, 1996; Leong et al., 2018)
Crocin	Yellow	Approved at quantum satis	E 160c	*Gardenia*	Water soluble	Low-intensity color	(EC, 2011; Hendry & Houghton, 1996)
Lutein	Yellow and oragne	Approved with combined maximum limit	E 161b	*Calendula*	Oil soluble	Low-intensity color	(EC, 2011; Hendry & Houghton, 1996; Janiszewska-Turak et al., 2016)
Betanin	Pink or red	Approved at quantum satis	E 162	*Beta vulgaris, Hylocereus polyrhizus, and red dragon fruit*	Water soluble	pH dependent (ideal: 5 - 6); very heat sensible; natural oxidation; light, Fe, Cu, and sulfur dioxide sensible	(EC, 2011; Hendry & Houghton, 1996; MacDougall, 2002)
Anthocyanin pH 1 Anthocyanin pH 4 Anthocyanin pH 8 Anthocyanin pH 12	Red Purple Green Yellow	Approved at quantum satis	E 163	The list of sources includes grapes, redcurrants and blackcurrants, raspberries, strawberries, apples, cherries, red cabbages, purple sweet potatoes and aubergines	Water soluble	Their color intensity is also pH-dependent being greatest at pH 1.0 and decreasing rapidly as pH rises. Some extracts react with proteins to form a haze or even a precipitate. Thermolabile (>50°C); light, oxidation, humidity, enzyme and sulfur dioxide sensible	(Abdullah, Lee, & Lee, 2010; EC, 2011; Jackman, Yada, Tung, & Speers, 1987; MacDougall, 2002)
Carminic acid pH <5 Carminic acid 7 < pH < 5 Carminic acid pH > 7	Orange Red Violet	Approved with combined maximum limit	E 120	*Coccus cacti* L.	Water soluble	Very low color intensity. Stable to light, heat, suphur dioxide and oxitation	(Amchova, Kotolova, & Ruda-Kucerova, 2015; EC, 2011; MacDougall, 2002)
Spirulina	Green to blue	Not approved	-	*Arthrospira platensis*	Water soluble	Thermolabile (> 40°C and < 0°C); light, sulfur dioxide, alcohol, pH below 3 sensible (ideal pH 7)	(Buchweitz, 2016; Chaiklahan, Chirasuwan, & Bunnag, 2012)
Blue compound from genipin	Blue	Not approved	-	*Genipa americana* L. and *Gardenia jasminoides*	Water soluble	Without a significant pH-dependent color; reaction between genipin and primary amine is essential; heat, light and storage stable; educts used can influence the color intensity	(Buchweitz, 2016; Janiszewska-Turak et al., 2016)
Caramel	Caramel	Approved at quantum satis	E 150a	Sugar	Water soluble	Very stable to light, heat, pH variations, suphur dioxide, and oxidation, but usually carry an electrical charge and thus may cause precipitation in the presence of oppositely charged molecules	(EC, 2011; Janiszewska-Turak et al., 2016; MacDougall, 2002)
Canthaxanthin	Red-orange	Not approved	E 161g	Bacteria, algae and some fungs	Oil soluble	Natural oxidation effect; thermal stable (>190°C); light and sufur doixide sensible	(Esatbeyoglu & Rimbach, 2017)

(*continued on next page*)

Table 2 (*continued*)

Colorant	Color obtained	Approbation status	E number	Primary sources	Solubility	Physical and chemical stability	References
Carbon black	Black	Approved at quantum satis	E 153	Derived from vegetable material, usually peat, by complete combustion to the insoluble carbon	Oil soluble	Very stable to light, heat, pH variations, suphur dioxide, and oxitation	(EC, 2011; Hendry & Houghton, 1996; Kühner & Voll, 2018)

color obtained depends on the pH and the acylation degree. Concerning the pH dependence, at pH below 2, the red flavilium cation predominates. At pH 4, there is a purple color. At pH 5, it is colorless (due to pseudo-base carbinol formation) and, at pH 6 the color is purple-blue (due to quinoid base formation). At pH 8, the color is green, and, at pH 12, it is yellow (Calderaro et al., 2020, pp. 177–195; Hendry & Houghton, 1996). Despite the range of possible colors by the medium's different pH values, pH variations throughout the process and storage in foods can lead to changes in the anthocyanin coloration, mischaracterizing the food matrix. In the presence of enzymes such as polyphenol oxidase (PPO), β-glucosidase, and anthocyanase, anthocyanins are hydrolyzed, releasing anthocyanidin, which is even more unstable. Anthocyanin can also be converted to carbinol (colorless compound) (Rodriguez-Amaya, 2016). Anthocyanins are degraded by ascorbic acid in the presence of oxygen through cleavage of the pyridinium ring and by exposure to light by a photooxidative mechanism (Tarone et al., 2020). Some molecules, called co-pigments, can also cause changes in anthocyanin color. Co-pigments may associate with anthocyanins forming different colors such as some flavonoids and alkaloids. Also, C-glycosylxanthones, pectin and tannin are other compounds reported as co-pigments (Asen, Stewart, & Norris, 1972; Roy & Rhim, 2020). Due to its high instability, the anthocyanin application presents itself as a significant challenge. Its encapsulation into biopolymer matrices may minimize its exposure to pH variations, light, oxygen, high temperatures, and interactions with undesirable co-pigments during the process and storage (Bryła, Lewandowicz, & Juzwa, 2015; Cabral et al., 2018; Carvalho et al., 2016; de Moura, Berling, Germer, Alvim, & Hubinger, 2018). Anthocyanins are approved by the FDA and European Commission Regulation (FDA, 2001). They are bioactive compounds with antioxidant, anti-allergic, anti-inflammatory, antiviral, antimicrobial, and anti-tumor potential, besides acting in the improvement of microcirculation and prevention of peripheral capillary fragility. Anthocyanins are associated with a low prevalence or relief of some disorders, such as cancer, cardiovascular diseases, diabetes, and obesity (Calderaro et al., 2020, pp. 177–195; Pojer et al., 2013). The main anthocyanins' applications are beverages, dairy products, mainly yogurts, confectionery products, and fruit preparation (Baines & Seal, 2012).

Betalains are natural water-soluble colorants divided into two groups: violet-red betacyanins (or betaines) and yellow-orange betaxanthines (Herbach, Stintzing, & Carle, 2006). The best sources of betalain are beet (*Beta vulgaris*) and red pitaya (*Hylocereus polyrhizus*) (Prieto-Santiago, Cavia, Alonso-Torre, & Carrillo, 2020). They are pigments approved by the FDA and European Commission Regulation (FDA, 2001). Betalains can be degraded by high temperature, pH, light, and metallic cations (Prieto-Santiago et al., 2020). Some studies have examined the microencapsulation of betalains, seeking to maintain their color properties (Kaimainen, Marze, Järvenpää, Anton, & Huopalahti, 2015; Karangutkar & Ananthanarayan, 2020; Mohammed, Iswarya & Nisha., 2021; Torres, Yumang, Jose, & Canillo, 2020). Although betalains present the same coloration over a wide pH range, from pH 3 to 7; pH conditions beyond that range can lead them to total degradation (Stintzing & Carle, 2004). Furthermore, in the presence of oxygen, betalain is more stable only between pH 5 and 6. Under anaerobic conditions, the lower pH values from pH 4 to 5 are more favorable. Oxygen and hydrogen peroxide can accelerate its degradation (Wasserman, Eiberger, & Guilfoy, 1984). Additionally, while light and oxygen caused deterioration of betalain by 15.6% and 14.6%, their simultaneous presence caused the decomposition of betalain by 28.6% (Von Elbe, Maing, & Amundson, 1974). Temperature can be considered the most crucial influencing factor in its stability. Even temperatures of approximately 50 °C may affect its stability (Herbach et al., 2006). Betalains play an essential role in human health due to their antioxidant, anticancer, antilipidemic, and antimicrobial activities (Gengatharan, Dykes, & Choo, 2015). They are used as a colorant in some candies, yogurts, ice creams, salad dressings, ready-made frosting, cakes, meat substitutes, drink mixes, gravy mixes, marshmallow candies, soft drinks, and gelatin desserts (Delgado-Vargas et al., 2000).

Additionally, curcumin is one of the most important and widely known curcuminoids derived from *Curcuma longa* rhizomes. It is also widely used as a food color that promotes yellow color (Martins et al., 2016). Approved by the FDA and European Commission Regulation, it is a colorant already used by the food industry (EC, 2013; FDA, 2001). However, curcumin has low water solubility, restricting its use (Sharma, Gescher, & Steward, 2005). Its sensitivity to moderate-high temperatures, light, basic and neutral pH, humidity, and oxygen makes curcumin very susceptible to degradation during processing and storage (Fang & Bhandari, 2011; Khanji et al., 2018). Thus, several studies were carried out to develop techniques for their protection in food matrices (Fang & Bhandari, 2011; Gómez-Estaca, Gavara, & Hernández-Muñoz, 2015; Mazzarino et al., 2012; Neves, Desobry -Banon, Perrone, Desobry, & Petit, 2019; Sahu, Kasoju, & Bora, 2008). Curcumin presents anti-inflammatory, antimicrobial, and anti-carcinogenic activities and can treat diabetes and skin diseases (Fang & Bhandari, 2011; Kocaadam & Şanlier, 2017). Curcumin is already applied in meat, fish, cakes, cookies, drinks, jams, ice cream, sausages, dairy products, and tomato sauces (Hendry & Houghton, 1996).

Other natural colorants shown in Table 2 are also applied, with significant limitations due to low chemical and physical stability. The major obstacle to the use of natural colorants is the lack of uniformity in the process. Since they are natural additives, many raw material variations can occur, making it difficult to standardize the color (Carocho et al., 2015; Martins et al., 2016). Additionally, during processing or storage, the color loss may vary differently with each product. Therefore, products with natural colorants present shorter shelf life to avoid standardization problems during storage (Buchweitz, 2016). More effective techniques are needed to ensure natural colorants' physical and chemical stability and maintain the final attractiveness of colored foods (Martins et al., 2016).

Despite the physical and chemical stability issues, these colorants are used in several products. There are growing strategies that seek to increase their stability. Naturally colored foods in yellow, red, and green can already be found in the market. However, since blue color matters, the scenario is scarce, and natural resources have low stability (Buchweitz, 2016). Currently, only anthocyanins and phycocyanins have legislation approved concerning their potential use as natural blue colorants. As previously, however, discussed, they present severe problems of stability and pH restriction. Acid foods that use temperatures above 60 °C in processing could not employ anthocyanins as a blue colorant.

Concerning the natural colorants utilization, the big gap in the natural colorant industry remains bright, intense shade options. For the natural food colored, the color intensity has always less vibrant hues comparing to artificial foods. This difference in color chroma brings the consumers a new color perspective in foods. They have to deal with

more pastel colors. On the other hand, whether consumers associate less intense colors in foods with healthiness since they are natural colorants, the pastel colors will be more attractive due to their health appeal (de Mejie, Zhang, Penta, Eroglu & Lila, 2020).

5. What has been scientific progress in the search for natural blue colorants?

Fig. 2 presents the number of papers published between 2015 and 2020 in the Scopus database using natural blue colorant/pigment/dye as the keywords. Considering consumers' awareness of synthetic colorants' issues, consumers will always avoid eating blue foods, increasing, in the same way, the interest in natural products due to their health benefits. Thus, the scientific and industrial community also raised their efforts to obtain natural blue colorants.

As we can see in Fig. 1, blue colors are typical in nature. However, they are challenging to apply as a natural colorant in some foods. Therefore, while extremely necessary, providing a natural blue colorant is also a challenge.

Sigurdson et al. (2017) studied obtaining blue colorant from anthocyanins. They found that chelating metals and a pH between 5 and 7 are necessary to maintain the color. The anthocyanins of red cabbage and purple sweet potatoes express a blue color-purple at pH 5 to 7 and blue at pH 8. However, they found that after 60 days at 23 °C, the blue color completely disappeared, showing extreme physical instability. Buchweitz, Brauch, Carle, and Kammerer (2013) formulated gels with blue colorant obtained from anthocyanins and verified the gels' blue color stability for 6 weeks at 23 °C low light. They were able to see considerable color losses during storage. Besides, in gels prepared with dairy products, due to calcium ions, the authors observed the aggregation of the chain of pectin and agar molecules that promoted the color intensity loss. In these formulations, very high doses of colorants were required. The last finding regarding the buttermilk and yogurt gels was that lactic acid annihilated the formation of ferric chelates of blue anthocyanin, making the color formation impossible. In general, for all gels, the value of the instrumental parameter -b*, which indicates the color blue, showed low intensity, in the range of approximately −2 to −9. Despite this, anthocyanins present no known adverse effects or restrictions. Due to anthocyanins' low toxicity, extracts rich in this flavonoid are widely applied as natural food colorants (Buchweitz, 2016).

Another issue inherent to anthocyanin utilization is the presence of off-flavors. Anthocyanin-rich extracts from red cabbage or red radish and sweet potatoes contain deriving from the glucosinolates degradation. In this sense, technological approaches have been proposed for the removal of odorous volatiles and their precursors, such as membrane filtration, flocculation, and adsorption on macroporous resins and ion exchangers (Müller-Maatsch, Gurtner, Carle, & Steingass, 2019; Chung, Rojanasasithara, Mutilangi, & McClements, 2015).

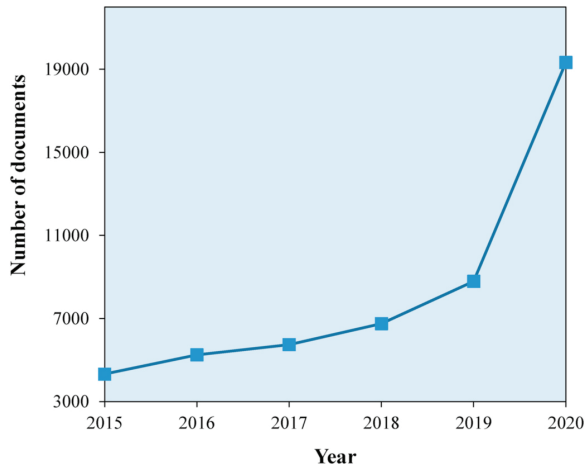


Fig. 2. The number of publications by year on natural blue colorants in the Scopus database (2015–2020).

Portisins are blue at acidic pH, and they are structures formed from flavonoids and anthocyanins, found in some aged wines (Mateus, Oliveira, Haettich-Motta, & de Freitas, 2004). However, it would require a greater supply of raw material for its industrial-scale recovery due to its low coloring power. There are still no studies with food applications using this colorant. Araújo et al. (2019) produced lipid microparticles to carry portisins, showed that the blue color obtained presented better physical stability when encapsulated. Nevertheless, the colored images showed a low coloring intensity. Finally, portisins have no toxic effect on humans (Buchweitz, 2016).

The *Gardenia jasminoides* plant contains the colorless iridoid genipin. After its recovery, blue colorants can be formed through reactions with primary amines (Brauch et al., 2016; Sakai et al., 2021). Although the blue color remained after 10 h at 60–90 °C, the blue compounds formed in this reaction have to be discovered to verify their possible effects on the human organism (Buchweitz, 2016; Paik, Lee, Cho, & Hahn, 2001). Due to the lack of knowledge about its chemical colorant composition, FDA and European Union have not yet approved its use as a food colorant (Buchweitz, 2016). Although promising, to date, there are no studies on the application of this colorant in food.

Phycocyanin is a blue protein complex found in blue-green algae of the cyanobacterial *Spirulina* species. It presents antioxidant and anti-inflammatory activities (Kilimtzidi, 2021). This colorant disadvantage is mainly in its high-cost production: the price of food-grade phycocyanin is approximately $ 500 per kg (Leema, Kirubagaran, Vinithkumar, Dheenan, & Karthikayulu, 2010). Phycocyanin also has limitations in high temperatures, light, and acidic media (Newsome et al., 2014). Phycocyanin is also poorly stable at room temperature and under cooling conditions. Only at 0 °C and pH 7 did this colorant present high physical stability (Mishra, Shrivastav, & Mishra, 2008). Additionally, its application occurs exclusively in acidic foods, which contain sugar or remain refrigerated, such as drinks, jams, chewing gum, and confectionery products (Chaiklahan et al., 2012). High concentrations of sugar and protein and low water content provide better conditions for its application (Chaiklahan et al., 2012; Mishra, Shrivastav, Pancha, Jain, & Mishra, 2010). Phycocyanin has been approved by the FDA to be used in sweets. For the European Union, spirulina extract is already considered a nontoxic food colorant (Buchweitz, 2016).

Natural indigo has been one of the oldest blue colors used in cosmetics since ancient times. Natural indigo comes from the *Indigofera* species. The fermentation of glucose from the leaves produces indoxyl compounds, which are oxidized, resulting in a greenish-blue color. Its low stability to light and high temperatures limits its application (Putri & Warsiki, 2019). At moderate temperatures such as 40 °C, the colorant is degraded. Additionally, for color maintenance, the pH must remain at 8–14 (Putri & Warsiki, 2019; Wahyuningsih et al., 2017). Thus, its application in food is not viable. The food industry applies natural indigo in smart packaging to indicate pH variations throughout product storage (Putri & Warsiki, 2019). *Indigofera* extracts do not present toxic effects. On the other hand, they showed antioxidant, analgesic, and anti-inflammatory activities (Gerometta, Grondin, Smadja, Frederich, & Gauvin-Bialecki, 2020). However, its use as a food colorant has not yet been thoroughly studied and approved.

Jespersen, Strømdahl, Olsen, and Skibsted (2005) compared the natural blue colorants phycocyanin and natural indigo produced after the reaction between primary amines and genipin extracted from *Gardenia*. According to them, the recommendation of indigo as a food colorant is not feasible due to its low water solubility and chemical instability to the light and food pH range. Gardenia blue exhibits excellent physical and chemical stability. It can be used as a food colorant, although it appears greenish-blue in solid applications.

The blue colorant derivatives of marennine are a blue-green synthesized by *Haslea ostrearia*; they are highly complex molecules and still undetermined (Pouvreau et al., 2006). The pigment is soluble in water and is blue-green only in acidic pH, and from pH 5, the compound is green (Gastineau et al., 2017, pp. 537–561; Pouvreau et al., 2008). However, the major problem of extracting colorants from microorganisms and algaes is the entire process's high cost (Dufossé, 2016). Additionally, as the chemical structure is still unknown, no studies have demonstrated the possible adverse effects on human health (Gastineau et al., 2017, pp. 537–561). To date, no studies have shown its application in food.

Some *Allium genus species*, such as garlic, onion, and leek spices, produce blue and yellow pigments during processing (crushing, cutting, maceration, or other processing) (Sigurdson et al., 2017). Isoalliin is converted enzymatically to 1-propenyl thiosulfinate, which reacts with amino acids to form colorant precursors. Allicin reacts to form blue and yellow colorants giving it a green color (Shin & Kyung, 2014). The formation of two colorants makes it challenging to isolate the compound responsible for the blue color, limiting its application. The extract also presents the garlic flavor, restraining its use in foods where this flavor is undesirable (Lee, Cho, Kim, & Lee, 2007).

6. What are the challenges and trends in the food industry, seeking blue food production?

Although there are natural blue sources, they have limitations for future applications on an industrial scale. Studies indicate that currently, only phycocyanin and anthocyanins, in the presence of chelating metals, and colorants derived from genipin would produce natural blue colorants (Buchweitz, 2016). However, there are still many drawbacks and limitations of these uses. First, colorants derived from anthocyanins have mainly temperature and pH range limitations. Second, the phycocyanin presents limitations in terms of physical and chemical stability and low coloring power. Finally, the compound derived from genipin presents limitations in legislation (Carocho et al., 2015). Colorants derived from genipin still need to be checked for food safety (Buchweitz, 2016). Studies still need to know the molecular structure formed by the reaction between genipin and primary amines. Later, colorant utilization may be approved (Buchweitz, 2016; Carocho et al., 2015; Faustino et al., 2019).

The lack of stability is a major potential problem in the broad application of natural colorants, an essential criterion in the food industry (Wrolstad & Culver, 2012). Several critical points lead to natural colorant degradation, from colorant extraction to food matrix storage. Colorant degradation affects both the intensity of its color, which damages the product's sensory attributes, and its bioactive compounds (Neves, Silva, & Meireles, 2019). The extraction stages, storage until use, application into the food and possible interactions, food processing, and storage of the final product; are factors that can degrade the colorant. Several factors that are present in all these stages, such as pH and its variations, light, temperature, processing method, and the existence of enzymes, sugars, proteins, oxygen, and metal ions in the food matrix can drastically affect the stability of natural colorants (Buchweitz, 2016; Carocho et al., 2015). Therefore, the use of natural colorants in food requires detailed knowledge of their chemical and physical stability. For example, the process temperature should not exceed 40 °C when anthocyanins are processed. During the entire process and storage, the matrix must remain within the desired color's specific pH range. In turn, phycocyanin should be applied only in acidic, sugary food matrices and with due protein content. Additionally, the production process must be taken at a temperature below 70 °C to avoid color damage.

Therefore, the entire industrial process involving natural colorants needs to be studied in detail to ensure color maintenance. Even concerning phycocyanin, its high production costs limit factors for its application (Carocho et al., 2015; Chaiklahan, Chirasuwan, Loha, Tia, & Bunnag, 2011). Based on academic literature searches, the cost of food-grade phycocyanin (purity greater than 0.7) is approximately $ 130 per g (Cisneros & Rito-Palomares, 2004). Compared to purple corn anthocyanin, which costs approximately $ 0.07 per g, the pigment extracted from spirulina is quite expensive (Somavat et al., 2018). The genipin extract (blue colorant precursor) does not yet have commercial value for food quality. However, economic feasibility studies for a pressurized liquid genipin extraction process report an average price that can reach approximately $ 0.1 per g (Náthia-Neves, Vardanega, & Meireles, 2019).

Despite the limitations related to price, stability, and lack of knowledge about the chromophore compound, natural blue colorants are the future for the food industry that seeks innovation and products that attract consumers' attention in the face of a competitive market. An article published in The New York Times, discussing food trends in 2020, highlighted: "Color of the Year: Blue", articulating that Pantone declared classic blue as its color of the year, and consequently will also play a huge role in new foods in 2020 and beyond (Severson, 2020). In general, the consumption of natural compounds has been encouraged in recent years due to their numerous health benefits. Along with this trend, the potential natural blue colorant quickly obtained, safe in health, and high coloring will be evidenced in developing new products. Consequently, the blue color will gradually gain prominence in the market and be more accepted by consumers.

7. How may genipap provide us with a future strategy for the blue color?

More promising than looking for other blue natural resources is to deepen the studies of the chromophore compounds formed that are still unknown, improve the stability, intensify the color, and even create strategies to reduce the already known dyes' costs. *Genipa americana* L. is known as jenipapo (Portuguese), jagua (Spanish), and bois de fer (French). It is a fruit from the *Rubiaceae* family, found mainly in Central and South America (Djerassi, Gray, & Kincl, 1960). From this fruit, in its initial stage of maturation, we can obtain genipin. This iridoid is an essential compound for forming the blue color from a spontaneous chemical reaction, in the presence of oxygen, with primary amines of amino acids, peptides, or proteins. The compound formed responsible for the blue color is believed to be a mixture of high molecular weight polymers (Buchweitz, 2016; Neri-Numa, Angolini, Bicas, Ruiz, & Pastore, 2018). Some studies are seeking to understand more deeply the cross-linking reaction between primary amines and genipin (Fiamingo & Campana-Filho, 2016; Kher, Udabage, McKinnon, McNaughton, & Augustin, 2007; Lin, Yu, Ai, Zhang, & Guo, 2020; Sun et al., 2016; Tamura et al., 2019). However, an important application is its potential to form a natural blue colorant.

Some studies on genipin extraction reported the formation of blue compounds (Brauch et al., 2016; Náthia-Neves et al., 2017; Neri-Numa et al., 2018). Patents reported obtaining the blue color from genipin with pure amino acids (Echeverry, Zapata, & Torres, 2011) and purified dimers (Cano, Lopez, Romero, Garcés, & Porras, 2016). Studies that carried out genipin recovery in ethanol and water obtained yellow or green colors (Náthia-Neves et al., 2017; Renhe, Stringheta, Silva, & Oliveira, 2009). There were not enough primary amines of the fruit in these cases to react with genipin to form the blue color. According to the two patents developed by Cano et al. (2016) and Echeverry et al. (2011), the blue color could be obtained only after adding purified amino acids or polymers. These components served as suppliers of primary amines to produce a blue color. Brauch et al. (2016) carried out a study with the blue colorant obtained by genipin and primary amine reactions. They compared gels' color stability with this compound and with synthetic colorants: Brillant Blue and Indigo Carmine. They concluded that the colorant produced by genipin presented a coloring power equivalent to that of the synthetics and even higher physical stability to light and storage time, with a half-life (t½) of 86–105 days, while Indigo Carmine presented a t½ of 9 days.

Forming a blue colorant from the addition of a pure compound, such as an amino acid or even primary amines, to react with genipin may not be economically attractive. In this sense, Neves, Strieder, Silva, and Meireles (2020) obtained a blue colorant from the milk and unripe *Genipa americana* L. pulp. Milk was used as a solvent, supplier of primary amines, and carrier of the blue color compounds. They obtained a highly intense blue color. Blue colorant-loaded milk seems to be the most promising natural blue option. It is excellent for several applications, mainly in dairy food formulations.

Despite the colorant from genipin as a precursor is already in use, it is still necessary to validate this possible ingredient's proper use since the blue compound is still unknown. The polymers obtained from the cross-linking between genipin and primary amines presented low cytotoxicity and high biocompatibility (Sung, Huang, Huang, & Tsai, 1999). However, the polymer formed depends on the protein used in this reaction (Cano et al., 2016). In this case, it is necessary to check for possible toxic effects of the polymer formed. The exact chemical composition of the blue compound formed is still unknown as well as the potential toxic effects on the organism. Consequently, it is still impossible to make this colorant available on the market.

Regarding genipin, there are few studies, controversial among themselves, on its toxicity. Mailloux, Adjeitey, and Harper (2010) demonstrated that genipin reduced the chemotherapeutic resistance in drug-resistant cells in the fight against cancer. Therefore, presenting potential as a drug sensitizing agent increases its effect on the body. Genipin can also inhibit the enzymatic activities of cytochrome P450 (which are responsible for oxidizing substances and making them more polar and water-soluble in the body) (Gao, Zhang, Cui, & Yan, 2014). The results by Neri-Numa, Torre, Oriani, Ruiz, and Pastore (2020) show that genipap extract at a dose of 25 μg/mL of solution with the solvent (methanol: water 80:20 (w/w)) and other extracted compounds (genipin1-β-gentiobioside, geniposide, and genipin acid) can be considered atoxic and with functional appeal. However, since the reaction consumes genipin, the focus should be given to studying the toxicity of the blue compound formed (Neves et al., 2020; Strieder, Neves, Silva, & Meireles, 2020). Even more in-depth knowledge of the reaction mechanism is necessary (Buchweitz, 2016). It is not yet clear, for example, whether the blue colorant is part of the protein that is cross-linked to genipin or whether there is the formation of a third chromophore compound. Such gaps in knowledge have to be filled to enable the use of this colorant.

The toxicity of the residual genipap pulp must be taken into account. In this scenario, Codignoto et al. (2017) demonstrated that the maximum non-cytotoxic concentration of the fruit extracts was 0.5 mg/mL. Assis (2015) evaluated acute (2000 mg/kg) and subchronic (100, 500, and 100 mg/kg for 30 days) toxicity in mice and classified the fruit as having both acute and subchronic low toxicity. On the other hand, they reported a potential effect of the fruit on cancer cells and melanoma treatment. The blue colorant-loaded milk is still being investigated in terms of toxicity, coloring power, food matrix interactions, and stability under some process conditions.

8. Conclusion

The use of non-synthetic additives is the food sector's current trend due to modern consumers' demand for food safety, reinforced by the health benefits of natural nutrients. We can currently observe an advance concerning the utilization of some natural colorants in the market, despite their physical and chemical stability limitations. The main natural colorants already used for the food industry are anthocyanins, betalains, chlorophylls, caramel, curcumin, carminic acid, lycopene, and carotenoids. Despite the noticeable advances regarding the replacement of artificial colors by natural colors, the natural blue color is still the most significant industry challenge. In addition to the stability concerns, none of the existing natural blue colorants reach the versatility, low cost, and intensity of synthetic blues.

Furthermore, some chemical structures responsible for the color are still undiscovered and require comprehensive knowledge to verify toxic effects on human health. Thus, after several studies, the colorant is approved and can be commercialized. Of the existing blue colorants, the compound formed from the reaction between genipin from *Genipa americana* L. and primary amines from milk bases may present great potential application due to its intensity, stability, versatility, and low cost. However, there is still a lack of toxicity effects, and its chemical structure is undiscovered. Thus, it is essential to fill in the gaps in this colorant's knowledge-seeking utilization. Finally, after the availability of a natural blue colorant with affordable, safe, and high color power, the food industry will highlight its products' new color. Consequently, the color blue will gain prominence in the market and be more accepted by consumers.

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CHAPTER 3

Experimental article I

Could milk be a primary amines supplier to produce the blue colorant?

Milk colloidal system as a reaction medium and carrier for the blue colorant obtained from *Genipa americana* L.

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Milk colloidal system as a reaction medium and carrier for the natural blue colorant obtained from the cross-linking between genipin and milk proteins

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ABSTRACT

A promising natural blue colorant was obtained from the cross-linking between genipin and milk proteins. Milk was simultaneously used to extract genipin from the unripe genipap (*Genipa americana* L.) and evaluated as a reaction medium and carrier for the novel blue colorant. The effects of the milk composition (skimmed, semi-skimmed, and whole) on the kinetic of blue color formation in the colorant-loaded milk samples during their cold storage time for 96 h were evaluated using their color parameters and free-genipin content. The reaction between milk proteins and genipin were evaluated by FTIR spectroscopy. In addition, the blue colorant-loaded milk samples were characterized according to their droplet size distribution, microstructure, and phase separation kinetics. The milk fat content influenced the genipin recovery while the milk with higher protein content contributed to obtaining a more intense blue color. Using whole milk with 3.0 g/100 g fat content favored the light scattering and, thus, a blue colorant with a more intense $-b^*$ and L^* values was obtained. On the other hand, the use of skimmed milk with 0.5 g/100 g fat content resulted in more consumption of genipin due to its more protein content for the reaction of blue color compounds formation. Thus, a more intense and darker blue coloration was observed with lower light scattering. The milk composition did not modify the phase separation kinetics of the blue colorant-loaded milk. Therefore, our results have demonstrated that the milk was a suitable medium for the reaction of blue color formation and also a good blue compounds carrier.

Industrial relevance: Consumer demands for natural colorants have increased in the last years.

Thus, the new food industry challenge is to develop novel healthy, safe and high-quality food products based on natural colorants. Currently, the colorant market does not have still available a natural blue colorant. Therefore, the development of a novel natural blue colorant from plant material could meet this worldwide demand increasing the added value of many products such as ice cream, dairy beverages, and candies.

1. Introduction

The global market of natural colorants has grown annually in the last 5 years (Cortez, Luna-Vital, Margulis, & Gonzalez de Mejia, 2017). Natural food colorants have received particular attention because they are potential replacers for synthetic additives (Martins, Roriz, Morales, Barros, & Ferreira, 2016). Also, natural colorants can present anti-inflammatory antimicrobial, anti-amyloid, and antitumor effects (Obulesu & Rao, 2011; Pan et al., 2010). Commonly used natural food colorants comprise anthocyanins, carotenoids, betalains, and chlorophylls, among others (Carocho, Morales, & Ferreira, 2015). However, among the available natural colorants in the market, the obtaining of a stable natural blue colorant is still a challenge for the food industry.

In this sense, the genipap (*Genipa americana* L.), a native fruit from Brazil, has been studied as a promising alternative for the production of a novel natural blue colorant (Brauch, Zapata-Porras, Buchweitz, Aschoff, & Carle, 2016; Náthia-Neves & Meireles, 2018; Náthia-Neves, Tarone, Tosi, Júnior, & Meireles, 2017). Its fruit has high levels of iridoids, such as genipin, which is an essential compound for the blue color formation from a spontaneous chemical reaction, on the presence of oxygen, with primary amine groups of amino acids, peptides or proteins (Fig. 1) (Brauch et al., 2016; Touyama et al., 1994).

The formation of the blue colorant is a result of an oxygen radical-induced polymerization and dehydrogenation of several intermediary colorants, producing water-soluble polymers (Bentes, de Souza, Amaya-Farfan, Lopes, & de Faria, 2015). This blue colorant has peculiar features like high stability to heat, light and pH changes (Bentes et al., 2015).

Genipin and amines interact by a cross-linking, which allows the formation of the blue color and include other reactions with a higher

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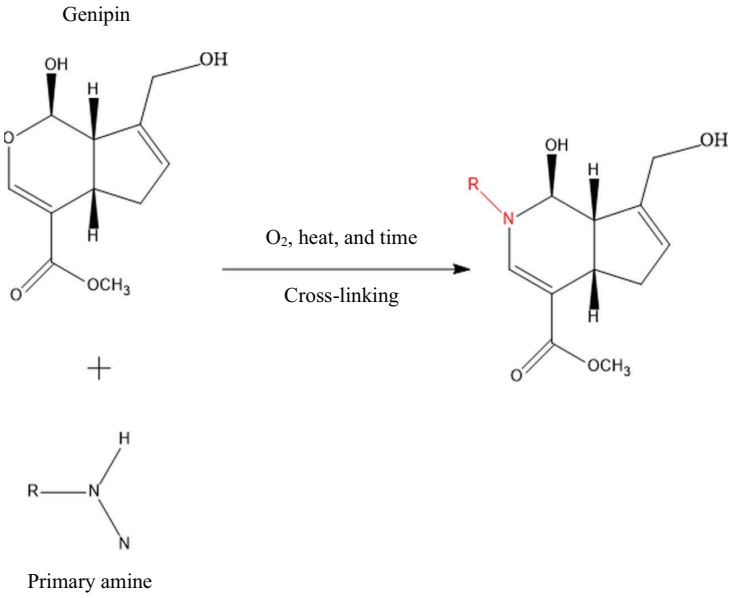

Fig. 1. Reaction between genipin and primary amine.

level of complexity. This interaction is based on genipin covalent cross-linker using several biopolymers of proteins and polysaccharides containing residues with primary amine groups (chitosan, gelatin, silk protein, collagen, casein, and others) (Brauch et al., 2016).

Casein is the major protein in bovine milk (approximately 80% of the total milk proteins). Casein molecules form micelle structures with a 200 nm hydrodynamic diameter on average (Walstra, Walstra, Wouters, & Geurts, 2005). They are potentially useful biopolymers to carrier different molecules and, therefore are the main compounds responsible for the encapsulating properties associated with the milk colloidal system (Khanji et al., 2015). Besides that, the milk is a complex system composed of other proteins that can also assist in carrying compounds. In this sense, the milk is an oil-in-water emulsion able to carry several active or functional compounds. Neves, Desobry-Banon, Perrone, Desobry, and Petit (2019) used the milk as a curcumin carrier and verified that it had the same potential for this purpose when compared to isolated casein.

The primary amine groups contained in caseins and whey proteins of milk are the reaction substrate for the formation of a blue color. Blue colorant formation increases linearly with increasing amino acid concentration, as providing excess primary amine groups (Brauch, 2016). Milk can then be used as an amine supplier to form a complex system. In addition, since milk can be used as a carrier of compounds, it also serves as a carrier of blue color components, providing a system to protect these ones and thus preserving the blue coloration. Therefore, the use of milk in the genipin recovery process has two functions: to ensure the rapid formation of the blue color due to amines in excess; also, to act as a carrier system since it has the potential to carry the complexes responsible for the blue color.

This complex system where the milk acts as a substrate for the blue color formation and its carrier has not yet been studied. The milk is a complex matrix with proteins, fat, sugars and minerals, therefore it is necessary, initially, to verify the influence of milk components on the formation of the blue color. The component with the most variation in the milk composition is fat, including natural variation and changes due to processing (Walstra et al., 2005). Currently, there are three kinds of milk on the market, with different fat levels which are the skimmed, semi-skimmed and whole milk. Thus, the aim of this study was to evaluate the potential of the milk colloidal system to provide a reaction medium for blue color formation from genipap extracts and to carry the novel colorant obtained. In addition, the effects of milk composition (skimmed, semi-skimmed, and whole) on the kinetics of blue color formation were evaluated.

2. Material and methods

2.1. Material

Unripe genipap (*Genipa americana* L.) was donated by "Fazenda Lagoa" (Ponte Alta do Tocantins, Brazil) and were stored at −24 °C until the experimental assays. Milk powder samples (skimmed, semi-skimmed, and whole) were obtained from a local market in Campinas. Skimmed, semi-skimmed, and whole milk were reconstituted using the ratio of 10 g of milk powder to 90 g of ultrapure water, respectively.

All the chemicals used in this study (ethanol, acetonitrile and formic acid) were of analytical grade. The HPLC standard of genipin (purity 98–100%) was purchased from Sigma-Aldrich (St. Louis, USA). Ethyl alcohol absolute with 99.5% purity was purchased from Anidrol (Diadema, Brazil). Acetonitrile was purchased from J. T. Baker (Phillipsburg, USA). Ultrapure water was supplied by a Milli-Q Advantage 8 Purifier System from Millipore (Bedford, USA). Formic acid (purity 98–100%) was purchased from Dinâmica (Diadema, Brazil).

2.2. Reconstituted milk characterization

The moisture content of the reconstituted milks was determined according to the methodology describe by Bradley (2010). Their fat content was determined by the Gerber method (British Standards Institution, 1955). The protein content was measured by Dumas combustion method using a conversion factor of 6.25 (Dumas VELP NDA 701, Usmate Velate, Italy). The calibration curve was made using rice protein standards. Table 1 presents the results for the reconstituted milk characterization.

Table 1
Characterization of the reconstituted milk (n = 3).

Reconstituted milk	Moisture (g/100 g)	Fat (g/100 g)	Protein (g/100 g)
Skimmed	92.38 ± 0.28	0.5 ± 0.01	3.84 ± 0.01
Semi-skimmed	92.36 ± 0.02	1.5 ± 0.05	3.02 ± 0.22
Whole	92.31 ± 0.14	3.0 ± 0.01	2.48 ± 0.16

2.3. High-intensity ultrasound-assisted production of blue colorant-loaded milk

The genipap was previously thawed at room temperature and ground in a conventional mechanical processor (Philips - Brasil LTDA, model RI1364/07, 400 W, Varginha, Brazil). Fifty grams of the whole fruit, without the peel, was milled for 5 min. For each experimental run, one fruit was used to perform all treatments aiming to eliminate the effect of different raw material.

We evaluated the effects of the milk composition (skimmed, semi-skimmed, and whole) on the blue color formation kinetics assisted by high-intensity ultrasound (HIUS) technology. HIUS treatment was used as a homogenization technique aiming to increase the reaction rate between the genipin and primary amine groups obtained from the genipap plant material and milk samples for the formation of blue compounds. We processed 5 g of ground genipap and 25 g of reconstituted milk in a 100 mL Becker with a removable inner metal basket to separate the genipap mass from the milk system after the HIUS processing. The HIUS treatment was performed in one-step with a 13-mm ultrasound probe diameter at 19 kHz and nominal power of 450 W for 5.56 min (Unique, 500 W, DES500, Indaiatuba, Brazil). For this, the probe contact height with the liquid medium was standardized to 15 mm. The HIUS processing was performed by inputting 5 kJ/g. This HIUS specific energy was chosen according to preliminary tests. The specific energy was calculated from nominal power according to Eq. (1). After the treatments, the samples were storage at 5 °C in a refrigerator (Consul, Joinville, Brazil). All experiments were performed in duplicate.

$$\text{Specific energy}\left(\frac{\text{kJ}}{\text{g}}\right) = \frac{\text{Nominal power (kW)} \times \text{Processing time (s)}}{\text{Mass (g)}} \quad (1)$$

2.4. Blue colorant-loaded milk characterization

2.4.1. Droplet size distribution

Droplet size distribution and mean diameter of the milk samples were determined at 25 °C by light scattering technique using laser diffraction (Malvern, Mastersizer 2000 Malvern Instruments Ltd., UK). The mean diameter of droplets was calculated based on the mean diameter of a sphere of similar area (superficial mean diameter - D_{32}, Eq. (2)). The polydispersity index (*span*) was calculated according to Eq. (3). The samples were analyzed by a wet method, with dispersion in water and refractive index of 1.52.

$$D_{32} = \frac{\sum n_i d_i^3}{\sum n_i d_i^2} \quad (2)$$

$$span = \frac{(d_{90} - d_{10})}{d_{50}} \quad (3)$$

where: d_i is the mean diameter of the droplets; n_i is the number of droplets; and d_{10}, d_{50}, and d_{90} are the diameters at 10%, 50% and 90% of cumulative volume, respectively.

2.4.2. Microstructure

The samples were poured onto microscope slides, covered with glass coverslips and observed using a Carl Zeiss Model Axio Scope A1 optical microscope (Zeiss, Gottingen, Ger-many) with the 100 × objective lenses and using immersion oil.

2.4.3. Phase separation kinetics

The kinetic stability of the blue colorant-loaded milk samples was evaluated using the near infrared backscattering profile technique. Each formulation was transferred to specific glass tubes and stored at 5 °C in a refrigerator (Consul, Joinville, Brazil) to measure the stability using a light backscatter scan analyzer Turbiscan LAB Expert (Formulaction®, Toulouse, France). The measurements were performed immediately after samples production (0 h) and for more than four days (24, 48, 72 and 96 h) of storage at 5 °C. The destabilization index (DSI) was calculated according to the Eq. (4) (Pereira et al., 2019a) by the software Turbisoft 2.0.0.28 (Formulaction®, Toulouse, France).

$$DSI = \sum_i \frac{|\sum_h scan_i(h) - scan_{i-1}(h)|}{H} \quad (4)$$

where $scan_i$ and $scan_{i-1}$ are the initial backscattering value and the backscattering value after the storage time, respectively; h is the height in the measuring cell; and H is the total sample height.

2.5. Fourier transform infrared spectroscopy

The reactions between milk proteins and genipin were evaluated using Fourier transform infrared (FTIR) spectroscopy. For this, genipin, milk powders, and the blue colorant-loaded milk samples were analyzed. These measurements were taken at room temperature, in the range of 400–4000 cm^{-1}, using a Fourier transform infrared Jasco 4100 spectrometer (Tokyo, Japan).

2.6. Kinetic of blue color formation

The blue color formation kinetics was monitored every 24 h starting right after the manufacturing (0 h) of blue colorant-loaded milk samples until 4 days of storage. The samples were analyzed in relation to their color parameters and free-genipin content.

2.6.1. Color parameters

The color parameters were determined by using a Hunter Lab colorimeter (model Color Quest II) with CIELab scale (L^*, a^*, and b^*) with reflectance mode. The color parameters were expressed in terms of lightness L^* (L^* = 0 for black and L^* = 100 for white) and chromaticity parameters a^* (green [−] to red [+]) and b^* (blue [−] to yellow [+]). From these parameters (L^*, a^*, b^*), the cylindrical coordinates C^* (chroma) and h^*(hue angle) were determined according to Eq. 5 and 6, respectively.

$$C^* = \sqrt{(a^{*2} + b^{*2})} \quad (5)$$

$$h^* = arctan\left(\frac{b^*}{a^*}\right) \quad (6)$$

2.6.2. Free-genipin content

The free-genipin content was quantified in the blue colorant-loaded milk samples by high-performance liquid chromatography (HPLC). An aliquot of 0.5 mL of each sample was dissolved in 1 mL of ethanol manually homogenized. Then, the mixture was centrifuged at 12,000 rpm for 15 min. Just after, an aliquot of 1 mL of supernatant was filtered using nylon membrane (0.45 μm) and injected . The HPLC-PDA (Waters, Alliance E2695, Milford, USA) system consists of a separation module (2695) with an integrated column heater, autosampler, and photodiode array (PDA) detector. The analysis was performed by a method developed and validated by Nathia-Neves, Nogueira, Vardanega, & Meireles (2018). Separation of the iridoids was carried out on a fused-core C18 column (Kinetex, 100 × 4.6 mm i.d.; 2.6 μm; Phenomenex, Torrance, USA). Using a mobile phase of water (A) and

acetonitrile (B) that were both acidified with 0.1% formic acid and the following gradient: 0 min, 99% A; 9 min, 75% A; 10 min, 99% A and 13 min, 99% A. The temperature and flow rate was 35 °C and 1.5 mL/min, respectively. The calibration curves of the iridoids were obtained at the range of 0.1–625 μg mL^{-1} for genipin (R^2 = 0.999). Free-genipin content was determined in relation to the weight of the genipap pulp used to produce the blue colorant-loaded milk sample (Eq. (7)). The dried genipap was regarding the genipap mass on a dry basis.

$$\text{Free-genipin content (mg/g)} = \frac{\text{Genipin (mg)}}{\text{Dried genipap (g)}} \quad (7)$$

2.7. Statistical analysis

Minitab® software 18 was used to evaluate the effects of the milk composistion on the kinetic stability of the blue colorant-loaded milk samples and the blue color formation kinetics by the analysis of variance (ANOVA) at a significance level of 95%.

3. Results and discussion

3.1. Blue colorant-loaded milk characterization

Table 2 presents the results for mean droplet size (D_{32}), cumulative volume (d_{10}, d_{50}, and d_{90}) and *span* values of the blue colorant-loaded milk samples obtained from the skimmed, semi-skimmed and whole milk (Table 1). The droplet size distribution was regarding the milk fat globules. The milk composition did not influence the D_{32} values (p-value = 0.33). The different milk fat content presented the same droplet size distribution. HIUS treatment promoted the homogenization of the milk samples due to the micro shear rate provided by the acoustic cavitation. The formation and subsequent collapse of microbubbles observed in the liquid medium when it is subjected to low-frequency and high-power ultrasound treatment are associated with extreme levels of highly localized turbulence. Thus, the fat globule is break up in small units after ultrasound treatment (Zhu et al., 2018). Similar results were reported by other authors, showing a reduction in the size of fat globules after HIUS processing (Bermúdez-Aguirre, Mawson, & Barbosa-Cánovas, 2008; Monteiro et al., 2018; Wu, Hulbert, & Mount, 2000). The span values express the polydispersity degree in the droplet size distribution of the milk samples. In this sense, smaller span values could be associated with more uniform and effective homogenization processing. According to these results, the HIUS processing led to the formation of a more uniform blue colorant-loaded milk sample (Silva et al., 2018).

Fig. 2 presents the optical microscopy of the reconstituted milk and blue colorant-loaded milk samples. According to the images, the samples presented fat globules and small particles in suspension. It was possible to note the effect of genipin on the microstructure of the system, in which the milk with genipin had blue micro points. The HIUS treatment had some important roles in the manufacturing of the blue colorant-loaded milk: HIUS assisted the genipin recovery from the plant material due to acoustic cavitation; and HIUS provided heating and turbulence effect. The thermal effect increases the reaction rate between genipin and primary amine groups. The turbulence acts on the homogenization of the milk samples by reducing the size of the fat globule. Thus, the HIUS processing provided better homogenization to the system. The difference in the milk fat content in the samples was observed in the images. A higher density of fat globules was observed for the samples with 3.0 g/100 g fat content (whole milk).

The multiple light scattering technique allows the detection of the destabilization phenomenon of systems before any effective visual observation (Trujillo-Cayado, Alfaro, Muñoz, Raymundo, & Sousa, 2016). Backscattering profiles (BS) *versus* the height of the measuring cell were obtained for the samples during 96 h of cold storage and are shown in Fig. 3. Immediately after the homogenization process (pink line), BS values did not change as a function of the height of the measuring cell, since fat droplets were dispersed uniformly throughout the colloidal system. Fig. 3 shows that there were slight differences in BS profiles of the blue colorant-loaded milk samples over storage time demonstrating their good kinetic stability. Milk has pronounced light-scattering properties due to the presence of fat globules (Walstra et al., 2005). The sample with 3.0 g/100 g fat content showed a superior backscatter. Higher fat content implies in more dispersed phase which interacts with the near-infrared light. The size of the fat droplets in the dairy system (Table 1) is in the light indent range, so higher content fat promotes higher BS.

Regarding phase separation kinetics, the higher is the DSI value, the greater is the phase separation or destabilization in the sample. The DSI values obtained for the skimmed, semi-skimmed and whole milk were 7 ± 1, 6.4 ± 0.1, and 6.0 ± 0.4, respectively. The milk composition did not influence DSI value (p-value = 0.56). Thus, fat content did not change the phase separation kinetics of the sample.

3.2. Reaction between milk proteins and genipin

The FTIR spectrum of all samples (Fig. 4) showed two bands in 4000–2000 cm^{-1} region, being abroad band centered at 3421 cm^{-1} assigned to hydrogen bonded O—H stretching vibrations and the second one a weak signal at 2927 cm^{-1} due to C—H stretching vibrations, including CH, CH_2, and CH_3 (Pereira et al., 2019). In detail, the band 1153 cm^{-1} represents the bending vibration of C-O-C. The broad band at 1620 cm^{-1} is mainly attributed to the OH bending mode of adsorbed water and protein amide (C=O) (Liu, He, Shankle, & Tewolde, 2016). Bands at 1249, 1500 and 1313 cm^{-1} are close to the absorptions of aromatic ring vibration, from genipin.

Amide *I* (1700–1600 cm^{-1}) corresponds mainly to C—O stretching vibrations of the peptide bonds and amide II (1600–1500 cm^{-1}) attributed to C—N stretching vibrations in combination with N—H bending (Markoska, Huppertz, Grewal, & Vasiljevic, 2019). The peak corresponding of C—N is more intense with genipin-milk.

The peaks at 1076 and 1035 cm^{-1} resulted from C—N stretching, which was higher in milk with genipin. This functional group (C—N) is created after the reaction of genipin with free primary amine groups (Fig. 1). Mi, Sung, and Shyu (2000) observed that the absorbance peak at 1076 and 1035 cm^{-1} was increased in intensity from the addition of genipin crosslinked with amines. The band at 1414 cm^{-1} was assigned to a ring stretching mode in the genipin molecule, which is higher in milk with genipin. The peaks at 1550 and 1710 cm^{-1} were due to protonated amine groups on the casein, which, in fact, after the reaction described in Fig. 1, larger amounts of protonated amines appear.

The peaks at ~800 to 1000 cm^{-1} resulted from carbohydrate ring (Lei et al., 2010). It was more intense in milk with genipin due to the presence of genipin molecule. The increase of O—H stretching at

Table 2
Effects of milk composition on the mean size and droplet size distribution.

Milk composition	D_{32} (μm)	d_{10} (μm)	d_{50} (μm)	d_{90} (μm)	*span* (−)
Skimmed	0.9 ± 0.1	0.30 ± 0.01	0.40 ± 0.01	0.65 ± 0.01	0.9 ± 0.1
Semi-skimmed	0.8 ± 0.1	0.3 ± 0.1	0.43 ± 0.03	0.67 ± 0.04	0.82 ± 0.03
Whole	1.0 ± 0.1	0.34 ± 0.01	0.5 ± 0.1	0.7 ± 0.1	0.83 ± 0.02

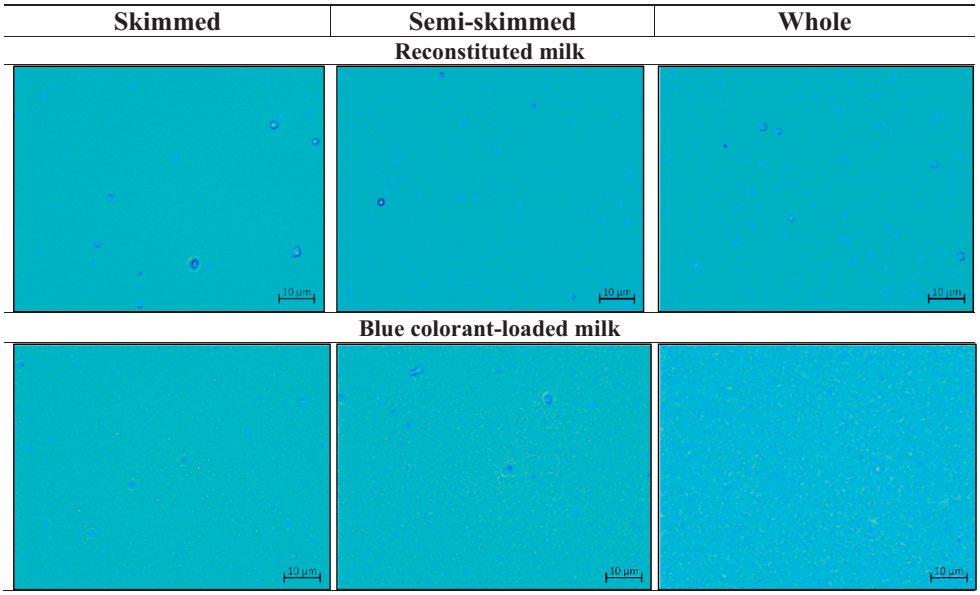

Fig. 2. Microstructure of the blue colorant-loaded milk samples.

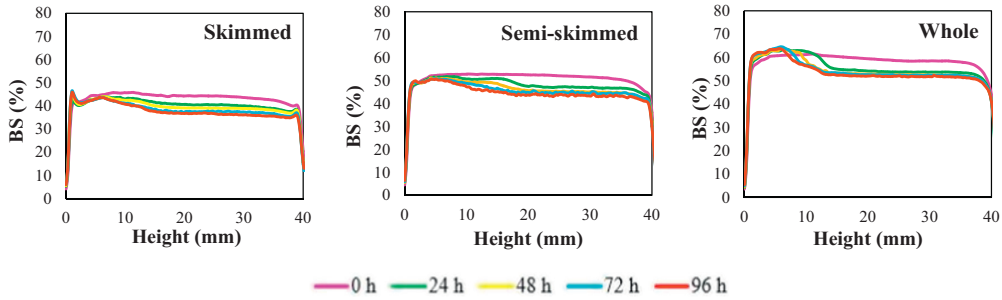

Fig. 3. Backscattering profiles of the blue colorant-loaded milk samples during their cold storage at 5 °C.

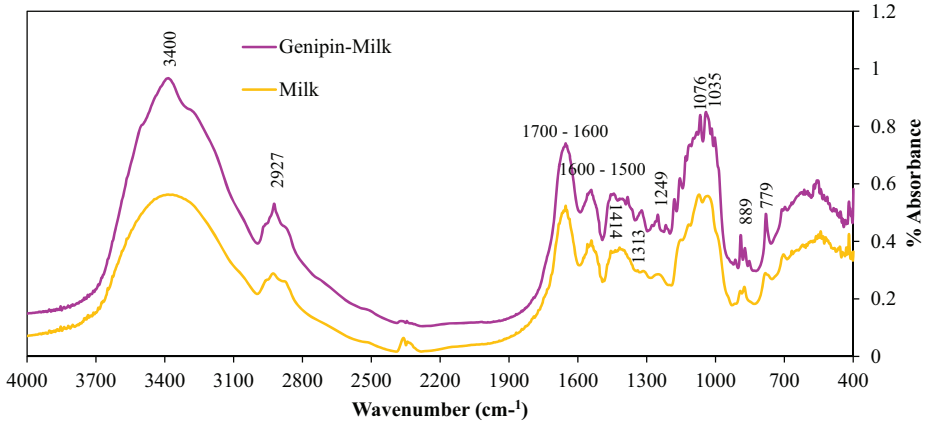

Fig. 4. FTIR spectra of the blue colorant-loaded milk and milk.

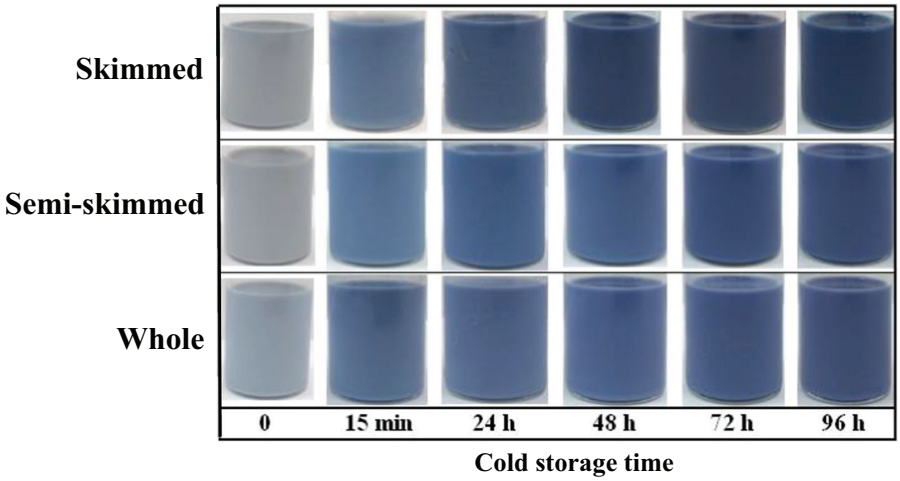

Fig. 5. Blue color formation kinetics in the blue colorant-loaded milk samples during their cold storage at 5 °C. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3400 cm^{-1} could be observed in milk with genipin, ascribed to OH of water, altered water-binding (O'Loughlin, Kelly, Murray, Fitzgerald, & Brodkorb, 2015).

The difference between the spectra of milk and genipin-loaded milk indicates the interaction between milk proteins and genipin, which promoted modification in functional groups, corroborating with the reaction shown in Fig. 1.

3.3. Effect of milk composition on the kinetic of blue color formation

The images of the blue colorant-loaded milk samples were obtained right after their HIUS processing (time 0), after 15 min and every 24 h until 96 h of cold storage time (Fig. 5). Blue color characteristic was obtained about 15 min after the HIUS processing. According to the images of the colloidal systems, the blue color became more intense during the cold storage time at 5 °C for 96 h. Regarding the milk composition, the skimmed milk sample resulted in a darker coloration, and initially presented grayer compared to the other milks. The whole milk was bluer since the initial time (0 h) compared to other kinds of milk after 96 h. The semi-skimmed milk showed an intermediate aspect in all days of cold storage.

The color differences among the blue colorant-loaded milk samples could be related to the difference in the amount of protein content for each reconstituted milk. In the same way that the skimmed milk presented the less amount of fat, it presented more protein content in relation to the semi-skimmed and whole milk. The formation of blue color compounds was directly related to the protein content since the reaction occurs between primary amine groups and genipin (Silva, Saint-Jalmes, de Carvalho, & Gaucheron, 2014).

Fig. 6 presents the results for the color parameters of the blue colorant-loaded milk samples during their cold storage. The milk composition altered the color parameters of the samples. For all parameters, changes between 0 and 24 h were observed. After 48 h, they were more stable. Probably the reaction that promotes changes in all parameters was more intense in the first days, working toward stability after 48 h. The L^* (lightness) parameter for the whole milk was higher in relation to the other kinds of milk in all cold storage days. The presence of fat globules acts scattering the incident light. The fat droplet size and the wavelength of the incident light determine this scattering efficiency (Postelmans, Aernouts, Jordens, Van Gerven, & Saeys, 2020). The 3.0 g/100 g fat content (whole milk) was enough to obtain a greater reflectance across the whole visible range, with a flatter long-wavelength region as indicated by the high L^* values. The visual perception of blueness darker associated with the 0.5 g/100 g fat content (skimmed) treatment may be due to the less content of dispersed fat globules (Joshi, 2000). In skimmed milk, the nature and population of scattering species are different resulting in decreased scattering in the long-wavelength region.

During the storage time, the L^* parameter decreased for all treatments, indicating the increase of the reaction between genipin and milk proteins. A similar trend for the blue color intensification may be associated with the decrease of b^* parameter. Negative values for the b^* parameter represent the blue coloration. Also, the h^* parameter in the range of ~250 also represents the blue color. Therefore, the milk sample presented bluer after the cold storage time, evidencing the increase in the formation of blue color compounds. The main changes in the color parameters associated with the blue color for all kinds of milk were mainly between 0 and 24 h. Thus, after 48 h the color of the system became stable. Regarding the milk fat content, the treatment with 3.0 g/100 g fat content favored the blue color formation because the fat on the system promoted more scattering of light, and thus the system color became more evident. Also, the fat promotes a more selective medium for the extraction of compounds, preventing the extraction of molecules more polar than genipin, such as geniposide, a yellow compound present in the genipap (Nathia-Neves, Nogueira, Vardanega, & Meireles, 2018) that could disturb the b^* parameter.

The negative values for the a^*parameter represent the green color. The cold storage increased the values of this parameter for all kinds of milk. However, the treatment with 0.5 g/100 g fat content favored green color. The increase in green tone interferes in the blue color. The saturation (C^*) increased until 24 h of cold storage and after that kept its values. The increase of fat content from 0.5 g/100 g to 1.5 g/100 g promoted an increase in color saturation of the blue colorant-loaded milk sample.

The free-genipin content in the milk samples was quantified during their cold storage at 5 °C (Fig. 7). These results correspond to non-reacted genipin content, because the reticulated genipin could not be measured by the performed methodology. According to the previous results discussed in the last section, the blue color is intensified on the samples during their cold storage time. The free-genipin content keeps reacting with the primary amine groups and milk proteins. Therefore, in all blue colorant-loaded milk samples occurred a significantly decreased in free-genipin content during their cold storage time. The decrease in free-genipin content was observed with the increasing

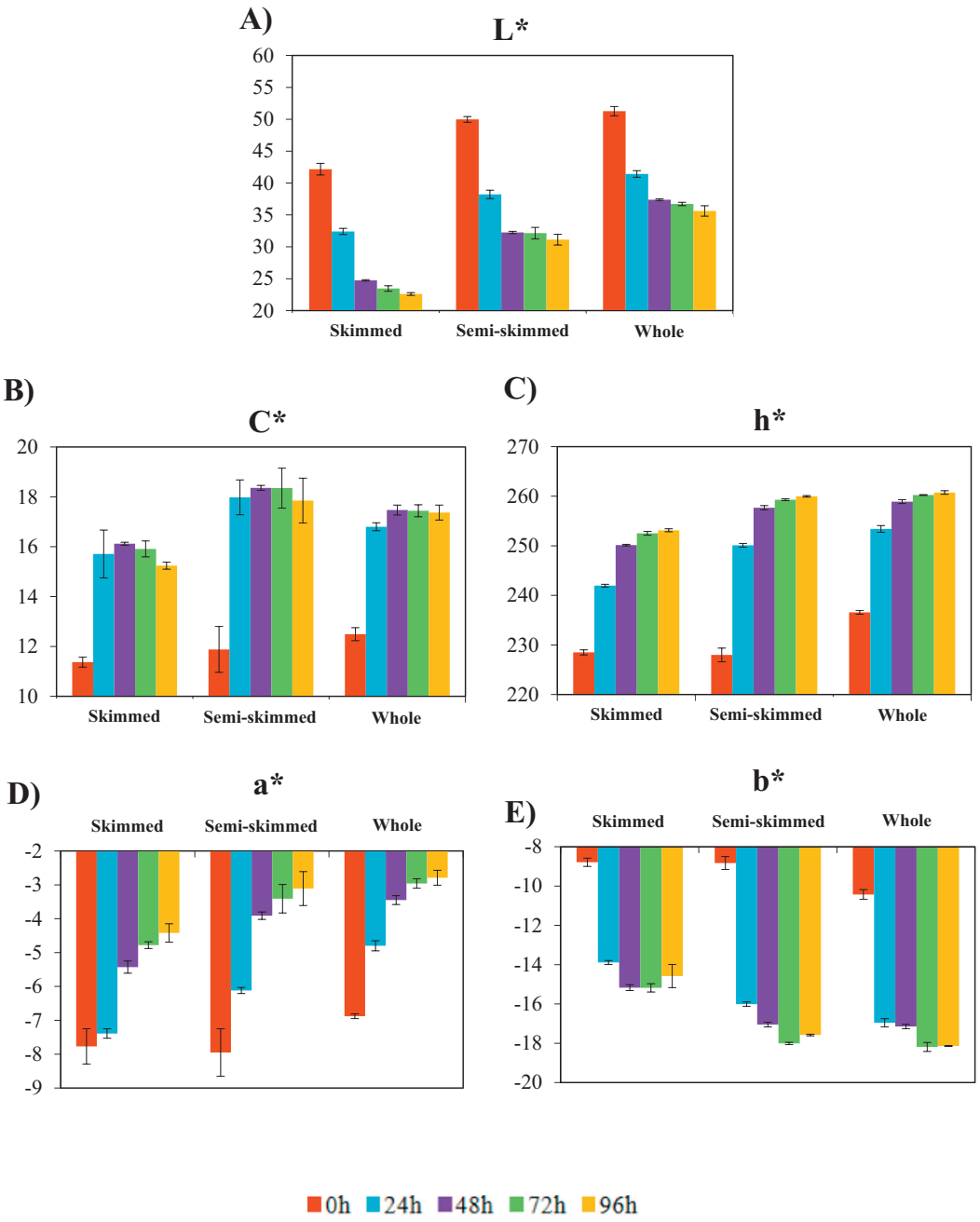

Fig. 6. Color parameters of the blue colorant-loaded milk samples during their cold storage at 5 °C: A) L*; B) C*; C) h*; D) a*; and E) b*. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

intensity of the blue color (Fig. 5). The blue color intensification rate decreased over storage time due to the decrease in free-genipin available to react with milk proteins. The decrease in free-genipin content in the milk samples was higher until 48 h. Similar behavior was observed for the blue color intensification during the storage time (Fig. 5). Gao et al. (2014) added different concentrations of genipin in chitosan to study the cross-linking of chitosan hydrogels with genipin. They observed that higher genipin concentrations promoted a more intense blue color.

The milk composition did not influence the free-genipin content in each cold storage day (p-value > 0.05). However, over the cold storage time, the amount of free-genipin content in whole milk was more stable in relation to the other kinds of milk. The skimmed milk had more protein content to react with genipin during the cold storage time, resulting thus in a more formation of blue color compounds.

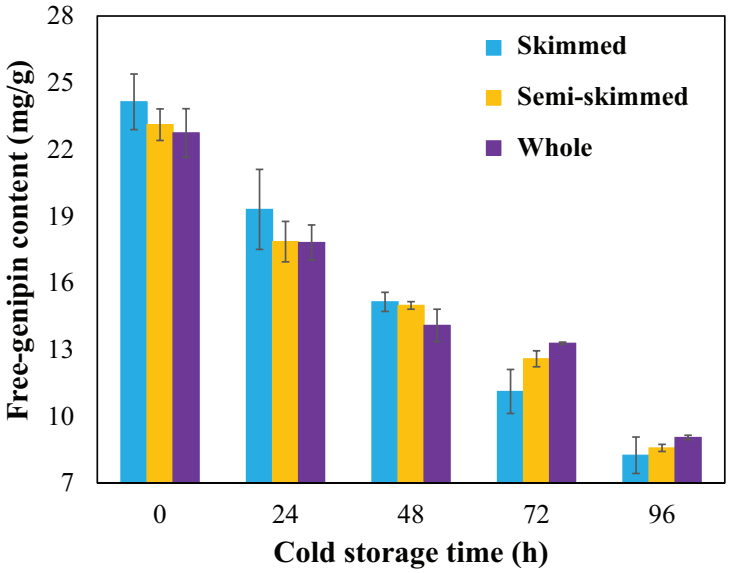

Fig. 7. Free-genipin content in the blue colorant-loaded milk samples during their cold storage at 5 °C.

4. Conclusion

A promising natural blue colorant was obtained from *Genipa americana* L. using the milk colloidal system as reaction medium and carrier for blue color compounds. Our findings demonstrated the high potential of the genipin obtained from genipap and the milk proteins to provide a novel blue colorant for food applications. The blue colorant was chemically stable during the cold storage time (96 h) exhibiting an intense blue color. The milk fat content influenced the genipin recovery while the milk with higher protein content contributed to obtaining a more intense blue color. Using whole milk with 3.0 g/100 g fat content favored the light scattering and, thus, a blue colorant with a more intense $-b^*$ and L^* values was obtained. On the other hand, the use of skimmed milk with 0.5 g/100 g fat content resulted in more consumption of genipin due to its more protein content for the reaction of blue color compounds formation. Thus, a more intense and darker blue coloration was observed with lower light scattering. Therefore, different blue color intensities were obtained just by changing the milk composition. However, the milk composition did not modify the phase separation kinetics of the blue colorant-loaded milk samples. Our results have demonstrated that the milk was a suitable medium for the reaction of blue color formation and also a good blue compounds carrier.

CRediT authorship contribution statement

Maria Isabel Landim Neves: Investigation, Formal analysis, Writing - original draft. **Monique Martins Strieder:** Investigation, Formal analysis. **Eric Keven Silva:** Investigation, Writing - review & editing. **M. Angela A. Meireles:** Resources, Supervision, Project administration.

Declaration of competing interest

The authors confirm that there are no known conflicts of interest associated with this publication.

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CHAPTER 4

Experimental article II

Any heat treated-milk could be used as a blue colorant amine supplier, reaction medium, and carrier?

Manufacturing natural blue colorant from genipin-crosslinked milk proteins: Does the heat treatment applied to raw milk influence the production of blue compounds?

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Manufacturing natural blue colorant from genipin-crosslinked milk proteins: Does the heat treatment applied to raw milk influence the production of blue compounds?

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ABSTRACT

The production of an innovative food ingredient obtained from unripe *Genipa americana* L. pulp and milk was studied in this paper. Natural blue compounds were produced from the crosslinking between genipin and milk proteins. They are a promising ingredient for the natural food colorant sector. In this regard, this paper aimed to evaluate the impact of heat treatments applied to raw milk on the production of blue compounds. Thermal treatments could change the milk protein conformation and the exposition of their amine groups affecting the crosslinking with genipin. For this, raw milk was subjected to different thermal treatments such as low-temperature and long-time (LTLT; 60 °C/30 min), high-temperature and short-time (HTST; 72 °C/15 s), ultra-high temperature (UHT; 145 °C/2 s), and spray drying (SD; inlet and outlet temperature: 165 and 79 °C, respectively). Thermally treated milk samples were used as solvents for the genipin extraction from unripe genipap pulp and suppliers of proteins to produce blue compounds. Blue colorants milk-based were compared concerning their FTIR spectra, free-genipin content, and color attributes. High temperatures impacted protein conformation, reducing the number of primary amines available for crosslinking. Free-genipin content after the reaction step was a good crosslinking indicator. The HTST-treated milk produced the bluest colorant due to its higher number of primary amines available to react with genipin. Our findings demonstrated that the heat treatment applied to raw milk could influence the production of blue compounds. These results are useful for the enhancement of the manufacturing process of natural blue food colorants.

1. Introduction

The replacement of artificial colorants by natural colorants is a big challenge for the worldwide food industry. Anthocyanins, betalains, and carotenoids can be used as natural alternatives for red, orange, and yellow colors, respectively. In contrast, natural blue colorants are scarce (Neves et al., 2021). In addition to the notorious chemical stability issues, none of the existing natural blue pigments reaches the versatility, low cost, and hue of synthetic blue colorants (Buchweitz, 2016; Chatham et al., 2020).

The rubiaceae *Genipa americana* L. is a typical fruit in tropical regions. Unripe genipap fruits present a high content of genipin, a colorless compound belonging to the class of the monoterpenoids iridoids (Náthia-Neves and Meireles, 2018; Strieder et al., 2021). Genipin reacts with primary amine groups from proteins and other biopolymers producing blue compounds (Silva et al., 2014). Among the available natural blue sources, the blue colorant produced from this crosslinking reaction has great potential for industrial application due to its color intensity, chemical stability, technological versatility, and low production cost compared to the other natural blue pigments (Brauch et al., 2016; Echeverry et al., 2011; Náthia-Neves and Meireles, 2018; Neri-Numa et al., 2018; Saint-Jalmes, de Carvalho, and Gaucheron, 2014). Fig. 1 presents the crosslinking reaction between genipin and casein. The primary amines from caseins and whey proteins could be the reagent for forming blue compounds (Neves et al., 2020; Strieder et al., 2020). Our research group has studied this complex system, where the milk acts as a reaction medium to produce the natural blue compounds. The blue colorant could not be formed without a source of primary amines. In this way, milk proteins are a potential source of primary amines. Thus, the protein structural conformation could influence the number of primary amines available for blue compounds formation reaction (Neves et al., 2020; Strieder et al., 2021). Changes in milk protein conformation due to different heat treatments applied to raw milk for its microbiological and enzymatic stabilization could affect the interactions with genipin.

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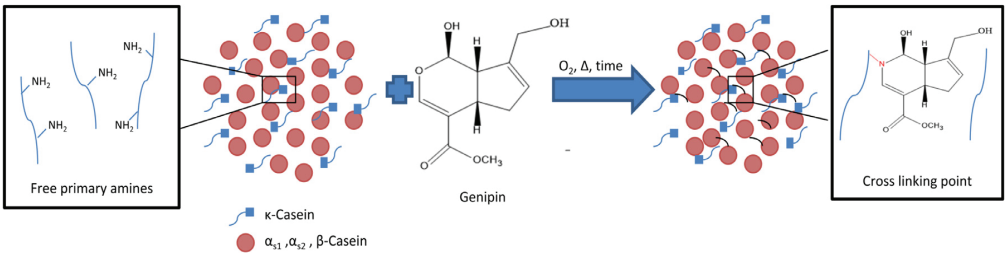

Fig. 1. Schematic illustration of the crosslinking between free primary amines from casein and genipin for obtaining the natural blue compounds.

Microbiological and enzymatic stabilization treatments applied to raw milk, such as pasteurization, are widely used in the dairy industry to ensure food preservation and safety (Chavan et al., 2016). The use of milk as an ingredient for products formulation is impracticable without a previous pasteurization treatment (Raikos, 2010). Consequently, the milk used as a reaction medium for the crosslinking with genipin should be previously stabilized with heat treatments. Pasteurization is a conventional technique in which a mild heat treatment is applied to raw milk to inactivate pathogenic and spoilage microorganisms and endogenous enzymes for extending the product shelf life. Low-temperature and long-time (LTLT) and high-temperature and short-time (HTST) are milder forms of thermal processing in which milk is heated at 60 °C for 30 min and 72 °C for 15 s, respectively. On the other hand, ultra-high temperature (UHT) is a severe thermal treatment that sterilizes milk by heating it at least 135 °C for 2 to 5 s to inactivate bacterial endospores (Chavan et al., 2016). These thermal treatments may result in the denaturation of native milk proteins. HTST and LTLT treatments have shown a minimum impact on major milk proteins such as caseins, β-lactoglobulin, and α-lactalbumin (Patel et al., 2006). While, after UHT treatment, both caseins and whey proteins may be affected, resulting in possible changes in the protein conformation (Chavan et al., 2016). Another standard microbiological stabilization treatment applied in milk is the spray drying (SD) technique. It is used to produce milk powder, thus facilitating the storage and transport of the milk (Nijdam and Langrish, 2006). In the SD treatment may occur the formation of protein complexes between whey protein and caseins (Gaiani et al., 2010; Murrieta-Pazos et al., 2012).

The differences in milk protein conformation after heat treatments or atomization processes may affect the formation of blue compounds. These microbiological and enzymatic stabilization treatments can change the bonding sites of free primary amines to react with genipin. In this context, what would be the impact of the stabilization treatments applied to raw milk on the formation of the natural blue colorant? Thus, the effects of stabilization treatments on the milk proteins and the availability of primary amines for producing natural blue compounds were studied. The natural blue colorant manufactured in this study could be further applied in several food products such as candies, drinks, bakery, confectionery, and dairy products.

2. Material and methods

2.1. Material

Unripe genipap *Genipa americana* L. was obtained in Ponte Alta do Tocantins, Brazil. The raw milk was obtained from the Sítio Santa Rita (Joaquim Egídio, Brazil). UHT milk was purchased at a local market in Campinas, Brazil. The milk manufacturer provided the thermal history of the UHT treatment. The chemicals used were of analytical grade. The HPLC standard of genipin with 99% purity was acquired from Sigma-Aldrich (St. Louis, USA). Ethyl alcohol absolute (99.5% purity), from Anidrol (Diadema, Brazil). Acetonitrile (99% purity), from J. T. Baker (Phillipsburg, United States). Formic acid with 98% purity, from Dinâmica (Diadema, Brazil). Ultrapure water was supplied by a Milli-Q Advantage 8 Purifier System from Millipore (Bedford, United States).

2.2. Skimming milk

Fat content was removed from raw milk to ensure the protein composition was the same for all milk samples. Thus, 250 mL of raw milk and UHT milk sample were centrifuged at 14,000 g for 20 min at 4 °C. The fat-cake layer was removed. The skimmed milk was centrifuged twice more using the same conditions for removing all fat content. The skimmed milk samples were stored at 10 °C immediately after their processing.

2.3. Skimmed milk characterization

The fat content of the skimmed milk was carried out by the Gerber method (British Standards Institution, 1989). Skimmed milk presented a fat content of 0.0 g/100 g ($n = 3$). The protein content was determined by the Dumas combustion method (conversion factor of 6.25) (Dumas VELP Scientifica NDA 701, Usmate Velate, Italy). Rice protein standards from VELP Scientifica (Usmate Velate, Italy) were used in the calibration curve. The protein content was 3.29 ± 0.05 g/100 g for raw milk and reconstituted milk (10 g of powder/100 g of milk) and 3.26 ± 0.02 g/100 g UHT milk ($n = 3$).

2.4. Milk stabilization treatments

The skimmed milk was subjected to LTLT, HTST, and SD treatment. Non-processed milk was named untreated.

2.4.1. LTLT treatment

LTLT-treated milk was obtained by applying 60 °C for 30 min. 250 mL of skimmed milk was heated in a water bath MA126/ BO (Marconi, Piracicaba, Brazil). The temperature of the milk sample was measured using a thermocouple, and the thermal history was recorded.

2.4.2. HTST treatment

Similar to LTLT treatment, HTST-treated milk was processed in a water bath with a temperature-time binomial of 72 °C and 15 s. The thermal milk history was recorded as described for the LTLT treatment.

2.4.3. Spray drying (SD)

Four hundred milliliters of raw skimmed milk were powdered in spray dryer equipment (Labmaq do Brazil, Ribeirão Preto, SP, Brazil). The SD treatment was performed inside a short cylindrical chamber of 0.215 m. The length was 0.5 m by a 0.5 mm nozzle mounted concentrically with the cylinder axis. The inlet temperature, feed rate, and airspeed were 165 °C, 8.1 mL/min, and 3.9 m/s, respectively. The outlet temperature was maintained at 79 ± 4 °C.

2.5. Manufacturing natural blue colorant from unripe genipap pulp and milk

Natural blue colorants were produced from crosslinking between genipin and milk proteins. The untreated, LTLT, HTST, UHT, and SD milk samples were used for manufacturing the natural blue colorant. These samples were used as solvent and reaction medium for the formation of blue compounds. Unripe genipap pulp was ground in a conventional processor model RI1364/07 400 W (Philips, Varginha, Brazil). For each experimental run, one fruit was used to perform all treatments seeking to eradicate the effect of raw material. However, for each process replicate, different genipap pulp was used.

High-intensity ultrasound (HIUS) processing was used as an extraction technique to recover genipin from the unripe genipap pulp. The milk samples were used as the solvent to promote, at the same time, the genipin extraction and crosslinking between genipin and primary amines. HIUS processing was performed using 5 g of milled unripe genipap and 25 g of skimmed milk in a 100 mL Becker. HIUS process conditions were described by Neves et al. (2020). After the extraction, the milk sample was separated from the genipap biomass using a nylon filter. Regarding the SD milk, 2.5 g of milk powder was reconstituted using 22.5 g of ultrapure water. This concentration was established to maintain the protein content similar to other milk samples used in this work.

2.6. Chemical changes in milk proteins

The chemical changes in milk proteins were evaluated using Fourier transform infrared (FTIR) spectroscopy. For this analysis, the blue colorants were previously lyophilized for 48 h in a workbench freeze-dryer, model L 101 (Liobras, São Carlos, Brazil). The measurements were carried out at 25 °C using a Fourier transform infrared Jasco 4100 spectrometer (Jasco, Tokyo, Japan). FTIR spectra were obtained in the range of 400 - 4000 cm^{-1} with a total of 10 scans at a resolution of 4 cm^{-1}.

2.7. Formation of natural blue compounds

After the HIUS processing, the samples were stored at 5 ± 2 °C in a refrigerator (Consul, Joinville, SC, Brazil). The formation of natural blue compounds was monitored until 72 h of cold storage (the time for the complete reaction and color formation). The blue colorants were analyzed regarding their free-genipin content and color parameters.

2.7.1. Free-genipin content

The free-genipin content was measured using high-performance liquid chromatography (HPLC) with the HPLC-PDA on a 2695D separation module (Waters Alliance, Milford, Connecticut, USA) with an integrated column heater, autosampler, and photodiode array (PDA) detector. The analytical validation method was described in detail by Nathia-Neves, Nogueira, Vardanega, and Meireles (2018). The limits of detection and quantification were 0.41 μg/mL and 1.63 μg/mL, respectively. Genipin showed a linear response from 0.41 - 625 μg/mL. The method's precision was evaluated regarding retention time and peak area for the intraday and interday. The relative standard deviation (RSD) was lower than 0.04% for retention time and lower than 0.67% for peak area for the intraday precision. The RSD was lower than 0.03% and 3.05% for retention time and peak area for interday precision, respectively. The accuracy was determined by analyzing the percentage of genipin recovery, ranging between 103.5 and 110.5%. The sample preparation was performed according to Neves et al. (2020). The genipin separation was accomplished on a fused-core Kinetex C_{18} column (150 × 4.6 mm i.d.; 2.6 μm) (Kinetex, Phenomenex, Torrance, USA). The calibration curve of genipin was arranged of 0.1 - 625 $\mu g\ mL^{-1}$ ($R^2 = 0.999$). Free-genipin content was determined concerning the weight of the genipap pulp used to produce the blue colorant sample (Eq. (1)). The dried genipap mass refers to genipap mass on a dry basis.

$$\text{Free} - \text{genipin content (mg/g)} = \frac{\text{Genipin (mg)}}{\text{Dried genipap mass (g)}} \quad (1)$$

2.7.2. Color parameters

Three milliliters of blue colorant sample were poured into rectangular quartz cuvettes with a volume of 3.5 mL. The color parameters were measured at 25 °C in an Ultra Scan Vis 1043 (Hunter Associates Laboratory, Reston, United States) using CIE (Commission Internationale de l'éclairage) coordinates L, a^*, and b^*, with reflectance mode. The differences between the samples regarding the milk treatments were evaluated concerning their color difference (ΔE) using $L\ a^*\ b^*$ coordinates, calculated according to Eq. 2.,

$$\Delta E_{Lab} = \sqrt{(\Delta L)^2 + (\Delta a^*)^2 + (\Delta b^*)^2} \quad (2)$$

The blueness index (BI) was calculated to compare the blue color intensity of the milk samples (Eq. (3)):

$$BI = 100 \times \left(\frac{(C_x \times X) - (C_z \times Z)}{Y} \right) \quad (3)$$

where $C_x = 1.3013$ and $C_y = 1.1498$ are illuminates (constants) for observer D_{65}; 10° X, Y, Z are color coordinates by the XYZ color scale (ASTM, 2014). BI values were validated using the blue color parameter standards (http://colormine.org/) according to Eq. 3.

2.8. Statistical analysis

The impact of the heat treatments and spray drying on the formation of natural blue compounds was evaluated by analysis of variance (ANOVA) at a significance level of 95% using Minitab®18 software. Tukey's test was used to determine statistical differences (p-value ≤ 0.05). All experiments were performed in duplicate.

3. Results and discussion

3.1. Milk stabilization treatments

This section discusses the effects of different stabilization treatments applied to milk samples on their protein conformation, amino acid composition, and the availability of primary amine groups for crosslinking with genipin. Fig. 2 presents the thermal history of the thermal treatments applied to raw milk samples. The time to achieve the preheating and heating step depended on the thermal treatment. Fig. 2a shows that the LTLT pasteurization remained in the heating process until 660 s. The heating step for HTST was until 720 s (Fig. 2b). UHT took less than 25 s to start the thermal treatment (Fig. 2c). After attaining this temperature, the milk samples passed through the holding time section, which in the figure is indicated by "thermal treatment". During this stage, for all treatments, the temperature of the milk sample was relatively constant, which assured the treatment efficiency. Regarding LTLT and HTST, the cooling temperature reaches 10 °C. UHT milk, after the treatment, reaches 25 °C and SD, 41 °C for the outlet temperature. The cooling process took 360 s for LTLT and HTST treatments and 16 s for the UHT.

The thermal treatment severity may promote conformational changes in proteins leading to a more random structure and irreversible changes resulting in protein aggregation and precipitation (Christiansen et al., 2020; Shanmugam et al., 2012). When milk is heated at temperatures above 65 °C, whey proteins expose their previously hidden hydrophobic groups. Subsequent unfolding, whey proteins could interact with themselves and k-casein to form protein aggregates (Raikos, 2010; Sathe and Sze, 1997). These changes at the molecular level may have an impact on -NH_2 availability, affecting thus their interaction with genipin. Also, the thermal treatment may result in changes in the quantification of amino acids, which result in amino-terminal groups available for crosslinking. Therefore, we focused on checking studies that have reported effects on the secondary structure of proteins and quantifying amino acids, which could affect the genipin access in milk protein to react with its primary amine groups.

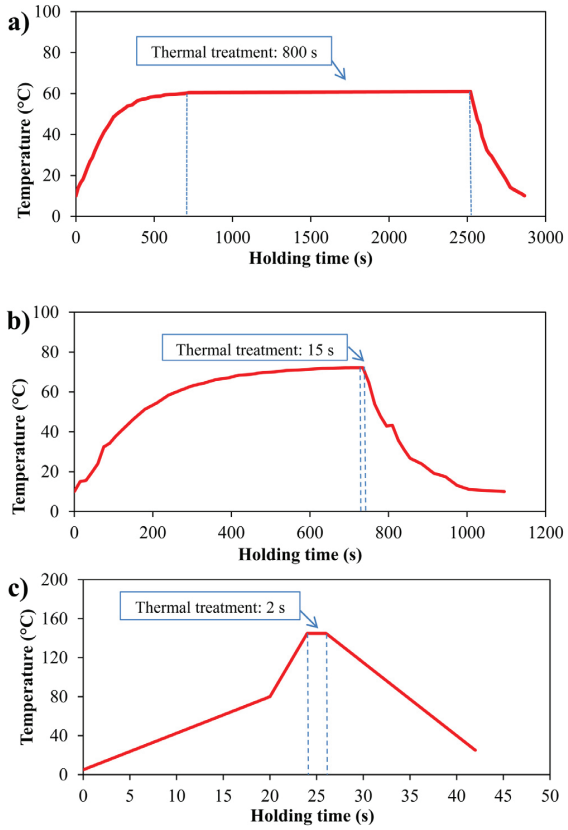

Fig. 2. Thermal histories of the heat treatments applied to the raw milk ($n = 2$): a) LTLT; b) HTST; and c) UHT.

Both binominals, 72 °C/15 s and 60 °C/30 min, have a minimal or no impact on the secondary structure of whey proteins not disturbing the supramolecular structure of the casein micelles (Bogahawaththa et al., 2018; Bogahawaththa et al., 2017; Qi et al., 2015). Bogahawaththa et al. (2018) studied the secondary protein structure of the skimmed milk after the HTST treatment by comparing it with raw milk. They observed a similar pattern of peaks between untreated and HTST-treated samples by FTIR analysis. Both milk samples showed prominent peaks representing α-helix (~1651 cm^{-1}) and intramolecular β-sheets (~1636 and 1624 cm^{-1}), which are common to the secondary structure of native whey proteins. Likewise, Qi et al. (2015) did not report differences between milk proteins of the raw milk and those subjected to HTST treatment. Bogahawaththa et al. (2018) also showed that the amount of native bovine serum albumin in HTST treated milk was about ~95% compared to the untreated milk sample by the enzyme-linked immune sorbent assay (ELISA) quantification technique. Silva-Hernandez et al. (2004) compared LTLT and HTST-treated milk and did not observe severe effects on milk proteins. However, the authors related a slight decrease in arginine and lysine content in LTLT-treated milk. Considine et al. (2007) reported that the β-Lactoglobulin, immunoglobulin G, lactoferrin, and bovine serum albumin are thermal sensitive milk proteins even when thermally treated at lower temperatures (65 °C) have their molecular structure changed.

Thus, the UHT treatment could be considered stabilization treatment more severe (145 °C/2 s) (Fig. 2c). In other words, the thermal treatment that would most damage the milk proteins during the holding time. Douglas Jr, Greenberg, Farrell Jr, and Edmondson (1981) reported that UHT treatment might contribute to 56% of whey protein denaturation. Furthermore, UHT thermal treatment could affect the structure of whey proteins and caused a significant loss of proteins' secondary structure (Qi et al., 2015). This thermal treatment also induced the solubilization of colloidal calcium phosphate and casein aggregation (Bogahawaththa et al., 2017). Accordingly, the more intense the thermal treatment is, the more protein modification may occur. These modifications could be protein aggregation, amino acid reduction, and protein denaturation, which could affect the amines availability for the crosslinking with genipin and, consequently, reducing the formation of blue compounds (Bogahawaththa et al., 2018, 2017; Devnani et al., 2020).

The SD process involves the rapid removal of water by entrainment. It can also be described as a coupled heat and mass transfer. The difference between the vapor pressure of the solvents and their partial pressure in the gas phase is the driving force of the drying process, which why hot air is used. The hotter the air, the drier it is and, therefore, the more efficient it to drag the water contained in the sample. The milk droplets do not reach high temperatures during the atomization process because the simultaneous airflow only transports water by mass transfer. Thus, the contact time between the hot air and the sample is short (20 to 60 s). It is not enough to heat the sample for more than 100 °C. Generally, the temperature of the powder is approximately 10 °C below the outlet air temperature (Schuck et al., 2005). Therefore, milk components are not thermally affected in the drying process. The milk proteins are most affected during the SD due to the whey protein and casein aggregation (Smithers and Augustin, 2012; Devnani et al., 2020).

3.2. Chemical changes in milk proteins

Fig. 3-a exhibits the FTIR spectra for natural blue colorant samples. The spectra for all samples showed one band centered at 3421 cm^{-1} assigned to hydrogen-bonded O–H stretching vibrations and a weak signal at 2927 cm^{-1} due to C–H stretching vibrations, such as CH, CH_2, and CH_3 . The band 1153 cm^{-1} represents the bending vibration of C-O-C. The broadband at 1620 cm^{-1} is mainly attributed to the OH bending mode of adsorbed water and protein amide (C = O) (Liu et al., 2016). Bands at 1249, 1500, and 1313 cm^{-1} are close to the absorptions of vibration of the aromatic ring from genipin (Mu et al., 2013). The peaks at 1550 and 1710 cm^{-1} corresponding to the protonated amine groups on the casein after the reaction, which could be seen in all treatments (Butler et al., 2003). The spectra do not present differences among the thermal milk treatments. Thus, all treatments allowed the reaction between genipin and amines.

The differences in protein structures related to the different stabilization treatments could be seen in the second derivative for the FTIR spectra (Fig. 3-b). Infrared absorption spectroscopy in the 1450 to 1700 cm^{-1} spectral region has been extensively used to study the secondary structure of proteins in the solid-state (Susi and Byler, 1983; Markoska et al., 2019). Mainly in the region between 1600 and 1700 cm^{-1} (amide I) which corresponds to C = O stretching of protein (Kher, Udabge, McKinnon, McNaughton, & Augustin, 2007).

FTIR spectrum may exhibit conformational changes in the secondary structure of the milk proteins, which could be linked to changes during their treatments (Kher et al., 2007). During thermal treatment, the most affected molecular structure was intermolecular β-sheets of β-lactoglobulin due to their involvement in denaturation and aggregation with caseins (Markoska et al., 2019). The changes in the UHT-treated milk spectrum showed that this thermal treatment might most affect the milk protein, causing denaturation, aggregation, and changes in amino acids proportion of the whey protein. Intermolecular β-sheets loadings identified at 1620 and 1683 cm^{-1} decreased in this treatment due to the β-Lactoglobulin denaturation (Markoska et al., 2019; Qi et al., 2015). The 1650 cm^{-1} band represents α-helical segments (Susi and Byler, 1983), presenting a decrease in this region affected by the structural rearrange due to UHT treatment which promoted a de-

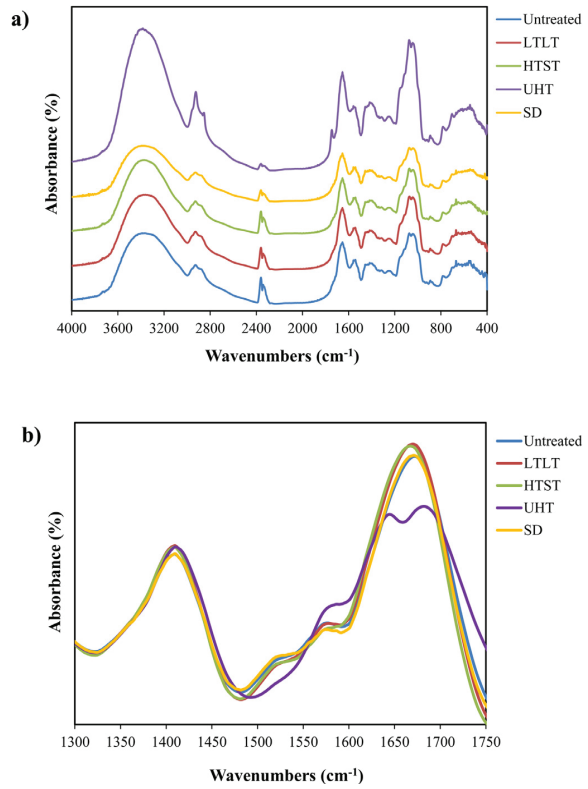

Fig. 3. Impact of the heat treatment applied to the raw milk on the chemical properties of the natural blue colorants: a) FTIR spectra of blue colorants (milk and genipin extracted with milk medium); b) Second derivative of the FTIR spectra.

naturation in these regions. Changes in the 1577 and 1525 cm^{-1} region have been associated with changes in the number of β-turns, observed in UHT-treated milk, also associated with the denaturation of proteins. β-turns after UHT treatment are more exposed and interact with k-casein (Markoska et al., 2019; Curley et al., 1998).

3.3. Natural blue compounds formation

Genipin spontaneously reacts with -NH_2. The most intense blue color can be observed after 72 h (Lin et al., 2020). Oxygen from the environment induces genipin polymerization, promoting the development of blue color compounds (Cui et al., 2017) (Fig. 1). Therefore, the time for this reaction depends on the natural oxygen diffusion into a liquid reaction medium. After the oxygen diffusion occurs a fast reaction of a nucleophilic attack promoted by a primary amine group from a polymeric structure to the C3 of genipin, which results in the opening of the dihydropyran ring and, subsequently, the attack on aldehyde group by the preformed secondary amine. Secondly, at a slower reaction rate, the nucleophilic substitution of the ester group of genipin to form an amide (Lin et al., 2020; Neves et al., 2020; Whitehead et al., 2019). Both reaction steps depend on the temperature, oxygen, reagent concentration, and time (Semenova, 2017). Fig. 4 presents the impact of the stabilization treatment applied to the raw milk on the free-genipin content of the natural blue colorants during their cold storage time. The free-genipin content indicates directly the genipin non-reacted with primary amine groups from proteins, which form the blue colorant. Truly, in this study, the primary amine groups are the limiting reactant whose

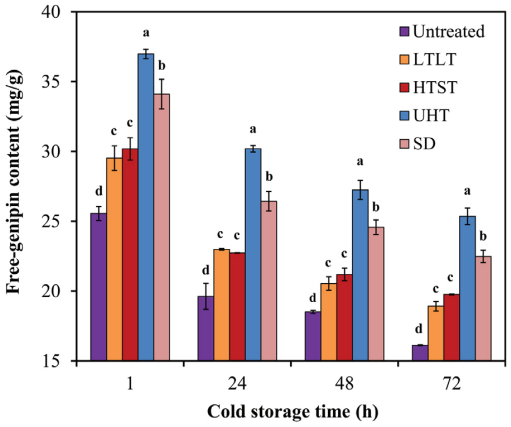

Fig. 4. Effect of the milk heat treatments on the free-genipin content ($n = 2$). Results are presented as mean ± standard deviation. a, b, c, d letters represent significant differences at the 5% significance level for samples evaluated with different milk heat treatments.

availability can be indirectly measured by the free-genipin level and blue color intensity. The UHT samples showed the highest free-genipin value (p-value < 0.001) due to the lower reaction rate with milk proteins. After UHT treatment, the SD sample presented higher free-genipin content. On the other hand, LTLT and HTST milk did not significantly differ in their free-genipin content. Untreated milk presented the lowest free-genipin content due to its most quantity of primary amine groups to react with genipin. Intense stabilization treatments such as UHT and SD may decrease amine availability used for the crosslinking reaction. Considering the three possible effects of these treatments, such as denaturation, protein aggregation, amino acid decrease, the protein denaturation, and aggregation may result in changes in protein exposure sites, decreasing the number of binding sites for genipin (Semenova, 2017). Also, changes in the proportion of amino acids can alter the number that has the largest amount of primary amine groups. In all cases, it results in higher free-genipin in the milk samples. Accordingly, the blue color compounds synthesis could be impaired.

The reduction of the free-genipin content is a reliable indicator for the production of blue compounds due to the crosslinking between genipin and primary amine groups from milk proteins. The blue colorant structure is unknown. Thus, the decrease of free-genipin content and the increase in blue color intensity are indirect measurements of blue colorant formation. Regarding the untreated milk, its free-genipin content after 1 h of contact with the milk was 25 ± 1 mg/g. After 72 h reached 16.00 ± 0.03 mg/g, corresponding to a reaction yield of about 36%. For the LTLT, HTST, UHT, and SD milk, the reaction yield was about 36%, 35%, 27%, 32%, respectively. These results could also demonstrate that the genipin and protein crosslinking increased. Furthermore, concurrently, the blue color compounds synthesis increased (Brauch et al., 2016). The blue color formation took a few days due to slow oxygen environmental diffusion into the cold liquid system.

The effects of the milk stabilization treatments on the production of natural blue compounds in the samples regarding their visual appearance during their cold storage are shown in Fig. 5. The photos were taken with samples of twenty milliliters placed in glass bottles. The blue color intensity increased during their cold storage; meanwhile, the free-genipin content was consumed. One hour after the HIUS processing, the blue color compounds formed from all treatments were already observed. Likewise, Fig. 5 could illustrate the sensorial differences among the treated milk. The naked eye could notice the impacts of the milk stabilization treatments on the formation of blue compounds. Moreover, the UHT treatment may damage the blue color formation, compared to

Table 1
Color parameters: Lightness (L), red-green parameter (a^*), yellow-blue parameter (b^*), hue (H), chroma (C), color difference (ΔE) and Blueness index (BI) of the blue colorant-loaded milk samples over their cold storage at 5 °C ($n = 3$).

Cold storage time (h)	Treatment	L	a^*	b^*	H	C	ΔE	BI
0	Untreated	39.7 ± 0.1^{d}	$(-)5.81 \pm 0.02^{a}$	$(-)7.0 \pm 0.1^{a}$	230 ± 1^{a}	9.1 ± 0.2^{a}	–	$(-)53 \pm 1^{a}$
	LTLT	40.9 ± 0.6^{c}	$(-)5.73 \pm 0.09^{a}$	$(-)6.6 \pm 0.2^{a}$	229.2 ± 0.1^{a}	8.7 ± 0.2^{b}	1.3 ± 0.1^{c}	$(-)50 \pm 1^{b}$
	HTST	40.2 ± 0.2^{d}	$(-)5.99 \pm 0.05^{a}$	$(-)7.19 \pm 0.02^{a}$	230.2 ± 0.1^{a}	9.36 ± 0.01^{a}	0.6 ± 0.3^{d}	$(-)53.6 \pm 0.3^{a}$
	UHT	46.3 ± 0.6^{a}	$(-)6.13 \pm 0.01^{b}$	$(-)3.9 \pm 0.1^{c}$	212 ± 1^{c}	7.2 ± 0.1^{c}	7.2 ± 0.1^{a}	$(-)34.2 \pm 0.1^{d}$
	SD	43.09 ± 0.02^{b}	$(-)6.15 \pm 0.08^{b}$	$(-)5.87 \pm 0.04^{b}$	223 ± 1^{b}	8.5 ± 0.1^{b}	3.6 ± 0.4^{b}	$(-)45 \pm 1^{c}$
24	Untreated	31.9 ± 0.4^{b}	$(-)7.85 \pm 0.02^{b}$	$(-)12.1 \pm 0.1^{a}$	236 ± 1^{a}	14.4 ± 0.1^{b}	–	$(-)100 \pm 2^{a}$
	LTLT	33.2 ± 0.7^{b}	$(-)8.1 \pm 0.1^{b}$	$(-)11.4 \pm 0.1^{b}$	234.6 ± 0.5^{a}	13.9 ± 0.2^{b}	1.4 ± 0.3^{c}	$(-)90 \pm 1^{c}$
	HTST	32.5 ± 0.4^{b}	$(-)8.09 \pm 0.02^{b}$	$(-)11.8 \pm 0.1^{a}$	235.8 ± 0.1^{a}	14.4 ± 0.1^{b}	0.6 ± 0.05^{d}	$(-)97.3 \pm 0.4^{b}$
	UHT	37.9 ± 0.2^{a}	$(-)8.91 \pm 0.07^{a}$	$(-)9.7 \pm 0.2^{c}$	227 ± 1^{b}	13.2 ± 0.2^{c}	6.5 ± 0.1^{a}	$(-)74 \pm 1^{d}$
	SD	33.8 ± 0.3^{b}	$(-)8.85 \pm 0.06^{a}$	$(-)12.5 \pm 0.4^{a}$	234 ± 1^{a}	15.3 ± 0.4^{a}	2.1 ± 0.2^{b}	$(-)99 \pm 1^{a}$
48	Untreated	31.4 ± 0.3^{c}	$(-)8.1 \pm 0.2^{d}$	$(-)16.41 \pm 0.07^{a}$	243 ± 1^{a}	18.3 ± 0.2^{ab}	–	$(-)128 \pm 1^{a}$
	LTLT	31.6 ± 0.6^{c}	$(-)8.8 \pm 0.1^{c}$	$(-)16.33 \pm 0.09^{a}$	241 ± 1^{a}	18.55 ± 0.04^{a}	0.71 ± 0.02^{c}	$(-)128 \pm 2^{a}$
	HTST	31.7 ± 0.9^{c}	$(-)8.8 \pm 0.2^{c}$	$(-)16.2 \pm 0.1^{a}$	241 ± 1^{a}	18.4 ± 0.1^{ab}	0.84 ± 0.04^{c}	$(-)127 \pm 1^{a}$
	UHT	37.7 ± 0.4^{a}	$(-)10.25 \pm 0.06^{a}$	$(-)13.09 \pm 0.05^{c}$	231.0 ± 0.1^{c}	16.6 ± 0.2^{c}	7.4 ± 0.1^{a}	$(-)95.5 \pm 0.1^{c}$
	SD	32.7 ± 0.2^{b}	$(-)9.57 \pm 0.05^{b}$	$(-)15.41 \pm 0.04^{b}$	238.4 ± 0.3^{b}	18.1 ± 0.1^{b}	2.25 ± 0.5^{b}	$(-)120.2 \pm 0.3^{b}$
72	Untreated	25.8 ± 0.5^{b}	$(-)6.7 \pm 0.3^{c}$	$(-)16.9 \pm 0.3^{a}$	247 ± 1^{a}	17.25 ± 0.07^{a}	–	$(-)154 \pm 2^{a}$
	LTLT	25.9 ± 0.5^{b}	$(-)7.2 \pm 0.2^{c}$	$(-)16.95 \pm 0.03^{a}$	245.3 ± 0.5^{a}	17.5 ± 0.4^{a}	0.5 ± 0.2^{c}	$(-)155 \pm 3^{a}$
	HTST	25.5 ± 0.6^{b}	$(-)7.4 \pm 0.1^{c}$	$(-)16.56 \pm 0.06^{a}$	244 ± 1^{a}	17.2 ± 0.8^{a}	0.80 ± 0.02^{c}	$(-)153 \pm 3^{a}$
	UHT	29.4 ± 0.3^{a}	$(-)9.2 \pm 0.4^{a}$	$(-)13.55 \pm 0.08^{c}$	234.2 ± 0.1^{c}	16.4 ± 0.4^{a}	5.5 ± 0.1^{a}	$(-)120 \pm 1^{b}$
	SD	25.8 ± 0.7^{b}	$(-)8.32 \pm 0.03^{b}$	$(-)15.8 \pm 0.1^{b}$	240.6 ± 0.1^{b}	16.9 ± 0.1^{a}	1.9 ± 0.4^{b}	$(-)146 \pm 3^{a}$

Mean values ± standard deviation. Values followed by different letters in the same column show differences by Tukey's test at 95% significance (p-value < 0.05).

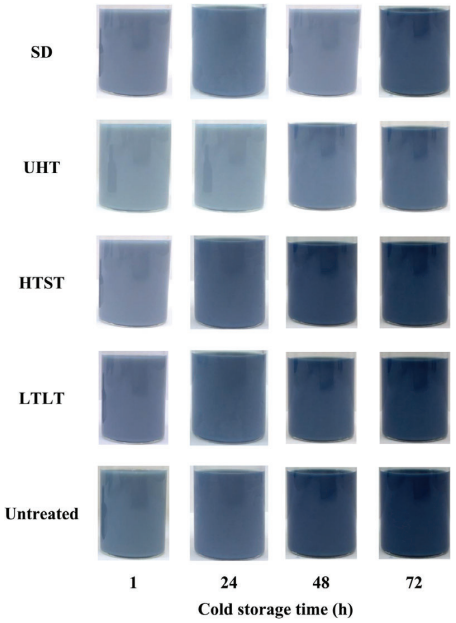

Fig. 5. Visual appearance of the blue color intensity during cold storage at 5 °C.

the others showing the bluest intensity. After UHT treatment, the SD milk showed a less intense blue color. From the first hour until 72 h, the HTST and LTLT-treated milk produced the most intense blue color in the milk system like the untreated milk.

Consonantly with the visual appearance, the color parameters L, a^*, b^*, H, C, ΔE, and *Blueness index* are presented in Table 1. The cylindrical coordinates H (hue) and C (chroma) were calculated using a^* and b^*. These results confirmed the formation of blue compounds and the influence of stabilization treatments on the color of milk samples. Lightness values were decreased in all treatments during the cold storage time (p-value < 0.001). Comparing 1 h and 72 h, the L parameter values in all milk samples decreased by around 60%. UHT milk was the lightest sample due to the high intensity of its thermal effect. Untreated and HTST samples allowed the blue color formation in the most, seeing that its darkest values. HTST thermal treatment less affected the protein conformation; consequently, the amines availability, presenting color results similar to untreated milk (Silva-Hernandez et al., 2004). The C parameter indicates the saturation of a color. It measures the intensity of the color (ASTM, 2014). For the untreated, LTST, HTST, and SD milk samples, the C parameter was the same and increased during cold storage. However, UHT sample presented the less saturation value (p-value < 0.001). The H parameter indicates hue value, varying between 0 until 360°, pointing to a color tone on the color wheel. The hue value from 230 to 250 indicates a blue color (ASTM, 2014). All milk samples were in a blue zone. A minimum H parameter was placed in the UHT sample (236.0 ± 0.4) and a maximum in untreated milk (247 ± 1).

The ΔE indicates the variation of the color parameters disturbed by stabilization treatment applied to milk samples. The UHT milk showed the high ΔE value (7.3 ± 0.1, after 1 h) (p-value < 0.001), due to its high thermal intensity, which could affect the availability of primary amine groups for production of blue compounds. Both types of milk LTLT and HTST presented a similar behavior to the untreated milk sample due to their low value of ΔE (1.3 ± 0.1 and 0.6 ± 0.3 after 1 h, respectively). A new parameter was proposed in this study: the blueness index (BI), which indicates the blue color intensity (Table 1). This index was elaborate based on the yellowness index, already reported in many other studies (Adams et al., 1999; Krokida et al., 1998; Neves et al., 2020; Zhao et al., 2019). This parameter enhanced the discussion of the blue color formation once that the most important parameter for this colorant ($-b^*$) could be elucidated. BI values increased by around 35% during the cold storage time for all milk samples. The bluest milk samples were untreated, LTLT, and HTST-treated, whose BI reached the values -154 ± 2, -155 ± 3, and -153 ± 3, respectively, after 72 h of cold storage. SD milk presented -146 ± 3, and UHT milk exhibited a lower BI value (-120 ± 1).

Therefore, in brief, the stabilization treatments applied to the milk samples impaired the blue color initial synthesis. However, it did not influence the increase in color intensity after 1 h until 72 h because the BI increase was the same for all milk samples (35%). The thermal treatment influenced the blue color formation because it changed the milk proteins and, consequently, the primary amines availability. Indeed, denaturation and/or aggregation of some proteins and changes in

amino acid proportion in UHT-treated milk modified the availability of primary amine groups to react with genipin to form blue compounds. Douglas Jr et al. (1981) reported that the UHT treatment decreased 10% in available lysine compared to untreated samples. It also exhibited lower values for alanine and cystine. These amino acids have free primary amine groups that could be available for the genipin reaction. Lin et al. (2020) studied the reaction between genipin and sugar beet pectin by analyzing the consumption of amino acids. They verified that the amino acid most consumed during the reaction was lysine. Thus, the thermal treatment that affects this amino acid could impair the reaction by promoting a loss in the reagent. Silva et al. (2014) also found higher lysine consumption in the reaction between genipin and casein. They also verified the importance of the accessibility of -NH_2 in the protein chain to enable the reaction. A decrease in these amino acids could negatively affect the production of blue color compounds. As stated, both in more free-genipin content and in less blue color intensity observed for the UHT treatment. Likewise, the SD sample resulted in intermediate blue color intensity because SD involves whey proteins and casein aggregation and probably decreasing the availability of primary amine groups for genipin interaction (Gaiani et al., 2010). Regarding HTST and LTLT treatment compared to the untreated milk, both thermal-treated milk samples showed a lower BI value due to the effect of the thermal pasteurization on the decrease of arginine and lysine content, decreasing the amino acid content slightly for providing primary amines available for the crosslinking.

4. Conclusion

A new natural blue colorant was manufactured in this work using unripe genipap pulp and milk. Our findings demonstrated that the microbiological and enzymatic stabilization treatment applied to the raw milk can influence the production of natural blue compounds. Thereby, the different milk stabilization treatments can impact the blue color intensity of the novel natural colorant obtained. The different stabilization treatments applied to raw milk like LTLT, HTST, UHT, and SD influenced the milk performance as a reaction medium for producing of blue compounds. Since this reaction is based on crosslinking between milk primary amine groups and genipin extracted from the unripe *Genipa americana* L. pulp. The formation of natural blue compounds depended on the severity of the milk stabilization treatment due to changes in protein structure, reducing the number of primary amine groups available to synthesize the blue compounds. Changes in protein structure were observed for the UHT milk sample, providing a less intensity blue color. In contrast, the mild thermal treatments LTLT and HTST resulted in blue colorant-loaded milk samples with the most intense blue color. Our results have contributed to elucidate technological issues associated with producing a natural blue colorant in liquid form. Its application in food products such as bakery products, dairy beverages, ice creams, candies, and many others, are promising due to its dairy composition.

Declaration of Competing Interest

The authors confirm that there are no known conflicts of interest associated with this publication.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.fufo.2021.100059.

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CHAPTER 5

Experimental article III

Why Genipa americana L. extract and milk are an ideal medium to produce the blue colorant?

Study of the reaction between genipin and amino acids, dairyproteins, and milk to form a blue colorant ingredient

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ABSTRACT

Currently, one of the biggest challenges of the colorant industry is to obtain natural blue colorants. Among the different options, a blue pigment can be formed by a crosslinking reaction between genipin and primary amine groups. However, at the industrial level, obtaining an ingredient from pure compounds, such as amino acids, is economically unfeasible. The present work aimed to study the reaction and kinetics of the blue color formation, starting the study with pure compounds (genipin and amino acids) to more complex and cheaper natural sources, such as *Genipa americana* L. fruits and milk. The reaction kinetics of the monomers/dimers for different amino acids reacting with genipin was evaluated, as well as the preferential amino acid, genipin:amino acid ratio and pH, to obtain the most rapid and intense blue color. Finally, the blue pigment formed using milk and its proteins was characterized by SDS-PAGE. The results suggest that the reaction kinetic is influenced by the type and concentration of the amino acid used and the pH of the medium, which could facilitate the further standardization of the industrial process. We also suggested milk as an excellent reaction medium to obtain the colorant from genipin as it presents an ideal pH and favorable amino acid composition to facilitate the reaction.

1. Introduction

Genipin is an iridoid obtained from unripe *Genipa americana* L. and *Gardenia jasminoides* J. Ellis fruits (both plants belong to the *Rubiaceae* family) (Tsutsumiuchi et al., 2021). This iridoid can react with primary amine groups from amino acids, peptides, or proteins, by crosslinking to form a blue colorant (Di Tommaso, David, Gomar, Leroy, & Adamo, 2014; Neves, Strieder, Silva, & Meireles, 2020). The colorant is a polymeric compound that is difficult to isolate and analyze separately. Consequently, several works have been focused on understanding the genipin and primary amine groups interaction. However, the molecular structure and formation mechanism have not been completely elucidated (Dimida et al., 2015; Tsutsumiuchi et al., 2021). Therefore, some authors worked with the simplest compounds (genipin and pure primary amine groups) to produce the colorant, obtaining more information about the reaction: dependence on the presence of oxygen, the strong influence of temperature and H^+, as well as the genipin:amino source ratio (Di Tommaso et al., 2014; Dimida et al., 2015; Fujikawa et al., 1987; Touyama et al., 1994).

The first study started when Fujikawa and co-authors (1987) elucidated the reaction between genipin and glycine at pH 7 and 80 °C during 4 h. The principal product discovered presented a molecular weight of 504 *m/z*, named genipocyanin G1. Later on, Touyama et al. (1994) proposed that the reaction consisted onthe attack by a nucleophilic amine group in the carbon atom in position 3, followed by a genipin dehydration to form a monomer and, finally, a dimerization reaction. Moreover, in a theoretical study, Di Tomasso et al. (2014) discovered many steps during the process until the blue color formation occurs.

Usually, two types of articles are found in the literature: those focused on understanding the colorant reaction mechanisms, and those focused on obtaining the blue colorant (Brauch, 2016; Náthia-Neves, Vardanega, & Meireles, 2019). Both studies are important because this promising natural colorant has great relevance due to its high coloring capacity and heat, pH and light stability (Bryś, Urbańska, & Olas, 2022; Neves, Strieder, Prata, Silva, & Meireles, 2021). According to Neves et al. (2021), after the blue colorant powder production with milk and *Genipa americana* L. fruits, the water-diluted sample presented stable even conditioned at a 2–8 pH range and at 100 °C during 30 min. Along

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with these scientific works, some patents based on blue colorant obtainment have been reported, such as a colorant derived from the raw juice obtained from *Genipa americana* L. and glycine (Echeverry, Zapata, & Torres, 2011), lysine (Song, Lan, Lv, Liu, & Cui, 2021), or *Genipa americana* L, watermelon, and some amino acids (Cano, Lopez, Romero, Garces, & Porras, 2014).

Despite the high number of studies focused on the blue color formation considering the addition of pure compounds, the colorant obtained from genipin and pure amino acids is not economically feasible seeking an industrial food application. Thus, a more available and less expensive supplier of primary amine groups and genipin would be more attractive to produce a food colorant. In this sense, Neves and co-authors (2020) reported the production of the colorant from *Genipa americana* L. fruit and milk. The last one was used as a primary amines supplier and a solvent to recover the genipin from the *Genipa americana* L. fruit. However, it is still unclear the reaction mechanism between milk proteins and genipin, making it difficult for industrial purposes. Hence, a complete understanding is needed, firstly, from a molecular standpoint (mechanism, kinetics, and preferential conditions of the reaction); and secondly, in a particle scale (influence of complex matrices on the production, possible interactions, and colorant characteristics). Understanding the reaction mechanism may also facilitate for further utilization of other matrices as a primary amine source. Additionality, if the chemical structures responsible for the color are not discovered, it is impossible to comprehend their toxic effects on human health and, consequently, to be approved concerning the regulatory aspects. In 2017, the pigment formed by using genipin and glycine was submitted to the 84th meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA) to revise its approbation and be applied in dairy products, ices, confectionery and beverages. However, because of the limited biochemical and toxicological databases and the low margin of exposure, the JECFA was unable to conclude the evaluation (JECFA, 2019). Moreover, the polymer formed depends on the protein used in the reaction (Cano et al., 2014), and therefore, it is necessary to check for possible toxic effects for each amine group supplier used.

Thus, the present work aimed to study the formation of the blue colorant based on the reaction between genipin and different primary amine groups suppliers (amino acids and proteins). For this, the effects of different amino acids, pH, and concentration of pure amino acids have been studied, as well as their combination with proteins present in *Genipa americana* L. fruits. Finally, the pigment formation after extraction of genipin from *Genipa americana* L. fruits using milk and its main proteins, as a solvent and reaction medium/amine supplier, has been evaluated.

2. Material and methods

2.1. Material and reagents

Glycine, lysine, aspartic acid, glutamic acid, and arginine, all with > 98% purity, were supplied by Sigma-Aldrich (Saint Luis, MO, USA). The HPLC standard of genipin was supplied by Acros Organics (Geel, Belgium) (purity > 98%). All the solvents used were of analytical grade. Ultrapure water was supplied by a Milli-Q Milipore® station (Billerica, MA, USA). Acetonitrile (ACN) (purity > 98%) and formic acid (purity 99–100%) were purchased from VWR Chemicals (Barcelona, Spain).

Unripe *Genipa americana* L. was obtained in Ponte Alta do Tocantins, Brazil. The fruits were partially lyophilized in a SuperModulyo Lyophilizator (Edwards, Crawley, England) and stored at −18 °C.

A local market provided spray-dried milk powder. It was reconstituted in 10 g of powder/100 g of milk. The casein and whey fraction were separated from the reconstituted milk by adding 2 mL of glacial acetic acid in 200 mL of milk (until obtaining a pH of 4.6) and centrifuging at 4000*g* for 20 min at 5 °C. The whey supernatant was collected, and the pH was adjusted to 6.5 by adding NaOH 1 M. Eighty grams of caseins were washed three times with acidulated H_2O (pH 4.6). After that, 240 mL of MilliQ water were added, and the pH was adjusted to 6.5 using NaOH 1 M. The system was sonicated in a water bath for 20 min for total casein solubilization.

2.2. Blue colorant production

2.2.1. Genipin and pure amino acids

Five amino acids were tested to react with genipin: lysine, glycine, aspartic acid, glutamic acid, and arginine. The reaction mediums were prepared by dissolving the different amino acids in MilliQ water (which conferred a pH corresponding to the amino acid pH) or by using phosphate buffer solution (PBS) to set the pH to 6.7. For that, 100 mL of a solution containing 50 mg of genipin and 50 mg of the amino acid were prepared and vortexed. Then, to verify the minimum amino acid concentration to produce the blue colorant, the same reaction medium was prepared by using the following amino acid concentration: 2, 1, 0.5, 0.1, 0.01 mg/mL in PSB (pH 6.7) with 0.5 mg/mL of genipin.

2.2.2. Genipa americana L. extract and pure amino acids

The extraction solvent and reaction medium to produce the blue colorant using *Genipa americana* L. fruits and pure amino acids were prepared by adding 100 mg of the amino acids (glycine, lysine, aspartic acid, glutamic acid, and arginine) in 28 g of water or PBS (pH 6.7). Genipin was recovered from 2 g of partially lyophilized unripe *Genipa americana* L. fruits using the different solvents. The extraction was performed according to Neves et al. (2020). A Branson 450 Digital Sonifier (Branson, Danbury, United States) was used to assist the genipin extraction process. After previous assays, the process was performed in 2 min, using a 12.6-mm probe diameter at 19 kHz, with 80% of amplitude (Nominal power: 360 W).

After, the same extraction procedure was performed using 100 mg of glycine and lysine in 27.9 g of PBS at different pH: 3, 4, 5, 6, 7, 8, and 9. Also, glycine and lysine at different concentrations (0.08, 0.17, 0.33, 0.67, 0.83, 1.33 and 3.33 mg/mL) in PSB (pH 6.7) were assayed.

2.2.3. Genipa americana L. extract and milk proteins

Milk, casein, and whey (solubilized in water) were used as extraction solvents to produce the blue colorant. Genipin was extracted from 2 g of partially lyophilized unripe *Genipa americana* L. with 28 g of milk, casein, or whey by ultrasound-assisted extraction as described in section 2.2.2. For each different solvent, the extract was produced until obtaining 150 g of liquid blue colorant. Subsequently, the blue colorant was atomized to obtain a powder: blue colorant in milk, blue colorant in casein, and blue colorant in whey.

A mini spray-dryer Buchi 190 (Buchi, Flawil, Switzerland) with a 1 mm nozzle mounted concentrically with the cylinder axis was used to perform the atomization process. The following conditions were used: 170 ± 5 °C as inlet temperature, outlet temperature in 100 ± 5 °C feed rate in 9 mL/min, and the atomizing airspeed in 3.9 m/s. The powders were cold-stored at 5 ± 2 °C.

2.3. Determination of visual aspects and color parameters

For the blue colorants produced using pure amino acids, 500 μL of the solution were added to a 24-well microplate conditioned in a water bath at 60 °C, and photos were captured every 5 min of the reaction to obtain the visual aspects.

The color parameters (CIE *Lab*) *L*, *a*, *b* and coordinates *X*, *Y*, *Z* of the blue colorants produced with the *Genipa americana* L. extract and pure amino acids after 24 h of their production were determined using the colorimeter Specord 210 (Analytik, Jena, Germany). At the same time, photos were obtained for all samples to visually follow the evolution of the reaction.

The whiteness index (*WI*) was calculated according to Eq. (1):

$$WI = L - 3b \quad (1)$$

where L and b are color coordinates by the CIE *Lab* scale.

The blueness index (*BI*) was calculated according to Eq. (2) (Neves, Strieder, Silva, & Meireles, 2021).

$$BI = 100 \times \left(\frac{(C_x \times X) - (C_z \times Z)}{Y} \right) \quad (2)$$

where $C_x = 1.3013$ and $C_y = 1.1498$ are illuminates (constants) for observer D_{65}; 10° X, Y, Z are color coordinates by the *XYZ* color scale (ASTM, 2014).

2.4. Determination of kinetics aspects

2.4.1. Spectrophotometer UV–VIS

The effect of the different amino acids and pHs of the reaction medium on the reaction rate with pure genipin was tested by adding 50 μL (0.5 μg/mL) of glycine, lysine, aspartic acid, glutamic acid, or arginine to 50 μL of genipin (0.5 μg/mL) and 100 μL of PBS (pH 6.7), or 100 μL of MilliQ water in a 96-well microplate. The absorbance was recorded using a multi-plate reader at λ = 240, 370, and 590 nm every 2 min for 60 min at 60 °C. The reaction rate (V_{mean}) was calculated, considering the points until 1 unit of absorbance intensity was obtained, using the Gen5™ version 2.0 Data Analysis software from BioTek Instruments (Winooski, VT, USA).

2.4.2. High-performance liquid chromatography-diode array detection-mass spectrometry (HPLC-DAD-ESI-MS)

Electrospray ionization (ESI) conditions to obtain the molecular weight of the blue colorant were optimized by direct infusion in the MS instrument in negative ionization mode. Each pure compound was dissolved to 0.5 mg/mL in MilliQ water with 0.1% formic acid (v/v) and analyzed. The ESI mass spectra were acquired using a Bruker ESQUIRE-2000 ion trap mass spectrometer (Bruker, Bremen, Germany) equipped with a gas nebulizer probe capable of analyzing ions up to *m/z* 650. The optimized ESI conditions were: flow rate of 300 μL/h; nebulizer gas flow pressure of 20 psi; turbo ions spray voltage of 2000 V; source temperature of 250 °C.

Then, 1.5 mg of genipin and 1.5 mg of the amino acid were solubilized in 3 mL of MilliQ water or PBS (pH 6.7) using a vortex for the blue colorant reaction kinetics. The samples were conditioned in a water bath at 60 °C. An aliquot of 60 μL was separated immediately after the solubilization (time zero) and every 15 min of the reaction (until 75 min), filtered and analyzed by HPLC-DAD-ESI-MS in negative ionization mode. The column used was a C18, 100 mm × 2.1 mm, 1.9 μm (Thermo Fisher Scientific, Vilnius, Lithuania). It was maintained at 35 °C during the analysis. Water (solvent A) and ACN (solvent B), both with 0.1% formic acid (v/v), were used as mobile phases. The flow rate was maintained at 0.25 mL/min with a sample injection of 10 μL. The solvent A gradient applied was initial 99%, followed by 75%, 7–8 min; 99%, 8–14 min. The area for each *m/z* was normalized to the total summed-area. A 206.7 *m/z* fragment from genipin was considered for genipin quantification.

2.5. Determination of complex size

2.5.1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed on the colorant samples produced using *Genipa ameriana* L. and milk proteins and on their milk basis, according to Miralles, Del Barrio, Cueva, Recio, and Amigo (2018). Powder samples were diluted in a buffer containing 1.6% (w/v) of SDS (Merck, Darmstadt, Germany), 0.05 M of Tris-HCl, pH 6.8 (Sigma-Aldrich), 8% (v/v) of glycerol (Panreac Química SAU, Castellar del Vallés, Spain) and 2% (v/v) of β-mercaptoethanol (Sigma-Aldrich, Burlington, MA, USA). The sample dilution was made according to its protein content for maintaining the same protein concentration in all samples (milk 1:30; caseins 1:30, whey 1:6). Samples were heated at 95 °C for 5 min, then loaded on 12% Bis-Trispolyacrilamide gels Criterion_XT (Bio-Rad, Hercules, CA, USA) and placed into a Criterion cell (Bio-Rad) with XT MES running buffer (Bio-Rad, CA, USA). Electrophoretic separation was run at 100 V for 5 min and 150 V for 45 min. Precision Plus Protein Standards (Bio-Rad, CA, USA) were used as standard molecular weight markers. Finally, the gel was stained with Coomassie Blue (Expedeon, Swavesey, United Kingdom).

2.5.2. Matrix-assisted laser desorption/ionization–time of flight mass spectrometry (MALDI-TOF MS)

MALDI-TOF MS spectra were acquired using an Autoflex SpeedTM Instrument (Bruker Daltonic, Bremen, Germany). The blue colorants produced using *Genipa americana* L. and milk proteins and their respective milk basis were analyzed. A transparent phase of a saturated solution of sinapic acid (Bruker Daltonic) in a mix of ACN and TFA in a volume ratio of 1:2 was used as a matrix and was produced according to Luque-Garcia, Zhou, Spellman, Sun, and Neubert (2008). Then, 1 μL was placed onto the Anchor ChipTM MALDI target (Bruker Daltonic); after, the samples were diluted 1:1000 (w/v) in Milli Q water (1 μL). Finally, the matrix and sample were air-dried at room temperature. For calibration, 1 μL of matrix and 1 μL of BTS were loaded on the target. The spectra were recorded in the positive linear mode according to Luque-Garcia et al. (2008). Five spectra (1000 shots at random positions on the same target place) were collected using the Protein Calibration Standard II (Bruker Daltonics).

2.6. Molecular structure and statistical analysis

The chemical structures were drawn using MarvinSketch software (ChemAxon, Budapest, Hungary). Statistical analyses were carried out using Minitab 18® software. The results are expressed as mean values ± standard deviation (SD) (three replicates). ANOVA using Tukey's test examined the differences between the mean values. The difference was considered statistically significant when the p-value was below 0.05.

3. Results

3.1. Reaction between genipin and pure amino acids

3.1.1. Visual aspects

The effect of the type of amino acid on the blue color intensity in the water medium is shown in Fig. 1**a**. The pH of the reaction medium was dependent on the correspondent amino acid, namely: 6 (glycine), 3 (aspartic and glutamic acid), 9 (lysine), and 10 (arginine). Fig. 1**b** shows the effect of the type of amino acid on the blue color intensity in PBS (pH 6.7). In general, for both reaction mediums (water and PBS), the colorant produced by lysine and arginine presented the most intense coloration, followed by glycine, and lastly, aspartic and glutamic acids. Glycine, at pH 6.7, presented a more intense coloration than at pH 6. Differently, for basic amino acids (lysine and arginine) in PBS (pH 6.7), the color was less intense than in water solution (pH 9 and 10, respectively). When acidic amino acids in water solution were used, the solution did not become blue. However, in PBS (pH 6.7), a low intense blue colorant was formed. Thus, the basic or controlled pH (6.7) of the medium favored the color formation.

The amino acid concentration also affected the blue color intensity obtained by using PBS solution (pH 6.7) (Fig. 2). With glycine, the colorant was formed with concentrations as low as 0.01 mg/mL, while the intermediate concentration (1 mg/mL) was the most favorable. However, a higher concentration (2 mg/mL) impaired the reaction. The colorant could not be formed by adding 0.01 mg/mL of lysine or arginine. For these two amino acids, the best concentration was 0.5 mg/mL, and above this concentration, the reaction was negatively affected. The same happened for aspartic and glutamic acids, where the best condition was an intermediate concentration (0.5 mg/mL). Hence, the best

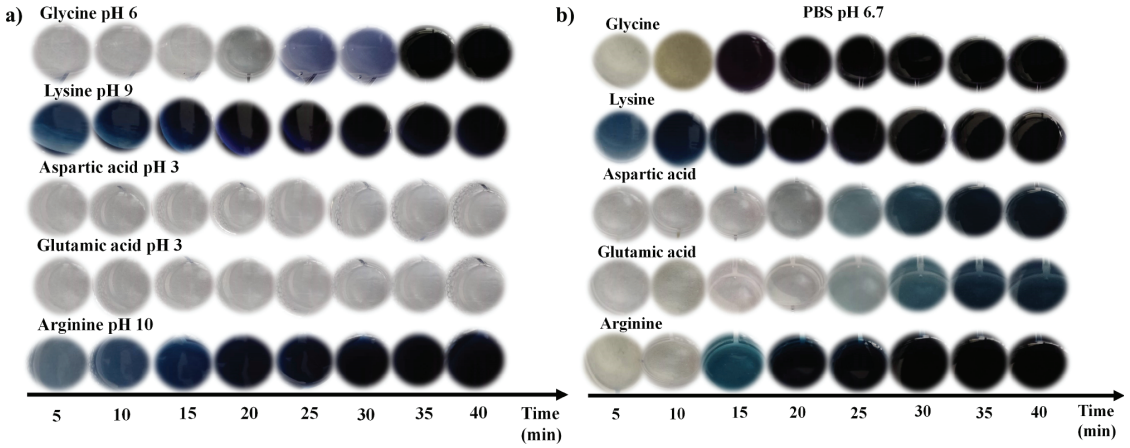

Fig. 1. Visual aspects of the blue colorant generation during 40 min at 50 °C, by using genipin and different amino acids (glycine, lysine, aspartic acid, glutamic acid, and arginine) in water (**a**) and in PBS (pH 6.7) (**b**). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

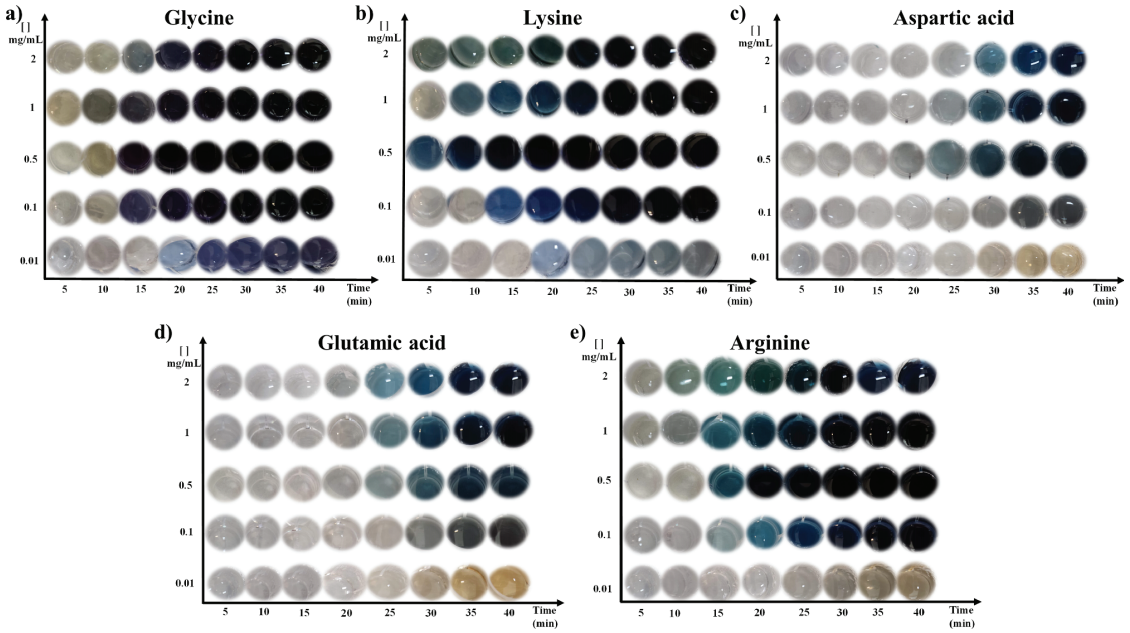

Fig. 2. Visual aspects of the blue colorant generation during 40 min at 50 °C in PBS (pH 6.7), by using genipin and glycine (**a**), lysine (**b**), aspartic acid (**c**), glutamic acid (**d**) and arginine (**e**) at different concentrations (0.01, 0.1. 0.5, 1, and 2 mg/mL). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

conditions to produce the colorant were obtained considering 0.5 mg/mL of genipin for all treatments, 1 mg/mL of glycine, and 0.5 mg/mL of arginine, lysine, aspartic and glutamic acids.

3.1.2. Kinetic aspects

Besides the color intensity, the type of amino acid and the pH of the medium also affect the reaction kinetics, as shown in Table 1. Regarding the UV–Vis Spectra analysis of the colorant, three maximum absorbance values were observed at 240, 370, and 590 nm. Genipin and genipin-amino acid monomer possessed the major absorption at 240 nm, as confirmed by HPLC-DAD-ESI-MS (*vide infra*). However, since the two molecules (genipin and genipin monomer) presented the same maximum absorbance, the kinetic calculations could not be performed at 240 nm. Moreover, at the beginning of the reaction, a maximum absorption peak was observed at 370 nm, which corresponds to a visually observed yellow color. As the reaction occurred, this peak decreased, and the maximum absorbance changed to 590 nm (corresponding to a visually observed blue color), continuously increased throughout the reaction. As illustrated in Table 1, the blue color reaction rate was faster than the yellow one. Visually, we could observe the yellow formation by using glycine in PBS (Fig. 1**b**).

The reaction rate with basic amino acids was faster in water medium than in PBS (pH 6.7). In contrast, acidic amino acids and glycine reaction rates were strongly increased at pH 6.7 for both yellow and blue color

Table 1
Effect of type of amino acid on blue colorant reaction rate (intermediate colorant formation, 370 nm, and blue colorant formation, 590 nm) by using PBS (pH 6.7) and MilliQ water. Letters indicate significant differences between the samples at the same wavelength after ANOVA with Tukey's Post-hoc, p-value < 0.05.

Reaction rate (absorbance/min)				
	370 nm		590 nm	
	PSB (pH 6.7)	Water	PSB (pH 6.7)	Water
Glycine	13.4 ± 0.9^{a}	0.3 ± 0.1^{c}	21.0 ± 1.0^{b}	0.5 ± 0.3^{c}
Lysine	14.6 ± 0.9^{a}	6.5 ± 0.2^{a}	39.0 ± 4.0^{a}	57.0 ± 2.0^{a}
Aspartic	2.5 ± 0.3^{c}	0.04 ± 0.01^{c}	1.0 ± 0.5^{c}	0.01 ± 0.01^{c}
Glutamic	2.9 ± 0.1^{c}	0.06 ± 0.01^{c}	1.9 ± 0.1^{c}	0.02 ± 0.01^{c}
Arginine	5.8 ± 0.2^{b}	3.1 ± 0.1^{b}	17.0 ± 1.0^{b}	23.0 ± 1.0^{b}

formation. Lysine was the amino acid in which the colorant was produced faster (<5 min), both in water and in PBS (p-value < 0.05), followed by arginine, in which the mixture became blue after 15 min.

Fig. 3 shows the reaction kinetic using glycine (Fig. 3**a-b**), lysine (Fig. 3**c-d**), and aspartic acid (Fig. 3**e-f**) determined by HPLC-DAD-ESI-MS. This experiment monitored the mass/charge ratio (*m/z*) of the monomers and dimers formed with genipin and the respective amino acid. Fig. 4 shows the molecules drawn (monomers and dimers). For the three amino acids studied, the monomer and dimer molecules formed were derived from the molecule of genipin by elimination of H_2O and replacement of an oxygen atom by an N-Me group from the amino acid. The dimer was formed from the combination of two monomers bonded from the carbon attached to the terminal –OH present in the monomer molecule, and the carbon 5 of the second monomer, by elimination of H_2O.

Fig. 3**a and b** illustrate the glycine/genipin kinetics in water medium and PBS (pH 6.7), respectively. For both mediums, a decrease in genipin and a monomer increase could be observed, mainly between 0 and 15 min of reaction; and the medium at pH 6.7 favored both genipin consumption and monomer production. The dimer initially presented a low intensity, and its production increased after 30 min of the reaction. The molecular weight calculated for the monomer and the dimer were 264.08 *m/z* and 506.13 *m/z*, respectively. The observed molecular weights in HPLC-DAD-ESI-MS were in good agreement, namely: 263.9 *m/z* (monomer), 281.7 *m/z* (monomer + H_2O), 504.9 *m/z* (dimer), and 521.9 *m/z* (dimer + H_2O). The controlled pH medium (PBS) promoted higher genipin consumption and higher monomer formation; here, the genipin was almost completely consumed, indicating a most favorable reaction condition (Fig. 3**b**).

Differently, in the reaction performed with lysine, genipin was completely consumed after 30 min of reaction in both water and PBS mediums (Fig. 3**c-d**), demonstrating lysine as the fastest reacting amino acid. At 15 min, a high genipin consumption was accompanied by a high monomer production. Compared to water, the reaction in PBS had less genipin consumption, and the monomer production was more visible (Fig. 3**d**) due to a slower polymerization reaction. The molecular

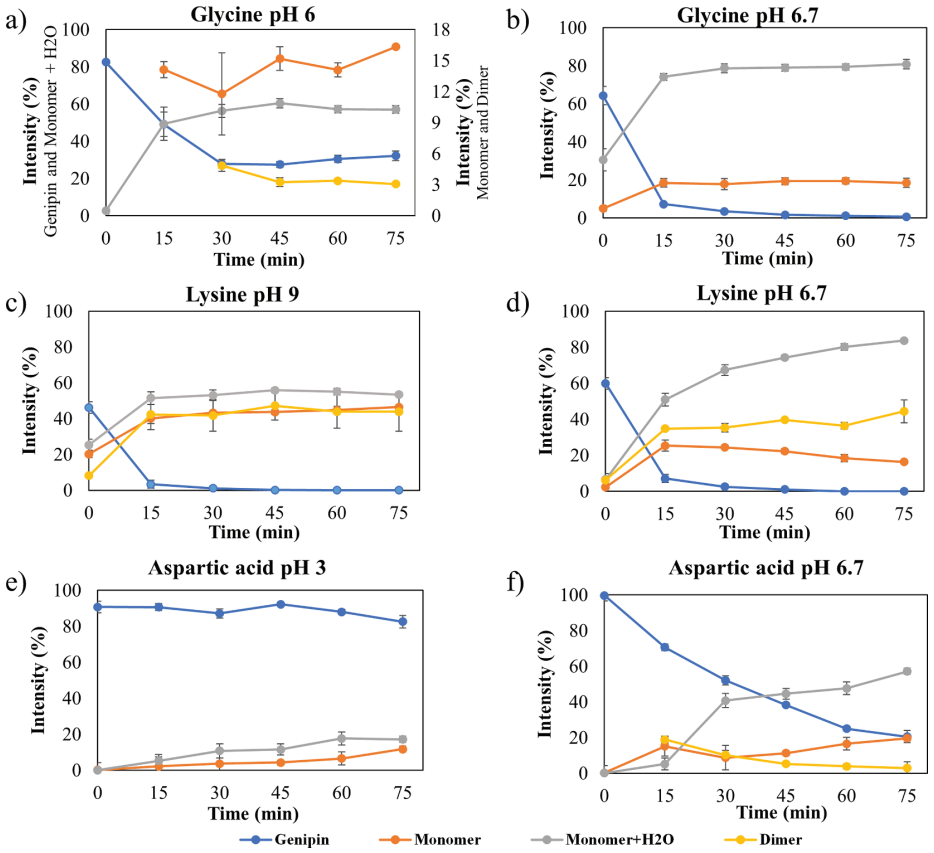

Fig. 3. Intensity representation of genipin, monomer, monomer + H2O and dimer molecules during the reaction time (75 min) using genipin and pure amino acids: glycine at pH 6 (**a**); glycine at pH 6.7 (PBS solution) (**b**); lysine at pH 9 (**c**); lysine at pH 6.7 (PBS solution) (**d**); aspartic acid at pH 3 (**e**); and aspartic acid at pH 6.7 (PBS solution) (**f**). Molecular mass of Genipin + Glycine monomer: 264.08 u; Genipin + Glycine dimer: 506.13 u; Genipin + Lysine monomer: 335.16 u; Genipin + Lysine dimer: 634.30; Genipin + Aspartic acid monomer: 322,09 u; Genipin + Aspartic acid dimer: 624.158 u.

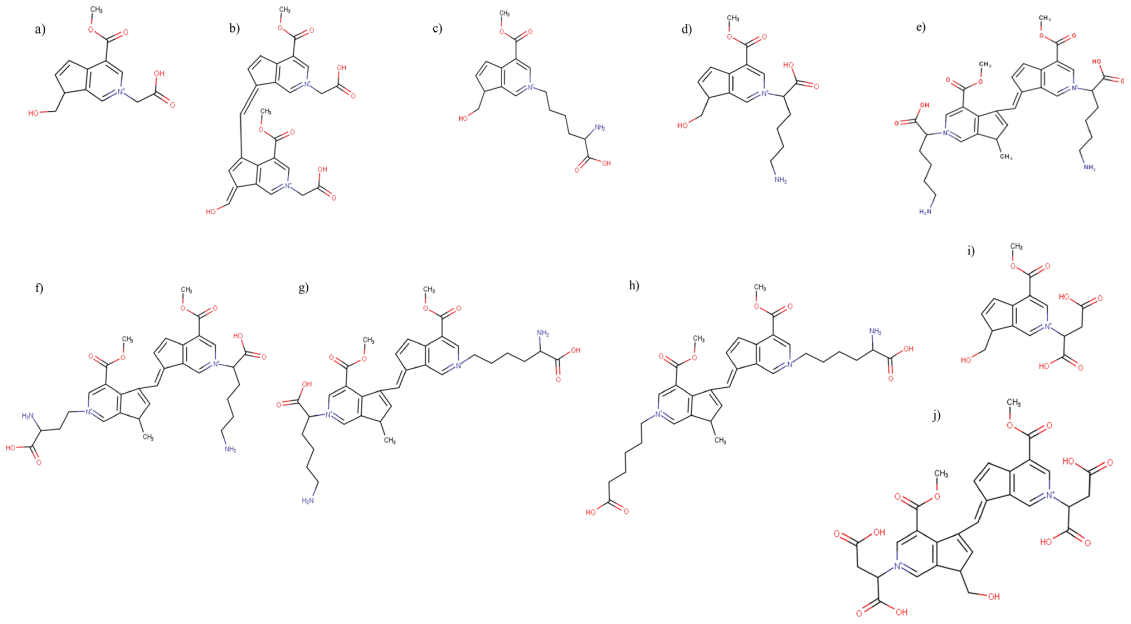

Fig. 4. Molecular formula and weight (mw) of (**a**) Genipin + Glycine monomer (mw = 264.08 u); (**b**) Genipin + Glycine dimer (mw = 506.13 u); (**c**) and (**d**) Genipin + Lysine monomer (mw = 335.16 u); (**e**), (**f**), (**g**), and (**h**) Genipin + Lysine dimer (mw = 634.30 u); (**i**) Genipin + Aspartic acid monomer (mw = 322.09 u); (**j**) Genipin + Aspartic acid dimer (mw = 624.16 u).

structure of two conformations of monomers and dimers is shown in Fig. 4. They present the molecular weight of 335.16 *m/z* (monomer), 352.9 *m/z* (monomer + H_2O), and 634.30 *m/z* (dimer).

As shown in Fig. 3**e-f**, acidic amino acids disfavored the reaction. In both media, the genipin consumption and monomer production was lower, and at pH 3, non-exist genipin consumption nor monomer production was observed. Under acidic conditions, a slower substitution reaction occurred in the ester group, resulting in the elimination of a methanol molecule. The molecular structure of the monomer and dimer is shown in Fig. 4, and they present the exact molecular weight of 322.09 *m/z* and 624.16 *m/z*, respectively.

Fig. 5 shows the HPLC-ESI-MS chromatograms obtained from the reaction between genipin and three different amino acids (glycine, lysine, and aspartic acid) after 30 min of reaction in water medium. As observed in Fig. 5**a**, two peaks could be detected: the most intense representing the genipin/glycine monomer and the genipin. The monomer's retention time (RT) was 10.3 min, and 11.5 min for genipin. Using lysine (Fig. 5**b**), the genipin could not be observed because it was consumed. Two monomer peaks were observed with the same mass (332.9 *m/z*). We hypothesize that these peaks correspond to two different monomer conformations due to the presence of two primary amine groups in lysine (Fig. 4). The differences in the RT of both species could be due to their slightly different polarities. In addition, the mass 206.7 *m/z* (corresponding to a genipin fragment) was also observed at 12.7 min. However, it is not from pure genipin (whose RT is 11.5 min). Finally, in the reaction with aspartic acid (Fig. 5**c**), only the genipin peak could be observed and presented the most intense peak compared to the other graphics mentioned above (RT = 11.5 min).

3.2. Reaction between Genipa americana L. extract and pure amino acids

3.2.1. Visual aspects and color parameters

Table 2 and Fig. 6 show the effect of type of amino acid on the blue color intensity of the colorant produced by *Genipa americana* L. extracts and different amino acids in water and PBS mediums. Lysine and arginine amino acids presented the most intense coloration in both reaction media. Nevertheless, the acidic amino acids (aspartic and glutamic acids) presented a less intense blue color (p-value < 0.05).

In general, all samples had a more intense blue color at pH 6.7, presenting a similar $-b$ value (p-value = 0.109). The bluest-dark colorants were produced when using glycine and lysine, as can be seen by the less luminosity (*L*) value. Glycine presented a blue-purple color, due to a higher *a* parameter (red tone) (p-value < 0.05). Also, lysine and arginine, both in water and PBS mediums, trended to be green, presenting $-a$ values (p-value < 0.05) (Table 2). In water, the highest blueness index (*BI*) was obtained with lysine (Fig. 6**a**); however, in PBS (Fig. 6**b**), glycine presented the highest *BI* (p-value < 0.05) due to its lower *L* value compared to lysine (p-value < 0.05).

Fig. 7**a** shows the effect of the pH medium on the blue color formation, using lysine and glycine as amine group suppliers. The reaction is impaired at low pH values, as seen by the slight blue color at pH 3 and 4. However, under basic conditions, the color was darker and tended to be green. The *BI* tendency shows that the pH range condition reached the maximum negative *BI* was 6–8. About the whiteness index (*WI*), a higher value was obtained at lower pHs; and from pH 6 onwards, the *WI* remained around the same range.

The effect of the amino acid concentration on the blue color formation using lysine and glycine is shown in Fig. 7**b**. At low concentrations (0.08 mg/mL) the solution was slightly blue. Both *BI* and *WI* values reached an ideal value when the amino acid concentration reaches 0.67 mg/mL and was constant when the concentration increases. A higher concentration of lysine promoted a green color, while higher glycine concentrations corresponded to a darker pigment.

3.3. The reaction between Genipa americana L. extract and milk proteins

3.3.1. Complex size

Fig. 8 shows the SDS-PAGE electrophoretic pattern of the milk, milk-colorant, casein, casein-colorant, whey, whey-colorant, and the genipin extract by ethanol. Bands corresponding to casein (~25 kDa), β-lactoglobulin (β-Lb) (18.4 kDa), and α-lactoalbumin (α-La) (14.4 kDa) were more visible with the pure dairy products. The bands corresponding to

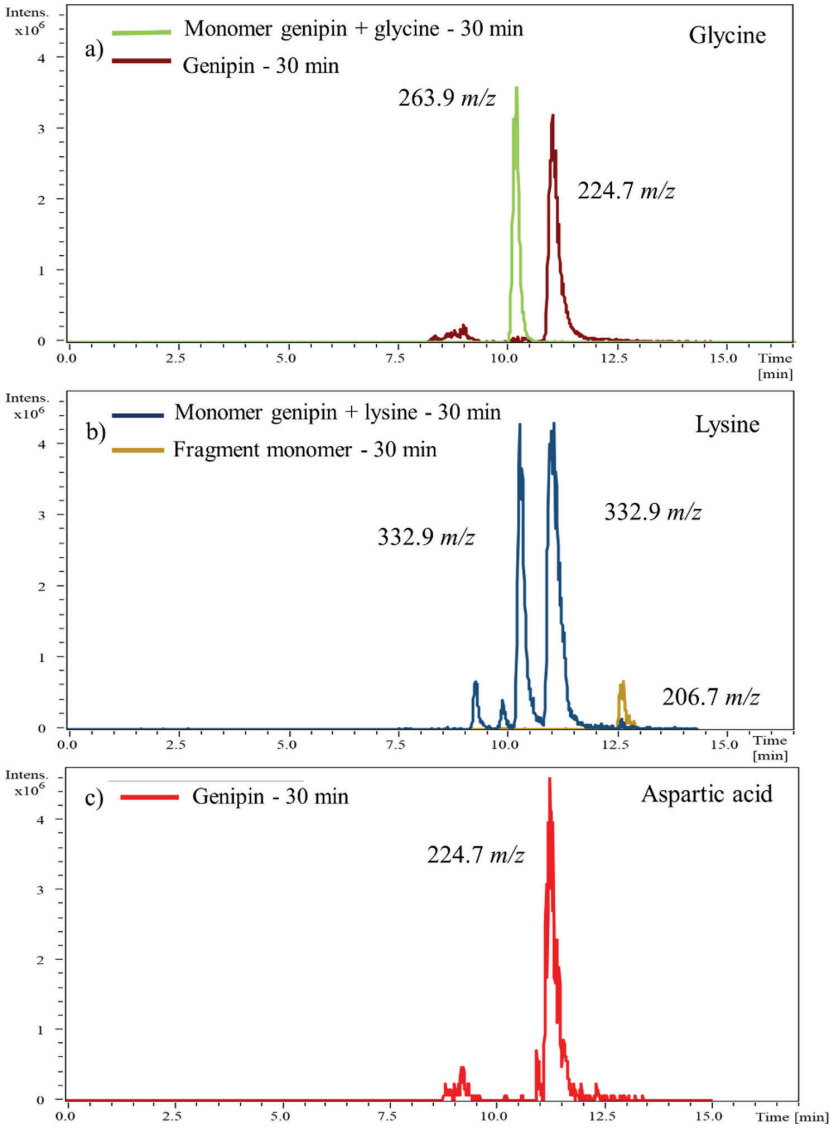

Fig. 5. HPLC-ESI Spectra after 30 min of the reaction between genipin and glycine (**a**), lysine (**b**), and aspartic acid (**c**).

Table 2
Color parameters of *Genipa americana* L. extracts with different amino acids (lysine, glycine, aspartic acid, glutamic acid, arginine) in water or PBS (pH 6.7). Letters indicate significant differences between the samples for each parameter after ANOVA with Tukey's Post-hoc, p-value < 0.05.

		L	a	b
Water	Lysine (pH 9)	36.9 ± 0.1^{d}	-4.4 ± 0.1^{b}	-23.1 ± 0.1^{a}
	Glycine (pH 6)	60.0 ± 1.0^{b}	8.8 ± 0.8^{c}	-22.0 ± 1.0^{a}
	Arginine (pH 10)	50.2 ± 0.2^{c}	-11.8 ± 0.1^{a}	-18.1 ± 0.2^{b}
	Asp Ac. (pH 3)	83.6 ± 0.2^{a}	-4.6 ± 0.2^{b}	-6.1 ± 0.2^{c}
	Glu Ac. (pH 3)	85.2 ± 0.3^{a}	-4.4 ± 0.1^{b}	-5.9 ± 0.1^{c}
PBS (pH 6.7)	Lysine	41.0 ± 0.1^{b}	-2.7 ± 0.1^{c}	-25.8 ± 0.4^{a}
	Glycine	37.1 ± 0.2^{c}	12.0 ± 0.9^{a}	-27.7 ± 0.7^{a}
	Arginine	45.4 ± 0.1^{a}	-6.3 ± 0.3^{d}	-25.7 ± 0.5^{a}
	Asp Ac.	45.1 ± 0.1^{a}	-1.3 ± 0.7^{c}	-26.8 ± 0.3^{a}
	Glu Ac.	42.0 ± 1.0^{ab}	-0.5 ± 0.1^{b}	-27.0 ± 0.4^{a}

these protein fractions were less visible with the respective colorants. In contrast, bands around 200 kDa could be observed. The 200 kDa bands correspond to the colorant complex, evidently caused by the cross-linking between genipin and proteins. This band was more pronounced in casein colorant. This result confirms that the size of the polymer (genipin and crosslinked milk proteins) can reach 200 kDa. Concerning the genipin extract, no bands could be observed, indicating no complex polymer formation.

Quantitative analysis by MALDI – TOF-MS provided further insights into the reactions between dairy proteins and genipin. As shown in **Figure S1**, all the proteins detected by MALDI-TOF-MS (casein fractions, whey proteins and milk proteins) decreased significantly in the presence of genipin due to the crosslinking reaction to form the complex protein-genipin polymer. Although we tried different conditions and MALDI matrices, this complex could not be observed in MALDI-TOF-MS, probably due to its very high molecular weight.

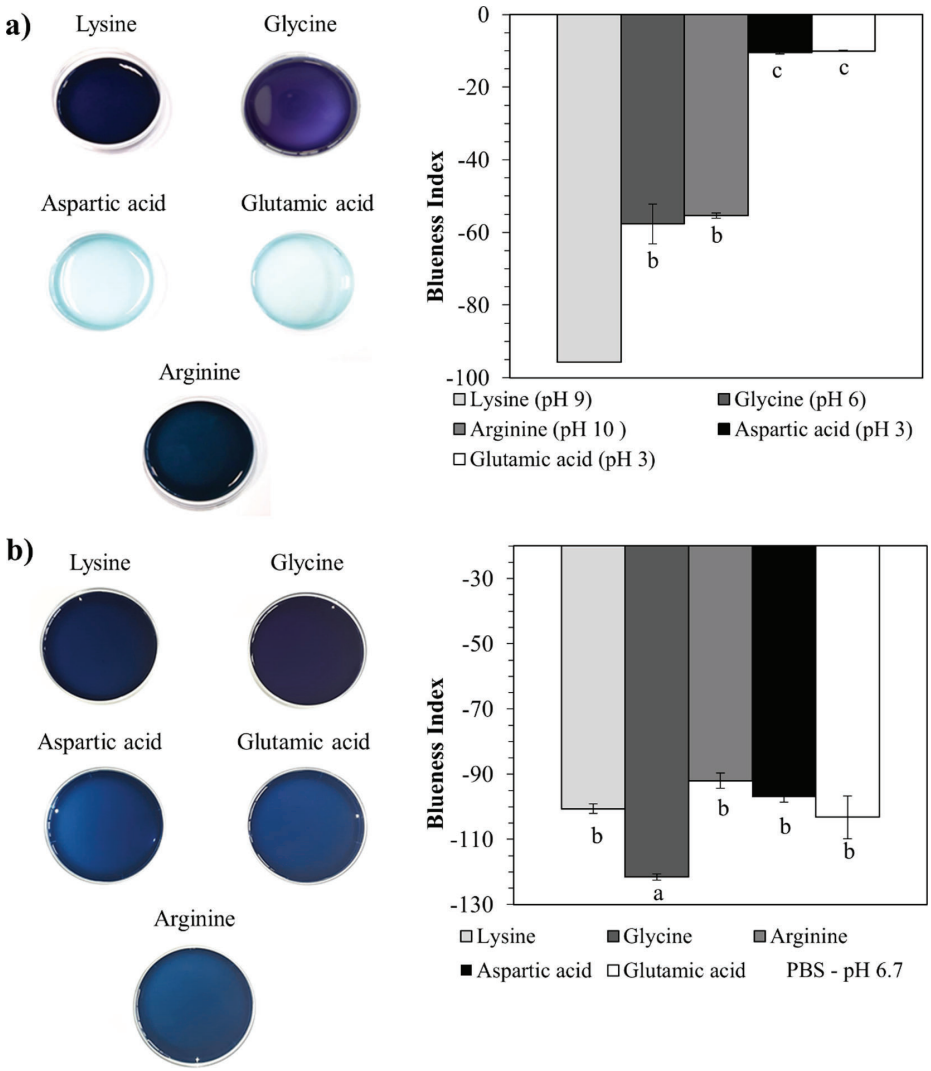

Fig. 6. Effect of the type of amino acid (lysine, glycine, aspartic acid, glutamic acid, and arginine) on the visual aspect of blue color intensity and blueness index parameters in water (**a**) and in PBS (pH 6.7) (**b**). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

4. Discussion

Starting with the most straightforward system (pure amino acids and pure genipin), it could be deduced that more than the type of amino acid, the pH in the medium is the primary factor influencing the intensity and the reaction kinetics for obtaining the blue colorant. The pH directly influences the quantity of NH_2 and NH_3^+ groups available. Because NH_3^+ cannot attack the genipin C3 carbon (Mi, Shyu, & Peng, 2005), when the $[NH_3^+/NH_2]$ ratio is higher, less reactive is the medium. This could explain the inexistent reaction with acidic amino acids at pH 3 because the NH_2 practically did not exist. Song et al. (2021) also studied the reaction between genipin and lysine, and they discussed the importance of having NH_2 and oxygen in the medium to promote a faster reaction. Analyzing the basic amino acids, lysine and arginine, they present different charges at pH 6.7 and pH ~ 10. In pH 6.7, the reaction is disfavored because the ratio $[NH_3^+/NH_2]$ is higher than at pH ~ 10, resulting in a higher blue color reaction rate at basic conditions. This relation also explains the consumption at different levels (lysine > arginine) in PBS (pH 6.7): the NH_2 pK_a values of lysine and arginine are 10.5 and 12.5, respectively. Thus, at 6.7 pH, the ratio $[NH_3^+/NH_2]$ for arginine is around 100 times higher than for lysine, becoming lysine more reactive than arginine. With lysine, the reaction rate was faster (the reaction became blue in less than 5 min), but with arginine, the mixture became blue in 15 min. Also, a strong difference in color and reactivity when using glycine in PBS or water was observed. In the last one (pH 6), glycine was less reactive despite the pH difference being only around 0.7. At pH 6, glycine is at its pK_a value ($NH_3^+ = NH_2$). At pH 6.7, glycine has more deprotonated amines (lower $[NH_3^+/NH_2]$ ratio).

Primary amine groups in amino acids have similar nucleophilicities (Brotzel & Mayr, 2007). Thus, the differences in the genipin attack reaction rate by the amino acids are mostly related to their number of primary amines and their structure. The higher primary amines content and the simplest molecular structure, the most intense is the blue color formed. Hence, the blue colorant produced using lysine and arginine

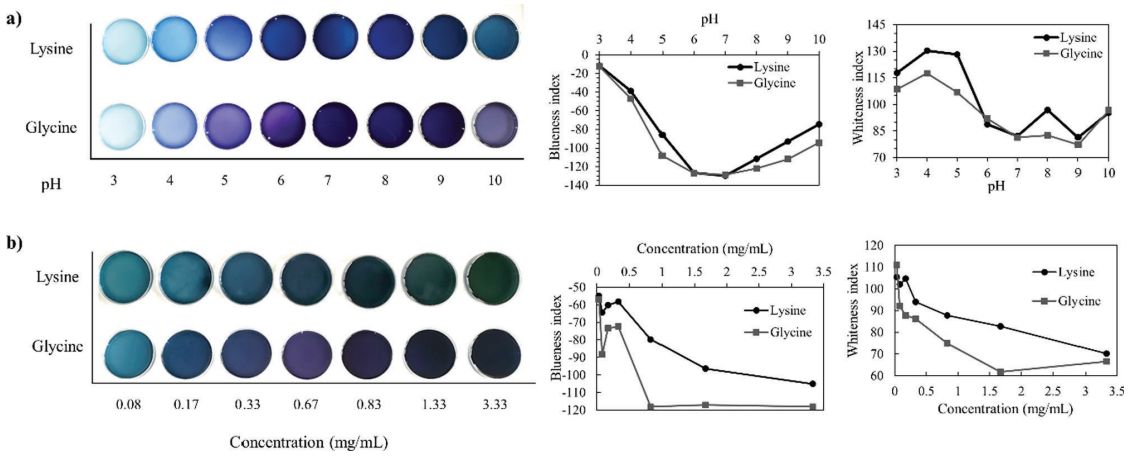

Fig. 7. Effect of the pH (**a**) and the amino acid concentration (**b**) on the blue color formation, using lysine and glycine as amine suppliers. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

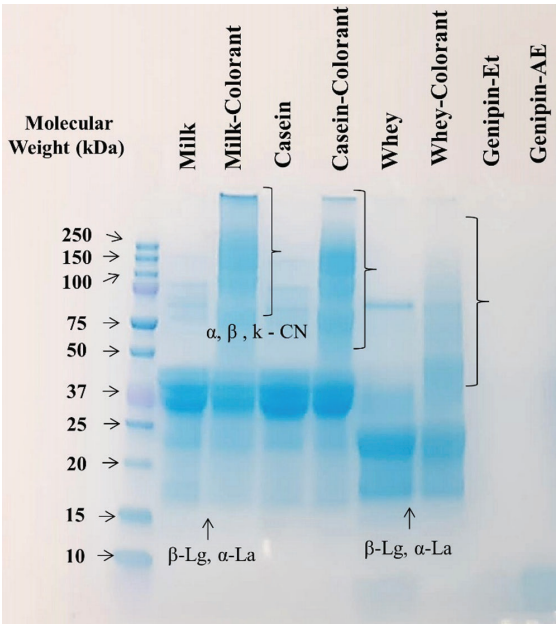

Fig. 8. SDS – PAGE patterns of milk, milk-colorant, casein, casein-colorant, whey, whey-colorant, and ethanolic extract of genipin. Samples were loaded as the following designated lanes: 1, molecular-weight markers (10 – 250 kDa); 2, milk; 3, milk-colorant; 4, casein; 5, casein-colorant; 6 whey; 7, whey-colorant, 8-Genipa americana extract in ethanol. β-Lg: β-lactoglobulin; α-La: α-lactalbumin, CN: Casein.

presented the most intense coloration because both amino acids contain two available primary amines to react with genipin. Regarding glycine, it was a highly reactive amino acid, despite having only one primary amine group, because of its small size and less steric effects.

Additionally, amino acids can present important differences in their pK_a values due to the surrounding microenvironment. This has been repeatedly observed when these pK_a values are experimentally determined for the isolated amino acids or for the amino acids being part of a macrostructure as a protein, polymer, etc. For instance, an important variation in the pKa values is induced by the electrostatic repulsion, as Cifuentes and Poppe (1994) discussed. Thus, a charged NH_3^+ group will bring about an important decrease of the pKa value of any nearest NH_2 group since its ionization will be more difficult due to electrostatic repulsion. In this sense, the crosslinking reaction between genipin and amino acids causes the ionization of amine groups (see Fig. 4), leading to important variations in the pK_a values of other neighboring chargeable groups (Bas, Rogers, & Jensen, 2008; Fitch, Platzer, Okon, Garcia-Moreno, & McIntosh, 2015). This effect could explain why similar amino acids with the same number of primary amines and carboxylic acids (such as arginine/lysine and aspartic acid/glutamic acid) show different reaction rates with genipin.

The amino acid concentration is another key aspect in reaction kinetics, being the equal molar proportion of genipin and amino acid the best condition. Some studies have observed a relationship between the molecular weight of neutral amino acids and their reactivity (Brotzel & Mayr, 2007). This may explain why glycine is more reactive than arginine; although arginine has two primary amines, its respective high molecular weight (174.20 g/mol) may negatively interfere in the reaction. Similarly, when comparing the two acidic amino acids with similar pK_a and the same number of primary amines, the aspartic acid is more reactive due to its lower molecular weight (133.11 g/mol) than glutamic acid (147.13 g/mol). However, other effects cannot be discarded, including electrostatic and steric effects once the polymer is generated.

When studying the best concentration for each amino acid, we observed that for glycine, it was necessary 1 mg/mL for 0.5 mg/mL of genipin. In contrast, the best concentration for the other amino acids was 0.5 mg/mL (Fig. 2). As glycine presents a lower molecular weight (75.03 g/mol) than genipin (226.08 g/mol), more than one mol must react with one mol of genipin. Contrarily, lysine, arginine, aspartic acid and glutamic acid have higher molecular weights than glycine, being more equimolar to genipin. This finding agrees with Fujikawa et al. (1987) who also found that the absorbance of the blue pigment was more intense for an equal molar concentration between genipin and the amino acids tested.

Analyzing the visual aspects combined with the HPLC-DAD-ESI-MS kinetics results, the monomer between genipin - amino acid was formed before the blue color was observed. This can be inferred by Fig. 3**a**, in which the monomer was already formed at 15 min, and Fig. 1, which shows that the sample color became blue after 25 min. Also, regarding HPLC-DAD-ESI-MS spectra results, the monomer has a maximum absorbance at 240 nm, proving that the monomer is colorless. As a result, the blue colorant is a structure based on a polymer with

repeated units, starting with at least two monomer units (dimer). The detail of this reaction, using glycine as an example of amino acid to attack the genipin, is presented in **Figure S2**. This figure shows the monomers and the two monomers union for the dimer formation. From the dimer formation, the polymerization occurred by the union of more monomers, using the same reaction mechanism.

In general, the reaction rate in this work was higher than what it is reported in the literature: Fujikawa and co-authors (1987) produced the colorant from genipin and amino acids at 80 °C for 4 h. Also, Tsutmiuchi and co-authors (2021) performed the reaction at 50 °C for 5 h. In our work, mainly if the most reactive amino acids (lysine, arginine, and glycine) were used, in less than 15 min the colorant was already formed.

According to the kinetic study (Table 1), the amino acids reacted with different reaction rates and intensities with genipin until the blue pigment was produced, and some steps proceeded. The first one is the monomer formation, and then the polymerizations are assembled starting by the dimer formation (Di Tommaso et al., 2014).

Regarding this study's second point of view, the first observation in the reaction between the *Genipa americana* L. extract and pure amino acids is that there was the same reaction tendency as when using pure genipin. However, other amino acids and proteins present in the *Genipa americana* L. fruit may affect the reaction and the blue color formation. In both cases, the reaction is mainly favored at pH 6–7, and lysine, arginine, and glycine were the best amino acids as amine suppliers. Regarding the *Lab* color parameters, the different amino acids promoted diverse blue tones. Glycine promoted a blue-purple color, and lysine and arginine (both in water and in PBS mediums) trended to be greener (Table 2). These differences may be explained regarding the molecular conformation of the monomer. Cano et al. (2014) have previously worked with methionine and valine to react with genipin extract, and they produced a blue-green colorant. It is important to note that methionine, valine and lysine present very different side-chain groups (S-methyl thioether, isopropyl chain or a primary amine, respectively) apart from their N-terminal amine group, which can promote different conformations responsible for producing the blue-green tone. In our study, we used arginine and lysine, both amino acids presenting primary amines in the N-terminal and side-chain groups, which might promote other different monomers conformations. Since it is a small molecule, glycine interacts with genipin differently than other amino acids (Fig. 3). Its dimers present different rearranged conformations (Fig. 4), affecting the color. Also, each amino acid is differently affected by the microenvironment, consequently producing diverse blue tones (Fitch et al., 2015). These observations confirmed that the reaction conformation might have a crucial effect on the tone of pigments formed.

Additionally, according to Slusarewicz, Zhu, and Hedman (2010), an opening ring of the genipin molecule occurs in an alkaline environment ($pH \geq 9$), and the equilibrium between closed and open-ring tautomers can impair the genipin reactivity. This effect can also result in a color change to a green-darker colorant. Hence, using *Genipa americana* L. extract, basic conditions may not be the best option to promote this reaction, being a neutral pH the most appropriate.

Despite the similar results obtained using pure and extracted genipin, the use of *Genipa americana* L. extract also implies extracting the amino acids and proteins existing in the fruit, which can also react (or have already reacted) with genipin. However, a previous study has demonstrated that no significant differences for all free amino acids are observed between either fresh and oxidized fruits, where a blue colorant is naturally formed (Bentes, de Souza, Amaya-Farfan, Lopes, & de Faria, 2015). This leads to two consequences: the first one, it is not possible to know the accurate quantity of amino acid and neither the genipin, so the equimolar point is unknown; secondly, it can facilitate the blue colorant formation regardless of the conditions used. For this reason, as shown in Fig. 7, even under acidic conditions, the blue color is still formed or has already existed.

Arriving to a particle scale, the complex colorant system using milk was obtained as in our previous works (Neves et al., 2020). However, the colorant using casein and whey as a reaction medium and amine supplier was not produced yet. Our previous studies have demonstrated milk as a genipin solvent extracting medium and, at the same time, an amine supplier for blue colorant production (Neves et al., 2020). However, the present study sheds some light on why cow's milk is an ideal reaction medium to the blue colorant production with genipin: milk presents a 6.7 pH. Under these conditions, some amino acids of milk proteins present available NH_2 to attack the extracted genipin. In this regard, the most basic amino acids are arginine ($pK_a = 12.48$) and lysine ($pK_a = 10.53$). These amino acids are important in the reaction because they present 2 primary amines on NH_2 conformation in their structure. Histidine is also considered basic amino acid ($pK_a = 6$), facilitating the reaction. Regarding the casein and whey amino acid composition, although they are acidic proteins, casein presents 4.1 g of arginine, 8.2 g of lysine, and 1.81 g of histidine per 100 g of isolated proteins; and whey presents 2.3, 9.1, and 1.41, respectively (Beach, Bernstein, Hoffman, Teague, & Macy, 1941; Gordon, Semmett, Cable, & Morris, 1949). Consequently, all of the amino acids in milk (at pH 6.7), present a lower $[NH_3^+/NH_2]$ ratio, which is preferential to produce the colorant. Regarding those amino acids with one amino group, the lower the pK_a of the NH_3 group is, the lower is the $[NH_3^+/NH_2]$ ratio of the amino acid. Thus, due to the equilibrate amino acid content, the presence of basic amino acids, and above all, its pH, makes milk an ideal reaction medium for the blue color formation. The isoelectric point is another standpoint to consider for understanding the effect of the available NH_2: the α-La isoelectric point is around 4.2, the β-Lg is 4.85, and 4.6 for caseins. In milk pH (6.7), these proteins present a negative charge, and, in general, tend to present a lower $[NH_3^+/NH_2]$ ratio. In parallel, Neves et al., 2020 have discussed the possibility to produce the colorant using milk as amine group supplier. However, after our present findings, we can observe that both casein and whey proteins could also be an ideal reaction medium to produce the blue colorant.

In SDS-PAGE results (Fig. 8), the complex formed by milk and casein proteins tend to present higher molecular weight than the complex formed when using whey proteins. Since caseins are approximately 2 times bigger than whey proteins (β-Lg and α-La), the complex formed with caseins would be bigger. Also, as observed in MALDI-TOF spectra (**Figure S1**), caseins were more consumed than whey proteins. Therefore, the reaction with caseins may probably be faster and more intense than with whey proteins. Regarding the reaction between casein and genipin, Li et al. (2022) used the genipin molecule to modify the casein aiming to increase its resistance. After, the casein-genipin complex was used as nano carrier. These authors highlighted the high molecular mass produced by the crosslinking of genipin in the casein structure.

The effect of the microenvironment on the amino acids pK_a variation is even more evident in complex systems, in which the crosslinking structure does not involve only pure compounds, but more complex whey and casein proteins in which other spatial effects including secondary, tertiary and quaternary structures can play an important role (Grimsley, Scholtz, & Pace, 2009). This effect would be different for whey and casein proteins. Whey proteins present a compact globular structure (Walstra, Walstra, Wouters, & Geurts, 2006), in which the primary amines available to react with genipin are only those deprotonated amines present outside the structure. On the other hand, caseins have a micellar quaternary structure, being less compact and less folded than whey proteins (Walstra et al., 2006). This structure provides the caseins with more external genipin-NH_2 binding sites (Rodriguez-Nogales, 2005). As they are more folded, whey proteins would present less NH_2 groups available for genipin attack than caseins. More importantly, the size of the proteins and the number of NH_2 groups (i.e., due to lysine and arginine amino acids, plus the terminal NH_2 group) will also have an important effect on the reactivity of the protein with genipin. For instance, β-Lg (a whey protein with a molecular weight of 19,883 Da) counts with 16 residues of lysine and 3 of arginine, very low numbers compared to the protein alpha-casein (with a molecular weight of 45,143 Da, 23 lysine residues and 26 arginine residues). Besides,

approximately 80 percent of the protein in milk is composed by casein proteins, while the other 20 percent is whey protein. These effects may explain why caseins completely react with genipin (according to MALDI-TOF-MS results, **Figure S1**) and why SDS-PAGE bands of ~ 200 kDa size were more intense in the casein colorant pattern (Fig. 8).

5. Conclusion

This study is the first investigation that combines three different scales of reagents to produce a natural blue colorant based on genipin studying the reaction mechanism behind it. We have established the factors that mainly influence the reaction rate and color intensity, indicating the monomers and dimers formed between pure genipin and amino acids as the main precursors and confirming that the products became blue due to the dimer formation. With *Genipa americana* L., the fruit's proteins and amino acids play a role and react with genipin and influence color production. The pH (6–7) combined with basic amino acids and the equimolar genipin:amino acid proportion were the most favorable conditions for producing the blue colorant. Concerning the most complex system (extracted genipin with milk and dairy proteins), caseins are the preferencial proteins to produce the colorant, and the size of the polymer formed due to the crosslinking reaction between extracted genipin and dairy proteins gave a molecular mass about 200 kDa.These results explained the mechanism of formation of the blue colorant and the effect of the type and amount of protein on the blue color reaction. Also, the milk and their proteins are an excellent medium for this reaction because their proteins present a good number of basic amino acids and an ideal pH of 6.7. Therefore, from this study, the promising blue colorant applicable as a natural food ingredient and obtained from *Genipa americana* L. fruits and dairy proteins could be better understood and valued. Additionality, the knowledge of this reaction mechanism opens new possibilities for the use of other matrices as amine suppliers for further applications.

CRediT authorship contribution statement

Maria Isabel Landim Neves: Conceptualization, Methodology, Formal analysis, Data curation, Writing – original draft, Visualization. **Alberto Valdés:** Conceptualization, Methodology, Formal analysis, Data curation, Writing – original draft, Visualization. **Eric Keven Silva:** Writing – review & editing. **Maria Angela A. Meireles:** Writing – review & editing, Supervision. **Elena Ibáñez:** Conceptualization, Resources, Writing – review & editing, Supervision. **Alejandro Cifuentes:** Conceptualization, Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2022.111240.

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CHAPTER 6

Experimental article IV

How are the characteristics of the colorant carried by prebiotic matrices regarding powder properties, colorant power, and stability?

Fructans with different degrees of polymerization and their performance as carrier matrices of spray dried blue colorant

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ABSTRACT

Inulin-type fructans with different degrees of polymerization (DPs) were used as wall materials for the blue colorant produced from the crosslinking between genipin and milk proteins. The impact of using fructooligosaccharides (FOS) with DP = 5 and inulins with DP ≥ 10 (GR-In) and DP ≥ 23 (HP-In) on the physical (microstructure, size, water activity, wettability, solubility, water adsorption, glass transition temperature, and color), chemical (free genipin retention and moisture), and technological (colorant power, pH stability, and thermal stability) properties of the powdered blue colorant was examined. Inulins were more efficient carriers as seen from the physical characteristics of the microparticles. FOS and GR-In promoted higher retention of free genipin than HP-In. Additionally, their lower DP influenced the rehydration proprieties as well as the color intensity and colorant power. The DP did not affect the physical stability of the colorant at different pH conditions or at high temperature. Our findings demonstrated that the DP of the fructan exhibited a strong impact on the blue intensity of the samples and also their rehydration capacity.

1. Introduction

Health concerns have increased in the last decade due to growing internet access worldwide. There is a widespread demand for quality life focused on health improvement or disease prevention by modern consumers. In this regard, new approaches have emerged in the design of functional foods. Likewise, the ingredients used to formulate these products have been revised to increase their potential and benefits for health and well-being (Dima et al., 2020). Thus, the food industry must develop novel products employing innovative ingredients such as prebiotics carbohydrates (Betoret et al., 2015; Li & Nie, 2016). Different functional carriers have been used such as proteins, functional lipids, and prebiotic carbohydrates (Celli et al., 2015). However, among them, the use of prebiotic carbohydrates has grown. They are indigestible by the human body after oral administration.

In contrast, the human gut microbiota is adept at fermenting a broad range of these carbohydrates (El Kaoutari et al., 2013). Through fermentative processes, prebiotic carbohydrates are converted into short chain fatty acids, which are crucial signaling molecules for transducing the gut microbiota's physiological functions to the host. Therefore, they are recognized as functional ingredients (Sun et al., 2020).

Among prebiotic carbohydrates, inulin-type fructans (fructooligosaccharides and inulins) have emerged as functional ingredients due to their ability to promote the growth of microflora in the digestive tract. Also, their technological properties include production low-calorie foods for diabetic patients and managing blood sugar levels (Pacheco et al., 2018). As biopolymers, they present high or medium molecular weight, could stabilize proteins, provide no flavor or color, and incorporate some compounds in their molecular structure. Thus, in general, they have techno-functional properties that enable their use as carrier systems (Femat-Castañeda et al., 2019; Fernandes et al., 2017; Silva et al., 2016). The utilization of fructans as wall materials in spray drying processes of target compounds depends on their degree of polymerization (DP), which can vary between 4 and 80. The fructan DP influences the physicochemical characteristics of spray dried products such as their morphology, rehydration and thermal behavior, physical stability, and chemical properties (Silva et al., 2016). Recently, fructans have been used to carry or encapsulate some compounds or microorganisms such as chlorophyll (Femat-Castañeda et al., 2019), oleuropein (Pacheco et al., 2018), *Lactobacillus acidophilus* (Raddatz et al., 2020), anthocyanins (Lacerda et al., 2016), antifungal thyme oil (Esquivel-Chávez et al., 2021), and betalains (Kuhn et al., 2020), to increase their functionality

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in food ingredients.

The functional ingredients that most demand a carrier matrix are natural colorants (Sigurdson et al., 2017). The carrier is in some cases necessary to enable the production, and later incorporation of the powdered colorant in food (Neves et al., 2021; Sigurdson et al., 2017). Among natural colorants, blue compounds are still the most challenging for the food industry because of their scarce sources in nature (Buchweitz, 2016). Neves et al. (2020) studied obtaining a blue colorant from the crosslinking between milk proteins and genipin extracted from unripe *Genipa americana* L. pulp. The blue compound formed from the reaction between genipin and primary amines present in milk has great potential for industrial application compared to other natural blue colorants (Neves et al., 2021; Rodriguez-Amaya, 2016). Since this blue compound is a food ingredient, a prebiotic carrier could design a new functional ingredient for food application with a healthy appeal. To ensure the use of an appropriate fructan-based carrier, the study of the impact of DP on the spray dried blue colorant system is a fundamental step.

In this context, this work aimed to investigate the impact of the fructan DP on the physical, chemical, and technological properties of spray dried blue colorant produced from the crosslinking between genipin and milk proteins. For this, fructooligosaccharides (FOS) with DP = 5 and inulins with DP ≥ 10 (GR-In) and DP ≥ 23 (HP-In) were used as carriers of the innovative blue colorant.

2. Material and methods

2.1. Production of blue colorant based on milk proteins and genipin

The liquid blue colorant was produced using unripe *Genipa americana* L. pulp and milk according to the methodology described by Neves et al. (2020). The blue compounds were obtained from the crosslinking between genipin and milk proteins. Genipin was extracted from unripe *Genipa americana* L. pulp using the milk as a solvent. The colloidal milk system was also used as a reaction medium for the reaction between genipin and milk primary amines. The production of the blue colorant-loaded milk was assisted by low-frequency ultrasound (Neves et al., 2020; Strieder et al., 2020).

2.2. Spray drying of blue colorant-loaded milk using fructans as carriers

The fructans used as wall material were obtained from chicory: Orafti®FOS, (FOS, DP = 5); Orafti®GR (GR-In, DP ≥ 10); and Orafti®HP (HP-In, DP ≥ 23) (SweetMix, Sorocaba, Brazil). The wall materials were previously characterized by Silva and Meireles (2015). Each carrier suspension was prepared by dissolving 15 g of fructan into 85 g of deionized water (Millipore®). All wall materials were heated at 80 °C to ensure complete solubilization of the fructan in deionized water. After solubilization, the suspensions were cooled to 40 °C. Then, 166.66 mL of the blue colorant-loaded milk (dispersed phase) was gradually added into 83.33 mL of cooled suspension containing FOS, GR-In, or HP-In (continuous phase). The addition was assisted by a rotor-stator type homogenizer model 713D (Fisatom, São Paulo, Brazil) at 2000 rpm for 5 min. The total concentration of solids in the system (wall material + blue colorant-loaded milk) was equal to 8.3 g of solids per 100 g of suspension. The proportion of solids was 1:2 (carrier: blue colorant-loaded milk, *w*/w). The control sample refers to the blue colorant-loaded milk without wall material, which was adjusted to achieve the same content of solids (8.3 g of solids per 100 mL of suspension).

Immediately after homogenization, a portion of 250 mL of the drying suspension was spray dried. Atomization was performed in an SD-06 spray dryer (LabPlant, Hunmanby, United Kingdom) inside a short cylindrical chamber (diameter 0.215 m, and length 0.5 m) by a 1 mm double fluid nozzle mounted concentrically with the cylinder axis. The inlet temperature was 180 °C, the feed rate was 8.1 mL/min, and the atomizing airspeed was kept at 3.9 m/s. The outlet temperature was maintained at 91 ± 2 °C. After their production, the blue colorant powders were stored at 25 ± 2 °C. The spray drying processes were performed in duplicate ($n = 2$).

2.3. Impact of DP of fructan on the physical and chemical properties

2.3.1. Microstructure

The particles microstructure was examined by scanning electron microscopy. The samples were placed on double-sided carbon tape (Ted Pella, Redding, United States) fixed to aluminum stubs. The images were captured under a table-type microscope, TM-4000 (Hitachi High Technologies, Tokyo, Japan), at 2500× magnification.

2.3.2. Particle size distribution

The particle size distribution was analyzed at 25 °C by using a laser diffraction Mastersizer 2000 (Malvern Instruments, Malvern, UK). The average size was determined in terms of volumetric mean diameter ($D_{4,3}$) (Eq. (1)). The polydispersity index was calculated by the span (Eq. (2)). The samples were analyzed by a wet method, with dispersion in water and a refractive index of 1.52.

$$D_{4,3} = \frac{\sum_{i=1}^{k} n_i.d_i^4}{\sum_{i=1}^{k} n_i.d_i^3} \quad (1)$$

$$span = \frac{d_{90} - d_{10}}{d_{50}} \quad (2)$$

where d_{10}, d_{50}, and d_{90} are the diameters at 10%, 50%, and 90% of the cumulative number, respectively, and n represent the number of particles.

2.3.3. Water activity, moisture content, solubility, and wettability

The water activity was determined with a Labtouch-water activity analyzer (Novasina, Lachen, Switzerland). The moisture content was evaluated according to AOAC method 925.10 (AOAC, Association of Official Analytical Chemists (AOAC), 1993). The water solubility and wettability were determined according to the methodology described by Schuck et al. (2012). The solubility was calculated from the volume of sediment (mL) remaining after two centrifugations for 5 min at 160 *g*. The insolubility index (*II*) is equal to the volume of sediment (mL) remaining after the second centrifugation. The solubility index (SI) (Eq. (3)), expressed as a %, is equal to:

$$SI = 100 - (2 \times II) \quad (3)$$

Wettability is the time for a powder to cross the water surface under given conditions. For this, 0.1 g of sample was dispersed over 100 mL of water into a 400-mL beaker at 25 °C.

2.3.4. Dynamic hygroscopicity under different relative humidity conditions

Two grams of powder was needed in a dynamic dewpoint isotherm (DDI) (Aqualab VSA-Meter group, Pullman, United States). The measurement parameters were 25 °C, airflow 100 mL/min, desorption direction until a water activity of 0.1, and after adsorption until a water activity of 0.9. The water content of the initial samples was predicted by option provided by the DDI software Moisture Analysis Toolkit (Meter group, Pullman, United States).

2.3.5. Physical structure

The physical structure of the powders was examined by X-ray diffraction using a PW 1710 diffractometer (Philips, Almelo, Netherlands) using the following conditions: Bragg-Brentano geometry (θ:2θ); radiation: Cu-Kα_1 (λ = 1.54178 Å); voltage: 40 kV; current: 30 mA; measurement intervals: 0.02°; acquisition time: 2 s; scan: 5 to 40° (2θ angular scan).

2.3.6. Glass transition temperature

The glass transition temperature (T_g) was determined using a differential scanning calorimeter DSC Q2000 (TA Instruments, New Castle, United States). The powder samples were maintained at 25 °C under a relative humidity of 56% provided by sodium bromide solution until equilibration (Greenspan, 1977). Two milligrams of sample, under a nitrogen atmosphere at 50 mL/min, was subjected to two heating gradients for constructing DSC curves: 25 °C to 110 °C with a scanning rate of 20 °C/min and isothermal period for 5 min (first run) and 25 °C to 250 °C (second run). The T_g was recorded in the second run at the midpoint of the glass transition range (Botrel et al., 2014).

2.3.7. Color attributes

Color attributes were determined by a CR-400 apparatus (Konica Minolta, Tokyo, Japan) using the CIELab scale (*L*, *a*, and *b*) with reflectance mode. The color difference (ΔE) was calculated (Eq. (4)) to evaluate the DP effect on the color attributes.

$$\Delta E_{Lab} = \sqrt{(\Delta L)^2 + (\Delta a)^2 + (\Delta b)^2} \quad (4)$$

where Δ is the difference between the powders obtained with different carriers and the powder control (without carrier).

2.3.8. Free genipin content

The free genipin content was determined in the blue colorant-loaded milk and spray dried blue colorant samples using high performance liquid chromatography (HPLC). An HPLC-PDA Alliance E2695 (Waters, Milford, USA) equipped with a Kinetex C18 column (150 × 4.6 mm id, 2.6 μm, Phenomenex, Torrance, USA) was used to quantify the genipin content. An aliquot of 0.5 mL of blue colorant-loaded milk or spray dried blue colorant reconstituted in ultrapure water (10 g/100 g of solution) was manually homogenized in 1 mL of ethanol. Then, the mixture was centrifuged at 12,000 rpm for 15 min. An aliquot of 1 mL of supernatant was filtered using a nylon membrane (0.45 μm) and injected into the column. The detailed method and quantification of free genipin were described by Neves et al. (2020). The moisture content of each microparticle was considered. Additionally, the solid corresponding to the carrier material was considered in the calculations.

2.4. Impact of DP of fructan on the technological properties

2.4.1. Colorant power

The colorant power was determined by adding 0.1 g of the spray dried blue colorant in volumetric flasks of 5, 10, 25, and 50 mL and then adding deionized water until the total volume of each flask was reached. The powders were totally solubilized. Then, 10 mL of the solutions with the colorant were placed in identical 5.5 cm diameter Petri dishes and analyzed by a CR-400 apparatus (Konica Minolta, Tokyo, Japan) with the CIELab scale (*L*, *a*, and *b*) in reflectance mode.

2.4.2. pH stability

The colorant stability was evaluated under various pH conditions. Two milliliters of the reconstituted blue colorant powder (0.005 g powder in 1 mL deionized water) were diluted into 4 mL of buffer solution of different pH values of 2, 3, 4, 5, 6, 7, and 8. Then, the solutions were mixed in a vortex system for 30 s. The powders were totally solubilized. The color intensity was analyzed in a UV–Vis DU73 spectrophotometer (Beckman Coulter, Brea, USA) at 600 nm.

2.4.3. Thermal stability

Three milliliters of the reconstituted blue colorant powder (0.005 g powder in 1 mL deionized water) were heated to 100 ± 2 °C for 30 min in a water bath. The color parameters were analyzed in a colorimeter CR-400 apparatus (Konica Minolta, Tokyo, Japan) with the CIELab scale (*L*, *a*, and *b*) in reflectance mode. A UV–Vis DU73 spectrophotometer (Beckman Coulter, Brea, USA) at 600 nm was also used for this analysis. For the UV–Vis analysis, the samples were diluted 2× in deionized water.

2.5. Statistical analysis

Free genipin content, color, particle size, moisture, water activity, and rehydration behavior were determined in triplicate. Glass transition temperature, colorant power, and physical stability were performed in duplicate. Analysis of variance (ANOVA) was performed using Minitab 18® software to verify the fructan DP's effects on spray dried blue colorants. The Tukey mean test examined the differences between the mean values obtained at a 5% significance level (p-value ≤0.05). Physical structure, dynamic hygroscopy, visual appearance, and morphology results were analyzed descriptively.

3. Results and discussion

3.1. Impact of DP of fructan on the physical and chemical properties

3.1.1. Microstructure and particle size distribution

Fig. 1 shows the effects of the DP of fructan on the microstructure of the spray dried blue colorants. All microparticles presented a smooth surface without cracks. These characteristics favor the retention of blue compounds and rehydration capacity (Fritzen-Freire et al., 2012; Carmo et al., 2018). The fructan DP influenced the powder morphology since the increase in DP decreased the agglomeration tendency and particle size. Spray dried blue colorants produced without wall material or with FOS were more agglomerated than those produced using GR-In and HP-In as carriers. In general, oligosaccharides are hygroscopic which increases the moisture content on the surface of the particles leading to their agglomeration (Carmo et al., 2018). FOS are fructans more hygroscopic than inulin. Higher agglomeration was observed in microparticles produced with FOS (Patel & Goyal, 2011). Verruck, Liz, Dias, Amboni, and Prudencio (2019) used goat's milk and/or inulin and/or FOS as encapsulating matrices of *Bifidobacterium* BB-12. They also observed agglomeration in their samples, especially for those produced with the addition of FOS. Fritzen-Freire et al. (2012) encapsulated *Bifidobacterium* BB-12 using skim milk as an encapsulating system. For some treatments, inulin and FOS were added as wall materials. Microparticles without a prebiotic matrix were similar to those with inulin and FOS, indicating that these carrier materials did not affect the morphology of the particles.

Fig. 1 also presents the influence of the fructan based carriers and their DP on the particle size distribution. The microparticles produced without the carrier exhibited higher $D_{4,3}$ values (20 ± 1 μm). The DP influenced only the volumetric diameter. An increase in the DP decreased the mean particle size (p-value <0.001). Spray dried blue colorants produced with FOS, GR-In, and HP-In presented $D_{4,3}$ values of 15 ± 1 μm, 13 ± 2 μm, and 12 ± 1 μm, respectively.

The DP of fructan did not influence all parameters of the particle size distribution. However, the control sample exhibited higher values for d_{10}, d_{50}, and d_{90} (p-value <0.001). Control powders presented a larger particle size, probably because the complex formed by genipin and primary amine generated a polymeric complex of high molecular weight, possibly higher than the carbohydrates added in the other samples. In general, all microparticles presented sizes within the ideal range to be applied to foods. Duongthingoc et al. (2013) recommended an average particle size below 40 μm to provide a good mouthfeel for food products.

3.1.2. Water activity, moisture content, solubility, and wettability

The addition of carrier material and DP of fructan did not influence the water activity (p-value = 0.639) (Table 1). Spray dried blue colorants showed water activity values between 0.07 ± 0.01 and 0.10 ± 0.04. As can be observed in Table 1, the addition of wall material and fructan DP did not affect the moisture content of the powdered blue colorant (p-

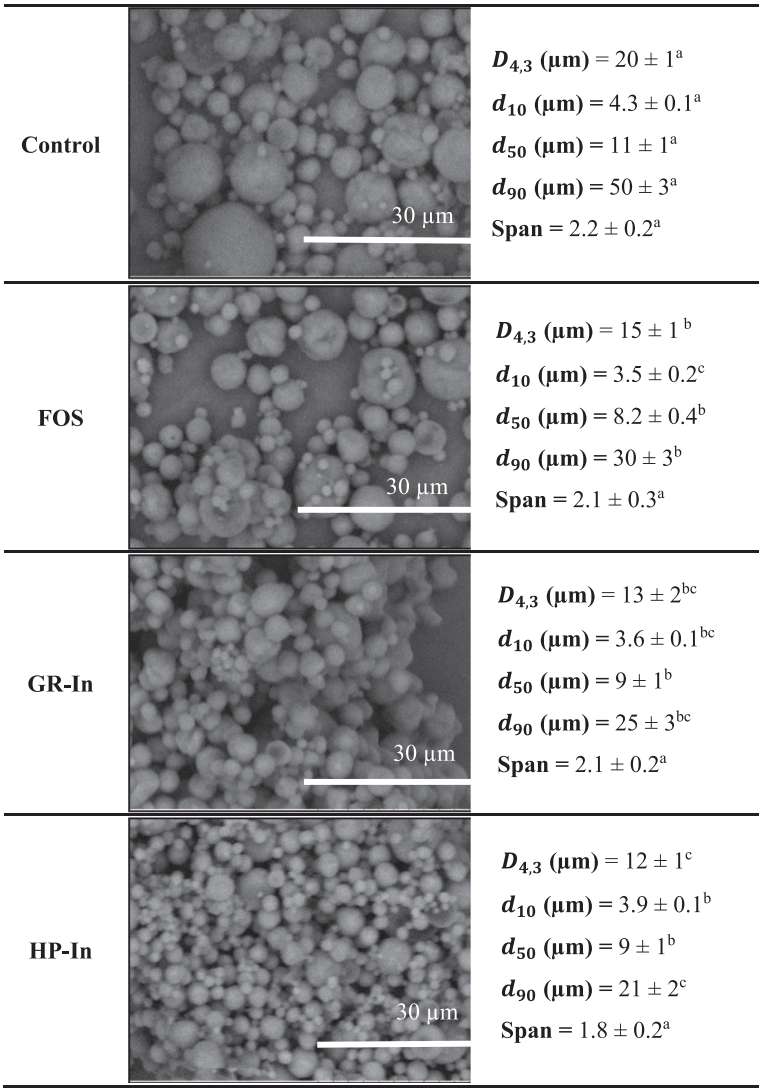

Fig. 1. Morphology of the spray dried blue colorants produced from fructans with different DP at 2500× magnification and particle size distribution. Control: powdered blue colorant without carrier material; FOS: powdered blue colorant carried by fructooligosaccharides; GR-In: powdered blue colorant carried by inulin DP ≥ 10; HP-In: powdered blue colorant carried by inulin DP ≥ 23. [a, b, c] letters represent significant differences at the 5% significance level. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

value = 0.171). The blue colorant powders presented moisture content between 2.2 ± 0.84 and 3.2 ± 0.7%.

Powdered ingredients are generally rehydrated before their use. Hence, their ability to reconstitute in water is essential for food formulation (Neves et al., 2021). Table 1 shows the rehydration behavior of the microparticles. The solubility and wettability of powder products depend on their composition and physical structure, and mainly regarding the affinity of their components with water (Crowley et al., 2016). Specifically, wettability depends more on the composition of the powder surface. Moreover, solubility is more dependent on physical structure such as particle size and morphology. The blue colorant powders presented some characteristics and properties interfering in their solubility and wettability. Spherical particles with small size present tendency to agglomerate (Fig. 1). Also, polymers with high molecular weights and the use of amorphous carbohydrates as carriers may impact the solubility and wettability of powdered products.

The carrier matrix influenced the rehydration properties of the blue colorant powders. Carbohydrates with low molecular weight, when dried, have an amorphous and hygroscopic structure (Crowley et al., 2016). The use of fructooligosaccharides increased the tendency of powdered milk to adsorb water. Particles with a high degree of aggregation compromise the rehydration properties. These powders, in general, have difficulties getting wet. Likewise, powders with high-fat content such as whole milk powder, have inferior rehydration properties than skim milk because of the expulsion of fat from the milk to the powder surface during storage, even for a short time (Phosanam et al., 2020). The surface composition of whole cow milk powder varied from 71% to 98% fat, 7.2% to 31% protein, and 0.8% to 13% lactose (Murrieta-Pazos et al., 2012). Powders containing inulin with DP ≥ 23 showed higher wettability values than other samples. Larger molecular chains could act as a physical barrier against water penetration, compromising the rehydration characteristics (Silva et al., 2016). Therefore, there are two distinct effects on powder wettability. The hygroscopicity of the carriers promotes particle aggregation reducing its ability to get wet. On the other hand, the carbohydrate molecular chain size contributes to lower agglomeration, preventing water penetration

Table 1
Effect of the degree of polymerization of fructan on the water activity, moisture content, solubility, wettability, and glass transition temperature of the spray dried blue colorants ($n = 6$).

Spray dried blue colorant	Water activity	Moisture content (%)	Wettability (s)	Solubility (%)	Glass transition temperature (°C)
Control	$0.10 \pm <0.01^{a}$	2.8 ± 0.8^{a}	320 ± 19^{b}	95 ± 1^{c}	83.5 ± 0.2^{c}
FOS	0.09 ± 0.01^{a}	3.2 ± 0.7^{a}	327 ± 25^{b}	99.2 ± 0.3^{a}	127 ± 1^{b}
GR-In	0.07 ± 0.01^{a}	2.4 ± 0.6^{a}	319 ± 11^{b}	98 ± 1^{b}	133 ± 3^{ab}
HP-In	0.10 ± 0.04^{a}	2.2 ± 0.8^{a}	376 ± 22^{a}	96 ± 1^{bc}	136 ± 2^{a}

Control: powdered blue colorant without carrier material; FOS: powdered blue colorant carried by fructooligosaccharides; GR-In: powdered blue colorant carried by inulin DP ≥ 10; HP-In: powdered blue colorant carried by inulin DP ≥ 23. Results are presented as mean ± standard deviation. [a, b, c] letters represent significant differences at the 5% significance level.

due to molecular affinity (Chever et al., 2017). The later effect explains the difficulty of all powders, including the control, in getting wet. The polymers formed from the crosslinking between genipin and milk proteins can also be present on the particle surface and with less affinity for water due to their molecular chain size.

High solubility values are directly related to small dust particles (see the d_{50}, approximately 9 μm, which is relatively small for food powders). Powders constituted by small particles tend to be cohesive, easy to solubilize, and hard to wet (Chever et al., 2017; Gnagne et al., 2017; Neves et al., 2019). In general, the particles showed similar solubility values found in the literature for powdered milk with particles of similar dimensions. Neves et al. (2019) observed that skimmed milk powder (d_{50}, approximately 15 μm) showed 95% solubility. The lower solubility of the control samples may be associated with high molecular weight polymers due to crosslinking. The use of carrier materials contributed to the solubility of all blue colorant powder samples. The fructan DP influenced the solubility of the samples (p-value = 0.013). Large molecules negatively interfere with particle solubility (Fan et al., 2018). The lower DP enhanced the solubility. The food industry commonly uses low molecular weight amorphous ingredients to improve powder solubility (Chever et al., 2017).

3.1.3. Dynamic hygroscopicity under different relative humidity conditions

The dynamic hygroscopicity behavior allows foreseeing the best storage conditions of the products. Water adsorption is the primary factor of spoilage in powdered products. Fig. 2 exhibits the results for water adsorption at 25 °C. FOS-based blue colorant powders showed greater hygroscopic capacity than other samples. Water adsorption in oligosaccharides of shorter molecular chain requires less energy (Silva et al., 2016). Thereby, the powdered blue colorant produced with FOS presented greater agglomeration as seen in Fig. 1. After 70% relative humidity, the spray dried samples showed higher water adsorption capacity, especially the samples with FOS. For relative humidity below 30%, all samples showed greater resistance to humidity raise. However, after 40% relative humidity, the samples showed an increase in adsorption capacity. Hence, the humidity range was critical for their physical stability. The higher the DP of the fructan, the lower was the water adsorption capacity, and the more stable the product would be. This effect is due to molecular length of the fructans, whose shorter chain contains more hydroxyl groups available for binding with water (Schaller-Povolny et al., 2000). The powdered blue colorant produced without carrier agent showed similar results that were observed for GR-In-based powders. This means that only using a matrix with a DP above 10 enhanced the stability of the products. A DP below 10 could end up damaging its stability. All powders remained visibly in the glassy state even at high relative humidity, which means that the microparticles could be added into foods without impairing the stability of the final products.

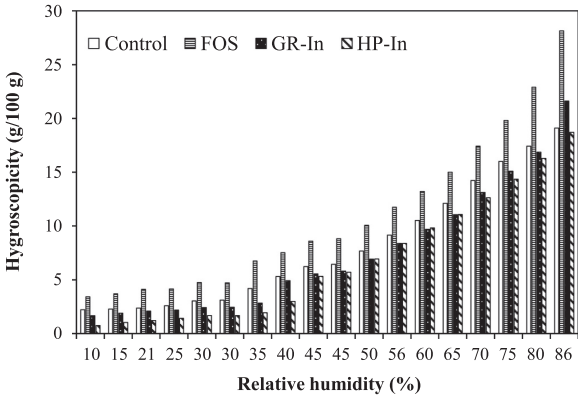

Fig. 2. Dynamic hygroscopicity under different relative humidity conditions at 25 °C. Control: powdered blue colorant without carrier material; FOS: powdered blue colorant carried by fructooligosaccharides; GR-In: powdered blue colorant carried by inulin DP ≥ 10; HP-In: powdered blue colorant carried by inulin DP ≥ 23. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.1.4. Physical structure and glass transition temperature

Fig. 3 shows the X-ray patterns of the spray dried blue colorants. All samples presented amorphous structures without crystalline regions. In general, amorphous solids are more prone to water sorption, more hygroscopic, and more susceptible to develop reactions (Fernandes et al., 2017). The storage of amorphous powders in a high relative humidity environment favors agglomeration or crystallization phenomena.

Table 1 presents the glass transition temperature (T_g). Powdered milk typically exhibits T_g values of approximately 65 °C at a relative humidity of 45% (w/w) (Jouppila & Roos, 1994; Schuck et al., 2012). In the present study, the high value of the glass transition observed in all

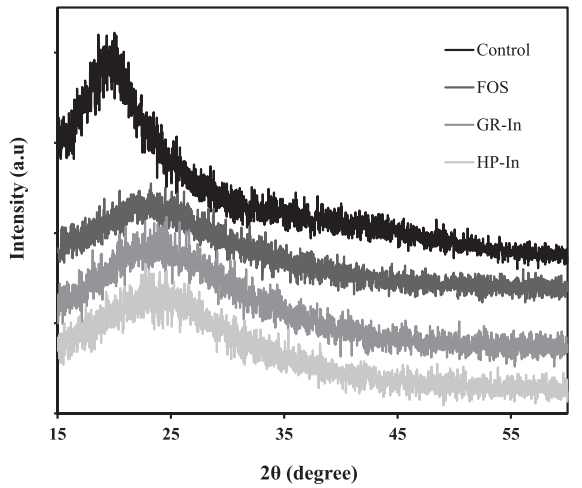

Fig. 3. X-ray patterns of the spray dried blue colorants: Control, FOS, GR-In, and HP-In. Control: powdered blue colorant without carrier material; FOS: powdered blue colorant carried by fructooligosaccharides; GR-In: powdered blue colorant carried by inulin DP ≥ 10; HP-In: powdered blue colorant carried by inulin DP ≥ 23. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

microparticles implies that the complex formed by genipin and primary amines led to the formation of a polymeric complex of high molecular weight according to the T_g value of the control microparticle (83 °C). High molecular weight polymers raise the T_g values (Jouppila & Roos, 1994; Schuck et al., 2012). Er et al. (2019) added transglutaminase to skimmed milk and produced spray dried milk. Higher transglutaminase proportions enhanced the crosslinking with caseins, increasing the molecular weight of the proteins besides increasing the T_g values of skimmed milk powder.

The wall materials contributed to the increase in T_g values compared to the control sample. HP-In-based powder showed a higher T_g value (p-value <0.001). We analyzed the glass transition temperatures of pure fructans. The commercial fructans used to produce the samples of FOS, GR-In, and HP-In exhibited T_g values of 132, 133, and 157 °C, respectively. The increase in the molecular chain of fructan increased the T_g values. Therefore, this same effect was observed for the T_g values of the fructan-based powdered blue colorants. The particles produced with biopolymers with high DP presented low moisture content, which contributes to powder redissolution (Lu et al., 2019).

3.1.5. Color attributes

Fig. 4 exhibits the impact of fructan DP on the spray dried blue colorants' color difference and visual appearance. According to the ΔE_{Lab} results, the fructan with the higher DP (HP-In) compromised the blue color intensity of the microparticles. HP-In is white when suspended in water. In this way, a mixture of white and blue decreased the blue intensity (p-value = 0.002) (González-Tomás et al., 2009). Likewise, HP-In-based spray dried colorants presented a more significant color difference compared to control sample (p-value = 0.009). Smaller polymeric chains can remain colorless, not interfering with the color intensity (Lacerda et al., 2016).

3.1.6. Free genipin content

Fig. 5 presents the free genipin content in the blue colorant-loaded milk and the spray dried blue colorants immediately after their production. Genipin interacts with milk primary amines and produces blue compounds right after its extraction from unripe genipap pulp. The milk is simultaneously used as a solvent extractor and supplier of primary amines for the reaction. However, part of the genipin stays in the colloidal system. The unreacted genipin was named free genipin content. More important than maintaining the free genipin content is maintaining the blue color. Nevertheless, as the compound responsible for the blue color is still unknown, the free genipin content can indirectly indicate the performance of the carrier matrix to carry the blue compound. A higher genipin loss can indicate that the fructan has a lower capacity to carry active compounds, which could be extended to the blue compounds.

The spray drying process promoted a decrease in the free genipin content. The genipin losses in the control sample were greater. Indeed, the carrier matrix was extremely important to retain more genipin after the drying process. Similar results were observed for the encapsulation of microorganisms. Microparticles produced with prebiotic carbohydrates as wall material were more efficient to protect the microorganisms (Raddatz et al., 2019).

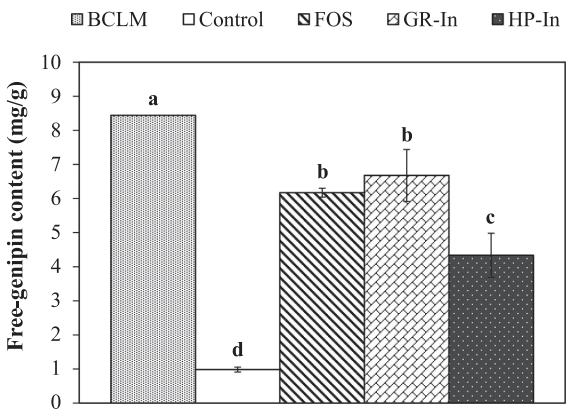

Fig. 5. Free genipin content of the blue colorant-loaded milk before its spray drying and the powdered blue colorants. BCLM: blue colorant-loaded milk; Control: powdered blue colorant without carrier material; FOS: powdered blue colorant carried by fructooligosaccharides; GR-In: powdered blue colorant carried by inulin DP ≥ 10; HP-In: powdered blue colorant carried by inulin DP ≥ 23. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

The DP of fructan influenced free genipin content (p-value = 0.001). The use of GR-In favored greater protection of genipin. Silva et al. (2016) studied inulins with DP ≥ 10 and DP ≥ 23 as wall materials for the encapsulation of annatto seed oil. They also observed better oil retention into the matrix using a lower DP. The authors claimed that the lower viscosity inherent in lower DP carriers promoted better retention of the compound due to its better homogenization of the liquid system (González-Tomás et al., 2009). Despite having a larger molecular size, which can provide a better barrier, a high DP did not help with the retention of some compounds (Silva & Meireles, 2015).

3.2. Impact of DP of fructan on the technological properties

3.2.1. Colorant power

Fig. 6 exhibits the visual appearance of the powdered colorants diluted

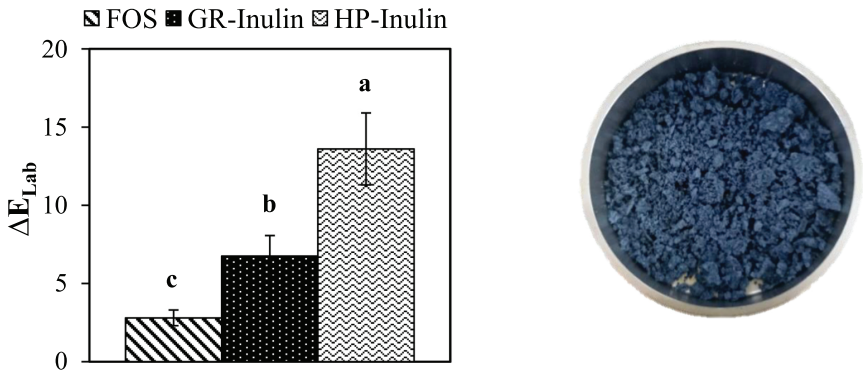

Fig. 4. Color difference (ΔE) between control and fructan-based spray dried blue colorants (n = 6) and visual appearance of the powdered control sample. FOS: powdered blue colorant carried by fructooligosaccharides; GR-In: powdered blue colorant carried by inulin DP ≥ 10; HP-In: powdered blue colorant carried by inulin DP ≥ 23.

in deionized water at the concentrations of 0.02, 0.01, 0.004, and 0.002 g/mL. The highest concentration (0.02 g/mL) is within the standards also seen in other natural colorants (Brauch et al., 2016; Buchweitz, 2016). This concentration needed to obtain a blue color can be applied on a large scale, considering that it is a natural and functional ingredient. The DP of fructan influenced the coloring power of the blue colorant. The color intensity of the colorants carried with FOS was higher, similar to the control. The use of a carrier matrix with a higher DP led to a lower blue color intensity, which may be due to the color interference of inulin in the powder.

3.2.2. pH stability

Table 2 exhibits the absorbance values at 600 nm of the reconstituted blue colorants that were conditioned in different buffer solutions with pH values ranging from 2 to 8. The different pH conditions changed the absorbance values for all samples of reconstituted blue colorant regardless of the DP of fructan. However, the most affected sample was that without carrier material (p-value $\leq$0.01). In control sample, the pH variation was more evident for each pH condition. Lower values of absorbance were observed at the most acidic pH (2 and 3) in the sample without a carrier. In this way, we can infer that the matrix contributed to the colorant stability at different pH conditions. A higher stability expands the possibilities to the application of this colorant in several food products regardless their pH range. Even in the industrial application of colorants, pH variations throughout the processing and storage will not affect the color of the final product. Usually, the stability of a natural colorant is pH-dependent, which impairs several applications (Buchweitz, 2016). In contrast, the powdered blue colorants presented excellent stability to different pH conditions, increasing their potential for application in various food products.

Table 2
Absorbance at 600 nm of the spray dried blue colorants reconstituted in water and subjected to media with different pH values (n = 4).

pH	Control	FOS	GR-In	HP-In
2	0.78 ± 0.02^{ef}	0.86 ± 0.03^{c}	0.90 ± 0.02^{ab}	0.88 ± 0.01^{b}
3	0.76 ± 0.03^{f}	0.91 ± 0.02^{a}	0.90 ± 0.01^{ab}	0.90 ± 0.02^{a}
4	0.86 ± 0.01^{b}	0.90 ± 0.02^{ab}	0.85 ± 0.03^{c}	0.82 ± 0.04^{b}
5	0.91 ± 0.02^{a}	0.90 ± 0.01^{ab}	0.91 ± 0.01^{a}	0.93 ± 0.04^{a}
6	0.8 ± 0.0^{de}	0.90 ± 0.01^{ab}	0.87 ± 0.02^{bc}	0.83 ± 0.02^{b}
7	0.82 ± 0.01^{cd}	0.87 ± 0.01^{bc}	0.88 ± 0.02^{abc}	0.85 ± 0.03^{b}
8	0.85 ± 0.01^{bc}	0.92 ± 0.01^{a}	0.89 ± 0.01^{ab}	0.84 ± 0.02^{b}

Control: powdered blue colorant without carrier material; FOS: powdered blue colorant carried by fructooligosaccharides; GR-In: powdered blue colorant carried by inulin DP $\geq$ 10; HP-In: powdered blue colorant carried by inulin DP $\geq$ 23. Results are presented as mean ± standard deviation. $^{a-f}$ Letters represent significant differences at the 5% significance level for treatment evaluated at different pH conditions.

3.2.3. Thermal stability

Table 3 shows the color parameters and the absorbance of the treated and untreated samples at 100 °C for 30 min. The heat treatment promoted a greater intensification of the color according to the increase in L values. The solutions became darker, highlighting the blue color, although there was no difference in the b value or absorbance. The DP of the fructans did not influence thermal stability. The carrier matrix did not enhance the stability at high temperatures because the colorant without the matrix is already thermally stable. Therefore, the thermally stable blue colorant produced in this study can be applied in formulations that may undergo intense thermal processes without color loss. This result is quite peculiar for a natural colorant since most natural colorants are not thermally stable (Albuquerque et al., 2020; Backes et al., 2020).

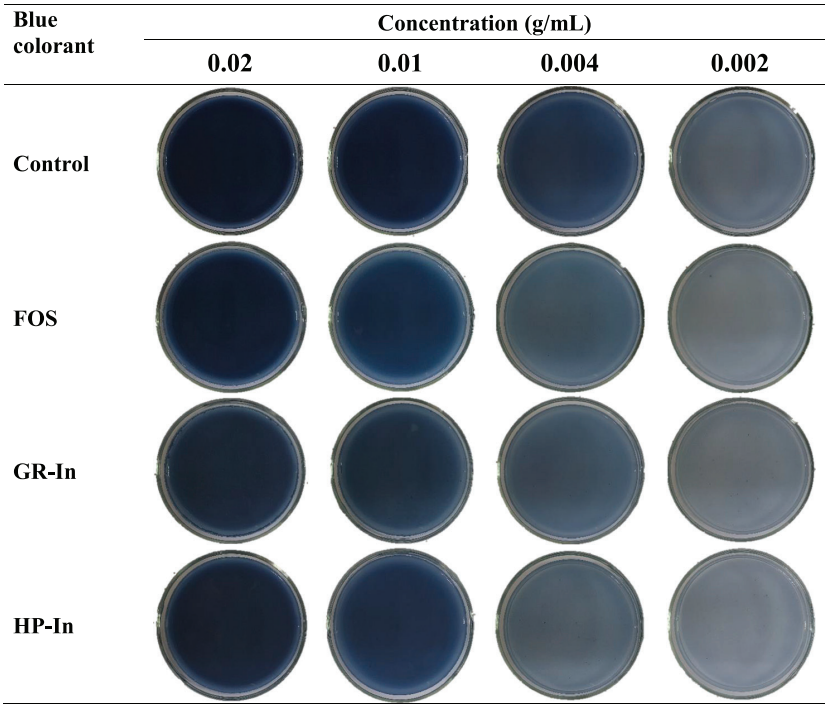

Fig. 6. Visual appearance of the reconstituted blue colorant powders at different concentrations. Control: reconstituted blue colorant without carrier material; FOS: reconstituted blue colorant carried by fructooligosaccharides; GR-In: reconstituted blue colorant carried by inulin DP $\geq$ 10; HP-In: reconstituted blue colorant carried by inulin DP $\geq$ 23.

Table 3
Color parameters and absorbance at 600 nm of powdered blue colorants reconstituted in water and subjected to heat processing at 100 °C/30 min (n = 4).

Blue colorant	Condition	*L*	*a*	*b*	Abs
Control	Untreated	22 ± 1	(−) 0.5 ± 0.2	(−) 6 ± 1	0.85 ± 0.01
	Treated	17 ± 1	(−) 0.3 ± 0.2	(−) 5 ± 1	0.81 ± 0.04
FOS	Untreated	26 ± 1	(−) 1.0 ± 0.1	(−) 5 ± 0	0.82 ± 0.03
	Treated	19.5 ± 0.2	(−) 0.2 ± 0.1	(−) 6 ± 0	0.80 ± 0.02
GR-In	Untreated	22 ± 1	(−) 1.3 ± 0.2	(−) 6 ± 0	0.87 ± 0.02
	Treated	19 ± 1	(−) 0.2 ± 0.0	(−) 6 ± 0	0.83 ± 0.02
HP-In	Untreated	23 ± 1	(−) 1.2 ± 0.2	(−) 5 ± 1	0.81 ± 0.02
	Treated	19 ± 1	(−) 0.1 ± 0.1	(−) 6 ± 0	0.80 ± 0.03

Control: powdered blue colorant without carrier material; FOS: powdered blue colorant carried by fructooligosaccharides; GR-In: powdered blue colorant carried by inulin DP ≥ 10; HP-In: powdered blue colorant carried by inulin DP ≥ 23. Lightness (*L*), red-green parameter (*a*), yellow-blue parameter (*b*).

4. Conclusion

In this study, the impact of the DP of fructan on physical, chemical, and technological properties of a blue colorant was evaluated. The blue colorant was stable at different pH conditions and high temperature. The DP influenced the sample characteristics. The morphology, size, water adsorption, and T_g values proved the effectiveness of inulin as a good wall material. FOS and GR-In showed more efficient for the retention of free genipin and regarding the rehydration properties. The use of a higher DP reduced the size of the particles and increased the T_g values. Fructans with lower DP were more susceptible to water adsorption, however, they presented better rehydration properties. The carriers with a lower DP (FOS and GR-In) produced spray dried blue colorants with greater blue color intensity. Reconstitution of these powdered colorants in water presented greater coloring power in the concentration range examined. Therefore, the use of carriers with lower DP, such as FOS and GR-In, was more promising for retention of the intensity of blue color and also its rehydration. This versatile blue colorant can be easily applied in dairy, confectionery, and bakery products.

CRediT authorship contribution statement

Maria Isabel Landim Neves: Investigation, Formal analysis, Writing - original draft.

Monique Martins Strieder: Investigation.

Ana Silvia Prata: Resources.

Eric Keven Silva: Conceptualization, Visualization, Methodology, Project administration, Writing - review & editing.

Maria Angela A. Meireles: Resources, Supervision.

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CHAPTER 7

Experimental article V

Could blue colorant improve its functionality being carried by xylooligosaccharides and be applied in some foodstuffs?

Xylooligosaccharides as an innovative carrier matrix of spray-dried natural blue colorant

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ABSTRACT

Xylooligosaccharides (XOS) are emerging prebiotic carbohydrates with a high potential to promote health and wellness due to their ability to modulate gut microbiota. Their use as a carrier of milk proteins-crosslinked genipin, an innovative natural blue colorant, was examined in this study. Fructooligosaccharides (FOS), a standard prebiotic carbohydrate, and maltodextrin (MD), a standard carrier material, were compared to XOS as wall materials. The effect of the carrier system on the powdered blue colorants was assessed regarding their size, morphology, free-genipin content, blue color intensity, moisture content, water activity, hygroscopicity, water adsorption, and physical structure. Their technological properties as food colorants were evaluated by incorporating in foodstuffs: *beijinho*, Chantilly, and muffin. XOS exhibited positive effects compared to other carriers evaluated. XOS-based microparticles presented higher free-genipin retention; smaller particle diameter equal to 6.5 ± 0.4 μm, and higher blue color intensity. The microparticles produced with XOS were more hygroscopic compared to other carrier matrices. However, similar to FOS and MD, the blue colorant carried with XOS presented an amorphous structure. XOS was the best carrier in food applications due to the less interference on the blue color in the powder colorant. The XOS microparticles could be easily incorporated into some food matrices. Therefore, spray-dried blue colorant using XOS presented significant innovative potential in many food products, acting as a functional ingredient, replacing artificial blue colors, and incorporating prebiotic properties.

1. Introduction

Xylooligosaccharides (XOS) are prebiotic oligosaccharides composed of chains of xylose residues, linked through β-(1,4)-xylosidic bonds (de Freitas, Carmona, & Brienzo, 2019). Depending on the xylan source and production process, XOS vary from 2 to 10 xylose units (Amorim, Silvério, Prather, & Rodrigues, 2019). XOS can be found in bamboo shoots, fruits, vegetables, milk, and honey (Aachary & Prapulla, 2011). The wide interest in incorporating XOS in food matrices aims to increase their functional properties (Farias, de Araújo, Neri-Numa, & Pastore, 2019; Hutkins et al., 2016). Food supplementation with prebiotic carbohydrates supports the control of health disorders like high cholesterol, high blood sugar, weight gain, and some allergies (Licht, Ebersbach, & Frøkiær, 2012). These carbohydrates are fermented by the gut microbiota and produce short-chain fatty acids related to improving several physiological effects, including intestinal function, absorption of minerals, and regulation of lipid and glucose metabolism (Dávila, Gullón, Alonso, Labidi, & Gullón, 2019). Also, prebiotic compounds can modulate the intestinal macrobiotic, improving the absorption of nutrients and protecting the organism from pathogens colonization (Hutkins et al., 2016; Raddatz et al., 2019). Along with the health benefits, XOS can be used in foods to improve their technological properties. Ferrão et al. (2018) applied XOS in cream cheese. This ingredient reduced the fluidity and the grained size and increased the elasticity and firmness of the product. Comparing to the other prebiotic carbohydrates, XOS have been preferred due to their organoleptic properties and better chemical stability in high temperatures up to 100 °C and different pH conditions (2.5–8) than inulin (IN) and fructooligosaccharides (FOS) (Aachary et al., 2011; Amorim et al., 2019). Additionally, XOS showed higher resistance to digestion and were more effective in stimulating *bifidobacteria* than FOS and IN (Aachary et al., 2011; Amorim et al., 2019). Because of their higher prebiotic efficiency, XOS present the smallest effective dosage required to be considered a functional ingredient. The minimum recommended intake of XOS per day is 1.4 g. In

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contrast, other prebiotic carbohydrates, such as FOS and IN, the recommended intake is 8 and 5 g per day, respectively. (Finegold et al., 2014). XOS are non-cariogenic and low-calorie, allowing their utilization in anti-obesity diets. They present advantages over IN regarding resistance to acid and high-temperature conditions in food processing, allowing their utilization in low-pH juices and carbonated drinks (Vazquez, Alonso, Domınguez, & Parajo, 2000).

Besides nutritional and health approaches, some prebiotic carbohydrates could exhibit the potential as a functional carrier agent by bioactive compounds (Farias et al., 2019). It could be a physically stable carrier to sustain *Lactobacillus paracasei* subsp. *paracasei* during storage and under the human gastrointestinal tract environment (Mantzouridou, Spanou, & Kiosseoglou, 2012). IN was evaluated as a suitable carrier for spray-dried ginger essential oil (Fernandes et al., 2017). FOS allowed greater viability of *L. rhamnosus* in coatings applied to blueberries (Bambace, Alvarez, & Moreira, 2019), in functional cream cheese (Speranza et al., 2018), green tea (de Souza et al., 2017), and in yogurt (Delgado-Fernández, Corzo, Olano, Hernández-Hernández, & Moreno, 2019). FOS presented similar proprieties as maltodextrin (MD) for carrier chlorophyll (Femat-Castañeda et al., 2019). Gum Arabic (GA) and FOS were used to encapsulate major phenolics on yerba mate extract with 80% efficiency (Pilatti-Riccio et al., 2019). Using prebiotic carbohydrates as carrier materials instead of MD and GA (the most common for this application) has gained force because they can be used in free-sugar products for diabetic people and promote health benefits. The most used carrier is MD, considered a commercial application standard. The degree of polymerization (DP) of the MD can vary between approximately 4 to 22. FOS is already used as a carrier. Although it is not yet widespread in the industry sector, it has nutritional advantages over MD because of its prebiotic character. The FOS DP varies between 3 and 10 (de Oliveira et al., 2011).

To date, FOS and IN-type fructans have dominated the prebiotics market. However, XOS have emerged commercially in Southeast Asia. The Asiatic market is the most significant consumption of XOS on a global scale. Nevertheless, XOS present potential growth in the world, as their techno-proprieties begin to be known. In 2018, the European Union approved the commercialization of XOS as a novel ingredient, based on European regulation 2015/2283. Recently, Prenexus Health (USA), also received GRAS certification for the production of XOS (Santibáñez et al., 2020; Saville & Saville, 2018). In this scenario, the XOS market can reach $ 130 million by 2025, growing from $ 94 million in 2018 with an annual growth rate of 4.1% during 2016–2018 (Singh, Singhania, Pandey & Larroche 2019). Despite these advantages comparing to other prebiotics and promising characteristics, XOS has not yet been used as a carrier for bioactive compounds, essential oils, and natural colorants, aiming to produce novel functional ingredients.

Among the known bioactive compounds, genipin is found in unripe genipap (*Genipa americana* L.), a native fruit to northern South America, the Caribbean, and southern Mexico (Ramos-De-La-Pena, Renard, Montañez, de la Luz Reyes-Vega, & Contreras-Esquivel, 2016). This fruit has been widely studied as a precursor to natural blue colorant, formed by the crosslinking between genipin and primary amines in amino acids, peptides, and proteins (Neri-Numa et al., 2020). Our research group recently developed a promising blue food colorant from the milk and genipap pulp (Neves, Strieder, Silva, & Meireles, 2020; Strieder, Neves, Silva, & Meireles, 2020). In this way, drying this colorant system could facilitate its application in food products. Regarding the healthy products sector, carrying this colorant with a prebiotic matrix can improve its market acceptability. The blue colorant powder could be incorporated in some foodstuffs like cakes, creams, pasta, ice cream, sweets, and others.

Therefore, this study aimed to evaluate the potential of XOS as carriers of a natural blue colorant obtained from the crosslinking between milk proteins and genipin. For this, MD and FOS were compared to XOS as wall materials for the production of spray-dried blue colorant. Also, the blue colorant powders with different carriers were incorporated in food matrices to verify their effect on the product's visual appearance.

2. Material and methods

2.1. Production of natural blue colorant

The natural blue colorant was produced from the crosslinking between milk proteins and genipin extracted from unripe genipap pulp according to the methodology described by Neves et al. (2020). For each 5 g of unripe genipap pulp were used 25 g of milk. The manufacturing procedure was performed until obtaining 600 g of the blue colorant-loaded milk. Then, all colorant fractions were mixed in a blender MX1500 (Waring, Dalton, United States).

2.2. Spray drying of natural blue colorant

MD, FOS, and XOS were used as wall materials for the production of blue colorant powder. MOR-REX® from non-genetically modified corn (MD, DP = 9) (Ingredion, Mogi Guaçu, Brazil). Orafti®FOS from chicory (FOS, DP = 5) (SweetMix, Sorocaba, Brazil). PreticX™ from non-genetically modified corn derived (XOS, DP = 2 to 6) (AIDP Inc., City of Industry, California, United States). PreticX™ samples were characterized by Silva, Arruda, Pastore, Meireles, and Saldaña (2020) regarding their xylooligosaccharides composition (30.2% xylobiose, 35.7% xylotriose, 23.9% xylotetraose, 6.1% xylopentaose, and 4.0% xylohexaose). FOS and XOS suspensions were separately prepared by dissolving 20 g of them into 80 g of deionized water (Millipore®) at 80 °C. After their complete dissolution, the suspensions were cooled to 40 °C. Twenty grams of MD were added into 80 g of deionized water, and the suspension was left to stand for 24 h at 25 °C. Two hundred grams (with 10.1 g of solids per 100 g of suspension) of natural blue colorant-loaded milk were gradually added to each suspension using a rotor-stator type homogenizer model 713D (Fisatom, São Paulo, Brazil) at 2000 rpm for 5 min. Total solids concentration (carrier + blue colorant) was equal to 10.35 g of solids per 100 g of total suspension. Each 100 g of suspension contained a 3.20 g dry mass of natural blue colorant-loaded milk. Then, 280 g of the suspension was dried in a spray dryer SD-06 (LabPlant, Hunmanby, United Kingdom). The atomization was performed in a short cylindrical chamber (of diameter 0.215 m, and length 0.5 m) by a 1 mm nozzle mounted concentrically with the cylinder axis. With 180 °C as inlet temperature, feed rate in 8.1 mL/min, and the atomizing airspeed in 3.9 m/s. The samples were stored at 25 ± 2 °C. The powdered blue colorants were produced in duplicate.

2.3. Characterization of spray-dried blue colorant

2.3.1. Morphology

The morphology was assessed by scanning electron microscopy. The samples were placed on a double-sided carbon tape (Ted Pella, Redding, United States) fixed to aluminum stubs. The images were captured under a microscope TM-4000 (Hitachi High Technologies, Tokyo, Japan) using a 2500 × magnification.

2.3.2. Particle size distribution

The particle size distribution was analyzed at 25 °C by light scattering technique using the laser diffraction Mastersizer 2000 (Malvern Instruments, Malvern, United Kingdom). The average size was characterized in terms of volumetric mean diameter ($D_{4,3}$) (Eq. (1)) and the polydispersity index calculated by the span (Eq. (2)). The samples were analyzed by a wet method, with dispersion in water and a refractive index of 1.52. The particle size distribution was determined according to d_{10}, d_{50}, and d_{90}, which are the diameters at 10%, 50%, and 90% of the cumulative number, respectively. The particle size distribution was measured in quintuplicate (n = 5).

$$D_{4,3} = \frac{\sum_{i=1}^{k} n_i . d_i^4}{\sum_{i=1}^{k} n_i . d_i^3} \quad (1)$$

Fig. 1. Visual appearance of spray-dried blue colorants with MD, FOS, and XOS as carrier matrices. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

$$span = \frac{(d_{90} - d_{10})}{d_{50}} \quad (2)$$

2.3.3. Free-genipin content

The free-genipin content in the powdered blue colorant samples was quantified before and right after their spray drying by high-performance liquid chromatography (HPLC) using an HPLC-PDA Alliance E2695 (Waters, Milford, United States). The methodology was developed and validated by Neves et al. (2020). Aliquots of 0.5 mL of liquid blue colorant and reconstituted colorant powder in ultrapure water (12.5 g/100 g of suspension) were dissolved in 2 mL of ethanol and manually homogenized. Then, the mixture was centrifuged at 12,000 rpm for 15 min. After, an aliquot of 1 mL of supernatant was filtered using nylon membrane (0.45 μm) and injected into the equipment. The free-genipin quantification was carried out according to Neves et al. (2020). The analysis was made in duplicate (n = 2).

2.3.4. Color

The color parameters were determined by a CR-400 apparatus (Konica Minolta, Tokyo, Japan) using the CIELab scale (*L*, *a*, and *b*) with reflectance mode. The blueness index (BI) was used (Eq. (3)) to evaluate the effect of the carrier matrix used concerning the blue color intensity:

$$BI = 100 * \frac{(C_x * X) - (C_z * Z)}{Y} \quad (3)$$

where C_x = 1.3013 and C_y = 1.1498 are illuminates (constants) for observer D65; 10°. Furthermore, X, Y, Z are color coordinates by the XYZ color scale (ASTM, 2014). The color measurement was performed in duplicate (n = 2).

2.3.5. Moisture content, hygroscopicity, and water activity

The moisture content was determined by the AOAC method 925.10 (AOAC, 1993). The hygroscopicity was measured according to Vardanega, Muzio, Silva, Prata, and Meireles (2019). Powder samples (1.0 g) were placed in a container with saturated NaCl solution (75.3% relative humidity) at 20 °C. The gain of weight due to the moisture adsorption was recorded daily until the thermodynamic equilibrium. The results were expressed as g of adsorbed moisture/100 g of sample. The water activity (a_w) was determined with the water activity analyzer Labtouch-a_w (Novasina, Lanchen, Switzerland). These analyses were performed in quadruplicate (n = 4).

2.3.6. Dynamic water adsorption under different relative humidity conditions

Two grams of powder was used in Dynamic Dewpoint Isotherm (DDI) (Aqualab VSA-Meter group, Pullman, United States). The parameters were the following: 0.1 to 0.9 a_w, 25 °C, airflow 100 mL/min. The desorption until 0.1 a_w and adsorption until 0.9 a_w. The DDI Moisture Analysis Toolkit software predicted the initial samples' water content (Meter group, Pullman, United States).

2.3.7. Physical structure

The physical structure of the samples was categorized by X-ray diffraction using a diffractometer PW 1710 (Philips, Almelo, Netherlands) using Bragg-Brentano geometry (θ:2θ) with radiation of Cu-Kα_1 (λ = 1.54178 Å), 40 kV of voltage and current of 30 mA. Measurements were obtained at intervals of 0.02° and acquisition time at 2 s, with verification from 5 to 40° (2θ angular scan).

2.4. Application of powdered blue colorants in foodstuffs

The spray-dried natural blue colorant produced with MD, FOS, and XOS were used to formulate three different foods. The same powdered sample was tested for each formulation to eliminate possible differences concerning the colorant manufacturing process.

2.4.1. Beijinho

A hundred grams of condensed milk, 20 g of milk cream, 1 g of butter, and 1.2 g of spray-dried blue colorant were heated and stirred for 6 min (until the mixture was easily detached from the pan's bottom). The control was made in the same way without the blue microparticles. After cooling, the dough was divided into 10 g portions, rolled in small spheres, and added grated coconut (typical pastry presentation).

2.4.2. Chantilly

A hundred thirty mL of fresh milk cream (with 35 g of fat in 100 g) and 1.2 g of spray-dried blue colorant were put in a food mixer for 2 min at low speed, then 15 g of sugar was added. After, the mixture was stirred for 2 min at medium speed. The control (without colorant) was produced equally.

2.4.3. Muffin

Four hundred grams of wheat flour, 200 g of milk, 100 g of sugar, three eggs, 70 g of buttermilk were mixture manually for 5 min. Later, eight portions with 130 g of the dough were separated into recipients. Two portions of dough were used as the control, and for the others, 1.2 g of spray-dried blue colorant was added to each recipient. After, 0.5 g of cake yeast was added to each container. The mixtures were placed in cupcake forms and taken to the oven at 180 °C for 25 min.

2.4.4. Color evaluation

The *beijinho* and muffin's color was measured in a portable colorimeter reading the values in three distinct cross-sect spots. Chantilly's color was measured in three distinct external points. The *L*, *a*, *b*, Δ*BI*, and ΔE_{Lab} parameters were used to evaluate color attributes. The BI values were determined according to section 2.3.2. The ΔBI values were calculated by the difference between the *BI* of the colored samples and the control sample. The total color difference (ΔE_{Lab}) between the

Table 1
Moisture content (g H_2O/100 g), hygroscopicity (g adsorbed H_2O/100 g), a_w, particle size (μm), and span values of the spray-dried natural blue colorants.

Microparticle	Moisture (g/100 g)	Hygroscopicity (g/100 g)	a_w	$D_{4,3}$ (μm)	d_{10} (μm)	d_{50} (μm)	d_{90} (μm)	Span
MD	1.6 ± 0.7^{b}	11.5 ± 1^{b}	0.08 ± 0.02^{a}	18 ± 4^{a}	3.8 ± 0.2^{a}	12.8 ± 0.3^{a}	22.2 ± 0.8^{a}	1.63 ± 0.04^{b}
FOS	1.5 ± 0.5^{b}	13.2 ± 1^{a}	0.09 ± 0.02^{a}	10 ± 2^{b}	2.5 ± 0.1^{b}	8.2 ± 0.2^{b}	17 ± 1^{b}	2.3 ± 0.1^{a}
XOS	2.8 ± 0.5^{a}	16.9 ± 1^{a}	0.08 ± 0.00^{a}	6.5 ± 0.4^{b}	2.3 ± 0.2^{b}	5.3 ± 0.4^{b}	11 ± 1^{b}	1.6 ± 0.1^{b}

Results are presented as mean ± standard deviation. [a, b] letters represent significant differences at the 5% significance level for samples evaluated with different carrier matrix.

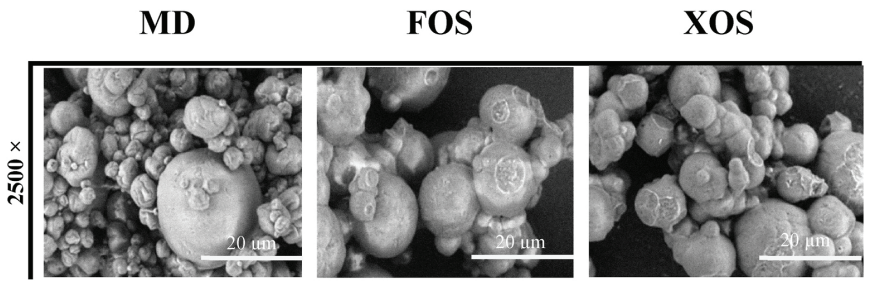

Fig. 2. Surface morphology of the blue colorant powders with different carrier matrix: MD, FOS and XOS.

colored and the control samples was calculated using Eq (4):

$$\Delta E_{Lab} = \sqrt{(\Delta L)^2 + (\Delta a)^2 + (\Delta b)^2} \quad (4)$$

2.5. Statistical analysis

The results of free-genipin content, color, particle size, moisture, a_w, dynamic water adsorption, hygroscopicity, and food color attributes were analyzed using Minitab 18® software to verify the effects of the carrier agents used by analysis of variance (ANOVA). Tukey's test examined the differences between the mean values obtained at a 5% significance level (p-value < 0.05). The morphology, X-ray diffractograms, and visual appearance were analyzed descriptively.

3. Results and discussion

3.1. Characterization of the spray-dried blue colorants

Fig. 1 presents the microparticles produced using MD, FOS, and XOS. Visually, the powders showed a blue color, with slight differences in intensity. The FOS and XOS matrices favored the obtaining of the highest blue intensity. The white color of the MD matrix and its larger molecular chain size impaired the blue colorant's intensity. Differently than FOS and XOS, which interfered less in the colorant color. All samples exhibited the same visual characteristics, with small particles and a high tendency to agglomerate.

3.1.1. Morphology and size distribution

Table 1 shows the size and distribution of the microparticles. Fig. 2 presents the images obtained by SEM of the microparticles produced using different carrier matrices. The particle size and morphology affect various powders' technological properties, such as bulk density, flowability, compaction, rehydration, and solubility. Likewise, these properties can influence the application of powdered ingredients in foodstuffs.

The atomization resulted in fine powders with a spherical shape, an irregular surface, and several indentations. Fine powders tend to have low wettability and more hygroscopic. The size range found in this study is expected for spray-dried powders produced in bench equipment (Etzbach et al., 2020; Dias et al., 2018). The carrier showed a significant effect on the $D_{4,3}$ of the particles (p-value = 0.001) (Table 1). The

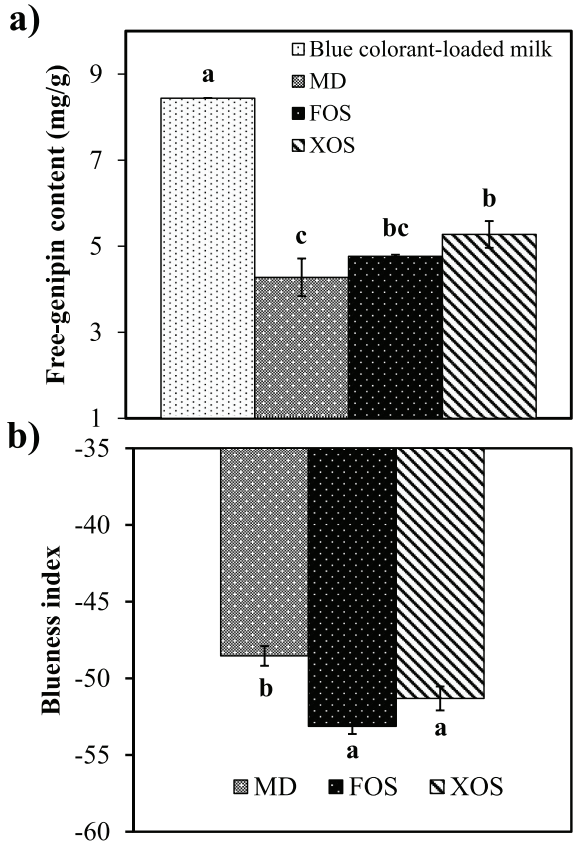

Fig. 3. Free-genipin content of the blue colorant-loaded mlk and the microparticles (a). The blueness index of the microparticles (b). Results are presented as mean ± standard deviation. a, b, c letters represent significant differences at the 5% significance level for samples evaluated with different carrier matrix.

particles with XOS and FOS had smaller sizes. Likewise, comparing to the size distribution, the particles with FOS and XOS presented means lower than MD (p-value = 0.012). The particle size is directly affected by the viscosity of the dispersion. Particles produced with MD tend to have higher viscosity due to the larger polymeric chain of this material. Nevertheless, XOS and FOS have smaller chain sizes. Their low viscosity enhances the liquid jet breakup, leading to smaller droplets and thus smaller particles (Neves, Desobry-Banon, Perrone, Desobry, & Petit, 2019; Dias et al., 2018). Consequently, XOS and FOS microparticles tend to be smaller than MD.

According to Fig. 2, there were no visual differences in the shape and surface of the blue colorant microparticles. However, the carrier material used affected the powders' morphology regarding the agglomeration behavior. The agglomeration and the formation of link bridges between the particles might be related to the low glass transition temperature of the powders (Dias et al., 2018). Low molecular weight polymers and monomers have been reported to have lower glass transition temperature values in their pure form. In comparison, longer chain molecules have higher glass transition temperature values (Shishir & Chen, 2017). According to other studies, the XOS matrix showed higher hygroscopicity, favoring the particle agglomeration (Zhang et al., 2018).

The images showed no evidence of pores or cracks, which is vital to ensure low permeability (Ortiz-Basurto, Rubio-Ibarra, Ragazzo-Sanchez, Beristain, & Jiménez-Fernández, 2017). Also, the particles produced with XOS presented morphological characteristics similar to the particles produced with MD and FOS.

3.1.2. Free-genipin content and color parameters

Fig. 3 presents the influence of the carrier matrix on the free-genipin retention and blueness index of the powdered blue colorants. We can also see the free-genipin content of the natural blue colorant-loaded milk just before its atomization. The genipin extracted from the genipap can reticulate with primary amines and produce the blue colorant or stay in a colloidal system. The last one is denominated free-genipin (Fig. 3-a). Since the chemical structure of genipin reticulated in milk proteins is still unknown, it could not be identified and quantified by chromatographic analysis. Thus, we use the free-genipin content as a parameter to indicate the carrier potential to retain and protect the genipin during the atomization process. The higher genipin loss indicates the less genipin carrier capacity by the matrix. Consequently, this effect also extends to the blue compound formed.

The microparticle with XOS showed a higher free-genipin content after drying, indicating XOS as the best carrier for the blue colorant comparing to MD. The FOS matrix was statistically equal to XOS and MD, indicating an intermediate carrier behavior. The XOS matrix presents the smallest molecular size and the highest polarity than MD. This characteristic probably contributed to a more significant interaction between XOS and genipin, helping in its better retention even under atomization conditions. Ortiz-Basurto et al. (2017) studied the microencapsulation of anthocyanin using MD and fructans of different DP as wall materials. The microparticles produced with fructans showed greater anthocyanin retention compared to MD. However, Araujo-Díaz, Leyva-Porras, Aguirre-Bañuelos, Álvarez-Salas, and Saavedra-Leos (2017) indicated that MD was more effective as a wall material than IN to protect the active materials due to the physicochemical properties of MD.

The blueness index (*BI*) measures the blue color intensity (Fig. 3-b). For all samples, *BI* presented the color characteristic of the blue colorant-loaded milk (Neves et al., 2020). It means that both the atomization process and the carrier did not de-characterize the colorant. Instead, there was a significant effect of the carrier matrix on the color of the powdered colorants (p-value = 0.013). In general, MD presented a more accentuated white color than FOS and XOS, interfering with the microparticle color. Indeed, the *BI* value of the microparticles with MD was the lowest (−49 ± 1).

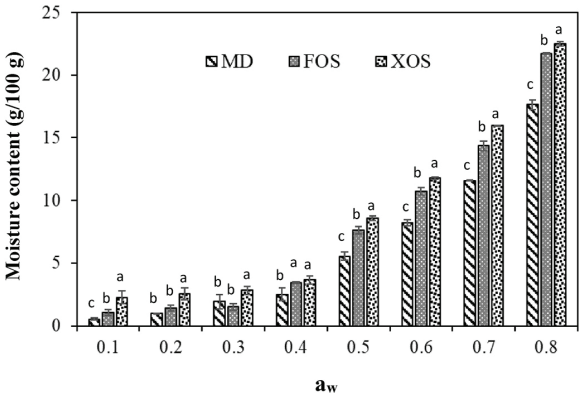

Fig. 4. Water adsorption for each a_w (0.1; 0.2; 0.3; 0.4; 0.5; 0.6; 0.7; 0.8) at 25 °C of the microparticles produced using the carriers: MD, FOS and XOS. Results are presented as mean ± standard deviation. a, b, c letters represent significant differences at the 5% significance level for samples evaluated with different carrier matrix.

The carrier cannot hide the compound's color without color interference and reacts with the colorant (Đorđević et al., 2015). Thus, the results observed for free-genipin content and BI indicated that the XOS molecular structure was favorable for their use as a carrier matrix of the blue colorant due to their less color interference in the microparticles and greater free-genipin retention.

3.1.3. Moisture content, hygroscopicity, and a_w

The moisture content, hygroscopicity, and a_w, of the spray-dried blue colorants are shown in Table 1. All formulations produced microparticles with less than 0.2 a_w and less than 3.4 g H_2O/100 g d.m. (dry matter) moisture content. Powders that reach values below these present suitable storage stability features and allow microbiological stability (Ortiz-Basurto et al., 2017). In general, microparticles prepared at higher drying temperatures and higher carrier concentrations present a lower a_w and moisture content. No significant difference in a_w was observed among the samples (p-value = 0.7). However, powders' moisture content varied from 1.5 ± 0.5 to 2.8 ± 0.5 g H_2O/100 g d.m. Among the powders, the one produced with XOS presented the highest value (p-value = 0.002). This property is possibly linked to its carbohydrate composition (Ortiz-Basurto et al., 2017). XOS presents a higher affinity for water, difficulting the drying process more than the other two matrices. Zhang et al. (2018) produced powders containing XOS using MD as a carrier. Their results showed that high XOS concentration became the powders more humid. They assumed, therefore, that XOS has a high-water retention capacity. The hygroscopicity results were according to moisture content. They are showing the higher water adsorption capacity of XOS among FOS and MD. The adsorption and water retention of a powder are crucial parameters in identifying drying and storage conditions and suitable packaging choices (Schuck, Jeantet, & Dolivet, 2012; Vardanega et al., 2019). According to Nurhadi, Andoyo, Mahani, and Indiarto (2012), powders with <20% hygroscopicity are regarded as acceptable as hygroscopic material. Our microparticles presented the % hygroscopicity compatible with this statement, varying from 11.5 to 16.9. Thakur and Nanda (2019) produced polyphenol-rich milk powder, and the hygroscopicity ranged from 13.10 to 18.20%. Hygroscopicity depends on a powder's composition, particularly in very hydrophilic components (proteins, carbohydrates, and others) and particle size (Schuck et al., 2012). In our study, XOS and FOS presented the highest hygroscopicity (p-value = 0.003) due to their smaller structure. They are chemically more attracted by water because they present more hydrophilic components. Microparticles produced with cladode mucilage and MD to carry yellow-orange

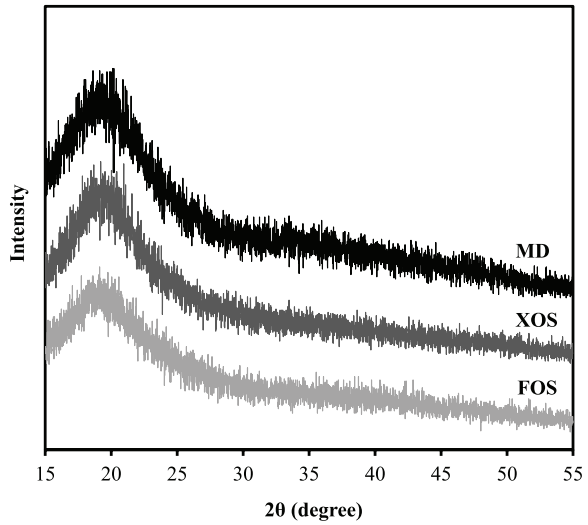

Fig. 5. X-ray patterns of the blue colorant powders with MD, FOS and XOS.

cactus pear pulp presented a hygroscopic range between 21.3 and 31.5%. This range was higher than our findings (Carmona, Robert, Vergara, & Sáenz, 2021).

3.1.4. Dynamic water adsorption under different relative humidity conditions

Fig. 4 shows the increase in the moisture content suffered by the microparticles with MD, FOS, and XOS due to the dynamic increase of a_w at 25 °C. In general, powders with a high amount of low molecular weight sugars contribute to higher water adsorption. Phisut (2012) mentioned that the transport agents' chemical structure explains the differences in water adsorption. In carbohydrates, the water adsorption phenomenon is attributed to the bonds between the hydrogen in the water molecules and the hydroxyl groups available in the substrate's amorphous regions and the crystalline regions of the surface. The greater intrinsic hygroscopicity of the sample leads to more significant water adsorption.

The particles with XOS showed greater ease in increasing the moisture content. Products with higher moisture adsorption tend to be more unstable and can quickly lose their powder properties. With a high probability of agglomeration, decreased glass transition temperature makes the product easily pass to a gummy state even at room temperature (Silva, Zabot, Bargas, & Meireles, 2016). The microparticles with XOS tend to adsorb water due to their smaller structure and greater affinity for water (Domian, Brynda-Kopytowska, Cieśia, & Ostrowska-Ligeza, 2017).

Until a_w at 0.3, the microparticles adsorbed water content in less than 5% of moisture. After 0.4 of a_w, the water adsorption was increasing quickly, which means that in a 40% humid or more, it can be a critical point to long-time storage of blue colorant powder (Domian et al., 2017).

3.1.5. Phase structure

Fig. 5 exhibits X-ray patterns of the microparticles. All samples presented the amorph structure without crystalline regions. In general, amorphous structures are disordered and produce dispersed bands without peaks of crystallization. Atomized products are usually amorphous due to insufficient crystallization time (Chiou & Langrish, 2007). Amorphous solids are generally more soluble than crystalline structures due to their bonds and molecules' low energy levels. However, on the other hand, they are more hygroscopic (Silva & Meireles, 2015). Additionally, the storage of amorphous powders in an environment of high relative humidity can lead to their agglomeration or crystallization.

The amorphous structure of the microparticles was due to the amorphous structure of the formed blue compound and the carrier matrices. Oligosaccharides are known to have an amorphous structure in the solid state (Botrel, de Barros Fernandes, Borges, & Yoshida, 2014; Glibowski & Pikus, 2011; Glibowski, Pikus, Jurek, & Kotowoda, 2014). As carriers of spray-dried compounds, MD and FOS are amorphous (Alves et al., 2014; Vardanega et al., 2019). This study showed that XOS are also amorphous as a carrier matrix. The advantage of carriers being amorphous solids is the ability to present stability of their structure in environments that do not reach high humidity. Contrary to crystalline solids in which they present polymorphic forms, changes in their structure can result in the expulsion of the material carried (Labuschagne, 2018).

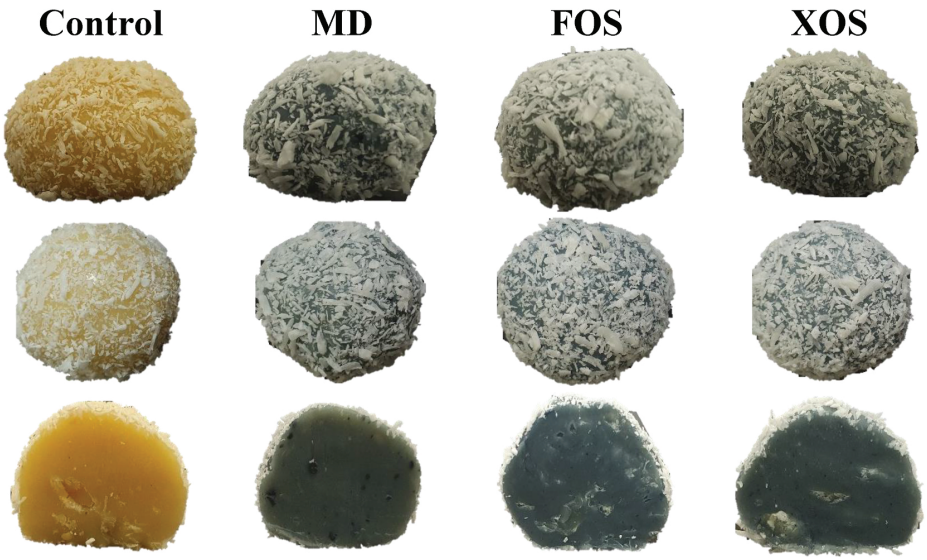

Fig. 6. Visual appearance of beijinho without colorant (control); incorporated with colorant with MD (MD); with FOS (FOS); and with XOS (XOS).

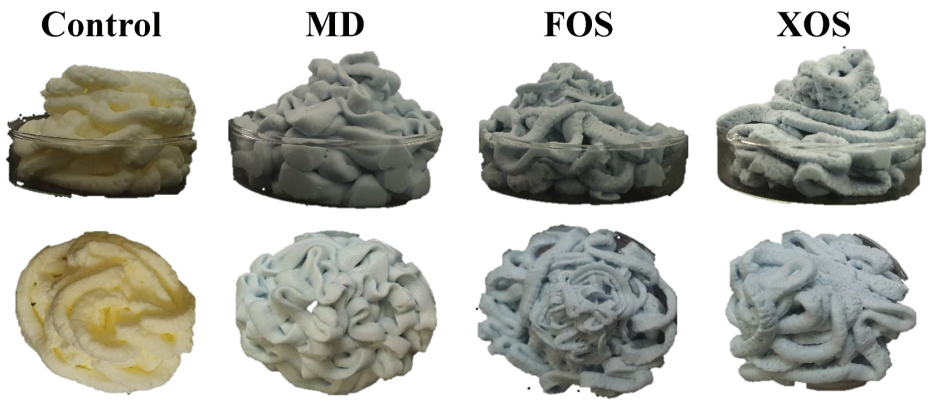

Fig. 7. Visual appearance of Chantilly without colorant (control); incorporated with colorant with MD (MD); with FOS (FOS); and with XOS (XOS).

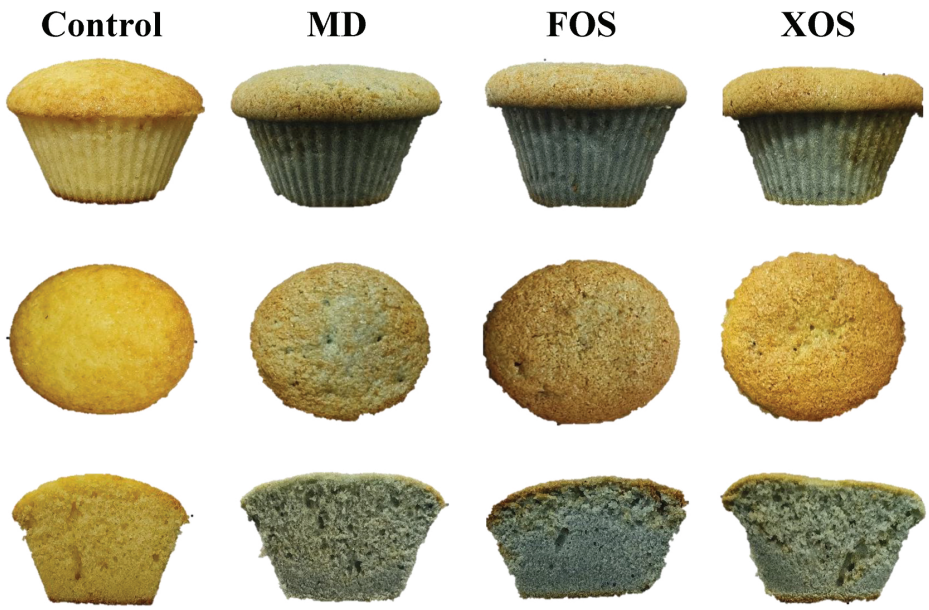

Fig. 8. Visual appearance of muffin without colorant (control); incorporated with colorant with MD (MD); with FOS (FOS); and with XOS (XOS).

3.2. *Application of the spray-dried blue colorants in foodstuffs*

Figs. 6–8 show the visual appearance of *beijinho*, Chantilly, and muffin, respectively. The product's visual appearance incorporated with MD, FOS, and XOS can be compared with the control sample (without colorant) (Control). The same amount of powdered blue colorant was incorporated in all products to eliminate the interference of colorant concentration. Although the same concentration was used, the *beijinho* was darker in color than the other products, followed by the muffin and Chantilly. Consequently, the colorant can be more intense in different food bases.

The *beijinho* production process reaches cooking and cooling temperatures. The colorant was incorporated before going through these processes, which indicates good stability at high temperatures (80 °C) and refrigeration (10 °C) (Chandan, 2011). The colorant with MD showed lesser ease of incorporation. Small particles that could not be properly solubilized can be observed (Fig. 6). Visually, the carrier did not influence the intensity of the blue color of the *beijinho*. The use of XOS to produce powdered blue colorant increases its healthiness by enriching it with these prebiotic carbohydrates.

Chantilly was produced with fresh cream, with 30% fat, being a food base with high difficulty incorporating hydrophilic compounds. The blue colorant particles were incorporated into the cream before being taken to the mixer (Fig. 7). It means that in dairy bases with high-fat content, it is possible to incorporate our blue colorant, regardless of the carrier matrix to be used (MD, FOS, or XOS). There was no difference in the Chantilly's visual appearance comparing the different carriers, showing the possibility of applying XOS as a blue colorant carrier in Chantilly. The positive results regarding the colorant's incorporation also indicate that the colorant is physically stable to the stirring process in which the product passes to be obtained.

Blue colorants were applied to the dough before being placed in the oven to produce the muffin. Thus, after the baking process, the blue color remained stable (Fig. 8). The colorant resisted the baking process, in which the center of the product can reach temperatures of approximately 100 °C for 25 min (Lostie, Peczalski, Andrieu, & Laurent, 2002).

Table 2
Color parameters and colored foods variations regarding the control samples.

Food	Parameter	Control	MD	FOS	XOS
Beijinho	***L***	71 ± 1^{a}	46 ± 2^{b}	46 ± 1^{b}	46 ± 2^{b}
	a	0.50 ± 0.02^{b}	-4.2 ± 0.1^{a}	-4.2 ± 0.2^{a}	-4 ± 1^{a}
	b	27 ± 1^{a}	-1.5 ± 0.4^{c}	-2.3 ± 0.1^{b}	-2.2 ± 0.1^{b}
	ΔE_{Lab}	–	38 ± 1^{a}	38 ± 1^{a}	39 ± 1^{a}
	ΔBI	–	-13 ± 1^{a}	-13 ± 1^{a}	-13 ± 1^{a}
Chantilly	***L***	94 ± 2^{a}	79 ± 1^{b}	78 ± 1^{b}	78 ± 1^{b}
	a	-2.0 ± 0.2^{c}	-3.3 ± 0.1^{b}	-4.4 ± 0.2^{a}	-4.2 ± 0.1^{a}
	b	13 ± 1^{a}	-3.0 ± 0.3^{c}	-4.1 ± 0.1^{bc}	-5.0 ± 0.1^{b}
	ΔE_{Lab}	–	23 ± 1^{a}	22 ± 1^{a}	24 ± 1^{a}
	ΔBI	–	-3.00 ± 0.01^{a}	-3.1 ± 0.1^{a}	-3.00 ± 0.03^{a}
Muffin	***L***	69 ± 2^{a}	54 ± 1^{b}	51 ± 2^{b}	54 ± 1^{b}
	a	-2.0 ± 0.1^{b}	-4.0 ± 0.2^{a}	-4.1 ± 0.2^{a}	-4.02 ± 0.01^{a}
	b	26 ± 1^{a}	4 ± 1^{b}	2.0 ± 0.4^{c}	5 ± 1^{b}
	ΔE_{Lab}	–	27 ± 1^{a}	27 ± 1^{a}	26 ± 1^{a}
	ΔBI	–	-7 ± 1^{a}	-7 ± 1^{a}	-7 ± 1^{a}

Results are presented as mean ± standard deviation. [a, b, c] letters represent significant differences at the 5% significance level in the same line for samples evaluated with different carrier matrix.

No variances were observed in the blue color intensity among the carrier matrices, being possible to apply XOS to carry the blue colorant in cakes.

Table 2 presents the *L*, *a*, and *b* parameters, the coordinates variation (ΔE_{Lab}), and *BI* variation (ΔBI) of the food colored compared to the control (without blue colorant). The external color analysis was one of the most critical analyses. Coloring was the intended main effect of the blue colorant. In general, analyzing the parameters *L*, *a*, and *b*, the products incorporated with XOS presented parameters slightly bluer. Therefore, they were the most recommended matrix to carry the colorant and highlight the color to the product. There were no significant differences in the blue color intensity variation (ΔBI) or the ΔE_{Lab} among the carrier matrices used. Evidencing the feasibility to use XOS in these products to carry the blue colorant.

4. Conclusion

This study put XOS in evidence as a new prebiotic wall material. XOS exhibited similar performance to FOS and MD as a carrier agent. These emerging prebiotic carbohydrates presented advantages compared to MD regarding their ability to carry the natural blue compounds in the powdered system and after incorporating them into foodstuffs. The powdered blue colorant produced with XOS was more hygroscopic and presented a smaller particle size resulting in a greater tendency to agglomerate and adsorb water. However, these characteristics are still within the limit to consider a potential carrier material. Similar to FOS and MD, microparticles with XOS presented an amorphous structure. Regarding food application, XOS presented excellent stability to the high-temperature process parallel to MD and FOS. Furthermore, the microparticles could be easily incorporated into a hydrophobic and hydrophilic food matrix. Thus, spray-dried natural blue colorant produced with XOS presented an innovative potential to be applied in several foods (dairy products, beverages, emulsions, candies, and others), contributing as a functional ingredient, replacing artificial blue colors, and incorporating prebiotic attributes.

CRediT authorship contribution statement

Maria Isabel Landim Neves: Investigation, Formal analysis, Writing - original draft., Monique Martins Strieder: Investigation., Ana Silvia Prata: Resources. Eric Keven Silva: Conceptualization, Visualization, Methodology, Project administration, Writing - review & editing., Maria Angela A. Meireles: Resources, Supervision.

Declaration of competing interest

The authors confirm that there are no known conflicts of interest associated with this publication.

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CHAPTER 8

General discussions

What did this thesis bring?

8. General discussion

Since color is one of the most important attribute for the sensory quality of a food, the colorant ingredient became a marketing tool to attract the consumers. Besides, nowadays, the natural ones gained force due to ensuring functional properties in foods. Comparing to other natural colors, blue is the biggest challenge since there are not many natural blue resources to provide a stable, low-cost colorant. Despite the natural blue sources such as *Gardenia jasminoides*, *Spirulina platensis, Indigofera, Haslea ostrearia, Genipa americana L.,* and *Allium*; all of them have at least some disadvantages that make their industrial application unfeasible (Sigurdson et al., 2017). The blue color can be produced by using anthocyanins, but only in the pH range of 6 to 7 and, in general, has low coloring power. Derivatives of phycocyanin and iridoids are also precursors of blue colorant. Lately, the Food and Drug Administration (FDA) permitted phycocyanin as spirulina extract. Still, its use is limited due to the problem of stability to high temperatures, light, and pH variations, in addition to its high-cost production (Pojer, Mattivi, Johnson, & Stockley, 2013; Somavat, Kumar, & Singh, 2018; Tarone, Cazarin, & Junior, 2020). The colorants derived from genipin extracted from *Genipa americana* and *Gardenia jasminoides* and primary amines reaction may be the most promissor blue colorant source (Brauch, Zapata-Porras, Buchweitz, Aschoff, & Carle, 2016; Buchweitz, 2016). For this reason, before carrying out this thesis, we have seen in the literature some works focused on understanding the crosslinking reaction with genipin and primary amines, for producing an intense dark blue colorant, using pure compounds (Fujikawa, Fukui, Koga, Iwashita, Komura, & Nomoto, 1987; Di Tommaso, David, Gomar, Leroy, & Adamo, 2014; Dimida, Demitri, Benedictis, Scalera, Gervaso, & Sannino 2015). There were also others works dedicated to extracting genipin from *Genipa americana* L. (Ramo-de-la-Pena, Renard, Wicker, Montanez, Garcia-Cerda & Contreras-Esquivel, 2014; Náthia-Neves, Vardanega, & Meireles, 2019). And others that used pure genipin reacted with different sources of primary amines, such as, Yang, Zhou, Wie, Zhu & Fan, (2012), who studied the reaction with egg proteins.

The interest in studying this reaction started with Fujikawa and co-authors (1987), who elucidated the reaction between genipin and glycine at controlled conditions: pH 7, 80 °C during 4 h. The major component elucidated was named *genipocyanin G1* with 504 of *m/z* - that from this thesis we now know that this compound is the dimer between genipin and glycine. And until the present year, 2021, articles are published aiming to clarify the production of the blue color (Tsutsumiuchi et al., 2021) and seek better ways to extract genipin (Strieder, Neves, Zabot, Silva,

Meireles, 2021). Despite years of study, some aspects such as: all reaction steps, the polymer complex formed, optimized reaction conditions, and influence of the source of primary amines and genipin are still not fully understood. Precisely because there are many unknowns regarding the reaction mechanism, this colorant is not yet used as a natural blue colorant for the food industry. Not understanding how a compound is formed makes its application difficult, as it avoids a better standardization of production conditions. Also avoids the use of a cheaper and a more available sources (primary amine and genipin) for large-scale production. In parallel, our review article clearly shows the lack of a natural blue colorant by the industry, which highlights the importance of this study. Thus, in addition to studying the crosslinking reaction that produces the natural color, making available a powdered colorant produced by available sources and with functional appeal has great value both from an academic and industrial approach.

In general, the great advantage from this thesis was the colorant production using not expensive and available raw materials, resulting a commercial-available colorant. Precisely, we developed a promising natural blue colorant from *Genipa americana* L. using the milk colloidal system as reaction medium and carrier for blue color complex. After having brought this idea, we started to deeply understand the effects of milk on blue colorant production. For that, first, we discovered the effect of the milk composition on the blue color formation, seeking to selected the best commercial milk to be used. The milk composition affected the genipin recovery because the milk with higher protein content contributed to obtaining a more intense blue color. The whole milk with 3.0 g/100 g of fat favored the light scattering producing a blue colorant with a more intense $-b$ and L values was obtained. On the other hand, the use of skimmed milk with 0.5 g/100 g fat content resulted in more consumption of genipin due to its more protein content for the reaction of blue color compounds formation. Consequently, a more intense and darker blue color was obtained with lower light scattering.

After, we discovered the most common thermal treated milks: LTLT, HTST, UHT, and SD influenced the potential of milk as a reaction medium to produce the colorant. Since this reaction is based on crosslink between milk primary amines and genipin; the formation of natural blue compounds depended on the milk treatment due to changes in protein structure, reducing the number of primary amine groups available to develop the blue compound. Thus, the less severe thermal treatments LTLT and HTST resulted in blue colorant-loaded milk samples with the most intense blue color.

Discovered that both the composition and thermal treatment affected the blue color intensity, we questioned how could be the influence regarding the reaction mechanism. For that, the study that combines three different scales of reagents to produce a natural blue colorant based on genipin studying the reaction mechanism behind it was carried out. We have established the factors that mainly influence the reaction velocity and color intensity. Therefore, some questions regarding the genipin-primary amine reaction could be clarified. Considering that there was controversy in the literature about whether the colorant was a polymeric complex (Butler, Ng & Pudney, 2003) or whether, with only one unit of primary amine and genipin, the color was produced (Touyama, Takeda, Inoue, Kawamura, Yatsuzuka, Ikumoto, Shingu, Yokoi, & Inouye, 1994; Di Tomasso et al., 2014). From this thesis we proved that the blue color is formed from the union of two genipin monomers with a primary amine, that is, the colorant is formed by repeated monomer units, starting from the dimer. We have also found that basic amino acids (arginine and lysine) are preferred to produce color, and the molar ratio of 1:1 genipin to amino acid appears to be ideal for the reaction. With *Genipa americana* L. extract, the ideal pH range is between 6-7. These optimal conditions mentioned favored not only the intensity of the color, but also the reaction velocity. These results further evidenced the use of milk as an extraction solvent, reaction medium and supplier of primary amines, as it presents a sufficient amount of basic amino acids and an ideal pH for the reaction. Also, concerning the most complex system (extracted genipin, milk and dairy proteins), the size of the polymer formed due to the crosslinking reaction between extracted genipin and dairy proteins gave a molecular mass average of about 200 kDa.

Knowing more deeply the blue color formation mechanism with genipin and milk proteins, the colorant micronization would be necessary for further applications. For its micronization, the addiction of a carrier matrix could increase the colorant chemical stability and facilitate the atomization process. The use of a prebiotic carriers could be a best option seeking to bring the functional characteristic of the blue colorant. For that, we first studied the influence of the DP of fructan on the chemical, physical, and technological properties of a blue colorant. The DP influenced the particle characteristics: the use of carriers with lower DP, such as FOS and GR-In, was more favorable for retention of the blue color and also its rehydration. Also, the addiction of a carrier matrix improved the powder characteristics and the blue colorant stabilization at different pH conditions and high temperature. Among the fructans studied as a carrier matrix, this thesis put XOS in evidence as a new prebiotic wall material. XOS exhibited similar performance to FOS and

MD as a carrier agent. These prebiotic carbohydrates presented better advantages compared to MD regarding their ability to carry the blue compound in the powdered system.

Additionally, we realized the needed to study the coloring power, stability and test its application in different foodstuffs. It is important to evaluate if the colorant can be applied in some food formulation with a minimal concentration, also, if the color do no change after be submitted to a high temperature treatment and extremely pH values. The natural colorant produced in this thesis presented a high coloring power comparing to some natural colorants. Its color did not change after be submitted at high temperatures and pH variations. Also, we could demonstrate the powder colorant can be easily incorporated into a hydrophobic and hydrophilic food matrix.

Apart from our discoveries, it is important to highlight that this thesis involved some interesting points, at the same time brought the solution of a blue colorant:

- The use of *Genipa americana* L. as an accessible source of genipin instead of using pure compound.
- The use of an emerging technology (HIUS) for the extraction of genipin, which increases the efficiency of extraction, for a shorter process time.
- Milk was used as a solvent for the extraction of genipin from *Genipa americana* L. at the same time, as a reaction medium and the supplier of primary amines from its proteins to form the blue color.
- HIUS technology, in addition to being efficient to extract genipin, would also favor the blue color reaction kinetics, providing temperature increase and homogenization.
- We have proven that commercial milk can be used for this purpose, which facilitates its industrial application.
- A colorant made with a milk base has advantages over colorants that are based only on the extract (aqueous or ethanolic). Advantages that are related to its micronization: drying a colorant in milk presents greater efficiency and yield, as well as better technological powder characteristics.
- We produced a functional powder colorant rich in prebiotics, due to the use of carriers such as FOS and XOS, which improved the technological characteristics of the powder and the stability of the colorant.

Therefore, this thesis added valuable information on the production of the blue colorant from genipin and primary amines, but, mainly, it brought the possibility of producing the colorant by accessible means that provide ideal reaction conditions, using emerging technologies and

enriched with functional ingredients. To finish, the particles could be properly applied in different food bases: *chantilly*, muffin and *beijinho*. These finds collaborated to conclude the particles could be applied to diverse bases during their formulation process, such as baking and mechanical agitation, without losing their color stability.

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CHAPTER 9

General conclusion and memorial of doctorate period

9.1 General conclusion

The bibliographic review developed in **Chapter 2** was the basis for understanding the current scenario of blue colorants in food sector and was fundamental for considerate the importance to develop a new blue colorant applicable to various foodstuffs. In addition, the sensory challenge regarding the idea that blue food is linked to an artificial food. Other relevant theme discussed in this chapter was about the real need in the current market to replace synthetic colorant by a natural one that has a high coloring power, stability and versatility. Finally, we highlight the blue colorant-loaded milk as a solution to replace the synthetics blue colorants and, after having a natural ingredient, a new concept concerning blue foods will may be change.

Chapter 3 revealed the potential of milk as a genipin solvent, reaction medium, and primary amine supplier for the reaction with genipin to form the natural blue colorant. The influence of the milk composition on the blue color formation was also confirmed: the whole milk promoted the formation of a bluer colorant, whereas the skimmed milk promoted the formation of a darker one. After understanding the milk composition influences, we discovered on the milk commercial treatments effects. For that, **Chapter 4** revealed the influence of heat milk processes commonly used by the food sector on the blue color reaction. The less intense treatments favored to a more intense color formation. The next chapter (**Chapter 5)** was important to deep the comprehension about genipin-primary amines reaction, we discovered the preferential amino acids, pH, and genipin:amino acid molar ratio conditions. These conditions clarified why the milk is an ideal reaction medium and primary amines supplier.

The colorant particle production, stability and powder colorant evaluation were developed in **Chapter 6.** We observed the importance to use a carrier to improve the blue colorant powder stability and powder properties. Also, the colorant reveled a stable ingredient even at high temperature and pH variation conditions. Then, in **Chapter 7**, we reveled the xylooligosaccharides (XOS) as potential carrier matrix. Which exhibited similar performance to fructooligosaccharide and maltodextrin as a wall material. We also could incorporate the particles into hydrophobic and hydrophilic foodstuffs formulations.

Thus, this thesis presented results that solve the food sector problem about find a stable, versatile and easy scale-up natural colorant to replace the synthetic ones. Spray-dried natural blue colorant produced with XOS presented an innovative potential to be applied in several foods (dairy

products, beverages, emulsions, candies, and others), contributing as a functional ingredient and incorporating prebiotic attributes.

9.2 Memorial of doctorate period

In March 2018, Maria Isabel Landim Neves entered the doctorate program in Food Engineering. Awarded with CAPES financial support. During her doctorate, she attended the subjects TP320 Thermodynamics; TP322 Transport Phenomena I; TP323 Transport Phenomena II; TP199 Seminars, TP121 Topics in Food Engineering, TP333 Experimental Planning and Process Optimization totaling 18 credits. She also participated as a volunteer Teaching Internship Program (PED C) with partial support activities for teaching TA431 Mechanics of Materials.

During her doctorate, she collaborated in the organization of 2 scientific events: 8th International Conference on Food Proteins and Colloids and V Iberoamerican Conference on Supercritical Fluids, presenting works related to her thesis. In which the second, she presented an oral work. She also participated in 2018 and 2020 as an evaluator of scientific works at the UNICAMP Internal Scientific Initiation Congress. In 2019, she participated in a complementary program in Kumamoto, Japan (Sakura program), in which she presented part of his thesis. In 2020 she served as student representative at the FEA Graduate Commission (CPG-FEA) and PED Commission. Finally, in 2021, she held a doctorate sandwich program at the Foodomics Laboratory, under the supervision of the Doctors Alejando Cifuentes, Elena Ibáñez, and Alberto Valdés. This period was financial supported by CAPES. During her stay, she participated in 2 scientific events: 6th PATh Spring Workshop and 6th Green & Sustainable Chemistry Conference presenting scientific works, the first conference cited was an oral presentation. During this time, she developed 2 papers as first author: one is part of this thesis (Chapter 5), and another was in collaboration with her colleagues (Appendix IV). She also has collaborations in others papers as a co-author. In addition, she co-supervised a final course project and a master's thesis.

Research related to the doctoral project resulted in 2 review articles published in the journals Food and Public Health (Appendix I) and Trends in Food Science & Technology; 4 experimental articles published in Innovative Food Science and Emerging Technologies, Future Foods, Carbohydrates & Polymers and Food Hydrocolloids; 2 articles submitted in Food Chemistry and Dyes and Pigments; and 1 patent (Appendix III). Also, she published 1 paper in a collaboration with her colleagues as first author (Appendix II). She is also a co-author of 5 other papers.

Below, are exposed an overview of the doctorate period (Figure 9.1).

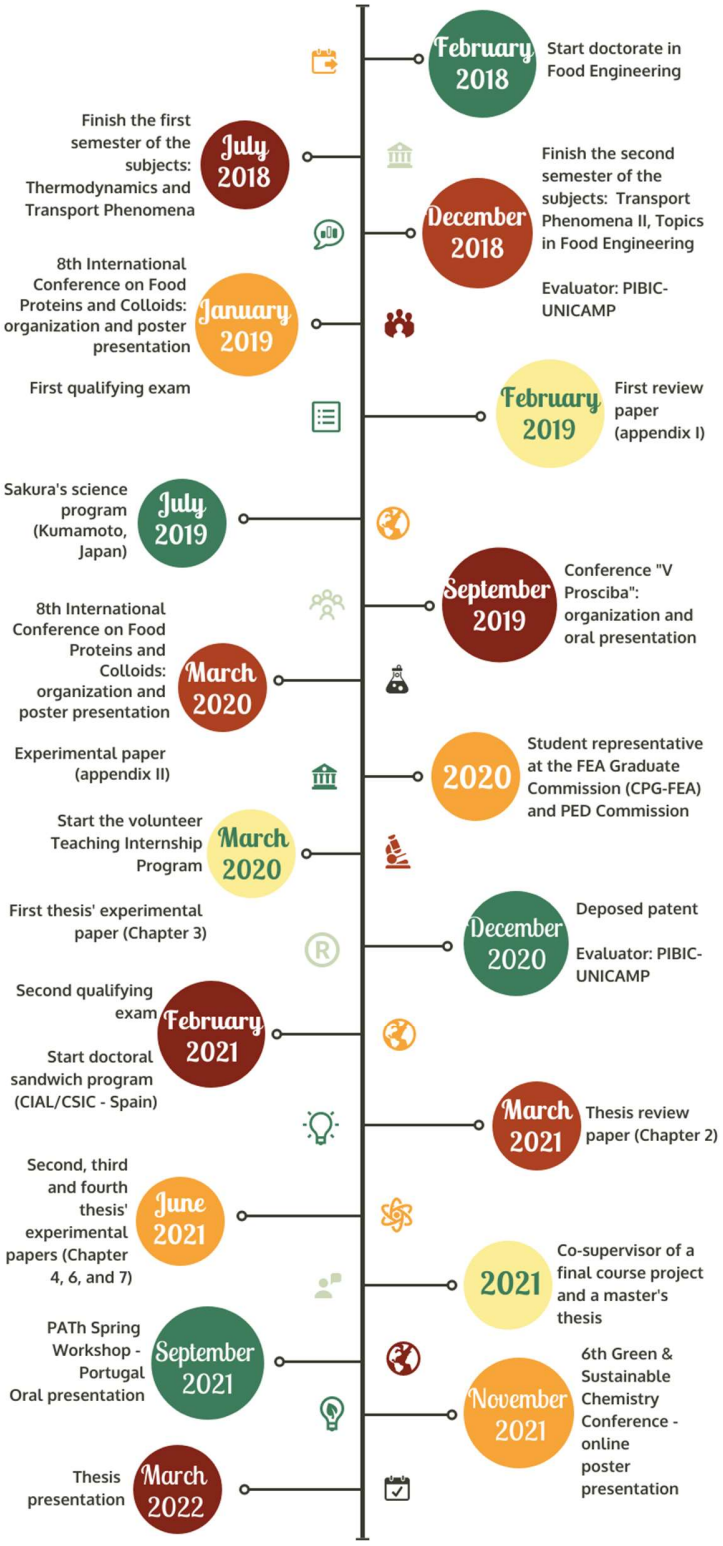

Figure 9.1. The doctorate period timeline.

Below, **in chronological order**, all the **articles published** in her academic trajectory:

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APPENDICES

10.1 Appendix I – Review article about natural food colorants

Trends and Challenges in the Industrialization of Natural Colorants

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Trends and Challenges in the Industrialization of Natural Colorants

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Abstract Consumer demands and their preferences for naturally derived colorants have increased exponentially and are widely associated with the image of healthy, safe and high-quality products, which constitutes a great challenge to food industries and related food science research institutions. Moreover, such demands have resulted in searches to increase the availability of these compounds. Despite the intense search for plant and microbial sources and efforts to increase extraction yield, few natural colorants have reached available the market. Physicochemical instability during the extraction and storage of natural colorants is a major limitation; some non-conventional extraction and encapsulation technologies are being advocated to minimize this problem. Thus, improving the techniques used for the encapsulation of sensitive or labile ingredients is important to make colorants and, consequently, their bioactive proprieties, accessible to consumers.

Keywords Nonconventional extraction, Encapsulation, Precipitation, Supercritical fluids

1. Introduction

Color additives can be natural or synthetic [1] and have a function to impart, restore, or standardize the color of food, making them sensorially more attractive to consumers. The production of synthetic colorants is less expensive than natural colors, and they have higher chemical stability without conveying flavor to products [2, 3]. However, one of the limiting factors associated with using synthetic colorants is their potential effects on human health, depending on the dose that is used. In addition to regulations, consumers also show a higher preference for food products that use natural ingredients [4]. These circumstances have strongly influenced the food sector, which emphasis on replacing the synthetic colorants with natural colorants in food products.

Currently, the production of natural colorants has tended to grow worldwide. It is expected that the global market will grow at an annual rate of 6.22% over the period 2015-2019 [5]. Commonly used natural food pigments include anthocyanins, carotenoids, betalains, and chlorophylls, among others [1, 5].

Natural food colorants have received particular attention, not only because they are potent substitutes for synthetic additives but also because they provide health and security benefits to consumers [2] such as their anti-inflammatory, antimicrobial, anti-amyloid, and antitumor proprieties. Additionally, these compounds have been associated with reductions in several diseases, such as diabetes and obesity [5].

A challenge for the industry is to keep these natural bioactive products available and stable until reaching the final consumer, ultimately to promote benefits to human health [2, 6]. Thus, significant academic and industrial research of methods to stabilize and expand the application possibilities for the various natural food colorants is ongoing. Most developments that food color manufacturers proclaim are vehicles for delivering established natural pigments into food products [7]. The challenges are to extract and transport the colorants using clean technologies. Thus, recent studies have focused on increasing the means of extraction and availability of bioactive compounds using non-environmentally harmful technologies [8-12].

Normally, the chemical stability of food pigments is markedly affected by several external factors [3], such as pH, temperature, light, oxygen, and solvents, as well as the presence of enzymes, proteins and metallic ions in food products.

Research in Food Science generally falls under the following topics for study: (1) influencing factors that improve the stability of natural colorants; (2) investigations of new raw materials to extract colorants or increase the pigment content; (3) assessing and improving stability; (4) industry processing effects on colorants, and (5) health effects [13].

The objective of this paper is to provide an updated overview that shows the trends of research to improve the

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availability of colorants, the challenges associated with this endeavor, and progress over the past 10 years (2008 – 2018).

2. Overview of Colorants in the Food Industry

The natural colorants market is projected to be $ 1.7 billion by 2020 [13]. Color is the first sensory attribute by which foods are judged by consumers, influencing the purchase decision. Consequently, it is widely used in the food industry to meet consumer expectations, due to their habitual association of color with the taste, smell or quality of the product.

Some natural colorants include: annatto, anthocyanins, betaines, betacyanins, chlorophylls, caramel, cochineal, curcumin and other phenolic compounds, carminic acid, genipin, lycopene and carotenoids [2, 14]. These natural colorants are applied in the food industry in the form of extracts or concentrates that contain natural raw materials of vegetable origin. The colorant is extracted from a part of the plant such as leaves, roots, flowers, rhizomes, peels or fruits [15], which are the most common sources. However, there are other sources of natural colorants such as algae, insects and microorganisms [16, 17].

To obtain the extracts, there are many choices based on the chemical characteristics of the colorant, the raw material, the vegetable part where the colorant is found and the final application of the pigment. The majority of the extraction processes use organic solvents [18]; however, attention should be paid to the use of nontoxic solvents when the colorant is intended for food. To select the most suitable solvent, it is important to determine the nature of compounds that are to be isolated or extracted. To extract hydrophilic compounds, polar solvents such as methanol, ethanol or ethyl-acetate can be used; for the extraction of lipophilic compounds, dichloromethane or a mixture of dichloromethane/methanol in ratio of 1:1 are frequently used [17]. Several methods including sonification, heating under reflux, and Soxhlet extraction are commonly used depending on the target compound's polarity and thermal stability. Some modern methods are also used for extraction, such as solid-phase microextraction, supercritical fluid extraction, pressurized-liquid extraction, microwave-assisted extraction, solid-phase extraction and surfactant-mediated techniques, which have advantages in terms of yield and easy collection of extracts [17, 19]. Now, due to decreasing fossil resources (such as petroleum) and rising energy prices, researchers are being challenged to find new technologies to reduce or eliminate the consumption of solvents during extraction [20]. In addition, many extractions demand nonrenewable energy sources that can introduce effluents into the atmosphere that contain substantial amounts of pollutants such as aldehydes and free fatty acids. Another disadvantage is that conventional extraction methods are time consuming: some processes require several hours to achieve a complete extraction [21]. Additionally, attention should be paid to the use of nontoxic solvents when the colorant is intended for food. Therefore, the transition to alternative, green techniques to replace conventional extraction methods is attractive as a research topic due to advantages that correspond to reduced energy needs and resorting to nonhazardous substitute solvents and renewable natural products while ensuring a safe and high-quality extracts. Some non-conventional extraction techniques provide such advantages and are also efficient technologies at low cost [18, 21-23].

In addition to obtaining colorants by plant matrices, microorganisms produce various coloring compounds [24]. The pigment most commonly used in industries is beta-carotene, which is obtained from a cyanobacteria. Microorganisms that have the ability to produce pigments in high yields include species of *Monascus, Paecilomyces, Serratia, Cordyceps,* and *Streptomyces,* for example [25]. However, due to difficulties with the introduction of microbial pigments on the market (because of the possibility of mycotoxin), only a small number of these colorants are produced industrially. There are also colorants that are obtained from animals. For example, Cochineal Carmine is obtained from the dried bodies of females of scaled insect species, namely, *Dactylopius coccus Cost* [26].

3. Stages for Maintaining the Accessibility and Availability of Colorants

There are several critical points throughout production, from extraction to storage of the colorant, that lead to its disintegration. The degradation of the colorant affects so much of its color that it sensorially damages the product, as well as its bioactive compounds. The preservation of these characteristics is a major challenge for science and the food industry. It is necessary, therefore, to separate each critical point, aiming at studying strategies to overcome these problems.

3.1. Extraction

The first critical point is related to natural colorant extraction. Some studies have been applied to optimize the extraction of natural pigments, in order to maintain their characteristics and avoid the loss of color and bioactive compounds in the pigments [27-32]. Table 1 shows different colorants, their origin, types of innovative extraction methods and processing conditions from recent studies.

Losses can occur based on how aggressive the extraction process is [27], as well as the temperature at which extraction occurs. The inadequate choice of solvent, inadequate choice of method and the complexity of the matrix from which the colorant should be extracted may hinder the extraction when there are strong domains of interaction between the matrix and the pigment [20, 33]. Therefore, it is necessary to

carefully study the characteristics of the matrix where the colorant is obtained and its interaction with the solvent. Similarly, knowing the characteristics of the colorant helps in choosing the solvent and the appropriate extraction method, as well as the maximum temperature that can be reached. It is important to emphasize the importance of being a nontoxic solvent, applicable in food, which limits the range of extraction options [15, 34]. It is possible to perceive the complexity of obtaining a suitable extraction method with conditions that will lead to an excellent extraction yield and maintains the characteristics of the colorant.

In this context, there are several extraction methods that focus on better preserving the pigment characteristics. Additionality more recent developments concern the use of non-thermal concepts to facilitate the extraction without risking overheating of the matrix while decreasing energy use. Therefore, there has been an increase in the application of non-conventional and emerging technologies to extract various classes of bioactive compounds. Another type of extraction method, aimed at reducing aggressive risk to the environment, named "green extraction" is a new concept that reduces energy consumption, allows the use of alternative solvents and ensures a safe and high-quality extract. Traditional techniques are time consuming, lack efficiency in extracting the target analytes and require large volumes of non-environmentally friendly organic solvents, sorbents and samples. Traditional solid liquid extraction (SLE) methods include Soxhlet extraction, maceration and turbo-extraction (high-speed mixing). On the other hand, supercritical fluid extraction (SFE), ultrasound-assisted extraction (UAE), microwave-assisted extraction (MAE), and pressurized liquid extraction (PLE) are examples of innovative green extraction techniques [50].

Table 1. Studies published on the application of non-conventional technologies to extract colorants compounds

Colorant	Species	Part of the plant	Extraction	Process conditions	Reference
Bixin	*Bixa orellana*	seeds	Ultrasound	Ethanol, 40 kHz, S/F: 19	[28]
Bixin	*Bixa orellana*	seeds	Low pressure solvent extraction	Ethanol, 95 min, S/F: 8, 333 K	[29]
Bixin	*Bixa orellana*	seeds	Microwave	Water, 900 W, 2450 MHz	[30]
Bixin	*Bixa orellana*	seeds	Low pressure solvent extraction	Ethanol, S/F: 8, 333 K	[31]
Bixin	*Bixa orellana*	seeds	Supercritical fluid extraction CO_2	S/F: 35, 333 K, 31 MPa	[35]
Bixin	*Bixa orellana*	seeds	Low-pressure solvent extraction	Ethanol, 20 min, S/F: 8, 333 K	[36]
Curcumin	*Curcuma longa*	rhizome	Supercritical fluid extraction H_2O	13 min, 413 K, 1.5 MPa, 0.7 mm of particles	[37]
Curcumin	*Curcuma longa*	rhizome	Pressurized liquids extraction	Ethanol, S/F: 9.5, 333 K, 10 MPa	[32]
Curcumin	*Curcuma longa*	rhizome	Ironic liquid based ultrasonic	[OMIM]Br concentration: 4.2 mol/L, liquid-raw ratio: 30 mL/g, 90 min, 250 W	[38]
Curcumin	*Curcuma longa*	rhizome	Supercritical water	100 min, 373 K, 3 MPa	[39]
Curcumin	*Curcuma longa*	rhizome	Microwave	Ethanol, 15 min, 348 K, 1500 W	[40]
Curcumin	*Curcuma longa*	rhizome	Ultrasound	5 min, S/F: 4.294 K, 150 W	[41]
Curcumin	*Curcuma longa*	rhizome	Ultrasound	35°C, S/F: 5, 250 W, 22 kHz,	[42]
Curcumin	*Curcuma longa*	rhizome	Ultrasound and microwave	Ethanol, 7 min, 80 W, 245 MHz	[43]
Anthocyanin	*Myrciaria cauliflora*	peel	Supercritical carbon dioxide	Ethanol (cosolvent), 333 K, 20 MPa	[44]
Anthocyanin	*Myrciaria cauliflora*	peel	Ultrasound	Ethanol, 10 min, S/F: 20, 303 K, 25 kHz, 150 W, (ultrasound bath: 2.7 L)	[45]
Anthocyanin	*Myrciaria cauliflora*	peel	Pressurized liquid extraction	Ethanol, 353 K, 5 MPa	[46]
Anthocyanin	Crocus sativus	petals	Solvent: ethanol	S/F: 20, 298.9 K, 24 h	[47]
Anthocyanin	Vitis labrusca B	peel	Supercritical carbon dioxide	Ethanol, 318 K, 16 and 18 MPa	[48]
Betacyanin	Beta vulgaris	red beets	Microwave	1:1: ethanol/water, 16.67 min, 800 W,	[49]

3.1.1. Supercritical-Fluid Extraction (SFE)

SFE technology is a viable solution for a clean and low-cost technology in which it is possible to recycle the solvent, as well as use non-toxic solvents with a high extraction efficiency [15]. When using this technology, it is possible to control the parameter changing conditions of extraction, providing the possibility of more applications of specific components and improving the efficiency of the extraction. The selection of these conditions depends on the compound, the matrix where the compound is found and the verification of the best parameter from the extraction efficiency.

SFE depends on the solvating properties of a supercritical fluid, which can be obtained by applying pressure and temperature above the critical point of a substance. Each compound will have a unique critical point [51]. This process consists of extraction of soluble substances from the solid substratum by the supercritical solvent, followed by separation of these compounds from the supercritical solvent after the expansion [15].

SFE depends on fluid conditions such as temperature and pressure, which are the most commonly altered parameters in the process for optimization. In addition, some extrinsic features such as the characteristics of the sample matrix, interaction with targeted analytes and many environmental factors [51]. With regard to the solvent, carbon dioxide is the solvent most widely used in SFE because it is reasonably safe, nontoxic and has feasible supercritical conditions with low temperature and pressure [52]. Supercritical CO_2 ($ScCO_2$) is also attractive because of its high diffusivity and its easily tunable solvent strength. It is possible to obtain $ScCO_2$ at 304.2 K and 7.39 MPa [15, 53].

3.1.2. Ultrasound-Assisted Extraction (UAE)

UAE is considered to be a nonconventional method that has demonstrated efficacy in extracting active compounds, introducing advantages such as low process times and high yields compared to traditional methods [54, 55]. Ultrasonic energy has been identified as an efficient tool to improve performance in different applications of analytical chemistry, such as the extraction of compounds. The improvement in extraction efficiency using ultrasonication is based on the phenomenon of cavitation, which occurs due to the application of low frequency ultrasound. This causes intense shear rates because it promotes the formation and subsequent collapse of microbubbles associated with extreme levels of highly localized turbulence [23, 56]. This ruptures the cell walls, facilitating a reduction in particle size and providing an increase in the surface area and channels of the solid for solvent access, resulting in higher mass transfer [23]. Additionally, although not detailed as a mechanism, the intense mixing effect generated by the propagation of ultrasound in the liquid medium contributes to enhancing mass transfer, greatly improving the solute transfer rate [54]. The capability of ultrasound to cause cavitation depends on its frequency and intensity; extract and solvent properties such as viscosity and surface tension; as well as temperature and pressure conditions [42]. For example, vapor pressure governs the intensity of collapse, whereas surface tension and viscosity govern the transient threshold of cavitation [54].

In general, scientific interest in the use of ultrasound to assist extraction has increased in the past decade [54]. UAE is a clean method that avoids the use of a large quantity of solvent and voluminous extraction vessels such as Soxhlet and maceration. The reduced environmental impact of UAE is clearly advantageous in terms of energy and time. The use of solvents is low, and the processing time at high power can be only minutes [23]. The application of ultrasound extends the range of solvent choice that may replace toxic organic solvents with GRAS (Generally Recognized as Safe) solvents. The selection of GRAS solvents provides several safety benefits and reduces the cost and environmental impacts of the extraction process. Ultrasound improves the extraction efficiency of solvents that have poor extractability using conventional methods [54].

3.1.3. Microwave-Assisted Extraction (MAE)

Microwaves are electromagnetic waves, generally used at 2.45 GHz, which induce heating inside the material via ionic conduction and dipolar rotation of molecules. Ionic conduction is the electrophoretic migration of ions when an electromagnetic field is applied [57]. Dipolar rotation is due to the alignment on an electric field of molecules that possess a dipole moment (either permanent or induced by the electric field) in both the solvent and the solid sample. This oscillation produces collisions with surrounding molecules and thus, the liberation of thermal energy into the medium [40].

In MAE, the moisture inside the cell is heated and its evaporation increases the porosity of the biological matrix, which in turn allows for better penetration of a solvent [58, 59]. The elevated temperature also generally increases solubility and improves yield. The main advantage is a reduction in the extraction time and solvent use. However, the high temperature can destroy the bioactive compounds [60]. As such, is reasonable to control these parameters to find a suitable extraction temperature (where the increase in extraction kinetics compensates the degradation reactions) is clear, as is the importance of contextualizing it with the tissue that constitutes the matrix [61]. Generally, to avoid overheating, low to moderate powers (coupled with longer extraction times) are used [62].

MAE allows for lower extraction times, requires less solvent and exhibits good reproducibility [58, 63]. On the other hand, the augmentation of compound diffusion from the matrix to the solvent, while potentially growing the extraction yield, also enables the extraction of non-targeted compounds [63].

In general, MAE has the following advantages: rapidity; reductions in solvent consumption; better chance of control, and automation. Microwave-assisted extraction is also

environmental friendly, since it requires less energy and can efficiently use nontoxic solvents [64].

3.1.4. Pressurized Liquid Extraction (PLE)

PLE, also known as accelerated solvent extraction (ASE), allows for the fast extraction of compounds with little solvent consumption [18]. In this method, high pressure is used to maintain the liquid solvents at higher temperatures (frequently, temperatures above the boiling point of the solvent), which allows for improvement in the solubility of the compounds, sample wetting and matrix penetration. These conditions improve analyte solubility and the kinetics associated with desorption from matrices [50, 65].

Extraction performed by PLE is an environmentally conscious technology that obtains high yields despite using significantly lower amounts of solvents than conventional technics. In addition, this process is selective because it is possible to extract either polar or nonpolar compounds, depending on the characteristics of the solvents that are used [50]. This process facilitates the use of environmentally friendly solvents, and therefore, PLE is considered to be a green technology. For example, PLE promotes water as an extraction solvent. Pressurized water extraction is a nonconventional method, and the major property of this technique is using temperature and pressure values above 373 K and 0.1 MPa (the atmospheric boiling point of water), as well as below the water critical point values (647 K, 22.1 MPa) [59].

3.1.5. Remarks

All these techniques are used to extract bioactive substances to shorten processing time, reduce solvent consumption, increase the extraction yield and improve the quality of extracts. They are all more efficient compared to conventional methods, providing a great argument for using these technologies for improving the availability of colorants while having a low impact on the environment. Consequently, these techniques have the ability to replace conventional systems.

There are studies that have also used combined methods [23]. The combination of methods can improve the efficiency of extraction. The combination of UAE and MAE is one of the most promising hybrid techniques for fast, efficient extractions. UAE promotes the release of soluble compounds from the plant by disrupting cell walls, facilitating solvent access to the cell contents. In addition, microwaves heat the entire sample very quickly, inducing the migration of dissolved molecules [23].

3.2. Food Process

When the colorant extract is incorporated into food matrices, sometimes the extract must be dried to facilitate its incorporation or storage until its use. During this procedure, it is important to verify that there is no sensory or bioactive compounds degradation. After the incorporation of the extract, the industrial processing unfortunately causes alteration, degradation, or even loss of food color. The factors that influence the stability of natural pigments during processing are pH, temperature, water activity, oxygen, metals, solvents, the presence of enzymes and ion radiation [13]. These factors are discussed below.

3.2.1. pH

Each pigment has an accepted pH range that does not cause degradation and the loss of properties (Table 2). The bioactive proprieties may not be affected, but the color can change with pH variation [66]. In general, at pH range of 3 to 5, suggested for use in acidic foods, can affect natural colorants [13]. For example, betalains are stable in extracts at pH 5, but below pH 3, the color of betanin moves toward violet, and above pH 7, the color shifts toward blue due to the longer wavelength [67].

3.2.2. Water Activity

Water activity controls the rate of biochemical conversion. Water activity affects the stability of colorants stability by controlling the water-dependent hydrolytic reactions for bond cleavage [67].

3.2.3. Temperature

Thermal processing is used in the development of different processed products. Temperature affects the stability of pigments, and increases in temperature result in degradation [68]. However, thermal degradation is also affected by the temperature range, the extent of heating, the presence of oxygen, and the concentration of pigments. The application of these colorants is limited in foods that require thermal processing such as pasteurization or blanching [69].

3.2.4. Light

Color is oxidized and degraded in presence of light. There is a reverse relationship between light intensity and colorant stability [70]. Immersion in UV and visible light excites electrons of the colorant chromophores to a more energetic state, initiating higher reactivity or lowered activation energy of the molecule [67].

3.2.5. Metal

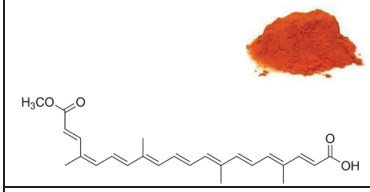
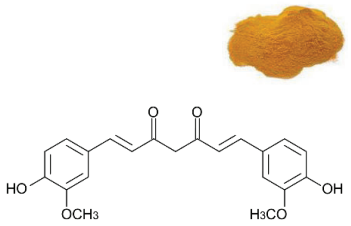
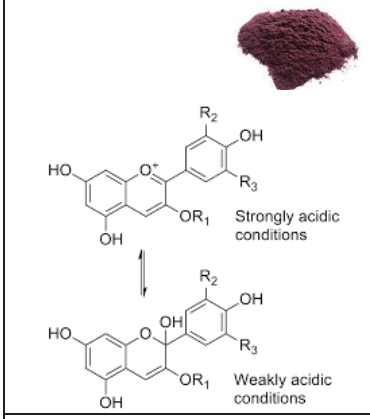
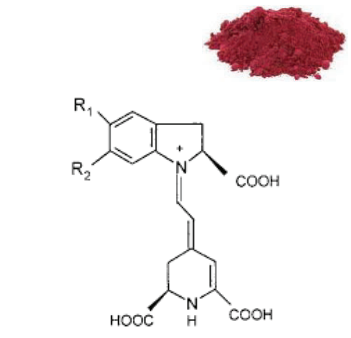
Some metal cations were identified to facilitate or accelerate pigment degradation, such as iron, copper, tin, aluminum, etc. An early study indicated that juice is less vulnerable to metal ions because of the presence of metal complexing agents. Chelating agents (citric acid and EDTA) were reported to stabilize colorants against metal-catalyzed degradation [67]. For example, the presence of metals assists as catalysts for the oxidation reactions of polyphenols and carotenoids [13].

In addition to the process during which the colorant is applied, the incorporation of bioactive compounds in different food matrices is limited by its inherent characteristics, such as flavor and incompatibility with the matrix, as well as the formulation and other ingredients that

are introduced, which can affect the stability of bioactive compounds [71].

In this way, it is important to protect the colorant or create strategies for it to be applied after the processing. Therefore, it is important to study the processing of the food and the tolerable range of bioactive compounds to prevent possible losses of natural pigment.

Table 2. Main natural pigments from different vegetable matrices and parameters that affect their stability

Colorant	Group	pH	Temperature	Light	Chemical compounds	Ref.
Bixin 	Carotenoids	Above 7.0 below 3.0	100°C	Affected at 1380 lux	Divalent metals	[73]
Curcumin 	Sesquiterpenes	Above 7.0	100°C	Affected at 1450 lux	Sulfur dioxide	[70, 74]
Anthocyanin 	Flavonoids	Change of color and structure	100°C	Affected and low sensitivity in pH 3.5	Di and trivalent metals	[66]
Betacyanin 	Betalains	Change of color	90°C	Affected in range of 2200 – 4400 lux	Di and trivalent metals, Sulfur dioxide	[67]

3.3. Storage

The fourth critical point is the stability of the extract during storage, given its sensitivity to variations in light, water activity and elevated temperatures. Stability during storage includes oxidative stability, thermal stability, hygroscopicity, etc. [72]. For this concern, modern food processing and packaging techniques have been developed to improve the shelf life of natural products [2, 13, 68]. Packaging shall protect the colorants from light, temperature, oxygen and other components which may react with the colorant. It is difficult for the colorant to remain stable in the environment, so it is necessary to create strategies for its protection.

3.4. Solution to Improve Availability after Extraction

After obtaining extracts using technologies that promote the lowest possible degradation to the compounds, the stabilization of the natural colorants for their application in food products is one of the biggest challenges for the productive sector. It is necessary to use other techniques that will provide physical and chemical stability, so as not to impair the color sensory characteristics during food processing and storage. As previously stated, the extract obtained passes through a drying process to facilitate its application, which we named micronization of the extract. It is a process of obtaining particles of the active compounds. Micronization can be accomplished by various techniques. But also, in search of obtaining a system that will confer protection to the colorant, the process of encapsulation is the most appropriate one. In which an encapsulating matrix retard or prevent degradations of the colorant.

Encapsulation technology has been used in the food industry to (i) overcome solubility incompatibilities between ingredients, (ii) protect sensitive ingredients such as natural colorants from degradation during processing and storage, (iii) increase their bioavailability, including the controlled release of encapsulated compounds, and (iv) provide an effective barrier for environmental and/or chemical interactions until release is desired [68]. Encapsulation can preserve a substance in a finely divided state and to release it as occasion demands. The encapsulation techniques makes the stabilization of many ingredients viable, expanding the application of functional products in the global market [19, 75-78].

Majority systems may encapsulate compounds with defensive particulate structures, such as colloidal forms (self-assembly), liquid droplets (emulsion), soft microparticles (microbeads), solid particles (dried) or co-precipitation using $ScCO_2$.

There are several encapsulation forms that have both advantages and limitations, and selection depends on the characteristics of the bioactive components (molecular weight, polarity) and, consequently, the desired physicochemical properties of the encapsulate, such as solubility, partitioning, and stability. In addition to the usual characteristics exhibited and shared with other food powders (e.g., particle size and surface morphology), the encapsulate should contain a significant amount of the bioactive component in a form that can be easily incorporated into food matrices, can protect the material against degradation (throughout the food processing chain and *in vivo*) to ensure that it will remain in its active form, and can be compatible with the food matrix in which it is applied. Other conditions that should also be considered when selecting the appropriate technique are the time, cost, and steps required for preparation, production volume, market requirement, and regulations [77, 79]. Therefore, it is important to evaluate the best technique that protects the active material and promotes some applications. In addition, the choice of the most suitable encapsulation process is also important for the permanence of the colorant properties during storage [71].

Although encapsulation technologies are widely studied by the scientific community and some their applied industrially, studies concerning the particle design with controlled physical properties (size, morphology, solubility, thermal stability, and others) still need to be developed. Particle engineering may lead to an increase in the effectiveness of the encapsulating system [80]. Some encapsulation technologies do not allow the control of the particle characteristics during the manufacturing process as the spray-drying process and the freeze-drying process, for instance. It occurs because the process parameters in these technologies have little influence on the technological properties of the particles formed. In addition, conventional technologies, as the spray-drying, are thermal processes performed at high temperatures (150 - 210ºC) or on the other hand are very expensive, as the freeze-drying technology.

Considering the deficiencies of most encapsulation techniques, the ideal process would be non-thermal and allow the production of particles with controlled properties through the engineering of particles. In this context, supercritical technology would be an ideal process.

Supercritical technology is attractive due to the characteristics of supercritical carbon dioxide ($ScCO_2$), with low critical properties that are relatively mild of 304.25 K and 7.39 MPa, which allow for the realization of a non-thermal encapsulation process that will consequently increase the availability of the colorant and respective bioactive actions. In addition, it allows for variable parameters that assist in the production of particles with controlled properties, such as size, morphology and composition [80]. It has been a trend to use supercritical technology for particle production because of these advantages. One more positive aspect of encapsulation by supercritical fluids is that the great interest in the use of supercritical technology lies in the possibility of varying the parameters of the process, such temperature, pressure, injector nozzle diameter, solvent characteristics, solubility of the solute in $ScCO_2$ and nature of the solute-solvent interaction [81]. Producing particles with various characteristics and the guarantee that the process does not risk de-stabilization of the encapsulated or micronized bioactive compounds. That is, the supercritical process

technology allows for the stabilization of bioactive compounds in a non-thermal way [82]. Another important advantage of the production of colorant particles by supercritical technology is that very small particles can be produced. This is because color strength is enhanced when the coloring matter is obtained in very small submicronic particles [82].

Another important aspect that makes supercritical technology an excellent alternative for the production of particles with controlled properties is associated with its versatility of operation and functions in the precipitation process. Supercritical fluid can act as a solvent (Rapid expansion of supercritical solution (RESS), Rapid expansion of a supercritical solution into a liquid solvent (RESOLV), Rapid expansion of supercritical solution into an aqueous solution (RESSAS) and Rapid expansion of supercritical solution with a nonsolvent (RESS-N); antisolvent: Gas antisolvent (GAS), Supercritical antisolvent (SAS), Aerosol solvent extraction system (ASES), Solution enhanced dispersion by supercritical fluids (SEDS) and Particles by compressed antisolvent (PCA)); cosolvent (Gas saturated solutions (PGSS), Gas assisted melting atomization (GAMA) and Depressurization of an expanded liquid organic solution (DELOS)); or extractor and antisolvent (Supercritical fluid extraction of emulsions (SFEE)) [90].

As in the extraction process, it is important that the particle production process is also safe for the environment. As with the processes mentioned above, if the GRAS solvent is used, it can be classified as Green Technology. In Table 3, we show examples of studies that have used supercritical technology for the precipitation of colorants.

There are still many studies that use micronization and encapsulation of natural colorants, despite being a technique that exists in the literature for more than 10 years [91, 92]. This demonstrates how much can still be exploited for the better availability of several colorants.

Table 3. Studies using supercritical fluid technology to obtain colorant particles

Process	Colorant	Encapsulant material	Better conditions	Reference
SAS	Curcumin	Eudragit® L100, Pluronic® F127, and polyvinylpyrrolidone or mixture	10 MPa, 313 K, solution flow 1 mL/min, CO_2 flow: 2 kg/h.	[78]
RESS	Anthocyanin	Polyethylene glycol	20 MPa, 313.15 K, mass of CO_2: 8.43 g.	[19]
GAS	Curcumin	*	10 MPa, 298 - 300 K, CO_2 flow: 20 mL/min	[83]
SEDS	β-Carotene	*	9.4 MPa, 308 K, solution flow: 1 mL/min, CO_2 flow: 1.5 kg/h	[84]
SEDS	β-Carotene	*	8 - 20 MPa, 313 K, solution flow: 1 mL/min, CO_2 flow: 40 mL/min	[85]
DELOS	Anthocyanin	Lecithin and cholesterol	30 MPa, depressurization: 9000 a/min, 323 K	[86]
SAS	Anthocyanin	biodegradable polymer polyvinylpyrrolidone	12.5 MPa, 308 - 313 K, solution flow: 6 mL/min, CO_2 flow: 2 kg/h	[87]
SAS	Curcumin	*	10 MPa, 313 K, solution flow: 500 g/h, CO_2 flow: 500 and 800 g/h	[88]
SF-CO_2	All-trans-lycopene	*	32 MPa, 323 K	[89]
SAS	Curcumin	Polyethylene glycol	10 MPa, 313 K, solution flow: 1 mL/min CO_2 flow: 0.5 and 1 kg/h	[9]

*Micronization (without encapsulant material)

4. Perspectives on Natural Colorants

In addition to the concern for maintaining stable colorant, the trends in obtaining colorants (in both the extraction and particle production processes) must be framed in the context of green technologies. The basic principles of green processes are as follows: (1) use of renewable plant resources; (2) use of "GRAS-solvents"; (3) low energy consumption; (4) study of the use of post extraction co-products; and (5) reduction of operations that produce waste and gases to atmosphere [24]. Therefore, scientific research should focus on green technology.

There is also great concern regarding the solvent to be used; the selection of a suitable solvent is based on the safety of those handling the extraction and is made to avoid toxicity, carcinogenicity, mutagenicity, absorption through the skin and respiratory system, safety of the process. In addition, the solvent must not be flammable or explosive, and must promote environmental protection and process sustainability (recycling and reuse capacity) [27].

There are no doubts that the natural world is rich in color pigments and that most plants have not yet been exploited for their coloring properties/abilities. Thus, new naturally derived food pigments are prepared to satisfy consumer expectations. In parallel with this, increasingly effective techniques are needed to retain the stability of natural food pigments and to ensure the final attractiveness of enriched-foodstuffs during the manufacturing and processing practices, as well as during storage [2].

In general, and despite current advances in the field of food science, many other natural sources of food pigments must be evaluated for their coloring properties, whereas for sufficient quantities they should be made available mainly for extraction and subsequent use [2].

Therefore, the search for new plant extracts and studies aimed at better stability will be the trend of future research,

ultimately to increase the possibility of using natural colorants and their bioactive properties.

5. Concluding Remarks

The interest in the health benefits of natural pigments is a strong justification to study ways of improving their retention as natural constituents of foods during processing and storage and for their use as food additives.

As a suggestion for improving studies of the availability of colorants, we suggest the following: (1) using clean technology for extraction and encapsulation; (2) focusing on parameter studies to find optimum particle production conditions; (3) considering a phase transitions study of the food matrices to have a better application destination, which implies making a more detailed characterization of the particles produced; (4) studying how the processes can affect the active compound; (5) studying the bioavailability of compounds and (6) considering the recycling of solvent ethanol.

Therefore, as observed in this review, we highlight that supercritical technology can meet the requirements for better availability of colorants. The technique can be used from extraction to pigment encapsulation. The technique has several advantages, such as the possibility of studying parameter variability and that supercritical fluid use is a nonthermal process, which do not degrade the pigment in any of the phases. These results demonstrate that this technology supports the demands of the market in improving the retention of natural colorants during processing and storage.

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10.2 Appendix II – Experimental article about biorefinery of curcumin

Biorefinery of turmeric (*Curcuma longa* L.) using non-thermal and clean emerging technologies: an update on the curcumin recovery step

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Biorefinery of turmeric (*Curcuma longa* L.) using non-thermal and clean emerging technologies: an update on the curcumin recovery step†

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In this study, a biorefinery for the processing of turmeric (*Curcuma longa* L.) based on clean and emerging technologies has been proposed. High-intensity ultrasound (HIUS) technology was evaluated as a promising technique for curcumin recovery aiming to improve its extraction yield and technological properties as a colorant. In addition, we evaluated the effects of process conditions on the turmeric biomass after the extractions. The process variables were the number of stages of extraction with ethanol (1, 3 and 5) and the solvent to feed ratio (S/F) of 3, 5, 7, 9 (w/w). The highest curcumin content (41.6 g/100 g extract) was obtained using 1 wash and a S/F of 5 w/w, while the highest curcumin yield (3.9 g/100 g unflavored turmeric) was obtained using 5 stages and a S/F of 7. The extracts obtained by solid–liquid extraction assisted by HIUS showed a yellow color (157 and 169 of yellowness index) more intense than those obtained by the pressurized liquid extraction technique (101 of yellowness index) and better yield results than low-pressure solid–liquid extraction (using the same processing time). Thus, it was possible to obtain a characteristic yellow colorant with high curcumin yield in a short process time (5 min of extraction) using HIUS technology. Besides that, SEM images and FTIR spectra demonstrated that the turmeric biomasses processed by HIUS technology were not degraded.

1. Introduction

Curcuma longa L., commonly known as turmeric, has been traditionally used as an antioxidant, antiseptic, wound healing, and anti-inflammatory agent.[1,2] In addition to its bioactive properties, curcumin has been used as a colorant, flavoring substance, and as a food preservative.[2,3] In light of the curcumin importance for food and pharmaceutical industries, many studies have searched for ways to obtain curcumin from turmeric, seeking extraction processes that result in high yields while maintaining its bioactive properties.[2,4–8]

The conventional process for obtaining turmeric powder, oleoresin and curcumin is presented in Fig. 1. In the conventional process, there is no full use of the vegetable matrix. Turmeric rhizomes are cooked in water and dried using air circulation dryers between 65 and 68 °C for obtaining turmeric powder by way an expensive and long process (48 h), in which the product is exposed to high temperature, light, and oxygen. Then, hexane solvent is added to the material for removing off-flavors generated by the cooking and drying process.[9,10] Therefore, the conventional processing makes necessary the use of toxic organic solvents. In this sense, a production model-based on clean technologies can be used to overcome the main drawbacks associated with the turmeric processing chain.

A biorefinery model for turmeric processing from green emerging technologies which enables the full utilization of *Curcuma longa* L. is presented in Fig. 2. Biorefinery represents the exploration of all fractions originated from a single raw material.[11] The turmeric biorefinery was studied by Silva, *et al.* (2018),[12] where turmeric biomass was evaluated after the integration of emerging processes. In the first step, the oil fraction was recovered from turmeric powder by supercritical technology using carbon dioxide as a solvent. In the sequence, the pigments were extracted from unflavored turmeric powder by using pressurized liquid extraction (PLE) with ethanol as solvent. The use of alternative techniques such as supercritical carbon dioxide and pressurized ethanol have been studied to overcome the limitations of the conventional processes. These emerging processes are environmentally friendly and assist in better compound integrity.[7,13]

The use of PLE technology to obtain curcumin extracts from turmeric powder using ethanol as a solvent was studied by Osorio-Tobón, *et al.* (2014).[7] In general, the PLE procedure involves the use of liquid solvents at moderate to high-temperatures and pressures below their critical point.[7,15]

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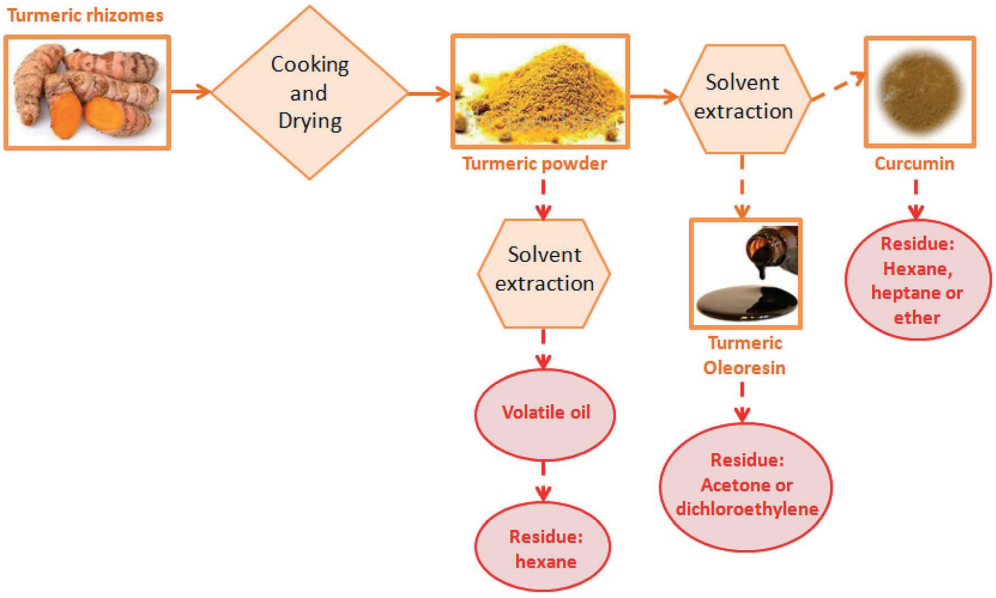

Fig. 1 Process flow diagram for the conventional turmeric processing chain.[9]

Curcumin is thermally unstable and can be degraded during longer extraction times even at moderate temperatures (60–80 °C).[16] To overcome the drawbacks associated with the thermal sensitivity of several bioactive compounds, the development of non-thermal extraction techniques based on process intensification approaches is required.[4,17]

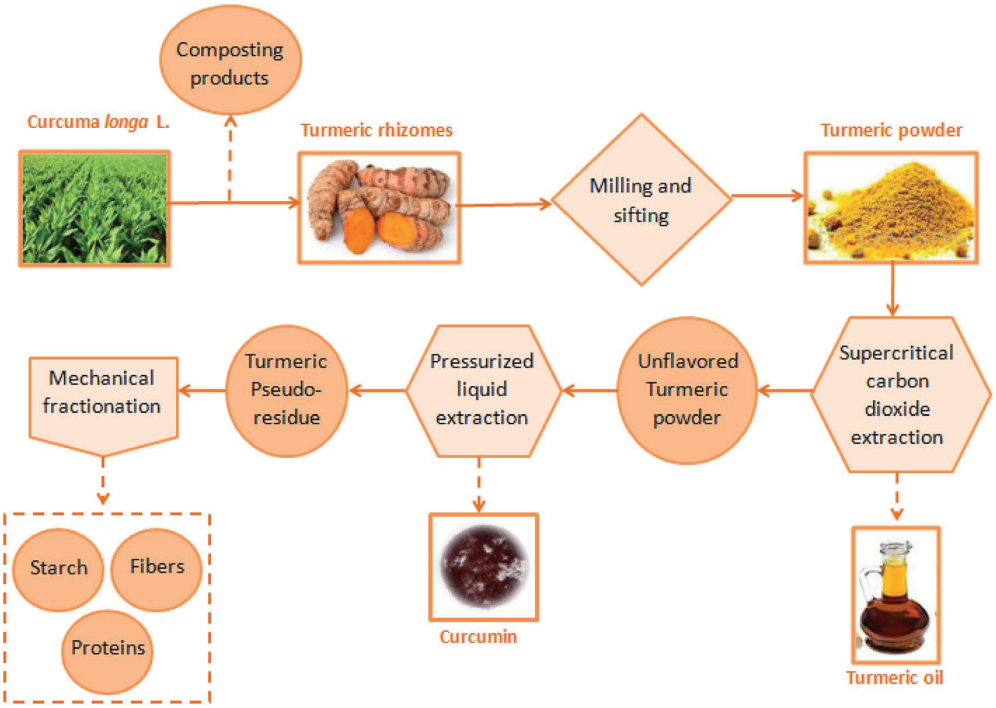

Fig. 2 Turmeric's biorefinery from clean emerging technologies.[7,14]

In this context, the solid–liquid extraction assisted by high-intensity ultrasound (HIUS) has proven to be an efficient emerging technology to recover phytochemical compounds from vegetable matrices, presenting advantages such as short processing time and high extraction yields compared to those obtained by conventional techniques.[18] The efficiency improvement of solid–liquid extraction assisted by HIUS is based on the cavitation phenomenon, that causes intense shear stress associated with extreme levels of localized turbulence.[18,19] The cavitation has a strong impact on the microstructure, causing ruptures that reduce the particle size and promotes better mass transfer. Also, the cavitation breaks up the surface of the cell walls, facilitating the solvent penetration into the cell and increasing the contact surface area between solid and liquid phases.[20,21] The use of solid–liquid extraction assisted by HIUS is environmentally and economically advantageous in terms of less residue generation and short processing time.[18] The recovery of curcumin assisted by HIUS technique can be a novel approach for the turmeric biorefinery, in which coproducts with high added value will be obtained by a non-thermal way with reduced processing time. HIUS technology requires low energy consumption to obtain high-quality phytochemical extracts and its low maintenance cost makes it economically profitable. HIUS technique is considered as the most feasible and economically lucrative large-scale application in the food field.[22] Nonetheless, previous work done by our research group has shown that the turmeric biorefinery is economically viable employing SFE followed by PLE, therefore, it will most certainly be economically viable when PLE is substituted by HIUS.[7,23] Osorio-Tobón, *et al.* (2016)[23] performed an economic evaluation of an integrated process using SFE, PLE, and supercritical antisolvent (SAS) process to produce high-quality products derived from turmeric processing chain such as turmeric essential oil and powdered curcuminoid-rich extract. The authors demonstrated that the scale-up led to an increase in process productivity and a decrease in the cost of manufacture for both the products and concluded that the integrated process is a feasible alternative and an attractive option to produce derivatives from turmeric.

Previous studies developed by our research group showed that the turmeric biomass obtained after the volatile oil extraction using supercritical technology and curcuminoids by PLE technique exhibited a high content of dietary fiber (30 g/100 g), leading the authors to propose some applications for this material, such as wall material for encapsulation processes; fat replacer in processed foods; substance for addition to gluten-free pasta, cakes or bread; food additive for gelatinization, hydrogel formation, and digestibility; for adjusting the viscosity, food additive which include dietary fiber input; novel types of biodegradable plastics; food additive rich in antioxidants because it might contain quantities.[12] Additionally, turmeric residue can be used for bioactive film production.[6]

Considering the full use of turmeric, the choice of non-thermal emerging extraction techniques with high extraction efficiency adds value to extracts and biomasses. Therefore, the solid–liquid extraction assisted by HIUS could be integrated into the turmeric biorefinery, replacing the PLE process in the curcumin recovery step. Since the HIUS process has a shorter processing time and lower process temperatures than PLE, besides a greater extraction efficiency. Thus, the aim of this study was to evaluate the HIUS as a potential step for curcumin recovery in a turmeric biorefinery approach that operates with non-thermal and clean emerging technologies. As well as to verify how the solid–liquid extraction assisted by HIUS conditions affect the characteristics of the resulting biomasses.

2. Material and methods

2.1. Turmeric

Turmeric rhizomes were donated by "Oficina das Ervas" (Ribeirão Preto, Brazil). The rhizomes were grounded in a knife mill (Marconi, model MA340, Piracicaba, Brazil) and were initially submitted to supercritical fluid extraction (SFE) for the volatile oil extraction at 60 °C and 25 MPa according to Carvalho, *et al.* (2014).[13] The global yield was 6.4 ± 0.1 g/100 g rhizomes and the ar-turmerone yield was 1.02 ± 0.01 g/100 g rhizomes. After the volatile oil extraction, the remaining solid material was used as the unflavored turmeric. The particle size distribution was determined using sieves from 9 to 80 mesh (WS Tyler, Wheeling, USA), being the average particle diameter of 0.53 ± 0.01 mm.

2.2. Solid–liquid extraction assisted by HIUS

The samples for the solid–liquid extraction assisted by HIUS were prepared using unflavored turmeric and ethanol 99.8% (Dinâmica, Brazil) to reach a final mass of 30 g. Solvent to feed ratios (S/F) of 3, 5, 7 and 9 (w/w) and different numbers of stages (1, 3, and 5 times) were the variables studied. The process conditions were evaluated through a randomized full factorial design (4 × 3), in duplicate, with a total of 24 experimental runs.

The samples were processed using a 13 mm diameter ultrasonic probe (Unique, Disruptor, 500 W, Indaiatuba, Brazil) at 19 kHz and nominal power of 400 W during 1 min for all experiments. The height of the contact between the ultrasound probe and the mixture was kept at 15 mm. For the experiments performed with 3 and 5 stages, after the extraction period (1 min) the mixture containing extract and solvent was replaced by fresh solvent 2 or 4 times, which means that for the experiments with 1, 3 and 5 stages, the total extraction time was 1, 3 and 5 min, respectively.

After the extractions, the extracts were separated from the biomasses by centrifugation at 2500 rpm for 5 min. The extracts were left under air circulation for 2 days at room temperature and pressure and the biomass for 4 h to evaporate the solvent. Afterward, both were oven-dried at 40 °C under vacuum for 6 h to eliminate the residual solvent. The biomasses were stored in a desiccator in amber packages until performing the further analyzes. The extraction yield was calculated according to eqn (1).

$$\text{Extraction yield (g/100 g)} = \frac{\text{extract mass (g)}}{\text{dry unflavored turmeric mass (g)}} \times 100 \quad (1)$$

The acoustic power provided by the ultrasound probe was determined by calorimetric methodology and HIUS efficiency was calculated according to eqn (2).[24]

$$\text{HIUS efficiency (\%)} = \frac{\text{acoustic power (W)}}{\text{nominal power (W)}} \times 100 \quad (2)$$

2.3. Low-pressure solid–liquid extraction and PLE process

For comparison purpose, conventional low-pressure solid–liquid extraction and PLE process were carried out to obtain curcumin from unflavored turmeric. The low-pressure solid–liquid extraction (named as control) was performed in a shaker (Marconi, MA 420, Piracicaba, Brazil) at 200 rpm for 5 min at 25 °C using a S/F of 7. PLE process was performed according to the best condition (60 °C, 10 MPa, S/F = 10) reported by Osorio-Tobón, *et al.* (2014).[7] The extracts and turmeric biomasses were stored for characterization as previously described for the samples obtained by solid–liquid extraction assisted by HIUS. These experiments were performed in duplicate.

2.4. Extracts and turmeric residues characterization

2.4.1. Curcumin quantification. The curcumin content was quantified in the turmeric extracts by high-performance liquid chromatography (HPLC) according to the method described by Osorio-Tobón, *et al.* (2014)[7] with some modifications. The individual compounds in the extracts were separated using a Kinetex C18 column (150 × 4.6 mm id, 2.6 μm, Phenomenex, Torrance, USA) maintained at 55 °C using a flow rate of 1.25 mL min^{-1}. The mobile phase consisted of water (solvent A) and acetonitrile (solvent B) both acidified with 0.1% (v/v) acetic acid at the following gradient: 0 min: 55% A; 3 min: 35% A; 5 min: 10% A; 7 min: 55% A. The curcumin was detected at 425 nm and identified by comparing its retention time and UV-vis spectra to the reference standards (curcumin ≥ 80%, Sigma Aldrich, St. Louis, USA). The curcumin contents and curcumin yields were calculated according to eqn (3) and (4), respectively.

$$\text{Curcumin content (g/100 g)} = \frac{\text{curcumin mass (g)}}{\text{extract mass (g)}} \times 100 \quad (3)$$

$$\text{Curcumin yield (g/100 g)} = \frac{\text{curcumin mass (g)}}{\text{dry unflavored turmeric mass (g)}} \times 100 \quad (4)$$

2.4.2. Color analysis. The color of the extracts and biomasses were characterized by a CR-400 apparatus (Konica Minolta, Inc., Japan). For each sample the color was measured in a Petri dish according to the three colors coordinates L, a^*, b^* of CIEL^*a^*b system. All measurements were taken of at least three points on the center and the periphery of the Petri dish filled with the turmeric biomass. To evaluation of the difference between samples, the yellowness index parameter was calculated according to the eqn (5).[25]

$$\text{Yellowness index} = 142.86\left(\frac{b^*}{L}\right) \quad (5)$$

2.4.3. Scanning electron microscopy (SEM). Morphological structures images of the biomasses were obtained in a Leo 440i scanning electron microscope with X-ray dispersive energy detector (LEO Electron Microscopy/Oxford, Cambridge, England). The images were recorded using two scales 250× and 5000×.

2.4.4. Fourier transform infrared spectroscopy (FTIR). Modifications of functional groups of the biomasses were analyzed by Fourier transform infrared spectroscopy FTIR (IRPrestige-21, Shimadzu, Kyoto, Japan). The samples were prepared in the proportion of 1 : 100 (sample/KBr) for reading. The spectra were recorded in the 4000–800 cm^{-1} region.

2.5. Statistical analysis

The effect of the process conditions on extraction yield, curcumin yield, curcumin content and color parameters of the biomasses and dry extracts was evaluated by analysis of variance (ANOVA) using the Minitab 16® software (Minitab Inc., State College, PA, USA) with a 95% confidence level (p-value ≤ 0.05).

3. Results and discussion

3.1. Acoustic power and energy efficiency of the HIUS process

Fig. 3 presents the temperature profile of the extraction system (unflavored turmeric + ethanol) subjected to the HIUS process. From the calorimetric method, the acoustic power, real power provided by the ultrasound probe to the extraction system, was 13 ± 1 W. According to eqn (2), the HIUS efficiency was and 3.1%. A similar result was reported by Shirsath, *et al.* (2017).[4]

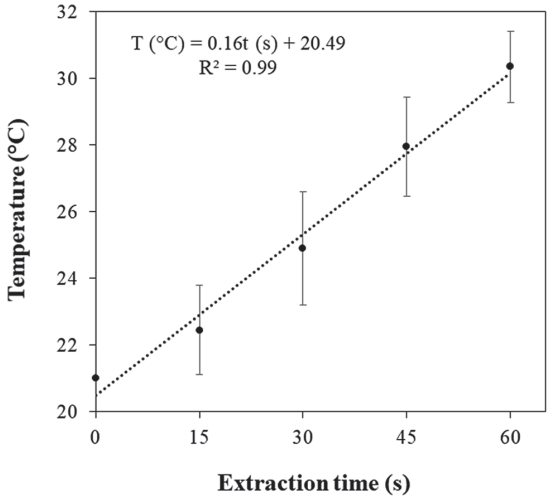

Fig. 3 Temperature profile for the solid–liquid extraction assisted by HIUS.

The authors verified an energy efficiency of 5.6% for the solid–liquid extraction assisted by HIUS of curcumin from *Curcuma amada* using a 20 mm probe diameter and employing a nominal power of 250 W.

In addition, the HIUS processing in the conditions performed in this study was a non-thermal process. It was observed an increase of only 10 degrees during the extraction time, reaching about 30 °C.

3.2. Extraction yield

Fig. 4 presents the extraction yield obtained by HIUS using different S/F and the number of stages. Only the number of stages significantly influenced (p-value < 0.001) the extraction yield where the higher yields were obtained for extractions using more than one wash reaching to 11.0 ± 0.2 (g/100 g) with 5 stages and an S/F of 9 w/w. This result suggests that the use of fresh solvent in each wash favored the extraction because minimized the solvent saturation with curcumin during the process. Besides that, the contact time between the biomass and solvent was increased with the increase in the number of stages, which can also favor the extraction.

3.3. Curcumin recovery

Fig. 5 presents the results for curcumin yield, where the highest value of 3.9 ± 0.4 g/100 g was obtained at S/F = 7 and 5 stages. These results agree with the literature since according to Kiamahalleh, *et al.* (2016),[26] turmeric rhizomes contain 2 to 5 g/100 g of curcumin. For curcumin yield, both the number of stages (p-value = 0.001) and interaction between the number of stages and S/F (p-value = 0.04) had a positive effect on the curcumin yield.

Wakte, *et al.* (2011)[2] obtained 2.1 g/100 g of curcumin yield after 8 h of extraction using acetone as a solvent in a Soxhlet apparatus. The same authors recovered 0.7 g/100 g using ethanol and a probe ultrasonic system at a nominal power of 150 W. The curcumin yields obtained in the present study were higher than that reported in literature probably as a result of the more intensive ultrasound specific energy applied, whereas the nominal power was 60% higher in this study.

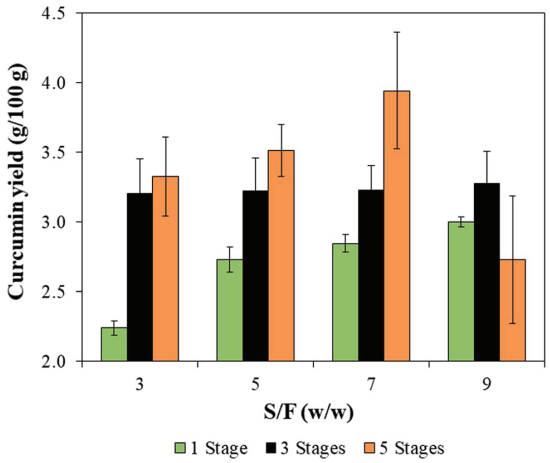

Fig. 5 Curcumin yields of the HIUS extracts obtained with different S/F and number of stages.

On the other hand, for the curcumin content, only the number of stages was significant (p-value < 0.001), the highest contents were obtained for the extractions employing only one wash, reaching up to 41.7 ± 0.3 g/100 g obtained at S/F = 5 (Fig. 6). This result is due to the lower amount of solvent used during the extraction, which in turn, minimize the dilution of curcumin. Although the use of one wash does not exhaust the curcumin content from the biomass, a more concentrated extract can be interesting in industrial applications. Besides that, the reduced extraction time (1 min) and the low amount of solvent used (S/F = 5 w/w) are the advantages of this process,

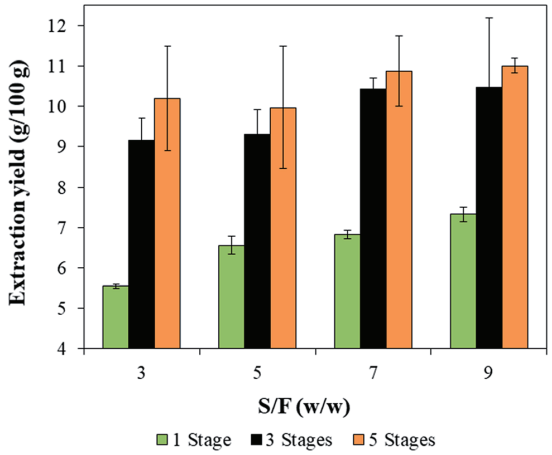

Fig. 4 Extraction yields obtained by solid–liquid extraction assisted by HIUS with different S/F and number of stages.

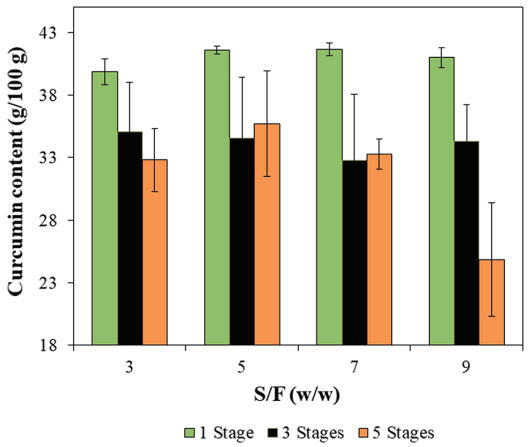

Fig. 6 Curcumin contents of extracts obtained by solid–liquid extraction assisted by HIUS with different S/F and number of stages.

considering that other solid–liquid extractions use higher S/F (10 w/w) and longer extraction times (about 1 h).[26] Thus, it was possible to obtain high values of curcumin content reducing the process time and the amount of solvent employed thus, reducing the residue generation.

3.4. Color of the turmeric residues

Another way of evaluating the efficiency of extraction of a colorant is verifying the color of the turmeric residues from which the colorant was recovered. The color of the turmeric residues according to the CIEL^*a^*b system are shown in Table 1.

It is possible to observe in Table 1 that the major color variation occurred for the coordinates a^* and b^*. In general, the a^* and b^* values were reduced by increasing the number of stages, indicating a reduction of red and yellow colors of biomasses, which means that the colorant was more efficiently extracted. The same was observed for the yellowness index values indicating the yellow color reduction in the biomasses. These results agree with curcumin yield that also demonstrated that increasing the number of stages enable the greater recovery of curcumin.

3.5. Comparison of the solid–liquid extraction assisted by HIUS with other extraction techniques

The extraction conditions assisted by HIUS that provided the highest curcumin content [S/F = 5 and one stage, named as HIUS (S/F: 5S : 1)] and curcumin yield (S/F = 7 and 5 stages, named as S/F: 7S : 1) were selected to be compared with the control and PLE processes and the results are presented in Fig. 7, 8 and Table 2.

The extraction yield obtained by HIUS (S/F: 7S : 5) was (10.8 ± 0.8) g/100 g, which was the same value obtained by PLE ((10 ± 2) g/100 g) (Fig. 7). The highest curcumin yield was also obtained by HIUS using S/F = 7 and 5 stages ((3.9 ± 0.4) g/100 g) and PLE ((3.4 ± 0.2) g/100 g) with results statistically equal

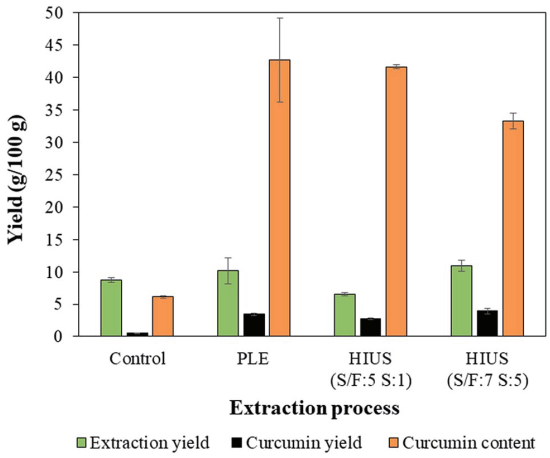

Fig. 7 Extraction yield, curcumin yield and curcumin content obtained by different extraction techniques.

(Fig. 7). It can also be observed that the condition that resulted in a similar value curcumin content to that obtained by PLE ((43 ± 10) g/100 g) was HIUS (S/F: 5S : 1) that resulted in (41.6 ± 0.3) g/100 g. Although HIUS and PLE achieved similar results of curcumin yield and curcumin content, the HIUS process was shorter than the PLE since HIUS had a processing time of about 5 min while PLE was about 1 h.

Although the HIUS and PLE techniques presented similar results of curcumin yield and curcumin content, the color characteristics of the dried extracts presented significant differences (Table 2 and Fig. 8).

The extracts obtained by the HIUS as well as the control process presented b^* values around 55 similarly to the values reported by Zheng, *et al.* (2017),[27] who verified values between 66 ± 3 and 68 ± 3 for curcumin aqueous solutions. On the other hand, PLE extract presented a b^* value of 18 ± 1, which does not

Table 1 Color parameters of the turmeric biomass obtained after HIUS processing[a]

HIUS conditions		Color parameter			Yellowness index average
S/F (w/w)	Number of stages	L	a^*	b^*	
3	1	58 ± 2	17.3 ± 0.9	69 ± 2	171
3	3	57 ± 2	13.9 ± 0.7	63 ± 2	158
3	5	57.7 ± 0.8	15.0 ± 0.5	63 ± 1	158
5	1	57 ± 2	16.5 ± 0.6	68 ± 2	169
5	3	59.1 ± 0.6	14 ± 1	66 ± 1	159
5	5	59 ± 1	14.3 ± 0.3	66 ± 2	159
7	1	59 ± 1	16 ± 1	69 ± 1	168
7	3	58 ± 1	14.5 ± 0.7	65 ± 1	160
7	5	58 ± 1	13.4 ± 0.7	63 ± 2	156
9	1	58 ± 2	15 ± 1	66 ± 2	163
9	3	58 ± 2	14.4 ± 0.3	65 ± 2	160
9	5	58 ± 1	14 ± 1	64 ± 1	158

[a] Mean values ± standard deviation (in triplicate, $n = 3$).

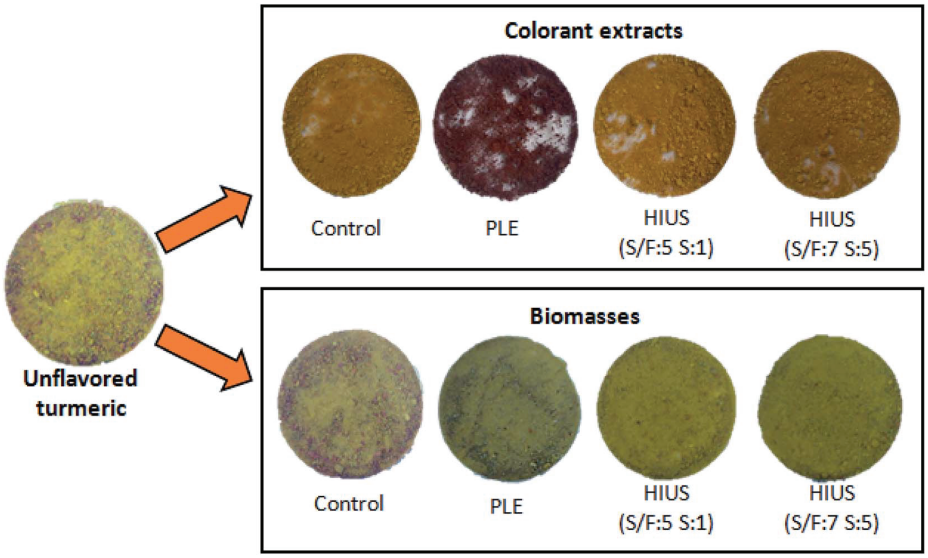

Fig. 8 Unflavored turmeric, dry extracts and biomasses obtained by the different extraction techniques.

characterize the yellow color of curcumin. In fact, the PLE extract presented a reddish color (Fig. 8 and Table 2) what can be a result of degradation process occurred due to the conditions in PLE process, which was of 60 °C for 1 h, which in turn, modified the extract coloration. Osorio-Tobón, *et al.* (2014)[7] observed that as the temperature increased in the PLE process, the curcuminoids content in the extracts decreased. The preservation of yellow color observed in the HIUS extracts (yellowness index in Table 2) can be attributed to the low temperature used in this process that reached up to (30 ± 1) °C.

Besides that, in Fig. 8 were showed the unflavored turmeric and biomasses images, wherein is possible observed visual differences in the particle size and color. Unflavored turmeric and control presented a bigger particles size and a more intense coloration than PLE and HIUS biomasses. These results were consistent with the results of SEM and color.

3.6. Biomass characterization

3.6.1. Morphology. Fig. 9 shows the SEM images for the surface of the unflavored turmeric and biomass obtained by control, PLE, HIUS (S/F: 5S : 1) and (S/F: 7S : 5). Although all samples presented an irregular shape, it can be observed in the micrographs that both HIUS processes caused morphological modifications in the biomasses. Analyzing the images at a magnitude of 250× (Fig. 9a), the size of the unflavored turmeric particles is larger than the biomasses because extractions processes promote rupture of the unflavored structure facilitating the release of the compounds. This effect was intensified when HIUS was applied due to the cavitation phenomenon.[18,28] The fragmentation is due to collisions between particles and shock waves created from collapsed cavitation microbubbles in the liquid medium. The consequence of the size reduction of particles by ultrasound is the

Table 2 Color parameters of the unflavored turmeric and colorant extracts and biomasses obtained by different extraction processes[a]

Product	Process	L	a^*	b^*	Yellowness index
Unflavored turmeric	—	55.4 ± 0.2^{a}	25.4 ± 0.4^{a}	67.4 ± 0.2^{a}	174
Dry extracts	Control	$45 \pm 3^{a,b}$	35 ± 2^{a}	54 ± 2^{a}	170
	PLE	26 ± 1^{b}	27.1 ± 0.4^{a}	18 ± 1^{b}	101
	HIUS (S/F: 5S : 1)	49 ± 3^{a}	27 ± 4^{a}	54 ± 3^{a}	157
	HIUS (S/F: 7S : 5)	$47 \pm 1^{a,b}$	35 ± 2^{a}	55 ± 2^{a}	169
Biomasses	Control	55 ± 2^{a}	16.3 ± 1.1^{b}	65 ± 2^{a}	165
	PLE	63 ± 2^{a}	$9.4 \pm 0.8^{b,c}$	52 ± 1^{b}	118
	HIUS (S/F: 5S : 1)	57 ± 2^{a}	16.5 ± 0.6^{b}	68 ± 2^{a}	169
	HIUS (S/F: 7S : 5)	58 ± 1^{a}	$13.4 \pm 0.7^{b,c}$	63 ± 2^{a}	156

[a] Mean values ± standard deviation (in duplicate, $n = 2$). Values followed by different letters in the same column show differences by Tukey's test at 95% significance (p-value < 0.05). Results are expressed in the dry base.

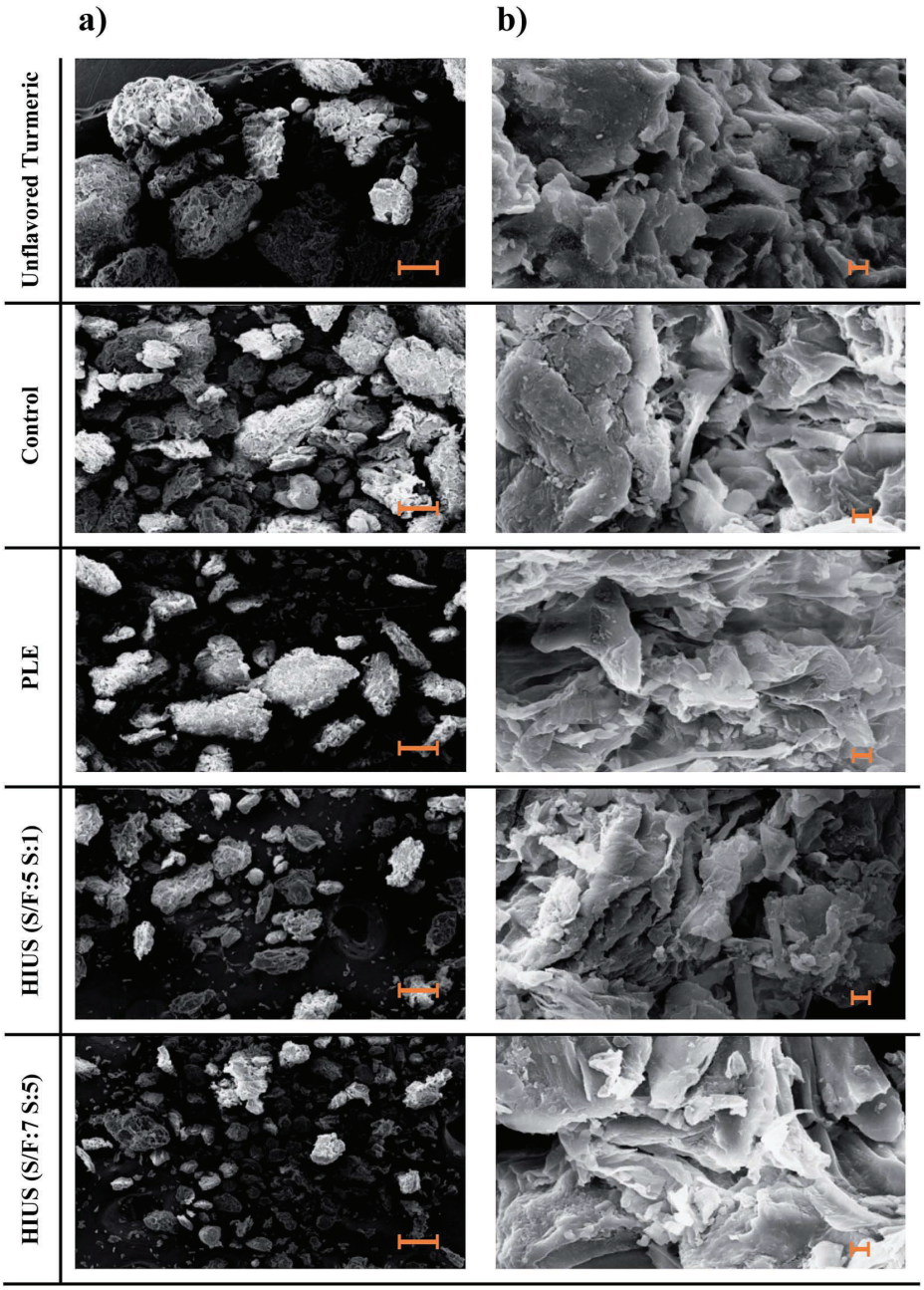

Fig. 9 Morphology of the unflavored turmeric powder (before curcumin extraction processes), and biomasses (control, PLE and HIUS). (a) 250× (100 μm), (b) 5000× (2 μm).

increase of the surface area of the solid, resulting in greater mass transfer and increase of extraction rate and yield.[18,29,30]

The micrographs at a magnitude of 5000× (Fig. 9b) show that, in general, the structure of the biomasses was not significantly altered by the extractions processes. This allows inferring that the processes do not affect its microstructure, which does not compromise its futures application. Comparing the different HIUS, it was observed that only the particle size was reduced in the HIUS (S/F: 7S : 5) due to the higher exposition to the acoustic cavitation, but there was no difference in the

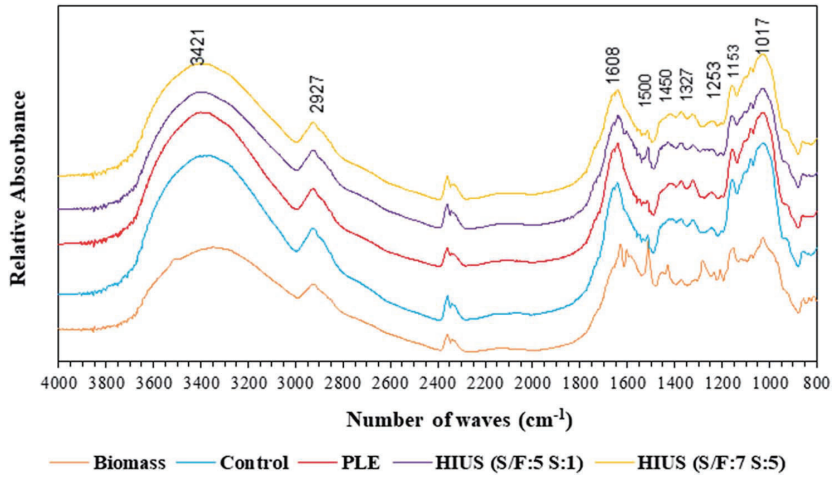

Fig. 10 Fourier Transform Infrared Spectroscopy (FTIR) spectra of turmeric biomass and biomasses obtained by control, HIUS and PLE processes.

structures of biomasses, which means that regardless of the choice of process, the biomasses characteristics are preserved.

Fig. 10 shows the FTIR spectrum of the unflavored turmeric and the biomasses obtained by the control, PLE and HIUS processes. It was possible to observe that different processes conditions did not alter the FTIR spectrum of the biomasses, demonstrating that the primary structure of the unflavored turmeric was preserved. This result can be attributed to the HIUS milder conditions (400 W for 1 and 5 min at 25 °C) used in the present study. The literature reports that HIUS process can alter the primary structure of biomass,[31,32] which usually occurs due to high powers and energies applied 302 W cm^{-2} and 100 W respectively.

The functional groups of the unflavored turmeric and biomasses were observed and the two bands in 4000–2000 cm^{-1} region, being abroad band centered at 3421 cm^{-1} assigned to hydrogen-bonded O–H stretching vibrations and the second one a weak signal at 2927 cm^{-1} due to C–H stretching vibrations, including CH, CH_2, and CH_3; these two bands are characteristics of all polysaccharides.[33] Considering that the turmeric biomass has 81% of carbohydrates,[12] the spectrum corroborates this information. The stretching peaks in the range of 950–1200 cm^{-1} was the characteristic absorbance of the polysaccharides.[34] The band 1153 cm^{-1} represent the bending vibration of C–O–C. The broad band at 1620 cm^{-1} is mainly attributed to the OH bending mode of adsorbed water and protein amide (C=O).[35] Bands at 1273, 1500 and 1313 cm^{-1} are close to the absorptions of aromatic ring vibration.[36] Bands at 1017 cm^{-1} are due to C–O stretching vibration.[37]

4. Conclusion

In this study, an upgrade for the turmeric biorefinery operating with clean emerging technologies was proposed. Our results demonstrated that HIUS processing was more efficient to recover curcumin from unflavored turmeric, with shorter processing time by a non-thermal way using mild temperature, which resulted in the maintenance of the color characteristics of the colorant. Although the curcumin yield and content obtained by HIUS process were the same obtained by PLE, the processing time of the HIUS was 12 times shorter than PLE. In addition, HIUS provided a dry extract with a yellow coloration characteristic of curcumin, differently from PLE that resulted in an extract with a reddish color. Therefore, the PLE process modified the curcumin coloration due to moderate temperature employed (60 °C), while HIUS reached 30 ± 1 °C. The biomasses obtained were not affected by the ultrasound treatment allowing their future applications, such as a gluten-free starch, modified starch, dietary fiber, natural encapsulating material, starchy material, and others.

Conflicts of interest

There are no conflicts to declare.

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10.3 Appendix III – Deposed Patent

PROCESSO DE PRODUÇÃO DE CORANTE NATURAL AZUL

INPI INSTITUTO NACIONAL DA PROPRIEDADE INDUSTRIAL

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Pedido nacional de Invenção, Modelo de Utilidade, Certificado de Adição de Invenção e entrada na fase nacional do PCT

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Dados do Depositante (71)

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10.4 Appendix IV – Manuscript about Genipin recovering

A synergic combination of natural deep eutectic solvents with high-intensity ultrasound extraction to recover genipin: optimization and evaluation of ready-to-use extract on its crosslinking and anti-neurodegenerative properties

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Abstract: In this paper, genipin, an important natural crosslinker and anti-neurodegenerative compound, was extracted from *Genipa americana* L. using an extraction process assisted by high-intensity ultrasound (HIUS) and natural deep eutectic solvents (NADESs). Extraction conditions were evaluated step-by-step to reach the best genipin recovery. The obtained ready-to-use genipin-NADES extract was tested regarding genipin crosslinking and anti-neurodegenerative capacity. The highest recovery was obtained using 40% water and 60% betaine:lactic acid NADES in molar ratio 1:3 (n/n) as solvent, a solvent:feed ratio of 19 (w/w), and HIUS nominal power of 180 W. NADES HIUS-assisted extraction showed to be a promising and efficient green extraction technique to obtain genipin from *Genipa americana* L. The ready-to-use genipin-NADES extract presented crosslinking capacity and anti-cholinergic activity. These results point that genipin-NADES extract can be directly applied in hydrogels for drug delivery, films, tissue engineering, and others. Also, it can be used in food, supplements, and medicines to enhance the neuro-protective effect.

Keywords: *Genipa americana* L; green solvent; optimum extraction; Alzheimer's disease; acoustic energy; betaine; NADES.

1. Introduction

Natural deep eutectic solvents (NADESs) have emerged as a new strategy to replace toxic, non-biodegradable, and flammable organic solvents, such as methanol, acetone and formic acid, for the extraction of plant metabolites (Gutiérrez, Alcalde, Atilhan, & Aparicio, 2020; Mišan et al., 2020). Even comparing to generally recognized as safe (GRAS) solvents, such as ethanol or water, NADESs have been revealed as more efficient extraction solvents (Benvenutti, Sanchez-Camargo, Zielinski & Ferreira, 2020). They are composed of, at least, a hydrogen bond acceptor (HBA) and a hydrogen bond donor (HBD), which are natural metabolites such as sugars, alcohols, organic acids, amino acids, etc., linked mainly by hydrogen bonds (Silva et al., 2021). However other type of interactions can be established between the components. NADESs have been attracting attention due to their physicochemical qualities (i.e. liquid state within wide temperature range, chemical or thermal stability) (Gutiérrez et al., 2020) as well as their low toxicity, high biocompatibility and easy preparation. Besides, as a result of HBA:HBD network formation, the solvation of target compounds is favored, which makes NADES ideal as green extraction solvents for the development of sustainable and efficient procedures (Benvenutti, Zielinski & Ferreira, 2019).

At the beginning of NADES utilization, the extract post-treatment, mainly the separation of the target compounds from the NADES, was considered an issue due to its inherent low vapor pressure, what makes difficult to recover the compounds from the extract solution (Dai, Verpoorte & Choi, 2014). However, since they are generally composed of natural and non-toxic substances, NADES may be directly incorporated in pharmaceutical, cosmetic and food formulations without demanding expensive purification steps, being a major advantage over conventional solvents (Mišan et al., 2020). Hence, nowadays, there are some works that consider the extracts from NADES as a ready-to-use material (Dai et al., 2020, Panić et al., 2019, Silva et al., 2021).

Often, to achieve an even greener and efficient process, the NADES solvent extraction is combined with an emergent technique, such as the high-intensity ultrasound (HIUS) extraction. This is an eco-friendly extraction tool due to the low requirements regarding solvent consumption and extraction time, which enhances the efficiency of the procedure in comparison with conventional techniques (Patil, Pathak & Rathod, 2021). The probe of HIUS in contact with the sample produces micro-turbulence in the medium, micro-jets, and micro-streaming that increase mass transfer and contact area between raw material and solvent. The

cavitation separates the desired compound from intracellular sources in a shorter period by increasing the interface among the solid matrix and extraction medium (Khadhraoui, Ummat, Tiwari, Fabiano-Tixier, Chemat, 2021; Strieder, Neves, Zabot, Silva & Meireles, 2021). Some previous works can be found in the literature using HIUS-assisted NADES extraction for the recovery of curcuminoids from *Curcuma longa* (Patil et al., 2021), flavonoids from *Fagopyrum esculentum* (Mansur, Song, Jang, Lim, Yoo, Nam, 2019), β-carotene from *Cucurbita maxima* (Stupar et al., 2021), and gingerols from *Zingiber officinale* (Hsieh, Li, Pan, Chen, Lu, Yuan, Zhu, Zhang, 2020).

Genipin is an iridoid found in unripe genipap (*Genipa americana* L.), a native fruit to northern South America, the Caribbean, and southern Mexico. This compound is a natural, biocompatible, and non-toxic cross-linker agent reacting with polymers and proteins' primary amines groups (Neri-Numa, Pessôa, Arruda, Pereira, Paulino, Angolini & Pastore, 2020). Crosslinking networks are generally used to produce hydrogels for drug delivery (Muzzarelli, 2009), films used in food packaging (Garavand, Rouhi, Razavi, Cacciotti & Mohammadi, 2017), engineering tissues (Mirzaei, Faridi-Majidi, Shokrgozar & Paskiabi, 2014), materials to increase emulsion stability (Lin, Yu, Ai, Zhang & Guo, 2020), etc. Regarding its health benefits, genipin is an anti-hypertensive, antioxidant, anti-inflammatory, and anticancer agent (Shetty & Sarkar, 2020). Moreover, some studies suggest that genipin presents anti-neurodegenerative effects since relieves the symptoms related to Alzheimer Disease (AD), which still lacks effective treatment (Huang, Wang, Li, Zhang, Ma, Zhu & Zhang, 2019). AD is considering a multifactorial and neurological disease, and it is characterized by cognitive deficiency, oxidative stress, neuro-inflammation, tau protein hyper-phosphorylation, and its aggregation into neurofibrillary tangles. The cognitive deficiency is a result of a progressive decline of acetylcholine neurotransmitter (ACh) in the synaptic cleft (Mushtaq, Greig, Khan & Kamal, 2014). Presently, there is not a treatment for this disease, nevertheless, the palliative treatment is based on the increasing of acetylcholine levels through an anti-cholinergic activity, inhibiting acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) enzymes (Mushtaq et al., 2014; Silman & Sussman, 2005).

To date, there is no work using NADESs as solvent for the HIUS-assisted extraction of genipin from its natural source. Thus, the aim of this research was to evaluate the application of the choline chloride (ChCl) and betaine (Bet)-based NADES combined with the most efficient extraction conditions to reach the highest genipin extraction rate and a ready-to-use genipin-NADES extract from *Genipa americana* L. Additionally, effect of the NADES on genipin crosslinking capacity and anti-cholinergic activity was investigated.

2. Material and Methods

2.1. Chemicals and material

All chemicals were of analytical reagent grade (except in those cases specifically indicated) and used as received. Acetonitrile (ACN) (purity 99.8%) and formic acid (purity > 99%) were purchased from VWR Chemicals (Barcelona, Spain). Deionized water was obtained from a Milli-Q Milipore® system A10 (Billerica, MA, USA). ChCl (98%), Bet (> 97%), lactic acid (LaAc) (> 90%), propylene glycol (ProGly) (99%), and propionic acid (ProAc) (99%), were acquired from Tokyo Chemical Industry (Tokyo, Japan), Genipin analytical standard was supplied by Acros Organics (Geel, Belgium) (> 98%). Standards of lysine, AChE type VI-S was from Electrophorus electricus, whereas BChE was from equine serum, acetylthiocholine iodide (ATCI), trizma hydrochloride (Tris-HCl), fluorescein sodium salt, and a 96 well acceptor plate (Catalog nº MATRNPS50) were obtained from Sigma-Aldrich (Madrid, Spain). The 4-(amino359 sulfonyl)-7-fluoro-2,1,3-benzoxadiazole (ABD-F) was purchased in Tokyo Chemical Industry (Tokyo, Japan).

Unripe genipap fruits were donated by "Fazenda Lagoa" (Ponte Alta do Tocantins, TO, Brazil). The entire fruits were partially lyophilized, grounded, and stored at -18 ºC until their use.

2.2. Synthesis of NADES

NADESs were prepared by mixing different HBDs and HBAs using a molar ratio (HBA:HBD) (n/n) of 1:2. Combinations of Bet and ChCl as HBAs with ProAc, ProGly, and LaAc as HBDs were synthesized. For those syntheses, NADES components were placed in a glass vial and stirred at 80 °C for 30 min, observing the formation of the homogeneous liquids (Adeyemi, Sulaiman, Almazroui, Al-Hammadi & AlNashef, 2020). The solvents were stored in a vacuum desiccator at 25 ± 2 °C. After NADESs preparation, they were combined with different amounts of water to prepare the extraction solvents.

2.3. NADES HIUS-assisted genipin extraction

The samples for the HIUS-assisted extraction were prepared using a solvent:genipap ratio to reach a final mass of 30 g, the solvent/feed ratio (S/F) (w/w) initially used was 14; also, the water proportion to form the solvent with NADES was initially 50%. Genipin extraction was carried out according to Neves, Strieder, Silva, and Meireles (2020), with some modifications. A 12.6 mm diameter ultrasonic probe 450 Digital Sonifier (Branson, Danbury, CT, United States) at 19 kHz and a nominal power of 315 W was employed at the beginning of the study.

The time of process was 3 min for all experiments. After selecting the most suitable NADES, different HBA:HBD ratios (1:2, 1:3 and 1:4) (n/n) were tested. Then, the S/F (29, 19, 14, 9) (w/w); and the $\%H_2O$ in the solvent formulation (30, 40, 50, 60 %) were evaluated. Afterwards, different HIUS nominal power was tested: 0, 180, 270, 315 and 405 W, referring to the amplitudes (%): 0, 40, 60, 70, 90, respectively. The acoustic power (real power) supplied to the model system (water) was determined by calorimetric assays according to Mason, Lorimer, Bates and Zhao (1994) and according to the sequence of Eq. 1, 2, and 3. For the respective nominal power, we had 0; 14.4 ± 0.5; 22.1 ± 0.2; 27.2 ± 0.7; and 33.6 ± 0.9 W, of acoustic power. Finally, the extracts were separated from the biomasses by a paper filtration. An aliquot of 200 µl was collected for each run, diluted 1:5 in water, and filtered through a 0.45 µm filter to quantify the extraction rate. The extracts were stored at -18 °C until performing the analyses.

$$Acoustic\ power\ (W) = mC_p(\frac{dT}{dt}) \quad (1)$$

$$Specific\ energy\ \frac{(kg)}{g} = (\frac{Acoustic\ power\ \times processing\ time}{mass}) \quad (2)$$

$$Ultrasound\ intensity\ \frac{(W)}{cm^2} = (\frac{Acoustic\ power\ \times 4}{\pi D^2}) \quad (3)$$

where, m is the sample mass (g); C_p is the specific heat of water (J/g °C), $\frac{dT}{dt}$ is the change in temperature along with the whole-time range (°C/s) determined by a polynomial curve fitting, and D is the diameter of the HIUS probe in cm.

Experiments were carried out by triplicated. For each step the same fruit was used, aiming to eliminate the effect of different raw materials.

2.4. HPLC-DAD method

Genipin extraction rate was determined using an HPLC-DAD Agilent 1100 series system (Agilent Technologies, Santa Clara, CA, United States). Genipin was separated using a column Poroshell 120, C_{18} (4.6 × 100 mm, 2.7 µm) (Agilent Technologies, Santa Clara, CA, United States). Separation was carried out according to Nathia-Neves, Nogueira, Vardanega, and Meireles (2018), using a mobile phase composed of water (A) and ACN (B), both acidified with 0.1% formic acid and applying the following gradient: 0 min, 99% A; 9 min, 75% A; 10 min, 99% A and 13 min, 99% A. Injection volume was 10µL. Column temperature was set at 35 °C and flow rate at 1.5 mL/min. Genipin was detected at 420 nm and identified by comparing its retention time and UV-vis spectra to the reference standard (St-Gp). The

extraction rate (mg/g of genipap mass) was calculated according to Eq. (4).

$$Genipin\ extracion\ rate\ = {genipin\ mass\ (mg)}/{genipap\ mass\ (g)} \quad (4)$$

For confirmation analyses, the St-Gp, genipin extracted with water (Gp-H_2O), genipin extracted with NADES (Gp-NADES), selected NADES, and St-Gp with NADES (St-Gp + NADES) were evaluated using a MS system from Bruker Daltonik (Bremen, Germany) with electrospray ionization (ESI) (negative mode) and an ion trap as analyzer. The extracts were diluted in water to reach the genipin concentration of 0.5 µg/mL St-Gp, NADES, and (St-Gp + NADES) were prepared based on the same concentration. ESI nebulizer gas flow was set at 20 psi; spray voltage at 2000 V and source temperature at 250 °C. The analytical column used was a C_{18}, (2.1 × 100 mm, 1.9 µm) (Thermo Fisher Scientific, Vilnius, Lithuania), at 35 °C. Water (solvent A) and ACN (solvent B), both with 0.1% formic acid (v/v), were used as mobile phases. Initial composition was established at 99% of A and 0.1% of B, this percentage was reduced until 75% of A at 7 min, maintaining this composition until 8 min. Then, it was increased up to 99%, until 14 min. The sample flow rate was set at 0.25 mL/min and sample injection volume was 10 µL. The area for each *m/z* was normalized to the total summed area.

2.5. Reverse Phase Liquid Chromatography-Quadrupole-Time of Flight Mass Spectrometry (RP/HPLC-Q-TOF MS/MS) analysis

NADES-genipin extract (Gp-NADES) samples obtained under optimized conditions were dissolved 1:10 in water to a final concentration of 1 mg/mL, and 2 µL (in triplicates) were injected into a HPLC (model 1290) coupled to a quadrupole Q-TOF (6540 series) from Agilent Technologies (Germany). The conditions used for compound separation and detection were the same as previously described (Dauber et al., 2022). For data processing, LC-MS raw data files were converted to ABF format and data processing was performed using MS-DIAL (v. 4.80) (Tsugawa et al., 2015). Peak area calculation was performed by combining data for different detected molecular species ($[M-H]^-$, $[M+Cl]^-$, $[M+FA-H]^-$ adducts). Total compound contribution (%) was calculated as compound area/Total Ion Current area ∗100, and standard deviation of the three replicates was calculated. All metabolites were identified as MSI level 2a following the Metabolomics Standard Initiative (MSI) guidelines (Blaženović, et al., 2019). The list of metabolites obtained was filtered removing unknown metabolites, metabolites with a maximum height below 1000 units or metabolites with a maximum height

below three times the average height in the extraction blanks (NADES).

2.6. NADESs characterization

The selected NADES and its pure constituents were analyzed in order to confirm the correct preparation of the extraction solvent. Fourier transformed-infrared (FT-IR) spectra was performed using a microscope IR Spotlight 200i (Perkin Elmer) obtaining the Attenuated Total Reflectance (ATR) with diamond crystal in the range of medium IR (4000-450 cm^{-1}) applying 20 scans, aperture of 8.94 mm, and resolution of 4 cm^{-1}. Viscosity study was carried out using a Viscosimeter VSM 3000 Stabinger (Anton Paar® GmbH), at 25 ºC using 7 mL of the selected NADES.

2.7. Effect of the presence of NADES on genipin properties

NADES-genipin extract (Gp-NADES) obtained under optimized conditions was analyzed with the aim of evaluating the influence of the NADES on genipin anti-neurodegenerative effect (based on its anti-cholinergic activity) and genipin crosslinking capacity. For that, Gp-NADES, Gp-H_2O, and St-Gp solution were evaluated and compared. Before the anti-cholinergic activity and crosslinking assays, the pH of the extracts was adjusted at 8 and 6 with NaOH 10 M, respectively (the amount added did not affect the concentration of the extracts).

2.7.1. Anti-cholinergic activity

The anti-cholinergic capacity of the genipin extracted by NADES was tested by *in-vitro* assays based on the AChE and BChE inhibitory capacity performed based on Ellman's method by fluorescent enzyme kinetics. The samples tested were the St-Gp, Gp-H_2O, and Gp-NADES. The method was carried out according to Sánchez-Martínez, Bueno, Alvarez-Rivera, Tudela, Ibañéz & Cifuentes (2021). A hundred µL of the sample at the concentrations of 200 µg–2000 µg/mL, 100 µL of buffer (150 mM Tris-HCl pH = 8) and 25 µL of 1.6 U/mL AChE or BChE in buffer were filled in a microplate of 96-well and then, incubated for 10 min. Afterwards, 25 µL of ABD-F (125 µM) in buffer and 50 µL of ATCI at a concentration of the K_M (Michaelis–Menten constant) value in H_2O were added. The K_M constant is the substrate concentration at which the reaction rate is half of the maximum velocity rate. The fluorescence was logged at $\lambda_{excitation}$ = 389 nm and $\lambda_{emission}$ = 513 nm every min for 10 min at 37 °C. This kinetic measurement seeks to obtain the V_{mean} value, which resembles enzymatic mean velocity achieved during kinetic measurement. Galantamine hydrobromide in

EtOH/H_2O (1:1, v/v) was used as the reference inhibitor for AChE and BChE enzymes (Sánchez-Martínez, Bueno, Alvarez-Rivera, Tudela, Ibañéz & Cifuentes, 2021). The K_M value was measured by adding 100 µL of ATCI at different concentrations (0.4 – 4 mM) in H_2O, 50 µL of H_2O, and 100 µL of buffer and starting the reaction by adding 25 µL of ABD-F (125 µM) in buffer and 25 µL of 1.6 U/mL AChE or BChE in buffer. V_{mean} and K_M were calculated by Gen5™ version 2.0 Data Analysis software from BioTek Instruments, Winooski, VT, USA. The 96-well was placed in a spectrophotometer reader Synergy HT (BioTek Instruments, Winooski, VT, USA).

2.7.2. Crosslinking capacity

The crosslinking reaction between genipin and primary amines from amino acids, proteins or polymers produces a blue pigment. The effect of the presence of the NADES on the genipin crosslinking capacity was evaluated by determination of the absorption modification of the solutions at 590 nm (wavelength corresponding to the blue). For this, lysine (lys), St-Gp; Gp-H_2O, and Gp-NADES were prepared at pH 6 and diluted to reach the same concentration (80 µg/mL). All extracts without and with the amino acid (used as amine supplier) were put in a 96-well microplate. Immediately, the microplate was read at 590 nm in a spectrophotometer Synergy HT (BioTek Instruments, Winooski, VT, USA) for the time 0 and each 2 min until 30 min. The results were expressed in $\Delta_{absorbance}$, which was calculated according to the Eq (5).

$$\Delta_{absorbance} = A_t - A_0 \qquad (5)$$

Where, A_t is the absorbance at time t, and A_0, correspond to the initial absorbance (time 0).

2.8. Statistical analysis

Statistical analysis was performed using Minitab 18® software. The results were expressed as mean values ± standard deviation (SD) (three replicates). ANOVA using Tukey's test examined the differences between the mean values. The difference was considered statistically significant when the p-value was below 0.05.

3. Results and Discussion

3.1. NADES selection

3.1.1. NADES components selection

Screening of six NADESs was carried out to select the most suitable for extracting genipin from *Genipa americana* L. All NADESs were based on ChCl or Bet in combination with

ProAc, ProGly or LaAc at a 1:2 molar ratio. As previously mentioned, all NADESs have a natural origin being widely distributed among living organisms, and thus, it leads to low-cost environmentally friendly NADES (Hammond, Bowron, Edler, 2016). The choice of these six solvents was based on the literature (Ivanović, Alañón, Arráez-Román, Segura-Carretero, 2018; Yu et al., 2021) in which terpenoids with similar characteristics as genipin were extracted using these combinations.

One of the main issues of using NADESs in extraction processes is their high viscosity. This characteristic leads to low mass transfer and low compound diffusion in extraction procedures (Zainal-Abidin, Hayyan, Hayyan & Jayakumar, 2017). In fact, even with organic acid-based NADESs, which present relatively high fluidity at room temperature, viscosity is significantly higher than that of the commonly used organic solvents (Sánchez, González, Salgado, Parajó & Domínguez, 2019). In this sense, the combination of NADES with water can reduce viscosity and, consequently, decrease surface tension and increase molecular diffusion. Thus, all experiments were conducted using 50% of water in NADES as standard conditions in an initial selection of the NADES.

Figure 1a) shows the effect of NADES combination on genipin extraction efficiency. We also performed a genipin extract using water, as a control. However, in HPLC-DAD analysis, the genipin content was not stable inter-day and the results was unconcluded. May because of some reactions occurred in genipin extract using the water as a solvent. Water recovers other compounds from the fruit, such as amines from amino acids and sugars which react with the genipin. Renhe, Stringhetam Silva and Oliveira (2002) extracted genipin by *Genipa americana* L using ethanol and water, seeking to obtain a natural blue colorant. They observed an unstable color in the water extract, probably due to the reactions occurring. This result also confirmed the importance to use NADESs as genipin solvent extraction to maintain the genipin stable, once NADES was a more selective solvent comparing to water. Additionality, as can be observed, the NADESs are efficient solvents because their hydrogen bond capability and electrostatic interactions with target components enhances compound recovery (Zainal-Abidin et al., 2017).

ChCl:ProGly was the less favorable to recover the genipin, followed by Bet:ProGly and ChCl:ProAc, which showed intermediate values of genipin recovery. To promote the highest genipin extraction efficiency, the best solvents were Bet:ProAc, Bet:LaAc, and ChCl:LaAc Despite the fact that they presented the same genipin recovery capacity, Bet:LaAc presented the smallest standard deviation; for this reason, it was selected for further studies. It should be noted that organic acid-based NADESs (e.g., ChCl:LaAc, Bet:LaAc, and Bet:ProAc) showed

a high capacity for extraction genipin. These results are in agreement with previous results using organic acid-based NADESs (García, Rodríguez-Juan, Rodríguez-Gutiérrez, Rios, & Fernández-Bolaños, 2016, Benvenutti et al., 2020), which confirmed that using such compounds as solvents effectively facilitates the extraction of bioactive compounds.

3.1.2. NADES molar ratio selection

After selection of the HBA and HBD for the NADES formulation, it is important to verify the proportion of both components. HBA and HBD proportion influences NADES stability and its physicochemical properties and, therefore, its extraction capacity, favoring or limiting the solubility of the target compound in the solvent. In this sense, Figure 1b) presents the effect of Bet:LaAc acid molar ratio (n/n) (1:2, 1:3, and 1:4) on genipin extraction rate. The LaAc concentration affected the potential of the NADES as a solvent (p-value = 0.003). Based on these results, the selected proportion to conduct the subsequent steps was 1:3 (Bet:LaAc).

3.2. NADESs characterization

Selected NADES Bet:LaAc was characterized by FT-IR to verify the establishment of the hydrogen bonds between both components and, consequently, the correct preparation of the new solvent. Hence, Figure S1 (Supplementary material) shows the FT-IR spectra of Bet (a), LaAc (b) and Bet:LaAc (1:3) NADES (c). The bands at 3359 and 3285 cm^{-1} in Figure S1a) are associated with the asymmetric and symmetric stretching of N-H bonds in the Bet structure while the bands at 1695 and 1616 cm^{-1} are characteristic of the asymmetric and symmetric stretching of carboxylate group present in its structure (Olivares, Martínez, Rivas, Calderón, Munita & Campodonico, 2018; Li, Xie, Liu, Liu, Wan, Liang & Zang, 2020). Figure S1b) shows a band at 3401 cm^{-1} associated with the stretching of hydroxylated group of the LaAc structure. The interaction of both molecules is established by the formation of a hydrogen bond between carboxylate group in Bet and the hydroxylated group of LaAc as a consequence of the strong electronegativity of the O^- of (COO^-) group and the positive charge of the H- in the OH- group. Such interaction modifies the stretching vibration of carbonyl and hydroxyl group bringing about a shift of the wavelength as can also be seen for both specific bands in Figure S1c) (Li et al., 2020).

Among the different characteristics of NADES, viscosity plays an important role in the extraction performance (Table S1 – Supplementary material). When solvent presents low viscosity, its diffusivity through the matrix is improved favoring the interactions target compound-NADESs and improving the extraction process. In this work, the viscosity of three

different Bet:LaAc molar ratios (1:2; 1:3 and 1:4) were evaluated at 25 ^{o}C. Results showed dynamic viscosities of 965.57 ± 3.51 mPa·s; 465.15 ± 1.21 and 311.47± 0.37 mPa·s, respectively. By decreasing the proportion of LaAc, an increase in viscosity was observed, which can hinder the effectiveness of HIUS extraction. Those values agree with other studies previously published in the literature in which similar data was reported (Gutiérrez, Alcalde, Atilhan & Aparicio, 2020; Sánchez et al., 2019)

The solvent viscosity directly affects the efficiency of the HIUS assisted extraction. When solvent viscosity is increased, cavitation bubbles within the fluid occurred more creepily and thus, the tensile strength of the liquid increases. Hence, these results suggest higher suitability of molar ratio 1:4 to perform the extraction procedure due to its low viscosity. However, other aspects such as the better formation and interaction between each NADESs components should be also considered. Hence, the 1:2 ratio impair the extraction due to the high viscosity and the 1:4 proportion was not suitable to generate an efficient NADES as a solvent; therefore, 1:3 ratio was finally selected.

3.3. Optimization of genipin extraction

In this section, a thorough step-by-step evaluation of the different parameters that could affect the extraction efficiency was performed in order to obtain the highest recovery of genipin. Those parameters were: solvent:feed ratio (S/F) (w/w) (29, 19, 14, and 9), proportion of water in the extraction solvent % (w/w) (30, 40, 50, and 60), and HIUS amplitude (%) (control, 40, 60, 70, 90).

3.3.1. Solvent/feed (S/F) ratio selection

Results of genipin recovery are presented in Figure 2a). The best S/F was 19 (1.5 g of genipap mass and 28.5 g of solvent) (p-value < 0.001), using the initial parameters not yet optimized (water in the extraction solvent % (w/w) 50; HIUS amplitude (%) 70). The equilibrium between the solvent and extractable material is important to achieve an adequate recovery of the target compound. When the amount of solvent is small, the NADES can only interact with part of the target compound. However, when the extractable material exceeds a certain value, NADES cannot interact with the extractable material to recover the target compound. Indeed, the selected proportion S/F of 19 allows a suitable interaction between the target compounds with the solvent, maximizing the extraction rate (Liu, Li, Fu, Zhang, Wang & Wang, 2019).

3.3.2. Study of water proportion on the extraction solvent

Water proportion on the solvent formulation by NADES can affect the efficacy of the final solvent (p-value = 0.001), as could be seen in Figure 2b). It has great importance to use the minimal quantity of NADES which reach a higher extraction efficiency to reduce the process costs. Water addition increases polarity, reduces surface tension and reduces viscosity of NADES. However, excessive addition of water can impair the hydrogen bond between the HBA and HBD, reducing NADES stability and hindering the interactions between the compound and NADES (Sánchez et al., 2019). Experiments were developed fixing the optimized S/F at 19 and using the HIUS amplitude not yet optimized at 70%. The minimal amount of water that reached the best extraction conditions was 40%. Using 30% of water the extraction was less efficient, probably due to the higher viscosity. From 50% of water onwards, the genipin recovery process becomes less efficient. Thus 40% was selected for further studies.

3.3.3. Evaluation of HIUS amplitude

After fixing the best extraction conditions regarding the NADES and solvent formulation, and solvent/feed proportion, we evaluated the best HIUS amplitude. Acoustic cavitation offered by HIUS resulted in a higher performance on genipin extraction from *Genipa americana* L., besides its diffusion into the Bet-based NADES. The effects of this phenomenon include heat and high shear rates, in which the last one promotes a vegetable cell rupture, facilitating the access by the solvent to recover the target compound. Thus, the acoustic energy promoted temperature and turbulence, and both favored the genipin recovery and diffusion in the NADES solvent (Strieder et al., 2021). The higher the power used in the HIUS process, the greater the acoustic cavitation. In fact, some authors directly apply the maximum nominal power to extract the target compound (Neves, Strieder, Vardanega, Silva & Meireles, 2020). However, studying the minimal nominal power that can be used to recovery the maximum target compounds, it is possible to reduce the extraction cost and energy consumption, according to sustainability premises. Figure 2c) shows the effect of applied nominal power on genipin recovery efficiency when previous selected parameters were fixed. A solid-liquid extraction without HIUS assistance was performed to observe the effect of the HIUS process on genipin extraction. The use of the HIUS-assisted extraction process influenced the recovery of genipin by the solvent (p-value < 0.001). However, even a nominal power of 180 W was enough to present better extraction yields. The result is very favorable from an energy point of view, as the lower power was already sufficient to promote the necessary acoustic

cavitation to increase the extraction efficiency. Higher energy in the HIUS process did not increase genipin extraction rate, probably because with 180 W the maximum recovery of the compound had already been reached.

3.4. Evaluation of solvent and matrix effect on genipin determination

Matrix effect is caused by the influence of matrix composition on the detection signal of the target analyte which can be enhanced or suppressed (Meerpoel et al., 2018). Hence, the genipin quantification can be impaired by this phenomenon. In this sense, Figure S2 (Supplementary material) shows the calibration curves for genipin in the concentration range (0-500 μg/mL) in water solvent and in *Genipa americana* L. extract obtained using water as extraction solvent, respectively. For the last one, the initial amount of genipin was quantified and excluded from the results.

As shown in Figure S2, matrix did not cause any effect on the genipin detection by HPLC-DAD, because the calibration curve of St-Gp in water and in genipap extract presented similarly slope.

In the same way, NADES effect on genipin signal was also evaluated by comparison of the peak areas obtained for genipin extract with and without NADES. As it is shown in Table S2 (Supplementary material), similarity in the range 80-100% was obtained in all experiments indicating that there is not significant influence of the NADESs on genipin signal that could preclude the correct determination of this compound in NADES extracts.

3.5. NADES HIUS-assisted genipin extraction validation

Genipin calibration curve ($y = (13.81 \pm 0.336)x + 95.36 \pm 130.91$) was obtained in the range 1–1000 μg/mL, reaching a determination coefficient of 0.9993. Repeatability of the method was evaluated inter-day and intraday. Relative standard deviation for intraday experiments was 8% and 11% for inter-day (n=5). Also, the identification of the genipin was confirmed by HPLC-(ESI)-MS (Figure 3). As can be observed, St-Gp peak appear at 11 min and is associated with two *m/z*: 224.7 and 206.7 and (green and red one, respectively), in which the first one represents the molecular ion (genipin exact mass: 226.084) and the second one is a genipin fragment related to the loss of a H_2O molecule from molecular ion (Chen et al., 2014). We could observe the same peaks at the same retention time (Rt) for the extracts Gp-NADES, Gp-H_2O, and St-Gp + NADES. These results confirm the effective extraction of genipin by using both H_2O and NADES solvents. For NADES spectra, three main peaks are present at a retention time of 14 min. Those peaks are associated with the *m/z* 304.8, 232.7, and 160.7:

blue, pink, and light blue chromatograms, respectively (Figure 3). Also, the samples in which NADES is present (Gp-NADES and St-Gp-NADES) show those peaks which should be related to NADES signal.

3.6. Identified compounds in NADES-genipin extract

Regarding the individual compounds identified by RP/HPLC-Q-TOF MS/MS, Table 1 summarizes the main compounds and their contribution to the extract studied, ordered based on their retention time. The most abundant compound was malic acid, follow by citric acid, mannitol and genipin. Other compounds such as geniposide and genipin 1-gentiobioside were also detected, that have been previously identified in other works which studied the *Genipa americana* L. methanol extract's compounds (Neri-Numa et al., 2020; Bente & Mercadante, 2014). These same authors did not find malic acid on their extracts. Probably NADESs present more capacity to recover this compound.

3.7. Effect of the presence of NADES on genipin properties

As mentioned, it is known that when NADES is used as an extraction solvent, its separation from the target compound is complex due to their low volatility (Dai et al., 2014). As the extract with NADES has been considered for some authors as a ready-to-use extract, is it important to test if the target compound will present the same properties in presence of NADES. For this reason, we tested the effect of the NADES on two important genipin properties: its anti-cholinergic activity and its crosslinking capacity.

3.7.1. Anti-cholinergic activity

It has been reported that genipin presents AChE and BChE inhibition capacity (Huang et al., 2019). Based on that, Gp-NADES and Gp-H_2O extracts, and St-Gp were tested to evaluate the effect in AChE and BChE inhibition capacity. For enzyme activation, it is necessary a buffer solution at pH 8. However, NADES extracts present pH 3.0 ± 0.2 due to the presence of lactic acid in their composition. For this reason, 10 M NaOH was added to the samples until reaching the desired pH 8. AChE and BChE enzymatic activities were expressed as % of inhibition using 66 µg/mL of genipin in each extract forms. As can be seen in Table 1, extracts obtained with NADES present higher AChE and BChE inhibition capacity than the genipin standard at the same concentration. In the same way, these values were also higher than the ones obtained for Gp-H_2O. These results demonstrate that not only the selected NADES did not impair the genipin's AChE and BChE activity, but can also enhance this

activity. This effect can be explained as a result of the use of natural compounds as NADESs constituents which are expected to promote a synergic bioactive effect. Additionally, the higher AChE inhibitory capacity found in NADES extract could be due to the extraction of other metabolites such as malic acid. Zengin and co-authors, (2018) have worked with wild edible silene species and found a high amount of malic acid. In parallel, all species analyzed presented AChE and BChE inhibitory capacity, and they related this propriety due the presence of malic acid. In our study, although the aqueous extract is less selective in recovering sugars and amino acids, the NADES solvent, unlike the aqueous one, extracted a high level of malic acid. Thus, different solvents to extract compounds by *Genipa americana* L. can extract different compounds. We can observe the effect of solvent on the recovery of different compounds by *Genipa americana* L comparing some literature works. For example, Neri-Numa and co-authors (2020) extracted compounds from *Genipa Americana* L. with methanol, and found mainly genipin, geniposide, and geniposide acid; however, Nathia-Neves, Nogueira, Vardanega & Meireles (2018) extracted by ethanol and obtained only genipin and geniposide.

3.7.2. Crosslinking capacity

In the presence of oxygen, genipin can spontaneously react with primary amines of amino acids, peptides, proteins, or other polymers which contain amines groups through a crosslinking process used for some technological applications (Neri-Numa et al., 2020). This reaction produces a blue pigment with a maximum absorbance at 590 nm (Neves, Silva & Meireles, 2021). Figure 4 presents the change in absorbance at 590 nm of the St-Gp, Gp-H_20, and Gp-NADES, the increase is a measure of the genipin's crosslinking capacity.

In the samples without lysine (St-Gp, Gp-H_2O, Gp-NADES), the absorbance did not increase, however, when lysine was added, an increase in the absorbance at 590 nm was observed in all samples (St-Gp + Lys, Gp-H_2O + Lys, Gp-NADES + Lys). This increase corresponds with the formation of blue color as a result of the crosslinking reaction with genipin. This result proves that NADES does not impair the crosslinking capacity of genipin and, therefore, can be maintained as a ready-to-use extract.

4. Conclusions

An extraction solvent composed of Bet-based NADES with lactic acid (1:3), and 40% of water (w/w) was the most promising solvent for genipin extraction. Also, the best S/F ratio was 19. This study also pointed out the HIUS as an intensification tool for NADES-based

extraction of genipin, even using 180 W of nominal power. Therefore, obtained results justify the use of NADES HIUS-assisted extraction as an alternative environment-friendly, acceptable cost and very efficient technique for genipin recovery. Additionally, obtained extract is ready-to-use since NADES not only does not impair genipin crosslinking capacity nor its anti-cholinergic activity but on the opposite, can enhance such its neuroprotective potential mainly due to the capacity to extract also malic acid from *Genipa americana* L.. Results obtained in this work point the ready-to-use Gp-NADES extract potential in food, supplements, medicines, and cosmetic applications.

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Figures:

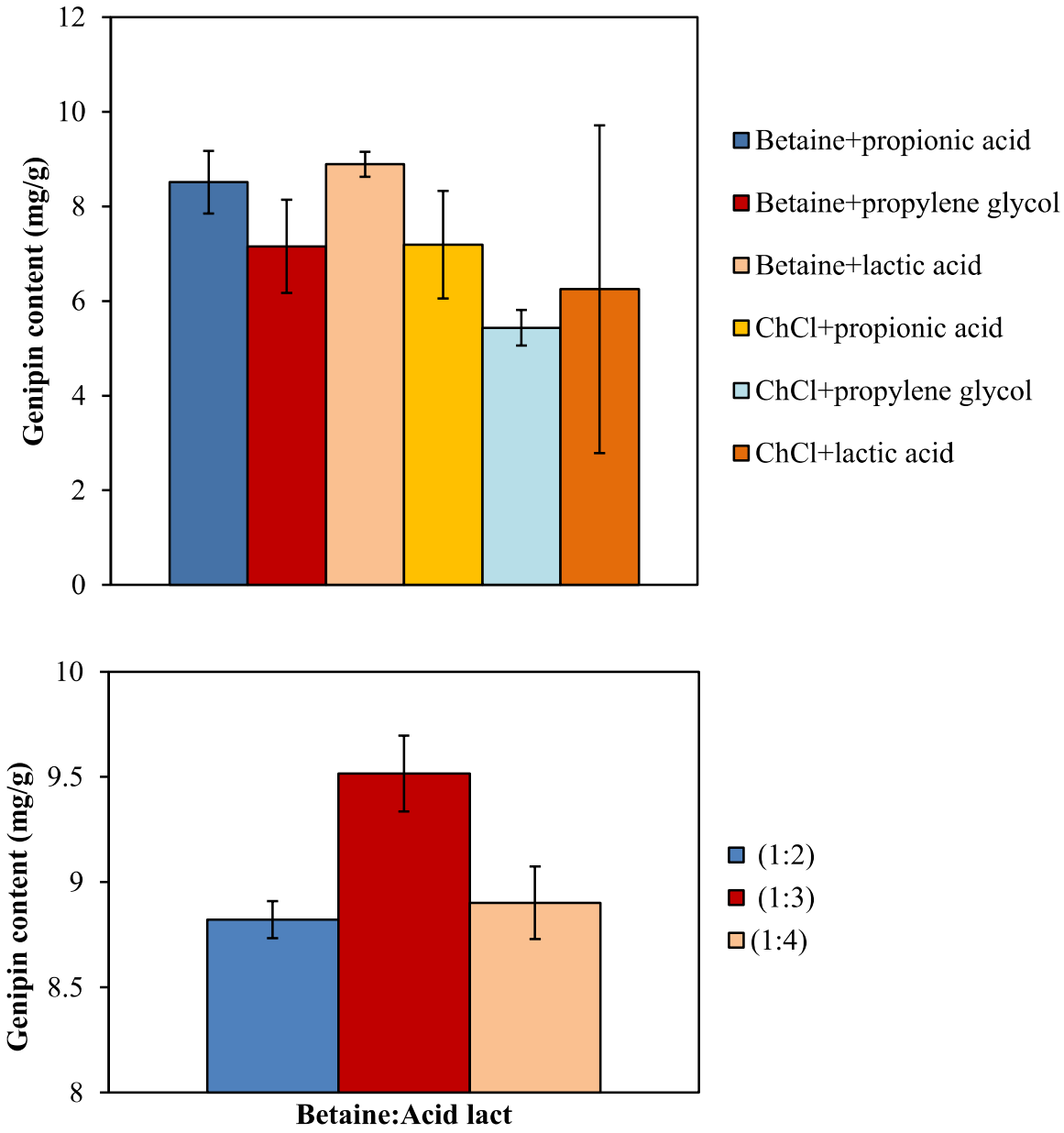


Figure 1. Extraction yields based on genipin extraction rate (mg/g of genipap) obtained by solid–liquid extraction assisted by HIUS. Experiments were carried out in triplicate using 315 W of nominal power, S/F (w/w) of 14, and 50% of water. **a)** with different NADES including combinations of Bet and ChCl as HBAs with ProAc, ProGly, and LaAc as HBDs; **b)** with different Bet:LaAc molar ratios (1:2; 1:3; 1:4).

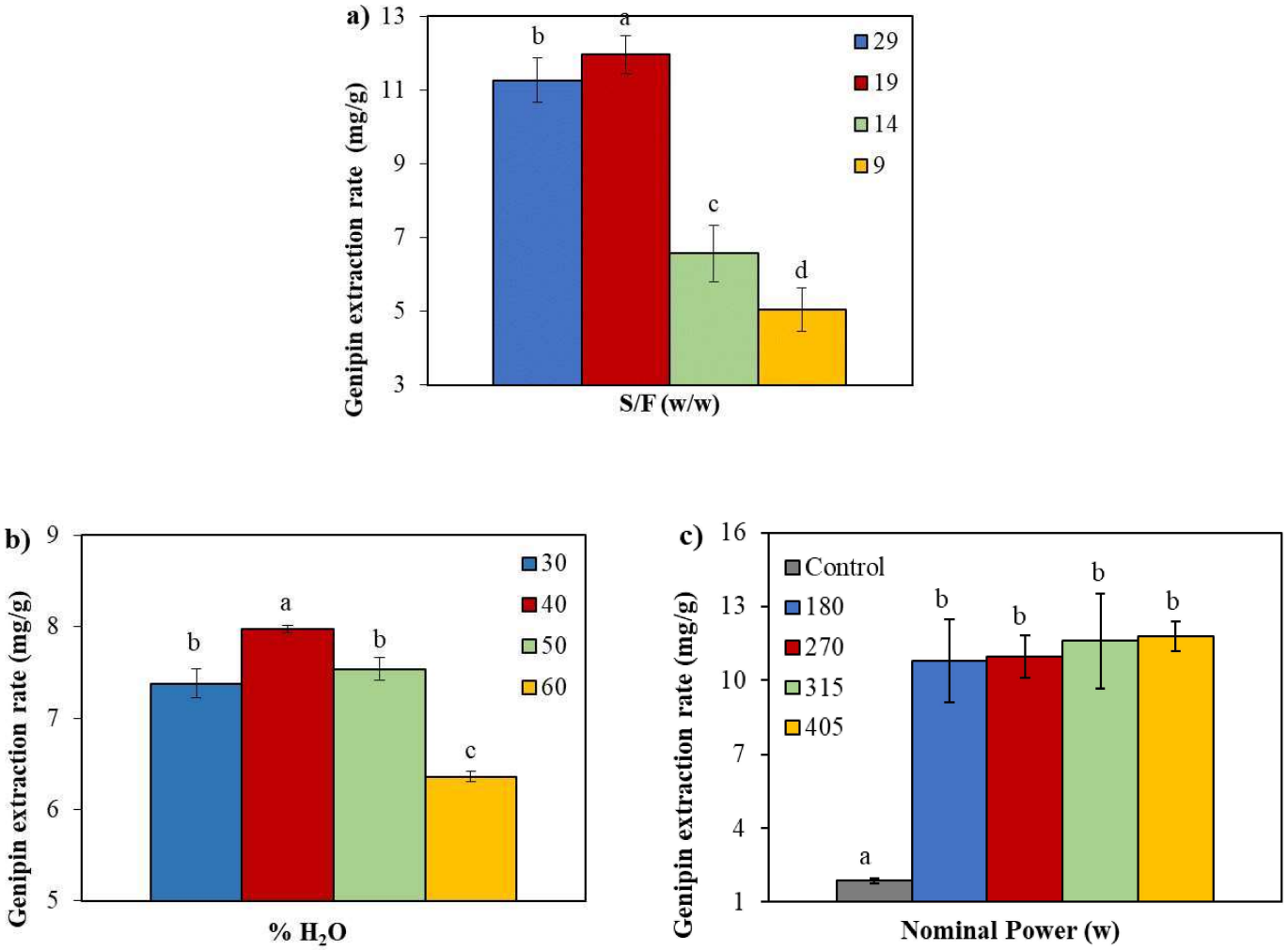
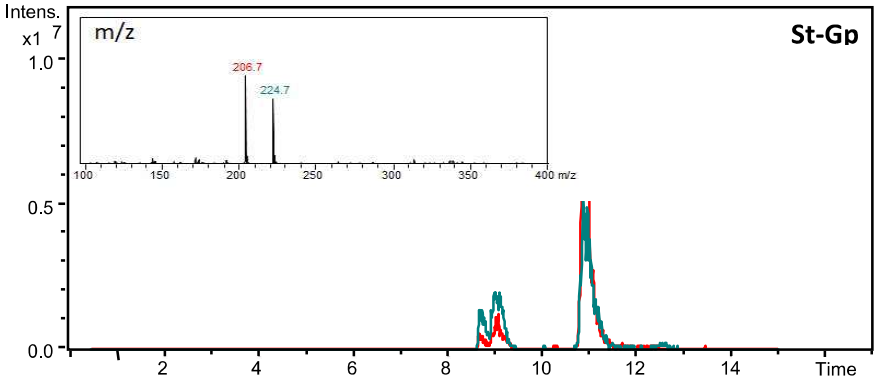


Figure 2. Extraction yields based on genipin extraction rate (mg/g of genipap) obtained by solid–liquid extraction assisted by HIUS. Experiments were carried out in triplicate using 1:3 Bet:LaAc, **a)** with different solvent/feed (w/w) ratio (S/F) (9,14, 19, 29) and fixing the % of water at 50% and the nominal power at 315 W; **b)** with different water concentration in the solvent (30, 40, 50, 60%) and applying 315 W, as nominal power and 19 as S/F, **c)** applying different nominal power on HIUS extraction and using 40% of water and 19 as S/F. Control sample: without HIUS process.

a)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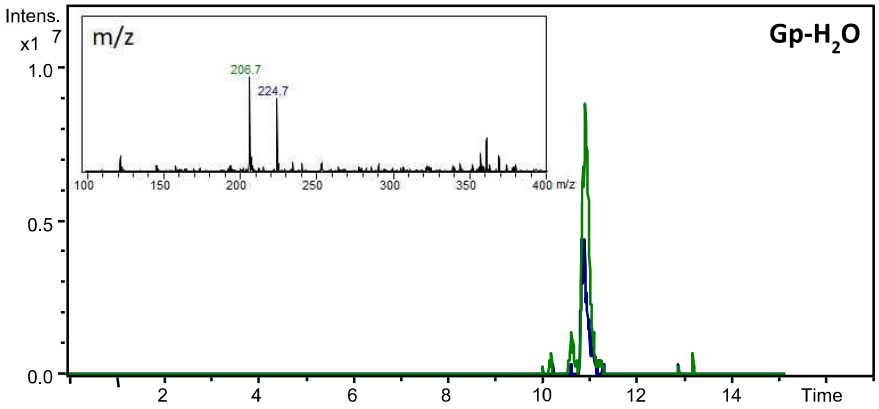
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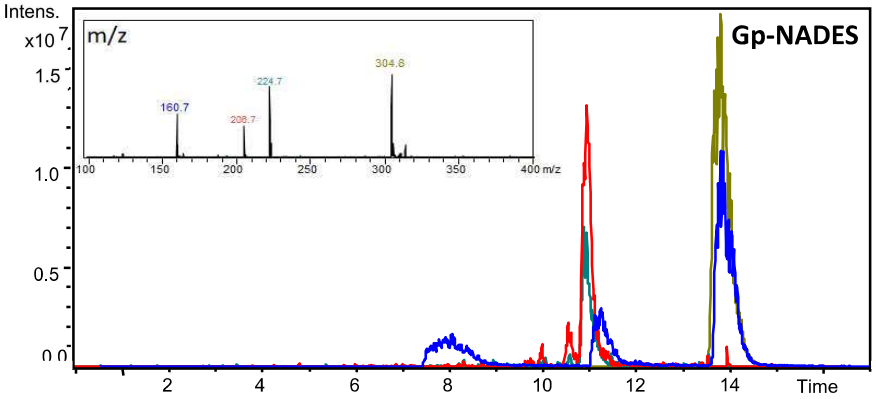
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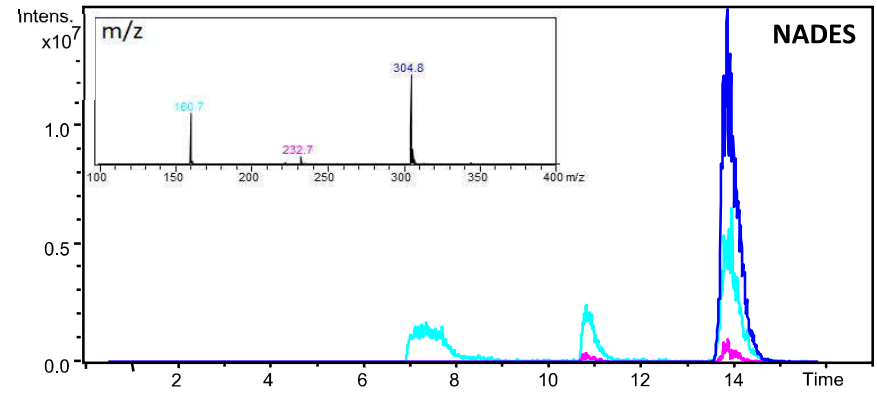
d)


Intens.
x10 7
1.0
0.5
0.0
m/z
160.7
232.7
304.8
100 150 200 250 300 350 400 m/z
NADES
2 4 6 8 10 12 14 Time

e)

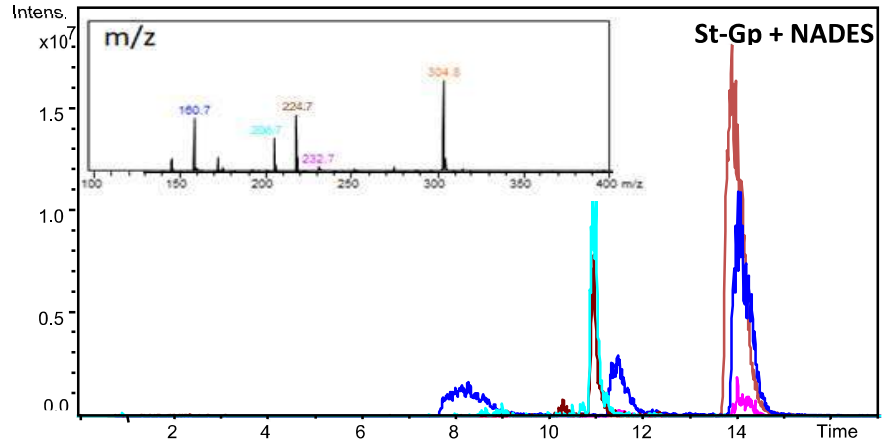

Intens.
x10 7
1.5
1.0
0.5
0.0
m/z
160.7
206.7
224.7
232.7
304.8
100 150 200 250 300 350 400 m/z
St-Gp + NADES
2 4 6 8 10 12 14 Time

Figure 3. HPLC-(ESI)-MS chromatograms (0.5 µg/mL of evaluated samples) **a)** St-Gp; **b)** genipin extracted by water (Gp-H_2O); **c)** genipin extracted by NADES (Gp-NADES); **d)** pure Bet:LaAc NADES; **e)** the mixture of standard genipin and Bet:LaAc NADES (St-Gp + NADES). Small boxes represent the m/z spectrum in each case.

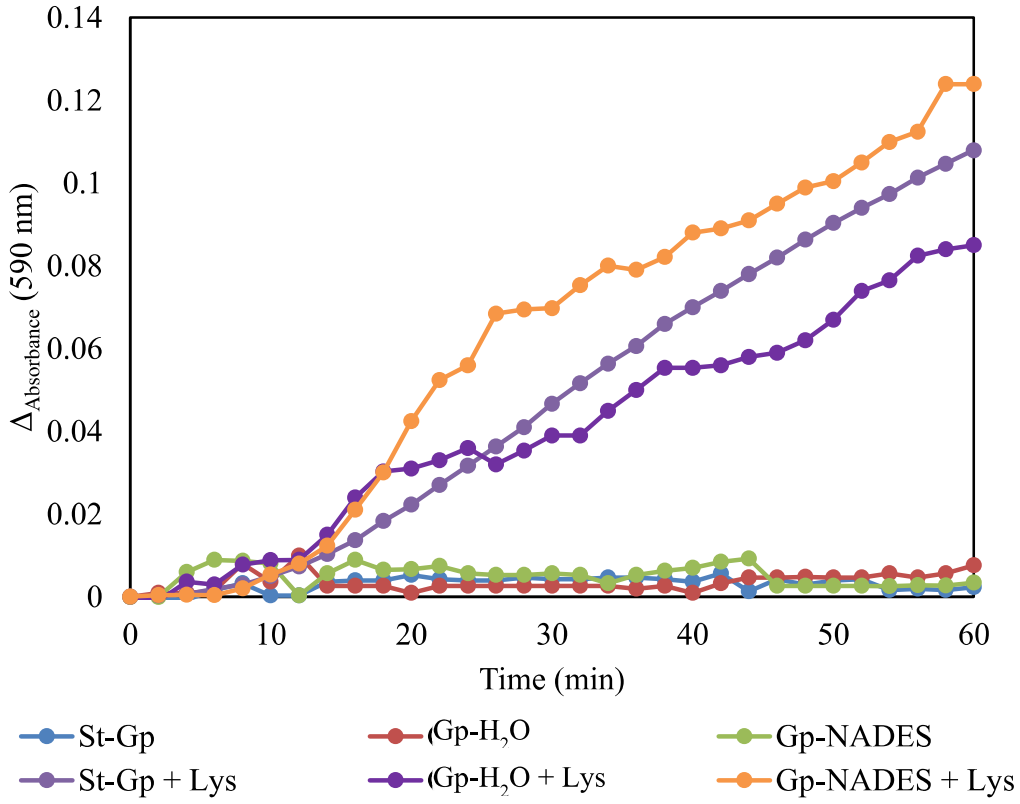


Figure 4. $\Delta_{Absorbance}$ at 590 nm of the genipin in standard form (St-Gp), and as extract with water and NADES as a solvent (Gp-H_2O) and (Gp-NADES), respectively. And, St-Gp, Gp-H_2O, Gp-NADES added with lysine (St-Gp + Lys), (Gp-H2O + Lys), and (Gp-NADES + Lys), respectively.

Tables:

Table 1. Tentatively identified compounds and total compound contribution (%) obtained from NADES-genipin extract and analyzed by RP/HPLC-ESI (-) Q-TOF-MS/MS.

Tentative identification	Retention time (min)	Formula	Total compound contribution (%)
D-Mannitol	0.551	$C_6H_{14}O_6$	12.80 ± 0.96
D-Arabinonic acid	0.555	$C_5H_{10}O_6$	1.69 ± 0.09
D-Gluconic acid	0.556	$C_6H_{12}O_7$	4.35 ± 0.39
Threonic acid	0.566	$C_4H_8O_5$	2.78 ± 0.10
D-Malic acid	0.688	$C_4H_6O_5$	35.43 ± 0.39
Citric acid	0.933	$C_6H_8O_7$	17.60 ± 0.83
2-Furoic acid	0.936	$C_5H_4O_3$	1.00 ± 0.23
D-Pyroglutamic acid	1.051	$C_5H_7NO_3$	4.14 ± 0.34
Citramalic acid	1.225	$C_5H_8O_5$	0.60 ± 0.04
Guanosine	1.894	$C_{10}H_{13}N_5O_5$	0.11 ± 0.01
3,4-Dihydroxybenzaldehyde	3.079	$C_7H_6O_3$	0.64 ± 0.06
Genipin 1-gentiobioside	3.837	$C_{23}H_{34}O_{15}$	0.15 ± 0.01
Geniposide	4.478	$C_{17}H_{24}O_{10}$	3.90 ± 0.12
Genipin	4.862	$C_{11}H_{14}O_5$	11.34 ± 1.09
Salicylic acid	5.652	$C_7H_6O_3$	0.21 ± 0.08
Phellopterin	6.375	$C_{25}H_{24}O_{12}$	0.16 ± 0.04
(9Z)-5,8,11-Trihydroxyoctadec-9-enoic acid	8.596	$C_{18}H_{34}O_5$	2.80 ± 0.14
9-hydroxy-10E,12Z-octadecadienoic acid	9.769	$C_{18}H_{32}O_3$	0.30 ± 0.09

Table 2. AChE and BChE inhibition (%) using the genipin (66 μg/mL) in standard form (St-Gp), extracted by H_2O (Gp-H_2O), and by NADES (Gp-NADES).

Genipin	% Inhibition	
(66 μg/mL)	AChE	BChE
St-Gp	47.2 ± 0.2[c]	61.0 ± 0.7
Gp-H_2O	61 ± 2[b]	59 ± 3
Gp-NADES	73 ± 10[a]	63 ± 2

Supplementary material for

A synergic combination of natural deep eutectic solvents with high-intensity ultrasound extraction to recover genipin: optimization and evaluation of ready- to-use extract on its crosslinking and anti-neurodegenerative properties

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[b] *Foodomics Laboratory, Department of Bioactivity and Food Analysis, Food Research Institute(CIAL-CSIC), Nicolás Cabrera 9, 28049 Madrid, Spain.*

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Contents:

Table S1. Dynamic viscosity and density of Bet:LaAc based NADES in different proportions.

NADES	Dynamic viscosity (mPa.s)	Density (g/cm^3)
Bet:LaAc (1:2)	965.15 ± 3.51	1.20 ± 0.00
Bet:LaAc (1:3)	465.15 ± 0.31	1.20 ± 0.00
Bet:LaAc (1:4)	311.47 ± 0.37	1.20 ± 0.00

Figure S1. Fourier Transform Infrared Spectroscopy (FTIR) spectra of betaine (Bet), lactic acid (LaAc), and the produced NADES using Bet and LaAc in the proportion of 1:3 (Bet:LaAc).

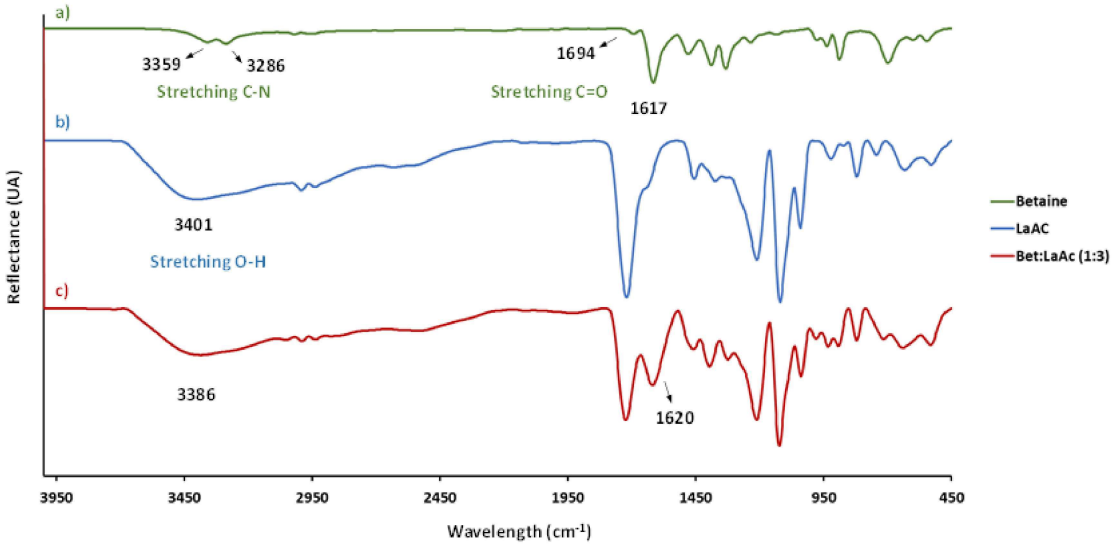


Figure S2. Calibration curves for genipin. St-Gp (Genipin standard) in water and St-Gp added ingenipap extract in water (St-Gp+Gp-H2O).

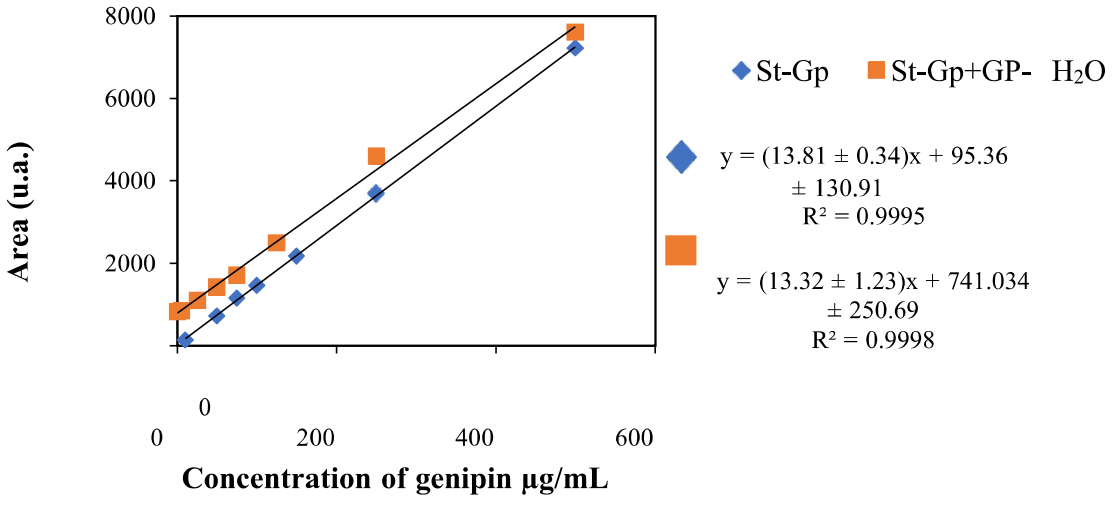
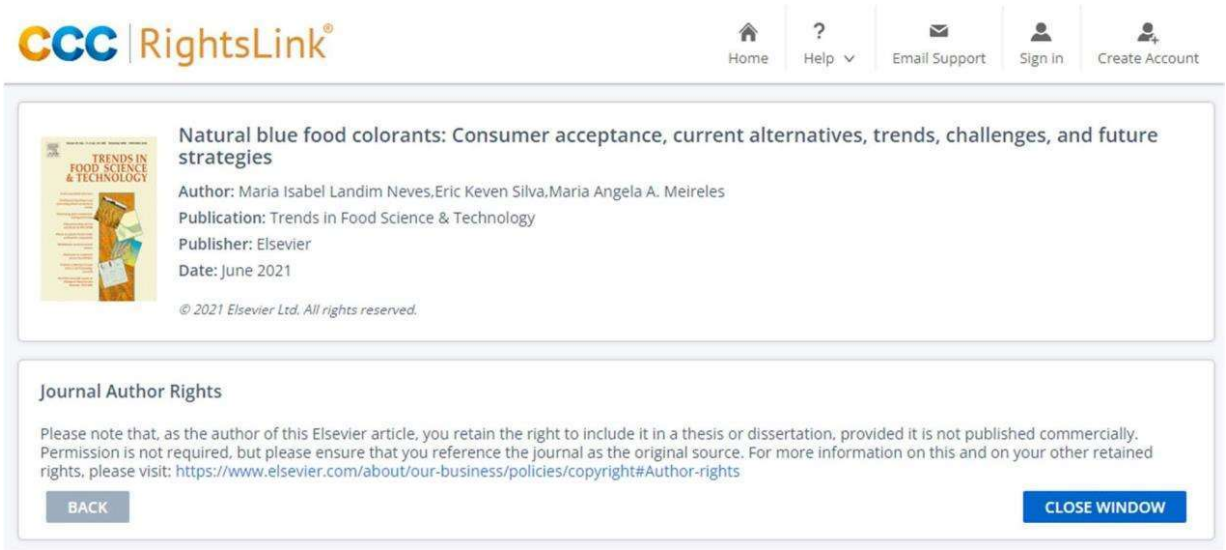


Table S2. Response of matrix coefficient calibration curves of the St-Gp in the function of NADESutilization.

Matrix coefficient calibration curves	ES
Bet:ProAc	97%
Bet:ProGly	101%
Bet:LaAc	103%
ChCl:ProAc	102%
ChCl:ProGly	92%
ChCl:LaAc	98%

10.5 Appendix V – General supplementary material

1. Supplementary material of chapter II



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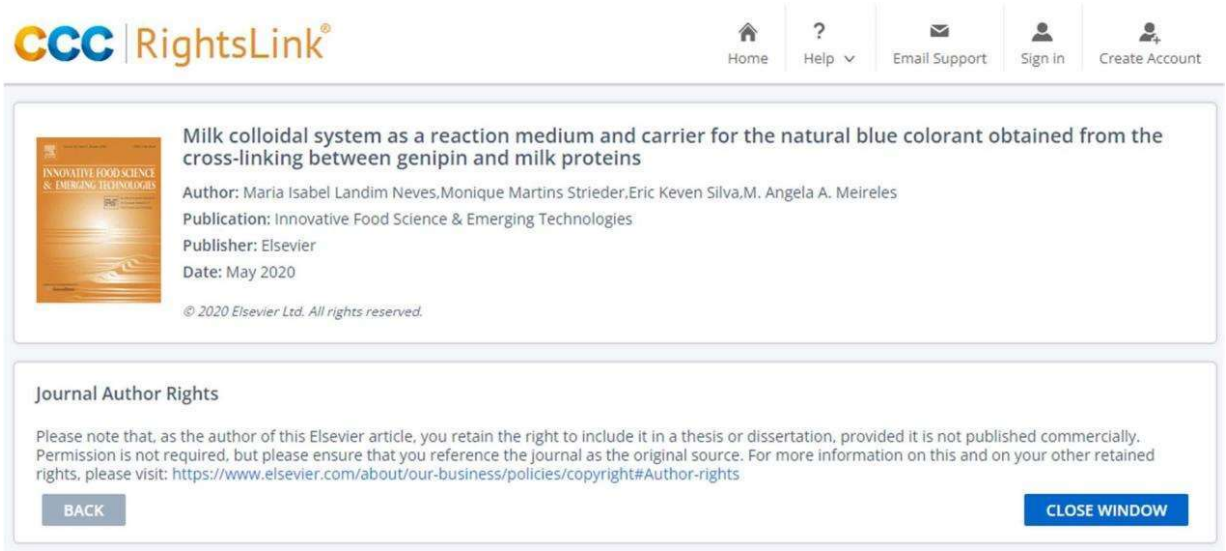
2. Supplementary material of chapter III



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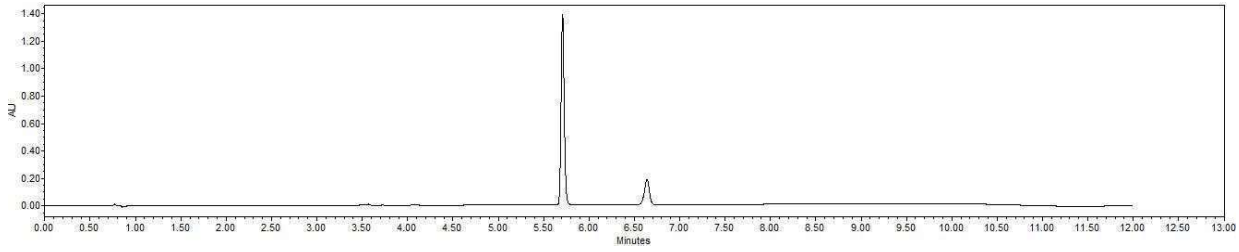


Figure A1. Chromatographic HPLC-PDA profile of the free-genipin content in the filtred bluecolorant-loaded milk system.

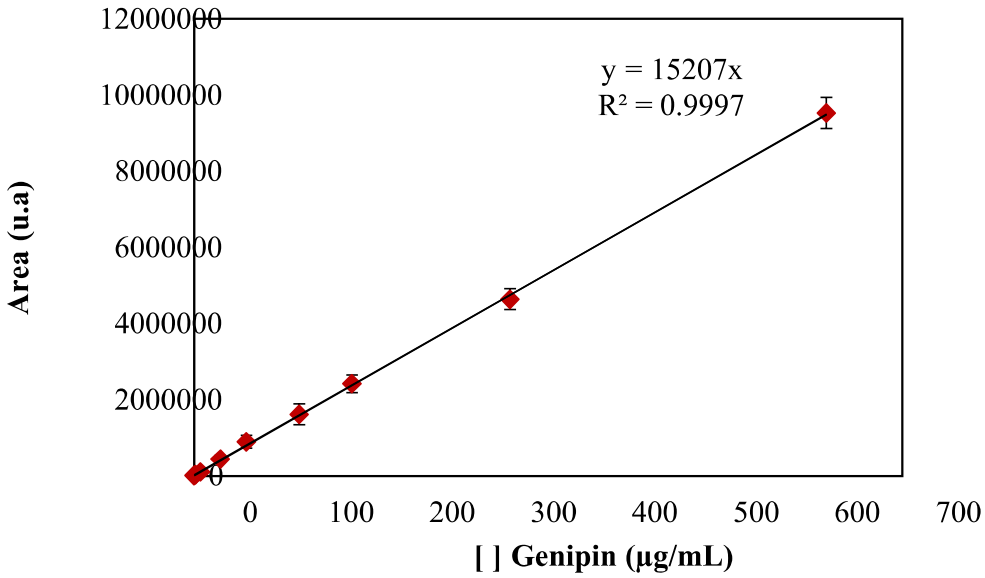
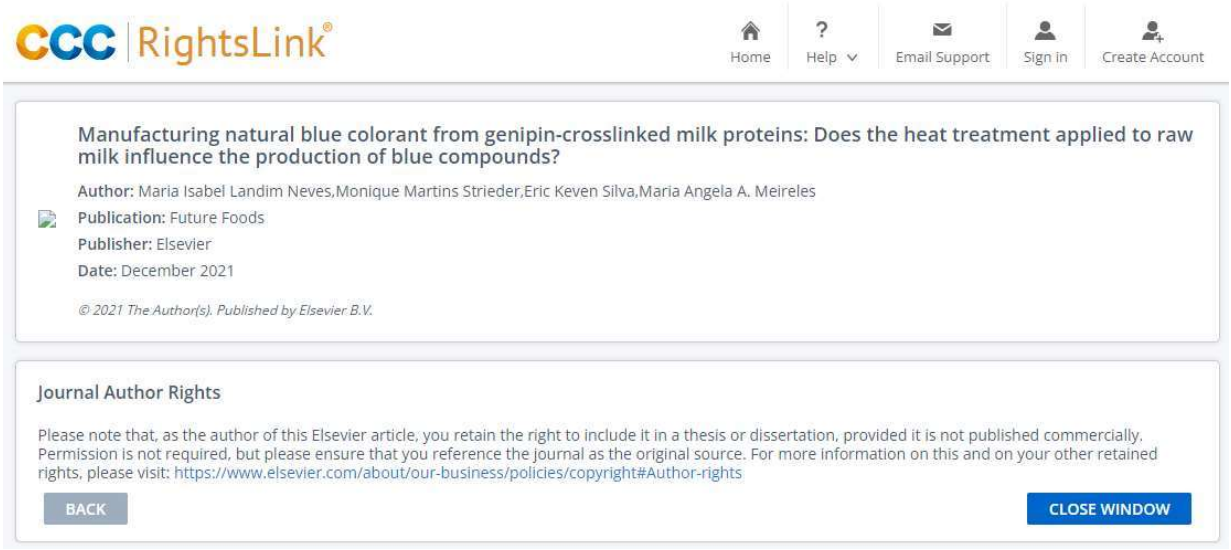


Figure A2. Calibration curve for standard genipin in water.

3. Supplementary material of chapter IV



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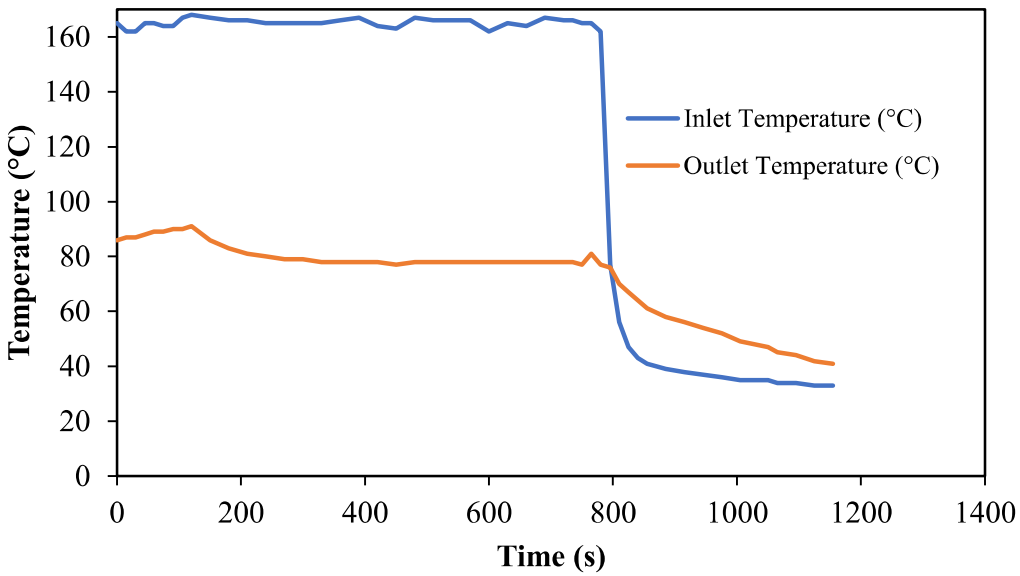
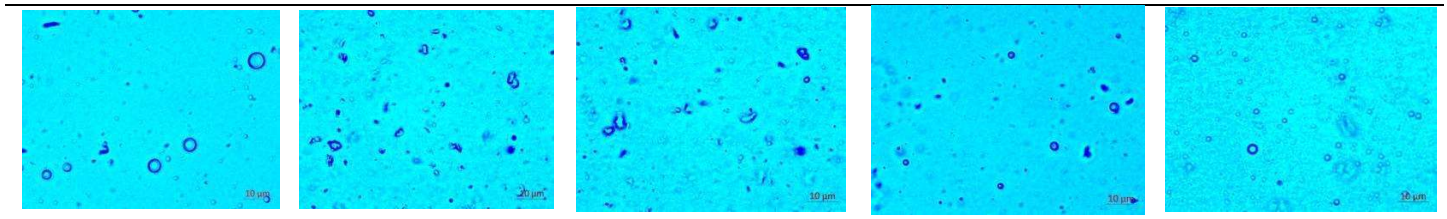


Figure A3. Thermal history of the spray-drying process applied to the raw milk (n = 2).



Control	LTLT	HTST	SD	UHT

Figure A4. Optical micrograph of the blue colorant-loaded milk system.

4. Supplementary material of chapter V

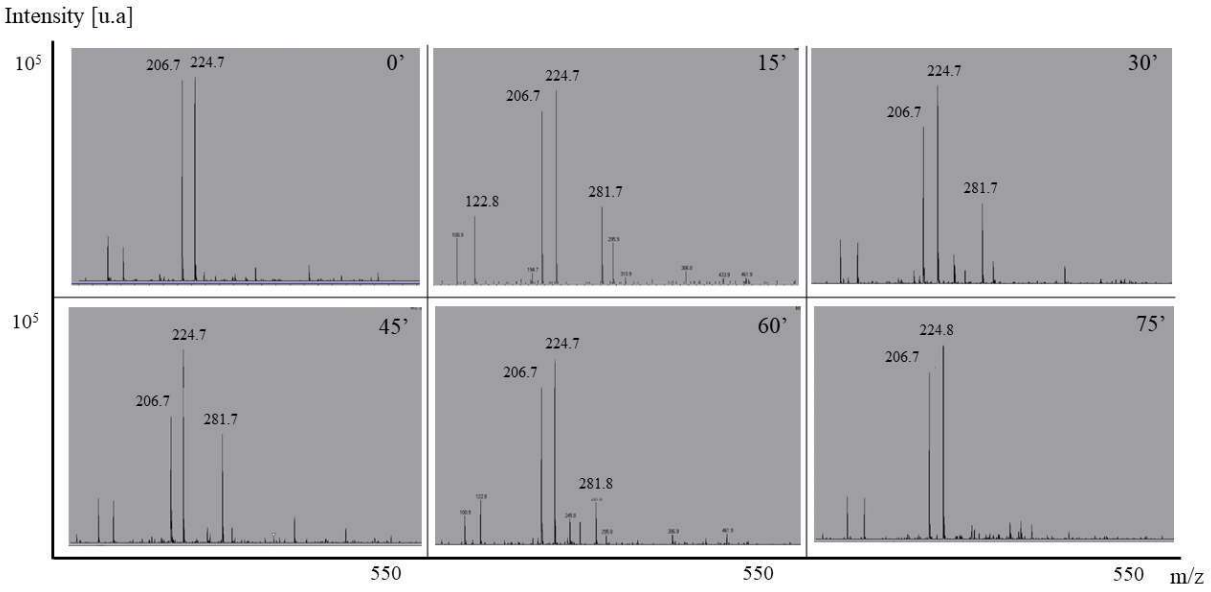
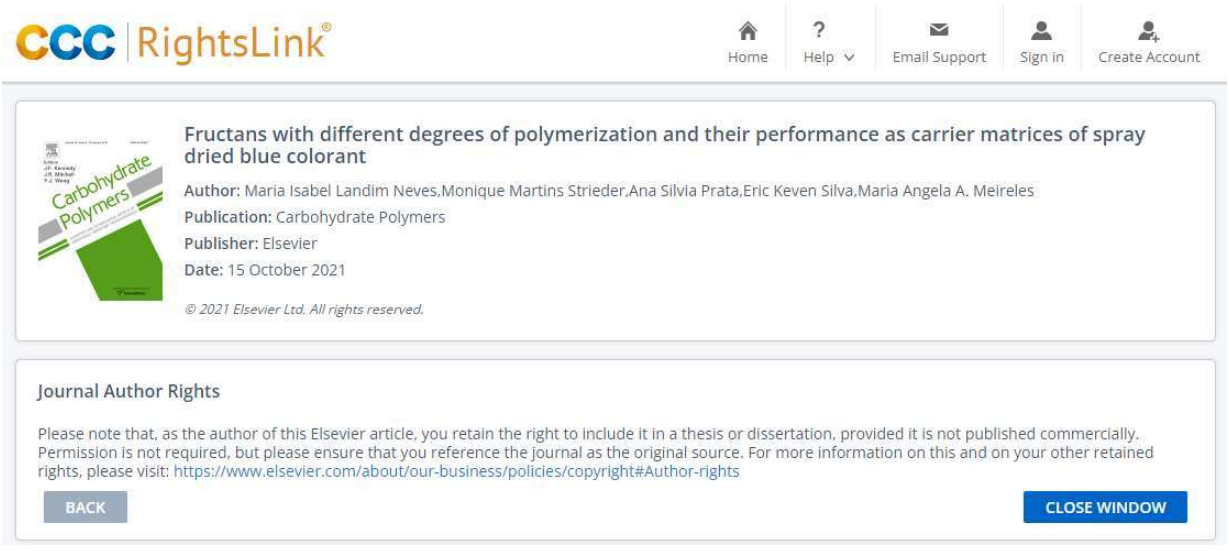


Figure A5. Kinetics of electrospray ionization (ESI-MS) direct infusion of genipin+glycine (0.5 mg/mL in MilliQ water with 0.1% formic acid (v/v)).

Figure A6. Visual aspects of milk, casein and whey; and their colorants: milk-colorant, casein-colorant, whey-colorant.

5. Supplementary material of chapter VI



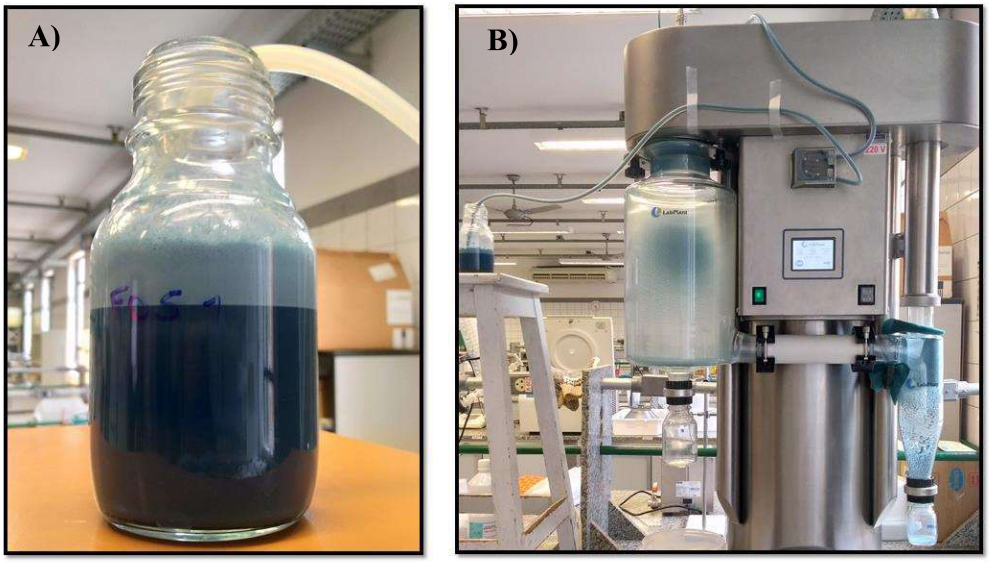
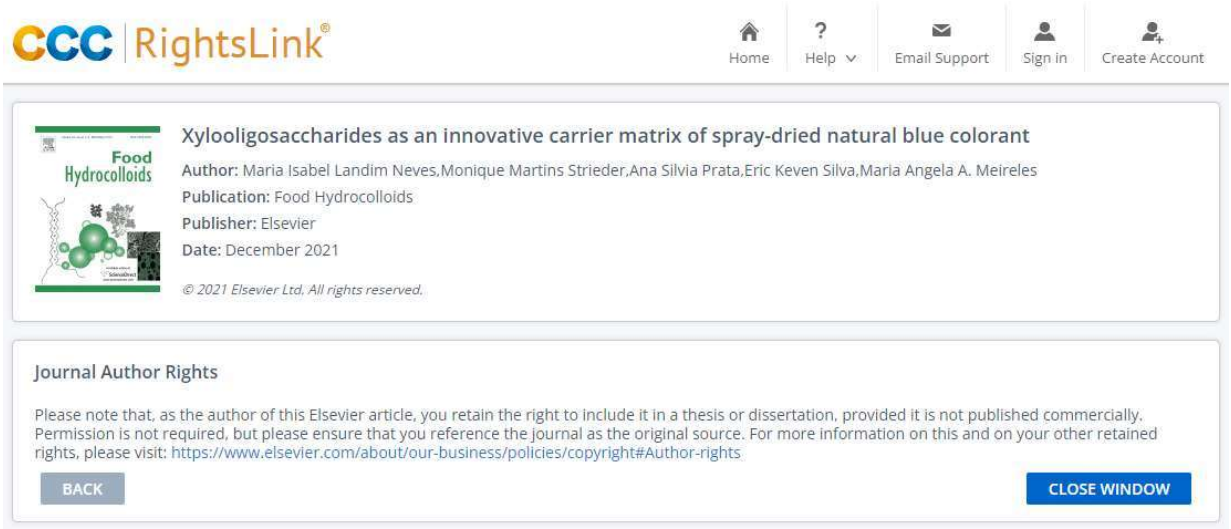


Figure A7. A) Blue colorant-loaded milk with FOS before be dried. **B)** Spray-drying process to produce the blue colorant powder.

6. Supplementary material of chapter VII



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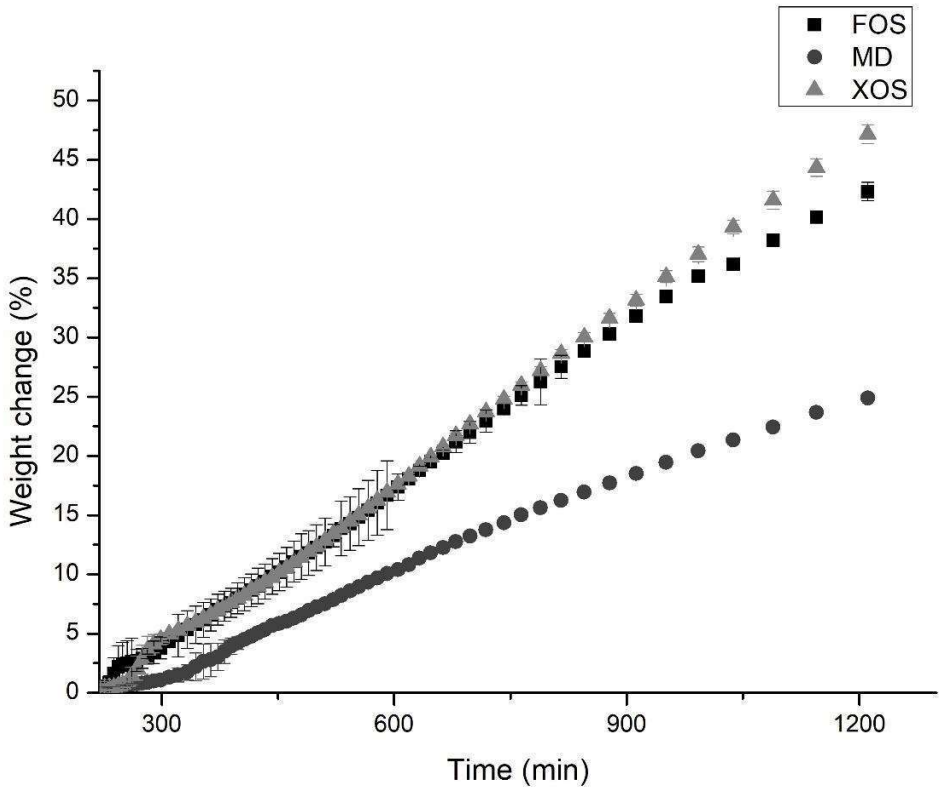

Figure A8. Adsorption kinetic of the blue colorant powders with MD, FOS and XOS.