

MESTRADO EM CONTROLO DA QUALIDADE
ESPECIALIZAÇÃO EM PRODUTOS DE COSMÉTICA E HIGIENE CORPORAL

Development of cosmetic formulations with olive pomace extracts

Raquel Sofia Martins Rodrigues

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Trabalho realizado sob a orientação de: Professora Doutora Beatriz
Oliveira, Doutor Hugo Almeida e Doutora Rita Alves

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DECLARAÇÃO DE REPRODUÇÃO

É AUTORIZADA A REPRODUÇÃO PARCIAL DESTA DISSERTAÇÃO (INDICAR, CASO TAL SEJA NECESSÁRIO, Nº MÁXIMO DE PÁGINAS, ILUSTRAÇÕES, GRÁFICOS, ETC.), APENAS PARA EFEITOS DE INVESTIGAÇÃO, MEDIANTE DECLARAÇÃO ESCRITA DO INTERESSADO, QUE A TAL SE COMPROMETE

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ABSTRACT

Hydroxytyrosol (HT) is a naturally occurring phenolic compound with antioxidant, anti-aging, anti-inflammatory and photoprotector activities. It is significantly present in olive by-products such as olive pomace (OP), so its recover from these by-products can provide a natural and sustainable ingredient with biological effects for cosmetic applications. In that sense, this study aimed to optimize the extraction of HT from OP, using eco-friendly solvents and low-cost procedures, and incorporate these extracts in a base cream. Optimized conditions were found using a 1:1 hydroalcoholic solvent (v/v) at an extraction temperature of 25 °C for 60 minutes. A pasteurization process significantly increased ($p < 0.05$) the HT yield. An extract was produced, under these conditions, showing FRAP values of $90.50 \pm 1.76 \text{ mmol}_{\text{FSE}}/100 \text{ g}$ and DPPH[•] scavenging ability of $11.73 \pm 0.89 \text{ g}_{\text{TE}}$ in 100 g OP (dw). TPC attained values of $8.78 \pm 0.37 \text{ g}_{\text{GAE}}/100 \text{ g OP (dw)}$ and HT extraction yield reached $738.43 \pm 7.48 \text{ mg}/100 \text{ g OP (dw)}$. Tyrosinase inhibition was found at $26.02 \pm 2.57 \%$ and elastase inhibition presented a value of $8.68 \pm 1.32 \%$. MTT assay showed a decrease in cell viability at concentrations equal and higher to 1 mg/ml. Microencapsulation of the extracts, by a spray-drying process, using pectin as encapsulation agent, was also attempted in order to promote phenolic stability and efficacy. Microcapsules showed a particle size of $1.42 \pm 0.18 \mu\text{m}$, as well as homogenous distribution, with an encapsulation efficiency of 97.01%. Formulations with non-encapsulated olive pomace extract (NE-OP) and microencapsulated olive pomace extract (ME-OP) were characterized based on their organoleptic, physicochemical and rheological properties, as well as microbial contamination. Accelerated stability testing was performed in $40 \pm 2 \text{ }^{\circ}\text{C}$ temperature and $75 \pm 5\%$ relative humidity for 3 months. Results showed that pectin incorporation had an impact in formulations' texture and pH. All creams presented shear-thinning behavior, however, viscosity had a significant decrease for ME-OP samples translating some physical instability. Color parameters also altered over time, but results were similar for both NE-OP and ME-OP formulations. No microbial contamination was found in the time period tested. Regarding total phenolics and HT and Tyr content, significant differences ($p < 0.05$) were found for the NE-OP creams which can suggest some phenolic degradation after storage. ME-OP creams appeared to protect phenolic compounds, but results were not conclusive as physical instability could have an impact on this evaluation. Further works should include different encapsulation techniques and/or polymeric carriers, as well as release and permeation studies in order to assess OP encapsulation feasibility in cosmetic formulations, with the ultimate purpose of guarantee safe and effective skincare products.

Key words: Olive pomace, Hydroxytyrosol, Cosmetic, Microencapsulation

RESUMO

O Hidroxitirosol (HT) é um composto fenólico de origem natural que demonstra atividade antioxidante, antienvelhecimento, anti-inflamatória e fotoprotetora. Encontra-se presente em quantidades apreciáveis nos subprodutos da azeitona, como é o caso do bagaço de azeitona (BA), sendo que a sua extração a partir deste subproduto pode representar um novo ativo sustentável para a indústria cosmética. Neste sentido, este estudo teve como objetivo otimizar a extração do HT, utilizando solventes ecológicos e métodos de baixo custo, e posteriormente incorporar o extrato num creme base. As condições otimizadas de extração foram estabelecidas utilizando como solvente uma mistura água:etanol (1:1, v/v), temperatura de extração de 25 °C e tempo de extração de 60 minutos. Um processo de pasteurização foi testado resultando num aumento da extração do HT. A análise antioxidante de um extrato otimizado, pelos métodos de FRAP e DPPH^{*}, originou resultados de $90,50 \pm 1,76 \text{ mmol}_{\text{ESF}}/100 \text{ g}$ e $11,73 \pm 0,89 \text{ g}_{\text{ET}}$ em 100 g de BA, respetivamente. O conteúdo fenólico total foi de $8,78 \pm 0,37 \text{ g}_{\text{EAG}}/100\text{g}$ de BA e a quantificação de HT atingiu os valores de $738,43 \pm 7,48 \text{ mg} /100 \text{ g}$ de BA. A inibição das enzimas tirosinase e elastase pelo extrato obteve valores de $26,02 \pm 2,57\%$ e $8,68 \pm 1,32\%$ respetivamente. O ensaio de MTT demonstrou que concentrações acima de 1 mg/ml do extrato produziam um decréscimo da viabilidade celular. A microencapsulação dos extratos de BA foi igualmente estudada, utilizando um método de *spray-drying* e pectina como agente de encapsulação, por forma a aumentar a estabilidade dos compostos fenólicos. As microcápsulas obtidas apresentavam homogeneidade e um tamanho de partícula de $1,42 \pm 0,18\mu\text{m}$, com uma eficiência de encapsulação dos ativos de 97,01%. Cremes com extrato não encapsulado (ENE) e microencapsulado (EM) foram testados considerando características organoléticas, físico-químicas, reológicas e contaminação microbiológica. As formulações foram sujeitas a um teste de estabilidade acelerado nas condições de temperatura de $40 \pm 2^\circ\text{C}$ e $75 \pm 5\%$ de humidade relativa por 3 meses. Todos os cremes apresentaram comportamento pseudoplástico, sendo notória a diminuição da viscosidade com a incorporação de EM o que pode evidenciar instabilidade física dos cremes. Existem alterações dos parâmetros da cor durante o tempo de estudo, com resultados semelhantes para os cremes com ENE e EM. Não houve evidências de contaminação microbiológica no período de tempo testado. Os resultados da análise fitoquímica sugerem que existe uma proteção dos compostos fenólicos com a encapsulação, no entanto o estudo não é conclusivo dada a instabilidade física destas formulações. Futuramente, outras técnicas de encapsulação e/ou polímeros poderão ser aplicados, assim como estudos relativos à libertação e permeação dos ativos por forma garantir segurança e efetividade dos produtos cosméticos com bagaço de azeitona.

Palavras-chave: Bagaço de azeitona, Hidroxitirosol, Cosmética, Microencapsulação

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LIST OF ABBREVIATIONS AND SYMBOLS (alphabetical order)

-	A20	Water-base extract stored at -20 °C
-	A4	Water-based extract stored at 4 °C
-	AA	Antioxidant Activity
-	Abs	Absorbance
-	BC	Base cream
-	CAE	Caffeic Acid Equivalents
-	CFU	Colony-Forming Units
-	COX-2	Cyclooxygenase-2
-	DAD	Diode Array Detector
-	DES	Deep Eutectic Solvents
-	DMEM	Dulbecco's Modified Eagle Medium
-	DMSO	Dimethyl sulfoxide
-	DPPH•	2,2-diphenyl-1-picrylhydrazyl radical
-	DSC	Differential Scanning Calorimetry
-	DSL	Dynamic light scattering
-	dw	dry weight
-	ECM	Extracellular matrix
-	EE	Encapsulation efficiency
-	ERT	Hydroalcoholic extract stored at room temperature
-	EtOH	Absolute ethanol
-	FBS	Fetal Bovine Serum
-	FD	Freeze-dried
-	FD-E	Freeze-dried dried olive pomace extract
-	FD-OP	Freeze-dried olive pomace
-	FLD	Fluorescence detector
-	FRAP	Ferric-Reducing Antioxidant Power
-	FSE	Ferrous sulphate equivalents
-	FTIR	Fourier Transform Infrared
-	fw	fresh weight
-	GAE	Gallic Acid Equivalents
-	GRAS	Generally Recognized As Safe
-	HaCaT	Immortalized Human Keratinocytes
-	HDF	Human Dermal Fibroblasts
-	HPLC	High Performance Liquid Chromatography
-	HT	Hydroxytyrosol

- HTST	High-Temperature-Short-Time
- IC₅₀	Half-maximal inhibitory concentration
- IL-1α	Interleukin-1 α
- IL-6	Interleukin-6
- iNOS	Nitric Oxide Synthase
- LC	Loading capacity
- LCT	Life Cycle Thinking
- LED-BL	Light-Emitting-Diode-generated Blue Light
- MCE	Mixed Cellulose Ester
- ME- OP	Microencapsulated olive pomace extract
- ME-OP	Encapsulated olive pomace extract
- MMPs	Matrix Metalloproteases
- MTT	3,4,5-dimethylthiazolyl-2–2,5-diphenyl-tetrazolium bromide
- NE-OP	Non-encapsulated olive pomace extract
- NP-OP	Non-pasteurized olive pomace
- NP-OP-FD	Non-pasteurized freeze-dried olive pomace
- O/W	Oil-in-water
- OMWW	Olive mil wastewaters
- OP	Olive pomace
- OPC	Olive pomace based creams
- OPE	Olive pomace extract
- PBS	Phosphate Buffered Saline solution
- PCA	Plate Count Agar
- PI	Polydispersity index
- P-OP	Pasteurized olive pomace
- P-OP-FD	Pasteurized freeze-dried olive pomace
- PTFE	Polytetrafluoroethylene
- RH	Relative Humidity
- RNS	Reactive Nitrogen Species
- ROS	Reactive Oxygen Species
- rpm	rotations per minute
- SDA-C	Sabouraud Dextrose Agar - Chloramphenicol
- SEM	Scanning Electron Microscopy
- SEM¹	Standard error of mean
- SLR	Solid to Liquid Ratio
- T0	Post-production time

- T1	1 month after storage
- T2	2 months after storage
- T3	3 months after storage
- TEAC	Trolox Equivalent Antioxidant Capacity
- TEM	Transmitting Electronic Microscopy
- TNF-α	Tumour necrosis factor
- TPC	Total Phenolic Content
- TPTZ	2,4,6-tri-(2-pyridyl)-S-triazine
- TSA	Trypticasein Soy Agar
- TSB	Trypticasein soy broth
- Tyr	Tyrosol
- UV	Ultraviolet
- v/v	volume/volume
- w/v	weight/volume
- w/w	weight/weight
- XRD	X-ray diffraction
- ΔE	Total colour difference
- <i>f</i>	Viscosity

CHAPTER I

OLIVE POMACE EXTRACTS: OPTIMIZATION BASED ON HY- DROXYTYROSOL AND TYROSOL QUANTIFICATION AND CHARACTERIZATION FOR COSMETIC APPLICATIONS

1. Introduction

1.1. Circular economy in the olive oil sector: by-products upcycling

In the last years, olive oil production has experienced a progressive increase, owing, not only to its unparalleled organoleptic qualities, but also to its recognized beneficial properties. These properties are related to the presence of high levels of fatty acids, particularly monounsaturated acids such as oleic acid, as well as other valuable components such as phenolics, phytosterols, tocopherols, and squalene (1). Olive oil plays a vital role in the Mediterranean diet, and its production is particularly relevant for the economy of Southern European countries like Spain, Italy, Greece, and Portugal (2). As production increases, waste impact is rising. Each year, an average of 5 million kg of olive residues are produced with enormous environmental and economic burden (3). The impact of waste disposal along with the depletion of resources and climate change concerns gives rise to the concept of circular economy. Principles of circular economy are based on recycling and upcycling, with the ultimate purpose of creating value from low-cost by-products, therefore contributing to sustainable patterns of production and consumption (4, 5). Recently, the agro-food industry has been investing great efforts to implement circular economy models (6). Olive oil production has a strong potential to transit to a circular rather than a linear economic model, since the created by-products are extremely rich in bioactive compounds, suitable for other industries re-use such as food, pharmaceutical or cosmetic (7). These olive by-products are mostly leaves, olive pomace (OP), olive stones and olive mill wastewaters (OMWW) (2).

Different by-products are generated depending on the extraction phase as seen in **Figure 1**. Leaves are the first by-product generated from the harvesting phase and by cleaning the olives prior to processing (1). Traditionally, olive oil was obtained by pressing, but nowadays olive oil extraction is accomplished by two or three phases centrifugation (8, 9). These two methods differ, in particular, by the amount of water used and the residues produced. The two-phase separation system is the most used by the olive oil industry as it represents an eco-friendlier process with lower water and energy consumption (8). In this process both olive oil and a humid semi-solid pomace (OP) with a moisture content up to 70% are obtained (1, 10). The three-stage system produces higher volumes of OMWW which are highly phytotoxic to soil and water. Both OP and OMWW are extremely pollutant, since they hold a high content of phenolic compounds, tanins and a low pH (10, 11). OP in particular, represents about 80% of the olive oil production in the two-phase systems (1). In the later stages of olive oil production, OP is discharged in large open-air containers as seen in **Figure 2**, where it is stored until a final step is performed, the extraction of OP residual oil. An oil-free residue, extracted olive pomace, remains as the final by-product produced (11).

Disposing of olive by-products is difficult and expensive, therefore strategies to harvest their potential are currently needed. In fact, around 98% of the phenolic content is found in by-products such as OP, since their hydrophilic nature influences their distribution in the water phase rather than the oil phase (12). Additionally, OP contains considerable amounts of cellulose (30%) and pectic polysaccharides (39%), a rich lipid fraction, especially in oleic acid (75% lipid content), as well as squalene, tocopherols and a rich mineral composition that can also be upcycled as sustainable ingredients for different purposes (2, 12, 13).

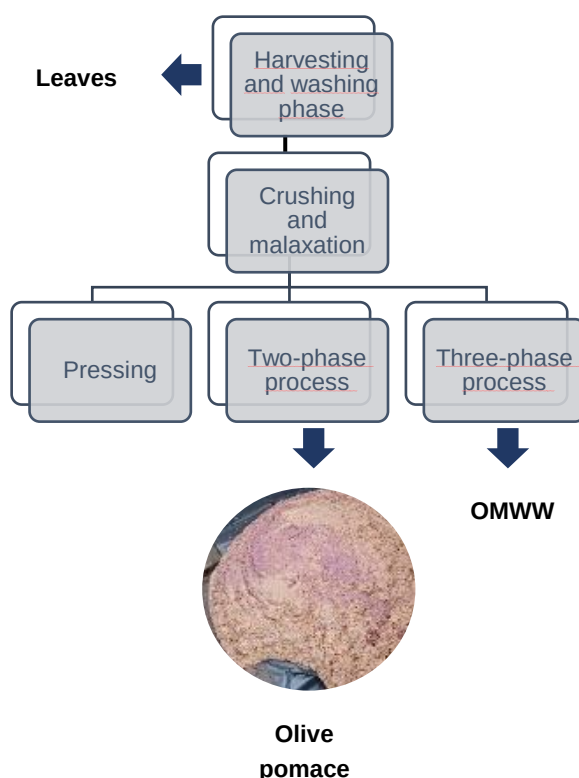


Figure 1. Extraction methods and different by-products in the olive oil production.



Figure 2. Different phases of the olive oil extraction process (Alfândega-da-Fé, Portugal).

1.2. Polyphenols: the role of hydroxytyrosol in skincare

Agro-industrial waste can represent, for the cosmetic field, an important natural source of biodegradable and eco-friendly ingredients. They possess numerous compounds that can exert either functional or cosmeceutical activities. Cosmeceuticals are cosmetic products placed between cosmetics and pharmaceuticals as they possess biologically active ingredients claiming to exert drug-like benefits with effects on skin structure and function. Cosmeceuticals are an expanding market, particularly in the anti-aging category, and many possess ingredients of natural origin. One of the great challenges with the rise of cosmeceuticals is that marketing claims are often unsupported by clinical or scientific data, so steps to improve clarity in efficacy claims are essential (14, 15).

Phenolic compounds constitute a large and important group of naturally occurring antioxidants and as mentioned before, are substantially present in olive by-products such as OP. Olive phenolics are greatly heterogeneous and include, mainly, phenolic alcohols (tyrosol and hydroxytyrosol) phenolic acids (caffeic acid, gallic acid and verbascoside), flavonoids (luteolin, apigenin and rutin), secoiridoids (oleuropein and ligstroside) and lignans (pinoresinol) (1). Hydroxytyrosol (HT) is the main phenolic found in OP, although some studies

reported other largely representative compounds such as oleuropein and tyrosol (16). Many variables such as the type of extraction process used, olive variety and geographical origin, harvest time and maturation stage can explain the phenolic content differences in this by-product (12, 16). HT is mainly produced from the hydrolysis of oleuropein which can occur in the first phases of olive processing, namely crushing and malaxation, but also through the extraction process or even during storage time (10). HT is a powerful antioxidant since its *ortho*-diphenolic structure stabilizes free radicals by hydrogen-atom-transfer, limiting cellular damage (10). There is also supporting evidence for its antioxidant benefits, as well as anti-inflammatory, antimicrobial and photoprotector activities as seen in **Table 1**. Many studies have evaluated the activity of phenolic substances in skin cells. Some have shown that HT can inhibit several skin enzymes and influence gene expression, suggesting that this compound may exhibit anti-aging effects (17).

To understand the aging process, it is necessary to distinguish between intrinsic (chronological) and extrinsic aging (exposure to environment) and comprehend the different factors involved. There is significant evidence that oxidative stress plays a major role in both the intrinsic as well as the extrinsic process of skin aging. Oxidative stress occurs from the imbalance between reactive oxygen and nitrogen species (ROS and RNS) production and the endogenous antioxidant defences. As this disparity between pro-oxidant and antioxidant factors persists, the cascade of cellular events and damage triggered by free radicals ultimately leads to chronic inflammation and upregulation of matrix metalloproteinases (MMPs) (18). MMPs are a group of enzymes that degrade structural proteins, such as collagen or elastin, and contribute to the normal turnover of the extracellular matrix (ECM). With aging, the normal balance between synthesis and degradation of the ECM components is compromised, resulting in skin aging visible signs such as loss of skin elasticity and wrinkles (18). As antioxidants protect skin against oxidative stress, they are frequently added to anti-aging formulations to prevent premature aging.

As skin is the body's first line of defence for environmental exposure, ultraviolet (UV) exposure is one of the main causes for extrinsic aging. Damages to the skin barrier from UV exposure create an inflammatory response with the production of cytokines (e.g., IL-1 α , IL-6, and TNF- α), proteolytic enzymes and ROS (18). Further, both UVA and UVB radiation can increase the production of MMPs (18). Pre-treatment of keratinocytes with 100 μ M HT have caused a decrease in intracellular ROS formation induced by ultraviolet UVB rays up to 47% (19). Also, negative effects from UVA radiation in different cells were ameliorated in a dose dependent manner, with reduced expression of MMPs, pro-inflammatory cytokines and protein damage (17, 20). Anti-aging effects on human dermal fibroblasts (HDFs) treated with UVA radiation, were assessed using an aging indicator (β -galactosidase) by Choi *et al.* (17). A decrease in β -galactosidase activity, pro-inflammatory molecules and MMP-1 and

MMP-9, with increasing HT concentrations (up to 10 μ M) was exhibited. The impact on blue light on skin is also a subject of increasing interest for the cosmetic industry as it has been shown to generate ROS and induce oxidative damage (21). Avola *et al.* (22) demonstrated that up to 50 μ g/ml HT had a protective effect, in skin keratinocytes and fibroblasts subjected to light-emitting-diode-generated blue light (LED-BL), by decreasing ROS formation and MMP-1 (collagenase) and MMP-12 (elastase) levels. Another visible sign of photoaging is related to pigmentary changes, namely focal hyper-pigmentation, as excessive production of melanin occurs in response to chronic exposure of skin to UV radiations (18). Tyrosinase inhibitors are usually the first choice for hyperpigmentation treatment, since tyrosinase is a crucial enzyme involved in melanin production (18). HT showed similar IC₅₀ results in tyrosinase inhibition (13.0 mmol/L) as one of the most common depigmenting agents, kojic acid (14.8 mmol/L) (23).

Table 1. Hydroxytyrosol skin related benefits documented in scientific literature (24).

Proprieties	Mechanisms
Antioxidant and anti-aging	Ability to scavenge free radicals and quelate metals (25, 26).
	Decrease in β -galactosidase expression (17).
	Elastase and collagenase inhibition (7, 17).
	Decrease in pro-inflammatory cytokines (17).
Anti-inflammatory	iNOS and COX-2 inhibition (27).
Antibacterial	Reduced growth rate in Gram ⁺ and Gram ⁻ bacteria (e.g., <i>Staphylococcus aureus</i> , <i>Pseudomonas aeruginosa</i> , <i>Escherichia coli</i>) (28, 29).
	Decrease in UVA-induced protein damage and proapoptotic stimulus in melanoma cells (20).
Photoprotector	Protective effect against UVB-induced genotoxicity (19).
	Protection against LED-BL skin induced damage (22).
Anti-pigmentation	Tyrosinase inhibition (23).

COX-2, cyclooxygenase-2, LED-BL, light-emitting-diode-generated blue light, iNOS, nitric oxide synthase, UVA, ultraviolet A, UVB, ultraviolet B.

1.3. Bioactive extraction from olive pomace

When producing an extract, the main focus resides in concentrating the biologically active constituents, promoting maximum efficiency with the lowest environmental and economic impact (14). Numerous studies have reported the extraction of bioactive compounds from OP. The extraction of these compounds can be performed by conventional or non-conventional extraction methods (1, 30). The increasing need for more efficient recovery

processes has led to the development of non-conventional methods that can reduce the extraction time, as well as solvent and energy consumption when compared to conventional extraction methodologies (13). In that sense, non-conventional techniques, such as ultrasound-assisted extraction, microwave-assisted extraction, pressurized liquid extraction and supercritical fluid extraction have been used in order to improve the extraction yield of phenolic compounds (16). Nevertheless, conventional liquid extraction simplicity, as well as reasonable efficiency and affordability, make this technique the first option as standard procedure for the extraction and concentration of different substances. Several studies approached different variables to phenolic extraction, based on total phenolic content (TPC) and antioxidant activity (AA), such as temperature, solvent, extraction time and solid to liquid ratio (SLR) (31). In order to maximize the extraction performance these parameters are frequently optimized.

When choosing an extraction solvent, it is important to consider its suitability for cosmetic use (32). Many extraction protocols include non-GRAS (Generally Recognized As Safe) solvents, that can be toxic for human use as well as environmentally harmful (3). Also, according to green chemistry principles, methodologies should aim to be environmentally friendly, therefore eliminating or reducing the use of dangerous, non-biodegradable and toxic chemicals at any stage of production (33). Solvent polarity is particularly critical for a successful extraction since it greatly determines the selectivity and distribution of the phenolic compounds in the extract (3). Usually, solvent mixtures in varying proportions produce higher extraction yields, due to an expanded polarity range (33).

Temperature is another variable that can have a great impact on the extraction performance. High temperatures generally favour the mass transfer process, by reducing viscosity and facilitating solvent penetration in the sample matrix. It also weakens the interactions between phenolic compounds and proteins or polysaccharides, increasing the rate of diffusion (3). On the other hand, if the target compound is highly labile, reducing the extraction temperature may be required.

Polyphenols are, in general, unstable and susceptible to degradation, therefore extraction time is also an important parameter to evaluate. Prolonged oxygen or light exposure can deteriorate phenolic compounds reducing the extract antioxidant ability (3).

Cepo *et al.* (34) found that the optimum conditions for OP extraction used a 60% ethanol solution in a SLR of 1:5 (w/v), at 70 °C for 120 minutes. It was reported that temperatures above 70 °C did not produce any additional benefit. TPC and AA increased, after 30 minutes extraction time, to an optimal value of 120 minutes, after which there was evidence of sample chemical degradation. HT content was found at 81.8 ± 0.41 g/Kg_{OP} (fresh weight, fw), TPC was 3.62 ± 0.03 mg Gallic Acid Equivalents (GAE)/g_{OP} (fw) and AA determination by Trolox Equivalent Antioxidant Capacity (TEAC) was 3.64 ± 0.03 mg Trolox equivalents (TE)/g_{OP} (fw).

Another study from Böhmer-Maas *et al.* (35) described that the highest TPC was obtained by using 40% methanol, 70 °C and 180 minutes (23061.2 mg_{GAE}/Kg) and the highest AA by Ferric-Reducing Antioxidant Power (FRAP) assay was found in the extract obtained with 40% methanol, 45 °C and 180 minutes extraction time (20405.5 mg_{TEAC}/Kg). However, the high toxicity of methanol makes it unfeasible for food or cosmetic applications (3).

Nunes *et al.* (36) reported higher values for 2,2-diphenyl-1-picrylhydrazyl radical, DPPH[•], scavenging ability (1403.1 µg_{TE}/ml), FRAP (9.40 µmol ferrous sulphate equivalents (FSE)/ml and TPC (487 µg_{GAE}/ml) at 40°C with a SLR of 1 g to 50 ml of a 1:1 ethanol:water solution (v/v) for 60 minutes.

Recently, another work aimed to extract phenolic compounds from OP using deep eutectic solvents (DES) based on choline chloride ([Ch]Cl) and carboxylic acids. In the optimized extraction conditions [Ch]Cl:malonic acid showed similar capacity to extract phenolic compounds as ethanol, with TPC values of 27.61 mg_{GAE}/g versus 25.32 mg_{GAE}/g (dry weight, dw). Optimized conditions found for ethanol and [Ch]Cl:malonic acid were comparable in temperature and solvent dilution in water (66.3 °C and 50 % w/w) only differing in the SLR, 30 g/L for ethanol extraction versus 13.18 g/L for DES extraction (37).

For cosmetic purposes and allegation claims extracts should be standardized to ensure a known concentration of the primary active compound, such as HT. It means a measurable quantity of the target compound is consistent in every batch produced (14). Ethanolic extracts are generally very complex mixtures since ethanol extracts a wide range of constituents from the plant material. This complexity may cause difficulties formulating, as well as stability issues (14). Purification techniques such as ultrafiltration, nanofiltration or reverse osmosis are commonly used (11). However, an extra step can represent an increase in cost/time and the combination of antioxidants present in the natural extract is usually more effective due to the synergistic effect produced (38).

Cosmetic safety is also an important parameter that should be evaluated. Natural products extract possesses a variety of chemical compounds whose interactions can cause adverse reactions. Even highly purified extracts contain compounds that are safe at low levels but can present toxicity at higher concentrations (14). In fact, a popular misconception resides in the fact that an ingredient natural origin means an absence of toxicity. In that sense, *in vitro* cytotoxic potential of extracts should be tested in several human cell lines prior to human use (15). In the case of cosmetic application *in vitro* models to test skin or eye irritation such as EpiSkin™ or SkinEthic™, can be used (39). These models mimic the skin biophysical properties and are able to determine cell viability, histology, and pro-inflammatory mediators release (72). *In vivo* assays, in human volunteers, like the occlusive patch test, are also usually performed to evaluate acute irritant reactions (39). Other

considerations such as possible interactions with other ingredients in the formulation and potential of skin penetration should also be considered (40).

2. Objectives

The first part of this work had the main objective of identifying the best conditions, through independent variables analysis, for the extraction of hydroxytyrosol (HT) and tyrosol (Tyr), regarding the principles of green chemistry, as well as economic and upscaling feasibility. Also, it aimed to promote a new strategy for OP re-use as a potential ingredient for cosmetic products characterizing these extracts safety, stability and biological activity. **Table 2** summarizes the key objectives in this work.

Table 2. Chapter I objectives.

Objectives	
1	To evaluate the influence of different variables such as solvent, SLR, temperature, extraction time and number, in the extraction yield of HT and Tyr in a conventional solid-liquid extraction.
2	To achieve a cost-effective and eco-friendly solution to HT extraction from OP for cosmetic purposes.
3	To assess microbiological safety from OP samples and extracts for cosmetic applications, as well as the efficiency of a pasteurization process.
4	To understand the potential of optimized extracts for cosmetic formulations regarding antioxidant activity and biological activities.
5	To determine extract safety for cutaneous application through an <i>in vitro</i> cytotoxicity assay.
6	To contribute to a circular economy model for the olive oil industry.

3. Material and Methods

3.1. Sample

Olive pomace was collected in a two-phase olive mill in Alfândega-da-Fé, Portugal, in November 2022. The sample was homogenized, divided in different batches and frozen at -80 °C.

3.2. Standards and reagents

Folin-Ciocalteu's reagent, sodium carbonate, 2,4,6-tri-(2-pyridyl)-S-triazine (TPTZ), ferric chloride, 2,2-diphenyl-1-picrylhydrazyl radical (DPPH[•]), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), gallic acid, heptahydrate ferrous sulphate and sodium acetate were acquired from Sigma-Aldrich (St. Louis, MO, USA). Glacial acetic acid was obtained from Fisher Chemical (Loughborough, England). Sodium carbonate decahydrate was purchased from Merck (Darmstadt, Germany). HPLC grade methanol was acquired from Panreac (Barcelona, Spain), and absolute ethanol from Honeywell Riedel-de Haën (Seelze, Germany). Hydroxytyrosol (2-(3,4-di-hydroxyphenyl)ethanol) ≥98% from Extrasynthese (Genay, France) and tyrosol (2-(4-hydroxyphenyl)ethanol) 98% from Aldrich (Steinheim, Germany) were used as standards in HPLC analysis. A Milli-Q water purification system (Millipore, Bedford, MA, USA) was used to treat and obtain the ultrapure water.

For enzymatic activities all reagents (Dimethyl sulfoxide, Tris-Hydrochloride, potassium phosphate), substrates (L-tyrosine and N-(methoxysuccinyl)-ala-ala-proval-4-nitroanilide) and enzymes (tyrosinase, elastase) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Extracts cytotoxicity evaluation was performed using MTT (3,4,5-dimethylthiazolyl-2,5-diphenyl-tetrazolium bromide) solution, from Sigma-Aldrich (St. Louis, MO, USA). Immortalized Human Keratinocytes (HaCaT cell line) were supplied by ATCC[®] and cultured in Dulbecco's Modified Eagle Medium (DMEM), supplemented with Fetal Bovine Serum (FBS) penicillin, streptomycin and amphotericin B, all provided by Sigma-Aldrich (St. Louis, MO, USA).

For microbiological studies sodium chloride was acquired from VWR Chemicals (Alfragide, Portugal). Mediums and broths were obtained from Liofilchem (Roseto degli Abruzzi, Italy) and mixed cellulose ester (MCE) membranes (0.45 µm pore size, 47 mm) were acquired from Frilabo, Milherós, Portugal). All solutions were sterilized in an autoclave.

3.3. Moisture evaluation

Sample moisture was determined using an infrared balance (KERN DBS, Baligen, Germany) in triplicate (**Figure 3**). Fresh OP, Pasteurized OP and freeze-dried OP were analysed.



Figure 3. Infrared balance (KERN DBS, Baligen, German).

3.4. Extract preparation

Extracts were prepared, in triplicate, by adding 35 ml of extraction solvent with different ethanol percentages (0, 30, 70 and 100%) to different masses (0.5, 1 and 2 g) of sample (fresh OP or freeze-dried OP). The mixture was continuously stirred at 600 rpm in a heating plate at different temperatures (room temperature, 25 and $40 \pm 1^\circ\text{C}$) and extraction times (15, 30, 60, 90 and 120 minutes). For assessment of the first variable effect (solvent) extracts were only vortexed 15 min at room temperature ($17 \pm 2^\circ\text{C}$). All extracts were centrifuged at 2500 rpm for 15 minutes and the supernatant filtered through a $0.45 \mu\text{m}$ PTFE membrane filter into a 2 mL injection vial for HPLC analysis.

3.5. Dried powders fractioning

Freeze-dried samples were sieved through three different mesh sizes (500, 250 and $125 \mu\text{m}$) using a vibratory sieve shaker (Retsch GmbH, Haan, Germany) set for 60 amplitude, for 5 minutes (**Figure 4**). Fractions were collected with particle sizes higher than $500 \mu\text{m}$ ($> 500 \mu\text{m}$), between 500 and $250 \mu\text{m}$ ($> 250 \mu\text{m}$), between 250 and $125 \mu\text{m}$ ($> 125 \mu\text{m}$) and inferior to $125 \mu\text{m}$ ($< 125 \mu\text{m}$).



Figure 4. Vibratory sieve shaker (Retsch ®).

3.6. Extract characterization

For antioxidant activity and total phenolics determination, 200 ml of OP extract was prepared following the best conditions tested. Extract was filtered (Whatman No. 4) and then concentrated in a rotary evaporator, Rotavapor® R-300 (BÜCHI Labortechnik, Essen, Germany) (**Figure 5**) and stored in several aliquots at -20°C. Before analysis, aliquots were homogenised and centrifuged at 10000 rpm for 5 minutes to ensure a limpid sample.

For cytotoxicity and enzymatic activities in this chapter, a freeze-dried extract (FD-E) was produced (Telstar Cryodos-80 Terrassa, Barcelona) (**Figure 6**), but the conditions are referred in **Chapter II**, as the SLR ratio used was not the same.



Figure 5. Rotavapor® R-300.



Figure 6. Freeze-Dryer Telstar®.

3.7. Hydroxytyrosol and tyrosol content analysis by HPLC-DAD-FLD

HPLC system was composed of an AS-2057 automated injector, a PU-2089 pump, a MD-2018 multi-wavelength diode array detector (DAD) and a fluorescence detector (FLD) FP-2020 (Jasco, Japan), programmed for excitation at 280 and emission at 330 nm (**Figure 7**). A chromatographic column (Zorbax-SB-C18; 250 × 4.6 mm, 5 µm; Agilent Technologies, Amstelveen, Netherlands) was used at 20 °C with a flow rate of 1 mL/min and an injection volume of 20 µL. Mobile phase comprised 1% acetic acid solution (A) and 100% methanol (B) in gradient elution mode. The gradient elution program used was the following: 0 min, 5% B; 20 min, 25% B; 22 min, 100% B; 27 min, 5% B. Calibration curves were prepared with HT and Tyr standards ranging from 0.25 to 200 µg/ml ($R^2 = 0.9998$).



Figure 7. HPLC-DAD-FLD system (Jasco Corporation).

3.8. Pasteurization

To assure microbiological safety, fresh OP was submitted to a pasteurization process in a Vorwerk Thermomix TM 31[®] for 15 seconds at 85 °C (**Figure 8**). Extraction, in triplicate of P-OP (pasteurized OP) was compared with NP-OP (non-pasteurized) for HT and Tyr quantification and microbiological analysis.



Figure 8. Vorwerk Thermomix TM 31[®].

3.9. Total phenolic content

The assessment of the phenolic content was performed according to Costa *et al.* (41) with modifications. Hence, 150 μL of Folin–Ciocalteu's reagent (1:10) and 120 μL of sodium carbonate (7.5%, w/v) were added to 30 mL of diluted extracts (1:100). The microplate was incubated at 45 °C for 15 min, protected from light. After 30 minutes at room temperature, absorbance was measured at 765 nm (Synergy HT Microplate Reader, BioTek Instruments, Inc., Winooski, VT, USA). The analysis was performed in triplicate. A calibration curve using gallic acid (5–100 mg/L, $R^2 = 0.9996$) was prepared. Results are presented in $\text{g}_{\text{GAE}}/100\text{g}$ OP and $\mu\text{g}_{\text{GAE}}/\text{ml}$ of extract.

3.10. Ferric-Reducing Antioxidant Power (FRAP)

The FRAP assay was carried out according to Costa *et al.* (42). Briefly, using a microplate, 35 μL of a diluted extract (1:100) were mixed with 265 μL of the FRAP reagent (0.3 M acetate buffer, 10 mM TPTZ solution and 20 mM of ferric chloride). The mixture was incubated at 37 °C, for 30 minutes, protected from light. Absorbance was read at 595 nm (Synergy HT Microplate Reader, BioTek Instruments, Inc., Winooski, VT, USA). The ferric-reducing antioxidant power was determined using a calibration curve prepared with ferrous sulphate (50–600 $\mu\text{mol/L}$, $R^2 = 0.9997$). The analysis was performed in triplicate. Results are presented in $\text{mmol}_{\text{FSE}}/100\text{g}$ OP and $\mu\text{mol}_{\text{FSE}}/\text{ml}$ of extract.

3.11. 2,2-Diphenyl-1-Picrylhydrazyl Radical (DPPH $\cdot$) Scavenging Ability

The radical scavenging potential was assessed according to Costa *et al.* (41). For that, 270 μL of an ethanolic DPPH $\cdot$ solution (6.0×10^{-5} mol/L) was mixed with 30 μL of the diluted extract (1:100). The absorbance decrease was measured every 2 min, at 515 nm, in order to observe the kinetic reaction (Synergy HT Microplate Reader, BioTek Instruments, Inc., Winooski, VT, USA). The reaction endpoint was reached at 20 minutes. Trolox was used as a standard to prepare the calibration curve (5.62–175.34 mg/L, $R^2 = 0.996$). The analysis was performed in triplicate. Results are presented in $\text{g}_{\text{TE}}/100\text{g}$ OP and $\mu\text{g}_{\text{TE}}/\text{ml}$ of extract.

3.12. Inhibition of Enzyme Activities

Elastase and tyrosinase inhibition assays were performed using freeze-dried extract (FD-E) in different concentrations (1-10 mg/ml), using 10% dimethyl sulfoxide (DMSO) as solvent. Inhibition percentage was calculated according to the following equation (**Eq.1**):

$$Inhibition (\%) = \frac{Abs_{control} - Abs_{sample}}{Abs_{control}} \times 100$$

Eq. 1. Enzyme inhibition % calculation.

3.12.1. Tyrosinase inhibition

Tyrosinase inhibition assay was based on Taghouti *et al.* (43) with some modifications. 20 µL of enzyme (tyrosinase, 1000 U/ml) and 170 µL of a solution containing L-tyrosine, phosphate buffer (50 mM, pH 6.5) and distilled water (10:10:9 ratio), were added to 10 µL of extract (1 and 10 mg/ml). Kojic acid (1 mg/ml) and DMSO 10% were used as positive and negative controls, respectively. The reaction was incubated for 10 minutes at 37 °C, and, after this time, the absorbance was read at 490 nm, in a microplate reader (Thermo-Fisher Scientific, Oporto, Portugal).

3.12.2. Elastase inhibition

Elastase inhibition assay was performed according to Taghouti *et al.* (43). Initially, 50 µL of each extract (1, 2.5, 5 and 10 mg/mL) were mixed with 160 µL of Tris-HCl buffer (0.20 mM, pH 8) and 20 µL of the substrate (0.80 mM, in Tris-HCl buffer) and incubated for 10 minutes at room temperature. Then, 20 µL of elastase (1 U/mL, in Tris-HCl buffer) was added and the microplate was incubated for 20 minutes at room temperature. Absorbance was read at 410 nm using a microplate reader (Thermo-Fisher Scientific, Oporto, Portugal). Tris HCL-buffer was used as negative control.

3.13. *In Vitro* Cytotoxicity Assay

3.13.1. Cell culture

Immortalized human keratinocytes (HaCaT cell line) were cultured in DMEM low glucose, containing L-glutamine, supplemented with 10% FBS, penicillin (100 U/ml), streptomycin (100 mg/ml), and amphotericin B (0.25 mg/ml). Culture medium was changed every 2–3 days and the culture was split every 7 days when it achieved 95% of confluence. For sub-culturing, the cells were removed enzymatically (0.25% trypsin–EDTA, 5 minutes, at 37 °C), split 1:3, and sub-cultured in plastic culture dishes (21 cm²; 60 mm; Corning Costar, Corning, NY).

3.13.2. MTT assay

MTT is a rather cost-efficient colorimetric technique, where the measurement strictly depends upon live cells. The tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, is reduced to formazan exclusively by the mitochondrial succinate dehydrogenase enzyme of viable cells.

Cytotoxicity was assessed for HaCaT cells exposed to different olive pomace extract (OPE) concentrations by performing a MTT assay. Those cells were seeded into 96-micro-well plates (10^4 cells/well – density) and incubated for 48 hours. After 2 days, the culture medium was substituted by medium without FBS with concentrations between 1 and 2000 $\mu\text{g/mL}$ of an extract solution (10 mg/ml, w/v). Cells with medium without OPE were used as control (water), and culture medium was used as reagent blank. After exposure time (24 hours), the culture medium was removed, and 100 μL of MTT working solution was added to each well. Plates were incubated for 4 hours, at 37 °C. After removing the reagent solution and rinsing with phosphate buffer saline solution (PBS), 100 μL of DMSO was added to dissolve the formazan crystals and the microplate was incubated for 1 hour, at 37 °C. The absorbance of the resulting solution was measured at 550 nm to determine cell viability, using the Varioskan Lux microplate reader (ThermoScientific, Dreieich, Germany). Experiments were performed in triplicate.

3.14. Extract stability

From the prepared extract (**Section 3.4**) several aliquots were taken before concentration in the rotary evaporator (1:1, v/v, hydroalcoholic extract) to be stored at room temperature for three months. After concentration, the water-based extract was stored at 4 and -20 °C for a three-month period. Each month, samples were submitted to HPLC-DAD-FLD analysis for HT and Tyr quantification, following the same procedure described in **Section 3.7**. The same samples were also submitted to microbiological count as described in the next section.

3.15. Microbiological studies

Microbiology analysis was performed, not only to establish the safety OP for cosmetic application, but also the efficacy of the pasteurization process. In that sense, FD samples and extracts from both NP-OP and P-OP were compared according to specific criteria (ISO 17516: 2016, Microbiological Guidelines for Cosmetic Products) (44). Also, extract

microbiological stability over time was also assessed. Results are expressed as colony-forming units (CFU) per ml or g of sample.

Mesophilic aerobic bacteria count, and yeast and mold count were performed using three different methods:

3.15.1. Pour plate method

1 mL of extracts and FD samples (1 g diluted in 9 g saline solution) were placed in a petri dish and about 25 mL of melted Trypticasein Soy Agar, TSA, (for bacteria count) or Sabouraud Dextrose Agar - Chloramphenicol, SDA-C, (for yeast and mold count), at approximately 50 °C, were added. Circular motions homogenized the mixture, and solidification occurred at room temperature. Plates were incubated at 37 °C for 24 hours (bacteria count) and 72 hours (yeast and mold count).

3.15.2. Surface Spreading Method

100 µL of FD samples (1 g diluted in 9 g of saline solution) was spread evenly over the agar surface of two different mediums, TSA and SDA-C. The same process was used for evaluating microbiological stability of water-based extracts stored at 4 °C and hydroethanolic extracts (1:1, v/v) stored at room temperature, each month, for a total of 3 months. Plates were incubated at 37 °C for 24 hours (bacteria count) and 72 hours (yeast count).

3.15.3. Membrane filtration

A 1:100 solution with FD samples from NP-OP and P-OP (particle size < 250 µm) and deionized sterile water was prepared. As the solution was fairly turbid, two centrifugation steps (5000 rpm, 10 minutes) were performed after sample dilution and the supernatant was collected. Then, using a filtration unit under vacuum (**Figure 9**), 25 and 50 ml were filtered through a MCE 0.45 µm pore size membrane. The membrane was placed in a petri dish with medium, Plate Count Agar (PCA), and incubated at 37 °C for 24 hours.



Figure 9. Filtration unit

According to ISO 17516: 2016 (44), identification and isolation of pathogenic bacteria is required for cosmetic products, namely *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Candida albicans*. The first step is enrichment in a non-selective broth to increase the number of microorganisms. The second step involves isolation on selective media followed by identification tests as shown in **Figure 10**. Accordingly, 1 g of each FD samples or 1 ml of extract was enriched in MacConkey broth, Trypticasein Soy Broth (TSB), peptone buffered water and Sabouraud broth depending on the bacteria to identify. TSB and Peptone buffered water were both used to see if any difference in bacterial growth occurred. Then, surface spreading method was applied, with 100 µL of the inoculated broth in selective mediums: Cetrimide agar, Mannitol Salt Agar, Sabouraud Dextrose Agar, MacConkey agar and TSA. As selected mediums only provide presumptive isolation, confirmation tests were performed.

For identification of *Staphylococcus aureus* a coagulase test was performed by inoculating a CFU in a test tube with rabbit plasma. Clot formation within 2 hours at 37 °C suggests a positive result.

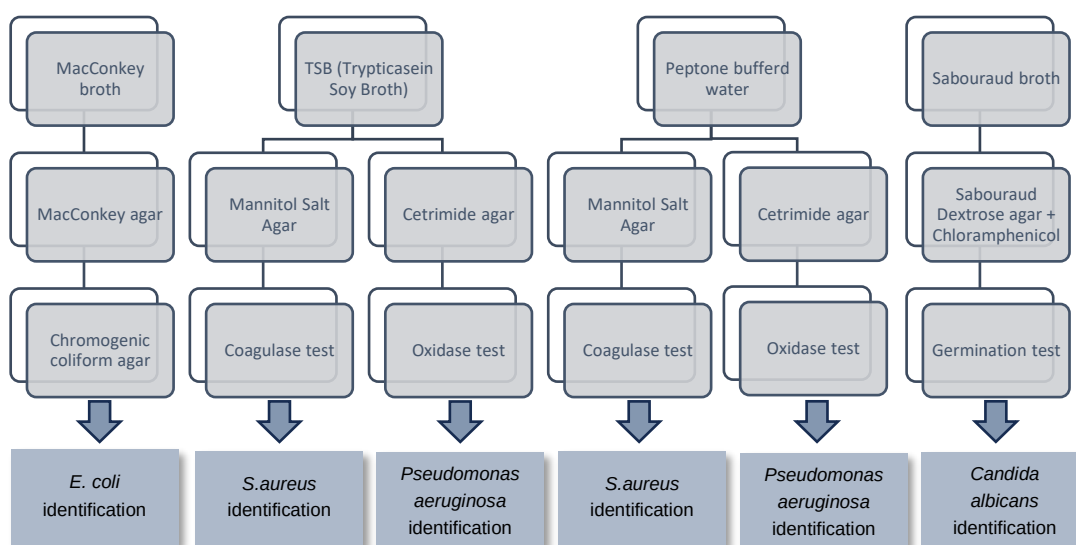


Figure 10. Flow chart used for isolation and identification of pathogenic bacteria in cosmetic products (based on ISO 21150:2015, ISO 22717:2015, ISO 22718:2015 and ISO 18416:2015).

3.16. Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics v.25 (IBM Corp., Armonk, NY, USA). Data are expressed as mean $\pm$ standard ($n=3$ or $n=6$). The one-way ANOVA was used to evaluate differences between samples, followed by Tukey's HSD post hoc test.

T-student test was also performed for cytotoxicity analysis. The level of significance for all hypothesis tests was 0.05.

4. Results/Discussion

4.1. Moisture content

Fresh OP presented a moisture percentage of $72.17 \pm 1.04\%$ similar to pasteurized OP ($69.35 \pm 0.54\%$). After freeze-drying, OP presents a $3.62 \pm 0.06\%$ moisture content. As OP water content is very significant freeze-drying process yield doesn't reach 30% (28.95%).

4.2. Extraction variables analysis

4.2.1. Solvent

Phenolic compounds are generally polar although, depending on the number and conjugation of the phenol groups, their hydro/lipophilicity ratio may vary (3). Therefore, polar solvents provide better extraction results. For this work, chosen solvents included water and ethanol at different proportions, since both solvents are considered non-toxic and dermatological safe (33). As shown in **Table 3** and **Figure 11** and **12**, similar results were obtained with 30:70, 50:50 and 70:30 ethanol/water ratios for HT and Tyr extraction, at room temperature, for 15 minutes, using 1 g of fresh OP and 35 ml of the different solvents.

Table 3. HT and Tyr extraction with different solvents, maintaining the same SLR, temperature and time.

Solvent	HT mg /100 g (fw)	HT mg /100 g (dw)	Tyr mg/ 100 g (fw)	Tyr mg/ 100 g (dw)
100% H ₂ O	32.20 ± 6.21^a	115.69 ± 22.30^a	21.42 ± 1.41^a	76.96 ± 5.08^a
30:70 EtOH:H ₂ O	56.24 ± 6.86^{bc}	202.10 ± 24.64^{bc}	21.83 ± 0.30^a	78.45 ± 1.07^a
50:50 EtOH:H ₂ O	65.03 ± 3.86^c	233.68 ± 13.85^c	20.33 ± 3.03^a	73.06 ± 10.89^a
70:30 EtOH:H ₂ O	57.20 ± 11.56^{bc}	205.53 ± 41.52^{bc}	20.18 ± 3.94^a	72.52 ± 14.16^a
100% EtOH	40.74 ± 10.16^{ab}	146.38 ± 36.49^{ab}	12.13 ± 1.29^b	43.58 ± 4.65^b

HT, hydroxytyrosol; Tyr, Tyrosol; EtOH, absolute ethanol; H₂O, deionized water; dw, dry weight; fw, fresh weight. Results presented as mean $\pm$ standard deviation ($n=3$). Different letters in each column represent significant differences between solvents ($p < 0.05$).

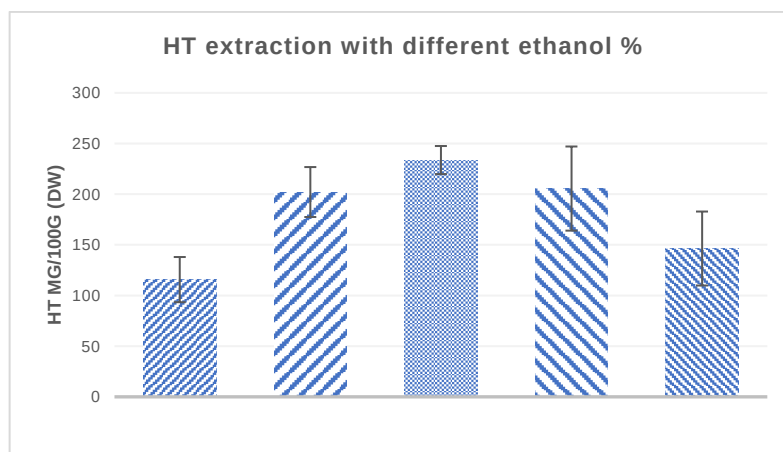


Figure 11. HT extraction with different ethanol:water ratios.

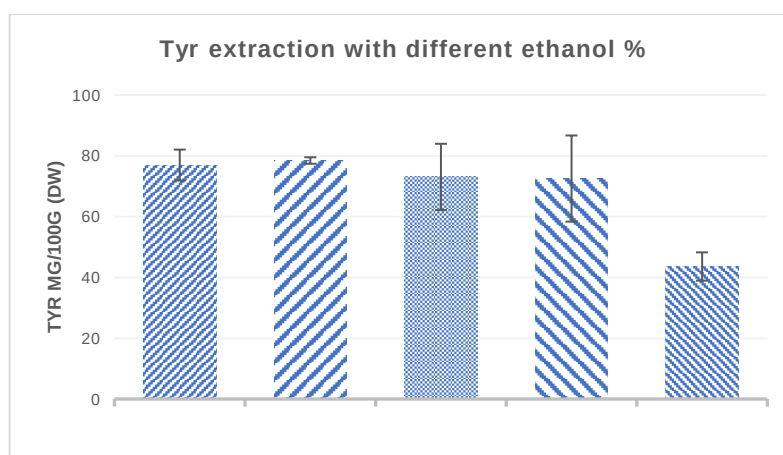


Figure 12. Tyr extraction with different ethanol:water ratios.

For further testing, 50:50 and 70:30 ratios were selected since the first provided the best extraction yield and the latter could have an added benefit by reducing microbiological contamination, a fact that should be considered in natural derived raw materials for cosmetic purposes.

4.2.2. Temperature and extraction time

Temperature and extraction time selection can also greatly influence phenolic extraction since they potentially can accelerate degradation of certain compounds, reducing overall antioxidant capacity of the extract and produce solvent evaporation (3). **Tables 4 and 5** show the results for HT and Tyr extraction varying temperature (room temperature, 25 and 40°C ± 1) and extraction time (15, 30 and 60 minutes) maintaining the solvent ratios selected previously as well as the SLR (1:35, w/v). For the 1:1 (v/v) hydroalcoholic solvent, values at room temperature were significant different ($p < 0.05$) compared to the higher

temperature results. 25°C for 60 minutes provided the best extraction yield, although statistical differences were not found ($p > 0.05$) between 25 °C and 40 °C for the same extraction period. Nevertheless, since the increase in temperature could result in a less cost-effective method, 25 °C was considered the optimum temperature for HT extraction. Similarly, for the 70% ethanol solution, 25°C temperature provided the best extraction results for HT, but no statistical differences were observed for Tyr extraction ($p > 0.05$). In order to understand if an extraction time increase could potentiate the extraction performance, extraction at 25 °C proceeded for 90 and 120 minutes with the 1:1 (v/v) solvent ratio (**Figure 13** and **14**). No statistical differences ($p > 0.05$) were found between these time periods and 60 minutes extraction time, suggesting further elongation of the extraction process did not contribute to its efficiency. However, for Tyr extraction an increase in extraction yield is verified after 90 minutes, which can be in agreement with previous studies in which TPC increased with extraction time up to 120 minutes (34). However, for the purpose of this study, since HT is the major compound with cosmetic application, maintaining a high extraction yield for this compound using a shorter time will be much more productive.

Table 4. HT and Tyr extraction with 50:50 (v/v) hydroethanolic solvent, SLR 1:35 (w/v) varying temperature and extraction time.

Temperature	Time	HT mg /100 g (fw)	HT mg /100 g (dw)	Tyr mg /100 g (fw)	Tyr mg /100 g (dw)
Room temperature	15'	36.14 ± 11.68 ^a	129.87 ± 41.95 ^a	17.32 ± 1.80 ^{abcd}	62.23 ± 6.47 ^{abcd}
	30'	40.49 ± 6.92 ^{ab}	145.48 ± 24.87 ^{ab}	16.00 ± 1.06 ^{abc}	57.48 ± 3.82 ^{abc}
	60'	33.28 ± 6.07 ^{ab}	119.60 ± 21.82 ^{ab}	13.72 ± 2.45 ^a	49.32 ± 8.82 ^a
25 ± 1 °C	15'	46.23 ± 5.15 ^{abc}	166.12 ± 18.51 ^{abc}	13.96 ± 0.77 ^{ab}	50.17 ± 2.78 ^{ab}
	30'	62.98 ± 3.37 ^{cde}	226.29 ± 12.11 ^{cde}	17.51 ± 0.82 ^{bcd}	62.93 ± 2.96 ^{bcd}
	60'	68.40 ± 2.53 ^e	245.79 ± 9.10 ^e	16.83 ± 0.57 ^{abcd}	60.49 ± 2.05 ^{abcd}
	90'	65.05 ± 4.65 ^{de}	233.75 ± 16.72 ^{de}	19.99 ± 1.50 ^{de}	71.82 ± 5.41 ^{de}
	120'	63.36 ± 1.37 ^{de}	227.66 ± 4.93 ^{de}	21.77 ± 0.95 ^e	78.24 ± 3.40 ^e
40 ± 1 °C	15'	56.86 ± 7.47 ^{bcde}	204.31 ± 26.86 ^{bcde}	17.15 ± 0.92 ^{abcd}	61.61 ± 3.29 ^{abcd}
	30'	49.81 ± 2.40 ^{abcd}	178.96 ± 8.64 ^{abcd}	18.28 ± 1.44 ^{cde}	65.70 ± 5.16 ^{cde}
	60'	58.32 ± 3.44 ^{cde}	209.56 ± 12.36 ^{cde}	17.65 ± 0.68 ^{cd}	63.78 ± 2.43 ^{cd}

HT, hydroxytyrosol; Tyr, Tyrosol; dw, dry weight; fw, fresh weight; Room temperature, 17°C ± 2.
Results presented as mean ± standard deviation ($n=3$).
Different letters in each column represent significant differences ($p < 0.05$).

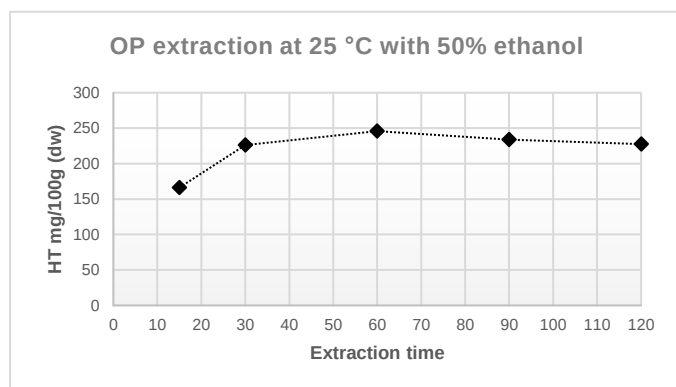


Figure 13. HT concentration with extraction time variation at 25 °C and 1:1 solvent ratio, and 1:35, w/v, SLR.

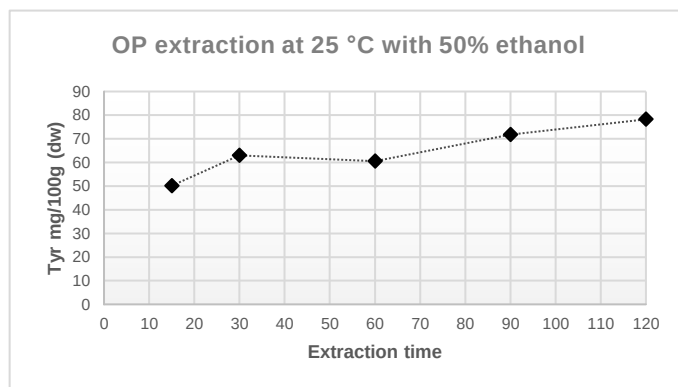


Figure 14. Tyr concentration with extraction time variation at 25 °C and 1:1 solvent ratio, and 1:35, w/v, SLR.

Table 5. HT and Tyr extraction with 70:30 (v/v) ethanol:water, SLR 1:35 (w/v), varying temperature and extraction time.

Temperature	Time	HT mg /100 g (fw)	HT mg /100 g (dw)	Tyr mg /100 g (fw)	Tyr mg /100 g (dw)
Room temperature	15'	46.47 ± 1.57 ^a	166.98 ± 5.64 ^a	17.81 ± 0.10 ^a	63.98 ± 0.38 ^a
	30'	48.10 ± 3.01 ^a	172.82 ± 10.82 ^a	16.77 ± 1.49 ^a	60.26 ± 5.35 ^a
	60'	49.38 ± 5.75 ^a	177.45 ± 20.67 ^a	16.58 ± 1.51 ^a	59.58 ± 5.43 ^a
25 ± 1 °C	15'	61.36 ± 2.20 ^b	220.49 ± 7.91 ^b	18.97 ± 1.81 ^a	68.16 ± 6.49 ^a
	30'	64.36 ± 7.61 ^b	231.27 ± 27.34 ^b	17.97 ± 1.15 ^a	64.56 ± 4.14 ^a
	60'	66.26 ± 0.83 ^b	238.09 ± 3.00 ^b	19.12 ± 1.94 ^a	68.71 ± 6.95 ^a
40 ± 1 °C	15'	45.06 ± 3.11 ^a	161.92 ± 11.17 ^a	15.54 ± 1.26 ^a	55.85 ± 4.52 ^a
	30'	49.00 ± 1.20 ^a	176.05 ± 4.43 ^a	16.41 ± 0.73 ^a	58.98 ± 2.61 ^a
	60'	49.29 ± 0.19 ^a	177.09 ± 0.67 ^a	16.81 ± 1.48 ^a	60.41 ± 5.31 ^a

HT, hydroxytyrosol; Tyr, Tyrosol; dw, dry weight; fw, fresh weight; Room temperature, 17 ± 2°C.

Results presented as mean $\pm$ standard deviation ($n=3$).
 Different letters in each column represent significant differences ($p < 0.05$).

4.2.3. Solid to liquid ratio

The solid to liquid ratio (SLR) is another important operating variable. The correct balance between sample and solvent could save resources and facilitate the post-processing of the extraction method, such as the concentration and/or purification phases (50). With fixed variables of solvent, temperature and extraction time assessed earlier, varying the sample mass to a fixed solvent volume of 35 ml showed significant differences ($p < 0.05$) in HT yield between 0.5 g/35 ml and the other ratios (**Table 6**). However, when duplicated the sample amount to 2 g no statistical differences were observed ($p > 0.05$). This suggests that the solution was saturated since no additional extraction for HT or Tyr is observed.

Table 6. HT and Tyr extraction with 50% ethanol at 25°C for 60 minutes, varying SLR.

SLR (w/v)	HT mg /100 g (fw)	HT mg /100 g (dw)	Tyr mg/ 100 g (fw)	Tyr mg/ 100 g (dw)
0.5/35	34.06 $\pm$ 2.28 ^a	122.39 $\pm$ 8.21 ^a	15.12 $\pm$ 1.39 ^a	54.33 $\pm$ 5.01 ^a
1/35	46.71 $\pm$ 2.29 ^b	167.84 $\pm$ 8.21 ^b	17.35 $\pm$ 0.33 ^a	62.35 $\pm$ 1.18 ^a
2/35	46.46 $\pm$ 4.81 ^b	166.93 $\pm$ 17.27 ^b	17.19 $\pm$ 1.03 ^a	61.78 $\pm$ 3.71 ^a

HT, hydroxytyrosol; Tyr, Tyrosol; SLR, solid to liquid ratio, w/v, weight/volume, dw, dry weight; fw, fresh weight. Results presented as mean $\pm$ standard deviation ($n=3$).
 Different letters in each column represent significant differences ($p < 0.05$).

4.2.4. Pasteurization

Natural products, due to their characteristics, are more susceptible to microbial contamination. In a previous study from this work group, different heat-treatments were applied to OP in order to minimize the natural microbiological load, aiming for its' use as a safe food ingredient (49). The same scrutiny should be applied for cosmetic products since they have to obey specific rules regarding microbial contamination such as ISO 17516: 2016 (44).

A High-Temperature-Short-Time (HTST) pasteurization (85°C for 15 seconds) was selected, since less heat time could minimize chemical changes. To determine the effect on HT and Tyr concentration, pasteurized OP (P-OP) was compared with non-treated OP (NP-OP) under the conditions previously tested (50% ethanol, 25 °C, 60 minutes extraction time and SLR 1:35, w/v). Results showed that the pasteurization process nearly doubled HT extraction yield, but no significant differences were observed for Tyr extraction ($p > 0.05$) (**Table 7**). In plants, phenolic compounds can be bound to cell wall components as polysaccharides and this affinity is dependent on polyphenols structures, concentrations,

temperature, pH, ionic strength, or the presence of proteins (50). Since high temperatures can weaken the interactions between phenolic compounds and other cell components, this effect could be more pronounced in HT, rather than Tyr, increasing the diffusion rate and consequently the extraction yield (36).

Table 7. Comparison in HT and Tyr content of pasteurized and non-pasteurized OP with 50% ethanol, at 25°C, for 60 minutes and SLR of 1:35 (w/v).

Sample	HT mg /100 g (fw)	HT mg /100g (dw)	Tyr mg/ 100 g (fw)	Tyr mg/ 100 g (dw)
P-OP	66.31 ± 7.21 ^a	216.33 ± 23.54 ^a	18.79 ± 3.04 ^a	61.31 ± 9.93 ^a
NP-OP	33.23 ± 3.87 ^b	119.41 ± 13.91 ^b	15.03 ± 0.94 ^a	54.01 ± 3.39 ^a

P-OP, pasteurized olive pomace; NP-OP, non-pasteurized olive pomace; HT, hydroxytyrosol; Tyr, Tyrosol; dw, dry weight; fw, fresh weight.

Results presented as mean ± standard deviation ($n=3$).

Different letters in each column represent significant differences ($p < 0.05$).

4.2.5. Particle size

Considering OP high water content, this by-product is easily susceptible to microbial degradation and enzymatic reactions that can minimize storage time and lead to chemical changes, reducing bioactive content and efficiency. Freeze-drying has been proposed as an adequate procedure to preserve polyphenols content and retain the functional potential of the extracts (3). In the next sections, freeze-dried OP (FD-OP) was used for HT and Tyr extraction, maintaining the optimized variables determined in the previous sections for fresh OP, only varying particle size, SLR and extraction number. Also, to confirm if the pasteurization process increases HT yield, fresh OP pasteurization before freeze-drying was performed and pasteurized FD samples were compared to non-pasteurized ones.

OP is an heterogenous biomass comprising pulp, husks and crushed olive stones (2). Phenolic compounds could have higher or less expression depending on the olive fruit matrix, since their primordial function is the to uphold plant integrity, protecting its survival. In that sense, the plants' outer layers usually contain higher phenolic content (50).

FD-OP was sieved, with different mesh sizes (500, 250 and 125 µm), obtaining different fractions as seen in **Figure 15**. Then, these fractions were subjected to the previous extraction process (SLR of 1:35, w/v, with 50% ethanol, at 25 °C for 60 minutes). Also, these samples were pasteurized before freeze-dried. Results are presented in **Table 8**. HPLC analysis demonstrated that significant differences ($p < 0.05$) occur between the extraction from FD-OP with particles over 500 µm and the other parcels, with a lower extraction yield for both HT and Tyr when particle size is higher than 500 µm. This fraction comprises

predominately small, crushed olive stones that remain in the olive pomace, which will contribute to the sample weight and add lower bioactive percentages per g.

Table 8. HT and Tyr extraction of FD-OP subjected to pasteurization, with 1:35 (w/v) SLR, 50% ethanol, 25°C and 60 minutes extraction time, varying particle size.

Particle size	HT mg /100g	Tyr mg/ 100 g
> 500 μm	116.33 $\pm$ 6.89 ^a	34.06 $\pm$ 1.34 ^a
250 - 500 μm	275.91 $\pm$ 17.31 ^b	86.01 $\pm$ 1.79 ^b
125 - 250 μm	285.48 $\pm$ 7.18 ^b	84.81 $\pm$ 1.75 ^b
125 μm	292.03 $\pm$ 7.70 ^b	88.03 $\pm$ 1.07 ^b

HT, hydroxytyrosol; Tyr, Tyrosol.

Results presented as mean $\pm$ standard deviation ($n=3$), per 100 g of freeze-dried OP.

Different letters in each column represent significant differences ($p < 0.05$).

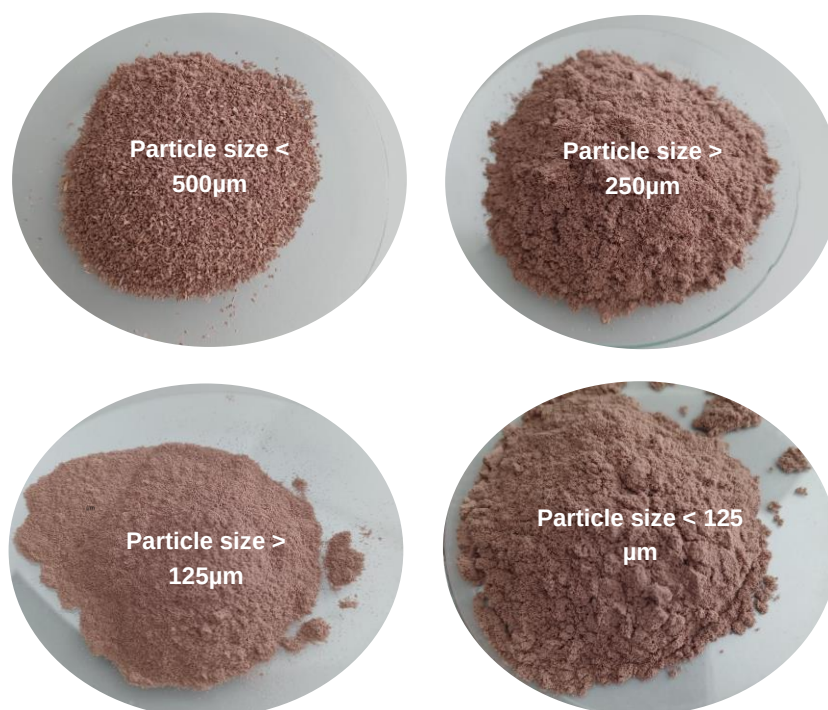


Figure 15. Freeze-dried OP fractions after sieving with different mesh sizes (500, 250 and 125 μm).

4.2.6. Pasteurization effect in freeze-dried samples

Since FD samples presented more reproducible extracts (after sieving OP powders are homogenous, meanwhile fresh OP can be more heterogeneous), the optimized extraction variables found in the earlier sections (50% ethanol, 25 °C for 60 minutes) with a 1:35 SLR (w/v) and particle size between 250 and 125 μm) were applied to FD samples subjected (P-

OP-FD) or not (NP-OP-FD) to the pasteurization process described in **section 3.8**. In **Table 9** it is possible to observe that significant differences ($p < 0.05$) are obtained for HT extraction yield between tested samples but not for Tyr extraction. These results corroborate the determined results from fresh OP samples (**Table 7**)

Table 9. Comparison in HT and Tyr content of pasteurized and non-pasteurized FD-OP samples, with extraction variables of 50% ethanol, 25 °C for 60 minutes, 1:35 SLR (w/v) and particle size between 250 and 125 μm .

Sample	HT $\text{mg}/100\text{ g}$	Tyr $\text{mg}/100\text{ g}$
P-OP-FD	285.16 $\pm$ 6.47 ^a	84.99 $\pm$ 3.28 ^a
NP-OP-FD	157.09 $\pm$ 5.82 ^b	79.07 $\pm$ 3.22 ^a

P-OP-FD pasteurized freeze-dried olive pomace; NP-OP-FD, non-pasteurized freeze-dried olive pomace; HT, hydroxytyrosol; Tyr, Tyrosol; dw, dry weight; fw, fresh weight.
Results presented as mean $\pm$ standard deviation ($n=3$), per 100g of freeze-dried sample.
Different letters in each column represent significant differences ($p < 0.05$).

4.2.7. Solid to liquid ratio/ number of extractions

Re-extraction was also attempted using different SLRs. Increasing the number of extraction cycles can potentially improve the extraction performance since fresh solvent is added, solubilizing remaining compounds. The optimized variables were maintained, including pasteurization treatment before freeze-drying and particle size sieving under 500 μm . Except for de 2 g sample there was no significant differences ($p > 0.05$) observed between the first and second extraction regarding HT and Tyr concentrations, suggesting that the first one mostly extracted these compounds (**Table 10**).

In conclusion, **Table 11** summarizes the findings in this work bearing in mind the optimized variables that achieve the highest HT yield in the most cost-effective manner.

Table 10. Influence of solid to liquid ratio and extraction cycles in HT and Tyr quantification, maintaining optimized variables (solvent, temperature, extraction time, pasteurization, and particle size).

Extraction cycles	One extraction			Two extractions		
SLR (w/v)	0.5/35	1/35	2/35	0.5/35+35	1/35+35	2/35+35
HT $\text{mg}/100\text{ g}$	515.03 $\pm$	475.11 $\pm$	301.35 $\pm$	537.78 $\pm$	498.73 $\pm$	525.74 $\pm$
	63.16 ^a	3.72 ^a	39.37 ^b	57.69 ^a	14.01 ^a	14.09 ^a
Tyr $\text{mg}/100\text{ g}$	108.99 $\pm$	105.71 $\pm$	64.58 $\pm$	112.38 $\pm$	114.20 $\pm$	112.73 $\pm$
	8.22 ^a	0.18 ^a	7.82 ^b	14.26 ^a	8.57 ^a	2.96 ^a

HT, hydroxytyrosol; Tyr, Tyrosol; SLR, solid to liquid ratio.
Results presented as mean $\pm$ standard deviation in ($n=3$), per 100g of freeze-dried sample.
Different letters in each line represent significant differences ($p < 0.05$).

Table 11. Optimized variables found for HT extraction.

Sample	SLR (w/v)	Particle size	Number of extractions	Solvent	Temperature	Time	Pasteurization
FD-OP	0.5/35	<500µm	1	50% EtOH	25°C	60'	85 °C 15''

SLR, solid to liquid ratio; FD-OP, freeze-dried olive pomace; EtOH, absolute ethanol.

4.3. Total phenolic content and antioxidant activity

In vitro antioxidant activity is one of the most common means for antioxidant potential evaluation of substances as free radical scavengers. In OP extracts, several phenolic compounds describe in **Section 1**, contribute to these properties. For this purpose, two spectrophotometric methods were used since they present different, but complementary mechanisms of action. The prepared extract featuring optimal conditions defined previously (**Table 11**) presented a FRAP of 90.50 ± 1.76 mmol_{FSE}/100 g and a DPPH' scavenging ability of 11.73 ± 0.89 g_{TE} in 100 g OP (dw). Total phenolic content attained 8.78 ± 0.37 g_{GAE}/100 g OP (dw) and HT and Tyr extraction yield reached 738.43 ± 7.48 and 180.88 ± 3.94 mg/100 g (dw), respectively (**Table 12**). HT /100 g presents a higher value when compared to the study of Nunes *et al.* (12) using similar parameters (SLR 1:50, w/v, with 50% ethanol, at 40 °C for 60 minutes), namely 738.43 ± 7.48 versus 238.42 ± 35.90 mg (dw). Although pasteurization process could partially explain these differences, geographical and climatical differences can also impact phenolic content, especially stress conditions, which can favor phenolic increase. **Table 13** represents the TPC, AA and HT and Tyr content /ml of extract.

Table 12. Total phenolic content, antioxidant activity and HT and Tyr quantification from an optimized extract per 100 g OP

TPC (g _{GAE} /100g)	FRAP (mmol _{FSE} /100 g)	DPPH' inhibition (g _{TE} /100 g)	HT (mg/100 g)	Tyr (mg/100 g)
8.78 ± 0.37	90.50 ± 1.76	11.73 ± 0.89	738.43 ± 7.48	180.88 ± 3.94

Results expressed in dry weight, as mean $\pm$ standard deviation (n=3).

Table 13. Total phenolic content, antioxidant activity and HT and Tyr quantification from an optimized extract per ml of extract.

TPC (µg _{GAE} /ml)	FRAP (µmol _{FSE} /ml)	DPPH' inhibition (µg _{TE} /ml)	HT (µg/ml)	Tyr (µg/ml)
1251.06 ± 53.19	12.50 ± 0.25	1671.85 ± 126.35	105.23 ± 1.07	25.78 ± 0.56

Results expressed as mean $\pm$ standard deviation (n=3).

4.4. Extract stability

At an industrial level is important to maintain quality standards that can assure raw materials safety and the preservation of their physical and chemical proprieties over time. In that sense, as production of extracts can occur at different phases than the formulation preparation, stability over time at different storage conditions of the extracts was assessed, both regarding microbiological content and HT and Tyr concentrations.

HPLC analysis showed, as seen in **table 14**, significant differences ($p < 0.05$) in the HT content for the water-based extract stored at 4 °C (A4) after three months storage, suggesting two months as the maximum period for this storage type. For room temperature analysis, the same extract before the concentration step (ERT), was analysed since ethanol has antimicrobial proprieties and an exclusively water-based extract would be prone to microbial contamination at that temperature. HT content diminished in 1 month period, hence this procedure should not be recommended for extract storage. At -20 °C the same water-based extract (A20) maintained its chemical properties for the 3 months period tested, making this the most efficient storage method. Regarding microbiological studies no microbiological growth was observed in all extracts in the time period tested. HT and Tyr content from the extract subjected to a freeze-drying process (FD-E) was also assessed, after reconstitution with deionized water. FD-E had been stored for 3 months at room temperature and protected from light. Similarly to what was observed with the extract stored at -20 °C, there was an increase in HT content after 3 months for the FD-E suggesting that there are chemical transformations of the phenolic content in the extract matrix as time progresses, potentiating HT extraction.

Table 14. Extract stability, for three months, based on HT concentration.

Time/Sample	HT (µg/ml)			
	A4	A20	ERT	FD-E
T0	105.23 ± 1.07 ^{ab}	105.23 ± 1.07 ^a	58.47 ± 3.04 ^a	206.05 ± 10.45 ^a
T1	107.34 ± 2.71 ^a	102.11 ± 4.97 ^a	41.92 ± 0.67 ^b	—
T2	102.24 ± 3.16 ^b	107.98 ± 5.37 ^a	43.27 ± 3.38 ^b	—
T3	63.97 ± 2.00 ^c	129.37 ± 8.00 ^b	42.05 ± 1.18 ^b	299.43 ± 6.40 ^b

HT, hydroxytyrosol; T0, after extract preparation; T1, 1 month storage; T2, 2 months storage; T3, 3 months storage; A4, water-based extract stored at 4 °C; A20, water-based extract stored at -20 °C; ERT, hydroalcoholic extract (1:1, v/v) stored at room temperature; FD-E, freeze-dried extract. Results expressed as mean ± standard deviation ($n=3$). Different letters in each column represent significant differences ($p < 0.05$).

Table 15. Extract stability, for three months, based on Tyr concentration.

Time/Sample	Tyr (µg/ml)			
	A4	A20	ERT	FD-E
T0	25.78 ± 0.40 ^a	25.78 ± 0.40 ^a	11.65 ± 0.08 ^a	75.24 ± 4.75 ^a
T1	35.09 ± 0.54 ^b	30.54 ± 0.37 ^b	15.06 ± 0.18 ^b	—
T2	37.58 ± 1.04 ^b	35.67 ± 0.79 ^c	19.19 ± 0.57 ^c	—
T3	33.80 ± 3.82 ^b	28.99 ± 0.68 ^d	16.98 ± 0.26 ^d	78.23 ± 1.25 ^a

Tyr, Tyrosol; T0, after extract preparation; T1, 1 month storage; T2, 2 months storage; T3, 3 months storage; A4, water-based extract stored at 4 °C; A20, water-based extract stored at -20 °C; ERT, hydroalcoholic extract (1:1, v/v) stored at room temperature; FD-E, freeze-dried extract.

Results expressed as mean ± standard deviation (n=3).

Different letters in each column represent significant differences ($p < 0.05$).

4.5. Microbiological studies

Raw materials, especially those of natural origin, can contribute to a significant level of microbial contamination to the finished product, consequently they should be tested beforehand using adequate methodologies. For FD-OP microbiological analysis three methods were used since they required different volumes. In fact, if facing low-level contamination samples, there is an increased chance of missing the contamination and returning a false negative result when small volumes are used. In that sense, the first step was performing surface spread of 100 µL in medium. As no microbiological growth was detected in both mediums, volume increase to 1 ml by pour plate method. In this case, only 1 CFU from NP-OP samples was counted. To increase the volume number, filtration of 25 and 50ml of NP-OP and P-OP diluted samples (1:100) was performed. Since the dried powder is not completely soluble, a suspension is formed, thereby CFU reading can be difficult as some residues are retained in the membrane. In that sense, a centrifugation step was performed before filtration. It is important to notice, since only the supernatant was filtered through the MCE membrane, the remaining pellet could possibly contain microorganisms adsorbed to this fraction, creating a false result. In fact, as seen in **Figure 16** no microbial growth was found for 25 or 50 ml in both pasteurized and non-pasteurized FD-OP samples. For extracts, only non-pasteurized FD-OP presented microbial growth (1 CFU). A triplicated was performed in TSA medium, presenting a final result of < 1 CFU/ml extract.

For pathogen identification, only Mannitol Salt Agar from FD-OP samples inoculated in peptone buffered water shown microbiological growth as seen in **Figure 17**. To differentiate strains of *S.aureus* from other species, a coagulase test was performed. Since no clot formation was observed, the result was negative, not confirming the presence of this pathogen in both FD-OP samples. Extracts shown no microbiological growth in the selective mediums.

Low contamination can be explained by the different processes OP was subjected to. As freeze-drying lowers water content, microbiological growth is impaired. Regarding extracts, ethanol addition can also mitigate microbiological contamination. Finally, OP has shown antimicrobial effect in different studies which can also account for these results (29).

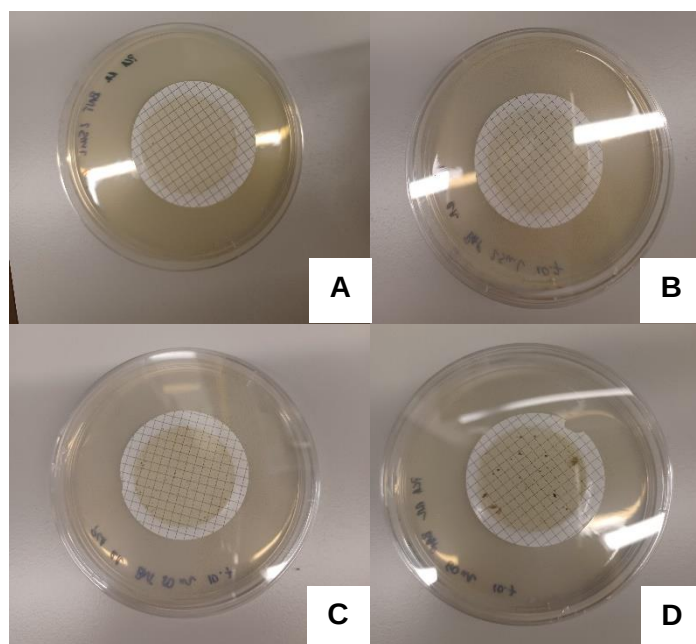


Figure 16. Membrane filtration of 25 ml of NP-OP solution (A), P-OP solution (B) and 50 ml of NP- OP solution (C) and P-OP solution (D) in Plate Count Agar medium.

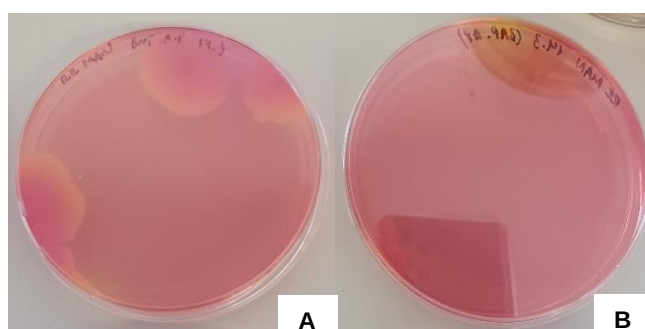


Figure 17. Mannitol Salt Agar medium inoculated with NP-OP (A) and P-OP (B) freeze-dried samples.

4.6. Enzyme inhibition

To date no studies on anti-tyrosinase and anti-elastase activities of OP extracts have been published. In fact, only extracts from olive leaves have been tested for anti-aging activity based on these enzymes. Nunes *et al.* (7) reported that an olive leave extract,

presenting a TPC of 6197 $\mu\text{g}_{\text{GAE}}/\text{ml}$ and an HT concentration of 1352 $\mu\text{g}/\text{ml}$, had an elastase inhibition of $100 \pm 0.1\%$ and $16 \pm 6\%$ for tyrosinase inhibition. Another study revealed that the highest concentration tested (5 mg/ml) of an olive leaves extract from a Portuguese cultivar was capable of inhibit elastase and tyrosinase activities by 82.5% and 50%, respectively. Nevertheless, as sample matrix changes, there can be different predominant phenolics, as well as interactions between components that may lead to different results.

In this study anti-tyrosinase assay provided an inhibition percentage of $26.02 \pm 2.57\%$ at the higher concentration tested (10 mg/ml), clearly inferior to the positive control, kojic acid ($92.06 \pm 0.36\%$). For elastase inhibition only the less concentrated sample (1 mg/ml) manifested enzyme inhibition ($8.68 \pm 1.32\%$). It is expected that the enzyme inhibition percentage increases with increasing extract concentration. However, more concentrated extracts presented colour and, since this assay is based on colorimetric determination, some interference in the absorbance read could be possible. Another explanation could reside in the fact that this extract is a complex mixture of components, not only phenolics, but other substances that can be extracted with the solvents used. In that sense, possible interactions that can exert an inhibitory anti-elastase or samples' absorption effects at the same wavelength of the assay could explain these results.

4.7. *In vitro* cytotoxicity evaluation

The analysis of olive pomace extracts (OPE) by the MTT method revealed that there were no statistically significant differences ($p > 0.05$) between the results obtained from 1 to 500 $\mu\text{g}/\text{ml}$ concentrations and the control group, as shown in **Figure 18**. Results represented are normalized to the control group (culture media without OPE). Its noticeable that only concentrations equal to or greater than 1000 $\mu\text{g}/\text{ml}$ were capable of affecting cell viability. The evidence of cytotoxicity of the extracts at doses above 1000 $\mu\text{g}/\text{mL}$ implies that caution must be taken when using OP extracts, since viability of skin cells may be affected at higher concentrations.

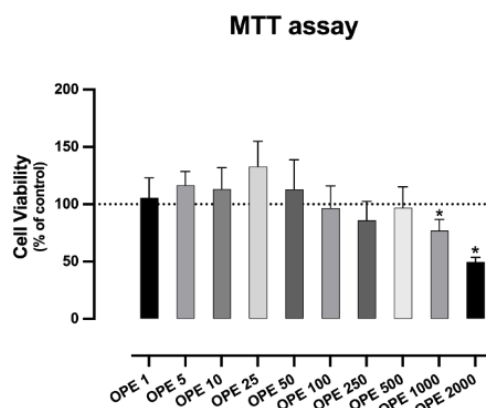


Figure 18. MTT assay in HaCaT cells after exposure to various concentrations of OPE (1 to 2000 µg/mL). Data depicted as mean $\pm$ SEM ($n=7$). * Significant differences $p > 0.05$.

5. Conclusion

As sustainability issues arises, more conscious decisions have to be made by key players in society. Eco-innovation with the selection of raw materials such as agro-food by-products can present an alternative in skincare products, diminishing waste and creating value. In this study we presented a sustainable and low-cost method for HT extraction from OP, since HT biological activity shows cosmetic potential. A pasteurization process was tested and, although no major differences were observed regarding microbiological inhibition, it exerted a remarkable effect in HT extraction yield. An extract produced after sample pasteurization in the optimized conditions (SLR 0.5:35, w/v, 50% ethanol, 25 °C and 60 minutes with FD samples particle size inferior to 500 µm) possess significant TPC content (8.78 ± 0.37 g_{GAE}/100 g) and AA activity using FRAP and DPPH^{*} methodologies (90.50 ± 1.76 mmol_{FSE}/100 g and 11.73 ± 0.89 TE/100 g, respectively). Tyrosinase inhibition was found at 26.02 ± 2.57 % and elastase inhibition presented a value of 8.68 ± 1.32 %, but purified extracts should also be tested since a lesser matrix effect could be experienced and higher values could possibly be obtained. Also, the high concentrations tested for this analysis could have an impact on skin keratinocytes, as MTT assay shows a decrease in cell viability at concentrations equal and higher to 1 mg/ml. In that sense, further testing is needed to ensure maximum efficacy without compromising safety for these extract's applications in cosmetic formulations. Extracts also showed microbiological safety and stability over time in -20 ° C storage, and after freeze-drying, for 3 months. For further studies, extract stability up to one year should be assessed due to olive pomace seasonality. Since olive production occurs 1 to 2 times/year, olive pomace obtention can be conditioned, hence extracts may need to be preserved for longer than 3 months.

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CHAPTER II

MICROENCAPSULATION OF OLIVE POMACE EXTRACTS AND INCORPORATION IN CREAMS

7. Introduction

7.2. A key word for cosmetic development: Sustainability

As consumers awareness over sustainable development grows, environmental and ethical issues are increasingly relevant factors in their purchase behaviour. Sustainable development aims to meet the current needs of the population without compromising future generations in this process, including three interconnected dimensions: economical, environmental and social (1). Therefore, a shift in industries patterns towards more eco-friendly and sustainable procedures tends to align with consumer views and preferences. Cosmetic industry is no exception, as great efforts have been made to rethink their mission and vision by implementing sustainable production and consumption solutions. Although consumers perceive products defined as natural or green as eco-friendlier and safer than synthetic alternatives, those products are not necessarily sustainable (2). Sustainability in the cosmetic industry should consider all possible product life-cycle impacts, from the initial design and raw materials choice, through manufacturing, packaging, distribution, consumer use and disposal (2, 3). Sustainable Life Cycle Thinking (LCT) model for cosmetic products is presented in **Figure 19**. However, it should be noted that a significant part of the environmental impact of a cosmetic product is defined at an initial stage, particularly in the sourcing of raw materials (2). Selection of raw materials should respect their environmental impact, bearing in mind its origin and biodegradability pattern, but also the extraction and/or purification processes used (4). Transforming waste materials into higher value substances (up-cycling) is an increasingly way to address the new eco-consumerism, by promoting sustainable and innovative ingredients from natural matrices with biological actions and providing a solution to waste disposal from different industries (5). Several studies have presented potential applications of agro-industrial and food derived by-products in sustainable cosmetics formulations. However, one of the main challenges in these sustainable alternatives is to achieve comparable quality and efficacy as other more common and studied ingredients. Since some limitations with their use could involve instability and formulation concerns, it is important to further investigate, not only the effectiveness and safety of these materials, but also their stability in the formulation and consumer acceptance.

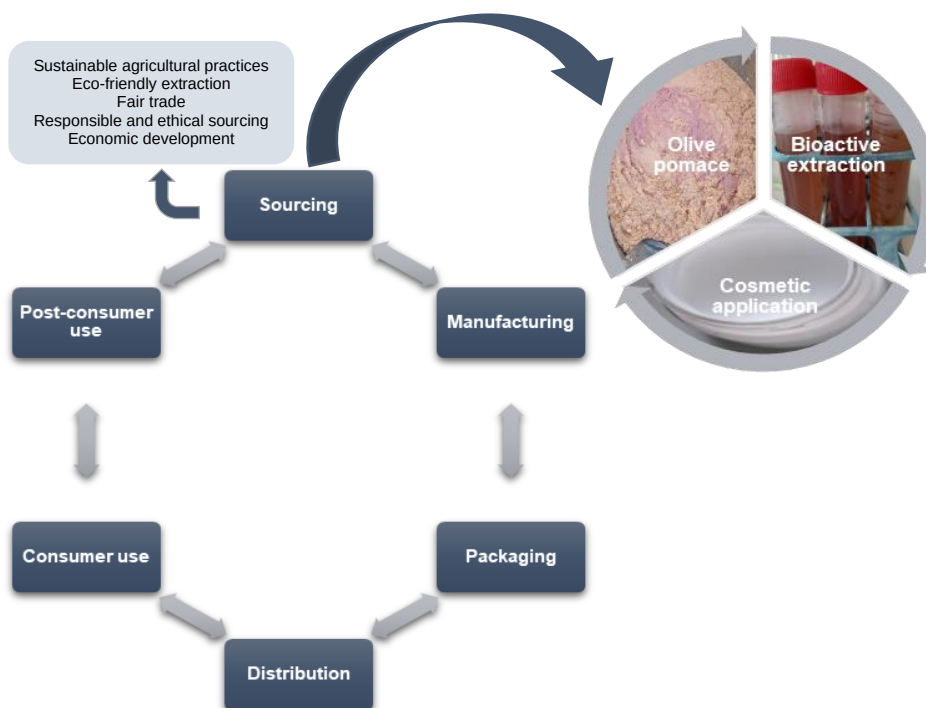


Figure 19. Life cycle assessment in cosmetic products (adapted from Bom et al.(2)) and olive pomace upcycling.

7.3. Microencapsulation of phenolic compounds

Polyphenols have been proposed as effective ingredients for skin care products with anti-aging properties, due to their anti-inflammatory effect, oxidative stress and UV damage prevention and dermal enzymes inhibition (6). However, in order to exert their skin beneficial effects these compounds must be released from the formulation and cross the epidermal barrier in sufficient amount to reach their target. Unfortunately, phenolic compounds can be unstable and present a low bioavailability in the skin (7). Exposure to light, oxygen, temperature and enzymatic activities can limit their efficacy (8). Encapsulation strategies are being constantly developed to face those limitations by increasing shelf-life and keeping their biological activities until and during consumer use. Furthermore, encapsulation technologies in the incorporation of certain active substances can also enhance bioactive skin penetration provide their controlled release and solve potential organoleptic constraints (9, 10).

Microencapsulation is a widely used encapsulation technique, particular for food and pharmaceutical purposes. It is based on the creation of a physical barrier that coats and protects micro-sized substances from the external environment and potentially allowing a controlled release of the bioactive compounds. According to their internal morphology these microparticles can be classified in microcapsules (if the entrapped core is surrounded by layer of the encapsulation agent) or microspheres (when the core is homogeneously

dispersed within the encapsulating material) (11). Although a variety of techniques have been reported for microencapsulation, spray-drying is one of the most commonly used methods for industrial applications. **Figure 20** demonstrates the spray drying process. In this technique an aqueous mixture containing the active ingredient and the encapsulating material is atomised in hot air, producing a final product in powder form, which improves microbiological stability, enhances water solubility and reduces degradation mechanisms (8, 12). Spray-drying has some advantages over other encapsulation methods as it is an economic, reproducible and fast process that allows a large-scale production in a continuous mode with a high encapsulation efficiency and a good stability of the finished product (11, 12). Nevertheless, it presents some limitations namely high energy consumption, unsuitability for thermolabile products, low yields of production due to particle loss on the drying vessel, difficult control of particle size and shape and stickiness of the final product (11, 12). Particle size may vary between 0.2 μm to 5 mm with this technique and particles can display various shapes depending on the encapsulation agent, core material, and process conditions (13). A large number of operative parameters in a spray-dryer can be optimized, which will influence the final powder characteristics, such as inlet temperature, feed flow and aspiration rate (12). The choice of an encapsulating agent and its concentration is also very important for the encapsulation efficiency and microparticles stability (11). Topical delivery of cosmetic active ingredients requires safe, non-toxic and effective means to reach the target destination sites within the skin. As such, the use of natural biodegradable polymeric carriers can potentiate bioavailability of the actives while being, at the same time, easily removed from the body via normal metabolic pathways (14). A large number of substances have been employed for spray-drying such as polysaccharides (for example, chitosan, starch, pectin, arabic gum, sodium alginate, carrageenan, maltodextrins and cyclodextrins) and proteins (such as sodium caseinate, whey protein or soybean protein). When selecting an encapsulation agent, considerations based on the physicochemical properties of the coating material and the entrapped substances, as well as the compatibility between the two, should be made (12). Release can also be affected by the choice of the encapsulation agent. Microparticles produced from spray-drying technique usually present a bioactive release controlled by solvents action and by diffusion. In that sense, mass diffusion through pores or throughout the polymer can be the primary release pattern or differences in osmotic pressure that can favour the bioactive release (11). Other processes include enzymatic cleavage, temperature or pH activated releases (12). Many microcapsules employed in cosmetics can also release their entrapped substances under the action of slight massage when applicated, without leaving any residue (14).

Several methodologies of microparticles assessment have been described in the literature such as scanning electron microscopy (SEM), transmitting electronic microscopy (TEM), Fourier transform infrared (FTIR) spectrophotometry, X-ray diffraction (XRD) and differential scanning calorimetry (DSC) (12). Besides physicochemical characterization of the microparticles, release profiles as well as antioxidant activity and storage stability should be evaluated.

Some examples of OP microencapsulation with spray drying technique can be found. Paini *et al.* (8) encapsulated phenolic compounds extracted from OP, by spray drying, using maltodextrin as coating agent at different concentrations. Variables such as inlet temperature and feed flow were evaluated in this work. Optimized conditions were established at an inlet temperature of 130 °C, maltodextrin concentration of 100 g/L and feed flow of 10 mL/min. Results presented an encapsulation efficiency of 76%, microencapsulation yield of 94%, and phenolic content of 39.5 mg caffeic acid equivalents (CAE)/g dry OP. Stability at different temperatures and light conditions for 70 days was also assessed. A reduction of 21% and 34% of the initial phenolic content was observed for the samples stored at 25 °C and 45 °C, respectively. Light exposure also had an impact in the microparticles phenolic content, as 14 days exposure time decreased its values from 40.0 ± 4.0 mg CAE/g to 33.1 ± 0.8 mg CAE/g dry OP.

Another study by Cepo *et al.* (15) encapsulated phenolic compounds from OP using cyclodextrin as coating agent. Encapsulated particles antioxidant activity was assessed in different biological models and food matrices and compared with synthetic antioxidant butylated hydroxyanisole (BHA). Cyclodextrin encapsulation enhanced the antioxidant activity of OP extracts by significantly increasing their polyphenolic content. Their antioxidant activity in the oil and meat models was comparable to that of BHA when applied at concentration levels of 0.1% and 2–3%, respectively.

In order to determine the optimum conditions for spray drying microencapsulation of an OP extract, Aliakbarian *et al.* (16) tested different maltodextrin concentrations (100–500 g/L), inlet-drying air temperatures (130–160 °C), feed flow rates (5.0–10.0 mL/min), and air flow rates (20–32 m³/h). Microencapsulation yield, moisture content, water solubility, polyphenol content, antioxidant activity and encapsulation efficiency were evaluated. At optimal conditions microencapsulation yield varied between 65–82% and encapsulation efficiency reached 92%. Total phenolic content and antioxidant activity were 38–52 mg CAE/g powder and 230–487 µg TE/g powder, respectively.

Although some of these studies regard incorporation of encapsulated phenolics in food matrices, the same encapsulation principles can be applied in for OP microencapsulation in cosmetic formulations. Microencapsulation can be a possible carrier system for OP, allowing to deliver active ingredients into the skin in a safe and targeted manner, to protect

compounds from oxidation and degradation, and to improve the organoleptic characteristics of cosmetic formulations (17).

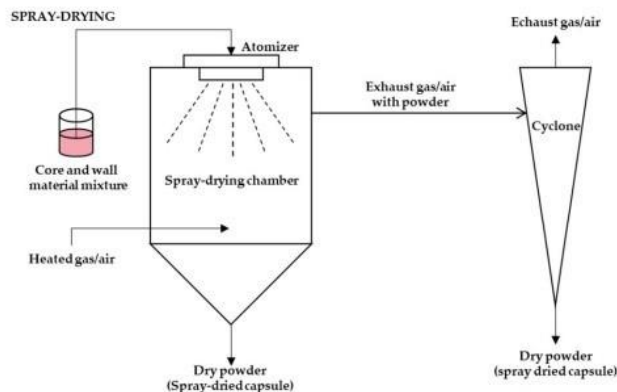


Figure 20. Schematic view of the spray-drying process (from Buljeta *et al.* (18)).

7.4. Quality control in cosmetic products

Formulating cosmetics with natural raw materials can pose technical challenges due to actives instability or interactions between those and the vehicle components, particularly when high concentrations of natural ingredients are used (19). Rheological analyses are very important for product development, optimization and quality control. Rheological studies, obtained in continuous and oscillatory conditions, can provide essential information on the structure and physical stability of the formulations. Moreover, viscosity determinations can assist on the evaluation of the final product in-use behaviour and predict sensory attributes related to the application phase (19).

The long-term stability of a cosmetic formulation is essential to evaluate a product's shelf-life. Stability testing ensures that a cosmetic product meets the intended physical, chemical, and microbiological quality standards, as well as functionality and aesthetics, when stored under appropriate conditions. European Union (UE) regulation for cosmetic and hygiene products (EC 1223/2009 Regulation) defines the need for stability information to be displayed on the product safety but does not specify the requirements to be met. Some recommended guidelines published, such as Cosmetic Europe Guidelines on Stability Testing for Cosmetic Products (20), can assist manufacturers to establish protocols for stability assessment. As a fast-paced and competitive market, cosmetic industries are pressured to launch new products and stability testing protocols can be time-consuming procedures. In that sense, accelerated stability testing can be very helpful to predict shelf-life in a faster

manner. Warmer temperature conditions will accelerate any reactions that may occur under normal shelf-life conditions (21). Parameters to be evaluated might include appearance, pH, viscosity, efficacy and microbial stability (20). Traditional accelerated testing could be accompanied by additional tests such as freeze-thaw (6 cycles of samples placed being between freezing and room temperatures), centrifuging and light/UV exposure, which can be useful for assessing possible transport/distribution impact (21).

Before a new cosmetic ingredient is placed on the EU market (EC 1223/2009 Regulation) safety profile evaluation is mandatory (22). Safety requirements for cosmetic ingredients include skin sensitization, skin/eye irritation, genetic toxicity and photo-toxicity (23). A wide range of *in vitro* models for safety testing of cosmetic products and cosmetic ingredients have been developed and adopted in test guidelines (22).

Cosmetic product claims are subject to a framework of regulation and should be evidence-based and not misleading (24). Methodological approaches to assess cosmetic efficacy comprise *in vitro* or *ex vivo* testing as well as evaluation on human volunteers (25). Instrumental measurements can be performed on subjects in controlled laboratory conditions/environment and include biometric cutaneous parameters evaluation such as skin hydration, roughness, firmness, elasticity among others (26). Sensory testing can be an extremely useful and relevant tool for developing cosmetic products and can be performed either by a trained expert panel, under medical supervision, or by consumer use (25). Contrary to pharmaceutical industries, cosmetics do not require active content evaluation as a routine part of quality control. However, as the cosmetic market evolves it makes sense to adapt and develop suitable assay methods that can assure quality and efficient cosmetics (27).

8. Objectives

The second part of this work aims to promote a new use for OP extracts, comprehending their cosmetic potential. In that sense, OP extracts were incorporated, for the first time, in a cream formulation. An encapsulation process was used to increase extract physical stability over time and was compared to the non-encapsulated extract in the same base formula. The impact of the incorporation of OP-extracts in the cosmetic formulation regarding physicochemical characteristics was evaluated. **Table 16.** summarizes the key objectives in this work.

Table 16 . Chapter II objectives

Objectives

1	To evaluate the influence of OP extracts incorporation in a base cream.
2	To characterize the cosmetic formulations considering organoleptic characteristics, microbiological safety, pH, rheological behaviour, TPC, HT and Tyr content as well as antioxidant activity in accelerated conditions for 3 months.
3	To assess microencapsulation efficiency, using pectin as encapsulation agent, for creams physical and chemical stability over time.
5	To produce cosmetic products based on OP upscaling principles, contributing for a new strategy for OP re-use and assessing the feasibility of this new sustainable ingredient for skincare.

9. Material and Methods

9.1. Sample

Olive pomace was collected in a two-phase olive mill in Alfândega-da-Fé, Portugal, in November 2022. The sample was homogenized, divided in different batches and frozen at -80 °C. Base cream was kindly provided by Mesosystem S.A., São Félix da Marinha, Portugal.

9.2. Standards and reagents

Folin-Ciocalteu's reagent, sodium carbonate, 2,4,6-tri-(2-pyridyl)-S-triazine (TPTZ), ferric chloride, 2,2-diphenyl-1-picrylhydrazyl radical (DPPH[•]), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), gallic acid, heptahydrate ferrous sulphate and sodium acetate were acquired from Sigma-Aldrich (St. Louis, MO, USA). Glacial acetic acid was obtained from Fisher Chemical (Loughborough, England). Sodium carbonate decahydrate was purchased from Merck (Darmstadt, Germany). HPLC grade methanol was acquired from Pancreac (Barcelona, Spain), and absolute ethanol from Honeywell Riedel-de Haën (Seelze, Germany). Hydroxytyrosol (2-(3,4-di-hydroxyphenyl)ethanol) ≥98% from Extrasynthese (Genay, France) and tyrosol (2-(4-hydroxyphenyl)ethanol) 98% from Aldrich (Steinheim, Germany) were used as standards in HPLC analysis. A Milli-Q water purification system (Millipore, Bedford, MA, USA) was used to treat and obtain the ultrapure water. Apple pectin was supplied from LABChem, Portugal. For microbiological studies sodium

chloride was acquired from VWR Chemicals (Alfragide, Portugal). Mediums were obtained from Liofilchem (Roseto degli Abruzzi, Italy). All solutions were sterilized in an autoclave.

9.3. Olive pomace extract preparation

A hydroalcoholic extraction was performed using 14.27 g of FD-OP (particle size < 500 μm) in 500 ml of ethanol:water solution (1:1, v/v) at 25 °C for 60 minutes, with constant stirring. Then, the extract was filtered (Whatman No. 4) under vacuum and ethanol was evaporated using a rotary evaporator (Rotavapor® R-300, BÜCHI Labortechnik, Essen, Germany). 126 ml were used for spray-drying and the remaining extract was frozen at - 80°C and freeze-dried (Telstar Cryodos-80 Terrassa, Barcelona), (**Figure 21**).



Figure 21. Freeze-dried OP extract (FD-OP).

9.4. Preparation of OP-loaded microcapsule and spray-drying conditions

OP-loaded microcapsule was prepared with 10% (w/v) in mass ratio of 1:7 (w/w) of OP extract to pectin. The pectin was first dissolved in ultrapure water and stirred for 2 hours at 55 °C and subsequently cooled down to 25 °C. The OP extract was added to the homogeneous solution of pectin, under stirring conditions, at 25 °C for 1 hour. The mixture of OP extract and pectin was fed to a mini spray-dryer B-290 BÜCHI (Flawil, Switzerland) with a standard nozzle (0.5 mm) to obtain the encapsulated powder (**Figure 22**.) The encapsulation procedure was optimized based on previously reported data with some modifications (28): inlet temperature of 120 °C, aspiration rate of 100%, air flow rate of 3.5 ml/min, and air pressure of 5.5 bar. The outlet temperature was obtained at 57 ± 3 °C. The injection process was performed, under stirring conditions, at room temperature to avoid aggregation and/or precipitations of its solid media. The encapsulated powder was collected in a falcon tube, sealed and protected from light, and stored before further analysis. The product yield (%) was calculated by the amount of the particles recovered from the collector divided by the total mass content of the initial feeding solution (**Eq.2**), (28).

$$\text{Yield (\%)} = \frac{\text{Total weight of encapsulated powder (g)}}{\text{Total weight of feeding solution (mass of OP extract + Pectin)}} \times 100$$

Eq. 2. Spray-drying yield calculation



Figure 22. BÜCHI Mini Spray Dryer (left image); final spray dried powder (right image).

9.5. Microcapsule characterization

9.5.1. Encapsulation Efficiency (EE%) and Loading Capacity (LC%)

The encapsulation efficiency (EE) was calculated by the amount of OP extract successfully entrapped in microcapsule according to content of HT as standard. The EE % is calculated by evaluating the total content of HT (the main compounds of OP extract) subtracted by the non-entrapped content of HT divided by total content of content of HT in OP extract (initially added in preparation steps) and reported in percentage. The LC was calculated based on EE multiple to theoretical content of EE (if 100% of OP-Extract was trapped inside the microcapsule). For that, 10 mg of microencapsulated particles were dissolved in 1 mL of solvent (mixture of ultrapure water and ethanol in a ratio of 30:70, v/v) and vortexed for 30 seconds, followed by centrifugation (1200 rpm, 3 minutes) according to Fathi *et al.* (28). The supernatant media was filtered by a 0.45 µm pore size PTFE filter for HPLC injection. The EE% and LC% was calculated using **Eq.3** and **Eq.4**, respectively (28):

$$EE\% = \frac{HT \text{ total amount} - HT \text{ non entrapped amount}}{HT \text{ total amount}} \times 100$$

Eq. 3. Encapsulation efficiency (%) calculation

$$LC\% = EE \times LC_{theoretical} \times 100$$

Eq. 4. Loading capacity (%) calculation

9.5.2. Particle size and zeta potential

Particle size and distribution, expressed as the polydispersity index (PI), were determined by dynamic light scattering (DLS) using a particle size analyser (Brookhaven Instruments Corporation, operated by particle sizing v.5 Brookhaven instruments software, Holtsville, NY, USA) (**Figure 23**). The encapsulated powder was dispersed in absolute ethanol before analysis. The particle size was characterized by mean size in volume and number, obtained in six runs of 1 min each at 21 °C. The electrical charge surface of the encapsulated powder, expressed as the zeta potential, was obtained with ZetaPALS (Zeta Potential Analyzer, Brookhaven Instruments Corporation, operated by the PALS Zeta Potential Analyzer v.5 Brookhaven Instruments software, Holtsville, NY, USA). The process was run in 6 cycle of 30 s each.



Figure 23. Particle size analyser, Brookhaven Instruments.

9.5.3. Scanning Electron Microscopy (SEM)

The encapsulated particle surface morphology was evaluated by Scanning Electron Microscopy, SEM (Fei Quanta 400 FEG ESEM/EDAX Pegasus X4M). The samples were subjected on carbon stub (double-sided adhesive tap), dried by N₂ and subsequently coated by gold (as electrical conductivity) under vacuum by using Jeol JFC 100 apparatus at Centro

de Materiais da Universidade do Porto (CEMUP). The morphology screening was performed with magnification of 2000, 5000 and 20,000x, for surface structure observation.

9.6. Creams formulation preparation

Non-encapsulated OP powder (NE-OP) and microencapsulated OP powder (ME-OP) at different concentrations (0.5, 1 and 2% for ME-OP creams and 0.07, 0.14 and 0.28% for NE-OP creams) were incorporated in a Meosystem® base cream (formula not shown), using an Unguator® EMP (Gako International GmbH, München, Germany), (**Figure 24**). Mixing conditions were set at level 5 speed for 1:30 minutes. 50 g of each cream were prepared. For comparison, percentages in NE-OP formulations represent the amount of extract presented in ME-OP creams, considering the 1:7 extract: pectin ratio (w/w) (**Table 17**).

Table 17. Concentration of OP-formulations.

% of microencapsulated extract	% of corresponding freeze-dried extract (non-encapsulated)
0.5	0.07
1	0.14
2	0.28



Figure 24. Unguator® EMP, Gako.

9.7. Accelerated stability study

Evaluation of the physicochemical stability was performed according to the method for accelerated stability assessment reported by the ICH guidelines (International Conference on Harmonization, 2003) (29). Each cream was stored for 3 months at 40 ± 2 °C and $75 \pm 5\%$ RH in a climatic chamber (Shjianheng SDJ- 150GSD, Jianheng Instrument Co. Shanghai,

China). Samples were analysed before entering the chamber, post-production time (T0), 1 month after storage (T1) and 3 months after storage (T3). Samples were tested for chemical stability (pH, HT and Tyr content, TPC and AA), organoleptic features (colour, homogeneity), rheological behaviour and microbiological contamination.

9.8. pH measurement

pH values were determined directly, at room temperature, using a pHmeter (PHS-3E, Mesulab Instruments, Guangzhou, China).

9.9. Differential scanning calorimetry (DSC)

Differential scanning calorimetry (DSC) was used to determine the thermodynamic stability of encapsulated particles in powder and cream media. The evaluations were performed by using DSC 200 F3-MAIA Netzsch®, USA (**Figure 25**). The samples were weighted (8.5 - 10 mg) in an aluminium pan and sealed. The system was programmed to heat from 15 °C to 235 °C, keeping 15 °C for 3 min and 235 °C for 2 min, applying a heating rate of 10 K/min and using an empty pan as reference. The analyses were carried out under nitrogen flow of 100 ml/min. The thermographs were analysed using the Netzsch Proteus® Software.



Figure 25. DSC 200, Netzsch®.

9.10. Rheological analysis

The rheological characteristics of the formulations were examined through continuous shear techniques using a Kinexus Rheometer (Malvern Instruments, Worcestershire, UK) and a geometry seen in **Figure 26**. All studies were performed at room temperature (25 °C).

The shear rate method was accomplished using shear rates from 0.1 s^{-1} to 100 s^{-1} . A three-step test was performed to evaluate formulations thixotropy. In this analysis a low shear rate is employed in phase one which is meant to replicate the samples at near rest behaviour. In phase two, a high shear rate is applied to replicate the breakdown of the sample's structure followed by the third stage which results from the samples viscosity recovery as the shear rate is dropped. Conditions set for this analysis were:

- Phase 1, shear rate at 0.1 s^{-1} for 60 seconds.
- Phase 2, shear rate at 100 s^{-1} for 30 seconds.
- Phase 3, shear rate at 0.1 s^{-1} for 5 minutes.

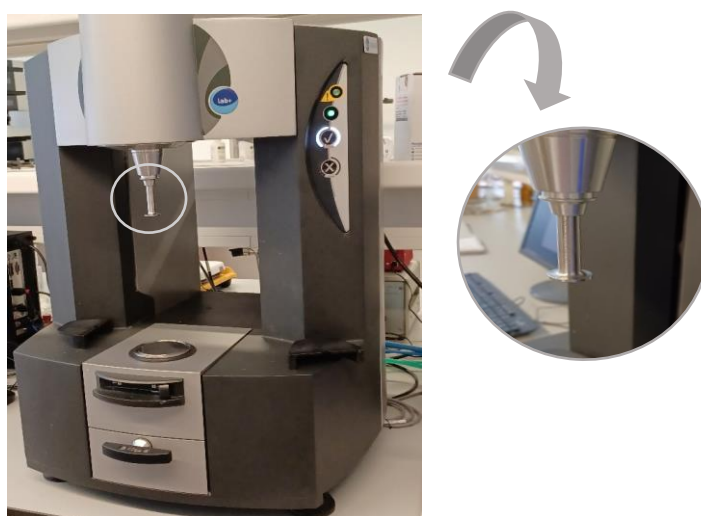


Figure 26. Kinexus Rheometer, Malvern Instruments.

9.11. Determination of color parameters

Colour tests were performed using a CR 400 colorimeter from Konica Minolta (Tokyo, Japan), (**Figure 27**). Each formulation was assessed in the C.I.E. system based on the measurement of three trichromatic components: L^* , a^* and b^* . Each colour was determined through three components: L^* (lightness), a^* (value between red and green) and b^* (value between yellow and blue). Colour changes over time were established by total colour difference (ΔE), according to **Eq.5** (30):

$$\Delta E_{T3/T0} = \sqrt{(L_{T3}^* - L_{T0}^*)^2 + (a_{T3}^* - a_{T0}^*)^2 + (b_{T3}^* - b_{T0}^*)^2}$$

Eq. 5. Total colour difference (ΔE) between T0 and T3 calculation

Where T0 and T3 are the times the samples were tested (post-production and 3 months after storage).

Differences in colour samples relative to the base cream in post-production analysis were calculated according to the same equation with modifications (**Eq.6**) (30):

$$\Delta E_{BC/OPC} = \sqrt{(L_{BC}^* - L_{OPC}^*)^2 + (a_{BC}^* - a_{OPC}^*)^2 + (b_{BC}^* - b_{OPC}^*)^2}$$

Eq. 6. Total colour difference (ΔE) between samples at T0 calculation

Where BC refers to the base cream and OPC are the different olive pomace-based creams.



Figure 27. Colorimeter CR-400, Konica Minolta.

9.12. Microbiological analysis

Each cream formulation was dissolved in saline solution (1:10 w/v) and 100 μ L were spread evenly over the agar surface of two different mediums, Trypticase Soy Agar (TSA) and Sabouraud Dextrose Agar – Chloramphenicol (SDA-C) for mesophilic aerobic bacteria count and yeast and mold count, respectively. Plates were incubated at 37 °C for 24 hours (TSA) and 72 hours (SDA-C). Analysis were made at T0, T1 and T3 and results are expressed as colony-forming units (CFU) per g of sample.

9.13. Extract preparation from creams

For TPC, AA characterization and HT and Tyr content evaluation of the prepared creams, hydroalcoholic extracts from these samples were performed according to same conditions of olive pomace extraction in this chapter (SLR of 1:35, w/v, with 50% ethanol, at 25 °C for 60 minutes). For ME-OP creams, a sample preparation step was performed prior to the extraction process to allow microparticle release. Since pectin dissolves in water 0.50 g of each cream were diluted in 2 ml of deionised water for 48 hours at room

temperature. Then, extraction proceeded with 17,50 ml of the extraction solution previous described. After the extraction, a centrifugation step (10000 rpm for 5 min) was performed, and the supernatant stored at -20°C until analysis. This process was repeated in each time period tested (0, 1 and 3 months).

9.14. Hydroxytyrosol and tyrosol content analysis by HPLC-DAD-FLD

HT and Tyr quantification was performed for extract characterization as well as creams chemical stability assessment. Chromatographic conditions followed the same procedure described in **section 3.7** from **Chapter I**. HPLC analysis of the extracts from cream formulations applied the same conditions used for OP extract with some modifications. Due to the sample's viscosity after extraction, a dilution with the eluent mixture (1:2, v/v) was performed and then the solution was filtered through a 0.22 µm PTFE filter into a 2 ml vial for analysis. Also, an amplification for the detected signal was performed by increasing the fluorescence gain up to 1000 times. Calibration curves were prepared with HT standards ranging from 0.25 to 200 ng/ml ($R^2 = 0.998$).

9.15. Total phenolic content and antioxidant activity

The same procedures described in **sections 3.9.**, **3.10.** and **3.11** from **Chapter I** were used for TPC and AA evaluation for extract characterization. For extract characterization the same linear range was used in all determinations as the extract produced in **Chapter I**.

For creams extracts, TPC and FRAP assay also followed the same methodology, and analysis were made at production time (T0) and after storage at accelerated conditions (T1 and T3). For TPC, calibration curves using gallic acid (5-100 mg/L) were prepared at T0 ($R^2 = 0.9996$), T1 ($R^2 = 0.9995$) and T3 ($R^2 = 0.9995$). Analysis was performed in triplicate and results are presented in mg_{GAE}/50g cream. For FRAP assay, calibration curves using ferrous sulphate (50-600 µmol/L) were prepared at T0 ($R^2 = 0.9996$), T1 ($R^2 = 0.9997$) and T3 ($R^2 = 1$). Analysis was performed in triplicate and results are presented in µmol_{FSE}/50g cream. DPPH• radical scavenging was assessed by % inhibition, according to **Eq. 7**:

$$\% \text{ inhibition} = \frac{Abs_{DPPH} - Abs_{sample}}{Abs_{DPPH}} \times 100$$

Eq. 7. DPPH• radical inhibition calculation

Where Abs refers to the absorbance of the DPPH• radical or cream sample.

9.16. Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics v.25 (IBM Corp., Armonk, 241 NY, USA). Data are expressed as mean $\pm$ standard. The one-way ANOVA was used to evaluate differences between samples, followed by Tukey's HSD post hoc test. The level of significance for all hypothesis tests was 0.05.

10. Results/Discussion

10.1. Extract characterization

Extract evaluation of antioxidant activity, total phenolic and HT and Tyr content is presented in **Tables 18** and **19**. The variables optimized in **Chapter I** were used for this extraction with only a variation in solid to liquid ratio. Here, a 1:35 (w/v) ratio was used instead of the optimized ratio of 0.5:35 (w/v) determined. In fact, similar values as the ones obtained in **Chapter I** are shown in **Table 19** when results are presented per 100 g of OP powder. When comparing results per ml the difference is doubled (**Table 18** versus **Table 13**) as the double mass is used, which results in an increase phenolic content and antioxidant activity/ml in this extract. Results per ml more accurately reflect on the antioxidant efficacy and phenolic content of the creams produced, so this extract was used for creams preparation.

Table 18. Total phenolic content, antioxidant activity and HT and Tyr content expressed per ml of extract.

TPC ($\mu\text{g}_{\text{GAE/ml}}$)	FRAP ($\mu\text{mol}_{\text{FSE/ml}}$)	DPPH' inhibition ($\mu\text{g}_{\text{TE/ml}}$)	HT ($\mu\text{g/ml}$)	Tyr ($\mu\text{g/ml}$)
2641.13 $\pm$ 68.39	26.75 $\pm$ 0.60	3316.30 $\pm$ 180.99	228.02 $\pm$ 1.97	71.92 $\pm$ 4.50

Results expressed as mean $\pm$ standard deviation ($n=3$).

Table 19. Total phenolic content, antioxidant activity and HT and Tyr content expressed per 100g of olive pomace (dw).

TPC ($\text{g}_{\text{GAE/100g}}$)	FRAP $\text{mmol}_{\text{FSE/100g}}$	DPPH' inhibition ($\text{g}_{\text{TE/100 g}}$)	HT (mg/100g)	Tyr (mg/100g)
9.25 $\pm$ 0.24	93.73 $\pm$ 2.11	11.62 $\pm$ 0.63	798.97 $\pm$ 6.91	252.00 $\pm$ 15.75

Results expressed in dry weight, as mean $\pm$ standard deviation ($n=3$).

10.2. Microencapsulation analysis

Preparation of OP-pectin microparticles was performed according to previous experiments. The spray drying parameters used were optimized for pectin encapsulation and the concentration of encapsulation agent was selected based on the sample oil content in order to reduce stickiness and increase spray drying yield.

Pectins are natural anionic, water-soluble linear polysaccharides whose main component is galacturonic acid. Pectin is widely used as an encapsulation agent in microencapsulation techniques, especially for food products. As cosmetic carriers they possess the advantage of being biocompatible, safe and inexpensive, since they can be obtained from wastes from agro-industrial processes (31, 32).

Particle size is one of the fundamental characteristics of a powdered ingredient. It is an important parameter to predict stability, and, for cosmetic use, it impacts on skin feel and delivery. The particle charge determination is also important to predict stability, since highly charged particles will be more stable, since electric repulsion will prevent aggregation (33). The polydispersity index (PI) is a measure of the heterogeneity of a sample based on size. Polydispersity can occur due to size distribution in a sample or agglomeration of the sample during isolation or analysis (34).

Table 20 presents the microparticles characterization for both empty pectin microparticles and the OP encapsulated powder. Particle size was determined in volume and number distribution. For OP-loaded microcapsule particle size was 1420.8 ± 178.0 nm (in volume distribution) showing a significant increase regarding empty microparticles 638.7 ± 152.4 (in volume distribution). Zeta potential decreased from empty microparticles to OP loaded microparticles (from -28.2 ± 1.68 to -10.46 ± 5.28) which can predict some long-term instability. PI values were below 0.2 which reveals homogeneous distribution (33). Particle yield depends on several parameters defined in the spray drying process, such as the feed solution characteristics, inlet, outlet temperature, and pressure (35). The spray drying yield was 53.15%, which may be considered good for a lab scale spray dryer (28). Some loss in product is expected due to difficulties in a complete recovery on the walls of the drying chamber. SEM analysis showed regular, spherical and smooth particles with no fissures implying that the encapsulation powder may be less permeable to gases and therefore oxidation (**Figure 28**). Encapsulated powder presented an EE of 97.01% and LC of 22.38%.

Table 20. Loaded (ME-OP) and unloaded (Pectin) microparticles characterization.

Sample	Yield	Particle size (nm)				ZP (mV)	EE (%)	LC %
		Volume	Number	kcps	PI			
Pectin	54%	638.7 ± 152.4	446.7	359.4	0.005	- 28.2 ± 1.68	_____	_____
ME-OP	53.15%	1420.8 ± 178.0	555.4	466.8	0.005	- 10.46 ± 5.28	97.01	22.12

ME-OP, microencapsulated olive pomace; PI, polydispersity index, ZP, zeta potential, EE, encapsulation efficiency; LC, loading capacity.

Results expressed as mean ± standard deviation (n=6).

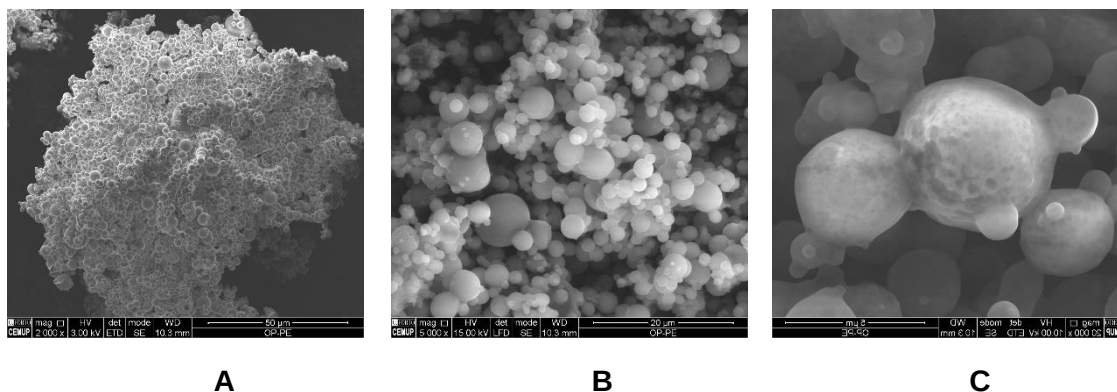


Figure 28. SEM micrographs of OP loaded microparticles. Magnification 2000× (A) 5000× (B) and 20,000× (C). Beam intensity (HV) 3kV (A), 15 kV (B) and 10 kV (C).

10.3. Creams evaluation after production (T0)

10.3.1. Organoleptic features

ME-OP creams show a homogenous appearance with the characteristic perfume of the base cream and a slight pink color that increases with powder concentration. NE-OP creams are very similar to the ME-OP creams except for the color that has a more brownish hue as shown in **Figure 29**.

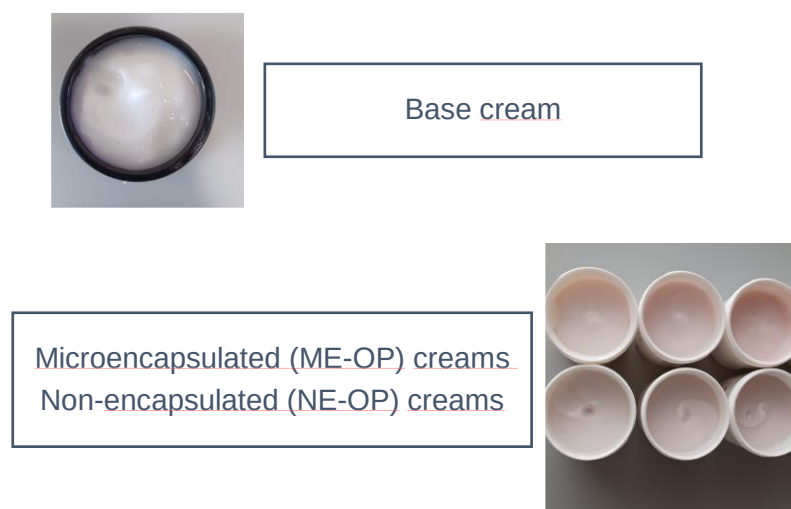


Figure 29. Base cream, ME-OP creams and NE-OP creams at post-production time (T0).

10.3.2. Differential scanning calorimetry (DSC)

Differential scanning calorimetry (DSC) is a thermal analysis technique which measures the difference in energy supplied to the substance and a reference material in function of temperature, both in terms of cooling and heating. Such measurements provide information about physical and chemical changes that include endothermic/exothermic events (36). Thermal analysis can be useful at the pre-formulation stage or in cosmetic quality control to obtain qualitative information on the active substances related to their stability (36).

Figures **30** and **31** show the DSC curves of pectin, spray-dried OP extract (ME-OP), freeze-dried OP extract (FD-E), base cream and ME-OP cream samples. Pectin displays endothermic events at 81.2 °C, 216.2 °C and 222.2 °C. FD-E, as a complex mixture, presents several events around 140-150 °C. For the microencapsulated powder there is a decrease in the 81.2 °C peak corresponding to pectin and no events corresponding to the OP extract suggesting its successful incorporation. All cream samples had a broad endothermic peak near 120 °C and no thermal events similar to those of pectin or FD-E were present, which suggest ME-OP extract dissolved within the cream matrix.

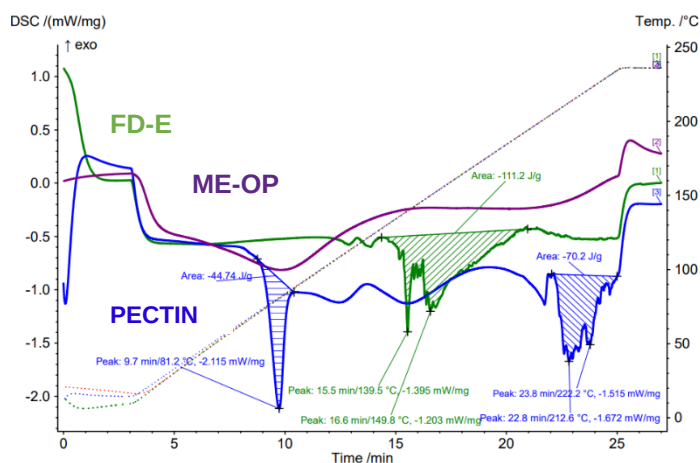


Figure 30. DSC thermograph for microencapsulated extract (ME-OP), freeze-dried extract (FD-E) and pectin.

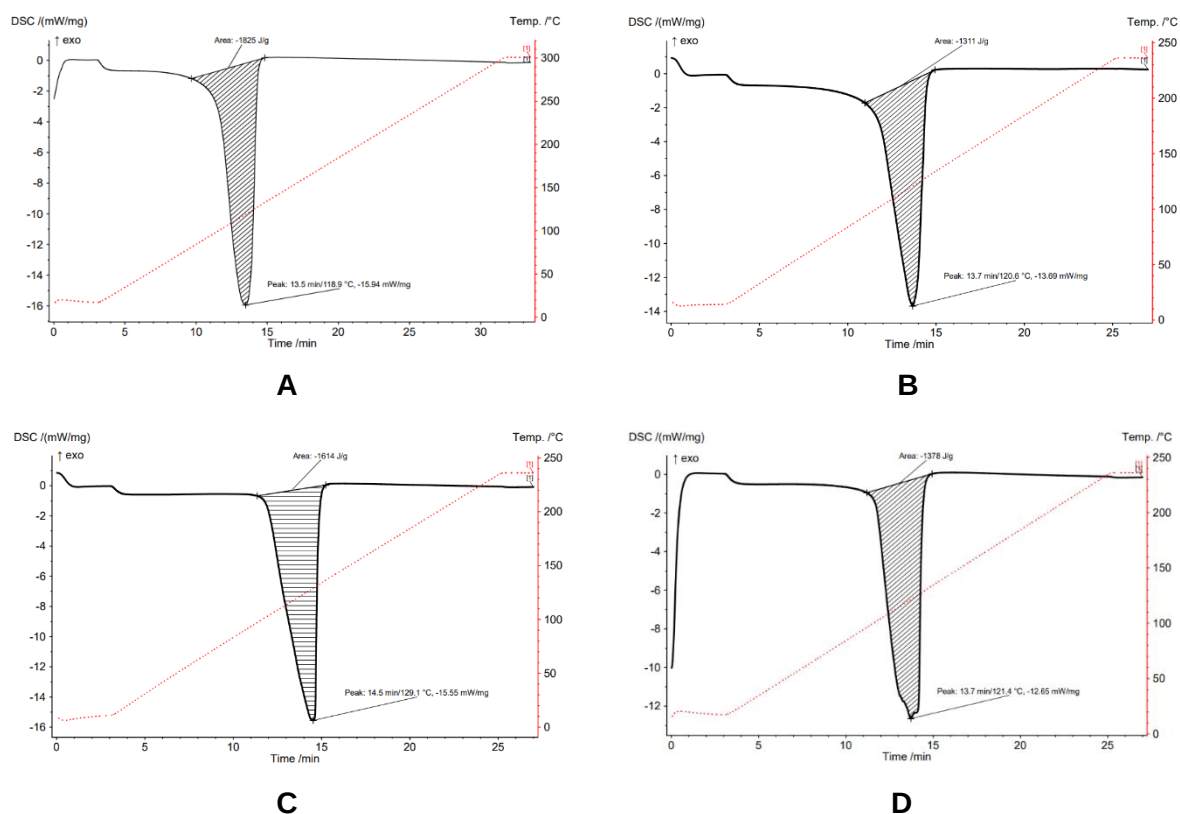


Figure 31. DSC thermographs for Base cream (A), ME-OP 0.5% (B), ME-OP 1% (C), and ME-OP 2% (D) creams.

10.3.3. pH measurement

Results for pH analysis are shown in **Figure 32**. Incorporation of NE-OP lead to a decrease in the formulation pH, when compared to the base cream, presenting a more skin compatible pH, since skin pH is slightly acidic (4.0-4.5). The acidic skin pH is a key factor in its barrier function influencing various factors such as composition of stratum corneum lipids and hydration, as well as the resident microflora (10). However, ME-OP creams presented values below 4 which could present itself as an undesirable condition for their usage. A lower pH can lead to sensitivity and redness with longer period use. This difference in pH from ME-OP creams could possibly be explained by pectin incorporation. As pectin is extracted from apple or citrus fruits in acidified water, typically at pH 1-3, it can possibly have an impact on this substance final pH (31). In fact, when preparing a solution with the pectin used for encapsulation (1:10, w/v in deionized water) it presented a pH of 2.88 ± 0.02 which is consistent with our conclusions regarding ME-OP formulations.

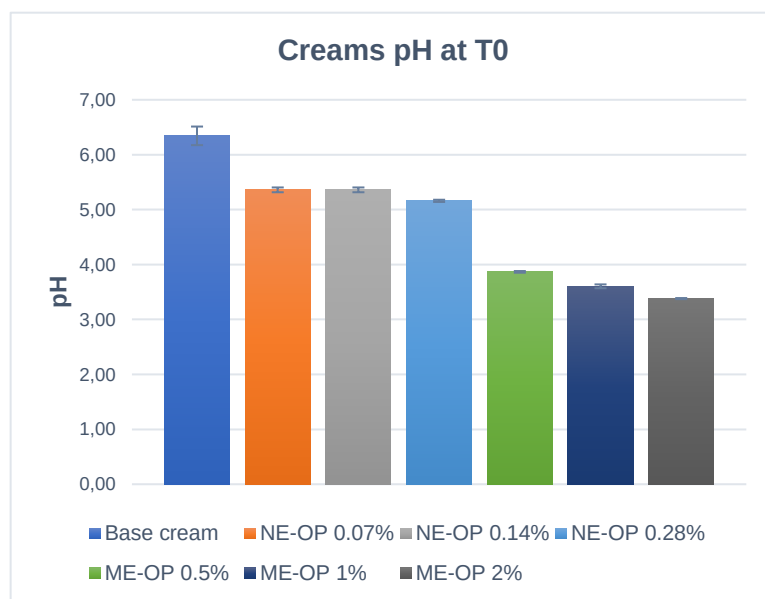


Figure 32. Formulations pH at post-production time (T0).

10.3.4. Phenolic content and antioxidant activity

In order to determine chemical stability of the formulations during storage time, creams' HT and Tyr content, as well as TPC and antioxidant activity, was assessed after preparation (T0). **Table 21.** reports the encountered values for these parameters.

Regarding TPC analysis, no significant changes ($p > 0.05$) were observed between the base cream and the lowest NE-OP concentrations creams (0.07 and 0.14%). The same was observed for ME-OP 0.5% cream. Extract concentrations in these formulations are

considerably low and the method used (Folin-Ciocalteu) has some disadvantages such as lack of selectivity and lower sensibility than other quantification methods (37). In fact, for HT and Tyr content assessment, it was possible to quantify these compounds, using HPLC analysis, in the formulations with the lowest extract concentrations with significant differences between samples ($p < 0.05$).

Similar values were found in FRAP assay for the samples with the same theoretical extract concentrations, except for the ME-OP 2% and NE-OP 0.28% formulations. In fact, ME-OP 2% cream showed significant differences for the majority of the parameters tested attaining the highest HT concentrations ($969.05 \pm 36.61 \mu\text{g}/50\text{g}_{\text{cream}}$) as well as TPC ($15.88 \pm 1.36 (\mu\text{mol}_{\text{FSE}}/50\text{g}_{\text{cream}})$) and antioxidant activity by FRAP analysis ($147.23 \pm 18.26 \text{ mg}_{\text{GAE}}/50\text{g}_{\text{cream}}$). DPPH[•] radical scavenging assay displayed no significant differences between samples, with low inhibition percentages. In fact, this assay revealed some variability and lack of reproducibility, particularly for ME-OP samples, so interferences cannot be discarded.

Table 21. Creams HT and Tyr content, TPC and AA (FRAP and DPPH[•] radical scavenging) at post-production time (T0)

Creams	HT ($\mu\text{g}/50 \text{ g}_{\text{cream}}$)	Tyr ($\mu\text{g}/50 \text{ g}_{\text{cream}}$)	TPC ($\mu\text{mol}_{\text{FSE}}/50 \text{ g}_{\text{cream}}$)	FRAP ($\text{mg}_{\text{GAE}}/50 \text{ g}_{\text{cream}}$)	DPPH [•] (Inhibition %)
Base cream	0 * a	0 *a	5.12 ± 0.95 a	16.54 ± 0.79 a	0.36 ± 0.00 a
NE-OP 0.07%	181.19 ± 38.02 b	92.29^{**}	5.87 ± 1.18 ab	47.78 ± 1.79 b	6.30 ± 0.47 b
NE-OP 0.14%	349.41 ± 6.28 c	110.13^{**}	8.12 ± 0.98 ab	69.42 ± 4.74 bc	8.23 ± 1.00 b
NE-OP 0.28%	710.06 ± 68.38 d	268.12 ± 74.63 c	12.76 ± 2.03 c	$117.71 \pm$ 11.43 d	$12.47 \pm$ 1.00 b
ME-OP 0.5%	370.17 ± 38.80 c	***	6.78 ± 0.54 ab	55.90 ± 7.67 b	$12.05 \pm$ 6.95 b
ME-OP 1%	780.82 ± 78.74 d	122.00 ± 12.60 b	12.48 ± 2.20 c	$87.12 \pm$ 21.45 c	$10.92 \pm$ 2.37 b
ME-OP 2%	969.05 ± 36.61 e	201.57 ± 35.79 bc	15.88 ± 1.36 d	$147.23 \pm$ 18.26 e	9.97 ± 5.49 b

ME-OP, microencapsulated olive pomace; NE-OP, non encapsulated olive pomace.

Results expressed as mean $\pm$ standard deviation ($n=3$, for Tyr and HT and $n=6$ for TPC and AA).

* Not determined (below detection limit)

** $n=1$, since other determinations had low resolution. Not usable for statistical analysis.

*** Below linear range used.

Different letters in each column represents significant differences between samples ($p < 0.05$)

10.3.5. Rheological evaluation

As mentioned previously rheological studies provide an important tool for cosmetic products quality assessment. Viscosity is as important parameter used to evaluate the consistency, thickness, and flow properties of the product. Preferably, a skin cream should have low viscosity at high shear to facilitate application, and high viscosity at low shear so it does not spill out of the container (38).

As presented by **Figure 33** all formulations present a shear-thinning behavior as viscosity progressively decreased under increased shear application. Formulations with these characteristics improve application and spreading, providing a pleasant sensory feeling and skin coverage (39). It is also noticeable that the incorporation of the OP extract diminishes the formulation viscosity. In fact, Nunes *et al.* (40) also reported that, when incorporating another olive by-product extract (olive leaves extracts) in O/W emulsions, it had an impact on viscosity and lead to a less cohesive structure. This effect is more pronounced with the ME-OP cream samples. As the concentration of extract:pectin powder increases, the formulation become less viscous, which can have an impact on the formulation stability. Three step flow analysis reveals that all creams instantly revert to its at-rest viscosity after shear removal, showing no thixotropy, a common behavior for more fluid formulations (**Figure 34**).

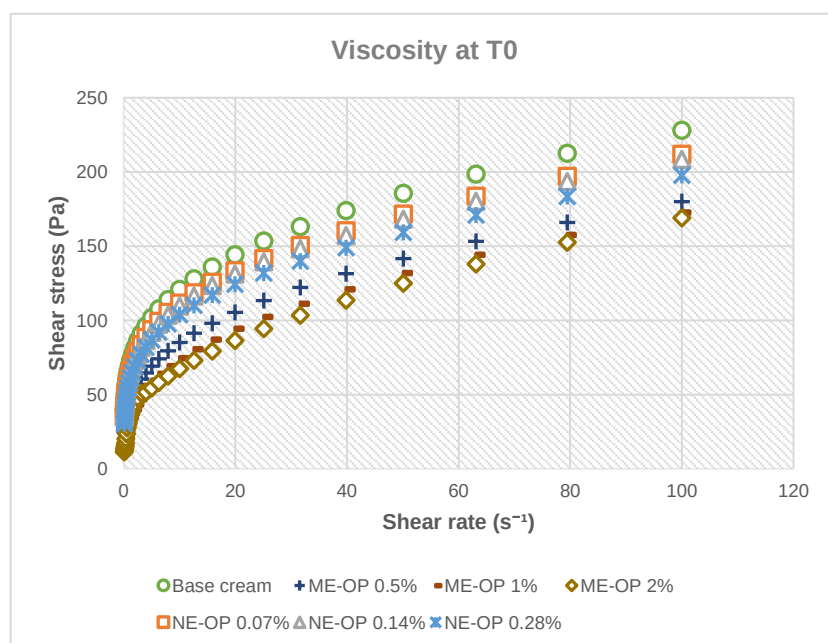


Figure 33. Shear stress/Shear rate curves for Base, NE-OP and ME-OP creams at post-production time (T0).

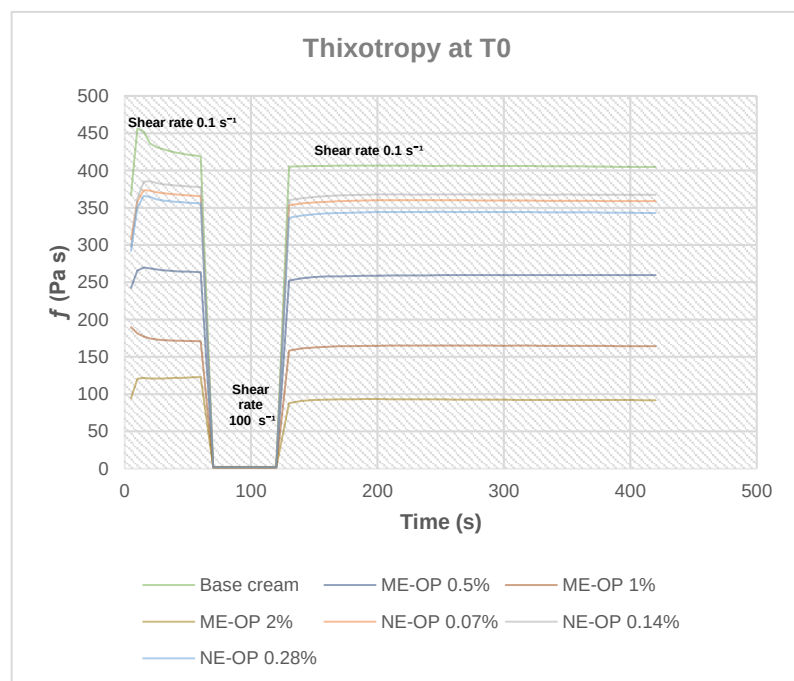


Figure 34. Three step flow analysis for Base, NE-OP and ME-OP creams at post-production time (T0).

10.3.6. Determination of color parameters

Table 22 summarizes the values of colour parameters determined and calculated for the formulated creams. With increasing concentrations of both NE-OP and ME-OP, values of parameter L^* decreased and values of parameter b^* increased. That represents a decrease in samples' lightness and an increase in the intensity of the yellow colour. It is noticeable that the total colour difference (ΔE), when comparing the base cream and the OP extract creams, is higher for the NE-OP formulations than the ME-OP formulations, which is consistent with the colour difference between the FD and ME extract (**Figure 21** and **Figure 22**).

Table 22. Creams colour evaluation at T0.

Creams	L^*	a^*	b^*	$\Delta E_{BC/OPC}$
Base cream	69.39 ± 0.13	-4.86 ± 0.01	0.88 ± 0.01	—
NE-OP 0.07%	60.80 ± 0.11	-4.03 ± 0.02	3.59 ± 0.02	9.04
NE-OP 0.14%	61.68 ± 0.09	-3.90 ± 0.02	5.76 ± 0.06	9.17
NE-OP 0.28%	54.37 ± 0.18	-3.60 ± 0.01	5.60 ± 0.06	15.79
ME-OP 0.5%	68.65 ± 0.02	-4.26 ± 0.01	3.94 ± 0.02	3.20
ME-OP 1%	66.55 ± 0.03	-3.64 ± 0.01	5.60 ± 0.02	5.64
ME-OP 2%	60.58 ± 0.02	-2.42 ± 0.01	7.56 ± 0.06	11.32

ME-OP, microencapsulated olive pomace; NE-OP, non encapsulated olive pomace; $\Delta E_{BC/OPC}$, total colour difference between base cream and olive pomace creams at T0
Results expressed as mean $\pm$ standard deviation ($n=3$).

10.4. Accelerated stability study

In this research, accelerated stability testing was used to examine the shelf-life of the formulations in harsher storage conditions (higher temperature and increased humidity) and in a shorter time period than real time stability. Rheology, pH, microbiological and chemical analysis were performed after 1 month storage and 3 months storage for all samples (Base, NE-OP and ME-OP creams).

10.4.1. Organoleptic features

After 1 month storage all formulations show identical smell and homogeneity to samples at post-production time. It is noticeable that all samples are more fluid except for the ME-OP 2% cream which presents a more jelly like texture. This sample presents the highest concentration of pectin in the formulation and pectin has gelling proprieties. Since water loss is probable with the increase in temperature, this formulation became more consistent. **Figure 35** represents the formulations at T1 and **Figure 36** displays the formulations visual characteristics at T3. After 3 months storage, all samples appear more fluid and ME-OP 2% and NE-OP 0.28% creams present a more intense color. ME-OP samples present some loss of homogeneity with some more translucent zones which can predict lower physical stability for the time period tested. Under high temperature, ME-OP creams may be instable and eventually led to some phase separation.

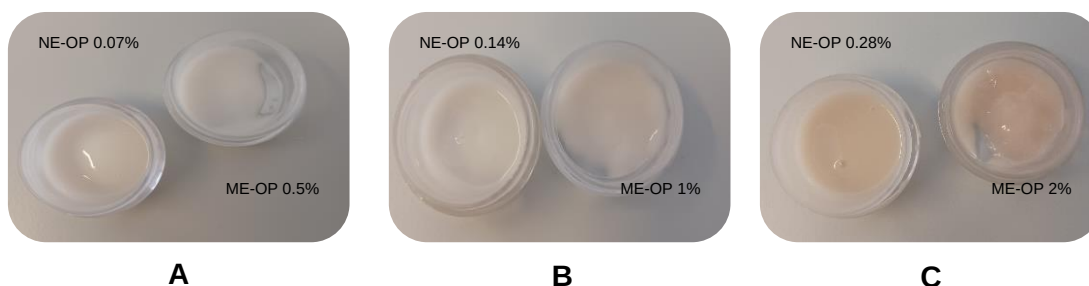


Figure 35. Creams after 1 month storage (T1). NE-OP and ME-OP samples comparison (0.07 and 0.5% (A), 0.14 and 1% (B) and 0.28 and 2% (C).

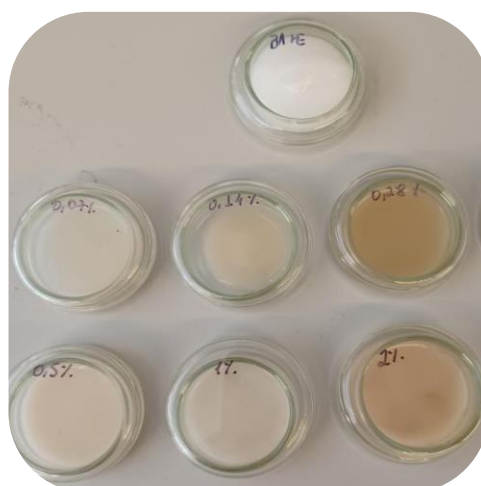


Figure 36. Creams after 3 months storage (T3). Base (1^o row), NE-OP samples (2^o row) and ME-OP samples (3^o row), with increasing concentrations (left to right).

10.4.2. Rheological evaluation

The rheological analysis over time reveals that all samples retained their shear-thinning behaviour with a significant decrease in viscosity after 1 month at 40 °C and 75% RH (**Figure 37.**). This fact is more accentuated for NE-OP samples. Similarly, to the base cream, NE-OP samples show a more pronounced viscosity decrease for T1 but a similar profile for T3 (**Figures 41 to 44**). Steady shear viscosity (at low shear rate, 0.1 s^{-1}) increased for ME-OP 2% formulation at T1 (113.9 Pa.s at T0 and 178.1 Pa.s at T1) which is consistent with visual evaluation. At T3, there is significant viscosity decrease for all ME-OP formulations (**Figure 39**) and more accentuated than NE-OP or base cream as presented in **Table 23**. Flow curves indicate that the deformation of the ME-OP formulations networks required lower shear stresses than those needed to deform NE-OP or base creams which can suggest a more instable formulation as concentration pectin:extract increased. It is also noticeable an increase in thixotropy in ME-OP creams in T1 (**Figure 38**) as structure recovery was not achieved as the shear rate is dropped. This is consistent with visual observation as formulations became more fluid not returning to their more consistent and jelly-like texture at low shear.

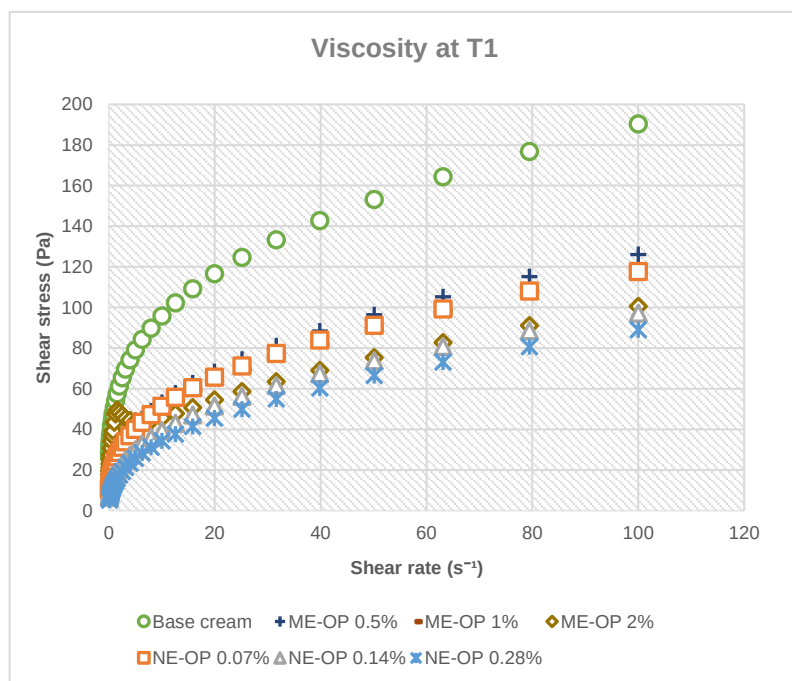


Figure 37. Shear stress/Shear rate curves for Base, NE-OP and ME-OP creams 1 month after storage at 40 °C and 75% RH (T1).

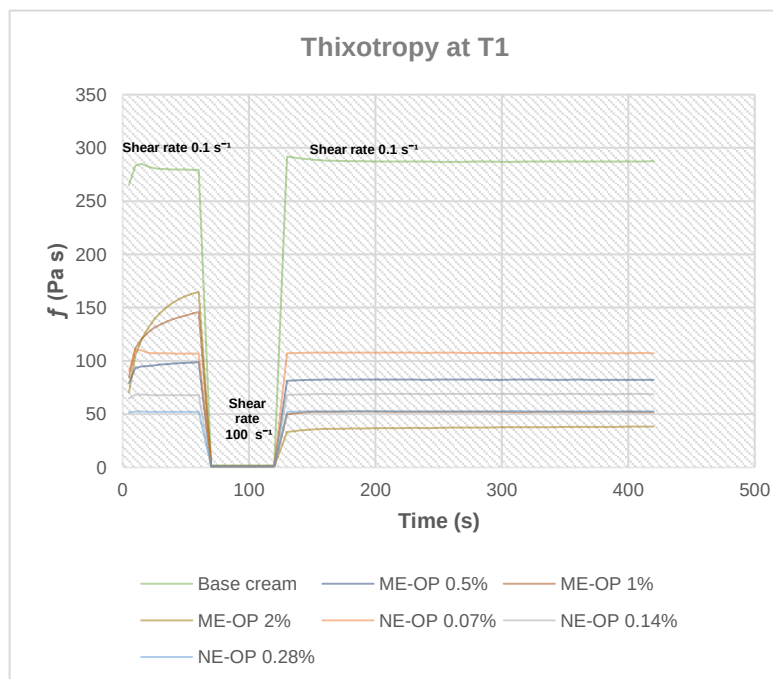


Figure 38. Three step flow analysis for Base, NE-OP and ME-OP creams 1 month after storage at 40 °C and 75% RH (T1).

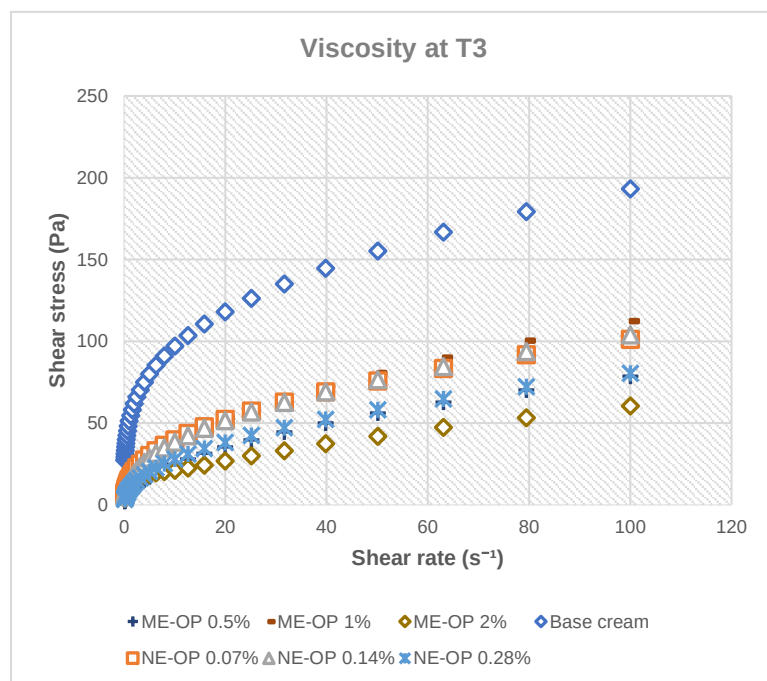


Figure 39. Shear stress/Shear rate curves for Base, NE-OP and ME-OP creams 3 months after storage at 40°C and 75% RH (T3).

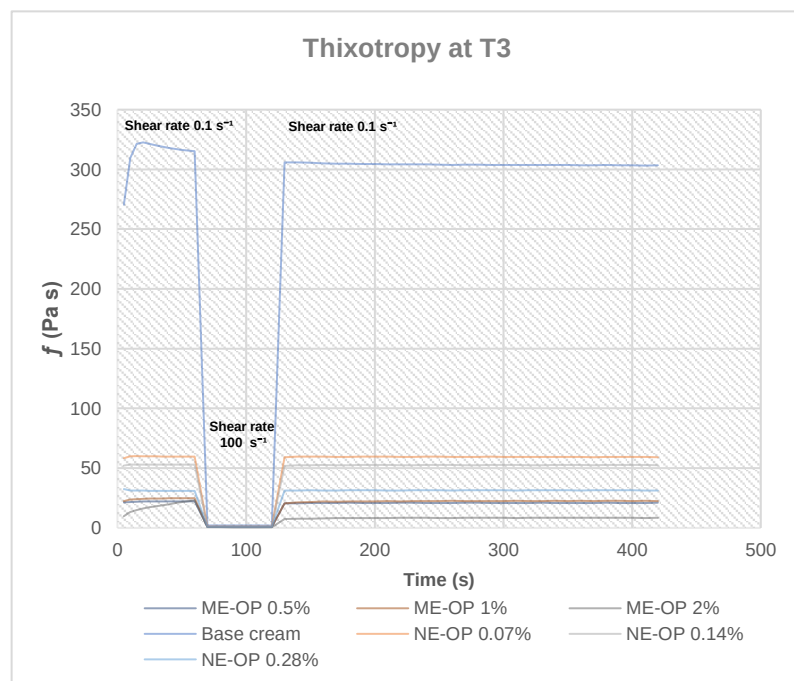


Figure 40. Three step flow analysis for Base, NE-OP and ME-OP creams 3 months after storage at 40°C and 75% RH (T3).

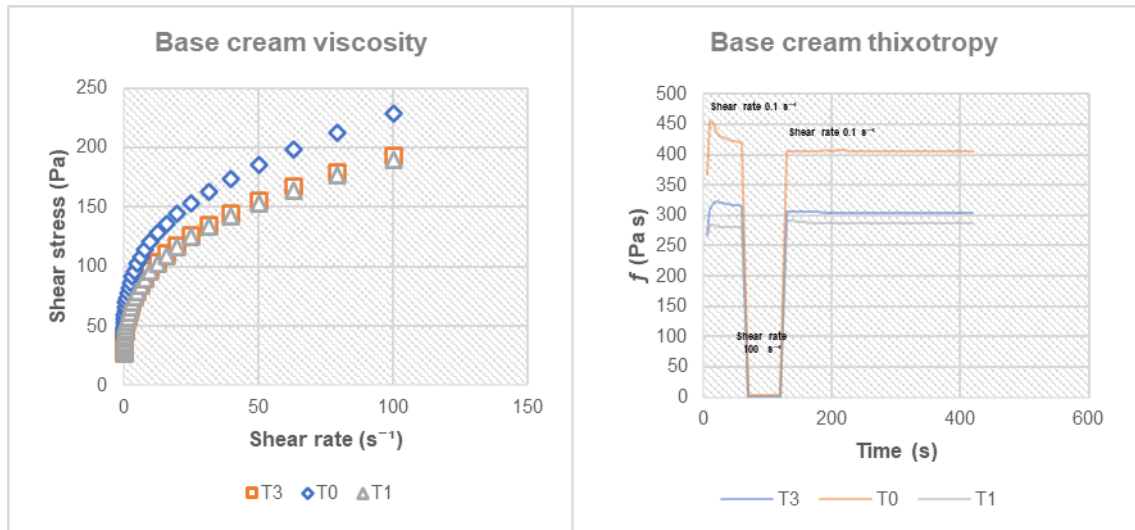


Figure 41. Shear stress/Shear rate curves and three step flow analysis for Base cream at different time periods.

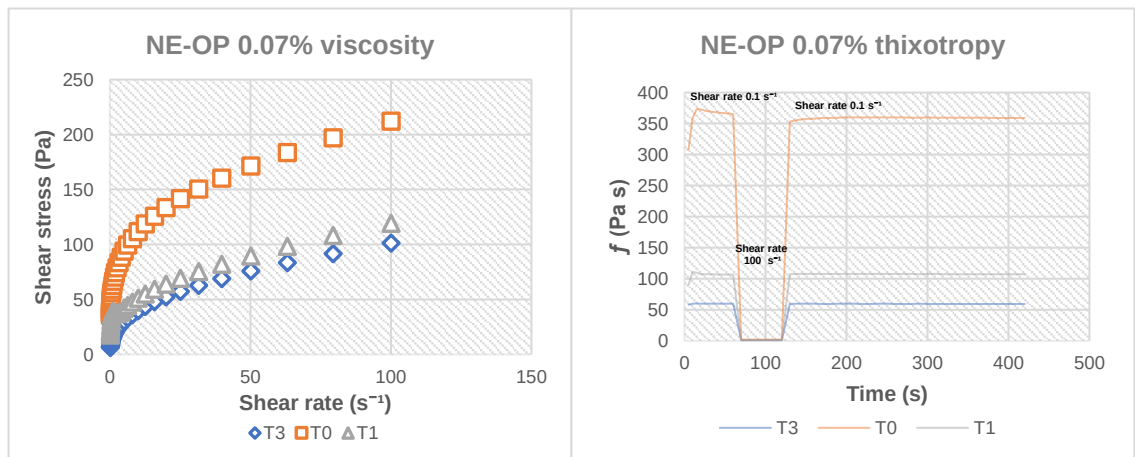


Figure 42. Shear stress/Shear rate curves and three step flow analysis for NE-OP 0.07% cream at different time periods.

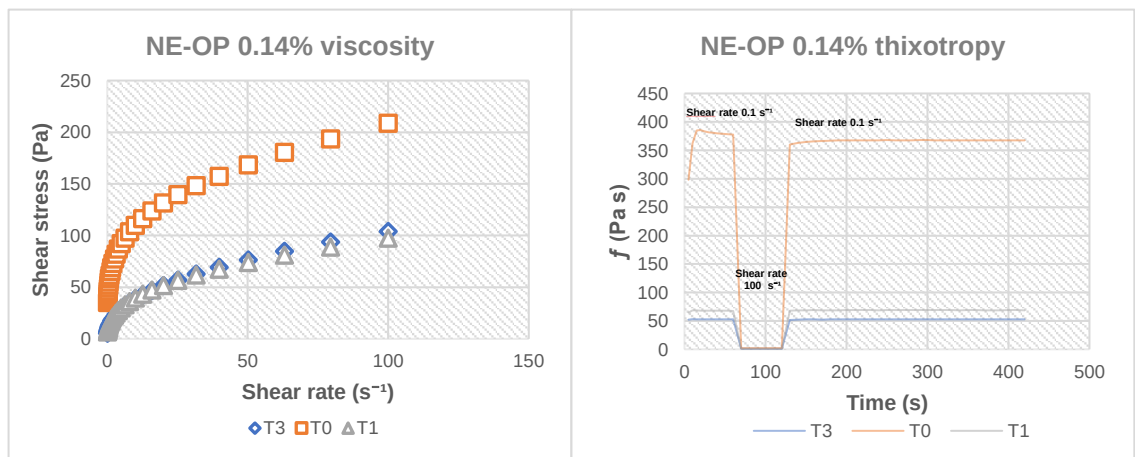


Figure 43. Shear stress/Shear rate curves and three step flow analysis for NE-OP 0.14% cream at different time periods.

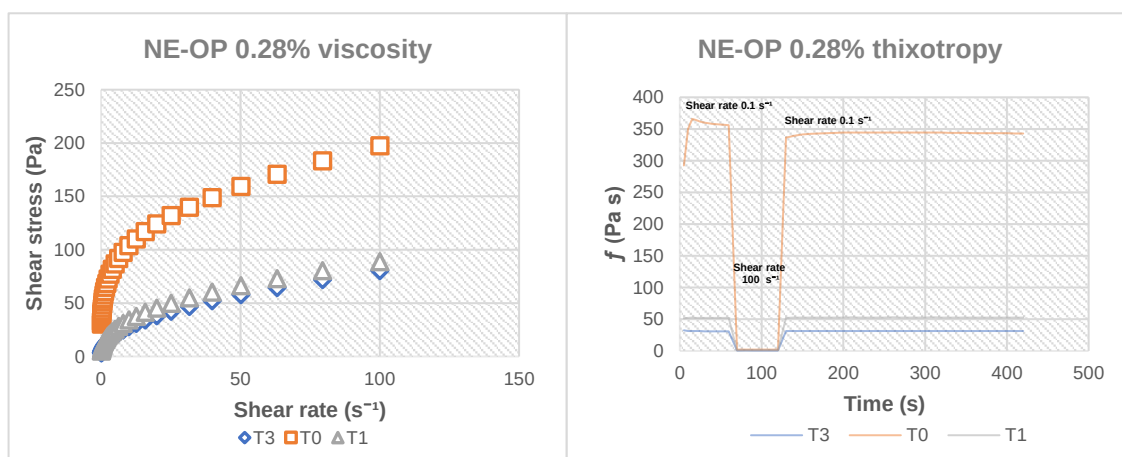


Figure 44. Shear stress/Shear rate curves and three step flow analysis for NE-OP 0.28% cream at different time periods.

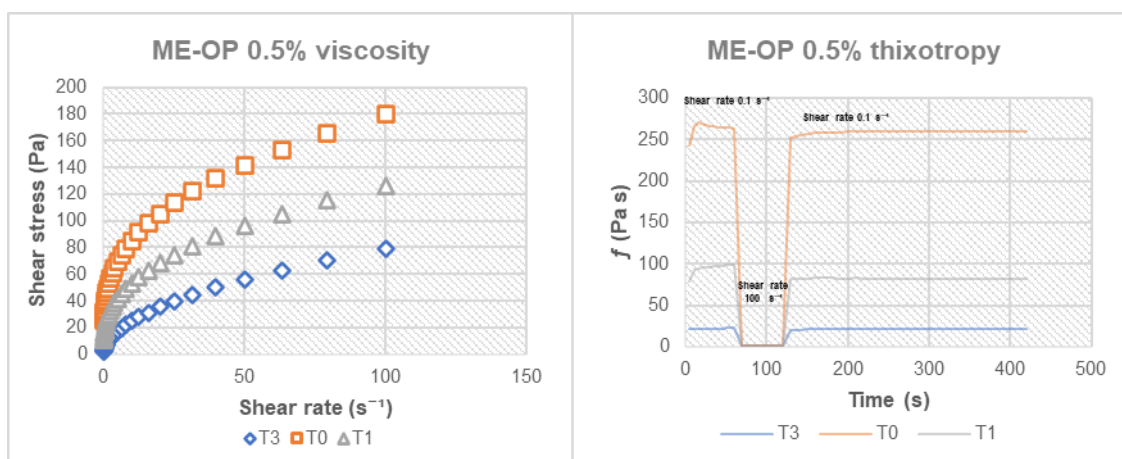


Figure 45. Shear stress/Shear rate curves and three step flow analysis for ME-OP 0.5% cream at different time periods.

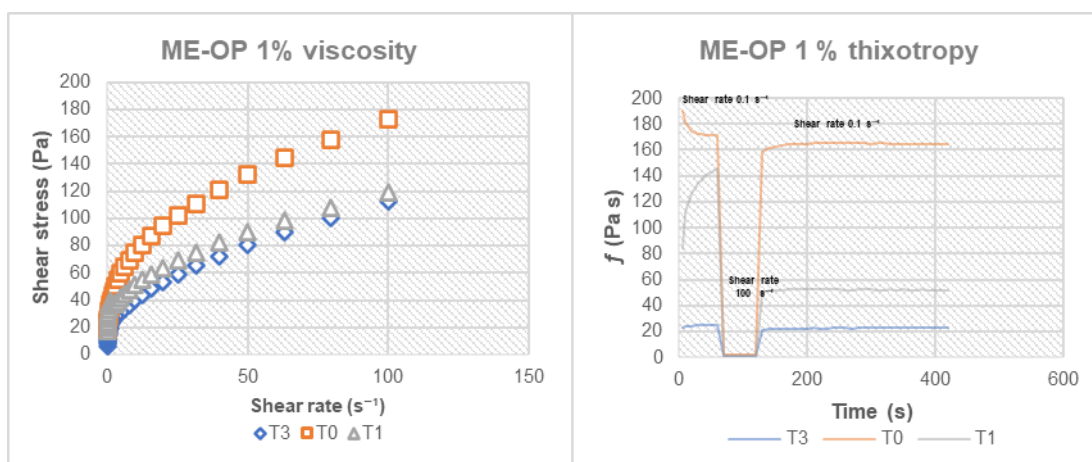


Figure 46. Shear stress/Shear rate curves and three step flow analysis for ME-OP 1% cream at different time periods.

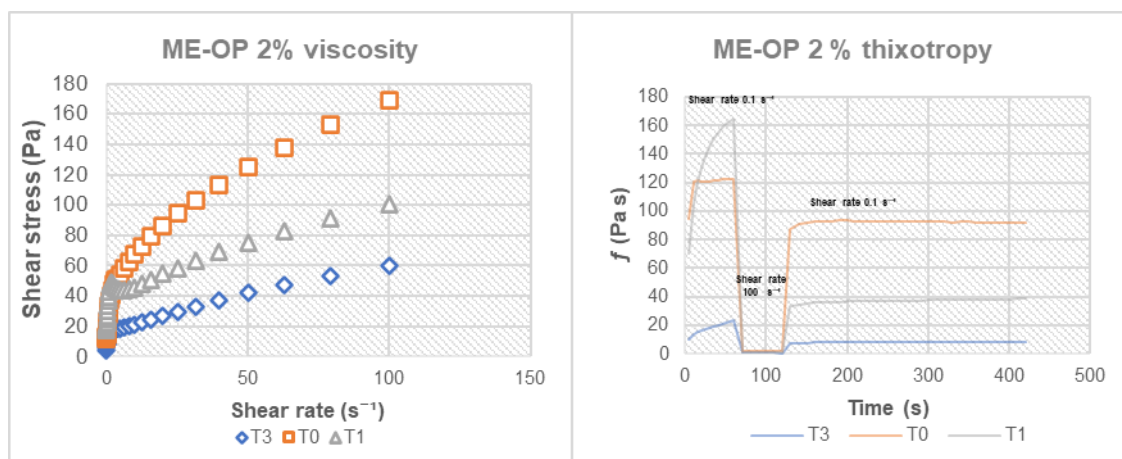


Figure 47. Shear stress/Shear rate curves and three step flow analysis for ME-OP 2% cream at different time periods.

Table 23. Creams viscosity values (f) at the highest shear rate (100 s^{-1}) for the different time periods tested.

Creams/Time	$f \text{ (Pa.s) at } 100 \text{ s}^{-1}$		
	T0	T1	T3
Base cream	2.281	1.903	1.931
NE-OP 0.07%	2.120	1.177	1.011
NE-OP 0.14%	2.084	0.973	1.039
NE-OP 0.28%	1.976	0.890	0.804
ME-OP 0.5%	1.799	1.261	0.785
ME-OP 1%	1.727	1.192	1.122
ME-OP 2%	1.689	1.005	0.603

10.4.3. pH measurement

In general pH values were very similar over the time periods tested (**Figure 48.**). The highest difference was presented by the base cream after one month of accelerated testing (6.34 to 5.23). NE-OP creams appeared to be more stable regarding pH values, and ME-OP creams cream registered a small increase at the end of the accelerated stability study particularly the 0.5% ME-OP concentration, with pH values more compatible with skin (3.87 to 4.55).

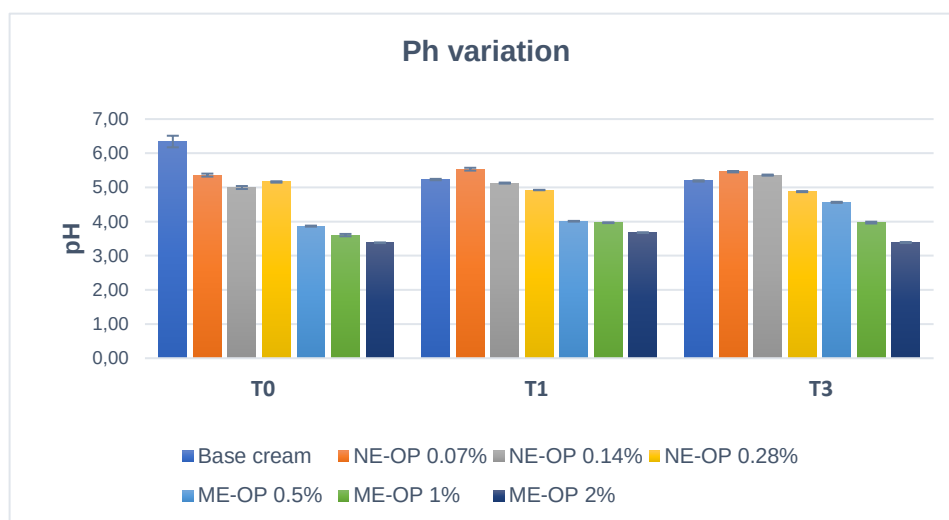


Figure 48. pH evaluation of base, NE and ME-OP creams at different times

10.4.4. Determination of color parameters

As seen in **Table 24**, L^* and b^* parameters increased with extract concentration for both NE and ME-OP formulations. It means that those creams presented a decrease in lightness and an increase in the yellow colour intensity. Extract encapsulation, based on this results, does not seem to have a major impact in color stability, as ΔE values are very similar between samples. However colour variations were higher for NE-OP and ME-OP creams compared to the base cream, after 3 months.

Table 24. Colour variation between Base, NE-OP and ME-OP creams in the 3-month period

Time	Color	Base cream	NE-OP 0.07%	NE-OP 0.14%	NE-OP 0.28%	NE-OP 0.5%	NE-OP 1%	NE-OP 2%
T0	L^*	69.39 ± 0.13	60.80 ± 0.11	61.68 ± 0.09	54.37 ± 0.18	68.65 ± 0.02	66.55 ± 0.03	60.58 ± 0.02
		- 4.86 ± 0.01	- 4.03 ± 0.02	- 3.90 ± 0.02	- 3.60 ± 0.01	- 4.26 ± 0.01	- 3.64 ± 0.01	- 2.42 ± 0.01
	a^*	0.88 ± 0.01	3.59 ± 0.02	5.76 ± 0.06	5.60 ± 0.06	3.94 ± 0.02	5.60 ± 0.02	7.56 ± 0.06
		0.88 ± 0.01	3.59 ± 0.02	5.76 ± 0.06	5.60 ± 0.06	3.94 ± 0.02	5.60 ± 0.02	7.56 ± 0.06
	b^*	69.75 ± 0.01	57.06 ± 0.01	54.86 ± 0.01	51.73 ± 0.00	61.39 ± 0.00	60.29 ± 0.04	56.77 ± 0.02
		- 4.39 ± 0.01	- 3.92 ± 0.01	- 3.68 ± 0.02	- 2.49 ± 0.01	- 3.80 ± 0.01	- 3.17 ± 0.05	- 1.88 ± 0.01
T1	L^*	2.01 ± 0.00	7.82 ± 0.12	10.21 ± 0.01	12.84 ± 0.01	5.23 ± 0.01	7.88 ± 0.04	11.27 ± 0.01
		2.01 ± 0.00	7.82 ± 0.12	10.21 ± 0.01	12.84 ± 0.01	5.23 ± 0.01	7.88 ± 0.04	11.27 ± 0.01
	a^*	- 4.39 ± 0.01	- 3.92 ± 0.01	- 3.68 ± 0.02	- 2.49 ± 0.01	- 3.80 ± 0.01	- 3.17 ± 0.05	- 1.88 ± 0.01
		- 4.39 ± 0.01	- 3.92 ± 0.01	- 3.68 ± 0.02	- 2.49 ± 0.01	- 3.80 ± 0.01	- 3.17 ± 0.05	- 1.88 ± 0.01
	b^*	2.01 ± 0.00	7.82 ± 0.12	10.21 ± 0.01	12.84 ± 0.01	5.23 ± 0.01	7.88 ± 0.04	11.27 ± 0.01
		2.01 ± 0.00	7.82 ± 0.12	10.21 ± 0.01	12.84 ± 0.01	5.23 ± 0.01	7.88 ± 0.04	11.27 ± 0.01

T3	L^*	65.35 ±	50.99 ±	49.37 ±	46.71 ±	57.37 ±	54.98 ±	51.21 ±
		0.03	0.01	0.00	0.01	0.01	0.05	0.18
	a^*	- 4.08 ±	- 3.78 ±	- 3.24 ±	- 3.00 ±	- 3.46 ±	- 4.38 ±	- 3.22 ±
		0.02	0.01	0.01	0.02	0.02	0.20	0.02
	b^*	2.76 ±	8.26 ±	11.70 ±	13.22 ±	10.58 ±	7.51 ±	11.48 ±
		0.01	0.01	0.03	0.01	0.01	0.01	0.29
$\Delta E_{T3/T0}$		4.52	10.87	13.68	10.82	13.11	11.75	10.19

ME-OP, microencapsulated olive pomace; NE-OP, non-encapsulated olive pomace; ΔE , total colour difference between T3 and T0.

Results expressed as mean ± standard deviation ($n=3$).

10.4.5. Phenolic content and antioxidant activity stability

Regarding HT content, significant differences ($p < 0.05$) were observed for NE-OP creams at the lowest concentrations (0.07 and 0.14%) after 3 months at 40 °C and 75% RH, since it was not possible to quantify HT at this phase, suggesting extract degradation after 3 months in these conditions. Regarding ME-OP creams, only ME-OP 1% cream showed a significant decrease ($p < 0.05$) in HT content at T3. For ME-OP 0.5% and ME-OP 2% creams no significant differences ($p > 0.05$) were observed during the time period tested. A possible explanation for this difference between microencapsulated samples could reside in a loss of encapsulation integrity leading to degradation and HT release in ME-OP 1% sample. As a non-entrapped substance, HT became more susceptible to the harsh stability testing conditions. TPC analysis for this sample also presents a decrease in phenolic content which is in line with this observation, since HT it's the primary phenolic compound present in OP. It is also noticeable that ME 0.5% creams present, at T3, a higher standard deviation between the cream extractions. Probably, since ME-OP creams at the end of the stability study presented less homogeneity, different cream samples used for different extracts would, most likely, not be reproducible. Antioxidant activity for all creams did not show significant decreases except for the NE-OP samples at T1. Although it is possible that, since this is a complex extract, other compounds are present that possess antioxidant activity and are more stable in the cream formulas, that can also be the case that the extraction method used for the creams is not reproducible. It is also feasible that degradation of polyphenols generates new polyphenolic compounds that retain antioxidant activity (41). Nevertheless, the cream extraction method used should be optimized and validated, since matrix interferences are possible, leading to dubious results.

Table 25. HT quantification in different formulations in 3 months storage.

HT ($\mu\text{g}/50 \text{ g cream}$)			
Creams/Time	T0	T1	T3
Base cream	0 * a	0* a	0* a
NE-OP 0.07%	181.19 $\pm$ 26.89 a	208.90 $\pm$ 38.16 a	0* b
NE-OP 0.14%	349.41 $\pm$ 4.44 a	409.13 $\pm$ 16.15 b	0* c
NE-OP 0.28%	710.06 $\pm$ 48.35 a	775.74 $\pm$ 31.48 a	569.62 $\pm$ 11.36 b
ME-OP 0.5%	370.17 $\pm$ 27.44 a	287.37 $\pm$ 5.87 a	286.62 $\pm$ 101.79 a
ME-OP 1%	780.82 $\pm$ 55.68 a	648.27 $\pm$ 91.80 a	110.65 $\pm$ 3.56 b
ME-OP 2%	969.05 $\pm$ 25.89 a	1024.19 $\pm$ 72.31 a	992.83 $\pm$ 80.00 a

ME-OP, microencapsulated olive pomace; NE-OP, non-encapsulated olive pomace.

* Not determined (below detection limit).

Results expressed as mean $\pm$ standard deviation ($n=3$).

Different letters in each line represents significant differences between time periods ($p < 0.05$)

Table 26. Tyr quantification in different formulations in 3 months storage.

Tyr ($\mu\text{g}/50 \text{ g cream}$)			
Creams/Time	T0	T1	T3
Base cream	0 * a	0 * a	0 * a
NE-OP 0.07%	92.29 **	107.65 $\pm$ 20.98 a	25.41 $\pm$ 5.41 b
NE-OP 0.14%	110.13 **	244.41 $\pm$ 2.36 a	122.18 $\pm$ 3.16 b
NE-OP 0.28%	268.12 $\pm$ 74.63 a	474.80 $\pm$ 56.33 b	663.26 $\pm$ 11.59 c
ME-OP 0.5%	***	40.52 $\pm$ 4.84 a	105.87 $\pm$ 17.24 b
ME-OP 1%	122.00 $\pm$ 12.60 a	215.09 $\pm$ 32.16 b	42.72 $\pm$ 2.58 c
ME-OP 2%	201.57 $\pm$ 35.79 a	235.81 $\pm$ 61.84 a	477.32 $\pm$ 60.88 b

ME-OP, microencapsulated olive pomace; NE-OP, non-encapsulated olive pomace.

* Not determined (below detection limit).

** $n=1$, since other determinations had low resolution. Not usable for statistical analysis.

*** Below linear range used.

Results expressed as mean $\pm$ standard deviation ($n=3$).

Different letters in each line represents significant differences between time periods ($p < 0.05$).

Table 27. Total phenolic content (TPC) analysis in different formulations in 3 months storage.

TPC ($\text{mg}_{\text{GAE}}/50 \text{ g cream}$)			
Creams/Time	T0	T1	T3
Base cream	5.12 $\pm$ 0.95 a	5.54 $\pm$ 0.37 a	0.84 $\pm$ 0.10 b
NE-OP 0.07%	5.87 $\pm$ 1.18 a	6.03 $\pm$ 0.91 a	2.42 $\pm$ 0.45 b
NE-OP 0.14%	8.12 $\pm$ 0.98 a	7.26 $\pm$ 0.71 a	5.50 $\pm$ 0.47 b
NE-OP 0.28%	12.76 $\pm$ 2.03 a	12.84 $\pm$ 2.17 a	9.77 $\pm$ 1.74 b
ME-OP 0.5%	6.78 $\pm$ 0.54 a	5.47 $\pm$ 0.84 a	10.28 $\pm$ 5.77 a
ME-OP 1%	12.48 $\pm$ 2.20 a	7.96 $\pm$ 0.83 b	8.03 $\pm$ 1.63 b

ME-OP 2% 15.88 ± 1.36^a 20.02 ± 3.49^b 23.85 ± 2.73^b

ME-OP, microencapsulated olive pomace; NE-OP, non-encapsulated olive pomace.

Results expressed as mean $\pm$ standard deviation ($n=6$).

Different letters in each line represents significant differences between time periods ($p < 0.05$).

Table 28. FRAP analysis of different formulations in 3 months storage.

FRAP ($\mu\text{mol}_{\text{FSE}}/50 \text{ g cream}$)			
Creams/Time	T0	T1	T3
Base cream	16.54 ± 0.79^a	10.77 ± 1.20^b	13.13 ± 0.78^b
NE-OP 0.07%	47.78 ± 1.79^a	61.11 ± 5.69^b	37.63 ± 1.43^c
NE-OP 0.14%	69.42 ± 4.74^a	97.68 ± 9.07^b	73.83 ± 5.25^a
NE-OP 0.28%	117.71 ± 11.43^a	158.71 ± 11.00^b	124.09 ± 14.75^a
ME-OP 0.5%	55.90 ± 7.67^a	49.25 ± 4.39^a	106.88 ± 48.92^b
ME-OP 1%	87.12 ± 21.45^a	91.34 ± 12.67^a	71.8 ± 16.21^a
ME-OP 2%	147.23 ± 18.26^a	188.93 ± 29.59^a	155.33 ± 69.99^a

ME-OP, microencapsulated olive pomace; NE-OP, non-encapsulated olive pomace.

Results expressed as mean $\pm$ standard deviation ($n=6$).

Different letters in each line represents significant differences between time periods ($p < 0.05$).

Table 29. DPPH[•] inhibition of different formulations in 3 months storage.

DPPH [•] Inhibition %			
Creams/Time	T0	T1	T3
Base cream	0.36 ± 0.00^a	0 ^{*a}	3.45 ± 0.51^b
NE-OP 0.07%	6.30 ± 0.47^a	8.57 ± 1.60^b	9.62 ± 1.23^b
NE-OP 0.14%	8.23 ± 1.00^a	12.85 ± 3.37^b	11.80 ± 1.53^b
NE-OP 0.28%	12.47 ± 1.00^a	13.39 ± 1.49^{ab}	15.52 ± 0.64^b
ME-OP 0.5%	12.05 ± 6.95^a	9.65 ± 1.96^a	13.47 ± 1.98^a
ME-OP 1%	10.92 ± 2.37^a	10.23 ± 2.20^a	12.33 ± 1.66^a
ME-OP 2%	9.85 ± 6.12^a	14.32 ± 1.24^{ab}	16.45 ± 0.88^b

ME-OP, microencapsulated olive pomace; NE-OP, non encapsulated olive pomace

* No inhibition

Results expressed as mean $\pm$ standard deviation ($n=6$).

Different letters in each line represents significant differences between time periods ($p < 0.05$).

10.4.6. Microbiological analysis

Microbiological stability analysis shown no microbial growth for bacteria, yeast or mold for every sample in each time period tested. Not only OP extracts are low contamination products, as demonstrated in **section 4.5.**, but the formulation also contains a preservative, hence showing its effectiveness regarding microbiological safety.

10.5. Microcapsule stability

Particle size (in volume and number distribution), zeta potential, EE and LC were determined at 3 months after storage at room temperature for both empty and OP-loaded microcapsule to assess their stability over time. An increase in particle size for both empty microparticles and encapsulated OP is noticeable. Zeta potential presents similar values after 3 months. EE% and LC % also suggest particle stability over time, with no considerable variations.

Table 30. Microparticle analysis in post-production time (T0) and 3 months after storage (T3).

Sample	Particle size (nm)				ZP (mV)	EE %	LC %
	Volume	Number	Kcps	PI			
Pectin (T0)	638.7 ± 152.4	446.7	359.4	0.005	- 28.2 ± 1.68	—	—
Pectin (T3)	994.9 ± 82.7	967	393.5	0.005	- 30.64 ± 2.86	—	—
ME-OP (T0)	1420.8 ± 178.0	555.4	466.8	0.005	- 10.46 ± 5.28	97.01	22.12
ME-OP (T3)	2849.3 ± 720.6	861.9	457.7	0.005	- 15.67 ± 1.5	98.13	22.38

ME-OP, microencapsulated olive pomace; PI, polydispersity index, ZP, zeta potential, EE, encapsulation efficiency; LC, loading capacity.
 Results expressed as mean ± standard deviation (n=6).

11. Conclusion

Nowadays, with the increasing trend of natural-based skincare products, there is great potential for the valorisation of agro-industrial by-products. Nevertheless, the design and formulation of novel cosmetic products can be a difficult task based on trial-and-error experimental procedures in order to achieve effective skin actions, stability and sensory appeal. In this work, we aimed to incorporate OP extracts in a base formula and evaluate its stability in accelerated conditions. A microencapsulation process was used to assess if significant differences in phenolic stability could exist. Rheology studies demonstrated that encapsulated (ME-OP) and non-encapsulated (NE-OP) formulations exhibit shear-thinning behaviour, although viscosity decreased over time, particularly for creams with encapsulated extract. As the encapsulating agent increased in the formulations, significant changes in organoleptic qualities, pH, feel and viscosity occurred. NE-OP creams appear to be more

physically stable and closer to base cream behaviour over time, and pH values are more compatible with cutaneous application. It is difficult to judge if microencapsulation diminished phenolic degradation since results are not conclusive. Nevertheless, HT and Tyr quantifications as well as TPC analysis suggest phenolic degradation over time in non-encapsulated samples which is consistent with literature reports of phenolic compounds being prone to environmental degradation. Although there is some evidence of less phenolic degradation (samples ME-OP 0.5 and 2%), pectin inclusion had an impact in the overall cream physicochemical attributes, ultimately leading to some formulation instability. Also, DSC analysis at 3 months period could have been helpful to understand if there was microcapsule degradation. From the ME-OP formulations, the 2% concentration presented the highest HT content and antioxidant activity (FRAP assay), but its suitability for cosmetic application is questionable, as pH values are quite low (below 4) and organoleptic and texture qualities could impair consumer acceptance. Besides, rheological evaluation shows that these formulations can be less cohesive and prone to instability, limiting their shelf-life. ME-0.5% appears to have better qualities regarding physical evaluation, organoleptic features and pH. However, the pectin:extract ratio used (7:1, w/w) diminishes the extract % in this formulation, which can have a negative impact in the effectiveness of the cream. In fact, creams biological activity should also be assessed, in future works, in order to predict effectiveness. In that sense, a different encapsulation method should be assessed that allows for increase % of extract as we concluded that concentrations over 2% of this microencapsulated powder are not feasible.

12. References

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CHAPTER III

FINAL CONCLUSIONS AND FUTURE PERSPECTIVES

13. FINAL CONCLUSIONS AND FUTURE PERSPECTIVES

The sector of cosmetics and personal care products is continuously challenged to develop formulations that include innovative, natural and sustainable bioactive ingredients with substantiated efficacy claims.

Numerous works have characterized different agro-industrial by-products for cosmetic applications, but there was a loophole regarding olive pomace application in skincare, despite various studies regarding its phenolic composition have showed enormous cosmetic potential.

This preliminary study shows that incorporation of olive pomace extracts in creams can provide *in vitro* antioxidant effects. Nevertheless, release studies and efficacy testing in cell cultures as well as biometric cutaneous parameters analysis in human volunteers can more accurately define the positive antioxidant effects in individuals skin health. The olive pomace extracts applied in the base formula used were quite low (not reaching 0.3%), thus their impact in formulation physical stability was not very considerable as rheology results are quite similar to the base cream in accelerated stability testing. However, some chemical changes observed at the end of the stability study in phenolic quantification and antioxidant activity can impair those creams shelf-life and efficacy. In that sense a microencapsulation methodology (spray-drying) was applied. Encapsulated extracts appear to increase phenolic stability, but the encapsulation agent selected (pectin) produced creams with some limitations, as viscosity, texture and feel as well as pH were significantly affected by the increase in pectin concentrations. Since pectin:extract ratio impaired the use of higher extract concentrations, different encapsulation agents should be tested and the encapsulation technique optimized in order to incorporate higher concentrations, bearing in mind efficacy but also possible toxicity. Also release studies should be performed as bioactive possible controlled release would be an asset for skin application.

Regarding this study methodologies, creams bioactive extraction should also be optimized in further works. Creams are extremely complex combinations of ingredients and interferences in the assays used or difficulties in compounds extraction could occur. Although the base cream was chosen for its simplicity, since it possesses few ingredients, the polymeric thickening agent used in this formula impacted the extraction process which difficulted the posterior analysis.