Universidade de Lisboa

Faculdade de Farmácia

Applications of Natural Deep Eutectic Solvents (NADES) in topical formulations

Ana Teresa Clérigo Fonseca

Dissertation supervised by Professor Joana Marques Marto and co-supervised by Professor Alexandre Babo de Almeida Paiva.

Biopharmaceutical Sciences

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ACKNOWLEDGMENTS

Esta dissertação marca o fim de uma etapa muito importante do meu percurso académico. Foi, sem dúvida, um ano cheio de desafios para enfrentar e novas etapas por descobrir e nada disto teria sido possível sem o apoio e contributo de diversas pessoas e instituições, às quais expresso o meu mais sincero agradecimento.

Em primeiro lugar, quero agradecer à minha orientadora, Professora Doutora Joana Marto, pela oportunidade de me juntar ao seu laboratório para desenvolver este projeto. A sua disponibilidade, profissionalismo, apoio e simpatia são para mim um exemplo de excelência académica e rigor científico que levarei comigo no futuro.

Ao meu co-orientador, Doutor Alexandre Paiva, pela prontidão e flexibilidade com que sempre me recebeu no seu laboratório, bem como pela importante oportunidade de colaboração com a Faculdade de Ciências e Tecnologia da Universidade Nova de Lisboa.

À Carolina Costa, quero agradecer pela disponibilidade e ajuda constante que me deu no início deste percurso, em particular durante o período em que trabalhei nas instalações da FCT, onde a sua ajuda foi fundamental.

À Professora Doutora Lídia Gonçalves, por toda a simpatia com que sempre me recebeu no seu laboratório durante a realização dos ensaios celulares *in vitro*.

Quero também agradecer à Aline, por toda a disponibilidade e ajuda durante a realização deste projeto. A sua simpatia, boa disposição e companheirismo foram uma constante fonte de motivação ao longo dos meses em que trabalhámos juntas. Mesmos nos momentos em que algo corria menos bem, soube sempre encontrar o lado positivo e transmitir energia para seguir em frente.

À Angélica, que na última etapa deste percurso esteve sempre disponível para me ajudar e esclarecer todas as dúvidas, expresso o meu mais sincero agradecimento.

À Sara, Rafaela, Matilde, Ana Neves, Joana e Ana Ruas, quero expressar o meu mais sincero agradecimento por me terem recebido tão bem. A entre ajuda, boa disposição, companheirismo e amizade que existe no DermoLab, é algo que vou levar para sempre comigo. O que poderia ter sido uma experiência difícil e stressante transformou-se em algo incrível, graças a todas as Dermogirls que tornaram cada dia mais leve e inesquecível!

Ao Tiago, que para além de meu namorado é também o meu melhor amigo, quero agradecer por todo o amor, carinho e apoio que sempre me deu, não apenas durante este período, em que tudo isso foi essencial, mas ao longo de todo o nosso percurso juntos.

À minha família, por terem estado sempre ao meu lado, a apoiarem-me e a motivarem-me para ser sempre melhor. Sem eles, nada teria sido possível. Por fim, aos meus queridos avós, Laura e Luís, obrigada.

Declaro ter desenvolvido e elaborado o presente trabalho em consonância com o Código de Conduta e de Boas Práticas da Universidade de Lisboa. Mais concretamente, afirmo não ter incorrido em qualquer das variedades de fraude académica, que aqui declaro conhecer, e que atendi à exigida referenciação de frases, extratos, imagens e outras formas de trabalho intelectual, assumindo na íntegra as responsabilidades da autoria.

ABSTRACT

The rising interest in sustainable, safe, and effective cosmetics has boosted the need for innovative raw materials that comply with environmental and regulatory standards. Thus, natural deep eutectic solvents (NADES) have emerged as a sustainable alternative to conventional solvents due to their non-toxicity, biodegradability, and biocompatibility. Considering this, this project aims to investigate the application of NADES in topical formulations, such as shampoos and O/W emulsions, to assess their safety and efficacy in topical formulations.

Five different NADES were developed, which were subsequently subjected to detailed physicochemical characterization, including rheological, textural, and surface analyses, and then incorporated into shampoo formulations. Compared to the controls, shampoos with NADES showed better structural recovery, improved sensory attributes, and acceptable *in vitro* safety profiles, as determined by MTT assays in HaCaT keratinocytes.

All five NADES were tested as stabilizers of O/W emulsions. However, after seven days, all emulsions showed phase separation, indicating that NADES must be combined with conventional emulsifiers for long-term stability. The role of NADES as eco-friendly extraction solvents was also studied, using lupin by-products. Using assays such as DPPH, FRAP, and TPC, it was possible to determine the antioxidant activity and quantify the phenolic compound content of the extracts resulting from NADES extractions. Human neutrophil elastase (HNE) inhibition and cytotoxicity tests were developed to determine the safety and efficacy of the extracts. The extract that performed best was incorporated into O/W emulsions, which were subjected to comprehensive structural, rheological, microscopic, and stability tests. The emulsions showed favorable droplet size, texture, and storage performance, maintaining safety and bioactivity.

This project highlights the multifunctionality of NADES in dermocosmetics, serving as ecological solvents, texture modifiers, and carriers of bioactive compounds, while aligning with green chemistry and circular economy principles. The use of agro-industrial by-products, such as lupin residues, enhances sustainability, reinforcing the potential of NADES in creating innovative and environmentally responsible cosmetics.

Keywords: NADES, sustainability, green solvents, shampoos, emulsions.

RESUMO

O crescente interesse por cosméticos sustentáveis, seguros e eficazes impulsionou a necessidade de matérias-primas inovadoras que cumpram as normas ambientais e regulamentares. Assim, os solventes eutéticos naturais profundos (NADES) surgiram como uma alternativa sustentável aos solventes convencionais devido à sua não toxicidade, biodegradabilidade e biocompatibilidade. Considerando isso, este projeto tem como objetivo investigar a aplicação de NADES em formulações tópicas, como champôs e emulsões O/W, para avaliar a sua segurança e eficácia em formulações tópicas.

Foram desenvolvidos cinco NADES diferentes, que foram subsequentemente submetidos a uma caracterização físico-química detalhada, incluindo análises reológicas, texturais e superficiais, e depois incorporados em formulações de champô. Em comparação com os controlos, os champôs com NADES apresentaram melhor recuperação estrutural, atributos sensoriais melhorados e perfis de segurança *in vitro* aceitáveis, conforme determinado por ensaios MTT em queratinócitos HaCaT.

Todos os cinco NADES foram testados como estabilizadores de emulsões O/W. No entanto, após sete dias, todas as emulsões apresentaram separação de fases, indicando que os NADES devem ser combinados com emulsionantes convencionais para estabilidade a longo prazo. O papel dos NADES como solventes de extração ecológicos também foi estudado, utilizando subprodutos de tremoço. Através de ensaios como DPPH, FRAP e TPC, foi possível determinar a atividade antioxidante e quantificar o teor de compostos fenólicos dos extratos resultantes das extrações NADES. Foram desenvolvidos testes de inibição da elastase neutrofílica humana (HNE) e citotoxicidade para determinar a segurança e a eficácia dos extratos. O extrato com melhor desempenho foi incorporado em emulsões O/W, que foram submetidas a testes estruturais, reológicos, microscópicos e de estabilidade abrangentes. As emulsões apresentaram tamanho de gotículas, textura e desempenho de armazenamento favoráveis, mantendo a segurança e a bioatividade.

Este projeto destaca a multifuncionalidade dos NADES em dermocosméticos, servindo como solventes ecológicos, modificadores de textura e transportadores de compostos bioativos, ao mesmo tempo que se alinha com os princípios da química verde e da economia circular. O uso de subprodutos agroindustriais, como resíduos de tremoço, aumenta a sustentabilidade, reforçando o potencial dos NADES na criação de cosméticos inovadores e ambientalmente responsáveis.

Palavras-chave: NADES, sustentabilidade, solventes verdes, champô, emulsões.

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ABBREVIATIONS

API – Active pharmaceutical ingredients

CMC – Critical micelle concentration

CYP – Cytochrome P450

DES – Deep Eutectic Solvents

DPPH – 2,2-diphenyl-1-picrylhydrazyl

FRAP – Ferric reducing antioxidant power

GMP – Good Manufacturing Practices

H2-DCFDA – 2,7' dichlorodihydrofluorescein diacetate

HBAs – Hydrogen bond acceptors

HBDs – Hydrogen bond donors

HEVO – Biosynthetically more highly evolutionary metabolites

HNE – Human neutrophil elastase

LVER – Linear-viscoelastic region

MTT – 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NADES – Natual Deep Eutectic Solvents

NPs – Natural products

O/W – Oil-in-Water

O/W/O – Oil-in-Water-in-Oil

PRIM – Biosynthetically primordial metabolites

RH - Relative humidity

ROS – Reactive oxygen species

SDS – Sodium dodecyl sulfate

SLS – Sodium lauryl sulfate

TCC – Total carbohydrate content

TPA – Texture profile analysis

TPC – Total phenolic content

W/O – Water-in-Oil

W/O/W – Water-in-Oil-in-Water

WPC – Whey protein concentrate

CHAPTER 1: Introduction

1.1 History and Evolution of Cosmetic Products

Cosmetics and personal care products have historically played a vital and diverse role in human society [1]. The origins of cosmetics can be traced back to ancient Egypt, where their primary functions were to enhance hygiene and provide health benefits [2]. Noteworthy examples include soap and eye cosmetics developed over 4.000 years ago, along with lip products that have been in use since 7000 BC [1]. During the Middle Ages, the use of cosmetics became a symbol of nobility, particularly given the limited hygiene practices of that era [3]. Ancient Egyptians formulated eye makeup from natural dyes extracted from minerals and plants, which not only enhanced appearance but also served as remedies for various eye diseases and skin conditions [4]. This historical relationship between cosmetics and pharmaceutical products has continued through the ages. However, it was not until the early 21st century that this connection became more pronounced, driven by the growing demand for cosmetic products that prioritize both safety and efficacy [5].

The demand for natural products is steadily increasing, particularly within the cosmetics industry. This increase can be attributed to growing concerns over the negative impacts that synthetic materials have on both consumer health and the negative effects of synthetic materials on both consumer and the environment. Natural products provide numerous benefits, including reduced water consumption, materials, and energy during production, minimal pollution to natural ecosystems, and often recyclable packaging. Currently, the cosmetics market is highly competitive, with product quality, efficacy, and safety becoming increasingly crucial [6].

A cosmetic product, as defined by the Regulation (EC) No. 1223/2009, refers to any substance or mixture intended for application on the external parts of the human body, including such as the skin, scalp, nails, lips, and external genitalia, as well as with the teeth or the mucous membranes of the oral cavity. Its primary purpose is to cleanse, perfume, alter appearances, protect, maintain good condition, or mask body odors. Cosmetic products encompass a wide range of varieties, which can typically be categorized as follows: 1. Personal hygiene cosmetics (such as soaps, deodorants, and shampoos), 2. Skin, hair, and integument cosmetics (including products for external intimate care), 3. Beautifying cosmetics (like perfumes and makeup), 4. Protective cosmetics (such as sunscreens and anti-wrinkle treatments), 5. Corrective cosmetics (including beauty masks and hair dyes), 6. Maintenance cosmetics (like shaving creams and moisturizing creams), and 7. Active cosmetics (such as fluoride toothpaste and antiseptics) [7].

In today's world, cosmetic products are used by individuals of all ages to enhance or modify their appearance without affecting the body's structure or function. According to the Environmental Working Group, women typically use an average of 12 cosmetic products daily, which collectively contain around 168 different chemicals. Globally, more than 3.000 chemicals are used to formulate a diverse range of fragrances in these products. In the United States alone, approximately 12.500 unique chemical ingredients are allowed for use in personal care items. As consumers become increasingly aware of the ingredients they apply to their skin, there is a growing demand for more sustainable and natural cosmetics. The synergy of active ingredients in cosmetics is essential for promoting skin

health and addressing concerns such as anti-aging, hydration, and acne treatment. As a result, cosmetics manufacturers are continually innovating to create new products that meet the evolving needs of consumers [8].

1.2 Cosmetic Formulation Strategies: From Shampoos to Emulsions

1.2.1 Shampoos

Since ancient times, people have used natural extracts and resources for cosmetic and healthcare purposes. As mentioned previously, there has been a significant increase in consumer demand for natural ingredients, especially in cosmetic products like shampoos, to replace synthetic compounds that may pose risks to health and the environment. In response to this demand and the need to reduce the use of microplastics, marketing trends are shifting towards a cosmetic industry, in this case focused on hair care, based on natural ingredients, often associated with a healthy lifestyle. Additionally, despite technological challenges related to permissible ingredients and their legislative concentration limits, the cosmetic sector is concentrating on researching innovative raw materials, strategies, and techniques for the formulation of new products that also provide a pleasing texture and skin feel. Many cosmetic industries are dedicated to hair care, producing safe products and eco-friendly packaging [9].

In general, shampoos are primarily designed to cleanse the scalp first and the hair second, contrary to the common belief held by most consumers. Shampoo is a relatively modern invention; until the mid-1930s, bar soap was the conventional method for cleaning the hair. However, this approach was somewhat unsatisfactory, as hard water - high in calcium and magnesium salts, combined with bar soap often left a residue that dulled the hair's appearance [10].

Thus, the progressive evolution of hair care products has been fundamentally driven by the need to facilitate cleansing, oil removal, lipid regeneration, and hydration of both the scalp and hair. This evolution has resulted in a diverse array of shampoo types [9,11]. The process of hair washing with shampoo constitutes a complex procedure, particularly considering that, on average, a female individual possesses approximately 4-8 square meters of hair surface that requires meticulous cleansing. While it is relatively easy to formulate a shampoo that removes all sebum from the hair and scalp, doing so would leave the hair frizzy, dry, and unattractive. Therefore, the main challenge in formulating a shampoo lies in achieving a balance: it must remove only enough sebum to create a clean appearance while retaining sufficient conditioning agents, which actually represent synthetic sebum, to enhance the beauty the cleaned hair [10].

1.2.1.1 Hair structure

Understanding the structure of hair is essential to comprehending its growth, resistance, and response to external agents, such as cosmetic products. A hair strand consists of three main parts: the bulb, the root, and the shaft. It is anchored within the pilosebaceous follicle in the dermis, as shown in Figure 1. The bulb, which is the deepest

extremity of the hair, is the region responsible for hair growth. It is connected to the dermal papillae, a region that is densely innervated and vascularized, facilitating the supply of essential nutrients for hair growth [9].

The root of the hair is firmly fixed within the hair follicle, which is situated between the bulb and the epidermal surface, marking the point where the hair transitions into the stem. Both the root and the stem are composed of three concentric layers: the medulla, the cortex, and outer cuticle. The medulla serves as the central core of the hair, while the cortex represents its largest and thickest component, playing a crucial role in determining many of the hair's mechanical properties. A key element of the cortex consists of keratin filaments, which include cysteine residues that readily form strong covalent disulfide bonds. This cross-linking between adjacent keratin chains significantly contributes to the hair's shape, stability, and texture [9,12].

The cuticle is a resilient layer of overlapping dead cells that serves as a protective barrier against the environment aggressions. A healthy cuticle appears smooth, promoting light reflection and reducing friction between hair strands, which contributes to the overall shine and texture of the hair [9]. The hair cuticle consists of three superimposed layers: the β-layer, the α-layer, and the epicuticle (also known as the f-layer). This outermost layer is composed of a hydrophobic lipid and functions as the primary defense against moisture loss and external damage. Together with the cortex, these layers play a crucial role in maintaining hair's shine and volume. When the epicuticle is compromised, whether through frequent friction or chemical treatments, this lipidic protection decreases, making the hair more prone to damage and fragility. Keratin is the main component of hair, and the hair fiber itself is a complex mixture of sulfur-rich proteins, lipids, water, melanin, and other elements, that collectively provide strength and color to the hair [9].

It is important to recognize that while hair texture and shine are typically associated with the surface properties of the hair, the hair's overall integrity is attributed to the hair cortex. To address these aspects, a variety of hair care products are available. These products aim to improve the structural integrity of hair fibers and enhance tensile strength, in addition to enhancing hair volume, reducing frizz, improving manageability, and promoting new hair growth [9,13]. It is particularly relevant that modern cosmetic products are formulated not only to cleanse the hair of sebum but also to restore and enhance hair physiological properties. For instance, intensive conditioning agents can temporarily "replace" the f-layer, thereby improving moisture retention in the cortex and reconstructing some of the diminished physical properties of the hair. As a result, increased hair shine emerges as one of the primary benefits offered by modern hair care products [9].

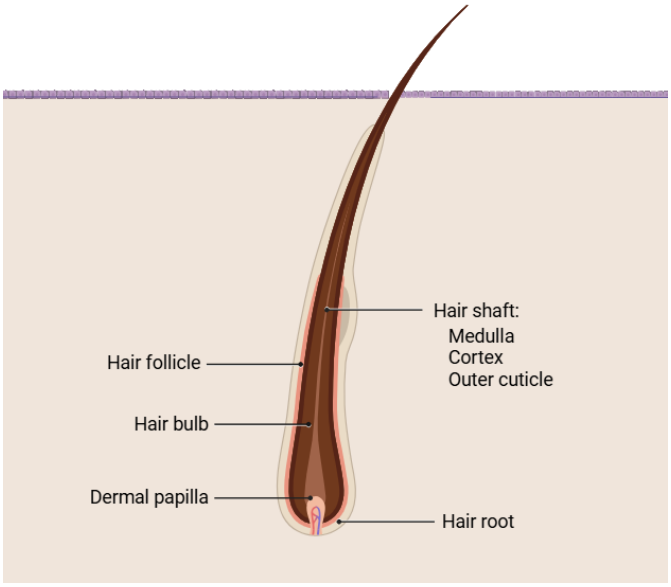


Figure 1 - Hair structure. Image created in BioRender.

1.2.1.2 Traditional and non-traditional shampoos

The most widely used cosmetic product for hair care is shampoo. A shampoo can be defined as a cosmetic formulation, conveniently packaged for application, that is typically used to cleanse the hair and scalp of dirt, residues from previously applied styling products, and environmental pollutants. To maintain the physiological balance of essential hair elements, enhance hair shine, and improve overall aesthetic appearance, the cleansing process must be gentle [9,14]. Furthermore, a shampoo should also be easily to rinsed out with water, generate an adequate amount of foam to meet consumer expectations, and be non-toxic and non-irritating to the hair and scalp, avoiding any side effects or irritation to the skin, hair, scalp and eye [9].

Among commercial shampoos, there are not only conventional cleansing and conditioning types but also specialized formulations that incorporate functional ingredients targeting specific hair concerns. Indeed, shampoo has largely moved beyond its basic role as a cleansing agent. In detail, these "specific" shampoos may contain antibacterial agents, natural essential oils, or extracts to address issues such as dandruff, dermatitis, and various other hair and scalp conditions. Given that the scalp is one of the most absorbent parts of the body, cosmetic products applied there have the potential to enter the bloodstream directly. Therefore, it is crucial to understand the effects of the ingredients used in shampoo formulations [9].

When it comes to "specific shampoos", there are also many categories tailored for different hair types: shampoos for "normal hair", "dry hair," and "oily hair." Shampoos formulated for "normal hair," which often contain sodium dodecyl sulfate (SDS) as the primary detergent cleansing agent, effectively cleansing the scalp. This type of shampoo is often suitable for chemically untreated hair as well. In contrast, shampoos intended for "dry hair" provide a gentle cleansing experience while offering excellent conditioning by utilizing amphoteric and anionic detergents known as sulfosuccinates. Lastly, shampoos designed for "oily hair," which is often characterized by significant sebum production,

may also include SDS or sulfosuccinates, although these ingredients can potentially lead to dryness in the hair fibers [9].

Beyond the typical liquid shampoos commonly used, there are also other shampoo types with distinct formats and textures. These notably include powder shampoo, lotion shampoo, solid gel shampoo, herbal liquid shampoo, solid cream shampoo, and aerosol foam shampoo [9]. The different types of non-conventional shampoos are shown in Figure 2.

Figure 2 - Types of non-conventional shampoos. Image created in BioRender.

1.2.1.3 Shampoo Ingredients

A shampoo is composed of various ingredients, each serving specific functions. These components ensure the product has a suitable consistency, maintains stability over time, and enhances overall user experience. Typically, the primary ingredients that constitute a shampoo are [10]:

- Detergents/surfactants: These ingredients are designed to remove environmental dirt, styling product residues, sebum, and dead skin cells from both the hair and scalp;
- Foaming agents: These allow the shampoo to form foam, as consumers often associate cleansing effectiveness with foam production, even though the two are not directly correlated;
- Conditioners: They provide softness and smoothness to the hair after the detergent has removed the sebum;
- Thickeners: These ingredients increase the viscosity of the shampoo, as consumers generally perceive thicker shampoo as being more effective than thinner ones;
- Opacifiers: There are added to make the shampoo opaque rather than translucent, serving as an aesthetic purpose rather than a cleaning function;
- Sequestering agents: Their primary function is to prevent the formation of soap residue on the hair and scalp when exposed to hard water, which is the fundamental difference between liquid shampoo and bar soap;
- Fragrances: These provide a pleasant scent to the shampoo, enhancing the consumer experience;
- Preservatives: These help prevent microbial and fungal contamination of the shampoo, both before and after the product is;

- Special additives: These treatment ingredients or marketing enhancements are added to offer additional benefits to the shampoo beyond merely cleaning the hair and scalp.

1.2.1.4 Surfactants

The primary cleansing ingredient found in shampoos is known as surfactant. These agents are synthetic, soap-free compounds characterized by both lipophilic and hydrophilic structures. The lipophilic end adheres to sebum and dirt, while the hydrophilic end enables the removal of the sebum with water [10]. Specifically, detergents can be classified as anionic, cationic, amphoteric, and non-ionic surfactants, according to their chemical structures [9]. Figure 3 summarizes all types of surfactants.

Anionic surfactants are characterized by their negatively charged hydrophilic polar group, which makes them highly effective at removing sebum and dirt. However, their potent cleansing properties can increase the negative electrical charges on the hair surface, resulting in frizz and friction. Consequently, to mitigate damage and achieve gentle detergency, secondary surfactants, such as non-ionic and amphoteric types, are commonly incorporated into formulations. Examples include ammonium lauryl sulfate, sodium lauryl sulfate and SDS [9,15].

Cationic surfactants feature a positively charged hydrophilic end and serve primarily as conditioners. They effectively neutralize the negative charges presented on the hair surface after washing, which helps to reduce frizz. Furthermore, due to their chemical nature, they also exhibit bacteriostatic properties. Common examples of this type of surfactant are benzalkonium chloride and quaternary ammonium salts [9,16].

Amphoteric surfactants are characterized by their ability to control charge based on the pH values of the solution. In other words, they function as anionic or cationic surfactants in alkaline or acidic solutions, respectively. Their mildness makes them dermatologically compatible, and they exhibit good foaming, detergency, and wetting properties. As a result, they are often employed to mitigate the harshness associated with anionic surfactants [9]. A few examples of this surfactant include cocamidopropyl betaine and sulfatobetaine [17].

Finally, non-ionic surfactants are characterized by the absence of electrical charge in aqueous solutions, owing to the lack of dissociable hydrophilic groups. Compared to other types of surfactants, they are less aggressive, which, along with their mild properties, makes them popular choices as emulsifiers and solubilizers in cosmetic formulations. They also act as surfactants, although with lower detergent power. Often derived from plant sources, examples include glucosides, citrates and fatty alcohols [9,18].

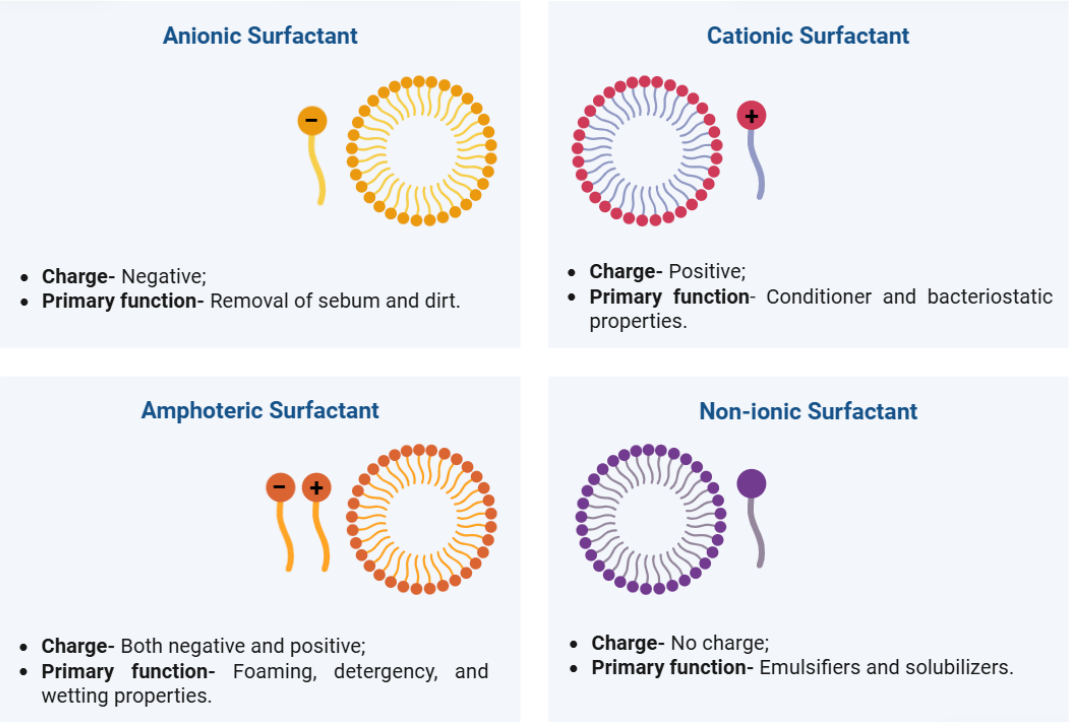


Figure 3 - Comprehensive overview of the various classes of surfactants, including anionic, cationic, amphoteric, and nonionic surfactants. Image created in BioRender.

1.2.1.4.1 Mechanisms of sebum removal by surfactants

Numerous reports indicate four distinct mechanisms of sebum removal, each relying on the amphiphilic nature of the surfactant molecules. The first mechanism is referred to as the "roll-up" mechanism, as shown in Figure 4A. This process is initiated when surfactant molecules migrate to the oil/water interface, effectively reducing interfacial tension. There, surfactant molecules gradually increase the contact angle between the oily residue and the hair strand from 0° to approximately 180°. As the angle increases, the forces that bind the oil to the hair are overcome, allowing the residue to detach and "roll up" into the aqueous phase. Simultaneously, the polar heads of the surfactants repel the dirt, facilitating its incorporation into micelles, where it remains solubilized and removed from the hair fiber, resulting in a clean surface [19,20].

The second mechanism is referred to as spontaneous emulsification (Figure 4B). This mechanism begins when the surfactant reduces the interfacial tension at the dirt/water boundary, however, it does not sufficiently increase the contact angle to completely detach the oil from the hair. Instead, the surfactant causes the dirt film to deform, resulting in the formation of small "buds" or emulsified droplets that remain partially adhered. Subsequently, agitation of the system through friction or rinsing provides sufficient energy to break these droplets, dispersing them in the water [20,21].

The third mechanism is known as the penetration mechanism or "soil-softening", represented in Figure 4C. This mechanism initiates when surfactant molecules, due to their hydrophobic character, penetrate into insoluble dirt particles, thereby locally reducing the interfacial tension at the dirt/water boundary. This penetration process results in the formation of an interfacial liquid-crystalline phase on the dirt's surface, weakening

its structure. Following this, agitation, either by friction or water flow, gradually removes this softened phase in layers, exposing new dirt that has not yet been affected. By repeating this cycle of surfactant penetration and agitated removal, the dirt can be completely dissolved and removed from the hair strand [20,22].

The fourth and final mechanism is known as the solubilization mechanism (Figure 4D). This mechanism occurs when the surfactant concentration reaches its Critical Micelle Concentration (CMC), prompting the molecules self-assemble into micelles featuring a hydrophobic core. These micelles approach the dirt at the oil/water interface, adsorbing onto its surface, and encapsulating the oily particles within their hydrophobic interior. Once encapsulated, the particles become soluble in the aqueous phase, allowing for an easy removal during rinsing and leaving the hair fiber clean [19,20].

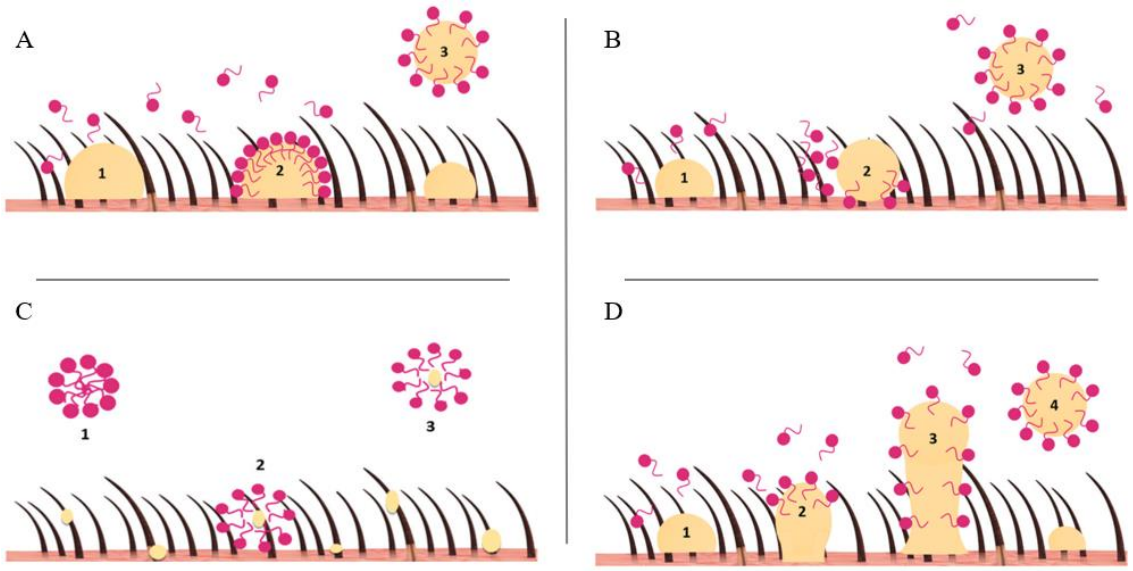


Figure 4 - Mechanisms of sebum removal by surfactants. Sebum is represented in yellow and surfactant molecules in pink. (A) Roll-up mechanism, where surfactants reduce interfacial tension, allowing the sebum layer to detach from the surface. (B) Spontaneous emulsification mechanism, involving the formation of fine sebum droplets stabilized by surfactant molecules. (C) Penetration mechanism, in which surfactants infiltrate the sebum matrix, facilitating its disruption. (D) Solubilization mechanism, where surfactants incorporate sebum into micelles, enhancing its removal [20].

1.2.1.4.2 Surfactant selection in shampoo formulation

The art of shampoo formulation involves blending detergents to achieve an optimal balance between effective cleansing and hair beautification. Typically, a combination of several detergents is used to reach the desired final outcome [10]. Generally, a primary surfactant, responsible for effective cleansing and usually comprises up to approximately 10% w/v, is paired with a cosurfactant, typically around 1–5% w/v. This cosurfactant enhances key characteristics such as foam formation and skin mildness. Primary surfactants are predominantly anionic, such as sodium lauryl sulfate (SLS), chosen for their strong cleansing properties. In contrast, cosurfactants are often amphoteric, such as betaines, owing to their tolerance to pH variations and their ability to improve the user experience without compromising formula's efficacy [20].

A study conducted by Sang *et al.* developed shampoos incorporating varying percentages of mangosteen peel extract, with different surfactants such as sodium cocoyl glutamate, sodium lauroyl sarcosinate, and cocamidopropyl betaine. The findings indicated that these formulations exhibited notable antioxidant and antimicrobial properties, along with impressive foam stability and cleansing effectiveness.[23].

In another study, Nunes *et al.* formulated shampoos using various sugar-derived surfactants, specifically the alkyl polyglucosides decyl glucoside and coco-glucoside. The results indicated that these shampoos, formulated with different surfactants, showcased enhanced washing performance and facilitated easier combing. This research highlights the promising potential for creating sustainable, effective, and safe shampoo formulations [24].

1.2.1.5 Surfactant Interactions and the Challenge of Skin Mildness in Shampoo Formulations

An ideal shampoo should effectively cleanse dirt from hair without causing skin irritation or dryness. Unfortunately, surfactant molecules tend to be indiscriminate in their cleansing action on the hair and scalp, often leading in the unintentional removal or alteration of beneficial proteins and lipids. Such detrimental interactions can manifest in several ways. Firstly, surfactant molecules can bind with proteins present in the hair and scalp, creating protein-surfactant complexes. This interaction inhibits these proteins from binding and retaining water molecules to their usual extent, resulting in a sensation of dryness post-washing. Additionally, the swelling action induced by these protein-surfactant interactions has also been observed to facilitate surfactant penetration into the dermis, potentially triggering adverse biochemical responses such as inflammation and irritation. Furthermore, the charge density of the micelles formed can contribute to protein denaturation, exacerbating irritation. Beyond protein interactions, shampoo surfactants can also engage with essential lipids, leading to their solubilization within the formed micelles. Studies indicate that the charge associated with the surfactant enables it to penetrate and adsorb onto the lipid bilayers of cell walls, thereby destabilizing them and triggering adverse biochemical reactions [20].

The term "skin mildness" is an important concept in the personal care industry, describing the extent to which various surfactant blends elicit responses from the skin during application. Products that generate fewer adverse reactions are thereby classified as "milder" on the skin, which enhances the overall user experience [20].

1.2.2 Emulsions

The term "emulsion" is primarily associated with formulations intended for internal use in the pharmaceutical industry. In contrast, those designed for external application are classified differently, such as lotions or creams. An emulsion can be defined as a two-phase system composed of two immiscible liquids. One of these liquids, known as the dispersed phase, is uniformly distributed as small globules throughout the second liquid, referred to as the continuous phase. Due to their inherent thermodynamic instability, emulsions require the incorporation of a third component, known as an

emulsifier, to enhance their stability. The emulsifier forms a thin film around the droplets of the dispersed phase, thus preventing separation. Both the dispersed and continuous phases can exhibit varying consistencies, ranging from liquid to semi-solid. As a result, pharmaceutical emulsions span from low-viscosity lotions to higher-viscosity creams, with the particle size of the dispersed phase typically ranging from 0.1 to 100 μm [25].

Emulsions play a crucial role in numerous industries, including pharmaceuticals, cosmetics, food, and agriculture. In the cosmetics industry, emulsions are particularly versatile and are extensively utilized in color cosmetics, as well as in skin and personal care products [26].

1.2.2.1 Types of emulsions

Pharmaceutical emulsions typically consist of mixtures that combine aqueous phases with various oils and fats. There are several types of emulsions, each designed for specific applications and characterized by distinct preparation methods, as shown in Figure 5. The most common types are oil-in-water (O/W) emulsions and water-in-oil (W/O) emulsions. O/W emulsions are generally preferred due to their non-greasy texture, quick spreadability, and ease of removal with water. In contrast, W/O emulsions have a greasier feel and are mainly used to reduce moisture loss from the skin's surface. Depending on their consistency and viscosity, these emulsions can be categorized into liquid formulations, lotions, semi-solid formulations, or creams [25].

In addition to the two primary types of emulsions, there are several others, including multiple emulsions, microemulsions, and Pickering emulsions. Multiple emulsions are complex systems that have proven to be safe for use in both cosmetic and pharmaceutical applications. This intricate type of emulsion involves the dispersion of O/W or W/O emulsions within another liquid medium. For instance, an oil-in-water-in-oil (O/W/O) emulsion contains tiny oil droplets dispersed within the water droplets of a W/O emulsion, while a water-in-oil-in-water (W/O/W) emulsion comprises water droplets suspended in the oil phase of an O/W emulsion. These emulsions are employed in various cosmetic products, such as skin moisturizers. They offer several advantages, including the ability to incorporate diverse active ingredients into different compartments. However, despite their significance, multiple emulsions encounter challenges related to thermodynamic instability and their intricate structure [25].

Microemulsions are systems composed of water, oil, and surfactant, resulting in a unified liquid solution that is both optically isotropic and thermodynamically stable. There are two primary types of microemulsions: O/W and W/O, similar to "conventional" emulsions. Due to their nanometric size, microemulsions enhance the delivery of active ingredients to deeper layers of the skin, while also stabilizing sunscreens and sensitive extracts, which extends the shelf life of products. Furthermore, they enable the creation of lightweight, easy-to-apply formulations that offer a gradual release of active ingredients, ultimately enhancing both the comfort and effectiveness of skincare routines [25].

Finally, there are Pickering emulsions, which utilize colloidal-sized solid particles as stabilizers, known as Pickering stabilizers. Recently, these emulsions have been

applied in various fields, including pharmaceuticals and cosmetics, such as creams and lotions, providing enhanced stability and a creamier texture [25,26].

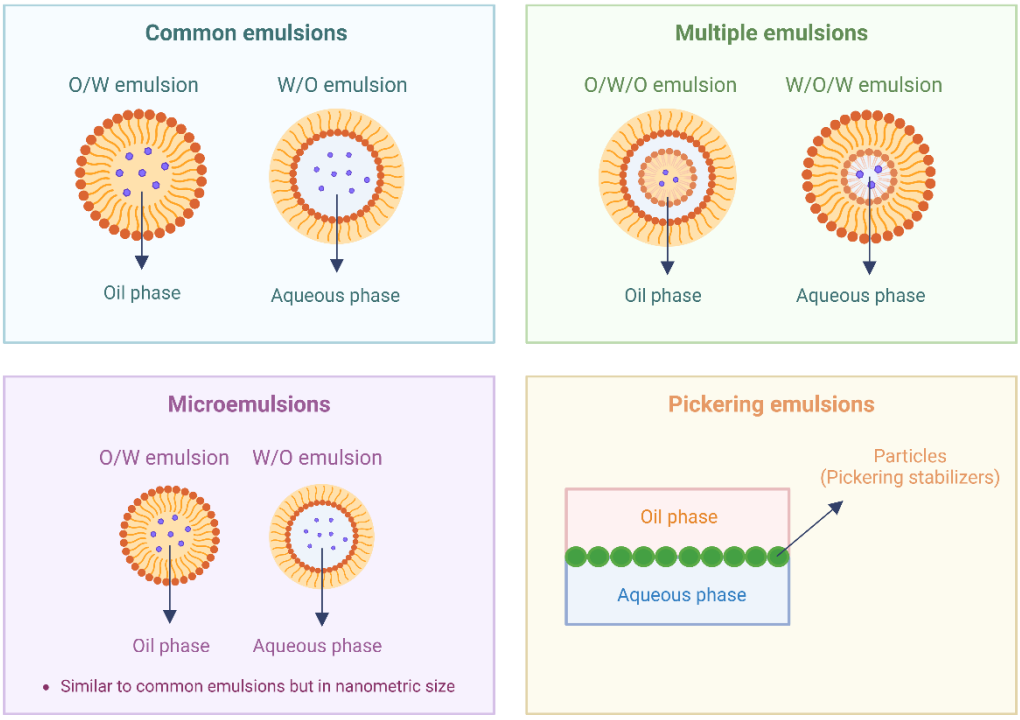


Figure 5 - Classification of emulsion systems. Representative examples include conventional emulsions—oil-in-water (O/W) and water-in-oil (W/O); multiple emulsions—oil-in-water-in-oil (O/W/O) and water-in-oil-in-water (W/O/W); microemulsions—O/W and W/O types; and Pickering emulsions, which are stabilized by solid particles rather than surfactants.

1.2.2.2 Selection of emulsion type

In cosmetics industry, the selection of O/W emulsions, W/O emulsions, or multiple emulsions such as W/O/W depends on both technical performance and the sensory experience of the user. O/W formulations, which represent approximately two-thirds of the emulsified products available on the market, are known for their ease of application and non-greasy finish, making them popular choices for day creams and body lotions. In contrast, W/O emulsions create a more substantial occlusive barrier, offering extended moisturization [27]. This makes them ideal for night creams and products intended for very dry skin, though they may leave a greasier sensation. Multiple emulsions (W/O/W) are employed when there is a need for a controlled release of hydrophilic active ingredients encapsulated within oil droplets is desired, rendering them especially suitable for serums and specialized treatments [27].

1.2.2.3 Emulsions ingredients

The formulation of an emulsion is influenced by the choice of ingredients, which ultimately dictate its final properties such as stability and viscosity. In cosmetic formulations, the selection of ingredients also depends on the active ingredients to be

incorporated, as these contribute to determining the function of the product. Bioactive ingredients can be sourced from plant extracts, vegetables, and their by-products [25,27].

Key components of an emulsion include emulsifiers, emollients, thickeners, antioxidants, preservatives, humectants, and water. Emulsifiers are essential for stabilizing the emulsion, as they prevent the coalescence of dispersed phase droplets. This stabilization enhances the emulsification process during production and allows for better control of rheological properties [27,28]. Emollients contribute to skin softness and help retain moisture by forming a protective barrier [29]. Thickeners increase the viscosity of emulsions, further aiding in their stabilization [28]. Antioxidants serve to protect against oxidation, consequently preventing skin degradation and premature aging [30]. Preservatives protect against microbial growth, extend shelf life, and may originate from either natural or synthetic sources [31]. Humectants, which are hydrophilic substances, form hydrogen bonds with water molecules to ensure the skin remains hydrated. Typically, they are combined with emollients to enhance their moisturizing effects [32]. Lastly, water functions as the universal solvent, facilitating the incorporation of other ingredients and enhancing the hydration and overall texture of the emulsion [28].

1.2.2.4 Emulsions stability

A critical parameter that influences the properties of emulsions is their stability, which poses a complex challenge for evaluation. The stability of an emulsion is characterized by the absence of coalescence within the dispersed phase, minimal creaming, and the preservation of its physical attributes, including elegance, odor, color, and overall appearance. Emulsion instability can be categorized into three principal phenomena: flocculation, creaming, and coalescence [25].

Flocculation is defined as the aggregation of small droplets within an emulsion into larger clusters, which can subsequently separate upon agitation without the individual droplets disintegrating. This phenomenon typically occurs when there is an excess of surfactant present in the continuous phase, creating attractive forces that draw droplets close together, increasing the likelihood of coalescence [25].

Creaming refers to the upward or downward migration of dispersed phase droplets, resulting in the formation of a distinct layer within the continuous phase, without the droplets merging. This layer can be redispersed upon agitation. In O/W emulsions, where the density of oil droplets is lower than that of water, the creamed layer rises to the surface. Conversely, in W/O emulsions, where water droplets are denser, creaming occurs in a downward direction. Enhancing the viscosity of the continuous phase is an effective strategy to mitigate this process [25].

Coalescence occurs when the existing electrical or mechanical barriers fail to prevent the merging of droplets, resulting in the formation of progressively larger aggregates. To prolong the stability of emulsions and delay the coalescence process, high molecular weight or high boiling point substances can be incorporated into the continuous phase, thus reinforcing the protective film surrounding the droplets [25].

1.2.2.5 Rheological analysis

Besides visual or physical-chemical analysis, the stability of emulsions can be evaluated by instrumental methods, with rheology being one of the most relevant techniques. Rheology is the scientific study of the flow and deformation properties of matter. Its primary objective is to analyze how materials behave when subjected to deformation due to applied tension. To assess the properties of a sample, rheometric tests are conducted, the most common of which are viscosity and oscillation tests. Viscosity quantifies a material's resistance to flow; higher viscosity indicates that the fluid will flow more slowly. On the other hand, oscillation tests evaluate the viscoelastic properties of a material by measuring its elastic and viscous responses when exposed to periodic deformations [33–35].

When defining the viscosity of a material, materials can display Newtonian or non-Newtonian behavior, depending on their flow characteristics. Newtonian fluids generally have a constant viscosity that remains unchanged regardless of the shear rate (the force applied), with water serving as a typical example. Conversely, non-Newtonian fluids do not maintain a constant viscosity; rather it varies with changes in the applied force. Several types of non-Newtonian behavior exist, including: pseudoplastic behavior, where viscosity decreases as the shear rate increases; dilatant behavior, in which viscosity increases with an increase in shear rate, and viscoelastic behavior, where a material can simultaneously exhibit both viscous (liquid) and elastic (solid) characteristics, depending on the applied force [34].

The relationship between the G' modulus (storage modulus, which represents the elastic component of a material) and G" (loss modulus, representing the viscous component) is essential for describing the viscoelastic behavior of materials. It indicates the balance between a formulation's elastic (solid) and viscous (liquid) properties. Generally, when G' is higher than G", the material demonstrates predominantly elastic behavior, characteristic of gels or emulsions. Conversely, when G" surpasses G', the material exhibits viscous behavior, typical of formulations like shampoos. When G' is equal to G", the point where the two modulus intersect is obtained, signifying a transition between liquid and solid states. In essence, this point illustrates that the material achieves a state of equilibrium between its solid and liquid properties [35].

1.3 Importance of Sustainability in the Cosmetics Industry

The cosmetics industry faces the challenge of balancing consumer expectations for both effectiveness and visual appeal, while also prioritizing consumer safety and environmental sustainability [8]. Research conducted by the Environmental Working Group reveals that the average woman utilizes approximately 12 cosmetic products each day, containing around 168 distinct chemical substances in total [8]. In response to public health concerns, the European Union implemented regulations that became effective on July 11, 2013. These regulations establish stringent criteria for toxicological assessments, require comprehensive documentation for all ingredients and ensure compliance with key legal regulations, including Regulation (EC) No. 1223/2009 and the CLP Regulation. [8,36].

When evaluating the complete life cycle of a cosmetic product, it is crucial to assess not only the efficacy of active ingredients- such as those aimed at anti-aging, moisturizing, or treating acne- but also to analyze carbon dioxide emissions, energy and water consumption, and the use of alternative sources to fossil fuels throughout the extraction of raw materials and the product's end-of-life stage [37]. In this context, the concept of “eco-innovation” becomes a crucial tool for assessing environmental impacts and risks at each stage of the production process [8,38].

Sustainability has emerged as a critical factor in consumers' purchasing decisions, with an increasing emphasis on natural and plant-based products [8,39]. However, the transition towards more sustainable ingredients presents substantial challenges. The replacement of synthetic compounds with natural, organic, or green chemistry alternatives requires extensive research and development to ensure that functionality, stability, and sensory performance are maintained. Moreover, many conventional ingredients lack direct substitutes, necessitating collaborations between laboratories and suppliers to conduct joint research [36,40]. The high costs associated with research and development, combined with the inconsistent availability of sustainable raw materials, pose significant challenges. Traditional ingredients often remain more cost-effective and easier to produce, complicating large-scale adoption [36,40]. Additionally, the process of introducing new products must comply with stringent safety and regulatory standards, which can be slow and bureaucratic. Lastly, internal resistance may also present a barrier, since many companies are reluctant to modify established formulations due to concerns over the commercial risks involved in using less familiar ingredients [36].

1.4 The Rising Demand for Green Solvents

The formulation of cosmetic products involves a diverse array of ingredients, such as oils, emollients, solvents, surfactants, preservatives, texturizing agents, pigments, fragrances, actives, and auxiliaries [41]. This diversity presents a significant challenge for transitioning to more sustainable and eco-friendly cosmetic products. A key component of these formulations is solvents, which play a crucial role in cosmetics by dissolving, diluting, and homogenizing all the ingredients in the formulation [42]. In addition, solvents enhance the efficacy and incorporation of active ingredients, making them versatile for applications that allowing better penetration of these ingredients into the skin or hair [42].

The cosmetics industry frequently utilizes a variety of conventional organic solvents, including dichloromethane, ethyl acetate, acetone, n-butanol, ethanol, and methanol [43]. However, concerns regarding the safety of these conventional organic solvents arise from the potential toxicity associated with improper extraction methods and the possibility of residual solvents in the final products [44]. Furthermore, these organic solvents are highly volatile, which posing a risk as contributors to atmospheric pollution and global warming [44].

The growing demand for sustainable solvents is largely driven by their status as the predominant component in industrial processes and product formulations, often exceeding all other compounds combined [45]. Consequently, solvents are recognized for

their potential to cause significant environmental harm due to their extensive use [45]. Typically, the solvents employed are highly flammable and generate smoke, with the exception being halogenated solvents and water [45].

A substance can be classified as a solvent only if it meets certain criteria. Firstly, it must exhibit high selectivity, meaning its polarity should align with that of the target compound. Additionally, the solvent should also be safe, meaning it must have low toxicity, and be non-explosive and non-flammable. Moreover, the solvent needs to be neutral and easily separable from the target compounds. A low viscosity is also essential, as it facilitates efficient mass transfer. Furthermore, the solvent should have a low boiling temperature to prevent the degradation of the compounds. Lastly, it is important for the solvent to be as economical as possible. Considering that many conventional solvents present significant risks to both health and the environment, such as being carcinogenic, mutagenic, teratogenic, neurotoxic, acid rain-forming, persistent in the environment, and susceptible to forming explosive peroxides, the demand for alternative solvents that meet all of these criteria has been steadily rising [43].

As previously mentioned, a significant drawback of solvents is their potential neurotoxicity. Solvents predominantly enter the human body through inhalation, and many can penetrate the skin. These compounds are primarily metabolized in the liver and tend to accumulate in lipid-rich tissues, such as the brain. Therefore, a chemical is considered neurotoxic if it is capable of inducing a consistent pattern of neuroanatomical changes or neural dysfunctions that result in neurochemical, neurophysiological, or behavioral effects [46]. It is crucial to understand that the absorption of solvents depends on various factors, including the state of the skin, the chemical composition and concentration of the solvent, the duration of exposure, and the surface area of the skin that is exposed. Moreover, the absorption of solvents is influenced by the specific air/blood partition coefficients of each solvent, which are determined by the permeability of the alveolocapillary membrane and the solvent's solubility in the blood [46].

The potential toxicity of a solvent is not only determined by its water solubility or lipophilicity, but also by its metabolization process. When solvents are absorbed, they undergo metabolism in the liver, ultimately being eliminated as metabolites in the urine or, to a lesser extent, in the bile. Solvent metabolism in the liver is a two-phase process: in phase I, the cytochrome P450 (CYP) enzyme complex generates reactive metabolites, which are then detoxified in phase II. As a result, the toxicity of solvents is influenced by both the inherent toxicity of the solvent itself and the potential toxicity of reactive intermediate metabolites. Moreover, the use of typical solvents may trigger alterations in the lipid composition of cellular membranes, which can disrupt intercellular communication and promote the production of reactive oxygen species (ROS). This, in turn, results in lipid peroxidation caused by free radicals, mitochondrial and nucleic acid damage, as well as contributing to oxidative stress [46].

Taking these factors into account, green solvents emerge as a viable alternative to traditional solvents. A green solvent is defined as one that minimizes environmental impact throughout the entire life cycle of a product or process, thereby posing minimal risk to both the environment and consumer health [45]. The advantages of green solvents are numerous; they are simple to produce, derived from eco-friendly materials and

processes, pose fewer risks, require minimal energy, and can be reused or naturally decomposed [43]. Among the array of green solvents available, Natural Deep Eutectic Solvents (NADES) have gained greater attention from the scientific community [43]. To qualify as a "green" solvent, it must adhere to specific standards, including safety, biodegradability, and recyclability [41].

NADES represent a new generation of solvents, classified as a subgroup of deep eutectic solvents (DES), that are composed exclusively of natural compounds [47]. NADES are produced by mixing two or more natural ingredients like sugars, sugar alcohols, polyalcohols, amino acids, organic acids, and organic bases in specific molar ratios, often with the addition of water, to create a eutectic liquid suitable for use in various applications, including cosmetic formulations [47]. A eutectic liquid is a unique system that exhibits a lower melting point than that of its individual components when mixed in a specific ratio [44]. Notably, NADES exhibit a significant reduction in melting point, allowing them to transform into liquid form at standard room temperature [44]. It is important to recognize that temperature and the proportion of the components are critical factors in a eutectic system [48]. The interaction between these components is due to intermolecular forces, more specifically hydrogen bonds, rather than covalent or ionic bonds [48].

In consideration of sustainability, an ideal green solvent should have zero ecological impact throughout its life cycle, while also enhancing process conditions and sustainability [49]. In line with the 12 principles of green chemistry, such solvents should prioritize waste prevention, atom economy, less hazardous chemical synthesis, the design of safer chemicals, the use of safer solvents and auxiliaries, energy efficiency, the use of renewable feedstock, the reduction of derivatives, catalysis, design for degradation, real-time analysis for pollution prevention, and inherently safer chemistry to prevent accidents [50]. It must be produced efficiently, both in energy and cost, using renewable raw materials that do not generate emissions, crate a carbon footprint, or waste [49]. When applied, this versatile solvent should improve process performance and minimize solvent loads during subsequent recycling processes due to its high stability [49]. Importantly, it should pose no risk to human health and the environment. Recovery and reuse should be simple and efficient [49]. Finally, once it reaches the end of its useful life, the solvent should be completely biodegradable, leaving no environmental residue [49].

1.4.1 NADES as Green Solvents

1.4.1.1 General characteristics of NADES

As previously mentioned, NADES offer an innovative solution as either solvents or active ingredients within the cosmetics, food and pharmaceutical industry. These eutectic mixtures typically consist of two or more components that interact through hydrogen bonds, resulting in a lower melting point than of each individual component [43]. The liquid mixtures are composed of both hydrogen bond acceptors (HBAs) and hydrogen bond donors (HBDs) [43]. NADES are derived from DES and are considered "natural" because their constituent components are primary metabolites found in plants

from which they are extracted, such as sugars, organic acids, organic bases, amino acids, among others [43].

NADES are increasingly recognized for their effectiveness in extracting natural substances to isolate targeted bioactive compounds [43]. Additionally, NADES are used as catalysts for enzymatic or chemical processes and as carriers for insoluble hydrophobic compounds in pharmaceutical applications [43]. As extraction solvents, NADES function through two distinct mechanisms of action: direct interaction with the targeted compounds, typically through hydrogen bonding, or indirect action by damaging cell walls, which facilitates the release of the desired compound from the plant matrix [43]. Furthermore, NADES have the unique capability to dissolve substances such as phenolic compounds and active pharmaceutical ingredients, consequently enhancing their accessibility [51]. This capability can be adjusted by modifying the HBA:HBD molar ratio, allowing for manipulation of the physicochemical characteristics of NADES [51]. As a result, these solvents prove to be highly adaptable in various industries, as previously stated [51].

1.4.1.2 Components of NADES

In general terms, the components of NADES can be categorized into two main chemical constituents: biosynthetically primordial metabolites (PRIM) and biosynthetically more highly evolutionary metabolites (HEVO) [48]. PRIMs are considered the most important elements of NADES and are composed of a mixture of compounds such as sugars, organic acids, amino acids and choline salts [48]. This group of elements is defined by its strong polarity, hydrophilicity and ability to establish hydrogen bonds with one another, all of which contribute to the distinctive liquid properties of NADES [48]. On the other hand, HEVO refers to the components that provide NADES with hydrophobic and electrostatic versatility [48]. These components may include terpenes, steroids, fatty acids, and other compounds that enhance the properties of NADES, allowing for their adaptation to various applications [48].

1.4.1.3 Preparation of NADES

As environmentally friendly solvents like NADES grow in popularity, it is crucial to comprehend their preparation process. To create NADES, the components must be mixed in precise proportions, followed by homogenization and/or heating treatments to establish entropy within the solution. [48]. In the heating method, the various components of the NADES are combined in specific ratios and then mixed at a designated temperature, usually 50ºC. This process increases entropy and encourages the formation of eutectics, resulting in a homogeneous and, in some cases, transparent liquid. Upon cooling to room temperature, a clear and consistent mixture indicates the successful production of the desired NADES. Conversely, the vacuum evaporation process involves two basic stages. First, the NADES components are dissolved in water, often through heating. Next, the water is extracted via vacuum evaporation, typically using rotary evaporators [48].

It is important to highlight that the physicochemical properties of NADES stem from the intermolecular forces that dictate the interactions between their constituents.

These intermolecular interactions lead to various properties, including low volatility, low melting points, non-flammability, low vapor pressure, acidity, customized polarity, chemical and thermal stability, and miscibility. These features position NADES as a promising alternative to conventional solvents in various fields of research domains, including chemical synthesis, separation, and extraction processes [51]. In light of this, the use of NADES provides an extensive array of possibilities for the development of tailor-made solvents with properties that are optimally suited for specific applications [52]. Given the large number of combinations that can be formed, the potential for obtaining novel solvents with desirable attributes is considerable [52].

1.4.1.4 Properties of NADES

NADES are becoming increasingly popular due to their numerous environmentally friendly properties, making them a great alternative to conventional solvents. Their most notable features are their renewability and biodegradability. Since all of their components are natural products (NPs), NADES can be biosynthesized and metabolized by almost all organisms, making them highly biocompatible. Compared to conventional organic solvents, the biodegradability of NADES helps to reduce potential environmental risks. Additionally, NADES can be easily recycled and reused, making them a sustainable choice [48]. The viscosity and conductivity of NADES are also properties that characterize them due to the hydrogen bonds and Van der Walls interactions that contribute to the viscosity of the liquid. However, compared to conventional solvents, NADES have the disadvantage of high viscosity. This high viscosity can hinder the diffusion coefficients of the analyzed substances, resulting in low mass transfer and prolonged extraction times [43,48]. However, this problem can be resolved by adding water to the NADES or increasing the temperature [43]. The amount of water in a NADES matrix plays a significant role in determining its viscosity and conductivity. Due to the relatively small quantity of water involved in their construction, NADES tend to exhibit higher conductivity [48].

Furthermore, NADES may exhibit either hydrophilic or hydrophobic characteristics, depending on their composition. They contain hydrophilic components that can establish hydrogen bonds using electronegative groups, making them miscible with polar solvents like water. However, the presence of hydrophobic components in NADES can lead to contradictory behavior, causing them to be immiscible with water. This duality between NADES hydrophilicity and hydrophobicity highlights the intricate nature of their miscibility and solubilization properties [48]. In addition, it is worth noting that NADES possess unique solubilization and stabilization properties owing to their ability to influence the solubility and stability of solutes through molecular association effects. This characteristic has been extensively studied in the academic literature and has important implications for various fields, including chemistry and biology [43]. While NADES may have some drawbacks, such as limited substance options and requiring significant research, they also offer multiple advantages. Their safe life cycle aligns with green chemistry principles, and they have a strong dissolving capacity, extraction selectivity, and long-term stability. Additionally, NADES is straightforward in formulating and offering an economically feasible process [51].

Due to their non-toxic properties, NADES applications have garnered significant interest in industries such as food, agrochemicals, cosmetics, and pharmaceuticals [47]. This interest has grown exponentially since the concept was first introduced and patented in 2011. Examples of NADES applications include extracting active compounds from medicinal plants, solubilizing active pharmaceutical ingredients (API), and producing plant extracts for use as ingredients in cosmetic formulations [47]. It is crucial to thoroughly investigate the integration of NADES as environmentally friendly solvents in cosmetic products and their impact on consumer health. This research is essential for creating safe, sustainable, and effective cosmetic formulations.

1.5 NADES Applications in the Cosmetics Industry

1.5.1 Exploring the potential of NADES for encapsulation of bioactive compounds and as surfactants

In recent years, NADES have emerged as highly versatile media for the solubilization and stabilization of sensitive bioactive molecules. Unlike traditional organic solvents, which often present toxicity concerns and limited efficacy in cosmetic or pharmaceutical contexts, NADES offer tunable polarity, low volatility, and biocompatibility, making them ideal candidates for novel encapsulation strategies [53].

Basar *et al.* explored this potential by encapsulating β-carotene, a lipophilic antioxidant known for its UV-protective, anti-inflammatory, and collagen-stimulating properties, in whey protein concentrate (WPC) capsules [53]. Recognizing that β-carotene's low solubility in aqueous and lipid systems and its susceptibility to photo-oxidation pose significant formulation challenges, the study employed NADES based on glucose, glycerol, or propanediol as alternative solvents. Through systematic stability and photo-oxidation assays, it was demonstrated that these NADES increased the β-carotene loading capacity of WPC capsules and generated emulsions that remained physically and chemically stable over extended periods. These findings underscore the capacity of NADES to enhance both the solubilization and protective encapsulation of water-sensitive bioactives [53].

Complementing this approach focused on solvents, Bryant *et al.* investigated the role of NADES as surfactants within W/O Pickering emulsions stabilized by cellulose nanofibrils. In their work, mixtures of NADES and water were emulsified with finely prepared cellulose nanofibrils and then monitored over 200 days at room temperature. The results revealed that NADES not only preserved the structural integrity of the nanofibrils but also contributed to exceptional emulsion stability, with no detectable phase separation or fibril degradation. Such long-term robustness positions NADES-based Pickering systems as promising platforms for sustained delivery of active ingredients in cosmetic formulations [54]. Overall, the findings indicate that the emulsions stabilized with cellulose nanofibrils remained stable for 200 days at room temperature, emphasizing the high stability of such emulsions in NADES and water. Additionally, the presence of NADES did not affect the structure of the cellulose nanofibrils, which supports their potential to maintain their structure and facilitate the stability of emulsions in NADES mixed with water [54].

1.5.2 Exploring the potential of NADES as extraction solvents for cosmetic formulations

In the current landscape of sustainable cosmetics, NADES have emerged as eco-friendly alternatives to conventional organic solvents. They enable the efficient and less toxic extraction of bioactive compounds from microbial and plant sources. Microalgae, particularly Spirulina, represent a renewable source of biomolecules with high cosmetic value, such as phycocyanin (an anti-pollution agent), carotenoids (possessing anti-aging and anti-inflammatory properties), and free fatty acids (moisturizing agents and skin microbiome modulators). The application of NADES reduces the environmental impact of extraction processes and optimizes the selectivity and yield in recovering these compounds of interest [55].

Wils *et al.* systematically explored this approach by designing various hydrophilic and lipophilic NADES formulations, utilizing ultrasound to extract pigments and lipids from Spirulina biomass [55]. The study evaluated the extraction efficiency of phycocyanin and free fatty acids and the effect of these formulations on skin cells. To achieve this, viability assays were conducted on keratinocytes, anti-inflammatory activity tests were performed on cells infected with *Staphylococcus aureus*, and bactericidal action assays were carried out against four common skin bacterial strains [55].

The results demonstrated that hydrophilic NADES maintained high cell viability (approximately 90%) in keratinocytes, while lipophilic NADES showed viabilities between 40% and 76% [16]. Regarding anti-inflammatory effects, only one formulation significantly reduced the mediators CXCL-8 and TNF-α, likely due to its high phycocyanin content. Regarding bactericidal activity, lipophilic preparations demonstrated the ability to reduce the viability of all tested strains, in contrast to the minimal effect observed with hydrophilic NADES. These findings highlight not only the extraction capacity of NADES but also their functional properties, such as anti-inflammatory and antibacterial properties, which make them promising candidates for the formulation of innovative cosmetic and dermatological products [55]. It is important to mention that the scalability of NADES-based extraction methods offers opportunities for the industrial production of cosmetic products, enabling more sustainable manufacturing processes. Their integration into large-scale formulations could reduce dependence on volatile organic solvents, lower production costs related to waste management, and support the development of eco-certified products that meet consumers demand for sustainable cosmetics [51].

1.5.3 The potential of reusing and extracting bioactive compounds for cosmetic formulations

In response to the growing environmental challenges and the urgent need to valorize agro-industrial by-products, recent research has investigated the use of NADES to recover high-value bioactive compounds from various food wastes for cosmetic applications. Punzo *et al.* focused on grape pomace, the solid residue from wine production, which is rich in polyphenols known for their antioxidant and anti-inflammatory activities [56]. By formulating three NADES systems, all based on betaine as a HBA paired with citric acid (BET-CA), ethylene glycol (BET-EG), or urea (BET-U)

as HBDs, they demonstrated that BET-CA achieved superior permeability in 3D models of human keratinocytes, indicating its promise as a dermal delivery vehicle for polyphenols. Cytotoxicity assays revealed that, at lower dilutions, all NADES formulations maintained keratinocyte viability, although higher concentrations induced a significant release of lactate dehydrogenase, highlighting the need to define safe usage limits. In addition, BET-CA uniquely reduced the pro-inflammatory mediators CXCL-8 and TNF-α in keratinocytes exposed to *Staphylococcus aureus*, a result attributed to its high phycocyanin content and suggestive of synergistic anti-inflammatory effects [56].

Based on the concept of food waste recycling, Yoo *et al.* evaluated NADES for extracting phenolic antioxidants, such as chlorogenic acid, ferulic acid, and catechin, from coffee residues [57]. Employing a suite of analytical techniques (HPLC quantification and DPPH, ABTS, and FRAP for radical scavenging assays), they found that NADES extractions yielded substantial phenolic content and robust antioxidant activity, comparable or superior to conventional solvents. These bioactives, including flavonoids such as quercetin, luteolin, and apigenin, exhibit reported antioxidant, anti-inflammatory, and antibacterial properties, positioning coffee residues as a sustainable source of multifunctional cosmetic ingredients [57].

Similarly, Chanioti *et al*. applied NADES to olive pomace, another abundant by-product, to isolate phenolic compounds such as caffeic acid, ferulic acid, and oleuropein [58]. When comparing NADES extractions with traditional solvent systems, the researchers observed an increase in total phenolic content and higher antioxidant activity in NADES extracts, as measured by the DPPH assay. Furthermore, the versatility of NADES combinations and extraction methodologies allowed for the selective recovery of distinct phenolic profiles, reinforcing the adaptability of these solvents to tailor the extract composition for specific cosmetic functions, including cellular protection against oxidative stress and modulation of inflammatory pathways [58].

Overall, these studies highlight NADES as eco-friendly and adjustable solvents that can efficiently extract and improve the functionality of bioactive compounds from food industry waste. Their proven ability to provide polyphenols, antioxidant pigments, and high-purity fatty acids, in addition to their intrinsic biocompatibility and bioactivities, underscores their potential to transform waste residues into value-added ingredients for advanced cosmetic and dermatological formulations.

1.6 The Potential of Lupin as By-product in the Cosmetics Industry

The cosmetics industry is increasingly focused on utilizing by-products to enhance the sustainability and innovation of cosmetic products. Accordingly, selecting ingredients is crucial, as it greatly influences skin health and the product's overall sustainability. However, making the right ingredient choices presents a challenge; it requires carefully assessing factors such as ingredient quality and safety, formulation stability, and alignment with consumer preferences. A significant challenge in this context is replacing non-sustainable synthetic ingredients with more sustainable natural alternatives [59].

A potential solution to address this challenge is using agro-industrial by-products, such as those from the lupin industry, as sustainable ingredients in cosmetics. This

approach is closely linked to the concept of upcycling, a sustainable practice that gives new life to by-products that would otherwise be discarded [60]. By extracting valuable substances from lupin by-products, it's possible to create new products with added value and beneficial health properties in an ecological way. This methodology is gaining traction for several reasons, one is that lupin seeds and their by-products are rich in health-promoting bioactive compounds that have applications in various industries, including dermocosmetics. For instance, lupin peptide extracts demonstrate anti-metalloproteinase activity, exhibiting properties such as anti-gelatinase and anti-collagenase, which have significant implications for the health of supporting tissues like the skin [59].

Overall, lupin is a type of legume native to the Mediterranean region and Latin America. There are many varieties of lupin, each with distinct chemical compositions. This is due to their genetic differences and the environmental ecosystem, encompassing temperature, season, sunlight, soil properties, and pathogens [61]. Generally, the chemical composition of lupin includes high levels of protein (36%-52%), fiber (30%-40%), oil (5%-0%), and oligosaccharides (6%). Lupin seeds comprise two protein classes: 15% albumin, which serves as a functional protein, and 85% globulin [59].

The most commonly cultivated lupin species in Europe are white lupin (*Lupinus albus L.*), narrow-leafed lupin (*Lupinus angustifolius*), and yellow lupin (*Lupinus luteus*) [59,62]. White lupin (*L albus*) is a cultivated legume that belongs to the Fabaceae family, reaching heights of approximately 120 cm. It features a robust stem and secondary roots that can penetrate the soil up to 1.5 meters deep. Like many legumes, white lupin can fix atmospheric nitrogen into ammonia, a process that enriches the soil for surrounding plants. This ability enables lupins to thrive in infertile, acidic, and sandy soils while enhancing the quality of poor soils. *L. albus* is rich in a variety of nutritional compounds, including proteins and amino acids, complex carbohydrates, polysaccharides, dietary fibers, fatty acids, macroelements, microelements, and phytochemicals. However, it is essential to note that white lupin also contains antinutritional factors such as phytates, alkaloids, protease inhibitors, lectins, and raffinose family oligosaccharides. Additionally, it can accumulate potential contaminants, including mycotoxins, pesticide residues, and heavy metals. Conversely, lupin peptides and other bioactive compounds exhibit considerable nutraceutical potential. They may function as appetite suppressants and contribute to reducing the risk of cardiovascular diseases, demonstrating hypolipidemic, antihypertensive, and antidiabetic properties. Furthermore, these compounds can enhance intestinal function and possess anticonvulsant effects [63].

1.6.1 The role of lupin in the cosmetics industry

Due to their sustainability and distinctive properties, the cosmetic industry has demonstrated a notable interest in lupin and its derived by-products. All by-products from the lupin industry exhibit cosmetic benefits, irrespective of their specific chemical compositions. These by-products are particularly well-suited for the formulation of topical products intended for skin repair and rejuvenation and for the prevention of various dermatological conditions, including acne, atopic dermatitis, and erythema [59]. The skin, the largest organ in the human body, plays a crucial role in safeguarding against internal and external factors. It consists of three primary layers: the epidermis, dermis,

and hypodermis. The skin continually adjusts to environmental changes, regulates temperature, and responds to the body's physiological needs [64]. However, the skin is vulnerable to chronic inflammatory conditions like psoriasis and atopic dermatitis, which can compromise its protective barrier. Additionally, photodamage accelerates aging, leading to wrinkles, pigmentation spots, and a decline in firmness. Intrinsic factors such as hormonal changes, oxidative stress, and genetic predispositions also play a significant role in skin aging, which is characterized by a reduction in collagen and subcutaneous fat [65,66]. These skin problems impact quality of life, leading to aesthetic concerns, diminished self-esteem, and social isolation. As a result, there has been a notable rise in demand for topical treatments aimed at delaying the signs of aging, including anti-aging, anti-wrinkle, and firming products [59,67]. Dermocosmetics, which incorporate bioactive ingredients, are gaining popularity due to their capacity to provide targeted therapeutic benefits [59].

Due to its properties, lupin can help mitigate the issues mentioned above. For example, lupin and its by-products possess antioxidant properties, mainly due to the presence of proteins, triterpenes, and various phenolic compounds, including flavonoids, isoflavonoids, tocopherols (vitamin E), ascorbic acid (vitamin C), phenolic acids, and phytosterols [68]. These bioactive substances effectively protect the human body against damage caused by ROS [59].

Additionally, lupin species are rich in bioactive substances with antimicrobial activity, making them potential preservatives for cosmetic applications [68]. These bioactive compounds effectively inhibit the proliferation of pathogenic microorganisms in cosmetic products, particularly those with water content, such as lotions and creams. By mitigating microbial contamination, these substances contribute to preserving product integrity and enhancing consumer safety. Moreover, the antimicrobial efficacy of lupin and its derivatives is intricately linked to their physicochemical properties, including charge, hydrophobicity, solubility, and molecular size [59].

Compounds derived from lupin exhibit anti-inflammatory properties. Specifically, lupin protein hydrolysates have been demonstrated to decrease the *in vitro* expression of pro-inflammatory cytokines, such as tumor necrosis factor (TNF-α) and interleukins IL-1β and IL-6, in THP-1 derived macrophages and osteoclasts [59,69,70].

Finally, compounds derived from lupin and by-products of the lupin industry, such as lupin peptides and lupeol, exhibit anti-aging properties that benefit the skin. Lupin peptides, sourced from protein hydrolysates, are multifunctional and interact with various molecular targets to influence a range of physiological outcomes, in contrast to monofunctional peptides. This versatility enhances their value as components in anti-aging cosmetic products [33,59].

In this scenario, lupin and its by-products are gaining prominence in dermocosmetics. Their antioxidant, antimicrobial, anti-inflammatory, and anti-aging properties, stemming from bioactive compounds, make them promising for treating various skin conditions [59].

1.7 Objectives

The aim of this thesis was to explore and evaluate the potential applications of NADES in cosmetic formulations, focusing on three specific areas: the direct incorporation of NADES in cosmetic formulations, particularly in shampoos to improve foam ability; their role as stabilizers in unstable O/W emulsions; and their use as solvents for extracting phenolic compounds. This research primarily involved the production and physical-chemical characterization of various NADES systems, including rheological, textural, and surface tension analyses.

Shampoos were formulated to investigate how NADES can improve their inherent properties. This included testing different NADES in shampoo formulations and assessing their rheological, textural, surface tension, contact angle, and foam height characteristics, as well as conducting stability tests.

Moreover, unstable O/W emulsions were prepared to explore the capacity of the selected NADES as emulsion stabilizers. NADES were also serve as solvents for extracting phenolic compounds from lupin waste, followed by chemical evaluations such as antioxidant tests, quantification of phenolic compounds, carbohydrate quantification, and stability tests. The resulting extracts were incorporated into O/W emulsions, which were tested for rheological behavior, texture analysis, droplet size, color determination, and stability tests.

Finally, *in vitro* assessments were conducted to evaluate the safety and efficacy of the extracts, shampoos, and emulsions. These included skin cytotoxicity tests, antioxidant capacity evaluations, and enzymatic tests using the HaCaT cell line.

CHAPTER 2: Materials and Methods

2.1 Materials

The following ingredients were used to prepare NADES: Betaine (≥99% purity) was acquired from Glentham Life Science (UK); Citric Acid (≥99.5%), Sucrose (≥99%), Glycerol (≥99.5%), Sorbitol (≥98%), Glucose (≥99.5%), Fructose (≥99%), were acquired from Sigma-Aldrich (USA).

The following ingredients were used to prepare the formulations: Oramix NS 10 (Decyl Glucoside), Oramix GB 10 (Decyl Glucoside (and) Cocamidopropyl Betaine), Montanov 202 (Arachidyl Alcohol (and) Behenyl Alcohol (and) Arachidyl Glucoside) were acquired from Seppic (France); Tegosoft LSE (Sucrose Cocoate), Tegosoft GC (PEG-7 Glyceryl Cocoate), Tego betain P 50 C (Cocamidopropyl Betaine), Rewoteric AMC (Sodium Cocoamphoacetate), Tego Betain 810 (Capryl/Capramidopropyl Betaine), Antil 200 (PEG-200 Hydrogenated Glyceryl Palmate (and) PEG-7 Glyceryl Cocoate), TEGOSOFT® DEC (Diethylhexyl carbonate), TEGIN® M Pellets (Glyceryl Stearate), TEGOSOFT® CT (Caprylic/capric triglyceride) were acquired from Evonik (Germany); Pharmaceutical Glycerin (≥99.5%) was acquired from LABCHEM (USA); Span 80 (Sorbitan Oleate) was acquired from Fluka (Switzerland); Tween 20 (Polysorbate 20) was acquired from Croda (UK); Karite Butter and Tocopherol were acquired from Plena Natura (Portugal); Almond Oil was acquired from Ceamed (Spain); Lanette O (Cetearyl Alcohol) was acquired from BASF (Germany).

The following ingredients were used for chemical analysis of the extracts: DPPH, FRAP Kit, Sodium carbonate solution were acquired from Sigma-Aldrich (USA); Folin-Ciocalteu reagent was acquired from MERK (Germany).

2.2 NADES Production

The first step of this project, carried out in collaboration with the NOVA School of Science and Technology, was the development of five Natural Deep Eutectic Solvent (NADES) systems. Based on the desired molar ratios and molecular weights, the appropriate amounts of each component were weighed using an analytical balance. The preparation involved precisely mixing the selected compounds in defined molar ratios. The resulting mixtures were then subjected to stirring and gentle heating, between 40 °C and 50 °C, until clear and homogeneous solutions were obtained. It is important to note that all chemicals were used as received, without further treatment or purification. A summary of the five synthesized NADES systems is presented in Table 1.

Table 1 - Overview of the NADES prepared, including their constituent components, abbreviation and respective molar ratios.

NADES	Abbreviation	Molar Ratio
Betaine:Citric Acid:Water	1	(1:2:2)
Betaine:Sucrose:Glycerol:Water	2	(2:1:3:5)
Betaine:Sorbitol:Water	3	(3:1:10)
Betaine:Glucose:Water	4	(5:2:10)
Glycerol:Fructose:Sorbitol:Water	5	(1:1:1:3)

2.3 NADES Characterization

2.3.1 NADES structure analysis

Structural evaluations were performed with a controlled stress Malvern Kinexus Rheometer (Malvern Instruments, Malvern, UK) using cone and plate geometry (truncated cone angle 4° and radius 40mm).

2.3.1.1 NADES viscosity determination

To evaluate the viscosity of the NADES, measurements were conducted across shear rate values by increasing the shear rate from 0.1 to 100 s^{-1}, at both 25ºC and 40ºC.

Additionally, the impact of temperature increase over time on the viscosity of the NADES was studied. For this analysis, the same cone-plate geometry was used, with measurements taken at temperatures between 25ºC and 40ºC.

2.3.1.2 NADES oscillatory properties determination

An oscillatory amplitude sweep and frequency testing was performed using this equipment. The amplitude sweep conditions used were shear strain between 0.01% and 100% with the frequency of 1 Hz. It was concluded that the LVER (linear-viscoelastic region) was at shear strain of 10. In the frequency testing the frequency range used was between 0.1 - 10 Hz with a shear strain of 10. The temperature sweep tests with oscillatory shear were performed with frequency of 1 Hz and shear strain of 1% at a 2.5 °C/min rate after loading the sample at 25 °C. The sample was heated from 25 °C to 40 °C while the G’ and G’’ moduli were determined.

2.3.1.3 Adhesiveness Test

The adhesive strength was measured using a plate-plate geometry, with a diameter of 20 mm and a gap of 0.2 mm. A toolkit was used with the conditions of 0.5 mm/s, 10 mm, and a gap of 10 mm. All samples were tested in 6 replicates each (n=6).

2.3.2 NADES texture analysis

To conduct a textural analysis of the NADES, a Texture Profile Analysis (TPA) test was performed using a texture analyzer (TA.XT plus, Stable Microsystems, Godalming Surrey, UK) equipped with a 5 kg load cell. A hemispheric probe was utilized, with a pre-test speed of 3 seconds, a test speed of 1 second, a distance of 3 mm, and a post-test speed of 3 seconds for NADES 1. For NADES 2 and 5, the post-test speed was extended to 2 seconds, while NADES 3 maintained a post-test speed of 1 second. All samples were tested in 6 replicates each (n=6).

2.3.3 NADES surface tension analysis

A force tensiometer (Attension Sigma 700/701, Biolin Scientific) was utilized to measure the surface tension of the NADES. Measurements were made using a platinum ring with 10 points, a start position of 0.5 mm, a return position of 0.5 mm, and a speen down of 30 mm/s. All samples were tested in 6 replicates (n=6).

2.4 NADES Incorporation in Shampoos

2.4.1 Formulation of shampoos

Firstly, basic shampoos were formulated with water, surfactant and the corresponding NADES, in order to understand how the NADES could influence the foam and the intrinsic characteristics of the shampoos. Table 2 represents the shampoo composition.

Table 2 - Quantitative formulation of shampoo systems incorporating NADES 1–5.

Ingredients (commercial name)	INCI	% (w/w)
Oramix NS 10	Decyl Glucoside	1
NADES	-	10
Water	Aqua	89

2.4.2 Foam Height analysis

To evaluate the foam height produced by each shampoo, a series of manual agitation procedures were conducted for approximately 10 seconds on the different formulations. The resulting foam height was measured in centimeters using a ruler at designated time intervals: 0 minutes, 1 minute, 2 minutes, 5 minutes, and 10 minutes after agitation.

2.4.3 Shampoo texture analysis

A TPA test was also carried out on the shampoo formulations. The method is described in section 2.3.2. The conditions used were a pre-test speed, a test speed, and a post-test speed of 1 second, and a distance of 5mm.

2.4.4 Shampoo surface tension and contact angle analysis

The surface tension of the shampoos was evaluated as described in section 2.3.3. The conditions used were a number of points of 10, a start position of 0.5 mm, a return position of 0.5 mm, and a speed down of 20 mm/s.

To assess the contact angle of each shampoo, an optical tensiometer (Attension Theta Flex, Biolin Scientific) was used, capable of dropping a drop and measuring the right and left angle of the drop. All samples were tested in 6 replicates each (n=6).

2.5 NADES Incorporation in the Final Shampoos

2.5.1 Development of a final shampoo formulation

Two of the five NADES produced were selected based on their properties, particularly their foam production and the results obtained from their characterization at the TPA test level, including measurements of surface tension and contact angle.

These selected NADES were then used to create a more complex final formulation of shampoo. Consequently, three shampoos were developed: a control shampoo, a shampoo incorporating NADES 2, and a shampoo incorporating NADES 5. The ingredients used for these formulations are outlined in Table 3. The shampoos were prepared by weighing the different ingredients and maintaining stirring and heating at 50°C until a homogeneous mixture was achieved.

Table 3 - Quantitative composition of the final shampoo formulations, including the control formulation and those incorporating NADES 2 and NADES 5.

Ingredients (commercial name)	INCI	% (w/w)
Tegosoft LSE	Sucrose Cocoate	2.9
Tegosoft GC	PEG-7 Glyceryl Cocoate	2.0
Oramix GB 10	Decyl Glucoside (and) Cocamidopropyl Betaine	12.5
Tego betain P 50 C	Cocamidopropyl Betaine	12.5
Rewoteric AMC	Sodium Cocoamphoacetate	11
Tego Betain 810	Capryl/Capramidopropyl Betaine	4
Antil 200	PEG-200 Hydrogenated Glyceryl Palmate (and) PEG-7 Glyceryl Cocoate	6
Glycerin or NADES	Glycerin	5
Abil T Quat 60	Silicone Quaternium-22	0.5
Water	Aqua	43.6

2.5.2 Final shampoo structure analysis

Evaluation of structural experiments was performed with a controlled stress Malvern Kinexus Rheometer (Malvern Instruments, Malvern, UK) using cone and plate geometry (truncated cone angle 4° and radius 40mm) with a controlled stress Kinexus Lab+ Rheometer.

2.5.2.1 Final shampoos viscosity determination

To assess the viscosity of the different shampoos, the viscosity measurement test was carried out at 25ºC, as described in section 2.3.1.1.

2.5.2.2 Final shampoos oscillatory properties determination

To evaluate the shampoos oscillatory properties, the oscillation frequency sweep tests were performed based on the method described in section 2.3.1.2.

2.5.2.3 Final shampoos thixotropic properties determination

The thixotropic behavior of the shampoos formulations was analyzed using a three-time interval sequence that analyzed the viscosity recovery behavior. This test comprises three phases: (1) constant shear rate of 0.1 s^{-1} for 30 seconds; (2) a shear rate of 50 s^{-1} for 30 seconds; and (3) with a shear rate of 0.1 s^{-1} for 5 minutes. Frequency was maintained at 1 Hz with a temperature of 25 ºC at all stages.

To complement this assay, the Ramp Up-Ramp Down thixotropic test was also performed. The measurements were carried out with a minimum shear rate of 0.1 s^{-1}, a maximum shear rate of 10 s^{-1} and a ramp time of 5 minutes, at 25ºC.

2.5.3 Final shampoos foam height analysis

The foam height of the different shampoos was examined as described in section 2.4.2.

2.5.4 Final shampoos texture analysis

The shampoos were tested for TPA using the method described in section 2.3.2. The conditions used were a pre-test speed of 1 second, a test speed of 2 seconds, a post-test speed of 1 second, and a distance of 7 mm.

In addition, the TPA test was also performed on the shampoo foam. The method is also described in section 2.3.2, and the measurement conditions were a pre-test speed, a test speed, and a post-test speed of 2 seconds, and a distance of 18 mm.

2.5.5 Final shampoos surface tension and contact angle analysis

The surface tension of the final shampoos was evaluated as described in section 2.3.3. The conditions used were a number of points of 10, a start position of 0.5 mm, a return position of 0.5 mm, and a speed down of 20 mm/s.

The contact angle of the different shampoos was also evaluated, as described in section 2.4.4.

2.5.6 *In vitro* safety analysis

2.5.6.1 Final shampoos cytotoxic assays

The cytotoxicity of the final shampoos was assessed using the MTT endpoint (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) as a metabolic assay, as described in [34]. In general, HaCaT cells (a spontaneously immortalized human keratinocyte cell line from CLS, Germany) were seeded in 96-well culture plates at a cell density of $2x10^5$ cells per well and allowed to adhere and stabilize for 24h. The culture medium used was RPMI 1640, supplemented with 10% fetal bovine serum, 100 units of penicillin G (sodium salt) and 100µg of streptomycin sulfate and 2mM L-glutamine, and incubated at 37ºC and 5% CO_2 in a humidified atmosphere. The cells were then incubated

with the shampoo samples in different concentrations (5 mg/mL, 2.5 mg/mL, 1.25 mg/mL, 0.625 mg/mL). Culture medium was used as a negative control and 1 mg/mL sodium dodecyl sulfate (SDS) as a positive control. After an exposure period of 24 hours, the medium was replaced with another containing 0.5 mg/mL MTT. After 3 hours of incubation at 37ºC and 5% CO2 in a humidified atmosphere, the formazan crystals were extracted with 100µL of DMSO. Finally, the absorbance was measured using a microplate reader (FLUOstar BMGLabtech, Ortenber, Germany) at 570 nm. Equation 1 was used to calculate the relative cell viability (%) compared to control cells,

$$\%\ Cell\ viability = \frac{Abs_{sample}}{Abs_{control}} \times 100 \quad (1)$$

where Abs sample is the absorbance of the blank (Control), and Abs sample is the absorbance of the samples.

2.5.7 Stability assay

Stability tests were carried out on the final shampoos at room temperature and 40°C ± 2°C/75% relative humidity (RH) ± 5% RH during a 3-month period. The parameters evaluated were pH and viscosity. The pH of each shampoo was measured using a pH meter (EutechTM Ion 2700 Meter, Thermo ScientificTM) at room temperature. Viscosity measurements were carried out as described in section 2.3.1.1. Stability was determined for all samples at 0, 30 and 90 days.

2.6 NADES Incorporation in O/W Non-Stable Emulsions

2.6.1 Development of O/W non-stable emulsions

Non-stable emulsions were formulated to investigate the use of NADES as emulsion stabilizers. Each emulsion incorporated a different NADES to assess their influence on emulsion stability. A total of six emulsions were developed: one control emulsion with glycerin, and five additional emulsions each containing different NADES (NADES 1, NADES 2, NADES 3, NADES 4, and NADES 5).

The initial step in the emulsions production process involved weighing the oil and water phases. Both phases were then heated in a thermostatic bath (Model 601, Nahita) at 80ºC. Emulsification occurred while the mixture was still hot, by gradually adding the oil phase to the water phase while manually stirring with a glass rod. This was followed by homogenization at approximately 10.000 rpm (ULTRA TURRAX T25, IKA) for one minute. Subsequently, manual stirring continued with the glass rod until a uniform and a total cooled mixture was obtained. Table 4 presents the composition for these emulsions.

Table 4 - Composition of the emulsion formulations, including the control emulsion and those containing NADES 1 through NADES 5.

Ingredientes (commercial name)	INCI	Function	% (w/w)
Oil phase			
Soybean oil	Glycine Soja Oil	Emollient	20.00
Span 80	Sorbitan Oleate	Emulsifier	3.75
Tween 20	Polysorbate 20	Emulsifier	1.25
Aqueous phase			
Phenoxyethanol	Phenoxyethanol	Preservative	1.00
Glycerin or NADES	Glycerin	Humectant	5.00
Water	Aqua	Solvent	69.00

2.7 NADES as Extraction Solvents

2.7.1 Extraction of phenolic compounds with NADES

NADES 2 and NADES 3 were chosen for the extraction of phenolic compounds from the lupin residues. The extraction of phenolic compounds from the lupin residue was carried out by adding the required amount of lupin residue according to the solid/liquid ratio (g/mL) and weighed using an analytical balance, for the respective volume of NADES. Three different particle sizes of residue were used: Small residue (250 μm), medium residue (1 mm), and large residue (> 1 mm).

The extraction was carried out for 1 hour using a water bath and stirring. The tubes were centrifuged for 15 minutes at 4000 rpm to ensure complete separation of the phases. The extracted samples were filtered through cotton and syringes to eliminate any remaining contaminants. The solid/liquid ratio studied was 1:10. The NADES were also evaluated at different extraction temperatures, 25ºC and 50ºC.

2.8 NADES, Extracts, and Residues Chemical Analysis

2.8.1 Antioxidant activity determination

2.8.1.1 DPPH assay

To evaluate the antioxidant potential of the NADES, the extracts, and the lupin residues, the DPPH (2,2-diphenyl-1-picrylhydrazyl) assay was carried out, as described in [34]. In general, antioxidant compounds react with DPPH by donating hydrogen, which reduces the compound. The color of the sample changes from violet to yellow as a result of this reduction. Briefly, the first step was to prepare the DPPH solution, in which 23 mg of DPPH was dissolved in 100 mL of pure ethanol and kept at 4°C for 24 hours. Next, the assay was performed in 96-well microplates, in which 396 μL of DPPH solution were combined with 4 μL (5 mg/mL) of the respective samples and incubated at room temperature for 40 minutes in the absence of light. Four concentrations (5 mg/mL, 2.5 mg/mL, 1.25 mg/mL, and 0.625 mg/mL) were tested in triplicate (n=3). A standard solution of Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) in the range of 2.4-43.2 μL/mL was used as a positive control, and DPPH only with the solvent was used as a negative control. The absorbance was measured at 517 nm using a

microplate reader (FLUOstar BMGLabtech, Ortenber, Germany). The following equation 2 was used to determine the percentage of inhibition:

$$\%\ inhibition = \frac{Abs_{DPPH} - Abs_{sample}}{Abs_{DPPH}} \times 100 \quad (2)$$

where Abs $_{DPPH}$ is the absorbance of the blank (Control), and Abs $_{sample}$ is the absorbance of the sample solution.

2.8.1.2 FRAP assay

The FRAP (Ferric reducing antioxidant power) assay was carried out using the Sigma-Aldrich FRAP assay kit [71]. The calibration curve was made by preparing a standard solution (180 µM) by mixing 20 µL of 1.8 mM Fe^{2+} Standard with 180 µL purified water. Another solution was then prepared by mixing 20 volumes of Reagent A, 1 volume of Reagent B, and 1 volume of Reagent C (20:1:1). In a 96-well plate, 200 µL of these solution was placed in the wells, along with 50 µL of each sample, at a concentration of 5 mg/mL. All samples were tested in duplicate (n=2). The absorbance was measured at 594 nm after 30 minutes of reaction, at room temperature and in the absence of light, using a microplate reader (FLUOstar BMGLabtech, Ortenber, Germany). The results were expressed as the concentration of Fe^{3+} (mM), calculated according to equation 3,

$$Concentration\ of\ Fe^{3+}(mM) = \frac{Abs\ sample - Abs\ blank}{slope} \times\ DF \quad (3)$$

Where Abs sample is the absorbance of the sample dilution, Abs blank is the absorbance of the blank, and DF is the dilution factor.

2.8.2 Total carbohydrate content (TCC) determination

The total content of carbohydrates in the samples was quantified using the phenol-sulphuric method, as described in [72]. Briefly, the calibration curve was prepared by preparing a stock solution in which 100 mg of D+ glucose monohydrate (Glu) and 100 mL of deionized water were added. Next, 500 µL of the diluted samples (7.81 mg/mL), 1.5 mL of H_2SO_4 (96%), and 300 µL of a 5% (w/w) aqueous phenol solution were added to a test tube. The mixtures were then stirred with a vortex and incubated for 5 minutes at 90ºC in a dry bath. The mixtures were then stirred again with a vortex, and after cooling to room temperature, 200 µL of each mixture was added to a well of a 96-well plate. For each sample, different concentrations were tested (7.81 mg/mL, 3.91 mg/mL, 1.95 mg/mL, 0.98 mg/mL, 0.49 mg/mL, and 0.24 mg/mL). Absorbance was measured at 490 nm using a microplate reader (FLUOstar BMG Labtech, Ortenber, Germany). The results were expressed as g Glu equivalents per 100 g dry mass of lupin (g sugar / 100 g lupin).

2.8.3 Total phenolic content (TPC) determination

The total phenolic content of the samples was determined using the Folin-Ciocalteau method [73]. In general, the first step consisted of preparing the calibration

curve, using gallic acid (GA) as the reference compound. A solution was prepared with 0.1 g GA , 10 mL ethanol, and a 100 mL volumetric flask was filled with deionized water. Next, the proteins in the samples had to be precipitated. To do this, 800 µL of the diluted samples (5 mg/mL) were mixed with 120 µL of 100% (w/w) trichloroacetic acid. The mixture was agitated and kept at -20ºC for 5 minutes, followed by storage at 4ºC for 15 minutes. After 15 minutes the mixture was centrifuged at 12.000 rpm for 15 minutes and the precipitate was discarded. After the proteins had been precipitated, 1.58 mL of deionized water and 100 µL of Folin-Ciocalteu reagent were added to 20 µL of the samples. The mixture was stirred using a vortex and incubated at room temperature for 5 minutes. 300 µL of previously prepared sodium carbonate solution was added and incubated for 30 minutes at 40ºC. After 30 minutes, 200 µL of each mixture was added to a well of a 96-well plate, and absorbance was measured at 750 nm using a microplate reader (FLUOstar BMGLabtech, Ortenber, Germany). Four concentrations were tested (5 mg/mL, 2.5 mg/mL, 1.25 mg/mL, and 0.625 mg/mL) and all samples were tested in triplicate (n=3). TPC data were expressed as g of GA per 100 g of lupin dry mass (g GA/100 g lupin).

2.9 NADES, Extracts and Residues *in vitro* Analysis

2.9.1 Cytotoxic assays

The cytotoxicity analysis of all the samples was determined as described in section 2.5.6.1.

2.9.2 Antioxidant capacity

The intracellular production of reactive oxygen species (ROS) in the cells was assessed with a fluorometric technique using the probe 2,7' dichlorodihydrofluorescein diacetate (H_2-DCFDA), as described in the work [34]. Subconfluent HaCaT cells seeded in 96-well plates were incubated for 30 minutes with 20 µM of H2-DCFDA at 37ºC. The culture medium was then removed and 90 µl of fresh medium was added to the cells exposed to the previously selected concentrations of the samples (5 mg/mL, 2.5 mg/mL, 1.25 mg/mL, and 0.625 mg/mL). Ascorbic acid was used as a positive control and culture medium was used as a negative control. All samples were tested in triplicate (n=3). The cells were incubated for 1.5h at 37ºC and 5% CO2 in a humidified atmosphere. Oxidative stress was induced in the cells using a solution of H_20_2 (500 µM) or by exposure to UV-B light (emission wavelength 321 nm) for 15 minutes. After exposure, ROS levels were determined by fluorescence (excitation wavelength 485 nm, emission wavelength 520 nm) using a fluorescence microplate reader (FLUOstar BMGLabtech, Ortenber, Germany). Data are reported as the percentage of ROS reduction, determined using the following equation:

$$\%\ ROS\ reduction = 100 - \frac{Florescence\ of\ unexposed\ control}{Florescence\ of\ exposed\ cells} \times 100 \qquad (4)$$

2.9.3 Human Neutrophil Elastase (HNE) Enzymatic Inhibition Assay

Human neutrophil elastase (HNE) fluorometric inhibition assays were carried out using the [34] procedure. Essentially, the assays were carried out in 200 µL of assay buffer (0.1 M HEPES pH 7.5 at 25 ºC), containing 20 µL of 0.17 µM HNE in assay buffer, 155 µL of assay buffer and 5 µL of each concentration of the different extract samples (1.25 mg/mL, and 0.625 mg/mL). To start the reaction, 20 µL of 200 µM of fluorogenic substrate (MeO-Suc-Ala-Ala-Pro-Val-AMC was added and the activity was monitored for 30 minutes at 25ºC using a microplate reader (FLUOstar BMG Labtech, Ortenber, Germany) with an excitation wavelength of 380 nm and an emission wavelength of 460 nm. The controls were as follows: (1) enzyme, (2) substrate, (3) enzyme with DMSO and (4) positive control with Sivelestat sodium salt hydrate. The assays were carried out in triplicate and presented as the log of the inhibitor concentrations versus the percentage of activity.

2.10 NADES, Extract and Residue Stability Assays

Based on the results obtained from the previous tests, NADES 2, extract small, and residue small (250 µm) were chosen for the stability tests. NADES 2 and extract small were prepared according to the methods described in sections 2.1 and 2.7.1. For the stability assay, the samples were tested under four different conditions: (1)- Sample stored at room temperature in the absence of light; (2)- Sample stored at room temperature with light; (3)- Sample stored in a heating oven at 40ºC, (4)- Sample stored in the fridge at 4ºC.

The parameters evaluated in this test were the color determination of the samples, using the CIELAB parameters L*, a* and b* which were determined with the Digital Color Meter program. In addition, other parameters were analyzed such as the DPPH assay, the FRAP assay and the determination of TPC, according to the methods described in sections 2.8.1.1, 2.8.1.2, and 2.8.3, respectively. Stability was determined for all samples at 0, 14, 30, 60 and 90 days.

2.11 NADES Incorporation in the Final O/W Emulsions

2.11.1 Development of a final O/W emulsion

In order to understand how the extract performs in a formulation, three different emulsions were developed: emulsion control, emulsion with the small extract and emulsion with NADES 2 (since the extract was obtained from an extraction with the NADES 2). In general, the emulsions were prepared as described in section 2.6.1. The ingredients used in these formulations are shown in Table 5.

Table 5 - Composition of the emulsion formulations, including the control emulsion, the emulsion containing the crude plant extract, and the emulsion incorporating NADES 2.

Ingredients (commercial name)	INCI	Function	% (w/w)
Oil phase			
Montanov 202	Arachidyl Alcohol (and) Behenyl Alcohol (and) Arachidyl Glucoside	Emulsifier	4.0
TEGOSOFT® DEC	Diethylhexyl carbonate	Emollient	2.5
TEGIN® M Pellets	Glyceryl Stearate	Consistency enhancer and Co-emulsifier	0.5
Lanette O	Cetearyl Alcohol	Emollient and Co-emulsifier	2.5
Almond oil	-	Emollient	2.0
Shea butter	-	Emollient	2.0
TEGOSOFT® CT	Caprylic/capric triglyceride	Emollient	5.0
Tocopherol	Tocopherol	Antioxidant	0.5
Aqueous phase			
Phenoxyethanol	Phenoxyethanol	Preservative	1.0
Glycerin or Extract or NADES	Glycerin	Humectant	5.0
Water	Aqua	Solvent	75.0

2.11.2 Final emulsions structure analysis

Structural experiments evaluation were performed with a controlled stress Malvern Kinexus Rheometer (Malvern Instruments, Malvern, UK) using cone and plate geometry (truncated cone angle 4° and radius 40mm) with a controlled stress Kinexus Lab+ Rheometer.

2.11.2.1 Final emulsions viscosity determination

The viscosity determination test of the different emulsions was performed at 25ºC, as described in section 2.3.1.1.

2.11.2.2 Final emulsions oscillatory properties determination

To assess the oscillatory properties of the emulsions, oscillation frequency sweep tests were carried out using the method described in section 2.3.1.2.

2.11.2.3 Final emulsions thixotropic properties determination

In order to understand the thixotropic behavior of the emulsions, the 3-step shear test was carried out, as described in section 2.5.2.3.

2.11.3 Final emulsions texture analysis

A TPA test was carried out to understand the textural properties of the different emulsions. This method can be found in section 2.3.2. The conditions used were a pre-test speed, a test speed, and a post-test speed of 2 seconds, and a distance of 7 mm.

2.11.4 Droplet size analysis

The droplet size distribution of the final emulsions was measured by light dispersion using the Malvern Mastersizer 2000 equipment (Malvern Instruments, Worcestershire, UK) coupled to a Hydro S accessory. Briefly, about 0.1 g of each emulsion was diluted in water to obtain the required turbidity (laser obscuration between 10-18%). All samples were tested 6 times (n=6).

2.11.5 Color determination

In order to determine the color of the emulsions, a Digital Color Meter program was used, as mentioned in section 2.10.

2.11.6 *In vitro* safety analysis

2.11.6.1 Final emulsions cytotoxic assays

To ensure the safety of the final product, the MTT endpoint test was carried out to determine the cytotoxicity of the emulsions, as described in section 2.5.6.1.

2.11.7 Stability assays

Stability assays were conducted on the final emulsions at both room temperature and 40°C ± 2°C/75%RH ± 5%RH over a duration of 3 months. The parameters evaluated included pH and viscosity. The pH of each shampoo was measured using a pH meter (Eutech™ Ion 2700 Meter, Thermo Scientific™) at room temperature. Viscosity measurements were carried out as described in section 2.3.1.1. The stability of all samples was assessed at 0, 30, and 90 days.

CHAPTER 3: Results and Discussion

3.1 NADES Selection and Development

The selection of NADES was based on the use of natural raw materials, recognized for their biocompatibility and applicability in cosmetic formulations. Simple components such as betaine, glycerol, and sugars were chosen due to their biocompatibility and safety. In addition, their low cost, high availability, and biodegradability make them suitable for large-scale use in the cosmetic industry. These compounds allowed the formation of stable deep eutectic solvents through hydrogen bond interactions, in which some components were predominantly hydrogen bond donors and others were hydrogen bond acceptors [43].

In general, all NADES resulted in a translucent and transparent mixture, as expected, except for NADES 4, which always had a turbid color, suggesting lower stability compared to the other NADES. In terms of handling, all NADES proved easy to work with, except for NADES 1, whose higher viscosity made it difficult to handle.

3.2 NADES Characterization

3.2.1 Rheological Analysis

To analyze the rheological characteristics of the various NADES produced, their viscosity was evaluated to determine which formulations possess the most suitable properties for application in shampoos and emulsions. A systematic study was conducted to explore how viscosity varies with different temperature conditions, namely at 25 °C (room temperature) and 40 °C, chosen to simulate typical conditions during product storage or application on the skin or scalp. Temperatures above 50ºC were not considered to avoid possible degradation of the NADES components. Furthermore, tests were performed to assess the oscillatory performance of the NADES. The results are presented in Figures 6 to 10.

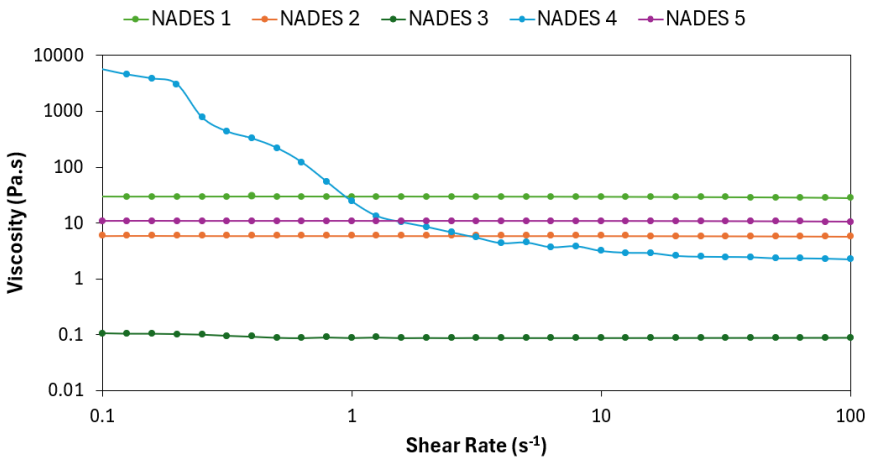


Figure 6 - Viscosity as a function of shear rate for the tested NADES at 25 °C.

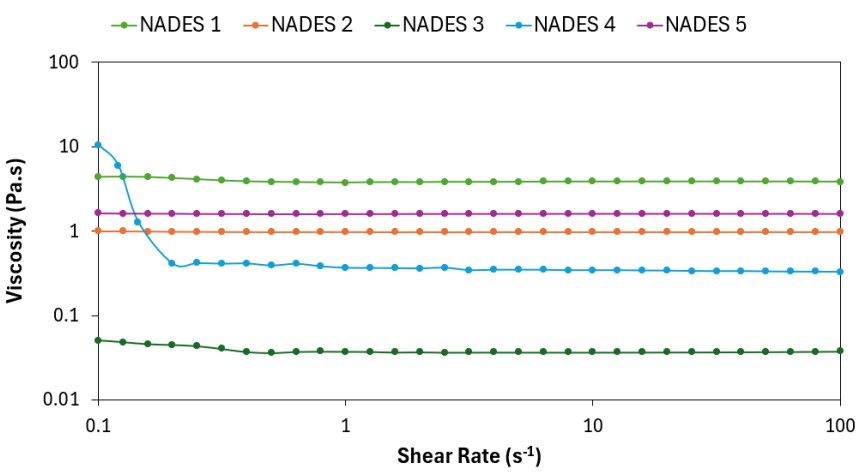


Figure 7 - Viscosity as a function of shear rate for the tested NADES at 40 °C.

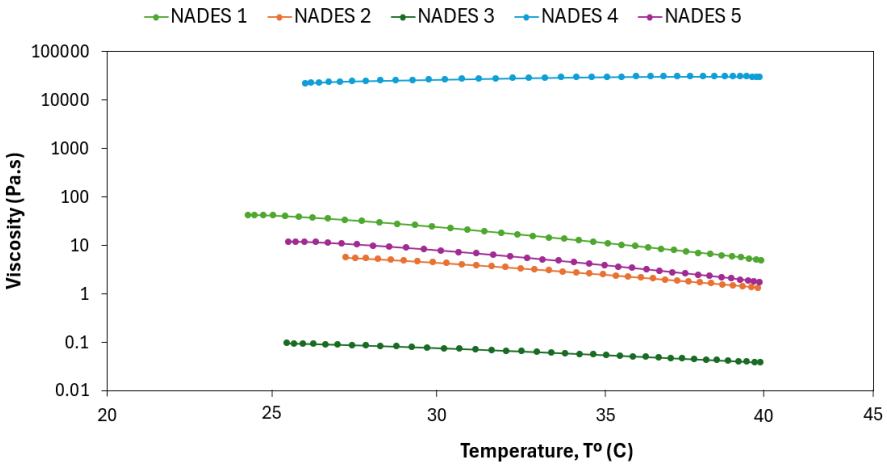


Figure 8 - Viscosity profiles of the tested NADES under a temperature ramp from 25 to 40 °C.

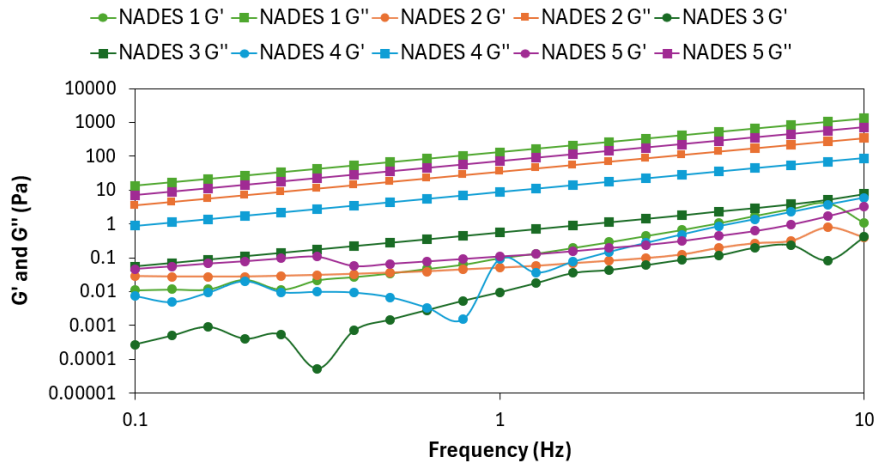


Figure 9 - Storage and loss moduli of the tested NADES as a function of oscillation frequency, at 25 °C.

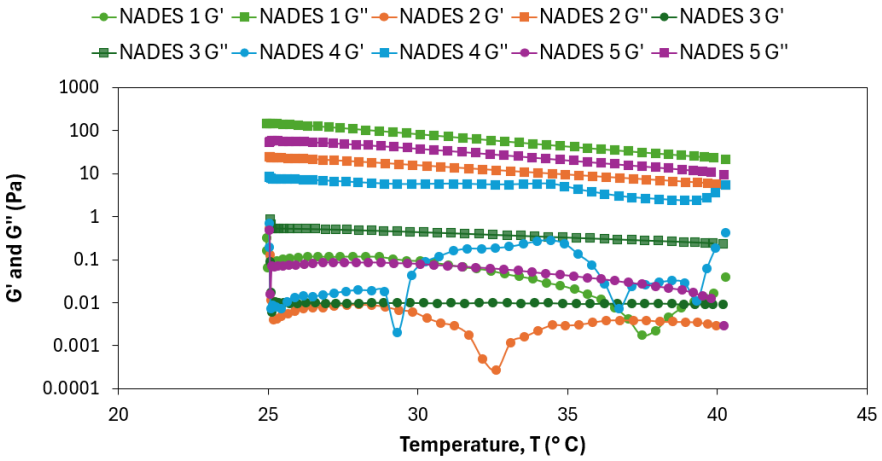


Figure 10 - Storage and loss moduli of the tested NADES as function of oscillatory temperature ramp from 25 to 40 °C.

As shown in Table 6, the adhesiveness of NADES was also determined. This test is crucial as it quantifies the adhesion strength of a formulation to the skin or other surfaces, offering relevant data on spreadability, texture, fixation, and application behavior [74].

Table 6 - Comparison of adhesiveness among the tested NADES (N.s) (n = 6, Mean ± SD).

	NADES 1	NADES 2	NADES 3	NADES 4	NADES 5
Adhesiveness - Area under force time curve (N.s)	0.45 ± 0.1	0.17 ± 0.02	0.13 ± 0.02	0.16 ± 0.02	0.23 ± 0.04

The analyzed NADES exhibits distinct rheological profiles that significantly influence their suitability for various formulations, including emulsions and shampoos. Notably, in terms of viscosity, as illustrated in Figures 6 and 7, NADES 1, 2, 3, and 5 demonstrate Newtonian behavior, characterized by a constant viscosity regardless of the shear rate, both at 25°C and 40°C. This rheological stability renders these NADES ideal for applications that require consistent performance in the presence of variable forces or temperature conditions. Conversely, NADES 4 displays pseudoplastic non-Newtonian behavior, evidenced by decreasing viscosity with increasing shear rates and notable thermal instability, which makes it less suitable for formulations that necessitate a uniform texture. As expected, all NADES exhibited a reduction in viscosity with elevated temperatures, attributable to diminished molecular interactions, as evidenced in Figure 8 [34].

Regarding oscillatory behavior, as shown in Figure 9, NADES 3 exhibited the lowest values of G′ and G″, indicating a fluid character with low mechanical resistance and predominantly liquid behavior, thus making it ideal for formulations that prioritize fluidity. In contrast, NADES 1, 2, and 5 showed higher and more balanced G′ and G″ values, signifying a more cohesive and stable structural integrity, which is desirable for products requiring mechanical resistance, such as creams. NADES 4, on the other hand,

maintained G" greater than G′ across all tested frequencies, reinforcing its characterization as a fluid and unstructured system.

Concerning viscoelastic thermal response, as illustrated in Figure 10, NADES 1 and 5 consistently maintained G′ values higher than G" even with increased temperature, indicating favorable elastic stability. In comparison, NADES 2 and 3 exhibited liquid and fluid behavior with minimal variation in rheological parameters. Again, NADES 4 demonstrated instability, characterized by marked oscillations in G", suggesting greater sensitivity to thermal fluctuations.

In terms of adhesiveness, as summarized in Table 6, NADES 1 and 5 demonstrated superior adhesion to surfaces, rendering them particularly suitable for creams that benefit from enhanced adherence on the skin. Conversely, NADES 2, 3, and 4 exhibited lower adhesiveness, thereby appearing more favorable for shampoos, where reduced adhesive interaction facilitates easier rinsing.

Overall, NADES 1 and 5 are recommended for emulsion formulations due to their moderate and stable viscosity, superior elasticity, structural cohesion, and enhanced adhesiveness. For shampoo formulations, NADES 2 and 3 are preferred options due to their low viscosity, predominantly liquid behavior, and reduced adhesiveness, which facilitate a smooth application that can be easily rinsed off.

3.2.2 Texturometer Analysis

In order to understand the textural profile of the different NADES, the TPA test was carried out to obtain the results of hardness, adhesiveness, springiness, cohesiveness, and resilience, as described in Table 7. This test allows to evaluate the sensory properties of a product, enabling prediction of consumer perception during product application.

Table 7 - TPA parameters obtained for the tested NADES, including hardness, adhesiveness, springiness, cohesiveness, and resilience (n = 6, Mean ± SD).

	Hardness (g)	**Adhesiveness (g.sec)**	**Springiness**	**Cohesiveness**	**Resilience**
NADES 1	-3.604 ± 0.926	-59.607 ± 8.85	0.992 ± 0.000	0.953 ± 0.050	0.027 ± 0.001
NADES 2	-1.267 ± 0.103	-10.834 ± 1.987	0.995 ± 0.002	0.852 ± 0.024	0.028 ± 0.009
NADES 3	-0.352 ± 0.154	-0.496 ± 0.587	5.066 ± 1.079 ±	1.267 ± 0.238	0.437 ± 0.057
NADES 5	-1.528 ± 0.034	-19.699 ± 2.083	0.950 ± 0.026	0.829 ± 0.023	0.017 ± 0.001

Overall, the different NADES exhibited distinct textural profiles that directly influenced their suitability for cosmetic applications. NADES 1 demonstrated the highest levels of adhesiveness, characterized by notable hardness and adhesiveness. This makes it particularly well suited for emulsions that require a thicker consistency. In contrast, NADES 3 was the softest and most elastic, displaying greater cohesiveness, which makes it ideal for shampoos due to its fluidity and ease of application. Meanwhile, NADES 2 and 5 showed intermediate characteristics, with NADES 5 resembling an emulsion more closely, while NADES 2 is better suited for lighter formulations, such as shampoos.

3.2.3 Tensiometer Analysis

The surface tension of the produced NADES was assessed using a force tensiometer. NADES 4 was excluded from the evaluation due to its instability, as shown in the rheological evaluation. Furthermore, NADES 1 was also ruled out, as it was determined that it cannot be reliably produced at lower room temperatures, directly impacting its reproducibility. As a result, NADES 1 was not considered a viable candidate for further investigation. Table 8 presents the surface tension values obtained for NADES 2, NADES 3, and NADES 5.

Table 8 - Surface tension measurements of NADES 2, NADES 3, and NADES 5 (n = 6, Mean ± SD).

	NADES 2	NADES 3	NADES 5
Surface Tension (N/m)	68.7 ± 0.0	76.2 ± 0.1	68.3 ± 0.2

Surface tension is the force that maintains molecules cohesion on a liquid's surface. A higher surface tension indicates that the liquid is more resistant to deformation and spreading, making it difficult to spread evenly on the skin [75]. Thus, when observing Table 8, it can be concluded that NADES 3 presented the highest surface tension, which favors internal cohesion and a more stable structure, making it more suitable for emulsions. NADES 2 and 5, with lower surface tension, facilitate spreading and application, and have desirable characteristics in shampoos and more fluid formulas.

3.3 NADES Incorporation in Shampoos

3.3.1 Foam Height

One of the most critical aspects of shampoo is its foam formation, as already discussed in the introduction section. This approach facilitates a comparative analysis of the foam stability and performance characteristics of the different shampoos. Taking this into account, the foam height test was carried out over time to understand which of the "basic" formulation shampoos containing the different NADES present the highest foam production, as shown in Figure 11.

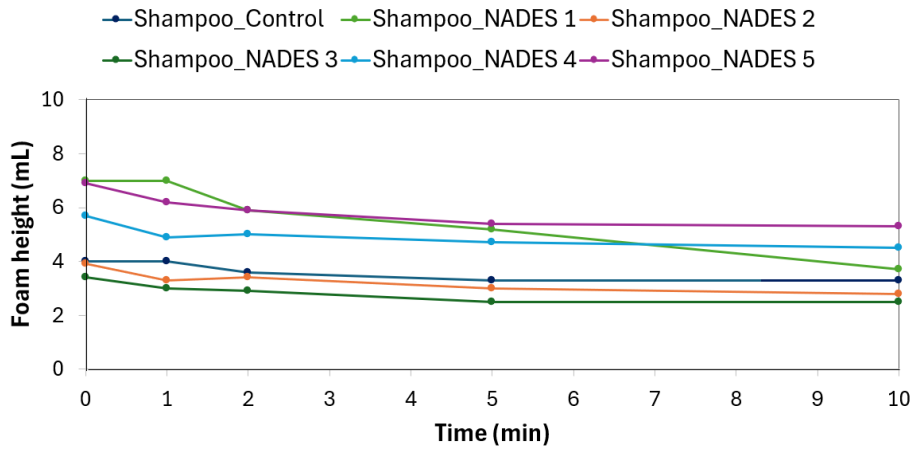


Figure 11 - Foam height of shampoo formulations containing 10% (w/w) of NADES (NADES 1 to 5), compared to a control formulation without NADES (Control) over a 10 minute period.

In general, according to Figure 11, the shampoo with NADES 1 and NADES 5 showed the highest foaming capacity. Therefore, these formulations may be considered more promising for further development. It is important to note that commercial shampoos typically present foam height between 6-9 mL after agitation, which places the performance of NADES 1 and NADES 5 within the expected range for acceptable formulations [76].

3.3.2 Texturometer Analysis

The TPA test was once again carried out in order to understand the textural characteristics of each of the shampoos. The results are shown in Table 9.

Table 9 - TPA parameters obtained on the shampoo formulations containing 10% (w/w) of NADES (NADES 1 to 5), compared to a control formulation without NADES (Control), including hardness, adhesiveness, springiness, chewiness, and resilience (n = 6, Mean ± SD).

	Hardness (g)	Adhesiveness (g.sec)	Springiness	Chewiness	Resilience
Shampoo Control	-0.058 ± 0.233	-1.518 ± 0.648	0.000 ± 0.000	0.000 ± 0.000	0.148 ± 0.031
Shampoo NADES1	-0.040 ± 0.250	-2.274 ± 0.957	0.013 ± 0.020	0.000 ± 0.000	0.045 ± 0.256
Shampoo NADES2	-0.123 ± 0.211	-2.001 ± 1,480	0.042 ± 0.045	-0.009 ± 0.048	0.005 ± 0.309
Shampoo NADES3	-0.076 ± 0.235	-1.693 ± 1.042	0.004 ± 0.009	0.000 ± 0.000	0.158 ± 0.044
Shampoo NADES5	-0.036 ± 0.141	-1.586 ± 0.840	0.000 ± 0.000	0.000 ± 0.000	0.145 ± 0.036

Among the formulations tested, the shampoo with NADES 2 stands out with the best overall performance, showing greater hardness, adhesiveness, and springiness compared to the control, which translates into a more structured texture that is pleasant to apply. The Shampoo with NADES 3 also showed improvements, with moderate adhesiveness, making it easier to use and rinse off. The shampoos with NADES 1 and 5 showed and higher adhesiveness, which can make the rinsing procedure more challenging. In conclusion, NADES 2 is the most promising option, combining structure with good sensory performance.

3.3.3 Tensiometer Analysis

The shampoos were also submitted to a surface tension test using a force tensiometer, while the contact angle of each shampoo was determined using an optical tensiometer, as described in Table 10 and Table 11, respectively. These tests are essential for evaluating the wettability and spreading properties of shampoo on the scalp or hair. Surface tension affects the foaming and cleaning capabilities of shampoo, while the contact angle provides insight into the product's affinity for various surfaces. These factors can influence consumer sensory perception and the overall effectiveness of the application [77,78].

Table 10 - Surface tension values of shampoo formulations containing 10% (w/w) of selected NADES (NADES 2, 3, and 5), compared to the control formulation without NADES (n = 6, Mean ± SD).

	Control	Shampoo NADES 2	Shampoo NADES 3	Shampoo NADES 5
Surface Tension (N/m)	26.2 ± 0.0	28.1 ± 0.0	29.3 ± 0.0	27.6 ± 0.0

Table 11 - Contact angle values of shampoo formulations containing 10% (w/w) of selected NADES (NADES 2, 3, and 5), compared to the control formulation without NADES (n = 6, Mean ± SD).

	Control	Shampoo NADES 2	Shampoo NADES 3	Shampoo NADES 5
Contact Angle (º)	29.85 ± 0.06	29.10 ± 0.43	22.81 ± 0.28	21.58 ± 0.55

According to Table 10, the control had the lowest value in terms of surface tension, indicating the best spreading and wettability. The shampoo with NADES 2 had a similar performance, followed by the shampoo with NADES 5, with slightly higher values. The shampoo with NADES 3, with higher surface tension, showed the lowest spreading capacity.

Analyzing the contact angle, when observing Table 11, the shampoo with NADES 5 stood out with the lowest value, indicating greater wettability and better spreadability, followed by the shampoo with NADES 3. The shampoo with NADES 2 presented an angle similar to the control, reflecting similar behavior.

Overall, shampoos with NADES 3 and 5 are the most promising in terms of wettability and spreadability, being more suitable for formulations that require good application on the skin or hair.

3.4 NADES Incorporation in the Final Shampoos

Upon characterizing the “basic” shampoos, composed mainly of water, surfactant, and the different NADES, the most effective NADES were selected for individual incorporation into a more complex shampoo formulation containing all the ingredients necessary to formulate an effective shampoo. Generally, NADES 2 demonstrated the best outcomes in terms of contact angle and TPA test, while NADES 5 exhibited the highest foam formation. Consequently, three final formulations were developed: a control shampoo, a shampoo incorporating NADES 2, and a shampoo incorporating NADES 5.

3.4.1 Rheological Analysis

In order to assess the rheological behavior of the various shampoo formulations, oscillatory, viscosity, and thixotropic measurements were conducted. Thixotropy tests are important as they assess how a product's viscosity varies with the application of force and how it recovers at rest. This property is essential to ensure that the product is easy to spread on the skin but returns to its stable consistency after application [79]. The results are shown in Figures 12 to 15.

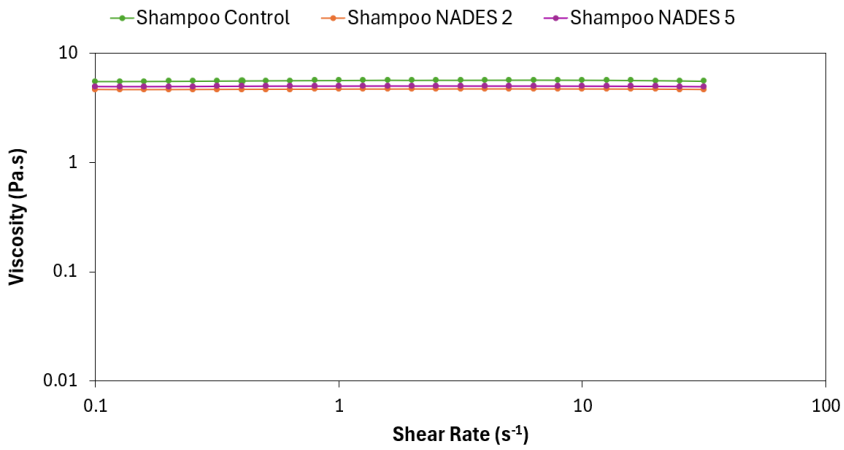


Figure 12 - Viscosity as a function of shear rate of shampoo formulations containing 10% (w/w) of selected NADES (NADES 2 and 5), compared to the control formulation without NADES at 25 °C.

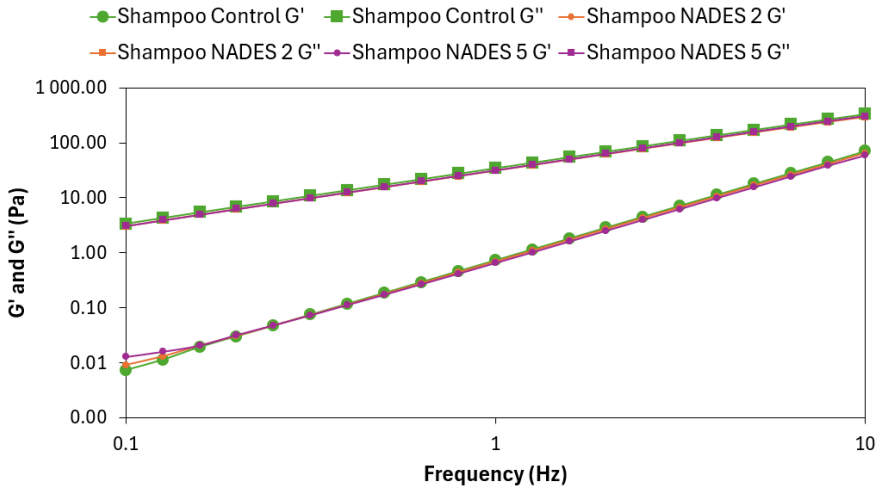


Figure 13 - Storage and loss moduli of the shampoo formulations containing 10% (w/w) of selected NADES (NADES 2 and 5), compared to the control formulation without NADES, as a function of oscillation frequency at 25 °C.

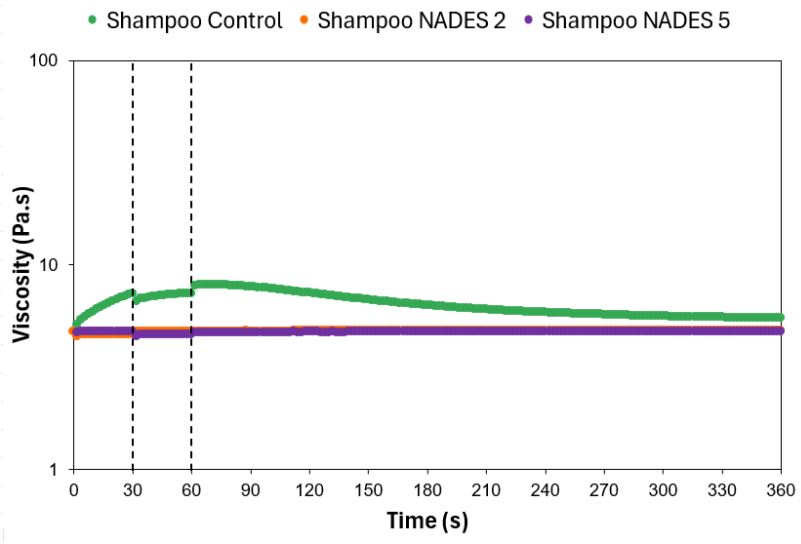


Figure 14 - Three-step rheological test of shampoo formulations containing 10% (w/w) of selected NADES (NADES 2 and 5), compared to the control formulation without NADES, performed to assess structural recovery and thixotropic behavior at 25 °C.

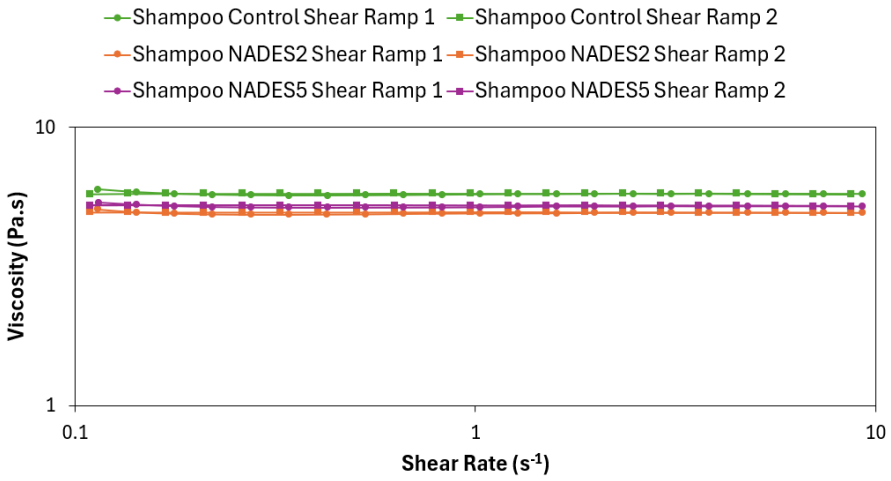


Figure 15 - Thixotropy test of shampoo formulations containing 10% (w/w) of selected NADES (NADES 2 and 5), compared to the control formulation without NADES, performed through a shear rate ramp-up/ramp-down protocol, at 25 °C.

The rheological studies enabled a detailed characterization of the behavior of the shampoos containing NADES in comparison with the control formulation, revealing clear differences in viscosity, structural integrity, and formulation stability. In the viscosity assessment, as shown in Figure 12, it was observed that all shampoos maintain a constant viscosity across the different shear rates applied, which indicates typical Newtonian behavior. The control shampoo presented the highest viscosity, while the shampoos with NADES 2 and NADES 5 were lighter and more fluid, with NADES 2 being the most fluid of all. This characteristic suggests that formulations with NADES may be easier to apply and spread.

Through oscillatory analysis, which evaluates the elastic and viscous modules, from Figure 13, it was found that all shampoos exhibit viscoelastic behavior, with an increase in G' and G" with frequency. However, the control shampoo recorded slightly higher values, reflecting a firmer internal structure. In contrast, shampoos with NADES proved to be softer, more flexible, and less structural rigidity.

In Figure 14, the three-step test was performed to evaluate the thixotropic behavior of the shampoos. This type of test measures viscosity in three phases: resting (phase 1), application with high shear rate (phase 2), and recovery (phase 3) [80]. The control shampoo showed thixotropic behavior, with a decrease in viscosity during phase 2 and partial recovery in phase 3, evidencing loss and restructuring of the internal matrix. On the other hand, shampoos with NADES 2 and 5 maintained their viscosity almost unchanged throughout the three phases, reinforcing their non-thixotropic behavior and greater resistance to structural deformation.

Finaly, the shear ramp test (up and down), observed in Figure 15, reinforced the stability of formulations with NADES. In this case, the curves of all shampoos remain practically overlapping. This reveals a non-thixotropic behavior, where the product's structure does not change with movement, and the viscosity remains stable throughout the cycle. In contrast, thixotropic behavior would be characterized by a decrease in viscosity with movement, followed by gradual recovery at rest, which is common in products that become more fluid when shaken or applied, such as some gels or emulsions [81]. The control shampoo maintains the highest viscosity, while shampoos with NADES, especially NADES 2, have a more fluid texture. Overall, the results indicate that all formulations are rheologically stable, with uniform consistency, without structural changes during use.

Collectively, the results indicate that shampoos with NADES, especially NADES 2, offer greater rheological stability, a more fluid texture, and predictable behavior during application, which are desirable characteristics in formulations such as shampoos.

3.4.2 Texturometer Analysis

TPA was also performed on the control shampoo and on the formulations containing NADES 2 and NADES 5. The corresponding results are presented in Table 12.

Table 12 - TPA parameters obtained on the shampoo formulations containing 10% (w/w) of NADES (NADES 2 and 5), compared to a control formulation without NADES (Control), including hardness, adhesiveness, springiness, chewiness, and resilience (n = 6, Mean ± SD).

	Hardness (g)	Adhesiveness (g.sec)	Springiness	Chewiness	Resilience
Shampoo Control	-0.673 ± 0.254	-51.105 ± 5.367	0.583 ± 0.054	-0.344 ± 0.182	0.008 ± 0.002
Shampoo NADES2	-0.182 ± 0.297	-24.696 ± 1.899	0.479 ± 0.070	-0.092 ± 0.143	0.013 ± 0.003
Shampoo NADES5	-0.271 ± 0.223	-21.074 ± 1.499	0.461 ± 0.041	-0.114 ± 0.103	0.014 ± 0.005

According to Table 12, the control shampoo exhibits higher values for adhesiveness and chewiness, indicating a stickier and more resistant formulation, with lower fluidity. In contrast, shampoos incorporating NADES 2 and NADES 5 demonstrate lower values for these parameters, making them easier to apply and rinse out. Notably, NADES 5 stood out with elevated levels of springiness, and resilience, suggesting a more elastic and stable structure during use. Meanwhile, NADES 2 presents intermediate performance, offering good levels of elasticity.

Additionally, a TPA test was conducted on the foam each shampoo generated to elucidate its textural properties. The examined characteristics included hardness, adhesiveness, springiness, and cohesiveness, as illustrated in Table 13.

Table 13 - TPA parameters obtained on the shampoo foam formulations containing 10% (w/w) of NADES (NADES 2 and 5), compared to a control formulation without NADES (Control), including hardness, adhesiveness, springiness and cohesiveness (n = 6, Mean ± SD).

	Hardness (g)	Adhesiveness (g.sec)	Springiness	Cohesiveness
Foam Shampoo Control	0.130 ± 0.113	-2.499 ± 0.510	0.105 ± 0.079	0.310 ± 0.219
Foam Shampoo NADES2	-0.032 ± 0.162	-2.465 ± 0.515	0.098 ± 0.089	0.186 ± 0.255
Foam Shampoo NADES5	-0.006 ± 0.191	-2.888 ± 0.435	0.126 ± 0.057	0.453 ± 0.249

The TPA of the shampoo foam, as presented in Table 13, reveals that while all shampoos exhibit similar characteristics, there are notable differences among them. The shampoo with NADES 5 demonstrates the most cohesive and elastic foam, indicating enhanced stability. In comparison, the shampoo with NADES 2 performs similarly to the control, maintaining a good structure. Although the control foam is balanced, it lacks some cohesiveness. In conclusion, shampoo with NADES 5 is distinguished by its superior foam structure, making it ideal for applications requiring greater stability.

3.4.3 Tensiometer Analysis

All the shampoos final formulations were also submitted to the surface tension and contact angle tests, as shown in Table 14 and Table 15, respectively.

Table 14 - Surface tension values of final shampoo formulations containing 10% (w/w) of selected NADES (NADES 2 and 5), compared to the control formulation without NADES (n = 6, Mean ± SD).

	Control	Shampoo NADES 2	Shampoo NADES 5
Surface Tension (N/m)	26.6 ± 0.8	26.9 ± 0.5	27.2 ± 0.3

Table 15 - Contact angle results of final shampoo formulations containing 10% (w/w) of selected NADES (NADES 2 and 5), compared to the control formulation without NADES (n = 6, Mean ± SD).

	Control	Shampoo NADES 2	Shampoo NADES 5
Contact Angle (º)	52.69 ± 0.15	50.24 ± 0.25	43.88 ± 0.08

The results shown in Table 14 indicate that the surface tension of shampoos containing NADES 2 and NADES 5 are not significantly different from that of the control formulation. Notably, the shampoo with NADES 5 presents a slight increase in surface tension, suggesting only minor differences in spreadability among the formulations.

Additionally, contact angle measurements presented in Table 15 show that the shampoo containing NADES 5 has the lowest contact angle, indicating enhanced wettability and improved surface spreading. In contrast, the control shampoo exhibits the highest contact angle, reflecting reduced interaction with surfaces.

Overall, these findings suggest that the formulation with NADES 5 provides superior wettability compared to the other tested shampoos.

3.4.4 Foam Height

The foam height test was evaluated on the different shampoos to determine which shampoo presents the highest foaming capacity, as shown in Figure 16.

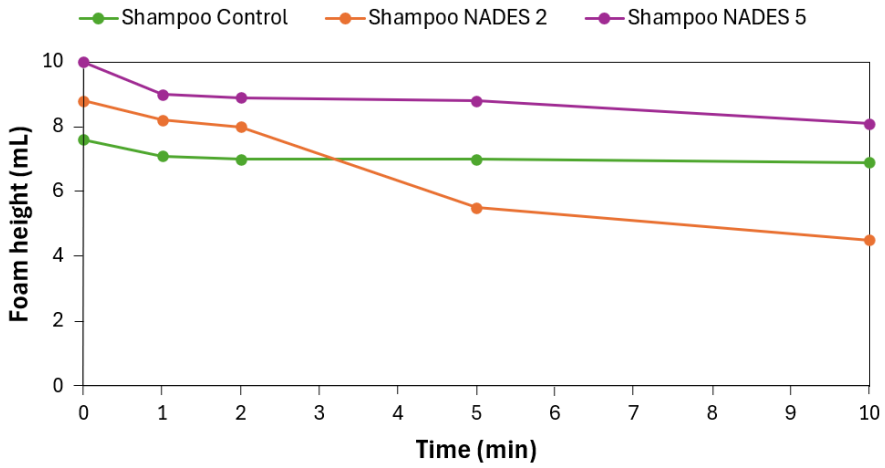


Figure 16 - Foam height of shampoo formulations containing 10% (w/w) of NADES (NADES 2 and 5), compared to a control formulation without NADES (Control) over a 10-minute period.

As discussed in section 3.3.1, the shampoo containing NADES 5 exhibited the highest foaming capacity. This characteristic may offer a marketing advantage and contribute positively to user satisfaction, given that consumers often associate abundant and rich foam with increased cleansing efficacy and overall product performance.

3.4.5 *In vitro* safety analysis

3.4.5.1 Final Shampoo cytotoxic analysis

To ensure the safety of the final shampoos, an MTT cytotoxicity assay was performed using HaCaT cells. This type of assay is essential as it assesses the potential toxic effects of a product on human skin cells. HaCaT cells are commonly used in these skin compatibility assays because they are a well-established *in vitro* model for human keratinocytes [82]. The results are shown in Figure 17.

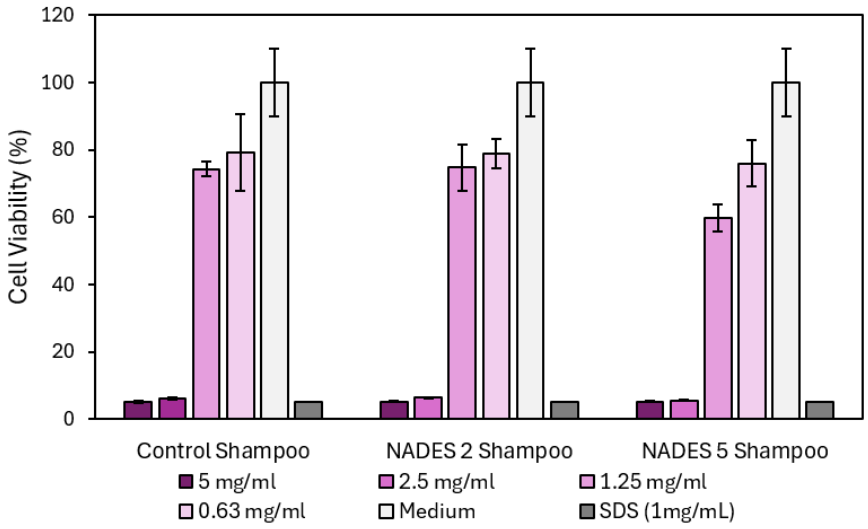


Figure 17 - Cell viability assays of HaCaT cells exposed to different concentrations of shampoo formulations containing 10% (w/w) of NADES (NADES 2 and 5), compared to a control formulation without NADES (Control)(Control)using MTT method.Medium was used as positive control and SDS as a negative control (n = 8, Mean ± SD).

Figure 17 presents the results from *in vitro* cytotoxicity assays performed on HaCaT cells, which assessed cell viability after exposure to various shampoo formulations. As expected, a decrease in cell viability was noted at higher concentrations (5 mg/mL and 2.5 mg/mL), attributable to the harmful effects of surfactants on cellular integrity [83].

In contrast, at lower concentrations (1.25 mg/mL and 0.63 mg/mL), all shampoo formulations exhibited high levels of cell viability, with the shampoo containing NADES 2 performing similar to the control shampoo. The shampoo with NADES 5 demonstrated slightly lower cell viability values.

In conclusion, the results indicate that shampoos containing NADES, particularly NADES 2, do not significantly elevate cytotoxicity in comparison to the control. This finding supports a favorable safety profile at concentrations representative of typical usage.

3.4.6 Stability Tests

To evaluate the stability of the shampoos over an extended period, comprehensive stability tests were conducted. pH and viscosity measurements were systematically recorded over a three-month timeframe. The findings are detailed in Table 16 and in Figures 18 to 20.

Table 16 - pH values of shampoo formulations (Control, NADES 2, and NADES 5) measured over a 90-day stability study at 25 °C and 40 °C. Measurements were taken at 0, 30, and 90 days after storage.

	0 days		**30 days**		**90 days**	
pH	**25ºC**	**40ºC**	**25ºC**	**40ºC**	**25ºC**	**40ºC**
Shampoo Control	5.74	5.74	5.71	5.13	6.55	7.30
Shampoo NADES 2	4.51	4.51	5.50	4.41	7.04	6.87
Shampoo NADES 5	5.64	5.64	5.62	5.24	6.62	6.59

The pH level is a critical parameter in hair care formulations, as it significantly influences both skin tolerance and the stability of the product. For a shampoo to be deemed safe and gentle, its pH should ideally range between 4.5 and 5.5, which is consistent with the physiological pH of the scalp [84].

Initially, both NADES 2 and NADES 5 shampoos exhibited pH values within this optimal range, with NADES 2 demonstrating a notably more acidic pH value. However, over time, all formulations displayed variations in pH, which were particularly pronounced at elevated temperatures of 40°C. This phenomenon can be attributed to the increased rate of chemical reactions and component degradation at higher temperatures.

After 90 days, only the shampoo containing NADES 5 maintained a pH close to the ideal range, particularly at 25°C. In contrast, the control shampoo and the shampoo with NADES 2 recorded pH values exceeding 6.5 and 7.0. Such elevated pH levels may compromise the product's conditioning properties and pose potential irritation risks for individuals with sensitive skin, suggesting that a buffer may be required to ensure long-term stability.

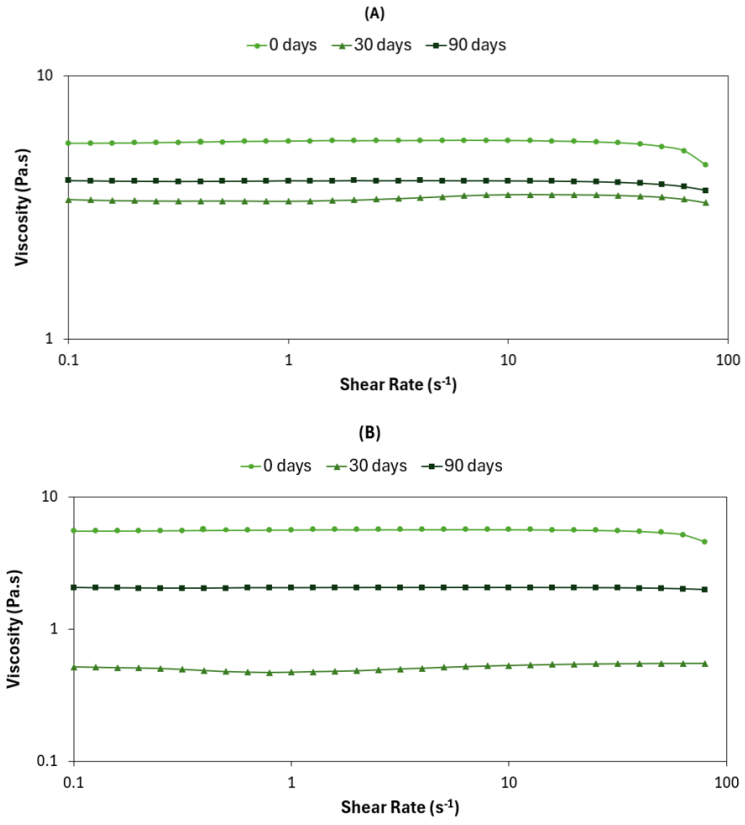


Figure 18 - Viscosity as a function of shear rate for the Control Shampoo stored at 25 °C (A) and 40ºC (B) over a 90-day stability study. Measurements were taken at 0, 30, and 90 days.

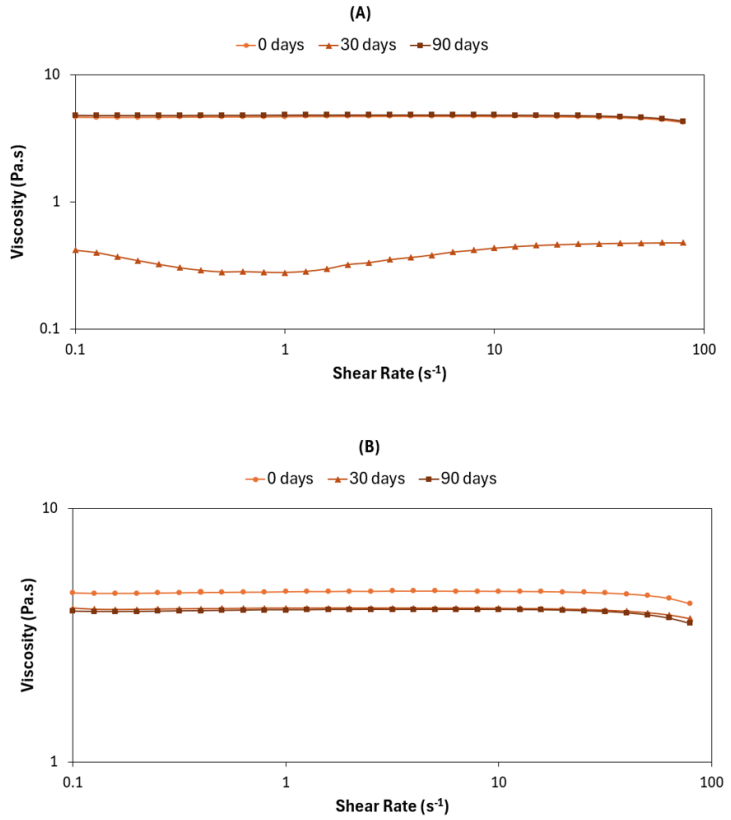


Figure 19 - Viscosity as a function of shear rate for the NADES 2 Shampoo stored at 25 °C (A) and 40ºC (B) over a 90-day stability study. Measurements were taken at 0, 30, and 90 days.

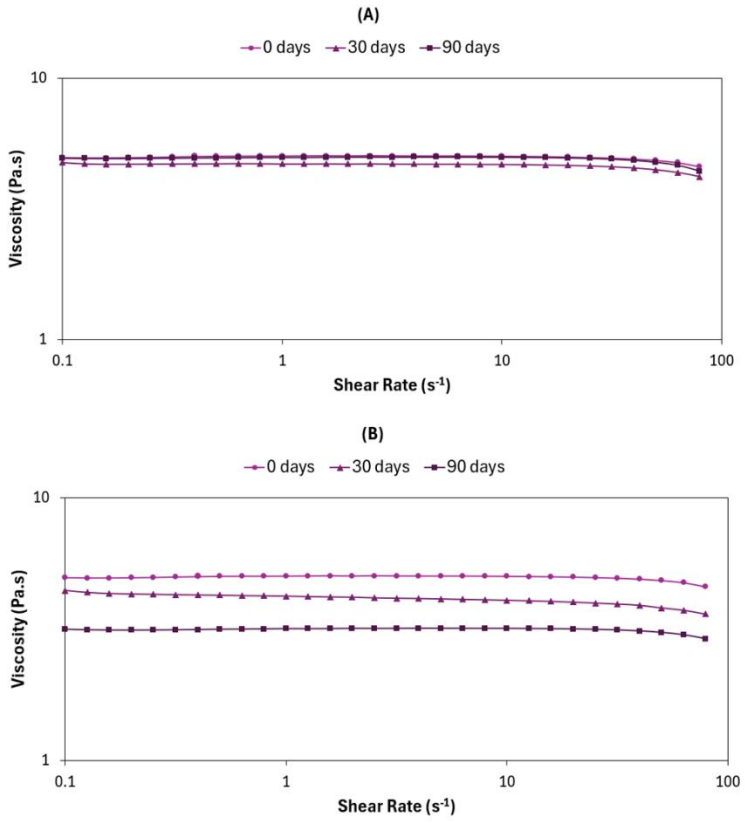


Figure 20 - Viscosity as a function of shear rate for the NADES 5 Shampoo stored at 25 °C (A) and 40ºC (B) over a 90-day stability study. Measurements were taken at 0, 30, and 90 days.

In general, the control shampoo, as observed in Figure 18, suffered the most changes over time, with a progressive and significant decrease in viscosity, especially when exposed to 40°C, thus indicating structural instability.

The shampoo containing NADES 2, represented in Figure 19, revealed a marked decline in viscosity after 30 days at 25°C, followed by a potential recovery at 90 days, which may indicate a temporary reorganization of its internal structure. In contrast, when exposed to 40°C, this formulation exhibited a gradual and consistent viscosity loss.

Conversely, the shampoo incorporating NADES 5, presented in Figure 20, maintained a relatively stable viscosity throughout the study, at both 25°C and 40°C, exhibiting only minimal variations. This consistent behavior suggests a more robust and resilient structural integrity that is less susceptible to fluctuations in time and temperature.

3.5 NADES Incorporation in Emulsions

3.5.1 Emulsions stabilization

The primary objective of incorporating various NADES into emulsions was to achieve an emulsion-stabilizing effect. As previously described, unstable emulsions were formulated, and the different NADES were added to each to evaluate their potential stabilizing abilities. The results are presented in Figure 21.

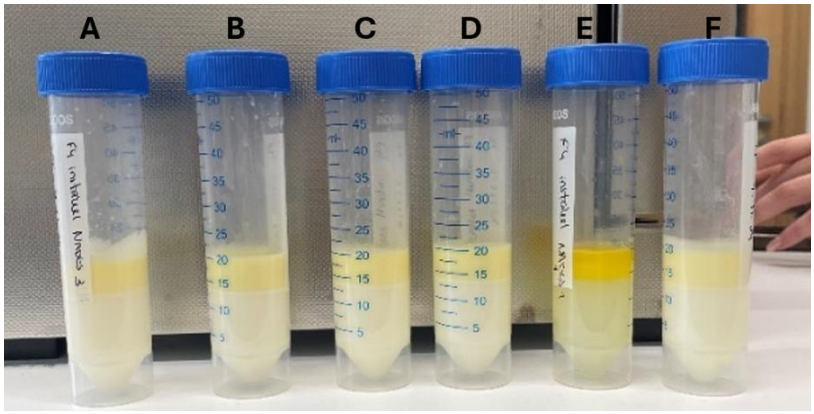


Figure 21 - Non-stable emulsions - Emulsion Control (A), Emulsion containing NADES 1 (B), Emulsion containing NADES 2 (C), Emulsion containing NADES 3 (D), Emulsion containing NADES 4 (E), and Emulsion containing NADES 5 (F).

The observed results indicate that NADES were ineffective in stabilizing the emulsions, as evidenced by the clear phase separation in all samples. The distinct layers between the oil phase (yellowish) and the aqueous phase (whitish) reveal that a homogeneous emulsified structure was not maintained over time. This separation points to physical-chemical instability and suggests low compatibility between the NADES and the other components of the emulsion.

3.5.2 NADES as extraction solvents

Of the five NADES produced, NADES 2 and NADES 3 were selected as solvents for extracting phenolic compounds from lupin residues. This decision was made because

NADES 2 and NADES 3 were preferred as extraction solvents due to their lower viscosity, which, according to existing literature, is associated with higher and more effective extraction yields [85]. Three different sizes of lupin residues were utilized for the extractions: small residue (250 µm), medium residue (1 mm), and large residue (greater than 1 mm). Table 17 displays the amount of extract obtained from each extraction.

Table 17 - Quantity of extract obtained from different NADES (NADES 2 and NADES 3) at 25 °C and 50 °C using residues of varying particle sizes (small, medium, and large).

		Residue Small	Residue Medium	Residue Large
NADES 2	**25ºC**	-	-	-
	50ºC	1.5 mL	1 mL	0.5 mL
NADES 3	**25ºC**	3.7 mL	3.5 mL	2.6 mL
	50ºC	3.75 mL	2.9 mL	1.9 mL

The results revealed that NADES 3 achieved a higher extraction yield under all conditions, with significantly greater volumes observed at both 25°C and 50°C. Conversely, NADES 2 did not produce any extract at 25°C, and its yields at 50°C were notably lower. This diminished performance may be attributed to its higher viscosity, which could hinder the diffusion and solubilization of the compounds present in the residues, particularly at lower temperatures. Similar results were described by Ling *et al.*, who studied DES as solvents for the extraction of biological macromolecules. In their study, the DES LGH (composed with lactic acid and glucose), with the lowest viscosity value, provided the highest extraction yield. In addition, they observed that certain extracted molecules, such as cinnamic acid, had higher solubility in low-viscosity DES [86]. Despite yielding smaller volumes, NADES 2 was not dismissed from further testing. The quantity of extract does not necessarily indicate the quality or concentration of the extracted phenolic compounds. Therefore, given its potential as a selective solvent for bioactive compounds, NADES 2 was retained for the subsequent characterization studies.

3.5.2.1 Antioxidant activity assays

The DPPH assay was performed to analyze the antioxidant activity of the extracts, their residues, and NADES 2 (solvent used to obtain the extract). Furthermore, a conventional solvent (water/ethanol) and its respective extract were used as controls. It is important to note that, when preparing the samples, since they need to be solubilized, four different ultrasound conditions were tested to optimize the protocol. The samples were tested without ultrasound (0 minutes), 5 minutes of ultrasound, 10 minutes of ultrasound, and 20 minutes of ultrasound. The results are presented in Table 18.

Table 18 - DPPH assay results of Extract Small, Residue Small, Extract Medium, and Residue Medium from NADES 2; conventional extract and solvent; DPPH (negative control) and Trolox (positive control), expressed as % radical scavenging activity. Samples were prepared at a concentration of 5 mg/mL and subjected to 0, 5, 10, and 20 minutes of ultrasound treatment (n = 3, Mean ± SD).

	% Radical scavenging activity				
	Final Concentration (mg/mL)	**0 min ultrasound**	**5 min ultrasound**	**10 min ultrasound**	**20 min ultrasound**
Extract Small	5	37.59 ± 0.69	5.08 ± 3.11	-	-
Residue Small	5	44.25 ± 4.99	45.66 ± 3.15	20.81 ± 8.42	-
Extract Medium	5	6.74 ± 0.01	7.58 ± 1.11	-	-
Residue Medium	5	27.58 ± 0.88	25.74 ± 0.10	57.62 ± 1.56	0.83 ± 7.88
Extract Large	5	21.13 ± 0.71	5.37 ± 0.63	-	-
Residue Large	5	26.84 ± 0.98	25.02 ± 1.30	32.94 ± 12.81	-
NADES 2	5	15.34 ± 2.27	15.12 ± 2.24	50.75 ± 4.43	-
Conventional Extract	5	19.20 ± 1.45	-	-	-
Conventional Solvent	5	-	-	-	-
DPPH	5	0.00 ± 0.94	0.00 ± 0.94	0.00 ± 0.94	0.00 ± 0.94
Trolox	5	94.28 ± 0.44	94.28 ± 0.44	94.28 ± 0.44	94.28 ± 0.44

"-" indicates no antioxidant activity.

In general, the best results were achieved using smaller residues (Residue Small), particularly without ultrasound (0 min). Notably, the extract small demonstrated a radical scavenging activity of 37.59%, marking the highest value among all tested samples. This performance can be attributed to the increased contact area of smaller residues with the solvent, which enhances the extraction of phenolic compounds.

In contrast, extracts derived from medium and large residues exhibited lower radical scavenging values, especially after 20 minutes of ultrasound exposure, during which very low or even negative activities were recorded. This suggests a degradation of bioactive compounds caused by excessive ultrasonic energy. Consequently, prolonged ultrasound application proved detrimental to antioxidant activity rather than beneficial.

When comparing these results with those obtained using conventional methods, it was observed that both the conventional extract and the conventional solvent presented significantly lower values (16.18% for the extract and negative values for the solvent). This highlights the contribution of NADES 2 to the antioxidant activity of the extracts. When tested alone without ultrasound, it demonstrated a radical scavenging activity of 15.34%, while the conventional solvent showed negative values under the same conditions. This result indicates that NADES 2 acts as a solvent and may have intrinsic antioxidant properties, unlike the conventional solvent.

Given that the ultrasound durations of 0 and 10 minutes yielded the most promising results, only these time points were utilized for the NADES 3 DPPH assay.

Additionally, residue large and its extract were excluded from further analysis. The outcomes of the DPPH assay for NADES 3 are presented in Table 19.

Table 19 - DPPH assay results of Extract Small, Residue Small, Extract Medium, and Residue Medium from NADES 2; conventional extract and solvent; DPPH (negative control); and Trolox (positive control), expressed as % radical scavenging activity. Samples were prepared at a different concentration of 5 mg/mL and subjected to 0 and 10 minutes of ultrasound treatment (n = 3, Mean ± SD).

	% Radical scavenging activity		
	Final Concentration (mg/mL)	**0 min ultrasound**	**10 min ultrasound**
Extract Small	5	7.29 ± 1.16	36.69 ± 1.30
Residue Small	5	44.25 ± 4.98	20.81 ± 8.42
Extract Medium	5	4.84 ± 0.07	39.14 ± 0.30
Residue Medium	5	27.58 ± 0.87	57.62 ± 1.56
NADES 3	5	32.16 ± 5.92	37.56 ± 14,98
DPPH	5	-	-
Trolox	5	94.28 ± 0.44	94.28 ± 0.44

"-" indicates no antioxidant activity.

The results obtained with NADES 3 revealed moderate radical scavenging activity, although lower than that observed with NADES 2 under similar conditions. Although NADES 3 showed some potential, especially with the medium residue at 10 minutes of ultrasound, the radical scavenging activity values were, in most cases, lower than those recorded with NADES 2. This difference may be related, for example, to the chemical composition of NADES 3, which may have limited the efficient extraction of phenolic compounds.

In addition to the DPPH assay, the FRAP assay was also performed in order to validate the DPPH results. In this assay, residue large and extract large were excluded. Table 20 represents the results obtained from this assay.

Table 20 - FRAP assay results of Extract Small, Residue Small, Extract Medium, and Residue Medium from NADES 2 and NADES 3; conventional extract and solvent; expressed as Fe^{2+} concentration (mM). Samples underwent 0 and 10 minutes of ultrasound treatment (n = 2, Mean ± SD).

	Concentration of F^{2+} (mM)	
	0 min	**10 min**
Extract Small NADES 2	0.03 ± 0.02	0.12 ± 0.00
Extract Small NADES 3	0.04 ± 0.02	0.06 ± 0.01
Residue Small	0.24 ± 0.03	0.01 ± 0.00
Extract Medium NADES 2	0.04 ± 0.02	0.02 ± 0.00
Extract Medium NADES3	0.03 ± 0.02	0.05 ± 0.00
Residue Medium	0.34 ± 0.02	0.13 ± 0.01
NADES 2	-	-
NADES 3	-	-
Conventional Extract	0.15 ± 0.08	-
Conventional Solvent	-	-

"-" indicates no antioxidant activity.

The results of the FRAP assay indicate that there are no significant differences between the extracts prepared with NADES 2 and NADES 3. The values obtained after 0 and 10 minutes of ultrasound are relatively low and closely comparable, regardless of the type of NADES used or the matrix (small or medium residue).

This lack of significant variation may be attributed to the specific nature of the FRAP assay, which measures only the reducing capacity of Fe^{3+} to Fe^{2+} and does not evaluate total free radical scavenging capacity. Consequently, the compounds within the extracts may exhibit antioxidant activity through alternative mechanisms, such as their ability to scavenge free radicals, as demonstrated in the DPPH assays. While the extracts show recognized antioxidant activity in other tests, the limited values obtained in the FRAP assay suggest that iron-reducing mechanisms do not primarily mediate this activity. Instead, it likely involves different types of antioxidant reactions. This underscores the importance of utilizing complementary methods to assess the antioxidant efficacy of natural extracts [87].

3.5.2.2 Total carbohydrate content (TCC) determination - Phenol-sulfuric method

The sugar determination test was carried out to analyze the amount of sugar in the samples. Determining the carbohydrate content provides a better understanding of the composition of NADES and extracts and their possible impact on the stability or performance of formulations. NADES 3 and its samples were not included in this test, as their overall results were worse than NADES 2 and its samples in the previous tests. The results are described below, represented in Table 21.

Table 21 - Sugar content determination results for Extract Small, Residue Small, Extract Medium, and Residue Medium from NADES 2, expressed as g sugar/100 g lupin. Samples were prepared at a concentration of 7.81 mg/mL and subjected to 0 and 10 minutes of ultrasound treatment, using the phenol–sulfuric acid method (n = 3, Mean ± SD).

	g sugar / 100 g lupin		
	Final Concentration (mg/mL)	**0 min**	**10 min**
Extract Small	7.81	296.52	296.52
Residue Small	7.81	2.68	1.29
Extract Medium	7.81	296.52	296.52
Residue Medium	7.81	-	-
NADES 2	7.81	296.52	296.52

"-" indicates no carbohydrate content.

The results of the TCC assay, presented in Table 21, reveal that the values observed in the extracts obtained using NADES 2 are primarily attributed to the solvent itself. In fact, NADES 2 is a sugar-based eutectic system, which explains the high carbohydrate values detected, even when the solvent is analyzed in isolation.

The extracts exhibit sugar contents strikingly similar to those of pure NADES 2. For instance, both extract small and extract medium show a carbohydrate content of 296.52 g of sugar per 100 g of lupin. This indicates that the sugars measured primarily

originate from the solvent rather than from the residue. Furthermore, the residues demonstrate very low or even negative sugar values, suggesting that the amount of sugars extractable from lupin under the tested conditions is negligible or possibly non-existent. This reinforces the conclusion that the values obtained from the extracts do not accurately reflect the sugar content of the residue, but rather the intrinsic composition of NADES 2.

3.5.2.3 Total phenolic content (TPC) determination

The Folin–Ciocalteu method was used to quantify the phenolic compound content in the samples. In general, phenolic compounds have been associated with several health benefits, derived from their anti-allergic, anti-atherogenic, anti-inflammatory, antimicrobial, antioxidant, and other properties. Plant-derived polyphenols have received increasing attention as substitutes for synthetic antioxidants, such as butylated hydroxyanisole and butylated hydroxytoluene, which are widely used by the food industry. The fact that synthetic antioxidants are carcinogenic and toxic to the liver has promoted the development and use of healthier and more biocompatible antioxidants of natural origin. As a result, they are useful not only for the food industry, but also for the pharmaceutical and cosmetic industries. Consumers increasingly demand innovative uses of natural compounds to treat signs of skin aging, such as wrinkles and hyperpigmentation. In response, manufacturers have launched organic product lines and natural solutions to treat a variety of skin problems and diseases. The production of polyphenol extracts from food by-products is promising for the cosmetics industry, which is continually searching for new, sustainable, and economical sources of functional ingredients. Polyphenols have been shown to have various effects on the skin, such as antioxidant, therapeutic, and anti-aging properties [88].

The 10-minute ultrasound condition was excluded from the analysis, and only samples without ultrasound treatment (0 min) were tested. The results are presented in Table 22.

Table 22 - Total phenolic content of Extract Small, Extract Medium, and Residue Medium from NADES 2, expressed as g gallic acid/100 g lupin. Samples were prepared at a concentration of 5 mg/mL and analyzed using the Folin–Ciocalteu method after 0 minutes of ultrasound treatment. E11 was used as a positive control (n = 3, Mean ± SD).

	g gallic acid / 100 g lupin	
	Final Concentrations (mg/mL)	**0 min**
Extract Small	5	0.29 ± 0.05
Extract Medium	5	0.34 ± 0.04
Residue Small	5	0.68 ± 0.11
Residue Medium	5	0.68 ± 0.01
NADES 2	5	0.05 ± 0.03
E11 (Positive Control)	5	12.22 ± 1.79
Conventional Extract	5	0.03 ± 0.00
Conventional Solvent	5	-

“-“ indicates no phenolic content.

Overall, the results indicated that the residues contained higher levels of total phenolic compounds than the extracts obtained using NADES 2. This suggests that, although NADES 2 is capable of extracting certain phenolic compounds, its efficiency is limited under the tested conditions.

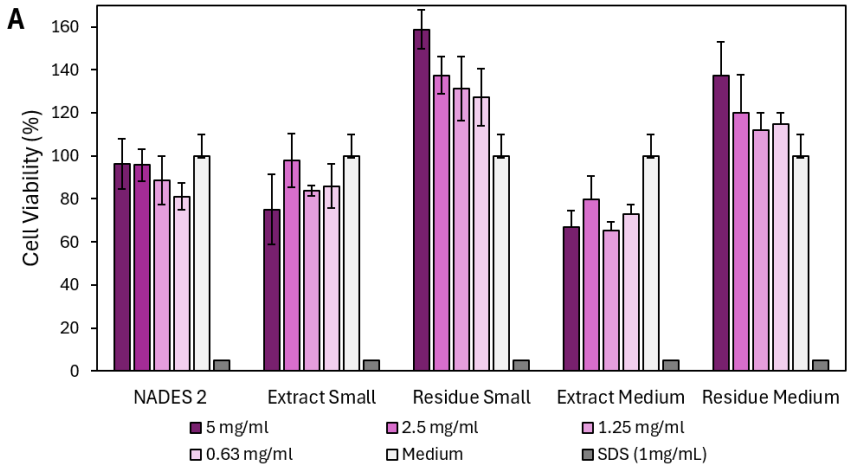
Additionally, the findings showed that NADES 2 tested in isolation exhibited minimal activity, with values at or near zero. This confirms that the phenolic compounds detected in the extracts originated from the residue, rather than from the solvent.

In contrast, the extracts obtained with conventional solvents displayed very low or even negative values, indicating low extraction efficiency. The positive control (E11) produced the highest values, as anticipated, thereby validating the sensitivity of the assay.

In summary, the data indicates that while NADES 2 can facilitate the extraction of phenolic compounds, a notable portion remains trapped in the residue. This underscores the importance of optimizing the extraction process to enhance phenolic yield.

3.5.2.4 *In vitro* safety and efficacy assays

To evaluate the safety and efficacy of the extracts, their residues, and NADES 2, a series of *in vitro* assays were performed, including the MTT cytotoxicity assay, antioxidant activity assays (ROS reduction), and human neutrophil elastase (HNE) enzyme inhibition tests. The results are presented in Figure 22.



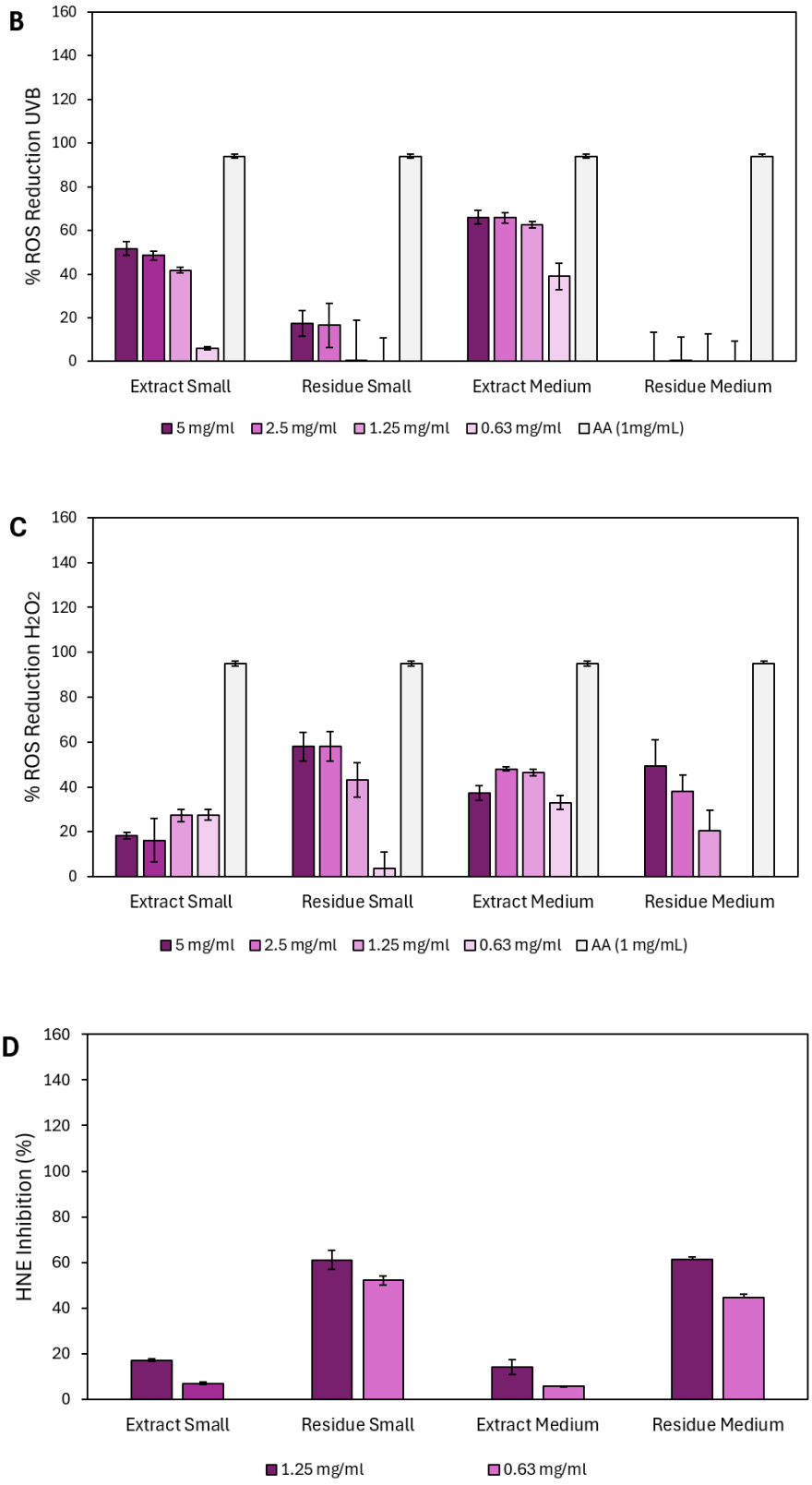


Figure 22 - *In vitro* safety and efficacy results of NADES 2, Extract Small, Residue Small, and Extract Medium, assessed through: A- MTT assay (cell viability), B- ROS reduction induced by UVB, C- ROS reduction induced by H_2O_2, and D- Human neutrophil elastase (HNE) inhibition. Samples were tested at different concentrations (mg/mL). Positive (Medium and Ascorbic Acid - AA) and negative (SDS) controls were included (n = 3, Mean ± SD).

All samples tested exhibited high levels of cell viability across nearly all concentrations assessed, indicating a favorable safety profile. Notably, extract small distinguished itself with a cell viability rate of 75.09 ± 16.16 % at 5 mg/mL, compared to extract medium, which recorded a rate of 66.78 ± 7.86 % at the same concentration. However, extract medium still remained within a safe range and showed no significant cytotoxicity. Furthermore, NADES 2 demonstrated excellent levels of cell viability at all tested concentrations, underscoring the safety of this solvent and its appropriateness for topical application. These results affirm the safe use of the formulations evaluated, including both the extracts and solvents.

The percentage reduction in ROS was evaluated using two distinct methods: exposure to UVB radiation (a physical method) and induction with H_2O_2 (a chemical method). Overall, the results demonstrated positive antioxidant activity across various samples, although variations were noted depending on the method employed. In the UVB radiation test, the small and medium extracts exhibited the highest ROS reduction values. Specifically, extract small at 5 mg/mL achieved a reduction of 51.75 ± 3.19 %, while extract medium at 5 mg/mL reached 66.00 ± 3.16 %. Conversely, when oxidative stress was induced with H_2O_2, the results were inverted; the small and medium residues demonstrated superior antioxidant performance, with residue small at 5 mg/mL performing at 58.03 ± 6.35 % and residue medium at 5 mg/mL at 49.42 ± 11.54 %. These differences in results can be attributed to the inherent chemical complexity of natural extracts. As these extracts are derived from biological sources, the composition of active compounds can vary significantly between extractions. Each sample may contain not only compounds with antioxidant properties, but also potentially antagonistic or pro-oxidant compounds. Consequently, the effectiveness of these extracts in neutralizing ROS may depend heavily on the specific nature of the extracted compounds, the extraction technique utilized, and the type of oxidative stress induced. As a result, the two methods are expected to yield different outcomes, complicating the achievement of a standardized antioxidant response [89]. Nevertheless, the obtained data confirms the antioxidant potential of the samples, emphasizing the varying effects based on the source of the extract, the type of residue tested, and the method of oxidative stress applied.

The results regarding elastase enzyme inhibition reveal that the residues display a significantly higher inhibition rate compared to the extracts. At a 1.25 mg/mL concentration, the residues exceed 60%, whereas the small and medium extracts show values of 17.74 ± 0.50 % and 14.09 ± 3.31 %, respectively. This data suggests that while the extracts have some capacity to inhibit elastase enzyme activity, their effectiveness is considerably limited in comparison to the residues. A possible explanation for this disparity may relate to the chemical composition of the residues, which could harbor a higher concentration of bioactive compounds that are more potent in inhibiting elastases, such as specific polyphenols or flavonoids that extraction methods may not capture effectively. Elastase inhibition is particularly significant for the protection of skin cells, as this enzyme influences the metabolism of elastic fibers. Its activity contributes to a decline in skin elasticity, ultimately leading to the formation of wrinkles during the aging process. Consequently, substances with the capability to inhibit elastase offer promising potential for preventing skin aging and for the formulation of dermocosmetic products with anti-aging benefits [90].

3.5.2.5 Stability Tests

Stability tests were performed to analyze the stability of the extract, its residue, and NADES 2 over time (0, 14, 30, 60, and 90 days). Thus, DDPH, FRAP, and TPC tests and color measurements were performed over three months. The results are presented in Tables 23 to 26.

Table 23 - DPPH assay results of NADES 2, Extract, and Residue stored under different conditions: room temperature without light, room temperature with light, 40 °C, and 4 °C, over a period of 0, 14, 30, 60, and 90 days. Results are expressed as % radical scavenging activity. DPPH (negative control) and Trolox (positive control) were included. Values represent mean ± standard deviation (n = 3, Mean ± SD).

			% Radical scavenging activity				
	Final Concentrations (mg/mL)		0 days	14 days	30 days	60 days	90 days
Room temperature without light	NADES 2	5	15.34 ± 2.27	-	23.09 ± 3.16	-	-
	Extract	5	37.59 ± 0.69	1.61 ± 0.52	-	14.54 ± 3.77	3.72 ± 0.92
	Residue	5	44.25 ± 4.99	18.35 ± 0.65	47.25 ± 0.63	14.26 ± 3.38	12.17 ± 0.88
Room temperature with light	NADES 2	5	15.34 ± 2.27	-	25.87 ± 2.53	0.08 ± 5.76	-
	Extract	5	37.59 ± 0.69	4.99 ± 1.30	-	-	6.01 ± 2.04
	Residue	5	44.25 ± 4.99	21.73 ± 1.24	41.56 ± 2.53	16.72 ± 4.11	5.01 ± 0.16
40 °C	NADES 2	5	15.34 ± 2.27	9.45 ± 0.21	2.28 ± 0.70	5.18 ± 2.94	-
	Extract	5	37.59 ± 0.69	-	-	2.44 ± 1.09	11.26 ± 2.10
	Residue	5	44.25 ± 4.99	18.25 ± 2.05	6.33 ± 0.06	5.18 ± 0.24	5.48 ± 0.51
4 °C	NADES 2	5	15.34 ± 2.27	-	24.73 ± 11.8	-	3.80 ± 1.43
	Extract	5	37.59 ± 0.69	-	-	0.53 ± 1.12	2.19 ± 2.02
	Residue	5	44.25 ± 4.99	25.03 ± 0.25	16.32 ± 1.20	19.05 ± 4.88	15.25 ± 3.61
DPPH		5	0.00 ± 0.94	-	-	-	0.61 ± 0.61
Trolox		5	94.28 ± 0.44	89.21 ± 0.20	82.04 ± 2.15	91.78 ± 0.07	74.58 ± 1.03

"-" indicates no antioxidant activity.

Table 24 - FRAP assay results of NADES 2, Extract, and Residue stored under different conditions: room temperature without light, room temperature with light, 40°C , and 4 °C, over a period of 0, 14, 30, 60, and 90 days. Results are expressed as Fe^{2+} concentration (mM) (n = 2, Mean ± SD).

		Concentration of F^{2+} (mM)				
		0 days	14 days	30 days	60 days	90 days
Room temperature without light	NADES 2	-	-	-	-	-
	Extract	0.03 ± 0.02	0.07 ± 0.08	0.01 ± 0.00	-	-
	Residue	0.24 ± 0.03	0.64 ± 0.02	0.47 ± 0.00	0.29 ± 0.01	0.42 ± 0.06
Room temperature with light	NADES 2	-	-	-	-	-
	Extract	-	0.03 ± 0.04	-	-	-
	Residue	0.24 ± 0.03	0.38 ± 0.22	0.45 ± 0.03	0.33 ± 0.02	0.41 ± 0.03

40 °C	**NADES 2**	-	-	-	-	-
	Extract	0.03 ± 0.02	0.07 ± 0.01	-	-	-
	Residue	0.24 ± 0.03	0.49 ± 0.21	0.15 ± 0.37	0.42 ± 0.02	0.47 ± 0.04
4 °C	**NADES 2**	-	-	-	-	-
	Extract	0.03 ± 0.02	0.05 ± 0.01	-	-	-
	Residue	0.24 ± 0.03	0.71 ± 0.02	0.56 ± 0.00	0.48 ± 0.02	0.42 ± 0.08

"-" indicates no antioxidant activity.

Table 25 - Total phenolic content determined by the Folin–Ciocalteu method for NADES 2, Extract, and Residue stored under different conditions: room temperature without light, room temperature with light , 40 °C , and 4 °C, over a period of 0, 30, 60, and 90 days. Results are expressed as g gallic acid/100 g lupin (n = 3, Mean ± SD).

		g gallic acid / 100 g lupin				
		Final Concentration (mg/mL)	**0 days**	**30 days**	**60 days**	**90 days**
Room temperature without light	**NADES 2**	5	0.05 ± 0.03	-	-	-
	Extract	5	0.29 ± 0.05	-	-	-
	Residue	5	0.68 ± 0.11	0.05 ± 0.08	0.10 ± 0.01	0.14 ± 0.02
Room temperature with light	**NADES 2**	5	0.05 ± 0.03	-	-	-
	Extract	5	0.29 ± 0.05	-	-	-
	Residue	5	0.68 ± 0.11	0.11 ± 0.08	0.11 ± 0.01	0.14 ± 0.02
40 °C	**NADES 2**	5	0.05 ± 0.03	-	-	-
	Extract	5	0.29 ± 0.05	-	-	-
	Residue	5	0.68 ± 0.11	-	0.15 ± 0.01	0.18 ± 0.01
4 °C	**NADES 2**	5	0.05 ± 0.03	-	-	-
	Extract	5	0.29 ± 0.05	-	-	-
	Residue	5	0.68 ± 0.11	0.04 ± 0.08	0.17 ± 0.00	0.22 ± 0.01

"-" indicates no phenolic content.

Table 26 - Color analysis of NADES 2, Extract, and Residue stored under different conditions: room temperature without light, room temperature with light, 40 °C, and 4 °C, over a period of 0, 14, 30, 60, and 90 days (n = 6, Mean ± SD).

			0 days	**14 days**	**30 days**	**60 days**	**90 days**
Room temperature without light	**NADES 2**	L *	76.46 ± 0.57	80.51 ± 0.47	80.81 ± 0.36	80.95 ± 0.60	80.97 ± 1.07
		a *	-2.74 ± 0.05	-4.04 ± 0.17	-3.92 ± 0.09	-3.75 ± 0.05	-3.56 ± 0.12
		b *	8.06 ± 0.07	6.90 ± 0.12	5.52 ± 0.19	6.17 ± 0.13	6.39 ± 0.28
	Extract	L *	59.19 ± 0.97	60.51 ± 0.59	64.35 ± 0.51	67.6 ± 1.46	66.70 ± 0.43
		a *	5.37 ± 0.34	4.99 ± 0.63	3.16 ± 0.13	2.13 ± 0.40	2.10 ± 0.10

		b *	37.61 ± 0.76	36.41 ± 0.32	32.43 ± 0.25	29.69 ± 0.80	29.32 ± 0.50
	Residue	L *	44.93 ± 0.96	44.48 ± 1.27	44.64 ± 1.46	45.41 ± 1.46	45.24 ± 0.70
		a *	8.31 ± 0.24	8.13 ± 0.46	8.22 ± 0.47	8.50 ± 0.36	8.37 ± 0.20
		b *	22.70 ± 0.56	22.10 ± 1.00	22.39 ± 1.30	23.00 ± 0.69	22.67 ± 0.70
Room temperature with light	**NADES 2**	L *	76.46 ± 0.57	80.15 ± 0.66	79.80 ± 0.78	80.28 ± 0.30	80.16 ± 0.14
		a *	-2.74 ± 0.05	-3.74 ± 0.09	-3.67 ± 0.08	-3.79 ± 0.04	-3.89 ± 0.05
		b *	8.06 ± 0.07	6.56 ± 0.13	5.34 ± 0.19	6.06 ± 0.12	6.25 ± 0.12
	Extract	L *	59.19 ± 0.97	62.99 ± 0.30	64.87 ± 0.79	73.16 ± 0.42	75.30 ± 0.61
		a *	5.37 ± 0.34	2.80 ± 0.18	2.19 ± 0.17	-0.50 ± 0.07	-1.42 ± 0.10
		b *	37.61 ± 0.76	28.75 ± 0.54	26.56 ± 0.60	19.79 ± 0.24	17.77 ± 0.50
	Residue	L *	44.93 ± 0.96	44.37 ± 1.57	43.74 ± 0.94	47.01 ± 0.80	45.27 ± 1.61
		a *	8.31 ± 0.24	7.83 ± 0.43	7.67 ± 0.33	8.31 ± 0.18	8.25 ± 0.40
		b *	22.70 ± 0.56	21.60 ± 1.29	20.36 ± 0.85	22.77 ± 0.50	21.87 ± 1.28
40 °C	**NADES 2**	L *	76.46 ± 0.57	80.41 ± 0.34	80.43 ± 0.61	80.49 ± 0.35	80.87 ± 0.99
		a *	-2.74 ± 0.05	-4.24 ± 0.03	-3.82 ± 0.09	-3.89 ± 0.06	-3.98 ± 0.11
		b *	8.06 ± 0.07	7.28 ± 0.15	5.50 ± 0.17	6.45 ± 0.32	6.36 ± 0.30
	Extract	L *	59.19 ± 0.97	60.85 ± 0.81	60.81 ± 0.92	66.70 ± 0.90	67.48 ± 0.91
		a *	5.37 ± 0.34	4.93 ± 0.26	4.75 ± 0.26	2.51 ± 0.10	2.08 ± 0.32
		b *	37.61 ± 0.76	36.45 ± 0.46	36.13 ± 0.62	30.50 ± 0.48	29.04 ± 0.87
	Residue	L *	44.93 ± 0.96	41.10 ± 1.25	41.93 ± 1.39	37.46 ± 0.69	40.14 ± 1.24
		a *	8.31 ± 0.24	6.60 ± 0.15	6.96 ± 0.40	6.73 ± 0.22	7.74 ± 0.28
		b *	22.70 ± 0.56	17.28 ± 0.80	18.46 ± 0.92	16.40 ± 0.48	18.74 ± 0.80
4 °C	**NADES 2**	L *	76.46 ± 0.57	80.03 ± 0.35	80.28 ± 0.43	80.43 ± 0.40	80.06 ± 0.32
		a *	-2.74 ± 0.05	-4.16 ± 0.05	-3.81 ± 0.08	-3.74 ± 0.07	-3.85 ± 0.03
		b *	8.06 ± 0.07	7.17 ± 0.09	5.60 ± 0.14	5.96 ± 0.30	6.40 ± .11
	Extract	L *	59.19 ± 0.97	59.69 ± 0.36	58.07 ± 0.24	65.85 ± 1.20	71.38 ± 1.50
		a *	5.37 ± 0.34	5.51 ± 0.08	6.03 ± 0.20	2.46 ± 0.34	0.25 ± 0.52
		b *	37.61 ± 0.76	37.56 ± 0.30	38.69 ± 0.64	29.62 ± 1.01	23.03 ± 1.91
	Residue	L *	44.93 ± 0.96	42.12 ± 1.00	41.64 ± 0.86	41.76 ± 1.43	41.57 ± 0.71
		a *	8.31 ± 0.24	6.63 ± 0.18	6.38 ± 0.24	6.72 ± 0.17	6.70 ± 0.20
		b *	22.70 ± 0.56	17.63 ± 0.42	17.14 ± 0.72	17.78 ± 0.68	17.91 ± 0.64

L*, lightness, a*, associated with changes in redness, and b*, associated with changes in yellowness blueness.

Table 23 presents the results of the DPPH assay conducted over 90 days under four different storage conditions. Overall, the values exhibited fluctuated over time without demonstrating a consistent trend of increase or decrease, suggesting a lack of significant and sustained alterations. For instance, NADES 2 increased from 15.34% on day 0 to 23.09% on day 30 at room temperature without light, but dropped to only 2.28%

on day 30 at 40°C. The extract showed marked instability, decreasing from 37.59% on day 0 to 6.01% on day 90 at room temperature with light. In contrast, the residue maintained a comparatively higher proportion of its activity, particularly under refrigeration, where values decreased from 44.25% on day 0 to 15.25% on day 90. This stability may suggest that lower storage temperatures are more effective in preserving the antioxidant activity of such samples. It is important to note that the DPPH assay itself may have certain limitations that could have influenced the variability of the results. The DPPH radical is sensitive to light and temperature changes, and its reactions with antioxidants depend on the chemical structure of the compounds and the duration of incubation. Thus, some of the observed variation in the results may be attributed not only to the degradation of antioxidant compounds over time, but also to the inherent sensitivity and limitations of the assay method itself [91].

Table 24 summarizes the stability results obtained from the FRAP assay, revealing that, overall, all samples exhibited very low, reducing activity values over the 90-day period, irrespective of the storage conditions. Both NADES 2 and the extract maintained values consistently close to zero, for example ranging between 0.01 and 0.07 mM Fe^{2+} across all conditions and time points, with no significant fluctuations. The residue, however, demonstrated comparatively higher values, rising from about 0.24 mM at day 0 and then stabilizing near 0.42 mM by day 90, both at room temperature and under refrigeration. Nonetheless, the results remained generally low, indicating limited antioxidant activity within this assay. As previously discussed, this lack of notable results may be attributed to the specific characteristics of the FRAP assay.

The phenolic compound quantification test results, presented in Table 25, indicate very low values across all samples, regardless storage conditions. NADES 2 and the extract consistently showed minimal levels, close to 0.03g gallic acid/100g with no relevant changes over the 90 days. The residue sample had slightly higher values, particularly at 4°C, where the results at day 0 were 0.68g gallic acid/100g, and despite decreasing over time, at day 90 the results remained at 0.22g gallic acid/100g. However, even in this case, the values remained lower, and no significant increases were observed.

Regarding the colorimetric measurements presented in Table 26, NADES 2 demonstrated notable stability over the 90-day period, regardless of storage conditions employed. This consistency suggests that NADES 2 maintains good visual stability over time. In contrast, the extract showed the most significant variation in its values, showing pronounced fluctuations across different time points and conditions. Notably, at 4°C, the values remained comparable to the initial readings, particularly at the 14 and 30-day marks. Regarding the residue, it displayed greater stability over time, particularly under room temperature conditions without light and at 4°C, where the values remained closer to the initially recorded levels.

3.6 Incorporation of the Extract into Emulsions

3.6.1 Emulsions Characterization

3.6.1.1 Rheological Analysis

Following the previous tests, the extract small derived from residue small using NADES 2 as the extraction solvent was selected for further study. As a result, three emulsions were formulated: a Control emulsion, an Emulsion containing Extract Small, and an Emulsion using NADES 2. The initial phase focused on characterizing these formulations, beginning with a rheological analysis to evaluate their viscosity, oscillatory and thixotropy performance. The results are illustrated in Figure 23 to 25.

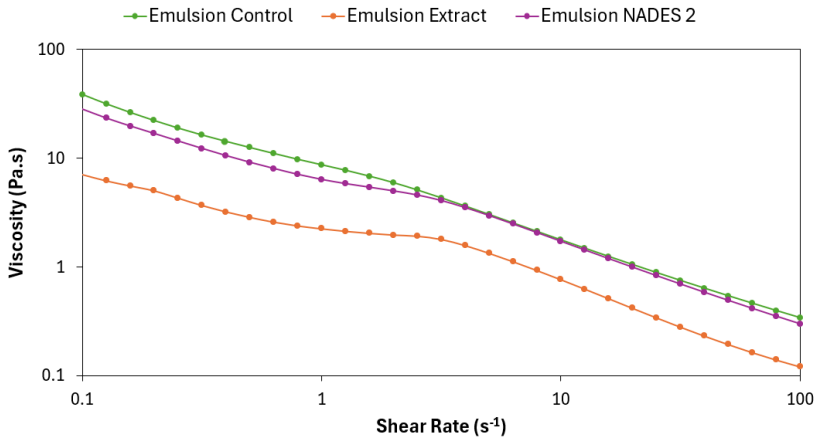


Figure 23 - Viscosity as a function of shear rate for the Control emulsion, Emulsion containing Extract Small (Emulsion Extract), and an Emulsion using NADES 2 (Emulsion NADES 2) at 25 °C.

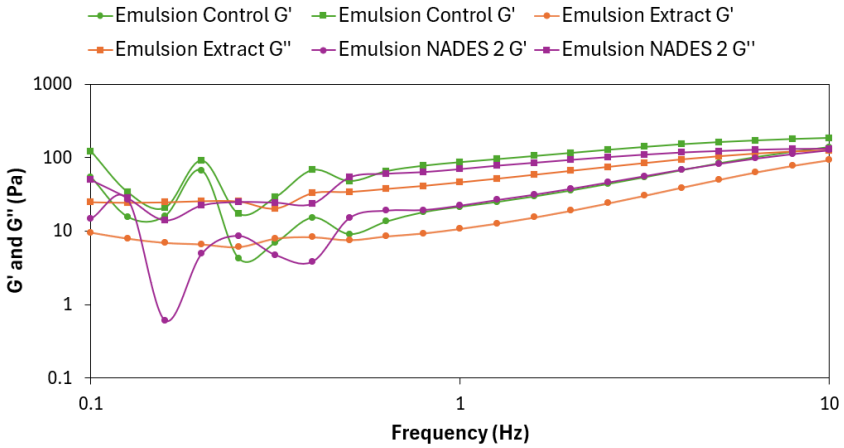


Figure 24 - Storage and loss moduli of the Control emulsion, Emulsion containing Extract Small (Emulsion Extract), and an Emulsion using NADES 2 (Emulsion NADES 2) as a function of oscillation frequency at 25 °C

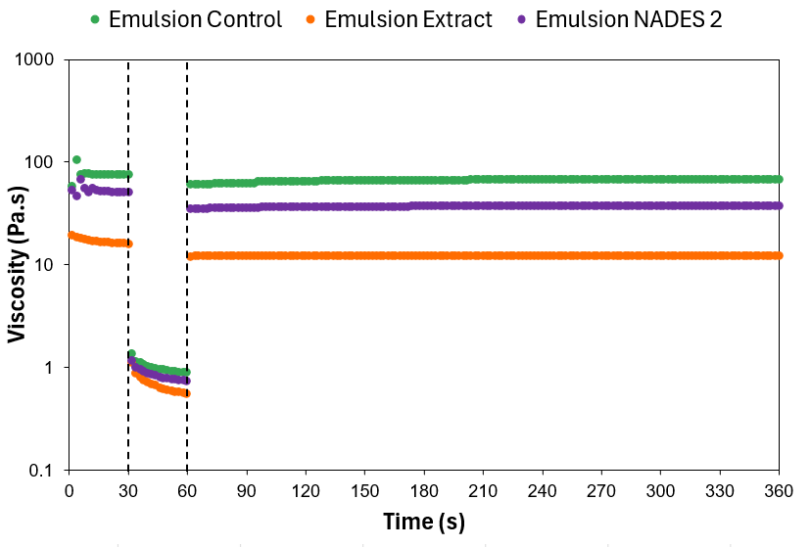


Figure 25 - Three-step rheological test of Control emulsion, Emulsion containing Extract Small (Emulsion Extract), and an Emulsion using NADES 2 (Emulsion NADES 2) performed to assess structural recovery and thixotropic behavior at 25 °C.

The rheological studies made it possible to characterize the behavior of emulsions containing a small extract and NADES 2 compared to the control emulsion, revealing distinct differences in viscosity, structure, and stability of the formulations.

As illustrated in Figure 23, the viscosity test demonstrated the typical behavior of a pseudoplastic non-Newtonian fluid, where viscosity decreases with an increasing shear rate. This behavior is expected in emulsified systems [92]. The control emulsion exhibited the highest viscosity throughout the entire range of shear rates, followed by the emulsion with NADES 2. In contrast, the emulsion containing the extract displayed the lowest viscosity, indicating a less cohesive and more easily deformable internal structure. From a sensory perspective, emulsions with moderate viscosities, such as the one formulated with NADES 2, are generally more appealing to users, as they spread more easily without excessive dripping. Although the extract emulsion is more fluid, it may be perceived as overly watery. On the other hand, the control emulsion, with its higher viscosity, may pose difficulties in achieving an even application on the skin.

In the oscillatory analysis shown in Figure 24, the control emulsion exhibited the highest values for both G′ and G″, signifying greater elasticity and structural stability. The emulsion containing NADES 2 displayed intermediate values, indicative of a moderately cohesive structure. In contrast, the emulsion with extract recorded the lowest values for both modules, indicating a more fluid and less elastic nature. However, its curves demonstrated the most stability and consistency, which suggests predictable behavior and a uniform texture. This consistency implies more reliable and uniform rheological properties, which can be beneficial from a sensory perspective, offering a light, homogeneous, and easy-to-spread texture. Therefore, while it may possess lower mechanical resistance, the emulsion with extract presents a smooth and stable profile that can be advantageous in cosmetic applications focused on comfort and ease of application.

Finally, as observed in Figure 25, the Three-step test was performed to evaluate the structural recovery capacity of the emulsions after being subjected to mechanical stress. The results show that phase 2, corresponding to the application of intense shear,

occurs between 0 and 50 seconds. The control emulsion shows the best recovery, reaching the highest viscosity, followed by the emulsion with NADES 2, which recovers partially. The emulsion with extract shows the lowest recovery, maintaining a low but stable viscosity, which suggests a less resistant but predictable and consistent structure, potentially advantageous for formulations that value lightness, uniformity, and good spreadability.

3.6.1.2 Texturometer Analysis

To understand the textural profile of the emulsions, the TPA test was carried out to obtain the results of hardness, adhesiveness, springiness, cohesiveness and resilience, as described in Table 27.

Table 27 - TPA parameters obtained for Control emulsion, Emulsion containing Extract Small (Emulsion Extract), and an Emulsion using NADES 2 (Emulsion NADES 2), including hardness, adhesiveness, springiness, cohesiveness, and resilience (n = 6, Mean ± SD).

	Hardness (g)	Adhesiveness (g.sec)	Springiness	Cohesiveness	Resilience
Emulsion Control	-0.316 ± 0.248	-19.647 ± 1.194	0.823 ± 0.018	0.844 ± 0.013	0.021 ± 0.002
Emulsion Extract	-0.306 ± 0.146	-12.331 ± 2.350	0.801 ± 0.029	0.780 ± 0.081	0.011 ± 0.002
Emulsion NADES 2	-1.634 ± 0.360	-64.979 ± 3.539	0.972 ± 0.002	0.754 ± 0.015	0.017 ± 0.000

Regarding the TPA test, the control emulsion exhibited the best cohesiveness and resilience, resulting in a stable texture that was easy to apply. The emulsion containing the extract displayed a similar profile to the control, though it was slightly less cohesive and resilient, with reduced adhesiveness, which may enhance application and rinsing processes. Conversely, the emulsion with NADES 2 demonstrated the highest springiness. However, it was also the most adhesive and sticky, with lower cohesiveness. This combination suggests a less pleasant texture that could make it challenging to spread. Overall, the control emulsion achieves the most favorable balance between structural integrity and sensory experience, while the extract may appeal to individuals who prioritize smoothness and minimal adhesiveness. Despite its high elasticity, the textural profile of the emulsion with NADES 2 is less ideal for enjoyable sensory applications.

3.6.1.3 Droplet Size

The droplet size of all the emulsions was determined to understand how this parameter can affect the stability of the emulsions. The result is described in Figure 26.

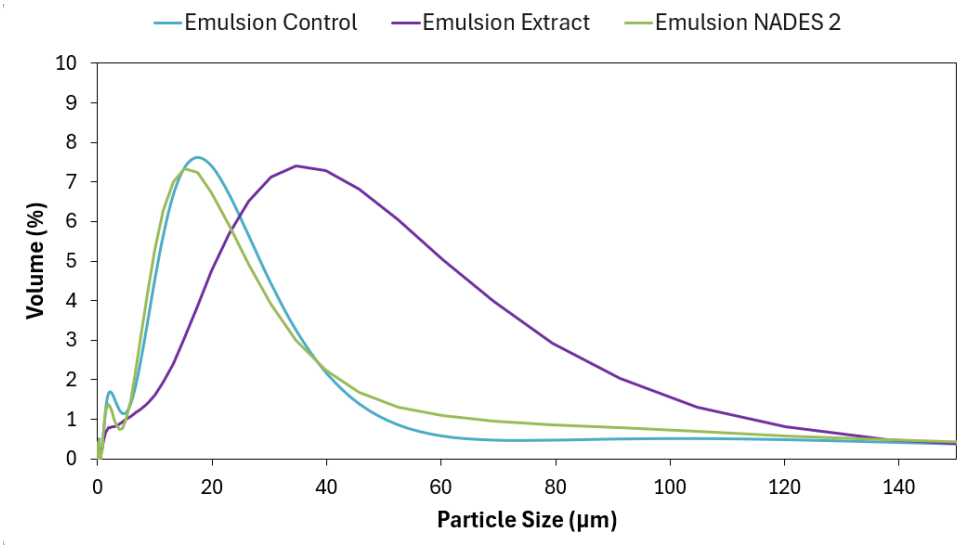


Figure 26 - Emulsions droplet size of Control emulsion, Emulsion containing Extract Small (Emulsion Extract), and an Emulsion using NADES 2 (Emulsion NADES 2).

Figure 26 illustrates the particle size distribution, which is essential for assessing the homogeneity and stability of emulsions. This parameter significantly influences the product's texture, appearance, and sensory performance [93].

Generally, it can be noted that the control emulsion and the emulsion containing NADES 2 exhibit very similar distributions, with a peak between 10 and 30 µm. This suggests a finer and more uniform distribution of droplets, typically associated with emulsions that are more stable and pleasant to the touch.

In contrast, the emulsion with extract shows a broader distribution, leaning towards larger particles, featuring a peak between 20 and 50 µm and a notable presence of droplets reaching up to 100 µm. This increased variability in particle size may indicate lower efficiency in the emulsification process or potential instability, which could adversely affect the final texture and appearance of the product. However, this profile may also contribute to a richer and more substantial texture, which can be advantageous in products designed for greater coverage or more nourishing applications. Consequently, each particle size profile can be tailored to meet the specific sensory and functional objectives of the formulation.

3.6.1.4 *In vitro* safety assays

3.6.1.4.1 Final Emulsion cytotoxic assays

To ensure the safety of the final emulsions, an MTT cytotoxicity assay was performed. The results are shown in Figure 27.

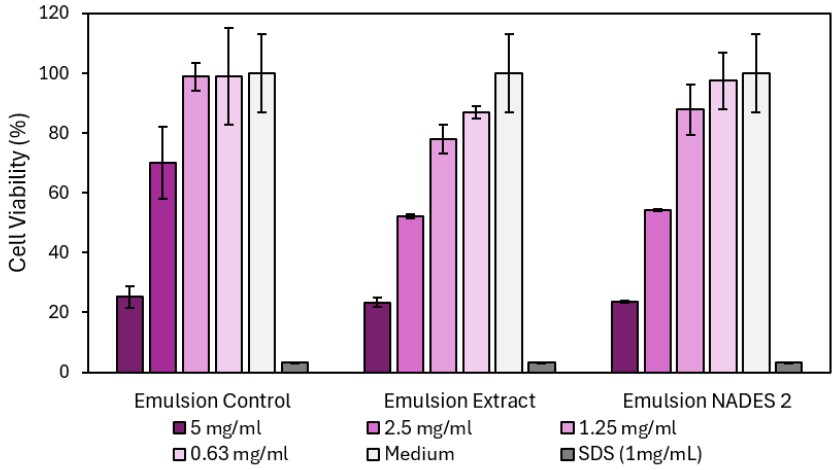


Figure 27 - Cell viability assays of HaCaT cells exposed Control emulsion, Emulsion containing Extract (Emulsion Extract), and Emulsion containing NADES 2 (Emulsion NADES 2), using MTT method. Medium was used as positive control and SDS as a negative control (n = 3, Mean ± SD).

The results of the MTT cell viability assay for HaCaT cells treated with various emulsions are summarized in Figure 27. A notable decrease in cell viability was observed when the concentration reached 5 mg/mL. In addition, using DMSO as a solvent to solubilize the samples also caused a decline in cell viability at higher concentrations.

On a positive note, cell viability began to improve steadily from 2.5 mg/mL, reaching over 70% at 1.25 mg/mL and 0.63 mg/mL. All emulsions showed promising safety profiles at these concentrations. Specifically, the NADES 2 emulsion achieved a cell viability of 87.9 ± 8.4 % at 1.25 mg/mL, very close to the control value. On the other hand, the extract emulsion demonstrated consistent cell viability at all concentrations tested, with a remarkable value of 77.9 ± 4.9 % at 1.25 mg/mL, highlighting its reliability and safety.

3.6.1.5 Stability Tests

To evaluate the stability of the emulsions over an extended period, comprehensive stability tests were conducted. Similarly to what was performed on the shampoo formulations, pH and viscosity measurements were systematically recorded over a three-month timeframe. The findings are detailed in Table 28 and in Figures 28 to 30.

Table 28 - pH values of emulsion formulations (Control, Extract, and NADES 2) measured over a 90-day stability study at 25 °C and 40 °C. Measurements were taken at 0, 30, and 90 days after storage.

	0 days		**30 days**		**90 days**	
pH	**25ºC**	**40ºC**	**25ºC**	**40ºC**	**25ºC**	**40ºC**
Emulsion Control	6.54	6.54	6.33	5.79	6.44	6.07
Emulsion Extract	6.15	6.15	6.12	6.01	6.07	6.61
Emulsion NADES 2	6.23	6.23	6.19	5.58	6.52	6.48

The pH value is an essential parameter in formulating cosmetic emulsions, influencing skin tolerance and product stability. Ideally, it should be between 4 and 6 [94].

Over 90 days, all emulsions showed pH variations, particularly at 40°C, due to the acceleration of chemical reactions at high temperatures.

All emulsions recorded values slightly above the ideal range, especially with time and increasing temperature. However, these values remain within a safe margin, without significantly compromising skin compatibility. The emulsions with NADES 2 and extract showed the most stable profiles, with a pH close to 6, while the control emulsion showed a more pronounced variation.

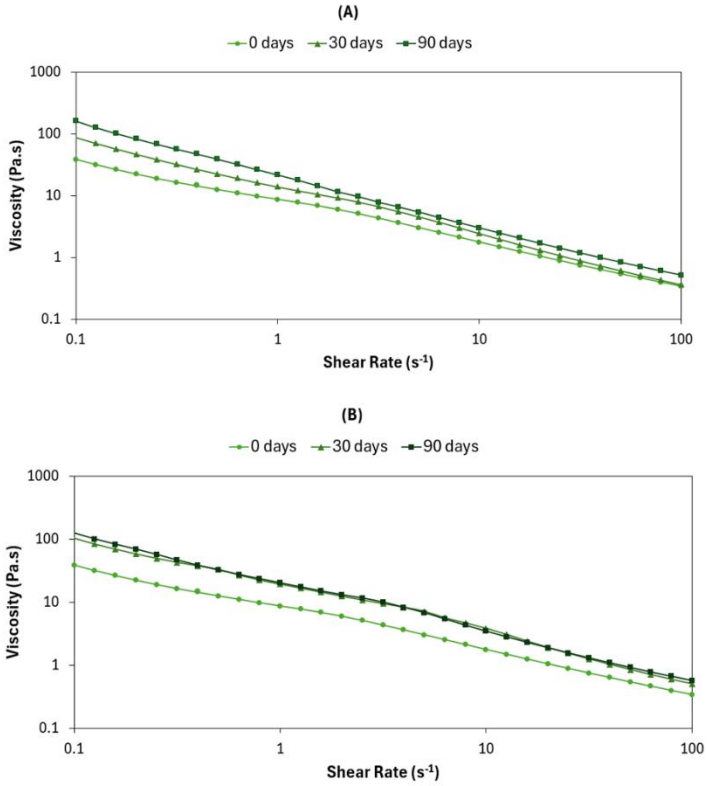


Figure 28 - Viscosity as a function of shear rate for the Control Emulsion stored at 25 °C (A) and 40ºC (B) over a 90-day stability study. Measurements were taken at 0, 30, and 90 days.

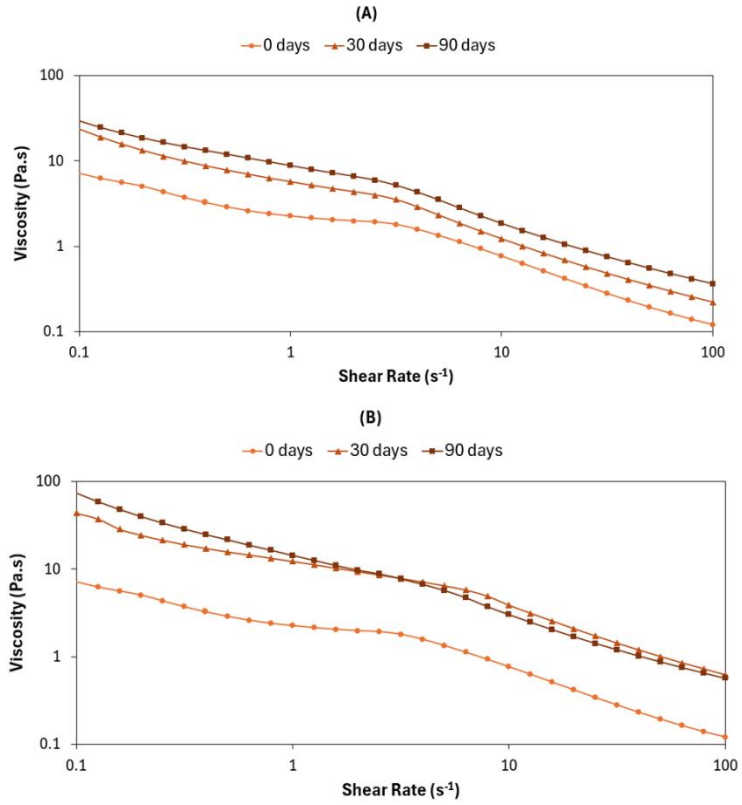


Figure 29 - Viscosity as a function of shear rate for the Extract Emulsion stored at 25 °C (A) and 40ºC (B) over a 90-day stability study. Measurements were taken at 0, 30, and 90 days.

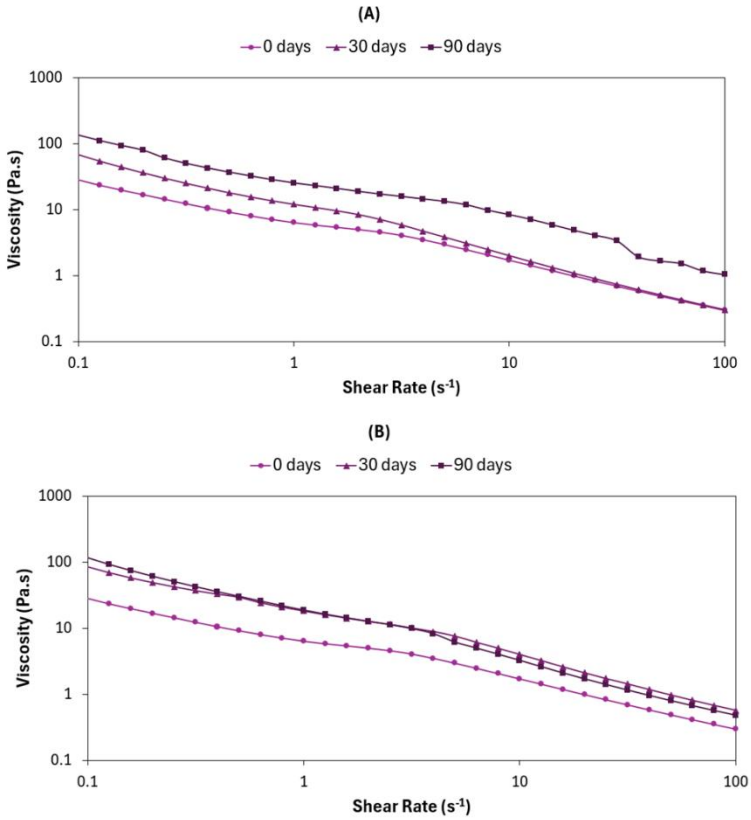


Figure 30 - Viscosity as a function of shear rate for the NADES 2 Emulsion stored at 25 °C (A) and 40ºC (B) over a 90-day stability study. Measurements were taken at 0, 30, and 90 days.

The analysis of the rheological stability of the emulsions over 90 days, at 25°C and 40°C, represented in Figures 28 to 30, revealed a gradual increase in viscosity in all formulations, indicating structural reinforcement over time.

In general, the control emulsion (Figure 28) showed consistent behavior at both temperatures, with a progressive and controlled increase in viscosity, a sign of good thermal resistance and structural integrity. This profile suggests that the emulsion maintains its functionality and ease of application over time, even under thermal stress.

Although initially more fluid, the emulsion with extract (Figure 29) showed the most significant variation over the 90 days, especially at 40°C. This significant increase in viscosity may indicate a positive internal reorganization of the colloidal matrix, becoming more structured and potentially more stable. This behavior is promising, demonstrating the emulsion's ability to adapt, gaining body and consistency over time.

On the other hand, the emulsion with NADES 2 (Figure 30) showed a marked increase in viscosity, especially at low shear rates and after 90 days. Although it maintains adequate pseudoplastic behavior, this more significant increase may influence the sensory experience, making the emulsion thicker.

CHAPTER 4: Conclusion

The primary goal of this dissertation was to assess the potential of various NADES in topical cosmetic formulations, including shampoos and emulsions, with a focus on innovation and sustainability. Furthermore, the study also explored the potential of NADES as an extraction solvent for by-products, highlighting their potential for valorizing agro-industrial by-products with cosmetic applications. As both consumers and industries increasingly seek safer, more sustainable, and natural alternatives to conventional solvents, NADES emerge as a promising solution, given their natural origin, versatile composition, and low toxicity. Five distinct NADES systems were developed and subjected to a comprehensive physical-chemical characterization. Rheological, surface tension, texture, and adhesiveness analyses enabled the selection of the most suitable NADES for incorporation into cosmetic products, namely NADES 2 and NADES 5 systems. These selected systems were then integrated into final shampoo formulations, with their performance evaluated through foam formation and stability tests, rheological analysis, surface tension, contact angle assessments, optical microscopy, and physical-chemical stability tests. The results demonstrated that NADES positively influence the sensory and functional attributes of shampoos, enhancing aspects such as viscosity, foaming capacity, and texture. Additionally, *in vitro* tests performed on HaCaT cells showed low levels of cytotoxicity, reinforcing the safety of using these solvents in skin contact applications.

Emulsions formulated with NADES exhibited significant physical instability, including visible phase separation over time. This observation suggests that the emulsions were unable to maintain a stable dispersion of droplets, indicating that NADES, when used as the sole stabilizing component, do not provide adequate interfacial protection to prevent destabilization mechanisms. As a result, their effectiveness as independent emulsion stabilizers appears limited, however, they may still prove valuable when used in conjunction with other stabilizing agents.

In addition, NADES 2 and NADES 3 were applied as solvents for extracting phenolic compounds from lupin residues. Overall, the results obtained from the *in vitro* tests demonstrated that the extracts exhibit antioxidant activity, confirming their effectiveness. Assays performed with HaCaT cells showed that the extract exhibited no cytotoxicity in the MTT test, thereby affirming its safety. The data obtained supports the applicability of NADES as innovative and sustainable excipients in cosmetic formulations, functioning not only as solvents but also as promoters of texture, foam, and stability over time. Furthermore, they can serve as extraction solvents and carriers for bioactive compounds.

When considering future prospects, it is crucial to explore further the long-term stability of formulations that integrate NADES and to evaluate their effectiveness under real-world conditions through *in vivo* testing. Furthermore, investigating new combinations of NADES components could facilitate the optimization of their rheological, sensory, and extraction properties, allowing for customization tailored to several types of topical formulations.

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