Development of Anti-Aging Cosmetic Formulations

Marta Caçador

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Universidade do Minho
Escola de Engenharia

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Mestrado em Biotecnologia

Trabalho efetuado sob a orientação do
Professor Doutor Artur Cavaco-Paulo
e da
Doutora Ana Isabel Sá Loureiro

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STATEMENT OF INTEGRITY

I hereby declare having conducted this academic work with integrity. I confirm that I have not used plagiarism or any form of undue use of information or falsification of results along the process leading to its elaboration.

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Resumo: Desenvolvimento de formulações cosméticas anti-envelhecimento

A indústria de cosméticos adquiriu uma enorme importância, sendo considerada uma área de negócios e investigação em constante expansão. A sociedade em que vivemos está cada vez mais preocupada com a saúde e a aparência, o que aumentou a procura por formulações anti-envelhecimento eficazes. O desenvolvimento de formulações para cuidados com a pele é um processo complexo na medida em que é necessário considerar várias características tais como a interação dos ingredientes, estabilidade, nível de hidratação, espalhamento e sensação na pele.

O objetivo deste trabalho foi o desenvolvimento e a caracterização de séruns com propriedades anti-envelhecimento. O foco do trabalho foi o desenvolvimento de diferentes séruns faciais, séruns aquosos e leitosos. O desenvolvimento dos séruns aquosos provou ser um processo mais rápido do que os séruns leitosos. Foram desenvolvidas seis formulações, três formulações base e três contendo os ingredientes ativos. A estabilidade das formulações desenvolvidas foi realizada através da avaliação de diferentes propriedades físicas e químicas durante três meses. As propriedades incluíam: determinação do pH, viscosidade, estabilidade a temperaturas extremas e avaliação das características organoléticas (odor, cor e aparência) a diferentes temperaturas (4°C, temperatura ambiente e 37°C). Foi também estudada a influência da luz e do material de embalagem na estabilidade das formulações. Os resultados obtidos mostraram que as Formulações A, tanto a base como com os ingredientes ativos, são estáveis em todas as condições. Em contraste, as Formulações B e C, não demonstraram ser estáveis principalmente as formulações que contêm ingredientes ativos (Formulações B2 e C2). Numa etapa final deste trabalho, após algumas otimizações, foram obtidas Formulações B1 e C1 mais estáveis através da inclusão de estabilizadores de emulsão e agentes quelantes.

Em conclusão, este trabalho contribuiu para o desenvolvimento de novas formulações cosméticas uma vez que foram obtidas com sucesso formulações base estáveis, que podem ser melhoradas para o efeito de anti-envelhecimento.

Palavras-chave: anti-envelhecimento, estabilidade, formulações cosméticas, ingredientes ativos, pH, reologia.

Abstract: Development of anti-aging cosmetic formulations

The cosmetics industry has acquired enormous importance and it is considered a constant expanding area of business and research. Our society is increasingly concerned with health and appearance, which has increased the demand for effective anti-aging formulations. The development of skin care formulations is a complex process because it is necessary to consider several characteristics such as the interaction of ingredients, stability, moisturizing level, skin spreading and sensation.

The goal of this work was the development and characterization of skin care formulations with anti-aging properties. The focus was the development of different face serums, aqueous and milky serums. The preparation of aqueous serums proved to be a faster and easier process than the milky serums. Six formulations were developed, three base formulations and three containing active ingredients. The stability of developed formulations was performed through the evaluation of different physical and chemical properties for three months. The properties included: determination of pH, viscosity, stability at extreme temperatures and evaluation of the organoleptic characteristics (odor, color and appearance) at different temperatures (4°C, room temperature and 37°C). It was also studied the influence of light and the packaging material in the formulation's stability. The results obtained showed that Formulations A, both the base and with the active ingredients, have stability in all conditions. In contrast, the Formulations B and C, did not show to be stable mainly the formulations containing active ingredients (Formulations B2 and C2). In a final step of this work, after some optimizations, more stable Formulations B1 and C1 were obtained by the inclusion of emulsion stabilizers and chelating agents.

In conclusion, this work contributes to the development of new cosmetic formulations since stable base formulations were successfully obtained, which can be improved for anti-aging purpose.

Keywords: cosmetic formulations, anti-aging, stability, active ingredients, rheology, pH.

Contents

List of Abbreviations and Symbols

$ – Dollar

€ – Euro

%(m/m) – Mass percentage by mass

µl – Microliter

γ – Shear strain

mPa.S – Millipascal second

AHA – Alpha hydroxy acids

ALA – Alpha lipoic acid

BHA – Beta hydroxy acids

DLS – Dynamic Light Scattering

DNA – Deoxyribonucleic Acid

G' – Elastic/storage modulus

G'' – Viscous/loss modulus

GAGs – Glycosaminoglycans

HA – Hyaluronic acid

HLB – Hydrophilic-lipophilic balance

Hz – Hertz

KDa – Kilodaltons

LVR – Linear viscoelastic region

NMF – Natural moisturizing factor

O/W – Oil in water

PCA – Pyrrolidone carboxylic acid

PDI – Polydispersity index

pH – Hydrogenic potential

Rpm – Revolutions per minute

RT – Room temperature

SC – Stratum corneum

UV – Ultraviolet

W/O – Water in oil

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1. State of Art

1.1 Cosmetics Industry

The cosmetics industry has acquired enormous importance over time and it is a constantly expanding area of business and research. According to Cosmetics Europe - The Personal Care Association, about 450 million Europeans use a wide range of cosmetics in their daily routines, such as shampoos, conditioners, perfumes, shaving cream, makeup, and creams (Brandt et al., 2011; Newburger, 2009). Specifically, there are seven categories of cosmetics and personal care products, referred to as oral care, skin care, sun protection, hair care, decorative cosmetics (makeup), body care, and perfumes (Creek et al., 2017). Based on the categorical division of cosmetic products, it is also possible to segment the market by the same categories. It is evident that skin care related products are the most sought after and used (**Figure 1**).

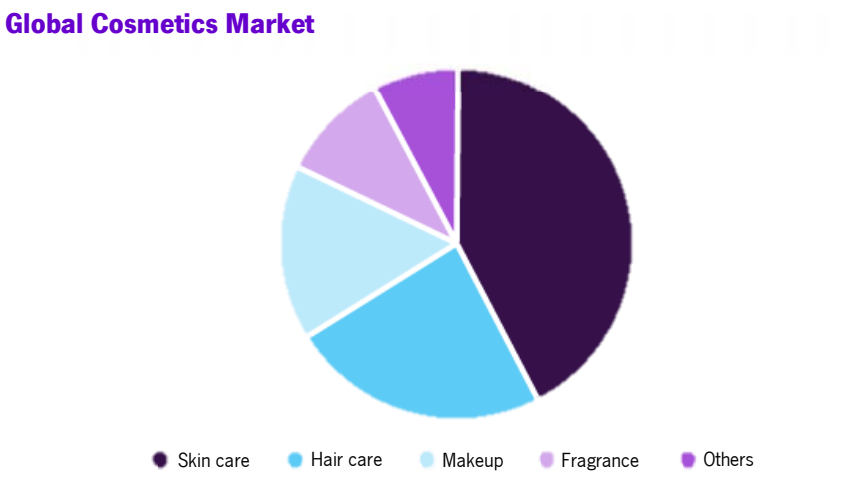


Figure 1.Market segmentation by major cosmetics categories. Adapted from Fortune Business Insights (2021).

The categories presenting the largest share in the European market concern skincare-related products (€20.39 billion euros); personal care products (€19.92 billion of euros); hair care products (€14.92 billion of euros); fragrances/perfumes (€12.289 billion of euros) and decorative cosmetics (€11.07 billion of euros) (**Figure 2**).

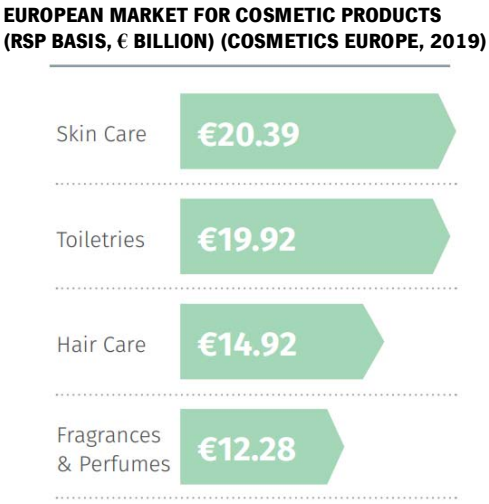


Figure 2. Representation of the European Cosmetics Products market, by category and in billion euros. Adapted from Cosmetics Europe (2019).

One of the main goals of the cosmetic industry, as far as skin care is concerned, is to meet the growing demand for anti-aging products. The assessment of the global anti-aging cosmetics market showed a value of $38.62 million in 2018, and this is expected to reach around $60 million by 2026 (Grand View Research,2021). The market for anti-aging products has many multinationals as well as domestic manufacturers operating in the market, making product prices highly competitive. The companies with the most market recognition in this area is L'Oréal Professional, Unilever, Procter & Gamble, Estée Lauder Inc, Beiersdorf AG, Shiseido CO., Coty Inc Johnson and Johnson Services (Statista, 2020).

In terms of monetary values, the cosmetics industry showed a global market of around $277.67 billion in 2020. Due to the negative impact that the pandemic had on the market, there was a slight decrease in the global market. However, values are expected to increase steadily (Fortune Business Insights, 2021).

1.2. The skin and its structure

Skin is the human body´s largest sensory and touch organ, and, like all other organs, it is vulnerable to aging. The skin is divided into several layers, as shown in **Figure 3**. The epidermis and dermis are the two primary layers of the skin. A third layer, the hypodermis or subcutaneous tissue, lies underneath the dermis (Ganceviciene et al., 2012; Mohiuddin, 2019a).

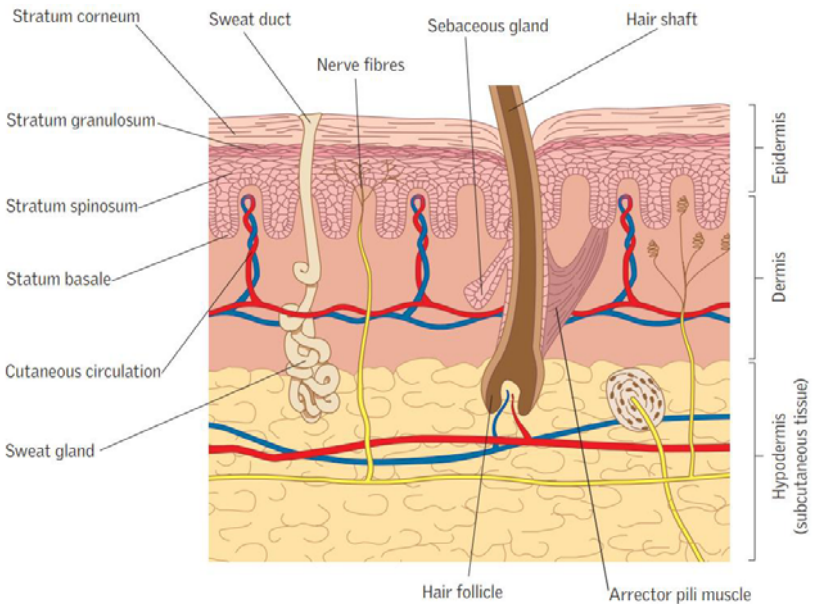


Figure 3. Diagram of human skin structure, showing the three main skin layers. Adapted from MacNeil (2007).

1.2.1. Epidermis

The epidermis is the most superficial and thinnest layer and due to its high lipid content, is responsible for determining which active ingredients can penetrate, as well as preventing excessive water loss (Ludovici et al., 2018). The epidermis is in turn divided into five layers: the stratum corneum (SC), stratum lucidum, stratum granulosum, stratum spinosum, and stratum basal. In the epidermis, there are keratinocytes (cells that produce keratin) and melanocytes (cells that produce melanin) that are arranged in the layers mentioned above (Knox & O'Boyle, 2021; Ludovici et al., 2018).

The SC, which is the outermost layer, is a barrier made up of dead cells. This layer of the epidermis is a complex matrix that contains proteins and lipids. It is composed mostly of three classes of lipids: ceramides, cholesterol, and non-esterified fatty acids, which are produced by sebocytes, keratinocytes, and the skin's own microbiome (Schmitt & Neubert, 2020; Van Smeden & Bouwstra, 2016; Wohlrab et al., 2018). The SC is also composed of low molecular weight and water soluble molecules that play an important role in maintaining skin hydration, which are called natural moisturizing factor (NMF) (Baumann, 2008; Rawlings & Harding, 2004; Verdier-Sévrain & Bonté, 2007). NMF molecules hydrate hydrophobic keratin, allowing it to retain water molecules and so maintain proper skin hydration. This permits the skin's flexibility to remain intact, protecting it from harm. (Baumann, 2008; Harding et al., 2000; Kwan et al., 2012; Verdier-Sévrain & Bonté, 2007). NMF consists mainly of free amino acids and various derivatives thereof, such as

pyrrolidone carboxylic acid (PCA), urocalcenic acid (a natural absorber of ultraviolet (UV) radiation), and inorganic salts, and sugars (Kwan et al., 2012).

The stratum lucidum is a smooth and apparently translucent layer of the epidermis located just below the SC and above the stratum granulosum. Stratum lucidum is composed by dead and flattened keratinocytes (Ita, 2020) and only the thick skin of the palms, soles, and fingers contains this thin layer of cells.

The stratum granulosum has a granular appearance, as the name implies, due to further changes in the keratinocytes as they are pushed off the stratum spinosum (Khavkin & Ellis, 2011).

The stratum spinosum is the layer above the stratum basal, and it is made up mostly of desmosome-attached keratinocytes and immunologically active Langerhans cells that come from the bone marrow (Nafisi & Maibach, 2018; Rochette et al., 2020).

The basal stratum is the deepest layer and is responsible for joining the epidermis to the basal lamina, below which is the dermis. The cells of the basal stratum connect to the dermis through collagen fibers. In the basal stratum, the keratinocytes are formed and then migrate to the SC, where they undergo differentiation, becoming corneocytes. This stratum also presents in its constitution other cells, such as melanocytes and Merkel cells, which work as receptors and are responsible for stimulating the sensory nerves of the brain (Draelos & Thaman, 2005).

1.2.2. Dermis

Dermis is responsible for most of the skin's functions, such as environmental perception, thermoregulation, immune defense, water storage, and mechanical stress (Baki & Alexander, 2015). The main structural components of the dermis are collagen and elastin, glycosaminoglycans (GAGs), and water. Collagen is the most abundant protein and forms a three-dimensional fiber network responsible for keeping the dermis resistant to traction. Elastin, in turn, provides the skin with its elastic properties. The space between the collagen and elastin fibers in the reticular layer is filled by hyaluronic acid (HA), a gelatinous molecule with high water retention capacity (Baki & Alexander, 2015; Draelos & Thaman, 2005). It has nerves, blood and lymph vessels, as well as other parts including hair follicles and sweat glands. Because the epidermis lacks blood vessels, its cells must obtain oxygen and nourishment from capillaries in the dermis (Cui et al., 2020). The basic cells in the dermis are fibroblasts, but histiocytes, mast cells, and adipocytes are also crucial for preserving the dermis' typical form and function (Rochette et al., 2020).

1.2.3. Hypodermis

The hypodermis lies just below the dermis and is considered the third layer of the skin. This layer consists mainly of adipose tissue and fibrous connective tissue. It also includes fibroblasts, nerves, blood vessels, macrophages, and cells that are part of the immune system. The essential functions are to store lipids that will serve as energy and protect the body, from both thermal and mechanical shocks (Voegeli, 2012).

1.3. Skin Aging

The aging process for the body's organs starts the moment a person is born, and the skin is no exception. As the largest organ in the body, the skin ages most visibly and obviously as a person gets older. Skin aging is a gradual and continuous process. It is divided into intrinsic aging, when associated with genetic, metabolic and hormonal changes, or extrinsic aging, due to external factors related to the environment and lifestyle (Dimitriu et al., 2019).

Intrinsic aging reflects chronological genetic changes in the body, in which the renewal of cells is slowed. As a result, cells stop dividing and enter a state of senescence. In general, all cells enter in senescence after a certain number of cell divisions, established a priori by the cell itself. However, with advancing age, cells responsible to produce keratin and fibroblasts that finished their replication cycles accumulate in the skin, accelerating the aging process. In this way, cell communication is altered, leading to tissue rigidity that, consequently, causes the appearance of wrinkles (Bonté et al., 2019; Dimitriu et al., 2019; Shin et al., 2019; Tobin, 2017; Wong & Chew, 2021). Intrinsic aging also includes hormonal changes, for example after a certain age estrogen production is reduced. This reduction affects the synthesis of HA, which makes the skin thinner and less hydrated (Ramos-e-Silva et al., 2013).

The main cause of extrinsic aging is photoaging because the skin exposure to UV rays induces numerous changes, at molecular and cellular level, which cause a rapid and dynamic disorder in the skin. In contrast, intrinsic changes occur slowly, producing generalized atrophy and few structural changes by the age of 50 (Ahmed et al., 2020). The effects of sunlight on the skin are profound and account for up to 90% of visible facial skin aging, especially in fair-skinned individuals. Although photoaging is the most prevalent extrinsic factor, ethnicity, anatomical variations, stress, tobacco and alcohol consumption, fatigue, among others, also contribute to premature skin aging (Ahmed et al., 2020; Wong & Chew, 2021).

1.3.1. Effects of Aging on Skin

Symptoms of aging include the appearance of fine lines, dryness of the skin, appearance of wrinkles, loss of skin elasticity, dullness and roughness, thickened epidermis, mottled discoloration (Ganceviciene et al., 2019). In addition to the visible changes, over the years, the skin also undergoes physiological changes at the biochemical level as well as changes in neurosensory perception, permeability, response to injury, repair capacity and possible increase in the incidence of some skin diseases (Khan, 2018; Mohiuddin, 2019b).

1.3.1.1. Biochemical Changes

pH is a physicochemical factor of high importance when it comes to biological processes, namely metabolic, molecular, and cellular regulation processes. Focusing only on the skin, the SC is especially affected by pH because it can interfere with its barrier function (Farage et al., 2018; Schulte to Brinke et al., 2021). Thus, the pH of the skin surface is slightly acidic and lies in the range between 4.1 and 5 (Schulte to Brinke et al., 2021). But as the deeper layers of the skin are reached, the pH value increases slightly. The acidic nature of the SC allows an antimicrobial activity and ensures that processes, such as lipid synthesis and desquamation, proceed normally. However, the pH value is easily influenced by endogenous and exogenous factors, such as eccrine and sebaceous secretions, anatomical sites, genetic predisposition, age, humidity, among others (Lukić et al., 2021; Schulte to Brinke et al., 2021; Surber et al., 2018). The daily use of cosmetics can contribute to the maintenance of skin health, for example by controlling the pH value of the skin. Moreover, in certain skin disorders, the use of topical products capable of correcting skin pH represent a good form of therapy (Abels & Angelova-Fischer, 2018; Hawkins et al., 2021). An increase in skin pH value increases the susceptibility to skin damage from infection, allergy and irritation. So, it is essential to take into consideration the pH value when are developed new cosmetic products.

Intrinsically aging skin also shows significantly reduced extracellular matrix protein expression in the lower and upper epidermal layers, inducing potential changes in normal skin structure and function. In addition, vitamin D synthesis slows down in aging skin, leading to a decreased concentration of this compound (Kazanci et al., 2017; Mohiuddin, 2019a).

1.3.1.2. Structural changes

The major cutaneous change observed in intrinsic aging is the reduction of contact between the epidermis and dermis layers. The loss of the epithelial extensions that project into the underlying connective tissue negatively affects the supply of nutrients, metabolites, and oxygen to the epidermis. Epidermis also loses its ability to produce cells, and the SC is not replenished as quickly as at a young age, resulting in dehydrated skin and therefore rougher and drier (Nigam & Knight ,2017). At the level of the dermis, with the advance of age, its thickness, as well as its volume, decreases, accompanied by a decrease in vascularity and in the amount of mast cells and fibroblasts. The number of GAGs and the amount of HA produced by fibroblasts decreases in the dermis from the age of 26. The collagen content decreases about 1% per year from adulthood onwards. The decrease in collagen and elastin content causes a gradual degradation of the dermis structure, which consequently loses its bond to the elastin fibers (Shin et al., 2019; Zullo et al., 2020). Regarding the hypodermis, the adipose tissue decreases, more specifically in the facial region, the back of the hands, and the shins. This change in the distribution of adipose tissue also influences the thermoregulation capacity, as well as decreases the capacity to help to mechanical shocks (Shah & Kennedy, 2018; Zhang & Duan, 2018). The skin changes caused by the aging are summarized in **Figure 4**.

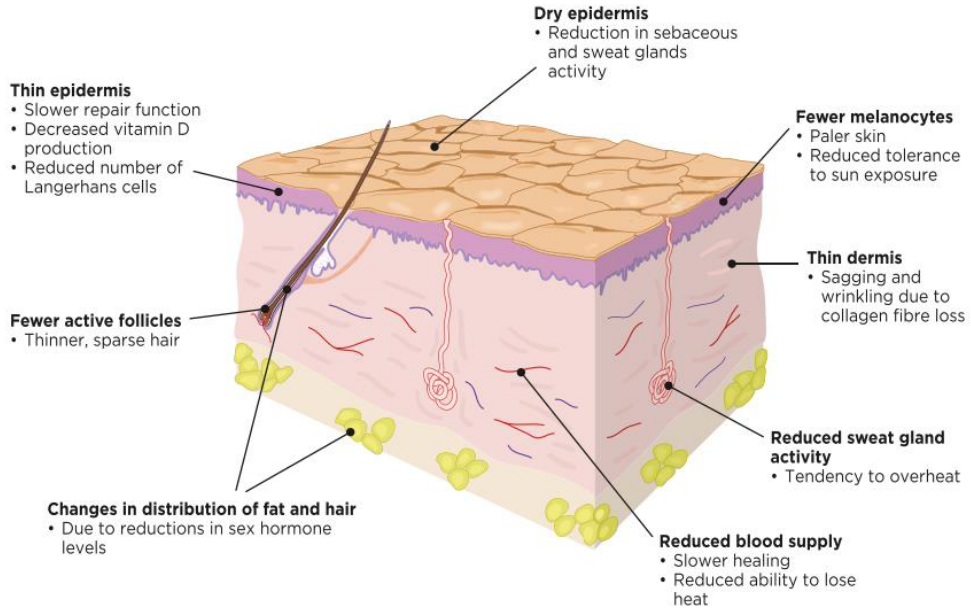


Figure 4. Schematic representation of the skin changes caused by aging. Adapted from Nigam & Knight (2017).

1.4. Cosmetic formulations for skin care

The most requested cosmetics are those related to skin care, more precisely, those targeting the facial skin and with anti-aging functions. The category of skin care cosmetics is very broad and includes anti-aging creams, eye creams, moisturizers, exfoliants, face masks, serums and sunscreens (Creek et al., 2017).

Serums are cosmetic products that present in its constitution a high concentration of active ingredients and whose purpose is to reach the deepest layers of the skin (Kaur et al., 2019). The products formulated in serum form may have antioxidant, moisturizing, smoothing, depigmenting, anti-aging functions, among others, according to the active ingredients present in formulation. Serums are usually applied in conjunction with a cream to provide the desired benefits to the skin. Unlike creams, serums are lighter and finer in texture, which gives the skin a lighter appearance, allowing them to be used in combination with other products (Barton et al., 2020; Ojha et al., 2019). The creams may include ingredients with occlusive capacity that form a protective barrier, preventing the loss of water to the skin surface and maintains its integrity and hydration. In the other hand, a serum acts to promote the fixation of water in the superficial layers of the skin (Rane et al., 2018). Serums are, in general, composed by few ingredients and is designed to maximize the availability of the active ingredient, which could be a vitamin, growth factor, or botanical extract, among others (Kaul et al., 2018; Mohiuddin, 2019b; Rane et al., 2018).

Serums can be divided in two categories: aqueous serums and emulsion-based serums. The first are composed exclusively of a single phase - the aqueous phase, and the second by an emulsion that is defined as a system of two or more immiscible liquid phases, one of which is dispersed as droplets in the other (Guzmán et al., 2022). The classification of emulsion systems differs according to the criteria. One of the distinctions is determined by the component that compose the continuous and dispersed phase (Figure 5). Since serums are mostly composed of water, they are usually oil-in-water (O/W) emulsions.

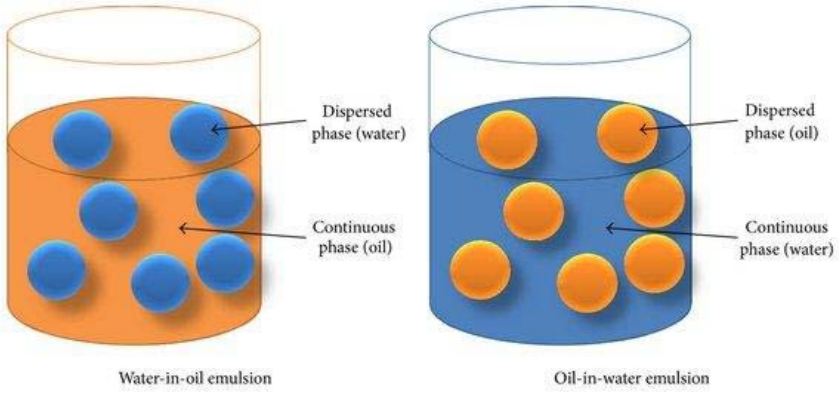


Figure 5.Classical emulsion systems of water-in-oil (W/O) and oil-in-water (O/W).

1.4.1. Base ingredients of cosmetic formulations

Stability and safety are two important characteristics that need to be guaranteed for the approval of a new cosmetic formulation. The presence of certain compounds is essential and/or can improve these two characteristics (Pinto et al., 2021). Water, preservatives, emollients, humectants, pH stabilizers, viscosity controllers, penetration enhancers, and emulsifiers, which are specific for O/W and water-in-oil (W/O) emulsions, can be found in serum formulations.

Water is the most abundant ingredient in lighter and more fluid formulations as serums. Due to the high-water content, serums are formulations that can be easily contaminated by microorganisms. Microbial contamination of cosmetics represents a health risk to consumers because contaminated formulations can lead to irritation or infection in skin (Halla et al., 2018). Therefore, the addition of **Preservatives** is essential to prevent the contamination of cosmetic products and to protect the consumer. In addition, preservatives are also able to protect cosmetics against any kind of damage or degradation caused by exposure to oxygen or UV light (Halla et al., 2018; Salvador & Chisvert, 2017). According to their chemical structure, the preservatives can be divided in different categories as represented in **Table 1**.

Table 1.Different preservatives commonly used in cosmetic formulations and their chemical classification.

Classification	Preservative
Organic Acids and their salts	2,4-hexadienoic and its salts, e.g. potassium sorbate; benzoic acid, its salts and esters, e.g. sodium benzoate, p-hydroxy benzoic acid and its esters, commonly known as parabens, e.g. Methyl Paraben;
Alcohols and phenols	2-phenoxyethanol bronopol
Heterocyclic compounds	Imidazolidynyl Urea
Quaternary ammonium salts	Methenamine 3-chloroallylochloride).
Phenols	Thymol, eugenol and carvacrol

Emollients are compounds responsible for a set of sensory and visual characteristics. Regarding skin sensation, they are directly linked to a feeling of softness, elasticity and spreadability (Mawazi et al., 2022). Relatively to visual perception, emollients can be responsible for the final finish of a cosmetic product on the skin, i.e., its glossy or matte appearance. Emollients consist essentially of lipids and oils with the ability to moisturize and enhance the skin's softness and flexibility. They fill the gaps resulting from the desquamation of the corneocytes (Chao et al., 2018; Sethi et al., 2016). Emollients are widely used in the development of cosmetic formulations and can be divided into four distinct groups according to their chemical family: hydrocarbons, fatty alcohols, esters, and silicones (Chao et al., 2018).

Humectants are hygroscopic compounds, which means that they attract water from both the epidermis and dermis, as well as from the environment when it has humid conditions. These characteristics highlight the important role of this type of ingredient in moisturizing the SC (Goodman, 2009). Honey, sorbitol, glycerin, panthenol, urea, gelatin, HA, AHA (glycolic acid, lactic acid, sodium pyrrolidine, carboxylic acid), propylene glycol, and butylene glycol are some examples of humectants used in cosmetic formulations (Nolan & Marmur, 2012; Purnamawati et al., 2017).

pH stabilizers are substances whose purpose is to keep the activity of cosmetic formulation components, as well as to keep the pH constant (Surber et al., 2018). Generally, pH adjustment and/or stabilization is achieved by the addition of lactic and citric acid, sodium acetate, sodium lactate, sodium citrate, and diammonium citrate (Bajkacz & Adamek, 2020; Du Plessis et al., 2018).

As already mentioned, serums can be emulsions, which are composed of leastwise two phases, an oily phase, and an aqueous phase. These phases are immiscible and one of the phases is suspended or dispersed in the other phase. **Emulsifiers** are added to the emulsions, providing the appropriate stability to the final formulations. Emulsifiers are a type of surfactants that can be

divided into four categories: cationic, anionic, non-ionic, and amphoteric. Surfactants are chosen for a particular purpose based on their hydrophilic and lipophilic groups, which is conveniently represented in the form of a scale called hydrophilic-lipophilic balance (HLB) (Alam et al., 2020). This system is based on a scale of 1 to 20, whereby the more hydrophilic emulsifier presents a higher HLB. HLB value dictates which specific activity each surfactant will exert in emulsions (**Table 2**) (Ashaolu, 2021; Guzmán et al., 2022).

Table 2. HLB values and correspondent emulsifier function. Adapted from Prieto-Blanco et al., 2018.

HLB Values	Emulsifiers Function
2-3	Anti-foaming agent
3-6	W/O emulsifying agents
7-9	Spreading and wetting agents
8-16	O/W emulsifying agents
13-15	Detergents
15-18	Solubilizing agents

Thickeners, also known as **viscosity controllers**, are key additives that influence the texture, consistency, and final function of a formulation (Baki & Alexander, 2015). Although their primary role is to raise the viscosity of the formulation, they can be classified as multifunctional additives since they influence a variety of product parameters. They can change the product's appearance and rheological qualities, as well as its sensory attributes and skin performance. In surfactant-based formulations, they can also suspend insoluble components like pigments, stabilize emulsions, and adjust foaming capabilities (Karsheva, 2007). Xanthan gum, a high molecular weight polysaccharide of natural origin produced from maize glucose syrup, is a frequently used thickening ingredient in cosmetic formulations. This ingredient is used to thicken and also to stabilize emulsion-based cosmetic formulations, because xanthan gum is resistant to enzymatic degradation and is stable across a wide pH and temperature range. When exposed to modest shear pressures, this ingredient has a high viscosity, allowing the maintenance of particles suspended and/or preventing bubbles from forming (Baki & Alexander, 2015; Beer, 2009).

Penetration enhancers are compounds responsible for increasing the permeability to cosmetic formulations through the SC, which is relatively impermeable to most of them (Gupta et al., 2019; Lane, 2013; Wiechers, 2005). Chemical, physical, enzymatic, and vesicular transporters are the four types of penetration enhancers. Chemical enhancers, such as terpenes, sulfoxides, laurocapram, pyrrolidones, fatty acids, fatty acid esters, fatty alcohols, alcohols, and glycols, are

the most common in this field. Solvents, such as ethanol and propylene glycol, are frequently employed as enhancement vehicles, not only because do they promote penetration but also are frequently utilized as co-solvents (Hu & He, 2021). The main advantages of chemical penetration enhancers are its low cost, it can be incorporated directly into cosmetic formulations, and does not require the use of additional instrumentation (Kim et al., 2020). Isopropyl myristate, a fatty acid ester, is another chemical enhancer commonly mentioned in the literature (Lane, 2013).

1.4.2. Active ingredients

Active ingredients are compounds that can be added to the base ingredients previously described, presenting specific purposes such as antioxidant, anti-acne, anti-wrinkle, or sunscreen, among others.

Antioxidants are used mainly for anti-aging purpose due to their ability to protect cell membranes and prevent oxidative stress in tissues by neutralizing toxic oxygen molecules and free radicals (Mohiuddin, 2019a). Free radicals are molecules or atoms with unpaired electrons, which translates into higher reactivity. These radicals seek to achieve stability and, to do so, steal an electron from another molecule, resulting in a chain reaction. This process induces damage in proteins, deoxyribonucleic acid (DNA) and lipids, having disastrous consequences for the body and can even lead to the development of severe diseases (Mohiuddin, 2019a). Antioxidants act as electron donors for free radicals, leading to their stability and preventing damage to other cellular compounds (Graf, 2010; Mohiuddin, 2019a). Vitamin A (retinol) and its derivatives, vitamin C (ascorbic acid), vitamin B5 (panthenol) and vitamin B3 (niacinamide), vitamin E (tocopherol), alpha lipoic acid (ALA), coenzyme Q10 (ubiquinone), and hydroxy acids are antioxidants commonly used in cosmetic (Addor, 2017; McCook, 2016; Turnbull, 2018).

Vitamins A, C, E and alpha lipoic acids slow down the aging process, preventing free radicals from oxidizing sensitive biological molecules or by reducing free radical formation (Ahmed et al., 2020; Draelos, 2010; Herranz-López & Barrajón-Catalán, 2020; Pavlou et al., 2021; Perricone, 2000; Zasada & Budzisz, 2019).

Coenzyme Q10 is already a molecule capable of preventing oxidative stress-induced cell apoptosis (Suter et al., 2016). Coenzyme Q10 is an anti-aging and anti-wrinkle antioxidant that protects the collagen and elastin manufacturing processes from harm.

Hydroxy acids are organic carboxylic acids classified into alpha hydroxy acids (AHAs) and beta hydroxy acids (BHAs) according to their molecular structure, and provide long-term cosmetic

benefits, such as improving skin firmness and elasticity and reducing lines and wrinkles (Babilas et al., 2012; Jarrar, 2015; Sathsarani & Wickramarachchi, 2021).

Niacinamide (vitamin B3) and panthenol (vitamin B5) belong to the family of B vitamins used in moisturizers to improve the skin's appearance. Niacinamide, also known as nicotinamide, is a water-soluble component present in a variety of foods, including whole grains and green leafy vegetables. When applied to the skin, it has been proven to provide a variety of advantages, including anti-inflammatory, depigmenting, and immunomodulatory qualities. This vitamin promotes collagen formation, which aids in the elimination of wrinkles associated with aging. Moreover, niacinamide inhibits the transference of melanosomes from melanocytes to keratinocytes, thereby reducing skin hyperpigmentation. When applied in aged skin, this vitamin enhances the skin's surface structure, smoothes wrinkles, and prevents photo carcinogenesis (Athawale et al., 2011; Berson et al., 2013; Wohlrab & Kreft, 2014). Panthenol, in turn, is converted into pantothenic acid, which is the active form of vitamin B5. This acid is important for promoting lipid formation in the skin, resulting in a barrier that aids wound healing. Vitamin B5 easily penetrates the skin barrier and is known for its moisturizing and soothing capabilities (Draelos et al., 2021).

Vitamin E is a fat-soluble molecule present in the skin and that can be found in various foods such as vegetables, seeds, and meat. It is the most abundant vitamin in the skin and is produced by the sebaceous glands in two forms: alpha-tocopherol or gamma-tocopherol. Vitamin E represents the skin's first line of defense against environmental stress (Johnson et al., 2021). The lipophilic property of vitamin E makes it suitable for cutaneous application and absorption. This vitamin acts in skin healing, protection against harmful germs, and have also moisturizing properties, reducing dehydration (Roškar, 2021). The antioxidant action of this molecule is due to its free hydroxyl group. When the rate of tocopherol production is lower than the rate of clearance, oxidative damage occurs. In this way, the presence of this vitamin is critical in the skin's defense against free radicals (Gamna & Spriano, 2021; Manela-Azulay & Bagatin, 2009).

Another important active ingredient greatly used in cosmetics for anti-aging purpose is the HA. This compound is a water-soluble, linear, anionic polysaccharide found in connective, epithelial, and neural tissue, and is one of the most prevalent substances in the extracellular matrix (Draelos et al., 2021). HA is in the interfaces of elastin and collagen fibers, which helps to preserve their right shape. As a result of the aging process, the link between HA and collagen and elastin fibers breaks down, causing wrinkles to develop. HA has become a crucial ingredient in the

cosmetic formulations because it is a key compound for keeping the integrity of the skin (Draelos et al., 2021; Neuman et al., 2015; Poetschke et al., 2016). HA is available in a variety of molecular weights and the main difference between the HA molecular weights is their ability to penetrate the skin at different depths. Molecules with a reduced molecular weight are smaller and can penetrate deeper layers of skin (Gallo et al., 2019; Muntean et al., 2019). On the other hand, molecules with higher molecular weight stay closer to the surface, where they can offer the most evident benefits in a shorter time frame. Although they cannot penetrate the skin barrier, high molecular weight HA forms a keratin film that can hydrate the skin and improve its elasticity, however, this film is easily eliminated (Kibbelaar & Shahidzadeh, 2021; Qiu et al., 2021; Ranjbar, 2021; Saha & Rai, 2021).

1.5. Stability of cosmetic products

The creation of a new cosmetic product is a lengthy process that includes the development of a formulation and the testing phases. Since cosmetics are targeted to specific parts of the body, which may be delicate, they need to be resistant and highly stable over time even throughout different adverse conditions (Korać et al., 2016).

Intrinsic factors, such as the nature of the product or the interaction between the different components, might impact the stability of a cosmetic product. Extrinsic factors, or external elements that the formulation is exposed, such as time, temperature, light, humidity, oxygen, vibration, and microbes, also impact the product's stability (**Table 3**) (Mohsin et al., 2016).

Table 3. Intrinsic and extrinsic factors that affect the stability of cosmetic formulations.

Intrinsic Factors	Extrinsic Factor
pH	Time
Reactions of oxidation-reduction	Temperature
Hydrolysis reactions	Light and Oxygen
Interactions between formulation ingredients	Containing material
Interactions between formulation ingredients and the packaging material	Microorganisms

Stability testing is defined as the process of evaluation of a given product to ensure that key attributes remain within acceptable guidelines. Stability testing is critical because it serves as a guide for the manufacturer during product development. These tests ensure that the product will be aesthetically pleasing to the consumer, determine that the product will function as intended and remain safe to use, and allows the company to avoid problems that may arise after the product has been marketed (Pokharana et al., 2018). Real-time cosmetic stability testing is not always

practicable due to the importance of time in the creation of a new cosmetic product. In certain cases, accelerated stability testing is a viable option (Bajaj et al., 2012; Chiari et al., 2012). There are numerous procedures for accelerated physical stability testing of emulsions that involve accelerated temperature and/or mechanical changes that occur naturally over shelf life. The first temperature-related test that must be performed is based on exposing the formulations to different temperatures, namely 4°C, room temperature (RT), and 37°C, for a period of three months. The second category of regularly used stability evaluation approaches is well-known for its capacity to destabilize emulsions, particularly those prone to coalescence. The most common test is the freeze-thaw test, where products are subjected to different temperatures, namely freezing followed by RT or higher temperatures, for 24-hour cycles that are repeated a minimum of three times (Berthele et al., 2014; Navarro-Pérez et al., 2021; Palareti et al., 2016).

According to ISO/TR 18891:2018, physical and chemical characteristics are quantifiable parameters previously established by the manufacturer. Firstly, the samples must be subjected to preliminary stability tests that include pH measurement, centrifuge test, and sensory tests (Tafuro et al., 2021). The evaluation of pH is done to ensure that the cosmetic formulations have a pH value compatible with skin pH. The centrifuge test is intended to determine whether phase separation occurs, and the absence of this phenomenon is desired. Since cosmetic emulsions are inherently unstable, it is critical to develop methods to keep the formulation in a minimally decent "stationary state" or "metastable condition" until the end of its storage (Waqas et al., 2017). Force of gravity, delayed changes in the crystalline structure of substances, or variations in formulation viscosity seem to be the most common causes of physical instability. Depending on the density difference between the two phases and the mass of the globules, the globules can collect in either the top (creaming) or lower layer (sedimentation). Creaming and sedimentation are processes that generate two separate layers inside the volume of an emulsion, with one layer containing more of the dispersed phase than the other (Guzmán et al., 2022; Semenzato et al., 2018). Another type of instability is flocculation, which occurs when internal phase droplets attach to one other and create tiny clumps (flocci). Flocculation can be reversed by agitation, increasing the emulsifier content, or adding a higher HLB emulsifier. Coalescence generally happens when an emulsion is formed with inadequate emulsifier. This technique is commonly performed after flocculation, in which the aggregates are converted into big and isolated droplets. Centrifugation and vibration tests are the most widely used tests to verify if physical changes occur, in order to predict the stability of the cosmetic formulations (Adejokun & Dodou, 2020; Estanqueiro et al., 2014; Tafuro et al.,

2021). The sensory tests aim to evaluate if the look, feel, and comfort of the formulation suits the customer's needs. Using this method, it is feasible to identify customer preferences in relation to specific items, being possible to adjust and improve formulations in order to enhance the product's acceptance (Ku & Jose, 2013). Sensory analysis provides information that other analytical processes cannot discover, such as customer approval and purchase intention (Filter et al., 2009; Gore et al., 2018; Moravkova & Filip, 2016). The formulation spreading is also an important point to attend when physical and chemical characterization has been done. The spreadability of a cosmetic is defined as its ability to cover an area of skin more or less quickly and is affected by molecular weight, viscosity, and chemical structure (Bom et al., 2020; Gore et al., 2018). When applied on human skin, cosmetics must spread readily without feeling oily or sticky.

Rheological characterization must be done since rheology may significantly influence how formulations are defined and controlled in terms of their in-use performance, stability, and usability. The flow curve of the cosmetic formulations, i.e., the plot of shear stress against shear rate or against viscosity, serves as the basis for rheological categorization (Huynh et al., 2021). Thus, to determinate the flow behavior, the formulation must be submitted to different shear rates at a constant temperature. The **Figure 6** shows the most common flow curves of fluids.

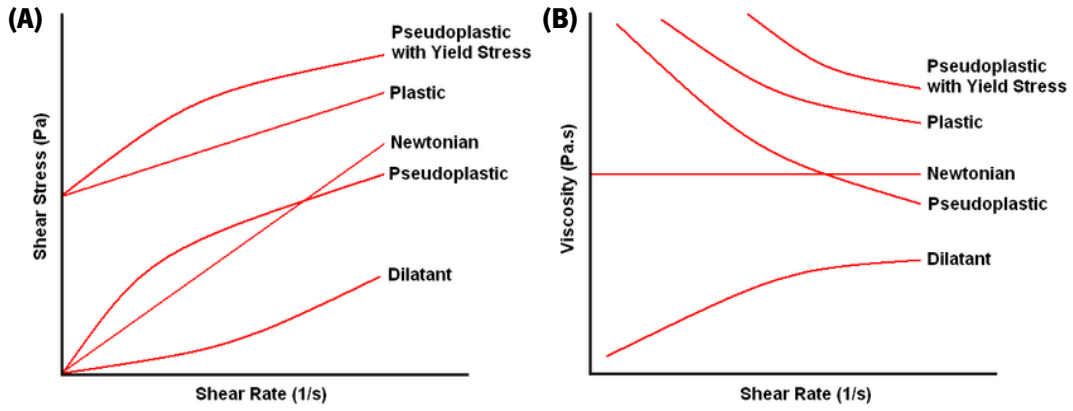


Figure 6. Typical flow curves of (A) Shear stress versus Shear rate (B) and Viscosity versus Shear rate. Adapted from Carlesso (2017).

Fluids are often classified into three types based on their flow characteristics: Newtonian fluids; time-independent non-Newtonian fluids; time-dependent non-Newtonian fluids. The Newtonian fluids have shear stress that is directly proportional to shear rate and hence viscosity that is invariant with shear rate or shear stress. Water, simple hydrocarbons, and dilute colloidal dispersions are examples of Newtonian fluids. Non-Newtonian fluids have viscosities that vary as a

function of shear rate or shear stress (Popova et al., 2019; Rehman et al., 2018; Yang & Du, 2020). **Table 4** summarizes the rheological behavior of non-Newtonian fluids.

Table 4. Characterization and examples of non-Newtonian fluids time-independent and time-dependent.

Rheological Behavior of Non-Newtonian Fluids	
Time-independent	Shear-thinning: viscosity decreases with increasing shear rate (plastic and pseudoplastic), e.g., blood, fruit juice concentrate, skin creams and lotions, shampoo. Shear-thickening: viscosity increases with shear rate (dilatant), e.g., concentrated corn starch suspensions, sand/water mixtures. Plastic: exhibit a yield stress, e.g., ketchup, toothpaste, hand cream
Time-dependent	Thixotropic: viscosity decreases in time, e.g., clay suspensions, blood, creams. Antithixotropic: viscosity increases in time, e.g., gypsum paste, concentrated latex dispersions.

Another important rheological analysis is the strain (stress) amplitude sweep test, which is an oscillatory rheological test that determines the degree of linearity of the formulation and is a suitable initial step in assessing the viscoelastic feature of the cosmetic formulations (Mohsin et al., 2016). The linear viscoelastic region (LVR) corresponds to the zone in which the formulation can maintain its structure when subjected to some force. Thus, this region allows infer the firmness of the cosmetic formulation, i.e., lower LVR indicates a less firm formulation, and higher LVR indicates a greater ability to maintain its structure when a force is applied (Adejokun & Dodou, 2019; Ho & Dodou, 2007). After definition of LVR with a strain sweep, the structure of a formulation may be further described with a frequency sweep at a strain less than the critical strain. In this test, the strain value is kept constant, only the frequency at which the samples are submitted is wide-ranging. Thus, it is possible to evaluate the elastic/storage modulus (G') and the viscous/loss modulus (G'') of the formulation. The G' represents the stored deformation energy, whereas the G" reflects the deformation energy lost (dissipated) during flow. If G' is greater than G'' the sample presents an elastic behavior in which the tan δ, determined by ***equation 1***, is less than 1, and if G''' predominates over G' then the behavior is viscous with tan δ greater than 1 (Chiari et al., 2012; Greenaway, 2010; Huang, 2019).

$$\boldsymbol{Equation\ 1}\text{: } \tan\delta = \frac{G''}{G'}$$

2. Materials and Methods

2.1. Development of skin care formulations

Based on the current serums on the market, efforts were made to develop aqueous and milky (emulsion-based formulation) serums. After several steps of optimization, where several ingredients were tested at different concentrations, three base formulations were achieved. Formulation A1 correspond to an aqueous serum and Formulations B1 and C1 to an emulsion-based formulations (oil-in-water emulsions). These three formulations were enriched with active ingredients, resulting in Formulations A2, B2 and C2.

Regarding the aqueous serums, namely A1 and A2, their production consisted in dissolving the different ingredients in water under mechanical stirring. The first stage was the weighing of the ingredients separately, which are described in **Table 5**. All ingredients were added individually to a glass beaker containing the water, and homogenized with the support of a mechanical stirrer, IKA RW 20 digital, at a speed of about 400 rpm.

Table 5. Composition of Formulations A1 and A2.

		A1	**A2**
	Ingredients	**% (m/m)**	
Phase A (Aqueous phase)	Water	90	87.55
	Thickener 1	0.4	0.4
	Humectant 1	2	2
	Humectant 2	4	4
	Preservative 1	1	1
	Active ingrediente 1	-	0.5
	Active ingrediente 2	-	2
	Preservative 2	0.5	0.5
	Emollient 1	2	2
	Fragrance	0.05	0.05

Relatively to emulsion-based formulations, different processes were used considering the needs of each emulsifier and the presence of active ingredients in formulations. For Formulation B1, stabilized by Emulsifier 1, the process was carried out at RT, and the aqueous and oil phases

were prepared separately. Both phases were weighed according to **Table 6**. Then, the aqueous phase was homogenized using a Ultraturrax IKA T50 digital Homogenizer up to a maximum speed of 9000 rpm, while the oily phase was placed under magnetic stirring. When completely homogenized, the phase B (oily phase) was carefully added to phase A (aqueous phase) at a stirring speed of about 600 rpm. After 5 minutes of homogenization with the mechanical stirrer, IKA RW 20 digital, it was obtained the emulsion, posteriorly the phase C were slowly added (200 rpm). Due to the addition of active ingredients, the preparation of Formulation B2 required greater care and several optimizations were performed. The ingredients that constitute the phase A were weighed individually and then added one by one in the following order: Water, Preservative 2, Humectant 1, Active ingredient 1, Active ingredient 2, Thickener 1, Humectant 2, and finally Preservative 1. On the other hand, the oil phase was weighed and placed on a magnetic stirrer. When both phases are completely homogenized, the oil phase was added to aqueous phase at 600 rpm. After 5 minutes, the emulsion was formed, and the phase C was incorporated at 200 rpm.

Table 6. Composition of Formulations B1 and B2.

		B1	B2
	Ingredients	**% (m/m)**	
Phase A (Aqueous phase)	Water	77	74.4
	Preservative 1	0.5	0.5
	Thickener 1	0.35	0.35
	Humectant 1	0.5	0.5
	Humectant 2	1	1
	Preservative 2	1	1
	Active Ingredient 1	-	0.3
	Active Ingredient 2	-	1
Phase B (Oil phase)	Emulsifier 1	2.5	2.5
	Emollient 2	3	3
	Emollient 2	3	3
	Emollient 4	3	3
	Emollient 5	5	5
	Active Ingredient 3	-	2
Phase C	Humectant 3	1	1
	Emollient 1	1.5	1.5
	Fragrance	0.05	0.05

Formulations C1 and C2 were developed according to the process mentioned above and their compositions are described in **Table 7**. This formulation requires a hot/cold process because the Emulsifier 2 is solid and requires to be heated to mix with the other ingredients of oil phase. Therefore, the oil phase was heated at 75°C under magnetic stirring to ensure homogeneity of the solution. Then, the oil phase was added to aqueous phase for emulsion formation, as previously described for Formulations B, and the phase C added to the emulsion.

Table 7. Composition of Formulations C1 and C2.

		C1	C2
	Ingredients	**% (m/m)**	
Phase A (Aqueous phase)	Water	79	75.2
	Preservative 3	1	1
	Thickener	0.35	0.35
	Humectant 1	0.5	0.5
	Humectant 2	1	1
	Preservative 2	0.5	0.5
	Active Ingredient 1	-	0.3
	Active Ingredient 2	-	1
Phase B (Oil phase)	Emulsifier 2	4	4
	Emollient 6	2	2
	Emollient 3	2	2
	Emollient 4	2	2
	Emollient 7	2	2
	Emollient 8	2	2
	Active Ingredient 3	-	2
Phase C	Humectant 3	1	1
	Emollient 1	1.5	1.5
	Humectant 4	1	1
	Fragrance	0.05	0.05

In a final part of this project, more optimizations were needed to increase the stability of developed formulations. Chelating agents and emulsion stabilizers were incorporated in aqueous phase of base formulations, and some modifications in the preparation processes were also tested.

2.2. Characterization of skin care formulations

2.2.1. Organoleptic properties

The formulations were evaluated for changes in color, odor, or spontaneous coalescence of the phases by visual and olfactory observation at different time points (Muntean et al., 2019).

2.2.2. pH measurement

The pH of each formulation was measured, with a digital pH meter (METRIA BENCH TOP PHMETER MODEL M92) on the day of its preparation and on predetermined time points (7, 15, 30, 60 and 90 days after preparation).

2.2.3. Centrifugation Test

1 gram of each formulation was centrifuged at 3000 rpm and 20°C for 30 minutes in a GYROZEN 1730R centrifuge. This procedure was performed at the day of preparation and after 30, 60 and 90 days of storage.

2.2.4. Determination of particles size

The size and surface charge, expressed as zeta-potential, of the particles in formulations were determined at RT by Dynamic light scattering (DLS) technique using a Malvern Zetasizer NS (Malvern Instruments) (Mondéjar-López et al., 2022). For this purpose, three replicates were prepared in which 20 µl of each formulation was diluted in 980 µl of water to allow a proper particle size reading. The surface charge was measured, using a specific cell for this purpose and a sufficient volume of sample to cover the electrodes of the cell. Formulation A1 and A2 were not submitted to this analysis because are not emulsion-based formulations.

2.2.5. Evaluation of Rheological Behavior

Rheological characteristics of all formulations were evaluated using a Discovery HR-1 hybrid rheometer (TA instruments) and TRIOS software. A plate geometry (40 mm) was employed to analyze the formulations at 20.0± 0.1°C. In steady-state flow, shear rates varied from 0.1 to 300s^{-1} for viscosity and thixotropy experiments. After determination of the linear viscoelastic region (LVR), varying the oscillation strain, the dynamic viscoelasticity was measured as a function of

frequency (Lukic et al., 2012; Tafuro et al., 2019). The LVR was first identified using an amplitude sweep test at a fixed value of frequency (1Hz), varying the strain (γ) from 0.1 to 1000%. The amplitude sweeps aids in determining the linearity of the material. The structure is intact below the critical stress level, and G' (storage modulus) and G'' (loss modulus) stay constant. A frequency sweep test at a fixed value of strain, which was identified in the LVR, was conducted in a range of 1000 to 0.01 Hz in order to study the materials inner structure and physical stability (Sciabica et al., 2021).

2.2.6. Determination of viscosity

The ROTAVISC viscometer (IKA Rotavisc lo-vi)) was used to determine the viscosity, in millipascals (mPa.S), of each formulation at RT. The force acting between two coaxial cylinders is measured by viscosity (spindles). This value was measured using a VOL-SP-2.1 spindle in two different ways: fixing the torque at 13% and fixing the speed at 200 rpm per minute.

2.3. Stability studies

2.3.1. Accelerated Stability Tests

A three-month stability study was performed, in which the organoleptic characteristics analysis, centrifugation test, viscosity, particle size and PDI and determination of pH were evaluated in day of formulations preparation (T0), 30 days (T30), 60 days (T60) and 90 days (T90) after preparation. The samples were stored at RT (25°C±2), in the fridge (4°C±2) and in the oven (37°C±2) (Agency, 2004; Kirkbride et al., 2021).

2.3.2. Light Radiation Exposure and Packaging Material test

The light stability of the developed formulations was also evaluated, at RT. All formulations were placed in glass containers and in plastic containers, both exposed to light. Simultaneously, they were placed in glass and plastic containers covered with aluminum foil. Characteristics such as pH, organoleptic characteristics, viscosity, phase separations and uniformity of the particles size were evaluated. Observations were made at the beginning of the experiment (day of preparation) and at the end of 30, 60 and 90 days of storage (Agency, 2004).

2.3.3 Freeze-thaw cycle testing

Developed formulations were submitted to three cycles consisting of 24 hours at RT and 24 hours at -10°C (Cekic et al., 2020). pH, organoleptic characteristics, viscosity, phase separation, particle size and zeta-potential were evaluated after these three cycles.

3. Results and Discussion

3.1. Characterization of developed formulations

3.1.1. Organoleptic properties

As any marketed cosmetic product, the stability of developed cosmetic formulations must remain unchanged during their shelf life. The control of the organoleptic characteristics is a simple but effective indicator to evaluate the stability of formulations. Immediately after preparation, the organoleptic characteristics of base formulations (A1, B1 and C1) and formulations containing active ingredients (A2, B2 and C2) were evaluated (**Table 8**).

Table 8. Organoleptic properties of Formulations A, B and C, after their preparation (time 0).

		Color	Odor	Aspect
Formulations A	**A1**	T	OF	H
	A2	T	OF	H
Formulations B	**B1**	B	OF	H
	B2	B	OF	H
Formulations C	**C1**	W	OF	H
	C2	W	OF	H

Legend: A1, B1 and C1: Base formulations; A2, B2 and C2: Formulations containing active ingredients; T: Transparent; B: Beige; W: White; OF: Odor according to the fragrance; H: Homogeneous solution.

It should be noted that Formulations C appear to be thicker and less fluid than the others and, considering that the main difference is the emulsifier, it is believed that this is responsible for their thickening.

The organoleptic analysis of the formulations was also performed 30, 60 and 90 days after preparation, being the formulations conditioned at RT, 4℃ and 37℃.

3.1.2. Measurement of pH

The pH of a solution is defined as the negative logarithm (ten base) of the concentration of free hydrogen ions (H^+) (Lukić et al., 2021) On a scale of 0 to 14, it is used to calculate the acidity–alkalinity ratio. To avoid disturbing the physiological pH of the skin, it is typically advised that skin care products have a pH of 5–5.5 (Wohlrab & Gebert, 2018). The pH measurement is an extremely important parameter in the development of cosmetic formulations, since these are

products that will be in contact with the skin. **Table 9** shows the pH values of the developed cosmetic formulations.

Table 9. pH values of Formulations A, B and C, after their preparation (time 0).

		pH values
Formulations A	A1	5.162
	A2	5.320
Formulations B	B1	4.980
	B2	5.052
Formulations C	C1	5.194
	C2	5.286

The pH of all formulations is in the desired range, around 5. According to the measured pH-values it is possible to see that the addition of active ingredients induces a slight increase of pH values.

3.2. Preliminary Stability Test

Techniques to assess the stability of cosmetic formulations often must be adapted to the type of formulation to detect any instability and/or changes in their properties (MakingCosmetics Inc, 2020). Centrifugation produces stress in the formulations, simulating an increase in the force of gravity, increasing the mobility of the particles, and anticipating possible instabilities. This force can result in precipitation, phase separation, formation of compact sediment (caking), coalescence, among others. This accelerated test allows a preliminary evaluation of the stability considering the sample's appearance.

Table 10 shows that no phase separation was observed after centrifugation of all developed formulations. These results indicate that all formulations are stable at the initial point (after preparation) and it was possible to proceed with the accelerated stability study.

Table 10. Appearance of developed formulations after centrifugation at 3000 rpm for 30 minutes at 20°C.

	Bases	With active Ingredients
Formulations A		
Formulations B		
Formulations C		

3.2.1. Particle Size

The skin acts as a barrier to the external environment, mainly due to the SC (Nishifuji & Yoon, 2013). For topical applications overcome the several skin layers, it is very important that the particles of cosmetic formulations have an appropriate size. High particle sizes are not compatible with the skin absorption and can also occur phenomena such as flocculation and coalescence in formulations (Jeong et al., 2001). Small particles ensure greater contact with the SC, facilitating compound permeation by increasing the partition coefficient between the carrier and the SC (Alvarez-Román et al., 2004; D. Gianeti et al., 2012), improving the depth of penetration. Regarding particle size, a slight increase in particle size was observed with the addition of the active ingredients, mainly for Formulations C (**Table 11**).

The degree of non-uniformity in a particle size distribution is referred to as "polydispersity" (or "dispersity," as suggested by IUPAC) (Danaei et al., 2018). The polydispersity index (PDI) value of formulations is obtained simultaneously with the particle size determination. The PDI value is a parameter that defines the particle size distribution in emulsions. PDI values between 0.2 and 0.3 indicate that formulations are moderately homogeneous, values of 0.0 are very homogeneous, while values of 1.0 show very heterogeneous formulations (Al-Othman, 2011; Saraf Pt Ravishankar et al., 2011; Tantra et al., 2010; Singh et al., 2019). PDI values of developed formulations were

mostly between 0.1 and 0.3, which indicate moderate homogeneity and is acceptable for this type of application (topical application).

Table 11. Particle size and PDI values of Formulations B and C, after their preparation (time 0).

	Formulations B		**Formulations C**	
	B1	**B2**	**C1**	**C2**
Mean of Particles Size (nm)	1480.67±270.43	1590.00±77.60	1740.00±181.02	2195,00±25.46
PDI	0,198±0,008	0,193±0,026	0,178±0,193	0,239±0,046

3.2.2. Rheological Behavior of Formulations

Rheological properties may be an indicator of changes produced by the addition of some ingredients, such as the active ingredients (Gianeti et al., 2012). Fluids can present different behaviors when submitted to rheological analysis. An ideal fluid will exhibit a behavior called Newtonian flow, where the relationship between shear stress and shear velocity is linear and the constant of proportionality is viscosity. However, there are many fluids that do not respect linearity and exhibit non-Newtonian behavior.

The rheological behavior of developed formulations was determined graphically by means of a "flow curve" which is obtained by shear stress (A) or viscosity (B) versus shear rate (**Figure 7**).

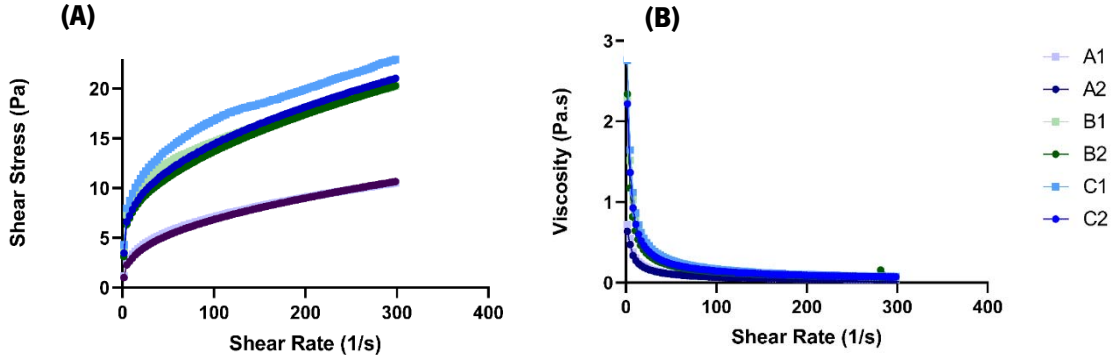


Figure 7. Rheological behavior of developed formulations through the analysis of **(A)** shear stress versus shear rate and **(B)** viscosity as a function of shear rate.

By analyzing the behavior of the curves, it is possible to infer that all formulations exhibit a pseudoplastic behavior with yield that is included in the shear-tinning group (**Figure 7A**), which is characteristic of creams/serums. The yield means that when a rising force is applied, the liquid does not begin to flow until a particular threshold is reached (Yang & Du, 2020). The behavior of a cosmetic formulation can be further corroborated by analyzing the graph of viscosity as a function of shear rate because each type of fluid exhibits a specific increase or decrease of viscosity when subjected to high shear forces. Pseudoplastic fluids, such as cosmetic emulsion formulations show a decrease of viscosity with the increase of shear rate, remaining almost stable from a certain value of shear rate. All developed formulations demonstrated this type of rheological behavior (**Figure 7B**)

The determination of the LVR is also an important rheological evaluation because gives insight into the firmness of cosmetic formulations. This linear region provides information about stability/firmness/structured of the serums. Longer LVR correspond to a more structured cream, whereas the shorter LVR indicate a less structured formulation (Adejokun & Dodou, 2019; Dabbaghi et al., 2021). The storage modulus G' is a measure of the deformation energy held by the formulation throughout the shear process, illustrating the elastic behavior of the material. The loss modulus G'' is a measure of the deformation energy used by the formulation during the shear process, representing the viscous behavior of the material. These two parameters allow the characterization of formulations in terms of rigidity and stability (Pinto et al., 2021; Wahab et al., 2020).

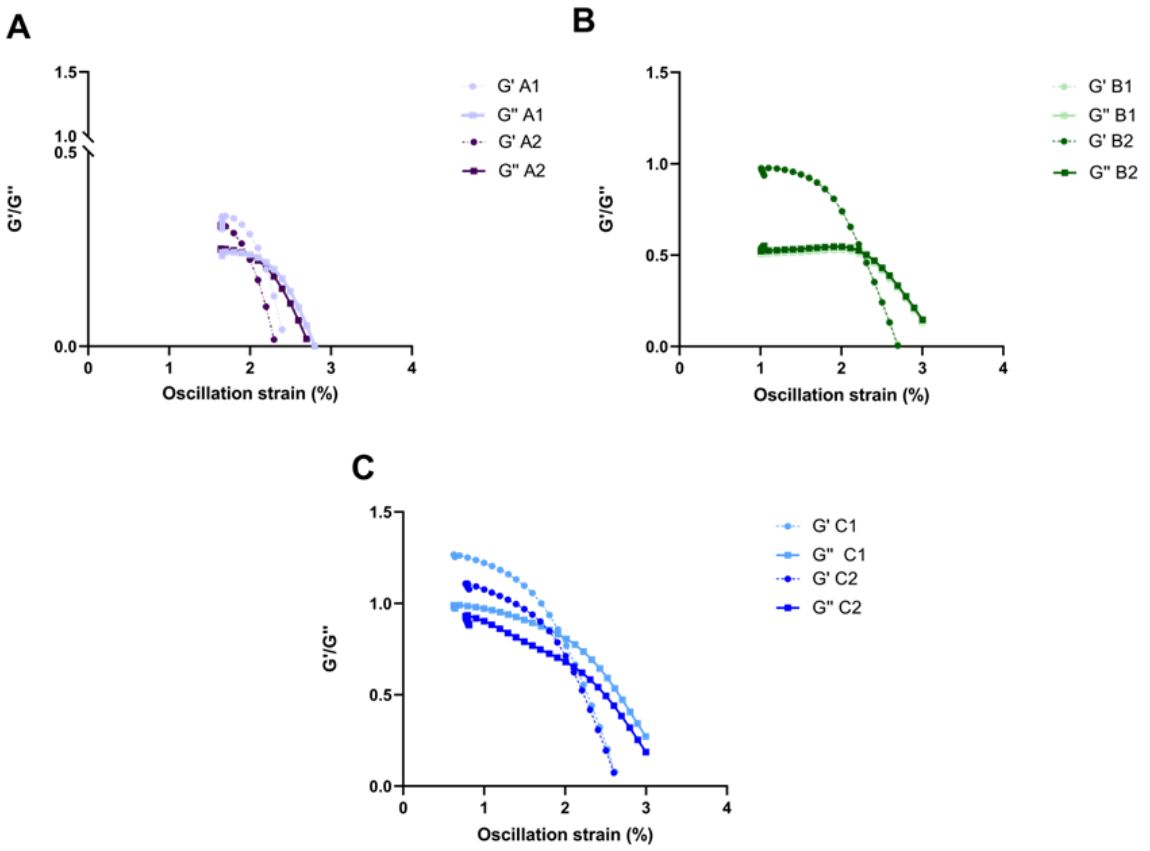


Figure 8. Graphical representation of storage modulus (G') and loss modulus (G'') as a function of oscillation strain of **(A)** Formulations A, **(B)** Formulations B and **(C)** Formulations C.

Figure 8 shows that G' predominates during almost all analysis relative to the G'', indicating that the developed formulations present an elastic behavior. Additionally, it can be stated that Formulation A1 is firmer compared to A2 because the G'' becomes predominant at higher oscillation strains. This result indicates that it is necessary to exert more force for the formulation pass from the elastic behavior to the viscous one. Relatively to Formulations C, it was observed that G' was higher at lower stress values for Formulation C1, indicating that this formulation has a greater internal structure than Formulation C2. However, the value at which the serums change their gel to liquid state was very similar. On the other hand, no significant differences were observed for Formulations B, indicating that the inclusion of active ingredients did not change the rheological behavior.

Considering the LVR of each formulation (**Figure 8**) was determined a value of oscillation strain to proceed with the frequency sweep test. This test provides information about the morphological identity, i.e., if the formulation is more elastic/bouncy, like a solid, or more viscous, like thin

oil/water (Adejokun & Dodou, 2020; Greenaway, 2010). Thus, frequency sweep test was performed for all developed formulations, and the graphics are represented in **Figure 9**.

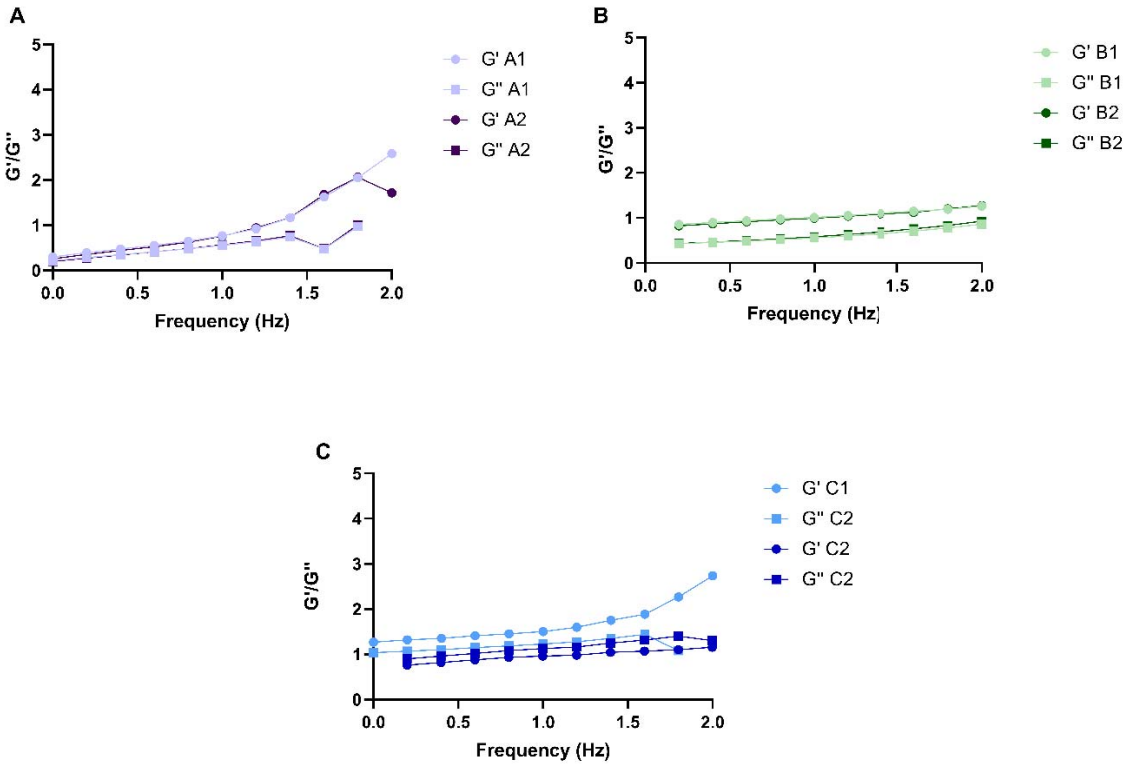


Figure 9.Graphical representation of frequency sweep test, in which curves of G' (loss modulus), G'' (storage modulus) were plotted as a function of frequency of **(A)** Formulations A, **(B)** Formulations B and **(C)** Formulations C.

The frequency sweep analysis revealed that the G' curve dominated the G'' curve over the entire frequency range for all developed formulations, as expected for viscoelastic cosmetic formulations (Kocen et al., 2017). When comparing the base formulations with the formulations containing active ingredients no significant differences were observed, indicating that their behavior and firmness in LVR are very similar, except for formulation C where in C1 G' always predominates while in C2, G'' predominates. In this way, the addition of anti-aging ingredients did not interfere with the way of formulation A and B behaves under different frequencies but influences C.

3.2.3. Viscosity of developed formulations

The assessment of viscosity, or flow resistance, of a formulation is an essential aspect of quality control. The viscosity of cosmetics and personal care products is often measured with a viscometer, which provides quick and easy viscosity testing. In general, spring-type viscometer readings should be taken between 10% and 100% torque. The viscosity of a formulation shows its resistance to flow, i.e., higher viscosity results in slower flow rate of the formulation (Pinto et al.,

2021; Surini et al., 2018) (Pinto et al., 2021; Surini et al., 2018). Low viscosity topical formulations clear quicker than viscous solutions. Furthermore, extremely viscous liquids might be irritating to the skin. In terms of permeation, lesser viscosity formulations should outperform thicker formulations (Karakucuk et al., 2021; Surini et al., 2018). In **Table 12** are represented the values of viscosity for all developed formulations.

Table 12. Viscosity values of developed formulations A, B and C, after their preparation (time 0).

		Viscosity ($mPa.s^{-1}$)
Formulations A	A1	76.5
	A2	69.1
Formulations B	B1	204.2
	B2	162
Formulations C	C1	194.2
	C2	174

Base formulations (A1, B1 and C1) have a slightly higher viscosity than the formulations containing active ingredients (A2, B2 and C2). The emulsion-based formulations (Formulations B and C) showed higher viscosity values compared to the aqueous formulations (Formulations A). This could probably be related to the fact that Formulations A are not emulsions and therefore does not need a surfactant, which leads to an increase in viscosity (Kim et al., 2020).

3.3. Accelerated stability tests

3.3.1. Influence of packaging material

The packaging material is an important part in the development of a new cosmetic product. Packaging may be characterized as an inexpensive way to give to a new product: protection, identity, information, containment, convenience, and compliance throughout storage, conveyance, and appearance. Packaging must be inexpensive while also protect the cosmetic product against climatic conditions and biological, physical, and chemical dangers (Muntean et al., 2019; Zadbuke et al., 2013). Despite its relevance, there is too little information regarding probable chemical-physical interactions between formulation and packaging because, unlike food packaging, cosmetic packaging is not regulated (Briasco et al., 2016).

Glass and plastic behave differently, and different types of plastic have varied features such as oxygen permeability, color fastness, and temperature resistance. For example, the stability of fragrance is also dependent on the type of material used to store the cosmetic product. Since plastic is the most used material, it is possible that some of the fragrance may migrate into it and consequently change the odor of the formulation (Bogdan et al., 2019; Juncan & Rus, 2018). In this way, the stability of developed formulations was evaluated using two different packaging material, plastic and glass (described as an inert material). All developed formulations were stored in glass and plastic containers at three different temperatures (4°C, RT and 37°C).

Table 13. Organoleptic characteristics and pH values of developed formulations stored during 30 days at different temperatures and in different packaging material, compared to time 0.

		Color			**Odor**			**Appearance**			**pH**		
		4°C	RT	37°C	4°C	RT	37°C	4°C	RT	37°C	4°C	RT	37°C
Formulations A	A1 in glass	T	T	T	OF	OF	OF	H	H	H	5.064	5.062	4.923
	A2 in glass	T	T	T	OF	OF	OF	H	H	H	5.217	5.213	5.070
	A1 in plastic	T	T	T	OF	OF	OF	H	H	H	5.119	5.070	4.864
	A2 in plastic	T	T	T	OF	OF	OF	H	H	H	5.297	5.220	5.012
Formulations B	B1 in glass	B	B	B	OF	OA	OA	H	H	H	4.895	4.899	4.799
	B2 in glass	B	B+	B+	OA	OA	OA	H	H	S	5.026	5.045	4.915
	B1 in plastic	B	B	B	OA	OA	OA	H	H	H	4.983	4.904	4.722
	B2 in plastic	B	B+	B+	OA	OA	OA	S	S	S	5.099	5.038	4.840
Formulations C	C1 in glass	W	W	W	OF	OF	OF	H	H	H	5.118	5.114	4.942
	C2 in glass	W	W	W	OF	OF	OF	S-	S-	S-	5.232	5.203	5.019
	C1 in plastic	W	W	W	OF	OF	OA	H	H	H	5.183	5.115	4.875
	C2 in plastic	W	W	W	OF	OF	OA	S-	S-	S-	5.290	5.203	4.950

Legend: A1, B1 and C1: Base formulations; A2, B2 and C2: Formulations containing active ingredients; T: Transparent; B: Beige; B+: Dark beige; W: White; OF: Odor according to the fragrance; OA: Different and agreeable odor; H: Homogeneous solution; S-: Some surface separation (a small amount of clear liquid forms on the surface); S: Evident phase separation.

Formulations A maintained their organoleptic properties regardless of the type of material used, i.e., the material had no influence on the odor and there was no change in the transparency of the formulations. The pH values also remained practically unchanged in both materials and at different temperatures (**Table 13**). Formulations B, in general, showed changes in fragrance intensity. In terms of color and appearance, only Formulation B2 showed some instability with a visual darkening and a slight phase separation. The color change and phase separation of Formulation B2 can be due to: (1) high concentration of Active ingredient 3; (2) molecular instability of Active ingredient 3, which is readily oxidized to an inactive state when exposed to air or light. Relatively to the odor, there was a decrease in the intensity of the fragrance, however, remaining pleasant. pH values of Formulations B did not change abruptly, indicating that the type of material used for packaging did not have impact on this parameter. Formulations C showed similar results to Formulations A, concerning to sample color, odor and pH values. However, Formulations C stored in plastic containers at 37°C showed a slight reduction in odor (**Table 13**). This reduction in fragrance can be related to the easy permeation into plastic and the fact that the fragrance become more volatile at 37°C. It was also possible to observe phase separation in the Formulation C2 in both type of packaging material. pH values remained practically the same for all temperatures and in both materials. In this way, the type of material used to store the developed serums has no significantly influence in odor, color, appearance, and pH of formulations.

After 60 days of storage occurred some changes relative to time zero, but they were very similar to the results obtained for 30 days. The main difference was observed in Formulation B2 stored at 37°C that compared to time zero showed a darker color. This difference is represented in **Figure 10**, comparing with the same formulation (Formulation B2) stored at 4°C and RT.

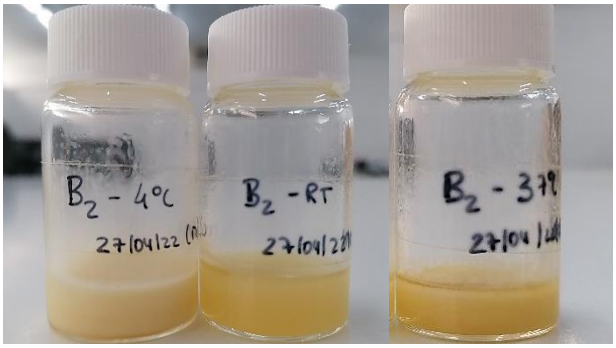


Figure 10. Visual comparison of Formulations B2 at 4°C, RT and 37°C after 60 days of storage, in glass containers.

Table 14 shows the results otained after 90 days of storage, where it is possible observe that only Formulations A remained stables, maintaining all initial organoleptic characteristics. Formulations B showed changes in their organoleptic characteristics, mainly Formulation B2 stored at 37°C. Formulation B2 stored at 4°C and RT, either in glass or plastic, retains its color and remained with same odor and appearance observed after 30 days of storage. Storage at 37°C demonstrated to be the worst condition for this formulation because it was observed a more evident phase separation, darker color and an unpleasant odor. In general, Formulations C maintained the color and odor at all temperatures and both type of containers. Relatively to pH values they remain constant.

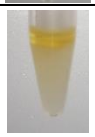
Table 14. Organoleptic characteristics and pH values of developed formulations stored during 90 days at different temperatures and in different packaging material, compared to time 0.

		Color			**Odor**			**Appearance**			**pH**		
		4°C	RT	37°C	4°C	RT	37°C	4°C	RT	37°C	4°C	RT	37°C
Formulation A	A1 in glass	T	T	T	OF	OF	OF	H	H	H	5.163	5.050	4.763
	A2 in glass	T	T	T	OF	OF	OF	H	H	H	5.307	5.182	4.894
	A1 in plastic	T	T	T	OF	OF	OF	H	H	H	5.176	4.995	4.814
	A2 in plastic	T	T	T	OF	OF	OF	H	H	H	5.202	5.070	4.934
Formulation B	B1 in glass	B	B	B	OA	OA	OA	H	H	H	5.010	4.881	4.633
	B2 in glass	B	B+	B++	OA	OA	OD	S	S	S+	5.121	5.071	4.681
	B1 in plastic	B	B	B	OA	OA	OA	H	H	H	5.004	4.893	4.669
	B2 in plastic	B	B+	B++	OA	OA	OD	S	S	S+	5.137	5.002	4.798
Formulation C	C1 in glass	W	W	W	OF	OF	OF	H	H	H	5.080	5.057	4.789
	C2 in glass	W	W	W-	OF	OF	OF	S-	S-	S-	5.140	5.116	4.854
	C1 in plastic	W	W	W	OF	OF	OF	H	H	H	5.191	5.048	4.834
	C2 in plastic	W	W	W-	OF	OF	OA	S-	S-	S-	5.234	5.121	4.894

Legend: A1, B1 and C1: Base formulations; A2, B2 and C2: Formulations containing active ingredients; T: Transparent; B: Beige; B+: Dark beige; W: White; OF: Odor according to the fragrance; OA: Different and agreeable odor; OD: Odor different and disagreeable; H: Homogeneous solution; S-: Some surface separation (a small amount of clear liquid forms on the surface); S: Evident phase separation; S+: more evident separation of phase

Another analysis performed during the stability studies was the centrifugation test, where all formulations were submitted to 3000 rpm for 30 minutes and the results are represented in **Table 15**.

Table 15. Results of centrifugation test Formulations A, B, and C stored different material containers at 3 temperatures for 90 days (T3).

		Formulations after centrifugation		
		4°C	22°C	37°C
Formulations A	A1 in glass			
	A2 in glass			
	A1 in plastic			
	A2 in plastic			
Formulations B	B1 in glass			
	B2 in glass			
	B1 in plastic			
	B2 in plastic			
Formulations C	C1 in glass			
	C2 in glass			
	C1 in plastic			
	C2 in plastic			

Formulations A were the only ones that did not present any type of separation/precipitation after centrifugation test, even after 90 days of storage at different temperatures and in both type of containers. These results were expected since these formulations are composed of only one phase, aqueous phase. Since the used ingredients are water miscible, it was easier to achieve stability in these formulations. Relatively to Formulations B, it was possible observe great differences between the base formulations and formulations containing active ingredients. Formulation B1 showed stability at all temperatures and both in glass and plastic containers, while Formulation B2 presented phase separation for all tested conditions, with no significant differences between them. The instability of Formulation B2 that contain the active ingredients was observed at the first time point (30 days of storage, data not shown). The base formulation B (Formulation B1) seems to be stable, and it is possible to conclude that the inclusion of the active ingredients is responsible by the formulation instability. The reason for this instability can be the addition process or the concentration of active ingredients and not their presence in formulations because there are several cosmetic products on the market that have these three active compounds. Finally, Formulations C had an identical behavior to Formulations B, but the separations observed in Formulation C2 is less evident compared to the separation obtained for Formulation B2. Only for the storage at 37°C is more evident the phase separation of Formulation C containing active ingredients, independently of the container's material. In a general way, this formulation does not show significant separation of phases when submitted to the centrifugation test, probably because the HLB value of emulsifier 2 is of 13, which indicate that these formulations have higher affinity for water and since they are mostly composed of water, it seems to improve their stability.

The particle size of the emulsion-based formulations (Formulations B and C) was also evaluated at 3 different time points (30, 60 and 90 days) of storage at 4°C, RT and 37°C in glass and plastic containers. **Figure 11** shows the particle size and PDI values of Formulations B and C after the preparation and at the end of each time point. It should be noted that the particle size and PDI of Formulations A were not measured because these formulations are not emulsions.

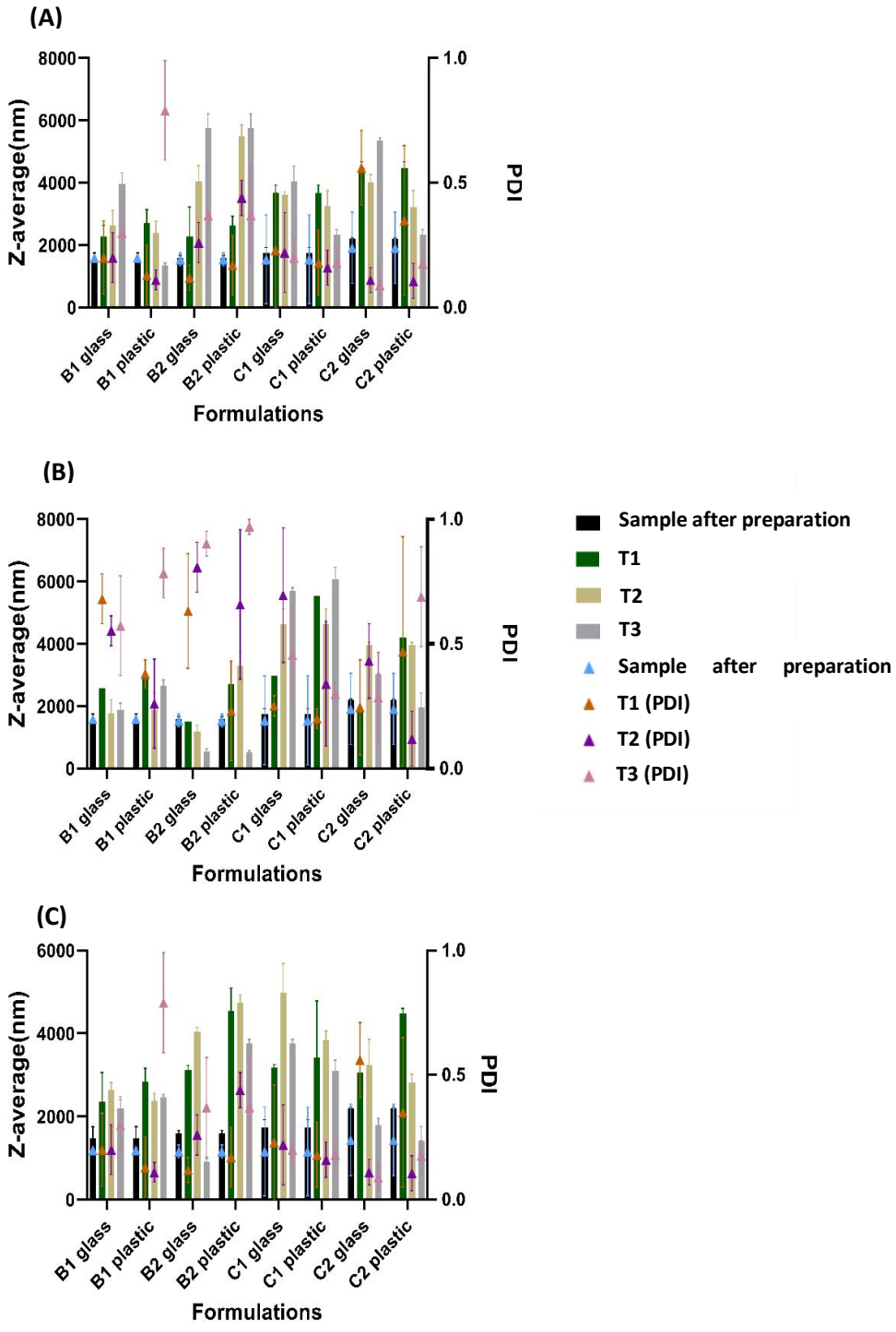


Figure 11.Particle size and PDI values of Formulations B and C stored in glass and plastic containers at **(A)** 4°C, **(B)** RT and **(C)** 37°C. For each sample was performed three replicates.

By analysis of **Figure 11**, it was observed that Formulation B1 did not show a great alteration of particles size over time, regardless of the material and storage temperature. However, the PDI values showed a significant increase in relation to the initial values, except for storage at 37°C. On the other hand, Formulation B2 presents several oscillations of values at 4°C and 37°C, the particle size increases throughout the 90 days of storage. At RT, there is a less marked increase in the first 60 days of the formulations conditioned in plastic while the ones in glass suffered an increase of the particle size. However, at all temperatures, it can be observed that the particle size is slightly larger in the formulations stored in plastic containers, although this is not a significant difference, except at 37°C after 90 days of storage.

Regarding Formulations C, both C1 and C2, demonstrated an increase of particles size after 30 days of storage, which remains practically unchanged regardless of the material for the storage at 4°C. However, Formulation C1, stored in plastic, showed a sharp increase in particle size at RT after 30 days and then remained constant over the remaining 60 days. Formulation C2, presents the same behavior pattern at all temperatures, which once again shows that in these formulations the type of material used as packaging did not influence the particles size.

To determine if the packaging material and temperature influence the viscosity of developed formulations, this parameter was also evaluated during the stability tests. The optimal viscosity of a cream is determined by its intended purpose, for example if it should form a protective layer on the skin or should penetrate fast (Kulawik-Pióro et al., 2019). A lipid-rich serum, for example, will have lower spreadability values due to its high viscosity and surface tension (a measure of cohesiveness). A serum with lower thickness, surface tension and viscosity values will have higher spreadability and easy skin penetration (Savary et al., 2013). **Table 16** shows the viscosity values of developed formulations that were stored in glass and plastic containers at 4°C, RT and 37°C for 30 (T1) and 60 days (T2). Due to the viscosity of the samples, in the consecutive viscosity evaluation occurred samples lost, being impossible the measurement of viscosity after 90 days of storage.

Table 16. Viscosity values of Formulations A, B and C stored at 4°C, RT and 37°C for 30 (T1) and 60 days (T2), in glass and plastic containers.

		T0	T1			T2		
			4°C	RT	37°C	4°C	RT	37°C
Formulations A	A1 in glass	76.5	82	80.3	77.3	78.8	83.2	83.2
	A1 in plastic		80.3	80.6	76.2	78.5	82.3	81.9
	A2 in glass	69.1	85	82	80.3	84.4	83.8	88.5
	A2 in plastic		80.3	78.5	83.8	84.4	79.1	83.4
Formulations B	B1 in glass	204.2	175.2	154.7	169.9	168.7	169.3	211.5
	B1 in plastic		165.8	141.8	140.6	163.2	144.7	137.1
	B2 in glass	162	171.7	189.2	163.5	174.9	171.9	183.4
	B2 in plastic		154.1	146.5	140	155.6	149.7	138.9
Formulations C	C1 in glass	194.2	226.1	207.2	239	208.6	203	256.3
	C1 in plastic		208.6	232.6	236.7	199.3	164.35	249.5
	C2 in glass	174	223.8	215.6	214.4	214.1	224.7	251.4
	C2 in plastic		219.7	203.3	187.5	217.7	202.4	247.2

According to the results, Formulations A do not present significant differences in viscosity values regardless of temperature and packaging material, even after 60 days. On the other hand, Formulations B, when stored at higher temperatures, namely at RT and 37°C present a decrease in viscosity when stored in plastic containers. On the other hand, the Formulations C, suffer an increase in viscosity independent of the storage material, being always higher in formulations stored in glass containers.

3.3.2. Influence of light exposure

The intrinsic photostability features of cosmetics products should be assessed to ensure that light exposure does not cause undesirable alterations. Cosmetics are often exposed to different light intensities from the point of sale to the place where the customer stores it. Therefore, it is of prime importance that cosmetic formulations are photostable (Abdassah et al., 2015; Kryczyk-Poprawa et al., 2019). Sunlight exposure can affect chemical integrity of formulations and some physical attributes, such as mechanical strength (e.g., brittleness) or color (e.g., fading). In addition, exposure to sunlight can cause oxidation of certain ingredients. Direct exposure to sunlight or UV radiation allows the evaluation of possible reactions between reactive compounds at certain wavelengths (Barel et al., 2014). Since the different materials used in the packaging of cosmetic formulations can interfere with radiation, samples in plastic and glass containers were placed near

a window with access to natural sunlight. As a control, the same samples (stored in plastic and glass containers) were placed near the window but fully covered with aluminum foil, avoiding direct sunlight. The organoleptic characteristics (color, odor, appearance) of the developed formulations remained unaltered after 30 days of storage, except for Formulations B and C2. Formulation B1 just showed alteration in odor (fragrance odor decrease). Formulations B2 become darker and fragrance odor also decreased and both formulations containing active ingredients (Formulation B2 and C2) did not present homogeneous appearance. Relatively to pH values, it was observed stability over time regardless covered with aluminum or not. No significant differences were observed after 90 days of storage (**Table 17**) when compared with the results obtained for 30 days of storage. It would be expected that formulations covered with aluminum would show more stability in terms of organoleptic characteristics. Aluminum provides a barrier against light, delaying the oxidation of the ingredients that constitute the formulations. However, no significant differences were observed between the covered and not covered formulations. Only Formulation B1 showed a decrease of its color, which became closer to white, when this formulation was not covered with aluminum (**Table 17**). Then, it is possible to infer that the type of packaging and the protection or not with aluminum had not a significant interference in the organoleptic characteristics of the developed formulations.

Table 17. Organoleptic characteristics and pH values of developed formulations stored for 90 days in glass and plastic containers covered or not with aluminum.

		Color	Odor	Appearance	pH
Formulations A	A1 in glass	T	OF	H	5.050
	A2 in glass	T	OF	H	5.182
	A1 in glass covered	T	OF	H	5.019
	A2 in glass covered	T	OF	H	5.150
	A1 in plastic	T	OF	H	4.995
	A2 in plastic	T	OF	H	5.070
	A1 in plastic covered	T	OF	H	5.026
	A2 in plastic covered	T	OF	H	5.202
Formulations B	B1 in glass	B-	OA	H	4.881
	B2 in glass	B+	OA	S-	5.011
	B1 in glass covered	B	OA	H	4.880
	B2 in glass covered	B+	OA	S	4.984
	B1 in plastic	B-	OA	H	4.893
	B2 in plastic	B+	OA	S	5.002
	B1 in plastic covered	B	OA	H	4.927
	B2 in plastic covered	B+	OA	S	5.009
Formulations C	C1 in glass	W	OF	H	5.057
	C2 in glass	W	OF	S	5.116
	C1 in glass covered	W	OF	H	5.035
	C2 in glass covered	W	OF	S-	5.110
	C1 in plastic	W	OF	H	5.048
	C2 in plastic	W	OF	S-	5.121
	C1 in plastic covered	W	OF	H	5.080
	C2 in plastic covered	W	OF	S-	5.140

Legend: A1, B1 and C1: Base formulations; A2, B2 and C2: Formulations containing active ingredients; T: Transparent; T-: Transparent plus dull; B: Beige; B-: Light beige; B+: Dark beige; W: White; C+: More White; C-: Less White; OF: Odor according to the fragrance; OA: Different and agreeable odor; OD: Odor different and disagreeable; H: Homogeneous solution; S-: Some surface separation (a small amount of clear liquid forms on the surface); S: Evident phase separation;

To determine if the exposure to sunlight have influence in stability of the developed formulations, centrifugation test was also performed with formulations covered with aluminum foil. It was observed the same behavior independently if the formulations were covered or not (**Table 18**). Only Formulations B2 and C2 demonstrated instability (phase separation) at the first time point evaluated (30 days of storage), although for Formulation C2 the separation was less

pronounced. Since the results obtained for 90 days of storage were very similar to other time points, it was decided to present only the results corresponding to the last time point (90 days).

Table 18. Results of centrifugations of developed formulations stored for 90 days in glass and plastic containers covered or not with aluminum.

		Formulations after centrifugation	
		Covered	**Uncovered**
Formulations A	A1 in glass		
	A2 in glass		
	A1 in plastic		
	A2 in plastic		
Formulations B	B1 in glass		
	B2 in glass		
	B1 in plastic		
	B2 in plastic		
Formulations C	C1 in glass		
	C2 in glass		
	C1 in plastic		
	C2 in plastic		

As mentioned before, particle size is essential to ensure the stability of formulations, so it is expected that no significant changes occur in these values.

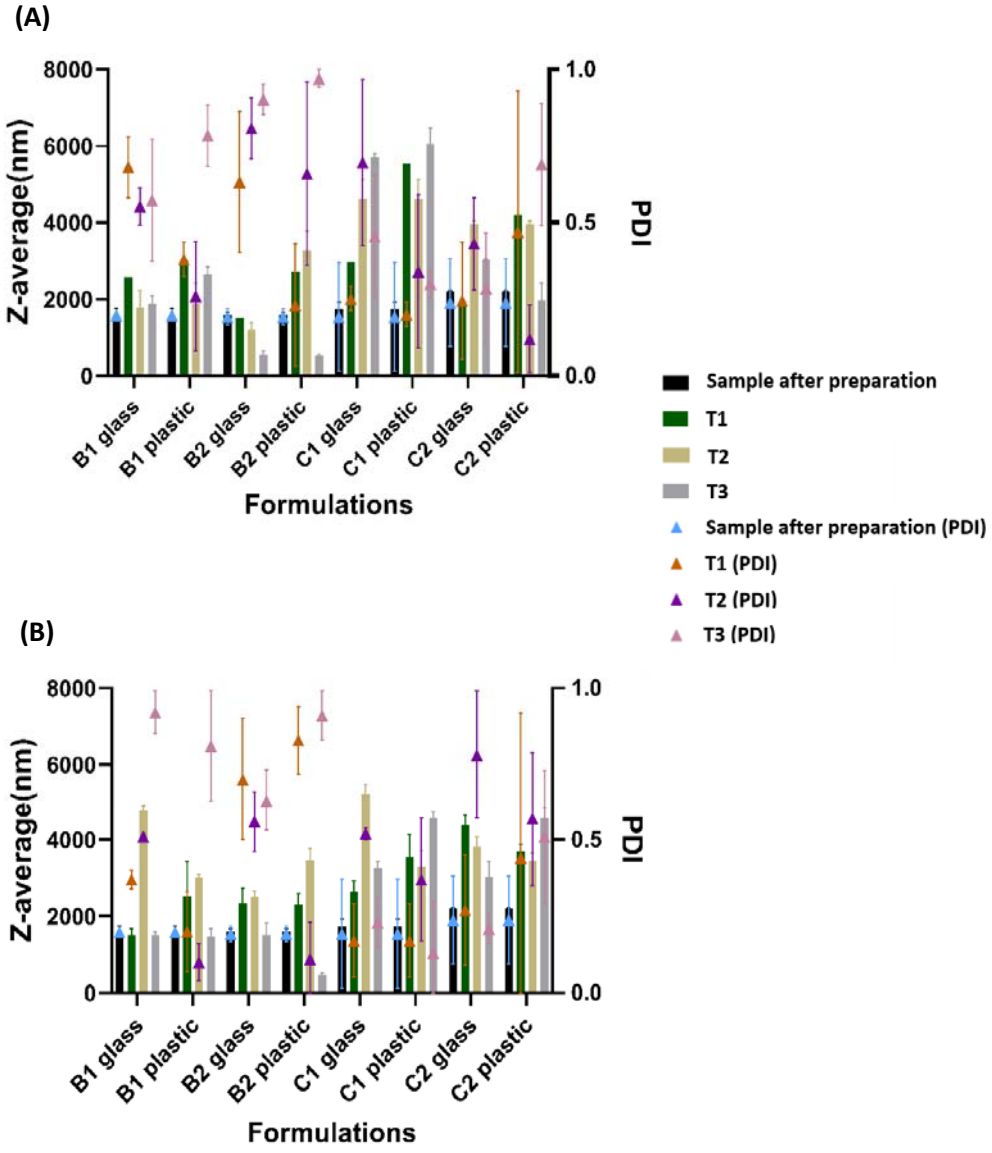


Figure 12.Particle size and PDI at 90 days of the formulations B and C. These formulations were stored in four different containers, glass and plastic and glass and plastic covered with aluminum foil at RT under sunlight. For each sample was performed three replicates.

Figure 12 showed that Formulation B1 does not show significant differences in particles size along 90 days of the study independently of being covered or not with aluminum. Only Formulation B1 stored in glass packaging and covered demonstrated an increase of particles size after 60 days of storage. However, at T3 (90 days) the particles size decreased again and, therefore, it could have been a problem in the measurement of a sample at 60 days of storage. Formulation B2 showed a final particles size much smaller than the initial one, which, together with the increase of PDI values, indicates that the Emulsifier 2 seems to lose its capabilities causing formulation instability. Formulation C1 showed a greater increase in particle size when stored without aluminum, relative to the covered ones. Formulation C2 presented a very similar behavior if covered or not with aluminum foil. In general, Formulations B demonstrated a greater increase of PDI values comparatively to Formulations C. In summary, developed formulations did not show any significant differences and a pattern when exposed directly or not to the sunlight.

Once again, the viscosity values of all formulations were determined and no significant differences from the initial value were observed (**Table 19**).

Table 19. Viscosity values of Formulations A, B and C stored at RT for 30 (T1) and 60 days (T2) in glass and plastic containers, covered and uncovered with aluminum foil.

		T0	T1		T2	
			Covered	Uncovered	Covered	Uncovered
Formulations A	A1 in glass	76.5	82.6	80.3	81.75	83.2
	A1 in plastic		84.4	82	78.2	83.8
	A2 in glass	69.1	78.5	80.6	78.2	82.3
	A2 in plastic		79.1	78.5	77.0	79.1
Formulations B	B1 in glass	204.2	172.8	154.7	171.95	169.3
	B1 in plastic		167	189.2	173.4	171.9
	B2 in glass	162	142.4	141.8	149.7	144.7
	B2 in plastic		147.1	146.5	145	149.7
Formulations C	C1 in glass	194.2	159.9	207.2	224.7	203
	C1 in plastic		212.1	215.6	221.5	224.7
	C2 in glass	174	147.1	232.6	202.4	164.35
	C2 in plastic		190.4	203.3	189.3	202.4

It is important to note that it was not established a specific range of viscosity, but it depends on the purpose of the formulation as well as the type of packaging that will be used. However, the low viscosity at high shear is crucial for serum formulations to generate a homogeneous thin layer

that will enter the skin more quickly and aid in the absorption of active components without feeling greasy or sticky.

Formulations A present viscosity values very similar to each other and the variations are not considered significant. In the case of formulations B, there is a slight decrease in viscosity. Formulations C suffer a decrease in viscosity at T1 when covered, but an increase in viscosity when directly exposed to sunlight. This increase is also visible at T2, that is, in general, Formulation C presents an increase in viscosity over time, regardless of being covered or not.

3.3.3. Freeze-thaw cycle testing

Temperature cycling and/or "freeze-thaw" testing can show some sorts of instabilities more quickly than constant-temperature storage (Cosmetics Europe, 2019). This type of test is particularly essential when the products meant to be exported abroad since they may be subjected to different harsh temperatures during shipping, and these tests confirm that the products can endure or not the high temperature variations. Developed formulations were subjected to three cycles of extreme temperatures and then the organoleptic properties evaluation, pH measurement and particle size determination were performed.

Table 20 shows the organoleptic properties and the pH values of developed formulations (bases and with active ingredients) after three cycles of freeze-thaw. The main difference observed was in the Formulation B2 that revealed one alteration in color (become darker).

Table 20. Organoleptic properties and pH values of developed formulations after three cycles of freeze-thaw.

		Color	Odor	Appearance	pH
Formulations A	A1	T	OF	H	5.122
	A2	T	OF	H	5.279
Formulations B	B1	B	OF	H	4.970
	B2	B+	OF	S	5.089
Formulations C	C1	W	OF	H	5.174
	C2	W	OF	H	5.272

Legend: A1, B1 and C1: Base formulations; A2, B2 and C2: Formulations containing active ingredients; T: Transparent; B: Beige; B+: Dark beige; W: White; OF: Odor according to the fragrance; H: Homogeneous solution S: Evident phase separation;

The centrifugation test was also performed and revealed that Formulations B and C (bases and with active ingredients) present instability when subjected to extreme temperatures. **Table 21** shows the images obtained after centrifugation and it is possible to observe one phase separation in the Formulations B and C. Formulations A did not have any evidence of instability, for example precipitation/sedimentation. The separation observed in the emulsion-based formulations can be due to the freeze of water molecules that tend to group or coalesce (Tian et al., 2022). Then a formulation is frozen and then thawed, the location of the water molecules can change a lot of the time, which is especially problematic for emulsion-based formulations. The growing ice squeezes out the oil droplets, causing the emulsion destabilization that result in a different texture when the formulation is thawed (Ghosh et al., 2006).

Table 21. Results of centrifugation test of Formulations A, B, and C after the freeze-thaw cycles.

		Formulations after centrifugation
Formulations A	A1	
	A2	
Formulations B	B1	
	B2	
Formulations C	C1	
	C2	

In order to determine if extreme temperatures influence the average size and PDI values of emulsion-based formulations (Formulations B and C), it was performed DLS analysis of formulations after the freeze-thaw cyles. **Figure 13** shows that Formulations B did not present significant differences in particle size, while Formulations C demonstrated an increase in particle size after the three freeze-thaw cycles. This formulations was the less stable at extreme temperatures because more aggregation of certain ingredients may have occurred. Relatively to PDI values, for all formulations the value remained close to 0.2.

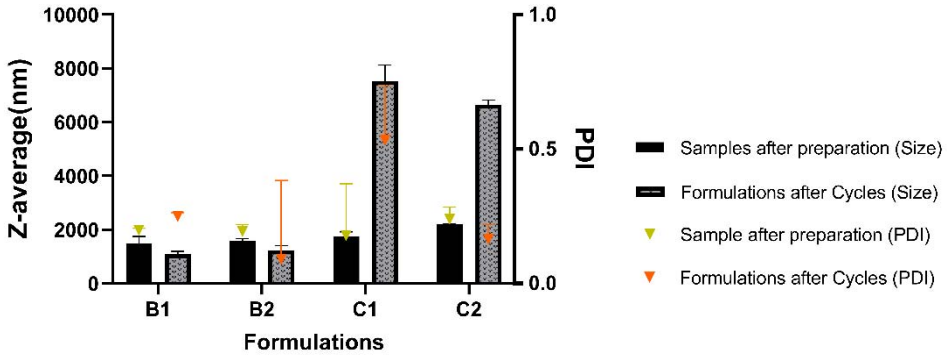


Figure 13. Particle size and PDI values of Formulations B and C after the freeze-thaw cycles. For each sample was performed three replicates.

Rheological evaluation of formulations after freeze-thaw cycles was performed in order to verify what happens to their viscosity behavior when exposed to extreme temperatures. When compared with the initial results there is an increase in shear stress with increasing shear rate in basically all the curves. While before the cycles, the curves for formulations B and C were very close, after the cycles it is already possible to observe a difference between them.

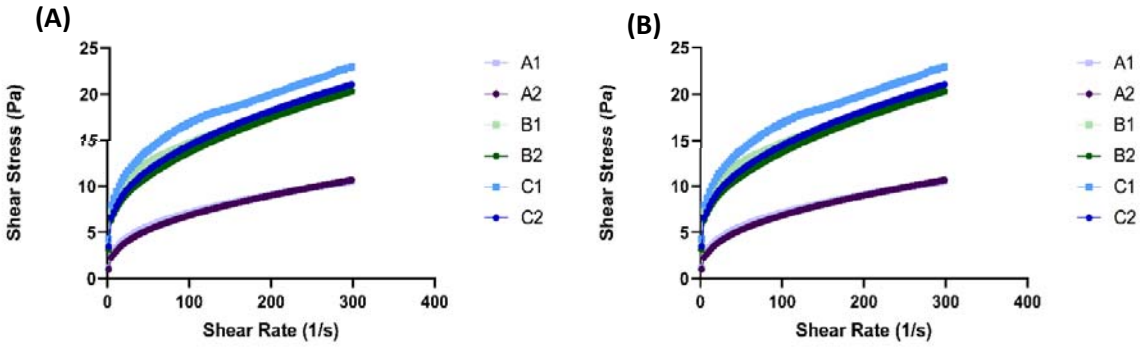


Figure 14. Rheological behavior of developed formulations through the analysis shear stress versus shear rate of the different formulations developed after exposure to three extreme temperature cycles. Graphics **(A)** are the results obtained before the temperature cycles, and graphics **(B)** are the results obtained after the cycles.

After the temperature cycle, there is an increase in viscosity and restructuration of the vesicles and lamellae in a more compact structure with an increased concentration of multilamellar tiny vesicles and other finely organized structures in the medium (Iliescu et al., 2005). However, cosmetic Formulation A remains the one with the lowest viscosity and cosmetic Formulation C the one with the highest viscosity. Thus, we can conclude that the formulations maintain their viscosity behavior when exposed to temperature cycles.

3.4. Optimizations for improve formulations stability

Considering the obtained results, Formulation B and C need to be optimized to improve their stability. This optimization began with the improvement of base formulations (Formulations B1 and C1). Several optimizations were performed, including small changes in the preparation method and addition of new ingredients (emulsion stabilizers and chelating agents). The formulations that demonstrate better results are presented in **Table 22**. The chelating agent contributes to emulsions stability due to their specific three-dimensional structure that can complex with metal ions responsible for premature serums deterioration (Cansell et al., 2010). Emulsion stabilizers are supporting agents that increase the stability of cosmetic formulas so that the water and oil components do not separate throughout time, preserving the function and effectiveness of active ingredients or fragrances (Barel et al., 2014).

Table 22. Composition of Formulations O.B1 and O.C1, which correspond to optimized Formulations B1 and C1 respectively.

		O.B1	**O.C1**
	Ingredients	**% (m/m)**	
Phase A (Aqueous phase)	Water	76.4	78.4
	Preservative 1	0.5	-
	Preservative 3	-	1
	Thickener	0.35	0.35
	Humectant 1	0.5	0.5
	Humectant 2	1	1
	Preservative 2	1	0.5
	Emulsion Stabilizer 1	0.1	0.1
	Emulsion Stabilizer 2	0.5	0.5
	Chelating Agent	0.1	0.1
Phase B (Oil phase)	Emulsifier 1	2.5	-
	Emulsifier 2	-	4
	Emollient 2	3	-
	Emollient 3	3	2
	Emollient 4	3	2
	Emollient 5	5	-
	Emollient 6	-	2
	Emollient 7	-	2
	Emollient 8	-	2
Phase C	Humectant 3	1	1
	Emollient 1	1.5	1.5
	Humectant 4	-	1
	Fragrance	0.05	0.05

Optimized formulations were submitted to freeze-thaw test because this test is faster to provide information about the formulation's stability. The organoleptic characteristics and the pH of optimized formulations were evaluated after completion of the three freeze-thaw cycles (**Table 23**).

Table 23. Organoleptic characteristics and pH values of the optimized Formulations B1 (O.B1) and C1 (O.C1) after three freeze-thaw cycles.

Optimization	Color	Odor	Appearance	pH
O.B1	B	OF	H	5.638
O.C1	W	OF	H	5.403

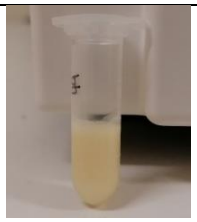
Legend: O.B1, O.C1: optimization of base formulations; B: Beige; W: White; OF: odor according to the fragrance; H: homogeneous solution.

Optimized formulations showed the same color, odor, and appearance than Formulations B and C before the optimizations. pH values are higher because the Emulsion stabilizer 1 is a strong base, increasing the final pH values, however, these the pH values are still within the desired values for topical application.

Centrifugation of Formulations O.B1 e O.C1, subjected to freeze-thaw cycles, demonstrated that the performed optimizations induced an increase of formulations stability.

Table 24 shows that both formulations did not present phase separation when subjected to the gravitational force of centrifugation. In this way, the optimizations resulted in stable base formulations that can be used for the development of anti-aging formulations.

Table 24. Results of centrifugation test of optimized formulations previously submitted to three freeze-thaw cycles.

	Formulations after Centrifugation
Formulation O.B1	
Formulation O.C1	

In a final stage of this master's thesis, the addition of active ingredients to these optimized base formulations was still tested. Several formulations were produced, and promising results were obtained when Active ingredient 3 was removed from the formulations, which can be substituted by a more stable derivative. In this way, with this work it was possible to develop promising formulations that can be used as base for the development of cosmetic formulations with anti-aging properties, as desired.

4. Conclusions

Nowadays, there is a greater concern with appearance and beauty, having the area of cosmetic skin care a growing demand. Anti-aging facial serums are a good option for skin care due to all their potentialities, such as the increased skin penetrability of the active ingredients and the feeling of lightness. For the development of this type of products, several features must be considered such as: visual appearance, level of hydration, fluidity, spreading and softness, among others. The development of aqueous serums allowed us to infer that the water percentage plays a crucial role in obtaining the light and non-greasy texture, which is characteristic of this type of serum. The addition of humectants revealed to be very important for the moisturizing and smoothing sensation of a serum. The development process of milky serums (emulsion-based serums) was much more time-consuming and laborious due to the complexity of the formulation. As this type of serum is obtained through the formation of an emulsion, the correct conjunction between the different ingredients is very important, especially regarding the emulsifiers.

The objective of this work was the development and characterization of cosmetic formulations, namely serums, with anti-aging properties. After several steps of optimization, six formulations were obtained and subjected to an exhaustive characterization that included preliminary stability test (centrifugation test), pH measurement and evaluation of the organoleptic characteristics. Additionally, it was also performed the stability evaluation along time, where several conditions were tested. The formulations had a pleasant color and odor as well as pH values were in the range of 4.9-5.4, which are compatible with the pH of stratum corneum. Subsequently, rheological characterization was also performed, which showed that all formulations exhibited a pseudoplastic behavior, as expected. The stability test demonstrated that Formulations A are the most homogenous, have a pleasing odor and color, and exhibit outstanding stability at all storage temperatures. The organoleptic characteristics of Formulations B and C presented some alterations during the stability test at different temperatures. The influence of light and the container material were also tested and it was observed that it did not influence the stability of Formulations A and C, but it clearly influences the odor and color of Formulations B. However, neither the sunlight exposition nor the packaging material had significant impact in the stability of developed formulations. Regarding the particle size and PDI values, also measured under the different storage conditions, it was observed that the developed formulations are very heterogeneous. Results also demonstrated that Emulsifier 2 lost the ability to maintain the emulsion stability. Freeze-thaw cycles were also performed, and

Formulations B and C (bases and containing active compounds) demonstrated loss of stability when subjected to extreme temperatures.

Finally, it was confirmed that the optimization of formulations by the addition of emulsion stabilizers and chelating agents resulted in an increased of formulations stability. Concluding, the work developed in this master thesis allowed the achievement of the proposed objectives and give a contribution towards the development of new cosmetic formulations for anti-aging purpose.

5. Future Perspectives

In the future, more optimizations will be performed to incorporate the active ingredients with anti-aging properties in the optimized base formulations. Despite the exhaustive characterization already performed, the developed formulations will be further investigated. The spreadability of developed formulations can be studied through texturometer. Formulations should be tested on the skin through the Human Repeat Insult Patch Testing test, evaluating allergic reactions and irritability. The evaluation of moisturizing power of formulations can be performed by determination of transepidermal water loss. Panel of volunteers can be also performed for sensorial evaluations of developed formulations.

6. References

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