Ricardo Alexandre Antunes Barras

Bachelor in Biochemistry

SuberSkin - plant defensive polymers in wound healing

Dissertation for the obtention of a master's degree in Biochemistry for Health

Supervisors

Prof. Dr. Cristina Silva Pereira, ITQB-NOVA

Dr. Abel Oliva, ITQB-NOVA

Co-supervisor

Dr. Joana Pais, ITQB-NOVA

January 2019

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Jury

President: Prof. Dr. Pedro Matias

Main opponent: Dr. Cláudia Santos

Vogal: Dr. Margarida Archer

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Para a minha família.

Acknowledgments

This work couldn't have been done without the support and care from my supervisors. Cristina Silva Pereira welcomed me into her laboratory and wonderful team – and I am thankful for the encouragement and support I received, not only in terms of scientific guidance, but also to purse this "*engenhocas*" side of mine.

Then again, a relevant part of this work was also performed in the BMD lab under the supervision of Abel Oliva, to whom I am thankful for all the support and guidance provided during this thesis.

Closely supporting me I have had Joana Pais, who ensured that everything was going well while reassuring and encouraging me.

Finally, I would also like to thank Sofia Souza for the brief time we spent together in the beginning of this thesis.

I would like to thank Prof. Teresa Catarino, who was always supportive and kind.

I would like to thank Pedro Lamosa (CERMAX) for the acquisition of NMR spectra; and Cristina Leitão (Small Molecule Analysis Facility) for the glycerol quantification through HPLC-RI.

This work also came with the opportunity to meet some incredible people and make new friends. I would like to thank Diego Hartmann and Patrícia Gonçalves for their help with the establishment of the basic methods of cell culture. I re-met Ana Amendoeira; and met Mafalda Pádua, Patrícia Zoio and Vânia Diogo, who accompanied me at the BMD lab (and somehow could put up with my karaoke sessions while working). Thank you to all of the people at the AEM lab, who were always kind and helpful. Bárbara Sousa, thank you for your time and company as well!

Also, while I resided in that magnificent place that is the AEM lab, an extraordinary agglomeration of people who feel like eating roasted chorizo occurred (except for our *Grã-Mestre*). Thus, the *Confraria do Chouriço* was born! Thank you to all of these amazing people, who have helped me so much throughout this time: Vanessa Correia, Rúben Rodrigues, Carlos Moreira, Artur Bento, Paula Guedes, Ana Tomé, Rita Escórcio, Celso Martins, Tiago Martins. Thank you for the words, the debates, the motivation and all of the good times we spent together.

I was lucky enough to have some friends at ITQB-NOVA, who dated from the start of my Biochemistry course, working on their own thesis projects alongside myself. Cheers, Diogo

“Carregaisen”, Miguel “Bernardase” and Cláudia “Pimpipona”! Thank you also to “Sergei” Torres, Rúben “Vanádio” and Catarina “Ressacas”. A big thank you to Nadine “Nata” Mota, who didn’t allow distance to mess with our friendship!

To all of the friends who were able to put up with me through all these years: André Rodrigues, Ivo Pina, Rute Brito, Rita Guardado, David Leitão, Diana Luís, Pedro Monteiro, Filipe Antunes, Angelina Kotyk, Mário Pequeno.

Por fim, nada disto teria sido possível sem o amor incondicional da minha família. Obrigado aos meus pais, que me massacram com amor, e às minhas irmãs Raquel e Rita e irmão Rafael por serem os melhores do mundo. E obrigado a ti, Catarina, por me apoiares e não me deixares desistir de mim.

Ricardo Barras acknowledges funding through the fellowship (068/BI/2017) granted by Fundação para a Ciência e Tecnologia through grant UID/Multi/04551/2013 (Research unit GREEN-IT "Bioresources for Sustainability").

Resumo

A tese focou-se na otimização do método desenvolvido no laboratório para a extracção de suberina com líquido iónico, usando hexanoato de colínio e decanoato de colínio. Construímos um reator para a extracção de suberina em pequena escala, com o qual se demonstrou que a agitação é fundamental para a eficiência da extracção. A extracção com hexanoato de colínio foi otimizada; a extracção com decanoato de colínio precisa de ser otimizada. Usámos ressonância magnética nuclear (RMN) de estado líquido, *gas chromatography coupled with mass spectrometry* (GC-MS), *high pressure liquid chromatography* (HPLC), *differential scanning calorimetry* (DSC) e *dynamic light scattering* (DLS) para caracterizar a suberina extraída. Conseguimos uma quantidade limitada de suberina com decanoato de colínio, devido à ineficiente recuperação do polímero. Uma das observações mais importantes foi feita recorrendo à técnica de RMN, que permitiu elucidar as várias configurações das ligações éster acil-glicerol presentes na suberina extraída (grau de *cross-linking*). A suberina obtida foi ainda usada para fabricar filmes impermeáveis, e este processo foi otimizado.

Observámos um impacto positivo da suberina na proliferação de queratinócitos humanos. Formulações distintas de suberina, com e sem adjuvantes, mostraram sempre a mesma tendência – um aumento da proliferação celular, mesmo para suberina sem excipientes. Testámos a utilização de polietileno glicol (PEG) para aumentar a dispersão das partículas de suberina. Observou-se um efeito negativo por parte do PEG na proliferação celular. Finalmente, estabelecemos um método para determinar o tempo de fecho para uma ferida artificial. Assim, estabeleceram-se métodos para testar o impacto na proliferação celular de várias formulações de suberina. O passo seguinte focará o estudo do impacto da suberina no tempo de fecho de uma ferida artificial, que esperamos que seja consistente com as nossas observações de aumento da proliferação celular na presença de suberina.

Palavras-chave: suberina, caracterização, hexanoato de colínio, decanoato de colínio, queratinócitos, proliferação

Abstract

A method for the extraction of suberin from cork using two different ionic liquids, cholinium hexanoate and cholinium decanoate, was optimised through the use of a home-built reactor. The use of stirring was essential to enhance the recovery yield of suberin. The extraction of suberin with cholinium hexanoate was fully optimised, whereas that with cholinium decanoate though promising needs further optimisation. Liquid state nuclear magnetic resonance (NMR), gas phase chromatography coupled to mass spectrometry (GC-MS), high pressure liquid chromatography (HPLC), differential scanning calorimetry (DSC), dynamic light scattering (DLS) were used to characterize the extracted suberin. Limited amounts of suberin samples extracted with cholinium decanoate were attained due to low recovery yields. The most remarkable observation was attained using NMR which allowed us to disclose the different configurations of acyl-glycerol ester bonds present in the extracted polymer (*i.e.* its degree of cross linking). The obtained suberin was used to cast waterproof films; this process was optimized.

Suberin was determined to have a positive impact on the proliferation of human keratinocytes. Cellular proliferation in the presence of suberin (comprising distinct formulations) displayed consistently the same trend – *i.e.* increase in cell proliferation. Polyethylene glycol (PEG) was used to reduce the polydispersity index of the suberin particles, since it can stabilise the polymer particles and limit their aggregation. The working-hypothesis is that PEG increases the overall surface contact area of suberin further stimulating keratinocyte proliferation. Instead, we have observed that PEG had a detrimental effect on cell proliferation. Finally, a method to determine the gap-closure rate of an artificial wound has been established. Therefore, all the conditions necessary to evaluate the impact of distinct suberin formulations have been established. The next step will be the analysis of suberin influence in the gap closure rate which we hypothesise to be in line with our previous observations on its ability to increase cell proliferation.

Key-words: suberin, characterization, cholinium hexanoate, cholinium decanoate, keratinocytes, wound-healing

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Abbreviations

[N_{1112OH}][Dec] – Cholinium decanoate

[N_{1112OH}][Hex] – Cholinium hexanoate

2D - Bidimensional

ATR-FTIR – Attenuated Total Reflectance Fourier Transform InfraRed spectroscopy

BCA - Bicinchoninic Acid

BPE – Bovine Pituitary Extract

CK - Cytokeratine

DAG – Di-acyl-glycerol

DLS – Dynamic Light Scattering

DMSO – Dimethyl Sulfoxide

DPBS – Dulbecco's Phosphate Buffered Saline

DSC – Differential Scanning Calorimetry

ECM – Extra-cellular matrix

EDTA – Ethylenediamine tetraacetic acid

EGF – Epidermal Growth Factor

ELISA - Enzyme-Linked Immunosorbent Assay

GC-MS – Gas Chromatography coupled with Mass Spectrometry

HCl – Hydrochloric acid

HEKn – Human Epidermal Keratinocytes from neo-natal foreskin

HPLC – High Performance Liquid Chromatography

HSE – Human Skin Equivalent

HSQC – Heteronuclear Single Quantum Coherence

IL – Interleukin

MAG – Mono-acyl-glycerol

MTT - 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NaOH – Sodium oxide

PDI – Polydispersion Index

PEG – Polyethylene glycol

PVP-I – Iodinated polyvinylpyrrolidone

RMN/NMR – Ressonância Magnética Nuclear / Nuclear Magnetic Resonance

TAG – Tri-acyl-glycerol

TNF-α – Tumor Necrosis Factor alpha

Introduction

Thesis problematic

1. Introduction to the thesis

1.1. The skin. The first barrier against the environment's threats.

Skin, the outermost organ in the human body, performs the role of the organism's barrier against the outer environment [5–8]. It performs many vital functions such as vitamin D synthesis [9,10] and body temperature regulation [10,11]. Importantly, as the main interest of the present thesis, the skin also protects the organism against microbial and pathogenic invasion [5–8]. This organ is the habitat of a diversity of microorganisms – the microbiota of the skin – which may, in some cases, contribute positively to the organism's overall well-being while in some other cases they may play the role of opportunistic pathogens [12]. When the skin is injured, these microorganisms may attempt to colonize the wound. The wound healing cycle can regenerate the skin, but the presence of pathogens may hinder its progression.

The long-term goal of this thesis is to propose a novel material for wound healing, aiming at enhancing the body's regenerative ability while also providing an antimicrobial barrier against potential pathogens, ultimately allowing for a better quality of life.

1.2. The wound healing cycle

When damage to the skin occurs, the organism starts a series of events which typically culminate in the regeneration of the affected skin and its functions. The wound healing process is commonly separated into four phases. These phases are not completely distinct and overlap with each other [13,14]. The four phases are mentioned below:

The haemostasis phase:

It is the immediate step after injury. Also called the coagulation phase, it serves to prevent blood loss and the contamination of the wound site. The blood vessels in the affected area constrict and platelets adhere to each other to close the gap in the walls of the blood vessel. Coagulation occurs to help maintain the platelets at the ruptured blood vessel.

The inflammatory phase:

The wound site, having been exposed to the outer environment, needs to be decontaminated to prevent infection. Transudate comprised of water, salts, nutrients and proteins leaks to the wound site allowing for the migration of specialized immune cells. Neutrophils are the first immune cells to migrate to the wound site, cleansing it of debris and bacteria. Macrophages then accumulate to help the phagocytosis of bacteria and damaged tissue [15].

The haemostasis/inflammatory phases often take 72h to finish [16].

The proliferate phase:

After wound debridement, the proliferative stage of wound healing begins. It is characterized by the increase in cell multiplication rates (fibroblasts, keratinocytes and endothelial cells) and the formation of an extracellular matrix which originates the granulation tissue [17]. This phase also emphasis cell-cell communication through secretion of cytokines and growth factors, such as the transforming growth factor-β family, interleukins and angiogenesis factors, for development of new blood vessels to feed the new cells proliferating at the wound site. This stage may take weeks to finish in a healthy person [17].

The remodelling phase:

Finally, a remodelling stage occurs with the objective of regaining the former tissue's functions and properties.

The balance between cell apoptosis and new cell production; the gradual degradation of profuse extra-cellular matrix and the replacement of immature type III collagen by mature type I collagen – play a crucial role in ensuring the success of this final phase of wound healing.

Out of the four phases described, the remodelling phase is the most prolonged one – taking from some months to years to finish, depending on the extent of the injury. Aberrations may lead to excessive wound healing or, in contrast, to the formation of a chronic wound [18].

1.3. Abnormalities in wound healing

When the wound healing stages are compromised, either due to immune deficiencies, disease or even due to persistent infection of the wound site, a chronic wound may develop. An acute wound is the typical wound which progresses through all the wound healing stages and starts the remodelling phase up to three months after the occurrence of the injury. When the wound fails to progress through the healing stages for more than three months, it is considered a chronic wound [19]. Chronic wounds are unable to progress through the wound healing stages – in fact, a chronic wound may exhibit all stages occurring at once, in different locations, in a disorderly manner, contributing to the mingling of destructive and constructive phenomena and continuously hindering healing [20–22]. Most chronic wounds may be categorized as one of three major types: diabetic ulcers, pressure ulcers and venous ulcers [23].

It may be of importance to note that a chronic wound is not necessarily a non-healing wound, but that it may progress into becoming one. The emphasis on this consideration is necessary because chronic wounds still have the window of opportunity to be managed and healed [19]. Non-healing wounds, as the name suggests, do not progress towards regeneration.

Different factors may lead to the formation of a chronic wound. As the organism ages, it also becomes duller in its ability to cope with external factors and this is also reflected in the wound healing cycle [24,25]. Population aging is currently a problem in most developed countries. Severely aged (or diseased) patients may also suffer from pressure ulcers (also known as bed sores) due to prolonged immobilization [26].

Another factor pertinent to wound healing is the health of the immune system. Immunocompromised patients are hindered in their ability to deal with infection and, therefore, wound contamination. This does not only represent a risk in terms of wound healing: the wound may become the gateway to systemic infection if left unattended.

Diabetes is also a risk factor for chronic wound formation. Data shows that 15% of all diabetic patients will be affected by diabetic foot ulcers, with reoccurrence being commonly higher than 70% within 5 years. Diabetes causes neuropathy and arterial damage, affecting most of the organism [27–29]. Diabetic ulcers display asynchronous wound healing stage progression: the same wound site displays different areas at different stages of the wound healing cycle. The wound enters stasis due to its inability to progress towards regeneration; the condition deteriorates, often leading to limb amputation [20,21,30]. Arterial damage caused by diabetes hinders blood circulation, leading to ischemia and hypoxia. The lowered levels of oxygen also have an impact on the progression through the wound healing cycle: oxygen is used not only to sustain the tissue but also to produce radicals which are used to cleanse the wound site of bacteria [31].

1.4. Core problematic

With obesity (a risk factor for diabetes) and diabetes currently being considered epidemics [32], as well as population aging, chronic wounds are expected to become even more prevalent in the future. In 2015, 13.3% of the Portuguese population suffered from diabetes (with a slightly higher prevalence in men). From 40-59 years of age, diabetes affected 12.7% of the population, increasing to 27% from 60 years onwards. The costs related to diabetic patients in Portugal signified 1% of the gross domestic product and 12% of expenses with health care. Globally, the trend observed is an increase in the cases of diabetes, reaching circa 650M in 2040 [33].

To tackle this problem, investment in wound healing treatment was increased [34]. Methods for wound management and new materials to aid wound healing were developed. Unfortunately, not all chronic wounds are alike and therefore there is not one solution that fits all cases. Currently, several products are available in the market, designed specifically to aid

wound healing: in this thesis, Septil® was used as a positive control in the preliminary formulation screening. Septil® is constituted by iodinated polyvinylpyrrolidone (PVP-I) and is a topical antiseptic [35,36].

Many new available materials seek to meet various criteria [37], such as keeping the wound site moist and actively aiding in wound debridement, and functionalization in order to enhance the natural wound healing processes. In this thesis, suberin is proposed as a novel material with potential for wound healing: it is a natural polyester, obtainable through green reactions [38] and from abundant sources. Previous studies have shown that suberin displays antimicrobial properties [39] and has the ability to be cast as films when extracted with a mild depolymerization method [40]. The subject of this thesis is to understand the potential of suberin as a wound healing agent, with emphasis on the production and characterisation of the material, and the definition of a suitable methodological approach for testing its wound healing capacity.

Chapter 1

Suberin extraction and characterization

2. Introduction

2.1. Materials for wound healing

The need for new solutions in the field of wound healing led to the development of more than 3000 products designed for the treatment of different kinds of wounds [34]. These materials aim at different aspects of the wound healing cycle to enhance the body's regenerative ability [34].

Dressings have been used since antiquity to promote wound healing. Examples of antique dressings are crushed plants (which could be mixed with clay or other materials), cloths soaked in oil and honey. Hippocrates (460-370 BC) has been mentioned to used wine and/or vinegar for wound debridement and treatment. Modern forms of wound dressings started to spread in the 20th century [41]. The first modern wound dressings were designed to keep the wound site dry and protected from microbial contamination [42], but advances in wound care eventually led to the realization that providing an adequately moist environment is preferable [43]. The maintenance of exudate is also important (e.g. through usage of a hydrophilic dressing capable of absorbing part of the exudate), because it allows for the migration of immune cells (e.g. neutrophils) and secretion of cytokines and growth factors [34].

Nowadays, modern wound dressings and materials are designed to enhance wound healing, not simply cover the wound. Many dressings are based on synthetic polymers and are categorized as passive, interactive or bioactive. Passive dressings cover the wound to isolate it and avoid microbial contamination. Interactive dressings may be semi-occlusive or occlusive and actively discourage microbial infection [44–47]. Bioactive dressings are derived from natural tissue and may be enriched in factors that typically promote wound healing (e.g. hyaluronic acid, collagen, chitosan) [48–55]. Their broad utilisation in the clinical set-up is largely hindered by the high cost of these materials and the requirement of a specialised management of the wound.

Briefly, modern available dressings are:

a) Semi-permeable films, composed of transparent and adherent polyurethane that allows gas exchange and is impermeable to bacteria [56];
b) Semi-permeable foams, constituted by hydrophilic and hydrophobic foams, that allow for gas exchange and absorption of wound exudate [57,58];
c) Hydrogels, insoluble hydrophilic materials made from synthetic polymers. Hydrogels have been reported as being adequate for all phases of wound healing, except for infected and heavily draining wounds [58];
d) Hydrocolloids, composed of an inner colloidal layer and an outer water-impermeable layer. Hydrocolloids are some of the most frequently used

dressings. Permeable to water vapor and impermeable to bacteria, they also absorb wound exudate and aid in debridement of the wound site [42,59];

e) Alginate dressings, derived from seaweed, are constituted by mannuronic and guluronic acid units that form a highly hydrophilic gel which limits exudation and minimizes bacterial contamination. They are reported to accelerate wound healing due to activating macrophages that produce tumour necrosis factor alpha (TNF-α), which initiates inflammatory signals [60];

f) Tissue-engineered skin substitutes. Human skin equivalents (HSE) mimic the skin and are composed of keratinocytes and fibroblasts on a collagen matrix. HSE secrete growth factors which encourage wound healing [34];

g) Medicated dressings. These dressings incorporate drugs which serve as antimicrobial agents, growth factors or debridement agents. Silver and iodine are commonly used as antimicrobial agents, while enzymes such as collagenase and papain are used to digest necrotic tissue;

h) Composites. These dressings are formed by distinct layers with distinct functions. The outermost layer protects the wound from microbial contamination, while the middle layer absorbs exudate and the innermost layer contacts with the wound site. It is designed to ease removal and avoid damaging newly formed tissue. These are also the most expensive dressings available.

2.2. Wound dressing ideal characteristics

Dressings should be selected considering the wound type. The dressing should promote tissue regeneration: provide an adequately moist environment, potentiate cellular migration, enhance angiogenesis and formation of connective tissue, allow gas exchange between the wound and the environment, be impermeable to microbial pathogens, maintain proper temperature, be non-adherent and easy to remove, sterile, non-toxic and non-allergenic [34,37]. It is also important that the wound dressing is cost-effective.

2.3. The polymer suberin

Suberin is a structural polyester found in higher plants (*e.g.* oak, specifically in cork), which is an insoluble polymer prior to extraction and extremely resistant to chemical and enzymatic approaches. It is abundant in the outer bark of *Quercus suber* (Figure 1), accounting for up to *ca.* 50% of its weight [61–65]. Suberin is a lamellar polyester with a supramolecular organization in the cell wall [66] that lines the internal and peripheral dermal tissues of the plant during secondary cell wall differentiation or as a response to damage [67]. Its chemical and structural complexity relates to its physiological function of apoplastic barrier for the plant, controlling exchanges of water, gases and solutes and protecting the plant from microbial contaminations and physical damage [68–72].

Figure 1 – *Quercus suber* tree. Photo taken in Bragança, Portugal.

In general, suberin is constituted by poly-aliphatic and poly-aromatic domains linked between each other and to other cell wall components [65,71,73–75]. The poly-aliphatic domain is a glycerol-based polyester of ω-hydroxyacids and α,ω-dicarboxylic acids [76] while the poly-aromatic domain is composed essentially by ferulic acid [75,77]. Usually, suberin is extracted using an alkaline hydrolysis which renders a soup of monomers and small oligomers that can be analysed using GC-MS analysis [1] (Figure 2). The host team developed a new method - ionic liquid based - for the extraction of suberin (introduced further ahead) that retrieves a highly esterified suberin [39,78]. The ionic liquid method cleaves specifically only one type of ester bonds, the acyl-glycerol esters and neither the aliphatic esters,[38,78,79] nor the aromatic ones (*personal communication*, A. Pinheiro). In other words, the ionic liquid method allows to recover suberin as a polymer. Therefore, to disclose its monomeric composition by GC-MS, after the extraction with the ionic liquid the polymer is submitted to a total depolymerization using *e.g.* alkaline hydrolysis. In both cases, using either the conventional or the ionic liquid method, the most

abundant groups found in cork suberin are the diacids and hydroxy-acids, typically with C18 and C22 chain lengths and glycerol [66].

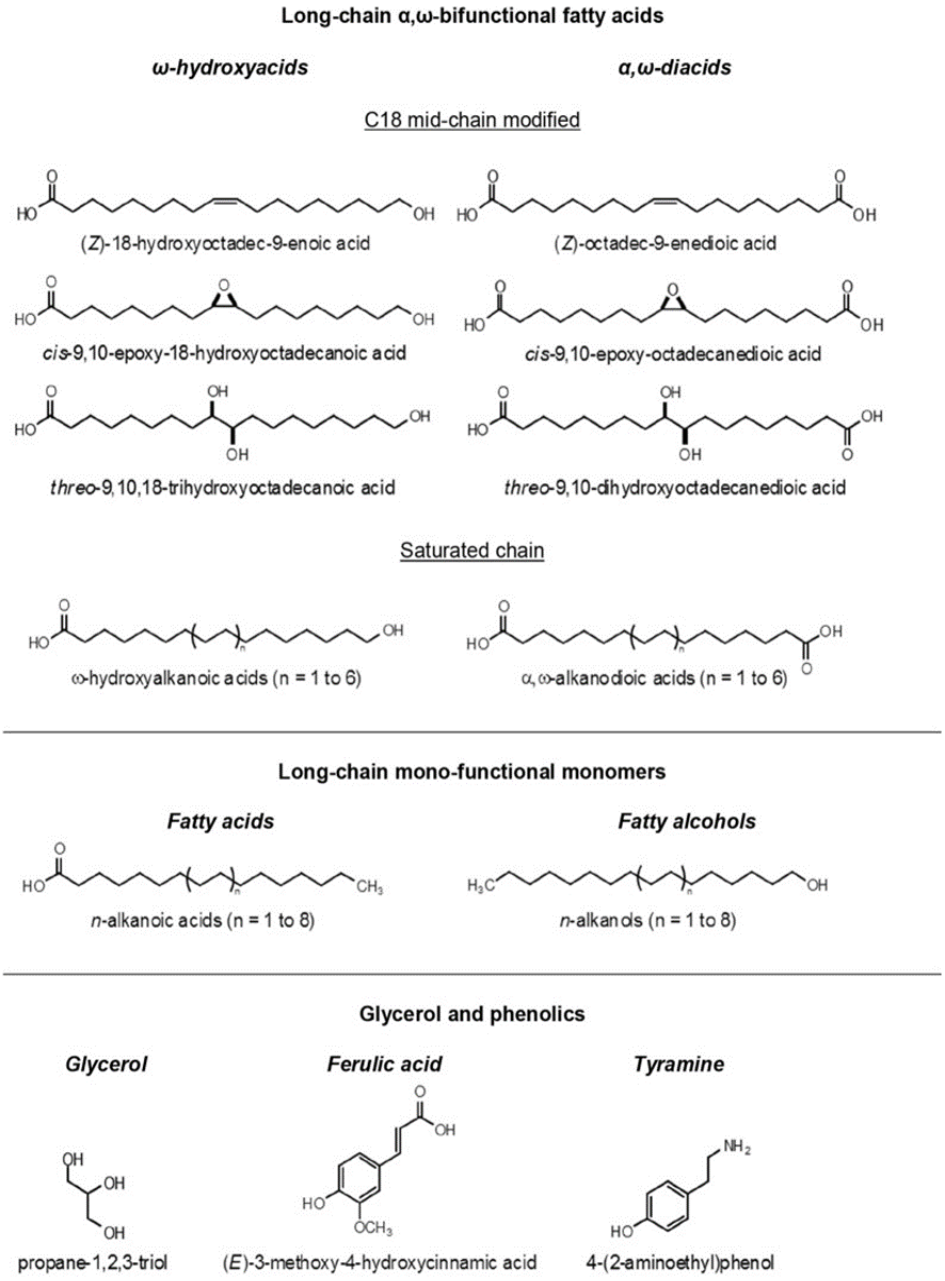


Figure 2 – Monomeric constitution of suberin. Adapted from [1].

Previous studies on cork suberin obtained using a partial hydrolysis (alkaline methanolysis) and by bidimensional nuclear magnetic resonance heteronuclear single quantum coherence spectroscopy (2D NMR HSQC) have disclosed the acyl-glycerol configurations of the extracted suberin oligomers [2]. In this thesis, the same approach was used to quantify the

proportion of each acyl-glycerol configuration present in the suberin extracted from cork with the ionic liquid method (Figure 3). Our team has recently solved the chemistry of the polymer extracted with cholinium hexanoate as well as that of the native cork suberin. The data showed that both 1,2,3-tri-acyl-glycerol (TAG) and 1,2-di-acyl-glycerol (1,2-DAG) are highly abundant in the native cork suberin (V. Correia *et al*., *unpublished data*). Therefore, both precursors undergo cleavage during the ionic liquid based catalysis, generating three additional configurations: 1,3-di-acyl-glycerol (1,3-DAG), 1-mono-acyl-glycerol (1-MAG) or 2-mono-acyl-glycerol (2-MAG) (V. Correia *et al*., *unpublished data*).

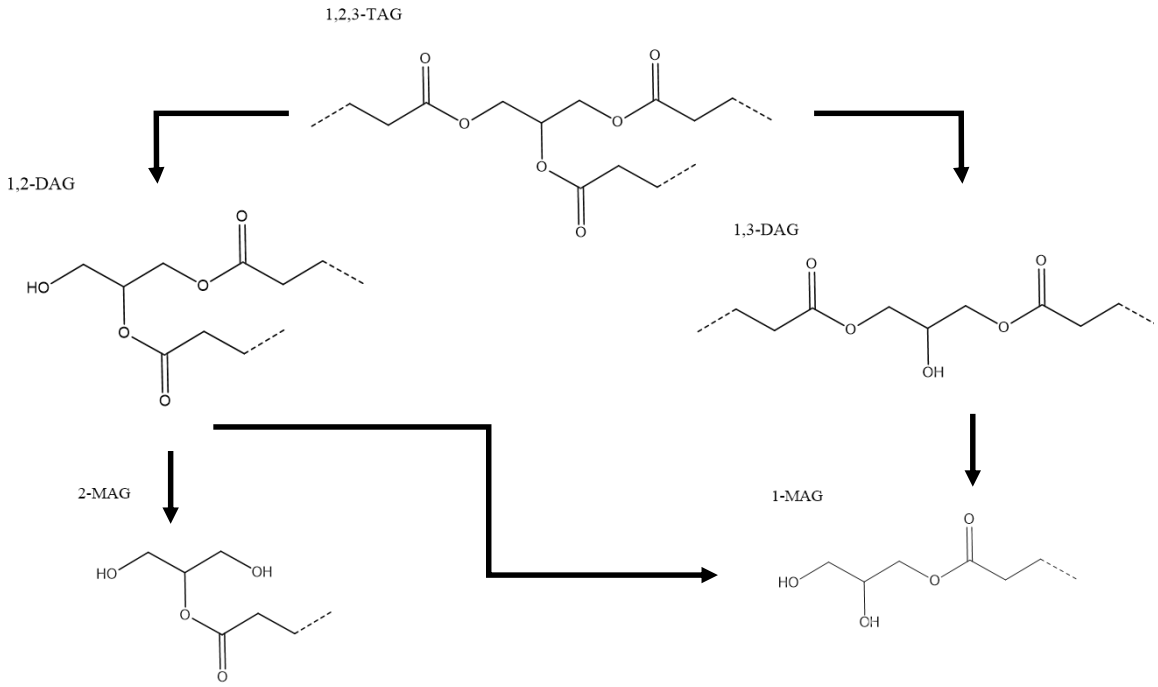


Figure 3 – Representation of the various configurations of the non-linear acyl-glycerol ester bonds that may be found in cork suberin. TAG and 1,2-DAG were shown to be present in suberin prior to depolymerization (unpublished data). Adapted from [2].

2.4. Ionic liquids

Ionic liquids are commonly defined as organic salts (composed only of ions) which are liquid at temperatures below 100ºC. These solvents comprise organic cations and either organic or inorganic anions. Also, either ion must be bulky or non-symmetrical to keep the solvent from solidifying [80,81].

An ionic liquid was first described in the 19th century by Gabriel and Weiner (ethanolammonium nitrate). Despite that, ionic liquids have reached the spotlight for research and industrial applications only in the last decade [82,83].

There are many aspects that cause this growing interest in ionic liquids. These solvents are considered environmentally friendly and safe due to their negligible vapour pressure [84]. Generally, they are non-inflammable, stable and recyclable but these considerations should not be taken for granted. The remarkable versatility of ionic liquids – the myriad of possible cations/anions combinations [82] – give way to the synthesis of many ionic liquids that may or may not fit the definition of an environmentally friendly solvent. This also means that ionic liquids can be designed to meet specific thermo-physical, chemical and biological characteristics [85–87]. The chemical environment created by an ionic liquid is also notable: the combination of Coulomb, van der Waals and specific interactions (*e.g.* H-bonding) makes it an outstanding solvent [88]. As mentioned, ionic liquids are also considered "greener" than many organic solvents, but this also depends on their constitution [89,90].

The ionic liquids used in this work, cholinium hexanoate and cholinium decanoate, are both considered environmentally friendly ionic liquids due to the presence of a benign cation in their constitution (cholinium). In fact, cholinium chloride is even classified as a provitamin in Europe and widely used as supplementation in animal feed [90]. The alkanoates anions used are also generally considered as safe. Previous studies done in the host lab showed that the cholinium alkanoates herein used present low toxicity and are biodegradable [38].

2.5. Extraction of suberin using a mild depolymerization method

In 2013 a new method for the extraction of suberin was developed in the host lab [78,79]. This new method affords a mild depolymerization of suberin, in contrast to the extensive depolymerization usually achieved through *e.g.* alkaline methanolysis. Extraction of suberin employing this method allowed the production of suberin films for the first time due to the capacity of the polymer moieties to spontaneously self-assemble *ex-situ* [39,40].

This method consists in the use of ionic liquids (namely, cholinium hexanoate) to achieve the cleavage of acyl-glycerol ester bonds in suberin. The dry ionic liquid (*ca.* 5 % water) is melted at 100 ºC and cork is added at a ratio of 10:1 (ionic liquid to cork). The melted ionic liquid then acts as a catalyst and a solvent: water, found in pockets, acts as a super-base and specifically hydrolyses only acyl-glycerol esters (not the linear esters). This specific cleavage allows for the extraction of a largely polymerised suberin polymer. By other words, the extracted suberin retains a polymeric structure closer to its original form, and the ability to spontaneously rearrange its supra-molecular structure *ex-situ* as a waterproof material [39,40].

The suberin films were shown to be non-soluble in water and to have antimicrobial properties [39]. Products composed essentially of suberin monomers which are obtained through

extensive depolymerization of the polymer are already used in some cosmetic products (*e.g.* SuberLift™) and advertised as having rejuvenating and lifting effects. This fact led to the interest of using suberin polymeric moieties, which can only be obtained when using the ionic liquid-based mild depolymerization method, as a dressing for wound healing, making use of the polymer's ability to form an impermeable and antimicrobial material.

3. Materials and Methods

3.1. Ionic liquid synthesis and quality assessment

Cholinium decanoate [$N_{1\,1\,1\,2\,OH}$][Dec] was purchased from Sigma-Aldrich.

Cholinium hexanoate [$N_{1\,1\,1\,2\,OH}$][Hex] was prepared by dropwise addition (1:1 ratio) of hexanoic acid (Acros Organics, 99 % v/v) to choline bicarbonate (Sigma, 80 % v/v in water), with constant stirring. During the reaction carbon dioxide and water are formed as secondary products. Stirring was maintained until CO_2 was completely released. The obtained [$N_{1\,1\,1\,2\,OH}$][Hex] was washed with diethyl ether (PanReac AppliChem ITW Reagents, 99.7 % v/v). This solvent was removed in a rotary evaporator (40 ºC, 50 mbar). Purity was evaluated through ^{1}H Nuclear Magnetic Resonance (NMR) in a Bruker 400 spectrometer using deuterium oxide (Acros Organics, 99.8 %) as solvent.

To dry the [$N_{1\,1\,1\,2\,OH}$][Hex] ionic liquid, first a rotary evaporator (50 ºC, 50 mbar) was used, followed by lyophilization (Labconco, -100 ºC, 0.008 mbar) .

Figure 4 depicts a representation of the cholinium cation and of the hexanoate and decanoate anions.

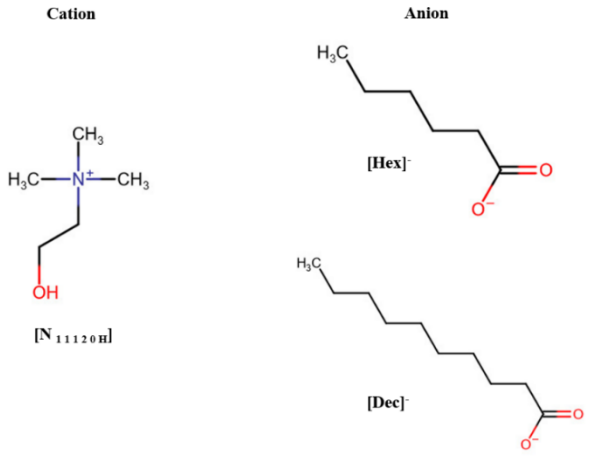


Figure 4 – Representation of the cholinium cation and of the hexanoate and decanoate anions. Structures drawn with *Reaxys.*

3.2. Source pre-treatment – Extractive removal

Cork (provided by *Amorim e Irmãos, SA)* was ground to 0.5 mm (Retsch ZM 200) and pre-treated prior to suberin extraction in order to remove not covalently bound molecules. Approximately 5 g of cork, which were weighed in extraction thimbles (Filtres Fioroni), were pre-treated using a Soxhlet extraction performed with solvents of increasing polarity: dichloromethane (Fisher, 99.9 % v/v), ethanol (PanReac AplliChem ITW Reagents, 99.5 % v/v) and bi-distilled water (8 h, 8 h and 24 h extraction times, respectively). Lyophilisation (Labconco, -100 ºC, 0.008 mbar) was used to dry the pre-treated cork source.

3.3. Suberin extraction

Pre-treated cork source was used to extract suberin using a mild and specific depolymerisation method developed in the host lab [39,40,78,79], using a stirred vessel specially designed for small scale reactions, built for this work (Figure 5).

Briefly, the ionic liquid was mixed with the pre-treated source (10:1 ratio) and allowed to react for 30 minutes or 2 hours, at 100 ºC with application of stirring at timed intervals (5 min ON, 15 min OFF). Dimethyl sulfoxide (DMSO) was then added to stop the reaction and reduce viscosity (80 mL per gram of cork source). Positive pressure filtration (Amicon) through a 0.45 µm porous membrane (Whatman), previously weighed, was used to separate the soluble fraction from the non-soluble fraction. The undissolved materials were recovered and centrifuged with bi-distilled water (several times) to remove DMSO and the ionic liquid. Finally, the recovered cork residues and the membrane filter were both lyophilized (Labconco, -100 ºC, 0.008 mbar) and weighed to calculate mass balances.

Figure 5 – Photo of the stirred vessel built for small scale extractions of suberin with the ionic liquid approach.

An excess of 1 L (per gram of cork source) of bi-distilled water was added to the soluble fraction (*i.e.* filtrated) and suberin allowed to precipitate (overnight, 4 ºC). Centrifugation (Beckman Coulter, 2452 x g) was used to recover suberin as a pellet. The pellet was centrifuged (Beckman Coulter, 5000 x g) with 1.1 L (per gram of cork source) bi-distilled water to remove any remains of DMSO and ionic liquid. Lyophilization (Labconco, -100 ºC, 0.008 mbar) was used to dry suberin. The recovered dry suberin was weighed to calculate mass balances and the extraction yield.

3.4. Suberin monomers identification and quantification by Gas chromatography-mass spectrometry (GC-MS)

An Agilent (7820A) gas chromatograph equipped with an Agilent (5977B) mass spectrometer (quadrupole) was used. The GC-MS was first calibrated with pure reference compounds (representative of the major classes of compounds present in suberin) relative to hexadecane (internal standard). Data acquisition was accomplished by MSD ChemStation (Agilent Technologies). Compounds identification was based on the equipment spectral library (Wiley-NIST) and on previously published data [64,79,91], focusing on their EI-MS fragmentation patterns and/or retention times. Suberin samples were subjected to alkaline hydrolysis prior to the methylation and silylation, to release their hydrolysable monomeric constituents. Briefly, suberin samples were treated with a solution of 0.5 M NaOH in methanol/water (1:1, v/v) at 95 ºC, for 4 hours. The mixture was cooled to room temperature, acidified to pH 3–3.5 with 1M HCl, and extracted three times by dichloromethane/water partition. The combined organic extracts were dried in a rotary evaporator, then trimethylsilylated, and analysed by GC-MS using a HP-5MS column with the following ramp temperature: 80ºC, 4ºC/min until 310 ºC, 310ºC during 15 min. Suberin monomers quantification was done with a calibration curve using pure standard compounds hexadecanoic acid, hexadecanedioic acid, pentadecanol and cinnamic acid (Sigma-Aldrich) within the quantification limits of 0.05 mg – 1mg for aromatics (cinamic acid) and 0.0052 – 0.104 mg for the rest of the compounds.

3.5. Glycerol quantification by High Performance Liquid Chromatography (HPLC)

Suberin samples were treated with a solution of 0.5 M NaOH in methanol/water (1:1, v/v) at 95 ºC, for 4 hours. The mixture was cooled to room temperature, acidified to pH 3–3.5 with 1M HCl and extracted three times by dichloromethane/water partition. The aqueous phase was analysed by HPLC, using an Alliance 2695 Waters chromatographer, connected to LKB 2142 Differential Refractometer (Bromma, Sweden). Data acquisition was accomplished with the

Empower 2 software (Waters Chromatography). Chromatographic separation was undertaken at 60 ºC using an Aminex HPX-87 column (300 x 7.8mm), 9 µm particle size (Bio-Rad, Hercules, California). Elution was carried out isocratically, at a flow rate of 0.5 mL/min and the sample volume was 20 µL. Glycerol quantification was done using a calibration curve within the quantification limits of 0.25-9.99 mg/mL.

3.6. Nuclear Magnetic Resonance spectroscopy (NMR) analyses of extracted suberin

1D (^{1}H) and 2D HSQC solution NMR spectra of the recovered suberin in deuterated DMSO (DMSO-d6) were acquired on an Avance III 800 spectrometer (Bruker, Rheinstetten, Germany) working at a proton operating frequency of 800.33 MHz, equipped with a three channel 5 mm inverse detection probe head with pulse-field gradients along the Z-axis. Analyses were run at 60 ºC using standard Brüker pulse programs. The obtained suberin spectra were analysed using MestreNova Software (MNova).

3.7. Differential Scanning Calorimetry (DSC) analysis of suberin

Standard differential scanning calorimetry (DSC) measurements were performed on a Q2000 (TA Instruments, USA), connected with a refrigerated cooling system. DSC tests were performed from -80 to 120 ˚C with Tzero pans and hermetically sealed lids, followed by a second test cycle under the same conditions (sample weight range: 7.5 – 10 mg; heating rate: 10 ˚C/min; purge gas: oxygen free nitrogen; gas flow rate: 50 cm^3/min). The characteristic peaks were analysed using Universal Analysis, version 4.4A software. Melting temperature (*Tm*) was determined as the minimum of the melting endothermic peak during the heating cycle.

3.8. Dynamic Light Scattering (DLS) analysis of suberin suspensions

DLS was used to measure the size distribution of suberin particles/aggregates in an aqueous suspension, with or without the addition of PEG 8000 0.5 % (w/v), in concentrations of suberin ranging from 6.25 to 100 µg / mL.

DLS measurements were performed using a Malvern Laser Particle Size analyser (ZETASIZER Nano ZS, UK) at 37ºC. Suberin suspensions were diluted (1/10) to calculate the average hydrodynamic diameter (d.nm) and the polydispersity index (PDI). All measurements were performed in triplicates using disposable plastic cuvettes.

3.9. Preparation of suberin films and pulverized films

The best conditions for the preparation of suberin films were assessed by varying the amount of suberin per 100 µL, as well as the type and duration of the homogenisation treatments applied to the aqueous suspensions of suberin prior to the fabrication of the films, namely agitation and sonication. Figure 6 shows a scheme of the various conditions assessed.

A suspension of suberin (extracted with [N_{1112OH}][Hex], reaction time 2h) was prepared in bi-distilled water. Quantities of suberin weighed are in accordance to Figure 6. The suspensions were subjected to the various conditions under testing and films were cast in plastic petri dishes. To do so, 30 µL of suberin suspension after homogenisation were deposited onto the plastic petri dish. Films were cast by evaporation of water at 30 ºC for 1 h, with no agitation, in a ventilated orbital shaker.

Pulverized suberin films were also produced to assess their effect on cell proliferation. Briefly, after cooling the suberin films with dry ice to *ca.* -80ºC, a tissuelyser (L1, QIAGEN) was used to pulverize the suberin films using metallic beads (50 oscillations/s, 1 min).

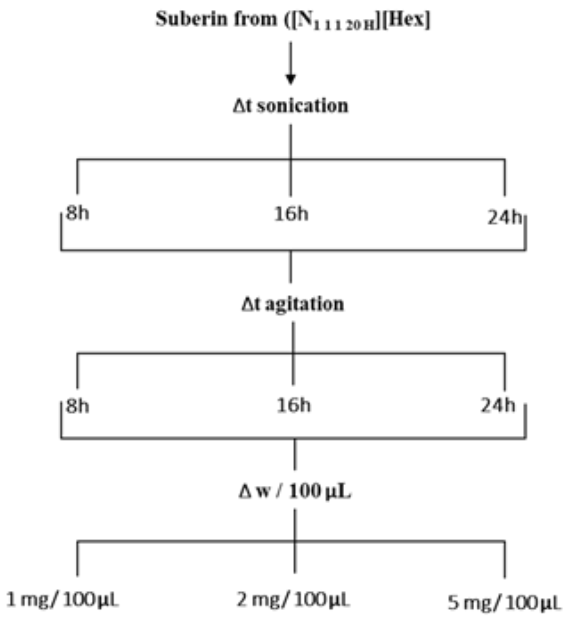


Figure 6 – Schematic representation of the various conditions tested for the preparation of suberin films.

3.10. Preparation of suberin formulations

Various suberin formulations were prepared to apply on cell proliferation assays. Figure 7 shows a schematic representation of the various conditions assessed.

Suspensions were prepared on Epilife broth medium (Cascade Biologics™ cat. no. M-EPI-500-CA), without supplementation. Adequate supplementation was added after sterilisation, according to the final concentrations described for the culture medium (see Materials and methods, Chapter 2).

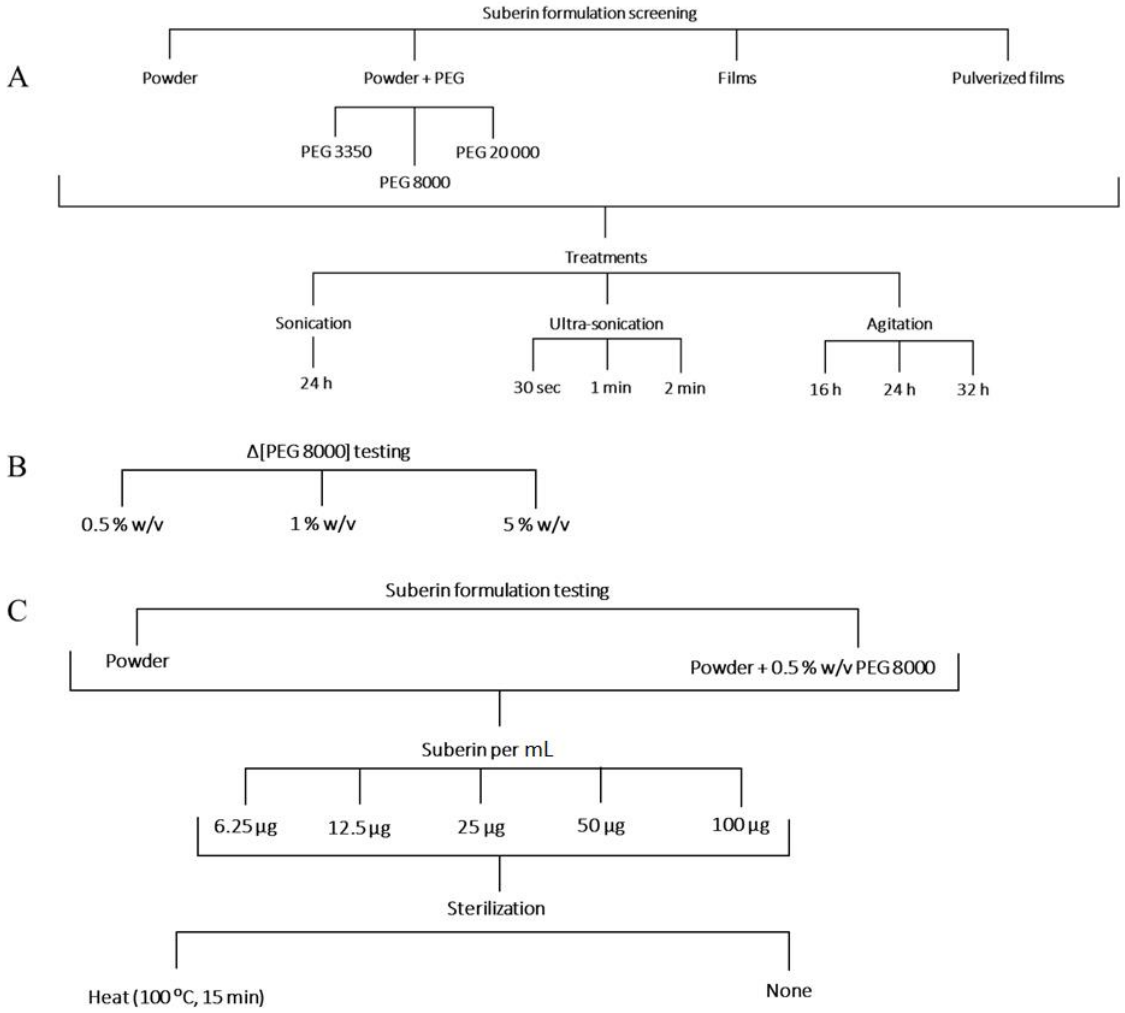


Figure 7 – Schematic representation of the various conditions assessed for testing suberin influence on cellular proliferation, A) the initial screening; B) assessment of PEG 8000 concentration effect on cell proliferation; C) assessment of suberin formulations, with the previously determined concentration of PEG 8000, effect on cell proliferation.

4. Results and discussion

4.1. Determination of the quality of the synthesis via NMR spectroscopy of [N $_{1\,1\,1\,2\,0\,H}$][Hex]

Two batches of [N $_{1\,1\,1\,2\,0\,H}$][Hex] were prepared in the laboratory. Samples were analysed through ^{1}H NMR spectroscopy to determine if the synthesis had been successful (Figure 8). The relative quantity of protons found in the anion and the cation show that the ratio anion to cation is of 1:1 and therefore, that the synthesis was successful.

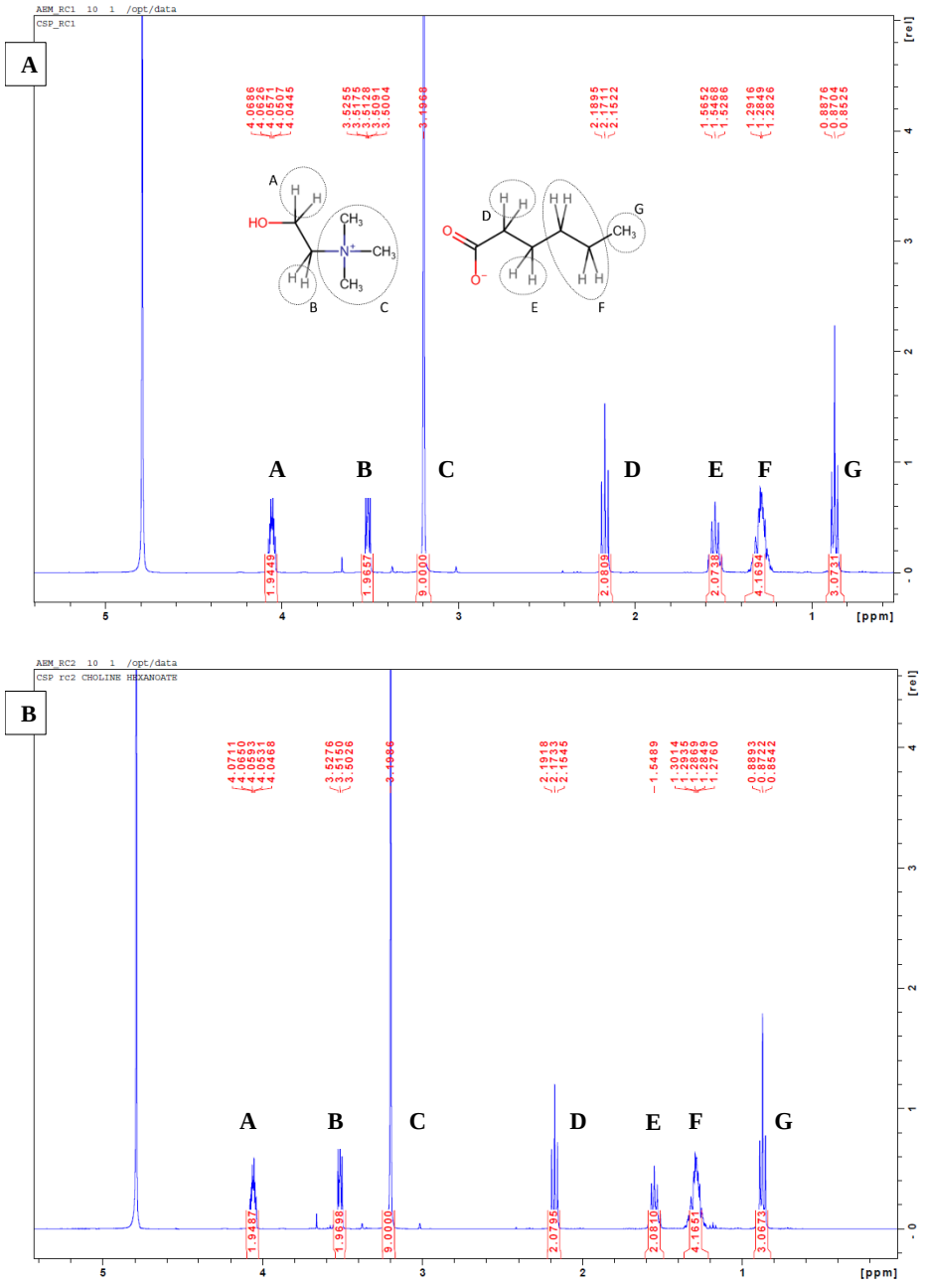


Figure 8 – ^{1}H NMR analysis of the two batches (A and B) of [N $_{1\,1\,1\,2\,0\,H}$][Hex] prepared *in-house*. A, B, C, D, E, F, G – NMR signal assignment..

4.2. Suberin extraction: analysis and characterization

Figure 9 shows a representation of the various techniques that were employed to characterize the suberin extracted with both ionic liquids.

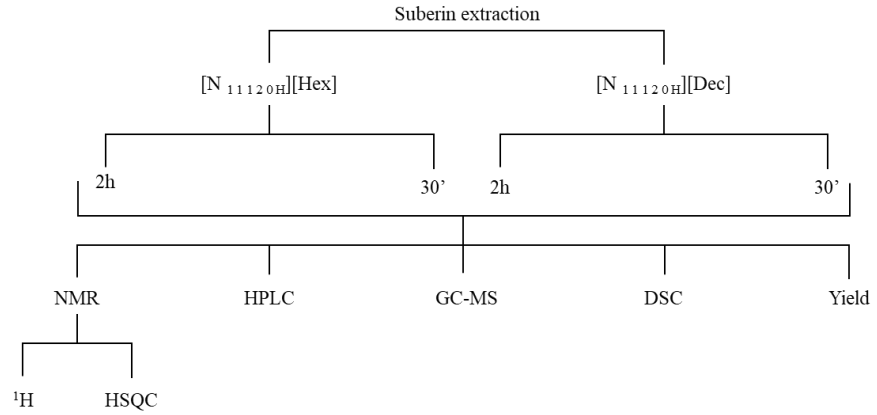


Figure 9 – Schematic representation of the various characterization assays performed and parameters measured with the suberin extracted using the ionic liquid extraction method.

4.2.1. Extraction mass balance

To determine the extraction yield of the various reactions performed with either ionic liquid, mass balances were calculated (*i.e.* masses of recovered suberin and of the insoluble fraction *versus* the initial cork mass). Table 1 shows the mass balances attained after 30 min or 2h of reaction time. As expected, an increase in the amount of extracted suberin was observed for the 2h reaction with [N 1 1 1 2 0H][Hex] compared to the 30 min reaction. Moreover, the reaction yield was substantially enhanced due to the application of stirring with the home-built reactor compared to reactions without agitation (*data not shown*).

Table 1 – Extraction mass balances for suberin extractions performed with [N 1 1 1 2 0H][Hex] and [N 1 1 1 2 0H][Dec] relatively to the initial mass. An increase in yield is found for a longer reaction time when using [N 1 1 1 2 0H][Hex], reaching an average of ~25% of the initial mass. Recovery of the materials extracted using [N 1 1 1 2 0H][Dec] was difficulted by the apparent low densities of the materials.

	[N 1 1 1 2 0H][Hex] 30 min	[N 1 1 1 2 0H][Hex] 2 h	[N 1 1 1 2 0H][Dec] 30 min	[N 1 1 1 2 0H][Dec] 2 h
Initial mass (g)	2.14 ± 0.05	2 ± 0.02	2 ± 0.01	1.99 ± 0.03
Insolubles (%)	87.53 ± 4.91	75.06 ± 7.92	83.79 ± 18.55	35.56 ± 18.84
Suberin (%)	2.89 ± 0.81	24.58 ± 6.45	8.63 ± 8.1	6.8 ± 1.17

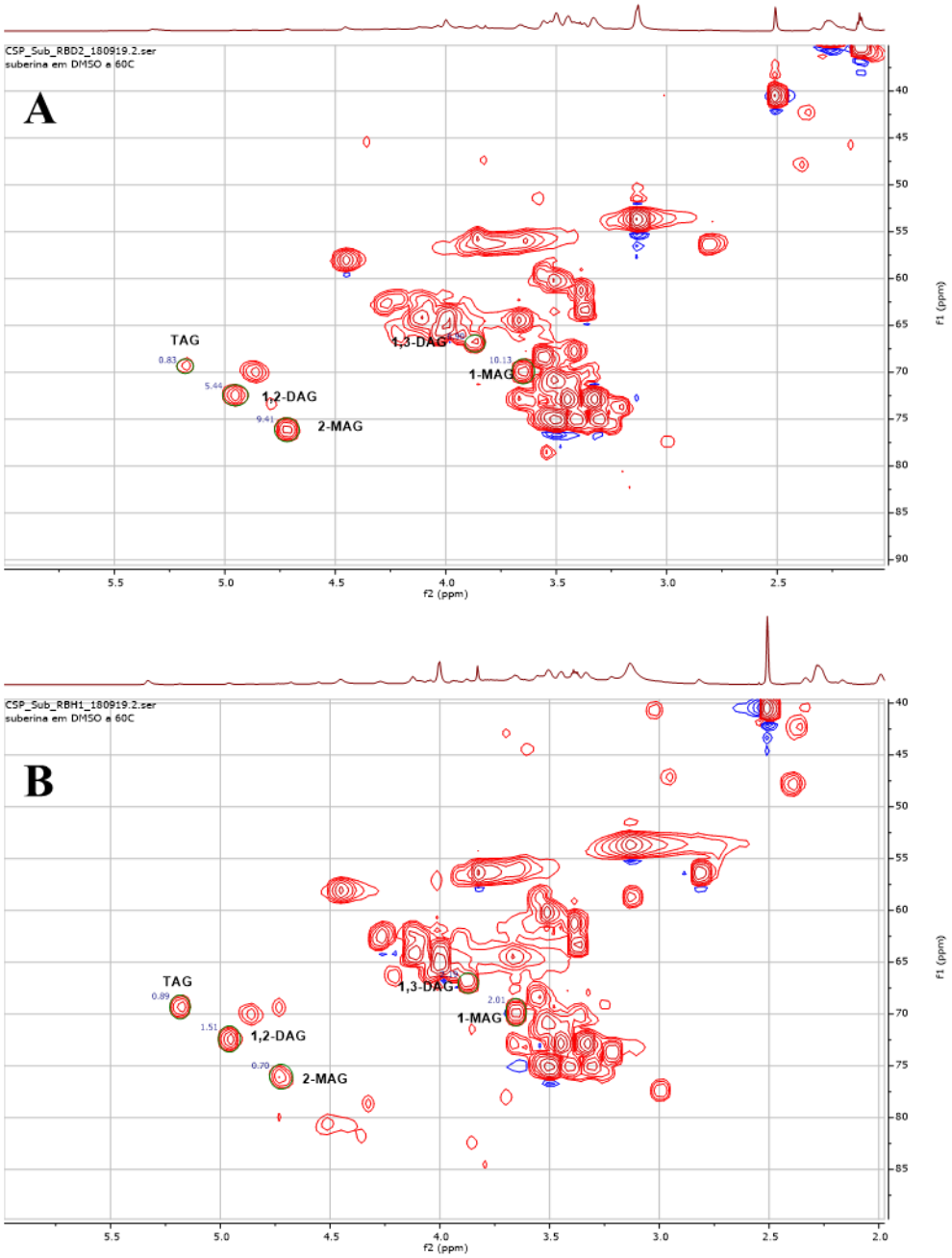
One possible explanation is that the application of agitation promotes the action of the ionic liquid catalyst by fine mixing of the reagents. The extraction yields apparently increased with the reaction time, consistent with the need to cleave some acyl-glycerol ester bonds to release suberin from the cork cell walls. More time points would be necessary to identify the minimal time required to extract all the available suberin. Moreover, the amount of glycerol released after the ionic liquid reaction would provide some additional information on the efficiency of

acyl-glycerol cleavage. This was not considered a priority since the main goal of the thesis was to characterise the recovered suberins, however the collected filtrates were kept at 4°C and will be used for chromatographic analysis of glycerol in the near future.

Additional assays performed in the lab on the ability of cholinium decanoate to cleave esters compounds (octyl octanoate and triacylglycerol) showed that this ionic liquid can also specifically catalyse the cleavage of acyl glycerol esters bonds and not of aliphatic esters, with yields comparable to that of cholinium hexanoate (A. Pinheiro *et. al*, unpublished). The interest of using this new ionic liquid for suberin extraction from cork – not yet optimised – is based on its commercial availability. In contrast to the observations made for [N_{1112OH}][Hex], the masses of suberin recovered when using [N_{1112OH}][Dec] were extremely low and similar at both time points. This was probably due to difficulties in recovering the suberin by centrifugation (especially that obtained after a 2 hour hydrolysis); it was very difficult to obtain a pellet since most of the extracted suberin floated in the supernatant. Therefore, the results obtained with the cholinium decanoate (Table 1) most likely reflect a major loss in the recovery step and cannot be regarded as an indication of lower efficiency of this reaction (*n.b.* the sum of the masses of suberin and of the insoluble fraction after a 2 hour reaction is much lower than that of the initial cork mass). Preliminary analyses of the attained insoluble fractions are on-going (ATR-FTIR) to qualitatively verify the degree of suberin removal. New extractions of suberin from cork with [N_{1112OH}][Dec] are necessary, probably using a second filtration step instead of centrifugation, to fully demonstrate the efficiency of this ionic liquid. On the other hand, the low density of the suberin attained with the [N_{1112OH}][Dec] would be consistent with efficient hydrolysis and recovery of a polymer with a low degree of cross-linking. To confirm this hypothesis, additional time points are necessary (using an optimised suberin recovery procedure).

4.2.2. Determination and relative quantification of acyl-glycerol ester configurations present in extracted suberin through ^{1}H and HSQC NMR spectroscopy

The various acyl-glycerol configurations present in the extracted suberins were determined and quantified using a suite of NMR methods. The different acyl-glycerol configurations herein considered are TAG, 1,2-DAG, 1,3-DAG, 1-MAG, 2-MAG (Figure 3). Figure 10 shows the various spectra obtained, except that of the suberin recovered using [N_{1112OH}][Dec] for 30 min since it was below the quantification threshold.



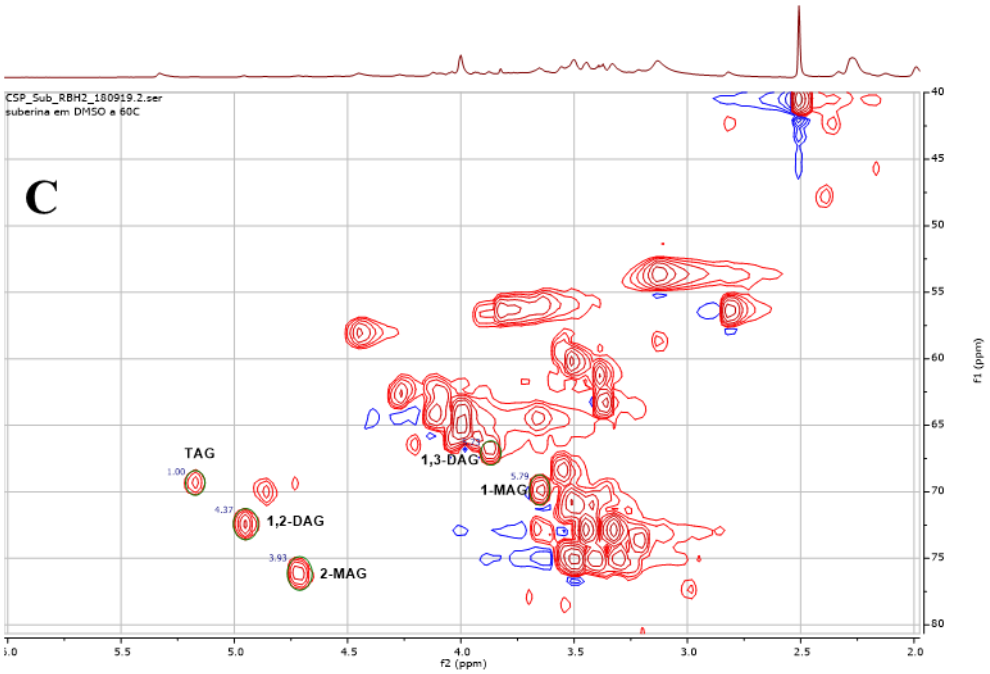


Figure 10 – 1H and HSQC NMR spectra of A – Suberin extracted with [N $_{1\,1\,1\,2\,0\,H}$][Dec], reaction time 2h; B – Suberin extracted with [N $_{1\,1\,1\,2\,0\,H}$][Hex], reaction time 30 min; C – Suberin extracted with [N $_{1\,1\,1\,2\,0\,H}$][Hex], reaction time 2h.

Figures 11 and 12 represent the relative abundance of the various acyl-glycerol configurations found in the extracted suberins. In general, the extracted suberins showed similar spectral features, irrespectively of the used ionic liquid and reaction time. For the 2 hour reactions, the suberin extracted with [N $_{1\,1\,1\,2\,0\,H}$][Dec] shows lower occurrence of precursors TAG and 1,2-DAG (V. Correia *et al.*, *unpublished data*) and higher of the two mono-acylglycerol configurations compared to [N $_{1\,1\,1\,2\,0\,H}$][Hex] (Figure 11). This suggests that the reaction with [N $_{1\,1\,1\,2\,0\,H}$][Dec] was indeed more extensive than that of [N $_{1\,1\,1\,2\,0\,H}$][Hex], regardless of its very low recovery yield as above discussed. However, this result should be considered preliminary and deserves further analysis to verify if the recovered suberin is representative or not due to major losses during the centrifugation step. Importantly, to fully understand the NMR information the amount of glycerol present in each suberin sample should also be considered (see Table 2 below).

In particular, for the suberins extracted with [N $_{1\,1\,1\,2\,0\,H}$][Hex] we observed a decrease in the precursor TAG and an increase in 2-MAG as the reaction time progresses (Figure 12). Figure 12 shows the relative abundance of the acyl-glycerol configurations found in suberin samples extracted with [N $_{1\,1\,1\,2\,0\,H}$][Hex], for a reaction time of 30 min and 2 h. A decrease in TAG and an increase in 2-MAG is observed, suggesting that the hydrolysis progresses as the reaction time increases. This observation shows that the hydrolysis progresses along time and has not reached a plateau at the 2 hour mark, as discussed above.

Figure 11 shows the relative abundance of the acyl-glycerol configurations found in suberin samples extracted with either [N $_{1\,1\,1\,2\,0\,H}$][Hex] or [N $_{1\,1\,1\,2\,0\,H}$][Dec], with a reaction time of 2 h. A higher abundance of 1-MAG and 2-MAG is observed for the [N $_{1\,1\,1\,2\,0\,H}$][Dec] extracted suberin, suggesting that the hydrolysis was more extensive when using this ionic liquid.

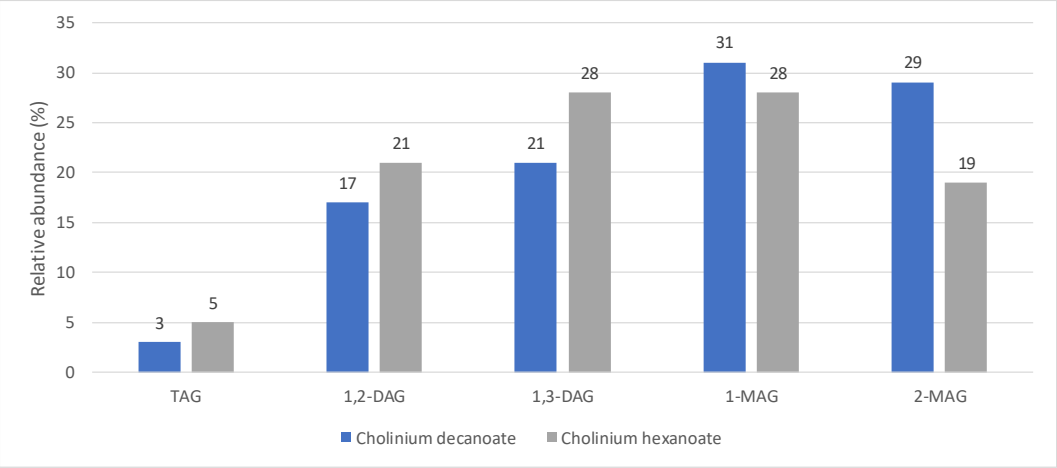


Figure 11 – Relative abundance of the acyl-glycerol configurations found in suberin samples extracted with either [N $_{1\,1\,1\,2\,0\,H}$][Hex] or [N $_{1\,1\,1\,2\,0\,H}$][Dec], with a reaction time of 2 h. A higher abundance of 1-MAG and 2-MAG is observed for the [N $_{1\,1\,1\,2\,0\,H}$][Dec] extracted suberin, suggesting that the hydrolysis was more extensive when using this ionic liquid.

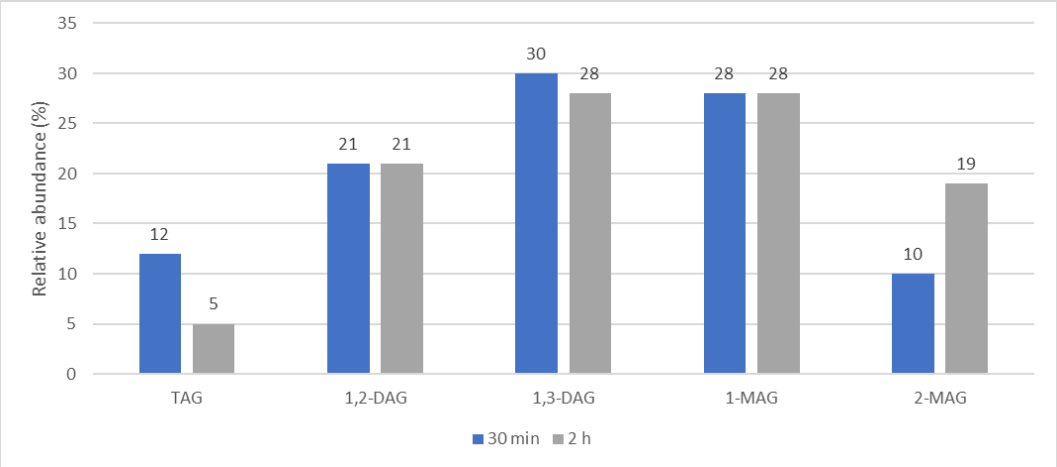


Figure 12 – Relative abundance of the acyl-glycerol configurations found in suberin samples extracted with [N $_{1\,1\,1\,2\,0\,H}$][Hex], for a reaction time of 30 min and 2 h. A decrease in TAG and an increase in 2-MAG is observed, suggesting that the hydrolysis progresses as the reaction time increases.

4.2.3. Determination of the glycerol content of the extracted suberin through HPLC

The amount of glycerol present in the distinct suberin samples was estimated by HPLC after the complete hydrolysis of the polymer. The results, which are depicted in Table 2, showed that the glycerol content of the extracted suberins was, in general, higher for samples obtained with longer reaction times. In particular, the glycerol content of the suberin samples extracted with [N_{1112OH}][Hex] was observed to increase with the reaction time. This is consistent with previous studies (unpublished) that suggested that longer extraction times may promote the extraction of suberin fractions presenting a higher degree of cross-linking (*i.e.* more recalcitrant). The glycerol content for suberin samples extracted after 30 min with [N_{1112OH}][Dec] or [N_{1112OH}][Hex] were observed to be similar. Even though the sample attained after 2 hours of hydrolysis in the presence of [N_{1112OH}][Dec] cannot be yet considered representative, it is interesting to note that nearly 60% of the quantified glycerol was found as 1-MAG or 2-MAG (Figure 12), whereas for [N_{1112OH}][Hex] the mono-acylglycerol configurations accounted for only 47% of the extant glycerol (Figure 11). As abovementioned, new samples need to be prepared to evaluate whether this result is an artefact created by the weak recovery of the polymer.

Table 2 – Glycerol content (mg/g of suberin) for the suberin extracted with either ionic liquid, at reaction times 30 min and 2 h, measured through HPLC. An increase in the glycerol content of suberin is observed for the reactions performed for 2 h, for both ionic liquids.

Sample	Glycerol (mg/g suberin)
[N_{111OH}][Dec] 30 min	34.65 ± 2.23
[N_{111OH}][Dec] 2 h	47.7 ± 2.07
[N_{111OH}][Hex] 30 min	31.52 ± 1.48
[N_{111OH}][Hex] 2 h	39.19 ± 0.85

4.2.4. Determination of the constitution of the extracted suberin using GC-MS

The extracted suberin samples were further hydrolysed using alkaline hydrolysis to release the monomeric constituents which were analysed using an established GC-MS method (see Materials and methods). In the following figures (Figures 13-21) the GC-MS results are depicted presenting first an overview of the most representative families of monomeric constituents and then presenting the distinct monomeric constituents per family. The results obtained for the suberins extracted with [N_{1112OH}][Dec] are also presented. However, these results will not be discussed because as abovementioned the recovered suberin might not be representative of the extracted suberin (*n.b.* due to significant losses during the centrifugation of the recovered polymer). The GC-MS data for [N_{1112OH}][Dec] herein presented are in general

difficult to correlate with those obtained for the other ionic liquid. New samples will be prepared after optimisation of a new recovery method as above mentioned.

On the other hand, the GC-MS results obtained for the suberin extracted with [N_{1112OH}][Hex] show, in general, that longer extraction times led to the recovery of suberins enriched in many monomeric constituents (comparison of a 30 min reaction with a 2 hour reaction). In particular, when considering the amount of each family of monomeric constituents, we observed an increase in mono hydroxy fatty acids and alkanedioic acids (Figure 13), in particular of 22-hydroxydecosanoic acid (Figure 16) and of octadecanedioic acid (Figure 19), respectively. The identification yields were as follows: 8.7% and 29.1% for the [N_{1112OH}][Hex] 30 min and 2 h extractions, respectively; and 56.4% and 19.9% for the [N_{1112OH}][Dec] 30 min and 2 h extractions, respectively.

Figure 14 (fatty acids) shows an increase in decosanoic acid content when the extraction time with [N_{1112OH}][Hex] increases, Figure 15 (fatty alcohols) shows an increase in 1-tetradecanol, Figure 17 (di-, tri-hydroxy fatty acids) displays an increase in 9(?),10(?),18-trihydroxyoctadec-12-enoic acid, Figure 18 (epoxy fatty acids) shows an increase in the epoxy content of the suberin samples, Figure 20 (steroids and triterpenes) displays an increase in betuline and betulinic acid and, finally, Figure 21 (aromatics) shows an augment in ferulic acid content.

The observed differences may be a natural consequence of the [N_{1112OH}][Hex] mediated catalysis that preferentially cleaves acyl-glycerol esters at the C2 position [79]. This would result in the preferential release of suberin fractions that are structurally bound to the cell wall by this type of bonds. Another explanation is that the suberin sample obtained after 30 min is less recalcitrant to hydrolysis and presents a lower degree of cross-linking than that obtained after a 2 hours hydrolysis with [N_{1112OH}][Hex]. One cannot ignore the possibility that the sample from the 30 min hydrolysis is also not representative due to a low recovery yield (below 10%). It is important to reinforce that some of the differences herein noticed are of foremost importance as they may provide valuable insights on the activity of specific groups of suberin in cell proliferation and wound healing. This is in part, the core of the present work, to demonstrate the potential of suberin in wound healing but also to extrapolate new hypotheses for future work in order to correlate suberin composition with activity – to be revisited in the near future, now that most of the methods and assays have been established to undertake these tasks.

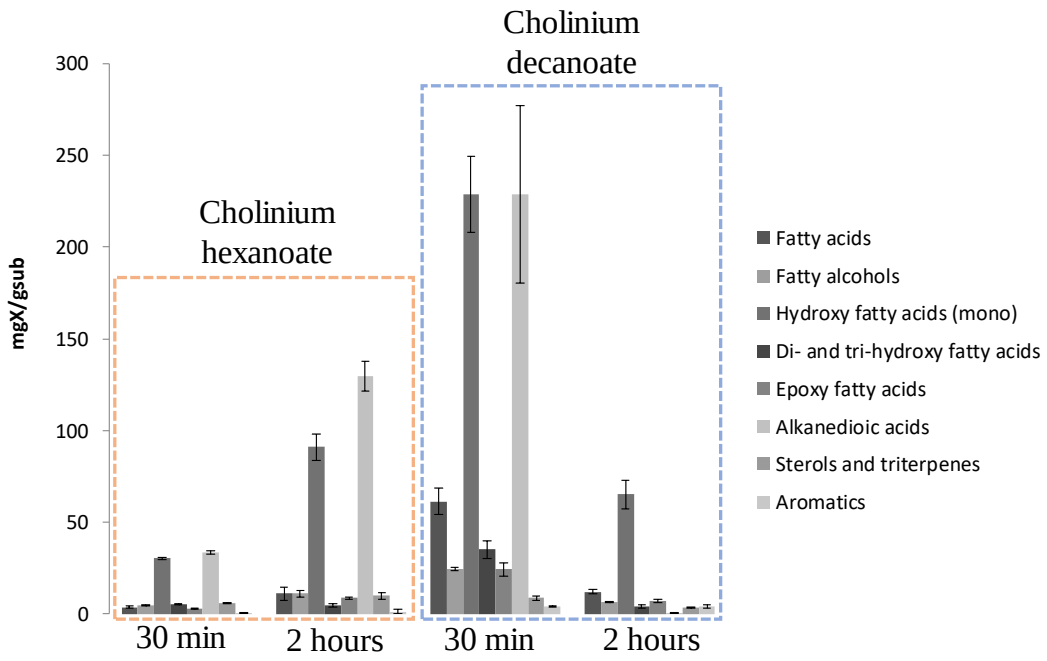


Figure 13 - Families of compounds identified in the suberin samples through GC-MS analysis. An increase in hydroxy fatty acids and alkanedioic acids is observed in the longer [N $_{1\,1\,1\,2\,0\,H}$][Hex] extraction time.

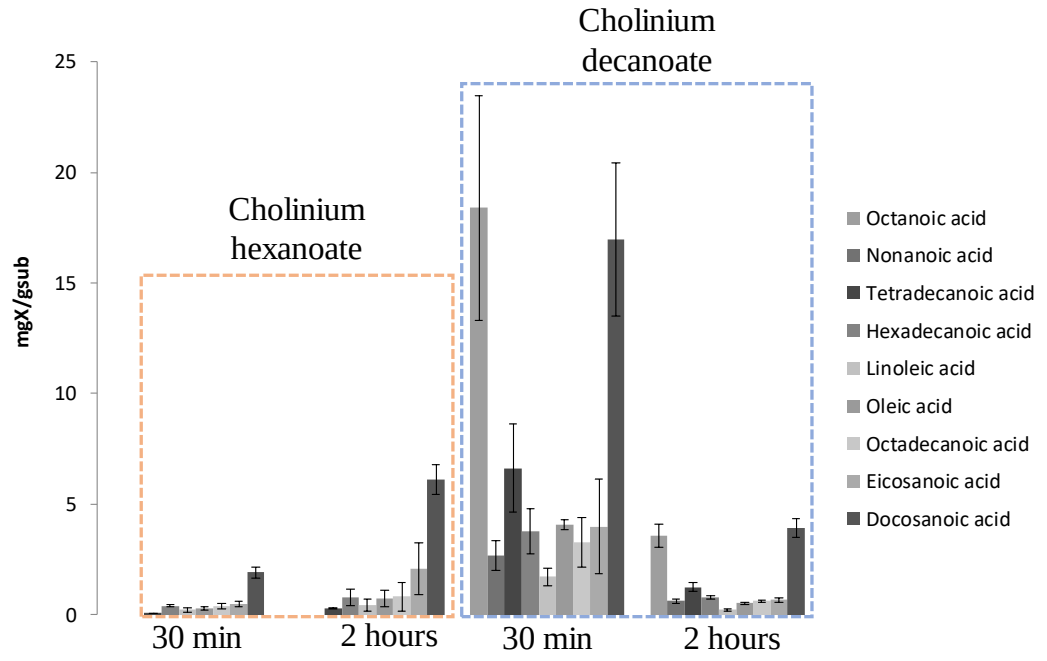


Figure 14 - Identification of the fatty acids found in the extracted suberin samples through GC-MS analysis. A major increase in docosanoic acid is observed for [N $_{1\,1\,1\,2\,0\,H}$][Hex] at 2 h extraction time.

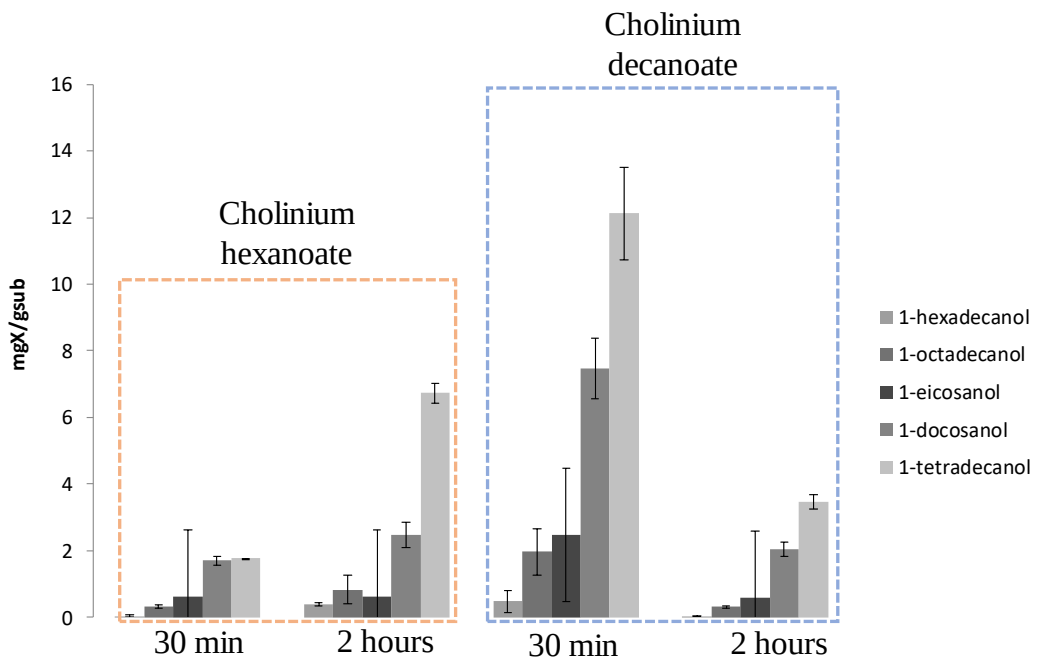


Figure 15 - Identification of the fatty alcohols found in the extracted suberin samples through GC-MS analysis. A major increase in 1-tetradecanol is observed for extraction time 2 h.

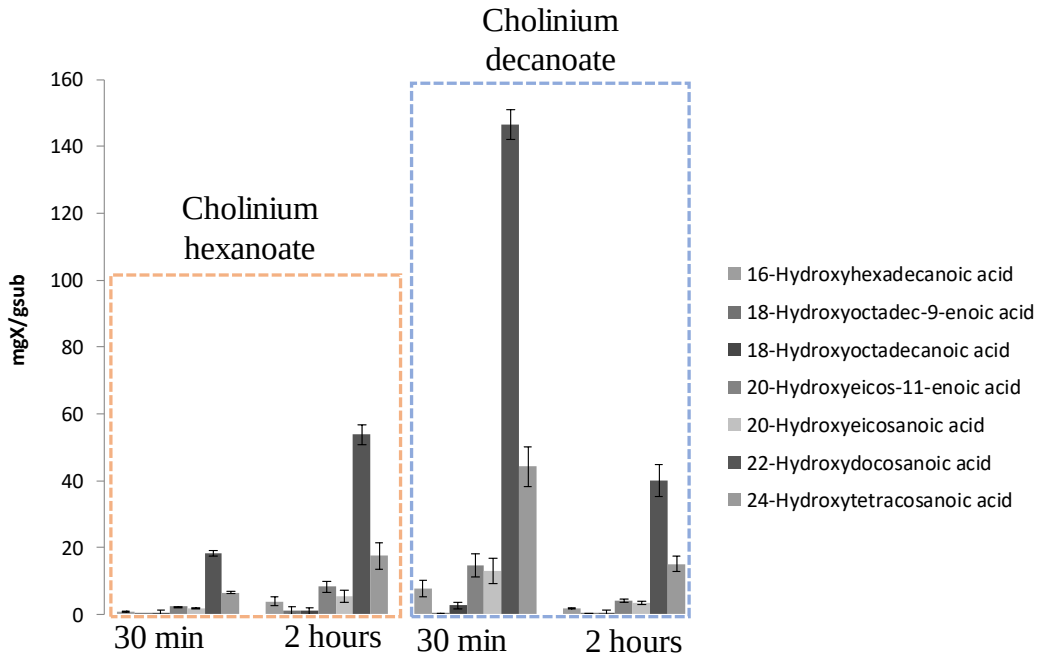


Figure 16 - Identification of the mono hydroxy fatty acids found in the extracted suberin samples through GC-MS analysis. A major increase in 22-hydroxydocosanoic acid is observed for [N $_{1\,1\,1\,2\,0\,H}$][Hex] at 2 h extraction time.

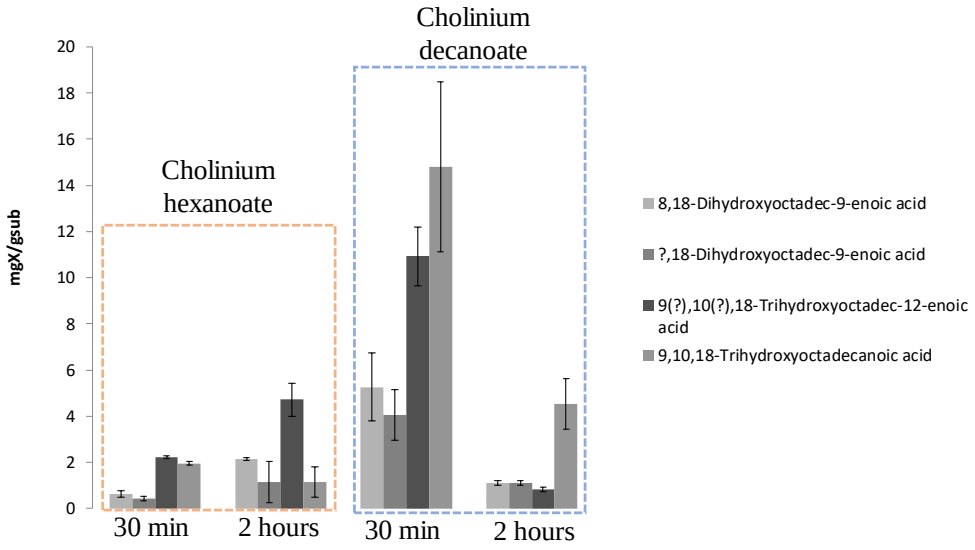


Figure 17 - Identification of the di- and tri-hydroxy fatty acids found in the extracted suberin samples through GC-MS analysis. All compounds increase at 2 h extraction time with [N_{1112OH}][Hex], with a more pronounced increase in 9(?),10(?),18-trihydroxyoctadec-12-enoic acid.

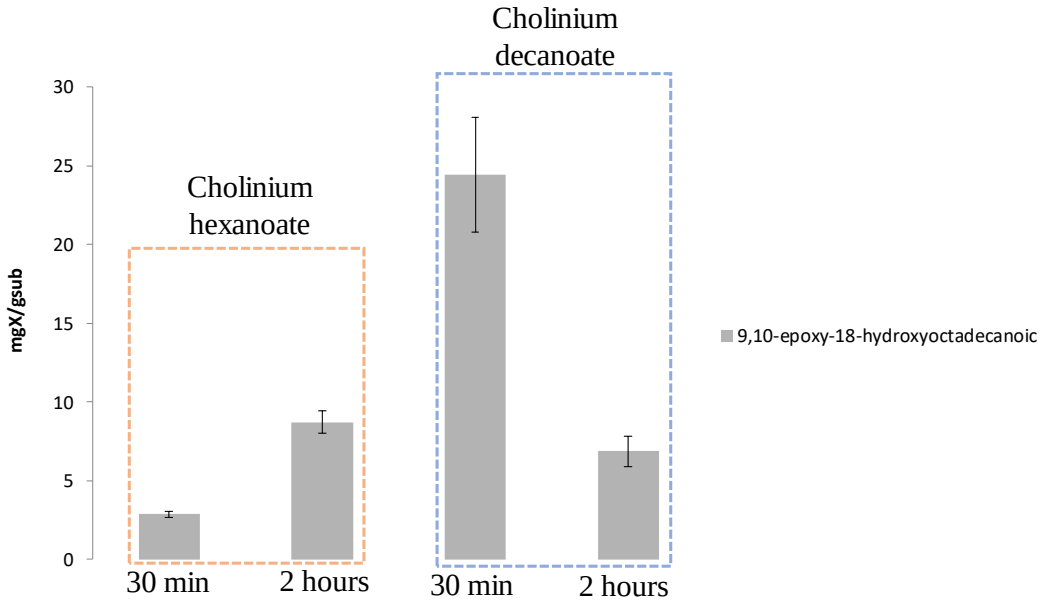


Figure 18 - Identification of the epoxy fatty acids found in the extracted suberin samples through GC-MS analysis. An increase in epoxy content is observed at the longer extraction time with [N_{1112OH}][Hex].

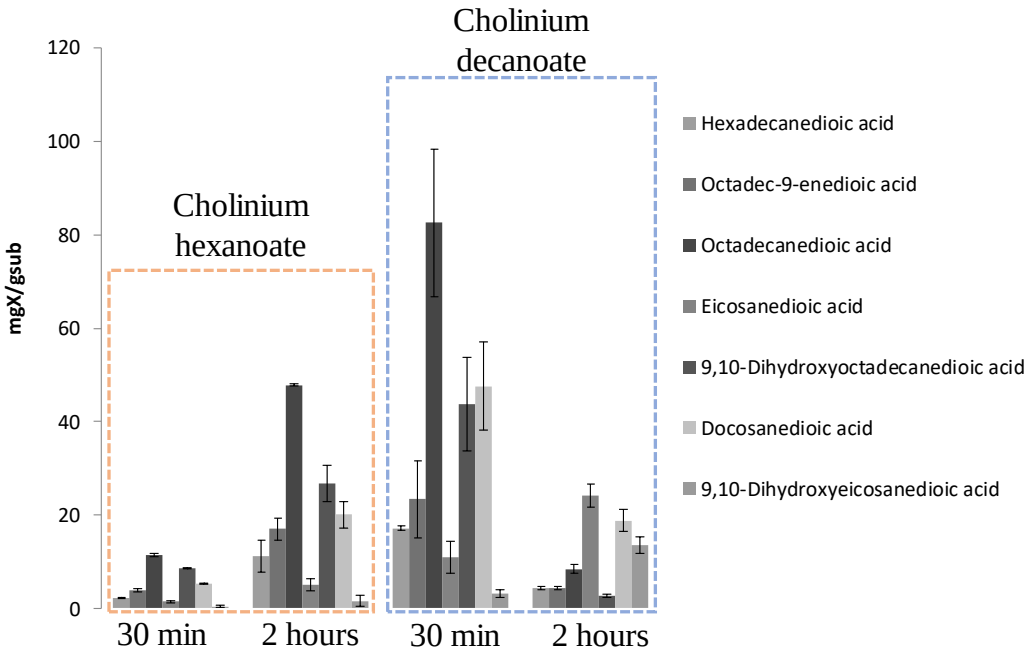


Figure 19 - Identification of the alkanedioic acids found in the extracted suberin samples through GC-MS analysis. An overall increase at 2 h is observed in all compounds for [N $_{1\,1\,1\,2\,0\,H}$][Hex], with a significant increase in octadecanedioic acid.

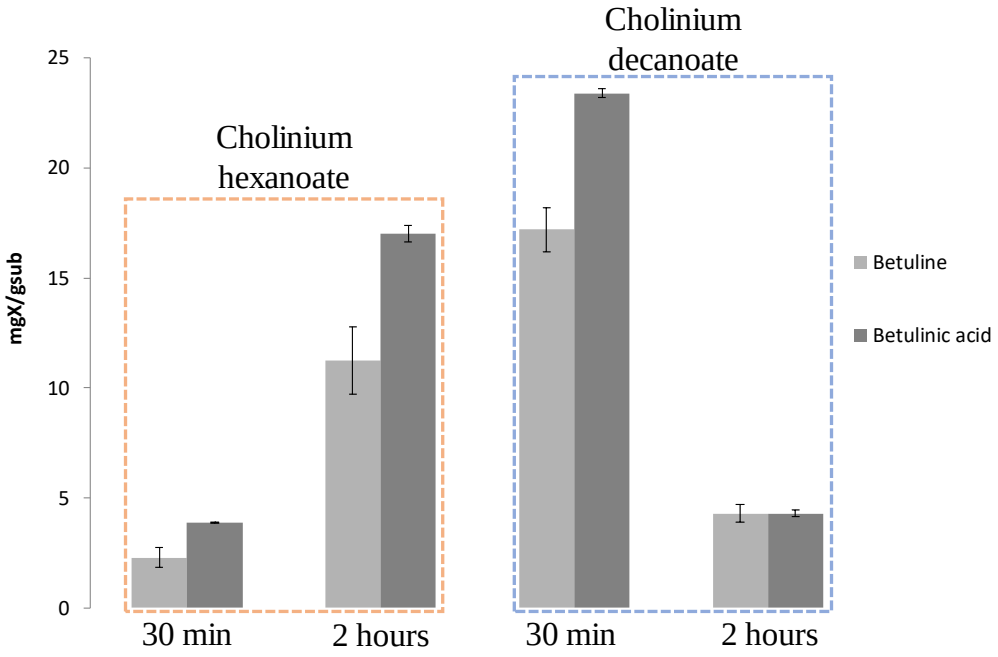


Figure 20 - Identification of steroids and triterpenes found in the extracted suberin samples through GC-MS analysis. Both betuline and betulinic acid show a significant increase for the suberin samples extracted with [N $_{1\,1\,1\,2\,0\,H}$][Hex] for 2 h.

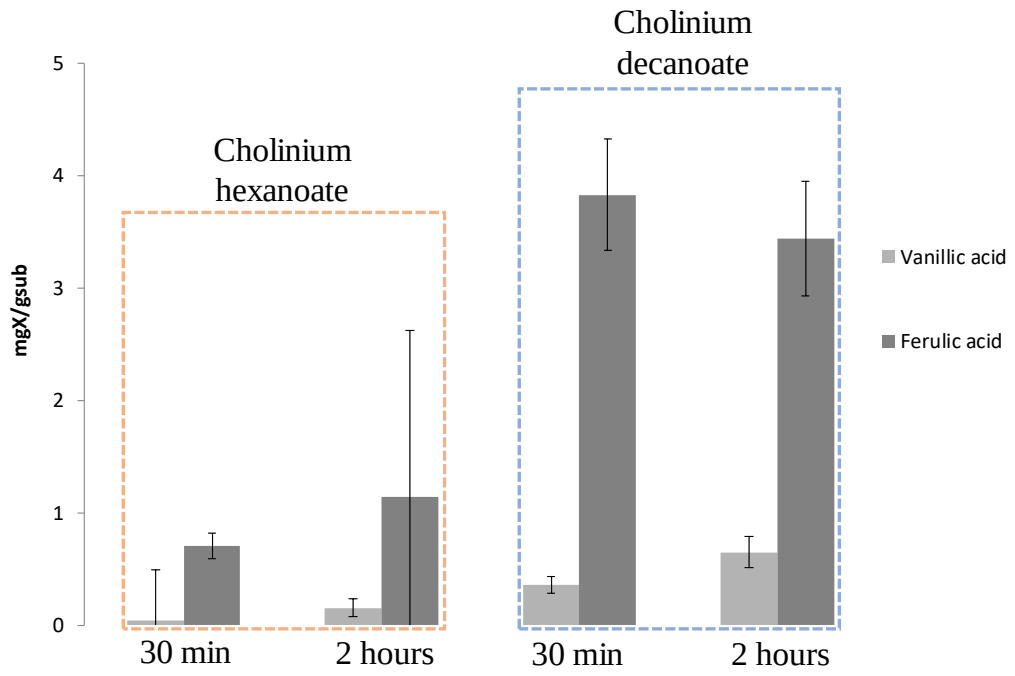


Figure 21 - Identification of aromatics found in the extracted suberin samples through GC-MS analysis. An increase in ferulic acid is observed for the suberin extracted with [N $_{1\,1\,1\,2\,0H}$][Hex] for 2 h, but the standard deviation between measured values is very high.

4.2.5. Determination of the thermal properties of extracted suberin via DSC

The thermal properties of the suberin samples extracted with either [N $_{1\,1\,1\,2\,0H}$][Hex] or [N $_{1\,1\,1\,2\,0H}$][Dec] were assessed through DSC. The DSC thermogram (Figure 22) shows that the suberin samples obtained with both ionic liquids present low crystallinity.

In Figure 22-A (first heating), the melting temperature for the suberin extracted with [N $_{1\,1\,1\,2\,0H}$][Hex] (2 h) is 71 ºC; whereas the melting temperatures for the suberin obtained with [N $_{1\,1\,1\,2\,0H}$][Dec], 30 min and 2 h, are 65 ºC and 75 ºC, respectively. The thermal profile verified for the suberin extracted with [N $_{1\,1\,1\,2\,0H}$][Hex] for 30 min shows that this sample possesses two different melting temperatures (54ºC, 75ºC), possibly being more heterogeneous compared to the other suberin samples. Observation of the crystallization and second heating thermograms (Figure 22-B, C) reveals minor differences in the thermal profile of the samples. The results observed are in accordance to the literature [1].

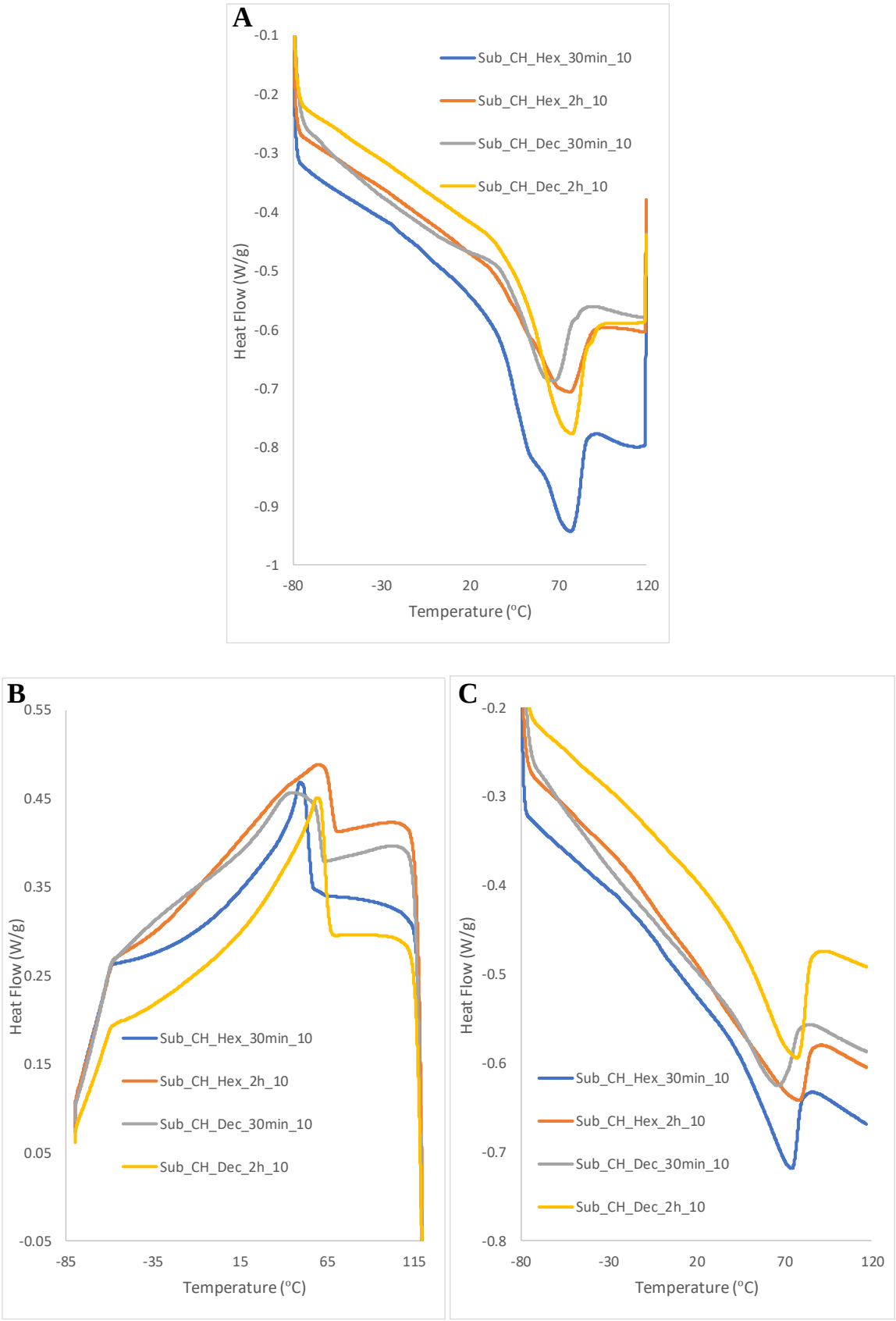


Figure 22 - Differential scanning calorimetry of the suberin samples extracted with either ionic liquid, at 30 min and 2 h extraction time. A – first heating; B – crystallization; C – second heating.

4.2.6. Analysis of suberin particle size distribution in formulations tested in cell culture via DLS

The particle size distribution and polydispersity index (PDI) of the suberin suspensions which were tested in the final cell proliferation assays were first analysed via Dynamic Light Scattering (DLS). The tested formulations were prepared with suberin extracted with [N_{11120H}][Hex] (2 h extraction time), with quantities ranging from 6.25-100 µg/mL. The effects of thermal sterilization and of the addition of PEG 8000 (final concentration 0.5 % w/v) were also assessed (Figures 23-26).

The results showed that the suberin polymer tends to aggregate when in an aqueous suspension; higher concentration of suberin in an aqueous medium resulted in the aggregation of polymer particles of larger average size (Figures 23 and 24). The thermal sterilization strongly influenced the aggregation of the polymer, seen in the formation of suberin particles of much lower average particle size (Figure 24). The aggregation of suberin is probably due to hydrophobic interactions since previous data demonstrated that the addition of salts does not reduce the size of the polymer particles (*unpublished data*).

The addition of PEG 8000 (0.5 % w/v) influenced the aggregation of the suberin particles in the aqueous solution (Figures 25 and 26) but the effects did not show a direct correlation with the concentration of suberin (Figures 25 and 26). In the suberin samples not submitted to thermal sterilisation, PEG addition possibly ensured some dispersion of the polymer, seen in the aggregation of suberin particles of a lower average size particle (Figure 25). However, in the suberin samples which were first thermally sterilised, the particle size was higher in the presence of PEG (Figure 26). One aspect to revisit soon is the stabilisation of the suberin particles in the presence of PEG, since in a standard aqueous medium suberin aggregation will most likely increase along time.

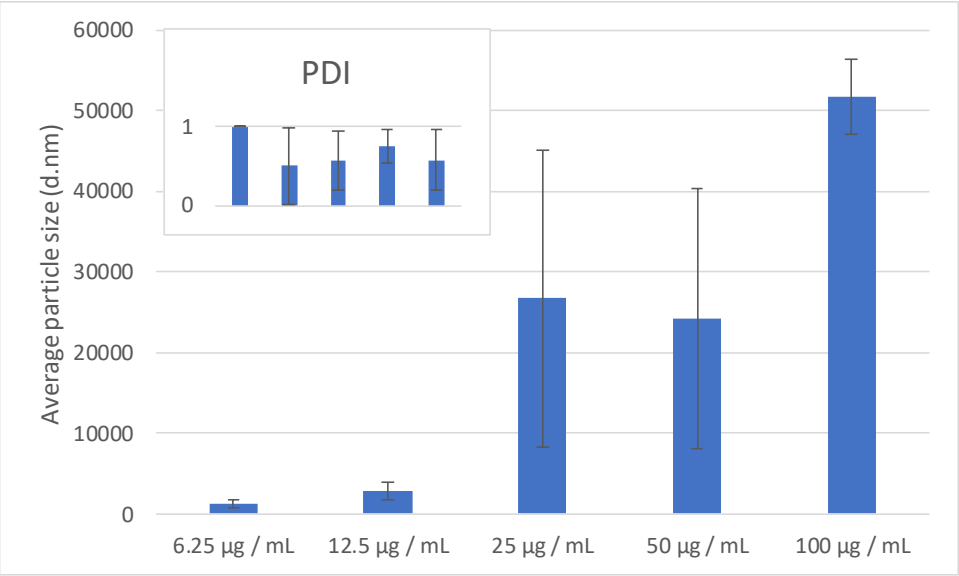


Figure 23 - Evaluation of the average particle size of suberin (non-sterilized) in aqueous solution; suberin concentrations ranging from 6.25 to 100 µg / mL. An increase in suberin concentration leads to an increase in particle size. Inlet: average PDI.

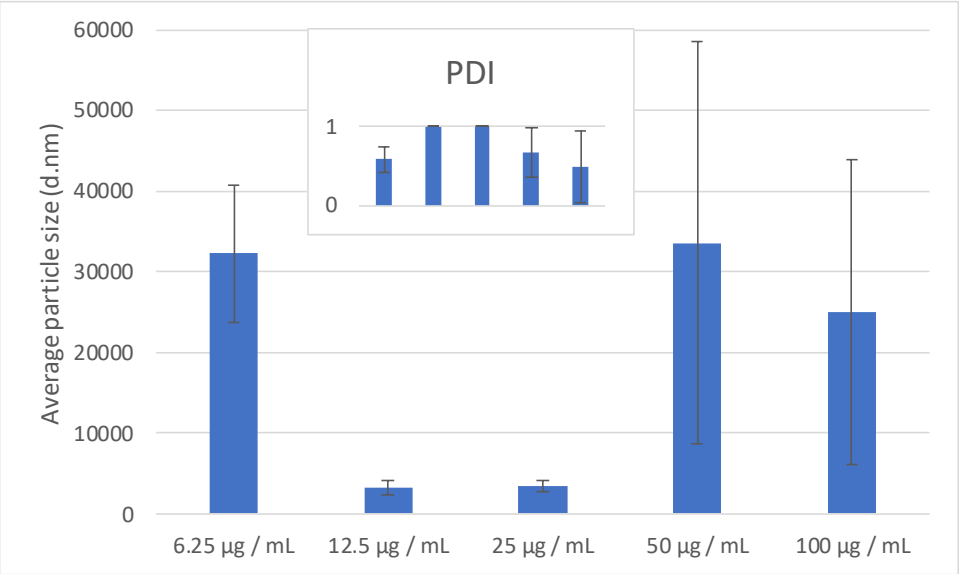


Figure 24 - Evaluation of the average particle size of suberin (non-sterilized) in aqueous solution supplemented with PEG 8000 0.5 % (w/v); suberin concentrations ranging from 6.25 to 100 µg / mL. In general, an increase in suberin concentration leads to an increase in particle size. Inlet: average PDI. In this case, the addition of PEG promoted a decrease in particle size when compared to non-sterilized suberin without PEG.

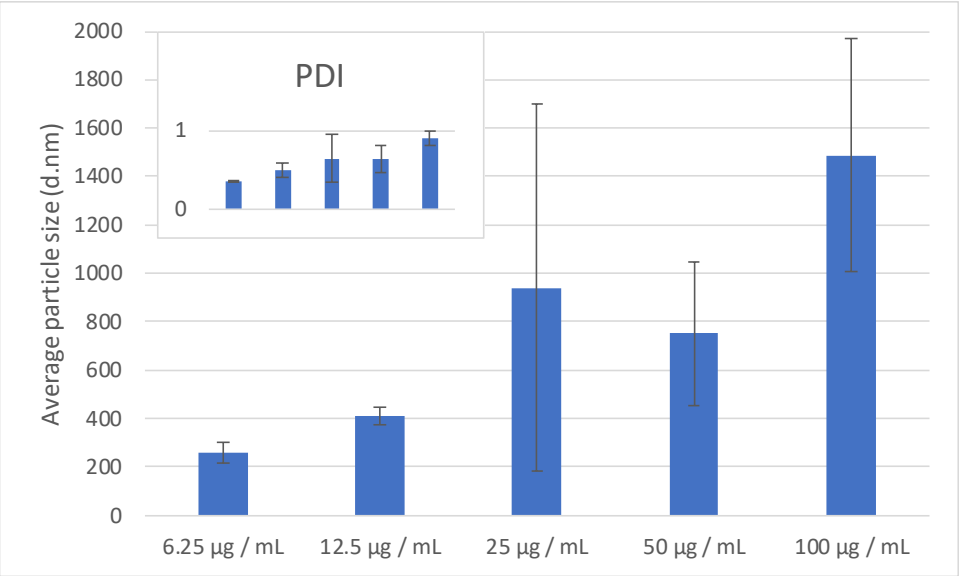


Figure 25 - Evaluation of the average particle size of suberin upon its thermal sterilization in aqueous solution; suberin concentrations ranging from 6.25 to 100 µg / mL. Notice that Y-axis maximum value is 2000 d.nm, several times lower than the maximum value of the other plots presented in this section. Inlet: average PDI. The presented results show that sterilized suberin tends to form smaller aggregates compared to non-sterilized suberin and to suberin formulations prepared with PEG 8000.

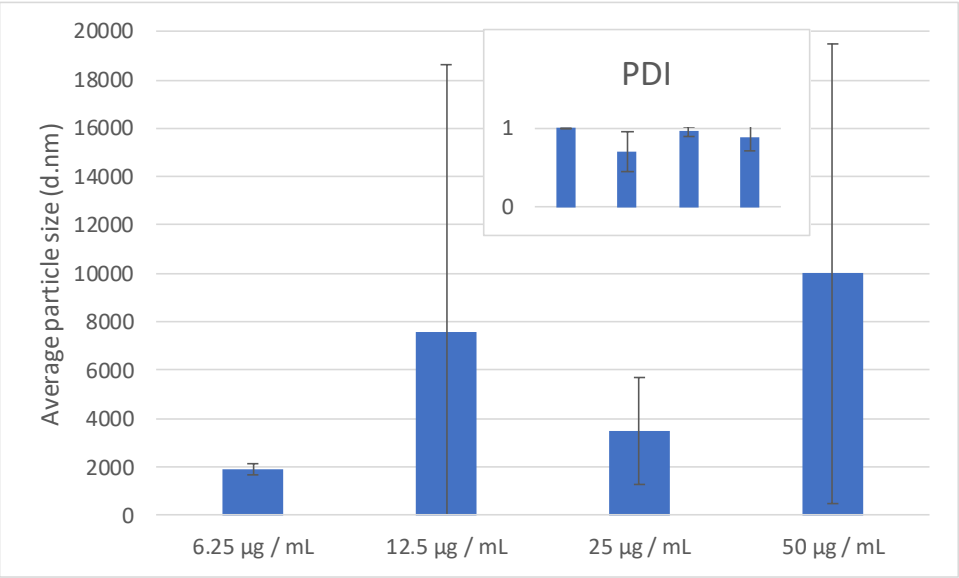


Figure 26 - Evaluation of the average particle size of suberin upon its thermal sterilization in an aqueous solution supplemented with PEG 8000 0.5 % (w/v); suberin concentrations ranging from 6.25 to 100 µg / mL. An increase in suberin concentration leads to an increase in particle size. Inlet: average PDI. The presented results show that sterilized suberin tends to form smaller aggregates, compared to the other conditions herein tested. The addition of PEG also leads to an increase in average particle size, compared to the sterilized suberin *per se* ..

4.2.7. Optimization of suberin films

Suberin extracted with [$N_{11\ 120H}$][Hex] was used to cast films. The fabrication was optimized to ensure recovery of films resistant to handling, hence suitable to be placed in contact with cell cultures. The suberin suspensions were homogenised prior to the casting using distinct physical treatments such as sonication or agitation. Some of these physical treatments have been tested before in the lab and were shown to have the potential to reduce the size of the suberin particles formed due to their aggregation in aqueous suspensions (*unpublished data*). Figure 27 shows that the best films were obtained when only agitation was applied to the suberin suspension. On the contrary, the use of sonication treatments originated brittle films which were difficult to recover and handle. The casting of the films can be influenced by the rate of water evaporation; herein we have used standardised conditions for the casting under defined conditions of temperature and humidity.

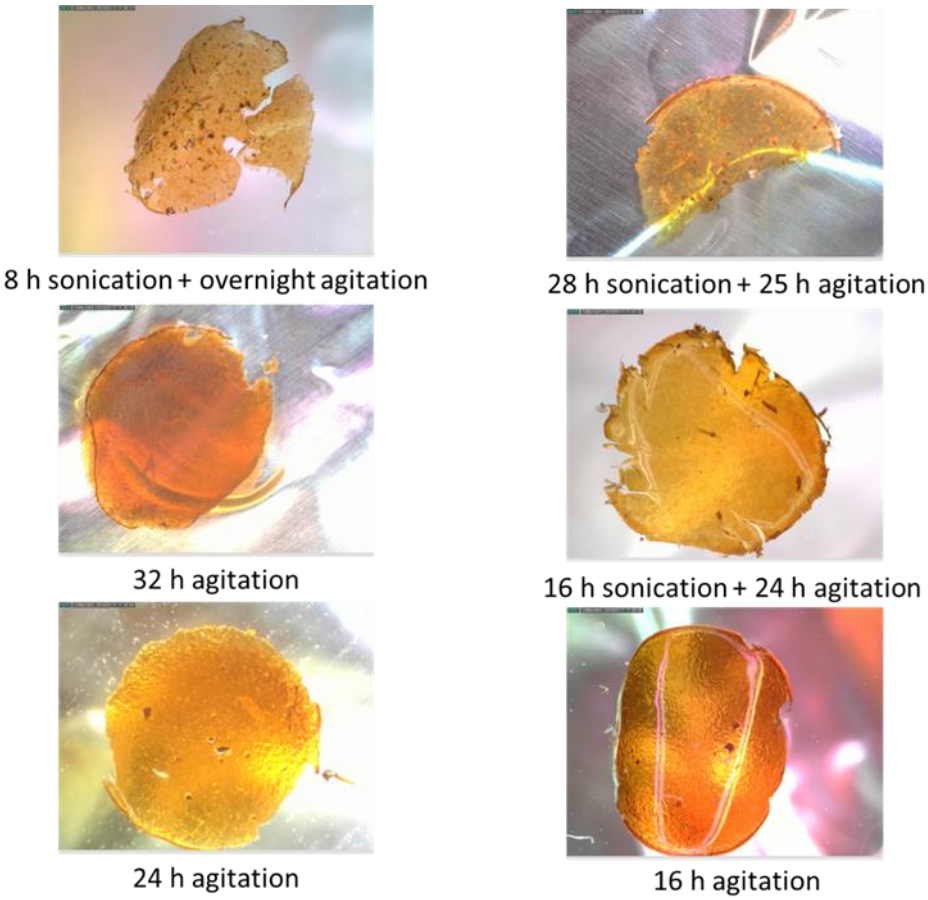


Figure 27 – Photos of the various suberin films cast. Only films that could be recovered from the surface were photographed. Agitated films are the best in terms of appearance and integrity. Films that were sonicated were shown to be more brittle and difficult to recover

5. Conclusions and future work

The results presented in this chapter have showed that it is possible to extract high quality suberin from cork using the previous established ionic liquid process in a small scale reactor. The use of stirring (not continuous) was crucial to achieve a good extraction yield. The reaction catalysed by [N_{1112OH}][Hex], which has been optimised before, progressed as expected. Nonetheless, for the first time in the host lab, we have tested the suitability of using as catalyst for suberin extraction from cork the commercial [N_{1112OH}][Dec]. The obtained results were promising, but the recovery of suberin by centrifugation was very inefficient, however that which was recovered (limited in amount and possibly not representative) was apparently free of contaminants (*e.g.* ionic liquid). Finally, the observation that suberin extracted with [N_{1112OH}][Dec] cannot be easily recovered by centrifugation (in opposition to that extracted by the other ionic liquid) together with a significant removal of the polymer fraction (mass of the insoluble residue) suggests that the ensuing polymer is less cross-linked that that isolated by the [N_{1112OH}][Hex]. These are exciting results as they suggest that the fine-tuning of the anion in the cholinium-based ionic liquid may be used to recover polymers with distinct degrees of cross-linking and density. This evidence opens new possibilities for the design of new isolation/fractionation methods aiming to isolate very homogenous fractions of the suberin polymer or to avoid its aggregation.

Therefore, future work concerning should focus on optimizing the recovery steps for suberin extracted with [N_{1112OH}][Dec] - for example by replacing the precipitation by membrane-based filtration; followed by a deep characterisation of its chemistry similar to that established for the suberin extracted with [N_{1112OH}][Hex]. Some changes in the reactor could be made as well, such as providing cooling to prevent overheating of the motor and allow for continuous stirring.

Chapter 2

In vitro determination of the wound healing potential of suberin

6. Introduction

6.1. *In vivo VS in vitro* models

New cosmetics and pharmaceutical products need to be extensively tested before release into the market. Strict laws in the European Union and in other countries, such as the United States of America, determine safety concerns that need to be cleared before a product is accepted for commercialization. The *in vivo* and *in vitro* approaches allow the testing of these products and the determination of the factors that may result in the acceptance or denial of a product.

In vivo, meaning "in a living organism", stands for the experiments and procedures that take place in a complex organism (*e.g.* mice, humans). As pros for this approach, the fact that a complex organism will reflect in more detail and with less bias the interaction with the drug. There are various limitations, such as the variability between organisms and the difficulty of setting up experiments, the fact that differences between species may lead to erroneous conclusions on the effects of a drug and the costs related to *in vivo* experimentation. Also, the risks *vs* benefits must be thoroughly assessed before conducting *in vivo* experiments, and therefore *in vitro* experimentation is needed as a first step before conducting an *in vivo* approach. While controllable to some extent, the *in vivo* approach is not as standardized as an *in vitro* approach.

In vitro means "in a glass" and stands for experimentation that is performed outside of an organism, in an experiment set-up to mimic or reflect part of an organism. The *in vitro* approach offers the pros of a controllable, easy to set up experimentation often presenting reduced costs. The tissues used for experimentation are also standardized for each experiment (*e.g.* keratinocytes sourced from the same donor and used at the same generation). The limitations of this approach are the fact that a whole organism is not reflected in an experiment, therefore, results may be biased due to a lack of interactions with other cells, tissues and fluids like in a whole organism. Due to this fact, products may be tested in a wide array of *in vitro* experiments comprising different tissue/organ models but must be tested *in vivo* in animals and humans prior to release. Still, great progress has been made to arrive at substitutes closer to the whole organism (*e.g.* organ-on-a-chip, human-on-a-chip). In the context of the present thesis we will take advantage only of *in vitro* methods, especially as our prime goal is to assess the potential use of suberin in wound management.

6.2. The skin

The skin is the largest organ of the human body and forms the integumentary system with the accessory structures hair, glands and nails. It performs various functions [92–94]:

- Barrier. It covers the whole body and protects it from environmental aggression. It prevents the entrance of external microorganisms that may cause disease while avoiding the loss of essential bodily fluids and water, preventing dehydration;
- Sensory. The nervous system found in the skin allow for the feeling of heat, cold, pressure and pain;
- Temperature regulation;
- Vitamin D production, essential for the metabolism of calcium and phosphorus;
- Excretion of waste through sweat.

The appearance of skin is also an important factor in diagnosing diseases that have an impact on its overall structure. The various functions performed by the skin are related to its intricate structure and inside interactions as well as with the other organs [92].

The following sections describe in more detail the constitution of the skin, as well as the reason to choose epidermal cells as *in vitro* models for wound healing.

6.3. Constitution of the skin

Figure 28 represents the various layers of the skin, along with some structures found in it.

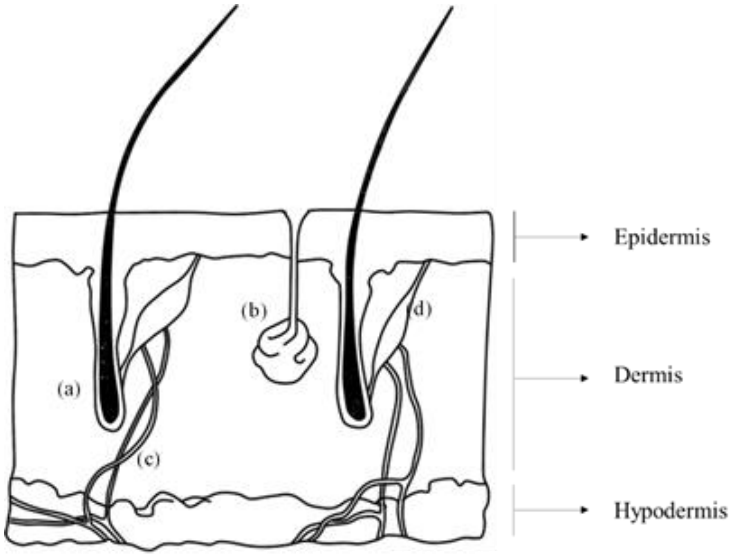


Figure 28 – Schematic representation of the layers that constitute the skin. Some structures found in the skin are also represented: (a) hair and hair follicle, (b) sweat gland, (c) blood vessels, (d) muscles. Adapted from [3].

6.3.1. The hypodermis

It is the innermost layer of the skin. It acts as a supporting layer, connecting the skin to the muscle and/or bone and includes blood vessels and nerves. It is mainly constituted by adipocytes, which store energy as fat and provide thermal insulation and shock-absorption. Macrophages and fibroblasts are also found in this layer of the skin [92,93,95].

6.3.2. The dermis

Found right above the hypodermis, the dermis is mainly constituted by fibroblasts and collagen type I (mature collagen). It accounts for *ca.* 90% of the weight of the skin and gives it structural integrity [92,93].

6.3.3. The epidermis

The outermost layer of the skin, the epidermis is mainly constituted by keratinocytes. It continuously renews itself: keratinocytes migrate from the basal layer (*stratum basale)* to the apical layer, where they arrive as dead cells composed mainly of keratins forming an impermeable barrier, called *stratum corneum*.

Keratinocytes produce keratin, a protein mixture which adds mechanical strength to the skin, hair and nails. Keratins have a structural role and belong to the family of intermediate filaments (cytoskeletal components). They may be divided as either acidic (type I, K9-K19) or basic-to-neutral (type II, K1-K8) and form filaments that contain both an acidic and a basic keratin [93,96]. These cells are responsible for the minimization of water loss through the skin and provide protection against microorganisms and abrasion on the skin's surface. The epidermis is commonly separated into 4-5 *strata* corresponding to the stages of differentiation of keratinocytes as they migrate to the outermost layer. These layers are, from the bottom to the top layer [93,96]:

- The *stratum basale.* This is the innermost layer of the epidermis and consists on a single layer of cuboidal keratinocytes that are linked between themselves through desmosomes and to the basement membrane by hemidesmosomes. The cells found in this *stratum* are mitotically active and are continuously dividing;
- The *stratum spinosum;*
- The *stratum granulosum;*
- The *stratum lucidum;*

- The *stratum corneum.* The outermost *stratum* of the epidermis, it is constituted by 25 or more layers of corneocytes (dead keratinocytes). Corneocytes are held together by corneodesmosomes. When these modified desmosomes break apart, the cells slough off from the surface of the skin.

Figure 29 depicts a representation of a transversal cut of the epidermis and the various layers that constitute it.

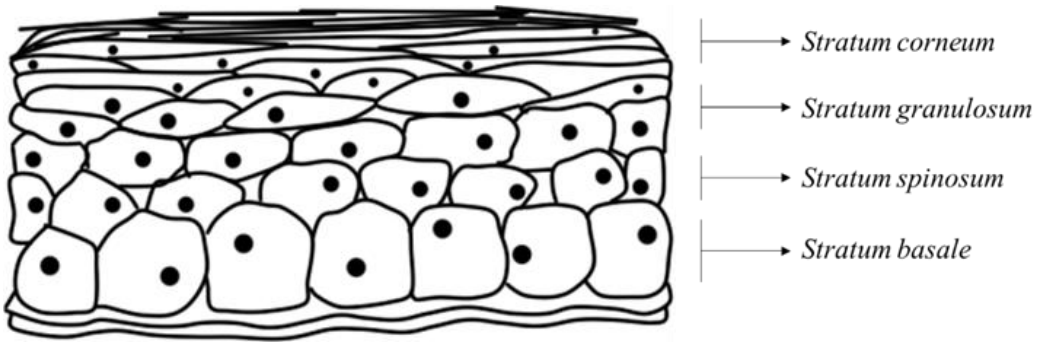


Figure 29 – Schematic representation of the *strata* found in the epidermis. *Stratum lucidum* is not represented. Adapted from [4].

6.4. Keratinocytes as *in vitro* models of wound healing

Keratinocytes are the main constituents of the epidermis, the outermost layer of the skin. They originate in the *stratum basale* and progress through the epidermis until they reach the *stratum corneum* as corneocytes, where they act as an impermeable barrier that protects the organism from the outer environment and microorganisms.

When the skin is wounded its structure and function are compromised. To achieve wound healing, re-epithelialization of the damaged tissue must occur. Keratinocytes are very important cells for re-epithelialization: they communicate with fibroblasts [97] and other cells [98], secreting cytokines that regulate inflammatory reactions (*e.g.* interleukin-17 [99]) and producing extracellular matrix (ECM). They play an important role in debridement of the wound: dead or damaged cells are substituted by live cells that promote healing at the wound edges. This makes keratinocytes important models (and our selected model) for the study of drugs that may affect the wound healing cycle [98].

7. Materials and methods

7.1. Culture of keratinocyte cells for proliferation and stock preparation

7.1.1. Materials

a) Human Epidermal Keratinocytes, neonatal (HEKn) ref. C-001-5C, GIBCO;
b) Culture medium: Epilife medium (Cascade Biologics™ cat. no. M-EPI-500-CA) supplemented with antibiotics (50 U/mL penicillin G and 50 µg/mL streptomycin) and HKGS (Cascade Biologics™ cat. no. S-001-5) final concentrations 0.2 % v/v Bovine Pituitary Extract (BPE), 0.2 ng/mL human recombinant Epidermal Growth Factor (EGF), 0.18 µg /mL hydrocortisone, 5 µg /mL insulin and 5 µg /mL transferrin;
c) Blocking solution: Epilife medium (Cascade Biologics™ cat. no. M-EPI-500-CA) containing 2% v/v foetal bovine serum (FBS)(cat. no. 1050-064, GIBCO);
d) Dulbecco's Phosphate Buffered Saline (DPBS), no calcium, no magnesium 500 mL, catalogue number:14190144, GIBCO;
e) Trypsin-EDTA 0.05% v/v, ref. 25300-054, GIBCO;
f) Cell cryopreservation solution: Epilife medium (Cascade Biologics™ cat. no. M-EPI-500-CA) containing 7 % dimethyl sulfoxide (DMSO)(cat. no. D2650-100ML, Sigma-Aldrich) and 10 % FBS (cat. no. 1050-064, GIBCO).

7.1.2. Cell culture protocol

The protocol used for the cell culture was adapted from De Vuyst et. al[100]

a) Cell thawing: a cryovial containing HEKn cells is recovered from liquid nitrogen storage and quickly thawed in a 37 ºC water bath. The cells are carefully pipetted onto 14 mL of warm culture medium and seeded in a 75 cm^2 T-Flask (VWR). After 24 h of incubation (NUAIRE, 37º C, 5%CO_2/95% air) cells are adherent and medium is changed for fresh medium. Medium is then changed every other day;
b) Cell passaging: when confluency reaches 70%/80% aspirate the culture medium and add 5 mL of DPBS to wash cell monolayer. Remove DPBS and add 3 mL of trypsin solution. Place keratinocytes in the incubator for 7-8 min, then carefully hit the side of the T-flask to help detach cells. Check cell detachment on the inverted microscope (NIKON Eclipse TE2000-S). If cell detachment is *ca*. 95%, add 12 mL of blocking solution. If cells are not detached, place T-flask in the incubator for another minute and check cell detachment again. Separate the cells in three 75 cm^2 T-flasks and add 10 mL culture medium to each (total volume of 15 mL). After incubating for 24 h, change for fresh medium;

c) Cell cryopreservation: after the passaging procedure, centrifuge cells at 335 x g for 10 min (Heraeus Sepatech). Remove supernatant and add cell cryopreservation solution in order to reach a final concentration of (at least) 10^6 cells/mL. Place 1 mL of cell suspension in each cryovial (Nalgene) and place cryovials in a slow cooling device such as Mr. Frosty™ (Nalgene) or CoolCell™ LX Freezing Container (Biocision)) in a -80 ºC freezer, overnight. Store cells in the gas-phase of a liquid nitrogen storage tank.

7.2. Trypan blue live/dead exclusion assay

The trypan blue live/dead exclusion assay was used to determine the number of live cells per mL.

An hemocytometer is used to count live cells after staining with trypan blue reagent (GIBCO, cat. no. 15250061). After passaging, cells are centrifuged at 335 x g for 10 min (Heraeus Sepatech) and the supernatant is removed. Cells are resuspended in 1-2 mL of warm culture medium and 20 µL are pipetted, after homogenizing, into an eppendorf. 20 µL of trypan blue reagent are then mixed with the cell suspension sample. 10 µL of the resulting solution are pipetted onto the hemocytometer and live cells are counted using an inverted microscope (NIKON Eclipse TE2000-S). The concentration of live cells is determined according to Equation A.

$$Average(cell\ counts) * Dilution\ Factor * 10\,000 = \frac{Cells}{mL} \qquad \textit{Equation A}$$

7.3. Cellular viability assays

7.3.1. Pierce's Bicinchoninic Assay (BCA)

Pierce's BCA total protein assay kit (Pierce, Rockford, IL) was used to determine the total amount of protein per well, according to the manufacturer's instructions with some adaptations.

Cells were seeded on 24-well plates with culture medium. After 24 h, medium was changed for testing formulations and incubated for 96 h. The medium was then aspirated and 200 µL of NaOH 0.1 M were added to each well to lyse all cells and left to incubate for 4 h.

Working reagent was prepared (50:1, reagent A to reagent B ratio). The provided albumin protein standards for the calibration curve were prepared (working range 5 to 250 µg/mL).

200 µL of working reagent were added to each well in a 1:1 working reagent to sample ratio. Samples were placed in an oven at 60 ºC for 30 min to enhance colour development. 100 µL of each sample were transferred to 96-well plates and absorbance was measured at 562 nm. All assays were performed in triplicate.

7.3.2. Cell viability assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay protocol used for the measurement of relative cell viability was adapted from the literature [101].

Cells were seeded (5000 cells/well) in 24-well plates with culture medium. After 24 h, medium was changed for testing formulations and incubated for 96 h.

A 5 mg/mL MTT solution was prepared and 50 µL were pipetted into each sample. Plates were wrapped in foil and placed in the incubator for 4 h. Medium was discarded, and the cell monolayer was gently washed with DPBS before adding 200 µL of DMSO to dissolve the formed formazan crystals. Plates were wrapped in foil and agitated for 1 h at 128 rpm. 100 µL of each sample were pipetted onto a 96-well plate to measure absorbance at 520 nm. All assays were performed in triplicate.

7.3.3. Gap-closure assay

CytoSelect's 24-well wound healing assay kit (Cell Biolabs, Inc) was used following the manufacturer's instructions.

Cells were seeded ($5x10^5$ cells/well) in 24-well plates, with inserts placed to create a gap. (Figure 30) Inserts were removed after 24 h and medium was changed for testing formulations. Gap-closure over time was monitored at regular intervals. Pictures were taken and Image-Pro Plus 7.0 and Image-J software was used to determine the distance between gap margins. The measured distances were then used to calculate the gap-closure rate.

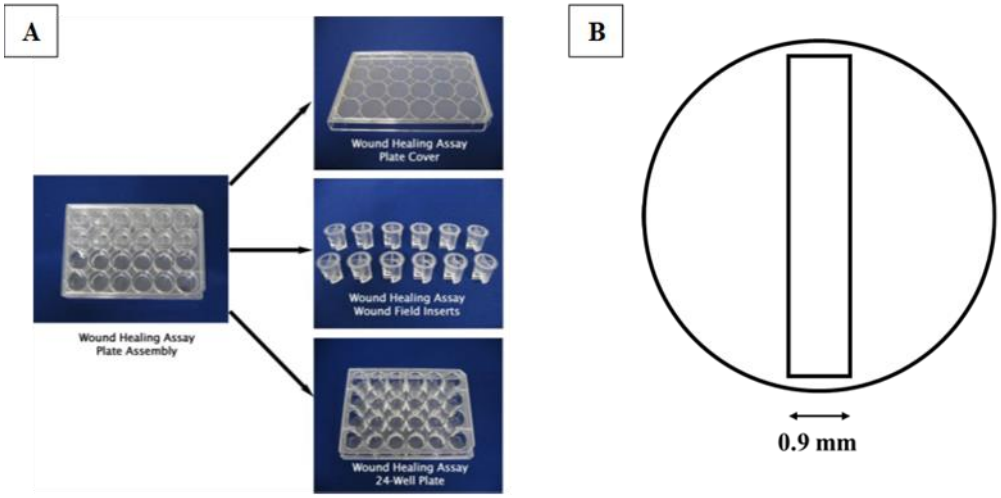


Figure 30 – A) CytoSelect's 24 well wound healing assay kit materials. B) schematic representation of a gap created in a cell monolayer with the inserts.

8. Results and discussion

8.1. Initial formulation screening

To analyse the influence of the polymer suberin on cell proliferation we have focussed on suberin samples which were extracted from cork for 2 hours with [N_{1112OH}][Hex]. To obtain a higher degree of homogenisation of the suberin suspensions we have applied distinct physical treatments (e.g. ultrasonication, sonication, agitation). Moreover, in addition to suberin suspensions (with or without the dispersion agent PEG), we also tested the effect of suberin films (Figure 31). In all these initial cell proliferation experiments the same suberin amount was used (approximatively 600 μg *per* well). Moreover, at this stage we have used the commercial powder Septil (composed of iodinated polyvinylpyrrolidone) as a positive control, which has been shown in previous studies to display some positive effect on cell proliferation [35,36].

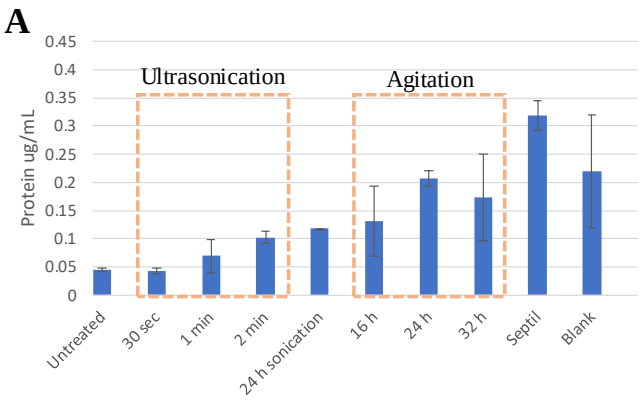
Cell proliferation was evaluated indirectly by the amount of total protein (see Materials and Methods). Proliferation refers to cell replication, which we did not measure – the assays herein performed were only correlated to proliferation, as these is a very preliminary screening. In the presence of suberin in suspension the level of cell proliferation was similar to that of the blank (negative control, *i.e.* culture medium) when prior to usage the suberin suspension was agitated for 24 hours or longer (Figure 31, A). For the untreated samples or those treated through ultra/sonication or agitated for a period below 24 hours, suberin apparently reduced cell proliferation. The only sample that stimulated cell proliferation above that of the blank was the positive control – Septil (consistent with previous publications [35]). However, the mechanism of action of Septil cannot be correlated to that of suberin which remains largely unknown. The use of a positive control at this stage served merely as an indicator of the functionality of these assays.

Surprisingly, when applying suberin as a film (instead of a suspension) we observed a significant increase in cell proliferation, above that of the blank and in some specific cases even above that of the Septil positive control (Figure 31, B). In particular, the best results were found for suberin films prepared with suberin suspensions treated for 32 hours with agitation. The application of a grinded film (*i.e.* pulverized), which was initially prepared with suberin suspensions treated for 16 hours with agitation then grinded using a tissuelyser, showed also positive effects on cell proliferation, slightly above that of the blank (Figure 31, C). These results may indicate that the spontaneous self-assembly of the polymer suberin [40] may augment its function as a cell proliferation agent.

PEG was used as a surfactant to enhance the dispersion of suberin fractions, even though the effect on the particle size of suberin at the concentrations used in these assays was not evaluated. To undertake this assay, we have used a suberin sample which was first

homogenised using 24 h of sonication followed by 24 hours of agitation. This treatment was chosen as it was observed in other assays to reduce the aggregation of suberin to particles of about 1 µm (*unpublished data*). The addition of PEG (50%) to the suberin suspension increased cell proliferation to levels comparable to those observed with the positive control, and above that of the blank (Figure 31, D). The tests done only with PEG suggested that PEG 3350 and 20000 did not affect cell proliferation, whereas PEG 8000 reduced cell proliferation to *ca.* half (Figure 31, D). For the subsequent experiments, we decided at this stage to pursue the usage of PEG 3350 and 8000. As mentioned in the introduction, suberin is a polymer composed of a myriad of long fatty acid chains. Since suberin is recovered as an oligomer with the method herein used, its molecular size is still considerable – therefore, the usage of a comparable size PEG (such as the 20000) is not recommended [102–104].

Although the results observed at this stage showed that suberin films were the most promising formulations, particularly the suberin film produced with a 32 h agitation treatment, the next assays focussed on the use of suberin suspensions stabilised through the use of PEG. This decision was taken because it was difficult to ensure reproducibility when placing the suberin films into the culture wells as the films rapidly adhere to the sides, top and/or bottom of the well. To allow a direct comparison of the follow-up experiments with this experimental dataset we kept the use of suberin samples which were first homogenised using 24 h of sonication followed by 24 hours of agitation, notwithstanding other physical treatments that influence suberin aggregation and also deserve further investigation.



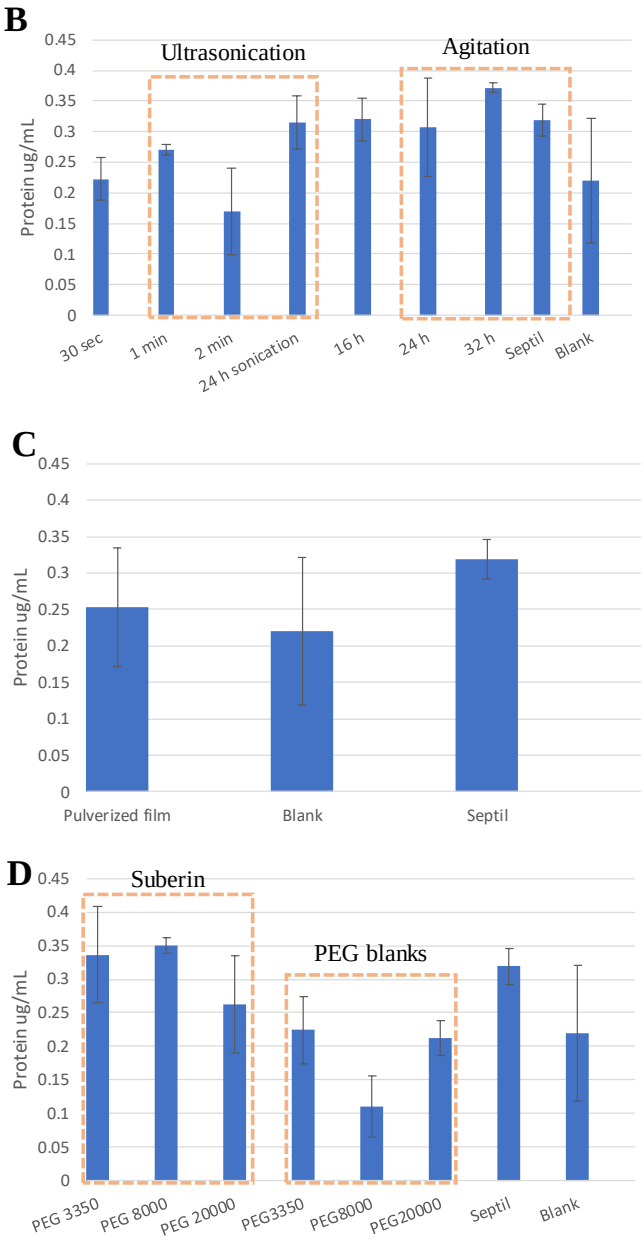


Figure 31 – Assessment of the effect of suberin formulations on cell proliferation which was inferred by the total protein amount (BCA assay). A – Impact of suberin submitted to distinct physical treatments on cell proliferation; B – Influence of distinct pre-treatments on the effect of the obtained suberin films on cell proliferation; C – Impact of a pulverised suberin film (retrieved from a suberin sample pre-treated with agitation for 16 hours) on cell proliferation; D – Impact of the addition of PEG on the effect of suberin (retrieved from a suberin sample pre-treated with agitation for 16 hours) on cell proliferation. The use of PEG was shown to boost cell proliferation on these preliminary screenings.

8.2. Assessment of the effects of the addition of PEG 3350 or PEG 8000 and sterilization on cell proliferation

To deepen the study of the effects of suberin on cell proliferation we decided to measure metabolic activity - MTT assay - in addition to total protein amount. This assay measures the metabolic rate of mitochondria as they metabolize tetrazolium into its insoluble formazan form.

It was introduced because it is another fast and easy way of assessing cellular viability that correlates with cellular proliferation. The effects of PEG on cellular proliferation were first tested (Figures 32-34). Both PEG 3350 and PEG 8000 were tested, at a 50 % (w/v) concentration, on sterilized supplemented media (Figure 32).

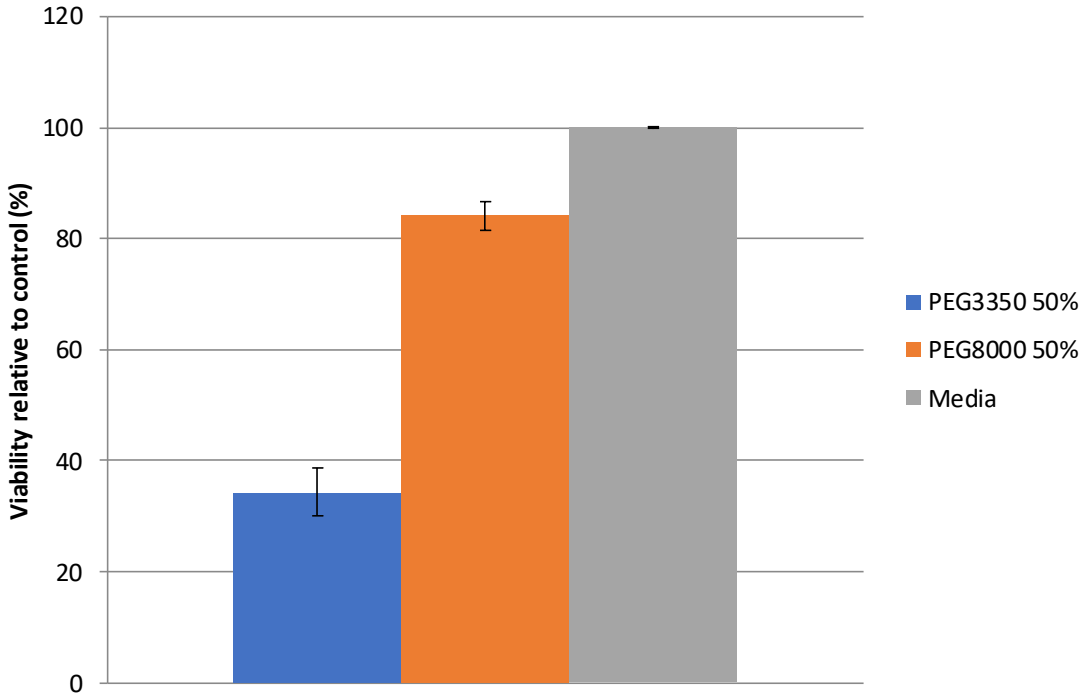


Figure 32 – Determination of the impact of PEG 50 % (w/v) on cell proliferation. The results show that PEG 3350 displays more toxicity when compared to the same (w/v) concentration as PEG 8000.

The results show that at this concentration PEG 8000 reduced cell proliferation only slightly but PEG 3350 had a rather negative impact. The negative impact of PEG 3350 could be circumvented by reducing its concentration to 1% (w/v); a direct correlation with the increase of PEG 3350 concentration and the reduction of cell proliferation was observed (Figure 33). PEG 8000 concentrations ranging from 0.5 % to 5 % (w/v) showed a low impact in cell proliferation (Figure 34) which was similar to that observed with a much higher concentration (50% w/v, Figure 32). In all the above cell proliferation assays (either using total protein or metabolic activity) the culture medium was subjected to a thermal sterilisation process.

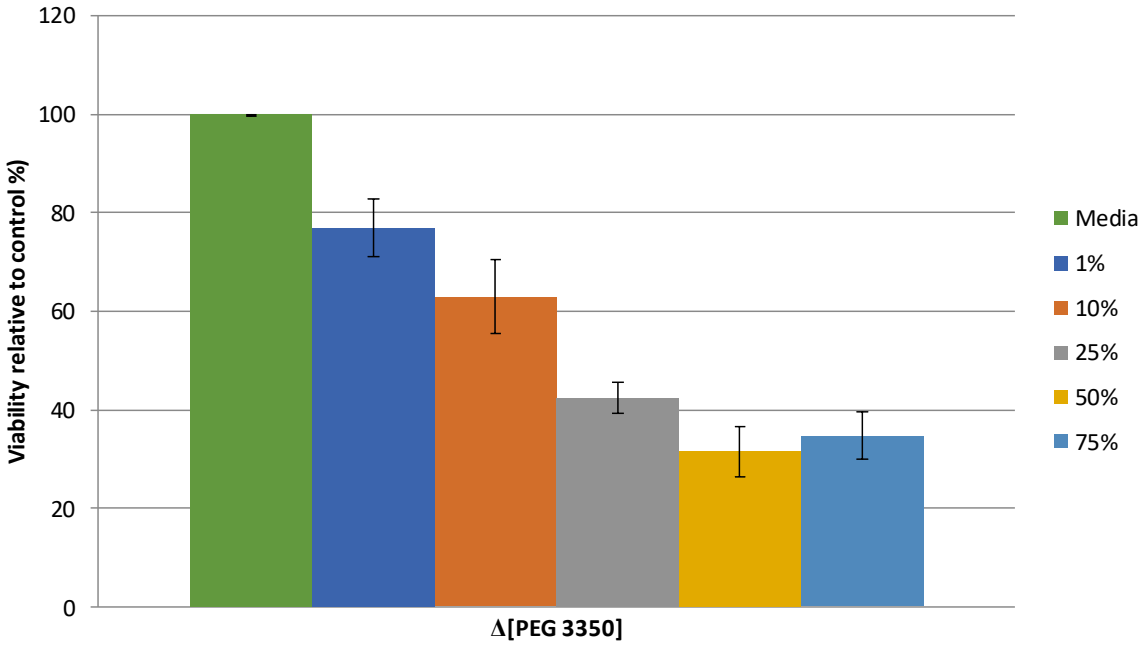


Figure 33 – Determination of the impact of PEG 3350 on cell proliferation. A range of concentrations (1-75% (w/v)) was tested. The results showed that PEG 3350 still displayed considerable toxicity at the lowest concentration tested.

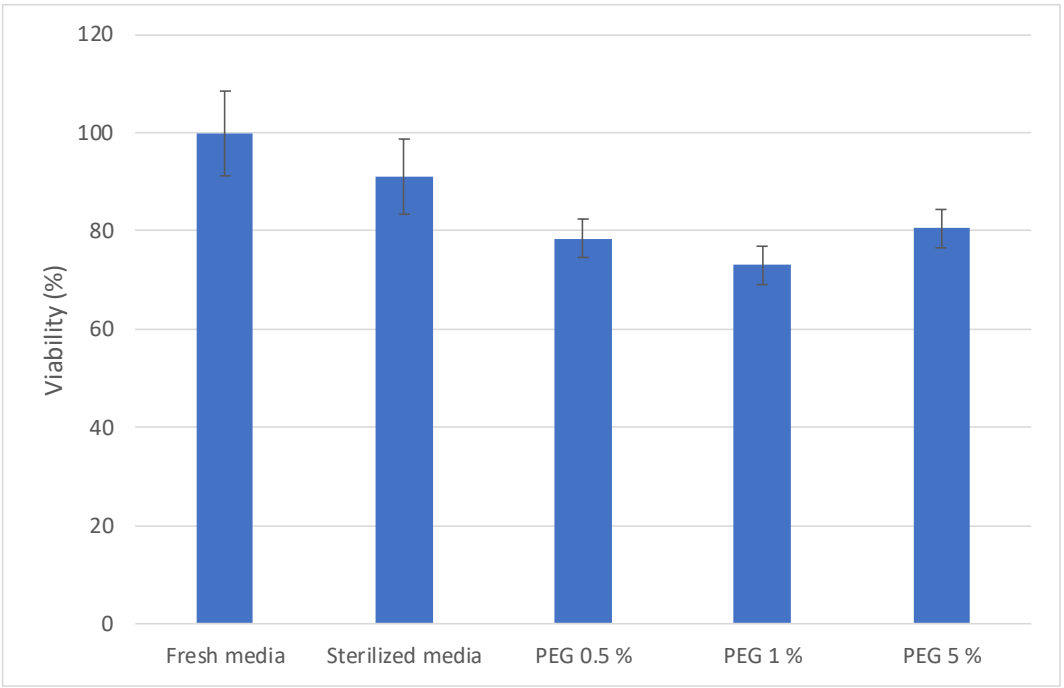


Figure 34 – Determination of the impact of PEG 8000 on cell proliferation. A range of concentrations (0.5-5% (w/v)) was tested. It was shown that sterilization also contributed to a decrease in cell proliferation.

At this stage, we hypothesised whether the thermal treatment of the culture media could be responsible for the reduction of cell proliferation observed, which was systematically below 20% (Figures 32-34). The thermal treatment could have led to the precipitation of proteins which are essential for cell growth. Regardless of that loss of nutrients, this aspect did not

hinder the identification of the stimulating effects of the medium supplements (either suberin or Septil), but their effect might have been underestimated. Indeed, the sterilisation of the culture media reduced cell proliferation by *ca.* 10% (Figure 34). To further understand the impact of sterilization on cellular proliferation, an array of assays comparing the effects of the sterilization of the media (with or without PEG 8000 0.5 % (w/v) (Figures 35 and 36) and of suberin (with concentrations ranging from 0-50 µg/mL) (Figure 37) were performed. Interestingly, we observed that if the addition of the HKGS supplementation (see Materials and methods) is done after the sterilization of the culture medium, no reduction in cell proliferation was observed (Figures 35 and 36). This procedure was therefore used in the remaining experiments that focussed the usage of suberin formulations.

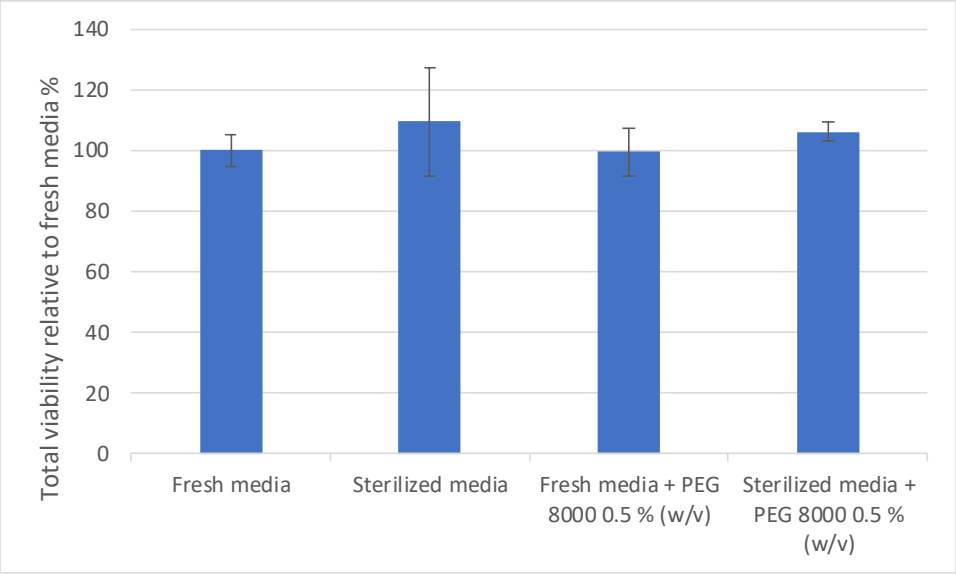


Figure 35 – Impact of the sterilization on cell viability. The results contradict the previously observed results. The variability is also high.

The ability of suberin to stimulate cell proliferation when inferred through the total protein content of the cells was made apparent (Figure 37). In fact, both the untreated and the thermally sterilised samples showed the same ability, promoting *ca.* 20% increase in cell proliferation at most of the concentrations tested. No correlation between the tested concentration and the total protein amount could be established. When the cellular viability was used as an indirect mean to measure cell proliferation the results were disappointing since suberin impact on cell proliferation remained undetected (Figure 37 A, non-sterilised suberin) or were negative (Figure 37 B). Because of this discrepancy we decided to resort also to cell count. The number of cells increased significantly when exposed to the non-sterilized suberin (Figure 37 A). This observation reinforces the idea that suberin might indeed stimulate cell proliferation, nonetheless the cell count showed many technical difficulties since we could not cover the

whole culture well with the used inverted microscope. A more robust and sophisticated microscopy-based method was established in the last stage of this thesis (see gap-closure protocol) to measure the rate of gap-closure instead of counting individual cells.

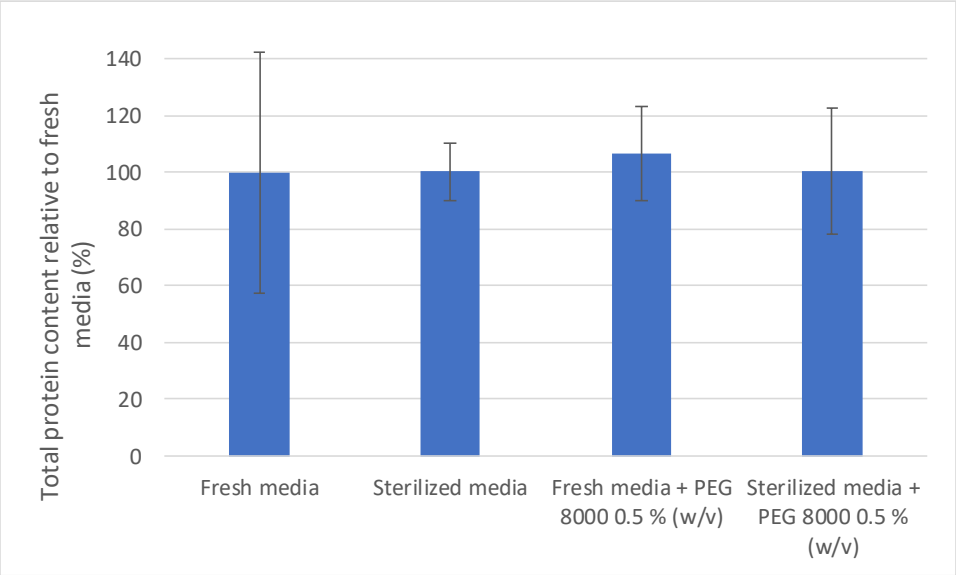
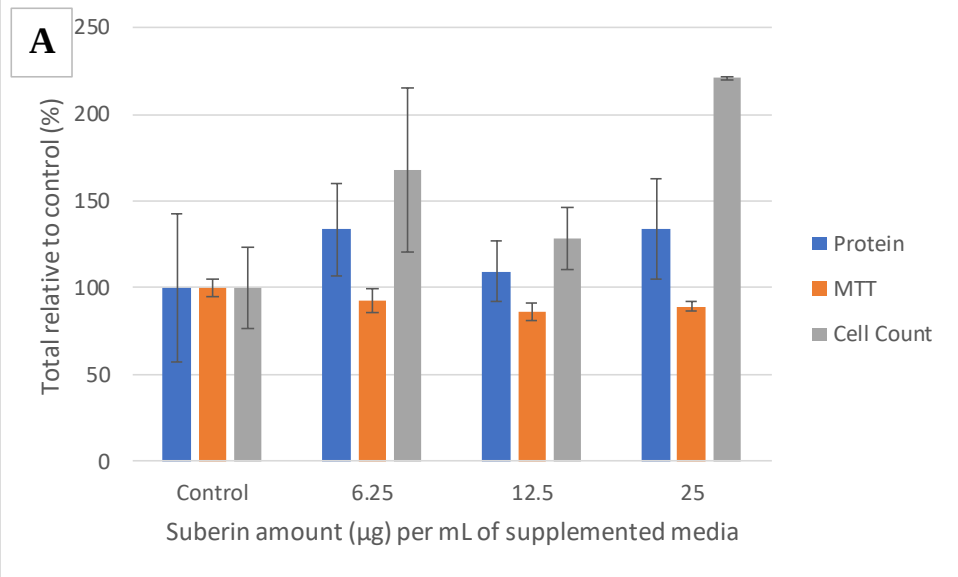


Figure 36 – Impact of the sterilization on total protein content. The results contradict the previously observed results.. The variability is also high.



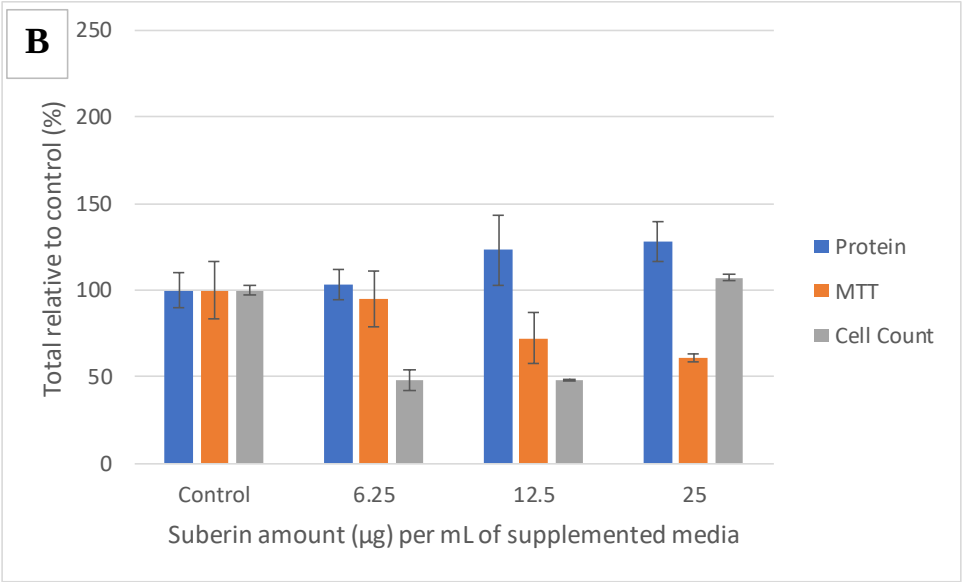


Figure 37 – Assessment of the effects of sterilization on cell proliferation. A – non-sterilized suberin; B – sterilized suberin. The total protein content (blue), viability (orange) and cell numbers (grey) were assessed. Although preliminary, these results indicate that sterilization most likely does not improve cell proliferation

To sum-up, at this stage the two methods used to measure cell proliferation showed somewhat conflicting results. We strongly believe that the cell viability is underestimating the suberin potential. Interestingly, the total protein measurements suggest that the sterilisation treatment of suberin did not affect its cell proliferation activity even though it leads to a major reduction in the particle size. Moreover, the sterilisation treatment is not necessary since we have systematically observed the absence of microbial contamination in all the experiments that used non-sterilised suberin. To further verify these conflicting findings, we have re-assessed suberin influence on cell proliferation measured though the metabolic activity assay. In these subsequent assays, we have covered a range of suberin concentrations all of which were stabilised by the addition of PEG 8000 (0.5%, w/v) which we previous established to not greatly affect cell proliferation (Fig. 34) (see also its impact on suberin particle size and polydispersity, section 4.2.6.).

8.3. Assessment of the effect of the final suberin formulations on cell proliferation

To verify whether the observed suberin effects on cell proliferation were concentration-dependent we decided to repeat some of the previous MTT assays covering a range of suberin concentrations from 6.25 µg/mL to 100 µg/mL. In all the assays, PEG 8000 at 0.5 % (w/v) was used as adjuvant (see Fig. 34).

The impact of suberin in the metabolic activity of the cells, therefore their proliferation, was measured relative to control conditions (culture medium). Surprisingly, suberin at most of the

tested concentrations was observed to stimulate the metabolic activity of the cells (Figure 38). In particular, 12.5 µg/mL of suberin were observed to promote an 80% increase in the metabolic activity of the cells relative to the control conditions. To validate these results, we have run two additional independent experiments which systematically showed suberin ability to stimulate the metabolic activity of the keratinocytes, despite the fact that the degree of increase relative to the control was not reproducible. This is possibly due to the aggregation of the suberin polymer when in an aqueous-based suspension, regardless of the use of PEG in an attempt to stabilise the polymer. The idea that the continuous aggregation of suberin possibly hinders its activity is so far the best explanation for the observations of our distinct experiments, especially the lack of effect of suberin in the metabolic activity of the cells when no PEG was used (Figure 37). Another explanation relies on the natural heterogeneity of this natural polymer which may be influencing these results. As abovementioned, naturally one of the next challenges will be to isolate chemically homogenous fractions of the polymer to identify fractions of distinct activity, if any. In addition, the results herein presented make clear that the particle size of suberin should be analysed prior to all tests as a simple standard method to evaluate the "shelf life" of the active samples.

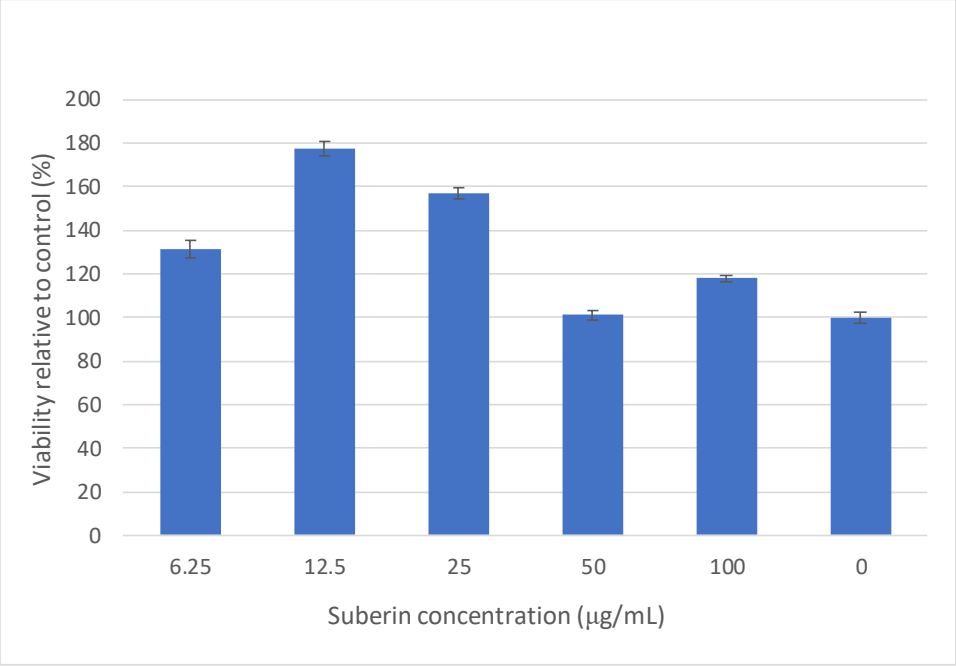


Figure 38 – Impact of suberin on cell proliferation. This assay showed that a dose of 12.5 µg/mL of suberin (with PEG 8000 0.5 % (w/v)) boosted cell proliferation by almost 80 %.

8.4. Microscopy-based analysis

To understand whether the presence of suberin had localized effects on cell proliferation (*i.e.* if it repelled cells) microscopic images were obtained at a 200x magnification on an

inverted microscope (Figure 39). We observed that cells did “not avoid” suberin, and while we could not ascertain if any cell-suberin binding occurred, it is visible on Figure 39 that many cells were proliferating contiguously to the suberin particles. These pictures were obtained from different areas of the same cell culture. Therefore, we can assume that suberin did not have a negative impact on the surrounding cells. This is a remarkable finding, especially since suberin can strongly hinder the proliferation of microbes [40] but apparently not of the keratinocytes, further suggestive of suberin as a wound healing agent. Moreover, we cannot exclude the idea of the potential exploitation of suberin as a scaffold for tissue regeneration, reinforcing the biotech potential of this biopolymer in wound healing.

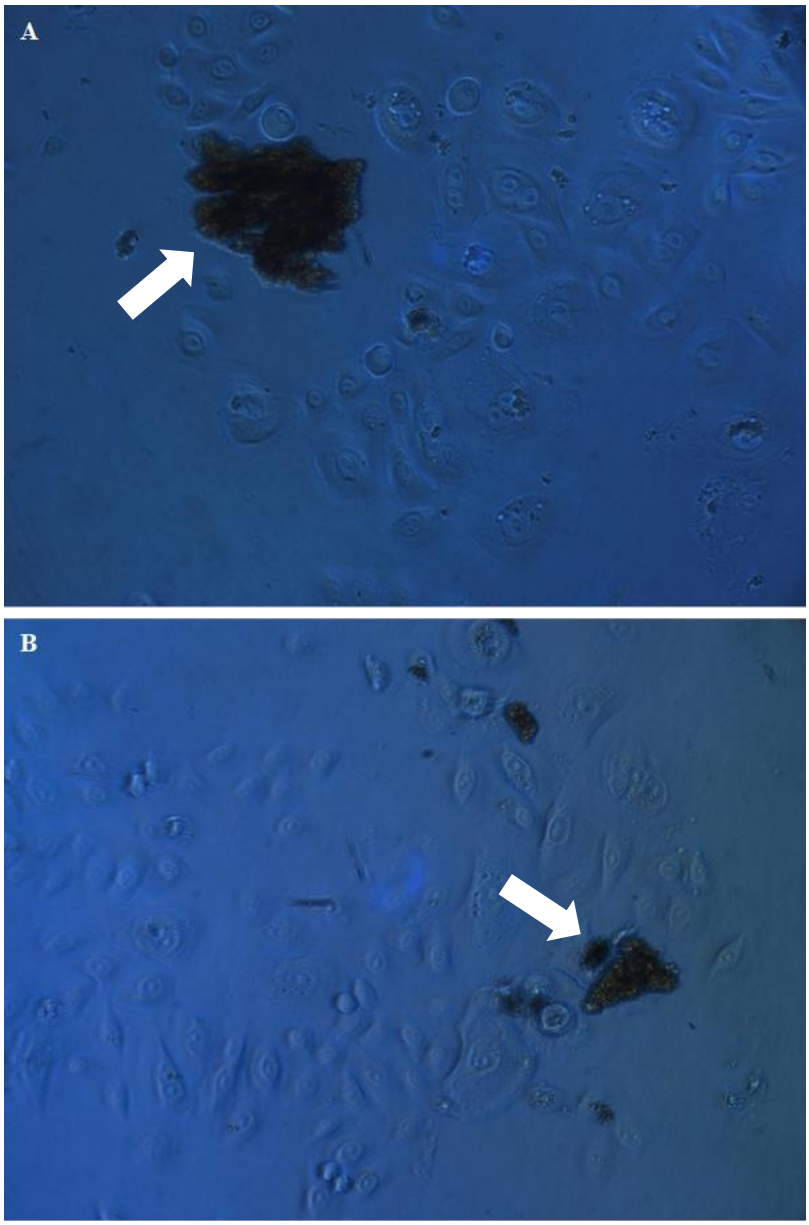


Figure 39 – Microscopic observation of keratinocytes when exposed to suberin (arrows). The photos demonstrate that the suberin *per se* does not inhibit cellular proliferation, as cells were recorded growing near it.

8.5. Impact of PEG 8000 0.5 % (w/v) on cell gap-closure rate

To define suitable experimental conditions to test how suberin impact the rate of wound healing (gap-closure assay) we first tested the rate of closure under control conditions. The closure times observed are critical to design future assays with suberin. The results of the gap-closure assay showed that cells not exposed to PEG 8000 0.5 % (w/v) had a higher gap-closure rate; *i.e.* closing the artificial wound faster than when exposed to PEG (Figure 40). This result needs to be analysed carefully, especially as lower PEG concentrations should be tested to identify a concentration that does not reduce significantly the closing rate. Herein, the addition of PEG delayed the closure of the artificial wound in *ca*. 10 hours.

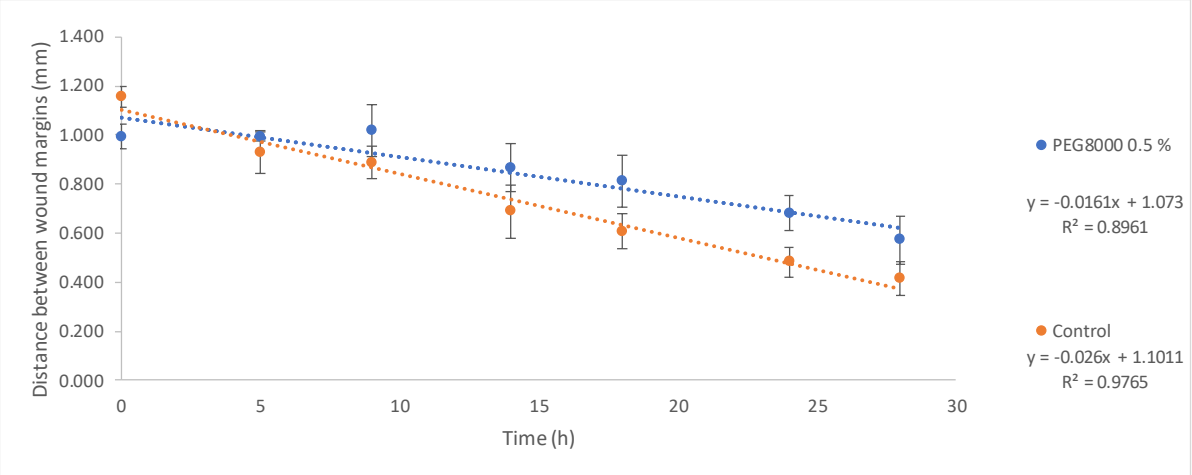


Figure 40 – Determination of gap-closure rate for control and PEG 8000 0.5 % (w/v) supplemented media. The control shows a higher rate of gap-closure, indicating that the PEG supplemented media hindered cell proliferation.

9. Conclusions and future work

The results presented in this chapter have shown that suberin has a positive impact on cell proliferation. The various assays herein performed have allowed us to establish a formulation containing both suberin and PEG 8000 that was capable to boost, in some cases up to 80%, cell proliferation. Naturally, the gap-closure protocol constitutes the next step of testing suberin wound healing capacity, but one needs to define a PEG concentration that does not affect the closure rate. As mentioned, the suberin tested in this chapter originates from a single batch of extraction with cholinium hexanoate, followed by a defined pre-treatment process (24 h sonication and 24 h agitation); therefore, the wound healing capacity of the other suberin samples (*e.g.* those extracted with cholinium decanoate) will be tested in the near future. The variability observed in the various cell proliferation assays may be related to the tendency of suberin to aggregate when in an aqueous suspension. More assays need to be undertaken to verify if PEG reduces the aggregation over time. Alternatively, the use of pulverised films could constitute an alternative to limit further aggregation and control the self-assembly which might also impact the activity. One interesting path to explore will be to consider the use of homogenous suberin fractions (*e.g.* in terms of density, size or weight) which can be prepared exploring distinct centrifugation and chromatographic methods. The use of more chemically homogenous samples will be essential to identify those specific fractions that are more suitable for wound healing. One aspect to analyse is the potential interest of suberin as scaffold for cell proliferation.

To further understand the effects of suberin on mammalian cell culture, we propose the use of additional cell lines of different tissues (such as fibroblasts, *i.e.* dermis) in the future. Moreover, we have planned to use immunological assays, *e.g.* Enzyme-Linked Immunosorbent Assay (ELISA) based assays to determine if suberin can lead to the release of inflammatory cytokines (*e.g.* IL-17), anti-inflammatory cytokines (*e.g.* IL-10) and regulatory cytokeratines (for keratinocyte assays, *e.g.* CK-10). These assays were not yet completed due to the lack of time but are herein considered a priority to fully understand the progress of the cell culture after exposure to suberin.

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