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Encapsulation of Natural Compounds in Hybrid Micelles for Topical Administration: Formulation and Stability studies

Beatriz Nibra Guedes

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**Encapsulation of natural compounds in hybrid micelles for
topical administration: Formulation and Stability studies**

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Faezeh Fathi.

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DE ACORDO COM A LEGISLAÇÃO EM VIGOR, NÃO É PERMITIDA A REPRODUÇÃO
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Abstract

With increasing concerns about skin's health and environment impact of cosmetics and other dermo pharmaceuticals, natural compounds and food by-products are increasingly sought after to treat numerous skin-related diseases. Rutin and resveratrol are non-toxic phytochemicals, found in various fruits and plants, with several biological properties of interest to the pharmaceutical and cosmetic industries, as they have antioxidant activity, anti-inflammatory, wound healing, antibacterial properties, among others. However, the low stability and low water solubility of these compounds, negatively affect their bioavailability, which limits their applications. To overcome these problems, new ways of increasing the beneficial effect of these compounds on the skin, such as their loading into delivery systems, have been considered. Olive pomace, on the other hand, is a by-product of the olive oil industry that has gain interest due to its high content of phenolic compounds, thus endowed with several beneficial properties, especially for the skin.

This work aimed to developed techniques to make possible the encapsulation of natural compounds in hybrid nanocarriers, namely hybrid micelles, and reusing the olive pomace to obtain an innovative dermocosmetic product capable of effectively delivering these bioactives.

To pursue this aim, micelles loaded with rutin and resveratrol were developed using hot homogenization and ultrasound techniques. Stability studies were carried out over 2 months to evaluate the physical and chemical stability of the formulations. Analysis of average hydrodynamic size, polydispersity index, zeta potential, morphological studies, thermal properties analysis and rheological studies were carried out. A hydrogel was prepared to which the micelles loaded with bioactives were added, together with olive pomace extract. Thermal, rheological and texture properties were analyzed.

The results of the analyzes demonstrated that the different samples of micelles prepared were stable during the analyzed period, with a size less than 90 nm, depicting a spherical morphology, high encapsulation efficiency and appropriate viscoelastic properties, with the formulation being more elastic than viscous. The hydrogel loaded with bioactives and olive pomace had viscoelastic results which indicated a gel-like structure. The viscosity of the different hydrogels was strongly dependent on the presence of micelles and olive pomace, since the hydrogels that contained olive pomace in their composition obtained considerably lower viscosity values, compared to the hydrogel samples without olive pomace. In the texture analysis, the results showed that hydrogels without olive pomace have a firmer and more cohesive matrix than hydrogels containing this by-product.

Key-words: Rutin; Resveratrol; Olive Pomace; Micelles; Hydrogel

Resumo

Com as preocupações crescentes acerca da nossa pele e do impacto ambiental dos cosméticos e outros produtos dermofarmacêuticos, compostos naturais e subprodutos alimentares são cada vez mais procurados para tratar inúmeras doenças relacionadas com a pele. A rutina e o resveratrol são fitoquímicos não tóxicos, encontrados em diversas frutas e plantas, que contêm diversas propriedades biológicas de interesse para a indústria farmacêutica e cosmética, devido à atividade antioxidante, anti-inflamatória, cicatrizante e propriedades antibacterianas que possuem, entre outras. No entanto, a baixa estabilidade e baixa solubilidade em água destes compostos afetam negativamente a sua biodisponibilidade, o que limita as suas aplicações. Para superar estes problemas, foram consideradas novas formas de aumentar o efeito benéfico destes compostos na pele, tais como o seu encapsulamento em sistemas de entrega. Já o bagaço de azeitona é um subproduto da indústria do azeite que tem ganhado interesse devido ao seu elevado teor de compostos fenólicos, dotado assim de diversas propriedades benéficas, especialmente para a pele. Este trabalho teve como objetivo desenvolver técnicas que possibilitem a encapsulação de compostos naturais em nanocarregadores híbridos, nomeadamente micelas híbridas, e a reutilização do bagaço de azeitona para a obtenção de um produto dermocosmético inovador capaz de libertar de forma eficaz estes bioativos. Para atingir este objetivo, micelas carregadas com rutina e resveratrol foram desenvolvidas utilizando técnicas de homogeneização a quente e ultrassom. Foram realizados estudos de estabilidade durante 2 meses para avaliar a estabilidade física e química das formulações. Foram realizadas análises de tamanho médio das partículas, índice de polidispersão, potencial zeta, morfologia, propriedades térmicas e reologia. Foi preparado um hidrogel ao qual foram adicionadas as micelas carregadas, juntamente com o extrato de bagaço de azeitona. Foram analisadas propriedades térmicas, reológicas e de textura. Os resultados das análises demonstraram que as diferentes amostras de micelas preparadas permaneceram estáveis durante o período analisado, com um tamanho inferior a 90 nm, com uma morfologia esférica, elevada eficácia de encapsulação e propriedades viscoelásticas adequadas, sendo a formulação mais elástica do que viscosa. O hidrogel carregado com bioativos e bagaço de azeitona apresentou resultados viscoelásticos que indicaram uma estrutura semelhante a um gel. A viscosidade dos diferentes hidrogéis foi fortemente influenciada pela presença de micelas e bagaço de azeitona, uma vez que os hidrogéis que continham bagaço de azeitona na sua composição obtiveram valores de viscosidade consideravelmente inferiores, em comparação com as amostras sem bagaço de azeitona. Na análise de textura, os resultados mostraram que os hidrogéis sem bagaço de azeitona apresentaram uma matriz mais firme e coesa.

Palavras-Chave: Rutina; Resveratrol; Bagaço de azeitona; Micelas; Hidrogel

List of Publications

Publications in international journals:

- I. **Guedes, B.N.**, et al. Rutin-loaded micelles: design and characterization of a new hybrid delivery system (submitted, June 2024).
- II. **Guedes, B.N.**, et al. Polyphenols-composed micelles: dual loading of resveratrol and rutin as nutrients for skin diseases (submitted, June 2024).
- III. **Guedes, B.N.**, et al. Development and characterization of eco-friendly hydrogels loading polyphenols-composed micelles (submitted, June 2024).
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- V. **Guedes, B.N.**, Krambeck K., Durazzo A., Lucarini M., Santini A., Oliveira M.B.P.P., Fathi F., Souto E.B. Natural antibiotics against antimicrobial resistance: sources and bioinspired delivery systems. *Brazilian Journal of Microbiology* (2024) <https://doi.org/10.1007/s42770-024-01410-1>.
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Intellectual property

- I. **Guedes, B.N.**, Fathi F., Oliveira M.B.P.P, Souto E.B.
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Abbreviations

3D	Three-dimensional
DD	Drug Delivery
DSC	Differential Scanning Calorimetry
EE	Encapsulation Efficiency
G'	Storage modulus
G''	Loss modulus
LC	Loading Capacity
OP	Olive Pomace
PDI	Polydispersity Index
RES	Resveratrol
ROS	Reactive Oxygen Species
SD	Standard Deviation
TEM	Transmission Electron Microscopy
Z-Ave	Average hydrodynamic size
ZP	Zeta Potential

Dissertation outline

The current Master dissertation is organized into five chapters, for which the content is outlined below.

Chapter 1, entitled “**Introduction and Objectives**” presents the central idea and main objectives of the master dissertation, giving the theoretical background behind the choice of developing eco-friendly, hybrid micelles for the loading of rutin and/or resveratrol, and also the rationale for choosing hydrogels as semi-solid base to develop a final product.

Chapter 2, entitled “**Rutin-loaded micelles: design and characterization of a new hybrid delivery system**”, is dedicated to the production, development and characterization of rutin-loaded hybrid micelles. It involves stability studies, such as the monitoring of hydrodynamic size, polydispersity index and zeta potential analysis over time, as well as the morphology studies, thermal properties studies, encapsulation efficiency, loading capacity and viscoelastic properties studies. The experimental work aimed to find methodologies that were capable of encapsulating the rutin efficiently, considering the use of green chemistry. Chapter 1 corresponds to the version of a manuscript submitted to a peer-review journal in June 2024.

Chapter 3, entitled “**Polyphenols-composed micelles: dual loading of resveratrol and rutin as nutrients for skin diseases**”, is dedicated to the production, development and characterization of resveratrol-loaded hybrid micelles and resveratrol-rutin-loaded hybrid micelles. To characterize the micelles, stability tests were performed over a period of two months, by analysis the hydrodynamic size, polydispersity index, zeta potential, morphology, thermal properties, encapsulation efficiency, loading capacity and rheological profiles. The experimental work was carried out based on green chemistry and the main goal was to effectively encapsulate the bioactive compounds within the micelles. Chapter 3 also corresponds to the version the manuscript submitted to a peer-review journal in June 2024.

Chapter 4, entitled “**Development and characterization of eco-friendly hydrogels loading polyphenols-composed micelles**”, is dedicated to the development and characterization of micelles-based Carbopol® 940 hydrogels. The development of semi-solid formulations bearing rutin-/resveratrol-loaded hybrid micelles is described, as well as the characterization by Differential Scanning Calorimetry analysis, rheological analysis and texture analysis. This chapter also presents confidential information regarding a patent application process for a formulation of rutin-resveratrol-olive pomace-loaded hydrogels, which was developed in this

work. Chapter 4 also corresponds to the version of a manuscript submitted to a peer-review journal in June 2024.

Chapter 5 gives the final conclusions and future prospects of the research that has been developed within the scope of this master work.

Chapter 1: Introduction and Objectives

1. Introduction

Skin is the largest organ in the human body counting with three layers: the epidermis, dermis, and the hypodermis, which vary significantly in their anatomy and function [1]. The skin is made of a complex network which serves as the body's primary line of defense against external antigens and unwanted influences like pathogens, UV light, and chemicals. It also regulates body temperature and the amount of water released into the environment [1, 2]. The superficial layer of the skin – epidermis – is considered the body's first barrier against trauma and xenobiotics. Skin integrity can be compromised through various mechanisms, such as surgery, acute injury and various physiological conditions [3]. This loss of integrity and normal functions of the skin is called a wound. Once a wound appears on the skin, there is a risk of a microbial infection and loss of fluids, which hinders the healing process [4]. However, our body has a natural physiological reaction to the injury, involving the vascular system, various types of cells, cytokines and mediators that provide the ability to quickly initiate the wound repair process [5]. This process requires the presence of a suitable environment to promote wound healing, such as redox environment. The interruption or absence of these processes leads to the loss of wound healing capacity, leading to their evolution into more complex and difficult to treat situations [6, 7].

Different phases are involved in this process, which are linked to each other and often overlapping [8]. Each occurs at different times and can last from minutes to several months [9, 10]. The wound healing process begins in the hemostasis phase, followed by the inflammation process, proliferation and finally, tissue remodeling [8].

a) Hemostasis

When a skin injury occurs, hemostasis is the first phase in the healing process. This phase involves the contraction of local blood vessels to reduce and subsequently stop the blood loss. For this to happen, platelets are involved to form a clot that limits blood flow in that place [11]. After this, platelet-derived SDF-1 α and platelet-derived growth factor are expelled by the clot, together with cytokines and transforming growth factor β 1, promoting the proliferation of inflammatory cells and thus beginning the phase of inflammation [4, 12].

b) Inflammation

After hemostasis, inflammation occurs and lasts, usually, around 3 days [13]. During this phase, there is vasodilation of blood-vessels, an increase in blood flow and an increase in vascular permeability, promoting the migration of neutrophils and monocytes to the site of interest [14]. First, neutrophils arrive at the wound. These eliminate possible pathogenic microorganisms present and remove debris [15, 16]. Circulating monocytes then clean the site by removing bacteria-contaminated neutrophils, damage matrix, non-functional host cells and

more debris. Once these processes are completed, angiogenesis and re-epithelialization begin [17-19].

c) Proliferation

After the inflammatory phase, the proliferation phase occurs, which involves three stages. First, re-epithelialization, then angiogenesis and, finally, the formation of granulation tissue [20, 21]. The first stage begins with the activation of growth factors and cytokines that cause the increase in various cells, such as stem cells, epithelial cells, fibroblasts and keratinocytes. Then, with the migration and increase of epithelial cells and fibroblasts, the old matrix is degraded by proteolytic enzymes, resulting in the formation of the new extracellular matrix and angiogenesis [22, 23]. Finally, after the formation of new blood vessels, the fibroblasts, cellular matrix and macrophages form granulation tissue. This is deposited at the base of the wound and allows the epidermis to be restored [24, 25].

d) Tissue Remodeling

After the steps described previously, this last one is the longest. Briefly, the tissue remodeling phase is a balance between three processes: synthesis, deposition and degradation. There is a large production of collagen in the normal network, followed by its deposition [26]. Then, there is an increase in the density and stability of the cellular matrix, with the segregation of new components [27].

2. Phytochemicals and natural compounds for wound healing

Because of their adaptability, safety and affordability, molecules produced from natural sources have been studied for a wide range of applications [28]. While a number of synthetic medications are effective in treating wounds, they also have drawbacks that make phytoconstituents appropriate alternatives [29]. Several studies have already proven the effectiveness of natural compounds in numerous skin problems, due to the therapeutic activities they have. These compounds can accelerate the wound healing process, preventing bacterial infections, as they have antioxidant, anti-inflammatory, antibacterial activities and the ability to synthesize collagen [30-33]. Plants represent a substantial source of natural compounds with interesting therapeutic properties, called secondary metabolites [34, 35]. Among them, polyphenols and phenolic compounds stand out. Phenolic compounds are extensively distributed in nature and have numerous beneficial actions related to the skin, being recently used in the fields of cosmetics and dermatology [36]. They promote wound healing, have antioxidant, antimicrobial, anti-inflammatory and photoprotective activity and increases the production and viability of collagen fibers [36-42].

2.1. Rutin

More and more focus is being paid to natural compounds derived from plants or fruits that have antibacterial, anti-inflammatory, and antioxidant qualities that can mediate healing [43]. Rutin is a non-toxic polyphenolic flavonoid present in different plants, such as passionflower, buckwheat, Betula leaves, green tea, apple, citrus fruits and neem [44-47], that can be useful in biomedical applications [48]. Rutin is highly appealing because of its many advantages, including its natural origin, numerous pharmacological activities, safety and a good cost-benefit profile [49, 50]. Several beneficial properties have been attributed to rutin, such as antiviral, antibacterial, antioxidant, antidiabetic, anticancer, antiallergic, anti-inflammatory, anti-arthritic, wound-healing, and neuroprotective properties [45, 51-54]. Rutin is regarded as an interesting, highly valuable flavonoid because of its possible preventive effect against a number of oxidative stress-related disorders [45], since this compound can decrease reactive oxygen species production in skin cells [55-57]. Rutin is also used in cosmetics, particularly as antioxidant in anti-aging products, and for formulations intended for allergic and sensitive skin [58, 59]. This flavonoid is lipophilic, and is soluble in organic solvents, such as pyridine, ethanol and methanol [60]. However, the low solubility and stability in water affects its bioavailability, which is therefore a huge disadvantage [61, 62]. As a result, rutin has an insufficient skin penetration rate and cannot be used efficiently in cosmetic products [63]. To overcome these problems new ways of increasing the beneficial effects of rutin on the skin have been considered, such as new delivery systems.

2.2. Resveratrol

Both external and internal drugs, primarily antibiotics, UV protection, antioxidants, anti-inflammatory, analgesics, and moisturizing agents, are used in the treatment of skin problems [64]. However, due to their limited efficacy, traditional medications frequently fall short of expectations and do not offer ongoing treatment for wound proliferation and anti-inflammation [65]. Plants and fruits are among the many natural sources that are frequently utilized in the treatment of many skin ailments. More recently, research has demonstrated that natural flavonoids and polyphenolic chemicals have numerous beneficial effects for the skin [66]. Resveratrol is a naturally occurring phytoalexin that is a non-flavonoid polyphenol molecule that plants make in response to pathogen attack [67, 68]. Strong antioxidant, anti-inflammatory, and immunomodulatory properties can be discovered in resveratrol, which is present in red wine, almonds, berries, and grape skin and seeds [69-71]. Resveratrol has also been shown to limit inflammation and stimulate angiogenesis in the healing of skin wounds [72, 73]. It has also been demonstrated to suppress the release of associated inflammatory cytokines and decrease the inflammatory phenotype of macrophages [74, 75]. Furthermore, because of resveratrol antioxidant and collagen-stimulating properties, it has shown promise

as antiaging and for UV protection of skin [76, 77]. Relevant to this work in particular, resveratrol has been the subject of much research in recent years regarding its effects on human pathogens. Numerous studies have shown that it has antimicrobial activity against a broad spectrum of bacteria, viruses, and fungi [78], and has been successful in treating a variety of skin disorders, such as acne [79], wounds [80] and melanoma [81].

The main issue with resveratrol that affects its usefulness in treating diseases is that, although being absorbed rather well (about 70%), it is extensively eliminated by the liver in the first pass metabolism, which results in low oral bioavailability [82]. In addition to its limited bioavailability, resveratrol is highly processed in the bloodstream and has an incredibly short half-life (8–14 min) [83]. Because the skin has a far lower rate of resveratrol metabolism than the liver, resveratrol administration through the skin is an appealing option to oral dosage for the treatment of topical and/or dermal conditions [84]. Compared to oral treatment, topical therapy has no risk of systemic side effects (provided that the drug is not aimed to be absorption into the blood circulation) and can deliver higher drug concentrations at the site of the disease, making it a more compelling and promising approach to treating skin diseases. Additionally, topical resveratrol is said to have a minimal potential for causing skin irritation [85, 86]. However, due to its hydrophobic nature and the difficulty in managing its fate in the skin, dermal targeting remains problematic [87].

Because of its low solubility and poor chemical stability, using nanotechnologies to create a delivery system that would enhance resveratrol's biological activities, without changing its chemical properties, is another way to improve the application of resveratrol-based therapy [88, 89]. The fields of medicine, cosmetics, and pharmacy are becoming increasingly interested in nanomedicine delivery technologies. Resveratrol has been proposed to be loaded into delivery systems to improve its stability, modify the release profile and promote its permeation in biological barriers [90]. One potential solution to the percutaneous penetration issue is the encapsulation of active ingredients in innovative delivery systems [91].

2.3. Olive Pomace

One of the oldest and most cultivated trees in the entire Mediterranean region is the *Olea europaea* L., olive tree [92]. This tree is native to Asia Minor and Syria and its products, from the olive oil to olives, are abundantly consumed in the Mediterranean diet [92-94]. However, the production of olive oil and, consequently, the industrialization of this sector, is associated with environmental problems due to high amount of potentially toxic waste through the region [95]. Waste such as olive leaves, wastewater and olive pomace (OP) are the most worrying wastes, due to a high load of toxic organic compounds and their low pH [96]. In particular, OP is a residue with a high amount of water, has an extremely low pH and stores most of the phenolic content of the olive in its composition [97, 98]. Although it has been demonstrated

that this by-product, as well as the phenolic compounds in its composition, has numerous beneficial and health-promoting activities, such as antioxidant [99-101], anti-inflammatory [102-104], photoprotective activities [100, 105-107], as well as applicability in the treatment of various skin diseases through of topical application [108, 109], this by-product is not biodegradable and becomes phytotoxic [92, 110]. Therefore, new ways of reusing this OP are sought and highlighted in several studies and its formulation in new dosage forms for topical application, such as hydrogels, is a promising example [96].

3. Hybrid micelles

For bioactive compounds to exhibit their health-promoting effects, they go through several processes before reaching their target [111]. These processes are the main factors that affect the biofunctional properties of bioactive compounds, called bioaccessibility and bioavailability [112]. Therefore, the assessment of these two factors has currently become a promising area of research. Bioaccessibility and bioavailability are influenced by several factors. Most plant bioactive ingredients degrade easily and their stability is affected by light, temperature, oxygen and storage. The bioavailability of hydrophobic compounds can be affected because they are poorly soluble in water, resulting in a poor dissolution, diffusion and, consequently, limited permeability of the compound [113]. The development of nanoformulations has been increasingly used in biomedicine, thus disadvantages shown by some compounds are overcome [114, 115]. Nanomedicine improved the absorption of compounds that are poorly soluble or insoluble in water, improved their bioavailability, provided prolonged release, effective delivery of active ingredients to specific locations, prevented interactions with other antagonistic compounds, etc. [116-118].

To transport compounds that are poorly soluble or insoluble in water, there are currently several lipid delivery systems for this purpose [119-124]. They have numerous advantages such as high affinity, high loading capacity, high capacity to cross membranes and high biocompatibility [125].

A supramolecular aggregate of a surfactant molecule, distributed in a liquid colloid with a particle width ranging from 5 to 100 nm, is called a micelle [126]. Lipid-core micelles are amphiphilic molecules with two regions of differing affinity for water that self-assemble to create normal-phase micelles. The hydrophobic molecules make up the center of the micelle, while the hydrophilic portion faces its exterior [127]. Lipid-core micelles are frequently proposed as delivery systems for water-insoluble molecules and pharmaceutical drugs since the micellar surface binds polar molecules while the center of the micelle can carry lipophilic pharmaceuticals [126, 128].

Micelles possess certain benefits over alternative drug delivery systems, particularly because of their size and structure. These advantages include their capacity to load both hydrophilic and lipophilic medications [129], the hydrophobic drugs are attached to the hydrophobic core, resulting in the production of a transparent aqueous solution that increases the drug's solubility [130, 131], the capacity to hold steady for extended periods of time inside the circulatory system [132], their active targeting capabilities make micelles target-specific, resulting in maximum delivery and minimal adverse effects [129], higher target tissue penetration, higher medication absorption, and better efficacy as a result of their size [133, 134].

The stabilization of lipid-core micelles can be reinforced with polysorbates (e.g., Tweens) that are hydrophilic to develop hybrid micelles.

4. Green production methods

As nanotechnology advances and gains more and more interest in different areas, the production of nanomaterials must keep pace with increased demand and technological advances. Thus, the problems associated with this mass production begin to emerge and has generated some concerns and interest in knowing the impact of these substances on the health of the planet, which is understood as the appropriate physical, mental and social well-being of human civilization and the environment [135-137]. Nanoparticles are synthesized using different methods, namely chemical, physical, photochemical and biological methods, each of these methods having its advantages and disadvantages [138]. Physicochemical methods are the least preferred, due to the excessive use of organic solvents applied in the synthesis of nanomaterials [139], the use of high pressure and high temperature conditions [140], volatile vapor [141] and the excessive production of carbon dioxide, which altogether contribute to the greenhouse effect [142]. These are risks and concerns that make the green synthesis gain ground. In comparison, biological methods are preferred in terms of ecology and sustainability [143], although the mechanism of synthesis is still a challenge [144].

5. Hydrogels

A family of three-dimensional (3D) polymer networks with a high capacity for water retention is called hydrogels [145, 146]. Almost any hydrophilic polymer can be converted into a hydrogel through chemical or physical cross-linking [147]. The chemical composition and tunable physical characteristics of hydrogels are very variable. Hydrogels are seen to be promising and have been used in a variety of biological contexts, including for tissue regeneration and drug delivery [148-151].

Four characteristics of hydrogels—biocompatibility, biodegradability, drug-loading capacity, and controlled drug release—are key to their effectiveness [152]. Their high porosity and suitability for water environments are additional benefits. They are particularly suitable for as drug delivery carriers because of this feature [153].

Different methods can be used to treat skin wounds. Compared to hydrogels, conventional treatments, such as bandages and gauzes, are being utilized less. Hydrogels are advantageous in the treatment of skin wounds because of their high-water retention capacity, which allows them to retain moisture in the wound and continually absorb exudate, as well as their mechanical characteristics that are compatible with biological tissues [154]. Because hydrogels are non-reactive with biological tissue, non-irritating, and optimally permeable to metabolites, they are categorized as appropriate for all four stages of the skin healing process (see section 1. of this chapter) [155]. Hydrogel dressings are therefore the subject of much research in an effort to speed up wound healing. Depending on the situation, a large range of hydrogel dressings can be used to treat various kinds of skin wounds, hastening the need to optimize wound care. Because of this property, hydrogels were able to enter the pharmaceutical market as wound dressing materials quite quickly. They can be found there as sheets, gels, or soaked gauze [156]. Therapeutic nanoparticles have been progressively mixed with various biomaterials to create nanohybrid systems for novel uses as nanotechnology has advanced [157, 158]. A typical example of a hybrid delivery system based on nanotechnology is a hydrogel that contains nanoparticles loaded with pharmacological moieties. That being said, a lot of emphasis has been paid to the loading of nanoparticles into hydrogels [159, 160].

Advanced wound care frequently uses polymer-based dressings because of their physical qualities, such as their ability to absorb water, their fluid affinity, their rheological qualities, and their suitability for delivering therapeutic agents while maintaining moisture on wound surfaces. This advanced wound care often entails the controlled administration of active substances to the injured site [161, 162].

However, if the cross-link density is changed, the hydrogel's porosity and water affinity can be readily adjusted. However, what really sets polymeric hydrogels apart as effective drug delivery systems for rapid wound closure is their ability to sustain a high local concentration of the active component over an extended period of time [163, 164]. Its three-dimensional network facilitates the effective distribution of bioactive substances to the region of interest while also improving cell adhesion and motility [165], as well as the deposition of extracellular matrix [166]. According to recent research, when hydrogels are swollen, they stop germs from penetrating the site and facilitate the delivery of useful chemicals there [164].

6. Objectives

General objective

Several works and intense research have been developed to improve the characteristics of bioactive compounds from natural sources, such as phytochemicals, with the help of nanotechnology. However, it remains essential to deepen scientific knowledge in this area, as more and more issues of environmental safety, green chemistry, and resistance of microorganisms to conventional medicine, are impacting our society and healthcare systems. The general objective of this work was to develop an innovative delivery product, enriched with biologically active components from natural sources –, rutin and resveratrol –, in a safe, controllable way, based on environmentally safe and eco-friendly chemistry, capable of being used in the research and development of new dermocosmetic products with nourishing properties.

Specific objectives

- Encapsulation of active compounds that are hydrophobic, with low solubility and poor chemical stability, with a focus on the dual loading of rutin and resveratrol, in new hybrid micelles.
- Evaluation of the physicochemical properties of the developed hybrid micelles.
- Evaluation of the stability of the developed hybrid micelles over time and at different storage temperatures.
- Incorporation of the enriched micelles into a gel, forming a final product.
- Evaluation of the physicochemical properties of the micelles-loaded hydrogel.
- Incorporation of the polyphenols-loaded micelles into a hydrogel, together with olive pomace, forming a final product for the consumer.
- Development of a stable, safe and eco-friendly final product.

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Chapter 2: Rutin-loaded micelles: design and characterization of a new hybrid delivery system

Abstract

In this work, we describe a new method for the loading of rutin into hybrid micelles to improve its low solubility and low bioavailability. Micelles composed of phospholipids combined with polysorbate and sorbitan monostearate were developed in a single step and their physicochemical stability monitored over a period of two months. Analyzes were carried out to determine average hydrodynamic size (Z-Ave), polydispersity index (PDI), zeta potential, viscoelastic profiles and differential scanning calorimetry. The average diameter of rutin-loaded micelles ranged from 72.6 ± 0.31 nm to 81.62 ± 2.18 and the blank micelles 77.95 ± 0.35 nm to 83.75 ± 1.05 nm. In general, the size of the micelles increases over the time and the same pattern can be observed in relation to the PDI value. Regarding the different storage temperature, it can be observed that both in the blank micelles and in the micelles loaded with rutin, the preparations stored at a temperature of 4°C, registered a micelle size value smaller than the micelles stored at 25°C. The PDI maintained a relatively low value and without major variations over the eight weeks, being a good prognosis for the good stability of the preparation. Corroborating these data, the zeta potential values recorded were high (in modulus) during the time studied, confirming the good stability of the preparations. The transmission electron microscopy study confirmed the spherical shape and smooth morphology of the developed micelles. The drug encapsulation efficiency reached 92.09% and loading capacity was 1.96%. The rheological analysis showed that both samples are more elastic than viscous. The DSC did not register the presence of peaks, corroborating that the sample has a liquid matrix.

Keywords: Rutin; Hybrid Micelles; Stability; Rheology.

1. Introduction

Because of their adaptability, safety, and affordability, molecules obtained from natural sources are being explored for a broad spectrum of applications [1]. Although several synthetic drugs are used as wound healing agents with promising results, they also have certain limitations associated to side effects that make phytoconstituents good alternatives [2]. New forms of treatment are being sought to address the current problems of conventional medicine, attracting more and more attention to natural products like plant- or fruit-derived chemicals that mediate the healing process through their antioxidant, antimicrobial and anti-inflammatory activities [3]. Rutin, also known as Vitamin P (**Figure 1**), is a polyphenolic flavonoid found in a variety of plants, including citrus fruits, buckwheat, *Betula* leaves, passionflower, green tea, and apples [4-7]. It thought to be a harmless substance with potential uses in biological and pharmacological applications [8].

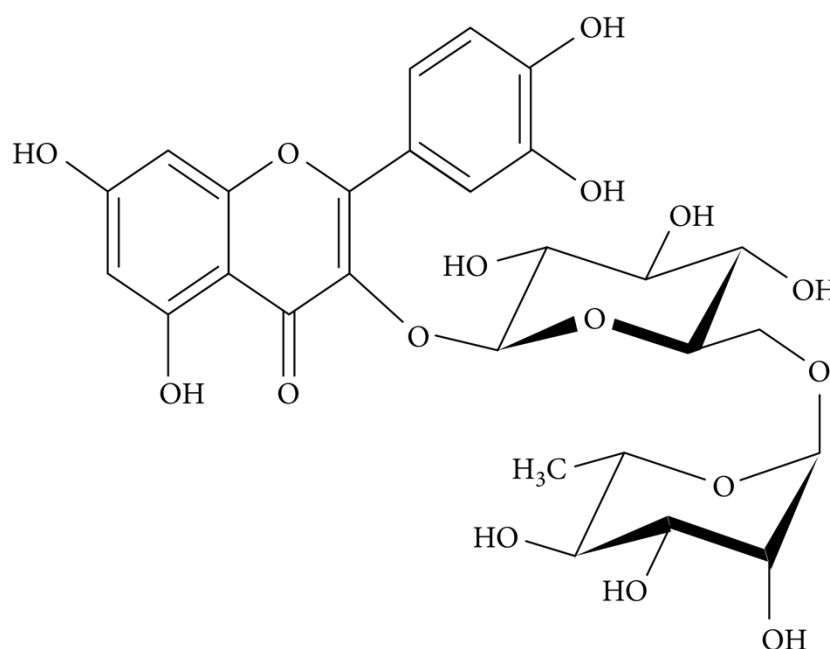


Figure 1. Chemical structure of rutin (reproduced after Enogieru, Adaze Bijou et al. (2018) [9], under the terms and conditions of the Creative Commons Attribution (CC BY) license.

Rutin is a very attractive compound due to its numerous beneficial features, such as its natural source, safety profile, cost-benefit and large number of pharmacological properties [10, 11]. Numerous studies demonstrate this flavonoid has anti-inflammatory, anti-allergic, antibacterial, anti-cancer, anti-viral, anti-wrinkle, wound-healing, and neuroprotective properties [5, 12-15]. Among the different pharmacological actions, rutin is also considered beneficial due to its preventive effect against a number of oxidative stress-related disorders

[5], because it decreases the level of reactive oxygen species (ROS) produced in macrophages and in other skin cells [16-18]. Owing to its ROS scavenging capacity, rutin has been proposed a smart ingredient in functional foods. Rutin is also used in dermocosmetic products, e.g. for anti-aging, allergic and atopic skin, mainly attributed again to its antioxidative properties [19, 20]. As a lipophilic ingredient, rutin is soluble in organic solvents (e.g., pyridine, methanol and ethanol) [21]. Some disadvantages related to this compound that hinders its incorporation in pharmaceutical dosage forms are its poor water solubility and stability, which consequently affect bioavailability [22, 23]. For the same reasons, poor penetration rate into the skin, and thus cannot be used efficiently in dermal products [24].

To overcome these problems new ways of increasing the beneficial effect of rutin on the skin, such as drug delivery systems, have been considered. The advances in nanotechnologies have allowed the creation of nanoformulations for problematic drugs creating new possibilities and opportunities in pharmaceutical and cosmetic industries [25]. Nanodelivery systems improve the bioavailability of drugs of low water solubility by increasing, their absorption and permeability through biological barriers [26]. The effective delivery to specific locations, controlled release, modification of organoleptic properties and prevention of interactions with other antagonistic components, are additional advantages of nanodrug delivery systems [27, 28].

Drugs that are water insoluble can be effectively delivered to various areas of the body using nanocarriers composed of lipid raw materials. There are currently a number of lipid-based nanocarriers being considered for pharmaceuticals, such as solid lipid nanoparticles [29], nanostructured lipid carriers [30], self-(micro) emulsifying drug delivery systems [31], solid lipid nanoparticles, micro and nanoemulsions [32], and hybrids nanocarriers that combine lipids and polymers, such as lipid nanocapsules [33] and lipid-core micelles [34].

These nanocarriers have several benefits, including high loading capacity, biocompatibility, strong affinity for drugs that are poorly soluble in water, which ultimately contribute to increase membrane permeation to loaded drugs [35].

A micelle is a supramolecular aggregate of surfactant molecules that are dispersed in a liquid and form a colloid with a diameter between 5 and 100 nm [36]. Lipid-core micelles, or normal-phase micelles, are created when molecules known as amphiphilic molecules—which contain two areas with varying affinity for water—self-assemble (**Figure 2**). In these amphiphilic molecules, the region facing the external surface of the micelle is called the hydrophilic region and the hydrophobic region of the molecules forms the core [37]. Because of this property, the micellar surface of lipid-core micelles attracts polar molecules whereas the inner phase of the micelle can carry lipophilic compounds, so they are often suggested as delivery systems for water-insoluble molecules and other pharmaceutical drugs [36, 38].

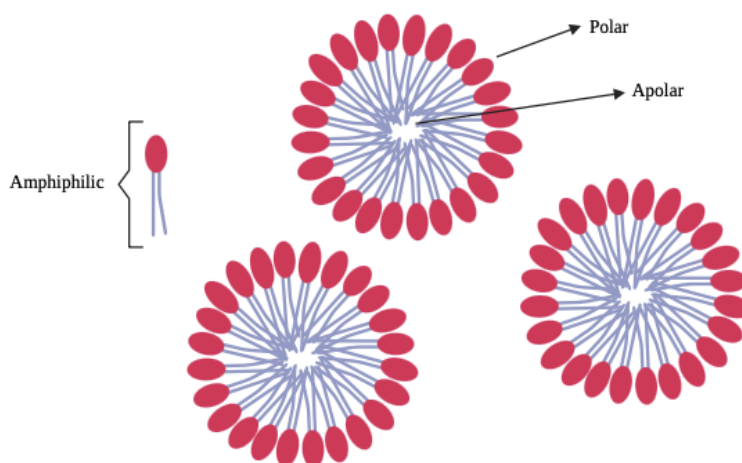


Figure 2. Structure of lipid-core micelles (authors own drawing).

Besides increasing the drug's solubility and bioavailability other concerns posed by manufacturing of such nanocarriers is the need to keep up with the growing demand and technological advancements as nanotechnology develops and attracts increasing attention in various fields. As a result, issues related to this mass production start to surface, raising questions about potential effects on the planet's health—that is, the appropriate physical, mental, and social well-being of human society and the environment and raising people's curiosity [39-41]. Several techniques are used to create a range of nanocarriers: chemical, physical, photochemical, and biological techniques. Each of these techniques has benefits and drawbacks [42]. Because of the excessive use of organic solvents in the production [43], the use of high pressure and high temperature conditions [44], the production of volatile components [45] and the excessive production of carbon dioxide, which contributes to the greenhouse effect [46], physicochemical methods are currently the least preferred. The green synthesis is gaining attraction to overcome these risks. Comparatively, green approaches are more favorable for ecology and sustainability [47], even though it is still difficult to upscale them [48]. By using renewable resources, and eco-friendly excipients to safeguard the environment, new type of nanocarriers can be proposed for drug delivery. The aim of this work was to develop a biocompatible and environmentally safe method to obtain micelles by a single step for the loading of the rutin, to improve its poorly-water soluble profile and enhance the flavonoid's bioavailability.

2. Materials and methods

2.1. Materials

Rutin was purchased from Acros Organics (Geel, Belgium). Polysorbate 80 (Tween®80) and Sorbitan monostearate 80 (Span®80) were obtained from Acofarma (Barcelona, Spain). L- α -Phosphatidylcholine was purchased from (Sigma-Aldrich, St. Louis, MO, USA). For all analyzes, Milli-Q water was used (water filtered through the Millipore system).

2.2. Production of micelles

In the production of aqueous micelle dispersions and to promote the encapsulation of lipophilic drugs in the micelles, hot shear homogenization technique and ultrasonication method were used, with some modifications. Rutin's concentration was 0.1% (w/w) which was mixed with 4.9% (w/w) of Soy Lecithin. These two ingredients formed the lipid phase. The aqueous phase contained 94% (w/w) of Milli-Q water and 1% surfactant (Tween®80 and Span®80). The compositions of the developed micelles are shown in **Table 1**. The aqueous phase was added to the lipid phase and both was heated under constant stirring until boiling. The resulting dispersion was subjected to high-speed stirring (7000 rpm) using an Ultra-Turrax for 5 minutes. Then, this pre-emulsion was subjected to a probe sonication for 5 minutes and 70% amplitude. The obtained formulations were split into two batches and stored at different temperatures (25°C and 4°C) for a period of 60 days, and physiochemically characterized. For comparison purposes, a blank sample formulation (without rutin) was also prepared following the same procedure without adding the drug to the inner lipid phase. The composition of the developed formulations is present in **Figure 3**.

Table 1: Composition of the developed hybrid micelles with and without rutin (Captions: F, sample with active ingredient; V, sample without active ingredient).

Sample	Rutin (%, w/w)	Soy Lecithin (%, w/w)	Polysorbate 80 (%, w/w)	Span 80 (%, w/w)	Water ad. (%, w/w)
F2	0.1	4.9	0.6	0.4	ad 100
V3	0.0	5.0	0.6	0.4	ad 100

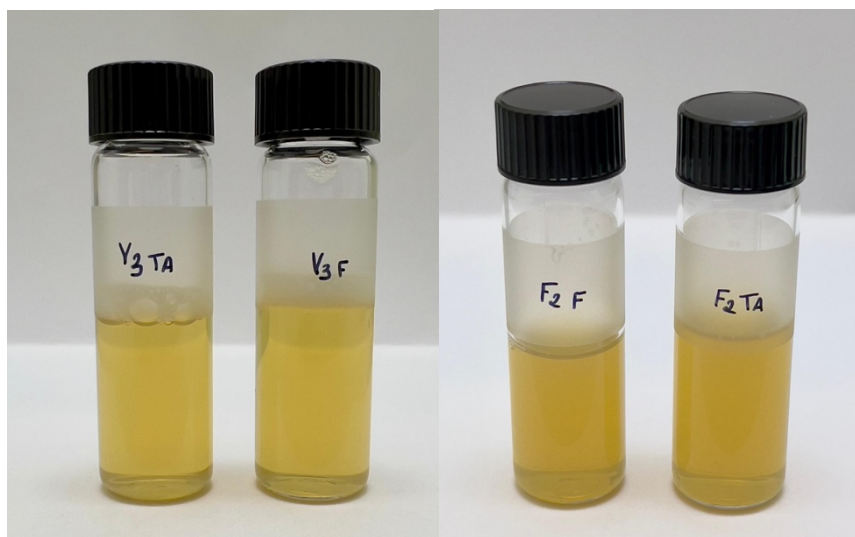


Figure 3. Photography blank micelles, V3 (left-hand side) and Rutin-loaded micelles, F2 (right-hand side) captured one day after production. (TA- room temperature; F- 4°C).

2.3. Hydrodynamic size, polydispersity and zeta potential

In a ZetaSizer series (Malvern Instruments, Malvern, UK), dynamic light scattering (DLS) was used to record the average hydrodynamic size (Z-Ave) and polydispersity index (PDI). Prior to the measurements, all samples were diluted (1:10) with Milli-Q water. All samples were analyzed in triplicate (10 runs per measurement). Data were analyzed at a temperature of 25°C and expressed as the arithmetic mean $\pm$ standard deviation (SD). The zeta potential (ZP), which is an indirect measure of the surface electrical charge of the particles was determined by measuring the electrophoretic mobility using a ZetaSizer series (Malvern Instruments, Malvern, UK). Samples were diluted (1:10) with Milli-Q water, the conductivity adjusted to 50 $\mu\text{S}/\text{cm}$, and subsequently analyzed at 25°C. To calculate the zeta potential, charge and mobility of the analyzed samples, the average of six runs, each one with ten cycles was obtained. After an electric field has been applied to a dispersion of particles, particles will migrate, with a speed proportional to the magnitude of the ZP, toward the oppositely charged electrode [49].

2.4. Transmission electron microscopy analysis

To observe the morphological characteristics, a transmission electron microscope (TEM) was used. A 20 mL sample was deposited for two minutes on a carbon film-coated 300 mesh copper grid (agar). Filter paper was used to absorb the leftovers. Each sample was negatively stained, for one to two minutes, with 2% w/v uranyl acetate. The grid was then allowed to air

dry. A Zeiss EM900 transmission electron microscope (Oberkochen, Baden-Württemberg, Germany) running at an 80 kV acceleration voltage was utilized for the grid analysis.

2.5. Differential Scanning Calorimetry

The thermal properties of the developed formulations were analyzed by differential scanning calorimetry using a DSC 200 F3 Maia® (NETZSCH, Selb, Germany). This equipment includes a twin oven with two settings. One position is taken by the crucible holding the sample for analysis. The empty reference crucible is positioned in the opposite location. Rutin-loaded micelle samples were weighed into an aluminum crucible containing approximately 5 mg of the flavonoid. After being closed, the crucible was placed in the oven and set to a temperature program that alternates between 10°C and 70°C at a heating rate of 10°C/min while nitrogen is flowed at a rate of 30 ml/min. Bulk rutin was subjected to the same conditions, yet up to a maximum temperature of 280°C (above the melting point temperature). A thermogram was generated and the melting onset temperature, enthalpy and melting point were recorded using the Proteus® 6.1.0B Thermal Analysis software (NETZSCH, Selb, Germany).

2.6. Encapsulation parameters (EE% and LC%)

Encapsulation efficiency (EE%) of rutin-loaded micelles was determined by calculating the ratio between the loaded flavonoid and the total amount of drug initially weighted to produce the micelles. The loading capacity (LC%) was determined by calculating the ratio between the loaded flavonoid and the total amount of lipids initially weighted to produce the micelles, using the following equations:

$$EE(\%) = \frac{\text{total amount of rutin} - \text{free amount of rutin}}{\text{total amount of rutin}} \times 100$$

$$LC(\%) = \frac{\text{total amount of rutin} - \text{free amount of rutin}}{\text{total amount of lipid}} \times 100$$

In order to determine the amount of free-rutin, micelle dispersions were placed into an ultrafiltration unit (10KDa Amicon Ultra; Merk Millipore®, Carrigtwohill, Ireland) and centrifugated at 3500 rpm for 30 minutes (Centrifuge Eppendorf, AG 5804, Hamburg, Germany). After centrifugation, a clear solution was obtained and 1 ml of the sample supernatant was collected, representing the unencapsulated drug (free rutin). The supernatant volume was diluted with 1 ml of methanol. A UV-Vis detector (Jasco V-650 Spectrophotometer, Tokyo, Japan) was used quantify the rutin by determining the absorbance of the sample at 206 nm and quantified against a calibration curve ($y = 0.1843x + 0.1154, r^2 = 0.9981$).

2.7. Rheological analysis

Using a cone-and-plate test geometry (plate diameter of 20 mm) and the Kinexus Lab + Rheometer from Malvern (Malvern, Worcestershire, UK), rheological study of micelle dispersions was carried out using the oscillation frequency sweep test. The analysis frequency range was from 0 to 10 Hz, and the stress amplitude was kept at 1 Pa. Each sample was placed into the lower geometry of the equipment in a sufficient amount. The upper geometry was then subjected to a torque, which increased the shear stress. The formulations underwent analysis at a temperature of $25\text{ }^{\circ}\text{C} \pm 1\text{ }^{\circ}\text{C}$, with a gap of 1 mm between the plates. A frequency sweep was performed in the oscillation mode, ranging from 0.1 to 10.0 Hz, with a strain of 1%. The rSpace for Kinexus Lab + program (version 1.75 Malvern Instruments, Worcestershire, UK) was utilized to collect the data.

3. Results and Discussion

The micelles were produced using Polysorbate 80 and Span 80 as stabilizers in the aqueous phase. The hydrodynamic size of the micelles was studied, as this parameter significantly contributes to the drug penetration into the skin. Blank micelles and micelles containing rutin were successfully produced and characterized by dynamic light scattering. The Z-Ave and PDI of all developed formulations (rutin-free and rutin-loaded micelles) were determined over time on day 1, and then 7 days, 15 days, 30 days, 45 days and 60 days after production. The Z-Ave of the blank micelles ranged from $77.95 \pm 0.35\text{ nm}$ to $83.75 \pm 1.05\text{ nm}$, and for the rutin-loaded micelles from $72.6 \pm 0.31\text{ nm}$ to 87.10 ± 6.38 over the timeframe of 60 days at different temperatures. The results are presented in **Table 2**.

Table 2. Z-Ave and PDI for blank and rutin-loaded micelles on the 1, 7, 15, 30, 45 and 60 days after the production.

Samples	Parameter	Day 1	Day 7	Day 15	Day 30	Day 45	Day 60
Blank micelles (25°C)	Z-Ave $\pm$ SD	78.88 $\pm$ 0.71	77.95 $\pm$ 0.35	82.17 $\pm$ 1.11	82.24 $\pm$ 0.43	82.93 $\pm$ 0.63	83.75 $\pm$ 1.05
	PDI	0.241 $\pm$ 0.009	0.251 $\pm$ 0.011	0.234 $\pm$ 0.002	0.243 $\pm$ 0.011	0.251 $\pm$ 0.004	0.242 $\pm$ 0.012
Blank micelles (4°C)	Z-Ave $\pm$ SD	79.23 $\pm$ 0.43	79.58 $\pm$ 1.14	78.95 $\pm$ 1.32	79.03 $\pm$ 0.33	80.01 $\pm$ 0.74	79.84 $\pm$ 0.53
	PDI	0.245 $\pm$ 0.006	0.243 $\pm$ 0.006	0.249 $\pm$ 0.003	0.246 $\pm$ 0.008	0.247 $\pm$ 0.011	0.248 $\pm$ 0.005
Rutin-loaded micelles (25°C)	Z-Ave $\pm$ SD	78.99 $\pm$ 0.86	79.57 $\pm$ 0.50	79.08 $\pm$ 0.48	78.58 $\pm$ 1.33	81.62 $\pm$ 2.18	87.10 $\pm$ 6.38
	PDI	0.251 $\pm$ 0.004	0.259 $\pm$ 0.007	0.265 $\pm$ 0.013	0.273 $\pm$ 0.008	0.295 $\pm$ 0.043	0.328 $\pm$ 0.066
Rutin-loaded micelles (4°C)	Z-Ave $\pm$ SD	78.29 $\pm$ 0.66	80.69 $\pm$ 0.53	81.08 $\pm$ 1.68	72.6 $\pm$ 0.31	78.54 $\pm$ 0.25	78.72 $\pm$ 2.01
	PDI	0.251 $\pm$ 0.004	0.251 $\pm$ 0.006	0.254 $\pm$ 0.008	0.253 $\pm$ 0.011	0.258 $\pm$ 0.002	0.265 $\pm$ 0.011

With the aging, the size of the micelles slightly increases and the same pattern could also be observed in relation to the PDI value, attributed to the dynamic behavior of the lipid vesicles when dispersed in water. However, 60 days after production, the increase was notably emphasized in the rutin-loaded micelles formulation when stored at 25°C, which also depicted the lowest zeta potential values. These events affected the stability of this sample (increase in PDI values), favoring the possible aggregation of particles and subsequent increase in their size.

Regarding the different storage temperatures, both in the blank micelles and in the micelles loaded with rutin, stored at a temperature of 4°C, registered a micelle size value smaller than the micelles stored at 25°C. After eight weeks, the variation in the size of micelles loaded with rutin at different temperatures was 87.1 ± 6.38 nm, for the preparation stored at 25°C and 78.72 ± 2.01 nm, at 4°C. Regarding the PDI, as previously mentioned, it maintained a relatively low value and without major variations over the eight weeks, being a good prognosis for the long-term stability of the micelles, as the developed micelles can then be considered monodisperse.

A material's surface, suspended particles, and macromolecules can all display electrical charge that can be indirectly determine by measuring the potential in the zeta layer. High values of the zeta potential (ZP), whether positive or negative, owing to the presence of electrostatic repulsion, indicate good physical stability of the dispersion. Understanding the mechanism underlying the drug-nanocarrier interaction can be estimated with the help of ZP determination [50, 51]. Formulations are usually considered stable when ZP values are close to or above +30 or -30 mV. In this study, the micelles present high negative values, showing good physical stability. ZP of empty micelles stored at 25°C was -59.18 ± 0.46 [mV] (day 1) to -60.79 ± 1.25 [mV] (day 60) and stored at 4°C was -56.74 ± 1.81 [mV] (day 1) to -58.31 ± 1.5 [mV] (day 60), whereas of rutin loaded-micelles stored at 25°C was -53.68 ± 2.13 [mV] (day 1) to -39.32 ± 4.74 [mV] (day 60) and stored at 4°C was -57.38 ± 0.50 [mV] (day 1) to -58.71 ± 1.86 [mV] (day 60). All of the results are presented in **Table 3**.

Table 3. Zeta Potential (ZP [mV] $\pm$ SD) for blank and rutin-loaded micelles on the 1, 7, 15, 30, 45 and 60 days after the production.

Samples	Storage Temperature (°C)	Day 1	Day 7	Day 15	Day 30	Day 45	Day 60
Blank micelles	25	-59.18 $\pm$ 0.46	-65.45 $\pm$ 1.79	-57.46 $\pm$ 3.17	-57.1 $\pm$ 1.16	-58.25 $\pm$ 0.29	-60.79 $\pm$ 1.25
Blank micelles	4	-56.74 $\pm$ 1.81	-60.36 $\pm$ 1.01	-59.73 $\pm$ 0.66	-59.02 $\pm$ 1.11	-60.61 $\pm$ 2.36	-58.31 $\pm$ 1.50
Rutin-loaded micelles	25	-53.68 $\pm$ 2.13	-55.18 $\pm$ 5.04	-49.72 $\pm$ 2.91	-48.22 $\pm$ 2.43	-45.29 $\pm$ 2.04	-39.32 $\pm$ 4.74
Rutin-loaded micelles	24	-57.38 $\pm$ 0.50	-58.67 $\pm$ 1.93	-55.42 $\pm$ 1.53	-61.74 $\pm$ 2.32	-59.93 $\pm$ 2.33	-58.71 $\pm$ 1.86

It was found that the blank micelles at the two storage temperatures, as well as the rutin-loaded micelles stored at 4°C exhibited a general tendency of a slight increase in ZP, in modulus, over time. However, rutin-loaded micelles stored at room temperature (25°C) showed a more notable variation, with a considerable reduction in ZP modulus. On day 1, the ZP value was -53.68 ± 2.13 [mV] while, eight weeks later, the ZP value recorded was -39.32 ± 4.74 [mV]. These data are in agreement with the results obtained for the hydrodynamic size and PDI.

TEM images shown in **Figure 4** depict the micelle morphology of loaded formulations with 0.1% rutin, showing a spherical shape and hydrodynamic size lower than 100 nm. These results support the data from the Z-Ave measurements recorded by DLS. In the DLS technique, the hydrodynamic diameters of the hydrated micelles were recorded, while the TEM analysis the data represents the size of the micelles in the dry state. The results suggest no without aggregation of micelles after their loading with rutin

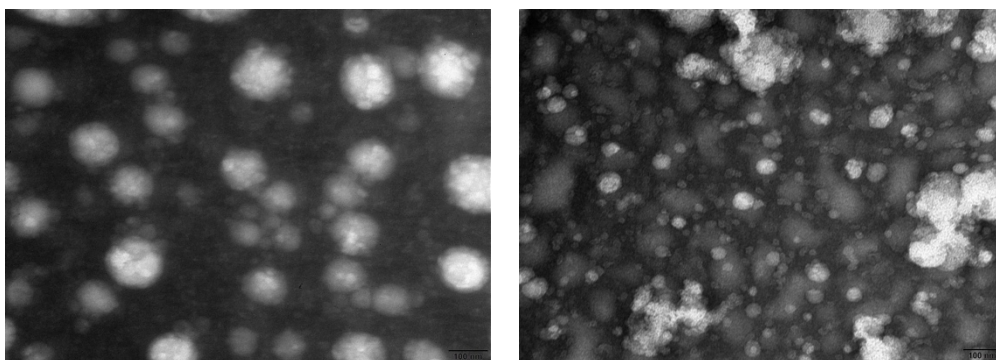


Figure 4. TEM picture of rutin-loaded micelles.

Figure 5 shows the differential scanning calorimetry (DSC) profile of the bulk rutin. Rutin shows three endothermic peaks at 162°C, 177.2°C and 212.3°C (this last value corresponds to the melting point). **Figure 6** shows the thermogram of the blank micelles and the rutin-loaded micelles stored at two different temperatures. The thermograms depicted in Figure 6 are typical of vesicles made from phospholipids, without endothermic peaks. These profiles also confirm no chemical interactions between the rutin and the micelles, as the blank sample and the micelle sample loaded with rutin have similar DSC profiles. No significant influence of the storage temperature was also registered, with the profiles being very similar among the samples stored at different temperatures.

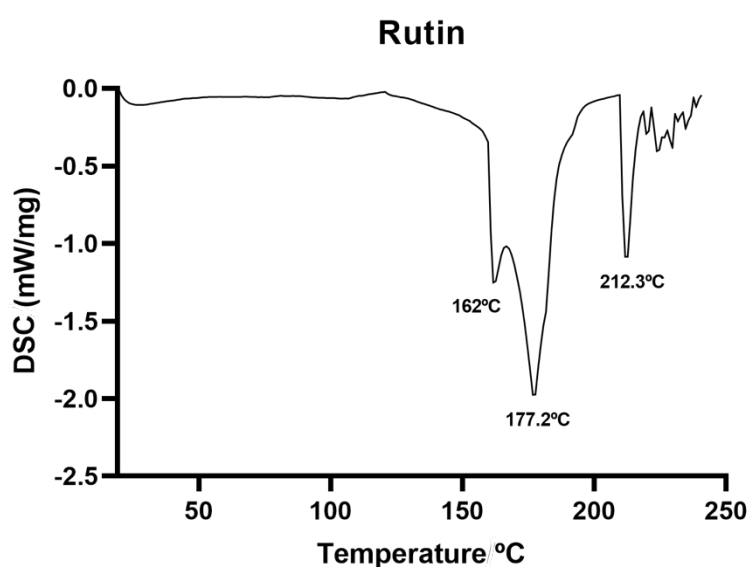


Figure 5. DSC analysis of bulk rutin.

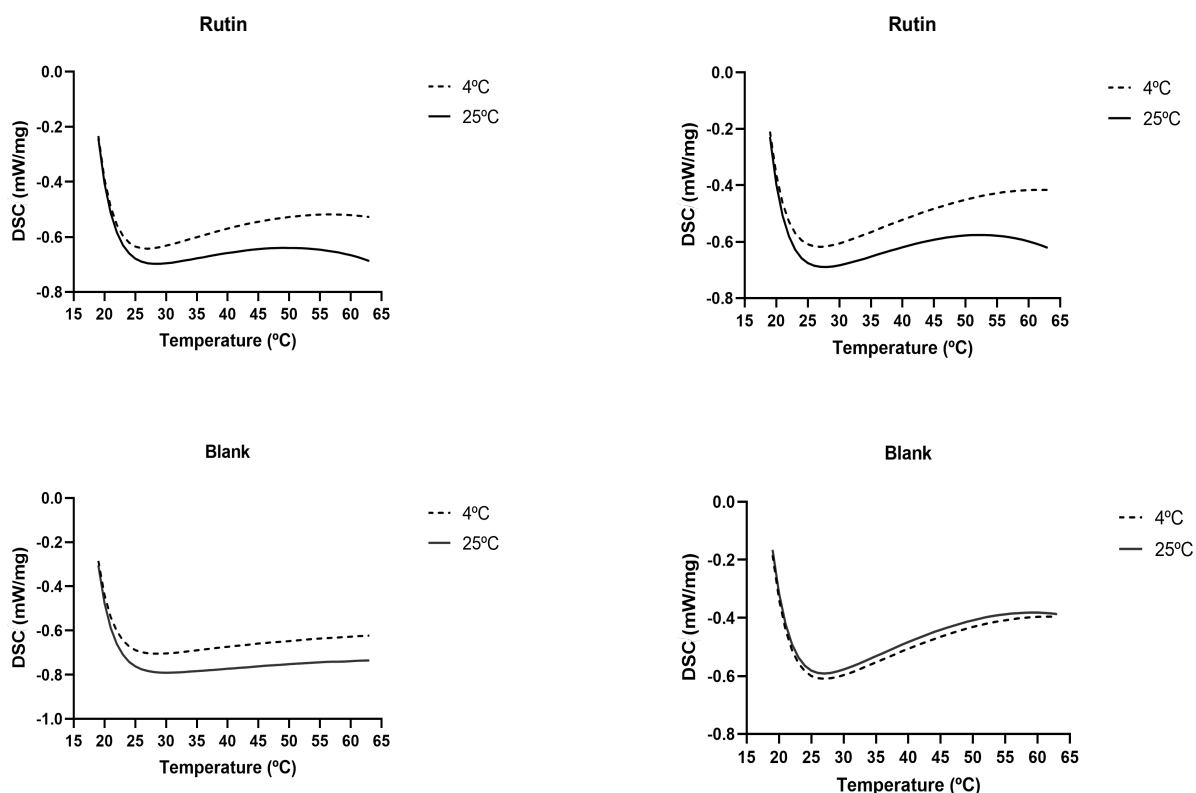


Figure 6. DSC analysis of blank and Rutin-loaded micelles, 24h after production (left-hand side) and 2 months after production (right-hand side).

The encapsulation efficiency (EE) and loading capacity (LC) were determined as quantitative and qualitative parameters of the newly developed production method. The EE reached 92.09% and the LC was 1.96%.

The rheology profiles of the developed formulations recorded 24 hours after production are shown in **Figure 7**. **Figure 8** shows the profiles of the samples recorded 2 months after production. For comparison purposes between blank sample and rutin-loaded micelles sample, the storage modulus (G'), loss modulus (G'') and shear viscosity at two different times, 24h after production and 2 months after production, were recorded. The storage modulus or elastic component quantifies the energy stored within the material during deformation and subsequently recovered after stress release, i.e., it resembles the amount of energy that the dispersion needs to be distorted, while the loss modulus or viscous component translates the energy lost during deformation. Storage modulus and loss modulus are key parameters used to characterize the viscoelastic behavior of materials.

As seen in all profiles, the elastic component was higher than the viscous component in the applied frequency range, both after 24h of production and after 2 months, regardless the different storage temperatures. When that happens, it means that the sample presents more elastic behavior than viscous behavior. When the frequency is low, the shear rate is also low.

This indicates that dispersions have a great capacity to preserve the medium's initial strength. When the frequency range increases, the shear rate increases as well, requiring more energy and raising the viscous and elastic components in the process. The G'' showed a lower dependence on the applied force, while the G' increased significantly in the frequency range highly dependent on it. This same dependence could be observed in the shear viscosity which also increases as the frequency increases. Comparing the viscosity of all formulations, it can be stated that samples stored at a temperature of 4°C has higher values than samples stored at 25°C after 24h of production. However, the shear viscosity values after 24 hours of storage, compared to the values after 2 months of storage, are different. In micelle samples loaded with rutin, the shear viscosity values increased after 2 months of preparation, compared to the values measured after 24h. The opposite occurred with the blank samples; the shear viscosity values decreased over time. The sample that presented the highest shear viscosity values after 24h was the blank sample at a storage temperature of 4°C, followed by the sample of micelles loaded with rutin stored at 4°C and then the sample of micelles loaded with rutin at a storage temperature of 25°C, finally the sample that presented the lowest values was the blank sample at a storage temperature of 25°C. After 2 months, the paradigm changed. These results may mean that the storage temperature is not a parameter that has an influence on the viscoelastic behavior of the studied samples.

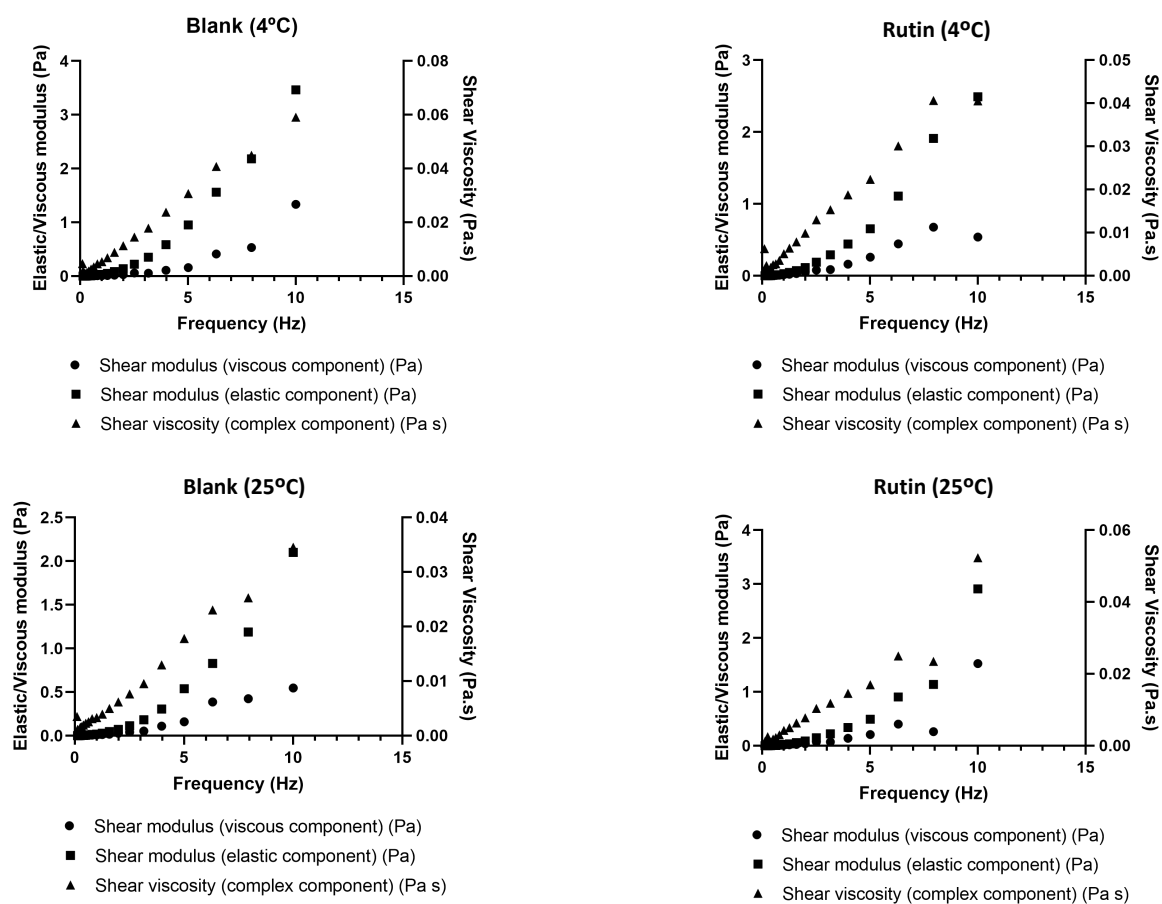


Figure 7. Rheological analysis of blank and Rutin-loaded micelles 24h after production.

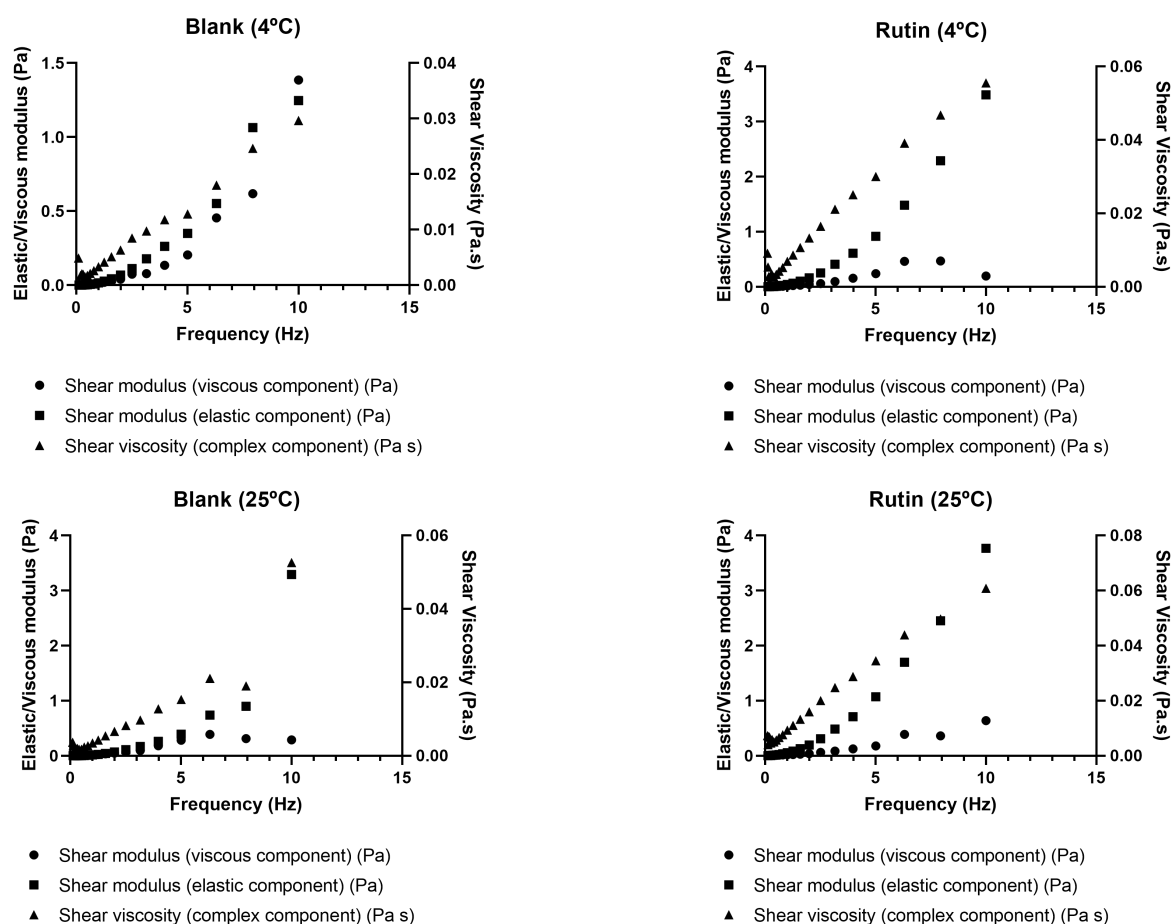


Figure 8. Rheological analysis of blank and Rutin-loaded micelles 2 months after production.

4. Conclusions

In this study, rutin-loaded micelles were successfully developed. Rutin-loaded micelles with appropriate size, high values of zeta potential, high encapsulation rate and good stability were obtained. The Z-Ave and ZP of the micelles remained practically the same after 2 months of production, yet the storage temperature influenced these parameters. This formulation had a notable increase in size and a decrease in the ZP value when stored at 25°C. In the rheological analysis, rutin-loaded micelles exhibited an increase of the shear viscosity values after 2 months of storage, compared to the values measured after 24h after production of the vesicles. The opposite occurred with the blank samples; the shear viscosity values decreased over time, suggesting that the presence of the bioactive compound influences the viscosity of the sample. The high EE and LC of rutin confirm the added-value of the developed method to overcome the problem of poor-water solubility drugs.

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Chapter 3: Polyphenols-composed micelles: dual loading of resveratrol and rutin as nutrients for skin diseases

Abstract

Nanotechnology is receiving increasing interest as an approach to develop controlled delivery systems for a great deal of drugs and bioactives, including natural compounds in order to increase their physicochemical stability. In this study, the physicochemical characterization of micelles loaded with resveratrol (F1) and micelles loaded with rutin and resveratrol (F4), and the stability of these formulations were studied. Analyzes were carried out to determine Z-Ave, PDI and ZP, 24 h after production and after 2 months of storage at two different temperatures (4°C and 25°C). Furthermore, rheological properties and thermal properties were recorded, also at two different times (24h later and 2 months later). The EE and LC were also analyzed. Samples F1 and F4 record Z-Ave and PDI values that are in the same range of values, with no noticeable differences between them. There were no marked changes over time in the Z-Ave and PDI values, thus confirming the good stability achieved in the formulations. Sample F1 showed an EE of about 98.69%, while sample F4 reached an EE of for 99.86% resveratrol and 98.31% for rutin. The viscoelastic profile showed that the G' was higher than the G'' in the applied frequency range, for both samples, demonstrating that they are more elastic than viscous. After 2 months, all loaded samples obtained higher viscosity values, showing that the presence of bioactive compounds increases the viscosity of the formulations. The DSC showed the absence of peaks, corroborating that in both samples no drug crystallization happened. The high EE values showed that the bioactive compounds are found within the micelles. The TEM study confirmed the morphology and shape of the produced micelles, which show potential for diverse biomedical applications.

Keywords: Resveratrol, Rutin, Natural compounds, Hybrid Micelles, Encapsulation, Nanotechnology.

1. Introduction

The treatment of skin disorders includes a variety of drugs and products that can be used either external or internal, such as UV protectors, antioxidants, anti-inflammatories, antibiotics, moisturizing agents, healing agents and analgesics [1]. However, these therapies considered conventional are often not sufficient to treat inflammation and proliferation of skin wounds [2]. As an alternative and/or complementary solution, plants and fruits are two of the natural sources of pharmacologically active ingredients, such as flavonoids and polyphenolic compounds, with several beneficial properties to improve the integrity and health of the skin [3]. Resveratrol (RES) (**Figure 1**) is a polyphenolic non-flavonoid compound and also a natural phytoalexin that is produced by plants during pathogen attack [4] and can be found in grapes (skin and seeds), nuts, berries and red wine [5].

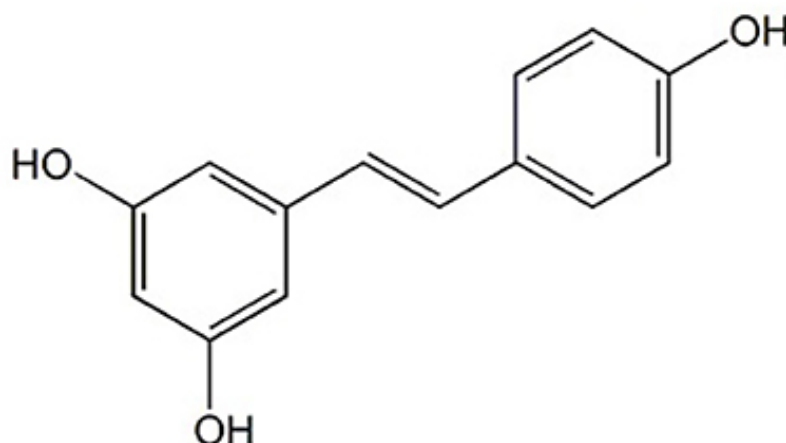


Figure 1. Chemical structure of Resveratrol (reproduced after Chedea, Veronica Sanda et al. (2021) [6], under the terms and conditions of the Creative Commons Attribution (CC BY) license.

RES has numerous beneficial properties that include anti-inflammatory and immunomodulatory activity promoting wound healing and angiogenesis [7-10]. It is also used in cosmetics as a photoprotector and anti-aging due to its collagen-stimulating and antioxidant properties [11, 12]. Of particular relevance to this work, RES has been widely studied in recent years for its antimicrobial activity against a wide range of microorganisms and successful treatments of various skin diseases such as wounds [13], acne [14] and melanoma [15]. Although RES is relatively well absorbed orally, it undergoes a first-pass hepatic elimination process, which results in low oral bioavailability and, in addition, it has a very short half-life,

around 8 to 14 minutes. These problems associated with RES are the main causes of its limited use in delivery systems [16, 17]. Therefore, the topical administration of RES is considered a suitable alternative over the oral route, since RES metabolism in the skin is significantly lower than in the liver and, consequently, a higher amount of bioactive may be delivered to the site of interest [18]. Furthermore, topical RES treatment is associated with a lower risk of side effects when compared to oral treatment and potential skin irritations due to are reported to be low [19, 20]. However, due to RES hydrophobicity and the difficulty in controlling its fate in the skin, topical treatment is still a challenge [21].

In order to widen the use of RES in various topical treatments, the use of nanotechnology is an approach to be considered, as this area is undergoing increasing development in the pharmaceutical and cosmetic industries [22, 23]. The low solubility and low chemical stability of RES are disadvantages which may be overcome when loaded into nanocarriers. The encapsulation of RES in nanocarriers will provide higher chemical stability, higher quantity of available compound, improved targeting to sites of interest, controlled release, less side effects and better skin penetration [24, 25].

Several lipid nanosystems are available, such as self-(micro) emulsifying drug delivery systems [26], nanoemulsions [27], nanostructured lipid carriers, solid lipid nanoparticles [28] and hybrid systems i.e., combining lipid materials and polymers, such as lipid nanocapsules and micelles with a lipid core.

A micelle is understood to be an aggregate or supramolecular structure of a surfactant molecule dispersed in a liquid colloid with a mean diameter ranging from 5 to 100 nm [29]. Lipid core micelles are normal phase micelles that are formed by molecules with two regions with different affinity to water, one hydrophilic that faces the outside and the other is hydrophobic facing the interior of the core [30]. These molecules with two regions with different affinities to water are called amphiphiles.

Due to their size and structure, micelles have several advantages as delivery system for bioactive compounds. As this structure is composed by amphiphilic molecules, two regions i.e., a hydrophilic region and a hydrophobic region, are generated making micelles able to carry lipophilic and hydrophilic compounds [31-33]. Micelles have the ability to remain stable for long periods of time, they are able to get the loaded bioactive to specific locations of interest, increasing their bioavailability and solubility [31, 34-36]. In this work, we describe the development of innovative hybrid micelles for resveratrol and tested the same systems for the dual loading of resveratrol and rutin (already formulated as described in Chapter 2), with respect to physicochemical stability, encapsulation parameters and rheological profile.

2. Materials and Methods

2.1. Materials

The materials used in the study included Polysorbate 80 (Acofarma, Barcelona, Spain), L- α -Phosphatidylcholine (Sigma-Aldrich, St. Louis, MO, USA), Resveratrol (Fagron, Barcelona, Spain), Rutin (Acros Organics, Geel, Belgium), and Milli-Q water was home supplied.

2.2. Production of micelles

In the production of aqueous micelle dispersions, the hot shew homogenization technique, followed by ultrasonication method. Samples of micelles loaded with resveratrol, micelles loaded with rutin and resveratrol and blank micelles (without any bioactive ingredient) were prepared. The compositions of the developed micelles are shown in **Table 1**.

Table 1: Composition of the prepared Resveratrol loaded micelles and resveratrol-rutin-loaded micelles (Captions: F, sample with active ingredient; V, sample without active ingredients).

Sample	Resveratrol (%, w/w)	Rutin (%, w/w)	Soy Lecithin (%, w/w)	Polysorbate 80 (%, w/w)	Water ad. (%, w/w)
F1	0.10	-	4.90	1.00	94.00
F4	0.05	0.05	4.90	1.00	94.00
V1	-	-	5.00	1.00	94.00

To produce the developed micelles, the aqueous phase was added to the lipid phase and both were heated under constant stirring until the mixture boiled. After that, the mixture was subjected to high-speed stirring using an Ultra-Turrax for 5 minutes at 7000 rpm and then subjected to a sonicator for 5 minutes with an amplitude of 70%. The resulting dispersions were stored at two different temperatures, 25°C and 4°C until further use. For the loaded micelles, the bioactives were added to the lipid phase prior mixing to the water phase. The dispersions were monitored and characterized for 60 days, as described in following sections.

2.3. Hydrodynamic size, polydispersity index and zeta potential

Average hydrodynamic size (Z-Ave) and size distribution (PDI) of loaded and blank micelles were determined by the dynamic light scattering method. For these analyzes the Zetasizer PRO (Malvern Instruments, Malvern, UK) was used. Prior to analysis, the samples were diluted with Milli-Q water (1:10) to achieve a suitable intensity. The zeta potential (ZP) was measured in the same device, after adjusting conductivity to 50 μ S by measuring the

electrophoretic mobility of the particles. The samples were all analyzed at 25°C and the data presented as mean $\pm$ standard deviation.

2.4. Transmission electron microscopy

The morphology and size of the developed micelles in their dry state were analyzed using transmission electron microscopy (TEM) (Zeiss EM900, Florida, USA). The device performed the analysis at 80 kV. For the analysis to carry out, 20 mL of sample was placed on a carbon film on a 300-mesh copper grid (agar) for 2 minutes. Excess solution was absorbed with filter paper and the grid was air dried. Subsequently, the samples were negatively stained with uranyl acetate solution (2% w/v).

2.5. Differential Scanning Calorimetry

Differential Scanning Calorimetry (DSC) analysis of the developed micelle samples was carried out using the DSC 200 F3 Maia ® equipment (NETZSCH, Selb, Germany). Approximately 5 mg of each sample was weighed into an aluminum crucible that was subsequently sealed, placing it in position 1. In position 0, an empty crucible was placed, representing the reference. The bulk resveratrol sample was heated from 10°C to 290°C (20°C above the melting point) and the bulk rutin sample was heated from 10°C to 280°C (20°C above melting point). The heating rate of both was 10°C per minute. Sample cells were purged with nitrogen (flow 30 ml/min). Samples of micelles were subjected to the same conditions, but up to a maximum temperature of 70°C. As a result, a thermogram was generated and the analysis of enthalpy data, melting start temperature and melting point was carried out using the software Proteus® 6.1.0B Thermal Analysis (NETZSCH, Selb, Germany).

2.6. Encapsulation parameters

The encapsulation efficiency (EE) and loading capacity (LC) of resveratrol in the F1 sample was determined after separation, by centrifugation, of the non-incorporated drug. The sample was deposited in an ultrafiltration unit (10KDa Amicon; Merk Millipore®, Carrigtwohill, Ireland) and centrifugation (Centrifuge Eppendorf, AG 5804, Hamburg, Germany) occurred at 3500 rpm for 30 minutes to isolate the particles from the suspension. Then, 1 ml of the clear supernatant was diluted with 1 ml of methanol and quantified against a calibration curve ($y = 0.1422x - 0.0524, r^2 = 0.9991$) to determine the drug concentration. A UV-Vis detector (Jasco V-650 Spectrophotometer, Tokyo, Japan) was used to measure the drug content at 306 nm. The same method was used for sample F4. As this sample contains two different active ingredients, rutin and resveratrol, to quantify it we need the calibration curves of the two substances, the one for resveratrol has already been mentioned above and the one for rutin is as follows: $y = 0.1843x + 0.1154, r^2 = 0.9981$ (See *Chapter 2, section 2.6*). Rutin was recorded at 355 nm.

The EE and LC were then calculated according to the following equations:

$$EE(\%) = \frac{M(T) - M(f)}{M(T)} \times 100L \quad LC(\%) = \frac{M(T) - M(f)}{M(lipid)} \times 100$$

where $M(T)$ is the total amount of resveratrol in micelles; $M(f)$ is the amount of free resveratrol in micelles dispersion and $M(lipid)$ is the total amount of lipid in micelles.

2.7. Rheological analysis

Rheological analyzes of the developed micelle samples were carried out using a Kinexus Lab + rheometer (Malvern, Worcestershire, UK), equipped with a 20 mm plate diameter probe. The test applied for the analysis was the oscillation frequency sweep test at a constant stress amplitude of 1 Pa. The frequency range used was 0 to 10 Hz. The samples were measured at 25°C, with a gape of 1 mm between the equipment plate and the probe. A small portion pf the sample was placed on the equipment plate and shear stress were applied. After analysis, data were collected using the rSpace for Kinexus Lab + software (version 1.75 Malvern Instruments).

3. Results and Discussion

All loaded micelles were produced with 4.9% (w/w) of lipid in their composition, namely, soy lecithin and 0.1% (w/w) of active compound which represent the lipid phase of the system. The aqueous phase was composed of 94% (w/w) of water and 1% (w/w) of surfactant (polysorbate 80). For blank micelles, the composition was the same, only the bioactive was not present. **Figure 2** depicts the photographs of the developed micelles, with a homogenous appearance.

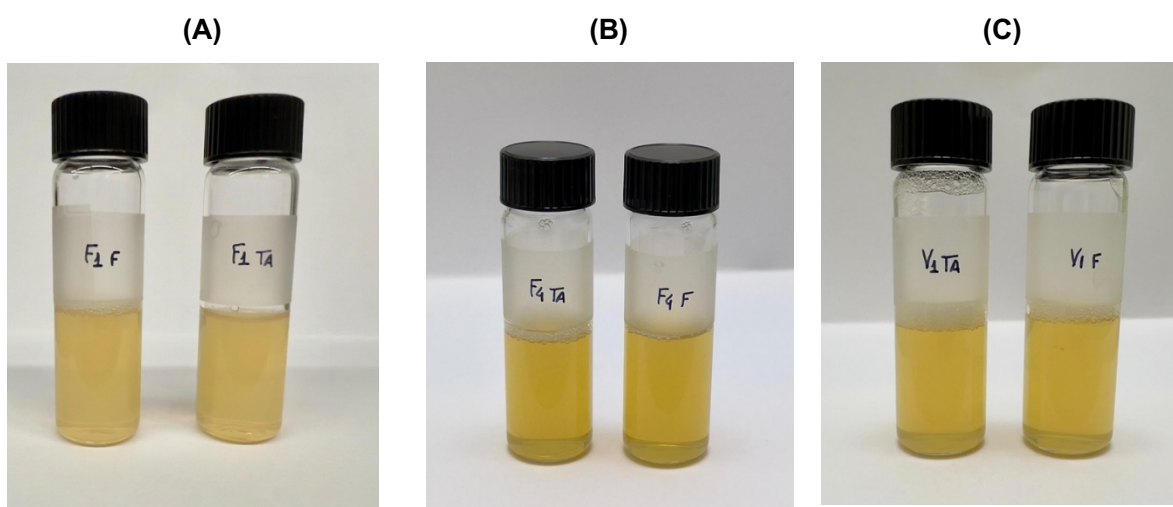


Figure 2. (A) Resveratrol-loaded micelles stored in the Fridge (F) at 4°C and at Ambient Temperature (TA) at 25°C; (B) Resveratrol-rutin-loaded micelles; (C) Blank micelles. Captions: F, Fridge at 4°C; TA, Ambient Temperature at 25°C; V, void (blank).

To evaluate the stability of the formulations and to determine the narrowness of the hydrodynamic size distribution, Z-Ave and PDI were analyzed. Once the micelles were produced, Z-Ave, PDI and ZP of all developed formulations were determined on day 1, day 7, day 15, day 30, day 45 and day 60 after production. Results 24 hours after the production (day 1) were obtained for the empty-micelles and loaded-micelles and reached about 64.34 ± 0.23 nm with PDI 0.272 ± 0.004 for blank micelles at 25°C and 65.37 ± 0.41 nm with PDI 0.278 ± 0.002 for blank micelles at 4°C. Micelles loaded with bioactives showed higher hydrodynamic size values. Resveratrol-loaded micelles had in Z-Ave 70.87 ± 1.05 nm with PDI 0.267 ± 0.004 when stored at 25°C and 69.47 ± 0.66 nm with PDI 0.273 ± 0.006 when stored at 4°C. The resveratrol-rutin-loaded micelles had a hydrodynamic size of 68.70 ± 0.41 nm with PDI 0.238 ± 0.008 when stored at 25°C and 70.41 ± 1.16 nm with PDI 0.276 ± 0.004 when stored at 4°C. As both samples presented a low PDI, it can be stated that the formulations were monodisperse. **Figure 3** shows the hydrodynamic size parameters and ZP values over storage time at different temperatures. The Z-Ave values did not undergo major changes. After eight weeks, the Z-Ave and PDI values recorded for each sample at day 60 were as follows: Blank micelles (25°C): Z-Ave 66.79 ± 0.72 nm with PDI 0.266 ± 0.009 ; Blank micelles (4°C): Z-Ave 69.19 ± 0.44 nm with PDI 0.305 ± 0.039 ; Resveratrol-loaded micelles (25°C): Z-Ave 72.04 ± 1.38 nm with PDI 0.293 ± 0.006 ; Resveratrol-loaded micelles (4°C): Z-Ave 69.64 ± 0.90 nm with PDI 0.266 ± 0.002 ; Resveratrol-rutin-loaded micelles (25°C): Z-Ave 68.92 ± 0.89 nm with PDI 0.265 ± 0.004 ; Resveratrol-rutin-loaded micelles (4°C): Z-Ave 69.73 ± 0.64 nm

with PDI 0.282 ± 0.005 . It should be noted that there were no marked changes over time in the Z-Ave and PDI values, thus confirming the good stability.

Regardless the storage conditions, for resveratrol-loaded micelles and resveratrol-rutin-loaded micelles, the Z-Ave values appeared to be practically identical to each other. The same can be said for blank micelles. PDI values were a little more inconsistent, however, no abrupt changes that could compromise the stability of the micelles were recorded. A high ZP value, such as $|\pm 30 \text{ mV}|$, is considered a value capable of providing good physicochemical stability to colloidal dispersion, since repulsive forces tend to prevent particle aggregation [37]. In this work, the results obtained are within the range of values described as being optimal for this parameter, in the blank samples and in the micelles loaded with bioactives, finding negative values of -41.96 to -64.18 mV, which is typical of lipid nanoparticles with values that provide adequate stability for the system. In general, over 2 months, ZP values remained within the range of acceptable values considered to be a good indicator of formulation stability; however, there were some variations comparing their values after 24h and after 2 months, namely, ZP of blank micelles was $-59.75 \pm 1.95 \text{ [mV]}$ (day 1) to $-59.75 \pm 0.72 \text{ (day 60)}$ when stored at 4°C and $-56.67 \pm 1.94 \text{ [mV]}$ (day 1) to $-57.63 \pm 1.49 \text{ (day 60)}$ when stored at 25°C, resveratrol-loaded micelles was $-61.62 \pm 0.70 \text{ [mV]}$ (day 1) to $-62.43 \pm 0.27 \text{ [mV]}$ (day 60) when stored at 4°C and $-59.08 \pm 2.38 \text{ [mV]}$ (day 1) to $-41.96 \pm 1.29 \text{ (day 60)}$ when stored at 25°C and resveratrol-rutin-loaded micelles was $-60.18 \pm 0.26 \text{ [mV]}$ (day 1) to $-63.11 \pm 2.34 \text{ [mV]}$ (day 60) when stored at 4°C and $-59.86 \pm 0.73 \text{ [mV]}$ (day 1) to $-57.2 \pm 2.05 \text{ (day 60)}$ when stored at 25°C (**Figure 4**).

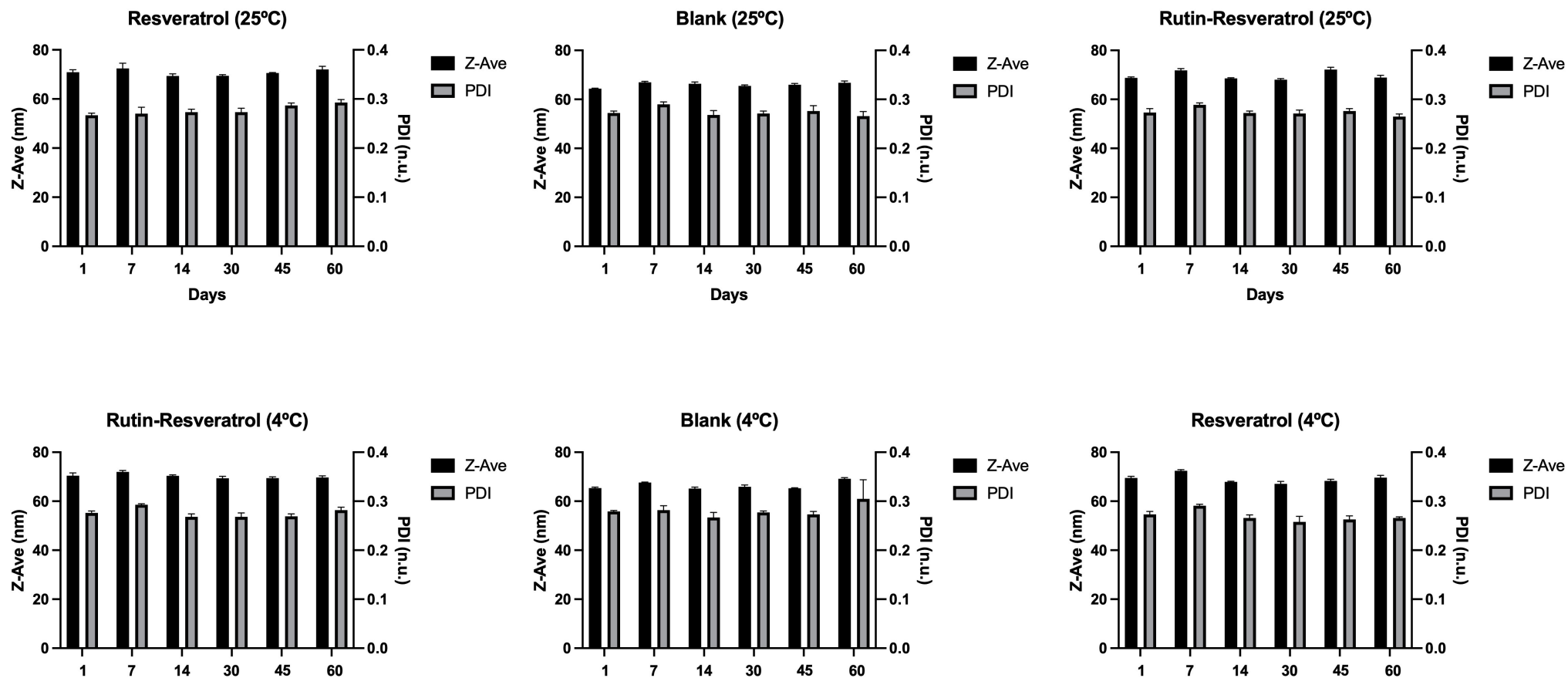


Figure 3. Z-Ave and PDI for blank, resveratrol-loaded micelles and resveratrol-rutin-loaded micelles on the 1, 7, 15, 30, 45 and 60 days after the production.

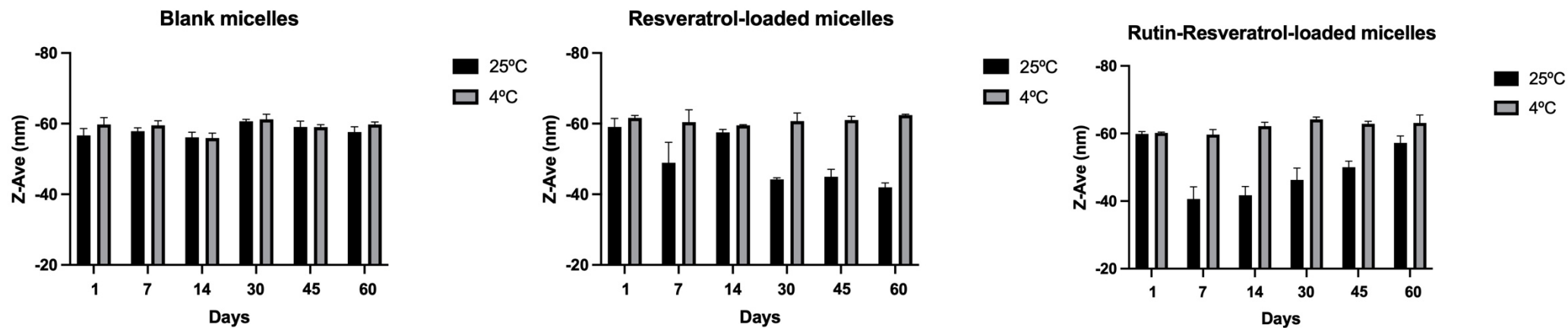


Figure 4. Zeta Potential for resveratrol-loaded micelles, resveratrol-rutin-loaded micelles and blank sample on the 1, 7, 15, 30, 45 and 60 days after the production.

In blank micelles, the ZP remained practically constant, regardless the storage conditions. The same cannot be said about micelles loaded with bioactives. A large difference was noted in the ZP values between the two micelles stored at distinct temperatures. For the micelles loaded with resveratrol stored at 4°C, the ZP had a similar behavior as the blank micelles, remaining stable over time. In micelles loaded with resveratrol stored at 25°C, there was a higher variation in values, and, in general, the ZP decreased (in modulus) over time. The same pattern occurred in the sample containing rutin- and resveratrol-loaded micelles on day one. Then, the ZP values gradually increased (in modulus) over time. This difference in behavior may mean higher instability when the formulation is stored at 25°C, compared to that stored at 4°C. It can be stated that the storage temperature of the samples is a governing factor for their stability, due to the different recorded values of the zeta potential in different temperatures of storage.

To obtain information about the morphology and shape of the developed micelles, TEM analysis was used. The images from the analysis showed micelles with a practically spherical shape with diameters below 100 nm in almost all micelles (**Figure 5**). The micelle sizes recorded in the TEM technique were slightly smaller than the values recorded in the DLS technique. This is due to the fact that in the TEM technique the micelles analyzed are in the dry state and in the DLS the micelles are in the hydrated state (hydrodynamic diameter), hence the size is larger. Nevertheless, the data collected by TEM images confirm and corroborate the data collected by DLS regarding the Z-Ave of resveratrol-loaded micelles.

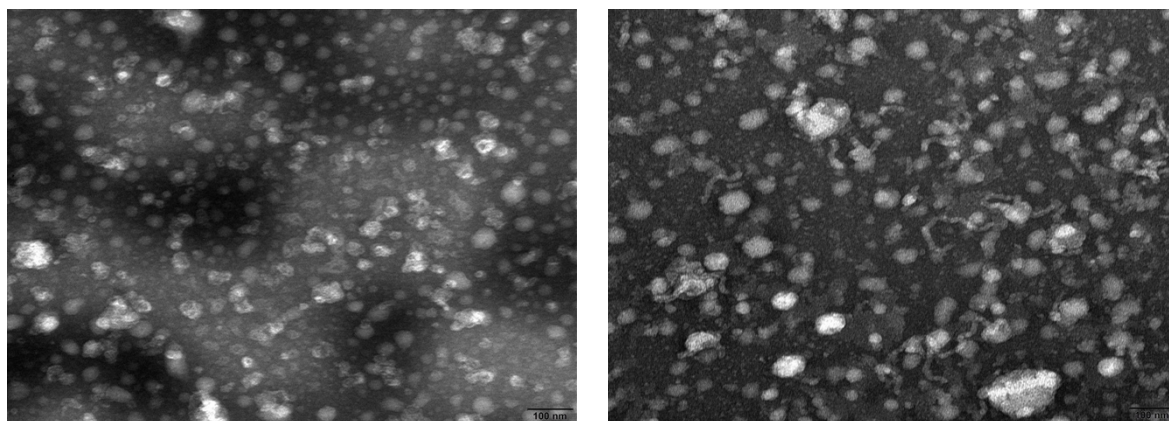


Figure 5. TEM picture of resveratrol-loaded micelles (left-hand side) and rutin-resveratrol-loaded micelles (right-hand side).

The thermogram of bulk resveratrol sample showed an endothermic peak at 270.9°C (this value corresponds to the melting point) and DSC analysis of bulk rutin sample showed three endothermic peaks at 162°C, 177.2°C and 213.3°C, respectively (**Figure 6**) (the last value corresponds to the melting point).

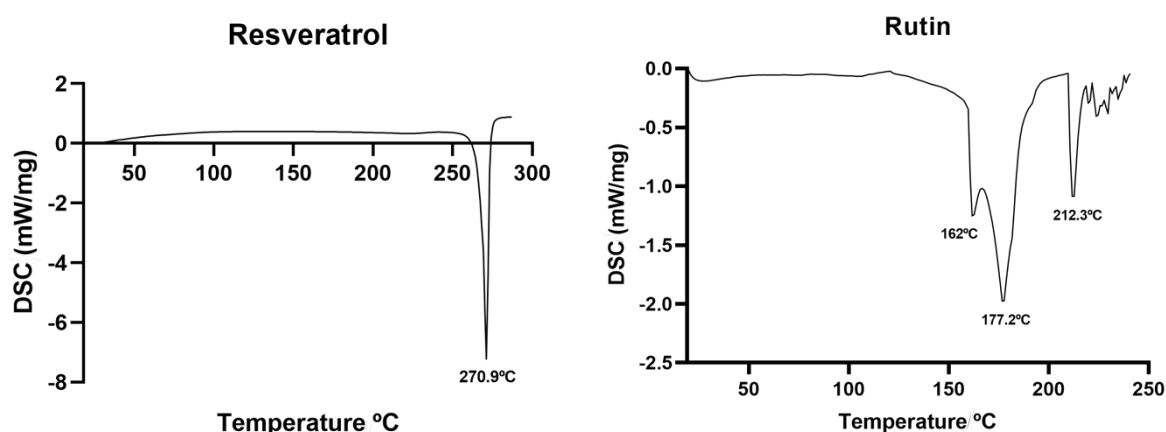


Figure 6. DSC analysis of bulk resveratrol and bulk rutin.

The thermal analysis of all samples, i.e., V1, F1 and F4 had similar DSC profiles, which indicates that there was no interaction of the active ingredient with the micelles, since the thermal analysis of the blank samples had an identical profile to the loaded micelle samples with resveratrol and rutin-resveratrol-loaded micelles (**Figure 7**). This shows that the bioactives are encapsulated in the micelles corroborating the idea that the system does not have a solid matrix but only a liquid matrix. The different variables presented, namely, storage temperature and times after production, did not show to be significant constraints for the study. EE and LC were calculated to estimate the amount of resveratrol and rutin encapsulated in the micelles. The EE reached 98.69% and the LC was 2.11% for F1 formulation. For F4, the same parameters were calculated to estimate the amount of rutin and resveratrol that were encapsulated in the micelles. For resveratrol in the F4 formulation, the EE was 99.86% and the LC was 1.01%; for rutin in the F4 formulation, the EE was 98.31% and the LC was 0.99%.

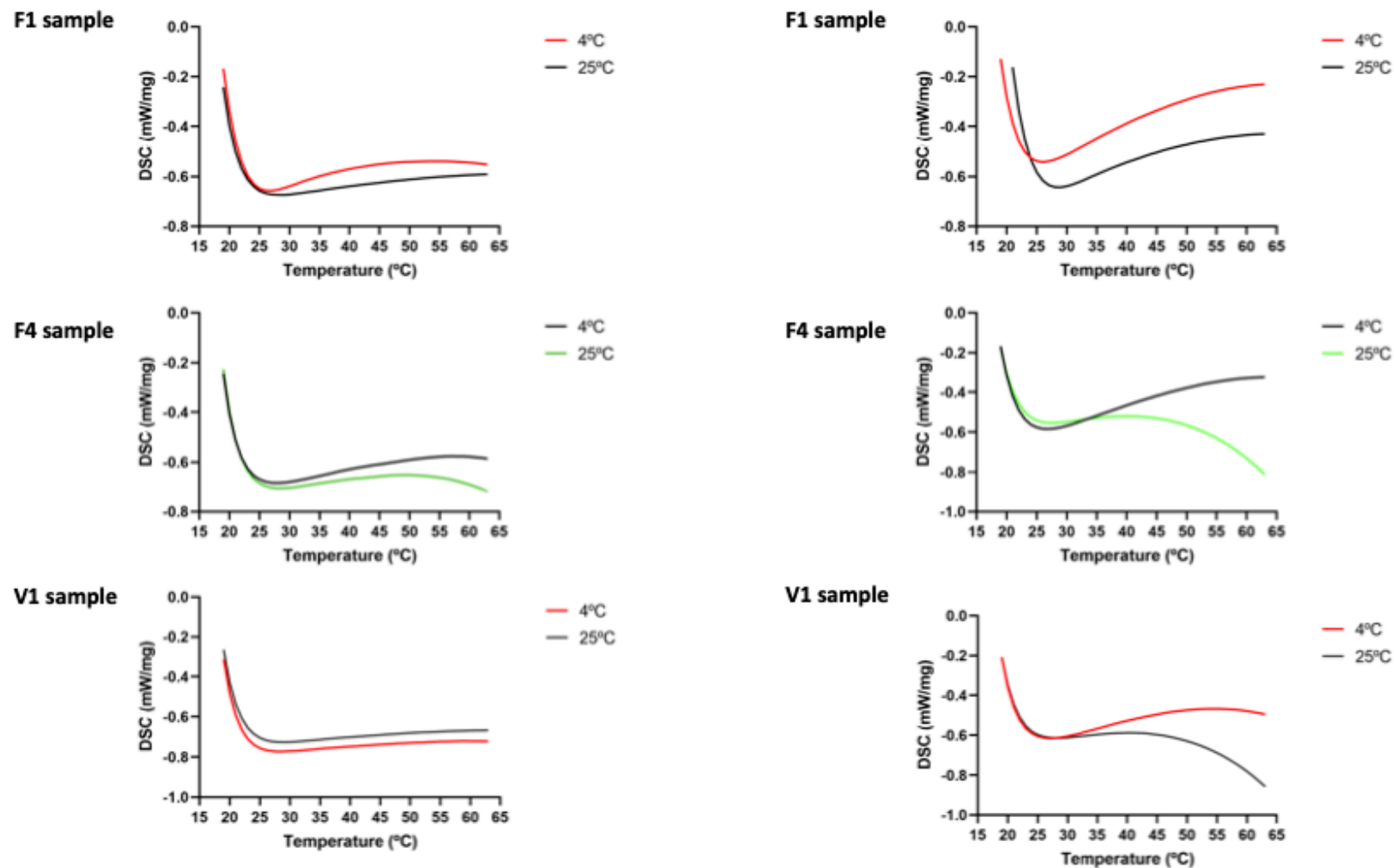


Figure 7. DSC analysis of resveratrol-loaded micelles (F1 sample), rutin-Resveratrol-loaded micelles (F4 sample) and blank sample (V1), 24h after production (left side) and 2 months after production (right side).

Rheological characteristics strongly influence physical and sensory properties of the system. **Figure 8** shows the rheological analysis, recorded after 24 hours of production, of blank micelles, micelles loaded with resveratrol and micelles loaded with rutin and resveratrol, and **Figure 9** shows the same test 2 months after production. The storage modulus G' or elastic component, the loss modulus G'' or viscous component and the shear viscosity are presented. As can be seen, in general, $G' > G''$, as shown in **Figures 8** and **Figure 9**. The G' was higher than the G'' in the measured frequency range, indicating a sample with more elastic characteristics. The G'' showed less dependence on the frequency, while the G' increased significantly in the frequency range from 0 to 10 Hz, highly depending on it. The rheological analysis of the micelles showed a behavior in which the shear viscosity increased with frequency and was highly dependent on it.

Based on the results, shown in **Figure 8**, when stored at a temperature of 4°C, both samples loaded with bioactive ingredients (F1 and F4) registered higher values of shear viscosity and G' than those stored at 25°C. Blank sample stored at 4°C recorded the highest shear viscosity and G' values after 24 hours of production. These results suggest that there is a certain influence of storage temperature on the viscoelastic behavior of the developed micelles, but only 24 hours after production. After 2 months of storage, the results changed. In both F1 and F4 samples, the shear viscosity values increased in comparison to the results recorded after 24h. Sample F1 after 2 months of production showed higher shear viscosity and G' values than F1 24 hours after production and the same happened for sample F4. The increase in viscosity after 2 months was higher in F4 than in F1. In F1, the viscosity values after 2 months slightly increased, as already mentioned. Compared to F4, the viscosity values recorded after 2 months suffered a high increase (more than double), which may suggest that the presence of two bioactive compounds may increase the viscosity of the micelles. In opposite to the results obtained in the first analysis, after 2 months of production, the samples loaded with bioactive ingredients presented higher shear viscosity and G' values than the blank samples. These results suggest that micelles loaded with bioactive ingredients gain shear viscosity over time, unlike blank samples, where viscosity decreased.

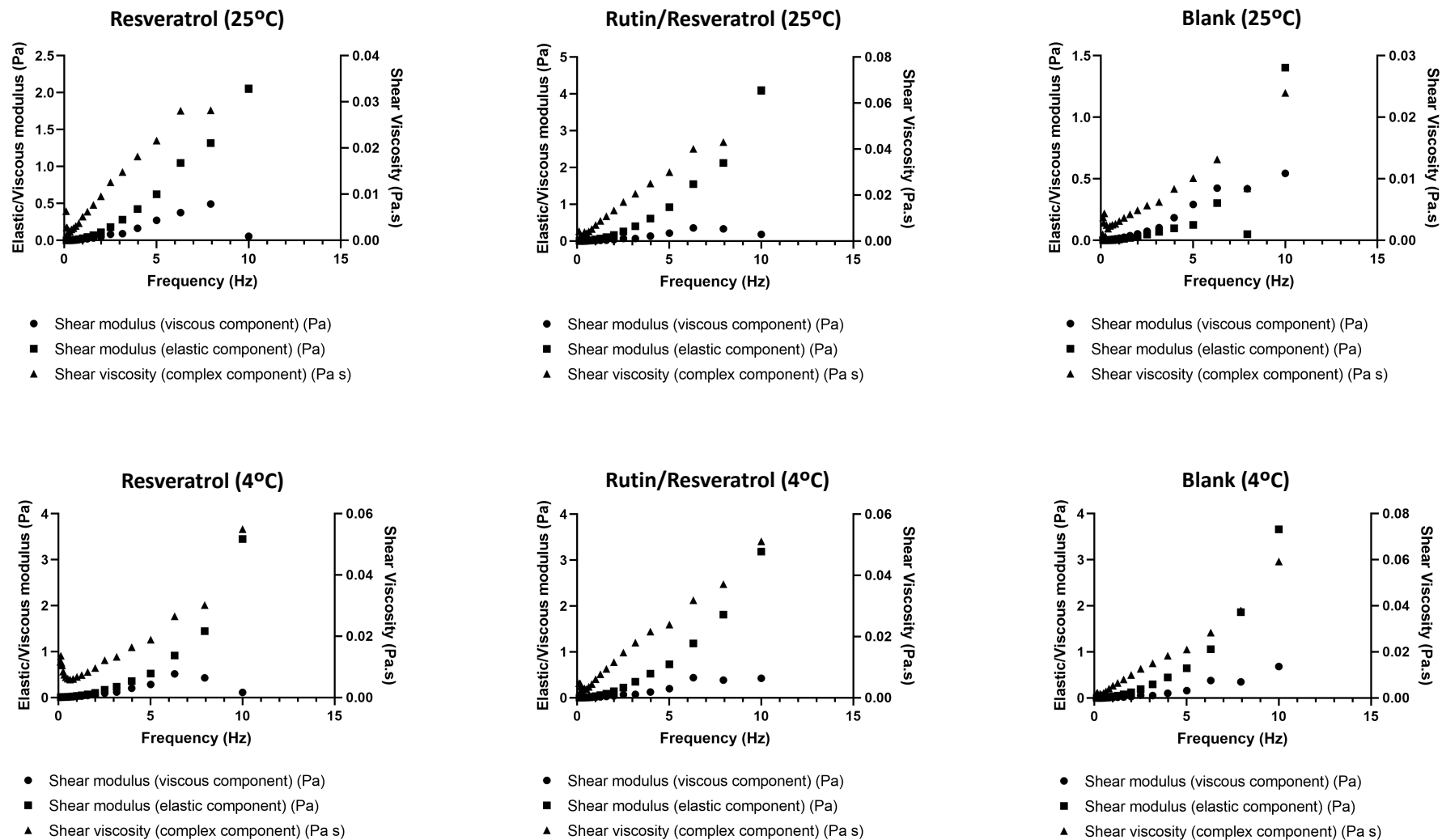


Figure 8. Rheological analysis for blank, resveratrol-loaded micelles and rutin-resveratrol-loaded micelles 24h after production.

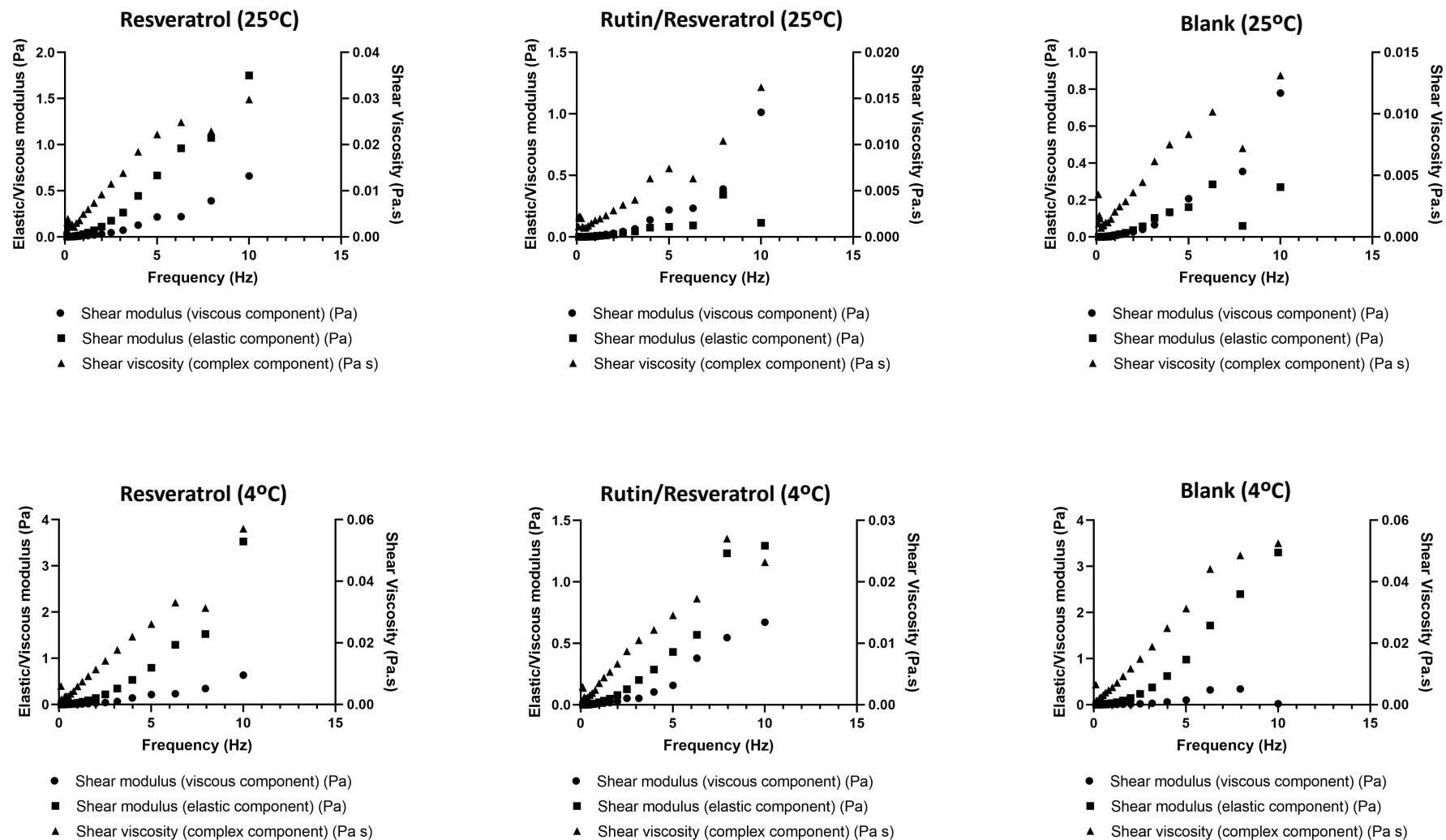


Figure 9. Rheological analysis for blank, resveratrol-loaded micelles and rutin-resveratrol-loaded micelles 2 months after production.

4. Conclusions

In the present work, resveratrol-loaded micelles and rutin-resveratrol-loaded micelles were successfully produced with suitable Z-Ave, PDI and ZP. In these analyzes it was possible to conclude that no considerable differences were recorded between the two types of developed micelles (F1 and F4), finding the results in the same range of values. In both types of micelles, the encapsulation efficiency and loading capacity were very high, indicating that the drugs were successfully loaded into the micelle's structures, overcoming the problems of insolubility of the drugs. Regarding the rheology analysis, significant difference in results between the developed micelles were found. The shear viscosity of F4 was found to be much higher than sample F1, two months after production, suggesting that the presence of more than one bioactive compound increases the viscosity of the system. Furthermore, the stability of the micelles was evaluated and all samples showed excellent long-term physical stability. The produced samples, F1 and F4, are thus considered promising delivery systems for the dual loading of resveratrol and rutin for therapeutic treatment of skin diseases.

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Chapter 4: Development and characterization of eco-friendly hydrogels loading polyphenols-composed micelles

Abstract

In this study, an ideal combination of hydrogel containing micelles loaded with rutin, resveratrol and the combination of both bioactives, was fabricated in the interest of combining the advantages of hydrogels for topical administration of drugs and of micelles for the delivery of poorly-soluble compounds in a combination therapy. First, micelles loaded with different bioactive compounds were synthesized using the hot shear homogenization and followed by ultrasonication, as previously describe by us (chapter 2 and chapter 3). The three types of developed micelles were dispersed in a carbomer 940-based hydrogel to obtain three distinct semi-solid formulations, which were then characterized with respect to thermal analysis, rheology and texture. Olive pomace-based hydrogels were also produced to be contain the same micelles as an alternative to respond the needs of zero waste and circular economy. The thermograms show no changes in the typical profiles of micelles when loaded into the hydrogels. The theologically analysis showed that the different produced hydrogels achieved the ideal properties of a semi-solid product for topical administration. The viscosity values of the hydrogels loaded with micelles and olive pomace (Hydrogels A) proved to be higher than the hydrogels without the addition of olive pomace (Hydrogels B), with this ingredient having a considerable effect on the viscosity of the final formulation. The texture analysis of both hydrogels A and B also exhibit the typical behavior expected of a semi-solid system.

Keywords: Hydrogel; Micelles; Rutin; Resveratrol; Olive Pomace.

1. Introduction

Hydrogels are a class of three-dimensional (3D) polymer networks with high water retention capacity [1]. As they composed by any hydrophilic polymer by chemical or physical cross-linking [2]. Hydrogels have a wide variety of physical properties and chemical compositions that can be tailored to a specific need, thus showing a good deal of applications, from studies of physiological and pathological mechanisms to tissue regeneration and drug delivery (DD).

Hydrogels have certain properties that make them highly suitable as drug delivery vehicles, such as being biodegradable and biocompatible, having the ability to carry different drugs, to maintain a high concentration of active ingredient for a long period, in the site of interest and modify the release profile of loaded drugs [3, 4].

In skin wounds, treatment can involve different approaches. More traditional treatments, such as bandages and gauzes, are being used less, unlike hydrogels, which are a new approach to tackle this problem. Hydrogels have several beneficial properties for treating skin wounds, since they are compatible with biological tissues, have the ability to retain a high amount of water due to their high porosity, and can simultaneously keep the wound moist and absorb exudate [5]. Recent studies have shown that swollen hydrogels prevent microorganisms from entering the wound and, at the same time, allow the movement of functional molecules into the wound [6]. Hydrogels are appropriate for all stages of the skin healing process - hemostasis, inflammation, cell migration/proliferation and maturation - since they are non-irritating, do not react with biological tissue and are permeable to metabolites [7]. Thus, hydrogel dressings are being widely studied for accelerated wound healing. To expedite the need to optimize wound treatment, there is a large variety of hydrogel dressings that can be applied to several types of skin wounds, depending on the need. This resulted in a quick entry of a range of dressing materials into the pharmaceutical market, such as sheets, saturated gauze or gels [8]. With the development of nanotechnology, nanoparticles loaded with bioactives of interest are being combined with other biomaterials to create innovative hybrid systems for new applications [9, 10]. That said and following this evolution, the addition of nanoparticles to hydrogels gaining even more interest [11, 12].

For bioactive compounds to achieve their health-generating effects, they go through a variety of processes before reaching their target [13]. These processes, called bioavailability and bioaccessibility mostly affect the biofunctional properties of bioactive compounds, therefore the evaluation of these processes has become an area of interest in research [14]. The development of DD through nanoparticle systems has grown significantly in biomedicine [15, 16]. With the development of nanomedicine, the creation of nanoformulations offers the advantage of increasing the bioavailability of active ingredients

[17]. Nanomedicine improves the absorption of poorly water-soluble compounds, resulting in a higher bioavailability of drugs, besides allowing to modify the release profile of drugs and to target them to specific locations, and thus reducing the risk of side effects due to systemic distribution of drugs [18-20]. In this work, we describe the development of semi-solid formulations using previously produced hybrid micelles containing resveratrol (F1) and rutin (F2) and their dual loading (F4), as described in the previous chapters. These two compounds are obtained from natural sources, namely plants and fruits, and both offer interesting activities of pharmacological and cosmeceutical interest. Their formulation into hybrid micelles aimed at overcoming the poor water solubility, poor chemical stability and low bioavailability, associated to resveratrol and rutin [21-24].

Olea europaea L., olive tree, is one of the oldest cultivated trees in the Mediterranean and it is native to Asia Minor and Syria [25]. In the Mediterranean diet, olive tree products such as olive oil and olives are widely consumed [26, 27]. However, the olive oil industry is associated with environmental issues, due to the high production of waste, mostly during the agricultural and product production phase [28]. The wastes that generate the greatest ecological concern due to high organic toxicity and low pH, include olive pomace (OP), olive leaves and olive mill wastewater [29]. OP is a semi-solid residue with a high amount of water in its constitution (60%) and has low pH. OP also contains a high content of phenolic compounds and retains most of the phenolic content of the olive [30, 31]. Only about 1–2% of the phenolic content is present in olive oil [30, 32]. However, this by-product becomes phytotoxic and non-biodegradable [25, 33]. Despite this, these by-products are of great interest to several industries due to their varied bioactivities and health-promoting properties, and new forms of reuse are being proposed. It has been demonstrated that these by-products, as well as their bioactive phenolic compounds, show anti-inflammatory [34-36], antioxidant [37-39] and photoprotective activities [38, 40-42]. Furthermore, their application has also been reported in cosmetics and dermatological products, for the treatment of skin diseases through different topical application systems [43, 44]. Hydrogels based on olive pomace are one example of these delivery systems.

Therefore, to value these olive by-products, their sustainable recovery has been highlighted in several research studies [29].

2. Materials and methods

2.1. Materials

The materials used in the study included Polysorbate 80 (Acofarma, Barcelona, Spain), Sorbitan monostearate 80 (Acofarma, Barcelona, Spain), L- α -Phosphatidylcholine (Sigma-Aldrich, St. Louis, MO, USA), Rutin (Acros Organics, Geel, Belgium), Resveratrol (Fagron,

Barcelona, Spain), Carbomer 940 (Fagron, Barcelona, Spain), Sodium Hydroxide (Sigma-Aldrich, St. Louis, MO, USA), Methylparaben (Acofarma, Barcelona, Spain), Propylparaben (Acofarma, Barcelona, Spain) and Propylene glycol (Acofarma, Barcelona, Spain). Olive pomace and Milli-Q water were home supplied.

2.2. Production of rutin- and resveratrol-loaded micelles

For the production of micelles, the hot shear homogenization technique, followed by ultrasonication method was used as previously described by use (chapter 2 and chapter 3). The compositions of the different prepared micelle formulations are shown in **Table 1**. Briefly, for all samples, F1, F2 and F4 the active compound concentration was 0.1% (w/w). This, mixed with soy Lecithin (L- α - Phosphatidylcholine), formed the lipid phase of the system. The aqueous phase was composed of Milli-Q water and surfactant (Polysorbate 80 and/or Span 80). To the lipid phase, the aqueous phase was added and both were heated under constant stirring until boiling. The resulting dispersion was stirred at high speed (7000 rpm) using an Ultra-Turrax (IKA, Staufen, Germany) for 5 minutes. This mixture was subsequently subjected to sonication with a probe for 5 minutes and an amplitude of 70%. Then, the developed samples were stored at room temperature until further use. The micelle preparation method used was the same for all samples F1, F2 and F4.

Table 1: Composition of rutin- and resveratrol-loaded micelles.

Sample	Resveratrol (%, w/w)	Rutin (%, w/w)	Soy Lecithin (%, w/w)	Polysorbate 80 (%, w/w)	Span 80 (%, w/w)	Water ad. (%, w/w)
F1	0.10	-	4.90	1.00	-	100
F2	-	0.10	4.90	0.60	0.400	100
F4	0.05	0.05	4.90	1.00	-	100

2.3. Production of rutin- and resveratrol- loaded micelles hydrogel

For the acceptance of a topical product, there are several important parameters to be considered, such as its easy application to the skin, the sensorial properties (brightness and amount of residue) and the appearance of the product. As micelle dispersions do not have an adequate consistency for application to the skin, as they are liquid systems, their transformation into semi-solid systems becomes an attractive solution for product acceptability, besides improving the stability of nanoparticles by reducing the risk of forming aggregates over shelf-life [45, 46]. Thus, hydrogels based on micelles (Hydrogel A and

Hydrogel B) were developed. Carbomer was used to improve the viscosity of the system, in order to obtain suitable consistency levels for topical application. First, a 2% (w/v) carbomer 940 gel was prepared with the aid of the Unguator® mixer (Gako Deutschland GmbH, Schesslitz, Germany). Carbomer 940, water and propylparaben were weighed and mixed at a speed of 2400 rpm for 30s. Propylparaben was used as a preservative and a 10% (w/v) solution of sodium hydroxide was used to adjust the pH value of the carbomer up to 6.5-7. These two constituents were added to the mixture under gentle stirring (600 rpm for 8 minutes and 30 seconds) to induce polymer gelation. The developed hydrogel was not stirred for 12 hours to eliminate the presence of any existing air bubbles. It was then kept at a controlled temperature of 2–8 °C for further studies. For the final preparation of the hydrogel loaded with micelles, the previously prepared 2% carbomer gel was used. The micelles were added and mixing was carried out for 1 minute at 600 rpm. The final formulation consisted of 50% of 2% carbomer gel and 50% of micelles (hydrogel A) and 50% of 2% carbomer gel, 49.7% of micelles and 0.3% of olive pomace (hydrogel B). The composition of all developed hydrogels is represented in **Table 2** and **Table 3**. **Figure 1** depicts the photography of all the produced hydrogels.

Table 2. Composition of 100g of 2% carbomer 940 gel.

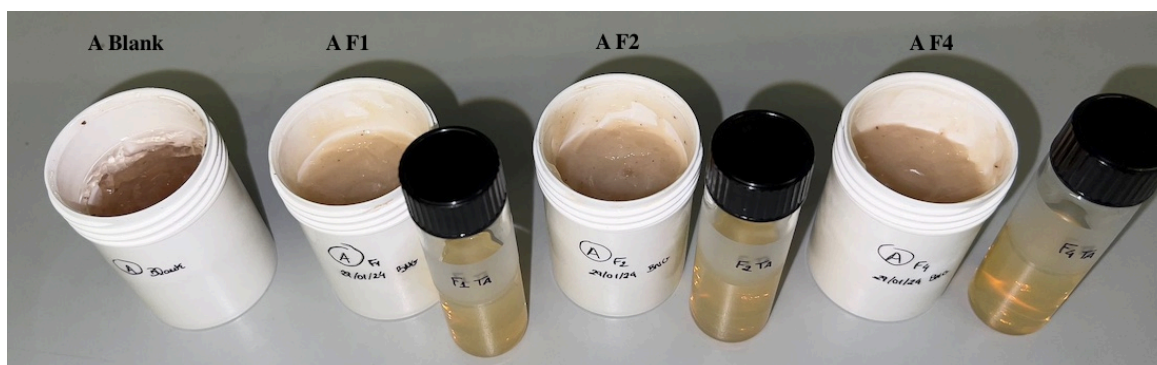
Carbomer 940 (g)	10% Sodium Hydroxide Solution (ml; w/v)	Propylparaben	Purified Water ad (g)
2	3.2	1.5	100

Table 3. Composition of hydrogels A and hydrogels B.

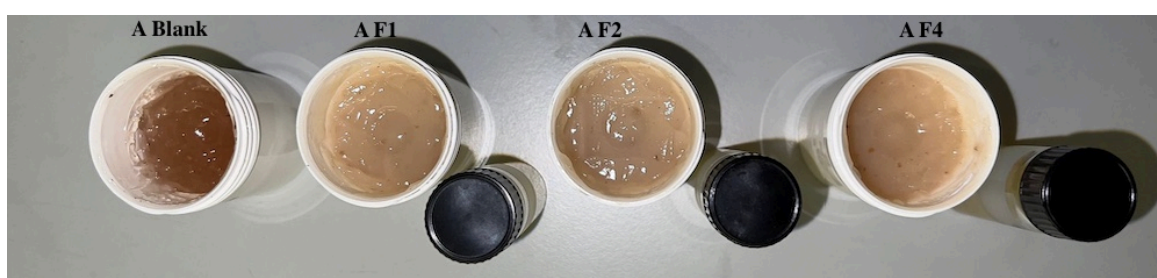
Materials	A Blank	A F1	A F2	A F4	B Blank	B F1	B F2	B F4
2% Carbomer 940 (g)	29.85	15	15	15	30	15	15	15
Olive Pomace (g)	0.15	0.15	0.15	0.15	-	-	-	-
Rutin-load micelles (g)		-	14.85	-	-	-	15	-
Resveratrol-load micelles (g)	-	14.85	-	-	-	15	-	-
Rutin+resveratrol- load micelles (g)	-	-	-	14.85	-	-	-	15

2.4. Differential Scanning Calorimetry

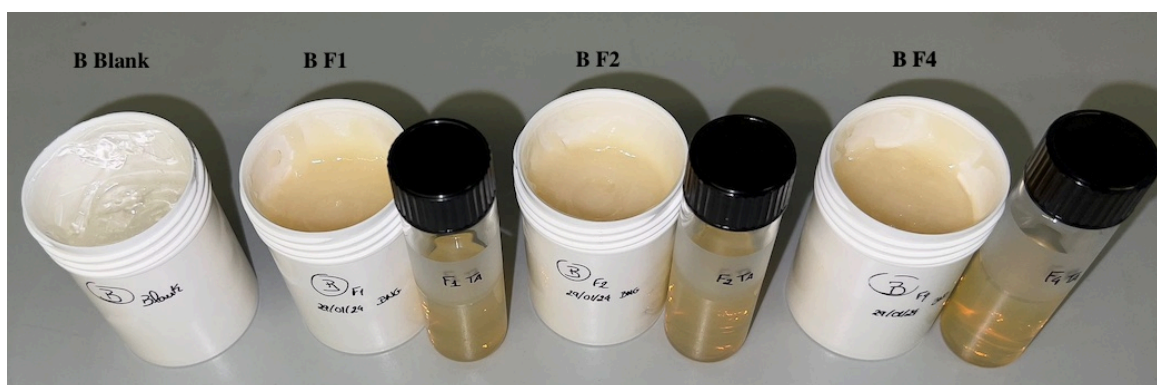
Differential Scanning Calorimetry analysis was used to ascertain the physical state of the hydrogel. This work was carried out on a DSC 200 F3 Maia® (NETZSCH, Selb, Germany). This equipment consists of an oven with two different positions. One position houses the crucible that contains the sample and the other position houses the reference crucible (position 0). The samples to be analyzed were weighed, approximately 5 mg into an aluminum crucible that was subsequently closed. The analysis consists of a temperature program between 20 °C and 70 °C, at a heating rate of 10 °C/min, to which the samples are subjected. As a result, and with the data to be analyzed, a thermogram was generated. The thermogram contains information such as enthalpy, melting onset temperature and melting point. Data analysis was performed using the thermal analysis software “Proteus® 6.1.0B” software (NETZSCH, Germany).



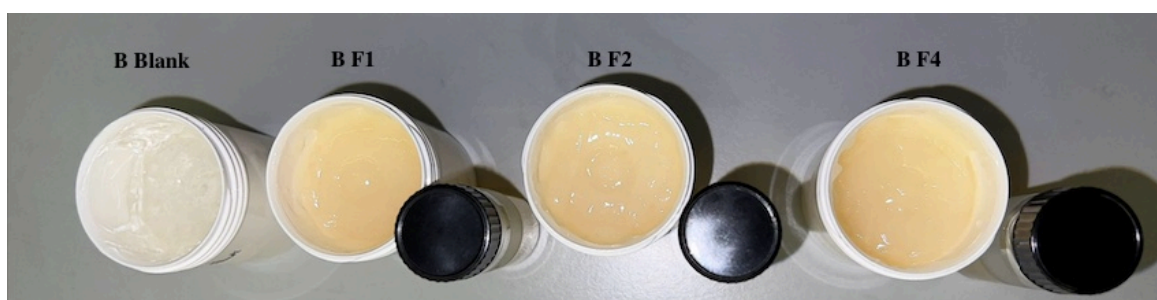
I



II



III



IV

Figure 1. Hydrogels A loaded with micelles samples F1, F2 and F4 and olive pomace (I and II) and hydrogels B loaded with micelles samples F1, F2 and F4 (III and IV) 24 hours after production.

2.5. Rheological analysis

The viscoelastic properties of the developed hydrogels were evaluated using a Rheometer Kinexus Lab + (Malvern, Worcestershire, UK). The hydrogels were measured at a temperature of 25°C with a spacing of 1 mm gap between plates. A portion of the sample was deposited on the lower plate of the equipment where a torque was subsequently applied, which promoted shear stress. The frequency used in the analysis was 0.1 to 10.0 Hz, in oscillation mode and 1% deformation. Data were compiled and collected using the software “rSpace for Kinexus Lab +” (version 1.75 Malvern Instruments).

2.6. Texture analysis

To analyze the texture of the different samples, a texture analyzer TA-XT2i© (Stable Micro Systems, Godalming, UK) and the software “Exponent” (version 6.1.12.0) were used. The sample was placed in the equipment and a P/0.5 ½ Dia Delrin Aoacque probe penetrated it with a 5 Kg load cell. The firmness and cohesiveness were evaluated at room temperature (18–25°C), for all samples. Data acquisition and analysis were performing using the software “Texture Expert®”.

3. Results and Discussion

The thermal properties of hydrogels A and B were analyzed by DSC and results are presented in **Figure 2**, showing that all samples presented similar endothermic profiles. From the analysis of the different hydrogel samples A and B, no peaks were recorded, corroborating the idea that the system does not have a solid matrix.

The oscillatory study (**Figure 2** and **Figure 3**) describes the system response as a function of frequency at constant shear strain and provides information on the storage modulus (elastic component, G'), loss modulus (viscous component, G'') and shear viscosity. The G' resembles the amount of energy that the dispersion needs to be distorted, while the G'' reflects the energy lost during deformation. As the frequency range increases, the shear rate also increases, thus requiring more energy and consequently increasing G'' and G' . The developed hydrogels demonstrated G' modulus values much higher than G'' modulus values throughout the entire frequency range, for all formulations tested. These data point to the presence of a gel-like structure and indicate that the system is more elastic than viscous.

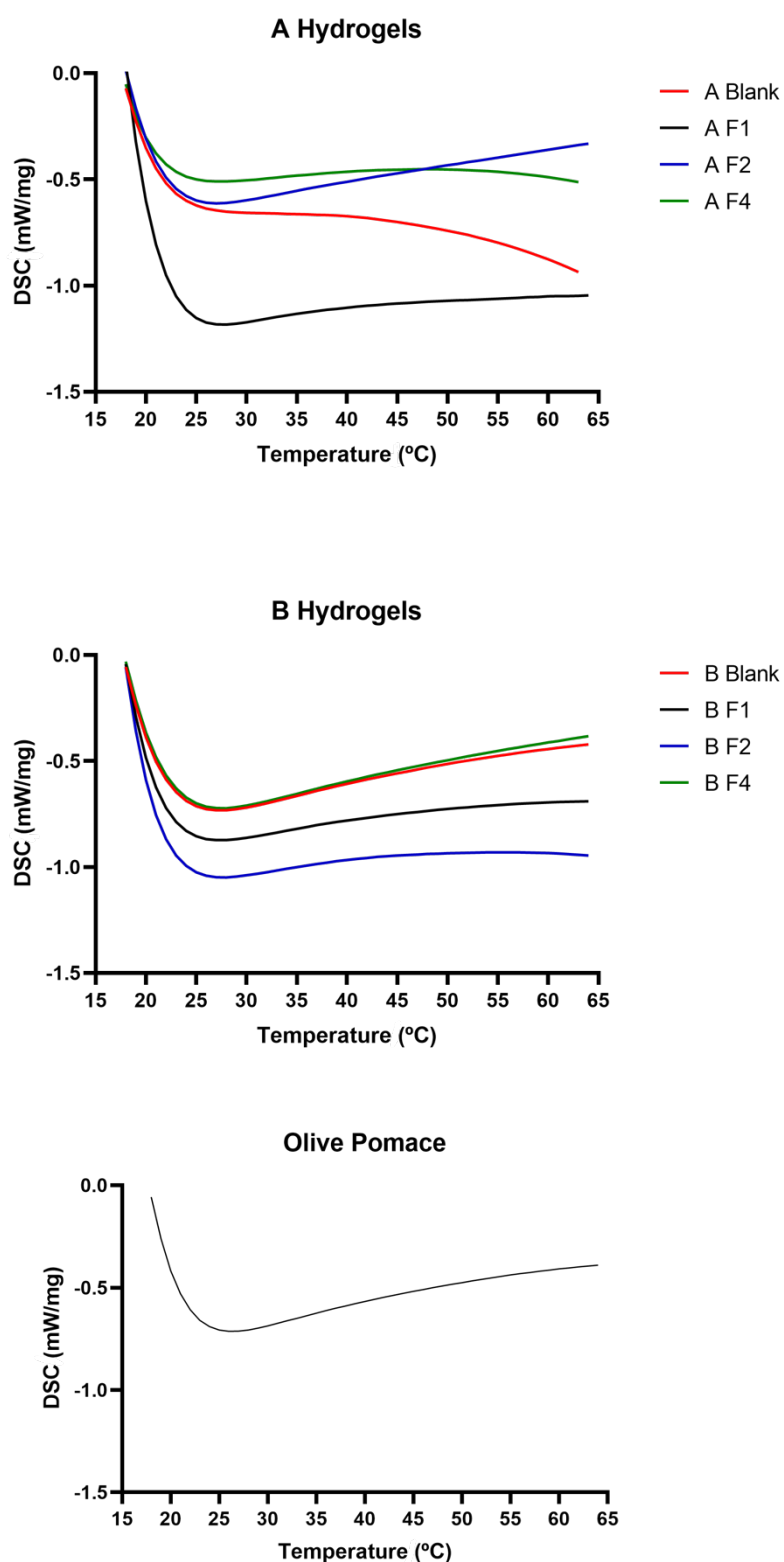


Figure 2. Differential Scanning Calorimetry analysis of the different samples.

It can also be observed that G' and G'' did not show any dependence on frequency, being practically constant with the increase of this parameter. The profiles were recorded as straight lines with a slight inclination, without ever crossing each other. This behavior is

considered typical of a semi-solid vehicle [47-49]. However, viscosity is frequency dependent as viscosity values decrease with increasing frequency. This behavior is typical of viscoelastic solids and can be found for standard topical dosage forms and indicate that the formulations can be easily rubbed onto the skin [50]. Comparing the viscosity of hydrogels loaded with micelles with blank hydrogels, it is seen that hydrogels loaded with micelles have higher values. The obtained results indicate that the blank hydrogels have a more rigid structure when compared to the hydrogels loaded with micelles. The viscosity of the hydrogels A and B was strongly dependent on the presence of micelles and olive pomace. In hydrogel A, the viscosity is considerably lower in all samples (A F1, A F2 and A F4) compared hydrogels B formulations (**Figure 4**), due to the presence of olive pomace which reduces the viscosity of the systems [51]. We could also see that in hydrogels B a greater distance was recorded between the modules G' and G'' for each formulation compared to hydrogels A. This is the result of a higher shear stress caused by the increase in viscosity.

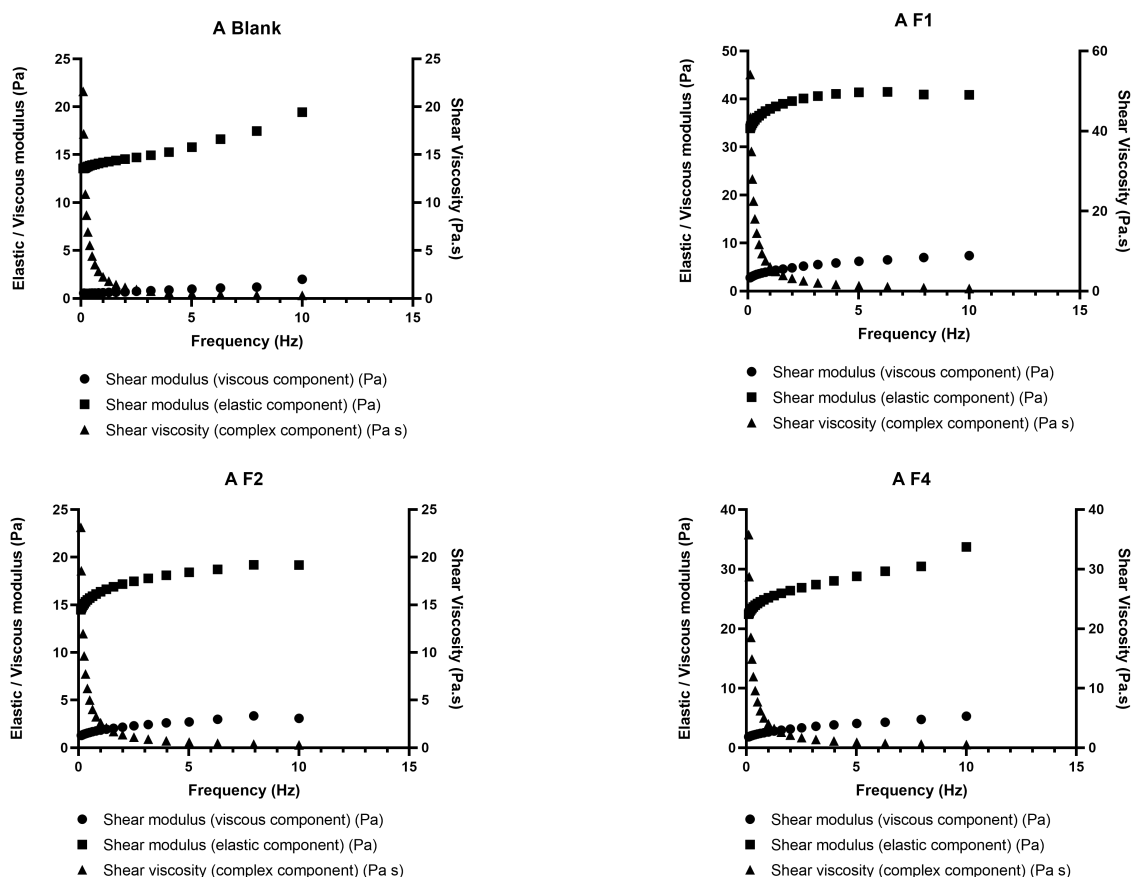


Figure 3. Oscillation frequency sweep test of Hydrogels A.

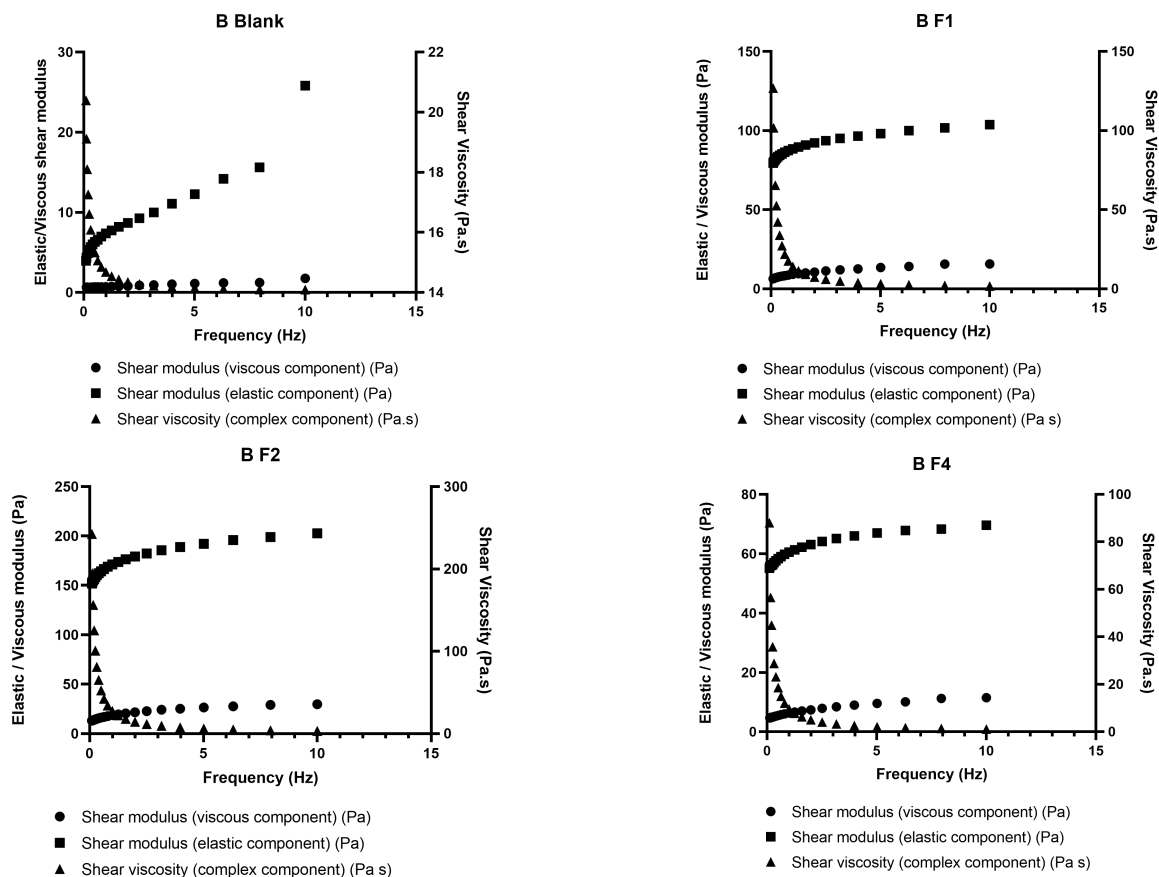


Figure 4. Oscillation frequency sweep test of Hydrogels B.

Texture can be understood as the physical characteristics perceived through touch and is related to the deformation caused by a force. These characteristics are analyzed and measured through distance, force and time. Through texture analysis we were able to obtain the necessary knowledge about some structure properties of the products and to predict the behavior of the formulation *in vivo* [52]. The texture analysis of topical pharmaceutical formulations is an important analysis because the acceptability of the product by the buyer/patient will depend, mainly, on its application and organoleptic attributes. o Texture analysis was carried out by compressing the different samples, with a specific probe, at a constant speed. As a result, a graph is generated in which the positive part represents the force necessary to penetrate the sample, while the negative part refers to the force necessary for the probe to be removed from the sample and return to its initial position. The maximum force recorded represents firmness and the negative force peak of the graph shows cohesiveness. Firmness measures the force required to produce deformation in the gel and measures the ability of the gel to resist the mucociliary clearance in the target area.

Cohesiveness measures the internal binding strength of the final hydrogel structure [53, 54]. The results obtained in relation to the texture analysis of different samples are presented in **Table 4**.

Table 4. Textural properties of the hydrogels.

Parameters	A Blank	A F1	A F2	A F4	B Blank	BF1	B F2	B F4
Firmness (N)	0.3305	0.2628	0.2470	0.2323	0.3049	0.2592	0.2745	0.2886
Cohesiveness (N)	-	-	-	-	-	-	-	-
	0.1328	0.1536	0.1391	0.1302	0.1427	0.1416	0.1538	0.1341

Observing the blank hydrogels with the hydrogels loaded with bioactives, it can be seen that the loaded hydrogels registered lower firmness values, i.e., the addition of micelles reduced the firmness of the system. The first positive region of the graph is related to firmness, higher values mean a firmer gel; it can be observed that both blank hydrogels are those with higher firmness force values (A Blank 0.3305 N and B Blank 0.3049 N), therefore being firmer and more rigid solid systems, suggesting that blank hydrogels are firmer compared to the other hydrogels. On the other hand, the two lowest firmness values recorded are both found in hydrogel A, in the samples A F2 and A F4, 0.2470 and 0.2323, respectively. The first negative force that appears in the graph is related to the cohesiveness of the gel, where a lower value indicates higher cohesion of the gel. It was observed that the two highest cohesiveness values are both from the hydrogel A, in samples A F1 and A F4, -0.1328 N and -0.1302 N, respectively. It can be observed that the cohesiveness values are lower, in the majority, in hydrogel B, being more cohesive than hydrogels A, except for sample B F1, which shows higher values compared to sample A F1. In summary, our results indicate that hydrogels B exhibit strong firmness, strong gel strength and better cohesiveness compared to hydrogels A, highlighting the interest in the use of olive pomace as a new ingredient of hydrogel formulations for skin administration.

4. Conclusions

The aim of this study was to prepare new natural-based hydrogels loaded with different natural compounds. In conclusion, hydrogels based on rutin-loaded micelles, resveratrol-loaded micelles, rutin-resveratrol-loaded micelles and containing olive pomace were successfully prepared, optimized and characterized, showing to be suitable for application

on the skin. Regarding the rheological analysis, the hydrogels presented G' values much higher than the G'' values across the whole frequency range, indicating a gel-like structure and showing that the system were more elastic than viscous, which is an important attribute for topical semi-solid products. It was also found that the viscosity of hydrogels A and B was strongly dependent on the presence of micelles and olive pomace, suggesting that the presence of olive pomace appears to reduce the viscosity of the systems. In the texture analysis, the results obtained showed that hydrogels containing olive pomace have a more rigid, firm and cohesive matrix than those hydrogels based only on carbomer. The results of this analysis agree with those of the rheological analysis and corroborate the interest in using by-products in pharmaceutical and cosmetic formulations.

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Chapter 5: Conclusions and Future Perspectives

Conclusions and Future Perspectives

Nowadays, concerns about skin health and appearance are part of our daily life, leading to the increasingly growing market of anti-aging products, wound healing, antioxidants and photoprotective products. At the same time, concerns about environmental health and the demand for natural products are also in the pipelines. That being said, the choice of cosmetic products with natural ingredients in their composition, as well as the use of by-products, meets the needs of users. Therefore, it is extremely important to find natural products with interesting and beneficial therapeutic activities for skin application.

Rutin and resveratrol are two compounds of natural origin, present in several fruits and plants. These two compounds are non-toxic and have several proven beneficial activities, such as antioxidant, anti-inflammatory, wound healing, antibacterial, among others. Just as the demand for natural products has increased in the latest years, the interest in using food by-products in different ways is also following this trend. Olive pomace is a by-product of olive oil production which, if left on soil, is toxic and non-biodegradable. However, this by-product has a high amount of phenolic content with numerous beneficial effects, particularly for the skin. The search for new ways to introduce these compounds and deliver them effectively is one of the challenges, and several studies include extensive research related to the use of nanocarriers, with hybrid micelles being one of the candidates, to overcome this problem.

The first experimental chapter (chapter 2) described the production of micelles loaded with rutin, using the hot shear homogenization technique followed by the ultrasound technique. The micelles resulting from this production pathway were subjected to different testes to monitor the stability of these vesicles, namely, determination of the mean hydrodynamic size, polydispersity index and zeta potential, on different days after production, as well as studies to determine thermal properties, rheological studies, morphology, encapsulation efficiency and loading capacity. Hybrid micelles with a size less than 90 nm were successfully obtained, with good chemical stability during the studied timeframe, with adequate viscoelastic behavior and high capacity to encapsulate the phenolic compound, rutin.

In the Chapter 3, the objective was to encapsulate resveratrol into hybrid micelles and test the potential use of the developed hybrid micelles for the dual loading of the two phenolic compounds, rutin and resveratrol. For the production of such micelles, the same above-mentioned techniques, i.e., the hot shear homogenization and the ultrasound, were used and the dual loaded micelles monitored. In this study, no considerable differences between

the two types of prepared samples (i.e., single-loaded versus dual-loaded) were noted. The viscoelastic behavior of the two formulations was also found to follow the same trend as rutin-loaded micelles (described in chapter 2), all showing a more elastic than viscous behavior. Over time, however, the viscosity of dual-loaded micelles formulation increased significantly in comparison to resveratrol-loaded micelles, suggesting that the presence of more than one bioactive compound increases the viscosity of the system. This study confirms that it is possible to produce micelles capable of successfully encapsulating both polyphenols. Both samples were produced with high encapsulation efficiency rates, had a size less than 75 nm and showed excellent long-term physical stability over 2 months.

Chapter 4 described the development of hydrogels containing different samples of micelles loading the selected polyphenols. The eight hydrogels produced were subjected to different analyses, namely, differential scanning calorimetry analysis, rheological and texture analysis. Rheological analysis highlighted that the prepared hydrogels were more elastic than viscous, in the analyzed frequency range. The presence of micelles and olive pomace was the factor that influenced the viscosity of the system. The presence of olive pomace reduced the viscosity of the hydrogels and, consequently, affected the texture, as the hydrogels with olive pomace in their composition were, in general, less rigid and less cohesive than the hydrogels without olive pomace.

Hydrogels containing rutin-load micelles, resveratrol-load micelles, rutin-resveratrol-load micelles and olive pomace were successfully prepared, optimized and characterized as being suitable for application on the skin.

Future perspectives include the assessment of cytotoxicity of the developed formulations in selected skin models and also the acceptance performance of the developed semi-solid formulations by volunteers.