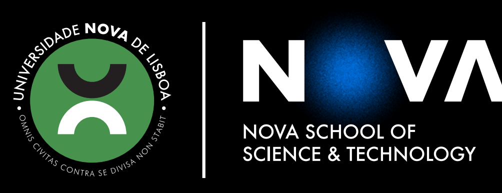

DEPARTAMENT OF CHEMISTRY

ANA CAROLINA RAMOS DA COSTA

BSc in Applied Chemistry

DESIGN OF A COSMETIC FORMULATION WITH A DEEP EUTECTIC SYSTEM COFFEE EXTRACT

MASTER IN BIOTECHNOLOGY

NOVA University Lisbon

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ANA CAROLINA RAMOS DA COSTA

BSc in Applied Chemistry

Adviser: Doutor Alexandre Babo de Almeida Paiva,
Assistant Researcher, NOVA School of Science and Technology

Co-advisers: Professora Doutora Joana Marques Marto,
Assistant Professor, Faculty of Pharmacy, University Lisbon

Examination Committee:

Chair: Professora Doutora Ana Cecília Afonso Roque,
Associate Professor , NOVA School of Science and Technology

Rapporteur: Doutora Márcia Filipa Carvalho dos Santos,
NOVA School of Science and Technology

Adviser: Doutor Alexandre Babo de Almeida Paiva,
Assistant Researcher, NOVA School of Science and Technology

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Design of a cosmetic formulation with a deep eutectic system coffee extract

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ABSTRACT

Over the world approximately are produced 6 million tons per year of spent coffee grounds (SCG). These are a valuable resource of bioactive compounds, such as antioxidant phenolic compounds, which makes them potential ingredients in topical formulations. One of the biggest problems in the cosmetic industry is the large quantities of conventional organic solvents used. A green sustainable alternative is using Deep Eutectic Systems (DES). This work aims to valorize SCG extracts, by optimizing the extraction process of phenolic compounds with antioxidant activity, using natural DES to be incorporated directly in novel and sustainable Eco Label topical formulations. Several DES were produced and evaluated for the green extraction of the bioactive compounds from SCG. Extracts were assessed for total phenolic content (TPC), antioxidant activity (AA), *in vitro* ROS scavenging activity, cytotoxicity assays in human keratinocyte HaCaT cells, and stability over time. The optimal extraction conditions were stirring at 50ºC, for 1h and a solid/liquid ratio 1:10 (g/ml). DES with acids in their composition were toxic against HaCaT cells, while the remaining DES showed low cytotoxicity. An optimal extract was obtained with a DES composed of Proline: Glycerol: Water, which had higher TPC (16.72 mg GAE/g SCG), high AA (EC_{50} =0.20 g/mL), high capacity to reduce ROS (> 75%), ability to inhibit HNE activity, and demonstrated stability upon a time, than conventional hydroalcoholic extraction. Oil-in-water (O/W) emulsions were developed according to the Eco-Label regulation. After preparation, emulsions were characterized for their organoleptic, physicochemical, rheological, and spreadability properties. With this work, it was possible to develop emulsions suitable for topical application containing SCG extracts, compatible with the skin and with biological properties. SCG extracts prepared with DES, namely the optimal DES extract, have valuable properties that benefit their upcycling in the cosmetic industry.

Keywords: Spent coffee grounds, Deep eutectic solvents, Extraction, Phenolic Compounds, Topical formulation

Resumo

No mundo são produzidos aproximadamente 6 milhões de toneladas por ano de borra de café (SCG). Esta é rica em compostos bioativos, como os compostos fenólicos antioxidantes, o que os torna potenciais ingredientes nas formulações tópicas. Um dos maiores problemas da indústria cosmética são as grandes quantidades de solventes orgânicos convencionais utilizados. Uma alternativa sustentável verde é o uso de Deep Eutectic Systems (DES). O objetivo deste trabalho é a valorização de extratos de SCG, através da otimização do processo de extração de compostos fenólicos com atividade antioxidante, utilizando DES naturais para serem incorporados diretamente em novas e sustentáveis formulações tópicas Eco-Label. Vários DES foram produzidos e avaliados para a extração sustentável dos compostos bioativos dos SCG. Os extratos foram avaliados quanto ao conteúdo fenólico total (TPC), atividade antioxidante (AA), atividade de redução de ROS *in vitro*, ensaios de citotoxicidade em células de queratinócitos humanos HaCaT e estabilidade ao longo do tempo. As condições ótimas de extração foram agitação a 50ºC, por 1h e proporção sólido/líquido 1:10 (g/ml). Os DES com ácidos na sua composição apresentaram toxicidade contra células HaCaT, enquanto os DES restantes apresentaram baixa citotoxicidade. O extrato ótimo foi obtido com DES composto por Prolina:Glicerol:Água o qual apresentou maior TPC (16.72 mg GAE/g SCG), elevada AA (EC_{50} = 0.20 g/mL), alta capacidade de redução de ROS (>75%), capacidade de inibir a atividade da enzima HNE, e demonstrou estabilidade ao longo do tempo, comparando com o extrato convencional hidroalcoólico. Emulsões óleo-em-água (O/A) foram desenvolvidas de acordo com o regulamento Eco-Label. Após a preparação, as emulsões foram caracterizadas quanto às suas propriedades organolépticas, físico-químicas, reológicas, e espalhabilidade. Com este trabalho foi possível desenvolver emulsões adequadas para aplicação tópica, compatíveis com a pele e com propriedades biológicas. Extratos de SCG preparados com DES, como o melhor extrato de DES, possuem propriedades que beneficiam o upcycling na indústria cosmética.

Palavas chave: Borra de café,Solventes eutéticos, Extração, Compostos fenólicos, Formulação tópica

CONTENTS

1 INTRODUCTION 1

1.1 The cosmetic industry 1

1.1.1 Sustainability in the cosmetic industry 4

1.2 Concept of circular economy and upcycling 6

1.3 Extraction of bioactives compounds from by-products 7

1.4 Deep eutetic systems (DES) 9

1.4.1 DES in the cosmetic industry 11

1.5 Valorization of bioactive compounds from by-products 13

1.5.1 Spent coffee grounds (SCG) 14

1.6 Objectives 18

2 MATERIALS AND METHODS 21

2.1 Materials 21

2.2 Extraction and chemical analyses 22

2.2.1 Spent coffee grounds (SCG) 22

2.2.2 Preparation of DES 22

2.2.3 Extraction with DES 23

2.2.4 Hydroalcoholic extraction 24

2.2.5 Determination of Total Phenolic Content 24

2.2.6 Determination of Antioxidant activity 25

2.2.7 *In Vitro* Efficacy and Safety Analysis DES and DES Extracts 25
2.2.7.1 Cell viability studies 25
2.2.7.2 Reactive Oxygen Species (ROS) Production Measurement 26
2.2.7.3 Human Neutrophil Elastase (HNE) Enzymatic Inhibition Assay 26
2.2.8 High-Performance Liquid Chromatography (HPLC) Analyses 27
2.2.9 Liquid chromatography–Mass spectrometry (LC-MS) Analyses 27
2.2.10 Stability assay 28
2.3 Topical formulations development 28
2.3.1 Formulation development strategy 28
2.3.2 Manufacturing process 31
2.4 Physical and chemical characterization of emulsions 31
2.4.1 Appearance, pH determination, and physical stability 31
2.4.2 Droplet size analysis 32
2.4.3 Structure analysis of emulsions 32
2.4.3.1 Rotational viscosity measurements 32
2.4.3.2 Oscillation frequency test 32
2.4.3.3 Adhesion test 32
2.4.3.4 Tribology 33
2.4.4 Spreadability 33
2.4.5 Microscopic analysis 33
2.4.6 Packaging performance tests - Criterion 5 from Ecolabel regulation 33
2.4.7 Stability assay 34
2.5 *In Vivo* Efficacy and Safety Analysis 34
2.5.1 Human Repeat Insult Patch Test (HRIPT) – Safety Evaluation 34
2.5.2 Biological Effects (Hydration Test) – Efficacy Evaluation 35
2.6 Upcycling the Spent coffee Grounds from this work 35
2.6.1 Physical characterization of SCG 36

2.6.2 SCG oil extraction and reuse of SCG 36
2.6.3 Development of scrub 37
3 RESULTS AND DISCUSSION 39
3.1 Extraction and chemical analysis 39
3.1.1 Spent coffee grounds 39
3.1.2 Deep Eutectic Systems (DES) 40
3.1.3 Spent Coffee Ground: Total phenolic content 40
3.1.4 Spent Coffee Ground: Antioxidant activity 43
3.1.5 *In Vitro* Efficacy and Safety Analysis 44
3.1.5.1 Cell viability Assays 44
3.1.5.2 Reactive Oxygen Species (ROS) Production Measurement 46
3.2 Final DES Spent coffee grounds extracts 48
3.2.1 Stability assay 48
3.2.1.1 Total phenolic content and Antioxidant activity 48
3.2.1.2 Liquid Chromatography–Mass Spectrometry (LC-MS) 52
3.2.1.3 High-Performance Liquid Chromatography (HPLC) 54
3.2.2 *In Vitro* Efficacy and Safety Analysis 55
3.2.2.1 Cell viability assays 55
3.2.2.2 Reactive Oxygen Species (ROS) Production Measurement 55
3.2.2.3 Human neutrophil elastase (HNE) Enzymatic Inhibition Assay 57
3.3 Topical formulations 58
3.3.1 Final formulations 58
3.4 Physical and chemical characterization of emulsions 59
3.4.1 Appearance, pH determination, and physical stability 59
3.4.2 Droplet size distribution analysis and microscope structure of emulsions 60
3.4.3 Structure analysis of emulsions 61
3.4.3.1 Rotational viscosity measurements 61

3.4.3.2 Oscillation frequency test ... 64
3.4.3.3 Adhesion test and Spreadability ... 64
3.4.3.4 Tribology ... 65
3.5 Packaging performance tests - Criterion 5 from Ecolabel regulation ... 66
3.6 Stability study ... 68
3.7 *In Vivo* Efficacy and Safety Analysis ... 72
3.7.1 Human Repeat Insult Patch Test (HRIPT) – Safety Evaluation ... 72
3.7.2 Biological Effects (Hydration Test) – Efficacy Evaluation ... 72
3.8 Upcycling the Spent coffee Grounds from this work ... 73
3.8.1 Physical characterization of SCG ... 73
3.8.2 SCG oil extraction and reuse of SCG ... 73
3.8.3 Development of scrub ... 74
4 CONCLUSION ... 75
5 AN APPENDIX ... 89
5.1 HPLC calibration curves ... 89
5.2 HPLC chromatograms results upon time for extract I ... 91
5.3 HPLC chromatograms results upon time for extract N ... 93
5.4 Droplet Size distribution upon time-Stability assay ... 96

LIST OF FIGURES

Figure 1.1-Diagram with phase behavior of melting point versus the molar ratio of solid components A and B. Adapted [44] 10
Figure 1.2- Chemical structure of chemical compounds present in SCG: A-Chlorogenic acid; B-Caffeine. Designed with *ChemDraw* 16
Figure 2.1-Scheme: validation and/or exclusion method for cosmetic formulations 29
Figure 2.2- Scheme: Circular Economy and Upcycling approach used in the work 36
Figure 3.1-Sample of Spent Coffee Grounds with origin in the French Press (FP) 39
Figure 3.2-Optimization of the extraction time with DES 2, with an SLR of 1:10, at 50ºC 43
Figure 3.3- The percentage of cell viability of HaCat cell line exposed to different concentrations of DES samples for 24h of exposition time (Mean±SD; n=9) 45
Figure 3.4- The percentage of ROS reduction in HaCaT cells (500 µM of H_2O_2) by the different samples of DES extracts at 10% and 5% V/V (Mean ±SD, n=9) 46
Figure 3.5-The percentage of ROS in the function of logarithm concentration of compounds in HaCaT cells after 500 µM of H_2O_2 exposition, for extracts A, I, M, N, and P, (Mean ±SD, n=9) 47
Figure 3.6-SCG extracts for stability assay, with SLR of 1:10, at 50ºC, for 60 min, with FP sample: 1-N, 2-I, and 3- Conv (Conventional Hydroalcoholic extract) 49
Figure 3.7-Stability assay-TPC and EC_{50} results for extracts I, N, and Conventional Hydroalcoholic extraction (Conv) 51
Figure 3.8-Mass spectrometry (MS) spectrum for sample EI (t=0) T.r.23.99 min corresponding to caffeine 52
Figure 3.9-Mass spectrometry (MS) spectrum for sample EI (t=0) T.r.7.6 min corresponding to CQA: A-Zoom on positive mode; B- Negative mode. 53

Figure 3.10- The percentage of cell viability by MTT endpoint, of HaCaT cell line exposed for 24h to 100, 50, 25, and 12.5 μg/mL of DES 11, 13, extracts I, N, and conventional hydroalcoholic. (Negative control was cell culture medium and positive control SDS 1mg/mL) (Mean ±SD, n=9) 55
Figure 3.11-The percentage of reduction of reactive oxygen species (ROS) formation (%) in HaCaT cells exposed to (A) hydrogen peroxide (500μM), or (B) UVB light for 15 min. An ascorbic acid solution (1 mg/mL) was used as positive control. (Mean ±SD, n = 9), for extracts I, N and conventional hydroalcoholic 56
Figure 3.12-The percentage of HNE activity in the function of logarithm concentration for extract I, N, and conventional hydroalcoholic (Mean ±SD, n = 2) 58
Figure 3.13-Macroscopic aspect of the validated formulations: 1- Placebo; 2-E13; 3-E11; 4-EI; 5-EN 59
Figure 3.14-Droplet size distribution of final emulsions (mean, n=6) 60
Figure 3.15-Images obtained by microscopy for the emulsions in study- A) Placebo with 40x magnification; B) E13 with 40x magnification; C) EN with 40x magnification; D) EI with 40x magnification; E) E11 with 40x magnification 61
Figure 3.16-Viscosity flow behavior of emulsions in study 62
Figure 3.17-Viscosity flow behavior of emulsions in study for stress ramp test 63
Figure 3.18-Results of the Oscillation frequency sweep test for emulsions in study 64
Figure 3.19-Results of Tribology test for emulsions in study 66
Figure 3.20-Particles from scrub characterization: A-Particle distribution (mean.n=6); B- Image obtained by microscopy with 20x magnification. 73

LIST OF TABLES

Table 1.1-Principal components of emulsions and their functions 2
Table 1.2-Types of DES. Adapted [31, 47] 10
Table 1.3-Example of comparison between the composition of Coffee beans and spent coffee grounds. Adapted [79] 15
Table 1.4-Examples of extraction techniques using DES from spent coffee grounds 18
Table 2.1-Summary of all the DES prepared. 23
Table 2.2-Qualitative and quantitative composition of emulsions: Placebo-Control; E13-Control with DES 13; E11-Control with DES 11; EI-With Extract from DES 13; EN- With Extract from DES 11 30
Table 2.3- Ingredients in SCG Scrub 37
Table 3.1-Elemental composition of SCG from FP.. 40
Table 3.2-TPC in mg GAE/g SCG present in the SCG extracts with different DES and same experimental conditions in time and temperature, 60 minutes and 50ºC and different solvent/solid ratio 41
Table 3.3- TPC in mg GAE/g SCG present in the SCG extracts, with the same SLR of 1:10 and time extraction of 60 minutes- optimization conditions with DES 2 42
Table 3.4-TPC for conventional extraction with a solution of water and alcohol (1:1), at 50ºC, for 60 minutes, with an SLR of 1:10. 43
Table 3.5-Determination of EC_{50} present in the SCG extracts with different samples............. 44
Table 3.6-Compounds quantification by HPLC in mg/g SCG for extracts................................ 54
Table 3.7-Droplet size distribution results of final formulations (mean±SD, n=6).................... 61
Table 3.8-Adhesive properties of the emulsions in study at 25ºC (mean±SD, n=6); Spreadability properties of the emulsions in study (mean±SD, n=3). 65

Table 3.9- Value of R (%) for different containers made with different materials: glass, plastic, and aluminum 67
Table 3.10-Stability test results for formulations placebo, E11, E13, EI and EN during 90 days at 25 and 40ºC 69
Table 3.11- Droplet size distribution (mean,SD, n=6) results for formulations placebo, E11, E13, EI and EN during 90 days at 25 and 40ºC 71
Table 3.12-Skin hydration results for Control area and emulsion EI (mean±SD, n=10). 72

ABBREVIATIONS AND SYMBOLS

CE	Circular Economy
ChCl	Choline chloride
CO_2	Carbon dioxide
CQA	Chlorogenic acid
DES	Deep eutectic systems
DMSO	Dimethyl sulfoxide
DPPH	2,2-diphenyl-1-picrylhydrazyl
GAE	Gallic acid equivalence
H_2O	Water
HaCaT	Human keratinocyte cell line
HBA	Hydrogen bond acceptor
HBD	Hydrogen bond donor
HNE	Human neutrophil elastase
HPLC	High-Performance Liquid Chromatography
ILs	Ionic Liquids
LCA	Life Cycle Assessment
LC-MS	Liquid chromatography–mass spectrometry
LCT	Life Cycle Thinking

MAE	Microwave assisted extraction
NaDES	Natural Deep Eutectic Systems
O/W	Oil in Water
Pa	Pascal
PLE	Pressurized liquid extraction
ROS	Reactive oxygen species
SCG	Spent coffee grounds
SDS	Sodium dodecyl sulfate
SFE	Supercritical fluid extraction
SLR	Solid-liquid ratio
SPF	Sun Protection Factor
SWE	Subcritical water extraction
UAE	Ultrasound-assisted extraction.
UV	Ultraviolet light
W/O	Water in oil
$\dot{\gamma}$	Shear Rate
σ	Shear Stress
η	Viscosity

1

INTRODUCTION

1.1 The cosmetic industry

Industrialization has led to an improvement in the quality of human life, however with the chemicals and processes used there is also the production of large quantities of waste, which are affecting the world as pollutants. [1] For the last two decades, the Cosmetic Industry has expanded approximately 4.5% per year worldwide, [2] and the European cosmetic market appraised by 80 billion in 2021.[3]

Cosmetics, are defined according to the European regulation on cosmetics [4] and cosmetic products for the skin have a variety of functions, like repairing damaged skin, reversing signs of aging, or providing ultraviolet protection, which is associated with ingredients with antioxidants.[5] Under the preceding regulations, several classes of personal care products and cosmetics are considered. Emulsions for the skin are an example of cosmetics. [4]

Emulsions were discovered in 1925 by William Seifriz and according to IUPAC they are a colloidal system consisting of two or more immiscible liquids, dispersed one in the other, the droplets of the internal phase in the external phase. These can be organized depending on the type of internal and external phases. If the internal phase is aqueous and the external phase oily, it is a water-oil emulsion (W/O), if the inverse is an oil-water emulsion (O/W). [6,7].

Oil-in-water emulsions are the most common as they are not greasy, spread quickly, and can also be simple cleaned with water. By contrast, W/O emulsions are considered greasy and are used to avoid evaporation of moisture from the skin surface. According to their consistency and viscosity, they can be categorized as liquid formulations, lotions, semi-solid formulations, or creams. Emulsions can be classified according to their droplet size, as macroemulsions or

nanoemulsions. The first ones have droplets larger than 1μm to 100μm, and the others have a range between 10 and 1000 nm. [8,9,10]

The development of formulations is dependent on the choice of materials, as they can affect the final product, for example, stability and viscosity. Plants, vegetables, and by-product extracts may be used as biologically active ingredients in cosmetic formulations. [8,9,10]Some of the main components of emulsions are outlined in Table 1.1.

Table 1.1-Principal components of emulsions and their functions

Name	Function	Reference
Water	Conductive medium.	[9]
Emulsifiers	Stabilize the emulsion, avoiding the coalescence of the dispersed phase droplets. Improve emulsification while manufacturing and control rheological properties.	[9]
Preservatives	Protect from microbial proliferation, increase shelf life, and may be natural or synthetic in origin.	[11]
Humectants	Hydrophilic substances create hydrogen bonds with water molecules to keep the skin moist. Are generally added to emollients to improve their ability to hydrate.	[12]
Emollients	Prevent water loss from the skin and create a physical barrier on the skin. Moisturizing ingredients which soften the skin.	[12]
Thickeners	Increase viscosity or stabilize the emulsion	[13]

Emulsions are considered thermodynamically unstable systems, and this is related to the high interfacial tension between the two immiscible phases. When the conditions are optimized, such as the concentration of the materials, the emulsification conditions, and the time required for mixing and rate, it is possible to have a stable emulsion. One of the most important factors in cosmetic emulsions is the sensory feeling in the skin. When formulating, the methodology must prevent phase separation, a long shelf-life of the product, the biodegradability of the materials, and biocompatibility with skin. Instability may be due to chemical changes in components or the presence of microorganisms. [13, 14] There are parameters such as pH, droplet size, rheology and spreadability that can be evaluated to infer about stability of the emulsion. Concerning the pH, it is mainly acidic to contribute to the normal functioning of the skin, so all of these topical formulations should have a pH value near to the range. [15]

Droplet size is a method that infers the stability of an emulsion. For an emulsion to be considered stable, droplets need to be smaller and evenly distributed. [9,15]

Rheology is a science that studies the rheological properties of matter, its deformity, and fluidity. Analyzes the behavior when a body undergoes a deformation by the action of stress. Rheometric tests can be conducted to determine the properties of a material, such as viscosity and oscillation. The types of flow are shear and extensional. Shear flows are the most common, the elements flow or shear over each other. Regarding extensional, flows to or away from each other. In order to have a shear flow, an external force takes action on the fluid, which has the form of shear stress, a force per unit area. [17, 18]

Viscosity (**η**) represents the resistance that a given substance presents to the flow or movement, when higher, lower the rate at which the fluid travels. It can be determined by the coefficient of proportionality between the shear stress (σ) and shear rate ($\dot{\gamma}$), and it is expressed in the S.I. system in Pa.s. [17, 18]

Depending on the properties of the flow, the materials can exhibit Newtonian or Non-Newtonian behavior. In the case of Newtonian behavior, the viscosity is constant and does not depend on the applied force, and one example is water. However, if the behavior is non-Newtonian, the viscosity is not constant, varying with the increase in applied force. These fluids can be classified as pseudoplastic, where the viscosity decreases with increasing shear rate, and dilatant, the opposite. It is also important to notice that the viscosity of an emulsion is higher, when the smaller the droplet size of the oil phase is and the smaller the dispersion of its distribution, making it stable. [17, 18, 19]

The oscillatory mode applies stresses that do not cause irreversible destruction of material structure. A small-amplitude oscillatory shear test, to measure viscoelastic properties, is performed. The sample is oscillated around its equilibrium position in a continuous cycle, equivalent to a 360° rotation. The relationship between the applied stress and the measured stress results in the complex modulus (G*), which measures the material's stiffness or resistance to deformation. This is constituted by the elastic modulus (G′), the real component of the complex modulus, as it represents the energy storage, added with the dissipative modulus (G″). When the elastic modulus is higher than the dissipative modulus, solid behavior predominates, and when the opposite happens, liquid behavior predominates. [17, 18]

Concerning the emulsification process, the rate of cooling or heating, and the order in which the components are blended may influence the emulsions. The conventional emulsification method is a hot process, on the other hand, cold emulsification is easier to control since there is no process of cooling down after heating, and the time of production reduces, besides the consumption of energy and water. With the aim of having a greener process, all

components must be liquids or soluble, which makes the process more advantageous. By reducing energy consumption, there are fewer CO_2 emissions, and less water waste, which in the end reduces the impact on the environment and carbon footprint. [19]

1.1.1 Sustainability in the cosmetic industry

A study showed that women use an average of 12 cosmetic products daily, containing 168 different chemicals. [20] Over 3000 chemicals are used to formulate a large range of fragrances used in products worldwide, and manufacturers are constantly developing new products to meet the needs of users. [20] According to recent news regarding the cosmetic sector, consumers are giving more attention to the effects their products have on the environment. Consequently, it is important to look for better alternatives to the ones used and understand their sustainability issues in the industry [21] There are problems related to the environment and human health, production yields, waste management, high consumption of raw materials, energy and water, the carbon footprint of the production chain, biodegradability, high costs, and also the non-green extraction techniques. [22]

Nowadays, since cosmetics started to have more requirements for being more natural and greener, with low ecological footprints, one of the biggest problems is the large quantities of solvents, chemicals, and raw materials used for all processes, such as extractions, and formulations. The most used are organic solvents, but the majority of them derive from the petrochemical industry which can cause environmental pollution. Considering they are used in cosmetic products, there are reports of how they can be dangerous to human health, causing sensitivity and irritability on the skin. [7,8] When toxic substances are used as solvents or other components, situations like disposing of these solvents end up polluting the planet. [24 ,25,26] For instance, about 400 million tons of hazardous waste are generated in the world each year. [26]

Sustainability metrics, such as the environmental factor (E factor), evaluates how "green" is the process. It represents the amount of waste produced in the process, excluding the cosmetic product, and it is the proportion of the mass of waste per mass of product. It also takes into consideration the chemical yield and includes all the reagents (excluding water), and solvent losses. In order to pollution and waste, there is a need to search for efficient processes and technologies for manufacturing.[22, 24]

Another factor to consider is to evaluate the stages in the life cycle of a product, and this concept is called Life Cycle Thinking. This concept is also associated with the Life Cycle Assessment (LCA) since it considers how all steps of the product life are interdependent, providing for the assessment of any potential environmental effects that cosmetics may have. [22, 24] Raw materials present in cosmetics are investigated in their biodegradability, bioaccumulation potential, and aquatic toxicity since there are some limitations.

When discussing the sustainability of the cosmetics industry, in addition to the raw materials and solvents used, packaging materials are also essential, due to the concerns about global warming, soil, and ecosystem damage, in addition to the increasing population worldwide. The most common is to find products with unnecessary layers, and with materials such as glass, paper, and plastics. There is a large use of plastic types of containers, however, their reuse and recycling happen in a very low percentage, especially compared to other materials like metals, paper, and glass. Further details, 25.8 million tons of plastic waste is produced in Europe each year, and slighter than 30% is collected to recycling. [22, 24 ,28]

Petrochemical plastics are more used due to some properties like heat sealability, large availability, low cost, and good barriers to oxygen and carbon dioxide, but with the disadvantage of not being biodegradable. The solution can be simple, applying the 3R's rule, reduce, recycle and reuse, suggesting industries to reduce CO_2 emissions, waste, and water consumption, and use recycled materials or alternatives such as biopolymers and plant-based plastics. A growing trend is the use of recycled materials and reusable or that can refilled packaging. [22, 24 ,28]

Nowadays we find that certain cosmetic products carry labels that may make them more attractive to consumers, as they read "Natural" or "Organic", existing a certain competition between products. These labels are named eco-labels and are considered a tool for consumers, to help select environmentally friendly products. Examples of European private certification organizations with standards for organic and natural products are *BDIH*, *Natrue*, *Ecocert*, *Greenlife*, and *COSMOS*, which operate independently and have their own rules for certification. [24, 29] However recently, in 2021, the European Commission published criteria covering cosmetic produced, regarding the EU Ecolabel. In order to qualify for the *EU Ecolabel*, according to the organization, it is necessary to take into account several criteria. [29] Examples are the impact on aquatic toxicity and biodegradability. For example, a product should be easily biodegradable or have lower aquatic toxicity. [29]

The indicated labels and their information must be clear and meaningful to attend consumers and influence them towards choosing green products. There are also excluded substances and others are subject to restrictions, regarding their hazard statements. Assure sustainable sourcing of oils, that there is adequacy for the purpose of a product is intended. [29] Another factor taken into account is the packaging, which should be in direct contact with the contents, including end-of-life information, and cream jars must have a 90% emptying level. [29] Consequently, new and sustainable production chains, as well as thoughtful consumption patterns are required.

1.2 Concept of circular economy and upcycling

The world population has been increasing, and expected growth will continue. A study showed that cities are producing 1.3 billion tonnes of waste each year, and is anticipated to be 2.2 billion tonnes by 2025. To diminish this negative effect, in particular for industries such as the chemical industry, which produce a lot of waste, in 1990 a new concept emerged, named "Green Chemistry". The main goals are minimizing the use and production of hazardous substances applied in chemical processes and improving the processes, to have a positive impact on the ecosystem. Another aim of Green Chemistry is to replace conventional solvents with renewable ones, along with the other twelve principles of green chemistry that Anastas and Werner presented in 1998.[30]

Generated waste has a negative impact on the planet, and because of this, it is necessary a serious action against it. Besides, according to the European Commission, Europe countries must change their approach to the production, consumption, recycling, and disposal of biological resources and one possible solution is converting to a circular economy (CE). [25, 32] The term appeared in the 1970s with the intention of decreasing the consumption of industrial production, however, this idea can be applied to any resource. The pioneers William McDonough e Michael Braungart used the expression "Cradle to Cradle", but it is possible to have a systemic approach as we talk about the CE. [32] A plausible definition is that it is the combination of reducing, reusing, and recycling activities in a systemic change, from a linear economy. [33] This economy provides better use of available resources, being an emerging essential growth model for sustainable development. [34] A certain industry must produce products that do not turn into useless waste when they reach the end of their useful life but can instead be transformed into new products. Another concept emerging, "Upcycling",

where waste materials are turned into more valuable ones, which can also be called a "second life", and the term was first used in 1994 by Reiner Pilz. [33, 36] The aim is to avoid throwing away useful materials and consuming unnecessary ones.

The concept is not the same as recycling, since this uses more energy, and upcycling is totally energy saving. The advantages are, for instance, minimal long-term effects on the environment, conserving resources sometimes limited, lower costs of production, and developing creativity. [33,36,37]

In the 21st century, big brands using renewable and recycled materials are becoming a growing trend, attracting even more customers. Industries are not producing a completely new product, and due to this, the costs are reduced, which also reduces greenhouse gas emissions. An industry benefits in terms of economic impact as mentioned before, which can be a market opportunity. In addition, can also have a social impact, becoming more popular for using a more environmentally friendly policy, developing opinions, changing cultures, and creating a sense of community through upcycling networks. [33,36,37]

However, there are known problems for certain big companies, for example, related to the current value and the quality of materials or products, inability to handle all types of materials, inconsistent supply of materials with controlled quality, and process complexity. Due to this, it is important in a world that is being affected by environmental problems, to change the current methods, and make these concepts more ordinary in our daily life. Only 8.6% of the world's economy is defined as circular, so the current goal is to move toward a circular and sustainable economy, especially for the industries which have the most negative impact on the planet, and the ones that produce a lot of waste. Namely, the cosmetic and chemical industries, which need greener and more sustainable alternatives for their processes, particularly for the extraction of bioactive compounds. [33, 36,37]

1.3 Extraction of bioactives compounds from by-products

The bioactive compounds are extracted using a variety of techniques, including pre-treatments which involve drying or grinding the material, and also the post-treatment. These operations are applicable to the extracts, which need separation, filtration, and purification, depending on the type or properties of the product. The separation to obtain a pure natural product has some challenges. If the mixture is too complex, and it is not possible to isolate the targeted compounds in just one step, it is necessary to develop more methods that allow

reducing the time to obtain a pure compound. In order to avoid the high consumption of water, energy, and time it is necessary to optimize the procedures. [38, 39,40]

A high amount of waste is produced when environmentally toxic chemicals or a lot of water are used. Due to the intense extraction conditions, the extract from this technique is not necessarily safe because it could contain impurities, denatured compounds, or residual solvents. [31]

The procedure to reuse wastes depends on their characteristics as well as the final product. The extraction needs to be considered green, by reducing or eliminating certain types of solvents, but also assure safety and quality. Ensuring a "green extraction" passes by searching for extraction methods that will use less energy, permit the use of alternative solvents, and guarantee both safety and quality of the extract and the finished product. [31]

Some of the conventional techniques used to obtain bioactive compounds are soxhlet extraction, maceration, hydrodistillation, and percolation. Nevertheless, all of them have drawbacks: high volume of chemicals needed, harmful organic solvents, the requirement of costly and high purity solvent, long extraction periods, low yields of extraction, and sometimes thermal decomposition of thermolabile ingredients or evaporation of a high amount of solvent. [41, 42]

The most common extraction techniques are considered non-green processes but the interest in greener approaches according to the principles of the circular economy is increasing, since the techniques are less expensive. The principles on which the green and non-conventional extraction methods follow the "Green Chemistry". Some examples are supercritical fluid extraction (SFE), subcritical water extraction (SWE), ultrasound-assisted extraction (UAE), and microwave-assisted extraction (MAE). In general, these techniques are eco-friendlier, enhance the global extraction yield, lower the organic solvent consumption, reduce the extraction time, have higher selectivity, and also cost less to operate without sacrificing the quality of the final extracts. [41, 42]

Extracts aiming to be used in the cosmetic industry, should follow the demands mentioned above, as they are to be in contact with humans, and the products cannot lose their properties, and one of the biggest problems is the solvents commonly used. [41, 43]

The selection of the solvent is critical following the green chemistry and the circular economy principles since it determines if it is a green extraction, even in situations where instead of the conventional methods, are used the non-conventional. It is important to ensure the durability of global processes, making sure the use 100% natural and renewable solvents

to minimize the environmental impact. However, for being considered a green extract, it must also be natural, with high quality and active compounds, high functionality, and adhere to the applicable legislation resulting in little impact on the environment. [42] With a market full, of promises of improving both general health and the ecological recycling problems, where value is given to waste and its value-added products, it is necessary to search and develop new alternatives to the existent solvents. [5, 43]

The range of polarities and physical properties of the bioactive compounds requires different solvents for processes such as extraction, separation, and purification. [43] A possible alternative to the existing solvents is the Ionic liquids (ILs). They are generated from ionic components which are combined by ionic bonds, have high thermal as well as chemical stability, are non-flammable, and have low vapor pressure. Despite these, the aim is to have the "greenest" solvent, and ILs can have a high cost, be toxic, and also most of the constituents are petroleum-derived. [31, 42]

1.4 Deep eutetic systems (DES)

The expression "eutectic" originated from the Greek word, eutēktos, which stands for low melting. Deep Eutectic Systems (DES) were described for the first time in 2003 by Abbott and his co-workers, these systems consist of eutectic mixtures, when two or more components usually solid at room temperature, change to liquid by the combination at a certain molar ratio.[44]

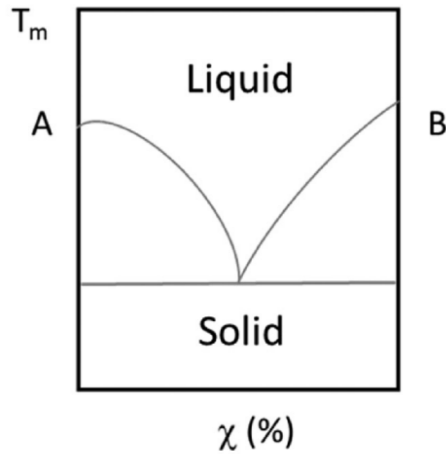


Figure 1.1-Diagram with phase behavior of melting point versus the molar ratio of solid components A and B. Adapted [44]

This can be explained by the hydrogen bond interactions between a hydrogen bond donor (HBD), such as carboxylic acids, alcohols and others, and a hydrogen bond acceptor (HBA). The capacity to increase their dissolution capability, is justified by the capacity to donate and accept protons and electrons, forming hydrogen bonds.[43, 46] Considering their composition, DES can be described by their type as represented in table 1.2.

Table 1.2-Types of DES. Adapted [31, 47]

Type	HBD and HBA
I	organic salts and metal salts
II	organic salts and metal hydrates
III	organic salts and hydrogen bond donors
IV	metal chlorides and hydrogen bond donors

DES have low vapor pressure, are non-volatile, non-flammable, miscible with water, and are easy to prepare. The major advantage when compared to conventional solvents is that they don't require purification and are made from low-cost compounds. DES have also low or negligible toxicity, are biodegradable and are easily recyclable. In opposition, these solvents have low conductivity due to their high viscosity (higher than 100 mPa.s). However, thus conductivity increases as the temperature increases, and viscosity decreases, which is because of weaker intermolecular interactions. [38, 48]

As mentioned before, the process of production of DES is considered easy and may include different techniques such as evaporation and heating along combining. This last one,

which is the most commonly used, consists of a emulsifying process under a settled stirring and heating process at certain temperature, and then a homogeneous solution is obtained. [30], [48] As a result of the optimal properties of DES as green solvents, such as biodegradability and sustainability, they have started to be applied in the agro-food industry, the beauty, and health care industry, and also in the natural ingredient industries. [48, 52]

There are other solvents that are a subcategory of DES, whose compounds occur naturally, the Natural Deep Eutectic Systems (NaDES). Examples of constituents are primary metabolites, such as organic acids, amino acids, and sugars, commonly present in living cells. NaDES most common base composition is choline chloride, containing organic acids or sugars such as HBD. It is important to have in mind that when are composed of organic acids are normally more acidic than sugars, which can be more suitable for some applications of these solvents. [50, 51,52]

Based on studies it is known that using different NADES it is possible to extract phenolic compounds from plants or flowers. This can be justified by the hydrogen bond interactions between the phenolic compounds and the NADES molecules. [51, 52] Due to their properties, most applications are in health, food, pharmaceuticals, and cosmetics. [51, 52]

Both DES and NADES have been receiving more attention and achieving consumer acceptance since they are green alternative solvents for the extraction of bioactives which can reduce the environmental impact. Even for industries that extract bioactive compounds giving them a second life in their products, there is still concern with the negative impact on the environment related to the use of conventional solvents. In order to make progress, and to have more sustainable methods, green alternatives are being more sought after and applied, such as Deep Eutectic Systems. [42,49,53]

Research [54, 55] showed a number of experiences where extraction efficiency with DES was higher than those with conventional organic solvents. Besides, another advantage of using NADES over conventional solvents, is that they can increase the stability of natural compounds during the extraction and storage. Compared with conventional solvents, it is obtained with DES a extract that maintains the biological effects and has no toxicity, assuring high quality and safe product. [54, 55]

1.4.1 DES in the cosmetic industry

Previously, it was concluded that a better alternative to solvents normally used in extraction are DES, and this is valid also for the cosmetic industry. As seen in the literature the

most studied HBA in DES, is choline chloride (ChCl). [41] Despite that, DES composed of choline salts, and their esters, have limitations in their application in the cosmetic industry. According to the European Commission's Inventory of Cosmetic Ingredients (2009), is possible to verify that many components are prohibited, including choline salts. [55] An alternative is betaine, which has similar properties and has also a thickening potential, a very interesting factor for the industry. [56] To assure a safe and efficient extraction it is important to study all the different components that can be used in the cosmetic sector, based on their correct characterization and evaluation. [57]

As mentioned before, DES has a lot of advantages when compared to conventional organic solvents, and for the cosmetic industry, some of them are very important. Since DES are not toxic and are composed of natural metabolites, they can be applied in the final formulations, avoiding the need for purification and other expensive processes. [31,58] In fact, DES can be produced and used for the extraction of valuable compounds such as phenolic compounds, which have excellent stability in sugar-based DES, making them a suitable approach for the food and pharmaceuticals industry. [5, 43,59]

Some companies already described how they succeeded in the development of extracts for a cosmetic formulation based on DES. A known company using this alternative is *Naturex*, which was founded in 1992 in France, as part of the company *Givaudan*. In 2016, they developed a patent for a new extraction process, that consisted of the extraction of plant-based active compounds, which was called "eutectigenesis". In order to develop Eutectys™, different mixtures of pure molecules with 25% of water (w/w), were created. This is a range of 100% natural and biodegradable cosmetic products, with six actives, and higher phytochemical profiles and biological activities, for example antioxidant, and anti-inflammatory, compared to the corresponding conventional hydroglycerine extracts. The technology uses NaDES as an alternative to the most common solvent for extraction, and the extracts from the collection are safe and the profiles of toxicity are according to the regulation.[60, 61,62]

With origin in France, *Gattefossé,* leads a provider of specialty ingredients and formulation solutions for the beauty and health care industries worldwide. The company started studying and working with NaDES in 2012, with the goal of ending up with 100% natural, innovative, and bio-based solvents for plant extraction that could be used in the cosmetic industry. [57] They developed and patented NADES adapted for the extraction of cosmetic active ingredients with components such as sugars and aminoacids.[62]

The company *L'Occitane en Provence*, founded in 1976 in France, has been studying for 20 years the immortelle flower. For the extraction method of the immortelle sap-like extract, they are now using the NaDES technology, which took them 7 years of research. In a skincare product, the extract promises to reduce redness, treat acne scars, restore the skin glow and also fight signs of age. [63]

As mentioned before, DES are considered non-toxic, therefore they are a better alternative compared to the most common solvents used to extract natural extracts, even though it is an area that needs even more research. [37] Cosmetic products are generally safe, as most people do not show any side effects, because of the intensive testing before release to the market. In the case of cosmetics with this type of extract, the common procedure without the quantitative composition evaluation is to evaluate the bioactivity and toxicity of the extracts in vitro studies.[47]

1.5 Valorization of bioactive compounds from by-products

The UNEP FOOD WASTE INDEX from 2021, reports that around 931 million tonnes of food waste were produced in 2019 from households, retail establishments, and the food service industry. The waste from certain industries such as the ones related to food has a certain value since some by-products also have high-value components. In order to face the needs of the present day and the demands of consumers and society, it is possible to contribute to minimal waste. [37, 65]

Agricultural activity is growing, according to population and consumption growth. Research proves that there is a global loss of about 30 to 40% of all the food produced for human consumption each year. [65] This in conjunction with insufficient waste management methods, developed an agricultural waste problem, producing approximately 700 million tonnes per year in Europe. For instance, one of the biggest problems associated with waste is the places where they are deposited, like landfills or even incineration, which all pollute the surrounding landscape, and release methane into the atmosphere. [65]

A strategy is using the circular economy and upcycling approach, in order to prolong the lifespan of agricultural products and by-products through waste recovery processes. In our days, it is possible to see some growing awareness, as some companies started to apply this, as they saw the potential of the by-products, due to their bioactive properties. [65] It can

also be used also in cosmetic and pharmaceutical applications, as reported in the literature. [67, 68]

The by-products from the agro-food industry are considered some wastes, that can be included in cosmetic products, with a lot of benefits. Some examples are peels and pomace of apple, grapes, citrus fruits, vegetable sources, spent coffee grounds, and a lot more, depending on the bioactivity that is expected from a certain product. [68]

1.5.1 Spent coffee grounds (SCG)

The history behind coffee started a long time ago, and it is now one of the most popular beverages and agricultural commodities in the world. It has been consumed for over a thousand years, and the drink that we see today has originated in the XVI century in Persia, and only in 1615, it was brought to Europe. Coffee beans have oring in the plant *Coffea L.* .Therefore, the most cultivated for commercial production is *Coffea arabica* with origin in Ethiopia, followed by *Coffea canephora var. robusta*, known as Arabica and Robusta. The first is produced in a higher percentage, around 70 and 75%, as it is considered to be of superior quality, the second is mostly used for producing soluble coffee extracts by the instant coffee industry, being more acid. [70, 71]

This industry is growing every year, as coffee is drunk by millions of people every single day. According to the International Coffee Organization, in years 2020 and 2021, around 166.63 million 60-kilogram bags of coffee were consumed over the world. [71] During the production process of coffee, more than 50% of the fruit is not used, and it is discarded, which makes companies end up having a lot of waste.[73,74, 75] Coffee is known for its aroma, flavor, color as well as health benefits, and its popularity results in an average 3.5 billion of cups of coffee being consumed every day. [70, 71,76, 77]

The coffee beverage is prepared by extraction with boiling water, and there are a lot of different ways to brew coffee worldwide. Some examples are the filtration–percolation method used in coffee machines, where the coffee is extracted by slow gravity percolation. In the known Expresso, coffee is extracted using Subcritical water, and the filtration is accelerated by the vapor to a pressure between 7 and 9 bar for a low period of time. Another example is the coffee made with a Moka Pot or the French press. However, all these different processes have different contributions to the chemical compositions of coffee brews, in which different technical conditions are applied. Examples are different coffee and water ratio, the water temperature, and used pressure. [70, 71]

Instant coffee is obtained by the extraction of roasted coffee. During the production of instant coffee or the coffee beverage, raw coffee powder is in contact with water and produces residues that are solid and called spent coffee grounds (SCG), estimated as 6 million tons per year. Around one kilogram of coffee produces two kilograms of wet SCG waste, these residues are fine particles, with high humidity, acidity, and organic load. [70, 71]

For example, the company *bio-bean* collects the SCG from coffee shops, restaurants as well as industries, and has industrialized the process of recycling them into biofuels and bio-mass. According to an estimate by the company, a quarter of a million tonnes of waste coffee grounds are generated each year. However, SCG for some do not have economic value, being discarded as waste or used as fertilizers, which is not in agreement with the circular economy approach, and this by-product is not being upcycled as it should be. [73, 76,78]

The SCG composition depends on the extraction, roasting process and the type of coffee beans, a comparison between SCG and the coffee bean is represented in table 1.3. Usually, they are both rich in polysaccharides, lignin, cellulose, and hemicellulose structures. There are also present micronutrients like minerals, and alkaloids such as caffeine and trigonelline. They are also composed of proteins, a number that is higher than the observed in the coffee beans, this can be justified by the presence of components that were not extracted during the preparation of instant coffee. However, it can be overvalued because of the presence of substances like alkaloids and amino acids containing nitrogen. [70, 71,76, 79]

Table 1.3-Example of comparison between the composition of Coffee beans and spent coffee grounds. Adapted [79]

Dry matter %	Green coffee bean	Spent coffee grounds
Carbohydrates	48-61	45-47
Cellulose	7.8	8.6-9.0
Hemicellulose	40	37-38
Mannose	22	23-26
Galactose	12	15
Arabinose	4	1.4-1.9
Lipids	10-16	9-16
Proteins	10	13-17
Total polyphenols	<14	1.7-3.5
Chlorogenic acids	4-14	0.1-0.8
Caffeine	1-2	0.5-1.2

One of the most important components are the phenolic compounds, which are antioxidant potentials found in high concentrations in plants, and have beneficial effects on human health. Phenolic acids may be divided into different groups, the hydroxybenzoic and hydroxycinnamic acids. In the first group, it is common to have the C6–C1 structure, like gallic acid, and in the second group, aromatic compounds like chlorogenic acid, and caffeic acid. Chlorogenic acid (CGA) is the antioxidant present in higher quantities in spent coffee grounds and contributes to the acidity of the coffee brew. [71, 76,81, 82]

A

B

Figure 1.2- Chemical structure of chemical compounds present in SCG: A-Chlorogenic acid; B-Caffeine. Designed with *ChemDraw*

As mentioned before using alternative solvents is a growing trend and there are also some examples of using DES for the extraction of phenolic compounds from SCG. In table 1.4 some examples are listed. Is possible to confirm that DES provides higher extraction yields when compared to conventional solvents, being a better alternative for example to the cosmetic industry, in conjunction with other non-conventional extraction methods. [70, 73,84]

The health benefits of phenolic compounds can be described as the protective action against diseases related to skin and others. The most known is the anti-aging feature and then the antioxidant, anti-inflammatory, antimicrobial, antiviral, anticancer, and anti-cellulite. [75] So, it can be concluded that SCG have applications in the cosmetic industry, and as a consequence, some cosmetic brands are using SCG in the composition of their products. [73,74]

When producing an emulsion with an extract rich in polyphenols it is important to assure that it is going to permeate the skin. These components have low solubility, which makes them precipitate in water, since they should not precipitate in the formulation, emulsion components like thickeners and emulsifiers promote their solubilization. [83] However, the incorporation of an extract rich in polyphenols into emulsions can influence their

rheological properties and stability, particularly, may occur a decrease in viscosity, which can be related to the interactions between components of the emulsion like emulsifiers. Nevertheless, the antioxidant properties can improve oxidative stability and the storage stability of emulsions. [83]

Since SCG have origin in coffee beans, they are also rich in caffeine, having emulsifying properties which could be interesting for some formulations. The oil extracted from SCG can be incorporated into emulsions, and studies showed that with 10% of the lipid fraction, it has a hydration action lowering water loss, and also increasing sebum levels. [76, 86]

The process of skin aging affects a lot of people and can be the result of exposure to ultraviolet light (UV), which causes a change in the skin components, collagen, and elastin, resulting in wrinkles. Another thing that can happen is oxidative stress, which produces reactive oxygen species (ROS) production in epidermal tissues. They are derived from molecular oxygen such as O_2 and H_2O_2, effective oxidants. [87, 88]

With the growing awareness of the impact industries have on the environment, and the need to reduce waste production, interest in by-products like the SCG has also grown. For example, a research article reported that they successfully extracted bioactive molecules from SCG using deep eutectic solvents. The oil was obtained and incorporated into topical formulations, showing improved skin lipids and hydration qualities. [87] However, for DES, there also are extractions processes reported in the literature but they have their composition compounds that cannot be used in the cosmetic industry such as Choline chloride, as it is shown in table 1.4

Table 1.4-Examples of extraction techniques using DES from spent coffee grounds

DES	Extraction Conditions	Yield	Conventional	Reference
ChCl:1,6-hexanediol (1:17)	Technique: UAE SLR: 2.6 mL/100 mg Temperature: 60ºC Water:30%	15.1 mg GAE/g SCG	Solvent:20% Ethanol Yield: 9.2 mg GAE/g SCG	[73]
Betaine:1,2-butanediol (1:5)	Technique: Thermo-shaker Time: 60 min SLR: 1:10 (g/mL) Temperature: 50ºC Other: 500 rpm	10.34 mg GAE/g SCG	Solvent: Ethanol Yield: 4.24 mg GAE/g SCG	[88]
Betaine:triethylene glycol (2:1)	Technique: UAE Time: 20 min SLR: 1.15 (g/mL) Temperature: 60ºC Water: 30%	0.67-4.64 mg g^{-1} of dry weight SCG	n.d.	[89]

1.6 Objectives

The aim of this thesis is to develop spent coffee grounds extracts using deep eutectic systems as an alternative to conventional solvents and to incorporate them into topical formulations with high bioactivity. These topical formulations are novel and sustainable o/w emulsions for skin application, following the EU Ecolabel criteria. The work will be divided into the following tasks:

- **Preparation of DES, SCG characterization, and DES extraction of valuable compounds from SCG**

In this study, DES, novel solvents, are going to be produced and used for the extraction of valuable compounds from SCG with different origins. SCG will be combined with the DES at different solid to liquid ratios and the extraction will take place in a stirred vessel at a controlled temperature. Samples will be taken at determined time points and characterized in

terms of phenolic content and antioxidant activity. The optimum extraction conditions and DES will be used for the following tasks.

- **Extract bioactivity evaluation**

To assess the biosafety of extracts, will be performed in vitro studies using human adult dermal spontaneously immortalized human keratinocyte cell line (HaCaT) namely a cell bioavailability assay (MTT) and Enzymatic Inhibition to Human Neutrophil Elastase (HNE). To evaluate the bioactivity of safe extracts regarding the anti-oxidant effects, HaCat cells will be exposed to specific conditions to stimulate an oxidant response in cells. Extracts with the best results will be evaluated in stability over time in total phenolic content and antioxidant activity (DPPH) and will be incorporated into the novel and sustainable topical emulsions.

- **Formulation development and characterization**

Topical formulations will be developed following the EU Ecolabel criteria and they will be fully characterized by different methods, such as macroscopic characterization; pH measurement; microcopy analysis; droplet size analysis; rheology; textural profile analysis; and spreadability. The emulsion presenting the best results will be submitted to *in vivo* studies, regarding the evaluation of safety and efficacy.

Finally, to follow the CE and Upcycling approach will be reused the leftover SCG. This will be possible, by characterizing the particles, extracting their oil, and incorporating both in a scrub.

2

MATERIALS AND METHODS

2.1 Materials

The following excipients were used to prepare the DES: Glucose (99.5%), Glycerol (99.5%), Lactic Acid (85%), Citric Acid (99.5%), Urea (99%) were acquired from Sigma-Aldrich (USA); Betaine (98%) was acquired from Glentham Life Science (UK); Proline (99%) was acquired from Alfa Aesar (USA); Malic Acid (99%) was acquired from Scharlau (Spain).

The following excipients were used for extraction and chemical analysis of the extracts: Ethanol (99.5%), Methanol (99.8%), Trichloroacetic acid (99.5%) were acquired from Scharlau (Spain); Folin-Ciocalteu reagent was acquired from MERK (Germany); Sodium carbonate solution, and DPPH were acquired from Sigma-Aldrich (USA).

The following excipients were used to prepare the formulations: MONTANOV™ 68 MB (Cetearyl Alcohol (and) Cetearyl Glucoside), EMOSMART™ V21 (C18-21 Alkane), EMOSMART™ C28 (C21-28 Alkane), LANOL 2681 (Coco-Caprylate/Caprate) were acquired from Seppic (France); SABOWAX CP MB (Cetyl Palmitate) was acquired from Sabo S.p.A. (Italy); Grape seed oil (Vitis Vinifera (Grape) Seed Oil), Mango Butter (Mangifera Indica (Mango) Seed Butter), Almond oil (Prunus Amygdalus Dulcis (Sweet Almond) Oil) were acquired from Plena Natura (Portugal); Acticire MB (Jojoba Esters (and) Helianthus Annuus (Sunflower) Seed Wax (and) Acacia Decurrens Flower Wax (and) Polyglycerin-3) and Compritolâ CG 888 Pellets (Glyceryl Dibehenate (and) Tribehenin (and) Glyceryl Behenate) was acquired from Gattefossé (France); pharmaceutical glycerin (Glycerin) was obtained from Laboratório EDOL (Portugal), Geogard™ 221 (Dehydroacetic Acid (and) Benzyl Alcohol) was acquired from Lonza (Switzerland); and Purified Water.

2.2 Extraction and chemical analyses

2.2.1 Spent coffee grounds (SCG)

Spent coffee grounds from four different origins were chosen as starting material for extractions: capsules from automatic espresso machines (CP), moka pot (MP), french press (FP), and a sample of SCG dried in 2020 from coffee capsules (CD). The samples were provided by users from Laboratory 427 of the Chemistry Department at the FCT campus and house wastes.

The residues CP, MP, and FP were dried in a heating oven at 80ºC for more than 48 hours until the moisture content was under 5 wt%. This moisture content was measured with a moisture analyzer (KERN DAB 200-2, KERN & SOHN GmbH). Dried SCG for the extractions were stored at room temperature in plastic bags protected from the light. For the sample from coffee dried in 2020, it was only measured the moisture content. Elementary analysis of SCG was performed at Laboratório de Análises, REQUIMTE-LAQV. In order to determine protein content, was used a nitrogen-to-protein conversion factor of 6.25. [90]

2.2.2 Preparation of DES

In order to study the influence of changing the solvent and any potential impact on viscosity, several DES were prepared.

According to the molar ratio and molecular weight, the required quantity of each component was measured using an analytical balance (PLJ 1200-3A-2020a, Kern & Sohn GmbH) with an accuracy of ±0.001 g. The components were heated on a water bath in a hot plate (RSLAB-1C, RS France Rogo-Sampaic) with a magnetic stirrer at approximately 40-50ºC until a homogeneous transparent liquid was formed. In table 2.1 can be found a summary of the synthesized DES.

Table 2.1-Summary of all the DES prepared.

Abbrev.	DES	Ratio	Abbrev.	DES	Ratio
1	Glucose:Glycerol:Water	(1:4:2)	**11**	Proline:Glycerol:Sorbitol:Water	(1:1:1:13)
2	Glucose:Lactic Acid:Water	(1:5:3)	**12**	Urea:Glucose:Proline:Water	(1:1:1:4)
3	Betaine:Citric Acid:Water	(1:1:3)	**13**	Proline:Glycerol:Water	(2:5:11.5)
4	Betaine:Citric Acid:Water	(1:1:7)	**14**	Malic Acid:Betaine:Water	(1:2:3)
5	Lactic Acid:Betaine	(2:1)	**15**	Betaine:Glycerol	(1:2)
6	Citric Acid: Proline: Water	(1:1:6)	**16**	Betaine:Glicerol: Water	(1:2:1)
7	Citric Acid: Proline: Water	(1:3:6)	**17**	Betaine:Glucose:Water	(5:2:10)
8	Malic Acid:Glycerol:Water	(1:1:1,25)	**18**	Proline:Glucose	(5:3)
9	Proline:Malic acid:Water	(1:1:3)	**19**	Betaine:Sorbitol:Water	(1.25:1.2:2)
10	Urea:Glucose:Water	(1:1:5)			

2.2.3 Extraction with DES

The extraction of phenolic compounds from SCG was performed adding the required amount of SCG according to the solid/liquid ratio (g/ml) and was weighed using an analytical

balance (Precisa 205 A, SuperBal-series) into the respective volume of DES. Samples were extracted at 1h and 50ºC, with a water bath and stir. The tubes were centrifuged for 10 minutes at 6000 rpm to enable complete phase separation. Before analysis, the extracted samples were filtered through cotton seringues to eliminate any remaining contaminants. To assess the impact of the extraction conditions on the total phenolic content and antioxidant capacity, a number of important operating parameters were changed.

The solid/liquid ratio studied was 1:10, 1:5 and 1:20. One DES was also evaluated at different time extraction at 15,30,45, and 180 minutes, as well as different temperatures, at 40º and 60ºC.

2.2.4 Hydroalcoholic extraction

As a comparative method, hydroalcoholic extraction of the four residues was performed. A solution of Ethanol and Distilled Water in the molar ratio of 1:1 was made by adding 25ml of water and 25ml of ethanol in a glass bottle, as previously made in the literature. [91] The extraction of phenolic compounds from SCG was performed by adding 1g of SCG according to the solid/liquid ratio, 1:10, and was weighed using an analytical balance (Precisa 205 A, SuperBal-series) into the 10ml of the solution. Samples were extracted for 1h at 50ºC with a water bath and stir. To enable complete phase separation, extracted samples were t filtered through a funnel with filter paper to eliminate any remaining contaminants.

2.2.5 Determination of Total Phenolic Content

The Folin-Ciocalteau method, which has previously been described, was used to determine the total phenolic content of the extracts. [90] Briefly, tubes were filled with 1580 µl of distilled water, 20 µl of the sample, and 100 µl of Folin-Ciocalteu reagent before being agitated on vortex, and then were 5 minutes at room temperature. Then to the mixture was added 300 µl of a sodium carbonate solution, followed by agitation. Tubes were placed bath at 40ºC during 30 minutes. After they were analyzed by UV/Vis spectroscopy using a spectrophotometer (GENESYS™ 50, Thermo Scientific™) at 750 nm. For some samples before the determination of phenolics, it was necessary to make a dilution (1:4) and the step of protein precipitation. (hydroalcoholic extract).

An amount of 120 mL of 100% (w/v) trichloroacetic acid was added to 800 mL of the sample, and the mixture was thoroughly mixed before being stored for 5 min at 20°C and subsequently during 15 min at 4ºC. After centrifugation (12000 rpm, 15 min) the precipitate was

discarded. The reference compound was gallic acid standard, and a standard calibration curve was created by plotting the sample absorbance as a function of concentration. By applying the gallic acid standard calibration curve, the absorbance of the samples was converted to phenolic content. The data for the total phenolic content were expressed as mg of gallic acid equivalent per gram of SCG (mg GAE/ g SCG).

2.2.6 Determination of Antioxidant activity

To evaluate the antioxidant potential of the phenolic extracts, the method of DPPH was used as previously described. [90] The antioxidant compounds react with DPPH by donating hydrogen, which reduces the compound. The sample's color, which was evaluated by the absorbance at 517 nm using UV/Vis spectroscopy, changed from deep violet to pale yellow as a result of this reduction. To make the stock solution, was dissolved 24 mg of DPPH in 100 mL of methanol, and then was kept it at -20°C for at least two hours. The absorbance was then determined at 517 nm by diluting the solution by adding 10 mL to 45 mL of methanol. The absorbance must be in the range of 1 and 1.1. The extracts were produced with serial dilutions (1:2,1:4,1:8,1:16,1:32,1:64). A mixture of 150µL of the diluted extracts and 4ml of DPPH solution was incubated in amber vials, at room temperature protected from light during 40 min, before measuring the absorvance at 517 nm. The procedure was the same with a sample of distilled water, and all the diluted extracts were made in triplicates. The following equation was used to evaluate the inhibition of the free radical by each sample.

$$\%\text{inhibition}=\frac{A_{DPPH}-A_{Sample}}{A_{DPPH}} \quad (1)$$

In the equation, A_{DPPH} is the absorbance of the blank and A_{Sample} is the absorbance of the sample in study. The half-maximum effective concentration (EC_{50}) was derived from the obtained inhibition curves to assess the antioxidant activity of the samples.

2.2.7 *In Vitro* Efficacy and Safety Analysis DES and DES Extracts

2.2.7.1 Cell viability studies

The cytotoxicity was assessed using the endpoint MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl- 2H-tetrazolium bromide) as metabolic assay as previously described in [92] Briefly,

the day before the experiment cells HaCaT (CLS, Germany), were seeded in 96 well tissue culture plates at a cell density of $2x10^4$ cells per well, in RPMI 1640 culture medium supplemented with 10% Fetal serum bovine, 100 units of penicillin G (sodium salt) and 100μg of streptomycin sulfate and 2mM L-glutamine, and incubated at 37 ºC and 5% CO_2 in a humidified atmosphere. Cells were incubated with the test samples at different concentrations (100, 50, 25, and 12.5 μg/mL), culture medium, and 1mg/mL of sodium dodecyl sulfate (SDS) as negative and positive controls, respectively. Following a 24-hour exposure period, the medium was replaced with one containing 0.5 mg/mL of MTT. The formazan crystals were extracted with 100μL of DMSO after 3 hours of incubation, and the absorbance was assessed using a microplate reader (FLUOstar Omega, BMGLabtech, Germany). The following equation was used to calculate the relative cell viability (%) when compared to control cells:

$$\text{Cell viability} = \frac{Abs_{Sample}}{Abs_{Control}}\ x\ 100 \quad (2)$$

2.2.7.2 Reactive Oxygen Species (ROS) Production Measurement

The intracellular production of reactive oxygen species (ROS) within cells was assessed with a fluorimetric technique using 2,7′ dichlorodihydrofluorescein diacetate (H2-DCFDA, Life Technologies, UK), as described in previous work [92] Briefly, HaCaT sub-confluent cells grown in 96 well plates were incubated for 30 min with 20 μM of H2-DCFDA in dark at 37°C. Media was removed and fresh medium was added to the cells before were exposed to different concentrations of samples and ascorbic acid (vitamin C) for 1.5 h. For induction of ROS in cells an Hydrogen peroxide (H_2O_2) solution (500 μM, 1h) or exposure to UVB light (emission wavelength 312 nm) for 15min were used. After exposure, ROS levels were determined at excitation 485 nm and emission 520 nm wavelengths using a fluorescence microplate reader (FLUOstar BMGLabtech, Germany) Data from 9 replicates are reported as the percentage of % of ROS reduction determined as 100-(fluorescence of exposed cells/fluorescence of unexposed control from the same experiment) x100.

2.2.7.3 Human Neutrophil Elastase (HNE) Enzymatic Inhibition Assay

Fluorometric inhibition assays for Human Neutrophil Elastase (HNE, Merck, Darmstadt, Germany) were performed as described in previous work. [92] Briefly, the assay was performed in 200 μL assay buffer (0.1 M HEPES pH 7.5 at 25°C) containing 20 μL of 0.17 μM HNE in assay buffer, 155 μL of assay buffer, and 5 μL of each concentration of tested samples.

The reaction was initiated by the addition of 20 μL of the fluorogenic substrate at 200 μM (MeO-Suc-Ala-Ala-Pro-Val-AMC), and the inhibition activity was monitored for 30 minutes at 25°C on a microplate reader (FLUOstar Omega; BMG Labtech, Germany) (excitation wavelength 380 nm and emission 460 nm). Assays were performed in duplicate and presented as a log of inhibitor concentrations versus the percentage of activity. IC_{50} values were determined by non-linear regression analysis in GraphPad PRISM® 5 software.

2.2.8 High-Performance Liquid Chromatography (HPLC) Analyses

The extracts were filtered with a Syringe Filter (13mm, 0.22μm, Nylon HPLC Syringe Filter, Labfil). HPLC was used with the aim of identifying and quantifying phenolic and other compounds from the extracts like caffeic acid, catechin, chlorogenic acid, epicatechin, ferulic acid, gallic acid, p-coumaric acid, caffeine, and quercetin. All the analyses were performed at Laboratório de Análises, LAQV-REQUIMTE. Following the method described in the literature [93], using the Dionex ICS-3000 system with Photodiode Array (PAD). The absorbance is measured using a wavelength of 280 nm with a UV / Vis detector. For the analysis was used a reverse-phase polymeric C18 column, Novapak C18 Waters 150 x 4 mm. The chromatographic separation was carried out using as mobile phase a solution with methanol: acetic acid: water with a molar ratio of 10:2:88 (v/v) as solvent A and with a molar ratio of 90:2:8 (v/v) as solvent B. This method has an injection volume of 20 μL and a flow rate of 1 mL/min. Chromatographic peaks were identified by comparing the retention times of samples with those of standard compounds. The concentration of each compound was calculated through the respective calibration curve. (An Appendix 5.1)

2.2.9 Liquid chromatography–Mass spectrometry (LC-MS) Analyses

LC-MS was used to identify and confirm the main components present in the extracts, previously identified by the HPLC analysis. All the analyses were performed at Laboratório de Análise, LAQV-REQUIMTE. For the chromatographic separation, a Nova-Pak C18 column with 150X3.6 mm. The instrumental conditions for the chromatograph were 0.40 mL min^{-1} (flow), 30ºC and 1μL (injection volume). The total run time was 50 min. The mobile phase was composed of solvent A (water with 0.1% formic acid) and solvent B (methanol with 0.1% formic acid). The gradient employed was 10% (v) B holding for 10 min, then 15% (v) B during 25 min, then 50% (v) B during 35 min, and then 50% (v) B during 30. min.

2.2.10 Stability assay

Extracts were produced according to the method describe before in 2.2.3, to see also if this could influence the results. As a comparative method, hydroalcoholic extraction was performed according to the method described in 2.2.4. Over time (days) the total phenolic compounds were quantified using the method described in 2.2.5, and the antioxidant activity with DPPH assay as described in 2.2.6.

For the stability assay samples were tested under four different conditions and stored in glass flasks as follows:

- Sample stored at room temperature in the light (RT);

-Sample stored at room temperature in the dark, covered in aluminum foil (RTD);

- Sample stored in the fridge at 4 °C (CT);

-Sample stored in a heating oven at 40ºC (HT).

The measurement of phenolic compounds was performed on all samples 0, 14, 30, 60, and 90 days after the determination of the reference data. Following the procedure outlined in 2.2.9, the antioxidant activity was also assessed for the same days after the reference analysis and HPLC analyses. Following the procedure previously described in 2.2.10, an LC-MS analysis was conducted to identify the principal compounds in the samples.

2.3 Topical formulations development

2.3.1 Formulation development strategy

Two of five different extracts, based on their properties, like highest TPC, antioxidant activity showed in DPPH assay and ROS scavenging properties, and constituent's properties were chosen in order to prepare topical formulations. Among different kinds of possible formulations, it was chosen an O/W emulsion for body care, considering different reasons: easier to comply with the Ecolabel regulation; the nature of the extracts, and their bioactivity.

With the aim of developing sustainable emulsions according to the Eco label regulation, it was necessary to make a search to understand what kind of ingredients could be used. The first step was to select an emulsifier, to stabilize the oil-water interface. Then to formulate the emulsion, was necessary to choose other ingredients, regarding viscosity, spreadability, and feel.The selection of the ingredients was performed using a validation and exclusion method, as described in figure 2.1. The emulsions were developed through the combination of different

ingredients, the final formulations are described in Table 2.2. For the emulsions with DES and DES extracts, was noticed that since they have in common with the placebo glycerol in the composition, could cause sob instability in the emulsions and change viscosity, and was decided to substitute the 5% of Glycerol with DES/Extract.

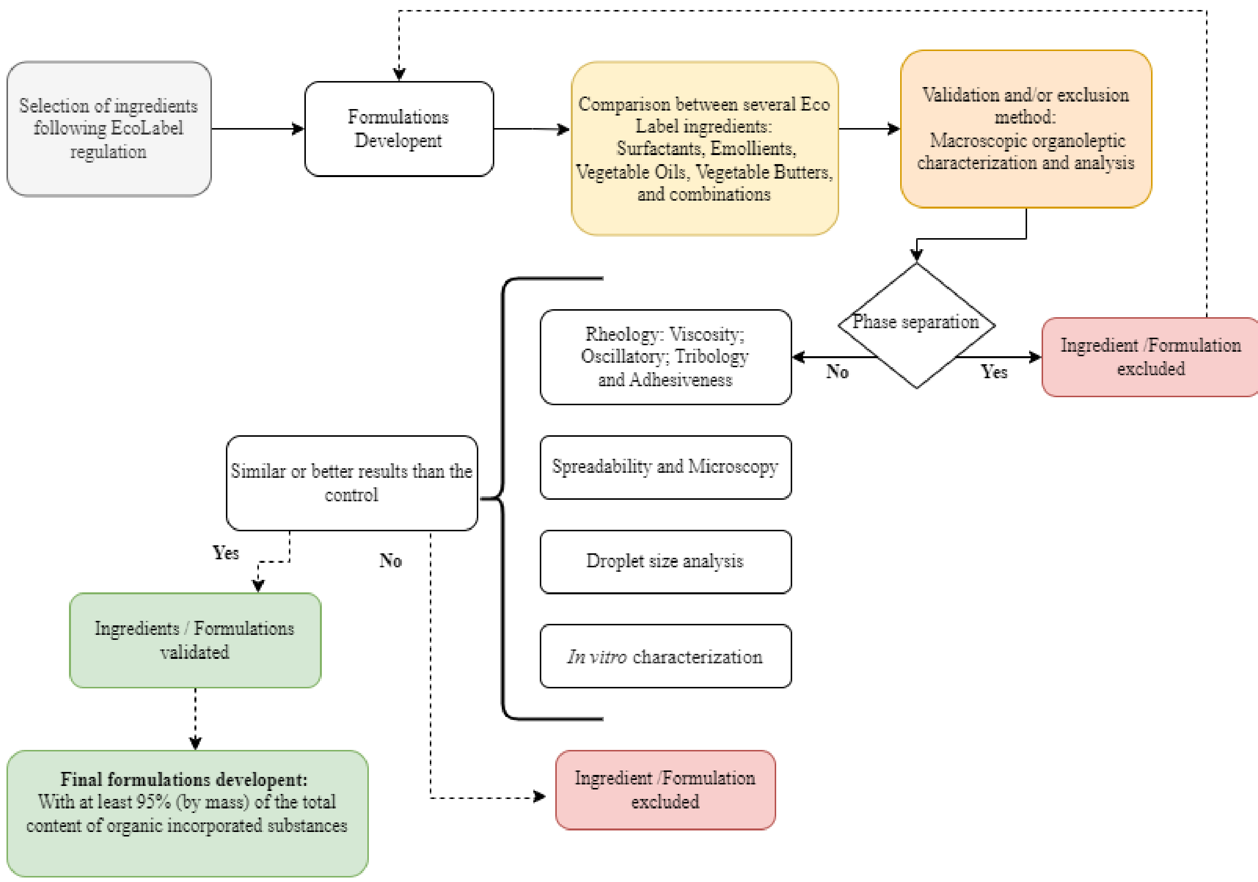


Figure 2.1-Scheme: validation and/or exclusion method for cosmetic formulations

Table 2.2-Qualitative and quantitative composition of emulsions: Placebo-Control; E13-Control with DES 13; E11-Control with DES 11; EI-With Extract from DES 13; EN- With Extract from DES 11

Phase	Comercial Name	INCI	Function	% (w/w)				
				Placebo	E13	E11	EI	EN
Oil	MONTANOV MB 68	Cetearyl Alcohol (and) Cetearyl Glucoside	Surfactant	5	5	5	5	5
	Emosmart V21	C18-21 Alkane	Emollient	5	5	5	5	5
	Emosmart C28	C21-28 Alkane	Emollient	5	5	5	5	5
	Lanol 2681	Coco-Caprylate/Caprate	Emollient	5	5	5	5	5
	Sabowax	Cetyl palmitate	Thickener	1	1	1	1	1
	Grape Seed Oil	Vitis Vinifera (Grape) Seed Oil	Emollient	1.5	1.5	1.5	1.5	1.5
	Mango butter	Mangifera Indica (Mango) Seed Butter	Emollient	1.5	1.5	1.5	1.5	1.5
	Acticire	Jojoba Esters (and) Helianthus Annuus (Sunflower) Seed Wax (and) Acacia Decurrens Flower Wax (and) Polyglycerin-3	Emollient	0.5	0.5	0.5	0.5	0.5
Aqueous	Geogard 221	Dehydroacetic Acid (and) Benzyl Alcohol	Preservative	0.8	0.8	0.8	0.8	0.8
	Glicerin	Glycerin	Emollient	5	-	-	-	-
	Water	Water	Conductive medium	69.7	69.7	69.7	69.7	69.7

Phase	Comercial Name	INCI	Function	% (w/w)				
				Pla-cebo	E13	E11	EI	EN
	DES I	Proline:Glycerol:H2O (2:5:11.5)	Solvent	-	5	-	-	-
	DES N	Proline:Glycerol:Sorbitol:H2O (1:1:1:13)	Solvent	-	-	5	-	-
	Extract I	DES I + SCG	Bioactive	-	-	-	5	-
	Extract N	DESN + SCG	Bioactive	-	-	-		5

2.3.2 Manufacturing process

The emulsions were developed through the preparation of an oil liquid phase and an aqueous phase. The ingredients were weight using an analytical balance (PLJ 1200-3A-2020a, Kern & Sohn GmbH) with an accuracy of ±0.001g. The components of the oil phase and aqueous phase were mixed in separate stainless-steel bowls, then melted at 85ºC in a water bath (Digital water bath Model 601 by Nahita). The emulsification phase was performed by slowly adding the oil phase to the aqueous phase and with high shear mixing at a rate of about 10000 rpm/min (IKA® T25 digital, ULTRA-TURRAX®) for 1 minute. Then was stirred at 600 rpm (Turbotest, VMI The Mixing Company) for 30 min and changing to an overhead stirrer (EUROSTAR 60 digital, IKA) with an R-turbine agitator until room temperature. Emulsions were stored in plastic jars. Additionally, DES/Extract (in the formulations E11, E13, EI, and EN) were added at room temperature by manual stirring.

2.4 Physical and chemical characterization of emulsions

2.4.1 Appearance, pH determination, and physical stability

The macroscopic appearance of each formulation was visually analyzed, and physical stability was evaluated after 24h.

Emulsions were centrifugated in a centrifuge (Heraus Sepatech, Medifuge) for 10 min at 4x1000 RPM/min to evaluate phase separation. Measurements were done for each emulsion,

in two periods of 10 minutes. The pH of each formulation was measured with the pH meter (Eutech™ Ion 2700 Meter, Thermo Scientific™), at room temperature. After the equipment's value had stabilized, the data were collected.

2.4.2 Droplet size analysis

The droplet size distribution of the emulsions was measured by light scattering using a Malvern Mastersizer 2000 (Malvern Instruments, Worcestershire, UK) coupled with a Hydro S accessory. About 0.1g of each formulation was diluted in 5 ml of water to achieve the proper turbidity.Corresponding to an obscuration between 10% and 20% was added the sample in the chamber with water using a stirrer at 1750 rpm. The data were expressed in terms of the relative distribution of the volume of droplets and given as diameter values corresponding to percentiles of 10%, 50%, and 90% (mean ± SD; n=6). Measurements were performed 24 hours after the preparation of the emulsions.

2.4.3 Structure analysis of emulsions

Structural experiments were performed with a controlled stress Kinexus Lab+ Rheometer (Malvern Instruments, Worcestershire, UK).

2.4.3.1 Rotational viscosity measurements

Rotational viscosity was determined using a cone-and-plate geometry. Measurements were carried out between 1 and 100 Pa on a logarithmic increment, ranging from 0.1 to 100s^{-1}. A shear stress ramp test was also made between 1 and 100 Pa, for 1 minute. All measurements were performed at 25ºC, 24 hours after the preparation of the emulsions.

2.4.3.2 Oscillation frequency test

Oscillation frequency sweep tests were performed using cone-plate geometry, and at frequencies ranging between 100 Hz and 0.01 with a shear strain of 0.1%. All measurements were performed at 25ºC, 24 hours after the preparation of the emulsions.

2.4.3.3 Adhesion test

To evaluate adhesion properties a pull-away test was performed at 25°C, using a plate-plate geometry, with a speed between 0.5 and 5.5 mm/s and a gap of 0.2mm. All measurements were performed at 25ºC, 24 hours after preparation of the emulsions.

2.4.3.4 Tribology

Tribology assay was performed using a three-ball-on-plate geometry. Measurements were carried out with a velocity between $1x10^{-4}$ and 50 rad/s, with a normal force of 0.5N and a lower sample thickness of 3mm. All measurements were performed at 25ºC, 24 hours after preparation of the emulsions.

2.4.4 Spreadability

The spreadability was evaluated as previously described [94]. Briefly, was measured using an analytical balance 1g of each emulsion, which was placed at the center of an acetate sheet. A second acetate sheet of the same size was placed over this one, and in the upper face of the sheet a weight of 200g was applied. The weight was removed after one minute, and the spread area's diameter (in cm) was calculated. The measurements were taken in triplicate (n=3) and performed 24 hours after the preparation of the emulsions.

2.4.5 Microscopic analysis

Brightfield optical microscopy observation of the emulsions was performed using a trinocular microscope (Y-TV55 lens, Nikon's Digital Eclipse C1) with a digital camera (MD-E3-6.3, MicrosCopiaDigital) connected, and in turn connected to a computer (SyncMaster BX2331, Samsung). The observation was carried out 24 hours after the production of the emulsions. For this, the samples were placed on slides, covered with coverslips, and observed under a microscope with 40x magnification.

2.4.6 Packaging performance tests - Criterion 5 from Ecolabel regulation

For the selection of the packaging material, a study was performed simulating the use of the product. The aim of this test was to access the impact of different packaging materials, plastic, glass, and aluminum, as well as different type of packages such as dispensers, jars, tubes, flip-flop bottles, and bottles without dispensers. Briefly, first weigh the empty packages using an analytical balance (PLJ 1200-3A-2020a, Kern & Sohn GmbH). After, the selected final formulation was packaged in the different packages, was again weighted, and lastly dispensed the product simulating the use of the product and weighing.

According to the Ecolabel regulation, namely criterion 5, the packaging of choice should have 90% of emptying level, and the residual amount (R) lower than 10%.[29] The aim of the test was to evaluate the amount of product that remained on the package by ratio, in order to understand which discarded a greater percentage of the product. This is calculated by the following equation 3, where R is the residual amount of the product in the container, m_1 is the massa corresponding the packaging and product (g), m_2 is the mass of the packaging and product residue in normal conditions of use (g) and m_3 is the mass of the packaging completely empty and clean (g). [29]

$$R=\frac{m2-m3}{m1-m3}x100(\%) \quad (3)\ [29]$$

2.4.7 Stability assay

The stability tests were performed on emulsions packaged in plastic jars and storage at room temperature and 40°C±75% of relative humidity (RH) during 3 months. The macroscopic appearance, the pH, the viscosity, the oscillation frequency test, and droplet size distribution were performed as described previously in chapters 2.4.1, 2.4.2, 2.4.3.1 and 2.4.3.2. For all samples, the stability was determined at 0, 14 days,30, 60, and 90 days.

2.5 *In Vivo* Efficacy and Safety Analysis

The protocols for *in vivo* studies that includes the safety, efficacy and sensorial evaluation were submitted and approved by the Ethical Committee of PhD Trials ® (https://phdtrials.com/), which is an International Contract Research Organization engaged in the clinical assessment of safety and efficacy of products for topical application.

2.5.1 Human Repeat Insult Patch Test (HRIPT) – Safety Evaluation

A safety evaluation study was performed using a Marzulli and Maibach Human Repeat Insult Patch Test (HRIPT) protocol. [95]As also described in previous work [96], briefly the emulsion was applied on the back of 51 healthy volunteers. For the induction period, a series n=9 was performed over a period of 3 weeks. At the product site, an occlusive patch containing 20 mg of the formulation was applied to the left side of the back where it remained for 48 h. A two-week rest period was observed without the application of the test material. During the

challenge period, new patches were prepared and fixed in the same manner as in the induction period. The patches were removed after 48 h and scoring of skin reactions was performed. The protocol was approved by the local Ethical Committee (MS/2017/4457/P22315) and respected the Helsinki Declaration and the AFSSAPS regulations on performing HRIPT studies on cosmetic products. The study was conducted under the supervision of a dermatologist who participated in the evaluation of irritation/allergic reactions to the emulsions.

2.5.2 Biological Effects (Hydration Test) – Efficacy Evaluation

A quantitative assessment of the hydration level of the *stratum corneum* (SC) was performed using a Corneometer ® CM 825 device. The Corneometer ®CM 825 determines the hydration level of the SC by measuring the change in dielectric constant due to the skin hydration surface. Measurements were performed on two areas on the ventral side of the forearm of each volunteer (n=10), using the devices at 0, 1,2, and 4 hours. The method was used in order to understand and compare the moisturizing cream effects, in an emulsion with a DES coffee extract. The emulsion was then applied on the forearms according to the schedule defined, and in one of the areas was not applied any product (control area).

2.6 Upcycling the Spent coffee Grounds from this work

In this work the concept of CE and upcycling is always present, as well as sustainability. One of the aims of this work was to optimize the extraction conditions, for extraction with DES. Therefore, the remaining SCG was used to extract oil and produce a scrub using the remaining particles of the SCG, as described in a scheme presented in figure 2.2.

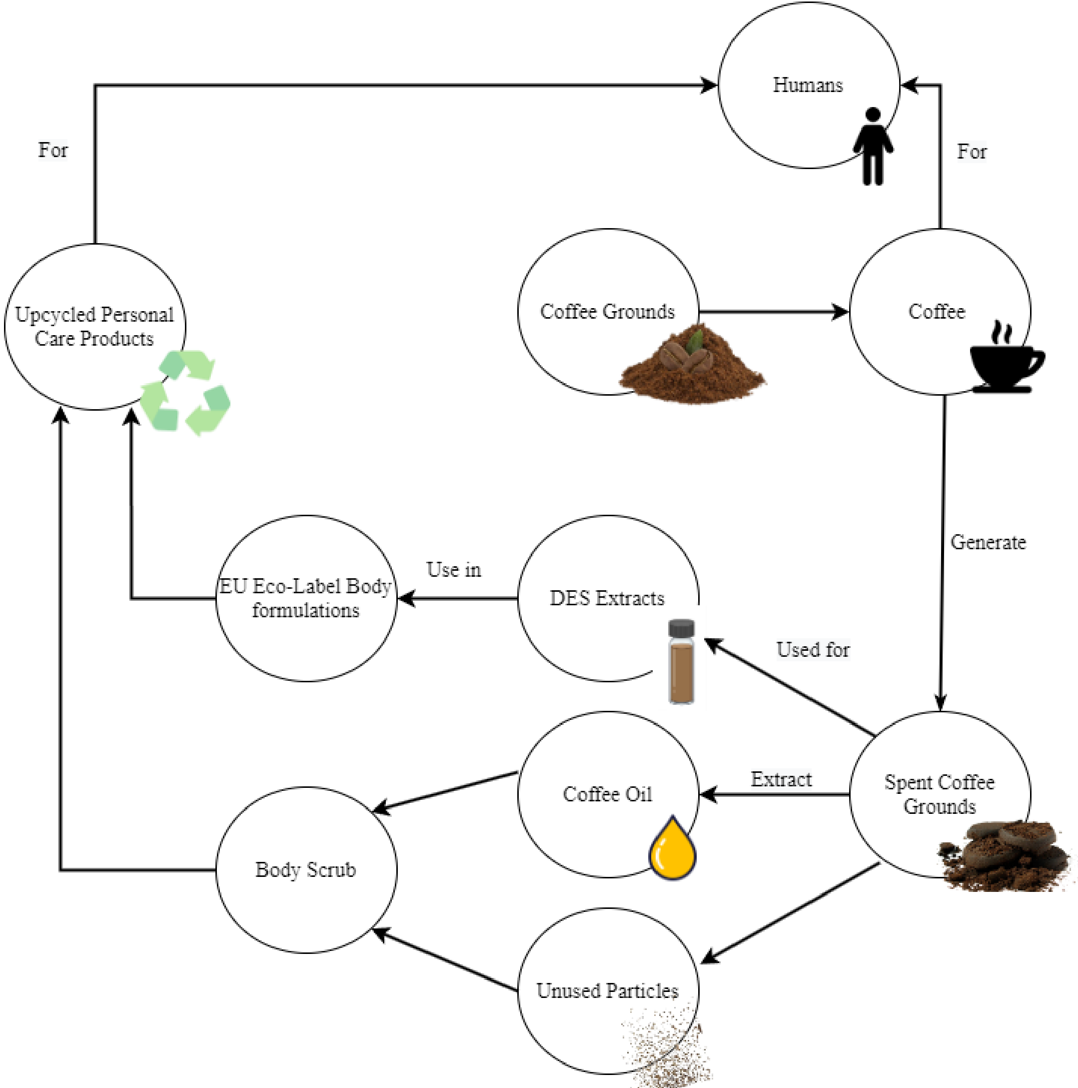


Figure 2.2- Scheme: Circular Economy and Upcycling approach used in the work

2.6.1 Physical characterization of SCG

The SCG particle used in the extraction of the oil and scrub were characterized as described above in chapters 2.4.2 and 2.4.5.

2.6.2 SCG oil extraction and reuse of SCG

For the sake of time hexane was used for the extraction of the oil fraction of SCG. The oil extracted with hexane has similar characteristics as extracted with supercritical CO_2. [97] Although hexane is not considered a green solvent, due to the amounts of oil needed for this part of the work and the short time available, hexane was used.

A simple extraction process similar to the one described in chapter 2.2.3 was used for the extraction method with DES. The extraction of oil from SCG was performed by adding the required amount of SCG according to the solid/liquid ratio (g/ml) of 1:10 and was weighed using an analytical balance (Precisa 205 A, SuperBal-series) into the respective volume of n-hexane. Samples were extracted for 5h and at 40ºC, with a water bath and stir. After oil extraction, the solid and liquid phases were separated via vacuum filtration and the extraction solvent was distilled via rotary evaporation (STRIKE 300, Steroglass) with a pressure of 368 Pa and at 45ºC.

2.6.3 Development of scrub

With the aim of developing a sustainable scrub for topical application was necessary to make combinations to understand which ingredients and in what percentage should be used. The SCG scrub was developed through the combination of different ingredients, being the final formulations described in table 2.3.

The process of producing the scrub started with measuring the required amount of the individual ingredients using an analytical balance (PLJ 1200-3A-2020a, Kern & Sohn GmbH) Then the butter and the oils were heated in a water bath (Digital water bath Model 601 by Nahita) at 50ºC and stirred until total homogenization. Then, the SCG, Vitamin E, and Geogard were added, while mixing, until total homogenization. The scrub was stored in a sustainable glass jar at room temperature.

Table 2.3- Ingredients in SCG Scrub

Comercial Name	INCI	Function	% (w/w)
-	Spent Coffee Grounds	Exfoliant agent	20
Vitamin E	Tocopherol	Antioxidant	0.4
Geogard™ 221	Dehydroacetic Acid (and) Benzyl Alcohol	Preservative	0.4
Mango Butter	Mangifera Indica (Mango) Seed Butter	Emollient	20
Almond oil	Prunus Amygdalus Dulcis (Sweet Almond) Oil	Emollient	29.6
-	SCG-Coffea Arabica (Coffee) Seed Oil	Emollient	29.6

3

RESULTS AND DISCUSSION

3.1 Extraction and chemical analysis

3.1.1 Spent coffee grounds

The material from all origins was dried in a heating oven and the moisture content was measured. For samples CP, MP, and FP, were 3,5 wt%, however for CD was 8 wt%. This can be justified by the degradation upon a time, and humidity from the place where stored, and that can have an influence on the results. The sample FP was characterized and the carbon, hydrogen, nitrogen, and sulfur content was determined by elementary analysis. The macroscopic aspect of this sample is figure 3.1 and the values obtained are expressed in table 3.1.

Figure 3.1-Sample of Spent Coffee Grounds with origin in the French Press (FP)

Table 3.1-Elemental composition of SCG from FP

% Carbon (C)	% Hydrogen (H)	% Sulfur (S)	% Nitrogen (N)	%Proteins
51.47	7.1	0.04	2.18	13.62

The sample is rich in Carbon, and this can be due to the chemical compounds present in coffee mentioned before, and also SCG are lignocellulose-rich waste, in carbohydrates, and proteins. And the results are in concordance with the ones represented in table 1.3, where content in proteins is between 13-17%. Sulfur content even in smaller amounts is important for the aroma and flavor.[98, 99]

3.1.2 Deep Eutectic Systems (DES)

The method mentioned before was used to prepare all the DES, however, DES 17 and 18 did not become liquid at room temperature. The extraction of phenolic compounds from SCG was performed according to the method previously described, which was chosen for being good for extractions with SCG. Some DES were more viscous than others, turning the method more difficult, resulting in recovering a small amount of extract or not being possible to separate. DES 19 was very viscous and was not possible to filtrate.

3.1.3 Spent Coffee Ground: Total phenolic content

In tables 3.2 and 3.3 is shown the total phenolic content is estimated by the FC method (TPC), with the aim of understanding which was the best DES for extraction, coffee samples, and conditions. The TPC of DES 14,15, and 16 was not possible to determine since the values of absorbance obtained were below the detection limit.

Comparing the results from table 3.2, it is possible to observe that the higher amount of water in CD influenced the TPC present in the extracts. The compounds must have degraded over time, due to this it was used for comparative analysis between other coffee samples, and to understand the efficiency of DES. From the results obtained the best DES for extraction of phenolic compounds from SCG was DES 2, which is composed of Glucose, Lactic Acid, and Water (1:5:3). This result is in agreement with the literature, in which it is mentioned that Sugar-organic acids-water mixtures are very efficient in extracting phenolic compounds.[44, 100]

Therefore, this was the DES used to optimize conditions and understand which would be the best to continue to be used for future extractions, and the results are shown in table 3.3. It was necessary to assess the SLR to be used, and for this was used CD with DES 2, with extraction of one hour and 50ºC. The values with different SLR were not that significantly different, and because of that, an SLR of 1:10, was used for the remaining studies, also considering the economic and environmental impact.

Table 3.2-TPC in mg GAE/g SCG present in the SCG extracts with different DES and same experimental conditions in time and temperature, 60 minutes and 50ºC and different solvent/solid ratio

DES	**Abrev. Coffee Extract**	**Solid-Liquid Ratio (g/ml)**	**TPC (mg GAE/g SCG) Sample CD**	**TPC (mg GAE/g SCG) Sample FP**
1	AX	1:20	0.97±0.24	
2	BX		12.9±1.50	
3	CX		2.81 ±0.61	
5	D		1.49 ±0.31	
2	BX_1	1:5	10.56±1.31	
1	A	1:10		2.44 ±0.13
2	B		10.4±0.80	21.68± 0.76
4	C		3.68±0.42	11.18 ±0.89
6	F		4.95 ±0.93	14.66±0.63
7	G		3.70 ±1.31	
8	K		6.17 ±0.12	13.32±0.31
9	L		5.38 ±0.33	10.66 ±0.2
10	M		4.67 ±0.10	11.35±1.0
11	N		5.90 ±0.77	14.33±0.61
12	P		8.70 ±0.30	16.8 ±1.1
13	I		3.59 ±1.1	13.2±0.7

Table 3.3- TPC in mg GAE/g SCG present in the SCG extracts, with the same SLR of 1:10 and time extraction of 60 minutes- optimization conditions with DES 2

<table>
<tr><th>Coffee Sample</th><th>Temperature (ºC)</th><th>TPC
(mg GAE/g SCG)</th></tr>
<tr><td>MP</td><td rowspan="2">50</td><td>10.40±0.70</td></tr>
<tr><td>CP</td><td>20.60±0.36</td></tr>
<tr><td rowspan="2">FP</td><td>40</td><td>19.83±1.0</td></tr>
<tr><td>60</td><td>21.69±1.1</td></tr>
</table>

The other samples, MP and CP were also tested, with an SLR of 1:10 (g/ml) and extraction of one hour at 50ºC. Comparing samples, the best results are obtained with FP, however, the difference is not significant, but it is more similar to the process of making instant coffee. The remaining experiments were made with FP.

Then was evaluated the used temperature, using an SLR of 1:10 (g/ml) and one hour of extraction. Comparing three different temperatures, 40, 50, and 60ºC, the values of TPC were very similar, nevertheless the best option in terms of results, economically and environmentally, is making extractions at 50ºC.

Another parameter in the study was time, for which extractions were done for 15, 30,45, 60, and 180 min, evaluating extraction yield. as possible to see in the graph in figure 3.2. A temperature of 50ºC and an SLR of 1:10 (g/ml) were always used. It is possible to see that after about 45 to 60 min, the values begin to stabilize, so it was decided to only perform extractions for 60 min. The optimal extraction conditions which are going to be used in the remaining work are using an SLR of 1:10 (g/ml), and extractions of one hour at 50ºC.

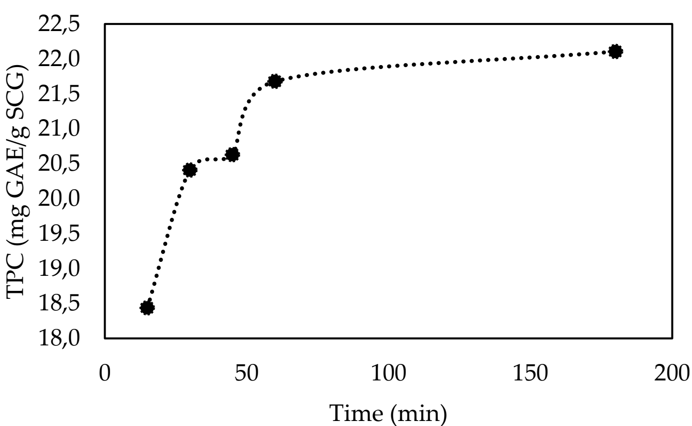


Figure 3.2-Optimization of the extraction time with DES 2, with an SLR of 1:10, at 50ºC

In order to compare the method, it was made a hydroalcoholic extraction for the four samples CD, MP, FP, and CP with the optimized conditions, and the values are expressed in table 3.4. These results confirm what was mentioned before for all samples.

Table 3.4-TPC for conventional extraction with a solution of water and alcohol (1:1), at 50ºC, for 60 minutes, with an SLR of 1:10.

Sample	TPC (mg GAE/g SCG)
CD	8.87 ± 1.41
MP	14.22 ± 1.27
FP	16.36 ± 0.79
CP	18.49 ± 1.09

3.1.4 Spent Coffee Ground: Antioxidant activity

The antioxidant activity was determined with the method of DPPH, The %inhibition was calculated, and the half-maximum effective concentration (EC_{50}) was determined. The values obtained for DES 2 with the optimized conditions, for samples CD, CP and FP are in table 3.5.

Table 3.5-Determination of EC_{50} present in the SCG extracts with different samples

Sample	EC_{50} (g/ml) DES 2	EC_{50} (g/ml) Conventional
CD	0.48±0.02	0.31±0.01
FP	0.23±0.00	0.18±0.01
CP	0.22±0.01	0.17±0.00

Since a lower value of EC_{50} indicates higher antioxidant activity, the samples from FP and CP have higher antioxidant activity with the same optimized extraction conditions. The results are in accordance with the TPC values obtained, which makes them the best option for future use. Regardless, the results for conventional extraction presented higher antioxidant potential than when compared with the green solvent, for the same samples. This can be explained by the properties of the solvents used for conventional extraction, ethanol, and water, such as the polarity. The mechanism of the free radical scavenging reactions may be influenced by the hydrogen bond's capability to accept or donate hydrogen atoms to free radicals, which may have an impact on the results of antioxidant capacity tests.[101]

3.1.5 *In Vitro* Efficacy and Safety Analysis

3.1.5.1 Cell viability Assays

Relative cell viability of HaCaT cells was measured by MTT reduction after 24h of exposition, for to three different concentrations (10, 2.5, and 0.6 % (V/V) of DES samples and the data are presented in figure 3.3.

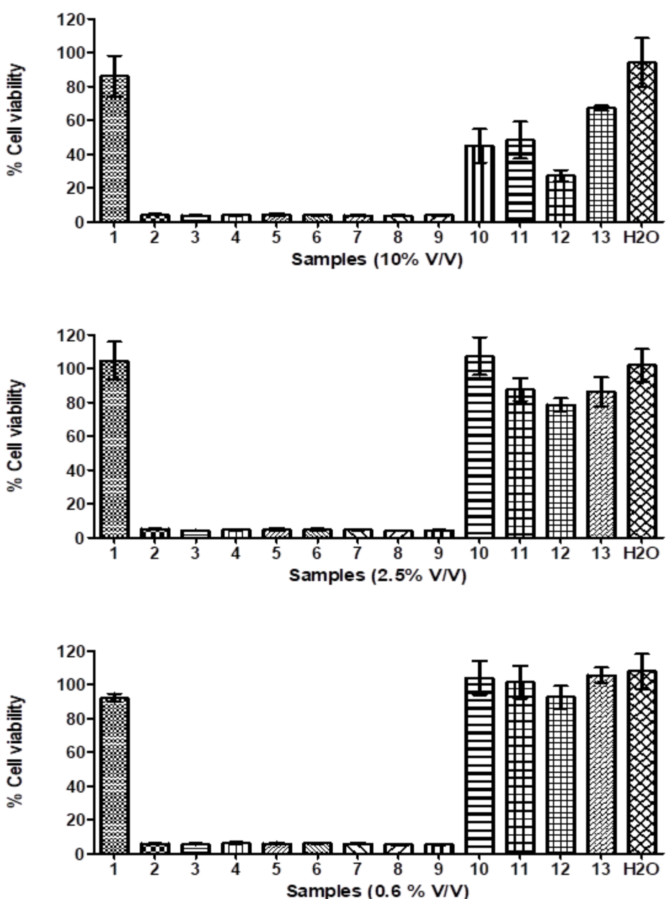


Figure 3.3- The percentage of cell viability of HaCat cell line exposed to different concentrations of DES samples for 24h of exposition time (Mean±SD; n=9)

The samples DES 2 to 9 are cytotoxic for all concentrations tested in HaCaT cell line with a percentage of cell viability lower than 10%, being this effect due to acid composition of DES samples that is in agreement with previously results, that demonstrate that, acids increase the overall toxicity, making them not suitable for cosmetic emulsions. [102]However, the DES samples 1,10,12 and 13, demonstrated more biocompatible with values of percentage of cell viability higher than 80% for samples tested at 0.6 and 2.5 %V/V. For DES sample 1, Increasing the concentration of samples to 10% V/V, did not impact in the percentage of cell viability. That can be explained by the biocompatible components (glucose and glycerol) of DES sample 1. Due to low cytotoxicity of DES samples 1,10,11,12 and 13 they can be preliminarily regarded as safe and suitable for incorporation in topical formulations. The DES 2 even with the highest values for TPC, cannot be used for this future work and was only used in order to optimize extraction conditions This can be justified by one of the components present in sample DES 2,

which is Lactic Acid, and as previously results showed, this sample was considered cytotoxic for all concentrations tested.

3.1.5.2 Reactive Oxygen Species (ROS) Production Measurement

The anti-ROS activity of the extracts A, I, M, N and P were tested in HaCaT cells exposed to H_2O_2, and the percentage of reduction of ROS between samples was determined (Figure 3.4). Selected extracts were from DES that did not present cytotoxicity in the cell viability assay. The samples that present the high capacity to reduce ROS are M, N and P but all present values higher than 75% at 5%V/V.

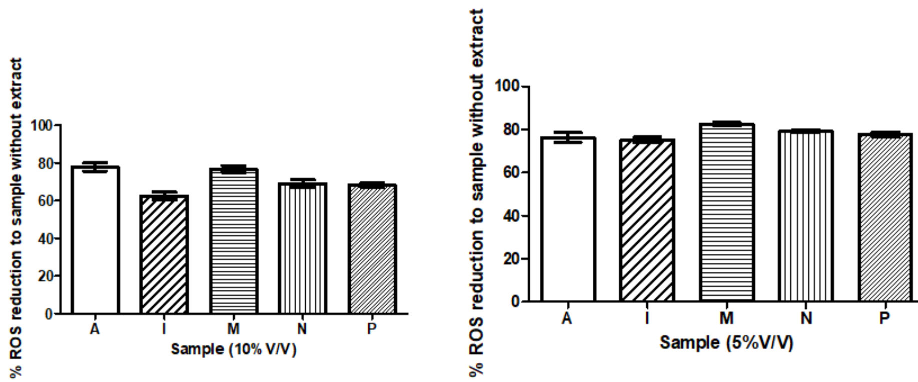


Figure 3.4- The percentage of ROS reduction in HaCaT cells (500 μM of H_2O_2) by the different samples of DES extracts at 10% and 5% V/V (Mean ±SD, n=9)

The concentration of samples that reduces in 50% of ROS is IC_{50} and was determined by non-linear regression analysis of the % ROS in the function of the logarithm of the concentration (figure 3.5). The data for the concentration that reduces the ROS in 50% for extracts are the following: 5.4±1.8 (A), 1.1±0.4 (I), 1.0±0.1 (M), 1.0±0.2 (N) and 1.5±0.3 (P) in μg/mL.

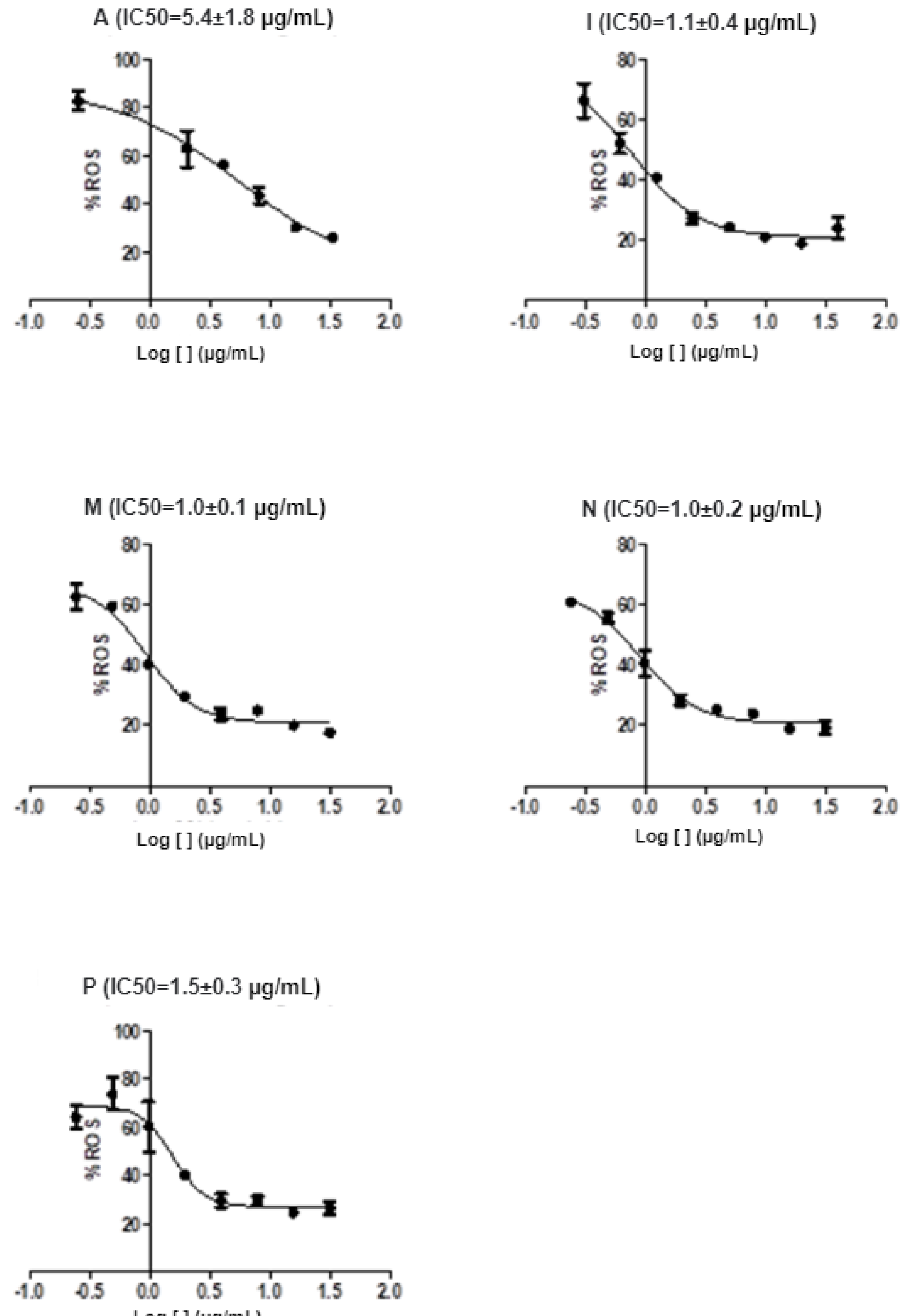


Figure 3.5-The percentage of ROS in the function of logarithm concentration of compounds in HaCaT cells after 500 μM of H_2O_2 exposition, for extracts A, I, M, N, and P, (Mean ±SD, n=9)

Comparing the values of concentration, the highest IC_{50} is for e extract A, followed by P. The samples M, N, and I present the lower values for IC_{50} indicating its higher antioxidant activity.

3.2 Final DES Spent coffee grounds extracts

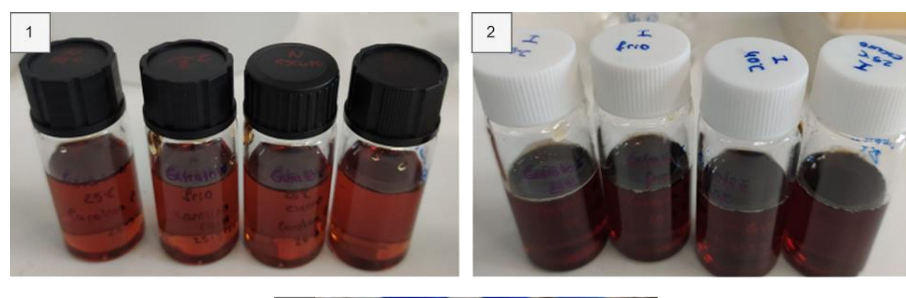
After evaluating all DES and respective extracts, it was necessary to choose one or two to continue the work, based on all the results. The remaining extracts were A, I, M, N, and P, however, was possible to choose only two for the stability assays, I and N. Extract A presented the best result for cell viability, however, had the lowest TPC, which influenced the antioxidant activity. The production time of DES 1 was the highest, which results in higher energy spent, being not the best economic and environmental option.

The remaining extracts have similar results, due to this, the decision was related to price, and impact on cosmetics. Proline according to literature, is expensive, but it is an amino acid with anti-aging properties, improves skin elasticity, and promotes skin repair and regeneration. Urea, according to the cosmetics glossary slows the loss of moisture, by increasing the water content of the top layers of the skin. However, it is reported that in cosmetics sometimes symptoms such as skin sensibilization are occasionally noticed, and at higher quantities, they may be more common. [103,104]Due to this it was decided to not select DES with Urea in their composition. As a consequence, the final extracts are I and N.

3.2.1 Stability assay

3.2.1.1 Total phenolic content and Antioxidant activity

The final extracts were I and N, and their stability over time was evaluated. A comparative method with a 1:1 solution of Water and Ethanol was also performed. The different extracts stored under different conditions are presented in figure 3.6.



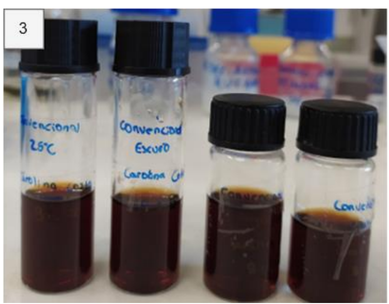


Figure 3.6-SCG extracts for stability assay, with SLR of 1:10, at 50ºC, for 60 min, with FP sample: 1-N, 2-I, and 3-Conv (Conventional Hydroalcoholic extract)

The sample storage conditions were considered to identify the changes in TPC and in antioxidant activity over time. At figure 3.7 are presented the TPC and EC_{50} for I, N and Conventional extract, for different stored conditions, in the form of graphs. In this way, it is better to see the evolution of the values over time. As previously referred, phenolic compounds present sensibilty to temperature and light exposure, therefore temperature was found to influence TPC and EC_{50} with variations observed throughout the entire storage period, as it is possible to see in figure 3.7.

The TPC data for samples kept at 40ºC (HT) decrease over time due to the rapid degradation of phenolic compounds in both DES and hydroalcoholic extract. After 90 days, the TPC values decreased 15% (I), 21% (N) and 32% (Conv). With high temperature, over time, as the TPC value decreases, the EC_{50} value increases, which demonstrates the lower antioxidant properties. At the end of the essay, EC_{50} increased by 5% (I), 54% (N) and, 38% (Conv). For the conventional hydroalcoholic extraction, the TPC value is lower than the I, however, EC_{50} is higher, which is in concordance with a lower TPC. However, as it is possible to observe in figure 3.11, for conventional extract, upon time was observed only after 14 and 30 days a decrease in TPC, and an increase in EC_{50}, which demonstrated that occurs a faster and more visible upon time degradation of the phenolic compounds, which confirms that is better using DES as an alternative to conventional solvents.

Furthermore, the EC_{50} values for samples I and N were stable over time (variations <10%) which means that antioxidant activity does not decrease upon time, which makes extracts stable.

TPC of samples RT and RTD for I showed good stability after 90 days, decreasing 2 and 1% respectively. For the same conditions, RT and RTD samples for N, decreased by 3%, and increased by 3%, respectively., oscillations that are considered normal and non-significant. At time 90 days, the samples kept at room temperature with and without light for the conventional extract possibly evaporated, due to their composition, and was not left enough quantity to perform the study. Nevertheless, after 60 days, for samples RT and RTD the TPC value decreased by 16 and 8.6%, respectively.

Comparing TPC for samples CT, stored at a lower temperature, after 90 days, sample I showed best stability results, decreasing only 0.7%, and the remaining increased by 8% (N) and decreased by 19%.

Comparing all the results, over the 90 days it is possible to notice that the most stable extract is I. As expected, the samples stored at higher temperature present higher EC_{50} values relative to the other samples, which is correlated to the decrease of the TPC. Phenolic compounds are then less degraded when samples are stored at lower (CT) and room temperatures (RT), as well as when are protected from light (RTD). In these conditions values of TPC and EC_{50} tend to stabilize, and not lose their antioxidant properties that can be used in cosmetic formulations upon time. As predicted the conventional extract degraded faster when compared to DES extracts, which proves they are a better alternative to conventional organic solvents for the extraction of phenolic compounds from SCG.

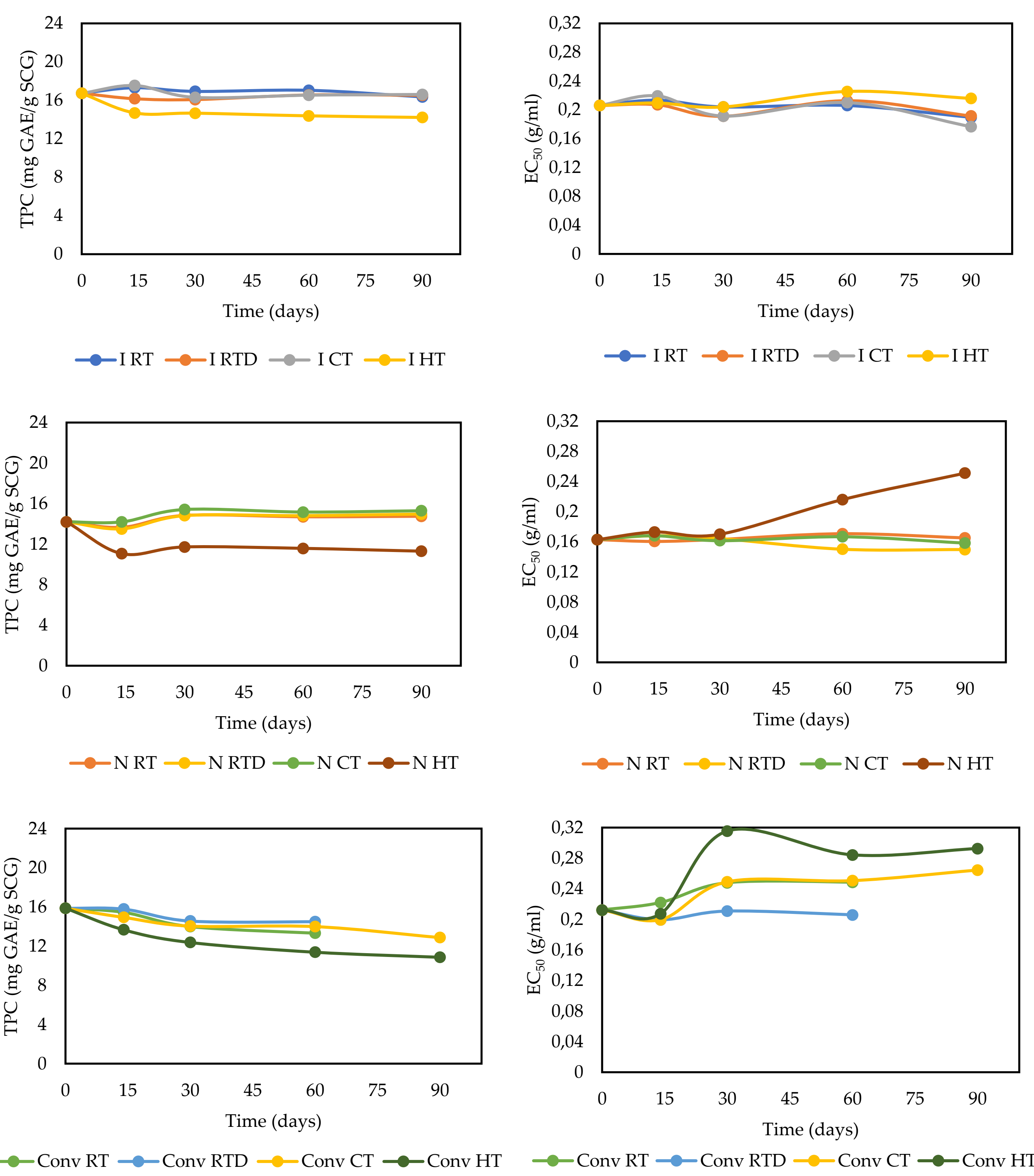


Figure 3.7-Stability assay-TPC and EC 50 results for extracts I, N, and Conventional Hydroalcoholic extraction (Conv)

3.2.1.2 Liquid Chromatography–Mass Spectrometry (LC-MS)

In order to identify the presence of the major compounds present in the two extracts I and N, an LC-MS analysis was performed to compare the mass of those compounds. The analysis was performed for sample EI at time zero, however, it is expected that extracts have in common the compounds present in higher concentrations. From the analysis was possible to identify caffeine (molecular mass of 194 u) as the compound present in higher quantities. The mass spectrum is presented in figure 3.8 and the results are only in positive mode since caffeine does not ionize well in negative mode and at retention time (T.r) 23.99 min.

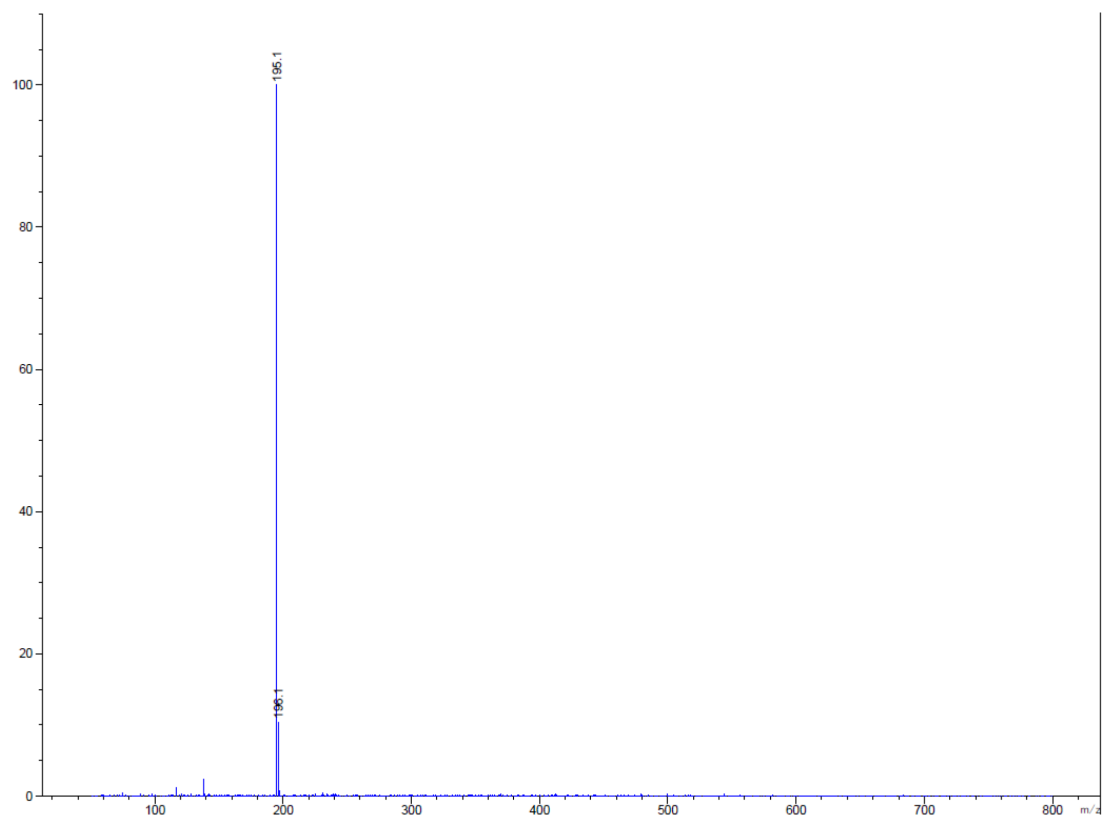


Figure 3.8-Mass spectrometry (MS) spectrum for sample EI (t=0) T.r.23.99 min corresponding to caffeine

The presence of chlorogenic acid (CQA molecular mass of 354 u) was possible to confirm, demonstrated at positive mode at a retention time of 7.6 min.; 15.4 min.; 18.5 min and 19.1 min. It is present ion 355 (m/z) correspondent to $[M+H]^+$. In negative mode, ion 353 (m/z) is present, which corresponds to the deprotonated molecule, [M-H]. These ions are strongly suggestive of the presence of chlorogenic acids. To demonstrate the data presented above, only the MS spectrum at the retention time of 7.6 min for positive and negative modes is shown in figure 3.9.

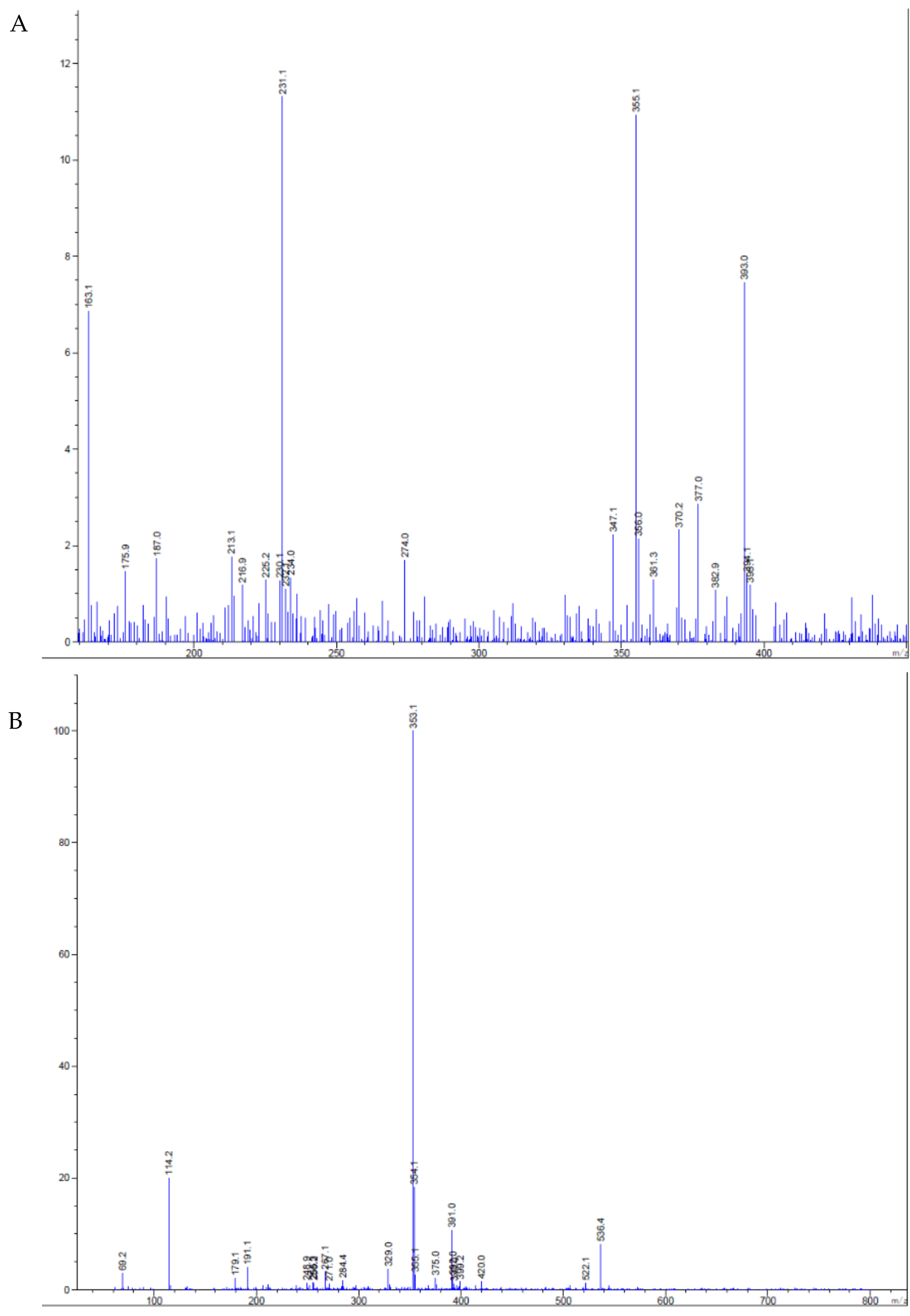


Figure 3.9-Mass spectrometry (MS) spectrum for sample EI (t=0) T.r.7.6 min corresponding to CQA: A-Zoom on positive mode; B- Negative mode.

3.2.1.3 High-Performance Liquid Chromatography (HPLC)

For the two extracts I and N was performed the quantification of the phenolic compounds by HPLC for time zero. For the conventional hydroalcoholic extract was not possible to do this quantification. The concentration of the compounds from extracts I and N is presented in table 3.6.

Table 3.6-Compounds quantification by HPLC in mg/g SCG for extracts

Compound	Concentration (mg/g SCG)	
	I	N
Gallic Acid	n.d	0.13
Caffeic Acid	0.05	0.06
Chlorogenic Acid	0.96	1.40
Caffeine	3.73	4.13
Ferulic Acid	0.08	n.d
Quercetin	0.04	0.05

The total content of phenolic compounds obtained by HPLC is different from with the Folin–Ciocalteu method, being much lower. This can be justified by the fact with the HPLC analyses were not identified all compounds. However, the compounds present at a higher percentage are Caffeine and Chlorogenic Acid, which is in concordance with the literature [79] in table 1.3, and the results obtained by LC-MS analysis

The HPLC analyses were also used to compare the profile upon time for the different samples, in order to understand if the compounds remain or start to degrade. The HPLC chromatograms for samples after 14 and 90 days are presented in An appendix 5.2 and 5.3 for extract I and N, respectively. Comparing only these two different times, it is possible to notice that for all samples, and conditions the profile is very similar. Are always highlighted two major peaks, that correspond to the two compounds Chlorogenic Acid and Caffeine present in higher quantities over time.

3.2.2 *In Vitro* Efficacy and Safety Analysis

3.2.2.1 Cell viability assays

The more promising DES extracts, N and I, and the conventional hydroalcoholic extraction were also evaluated and DES 11 and 13 ate different concentration In HaCaT cell line using MTT assay for 24h of exposition. Data percentage of cell viability are presented in figure 3.10.

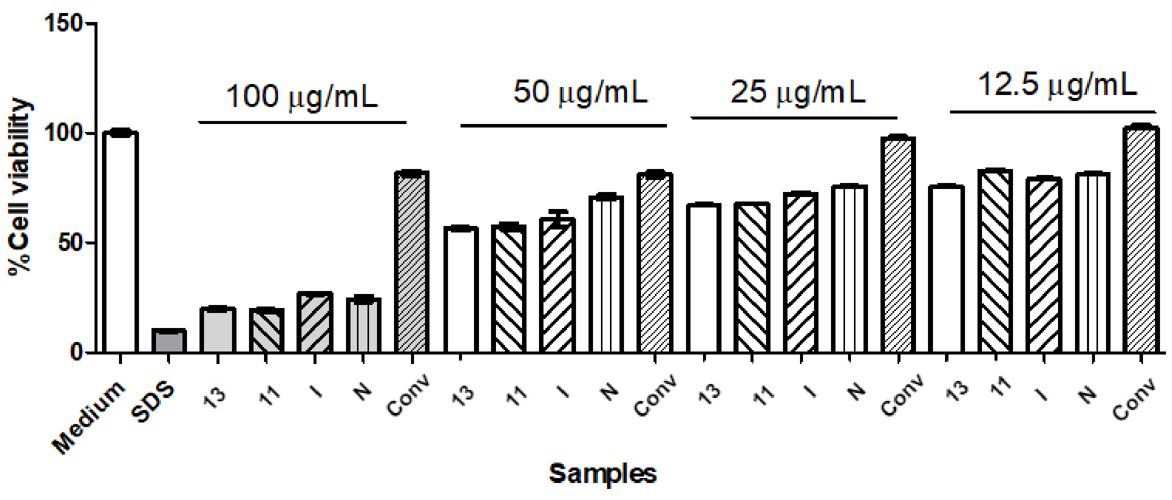


Figure 3.10- The percentage of cell viability by MTT endpoint, of HaCaT cell line exposed for 24h to 100, 50, 25, and 12.5 µg/mL of DES 11, 13, extracts I, N, and conventional hydroalcoholic. (Negative control was cell culture medium and positive control SDS 1mg/mL) (Mean ±SD, n=9)

The percentage of cell viability decrease with the increase of sample concentration for all samples tested in agreement with the previous results. The conventional extract (Conv), from a solution with ethanol and water, presents higher cell viability and was used as a comparative control. From this data the results show that DES extracts from SCG can be suitable for topical application, presenting percentage of cell viability higher than 50%, for concentrations ≤50µg/ml.

3.2.2.2 Reactive Oxygen Species (ROS) Production Measurement

The DES extracts, N and I, and respective the DES 11 and 13, comparing with the conventional hydroalcoholic extraction, were analyzed in terms of the capacity to prevent the ROS production, in HaCaT cell line, induced by 500 µM, H_2O_2 or by exposure to UVB light (figure 3.11).

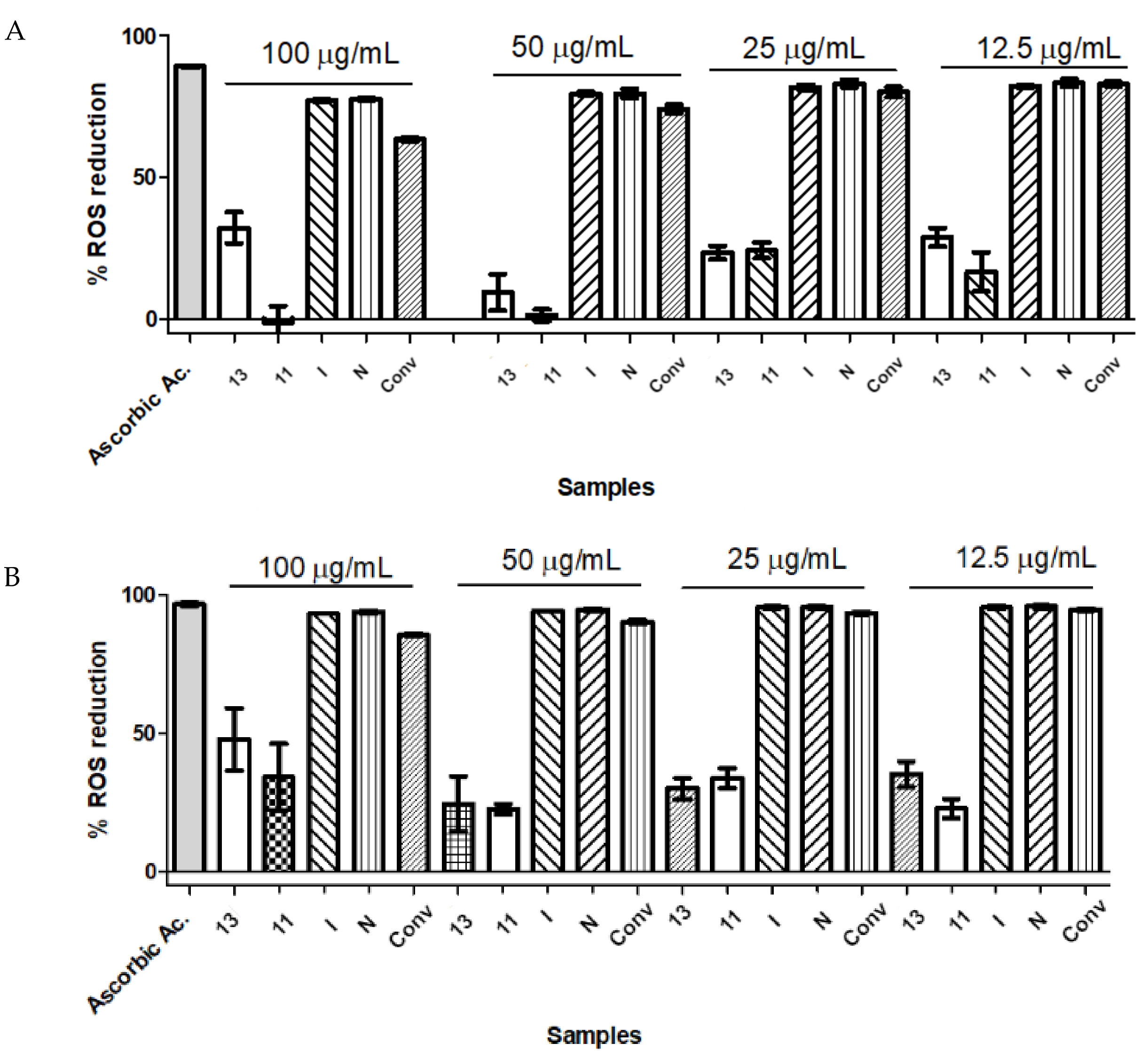


Figure 3.11-The percentage of reduction of reactive oxygen species (ROS) formation (%) in HaCaT cells exposed to (A) hydrogen peroxide (500µM), or (B) UVB light for 15 min. An ascorbic acid solution (1 mg/mL) was used as positive control. (Mean ±SD, n = 9), for extracts I, N and conventional hydroalcoholic

The samples that present the high capacity to reduce ROS are the extracts with DES and conventional, namely I, N and, Conv, with values of %ROS reduction higher than 70%, values similar to the positive control (Ascorbic acid solution). The best results obtained in the reduction of ROS induced by UV are expected by presence of the compounds from the SCG, such as phenolic compounds, which have antioxidant properties. [76,85]

In cells exposed to UV, the % of ROS reduction with extracts were not significantly different from each other. Cells exposed to H_2O_2 the % of ROS reduction with DES extracts, I and N were higher than the one observed for the conventional extract. Since in both conditions,

extracts I and N were able to reduce the ROS formation by more than 80%, can be explored for topical application.

3.2.2.3 Human neutrophil elastase (HNE) Enzymatic Inhibition Assay

The results of enzyme activity inhibition for HNE are shown in figure 3.12. The values of IC_{50} for extracts are: 26.7±1.2 (I), 28.9±1.3(N), and 3.6±1.2 (Conv) in µg/mL. The DES extracts I and N presented higher IC_{50} values than the conventional hydroalcoholic extracts, which makes them less potent inhibitors of HNE. Conventional hydroalcoholic extract presented a better result, but in this work is being used as a comparative method.

It is important to point out that even requiring higher concentrations to inhibit enzymes, DES extracts were active, that allow to explore it in an application that prevents the skin problems associated with high HNE activity. It has been described that inhibitors of elastase enzyme may help to prevent the loss of skin elasticity as well as skin aging.[105] For future studies would be interesting to study the enzymatic inhibition activity against other enzymes involved in skin problems.

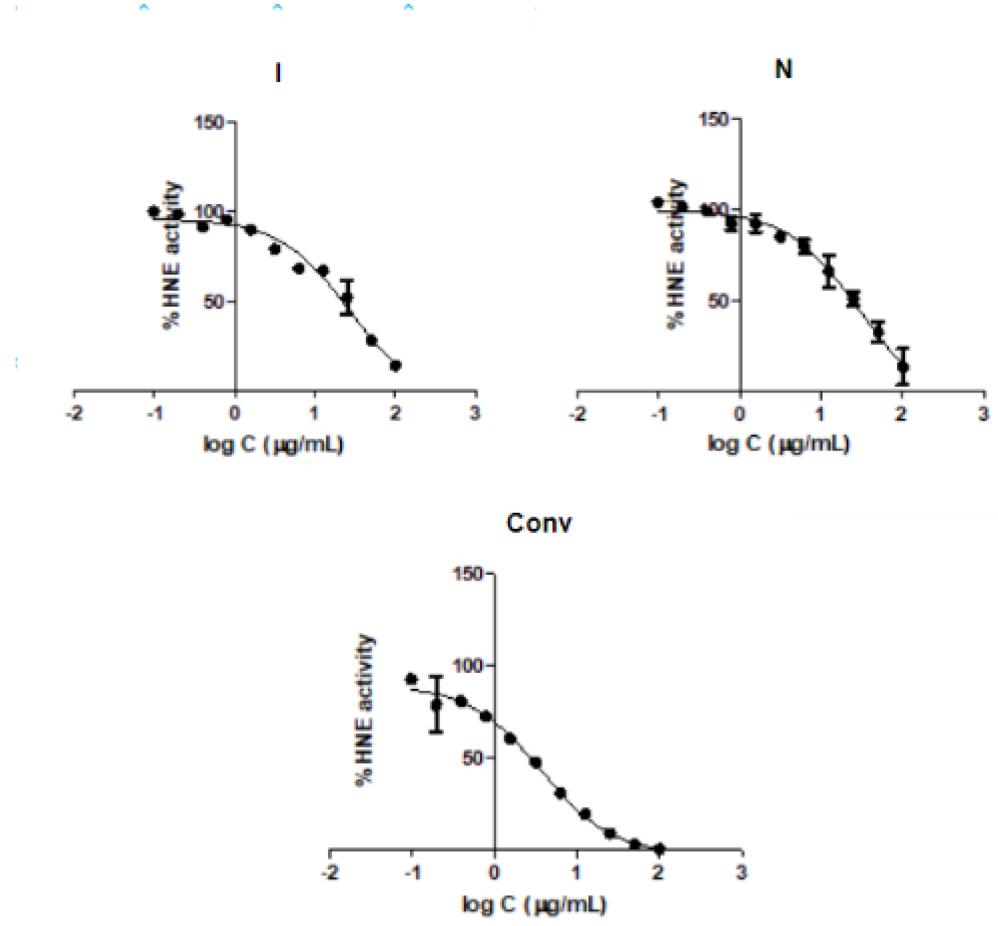


Figure 3.12-The percentage of HNE activity in the function of logarithm concentration for extract I, N, and conventional hydroalcoholic (Mean ±SD, n = 2)

3.3 Topical formulations

3.3.1 Final formulations

The DES 11 and 13, and respective extracts N and I were chosen to be incorporated in topical formulations, as bioactive ingredients. The purpose of this study was to analyze the properties of DES as a solvent and the bioactive activity present in the extracts when applied to cosmetic emulsion. As a control, a placebo without DES and extracts was produced. In order to select all ingredients for the emulsions according to the Ecolabel regulation, was followed criteria 3 and 4, which refer to the biodegradability and aquatic toxicity and the excluded or restricted substances, respectively. [29]

The emulsions stabilized with Montanov 68 MB presented a homogeneous appearance, white color very similar to normal body creams, and in terms of after feel of application it was the best. According to the literature, Montanov 68 MB is reported to generate liquid crystals surrounding the oil droplets, which makes emulsions less likely to coalesce.[106] Based on this result it was decided to use this emulsifier to stabilize the eco-label emulsions.

In literature is reported that an oily phase containing different long-chain alkanes instead of short-chain emollients is more stable. [107], [108] This can be justified by the fact that alkanes can inhibit coalescence, which results in the prevention of the interactions between emulsion droplets and assuring pH values in a suitable range. In this work, 2 emollients were selected C18-21 Alkane and C21-28 Alkane, which are long-chain alkanes with medium viscosity. A sustainable preservative ,Geogard 221, (*Dehydroacetic Acid (and) Benzyl Alcohol*) was selected with the aim of preventing any alteration caused by microorganisms and contamination during production, storage, and consumer use. [109] Emulsions in study are E11 and E13 (with DES 11 and 13); EI and EN (DES extract from SCG with DES 13 and with DES 11); and placebo as a control.

3.4 Physical and chemical characterization of emulsions

3.4.1 Appearance, pH determination, and physical stability

Regarding the organoleptic characteristics, all formulations presented a homogeneous appearance with bright white color. EI and EN emulsions presented a homogeneous appearance, but with a smooth yellow color due to the presence of the spent coffee ground extracts. These results can be confirmed in figure 3.13. Emulsions did not present phase separation in the centrifugation tests.

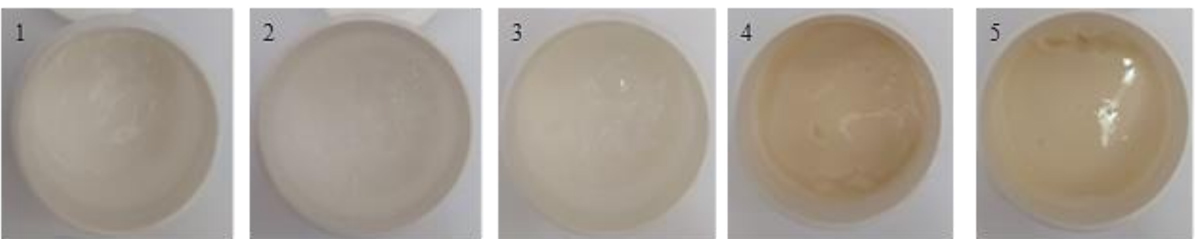


Figure 3.13-Macroscopic aspect of the validated formulations: 1- Placebo; 2-E13; 3-E11; 4-EI; 5-EN

It is important to measure pH values of topical products to guarantee that they respect the acidic physiologic pH of the skin and their stability. The pH of formulations ranges

between 3.98 and 4.58, which is required to be adjusted to the acceptable pH range (4.5-6.0), for the skin, with a solution of NaOH 5%. After the adjustment, the pH range was between 5.18 and 5.40. It was noticed that adding DES and extracts increased the pH of the emulsions, being more suitable for topical application.

3.4.2 Droplet size distribution analysis and microscope structure of emulsions

Small droplet size and tight distribution results in a more stable emulsion. [9] Concerning the results shown in figure 3.14, all emulsions presented a bimodal distribution. Results in table 3.7, concerning the droplet size results, show that the droplet size is similar for some emulsions considering the standard deviation. It is possible to observe that the addition of 5% of extracts increases the droplet size. Since none of the emulsions showed phenomenon of instability, and the distribution is very similar, all emulsions can be considered stable.

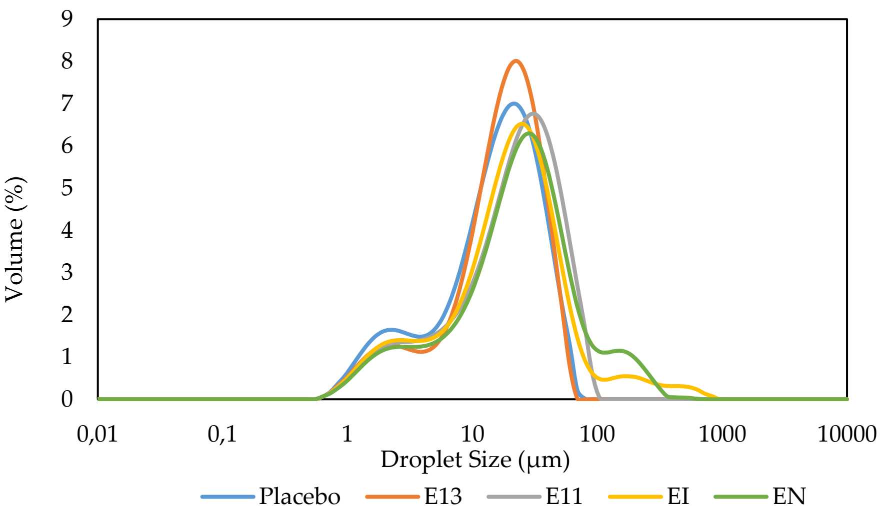


Figure 3.14-Droplet size distribution of final emulsions (mean, n=6)

Table 3.7-Droplet size distribution results of final formulations (mean±SD, n=6)

Emulsion	Droplet size distribution (μm)			
	d(10)	d(50)	d(90)	Span
Placebo	2.37±0.00	15.17±0.02	35.9±0.16	2,21±0,01
E13	2.95±0.01	16.70±0.04	35.7±0.1	1,96±0,00
E11	2.92±0.01	20.71±0.06	50.6±0.27	2,30±0,01
EI	2.83±0.06	18.97±0.44	55.35±8.45	2,76±0,37
EN	3.19±0.11	21.9±0.75	73.62±21.5	3,18±0,85

The images obtained using the microscope, are represented in figure 3.15. Observing the images it is possible to affirm that the O/W emulsions present uniformly distributed droplets from different sizes. Comparing the images from the five emulsions, there is not visible a significant difference between them.

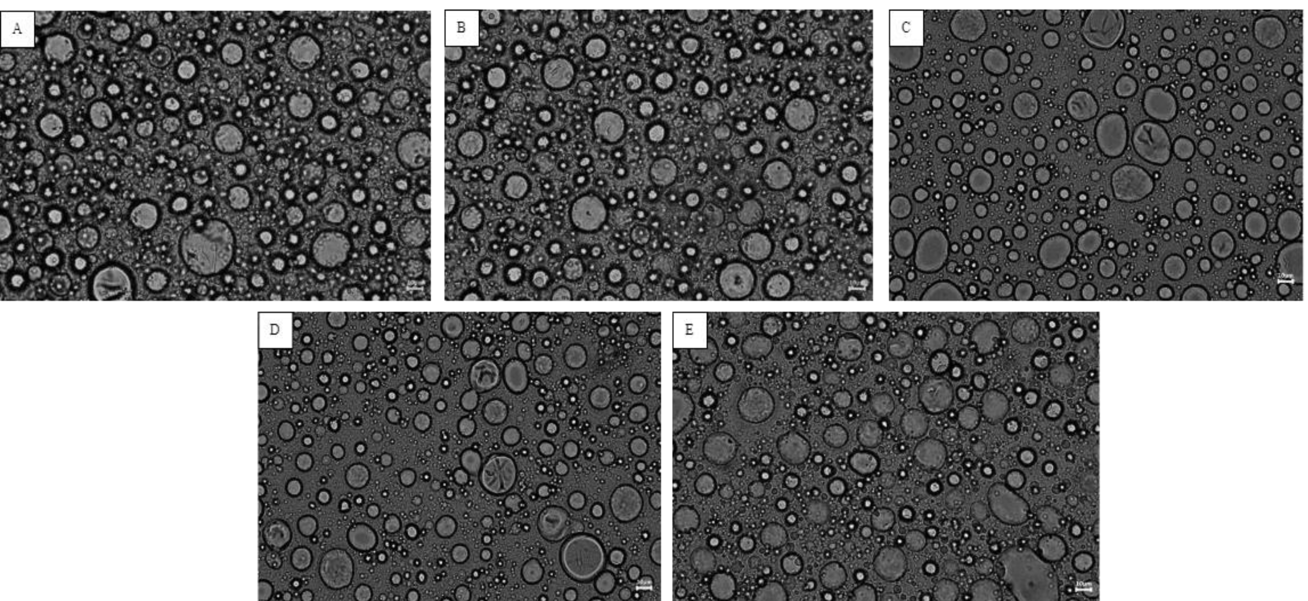


Figure 3.15-Images obtained by microscopy for the emulsions in study- A) Placebo with 40x magnification; B) E13 with 40x magnification; C) EN with 40x magnification; D) EI with 40x magnification; E) E11 with 40x magnification

3.4.3 Structure analysis of emulsions

3.4.3.1 Rotational viscosity measurements

The viscosity of the five emulsions was measured, and the results are represented in Figure 3.16, which shows the flow curves (shear stress function of shear rate). The addition of

DES and extracts to the placebo emulsion did not significantly change the viscosity. A similar flow curve was observed, where the apparent viscosity decreases simultaneously with the increase in shear rate. Since the viscosity is not constant and varies as a function of the applied shear rate or shear stress, emulsions can be classified as shear-thinning fluids.[17, 18,19]

According to Zillich, O V et al [83] the incorporation of an extract rich in polyphenols into emulsions influenced their rheological properties and stability, namely, decreasing the viscosity. The justification could be related to the interactions between extracts and components of the emulsion, such as the emulsifiers. However, in this study, this phenomenon did not happen, but since extracts are rich in antioxidant properties, they can improve oxidative stability and storage stability of emulsions. [83]

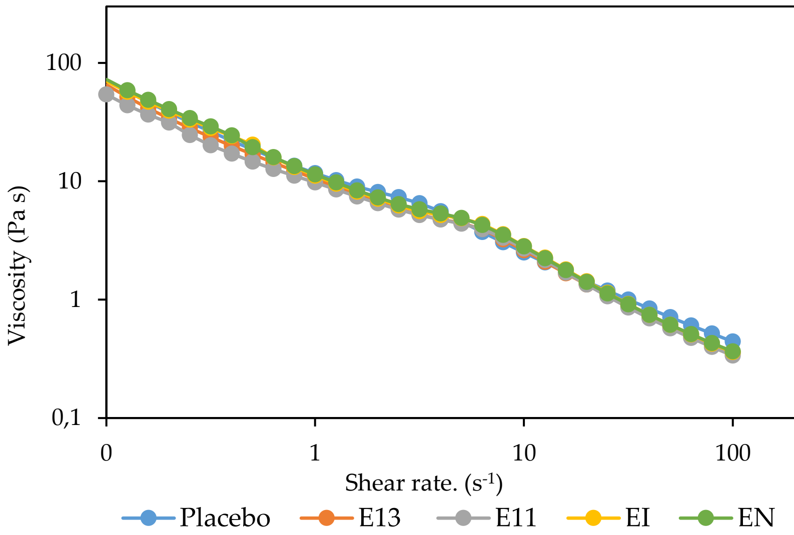


Figure 3.16-Viscosity flow behavior of emulsions in study

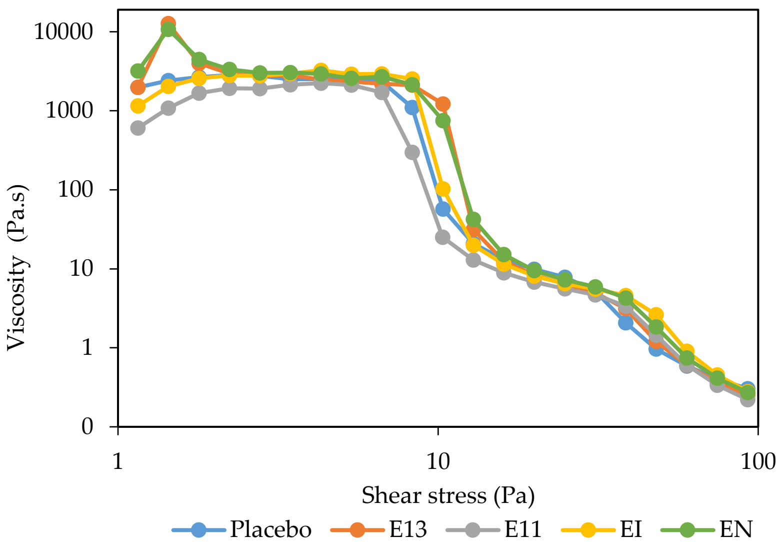


Figure 3.17-Viscosity flow behavior of emulsions in study for stress ramp test

In this research study it was determined the yield stress of emulsions by means of a shear stress ramp test. The test simulates the primary skin feeling, corresponding to the moment when the product is first applied to the skin, namely the sensations at the start of the application. [110] In the test, are correlated shear stress and dynamic viscosity, as well as the influence in consistency which is determined by the maximum viscosity of the product. [110] The results are expressed in figure 3.17. For all emulsions, there is a different initial viscosity, but at certain shear stress tends to be more constant, and then decreases.

Therefore, the thickness of the emulsion decreases as it is rubbed into the skin, which is related to absorption. The results are in concordance with the ones presented in figure 3.16, which represents the secondary skin feeling. The sensation is related to the feeling on the skin after it has been applied, evaluating the absorption capacity. There is a correlation between the value of the viscosity and shear rate, at the end of the application to the skin. [110]

Emulsion E11 according to the results is less viscous and more fluid compared with the others. This emulsion a has tendency to absorb more quickly into the skin. An opposite result is formulation EN and E13, with higher viscosity, require more time to break their structure at higher shear stress. [110] The flow properties can correlate to the ease of applying and spreading on the skin. According with the literature, an emulsion for the skin should have low viscosity at high shear (easier to apply) and high viscosity at low shear, in order for not spill out of the packaging , which is in concordance with the results. [111]

3.4.3.2 Oscillation frequency test

The results obtained for the oscillation frequency test are expressed in figure 3.18. Regarding these, in all formulations, the G'> G'', which means formulations have the elastic module superior to the viscous module and present a strong network that allows suitable spreadability, adhesion, and tackiness for skin application. Additionally, in formulations with DES and extracts, these results confirmed that these emulsions are more structured than the control, and solid behavior predominates. [17, 18]

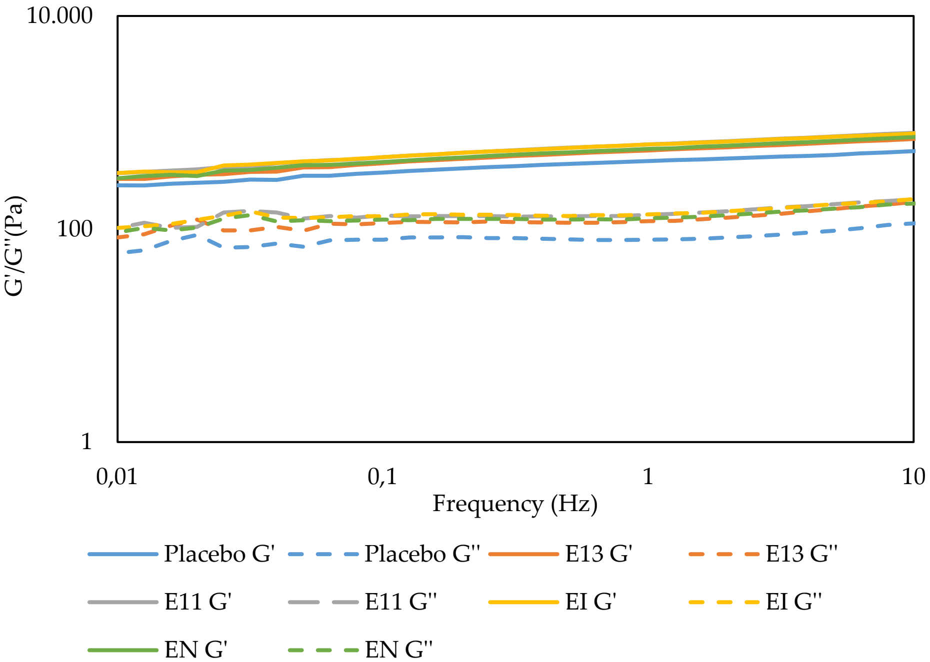


Figure 3.18-Results of the Oscillation frequency sweep test for emulsions in study

3.4.3.3 Adhesion test and Spreadability

Adhesive strength was performed at 25ºC with the aim of simulating how the formulations behaved in terms of adhesion, when used daily and when are removed from the packaging. The results on Table 3.8 show similar adhesion and tackiness properties to the control. In terms of spreading behavior all exhibit, a similar behavior to the control and these results are in accordance with the rheological results.

Table 3.8-Adhesive properties of the emulsions in study at 25ºC (mean±SD, n=6); Spreadability properties of the emulsions in study (mean±SD, n=3).

Formulation	Peak Normal Force (N)	Time for Force to reduce by 90% of peak (s)	Area under force time curve (N.s)	Diameter of spread area (cm)
Placebo	-0.394±0.011	-0.039±0.001	0.231±0.020	6.98±0.03
E13	-0.454±0.018	-0.045±0.002	0.257±0.012	7.46±0.16
E11	-0.431±0.008	-0.043±0.001	0.240±0.013	7.05±0.01
EI	-0.461±0.013	-0.046±0.001	0.261±0.012	7.3±0.22
EN	-0.453±0.009	-0.045±0.001	0.257±0.004	6.7±0.16

3.4.3.4 Tribology

Tribology, a sub-category of rheology, is an emerging technique for texture studies, and is important to the human perception of applying a topical formulation. The measurement represents the forces necessary to start or continue a sliding motion of the sample on a defined area.[112]The results obtained for tribology are presented in figure 3.19. A force value was selected for all emulsions, representing the force needed to spread into the skin at a lower speed. The values (N m) corresponding to this force to the lowest speed ($1.23E^{-4}$ (t)(rad/s) for each emulsion are $2.69E^{-5}$ (Placebo), $1.97E^{-5}$ (E13), $3.33E^{-5}$(E11), $3.70E^{-5}$ (EI) and $3.39E^{-5}$ (EN).

Curves for all formulations are similar and compared with the values presented above, the difference is not significant. This is in concordance with the previous results, where was not observed a noticeable difference between placebo and emulsions with DES and extracts.

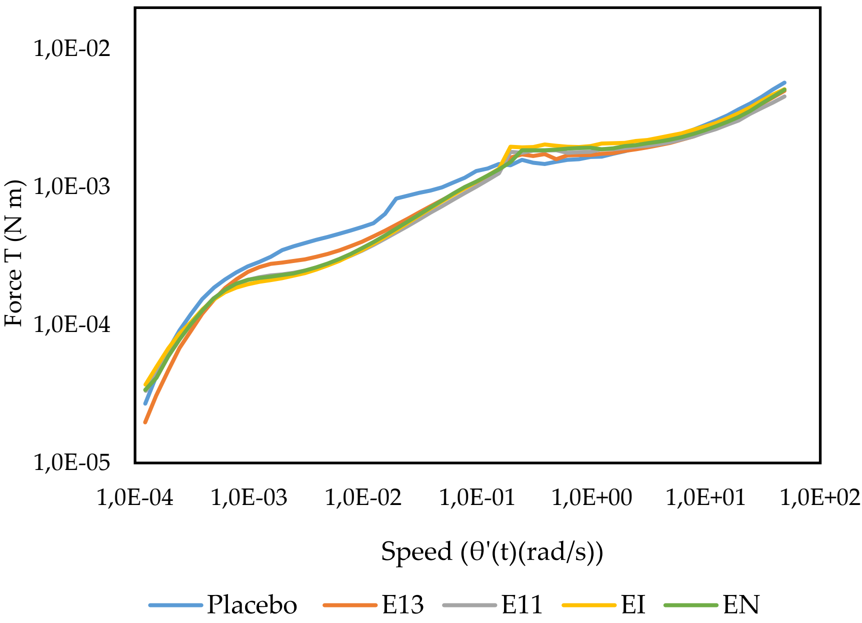


Figure 3.19-Results of Tribology test for emulsions in study.

3.5 Packaging performance tests - Criterion 5 from Ecolabel regulation

The results obtained for the residual amount of the product in the containers, R, are presented in table 3.9 Due to consistency and viscosity of emulsions, when trying the packages were found some difficulties in the packages with a dispenser, being R>29%. On bottles without a dispenser was found the same problem, and was not obtained an R<10%. As predicted, the best packaging for the emulsion under study are jars, either plastic, glass, or aluminum, which all obtained an R< 2%. The two tubes in the study also showed good results, plastic, and aluminum, with an R <10%.

Glass could be chosen, however, due to the weight will not be an option. In terms of choices, plastic and aluminum packages were considered. When a container allows the product to be exposed, higher is the probability of being contaminated, as happens in large mouth jars. As the consumer uses and applies the cream, the product can be contaminated by microorganisms present in the hands and body. This was taken into account when the emulsions were formulated, in particular the preservative system, in order to be able to compensate for frequent consumer exposure and resist microbial contamination. Some requirements for the packaging of sustainable products are: easily accessible; minimizes product exposure; restricts or minimizes direct contact with delivery mechanism; compatible with product and

preservative system; composed of recyclable or sustainable materials and aesthetically appealing. [113] Due to these criteria, it was decided to use a recyclable plastic jar with a refill because functionally and aesthetically.

Table 3.9- Value of R (%) for different containers made with different materials: glass, plastic, and aluminum

Material	Packaging	R (%)	Material	Packaging	R (%)
Glass	Jar	1.9	Plastic	Without dispenser	16.5
Aluminum	Jar	0.4		Flip-Flop	10.6
Plastic	Jar	0.4		Tube	8.3
	Dispenser	29.4	Aluminum	Tube	9.1

3.6 Stability study

In order to study the physical and chemical stability of formulation upon time, a stability study was performed at different time points considering two different conditions of storage. Concerning the organoleptic characteristics, all emulsions presented a homogeneous appearance, with no visual sign of instability.

It was noticed, after 14 days, a little change in color of a placebo, E13, E11 kept at 40ºC. This might be due to the oily mild oxidation which is promoted at higher temperatures. [114] In future work, to avoid this must be added an antioxidant, such as tocopherol, also known as Vitamin E, which can inhibit lipid peroxidation by scavenging free radicals. Since this does not happen in emulsion EI and EN, demonstrates the potential antioxidants present in the extract. [115, 116]

On table 3.10 are presented the pH values for all emulsions upon time. Emulsions stored at 25ºC and 40ºC, pH values decrease (between 5% for EI and EN; and 10% for E11,13 and placebo after 90 days) and might be due to the production of acid by-products, or the composition of the preservative. [115] The selected preservative is composed of Dehydroacetic Acid and Benzyl Alcohol which can cause the pH of formulations to drift downwards. A strategy in future work to assure the pH range and the stability of formulation should be to add a pH buffer, to adjust the acidity of a product, such as sodium citrate.

Table 3.10-Stability test results for formulations placebo, E11, E13, EI and EN during 90 days at 25 and 40ºC

Formulations	Conditions	25ºC				40°C±75%			
	Time (days)	pH	η (Pa.s)*	G′ (Pa)**	G″ (Pa)**	pH	η (Pa.s)*	G′ (Pa)**	G″ (Pa)**
Placebo	0	5.30	18.6	409.3	79.98	5.30	18.6	409.3	79.98
	14	4.34	22.01	513	116.7	5.31	19.46	362.2	57.2
	30	4.94	35.56	588.2	139.1	5.03	25.84	838,7	183.6
	60	4.83	21.52	634.3	157	-	-	-	-
	90	4.80	21.74	684.1	172	-	-	-	-
E13	0	5.37	16.91	510	114.3	5.37	16.91	510	114.3
	14	4.94	20.01	525.6	134.3	5.61	18.94	437.3	91.63
	30	5.02	20.32	472.4	124.9	5.29	16.31	414.1	86.1
	60	4.99	23.65	519.3	190.2	5.16	15.97	345.5	68.12
	90	4.89	32.24	779.7	241.2	4.99	17.02	424.3	87.68
E11	0	5.40	14.68	579.2	131.4	5.40	14.68	579.2	131.4
	14	5.12	18.04	497.9	133.1	5.46	17.92	453.5	97.09
	30	5.02	20.66	584.1	166.1	5.06	17.88	502.1	107.8
	60	5.05	21.66	558.1	158.4	5.01	17.96	505.8	117.1
	90	4.83	19.25	684.1	172	4.88	22.26	685.1	172
EI	0	5.26	20.43	577.5	132	5.26	20.43	577.5	132
	14	5.03	19.57	619.9	166.9	5.26	15.28	471.7	97.54
	30	5.06	20.41	650.6	183.2	5.16	16.18	555.9	118.5
	60	5.00	19.71	521.6	148.4	4.99	17.17	430.3	92.75
	90	4.99	19.72	519.7	147.1	4.92	18.99	438.4	93.98
EN	0	5.18	19.37	525.5	122.7	5.18	19.37	525.5	122.7
	14	5.08	20.04	604.8	169.2	5.20	18.76	563.7	122.7
	30	5.15	19.05	573.1	162.4	5.09	17.14	518.9	116.2
	60	5.01	18.23	507.9	148.1	4.97	17.31	446.1	96.73
	90	4.97	20.51	526.6	158.2	4.89	19.82	582.3	137.8

*Apparent viscosity determined at shear rate= 0.5012s^{-1}

**Elastic and Plastic Module at frequency= 0,5012Hz

Regarding the viscosity behavior over time, the results showed that viscosity tend to oscillate over time, independently of storage conditions. These oscillations of viscosity are justified by at a higher temperature, oil molecules have higher energy from heat, which makes them less viscous, and oil can flow easily. At 25ºC, emulsions tend to have a higher viscosity when compared to time zero, however, remain more constant. After 60 days, the placebo stored at 40ºC lost water, and the texture changed and did not continue for future studies. When stored at 25ºC and 40ºC, the viscosity of emulsions EI and EN demonstrates a more

constant profile. For emulsion EI, viscosity decreased after 90 days by 3.4 and 7% at 25ºC and 40ºC respectively. Regarding emulsion EN, the viscosity increased after 90 days by 6 and 2.3%, at 25ºC and 40ºC respectively. The observed oscillations could also be explained by the behavior of some components of formulations for the different conditions, which could affect the viscosity of the final product.

Concerning oscillatory results for all formulations presented in table 3.10, the G'> G'', presents a strong network that allows suitable spreadability, adhesion, and tackiness for skin application. Emulsion EI and EN present higher elastic and viscous modules which confirmed that these emulsions are structured and solid behavior predominates. The values of G' and G'' are lower at a higher temperature, which agrees with the possibility of in these conditions formulations are less stable. [17, 18]

The results for the droplet size distribution for all formulations upon a time are presented in table 3.11. When stored at 40 ºC, the emulsions presented a higher oscillation in values when compared to time zero. Nevertheless, the results from the stability assays demonstrated, that the addition of the DES and extracts, tends to stabilize the topical formulations being the best in particular the EI.

Table 3.11- Droplet size distribution (mean,SD, n=6) results for formulations placebo, E11, E13, EI and EN during 90 days at 25 and 40ºC

Formulations		25ºC				40°C±75%			
	Time (days)	d(10)	d(50)	d(90)	Span	d(10)	d(50)	d(90)	Span
Placebo	0	2.37±0.00	15.17±0.02	35.9±0.16	2.21±0.01	2.37±0.004	15.17±0.02	35.9±0.16	2.21±0.01
	14	2.87±0.03	18.34±0.22	44.65±1.49	2.28±0.05	2.66±0.08	17.2±0.56	100.0±18.3	5.62±0.9
	30	2.46±0.03	16.62±0.24	42.64±1.21	2.42±0.04	2.10±0.01	13.28±0.08	33.08±0.48	2.33±0.02
	60	2.76±0.03	18.62±0.07	48.7±0.22	2.46±0.01	-	-	-	
	90	3.11±0.04	20.41±0.23	50.18±0.96	2.30±0.01	-	-	-	
E13	0	2.95±0.01	16.70±0.04	35.7±0.1	1.96±0.001	2.95±0.01	16.70±0.04	35.7±0.1	1.96±0.0
	14	3.29±0.01	18.31±0.05	39.81±0.13	1.99±0.002	3.02±0.18	19.1±0.80	72.48±28.8	3.8±1.4
	30	3.09±0.03	18.46±0.14	40.895±0.60	2.05±0.02	2.44±0.2	14.35±0.05	32.57±0.20	2.10±0.01
	60	3.23±0.06	19.28±0.27	50.58±2.16	2.45±0.07	2.51±0.01	15.43±0.03	35.71±0.09	2.15±0.00
	90	3.70±0.25	20.02±1.0	44.1±1.1	2.06±0.04	2.06±0.01	16.57±0.30	39.1±1.4	2.20±0.04
E11	0	2.92±0.01	20.71±0.06	50.6±0.27	2.30±0.01	2.92±0.01	20.71±0.06	50.6±0.27	2.30±0.01
	14	4.16±0.04	27.9±0.1	65.46±1.36	2.19±0.12	3.09±0.14	23.6±1.28	71.8±12.76	2.88±0.37
	30	3.67±0.03	27.58±0.17	62.05±0.44	2.12±0.003	2.76±0.02	21.26±0.12	57.72±0.40	2.59±0.01
	60	3.91±0.03	27.7±0.16	64.6±0.45	2.18±0.003	2.89±0.09	23.95±082	62.74±1.5	2.49±0.12
	90	3.51±0.20	30.77±0.35	72.64±0.18	2.24±0.03	2.85±0.04	24.4±0.34	62.1±0.77	2.42±0.00
EI	0	2.83±0.06	18.97±0.44	55.35±8.45	2.76±0.37	2.83±0.06	18.97±0.44	55.35±8.45	2.76±0.37
	14	3.0±0.01	20.3±0.06	46.5±0.28	2.14±0.01	2.92±0.10	20.58±0.80	55.72±6.0	2.56±0.19
	30	3.29±0.02	21.79±0.09	49.13±.0.29	2.10±0.01	2.18±0.1	14.9±0.16	42.06±0.45	2.68±0.03
	60	3.37±0.03	23.2±0.13	54.02±0.62	2.18±0.01	2.52±0.01	18.05±0.11	47.5±0.37	2.49±0.01
	90	3.76±0.05	24.7±0.12	58.4±0.3	2.21±0.00	2.62±0.02	20.3±0.16	51.1±0.26	2.39±0.00
EN	0	3.19±0.11	21.9±0.75	73.62±21.53	3.18±0.85	3.19±0.11	21.9±0.75	73.62±21.5	3.18±0.85
	14	3.23±0.03	21.1±0.11	48.82±0.36	2.16±0.01	2.40±0.14	16.19±0.51	47.24±3.54	2.77±0.13
	30	3.35±0.01	21.70±0.05	50.30±0.11	2.16±0.00	2.81±0.23	18.62±0.18	49.32±0.78	2.50±0.02
	60	3.14±0.06	23.4±0.35	56.31±1.33	2.26±0.02	2.53±0.02	18.2±0.18	50.3±0.59	2.62±0.01
	90	3.85±0.5	28.7±0.84	96.1±18.1	3.19±0.5	2.75±0.1	20.3±0.57	56.5±4.4	2.64±0.13

3.7 *In Vivo* Efficacy and Safety Analysis

3.7.1 Human Repeat Insult Patch Test (HRIPT) – Safety Evaluation

During the HRIPT study, were not observed any reaction, skin irritation or sensibilization on the first 3 weeks of contact and after the final contact. Therefore, for emulsion EI, was obtained a good skin compatibility.

3.7.2 Biological Effects (Hydration Test) – Efficacy Evaluation

A skin hydration method was used in order to assess the hydration performance of the EI emulsion. The data is shown in table 3.12.

The results demonstrate that one application of the emulsions increased the moisture content over 4 hours, resulting in higher corneometry values. In the control area, the results considering the standard deviation value demonstrate that were not many oscillations. As a consequence, indicates that the emulsion in study has the ability to increase skin hydration.

However, for future work, should be interesting to perform this method for a longer period of time in order to study the influence that time has on the properties of emulsions regarding the hydration capacity.

Table 3.12-Skin hydration results for Control area and emulsion EI (mean±SD, n=10).

Time (h)	**Skin hydration (AU)***	
	Control Area	**EI**
0	35.73±6.33	61.65±12.2
1	37.11±6.61	51.60±9.97
2	35.55±5.64	51.10±9.54
4	37.29±7.51	47.26±7.67

*AU-Arbitrary units

3.8 Upcycling the Spent coffee Grounds from this work

3.8.1 Physical characterization of SCG

In order to demonstrate the feasibility of the SCG particles, as proof of concept it was then characterized. Figure 3.20 presents the particle size distribution and a microscope image of the particles.

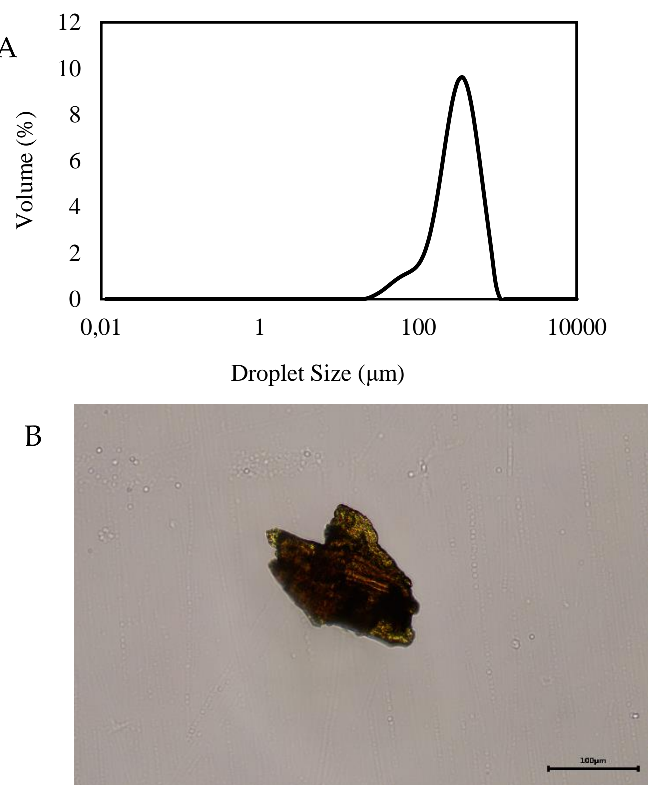


Figure 3.20-Particles from scrub characterization: A-Particle distribution (mean.n=6); B- Image obtained by microscopy with 20x magnification.

The particles size distribution for the SCG is: for d(10) 113.2±2.0 μm; for d(50) 298.2±6.3μm; for d(90) 575.1±15.4μm and Span value is 2.12±0.1 μm. According to the literature [117] the particle size from SCG ranges between 100 and 500 μm, which is in concordance with the results obtained.

3.8.2 SCG oil extraction and reuse of SCG

In order to follow the concept of upcycling and CE was extracted the oil from the unused SCG in this research work. The recovery of the oil was performed, and it was obtained a yellow to orange viscous vegetable oil, with a yield of 10%, which is a low value. As mentioned before,

a green technology should be used, to avoid using conventional organic solvents, namely supercritical carbon dioxide extraction, and as reported in the literature the extraction yield of spent coffee oil at 250 bar and 508ºC was 12.1%.[87]

3.8.3 Development of scrub

The extracted oil was used in the development of the SCG scrub, as well as the unused SCG particles. The composition of the scrub remained very simple, with similar ingredients to the emulsions previously developed, because the aim in the future is to assure that the scrub could also be considered an Eco-Label product. However, this would only be possible if the used SCG oil was extracted with green technology or with a green solvent. Considering that was used n-Hexane the scrub cannot be considered an Eco-Label. The scrub obtained was a dark brown color and with a thick consistency. The results demonstrated the benefits of the extraction of coffee Oils from SCG, and their application in a body scrub. In the future should be evaluated more studies to characterize completely the scrub, and optimize conditions.

4

CONCLUSION

Some of the available by-products are a potential source of bioactive compounds to be applied in the pharmaceutical and cosmetic industries. Coffee is one of the beverages that people consume the most worldwide, and the spent coffee grounds are a by-product of this industry.The SCG are rich in antioxidant compounds. The aim of this work was the valorization of SCG, one of the most abundant residues in the coffee industry, using DES for the extraction of value-added products,such as phenolic compounds.

Before starting the extractions, it was necessary to produce several different DES, using aminoacids, acids, and sugars, and then evaluate them for the green extraction of phenolic compounds from SCG, comparing the value of TPC measured by the Folin-Ciocalteu method.

The higher content in phenolic compounds was obtained with DES 2 formed by Lactic Acid:Glycerol:Water and it was used for optimizing the extraction conditions. To evaluate the influence of temperature, time, and SLR, three temperatures (40, 50, 60°C), five times, (15, 30, 45, 60, 180 minutes), and two SLR (1:10, 1:5, 1:20 g/ml) were tested. From this work, it was possible to conclude that the best results were obtained at 50°C, 60 min, and with an SLR of 1:10. In fact, the results were similar, and this was the best option in terms of results, economically and environmentally.

The cell viability with all DES samples was evaluated, and those with acids in their compositions are cytotoxic for all concentrations tested in HaCaT cell line, and were not used for the following work. Although, DES A, M, N, P, and I, demonstrated more biocompatible values of percentage of cell viability higher than 80%, which is considered safe and suitable for incorporation in topical formulations. After evaluating all DES and respective extracts, it was necessary to choose only two to continue the work based on all the results, and the final

extracts were I and N, with percentage of cell viability higher than 50%, for concentrations ≤50μg/ml.

In the stability assay, was concluded that phenolic compounds are sensitive to temperature and over time the TPC values in different conditions tend to decrease. The EC_{50} values for the samples were similar, which means that antioxidant activity does not decrease over time, which makes extracts very stable. Nevertheless, for samples stored at higher temperatures as the EC_{50} value increases the TPC value decreases, which demonstrates lower antioxidant properties. For the conventional hydroalcoholic extraction, the values for TPC decreased faster, as the EC_{50} increased which means that is occurring degradation of the compounds present in the sample. In conclusion, DES I, presented higher stability over time in TPC and EC_{50} values, with variations under 10%. Furthermore, Extract I, composed of Proline:Glycerol: Water obtained the highest TPC of 16.72 mg GAE /g SCG.

The LC-MS analysis showed that the two major compounds in the extracts were caffeine and chlorogenic acid. With the HPLC analysis was possible to quantify these two compounds present in higher concentration and others in smaller concentrations. In extract I was quantified 0.96 and 3.73 mg/g SCG of caffeine and chlorogenic acid respectively. Additionally, HPLC results confirmed that after 90 days were still present these two compounds.

In cells exposed to UV, the % of ROS reduction with extracts I and N were not significantly different, and in cells exposed to H_2O_2, the % of ROS reduction was higher than the one observed for the conventional extract. Additionally extracts I and N demonstrated the ability to inhibit HNE, which is very interesting in topical formulations, to prevent the skin problems associated with high HNE activity.

Extract I, was considered the extract with better results, and higher stability, as an alternative to conventional organic solvents for the extraction of phenolic compounds from SCG.

According to the Eco-Label regulation were developed topical formulations with incorporated DES and respective extracts I and N. The results were compared and the overall results showed that formulations are suitable for topical application. The prepared formulations resulted in a semi-solid emulsion, with good characteristics regarding of chemical, physical, rheological, and stability properties. For the oscillatory test, in all formulations, the G'>G'', suggests a strong network that allows suitable spreadability and adhesion for skin application, which was further supported by results obtained in spreadability and tribology assays. Concerning the stability assay, first it was noticed a decrease in pH values, and a strategy in future

work to assure the pH range and the stability of formulation should be to add a pH buffer, to adjust the acidity of a product and Vitamin E, to inhibit lipid autoxidation.

The emulsion EI demonstrated a lower potential for irritation, biocompatibility, and increased moisture content of the skin over 4 hours. DES were considered a better sustainable alternative to conventional organic solvents following the results. Their extracts could be applied directly in the final formulation, circumventing the need for other processes. As a consequence, the main aim of this work was achieved.

Finally, and concluding was also finished the life cycle of the SCG used, is in total concordance with the circular economy and upcycling approach. This was possible due to the extraction of oil from the unused SCG, and the remaining particles were incorporated into a body scrub. The results were proof of concept, for future work, to prove the value of the CE concept and the cosmetic application of SCG oil and the raw material. However, needs to be optimized to have the greenest process and higher yield.

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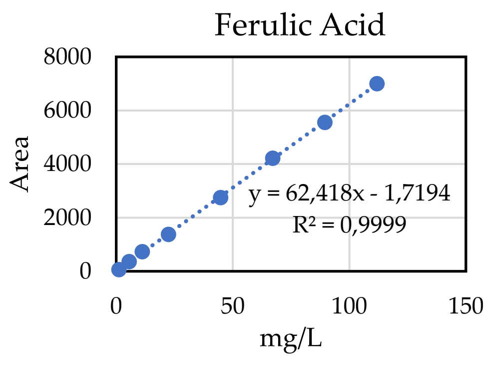
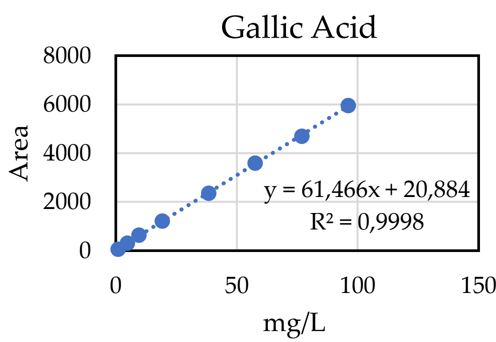
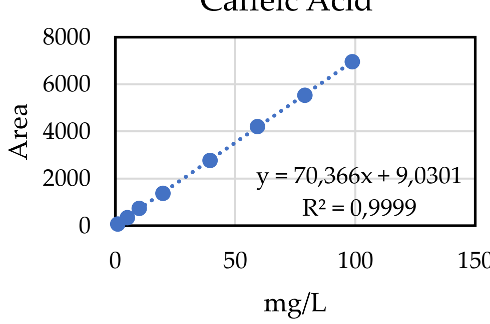
5

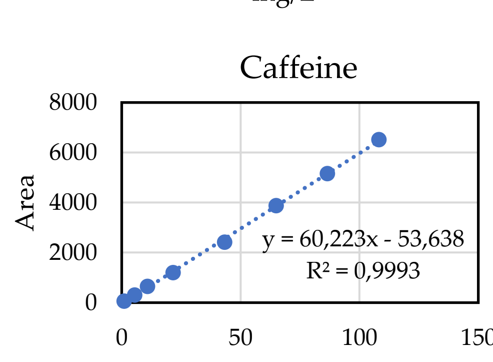
An Appendix

5.1 HPLC calibration curves



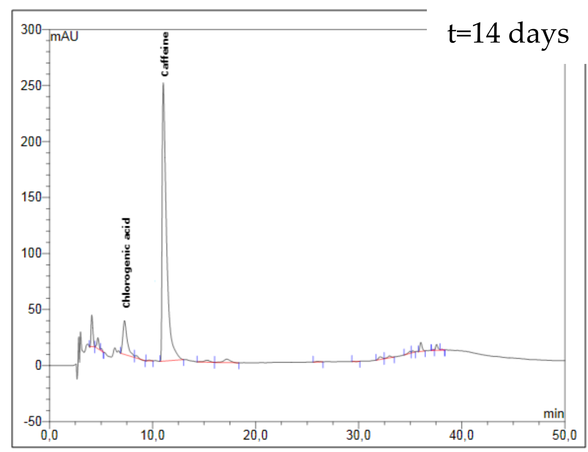


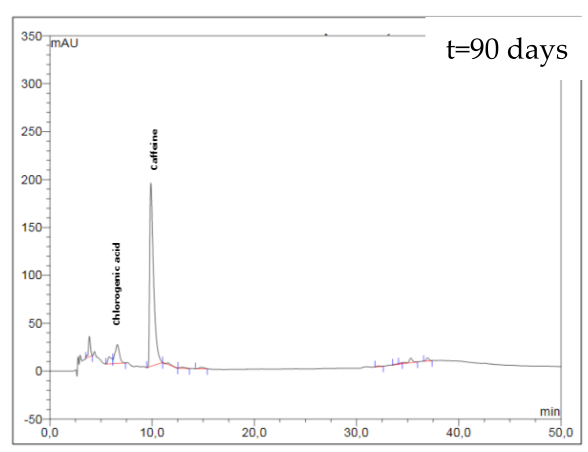




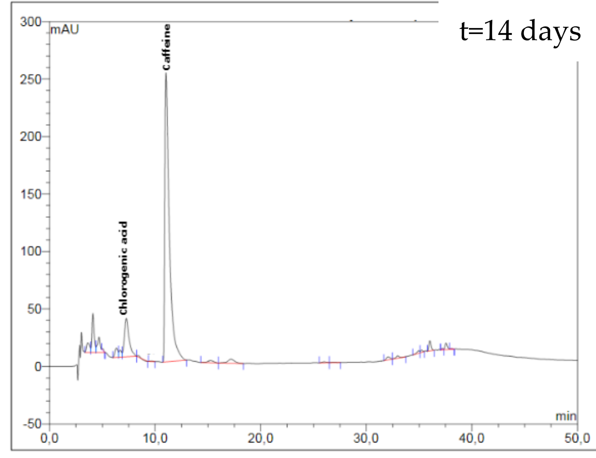
5.2 HPLC chromatograms results upon time for extract I

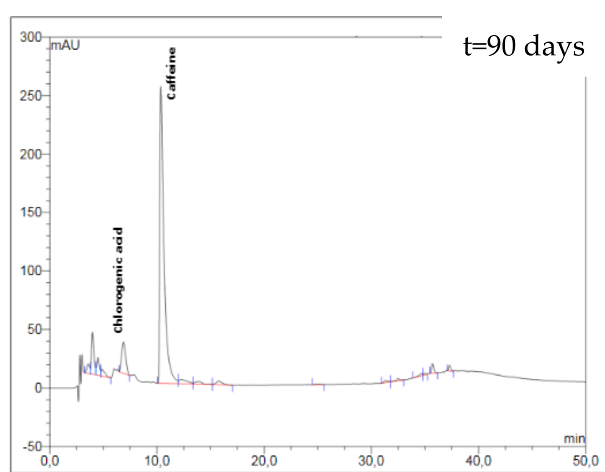
Samples stored at 25ºC



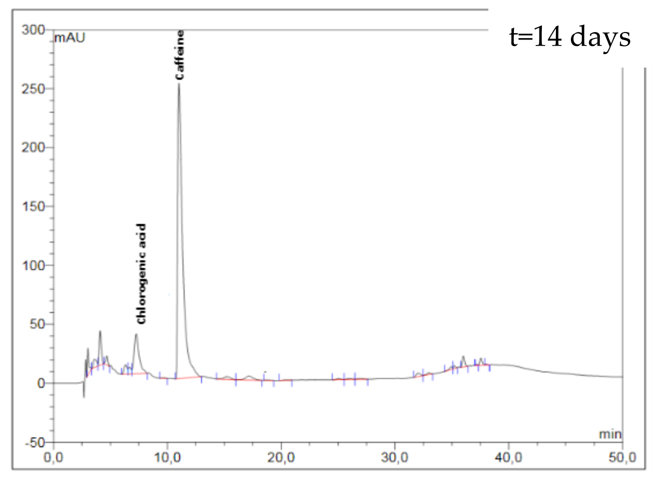


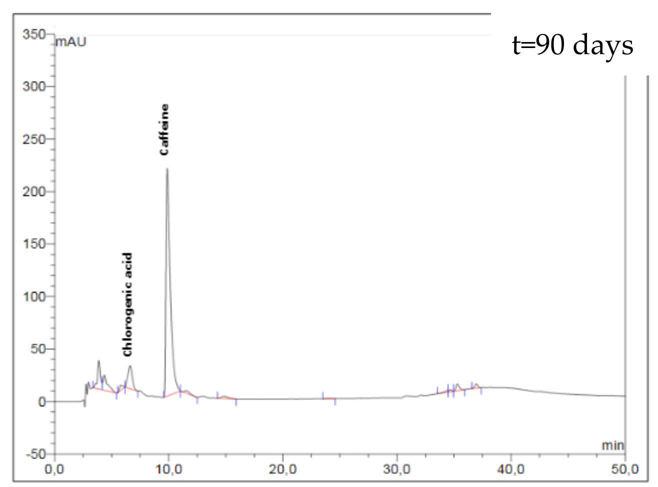
Samples stored at 25ºC without light



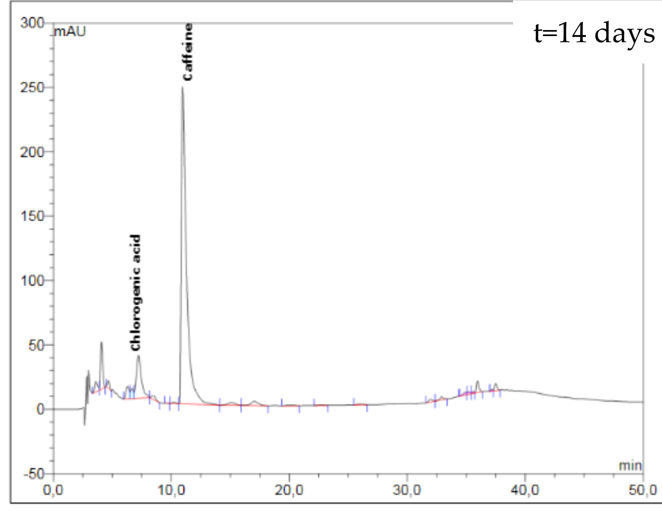


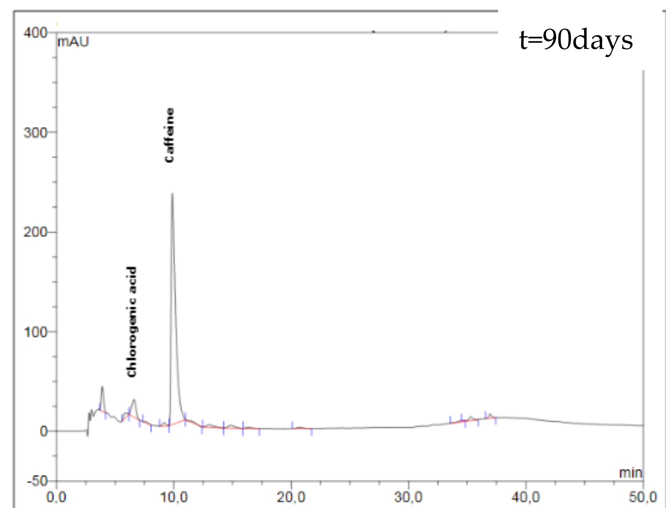
Samples stored at 4ºC





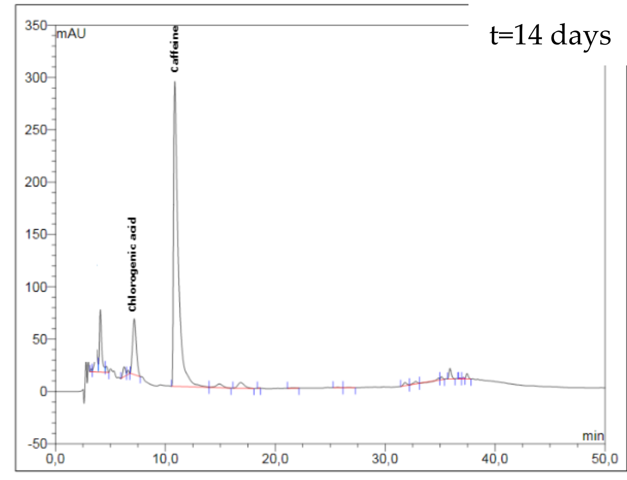
Samples stored at 40ºC

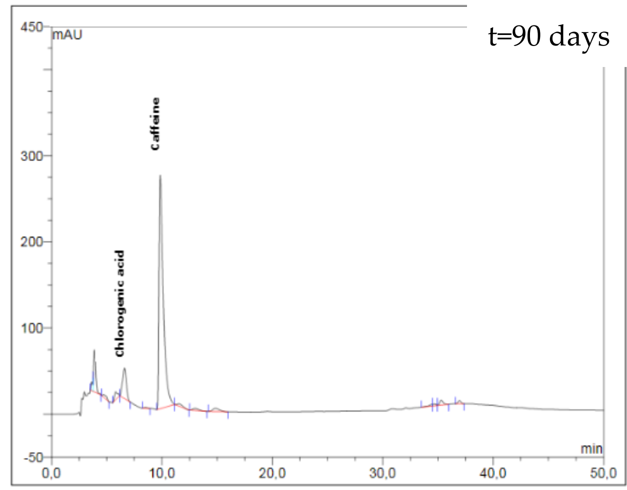




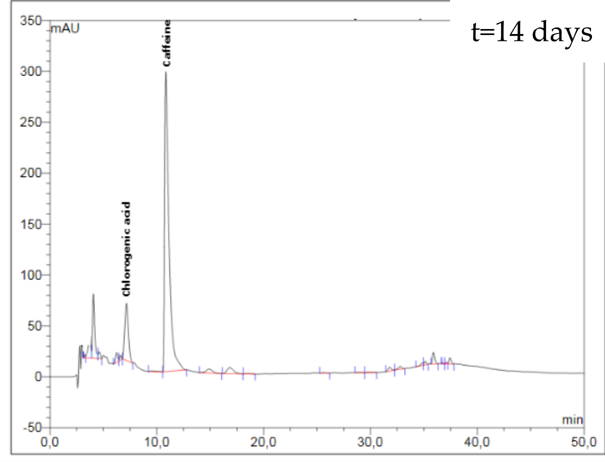
5.3 HPLC chromatograms results upon time for extract N

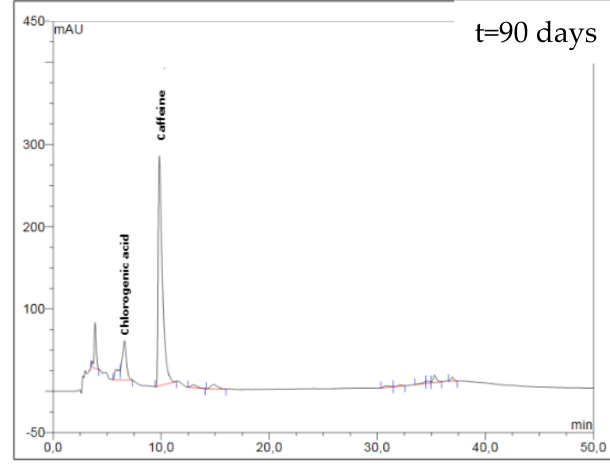
Samples stored at 25ºC



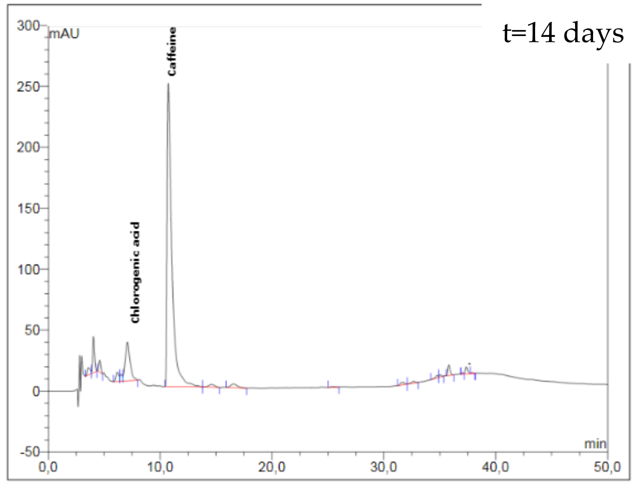


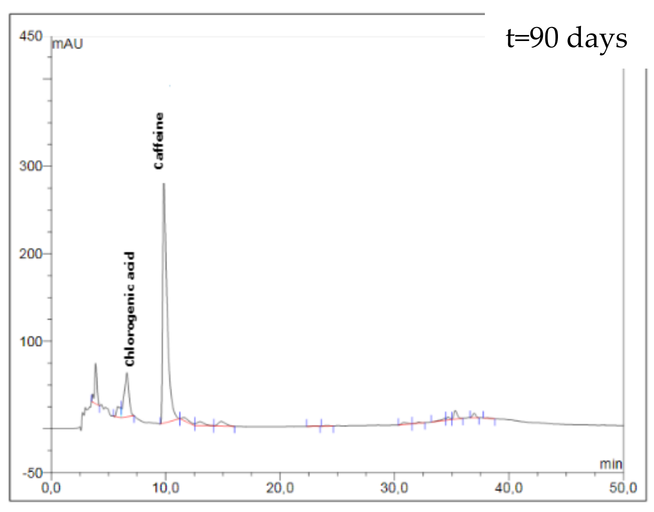
Samples stored at 25ºC without light



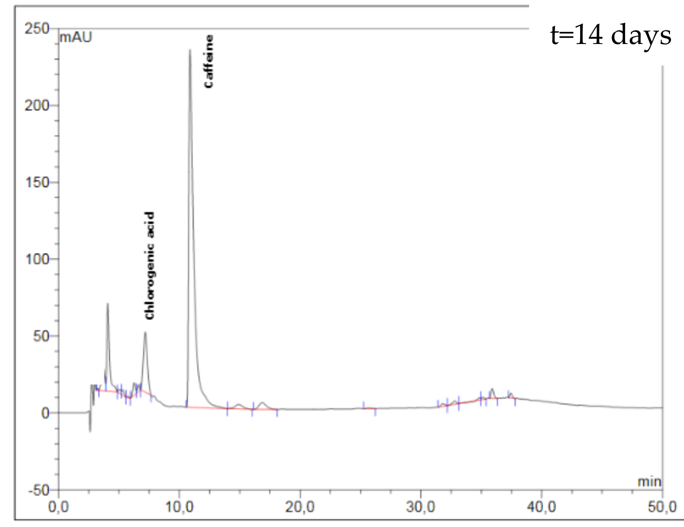


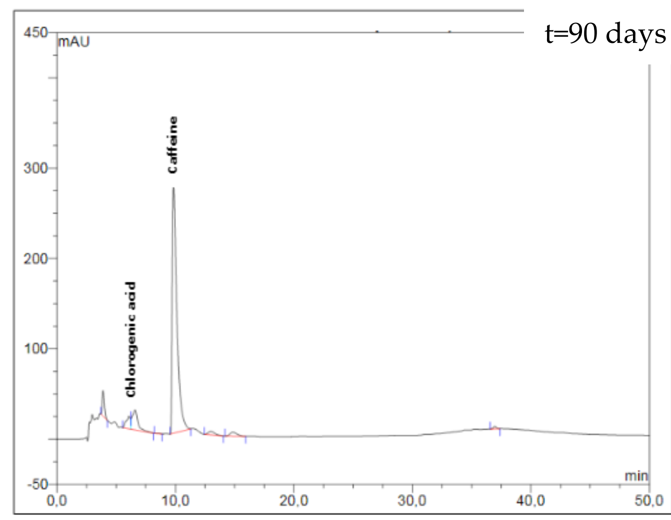
Samples stored at 4ºC



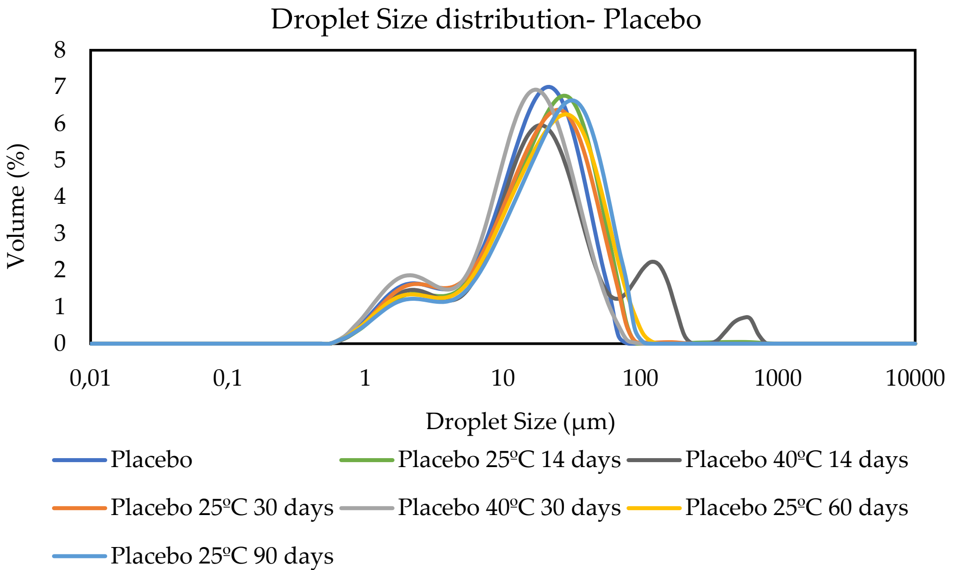


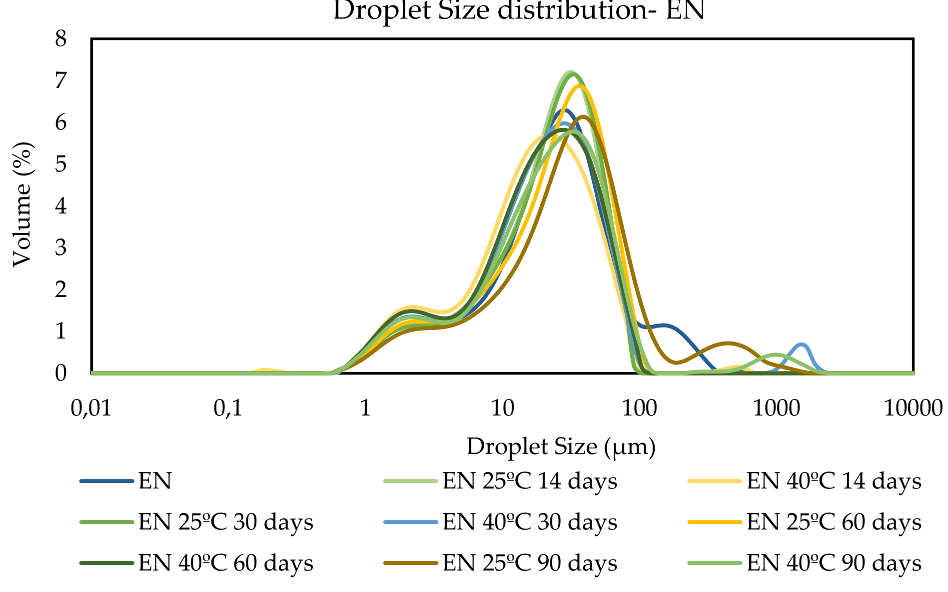
Samples stored at 40ºC

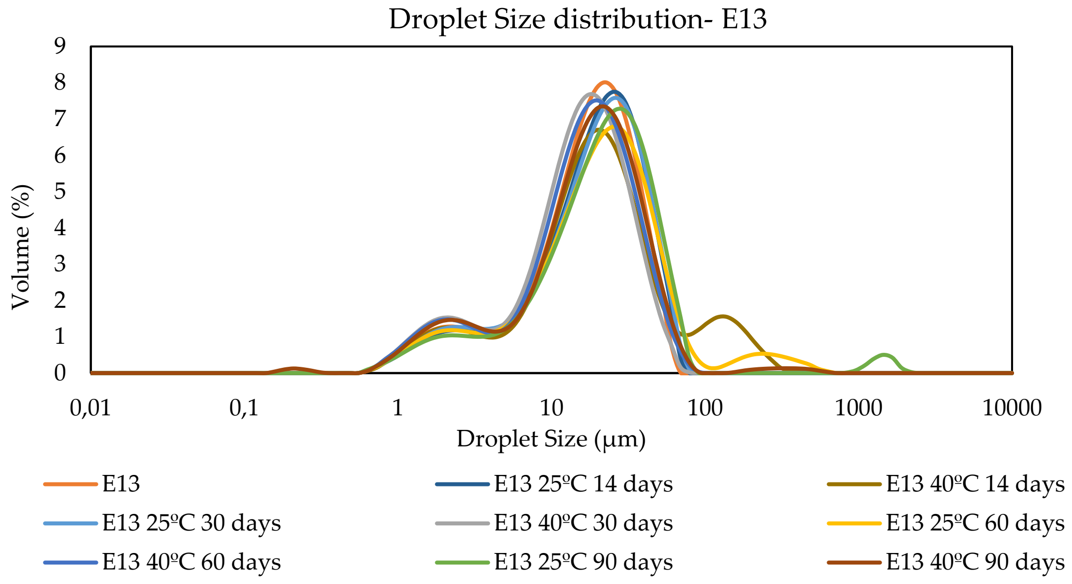


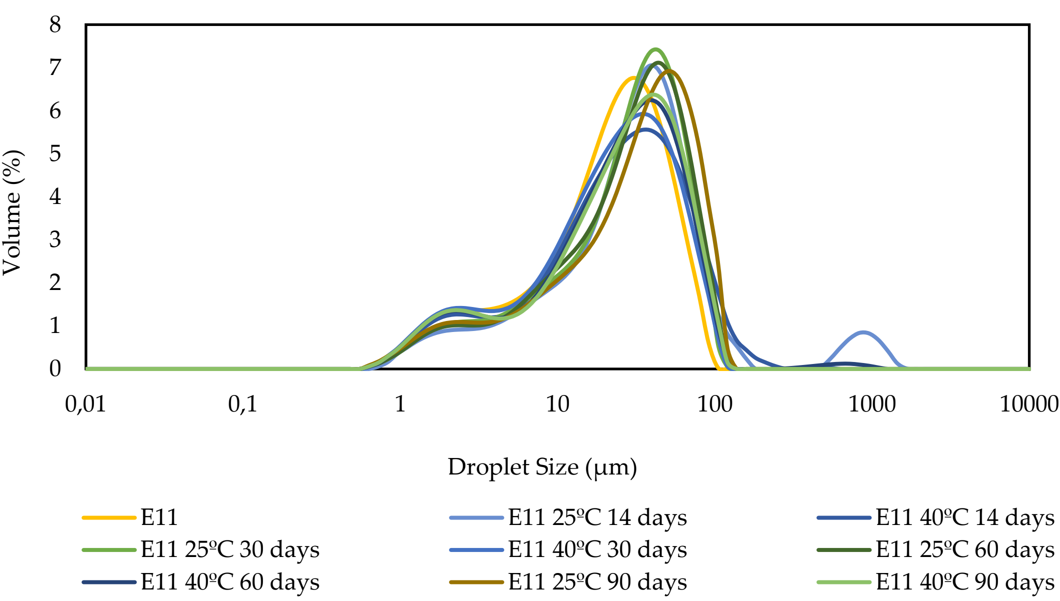


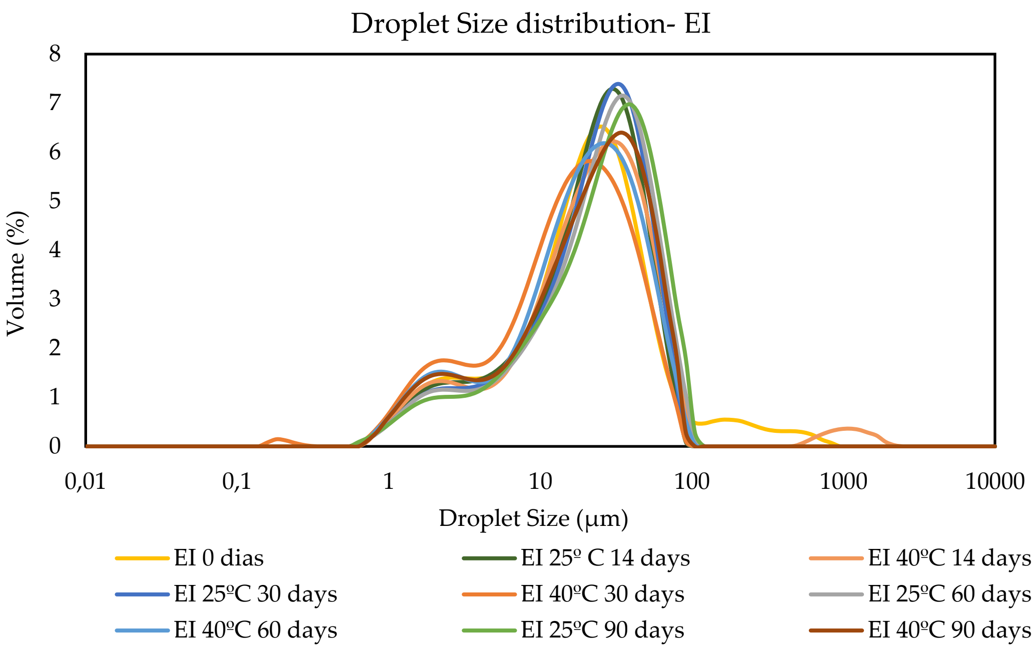
5.4 Droplet Size distribution upon time-Stability assay






Droplet Size distribution- E13
Volume (%)
9
8
7
6
5
4
3
2
1
0
0,01
0,1
1
10
100
1000
10000
Droplet Size (µm)
E13
E13 25ºC 14 days
E13 40ºC 14 days
E13 25ºC 30 days
E13 40ºC 30 days
E13 25ºC 60 days
E13 40ºC 60 days
E13 25ºC 90 days
E13 40ºC 90 days


Droplet Size distribution- E11
Volume (%)
8
7
6
5
4
3
2
1
0
0,01
0,1
1
10
100
1000
10000
Droplet Size (µm)
E11
E11 25ºC 14 days
E11 40ºC 14 days
E11 25ºC 30 days
E11 40ºC 30 days
E11 25ºC 60 days
E11 40ºC 60 days
E11 25ºC 90 days
E11 40ºC 90 days



Droplet Size distribution- EI
Volume (%)
8
7
6
5
4
3
2
1
0
0,01
0,1
1
10
100
1000
10000
Droplet Size (μm)
EI 0 dias
EI 25º C 14 days
EI 40ºC 14 days
EI 25ºC 30 days
EI 40ºC 30 days
EI 25ºC 60 days
EI 40ºC 60 days
EI 25ºC 90 days
EI 40ºC 90 days

2022

ANA COSTA

DESIGN OF A COSMETIC FORMULATION WITH A DEEP EUTECTIC SYSTEM COFFEE EXTRACT

NOVA
NOVA SCHOOL OF SCIENCE & TECHNOLOGY