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VICTOR JESÚS AREDO TISNADO

Formation of pigment-loaded lipid particles using supercritical CO_2

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I devote this work to my family: Jesus, Rosa,
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"And now here is my secret, a very simple secret: It is only with the heart that one can see rightly; what is essential is invisible to the eye."

Antoine de Saint-Exupéry

RESUMO

AREDO-TISNADO, V. J. **Formação de partículas lipídicas carregadas com pigmentos usando CO_2 supercrítico**. 2022. 124 p. Tese (Doutorado) – Faculdade de Zootecnia e Engenharia de Alimentos, Universidade de São Paulo, Pirassununga, SP, Brasil, 2022.

Partículas lipídicas têm sido sugeridas como veículos de entrega eficientes para compostos bioativos. Nesse contexto, a formação de partículas mediante tecnologias de CO_2 supercrítico é promissora, pois geralmente não utiliza solventes orgânicos e emprega temperaturas moderadas. Entretanto, a aplicação prática dessas tecnologias em pigmentos de interesse para a indústria alimentícia é limitada, por exemplo, pelos poucos materiais carreadores compatíveis. Esta tese teve como objetivo estudar a formação de partículas lipídicas carregadas de pigmento usando tecnologia de CO_2 supercrítico. A fase experimental estudou: a solubilidade da cera de abelha, óleo de castanha-do-Brasil e sua mistura em CO_2 supercrítico a 60 °C e 150-300 bar para entender seu uso como materiais carregadores em CO_2 supercrítico; a formação de micropartículas de cera de abelha carregadas com óleo de castanha-do-Brasil usando Partículas de Soluções Saturadas de Gás (PGSS) com CO_2 supercrítico a 60 °C e 150-300 bar para explorar a factibilidade técnica do processo; a formação de carreadores lipídicos estruturados com extrato de cúrcuma usando PGSS com CO_2 supercrítico a 60 °C e 300 bar, e misturas de cera de abelha e óleo de castanha-do-Brasil como carregador para propô-lo como ingrediente inovador para a indústria alimentícia; o comportamento do colágeno hidrolisado em CO_2 supercrítico a 40-60 °C e 100-250 bar para ideação de novos sistemas carregadores; o revestimento de cera de abelha de partículas de colágeno hidrolisado carregadas com extrato de mirtilo usando CO_2 supercrítico a 60 °C e 300 bar e avaliando formulações para sugerir um novo ingrediente alimentar. A solubilidade em CO_2 supercrítico dos lipídeos aumentou quando a pressão aumentou (R^2>0,90, p<<0,05). Micropartículas de cera de abelha carregadas com óleo de castanha-do-Brasil foram efetivamente formadas usando PGSS e observou-se que seu tamanho e densidade aparente foram afetados inversamente pela pressão do CO_2. Misturas de cera de abelha e óleo de castanha-do-Brasil, mantendo uma proporção de material sólido/líquido de 1:1 (p/p) na formulação, são transportadores úteis para carregamento uniforme de extrato de cúrcuma. Esses carregadores lipídicos estruturados com extrato de cúrcuma eram pós amarelos semelhantes a esponjas. O colágeno hidrolisado tem baixa interação com o CO_2 supercrítico,

pois tem solubilidade constante (0,5-0,6 g/kg CO_2) e não expande seu volume, o que o torna interessante para impregnação. As partículas de colágeno hidrolisado carregadas com extrato de mirtilo revestidas com cera de abelha formuladas com 6,33% de cera de abelha, 10,67% de extrato de mirtilo e 83% de colágeno hidrolisado destacam por apresentarem alto teor de antocianina total (0,42 mg cianidina-3-glicosídeo/g), alta variação de cor (29,8) e reduzido grau de solubilização em água (72,9%). Os resultados demonstraram que a formação de partículas lipídicas carregadas de pigmento usando a tecnologia de CO_2 supercrítico é tecnicamente fatível. Tanto os carregadores lipídicos estruturados com extrato de cúrcuma quanto as partículas de colágeno hidrolisadas carregadas com extrato de mirtilo são produtos com aplicação tecnológica potencial como pigmento antioxidante. Esses resultados podem ser referência para a formação de partículas carregadas com outros bioativos usando CO_2 supercrítico que serão aplicadas como ingrediente no enriquecimento/formulação de produtos alimentícios tradicionais e inovadores.

Palavras-chave: Alimentos em polvo. Cera de abelha. Curcumina. Micronização supercrítica.

ABSTRACT

AREDO-TISNADO, V. J. **Formation of pigment-loaded lipid particles using supercritical CO_2**. 2022. 124 p. Ph.D. Thesis – Faculty of Animal Science and Food Engineering, University of Sao Paulo, Pirassununga, SP, Brazil, 2022.

Lipid particles have been suggested to be efficient delivery vehicles for bioactive compounds. In this context, particle formation by supercritical CO_2 technologies is promising because it generally does not use organic solvents and employs moderate temperatures. However, the practical application of these technologies in pigments of interest for food industry is limited, for example, by the few compatible carrier materials. This thesis aimed to study the formation of pigment-loaded lipid particles using supercritical CO_2 technology. For which experimental phase focused on studying: the solubility of beeswax, Brazil nut oil and their mixture in supercritical CO_2 at 60 °C and 150-300 bar to understand their use as carrier materials in supercritical CO_2 technology; the formation of Brazil nut oil-loaded beeswax microparticles using Particles from Gas Saturated Solutions (PGSS) with supercritical CO_2 at 60 °C and 150-300 bar to explore the technical feasibility of the process; the formation of turmeric extract-loaded structured lipid carriers using PGSS with supercritical CO_2 at 60 °C and 300 bar, and mixtures of beeswax and Brazil nut oil as carrier material to propose it as an innovative ingredient for the food industry; the behaviour of hydrolysed collagen powder in supercritical CO_2 at 40-60 °C and 100-250 bar for design of new carrier systems; the beeswax coating of blueberry extract-loaded hydrolysed collagen particles using supercritical CO_2 at 60 °C and 300 bar and assessing formulations to suggest a new food ingredient. The solubility in supercritical CO_2 of lipid materials increased when pressure increased (R^2>0.90, p<<0.05). Brazil nut oil-loaded beeswax microparticles were effectively formed by PGSS process and it was observed that its size and bulk density were affected inversely by CO_2 pressure. Mixtures of beeswax and Brazil nut oil, maintaining a solid/liquid material ratio of 1:1 (w/w) in the formulation, are useful carriers to uniform loading of turmeric extract. This turmeric extract-loaded structured lipid carriers were yellow, free-flowing and sponge-like powders. Hydrolysed collagen powder has low interaction with supercritical CO_2 since it has constant solubility (0.5-0.6 g/kg CO_2) and does not expand volumetrically, which makes interesting for impregnation applications. The blueberry extract-loaded hydrolysed collagen particles coated with beeswax formulated with 6.33% of beeswax, 10.67% of blueberry extract and 83% of hydrolysed collagen is

recommended because it had high total anthocyanin content (0.42 mg cyanidin-3-glucoside /g), high colour variation (29.8), and reduced degree of solubilisation in water (72.9%). The results of this thesis demonstrated that the formation of pigment-loaded lipid particles using supercritical CO_2 is technically feasible. Both turmeric extract-loaded structured lipid carriers and blueberry extract-loaded hydrolysed collagen particles are products with potential technological application as an antioxidant pigment. In addition, these results can serve as a reference for the formation of particles loaded with other bioactive substances by means of supercritical CO_2 technology that will be applied as an ingredient in the enrichment/formulation of traditional and innovative food products.

Keywords: Beeswax. Carbon dioxide. Curcumin. Food powders. Supercritical micronisation.

FIGURES

CHAPTER V: Turmeric extract-loaded structured lipid carriers by Particles from Gas-Saturated Solutions

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CHAPTER I: Introduction, Objectives and Thesis Structure

1. Introduction

Nowadays, the consumption of bioactive substances is considered very important for health care. For this reason, several products from the food, cosmetic and pharmaceutical industries have incorporated these substances (KUREK et al., 2022; LIU et al., 2022). In this context, particle formation technologies are used to stabilise and facilitate the incorporation of bioactive substances in products (MEHTA et al., 2022). Interesting particle types are lipid particles such as solid lipid particles and structured lipid carriers that differ from other particles by the use of lipid-encapsulating agents (also called lipid carriers materials) that remain solid at room temperature capable of loading and protect bioactive substances (NAHUM; DOMB, 2021; KATOPODI; DETSI, 2021).

In this context, the formation of lipid particles using supercritical CO_2 is recommended because the use of a low cost and low toxicity solvent, operation at moderate temperatures and an inert atmosphere that prevents the degradation of bioactive substances (WANG et al., 2021; NIKOLAI et al., 2019). In addition, it is possible to adjust the properties of the supercritical fluid through pressure and temperature changes to process different materials and control the size and morphology of the particles (SOH; LEE, 2019; FAHIM et al., 2014).

Particle formation by particles from gas saturated solutions (PGSS), also known as supercritical fusion micronisation (MÜNÜKLÜ; JANSENS, 2007), is the main supercritical technique used for the formation of solid particles from lipids and structured lipid carriers (GANESAN; NARAYANASAMY, 2017). This technique basically consists of melting the carrier material mixed with the bioactive substance in an autoclave in contact and saturation with supercritical CO_2 at constant pressure and temperature, then precipitating-micronizing the mixture through a nozzle at atmospheric pressure (COCERO et al., 2009; ESFANDIARI, 2016).

Although supercritical CO_2 technologies are ideal for processing lipids, there is little research on their use in the formation of solid lipid particles and structured lipid carriers. Its industrial application is likely to be limited by the lack of low-cost, process-compatible carrier materials. Therefore, studies on alternative lipid carrier materials could facilitate a better

understanding of the process, facilitating scalability and applicability in the bioactive substance industry.

In this sense, beeswax could be an alternative carrier material for the formation of lipid particles because it melts in contact with supercritical CO_2 at moderate temperatures and solidifies under ambient conditions. Beeswax is a material produced by bees in their specialised glands for use in the construction of honeycombs (BOGDANOV, 2004). The chemical composition of this material is diverse in lipids, being a mixture of hydrocarbons, free fatty acids, monoesters, diesters, triesters, hydroesters, hydroxypolyesters, fatty acid polyesters and some unidentified compounds (MAIA; NUNES, 2013).

Pure beeswax or mixed with materials such as cocoa butter (ATTAMA; SCHICKE; MÜLLER-GOYMANN, 2006) or goat fat (ATTAMA; SCHICKE; MÜLLER-GOYMANN, 2007; ATTAMA; MÜLLER-GOYMANN, 2008) were explored in the formation of solid lipids particles loaded with bioactive substances such as ketoprofen (ÜNER et al., 2005; KHERADMANDNNI et al., 2010), flurbiprofen (BAVISKAR et al., 2012), cinnamic acid (ROSITA et al., 2014), geranic acid, ursolic acid, forskolin (LASOŃ et al., 2016) and vitamin D3 (DEMIRBILEK et al., 2017). Regarding supercritical technology, beeswax was studied in encapsulation with menthol (ZHU et al., 2010), and in our study with Brazil nut oil (AREDO et al., 2021).

Substances of interest to the natural products industry are vegetable pigments. However, to our knowledge, not much research is available on the formation of pigment-loaded lipid particles using supercritical CO_2 technology. Therefore, more efforts are needed to make its industrial application possible.

Turmeric rhizome extract (Curcuma longa L) is receiving increasing attention in the scientific community, because it is rich in curcumin, a polyphenolic compound extremely useful as a yellow pigment, which has a broad spectrum of bioactivity (antioxidant, anti-inflammatory, antimicrobial and anticancer) (GANESAN, 2018; MALAEKEH-NIKOUEI; SALARBASHI, 2018). However, this pigment is unstable and degrades in the presence of light, oxygen, and digestive tract conditions (LEE et al., 2014; SUN et al., 2013). In this context, the formation of turmeric extract-loaded lipid particles has a high potential to stabilise and improve the bioavailability of curcumin compared to the formation of polymeric particles due to the hydrophobic nature of the pigment (MALAEKEH-NIKOUEI; SALARBASHI, 2018; LEE et al., 2014). In this context, the formation of high-quality particles is required, which can be

achieved using safe, low-cost, alternative encapsulating agents, and organic solvent-free technologies such as PGSS.

Another supercritical CO_2 technology that uses lipid materials in particle formation is the supercritical fluid-based coating process proposed in the early 2000s (SANTOS et al., 2002). It consists of the contact of protein particles with a lipid material solubilised in supercritical CO_2, which remains on the surface of the particles after depressurisation of the system (SANTOS et al., 2002). In this context, the application of this process in pigment-loaded hydrolysed collagen powder, which is an emerging carrier system formed by supercritical CO_2 technology (AREDO et al., 2019; AREDO; PASSALACQUA; OLIVEIRA, 2022), could be useful for design new powder ingredients.

In this context, the formation of particles loaded with blueberry extract (Vaccinium corymbosum L.), is of interest to the industry as it is a natural product rich in anthocyanin pigment, which has pharmacological activity (antioxidant and anti-inflammatory) (LIU, et al. 2021).

Therefore, the formation of pigment-loaded lipid particles using supercritical CO_2 technology could be applied as a food ingredient with technological function as pigment and antioxidant.

2. Objectives

2.1. General

The main objective of this research is to study the formation of pigment-loaded lipid particles using supercritical CO_2 technology.

2.2. Specifics

- To perform solubility measurements of beeswax, Brazil nut oil, and beeswax/ Brazil nut oil mixtures in supercritical CO_2;
- To produce solid lipid particles from a mixture of beeswax and Brazil nut oil (study of PGSS process conditions);

- To study the formation of turmeric extract-loaded particles using a mixture of beeswax and Brazil nut oil as carrier material;
- To explore the behaviour of hydrolysed collagen particles in supercritical CO_2 as carrier material;
- To evaluate the production of hybrid particles consisting of hydrolysed collagen particles loaded with blueberry extract and coated with beeswax by supercritical CO_2 treatment.

3. Thesis structure

This doctoral thesis is structured in chapters.

Chapter I presents an introduction to the research topic, the main objective and specific objectives, as well as the structure of the doctoral thesis.

Chapter II contains a brief review entitled "Formation of Solid Lipid Particles and Structured Lipid Carriers using Supercritical CO_2" that contextualise the topics covered in this study, which includes, binary/phase (lipid + CO_2) studies, lipid micronisation and the formation of solid lipid particles and structured lipid carriers loaded with bioactive compounds.

Chapter III is entitled "Solubility of beeswax, Brazil nut oil and their mixture in supercritical CO_2" and analysed data about the behaviour in supercritical CO_2 of the lipid materials of interest for this thesis.

Chapter IV is part of the study entitled "Formation of edible oil-loaded beeswax microparticles using PGSS – Particles from Gas-Saturated Solutions" published in The Journal of Supercritical Fluids. That study explored the technical feasibility of formation of pure beeswax microparticles and Brazil nut oil-loaded beeswax microparticles by PGSS as supercritical CO_2 technology to be used as potential carrier systems.

Chapter V is entitled "Turmeric extract-loaded structured lipid carriers by Particles from Gas-Saturated Solutions" and explored some formulations of turmeric extract/beeswax/Brazil nut oil for formation of yellow microparticles that could be used in food pigmentation.

Chapter VI is part of the study entitled "Hydrolysed collagen as carrier material for particle formation via supercritical CO_2 impregnation" published in The Journal of Supercritical Fluids. That study proposed that the low interaction of commercial hydrolysed collagen

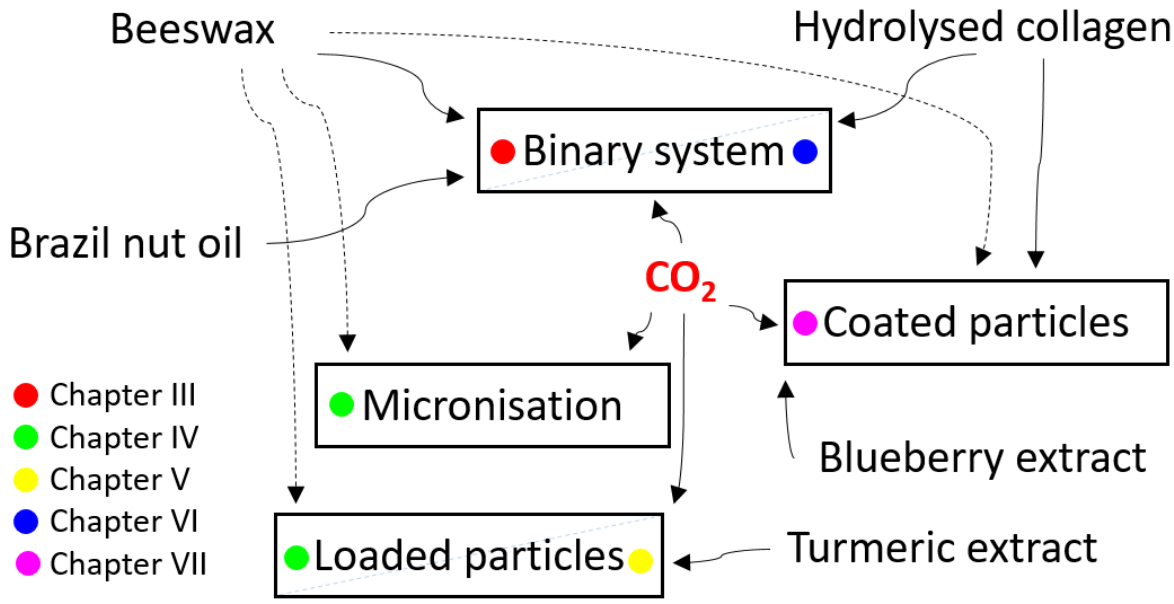
powder with supercritical CO_2 is desirable to develop potential applications as carrier material of bioactive compounds using supercritical CO_2 impregnation.

Chapter VII is entitled "Beeswax coating on blueberry extract-loaded hydrolysed collagen microparticles by supercritical CO_2 treatment" and evaluated the reduction in water solubilisation of hydrolysed collagen microparticles loaded with blueberry extract as an effect of beeswax coating. These particles could be of interest for anthocyanin enrichment in functional food products.

Finally, **Chapter VIII** brings the general conclusions of the Doctoral Thesis and suggestions for further studies.

Figure 1 schematically presents the materials and topics of the experimental chapters of this Doctoral Thesis to facilitate readers' understanding.

Figure 1 - Schema of the materials and topics of the experimental chapters



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CHAPTER II: Formation of Solid Lipid Particles and Structured Lipid Carriers using Supercritical CO_2

(No submitted)

CHAPTER II: Formation of Solid Lipid Particles and Structured Lipid Carriers using Supercritical CO_2

Abstract

Solid lipid particles and structured lipid carriers are suggested as efficient carriers of lipophilic bioactive compounds. Supercritical CO_2 technologies, mainly Particles from Gas-Saturated Solutions (PGSS), are an advantageous alternative among conventional techniques for the formation of these type of particles due to its low thermal impact. Although these technologies are currently little used for this purpose, they had high potential due to the growing consumer demand for high-quality natural products. Selected examples were revised and the achievements and challenges were summarised.

Keywords: bioactive, carrier system, encapsulation, particle formation, supercritical fluid.

1. Introduction

The increases in illness related to dietary intake, such as cardiovascular disease, and some types of cancer, has led to the development of health-promoting products loaded with bioactive compounds. However, many bioactive compounds are highly lipophilic, resulting in poor absorption and limited bioavailability, which creates difficulties associated with their inclusion in products from the pharmaceutical, food, and cosmetic industries (COMUNIAN et al., 2021; MEHTA et al., 2022; KUREK et al., 2022).

In this context, the production of carrier systems appears as a strategy to overcome the limitations in the use of lipophilic bioactive compounds. Types of carrier systems proposed for these compounds are lipid particles such as solid lipid particles and structured lipid carriers. Solid lipid particles consist of the use of solid lipid carriers, while structured lipid carriers consist of the use of a mixture of liquid lipid with solid lipid. In this sense, these particles are recommended to protect hydrophobic bioactive substances given their high compatibility with lipid carriers (ÜNER, 2006; KATOUZIAN et al., 2017; NAHUM; DOMB, 2021).

The historic evolution of knowledge on applications and techniques used for the formation of these particles such as high-pressure homogenisation, high-speed homogenisation and ultrasonication, emulsification-evaporation, microemulsion, phase inversion temperature, solvent diffusion and solvent injection were reviewed in many works (MÜLLER et al., 2000; ÜNER, 2006; WEISS et al., 2008; MEHNERT; MÄDER., 2012, EKAMBARAM et al., 2012; TAMJIDI et al., 2013, JAISWAL et al., 2016; ADITYA; KO, 2015, KATOUZIAN et al., 2017, GANESAN; NARAYANASAMY, 2017; GANESAN et al., 2018; GORDILLO-GALEANO; MORA-HUERTAS, 2018; ZHONG; ZHANG, 2019; APOSTOLOU et al., 2021; NAHUM; DOMB, 2021). Nonetheless, the use of supercritical CO_2 was little reviewed as a method for the formation of these particles (GANESAN; NARAYANASAMY, 2017).

On the other hand, specific reviews on particle formation by supercritical technologies have not paid much attention to solid lipid particles and structured lipid carriers (PADRELA et al., 2018; KUMAR, R. et al. 2021). However, supercritical CO_2 is a promising alternative for the formation of these particles in the context of high consumer interest in natural products and stricter government regulations on the use of organic solvents like hexane (TEMELLI, 2009), and technological advantages such as the use of low-cost solvent, not using water to mix the carrier material and the bioactive substance, operation at moderate temperatures and inert atmosphere (without oxygen) that prevents the degradation of bioactive substances. In addition, it is possible to adjust the properties of the supercritical fluid through pressure and temperature changes to process different materials and control the size and morphology of the particles (WANG et al., 2021; AHANGARI et al., 2021; PADRELA et al., 2018).

Thus, the objective of this review was to carry out an analysis of the works that used supercritical CO_2 technologies in the formation of solid lipid particles and structured lipid carriers.

2. Solid lipid particles and Structured lipid carriers

Solid lipid particles were proposed in 1991. Solid lipid particles are produced from a solid lipid or a mixture of solid lipids at the final application temperature (body/room temperature) (MÜLLER et al., 2000). It consists of lipid droplets that crystallise and have an organised crystalline structure with the bioactive components housed within the lipid matrix (ÜNER,

2006; WEISS et al., 2008). In 2002, liquid lipid-loaded solid lipid particles, also known as structured lipid carriers, were proposed for an apparently easy solubilisation of the lipophilic bioactive compound in the liquid lipid, which remains trapped in the crystal lattice of the solid lipid (MÜLLER et al., 2002).

The advantages and disadvantages of solid lipid nanoparticles and structured lipid carriers have been extensively reviewed (ÜNER., 2006; TAMJIDI et al. 2013; KATOUZIAN et al., 2017; APOSTOLOU et al., 2021). In short, both do not use organic solvents and can use natural food lipids that minimise the danger of bio-toxicity. The main disadvantages of solid lipid particles are unexpected transitions in fatty crystal structures leading to expulsion of core materials during storage and low bioactive incorporation capacity, which are overcome in structured lipid particles by the presence of liquid lipids. In addition, in both systems there is concern about the negative effect of the materials on the sensory properties of foods with incorporated particles and their stability during food processing and storage.

3. Supercritical CO_2

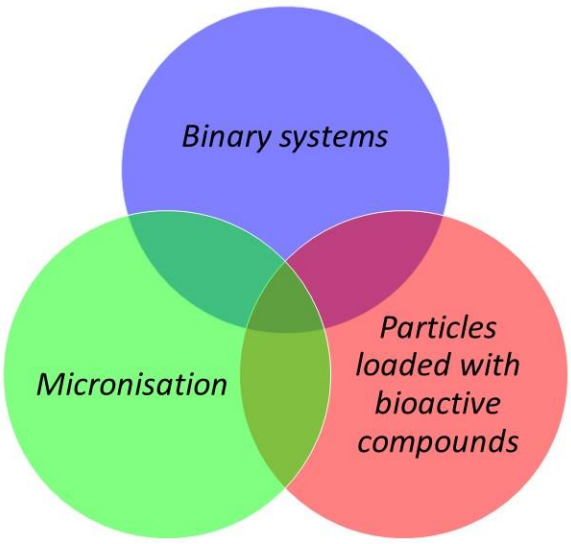
A supercritical fluid is a pure substance that is above its critical temperature and pressure (NIKOLAI et al., 2019; SILVA et al., 2020). Its physical properties are between the liquid and gas phase and are easily adjustable by small changes in temperature and pressure, especially when carried out near the critical point (SILVA et al., 2020; AHANGARI et al., 2021). It is an excellent solvent characterised by a viscosity nearby to that of gas, an intermediate diffusivity, and density similar to that of liquid (NIKOLAI et al., 2019) that allows it to easily diffuse through solid matrices and dissolve materials (AHANGARI et al., 2021). Some compounds that are applied in supercritical technology are CO_2, water, nitric oxide, ammonia, ethane, propane (PAVLOVA et al., 2022).

Supercritical CO_2 is the most widely used supercritical fluid because it easily reaches its critical temperature (31.2 °C) and its critical pressure (7.38 MPa) (SILVA et al., 2020). CO_2 has advantages such as being generally recognised as safe, non-flammable, non-corrosive, non-toxic, inert to oxidation reactions, readily available in high purity, easy to remove as it is a gas at room temperature and atmospheric pressure, low cost because it is naturally available in the atmosphere, environmentally friendly as it can be obtained from existing industrial

processes without major contribution to the greenhouse effect (NIKOLAI et al., 2019; SILVA et al., 2020; AHANGARI et al., 2021). Furthermore, CO_2 is a small linear molecule that diffuses more rapidly than bulkier conventional liquid solvents and is quite miscible with a variety of organic solvents (NIKOLAI et al., 2019). Since pressurised CO_2 is non-polar in nature, the solubility of highly polar compounds in CO_2 is expected to be low. To overcome this, it is possible to modify the selectivity and solubility of these compounds in supercritical CO_2 by adding polar co-solvents such as ethanol (UWINEZA; WAŚKIEWICZ, 2020). Consequently, the properties of supercritical CO_2 make it suitable for various applications in the pharmaceutical-food area, such as the extraction of target compounds, particle formation, extrusion, drying and inactivation of microorganisms and enzymes (WANG et al., 2021).

Regarding the works on the use of supercritical CO_2 in lipid materials that are of interest for this review, these can be grouped into studies of phase/binary systems, micronisation, and particles loaded with bioactive compounds (Figure 1). These studies can be independent or interrelated depending on the objectives of the authors.

Figure 1 - Types of studies in lipid materials that are of interest for this review



Source: own authorship.

4. Phase studies (solid lipid + CO_2)/binary studies

This section mentions studies of binary or phase systems that were used to design or explain studies of micronisation and formation of particles loaded with bioactive compounds.

Some topics studied were the phase transition (solid-liquid-gas) of cocoa butter in supercritical CO_2 (KOKOT et al., 1999), the solubility of carbon dioxide in commercial lipid-based biocarriers (SOUSA et al., 2006), the binary solid–liquid–gas equilibrium of the tripalmitin and CO_2 (LI et al., 2006), the liquid-vapour curves and solubility of systems of supercritical CO_2 with hardened rapeseed oil, tripalmitin and hydrogenated castor oil (MÜNÜKLÜ et al., 2006), the melting and solidification temperatures of tristearin and a tristearin–phosphatidylcholine–dioctyl sulfosuccinate in CO_2 at 70 bar (SPILIMBERGO et al., 2006), the melting temperature, solubility of CO_2, density and viscosity of CO_2-saturated cocoa butter mixtures at high pressures (VENTER et al., 2007), density and volumetric expansion of canola oil and its blend with fully-hydrogenated canola oil in supercritical CO_2 (JENAB; TEMELLI, 2012), and the melting point depression of solid lipids in pressurised carbon dioxide (CIFTCI; TEMELLI, 2014).

5. Micronisation studies

Particle formation technologies allow the micronisation of materials and the production of particles loaded with bioactive compounds. PGSS is the main supercritical CO_2 process mentioned for the formation of solid lipid particles and structured lipid carriers in the absence of organic solvents. However, there is a difficult to differentiate this process from the Rapid Expansion of Supercritical Solutions (RESS) process, especially in applied research that does not cover thermodynamic aspects, since in both processes fine particles are obtained by precipitation after a rapid expansion as a consequence of the drastic reduction in solubility.

In this sense, Alessi et al. (1996) explained that in both processes the systems to be processed are mainly binary systems (material + supercritical CO_2), which, at a given temperature and pressure, are unstable and will originate a two-phase system. At high temperature (above the melting point of the solute) these two phases are liquid: one is richer in supercritical CO_2 with a low solute content, and the second is the liquid material in which the supercritical CO_2 is dissolved. In the PGSS process, the solute-rich phase is then depressurised through a nozzle and is capable of processing a concentrated solution of the material of interest with high productivity (WEIDNER et al., 1994). In the RESS process, equilibrium between solute and supercritical fluid is reached at temperatures well below the melting point of the solute, so the two phases are the solid phase (almost pure solute) and the

supercritical phase (in which the solute is partially dissolved). The diluted supercritical phase is then depressurised to precipitate solid particles (KUMAR et al. 2021).

The researchers were interested in studying the micronisation of solid lipid materials as a control to explain the characteristics of the particles when other substances are present in the mixture (AREDO et al., 2021) and to develop processes with technical and economic feasibility generating new concepts for applications in different areas (YANG; CIFTCI, 2016; WEIDNER., 2009). In this sense, some reported topics were the thermodynamic analysis of PGSS micronisation processes of tristearin (ELVASSORE et al., 2003), production of micronised cocoa butter particles (LETOURNEAU et al., 2005), the formation of microcomposites theophylline/hydrogenated palm oil (RODRIGUES et al., 2004), creation of hollow solid lipid particles (YANG; CIFTCI, 2016), the thermodynamic balance of the expansion of Precirol® (CALDERONE et al., 2007), the particle formation of rapeseed oil (MUNUKLU; JANSENS, 2007) and milk fat (LUBARY et al., 2011) using supercritical melt micronisation process as alternative name of PGSS process, the influence of temperature and pressure during PGSS micronisation and storage time on degree of crystallinity and crystal forms of monostearate and tristearate (MANDŽUKA; KNEZ, 2008), and formation of solid lipid microparticles from fully hydrogenated canola oil using supercritical CO_2 (CIFTCI; TEMELLI, 2016).

6. Studies of particles loaded with bioactive compounds

Particle formation by supercritical CO_2 technologies is expected to be advantageous for processing bioactive compounds because the particles are formed directly from mixtures of solid lipids and bioactive substances without an emulsion formation step, temperatures are relatively milder, uses water in the process and the particle size is mainly influenced by the diameter of the nozzle and the expansion pressure allowing a better control of the process (YANG; CIFTCI, 2017). However, there is little research on the formation of solid lipid particles and structured lipid carriers loaded with bioactive compounds using these technologies (Table 1). It could be explained by the high costs of the technology and the lack of low-cost lipid carrier materials compatible with the process, which could facilitate scalability and applicability in the bioactive substance industry.

Table 1 - Main research on formation of solid lipid particle and structured lipid carriers by supercritical technology

Lipid carrier material	Substance of interest	P (bar)	T (°C)	t (h)	Nozzle (mm)	Size (µm)	Efficiency (%)	Source
Palm fat	Elderberry juice	100	60	2	0.75	7-18	–	Bánvölgyi et al. (2016)
Fully hydrogenated canola oil	Spearmint essential oil	122-300	60	1	0.1-14.3	2.7-4.8	96	Ciftci and Temelli (2016)
Fully hydrogenated canola oil	Vitamin B2	100-250	65	1	3.2	61-0.8	12-48	Couto et al. (2017)
Glyceryl monostearate and waxy triglyceride	Caffeine, glutathione and ketoprofen	130	72	1	0.6	_	21.2-95.6	García-Gonzáles et al. (2010)
Trimiristin and gelucire	Bovine serum albumin	200	45	1	–	492-704	–	Santos et al. (2003)
		200	35-45	1	–	125-500	–	Santos et al. (2002)
Hydrogenated palm oil	Theophylline	120-180	60	–	1.8	3	–	Rodrigues et al. (2004)
Tristearin	Insulin	150	45	0.5	0.1	0.08-0.12	95	Salmaso et al. (2009a)
Glyceryl Monostearate	Caffeine	130	62	_	0.3	5.49	–	Sousa et al. (2007)
Fully hydrogenated soybean oil	Peppermint Essential Oil	200	57	1	0.05	6	39-47.5	Yang and Ciftci (2016)
polyethylene glycol	Ribonuclease A	130-140	60-65	–	–	5	30-35	Vezzù et al., 2010
Beeswax	Menthol	60-200	60	_	0.08	2-50	60	Zhu et al. (2010)

Source: own authorship.

Some applications for the formation of solid lipid particles and structured lipid carriers using these technologies are the formation of particles by PGSS of glyceryl monostearate and silanized TiO_2 loaded with caffeine, glutathione and ketoprofen with sponge-like morphology (GARCÍA-GONZÁLEZ et al., 2010), the production by PGSS of glyceryl monostearate particles loaded with caffeine (140 mg/g) with the appearance of aggregates of non-spherical porous needles larger than 50 µm (SOUSA et al., 2007), particle formation by PGSS of tristearin, phosphatidylcholine and polyethylene glycol loaded with Robonuclease A with an efficiency of 82% and an average size of 5 µm (VEZZÙ et al., 2010), the evaluation of an integrated process to produce anthocyanin-enriched palm fat particles from elderberry juice (BÁNVÖLGYI et al., 2016), evaluation from preformulation to in vivo studies of a structured

lipid carrier containing lidocaine (RIBEIRO et al. al., 2017), encapsulation of menthol in beeswax by PGSS (ZHU, et al., 2010), development of free-flowing mint essential oil-loaded hollow solid lipid microparticles and nanoparticles by carbon dioxide atomization (YANG; CIFTCI, 2016b), and production of solid lipid nanoparticles from vitamin B2-loaded hydrogenated canola oil by PGSS (COUTO et al., 2017).

Other studies used variants of these technologies such as supercritical fluid extraction of emulsions for the production of solid lipid nanoparticle suspensions of tripalmitin, tristearin, or Gelucire loaded with ketoprofen (CHATTOPADHYAY et al., 2007), modified supercritical gas-assisted melting atomisation process for the production of solid lipid submicron particles tristearin loaded with insulin (SALMASO et al., 2009ab), supercritical CO_2 impregnation for the formation of poly(methyl methacrylate)–poly-ε-caprolactone–cholesterol microspheres (ELVIRA et al. ., 2004), and supercritical fluid assisted emulsion-diffusion for the production of lipid nanoparticles of stearic acid (CAMPARDELLI et al. al., 2013).

Studies that are not specifically on solid lipid particles and structured lipid carriers but can be considered precursors to those in supercritical technology, focused on the microencapsulation of protein particles within lipids using supercritical fluid-based coating technology. In this context, Santos et al. (2002) proposed trimyristin (Dynasan®) or Gelucire® coating of bovine serum albumin crystals (solid protein particles) using supercritical fluid technology. A discontinuous coating of crystalline microneedles was obtained using Dynasan®. A more homogeneous coverage was achieved with Gelucire®, explained by the crystallisation of Dyanasan® (a pure triglyceride). The best advantage of this process compared to other supercritical processes is that the active material that is coated remains in its solid state, as it does not require solubilisation and does not undergo any degradation to bovine serum albumin. Based on this work, specific studies of process parameters (THIES et al., 2003), influence of solubility (SANTOS et al., 2003a) was carried out, which were applied in the improvement of the coating process and tested in bovine serum albumin crystals (SANTOS et al., 2003b).

7. Characterisation of particles

Particle characteristics can be altered as a result of process conditions such as pressure, temperature, agitation, and nozzle size, and quality and quantity of carrier material, bioactive compounds, and additives. In this sense, the characterisation of these particles requires the selection of appropriate techniques considering their size scale and nature of the materials (ANDONOVA; PENEVA, 2017). Regarding the physical characterisation of lipid particles, dynamic light scattering and laser diffraction are recommended to determine the size and size distribution of the particles, scanning electron microscopy and transmission electron microscopy are used to observe the surface morphology of the particles, and X-ray diffraction and differential scanning calorimetry are applied to study the degree of crystallinity and lipid modification in the particles (WOLLENWEBER et al., 2018).

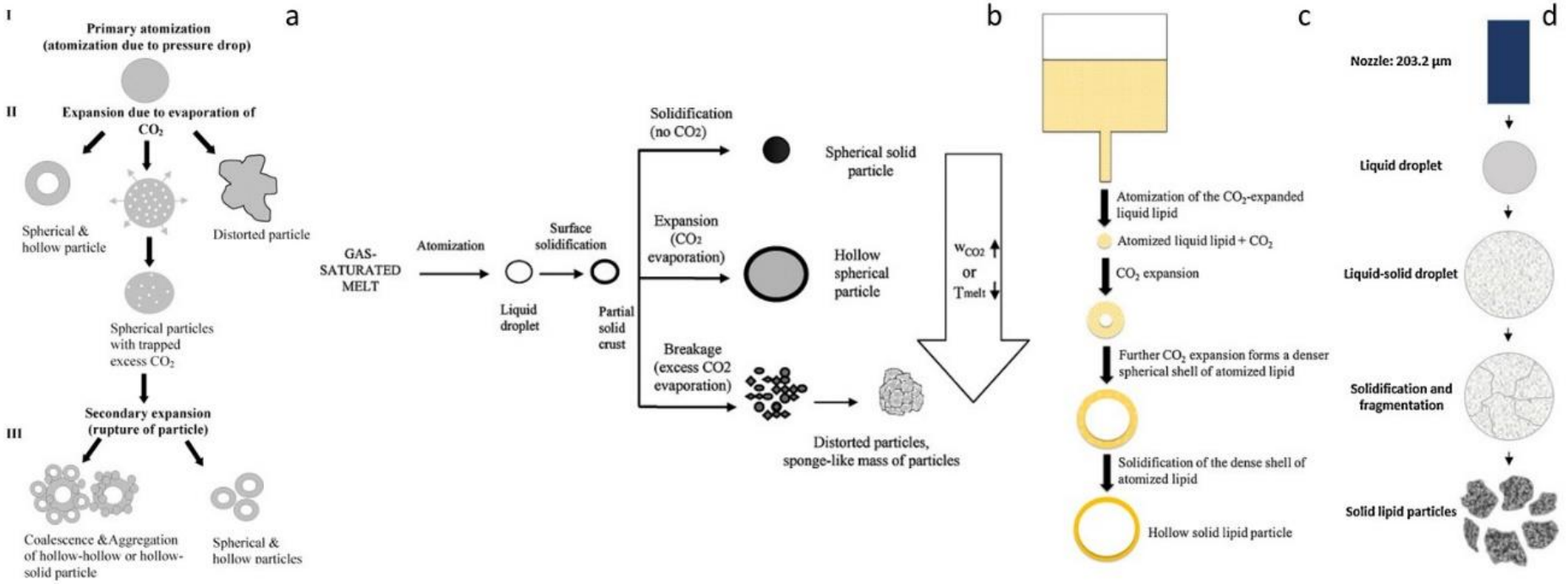
8. Mechanisms of formation

There are mechanisms of formation (Figure 2) that explain morphologies such as solid sphere, hollow sphere, and sponge that can be expected in the formation of solid lipid particles and structured lipid carriers by supercritical CO_2 technologies (MÜNÜKLÜ; JANSENS, 2007; LUBARY et al., 2011; YANG; CIFTCI, 2016; AREDO et al., 2021). These mechanisms of formation focused on the instantaneous alterations that occur in the CO_2-saturated lipid mixture when it is atomised through a nozzle, which are mainly the expansion of the droplet by CO_2 evaporation at atmospheric pressure and solidification of lipids by heat loss.

The distorted and sponge-like appearance of the particles is a consequence of the fact that the solidification of the lipids begins before all the dissolved CO_2 has evaporated. It can occur when the supercritical CO_2 process is performed at high pressures and at a temperature closer to the melting temperature of the lipid (MÜNÜKLÜ; JANSENS, 2007; LUBARY et al., 2011; AREDO et al., 2021).

On the other hand, solid-sphere and hollow-sphere morphologies are expected with reduced CO_2 concentration in the molten lipid, allowing CO_2 evaporation as the lipids solidify. It occurs at relatively low pressures and temperatures considerably above the melting temperature of the lipid (MÜNÜKLÜ; JANSENS, 2007; LUBARY et al., 2011; YANG; CIFTCI, 2016).

Figure 2 - Mechanisms of formation of solid lipid particles and structured lipid particles proposed by: (a) Münüklü and Jansens (2007), (b) Lubary et al. (2011), (c) Yang and Ciftci (2016), and (d) Aredo et al. (2021)



Source: own authorship.

10. Conclusions and future perspectives

Solid lipid particles and structured lipid carriers are interesting alternatives of powdered carrier systems for lipophilic bioactive compounds. Supercritical CO_2 technologies can be used for formation of these particles with high quality and safety to be used in food, pharmacy and cosmetic industries.

Studies of solid lipid particles and structured lipid carriers loaded with bioactive compounds with the aim of further understanding their process were based on phase/binary and micronisation studies.

It is well known that supercritical CO_2 technologies are ideal for lipid processing (TEMELLI, 2009). In this sense, there is an expectation of increasing the use of supercritical technologies for the particle formation of lipid materials loaded with bioactive compounds that will be applied as powdered ingredients in innovative food products (TEMELLI, 2018). Other options are the design of nanostructured photoprotective formulations for UV protection using vegetable oils (BADEA et al., 2015; NICULAE et al., 2014).

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CHAPTER III: Solubility of beeswax, Brazil nut oil and their mixture in supercritical CO_2

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CHAPTER III: Solubility of beeswax, Brazil nut oil and their mixture in supercritical CO_2

Abstract

The solubility of beeswax, Brazil nut oil and their mixture in supercritical CO_2 at different pressures and contact times was studied. The solubility measurements of beeswax were made in ranges of pressure of 120-330 bar and contact time 35-205 min following a composed central rotatable design approach for comparison with a factorial design approach. The solubility measurements of Brazil nut oil and its mixture with beeswax (1:1 w/w) were carried out in ranges of pressure of 150-300 bar and contact time of 60-180 min following a factorial design approach. The comparison of approaches revealed that factorial design was a simple way to study solubility. The solubility of these materials was solely dependent on pressure ($p<0.05$). Good linear fits between the solubility of these materials and pressure were observed ($R^2>0.90$, $p<<0.05$). These results could help the application of these materials in the formation of lipid particles using supercritical CO_2 technology.

Keywords: bioactive, carrier system, encapsulation, particle formation, supercritical fluid.

1. Introduction

Supercritical CO_2 technology has different applications such as extraction, purification, particle formation, and reaction (NIKOLAI et al., 2019). This technology has advantages such as negligible negative impact on the environment, use of medium temperatures, low-cost solvent, better quality and safety of final products. Therefore, supercritical CO_2 is investigated as an alternative to organic and environmentally destructive solvents for processing many materials (WANG et al., 2021; POLIKHRONIDI et al., 2019; KNEZ et al., 2019).

One of the material properties that is necessary to know for design applications with supercritical CO_2 technology is the solubility (GÜÇLÜ-ÜSTÜNDAĞ; TEMELLI, 2004; VALLE et al., 2012). It because understanding the solubility behaviour of the material in supercritical CO_2 helps define the process parameters such as temperature, pressure, proportions of CO_2:materials, use of co-solvents, etc. For example, solubility of coating materials in supercritical CO_2 such as waxes (paraffin, beeswax, carnauba wax), pure triglycerides

(tricaprin, trimyristin, tripalmitin, tristearin) and a mixture of glycerides and fatty acid esters (Gelucire®) under different conditions of temperature and pressure was studied to select appropriate coating materials for the implementation of a supercritical CO_2-based coating process of protein crystals (SANTOS et al., 2003).

A natural, safe and low-cost material is beeswax, which is interesting in applications of supercritical CO_2 technology, since in addition to being a coating material, it is used against embrittlement in polylactic acid foams (LIN et al. al., 2013), as an impregnation material on cellulose substrates (HUTTON-PRAGER et al., 2021), as a carrier material for solid lipid particles loaded with menthol (ZHU et al., 2010) and in structured lipid particles mixed with edible oils such as avocado oil and Brazil nut oil (AREDO et al., 2021).

In the context of limited data on the solubility of beeswax in supercritical CO_2 under a variety of conditions (SANTOS et al., 2003; AREDO et al., 2021), it is necessary to generate data, for example, on the effect of time on solubility, which is little discussed, set from little pre-experimental evidence, set from the review of the literature or even set arbitrarily (CORNELIO-SANTIAGO et al., 2017).

Solubility measurements of beeswax and other lipid materials in supercritical CO_2 are usually performed following a factorial approach, however, the response surface approach could be interesting to compare conclusions (BEGAN et al., 2000). This is because in using the response surface approach, it is not necessary to perform a full set of experiments on the full range of variables, which means less experimentation. It also has the advantage that it is possible to obtain a response equation to easily calculate the values of the property of interest (SICHE et al., 2015).

Based on the above, this research aimed to study the measurement of the solubility of beeswax, Brazil nut oil and their mixture (1:1 w/w) in supercritical CO_2 at different pressures and contact times.

2. Material and methods

2.1. Material

Beeswax from a farm located in Pirassununga (São Paulo, BR) and Brazil nut oil extracted from commercial Brazil nuts (Bertholetia excels H.B.K.) from Para (BR) were the lipid materials

of interest. The supercritical CO_2 extraction process and the characteristics of Brazil nut oil were studied by Cornelio-Santiago (2019). CO_2 (99%; Linde, Sertãozinho, Brazil) was used as supercritical fluid.

2.2. Solubility of lipids in supercritical CO_2

Experimental measurement of solubility of lipids (beeswax, Brazil nut oil and mixture of beeswax and Brazil nut oil (1:1 w/w)) in supercritical CO_2 (g/kg of CO_2) was based on the static method of Villegas et al. (2021) and carried out using equipment at the Laboratory of High Pressure Technology and Natural Products (LTAPPN) of the Faculty of Animal Science and Food Engineering of University of São Paulo (FZEA/USP) (Pirassununga, BR). The experimental measurement initially consisted of contact between lipid material (5 g) dispersed on glass beads and supercritical CO_2 at fixed conditions of pressure and temperature into an equilibrium cell. After a fixed contact time, CO_2 inlet and outlet valves of the equilibrium cell were simultaneous opened to shift a sample of solution in equilibrium (lipid material + supercritical CO_2) to a known volume collector (8.58 cm^3). The line of the collector was depressurised and washed with pressurised ethanol to remove the remaining material contained in the collector. Finally, for complete evaporation of ethanol, the mixture of beeswax and ethanol was placed in an oven at 105 °C until a constant weight was attained.

The solubility of lipids in supercritical CO_2 (g/kg of CO_2) was calculated as follows: mass of solubilised lipid material/ (collector volume*solution density). The solution density was replaced by the CO_2 density because the CO_2 is present in greater amount in the mixture.

2.3. Conditions of interest and analysis approach

The conditions for solubility measurement were based on the potential application of beeswax as a carrier material in the formation of solid lipid particles and structured lipid carriers by supercritical CO_2 technologies. The CO_2 temperature of interest was 60 °C (ZHU et al., 2010), because tests revealed that beeswax required temperatures slightly below 60 °C to melt in supercritical CO_2 (Appendix 1). The CO_2 pressures of interest considered as reference pressures of 122-300 bar to explore the effect of different CO_2 properties (CIFTCI; TEMELLI, 2016; COUTO; ALVAREZ; TEMELLI, 2017). The contact times of interest were defined on the

basis that this parameter is chosen arbitrarily and varies from 30 (SALMASO et al., 2009) to 120 min (BÁNVÖLGYI et al., 2016) in studies of lipid particle formation.

Based on these considerations, a central composite rotating design approach (2^2 factorial points + 2*2 axial points + 3 central points) of response surface methodology was used as a way to explore a wide range of variables, such as pressures of 150-330 bar and contact times from 35 to 225 min. It was used to determine the influence of CO_2 pressure and contact time on the solubility of beeswax in supercritical CO_2 (Table 1). This approach was carried out to verify what was observed by Aredo et al. (2021) in a factorial approach.

On the other hand, the solubility measurements of Brazil nut oil and of the mixture of beeswax and Brazil nut oil (1:1 w/w) in supercritical CO_2 were carried out considering the limits of the factorial approach of the approach of central compound rotary design in beeswax. In this sense, CO_2 pressures of 150, 200, 250 or 300 bar and contact times of 60 min, 120 min or 180 min were evaluated.

The relationship between beeswax solubility with CO_2 pressure and CO_2 density was studied to verify the linearity reported for other lipid materials (SANTOS et al., 2019).

2.4. Statistical analyses

Statistical analyses were performed using Statistica® software (version 12.0, StatSoft, USA). The analysis of variance of means was performed to determine possible significant differences. For the comparison of means, the Tukey test was used (p=0.05). Analysis of the central composite rotating design approach consisted of an analysis of variance and effects (p = 0.05) and model coefficients.

3. Results and discussion

3.1. Central Composite Rotatable Design approach

The solubility of beeswax in supercritical CO_2 (60 °C) at different pressures (120-330 bar) ranged from 1.5 ± 0.1 g of beeswax/kg of CO_2 at 120 bar and 120 min to 5.0 ± 0.6 g of beeswax/kg of CO_2 at 330 bar and 120 min (Table 1).

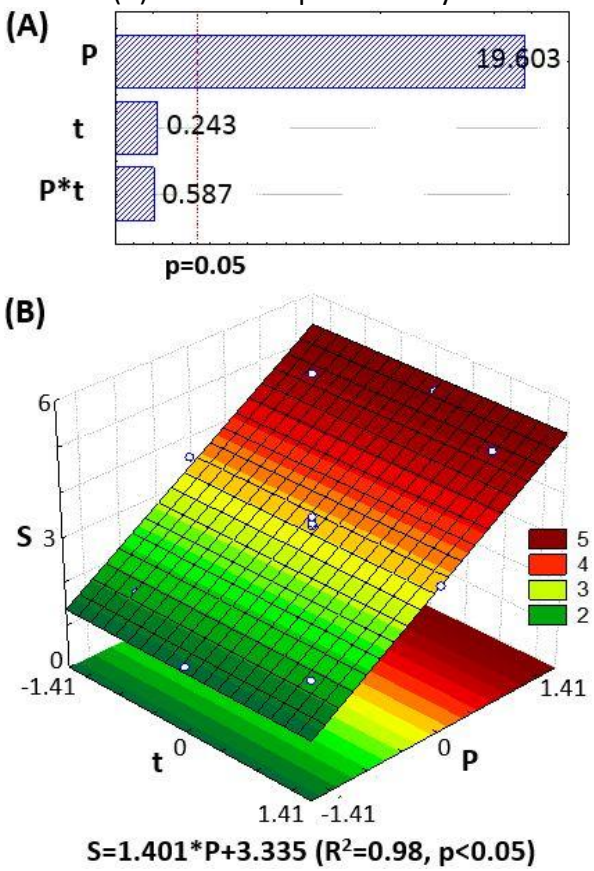
Table 1 - Solubility of beeswax (g/Kg CO_2) in supercritical CO_2 (60 °C) at different pressures and contact times

Points	Pressure (bar)	Time (min)	Solubility (g/Kg CO_2)	Relative error (%)
Composite central design				
1	150 (-1)	60 (-1)	$1.8 \pm 0.2^{cd,*}$	7.2
2	150 (-1)	180 (1)	$1.7 \pm 0.1^{cd,*}$	11.5
3	300 (1)	60 (-1)	$4.9 \pm 0.1^{a,*}$	3.7
4	300 (1)	180 (1)	$4.9 \pm 0.5^{a,*}$	2.8
5	120 (-1.41)	120 (0)	1.5 ± 0.1^{d}	8.7
6	330 (1.41)	120 (0)	5.0 ± 0.6^{a}	6.6
7	225 (0)	35 (-1.41)	3.3 ± 0.6^{abc}	0.6
8	225 (0)	205 (1.41)	3.5 ± 0.0^{abc}	4.7
9-11	225 (0)	120 (0)	3.26; 3.33; 3.48 = 3.4 ± 0.1^{abc}	0.6
Validation				
12	200 (-0.33)	60 (-1)	$3.0 \pm 0.3^{bcd,*}$	2.7
13	200 (-0.33)	180 (1)	$2.8 \pm 0.1^{bcd,*}$	1.8
14	250 (0.33)	60 (-1)	$4.0 \pm 0.6^{ab,*}$	4.8
15	250 (0.33)	180 (1)	$4.0 \pm 0.3^{ab,*}$	5.6

Values expressed as means ± SD; n = 2 for 1-8 and 12-15 points. Different letters mean significant differences ($p < 0.05$). *Data from Aredo et al. (2021). Source: own authorship.

Effect analysis (Figure 1A) revealed that the solubility of beeswax depended uniquely on CO_2 pressure ($p < 0.05$). By referencing the response surface (Figure 1B), it can be inferred that the solubility of beeswax in supercritical CO_2 can be described by a linear model that only considers the CO_2 pressure (R^2=0.98, $p < 0.05$) with a good fit for experimental and validation points and a relative error lower than 12% (Table 1). These results revealed that beeswax applications with supercritical CO_2 (60 °C and 120-330 bar) could be conducted in a short contact time to minimise the degradation of heat sensitive bioactive compounds that may constitute some food/pharmacy/cosmetic products in future research.

Figure 1 - Statistical analysis of influence of pressure and contact time on solubility of beeswax in supercritical CO_2 by central composite rotatable design: (A) effects analysis and (B) surface response analysis



Source: own authorship.

3.2. Factorial approach for solubility of lipids in supercritical CO_2

The solubility in supercritical CO_2 (60 °C) at different pressures (150−300 bar) ranged from 1.7 ± 0.1 g /kg of CO_2 at 150 bar and 180 min to 4.9 ± 0.5 g /kg of CO_2 at 300 bar and 180 min, from 0.8 ± 0.1 g /kg of CO_2 at 150 bar and 120 min to 6.1 ± 0.2 g /kg of CO_2 at 300 bar and 180 min, from 0.9 ± 0.2 g /kg of CO_2 at 150 bar and 60 min to 6.8 ± 0.7 g /kg of CO_2 at 300 bar and 60 min for beeswax, beeswax + Brazil nut oil (1:1 w/w) and Brazil nut oil, respectively (Table 2). It was observed that the solubility of beeswax, beeswax + Brazil nut oil (1:1 w/w) and Brazil nut oil depended uniquely on CO_2 pressure ($p < 0.05$) and that 60 min of contact is enough to reach the equilibrium of the system. Thereby, supercritical CO_2 process using these materials could be carried out in 60 min of contact time.

Table 2 - Solubility of beeswax, beeswax + Brazil nut oil (1:1 w/w) and Brazil nut oil in supercritical CO_2 (g/Kg CO_2) measured at 60, 120 and 180 minutes at different pressures

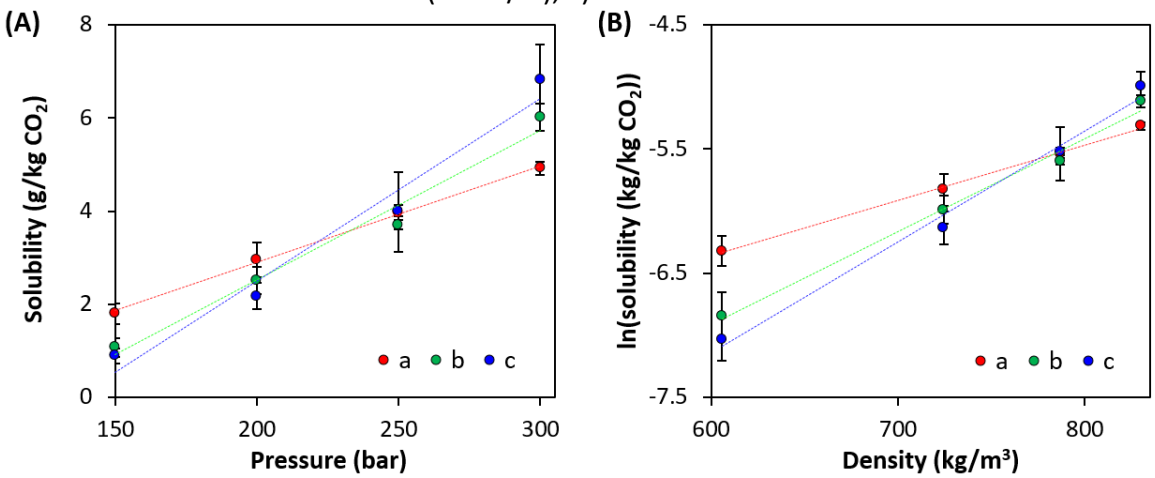
Sample	Pressure (bar)	Time (min)		
		60	120	180
Beeswax	150	$1.8 \pm 0.2^{c,*}$	_	$1.7 \pm 0.1^{c,*}$
	200	$3.0 \pm 0.3^{bc,*}$	_	$2.8 \pm 0.1^{bc,*}$
	250	$4.0 \pm 0.6^{ab,*}$	_	$4.0 \pm 0.3^{ab,*}$
	300	$4.9 \pm 0.1^{a,*}$	_	$4.9 \pm 0.5^{a,*}$
Beeswax + Brazil nut oil (1:1 w/w)	150	1.1 ± 0.2^{d}	0.8 ± 0.1^{d}	0.9 ± 0.3^{d}
	200	2.5 ± 0.3^{c}	2.2 ± 0.1^{c}	2.6 ± 0.1^{c}
	250	3.7 ± 0.1^{b}	3.7 ± 0.4^{b}	3.9 ± 0.3^{b}
	300	6.0 ± 0.3^{a}	6.0 ± 0.1^{a}	6.1 ± 0.2^{a}
Brazil nut oil	150	0.9 ± 0.2^{c}	1.2 ± 0.1^{c}	1.3 ± 0.1^{c}
	200	2.2 ± 0.3^{c}	2.1 ± 0.1^{c}	2.3 ± 0.2^{c}
	250	4.0 ± 0.1^{b}	4.0 ± 0.5^{b}	4.2 ± 0.4^{b}
	300	6.8 ± 0.7^{a}	6.7 ± 0.5^{a}	6.6 ± 0.7^{a}

Values expressed as means ± SD; n = 2. For each material, in each column, different letters mean significant differences ($p < 0.05$). In each row, no significant differences were found ($p > 0.05$). * Data from Aredo et al. (2021) included for comparison purposes. Source: own authorship.

Analysis of the relationship between CO_2 pressure and solubility of beeswax, mixture of beeswax and Brazil nut oil (1:1 w/w), and Brazil nut oil (Figure 2A) revealed a good linear adjustment ($R^2 > 0.91$, $p < 0.05$). Furthermore, a refined linear adjustment ($R^2 > 0.93$, $p < 0.05$) explained the relationship between CO_2 density and solubility values of beeswax, mixture of beeswax and Brazil nut oil (1:1 w/w), and Brazil nut oil expressed as a natural logarithm (Figure 2B), which suggests the adequacy for the adjustment to phase equilibrium equations proposed by Chrastil (1982) and Mitra and Wilson (1991). Solubility of beeswax measurements at other temperatures (> 60 °C) will be necessary for phase equilibrium studies for thermal-tolerant applications such as beeswax purification using supercritical CO_2.

The solubility of mixture of beeswax and Brazil nut oil (1:1 w/w) was found to be intermediate between that of beeswax and Brazil nut oil (Figure 2). It can be described as an additive effect with a slight deviation towards the values of Brazil nut oil (liquid lipid). For practical applications where there is interest in the solubilisation of the components in supercritical CO_2 at similar levels, the pressures between 200-250 bar are suggested as useful to obtain products with similar composition to the initial formulation. It explains why the Brazil-nut oil-loaded beeswax particles formed with supercritical CO_2 (60 °C and 150-300 bar) have similar Fourier transform infrared spectra that the initial mixture (AREDO et al. 2021).

Figure 2 - Relationship analysis: (A) solubility of lipids (g/kg CO_2) vs CO_2 pressure (bar) and (B) ln(kg/kg CO_2) vs CO_2 density (kg/m^3) for a) beeswax, b) mixture of beeswax and Brazil nut oil (1:1 w/w), c) Brazil nut oil



Values expressed as means ± SD; n = 2. Measurements at 60 min were used in the analysis. Dashed lines are linear regressions fitted for Beeswax: Solubility = -1.256 + 0.021*pressure (R^2=0.918, p=0.00) and ln(solubility) = -9.036 + 0.004*density (R^2=0.933, p=0.00); Mixture of beeswax and Brazil nut oil (1:1 w/w): Solubility = -3.874 + 0.032*pressure (R^2=0.970, p=0.00) and ln(solubility) = -11.389 + 0.007*density (R^2=0.976, p=0.00); Brazil nut oil: Solubility = -5.357 + 0.039*pressure (R^2=0.954, p=0.00) and ln(solubility) = -12.506 + 0.009*density (R^2=0.976, p=0.00). Source: own authorship.

The observation of the positive relationship between CO_2 pressure with the solubility of beeswax and mixture of beeswax and Brazil nut oil (1:1 w/w) in supercritical CO_2 (Figure 2A) is interesting for understanding applications of beeswax. For example, since the CO_2 pressure had a negative and significant correlation with the size and bulk density of beeswax particles and Brazil-nut oil-loaded beeswax particles (AREDO et al. 2021), it can be suggested that the higher solubility of beeswax and Brazil nut oil (1:1 w/w) in supercritical CO_2 at higher CO_2 pressures could lead to the reduction of the size and bulk density of the Brazil-nut oil-loaded beeswax particles.

4. Conclusions

The solubility of beeswax, mixture of beeswax and Brazil nut oil (1:1 w/w), Brazil nut oil in supercritical CO_2 (60 °C) was not affected by the contact time (60-180 min), but it was highly dependent on pressure (150-300 bar). In this sense, good linear adjustment was observed for the solubility vs CO_2 pressure and solubility vs CO_2 density (606-830 kg/m^3).

A response surface approach can be used to explore the effect of pressure and contact time on beeswax solubility in supercritical CO_2 with conclusions similar than those of a factorial approach.

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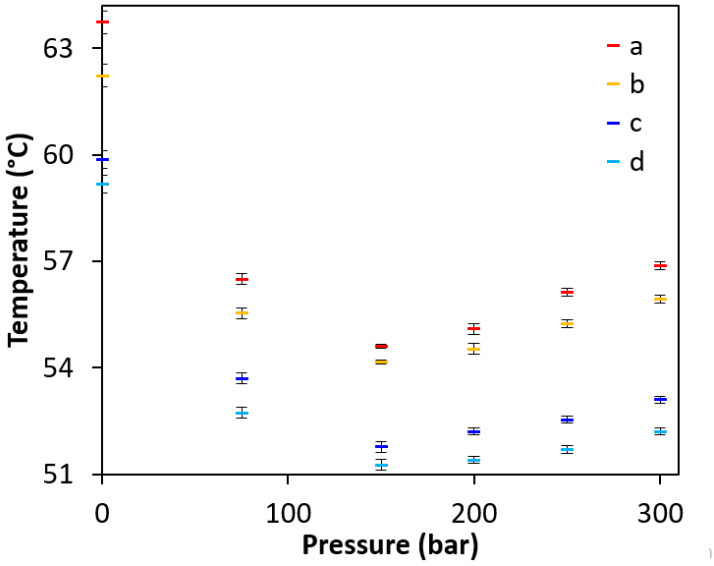
Appendix 1

Melting and solidification temperatures of lipids in supercritical CO_2

A phase monitor (SFC/RESS, Thar Instruments Co./Waters, Pittsburgh, USA) was used for observation of melting temperature (T_m) and solidification temperature (T_s) of beeswax and beeswax-Brazil nut oil mixture (1:1 w/w) in supercritical CO_2. The sample was melted at 90 °C to eliminate crystal memory, and about 0.2 g was placed in a clear glass tube (0.3 cm in diameter and 4.0 cm in height). The tube was placed into the high-pressure cell (5.5 cm in diameter and 5.8 cm in height) in a position that allowed it to be viewed through the sapphire window.

The high-pressure cell was heated until the entire sample melted at atmospheric pressure. For T_s identification, the cell was cooled down slowly (about 0.5 °C/min) until sample opacity was visually observed. Then, the sample was cooled down for extra 3 °C above T_s. At, this temperature, the sample awaited 10 minutes for equilibration. Subsequently, for T_m observation, the cell was heated slowly (about 0.5 °C/min) until the first brightness was visually observed. This process was repeated for the pressures studied that ranged from 0 bar to 300 bar.

Figure 3 - Melting temperature (T_m) and solidification temperature (T_s) of lipids: T_m (a) and T_s (b) of beeswax, and T_m (c) and T_s (d) of a mixture of beeswax and Brazil nut oil (1:1 w/w)



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The T_m and T_s of these lipids (Figure 3) decreased with increasing pressure from 0 to 150 bar, while, these temperatures increased with the increase in pressure from 150 to 300 bar. In this sense, it can be affirmed that these lipids remained melted in supercritical CO_2 processes at 60 °C (75-300 bar). As expected, the mixture of beeswax and Brazil nut oil (1:1 w/w) had lower T_m and T_s than pure beeswax. It is explained by the presence of unsaturated fatty acids in Brazil nut oil that causes the liquid state of this lipid material at mild temperatures. The results can serve as reference for the use or store of products based on these lipids, since exposure to temperatures higher than their T_m can irreversibly alter physical characteristics like appearance.

CHAPTER IV: Formation of edible oil-loaded beeswax microparticles using PGSS – Particles from Gas-Saturated Solutions

(This chapter is part of an article published in The Journal of Supercritical Fluids)

CHAPTER IV: Formation of edible oil-loaded beeswax microparticles using PGSS – Particles from Gas-Saturated Solutions

Abstract

This research aimed to study the formation of edible oil-loaded beeswax microparticles using the Particles from Gas-Saturated Solutions (PGSS) process. Lipid binary mixtures (1:1 w/w) of beeswax with Brazil nut oil was studied as model. The PGSS process consisted of contacting the lipid binary mixture with supercritical CO_2 in an autoclave at pressures from 150 to 300 bar at a temperature of 60 °C with an agitation at 1250 rpm for 1 h and the subsequent expansion of the solution (lipids + CO_2) through a nozzle of 203.2 µm. The resulting edible oil-loaded beeswax microparticles had a sponge-like morphology and sizes of 96−128 µm. Crystallinity and internal physical structure of the particles suggested uniform incorporation of edible oil into the crystalline lattice of beeswax. These particles could formulate ingredients for functional food products.

Keywords: Brazil nut oil, carbon dioxide, encapsulation, micronisation, supercritical melt micronisation.

1. Introduction

Currently, consumption of bioactive compounds is considered very important in facets of health care; however, most bioactive compounds isolated from natural sources are degradable due to the effect of environmental conditions (SANTOS; RIBEIRO; SANTANA, 2019; KATOUZIAN et al., 2017). Particle formation is used as a strategy to stabilise and facilitate the incorporation of bioactive compounds into food (SANTOS; RIBEIRO; SANTANA, 2019), cosmetic (JOSE; NETO, 2019) and pharmaceutical products (GANESAN et al., 2018). The particle formation is achieved using different technologies and carrier materials depending on the type of bioactive compound and desirable application (KATOUZIAN et al., 2017; ĐORĐEVIĆ et al. 2015).

Particle formation of lipophilic bioactive compounds represents a challenge to overcome because it requires compatible carrier materials. In this context in the early 1990s,

solid lipids began to be used as carrier material. This led to the formation of solid lipid particles, which have been suggested as efficient carrier systems when compared to liposomes and emulsions. In the early 2000s, solid lipid particles loaded with liquid lipids, also known as structured lipid carriers, were proposed as an apparently easy solubilisation of the lipophilic bioactive compound into the liquid lipid, which remains entrapped into the crystalline lattice of the solid lipid (MÜLLER; RADTKE; WISSING, 2002).

There are different technologies to form solid lipid particles such as high-pressure homogenisation, solvent emulsification evaporation, coacervation, microemulsion cooling, ultrasonification, among others (GANESAN; NARAYANASAMY, 2017). Nevertheless, particle formation using supercritical CO_2 is recommended because it operates at moderate temperatures and in an oxygen free atmosphere. Moreover, it allows control of particle characteristics such as morphology and size by changing the pressure and temperature of CO_2 (KNEZ et al., 2019; MATOS et al., 2019). Particles from Gas-Saturated Solutions (PGSS) stands out among other supercritical techniques because it generally does not need organic solvents (COCERO et al., 2009). This technique consists of the saturation of edible molten fats with supercritical CO_2 that are subsequently expanded through a nozzle (MÜNÜKLÜ; JANSENS, 2007).

Beeswax is a safe, low-cost and compatible carrier material for formation of solid lipid particles. This material is a natural fatty product used since ancient times in different applications. It contains over 300 different substances and consists mainly of esters of long chain fatty acids and alcohols and small quantities of hydrocarbons, acids and other substances (TULLOCH; HOFFMAN, 1972; TULLOCH, 1980; BOGDANOV, 2004). Beeswax was studied in formation of solid lipid particles loaded with menthol by PGSS (ZHU et al., 2010) and vitamin E by melting-emulsion solidification when mixed with medium-chain triglycerides, coconut oil or avocado oil (SOUZA et al., 2020). To our knowledge there are no studies of edible oil-loaded beeswax particles using supercritical technology. Brazil nut oil is an edible oil with high commercial value which composition in fatty acids, polyphenols, phytosterols and squalene suggests benefits for human health (CICERO et al., 2018). This edible oil could help in the formation of particles with improved bioactivity that have potential as powdered ingredients for functional foods.

In light of the possibilities, this research aimed to study the formation of edible oil-loaded solid lipid particles from beeswax using the PGSS process at different pressures.

2. Materials and methods

2.1. Materials

Beeswax from a farm located in Pirassununga (São Paulo, BR) was used as solid lipid material. Brazil nut oil extracted from commercial Brazil nuts (Bertholletia excelsa H.B.K.) from Para (BR) was the edible oil used as model of liquid lipid material. The extraction of Brazil nut oil was performed with supercritical CO_2 at 70 °C and 350 bar. Description of the extraction process and characteristics of the Brazil nut oil were previously studied by Cornelio-Santiago (2019). CO_2 was used as supercritical fluid (99 %; Linde, Sertãozinho, BR).

2.2. Particle formation

A 1:1 (w/w) ratio of solid lipid (beeswax): liquid lipid (Brazil nut oil) was used for the particle formation based on the ratios studied by Attama et al. (2006), and preliminary tests demonstrated particle melting at room temperature (25 °C) when there is more liquid lipid than solid lipid in the particle ratio. The particle formation process was carried out using an SFC/RESS (Thar Instruments Co., Ltd., Pittsburgh, PA, USA), which was previously described by Machado et al. (2016). This experiment is the first stage of a research and replicas of the particle formation process were not made. In this stage it is intended to define the process conditions and mainly the composition of the mixture of beeswax and vegetable oil that will be used in the impregnation of active compounds. Some preliminary tests (data not shown) performed on this equipment showed that the particles produced in the same conditions have the same characteristics (morphology, size and apparent density). In addition, the operation system is computerised and allows a reliable control of the process parameters (pressure and temperature).

The particle formation process consisted of two steps: 1) the addition of 4 g of beeswax and 4 g of edible oil (Brazil nut oil) into an autoclave (133.5 cm^3) to contact with supercritical CO_2 at a temperature of 60 °C and pressures of 150, 200, 250 or 300 bar for 1 h of agitation at 1250 rpm; 2) the expansion of the solution (lipids + CO_2) through a nozzle of 203.2 µm inside a chamber. The particles were stored in refrigeration (4 °C) in Petri dishes covered with aluminium foil. The experimental conditions were based on those commonly used in the

formation of solid lipid particles by supercritical technology (CIFTCI; TEMELLI, 2016; COUTO; ALVAREZ; TEMELLI, 2017; YANG; CIFTCI, 2016), and the experiments were focused to study the effect of CO_2 pressures on the properties of the particles. In addition, pure beeswax particles were formed using the same process as a control study.

2.3. Particle characterisation

2.3.1. Morphology

A scanning electron microscope (TM 3000, Hitachi, Tokyo, JP) from Food Technology Laboratory (LTA) of FZEA/USP was used to analyse particle morphology. For this, the particles were gently placed in a double carbon tape (Ted Pella, USA) fixed in aluminium stubs. The images were captured with 5 kV voltage acceleration at a current of 1.750 mA (PELISSARI et al., 2016).

2.3.2. Size distribution, bulk density and statistical analysis

The distribution of particle size was determined by laser light diffraction equipment (Sald-201 V, Shimadzu, Tokyo, JP) from Biopolymers Technology Laboratory (BIOPOLITEC) of FZEA/USP. The particles were manually and gently suspended in a liquid medium (solution of 1:1 (vol/vol) water:ethanol) at room temperature. The particle suspensions were placed in quartz cuvettes for measurements at absorbance levels ranging from 0.1 to 0.2 (VILLEGAS et al., 2020). The behaviour of the particles in water and ethanol was the basis for defining the liquid medium to suspend the particles. The particles are insoluble in water and were shown uniformly suspended but partially solubilised in anhydrous ethanol. Thus, a solution of 1:1 (vol/vol) water:ethanol allowed obtaining of stable and homogenous particle suspensions without significant solubilisation of the particles, which are desirable characteristics for laser light diffraction technique (VILLEGAS et al., 2020). The particle size measurements were performed in triplicate immediately after suspending them in the water: ethanol solution.

The Rosin-Rammler function was used for the analysis of the distribution of particle size. This function describes the normalised and cumulative size distribution as follows: $\Upsilon = 1 - \exp(-(X/X_R)^n)$, where "$\Upsilon$" is the fraction finer than size "X", "X_R" parameter that is the 63.21 percentile of the particles size and the "n" parameter that represents the narrowness of the

distribution (YAN; BARBOSA-CÁNOVAS, 1997), from which descriptors of the size distribution such as 10 percentile (D10), 50 percentile (D50) and 90 percentile (D90) were estimated.

Bulk density (mg/mL) was determined by weighing the mass of particles that can fill a volume of 20 mL in a test tube. These measurements were done in quintuplicate.

Statistical analyses were performed using Statistica® software (version 12.0, StatSoft, USA). Analysis of variance was performed to determine whether CO_2 pressure influences on size and bulk density of the particles. Tukey's test ($p < 0.05$) was used for the multiple comparison of means. The Pearson correlation coefficient (r) and p value were used to assess the correlation between CO_2 pressure and size and bulk density of the particles.

2.3.3. Polymorphism

Polymorphism was studied in diffractograms using an X-ray diffraction (Miniflex 600, Rigaku, JP) from Multi-User Materials Characterisation Laboratory (MultMat) of FZEA/USP. The diffractograms were obtained at room temperature (25 °C) using copper radiation kα (λ =1.5405 Å), a voltage of 40 kV, a current of 30 mA, an angular velocity of 0.05˚/s at angles ranging from 3 to 50˚ (PELISSARI et al., 2016).

2.3.4. Thermal properties

Thermal peaks were investigated with thermograms obtained by a differential scanning calorimeter (DSC-TA2010, Thermal Analysis Instruments, New Castle, DE, USA) from LTA of FZEA/USP. The procedure was based on Machado et al. (2016), in which a known quantity of sample was weighed, packed in sealed crucibles and heated at 5 °C / min between −100 °C and 150 °C in an inert atmosphere of nitrogen (45 mL N_2 / min).

2.3.5. Chemical structure

The study of the chemical structure was carried out using a Fourier transform infrared spectrometer (Spectrum One, Perkin Elmer, Norwalk, CO, USA) from LTA of FZEA/USP. Samples were added directly to the equipment in a Universal ATR Sampling Accessory to perform 40 scans at a resolution of 4 cm^{-1} in the spectral range of 4000−650 cm^{-1}.

2.3.6. Internal physical structure

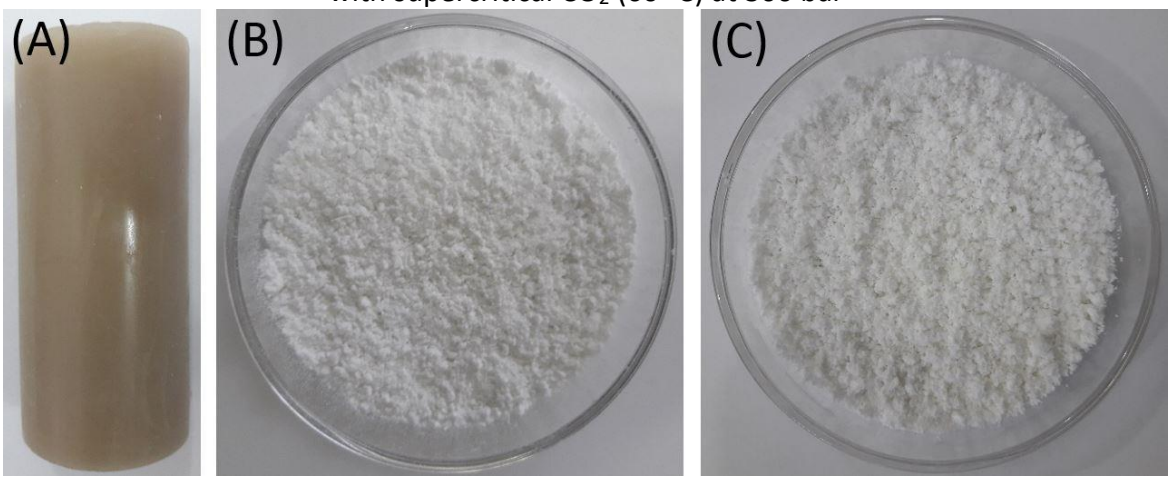
The particles were characterised according to their internal physical structure using a confocal fluorescence microscope (LSM 780, Zeiss, Heidelberg, GE) with two-photon excitation (laser titanium: 800 nm sapphire, 80 MHz, 100 fs pulse), an objective lens (20x; 0.8NA; wd =0.5 mm (air)) and high-sensitivity GaAsP detectors for spectral imaging at 400–700 nm (AREDO et al., 2019). This analyse was carried out by the optics group of the São Carlos Institute of Physics/USP.

3. Results and discussion

3.1. Macroscopic characteristics and morphology

Solid beeswax had a glossy appearance and a uniform green-brown colour (Figure 1A), which could be influenced by the ecosystem and bee's type (TULLOCH; HOFFMAN, 1972). The beeswax particles (Figure 1B) and the Brazil nut oil-loaded beeswax particles (Figure 1C) exhibited a white matte colour. The beeswax particles were free-flowing. The edible oil-loaded beeswax particles were not as free-flowing as beeswax particles, nevertheless, these particles were relatively easy to be deagglomerated. It is worth noting that no differences were observed in the macroscopic characteristics of the particles caused by the used CO_2 pressure.

Figure 1 - Physical appearance: (A) solid beeswax, (B) beeswax particles formed with supercritical CO_2 (60 °C) at 300 bar, and (C) Brazil nut oil-loaded beeswax particles formed with supercritical CO_2 (60 °C) at 300 bar



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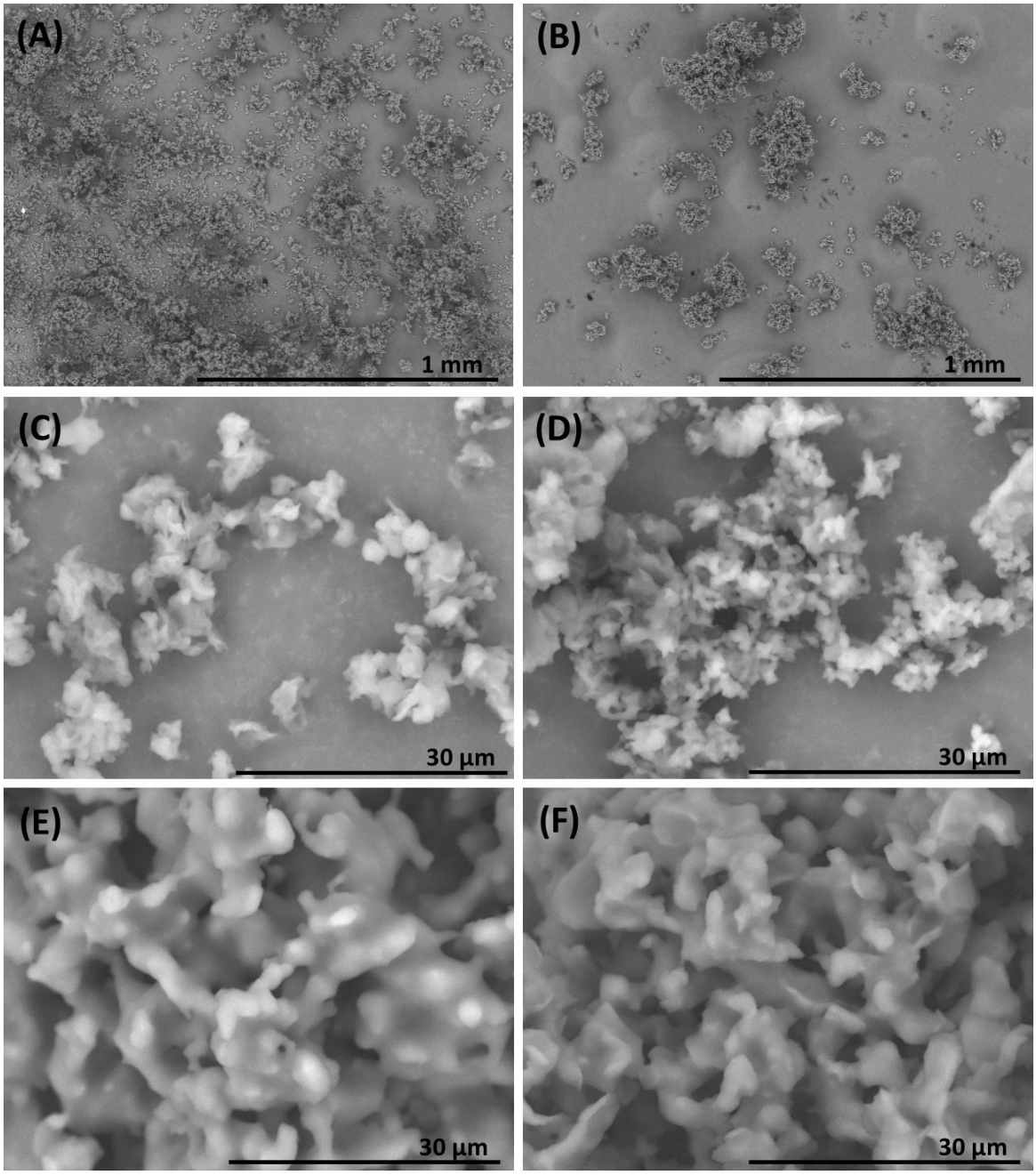
The particles had the appearance of sponge-like structures (Figure 2). The beeswax particles presented many fragments (Figure 2A), while the Brazil nut oil-loaded beeswax particles (Figure 2B) looked better defined with a low presence of fragments. CO_2 pressure did not cause the particles to have perceptible differences in their morphology. The morphological analysis of the sponge-like structures revealed that the beeswax particles formed with supercritical CO_2 (60 °C) at 150 bar were composed by semi-merged and distorted spheres (Figure 2C) and were similar to those from particles formed at pressures of 200 and 250 bar. Conversely, at 300 bar the semi-merged and distorted spheres looked more complex because they had lobed edges (Figure 2D). In the case of edible oil-loaded beeswax particles, it was noticed that they were composed of elliptical and merged lobules. No morphological alterations of the lobules were observed from CO_2 pressure Brazil nut oil-loaded beeswax particles (Figure 2E,F).

Figure 2 - Morphology of the particles formed with supercritical CO_2 (60 °C) at different pressures: (A) beeswax at 150 bar, (B) Brazil nut oil-loaded beeswax at 300 bar, (C) beeswax at 150 bar, (D) beeswax at 300 bar, (E) Brazil nuit oil-loaded beeswax at 150 bar, and (F) Brazil nuit oil-loaded beeswax at 300 bar



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3.2. Size distribution, bulk density and correlation analysis

The size distribution of the particles (Figure 3) was successfully explained by the Rosin-Rammler function (R^2 minimum = 99.75%) (Table 1); therefore, the Rosin-Rammler parameters (n and X_R) and estimated descriptors (D10, D50 and D90) were reliable for comparing the size distributions of the particles. The size distribution of the beeswax particles was not significantly affected by CO_2 pressure and was similar to the size distribution of the Brazil nut oil-loaded beeswax particles formed with supercritical CO_2 (60 °C) at 300 bar. These samples presented a size less than those of the Brazil nut oil-loaded beeswax particles that were formed with supercritical CO_2 (60 °C) at 250 bar, which in turn presented a size less than that of the Brazil nut oil-loaded beeswax particles formed with supercritical CO_2 (60 °C) at 150 and 200 bar.

Figure 3 - Particle size distribution of the size of the particles formed with supercritical CO_2 (60 °C) at different pressures: beeswax at (a) 150 bar, (b) 200 bar, (c) 250 bar and (d) 300 bar; Brazil nut oil-loaded beeswax at (e) 150 bar, (f) 200 bar, (g) 250 bar and (h) 300 bar.

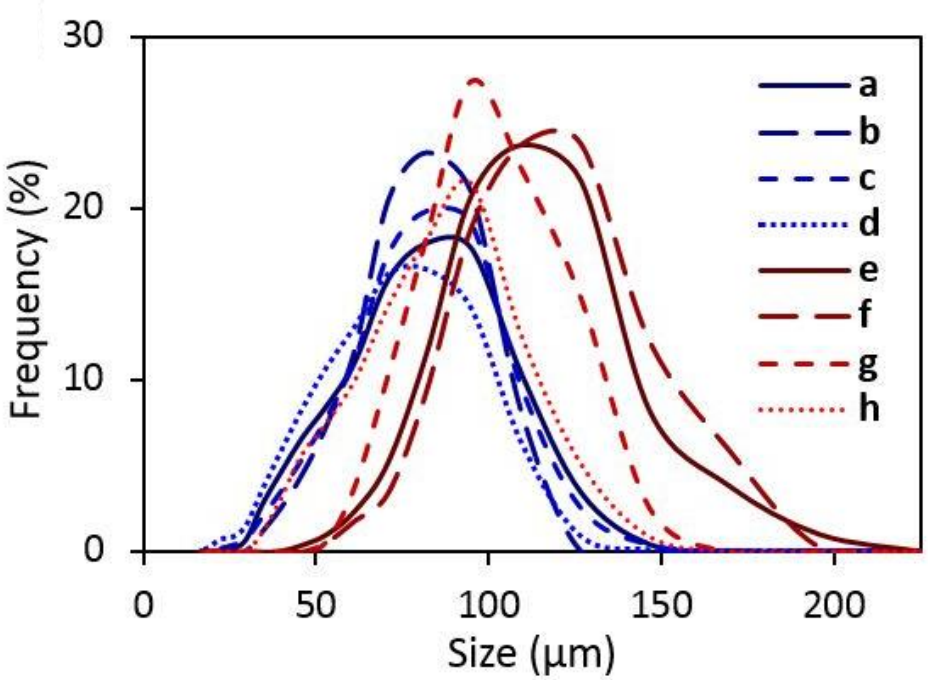

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Table 1 - Analysis of the size distribution of beeswax and Brazil nut oil-loaded beeswax particles formed with supercritical CO_2 (60 °C) at different pressures

Particles	Pressure (bar)	Rosin-Rammler parameters		R^2 (%)	Estimated descriptors		
		n	X_R (µm)		D10 (µm)	D50 (µm)	D90 (µm)
Beeswax	150	3.6 ± 0.5^{ab}	91 ± 4^{bc}	99.90	49 ± 3^{b}	82 ± 3^{b}	115 ± 7^{bc}
	200	4.5 ± 0.2^{ab}	89 ± 1^{bc}	99.84	54 ± 2^{b}	82 ± 1^{b}	107.3 ± 0.1^{c}
	250	3.6 ± 0.7^{b}	88 ± 4^{bc}	99.94	46 ± 5^{b}	79 ± 3^{b}	112 ± 8^{bc}
	300	3.4 ± 0.6^{b}	82 ± 2^{c}	99.93	42 ± 6^{b}	74 ± 3^{b}	106 ± 3^{c}
Brazil nut oil-loaded beeswax	150	5 ± 1^{ab}	127 ± 11^{a}	99.84	82 ± 7^{a}	118 ± 10^{a}	150 ± 17^{ab}
	200	6 ± 1^{ab}	133 ± 11^{a}	99.86	87 ± 4^{a}	124 ± 9^{a}	155 ± 17^{a}
	250	6.2 ± 0.8^{a}	113 ± 9^{ab}	99.89	78 ± 4^{a}	106 ± 8^{ab}	129 ± 13^{abc}
	300	4.0 ± 0.4^{ab}	97 ± 9^{bc}	99.84	55 ± 2^{b}	88 ± 7^{b}	120 ± 13^{abc}

Values expressed as means ± SD; n = 3. In each column, different letters mean significant differences ($p < 0.05$). Source: own authorship.

The bulk density of beeswax particles varied from 48 to 73 mg/mL, while the bulk density of edible oil-loaded beeswax particles varied from 80 to 145 mg/mL (Table 2). This indicated that the edible oil-loading increased the bulk density. It was observed that using supercritical CO_2 (60 °C) at 300 bar, the Brazil nut oil-loaded beeswax particles had a reduced bulk density when compared to those formed with Brazil nut oil at lower CO_2 pressures (Table 3). Therefore, the CO_2 pressure and the edible oil must be taken into account for particle design with a reduced bulk density. This characteristic is desirable in solid lipid particles to create healthy food products (with reduced calories) because implies that a fixed volume of product can be occupied by a minimal amount of lipid materials (GUDEMAN; YAN; CIFTCI, 2019).

Table 2 - Bulk density of the particles

Particles	Pressure (bar)	Bulk density (mg/mL)
Beeswax	150	73 ± 1^{e}
	200	60 ± 2^{f}
	250	48 ± 1^{h}
	300	54 ± 2^{g}
Brazil nut oil-loaded beeswax	150	145 ± 2^{a}
	200	105 ± 3^{c}
	250	125 ± 2^{b}
	300	85 ± 4^{d}

Values expressed as means ± SD; n = 5. Different letters mean significant differences ($p<0.05$). Source: own authorship.

The CO_2 pressure had a negative and significant correlation with the size and bulk density of beeswax particles and Brazil-nut oil-loaded beeswax particles (Table 3). These results suggested that the higher CO_2 pressures could lead to the reduction of the size and

bulk density of the Brazil-nut oil-loaded beeswax particles, and vice-versa, which could be useful for the process design of these particles.

Table 3 - Correlation analysis of CO_2 pressure vs size and bulk density of the particles

Particles	Size (μm)*		Bulk density (mg/mL)	
	r	p	r	p
Beeswax	-0.72	0.01	-0.83	0.00
Brazil nut oil-loaded beeswax	-0.75	0.01	-0.79	0.00

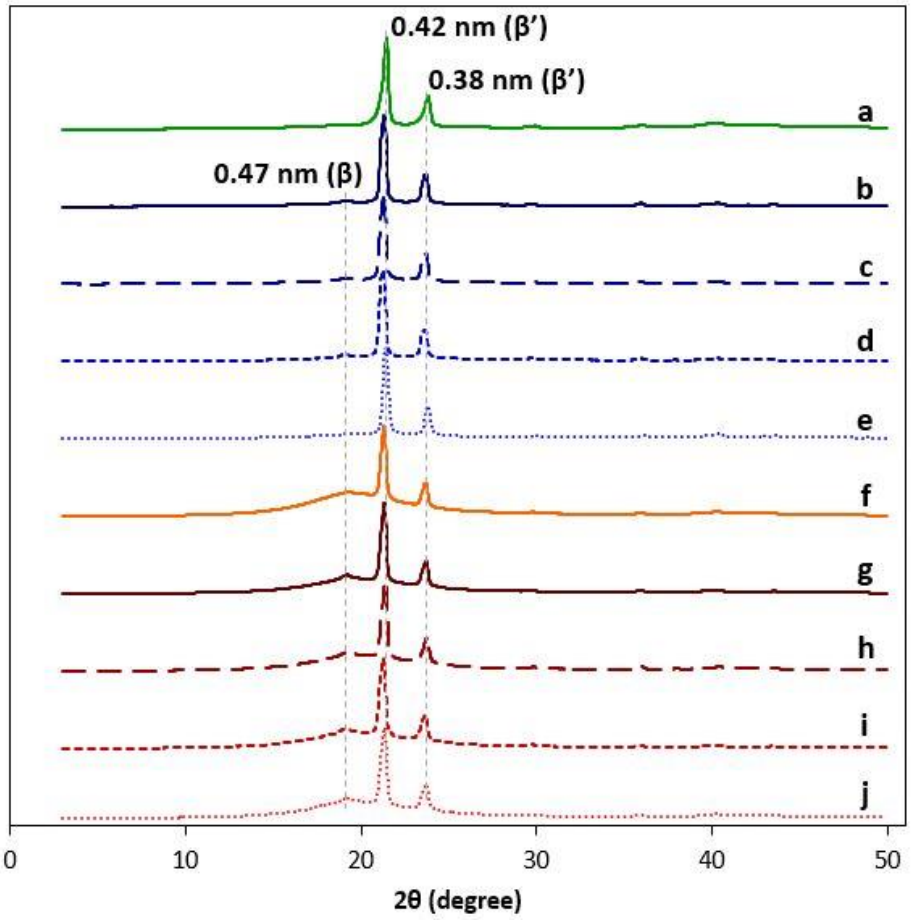
r: correlation coefficient, p: p value. *50 percentile values were used in the analysis. Source: own authorship

On the basis that beeswax was the solid lipid used as carrier material, it could be expected that the trends on size and bulk density observed in beeswax particles formed with supercritical CO_2 at different pressures remain in the edible-oil loaded beeswax particles, such as observed for the Brazil nut oil-loaded particles. However, other edible oil-loaded beeswax particles could exhibit different trends than expected. It is not possible to suggest an expected behaviour for edible oil-loaded beeswax particles, so individual tests are needed for each edible oil of interest.

3.3. Polymorphism and thermal properties

The X-ray difractogram of beeswax (Figure 4) showed two main interplanar distances (d-spacing) of 0.42 nm and 0.38 nm, which are expected for this material and are indicators of a predominance of β' polymorphism (ATTAMA; SCHICKE; MÜLLER-GOYMANN, 2006; ATTAMA; SCHICKE; MÜLLER-GOYMANN, 2007; SOLEIMANIAN et al., 2018) and typical of an orthorhombic subcell (JENNING; THÜNEMANN; GOHLA, 2000). The beeswax particles formed with supercritical CO_2 (60 °C) at CO_2 pressures of 150−300 bar did not evidence alterations of the crystallinity caused by the CO_2 pressure; however, in addition to the β' polymorphism, a trace of β polymorphism (0.47 nm) was noticed in these samples. The β polymorphism (0.47 nm) was poorly defined in the physical mixtures of beeswax with Brazil nut oil, while it was present in the edible oil-loaded beeswax particles formed with supercritical CO_2 (60 °C) at CO_2 pressures of 150−300 bar, which in turn did not present perceptible alteration caused by the CO_2 pressure. These results suggest that the particle formation process enhanced the β polymorphism of the beeswax and must be taken into account in stability studies of these particles.

Figure 4 - Crystalline patterns: (a) beeswax; beeswax particles formed with supercritical CO_2 (60 °C) at pressures of (b) 150 bar, (c) 200 bar, (d) 250 bar and (e) 300 bar; (f) physical mixture of beeswax and Brazil nut oil; Brazil nut oil-loaded beeswax particles formed with supercritical CO_2 (60 °C) at pressures of (g) 150 bar, (h) 200 bar, (i) 250 bar (j) 300 bar.

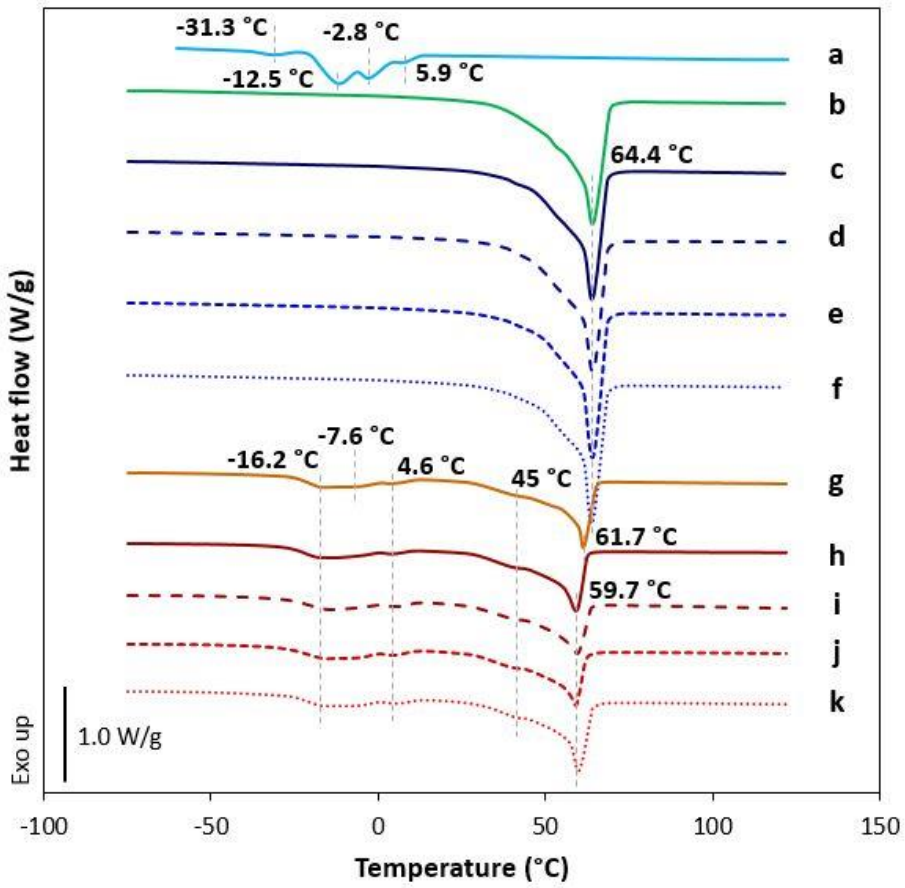


Source: own authorship.

The thermal profile of Brazil nut oil (Figure 5) was composed by a weak peak observed at -31.3 °C and a broad peak comprised of three peaks at -12.5 °C, -2.8 °C and 5.9 °C. The thermal profile of beeswax revealed that the melting temperature (Tm) of this material was 64.4 °C (Figure 5), which, as expected, remained unchanged in the beeswax particles formed with supercritical CO_2. The analysis of the thermal profile of the physical mixture of Brazil nut oil and beeswax (1:1 w/w) (Figure 5) revealed that: 1) the thermal peaks from Brazil nut oil changed to a broad peak that showed two weak peaks at -16.2 °C and 4.6 °C, 2) the Tm was reduced to 61.7 °C and, 3) the appearance of a weak shoulder at 45 °C. Thus, it can be suggested that the mixing may alter the individual thermal properties of the solid lipid and liquid lipid. The thermal profile of Brazil nut oil-loaded beeswax particles formed with supercritical CO_2 (Figure 5) was not altered by the used CO_2 pressure; however, when

compared to the thermal profile of the physical mixture, they showed a reduction in Tm to 59.5–60.2 °C. These results can be related to the changes in crystallinity caused by the particle formation process with supercritical CO_2.

Figure 5 - Thermal curves: (a) Brazil nut oil, (b) beeswax; beeswax particles formed with supercritical CO_2 (60 °C) at pressures of (c) 150 bar, (d) 200 bar, (e) 250 bar and (f) 300 bar; (g) physical mixture of beeswax and Brazil nut oil; Brazil nut oil-loaded beeswax particles formed with supercritical CO_2 (60 °C) at pressures of (h) 150 bar, (i) 200 bar, (j) 250 bar (k) 300 bar

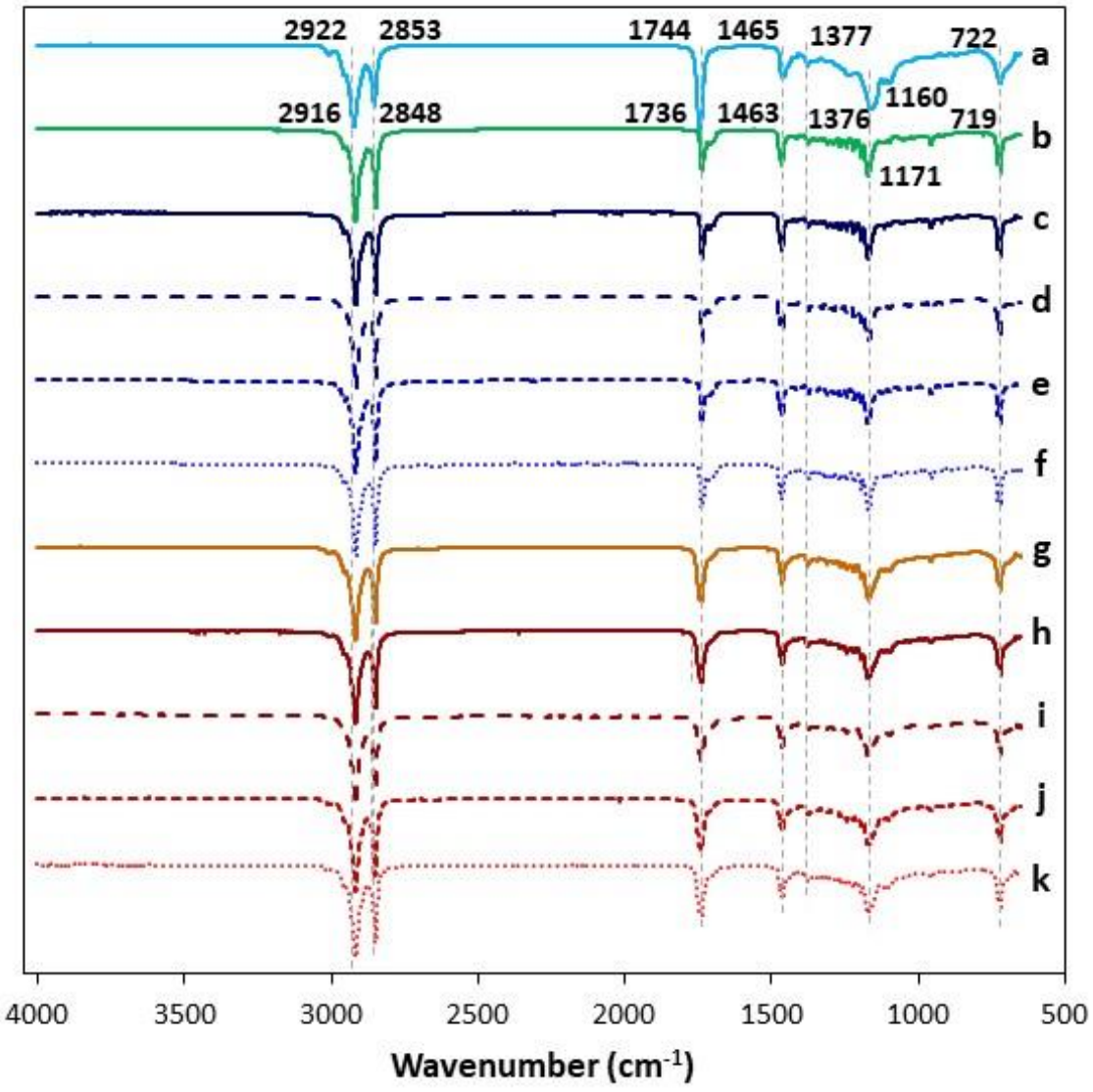


Source: own authorship.

3.4. Chemical structures

The main spectral peaks of Brazil nut oil (Figure 6) were similar to those expected in edible oils. Very strong peaks were observed near to 2924 cm^{-1}, 2853 cm^{-1} and 1746 cm^{-1}, which were attributable to the asymmetric stretching of –C–H (CH_2), symmetric stretching of –C–H (CH_2) and stretching of –C=H (ester), respectively (GUILLEN; CABO, 1997) [33]. Moreover, medium or strong peaks closer to 1465 cm^{-1}, 1377 cm^{-1}, 1163 cm^{-1} and 723 cm^{-1} were observed. These were attributable to the bending (scissoring) of single –C–H (CH_2, CH_3), symmetric bending of –C–H (CH_3), stretching of –C–O or bending of –CH_2– and bending (rocking) of –$(CH_2)_n$– (GUILLEN; CABO, 1997). The main spectral peaks of beeswax (Figure 6) were in agreement to the reported spectra for this material in another study (RIBEIRO et al., 2017). The positioning of main spectral peaks of beeswax was similar to that observed in Brazil nut oil, so it can be inferred that the same functional group vibrations of the edible oil were present in this lipid material. The beeswax spectra were not altered after processing with supercritical CO_2 (60 °C) at pressure of 300 bar (Figure 6). The spectra of the physical mixture of beeswax and Brazil nut oil (Figure 6) evidenced overlapping in the spectral peaks of their constituents and were identical to the spectra of edible oil-loaded beeswax particles formed with supercritical CO_2 (60 °C) at different pressures (Figure 6). Thus, it can be affirmed that the physical mixing and supercritical process did not chemically alter the beeswax and the edible oils, and that the chemical composition of the edible oil-loaded beeswax particles is identical to their respective initial physical mixture. It could imply that the process reaches a mixing or impregnation efficiency of edible oil in beeswax close to 100 %. Nonetheless, this parameter must be quantitatively determined by an analytical method, the development of which is complex work due to the difficult of separating edible oil and beeswax. Both materials are not pure substances, they are mixtures of different lipid molecules and the supercritical process leads to a stable and homogenous mixture of them. In this way, the mixture of edible oil and beeswax could be considered a “new material” since it is expected to have intermediate physical properties to both materials.

Figure 6 - Fourier transform infrared spectra: (a) Brazil nut oil, (b) beeswax; beeswax particles formed with supercritical CO_2 (60 °C) at pressures of (c) 150 bar, (d) 200 bar, (e) 250 bar and (f) 300 bar; (g) physical mixture of beeswax and Brazil nut oil; Brazil nut oil-loaded beeswax particles formed with supercritical CO_2 (60 °C) at pressures of (h) 150 bar, (i) 200 bar, (j) 250 bar (k) 300 bar.



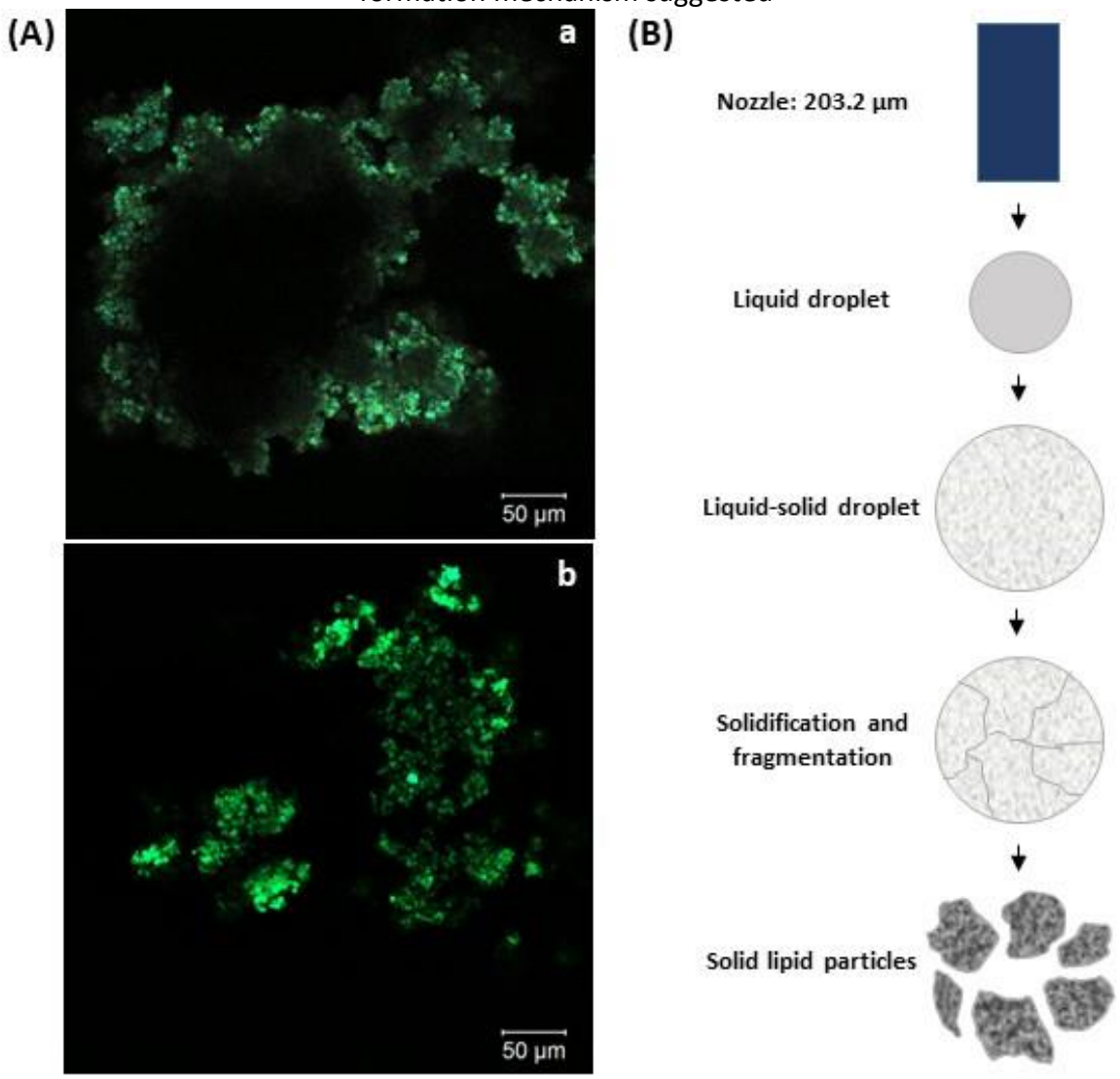
Source: own authorship.

3.5. Internal physical structure and mechanism of formation

The Brazil nut oil-loaded beeswax particles had green fluorescence emission (Figure 7Aa) with a maximum intensity at 513 nm that is difficult to attribute to some pigment compounds due to this edible oil is sensory perceived as very light-coloured. Curcumin (Sigma-Aldrich, Germany) was added to the Brazil nut oil-loaded beeswax particles to enhance their green fluorescence emission, seeking a better evaluation of the internal physical structure.

Axial plane images of the particles revealed that the internal physical structure (Figure 7A) was in accordance with the sponge-like morphology observed by scanning electron microscopy (Figure 2). Moreover, it could be noted that the fluorescence emission in an axial plane was relatively uniform for Brazil nut oil-loaded beeswax particles (coloured with curcumin) (Figure 7Ab). This is evidence of uniform distribution of the edible oils into the crystalline lattice of the beeswax.

Figure 7 - A) internal physical structure of the particles: (a) Brazil nut oil-loaded beeswax formed with supercritical CO_2 at 300 bar and 60 °C, and (b) Brazil nut oil-loaded beeswax formed with supercritical CO_2 at 300 bar and 60 °C coloured with curcumin; B) Particle formation mechanism suggested



Source: own authorship.

A mechanism of formation of edible-loaded beeswax particles was proposed (Figure 7B) based upon the results and mechanisms for formation of solid lipid particles proposed by Münüklü and Jansens (2007), Lubary et al. (2011) and Yang and Ciftci (2016). These studies explain the most prevalent morphologies (solid sphere, hollow sphere, and sponge-like) that can be expected in the formation of solid lipid particles by supercritical processing. In the present mechanism after the time of contact with CO_2 in an autoclave under agitation, the lipid binary mixture saturated with CO_2 is atomised through a nozzle. When these liquid lipid droplets rich in CO_2 are formed, they suffer instantaneous alterations due to expansion caused by CO_2 evaporation in atmospheric pressure and solidification of lipids caused by heat loss. As the PGSS process was performed at high pressures (which increase the CO_2 concentration in the molten lipid mixture) and at a temperature closer to the minimum Tm of the binary lipid mixture in pressurised CO_2, the lipid solidification started before all dissolved CO_2 evaporates (MÜNÜKLÜ; JANSENS, 2007; LUBARY et al., 2011). These lead to a random fragmentation of the expanded and solidified droplets enhanced by the high crystallinity of beeswax (Figure 4) that offers low permeability and low flexibility to the CO_2 evaporation. Finally, these alterations confer the irregular and sponge-like morphology observed in the particles (Figure 2, Figure 7A).

Edible oil-loaded beeswax particles with solid-sphere and hollow-sphere morphologies could be accomplished in further studies with a PGSS process at relatively low pressures and high temperatures (> 60 °C). These conditions reduce the CO_2 concentration in the molten lipid mixture allowing controlled evaporation of CO_2 as the lipids solidify (MÜNÜKLÜ; JANSENS, 2007; LUBARY et al., 2011).

4. Conclusions

Beeswax particles and edible oil-loaded beeswax particles were formed with supercritical CO_2 (60 °C) at pressures of 150−300 bar and resembled sponge-like microstructures. The particles did not have perceptible differences in their morphology caused by CO_2 pressure. Properties like size and bulk density could be affected inversely by pressure; however, this trend must to be verified for each liquid lipid that will be mixed with beeswax. The thermal properties, polymorphism, and chemical structure of the particles were not affected by the CO_2 pressure. Properties like morphology, polymorphism, thermal peaks,

chemical structures and physical internal structure suggest uniform incorporation of edible oil into the crystalline lattice of beeswax.

These results could be used for the design of beeswax particles loaded with other lipophilic compounds by PGSS to be used in the formulation of innovative and functional food products.

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CHAPTER V: Turmeric extract-loaded structured lipid carriers by Particles from Gas-Saturated Solutions

(No submitted)

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Abstract

The formation of turmeric extract-loaded structured lipid carriers (TE-SLCs) by Particles from Gas-Saturated Solutions (PGSS) process was studied. The carrier materials were beeswax as solid lipid and Brazil nut oil as liquid lipid. The mixtures of interest were beeswax: Brazil nut oil: turmeric extract ratios of 4:3:1, 2:1:1, and 4:1:3 (w/w/w). The PGSS process consisted of contacting the ternary mixture with supercritical CO_2 in an autoclave at pressure of 300 bar at a temperature of 60 °C with agitation at 1250 rpm for 1 h and subsequent expansion of the solution through a nozzle of 203.2 µm. The resulting TE-SLCs had a sponge-like morphology and mode sizes of 82-196 µm. The internal physical structure, polymorphism and thermal properties suggested uniform incorporation of amorphous turmeric extract mixed with Brazil nut oil into the crystalline lattice of beeswax. The TE-SLCs could be used as functional pigment for food products.

Keywords: Beeswax; Brazil nut oil; Natural pigment; Particle formation; Supercritical CO_2.

1. Introduction

Turmeric rhizome (Curcuma longa L) is rich in curcumin (diferuloylmethane), a polyphenolic compound (JIANG et al. 2021). Nowadays, turmeric extract and curcumin are included in many products (MUNEKATA et al., 2021; RADULY et al., 2021; RAFIEE et al., 2019) because curcumin is useful as a yellow pigment and has a wide spectrum of biological activities as antioxidant, anti-inflammatory, immunomodulatory, anticarcinogenic, antitumor, antidiabetic, antibacterial, and neuroprotective (FAROOQUI; FAROOQUI, 2019; ZIELIŃSKA et al., 2020; MEMARZIA et al., 2021; SADEGHIAN et al., 2020). However, the use of this pigment is not easy as it is limited by its hydrophobicity and sensitivity to light and oxygen that affect its bioavailability and biological efficacy (RASHWAN et al., 2022).

The formation of lipid particles loaded with turmeric extract is suggested as a strategy to facilitate the versatile inclusion of the curcumin in products (RAFIEE et al., 2019; RASHWAN

et al., 2022). There is wide investigation on the production of turmeric extract/curcumin-loaded lipid particles by conventional technologies such as microemulsion and high shear homogenisation with good results in terms of preserving bioactivity and bioavailability with demonstrated health benefits (DIZAJ et al., 2022; RAFIEE et al., 2019). Regarding non-conventional technologies, Particles from Gas-Saturated Solutions (PGSS) stand out for their low thermal impact and the use of supercritical CO_2 as safe medium to produce high quality lipid particles. This technology allowed to form lipid particles loaded with curcumin using tristearin (PEDRO et al., 2016) and polyethyleneglycol (PERKO et al., 2015) as carrier materials.

Despite the availability of studies on lipid particles loaded with turmeric extract /curcumin, it is important to test safe and low-cost carrier materials to develop particles with different characteristics that allow different applications. An alternative carrier material with high compatibility with the PGSS process is a mixture of beeswax and Brazil nut oil that constitute lipid particles called structured lipid carriers (AREDO et al., 2021). This carrier system is more advantageous than solid lipid particles due to its high loading capacity (ASHKAR et al., 2022).

Thus, this study aimed to study the feasibility of formation of turmeric extract-loaded structured lipid carriers (TE-SLCs) using PGSS process and mixtures of beeswax and Brazil nut oil as carrier material.

2. Material and methods

2.1. Material

CO_2 (99%) (Linde, Sertãozinho, SP, Brazil) was the supercritical fluid for PGSS process. Beeswax from a farm located in Pirassununga (São Paulo, Brazil) was used as solid lipid material. Brazil nut oil extracted from commercial Brazil nuts (Bertholletia excelsa H.B.K.) from Pará (Brazil) was used as liquid lipid material. The extraction of Brazil nut oil was performed with supercritical CO_2 at 70 °C and 350 bar. The extraction process and characteristics of the Brazil nut oil were studied by Cornelio-Santiago (2019). Turmeric roots from São Paulo (São Paulo, Brazil) were dried in a forced-air oven (Marconi, MA 035, Brazil) at 40 °C until constant weight, then ground in a knife mill (Marconi, MA 340, Brazil). The supercritical CO_2 extraction was performed at 300 bar pressure and 70 °C with CO_2 flow rate of 10 g/min and a flow rate

of 15 g/min of ethanol (AppliChemPanreac, Darmstadt, Germany) as co-solvent. The extraction process and characteristics of the turmeric extract composed of hydrophobic and/or oily substances were studied by Carvalho et al. (2020).

2.2. Particle formation

A 1:1 (w/w) ratio of solid lipid (beeswax): liquid material (Brazil nut oil and turmeric extract) was used for the particle formation based on the proportions studied in other works (ATTAMA et al. 2006; AREDO et al 2021). This with the intention of avoiding particle melting at room temperature (25 °C) when there is more liquid material than beeswax in the formulation. In this sense, formulations of mixtures with 4:3:1, 2:1:1, and 4:1:3 (w/w/w) ratios of beeswax: Brazil nut oil: turmeric extract were of interest. The PGSS process was carried out using an SFC/RESS (Thar Instruments Co., Ltd., Pittsburgh, PA, USA), which was previously described by Machado et al. (2016). The process consisted of contacting the mixture with supercritical CO_2 (300 bar and 60 °C) in autoclave (133.5 cm^3) with agitation at 1250 rpm. After 1 hour, the CO_2-rich mixture passed through a nozzle (203.2 μm) and precipitated into a particle chamber. The particles were stored under refrigeration (4 °C) in Petri dishes covered with aluminium foil (AREDO et al 2021).

2.3. Particle characterisation

The characterisation of the TE-SLCs was carried out according to the methodologies followed by Aredo et al (2021). Briefly, the morphology was studied by using a scanning electron microscope (TM 3000, Hitachi, Tokyo, Japan) at acceleration voltage of 5 kV. The size distribution was determined using a laser light diffraction equipment (Shimadzu Sald-201V, Tokyo, Japan) and ethanol:water (vol/vol) solution as suspension medium. The polymorphism was examined by X-ray diffraction (Miniflex 600, Rigaku, Japan). Thermal properties were investigated using a differential scanning calorimeter (DSC-TA2010, Thermal Analysis Instruments, New Castle, DE, USA). Internal physical structure was analysed by a confocal florescence microscope (LSM 780, Zeiss, Heidelberg, Germany) in spectral images at 400-700 nm. In addition, instrumental colour (L*, a*, and b* parameters) of TE-SLCs was determined using a colorimeter (Mini Scan XE; Hunterlab, Reston, VA, EUA).

2.4. Statistical analysis

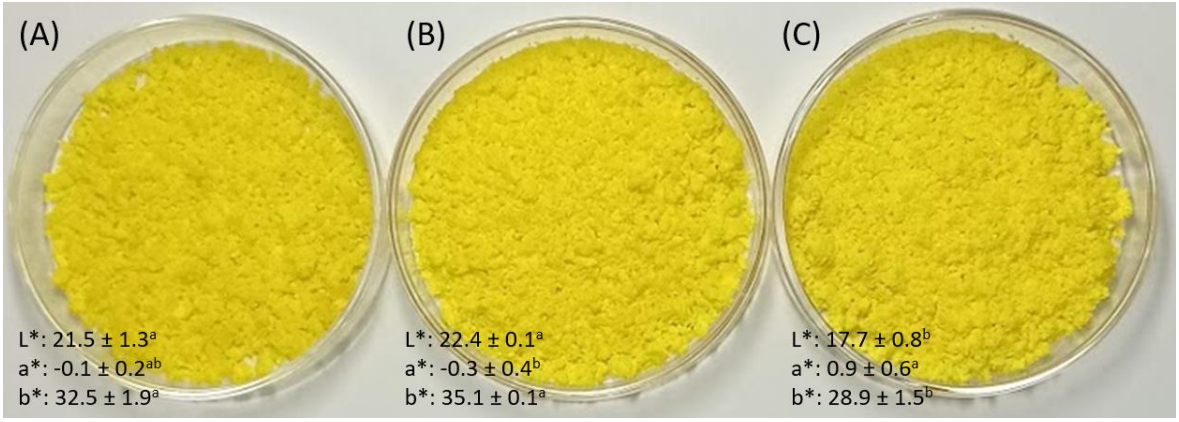
An analysis of variance of means was performed to determine if there were significant differences. Tukey's test ($p < 0.05$) was used to compare means. This analysis was performed in Statistica software (version 12.0, StatSoft, USA).

3. Results and discussion

3.1. Macroscopic characteristics of the particles

The TE-SLCs were free-flowing and yellowish powders with minimal macroscopic differences between formulations (Figure 1). Based on instrumental colour measurements, TE-SLCs formulated with 4:1:3 beeswax: Brazil nut oil: turmeric extract showed slight reduction in L* parameter, increase in a* parameter, and reduction in parameter b* compared to the other formulations, which is an effect of the high presence of turmeric extract in this formulation (Figure 1).

Figure 1 - Physical appearance of turmeric extract-loaded structured lipid carriers formulated with different beeswax: Brazil nut oil: turmeric extract ratios (w/w/w): (A) 4:3:1, (B) 2:1:1, and (C) 4:1:3



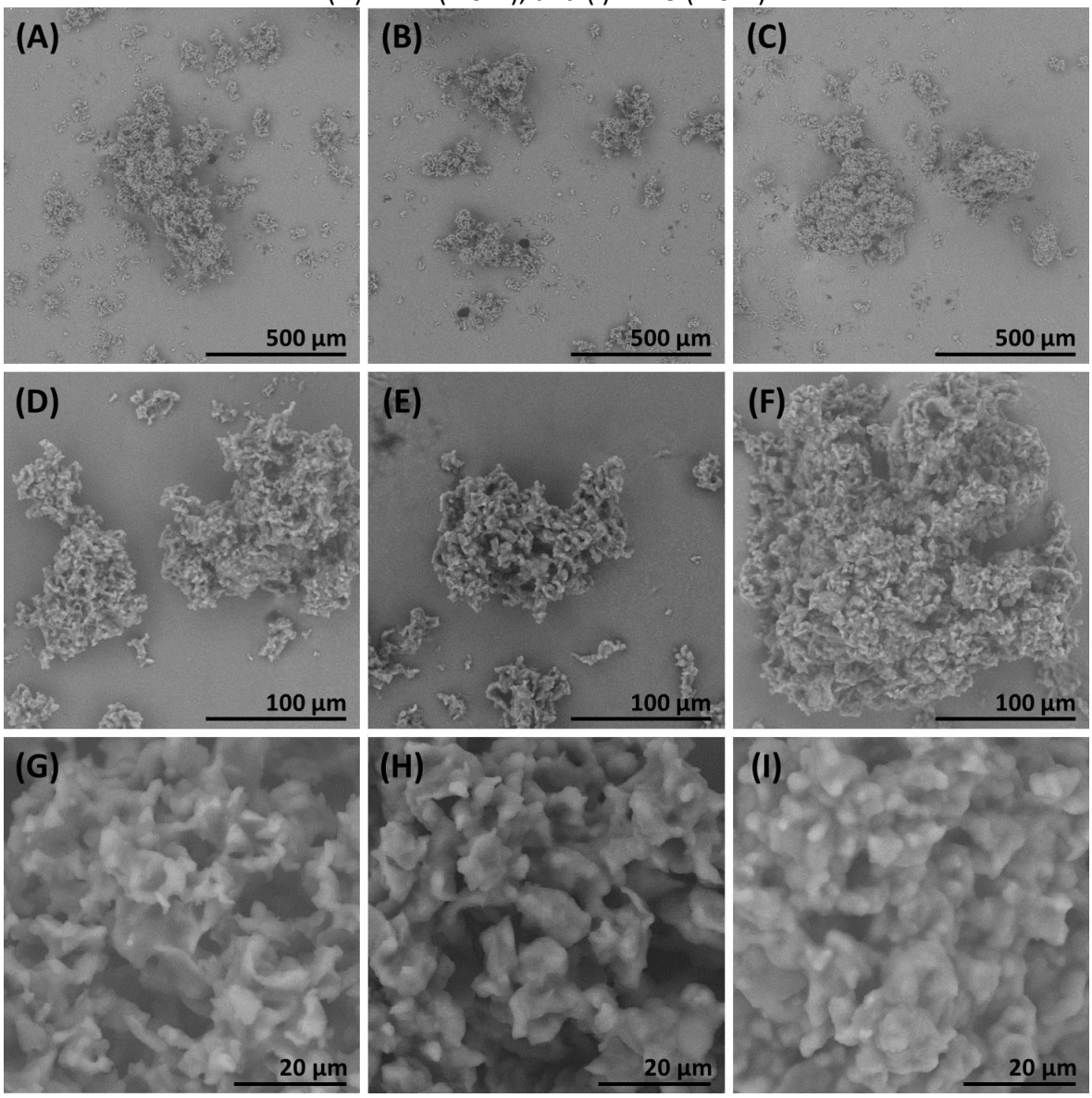
L*, a*, and b* are parameters of instrumental colour (CieLab) expressed as mean ± standard deviation, n=3. In each colour parameter, different letters mean significant differences ($p<0.05$). Source: own authorship.

3.2. Morphology and size of the particles

The TE-SLCs were sponge-like microstructures with irregular boundaries (Figure 2A-F) and free fragments (Fig 2A-C) with no major morphological differences between formulations. The observation of the surface of TE-SLCs revealed that the microstructures were composed of merged lobules, which appeared more regular when more turmeric extract was used in the formulation (Figure 2G-I). This suggest that morphology of the lobules was dependent on the formulation.

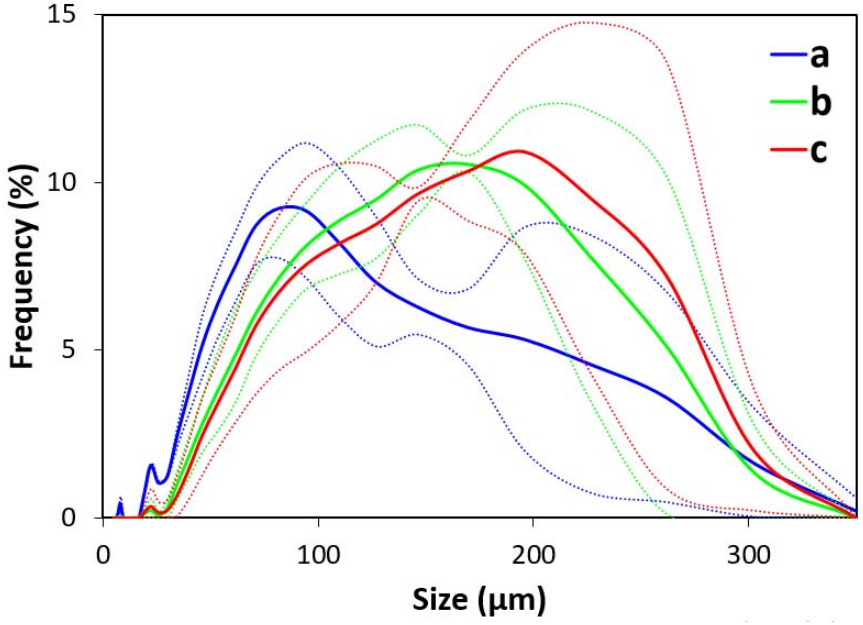
The size distributions of the TE-SLCs had a wide range (approximately 5-350 µm) and appeared to be relatively unimodal and overlapped between treatments (Figure 3). These characteristics were consistent with observations by scanning electron microscopy (Figure 2A, 2B and 2C). The mode as a descriptor of the TE-SLCs size distributions was considered for comparison. The mode size values were 82, 170, and 196 µm for TE-SLCs formulated with proportions of beeswax: Brazil nut oil: turmeric extract of 4:3:1, 2:1:1, and 4:1:3, respectively, suggesting that a greater presence of turmeric extract in the formulations increased the size of the TE-SLCs.

Figure 2 - Morphology of turmeric extract-loaded structured lipid carriers formulated with different beeswax: Brazil nut oil: turmeric extract ratios (w/w/w): (A) 4:3:1 (100x), (B) 2:1:1 (100x), (C) 4:1:3 (100x), (D) 4:3:1 (500x), (E) 2:1:1 (500x), (F) 4:1:3 (500x), (G) 4:3:1 (2.0kx), (H) 2:1:1 (2.0kx), and (I) 4:1:3 (2.0kx)



Source: own authorship.

Figure 3 - Size distribution of turmeric extract-loaded structured lipid carrier formulated with beeswax: Brazil nut oil: turmeric extract ratio (w/w/w) of (a) 4:3:1, (b) 2:1:1, and (c) 4:1:3



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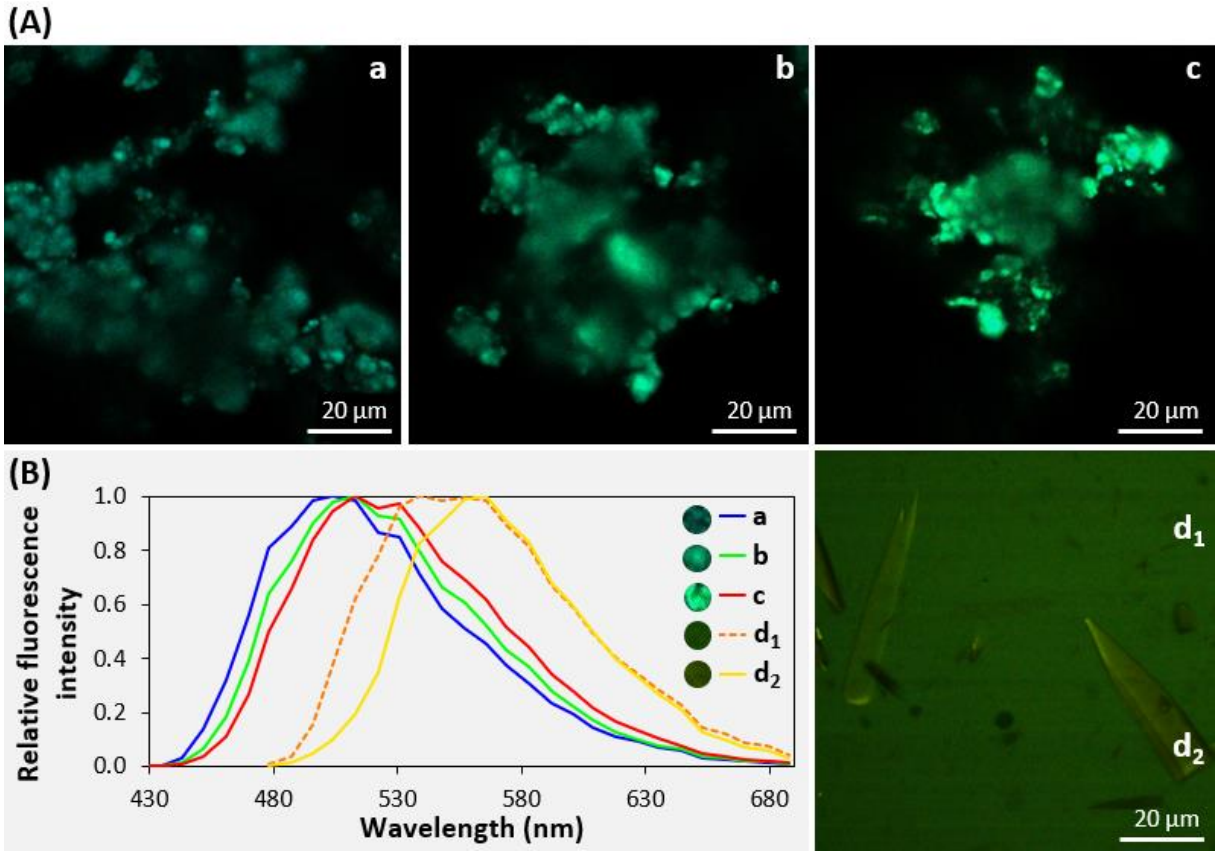
3.3. Internal physical structure

Axial plane images of the TE-SLCs allowed the analysis of internal physical structure (Figure 4Aa-c). TE-SLCs were observed to verify internal appearance of sponge-like microstructures composed of elliptical lobules as seen on surface (Figure 2). The fluorescence emission in each axial plane was relatively similar throughout the internal physical structure of the TE-SLCs (Figure 4Aa-c), suggesting uniform loading of turmeric extract. The turmeric extract had microcrystals and amorphous fractions (Figure 4Ad). In the absence of evidence of microcrystals in the TE-SLCs (Figure 4Aa-c) and detection of a crystalline residue in the autoclave after particle formation (results not shown), it can be inferred that the amorphous fraction of turmeric extract was actually incorporated. It supported by the analysis of fluorescence emission spectra (Figure 4B), which showed that when more turmeric extract was used in the TE-SLCs formulation, their spectra appeared relatively more similar to the amorphous fraction of pure turmeric extract.

Figure 4 - Internal physical structure (A) and fluorescence emission spectra (B) of turmeric extract-loaded structured lipid carrier formulated with beeswax: Brazil nut oil: turmeric extract ratio (w/w/w) of (a) 4:3:1, (b) 2:1:1, and (c) 4:1:3; (d_1) amorphous and (d_2) crystalised compounds from turmeric extract



Source: own authorship.

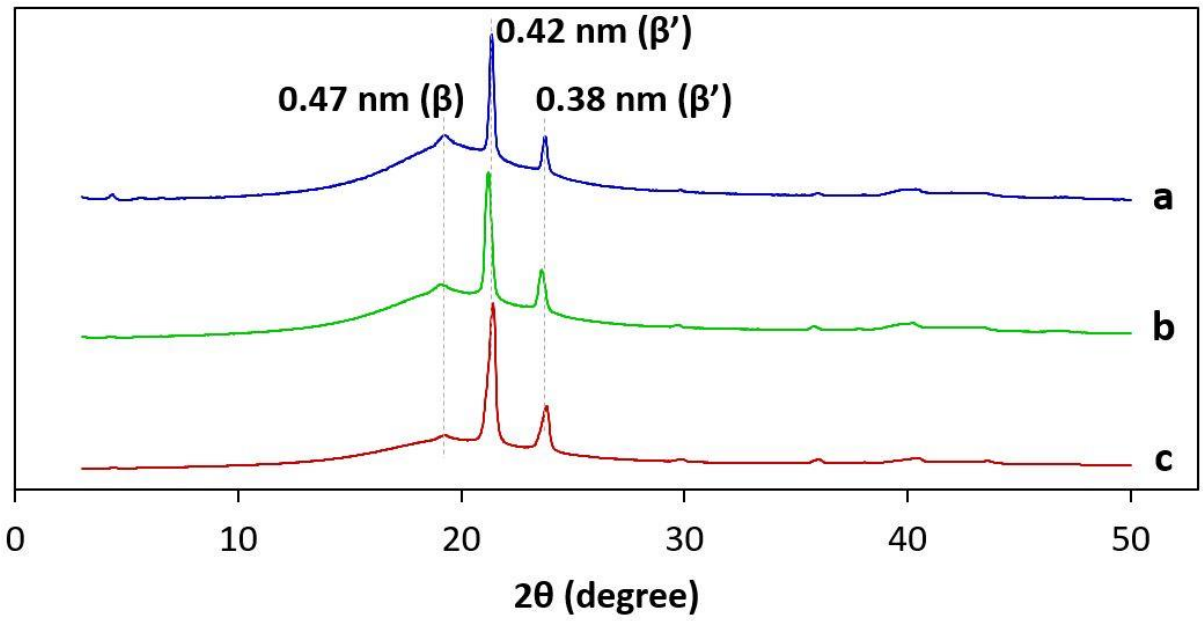
3.4. Polymorphism and thermal properties

The X-ray difractograms of TE-SLCs (Figure 5) exhibited two predominant peaks corresponding to interplanar distances (d-spacing) of 0.42 nm and 0.38 nm, which are indicators of β′ polymorphism of beeswax (SOLEIMANIAN et al., 2018). A trace of β polymorphism (0.47 nm) was observed, which could be caused by the presence of Brazil nut oil in the formulation and the effect of the supercritical treatment (AREDO et al., 2021). This based on the peaks intensity, where the β′ polymorphism appeared sharper and the β polymorphism appeared reduced when more turmeric extract was present in the TE-SLCs formulation.

The crystalline patterns were consistent with the thermal properties of the TE-SLCs (Figure 6) because the general reduction of the temperatures values of the thermal peaks was observed when more turmeric extract was present in the formulation of the TE-SLCs.

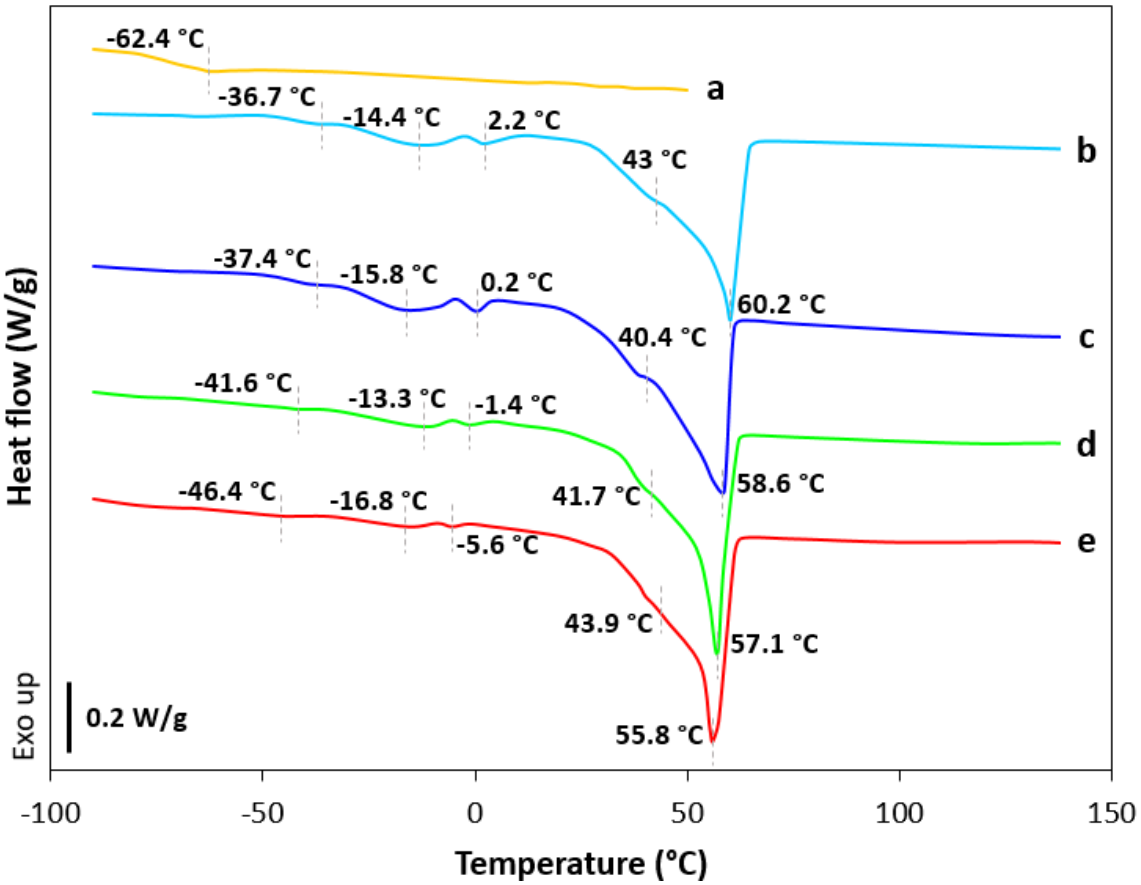
Based on the studied characteristics of the TE-SLCs, it can be stated that as more turmeric extract was used in the formulation, more amorphous turmeric extract mixed with Brazil nut oil was uniformly incorporated into the beeswax crystal lattice.

Figure 5 - Crystalline patterns of turmeric extract-loaded structured lipid carrier formulated with beeswax: Brazil nut oil: turmeric extract ratio (w/w/w) of (a) 4:3:1, (b) 2:1:1, and (c) 4:1:3



Source: own authorship.

Figure 6 - Thermal curves of (a) turmeric extract, (b) physical mixture formulated with beeswax: Brazil nut oil: turmeric extract ratio (w/w/w) of 4:3:1; turmeric extract-loaded structured lipid carrier formulated with beeswax: Brazil nut oil: turmeric extract ratio (w/w/w) of (c) 4:3:1, (d) 2:1:1, and (e) 4:1:3



Source: own authorship.

4. Conclusions

TE-SLCs formulated with 1:1 (w/w) ratio of solid lipid (beeswax): liquid material (Brazil nut oil/curcumin) were produced by PGSS using supercritical CO_2 at 300 bar and 60 °C. The TE-SLCs were yellowish powders with morphology of sponge-like microstructures composed of merged lobules. Characteristics of TE-SLCs such as internal physical structure, polymorphism and thermal properties evidenced that the use of more turmeric extract in the formulation led to more amorphous turmeric extract uniformly incorporated into the beeswax crystalline lattice.

The TE-SLCs could have potential as an alternative carrier system to facilitate incorporation of turmeric extract/curcumin in food, cosmetic and pharmaceutical products.

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CHAPTER VI: Hydrolysed collagen as carrier material for particle formation via supercritical CO_2 impregnation

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CHAPTER VI: Hydrolysed collagen as carrier material for particle formation via supercritical CO_2 impregnation

Abstract

This work investigated hydrolysed collagen (HC) powder as carrier material for particle formation via supercritical CO_2 impregnation. HC solubility and volumetric expansion in supercritical CO_2 (ρ: 605.8-834.8 g/L) were measured. HC had constant solubility (0.5-0.6 g/kg CO_2) and no volumetric expansion was visibly observed. Since HC showed low interaction with supercritical CO_2 under different conditions and preserved its powder appearance, it is suggested to be used as carrier of lipophilic bioactive compounds in particle formation via supercritical CO_2 impregnation at moderate temperature (40-50°C).

Keywords: Bioactive compounds, Carbon dioxide, Encapsulation, Powdered food, Wall material.

1. Introduction

Currently, the consumption of natural compounds with biological activity, also known as bioactive compounds, is highly recommended to preserve human health due to their antioxidant, antimicrobial, anticancer, and/or anti-inflammatory effects. However, the intake requirement of specific bioactive compounds cannot always be met with traditional diet (DIMA et al., 2020; SOTO; GONZÁLEZ; GALMARINI, 2021). In this sense, delivery systems for bioactive compounds is a technological alternative to facilitate their consumption as powdered health supplements or their use as powdered ingredients to enrich food products (DIMA et al., 2020; YE; GEORGES; SELOMULYA, 2018). Furthermore, it is known that particle formation can reduce the degradation of bioactive compounds because the carrier material, generally biopolymers and their derivatives, offers a solid structure that acts as a physical barrier to light and oxygen (RAY; RAYCHAUDHURI; CHAKRABORTY, 2016; SAMPATHKUMAR; TAN; LOO, 2020).

A carrier material for particle formation must meet certain requirements, such as safety, commercial availability, low cost and compatibility with the technological process and the

bioactive compound of interest (RAY; RAYCHAUDHURI; CHAKRABORTY, 2016; YE; GEORGES; SELOMULYA, 2018). In addition, it is desirable that the carrier material have some bioactivity to design particles with high bioactivity spectrum (TARONE et al., 2021). Therefore, different biomaterials have been tested to achieve these purposes.

Hydrolysed collagen (HC) is a biomaterial consisting of a mixture of peptides with low molecular weight that can be produced by enzymatic hydrolysis of natural collagen (LEÓN-LÓPEZ et al., 2019; MIRANDA; WEIMER; ROSSI, 2021). Peptides in HC are biologically active and provide health benefits such as prevention of skin aging (MIRANDA; WEIMER; ROSSI, 2021) and osteoporosis (DANEAULT et al., 2017), which has led to the application of HC as biomaterial, initially in the preparation of bioactive films (PEI et al., 2013). Regarding bioactive particles/capsules, HC powder was proposed as carrier material for lycopene dispersed in sunflower oil (LSO). This carrier system can be called HC particles loaded with LSO (HC-LSO) and was formed via supercritical CO_2 impregnation process at 150/250 bar and 50 °C, and HC/LSO ratio of 10:1 (w/w) (AREDO et al., 2019).

In this context, behavioural analysis of HC in supercritical CO_2 to determine its solubility and volumetric expansion could show how they interact and allows the understanding of the application of HC as carrier material (YASUJI; TAKEUCHI; KAWASHIMA, 2008; VILLEGAS et al., 2020; WEIDNER; STEINER; KNEZ, 2020). This is in the context of increasing attention to particle formation via supercritical CO_2 impregnation due to advantages such as oxygen-free atmosphere, moderate processing temperatures, absence of water to suspend or mix materials, and the adjustment of solvent properties of supercritical CO_2 by changes in pressure and temperature (KLETTENHAMMER et al., 2020; SANTOS et al., 2020; GAÑAN et al., 2020).

Thus, this work was conducted to evaluate the application of HC powder as carrier material in particle formation via supercritical CO_2 impregnation by evaluating its solubility and volumetric expansion in supercritical CO_2.

2. Material and methods

2.1. Chemicals

HC powder (Peptinex®, Gelnex™, Itá, SC, Brazil) was used as carrier material. CO2 (99%) (Linde, Sertãozinho, SP, Brazil) was the supercritical fluid. Anhydrous ethanol (99.9%) (AppliChemPanreac, Darmstadt, Germany) was used as co-solvent.

2.2. Behaviour of the carrier material in supercritical CO_2

The behavioural analysis consisted of determining the HC solubility in supercritical CO_2 and volumetric expansion in supercritical CO_2 as an indirect observation of solubility of supercritical CO_2 in HC.

The supercritical CO_2 conditions of interest for the behavioural analysis were those studied in the formation of HC-LSO such as 150 bar and 50 °C, 150 bar and 60 °C, 250 bar and 50 °C, and 250 bar and 60 °C (AREDO et al., 2019) and less severe conditions such as 140 bar and 50 °C, 120 bar and 45 °C, and 100 bar and 40 °C (NEROME et al., 2013).

2.2.1. Solubility in supercritical CO_2

HC solubility in supercritical CO_2 (g of HC/kg of CO_2) was measured using method and equipment described by Villegas et al. (2020). Briefly, 5 g of HC mixed with glass beads (4 mm) were placed in an equilibrium cell (300 mL) and contacted with supercritical CO_2 under fixed conditions of pressure and temperature. Clean glass beads and HC mixed with glass beads were inserted at the ends and centre of equilibrium cell, respectively. It is to reduce the possibility of undissolved material being pushed into the collector vessel due to pressure differential. It is worth mentioning that the glass beds reduced the available volume of equilibrium cell to approximately 120 mL. After a fixed contact time, a sample of the solution (HC plus supercritical CO_2) was shifted to collector of known volume (8.58 cm^3). The collector was depressurised and washed with water and ethanol to transfer HC to a flask. Finally, the flask content was dried to determine the HC mass by gravimetry. HC solubility in supercritical CO_2 was calculated as follows: mass of HC/(volume of the collector*CO_2 density). The mixture density was considered as the of CO_2 density, since the solvent is in greater quantity in the mixture.

Measurements were carried out in two sets: 1) HC solubility in supercritical CO_2 was measured at 140 bar and 50 °C (intermediate condition) and at different contact times (30 min, 45 min, 60 min (VILLEGAS et al., 2020), and 120 min). It was performed to evaluate and define the contact time required to measure the HC solubility in supercritical CO_2. 2) using the defined contact time, HC solubility in supercritical CO_2 was measured at the other pressures and temperatures of interest. The solubility measurements were performed in duplicate.

2.2.2. Volumetric expansion in supercritical CO_2

HC volumetric expansion in supercritical CO_2 was studied in SFC/RESS equipment (Thar Instruments Co./Waters, Pittsburgh, PA, USA). This equipment was previously described by Machado et al. (2016) and was used as a phase monitor for this investigation because it has a view cell (autoclave with sapphire window), which allows the sample visualisation. For observations, about 0.2 g of HC were placed in clear glass tube. The tube was placed into the autoclave in position that allowed its observation using camera through the sapphire window. The sample volume was observed under each supercritical CO_2 condition of interest after the contact time defined in the HC solubility study. Observations of HC volumetric expansion in mixtures of supercritical CO_2 with ethanol (5% w/w) were performed to study the effect of this co-solvent on the process. For this, ethanol was added manually instants before closing the autoclave.

2.3. Statistical analysis

Analysis of variance of means was performed to determine the possible presence of significant differences. Then, the Tukey test ($p < 0.05$) was used to compare means. This analysis was carried out in Statistica software (version 12.0, StatSoft, USA).

3. Results and discussion

3.1. Solubility in supercritical CO_2

At 140 bar and 50 °C, HC solubility in supercritical CO_2 measured at different contact times (from 30 to 120 min) was 0.5-0.6 g / kg of CO_2 (Table 1) without significant differences, which suggests that the phase equilibrium could have been reached in contact time of less than 30 min. Based on these results, HC solubility measurements under other supercritical CO_2 conditions were carried out at contact time of 45 min to ensure phase equilibrium.

HC powder solubility in supercritical CO_2 at different densities (605.8 to 834.8 g/L) was 0.5-0.6 g/kg of CO_2 (Table 1) without significant differences, which results are not consistent with the knowledge about polymer solubility in supercritical CO_2, where it is expected that the higher the CO_2 density, the greater the polymer solubility (KHOSRAVI-DARANI et al., 2003).

Values are affected by intermolecular forces in operation between solvent-solvent, solvent-polymer segment, and polymer segment-segment pairs in the solution (RINDFLEISCH; DINOIA; MCHUGH, 1996). Therefore, the constant HC solubility in supercritical CO_2 indicate that there is a limited number of molecules of the material that effectively interacts with CO_2, which is logical because this material is highly hydrophilic (AREDO et al., 2019). The interaction that leads to solubilisation in supercritical CO_2 is expected when molecules are hydrophobic (VILLEGAS et al., 2020). Since collagen is a protein composed of polar and apolar amino acids (KNUPP; SQUIRE, 2001), it is possible that, as result of the hydrolysis process, HC may have a small number of molecules with predominant hydrophobic character (caused by apolar amino acids), which are solubilised in supercritical CO_2.

Table 1 - Solubility of hydrolysed collagen in supercritical CO_2

CO_2 condition	CO_2 density (g/L)	Contact time (min)	Solubility* (g/kg CO_2)
140 bar and 50 °C	673.6	30	0.6 ± 0.1
		45	0.6 ± 0.2
		60	0.5 ± 0.1
		120	0.5 ± 0.2
120 bar and 45 °C	659.1	45	0.5 ± 0.1
100 bar and 40 °C	629.3		0.6 ± 0.2
150 bar and 50 °C	701.1		0.6 ± 0.1
150 bar and 60 °C	605.8		0.6 ± 0.1
250 bar and 50 °C	834.8		0.5 ± 0.1
250 bar and 60 °C	787.0		0.6 ± 0.1

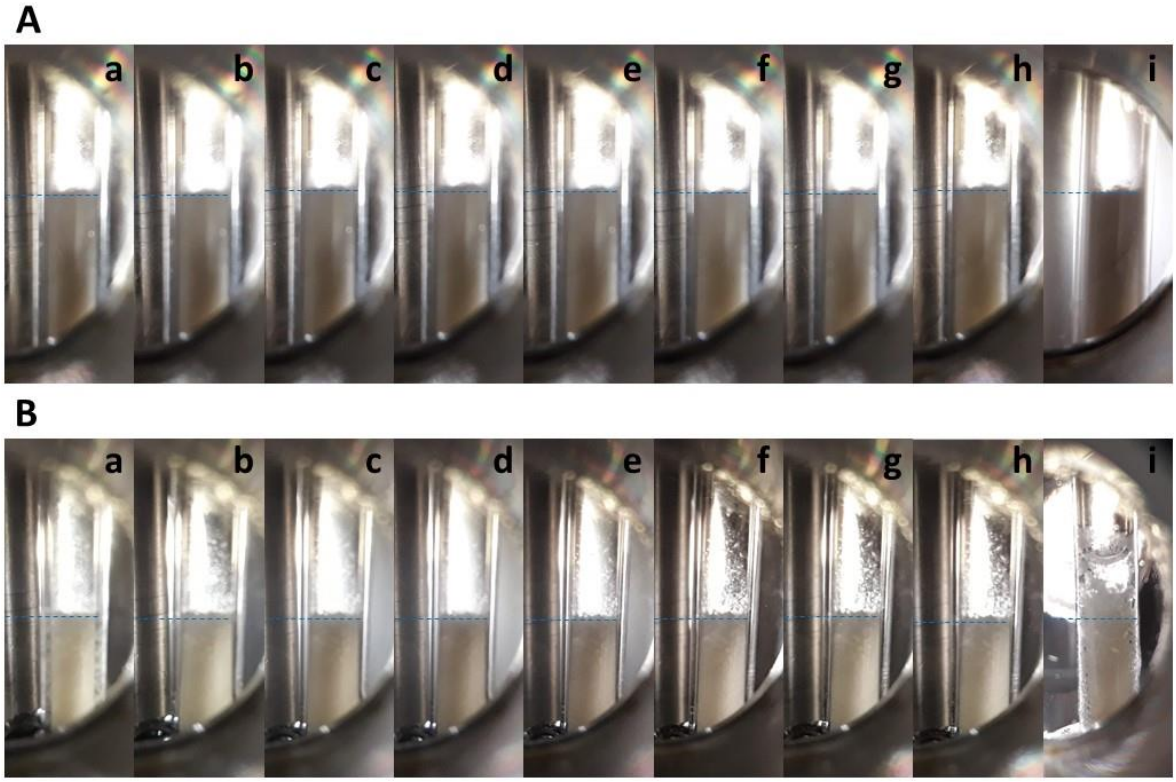
*No significant differences were observed ($p<0.05$). The measurements were performed in duplicate. Values expressed as mean ± standard deviation. Source: own authorship.

A quantitative comparison of solubility in supercritical CO_2 of HC with other biomaterials that have been studied with supercritical technology such as polyhydroxybutyrate, whose solubility varies from 0.47 g/kg of CO_2 (122 bar and 45 °C, ρ: 667 g/L) to 2.23 g/kg of CO_2 (243 bar and 55 °C, ρ: 805 g/L) (KHOSRAVI-DARANI et al., 2003) and polylacticacid, whose solubility varies from 0.56 (150 bar and 55 °C, ρ: 655 g/L) to 1.23 (250 bar and 55 °C, ρ: 811 g/L) (TOM; DEBENEDETTI, 1991) lead to affirm that HC solubility in supercritical CO_2 is similar to that of other biomaterials at the lowest density (605.8 g/L) but lower at the highest density (834.8 g/L).

3.2. Volumetric expansion in supercritical CO_2

The volumetric expansion is expected due to the solubilisation of supercritical CO_2 in polymers; however, HC volume did not visibly increase neither in pure supercritical CO_2 (Figure 1Aa-h) nor in supercritical CO_2+ ethanol (Figure 1Ba-h). Studies in starch powder yielded the same result (VILLEGAS et al., 2020; SANTOS et al., 2020; COMIN; TEMELLI; SALDAÑA, 2012). One explanation for this is that the material-CO_2 interaction is not high enough to be macroscopically evidenced by this method. An interesting behaviour was observed during the depressurisation of the supercritical CO_2+ethanol medium (Figure 1Bi), CO_2 bubbles passed through the condensed ethanol phase, which can be attributed to the CO_2 dissolved in ethanol at high pressure.

Figure 1 - Phase behaviour of the hydrolysed collagen powder in pure supercritical CO_2 (A) and supercritical CO_2 + ethanol (5% w/w) (B) at: (a) 1.01 bar and 25 °C, (b) 100 bar and 40 °C, (c) 120 bar and 45 °C, (d) 140 bar and 50 °C, (e) 150 bar and 50 °C, (f) 150 bar and 60 °C, (g) 250 bar and 50 °C, (h) 250 bar and 60 °C, and (i) during rapid depressurisation



Source: own authorship.

Despite the non-negligible evidence of material-CO_2 interaction measured as solubilisation of HC in supercritical CO_2 (Table 1), this was not perceived in the view cell as a volume reduction due to migration of free molecules from HC to the supercritical CO_2, or a change in the general appearance of HC (Figure 1). The view cell has changes in opacity under supercritical CO_2 conditions when compared to ambient conditions. These changes were slight and difficult to distinguish when the medium was pure supercritical CO_2 (Figure 1Aa-h). Nonetheless, opacity varied considerably depending on the supercritical CO_2 condition when ethanol was present (Figure 1Ba-h). These observations are only attributable to the medium and are not related to the material tested due to the high medium/material ratio (w/w).

Based on HC solubility and volumetric expansion results in supercritical CO_2, it can be inferred that: 1) HC has no potential as a carrier material for particle formation via rapid expansion of supercritical solutions-RESS process because it requires high solubilisation of the carrier material in supercritical CO_2 (DEBENEDETTI et al., 1993). 2) HC application in particles from gas saturated solutions-PGSS process is not feasible because it requires considerable CO_2 solubilisation in the carrier material, which generally occurs when the carrier material melts in the medium (WEIDNER; STEINER; KNEZ, 2020). 3) Behavioural studies of HC with other co-solvents could be performed to evaluate its possible use as a carrier material for particle formation via supercritical antisolvent (SAS) process, since investigation in protein-supercritical CO_2-co-solvent systems revealed promising results for this purpose (VARAEE et al. 2019; YEO et al. 1993). So, HC application as a carrier material for particle formation via supercritical micronisation/precipitation/expansion processes cannot be recommended without further research.

HC powder is comprised of porous particles with a uniform chemical composition and a predominantly polar character (AREDO et al., 2019). In this sense, it may be mentioned that the observed low interaction (constant solubility and no volumetric expansion) of the HC powder with supercritical CO_2 is result of the inherent properties of the material and is not caused by limited contact. These properties of HC powder are desirable for loading of bioactive compounds with slight or no alterations in the physical structure or morphology of the powder material via supercritical CO_2 impregnation (SANTOS et al., 2020). This process is feasible when the supercritical CO_2 solubilises the bioactive compound of interest and distributes it into the physical structure of the carrier material, for which, the bioactive compound must have more affinity for the carrier material than for the supercritical CO_2

(AREDO et al., 2019; SANTOS et al., 2020). Moreover, supercritical CO_2 impregnation is considered a less complex process compared to other previously mentioned supercritical processes (PERRUT; JUNG; LEBOEUF, 2005).

4. Conclusions

HC powder is a material that has low interaction with supercritical CO_2 since constant solubility and no volumetric expansion were observed at different conditions. In this sense, it has potential as carrier material for particle formation via supercritical CO_2 impregnation. However, it is not useful as carrier material for particle formation via supercritical micronisation/precipitation/expansion processes such as RESS, PGSS and SAS without further research that include the measurement of solubility of HC in supercritical CO_2 + co-solvents.

Further research is needed to determine the molecular properties of HC and explain how they influence its behaviour in supercritical CO_2. Moreover, studies are recommended to demonstrate the application of HC powder as carrier material for particle formation of different lipophilic and thermosensitive bioactive compounds by supercritical CO_2 impregnation under mild conditions.

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CHAPTER VII: Beeswax coating on blueberry extract-loaded hydrolysed collagen microparticles by supercritical CO_2 treatment

(No submitted)

CHAPTER VII: Beeswax coating on blueberry extract-loaded hydrolysed collagen microparticles by supercritical CO_2 treatment

Abstract

This study explored the beeswax coating on blueberry extract-loaded hydrolysed collagen microparticles using supercritical CO_2. Beeswax was mixed with blueberry extract-loaded hydrolysed collagen microparticles in autoclave with supercritical CO_2 (300 bar and 60 °C) under agitation at 1250 rpm for 1 hour. A simplex-centroid design was used to optimise the formulation by exploring the effect of beeswax (2-15%), blueberry extract (2-15%) and hydrolysed collagen (83-96%) on total anthocyanin content, colour variation, and degree of solubilisation in water. Models successfully explained the responses (R^2>75%, $p<0.05$) revealing that coated microparticles formulated with 6.3%, 10.7% and 83% of beeswax, blueberry extract and hydrolysed collagen, respectively, had high total anthocyanin content (0.42 mg cyanidin-3-glucoside/g), high colour variation (29.8), and reduced solubility in water (72.9%). These coated microparticles were purple-pigmented with slight agglomeration and evidenced effective incorporation of blueberry extract. Beeswax coating using supercritical CO_2 has potential for designing food ingredients.

Keywords: Anthocyanin; Carbon dioxide; Coated particles; Powdered ingredient; Vaccinium corymbosum L.

1. Introduction

Currently, the blueberry (Vaccinium corymbosum L.) is a highly appreciated fruit with an increase in its commercialisation related to its pleasant sensory characteristics and health-promoting or nutraceutical characteristics (YUAN; SUN, 2022). One of the main phytochemicals of interest in this fruit are anthocyanins for their biological activities as antioxidant and anti-inflammatory that prevent, for example, cardiovascular diseases. However, blueberry anthocyanins are still little used in food industry due to their instability and difficulty in bioabsorption (YANG et al. 2022; LIU et al., 2021).

One way to facilitate the use of anthocyanins is through the development of applications of blueberry anthocyanin-rich extracts following technological alternatives to improve their stability and bioavailability (CAI et al., 2021). In this context, the application of supercritical CO_2 technologies is suggested as a safety and low thermal impact alternative to produce microparticles loaded with plant extracts using a carrier material that acts as a physical barrier to potentially protect bioactive compounds from environmental conditions (MACHADO et al., 2022).

One supercritical CO_2 technology for particle formation is the supercritical fluid-based coating process, which involves contacting protein particles with a lipid material solubilised in supercritical CO_2 to create a lipid coating on the particles after depressurisation of the system (SANTOS et al., 2002). This technology was studied in other works (THIES et al., 2003, SANTOS et al., 2003a, SANTOS et al., 2003b) and suggested beeswax as a natural coating material.

Since beeswax is solubilised in supercritical CO_2 (AREDO et al., 2021). It could be useful for designing new powdered ingredients by coating protein-based particles loaded with plant extracts. In this sense, a protein-derived material compatible with the supercritical CO_2 process is hydrolysed collagen microparticles. It is interesting for its biological activity and technological advantage in preserving its porous physical structure for loading bioactive compounds (AREDO et al., 2019; AREDO et al., 2022).

Thus, the aim of this study was to explore the technical feasibility of beeswax coating on blueberry extract-loaded hydrolysed collagen microparticles using supercritical CO_2.

2. Material and methods

2.1. Materials

Beeswax from a local farm from Pirassununga – Sao Paulo (Brazil) was used as coating material. HC powder (Peptinex®, Gelnex™, Itá, SC, Brazil) was used as carrier material. Commercial Blueberry fruits (Vaccinium corymbosum L.) from Trujillo (Peru) in 125 g plastic “clam-shell” containers were the source of extract rich in anthocyanin of interest. CO_2 (99%) (Linde, Sertãozinho, SP, Brazil) was the supercritical fluid, and anhydrous ethanol (99.9%) (AppliChemPanreac, Darmstadt, Germany) was used as solvent for the blueberry extract.

2.2. Blueberry extract

Blueberry fruits (Vaccinium corymbosum L.) of the Biloxi variety were manually and visually selected considering their uniform blue colour, uniform firmness and absence of damage (ARTEAGA et al., 2022). A known quantity of sample (16.16 g) was then liquefied with ethanol. This mixture was vacuum filtered (400 mm Hg) through quantitative filter paper. The extract (25 mL) was concentrated on a rotary evaporator to a known concentration of solids. The extract was kept with ethanol because it is more practical for the process by helping in the distribution of the extract in the porous carrier material (hydrolysed collagen).

2.3. Particle formation

The process began with the manual mixing of blueberry extract and hydrolysed collagen microparticles with an average size of 77 µm (AREDO et al., 2019). Then, the mixture and the beeswax were put in contact with supercritical CO_2 (300 bar and 60 °C) in an autoclave with agitation at 1250 rpm for 1 hour to achieve high solubilisation of the coating material (AREDO et al., 2021). Finally, the system was slowly depressurised and the coated microparticles were collected from inside the autoclave. Particle formation was carried out in a SFC/RESS (Thar Instruments Co./Waters, Pittsburgh, PA, USA).

2.4. Particle characterisation

2.4.1. Total anthocyanin content

The pH differential method was adapted from Arteaga et al. (2022). 0.3 g of microparticles were weighed into a 50 mL becker, 10 mL of distilled water were added. The mixture was placed in a magnetic stirrer for 5 min with a 0.7 cm magnetic bar of (60% of speed at room temperature). The mixture was filtered in a 125 mL kitasato flask on C40 quantitative filter for 5 min without vacuum. The filtered liquid was placed in 15 mL falcon tube. Then, 0.6 mL of filtered liquid was added in four test tubes of 4 mL to be contacted with solutions of pH of 1 and 4.5. The test tubes were vortexed in darkness for 30 min. Absorbance were measured in a spectrophotometer UV-vis (Metash UV 5100, China) at 520 and 700 nm.

The total anthocyanin content (mg cyanidin-3-glucoside/g) was determined as follows: (calculated absorbance*molecular weight of cyanidin-3-glucoside (449.2 g/mol)*dilution factor (5)*volume of the solution (0.01 L)*1000)/(molar absorption coefficient of cyanidin-3-glucoside (26900 L/mol.cm)*cuvette optical path length (1 cm)*initial mass of the sample (g)). The calculated absorbance was: (absorbance measured at 520 nm in pH 1 - absorbance measured at 700 nm in pH 1) – (absorbance measured at 520 nm in pH 4.5 - absorbance measured at 700 nm in pH 4.5).

2.4.2. Degree of solubilisation in water (%)

Based on method of Santos et al. (2020). 0.3 g of microparticles were weighed in 50 mL falcon tubes and mixed with 20 mL of distilled water at room temperature, to avoid melting the beeswax, in a vortex for 10 seconds. The mixture was then vacuum filtered through filter paper. The filtered liquid was dried in Petri dishes until constant weigh. The degree of solubilisation was expressed as the percentage of the mass solubilised in water.

2.4.3. Instrumental colour and colour variation

Instrumental colour (L*, a*, and b* parameters) was determined using a colorimeter (Mini Scan XE; Hunterlab, Reston, VA, EUA). The colour variation-ΔE was used to measure how pigmented the carrier material was by the blueberry extract. It was calculated as the Euclidean distance between the instrumental colour of hydrolysed collagen microparticles and that of coated microparticles.

2.5. Statistical approach

A simplex-centroid design consisting of ten formulations was used to explore the effect of beeswax (2-15%), blueberry extract (2-15%) and hydrolysed collagen microparticles (83-96%) on total anthocyanin content (mg of cyanidin-3-glucoside/g), degree of solubilisation in water (%) and instrumental colour variation-ΔE (Table 1). The limits of the components of the formulations were based on the observation of a limited loading capacity of hydrolysed collagen microparticles (AREDO et al., 2019; AREDO et al., 2019) and preliminary tests that revealed that a percentage of 10% (w/w) can reduce significantly the degree of solubilisation of hydrolysed collagen microparticles in water.

The effects of individual components, binary and ternary interactions (p=0.05) were analysed with iterative removal of non-significant effects. An analysis of variance of the models was performed. Response surfaces were generated for models with R^2>70% and p<0.05 for graphical interpretation and optimisation. This analysis was performed in Statistica software (version 12.0, StatSoft, USA).

2.6. Particle characterisation of optimised microparticles

A formulation that optimises total anthocyanin content (mg of cyanidin-3-glucoside/g), degree of solubilisation in water (%) and instrumental colour variation-ΔE in beeswax coated blueberry extract loaded hydrolysed collagen microparticles was experimentally validated.

The optimised coated microparticles were further characterised according to methodologies followed by Aredo et al. (2019) and Aredo et al. (2021). Briefly, the morphology was studied using a scanning electron microscope (TM 3000, Hitachi, Kyoto, Japan) at accelerating voltage of 5 kV. Internal physical structure was analysed by a confocal florescence microscope (LSM 780, Zeiss, Heidelberg, Germany) in spectral images at 400-700 nm. Polymorphism was examined by X-ray diffraction (Miniflex 600, Rigaku, Japan) at angles from 3 to 50°. Thermal properties were investigated using a differential scanning calorimeter (DSC-TA2010, Thermal Analysis Instruments, New Castle, DE, USA) at temperatures from -100 °C to 150° C. Chemical structure was analysed with a Fourier transform infrared spectrometer (Spectrum One, Perkin Elmer, Norwalk, CO, USA) in the spectral range of 4000-650 cm^{-1}.

Table 1 - Total anthocyanin content, degree of solubilisation in water, instrumental colour parameters (L*, a* and b*) and colour variation (ΔE) in different formulation for beeswax coated blueberry-extract loaded hydrolysed collagen microparticles produced by supercritical CO_2 treatment at 300 bar and 60 °C

Mixture	Formulation			Responses					
	Beeswax (%)	Blueberry extract (%)	Hydrolysed collagen (%)	Total anthocyanin content (mg of cyanidin-3-glucoside/g)	Degree of solubilisation in water (%)	L*	a*	b*	ΔE **
1	2 (0)	2 (0)	96 (1)	0.08 ± 0.00	82.2 ± 1.3	72.03 ± 0.02	-0.42 ± 0.04	3.27 ± 0.05	13.2
2	15 (1)	2 (0)	83 (0)	0.07 ± 0.00	30.5 ± 2.2	69.40 ± 0.01	0.03 ± 0.01	2.51 ± 0.03	15.8
3	2 (0)	15 (1)	83 (0)	0.59 ± 0.02	77.6 ± 0.2	56.18 ± 0.03	0.79 ± 0.07	-0.17 ± 0.06	28.9
4	8.5 (1/2)	2 (0)	89.5 (1/2)	0.08 ± 0.00	33.0 ± 0.5	68.62 ± 0.00	0.03 ± 0.10	1.89 ± 0.03	16.8
5	2 (0)	8.5 (1/2)	89.5 (1/2)	0.33 ± 0.00	81.6 ± 1.6	61.12 ± 0.01	1.21 ± 0.02	-2.09 ± 0.09	25.3
6	8.5 (1/2)	8.5 (1/2)	83 (0)	0.35 ± 0.01	62.9 ± 2.8	56.89 ± 0.01	1.21 ± 0.06	-1.51 ± 0.05	28.8
7	6.33 (1/3)	6.33 (1/3)	87.34 (1/3)	0.27 ± 0.01	64.4 ± 2.5	58.45 ± 0.02	1.14 ± 0.04	-2.00 ± 0.06	27.6
8	4.16 (1/6)	4.16 (1/6)	91.68 (2/3)	0.17 ± 0.01	75.3 ± 1.7	64.26 ± 0.03	0.69 ± 0.03	-0.72 ± 0.03	21.9
9	10.67 (2/3)	4.16 (1/6)	85.17 (1/6)	0.15 ± 0.03	61.9 ± 2.9	63.49 ± 0.01	0.93 ± 0.04	-1.43 ± 0.13	23.0
10	4.16 (1/6)	10.67 (2/3)	85.17 (1/6)	0.43 ± 0.01	73.0 ± 1.6	54.18 ± 0.01	1.38 ± 0.01	-2.05 ± 0.02	31.5

** It was calculated using the instrumental colour of pure hydrolysed collagen microparticles as reference (L*: 82.92 ± 0.01, a*: -0.41 ± 0.05 and b*: 10.72 ± 0.06). Source: own authorship.

3. Results and discussion

3.1 Macroscopic appearance of coated microparticles

All the tested formulations produced microparticles (Figure 1). These beeswax coated blueberry extract-loaded hydrolysed collagen microparticles appeared purple-pigmented with rough texture (Figure 1a-k) compared to commercial hydrolysed collagen microparticles used as carrier material (Figure 1l). As expected, the purple pigmentation intensity was variable and dependent on the fraction of blueberry extract used in the formulation.

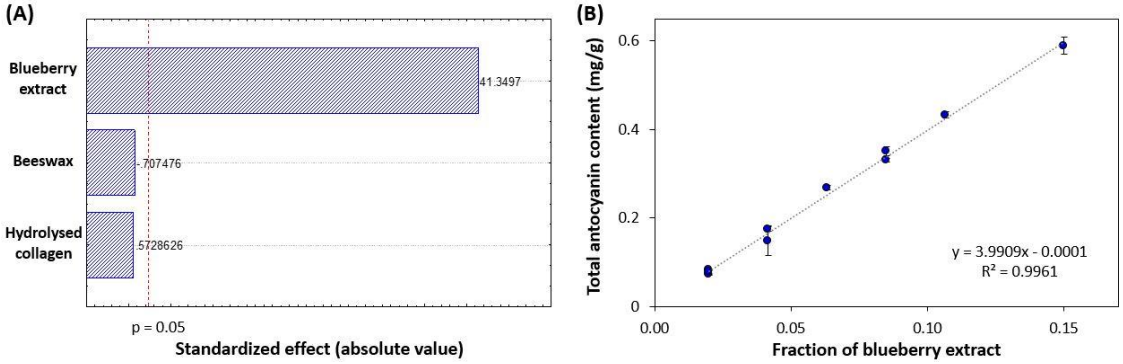
Figure 1 – Physical appearance of microparticles formulated with different percentages of beeswax, blueberry extract and hydrolysed collagen (%, %, %): (a-j) experimental runs, (k) optimised coated microparticles, and (l) pure hydrolysed collagen

Source: own authorship.

3.2. Total anthocyanin content and degree of solubilisation in water of the coated microparticles

The total anthocyanin content of the coated microparticles was affected by the fraction of blueberry extract used in the formulation (Figure 2A). This result is logical since hydrolysed collagen and beeswax are not sources of anthocyanin and suggest that these formulation components did not appear to have some effect on degradation or preservation of anthocyanin by supercritical CO_2 treatment. In this sense, a response surface analysis was not necessary. A linear relationship analysis (Figure 2B) confirmed the expected dependence between fraction of blueberry extract and total anthocyanin content.

Figure 2 - Statistical analysis of total anthocyanin content of coated microparticles: (A) analysis of the effect of mixture components, and (B) relationship with fraction of blueberry extract in the mixture

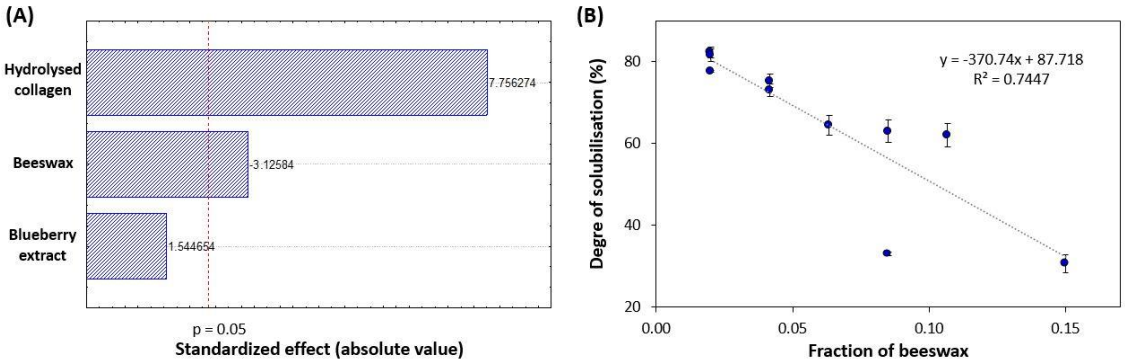


Source: own authorship.

The degree of solubilisation in water of the coated microparticles was mainly affected by the fraction of hydrolysed collagen in the formulation in a positive way, and negatively affected by the fraction of beeswax used in the formulation (Figure 3A). This result is explained by the total degree of solubilisation of hydrolysed collagen in water (AREDO et al., 2019) and the null solubilisation of beeswax in water (AREDO et al., 2021). The response surface analysis was disregarded, since an analysis of linear relationship between fraction of beeswax and degree of solubilisation (Figure 3B) allowed to detect a limited reduction of the degree of solubilisation in water to 61.9% in the coated microparticles when the fraction of blueberry extract is not minimal in the mixture (Table 1). In this sense, it can be stated that the use of more than 6.33% of beeswax in the formulation is not necessary to reduce the degree of solubilisation of the coated microparticles in water (Figure 3B). On the basis of this result, it

can be inferred that the beeswax coating was not uniform specially when blueberry extract is not minimal in the formulation affecting the adherence of beeswax to hydrolysed collagen microparticles used as carrier.

Figure 3 - Statistical analysis of degree of solubilisation in water (%) of coated microparticles: (A) analysis of the effect of mixture components, and (B) relationship with fraction of beeswax in the mixture



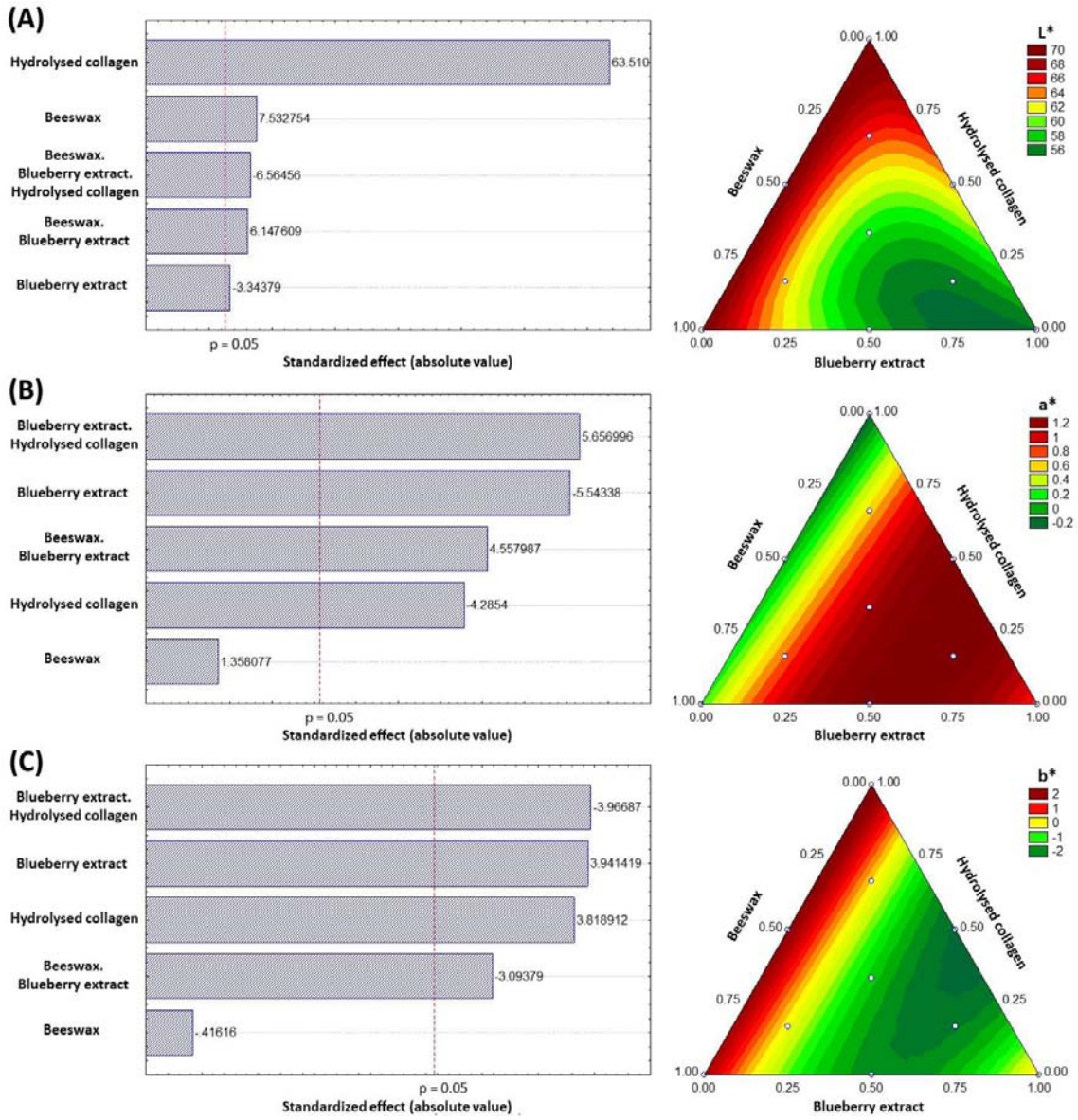
Source: own authorship.

3.3. Instrumental colour, colour variation and optimisation of the formulation

The analysis of effects in instrumental colour parameters (L*, a* and b*) revealed that the components of the mixture and their interactions can affect these values (Figure 4). In parameter L* (Figure 4A), it was noted that the three components had significant effect on this colour parameter and that the values are negatively influenced by the ternary interactions. In parameter a* (Figure 4B) and parameter b*(Figure 4C), it was observed that beeswax did not affect these parameters and that the significant binary interactions (blueberry extract x hydrolysed collagen and beeswax x blueberry extract) had similar but opposite effect to the hydrolysed collagen and blueberry extract.

It is worth mentioning that the construction of valid models (R^2>89%, $p<0.05$) and response surface analysis for instrumental colour parameters (L*, a* and b*) were carried out for predictive purposes and not for optimisation. In this sense, instrumental colour variation analysis is a way of capture information from this type of data when maximum pigmentation is requested.

Figure 4 – Statistical analysis (effects analysis and contour diagram) of instrumental colour parameters of coated microparticles: (A) L* parameter, (B) a* parameter and (C) b* parameter



The contour diagrams are representation of the following equations, where beeswax is BW, blueberry extract is BE, and hydrolysed collagen is HC.

$L^* = 76.8*BW - 34.1*BE + 76.4*HC + 24895.6*BW*BE - 31692.8*BW*BE*HC$ (R^2= 99.06, p=0.00)

$a^* = 3.339*BW - 207.437*BE - 1.319*HC + 208.818*BW*BE + 259.168*BE*HC$ (R^2= 95.52, p=0.00)

$b^* = -5.275*BW + 760.48*BE + 6.06*HC - 730.818*BW*BE -937.058*BE*HC$ (R^2= 89.24, p=0.01)

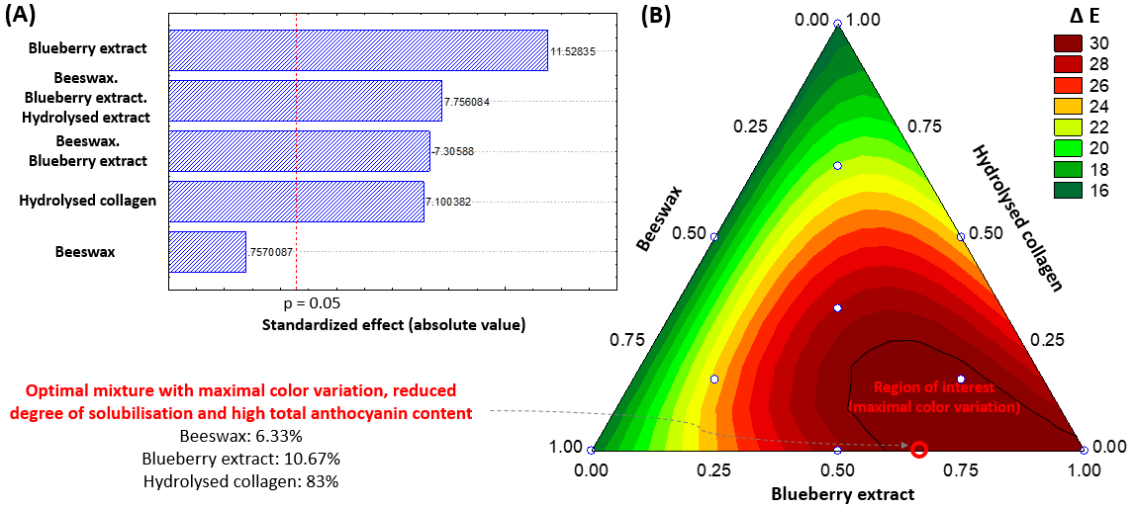
Source: own authorship.

Instrumental colour variation analysis (Figure 5) revealed that in terms of effects (Figure 5A) the blueberry extract, as effective contributor of pigments, was the most influential on the pigmentation of hydrolysed collagen microparticles. In addition, the positive and significant effect of the ternary interaction evidenced that pigmentation in the coated microparticles is complex and influenced by the components of the formulation. The analysis

of the model (R^2=99.09%, p<0.05) and its respective response surface graph (Figure 5B) allowed to identify a region of interest where there was the maximum instrumental colour variation as a characteristic of interest for potential consumers of beeswax coated blueberry extract-loaded hydrolysed collagen microparticles.

The optimisation goal was to maximise total anthocyanin content, minimise degree of solubilisation in water and maximise instrumental colour variation of beeswax coated blueberry extract-loaded hydrolysed collagen microparticles. However, it was not possible to find a region that met these requirements since each characteristic has a different behaviour (Fig 2B, 3B and 5B) and they cannot be graphically overlapped, which is the common step in optimisation using response surface analysis. In this sense, the region of interest of instrumental colour variation (Figure 5B) was taken as a reference to identify a formulation with desirable characteristics as a high total anthocyanin content and a low degree of solubilisation in water. Since the percentage limit beeswax needed of 6.33% (0.33 as coded value) to reduce the degree of solubilisation of the coated microparticles in water (Figure 3B) was in the region of interest (Figure 5B), the percentage of blueberry extract was fixed at 10.67% (0.67 as coded value) for a higher load of anthocyanin, while the percentage of hydrolysed collagen was 83% (0.33 as coded value) to complete the 100% of the formulation.

Figure 5 - Statistical analysis of instrumental colour variation (ΔE) of coated microparticles: (A) effects analysis and (B) contour diagram with definition of optimal formulation



The contour diagram is a representation of the following equation, where beeswax is BW, blueberry extract is BE, and hydrolysed collagen is HC.

$$\Delta E = 7.8*BW + 118.6*BE + 8.6*HC - 29852.5*BW*BE + 37782.3*BW*BE*HC \quad (R^2 = 99.09,\ p=0.00)$$

Source: own authorship.

3.4. Characteristics of the optimised coated microparticles

The characteristics of the optimised coated microparticles (Table 2) were predictable (maximum error 9.1%) using the respective model for each response (Figures 2-5). The notable difference between the observed and predicted values of the degree of solubilisation in water could indicate that there is an interference of the percentage of blueberry extract in the beeswax coating.

Table 2 – Experimental validation of the responses of interest in the optimised coated microparticles

Response	Observed	Predicted	Error (%)
Total anthocyanin content (mg of cyanidin-3-glucoside /g)	0.42 ± 0.01	0.40	1.3
Degree of solubilisation in water (%)	72.9 ± 0.6	66.3	9.1
L*	55.69 ± 0.02	55.05	1.1
a*	1.06 ± 0.02	1.31	0.3
b*	-1.39 ± 0.04	-1.93	0.5
Colour variation (%)	29.84	30.28	1.5

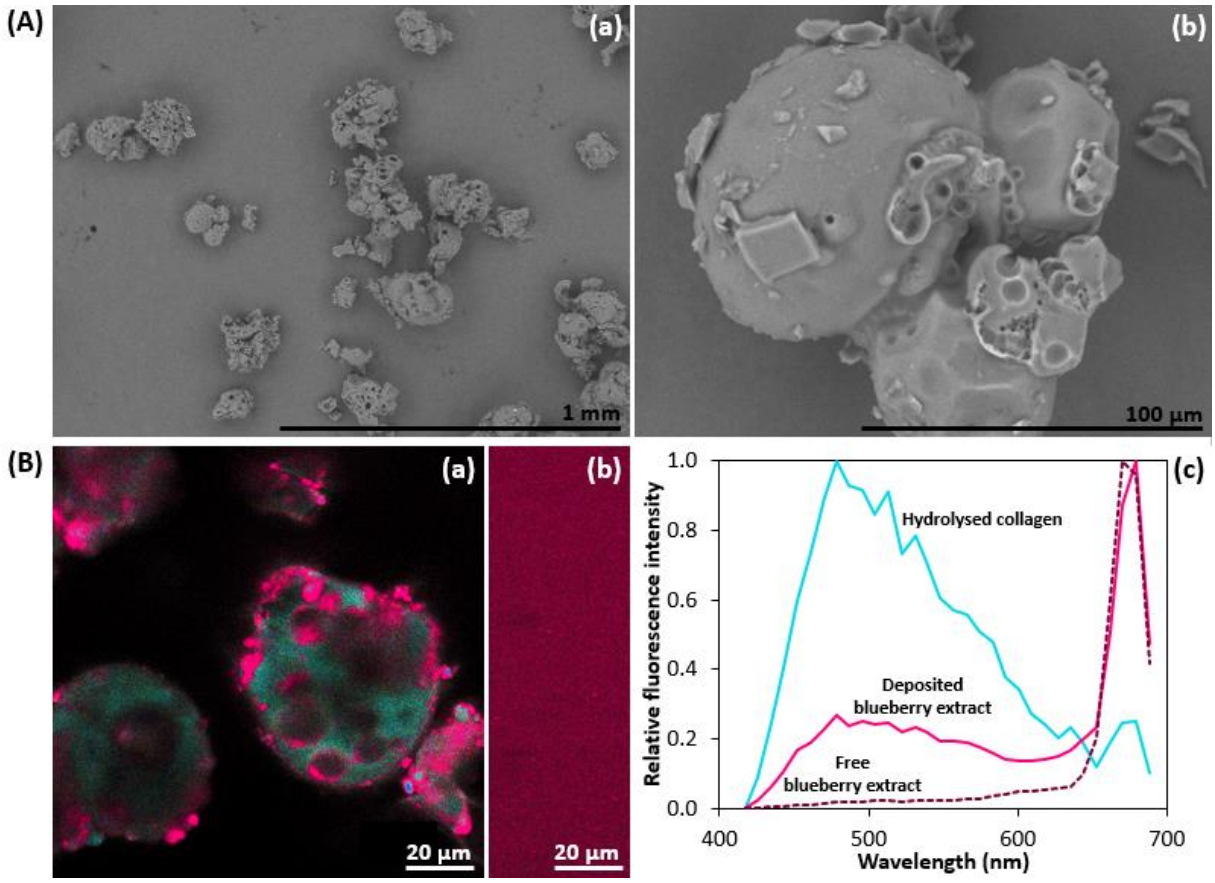
Source: own authorship.

Further characterisation revealed that optimised coated microparticles were slightly agglomerated (Figure 6Aa), which can be attributed to the beeswax used as coating material on microparticles surface. The agglomerates (Figure 6Ab) were composed of regular microparticles with fragments, which are typical of commercial hydrolysed collagen microparticles used as carrier material (AREDO et al., 2019). It could explain the sensory perception of rough texture in the formed microparticles (Figure 1a-k). The physical structure of hydrolysed collagen microparticles was preserved (Figure 6Ab) since were not observed fissures and morphological deformations, which is explained by the low interaction of the carrier material with supercritical CO_2 (AREDO et al., 2022). This characteristic implies that hydrolysed collagen microparticles is interesting for coating process because it allows predicting the morphology of the resultant particles.

The analysis of the internal physical structure through axial plane images (Figure 6Ba-b) and fluorescence emission spectra (Figure 6Bc) was based on the comparison between optimised coated microparticles and free blueberry extract. It was evidenced that there are regions of optimised coated microparticles with predominance of hydrolysed collagen spectra over blueberry extract spectra and vice versa. In this sense, the presence of blueberry extract

could be described as “deposits” in hydrolysed collagen microparticles. The blueberry extract deposits were found mainly in surface/external solid regions and some pores of the regular hydrolysed collagen microparticles, while in fragments these appeared to be contiguous. This observation suggests that the size and morphology of hydrolysed collagen microparticles affected the incorporation of the extract, which is logical considering that the loading process was manual and not enhanced by the supercritical process since blueberry extract is hydrophilic and is not soluble in supercritical CO_2.

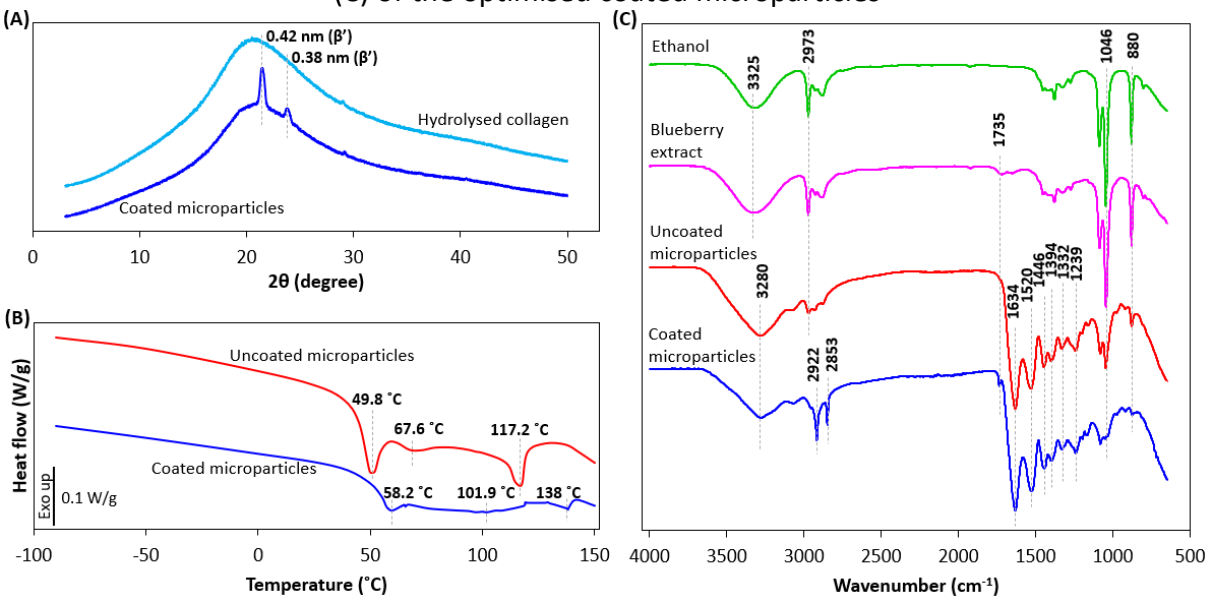
Figure 6 - (A) Morphology of optimised coated microparticles (a) at 100x and (b) at 1.0kx; (B) internal physical structure of (a) optimised coated microparticles and (b) free blueberry extract; and (c) fluorescence emission spectra



Source: own authorship.

The diffractogram of the optimised coated microparticles compared to that of hydrolysed collagen (Figure 7A) showed predominance of β′ polymorphism typical of beeswax (SOLEIMANIAN et al., 2018). Regarding the thermal properties (Figure 7B), the reduction of the depth of the peaks and changes in the position of the thermal peaks were observed, which can be attributed to the presence of highly distributed beeswax due to the treatment with supercritical CO_2. The analysis of chemical structure evidenced that the optimised coated microparticles had absence of ethanol functional groups from blueberry extract at 3325 cm^{-1}, 2973 cm^{-1}, 1046 cm^{-1}, 880 cm^{-1} (ALDIYAROV et al., 2009) and presence of functional groups of solid components such as blueberry extract anthocyanins at 1735 cm^{-1} (TAO et al. 2017), beeswax at 2922 cm^{-1} and 2853 cm^{-1} (AREDO et al., 2020), and hydrolysed collagen microparticles at 1634 cm^{-1}, 1520 cm^{-1}, 1446 cm^{-1}, 1394 cm^{-1}, 1332 cm^{-1} and 1239 cm^{-1} (AREDO et al., 2019), which suggests that there is no significant chemical alteration in the solid components caused by the process and that the ethanol was removed during depressurisation.

Figure 7 - Crystalline patterns (A), thermal curves (B), and Fourier transform infrared spectra (C) of the optimised coated microparticles



Source: own authorship.

4. Conclusions

Beeswax coated blueberry extract-loaded hydrolysed collagen microparticles were produced by supercritical CO_2 treatment at 300 bar and 60 °C. The microparticles appeared to be purpled pigmented hydrolysed collagen microparticles. This study recommends the production of coated microparticles formulated with 6.3%, 10.7% and 83% of beeswax, blueberry extract and hydrolysed collagen, respectively to achieve a high total anthocyanin content (0.42 mg of cyanidin-3-glucoside/g), reduced solubility in water (72.9%) and high colour variation (29.8).

The results suggest that beeswax coating using supercritical CO_2 could be applied as alternative process for design innovative beeswax coated microparticles for food products.

Acknowledgements

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CHAPTER VIII: General conclusions and suggestions for further studies

CHAPTER VIII: General conclusions and suggestions for further studies

General conclusions

The results and observations presented in the chapters of this Doctoral Thesis led to the following general conclusions:

- The solubility in supercritical CO_2 of beeswax, Brazil nut oil, and their mixture increased when pressure increased.
- Brazil nut oil-loaded beeswax microparticles formed by PGSS had sponge like morphology.
- Formation of turmeric extract-loaded microparticles by PGSS is technically feasible
- Hydrolysed collagen particles had low interaction with supercritical CO_2
- Beeswax covering on blueberry extract hydrolysed collagen microparticles reduce partially the solubility in water of the particles.

Suggestions for further studies

This thesis was mainly exploratory in nature and focused on demonstrating the technical feasibility of the proposed processes and the physical characterisation of the products. Indeed, there are different investigations that could be derived from this work, here are some suggestions for further studies:

- Determination of stability, digestibility, bioaccessibility and biological activity of the formed particles.
- Analysis of the incorporation of the formed particles into food, cosmetic and pharmaceutical products.
- Validation of process for other liquid lipids-beeswax mixtures as carrier materials and food extracts and drugs as core materials.
- Thermodynamic approach on the phase behaviour of carrier materials in supercritical CO_2.
- Estimation of the economic viability of the proposed processes.

Postgraduate Memory

Timeline

2015-2017: Master Degree in Food Engineering, FZEA/USP.
2018-2022: Doctorate Degree in Food Engineering, FZEA/USP.

Scientific articles published

1) **Victor Aredo**, Estela Selaro Passalacqua, Sebastião Pratavieira, Alessandra Lopes de Oliveira. Formation of lycopene-loaded hydrolysed collagen particles by supercritical impregnation. LWT 110 (2019) 158-167. doi.org/10.1016/j.lwt.2019.04.055.
2) Maria Eugenia Villegas, **Victor Aredo,** Kayque J. E. Asevedo, Rodrigo V. Lourenço, Reinaldo C. Bazito, Alessandra Lopes de Oliveira. Commercial Starch Behavior When Impregnated with Food Additives by Moderate Temperature Supercritical CO_2 Processing. Starch-Stärke (2020) 1900231. doi.org/10.1002/star.201900231.
3) Debora Nascimento e Santos, **Victor Aredo**, Reinaldo C. Bazito, Alessandra Lopes de Oliveira. Water free incorporation of shark liver oil into starch microparticles by supercritical CO_2 impregnation at low temperature. Journal of Food Process Engineering 43 (2020) e13541. doi.org/10.1111/jfpe.13541.
4) **Victor Aredo**, Gabriela Marques Bittencourt, Eliria M. J. A. Pallone, Francisco E. G. Guimarães, Alessandra Lopes de Oliveira. Formation of edible oil-loaded beeswax microparticles using PGSS-Particles from Gas-Saturated Solutions. The Journal of Supercritical Fluids 169 (2021) 105106. doi.org/10.1016/j.supflu.2020.105106.
5) **Victor Aredo**, Estela Selaro Passalacqua, Alessandra Lopes de Oliveira. Hydrolysed collagen as carrier material for particle formation via supercritical CO_2 impregnation. The Journal of Supercritical Fluids (2022) 105647. doi.org/10.1016/j.supflu.2022.105647.

Abstracts published in scientific events

1) **Victor Aredo**, Kayque Egg Asevedo, Heber P. C. Santiago, Maria E.V. Gomez, Alessandra Lopes de Oliveira. *Solubility of some biological polymers and oligomers in High Pressure CO_2*. Annals of *II Congreso Agroindustrial de Investigación Y Responsabilidad Social* (II CAIRS), Trujillo, Peru, 2016.
2) **Victor Aredo**, Estela Selaro Passalacqua, Alessandra Lopes de Oliveira. *Effect of supercritical CO_2 on the physical structure of commercial hydrolyzed collagen powder*. Annals of *XI Congreso Iberoamericano de Ingeniería de Alimentos* (XI CIBIA), Valparaíso, Chile, 2017.
3) Heber P. C. Santiago, Christiane E. C. Rodrigues, Nilson Ferreira, **Victor Aredo**, Alessandra Lopes de Oliveira. *Optimization of the pressurized liquid extraction of oil enriched with omega-6 and -9 from brazil nuts using the response surface analysis*. Annals of *XI Congreso Iberoamericano de Ingeniería de Alimentos* (XI CIBIA), Valparaíso, Chile, 2017.
4) **Victor Aredo**, Estela Selaro Passalacqua, Alessandra Lopes de Oliveira. *Supercritical impregnation of lycopene in commercial hydrolyzed collagen powder*. Annals of *12 Simpósio Latinoamericano de Ciência de Alimentos* (12 SLACA), Campinas, SP, Brazil, 2017.
5) **Victor Aredo**, Sebastião Pratavieira, Alessandra Lopes de Oliveira. *Incorporation of lycopene in hydrolyzed collagen powder by supercritical CO_2: a laser confocal microscopy study*. Annals of *12 Simpósio Latinoamericano de Ciência de Alimentos* (12 SLACA), Campinas, SP, Brazil, 2017.
6) **Victor Aredo**, Estela Selaro Passalacqua, Alessandra Lopes de Oliveira. *Particle formation of lycopene in hydrolyzed collagen powder using supercritical CO_2*. Annals of *31st EFFoST International Conference*, Stiges, Spain, 2017.
7) Estela Selaro Passalacqua, **Victor Aredo**, Alessandra Lopes de Oliveira. *Misturas para a formação de partículas de licopeno em colágeno hidrolisado usando CO_2 supercrítico*. Annals of *25 Simpósio Internacional de Iniciação Científica da Universidade de São Paulo* (25 SIICUSP), Pirassununga, SP, Brazil, 2017.

8) **Victor Aredo**, Gabriela Marques Bittencourt, Eliria M. J. A. Pallone, Alessandra Lopes de Oliveira. *Formation of Brazil nut oil-loaded beeswax particles using supercritical melt micronization*. Annals of *Iberoamerican Conference on Supercritical Fluids* (V PROSCIBA), Campinas, SP, Brazil, 2019.
9) **Victor Aredo**, Alessandra Lopes de Oliveira. *Solubility of beeswax in supercritical CO_2 at low temperature*. Annals of *Iberoamerican Conference on Supercritical Fluids* (V PROSCIBA), Campinas, SP, Brazil, 2019.
10) Gabriela Marques Bittencourt, **Victor Aredo**, Eliria M. J. A. Pallone, Alessandra Lopes de Oliveira. *Physical evaluation of beeswax particles with avocado oil (1:1) formed via RESS*. Annals of *Iberoamerican Conference on Supercritical Fluids* (V PROSCIBA), Campinas, SP, Brazil, 2019.
11) **Victor Aredo**, Alessandra Lopes de Oliveira. *Determination of specific size of supercritical CO_2 formed solid lipid microparticles by computer image analysis*. Annals of *13º Simpósio Latino Americano de Ciência de Alimentos* (13 SLACA), Campinas, SP, Brazil, 2019.
12) Geraldo, H. O. Soares, **Victor Aredo**, Alessandra Lopes de Oliveira. Particle formation of beeswax/brazil nut oil mixtures using supercritical CO_2 - a preliminary study. Annals of *13º Simpósio Latino Americano de Ciência de Alimentos* (13 SLACA), Campinas, SP, Brazil, 2019.
13) **Victor Aredo**, Eliria M.J.A. Pallone, Francisco E.G. Guimarães, Alessandra Lopes de Oliveira. Formation of turmeric extract-loaded structured lipid carriers by supercritical melt micronisation. Annals of *14º Simpósio Latino Americano de Ciência de Alimentos* (14 SLACA), Campinas, SP, Brazil, 2021.
14) **Victor Aredo**, Hubert Arteaga, Eliria M.J.A. Pallone, Francisco E.G. Guimarães, Alessandra Lopes de Oliveira. Beeswax coating on blueberry extract-loaded hydrolyzed collagen particles using supercritical CO_2. Annals of *14º Simpósio Latino Americano de Ciência de Alimentos* (14 SLACA), Campinas, SP, Brazil, 2021.

Participations as speaker in scientific events

1) **Victor Aredo.** Conference: "*Tecnologías de fluidos supercríticos para la extracción y formación de partículas de sustancias bioactivas"*. *II Congreso Agroindustrial de Investigación Y Responsabilidad Social* (II CAIRS), Universidad Nacional de Trujillo, Trujillo, Peru, 2016.
2) **Victor Aredo**. Conference: "*Potencial de los fluidos supercríticos en la innovación agroindustrial"*. *IV Jornada Peruana Internacional de Investigación en Ingeniería – JP3I*, Universidad Nacional de Trujillo, Trujillo, Peru, 2020.
3) **Victor Aredo**. Conference: "*Tecnología de impregnación supercrítica para la innovación en agroindustrias"*. *1er Simposio Internacional "Avances e Innovación en el Sector Agroalimentario"*, Universidad Nacional Autónoma de Chota, Chota, Peru, 2020.
4) **Victor Aredo**. Conference: "*Rol de la tecnología de CO_2 supercrítico en la encapsulación de compuestos bioactivos"*. *II Simposio Internacional "Avances e Innovación en el Sector Agroalimentario: en Tiempo de Pandemia"*, Universidad Nacional Autónoma de Chota, Chota, Peru, 2021.
5) **Victor Aredo**. Conference: "*Formación de Microcápsulas de Pigmentos usando Tecnología Supercrítica"*. *XXI Congreso Nacional de Estudiantes Ingeniería Agroindustrial*, Universidad Nacional Autónoma de Chota, Chota, Peru, 2022.

Participation as reviewer for scientific journals

1) Revista UDCA Actualidad & Divulgación Científica. 2 reviews. Bogota, Colombia. 2018.
2) Brazilian Journal of Food Technology. 2 reviews. Campinas, SP, Brazil. 2018.
3) Scientia Agropecuaria. 1 review. Trujillo. Peru. 2022.
4) European Journal of Lipid Science and Technology. 1 review. Weinheim, Germany, 2022.
5) Journal of Food Engineering. 11 reviews. London, United Kingdom. 2019-2022.

Participation of the Teaching Improvement Program (*Programa de Aperfeiçoamento de Ensino* – PAE)

1) *Refrigeração e cadeia de frio* (Refrigeration and cold chain). Food Engineering undergraduate program (2nd semester, 2016). FZEA/USP, Pirassununga, SP, Brazil. Responsible professor: Dr. Alessandra Lopes de Oliveira.
2) *Termodinâmica* (Thermodynamics). Biosystems Engineering undergraduate program (1st semester, 2019). FZEA/USP, Pirassununga, SP, Brazil. Responsible professor: Dr. Alessandra Lopes de Oliveira.
3) *Fenômenos de Transporte II* (Transport Phenomena II). Food Engineering undergraduate program (2nd semester, 2019). FZEA/USP, Pirassununga, SP, Brazil. Responsible professor: Dr. Izabel Freitas Moraes.

Participation as examiner

1) 26º Simpósio Internacional de Iniciação Científica da Universidade de São Paulo (26 SIICUSP), Food Engineering area (1st stage). Pirassununga, SP, Brazil, 2018.
2) 26º Simpósio Internacional de Iniciação Científica da Universidade de São Paulo (26 SIICUSP), Agrarian science area (International stage). São Paulo, SP, Brazil, 2018.
3) 27º Simpósio Internacional de Iniciação Científica da Universidade de São Paulo (26 SIICUSP). Food Engineering area (1st stage). Pirassununga, SP, Brazil, 2019.
4) Jury of the Supervised Internship I report and defense, Logistic area, undergraduate student: Estela Selaro Passalacqua, FZEA/USP, Pirassununga/SP, Brazil, 2021.
5) II Congresso Brasileiro Online de Ciência dos Alimentos (II CONBRACA). Brazil, 2021.
6) 30º Simpósio Internacional de Iniciação Científica da Universidade de São Paulo (30 SIICUSP). Food Engineering area (1st stage). Piracicaba, SP, Brazil, 2022.
7) XXI Congreso Nacional de Estudiantes de Ingeniería Agroindustrial (XXI CONEIA). Peru, 2022.