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BY-PRODUCTS FOR PHOTOPROTECTION IN COSMETICS: A CASE STUDY FOR CHESTNUT SHELLS

BEATRIZ MOREIRA FRANCO
MASTER THESIS IN CHEMICAL ENGINEERING
PRESENTED TO THE FACULTY OF ENGINEERING
OF THE UNIVERSITY OF PORTO

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By-Products for Photoprotection in Cosmetics: A Case Study for Chestnut Shells

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Abstract

Over the last decade, evidence of the harmful effects of excessive ultraviolet (UV) radiation on the skin has accumulated. As a result, sunscreen is a well-established and highly recommended method for preventing sun damage to the skin. However, certain UV filters in commercial sunscreen products, have raised concerns about their safety and efficacy due to their low photostability and potential toxicity to humans and the environment. The research on natural products aims to develop novel, effective, and safer photoprotective agents for commercially available cosmetic products. On the other hand, the market for repurposing food industry by-products - such as chestnut shells (CSs) - to create high-value products with a focus on health and sustainability is growing. These agricultural and industrial wastes have been identified as sources of phenolic compounds with high bioactive potential. Additionally, research indicates that the desire for the perfect tan is the primary reason most people expose themselves to solar radiation for long periods, which research also suggests is harmful to the skin. Incorporating natural pigments derived from carotenoids into topical formulations may assist individuals in achieving their goals without endangering their health. Therefore, the primary aim of this dissertation was to assess the antioxidant and photoprotective properties of chestnut shell extracts in topical formulations and determine the efficacy of incorporating annatto seeds (ASs) pigment as a tan accelerator. Initially, two phenolic extracts of CSs were obtained, one from an original chestnut shell (OCS) sample and the other from a lyophilized chestnut shell (LCS) sample using solid-liquid extraction, with a Soxhlet apparatus, and ethanol as the solvent. The yields obtained for the OCS and LCS were 26.79 % and 26.12 %, respectively. Also, the pigments extract from ASs was extracted using a conventional solid-liquid extraction method with acetone, resulting in a yield of 6.39 %. Secondly, the antioxidant capacity (AC) was determined following the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay. Both phenolic extracts exhibited a 88.99 and 79.88 percentage of DPPH inhibition ($p < 0.05$), with IC_{50} values of 13.52 and 16.97 $\mu g \cdot mL\ DPPH^{-1}$ ($p > 0.05$). Furthermore, seven formulations of O/W creams were produced with minor changes in composition and quantity. The additives phenolic extract (natural UV filter) and annatto seed extract (natural pigments) were incorporated into the formulations, and BHT (synthetic antioxidant) and oxybenzone (synthetic UV filter) were used as positive controls in order to compare the antioxidant capacity and photoprotective properties. The stability of the formulations was determined over 21 days, and it was verified that they remained stable throughout the study period. Finally, the sun protection factor (SPF) of the formulations containing the phenolic extract or the synthetic UV filter was calculated and compared. The results highlight that CS extract may have some photoprotective properties and is an excellent source of antioxidants. However, since these were preliminary studies, additional research is recommended.

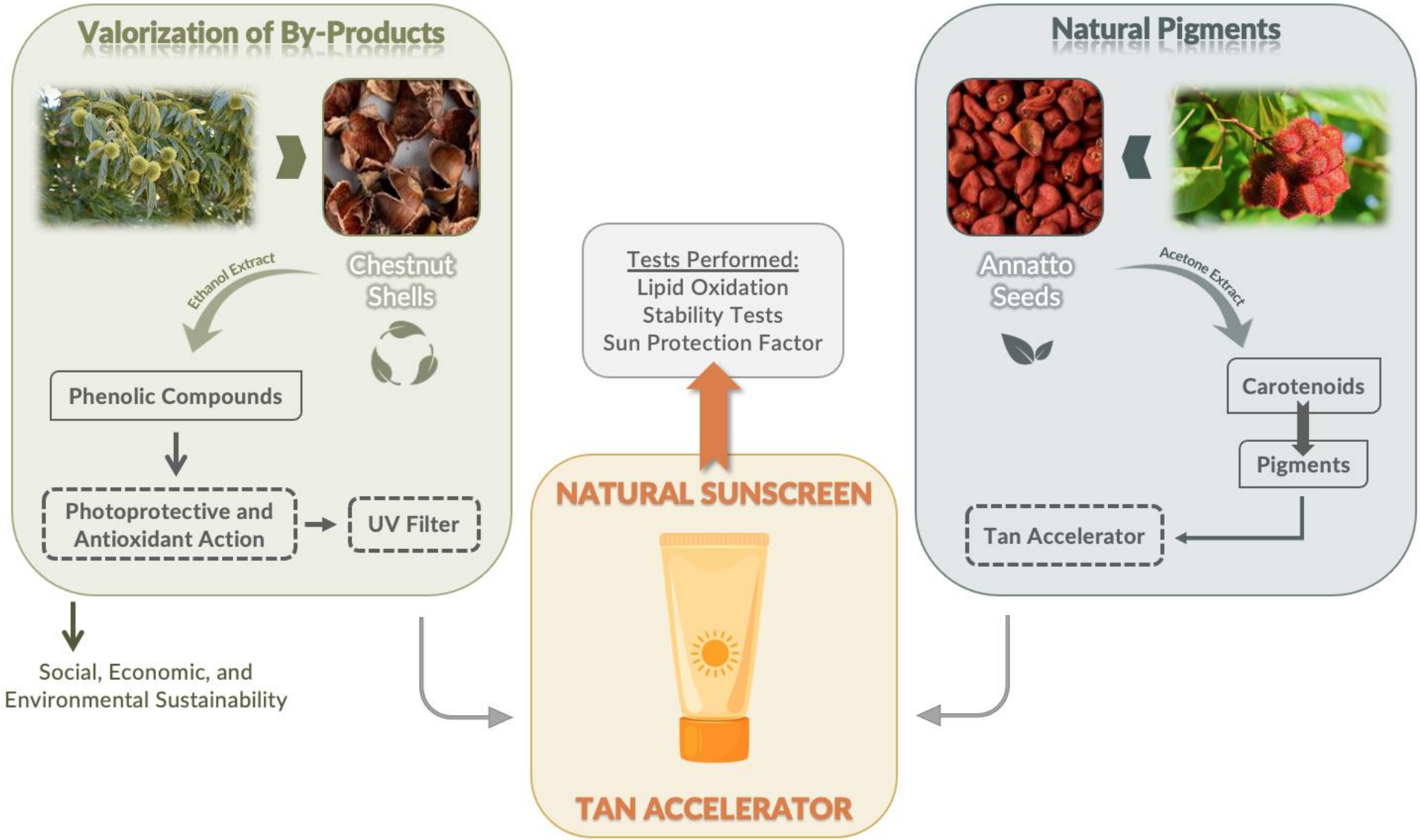
Keywords: By-products; Chestnut Shells; Photoprotection; Antioxidants; Sunscreen; Pigments

Resumo

Ao longo da última década, houve um crescimento da evidência científica quanto à nocividade da exposição excessiva à radiação ultravioleta (UV). Assim, o uso de protetor solar é um método já muito usado e recomendado para a prevenção dos danos na pele causados pelo sol. No entanto, alguns filtros UV presentes nos protetores solares comercializados têm sido questionados quanto à sua segurança e eficácia, devido à sua baixa fotoestabilidade e potencial toxicidade, tanto para o ser humano como para o ambiente. A pesquisa de produtos naturais visa ao desenvolvimento de agentes fotoprotetores, eficazes e seguros, que possam ser integrados em produtos cosméticos comercializados. Por outro lado, é inegável o crescimento do mercado de reaproveitamento de subprodutos alimentares – como cascas de castanha (*chestnut shells*, CSs) – para a criação de novos produtos, com foco na saúde e na sustentabilidade. Os desperdícios agrícolas e industriais foram identificados como fontes de compostos fenólicos com um forte potencial bioativo. Adicionalmente, há evidências que classificam o desejo de bronzear como a principal razão para a exposição voluntária da maioria da população à radiação solar por longos períodos temporais, atividade que, por sua vez, representa um perigo para a saúde da pele, de acordo com diversas investigações. A incorporação de pigmentos naturais derivados de carotenoides em formulações tópicas pode representar uma maneira de potenciar os resultados de bronze desejados, sem prejudicar a saúde. O principal objetivo desta dissertação foi a análise das propriedades antioxidantes e fotoprotetoras do extrato de casca de castanha (*Castanea Sativa Mill*) em formulações tópicas, assim como a determinação da eficácia da incorporação do pigmento de sementes de *Bixa orellana* (*annatto seeds*, ASs) como um acelerador de bronzeado. Inicialmente, obtiveram-se dois extratos fenólicos de casca de castanha – um de uma amostra de casca de castanha original (*original chestnut shell*, OSC), e outro de uma amostra de casca de castanha liofilizada (*lyophilized chestnut shell*, LCS) – através da extração sólido-líquido usado um extrator Soxhlet, sendo o solvente de extração o etanol puro. Os rendimentos de OCS e LCS obtidos foram, respetivamente, de 26,79 % e 26,12 %. A extração de pigmento das ASs foi realizada com recurso a uma técnica convencional de sólido-líquido com acetona, resultando num rendimento de 6,39 %. Em seguida, a capacidade antioxidante (*antioxidant capacity*, AC) foi determinada de acordo com o ensaio 2,2-diphenyl-1-picrylhydrazyl (DPPH). Ambos os extratos fenólicos apresentaram uma percentagem de inibição de DPPH de 88,99 e 79,88 % ($p < 0,05$), com valores IC_{50} de 13,52 e 16,97 $\mu g \cdot mL\ DPPH^{-1}$ ($p > 0,05$). Adicionalmente, foram produzidas sete emulsões do tipo óleo em água (O/W) sem alterações significativas à sua composição e quantidade. Os aditivos de extrato fenólico (filtro UV natural) e de extrato de semente de urucum (pigmento natural) foram incorporados nas formulações, e foi utilizado BHT (antioxidante sintético) e oxibenzona (filtro UV sintético) como controlos positivos para a comparação da capacidade antioxidante e das propriedades fotoprotetoras. A estabilidade das emulsões foi avaliada durante 21 dias, mantendo-se estáveis durante longo do período de estudo. Finalmente, calcularam-se e compararam-se os fatores de proteção solar (*sun protection factor*, SPF) das formulações contendo o extrato fenólico e o filtro UV sintético. Os resultados salientam a possibilidade do extrato de casca de castanha ter propriedades fotoprotetoras e de ser uma ótima fonte de antioxidantes. No entanto, estes estudos são ainda preliminares, pelo que é recomendada a realização de investigação adicional.

Palavras-chave: Subprodutos Agricolas; Casca de Castanha; Fotoproteção; Antioxidantes; Protetor Solar; Pigmentos

Graphic Abstract



Declaration

I, Beatriz Moreira Franco, hereby declare, under word of honour, that this work is original and that all non-original contributions are indicated and due reference is given to the author and source.

(Beatriz Moreira Franco)

Porto, May 2022

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Notation and Glossary

P	Pressure	Pa / mbar
s	Spreadability	cm
t	Time	s / min / hr / day
T	Temperature	ºC
d	Diameter	mesh
$\bar{x}$	Mean	

Greek Letters

μ	Viscosity	mPa·s
σ	Standard deviation	
λ	Wavelength	nm

List of Acronyms

ABTS	2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
AC	Antioxidant Capacity
AO	Antioxidant
AP	Annatto pigments
AS	Annatto seed
BAC	Bioactive compound
BHT	Butylated hydroxytoluene
CS	Chestnut shell
DNA	Deoxyribonucleic acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
FDA	US Food & Drug Administration
FRAP	Ferric Reducing Antioxidant Power
GRAS	Generally Recognized As Safe
HPLC	High-Performance Liquid Chromatography
IR	Infrared radiation
INCI	International Nomenclature Cosmetic Ingredient
LCS	Lyophilized chestnut shell
LO	Lipid oxidation
MAE	Microwave-assisted extraction
MDA	3 Malondialdehyde
MED	Minimal erythema dose
O/W	Oil-in-water
OCS	Original chestnut shell

OMC	Octyl methoxycinnamate
OTC	Over-the-counter
PC	Phenolic compound
PE	Phenolic extract
pH	Potential of hydrogen
PV	Peroxide value
ROS	Reactive oxygen species
SLE	Solid-liquid extraction
SPF	Sun protection factor
SWE	Subcritical water extraction
TBA	Thiobarbituric acid
TPC	Total phenolic content
UAE	Ultrasound-assisted extraction
UV	Ultraviolet
USAN	United States Adopted Name
VIS	Visible
W/O	Water-in-oil

1 Introduction

The skin is the body's largest and most exposed organ (Mitsui, 1997). It can reveal our age, way of life, origin, and state of health (Surber & Kottner, 2017). Nowadays, people are just as concerned with their appearance as they are with their health, making it difficult to disentangle the two concepts (Faria-Silva et al., 2020). The cosmetics and pharmaceutical industries offer a wide variety of skincare products designed to cleanse, repair, smooth, scent, beautify, protect, and treat our skin (Surber & Kottner, 2017). Body care cosmetics encompass a variety of items, including body creams and lotions, shower gels, perfumes, deodorants, sunscreens, toothpaste, and moisturizers. Additionally, Europe is considered to have the world's largest cosmetics market (COLIPA, 2017a, 2017b).

Furthermore, sunscreens are one of the most important categories in cosmetics since they provide protection against the sun, preventing the harm caused by solar radiation. While sunscreens are regulated as cosmetic items in Europe, they are regulated as over-the-counter (OTC) drugs in other regions, such as the United States, because they claim to prevent disease or skin injury (Engeler-Plischka, 2014).

The solar spectrum is measured in wavelengths and can be broken into three ranges: ultraviolet (UV), visible (VIS) and infrared radiation (IR) (Figure 1).

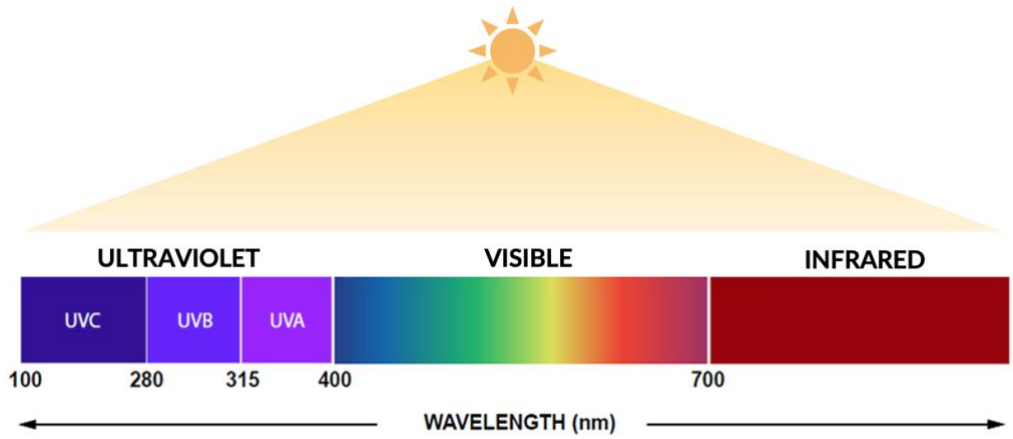

Figure 1- Solar Electromagnetic Spectrum (adapted from eyescreen, 2022).

Several studies have discovered numerous detrimental consequences of UV radiation on the skin, including ageing, wrinkles, sunburns, erythema, pigmentation issues, cutaneous degeneration, and various types of skin cancer (Donglikar & Deore, 2016). Moreover, exposure to sunlight also results in the production of free radicals. These molecules can cause damage to the body's cells, raising the chance of developing skin diseases. As a result, sunscreens containing antioxidants generate substantial interest due to their ability to neutralize free radicals (Lorigo & Cairrao, 2019; Ntohogian et al., 2018).

Ultraviolet radiation reaching the Earth's surface has grown dramatically in recent years as a result of the depletion of the ozone layer, which prevents UV radiation from entering the atmosphere (Gwak et al., 2021). As customers' first line of defence against the sun, this rise has elevated sunscreens to one of the most sought-after beauty items. Furthermore, UV radiation comprises three categories depending on the wavelengths, as illustrated in Figure 2 (Lorigo & Cairrao, 2019).

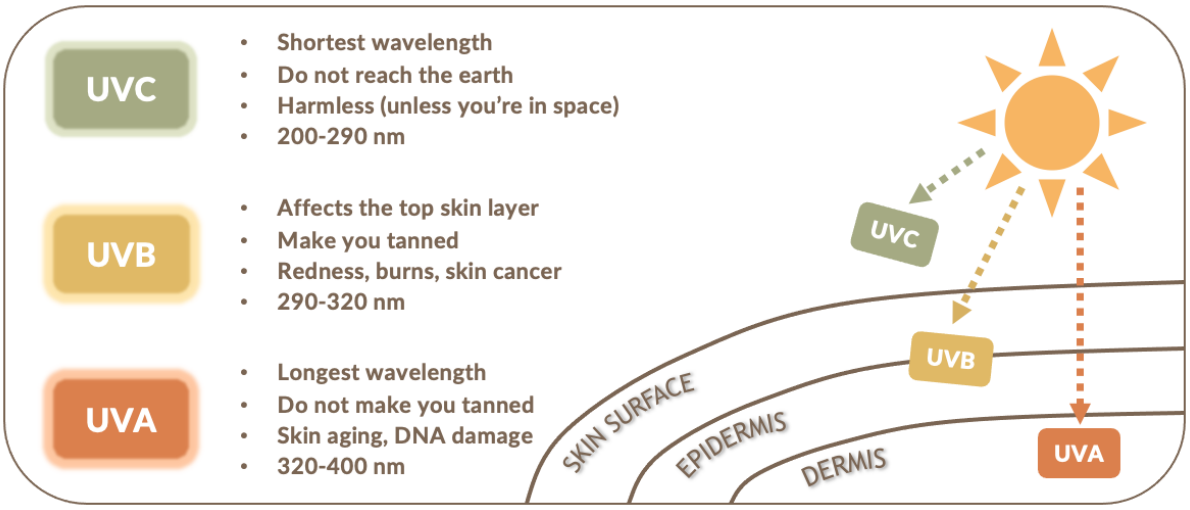

Figure 2- Types of UV radiation (adapted from Suntribe, n.d.).

Ideally, sunscreen formulations should contain UV filters that offer complete protection against both short-wavelength UVB radiation and long-wavelength UVA radiation (Ng'etich & Martincigh, 2021). As described in Figure 3, these sunscreens can be physical or chemical in nature, depending on the type of filter utilized (Gwak et al., 2021).

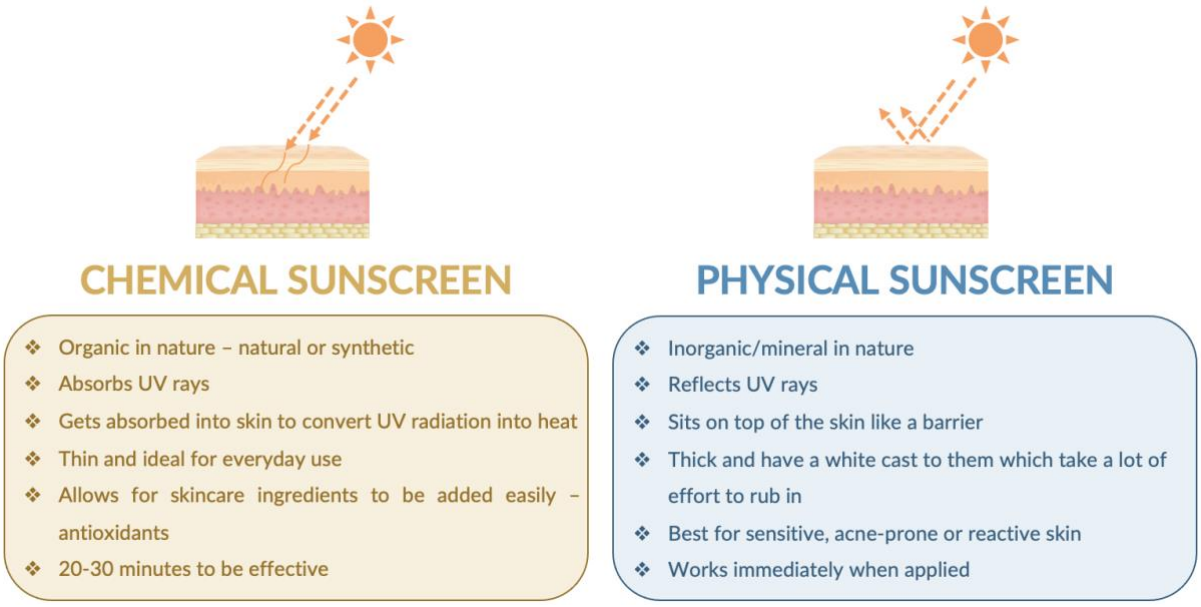

Figure 3- Chemical Sunscreen vs Physical Sunscreen (adapted from Shailaja, 2019).

The literature is full of information regarding the possible dangers UV filters in sunscreens may pose to human health and the environment (Ginzburg et al., 2021; Ramos et al., 2015). Moreover, sustainability is gaining popularity and is viewed as a necessary component of a brighter future because it emphasizes the development of new products without depleting natural resources. As a result, consumers prefer products that include in their constitution natural ingredients that efficiently block UVA and UVB radiation to provide the best possible skin protection. Consequently, the cosmetics industry is pursuing a more

sustainable business model to safeguard the natural environment, and human and ecological health (Korać & Khambholja, 2011; Ntohogian et al., 2018; Pinto, Cádiz-Gurrea, Garcia, et al., 2021).

Recently, new active ingredients for cosmetic purposes derived from agricultural and food by-products have been studied, not only to address pollution and environmental concerns but also to capitalize on bioeconomy concepts that seek to add value to food wastes, particularly fruits and vegetables. Due to the fact that the majority of these components are natural compounds with moisturizing, antibacterial, antioxidant, wound healing, anti-inflammatory, anti-cancer, anti-ageing, or cell rejuvenating properties, they are of great value in cosmetics (Donglikar & Deore, 2016).

Furthermore, Portugal is a major producer of olives, grapes, oranges, and tomatoes, and is a major driver of the fruit economy's development through the chestnut business. Chestnut shells (CSs) are an abundant and mostly underutilized by-product of the chestnut peeling process. The abundance of bioactive compounds (BACs) bolsters the case for shells to be valued as a source of natural antioxidants (Faria-Silva et al., 2020; Pinto, Cádiz-Gurrea, Garcia, et al., 2021).

Numerous studies have revealed that many people expose themselves to sunlight primarily to tan. Consumers are on the lookout for the ideal tan for various reasons. The primary goal is to look and feel better. Additionally, annatto seeds (AS) have sparked substantial scientific interest since evidence from ancient times indicates that Indians used the pigment produced by annatto seeds to paint and darken their skin for ornamental purposes, as well as to treat burns and as a sunscreen (Caldas et al., 2020). Thus, it is worth researching the possibility of developing natural sun protection solutions with tan-enhancing characteristics to satisfy consumer desire (Dennis et al., 2009; A. M. Rodrigues et al., 2017).

Therefore, the main goals of this study consisted in:

- Developing a sunscreen with a high percentage of natural ingredients and tan accelerator qualities
- Valorising agricultural by-products:
    - Chestnut Shell (CS) Extract – rich in phenolic compounds (PCs) with value-added properties
- Obtaining natural pigment from Annatto Seed (AS) Extract with tan accelerator properties
- Incorporating the by-product extracts in an oil-in-water (O/W) formulation, studying the antioxidant properties, photostability, and sun protection factor (SPF), and comparing with other formulations containing a synthetic antioxidant and UV filter, BHT and oxybenzone, respectively.
- Incorporating the AS extract into a sunscreen formulation and comparing its tanning impact following sun exposure.

1.1 Organization of the Dissertation

This dissertation is comprised of six chapters. The first chapter corresponds to the "Introduction," which includes a general framing of the issue and a study overview. The second chapter, "Context and State of the Art," introduces the subject, focusing on sunscreens and UV filters. It addresses the topic of agricultural

by-products, the antioxidant capacity (AC) and photoprotection properties of the extracts, and the legislation governing their use as UV filters in formulations. Additionally, the use of pigments extracted from annatto seeds is explored. The third chapter, "Materials and Methods," offers information about the materials and equipment utilized in the project, as well as the processes followed. Chapter four, "Results and Discussion," summarizes the findings and discusses them, while also assessing potential problems and challenges encountered throughout the course of the work. The fifth chapter discusses the dissertation's conclusions, while the sixth chapter discusses the dissertation's limits and ideas for further work. Finally, it includes the "References" and "Appendixes" sections, which contain supplementary information on certain contents.

2 Context and State of the Art

2.1 Sunscreens

There is no exact knowledge of when humans began protecting their skin from the sun. Our dark-skinned ancestors in Africa benefited from natural melanin, which protected them from sunburn. As humans migrated to cooler climates, they clothed themselves to avoid frostbite, eradicating their natural skin pigmentation that functioned as sunscreen. While ancient cultures were likely unaware of the science behind UV rays and sun damage, the sunburns and tans they obtained as a result of sunshine exposure were most likely a cause for concern. According to some sources, Egyptians, Ancient Greeks, and Native Americans attempted to develop some form of sun protection by combining oils, extracts, and spices to create pastes that would allow them to work and play during daytime hours (Aldahan et al., 2015; Svarc, 2015).

Following World War I, fashion called for tanned bodies, which were associated with excellent health. As a result, oils became available as cosmetics to "protect" the body, but lacked significant UV protection. In 1935, the first radiation filtering product was introduced, followed by many UV-protective items. However, the majority of these products provided protection only from UVB radiation, as UVA radiation was previously believed to be harmless. At the turn of the twenty-first century, it became obvious that UVA radiation was dangerous, so broad-spectrum (UVA/UVB) sunscreens began to be commercialized. In 1988, a statement was issued claiming that there is no safe way to tan. This marked the beginning of the shift of tanning from fashionable to a health hazard (Svarc, 2015).

Sunscreens and conceptions of sun protection have evolved throughout time, and we now have a greater understanding of how radiation affects the skin. Nonetheless, there are discrepancies in the set of UV filters permitted for use in various parts of the world, as well as in the testing procedures and scales used to define the various UVB/UVA protection balancing requirements. Moreover, many of the ingredients used today were developed decades ago and may be harmful to our health (Aldahan et al., 2015; Svarc, 2015).

Despite increased awareness of the risk of skin cancer and sunburns, tanning did not become unfashionable. Numerous studies have demonstrated that a strong desire to tan is the primary reason people expose themselves to the sun rather than finding cover and wearing protective gear, and hence sunscreens have been identified as the first line of defence against the sun. Nevertheless, some people reduce their sunscreen use if they believe their risk of sunburn is reduced and their skin has begun to tan. Avoiding sunburns by striking a balance between sun protection and tanning appears to be their method. Therefore, there is a potential need to address the priority placed on a tanned appearance, as individuals may risk jeopardizing their health in pursuit of the perfect tan (Dennis et al., 2009; A. M. Rodrigues et al., 2017).

Suncare products are unquestionably essential to mitigate the detrimental effects of solar radiation, and as such, their efficacy and safety are critical for both human health and the environment (Romanhole et al., 2020).

The restricted number of UV filters available as active components in sunblock is problematic, especially given the general perception of sunscreen safety (Ginzburg et al., 2021; Ramos et al., 2015). Thus, for ultimate skin protection, novel concepts must develop that effectively block the UV rays without causing adverse consequences to humans while still allowing them to tan (Faria-Silva et al., 2020; Ntohogian et al., 2018).

A significant percentage of the world's population has grown increasingly fond of cosmetics containing naturally occurring ingredients that perform active tasks on the skin and would pay extra for a cosmetic that offers additional skin advantages. Such treatments may employ pigments and antioxidants, with the former acting as a micromirror in the human skin, reflecting sunlight in the UV spectral regions. These naturally occurring sunscreen compounds with pigment and antioxidant (AO) characteristics may be derived from plant extracts or food by-products and are being investigated to create healthier and more environmentally friendly cosmetics (Faria-Silva et al., 2020; Ntohogian et al., 2018).

2.1.1 Commercially Available Formulations

Numerous sunscreen formulations are available on the market; some are more effective and safer than others. They are geared toward meeting the unique demands of various parts of the population since the best vehicle is determined by skin type, age, personal preferences, and filter type (organic or inorganic). The most frequently used formulations are depicted in Figure 4 (Korać & Khambholja, 2011; Romanhole et al., 2020).

Figure 4- Commercially available sunscreen formulations.

Emulsions are the optimal vehicle for sunscreens and have been the world's most popular type of protection. They are available in oil-in-water (O/W) or water-in-oil (W/O) formulations. Consumers prefer O/W products because they are easy to use, dry quickly, and are non-occlusive. Additionally, organic filters and inorganics may be present. The disadvantage is that they deteriorate when exposed to water, necessitating frequent reapplication (Romanhole et al., 2020).

Along with finding the appropriate formula, selecting products with suitable ingredients can significantly increase their effectiveness. As a result, incorporating natural ingredients in sunscreen products may be optimal. Consumers are in demand of an all-in-one sunscreen product that is non-toxic, non-allergic, moisturizing, antioxidant, tan activator, and protects against both UVA and UVB rays with a high SPF number. In Figure 5, the typical components of an O/W sunscreen formulation are depicted, along with their functions (Donglikar & Deore, 2016).

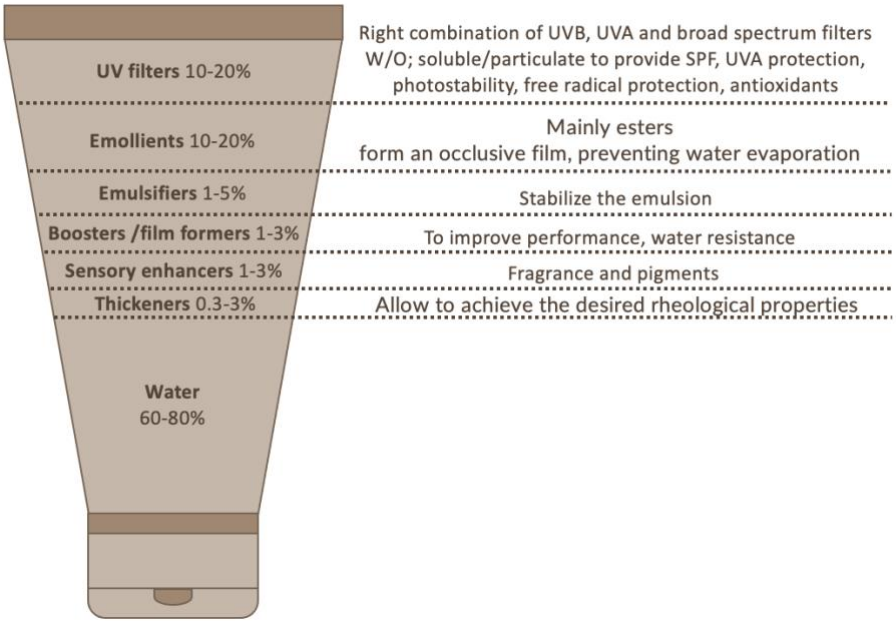

Figure 5- Typical components of an O/W sunscreen formulation (adapted from CareCreations, n.d.).

2.1.1.1 UV Filters

UV filters can be classified into two groups according to their nature: inorganic filters and organic filters. Zinc oxide (ZnO) and titanium dioxide (TiO2) are inorganic filters that offer broad-spectrum protection from UVA and UVB rays. They are soft enough for daily use because they rarely irritate the skin and are being touted as safer alternatives to organic filters. However, due to the scattering effect, they frequently generate a so-called "whitening" effect when applied to the skin, which has a detrimental effect on the aesthetics and efficacy of sunscreen lotions. Furthermore, while inorganic filters are considered to be a safer option than organic filters, there is evidence that UV irradiation of metal oxides in these products can result in hazards. Studies have confirmed that the generation of reactive oxygen species (ROS) and degradation of organic compounds can occur and that there is no data to support their long-term safety (Ginzburg et al., 2021; Korać & Khambholja, 2011). According to Romanhole et al., 2020, ZnO can have a negative effect on the marine environment by causing coral bleaching and destruction. Additionally, Ginzburg et al., 2021 claims that ZnO particles may contribute to sunscreen toxicity in hitherto unknown ways.

Photochemistry can occur in formulations including both ZnO and organic UV filters, resulting in a considerable reduction in UVA protection due to organic filter breakdown. Additionally, they can generate toxicity-inducing photodegradation products (Ginzburg et al., 2021).

The majority of organic sunscreens are made of many synthetic compounds, each of which blocks a particular wavelength of UV light, as the majority of them do not filter the entire UV spectrum. Furthermore, the safety of many synthetic chemical filters often employed in sunscreen lotions has not been verified, as has their influence on the environment (Huang et al., 2021; Korać & Khambholja, 2011).

Generally, the synthetic compounds employed in sunscreens are active in the UVB region, as only a few inhibit UVA. The best UV filter combines both organic and inorganic active ingredients. Combinations

of avobenzone and inorganic filters are not approved in the United States of America due to reports that avobenzone is unstable when combined with inorganic filters, owing to photodegradation (Korać & Khambholja, 2011).

The bulk of sunscreen products contains octyl methoxycinnamate (OMC), an organic UVB filter. According to studies, this compound has endocrine disruptive qualities in humans that are capable of causing DNA damage due to the photo instability of UVB filters and is a threat to coral reefs. As a result, the safety of this organic filter is a significant worry for human health (Ginzburg et al., 2021; Lorigo & Cairrao, 2019).

The state of Hawaii banned the sale and distribution of sunscreen formulas containing oxybenzone or octinoxate because of the potential danger to coral reefs, while the European Union cut the permitted concentration of oxybenzone in cosmetics from 10 % to 6 % in 2017 (Ginzburg et al., 2021; Huang et al., 2021; Romanhole et al., 2020).

According to the author Donglikar & Deore, 2016, with exposure to sunshine, many sunscreen ingredients degrade and lose their photoprotective qualities, reducing the product's efficacy. Additionally, sunscreens must remain on the epidermis's surface to ensure efficacy and provide the best protection against UVA/UVB rays. Recent research has revealed that specific UV filters in certain formulations may be absorbed through the skin, affecting their effectiveness and safety and posing a risk to consumers once they reach the systemic circulation. This absorbance may have various detrimental effects including the induction of apoptosis and a rise in mutagenesis or estrogenic activity, resulting in the rapid proliferation of cancer cells (Klimová et al., 2013; Romanhole et al., 2020).

The most frequently used synthetic organic UV filters are portrayed in Figure 6.

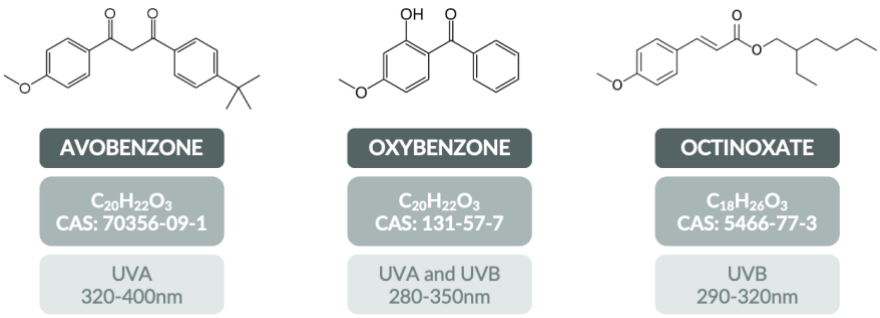

Figure 6- Most common Synthetic Organic UV Filters (adapted from Technologies, 2020).

Over the last few years, the sunscreen industry has concentrated on developing new products containing compounds of natural origin, such as polyphenols and carotenoids, as studies have revealed they are more effective than synthetic compounds in the long run, particularly against free radical-induced skin damage, in addition to their increased UV assimilation and AC (Donglikar & Deore, 2016; He et al., 2021).

2.2 Antioxidants

The main destructors of skin are oxygenated molecules, most commonly referred to as free radicals. Antioxidants are frequently employed in the beauty industry to encourage the skin's natural ability to heal

and rebuild itself, as the human body is continually subjected to oxidizing agents such as UV radiation and environmental toxins. Since antioxidants are substances capable of neutralizing UV-induced free radicals, their usage as UV filter stabilizers represents a novel photoprotective technique. Additionally, plants include a variety of additional compounds that are beneficial for skincare (Korać & Khambholja, 2011; Lorigo & Cairrao, 2019).

Despite the widespread use of synthetic antioxidants such as BHT (butylated hydroxytoluene) in cosmetic formulations, there is rising concern about generating sustainable cosmetics. Thus, natural AOs derived from plants, fruits, and even by-products have been studied for their antioxidant activity in recent years in an attempt to develop effective topical photoprotective treatments. As a result, natural antioxidants are increasingly being included in commercial skincare products, and plant phenolics are being considered for their potential to protect the skin from the damaging effects of UV radiation (S. Ferreira, 2021; Korać & Khambholja, 2011).

2.2.1 Phenolic Compounds

Phenolic compounds are used in cosmetics due to their antioxidant, antibacterial, and anti-ageing properties. Additionally, PCs are the primary class of secondary metabolites found in plants, and their ability to scavenge free radicals are a result of their high AO properties (Khatri et al., 2019; Minatel, 2017). Due to their natural origin and low toxicity, PCs are a viable tool for preventing and treating the causes and effects of skin ageing, skin disorders, and skin damage caused by UV radiation, including wounds and burns (Działo et al., 2016; Fernandes et al., 2021). The amount of PCs found in plants varies according to various circumstances, including cultivation technique, growing conditions, harvesting, processing, and storage. The main properties of phenolic compounds are illustrated in Figure 7.

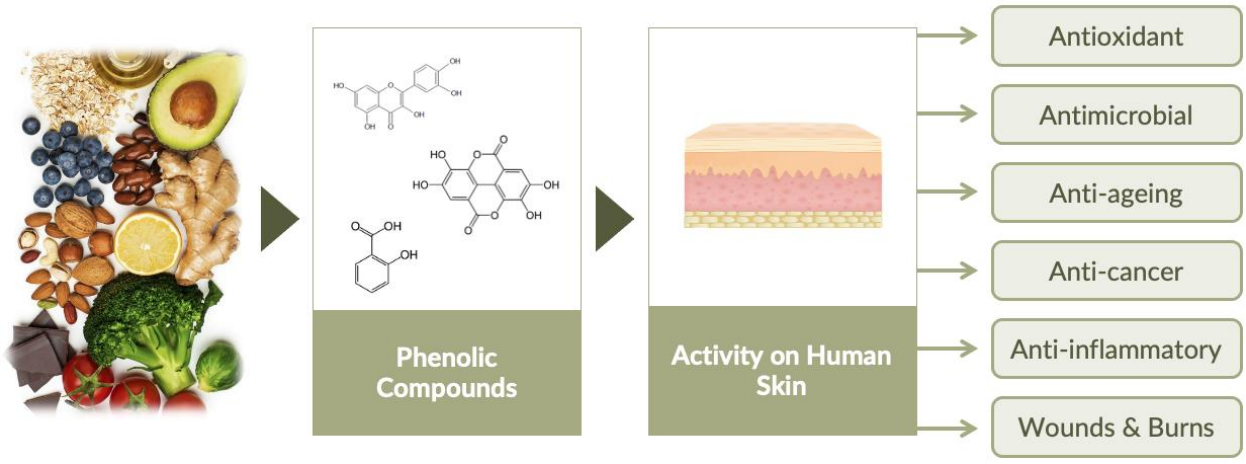

Figure 7- Natural sources and properties of phenolic compounds.

Phenolic compounds are a varied collection of compounds defined by the presence of a hydroxyl group (-OH) attached to one or more aromatic rings (phenolic acids or polyphenols). These substances are members of a broad and diversified class of chemical compounds that are commonly classified into two

groups: simple phenols and polyphenols. The classification of the various PCs classes is depicted in Figure 8 (Khatri et al., 2019; Minatel, 2017).

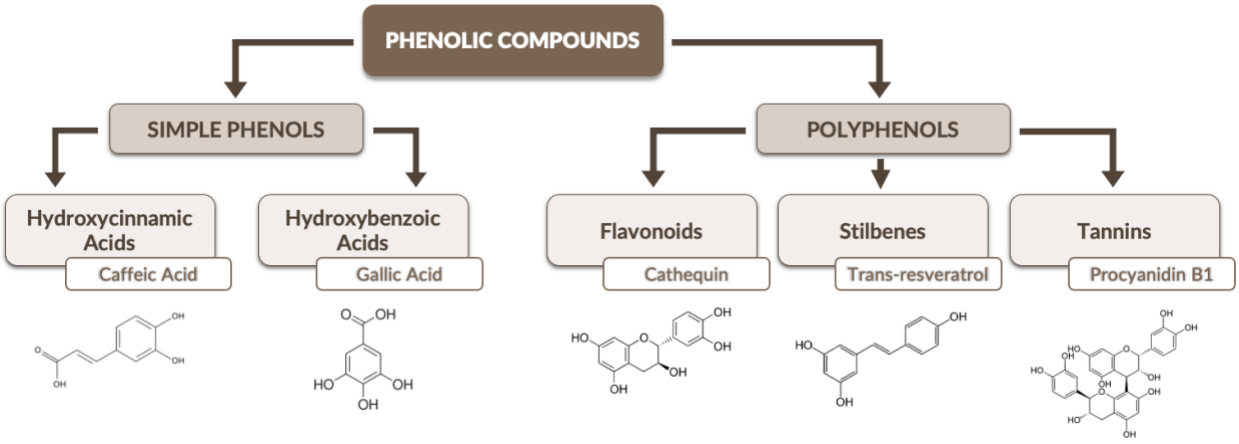

Figure 8- Schematic classification of phenolic compounds (adapted from S. M. Ferreira & Santos, 2022*).*

Flavonoids are the most abundant and diversified class of PCs found in plants. The other phenolic compounds typically found in plants are tannins, which can be found in two forms: hydrolysable tannins (produced during the phenolic acid pathway via sugar polymerization) and condensed tannins (combination of flavonoids) (Działo et al., 2016).

Phenolic acids are used in cosmetics due to their ability to combat oxidative stress, which accelerates cell ageing. Additionally, phenolic compounds such as resveratrol and quercetin can protect DNA from harm by reducing free radical production. Furthermore, plant extracts rich in PCs have a better capacity for UV absorption and antioxidant activity than synthetic products and can be used as organic UV filters.

It is critical to determine whether natural extracts have photoprotective properties during exposure to UV radiation before being used as UV filters in sunscreen compositions (Fernandes et al., 2021; He et al., 2021). Thus, there is an opportunity to study the antioxidant properties of the phenolic compounds present in chestnut shells and potentially incorporate them into sunscreens, thereby increasing their commercial value (Korać & Khambholja, 2011).

2.3 Colourants

The most common colourants are dyes and pigments (Figure 9), which are used to add or change the colour of something. They are widely utilized in various sectors, including textiles, medicines, food, cosmetics, plastics, paints, inks, photography, and paper. Dyes are coloured compounds that are soluble or dissolve in water during the application process and impart colour by selective light absorption. Pigments are coloured, colourless, or fluorescent, finely split organic or inorganic solids that are often insoluble and mostly unaffected chemically by the vehicle or medium in which they are introduced. In contrast to dyes, which are nearly entirely defined by their chemical structure, pigments' colouristic features are also

determined by the physical characteristics of their particles. Additionally, pigments act by scattering radiation of a particular wavelength (Gürses et al., 2016, 2021).

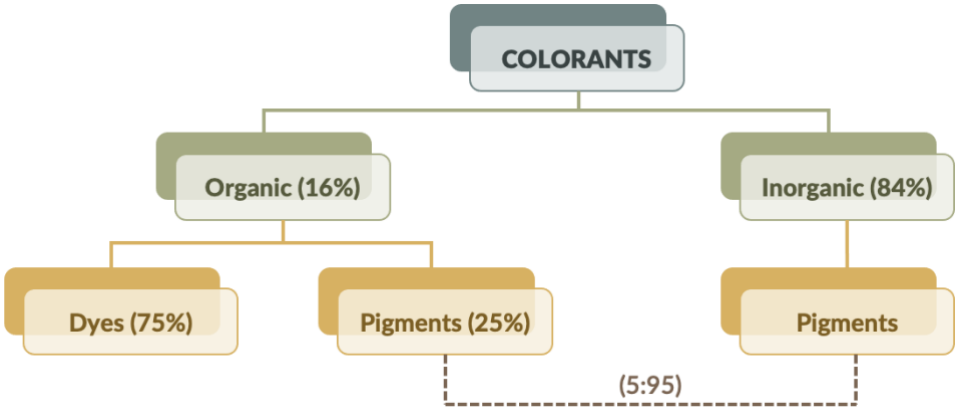

Figure 9- Most essential colourants.

Increased awareness of the health risks and toxicity associated with synthetic colourants encourages several sectors to incorporate natural colourants into an expanding variety of processed products. Natural dyes and pigments are gaining popularity due to the valuable characteristics associated with some of these colourants. While synthetic dyes and pigments have lower production costs and greater stability, the number of synthetic additives permitted in developed countries continues to decline each year, increasing the use of AO colourants such as carotenoids and anthocyanins by various industries (Santos et al., 2011). Natural pigments are found in plants, seeds, fruits, bark, and roots that include various colours responsible for light absorption. The colour appearance of fruits and vegetables is determined by a few basic essential components such as flavonoid, chlorophyll, and carotenoid (Sunder Sharma et al., 2021).

2.3.1 Carotenoids

Carotenoids are among the most abundant natural pigments, with over 600 distinct compounds identified to date, the most abundant being β-carotene (Stahl & Sies, 2004). Carotenoids are widely used in the food, pharmaceutical and cosmetic industries and are typically found in fruits, flowers, petals, and microbes. They range in hue from red to yellow to orange (Santos et al., 2011; Sunder Sharma et al., 2021).

Carotenoids have gained significant attention in recent years from researchers and the general public due to their potential health-promoting properties. In general, two mechanisms exist for carotenoid accumulation: systemic administration via carotenoid-rich food or supplementation and topical application of AOs via creams and lotions (Darvin et al., 2011). They are primarily located in the skin's epidermis and act as a barrier against several environmental stresses. Human experiments have demonstrated that a carotenoid-rich diet can help prevent and improve skin ageing (Balić & Mokos, 2019).

Furthermore, three pigments contribute to the skin's colour: haemoglobin, carotenoid, and melanin (Pezdirc et al., 2018). Significant yellowing of the skin is typically observed in carotenoid-rich dietary intervention trials due to carotenoid pigment build-up in the skin (Alaluf et al., 2002).

2.3.2 Annatto (Bixa Orellana L.)

Annatto is the fruit of urucuzeiro, a South American native plant cultivated in Asia and Africa. Although this seed is grown in various tropical nations, Peru and Brazil are the leading producers. Bixin is the primary carotenoid pigment, accounting for 70-80 % of the seed coat (Pattanaik et al., 2018). It is a red-orange carotenoid found exclusively in this plant and has considerable socio-economic potential. Additionally, Bixin is a widely utilized compound in the textile, pharmaceutical, cosmetic, and food industries (Santos et al., 2011).

Germano et al., 1997 state that annatto pigments (AP) are cutaneously tolerable, as evidenced by the absence of skin irritation and sensitization in treated rabbits. The authors investigation concluded that topical application of AP was safer than oral consumption of meals dyed with annatto seeds, which can result in hypersensitive reactions in some cases. Additionally, the authors claim that histochemical investigations revealed a low degree of skin penetration, implying that the use of AP in cosmetic products may be encouraged to battle skin infections or for aesthetic purposes (Germano et al., 1997).

According to the Scopus® Database, around 192 papers have been published in the last decade with the term «annatto» in the title. When the search is expanded to include the article title, abstract, and keywords, 400 papers are returned. Additionally, when the term «colourant», «dye», or «pigment» is included in the search, there are «63», «112», and «72» publications published, respectively (this information was gathered in May 2021.) (Brudzyńska et al., 2021). As a result, there is interest in investigating the idea of employing annatto pigments as a tan accelerator in a sunscreen composition.

2.4 By-Products

Nowadays, consumers are concerned not only with their physical appearance and well-being but also with their ecological footprint. Furthermore, “Green Beauty” is the new approach to consumers who desire green cosmetics with natural and organic ingredients without synthetic preservatives, pigments, silicones or fragrances. In recent years, studies have explored food-derived components to obtain “healthier” cosmetics since there is a belief that we are surrounded by food capable of having effects on the human body, including the skin. For example, fruits and vegetables have many variable antioxidants that create the opportunity to substitute synthetic compounds currently in use in the cosmetic industry (Faria-Silva et al., 2020).

Moreover, due to the recommendation of the circular economy implementation, an approach to economic growth that is in line with sustainable environmental and economic development, a large number of food industry wastes are being recovered as value-added products to be incorporated into cosmetic products. This has become a significant trend in the beauty industry since these agro-industrial residues represent a sustainable and rich source of several BACs (Faria-Silva et al., 2020). The circular economy of chestnuts is illustrated in Figure 10.

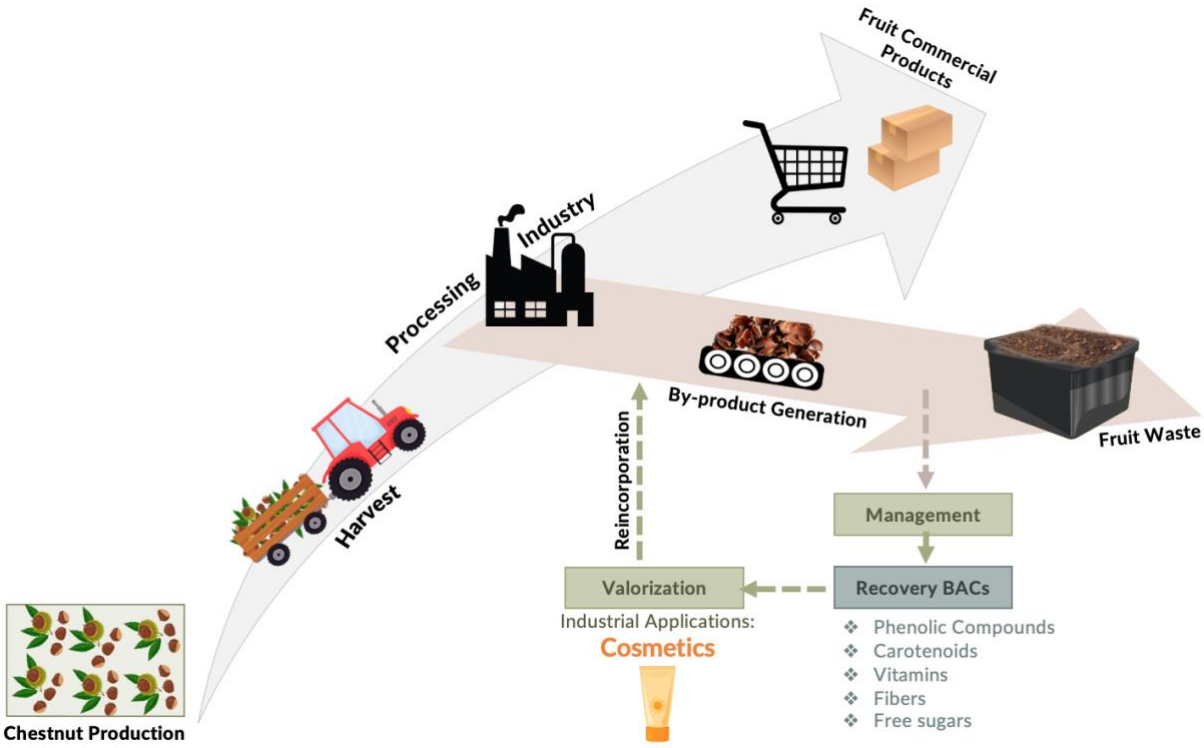

Figure 10- Circular Economy of Chestnuts.

The re-use of industrial by-products for high value-added products in the context of health and well-being is a growing market that promises to improve our general health and ecological recycling issues. However, these ingredients need to undergo several tests to prove their safety. Moreover, the BACs content of natural products varies significantly depending on the harvest time and storage conditions. This fact directly affects the quantity and quality of food-derived compounds and thus the effect of cosmetic products (Faria-Silva et al., 2020).

Based on these principles, a growing body of literature has analysed the potential use of by-products from different food industries in cosmetic products, usually considering each country's natural and traditional foods (Pinto, Cádiz-Gurrea, Garcia, et al., 2021). F. Rodrigues, Pimentel, et al., 2015 found that the olive oil industry generates large amounts of olive by-products that are extremely rich in bioactive compounds such as antioxidants, fatty acids and minerals. Therefore, they can become a source of possible active ingredients for cosmetic products with different claims, such as anti-ageing or hydration. Thus, it is mandatory to potentiate their re-use and valorisation in the cosmetic field (F. Rodrigues, Pimentel, et al., 2015). Furthermore, other studies demonstrate that the phospholipid complex of orange peel and liquorice extracts exhibited good potential as an anti-ageing cosmeceutical and can either be used alone or as an additive to an anti-ageing formulation. Also, orange peel cold-pressed oil can be used as a food-derived functional raw material for skin protection as it effectively inhibits the skin collagen degeneration in mouse skin, thus protecting the skin from UVB-induced photoaging (Damle & Mallya, 2016; Li et al., 2020). Pinto, Cádiz-Gurrea, Garcia, et al., 2021 emphasize the skin effects and safety of chestnut shell extract and their potential to be incorporated into cosmetic formulations as anti-ageing ingredients (Pinto, Cádiz-Gurrea, Garcia, et al., 2021).

2.4.1 Chestnut Shells

The European chestnut (*Castanea sativa* Mill.) belongs to *Fagaceae* family and the genus *Castanea* (Costa, 2018b). This tree can be explored for various purposes; however, chestnuts are mainly cultivated for fruit production in Portugal. According to 2010 data, China is the world's top chestnut grower, accounting for more than 80 % of global production. Portugal has the fifth-largest harvest area and is the seventh-largest producer of chestnuts, accounting for approximately 1 % of total production (Costa, 2018a).

According to accessible information and productivity data from the sector's key associations, the production of chestnuts in Portugal was approximately 34 000 tons per year in 2018. Additionally, most chestnut tree areas are concentrated in the vicinity of Bragança, Vila Real, and Viseu (Instituto Nacional de Estatística, 2019). Figure 11 depicts the distribution map of chestnut trees in Portugal.

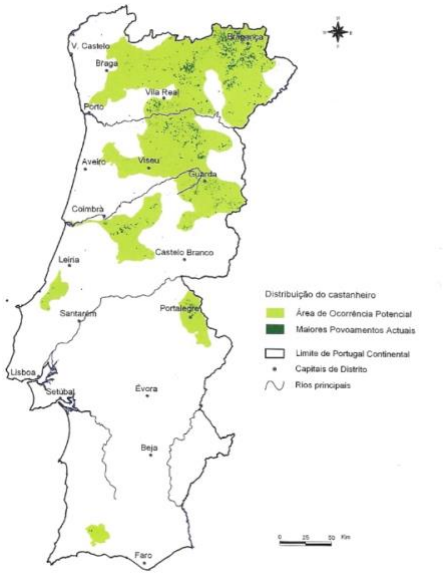

Figure 11- The distribution card of chestnut trees in Portugal (Capelo & Catry, 2007).

Large quantities of by-products, primarily shells, are produced during chestnut processing. Chestnut shells are an abundant and underexploited by-product of the chestnut peeling process, mostly reused as fuel. The shell alone accounts for around 20 % of the overall weight of the chestnut. Several publications have described the richness of chestnut shell extract in phenolic compounds, mainly phenolic acids (specifically gallic and ellagic acids), flavonoids (rutin, quercetin and apigenin) and tannins (condensed and hydrolysable) (Cacciola et al., 2019; M. C. B. M. De Vasconcelos et al., 2010; M. do C. B. M. de Vasconcelos et al., 2010; Gullón et al., 2018; Lameirão et al., 2020; Pinto, Vieira, Peixoto, Freire, et al., 2021; F. Rodrigues, Santos, et al., 2015; Silva et al., 2020). The abundance of BACs bolsters the value of shells as a natural source of antioxidants, highlighting the potential benefits of producing high-value-added products with appealing aesthetic uses in the cosmetic industry. The use of this by-product is likely to increase earnings, reduce pollution costs, and enhance social, economic, and environmental sustainability (Braga, 2014; Pinto, Braga, et al., 2020).

2.5 Extraction of Phenolic Compounds

The phenolic compounds found in plants exhibit a significant degree of structural diversity, making it almost impossible to find an extraction process suitable for all of these compounds. Numerous researches are now being conducted to examine the various present approaches for PCs extraction. The primary approach used is solid-liquid extraction (SLE), which, while providing excellent yields, has certain drawbacks, including excessive solvent usage. Recent advancements in extraction techniques, on the other hand, have emphasized the importance of creating solutions that safeguard the environment and consumers while yet enabling the economically viable production of high-quality extracts. Thus, the adoption of "cleaner" and "greener" extraction techniques such as supercritical extraction and ultrasound-assisted extraction becomes ideal (S. Ferreira, 2021). Table 1 outlines the major advantages and downsides of each of these techniques.

Table 1- The advantages and disadvantages of various extraction methods.

Extraction Method	Advantages	Disadvantages
Solid-Liquid Extraction	- Widely used method; - Simple procedure; - High yields extraction.	- Possibility of extraction of undesirable compounds; - Not suitable for thermosensitive compounds.
Ultrasound-Assisted Extraction	- Fast process; - High-efficiency method; - Suitable for thermosensitive compounds.	- Non-uniform wave distribution; - Decreased potency with time.
Supercritical Fluid Extraction	- Non-toxic solvent; - Reduced extraction time; - Suitable for thermosensitive compounds.	- High equipment costs; - High operating costs.
Microwave-Assisted Extraction	- Simple method; - Reduced extraction time; - Lower solvent consumption.	- High equipment costs.
Pressurized Liquid Extraction	- Fast technique; - Low solvent consumption; - Easy to automate.	- Low selectivity; - High equipment cost.
Enzyme-Assisted Extraction	- High extraction yields; - Eco-friendly technique, as it does not require solvents.	- Enzyme performance is easily affected by pH and temperature.

Source: S. Ferreira, 2021; Pinto, Cádiz-Gurrea, Vallverdú-Queralt, et al., 2021.

2.5.1 Solid-Liquid Extraction

For several decades, conventional extraction procedures (e.g., Soxhlet extraction) were the primary method for separating target compounds from natural sources. Conventional extraction, sometimes called solid-liquid extraction, involves direct contact between the solvent and the solid matrix in order to recover soluble compounds. Phenolic compounds' solubility is highly impacted by their chemical structure and the solvents' polarity. Additionally, experimental parameters like temperature, duration, solid/liquid ratio, and sample particle size should be carefully chosen to optimize target molecule recovery. Most research on chestnut shells has relied on organic solvents, elevated temperatures, and extended extraction times to obtain PCs via conventional extraction methods (Pinto, Cádiz-Gurrea, Vallverdú-Queralt, et al., 2021).

Selecting the solvent for extraction is a critical step, as it must address a number of factors, including solubility, selectivity, cost, safety, and sustainability - a factor that is becoming increasingly important in the modern era. Due to the polar character of these molecules, alcoholic solvents are quite effective for the extraction of phenolic compounds. Thus, ethanol is an appropriate solvent for the extraction of PCs since it is an FDA (US Food & Drug Administration) approved GRAS (Generally Recognized As Safe) solvent, which allows for its safe use in the cosmetic sector. Additionally, its low cost, biodegradability, and low toxicity make it a widely used solvent (S. Ferreira, 2021).

Furthermore, temperature and time of extraction are critical elements. On the one hand, increasing temperature facilitates extraction by increasing the solute's solubility and diffusion coefficient. On the other hand, when the temperature increases, the surface tension and viscosity of the solvent drop, allowing it to penetrate deeper into the solid matrix's interior layers. In terms of particle size, it appears as though the extraction produces more accurate findings with smaller particles. Indeed, when the particle size reduces, the surface area increases, increasing the time the solvent has in contact with the matrix and therefore improving extraction (S. Ferreira, 2021).

Solid-liquid extraction with a Soxhlet apparatus is a technology that is frequently used to extract PCs from by-products. The extraction procedure is straightforward; the sample is placed in a paper cartridge within the extractor, and the solvent is evaporated and condensed until it comes into contact with the solid component. The process is repeated until the extraction is complete. Soxhlet extraction is a simple procedure with a high degree of consistency and efficiency (S. Ferreira, 2021).

Apart from the extraction technique and solvent used, chestnut shells' varieties and geographical origins may significantly affect the extract's properties. For example, F. Rodrigues, Santos, et al., 2015 reported that the hydroalcoholic extract of Trás-os-Montes shells had the highest antioxidant activity and the highest total phenolic content (TPC) when compared to shells from other Portuguese regions. Therefore, it is critical to evaluate the extraction's efficacy in removing the desired compounds following its completion. Determining the extracts' antioxidant capacity (AC) is a frequently employed strategy (S. Ferreira, 2021; Pinto, Cádiz-Gurrea, Vallverdú-Queralt, et al., 2021).

2.6 Antioxidant Capacity

The antioxidant capacity of antioxidant compounds is related to their ability to protect biological systems from potential reactive oxygen or nitrogen species reactions. It is an efficient method for comparing the efficacy of various PCs (antioxidant) through different extraction techniques. Antioxidant capacity is primarily determined through two types of tests: those that involve lipid peroxidation and those that involve the capture of electrons or free radicals. In the case of chestnut by-products, the second technique is the most common and used as standardized methods are the DPPH (2,2-diphenyl-1-picryl-hydrazyl) and ABTS (2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) tests. While these are the most frequently used, other tests exist, such as the Ferric Reducing Antioxidant Power (FRAP) test (S. Ferreira, 2021; Pinto,

Cádiz-Gurrea, Vallverdú-Queralt, et al., 2021). Table 2 summarizes the advantages and disadvantages of the previously discussed methods for determining antioxidant capacity.

Table 2- The Advantages and Disadvantages of Methods for Determining Antioxidant Capacity.

Methodology	Advantages	Disadvantages
DPPH (2,2-diphenyl-1-picryl-hydrazyl)	- Easy, simple and fast technique; - Only needs a UV/Vis spectrophotometer to be performed; - Possibility of purchasing the DPPH radical instead of producing it.	- The reaction time is not exact; - The DPPH radical is not always very reactive due to its stability and stereo hindrance.
ABTS (2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)	- Cheap and easy to use technique; - It is not sensitive to pH, allowing the study of its effect on the AC; - Quick reaction.	- Extra step to form the free radical from the ABTS salt; - The free radical is not stable for long periods; - It is not a standardized method, making it difficult to compare results.
FRAP (Ferric Reducing Antioxidant Power)	- Simple, fast and cost-effective; - Used globally on a large scale; - No specialized equipment is required.	- Results may vary; - Possible interferences; - Temperature-sensitive; - pH-sensitive.

Source: S. Ferreira, 2021; Munteanu & Apetrei, 2021.

The DPPH assay is a spectrophotometric technique that allows for the rapid and simple determination of antioxidant capacity using DPPH as a reagent. This compound contains a stable free radical that accepts a hydrogen atom donated by an antioxidant (reducing agent). The colour of the solution changes from purple to yellow as a result of this reaction.

Thus, the antioxidant capacity of a solution can be determined by measuring the decrease in its absorbance at a wavelength (λ) of 517 nm. Due to the fact that antioxidant capacity is proportional to the amount of DPPH removed from the solution, the lighter the colour of the solution, the greater the antioxidant capacity of the extract or compound analysed. To ensure consistency, the results must be expressed in Trolox equivalents – a molecule similar to vitamin E that is soluble in water (S. Ferreira, 2021; Munteanu & Apetrei, 2021). The reaction between DPPH and the AO agent is depicted in Figure 12.

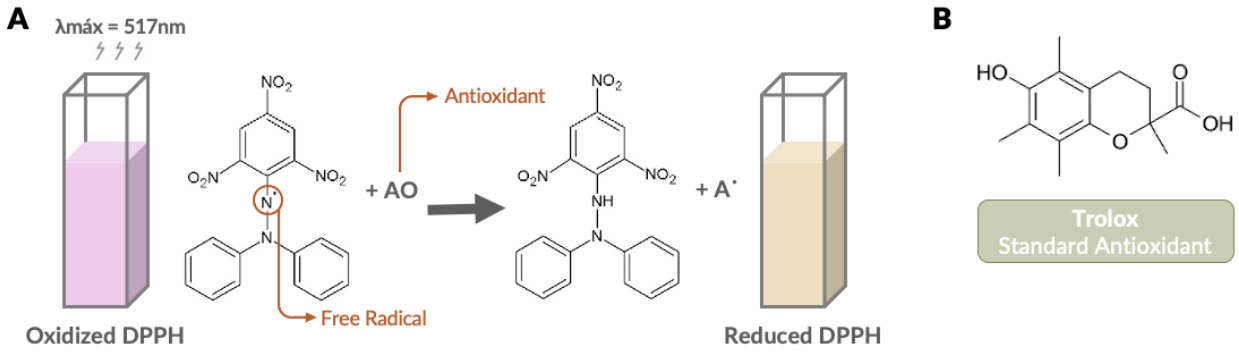

Figure 12- Schematic representation of the reaction between DPPH and the antioxidant agent, AO, (A) and Trolox molecule, used to normalize the results (B).

2.7 Extraction of Pigments

Conventional techniques, such as SLE, are straightforward, low-cost, and simple to use. As a result, these techniques have been widely applied to extract essential oils, bioactive compounds, and natural pigments from a variety of natural sources. The efficiency of conventional extraction procedures is directly related to a solute's solubility in an extraction solvent. Moreover, extraction temperature also significantly affects extraction efficiency (Ngamwonglumlert et al., 2017). Nevertheless, these procedures require a large amount of solvent, long extraction time and may lead to significant pigment degradation due to heat. Thus, to overcome these drawbacks, conditions of short extraction times using environmentally friendly pressurized solvents, such as supercritical CO_2 and pressurized ethanol, have been successfully used to obtain antioxidant dyes and pigment-rich extracts (Santos et al., 2011).

2.8 Sunscreen Stability

To validate a cosmetic product and enable its commercialization, data on its stability must be gathered to ensure consumer safety during use. Thus, it is necessary to subject all of these products to a set of stability tests that determine whether physical, chemical, and microbiological parameters are within acceptable ranges. Additionally, it should be determined whether any factors could jeopardize the product's use over time. By conducting these tests, it is possible to determine the product's shelf life and the optimal transport, storage, and use conditions. Tests are classified into three broad categories: tests on the packaging, physical-chemical properties, and microbiology (S. Ferreira, 2021). Table 3 summarizes several of these tests.

Table 3- Several of the stability tests required in cosmetics and their considerations.

Tests		Conditions
Physical-Chemical	Organoleptic Characteristics	A range of colours and/or scents must be selected. If a product falls outside of the acceptable range, it must be deemed unsatisfactory.
	PH	Check if pH values remain within the accepted values (compatible with the skin's pH): 4.50 – 6.
	Viscosity	It contributes to ensuring that this parameter remains constant throughout time.
Thermal Stability - Freeze-thaw cycles"		It detects suspension issues, emulsion instability, and packaging issues.
Mechanics - Centrifuge Test		They assist in predicting whether product movement during shipping will affect the product and determine whether the emulsion will separate.
Lipid Oxidation		It allows the analysis of the product's oxidation susceptibility.
Antimicrobial		It enables the prediction of target microorganisms' potential to proliferate and grow in cream.
Skin Compatibility - Patch		It helps manufacturers ensure that the product does not cause severe skin irritation.
Skin Compatibility – Cumulative Irritation Test		It determines whether a product has a sensitizing effect on the skin.
Hydration		It determines the product's hydration period.

Source: S. Ferreira, 2021.

2.9 Regulations

Sunscreen goods are classified as cosmetic products under the European Cosmetic Regulation No 1223/2009 (Couteau & Coiffard, 2010). Furthermore, Table 4 lists some of the UV filters that have been approved in Europe for use in cosmetic products and their respective concentration limits in sunscreens.

Table 4- UV filters approved in Europe for use in cosmetic products and their concentration limits in sunscreens.

USAN	INCI	Concentration Limits in Sunscreens (%)
Avobenzone	Butyl Methoxydibenzoylmethane	5
Oxybenzone	Benzophenone-3	6
Octinoxate	Ethylhexyl Methoxycinnamate	10
Octocrylene	Octocrylene	10
Zinc Oxide	Zinc Oxide	25
Titanium Dioxide	Titanium Dioxide	25

Source: Couteau & Coiffard, 2010.
USAN- United States Adopted Name; INCI- International Nomenclature Cosmetic Ingredient.

The effectiveness of sunscreen products should be specified on the label by adding the SPF and referring to the appropriate level of protection, such as "low," "medium," "high," or "very high". Moreover, the efficacy of sunscreens should be adequately validated by standardized and consistent testing procedures.

Moreover, pigment concentrations vary per product, ranging from 1 % in liquid soap to 60 % in pressed powders. Body creams can contain between 1 % and 5 % pigments. (JustPigments, 2022).

2.9.1 Sun Protection Factor

The sun protection factor (SPF) indicates how well a sunscreen protects the skin from UVB rays, the type of radiation that causes sunburn, damages the skin, and may contribute to the development of skin cancer. Besides, the SPF of a sunscreen is often described as the amount of UV energy required to produce a minimal erythema dose (MED) on protected skin divided by the amount of UV energy required to produce a MED on unprotected skin. The higher the SPF of a product, the more effective it is at preventing sunburn (Figure 13). Nonetheless, it is vital to standardize the procedures used to determine these products' SPF (Dutra et al., 2004). In general, sunscreens with SPF values of 2–12 provide minimal protection, those with SPF values of 12–30 provide moderate protection, and those with SPF values more than 30 provide maximum protection (Ntohogian et al., 2018).

The photoprotection provided by topical sunscreens against solar UV radiation exposure can be assessed in vivo or in vitro, and it is most effectively determined through photo testing on human volunteers. This method has been used for many years, and while it is valuable and exact, it is a time-consuming, complex, and expensive process, especially when information about protection against long-wavelength (UVA) radiation is necessary. As a result, much effort has been expended on developing in vitro methodologies for evaluating the photoprotective properties of sunscreen compounds. In vitro procedures are classified into two broad categories: methods for measuring UV radiation absorption or

transmission through sunscreen product films in quartz plates or biomembranes and methods for determining the absorption characteristics of sunscreen agents using spectrophotometric analysis of dilute solutions (Dutra et al., 2004). In-vitro SPF determination (absorbance measurement) by UV-Spectrophotometer is a simple and frequently used method (Donglikar & Deore, 2016).

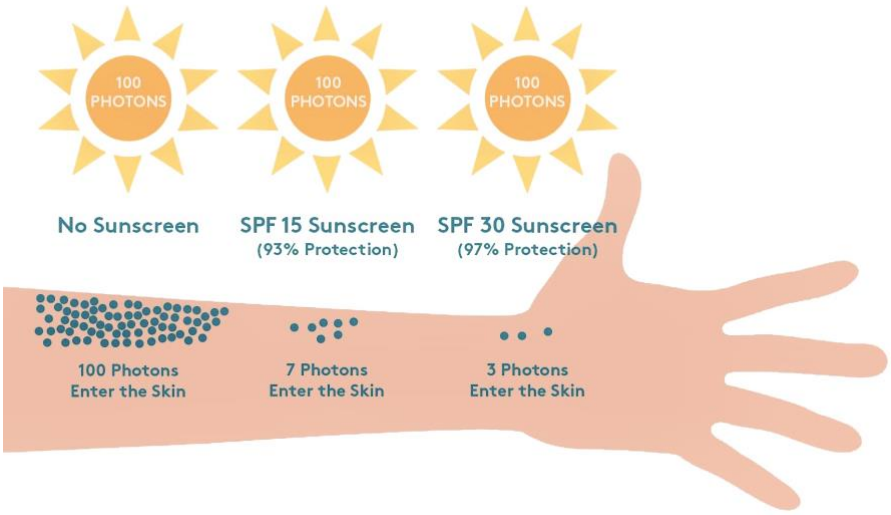

Figure 13- How effectively a sunscreen protects the skin from UVB rays according to SPF value (Deepika Chakraborty, 2020).

3 Materials and Methods

A sunscreen formulation was produced in this study, and its stability was evaluated. To obtain the phenolic extract (PE), BACs were extracted from agricultural residues, specifically the chestnut shells. Additionally, pigments were extracted from annatto seeds. The antioxidant capacity and SPF were determined. The project's many steps are summarized in Figure 14.

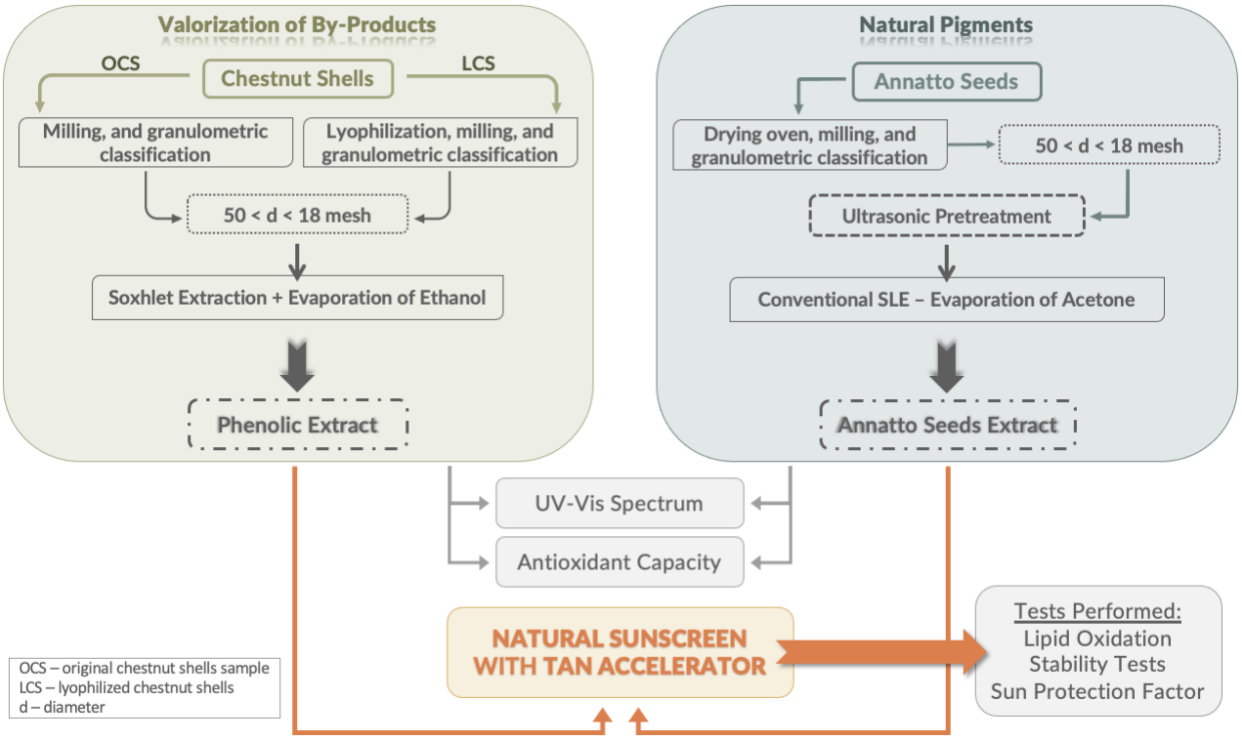

Figure 14- General scheme of the project.

3.1 Chemicals and Reagents

All chemical reagents, solvents, and standards were of analytical reagent grade and were purchased commercially. They were used as received or dried according to conventional protocols. Agromontenegro, located in Serra da Padrela (Alto Trás-os-Montes, Portugal), kindly provided the chestnut shell samples in October 2021. Annatto seeds were purchased from TodoEspecias (Sevilla, Spain), which stated they were originally from Brazil. The solvents for the extractions were pure ethanol (Ref. 1.02371.1000, C_2H_6O, CAS 64-17-5) and pure acetone (C_3H_6O, CAS 67-64-1) from VWR (Fontenay-sous-Bois, France). DPPH (Ref. D9132, $C_{18}H_{12}N_5O_6$, CAS 1898-66-4) was used to determine the antioxidant capacity and was purchased from Sigma Aldrich (St. Louis, MO, USA). The reagents used for the production of the formulations were glycerine (Ref. COSM-01216), xanthan gum (Ref. GOMA-00762), coconut oil (Ref. INCOCO-00185), sweet almond oil (Ref. ACEI-01886), betaine (Ref. COR-COSM-00969) acquired from GranVelada (Zaragoza, Spain), soy lecithin (Ref.L0023, CAS 8002-43-5), purchased from TCI (Tokyo, Japan), butylated hydroxytoluene (BHT) (Ref. 47168, $C_{15}H_{24}O$, CAS 128-37-0) bought from Sigma Aldrich (St. Louis, MO, USA) and benzophenone-3 (oxybenzone) (Ref. H36206, $C_{14}H_{12}O_3$, CAS 131-57-7) purchased

from Sigma-Aldrich (Lyon, France). For the lipid oxidation (LO) tests, barium chloride dihydrate (Ref.217565, $BaCl_2.2H_2O$, CAS 10326-27-9) and iron chloride III (ref. F2877, $FeCl_3.7H_2O$, CAS 10025-77-1) acquired from Sigma Aldrich (St. Louis, MO, USA), iron sulphate (II) heptahydrate (Ref. 24244.232, $FeSO_4 \cdot 7H_2O$, CAS 7782-63-0) and hydrochloric acid (Ref. 20255.290, HCl, CAS 7647-01-0) were purchased from VWR (Fontenay-sous-Bois, France), ammonium thiocyanate (Ref. A10632, CH_4N_2S, CAS 1762-95-4) acquired from Alfa Aesar (Haverhill, MA, USA) and chloroform (Ref. 438607, CH_3Cl, CAS 67-66-3) and methanol (Ref. 414816, CH_3OH, CAS 67-56-1) were bought from Carlo Erba (Barcelona, Spain). Water was deionized and ultra-purified using a Merck Millipore Mill-Q water purification equipment (Billerica, MA, USA) with a resistance of 18.20 Ω.

3.2 Equipment

Lyophilization of the chestnut shells was carried out in a freeze-dryer (SP Scientific, New York, USA), while annatto seeds were dried in a titration drying oven (Buchi GKR-51, Switzerland). Weighing's were performed using a Mettler Toledo AG245 analytical balance scale (Columbus, OH, USA). A digital heated ultrasonic cleaner bath (VWR Ultrasonic Cleaner 220V, Spain) and a cooling bath (JP Selecta, Barcelona, Spain) were used to aid in the extractions. Evaporation of the extraction solvent was carried out in a rotary evaporator (Böchi R-200, Flaiwil, Switzerland). A microplate reader (Synergy, HT, Biotek, USA) was used to read the absorbance of the antioxidant capacity assays. A UV-Vis spectrophotometer (UV-3100PC Spectrophotometer, China) was used to determine the absorption spectrum of extracts and formulations. For the production of the formulations, a thermostatic bath (Fontenay-sous-Bois, France), a high-performance homogenizer (IKA T18 Digital ULTRA-TURRAX®, Staufen, Germany), a stirring plate (AREX Digital, VELP Scientifica, Monza, Italy) were used. For stability tests, a digital pH meter (XS Instruments, Capri, Italy), a centrifuge (Centrifuge 5424, Eppendorf, Hamburg, Germany) and a modular compact rheometer (MCR 72/92 Anton Paar) were used.

3.3 Treatment of Chestnut Shells

The company Agromontenegro dried the shells for six days at a temperature of 50 ºC prior to providing them for this project. Part of them were subject to pre-treatment, lyophilized at -80 °C, at a pressure of 1.30 Pa, for three days. Following that, the original chestnut shell (OCS) sample and the lyophilized chestnut shell (LCS) sample were milled in a coffee mill and then sieved to obtain grains of equal size, aiming for particles between 18 and 50 mesh. Then, the shells were sealed in flasks and stored in the dark at room temperature until extraction.

3.4 Treatment of Annatto Seeds

Annatto seeds were dehydrated for 6 hours at a temperature of 50 ºC and an atmospheric pressure of 800 mbar. Subsequently, the seeds were milled in a coffee mill and then sieved to obtain grains of equal

size, aiming for particles between 18 and 50 mesh. The shells were then stored in flasks and maintained at room temperature in the dark until extraction.

3.5 Obtaining the Chestnut Shell Phenolic Extract

In order to extract the phenolic compounds present in chestnut shells, continuous solid-liquid extractions with a Soxhlet apparatus were performed with 210 mL of absolute ethanol for a chestnut shell sample of 7 g, a 1:30 ratio (w/V). The extractions were performed in triplicates. The solvent was poured into a 500 mL round bottom flask along with boiling regulators, and the sample was placed within a 200 mL Soxhlet inside a filter paper cartridge. The equipment was assembled by inserting the Soxhlet in a reflux position between the round-bottom flask and the condenser (Figure A.2.1). The CS phenolic compounds were extracted with ethanol, and this process was carried out at a temperature of 80 ºC for 90 minutes. Ethanol was evaporated using a rotary evaporator (BUCHI Laboratories, Flawil, Switzerland), with a bath temperature of 45 °C and a stream of nitrogen to remove the solvent present in the extract (S. M. Ferreira et al., 2022). Afterwards, the vials were weighed and stored. The extraction yield was determined using Equation (A 2.1). A one-way statistical analysis of variance (ANOVA) was performed to compare the acquired yield values by calculating the *p*-value (95% confidence), with *p*-values greater than 0.05 indicating no significant difference between extraction times – Appendix A.1.

3.6 Obtaining the Annatto Seed Extract

The extraction of pigments was carried out through a conventional SLE. For the extraction, approximately 117 mL of acetone was used for 29 g of sample, resulting in a ratio of 1:4 (w/V). The solvent and sample were poured into a 250 mL round bottom flask along with boiling regulators. Before the extraction, the sample was ultrasonicated for 15 minutes. Then, the equipment was assembled by inserting the condenser into the round-bottom flask. The extraction was carried out at a temperature of 50 ºC for 90 minutes. Following extraction, the extract was filtered through Whatman filters and then acetone was evaporated using a rotary evaporator with a bath temperature of 45 ºC (Figure A.3.1). After complete evaporation with a stream of nitrogen, the vials were weighed and stored. The extraction yield was determined using Equation (A 3.1).

3.7 Antioxidant Capacity – DPPH Method

The antioxidant capacity test was carried out according to the literature (S. M. Ferreira et al., 2022) for the three extracts. Initially, a solution of the DPPH radical at 0.2 mM in methanol was prepared by dissolving 39.4 mg of DPPH in 1 mL of methanol in an Eppendorf tube. Subsequently, the mixture was stirred vigorously using a vortex and, finally, after achieving homogeneity, it was transferred to a volumetric flask, where the sample volume was brought up to 500 mL through the addition of methanol. To each well of the microplate (except wells A2 to A9 and wells in columns 10 and 11), 100 µL of methanol was added, followed by the addition of 200 µL of 3 $g \cdot L^{-1}$ avocado peel extract solution in 2 % DMSO

aqueous solution, in wells A2 to A9. The sample was diluted eight times using the microplate dilution method, where 100 µL were taken from the first row of columns 2 to 9 and transferred to the corresponding wells in the second row. The same was performed from the second row to the third and so on until reaching the last row, where 100 µL were discarded. In column 12, 100 µL of the DPPH solution was added to the first well, and the dilutions were made from that well. Afterwards, 100 µL of the DPPH solution was added to each well of the plate, except for rows 2 and 6, where methanol was added instead. Finally, the plate was covered with the lid to avoid evaporation, wrapped in aluminium foil for light isolation and left to incubate for 30 min at room temperature. After the incubation period, absorbances were measured at 517 nm using a microplate reader (Synergy, HT, Biotek, Winooski, VT, USA). Appendix B contains the equation used to calculate the percentage of DPPH inhibition (% I) – Equation B 1.1 – and an image of a prepared microplate. The IC_{50} percentage - the necessary concentration of the extract to inhibit 50 % of DPPH - for each extract was determined using the linear regression equation derived from the absorbance *vs* the concentration plot.

3.8 UV-Vis Spectrum

The absorption spectrum of the extracts was obtained using a UV-3100PC spectrophotometer. To measure the absorbance values of the phenolic extracts, 0.08 g of the original chestnut shell (OCS) extract and 0.08 g of the lyophilized chestnut shell (LCS) extract were each dissolved in 40 mL of ethanol. Then, using ethanol as a blank, the absorbance values in the UV spectrum were determined for each solution. Additionally, to obtain the annatto seed (AS) extract absorption spectrum, 0.01 g of annatto seed extract was dissolved in 10 mL of acetone. After, 1 mL of the solution was transferred to a 10 mL volumetric flask and the volume completed with acetone. Finally. 0.50 mL of the new solution was transferred to a 10 mL volumetric flask, and the volume completed with acetone. Next, using acetone as a blank, the absorbance values were determined in the Visible spectrum for the AS extract. The UV-Vis spectrums are illustrated in Appendix A - Figure A 2.2 and Figure A 3.2.

3.9 Formulations Production

In order to evaluate the OCS, LCS and AS extracts in a cosmetic formulation and their effects on the formulation's stability, seven O/W emulsions were produced. The concentrations of the various ingredients used in the formulation are listed in Table 5. The various formulations were designated F1 to F7 based on the modifications made to the formulation. Phase A (aqueous phase) - made up of ultrapure water, glycerine, and xanthan gum - and phase B (oily phase) - made up of coconut oil, sweet almond oil, soy lecithin, and betaine - were separately heated to 70 ºC and blended. Then, because this is an O/W product, phase B was added to phase A while maintaining the stirring and temperature. These were then stirred and homogenized with a T 18 digital ULTRA-TURRAX® with stirring speeds at 12.000 rpm until complete homogenization was visually verified (approximately 2 minutes) – Appendix A.4.

For formulations with additives, the stabilizers were added to formulations after cooling until room temperature and stirred again for a few minutes until homogenization was visually observed. The percentage of additives added to the formulations is presented in Table 5; these values were added by reducing the corresponding percentage of water. Since F1 formulation was utilized as a blank (negative control), no additional components were added. To the F2 and F3 formulations, a commercial antioxidant (BHT) and commercial UV filter (oxybenzone) were added to allow these formulations to be used as standards, respectively. To see whether a rise in concentration resulted in alterations, chestnut shell phenolic extract was added to F4 and F5 at varying concentrations. In addition to the phenolic extract, annatto seed extract was added to the F6 formulation to determine if pigments affected tanning. Finally, only annatto seed pigments were added to the F7 formulation. It should be noted that the concentrations (%) used are comparable to those described in the literature and are within the legal limits.

Table 5 - Formulation and content of the various formulations produced.

	Ingredients	Function	F1	F2	F3	F4	F5	F6	F7
Phase A	Ultrapure Water	Solvent	73.60	73.50	73.00	73.10	72.60	72.85	73.35
	Glycerine	Humectant	7.60						
	Xanthan Gum	Thickening Agent	0.60						
Phase B	Coconut Oil	Emollient	7.60						
	Sweet Almond Oil	Emollient	6.80						
	Soy Lecithin	Emulsifier	2.70						
	Betaine	Secondary Emulsifier	1.10						
Formulation Stabilizers	BHT	Synthetic Antioxidant	-	0.10	-	-	-	-	-
	Oxybenzone	Synthetic UV filter	-	-	0.60	-	-	-	-
	Chestnut Shells Phenolic Extract	Natural Antioxidant and UV Filter	-	-	-	0.50	1.00	0.50	-
	Annatto Seeds Pigments Extract	Tan Accelerator	-	-	-	-	-	0.25	0.25

BHT- Butylated hydroxytoluene

3.10 Stability Tests

Following the manufacture of the various formulations, it was required to assess their stability using a set of tests. It should be emphasized that all tests were conducted at room temperature (20 – 25 ºC), except for the thermal stability test.

3.10.1 pH Test

A digital pH meter was used to determine the pH of the samples under study, directly measuring the value without dissolving the sample. The pH values of the various formulations were determined 7, 14, and 21 days following storage (Navarro-Pérez et al., 2021). The equipment used is detailed in Appendix C.1.

3.10.2 Viscosity

The rheological behaviour of the samples was analysed using a plate-plate configuration with a 40 mm diameter and a 1 mm gap. The sample was added to the lower plate, and the apparent viscosity (mPa·s) was measured against different shear rates (s^{-1}), at a constant temperature of 25 ± 0.1 °C. The viscosity of the different samples was measured on day 7 and day 21 after their production. Appendix C.1 shows the equipment used.

3.10.3 Skin Compatibility

The patch test was used to determine skin compatibility. A small amount of the formulations was applied to the inner part of a previously cleansed arm, followed by a patch. It was monitored for 24 hours for any form of reaction, such as itching, inflammation, or redness (Navarro-Pérez et al., 2021).

3.10.4 Spreadability Test

The spreadability determination was carried out in the manner described by Chen et al., 2016, with minor modifications. Between two glass slides, 0.15 g of sample was placed, and then a 20.0 g weight was carefully positioned in the centre of the upper slide. The weight was removed after 1 minute, and the diameter of the spread sample was measured. This was performed 7 days, 14 days, and 21 days after the formulations were produced.

3.10.5 Centrifugation Test

For the physical stability evaluation, the formulations were subjected to centrifugation at 5000 rpm for 10 min. Physical stability was evaluated by visually verifying if phase separation and colour changes occurred (S. M. Ferreira et al., 2022). Appendix C.1 shows the equipment used.

3.10.6 Thermal Stability Test

Freeze-thaw cycles were used to determine thermal stability. The formulations were left to incubate overnight at 50 °C, followed by a 20 °C resting phase during the following day, then a low-temperature phase with an overnight incubation at 5 °C, and finally another resting phase the following day at 20 °C. Thermal stability was evaluated by visually verifying textural, consistency and colour changes (S. M. Ferreira et al., 2022).

3.11 Lipid Oxidation

Lipid oxidation was assessed by measuring the Peroxide Value (PV) using the method described by (S. M. Ferreira & Santos, 2022). Initially, 0.10 g of sample were mixed in 9.80 mL of a 7:3 (*v*/*v*) solution of chloroform-methanol, and the final mixture was vortexed for 2 to 4 s. Afterwards, 50 µL of an ammonium thiocyanate solution was added and vortexed for 2 to 4 s, followed by 50 µL of an iron (II) solution. The samples were incubated for 5 min at room temperature, and absorbance was measured using a UV-Vis spectrophotometer at 500 nm and compared to the absorbance of the blank (which contains all reagents except the sample). Peroxide value (PV) was calculated using Equation (C 2.1). The peroxide value was determined for the formulations F1 (negative control), F2 (antioxidant positive control), F4 and F5 (F3 was excluded due to the absence of antioxidant additives, while F6 and F7 formulations were excluded because, in this project, annatto seeds extract was only studied for pigments purposes).

3.12 Sun Protection Factor – Preliminary Studies

The SPF was determined using the method described by Donglikar & Deore, 2016, with some minor changes. Except for F2, F6 and F7, all samples were weighed (1 g), transferred to a 100 mL volumetric flask, diluted to volume with ethanol, ultrasonicated for 5 minutes, and then filtered through Whatman filters. A 5 mL sample was transferred to a 50 mL volumetric flask and diluted to volume with ethanol for each formulation. The absorption spectrum was determined between 250 and 400 nm (Appendix D.1). Then, using the Mansur equation represented in Appendix D.2, the SPF values of the formulations were calculated. The SPF was determined for the formulations F1 (negative control), F3 (UV filter positive control), F4 and F5 (F2 was excluded due to the absence of UV filter additives, while F6 and F7 formulations were excluded because, in this project, ASE was only studied for pigment purposes).

3.13 Photoprotection Evaluation – Preliminary Studies

F1, F3, F4, F5, and F6 formulations were applied to the inner arm of volunteers who were subsequently exposed to sunlight or subjected to photo testing for one hour. To assess how the various formulations affected the skin's reaction to UV light exposure, a photograph was taken before and after 2 hours of sun exposure.

3.14 Packaging, Destination and Treatment of Waste

The liquid waste generated was primarily composed of organic solvents such as ethanol, acetone, n-hexane, methanol, and chloroform. These residues were collected in closed containers with adequate labelling and stored away from light and ignition sources for subsequent waste treatment by EcoFEUP (FEUP's Environmental Management System).

4 Results and discussion

4.1 Phenolic Extract of Chestnut Shells

Extraction is the most critical step in the process of isolating BACs (such as phenolic compounds) from natural matrices (Pinto, Silva, Freitas, Vallverdú-Queralt, et al., 2021). The PCs present in chestnut shells were extracted using a solid-liquid extraction technique with a Soxhlet apparatus, with pure ethanol as the extraction solvent. As previously stated, Soxhlet extraction offers some attractive advantages over other extraction methods. It is a simple procedure that requires low-cost equipment and enables the extraction of a large amount of material without requiring filtration afterwards. Despite its drawbacks, such as the duration of the extraction and the amount of solvent utilized, this approach is nevertheless widely employed in many laboratories (ŞENGÜN, 2018).

Moreover, because phenolic compounds are relatively polar compounds, they are best extracted using alcoholic solvents. While the literature indicates that ethanolic extraction provides the maximum yield, aqueous extracts demonstrate the highest total phenolic content (TPC) (Fernández-Agulló et al., 2014; Pinto, Silva, Freitas, Vallverdú-Queralt, et al., 2021). A possible explanation for the higher extraction yield obtained with ethanolic extracts is the presence of interfering substances such as nonphenolic compounds (e.g., sugars and lignins) or PCs that do not exhibit significant antioxidant capacity (Pinto, Silva, Freitas, Vallverdú-Queralt, et al., 2021). Although the literature indicates that aqueous solutions enable higher TPC, this option was not chosen due to the complexity, energy and time required to eliminate the water contained in the sample upon extraction. Thus, ethanol was selected as the solvent since it is generally recognized as safe (GRAS) and has a low boiling point, thereby lowering energy expenditures and simplifying the solvent removal procedure.

Furthermore, S. M. Ferreira et al., 2022 reported that 1.5 hours of continuous solid-liquid ethanol extraction using a Soxhlet apparatus at a 1:36 ratio resulted in a high yield extraction of avocado by-products. As a result, a ratio of 1:30 (w/V) was used for a 1.5 hour extraction. The extraction temperature was set to 80 ℃, as this temperature is favourable for extraction and is close to the boiling point of ethanol. The yield values of the phenolic extract produced by SLE with Soxhlet are shown in Table 6.

Table 6 - Yield of the extraction of phenolic compounds from Chestnut Shells, using solid-liquid extraction (Soxhlet method), for an extraction time of 90 minutes.

Chestnut Shells						
No	**Original Chestnut Shell (OCS) Sample**			**Lyophilized Chestnut Shell (LCS) Sample**		
	Shell (g)	**Extract (g)**	**Yield (%)**	**Shell (g)**	**Extract (g)**	**Yield (%)**
1	7.03	1.83	26.10	7.02	2.03	28.99
2	7.02	2.04	29.06	7.01	1.72	24.48
3	7.00	1.76	25.20	7.01	1.74	24.89
$\bar{x} \pm \sigma$	-	-	**26.79 ± 1.65**	-	-	**26.12 ± 2.04**

The yield of phenolic compounds extracted from OCS was on average 26.79 %, while the yield of phenolic compounds extracted from LCS was on average 26.12 %. Given that the OCS were not lyophilized but produced a slightly higher amount of extract than the LCS, this indicates that the Agromontenegro company dehydrated the OCS quite efficiently and that lyophilization had no effect on the yield. A statistical analysis of variance (ANOVA) of the yield data reveals that the difference in these extraction yields is not significant ($p > 0.05$). Table 7 summarizes the yields of phenolic compounds extracted from chestnut shells using various extraction techniques.

Table 7 – Examples of yields of Phenolic Compounds from Chestnut Shells using different extraction techniques stated in the literature.

Extraction Methodology	Yield (%)	Reference
Maceration with ethanol:water (1:1) at 50 °C for 30 min using a solid/liquid ratio of 1:20 (w/V)	12.57 – 13.67	F. Rodrigues, Santos, et al., 2015
Extraction performed in boiling water for 60 min using 5 % (w/V) of shells and water as solvent	5.20	Cacciola et al., 2019
Extraction was performed at different temperatures (25, 50 and 75 °C) for 60–120 min using different solvents (water, 50 % methanol and 50% ethanol) at a solid/liquid ratio of 1/10 (w/V)	Aqueous: 2.91 – 8.72 50 % Ethanol: 4.17 – 13.27 50 % Methanol: 4.33 – 12.09	Fernández-Agulló et al., 201
SFE (CO_2 and ethanol (co-solvent)) 90 min	0.55 – 1.12	Pinto, Cádiz-Gurrea, et al., 2020
Extraction with water in SWE equipment at a solid/liquid ratio of 1:10 (w/V) at a pressure of 40 bar and stirring of 200 RPM	6.70 – 9.19	Pinto, Vieira, Peixoto, Freire, et al., 2021
Extraction with water using UAE probe at a solid/liquid ratio of 1:20 (w/V) at an amplitude of 50 %	7.30 – 16.10	Lameirão et al., 2020
Extraction with water and ethanol at different concentrations (25, 50, 75 and 100 %) using MAE equipment at a solid/liquid ratio of 1:10 (w/V)	6.52– 13.02	Pinto, Vieira, Peixoto, Freire, et al., 2021
Conventional extraction using water under magnetic stirring (150rpm) at room temperature during 1h	6.43 - 12.59	Barreira et al., 2010

Source: Pinto, Cádiz-Gurrea, Vallverdú-Queralt, et al., 2021
UAE- Ultrasound-assisted extraction; MAE- Microwave-assisted extraction; SWE- Subcritical water extraction; SFE- Supercritical fluid extraction

As seen in Table 7, the yields of this study were more significant than the yield values obtained in other studies. To our knowledge, this is the first study that used SLE with a Soxhlet apparatus to extract phenolic chemicals from chestnut shells, and hence our results cannot be compared to others obtained using the same technique. However, S. M. Ferreira et al., 2022 obtained a phenolic extract yield of 19.45 % from avocado peels using the Soxhlet method for 1.5 hours and ethanol as solvent. This finding demonstrates that this extraction process generally results in a higher extraction yield of agricultural by-products. While the extraction yield in the present study was still higher than the one obtained in the reported studies, this could be attributable to the fact that we used different particle sizes. The authors employed particles with a mesh size of 50–100, whereas we used particles with a mesh size of 18–50. As the surface area of the particle increases, the interaction with the solvent increases, so using smaller-sized particles can shorten the extraction period (ŞENGÜN, 2018).

The extraction efficiency is dependent on a number of variables, including temperature, time, solvent polarity, and pH (Lameirão et al., 2020). According to Fontana et al., 2013, increasing the sample's solvent volume and the mass ratio increases extraction yield. This is consistent with the results obtained in this

work, as the yields in Table 7 were obtained using 1:10 and 1:20 ratios, resulting in lower extraction yields than our 1:30 ratio. However a solvent that allows for a high yield extraction can result in low-quality extracts containing fewer polyphenols or diminished antioxidant properties (Pinto, Silva, Freitas, Vallverdú-Queralt, et al., 2021). In contrast to the extraction yield, Pinto, Silva, Freitas, Vallverdú-Queralt, et al., 2021 state that increasing the ethanol percentage used in the extractions generally resulted in a decrease in total phenolic content. As a result, it is critical to determine the extract's properties, such as antioxidant activity, to ascertain its purity.

F. Rodrigues, Santos, et al., 2015 determined that the extraction yield of chestnut shells obtained from the same region as the present study, Trás-os-Montes, was 13.70 %. This could be due to the fact that the authors chose a mixture of water:ethanol as solvent, a lower temperature, and reduced the duration of the extraction. In this study, trials demonstrate that the extraction process and conditions are more advantageous in terms of extraction yields when compared to the author's results. However, we cannot confirm that our extract has a similar TPC because we did not do this assay.

As a matter of fact, the effectiveness of extraction is influenced by the chemical composition of the phytochemicals, the extraction methods utilized, the particle size of the sample, the solvent used, and the presence of interfering compounds (Pinto, Silva, Freitas, Vallverdú-Queralt, et al., 2021).

4.2 Antioxidant Capacity of Chestnut Shells

The antioxidant capacity of each sample was investigated by DPPH assay. A 2 % DMSO aqueous solution was prepared and added to various dilutions of the sample extracts, as these had extremely opaque hues that make reading the absorbance impossible. The antioxidant activity was quantified as the percentage of DPPH inhibition (% I) and the sample concentration required to reduce the initial DPPH radicals concentration by 50 % (IC_{50}). The AC of the phenolic extracts is listed in Table 8. It should be noted that a lower IC_{50} value indicates a higher inhibition percentage of DPPH and, thus, a better AO activity.

Table 8 - Antioxidant capacity of chestnut shell phenolic extract, by DPPH (2,2-diphenyl-1-picryl-hydrazyl) assay.

Chestnut Shells – Antioxidant Capacity: DPPH method				
No	**OCS**		**LCS**	
	% I	**IC_{50} (%)**	**% I**	**IC_{50} (%)**
1	89.13	11.01	80.51	17.73
2	89.27	14.94	79.30	17.50
3	88.59	14.62	79.83	15.68
$\bar{x} \pm \sigma$	**88.99 ± 0.29**	**13.52 ± 1.78**	**79.88 ± 0.49**	**16.97 ± 0.92**

OCS- Original chestnut shell samples; LCS- Lyophilized chestnut shells.

Chestnut shell extracts exhibited a remarkable DPPH scavenging ability. Moreover, the IC_{50} values for both extracts were significantly similar ($p > 0.05$). However, this was not the case for the DPPH inhibition percentages, which significantly differed across extracts ($p < 0.05$). The OCS extract showed the highest antioxidant potential because a low IC_{50} is associated with high AO power. According to different authors,

natural extracts containing a high concentration of total phenolic content also display significant AO activities (Pinto, Silva, Freitas, Vallverdú-Queralt, et al., 2021). Therefore, these results are in line with expectations. As previously stated, extractions with 100 % water as the solvent often have the highest TPC value than those with 100 % ethanol. As a result, because the OCS most likely had a superior humidity %, it is likely that they have more TPC and thus stronger antioxidant activity.

By comparing the acquired results to those reported by other authors for the same by-product, it is possible to confirm that the antioxidant activity of the CS extracts analysed in this project were significantly higher. For instance, Pinto, Cádiz-Gurrea, et al., 2020 investigated the effects of temperature, pressure and co-solvent percentage on the AO activity of chestnut shell extracts by SFE, and the DPPH inhibition percentages ranged between 27.98 % and 53.04 %. Additionally, Pinto, Silva, Freitas, Vallverdú-Queralt, et al., 2021 determined the antioxidant capacity of CSs MAE extracts utilizing water and various percentages of ethanol (25%, 50%, 75%, and 100%) as eco-friendly extraction solvents. The results demonstrated a notable DPPH scavenging ability, with the IC_{50} ranging between 47.80 µg/mL (aqueous extract) and 101.01 µg/mL (100% ethanolic extract). Due to the fact that the IC_{50} is inversely proportional to the AC, the aqueous extract showed the greatest $DPPH^{\bullet}$ quenching activity (Pinto, Silva, Freitas, Vallverdú-Queralt, et al., 2021). Also, Lameirão et al., 2020 analyse the effects of time and temperature on the AO activity of CS extracts using UAE. According to the authors, the IC_{50} determined for the DPPH assay ranged from 43.70 µg/mL (extraction 7, 34 °C, 25 min) to 63.70 µg/mL (extraction 5, 55 °C, 4 min).

Although extraction technology, conditions, and solvents had a noticeable effect on BACs recovery and AO activity, the significant differences between the results obtained and those reported by other authors could also be explained by differences in environmental conditions, such as temperature, humidity, soil quality, water availability and biodiversity (Pinto, Cádiz-Gurrea, Vallverdú-Queralt, et al., 2021).

Overall, these results revealed the high potential of the assayed by-product as a new source of AO compound. As determined by the DPPH assay, the high AO activity of extracts is most likely due to a high concentration of TPC; hence, it is possible to conclude that SLE with Soxhlet was effective.

4.3 Extraction of Pigments from Annatto Seeds

The pigments from annatto seeds were extracted in this study utilizing a conventional SLE method with acetone. This technique was chosen since Pattanaik et al., 2018 stated that bixin extraction using various solvents is more cost-effective and efficient than other developed techniques such as microwave, ultrasonic, and supercritical carbon dioxide. Additionally, the authors assert that a moderately polar solvent such as acetone aids in the extraction of bixin from ASs as compared to highly non-polar or polar solvents such as hexane or ethanol, respectively. This conclusion could be explained by bixin being more soluble in medium polar solvents than in polar or non-polar solvents. Moreover, some researchers reported maximum bixin extraction using medium polar solvents like acetone. Also, hot extraction speeds up the process, resulting in a shorter time frame and increased yield of bixin (Pattanaik et al., 2018). As a result, acetone was selected as the solvent for this extraction. Table 9 summarizes the yields obtained.

Table 9 - Yield of Annatto Seed Extract, using a conventional solid-liquid extraction method with acetone at 50 ºC, for an extraction time of 90 minutes.

Annatto Seeds			
No	**Seeds (g)**	**Extract (g)**	**Yield (%)**
1	29.33	1.84	6.26
2	29.11	1.81	6.21
3	29.11	1.95	6.69
		$\bar{x} \pm \sigma$	**6.39 ± 0.21**

The yield of annatto seed extract was, on average, 6.39 %. In an experiment conducted by Pattanaik et al., 2018, ASs from two different geographical regions in India yielded 9.21 % and 9.20 % of AS extract. Pattanaik et al.'s study used the same temperature (50 ºC) and solvent (acetone) as this paper but used a SLE technique with a Soxhlet apparatus. Moreover, the authors' extraction time was significantly longer (12 hours). When yields are compared, Pattanaik et al., 2018 extractions are significantly higher. This result could be the consequence of many factors. As discussed in Chapter 4.1, extraction efficiency relies on various variables, including the extraction method and time used, as well as the sample's origin, impurity content, and particle size.

Furthermore, even though the purpose of this project is not to determine the antioxidant activity of annatto seed extract because this component's primary function is to provide pigment to formulations, the AO activity was analysed. As a result, AS extract exhibited an IC_{50} of 181.69 µg/mL and a DPPH inhibition percentage of 20.01 %. These values are contrary to those obtained with chestnut shell extracts, as the IC_{50} is much higher and the DPPH inhibition percentage significantly lower. Due to the fact that the IC_{50} is inversely proportional to the antioxidant capacity, it is possible to conclude that AS extract does not possess a high level of antioxidant power, at least under the extraction process and conditions used in this study.

4.4 UV-Vis Spectrum

The UV-Vis spectrums were obtained for the chestnut shell (OCS and LCS) and annatto seed extracts. This technique determines the amount of discrete UV or visible light absorbed by a sample compared to a reference or blank sample. Appendix A contains the extracts' absorption spectrum.

Chestnut shell extracts (OCS and LCS) exhibit a maximum absorbance peak at 273 nm. Vázquez et al., 2009 investigation discovered that chestnut shell extracts exhibit a single absorbance maximum at 270 nm, which agrees with the result obtained. As previously stated, 273 nm corresponds to UVC radiation, implying that this extract will be incapable of protecting against UVB or UVA radiation. However, 273 nm is very close to the UVB absorbance range, and CS extract might even have compounds that absorb in the UVB zone, but only in tiny amounts; therefore, a peak with maximum absorbance in that zone is not evident. Also, this result can vary as the properties of CS extracts are influenced various factors, as pointed out previously. Moreover, UV-Vis spectrophotometry analysis reveals that the annatto seeds

extract contains three absorbance maxima at 432 nm, 456 nm and 486 nm. These findings are remarkably similar to those reported by Rahmalia et al., 2015.

4.5 Formulations Production

The formulations developed in the scope of this project were based on the article of S. M. Ferreira & Santos, 2022, with some slight modifications. The present formulation includes only the essential ingredients to create a cream in order to interpret the effect of the antioxidant activity and SPF after adding the extracts or synthetic compounds. The quantities (%) of all used ingredients are similar to those reported in the literature and follow the legislation.

As indicated previously in Chapter 3.8, 7 O/W formulations were developed. Synthetic antioxidant (BHT) was added at a concentration of 0.10 % to formulation F2. This ingredient was chosen since it was necessary to develop an experiment using a synthetic AO to compare it to the formulations containing the phenolic extract and AS extract. Additionally, this is one of the most widely used commercial AOs in the cosmetic industry, having been FDA approved (Lanigan, R. S., & Yamarik, 2002). To study the SPF of formulations containing PE, a formulation comprising a synthetic UV filter was produced. Thus, 0.60 % of oxybenzone – the maximum percentage allowed by cosmetic regulation in Europe - was added to F3. This was chosen as the synthetic UV filter because, as previously indicated, it is one of the most widely utilized in commercial sunscreens worldwide to this day. After establishing the percentage of oxybenzone, it was decided to conduct two tests with the phenolic extract that showed the best antioxidant capacity - OCS: one using approximately the same amount as the synthetic UV filter – 0.50 % – (F4), and another using twice as much – 1 % – (F5), in order to determine whether a higher percentage of PE had any effect on formulations. In F6, PE (0.50 %) and AS extract (0.25 %) were added to determine whether the addition of pigments influenced tanning. Finally, 0.25 % AS extract was added to the F7 formulation. Because AS extract is strongly pigmented and these components typically range between 1 and 5 % in emulsions, an intermediate value of 0.25 % was chosen. All formulations developed are represented in Figure 15.

Figure 15- Formulations developed throughout the project (F1 to F7).

4.6 Stability Tests

After manufacturing the formulations (F1–F7), their stability was determined. As a result, for 21 days, they were subjected to various tests. Appendix C comprises the equipment used to conduct the experiments and the pH and spreadability test values obtained throughout the analysis period.

Organoleptic qualities, such as colour and smell, stayed relatively unaltered throughout the research. Formulations F1 and F2 had a beige colour, F3 had a slightly more white colour than the latter, while formulations F4 and F5 had an old rose colour due to the presence of the CS phenolic extract. Since F5 included double the quantity of phenolic extract found in F4, this formulation had a deeper tone. F6 and F7 had a yellowish-orange colour due to the addition of annatto pigments. In terms of fragrance, formulations F1 to F3 were odourless, F4 and F5 had a light aroma, and formulations F6 and F7 had a more powerful fragrance. Only formulations containing phenolic extract and AS extract demonstrated a slight change in aroma, with a somewhat more pungent scent. In terms of appearance, the formulations containing annatto seed pigment were not homogeneous since particles were discovered throughout the analysis period. The remaining formulations lacked visible particles, indicating that the compositions were homogeneous.

Accelerated stability tests were used to determine the formulations' stability. The results of the centrifugation test are shown in Figure 16. At the end of the centrifugation test, it was observed that none of the formulations demonstrated phase separation, indicating that the emulsions were stable. However, formulations including AS extract demonstrated a deposit of the undissolved granules.

Additionally, the formulations were subjected to a thermal stability test, a standard procedure for simulating long-term shelf life. The results indicated that no phase separation occurred during these stability tests and that there were no significant changes in viscosity or colour (data not shown). As a result, it can be stated that the prepared emulsions exhibit exceptional long-term stability, while their properties remain unaffected by temperature changes.

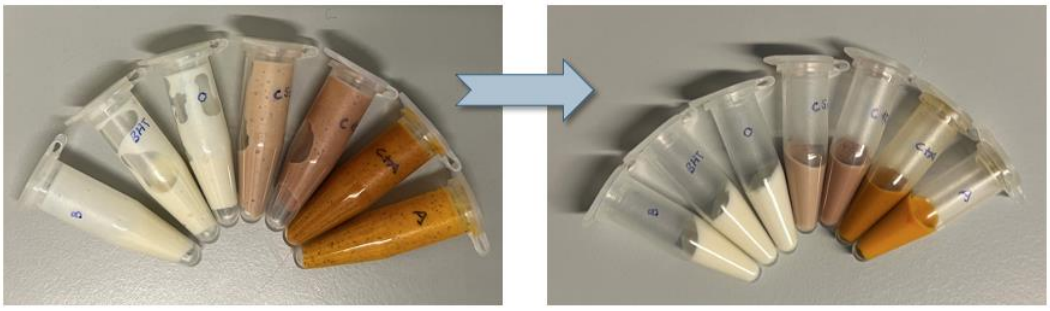

Figure 16- Results obtained for the centrifuge test.

Regarding the remaining physicochemical properties, such as pH, spreading, and viscosity, Figure 17 depicts their alterations during 21 days. This analysis reveals that there were some minor oscillations in all physiochemical characteristics. After 14 days of formulation production, the pH value of all formulations increased. However, on day 21, it returned to values comparable to the pH recorded on day 7. When each formulation is analysed, it becomes clear that the formulations containing the PE (F4 to F6) have a higher acidic pH than the others. The values, however, are within the ideal range of 4.50 to 6. Only F3, which contains oxybenzone, fell outside the ideal range.

Additionally, when comparing the viscosity of formulas F2 through F7 to F1, one can deduce that F2, F3, and F7 were less viscous. On the other hand, the formulations including phenolic extract, exhibited an increase in viscosity. Given that it appears as adding chestnut shell extract enhances viscosity, increasing

the amount of CS extract should result in an even more viscous formulation or at least a formulation with similar viscosity. However, F4, which had half the quantity of extract as F5, is relatively more viscous. This result may be because, in order to incorporate the extract, the agitation was slightly increased, which may have resulted in the development of air bubbles in F5, which can affect viscosity. As expected, the formulation including both CS extract and AS extract had a higher viscosity than F1 but were less viscous than formulations having only phenolic extract, which makes sense given that F7, the formulation containing annatto extract solely, has a lower viscosity than F1. In terms of spreadability, the results appear to be consistent with viscosity. When comparing day 7 and day 21, it is feasible to see that the spreadability and viscosity of all formulations, except F3, decreased. It makes sense that a decrease in one of these attributes would result in lowering the other since the more viscous a fluid is, the less it will spread under the same conditions. Between days 7 and 21, the viscosity of F3 remained constant, and hence its spreadability remained constant.

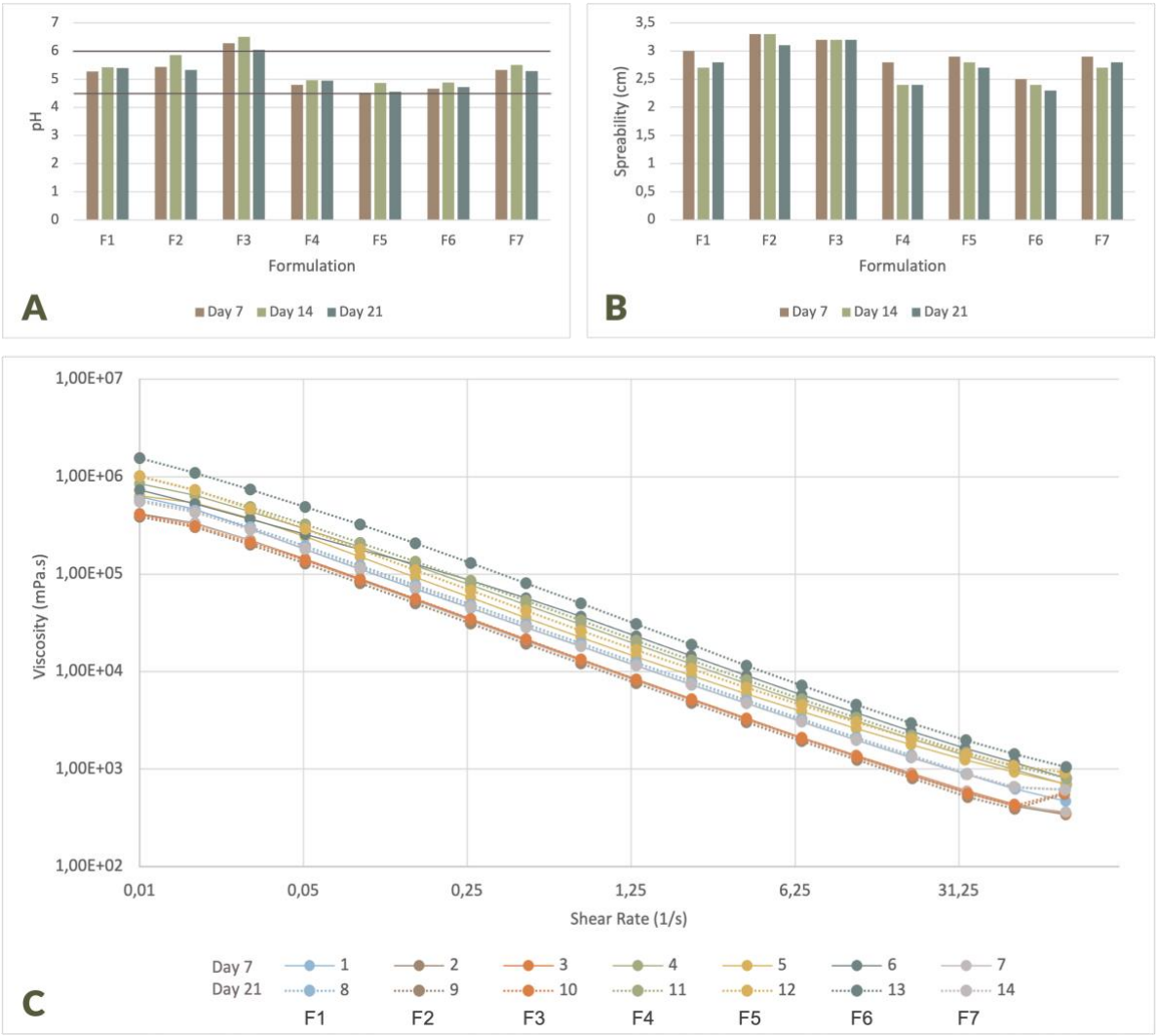

Figure 17- Results obtained for the various stability tests: A – pH test; B - Spreadability test; C - Viscosity (mPA.s) vs Shear Rate (1/s).

Finally, formulations were evaluated for compatibility with the skin to ensure that the created product did not induce any allergic reaction, such as irritation, redness, itching, or inflammation. The test was

conducted on seven individuals, and no reaction was seen. As a result, it is feasible to state that the sunscreen created is skin-compatible.

4.7 Lipid Oxidation

Certain components in the formulations produced are prone to lipid oxidation (LO). This is a common occurrence in formulations containing lipid-based components that alter the ultimate quality of the products. Lipid oxidation results in the development of undesirable odours and colour changes, as well as a reduction in the product's shelf life and performance. There is no precise method for determining the degree of oxidation of the formulations due to the complexity of the analysis. However, one of the most often utilized tests in the cosmetics industry is the determination of the peroxide value (PV). By quantifying the amount of peroxides present in the formulation, this value assists in evaluating the degree of fat and oil oxidation. Due to the fact that the substances are primary products of oxidation, their presence in the sample indicates that the product was oxidized (S. M. Ferreira & Santos, 2022). Peroxide values for various samples are shown in Figure 18.

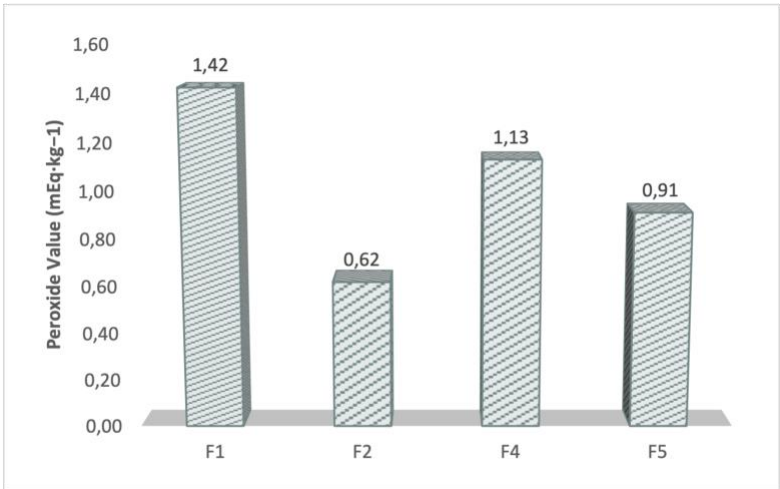

Figure 18- Peroxide value (PV), in milliequivalents of O_2 per sample kilogram, for the evaluated formulations: F1 – blank; F2 – BHT; F4 – Chestnut Shell Phenolic Extract 5% and F5 – Chestnut Shell Phenolic Extract 10%.

The peroxide value was determined in order to evaluate the efficacy of the phenolic extracts in lipid oxidation, in comparison with the synthetic antioxidant. A high PV value indicates that the extension of products lipid oxidation was high (S. M. Ferreira & Santos, 2022). As shown in Figure 18, F1 (base formulation) has the highest PV, implying that, as expected, this sample displayed a higher degree of LO. This result was anticipated, as no antioxidant agent was added, and hence no oxidation was prevented. Meanwhile, F2, the standard, has a lower PV due to the addition of BHT (synthetic antioxidant), which prevents and delays LO, hence preventing the formulation from deterioration. Antioxidants present in the formulation react with free radicals, reducing the oxidation of the products. On the other hand, formulations F4 and F5 — to which CS phenolic extract was added — had a greater PV value than F2. Nonetheless, the

readings are significantly lower than F1 (without the antioxidant), demonstrating that phenolic extracts can both prevent and postpone oxidation.

The results indicate that the addition of BHT (F2) to the formulation helps to prevent the formulations' lipids from oxidizing more. Nonetheless, formulations F4 and F5 demonstrated a high degree of resistance to oxidation. Notably, because BHT is a synthetic antioxidant, cosmetic regulation limits its use to 0.1–0.5 %. (S. M. Ferreira & Santos, 2022). Due to the fact that PEs may contain additional beneficial chemicals, such as vitamins, they can be added in greater quantities than BHT, hence increasing the formulations' resistance to oxidation. Although the literature is sparse, the results obtained indicate that the extent of LO was reduced. However, just because the PV is low does not mean that there has been no oxidation. As a result, in order to acquire more reliable and full information about the degree of LO in the formulations, PV determination should be supplemented with secondary oxidation product analysis. The most often used method for assessing MDA is the thiobarbituric acid (TBA) test, which involves spectrophotometric determination of the pink complex generated when TBA reacts with MDA (Thomsen et al., 2018).

As a result of the findings, phenolic extracts derived from chestnut shells may exhibit a strong antioxidant potential and possibly have the ability to protect the formulations from deterioration and oxidation. Additionally, it demonstrates that these extracts may work as antioxidants and may be used in place of synthetic compounds such as BHT, a commercial AO.

4.8 Sun protection Factor – Preliminary Studies

The SPF value was used to determine the efficacy of the prepared sunscreen emulsions in terms of UV radiation protection properties. The SPF values calculated for the prepared sunscreens are listed in Table 10. To calculate these values, the absorbance values at specific wavelengths were required; the absorption spectrum of these formulations is shown in Appendix D.

Table 10- SPF values for the formulations determined by the Mansur equation – Appendix D.

Formulation	F1	F3	F4	F5
SPF	0.02	3.75	0.23	0.85

F1- Blank; F3- Oxybenzone 6%; F4- Chestnut Shell Phenolic Extract 5%; F5- Chestnut Shell Phenolic Extract 10%.

After analysing Table 10, it is obvious F1 has no SPF, which makes sense given that it is the negative control. F3 formulations which contains a commercial UV filter exhibits a SPF of 3.75. When this value is compared to the values obtained for formulations containing phenolic extract from chestnut shells (F4 and F5), it is clear that F3 has a significantly higher SPF. Additionally, when comparing F4 to F5, it appears as though increasing the amount of CS phenolic extract resulted in an increase in photoprotection. One could conclude that chestnut shell extract possesses some photoprotective properties and could be used in topical formulations.

4.9 Photoprotection Evaluation – Preliminary Studies

The reactivity of a volunteer's skin to sunscreen formulations before and after one hour of exposure to sunlight is depicted in Figure 19. Because formulation F1 had no photoprotective ingredient, it was used as the negative control. As expected, the area of skin that received this formulation was the most irritated by UV rays. The F3 formulation showed the best response to UV light, which is understandable given its market status as a synthetic sunscreen. Nonetheless, formulas F4 and F5 appear to be moderately effective at protecting the skin from the sun, as the arm area where they were applied is less red than the area where formulation F1 was applied. Additionally, F6, a formulation containing chestnut shell phenolic extract and annatto seed extract, appears to be quite promising, as it seems that, following F3, this formulation best protected the skin and imparted a more tanned tone, rather than the reddish tone gained by the other formulations. While these are preliminary studies, they suggest that chestnut shell phenolic extract and annatto seed extract may be used as a source of photoprotection and aid in the pursuit of the perfect tan without putting one's health in danger.

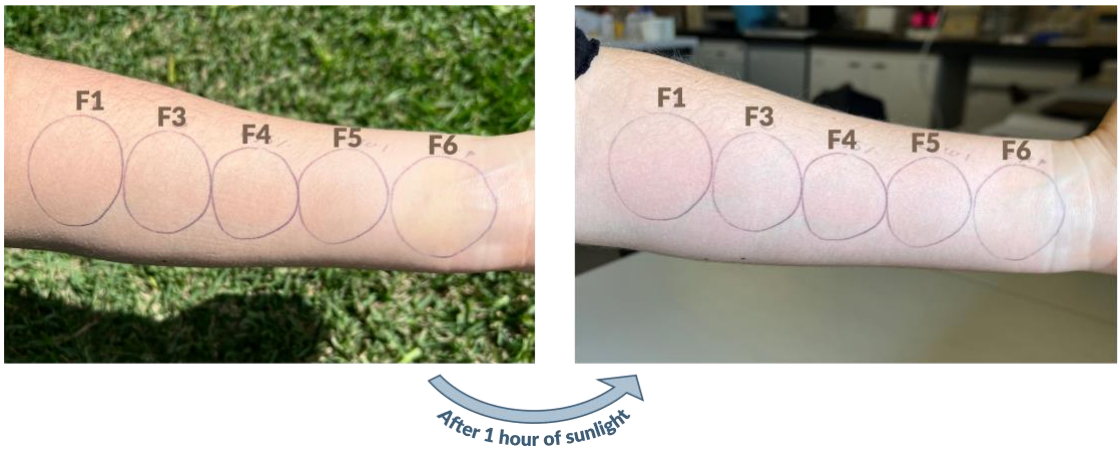

Figure 19- Volunteers' skin reaction before and after being exposed to sunlight for 1 hour: F1- negative control, F3- formulation containing 6 % of oxybenzone (synthetic UV filter) – positive control; F4- formulation containing 5 % of chestnut shell extract; F5- formulation containing 10 % of chestnut shell extract; F6- formulation containing 5 % of chestnut shell extract and 2.5 % of annatto seed extract.

5 Conclusion

The primary purpose of this dissertation was to investigate the feasibility of valuing agricultural by-products – chestnut shells – by incorporating them into a cosmetic product, specifically a sunscreen. Additionally, the incorporation of natural pigments to act as a tan accelerator was investigated. To extract phenolic compounds from CSs, two distinct methods for shell preparation were used. A portion of the shells was preserved as received in the original sample (OCS), while the remainder was lyophilized (LCS). The phenolic compounds in chestnut shells were extracted using the solid-liquid extraction method with a Soxhlet apparatus, with absolute ethanol, and yields of 26.79 % and 26.12 % were obtained, respectively. Although the composition of total phenolic compounds has not been determined, the literature indicates that CSs are abundant in phenolic compounds with antioxidant properties such as phenolic acids, flavonoids, and tannins. Additionally, pigments from annatto seeds were extracted using a conventional solid-liquid extraction method with acetone, and a 6.39 % yield was obtained. While this extract may not be a pure pigment and may contain additional components, the literature indicates that the primary pigment found in annatto seeds, Bixin, accounts for approximately 70-80 % of the extract.

The antioxidant capacity of the phenolic extracts was determined using the DPPH method and showed a percentage of DPPH inhibition of 88.99 % (OCS) and 79.88 % (LCS), and an IC_{50} of 13.52 (OCS) and 16.97 (LCS) $\mu g \cdot mL\ DPPH^{-1}$, indicating that the extract has a high antioxidant capacity. While the TPC composition was not determined, the literature states that an extract with high antioxidant capacity contains a significant amount of TPC.

Additionally, the extracts were incorporated into O/W formulations in different quantities to evaluate their antioxidant capacity, stability and sun protection factor (SPF). The pH of the formulations containing the extracts revealed values between 4.50 and 6, which is within the normal range for topical skincare products. Viscosity and spreadability were directly proportional, and the formulations remained stable during the 21 days of the study. In terms of lipid oxidation, the formulations containing the extracts had a lower oxidation level than the formulations without additives but had a higher level of oxidation when compared to BHT, a synthetic antioxidant. Nonetheless, the phenolic extract revealed a high antioxidant capacity, demonstrating that CSs are valuable because it enables the extraction of antioxidants from natural sources.

Finally, preliminary SPF studies revealed that formulations containing CS extract provide significantly less photoprotection than formulations containing the commercial synthetic UV filter, oxybenzone, for the same mass concentration. However, it was observed that increasing the concentration of the CS extract in the formulation resulted in a nearly fourfold increase in photoprotection, implying that the higher the extract concentration, the more the formulation protects against UV rays.

Overall, the results support the hypothesis that CSs may be a valuable source of phenolic compounds with antioxidant and photoprotective properties. As a result, CS extracts could be used in various personal care products, including daily moisturizers. Furthermore, the use of phenolic compounds extracted from CSs is critical for complying with circular economy principles. This is particularly relevant in Portugal,

where the chestnut industry is one of the main motors of the county's fruit economy development, therefore generating large amounts of by-products that enable the extraction of a significant quantity of value-added ingredients for use in cosmetics. Nonetheless, given the preliminary nature of these studies, additional research is needed to determine how CS extract can protect skin from UV damage and how annatto seed pigments can assist in tanning.

6 Assessment of the work done

6.1 Objectives Achieved

The work completed for this dissertation met the initial objectives. It was possible to extract value-added ingredients from agricultural by-products – chestnut shells – and demonstrate that their phenolic extract is rich in bioactive compounds, such as antioxidants. Moreover, while CSs may demonstrate some degree of photoprotection, additional research is necessary to substantiate this, as the timeframe allowed only for preliminary studies.

6.2 Future Suggestions

As a future work, it is suggested that the SLE method for CSs be optimized by examining several parameters that affect the extraction yield, including the extraction time, particle size, and type of extraction solvent, specifically the effect of water and ethanol:water mixtures on extraction. Additionally, HPLC/DAD analysis should be used to determine the TPC content. Moreover, it is recommended to employ additional methods for determining antioxidant activity, such as the ABTS and FRAP methods. Additionally, microencapsulation of the phenolic extract would be essential, as the antioxidant properties that make it attractive to the cosmetic industry are also its limitations, as they are prone to degradation and oxidation. Thus, employing this technique would increase the stability and protection of the compound while also allowing for a controlled release of the bioactive compounds.

Likewise, it is recommended to conduct additional studies on the formulations' stability, specifically in terms of lipid oxidation, using methods such as the malondialdehyde test, sun protection factor, light stability, and skin compatibility tests. Also, to determine the antibacterial activity, the disk diffusion test should be utilized. Moreover, natural extracts should also be tested for toxicity to determine the maximum percentage that can be added to formulations.

Finally, it would be interesting to investigate the incorporation of annatto seed oil into formulations rather than the extract, as the extract demonstrated a low solubility in formulations. Also, to better understand its effect when applied to the skin, in vitro human testing is suggested by inducing artificial UV light after applying formulations to the skin. To determine the effect of formulations containing annatto seed oil, colour measurements of the skin before and after UV exposure using the CM700d spectrophotometer or PR650 SpectraScan Colourimeter are ideal.

6.3 Final Assessment

Sustainability and natural cosmetics are two fields that have always intrigued me because they involve developing products that meet our beauty needs without causing harm to people or the environment. This project not only allowed me to research an area of interest and possibly contribute to a more sustainable approach to cosmetics but also to develop new laboratory skills and work methodology. Additionally, I was able to put many of the soft and hard skills that I acquired during my time at FEUP to use. Finally, this

study taught me that there is a vast array of agricultural by-products that enable the extraction of value-added properties that can be incorporated into cosmetics while minimizing waste and contributing to a circular economy.

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Appendix A - Experimental Procedure

A.1 Statistical Analysis

The statistical data were analysed using the arithmetic mean, which is based on the sum of the obtained values divided by the number of trials (n = 3), according to Equation (A 1.1). The standard deviation associated with these mean values was also calculated; this statistic indicates the average amount of variability in the dataset and was determined using Equation (A 1.2).

$$\overline{\boldsymbol{\varkappa}} = \frac{\sum_{i=1}^{n}(Valor)i}{n} \quad \text{(A 1.1)}$$

$$\sigma = \sqrt{\frac{\sum_{i=1}^{n}(Valor)i - \overline{\boldsymbol{\varkappa}}}{n-1}} \quad \text{(A 1.2)}$$

In order to validate the final results, they were submitted to statistical analysis, by performing a Student's t test, with a confidence level of 95 %, using the function *TEST.T(array1; array2; tails; type*), a *Microsoft Excel* tool. It is important to note that, the number "hypothesize" is the difference between the two population means. In this case, **0** is chosen in order to test whether or not the difference between the two populations means is 0. Thus, for *p* values larger than 0.05, the null hypothesis is not rejected. This indicates that there is a lack of adequate evidence to conclude that the two population means are different.

A.2 Extraction of Bioactive Compounds from Chestnut Shells

The extraction yield was calculated through the ratio between the weight of dry extract after extraction ($w_{extract}$) and the weight of the CS sample before extraction (w_{CS}), as shown in Equation (A 2.1).

$$\%\, Yield = \frac{w_{extract}}{w_{CS}} \times 100 \quad \text{(A 2.1)}$$

The chestnut shells supplied by Agromontenegro, the equipment used for Soxhlet extraction, the CS extract prior to evaporation, and the CS extract during evaporation in the rotary evaporator are depicted in Figure A 2.1.

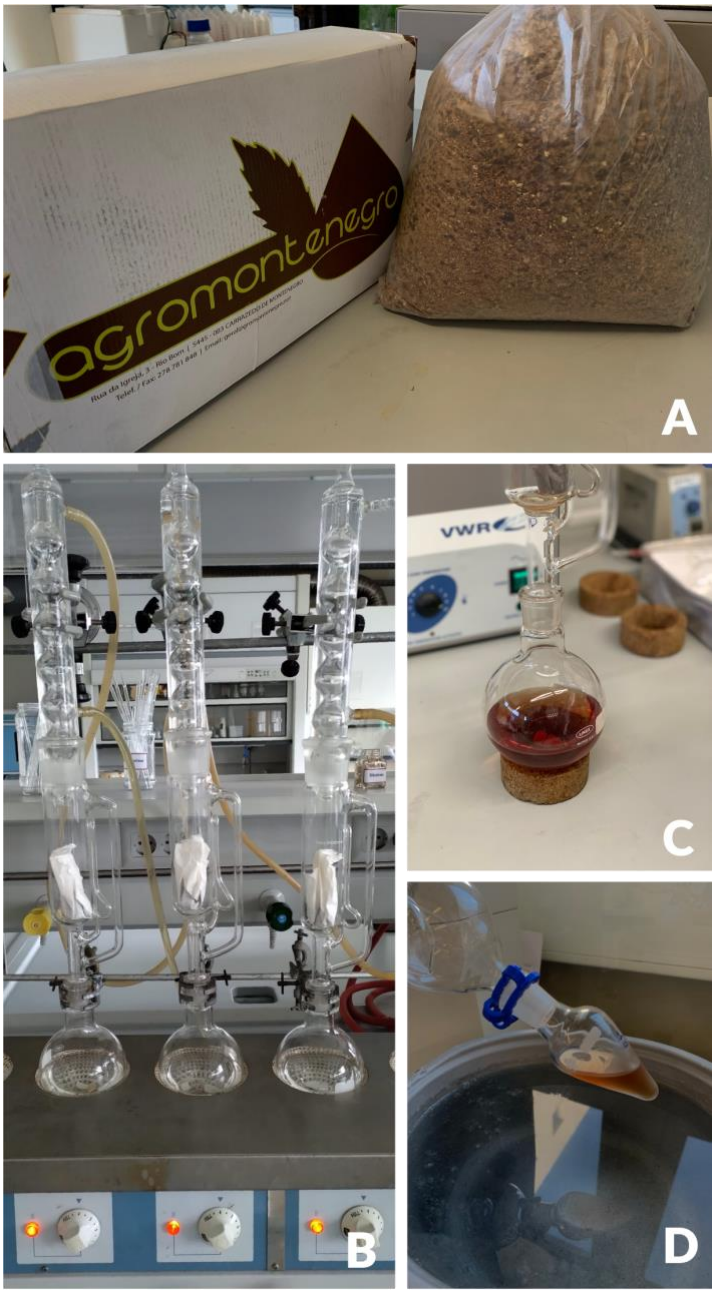

Figure A 2. 1- Chestnut shells supplied by Agromontenegro after being dehydrated for 6 days at 50 ºC (A); Assembly of solid-liquid extraction using the Soxhlet method, to obtain the chestnut shell phenolic extract (B); Chestnut shell extract prior to evaporation (C); and chestnut shell extract during evaporation in the rotary evaporator (D).

The UV spectrum of chestnut shell extracts (OCS and LCS) is depicted in Figure A 2.2.

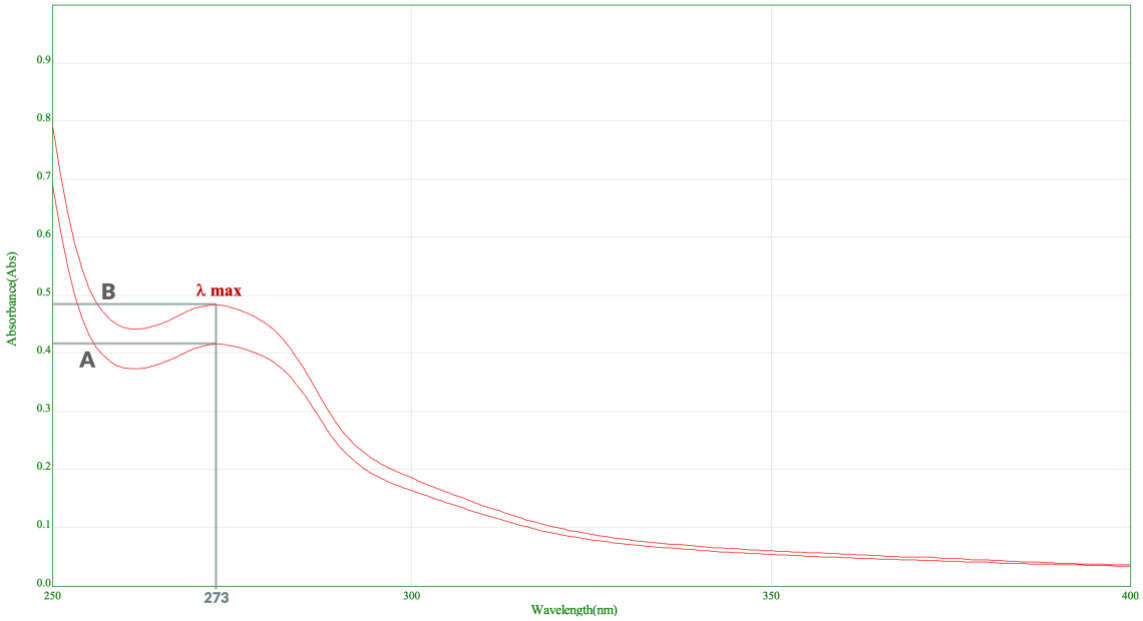

Figure A 2. 2- Absorption spectrum of chestnut shell extract: Original Chestnut Shell (OCS) sample (A); Lyophilized Chestnut Shell (LCS) sample (B). Both extracts have a λmax=273 nm.

A.3 Extraction of Pigments from Annatto Seeds

The extraction yield was calculated through the ratio between the weight of dry extract after extraction ($w_{extract}$) and the weight of the AS sample before extraction (w_{AS}), as shown in Equation (A 3.1).

$$\%\, Yield = \frac{w_{extract}}{w_{AS}} \times 100 \qquad \text{(A 3.1)}$$

Figure A 3.1 shows the glass oven used to dry the annatto seeds before solid-liquid extraction, the annatto seeds, the ultrasonic pre-treatment of AS and solvent before SLE, the filtration of the extract obtained after SLE, and the AS extract being evaporated in the rotary evaporator.

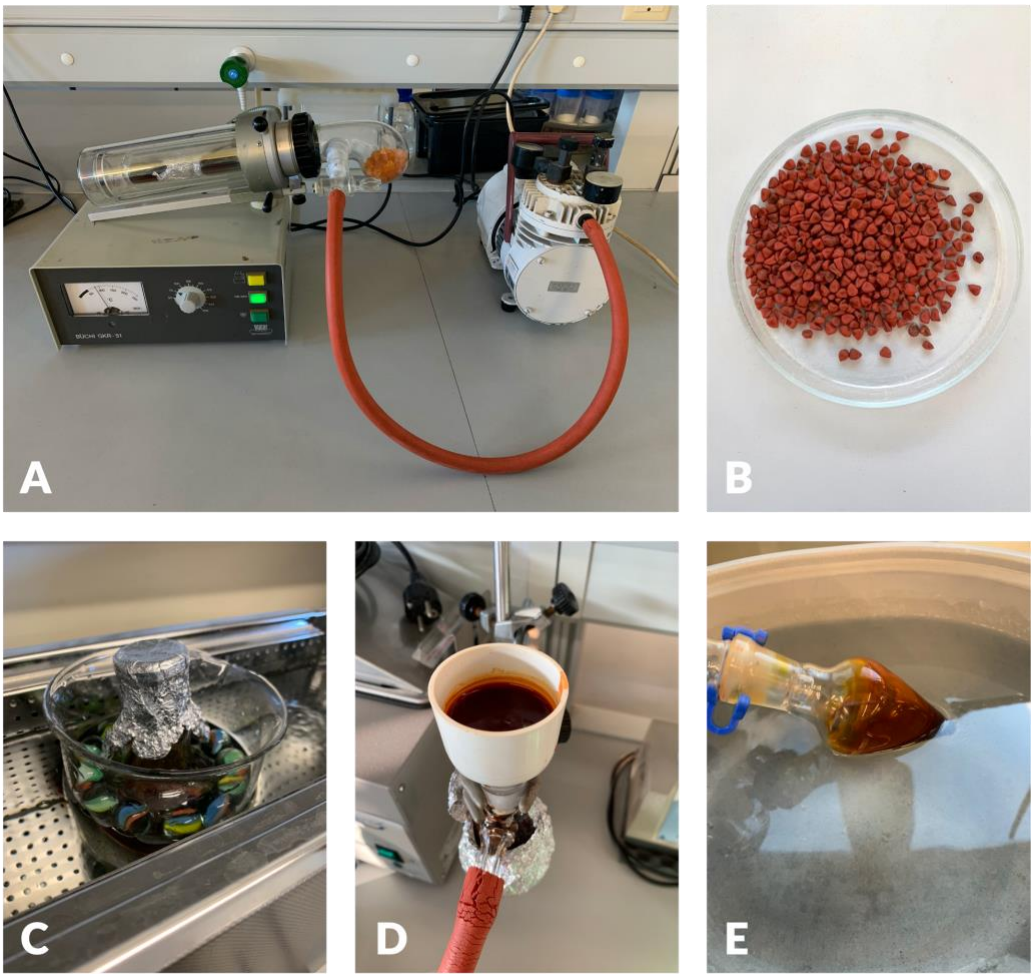

Figure A 3. 1- Annatto seed granules being dried in a titration drying oven (Buchi GKR-51) before solid-liquid extraction (A); Annatto seeds (B); Ultrasonication of annatto seeds and acetone before conventional solid-liquid extraction (C); Filtration of annatto seed extract obtained after solid-liquid extraction (D); and annatto seed extract being evaporated in the rotary evaporator (E).

Figure A 3.2 illustrates the UV spectrum of annatto seed extract.

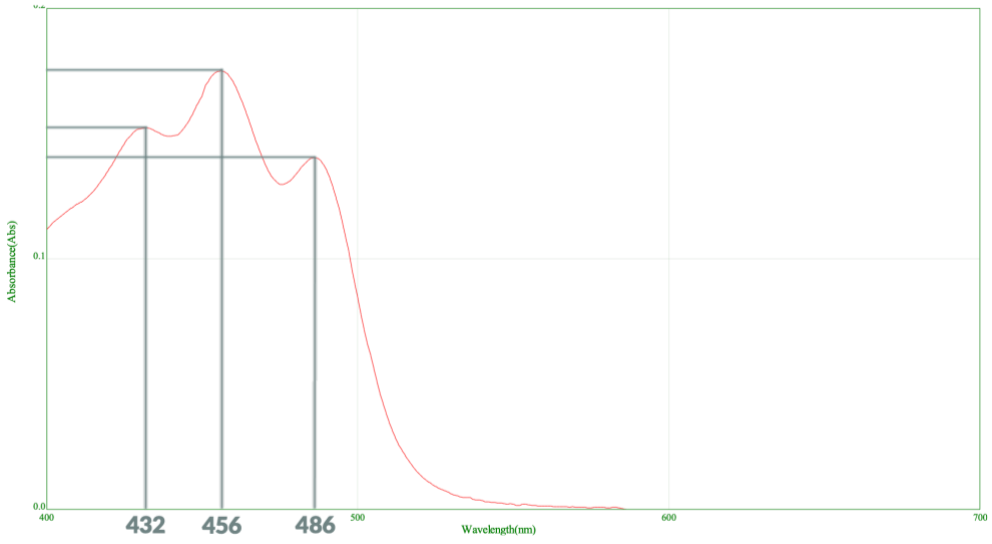

Figure A 3. 2- Absorption spectrum of annatto seed extract, λmax=432;456;486 nm

A.4 Sunscreen Production

The various formulations were produced using a thermostatic bath to heat the two phases, which was followed by a high-performance homogenizer. Figure A 4.1 illustrates the thermostatic bath and the formulations before and after being subjected to the high-performance homogenizer.

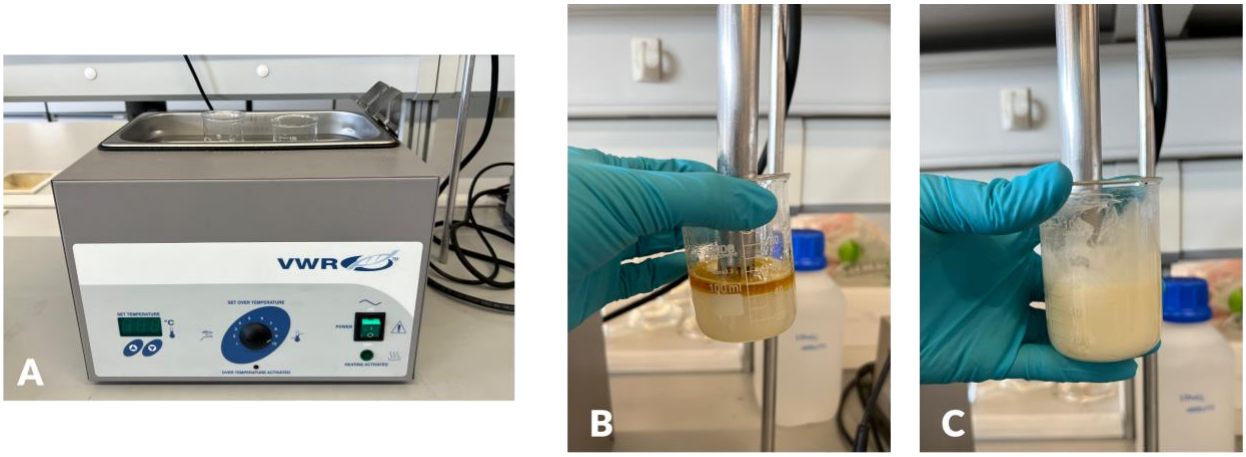

Figure A 4. 1- Thermostatic bath used to heat the two phases (A); O/W emulsion before being subject to the high-performance homogenizer (B); and homogenized formulation (C).

Appendix B - Antioxidant Capacity

B.1 DPPH Method

To perform the DPPH method, it was necessary to use a microplate for the subsequent measurement of absorbance values at λ = 515 nm. A microplate assembly is represented in Figure B 1.1.

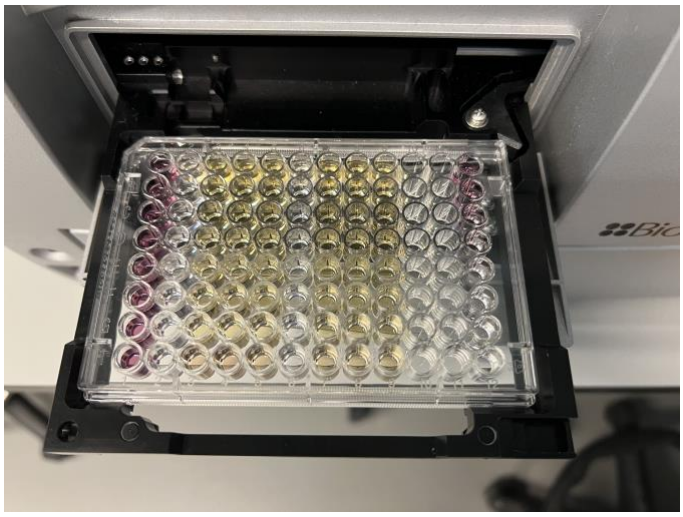

Figure B 1. 1- Microplate used to perform the DPPH method..

Equation (B 1.1) was used to calculate the percentage of DPPH inhibition (% I):

$$\%\, I = \frac{Acontrol - Asample}{Acontrol} \times 100 \qquad \text{(B 1.1)}$$

Where, $A_{control}$ is the absorbance of the control, that is, the well containing DPPH and no sample, and A_{sample} is the absorbance of the sample (difference between the absorbance of the sample with DPPH and the sample with methanol).

Appendix C - Formulations Evaluation

C.1 Stability Tests

Following the formulations' production, it was necessary to assess their stability. Thus, in Figure C 1.1, some of the equipment used to conduct these tests is illustrated.

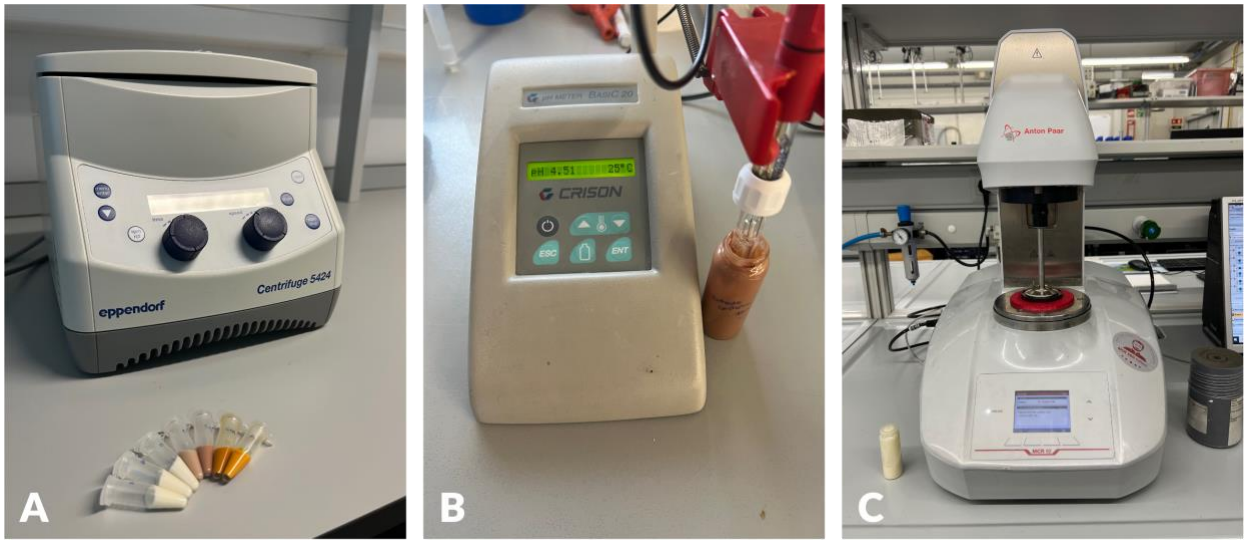

Figure C 1. 1- Equipment used to carry out the stability tests. A – Centrifuge (Centrifuge 5424, Eppendorf); B – pH meter (XS Instruments); C – Rheometer (MCR 72/92 Anton Paar).

The pH, viscosity, and spreadability of the formulations were determined over a 21-day period. The values for the pH and spreadability parameters obtained during the analysis period are listed in Table C 1.1.

Table C 1. 1- pH and spreadability (cm) values over a 21-day period for the different formulations produced.

Formulation	pH			Spreadability (cm)		
	Day 7	Day 14	Day 21	Day 7	Day 14	Day 21
F1	5.27	5.42	5.39	3.00	2.70	2.80
F2	5.43	5.85	5.33	3.30	3.30	3.10
F3	6.27	6.50	6.05	3.20	3.20	3.20
F4	4.80	4.96	4.95	2.80	2.40	2.40
F5	4.51	4.87	4.56	2.90	2.80	2.70
F6	4.67	4.88	4.72	2.50	2.40	2.30
F7	5.33	5.50	5.28	2.90	2.70	2.80

C.2 Lipid Oxidation

Regarding the oxidative stability of the formulations, this parameter was evaluated with the determination of the peroxide value (PV), determined using Equation (C 2.1).

$$Peroxide\ Value = \frac{A_{sample} - A_{blank} \times 41{,}52}{55{,}84 \times w_s \times 2} \quad \text{(C 2.1)}$$

Where A_{sample} and A_{blank} refer to the absorbance values of the sample and the blank, respectively; 12.26 is the value of the slope of the calibration curve Fe^{3+} vs. absorption, w_s corresponds to the weight of sample added (g), 55.84 is the molar mass of iron and the division by 2 gives milliequivalents of peroxides instead of milliequivalents of oxygen.

Appendix D - Sun Protection Factor

D.1 Sunscreens UV Spectrum

Figure D 1.1 shows the UV spectrum of the sunscreens.

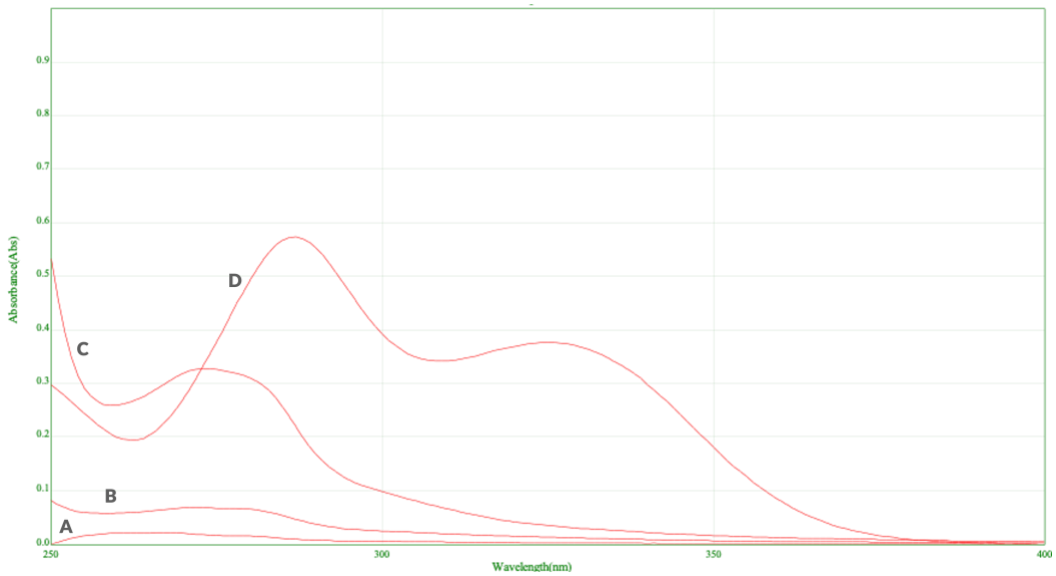

Figure D 1. 1- Absorption spectrum used to determine the sun protective factor (SPF) of formulations: A- formulation F1 (blank); B- formulation F4 (5% of chestnut shell extract); C- formulation F5 (10% of chestnut shell extract); D- formulation F3 (6% of oxybenzone).

Figure D 1.2 illustrates the UV-Vis spectrophotometer used to obtain the absorption spectrums.

Figure D 1. 2- Spectrophotometer used to obtain the absorption spectrum - UV-3100PC Spectrophotometer.

D.2 SPF Calculation

The Mansur equation was used to determine the SPF values of the formulations:

$$\text{SPFspectrophotometric} = CF \times \sum_{290}^{320} EE(\lambda) \times I(\lambda) \times Abs(\lambda) \qquad \text{(D 2.1)}$$

where: EE (I)–erythemal effect spectrum; I (l)–solar intensity spectrum; Abs (l)–absorbance of sunscreen product; CF-correction factor (=10). EE*I values are constant and given in Table D 2.1.

Table D 2. 1- Normalized product function used in the calculation of SPF.

Wavelength (λ) in nm	EE*I (normalized)
290	0.0150
295	0.0817
300	0.2874
305	0.3278
310	0.1864
315	0.0839
320	0.0180
	1

Source: Donglikar & Deore, 2016