University of Camerino

International School of Advanced Studies

Ph.D. course in Chemical and Pharmaceutical Sciences and Biotechnology

Curriculum in Pharmaceutical, Nutraceutical and Food Sciences

XXXVI cycle

Characterization and application of excipients used in galenic preparations

Doctoral thesis

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AA.YY. 2020-2024

Table of Contents

INTRODUCTION

1. INTRODUCTION

1.1. Galenic

The term "Galenic" originates from Galen, an ancient Greek physician who initiated the practice of formulating his own remedies through the blending of fundamental substances. The Galenic involves the processes of formulating and preparing a medication in terms of its form and/or dosage. This constitutes a crucial and exclusive skill set within the domain of the pharmacist. [1]

1.2. History of Galenic

Historically, pharmacists (Figure n.1) played a key role in medicine, being the sole creators of medications. They were as preparers, executing doctors' prescriptions using plant-based

medicines and other naturally derived substances. The pharmacist held the monopoly on the drug, with a perfect and comprehensive knowledge of the medication.

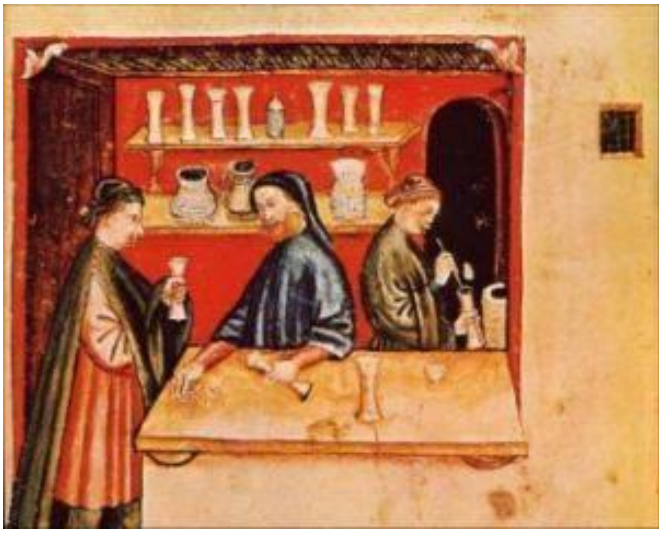

Figure n.1 *The ancient Galenist*

In the mid-19th century, the pharmacist, thanks to his skills in pharmacology, started researching new substances, thereby contributing to the birth of pharmacology. The active principles were then isolated and identified as a result.

Until that time, there were no ready-made medications, and the pharmacist had to compensate for this deficiency by formulating galenic preparations. Pharmacies began selling, in addition to traditional remedies, a wide range of new pre-packaged products, and for the first time in their history, they started producing their own specialties, that is, products ready for the treatment of mild conditions, with fancy names, unique and distinctive to that pharmacy, offered to the public without the need for a medical prescription. (Figure n.2)

Figure n.2 *The counter of an ancient pharmacy*

In the early 1900s, there were further discoveries in the pharmaceutical field that led to a significant transformation of galenic preparations. Many herbal drugs and historical active ingredients were replaced by the use of new molecules synthesized on an industrial scale. These substances were supplied to the pharmacist in powder form, and then, the pharmacist transformed them into the most appropriate pharmaceutical dosage form.

During the First World War, difficulties in supply and procurement of new substances led to a return to traditional products of plant origin and a temporary increase in galenic preparations.

During those years, however, the research and development of new drugs continued.

From that moment, only the industry was able to provide the ready-to-use medicine, with their pharmaceutical dosage form, packaging, and name. Industrial formulations gained increasing prominence in pharmacies, leading to a consequent loss of importance for galenic preparations. The pharmacist lost his crucial role and became a mere dispenser. The pharmaceutical industry became a protagonist in the field of pharmacology.

So, with the rise of the pharmaceutical industry, the significance of the galenist's role has diminished.

1.3. The revival of galenic preparations

Nowadays, the benefits of personalized medication are significant enough to revive the position of the “pharmacist preparer”, commonly referred to in the Good Compounding Practice (NBP) guidelines of the Pharmacopoeia. [2]

The benefits associated with utilizing a galenic medication dispensed by a pharmacist include:

- Considers the patient's age, allergies, and tolerance to active or excipient ingredients
- May involve incorporating different excipients
- May involve the simultaneous incorporation of two or more actives
- The active component exhibits chemical and/or physical instability, making it unsuitable for industrial production
- It is desired to improve the therapeutic compliance
- The dosage can be adjusted or set specifically for an individual patient
- The pharmaceutical form or industrial medicinal product that best suits the requirements is not currently accessible on the market

So, galenic medications offer extensive customization based on patient requirements and doctor's recommendations, while adhering to prevailing legislation.

The pharmacist is authorized to formulate galenic preparations exclusively within the designated galenic laboratory, which must adhere to specified adequacy standards and possess the equipment and substances outlined in the official pharmacopoeia.

The adequate competence of the prescribing doctor and the preparing pharmacist is indispensable because there is a need to ensure quality, effectiveness, and safety.

The pharmacist can prepare two types of products:

- Galenic preparations formulated only upon the arrival of the patient who provides a prescription issued by a doctor. The preparation of the medication prior to the arrival of the prescription is prohibited, and the responsibility is shared between the doctor and the pharmacist.
- Galenic preparations formulated within a pharmacy in accordance with the guidelines of a valid pharmacopoeia in a Member State of the E.U., with the purpose of being directly dispensed to patients within that specific pharmacy. The pharmacist

is allowed to prepare them at any time, with a maximum quantity not exceeding 3 kg of the preparation.

Furthermore, within the pharmacy, it is possible to formulate preparations containing herbal derivatives and cosmetic product. [3]

Formulations containing herbal derivatives must adhere to Good Compounding Practice (NBP), utilizing herbal products listed on the Ministry of Health's website and ensuring they do not exceed the recommended dosages per dose and per day as specified for market supplements.

Pharmacies have the option to formulate cosmetic products, but this should be done in a separate laboratory distinct from the one used for pharmaceutical production, ensuring compliance with the specific legal requirements.

1.4. Pharmaceutical dosage forms

The active ingredient is never administered alone, but it is always combined with one or more substances, known as excipients, for the formulation of the final product. Excipients play a crucial role in formulating the preparation, ensuring its quality and safety, and are significant in facilitating the delivery of the drug to the site of action. [4],[5]

Therefore, selecting the appropriate substances is vital in the manufacturing process of the medication, particularly in the pharmacy's preparation.

Pharmaceutical dosage forms (Figure n.3) are categorized into three primary systems:

- Solid (tablets, capsules, granulates…)
- Semi-solid (gels, ointments, creams, lotions…)
- Liquid (syrups, solutions, emulsions, eye drops…)

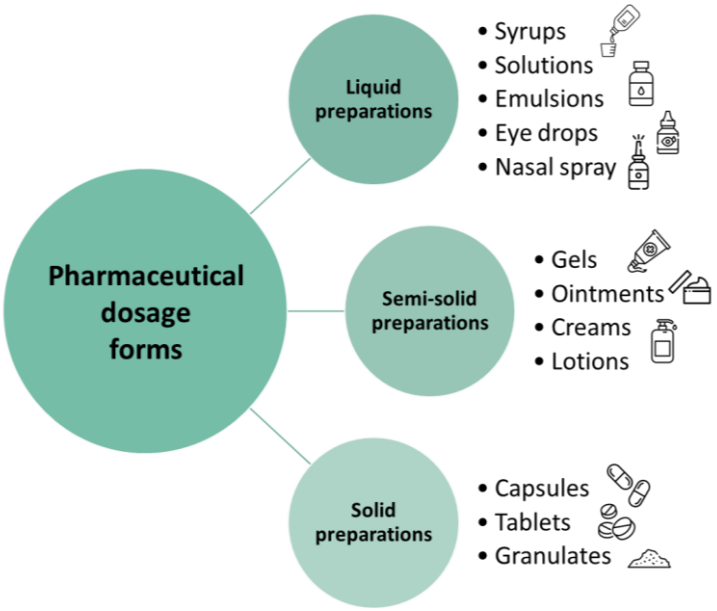

Figure n.3 *Pharmaceutical dosage forms*

In this study, the pharmaceutical dosage forms under consideration include water dispersions, emulsions, and semi-solid formulations.

1.5. Emulsion

Emulsions are dispersed systems comprising two immiscible liquids, where one is dispersed in the other in the form of droplets, creating a two-phase structure.

The components constituting an emulsion are:

- the internal phase (or discontinuous phase) → it constitutes the dispersed liquid phase,
- the external phase (or continuous phase) → it represents the phase in which the internal phase is dispersed.

Water and oil represent the prototypes of two immiscible liquids characterized by opposite polarities. In cases where water serves as the internal phase, the resulting emulsion is referred to as water in oil W/O emulsion, whereas when it constitutes the external phase, the emulsion is termed oil in water O/W emulsion.

Simply shaking vigorously would be sufficient to create an emulsion. However, because the cohesive forces among the molecules of the single liquid are stronger than those between two different liquids, the two phases are inclined to separate. Consequently, these systems are highly unstable and, as a result, require the presence of substances known as emulsifiers, which are hydrocolloids and surfactants. [6]

Emulsion stability

One of the characteristics of emulsions is the tendency of the two phases to re-aggregate. This leads to phenomena such as:

- Flocculation occurs when a phase tends to aggregate into small groups, referred to as flakes. In this case, the emulsion can be easily reconstituted by simple shaking;
- Coalescence occurs when the flocculate aggregates into compact, coarse particles, resulting in the irreversible separation of the two phases;
- Creaming occurs when the dispersed phase concentrates on the surface or at the bottom of the preparation. Shaking is necessary to restore the emulsion to its initial state, ensuring homogeneity.

Excipients employed in formulating emulsions

1. *Surfactants* → Compounds comprising a lipophilic, nonpolar segment composed of a lengthy hydrocarbon chain, along with a hydrophilic part consisting of polar groups.
2. *Hydrocolloids* → Linear-structured macromolecules with hydrophilic groups that can retain water molecules, resulting in increased viscosity. They can be classified into: natural, including gums, starches, alginates, agar, guar, gelatin, and bentonite; semi-synthetic, such as cellulose derivatives; synthetic, such as povidone and polyvinyl alcohol.
3. *Antioxidants* → the most commonly utilized ones include the propyl, octyl, and dodecyl esters of gallic acid, as well as butylhydroxyanisole and butylhydroxytoluene.

4. *Preservatives* → In general, oil-in-water emulsions are more susceptible to microbial contamination. Commonly employed options include esters of p-hydroxybenzoic acid and parabens.
5. *Humectants* → They are included to minimize water evaporation, both within the container and following application. The most commonly utilized compounds for this purpose are glycerol, propylene glycol, and sorbitol. [6]

1.6. Semi-solid formulations for topical application

Semi-solid formulations, upon applied to the skin, are designed for the localized or transdermal release of active ingredients, or they may exhibit emollient and/or protective properties, as stated in the pharmacopoeia. Semi-solid formulations are composed of a base, either simple or compounded, which can be constructed from natural or synthetic substances, possessing either hydrophilic or lipophilic characteristics.

The primary classifications of semi-solid formulations include:

- Ointments
- Creams
- Gels
- Pastes

The semi-solid consistency of these products is contingent on their three-dimensional structure, achieved through the use of various substances, either individually or in combination, commonly referred to as excipients.

1.6.1. Most common excipients for semi-solid preparations

The excipients employed in formulations for skin application must be inert, stable, non-irritating or sensitizing, and capable of promptly releasing the drug.

Based on their characteristics, they can be divided into lipophilic and hydrophilic excipients.

Lipophilic excipients that do not incorporate water

1. *Triglycerides*:

Natural oils and fats: They are composed of glycerol esters with monocarboxylic acids ranging from 10 to 18 carbon atoms, both saturated and unsaturated. The main issue in their use is rancidity.

Synthetic oils: They are glycerol esters with saturated fatty acids. They exhibit greater stability against oxidation.

Modified oils: They are hydrogenated triglycerides. They show increased resistance to rancidity and have a solid consistency at room temperature.

Butters: They are triglycerides composed of a mixture of saturated and unsaturated fatty acids. The semi-solid consistency is attributed to the presence of both liquid and solid glycerides.

2. *Esters of liquid fats*: They result from the combination of a fatty acid with an alcohol.
3. *Waxes*: They are monoesters, of plant or animal origin, obtained by condensation of acids with long-chain fatty alcohols. They have occlusive and anti-dehydrating properties, are soluble in organic solvents, and do not undergo oxidation.
4. *Hydrocarbons*: They are obtained from the residues of petroleum distillation. They are mixtures of n-paraffins, branched paraffins, and hydroaromatic hydrocarbons, with fluid, semi-solid, and solid consistencies, including paraffins and petrolatum. The advantage of paraffin is its compatibility with almost all chemical compounds and its high stability against atmospheric agents.
5. *Silicone polymers*: They are synthetic products, organopolysiloxanes with high chemical and physical stability. Depending on the chain length, number of bonds, and type of linked groups, silicones can be in the form of solid or semi-solid oily substances. When applied to the skin, they form persistent films with protective action.

Lipophilic excipients that incorporate water

The excipients within this category are referred to as adsorption bases. They are excipients capable of incorporating a certain amount of water and/or aqueous solutions, thus forming water-in-oil emulsions. This class includes both emulsifying bases and emulsified bases.

1. *Lanolin*: It is an anhydrous excipient, obtained through extraction and purification from wool grease. While it can incorporate a significant amount of water, it has an unpleasant odor, may lead to allergic reactions, and is susceptible to oxidation.
2. *Lanolin alcohols*: They are obtained by alkaline hydrolysis of lanolin and separation of the fraction containing cholesterol and other sterolic and triterpenic alcohols. They are often preferred over lanolin.
3. *Hydrophilic Petrolatum* of USP 23: It is composed of cholesterol, stearyl alcohol, white wax, and white petrolatum.
4. Lanolin/petrolatum: They are mixtures in various ratios of lanolin and petrolatum. They are employed because they can incorporate up to approximately 50% of water and exhibit better technological characteristics.

Hydrophilic excipients

1. *Polyethylene glycols* (PEGs): In the presence of sodium hydroxide, under specific conditions, ethylene oxide reacts with ethylene glycol, forming PEGs. Those with a molecular weight lower than 1000 are liquids at room temperature, while those with higher molecular weight are waxy solids. They serve as excellent solvents for many compounds, and when applied to the skin, they form non-occlusive films.
2. *Hydrocolloids*: They are macromolecular compounds, both organic and inorganic, capable of thickening water. Among these, we have:

- *Bentonite*: It is a hydrated aluminum silicate. In water, it increases viscosity or forms gels. It is therefore used as a stabilizer in oil-in-water emulsions or as an excipient for ointments.
- *Carbomer*: It is a synthetic polymer obtained by polymerization of acrylic acid. It is neutralized with alkali hydroxides, amino acids, and organic polar amines, dissolves in water, forming a gel. It is used as a thickening, dispersing, and gelling agent. [3]

1.6.2. Ointments

Ointments are a monophasic system in which solid or liquid substances can be dispersed. They are divided into:

- *Hydrophobic ointments* → They can absorb only small amounts of water. The most commonly used excipients are waxes, synthetic glycerides, vegetable oils, and paraffins. When applied, they have an occlusive action, allowing better penetration of the active ingredient. They have emollient properties and promote hydration, but are not easily removed with water.
- *Water-absorbing ointments* → They can absorb significant amounts of water. They are formulated by adding an emulsifier to the same bases used for lipophilic ointments. Consequently, they form either oil-in-water or water-in-oil emulsions depending on the type of excipient.
- *Hydrophilic ointments* → They are capable of absorbing a significant amount of water. They are formulated using bases that are water-miscible, such as mixtures of liquid and solid macrogols. They are used in the treatment of scaly and keratotic dermatoses. [1]

1.6.3. Creams

As outlined in the pharmacopoeia, creams are complex formulations comprising both aqueous and oily phases. Essentially, they are emulsions in which the dispersing phase, gelling, provides consistency. [2]

They are categorized into:

- *Hydrophobic creams* → Their continuous phase is the lipophilic phase, incorporating water-in-oil (W/O) emulsifiers such as sorbitan esters and monoglycerides. Applied to the skin, they form a continuous film and have nourishing, emollient, and moisturizing properties.
- *Hydrophilic creams* → Their continuous phase is the aqueous phase, incorporating oil-in-water (O/W) emulsifiers such as sodium or triethanolamine soaps, fatty alcohol sulfates, and polysorbates. They are easily absorbed when applied to the skin, and due to the rapid evaporation of water, they provide a refreshing effect.

The primary components of a cream can be divided into four categories:

- components of the hydrophilic phase
- components of the lipophilic phase
- gelling agent
- surfactant

In addition to these components, antioxidants are introduced to prevent the oxidation of sensitive substances, while preservatives are included to inhibit microbial proliferation in the aqueous phase.

1.7. Surface Tension

The surface tension is the cohesive force exerted between the surface molecules of a liquid. It is the energy required to increase the surface area by a unit quantity. Surface tension is greater when the forces between the particles are stronger.

The surface tension is expressed, in units of the International System, in N/m.

In the pharmaceutical field, the most commonly employed methods for measuring surface tension include the weight-of-drops method and the du Nouy tensiometer method.

1.8. Surfactants

Surfactants are compounds characterized by an apolar, lipophilic section, known as the "tail," composed of aliphatic chains or aromatic groups, and a hydrophilic portion, called the "head," consisting of polar groups like carboxyls, hydroxyls, amino, sulfuric, and others.

Surfactants are substances that reduce the surface tension between two phases, such as between two liquids, a gas and a liquid, or a liquid and a solid.

Based on their chemical structure, surfactants can be categorized into:

Anionic surfactants → Within an aqueous solution, molecules of anionic surfactants carry negative charges. The ionized part may consist of a carboxylate, sulfate, sulfonate, or phosphate group. Examples include soaps, sulfonic esters and sulfates (SDS), sulfonates.

Cationic surfactants → These are positively charged molecules. The majority of cationic surfactants are represented by quaternary ammonium compounds, such as benzalkonium chloride.

Amphoteric surfactants → They contain both anionic and cationic groups within the molecule. Examples include lecithin, casein, and gelatin.

Non-ionic surfactants → They do not ionize in aqueous solution. Among these, we have partially esterified polyvalent alcohols with fatty acids (Glyceryl monostearate), sorbitan esters (Spans), esters and ethers of polyethylene glycols, and polysorbates (Tweens). [7]

1.9. Aim of the project

This project is divided into two parts. The first part concerns the characterization and application of different types of keratins, while the other part focuses on the application of cetyl alcohol, glyceryl monostearate and sorbitan monostearate, already studied and commonly used, in oily systems capable of incorporating water, without the help of commonly used additional surfactants.

The common thread in these two works concerns the applied aspect, represented by the formulation of the most common pharmaceutical dosage forms.

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2. KERATINS

Over the years, there has been a growing interest in the research of materials that could replace traditional compounds derived from petroleum. The excessive use of petroleum poses environmental and non-environmental pollution problems, and it is, also, a finite source. For this reason, attention has shifted towards renewable, biodegradable, and sustainable resources for the development of polymeric materials. The choice falls on biopolymers, such as carbohydrates, proteins, and lipids, as they are abundantly available, renewable, biodegradable, and biocompatible. Among these, proteins are favored due to their characteristics and non-toxic nature, serving as a biological resource for the production of biomaterials. Among the most commonly used proteins are keratin, collagen, albumin, gelatin, and fibroin.[1], [2], [3]

2.1.Keratin

Keratin, a fibrous structural protein in epithelial cells [4], is the most abundant and important biopolymer in animals, after collagen. [5]

Keratinous materials, comprising organized keratinized cells filled with fibrous proteins (keratins), represent natural polymeric composites characterized by an intricate hierarchical

structure. This structure includes the polypeptide chain structure, filament-matrix structure, lamellar structure, and sandwich structure.[6] Specifically, keratin plays a crucial role as a fundamental element in various biological structures, including human hair, nails, claws, hooves, horns, scales, beaks and feathers. [7] It consists of scleroproteins, which are highly specialized fibrous proteins, and is generated by epithelial cells in higher vertebrates such as reptiles, birds, mammals and humans. [8]

2.2. History

The first utilization of keratin for medical purposes in history dates back to the sixteenth century, attributed to a Chinese herbalist named Li Shi-Zhen. Throughout her lifetime, she authored 800 books, detailing thousands of therapeutic prescriptions. Among the numerous substances she described, there was one named Xue Yu Tan, also known as Crinis Carbonisatus, which is made from powdered ash derived from pyrolyzed human hair. It was employed to expedite wound healing and blood clotting. The term "keratin" emerges in literature around 1850, used to denote the substance constituting hard tissues like animal horns and hooves. The term originates from the Greek word "*kera*", which means horn. [9]

Throughout the centuries, research initially emphasized the development of keratin extraction methods from various sources, for example, one of the early publications on methods for extracting keratin, a patent [10] from 1905. Subsequently, the focus shifted to the exploration of keratin's structure and function.

Since the 1970s, there has been an increase in publications regarding the improvement of extraction methods and characterization, which have led to the development of various applications, especially in the medical field. [11], [12]

2.3. Structure

Keratins are included in a category of insoluble proteins and belong to the superfamily of intermediate filament proteins, and normal tissue structure and function depend on their essential role. [13]

By employing X-ray diffraction (initial studies by Astbury and Street [14]), it became possible to categorize the keratin structure into two mainly patterns, which are the alpha-

helical conformation for hard keratins, and the beta-sheet structure. Alpha-keratin, present in mammals, serves as the main component in substances like wool, hair, nails, hooves, horns, and the stratum corneum of the skin. The predominant structure in the hard tissues of birds and reptiles, such as feathers, claws, beaks, scales, and reptilian claws, is the beta-form. [11]

Keratin can be also categorized into soft and hard types based on its sulfur content. Although both hard and soft keratin exhibit similar peptide structures with coiled-coil conformations, they differ in their amino acid compositions. Notably, hard keratin, with 5% sulfur, exhibits high cysteine content, low glycine content, and possesses structural durability and strength. It is present in structures such as hair, horns, feathers, nails, and tongue papillae. On the other hand, soft keratin, containing 1% sulfur, demonstrates less consistency and is easily fragmentable. In terms of secondary protein, it is found in epidermal tissue.[15], [16]

2.4. Sources

Key sources of keratin from livestock encompass sheepskins, goatskins, cattle hides, feathers, hairs, and buffalo hides. The skin and associated structures like feathers, wool, nails, hooves, hair, scales, and the stratum corneum represent the most abundant reservoirs of keratin (Figure n.1). Extraction can be performed from animal horns and hooves, wool, feathers, as well as human hair.[17]

Figure n.1 *Keratin sources* [18]

The keratin derived from hair has, compared to that obtained from other sources, a higher content of cysteine residues, forming more resistant structures through the formation of intermolecular disulfide bonds. It also features cellular binding motifs, such as leucine-aspartic acid-valine and glutamic acid-aspartic acid-serine binding residues. Approximately 300,000 t of hair are discarded worldwide every year, making it an excellent resource for keratin extraction, which can then be applied as an alternative biomaterial. [19]

2.5. Hair keratin

Human hair keratin (Figure n.2) referred to as hard keratin, consisting of 65-96% proteins, 1-9% lipids, 3% melanin, and other minor compounds. Keratin proteins can be categorized into three main groups: alpha-, beta-, and gamma-keratins. In alpha-keratin, the polypeptide chains adopt alpha-helical arrangements, while in beta-keratin, they assume a pleated beta-sheet configuration. Alpha-keratins feature an alpha-helical tertiary structure, with relatively low sulfur content, and an average molar mass spanning from 60 to 80 kDa. They are located within the cortex of the hair fiber. On the contrary, beta-keratins, primarily serving protective functions, make up the majority of the cuticle. Gamma-keratin is characterized by its

globular protein structure. These proteins are rich in sulfur amino acids and typically have a lower molecular mass ranging from 10 to 15 kDa compared to other forms. [20], [21]
The hair fiber consists of three components: the cuticle, the cortex, and the cell membrane complex. The cuticle primarily comprises β-keratins, whereas the cortex is a mix of α-keratins (50%-60%), matrix proteins (20%-30%), and melanin granules. The matrix's keratin-associated proteins are high-sulfur α-keratins with a molecular weight ranging from 10 to 25 kDa. They play a crucial role in binding filaments together and preserving the structure of the hair fibers. [22]

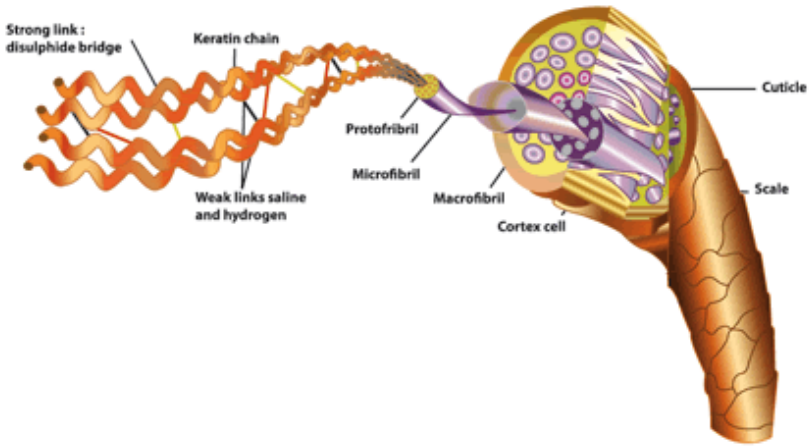

Figure n.2 *Hair keratin structure* [23]

2.6. Extraction methods

Over the years, various extraction methods for keratin from diverse sources have been developed and continually improved to obtain larger quantities of keratin-containing materials. Given the high content of keratin, hair and wool represent the most used starting material for keratin extraction.
Below are reported some examples of extraction methods [24], [25], [26].

Chemical Hydrolysis

Chemical hydrolysis is usually accompanied by the heating of the material to achieve high efficiency, although elevated temperatures tend to degrade amino acids. The yield of

hydrolysis depends on factors such as pH, temperature, reaction time, and the concentration of acid or base used.
The oxidation of keratin is achieved by using peracetic acid, ammonia, and hydrochloric acid. This method is primarily employed for extracting keratin from hair and wool.

In Brown et al.'s work [27], the use of denaturing agents such as 2-mercaptoethanol or sodium metabisulfite and urea was evaluated for the reduction process, as they target all the bonds in keratin fibers.

Enzymatic and microbial treatment

Microorganisms such as mesophilic fungi, actinomycetes, and some species of Bacillus assist in keratin extraction because they can secrete keratinolytic and proteolytic enzymes called keratinases, which are used for the bioconversion of keratin waste. Keratin is extracted from feathers and wool through the action of the enzyme Savinase. Enzymatic hydrolysis requires low energy but is carried out using reducing agents that break the disulfide bonds. Keratin degradation occurs through proteolysis, sulfitolysis, and deamination. The first two processes lead to the cleavage of disulfide bonds due to the release of sulfite material from microorganisms. Deamination occurs when microbial enzymes attack insoluble keratinous protein substrates, releasing molecules with free amino groups.

Dissolution in ionic liquids

Ionic liquids are salts composed of an organic cation and various organic and non-organic anions. They exhibit unique characteristics such as low vapor pressure, high ionic conductivity, high thermal stability, solubility for specific solutes, and they are neither volatile nor flammable. Thanks to all these features, they are widely used in many applications such as solvents for polymers, facilitating the solubilization of materials like silk, wool, cellulose, and chitin.

Microwave irradiation

In this technique, the activation energy required for extraction is not high, and the sample undergoing analysis is heated and degraded uniformly and rapidly. Examples of this

extraction method are reported by Bertini et al.[28], where the extraction is carried out at 180 degrees for 30 minutes, yielding 31%. Feroz et al.[12] extracted keratin from feathers by subjecting them to a temperature of 160-200°C for 20 minutes, with a yield of 71.83%.

Thermal hydrolysis or superheated process

This extraction method involves treating the sample with water under specific temperature and pressure conditions until the transformation of proteins into oligopeptides occurs. Initially, there is denaturation of the network of intermediate filaments, followed by the breaking of disulfide bonds that hold the keratin fibers together. An example of extraction was carried out under constant pressure, heating up to 200 °C, resulting in a yield of approximately 70%[29].

Steam explosion process

Steam explosion is an extraction technique that uses high-pressure saturated steam for short intervals, typically 1-10 minutes. The material is maintained at a temperature of 180-230°C in the reactor. Towards the end of the process, the pressure is rapidly lowered, causing decompression and consequently breaking the fibers of the material. The result is influenced by various factors related to temperature, resistance time, particle size, and humidity. This extraction technique was first used by Miyamoito et al., in 1982, for the extraction of keratin from wool, successfully converting 80% of the wool to a pepsin digestible material.

The steam flash-explosion (SFE) represents an evolved version of steam explosion, utilized as a pre-treatment method to improve the solubility of feathers in water and other solvents. In this procedure, steam pressure is sustained at 1.4–2 MPa for 30 seconds to 5 minutes, followed by a rapid generation of the explosion.

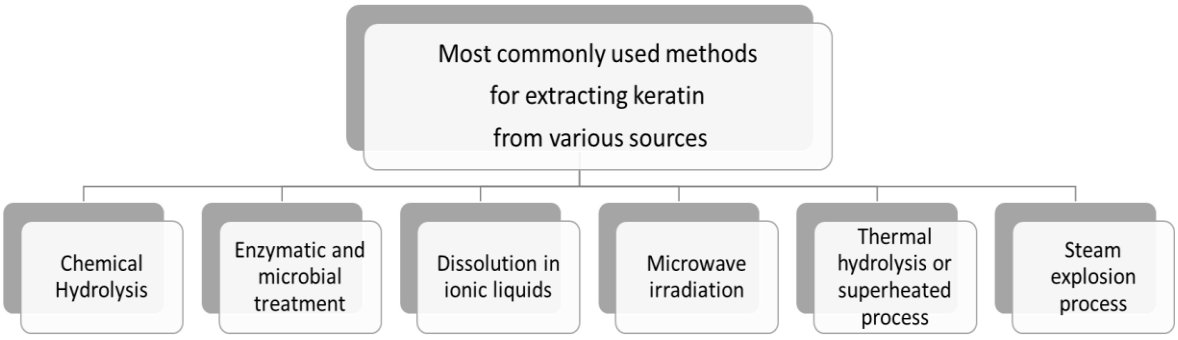

Figure n.3 *Most common methods of Keratin extraction*

2.7. Biomedical, pharmaceutical applications

Keratin-derived materials hold great potential for transforming the field of biomaterials, owing to their inherent biocompatibility, biodegradability, mechanical strength, and widespread availability.[9]

The key features of keratin-based biomaterials include the ability to self-assemble, biocompatibility, biodegradation, and support for cell proliferation. The self-assembly property has been studied at both the nano and macro scales, enabling polymerization into porous scaffolds. Some types of biomaterials have demonstrated the ability to support cell attachment due to the presence of cell-binding motifs. Numerous studies have been conducted for the fabrication of new biomaterials containing keratin in the form of films, sponges, fibers, gels, and scaffolds. [12], [22], [30]

Keratin has been investigated also for its potential applications in biomaterials, including wound healing[31], bone regeneration[32], hemostasis[33], and more recently, in the repair of peripheral nerves[34].

Keratin has also been utilized in the formulation of keratin nanoparticles, proving to be effective in drug delivery, as demonstrated in the work of Wu et al. [35].

2.8. Cosmetic application

Hydrolyzed keratin is also used in the formulation of cosmetic products, particularly for hair and skin treatment. Keratin peptides enhance the mechanical and thermal properties of hair, as well as moisture, making it shinier and softer. At the skin level, keratin peptides have moisturizing effects and can reinforce the barrier function. Furthermore, once solubilized, they can strengthen the nail plate by binding to the nail. [24], [36]

2.9. Other application

Keratin, from feathers, having a substantial nitrogen content, is also utilized as a fertilizer. Feather hydrolysate, having a composition similar to soy and cottonseed proteins, is used as a dietary supplement for ruminants. Therefore, keratin is employed as a protein in the preparation of animal feed. Keratin waste can be employed in leather tanning procedures, with keratin hydrolysate serving for filling and retention in the leather processing stages. Additionally, feathers can act as a yarn-sizing agent, improving weaving performance while enhancing the tensile strength and abrasion resistance of the yarns. [37]

2.10. Aim of the work

The work is focused on the extraction of keratin from human hair using three methodologies, while simultaneously studying commercial hydrolyzed keratins. Both types of keratins were chemically and physically characterized through a series of analyses, both in solid-state and in aqueous solution. Subsequently, they were evaluated as potential alternative surfactants in pharmaceutical preparations, such as emulsions.

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2A. EXTRACTED KERATINS

2A.1. MATERIALS

Human hair from a mix of individuals was collected from a local hairdresser salon. Sodium hydroxide (Riedel-de Haen, DE), Urea (Carlo Erba, Milano, IT), sodium disulfite (Carlo Erba, Milano, IT), sodium dodecyl sulfate (AppliChem, Darmstadt, DE); Hexane (Carlo

Erba, Milano, IT) were used for keratin extraction. Dialysis was performed using a membrane 6-8 kD (Spectra/Por molecular porous membrane tubing, Fisher Scientific, Milano, IT). Glyceryl monooleate (Peceol, Gattefossè, Saint-Priest, FR), Ketoconazole (Farmalabor, Milano, IT) and Sepimax-zen© (Seppic, Milano, IT) were used for the formulation of emulsion with the extracted keratin.

2A.2. METHODS

2A.2.1.Keratin Extraction from Hair

Keratins were obtained from a hair batch through three distinct techniques: extraction utilizing Sodium Hydroxide 0.5 M, extraction involving Urea 8 M - Sodium Dodecyl Sulfate 0.2 M - Sodium metabisulfite 0.5 M, and extraction using Urea 8M – Sodium Metabisulfite 0.5 M.

10 grams of untreated hair were subjected to incubation in 300 milliliters of three distinct solutions, which included 1) NaOH 0.5 M 2) Urea 8 M, SDS 0.2 M, $Na_2S_2O_5$ 0.5 M and 3) Urea 8 M, $Na_2S_2O_5$ 0.5 M. The extraction process involved heating in an oil bath at 80°C for one hour for the first method, and at 100°C for 8 hours for the remaining two methods.

Following hydrolysis, the solution underwent filtration with filter paper and was subsequently transferred into a separating funnel by introducing a quantity of hexane to enhance the extraction efficiency.

Dialysis membranes, featuring pores ranging from 6-8 kDa, were filled with the filtered solution and immersed in distilled water. They were left to undergo dialysis for a minimum of 15-20 days at room temperature, facilitating the elimination of salts formed during the extraction process.

The solution, now free from salts, was centrifuged for 10 minutes at 13000 rpm, leading to the removal of the precipitate. The solution was then placed in a flask in the freezer at -20°C overnight and subsequently at -80°C for two hours. Following this, it was transferred into the freeze-dryer to obtain lyophilized keratin powders.

2A.2.2.Elemental Analysis

The elemental analysis revealed the proportion of each element in both commercially hydrolyzed keratins and extracted keratins.

It is an analytical technique used to determine the quantities of carbon, hydrogen, nitrogen, and sulfur present in a compound. The analysis is based on the combustion of the sample: during combustion, the sample produces uniform gaseous compounds consisting of the elements carbon, hydrogen, nitrogen, and sulfur. These combustion products are determined using gas chromatography, and the ratio of elements in the original sample is consequently determined. The quantities of C, H, N, and S can be determined simultaneously.

The instrument used was the Flash 2000 (Organic Elemental Analyzer), which provided information on the carbon, hydrogen, nitrogen, and sulfur content in the keratin powder.

2A.2.3.Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Electrophoresis is an electrokinetic technique that allows the separation of charged molecules following the application of an electric field. Proteins have ionizable groups, so in solution they are electrically charged species. Under the force exerted by the external electric field (electrostatic Coulomb force) these molecules migrate to cathode or anode according to their charge, negative or positive respectively. In this type of electrophoresis, migration occurs on a matrix formed by polyacrylamide molecules, used to separate any type of protein. This matrix is usually prepared by polymerizing acrylamide monomers in the presence of small quantities of N, N'-methylene bis-acrylamide. Polymerization begins when persulfate and TEMED are added. TEMED decomposes the persulfate ion, producing free radicals and polymerize the acrylamide. Polyacrylamide is then able to form gels in water. It should be noted that the sample to be separated must be dissolved in the same buffer used to saturate the gel, ensuring homogeneous conditions during electrophoretic separation.

The samples were added with Sample Buffer then boiled at 100 °C for 5 minutes in a water bath to promote protein denaturation. The migration speed of the sample components will depend on their shape, size, and current intensity. An "Electrode buffer" is also used, which allows maintaining the electric field, and is formed by both the buffer used to prepare the gel and the one used to run the sample. Electrophoresis on polyacrylamide gel can be performed in both native and denaturing conditions. The latter, which was performed in this study,

requires the use of an anionic detergent – commonly Sodium Dodecyl Sulfate, for this reason it is referred as SDS-PAGE. In SDS-PAGE, the SDS anionic detergent denatures the proteins giving them a uniform negative charge. Therefore, proteins in a sample will result in having a similar shape (since they have been denatured) and a similar net charge. Therefore, proteins will migrate from the negative to the positive pole with a speed that depends only on their molecular weight.

Two types of gels were prepared:

- The stacking gel, situated at the top of the gel, possesses a porous structure with large pores, facilitating the entry of all proteins and priming them for the electrophoretic process. Additionally, the stacking gel contains wells where samples are loaded.
- The running gel, positioned below the stacking gel, typically contains a higher polyacrylamide concentration (ranging from 7.5% to 15%), tailored to the size of the proteins to be separated. It acts as a medium through which proteins migrate during the electrophoretic process.

Once the two types of gel were prepared and inserted on the appropriate support, the samples (previously treated with a buffer containing β-mercaptoethanol and SDS and heated at 100 ° C for 5 minutes to completely denature the proteins) were loaded into the wells on the Stacking gel.

In this study the SDS-PAGE was performed, as described by Laemmli (1970), under denaturing conditions with a 15% acrylamide-bis-acrylamide solution, using the Mini Protean III® instrument (Bio-Rad, gel size 7x8 cm x0.75mm). The markers used were Bio-Rad Low-molecular weight (phosphorylase b, 97.4 kDa; serum bovine albumin, 66.2 kDa, ovalbumin, 45.0 kDa; carbonic anhydrase, 31.0 kDa; trypsin inhibitor, 21, 5 kDa; lysozyme, 14.4 kDa).

20 µL of keratin 2 mg/mL were incubated with the denaturing solution. Electrophoresis occurred at 200 V for 40 minutes. At the end, the gel was removed from the gel holder, placed in a plastic tray and covered with Coomassie Blue dye (0.1% Coomassie Brilliant Blue R250 in 50% methanol and 10% acetic acid) for one hour. Then the Coomassie Blue dye was discarded, after which Destain I bleaching solution (50% methanol and 10% acetic acid) was added for about 3 hours.

2A.2.4.Differential Scanning Calorimetry (DSC)

Differential scanning calorimetry (DSC) analyses were conducted through the DSC 8500 Perkin Elmer instrument to determine the temperature and heat flow associated with various transitions in the sample. These transitions included melting enthalpies, glassy transitions, and crystallization kinetics of polymeric materials. Each sample, weighing approximately 4 mg, was placed in sealed pans and subjected to heating from 20°C to 300°C at a rate of 10°C per minute.

2A.2.5.Thermogravimetric Analysis (TGA)

Thermogravimetric analysis (TGA) is an experimental technique that involves measuring the weight, or more precisely, the mass of a sample in relation to its temperature or time. Analyses were carried out through the Simultaneous Thermal Analyzer STA 6000 instrument, which allows samples to be subjected to a constant heating rate or held at a consistent temperature. In this investigation, samples underwent heating from 30 °C to 700 °C at a ramp rate of 10 °C/min under nitrogen flow.

2A.2.6.Tensiometric Analysis

The tensiometric analyses were performed using the De-Nouy ring method with the DCA-100 tensiometer. This method was employed to investigate the surface properties of surfactants and their capacity to reduce water-air interfacial tension.
Keratin solutions were prepared at various concentration, from 0.00097 mg/mL up to 4 mg/mL.
The analyses proceeded from the least concentrated to the most concentrated solutions.
A total sample volume of 60 mL was used for each analysis at a temperature of 20°C. For each concentration, three surface tension values were measured and subsequently averaged.

2A.2.7.Conductimetric Analysis

Conductimetric analysis involves measuring the electrical conductivity of electrolytic solutions. In this research, the CRISON microCM 2200 conductivity meter was employed to assess the conductivity of keratin solutions. The samples were analyzed at increasing concentration, from 0.00097 mg/mL up to 4 mg/mL, beginning with the most diluted and progressing to the most concentrated, all conducted at 25°C.

2A.2.8.Cytotoxicity Test

Cellular viability test

Cell viability was established by examining cells under a phase contrast microscope with the 0.4% PBS Tripane Blue test. It allows to discriminate vital cells from dead cells as it is taken only by the latter. The cell count is performed with the Fuchs-Rosenthal counting chamber: 30 µL of cell suspension are mixed with 30 µL of Tripane Blue solution. The total number of cells, obtained by multiplying the average value of the count by the correction factor depending on the characteristics of the counting chamber, represents the number of cells contained in one mL of cell suspension.

Cytotoxicity test: MTT assay

100 µL of the cell suspension, with a density of 2 x 10^4 cells / mL, were sown in 96-well plates. After 24h, they were added to the medium at different concentrations in a range between 1.95 and 2000 µg/mL.

The plates were incubated for 72h. Proliferation was determined using the MTT colorimetric method. The MTT assay (3-(4.5-dimethylthiazol-2 yl)-2.5-diphenyltetrazolium bromide) exploits the ability of mitochondrial dehydrogenases to reduce tetrazolium salt to give rise to formazano crystals.

MTT was solubilized in PBS (2.7 mM KCl, 1.5 mM KH_2PO_4, 137 mM NaCl, 8 mM Na_2HPO_4) at a concentration of 5 mg/mL and filtered. After 72 hours of incubation, 10 microliters of MTT solution were added to each well and the plate was incubated at 37°C for a further 4 hours in a humidified environment to prevent evaporation.

The intracellular reduction of tetrazolium salts by the succinate dehydrogenase enzyme determines the formation of blue formazano crystals at the bottom of the wells. At the end

of the incubation time the supernatant was removed and 100 μL of dimethyl sulfoxide (DMSO) per well were added. The plate was shaken for about 15 minutes in order to solubilize the formed crystals and allow the absorbance of the colored solution to be measured at the FLUOstar Omega spectrophotometer (BMG Labtech) at 540 nm wavelength.

Cell viability was calculated as the percentage ratio of the absorbance of the sample to the control.

2A.2.9.Keratin Application

Emulsion Preparation

Emulsions are mixtures of two immiscible liquids. In this investigation, O/W emulsions have been created, with Peceol serving as the dispersed phase and water acting as the continuous phase. The surfactant agent was extracted keratin. Ketoconazole has been chosen as the active ingredient, as it is an antifungal and is found in shampoos used for the treatment of seborrheic dermatitis. Ketoconazole has shown solubility in oil, whereas keratin exhibits solubility in water. The emulsion was formed by mixing the two phases through Ultra-turrax at 9500 min $^{-1}$ for 5 minutes. The sample was placed on a heating plate at 40 degrees under stirring for one hour. Subsequently, it was homogenized once more using an Ultraturrax for an additional 5 minutes. The emulsion was subsequently thickened using Sepimax-Zen© to adjust its viscosity.

Microscope Analysis

Microscopic analyses were conducted to observe the stability of emulsions over time and assess the potential emulsifying properties of keratin. The instrument utilized for these analyses was the MT 9200 microscope from Meji, which was equipped with a camera (Invenio 3s) and had a magnification of 10x.

Test Hair Regrowth Promotion

Study design:

The therapeutic effect of Ketoconazole and Keratin was tested on hair regrowth promotion. Twenty-four adult Wister Albino mice were used (age= 6-7 weeks and weight= 25-35 g), obtained from Animal House of faculty of pharmacy at Ain Shams university. Prior to initiating the test, the mice underwent a 7-day acclimatization period (temperature 25°C, 12/12 light and dark cycles), after which they were randomly allocated into four groups.

- Group I: control group with no treatment
- Group II (standard group): treated with Commercial cream (Nizoral®)
- Group III (T1): treated with Ketoconazole cream
- Group IV (T2): treated with Ketoconazole and keratin cream

The dorsal skin of mice (2 cm x 2 cm area), in telogen phase, was shaved 24 hours before the experiment, using a clipper. The treated area was the shaved dorsal skin, with the control group not undergoing any treatment. The creams under examination were applied topically to the shaved regions every day for a duration of 21 days.

Photographs of dorsal areas were taken for each group using a digital camera on days 1, 7, 10, 14, and 21. The assessment of hair regrowth activities in various groups was conducted by examining both the condition of hair growth and the length of new hair. The qualitative evaluation of hair growth involved visually observing two parameters: hair growth initiation (the shortest time to initiate noticeable hair growth) and hair growth completion time (the minimum time taken to fully cover the denuded skin region with new hair).

Three weeks post-treatment, mice were euthanized through cervical dislocation following anesthesia, and skin samples were collected. They were fixed in 10% neutral buffered formalin for histopathological analysis. Tissue sections were stained by Hematoxylin and Eosin (H&E) and inspected by using light microscope (Axiostar plus, Zeiss, NY).

The percentage of hair follicles in the telogen (terminal stage) and anagen (growth phase) phases, along with the skin thickness, was assessed.

2A.3. RESULTS AND DISCUSSION

2A.3.1. Elemental Analysis

Elemental analysis enabled the examination of the chemical composition of both commercial and extracted keratins, focusing on the detection of carbon, hydrogen, nitrogen, and sulfur content.

Table n.1 *Chemical composition of commercial and extracted keratins*

	Commercial Keratin	**Keratin (NaOH)**	**Keratin (Urea-SDS-$Na_2S_2O_5$)**	**Keratin (Urea-$Na_2S_2O_5$)**
Nitrogen %	15.58	12.45 ±0.20	12.48 ± 0.09	13.40 ± 0.19
Carbon %	43.84	47.49 ± 0.19	39.81 ± 0.20	39.56 ± 0.14
Hydrogen %	6.77	6.49 ± 0.03	5.90 ± 0.23	5.73 ± 0.18
Sulfur %	0.50	1.06 ± 0.01	8.17 ± 0.06	7.93 ± 0.31

The Table n.1 illustrated that the levels of nitrogen, carbon, and hydrogen were quite comparable across the various keratins. However, the sulfur percentage was notably low in commercial keratin and higher in the others, particularly in the keratin extracted in the presence of SDS. This difference depended on the extraction method used. In fact, in the presence of SDS, sulfur values were higher and similar to each other, while in the case of keratin extracted with NaOH, the quantity was lower, more similar to those of commercial keratin.

2A.3.2.Polyacrylamide gel electrophoresis (SDS-PAGE)

Polyacrylamide gel electrophoresis (SDS-PAGE) was employed to verify the presence of keratin in the samples by assessing their molecular weight, comparing them to both commercial keratin and a reference standard.

The literature contains information on the molecular weights of keratins determined through electrophoresis, revealing the identification of two prominent bands (45-62 kDa) and several less distinct bands (10-20 kDa and 6-9 kDa).

To ensure the reliability of the results, two electrophoretic analyses were conducted. In the initial analysis, samples comprised non-dialyzed solutions, while in the subsequent analysis, solutions were prepared from the end product obtained through the processes of dialysis and lyophilization. The concentration of the solutions was maintained at 3 mg/mL.

Legend:

- Standard
- I = Commercial Keratin
- II = Keratin extracted with NaOH
- III = Keratin extracted with Urea – SDS – $Na_2S_2O_5$
- IV = Keratin extracted with Urea – $Na_2S_2O_5$

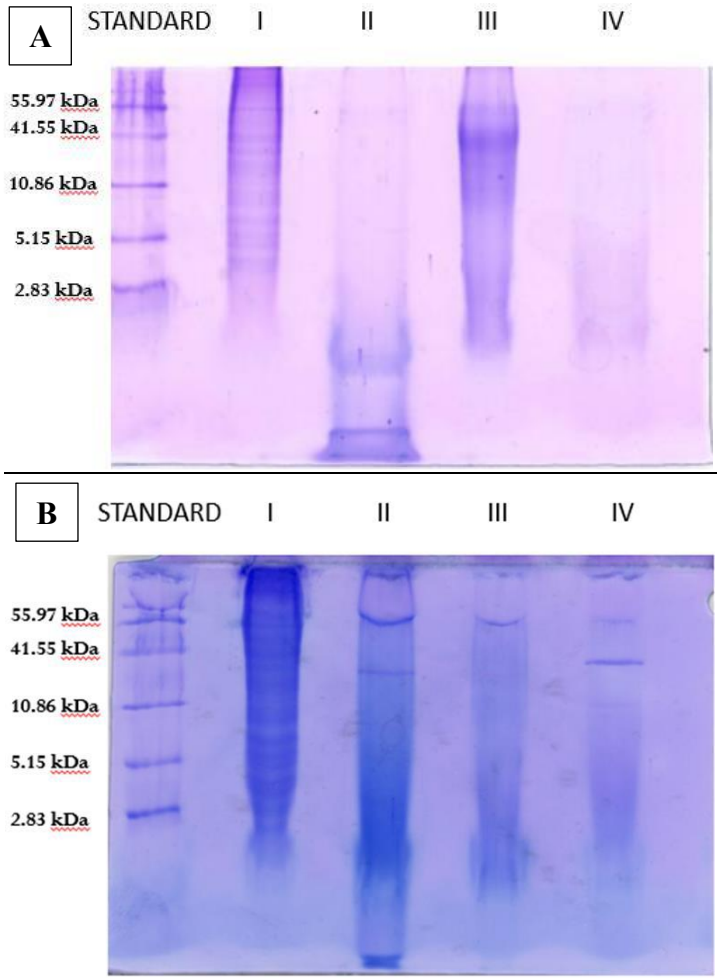


Figure n.1 *Electrophoretic gel before (A) and after (B) dialysis*

Commercial keratin exhibited numerous bands at various kDa values, suggesting the presence of protein fragments with diverse molecular weights. This observation was evident in both electrophoretic gels.

The keratin extracted using NaOH displayed a tail at the end of the stroke before dialysis, demonstrating the existence of low molecular weight peptides. Conversely, after dialysis, it exhibited distinctive bands.

The keratin extracted with Urea - SDS - $Na_2S_2O_5$ exhibited two discernible bands before dialysis within the 41-63 kDa range. However, post-dialysis, only a single band was observable.

These bands were indicative of the electrophoretic keratin patterns documented in the literature.

For the keratin extracted with Urea - $Na_2S_2O_5$, no bands were observable before dialysis; however, after dialysis, distinct bands became visible.

2A.3.3.Thermal Analyses (DSC/TGA)

Differential scanning calorimetry (DSC) and thermogravimetric analysis (TGA) enabled the assessment of endothermic transitions related to various keratins, aiming to establish the extent of their differences.

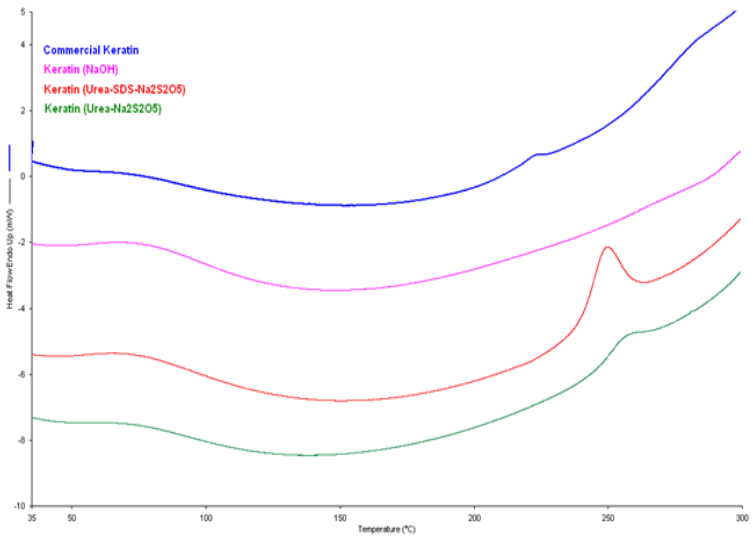


Figure n.2 *Differential scanning calorimetry (DSC) of commercial and extracted keratins*

DSC analysis revealed a consistent initial water loss between 50°C and 100°C for all keratins. Additionally, each plot exhibited a subsequent endothermic transition associated with protein denaturation. In the case of commercial keratin, this transition occurred at around 220°C, while for the extracted keratins, it took place at around 250°C. Among the three extracted keratins, the transition was more pronounced in the sample extracted with a solution of urea, SDS, and $Na_2S_2O_5$, suggesting that SDS played a role in preserving the keratin structure during extraction.

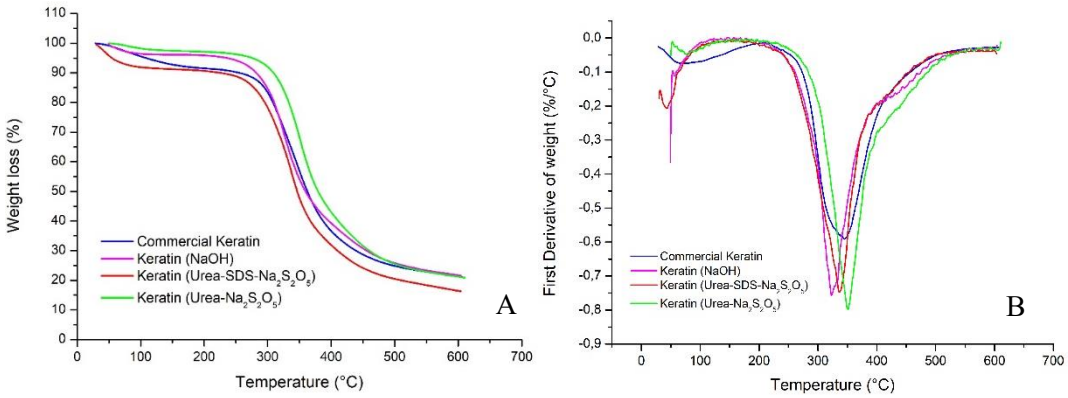


Figure n.3 *Thermogravimetric analysis (TGA) of commercial and extracted keratins(A) and its first derivative plot(B)*

TGA results corroborated the findings from DSC analysis. An initial weight loss of less than 10% was observed, attributed to water loss. Subsequently, a significant weight loss occurred, indicating protein degradation, within the temperature range of 300°C to 400°C. Notably, there was no discernible difference between the commercial keratin and the extracted keratins, suggesting that the extraction methods did not impact the analysis.

2A.3.4.Tensiometric and Conductimetric Analyses

A stock solution of keratin, solubilized in water, was prepared, and from this, by dilution, solutions at different concentrations were obtained, ranging from 4 mg/mL to a concentration of 0.00097 mg/mL. These solutions were then analyzed in increasing order of concentration, to assess their adsorption capacity at the water-air interface using tensiometric and conductometric analyses.

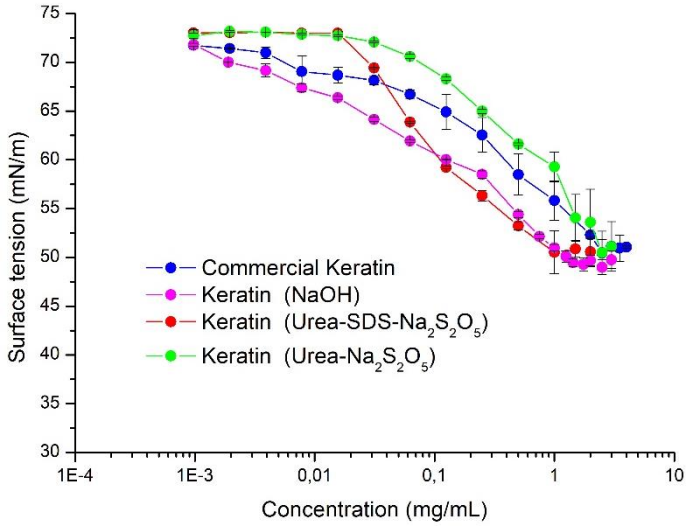


Figure n.4 *Variation of surface tension between solutions of different keratins*

At low concentrations of keratin, surface tension values closely resembled that of water, approximately 70 mN/m. In this scenario, keratin concentration exhibited minimal influence on surface tension.

With increasing concentration, there was a gradual reduction in surface tension, reaching values around 50 mN/m. The resulting profiles exhibited similarities across different keratins, suggesting that the extraction methods had no discernible effect on the surface tension of the keratin.

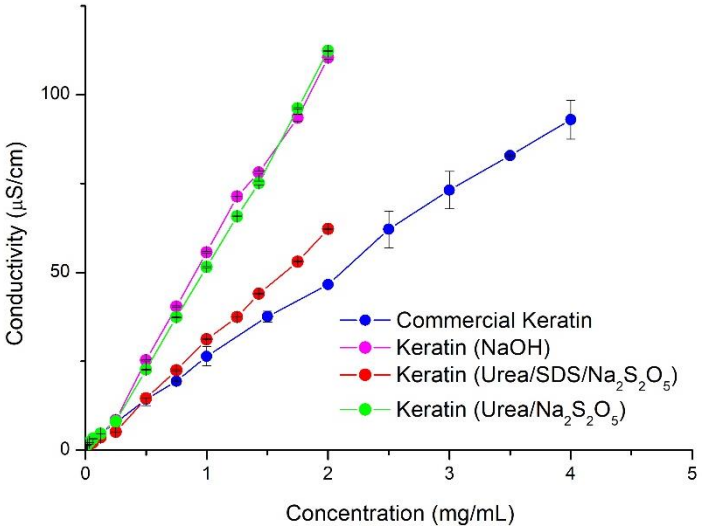


Figure n.5 *Conductivity trend of commercial and extracted keratins*

Similarly, the measured conductivity values for all samples (Figure n.5) were consistently below 100 µS / cm, indicating the successful and effective completion of the dialysis process.

2A.3.5.Cytotoxicity Test

To carry out a preliminary evaluation of keratin cytotoxicity, the human tumor cell line A431 (Squamous epidermis carcinoma) was used as cell culture.

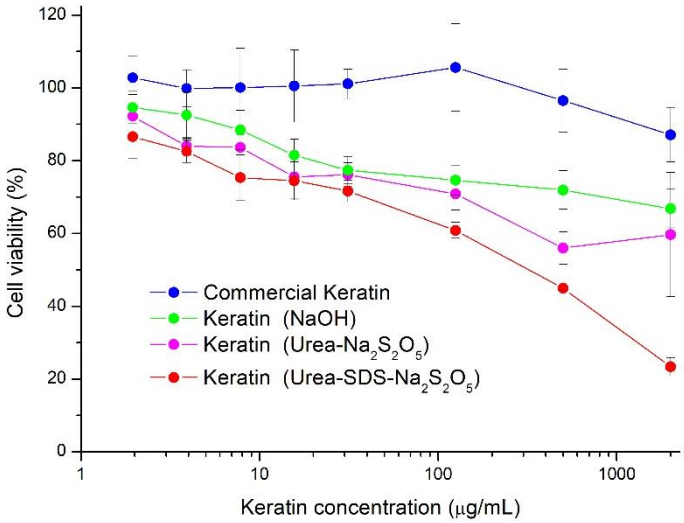


Figure n.6 *Keratin toxicity using human cancer cells A431*

As shown in Figure n.6, for the commercial keratin, cell viability remained consistently high at around 100% across all tested concentrations (up to 2 mg/mL). A minor decline in cell viability, approximately 70%, was noted for keratin extracted using NaOH or Urea-$Na_2S_2O_5$. However, in the case of keratin extracted with Urea-SDS-$Na_2S_2O_5$, a substantial decrease in cell viability was observed, reaching as low as 20% at concentrations exceeding 2 mg/mL. This decline could be attributed to the presence of SDS.

2A.3.6.Keratin Application

Emulsion Preparation

After conducting preliminary tests on the extracted keratins, it was chosen to employ the keratin extracted in the presence of NaOH. This decision was based on the observation that it exhibited similar even better characteristics to the other variants, and additionally, it was obtained in larger quantities. Experiments were conducted to assess the solubility of ketoconazole in oil, aiming to determine the optimal concentration required for formulating the emulsion.

The final formula of the emulsion (Figure n.7) was thus presented: Ketoconazole 1% w/w, Oil (Peceol) 10% w/w, Keratin 10% w/w, and water 79% w/w.

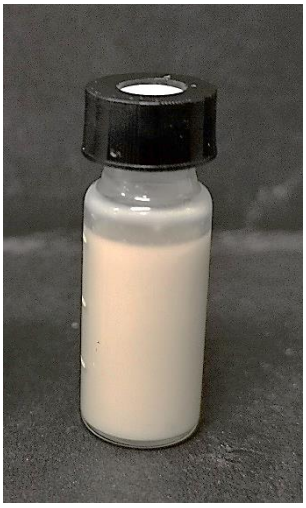


Figure n.7 *Ketoconazole and Keratin emulsion*

The amounts of oil were chosen to facilitate the solubilization of the active ingredient, while the quantities of keratin served to stabilize the O/W emulsion and simultaneously enhance the solubilization of Ketoconazole.

The emulsion was formed by dissolving Ketoconazole in oil and keratin in water. Using an Ultra-Turrax, the oily phase was gradually introduced into the aqueous phase to ensure proper homogenization. To enhance the solubilization of the active ingredient, the emulsion was placed on a heating plate at 40°C with stirring for one hour. Subsequently, it underwent further processing with the Ultra-Turrax.

After determining the concentration of the various components a cream, as final product was formulated. This cream was essentially the emulsion to which a thickening agent has been added, in order to increase its consistency. The selected thickening agent was Sepimax-Zen (1% w/w), and it was incorporated into the emulsion by solubilizing it.

This formulation was tested to assess the combined therapeutic impact of Ketoconazole and keratin on hair regrowth promotion.

Microscope analysis

Microscopic analysis enabled the confirmation that each component of the emulsion was effectively in solution. Simultaneously, it provided insights into the surfactant capabilities of keratin.

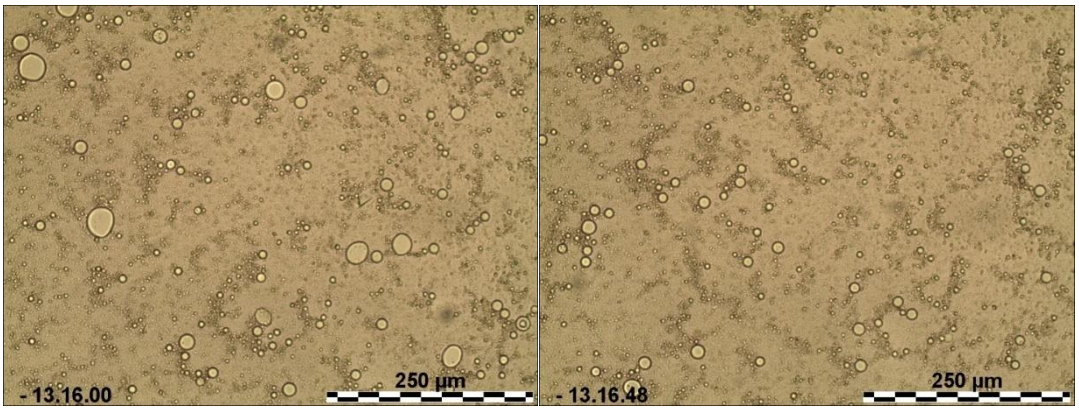


Figure n.8 *Microscopic observation of the emulsion containing Ketoconazole and Keratin*

Figure n.8 demonstrates the absence of crystals, indicating the homogeneity of the obtained emulsion. The presence of droplets, however, was attributed to the emulsifying properties of keratin.

Test Hair Regrowth Promotion

The formulation containing Ketoconazole and Keratin was tested to evaluate its therapeutic effect on hair regrowth promotion.

Regarding qualitative and quantitative hair growth, the hair growth initiation time in the various groups was determined, which corresponded to the photographs taken during the test. Additionally, the extent of hair length was also assessed.

Table n.2 *Initiation and completion time hair growth*

	Initiation time (days)	**Completion time (days)**
Control group	10	21
Nizoral cream	8	21
Ketoconazole cream	6	14
Ketoconazole + Keratin cream	4	14

As shown in Table n.2, in the control group, hair growth initiation was observed in the 2nd week (on the 10th day) within the denuded area. Similarly, in the commercial product (Nizoral®), hair growth initiation occurred on the 9th day. In contrast, the initiation of hair growth was significantly accelerated with the application of Ketoconazole cream (T1) and Ketoconazole and keratin cream (T2). Both treatments led to a rapid induction of hair growth on the 6th and 4th days, respectively. Complete hair growth was achieved on the shaved area after 2 weeks in all treatment groups (II-IV), except for the control group (Group I), which exhibited complete hair growth at the end of the experiment (21 days).

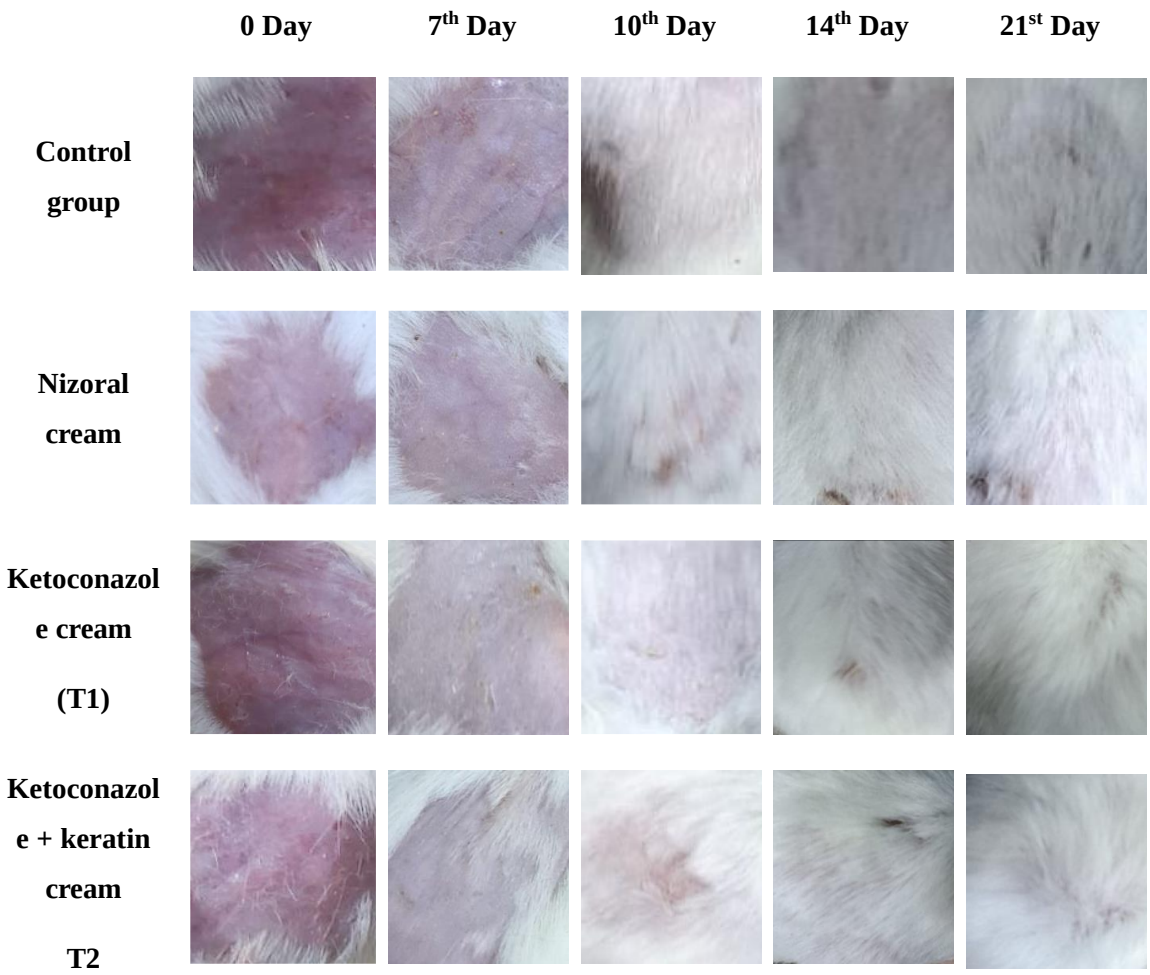


Figure n.9 *Photographs of dorsal areas representing hair growth*

In terms of quantitative analysis, the length of hair for the tested mice was measured as detailed in Figure n.10. Following the administered treatments, the hair length recorded at 10, 14, and 21 days exhibited a significant increase compared to the normal control group (Group I; $p < 0.05$). T2 demonstrated the highest hair length in comparison to both the commercial cream and T1. Interestingly, no statistical difference in hair growth was detected between the commercial cream and T1 on all tested days ($p > 0.05$). However, a notable difference in this parameter between T1 and T2 was observed at 14 and 21 days ($p < 0.05$), while the increase in hair length at 10 days was not quite significant between T1 and T2 ($p > 0.05$).

These findings indicate the superiority of T2 over other treatment groups in revitalizing hair growth in the tested mice.

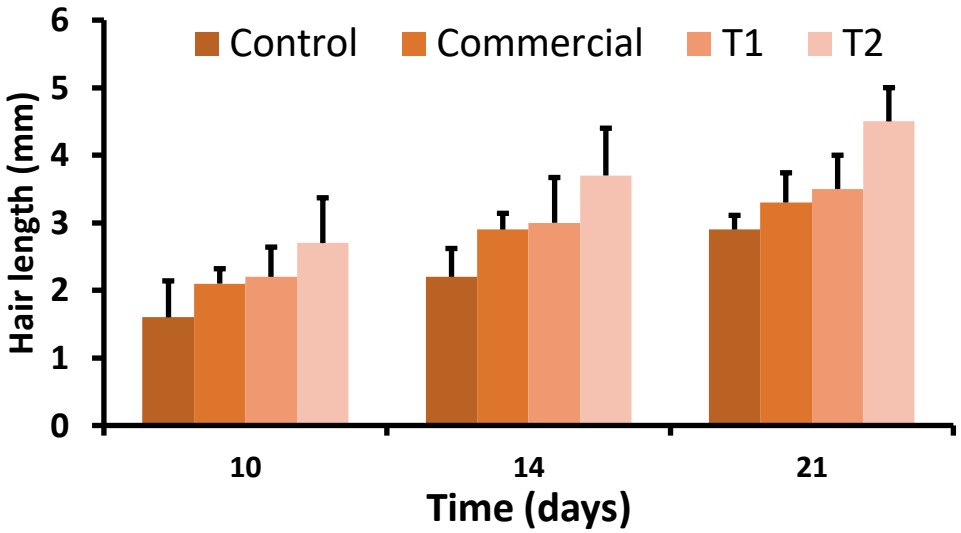


Figure n.10 *Hair length*

Histopathology

Microscopic examination of skin sections from the control group (Figure n.11) revealed normal skin with numerous hair follicles in telogen phase. The commercial drug treated group (Figure n.12) showed a marked increase in the numbers of hair follicles in anagen phase when compared to control group. Regarding T1 group (Figure n.13), despite the presence of few follicles in telogen phase at the deep dermis, the numbers of anagen follicles were greatly increased when compared to the control group. Likewise, the percentage of anagen phase follicles was significantly increased in T2 group (Figure n.14).

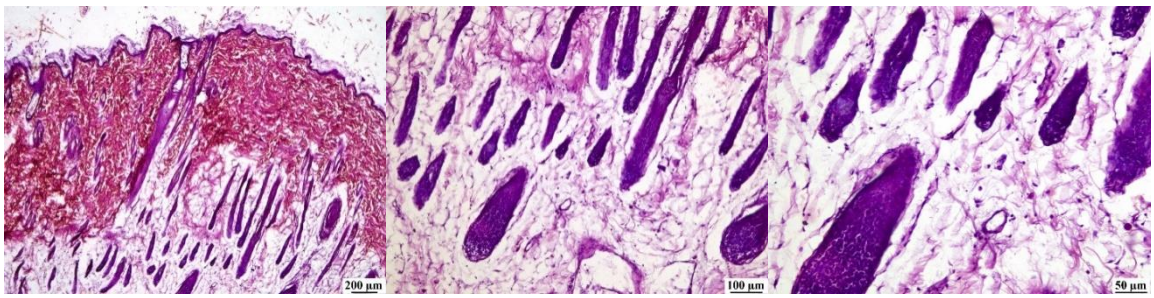


Figure n.11 *Photomicrograph of skin control group, showing numerous hair follicles in telogen phase in increasing magnification*

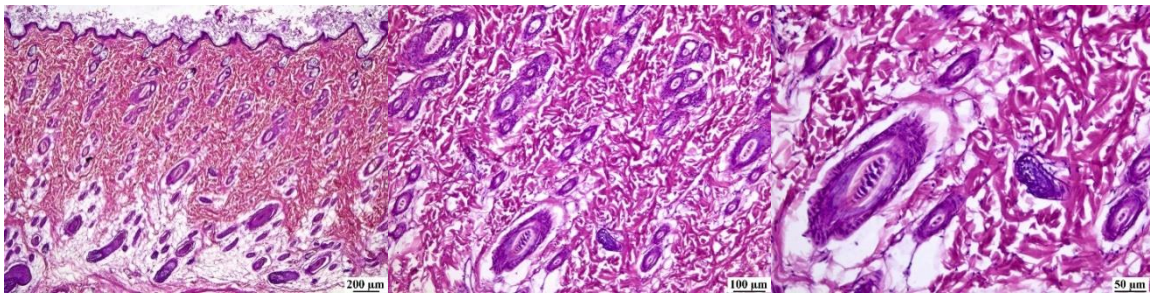


Figure n.12 *Photomicrograph of skin commercial drug group showing increased number of anagen follicles with few telogen follicles, in increasing magnification*

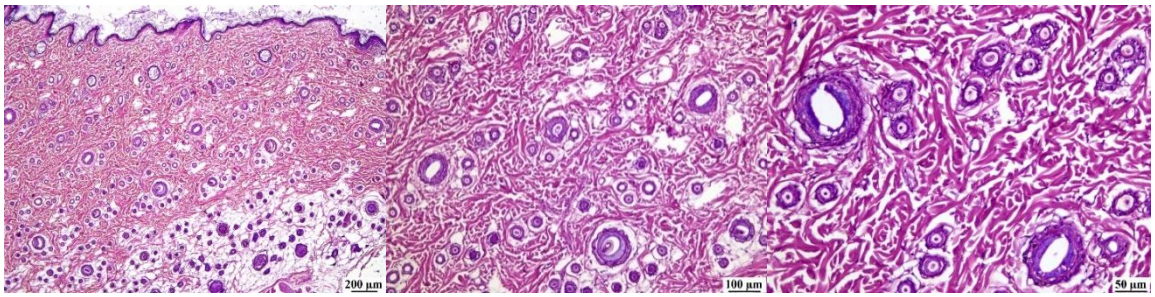


Figure n.13 *Photomicrograph of skin T1 group showing increased number of anagen follicles with few telogen follicles, in increasing magnification*

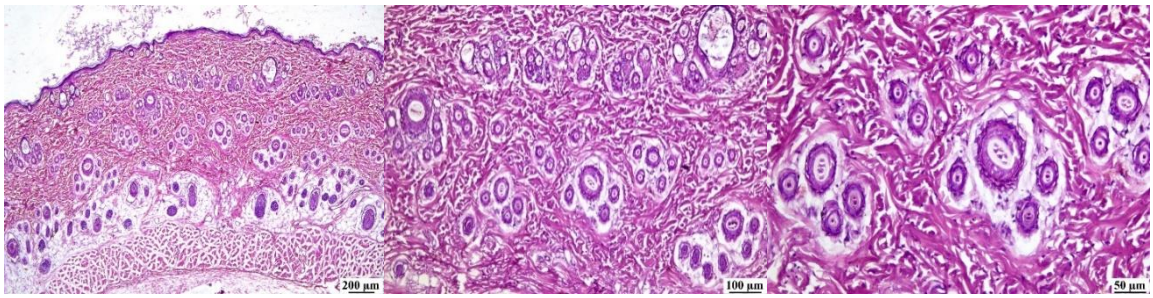


Figure n.14 *Photomicrograph of skin T2 group showing increased numbers of hair follicles in anagen phase, in increasing magnification*

As shown in Figure n.15 Control group showed the least number of anagen follicles. A significantly higher percentage of anagen follicles were detected in commercial and both treated groups when compared to the control group. T2 treated group exhibited the highest value in anagen follicles followed by that detected in T1 group.

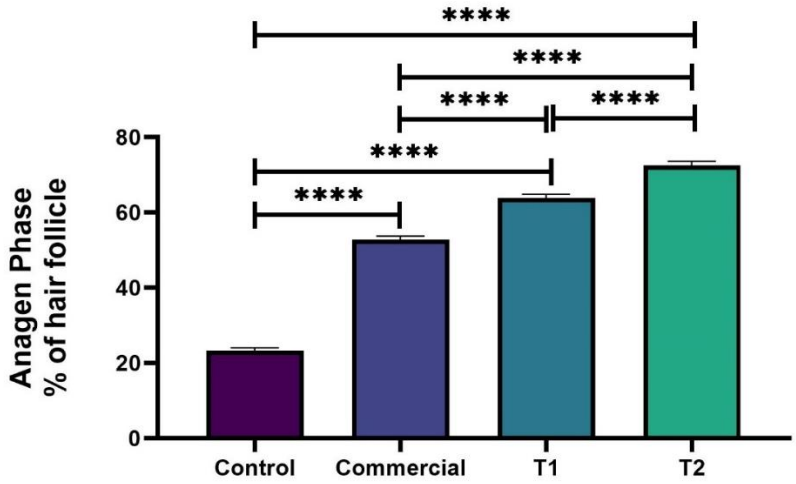


Figure n.15 *Anagen phase % of hair follicle of different experimental group. The asterisks represent the result of ANOVA followed by Tukey test: **** p-value < 0.0001*

The percentage of telogen phase follicles (Figure n.16) significantly decreased in all treated groups when compared to the control group. The least value of telogen follicles was detected in T2 group followed by that detected in T1 and standard groups.

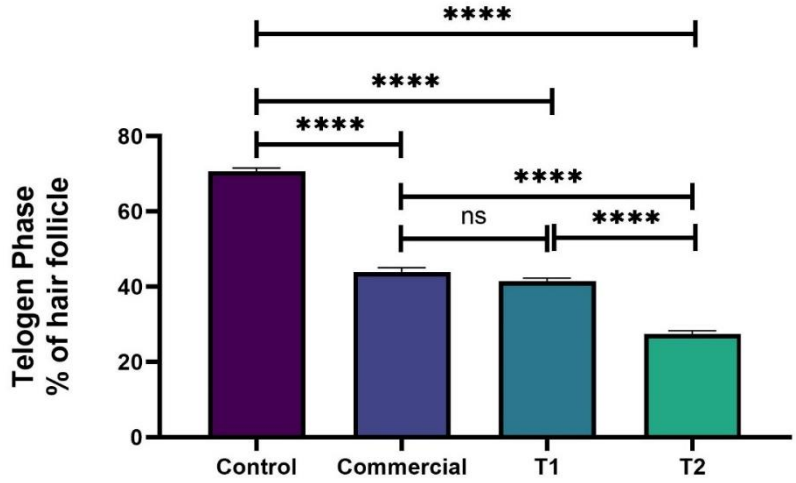


Figure n.16 *Telogen phase % of hair follicle of different experimental groups. The asterisks represent the result of ANOVA followed by Tukey test: **** p-value < 0.0001; ns > 0.05*

As shown in Figure n.17, the skin thickness was significantly increased in all treated groups. T2 group exhibited the highest value in skin thickness followed by T1 group.

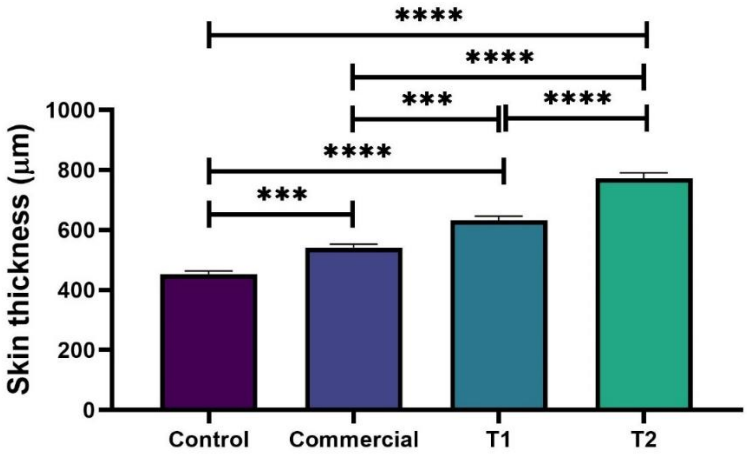


Figure n.17 *Skin thickness of different experimental groups. The asterisks represent the result of ANOVA followed by Tukey test: **** 0.001 < p-value < 0.0001*

2A.4. CONCLUSION

The study aimed to explore the application of keratin as an environmentally friendly surfactant in pharmaceutical formulations, specifically for oil-in-water (O/W) emulsions.

So, the objective was to utilize human hair as an alternative source of functional substances that could serve as biomarkers in various fields, with keratin being one of these substances.

Keratin extraction from the hair was achieved through three different methods: extraction utilizing NaOH 0.5 M, extraction involving Urea 8 M - SDS 0.2 M - $Na_2S_2O_5$ 0.5 M, and extraction using Urea 8M – $Na_2S_2O_5$ 0.5 M.
Based on the conducted analysis, all three methods proved to be effective in the keratin extraction process.

Additional examination of the extracted keratins revealed that the peptidic composition of keratins had an impact on their physical and chemical properties.

The tensiometric test revealed the effective surfactant capability of keratins extracted from hair, demonstrating promising activity in reducing surface tension by adsorbing at the air-water interface. This was further validated through the preparation of emulsions, in which keratin served as a stabilizing agent.

The emulsion, containing ketoconazole as the active ingredient and keratin extracted with NaOH acting as surfactant, remained homogeneous and stable over time.

Subsequently, the formulation proved to be effective in hair regrowth promotion. Indeed, compared to the control group and the groups treated with the commercial product and the cream containing only Ketoconazole, the group treated with the Ketoconazole and keratin cream exhibited superior hair regrowth.
This was evident in terms of time, indicating a quicker initiation and completion time of full hair growth. Additionally, it was observed at the level of maximum hair length reached, which was greater than that of the other groups. Furthermore, in terms of histopathological examination, the skin exhibited a higher count of follicles in the anagen phase, a lower count in the telogen phase, and also displayed greater thickening.

In summary, this study, through analyses and applications, demonstrated that extracted keratin can be served as a surfactant for the formulation of pharmaceutical products.

COMMERCIAL HYDROLYZED KERATINS

2B. COMMERCIAL HYDROLIZED KERATINS

2B.1. MATERIALS

The commercial hydrolyzed keratins, Galeno (Prato, IT) keratin, Keliwool keratin (Kelisema, Como, IT), Variker 100 keratin (Fagron, Bologna, IT) were used for the characterization and the formulation of water dispersions and emulsions. Ethyl oleate (Croda, Pavia, IT) was used for the formulation of emulsion with the commercial keratins.

2B.2. METHODS

2B.2.1. Elemental Analysis

Elemental analysis allows for the identification of the chemical composition of materials. It is an analytical technique used to determine the quantities of carbon, hydrogen, nitrogen, and sulfur present in a compound. The analysis is based on the combustion of the sample: during combustion, the sample produces uniform gaseous compounds consisting of the elements carbon, hydrogen, nitrogen, and sulfur. These combustion products are determined using gas chromatography, and the ratio of elements in the original sample is consequently determined. The quantities of C, H, N, and S can be determined simultaneously.
The evaluation of hydrogen, nitrogen, carbon, and sulfur content in commercial keratins powder has been assessed, through the Flash 2000 Organic Elemental Analyzer.

2B.2.2. Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Electrophoresis is an electrokinetic technique that allows the separation of charged molecules following the application of an electric field. Proteins have ionizable groups, so in solution they are electrically charged species. Under the force exerted by the external electric field (electrostatic Coulomb force) these molecules migrate to cathode or anode according to their charge, negative or positive respectively. In this type of electrophoresis, migration occurs on a matrix formed by polyacrylamide molecules, used to separate any type of protein. This matrix is usually prepared by polymerizing acrylamide monomers in the presence of small quantities of N, N'-methylene bis-acrylamide. Polymerization begins when persulfate and TEMED are added. TEMED decomposes the persulfate ion, producing free

radicals and polymerize the acrylamide. Polyacrylamide is then able to form gels in water. It should be noted that the sample to be separated must be dissolved in the same buffer used to saturate the gel, ensuring homogeneous conditions during electrophoretic separation.

The samples were added with Sample Buffer then boiled at 100 °C for 5 minutes in a water bath to promote protein denaturation. The migration speed of the sample components will depend on their shape, size, and current intensity. An "Electrode buffer" is also used, which allows maintaining the electric field, and is formed by both the buffer used to prepare the gel and the one used to run the sample. Electrophoresis on polyacrylamide gel can be performed in both native and denaturing conditions. The latter, which was performed in this study, requires the use of an anionic detergent – commonly Sodium Dodecyl Sulfate, for this reason it is referred as SDS-PAGE. In SDS-PAGE, the SDS anionic detergent denatures the proteins giving them a uniform negative charge. Therefore, proteins in a sample will result in having a similar shape (since they have been denatured) and a similar net charge. Therefore, proteins will migrate from the negative to the positive pole with a speed that depends only on their molecular weight.

Two types of gels were prepared:

- The stacking gel, situated at the top of the gel, possesses a porous structure with large pores, facilitating the entry of all proteins and priming them for the electrophoretic process. Additionally, the stacking gel contains wells where samples are loaded.
- The running gel, positioned below the stacking gel, typically contains a higher polyacrylamide concentration (ranging from 7.5% to 15%), tailored to the size of the proteins to be separated. It acts as a medium through which proteins migrate during the electrophoretic process.

Once the two types of gel were prepared and inserted on the appropriate support, the samples (previously treated with a buffer containing β-mercaptoethanol and SDS and heated at 100 ° C for 5 minutes to completely denature the proteins) were loaded into the wells on the Stacking gel.

In this study the SDS-PAGE was performed, as described by Laemmli (1970), under denaturing conditions with a 15% acrylamide-bis-acrylamide solution, using the Mini Protean III® instrument (Bio-Rad, gel size 7x8 cm x0.75mm). The markers used were Bio-Rad Low-molecular weight (phosphorylase b, 97.4 kDa; serum bovine albumin, 66.2 kDa,

ovalbumin, 45.0 kDa; carbonic anhydrase, 31.0 kDa; trypsin inhibitor, 21, 5 kDa; lysozyme, 14.4 kDa).

20 µL of keratin were incubated with the denaturing solution. The electrophoresis took place at 200 V for 40 minutes. At the end of the run, the gel was removed from the special support, placed in a plastic tray, and covered with Coomassie Blue dye (0.1% Coomassie Brilliant Blue R250 in 50% methanol and 10% acetic acid) for one hour. Subsequently, the Coomassie Blue dye was discarded, Destain I bleaching solution (50% methanol and 10% acetic acid) was added to the gel and left for about 3 hours.

2B.2.3. Differential Scanning Calorimetry (DSC)

Differential scanning calorimetry (DSC) analyses were performed through the DSC 8500 Perkin Elmer instrument to quantify temperature and heat flow related to transitions in the sample. The measurements included melting enthalpies, glassy transitions, and crystallization kinetics of polymeric materials. Approximately 4 mg of the samples were enclosed in sealed pans and heated from 20°C to 300°C at a rate of 10°C/min.

2B.2.4. Thermogravimetric Analysis (TGA)

Thermogravimetric analysis (TGA) is an experimental method where the mass of a sample is measured as a function of temperature or time. The analyses were conducted using the Simultaneous Thermal Analyzer STA 6000 instrument, which allows samples to be heated at a constant rate or maintained at a constant temperature. In this study, samples were subjected to heating from 30°C to 700°C at a rate of 10°C/min under a nitrogen flow.

2B.2.5. Infrared Spectroscopy

Infrared (IR) spectroscopy assesses the absorption of infrared radiation by chemical bonds within a substance. This technique is valuable for studying the vibrational modes of molecules, providing insights into their structural composition and functional groups. The evaluations were performed using the FT-IR Spectrometer Frontier to analyze the commercial hydrolyzed keratin.

2B.2.6. Tensiometric Analysis

Tensiometric analyses were performed using the De-Nouy ring method with the DCA-100 tensiometer. This method was employed to investigate the surface properties of surfactants in terms of their capacity to reduce water-air interfacial tension.

Keratin solutions were prepared at various concentrations ranging from 0.00097 mg/mL to 8 mg/mL and analyzed in ascending order of dilution. The analyses were carried on 60 mL of the sample at 20 °C. For each concentration, three surface tension values were measured and mean and standard deviation were calculated.

2B.2.7. Conductimetric Analysis

Conductimetric analysis involves measuring the electrical conductivity of electrolytic solutions. In this investigation, the CRISON microCM 2200 conductivity meter was utilized to assess the conductivity of solutions containing keratin. A stock solution of keratin dissolved in water was formulated to achieve a concentration of 8 mg/mL. Subsequently, dilutions in water were prepared from this stock solution, spanning concentrations from 8 mg/mL to 0.00097 mg/mL. The resulting solutions were sequentially analyzed, starting from the least concentrated to the most concentrated.

2B.2.8. Commercial Keratins Applications

Water Dispersions

Keratin water dispersions were prepared by blending 20 grams of commercial hydrolyzed keratin with varying volumes of water to facilitate keratin hydration. The resulting dispersion concentrations were 0.5% w/w, 1% w/w, 2.5% w/w, 5% w/w, and 10% w/w.
To inhibit mold formation during the analysis duration, water treated with 0.2% w/w sodium benzoate was employed.
Water dispersions were formulated with identical concentrations for all commercial keratins.

Emulsions

Emulsions represent metastable colloids composed of two immiscible fluids, with one being dispersed into the other through the inclusion of surfactant agents. In this research, the two

immiscible fluids consisted of ethyl oleate (dispersed phase) and preserved water (dispersing phase). Hydrolyzed keratin served as the surfactant agent.

Emulsions of commercial hydrolyzed keratin were formulated, incorporating oil concentrations of 2.5% w/w, 5% w/w, and 10% w/w, respectively. The keratin concentrations ranged from 0.5% w/w to 10% w/w. The emulsions were prepared using an Ultra-turrax for 5 minutes at 9500 min^{-1}. Ethyl oleate was introduced into the previously prepared keratin solution during the Ultra-turrax operation.

The amounts of commercial hydrolyzed keratin, water, and oil were determined for each individual emulsion to achieve a final product of 20 g for Galeno keratin and 5 g for Kelisema keratin.

Furthermore, to prevent mold formation over time, water treated with 0.2% w/w sodium benzoate was utilized

Microscope Analysis

Microscopic analyses were conducted to observe the long-term stability of the emulsions and to investigate the potential emulsifying properties of keratin. The microscope utilized for these analyses was an MT 9200 microscope (Meji), equipped with a camera (Invenio 3s) and set to a magnification of 10x.

Rheological Analysis

Rheological analyses were performed using the Kinexus lab + rheometer equipped with cone/plate geometry CP4/40. These analyses aimed to examine the viscoelastic properties of keratin dispersions and emulsions. Specifically, the analysis facilitated the correlation between the material's sliding or deformation and the force (stress) applied to the samples or the temperature.

Four distinct analyses were carried out, including viscometry test, oscillation stress sweep, frequency sweep test, and temperature test.

The viscometry test was conducted on keratin dispersions at the concentrations of 0.5% w/w, 1% w/w, 2.5% w/w, 5% w/w and 10% w/w at a temperature of 25 °C and a shear rate of 10-100 s^{-1}.

The oscillation stress sweep test was conducted on keratin formulations at concentrations ranging from 0.5% w/w to 10% w/w. The analysis was conducted at 25°C, with a shear stress of 1 Pa and a frequency of 1 Hz. Graphs generated from the analysis illustrated the correlation between the applied stress and either the G' modulus or the complex viscosity.

The frequency sweep test was conducted on keratin dispersions with concentrations ranging from 0.5% w/w to 10% w/w at a temperature of 25°C, utilizing a frequency range of 10-0.1% Hz and a shear stress of 5 Pa.

Temperature sweep tests were conducted on keratin preparations with concentrations ranging from 0.5% w/w to 10% w/w. The samples underwent a gradual temperature increase, starting from 20°C up to 50°C, with a ramp rate of 0.5 °C/min. The tests were performed at a frequency of 1 Hz and a shear stress of 1 Pa.

Rheological analyses were applied to characterize both water dispersions and emulsions, each incorporating commercial keratins.

2B.3. RESULTS AND DISCUSSION

2B.3.1. Elemental Analysis

Elemental analysis enabled the observation of the percentage composition of commercial keratins, including nitrogen, carbon, hydrogen, and sulfur.

Table n.1 *Chemical composition of commercial keratins*

	Keratin – GALENO	**Keratin - FAGRON**	**Keratin – KELISEMA**
Nitrogen %	15.58	13.3	12.16
Carbon %	43.84	39.88	41.29
Hydrogen %	6.77	6.99	5.86
Sulfur %	0.50	/	2.45

Table n.1 illustrated that the composition of the commercial keratins was comparable, with the noteworthy distinction found in Fagron keratin, which lacked sulfur. This variation could be attributed to the high degree of hydrolysis in this specific keratin.

2B.3.2. Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Commercial hydrolyzed keratins were analyzed using polyacrylamide gel electrophoresis (SDS-PAGE) to detect the presence of keratins and compare their molecular weights with those of a reference standard.

The literature provides data on the molecular weights of keratins, determined via electrophoresis, which reveals the presence of two prominent bands (45-62 kDa) and several less clearly defined bands (10-20 kDa and 6-9 kDa).

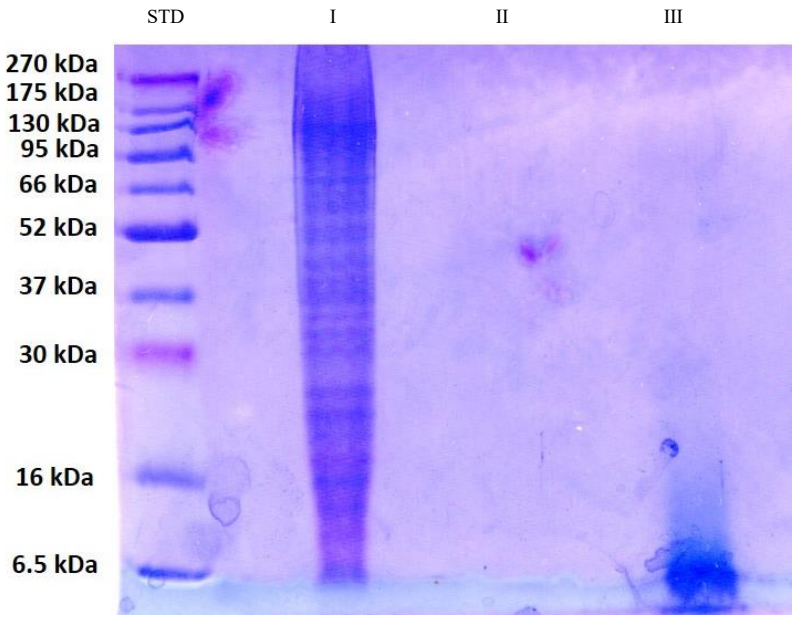


Figure n.1 *Electrophoretic gel of commercial hydrolyzed keratins*

Legend:

- STD = Standard (Reference)
- I = Galeno Keratin
- II = Fagron Keratin
- III = Kelisema Keratin

Galeno exhibited a continuous band, indicating the presence of keratins with varying molecular weights.
In contrast, Fagron showed no band, suggesting a molecular weight lower than 6.5 kDa.
Kelisema exhibited a tail at the end of the stroke, commencing around 16 kDa. This indicated the relatively low molecular weight of this keratin.

2B.3.3. Thermal Analyses (DSC/TGA)

The application of Differential Scanning Calorimetry (DSC) and Thermogravimetric analysis (TGA) has facilitated the identification of thermal transitions, which occurred in commercial keratins under a gradual increasing in temperature, enabling a comparative analysis.

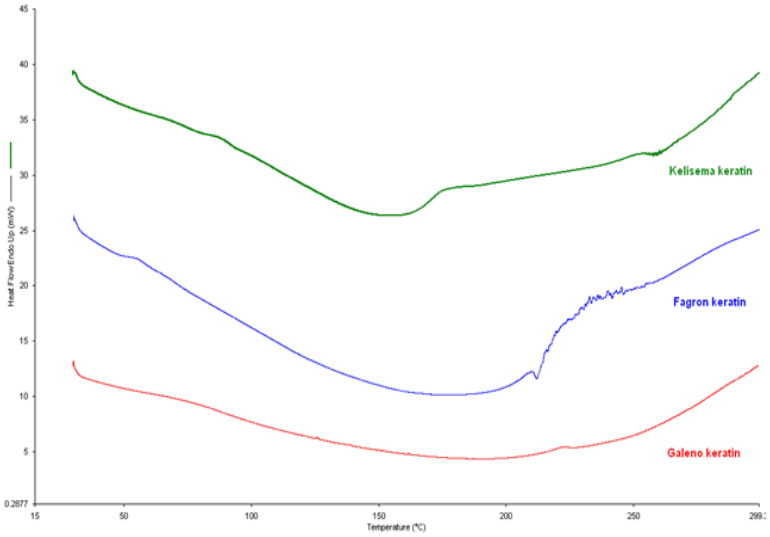


Figure n.2 *DSC analysis of Galeno, Fagron and Kelisema keratin showing thermal transition*

The DSC analysis graph (Figure n.2) revealed a common pattern among all keratins, characterized by an initial water loss between 50 °C and 100 °C. Additionally, each curve exhibited a subsequent endothermic transition associated with the protein denaturation process. In the case of commercial keratins, this denaturation transition typically took place at around 200 °C.

Within the three commercial keratins, the denaturation transition was particularly pronounced in Fagron keratin, likely attributable to its lower molecular weight.

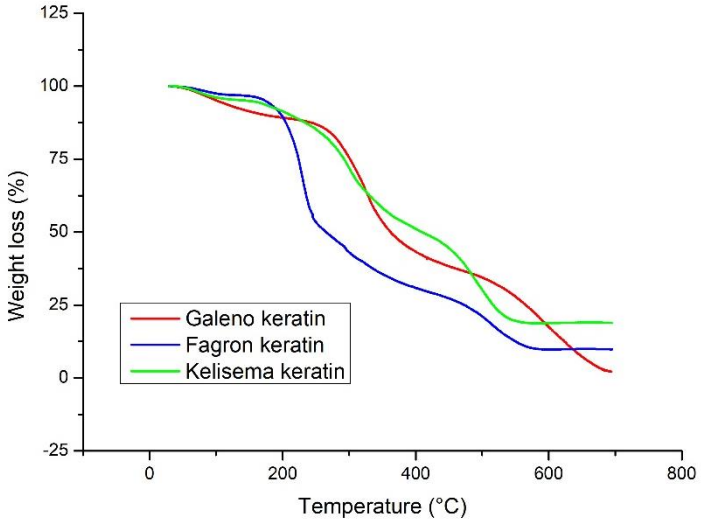


Figure n.3 *TGA analysis of Galeno, Fagron and Kelisema keratin depicting changes in mass with increasing temperature*

Through thermogravimetric analysis (Figure n.3), it was noted that an initial weight reduction occurred at 100 °C–150 °C, attributed to water loss, followed by a subsequent weight loss between 200 °C and 600 °C for commercial keratins, linked to the degradation of the protein.

Notably, Fagron exhibited a more significant weight loss compared to the other keratins, likely due to its substantially lower molecular weight, rendering it more susceptible to degradation.

The findings from TGA analysis corroborated the observations made previously through DSC.

2B.3.4. Infrared Spectroscopy

Figure n.4 displayed the IR spectra of commercial keratin powders, revealing vibrations associated with characteristic protein bands.

The literature contains information regarding the absorption bands related to the vibrations of the bonds that have been examined. [1]

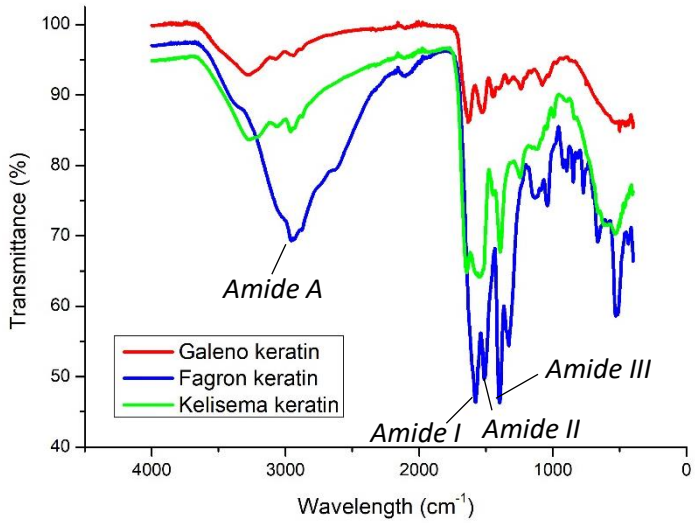


Figure n.4 *IR spectroscopy analysis of commercial keratins revealing characteristic vibrational frequencies*

The absorption band around 3300 cm^{-1} is related to the stretching vibration of N-H and OH bonds, specifically linked to Amide A.

Stretching vibrations of C=O bonds spanning from 1600 to 1700 cm^{-1} were indicative of the Amide I band, providing insights into the secondary structure of keratin.

The bending vibration of N-H in Amide II was evident at 1520 cm^{-1}.

Additionally, the stretching vibrations of -N and -H, along with the bending vibrations of N-H and C=O around 1220-1300 cm^{-1}, were associated with Amide III.

2B.3.5. Tensiometric Analysis

One of the primary analyses crucial for characterizing keratins was tensiometric analysis, which assessed their capability to reduce surface tension when adsorbed at the air-water interface.

The surface tension of keratin solutions, prepared for each sample, was examined in increasing concentrations ranging from 0.00097 mg/ml to 8 mg/ml.

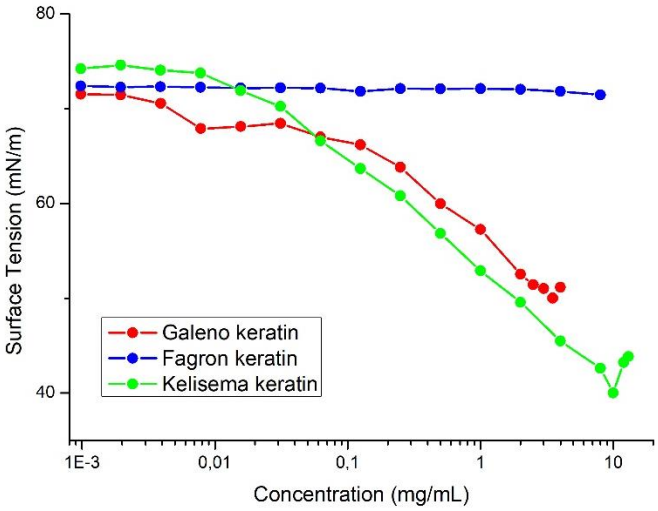


Figure n.5 *Tensiometric analysis of commercial keratins showing changes in surface tension*

In Figure n.5, it was evident that surface tension values closely align with those of pure water (approximately 70 mN/m) when keratin solutions had low concentrations.

The surface tension of Fagron remained constant at 70 mN/m. This suggested that, despite an increase in concentration, this particular keratin was no able to reduce the surface tension.

Conversely, surface tension values for Galeno and Kelisema keratin solutions confirmed that keratin led to a reduction in surface tension below that of pure water. Depending on the amount of keratin present, the surface tension decreased from that of pure water (71.8 mN/m) to a range of 46.31–42.14 mN/m. Notably, the surface tension curve for Galeno keratin exhibited a sudden decline compared to the curve for Kelisema keratin.

2B.3.6. Conductimetric Analysis

Solutions containing Galeno, Fagron, and Kelisema keratins were examined, ranging from the least concentrated to the most concentrated, to evaluate the relationship between electrical conductivity and concentration.

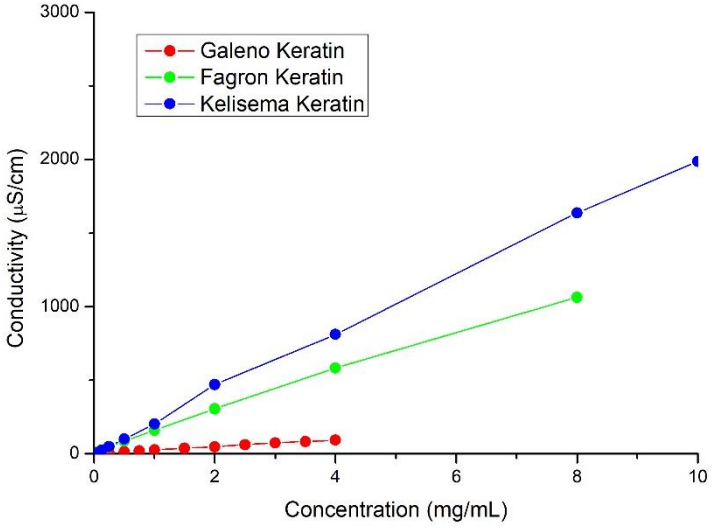


Figure n.6 *Variations in electrical conductivity as a function of concentration in commercial Keratins*

Figure n.6 illustrated that at low concentrations, nearly zero conductivity was measured for all commercial hydrolyzed keratins. However, at a concentration of 0.25 mg/mL, the conductivity increased for Fagron and Kelisema keratin, while for Galeno keratin, the conductivity remained comparatively lower, likely attributed to its higher molecular weight.

2B.3.7. Commercial Keratins Applications

Water Dispersions

Water dispersions were prepared at varying keratin concentrations, from 0.5% w/w to 10% w/w, utilizing preserved water. These concentrations were employed both for Galeno and Kelisema keratins. Fagron keratin was excluded from the preparation process based on data indicating its unsuitability as a surfactant and/or stabilizer in formulations.
Photographs of all samples were taken 24 hours after being prepared.

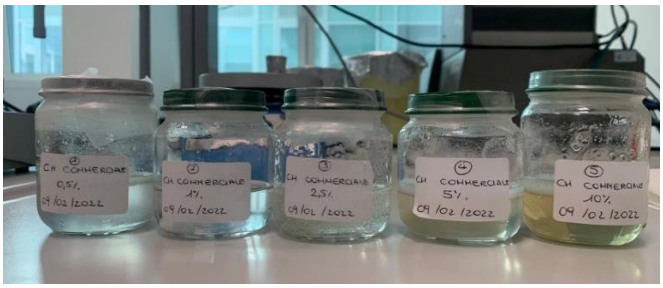


Figure n.7 *Galeno keratin dispersions at 0.5% w/w, 1% w/w, 2.5% w/w, 5% w/w, and 10% w/w*

Figure n.8 *Kelisema keratin dispersions at 0.5% w/w, 1% w/w, 2.5% w/w, 5% w/w, and 10% w/w*

Figures n.7 and n.8 showed water dispersions prepared with increasing concentrations of Galeno and Kelisema keratin.

From these pictures, it was evident that with an increase in keratin concentration, the dispersions shifted from being almost transparent to assuming a gradually more pronounced yellowish color, along with the emergence of foam on the surface, validating their surfactant activity.

Emulsions

Oil in water emulsions containing keratin were formulated by incorporating ethyl oleate as oily phase at varying concentrations, 2.5% w/w, 5% w/w and 10% w/w. The keratin, ranging from 0.5% w/w to 10% w/w, was solubilized in preserved water to form the aqueous phase. The emulsions were prepared by gradually introducing the oily phase into the aqueous phase under by mixing with ultra-turrax.

Identical concentrations and the same procedure were applied for both Galeno and Kelisema keratin. Fagron keratin was excluded from the preparation process due to data suggesting its unsuitability for use as a surfactant and/or stabilizer in formulations.

Figure n.9 *Galeno keratin emulsions at 0.5% w/w to 10% w/w and ethyl oleate 2.5% w/w*

Figure n.10 *Galeno keratin emulsions at 0.5% w/w to 10% w/w and ethyl oleate 5% w/w*

Figure n.11 *Galeno keratin emulsions at 0.5% w/w to 10% w/w and ethyl oleate 10% w/w*

As depicted in Figure n.9-11, the emulsions exhibited an increase in viscosity with the rising concentration of Galeno keratin. Notably, there was a noticeable transition towards semi-solid systems, beginning at a keratin concentration of 2.5% w/w and progressing to the formation of a solid system at a concentration of 10% w/w.

After 24 hours, the preparations appeared stable, uniform, and free from coalescence or phase separation.

Figure n.12 *Kelisema keratin emulsions at 0.5% w/w to 10% w/w and ethyl oleate 2.5% w/w*

Figure n.13 *Kelisema keratin emulsions at 1% w/w to 10% w/w and ethyl oleate 5% w/w*

Figure n.14 *Kelisema keratin emulsions at 2.5% w/w to 10% w/w and ethyl oleate 10% w/w*

Kelisema keratin emulsions, specifically those containing 0.5% w/w keratin and 2.5% w/w ethyl oleate, 1% w/w keratin and 5% w/w ethyl oleate, and 2.5% w/w keratin and 10% w/w ethyl oleate (as shown in Figure n.12-14), exhibited phase separation with visible oil stains on the surface.

On the contrary, the remaining emulsions were stable with only a slight creaming observed on the surface.

Microscope Analysis

Microscope analysis enabled the detection of oil droplets within emulsions and the assessment of their size variations with increasing concentrations of the surfactant and oil.

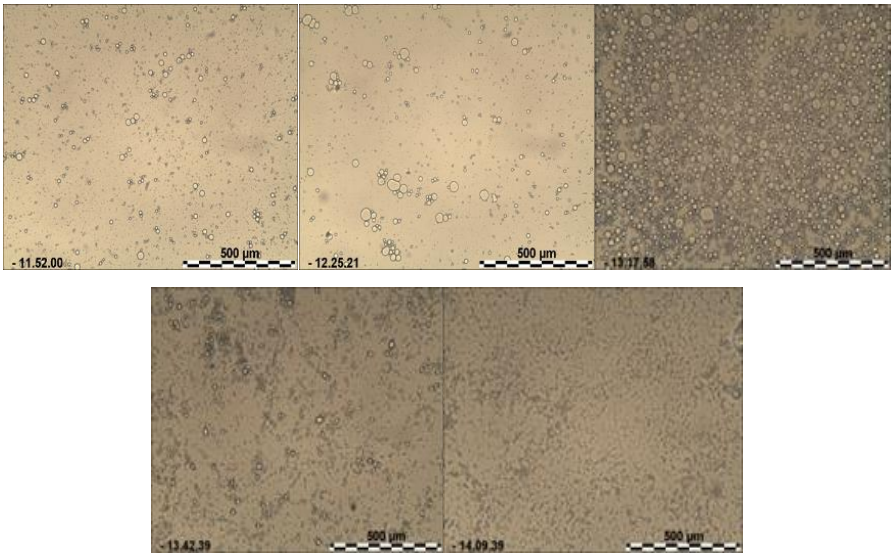


Figure n.15 *Microscope photo of Galeno keratin emulsions 0.5% w/w to 10% w/w and ethyl oleate 2.5% w/w*

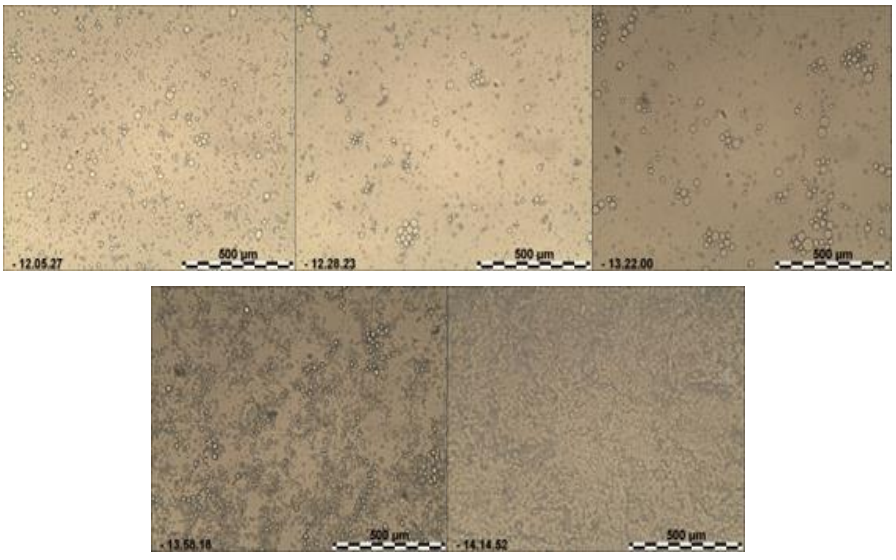


Figure n.16 *Microscope photo of Galeno keratin emulsions 0.5% w/w to 10% w/w and ethyl oleate 5% w/w*

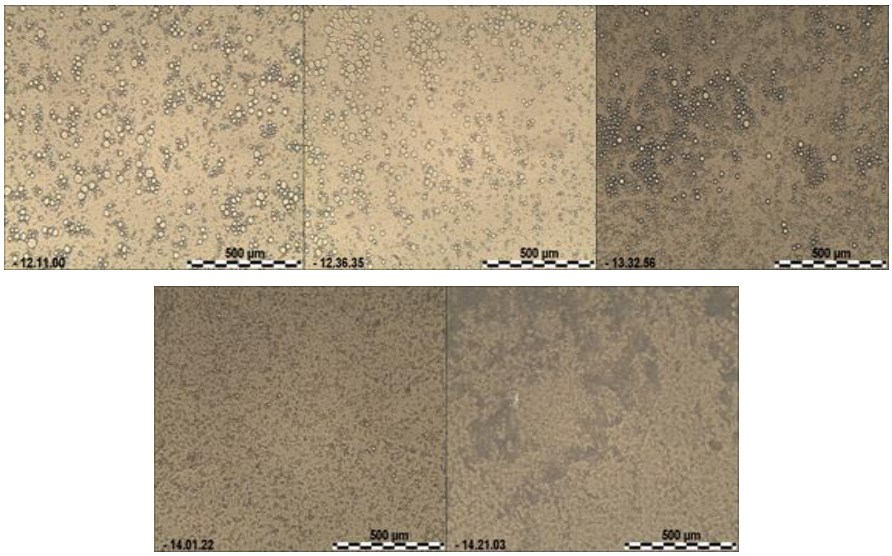


Figure n.17 *Microscope photo of Galeno keratin emulsions 0.5% w/w to 10% w/w and ethyl oleate 10% w/w*

Figures n.15-17 showed emulsions at different concentrations of Galeno keratin and ethyl oleate. The findings indicated that an elevated keratin concentration led to a reduction in the number of drops observable under optical microscopy, likely attributable to an increase in sample viscosity.

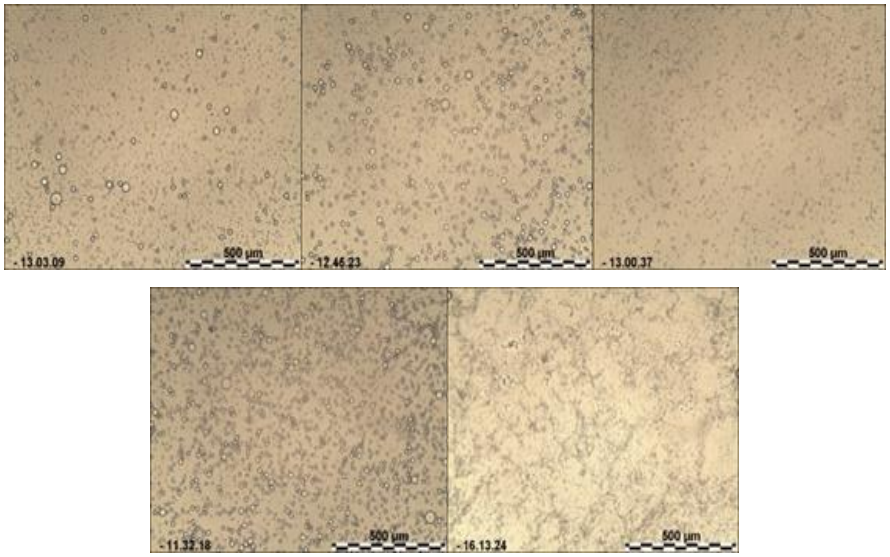


Figure n.18 *Microscope photo of Kelisema keratin emulsions 0.5% w/w to 10% w/w and ethyl oleate 2.5% w/w*

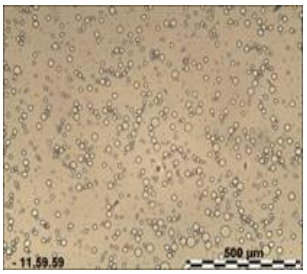


Figure n.19 *Microscope photo of Kelisema keratin emulsion 5% w/w and ethyl oleate 5% w/w*

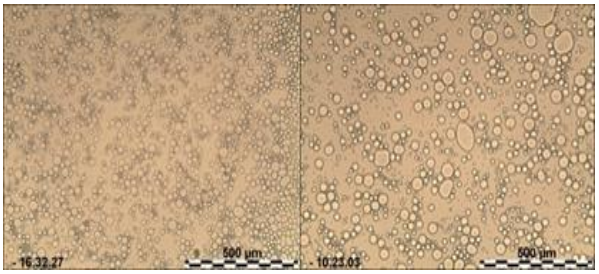


Figure n.20 *Microscope photo of Kelisema keratin emulsions 5% w/w – 10% w/w and ethyl oleate 10% w/w*

Conversely, for Kelisema keratin emulsions, an increase in oil concentration resulted in larger oil droplets, as illustrated in Figures n.18-20.

Rheological Properties of Galeno Keratin Applications

The rheological characterization of preparations containing Galeno keratin were performed to understand how samples behave under stress, aiming to assess their rheological properties.

Galeno keratin water dispersions

Viscoelastic properties of 2.5% w/w, 5% w/w, and 10% w/w of Galeno keratin water dispersions were evaluated through stress sweep and frequency sweep tests. Conversely, samples containing 0.5% w/w and 1% w/w of keratin were analyzed through viscometry tests, as they exhibited more liquid-like characteristics. Moreover, a temperature test was conducted on all water dispersions containing Galeno keratin concentrations ranging from 0.5% w/w to 10% w/w.

- Oscillation stress sweep test of Galeno keratin water dispersions

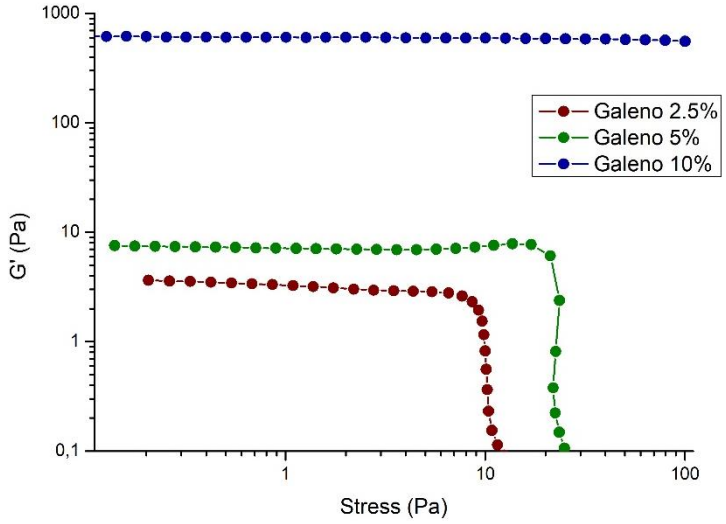


Figure n.21 *G' trend of Galeno water dispersions at 2.5% w/w, 5% w/w and 10% w/w*

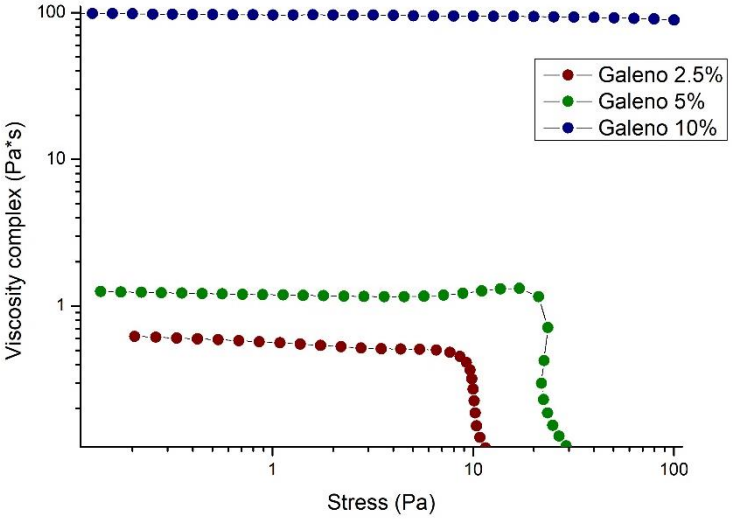


Figure n.22 *Complex viscosity trend of Galeno keratin water dispersions at 2.5% w/w, 5% w/w and 10% w/w*

The Figure n.21 illustrated a decreasing trend in the elastic modulus (G') at the concentration of keratin going to 2.5% w/w to 5% w/w, starting from a stress value of 20 Pa. At these concentrations, the sample structure breakdown. Conversely, the dispersion with a keratin concentration of 10% w/w maintained constant elastic modulus values.

An identical rheological behavior can be underscored by presenting the outcomes concerning the alteration of complex viscosity versus stress, as shown in Figure n.22.

- Frequency sweep test of Galeno keratin water dispersions

In this test, the G' and G" values of Galeno keratin dispersions were assessed at stress values of 5 Pa within a frequency range spanning from 10 to 0.1 Hz.

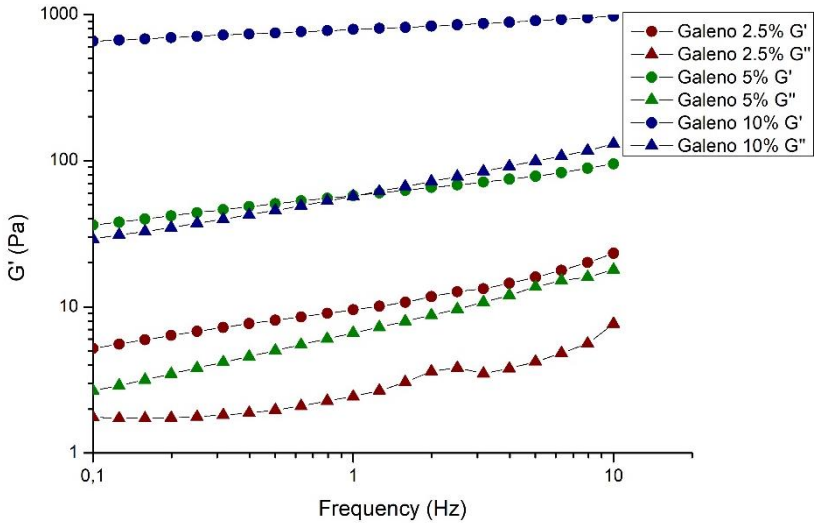


Figure n.23 *Frequency trend of Galen keratin dispersions*

Throughout the examined frequency range, the G' values consistently exceeded the G" values in all formulations (Figure n.23), indicating a prevalence of the elastic component. No crossover points were observed within the analyzed frequency range, indicating that the samples can be defined as a gel from a rheological standpoint.

- Viscometry test of Galeno keratin water dispersions

Viscometry analysis was carried out on dispersions of Galeno keratin at 0.5% w/w and 1% w/w.

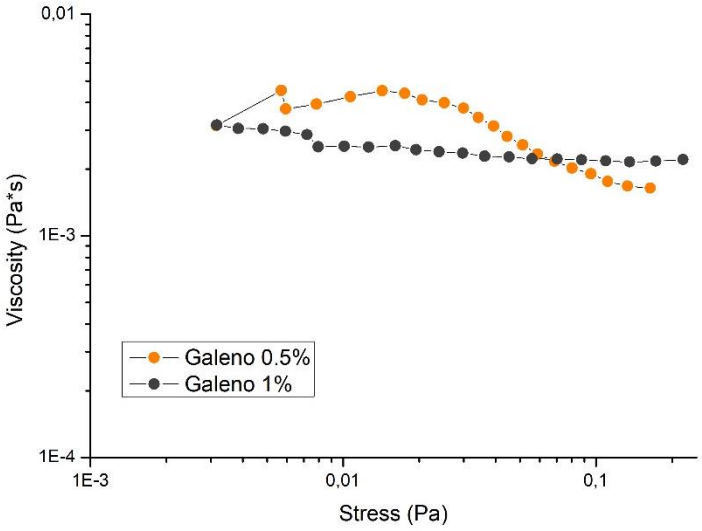


Figure n.24 *Viscosity of Galeno keratin dispersions at 0.5% w/w and 1% w/w*

In graph (Figure n.24), a Newtonian fluid behavior was evident for keratin concentrations at 0.5% w/w and 1% w/w. These dispersions exhibited consistent viscosity values within the specified stress range for the analysis.

- Temperature test of Galeno keratin water dispersions

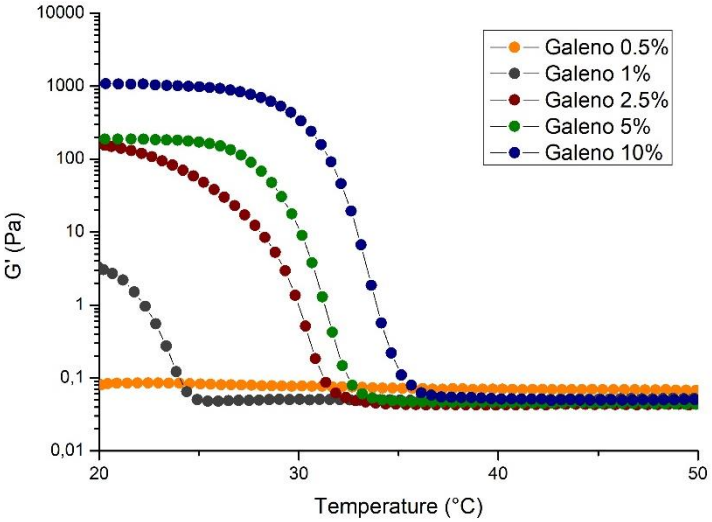


Figure n.25 *G'-temperature trend of Galeno keratin at 0.5% w/w to 10% w/w*

Figure n.25 illustrated that the dispersion with a keratin concentration of 0.5% w/w remained unaffected by temperature, as it was already in a liquid state. Dispersions with keratin concentrations of 2.5% w/w, 5% w/w, and 10% w/w exhibited a decrease in consistency between 30 and 35 °C, and at 35 °C, all dispersions became liquid.

Galeno keratin emulsions

Galeno keratin emulsions were evaluated through stress sweep and frequency sweep tests, examining samples containing 2.5% w/w, 5% w/w, and 10% w/w of keratin, as well as 2.5% w/w, 5% w/w, and 10% w/w of ethyl oleate. The emulsions containing 0.5% w/w and 1% w/w of keratin, combined with 2.5% w/w, 5% w/w, and 10% w/w of ethyl oleate, were analyzed through viscometry test.

Below are the analyses conducted, with their respective plots.

- Oscillation stress sweep test of Galeno keratin emulsions

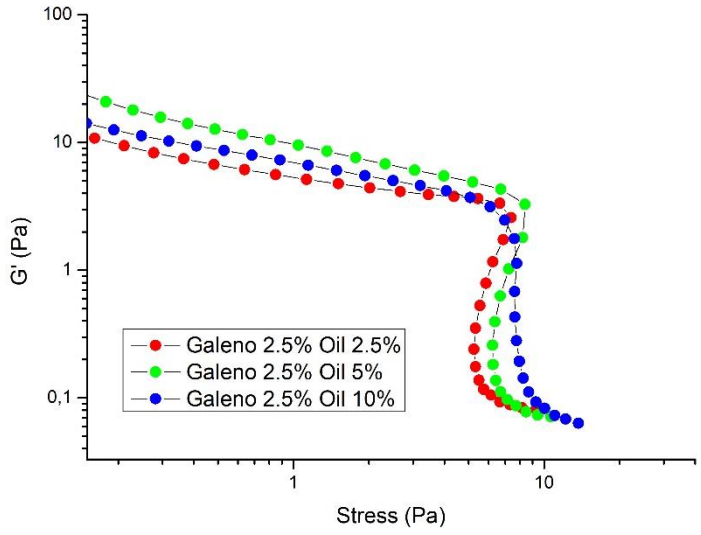


Figure n.26 *G ' trend of Galeno keratin emulsions at 2.5% w/w with ethyl oleate 2.5% w/w, 5% w/w and 10% w/w*

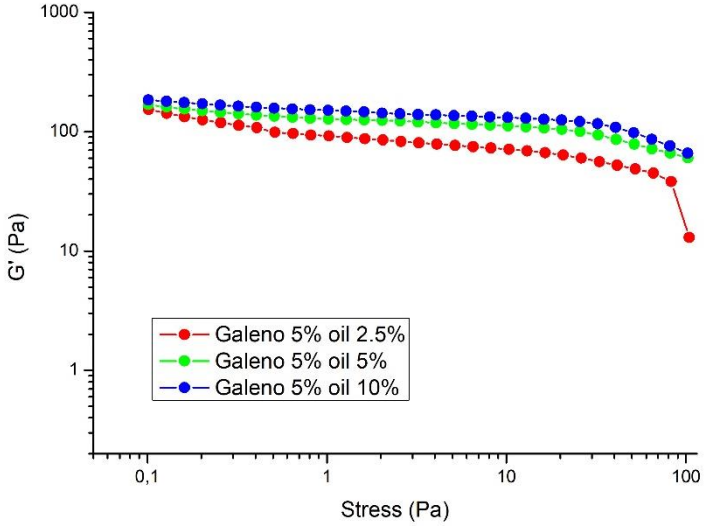


Figure n.27 *G ' trend of Galeno keratin emulsions at 5% w/w with ethyl oleate 2.5% w/w, 5% w/w and 10% w/w*

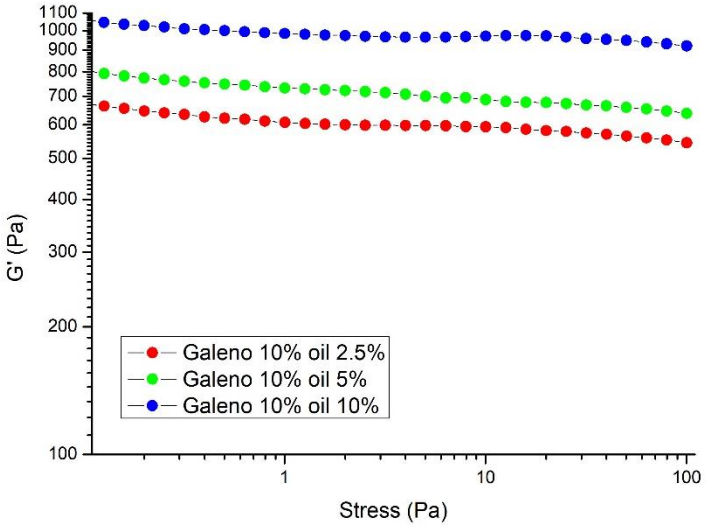


Figure n.28 *G ' trend of Galeno keratin emulsions 10% w/w with ethyl oleate 2.5% w/w, 5% w/w and 10% w/w*

The rheological findings, as depicted in Figure n.26 - n.28, indicated that the inclusion of ethyl oleate in the formulations did not exert a significant impact on the G' values.

- Frequency sweep test of Galeno keratin emulsions

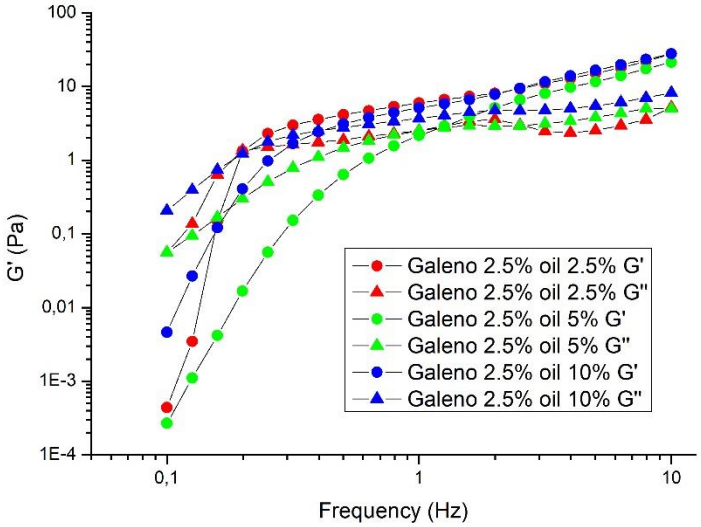


Figure n.29 *Frequency trend of Galeno keratin emulsions with 2.5% w/w with ethyl oleate 2.5% w/w, 5% w/w and 10% w/w*

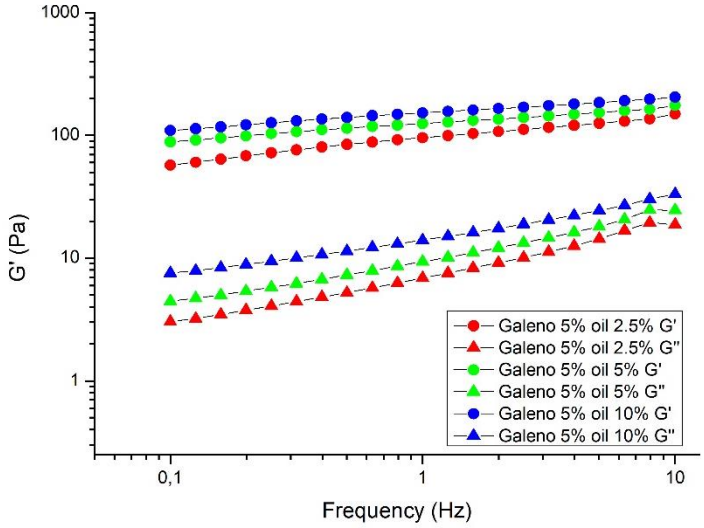


Figure n.30 *Frequency trend of Galeno keratin emulsions with 5% w/w with ethyl oleate 2.5% w/w, 5% w/w and 10% w/w*

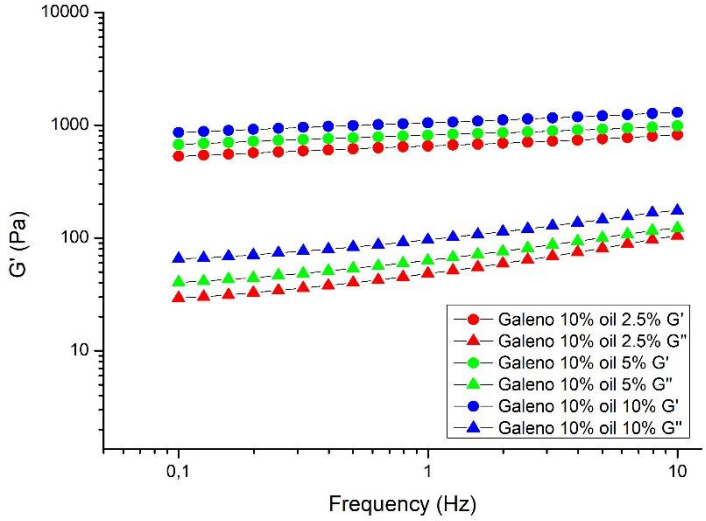


Figure n.31 *Frequency trend of Galeno keratin emulsions 10% w/w with ethyl oleate 2.5% w/w, 5% w/w and 10% w/w*

The G' values, observed in Figure n.29- n.31, as a function of frequency, remained constant following the incorporation of ethyl oleate into the formulations.

- Viscometry test of Galeno keratin emulsions

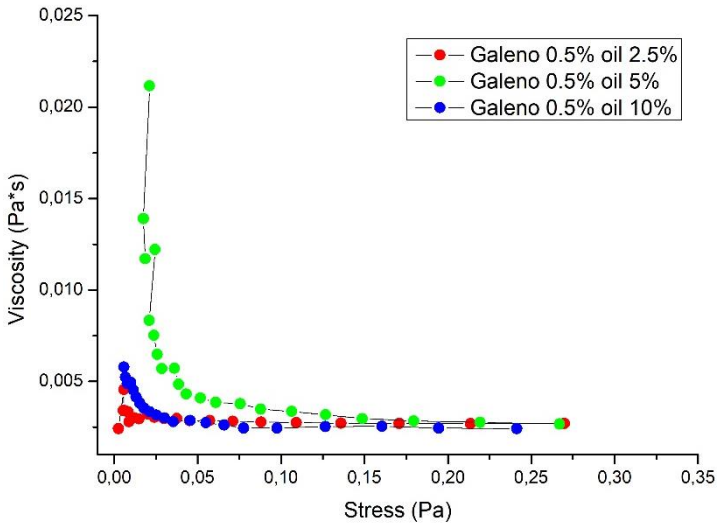


Figure n.32 *Viscosity of Galeno keratin emulsions at 0.5% w/w with ethyl oleate 2.5% w/w, 5% w/w and 10% w/w*

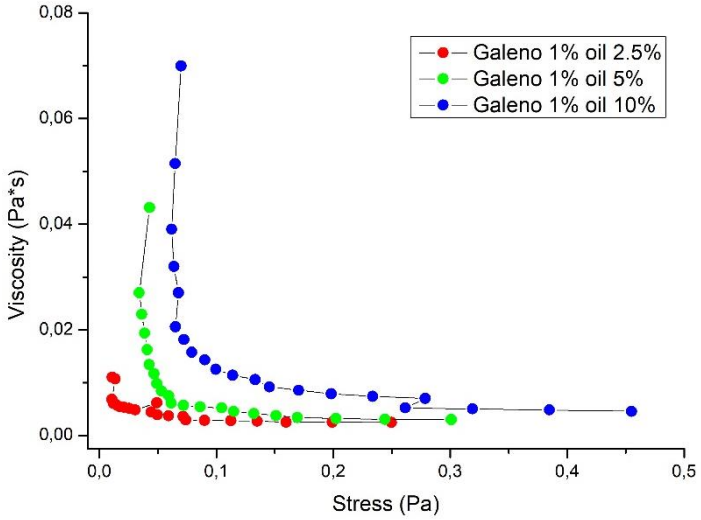


Figure n.33 *Viscosity of Galeno keratin emulsions at 1% w/w with ethyl oleate 2.5% w/w, 5% w/w and 10% w/w*

The introduction of ethyl oleate into the formulations did not affect viscosity values, as demonstrated in Figure n.32 and n.33, in comparison to the viscosity of the earlier tested keratin dispersions.

Rheological Properties of Kelisema Keratin Applications

As for preparations containing Galeno keratin, water dispersions and emulsions containing Kelisema keratin were analysed through a rheometer in order to assess their rheological properties.

Kelisema keratin water dispersions

The viscometry test was conducted on the water dispersions containing Kelisema keratin, which were primarily liquid samples, to assess their viscosity. The analysed samples had different concentrations, ranging from 0.5% w/w to 10% w/w of keratin.

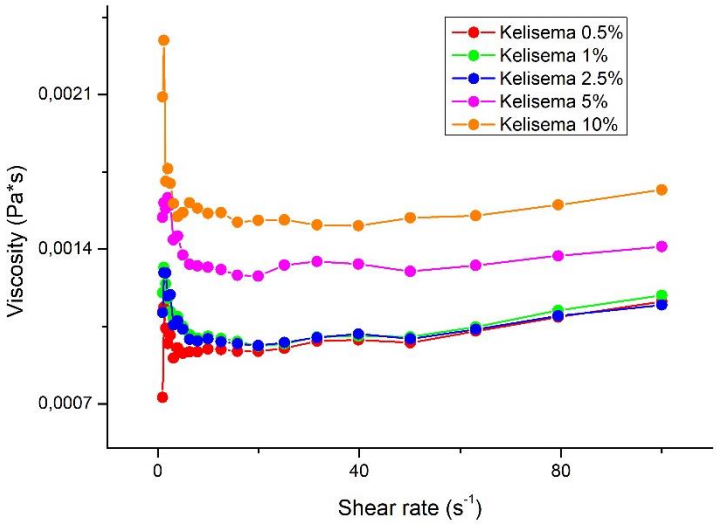


Figure n.34 *Viscosity trend of Kelisema keratin water dispersions at 0.5% w/w, 1% w/w, 2.5% w/w, 5% w/w and 10% w/w*

The graph (Figure n.34) indicated that, despite the rising concentrations of keratin, viscosity was not affected. In fact, as observed above, the samples exhibited a relatively liquid consistency.

Kelisema keratin emulsions

Viscometry tests was performed on emulsions containing Kelisema keratin. The samples consisted of 2.5% w/w and 5% w/w of keratin, along with 2.5% w/w, 5% w/w, and 10% w/w of ethyl oleate.

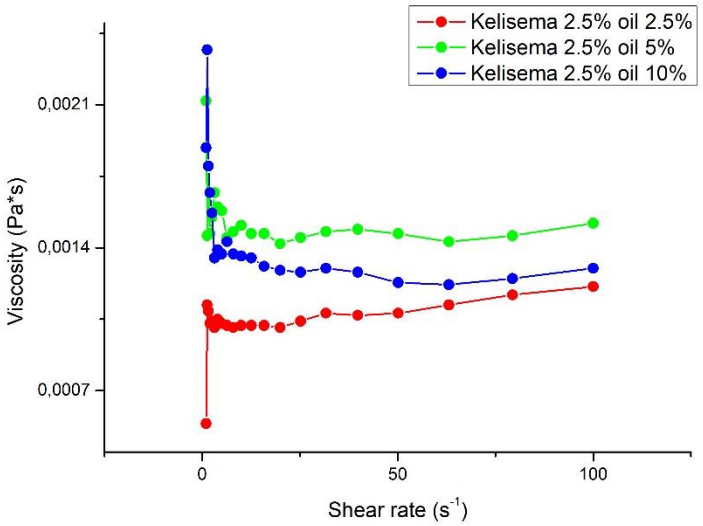


Figure n.35 *Viscosity trend of Kelisema keratin 2.5% w/w with ethyl oleate 2.5% w/w, 5% w/w and 10% w/w*

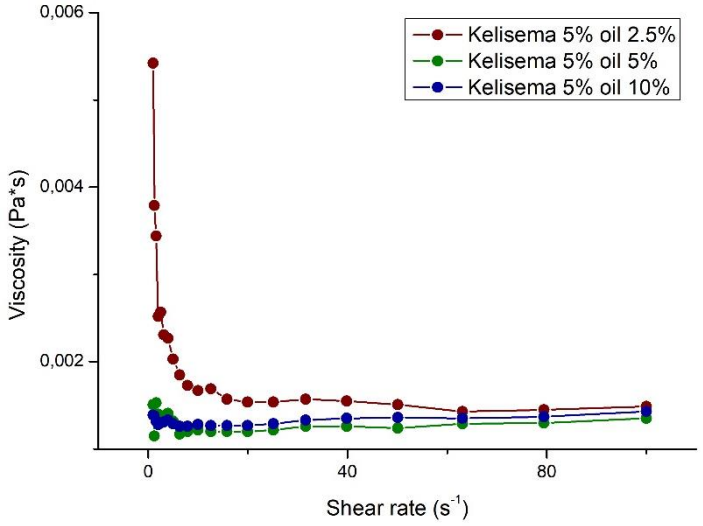


Figure n.36 *Viscosity trend of Kelisema keratin 5% w/w with ethyl oleate 2.5% w/w, 5% w/w and 10% w/w*

The viscometry test results (Figure n.35 and n.36) indicated that the presence of ethyl oleate did not affect the viscosity of the emulsions. Additionally, there were minimal variations observed among the different percentages of keratin.

2B.4. CONCLUSION

The study is focused on the physicochemical characterization of commercial keratins. The main objective was to assess their potential use as surfactants for pharmaceutical preparations, particularly in the experimentation of water dispersions and O/W emulsions.
Three commercial keratins were examined in this project were Galeno, Fagron and Kelisema keratin.

This study revealed that the peptide composition of the keratins impacted their physical and chemical attributes. The primary factor distinguishing them was their varying molecular weights.
The different molecular weights of keratins were due to the extraction method used. This allowed for differentiation among commercial keratins as well as between these and extracted keratins. Additionally, molecular weight influenced the characteristics of keratins, in terms of their ability to lower surface tension and thicken an aqueous medium.

Another noteworthy aspect was the tensiometric analysis, which revealed that commercial keratins were able to reduce surface tension. However, Fagron keratin, in contrast, was unable to lower surface tension. This rendered it unsuitable for the use as a stabilizer in pharmaceutical preparations.

The outcome derived from the tensiometric analysis was then observed in formulation of emulsions, where keratin served as a stabilizer and ethyl oleate used as the oily phase. Throughout the observation period, emulsions prepared with varying oil concentrations remained stable, exhibiting differences only in sample consistencies, which can be attributed to the distinct molecular weights of keratins used in the formulations.
Based on the acquired data, it can be concluded that keratin can be applied as alternative surfactants in the development of pharmaceutical formulations.

WATER ABSORBENT LIPOPHILIC BASES

3.1. INTRODUCTION

In the dynamic landscape of the pharmaceutical industry, topical formulations play a crucial role in addressing a myriad of dermatological and transdermal challenges. Among these, pharmaceutical creams stand out as versatile and widely employed dosage forms.

3.1.1. Semi-solid preparations

As reported in the 12th edition of the official Italian Pharmacopoeia, semi-solid preparations are intended for the local or transdermal release of the active ingredient, or they can have emollient or protective action. One-phase or multiphase systems can be formed by bases that are made of natural or synthetic substances. They may contain suitable additives such as antimicrobials, antioxidants, stabilizers, emulsifiers, thickeners and substances that increase absorption.[1]

Semi-solid formulations are named for their capacity to exist in a solid-like state at room temperature, transitioning into a liquid-like state upon application due to the friction on the skin. Within these categories of pharmaceutical formulations, creams are included. They serve multiple purposes, being utilized for the treatment of wounds and skin diseases, while also contributing to maintaining youthful skin. [2] [3] [4]

Creams offer several advantages compared to other formulations. They allow the incorporation of both hydrophilic and lipophilic components, enabling a more extensive release of incorporated medications. Additionally, creams provide the flexibility to control their rheological properties. [5]

The excipients employed in the formulation of semisolid dosage forms exhibit diversity in both their physical and chemical attributes.[6]

Emulsifiers are solid substances and macromolecules with amphiphilic properties that stabilize two-phase, immiscible systems by diminishing the surface tension at the interface. Commonly employed emulsifiers for pharmaceutical emulsions include macrogol, cetostearyl ethers, polysorbates, sorbitan monostearates, PEG-stearates, and sodium lauryl sulfate. They can be utilized independently or in conjunction with a co-emulsifier to enhance their effectiveness. [6] [7]

Emulsifiers stand out as crucial elements in these formulations. The selection of the appropriate emulsifier not only ensures the stabilization of the formulation but also significantly contributes to its overall success. [8] [9]

Several emulsifiers are available for selection, but this project specifically concentrates on three of them: Cetyl alcohol, Glyceryl monostearate, and Sorbitan monostearate.

3.1.2. Cetyl Alcohol

Cetyl Alcohol ($C_{16}H_{34}O$) (Figure n.1), also known as 1-hexadecanol or n-hexadecyl alcohol, is a C-16 fatty alcohol that is derived from natural sources, such as coconut or palm oil, or synthesized from petroleum. It is a waxy substance that appears as a white or creamy solid at room temperature, with a melting point at 50°C, and it is insoluble in water and soluble in alcohols and oils.

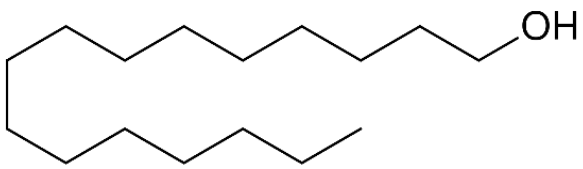


Figure n.1 *Cetyl Alcohol $C_{16}H_{34}O$ formula*

Cetyl alcohol is widely used in various industries, including cosmetics, personal care products, pharmaceuticals and food. [10] [11]
It has many functions and uses: emollient, which means it helps to soften and smooth the skin; emulsifier, it helps to prevent the separation of oil and water components in formulations; thickener, it contributes to the viscosity and thickness of products; surfactant, it can have surfactant-like properties and help to improve the spreadability; stabilizer, it helps to maintain the integrity and the stability of the product over time; opacifying agent, in some formulations it used to add opacity to the product.
It is important to note that cetyl alcohol is generally considered safe for the use in pharmaceutics, cosmetics and food, according to the FDA Code of Federal Regulations. [12]
The hydrating qualities of cetyl alcohol render it a fitting emulsifying and stabilizing agent in pharmaceutical formulations. It is found also in washable ointment bases because of its dispersing capabilities and stabilizing properties. [11]

3.1.3. Glyceryl Monostearate

Glyceryl monostearate ($C_{21}H_{42}O_4$) (Figure n.2), also known as GMS or monostearin, is a compound consisting of glycerol (a trihydric alcohol) and stearic acid (a saturated fatty acid). It is a hygroscopic flaky powder, appearing white, odorless, and possessing a sweet taste, with a melting point at 60°C.

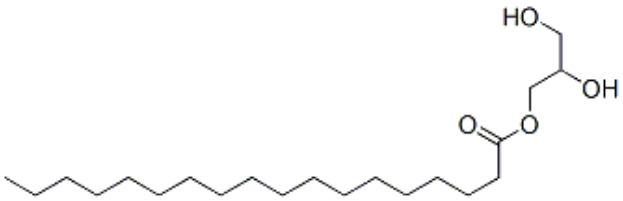


Figure n.2 *Glyceryl Monostearate $C_{21}H_{42}O_4$ formula*

Due to its versatile characteristics, glyceryl monostearate finds diverse applications across various fields. It is used as emulsifier and thickener in food industry as it aids in the stabilization of mixtures and it enhances the texture and the mouthfeel of certain food items. In cosmetic products it is used as emulsifier and stabilizer because it helps to create stable emulsions, ensuring consistent texture and preventing separation, and also as a moisturizer for its emollient properties. In pharmaceutical industry it can be used as binder and coating agent; in industrial applications it is used as plasticizer, enhancing the flexibility and moldability of some materials, and also as a softening agent.

The specific use of glyceryl monostearate can vary based on its concentration, the formulation of the product, and the industry in which it is applied. [13] [14] [15] [16]

Widely employed in cosmetics, foods, as well as oral and topical pharmaceutical formulations, Glyceryl monostearate is generally recognized as a non-toxic and non-irritating substance. [16]

3.1.4. Sorbitan Monostearate

Sorbitan monostearate ($C_{24}H_{46}O_6$) (Figure n.3), alternatively referred to as SMS or Span 60, is a non-ionic surfactant that is an ester derived from sorbitan (a derivative of sorbitol) and stearic acid. [17] [18]

It typically appears as a waxy or powder solid at room temperature, with a melting point of 60°C. [19]

Its physical state can depend of factors such as the specific formulation, purity and any additives present. In general, it is a creamy to light tan colored solid with a mild odor.

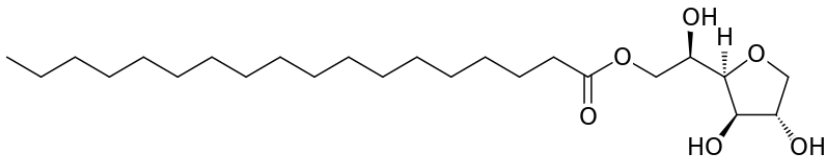


Figure n.3 *Sorbitan Monostearate $C_{24}H_{46}O_6$ formula*

It has surfactant properties, so it is commonly used as an emulsifier in the food, cosmetic, and pharmaceutical industries.

Sorbitan monostearate serves several purposes: in cosmetic products, it is used as emulsifier and stabilizer, so to create stable formulations with smooth texture; in pharmaceuticals, it is used as emulsifying agent, to improve the consistency and stability of pharmaceutical products; in food industry it is used to stabilize emulsions in food products.

Sorbitan, much like other esters, is generally regarded as a substance that is neither toxic nor irritating. Nevertheless, sporadic cases have been documented where hypersensitive skin reactions occurred after the application of products containing sorbitan esters topically. [16]

3.1.5. Viscosity

In this study, our primary focus lies on examining the viscosity of materials by subjecting them to viscometry tests using a rheometer.

Viscosity is an expression of a liquid's internal resistance to flow and is measured in Pa*s. When referencing viscosity, it is crucial to specify the temperature of the determination, as viscosity varies with temperature fluctuations.
Fluids can be categorized into Newtonian and non-Newtonian types. Newtonian fluids exhibit consistent and linear viscosity, whereas non-Newtonian fluids experience viscosity changes based on alterations in the speed gradient at a constant temperature. The graphical representation of the relationship between shear rate and shear stress is known as the flow curve. [20]

Common rotational tests involve viscosity functions that rely on shear rate, shear stress, time, or temperature. The outcomes of such tests can be presented through a flow curve diagram, illustrating the resultant shear stress values, and also as the corresponding viscosity function. A flow curve measurement on a rotational viscometer/rheometer involves incrementally increasing shear rate and determining the respective shear stress for each level. Materials may exhibit various flow behaviors, including ideally viscous, shear-thinning, and shear-thickening behavior.

3.1.6. Aim of the work

The aim of this study is to formulate and characterize lipophilic bases with the ability to incorporate water. The research is centered around developing three bases, namely cetyl alcohol with paraffin oil, glyceryl monostearate with paraffin oil and sorbitan monostearate with paraffin oil.

3.2. MATERIALS

Lipophilic bases were prepared using Cetyl Alcohol (Farmalabor, Milano, IT), Glyceryl Monostearate (Farmalabor, Milano, IT), Sorbitan Monostearate (Farmalabor, Milano, IT) and paraffin oil (Acef, Piacenza, IT).

3.3. METHODS

3.3.1. Bases Preparation

Emulsifiers, including cetyl alcohol, glyceryl monostearate, and sorbitan monostearate, were used to create lipophilic bases. These bases were formulated by blending each emulsifier with paraffin oil. The preparation of oily systems involved melting the emulsifier-paraffin oil mixture on a heating plate at temperatures ranging from 60°C to 80°C. Emulsifier concentrations varied from 5% w/w to 90% w/w.

3.3.2. Samples Preparation

For each lipophilic base variant, incremental amounts of distilled water were introduced to various emulsifier concentrations, reaching a maximum water concentration beyond which phase separation occurred. The process involved melting the solid excipient, mixed with paraffin oil, on a heating plate at temperatures ranging from 60°C to 80°C. After cooling, progressive additions of pure water, ranging from 5% w/w up to the maximum quantity tolerated by each system, were mixed until stable samples were achieved.

3.3.3. Rheological Analysis

Rheological analyses were conducted through the Kinexus lab + rheometer equipped with a flat-cone geometry CP4/40. The purpose was to evaluate the viscoelastic properties of lipophilic bases, both with and without the addition of water. Notably, viscosity, a key parameter indicating a fluid's resistance to flow, was analyzed. The viscometry test was carried out at a temperature of 25 °C and a shear rate ranging from 0.1 to 100 s^{-1}.

The Power Law model represented a generalized framework, providing a fundamental correlation between viscosity (ν) and strain rate ($\dot{\gamma}$). Within this model, viscosity is constrained by both a minimum value (ν min) and a maximum value (ν max).

The relation is given as: $\nu = k \cdot \dot{\gamma}^{n-1}$

- K is the flow consistency index (m^2/s)
- $\dot{\gamma}$ is the shear strain rate (s^{-1})
- n is the flow behavior index

Based on the flow behavior index, n:

- if $0<n<1$: the fluid shows pseudo-plastic or shear-thinning behavior. A smaller value of n means a greater degree of shear-thinning
- if $n=1$: the fluid shows Newtonian behavior
- if $n>1$: the fluid show dilatant or shear-thickening behavior with higher value of n resulting in greater thickening

3.3.4. Differential Scanning Calorimetry (DSC)

Differential scanning calorimetry (DSC) analyses were performed through the DSC 8500 Perkin Elmer instrument to quantify temperature and heat flow related to transitions within the sample. The analysis focused on determining melting enthalpies, glassy transitions, and crystallization kinetics of polymeric materials. Approximately 4 mg of each sample was enclosed in sealed pans and subjected to a heating process ranging from -20°C to 80°C, employing a ramp rate of 10°C/min.

3.4. RESULTS AND DISCUSSION

3.4.1. Bases Preparation

Lipophilic bases were formulated by blending the emulsifiers—cetyl alcohol, glyceryl monostearate, and sorbitan monostearate—with paraffin oil. The process involved melting the emulsifier at temperatures between 60°C and 80°C on a magnetic stirring heating plate, combined with paraffin oil. The concentrations of all emulsifiers ranged from 5% w/w to 90% w/w. However, after formulating the mixtures, only specific concentrations were viable for analysis, as systems with high concentrations became impractical to assess, because of the sensitivity of the employed instrument. The specified concentration ranges were as follows: cetyl alcohol (CA) from 5% w/w to 25% w/w, glyceryl monostearate (GM) from 5% w/w to 20% w/w, and sorbitan monostearate (SM) from 5% w/w to 25% w/w.

Table n.1 *Oily bases composition (w/w)*

Paraffin Oil	Cetyl Alcohol	Glyceryl Monost.	Sorbitan Monost.
95%	5%	5%	5%
90%	10%	10%	10%
85%	15%	15%	15%
80%	20%	20%	20%
75%	25%	/	25%

3.4.2. Samples Preparation

Once the maximum analyzable concentration for each emulsifier was determined (CA up to 25% w/w, GM up to 20% w/w and SM up to 25% w/w), incremental amounts of distilled water were introduced to achieve samples that remained free of phase separation and exhibited stability over time.

The preparation process involved melting the emulsifier mixed with paraffin oil on a heating plate at temperatures ranging from 60°C to 80°C. After cooling, pure water was added,

starting from 5% w/w of water and incrementally reaching the maximum concentration specified for each system.

In systems containing cetyl alcohol, pure water was added up to 15% w/w when the emulsifier concentrations were 5% w/w to 20% w/w. Conversely, for emulsifier concentration of 25% w/w, it was possible to reach the 17.5% w/w of water.

In systems containing glyceryl monostearate, water additions of up to 20% w/w were possible for emulsifier concentrations of 5% w/w and 10% w/w. For emulsifier concentration of 15% w/w, it was possible to include up to 30% w/w water. Lastly, in systems with 35% w/w glyceryl monostearate, water was added up to 35% w/w.

In samples containing sorbitan monostearate, it was possible to add up to 40% w/w deionized water across all concentrations (5% w/w to 25% w/w).

Regarding sorbitan monostearate, it was unsurprising that 25% w/w could accommodate up to 40% w/w of water, but what was more unexpected, yet still encouraging, was that even with just 5% w/w of surfactant, it could reach up to 40% w/w water content.

All samples are resumed in Table n.2.

Table n.2 *Samples containing Cetyl Alcohol(A) / Glyceryl Monostearate(B) / Sorbitan Monostearate(C) + paraffin oil + % water composition (w/w)*

A

Cetyl Alcohol	H_2O			
5%	5%	10%	15%	/
10%	5%	10%	15%	/
15%	5%	10%	15%	/
20%	5%	10%	15%	/
25%	5%	10%	15%	17,5%

C

Sorbitan Monost.	H_2O				
5%	5%	15%	25%	35%	40%
10%	5%	15%	25%	35%	40%
15%	5%	15%	25%	35%	40%
20%	5%	15%	25%	35%	40%
25%	5%	15%	25%	35%	40%

B

Glyceryl Monost.	H_2O						
5%	5%	10%	15%	20%	/	/	/
10%	5%	10%	15%	20%	/	/	/
15%	5%	10%	15%	20%	25%	30%	/
20%	5%	10%	15%	20%	25%	30%	35%

3.4.3. Rheological Properties

Viscosity was a key parameter in describing samples flow behavior. Rheological analysis has enabled the identification of the extent to which cetyl alcohol, glyceryl monostearate and sorbitan monostearate could increase the viscosity of the oily phase, while also enabling the identification of the maximum concentration of emulsifier that could be analyzed.

Rheological analysis also allowed the determination of systems behaviour when water was added.

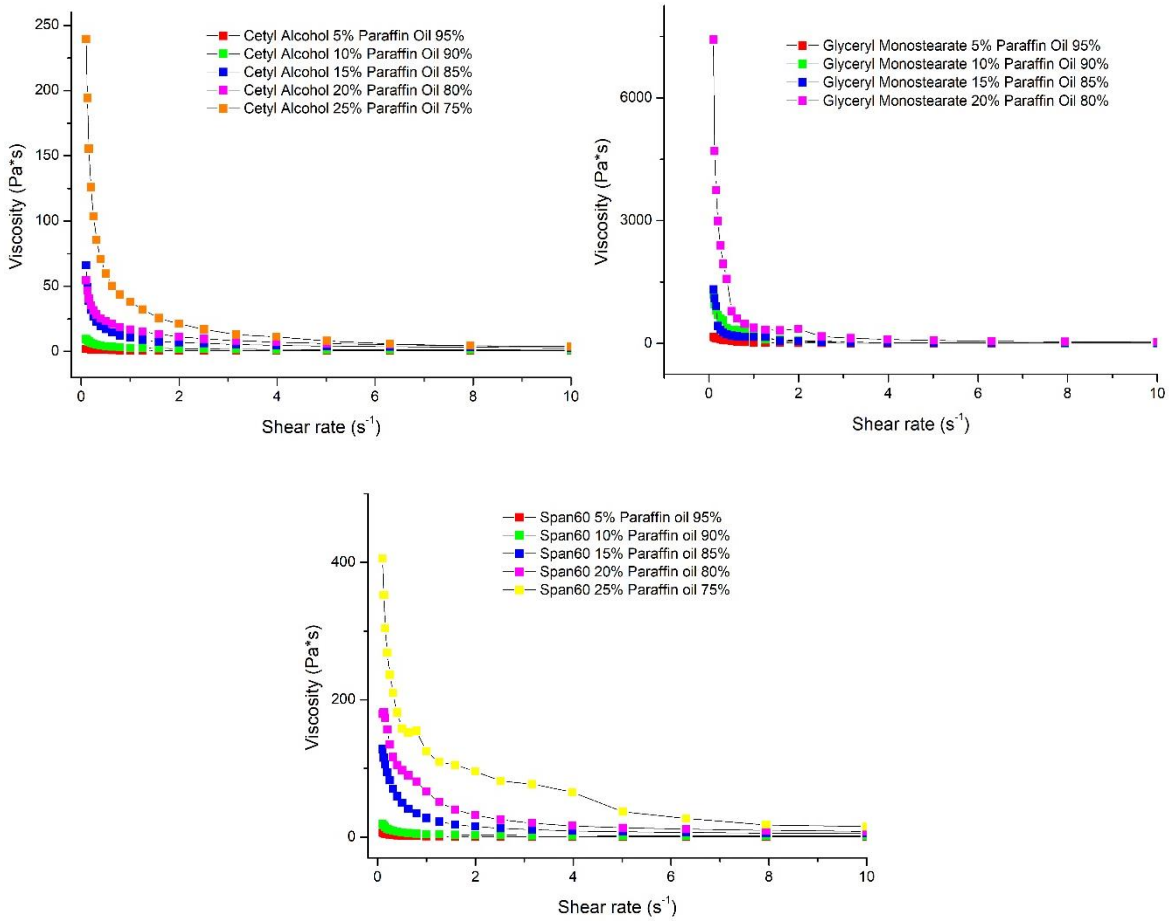


Figure n.4 *Flow curves of cetyl alcohol, glyceryl monostearate and sorbitan monostearate oily bases*

Cetyl alcohol was analyzable up to 25% w/w, glyceryl monostearate up to 20% w/w, and sorbitan monostearate up to 25% w/w. This limitation arose because concentrations beyond

these levels made samples not analyzable by the instrument, and, simultaneously, unsuitable for spreading on the skin.

The viscometry test was used to analyze the viscosity of the three lipophilic bases. The power law fitting provided by the method, could determine the relative viscosity trends, but it did not provide the specific value indicating the consistency of the sample at rest. For this purpose, the viscosity value at 0.1 s^{-1} share rate has been selected to compare all samples because very close to the imaginary value at rest.

Table n.3 *Viscosity values of Cetyl Alcohol(A), Glyceryl Monostearate(B) and Sorbitan Monostearate(C) oily bases*

A	POWER LAW	SHEAR RATE 0.1 s^{-1}
CETYL ALCOHOL	K Index	Viscosity (Pa*s)
0%	/	0.1
5%	0.8799	1.989
10%	3.01	9.541
15%	11.55	66.03
20%	14.94	54.44
25%	30.66	239.5

B	POWER LAW	SHEAR RATE 0.1 s^{-1}
GLYCERYL MONOST.	K Index	Viscosity (Pa*s)
0%	/	0.1
5%	22.74	160.4
10%	101.8	1200
15%	106.2	1321
20%	479	7424

C	POWER LAW	SHEAR RATE 0.1 s^{-1}
SORBITAN MONOST.	K Index	Viscosity (Pa*s)
0%	/	0.1
5%	1.733	6.834
10%	5.346	19.82
15%	28.28	128.3
20%	50.37	179.5
25%	98.73	406.1

The results of the rheological analysis (Figure n.4) were summarized in Table n.3, which illustrated the variation in viscosity of the lipophilic bases with an increasing amount of emulsifier.

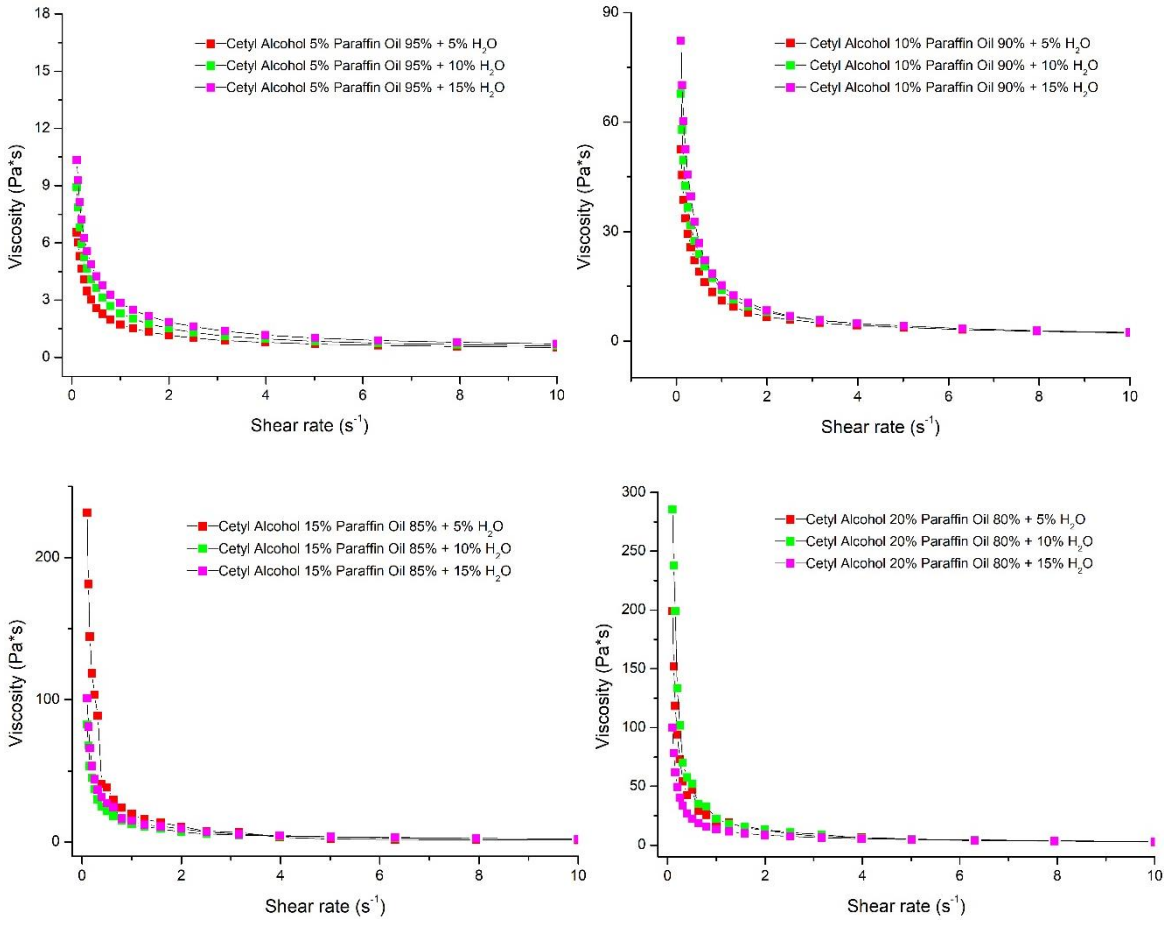
Each table (Table n.3 A, B, C) displayed, as its initial value, the starting viscosity of the system with 0% emulsifier, representing the viscosity of paraffin oil, 0.1 Pa*s. [20]

In all three systems, viscosity demonstrated an upward trend corresponding to the rise in emulsifier quantity. Glyceryl monostearate exhibited the highest thickening ability among the three thickening agents, to a lesser extent sorbitan monostearate, and cetyl alcohol having the least thickening ability.

After having ascertained the viscous properties of the three base systems, the rheological properties and viscosity of systems obtained by water addition were also assessed.

3.4.3.1. Water addition to Cetyl Alcohol / Paraffin oil system

The rheometer was used to analyze all groups of cetyl alcohol samples with varying water concentrations. This was done to assess the viscosity of each sample and determine the extent to which the presence of water impacts their consistency in comparison to the initial system.



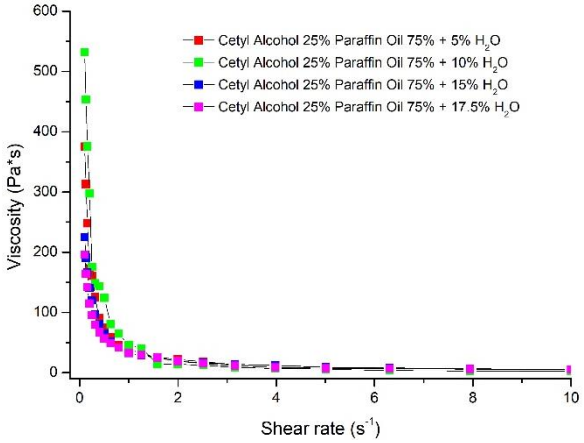


Figure n.5 *Viscosity trend of 5% w/w, 10% w/w, 15% w/w, 20% w/w and 25% w/w of cetyl alcohol with % of water*

As evident from Figure n.5, the introduction of water did not significantly alter the profile of the various samples. All systems remained pseudoplastic. However, its addition simultaneously resulted in an increase in viscosity compared to that of the lipophilic base.

Table n.4 *Viscosity values of cetyl alcohol/paraffin oil + % water*

Cetyl Alcohol 5%			Cetyl Alcohol 10%		
	POWER LAW	SHEAR RATE 0.1 s^{-1}		POWER LAW	SHEAR RATE 0.1 s^{-1}
% H_2O	K Index	Viscosity (Pa*s)	% H_2O	K Index	Viscosity (Pa*s)
0%	0.8799	1.989	0%	3.01	9.541
5%	1.832	6.551	5%	11.04	52.52
10%	2.36	8.919	10%	13.26	67.83
15%	2.819	10.34	15%	14.77	82.28

Cetyl Alcohol 15%			Cetyl Alcohol 20%		
	POWER LAW	SHEAR RATE 0.1 s^{-1}		POWER LAW	SHEAR RATE 0.1 s^{-1}
% H_2O	K Index	Viscosity (Pa*s)	% H_2O	K Index	Viscosity (Pa*s)
0%	11.55	66.03	0%	14.94	54.44
5%	19.83	231.6	5%	22.01	199.1
10%	13.01	166.5	10%	22.22	285.9
15%	15.28	101.3	15%	14.68	100

Cetyl Alcohol 25%		
	POWER LAW	SHEAR RATE 0.1 s^{-1}
% H_2O	K Index	Viscosity (Pa*s)
0%	30.66	239.5
5%	34.73	532.1
10%	34.36	375.4
15%	35.51	225.5
17.5%	32.2	196

Samples containing cetyl alcohol/paraffin oil could incorporate a maximum of 17.5% w/w pure water, and this addition altered the viscosity of the system.

The graphs (Figure n.5) depicted the viscosity trend of the samples containing 5% w/w, 10% w/w, 15% w/w, 20% w/w, and 25% w/w of cetyl alcohol with increasing amounts of deionized water. A reduction in viscosity could be expected, as water is a liquid component. On the contrary, there was a reverse effect. Specifically, an initial rise in viscosity occurred with low quantities of water, followed by a subsequent decrease at higher water content. This decrease became more evident with higher concentrations of cetyl alcohol, while it was less pronounced or absent at lower emulsifier concentrations. In fact, at a 5% w/w of cetyl alcohol, viscosity increased proportionally to the added water.

This was probably attributed to a structural modification associated with the altered interaction among the three molecules (cetyl alcohol, paraffin oil and water). We could not define the internal structure as we lacked the necessary instruments for accurate analysis.

3.4.3.2. Water addition to Glyceryl Monostearate / Paraffin oil system

Rheological analyses were conducted to examine the behavior of samples containing varying concentrations of glyceryl monostearate in the presence of water.

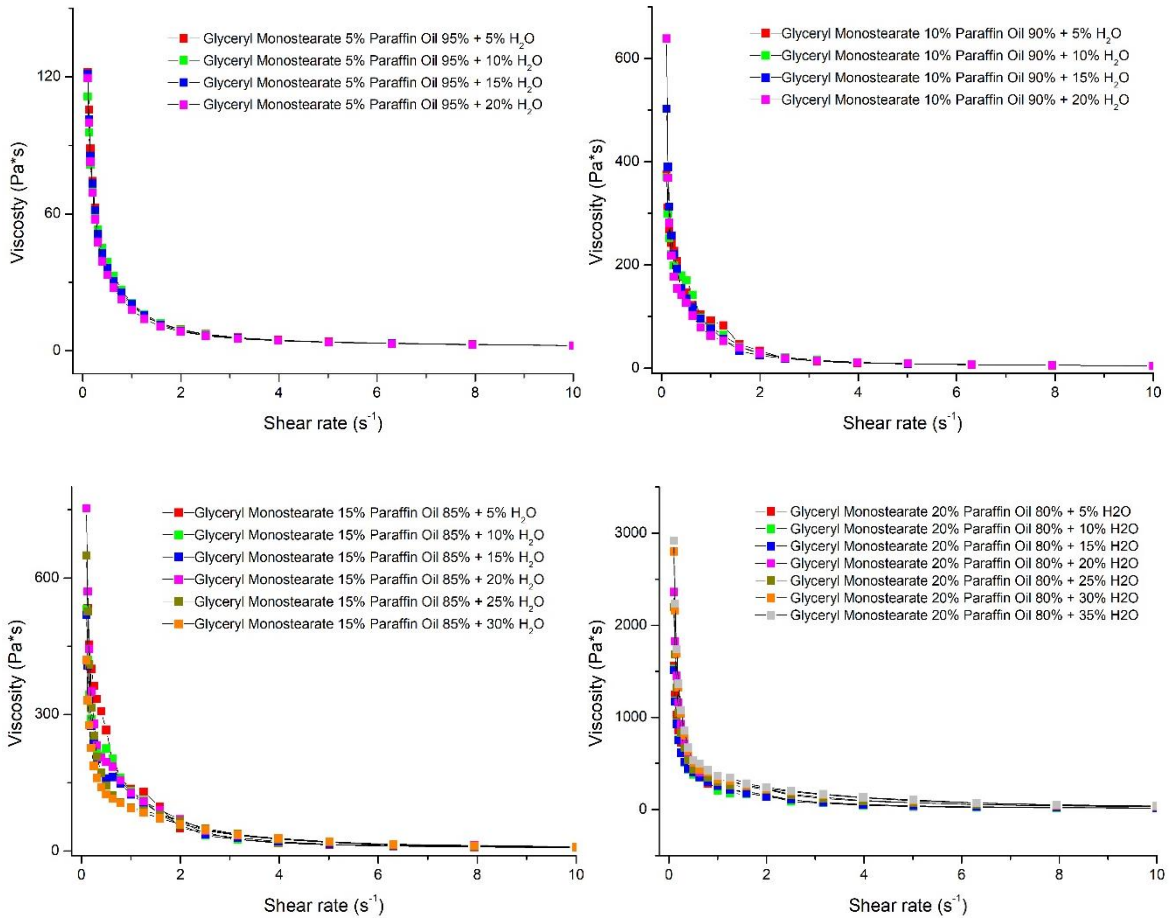


Figure n.6 *Viscosity trend of 5% w/w, 10% w/w, 15% w/w and 20% w/w of glyceryl monostearate with % of water*

Similar to the observations with cetyl alcohol, in these samples, the different percentages of water did not significantly alter the rheological behaviour of the samples.

Table n.5 *Viscosity values of glyceryl monostearate/paraffin oil + % water samples*

Glyceryl Monostearate 5%			Glyceryl Monostearate 10%		
	POWER LAW	SHER RATE 0.1 s^{-1}		POWER LAW	SHEAR RATE 0.1 s^{-1}
% H_2O	K Index	Viscosity (Pa*s)	% H_2O	K Index	Viscosity (Pa*s)
0%	22.74	160.4	**0%**	101.8	1200
5%	18.62	122.1	**5%**	57.39	374.5
10%	18.32	111.3	**10%**	57.15	369.9
15%	18.29	121.2	**15%**	56.82	502.7
20%	17.44	119.5	**20%**	54.21	638.6

Glyceryl Monostearate 15%		
	POWER LAW	SHEAR RATE 0.1 s^{-1}
% H_2O	K Index	Viscosity (Pa*s)
0%	106.2	1321
5%	95.52	649.8
10%	84.26	531.1
15%	80.39	518.6
20%	86.68	753.2
25%	83.13	649.2
30%	81.68	619.7

Glyceryl Monostearate 20%		
	POWER LAW	SHEAR RATE 0.1 s^{-1}
% H_2O	K Index	Viscosity (Pa*s)
0%	479	7424
5%	190.5	1665
10%	226.7	2098
15%	303.4	2745
20%	291.5	2362
25%	275.8	2196
30%	324.1	2800
35%	347.4	2918

Samples containing glyceryl monostearate/paraffin oil could accommodate a maximum of 35% w/w pure water, and this addition altered the viscosity. The rheological analysis revealed that the addition of water resulted in a reduction in viscosity compared to the oily base system. However, the viscosity of the samples was not significantly influenced by the quantity of added water.

In general, for samples containing glyceryl monostearate, upon the addition of a small amount of water, a sudden decrease in viscosity occurred, followed by a marginal increase in viscosity. This observation confirmed that the addition of water led to a reduction in viscosity (Table n.5).

This could suggest that the bound water was low but that there were scattered droplets. In reality, as we will see from the subsequent thermal analysis, the percentage of bound water is still significant. Anyway, these were systems that, in some way, were able to incorporate a considerable amount of water, up to 35% w/w.

3.4.3.3. Water addition to Sorbitan Monostearate / Paraffin oil system

Increasing quantities of water (up to 40% w/w) were added to lipophile bases containing up to 25% w/w sorbitan monostearate and then subjected to rheological analysis to assess viscosity and determine the influence of water presence in the structure of the base.

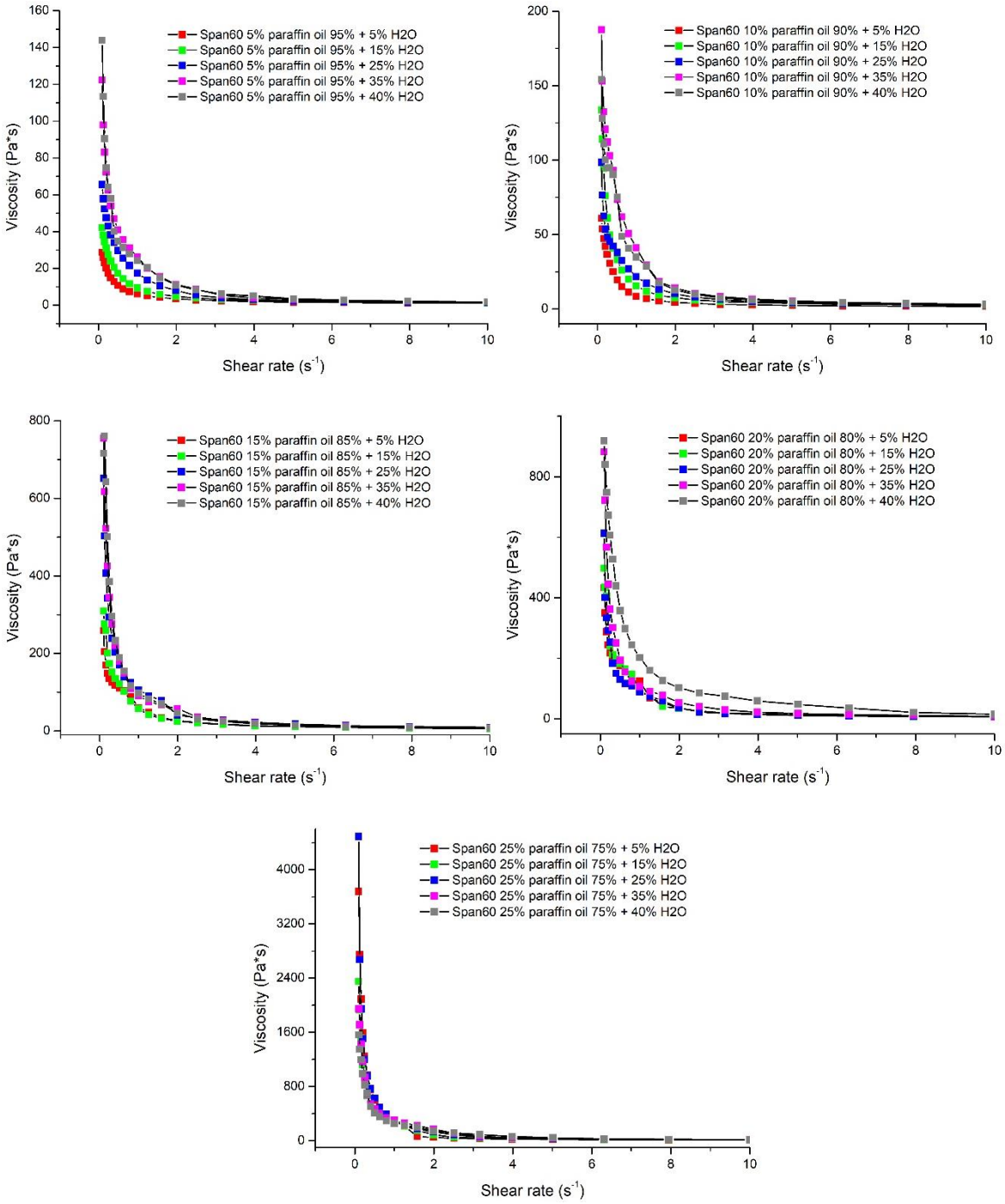


Figure n.7 *Viscosity trend of 5% w/w, 10% w/w, 15% w/w, 20% w/w and 25% w/w sorbitan monostearate + % water samples*

Table n.6 *Viscosity values of sorbitan monostearate / paraffin oil + % water samples*

SORBITAN MONOSTEARATE 5%		
	POWER LAW	SHEAR RATE 0.1 s^{-1}
% H_2O	K Index	Viscosity (Pa*s)
0%	1.733	6.834
5%	6.099	28.78
15%	8.688	42.01
25%	12.97	65.56
35%	18.56	122.3
40%	18.77	144.1

SORBITAN MONOSTEARATE 10%		
	POWER LAW	SHEAR RATE 0.1 s^{-1}
% H_2O	K Index	Viscosity (Pa*s)
0%	5.346	19.82
5%	10.32	60.97
15%	17.57	133.8
25%	17.86	158.47
35%	30.13	187.7
40%	27.1	194.2

SORBITAN MONOSTEARATE 15%		
	POWER LAW	SHEAR RATE 0.1 s^{-1}
% H_2O	K Index	Viscosity (Pa*s)
0%	28.28	128.3
5%	48.36	259.2
15%	51.81	310.1
25%	85.47	651.6
35%	88.16	682.6
40%	91.85	715.9

SORBITAN MONOSTEARATE 20%		
	POWER LAW	SHEAR RATE 0.1 s^{-1}
% H_2O	K Index	Viscosity (Pa*s)
0%	50.37	179.5
5%	70.13	433.5
15%	71.2	497.7
25%	68.45	612.9
35%	97.18	882.9
40%	101.8	918

SORBITAN MONOSTEARATE 25%		
	POWER LAW	SHEAR RATE 0.1 s^{-1}
% H_2O	K Index	Viscosity (Pa*s)
0%	98.73	406.1
5%	194.2	2353
15%	202.4	3679
25%	263.1	4491
35%	230.1	1946
40%	205.8	1565

Formulations containing sorbitan monostearate/paraffin oil could tolerate up to 40% w/w of pure water, resulting in a greater modification in viscosity compared to the oily system, similar to what occurred with cetyl alcohol samples. The tables (Table n.6) revealed an increase in viscosity compared to the oily base. In samples with 5% w/w, 10% w/w, 15% w/w and 20% w/w of sorbitan monostearate, viscosity gradually rose with increasing water

content. Conversely, in samples containing 25% w/w of the surfactant, viscosity initially increased but subsequently decreased with higher water concentrations.

3.4.4. Thermal Properties

Differential scanning calorimetry (DSC) provided valuable information about the thermal properties and behaviour of the materials.

Preliminary analyses were conducted on cetyl alcohol, glyceryl monostearate and sorbitan monostearate. The goal was to identify their respective melting points and observe any alterations when each of them was added to paraffin oil.

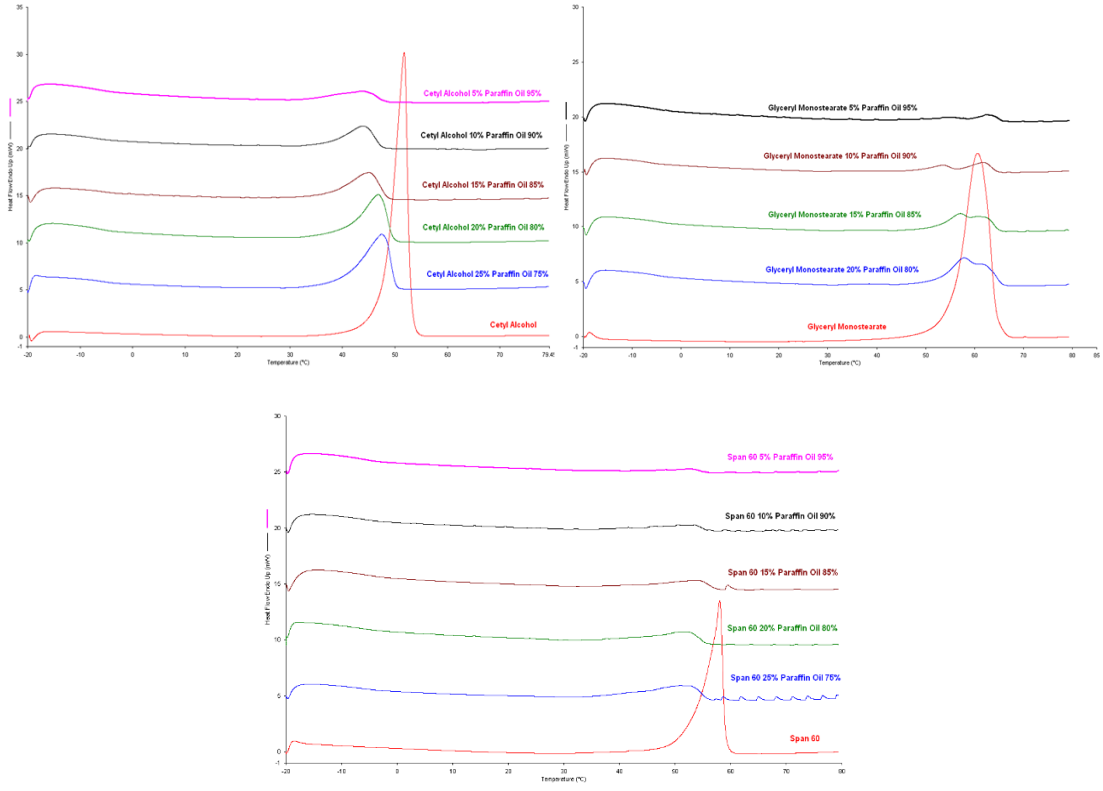


Figure n.8 *Thermal properties of cetyl alcohol, glyceryl monostearate and sorbitan monostearate oily bases*

The Figure n.8 illustrated peak variations of the three long chain surfactants, alone and added to paraffin oil. From the graph, it was evident that, when combined with paraffin oil, the

peak associated with cetyl alcohol remained discernible even at low concentrations. This persistence was probably attributed to the tendency of cetyl alcohol to maintain a crystalline structure. In contrast, with the other two surfactants, particularly sorbitan monostearate, the peak tended to decrease at lower concentrations. There were therefore different interactions with paraffin oil.

After having examined the thermal characteristics of the surfactant/oil bases, their interaction with different percentages of water added, according to previously reported tables (Table n.2 A,B,C) were assessed, in terms of free and bound water.

The quantification of both free and bound water involved correlating the enthalpy of the water peak with the corresponding percentage of water in the sample, in comparison to the enthalpy of free water.

The percentage of free and bound water was calculated comparing the enthalpy of water peak analyzed for each sample to that of the total pure water, considering the actual percentage of water within the sample.

Free and bound water were calculated according to the following equation:

$$X = \frac{\Delta H / \%}{\Delta H_0}$$

- X → Percentage of free water
- ΔH → Entalpy of water peak in the sample
- % → Percentage of added water in the sample
- ΔH_0 → Entalpy of free water

3.4.4.1. Water addition to Cetyl Alcohol / Paraffin oil system

The samples, composed of cetyl alcohol/paraffin oil base with the addition of water, were examined using DSC (Differential Scanning Calorimetry) to evaluate the levels of free and bound water in each sample.

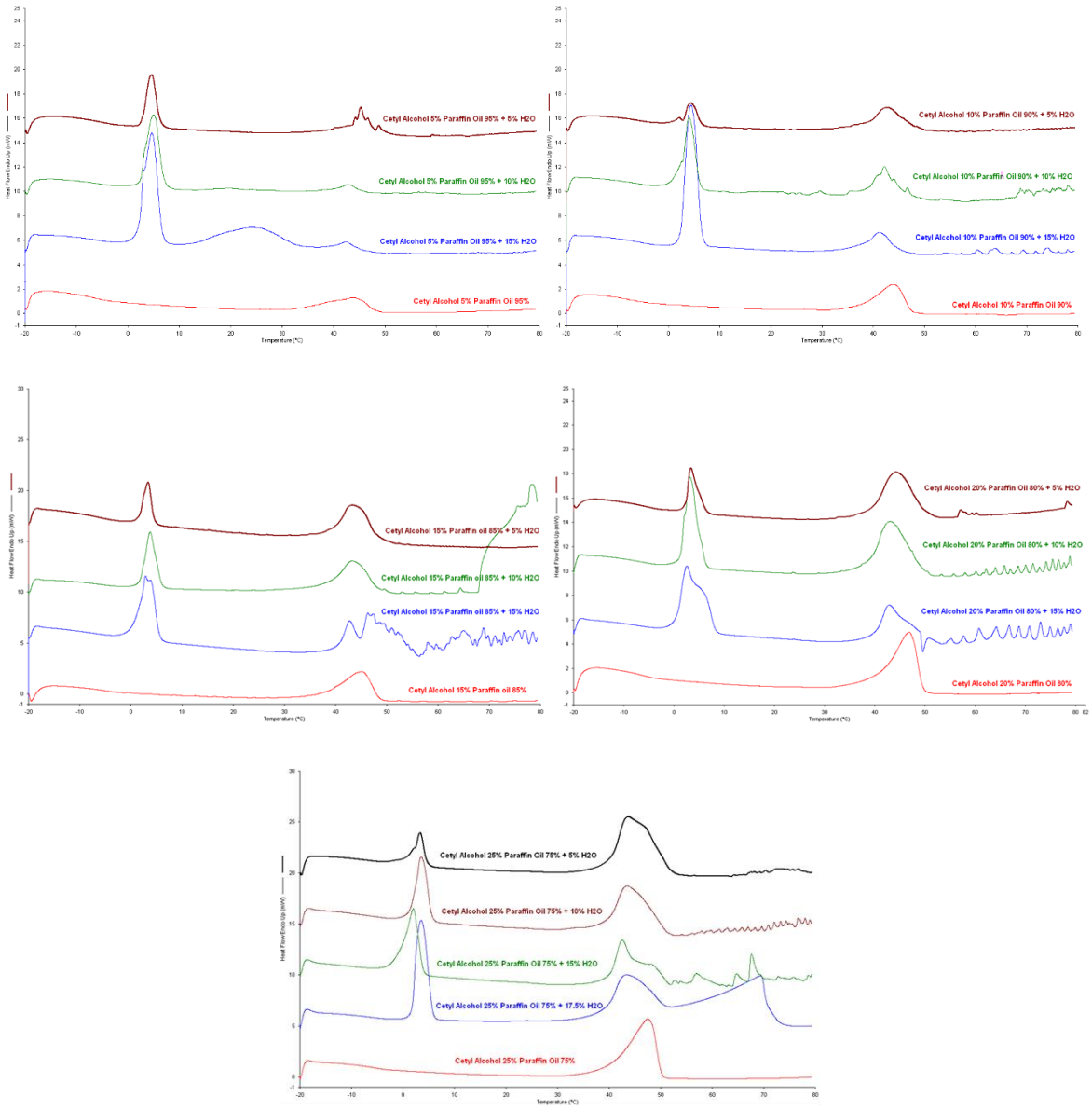


Figure n.9 *Thermal properties of cetyl alcohol / paraffin oil + % water samples*

Table n.7 F*ree and bound water values in cetyl alcohol/paraffin oil samples*

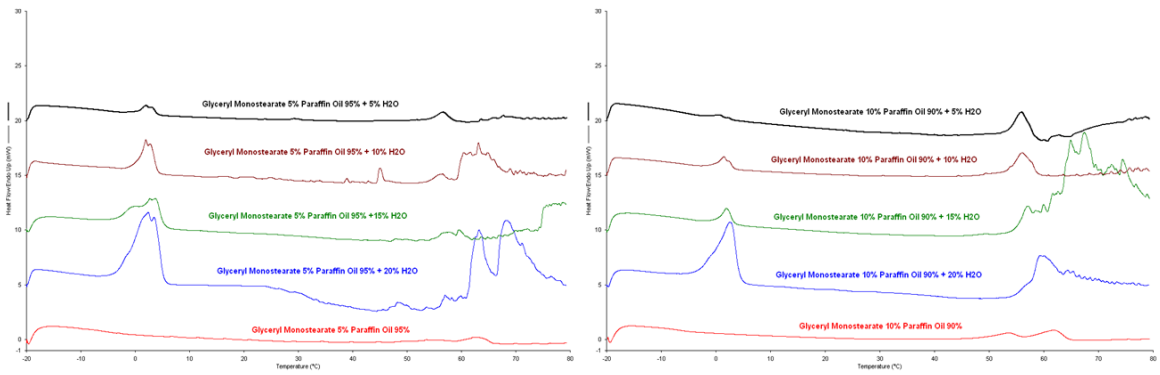
CETYL ALCOHOL	H_2O			
	5%	**10%**	**15%**	**17.5%**
5%	71.9% free 28.1% bound	78.26% free 21.74% bound	80.79% free 19.21% bound	/
10%	59.57% free 40.43% bound	74.88% free 25.12% bound	76.46% free 23.54% bound	/
15%	58.77% free 41.23% bound	69.28% free 30.72% bound	72.36% free 27.64% bound	/
20%	53.95% free 46.05% bound	65.55% free 34.45% bound	69.5% free 30.5% bound	/
25%	42.68% free 57.32% bound	58.44% free 41.56% bound	60.46% free 39.54% bound	68.42% free 31.58% bound

Data derived from the DSC analysis of the samples containing cetyl alcohol was presented in Table n.7. As the concentration of cetyl alcohol increased, the percentage of bound water with the system also increased, resulting in a corresponding decrease in the free water content. Simultaneously, the percentage of bound water decreased in comparison to the free water as the quantity of added water increased in the various samples.

This indicated and confirmed the limited capacity to incorporate water, as previously noted through rheological analysis, within cetyl alcohol / paraffin oil systems.

3.4.4.2. Water addition to Glyceryl monostearate / Paraffin oil system

Thermal analysis was conducted on the entire range of samples containing glyceryl monostearate to determine the quantities of bound and free water present.



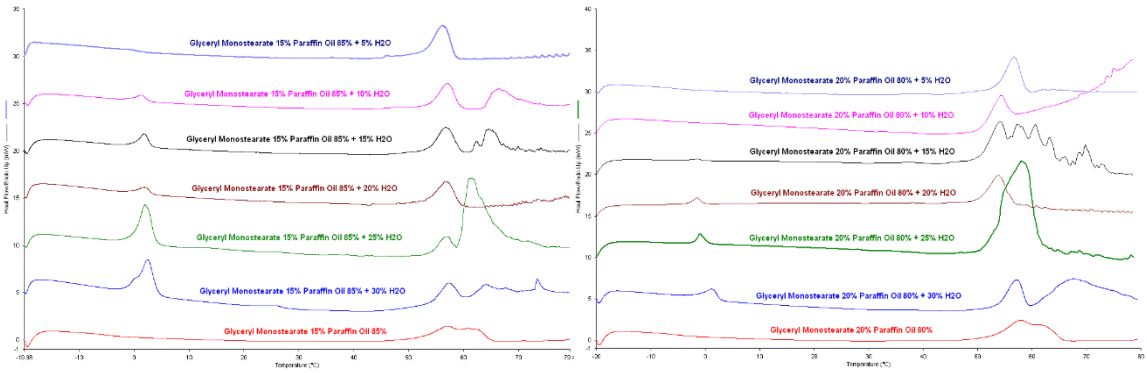


Figure n.10 *Thermal properties of glyceryl monostearate / paraffin oil + % water samples*

Table n.8 *Free and bound water values in glyceryl monostearate/paraffin oil samples*

GLYCERYL MONOST.	H_2O					
	5%	**10%**	**15%**	**20%**	**25%**	**30%**
5%	19.69% free 80.31% bound	34.25% free 65.75% bound	38.41% free 61.59% bound	58.02% free 41.98% bound	/	/
10%	6% free 94% bound	9.83% free 90.17% bound	8.24% free 91.76% bound	46.77% free 53.23% bound	/	/
15%	100% bound	7.1% free 92.9% bound	7.71% free 92.29% bound	10.6% free 89.4% bound	20% free 80% bound	25.7% free 74.3% bound
20%	100% bound	100% bound	0.57% free 99.43% bound	2.24% free 97.76% bound	9.1% free 90.9% bound	26.89% free 73.11% bound

The Table n.8 illustrated the variations in the percentage of free and bound water in samples containing quantities ranging from 5% w/w to 20% w/w of glyceryl monostearate. In samples with 5% w/w and 10% w/w of the emulsifier, the system could incorporate a maximum of 20% w/w of pure water, while for samples containing 15% w/w and 20% w/w of glyceryl monostearate, the maximum water content was 30% w/w.

The Table n.8 showed that the percentage of water bound to the system increased with an increase in glyceryl monostearate concentration, concurrently diminishing as the added water quantity became greater (up to 20%-30% w/w). Additionally, it was observable that for samples with 15% w/w and 20% w/w of the emulsifier and 5% w/w and 10% w/w of water, the percentage of added water was entirely incorporated into the system.

Therefore, it could be stated that glyceryl monostearate / paraffin oil systems, owing to the presence of glyceryl monostearate, not only exhibited a notable capacity for water

incorporation but also the water was largely bound to the structures formed by the surfactant within the oil. This also elucidated the impressive water incorporation capability.

3.4.4.3 Water addition to Sorbitan Monostearate / Paraffin oil system

DSC analysis was performed to determine the percentage of free and bound water in samples containing sorbitan monostearate.

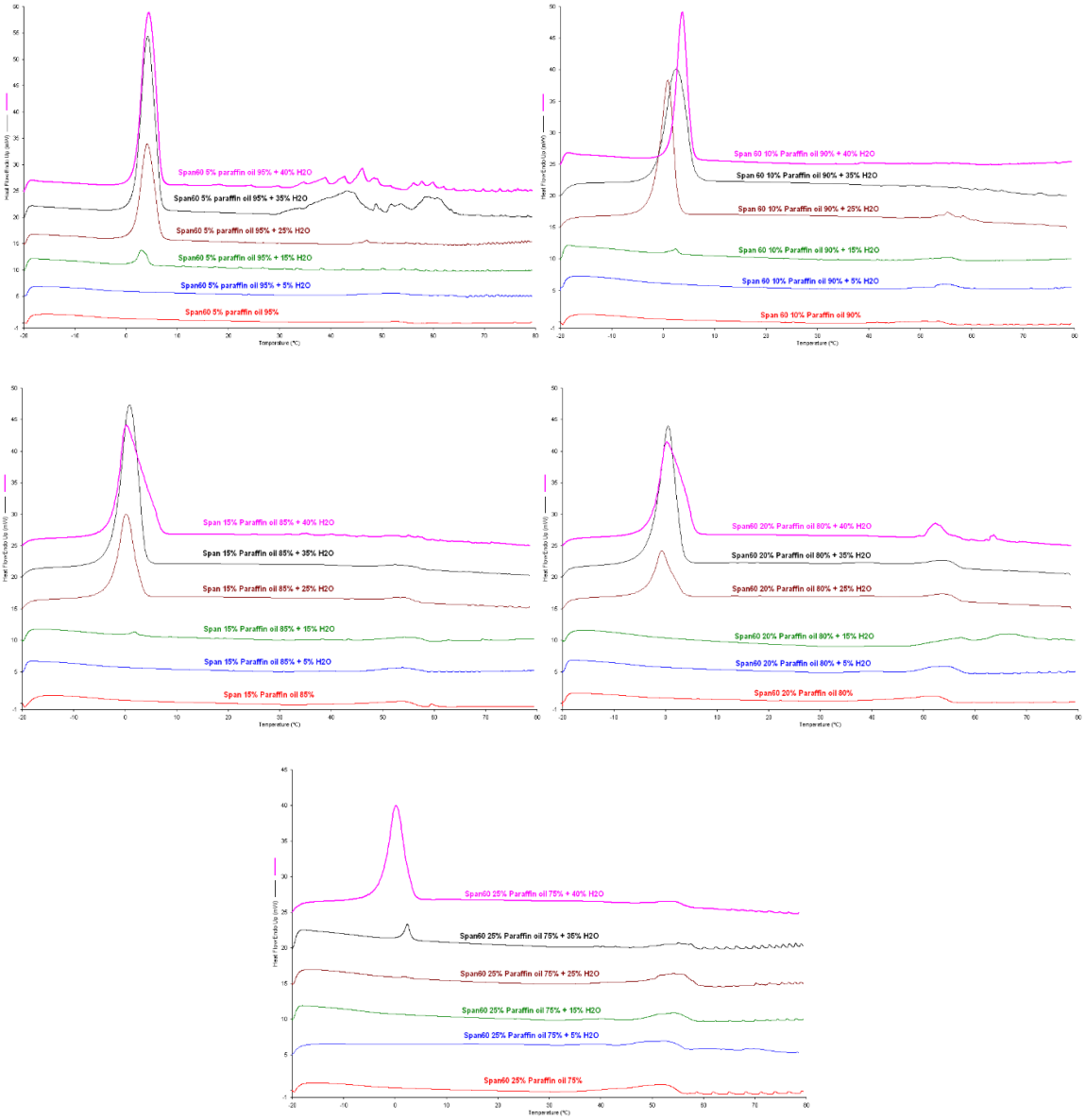


Figure n.11 *Thermal properties of sorbitan monostearate/ paraffin oil + % water samples*

Table n.9 *Free and bound water values in sorbitan monostearate/paraffin oil samples*

SORBITAN MONOST.	H_2O				
	5%	**15%**	**25%**	**35%**	**40%**
5%	100% bound	13.14% free 86.86% bound	81.97% free 18.03% bound	92.12% free 7.88% bound	96.03% free 3.97% bound
10%	100% bound	3.31% free 96.69% bound	57.97% free 42.03% bound	82.38% free 17.62% bound	87.07% free 12.93% bound
15%	100% bound	2.6% free 97.4% bound	44.1% free 55.9% bound	70.37% free 29.63% bound	81.3% free 18.7% bound
20%	100% bound	100% bound	32.7% free 67.3% bound	62.02% free 37.98% bound	75.45% free 24.55% bound
25%	100% bound	100% bound	0.41% free 99.59% bound	12.97% free 87.03% bound	65.85% free 34.15% bound

The data obtained from the analysis through differential scanning calorimetry (Figure n.11) has been reported in Table n.9. The combination of sorbitan monostearate and paraffin oil, at various concentrations, can incorporate up to 40% w/w of water without undergoing phase separation. The pattern observed in the percentage of free and bound water in various samples aligned with that of other studied emulsifiers. Specifically, the percentage of bound water increased with the rise in sorbitan monostearate concentration and concurrently decreased with the increase of the total amount of water. Furthermore, it is important to emphasize that this oily system could bind the largest amount of water than the other two surfactants. In fact, there were more samples that exhibit 100% bound water (all concentrations of sorbitan monostearate with the addition of 5% w/w of water, and 20% – 25% w/w of sorbitan monostearate with 15% w/w of water).

Based on the data acquired, it could be concluded that the systems involving sorbitan monostearate and paraffin oil, facilitated by sorbitan monostearate, exhibited the highest capacity not only for water absorption but also for retaining it nearly entirely as bound water.

3.5. CONCLUSION

This project resulted in the development of three lipophilic bases that can absorb a significant amount of water, avoiding phase separation and maintaining stability over time.

Rheological analysis revealed that for each type of lipophilic base, viscosity rose with the increase in emulsifier concentration. It also enabled the identification of the maximum concentrations of emulsifiers that could be investigated, ensuring a consistent availability of analyzed samples. This was particularly important for future applications, as it ensured that once the active ingredient is added, the samples will be easily spreadable on the skin. Among the selected emulsifiers, glyceryl monostearate demonstrated the highest thickening ability of the oily phase, followed by sorbitan monostearate and, lastly, cetyl alcohol. In most instances, the viscosity of the samples increased following the addition of water, especially in the cetyl alcohol/paraffin oil and sorbitan monostearate/paraffin oil systems. In the case of glyceryl monostearate, the addition of water resulted in a decrease in viscosity compared to that of the oily system.

The outcomes derived from the formulation and analysis of the samples indicated that sorbitan monostearate has exhibited greater effectiveness in incorporating water into the system compared to other emulsifiers, particularly in comparison to cetyl alcohol.

DSC analysis has revealed the variations in the thermal transitions of the emulsifier when introduced into an oily system at escalating concentrations: sharp peaks were not consistently obtained. Thermal analysis has also facilitated the assessment of the water incorporation capacity of the three oil systems. In the analyzed samples, the percentage of bound water escalated with an increase in emulsifier concentration.

Sorbitan monostearate systems exhibited a greater number of samples with the ability to fully bind water, with glyceryl monostearate exhibiting this capability to a lesser extent.

The systems involving sorbitan monostearate / paraffin oil demonstrated the capability not only to incorporate water but also to retain it almost entirely as bound water.

This characteristic, combined with the observation, through rheological analysis, that the presence of water contributed to an increase in viscosity and consequently enhanced the semi-solid nature of the system, sorbitan monostearate resulted as the most favorable choice for formulating W/O creams.

In summary, we can delineate the following descending order based on characteristics:

Thickening ability of the oily phase →

1) Glyceryl Monostearate
2) Sorbitan Monostearate
3) Cetyl Alcohol

Ability to incorporate water →

1) Sorbitan Monostearate
2) Glyceryl Monostearate
3) Cetyl Alcohol

Further increase in viscosity in the presence of water →

1) Sorbitan Monostearate
2) Cetyl Alcohol
3) Glyceryl Monostearate

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