DESIGN AND DEVELOPMENT OF
NANOSTRUCTURED FORMULATIONS FOR
COSMECEUTIC APPLICATIONS

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TRIENNIO 2012-2015
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Introduction

Nowadays the care of the exterior appearance and the purchase of cosmetic products that preserve the health and human well being, satisfying the relationship health = well being = beauty, are covering an increasingly important role. In this context, the present PhD project, developed in collaboration with Eurosocap srl company, world leader in the production of hair extensions, is aimed at the design and the development of highly innovative trichological formulations for cosmeceutic applications.

In the first part of this work, the interest is focused on the optimization of formulations based on the use of new components, safe for the health of customers and more eco-friendly. Several cosmetic formulations for hair care were prepared. In these formulations main components are surfactants, molecules that spontaneously self-organize into a variety of nanostructures, from micelles to vesicles. Other important components in cosmetic formulations are preservatives whose function is to avoid the product degradation. Considering that the surfactants themselves present bacteriostatic effects, it was evaluated if preservatives are actually needed in trichological formulations. For this reason, a prototype of a shampoo and of a conditioner were formulated both without preservatives and with preservatives in order to underline microstructural differences and to obtain microbiological information, through
respectively Electron Paramagnetic Resonance (EPR) studies and a specific biological test, called Challenge test. The second part of this thesis concerns the evolution from cosmetic to cosmeceutic formulations. Cosmeceutic products are cosmetic-pharmaceuticals hybrids which can be applied topically as cosmetics but that contain physiologically active substances such as restructuring vitamins, growth factors, enzymes or antioxidants. In this project a particular attention is given to a powerful antioxidant, the resveratrol in order to contrast the damaging effects of oxidative stress on the hair structure. The aim is to insert this molecule, poorly soluble in water, into adequate nano-vehicles so as to preserve the resveratrol efficacy, its stability and to allow the gradual release of this substance on the hair surface. Nano-vehicles, compatible with a typical hair formulation, are represented by catanionic vesicles, aggregates formed by an anionic and a cationic surfactant. In particular two surfactant very common in hair formulations were chosen: Sodium Dodecyl Sulfate (SDS) and Cetrimonium Chloride (CTAC). Catanionic vesicles obtained by mixing the two surfactant solutions at different molar ratios were characterized through Dynamic Light Scattering (DLS), Small Angle Neutron Scattering (SANS) and Electron Paramagnetic Resonance (EPR) measurements in order to describe the phase behavior for the catanionic mixture SDS-CTAC-water, both in the cationic rich-side and in the anionic rich-side.
Then, UV measurements were executed so as to evaluate resveratrol solubility in CTAC, SDS and in catanionic vesicles solutions and spectrofluorimetry analyses were carried out to obtain information about the chemical environment in which the antioxidant is located.

Finally, EPR studies were performed in order to evaluate the ability of resveratrol as radical scavenger in surfactants aggregates.

Overall, this thesis represents an attempt to build a reliable scientific and technological basis for the development of cosmeceutic formulations for hair care.
Chapter 1 – An overview of hair: structure and chemical composition

1.1 Introduction

Hair structure is much more complicated than it appears. Hair is a characteristic of all mammals and in humans grows over a large percentage of the body surface. The hair has a cylindrical structure, highly organized, formed by inert cells, most of them keratinized and distributed following a very precise and predefined design. Hair forms a very rigid structure in the molecular level which is able to offer the fiber both flexibility and mechanical resistance. Hair is considered as a dead matter and it is only alive when it is inserted in the scalp. When the thread emerges, it becomes dead matter although it appears to be growing since the fiber follows increasing its length by a speed of about 1.0 cm/month. Human hair has about 65-95% of its weight in proteins, more 32% of water, lipid pigments and other components. Chemically, about 80% of human hair is formed by a protein known as keratin with a high grade of sulfur, coming from the amino acid cysteine, which is the characteristic to distinguish it from other proteins. Keratin is a laminated complex formed by different structures, which gives the hair strength, flexibility, durability, and functionality [1]. Keratin fibers consist of long, tightly bound molecular chains held together in many different ways from covalent bonds to weaker
interactions such as hydrogen bonds, coloumbian interactions, van der Walls interactions and hydrophobic bonds. These interactions provide the fiber with remarkable resistance to various environmental constraints and attacks, such as friction, tension, flexion, UV radiation and chemical insult.

1.2 Anatomy of hair fiber

Human hair grows from large cavities or sacs called follicles. Hair follicles extend from the surface of the skin through the stratum corneum and the epidermis into the dermis (Fig.1).

Fig.1 : overview of integument system

Connected to hair follicles, there are the sebaceous glands that in humans are distributed throughout all the skin and found in greatest abundance on the face and scalp whereas they are absent from the palms and soles. Together with hair
follicle and arrector pili muscles, sebaceous glands form the pilosebaceous unit.

Sebaceous glands are microscopic glands which secrete an oily substance (sebum) in the hair follicles to lubricate the skin and hair of animals. Their function within the epidermis is to prevent the skin from dehydration and protect the body against infections and physical, chemical and thermal assaults of the environment. The main components of human sebum are triglycerides, fatty acids, wax esters and squalene. The production of sebum is regulated throughout life, and decreases dramatically with age [2].

In a fully formed hair fiber, it is possible to distinguish three main structures: cuticle, cortex and medulla (Fig.2).

![Section of a human hair fiber.](image)

Cuticle is the outer layer and protects inner layers. It is the most important component of the human hair, since it may be more or less affected by cosmetic treatments. On the cuticle, cosmetic products, such as conditioners, hair sprays, mousses
and gels are deposited. There are approximately 8 to 11 layers of cuticle, which are overlapped in the distal direction of the thread, depending on the type, condition, and the length of the hair. Each layer is formed by only one cell. Each cell of the cuticle contains a thin external membrane, the *epicuticle* which is a protein coat covered by a strong lipid structure. The other layers are:

- **Layer A**: a resistant structure containing cystine (>30%). The cross links of proteins, in this layer, not only give physical resistance but also makes them relatively resistant to chemical attack. These properties protect the fiber against both mechanical and chemical attacks.

- **Exocuticle**: also known as *Layer B*, corresponds to 55% of the cuticle area and it is rich in cystine (~15%), and it is physically rigid (but less intensively than *Layer A*).

- **Endocuticle**: with low grade of cystine (~3%), it is much softer than the superior layers and there are evidences that it swells with water.

- **Cellular membrane complex (CMC)**: these cells separate all the cells in the cuticle. The 18-methyl eicosanoic acid (18-MEA) is one of the most important lipid components of the CMC. This lipid is also bound in a covalent way to the hair external surface. 18-MEA is the main lipid in the hair composition contributing to the wet and dry combing proprieties.
• **Epicuticle**: this is considered the most important part of the cuticle and it is the most external layer. It is composed by \(\sim 25\%\) lipids and \(\sim 75\%\) proteins. Lipids involving mainly the 18-methyl eicosanoic acid form a hydrophobic region. This cuticle hydrophobic aspect is important because hydrophobic materials as silicones, fat alcohols, oils and polymers have great affinity for hydrophobic surfaces.

Overall, the condition of the cuticle is responsible for the hair visual and tactile properties; moreover it has the potential to assist in diagnosis of health disorders and may also be used forensically to provide information on the identity of the hair’s owner [1].

The cuticle layers surround the cortex that contains the major part of the fiber mass. The cortex is made up of cortical cells, which comprise the macrofibrils, long filaments oriented parallel to the axis of the fiber. Each macrofibril consists of intermediate filaments (IF), known also as microfibrils, and the matrix. The matrix proteins that surround the IFs through intermolecular disulfide bonds act as a disulfide cross-linker holding the cortical superstructure together and conferring high mechanical strength, inertness and rigidity to keratin fibres.

Dispersed throughout the structure of the cortex in granular form are the melanin pigment particles. The number,
chemical characteristics and distribution pattern of these cells determine the colour of the hair [3] (Fig.2).

Fig.2: Details of cortex and cuticle structures in hair fiber.

Thicker hairs often contain one or more porous regions called the medulla, located near the center of the fiber. The medulla is a thin cylindrical layer containing high lipid concentration and poor cystine. Its function is not yet completely elucidated, although its cells may become dehydrated and its spaces may be filled with air, which affects both color and shine in white, brown and blond hair. In human hair, however, the medulla is typically found only in terminal hair and may be continuous, discontinuous or even absent [4].

1.3 Biology of human hair

Hair is an important feature of mammals, where hair shafts fulfill a number of different functions such as thermoregulation, collection of sensory information,
protection against environmental trauma, social communication and camouflage. Each of us displays an estimated total number of 5 million hair follicles (HF), of which 80,000–150,000 are located on the scalp.

1.3.1 Hair follicle life cycle

The HF is one of the most complex mini-organs of the human body with the capacity to reconstitute itself. Follicle morphogenesis regularly occurs only during embryonic development, so each mammal is born with a fixed number of follicles that normally does not increase afterwards, although folliculoneogenesis can take place during wound healing.

The hair growth cycle describes the changing histological morphology of the shaft and of the follicle over time. Starting with anagen (rapid growth and hair shaft elongation), the follicle and its shaft progress through catagen (involution and apoptosis-driven regression), telogen (resting) and finally exogen (shedding).

Fig 3: Illustration of growth hair fiber.
In anagen, the growth phase of the hair cycle, hair undergoes morphological and molecular events similar to fetal HF morphogenesis. Many key molecular regulators of hair biology not only activate morphogenesis, but also regulate anagen induction and duration. In this phase, epithelial bulge stem cells differentiate into the various cell types that will reconstitute the entire hair shaft. Hair shaft synthesis and melanin production in melanocytes (melanogenesis), only take place in anagen. Pigmentation begins after the initiation of shaft formation and ends before this process is terminated, which causes the shaft to have an unpigmented tip and root. Anagen ends with a regulated involution of the HF, which is accompanied with apoptosis and terminal differentiation of cells, a period designated as catagen. The first sign of catagen is the cessation of melanin production in the hair bulb and apoptosis of follicular melanocytes, derived from melanocytic stem cells of the secondary hair germ. Programmed cell death of these stem cells seems to play an important role in hair graying.

During catagen, metabolic activity slows down whereas the anagen stage is characterized by intense metabolic activity in the hair bulb. The catagen stage lasts for only a few weeks. After regression, the HF enters telogen, a phase of relative quiescence regarding proliferation and biochemical activity. The follicle remains in this stage until it is reactivated by intra- and extrafollicular signals.
Once the growth cycle is complete and the phase of telogen reaches the end, the hair shaft will be shed. This process, exogen or teloptosis, is independent of a possible new HF cycle. In fact, it is most common in mammals that a new hair shaft regrows before the resting shaft sheds, assuring the animal is never completely naked.

All body hairs undergo a similar life cycle, although its extent, the duration of its phases and the length of individual shafts vary between different body areas and between individuals, depending on genetic programming, genre, age and health status [3].

1.4 Chemical composition of hair

Human hair is a complex tissue consisting of several morphological components and each component consists of several chemical species. For a clear understanding of its chemical and physical behavior, it is necessary to keep in mind that this fiber is an integrated system wherein several or all of its components can act simultaneously.

Depending on its moisture content (up to 32% by weight), human hair consists of approximately 65 to 95% proteins, principally keratins. There are different subtypes of keratin proteins, some are called "soft" keratins and others are "hard" keratins. Basically, soft keratins are found in the skin and are relatively easy to break down whereas hard keratins are very resistant to degradation. Hard keratins don't dissolve in water and they are highly resistant
to proteolytic enzymes. Hair fiber is mostly made from hard keratin proteins. Through specific techniques, different scientists have analyzed aminoacidic composition of these proteins in the cuticle, cortex and medulla. Bradbury et al. have suggested that the cuticle of human hair contains more cystine, cysteic acid, proline, serine, threonine, isoleucine, methionine, leucine, tyrosine, phenylalanine, and arginine than whole fiber.

Also the two main fractions of the hair cuticle, the exocuticle and the endocuticle, have been separated after enzymatic digestion and analyzed. Their chemical compositions are quite different. The proteins of the exocuticle and its A-layer are highly cross-linked by cystine (more than 30%) and, therefore, are extremely tough and resilient. In contrast, the proteins of the endocuticle contain very little cystine (3 to 6%) and relatively large amounts of the dibasic and diacidic amino acids. As a result of these large compositional differences, these two layers of the cuticle can be expected to react differently to permanent waves, bleaches, and even water and surfactants. With regard to the epicuticle and in particular the cell membrane complex was found to be rich in the dicarboxylic amino acids, aspartic acid, and glutamic acid. Analyzing the cortex, it is rich in cystine (although there is less cystine in cortex than in cuticle), but it is richer in diacidic amino acids, lysine and histidine than the cuticle. The two main components of cortex, the intermediate filaments
and the matrix, are very different in chemical composition. The intermediate filaments are rich in leucine, glutamic acid, and those amino acids that are generally found in alpha-helical proteins, although small quantities of cystine (~6%), lysine, and tyrosine are also regularly arranged in the intermediate filaments. On the other hand, the matrix is rich in cystine (about 21%), proline, and those amino acids that resist helix formation.

About the medulla, complete chemical analysis of this part of human hair fibers has not been reported. Studies of the medulla of human hair are complicated because it has poor solubility and it is difficult to isolate. For this reason, most of the experimental work on medulla has been on African porcupine quill, horse hair, or goat hair medulla rather than medulla of human hair fiber.

Also lipids are important in the human hair fiber. Sakamoto et al. claim that fatty acids and wax esters are the main components of the internal lipids of human hair. Hilterhaus-Bong and Zahn also find fatty acids, cholesteryl esters, and wax esters as main components. Negri et al. have shown that the outer lipid layer of epicuticle is predominately composed by 18-methyleicosanoic acid, a branched fatty acid consisting of 21 carbon atoms, and this fatty acid represents about 25% of the epicuticle. These authors have proposed a model of the epicuticle wherein the fatty-acid layer is connected to the underlying fibrous protein layer through thioester linkages involving the cysteine residues of the protein.
In the cell membrane complex, the fatty acids are predominately palmitic, stearic, and oleic acids. Hoting and Zimmerman have shown that the cell membrane complex lipids of hair fibers are degraded more by visible light, although these are also degraded by UV-A and by UV-B light helping to explain the weakened cell membrane complex and the multiple step fractures observed in sunlight oxidized hair. Further, these scientist have also demonstrated that the cell membrane complex lipids of chemically bleached hair are more readily degraded than the lipids of chemically unaltered hair [4].

However, much of our knowledge of hair lipids comes from “fat solvent” studies. Depending on the solvent used, different results are obtainable; ethanol removes more lipids from hair than solvents such as benzene, ether and chloroform [5]. The data (1 to 9% extracted hair lipid) represent total matter extracted from hair clippings of individual men and women. Presumably, the principal material in these extracts is derived from sebum and consists primarily of free fatty acids and neutral fat (esters, waxes, hydrocarbons, and alcohols).

Gloor classifies the different components of sebum into six convenient groups: free fatty acids (FFA), triglycerides (TG), free cholesterol (C), cholesterol and wax esters (C & WE), paraffins (P), and squalene (S). The composition of the sebaceous secretion changes with age. Nicolaides and Rothman have shown that the paraffinic hydrocarbon content of sebum is highest in children, lower in men, and lowest in
women. These same two scientists have also shown that the squalene content of the hair lipid of children, approximately 1.35% of the total lipid content is about one-fourth that of adults. In addition, the cholesterol content of the hair lipid of children is less than that from adults: 3.7% versus 12.2% whereas there is no difference with regard to fatty alcohol content of human hair lipid.

Another component present in human hair fiber is water. The amount of moisture in hair plays a critical role in its physical and cosmetic properties. Hydrophilic side chains (guanidino, amino, carboxyl, hydroxyl, phenolic, etc.) and peptide bonds of keratin fibers contribute to water sorption. Leeder and Watt in a very interesting study conclude that the binding of water by amino and guanidino groups is responsible for a large percentage of the water sorption capacity of keratin fibers, especially at low humidities. On the other hand, Breuer concludes that the peptide bonds are preferential sites for hydration. At low relative humidities, water molecules are principally bonded to hydrophilic sites of the fiber by hydrogen bonds. As the humidity increases, additional water is sorbed, producing a decrease in the energy of binding of water already associated with the protein. At very high relative humidities, above 80%, multimolecular sorption (water on water) becomes increasingly important [4].

In general humidity is an important factor when considering hair beauty and styling. Air humidity affects hair form and structure at the level of hydrogen bonds. A humidity increase
of 30–70% will augment by twofold the water content of hair, thereby increasing its volume by more than 20%. This influx of water eventually causes the hair fibers to swell, which results in friction between fibers and an additional increase in volume and frizz, changing hair appearance [3].

In human hair there are also trace elements. There are a number of studies describing the quantitative determination of various elements of human hair other than carbon, hydrogen, nitrogen, oxygen, and sulfur. In particular, the inorganic constituents of human hair appear to be receiving some attention because of their potential in diagnostic medicine and in forensic science. The mineral content of human hair fibers is generally very low (less than 1%), and it is difficult to determine whether this inorganic matter is derived from an extraneous source or whether it arises during fiber synthesis. Regardless of its origin, the principal metal content of human hair probably exists as an integral part of the fiber structure (i.e., as salt linkages or coordination complexes with the side chains of the proteins or pigment), although the possibility of mineral deposits as in soap deposition also exists.

Among the trace elements reported in human hair are Ca, Mg, Sr, B, Al, Na, K, Zn, Cu, Mn, Fe, Ag, Au, Hg, As, Pb, Sb, Ti, W, V, Mo, I, P, and Se.

From a study involving quantitative analysis of 13 elements in human hair and in hair wash solutions, Bate et al. concluded that a large portion of the trace elements in the
hair they analyzed were due to sweat deposits. In the case of metals, the water supply generally provides calcium and magnesium to hair. Common transition metals such as iron and manganese can also deposit in hair from the water supply. Other sources of metals in hair are diet, air pollution and metabolic irregularities. Metal contamination can also arise from hair products that provide zinc or selenium (antidandruff products), potassium, sodium, or magnesium (soaps or shampoos), and even lead from lead acetate-containing hair dyes. Concentrations of metals such as cadmium, arsenic, mercury and lead in hair tend to correlate with the amounts of these same metals in internal organs. For this reason the human hair can be considered as a diagnostic tool. For example, a study by Capel et al. indicated significantly higher concentrations of cadmium in hair from dyslexic children than in a normal control group [4]. Hair analysis is also being considered as a screening tool for diabetes because low levels of chromium in the hair have been demonstrated in victims of juvenile-onset diabetes.
References


Chapter 2 - Cosmetic formulations for hair care

In the second half of the twentieth century, the desire for products that improve the look and feel of hair has created a huge industry for hair care. Many scientists have focused on the physical and chemical properties of hair to develop products which get better the health, feel, shine, color, softness, and overall aesthetics of hair. For this reason, today the world is full of cosmetic formulations for hair care, principally shampoos and conditioners that represent the highest volume of products sold in personal care.

The primary function of shampoos is to clean both the hair and the scalp by removing effectively dust, soils and excessive sebum. Shampoos have also important secondary functions such as dandruff control, mildness (baby shampoos) and conditioning. In particular, this last function has become even more important with the advent of silicone-containing shampoos and some shampoos containing cationic polymers. With regard to the hair conditioners, their primary function is to make the hair easier to comb. Ease of combing depends on lubrication of the fiber surface. This action is accomplished by the sorption or binding of lubricating or conditioning ingredients onto the hair surface. Secondary benefits such as preventing flyaway hair, making shine the hair and protecting the hair from further damage by forming a thin
coating over the fibers are also important functions of hair conditioners [1].

In this chapter main components present into shampoos and conditioners will be described. Thus, a particular attention will be dedicated to the preservatives commonly present in trichological formulations and a new preservative, based on colloidal silver, will be presented.

2.1 General formulations for shampoos and conditioners

Shampoos consist of several types of ingredients generally containing many of the following types of components:
- primary surfactant for cleaning and foaming;
- secondary surfactant for foam and/or viscosity enhancement;
- viscosity builders: gums, salt, amide;
- solvents to clarify the product or to lower the cloud point;
- conditioning agents;
- opacifier for visual effects;
- acid or alkali for pH adjustment;
- colors for visual effects;
- fragrance;
- preservative;
- active ingredients (e.g., antidandruff agents).

Hair conditioners, on the other hand, are very different compositionally from shampoos. These are usually composed of many of the following types of ingredients:
- oily and/or waxy substances including mineral oil, long chain alcohols, and/or triglycerides or other esters including true oils and waxes, silicones, and/or fatty acids;
- cationic substances consisting of mono-functional quaternary ammonium compounds or amines or even polymeric quaternary ammonium compounds;
- bridging agents to enhance the adsorption of hydrophobic ingredients to the hair;
- viscosity builders;
- acid or alkalies for pH adjustment;
- colors and preservatives.

In both types of formulations, the key component, present in the highest amount, is represented by water.

### 2.1.1 Surfactants

Surfactants are amphiphilic molecules containing a hydrophilic part (the head) and a hydrophobic part (the tail) (Fig.1). These molecules spontaneously self-organize into a variety of structures, from micelles to vesicles, over a specific surfactant concentration, known as \textit{cmc (critical micelle concentration)}. 
Surfactants can be divided in ionic and non-ionic. A non-ionic surfactant don't have charge groups in its head (e.g. ethoxylate, amide and ester groups). The head of an ionic surfactant carries a net charge. If the charge is negative, the surfactant is more specifically called anionic; if the charge is positive, it is called cationic. If a surfactant contains a head with two oppositely charged groups, it is termed zwitterionic.

Anionic surfactants contain anionic functional groups at their head, such as sulfate, sulfonate, phosphate, and carboxylates. Examples of anionic surfactants, commonly present in cosmetic formulations for hair care, are ammonium lauryl sulfate, sodium lauryl sulfate or sodium dodecyl sulfate (SDS) and the related alkyl-ether sulfate sodium laureth sulfate, also known as sodium lauryl ether sulfate (SLES). In most modern shampoos, the primary cleansing agents are anionic surfactants which are known to be good in cleaning. Dirt and greasy residue are removed from the hair and scalp by these surfactants, making them the most important part of the shampoo. Surfactants have great lathering capabilities and
rinse off very easily. In general, anionic surfactants clean the hair through the following process: surfactant molecules encircle the greasy matter on the hair surface. The molecule end which is soluble in greasy matter buries itself in the grease while the water-soluble molecule end is faced outward with a negative charge. Since the hair fibers are negatively charged as well, the two negative charges repel each other. Thus, the greasy matter is easily removed from the hair surface and rinsed off (Fig.2).

![Fig.2: Anionic surfactants action on uncleaned hair.](image)

Regarding cationic surfactants the hydrophilic part is often represented by quaternary ammonium ion. These surfactants are primary surfactants in hair conditioners. The negative charge of the hair is attracted to the positively charged conditioner molecules, which results in conditioner deposition on the hair. This is especially true for damaged hair, since damaging processes result in hair fibers being even more negatively charged. The attraction of the conditioner to hair results in a reduction of static electricity on the fiber surfaces,
and consequently a reduction in the “flyaway” behavior. The conditioner layer also flattens the cuticle scales against each other, which improves shine and color. The smooth feel resulting from conditioner use gives easier combing and detangling in both wet and dry conditions [2].

For the zwitterionic surfactants the charge of the hydrophilic part is controlled by the pH of the solution. This means that they can act as anionic surfactant in an alkalic solution or as cationic surfactant in an acidic solution. These molecules, together with non ionic surfactants, are often used as co-surfactants both in shampoos and conditioners.

2.1.2 Viscosity builders
Viscosity builders are substances whose main function is to decrease the fluidity products in order to obtain cosmetic formulations for hair care that can be collected in the palm of your hand. To control the viscosity of many shampoos, salt is added to the surfactants mixture. The interactions between salt and the long chain surfactants transforms the small spherical micelles of the surfactants into larger rod-like or lamellar or even liquid-crystalline-type structures that increase the viscosity of the liquid shampoo. If one plots the salt concentration versus the viscosity in a such system, one typically finds an optimum for the maximum viscosity (Fig.3).
Above this optimum salt concentration, additional salt decreases the viscosity. In developing such a system in which viscosity is controlled by salt addition, it is recommended that one select the appropriate salt concentration on the ascending part of the viscosity-salt concentration curve. Nevertheless, many light-duty liquid products and some shampoos are formulated on the descending part of the curve. Furthermore, it is important to remember that impurities such as salt contaminants in surfactants must be carefully controlled to obtain the appropriate viscosity when salt control is employed. In addition to the salt as viscosity builder, there are also other substances such as polymeric gums, alkanolamides, betaines and amine oxides.

Polymeric gums such as methyl cellulose or hydroxy ethyl cellulose have also been used in shampoos to help control viscosity. In this case, the polymers interact with the
surfactants forming even larger more cohesive aggregates of higher viscosity.

Alkanolamides interact similarly and are very effective in reducing surfactant head group repulsion, thereby allowing even larger and more cohesive aggregates of higher viscosity. Some surfactants such as betaines and amine oxides can interact similarly to help increase viscosity of anionic surfactant systems. In such systems, the salt concentration is also helpful to viscosity control. Solvents such as propylene glycol, glycerine or other alcohols are sometimes used in shampoos to help solubilize or to clarify product or to lower cloud-clear points. Such ingredients often tend to lower product viscosity and are sometimes used for this purpose alone [1].

2.1.3 Conditioning agents: silicones

Many shampoos have certain levels of conditioning agents which mimic the functions of a full conditioner product [3]. Among these, silicones are the most used. Silicones are a family of polymers whose origins lie in the mineral quartz, or silica, which has the chemical formula SiO₂. Through reactions such as hydrolysis and condensation, the basic Si-O-Si-O chain or backbone structure of silicone is formed. The addition of different functional groups results in a variety of silicones with various properties [4]. Silicones have become standard ingredients in many conditioning shampoos for the smooth, silky hair feel that they confer. Among silicones used
in cosmetic formulations for hair care, there are cyclomethicones, dimethicones and amodimethicones. The cyclic silicones offer temporary conditioning and are generally used to formulate products with detangling properties.

Dimethicones or Polydimethylsiloxanes (PDMS) have excellent lubricating properties and are utterly water-insoluble. They form very hydrophobic films on the hair surface and this is the reason why PDMS are frequently found in rinse conditioners, air sprays and styling mousse [5]. Regarding amodimethicones, also known as amine-functionalized silicones, the methyl groups attached to the dimethyl siloxane polymers are partly replaced with organic amine groups. The essentially basic nature of the groups of primary amines causes this silicone polymer to develop a positive net charge in aqueous systems within a wide range of pH, ranging from 1 to 11.5. The charge density of the polymer can be varied by changing the placement and quantity of the amine groups. A polymer with greater charge density will be more substantive to the hair than one with lesser charge density. This silicone family has broad application in hair treatments, especially when the hair is heavily damaged. Therefore, emulsions based on aminofunctional silicones have been developed, and these materials are especially useful for ease of formulation [4]. Once the amine-functional silicone is deposited onto the surface of the hair, it spreads out and forms a cross linked film when it dries. This cross linked film
can last through several washings, which is considered to be advantageous in most applications. A unique property of these polymers is that once in place on the surface of the hair, they repel further deposition of amine-functional polymers on top of the existing layer, preventing buildup. This cross-linked film seals moisture inside the hair shaft, holding the cuticle flat and providing excellent wet and dry combability. An additional benefit of these silicones over other cationic polymers (such as polyquats) is their high refractive index, which gives the hair a high degree of gloss and shine.

Silicones, including amodimethicone, also protect from thermal damage resulting from styling tools, such as hot rollers, curling irons, and blow dryers. This phenomenon is due to their very low thermal conductivity, much lower than water, glycerin, or mineral oil. This reduces heat transfer through the hair surface to the cortex of the hair. Very high temperatures found when styling or processing hair (sometimes as high as 100°C to 160°C) are capable of vaporizing water contained within the cortex. It is extremely important to maintain proper hydration of the hair because water has a very high specific heat which helps protect the hair from getting too hot. Hair that reaches too high temperatures can suffer permanent damage to the delicate keratin fibrils in the cortex. Therefore, a protective layer of amodimethicone on the hair surface can help prevent or reduce damage done to keratin structure.
Nowadays, silicones continue to be essential ingredients in hair care formulations, although new substances with similar functions, come to market each year. Their versatility meets a wide range of consumer needs, both in terms of sensory effects and performance. Thus, even now silicones are considered the best high-performance conditioning polymers currently available to the hair-care product formulator.

2.1.4 Foam boosters
One essential attribute of a shampoo is its ability to produce a rich foam. Most people equate foaming with cleansing and think that higher is amount of foam, cleaner is the hair. Although this is not true, it is better to formulate shampoos forming foam. For this reason, foam boosters or foam stabilizers such as gums (e.g. guar or xanthan), sarcosinates and lactylates are often used. Also betaines, amphoteric surfactants, are commonly used as foam boosters in various products to improve their foamability and foam stability.

2.1.5 pH controllers
The pH of shampoos has been shown to be important for improving and enhancing the qualities of hair, minimizing irritation to the eyes and stabilizing the ecological balance of the scalp [6]. Mild acidity prevents swelling and promotes tightening of the scales, thereby inducing shine. Therefore, cosmetic formulations for hair care are often formulated at pH acid, between 4.5 and 5.5, by using citric or lactic acid. The
pH control is also required in order to allow the correct activity of some preservatives.

2.1.6 Fragrance and aesthetic additives

The fragrance in cosmetic formulations for hair care represents the reason of the choice by the consumers. They like smelling the fragrance and they want it remains on the hair and gives a feeling of freshness and cleanliness.

In addition to the fragrance, there are other aesthetic additives which play an important role in the appearance of products such as opacifiers or pearling agents and solvents to clarify or to lower the cloud point.

Opacifiers and pearling agents allow to obtain formulations with a richer appearance and creamier. Opacifiers such as ethylene glycol distearate or soap-type opacifiers are often used in conditioning shampoos, for visual effects, to provide the perception that something is deposited onto the hair for conditioning it [1].

On the other hand, some solvents such as propylene glycol, glycerine or other alcohols have opposite function compared to the opacifiers because they increase the transparency of products.

Overall, these additives increase the number of products for hair care on the market, ensuring a wide choice for consumers.
2.1.7 Preservatives

Preservation of cosmetic products against microbial contamination is important because such contamination can lead to product degradation. So it is necessary to preserve these products against microbial contamination at the time of manufacture and to ensure that cosmetic formulations are preserved for a reasonable period of time thereafter.

Some formulations are inherently more difficult to preserve than others. This problem is mainly connected to the water content, optimum substrate for microbial growth. For this reason, more water is present in a product, more difficult it is to preserve. In addition, some ingredients are more difficult to preserve than others. For example, plant extracts, vitamins, and some nonionic detergents are generally more difficult to preserve than other types of ingredients [1].

The use of preservatives is essential in most products not only to prevent product spoilage caused by microorganisms but also to protect the product from inadvertent contamination by the consumer during use.

In general, preservatives may have bacteriostatic or bactericide effects. The former contrast the microbial growth, the latter kill directly microbial agents.

However, the preservation of cosmetic formulations is rather complex and depends on several factors, among which:

- formulation type (solution, emulsion, cream, lotion, etc);
- factors affecting the microbial growth (nutritive substances, moisture level, pH, temperature, etc);
- factors affecting preservatives efficacy (preservatives concentration, contamination nature, presence of incompatible or inactivating substances);
- purity of raw materials, cleaning of equipments and local places of manufacturing.

Therefore, a potential contamination can depend on physical, chemical, microbiological and enzymatic phenomena.

In cosmetic formulations, also color, smell or viscosity changes can indicate a degradation product due to the presence of microbic agents, especially in aqueous formulations.

In order to avoid these problems that invalidate the quality of products, it is important to select a suitable preservative or even more than one, depending on factors above mentioned.

An ideal preservative should have the following characteristics:
- broad spectrum of action and a good effectiveness at low concentrations;
- solubility in water and high ripartition coefficient water/oil so that it places at water/oil interface where the most of microorganisms proliferate;
- compatibility with other ingredients present inside cosmetic formulations;
- inability to induce color or smell changes to formulations;
- ease and safety of use;
- high skin tolerance so as to reduce contact sensitization effects.

Unlikely a single preservative is able to satisfy all these properties. For this reason, in most products for hair care, a combination of more preservatives is used. Among them, some preservatives [7] commonly used in shampoos and conditioners are:

- **Benzoic acid and sodium benzoate**

![Chemical structure](image)

These preservatives are good fungicide and moderate bactericide on Gram+ and Gram-. Their action is carried out between pH 3 and pH 5. Except salts, benzoic acid derivatives are poorly soluble in water and are incompatible with anionic and non ionic surfactants and proteins. Besides, minimum inhibitory concentration (MIC) values are high.
• **Sorbic acid** and **potassium sorbate**

![Sorbic acid](image)

These preservatives are natural substances. The spectrum of action is narrow; they have effects only on fungi at pH between 4 and 5.

• **Methyl-, Ethyl-, Propyl-, Butyl-, Isobutylparaben**

![Paraben](image)

These preservatives are good fungicide and moderate bactericide on Gram+ and Gram-. Their action is carried out between pH 3 and pH 6.5. Except salts, parabens are poorly soluble in water and are incompatible with anionic and non ionic surfactants and proteins. Besides, MIC values are high. The European Economic Community (EEC) prohibits the use of parabens above 0.8% [1].
• *Phenoxyethanol*

![Phenoxyethanol structure](image)

It is a good bactericide and fungicide and it operates between pH 3 and 7. Its MIC values are high and it is poorly soluble in water. Often it is used with parabens.

• *Benzyl Alcohol*

![Benzyl Alcohol structure](image)

It is natural compound and it has effects on Gram + at pH above 5. It is incompatible with anionic surfactants.
• *Deydroacetic acid* and *sodium dehydroacetate*

They have a discreet spectrum of action. These preservatives are active between pH 5 and 6.5 but their use is banned in spray.

• *Methylisothiazolinone, Chloromethylisothiazolinone*

Isothiazolinones are characterized by a wide spectrum of action. These preservatives are instable at pH above 8 and they can be used in presence of anionic, cationic, non ionic surfactants and proteins. On the other hand, they can trigger contact sensitization effects.
• **DMDM hydantoin**

![DMDM hydantoin molecule](image)

DMDM hydantoin is a formaldehyde releaser preservative, so potentially irritating on skin and eyes. It is a strong bactericide but it is a weak fungicide. It has effects on microbial agents between pH 3.5 and 10. Besides, it is compatible with anionic, non ionic, cationic surfactants and proteins.

• **Imidazolidinyl urea**

![Imidazolidinyl urea molecule](image)

This preservative is a strong bactericide while it is ineffective against fungi. Its activity is carried out between 3.5 and 8. It is a formaldehyde releaser. For this reason it can cause
collateral reactions which include skin irritation, eye irritation, and contact sensitization. It is a thermolabile compound, compatible with anionic, cationic, non ionic surfactants and proteins.

2.1.7.1 An innovative preservative: the colloidal silver
In addition to common preservatives until now mentioned, a preservative composition for cosmetic products comprising electrolytic colloidal silver has been recently patented by italian inventors [8].

The metallic silver is an effective antibacterial agent, known for its positive effects against microorganisms since the times of ancient Greece. The antimicrobial action of metallic silver is due to its capacity to penetrate bacterial and fungal membranes of unicellular organisms, binding then to proteins and respiratory enzymes and to nucleic acids, blocking the survival systems of the microorganisms. Forms of silver such as colloidal silver, silver sulfadiazine, silver arsphenamine, silver nitrate or other salts have been used for therapeutic purposes, but the addition of silver in cosmetic products is usually prohibited due to alteration of the stability and organoleptic characteristics of the product. At the same time, the use of small amounts of silver is instead ineffective in exhibiting a preservative effect. Further, although silver does not have a toxic action at the level of mammalian cells, the prolonged taking of this metal by superior organisms has some unwanted effects, due to which several countries,
including the United States and Australia, have prohibited the therapeutic use and sale of products containing silver. The most evident of these effects is argyria, a condition in which the skin of the affected person assumes irreversibly an unsightly albeit harmless blue-gray coloring.

The use of this new preservative composition in cosmetic products as known from Italian patent No. 1307312 obviates the problems of skin and eyes irritation, common in other preservatives. This composition has no allergic or irritating effects and it is characterized by a superior antimicrobial effect. Besides, it can be included in a wide range of cosmetic products in their various forms and it keeps unchanged the organoleptic characteristics and the stability of the cosmetic product despite higher effectiveness against micro-organic flora.

The preservative composition comprises:

0.1 to 1 ppm of electrolytic colloidal silver with respect to the cosmetic product;
5 to 15% by weight of glycerin with respect to the weight of the cosmetic product;
0.01 to 0.5% by weight of chelating compound (e.g. ethylenediaminetetraacetic acid (EDTA)) with respect to the weight of the cosmetic product;
a dynamic buffer for keeping the pH around 4.50-6.00.

Electrolytic colloidal silver is the only form of silver approved as a supplement by the Food and Drug Administration. The
integration of electrolytic colloidal silver with the other components of the preservative composition allows to use a reasonably low quantity of silver, such as to not induce side effects such as for example argyria. Further, since electrolytic colloidal silver is the most powerful known oligodynamic metal with antibacterial properties, antimicrobial effectiveness is ensured even at extremely low concentrations. Advantageously, the reduced quantity of silver does not alter the organoleptic characteristics of the cosmetic product to which it is added.

Furthermore, the highest effectiveness against microorganisms of this preservative composition ensures, in the cosmetic products to which it is added, an extension of the period after opening (PAO).

It has been also observed that the use of electrolytic colloidal silver allows to formulate the preservative composition with a reduced quantity of glycerin, lower than the value generally believed to cause irritation (comprised between 30% and 35%), making such preservative composition suitable for application in cosmetic formulations in which it is fundamental to keep low the contents of polyalcohols in order to improve the pleasantness of the product.

In the same way, the presence of electrolytic colloidal silver allows to formulate the preservative composition with a reduced quantity of EDTA (0.01-0.5%), allowing to use the composition also in cosmetic products in which the quantity of EDTA is prohibited or limited.
At last, the composition further comprises a dynamic buffer for keeping the pH around 4.50-6.00. For example, the buffer can be a citric-phosphate buffer. Preferably, the pH is kept around 5.00-5.50. This dynamic buffer ensures that the pH is kept at the preset values, contrasting the changes that microorganisms would be capable of producing on the composition to promote their growth.

Generally, the choice of a suitable preservative represent a critical point in the development of cosmetic products. Therefore, in the first part of experimental section of this work, it will be discussed if these ingredients are actually needed in some formulations opportunely developed for hair care, and it was evaluated preservatives effect on microstructural properties of such formulations.
References


Chapter 3 – Evolution from cosmetic to cosmeceutic formulations for hair care: the resveratrol, a powerful antioxidant

Today a new hot topic in the cosmetic industry are 'cosmeceuticals', which are the fastest growing segment of the natural personal care industry.
The term 'cosmeceutical' is attributed to Dr. Albert Kligman who indentified a hybrid category of products between drugs and cosmetics in the late 1970s. It is now commonly used to describe a category of cosmetic products claimed to have biologically active ingredients with medicinal or drug-like benefits.
Cosmeceuticals (or alternatively, cosmaceuticals) are topical cosmetic-pharmaceutical hybrids intended to enhance the beauty through ingredients that provide additional health-related function or benefit. They are applied topically as cosmetics, but contain active ingredients such as antioxidants, growth factors, vitamins or enzymes and they are marketed to give a more youthful appearance to the skin and hair. These products are at the juncture where wellness meets beauty and growing use by consumer is indicative of their popularity.
Traditional cosmeceuticals involve the topical application of biologically active ingredients that affect the skin barrier and overall skin health. The ability of these ingredients to
enhance skin functioning depends on how they are formulated into products which have to preserve the integrity of the active ingredient, deliver it in a biologically active form to the skin, reach the target site in sufficient quantity to exert an effect, and properly release it from the carrier vehicle. Cosmeceutically active ingredients are constantly being developed by big and small corporations engaged in pharmaceuticals, biotechnology, natural products and cosmetics, while advances in the field and knowledge of skin biology and pharmacology have facilitated the development of novel active compounds more rapidly. Many substances, either chemically synthesized or extracted from plants or animals, can be used as functional ingredients. Nowadays, many cosmetic products with biologically active ingredients have been developed and marketed, though there are discrepancies about their regulations and approvals by the government. In fact, cosmeceuticals are not regulated by the U.S. Food and Drug Administration (FDA) and thus are not subject to premarket requirements for proof of safety or efficacy. Comeceuticals products often are tested through in vitro studies using silicone replicas of skin and clinical trials are usually supported by the cosmetic companies themselves. The rigorous testing required for pharmaceuticals is not mandatory for cosmeceutical products. So, it is often difficult for consumers to determine whether claims about the actions or efficacies of cosmeceuticals are valid unless the product has been approved by the FDA or equivalent agency.
However, the recent explosion and fast-paced growth of the cosmeceutical market have left both consumers and dermatologists confused. Patients often receive unregulated information and efficacy claims for products from the internet and the media.

Moreover, because many ingredients often are derived from plants, consumers may have a false sense of security that these “all-natural” products do not have potential adverse effects. In some instances, these products may not achieve the results they claim but they offer some benefit to the skin through an emollient effect. Indeed, it is often difficult to separate the effects of the moisturizer vehicle from the effects of the added active ingredient in cosmeceuticals.

Therefore, it is important for dermatologists to understand the theoretical mechanisms of action of cosmeceuticals. Such information will enable dermatologists to be better equipped to help patients navigate the often muddy waters of the ever-growing sea of products and to manage realistic expectations as they consider adding cosmeceuticals to their skin care regimen.

Considerable overlap exists in the purported actions of various products presently on the market. This update divides generally cosmeceuticals into the following broad categories: antioxidants, growth factors, peptides, anti-inflammatories, botanicals, polysaccharides, and pigment-lightening agents [1-3].
In this work a particular attention is given to a powerful antioxidant, the resveratrol, often present in cosmeceutical products for skin care but absent in hair cosmeceutical products.

The basic idea of this project is to develop hair care formulations, such as conditioners or shampoos, adding resveratrol inside them through suitable delivery systems represented by micelles or vesicles of common surfactants in order to allow the gradual release of this substance on hair surface and contrast oxidative stress effects, responsible of graying and loss hair.

3.1 The resveratrol

Resveratrol (3,5,4’-trihydroxy-stilbene) is a polyphenolic compound from the stilbens family, which is mainly found in grape skins, peanuts, berries and other foods that are commonly consumed as a part of human diet. The compound was first isolated from the root of *Polygonum cuspidatum*, a plant used in traditional Chinese and Japanese medicine. It is a phytoalexin synthesized by a wide variety of plant species in response to stress as a defence mechanism against fungal, viral, bacterial infections and damage from exposure to ultraviolet radiation (UV). Its relatively high concentration in red wine (0.1-14.3 mg/L) has led to its being proposed as the main protagonist for the so called “French paradox”; that is, despite fat-rich diets, mortality from coronary heart disease is
lower in France than in other countries due to the moderate consumption of red wine.

*In vitro* and *in vivo* studies suggest that resveratrol has a number of potential health benefits, including anti-carcinogenic, anti-inflammatory, anti-obesity, and heart/brain protective effects. Resveratrol may help combat coronary heart diseases by inhibiting myocardial infarction, arrhythmias, hypertension, hypertrophy, fibrosis, atherosclerosis, and thrombosis. Numerous biochemical mechanisms have been proposed to account for this ability, including antioxidant activity, modulation of nitric oxide biosynthesis and activity, alteration of cell-to-cell interactions, and inhibition of platelet COX-1. The ability of resveratrol to combat certain brain diseases has been attributed to its antioxidant activity, interference with signaling pathways, and ability to elevate sirtuin production in the brain. Resveratrol has also been shown to inhibit certain types of cancer, which has been attributed to its ability to induce apoptosis, activate caspase, modulate signaling pathways, decrease cell proliferation, arrest cell cycles, and decrease metastasis. Resveratrol has proven useful in the management of obesity through its ability to decrease lipid synthesis, increase lipolysis, and reduce lipid accumulation. Finally, resveratrol has been shown to have anti-inflammatory effects, which is partly attributed to its ability to decrease COX-2 expression [4]. A summary of health benefits of resveratrol is shown in Fig.1.
Despite the potential health benefits of resveratrol, its utilization in pharmaceutical, nutraceutical and cosmeceutical industry is currently limited due to its poor water solubility, low bioavailability and chemical instability. Resveratrol is a fat-soluble compound that is also soluble in ethanol at ~ 50mg/mL (~ 200mM) and in DMSO at ~ 16mg/mL (~ 70mM) but only slightly soluble in water (~ 0.13mM) and for this reason it is considered “practically insoluble” in water according to the European Pharmacopeia definition [5]. Consequently it is difficult to incorporate relatively high levels of resveratrol into aqueous base cosmetic products. In addition resveratrol is sensitive to chemical degradation and it may be chemically degraded when exposed to elevated temperatures, pH changes, ultraviolet light or certain types of enzymes. Chemical degradation often involves the isomerization of resveratrol [4]. In fact resveratrol exists as
two structural isomers: cis- (Z) and trans- (E) as shown in Fig.2.

![Chemical structure of trans-resveratrol and cis-resveratrol](image)

**Fig.2: Chemical structure of trans-resveratrol and cis-resveratrol**

The *trans*-isomer is biologically more active than the *cis*-isomer, probably due to its non-planar conformation. *Cis*-resveratrol has been reported to be unstable and is therefore not available commercially. In fact, when protected from light, *trans*-resveratrol is stable for at least 42h and for at least 28 days in pH 1–7 buffers, whereas the *cis* form is only stable at neutral pH when completely shielded from light.

Resveratrol is an extremely photosensitive compound: Vian et al. demonstrated that 80–90% of the *trans*-resveratrol in solution was converted to *cis*-resveratrol if exposed to light for 1 h. Trela and Waterhouse also reported that *trans*-resveratrol was susceptible to UV-induced isomerization: when pure *trans*-resveratrol was irradiated for 120 min at 366 nm, 90.6% was converted to *cis*-resveratrol [5].
3.1.1 Antioxidant and prooxidant properties of resveratrol

Biological properties of resveratrol are linked to its potential of acting either as an antioxidant or a prooxidant agent. Similar to most polyphenols, resveratrol has intrinsic antioxidant activity due to the direct quenching of reactive oxygen/nitrogen species (ROS/RON). Biological systems control oxidative stress via a variety of anti-oxidative mechanisms that restrict the reactivity of oxidation catalysts and free radicals. The antioxidant activity might explain the role of resveratrol in cancer chemoprevention, due to scavenging of free radicals involved in the peroxidation of membranes and oxidative damage of DNA. Resveratrol is both a free radical scavenger and a potent antioxidant because of its ability to promote the activities of a variety of antioxidant enzymes. The ability of the polyphenolic compounds to act as antioxidants depends on the redox properties of their phenolic hydroxy groups and the potential for electron delocalization across the chemical structure [6]. In fact, the structural requirement considered essential for effective radical scavenging by resveratrol is the presence of para-hydroxyl group in phenolic ring and conjugated double bond that makes the electrons more delocalized [7].

Theoretical studies on the antioxidant action have shown that hydrogen abstraction from the 4’-OH group in para position of phenolic ring is more favored than from 3-OH or 5-OH
positions of catecholic ring. Therefore, it is of interest to extend the research and study the structure-activity relationship of resveratrol oriented analogues by the introduction of electron-donating (ED) and electron-withdrawing groups (EW) in the ortho or para position of 4’-OH. On the other hand, phenol antioxidants react with free oxygen-centered and nitrogen-center radicals (X·) via three different mechanisms (Fig.3):

1) a one step hydrogen atom transfer from phenol to X· (HAT mechanism); 
2) a sequential proton loss electron transfer process from phenoxide anion to X· (SPLET mechanism); 
3) an electron transfer process from ArOH to X· followed by proton transfer (ET-PT mechanism) [8].

Fig.3: Antioxidative mechanisms of phenol antioxidants [8].

Each antioxidant, including resveratrol, is a redox species, protecting against free radicals in some circumstances and promoting free radical generation in others. Copper is one of the most redox-active metal ions present in living cells. In the presence of Cu(II), resveratrol is able to become a prooxidant
producing reactive oxygen species (ROS), which in turn are able to damage DNA. Fukura and Miyata first reported the prooxidant activity of resveratrol in a plasmid-based DNA cleavage assay in the presence of transition metal ions such as copper. Approximately 20% of copper is located in the nucleus and is closely associated with DNA bases, in particular, guanine. Besides, it has been shown that the concentration of copper is greatly increased in various malignancies. Copper ions from chromatin can be mobilized by metal-chelating agents, giving rise to internucleosomal DNA fragmentation, a property that is the hallmark of cells undergoing apoptosis.

The cytotoxic mechanism of resveratrol probably involves mobilization of endogenous copper ions, possibly chromatin bound copper [8]. The prooxidant mechanism of resveratrol is based on the acidity of the phenol group, involving a proton loss from the OH in para position, to give a phenoxide anion which participates in a redox reaction and gives a phenoxide radical while Cu(II) is reduced to Cu(I). Molecular oxygen is able to oxidize Cu(I) by conversion into a superoxide anion, the latter being able to generate hydrogen peroxide, which induces DNA damage directly or after conversion into a hydroxyl radical.

Therefore, resveratrol may open new perspectives in cancer therapy. By blocking survival and anti-apoptotic mechanisms or causing DNA degradation, as a consequence of its prooxidant action, resveratrol can sensitize cancer cells,
which may result in synergistic anti-tumour activities when resveratrol is combined with conventional chemotherapeutic agents or cytotoxic compounds. However, further insights into the signalling network and interaction points modulated by resveratrol may provide the basis for novel discovery programs to exploit resveratrol for the prevention and treatment of human diseases.

3.2 Oxidative stress: effects on hair

Reactive oxygen species (ROS) are continuously produced by the body’s normal use of oxygen such as respiration and some cell mediated immune functions. ROS include free radicals such as superoxide anion radicals (O$_2^-$), hydroxyl radicals (OH·), and non free radical species such as hydrogen peroxide (H$_2$O$_2$) and singlet oxygen (¹O$_2$). ROS are produced during normal physiologic events and can easily initiate the peroxidation of membrane lipids, leading to the accumulation of lipid peroxides. ROS at physiological concentrations may be required for normal cell functioning, but higher concentrations are capable of damaging crucial biomolecules such as nucleic acids, lipids, proteins, polyunsaturated fatty acids, and carbohydrates and may cause DNA damage that can lead to mutations. Hence, if ROS are not effectively scavenged by cellular constituents, they can stimulate free radical chain reactions,
subsequently damaging the cellular biomolecules and finally leading to disease conditions [9].

Experimental evidence supports the hypothesis that oxidative stress plays a major role in the ageing process. Reactive oxygen species are generated by a multitude of endogenous and environmental challenges but the body possesses endogenous defence mechanisms, such as antioxidative enzymes and non-enzymatic antioxidative molecules, protecting it from free radicals by reducing and neutralizing them. With age, the production of free radicals increases, while the endogenous defence mechanisms decrease. This imbalance leads to the progressive damage of cellular structures, presumably resulting in the ageing phenotype. Ageing of hair manifests as decrease of melanocyte function or graying, and decrease in hair production or alopecia. There is circumstantial evidence that oxidative stress may be a pivotal mechanism contributing to hair graying and hair loss.

Ageing is a complex process involving various genetic, hormonal, and environmental mechanisms. As the rest of the skin, the scalp and hair are subject to intrinsic or chronologic ageing, and extrinsic ageing due to environmental factors. Both occur in conjunction with the other and are superimposed on each other. Intrinsic factors are related to individual genetic and epigenetic mechanisms with inter-individual variation. Examples of intrinsic factors are familial premature graying and androgenetic alopecia (AGA).
Extrinsic factors include ultraviolet radiation (UVR), smoking, and nutrition [10].
It is well known that the UV components of sunlight damage human hair. UV irradiation (mainly UV-B) attacks both the melanin pigments and protein fractions (keratin) of hair. The effects of UV-B irradiation can be severe, resulting in the breakdown of disulfide bonds inside the hair fiber and on the surface of the cuticle. However, UV-A irradiation mainly produces free radical/reactive oxygen species (ROS) through interaction with endogenous photosensitizers. Studies have shown that photo-oxidation of hair fiber involves the fracture of C-S linkages from proteins, oxidation of internal lipids, and melanin granules and tryptophan degradation of keratin. Moreover, exposure to sunlight leads to hair decoloration due to melanin oxidation via free radicals [11].
Hair decoloration or its graying have undeniable psychosocial and socioeconomic implications, especially in today's world, where humans are confronted with increasing pressure to stay "forever young and vital". Hence, premature graying in particular has long attracted researchers and industry alike with scientific as well as commercial targets.
Numerous mechanisms were suggested for hair graying, including the loss of melanocyte stem cells or their failure to differentiate, melanocyte migration defects, anagen defects, melanocyte apoptosis and pigmentary machinery malfunction or loss. One key issue that is in common to these processes is oxidative damage [12]. So far, the biological process of hair
graying has been attributed to the loss of the pigment-forming melanocytes from the aging hair follicle, including the bulb and the outer root sheath. In this context, it is of interest that activity of hair bulb melanocytes is under cyclic control. Both melanogenesis and hair shaft production take place in the anagen phase of the hair cycle. Toward the end of this phase, the pigment forming melanocytes retract their dendrites and stop melanogenesis, which is followed by apoptosis driven regression in the catagen phase and the final resting telogen phase. Theories for the gradual loss of pigmentation include exhaustion of enzymes involved in melanogenesis, impaired DNA repair, loss of telomerase, antioxidant mechanisms, and antiapoptotic signals including the loss of Bcl-2 and decreased stem cell factor. Recent studies have also demonstrated that melanocytes in the graying human hair bulb show vacuoles prior to their loss, as an indicator of increased oxidative stress. In addition, several of the steps in melanin production yield H₂O₂ and other free radicals. This places melanocytes under a higher oxidative stress load than for example keratinocytes, which is the most prominent in hair follicle melanocytes because they produce large quantities of melanin throughout the anagen phase of the hair cycle. Moreover, decline of melanogenesis is associated with loss of tyrosinase activity, which affects in turn the rate-limiting step in melanogenesis. It is of interest that low H₂O₂ concentrations increase tyrosinase activity, while high
concentrations irreversibly deactivate the enzyme. Abundant evidence indicates that many proteins and peptides, including the H$_2$O$_2$ reducing enzyme catalase as well as the two repair mechanisms for free and bound methionine sulfoxide (Met-S=O), methionine sulfoxide reductases A and B (MSRA and MSRB) are structurally damaged and functionally altered by H$_2$O$_2$ mediated oxidation [13,14].

The scenario of these events is summarized in Fig.4.

Fig.4: H$_2$O$_2$ effects on melanogenesis process [13].

In detail, in the brown hair follicle, H$_2$O$_2$ is generated in the micromolar range, which can activate transcription of many proteins, including catalase, tyrosinase, MSRA and MSRB. On the other hand, in the presence of millimolar H$_2$O$_2$ concentrations, oxidation of Met, Cys, Trp, and residues in protein sequences are taking place, consequently altering the
tertiary structures. These structural changes often lead to deactivation of the affected protein/enzyme. This finding has been documented for catalase, MSRA, and MSRB. Low catalase levels and activities have been documented in the gray hair follicle, which in turn leads to increased H₂O₂ levels. Studies show that tyrosinase activity is interrupted due to oxidation of Met 374 in the enzyme active site by this ROS. The resulting Met-S=O cannot be repaired, because MSRA and MSRB are also deactivated by H₂O₂. The same scenario applies for catalase. Therefore, a shift in the H₂O₂ redox balance can significantly alter melanogenesis in the human hair follicle [13]. The concept of reduced catalase activity and hydrogen peroxide accumulation was further expanded to studies of hair graying. Schallreuter et al. provided detailed documentation on the deregulation of the innate antioxidant system of grey hair bulbar melanocytes and the subsequent accumulation of hydrogen peroxide at millimolar concentrations in the grey and white hair shafts. Interestingly, a significant reduction in catalase expression, in the expression of methionine sulfoxide reductases and in the functional loss of repair activity was demonstrated throughout the entire hair follicles, not only in the melanocytes. The ROS-induced greying concept therefore was expanded by these studies to include an oxidative insult to the whole affected follicle, not only to its bulbar melanocytes.
Currently, researches in this field support the proposed hypothesis of a “free radical theory of graying” and suggest that melanocytes in the hair follicle are highly susceptible to endogenous and exogenous oxidative stress.

The mechanisms of hair graying are still heavily studied, and the role of oxidative stress is likely to be only one piece of the comprehensive story. More detailed studies are needed to better understand the graying process and to possibly enable practical modulation. A comprehensive understanding of the loss of the enzymatic antioxidant capacity, and in particular of catalase, could provide more clues to preventive and therapeutic strategies. Better understanding of the susceptibility of melanocytes to oxidative stress at the different follicular locations might provide some indication of possible ‘antigraying’ acts [12].

In this work, a powerful antioxidant such as resveratrol was chosen in order to contrast oxidative stress effects on hair, by adding it in adequate formulations for hair care; in particular, it was thought to carry this substance on hair surface by using micelles of anionic and cationic surfactants or catanionic vesicles so as to increase the solubility in water and avoid the chemical degradation of this active compound. The results obtained will be discussed in the second part of experimental section of this thesis.
References


Experimental section
Chapter 4 – Design and development of hair care formulations: structural and microbiological analysis

In the first part of this PhD project, several cosmetic formulations for hair care were developed in order to obtain innovative, highly effective and more eco-friendly products. In particular, a prototype of shampoo (S17F13) and of conditioner (B14F13) were formulated. For these formulations, more eco-friendly and mild surfactants were chosen so as to reduce environmental pollution [1,2] and skin sensitization reactions.

In the main hair conditioners on the market, Cetrimonium Chloride (CTAC) and Behentrimonium Chloride (BTAC) are surfactants more used. On the other hand, these mono alkyl cationic surfactants are characterized by a strong aquatic toxicity. For this reason, another primary surfactant was used in the conditioner formulated. It is known as AMIDET APA-22 but its INCI (International Nomenclature Cosmetic Ingredient) name is Behenamidopropyl Dimethylamine whose surfactant property is excellent. This substance consists of C22 alkyl chain, amidopropyl functional group and dimethyl tertiary amine group [3] (Fig. 1). This surfactant is more compatible with the aquatic environment compared to other cationic surfactants with mono long alkyl chains and it is characterized by a higher biodegradability.
At the same time, in the most of shampoos present on market, the primary surfactant principally used is Sodium Laureth Sulfate (SLES), known for its undisputed detergency properties but characterized by irritating effects on the skin and the scalp. (Fig.2).

Therefore, this compound was substituted in the formulation S17F13 with more mild anionic surfactants such as Sodium Lauroyl Sarcosinate and Disodium Laureth Sulfosuccinate (Fig.3).
**Surfactant** | **Chemical structure**
--- | ---
Sodium Lauroyl Sarcosinate | ![Chemical structure](image)
Disodium Laureth Sulfosuccinate | ![Chemical structure](image)

![Fig.3: Mild surfactants used in shampoos.](image)

Furthermore, the two hair care formulations were enhanced with other ingredients, listed in the chapter 2, and were tested on hair samples so as to evaluate their efficacy.

### 4.1 Design and development of formulation S17F13

The shampoo S17F13 was formulated by using the ingredients, shown in Table 1. All ingredients were purchased from Acef spa and used without further purification. During the formulation, two different phases, named A and B, composed by different ingredients, were prepared and mixed with further substances in order to obtain final products. The phase A was obtained by dissolving Glyceryl Stearate Self-Emulsifying (2%) in hot distilled water. This phase was maintained under stirring for 15 minutes in order to obtain the complete solubilization of Glyceryl Stearate Self-Emulsifying.
The phase B was obtained by mixing at 70 °C Sodium Lauroyl Sarcosinate (18%), Disodium Laureth Sulfosuccinate (8%), Disodium Cocoamphodiacetate (6%) and PEG/PPG-20/6 Dimethicone (2%). Also this phase was maintained under stirring for 15 minutes in order to obtain a homogeneous phase. Therefore, the phase A was slowly added to phase B under stirring and the temperature of mixture was decreased at 40 °C. In sequence thermolabile components were added to the mixture, among which Polyquaternium-44 (0.6%), PEG-18/Glyceryl olate/cocoate (1.8%), pearlizing agent (1.8%), opacifying agent (0.5%), Cocamidopropyl betaine (7%) and fragrance (0.3%). At the end, the formulation S17F13 was acidified with lactic acid up to pH 5.0.

<table>
<thead>
<tr>
<th>Name</th>
<th>Chemical structure</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protelan LS 9011</td>
<td><img src="image1.png" alt="Chemical structure" /></td>
<td>Mild anionic surfactant with high foaming power, typically used in soft formulations.</td>
</tr>
<tr>
<td>Sodium Lauroyl Sarcosinate</td>
<td><img src="image2.png" alt="Chemical structure" /></td>
<td></td>
</tr>
<tr>
<td>% (w/w)</td>
<td>18</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Name</th>
<th>Chemical structure</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rewopol SBFA 30b</td>
<td><img src="image3.png" alt="Chemical structure" /></td>
<td>Mild anionic surfactant with high foaming power, used with other mild surfactants.</td>
</tr>
<tr>
<td>Disodium Laureth Sulfosuccinate</td>
<td><img src="image4.png" alt="Chemical structure" /></td>
<td></td>
</tr>
<tr>
<td>% (w/w)</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>Name</td>
<td>Chemical structure</td>
<td>Function</td>
</tr>
<tr>
<td>---------------------</td>
<td>--------------------</td>
<td>--------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Tegobetaina F50</td>
<td><img src="" alt="Chemical structure" /></td>
<td>Amphoteric surfactant, used as secondary surfactant in combination with anionic surfactants to reduce their aggressiveness.</td>
</tr>
<tr>
<td>Cocamidopropyl betaine</td>
<td><img src="" alt="Chemical structure" /></td>
<td>Amphoteric surfactant, used as secondary surfactant in combination with anionic surfactants to reduce their aggressiveness.</td>
</tr>
<tr>
<td>Disodium Cocampidiacetate</td>
<td><img src="" alt="Chemical structure" /></td>
<td>Amphoteric surfactant, used as secondary surfactant; it improves conditioning on skin and hair at a low pH.</td>
</tr>
<tr>
<td>Antil 171</td>
<td><img src="" alt="Chemical structure" /></td>
<td>Highly effective liquid thickening agent; it may be incorporated directly in each stadium of the production process and shows solubilizing properties.</td>
</tr>
<tr>
<td>Abil B 88183</td>
<td><img src="" alt="Chemical structure" /></td>
<td>Non-ionic copolymer produced by linking of long-chain polyether groups with polydimethyl siloxane. It forms a creamy foam. The presence of PEG and PPG increases its solubility in water whereas the dimethicone improves combability and shine of hair.</td>
</tr>
<tr>
<td>Name</td>
<td>Chemical structure</td>
<td>Function</td>
</tr>
<tr>
<td>-----------------------------</td>
<td>--------------------</td>
<td>------------------------------------------------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Luviquat ultracare</td>
<td><img src="image" alt="Chemical structure" /></td>
<td>Effective conditioning polymer for cleansing formulations. It provides wet and dry combability, a pleasant hair feel and is a lightweight conditioner with no build-up.</td>
</tr>
<tr>
<td>INCI</td>
<td>Polyquaternium-44</td>
<td></td>
</tr>
<tr>
<td>% (w/w)</td>
<td>0.6</td>
<td></td>
</tr>
<tr>
<td>Name</td>
<td>Chemical structure</td>
<td>Function</td>
</tr>
<tr>
<td>Glyceryl Monostearate Self-Emulsifying</td>
<td><img src="image" alt="Chemical structure" /></td>
<td>Self emulsifier that acts as stabilizer and thickener in o/w emulsions.</td>
</tr>
<tr>
<td>INCI</td>
<td>Glyceryl Monostearate SE</td>
<td></td>
</tr>
<tr>
<td>% (w/w)</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Name</td>
<td>Chemical structure</td>
<td>Function</td>
</tr>
<tr>
<td>Tegopearl B48</td>
<td><img src="image" alt="Chemical structure" /></td>
<td>Used as pearlizing agent, it possesses good thickening properties.</td>
</tr>
<tr>
<td>INCI</td>
<td>Cocamidoproyl betaine, Glycol distearate, Cocamide MEA, Cocamide DEA</td>
<td></td>
</tr>
<tr>
<td>% (w/w)</td>
<td>1.8</td>
<td></td>
</tr>
<tr>
<td>Name</td>
<td>Chemical structure</td>
<td>Function</td>
</tr>
<tr>
<td>Water</td>
<td><img src="image" alt="Chemical structure" /></td>
<td>It is a main component in shampoos, being aqueous formulations.</td>
</tr>
<tr>
<td>INCI</td>
<td>Water</td>
<td></td>
</tr>
<tr>
<td>Water</td>
<td><img src="image" alt="Chemical structure" /></td>
<td></td>
</tr>
<tr>
<td>% (w/w)</td>
<td>to 100</td>
<td></td>
</tr>
</tbody>
</table>

Table 1: Main ingredients used in formulation S17F13.
4.2 Design and development of formulation B14F13

The conditioner B14F13 was formulated by using the ingredients, shown in Table 2. All ingredients were purchased from Acef spa and used without further purification. Also for this formulation, an oil phase and water phase were prepared. The oil phase was obtained by heating in a water bath at 70 °C and under stirring Butyl Stearate (2%), Behenamidopropyl Dimethylamine (9%) and Cetearyl Alcool (3%), in order to obtain a homogeneous phase. Therefore, the water phase, composed by hot distilled water, was slowly added to the oil phase so as to have an emulsion O/W. Subsequently the temperature of the mixture was decreased at 40 °C; so Cocamidopropyl betaine (4%), hydrolyzed wheat proteins (2%) and the fragrance (0.3%) were added to the mixture. At the end, the formulation B14F13 was acidified with lactic acid up to pH 4.5.

<table>
<thead>
<tr>
<th>Name</th>
<th>Chemical structure</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crodamol BS-LQ-(RB)</td>
<td></td>
<td>Semi-solid emollient, emulsifier; it acts primarily as lubricant on the</td>
</tr>
<tr>
<td>INCI</td>
<td></td>
<td>skin’s surface, which gives the skin a soft and smooth appearance and it</td>
</tr>
<tr>
<td>Butyl Stearate</td>
<td></td>
<td>dissolves waxes and fats.</td>
</tr>
<tr>
<td>% (w/w)</td>
<td>Butyl Stearate</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Name</td>
<td>Chemical structure</td>
<td>Function</td>
</tr>
<tr>
<td>-------------------------------------</td>
<td>--------------------</td>
<td>--------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Amidet APA22</td>
<td><img src="image1.png" alt="Chemical structure" /></td>
<td>It has both cationic and non-ionic characteristic and is very compatible with anionic surfactants. It imparts smooth detangling, improves dry comb and fly away; excellent conditioning agent.</td>
</tr>
<tr>
<td><strong>INCI</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Behenamidopropyl Dimethylamine</td>
<td><img src="image2.png" alt="Chemical structure" /></td>
<td></td>
</tr>
<tr>
<td><strong>% (w/w)</strong></td>
<td>9</td>
<td></td>
</tr>
<tr>
<td><strong>Name</strong></td>
<td><strong>Chemical structure</strong></td>
<td><strong>Function</strong></td>
</tr>
<tr>
<td>Tegobetaina F50</td>
<td><img src="image3.png" alt="Chemical structure" /></td>
<td>Amphoteric surfactant, used in conditioners for its detergency properties</td>
</tr>
<tr>
<td><strong>INCI</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cocamidoproyl betaine</td>
<td><img src="image4.png" alt="Chemical structure" /></td>
<td></td>
</tr>
<tr>
<td><strong>% (w/w)</strong></td>
<td>4</td>
<td></td>
</tr>
<tr>
<td><strong>Name</strong></td>
<td><strong>Chemical structure</strong></td>
<td><strong>Function</strong></td>
</tr>
<tr>
<td>Alcool Cetil-stearilico TA 1618</td>
<td><img src="image5.png" alt="Chemical structure" /></td>
<td>A mixture of fatty alcohols, consisting predominantly of cetyl and stearyl alcohols. It is used as an emulsion stabilizer, opacifying agent, and foam boosting surfactant, as well as a viscosity-increasing agent.</td>
</tr>
<tr>
<td><strong>INCI</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cetearyl Alcool</td>
<td><img src="image6.png" alt="Chemical structure" /></td>
<td></td>
</tr>
<tr>
<td><strong>% (w/w)</strong></td>
<td>3</td>
<td></td>
</tr>
<tr>
<td><strong>Name</strong></td>
<td><strong>Chemical structure</strong></td>
<td><strong>Function</strong></td>
</tr>
<tr>
<td>Proquat wheat</td>
<td><img src="image7.png" alt="Chemical structure" /></td>
<td>It strengthens the hair to the roots, increases untangling, repairs the damaged hair follicles and makes the hair soft and bulky.</td>
</tr>
<tr>
<td><strong>INCI</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hydrolized Wheat Proteins and other ingredients</td>
<td><img src="image8.png" alt="Chemical structure" /></td>
<td></td>
</tr>
<tr>
<td><strong>% (w/w)</strong></td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Name</td>
<td>Chemical structure</td>
<td>Function</td>
</tr>
<tr>
<td>------------</td>
<td>--------------------</td>
<td>--------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Water</td>
<td><img src="https://via.placeholder.com/150" alt="H2O" /></td>
<td>It is a main component in shampoos, being aqueous formulations.</td>
</tr>
</tbody>
</table>

| INCI Water | % (w/w) to 100 |

Table 2: Main ingredients used in formulation B14F13.

### 4.3 Preservatives used in formulations S17F13 and B14F13

Often in the most of hair care formulations different types of preservatives are used in order to avoid the product microbial contamination.

In particular, for the formulations S17F13 and B14F13, two kinds of preservatives were considered: the first is a well known preservative, named Acnibio Ac, a mixture of methylchloroisothiazolinone and methylisothiazolinone, whose exploitation in commercial products is very well assessed; the second is an innovative preservative, containing the electrolytic colloidal silver, recently patented by italian inventors [4]. In this work, the inclusion of Acnibio Ac in the product is highlighted with the suffix Ac, while the suffix Ag is used for the product including colloidal silver.

Generally, all preservatives are characterized by a strong aquatic toxicity. For this reason the two kinds of preservatives were used in very low concentrations: 0,1% for the Acnibio Ac and 0.5 ppm for electrolytic colloidal silver with respect to the weight of the cosmetic product. Other
studies were performed on the same formulations by using 0.1 ppm and 0.005 ppm of electrolytic colloidal silver with respect to the weight of the cosmetic product so as to observe if even lower concentration of colloidal silver are effective to contrast microbial contamination.

In order to evaluate if these components are actually needed in trichological formulations, the shampoo and the conditioner S17F13 and B14F13 were prepared both without preservatives and with above mentioned preservatives. Therefore, first EPR analysis were performed on all hair formulations so as to underline microstructural differences induced by the presence of preservative; second, microbiologic analysis and a specific biologic test, called Challenge Test were performed in order to analyze the effectiveness of preservatives, used in both formulations.

4.4 EPR measurements

EPR spectra were recorded with a 9 GHz Bruker Elexys E500 spectrometer (Bruker, Rheinstetten, Germany). Samples were placed in 25 μl glass capillaries and flame sealed. The capillaries were placed in a standard 4 mm quartz sample tube containing light silicone oil for thermal stability. All the measurements were performed at 25 °C. Spectra were recorded using the following instrumental settings: sweep width, 120G; resolution, 1024 points; time constant, 20.48 ms; modulation frequency, 100 kHz; modulation amplitude, 1.0 G;
incident power, 6.37 mW. Several scans, typically 16, were accumulated to improve the signal to-noise ratio.

4.5 Challenge Test

The evaluation of the effectiveness of a preservative is one of the most critical stages in the formulation process of cosmetic products. According to the European Regulation 1223/2009, evaluation “in vitro” microbiological stability is carried out in accordance with method ISO (International Organization for Standardization) 11930:2012 [5,6]. This ISO comprises:

- a preservative efficacy test;
- a procedure for evaluating the overall antimicrobial protection of a cosmetic product, based on a risk assessment described in ISO 29621[7].

The analysis of the microbiological risk assessment is based on an overall approach. In particular, it integrates variables such as characteristics and composition of the formulation, its production conditions and the characteristics of the packaging in which the formulation will be delivered to the market place.

The preservative efficacy test, also named Challenge Test, represents one of the most suitable and reliable methods used to evaluate the microbiological stability of a cosmetic product. This test is carried out by contaminating the product with microorganisms from several species and by subsequently
assessing the change in the microbial load by plate counting
the number of surviving microorganisms at defined intervals
during a period of 28 days.

The Challenge Test steps can be so summarized:

- Preparation of microorganisms for the inoculum;
- Inoculation of samples;
- Check of surviving microorganisms at pre-determined
  intervals (7, 14 and 28 days);
- Evaluation of the results.

The test is performed using the following strains as test
microorganisms (Table 3).

<table>
<thead>
<tr>
<th>Bacteria Gram Positive</th>
<th>Staphylococcus aureus ATCC* 6538</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacteria Gram Negative</td>
<td>Pseudomonas aeruginosa ATCC* 9027</td>
</tr>
<tr>
<td>Bacteria Gram Negative</td>
<td>Escherichia coli ATCC* 8739</td>
</tr>
<tr>
<td>Fungi (Yeasts)</td>
<td>Candida Albicans ATCC* 10231</td>
</tr>
<tr>
<td>Fungi (Molds)</td>
<td>Aspergillus brasiliensis ATCC* 16404</td>
</tr>
</tbody>
</table>

* ATCC (American Type Collection Control).

Table 3: Test microorganisms used in Challenge Test in
accordance with ISO 11930:2012.

In order to perform the test, it is so necessary to prepare
calibrated microbial suspensions (inocula) having determined
concentration at $T_0$. In more details, the microbial count of
inoculum should be $10^7$-$10^8$ CFU/ml for bacteria and $10^6$-$10^7$ CFU/ml for fungi.

Subsequently, the test samples are contaminated with these inocula and incubated in specific conditions for 28 days; so the controls for the verification of the possible reduction of microorganisms by the preserving system are performed after 7, 14 and 28 days from the inoculation.

The method ISO 11930:2012 provides two criteria for the evaluation of the efficacy of the preserving system:

- **Criterion A** that is assigned if obtained results satisfy at the same time all the following conditions:
  - after 7 days, a reduction of at least 3 log units for the bacteria and at least 1 log unit for *Candida Albicans* must be observed;
  - after 14 days, a reduction of at least 3 log units for the bacteria (without any increase with respect to the previous time), at least 1 log unit for *Candida Albicans* (without any increase with respect to the previous time) and no increase with respect to time zero for *Aspergillus brasiliensis*;
  - after 28 days, a reduction of at least 3 log units for the bacteria (without any increase with respect to the previous time), at least 1 log unit for *Candida Albicans* (without any increase with respect to the previous time) and at least 1 log unit for *Aspergillus brasiliensis* (without any increase with respect to the previous time).
- **Criterion B** that is assigned if obtained results satisfy at the same time all the following conditions:
  after 14 days, a reduction of at least 3 log units for the bacteria, at least 1 log unit for *Candida Albicans* and no increase with respect to time zero for *Aspergillus brasiliensis*;
  after 28 days, a reduction of at least 3 log units for the bacteria (without any increase with respect to the previous time), at least 1 log unit for *Candida Albicans* (without any increase with respect to the previous time) and no increase for *Aspergillus brasiliensis*.

In the Table 4 a summary of evaluation criteria is shown.

<table>
<thead>
<tr>
<th>Microorganisms</th>
<th>Bacteria</th>
<th>C.albicans</th>
<th>A.brasiensi</th>
<th>A.brasiliensis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sampling time</td>
<td>T7</td>
<td>T14</td>
<td>T28</td>
<td>T7</td>
</tr>
<tr>
<td>Criterion A</td>
<td>≥ 3</td>
<td>≥ 3</td>
<td>≥ 1</td>
<td>≥ 1</td>
</tr>
<tr>
<td></td>
<td>and NI</td>
<td>and NI</td>
<td>and NI</td>
<td>and NI</td>
</tr>
<tr>
<td>Criterion B</td>
<td>n.p.d</td>
<td>≥ 3</td>
<td>n.p.</td>
<td>≥ 1</td>
</tr>
<tr>
<td></td>
<td>and NI</td>
<td></td>
<td></td>
<td>and NI</td>
</tr>
</tbody>
</table>

a) in this test, an acceptable range of deviation of 0.5 log is accepted.
b) NI: no increase in the count from the previous contact time.
c) \( R_x = 0 \) when \( \log N_0 = \log N_x \) (no increase from the initial count);
   \( N_0 = \) number of microorganisms inoculated at time \( T_0 \);
   \( N_x = \) number of surviving microorganisms at each sampling time \( T_x \).
d) n.p.= not performed

**Table 4: Evaluation criteria for the preservation efficacy test (ISO 11930:2012).**

If at the end of the Challenge test the sample is in accordance with criterion A, the microbiological risk is acceptable and the
cosmetic product is considered protect against the microbial proliferation and it is not necessary to consider other factors that are independent from the formulation.

On the other hand, if the sample is in accordance with criterion B, the microbiological risk is acceptable, thereby the level of protection is acceptable if the risk analysis demonstrates the existence of control factors not related to the formulation, for example a protective packaging in order to reduce the microbiological risk.

If at the end of the challenge test the sample does not satisfy neither criterion A nor the criterion B, the status of the product shall be evaluated solely according to the microbiological risk assessment. For example, a product in single-dose units can be considered a tolerable microbiological risk, provided that the microbiological quality of the finished product is assured at the time of release.

Overall, the microbiological safety of a cosmetic product depends on a combination of several factors, not only based on the results of the Challenge test, but also on the formulation characteristics, production conditions and final packaging in accordance with good manufacturing practice (GMP)[8].
4.6 Results and discussion

4.6.1 EPR results

The EPR analysis were performed by using two probes as spin labels: 5-DSA (5-Doxyl-stearic acid) and TEMPOl (4-Hydroxy-TEMPO) (Fig.4).

Cyclic nitroxides are often used as spin-probes due to their remarkable stability and to the localization of the spin density on the NO moiety, which allows extracting several information on the probe solubilization site from the EPR spectrum. In particular, 5-DSA probe gives information about the nature of molecular aggregates inside the formulations whereas TEMPO probe allows to obtain information about the aqueous environment in which it is located. Both the probes were used at a concentration 10^{-4} M and were added to the samples of formulations without preservatives and with preservatives. Regarding to S17F13 formulations, the comparison among spectra of 5-DSA probe is shown in Fig.5.
Fig. 5: Spectra of 5DSA probe in shampoo without preservatives (S17F13) and with preservatives (S1F13 Ac and S17F13 Ag).

Analyzing the spectra, no particular differences are present. In all cases, the spectra lineshape clearly shows an anisotropic motion of the probe, thus indicating that surfactants present in the shampoo form multilamellar vesicles.

Also the spectra of TEMPOI probe in shampoo without preservatives and with preservatives don't show particular differences, as shown in Fig.6.
Fig. 6: Spectra of TEMPO1 probe in shampoo without preservatives (S17F13) and with preservatives (S17F13 Ac and S17F13 Ag).

In particular, the motion of probe is isotropic in all cases, with a less intense third peak at high field; in general, a broadening of peaks is evident and this result indicates that the probe is located in a more viscous environment. Regarding to B14F13, B14F13 Ac and B14F13 Ag, the comparison among spectra of 5DSA probe is shown in Fig. 7. Also in this case, the spectra are similar and the lineshape indicates that in conditioner the surfactants form large micellar aggregates.
Fig. 7: Spectra of 5DSA probe in conditioner without preservatives (B14F13) and with preservatives (B14F13 Ac and B14F13 Ag).

In particular, the motion of probe is slow isotropic as indicated by the peaks at low and high field that are slightly broadened.

Finally, the comparison among spectra of TEMPOI probe in conditioner without preservatives and with preservatives is shown in Fig. 8.

The motion of probe is isotropic and narrow lines are observed. This result indicates that the aqueous environment in which the probe is located is less viscous than that found in S17F13 formulations.
Fig. 8: Spectra of TEMPO\textsubscript{1} probe in conditioner without preservatives (B14F13) and with preservatives (B14F13 Ac and B14F13 Ag).

All these results highlight that both in S17F13 and B14F13 formulations, the preservatives don't induce microstructural differences with respect to the samples not preserved.

### 4.6.2 Microbiologic analyses

In addition to EPR results, microbiological analyses were performed on the same formulations at the Department of Biological Science of the University “Federico II” in collaboration with research group of Prof. Marco Guida, in order to compare microbial concentration overtime and through Challenge test.
It is to be noted that since both S17F13 and B14F13 were prepared from commercial raw materials and following common industrial protocols, they are not sterile. A total microbial count was carried out at time zero ($t_0$) and after 30 days ($t_{30}$) both on formulations without preservatives and with Acnibio Ac and colloidal silver at specific concentrations, respectively 0.1% and 0.5 ppm with respect to the weight of the cosmetic product. As shown in Fig.9 and Fig.10, in the absence of preservatives, the total microbial concentration decreases overtime in shampoo and not in the conditioner. In the presence of preservatives no microbial initial contamination or growth was observed. Thereof, it can be deduced that preservatives are essential for B14F13 while their effective need in S17F13 has to be assessed.

![Total microbial count S17F13](image)

**Fig.9:** The total microbial count in S17F13 shampoo without and with preservatives.
Fig. 10: The total microbial count in B14F13 conditioner without and with preservatives.

Moreover the Challenge test was performed not only on these formulations but also on those preserved with colloidal silver at concentration 0.1 and 0.05 ppm with respect to the weight of the cosmetic product. After an appropriate contamination with microorganisms listed in Table 3, the capacity of defending against microbial attack of cosmetic formulations was verified; so the reduction of the total viable count was monitored, for each tested microbial strain, within a given time span, according to the criteria of acceptability emanated by ISO 11930:2012.

In order to satisfy the criteria of evaluation of preserving system provided by European Pharmacopoeia 7th edition [9,10] (Table 5), the microbial count for each tested microbial strain was performed also after 2 days from inoculation, in addition to 7, 14 and 28 days.
Log reduction values \((R_x = \log N_0 - \log N_x)\)

<table>
<thead>
<tr>
<th>Criteria</th>
<th>Log reduction values</th>
<th>(T_2)</th>
<th>(T_7)</th>
<th>(T_{14})</th>
<th>(T_{28})</th>
</tr>
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<tbody>
<tr>
<td><strong>Bacteria</strong></td>
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<td>(\geq 2)</td>
<td>(\geq 3)</td>
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<tr>
<td></td>
<td>B</td>
<td>-</td>
<td>-</td>
<td>(\geq 3)</td>
<td>NI</td>
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<tr>
<td><strong>Fungi</strong></td>
<td>A</td>
<td>-</td>
<td>-</td>
<td>(\geq 2)</td>
<td>NI</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>-</td>
<td>-</td>
<td>(\geq 1)</td>
<td>NI</td>
</tr>
</tbody>
</table>

\(N_0\) = number of microorganisms inoculated at time \(T_0\);
\(N_x\) = number of surviving microorganisms at each sampling time \(T_x\).
NI: no increase in the count from the previous contact time

**Tab.5: Evaluation criteria for the preservation efficacy test (Ph.Eur.7).**

For all cosmetic formulations analyzed, the log reduction values were calculated and compared to the minimum values required for evaluation criterion A or B, reported in ISO 11930 (table 4) and in Ph.Eur.7 (table 5).

Regarding S17F13 formulation, the log reduction values of all the samples contaminated with *Staphylococcus aureus* are in accordance with Criterion A, both for ISO 11930 and for Ph.Eur.7 (table 6).

<table>
<thead>
<tr>
<th>Log reduction values (\textit{S.aureus})</th>
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<th>S17F13 Ag0,05</th>
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<td>5,67</td>
<td>5,67</td>
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</tr>
<tr>
<td>(t_{14})</td>
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<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>(t_{21})</td>
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<td>(t_{28})</td>
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</table>

**Acceptance criteria**

<table>
<thead>
<tr>
<th></th>
<th>ISO11930</th>
<th>Ph.Eur.7</th>
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</thead>
<tbody>
<tr>
<td><strong>A</strong></td>
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</tr>
</tbody>
</table>

**Tab.6: Log reduction values obtained and acceptance criteria satisfied for the samples of shampoo contaminated with \textit{S.aureus}.**
The trend of microbial concentration during the incubation time in S17F13 formulations contaminated with *S.aureus* without preservatives, with colloidal silver and with Acnibio Ac is shown in Fig.11.

![Ch. Test Staphylococcus aureus-Shampoo](image)

**Fig.11:** The trends of microbial concentration at different times in S17F13 formulations contaminated with *S.aureus* without preservatives, with colloidal silver and with Acnibio Ac.

Similarly, the different samples of shampoo are contaminated with *P.aeuriginosa, E.coli, A.brasiliensis e C.albicans* and for all formulations, the log reduction values obtained are in accordance with criterion A, both for ISO 11930 and for Ph.Eur.7, as shown respectively in Table.7,8,9,10.

The trends of microbial concentration during the incubation time in S17F13 formulations contaminated with *P.aeuriginosa, E.coli, A.brasiliensis e C.albicans* are shown respectively in Fig.12,13,14 and Fig.15.
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<th>S17F13 Ag0,1</th>
<th>S17F13 Ag0,5</th>
<th>S17F13 AC</th>
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<tr>
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<td>5,34</td>
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<td>-</td>
<td>-</td>
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</tr>
<tr>
<td>t14</td>
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<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>t21</td>
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<td></td>
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</table>

Tab.7: Log reduction values obtained and acceptance criteria satisfied for the samples of shampoo contaminated with *P.aeruginosa*.

**Fig.12:** The trends of microbial concentration at different times in S17F13 formulations contaminated with *P.aeruginosa* without preservatives, with colloidal silver and with Acnibio Ac.
Tab.8: Log reduction values obtained and acceptance criteria satisfied for the samples of shampoo contaminated with *E.coli*.

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<th>S17F13 Ag0,1</th>
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<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>t14</td>
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</table>

Fig.13: The trends of microbial concentration at different times in S17F13 formulations contaminated with *E.coli* without preservatives, with colloidal silver and with Acnibio Ac.
Tab. 9: Log reduction values obtained and acceptance criteria satisfied for the samples of shampoo contaminated with *A. brasiliensis*.

<table>
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<th>S17F13 Ag0,5</th>
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Acceptance criteria

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</table>

Fig. 14: The trends of microbial concentration at different times in S17F13 formulations contaminated with *A. brasiliensis* without preservatives, with colloidal silver and with Acnibio Ac.
Tab. 10: Log reduction values obtained and acceptance criteria satisfied for the samples of shampoo contaminated with *C. albicans*.

<table>
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<tr>
<td>t₇</td>
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<td>4.83</td>
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<tr>
<td>t₁₄</td>
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<td>4.83</td>
<td>-</td>
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</tr>
<tr>
<td>t₂₁</td>
<td>-</td>
<td>-</td>
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<td>-</td>
<td>-</td>
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<td>t₂₈</td>
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**Acceptance criteria**

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<tr>
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</table>

Fig. 15: The trends of microbial concentration at different times in S17F13 formulations contaminated with *C. albicans* without preservatives, with colloidal silver and with Acnibio Ac.
Overall, in all samples of shampoo, even without preservatives, the microbiological risk is considered to be tolerable and the chosen preservatives show efficacy against tested microorganisms.

Regarding to B14F13 samples, the results of Challenge test do not always satisfy the acceptance criteria, both of ISO 11930 and Ph.Eur.7.

In particular, the log reduction values of all the samples of conditioner contaminated with *Staphylococcus aureus* are in accordance with Criterion A, both for ISO 11930 and for Ph.Eur.7 (Table 11).

<table>
<thead>
<tr>
<th></th>
<th>B14F13</th>
<th>B14F13 Ag0,05</th>
<th>B14F13 Ag0,1</th>
<th>B14F13 Ag0,5</th>
<th>B14F13 AC</th>
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</thead>
<tbody>
<tr>
<td>t&lt;sub&gt;2&lt;/sub&gt;</td>
<td>5,67</td>
<td>5,67</td>
<td>5,67</td>
<td>5,67</td>
<td>5,67</td>
</tr>
<tr>
<td>t&lt;sub&gt;7&lt;/sub&gt;</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>t&lt;sub&gt;14&lt;/sub&gt;</td>
<td>-</td>
<td>-</td>
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<td>-</td>
<td>-</td>
</tr>
<tr>
<td>t&lt;sub&gt;21&lt;/sub&gt;</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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**Acceptance criteria**

<table>
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<tr>
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</tr>
<tr>
<td>A</td>
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<td>A</td>
</tr>
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</table>

Tab.11: Log reduction values obtained and acceptance criteria satisfied for conditioner samples contaminated with *S.aureus*.

The trends of microbial concentration during the incubation time in B14F13 formulations contaminated with *S.aureus* without preservatives, with colloidal silver and with Acnibio Ac are shown in Fig.16.
Fig. 16: The trends of microbial concentration at different times in B14F13 formulations contaminated with *S. aureus* without preservatives, with colloidal silver and with Acnibio Ac.

As regards the samples of conditioner contaminated with *P. aeruginosa*, *E. coli* and *A. brasiliensis*, the Challenge test shows strongly different results among the sample without preservatives and the ones with preservatives. In fact, in absence of preservatives, the conditioner does not satisfy the acceptance criteria of ISO 11930 and Ph.Eur.7 whereas the preserved samples satisfy both criteria, as shown in Table 12, 13 and 14.

The trends of microbial concentration during the incubation time in B14F13 formulations contaminated with *P. aeruginosa*, *E. coli* and *A. brasiliensis* without preservatives, with colloidal silver and with Acnibio Ac are shown in Fig.17, 18 and 19.
Tab.12: Log reduction values obtained and acceptance criteria satisfied for conditioner samples contaminated with \textit{P.aeruginosa}.

<table>
<thead>
<tr>
<th></th>
<th>B14F13</th>
<th>B14F13 Ag0,05</th>
<th>B14F13 Ag0,1</th>
<th>B14F13 Ag0,5</th>
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<tr>
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<tr>
<td>t21</td>
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<td>-</td>
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Acceptance criteria

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<tr>
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Fig.17: The trends of microbial concentration at different times in B14F13 formulations contaminated with \textit{P.aeruginosa} without preservatives, with colloidal silver and with Acnibio Ac.
Tab. 13: Log reduction values obtained and acceptance criteria satisfied for conditioner samples contaminated with *E. coli*.

<table>
<thead>
<tr>
<th></th>
<th>B14F13</th>
<th>B14F13 Ag0,05</th>
<th>B14F13 Ag0,1</th>
<th>B14F13 Ag0,5</th>
<th>B14F13 AC</th>
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<tbody>
<tr>
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<td>2,16</td>
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<td>t14</td>
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Acceptance criteria

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</table>

Fig. 18: The trends of microbial concentration at different times in B14F13 formulations contaminated with *E. coli* without preservatives, with colloidal silver and with Acnibio Ac.
Tab.14: Log reduction values obtained and acceptance criteria satisfied for conditioner samples contaminated with *A. brasiliensis*.

<table>
<thead>
<tr>
<th></th>
<th>B14F13</th>
<th>B14F13 Ag0,05</th>
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<tr>
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Acceptance criteria

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Ch.test *Aspergillus brasiliensis*-Conditioner

Fig.19: The trends of microbial concentration at different times in B14F13 formulations contaminated with *A. brasiliensis* without preservatives, with colloidal silver and with Acnibio Ac.
Finally, all conditioner samples contaminated with *Candida Albicans* satisfy the acceptance criteria, both of ISO 11930 and Ph.Eur.7, as indicated by the log reduction values in Table 15.

<table>
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<th>Log reduction values (<em>C.albicans</em>)</th>
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<td>2.38</td>
<td>4.83</td>
<td>4.83</td>
<td>4.83</td>
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</tr>
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<td>t28</td>
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</table>

Tab.15: Log reduction values obtained and acceptance criteria satisfied for conditioner samples contaminated with *C.albicans*.

The trends of microbial concentration during the incubation time in the samples of conditioner contaminated with *C.albicans* without preservatives, with colloidal silver and with Acnibio Ac are shown in Fig.20.
Fig. 20: The trends of microbial concentration at different times in B14F13 formulations contaminated with *C. albicans* without preservatives, with colloidal silver and with Acnibo Ac.

Overall, the conditioner without preservatives fails the Challenge test since in the most of cases the acceptance criteria do not satisfy.

On the contrary, the samples of conditioner with Acnibo Ac and with colloidal silver in different concentrations are protected against microbial proliferation and therefore they can be delivered on the market.

### 4.7 Conclusions

The obtained results through EPR studies show that the preservatives do not change the microstructural properties of hair formulations developed. On the other hand, the differences in spectra lineshape between the shampoo and the
conditioner are exclusively dependent on the type of chosen surfactants and on their chemical-physical properties. Besides, the microbiological analyses and in particular the Challenge test highlight that preservatives could also not be added in the shampoo S17F13 whereas their presence is fundamental in the conditioner B14F13. This result is probably due to the higher content of water in the conditioner compared to the shampoo. Secondly, the use of colloidal silver at a concentration 0.05 ppm with respect to the weight of cosmetic product in the conditioner should be carefully evaluated since it represents a borderline situation. For this reason, higher concentrations of colloidal silver, as reported in Italian patent No. 1307312 [4], should be used in B14F13 formulation in order to obtain a cosmetic product protected against microbial proliferation which can be delivered on the market.
References


Chapter 5 - Catanionic self-aggregates as antioxidant nanovehicles for cosmeceutical applications

Formulations used as cosmetics for personal care and detergency are often based on mixtures of surfactants. For instance, formulations for hair care are based on mixtures of anionic (prevalent in shampoos) and cationic (prevalent in conditioners) surfactants. Mixtures are preferred to pure surfactants because production costs are lower, and, in many applications, mixtures of dissimilar surfactants exhibit performances superior to those of the single constituents [1]. From a microstructural viewpoint, aqueous mixtures of oppositely charged surfactants are a fascinating topic because, depending on composition, concentration and molecular features, they are able to form diversified micro- or nano-scale structures, such as vesicles, micelles, cubic mesophases, lamellae [2,3,4]. In dilute solutions, catanionic vesicles form [5], which have been proposed as promising tools for delivery of actives [6].

Considering the wide use of mixtures of oppositely charged surfactants in trichological formulations and their ability to form spontaneously catanionic vesicles, the idea of this second part of PhD project is to explore the possibility to incorporate trans-resveratrol, a known antioxidant, into this kind of
amphiphilic nano-aggregates, thus building a reliable platform for the development of a new class of cosmeceuticals. For this reason, a mixture of a cationic surfactant amply used in trichological products, such as cetyltrimethylammonium chloride (CTAC, commercially available as cetrimonium chloride), and the most representative of anionic surfactants, sodium dodecyl sulphate (SDS, commercially available as sodium lauryl sulphate) is used. Therefore in this second part of experimental work it is described: i) the chemico-physical characterization of the ternary system water-CTAC-SDS through the determination and analysis of the phase diagram, obtained by means of Dynamic light scattering (DLS), Small angle neutron scattering (SANS) and Electron Paramagnetic Resonance (EPR) measurements; ii) a study of the solubility of resveratrol in different nano-vehicles formed by CTAC, SDS and their mixtures, as quantitatively assessed by UV and fluorescence spectroscopy; iii) the determination of the antioxidant activity of resveratrol when incorporated into these nano-vehicles, as evaluated by EPR.

5.1 Materials and methods

5.1.1 Materials and preparation of surfactant mixtures. SDS, CTAC, 5-doxyl stearic acid (5-DSA) and 4-hydroxy-2,2,6,6-tetramethyl-1-piperidinyloxy (TEMPO) of high purity were purchased from Sigma Aldrich whereas trans-
resveratrol (≥98% pure) was purchased by Cayman Chemical Company. All materials were used without further purification. Millipore water was used for all samples, with the only exception of samples for SANS measurements where D$_2$O (purchased from Sigma Aldrich, isotropic enrichment >99.8%) was used. All surfactant mixtures were prepared by proper mixing/dilution of two stock concentrated solutions of CTAC and SDS at 0.0625 and 0.41 mol kg$^{-1}$, respectively. Samples at total surfactant concentration 0.01, 0.02 and 0.03 mol kg$^{-1}$ and with different anionic/cationic surfactant molar ratios were prepared. In all the cases, a short (5 min) sonication step in a thermostatic bath at 40 °C was necessary to promote mixing of surfactants.

5.1.2 Surface Tension Measurements. Critical aggregation concentrations (cac) for two catanionic mixtures (aqueous CTAC-SDS 70:30 and 30:70 mixtures) were determined by surface tension measurements. The surface tension of the aqueous mixtures, $\gamma$, was measured with a SIGMA 70 tensiometer using the Du Noüy ring method. Successive aliquots of a stock catanionic mixture, freshly prepared in Millipore water, were added to the vessel with a known volume of dilute solution. By this way, the total surfactant concentration was gradually increased while the surfactant ratio was kept constant. The cac was calculated as the inflection point of the plot reporting $\gamma$ as a function of the total surfactant concentration.
5.1.3 Dynamic Light Scattering. DLS measurements were performed with a home-made instrument composed of a Photocor compact goniometer, a SMD 6000 Laser Quantum 50 mW light source operating at 5325 Å, a photomultiplier (PMT-120-OP/B) and a correlator (Flex02-01D) from Correlator.com. The experiments were carried on at the constant temperature (25.0 ± 0.1) °C, by using a thermostatic bath, and at the scattering angle θ of 90°. The scattered intensity correlation function was analyzed using a regularization algorithm [7]. The diffusion coefficient of each population of diffusing particles was calculated as the z-average of the diffusion coefficients of the corresponding distribution [8]. Considering that the mixtures are quite dilute, the Stokes–Einstein equation, which rigorously holds at infinite dilution for non-interacting spherical species diffusing in a continuous medium, was used to evaluate the hydrodynamic radius, $R_H$, of the aggregates from their translation diffusion coefficients, $D$.

5.1.4 Small Angle Neutron Scattering. SANS measurements were performed at 25 °C with the KWS2 instrument located at the Heinz Meier Leibnitz Source, Garching Forschungszentrum (Germany) [9]. Neutrons with a wavelength spread $\Delta \lambda / \lambda \leq 0.2$ were used. A two-dimensional array detector at three different wavelength (W), collimation (C), sample-to-detector distance (D) combinations
(W₇ÅC₈mD₂m, W₇ÅC₈mD₈m and W₁₉ÅC₈mD₈m), measured neutrons scattered from the samples. These configurations allowed collecting data in a range of the scattering vector modulus $q = 4\pi/\lambda\sin(\theta/2)$ between 0.0019 Å⁻¹ and 0.279 Å⁻¹, with scattering angle. The samples were contained in a closed quartz cell, in order to prevent the solvent evaporation. Each measurement lasted for a period sufficient to obtain ~ 2 million counts. The raw data were corrected for background and empty cell scattering. Detector efficiency correction, radial average and transformation to absolute scattering cross sections $\frac{d\Sigma}{d\Omega}$ were made with a secondary plexiglass standard [10,11].

Thus, the obtained absolute scattering cross sections $\frac{d\Sigma}{d\Omega}$ data were plotted as function of $q$. Generally, the dependence of $\frac{d\Sigma}{d\Omega}$ from the scattering vector can summarized as in following equation:

$$\frac{d\Sigma}{d\Omega} = n_p P(q)S(q) + \left(\frac{d\Sigma}{d\Omega}\right)_{incoh}$$

where $n_p$ is the number of scattering objects, $P(q)$ and $S(q)$ are respectively the form factor and the structure factor, and the last term takes into account the incoherent scattering mostly due to the presence of hydrogen atoms within the sample.

The form factor is responsible for the shape, size distribution of the scattering particles, while a contribution of the structure factor can be considered when an interparticle correlation exists. The structural information contained in
both the form and the structure factor can be extrapolated by choosing an appropriate model to fit the obtained experimental data [12].

The scattered intensity of CTAC solution was fitted considering the micelles as interacting charged cylinders according to the following equation and using the Hayter and Penfold RMSA for the structure factor [13].

\[
P(Q) = \frac{\text{scale}}{V} \frac{2\Delta\rho V \sin \left( \frac{QL \cos \frac{\alpha}{Z}}{2} \right) J_1(Qr \sin \alpha)}{QL \cos \frac{\alpha}{Z}} \frac{Qr \sin \alpha}{Qr \sin \alpha}
\]

where \(\Delta\rho\) represent the scattering length density contrast, \(a\) is the angle between the cylinder axis and the scattering vector \(Q\), and \(V, L\) and \(r\) are the cylinder volume, length and radius respectively. \(J_1\) is the first order Bessel function.

The scattered intensity of SDS solution was fitted considering the micelles as interacting charged rigid spheres. The following equation was used for the form factor and the Hayter and Penfold RMSA was introduced in the model function in order to take into account the structure factor.

\[
P(Q) = \frac{\text{scale}}{V} \left[ 3V \left( \frac{\Delta\rho}{Qr^3} \right) \left( \sin Qr - \cos Qr \right) \right]^2
\]

where \(r\) represents the sphere radius.

Finally, for the CTAC-SDS vesicles the following equation for the form factor was used:

\[
P(Q) = \frac{2 \left( \Delta\rho \right)^2}{Q^2} \left( 1 - \cos Q\delta \right)
\]

where \(\delta\) is the thickness of the vesicle lamellae.
5.1.5 Electron Paramagnetic Resonance Spectroscopy. 5-doxyll stearic acid (5-DSA) was introduced in the surfactant aqueous mixtures as spin probe because it is a surfactant able to co-aggregate with CTAC and/or SDS. Spin labelled samples were prepared as follows: a small aliquot of 5-DSA in ethanol at 1 mg ml\(^{-1}\) was poured in a vial; a thin film was produced by evaporating the solvent with dry nitrogen gas and final traces of solvent were removed by subjecting the sample to vacuum desiccation for at least 3 h; then the probe film was hydrated with the surfactant mixture and incubated for 10 min. EPR spectra were recorded on a 9 GHz Bruker Elexys E-500 spectrometer (Bruker, Rheinstetten, Germany). Capillaries filled with 10 μl of samples were placed in a standard 4 mm quartz tube, also containing light silicone oil to assure thermal stability. The temperature of the sample was kept constant at 25 °C during the measurement. Instrumental settings were as follows: sweep width 6000 G; resolution 1024 points; modulation frequency 100 kHz; modulation amplitude 1.0 G; time constant 20.5 ms, incident power 6.4 mW, Gain 60 dB. With the aim to improve the signal-to-noise ratio, 16 scans were accumulated in all the cases.

The antioxidant activity of t-RESV in CTAC aggregates was also evaluated by EPR, monitoring the decreasing of the signal due to the free radical TEMPOL in the presence of t-RESV. Samples containing an excess amount of t-RESV in water and in CTAC micelles at total surfactant concentration 0.03 mol kg\(^{-1}\) were prepared. In addition a sample containing
alone CTAC micelles at total surfactant concentration 0.03 mol kg\(^{-1}\) was prepared as control. In all samples a fixed quantity of TEMPOL at 10\(^{-4}\) M concentration was present. After the samples were kept at 298 K for 24 hours, covered with aluminium foil in order to avoid t-RESV degradation, the supernatant of each sample was taken and analysed as previously described. Instrumental settings were set as follows: sweep width 6000 G; resolution 1024 points; modulation frequency 100 kHz; modulation amplitude 1.0 G; time constant 20.5 ms, incident power 6.4mW, Gain 40 dB.

5.1.6 UV-Vis Spectroscopy. t-RESV solubility in CTAC and SDS micelles, as well as in CTAC-SDS vesicles at 70:30 and 30:70 molar ratio, was evaluated determining the concentration of t-RESV in the different mixtures by means of UV spectroscopy [14]. Spectra were collected at 25 °C on a Jasco 530 UV–Vis spectrophotometer (Jasco Inc., Easton, MD), in the 500 -220 nm range, with a band width of 1 nm, scanning speed of 200 nm/min and using a 0.1 cm path length of the cell. Each experiment was repeated thrice. Samples were prepared by adding an excess amount of t-RESV to 2 g of the aqueous surfactant mixtures. The total surfactant molality ranged from 0 to 0.03 mol kg\(^{-1}\). The samples were shaken for 24 h in a thermostated bath at 25 °C. To minimise photochemical degradation of t-RESV the vials were covered with aluminium foil. Once equilibrium was reached, suspensions were kept at rest for 1 hr after which undissolved
t-RESV powder settles at the vial bottom. The supernatants were separated, opportunely diluted and finally analysed. The procedure did not include a filtration or centrifugation step, to avoid possible perturbation and/or partial separation of the surfactant aggregates.

With the aim to obtain a very careful determination of t-RESV concentration in the different samples, the molar extinction coefficients of t-RESV in CTAC and SDS mixtures was calculated. They are scarcely sensitive to the presence of surfactants. Moreover, the maximum solubility of t-RESV in water was determined by UV measurements.

5.1.7 **Fluorescence Spectroscopy.** Since maximum fluorescence emission is highly sensitive to polarity of the solvent, with the aim to verify that t-RESV experiences the more hydrophobic environment provided by the surfactant aggregates, fluorescence spectra in different solution conditions (i.e. in the presence of CTAC micelles, SDS micelles, mixed catanionic vesicles and in water) was recorded.

Fluorescence spectra were recorded with a thermostatically controlled JASCO FP-750 spectrofluorometer using a 1 cm path length of the cell, at 25 °C. The t-RESV was selectively excited at 318 nm and the emission was recorded from 330 to 500 nm at a scanning speed of 250 nm/min, with a 5 nm emission slit width and corrected for background signal.
5.2 Results and discussion

5.2.1 CTAC-SDS-Water Phase Diagram. The ternary phase diagram of the diluted CTAC-SDS-water system at 25 °C is shown in Errore. L'origine riferimento non è stata trovata..

The sample compositions are reported in weight percent concentrations. Two triangle sides correspond to the binary systems: the bottom side corresponds to the SDS-water mixtures, while the left one corresponds to the CTAC-water mixtures. Both binary systems present an isotropic micellar phase; the critical micelle concentration (cmc) values, reported in the literature [15,16], are quoted in the diagram and reported in Errore. L'origine riferimento non è stata trovata.. The third side, on the right, corresponds to ternary mixtures in which the total surfactant concentration is 1% by weight, while the surfactant composition ranges from pure SDS to pure CTAC.

The blue dotted-dashed line indicates the CTAC-SDS equimolar ratio; samples lying above the line present an excess of CTAC, whereas the ones lying below have an excess of SDS. The distinction between isotropic and anisotropic phases and the identification of the phase diagram areas where these phases coexist have been obtained by DLS, SANS and EPR measurements [17,18], as detailed below. The green, red and black dots indicate the mixtures at 0.01, 0.02 and 0.03 mol kg⁻¹ total surfactant concentration, respectively, which have been prepared and analysed.
Fig.1: Phase diagram of the ternary system CTAC-SDS-water at 25 °C. Experimental points are marked with dots. The catanionic precipitate region is marked explicitly; M⁺ and M⁻ indicate cationic and anionic micelles, respectively; V⁺ and V⁻ indicate cationic and anionic vesicles, respectively; M⁺V⁻ indicates the multiphase region where anionic micelles and vesicles coexist. The cmc values for SDS and CTAC, as reported in the literature, are explicitly shown on the corresponding axes.

Even in this high dilution region, the existence of different phases is observed, both as single phases and as biphasic equilibrium mixtures. In equimolar mixtures of the two surfactants a catanionic precipitate forms. Moving from the equimolar line towards the CTAC and SDS binary system sides, a qualitatively similar phase behaviour is observed.
Upon addition of either one of the two surfactants, the catanionic precipitate dissolves and a vesicular suspension forms. A stringent definition of the coexistence regions between precipitate and vesicle areas is beyond the scope of this work, and for this reason the phase boundaries are blurred. The vesicular suspensions are slightly bluish, as often found for catanionic vesicles. Further addition of surfactants results in the formation of mixed micelles, whose stability region extends close to the axes representing the binary systems. The region in which vesicles and micelles coexist is clearly detectable only in the SDS-rich side.

The CTAC-SDS-water phase behaviour depends on the alkyl chain length and symmetry [19]. Specifically, the asymmetry between the two components (16 vs 12 carbon atoms) determines a relatively small precipitation area, close to the equimolarity line, favouring the formation of vesicles [20]. Because of the strong synergistic effects typical of mixtures of oppositely charged surfactants, vesicles start to form at very low surfactant concentration, as proved by the values of critical aggregation concentration (cac) of the two catanionic mixtures CTAC-SDS 70:30 and 30:70, determined by surface tension measurements and reported in Table 1. In both cases, the cac’s are about two orders of magnitude lower than the cmc’s of the pure surfactants.
<table>
<thead>
<tr>
<th></th>
<th>CMC/CAC (mol Kg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTAC</td>
<td>1.1*10⁻³</td>
</tr>
<tr>
<td>CTAC-SDS 70:30</td>
<td>2.9*10⁻⁵</td>
</tr>
<tr>
<td>CTAC-SDS 30:70</td>
<td>8.8*10⁻⁵</td>
</tr>
<tr>
<td>SDS</td>
<td>9.5*10⁻³</td>
</tr>
</tbody>
</table>

Tab.1: cmc and cac of CTAC, SDS and their mixtures.

Identification of surfactant aggregate morphology was initially based on DLS measurements. This analysis allowed to detect the number of different populations of aggregates present in each sample and, on the basis of the mean hydrodynamic radius, to discriminate between micelles and vesicles. Some examples of DLS profiles of several mixtures at 0.03 mol Kg⁻¹ concentration are reported in Errore. L’origine riferimento non è stata trovata. With the only exception of the CTAC-SDS 10:90 mixture, a single population is observed, whose size distribution is more or less broadened depending on the cases. Representative mean hydrodynamic radii evaluated from these measurements are collected in Table 2. For pure surfactant mixtures, small aggregates are found, which can be identified as micelles, whereas in the mixtures of both surfactant larger aggregates are observed, which correspond to catanionic vesicles. These vesicles coexist with micelles in the CTAC-SDS 10:90 mixture.
Fig.2: Intensity weighed hydrodynamic radius distributions of different surfactant mixtures at 0.03 mol kg\(^{-1}\). Distributions corresponding to micelles and vesicles are differently marked with full or empty circles, respectively.

<table>
<thead>
<tr>
<th></th>
<th>(R_H) (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTAC</td>
<td>1.7 ± 0.5</td>
</tr>
<tr>
<td>CTAC-SDS</td>
<td></td>
</tr>
<tr>
<td>90:10 mol/mol</td>
<td>37 ± 3</td>
</tr>
<tr>
<td>CTAC-SDS</td>
<td></td>
</tr>
<tr>
<td>70:30 mol/mol</td>
<td>60 ± 3</td>
</tr>
<tr>
<td>CTAC-SDS</td>
<td></td>
</tr>
<tr>
<td>30:70 mol/mol</td>
<td>61 ± 2</td>
</tr>
<tr>
<td>CTAC-SDS</td>
<td></td>
</tr>
<tr>
<td>10:90 mol/mol</td>
<td>1.8 ± 0.2</td>
</tr>
<tr>
<td>SDS</td>
<td>1.4 ± 0.5</td>
</tr>
</tbody>
</table>

Tab.2: Mean Hydrodynamic Radius of CTAC, SDS and their mixtures at 0.03 mol kg\(^{-1}\) total surfactant concentration.
Analysis of the samples at lower total surfactant concentration (0.01 and 0.02 mol Kg\(^{-1}\)) gave similar results.

In both the cationic- and the anionic- rich side, the vesicle dimension at a given CTAC-SDS ratio is not affected by the total surfactant concentration. In the cationic rich side, the stability domain of the vesicular aggregates is large enough to analyse the dependence of the aggregate dimension on the CTAC-SDS molar ratio (Figure 3).

![Graph showing dependence of aggregate hydrodynamic radius on CTAC-SDS molar ratio.](image)

**Fig.3: Dependence of the aggregate hydrodynamic radius on CTAC-SDS molar ratio.**

Irrespective of surfactant concentration, the maximum radius of the vesicles (corresponding to the minimum curvature of their surface) is reached at CTAC-SDS 80:20 molar ratio. At higher ratios, the surfactant packing is hindered by the electrostatic repulsion between the excess of positively
charged CTAC head groups. At lower ratios, the mismatching between the tail length of CTAC and SDS disturbs the molecule self-organization.

A deeper insight into the structural features of surfactant aggregates was obtained by means of SANS. In particular, the pure surfactant mixtures and the 70:30 and 30:70 ones at 0.03 mol Kg\(^{-1}\) were analyzed. The scattering curves show a completely different profile for the pure surfactants and for the catanionic mixtures, as shown in Figure 4.

![Figure 4: Neutron scattered intensity for different surfactant mixtures at 0.03 mol kg\(^{-1}\). Fitting curves are reported as black lines.](image)

The presence of a pick in the low q-region of SANS curves for pure CTAC and SDS aqueous mixtures indicates that a
structure factor, arising from inter-aggregates interactions, has to be included in the fitting model together with the proper form factor. For pure SDS mixtures, the fitting of scattering data was performed according to the model of charged rigid spheres. The radius and the total charge of the micelles are reported in Table 3. In the case of CTAC micelles, very good results were obtained by fitting the data with the model of interacting charged cylinders. The total charge, the length and the radius of the cylindrical micelles are also reported in Table 3.

<table>
<thead>
<tr>
<th>Aggregate type</th>
<th>Charge (e)</th>
<th>Radius* (nm)</th>
<th>Length (nm)</th>
<th>Bilayer thickness (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTAC Cylindrical micelles</td>
<td>17 ± 1</td>
<td>1.9 ± 0.5</td>
<td>5.4 ± 0.8</td>
<td>-</td>
</tr>
<tr>
<td>CTAC-SDS 70:30 mol/mol Unilamellar vesicles</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>3.6 ± 0.6</td>
</tr>
<tr>
<td>CTAC-SDS 30:70 mol/mol Unilamellar vesicles</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>3.0 ± 0.2</td>
</tr>
<tr>
<td>SDS Spherical micelles</td>
<td>9 ± 1</td>
<td>1.7 ± 0.3</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*Sphere radius in the case of SDS micelles and cylinder radius in the case of CTAC micelles.

Tab.3: Structural features obtained from the SANS data analysis of both pure CTAC and SDS solutions and their mixtures at 0.03 mol kg⁻¹ total surfactant concentration.

SANS analysis well highlights structural differences between CTAC and SDS micelles, not only in terms of shape, cylinders vs. spheres, but also in terms of dimensions, being CTAC micelles significantly larger than SDS ones.
For what concerns the CTAC-SDS catanionic aggregates, SANS measurements indicate that, at both the analysed surfactant ratios, they are unilamellar vesicles. Indeed, as shown in Figure 4, a predominant form factor characterizes all the corresponding scattering profiles. From the fitting of the data, the thickness of the bilayers for the different mixtures (Table 3) was obtained. The CTAC-SDS 30:70 vesicles appear to be characterized by a slightly thicker bilayer than the 70:30 ones.

The ordering and dynamics of the surfactant molecules in the different nano-aggregates they form was investigated by EPR measurements, using 5-DSA as molecular probe. 5-DSA is an amphiphilic molecule bearing the nitroxide group close to the hydrophilic head group, as shown in the inset of Figure 5. Consequently, it inserts in the surfactant aggregates monitoring the environment just underneath the interface.

In Figure 5 the EPR spectra of 5-DSA in the same samples investigated by SANS are shown. In pure SDS solutions, an almost isotropic three-line lineshape is observed, indicating that the probe rotational motion is relatively unhindered, and is similar along the three molecular axes. In pure CTAC solutions, the 5-DSA spectrum is broadened, as emerges from the inspection of the high-field line. This indicates that the motion of the reporter group is slowed down by a more viscous microenvironment. In turn, this behaviour could be linked to the better ordering of surfactants tails in the bulkier
cylindrical aggregates formed by CTAC with respect to the smaller spherical ones formed by SDS. In catanionic mixtures, the 5-DSA spectra definitely assume an anisotropic lineshape, highlighted by the shoulder of the low-field maximum and the splitting of the high-field minimum. This evidence indicates that the rotational motion along the three axis is different. Similar results are usually found for bilayered surfactant arrangements, typical of vesicles, in which the motion along the direction perpendicular to the aggregate surface is different from that along the other two axes.

Fig.5: EPR spectra of 5-DSA in CTAC and SDS aqueous solutions and in mixed surfactant solutions at total concentration 0.03 mol kg\(^{-1}\).
A quantitative analysis of 5-DSA spectra was realized determining the order parameter, $S$, and the hyperfine coupling constant, $\alpha'_N$ as reported in the literature [21]. $S$ is a measure of the local orientational ordering of the radical group with respect to the normal to the aggregate surface, while $\alpha'_N$ is an index of the micropolarity it experiences. The values collected in Table 4 clearly show the significant ordering increase in going from SDS to CTAC micelles. Moreover, cationic-rich vesicles are better microstructured than anionic-rich ones. It is also interesting to observe that among the different aggregates, SDS micelles present a more polar local environment, possibly related to the penetration of water molecules among the disordered surfactant tails.

<table>
<thead>
<tr>
<th></th>
<th>$S$</th>
<th>$\alpha'_N$ (G)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTAC</td>
<td>0.14 ± 0.02</td>
<td>13.6 ± 0.1</td>
</tr>
<tr>
<td>CTAC-SDS 70:30 mol/mol</td>
<td>0.30 ± 0.03</td>
<td>13.8 ± 0.2</td>
</tr>
<tr>
<td>CTAC-SDS 30:70 mol/mol</td>
<td>0.24 ± 0.02</td>
<td>13.4 ± 0.2</td>
</tr>
<tr>
<td>SDS</td>
<td>0.066 ± 0.005</td>
<td>15.3 ± 0.1</td>
</tr>
</tbody>
</table>

Tab.4: Order parameter and the hyperfine coupling constant values for CTAC, SDS and their mixtures at 0.03 mol kg$^{-1}$ total surfactant concentration.

5.2.2 t-RESV Solubilization by CTAC-SDS aqueous mixtures. The solubility of t-RESV in CTAC and SDS micelles, as well as in the vesicles formed in the surfactants
mixtures, was evaluated by UV-VIS spectroscopy. As an example, UV-Vis spectra of t-RESV in saturated CTAC solutions at different surfactant concentration, properly diluted after removal of excess solid, are reported in Figure 6. All the spectra show a broad band around 310 nm, given by the overlapping of two peaks and due to $\pi\rightarrow\pi^*$ transitions involving the benzene rings of the molecule [22]. Incidentally, these spectral features are typical of t-RESV [23] and assures that in analysed samples no isomerization to cis-resveratrol occurred. As the surfactant concentration is increased, a significant enhancement of the absorbance is observed, a reflection of the increased chromophore concentration in solution.

![UV-Vis spectra of t-RESV in different aqueous solutions of CTAC at different concentration, after dilution 1:30.](image)

**Fig.6:** UV-Vis spectra of t-RESV in different aqueous solutions of CTAC at different concentration, after dilution 1:30.
Similar results are obtained in the presence of SDS micelles and CTAC-SDS vesicles.

It is known, and has been also re-verified, that in water a maximum value of absorbance is obtained, less than those observed in surfactant mixtures, due to the limited solubility of this molecule in water [24].

Initially, the molar extinction coefficient in solutions of CTAC and SDS at surfactant concentration 0.03 mol kg\(^{-1}\) was determined, in order to calculate with high accuracy the concentration of t-RESV in the different surfactant aggregates. Therefore, a stock solution of trans-resveratrol in ethanol was added to CTAC or SDS solutions, and for each sample at a known concentration of t-RESV, and at constant concentration of the surfactant, the UV-Vis spectrum was recorded. The molar concentration of t-RESV in the different conditions was determined by UV spectroscopy applying the Lambert-Beer law and using the calculated molar extinction coefficients at 304 nm. By plotting the absorbance values at 304 nm as a function of t-RESV molar concentration, the \(\varepsilon\) values were determined (Table 5). As emerges from the table, the molar extinction coefficient values in CTAC and SDS solutions are quite similar to each other and to the known value in water [25]. However, in UV analyses the CTAC \(\varepsilon\) value was used for CTAC rich mixtures, and, similarly, the SDS \(\varepsilon\) value for SDS rich ones.
<table>
<thead>
<tr>
<th></th>
<th>ε(M⁻¹ cm⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTAC</td>
<td>27700± 1400</td>
</tr>
<tr>
<td>SDS</td>
<td>30500± 1500</td>
</tr>
<tr>
<td>Water</td>
<td>30335</td>
</tr>
</tbody>
</table>

Tab.5: Molar extinction coefficient of t-RESV in CTAC and SDS aqueous solutions at a surfactant concentration 0.03 mol kg⁻¹, as determined by UV measurements. The literature value in water is also reported for comparison.

Subsequently, with the aim to clearly evaluate t-RESV solubilisation by CTAC and SDS micelles, and by CTAC-SDS vesicles at 70:30 and 30:70 molar ratio, and to properly compare solubilisation by the different surfactant mixtures, the molar solubilisation capacity, χ was determined. This parameter is defined as the number of moles of t-RESV that can be solubilized by one mole of aggregated surfactants [14]. It can be calculated as:

\[
\chi = \frac{[t-RESV] - [t-RESV]_w}{[\text{surfactant}] - \text{cac}}
\]

where [t-RESV] represents the molar concentration of t-RESV in the mixture, [t-RESV]_w is the maximum solubility of t-RESV in water, [surfactant] is the concentration of the surfactant, which is CTAC, SDS or their mixtures, and cac is the corresponding critical aggregate concentration (for micelles the cmc has been considered) [12]. The values of χ parameter thus calculated are represented in Figure 7 and reported in Table 6.
Fig. 7: t-RESV solubility as function of surfactant concentration.

<table>
<thead>
<tr>
<th></th>
<th>X</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTAC</td>
<td>0.32 ± 0.02</td>
</tr>
<tr>
<td>CTAC-SDS 70:30</td>
<td>0.127 ± 0.005</td>
</tr>
<tr>
<td>CTAC-SDS 30:70</td>
<td>0.094 ± 0.003</td>
</tr>
<tr>
<td>SDS</td>
<td>0.052 ± 0.003</td>
</tr>
</tbody>
</table>

Tab. 6: Molar Solubilisation Capacity, χ, of t-RESV in SDS or CTAC Micelles and in CTAC-SDS Vesicles, as Determined by UV Spectroscopy.

It clearly emerges that t-RESV can be efficiently solubilized by surfactant aggregates and that it is more soluble in cationic micelles and cationic rich vesicles than in the
corresponding anionic aggregates. This could be due to a better hydrophobic interaction with the longer chain of CTAC with respect to SDS (16 vs 12 carbon atoms) or to a possible cation-pi interaction between the aromatic molecule and the cationic surfactant.

Finally, we verified that t-RESV experiences the more hydrophobic environment provided by the surfactant aggregates by fluorescence spectroscopy. Fluorescence spectra of t-RESV are reported in Figure 8. An intense band centred at 400 nm characterizes emission spectrum of t-RESV in water. A significant blue-shift is observed in all the samples containing surfactant aggregates, proving that in these conditions t-RESV is in an apolar environment, such as micelles or vesicles.

Fig.8: Comparison among fluorescence emission spectra of t-RESV in water, CTAC, SDS and their aqueous mixtures.
This result confirms that the t-RESV has a strong affinity for surfactant tails, so it can be inserted among them, both in CTAC and SDS micelles as well as in catanionic vesicles.

5.2.3 t-RESV radical scavenging activity. TEMPOL is a stable water-soluble nitroxide free radical characterized by a well-defined EPR spectrum consisting of three hyperfine lines due to the interaction of unpaired electron with the nitrogen nucleus [26,27]. The presence of antioxidant compounds reduces the intensity of the EPR spectrum of TEMPOL due to the scavenging reaction taking place between the radical and the antioxidant. This method has been already used to evaluate the scavenging activity of antioxidants contained in foods [28] and its suitability has been proved. Considering that the cationic aggregates are the best solubilizing agent for t-RESV, therefore the most suitable nano-vehicles for cosmeceutical formulations, the scavenging activity of t-RESV in CTAC micelles in presence of TEMPOL was evaluated.

In Figure 9 the EPR spectrum of TEMPOL, in the presence of t-RESV in water is reported as control. Furthermore, the spectra in CTAC at total concentration of 0.03 mol kg$^{-1}$ both in presence and absence of t-RESV, are shown. As it can be seen, t-RESV in water does not affect the signal intensity of TEMPOL whereas it is strongly reduced by t-RESV solubilized in CTAC micelles. This result confirms that the antioxidant capacity of t-RESV is not affected by the surfactants aggregates, rather it is strengthened. The
capability of trans-resveratrol as radical scavenger depends on its concentration. For this reason, its activity is better into CTAC aggregates where the trans-resveratrol concentration is higher than in water.

Fig.9: Effect of t-RESV on the EPR TEMPOL spectra obtained at room temperature. The spectra correspond to: a) CTAC micelles at a total surfactant concentration 0.03 mol Kg\(^{-1}\) (dotted black line); b) t-RESV in water (dotted blue line); c) t-RESV in CTAC micelles at a total surfactant concentration 0.03 mol Kg\(^{-1}\) (red line).
5.3 Conclusions

Overall, the obtained results show that t-RESV is better solubilized in CTAC and SDS micelles as well as in CTAC-SDS vesicles with respect to water, showing a preference for positively charged aggregates, as quantitatively assessed by UV and fluorescence spectroscopy. The increased solubility of t-RESV in these nano-aggregates allow to develop formulations for hair care without using other solubilizers, harmful substances for humans and environment often used in order to facilitate the addition of biologically active compounds. Furthermore, t-RESV exerts a potent antioxidant and radical-scavenging activity, as demonstrated by EPR measurements. In fact, its antioxidant capability is strengthened in presence of surfactant aggregates, in particular CTAC micelles, because a higher concentration of t-RESV solubilizes in these surfactant aggregates. These results definitely point to the possibility to design effective cosmeceutic formulations based on catanionic surfactant mixtures.
References


[15] Khan, A.M.; Shah, S.S.: Determination of critical micelle concentration (Cmc) of sodium dodecyl sulfate (SDS) and the


